A NEW METHOD FOR THE PREPARATION OF SKELETAL MUSCLE RELAXING FACTOR*

C. J. Parker, Jr. and J. Yun
Department of Physiological Chemistry
Wayne State University College of Medicine
Detroit, Michigan

Received February 7, 1963

Skeletal muscle relaxing factor is produced by the so-called relaxing factor granules (muscle microsomes) in the presence of ATP and Mg^{++} (Parker and Gergely, 1960). Relaxing factor is separated from the granules by ultracentrifugation for 2 hours. Because of the time required and the fact that many such preparations are only slightly active, this method of preparation is unsuitable for routine study. To overcome these difficulties a more rapid procedure has been developed which requires about 10 minutes and yields relaxing factor of high activity. Advantage is taken of the fact that relaxing factor granules are inactivated by organic mercurials (Makinose and Hasselbach, 1960). Granules are incubated 2 minutes with ATP and Mg^{++} to allow the formation of relaxing factor. The granules are then inactivated by the addition of either mersalyl or p-mercuribenzoate (PMB). The mixture can be assayed directly for relaxing factor activity and does not require the time-consuming removal of granules.

Rabbit skeletal myofibrils and relaxing factor granules were prepared according to Gergely et al. (1959). Relaxing factor activity was assayed by measuring the inhibition of myofibrillar ATPase activity.

*This work was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S. (Grant No. A 5311).

It can be seen in Table I that if granules, in the absence of either ATP or Mg^{++}, are inactivated by either mersalyl or PMB they will no longer inhibit myofibrillar ATPase activity (Exp. 3, 4, 6, 7). If, however, relaxing factor granules are incubated with ATP and Mg^{++} prior to inactivation, relaxing factor activity is observed (Exp. 2, 5). This activity is due to the relaxing factor formed before inactivation with mersalyl or PMB. The data in Table I show that the activity of relaxing factor prepared in this fashion is comparable to that observed with the complete granular system (Exp. 1).

TABLE I

The Inactivation of Relaxing Factor Granules by Mersalyl and p-Mercuribenzoate

The incubation mixture contained 20 mM Tris at pH 7.0, 50 mM KCl, 5 mM Mg^{++}, 5 mM ATP, 2.5 mM oxalate, 0.01 mM mersalyl or PMB where indicated and 0.1 mM cysteine; final volume, 2.0 ml; granule concentration, 59 µgms in experiments with mersalyl and 194 µgms in experiments with PMB. The order of addition of ATP, Mg^{++} and mercurial is given in the Table. The incubation time prior to the addition of inactivator was 2 minutes. The mixture was allowed to stand 10 minutes after the addition of mercurial prior to the addition of cysteine. Relaxing factor activity was assayed by adding myofibrils to the system. The reaction was stopped after 5 minutes by addition of 2 ml of 10 per cent trichloroacetic acid. Inorganic phosphate was determined in the protein free supernatant by the method of Fiske and SubbaRow. Relaxing factor activity is expressed as per cent inhibition of myofibrillar ATPase activity.

Exp.	Additions			Per Cent Inhibition
	Before Inactivation	Inactivator	After Inactivation	
1	ATP, Mg^{++}	-	Cysteine	87.3
2	ATP, Mg^{++}	Mersalyl	Cysteine	78.3
3	Mg^{++}	Mersalyl	Cysteine, ATP	0.0
4	ATP	Mersalyl	Cysteine, Mg^{++}	12.6
5	ATP, Mg^{++}	PMB	Cysteine	80.7
6	Mg^{++}	PMB	Cysteine, ATP	5.0
7	ATP	PMB	Cysteine, Mg^{++}	3.8

Both mersalyl and PMB inhibit myofibrillar ATPase activity. It is necessary, therefore, to add cysteine to the system after inactivation of the granules to remove the excess mercurial. Neither 0.1 mM cysteine nor 0.1 mM cysteine plus 0.01 mM mersalyl or PMB affects myofibrillar ATPase activity (see Table II). It is apparent from the results in Table I that cysteine does not reverse the inactivation of granules by mersalyl or PMB.

TABLE II

The Effect of Cysteine, Mersalyl and p-Mercuribenzoate on Myofibrillar ATPase Activity

The experimental conditions are the same as those described in Table I. Cysteine and cysteine plus mercurial were added to the system prior to the addition of myofibrils.

Additions	ATPase Activity μmoles Phosphate per Minute per mg Protein
None	0.35
0.1 mM Cysteine	0.36
0.1 mM Cysteine + 0.01 mM Mersalyl	0.34
0.1 mM Cysteine + 0.01 mM PMB	0.36

The inactivation of relaxing factor granules by mersalyl is 50 per cent complete in 3 minutes; complete inactivation requires 6 to 7 minutes. Inactivation with PMB is somewhat faster; 70 per cent in 1 minute, 100 per cent in 4 minutes. In the work described above an inactivation time of 10 minutes was used with both mersalyl and PMB. The short period of incubation required to produce relaxing factor prior to inactivation of the granules and the short time required for inactivation indicates that relaxing factor is produced less

than 10 minutes. The longer incubation periods used by others (Parker and Gergely, 1960; Briggs and Fuchs, 1960; and Stam and Honig, 1962) for preparing the factor by the centrifugal method appear to be unnecessary.

The use of organic mercurials for separating relaxing factor from the granular enzyme system that produces it circumvents the disadvantages inherent in the centrifugal method of preparation. The procedure as outlined is rapid and suitable for routine study of relaxing factor.

We are grateful to Dr. Dana Dabich for her helpful criticism in the preparation of the manuscript.

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