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The Inhibitory Action of Relaxing-Factor Preparations on the Myofibrillar Adenosine Triphosphatase

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Marsh (1952) reported that whole-muscle homogenates contained a factor (known as the Marsh factor, Marsh–Bendall factor or relaxing factor) which prevented adenosine triphosphate from inducing the normal synaeresis which occurred on addition of the nucleotide to washed muscle-cell fragments. Under certain conditions in the presence of the factor an elongation of the fibre fragments took place and the process was considered to be analogous to relaxation. In whole-muscle homogenates Marsh also observed that the adenosine-triphosphatase activity of the myofibrils was suppressed and subsequent work by others (Bendall, 1953; Bozler & Prince, 1953) showed that relaxation in muscle-model systems was associated with a low rate of hydrolysis of adenosine triphosphate. A number of investigators have demonstrated that so-called relaxing-factor preparations can also inhibit the Mg^{2+}-ion-activated adenosine triphosphatase of isolated myofibril preparations (Hasselbach & Weber, 1953; Portzehl, 1957*a*; Bendall, 1958).

The nature of this relaxing factor has been a matter of some controversy and although various phosphorylating systems (Bendall, 1954; Goodall & Szent-Györgyi, 1953; Lorand, 1953) have been claimed to possess activity it is now clear that particulate material can be sedimented from homogenates of rabbit skeletal muscle which will induce the relaxation of fibre models in the presence of adenosine triphosphate (Ebashi, Takeda, Otsuka & Kumagai, 1956; Portzehl, 1957*a*) and simultaneously inhibit the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase. These prepara-

tions do not require the presence of transphosphorylase systems (Portzehl, 1957*b*; Briggs, Kaldor & Gergely, 1959), although some workers (Molnar & Lorand, 1959) consider that such systems can potentiate their action.

Relaxing-factor preparations sedimented from muscle homogenates have been considered to be microsomal in nature (Molnar & Lorand, 1959), although the only evidence for this appears to be the fact that they require conditions for sedimentation which are comparable with those used to sediment the microsome fraction from other tissues.

This paper is concerned with an investigation of the distribution of the relaxing factor, assayed by its inhibitory action on the myofibrillar adenosine triphosphatase, in myofibril-free homogenates of rabbit and pigeon skeletal muscle. The properties of the preparation and its requirement for potentiating substances have been examined to throw light on the mechanism of inhibition of the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase.

METHODS

Preparation of granules of rabbit-skeletal muscle

Fractionation. Chilled minced back and leg muscles (30 g.) of the rabbit were homogenized for 2 min. at full speed in the MSE Ato-Mix blender (Measuring and Scientific Equipment Ltd.) in 300 ml. of either 0·25 M-sucrose or 0·08 M-KCl, each containing 0·039 M-borate buffer, pH 7·1 (Perry, 1953). After standing for 15 min. the homogenate was centrifuged at 2400 *g* for 25 min., the sediment of myofibrils and debris was discarded and the turbid supernatant centrifuged at 2400 *g* for a further 10 min. Three fractions were sedimented in succession from the myofibril-free supernatant as follows: (i) 30 min. at 8000 *g* (2400–8000 *g* fraction); (ii) 60 min. at 20 000 *g*, sometimes 23 000 *g* (8000–20 000 *g* or 8000–23 000 *g* fractions respectively); (iii) 60 min. at 90 000 *g* (20 000–90 000 *g* or 23 000–90 000 *g* fractions respectively).

In each case the sedimented material was carefully resuspended in a volume of medium equal to one-half of that of the original supernatant from which it was obtained, washed by resuspension in medium and sedimented by centrifuging at 15 000 *g* for 40 min. for fraction (i) and at 90 000 *g* for 60 min. for fractions (ii) and (iii). Finally the pellets were resuspended in one-twentieth of the volume of the original supernatant and stored at 0°.

'*Routine granule preparations*'. Minced back and leg muscles (120 g.) were homogenized with 300 ml. of 0·08 M-KCl–0·039 M-borate buffer, pH 7·1. Myofibrils and cell residues were removed by centrifuging at 2400 *g* for 25 min.; the supernatant was centrifuged for 60 min. at 20 000 *g*, the granules were washed by resuspension and centrifuging for 60 min. at 90 000 *g* and finally resuspended in medium of volume equal to one-sixth to one-eighth of the volume of supernatant from which they were obtained. All manipulations for granule preparations were carried out at 0°.

Preparation of granules of pigeon-breast muscle. Finely sliced pigeon-breast muscle (10–20 g.) was homogenized in the Potter homogenizer for 2 min. with 9 vol. of 0·25 M-sucrose–0·039 M-borate buffer, pH 7·1. The homogenate was centrifuged for 10 min. at 700 *g* to sediment myofibrils and cell residues. Some mitochondria were also sedimented by this treatment. The turbid supernatant was centrifuged at 8000, 23 000 and 90 000 *g* and fractions were prepared as described for rabbit muscle.

Preparation of myofibrils and muscle proteins. Myofibrils were prepared in 0·1 M-KCl–0·039 M-borate buffer as described by Perry & Corsi (1958). Natural actomyosin was extracted from isolated myofibrils and reprecipitated three times by the method of Perry & Corsi (1958). Synthetic actomyosin was prepared by combining (in equal proportions) myosin, prepared as described by Perry (1955), and F-actin, made by the method of Straub (1943).

Determinations of adenosine triphosphatase. Assays were carried out at 25° in a total volume of 2 ml. containing 40 mM-histidine–HCl buffer, pH 7·0, 5 mM-adenosine triphosphate (ATP; sodium salt), 5 mM-$MgCl_2$ and, unless otherwise indicated, 5 mM-dipotassium oxalate. When granules in 0·25 M-sucrose–0·039 M-borate buffer were used the assay medium was brought to a final concentration of 0·25 M with respect to sucrose. Tubes were pre-incubated and the reaction was started by the addition of 0·2 ml. each of granule and myofibril suspensions. Assays were carried out on myofibrils and granules incubated together and separately. Adjustment was made to ensure that the salt concentration was identical in each incubation and appropriate controls were also carried out. The reaction was stopped, usually after incubation for 5 min., by the addition of 1 ml. of 15% (w/v) trichloroacetic acid. Inorganic phosphate was estimated by the Fiske & Subbarow (1925) method with a 608 filter on the EEL colorimeter. The percentage inhibition of the myofibrillar adenosine triphosphatase was given by:

$$\frac{\text{Sum of inorganic P liberated by myofibrils and granules incubated independently} - \text{inorganic P liberated by myofibrils and granules incubated together}}{\text{inorganic P liberated by myofibrils alone}} \times 100.$$

In the oxalate systems the amounts of granules added were such that their intrinsic adenosine-triphosphatase activity was very low (< 5% of myofibrils). To compare the intrinsic adenosine-triphosphatase activity of the granular fractions, assays were carried out at different enzyme activities in the same medium as was used for the studies of inhibition of myofibrillar adenosine triphosphatase, to which 0·4 mM-dinitrophenol was added. All assays of the adenosine-triphosphatase-inhibitory activity of granules for fractionation studies in the absence of oxalate or other activator and of the adenosine-triphosphatase activity of the granules were carried out on the day of preparation. Assay of the adenosine-triphosphatase-inhibitory activity of rabbit-muscle granules in the presence of oxalate for fractionation studies was usually carried out on the day after preparation, as it was found that activity in the presence of oxalate did not alter significantly over this period. All estimations of pigeon-granule myofibrillar adenosine-triphosphatase-inhibitory activity were carried out on the day of preparation and in the presence of 5 mM-oxalate.

Succinic-dehydrogenase activity. This was measured manometrically as described by Slater (1949), immediately after preparation of the fractions.

Ribonucleic acid analyses. Determinations of ribonucleic acid content of granular fractions were carried out as described by Perry & Zydowo (1959). Total P and ribose estimations were made on the final hot 10% (w/v) perchloric acid extracts of the granules.

RESULTS

Distribution of relaxing-factor activity

Rabbit muscle. Investigations of the distribution of the activity of granule preparations in inhibiting the Mg^{2+}-ion-activated myofibrillar (adenosine-triphosphatase-inhibitory) activity were confined to studies on the turbid supernatants obtained when the myofibrillar fraction had been removed from cell-free homogenates of skeletal muscle. The results were essentially the same whether the homogenates were made in the KCl–borate or the sucrose–borate solutions (Fig. 1), although, in general, yields of granules were greater in KCl–borate solution.

Usually three fractions, so-called 2400–8000, 8000–20 000 and 20 000–90 000 *g*, were collected (see Methods section) and for a given fraction the specific inhibitory activity, i.e. the amount of granule nitrogen required to produce 50% inhibition of the myofibrillar adenosine triphosphatase, was remarkably constant and independent of the particular sample of muscle from which the fraction was obtained. If the percentage inhibition of the adenosine triphosphatase was plotted against granular nitrogen a response curve was obtained which was characteristic for each fraction (see Fig. 1). It will be seen that about 3·2 μg. of granule nitrogen gives 50% inhibition with the 2400–8000 *g* fraction, whereas about twice as much nitrogen is required for a similar response with the 8000–20 000 *g* fraction. The 20 000–90 000 *g* fraction was much less active on a nitrogen basis, and it was estimated that 90% of the total activity sedimented by the three centrifugings was obtained in the 2400–8000 and 8000–23 000 *g* fractions. The distribution of adenosine-triphosphatase-inhibitory activity was similar when measured with high concentrations of the fresh granules in the absence of 5 mM-potassium oxalate or with much lower concentrations in the presence of this substance (see section on effect of oxalate).

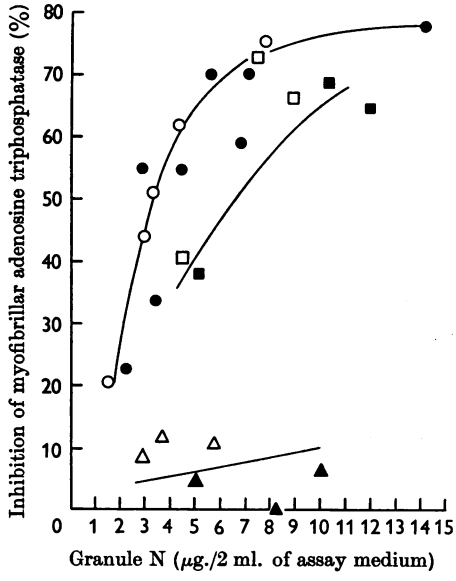

Fig. 1. Inhibitory activity of granular fractions sedimented from rabbit-muscle homogenates on rabbit myofibrillar adenosine triphosphatase. Assay conditions were as described in the Methods section. (1) 2400–8000 *g* fraction: ○, isolated in sucrose–borate; ●, isolated in KCl–borate; seven different preparations. (2) 8000–20 000 *g* fraction: □, isolated in sucrose–borate (at 23 000 *g*); ■, isolated in KCl–borate; four different preparations. (3) 20 000–90 000 *g* fraction: △, isolated in sucrose–borate; ▲, isolated in KCl–borate; four different preparations.

No clear separation in the distribution of succinic-dehydrogenase activity and adenosine-triphosphatase-inhibitory activity could be demonstrated between the three fractions. Although there was appreciable adenosine-triphosphatase-inhibitory activity in the lighter fractions, which were poorer in oxidative activity, the highest specific activity with respect to both properties was found in the 2400–8000 *g* fraction (Table 1).

In contrast with the above-mentioned properties no significant pattern of distribution of adenosine-triphosphatase activity or of ribonucleic acid content between the three fractions was apparent. Usually a total of about 0·34 mg. of nitrogen in the sucrose extracts, and 0·58 mg. of nitrogen in the KCl–borate extracts, per g. wet wt. of original muscle, was sedimented in the three fractions and distributed between them in a fairly constant manner (Table 1).

Pigeon-breast muscle. Homogenates of pigeon-breast muscle are very much richer in large granules than are similar preparations from rabbit skeletal muscle. These granules sedimented at relatively low speeds and to avoid too much loss in the preparation of the supernatants, myofibrils were removed by centrifuging in a lower centrifugal field than that used in similar preparations from rabbit muscle (see Methods section).

Granules of high specific activity for inhibition of rabbit myofibrillar adenosine triphosphatase were obtained in both the 8000–23 000 *g* fraction and the 23 000–90 000 *g* fraction. The most active pigeon

Table 1. *Inhibition of myofibrillar adenosine triphosphatase and other properties of granular fractions sedimented from homogenates of rabbit skeletal muscle*

Figures given are average values; numbers of experiments are shown in parentheses. Inhibition was measured under the usual assay conditions with about 0·2–0·6 mg. of myofibrillar N. For determination of succinic-dehydrogenase activity the three fractions prepared were: 2400–8000 *g*; 8000–20 000 *g*; 20 000–90 000 *g*. Assays of adenosine triphosphatase were done at 25°; succinic-dehydrogenase assays were done at 30°. Q_{O_2}: μl. of O_2/hr./mg. of N. Q_P: μl. of P/hr./mg. of N.

Homogenizing medium	Fraction	Distribution of total N (% of total sedimented)	Amount of granules to produce 50% inhibition (μg. of N)	Succinic-dehydrogenase activity (Q_{O_2})	Adenosine-triphosphatase activity (Q_P)	Ribonucleic acid content (mg. of ribonucleic acid P/g. of total N)
0·25 M-Sucrose, 0·039 M-Borate	2 400–8 000 *g*	20 (3)	3·2 (4)	21·8 (3)	2016 (1)	5·3 (3)
	8 000–23 000 *g*	50 (3)	5·5 (2)	3·8 (2)	1344 (1)	4·3 (3)
	23 000–90 000 *g*	30 (3)	20·0 (2)	0·9 (1)	2173 (1)	6·0 (3)
0·08 M-KCl, 0·039 M-Borate	2 400–8 000 *g*	21 (3)	3·5 (3)	—	—	—
	8 000–20 000 *g*	58 (3)	7·0 (2)	—	—	—
	20 000–90 000 *g*	21 (3)	85·0 (2)	—	—	—

Table 2. *Inhibition of myofibrillar adenosine triphosphatase and other properties of granular fractions sedimented from homogenates of pigeon-breast muscle*

Fractionation was carried out in 0·25 M-sucrose–0·039 M-borate buffer. Figures given are average values; numbers of experiments are shown in parentheses. Assays were carried out on fresh granule preparations under the same conditions as used for results shown in Table 1. Adenosine-triphosphatase assays were done at 25°; succinic-dehydrogenase assays were done at 30°. Q_{O_2} and Q_P are defined in Table 1.

Fraction	Distribution of total N (% of total sedimented)	Amount of granules to produce 50% inhibition (μg. of N)	Succinic-dehydrogenase activity (Q_{O_2})	Adenosine-triphosphatase activity (Q_P)
700–8 000 *g*	70 (5)	45 (2)	152 (2)	5420 (1)
8 000–23 000 *g*	—	20 (2)	—	—
	30* (5)	—	18* (2)	3810* (1)
23 000–90 000 *g*	—	20 (2)	—	—

* Estimated on fraction sedimented after centrifuging the supernatant, obtained after the 700–8000 *g* fraction was removed, for 60 min. at 90 000 *g*.

granules were, however, only about one-sixth as active on a nitrogen basis as the best preparations from rabbit tissue. In pigeon-breast-muscle preparations practically the whole of the succinic-dehydrogenase activity was sedimented in the 700–8000 *g* fraction. Although the specific adenosine-triphosphatase-inhibitory activity in this fraction was the lowest the total activity present was estimated to represent about half the total activity sedimented in all three fractions (see Table 2). Further, the range in specific activity between the fractions was less than with rabbit muscle.

Although the points made above clearly emerged, compared with those obtained with rabbit preparations the results with pigeon-breast muscle were much less consistent. In these studies pigeon-breast granules were assayed with rabbit myofibrils. This suggests that the effect is a general feature of muscle-granule and myofibril systems irrespective of their source.

Effect of oxalate

Relatively concentrated granular fractions isolated from rabbit muscle inhibited the adenosine-triphosphatase activity of myofibrils when the assay medium contained, in addition to buffer, only 5 mM-ATP and 5 mM-$MgCl_2$. It was observed, however, as reported by other workers, that certain substances had a marked potentiating action on the adenosine-triphosphatase-inhibitory activity. Oxalate, which was introduced into relaxing-factor systems by Portzehl (1957*a*), was particularly effective and at its optimum concentration (5 mM) the standard granule preparations (2400–20 000 *g* fraction) produced 50% inhibition of the myofibrillar adenosine triphosphatase at one-thirty-third of the nitrogen concentration required in its absence. Thus for 50% inhibition in the presence of oxalate under the normal assay conditions 7 μg. of 2400–20 000 *g* granule-fraction nitrogen was required, whereas for 50% inhibition in the absence of

oxalate, but under otherwise identical conditions, 230 μg. of nitrogen had to be present. Oxalate alone slightly depressed the adenosine-triphosphatase activity of myofibrils but for its potentiating effect to be apparent both granules and myofibrils were required to be present (Fig. 2). Oxalate could not be replaced by succinate, malonate, sulphite or hypophosphite. Citrate had some potentiating activity but was much less effective than oxalate. Pyrophosphate was comparable in effect to oxalate but at equivalent concentrations greater activity was always obtained in the presence of oxalate (Fig. 2). A further difference was that pyrophosphate had a greater depressing effect on the myofibrillar adenosine triphosphatase when this was measured in the absence of the granules. This was possibly due to the higher ionic strength of pyrophosphate solutions compared with oxalate solutions of equal concentration.

Low concentrations of calcium are well known to inactivate rabbit-muscle relaxing-factor preparations (Bozler, 1952; Bendall, 1953). Under the conditions employed in this work 0·1 mM-$CaCl_2$ completely removed the adenosine-triphosphatase-inhibitory activity of granule preparations used at concentrations of about 300 μg. of granule nitrogen-assay (Fig. 3), but this effect was prevented by the presence of 5 mM-potassium oxalate in the incubation medium. In the presence of 0·1 mM-$CaCl_2$ the intrinsic adenosine triphosphatase of the granules was increased, an effect which was likewise prevented by oxalate.

These results suggested that the action of oxalate could be explained by its property of combining with calcium and effectively removing it from the enzymic system. It was noticed in earlier experiments that often the inhibitory activity of granule preparations tested in the absence of oxalate fell off markedly on storage overnight at 0°. Similar changes in activity measured in the absence of oxalate occurred on incubation for 6 hr. at 35° in the presence of 2 mM-KCN. In both cases on assay in the presence of oxalate (at the same or at a very much lower nitrogen concentration) very little or no fall in activity could be detected (Table 3 and Fig. 4). Also associated with this apparent decrease in inhibitory activity was an increase in the endogenous adenosine-triphosphatase activity of the granules. The results were com-

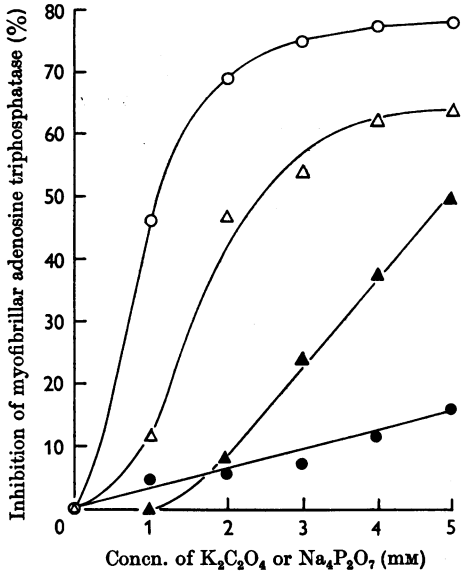

Fig. 2. Effect of dipotassium oxalate and tetrasodium pyrophosphate on: (*a*) Mg^{2+}-ion-activated adenosine triphosphatase of myofibrils; (*b*) inhibitory activity of rabbit-muscle granules on the myofibrillar adenosine triphosphatase. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (29·3 μg. of N in the 0·2 ml. of suspension taken) in a total assay volume of 2 ml. Assay conditions were similar to those described in Fig. 1, except that varying concentrations of oxalate in pyrophosphate were present. ○, Myofibrils and granules incubated with oxalate; ●, myofibrils alone incubated with oxalate; △, myofibrils and granules incubated with pyrophosphate; ▲, myofibrils alone incubated with pyrophosphate.

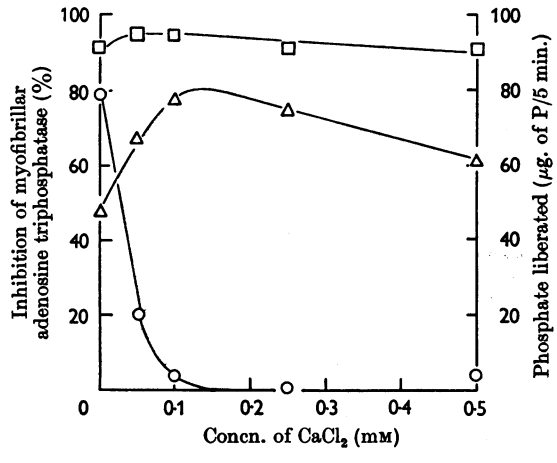

Fig. 3. Effect of $CaCl_2$ on the Mg^{2+}-ion-activated adenosine-triphosphatase activities of myofibrils and granules incubated independently and together. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (336 μg. of N in the 0·2 ml. of suspension taken) in 2 ml. of assay medium. Assay conditions were similar to those of Fig. 1 except that oxalate was omitted and varying concentrations of $CaCl_2$ were added. □, Myofibrillar adenosine triphosphatase (expressed as μg. of P liberated); △, granule adenosine triphosphatase (expressed as μg. of P liberated); ○, adenosine triphosphatase of myofibrils and granules combined (expressed as percentage inhibition of myofibrillar adenosine triphosphatase).

Table 3. *Effect of aging and calcium chloride on the adenosine-triphosphatase-inhibitory action of rabbit-muscle-granule preparations*

Conditions were as described in the Methods section.

Granule preparation	Dilution used	Granular N used (μg./assay)	Conditions	Oxalate absent: Inhibition of myofibrillar adenosine triphosphatase by granules (%)	Oxalate absent: Intrinsic adenosine-triphosphatase activity of granules (μg. of P/5 min.)	5 mM-Oxalate present: Inhibition of myofibrillar adenosine triphosphatase by granules (%)	5 mM-Oxalate present: Intrinsic adenosine-triphosphatase activity of granules (μg. of P/5 min.)
A	Undiluted	—	Fresh	87	68·5	—	—
		—	Aged 8 days	20	212	88	37·5
A	50-fold	—	Fresh	—*	—*	64	3·2
		—	Aged 8 days	—*	—*	48	6·4
B	Undiluted	330	Fresh	79	60·5	89	56
		330	Aged 4 days	23	67·5	81·5	49
B	30-fold	11	Fresh	—*	—*	60	2·8
		11	Aged 4 days	—*	—*	59	2·4
C	Undiluted	—	Control	35	52	—	—
		—	6 hr. at 35°	15	82	91	42
C	35-fold	—	Control	—*	—*	64	1·6
		—	6 hr. at 35°	—*	—*	62	1·6
D	Undiluted	336	Control	79	48	—	—
		336	0·1 mM-$CaCl_2$ in assay	3	77	—	—
E	Undiluted	352	Control	70	76	90	72
		352	0·1 mM-$CaCl_2$ in assay	2	106·5	89	76

* Granule suspension was too dilute for assay in the absence of oxalate.

patible with the slow liberation from the granules on aging of some substance, possibly calcium, which destroyed the adenosine-triphosphatase-inhibitory activity and whose effect could be removed by oxalate.

When granules which had been stored for 3 days at 0° and then washed three times with KCl–borate buffer were assayed in the absence of oxalate they were found to possess greater adenosine-triphosphatase-inhibitory activity than an unwashed control sample of granules stored under identical conditions. In fact little loss of activity was observed in the repeatedly washed granule samples. Further, the inhibitory activity of granules dialysed against KCl–borate buffer containing 2 mM-KCN for 2 days fell off much less than an undialysed control sample stored in the presence of 2 mM-KCN. These results suggested that the decrease in inhibitory activity observed when granule preparations were allowed to age was due to the release of a dialysable substance which had the property of destroying the adenosine-triphosphatase-inhibitory activity of the granules.

An affinity of active granules for calcium was further suggested by the following experiment. Granules were suspended in KCl–borate buffer containing 10 mM-$CaCl_2$ for 1 hr. and then subsequently washed by repeated centrifuging and resuspension in KCl–borate buffer. On assay in the absence of oxalate after storage overnight the treated granules had considerably lower activity than did a control sample treated in an identical manner, except for the fact that the original KCl–borate buffer medium used for the initial dilution did not contain $CaCl_2$. In the presence of oxalate both samples had the same high inhibitory activity.

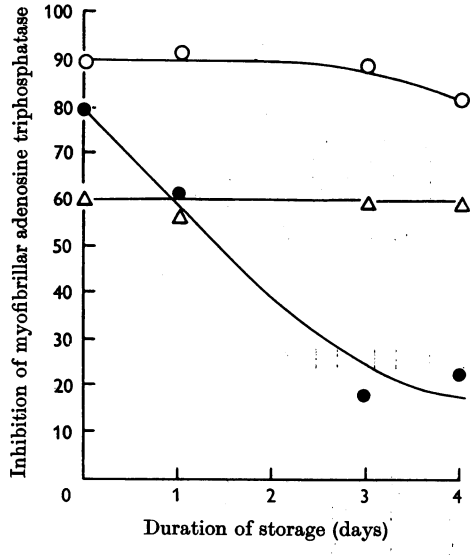

Fig. 4. Effect of storage at 0° on the adenosine-triphosphatase-inhibitory action of rabbit-muscle granules. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (330 μg. of N in the 0·2 ml. of suspension taken, 11 μg. of N when diluted) in assay conditions identical to those of Fig. 1 except that oxalate was omitted in one series of experiments. ○, Myofibrils and concentrated granules with 5 mM-oxalate; ●, myofibrils and concentrated granules, oxalate omitted; △, myofibrils and diluted granules with 5 mM-oxalate.

Some properties of the granular preparation

It was not found possible to prepare an active acetone-dried powder, but freeze-dried granule suspensions retained activity after storage for several weeks.

By extraction with Weber's solution (0·06M-KCl, 0·01M-Na_2CO_3, 0·04M-$NaHCO_3$) up to 25% of the total granular nitrogen was extracted but the adenosine-triphosphatase-inhibitory activity was unimpaired and remained associated with the insoluble residue. Treatment of a suspension of active freeze-dried granules with 0·3% of sodium deoxycholate resulted in destruction of the adenosine-triphosphatase-inhibitory activity. Subsequent centrifuging of the largely clarified suspension for 1 hr. at 15 000 *g* resulted in 67% of the total granule protein remaining in the clear supernatant. This supernatant, too, had no inhibitory activity. Similarly, treatment with 0·1 or 0·03% of deoxycholate did not yield active supernatants. The whole-granule suspension in 0·03% deoxycholate still retained activity, however. The removal of deoxycholate by dialysis did not alter these findings.

All granule preparations possessed adenosine-triphosphatase activity (see Tables 1 and 2), which was usually fully activated (Chappell & Perry, 1954) as very little stimulation was produced by 0·4 mM-dinitrophenol.

With a fixed amount of a granule preparation it was observed that the concentration of myofibrillar nitrogen could be increased by at least 200% without changing significantly the percentage inhibition of the myofibrillar adenosine triphosphatase, although the amount of hydrolysis of adenosine triphosphate ranged from 1·1 to 3·5 μmoles.

Inhibition was not affected by prior extraction of the actomyosin, for inhibition comparable with that obtained with myofibrils was obtained with natural and synthetic actomyosins (cf. Mueller, 1960). In these cases assays were carried out at somewhat lower ionic strength, as the Mg^{2+}-ion-activated adenosine triphosphatase of these systems was rather low in the usual incubation medium.

Neither Mg^{2+}-ion-activated myofibrillar inosine-triphosphatase activity nor uridine-triphosphatase activity was inhibited by granular preparations

Table 4. *Comparison of the inhibitory action of rabbit-muscle-granule preparation on the hydrolysis of adenosine triphosphate, inosine triphosphate and uridine triphosphate by myofibrils*

Incubation was carried out for 5 min. at 25° in a total volume of 2 ml. containing 0·2 ml. of granules or 0·2 ml. of myofibrils or both, 2·5 mM-$MgCl_2$, 5 mM-potassium oxalate, 40 mM-histidine–HCl buffer, pH 7·0, and 2·5 mM-nucleotide. ITP, Inosine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate.

Granule preparation	Granule N (used/assay) (μg.)	Substrate	Inhibition of myofibrillar nucleotidase by granules (%)	Myofibrillar-activity nucleotidase (μg. of P/5 min.)	Granular-activity nucleotidase (μg. of P/5 min.)
A (2400–90 000 *g*)	100	ITP	0	37	13·4
	100	ATP	79	90·5	14·8
	10	ITP	0	37	1·1
	10	ATP	36	90·5	2·1
B (2400–8000 *g*)	48	UTP	0	63	5·7
	48	ATP	76	84	8·5
	12	UTP	0	63	1·8
	12	ATP	41	84	2·2

which, under otherwise identical conditions, strongly inhibited the myofibrillar adenosine triphosphatase (Table 4).

DISCUSSION

Almost without exception all studies on relaxing-factor preparations reported in the literature have been confined to those isolated from rabbit skeletal muscle (cf. Bendall, 1954). In so far as the inhibitory action of muscle-granule preparations on the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase is an aspect of relaxing-factor activity, the present study provides evidence of the existence of a similar relaxing-factor system in pigeon-breast muscle. This is a highly oxidative tissue much richer in mitochondria than is rabbit skeletal muscle. In both tissues adenosine-triphosphatase-inhibitory activity was a property of all three of the granular fractions isolated although the specific activity was not identical in the fractions isolated from a given muscle type. There are suggestions that the structures with which the factor activity is associated are slightly different in pigeon-breast muscle compared with rabbit skeletal muscle. In homogenates of the former tissue material of highest specific activity was in the fractions containing slower-sedimenting components, whereas with the latter muscle the fraction with the faster-sedimenting components had the highest specific activity. In both rabbit and pigeon muscle the bulk of the oxidative activity and the major portion of the total sedimented-factor activity were associated with the heaviest-granule (mitochondrial) fraction.

With rabbit-muscle preparations at least it is clear that caution must be used in concluding that relaxing-factor preparations are comparable with the microsome fractions of other tissues. In rabbit muscle the fraction which sedimented under conditions which might be supposed to sediment microsomes, i.e. 23 000–90 000 *g* fraction, contained only 10% of the total sedimented relaxing-factor activity. Evidence has been provided by Perry & Zydowo (1959) that, unlike the microsome fraction of most other tissues, fractions isolated in a similar manner from muscle are not rich in ribonucleic acid, the bulk of which is sedimented with the myofibrillar fraction. Molnar & Lorand (1959) report that under their conditions they could detect little or no adenosine-triphosphatase-inhibitory activity in the material which was sedimented from 0·15 M-KCl extracts in 30 min. at 11 000 *g*. In our hands a similar fraction, the 2400–8000 *g* fraction, assayed in the presence of oxalate was 25 times as active on a nitrogen basis as the so-called microsome fraction of Molnar & Lorand (1959) assayed in the presence of a potentiating phosphokinase system. Although the maximum yield of factor activity is associated with the mitochondria-rich fractions it does not follow that the factor is part of these structures, for active fractions with little or no succinic-dehydrogenase activity can be separated from both rabbit and pigeon muscle. This suggests that the normal muscle-mitochondrial fraction is heavily contaminated with elements containing the relaxing factor. Whatever these structures are, the factor activity is tightly associated with them. It is possible that the activity is associated with the reticular structures seen in electron-microscope studies of muscle (Porter & Palade, 1957) and which may play a role in conducting into the interior of the cell those changes taking place at the membrane which initiate contraction (see Huxley & Straub, 1958).

The questions of the role of dialysable cofactors (Briggs *et al.* 1959) or of transphosphorylase systems

(Molnar & Lorand, 1959) in relaxing-factor systems has not been investigated. Nevertheless, the observation that granule preparations on standing lose activity which can be restored by the addition of oxalate is possibly pertinent to these problems. Also it should be pointed out that the activity of factor preparations potentiated by cofactors from muscle extracts or by transphosphorylase is considerably less than the activity obtained in the presence of oxalate alone. The specific activity obtained for the heaviest fraction isolated from rabbit muscle when assayed in the presence of oxalate is the highest yet reported in the literature. It is significant that pyrophosphate, which behaves in a manner similar to oxalate, has many similarities in behaviour to the dialysable cofactor (Briggs *et al.* 1959). It could be that the cofactors act by decreasing in some way the effect of the inhibitory substance present in factor preparations which appears to increase in amount on standing and is rendered ineffective by oxalate.

The nature of this substance which is present in normal granule preparations and which is responsible for the decrease in activity on aging is not known. There is, however, strong presumptive evidence that it is calcium. Isolated heart sarcosomes possess a strong affinity for this cation and it has been shown that all the calcium originally present in the muscle may be concentrated in these structures on isolation. There is evidence, however, that the accumulation of calcium in the isolated sarcosomes may not necessarily indicate the distribution of this metal *in situ* (Slater & Cleland, 1953).

There are certain similarities between the properties of the preparations inhibitory to adenosine triphosphatase and oxidative-phosphorylating systems. In both cases the effects of aging, protection from calcium effects by certain binding reagents and the action of deoxycholate are comparable. It seems unlikely, however, that the inhibitory action of relaxing-factor preparations is apparent rather than real, owing to a resynthesis of ATP by oxidative-phosphorylating systems which may be present. Relaxing-factor action can be demonstrated in well-washed and aged preparations which would presumably be free from any endogenous oxidizable substrate. Further inhibition of adenosine triphosphatase occurs in the presence of 0·5 mM-2:4-dinitrophenol and in some cases can be demonstrated in the less-readily sedimented granular fractions which contain few mitochondria.

From the experimental data presented above it can be calculated that to bring about 50% inhibition of the adenosine-triphosphatase activity of 1 mole of myosin (mol.wt. 400 000) in the myofibrillar form 14 600 g. of total granular protein is required. As presumably only a fraction of this granular protein has factor activity it seems unlikely that it could depress the myofibrillar adenosine-triphosphatase activity by binding enough magnesium to alter the magnesium/ATP ratio to an inhibitory level, as was suggested earlier (Perry, 1956; Perry & Grey, 1956). A more plausible explanation is that the factor inhibits by binding the trace of metal which appears to be essential for the Mg^{2+}-ion-activated adenosine triphosphatase of myofibrils. Such a mechanism was suggested earlier to explain the inhibitory action of ethylenediaminetetra-acetate on the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase at concentrations which were only 1–2% of that of the added magnesium (Perry & Grey, 1956). Binding of small amounts of this essential metal by excess of ATP could also explain the substrate inhibition obtained with the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase. The fact that this substrate inhibition is relieved by traces of calcium which are in themselves insufficient to activate the enzyme (Perry & Grey, 1956) suggests that calcium is the cation required in trace amounts for the Mg^{2+}-ion-activated adenosine triphosphatase. Recently Weber (1959) has independently expressed such a view and provided some experimental evidence in support of it. Further evidence that there are special aspects of the Mg^{2+}-ion-activated hydrolysis of ATP by myofibrils which are of fundamental significance in the mechanism of the relaxing-factor action are the results with inosine triphosphate and uridine triphosphate. Inhibition of the hydrolysis of these nucleotides by excess of substrate is very much less marked than with ATP (Perry & Grey, 1956). Significantly in the present study it has been shown that the myofibrillar adenosine triphosphatase was inhibited by the factor preparations, whereas the inosine triphosphatase and uridine triphosphatase were not, under the conditions studied. Hasselbach (1956) showed that these two nucleotides did not induce relaxation in glycerated fibres.

Taking all these facts into consideration a possible mechanism for the inhibitory action of factor preparations towards adenosine triphosphatase is as follows. It is postulated that the factor has a strong affinity for the trace of metal (possibly calcium) which is essential for the Mg^{2+}-ion-activated adenosine triphosphatase of the myofibril. In resting muscle this is bound strongly and the myofibrillar adenosine triphosphatase is at a low activity. It appears that the requirement for this metal is a feature of substrates with the 6-amino group, for inhibition of inosine triphosphatase and uridine triphosphatase under otherwise identical conditions does not occur. On stimulation there is a momentary liberation of calcium which either provides directly the trace of metal required for the Mg^{2+}-ion-activated hydrolysis of

ATP or by saturating the binding centres of the factor displaces from it the trace of metal essential for that purpose. On return to the resting state all of the calcium or activating metal is once more rendered unavailable to the myofibrillar system.

SUMMARY

1. The bulk of the myofibrillar adenosine-triphosphatase-inhibitory activity was found to be associated with the heavier fractions sedimented from rabbit-muscle extracts. In such extracts highest specific adenosine-triphosphatase-inhibitory activity was associated with the granule fraction of highest oxidative activity.

2. Granules isolated from pigeon-breast muscle had a considerably lower specific adenosine-triphosphatase-inhibitory activity than those from rabbit muscle and the more active fractions were sedimented less readily.

3. Oxalate, pyrophosphate and citrate potentiated the adenosine-triphosphatase-inhibitory activity of granules; succinate, malonate, hypophosphite and sulphite had no such action.

4. The loss of inhibitory activity which occurred on aging at 0° and in the presence of low concentrations of calcium chloride could be reversed by oxalate.

5. Myofibrillar inosine-triphosphatase and uridine-triphosphatase activities were not inhibited by granular preparations which strongly inhibited adenosine-triphosphatase activity under otherwise similar conditions.

6. Synthetic and natural actomyosin adenosine-triphosphatase activities were inhibited by the granular preparations.

7. A hypothesis for the mechanism of inhibition is suggested on the basis of these findings and earlier studies on the myofibrillar adenosine triphosphatase.

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