RELAXING FACTORS IN MUSCLE

J.R. Bendall

Low Temperature Research Station,
Downing St., Cambridge

Received November 14, 1960

Marsh (1951, 1952) was the first to demonstrate the presence in muscle of a labile relaxing factor, which had the properties a) of bringing about swelling of muscle fibre or fibril fragments in the presence of ATP and $MgCl_2$; b) of bringing about relaxation of loaded fibre-models and c) of inhibiting the Mg-activated ATP-ase activity of these systems (Bendall, 1953; Hasselbach & Weber, 1953). Since this early work it has been abundantly demonstrated, particularly under heading c), that all of this so-called relaxing-activity is contained in the granules which can be sedimented from fibre-free homogenates of muscle at a centrifugal force of about 23,000 g (Portzehl, 1957; Bendall, 1958b; Nagai, Makinose & Hasselbach, 1960; Baird & Perry, 1960). Further, Nagai *et al* and Baird & Perry have shown that the full effect can be obtained at the very low ratio of granular to fibrillar nitrogen of 2 to 100, which in many experiments represents an actual concentration of granular protein of 50 p.p.m.

It is to be noted that most of these workers have used exhaustively washed preparations of fibrils of $< 1\mu$ in diameter, to test the 'relaxing' activity of the granular fractions, because only in that way can the fibrillar test material be completely freed of its intrinsic granules. (Briggs & Portzehl, 1957; Bendall, 1958b).

In contradiction to this seemingly unshakeable proof of the identity of the relaxing factor with the very fine granular material of the sarcoplasm, Marsh (1960) has recently claimed that the relaxing factor is really α-glycerophosphate which is fully active only in the presence of an equimolar amount of inorganic phosphate. The optimal concentration of each of these substances was found to be 0.5 to 1.0 x 10^{-3}M, or 5 to 10 micromoles of each per 10 ml of reaction mixture containing the equivalent of 2 g of fresh muscle ($\equiv \sim$ 220 mg fibrillar protein). At this concentration, it is claimed, the ATP-ase activity of the fibre or fibril particles is reduced to about one third in the first 30 seconds of the reaction and to about one tenth later. It is to be noted that Marsh describes his system as containing medium-sized fibre pieces, not fibrils. This may indeed have an important bearing on the result.

I have repeated this work using washed suspensions of fibrils from pork and rabbit muscle, prepared in the same manner as those of Portzehl (1957), Nagai et al (1960) and Baird & Perry, (1960). The buffer used throughout consisted of 40 mM imidazole together with 108 mM KCl, at pH 7.20 (final concentrations). The control reaction mixture contained 4 mM ATP and 4 mM $MgCl_2$ in addition to buffer. Further additions were made, where noted in the table, as follows:- Solutions of sodium α-glycerophosphate (Fluka product) and potassium dihydrogen phosphate, adjusted to pH 7.2 with NaOH, were added each to a final concentration of 1 mM; $CaCl_2$ was added to a final concentration of 0.2 mM, and EDTA to a concentration of 1 mM. All reagents were of Analar grade, and all, with the exception of $CaCl_2$, were found by flame spectrometric analysis to contain $<$ 1 p.p.m. of calcium.

In the experiments at 18.5°C, the amount of fibrils added

to 36 mls of reaction mixture was equivalent to 2.4 g of fresh muscle ($\equiv \sim$ 220 mg of fibrillar protein in the case of pork muscle, and $\sim$ 270 mg in the case of rabbit). In the experiments at 35°C the amount of fibrils per 36 ml. of reaction mixture was reduced to $\frac{1}{4}$ of this value, because of the much higher rates of splitting of ATP. It will be noted that the ratio of α-glycerophosphate + phosphate to fibrils was considerably greater in these experiments than in those of Marsh (1960), although their concentration was identical. The results are given in the table in terms of the steady velocities of splitting of ATP in μmoles ATP split per second per g fibrillar protein. At 18.5°C, the velocity of splitting was constant from the 5th to the 120th second of the reaction, in every case. At 35°C, however, the velocity fell off as the reaction proceeded, because of inhibition by the products ADP and inorganic phosphate. The course of the reaction, from the 5th second onward, could however be described approximately by the equation:-

$$V_m t = a \ln \frac{a}{a - x}$$

where a = initial amount of ATP in μmoles per g fibrillar protein,

$a - x$ = amount of ATP present at time, t.

Hence, the steady velocities given in the table represent this theoretical value, V_m.

It is apparent from the table that addition of α-glycerophosphate and phosphate in the concentrations prescribed by Marsh has no inhibitory effect on the Mg-activated splitting of ATP under any of the conditions studied, whereas the well recognised inhibitor, EDTA, (Perry & Grey, 1956; Bendall, 1958a) at the same concentration of 1 mM considerably inhibits

TABLE

Steady velocities of splitting of ATP by fibril suspensions under various conditions

V_m in μ moles/sec/g fibrillar protein:-

	Control	Control + 0.2 mM $CaCl_2$	Control + 1 mM α-glyc-P + 1 mM phosphate	Control + 1 mM EDTA
Pork fibrils				
18.5°C	1.57	1.84	1.60	0.50
35°C	17.30	16.50	17.30	9.00
Rabbit fibrils				
18.5°C	2.40	4.20	2.40	0.20

the splitting by pork myofibrils, and almost completely that by rabbit myofibrils. It is probable that the control fibrils in these experiments contained some intrinsic granular relaxing factor, since their ATP-ase activity at 18.5°C was stimulated by addition of traces of Ca ions, although not so at 35°C (cf. Bendall, 1953). Because of this, one would have thought that the action of α-glycerophosphate would have been potentiated rather than suppressed. One can only conclude from these results that α-glycerophosphate, whatever may be its effect on fibre fragments, is not a relaxing substance per se, because it is unable to act under the critical test conditions with intact fibrils, where the natural relaxing factor of the sarcoplasmic granules is known to be fully effective. It is in any case most improbable, even if α-glycerophosphate were an effective inhibitor, that the granules could contain sufficient of it to account for their high activity at the low concentrations employed.

Mr. C. C. Ketteridge carried out the experimental work described in this note.

REFERENCES

Baird, G.D. & Perry, S.V. (1960) Biochem.J. 77, 262.

Bendall, J.R. (1953) J.Physiol. 121, 232.

Bendall, J.R. (1958a) Arch.Biochem.Biophys. 73, 283.

Bendall, J.R. (1958b) Nature, 181, 1188.

Briggs, F.N. & Portzehl, H. (1957) Biochim. et Biophys.Acta, 24, 483.

Hasselbach, W. & Weber, H.H. (1953) Biochim. et Biophys.Acta, 11, 160.

Marsh, B.B. (1951) Nature, 167, 1065.

Marsh, B.B. (1952) Biochim. et Biophys.Acta, 9, 247.

Marsh, B.B. (1960) Biochem.Biophys.Research Comm. 3, 233.

Nagai, T., Makinose, M. & Hasselbach, W. (1960) Biochim. et Biophys.Acta, 43, 223.

Portzehl, H. (1957) Biochim. et Biophys.Acta, 24, 474.

Perry, S.V. & Grey, T.C. (1956) Biochem.J. 64, 184.