IDENTIFICATION OF THE MUSCLE RELAXING FACTOR

B.B. Marsh

Meat Industry Research Institute of New Zealand, Box 345, Wellington, New Zealand.

Received August 8, 1960

The muscle relaxation factor discovered some years ago (Marsh, 1951, 1952) was first detected by means of a "centrifuge test" (Marsh, 1955) in which homogenized muscle fibres were observed to swell on addition of adenosine triphosphate (ATP) when the factor was present, but to shrink when the factor was absent. Swelling and shrinkage were correlated with lengthening and shortening respectively. ATP-ase activity was lower in presence of the factor than in its absence. The factor appeared to be very labile. Since then a number of relaxation factors have been detected, and two of these (Bendall, 1954; Briggs and Portzehl, 1957). have been claimed to be identical with the original factor. The extensive recent literature on relaxation has been ably summarised by Briggs, Kaldor and Gergely (1959).

With only one exception (Bendall, 1958) the later relaxation factors have been detected by length or tension changes in glycerinated muscle fibres or by changes in ATP-ase activity. Following the claim (Bendall, 1954) that myokinase in the presence of Mg ions was identical with the original factor, it was found in this laboratory that myokinase possessed no factor activity at all when the centrifuge test

was applied. It thus became clear that despite its inelegance and its ability to provide only semi-quantitative results, the centrifuge test was at least fairly specific for the original relaxation factor. For this reason the search for the factor has been continued, using this test as the sole means of detection.

Because of a scarcity of rabbits, beef has been used throughout this work; preparation of factor extract and fibres started within one hour post-mortem. 100 g. sterno-cephalicus muscle were homogenized for 45 seconds in a Waring Blendor in 250 ml. of 0.16 M KCl containing 0.002 M sodium iodoacetate. After the homogenate had been centrifuged at about 1600 g. for an hour, the supernatant extract was removed and frozen till required, and the fibres were washed six times, with alternate centrifuging, in 0.16 M KCl. Finer particles were decanted and discarded after centrifuging for 20-30 seconds; very coarse material was removed after settling from a dilute suspension. The medium-sized fibre pieces were suspended in 0.16 M KCl and stored at $2^{\circ}C$. In all other respects the method of detection of factor activity followed that reported earlier (Marsh, 1952). Schwarz crystalline disodium ATP was used.

Early in the investigation it became obvious that it was the fibre suspension rather than the factor which was unstable. Extracts apparently containing no factor activity were tested with a fresh fibre preparation and were found to respond positively to the centrifuge test. Thereafter, fresh fibre homogenates were prepared every 2-4 days. It was also found that factor preparations inactivated by heat, acid, or age could be reactivated and stabilised by passage through a cation exchange resin in either K^{+} or H^{+} form, provided Mg ions were added back. The factor was then observed to be

quite remarkably stable; appreciable activity persisted despite either one hour in N HCl at 100°, or 10 minutes in 0.2 N KOH at 100°.

Concentration of the factor was achieved in the following manner, its presence being verified at each stage after adjustment of KCl concentration to 0.16 M, of pH to 7.2-7.4, and of magnesium ion to 2 m M. The original supernatant extract was deproteinised with trichloroacetic acid, and the water-soluble, alcohol-insoluble barium compounds were precipitated (Le Page, 1957). The precipitate was vacuum-dried and taken up in water with a trace of HCl (pH of solution about 5.5). Barium was removed by passage of the solution through Amberlite (H^+) resin, and acid-washed Norit activated carbon (1 g. per l. original extract) was added. After removal of the Norit by centrifuging, the solution was neutralised with KOH and diluted to about the volume of the original extract. It was then passed through a column of De-Acidite FF anion exchange resin (60-150 mesh), and the column was eluted with 0.01 N HCl. The eluate was collected until a drop on a glass slide left no charred residue after gentle heating over a bunsen burner. The solution was concentrated by readsorption on the same column after regeneration with HCl, and was eluted this time with 0.16 N HCl. The eluate was neutralised with KOH, and 6 volumes of acetone were added. Centrifuging yielded a pale yellow syrup (1-2 g. per l. original extract) which proved highly active in the centrifuge test.

Ascending paper chromatography of this material (acid-washed Whatman No.1 paper, 16 hours at 18°) was carried out in ethyl acetate-acetic acid-water 3:3:1 (Mortimer, 1952). Phosphate detection by the method of Bandurski and Axelrod (1951) revealed three slow spots (R_f values .14-.23), two of

intermediate speed (R_f values .33 and .39) the faster of which was orthophosphate, and one fast spot (R_f .63). A similar chromatogram, heavily loaded with a streak of syrup, was developed, dried, and cut into zones corresponding to the three phosphate areas. The zones were eluted and tested for factor activity after adjustment of KCl and Mg ion concentrations and pH. Full activity was observed in the zone of intermediate speed (R_f .30-.45). No activity was detected in the other areas.

Further spots of the active eluted material were run in the same solvent and also in the methanol-ammonia-water (6:1:3) solvent of Bandurski and Axelrod (1951). In both solvents the two principal spots of the eluted material corresponded exactly in position to marker spots of orthophosphate and α-glycerophosphate (GP). This evidence, and that provided by the remarkable resistance of factor activity to acid and alkaline hydrolysis, suggested that the factor was GP, but no factor activity was detected when GP (plus Mg ions) was tested in the centrifuge. Since orthophosphate had been present in both the syrup and the active material eluted from the paper chromatogram, centrifuge tests were carried out on a solution of GP, orthophosphate and Mg ion; full activity was observed. Orthophosphate, either alone or with Mg ions, was completely inactive. For full activity about 5-10 micromoles each of GP and orthophosphate are required per tube (containing the fibres of about 2 g. muscle in 10 ml. suspension). Minimal Mg ion concentration is not yet known; it is below 20 micromoles per tube.

Determinations of fibre ATP-ase activity (Marsh, 1959) in presence and absence of GP plus orthophosphate (Mg ion present in both) have confirmed the earlier claim (Marsh, 1952) that

low ATP-ase activity accompanies factor activity. During the first 30 seconds (10^{o}), the rate of orthophosphate production was reduced to about one third, and after this time to about one tenth, of that in presence of Mg ion alone.

The regulatory function of GP suggested by the present work is interesting in view of the wide-spread distribution of this substance in tissues, its intimate relationship with glycolysis, the large difference between α-glycerophosphate dehydrogenase levels in normal and malignant tissues (Ciaccio, Keller and Boxer, 1960), and the appreciable effects caused by exercise, anoxia and starvation on the relatively very high GP content of housefly thorax (Winteringham, 1960). Further work is clearly needed to elucidate the mechanism of control by GP in muscle, and its possible role in other tissues.

The author is grateful to his colleagues, Drs. C.L. Davey and R.H. Locker, for very helpful advice and criticism; to Professor N.L. Edson of the University of Otago for a gift of sodium α-glycerophosphate, and to the Wellington Meat Export Co. for providing muscle in a pre-rigor condition.

REFERENCES

Bandurski, R.S. and Axelrod, B. (1951). J. Biol. Chem., 193, 405.

Bendall, J.R. (1954). Proc. Roy. Soc. B, 142, 409.

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

Briggs, F.N., Kaldor, G. and Gergely, J. (1959). Biochim. Biophys. Acta, 34, 211.

Briggs, F.N. and Portzehl, H. (1957). Biochim. Biophys. Acta, 24, 482.

Ciaccio, E.I., Keller, D.L. and Boxer, G.E. (1960). Biochim. Biophys, Acta, 37, 191.

Le Page, G.A. (1957). In "Manometric Techniques" by W.W. Umbreit, R.H. Burris and J.F. Stauffer, Burgess Publishing Co., Minneapolis, p.268.

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

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

Marsh, B.B. (1955). J. Sci. Instr., 32, 205.

Marsh, B.B. (1959). Biochim. Biophys. Acta, 32, 357.

Mortimer, D.C. (1952). Can. J. Chem., 30, 653.

Winteringham, F.P.W. (1960). Biochem. J., 75, 38.