DIFFERENCES IN TEMPERATURE DEPENDENCE OF MUSCLE CONTRACTILE PROPERTIES AND MYOFIBRILLAR ATPase ACTIVITY IN A COLD-TEMPERATE FISH

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SUMMARY
1. Single muscle fibres were isolated from the fast myotomal muscle of the teleost Myoxocephalus scorpius L. and chemically skinned with 1% Brij. Maximum Ca2+-activated force (P0) increased from 14.5 ± 1.1 N cm⁻² at 2°C to 19.1 ± 1.8 N cm⁻² at 15°C (mean ± s.e.). Maximum contraction velocity was determined by Hill's slack-test method (Vo) and by extrapolation from force-velocity (P-V) relationships (Vm). There was a linear relation between log₁₀ Vo and temperature below 15°C (Q₁₀ = 1.9, P < 0.01). The force-velocity characteristics of the fibres were determined at 2°C and 20°C. Points below 0.6 P0 on the P-V curve could be fitted by a linear form of Hill's equation. Extrapolated Vmax values were 0.55 muscle lengths s⁻¹ (L₀ s⁻¹) at 2°C and 1.54 L₀ s⁻¹ at 20°C. Curvature of the P-V relationship was independent of temperature.

2. The Mg²⁺, Ca²⁺-ATPase activity of Triton-X 100 extracted myofibrils was determined under similar ionic conditions to those used in skinned fibre experiments. (Ionic strength 0.16 mmol l⁻¹, pMgATP 2.5). A linear relationship between log₁₀ ATPase and temperature was only obtained below 15°C (P < 0.001). Above 15°C, the Q₁₀ for ATPase decreased significantly. The Q₁₀(0-15°C) for ATPase activity (3.9) was significantly higher than for unloaded contraction velocity. Supercontraction of isolated myofibrils to very short sarcomere lengths and differences in the mechanical constraints for crossbridge cycling between the preparations probably account for the lack of proportionality between these two parameters.

INTRODUCTION
The Mg²⁺-ATPase activity of fish myofibrils assayed at 0°C and physiological ionic strength under conditions approaching Vmax is highly correlated with environmental temperature (Johnston, Walesby, Davison & Goldspink, 1975; Johnston & Walesby, 1977, 1979). For example, at 0°C the ATP turnover rate of myofibrils isolated from the fast muscle of an antarctic species, Champsocephalus gunnari, (environmental
temperature —1.5 to +2°C) is 1.1 s⁻¹ compared to 0.05 s⁻¹ for similar preparations from *Oreochromis alcalicus grahami*, a fish adapted for life in a thermal hot spring (35–42°C) (Johnston et al. 1977). Increases in the catalytic efficiencies of cold-adapted actomyosins at low temperatures are associated with a decreased free energy of activation (ΔG‡) and adjustments in the relative contribution of enthalpy (ΔH‡) and entropy (ΔS‡) activation parameters (Johnston & Walesby, 1977, 1979; Johnston et al. 1977).

Unloaded contraction velocities of vertebrate muscles are generally proportional to their actin-activated myosin ATPases (Bárany, 1967). This relationship has repeatedly been used to make inferences about contractile properties from measurements of ATPase activity. However, for frog sartorius and tortoise iliofibularis muscle Barany found that at temperatures below 10°C, the parallel between contraction velocity of whole muscles and Mg\(^{2+}\)-ATPase of isolated myofibrils was only maintained if biochemical measurements were made at very low ionic strength (0·014 mmol l⁻¹). At more physiological ionic strengths (0·12–0·15 mmol l⁻¹) decreases in temperature produced proportionally greater reductions in ATPase activity than contraction velocity. For example, for tortoise iliofibularis muscle Mg\(^{2+}\)-activated myofibrillar ATPase increased 15-fold between 0 and 22°C compared to a four-fold increase for contraction velocity.

Maximum contraction velocity for mechanically skinned frog fibres at 5–7°C has been reported to decrease progressively when the KCl concentration is reduced below 140 mmol l⁻¹ (Thames, Teichholz & Podolsky, 1974). In contrast at 1°C Gulati & Podolsky (1978) found that the force-velocity relation of frog fibres was unaffected by ionic strength. Both groups of workers found a progressive increase in maximal isometric tension with decreasing ionic strength (Thames et al. 1974; Gulati & Podolsky, 1978).

Skinned fibres provide a useful preparation for mechanical studies since the three-dimensional structure of the filament lattice is preserved, yet it is possible to alter the ionic composition of the solutions bathing the myofilaments. In the present study, the effects of temperature on contraction velocity of skinned muscle cells has been compared with that for the Mg\(^{2+}\)-ATPase of isolated myofibrils measured under equivalent ionic conditions. Preparations were isolated from fast trunk muscle of the bullrout, *Myoxocephalus scorpius* L., a common bottom-living fish widely distributed in shallow water (4–60 m) along most sea coasts of northern Europe. The seasonal variation in body temperature experienced by this species is around 2–17°C.

**METHODS**

All experiments were carried out on fast fibres isolated from the myotomal muscles of the bullrout (*Myoxocephalus scorpius* L.) (25 fish; length 23·6 ± 1·2 cm; weight 238 ± 30 g; mean ± s.e. of mean). Fish were maintained in the laboratory in tanks of filtered, recirculated sea water at 10 ± 0·5°C (range).

**Preparation of myofibrils**

Around 3 g of fast (white) fibres were dissected from the epaxial myotomes from the anterior one-third of the trunk. Care was taken to avoid inclusion of slow (red) muscle...
Muscle was cut into small pieces with scissors and homogenized in 10 vol. of 0.1 mol l\(^{-1}\) KCl, 10 mmol l\(^{-1}\) imidazole-HCl, 5 mmol l\(^{-1}\) EDTA, pH 7.56 (at 0°C). Homogenization was for 3 × 15 s (at 0°C) using a Polytron homogenizer set at speed position 3-5. Between each burst of homogenization, the preparation was cooled in ice for 1 min. All subsequent operations were carried out at 0°C. The homogenate was centrifuged at 2000 \(g\) for 10 min and the pellet resuspended and washed twice in 20 vol. 0.1 mol l\(^{-1}\) KCl, 10 mmol l\(^{-1}\) imidazole-HCl, pH 7.56 (at 0°C). The washed pellet was resuspended in 5 vol. (v/v) Triton X-100, 0.1 mol l\(^{-1}\) KCl, 10 mmol l\(^{-1}\) imidazole-HCl, pH 7.56 (at 0°C) and left on ice for 2 h. Triton treatment serves to dissolve muscle membranes without affecting the kinetics of the myofibrillar ATPase (Solaro, Pang & Briggs, 1971). Following detergent treatment the preparation was centrifuged at 2000 \(g\) for 10 min and the pellet washed three times in 0.1 mol l\(^{-1}\) KCl, 10 mmol l\(^{-1}\) imidazole-HCl (pH 7.56 at 0°C) to remove the Triton. Myofibrils were subsequently prepared from the pellet by differential centrifugation as described previously (Johnston & Walesby, 1977).

**Assays for ATPase activity**

All measurements of ATPase activity were performed on the same day that the myofibrils were prepared. The basic incubation medium contained (mmol l\(^{-1}\)): 20 imidazole; 110 KCl; 3 MgCl\(_2\); 5 EGTA; 5 CaCl\(_2\); 2.5 ATP and 0.25 mg mg\(^{-1}\) myofibrils, pH 7.2 (at 20°C). Assays were initiated by addition of ATP to preincubated myofibrils and stopped by addition of an equal volume of 10% trichloroacetic acid. Incubation periods were chosen following a preliminary series of experiments to ensure the phosphate release was linear with respect to time. In some assays, either 5 mmol l\(^{-1}\) CaCl\(_2\) was omitted from the incubation medium or one of the following specific inhibitors of possible contaminating ATPases was added: 1.0 mmol l\(^{-1}\) ouabain (Na,K-ATPase), 0.25 mmol l\(^{-1}\) oligomycin (F-1 mitochondrial ATPase). Following addition of trichloroacetic acid, precipitated protein was removed by centrifugation and Pi determined in the supernatant (Rockstein & Herron, 1955). All assays were conducted in duplicate with the inclusion of appropriate controls and reagent blanks. Protein was determined by a micro-biuret method (Itzhaki & Gill, 1964).

**Fibre preparation**

Small strips of fast muscle were dissected from the deep portion of the anterior epaxial myotomes and pinned at their resting lengths on cork strips. The initial dissection was carried out in ice-cold teleost Ringer containing (mmol l\(^{-1}\)): NaCl 142.2; KCl 2.7; CaCl\(_2\) 2.0; MgCl\(_2\) 0.45; NaHCO\(_3\) 10; NaH\(_2\)PO\(_4\) 3; pH 7.4 (at 10°C). Bundles of ten to twenty fibres were gently dissected from a myotome by cutting them free from the myosepta. Fibre bundles were transferred to a 2 mm deep glass trough containing silicone oil (B.D.H. MS 550). A small drop of basic relaxing solution (see below) was injected into the oil around the fibres. All subsequent operations were carried out on an ice-cooled dissection block using a high power binocular microscope. Single fibres were dissected and transferred to the apparatus using jewellers' forceps. The thin covering of silicone oils helps prevent dehydration during transference of the fibre to the apparatus. This was usually complete in 15–30 s.
**Solutions**

The basic relaxing medium contained (mmol l\(^{-1}\)l): 110 KCl; 3 MgCl\(_2\); 5 EGTA \(N,N^1\)-tetraacetic acid; 10 phosphocreatine; 2-5 ATP; 10 imidazole; pH 7-2 at 20°C. Creatine kinase in solid form was added to a final concentration of 20 units ml\(^{-1}\) prior to each experiment. The skinning solution consisted of the basic relaxing medium containing a non-ionic detergent, 1 % Brij 58 (polyoxyethylene 20 cetyl ether).

Activating solution was made by addition of 4-0–5-0 mmol l\(^{-1}\) CaCl\(_2\) (B.D.H. 1 mol l\(^{-1}\) volumetric solution) to the standard relaxing solution. Ionic compositions were calculated using an iterative computer programme as used by White & Thorson (1972). The total ionic strength varied from 164–169 mmol l\(^{-1}\) and the added CaCl\(_2\) was adjusted at each temperature to give a free Ca\(^{2+}\) concentration of 15–20 \(\mu\)mol l\(^{-1}\). Correction was made for changes in \(K_{\text{app}}\) CaEGTA at different temperatures as described by Godt & Lindley (1982) (stability constant CaEGTA/Ca.EGTA = \(8.84 \times 10^{10}\) mol\(^{-1}\) at 22°C). Preliminary experiments established that this Ca\(^{2+}\) concentration was in excess of that required to produce maximal tension. Free concentrations (mmol l\(^{-1}\)) of Mg\(^{2+}\) and MgATP\(^{2-}\) were in the range 0-50–0-53 and 2-26–2-30, respectively. In all experiments, the pH was adjusted to 7-2 at 20°C and allowed to vary freely with temperature. Imidazole was chosen as the buffer since it has a \(\Delta pH/\Delta T\)\(^{\circ}\)C which is similar to that observed in fish muscle *in vivo* (~0-018 pH units/\(^{\circ}\)C: Heisler, Weitz & Weitz, 1976; Reeves, 1977).

**Step-tension releases**

The contraction velocity of lightly loaded fibres (<0-02 P/P\(_0\)) was determined at temperatures from 0–20°C by giving step-tension releases using an isotonic lever pivoted about a galvanometer coil. Different after-loads were applied to the fibre by increasing the galvanometer coil current. The apparatus and method of recording isotonic releases has already been described in detail (Altringham & Johnston, 1982). The chamber consisted of a series of three water-jacketed baths. Temperature was controlled by circulating refrigerated coolant through the jacket of the chamber (±0-1°C). Measurements of contraction velocity were taken over the second 50 ms following release; the first 50 ms of shortening was obscured to varying extents by oscillations of the lever. The full force-velocity relationship of fibres was determined at 2°C and 20°C. Extrapolation of P-V data fitted to Hill's (1938) equation permitted estimation of maximum contraction velocity (\(V_{\text{max}}\)).

**Slack test method for determining unloaded contraction velocity (\(V_0\))**

Unloaded contraction velocity was measured by the slack test method (Hill, 1970). The experimental chamber was constructed from Perspex and contained three water-jacketed troughs as previously. Fibres were mounted directly between two glass hooks (110 \(\mu\)m tip diameter). One hook was glued to the silicon beam of an AE801 strain gauge element (AME Horton, Norway). The element was held rigidly in a screened Perspex adaptor, mounted on a one-way micromanipulator which allowed the distance between the hooks to be adjusted. The output of the element was fed
Fish myofibrils

Bridge circuit and amplifier. Sensitivity was typically 6V mN⁻¹, noise was <5 mV, and drift <1 mV h⁻¹. The other hook was attached to a stout balsa wood beam which was glued to the centre of a loud-speaker coil from which most of the supporting core had been removed. A flag of aluminium foil attached to the beam was arranged to interrupt a series of infra-red photodiodes and emitters allowing the position of the beam to be monitored. A series of 11 pre-set length steps could be selected. Velocity feedback of the signal from the photodiodes was employed to obtain step-length changes without ringing. Tension and muscle length were monitored using a strip chart recorder (JJ Instruments, Southampton) and storage oscilloscope (Tektronix 5113), respectively. A typical step release was achieved in <1.5 ms.

Maximally activated fibres were given a release (L₁) of sufficient magnitude (usually 50–100 µm) so as to abolish the tension. The interval between the attainment of zero tension and the beginning of tension redevelopment was measured. Fibres were re-extended to their original length. At 30 s intervals, further releases of increasing magnitude were given. In each case, the time to redevelop tension (ΔT) was measured (Fig. 1). Slack or unloaded contraction velocity (V₀) was obtained from the slope of a plot of ΔL versus ΔT. Usually four cycles of release and re-extension were given for each fibre. In some experiments, fibres were transferred to relaxing solution after each release and prior to extension. No difference was found in V slack between fibres re-extended in the activated or relaxed states.

**Experimental protocol**

In both types of experiment, fibre segments 1.9–3.2 mm in length were wrapped around glass hooks and attached using Plexiglas acetone glue (Altringham &

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Fig. 1. A typical record from a 'slack test' determination of maximum contraction velocity showing a series of four-step length changes (L) each of 50 µm. The direction of shortening is downwards. The fibres go slack for subsequent releases and the time taken for tension redevelopment (ΔT) between successive length steps is measured. Tension redevelopment is upwards. Temperature was 10°C.
Fibres were skinned by a 30 min incubation in standard relax solution containing 1% Brij 58. They were subsequently transferred to relaxing solution without Brij 58 for 10–15 min.

In both types of apparatus the bottom of each trough was made of glass. Sarcomere length was measured using a He-Ne laser. Sarcomere length was initially set to 2.3 μm and (if necessary) readjusted after release. Fibre length and diameter were measured in situ.

**RESULTS**

Effects of temperature on myofibrillar ATPase activity

The Ca$^{2+}$-sensitivity of myofibril preparations was 85–95% over the temperature range 0–15°C but decreased somewhat at higher temperatures. Activity of Mg$^{2+}$, Ca$^{2+}$-myofibrillar ATPase at 20°C was not significantly reduced following the inclusion of a variety of specific inhibitors of non-myofibrillar ATPases (see Methods). A 2h incubation of myofibrils with 1% (v/v) Triton X-100 apparently reduces non-myofibrillar ATPases to a very low level (see also Solaro et al. 1971).

Mg$^{2+}$,Ca$^{2+}$-ATPase activity increased 7.7 times between 0 and 15°C. A linear relationship between log$_{10}$ ATPase activity and temperature was found between 0 and 15°C ($P < 0.001$; $Q_{10(0-15°C)} = 3.9$) (Fig. 3). Activation enthalpy ($\Delta H^\ddagger$) calculated from the corresponding Arrhenius plot was $89.5 \pm 3.4$ KJ mol$^{-1}$). Above 15°C, the $Q_{10}$ for ATPase activity decreased significantly as has been reported previously (Johnston & Walesby, 1977). Skinned fibres were not viable above 20°C (see below), therefore no attempt was made to correlate ATPase activity and contraction velocity over this higher range of temperatures.

![Fig. 2. Effects of temperature on maximum Ca$^{2+}$-activated tension development by single fast fibres. The number of fibres used is shown in brackets.](image)
**Fish myofibrils**

The effects of repeated activation-relaxation cycles on steady-state tension development at 10°C were investigated. No increase in resting tension was observed even after 12 activations. Isometric tension decreased with each activation, reaching around 0.75 of maximum tension (P₀) following five activations. Inclusion of 0.5 mmol L⁻¹ dithiothreitol in both skinnng and activating solutions did not significantly affect the decline in tension with successive activations.

In subsequent experiments, measurements of isometric tension were only taken from the first activation. P₀ increased slightly from 2 to 15°C (14.5 ± 1.1 to 19.1 ± 2.8 N cm⁻²) and then decreased somewhat (Fig. 2). Isometric tensions at 2, 5, 10 and 20°C were not statistically different (P > 0.05).

**Measurements of unloaded contraction velocity by the slack test method (V₀)**

V₀ (in lengths per second: L₀ s⁻¹) increased 1.8 times between 1°C (0.41 L₀ s⁻¹) and 10°C (0.75 L₀ s⁻¹). A plot of log₁₀ V₀ versus temperature was linear between
0–15°C (Fig. 3). Some evidence was obtained for a reduction in the Q\textsubscript{10} for contraction velocity at higher temperatures (Fig. 3). However, fibres were not viable at temperatures above 20°C, showing the development of resting tension and a rapid decline in Ca\textsuperscript{2+}-activated force development. Little useful information can therefore be obtained about the temperature dependence of contraction velocity at these higher temperatures, which are outside the normal range experienced by this species in its natural environment.

**Step-tension releases**

Measurements of contraction velocity for lightly loaded fibres (P/P\textsubscript{0} < 0.02) were found to be similar to values obtained from measurements of V\textsubscript{0}. Log\textsubscript{10} of unloaded contraction velocity showed a linear relationship with temperature in the range 1–15°C (P < 0.01) (Fig. 3). The Q\textsubscript{10}(0–15°C) for unloaded contraction velocity was 1.9, significantly less than the Q\textsubscript{10} for myofibrillar ATPase activity over this temperature range (3.9) (P < 0.01).

The force-velocity (P-V) characteristics of these fibres were determined at 2 and 20°C (Fig. 4). Points below 0.6 P\textsubscript{0} on the P-V curve could be fitted to a linear form of Hill’s equation (1938).

\[(P + a)v = b(P_0 - P),\]

where P = load, P\textsubscript{0} = maximum isometric tension, v = velocity and a and b are constants.
Table 1. Force-velocity relationship of white fibres isolated from myotomal muscle of *Myoxocephalus scorpius* (L.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2</th>
<th>20</th>
</tr>
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<tbody>
<tr>
<td>( V_{\text{max}} ) (( \text{L}_0 \text{s}^{-1} ))</td>
<td>0.55</td>
<td>1.54</td>
</tr>
<tr>
<td>( a/P_0 )</td>
<td>0.064</td>
<td>0.055</td>
</tr>
<tr>
<td>( b(\text{L}_0 \text{s}^{-1}) )</td>
<td>0.035</td>
<td>0.065</td>
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Extrapolated \( V_{\text{max}} \) values were 0.55 \( \text{L}_0 \text{s}^{-1} \) at 2°C and 1.54 \( \text{L}_0 \text{s}^{-1} \) at 20°C. Values for \( a/P_0 \) were independent of temperature (Table 1; Fig. 4).

**DISCUSSION**

Maximal Ca\(^{2+}\)-activated force (\( P_0 \)) generated by *Myoxocephalus scorpius* fast fibres is only 25% higher at 15 than at 2°C (Fig. 2). Similar results have been obtained for mechanically skinned frog muscle fibres where an increase in temperature from 4 to 22°C increased \( P_0 \) by 55% (Godt & Lindley, 1982). In contrast, much greater increases in maximal Ca\(^{2+}\)-activated force with temperature have been reported for skinned muscle cells isolated from rat fast and slow muscles (Stephenson & Williams, 1981). For example, with increasing temperature \( P_0 \) increased more than ten times between 0 and 5°C (1 N cm\(^{-2}\) to 10 N cm\(^{-2}\)), three times between 5 and 22°C, but only slightly over the range 25-35°C (Stephenson & Williams, 1981). These differences in the temperature dependence of tension development are probably related to adaptations in the actomyosins for function at different body temperatures (Johnston & Walesby, 1979). Rapid tension transient experiments with glycerinated fibres from the giant water-bug (*Lethocerus*) have provided evidence that the number of attached cross-bridges does not change with temperature in the range 5-35°C (Kuhn et al. 1979). Ford, Huxley & Simmons (1977), working with intact frog fibres, found that during a tetanus instantaneous stiffness (a function of the number of attached cross-bridges and the stiffness per cross-bridge) increased much less than tension over the range 0–8°C. Both of these observations suggest that the force generated per attached cross-bridge probably increases with increasing temperature.

Values for \( a/P_0 \) of *M. scorpius* fibres were similar at 2°C and 20°C indicating that the curvature of the P-V relationship was largely unchanged over this temperature range (Table 1). Similar results have been obtained for whole rat extensor digitorum longus muscle between 25 and 35°C (\( a/P_0 \) 0.39–0.42) (Ranatunga, 1982). In contrast, values for \( a/P_0 \) for rat soleus muscle increased progressively from 0.14 at 20°C to 0.21 at 35°C (Ranatunga, 1982). According to A. F. Huxley’s (1957) cross-bridge model, a decrease in the ratio of the constants for net cross-bridge attachment to detachment during isotonic shortening reflects an increased curvature of the P-V relationship (smaller values of \( a/P_0 \)). Values for \( a/P_0 \) for fish fast muscle are in the range 0.06–0.21 and are towards the lower range of values reported for other vertebrate fast muscles (Altringham & Johnston, 1982).

There is a variety of biochemical, structural and mechanical evidence for refractory
state models of the cross-bridge cycle during contraction at constant velocity (Tregear & Marston, 1979; Eisenberg & Green, 1980). These involve initial fast steps of myosin detachment and ADP hydrolysis to form a tight ligand complex in M.ADP.Pi which subsequently reattaches to give a transient intermediate AM.ADP.Pi which is only slowly converted to AM.ADP.Pn. There is evidence that the relative lifetimes of these states depends on rate constants which are themselves a function of the muscles’ extension. X-ray diffraction data has shown that binding of the ATP analogue, 5-adenyl imidodiphosphate (AMP-PNP) causes a slight increase in muscle length which has been interpreted as an increase in the preferred angle of the cross-bridge from 45° to nearer 90° (Marston, Tregear, Rodger & Clarke, 1978). Following the mechanically effective event, the products are released and the cycle is ready to begin again (Eisenberg & Green, 1980). A feature of such models is that factors affecting muscle length and/or internal cross-bridge load will influence the relative lifetimes of AM.ADP.Pi and AM.ADP.Pn and hence the rate constant of the limiting step in contraction. Thus ATP splitting by isolated myofibrils in free solution and isotonic contractions of skinned muscle cells differ considerably with respect to the mechanical constraints on cross-bridges.

Although the speed of shortening of intact frog fibres is independent of sarcomere lengths over the range of 1.6–3.0 μm (Edman, 1975), there is evidence that myofibrils in solution undergo supercontraction to an extent that may interfere with the normal cross-bridge cycle (Perry & Grey, 1956). In addition, analysis of isotonic velocity transient in a variety of species and fibre types has provided evidence for a progressive deactivation with increased shortening (Aidley, 1965; Floyd & Smith, 1971; Lannergren, 1978; Altringham & Johnson, 1982). Decreases in contraction velocity with increased shortening are particularly marked in slow fibres, and at submaximally activating Ca²⁺ concentration or high loads (Brenner, 1980; Altringham & Johnston, 1982).

On the above grounds, it is not surprising that there is not a tight correlation between contraction velocity and ATP splitting in the two preparations. Skinned muscle cells would appear to offer a good model for studying temperature effects on contractile proteins under defined mechanical states. In order to achieve maximum swimming speeds during prey capture or predator escape, fish recruit the bulk of their fast muscle fibre (Bone, 1966; Johnston, 1981). It is of interest that burst swimming has been reported to have a relatively low Q10 (1.3–2.0) in cold temperature marine fish (Bennett, 1978; Wardle, 1980) within the range reported for fast muscle contraction velocity in the present species.

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REFERENCES

