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5′-NUCLEOTIDASE: AN ECTO-ENZYME OF FROG SKELETAL MUSCLE

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Summary

Using ^{14}C-labeled AMP and IMP as substrates, 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was detected at the external surface of frog skeletal muscle with the active site facing toward the extracellular space. The enzyme was firmly bound to the muscle membrane. Its activity was dependent on Ca^{2+} or Mg^{2+} and was inhibited by non-radioactive ribonucleoside 5′-monophosphates, or theophylline, while adenosine 3′-monophosphate and *p*-nitrophenylphosphate had little or no effect. 5′-Nucleotidase with similar properties was also found in the isolated plasma membrane fraction of the muscle.

Introduction

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been shown to be localized or enriched in the plasma membranes of various cell types [1—8]. In cardiac and skeletal muscle this enzyme is believed to be responsible for the production of adenosine (from AMP) [9—11], the nucleoside which cases vasodilation. The regulation of the enzyme by adenine nucleotides is postulated to be a mechanism for the autoregulation of regional blood flow in myocardial and skeletal muscle [9,12,13]. Recently, evidence is accumulating that, in isolated intact cells, 5′-nucleotidase is an ecto-enzyme [6,14,15]; it appears to be localized on the plasma membrane with the active site facing the external medium not the cell cytoplasm. To our knowledge there is no information available about the orientation of the enzyme in cells of organized tissues. Elucidation of the topology of the enzyme in the plasma membrane of muscle cells is of the utmost importance in understanding the in vivo regulation of the enzyme. The experiments reported here were designed to

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demonstrate that muscle plasma membrane contains a nucleotidase which faces outwards in the intact fibre.

Previous investigations [16,17] from this laboratory have demonstrated the presence of an enzyme system, the activity of which is expressed at the external surface of intact frog skeletal muscle. This system converts ATP → ADP → AMP → IMP and the properties of the three enzymes (ATPase, adenyl kinase and AMP deaminase) have been intensively studied. In addition, cyclic AMP phosphodiesterase activity has been detected at the muscle surface [18]. The present report demonstrates that 5′-nucleotidase is also an ecto-enzyme of frog muscle.

Methods

The preparation and incubation of intact muscle bundles have been described previously [16—18]. For all experiments muscles were soaked overnight at 4°C in a Ringer's solution buffered with Tris · HCl (Ringer/Tris medium) [18] to permit complete recovery from dissection. All incubations were carried out at 20 ± 0.5°C in the basic or a modified Ringer/Tris medium with pure O_2 as the gas phase. When intracellular compounds were measured, the muscle was instantaneously frozen in isopentane (cooled in liquid N_2) and the labeled compounds extracted and separated by chromatography [24]. In these experiments D-[^{3}H]sorbitol (plus 1 mM unlabeled D-sorbitol) was used as a marker for the extracellular space. To measure nucleotidase activity, samples (10—15 μl) of the media in which the muscles were being incubated were removed at various times, spotted on Whatman 3MM chromatography paper, and chromatographed along with marker solutions using the solvent system of Aras et al. [19]. The spots containing AMP, IMP, adenosine and inosine were located using ultraviolet light, cut out and the radioactivity at each spot was counted in a liquid scintillation spectrometer (Nuclear Chicago Mark I) using a toluene-based scintillator solution. Using the initial specific activity of ^{14}C-labeled substrate in the incubation medium, the radioactivity of each compound was converted to concentration units (μM).

Isolated plasma membranes of skeletal muscle were prepared from bullfrogs (*Rana catesbiana*) using the method of Boegman et al. [20]. Three fractions were collected from the final continuous sucrose gradient (15—35%, d = 1.060—1.132). Fraction III (near the top of the gradient tube) was used for studying 5′-nucleotidase since it was devoid of 5′-AMP deaminase and contained the highest (Na^+ + K^+)-Mg^{2+}-ATPase activity (ouabain inhibition of the total ATPase, 70—80%) of all of the fractions. Assays for 5′-nucleotidase were carried out at 37°C in a total volume of 1.0 ml containing 50 mM Tris · HCl (pH 7.5) and various substances as described. The reaction was initiated by adding the membrane suspension to the assay tubes after equilibration at 37°C. After 15 min, samples (100 μl) of assay media were mixed with equal volumes of Tris · HCl buffer containing AMP (2 mM) and adenosine (2 mM) which were added to improve the chromatography and to retard the reaction by diluting the labeled substrate. The tubes were then placed in a boiling water bath for 2.5 min. After cooling, 5-μl samples were spotted on polyethyleneimine-impregnated cellulose thin-layer chromatography plates on aluminum sheets. The

nucleotides and nucleosides were separated with glass distilled water [21]. The spots were located using ultraviolet light, were cut out and counted.

Results and Discussion

Before we could conclude from media analyses that there was a sarcolemmal nucleotidase facing outwards, it was necessary to know that the substrate did not enter the muscle. In experiments to be published in detail elsewhere (Woo, Y.T., Manery, J.F., Riordan, J.R. and Dryden, E.E., unpublished), we studied the uptake by frog muscles of purified [^{14}C]AMP and [^{14}C]IMP; ^{3}H-labeled sorbitol was used to measure the extracellular spaces and, applying the double-label counting techniques of Dryden and Manery [22], the intracellular concentrations of labeled compounds were estimated. At IMP concentrations of 3.0 mM, no significant amount of ^{14}C was found inside the muscle cells. At AMP concentrations of 3.0 mM, only 0.13 ± 0.02 μmol (per g of muscle, average ±S.E. of four determinations) of ^{14}C-labeled compounds were inside the cell after 30 min of incubation. At the more sensitive μM concentrations (Figs 1B and 2B), the absence of entry of the nucleotides into the muscle cells is even more striking. Thus, the external concentrations of IMP and of AMP were not significantly altered by their entry into cells.

No ecto-nucleotidase activity could be detected with either AMP or IMP at concentrations of 3 mM, since little or no labeled adenosine or inosine was found in the incubation medium. When AMP (3 mM) was used as substrate the degradation product in the medium was almost exclusively IMP. These results are in good agreement with our earlier observations [16,17]. When micromolar concentrations of [^{14}C]AMP or IMP were used, however, the 5′-nucleotidase activity was readily detectable. The experiments are highly reproducible; only representative data are presented in the figures. Fig. 1A depicts that AMP at a concentration of 3.86 μM was rapidly degraded to IMP and adenosine showing the activity of AMP deaminase and of 5′-nucleotidase on AMP and IMP at the muscle surface. The data suggest that after an initial build-up of IMP and adenosine, some portion of both compounds was converted to inosine. This conversion results from the activity of the external 5′-nucleotidase and, in part at least, to the activity of an adenosine deaminase (to be published elsewhere). It is obvious, however, that there is an appreciable loss of the initial radioactivity from the medium over 2 h. The loss can be attributed to the entry of inosine and adenosine into muscle cells [23,24]. Further degradation to hypoxanthine did not occur in the external medium since no significant amount of hypoxanthine was found there.

In the absence of Ca^{2+} and Mg^{2+} and the presence of EDTA (1 mM), there was no nucleotidase activity; AMP was degraded to IMP (Fig. 1B). A similar graph showing inhibition of nucleotidase activity was obtained when non-radioactive IMP (1 mM) was included in the incubation medium (Ringer/Tris medium) along with [^{14}C]AMP. IMP was the only product found. These results demonstrated the presence of AMP deaminase, adenosine deaminase and nucleotidase activities at the muscle surface, also the dependence of the nucleotidase on divalent cations and the blocking of the degradation of labeled nucleotides by unlabeled IMP.

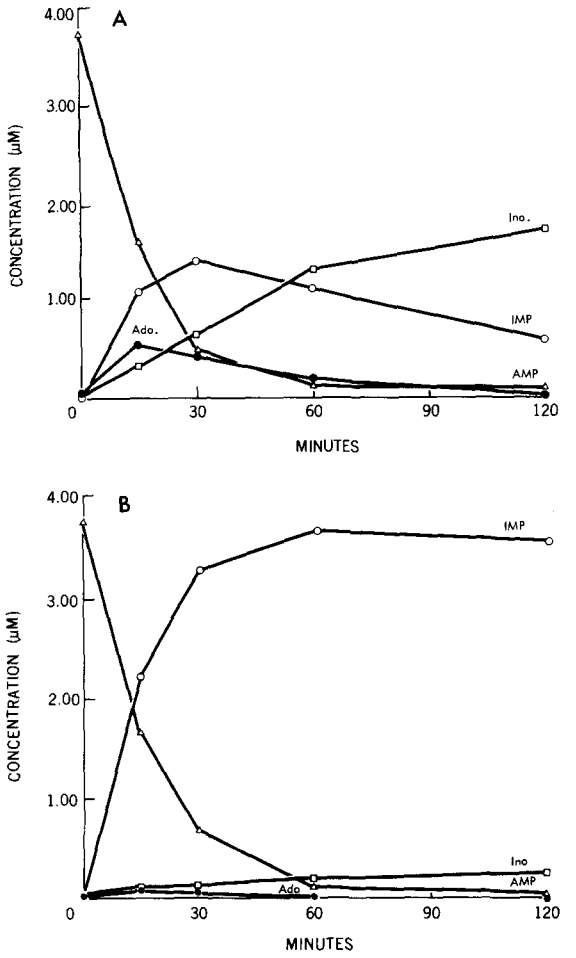
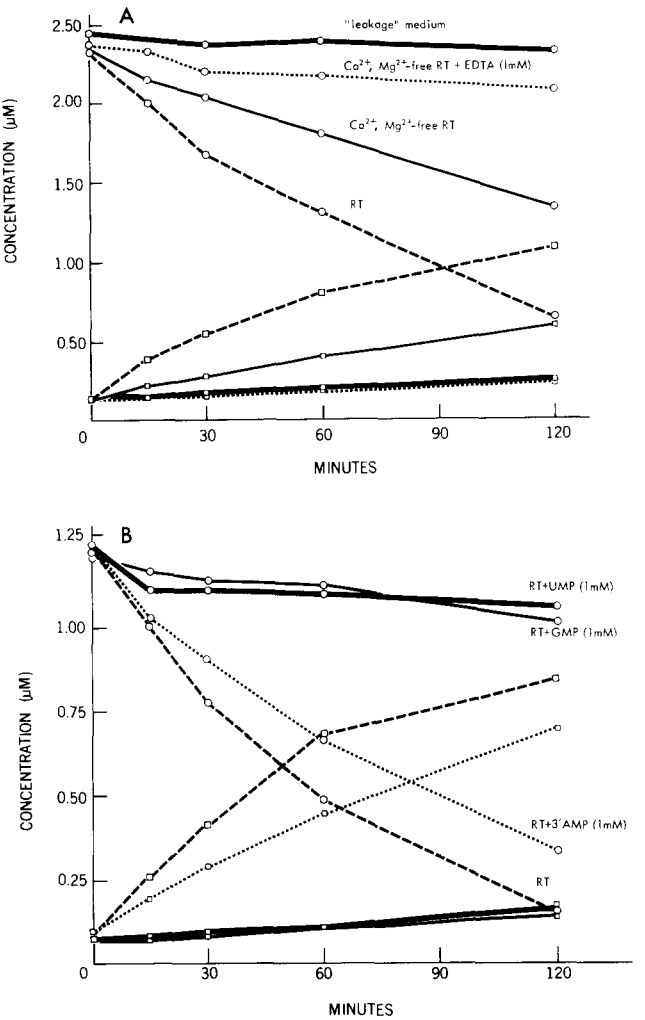


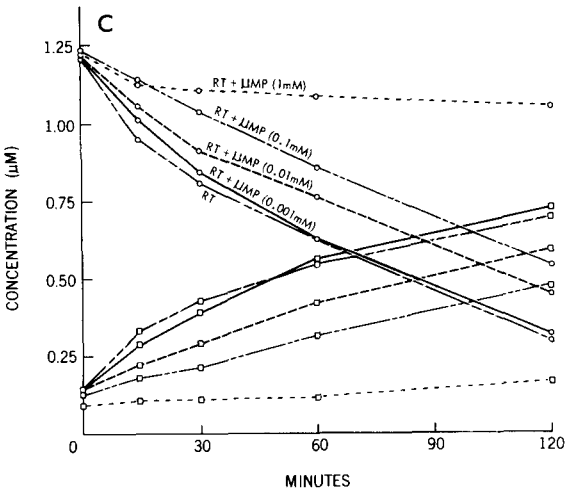
Fig. 1. Degradation of AMP in medium surrounding intact muscles. (A) Degradation of AMP (3.86 μM) in Ringer/Tris medium. After overnight soaking in Ringer/Tris medium, four muscles (total weight, 0.27 g) were incubated in 1.5 ml of Ringer/Tris medium containing [^{14}C]AMP (0.22 μCi/ml, spec. act. 56 Ci/mol). Samples (15 μl) of medium were removed at various times, and chromatographed along with marker solutions. Inosine, adenosine, IMP and AMP spots were located and the radioactivity measured; the concentrations were calculated using the initial specific activity of AMP. Ado, adenosine; Ino, inosine. (B) Degradation of AMP (3.98 μM) in Ca^{2+}-, Mg^{2+}-free Ringer/Tris medium containing EDTA (1 mM). The experimental procedure was the same as in A except that the muscles were soaked overnight in Ca^{2+}-, Mg^{2+}-free Ringer/Tris medium.

To circumvent the complications caused by AMP deaminase and adenosine deaminase when AMP was the substrate, experiments were carried out using [^{14}C]IMP. Fig. 2A shows that IMP was readily dephosphorylated in the incubation medium (Ringer/Tris medium) with the concomitant appearance of inosine. Part of the inosine produced did not accumulate in the medium because it crossed the cell membrane and was incorporated or accumulated inside the cell [23,24]. The nucleotidase activity appeared to depend on the presence of Mg^{2+} or Ca^{2+}. Its activity in Ca^{2+}-, Mg^{2+}-free Ringer/Tris medium was considerably less than that in Ringer/Tris medium. In the presence of EDTA

(1 mM), the nucleotidase activity was virtually absent. To determine whether the nucleotidase activity was firmly bound to the muscle surface or leaked out of the muscles, 'leakage' experiments were carried out (Fig. 2A). The complete absence of any hydrolysis of IMP to inosine showed that the 'leakage' medium was devoid of nucleotidase activity. The nucleotidase had remained bound to the muscle surface during the 60 min incubation of muscles without substrate.

To demonstrate the specificity of the nucleotidase, its activity was tested in the presence of various compounds. Fig. 2B shows that in the presence of UMP (1 mM) or GMP (1 mM), which are common substrates for 5′-nucleotidase, the dephosphorylation of [^{14}C]IMP was almost completely inhibited. The inhibitory effect of UMP on 5′-nucleotidase was apparently concentration-dependent (Fig. 2C) suggesting competitive inhibition. In contrast, adenosine 3′-monophosphate (1 mM), which is not a substrate for 5′-nucleotidase, only





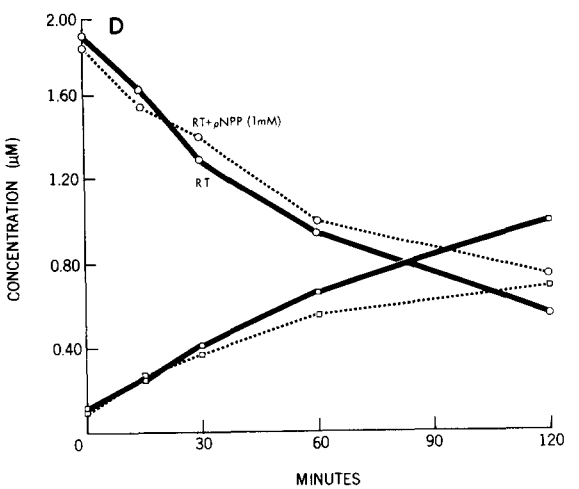


Fig. 2. Characteristics of the muscle surface nucleotidase using IMP as substrate. Open circles refer to IMP and open squares to inosine. The contents of the media are differentiated by line symbols. (A) Effect of Ca^{2+} and Mg^{2+}. Muscles were incubated in Ringer/Tris medium or modified Ringer/Tris medium containing IMP (2.32—2.47 μM). [^{14}C]IMP was 0.077—0.083 μCi/ml (spec. act. 33 Ci/mol). In the 'leakage' experiments, muscles were first incubated in Ringer/Tris medium for 60 min without substrate, then were removed and [^{14}C]IMP (0.08 μCi/ml) added to the 'leakage' medium. The incubation was allowed to continue for the time indicated. Samples of medium were removed at various times and analyzed as described in the legend to Fig. 1A. Ca^{2+}- and Mg^{2+}-free Ringer/Tris medium contained no added Ca^{2+} and Mg^{2+}. (B) Effect of ribonucleoside monophosphates. Muscles were incubated in Ringer/Tris medium containing IMP (1.21–1.23 μM) with [^{14}C]IMP, in the presence or absence of non-radioactive nucleotide as indicated in the figure. (C) Effect of UMP. Muscles were incubated in Ringer/Tris medium containing IMP (1.21–1.24 μM) with [^{14}C]IMP, in the presence or absence of UMP as indicated. (D) Effect of *p*-nitrophenylphosphate (*p*NPP). Muscles were incubated in Ringer/Tris medium containing IMP (1.78—1.83 μM) with [^{14}C]IMP, in the presence or absence of 1 mM *p*-nitrophenylphosphate. (RT, Ringer/Tris medium.)

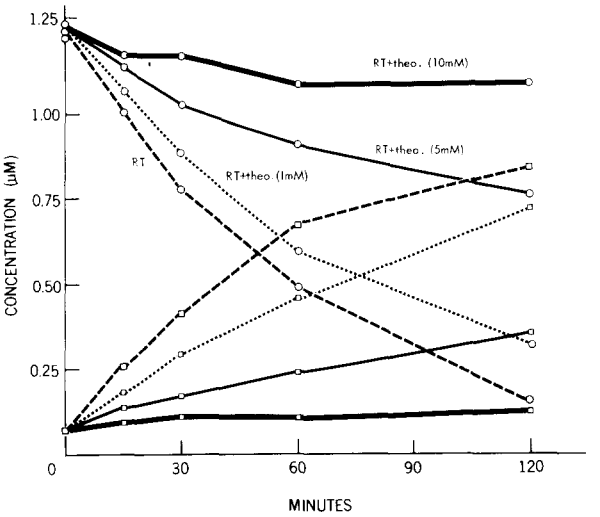


Fig. 3. Effect of theophylline. Muscles were incubated in Ringer/Tris medium containing IMP (1.23—1.24 μM) and [^{14}C]IMP, in the presence or absence of theophylline as indicated. Open circles refer to IMP and open squares to inosine. The contents of the media are indicated by line symbols. (RT, Ringer/Tris medium.)

slightly inhibited the nucleotidase activity. Furthermore, *p*-nitrophenylphosphate (1 mM), which was shown to prevent the hydrolysis of AMP by non-specific phosphates [14], had no effect on the hydrolysis of IMP (Fig. 2D). We concluded that the nucleotidase activity was that of 5′-nucleotidase and not that of non-specific phosphatases.

Theophylline, a well known inhibitor of cyclic AMP phosphodiesterase, also inhibits other cell activities, such as uptake of nucleosides and other cell nutrients [23—26]. Fig. 3 shows that theophylline also inhibited the muscle surface 5′-nucleotidase. At a concentration as low as 1 mM, some inhibition was observed, while at 10 mM, inhibition was almost complete. The mechanism of the theophylline effect remains to be elucidated.

To consolidate our finding that 5′-nucleotidase is an ecto-enzyme firmly bound to the muscle surface, plasma membrane was isolated from bullfrog skeletal muscle and its 5′-nucleotidase activity tested. Four different membrane preparations were investigated. Among all of the plasma membrane fractions tested (including the KCl and sucrose pellets), 5′-nucleotidase activity followed the distribution of the $(Na^+ + K^+)$-Mg^{2+}-ATPase. The enzyme was stable when stored at —20°C and was easily detected using micromolar concentrations of substrates. Table I summarizes the data on the Fraction III of one of the preparations. In good agreement with the data on intact muscles, the 5′-nucleotidase was selectively inhibited by 5′-ribonucleotides but was only slightly inhibited by adenosine 3′-monophosphate. The enzyme was inhibited by theophylline while *p*-nitrophenylphosphate had relatively little effect; it was dependent on divalent cations (Mg^{2+}, Ca^{2+}) (see refs 4, 12 and 13).

Since both enzymes studied (that bound to the intact muscle and that of the isolated plasma membrane) could be detected only at low substrate concentrations (μM), we suggest that a characteristic nucleotidase isoenzyme is situ-

TABLE I

EFFECTS OF VARIOUS AGENTS ON 5'-NUCLEOTIDASE ACTIVITY IN ISOLATED PLASMA MEMBRANES

A. Isolated plasma membranes (1.2 μg protein N) were incubated at 37°C for 15 min in 1.0 ml of 50 mM Tris · HCl buffer (pH 7.5) containing 5 mM Mg^{2+}, 10.4 μM AMP with [^{14}C] AMP (0.58 μCi/ml, spec. act. 56 Ci/mol) and 1 mM of non-radioactive nucleotide as indicated in the table. The control was without added non-radioactive nucleotides. After the incubation, the samples were treated and analyzed as described in the text. B. Isolated plasma membranes (2.4—2.6 μg protein N) were incubated in 1.0 ml of 50 mM Tris · HCl buffer (pH 7.5) containing 1.7—2.0 μM AMP with [^{14}C] AMP (0.10—0.12 μCi/ml, spec. act. 56 Ci/mol) and various agents as indicated in the table. The control contained 5 mM Mg^{2+}.

Agents	Relative activity	Agents	Relative activity
A. Ribonucleoside monophosphates (1 mM)		B. Mg^{2+} and other agents	
1. Control	100*	1. Control	100**
2. + 3'-AMP	76.9	2. + *p*-nitrophenylphosphate (1 mM)	80.9
3. + CMP	3.8	3. + theophylline (10 mM)	36.6
4. + UMP	5.7	4. No Mg^{2+} + EDTA (1 mM)	1.9
5. + GMP	4.5	5. No Mg^{2+}	17.7
6. + IMP	7.7	6. Mg^{2+} (1 mM)	83.8
		7. Mg^{2+} (2.5 mM)	94.8
		8. Mg^{2+} (10 mM)	113
		9. No Mg^{2+} + Ca^{2+} (5 mM)	77.5

* 1.38 nmol AMP hydrolysed/mg protein N per 15 min.
** 0.21 ± 0.04 nmol AMP hydrolysed/mg protein N per 15 min (average ± S.E. of five determinations).

ated there (see refs 27 and 28). The possibility that it is an isoenzyme with a low turn-over rate and a high affinity for the substrate should be explored. Our difficulty in detecting activity at high substrate concentrations can be reasonably attributed to either lack of sensitivity of the method at these concentrations or to substrate inhibition. Severson et al. [29] observed activity in their plasma membrane preparation from rabbit muscle only at μM substrate concentrations (calculated from their data). Much of their enzyme, however, had been lost by solubilization during the preparation. No such loss occurred in our experiments since no activity 'dissolved' off the muscle into the medium.

The finding of extracellular localization of the 5'-nucleotidase adds to the growing volume of literature on ecto-enzymes capable of catabolizing nucleotides [5,6,16—18,28]. Although the skeletal muscle ecto-5'-nucleotidase is obviously situated facing the extracellular space its exact histological location is unknown. From histochemical studies it appeared to be bound to the lining of compartments open to the extracellular space; its highest activity was in the region of small blood vessels [11]. In this position it can readily degrade circulating nucleotides (to which muscle cells are relatively impermeable) to nucleosides which then enter the intracellular pool. Muscles in negative oxygen balance (ischemic or contracting) must become permeable to ATP since it is found in the surrounding medium of isolated frog muscles [30] and in the venous effluent from human forearm muscles [31]. In addition such hypoxic muscles have been reported to degrade their nucleotides, produce adenosine and release it into the circulation [10,11]. Although ATP is a powerful vasodilator, its effect must be transient because of the active ecto-ATPase of skele-

tal muscle [16,17]. We suggest that the nucleotides released into the extracellular space are hydrolysed by the ecto-enzymes and that 5′-nucleotidase produces adenosine (with a longer effective time than ATP) in a strategic position for the dilation of the blood vessels. Thus oxygen diffusion into oxygen deprived muscles would be facilitated in a sustained fashion.

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