Effect of Exogenous Adenosine Triphosphate on the Metabolic State of the Excised Hypothermic Dog Heart

By Margita Fedelešová, Ph.D., Attila Ziegelhoffer, Ph.D., Ernst-Georg Krause, Dr.rer.nat., and Albert Wollenberger, Ph.D.

ABSTRACT

Solutions of adenosine triphosphate (ATP) were injected into the left coronary artery of isolated nonperfused dog hearts kept for 30 to 180 minutes at temperatures varying from 4 to 34°C. The amount of ATP administered varied from 0.5 to 7 μmoles/g heart. The left ventricles of the ATP-treated hearts had a higher content of adenine nucleotides and of phosphocreatine than did the left ventricles of control hearts not exposed to ATP. This effect was temperature-dependent and was maximal at 14°C. Glycogen disappearance in the hypothermic myocardial tissue was markedly slowed in a dose-dependent fashion by the administration of ATP. Injections of adenine and adenosine were without effect.

An analysis of the intra- and extracellular distribution of simultaneously administered adenosine-8-14C-triphosphate and adenosine triphosphate-α-32P shows that the injected ATP was mainly split into ADP, AMP, adenosine, and inorganic phosphate and indicates that a minor percentage of these fission products entered the myocardial cells, some of the ADP and AMP being rephosphorylated there to ATP. The results suggest that intravascular introduction of ATP into the arrested hypothermic heart might help in the survival of the organ.

ADDITIONAL KEY WORDS

ATP penetration into cells  tissue storage  hypothermia
heart conservation  myocardial glycogenolysis

Adenosine triphosphate (ATP) holds a key position as an energy donor in many cellular processes. In the myocardium the restitution of this compound from adenosine diphosphate (ADP) normally takes place mainly through oxidative phosphorylation in the mitochondria. During hypoxia the resynthesis of ATP associated with the glycolytic decomposition of the myocardial glycogen reserve and of glucose is not rapid enough, at least not in the beating, warm-blooded heart, to keep pace with continuing ATP hydrolysis. The resulting energy deficiency leads to a weakening of the heart and, eventually, to an irreversible decline and cessation of all cardiac activity.

It has repeatedly been shown that the impaired performance of the hypoxic myocardium can be improved and made normal by administration of ATP (1-3). Marshall and Andrus (2) observed that ATP exerted a stronger positive effect on the hypoxic hypodynamic frog heart than did ADP; both substances had no effect on the aerobic organ. Anders et al. (4) arrived at similar conclusions in their experiments on fatigued and anoxic skeletal muscle of the rat. According to Harary and Slater (3) rhythmically pulsating single cells derived from the rat heart can be arrested by monoiodoacetate and oligomycin, inhibitors of anaerobic and aerobic energy production, respectively. Addition of ATP overcomes this inhibition, and the cells pulsate...
again at normal frequency. Studies conducted in one of our laboratories (5) showed that the loss of glycogen and phosphocreatine from the isolated hypothermic dog heart could be mitigated by injection of ATP into the coronary arteries.

The results cited above indicate that it may be possible, under certain conditions, to restore impaired cellular function and metabolism in the myocardium by supplying exogenous ATP. They raise the question whether ATP and its breakdown products may be able to enter the myocardial cells from blood in the coronary vessels.

The present paper is a report on the effect of exogenous ATP on the metabolic state of the isolated dog heart subjected to various periods of hypoxia in combination with different degrees of hypothermia. An attempt was made to measure, using doubly labeled ATP, the distribution of the nucleotide in the myocardial tissue following its injection into a coronary artery. Some of the findings presented here were briefly reported in a preliminary communication (6).

**Methods**

**Isolation and Storage of the Heart.**—Adult mongrel dogs weighing 8 to 14 kg were used. The animals were anesthetized with thiopental, 30 mg/kg iv. Heparin was injected intravenously in a dose of 3 mg/kg body weight. The thorax was then opened in the fourth right intercostal space, and the heart was exposed, excised, and transferred to a storage vessel as described earlier (5). The entire procedure was completed in 25 to 30 seconds. The hearts were kept in Krebs-Ringer bicarbonate solution for 30 to 180 minutes at 4, 14, 24, or 34°C. In some experiments the temperature of the myocardium was measured by means of iron-constantan thermocouples (see Fig. 1).

**Administration of ATP into the Left Coronary Artery.**—After storing the heart for 15 minutes, 0.5 to 7.0 μmoles of ATP,1 dissolved in 2 to 4 ml of water, were injected per gram of heart into the left coronary artery at a rate of about 0.7 ml/min. In two series of experiments 5.5 μmoles of adenosine or adenine per gram of myocardium were injected in the same manner.

In another set of experiments a mixture of 14C- and 32P-labeled ATP was injected. The 14C label was in the C-8 atom of the purine nucleus, and the 32P label was in the α-phosphate group. The specific radioactivities were 0.05 μc of 14C and 0.05 μc of 32P per gram of myocardium. The total amount of ATP injected was 5.5 μmoles/g myocardium. Twenty minutes after the injection, Krebs-Ringer bicarbonate solution at a temperature of 14°C was infused in 10 portions of 20 ml each into the left coronary artery, and the perfusate flowing from the coronary sinus was collected in fractions through a catheter. The infusion lasted 5 minutes. Samples of the left ventricle were taken for determination of radioactivity.

**Washout of 131I-Labeled Serum Albumin.**—131I-labeled serum albumin was used to estimate the volume of liquid required to wash the coronary vessels free of administered fluid. One microcurie of 131I serum albumin (Radiochemical Centre, Amersham, England; specific activity 0.075 μc/mg) was dissolved in 4 ml 0.9% NaCl solution and injected in the same way into the coronary arteries of hearts cooled to 14°C as was the ATP solution. After 25 minutes of storage at 14°C, intermittent washing of the coronary system was begun with Krebs-Ringer bicarbonate solution at a pressure of 60 to 80 mm Hg. The perfusate was

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1C. F. Boehringer and Sons, Mannheim.
2Adenosine-8-14C-triphosphate (Na salt), specific activity 21 mc/mmmole, was a product of the Radiochemical Centre, Amersham, England; specific activity 0.075 μc/mg was dissolved in 4 ml 0.9% NaCl solution and injected in the same way into the coronary arteries of hearts cooled to 14°C as was the ATP solution. After 25 minutes of storage at 14°C, intermittent washing of the coronary system was begun with Krebs-Ringer bicarbonate solution at a pressure of 60 to 80 mm Hg. The perfusate was
collected from the coronary sinus in 8-ml fractions, and samples of the left ventricular muscle were taken.

**Tissue Extraction and Chemical Determinations.**—With the exception of the experiments with radioactive ATP, portions of the left ventricular musculature were instantly frozen with a cooling clamp (7). In the hearts treated with labeled ATP, three adjacent pieces of tissue were excised from the left ventricular wall of the washed hearts (see above) with a pair of scissors, washed twice with Krebs-Ringer bicarbonate solution, briefly blotted dry between filter papers, and frozen in liquid nitrogen.

The tissue was extracted with ice-cold 0.5N perchloric acid (8). Orthophosphate and phosphocreatine were determined by the method of Wahler and Wollenberger (9). Xanthine and hypoxanthine were determined by the method of Jørgensen (10). Other acid-soluble metabolites were determined by enzymatic methods as in a previous study (11). Glycogen was determined by the method of Isselhard et al. (12). The tissue extracts or perfusates (or both) in the isotope experiments were passed through a 1x5 cm column of Dowex 1 X 10/200 to 400 mesh, Cl− form, for separation of nucleotides (13). For the separation of orthophosphate, columns washed with 0.01M Na₄P₂O₇ were used (14). Nucleosides and nucleotides were determined by measuring the optical density of the appropriately diluted column effluents at 260 nm, using a model DU Beckman spectrophotometer. The tissue content of the metabolites is stated in μmoles/g of wet tissue.

**Measurements of Radioactivity.**—For the measurement of 14C and 32P, 1 ml each of the perfusates, or neutralized perchloric acid extracts of the washed tissue, or both were added to 10 ml of a mixture that contained, per 1 liter of dioxane, 5 g of 2,5-diphenyloxazole, 0.4 g of 2,2'-phenylen-bis-5-phenyloxazol, and 100 g of naphthalene. The measurements were performed in a Nuclear Chicago model Mark I scintillation spectrometer. The quotient 32P/14C obtained from the counts served as an approximate measure of at least the partial intactness of the administered ATP (see below). The radioactivity in the perfusate fractions following injection of 131I serum albumin was determined in the AMES (France) Volemetron flow counter. The sensitivity of the method was sufficient to detect as little as 0.1% of the administered radioactivity. The activity in the neutralized perchloric acid extracts of left ventricular muscle samples of the washed hearts was measured in a Geiger-Müller counter.

<table>
<thead>
<tr>
<th>Admixed material</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>Phosphocreatine</th>
<th>Orthophosphate</th>
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<tr>
<td>0</td>
<td>0.02 ± 0.01</td>
<td>5.8 ± 0.48</td>
<td>2.3 ± 0.19</td>
<td>2.95 ± 0.28</td>
<td>13.33 ± 1.24</td>
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EFFECT OF ATP ON THE ADENYLIC ACID SYSTEM AND ON PHOSPHOCREATINE

Earlier experiments (5) on dog hearts had showed that an injection of 0.5 μmole of ATP/g into the coronary arteries improves the metabolic state of the arrested organ cooled to 4°C. In continuation of this work, ATP was administered in varying quantities to hearts kept at 4, 14, 24, and 34°C. As can be seen from Figure 1, a temperature of 4°C was reached in the interior of the ventricular muscle approximately 15 minutes after the freshly excised heart had been placed in the cooling bath. In the experiments described below, ATP was administered after the heart had been cooled to the stated temperature. The data compiled in Table 1 indicate that after a 3-hour storage at 4°C the levels of ATP, ADP, adenosine monophosphate (AMP), and phosphocreatine were higher in ATP-treated than in control hearts. The effect of ATP increased with increasing dose of the nucleotide (Fig. 2), although there was no strictly linear dose-effect relationship. In the range between 1.5 and 7 μmoles of administered ATP per g, the sum of adenine nucleotides found in the heart rose by 0.3 μmoles/1 μmole of administered ATP. Similarly, the phosphocreatine content increased by 0.13 μmole/1 μmole of ATP added. Further increases in the amount of injected ATP produced no additional accumulation of adenine nucleotides and phosphocreatine in the heart muscle. The content of orthophosphate was reduced in proportion to the quantity of added ATP.

Figure 3 shows the influence of the time of storage on the content of ATP and ADP in the control hearts and in the hearts treated with 5.5 μmoles of ATP/g. These experiments, which were conducted at 14°C, yielded results similar to those just described, the maximum difference between the control and experimental group being obtained after a storage time of 180 minutes.

The effect of varying the temperature at a set storage time and a set quantity of exogenous ATP is shown in Table 2. It is...
EFFECT OF EXTERNAL ATP ON CARDIAC METABOLISM

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0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00

Evident that the greatest effect of the added ATP is exerted at a storage temperature of 14°C.

Since severe oxygen deficiency causes the breakdown of ATP beyond the nucleoside monophosphate stage (15), it was of interest to determine whether products such as hypoxanthine and xanthine were accumulating in the stored hearts. After storage of the isolated organs for 30 minutes at 24°C, the sum of hypoxanthine and xanthine was 1.1 ± 0.12 µmoles/g myocardium in the control hearts and 0.7 ± 0.13 µmoles/g in the hearts treated with ATP.

Injection of adenosine or adenine into the coronary artery caused no increase in the content of myocardial adenine nucleotides or of phosphocreatine.

EFFECT OF ATP ON THE CONTENT OF GLYCOGEN, LACTATE, AND PYRUVATE

Hearts supplied with ATP sustained a smaller loss of glycogen during a 3-hour storage at 4°C than did the control organs.

![Figure 4](http://circres.ahajournals.org/)

**Figure 4**

The influence of varying concentrations of exogenous ATP on the glycogen content of the heart muscle following storage for 180 minutes at 4°C. Each column represents the mean ± SD for 10 experiments. Following injection of ATP, 5.5 µmoles/g, there was a significant change in glycogen content (P < 0.01).
Influence of external ATP on the glycogen content of the myocardium as a function of the duration of storage. The temperature of the Krebs-Ringer bicarbonate solution was 4°C. Empty column: without ATP. Hatched column: with ATP, 5.5 μmoles/g.

(Fig. 4). The effect of the added ATP was dose-dependent, the myocardial glycogen level being elevated to 20% above the control value after administration of 0.5 and 1.5 μmole ATP/g heart and to 115% above the control level after 7.0 μmole ATP/g.

As was to be expected, myocardial glycogen levels decreased with the storage time (Fig. 5). However, at any given time, the ATP-treatment hearts were found to be richer in glycogen than were the corresponding controls. At a fixed dose of 5.5 μmoles/g of ATP and a fixed storage time of 30 minutes, the glycogen-sparing action of ATP depended somewhat on the storage temperature, the greatest sparing effect being exerted at 24°C (Fig. 6). The quantities of lactate and of pyruvate accumulating in the myocardium during cold storage were not significantly altered by administration of 0.5 to 7.0 μmoles of ATP/g heart.

In the experiments with 14C-and 32P-labeled ATP we checked, to begin with, to what extent the radioactivity could be washed out 20 minutes after administration of the labeled nucleotide. Washing consisted of a discontinuous perfusion of the coronaries with inactive Krebs-Ringer bicarbonate solution. A typical example of the kinetics of 32P washout is shown in Figure 7. After perfusing the heart nine times with 20-ml volumes, less than 2% of the initial 32P activity appear in the perfusate. The curve is resolved into two exponential components, indicating that there exist at least two different compartments in the cardiac tissue in which the 32P of the injected ATP is distributed. The infusion volume needed for removing 50% of the administered 32P (V1/2) from each of these two compartments can be calculated from Figure 7 to be, in a heart weighing 100 g, 21 ml for compartment I and 54 ml for compartment II. Compartment I may represent the intravascular space, and compartment II the interstitial space plus, possibly, a small fraction of the intracellular space.

The experiments with 131I-labeled serum albumin provided evidence that the volume of washing fluid required to clear the vascular...
The course of washout of $^{32}$P from the hypothermic myocardium during successive perfusion of the coronary vessels with 20-ml volumes of inactive Krebs-Ringer bicarbonate solution. Aliquots of the perfusates from the coronary sinus were used for the measurement of the radioactivity.

The bed of administered radioactivity is considerably smaller than the volume of the perfusion fluid used in the above experiments with the labeled ATP. Thus, following injection of $^{131}$I serum albumin into a heart weighing 80 g, perfusion with 40 ml of Krebs-Ringer bicarbonate solution sufficed for complete elimination of the administered radioactivity. In another heart weighing 100 g the corresponding volume was 60 ml.

In Figure 8 we present the ratios between the $^{32}$P and $^{14}$C counts in the injected solution, in the perfusate, and in the perfused tissue. The data indicate that both isotopes entered the tissue. Since the ratio of the two isotopes in the perfusate, which may be taken to reflect the activity in the extracellular space, differs considerably from the ratio in the tissue extracts, it is very unlikely that the radioactivity in the extracts represented merely the activity in the extracellular space. Moreover, the higher value of the ratio in the perfusate suggests that ATP had been partly split here to the stage of adenosine and phosphate and that the nucleoside or its decomposition products can permeate the cell membrane more quickly than does the phosphate moiety.

Table 3 lists the radioactivities in the perfusates collected before tissue sampling and in the perfused and washed myocardium after hypothermic storage. For the calculation of the counts/min/ml of intracellular water, we assumed that 25% of the cardiac tissue volume represents extracellular space and that there are no substantial differences between the $^{14}$C and $^{32}$P activities of the perfusate and interstitial space. On this assumption 5 to 10% of the $^{32}$P and $^{14}$C activities administered has penetrated into the cells, corresponding, on the average, to 0.25 μmole phosphate and 0.5 μmole of purine base per g myocardium. These values are considerably lower than the increment in nucleotides found after adminis-
### TABLE 3

<table>
<thead>
<tr>
<th>Dog</th>
<th>Volume of perfusion (ml)</th>
<th>Perfusate</th>
<th>Myocardium</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$^3$P</td>
<td>$^4$C</td>
</tr>
<tr>
<td>H3</td>
<td>200</td>
<td>3300</td>
<td>4650</td>
</tr>
<tr>
<td>H4</td>
<td>380</td>
<td>397</td>
<td>593</td>
</tr>
<tr>
<td>H6</td>
<td>200</td>
<td>755</td>
<td>1230</td>
</tr>
</tbody>
</table>

*The tenth washout fraction for experiments H3 and H6; the nineteenth fraction for experiment H4.

### TABLE 4

<table>
<thead>
<tr>
<th>Radioactivity (counts/min/E$_{260nm}$)</th>
<th>$^{32}P$</th>
<th>$^{14}C$</th>
<th>$^{32}P/^{14}C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>230</td>
<td>230</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP</td>
<td>54</td>
<td>77</td>
<td>0.7</td>
</tr>
<tr>
<td>ATP</td>
<td>87</td>
<td>50</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The specific radioactivities of $^{32}P$ and $^{14}C$ in the administered ATP were 1340 and 1060 counts/min/E$_{260nm}$ respectively, yielding a $^{32}P/^{14}C$ quotient of 0.84. The administered solution contained also some ADP which accounted for 18% of the light absorption at 260 nm. The $e^{32}P$ and the $^{14}C_8$ of this ADP yielded 402 and 1270 counts/min/E$_{260nm}$ respectively ($^{32}P/^{14}C = 0.32$).

### Discussion

In the present experiments we attempted to evaluate the changes in the metabolic state of the isolated hypothermic heart after intracoronary administration of ATP and to determine the distribution of the nucleotide in the heart using ATP labeled with $^{32}P$ and $^{14}C$. In the light of the present results, of our earlier findings (5) of positive effects of administered ATP on cardiac metabolism and function, and of the findings of other authors (2, 4, 16), to what extent does the widely accepted notion of the impermeability of the cell membrane to ATP (17) require revision?

The present results show that the positive effect of exogenous ATP on the metabolic state of the stored isolated heart varies with the amount of ATP administered, the temperature, and the time of storage. In assessing the significance of the present results, the observed effects of the ATP on the glycogen content of the stored hearts are of special interest. Under all conditions examined, the administration of ATP partly prevented or reduced the loss of this cellular energy reserve. This effect may have been due to a reduced energy requirement of the myocardial cells or it may have been the result of a specific regulative influence of the administered ATP or of one or more of its breakdown products on glycolytic metabolism, the major energy-yielding progress in hypoxic myocardium.

The known rate-limiting reactions in anaerobic carbohydrate catabolism, the phosphorylase (18, 19) and phosphofructokinase (20) steps, are markedly influenced by adenine nucleotides, AMP causing an acceleration and ATP a deceleration of metabolite
EFFECT OF EXTERNAL ATP ON CARDIAC METABOLISM

flow in this metabolic chain. Since the higher glycogen values of the hearts pretreated with ATP are indicative of a slowing of glycogenolysis and since this process takes place inside the cell, an increase in intracellular ATP in the ATP-treated hearts may at least in part have been responsible for the observed effect on the myocardial glycogen level.

The results of the "over-all" determinations of the nucleotide content in the hearts treated with ATP constitute only indirect evidence for a possible penetration of adenine nucleotides into the cardiac cells. However, the increase in phosphocreatine after intracoronary administration of ATP definitely represents a change taking place inside the cell, because cardiac phosphocreatine is an intracellular constituent. Determinations of the pH of heart perfusate collected 20 minutes after ATP administration yielded values lower than 5. It appears highly improbable that ATP-creatine phosphokinase, in case it had leaked out of the myocardial cells (21), became catalytically active in the coronary fluid to a significant extent, because the equilibrium of the Lohmann reaction,

\[ \text{ATP} + \text{creatine} \rightarrow \text{ADP} + \text{phosphocreatine} \]

is strongly displaced to the left at a pH below 7 (22). The activity of the enzyme in the perfusate of the stored hearts was not determined. Since in these experiments the duration of the hypothermic storage did not exceed 40 minutes, it appears doubtful that appreciable quantities of creatine phosphokinase were lost by the hearts. According to serum analyses (21), losses of this enzyme from normothermic infarcted myocardium do not occur in the first 2 hours following coronary occlusion.

The slowing of glycogen breakdown and the partial restitution of the phosphocreatine level may be taken as an expression of regulatory cellular processes and could perhaps be explained on the ground that the ATP introduced intra-arterially penetrates into the cell as ATP and AMP and flows into the cellular pool of these energy-rich compounds.

The observed distribution of $^{14}$C and $^{32}$P (Table 4), which at the beginning of the tissue storage were present predominantly as adenosine-$8^{-14}$C-triphosphate and adenosine triphosphate-$\alpha^{-32}$P, suggests that some ADP and AMP permeate into the cells of the hypothermic and hypoxic myocardium without dephosphorylation. But a part of the radioactive ADP fraction could have split off from ATP during passage through the membrane, in accordance with observations made by Hoffmann and Okita (23) in the normothermic guinea-pig heart. According to Antoni et al. (17), ATP added to myocardial fibers is broken down at the fiber surface to ADP and AMP. Williamson (24) found high activities of specific and nonspecific exophosphatases in the myocardium of the rat. However, our analyses of some of the perfusates of the stored hearts show that the splitting activities of these enzymes (if they were indeed responsible for the observed fission) were far from sufficient under the prevailing hypothermic conditions to split all the ATP present.

Two factors that may tend to suppress exophosphatase activity are hypothermia and the removal of calcium and magnesium ions by the chelating effect of ATP (see below). The presence of ATP in the perfusate leaving the heart 15 to 20 minutes after its administration also supports the belief that exophosphatase activity was low. Thus there must have existed after addition of ATP, both for this compound and probably also for ADP, a concentration gradient between the interstitium and the myocardial cells which favored permeation of these compounds into the cell and which lasted throughout the entire storage period. According to Bedo (25), hypothermia and anoxia can lead to changes in cell permeability. The finding of Khairallah and Mommaerts (26) that inosine monophosphate leaves myocardial tissue under hypoxic conditions points to the same conclusion.

It has been argued (17) that the polar nature of the nucleotides precludes their penetration through the cell membrane. This argument does not necessarily hold true for the present experiments, because at the prevailing pH of 5 or less these molecules are little dissociated. Cardos (27) has found
conditions—among them low pH and temperature—under which the erythrocyte membrane, which is usually impermeable to ATP, will permit passage of this molecule. Mayer and Avi-Dor (28) have reported that external ATP can enter the cells of Eschericia coli and is available there for the activation of glutamino-carbamyl phosphate synthetase, but not for the enzymes involved in active ion transport.

The well-known chelating properties of ATP may also be considered in the interpretation of our results. In all likelihood the administered ATP formed strong complexes with available free calcium and, especially, magnesium ions and removed them from the intravascular space. It is more difficult to say how the cellular Mg$^{++}$ and Ca$^{++}$ were affected. In any event, it is unlikely that a chelating effect of the administered ATP was of much importance to its observed metabolic action, for in another series of experiments (29), in which the ATP was dissolved in a volume of Krebs-Ringer bicarbonate solution containing Mg$^{++}$ and Ca$^{++}$ in excess, similar metabolic effects were observed.

An important element in the described beneficial effects of administered ATP may have been the prevention of an irreparable cellular loss of the purine moiety of the molecule which is usually seen in prolonged hypoxia (15, 30). Benson et al. (30) have shown that the low nucleotide level in dog myocardium which has been kept anoxic for more than 45 minutes at 30°C cannot be raised to normal by perfusion with oxygenated blood, while phosphocreatine may be elevated to the original level. Thus intra-arterial administration of ATP appears to be, in more than one respect, a promising method for the preservation and resuscitation of the arrested hypothermic heart.

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