Early detection of myocardial infarction in conscious dogs by analysis of plasma MM creatine kinase isoforms*

Hideaku Hashimoto, M.D., Dana R. Abendschein, Ph.D., Arnold W. Strauss, M.D., and Burton E. Sobel, M.D.

ABSTRACT To determine whether myocardial infarction could be detected early after onset by analysis of subforms of the MM isoenzyme (isoforms) of creatine kinase (MM CK) in plasma, we subjected eight conscious dogs to coronary occlusion and quantified isoforms in serial plasma samples by chromatofocusing. The fractions of MM_A (isoelectric point [pl] = 7.91), MM_B (pl = 7.74), and MM_C (pl = 7.51) in plasma samples before coronary occlusion averaged 11.4 ± 4.8% (SD), 22.3 ± 5.5%, and 66.3 ± 9.6% of total MM CK activity. The fraction of MM_A, the isoform of MM CK found in myocardium, increased significantly in plasma 1 hr after coronary occlusion, reached a maximum of 49.7 ± 8.0% in 4.1 ± 1.3 hr, and returned to baseline in 12.0 ± 2.3 hr. The fraction of plasma MM CK activity attributable to MM_C, an isoform formed slowly in plasma from MM_A via MM_B as an intermediate, decreased significantly within 1 hr, reached a minimum of 14.0 ± 4.1% in 4.8 ± 1.1 hr, and returned to baseline in 13.0 ± 2.9 hr after coronary occlusion. Total CK activity did not increase significantly until later, i.e., 5 hr after occlusion, and peaked at 1371 ± 530 IU/liter in 10.9 ± 1.9 hr. Within the first 4 hr after coronary occlusion, MM_A consistently comprised more than 20% of plasma MM CK activity despite insignificant increase of total CK. Changes in isoform proportions were consistent and independent of peak total CK activity and of cumulative CK release over a 10-fold range. Thus initial CK release indicative of infarction is detectable within 1 hr after the onset of ischemia by quantification of plasma MM CK isoforms.


EARLY DIAGNOSIS and accurate timing of the onset of initial and recurrent myocardial infarction are useful for patient selection and assessment of interventions such as coronary thrombolysis designed to salvage jeopardized myocardium. The diagnosis of infarction is frequently established by detection of elevation of the MB isoenzyme of creatine kinase (CK) (EC 2.7.3.2) in plasma. Characterization of total CK and MB CK time-activity curves has been useful for estimation of infarct size. However, plasma CK activity does not generally exceed the normal range for several hours after the onset of ischemia. Furthermore, time-activity curves vary with infarct size so that early detection and timing of infarction are difficult to assess by analysis of CK activity alone.

Recently, subforms of individual isoenzymes of CK (isoforms) distinguished by differences in isoelectric points (pl) have been found in plasma after myocardial infarction. Isoforms in plasma were soon recognized to reflect not only the form released into plasma from tissue but also conversion of one form to another in plasma in vitro or in vivo. Using a quantitative chromatofocusing assay that avoided inoppreparative denaturation, we have characterized the kinetics of conversion. The isoform in either normal or necrotic myocardium was found to be MM_A (pl = 7.91). The MM_B (pl = 7.74) and MM_C (pl = 7.51) isoforms evolved sequentially from MM_A in a consistent, time-dependent fashion in plasma in vitro and in vivo. These forms appear to correspond to those separated by isoelectric focusing (IEF) that have been designated with numerical subscripts, but differ in apparent isoelectric points: MM_A corresponds to MM_1 (pl = 6.86.

From the Cardiovascular Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis. Supported in part by NIH grant HL-17645, SCOR in Ischemic Heart Disease. Address for correspondence: Burton E. Sobel, M.D., Director, Cardiovascular Division, Washington University School of Medicine, 660 South Euclid Ave., Box 8086, St. Louis, MO 63110. Received May 29, 1984; revision accepted Nov. 8, 1984. *All editorial decisions for this article, including selection of reviewers and the final disposition, were made by a guest editor. This procedure applies to all manuscripts with authors from the Washington University School of Medicine.
by IEF\(^1\); MM\(_h\) corresponds to MM\(_s\) (pl = 6.49); and MM\(_c\) corresponds to MM\(_d\) (pl = 6.24).

Because the peak activity of tissue isoform in plasma after infarction has been found to occur several hours before the time of occurrence of peak total CK activity,\(^8\) it seemed likely that changes in the relative proportions of isoforms in plasma after myocardial infarction might occur before total CK activity became significantly elevated. This study was performed to determine whether or not analysis of changes of MM CK isoform patterns after coronary arterial occlusion in conscious dogs facilitated early detection of infarction.

Methods

Animal preparations. Fifteen male mongrel dogs weighing 16 to 23 kg were anesthetized with pentobarbital (30 mg/kg iv), and a left thoracotomy was performed aseptically via the fifth intercostal space. A snare made from 20 pound test nylon line within a polyethylene (PE240) tube was looped around the left anterior descending or left circumflex coronary artery distal to the first major branch. Its end was coiled in a subcutaneous pocket and the chest was closed. An external jugular vein was catheterized with silicone rubber (Silastic) tubing filled with 1000 U/ml heparin. The distal end of the catheter was exteriorized posteriorly.

Seven days after surgery, when plasma total CK activity had returned to normal (<100 IU/liter), 250 ml of blood was obtained through the jugular venous catheter and stored at 4°C in fresh acid citrate dextrose (ACD) (15 ml/100 ml blood of 80 mM sodium citrate, 35 mM citric acid, and 120 mM dextrose). To avoid hypovolemia, an equal volume of saline was infused intravenously.

Three days after collection of blood the dogs were supported in a sling, and electrocardiographic monitoring was initiated. The coronary snare was exteriorized through a small incision, lidocaine (30 mg iv) was given, and the snare was tightened to produce coronary occlusion verified by ST segment changes. Lidocaine was administered as needed to control arrhythmias. The dogs showed no signs of distress or agitation after coronary occlusion and were returned to their cages within 3 hr. Heparinized blood samples (15 U/ml) were drawn immediately before coronary occlusion (time 0), hourly for 1 to 16 hr, every 2 hr until 24 hr, and 30, 36, and 48 hr after coronary occlusion. To maintain constant vascular volume, blood samples were replaced with equal volumes of filtered, citrated blood acquired previously from the same animal. Blood was drawn to clear the dead space of the venous catheter before acquisition of each sample. Samples were maintained at 4°C to preclude isoform conversion in vitro\(^1\) and centrifuged immediately for 10 min at 1900 g. The plasma was supplemented with 5 mM \(\beta\)-mercaptoethanol (final concentration). Total CK activity was measured and samples were stored at -70°C before isoform analysis.

Autopsies were performed after each experiment. Recent myocardial infarction and complete coronary arterial occlusion were documented in each case.

Total CK activity and enzymatically estimated infarct size. Total CK activity was assayed spectrophotometrically at 37°C with a coupled enzyme system,\(^1\) CK S.V.R. reagents (Calbiochem-Behring, La Jolla, CA), and a GEMENI miniature centrifugal analyzer (Electro-Nucleonics, Fairfield, NJ). Results were expressed as international units per liter at 30°C. Assays were performed with and without creatine phosphate to exclude apparent CK activity attributable to adenylate kinase or other non-CK moieties.

Infarct size was estimated by calculating cumulative CK release from the total CK time-activity curve as previously described.\(^8\) The value used for the CK disappearance rate constant was \(0.0045 \text{ min}^{-1}\).

Assay of MM CK isoforms. Plasma MM CK isoforms were separated by chromatofocusing as previously described.\(^1\) The best resolution of isoforms was obtained when the sample applied to the chromatofocusing column contained more than 500 mIU of CK activity and less than 50 mg of total protein. Accordingly, plasma samples with CK activities less than 1000 mIU/ml and a total protein concentration of more than 60 mg/ml were pretreated to reduce non-CK protein and concentrate enzyme protein.

When total CK activity was greater than 1000 IU/liter, non-CK protein (primarily albumin) was removed from the plasma sample by Blue Sepharose (CL6B; Pharmacia, Piscataway, NJ) affinity chromatography.\(^1\) A plasma volume with at least 1000 mIU of activity was diluted fivefold with 50 mM sodium phosphate buffer at pH 5.8 containing 2 mM \(\beta\)-mercaptoethanol, 0.4 mM EDTA, and 5 mM magnesium chloride. After centrifugation at 1900 g for 10 min, the supernatant fraction was applied to either 12 ml (1 to 2.5 ml of plasma) or 80 ml (3 to 10 ml of plasma) Blue Sepharose columns previously equilibrated with the pH 5.8 buffer.

Selective elution of CK protein from Blue Sepharose was accomplished with 50 mM sodium phosphate buffer at pH 8.2 containing 5 mM \(\beta\)-mercaptoethanol, 0.2 mM EDTA, and 5 mM magnesium chloride. Fractions containing CK activity were pooled in filtration membrane cones (CF25; Amicon, Danvers, MA) and concentrated to 0.6 to 0.7 ml by centrifugation for 30 min at 950 g (4°C). The cones were washed three times with 100 μl of chromatofocusing equilibration buffer (pH 8.95) to recover virtually all of the CK activity and the total volume (<1 ml) was applied to the chromatofocusing column. Blue Sepharose chromatography and concentration of the sample removed 80% to 90% of the total protein in the plasma aliquot without appreciable loss of CK activity.

When total CK activity in the plasma sample was less than 100 IU/liter, a 10 to 15 ml aliquot of plasma was applied to an 80 ml Blue Sepharose column. The pH 8.2 eluent was passed through a 5 ml protein A Sepharose column (CL4B; Pharmacia, equilibrated with sodium phosphate buffer at pH 7.5 containing 2 mM \(\beta\)-mercaptoethanol) to remove additional non-CK protein, primarily globulin.

CK fractions were pooled and concentrated in filtration membrane cones to a volume of less than 1 ml for chromatofocusing. Protein A Sepharose removed 50% to 60% of the non-CK protein remaining after Blue Sepharose chromatography. Recovery of CK activity exceeded 90% and was comparable for each MM CK isoform.

After reduction of the non-CK plasma proteins and concentration of the CK activity, pH of the sample was adjusted to 8.8 and the sample was applied to the chromatofocusing column as previously described.\(^1\) Fractions of each of the three MM CK isoforms were expressed as percentages of total MM CK activity in the original plasma sample. Because specific enzyme activity is the same for each MM isoform\(^1\) and because the release of MB and BB CK from heart is minimal in dogs undergoing infarction,\(^8\) absolute enzyme activity of each MM isoform in the plasma sample was calculated by multiplying measured total CK activity by the percentage of MM CK attributable to each MM isoform.

Accuracy and precision. Hemodynamic changes associated with frequent blood sampling were minimized by concomitant infusion of equal volumes of autologous, whole blood anticoag...
ulated with ACD. Because ACD binds calcium, we excluded possible effects of the concentrations of ACD in transfused blood on the rate of isoform conversion in vitro. Purified MM \textsubscript{A} CK was obtained from canine myocardium as previously described\textsuperscript{15} and incubated at 37°C with canine plasma obtained before or 5 hr after coronary arterial occlusion. The 5 hr plasma sample was obtained after serial transfusions of ACD-treated blood (160 ml total volume). During 4 hr of incubation the proportions of isoforms in control plasma and in plasma containing ACD were indistinguishable.

To determine whether the Blue Sepharose or protein A Sepharose procedures affected proportions of isoforms in samples, plasma supplemented with known amounts of each of the three purified MM CK isoforms was applied to Blue Sepharose and protein A Sepharose columns. For both columns, the percentages of isoforms in the eluent were unchanged in comparison with values in the sample applied to the column. We have shown previously that chromatofocusing per se does not affect isoform proportions.\textsuperscript{11}

The reproducibility of the isoform analytical system combining chromatofocusing with one or both of the affinity chromatography steps was tested over several days by repeated analyses (two to five per sample) of samples with high (1260 IU/liter), moderate (623 IU/liter), or low (<100 IU/liter) CK activity.

**Results**

Among 15 dogs subjected to coronary arterial occlusion, five survived for at least 48 hr. Ten died because of ventricular fibrillation occurring 10 min to 17 hr after occlusion. Data from three dogs that died only after total CK activity had reached a maximum are included in the results. Complete coronary occlusion and recent myocardial infarction were verified at autopsy in each case.

The reproducibility of the isoform analyses was documented in repeated assays over several days of samples containing the entire range of total CK activities encountered in this study. Maximal differences encountered for the same sample analyzed sequentially were less than 5%. No isoform-specific differences were evident.

**CK time-activity curves.** Total CK activity in plasma samples before occlusion was consistently less than 100 IU/liter (62 ± 16, range 46 to 90) and did not differ significantly from CK activity in separate, normal, unoperated dogs (69 ± 22 IU/liter, n = 7). Total CK activity began to exceed 100 IU/liter (taken as the upper limit of normal based on the mean plus 2 SDs of values in samples from normal dogs) between 2 and 5 hr after coronary occlusion. Time-activity curves of total CK exhibited considerable but expected variation from dog to dog (figure 1) because of differences in the extent of infarction sustained. Thus cumulative CK release varied over a 10-fold range. Total CK activity peaked to 421 at 1873 IU/liter, 8 to 14 hr after coronary occlusion (table 1).

Time-activity curves of MM CK isoforms showed a consistent sequence of changes despite differences in infarct size. Thus the activity of MM\textsubscript{A} increased promptly and reached a maximum early. Changes in activities of MM\textsubscript{B} and MM\textsubscript{C} followed sequentially. In keeping with the association of increases in MM\textsubscript{A} activity and total CK release, it is not surprising that the configuration of MM\textsubscript{A} time-activity curves reflected the configuration of the total CK time-activity curves (figure 1). Consequently, configurations of MM\textsubscript{B} and MM\textsubscript{C} time-activity curves were reflections also of the configuration of the total CK time-activity curves.

![Figure 1](http://circ.ahajournals.org/)

**FIGURE 1.** Activities of total CK and MM\textsubscript{A} and MM\textsubscript{C} CK isoforms after coronary occlusion in eight dogs. Numbers on each line identify individual dogs corresponding to those listed in table 1. Because the contribution of MB CK was modest (<9% of total CK activity) and because BB CK was not detectable in plasma samples analyzed by electrophoresis, the activities of isoforms were expressed as a fraction of total CK activity.
TABLE 1
Total CK and MM CK isoform profiles after coronary occlusion

<table>
<thead>
<tr>
<th>Dog</th>
<th>Time to CK &gt; 100 IU/l (hr)</th>
<th>Time to maximal CK activity (IU/l)</th>
<th>Maximal CK activity (IU/l)</th>
<th>Cumulative CK released (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>12</td>
<td>1773</td>
<td>5989</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>9</td>
<td>1857</td>
<td>6362</td>
</tr>
<tr>
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</tr>
<tr>
<td>4</td>
<td>3</td>
<td>14</td>
<td>1873</td>
<td>7658</td>
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<tr>
<td>8</td>
<td>4</td>
<td>11</td>
<td>943</td>
<td>2874</td>
</tr>
</tbody>
</table>

Mean ± SD 3.5 ± 1.1 10.9 ± 1.9 1371 ± 530 4200 ± 2329

MM CK isoform curves. In contrast to the absolute activities of individual MM CK isoforms, the time-dependent changes in the relative proportions of isoforms were similar in each dog and independent of total CK activity (figure 2 and table 2). The proportions of MM\textsubscript{A}, MM\textsubscript{B}, and MM\textsubscript{C} in control samples before coronary occlusion were 11.4 ± 4.8% (SD, n = 5), 22.3 ± 5.5%, and 66.7 ± 9.6%. In each dog, MM\textsubscript{A} was not greater than 17% of total MM CK in the control sample nor was MM\textsubscript{C} less than 54%. The proportion of MM\textsubscript{A} increased quickly and significantly exceeded control as early as 1 hr after coronary occlusion while total CK activity remained within the normal range (figure 3). The fraction of MM\textsubscript{A} reached a maximum of 49.7 ± 8.0% (SD) of total MM CK 4.1 ± 1.3 hr after occlusion, i.e., 6.8 ± 2.1 hr earlier than the time of occurrence of maximal total CK activity. The proportion of MM\textsubscript{A} in plasma remained elevated significantly throughout the first 8 hr after coronary occlusion (figure 3) and returned to control (<20%) in 12.0 ± 2.3 hr (table 1). The proportion of MM\textsubscript{C} exhibited a mirror-image time course in comparison with that of MM\textsubscript{A}, with a phase lag reflecting the slow generation of MM\textsubscript{C} from MM\textsubscript{B} through the MM\textsubscript{B} intermediate. The fraction of MM\textsubscript{C} began to decrease 1 hr after coronary occlusion and reached a minimum of 14.0 ± 4.1% in 4.8 ± 1.1 hr. The proportion of MM\textsubscript{C} remained significantly decreased for 10 hr after coronary occlusion (figure 3) and returned to control (MM\textsubscript{C} > 50%) in 13.0 ± 2.9 hr (table 1). Changes in the

FIGURE 2. Proportions of MM\textsubscript{A} and MM\textsubscript{C} after coronary occlusion in eight dogs.

TABLE 2
Lack of dependence of proportions of isoforms on total CK activity

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Correlation coefficient\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent MM\textsubscript{A} and total CK activity</td>
<td>.190</td>
</tr>
<tr>
<td>Time to maximal MM\textsubscript{A} and time to maximal total CK</td>
<td>.901</td>
</tr>
<tr>
<td>Maximal MM\textsubscript{A} and maximal total CK</td>
<td>.601</td>
</tr>
<tr>
<td>Maximal MM\textsubscript{B} and cumulative CK released</td>
<td>.560</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All correlations were statistically insignificant (n = 8).
fractional contribution of MM_B to overall MM activity paralleled changes in the fraction of MM_A, with the more modest maximal deviation from control resulting in a blunted peak. MM_B reached a maximum of 44.6 ± 2.3% in 6.5 ± 2.4 hr after coronary occlusion and returned to baseline in 15.0 ± 3.5 hr.

The times to maximal MM_A or minimal MM_C proportional values were entirely independent of the time to maximal total CK activity and of cumulative CK release (table 2). Thus the time course and configuration of curves of percentages of MM_A or MM_C were independent of enzymatically estimated infarct size.

Discussion

The results of this study indicate that changes in the relative proportions of the three MM CK isoforms in plasma occur rapidly, with a significant rise of MM_A and a significant decrease of MM_C, evident as early as 1 hr after coronary occlusion. These early changes reflect ingress into the circulation of MM_A from myocardium. The modest increase of total CK activity accompanying early ingress into plasma of MM_A is not necessarily sufficient to elevate total CK above the upper limit of normal.

Chromatofocusing was used to quantify MM CK isoforms in this study because we previously found that it separated the isoforms without denaturing CK or producing artifactual sub-bands that may be encountered with procedures such as isoelectric focusing because of heating and ampholyte-protein complexing. Blue Sepharose and protein A Sepharose affinity chromatography efficiently removed non-CK protein from plasma and permitted separation of isoforms in samples with low CK activity. The reproducibility of the isoform analyses was satisfactory over the entire range of CK activities encountered.

Irreversible myocardial injury, manifest histologically, may occur as early as 20 min after the onset of ischemia. Thus initial release of MM_A CK into the circulation may begin soon after the onset of coronary occlusion. Because the net ingress of MM_A into the circulation is modest initially and because concomitant clearance of circulating CK tends to blunt any increase in total plasma CK, it is likely that the most prominent early manifestation of MM_A ingress would be an increase in the proportion of MM_A. This proportion is particularly sensitive to ingress of MM_A because the fractional contribution of MM_A to total MM is low in control plasma as a result of the prevailing conversion of circulating MM_A to MM_C. Because the formation of MM_C occurs slowly from MM_A via MM_B with a considerable time lag while MM_C continues to be cleared from the circulation, the small initial increase of MM_A produces a large change in the fractional contribution of MM_A to total MM CK activity with an associated decrease in the fractional contribution of MM_C. As MM_A release from the heart increases and begins to exceed the rate of CK clearance from blood, total plasma CK activity increases more strikingly. However, the relative contribution of MM_A to total MM in plasma decreases after peaking within 2 to 6 hr and returns to baseline within 8 to 14 hr (table 1). Thus recurrent ingress of MM_A into the circulation associated with early, recurrent infarction may be detectable before detection of a secondary rise in total CK activity.

Although total and MB CK activities exhibit time-dependent changes after myocardial infarction, they do not provide precise estimates of the time of onset of

FIGURE 3. Mean plasma total CK activity and proportions of MM_A and MM_C. Points represent means ± 1 SD (n = 5). *p < .01 compared with control (time 0) by Tukey's range test.

Vol. 71, No. 2, February 1985
infarction. Absolute values at any interval after onset are dependent on infarct size. In contrast, the temporal changes in proportions of MM CK isoforms in plasma observed in this study are consistent regardless of enzymatically estimated infarct size (table 2). Their pattern reflects the consistency of kinetics of isoform conversion over a wide range of total CK activity. Once MMₐ has been released from the myocardium into the circulation, it is converted to MM₈ and MM₉ according to kinetics that do not depend markedly on prevailing levels of CK activity.

MM CK in the dog heart consists primarily of MMₐ (95%). Myocardial MMₐ is not converted to either MM₈ or MM₉ in normal myocardium or ischemic tissue undergoing necrosis. Thus the time course of appearance of MMₙ in the circulation may provide an index of the duration of release of MMₐ from the myocardium.

Our findings indicate that proportions of MMₐ in plasma exceeding baseline (>20%) but associated with normal total CK activity (<100 IU/liter) reflect an onset of infarction within 4 hr (table 1). Conversely, baseline values of MMₐ associated with elevated total CK activity reflect an onset of infarction more than 14 hr previously (figure 3). The combination of elevated MMₐ and elevated total CK reflects an onset of infarction within 2 to 14 hr. Marked MMₐ elevations (>40%) associated with increased total CK reflect an onset of infarction of 2 to 7 hr previously. With an onset of infarction more than 8 hr before acquisition of the blood sample, MMₐ was consistently less than 40% of total MM CK in each dog (figure 2). Thus analysis of MM CK isoforms and total CK activity in a single plasma sample provides an index of the time of onset of infarction.

Control plasma samples exhibited predominantly MM₉ and small amounts of MM₈ and MM₉ compatible with previous findings with canine and human plasma. The presence of some MMₐ probably reflects release of CK from skeletal muscle and other tissues associated with physiologic turnover. The high proportions of MM₈ and MM₉ appear to reflect persistent conversion from MM₉ and differences in rates of clearance of individual isoforms from plasma in vivo. Clearance is fastest for MM₈ and slowest for MM₉.

The time-activity curves of the MM CK isoforms in this study resemble those reported in patients with acute myocardial infarction. This is not surprising since MM₉ is the dominant isoform in human as well as in canine myocardium. Isoform conversion in human plasma in vitro is approximately 50% as rapid as conversion in dog plasma, but the sequence of conversion (MM₉ to MM₉ via an MM₈ intermediate) and its irreversibility are similar in the two species (unpublished observations). Thus it is likely that estimation of the time of onset of myocardial infarction can be accomplished in patients by analysis of the proportions of MM CK isoforms and total CK activity in one or a small number of plasma samples. On the other hand, the specificity of MM₉ or of any MM CK isoform as a criterion for the diagnosis of myocardial infarction is less than that of the MB CK isoenzyme because MM CK may be released from tissues other than the heart. Nevertheless, because analysis of plasma MM CK isoforms appears to permit very early detection of irreversible myocardial injury, development of rapid methods of isoform analysis suitable for routine clinical analyses should facilitate early diagnosis of myocardial infarction.

We thank Ann Grace for preparing purified myocardial MM₉, Keith Candidotti and Bill Wolz for technical assistance, and Barbara Donnelly for secretarial help.

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_Circulation._ 1985;71:363-369
doi: 10.1161/01.CIR.71.2.363

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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