4-Heptanone is a metabolite of the plasticizer di(2-ethylhexyl) phthalate (DEHP) in haemodialysis patients

Hans Günther Wahl¹, Qunfa Hong², Sibylle Hildenbrand³, Teut Risler⁴, Dieter Luft² and Hartmut Liebich²

¹Department of Clinical Chemistry and Molecular Diagnostics, Klinikum der Philipps-Universität Marburg, Marburg, ²Department of Endocrinology, Metabolism and Clinical Chemistry, ³Institute of Occupational and Social Medicine, and ⁴Department of Nephrology, Internal Medicine Universitätsklinikum Tübingen, Tübingen, Germany

Abstract

Background. There is an ongoing discussion about the risks of di(2-ethylhexyl) phthalate (DEHP) exposure for the general population as well as for specific subgroups in various medical settings. Haemodialysis patients certainly belong to the group with the highest exposure taking into account the repeated treatments over a long period of time. Many studies have shown that DEHP metabolites are more active with regard to cellular responses than DEHP itself. Although 4-heptanone has been shown to be a DEHP metabolite in rats, this has never been tested in humans. On the other hand, 4-heptanone was reported to be associated with diabetes mellitus.

Methods. After establishing analytical methods for all postulated metabolites, we analysed (i) plasma samples from 50 patients on haemodialysis and 50 controls; (ii) urine samples from 100 diabetic patients and 100 controls; and (iii) urine samples from 10 controls receiving DEHP intravenously.

Results. 4-Heptanone concentrations in urine did not differ between controls (128.6 ± 11.4 μg/l, mean ± SEM) and diabetic patients (131.2 ± 11.6 μg/l) but were significantly elevated in plasma from haemodialysis patients (95.9 ± 9.6 μg/l) compared with controls (10.4 ± 0.5 μg/l). Exposure to DEHP led to a significant increase (P < 0.001) of the metabolite 4-heptanone and all the proposed intermediates in urine of healthy persons within 24 h.

Conclusions. These studies show that 4-heptanone is not associated with diabetes but is a major DEHP metabolite in humans. Studies concerning the toxicity of DEHP in haemodialysis patients and other highly exposed groups should therefore include 4-heptanone together with DEHP and its primary metabolites mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol.

Keywords: DEHP; diabetes mellitus; haemodialysis; 4-heptanone; in vivo metabolism

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is the major plasticizer used in polyvinyl chloride (PVC) products to achieve the desired softness and stability for specific applications. It is incorporated into electrical cables, clothing, wall coverings, flooring and many medical devices, where it may constitute up to 40% by weight. Medical devices with the highest DEHP content include dialysis tubings, infusion sets and storage bags for blood, parenteral nutrition and continuous ambulatory peritoneal dialysis (CAPD) fluids [1]. Since DEHP is not chemically bound to the PVC polymer, it can leach out easily. Already in the late 1960s the lipophilic DEHP was shown to leach from PVC tubings into the liquids passing through it, especially in the case of blood and other lipid-containing fluids [2]. At special risk are patients on haemodialysis, with exposures reported to be between 9 and 360 mg/patient for one session [3–6]. In order to reduce potential health risks arising from these chronic DEHP exposures, a great deal of attention has been paid recently to alternative materials [7]. Meanwhile, plasticizer-free CAPD bags are commercially available [8].

DEHP produces a wide spectrum of toxic effects in animals and multiple organ systems including the liver, reproductive tract, kidney, lung and heart (reviewed in [5]). Many studies [6,9–11] have shown that mono (2-ethylhexyl) phthalate (MEHP) and other DEHP metabolites may be even more active with regard
to cellular responses than DEHP itself, including peroxisome proliferation, stimulation of peroxisome proliferator-activated receptors (PPARs) \(\alpha\) and \(\gamma\), nephrotoxicity or suppression of cell proliferation. It is therefore important to identify all possible DEHP metabolites in humans for further toxicological and molecular genetic studies.

**In vivo** DEHP is rapidly hydrolysed to MEHP and 2-ethylhexanol, which is oxidized to 2-ethylhexanoic acid and further \(\omega\)- and \(\omega\)-1-oxidation products (Figure 1) [12]. Metabolites due to \(\omega\)- and \(\omega\)-1-oxidation of the MEHP side chain (Figure 1) were first reported in rats [9] and later also in man [13]. Albro [12] found 2- and 4-heptanone as \(\beta\)-oxidation products of DEHP-

Fig. 1. \(\beta\)-Oxidation pathway of DEHP metabolites. DEHP is hydrolysed to MEHP and 2-ethylhexanol, which is oxidized to 2-ethylhexanoic acid. Metabolites due to \(\omega\)- and \(\omega\)-1-oxidation of the MEHP side chain and 2-ethylhexanoic acid are indicated. \(\beta\)-Oxidation products of 2-ethylhexanoic acid are 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone. \(\beta\)-Oxidation of the ethyl side chain with the metabolite 2-heptanone is marked.
derived 2-ethylhexanoic acid in urine of rats with the then still postulated intermediates 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid (Figure 1). On the other hand, elevated levels of 4-heptanone in urine and serum from diabetic patients were reported and correlated to diabetic conditions [14], although other investigators did not see this elevation [15]. Liebich and Wall [16] also reported elevated levels of 4-heptanone in serum from haemodialysis patients.

In a pilot study [17,18], we found elevated levels of 2-ethylhexanol, 4- and 2-heptanone in patients on haemodialysis (breath and plasma) and in patients from intensive care units (breath, plasma and urine). After establishing new and more sensitive gas chromatography–mass spectrometry (GC-MS) methods for the analyses of the proposed DEHP metabolites, we therefore designed the here reported studies to clarify the role of 4-heptanone in haemodialysis patients.

**Subjects and methods**

The studies were conducted in accordance with the Declaration of Helsinki. Each subject was informed about the purpose and the scope of the particular part of the study. In the case of the ‘DEHP metabolism *in vivo*’ study where 1000 ml of isotonic saline solution were administered intravenously (i.v.), written informed consent was obtained from each subject. In the case of the ‘Haemodialysis’ and ‘Diabetes’ studies where analyses were performed from each subject. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study. In the case of the ‘DEHP metabolism *in vivo*’ study.

**Diabetes mellitus study**

This study was done to repeat the old studies [14,15] with a new and more sensitive GC-MS method (see below). Also, this time only diabetic patients from the out-patient clinic were enrolled in the study. First morning urine samples from 100 diabetic patients and 100 healthy controls were collected and analysed for 4-heptanone. Diabetic patients had a diabetes history of at least 2 years. Both type 1 (n = 15) and type 2 (n = 85) diabetic patients were included in this study. Urine aliquots were taken from samples at the outpatient clinic. The patients did not receive any dialysis treatment or any i.v. infusions. Urine creatinine was measured to account for the different concentrations. Plasma glucose and HbA1c were measured in samples from patients and controls to ensure the controls were not diabetic. The controls were healthy volunteers without specific DEHP exposure.

**Haemodialysis study**

This study was done to repeat the old pilot study [16] with more patients and with a new and more sensitive GC-MS method (see below). Plasma samples (EDTA) from 50 haemodialysis patients after a 2 day interdialytic interval (before and after a 4h dialysis session, Gambro Polyflux 14L and 17S, Fresenius Polysulfone F6 and F60) and from 50 healthy controls were analysed for 4-heptanone. Haemodialysis patients were from the local dialysis centre and from the Department of Internal Medicine III of the University of Tübingen with a dialysis history of ≥1 year. Both diabetic and non-diabetic patients were included in this study. Plasma aliquots were taken from the monthly taken blood draw. Plasma glucose and HbA1c were measured in samples from patients and controls to ensure the controls were not diabetic. The controls were healthy volunteers without specific DEHP exposure.

**DEHP metabolism *in vivo***

Ten healthy adults with no specific DEHP exposure were included in this part of the study. It was designed to test the hypothesis that 4-heptanone is a metabolite of DEHP in humans. In this case, an infusion of a defined amount of DEHP would increase the amount of 4-heptanone excreted in urine. It has been shown before that soft PVC medical infusion sets contain DEHP [1]. By infusing 1000 ml of isotonic saline from a PVC infusion bag with four in-line-connected regular infusion sets (diameter 3 × 4.1 mm, 4 × 1.4 m length, Oriplast GmbH, Germany), leached DEHP and components (e.g. MEHP) were thereby administered i.v. The total amount of DEHP was estimated by GC-MS analysis of infused aliquots from the beginning and the end of the infusions, as the concentration of leached DEHP decreased during the infusion.

Urine was collected for 24 h before and after the infusion. The subjects were told not to change their daily routine during the study period in order to minimize environmental confounding. Urine aliquots were analysed for the content of 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone.

**Quantification of 4-heptanone**

Analysis of 4-heptanone in urine and plasma was done by headspace GC-MS [GC-MS HP 5890 series II/HP 5971 A, Hewlett Packard, Avondale, USA; cold injection system (CIS)-3 and multipurpose sampler, Gerstel GmbH, Mülheim, Germany] as previously reported [19]. Briefly, headspace sampling was performed with a gas-tight syringe and combined with a temperature-controlled CIS. GC vials filled with 1 ml of acidified urine were used as headspace sampling vials. For the analysis of plasma, 1 ml of acidified and sodium chloride-saturated EDTA plasma was used. GC-MS analysis was done in the selected ion monitoring (SIM) mode.

**Quantification of 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine**

Analysis of the three proposed DEHP metabolites in urine was done by GC-MS (GC-MS HP 5890 series II/HP 5971 A, Hewlett Packard) as previously reported [20]. Briefly, after oxidation with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride and sample clean-up with Chromosorb P-filled glass tubes, all three organic acids were converted to their *tert*-butyldimethylsilyl derivatives. Quantification was done with trans-cinnamic acid as internal standard and GC-MS analysis in SIM mode.

**Quantification of DEHP and metabolites in saline infusates**

At the beginning and at the end of the isotonic saline infusion, 30 ml of solution were drawn from the set. Analysis
of DEHP in the infusates was done by stir bar sorptive extraction (SBSE) and Thermal Desorption GC-MS (GC-MS, Hewlett Packard; CIS-3 and thermodesorption TDS-2, Gerstel GmbH). Briefly, a small stir bar (10–20 mm length, 1.3 mm i.d.) coated with polydimethylsiloxane (Twister, Gerstel GmbH) was placed directly in the sample and stirred for 1 h. The stir bar was removed and placed into a thermal desorption unit. Heating of the stir bar released the extracted compounds into a GC-MS system for subsequent analysis in SIM mode. Analysis of MEHP in the infusates was done as their trimethylsilyl derivatives by GC-MS [21]. Briefly, monon(n-octyl)phthalate was used as internal standard and GC-MS analysis was performed in SIM mode.

Creatinine, plasma glucose, HbA1c and all other measurements reported here were done in the Zentrallabor Medizinische Klinik IV at the University of Tübingen. Analyses in this laboratory are conducted in accordance with the German ‘Richtlinien der Bundesärztekammer’ and good laboratory practice.

Statistical analyses

Statistical analyses were done using sigma plot software. Tests used were two-sample t-test, Kolmogorov–Smirnov two-sample test and Spearman correlation matrix where applicable.

Results

Diabetes mellitus study

As 11 controls with glucose impairment (elevated blood glucose or HbA1c) had to be excluded from the study, 89 non-diabetic controls (38.5±17.3 years, mean±SD; 49 females, 40 males) and 100 diabetic patients (55.5±14.3 years, 44 females, 56 males) with HbA1c values of 8.0±1.4% and plasma glucose of 8.9±3.1 mmol/l (mean±SD) were finally included in this study. There was no significant difference (two-sample t-test) in the 4-heptanone concentrations in urine between controls (128.6±11.4 µg/l, mean±SEM) and diabetics (131.2±11.6 µg/l). The correction for creatinine resulted in 124.4±11.6 µg/g (heptanone/creatinine, mean±SEM) for the controls and 139.5±18.5 µg/g for the diabetic patients, with no significant difference between the two groups (Figure 2). There was also no significant difference when the median (Kolmogorov–Smirnov two-sample test) was used instead of the mean. There was no correlation (Spearman correlation matrix) between the 4-heptanone concentration in urine and plasma glucose, HbA1c or period of diabetes in the patient group. As expected, there was a strong correlation between plasma glucose and HbA1c ($r_s = 0.544$).

Haemodialysis study

Fifty haemodialysis patients (58.6±12.3 years, 23 females and 27 males, haemodialysis treatment 5.3±4.9 years, mean±SD) and 50 healthy controls (43.8±12.5 years, 28 females and 22 males) were included in this study. There were significant ($P<0.001$) higher 4-heptanone plasma levels (mean±SEM) in both pre- and post-dialysis samples compared with the control group: controls 10.4±0.5 µg/l, pre-dialysis 95.9±9.6 µg/l and post-dialysis 37.9±2.7 µg/l. There was a substantial 4-heptanone elimination of 55.6±2.2% (mean±SEM) for all patients by haemodialysis. No significant difference in 4-heptanone concentrations was found between diabetic ($n = 23$, HbA1c 7.3±1.4%,

![Fig. 2. 4-Heptanone concentration in urine from diabetic patients and controls. There was no significant difference (two-sample t-test) in the 4-heptanone concentrations (mean±SEM) in urine between controls (128.6±11.4 µg/l, n = 89) and diabetics (131.2±11.6 µg/l, n = 100). Correction for creatinine (µg 4-heptanone/g creatinine) as well as calculating the median (Kolmogorov–Smirnov two-sample test) did not change the results. The horizontal line within each box represents the median, the lower and upper borders the 25th and 75th, and the T bars the 10th and 90th percentiles, respectively. Outliers are depicted as single points.](image-url)
mean ± SD) and non-diabetic haemodialysis patients in both pre-dialysis (diabetic 87.4 ± 16.1 µg/l and non-diabetic 103.4 ± 12.4 µg/l, mean ± SEM) and post-dialysis (diabetic 39.7 ± 4.7 µg/l and non-diabetic 36.4 ± 3.5 µg/l) samples (Figure 3). Also, no correlation was found for 4-heptanone plasma concentration and plasma glucose or HbA1c.

**DEHP metabolism in vivo**

Ten healthy controls (five females, five males, 28.7 ± 5.8 years, mean ± SD) were enrolled in this study. As DEHP and its metabolites are ubiquitous, care was taken to balance every metabolite before and after the DEHP-loaded infusion. An estimation of 4-heptanone production was achieved on the molar basis of its precursor 2-ethylhexanol (Figure 1). GC-MS analysis of the saline infusate showed the absence of 4-heptanone and revealed the mean loading of a total of 2352 nmol 2-ethylhexanol equivalents per person resulting from DEHP, MEHP and 2-ethylhexanol in the bag and tubings. The total amount of DEHP infused per person in this study was <0.5 mg compared with up to 360 mg/patient during one haemodialysis treatment [5,6]. On a molar basis, the measured concentrations of DEHP metabolites in urine were converted to nmol/24 h. There was a significant increase (P < 0.001) in the metabolite 4-heptanone of 914 ± 187 nmol/24 h (mean ± SEM) and in the sum of both the total 2-ethylhexanol oxidation products (oxidation-equivalents: sum of 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone) of 1387 ± 113 nmol/24 h (P < 0.0001) and the 2-ethylhexanoic acid β-oxidation products (β-oxidation-equivalents: sum of 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone) of 1353 ± 103 nmol/24 h (P < 0.0001) after the DEHP-loaded infusion. Moreover, in each of the 10 individuals, there was an individual increase of the metabolite 4-heptanone between 302 and 2351 nmol/24 h (Figure 4). The intermediate metabolites showed a more complex pattern, with both decreases and increases in the different oxidation states (Figure 4).

**Discussion**

As 4-heptanone was found to be a DEHP metabolite in rats [12] and diabetic and haemodialysis patients were reported to have increased 4-heptanone levels [14,16], we set up these studies to clarify the origin of 4-heptanone. Twenty-five years after the first report, we were able to repeat the old studies [14–16] with an improved GC-MS method for the quantification of 4-heptanone [19] in urine and plasma. The investigation of 100 diabetic patients and 89 controls revealed no significant difference of 4-heptanone concentrations in urine. Moreover, there was no correlation between the 4-heptanone concentration in urine and plasma glucose or HbA1c. In the haemodialysis patients group, there was no significant difference of 4-heptanone concentrations in plasma between diabetic and non-diabetic patients and, again, no correlation was found for 4-heptanone in plasma with plasma glucose or HbA1c.

We suspect a higher DEHP exposure of the patients by medical devices in the older studies [14–16] as all diabetic patients were hospitalized at the time of investigation, whereas the control group was not a non-diabetic hospitalized group but healthy co-workers. This reasoning is confirmed by two others facts. In the old study, in addition to 4-heptanone, cyclohexanone was always found at highly increased levels without a possible explanation from the authors then [14,15]. As cyclohexanone is a solvent sealer typically found in DEHP-containing PVC tubings [1], this is further proof of exposure. Another study found, in addition to 4-heptanone, an octanol isomer [15] that could not be identified at that time, but according to the published mass spectrum it probably was 2-ethylhexanol. Again, 2-ethylhexanol as a primary metabolite of DEHP would be further proof of exposure. When the authors found elevated 4-heptanone, 2-ethylhexanol and cyclohexanone, they actually were reporting DEHP metabolites and other PVC-based components for the first time in patients without knowing it.

Although the reported DEHP exposures for haemodialysis treatment varies greatly between 9 and 360 mg/patient for one session [5,6] this group certainly belongs to the highest exposed, taking into account the repeated treatments over a long period of time. These differences might be due to both analytical problems and difficulties in calculating the amount of DEHP actually retained [6]. We analysed the plasma from 50 controls and 50 pre- and post-dialysis patients. The haemodialysis patients had significantly higher 4-heptanone values both pre- (95.9 ± 9.6 µg/l) and post-dialysis (37.9 ± 2.7 µg/l) compared with controls (10.4 ± 0.5 µg/l). This is in good agreement with the original pilot study [16] where elevated blood levels of 4-heptanone in the range from 40 to 110 µg/l were found in five patients on haemodialysis. If diabetes
mellitus were the cause of 4-heptanone, the group of diabetic patients on haemodialysis should have the highest values. However, there were no differences between diabetic and non-diabetic haemodialysis patients in both pre-dialysis (diabetic 87.4 ± 16.1 µg/l and non-diabetic 103.4 ± 12.4 µg/l, mean ± SEM) and post-dialysis (diabetic 39.7 ± 4.7 µg/l and non-diabetic 36.4 ± 3.5 µg/l) samples. There was a substantial 4-heptanone elimination of 55.6 ± 2.2% (mean ± SEM) for all patients by haemodialysis. This is in good agreement with the small molecule size of 4-heptanone (mol. wt 114 Da) and the fact that it is a final metabolite of DEHP with several steps of hydrolysis and oxidation in between (Figure 1). The duration of the haemodialysis treatment would not be long enough to give rise to measurable amounts of 4-heptanone. Instead, as one would expect, there is a decrease of the already existing 4-heptanone (elimination). In the next interdialytic interval, the newly infused DEHP will then be completely metabolized to MEHP and 2-ethylhexanol, and, after several more steps (Figure 1), will finally be metabolized to 4-heptanone, thereby causing the elevated levels. There was a moderate positive correlation ($R^2 = 0.66$) for dialytic clearance of 4-heptanone.

**Fig. 4.** DEHP metabolism in vivo. DEHP metabolites (nmol/24 h) in urine before and after an i.v. DEHP load (data from 10 healthy controls). Oxidation-equivalents: sum of 2-ethylhexanol oxidation products 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone; β-oxidation-equivalents: sum of 2-ethylhexanoic acid β-oxidation products 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone.

2-Ethyl-hexanoic acid ($p = 0.188$) 2-Ethyl-3-hydroxyhexanoic acid ($p = 0.384$)

2-Ethyl-3-oxo-hexanoic acid ($p = 0.083$) 4 - Heptanone ($p < 0.0005$)

Oxidation - Equivalents ($p < 0.0001$)  β - Oxidation - Equivalents ($p < 0.0001$)
and plasma concentration of 4-heptanone. 4-Heptanone is also eliminated via breath, where we were able to detect 4-heptanone together with 2-ethylhexanol by thermodesorption GC-MS [17]. Based on the 4-heptanone calibration, we found 2-heptanone concentrations close to or below the detection limit of 2 μg/l in plasma of the controls compared with 11.9 ± 1.0 μg/l pre- and 7.5 ± 0.5 μg/l post-dialysis. The isomer 2-heptanone would arise from β-oxidation of the ethyl side chain of 2-ethylhexanol (Figure 1) and is further evidence for the proposed DEHP metabolism.

The now postulated metabolic pathway for 4-heptanone in humans (Figure 1) is the same as that published by Albro in 1975 for rats [12]. Analytical methods for all metabolites in question were first established and we now could show 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone to be regular constituents of human urine [19,20]. Recently, 4-heptanone and 2-ethyl-1,6-hexanediolic acid were found in urine from babies exposed to DEHP [22] and the authors postulated them to be DEHP metabolites. Interestingly, a baby with a deficiency of 2-methylbranched chain acyl-CoA dehydrogenase (proposed enzyme for this oxidation) had barely detectable 4-heptanone levels in urine despite equivalent DEHP exposure [22].

In order to test the hypothesis of 4-heptanone as a major DEHP metabolite in humans, healthy controls were exposed to a DEHP load by infusing isotonic saline through regular DEHP-containing PVC tubings. There were significant increases in the final metabolite 4-heptanone and in the sum of all β-oxidation products in each of the 10 individuals. Based on the 4-heptanone mean increase of 914 nmol and mean DEHP load of 2352 nmol 2-ethylhexanol equivalents, this results in an estimated DEHP metabolism of 39% for 4-heptanone without taking into account the elimination via breath [17]. The same estimation for all three β-oxidized metabolites showed a DEHP metabolism of 58% compared with 25% β-oxidized metabolites found in rats [12] where ω- and ω-1-oxidation is predominant. The fact that only 12–14% of a dose of DEHP given orally to healthy volunteers was recovered as MEHP metabolites [23], and that high blood concentrations of phthalic acid in haemodialysis patients seems to be in agreement with this high rate of β-oxidation in humans. These species-dependent differences in DEHP metabolism could be one of the reasons for the known species differences in peroxisome proliferation and hepatocarcinogenesis [9] in addition to a species difference in PPAR expression [24]. Also, recently it was shown that DEHP requires metabolism to exert its PPAR-dependent effect [10]. While DEHP did not show any activation, 2-ethylhexanoic acid activated PPARα, and MEHP activated both PPARα and γ [10].

Haemodialysis patients are exposed to high amounts of DEHP and its metabolites in two ways. In addition to a primary high exposure due to the repeated treatments over a long period of time, they suffer from reduced and late elimination. In the ongoing discussion about the risk of DEHP exposure of the population of haemodialysis patients at high risk, the here identified β-oxidation metabolites with 4-heptanone as a final product certainly should be included. The role of these metabolites in DEHP toxicity and PPAR activation merits further investigation.

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