Semicarbazide Formation in Azodicarbonamide-Treated Flour: A Model Study

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Semicarbazide was previously found in foods that were in contact with rubber gaskets foamed at high temperatures with a blowing agent azodicarbonamide. Because azodicarbonamide is an approved flour additive in certain countries, we set out to ascertain if semicarbazide is formed during the baking process from flours containing that additive. The levels of semicarbazide in baking flour treated with azodicarbonamide and bread baked from such flours were determined by isotope dilution ($^{13}C^{15}N_2$-semicarbazide) liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS). The samples were homogenized with HCl, extracted with *n*-pentane, derivatized with 2-nitrobenzaldehyde, and the derivative was extracted with ethyl acetate. After solvent exchange to 10% acetonitrile in water containing 0.1% acetic acid, the samples were analyzed using a 2.1 mm × 150 mm C18 column eluted with 2 mM ammonium formate in water/methanol (40:60). Semicarbazide was formed during the dry heating of commercial azodicarbonamide-containing flours at temperatures of 150−200 °C reaching levels of 0.2 mg/kg. Similar levels of semicarbazide were found in the crusts of breads made from azodicarbonamide-treated flour.

KEYWORDS: Semicarbazide; azodicarbonamide; 2-nitrobenzaldehyde; flour; LC-MS/MS

INTRODUCTION

Recently, it was discovered that trace levels of semicarbazide found in foods can originate from the gaskets on the lids used to seal glass bottles and jars as these gaskets are foamed, to ensure a tight seal, with the blowing agent azodicarbonamide (*1*). That finding prompted us to investigate the possibility of the formation of semicarbazide through the use of azodicarbonamide-treated baking flour. Azodicarbonamide is approved for use in flour in Canada, at a maximum level of 45 mg/kg, and in several other countries due to its dough-improving properties (*2, 3*); however, its use in the European Union has been discontinued (*4*). Semicarbazide was shown to have weak carcinogenic and genotoxic activities in animals (*5*). Azodicarbonamide decomposes (the azo double bond is reduced to a hydrazo moiety) relatively quickly in the wet flour to biurea, an essentially nontoxic compound; however, azodicarbonamide is stable in dry flour (**Figure 1**). The flour used for the sprinkling of baking goods and baking sheets to prevent adhesion might be reasonably expected to follow the high-temperature decomposition pattern observed in the process of producing gaskets and thus might produce semicarbazide.

At the beginning of our investigation, no detailed information was available about semicarbazide originating from the use of azodicarbonamide-containing flour; however, there was a report
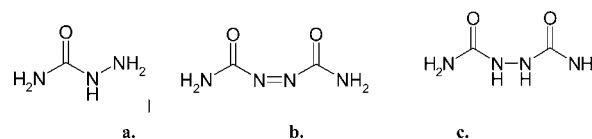

Figure 1. Chemical structures of (**a**) semicarbazide, (**b**) azodicarbonamide, and (**c**) biurea.

(*7*) indicating spurious findings of semicarbazide in foods. Semicarbazide is a metabolite of the antibiotic agent, nitrofurazone; however, it is unlikely that use of that antibiotic could explain all of these findings. When our initial experimental work was completed, the work of Pereira et al. (*8*) was published. The results were obtained by liquid chromatography tandem mass spectrometry (LC-MS/MS) with 2-nitrobenzaldehyde derivatization and the use of 3-amino-2-oxazolidone as an internal standard. These authors established that the concentration of semicarbazide in azodicarbonamide-treated flour, after hydrolysis and derivatization, was around 2−5 ng/g. After azodicarbonamide was spiked at a level of 10 μg/g to the wet azodicarbonamide-free flour and a similar workup, they found semicarbazide at a 12 ng/g level. Pereira et al. (*8*) thus postulated that biurea resulting from decomposition of azodicarbonamide undergoes a further transformation to semicarbazide. In that study, the flour did not undergo a heat treatment. In our study, the flour was heated to temperatures expected during the baking process and the bread made from azodicarbonamide-containing flour was baked at commonly used conditions. For the analysis

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of semicarbazide, we implemented a modified LC-MS/MS method (*6*) based on the derivatization of semicarbazide with 2-nitrobenzaldehyde but with the added benefit of using an isotopically labeled $^{13}C^{15}N_2$-semicarbazide.

MATERIALS AND METHODS

Chemicals. *n*-Pentane (pesticide grade) and methanol (HPLC grade) were obtained from EM Science (Gibbstown, NJ). Water was obtained from a MilliQ Gradient A10 purification system (Millipore). All other reagents were of analytical grade. Semicarbazide hydrochloride (99+%), azodicarbonamide (97%), and 2-nitrobenzaldehyde (98%) were from Aldrich (Milwaukee, WI), biurea was from TCI America (Portland, OR), and the semicarbazone of 2-nitrobenzaldehyde was from Witega (Berlin, Germany). The labeled standard of $^{13}C^{15}N_2$-semicarbazide hydrochloride (98%) was from Witega. Stock and spiking $^{13}C^{15}N_2$-semicarbazide hydrochloride solutions (200 and 5 μg/mL) solutions were prepared in water while calibration solutions were made up in methanol/water (1:1).

Foods. All food samples used in this study were purchased locally.

Equipment/Materials. The model 1100 HPLC consisted of an autosampler, binary pump, degasser, and column oven (Agilent, Palo Alto, CA). The triple quadrupole tandem mass spectrometer was a Quattro-Ultima (Micromass Inc., Manchester, United Kingdom) with a MassLynx version 3.5 data system (Micromass). Other equipment: analytical column, 2.1 mm i.d. × 150 mm, 3 μm, C18 Genesis (Jones Chromatography, Mid Glamorgan, United Kingdom); centrifuge tubes, FEP, 50 mL (Nalgene, Rochester, NY); centrifuge filter 0.22 μm, Nanosep MF, (Pall Corporation, MI).

Liquid Chromatograph-Mass Spectrometer Operating Conditions (MS/MS Mode). Mobile phase, 60% methanol in 2 mM aqueous ammonium formate (isocratic); flow rate, 0.15 mL/min; injection volume, 10−20 μL; column temperature, 28 °C; autosampler temperature, 10 °C; ionization mode, positive ion electrospray; desolvation gas temperature, 250 °C; source temperature, 120 °C; desolvation gas flow, 555 L/h; cone gas flow, 150 L/h; collision gas pressure, 2.6 × 10^{-3} mbar (argon); resolution settings, around 80% valley separation for both quadrupoles; ion energies, 0.2 and 1.0 V for quadrupoles 1 and 2; precursor ion → product ion transitions in multiple reaction monitoring (MRM), labeled semicarbazide derivative m/z 212 → 195 (collision energy 10 eV); m/z 212 → 168 (9 eV); m/z 212 → 134 (11 eV); native semicarbazide derivative m/z 209 → 192 (10 eV); m/z 209 → 166 (9 eV); m/z 209 → 134 (12 eV); the cone voltage was 40 V for all MRM transitions; dwell time for each MRM transition, 0.2 s; and interchannel delay, 0.1 s.

Typical Food Sample Extraction. The sample was ground, if necessary, in a blender. A subsample (1 g) was homogenized (Polytron) with 9 mL of 0.125 N HCl in a centrifuge tube together with 20 μL of 5 μg/mL isotopically labeled semicarbazide. The homogenate was extracted (phase separation assisted by centrifugation) with *n*-pentane (2 × 5 mL), and a solution (0.2 mL) of 2-nitrobenzaldehyde (30 mg in 4 mL of methanol) was added. The mixture was shaken on a rotary shaker for 16 h at 37 °C, the pH was adjusted to between 6.5 and 7 with 0.3 M trisodium phosphate and extracted (assisted by centrifugation) with ethyl acetate (2 × 4 mL). The ethyl acetate was removed under a stream of nitrogen at 40 °C, and the residues were dissolved (assisted by ultrasonication) in 10% acetonitrile in water containing 0.1% acetic acid (0.5 mL), filtered through a 0.22 μm spin filter, and analyzed by LC-MS/MS.

Laboratory Baking Experiments. *Heating of Flour.* Flour (5 g) with and without azodicarbonamide additive was spread evenly in a 100 mm diameter Petri dish and heated for 30 min in a lab convection oven at 150, 175, 200, or 225 °C. Alternatively, flour (5 g) was spread evenly in a 100 mm diameter Petri dish and heated for 3 min in a toaster oven set on the high broiling setting.

Baking of Bread. Bread was prepared using a typical recipe (flour, yeast, butter, sugar, milk, salt, and water); some loaves were slightly sprinkled on top with flour and baked in a loaf pan in an oven for 30 min at 200 °C.

Preparation of Spiked Flour. Azodicarbonamide-free flour D (2 g) was spiked with 10 mg of azodicarbonamide concentrate (1.1% in the same flour) and thoroughly mixed using an agate mortar to give a mixture containing azodicarbonamide at the level of 56 μg/g.

Preparation of 2-Nitrobenzaldehyde $^{13}C^{15}N_2$-Semicarbazone. $^{13}C^{15}N_2$-Semicarbazide hydrochloride (30 mg) and sodium acetate (50 mg) were dissolved in 0.75 mL of water, a solution of 2-nitrobenzaldehyde (50 mg) in 0.75 mL of methanol was added, and the mixture was left at room temperature overnight. The precipitate was washed three times with 1 mL of water and two times with 1 mL of methanol, crystallized from hot DMSO/water (v/v approximately 3:1), and dried in vacuo over phosphorus pentoxide at 100 °C. Yield: 33 mg, 59%. Estimated purity by ^{1}H NMR ≥ 96%.

Model Reaction Conditions: Influence of Moisture. The azodicarbonamide-containing flour (5 g) was mixed with 1 mL of water, spread evenly in a 100 mm diameter Petri dish and, after 1 min, was heated for 30 min in a lab convection oven at 200 °C hard return. Precision data for the method were obtained by processing six replicates of the bread sample.

RESULTS AND DISCUSSION

The LC-MS/MS method for analysis of semicarbazide developed in this study was patterned after earlier work (*9−11*) in which the determination of total (bound and free) semicarbazide (and/or other amino metabolites of nitrofuran antibiotics) was accomplished after acid hydrolysis and derivatization with 2-nitrobenzaldehyde.

To increase the accuracy, our analysis was done by isotope dilution using $^{13}C^{15}N_2$-semicarbazide. To avoid preparation of standards by derivatization in situ and to improve the consistency from batch to batch, we synthesized the $^{13}C^{15}N_2$-semicarbazone of 2-nitrobenzaldehyde from labeled semicarbazide. Our method also incorporated extraction of the sample with *n*-pentane in its prederivatization step. This defatting procedure is not likely to remove significant amounts of any free semicarbazones already present in the sample, since losses of spiked 2-nitrobenzaldehyde semicarbazone, which could be expected to serve as a reasonable surrogate, were less than 1%. The derivatizing agent, 2-nitrobenzaldehyde, was dissolved in methanol, not in DMSO as described in previous papers. We also determined by NMR that 2-nitrobenzaldehyde is stable in methanol at room temperature for at least a week, thus obviating the need for preparation of fresh solutions.

After extraction of the derivative with ethyl acetate, the samples were redissolved in water containing a relatively low amount of organic phase (10% acetonitrile plus 0.1% acetic acid) (*11*) and analyzed using a C18 column eluted with 2 mM ammonium formate in water/methanol (40:60). The MRM chromatograms of a mixture of 25 ng/g 2-nitrobenzaldehyde derivatives of semicarbazide (M_w = 208.2) and $^{13}C^{15}N_2$-semicarbazide (M_w = 211.2) standards (retention time, 4.5 min) are shown in **Figure 2**. An ion transition of m/z 209 → 166 was used for quantification of native semicarbazide while the ion transition of m/z 212 → 168 was used for the isotopically labeled semicarbazide.

The absolute recovery of our internal standard from flour samples was in the range of 30−40%. We attribute such a low recovery to the significant amount of reactive carbonyl compounds present in samples, the concentration of which is expected to increase after heating of the flour. These reactive carbonyls might then compete with 2-nitrobenzaldehyde in the derivatization reaction. It appeared that such a process might actually be taking place, because a 2.5-fold increase in the amount of 2-nitrobenzaldehyde added afforded a 2-fold increase in recoveries. The relative standard deviation (RSD) of 3% was obtained when six replicates of bread slices (with an average
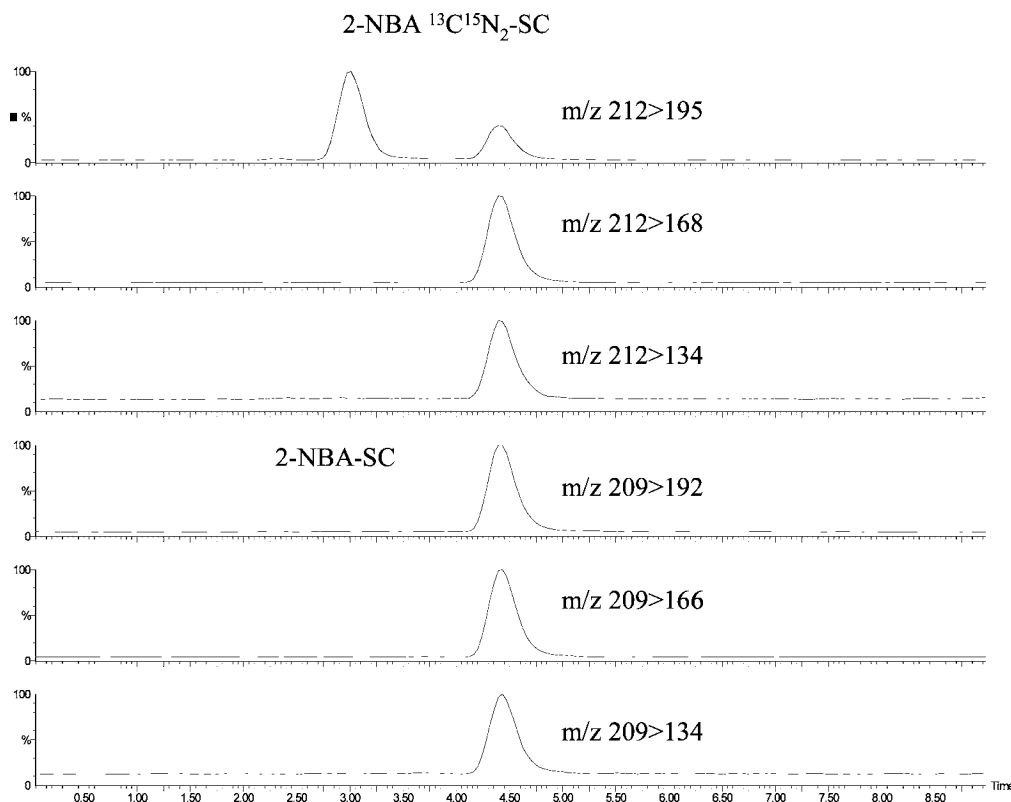
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2-NBA $^{13}C^{15}N_2$-SC

Figure 2. MRM chromatograms of a mixture of 25 ng/mL 2-nitrobenzaldehyde semicarbazone (2-NBA-SC) and 25 ng/mL 2-nitrobenzaldehyde $^{13}C^{15}N_2$-semicarbazone (2-NBA-$^{13}C^{15}N_2$-SC) standards.

Table 1. Effects of Wheat Flour Type and Processing on Yield of Semicarbazide (SC)

flour (type)	additive: ADA	SC[a] (ng/g)	SC[b] (ng/g)	SC[c] (ng/g)
unbleached "A"	yes	140	70	3
whole wheat "B"	yes	160	130	3.1
whole wheat "C"	yes	170	61	2.4
whole wheat "D"	no	<5	NA[d]	<0.5
unbleached "E"	no	<5	NA	<0.5
organic	no	<5	NA	<0.5
organic whole wheat	no	<5	NA	<0.5

[a] Heating in convection oven: 200 °C for 30 min. [b] Heating in toaster oven: broiling for 3 min. [c] Raw flour, no heating. [d] NA, not analyzed.

semicarbazide concentration of 28 ng/g) were analyzed. The five point calibration curve containing native 2-nitrobenzaldehyde semicarbazone at 1, 5, 12.5, 25, and 125 ng/mL and labeled 2-nitrobenzaldehyde $^{13}C^{15}N_2$-semicarbazone at 25 ng/mL was linear with $r^2 = 0.9996$.

MRM chromatograms of a baked bread crust sample containing 135 ng/g semicarbazide are shown in **Figure 3**. Using a signal-to-noise criterion of 3:1 (peak-to-peak noise definition) at the m/z 209 → 166 transition, the limit of detection was calculated as approximately 2 pg of standard injected on-column, which translates to 0.1 ng/g in the sample. The RSD for repeated injections (within-run precision) of the bread sample containing semicarbazide at the 25 ng/g level was 1.5% ($n = 9$).

To test the hypothesis that the azodicarbonamide present in the flour might decompose to semicarbazide, we heated five commercial wheat flour samples (plus two organic), three of which had listed azodicarbonamide as an additive, at 200 °C and analyzed them afterward for semicarbazide (**Table 1**). It is clear that semicarbazide forms in large quantities only in those flours that were fortified with azodicarbonamide. In nontreated

flours, after heat treatment, we found minor peaks at the retention time close to that of 2-nitrobenzaldehyde semicarbazone; these might indicate either chromatographic interferences and/or trace cross-contamination with azodicarbonamide in flour mills. The concentration of azodicarbonamide in flours was not known, but industry practices point to a likely 20 μg/g level. The concentration of semicarbazide in raw flours containing azodicarbonamide (without heat treatment) was in the range of 3 ng/g, consistent with observations of Pereira et al. (*8*). Azodicarbonamide-fortified flours were also subjected to broiling using a toaster oven with a top heating element. Because of the difficulty of positioning, samples of flours in this experiment (unlike one using a convection oven) showed varied discoloration. A cursory examination suggested that the degree of browning correlated with the amount of semicarbazide present in the heated flour sample. While azodicarbonamide-containing flours heated in a convection oven produced comparable levels of semicarbazide, flours with more broiling-induced browning showed reduced levels of semicarbazide.

To further elucidate the fate of azodicarbonamide during baking, the same azodicarbonamide-containing flour (flour A) was dry-heated at temperatures of 150, 175, or 225 °C and also heated in the presence of moisture (**Table 2**). An increase in the temperature beyond 200 °C dramatically reduced the semicarbazide yield, presumably by either decomposition of formed semicarbazide or reaction of semicarbazide with decomposition products of flour. It is claimed (*2*) that essentially all of the azodicarbonamide present in the wet dough will react as an oxidant and convert to inert biurea in less than 30 min. Azodicarbonamide is stable in dry flour; however, certain baking practices limit the exposure of flour to water and thus might limit the extent of such a transformation. To establish the effects of moisture on semicarbazide formation, an experiment was conducted as follows: 5 g of flour A was mixed with 1 mL of
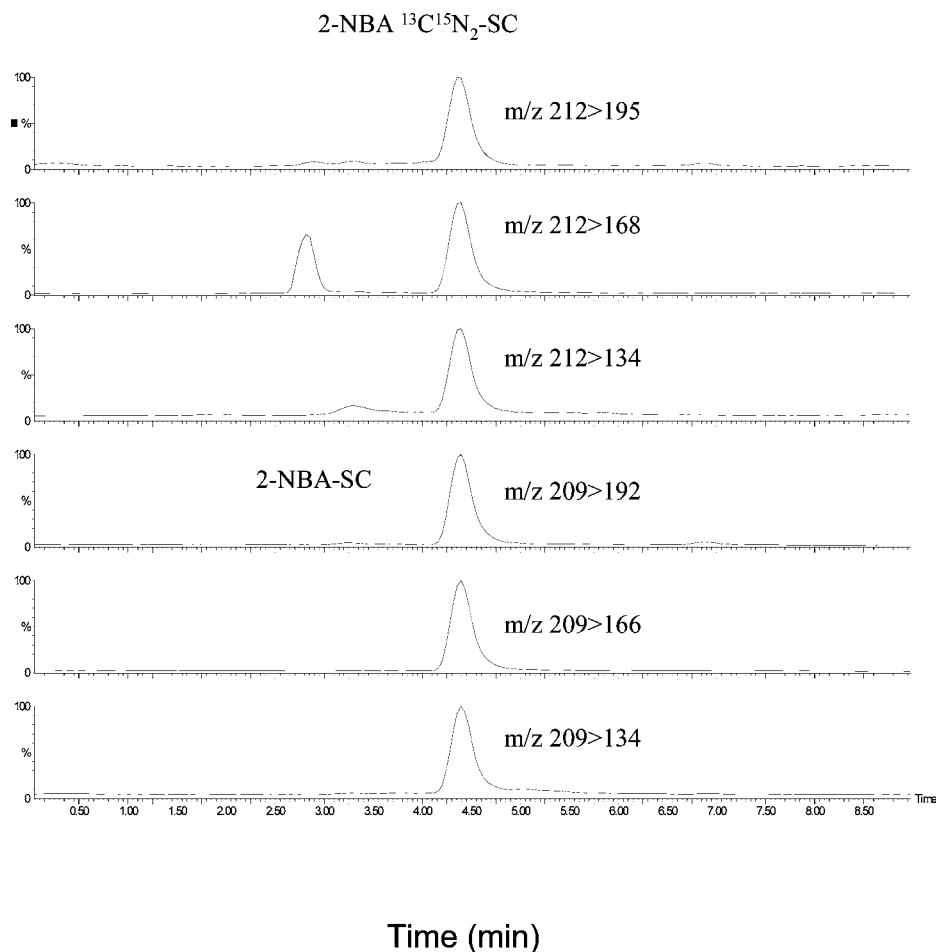
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Time (min)

Figure 3. MRM chromatograms of a baked bread crust sample with a concentration of semicarbazide in the sample of 135 ng/g.

Table 2. Effects of Conditions on Yield of Semicarbazide (SC)

condition[a]	SC (ng/g)	SD[b]
dry flour (150 °C)	61	
dry flour (175 °C)	231	19
dry flour (200 °C)	53	1.4
dry flour (225 °C)	7.1	
wet flour (200 °C)	92	9.4
bread crust[c]	135	
bread crust[d]	106	
bread slice[c]	28	
bread (center)[c]	9.8	

[a] Heating in convection oven for 30 min. [b] Standard deviation $n = 3$. [c] Bread sprinkled with flour before baking. [d] Bread not sprinkled with flour before baking.

water (wet flour), and after 1 min, it was baked at 200 °C. Flour heated in the presence of moisture had slightly higher levels of semicarbazide as compared to dry heating. This fact might be explained by the cooling effect of water and thus reduction of the decomposition of semicarbazide. Alternatively, a hydrolysis of biurea formed from azodicarbonamide might be taking place, as postulated by Pereira et al. (*8*).

Flour A was also used to prepare a typical loaf of bread by yeast fermentation and subsequent baking at 200 °C. Three subsamples of the bread were analyzed as follows: a cross-section slice, the top crust, and the bread interior. The moisture contents in these bread subsamples were 38, 25, and 43%, respectively. From results in **Table 2**, it is evident that formation of the semicarbazide is encouraged on the surface of the bread, likely because of a higher temperature.

The commercial standard of azodicarbonamide used for spiking experiments was purified before use as it contained 1500

μg/g of semicarbazide. The azodicarbonamide was crystallized from boiling water (approximately 1 g per 1 L). The crystallization resulted in the lowering semicarbazide content to 64 μg/g. The azodicarbonamide free flour was spiked at the 56 ppm level, which was close to the maximum 45 ppm allowable under regulation. The residual semicarbazide present in azodicarbonamide would then translate to less than 4 ng/g in the flour assuming a 100% recovery. After the flour was heated for 30 min at 200 °C and subsequently analyzed, semicarbazide was found at 550 ng/g. The similar spiking experiment with flour A containing azodicarbonamide resulted in semicarbazide at 700 ng/g. Using these data, we calculated that flour A contained azodicarbonamide at approximately 15 μg/g.

It is clear from our experiments that azodicarbonamide can be converted under conditions of the baking process to semicarbazide, with yields greater than 0.1%, which are much higher than observed by Pereira et al. (*8*) during room temperature reactions of azodicarbonamide. The toxicological significance of our findings is to be determined. We are currently employing our model system in the investigation of parameters and mechanisms responsible for the formation of semicarbazide in foods.

NOTE ADDED IN PROOF

Two publications related to the sources and mechanism of the formation of semicarbazide appeared while the manuscript was under review (*12, 13*).

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