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The mechanism of action of hexahydro-1,3,5-triethyl-*s*-triazine

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SUMMARY

The mechanism of antimicrobial action of hexahydro-1,3,5-triethyl-*s*-triazine (HHTT) was studied using the HHTT-resistant isolate, *Pseudomonas putida* 3-T-15², its HHTT-sensitive, novobiocin-cured derivative, *P. putida* 3-T-15² 11:21, *P. putida* ATCC 12633, *Pseudomonas aeruginosa* PA01 and *Escherichia coli* J53(RP4). HHTT was oxidized by *P. putida* 3-T-15², while respiration of *P. putida* 3-T-15² 11:21 was inhibited by HHTT. Chemical assays showed that HHTT released formaldehyde. *P. putida* 3-T-15² was highly resistant to formaldehyde, while *P. putida* 3-T-15² 11:21 was highly sensitive to formaldehyde. Both HHTT and formaldehyde acted similarly to inhibit proline uptake in bacterial cells and to inhibit the synthesis of the inducible enzymes, β-galactosidase and glucose-6-phosphate dehydrogenase. HHTT did not have uncoupler-like activity. *P. putida* 3-T-15² used either HHTT or ethylamine, a component of HHTT, as a nitrogen source for growth, but neither HHTT, ethylamine or formaldehyde served as a carbon and energy source for growth. We concluded that a major mechanism of antimicrobial action of HHTT was through its degradation product, formaldehyde.

INTRODUCTION

Recent work from our laboratory has focused on microbial resistance to industrial biocides. Candal and Eagon [5] studied 16 isolates of *Pseudomonas* strains that showed various resistance profiles to four industrial biocides and to certain heavy metals. These organisms were cultured from biocide-treated kaolin slurries and, in one instance, from paint. Many of the isolates were resistant to antimicrobial agents to which they had no known prior exposure. One to four plasmids were detected in each of the biocide-resistant strains. Recently, Hall and Eagon [10] presented indirect evidence that resistance to one of the four biocides tested, hexahydro-1,3,5-triethyl-*s*-triazine (HHTT) was plasmid-mediated in at least one *Pseudomonas putida* isolate.

Many industries depend upon the use of biocides to control unwanted microbial growth. Thus, the occurrence of biocide-resistant organisms in products of such industries presents a serious problem. Moreover, the use of biocides is regulated in some cases by the Food and Drug Administration (FDA), in other cases by the Environmental Protection Agency (EPA), and, in still other cases, by both regulatory agencies. This severely limits the

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numbers and types of biocides that an industry is permitted to use. Curiously, little information has been reported in the literature on the mechanism of antimicrobial action of industrial biocides. Even less information has been reported on the mechanism of microbial resistance to biocides. With this in mind, we selected HHTT as a model system to study. The purpose of the experiments in this article, therefore, was to elucidate the mechanism of antimicrobial action of HHTT.

MATERIALS AND METHODS

Organisms. The HHTT-resistant organism used in this study was one of several *Pseudomonas* strains originally isolated from contaminated kaolin slurries that had been treated with industrial biocides [5]. This organism was later identified as *Pseudomonas putida* [10] and given the strain designation 3-T-15^2. The HHTT-sensitive, novobiocin-cured derivative of this strain was designated *P. putida* 3-T-15^2 11:21 [10]. *P. putida* ATCC 12633, *Pseudomonas aeruginosa* PA01 and *Escherichia coli* J53(RP4) were also used in this study as control and test organisms and for comparative purposes.

P. putida 3-T-15^2 was maintained on Luria (L) agar plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar and deionized water to 1 liter) containing in final concentration 500 ppm HHTT (v/v). All other organisms were maintained on L-agar slants stored at room temperature and transferred every 2–3 weeks.

Cultivation of organisms. The organisms were cultivated aerobically on a rotary shaker at 30°C in the chemically defined basal salts medium previously reported [9] and supplemented with either 11 mM glucose (BSG) or 40 mM succinate (BSS). The organisms were harvested in late exponential phase by centrifuging (4°C) at 8000 × *g* and washing with 0.01 M potassium phosphate buffer, pH 7.5. These washed cells were used in the subsequent experiments with the exception of the β-galactosidase experiment.

Formaldehyde determinations. HHTT was examined for formaldehyde release using both the 2,4-pentanedione reagent [16] and the phenylhydrazine hydrochloride reagent [19]. Reagent grade formalin was used as the formaldehyde standard.

Respirometry. The effect of HHTT on oxygen uptake in *P. putida* 3-T-15^2 and 3-T-15^2 11:21 was studied by the manometric technique [20] using a Gilson IC-14 differential respirometer (Gilson Medical Electronics, Middleton, NJ). For this study, washed BSG-grown cells were suspended in 0.01 M potassium phosphate buffer, pH 7.5, to a concentration of approx. 1 mg protein/ml buffer. Each reaction flask contained in final concentration: 1 ml cell suspension; 1.5 ml 0.01 M potassium phosphate buffer, pH 7.5; 5 μmol glucose (when used), and various concentrations of HHTT as appropriate in a total volume of 3 ml. All flasks contained 0.2 ml of 20% (w/v) KOH in the center well to absorb CO_2.

Proline uptake. The effect of formaldehyde and HHTT on the uptake of [^{14}C]proline was determined by the conventional membrane filtration procedure. The organisms were grown in BSG, washed in 0.01 M phosphate buffer, pH 7.5, and suspended in the same buffer to a density of 1 g wet weight/20 ml buffer. Incubation mixtures, held in 16 × 125 mm borosilicate tubes on a reciprocal shaking water bath a 30°C, contained in final concentration as appropriate: 10 μM [^{14}C]proline (5 μCi/μmol), 125 ppm (0.73 μmol) HHTT, 31 ppm (1 μmol) formaldehyde, 0.2 ml cell suspension, and 0.01 M phosphate buffer, pH 7.5, to a final volume of 1 ml. Samples were removed at intervals, washed on membrane filters and radioactivity determined as previously described [11].

Intracellular ATP determinations. Washed BSS-grown cells of *P. aeruginosa* PA01 were suspended to the original volume in 0.01 M phosphate buffer, pH 7.5, and incubated on a rotary shaker for 2 h at 30°C to reduce endogenous energy sources. Cells were centrifuged (4°C) at 8000 × *g*, washed in 0.01 M phosphate buffer, pH 7.5, suspended to the original volume in the same buffer and kept aerated on a magnetic stirrer at ambient temperature until used. In a reaction mixture of 10 ml final volume, 2 ml of cell suspension (containing between 0.5 and 1 mg protein/ml buffer) were incubated with either

0.01 M phosphate buffer, pH 7.5, alone or with the addition of 40 mM succinate containing, as appropriate, 0.73 μmol HHTT or 50 μM carbonyl cyanide chloro-*m*-phenylhydrazone (CCCP).

Samples of 1 ml were removed at intervals over a 5-min period and ATP was extracted in boiling 0.1 M sodium bicarbonate buffer plus ethylenediaminetetraacetate (EDTA), pH 8.5, and intracellular ATP was measured using the firefly tail luciferin-luciferase reaction according to the procedure of Bancroft et al. [3].

Minimal inhibitory concentration (MIC). MIC determinations in liquid media were done by the two-fold serial dilution tube method of Anderson [1] using Antibiotic Medium 3 (Difco Laboratories, Detroit, MI). The results were recorded after 72 h of incubation at 32°C.

Assay for β-galactosidase (EC 3.2.1.23). Cells of *E. coli* J53(RP4) were grown overnight at 37°C in 200 ml of basal medium, pH 7.2, containing: 7 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2 \cdot 6H_2O$, 2.5 g vitamin-free casein hydrolysate, and deionized water to 1 l. Cells were harvested in late exponential phase by centrifuging (4°C) at 8000 × *g*, washed with 0.05 M sodium phosphate buffer, pH 7.5, and centrifuged again. Cells were suspended to the original volume in the same buffer and aerated on a rotary shaker for 10 min at 37°C. The cell suspension was then separated into five flasks (40 ml each) and incubated on a rotary shaker at 37°C to which, in final concentration, 10 mM lactose were added, and, as appropriate, 125 ppm (0.73 μmol) HHTT, 31 ppm (1 μmol) formaldehyde, or 50 μM chloramphenicol. Samples were removed at 15-min intervals over a 2-h period and assayed for β-galactosidase according to the procedure described by Dobrogosz [7].

Assay for glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Washed BSS-grown cells of *P. putida* ATCC 12633 were incubated on a rotary shaker at 32°C in 11 mM glucose in 0.01 M potassium phosphate buffer (pH 7.5) and containing in final concentration as appropriate: 125 ppm (0.73 μmol) HHTT, 31 ppm (1 μmol) formaldehyde, or 300 μM chloramphenicol. Samples of 200 ml were removed at intervals over a 3-h period. The samples were centrifuged, washed with 0.01 M phosphate buffer, pH 7.5, and suspended in 5 ml of the same buffer. Cell extracts of the samples were prepared by passing the cells twice through a French pressure cell at 15000 lb/in^2. Cell debris and intact cells were removed by centrifuging (4°C) for 30 min at 20000 × *g*. The supernatant fluid was assayed for glucose-6-phosphate dehydrogenase according to the method of Maurer et al. [14].

Other. Protein was determined by the Lowry procedure as modified by Markwell et al. [13]. [^{14}C]Proline was purchased from New England Nuclear, Boston, MA. Formalin (37% formaldehyde), reagent grade, was obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals in the highest state of purity were purchased from commercial sources.

RESULTS

Effect of HHTT on respiration

In order to determine what effect HHTT might have on respiration, manometric studies were carried out to measure oxygen consumption. As shown in Fig. 1, HHTT did not inhibit the oxygen uptake by HHTT-resistant *P. putida* 3-T-15[2]. Instead, oxygen uptake due both to endogenous respiration and to glucose catabolism was increased in the presence of HHTT, and oxygen uptake increased as the HHTT concentration increased (Fig. 1A and C). In contrast, HHTT inhibited endogenous respiration and strongly inhibited respiration due to glucose catabolism by HHTT-sensitive, novobiocin-cured *P. putida* 3-T-15[2]11:21 (Fig. 1B and D). These data strongly suggest, therefore, that the HHTT-resistant strain was able to metabolize HHTT.

Determination of formaldehyde release by HHTT

Experiments were done to determine whether HHTT was a formaldehyde-releasing compound. As shown in Table 1, between 2.1 and 2.4 mol of formaldehyde were released per mol of HHTT. The theoretical yield is 3 mol of formaldehyde per mol of HHTT. As controls, a known formaldehyde-releasing agent, 3,5-dimethyltetrahydro-1,3,5-(2*H*)-

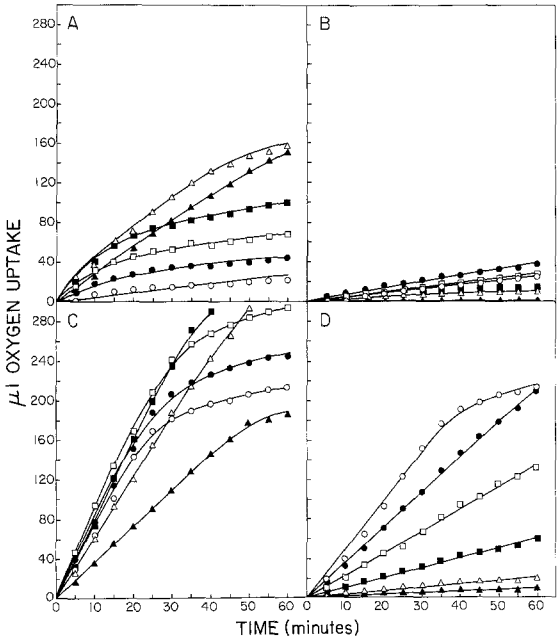

Fig. 1. Endogenous respiration of HHTT-resistant *P. putida* 3-T-15[2] (A) and of HHTT-sensitive, novobiocin-cured *P. putida* 3-T-15[2] 11:21 (B), and glucose-driven respiration of *P. putida* 3-T-15[2] (C) and of *P. putida* 3-T-15[2] 11:21 (D) in the absence and presence of HHTT as indicated. In addition to cells, buffer and glucose, when used, the reaction flasks contained: ○, no HHTT; ●, 1.095 μmol HHTT; □, 2.19 μmol HHTT; ■, 4.38 μmol HHTT; △, 8.76 μmol HHTT; ▲, 17.55 μmol HHTT. Respiration was calculated as μl oxygen uptake/mg protein. See Materials and Methods for further details.

thiadiazine-2-thione (DTT) [4], and a biocide not reported to release formaldehyde, 1,2-benzisothiazolin-3-one (BIT), were also used. Between 1.3 and 1.6 mol of formaldehyde were released per mol of DTT, the theoretical yield being 2 mol of formaldehyde per mol of DTT, and no formaldehyde was released by BIT (Table 1).

MIC of HHTT and of formaldehyde

Since HHTT was shown to release formaldehyde, MIC determinations were made on the various test organisms to compare their susceptibility to both HHTT and formaldehyde. The results are indicated in Table 2. HHTT-resistant *P. putida* 3-T-15[2] was shown to be highly resistant to both HHTT and formaldehyde and novobiocin-cured, HHTT-sensitive *P. putida* 3-T-15[2] 11:21 was extremely sensitive to both HHTT and formaldehyde.

Table 1

Analysis of formaldehyde release by three industrial biocides using two analytical procedures

Biocide	mol formaldehyde released/mol biocide	
	phenylhydrazine-HCl reagent[a]	2,4-pentanedione reagent[b]
HHTT[c]	2.4	2.1
DTT[d]	1.3	1.6
BIT[e]	0	0

[a] Final pH of assay was 6.34.
[b] Final pH of assay was 12.8.
[c] Hexahydro-1,3,5-triethyl-*s*-triazine, a pure technical product with some water of reaction present.
[d] 3,5-Dimethyl-1,3,5-(2*H*)-tetrahydrothiadiazine-2-thione, a 24% solution plus 9% isopropanol (in final concentrations) dissolved in 10% NaOH.
[e] 1,2-Benzisothiazolin-3-one, a 35% solution (in final concentration) in 25% ethylenediamine.

Effect of HHTT and formaldehyde on proline uptake

Both HHTT and formaldehyde strongly inhibited proline uptake in *P. putida* ATCC 12633 (Fig. 2) and in *P. aeruginosa* PA01 (data not shown). Proline is taken into these organisms by active transport energized by the proton motive force [21]. Thus, since HHTT inhibited oxygen uptake in sensitive organisms (Fig. 1), this effect could be due to

Table 2

MIC values of HHTT and formaldehyde against organisms used in this study

Organism	MIC values (ppm of agent)	
	HHTT	formaldehyde
P. putida 3-T-15[2]	1000	1000
P. putida 3-T-15[2] 11:21	31	16
P. putida ATCC 12633	125	31
P. aeruginosa PA01	125	31
E. coli J53(RP4)	125	31

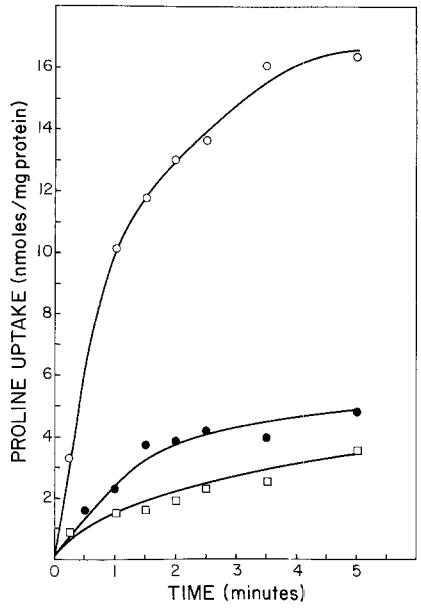

Fig. 2. Inhibition of proline uptake in *P. putida* ATCC 12633 by formaldehyde and by HHTT. Symbols: ○, no formaldehyde or HHTT added; ●, 1 mM formaldehyde; □, 0.73 mM HHTT. See Materials and Methods for further details.

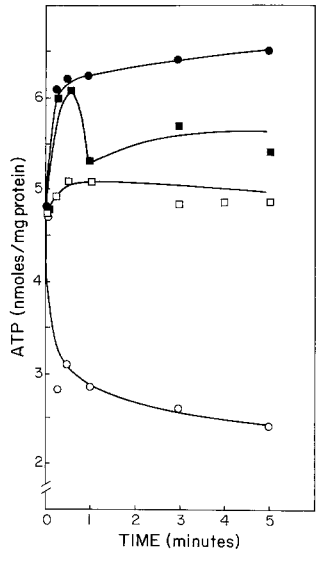

Fig. 3. Effect of HHTT on intracellular ATP levels in *P. aeruginosa* PA01. Symbols: □, buffer only; ●, 40 mM succinate; ○, 40 mM succinate plus 50 μM CCCP; ■, 40 mM succinate plus 0.73 μmol HHTT. See Materials and Methods for further details.

the inability of the membrane to form a proton motive force required for proline transport.

Effect of HHTT on intracellular ATP level

Certain ring-structured antimicrobial agents act as uncouplers (i.e., as proton ionophores or proton conductors that collapse the proton gradient across the membrane). Thus, experiments were carried out to determine whether HHTT had uncoupler activity by determining its effect on intracellular ATP formation or depletion. *P. aeruginosa* PA01 was used as the test organism for the results shown in Fig. 3. The results indicated that HHTT did not act as an uncoupler. When an authentic uncoupler, CCCP, was added to the reaction, there was an immediate and rapid depletion of intracellular ATP.

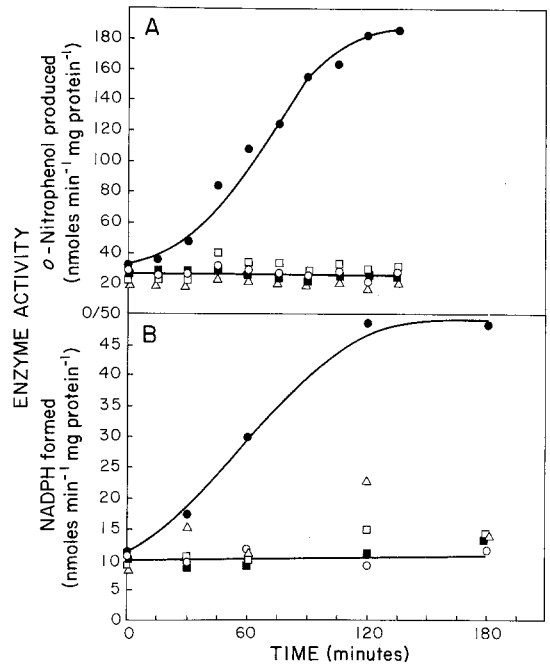

Fig. 4. Inhibitory effect of HHTT and of formaldehyde on the induction of β-galactosidase by *E. coli* J53(RP4) (A) and on the induction of glucose-6-phosphate dehydrogenase by *P. putida* ATCC 12633 (B). Symbols: ○, buffer only; ●, buffer plus 10 mM lactose or 11 mM glucose as indicated; ■, buffer and substrate plus 0.73 mM HHTT; □, buffer and substrate plus 1 mM formaldehyde; △, buffer and substrate plus 50 μM chloramphenicol (A) or 300 μm chloramphenicol (B). See Materials and Methods for further details.

When the organisms were incubated in buffer alone, the intracellular level of ATP remained at a steady level. Addition of succinate stimulated ATP production. Addition of both succinate and HHTT resulted in a transient increase in ATP followed by a small decrease of ATP to a steady-state level. These experiments were also carried out with *P. putida* ATCC 12633 with closely parallel results (data not shown). We interpret these results to indicate that the inability of these organisms to synthesize ATP in the presence of HHTT was most likely due to the inhibition of respiration by HHTT (see Fig. 1B and D).

Effect of HHTT and formaldehyde on inducible enzyme formation

In an effort to measure the effect of HHTT and formaldehyde on protein synthesis, the effect of these two agents on the synthesis of two inducible enzymes was determined. The well-documented inducible β-galactosidase of *E. coli* was selected as one of the enzymes, while the inducible glucose-6-phosphate dehydrogenase of *P. aeruginosa* and *P. putida* [12] was chosen as the other enzyme. Thus, the results shown in Fig. 4 using *P. putida* ATCC 12633 clearly show that neither inducible enzyme was synthesized in the presence either of HHTT or of formaldehyde. The same results were obtained when *P. aeruginosa* PA01 was used (data not shown).

Use of HHTT and ethylamine as a nitrogen and carbon source for growth

HHTT-resistant *P. putida* 3-T-15[2] could use HHTT as a nitrogen source for growth when HHTT was used in BSG medium as the nitrogen source instead of the $(NH_4)_2SO_4$ normally used (data not shown). Moreover, *P. putida* 3-T-15[2] also used ethylamine, a component of HHTT, as a nitrogen source for growth. This is further evidence that HHTT degrades to its component parts, ethylamine and formaldehyde. However, neither HHTT, nor ethylamine nor formaldehyde could serve as a carbon source for growth of *P. putida* 3-T-15[2].

DISCUSSION

The objective of this study was to determine the mechanism of antimicrobial action of HHTT. Thus, we presented evidence that: (a) HHTT was metabolized by HHTT-resistant *P. putida* 3-T-15[2] as shown by oxygen uptake in respirometric experiments, but HHTT inhibited oxygen uptake by HHTT-sensitive, novobiocin-cured *P. putida* 3-T-15[2] 11:21; (b) in chemical assays for release of formaldehyde, HHTT was shown to degrade to yield formaldehyde to give 70–80% of the theoretical yield; (c) HHTT-resistant *P. putida* 3-T-15[2] was also highly resistant to formaldehyde while HHTT-sensitive, novobiocin-cured *P. putida* 3-T-15[2] 11:21 was highly sensitive to formaldehyde; (d) both HHTT and formaldehyde inhibited proline uptake in *P. putida* ATCC 12633 and in *P. aeruginosa* PA01 more or less equally, suggesting inhibition of active transport; (e) although HHTT partially inhibited oxygen uptake in sensitive organisms, it did not act as an uncoupler in that intracellular ATP was neither depleted nor produced appreciably when cells were incubated with HHTT; (f) both HHTT and formaldehyde inhibited the induction of β-galactosidase and glucose-6-phosphate dehydrogenase; and (g) HHTT-resistant *P. putida* 3-T-15[2] could use either HHTT or ethylamine, a component of HHTT, as a nitrogen source for growth, but neither of these substances could be used as a carbon source for growth nor could formaldehyde, the second component of HHTT.

Our data, therefore, showed that both HHTT and formaldehyde demonstrated parallel effects. Thus, we conclude that a major mechanism of antimicrobial action of HHTT is through its degradation product, formaldehyde. Whether HHTT itself has antimicrobial activity could not be discerned from our experiments.

It should be mentioned that even though neither HHTT, nor ethylamine nor formaldehyde could be used as a carbon source for growth, this does not preclude one or more of these substances being oxidized by *P. putida* 3-T-15[2]. Thus, this would be an explanation for the oxygen uptake noted when this

organism was incubated with HHTT. Indeed, as is shown in our accompanying paper [8], *P. putida* 3-T-15[2] has an active formaldehyde dehydrogenase.

There has been no overall agreement whether HHTT releases formaldehyde under practical conditions of use as an antimicrobial agent. Although HHTT is often referred to as a formaldehyde-releasing compound [4,17], other authors have presented evidence that HHTT does not release formaldehyde [6]. While HHTT is generally regarded to be stable under alkaline conditions it has also been reported, conversely, that formaldehyde-releasing preservatives in general are unstable at high pH [4]. Nevertheless, under the conditions of dilution and assay used in the experiments reported herein, HHTT was found to readily degrade to formaldehyde. Our experiments, however, did not permit us to discern whether HHTT-resistant *P. putida* 3-T-15[2] could degrade HHTT through enzymatic action.

The objective of this study was not to study the mechanism of action of formaldehyde. There is considerable information, however, on this subject. Thus, formaldehyde has been reported to cross-link proteins through the amino groups of amino acids. There is also suggestive evidence that formaldehyde reacts with the amino groups of nucleic acids and nucleoproteins. In the case of mammalian DNA, formaldehyde is thought to be able to induce single-strand breakages (for reviews see Refs. 2, 15 and 18).

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