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ALLYLGLYCINE: INTRANIGRAL EFFECTS AND REAPPRAISAL OF ACTIONS ON THE GABA SYSTEM

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Abstract—The interaction of allylglycine (administered intraperitoneally, intraventricularly and intranigrally) with the γ-aminobutyric acid system was studied in a number of rat brain regions. The effects of allylglycine appeared to result from reduced availability of brain γ-aminobutyric acid through inhibition of glutamate decarboxylase activity. Allylglycine was a more effective inhibitor of glutamate decarboxylase *in vivo* than *in vitro*, and could be metabolized to 2-keto-pent-4-enoic acid. Sublethal doses of intraventricular allylglycine had no effect on γ-aminobutyric acid metabolism. Intranigral allylglycine initially produced contralateral circling behaviour accompanied by elevated dopamine turnover in the ipsilateral striatum—seizure activity was subsequently noted. Observed actions of allylglycine probably result from the combined actions of the parent amino acid and the keto-acid metabolite.

Allylglycine (2-aminopent-4-enoic acid) is a simple unsaturated amino acid possessing convulsant properties [1, 2]. The delayed onset of allylglycine-induced convulsions has generally been considered to be a consequence of a time-dependent enzyme inhibition, which appears to involve brain glutamate decarboxylase (EC 4.1.1.15, GAD) [3, 4]. Since GAD is the enzyme responsible for γ-aminobutyric acid (GABA) biosynthesis, the subsequent reduced levels of this major inhibitory neurotransmitter [5] may explain the observed convulsions. Indeed, since ultrastructural changes accompany allylglycine-induced reductions in GABA and GAD levels [3, 6], the drug has been suggested as a pharmacological tool for studying GABA-utilizing synapses [6]. Because monoamine neurotoxins (e.g. 6-hydroxydopamine and 5,7-dihydroxytryptamine) have been so useful in the study of neurotransmitter function, we have examined allylglycine as a possible neurotoxin for the destruction of GABA-utilizing neurons and reinvestigated its mechanism of action.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–200 g) of the Charles River stock were used in all experiments. DL-C-allylglycine (Sigma, St. Louis, U.S.A.) was administered intraperitoneally at a dose of 1.30 mmol. kg^{-1} (150 mg. kg^{-1} in 0.9% saline) in a volume of 3 ml/kg. All other drugs were administered intraperitoneally in a volume of 2 ml/kg.

Glutamate decarboxylase. Freshly dissected tissue was homogenized in 10 vol of ice-cold 5 mM sodium phosphate pH 7.4, containing 0.5 mM pyridoxal phosphate, 0.5 mM dithiothreitol and 0.25% (w/v) Triton X-100. GAD activity was estimated in 100 μl portions by a column chromatographic method [7]. The assay was linear with respect to incubation time and amount of homogenate employed, and the final glutamate concentration *in situ* was 1.67 mM (0.2 μCi of [3-^{3}H]-L-glutamate [New England Nuclear, Boston, U.S.A.] per sample).

GAD activity was also estimated by carbon dioxide trapping. Portions of the above 10 vol homogenate (100 μl) plus 100 μl of homogenizing buffer were incubated with 200 μl of 25 mM sodium phosphate and 200 μl of 5 mM glutamic acid (1.67 mM final concentration, 0.2 μCi of [U-^{14}C]glutamate (NEN) per sample) at 37° for 30 min. Glacial acetic acid (200 μl) was then injected and the $^{14}CO_2$ evolved trapped by 200 μl of NCS tissue solubilizer (Amersham/Searle, Arlington Heights, U.S.A.) and a paper wick in a well, after a further 1 hr incubation at 37° and being allowed to stand overnight. The assay was linear with respect to incubation time and amount of homogenate employed.

GAD activities were also estimated by both procedures at a final glutamate concentration of 10 mM *in situ* (in these studies 0.4 μCi of radioactive glutamate was used per sample). Kinetic studies were carried out using the chromatographic assay described above with 10 and 20 mM allylglycine present during the preincubation with enzyme (5 min), and glutamate (0.667 mM–1.67 mM) being added to start the assay.

GABA transaminase. GABA transaminase (EC 2.6.1.16, GABA-T) activity was determined in 100 μl portions of homogenates employed for GAD measurements by measuring the coupled formation of succinate from GABA as described previously [7].

GABA. Freshly dissected tissue was rapidly frozen on dry ice and levels were routinely determined by a fluorimetric procedure [8], although the enzymatic fluorimetric assay [9] was employed in initial studies—cell free GABASE was purchased from Worthington Biochemical Company, N.J.

Pyridoxal phosphokinase and pyridoxal phosphate. Pyridoxal phosphate was extracted and assayed by a

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procedure which utilizes the pyridoxal phosphate dependence of tyrosine apodecarboxylase (*Streptococcus faecalis*, Sigma), with the quantitation based on the evolution of $^{14}CO_2$ from [U-^{14}C]-L-tyrosine (NEN) [10, 11].

GABA and amino acid synthesis. Three days after the chronic implantation of cannulae into the right lateral ventricle, rats were pretreated with intraperitoneal allylglycine (or saline) for 1 hr and infused via the intraventricular cannulae and a microsyringe with 20 μl of 0.9% saline containing [U-^{14}C]-D-glucose (20 μCi, NEN) or [2-^{14}C]acetate (50 μCi, NEN) followed by 5 μl of saline. After 10 min the rats were sacrificed, the hippocampi, cerebral cortices and cerebella rapidly dissected and frozen on dry ice before homogenization in 10 vol of ice-cold 0.6 M aqueous perchloric acid. Homogenates were centrifuged and portions of the supernatant were applied to an Amberlite CG 120H$^+$ column (0.6 × 3 cm), which was washed to neutrality and eluted with 3 ml of 50% ethanolic 2 M triethylamine to isolate the amino acid fraction. This eluate was dried down under a stream of warm air, taken up in 5 mM sodium bicarbonate pH 10.2 and analysed by microdansylation. This procedure employs [^{3}H]dansyl chloride and the ^{14}C/^{3}H ratio of each amino acid is an index of specific activity—full details are given elsewhere [12].

GABA synthesis was also investigated using the accumulation of GABA after aminooxyacetic acid administration [13]. Aminooxyacetic acid (hemihydrochloride, AOAA) was administered intraperitoneally at a dose of 70 mg kg^{-1} (in 0.9% saline, adjusted to pH 7.4 with aqueous sodium hydroxide). Two studies were carried out: (1) allylglycine or vehicle was administered 15 min before AOAA and the animals sacrificed 1 hr after AOAA; (2) allylglycine was administered 1 hr after AOAA and the animals sacrificed 1 hr later. GABA was estimated as described earlier. Preliminary studies had shown that at this dosage AOAA inhibited cortical GABA-T activity by at least 90 per cent over 2 hr while GAD activity was virtually unaffected, and that GABA accumulation was linear in hippocampus, cortex and cerebellum.

Intraventricular and intranigral injections. The procedure of Noble *et al.* [14] was used for intraventricular injections with rats being anaesthetized with choral hydrate (400 mg. kg^{-1}), and allylglycine being injected in 20 μl of 0.9% saline. For intranigral injections rats were anaesthetized with choral hydrate or ether (see Results) and unilateral injections (2 μl injected over 2 min) were made with a stereotaxically positioned microsyringe (A 2.1, L 2.0, D + 2.5; 15). Animal behaviour was observed immediately after recovery from anaesthesia and circling was examined in both a rotometer and open field.

Dopamine turnover. Rats received α-methyl-*p*-tyrosine methyl ester hydrochloride (315 mg. kg^{-1}) or saline intraperitoneally, in addition to intranigral allylglycine or saline, and were sacrificed according to the experimental schedule (see Table 3 for details). Corpora striata were quickly removed, frozen on dry ice and weighed before homogenization in 1 ml of ice-cold 0.4 M perchloric acid. After the addition of ethylenediamine tetraacetate and sodium metabisulphite, the striatal homogenate was adjusted to pH 8.5 and the dopamine isolated from an alumina column [16]. Dopamine was assayed by a fluorometric procedure [17]. The recovery of dopamine was 88.0 ± 5.9 (n = 15) per cent and reported values are not corrected for recovery.

Metabolism of allylglycine. Whole rat brains were homogenized in 5 vol of ice-cold 5 mM potassium phosphate pH 8.2, containing 0.1 M dithiothreitol, 50 μM pyridoxal phosphate and 0.2% Triton X-100, and the crude homogenate was dialysed overnight against two changes of the same buffer. Branched chain amino acid aminotransferase activity was determined using 250 μl portions of the dialysed homogenate in the assay procedure of Ari and Ichihara [18] with L-leucine, DL- and L-allylglycine being compared.

The procedure of Lichtenberg and Wellner [19] was used to examine DL- and L-allylglycine as substrates for snake venom L-amino acid oxidase (Crotalus adamenteus, type IV, Sigma) relative to L-leucine.

RESULTS

Intraperitoneal allylglycine and GABA neurochemistry. Intraperitoneal administration of allylglycine at the dose employed by Alberici *et al.* [3]; 150 mg. kg^{-1}, 1.30 mmol. kg^{-1}) caused a substantial reduction (35–55%) in the GABA content of hippocampus, cortex and cerebellum, although GAD activity was decreased only approximately 15 per cent relative to control in the same regions (see Table 1). GABA-T activity was never significantly affected.

Table 1. Intraperitoneal allyl glycine and GABA metabolism

	GABA Level	GAD Activity	GABA-T Activity
Hippocampus	1.63 ± 0.24*	18.16 ± 3.09	5.60 ± 1.16
	(3.10 ± 0.49)	(20.90 ± 1.32)	(6.14 ± 0.76)
Cortex	1.39 ± 0.24	17.95 ± 0.60†	5.42 ± 0.56
(parietal)	(2.09 ± 0.25)	(20.50 ± 0.64)	(5.70 ± 0.56)
Cerebellum	0.79 ± 0.15†	14.70 ± 0.41†	7.45 ± 0.94
	(1.81 ± 0.12)	(17.38 ± 0.25)	(7.95 ± 0.57)

Values are the mean ± S.E.M. of 3–9 animals and were determined as described in the text. Rats were sacrificed after the first running fit produced by a single intraperitoneal injection of allyl glycine (1.30 mmol.kg^{-1}). Values in parentheses represent saline controls: units for GABA μmol.g^{-1} wet wt, GAD μmol.g^{-1}.hr^{-1} and GABA-T μmol.g^{-1}.hr^{-1}. Further details are given in the text.

*0.05 < P < 0.10.

†P < 0.01 relative to control.

The allylglycine inhibition of GAD activity was further investigated because a number of previous workers have described this action [2, 4, 20]. Enzyme activity was studied by both the column chromatographic separation of newly synthesized GABA and by carbon dioxide trapping at glutamate concentrations of 1.67 and 10 mM *in situ* (see Methods). Intraperitoneal allylglycine (150 mg. kg^{-1}) never reduced GAD activity in hippocampal, cortical and cerebellar homogenates by more than 24 per cent of control (range of inhibition 5–24%) using either assay procedure.

Kinetics of GAD inhibition. Kinetic analyses of the inhibition of GAD activity at varying glutamate concentrations were consistent with a competitive action (not shown), when allylglycine was either added to the incubation mix or when the drug had been administered intraperitoneally, and when a crude cortical homogenate was employed as the enzyme preparation. However, the studies with *in vitro* addition of allylglycine suggested a non-linear competitive inhibition, although only two drug concentrations (10 and 20 mM) were employed. The omission or addition of pyridoxal phosphate to the standard incubation mix (see Methods) did not change the extent of inhibition of GAD after intraperitoneal allylglycine, and *in vitro* L-allylglycine was responsible for all of the inhibition observed wih the DL-racemate. It was of some interest that intraperitoneal allylglycine (150 mg. kg^{-1}, approximately 2.1 mM if uniformly distributed in body fluids) caused 20 per cent inhibition of cortical GAD activity, while *in vitro* 10 mM allylglycine effected only 33 per cent inhibition of enzyme activity.

Vitamin B_6 metabolism. Although allylglycine caused a 35 per cent reduction of hippocampal pyridoxal phosphokinase activity, much smaller decreases were found in cortex and cerebellum—none of the reductions was statistically significant. The levels of pyridoxal phosphate in the same brain regions were never altered by the small inhibition of pyridoxal phosphokinase activity (see Table 2).

Allylglycine convulsions. Convulsions following allylglycine administration took the form of a series of running fits followed by periods of partial rigidity and seizures, and eventually by death [1–3]. Times of onset of the first running fits were remarkably consistent from experiment to experiment and results from several studies were pooled; mean ± S.E.M., 86 ± 2 (17) min. Pyridoxal phosphate (0.125 mmol. kg^{-1}) or pyridoxine (0.25 mmol. kg^{-1}) pretreatments did not prevent or significantly alter the time of onset of first convulsions. Aminooxyacetic acid (30 mg. kg^{-1}, 0.24 mmol. kg^{-1}, 3 hr pretreatment) delayed the onset (126 ± 10 (4) min, $P < 0.001$), but did not prevent convulsions, in agreement with previous findings [21].

Table 2. Allylglycine and vitamin B_6 metabolism

	Pyridoxal phosphokinase	Pyridoxal phosphate
Hippocampus	87.55 ± 10.14	0.67 ± 0.03
	(136.67 ± 21.63)	(0.77 ± 0.06)
Cortex	71.08 ± 14.45	0.75 ± 0.05
	(86.66 ± 10.08)	(0.75 ± 0.04)
Cerebellum	95.86 ± 16.97	0.89 ± 0.07
	(111.44 ± 16.78)	(0.88 ± 0.04)

All values are the mean ± S.E.M. of 3 animals and were determined as described in the text. Values in parentheses represent saline controls: units for pyridoxal phosphokinase nmol.g^{-1}.hr^{-1} and pyridoxal phosphate $\mu g.g^{-1}$ wet wt. Further details are given in the footnote to Table 1.

Actions on amino acid synthesis. Actions on GABA synthesis were demonstrated only when GABA accumulation was followed after GABA-T inhibition by aminooxyacetic acid (see Table 3) and were more pronounced in cortex than hippocampus. Alterations using this procedure were found only by administering AOAA after allylglycine—administration before allylglycine appeared to minimize GABA depletions and synthesis changes were not detected. GABA synthesis was decreased in hippocampus and cortex, but elevated in cerebellum.

Allylglycine had little effect on GABA synthesis when studied after intraventricular infusion of [^{14}C]-glucose or acetate. With [^{14}C]glucose, which preferentially labels neuronal metabolism [12, 22], the glutamine/glutamate specific activity ratio was unchanged, indicative of no differential effect on glutamate compartmentation. However, in all three areas examined the alanine specific activity index was elevated, with the increase over the regions being 23 ± 7 ($n = 3$) per cent of control. This observation, when considered with the reduced incorporation of label from [^{14}C]glucose into total amino acids suggested a decreased carbon flux into the citric acid cycle under the influence of allylglycine. When [^{14}C]acetate, a preferential label for glial metabolism [12, 22], was the precursor, the glutamine/glutamate specific activity ratio was reduced in hippocampus, cortex and cerebellum (average decrease 25 ± 4 ($n = 3$) per cent of control). This change was attributable to increased labelling of glutamate, since the specific activity index was increased (41 ± 5 ($n = 3$) per cent of control) while the corresponding glutamine values were unchanged—concurrently the aspartate specific activity index was also increased (average increase 79 ± 33 ($n = 3$) per cent of control).

Intraventricular and intranigral allylglycine. Rats receiving 500 μg of intraventricularly administered allylglycine showed the first physical symptoms at 2 hr of brief twitches and transient rigidity—no long lasting rigidity was observed. When administered at higher doses (2 and 5 mg), the drug produced effects similar to those seen after intraperitoneal administration, with death occurring within a few hr. Hippocampal, cortical and cerebellar GAD activity in whole tissue homogenates was unaffected by 500 μg of intraventricular allylglycine when estimated 2½, 15 and 40 hr after injection.

Allylglycine (200 μg) was unilaterally injected into the zona reticulata of the substantia nigra of rats under choral hydrate anaesthesia in initial studies. All animals displayed postural asymmetry on removal from the stereotaxic frame, with the upper path of the body contorted contralaterally (away from the side of injection). Within 30–60 min approximately ⅓ of the animals displayed spontaneous tight contralateral circling at 5–10 turns. min^{-1}. The head became increasingly twisted and ipsilaterally contorted until

Table 3. Allylglycine and GABA synthesis

	GABA rate of synthesis		GABA specific activity index	
	AOAA (PRE)	AOAA (POST)	[^{14}C]Glucose	[^{14}C]Acetate
Hippocampus	1.08 ± 0.45	1.00 ± 0.38	552	10
	(1.20 ± 0.50)	(1.35 ± 0.31)	(842)	(9)
Cortex	0.89 ± 0.18	0.31 ± 0.17	611 ± 14	10
	(0.88 ± 0.25)	(0.84 ± 0.30)	(546 ± 17)	(9)
Cerebellum	0.62 ± 1.18	0.86 ± 0.46	860 ± 63	19
	(0.67 ± 0.32)	(0.46 ± 0.31)	(817)	(26)

Values are the mean ± S.E.M. of 6 animals for AOAA studies and 2 or 3 for [^{14}C]glucose and acetate studies. AOAA (PRE) refers to AOAA administrated 1 hr before allylglycine, the rats being sacrificed 1 hr later, while AOAA (POST) refers to AOAA administered 15 min after allylglycine, the rats being sacrificed at 75 min. Values in parentheses represent saline controls. Units for rate of GABA synthesis μmol.g^{-1} wet wt.hr^{-1}. GABA specific activity indices have been multiplied by 10^3. Further details are given in the text.

of bursts of complete ipsilateral body rolling, which was eventually replaced by running fits and rigidity.

Circling after intranigral allylglycine was further investigated by unilateral stereotaxic injection in rats lightly anaesthetized with ether. All rats circled contralaterally with a mean frequency of 7.6 ± 1.4 ($n = 4$) turns. min^{-1} during the 10–15 min post injection and rearing was occasionally noted. A quiescent period of reduced circling followed and tumbling commenced at 40–45 min. Saline-injected controls showed no characteristic behaviour.

When the action of intranigral allylglycine on striatal dopamine turnover was investigated using the synthesis inhibitor α-methyl-*p*-tyrosine, tumbling did not appear to be a consequence of a selective action on dopamine turnover, whereas elevated turnover was found during the period of circling (see Table 4). Thus unilateral allylglycine effected an increased turnover of dopamine in the ipsilateral striatum relative to that seen in saline-treated controls or the contralateral striatum (not shown). Concomitantly the steady state GABA level in substantia nigra, 6.98 ± 0.22 ($n = 3$) μmol. g^{-1} wet wt, was unchanged relative to control, 7.47 ± 0.32 ($n = 3$). Nigral GAD activity, when estimated 45 min, 24 and 48 hr post injection, was also unaffected by allylglycine.

Metabolic conversion of allylglycine. When tested as a substrate for brain branched chain amino acid aminotransferase, allylglycine appeared to be a weak amino donor relative to leucine, one of the best substrates [18]. DL-Allylglycine was approximately $\frac{1}{10}$ as active as leucine as an amino donor with all of the activity attributable to the L-isomer. However, allylglycine was an excellent substrate for snake venom L-amino acid oxidase with the L-isomer and DL-racemate being respectively 103 and 71 per cent as active as leucine. This finding suggests that both D- and L-isomers are metabolized by L-amino acid oxidase, and that the L-isomer is approximately $2\frac{1}{2}$ times more active than the D-form.

DISCUSSION

Allylglycine, although it can produce a large fall in brain GABA levels (approximately 50 per cent in the regions examined), was found to only produce a weak inhibition of brain GAD activity (inhibition 5–24 per cent of control). Many convulsants reduce brain GAD activity by interfering with the synthesis of pyridoxal phosphate. Our results indicate that whatever the mechanism of inhibition of GAD activity following allylglycine administration, it certainly does not involve vitamin B_6 metabolism. 3-Mercaptopropionic acid, another convulsant, also appears

Table 4. Striatal dopamine after intranigral allylglycine

	Seizure Activity		Circling Behaviour	
	Striatal Dopamine			
	Level	Δ	Level	Δ
Saline	7.35 ± 0.34	—	8.25 ± 0.34	—
Saline + α mpt	3.72 + 0.34†	1.82	7.88 + 1.16*	0.74
Allylglycine	8.05 ± 0.56	—	9.38 ± 0.63	—
Allylglycine + α mpt	4.05 ± 0.51†	2.00	6.97 ± 0.27†	4.82

All values are mean ± S.E.M. of 6 or 7 determinations. Saline or allylglycine was injected unilaterally into the pars reticulata of substantia nigra. In studies of dopamine levels in the period of seizure activity (tumbling), choral hydrate was the anaesthetic and intraperitoneal α-methyl-*p*-tyrosine methyl ester hydrochloride (α mpt) or saline was administered 1 hr after allylglycine and rats sacrificed 2 hr later. Ether was the anaesthetic employed for studies of circling: α mpt or saline was coadministered with allylglycine and rats were sacrificed 30 min later. Units for dopamine, μg.g^{-1} wet wt. Δ is the change in dopamine level (turnover) after α mpt: units μg.g^{-1} wet wt. hr^{-1}. Further details are given in the text.

*Not significant.

†$P < 0.005$ relative to allylglycine or saline.

not to reduce GAD activity via pyridoxal phosphate [2, 23]. These two drugs differ markedly in their latency of response, 3-mercaptopropionic acid often producing seizures less than 5 min after intraperitoneal administration [2, 23]. The delayed onset of allylglycine-induced convulsions is probably a consequence of a slow transport into brain and of the time necessary to form sufficient active metabolite within GABAergic neurones for GAD inhibition. Once into brain, allylglycine is probably avidly accumulated by the "large neutral" amino acid transport system into intracellular sites [24], where it rapidly undergoes metabolic conversion. Our finding that this amino acid was a more potent inhibitor of GAD *in vivo* than *in vitro* further suggested conversion to a more active metabolite. Although inhibition of GAD activity and reduction of GABA levels are clearly important for allylglycine-induced convulsions, concurrent actions on intermediary metabolism, including energy production, should not be ignored. Our studies of amino acid synthesis indicate a reduced carbon flux into the citric acid cycle and a consequent depletion of energy reserves after allylglycine (cf. [25]). It is of some interest that two unsaturated carboxylic acids closely related to allylglycine both inhibit fatty acid oxidation such that oxidizable substrates become depleted and energy stores exhausted [26].

Allylglycine should be rapidly deaminated by brain L-amino acid oxidase to 2-keto-pent-4-enoic acid. Recently an enhanced inhibition of GAD activity by D- and L-allylglycine in the presence of amino acid oxidase has been reported [27]. According to a preliminary report 2-keto-pent-4-enoic acid is a potent inhibitor of brain GAD activity (K_i 0.1–1 μM) and a powerful convulsant [28]. However, the observed actions of allylglycine probably result from the combined actions of both the parent amino acid and its keto-acid metabolite.

Circling behaviour in rodents has been widely used as an index of the activity of nigrostriatal dopaminergic neurones [29, 30]. A number of reports have indicated that intranigral administration of drugs with actions on GABAergic neurones can elicit circling behaviour [31–33]. This circling may be a consequence of alterations in the firing rate or synaptic functioning of GABAergic neurones of the striato (pallido)-nigral pathway [34–37], which can modulate the activity of dopaminergic nigrostriatal neurones. Thus, this circling should be blocked by neuroleptic drugs and associated with alterations in striatal dopamine synthesis. Our behavioural observations with intranigral allylglycine, when supported by biochemical studies demonstrating an elevated striatal dopamine turnover, suggested that allylglycine-induced circling was a specific result of modulation of impulse flow in nigrostriatal neurones. Inhibitors of GAD have not been previously administered by the intranigral route, but further studies in our laboratory have revealed that other GAD inhibitors (3-mercaptopropionic acid, semicarbazide) also produce contralateral circling upon intranigral administration [38]. The seizure activity which follows circling also supports other evidence for the involvement of nigral GABA stores in seizure activity [39].

Although allylglycine is clearly a potent drug with actions on GABAergic mechanisms, its effects at sublethal doses after direct injection into brain or intraventricular admnistration are transient. The general toxicity of the drug, when coupled with the inability to produce long-term actions on GABA metabolism, indicates that allylglycine does not satisfy the criteria necessary for an effective neurotoxin. Nevertheless, the drug is of particular pharmacological interest and further investigations of the interaction of 2-keto-pent-4-enoic acid with GAD are certainly warranted.

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