

DRUG BIOCHEMISTRY

DIFFERENT RESPONSE OF ANTIOXIDANT DEFENSE SYSTEM TO
ACAMPROSATE IN ETHANOL PREFERRING AND NON-PREFERRING RATSMAŁGORZATA EWERTOWSKA¹, PRZEMYSŁAW Ł. MIKOŁAJCZAK²,
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Abstract: The aim of the study was to investigate whether acamprosate, an agent attenuating relapse in human alcoholics, might modulate antioxidant status in rats chronically administered ethanol. Male Wistar rats were presented with a free choice paradigm between tap water and ethanol solution for three months to distinguish two groups of animals, preferring (PRF) and non-preferring (NPF) ethanol. Then, rats were administered acamprosate, 500 mg/kg/day, per os, for 21 days. The hepatic level of enzymatically-driven lipid peroxidation was enhanced by ethanol in PRF and NPF rats by 67 and 82%, respectively. Unstimulated microsomal lipid peroxidation was increased solely in NPF rats by 33%. Acamprosate caused 36% increase in stimulated lipid peroxidation only in NPF animals. The activities of all hepatic antioxidant enzymes examined: superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase were decreased in rats treated with ethanol by 30 to 64% as compared to controls, however, this decrease was more distinct in ethanol preferring rats. Administration of acamprosate further reduced the activity of antioxidant enzymes only in NPF rats: catalase by 47%, glutathione peroxidase and glutathione S-transferase by 37% and glutathione reductase by 33%. No effect of acamprosate on 4-nitrophenol hydroxylase, a marker of CYP2E1 activity, was observed. As acamprosate enhanced oxidative stress only in the rats non-preferring ethanol, it could be expected that these adverse effects are not demonstrated in alcohol-dependent humans treated with acamprosate.

Keywords: ethanol, acamprosate, lipid peroxidation, antioxidant enzymes

It is generally accepted that oxidative stress plays an important role in ethanol toxicity. The ability of acute and chronic ethanol treatment to increase production of reactive oxygen species (ROS) and enhance peroxidation of lipids as well as oxidative damage of protein and DNA has been demonstrated in a variety of systems, cells and species including humans. Various pathways play a key role in ethanol-induced oxidative stress e.g., redox state changes (decrease in the NAD⁺/NADH ratio) produced as a result of ethanol oxidation, effects on antioxidant enzymes, one electron oxidation of ethanol to the 1-hydroxyethyl radical. It has been shown that CYP2E1 which is specifically involved in ethanol oxidation has a high oxidase activity and plays a crucial role in the microsomal generation of ROS and of ethanol-derived free radicals (1, 2).

Acamprosate (calcium 3-acetamido-1-propanesulfate; CAS number 77337-73-6) (AC), a structural analog of α -aminobutyric acid and homolog of taurine, has been shown to attenuate relapse in human alcoholics (3, 4). There is some evidence suggesting that AC affects glutamatergic receptor system and may act as “partial co-agonist” directly on spermidine-sensitive site of the N-methyl-D-aspartate (NMDA) receptor or *via* metabotropic receptors type 5 (mGluR5) but many aspects of its pharmacological profile are still unknown (5). Moreover, results of some research suggest that, as a taurine analog, AC can act as a ligand for taurine receptors (6). It was also noted that AC administration to laboratory animals significantly increased taurine levels in the brain (7).

Taurine has been shown to be tissue protective in many models of oxidative injury. For example,

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protective effects of taurine against hepatic steatosis and lipid peroxidation were demonstrated in rats administered ethanol (8). Taurine protects hepatocytes against H₂O₂-induced damage (9) and inhibits tert-butylhydroperoxide-induced damage to lipids in rat liver slices (10). It has been found that taurine analog, acamprosate, acts as antioxidant/free radical scavenger in the brain of rats intoxicated with alcohol (11).

The present study was undertaken to assess whether acamprosate can also prevent oxidative damage in the liver of alcoholized rats. We have used a model similar to that applied in our previous experiments, namely selectively bred alcohol-preferring and non-preferring rats (12, 13). This model has been introduced to investigate the neurobehavioral backgrounds for alcoholism and to develop efficacious therapeutic treatment (14).

The aim of the present study was to investigate whether acamprosate might modulate antioxidant status i.e., antioxidant enzymes, microsomal lipid peroxidation and GSH level in the liver of rats chronically administered ethanol.

MATERIALS AND METHODS

Acamprosate (AC) (tabl. 333 mg) was purchased from Campral, Lipha S.A., France, whereas the rest of chemicals were from Sigma Aldrich, Poland.

Experimental design

Thirty male Wistar rats (180 ± 10 g body weight) obtained from certified supplier (Laboratory Animals Breeding, Brwinów, Poland) were used in the experiment. Rats were housed individually in standard plastic cages with stainless steel covers, kept in an animal facility on reversed 12 h light-dark cycle at 20 ± 1°C, controlled humidity (65%) and circulation of air, fed Labofeed diet (ISO 9001) and tap water *ad libitum*.

The rats were presented with a free choice paradigm between tap water and ethanol solution (12% w/w) for three month with two 2-week withdrawal periods after the first and the second month. This procedure – preference development – permitted distinction of two groups of ethanol-drinking animals: (i) rats with a mean intake of ethanol about 4.9 g/kg b.w./day, preferring alcohol (PRF) and (ii) rats with a mean intake of ethanol about 1.2 g/kg b.w./day, non-preferring alcohol (NPF). Additionally, for comparative purposes, throughout the whole period of chronic ethanol treatment, an ethanol-naive control group of animals received

only tap water. Both types of ethanol-drinking rats were divided into 2 subgroups, 6 rats each. In the second part of the experiment, one subgroup of PRF and one subgroup of NPF rats were treated with acamprosate (500 mg/kg b.w./day, *p.o.*, suspended in 1% methylcellulose solution) for 21 days. Two subgroups of animals, PRF and NPF, were treated *p.o.* with 1% methylcellulose solution alone for 21 consecutive days.

During the last week of drug treatment, ethanol and total fluid (sum of water and ethanol solution) intakes were measured and expressed in g/kg/day. The body weight of animals was measured after the drug treatment period.

At the end of the experiment, animals were anesthetized with ketamine (160 mg/kg b.w.; *i.p.*) and sacrificed by decapitation. The livers were removed, perfused with ice-cold 1.15% KCl and homogenized in buffered sucrose solution (Tris, pH 7.55). Microsomal and cytosol fractions were prepared by differential centrifugation according to the standard procedure. Protein concentration in the fractions was determined using Folin-Ciocalteu reagent (15). Liver homogenate for glutathione determination was prepared in phosphate buffer, pH 7.4.

The experiment was performed according to the Local Animal Ethics Committee guidelines for animal experimentation.

Biochemical assays

Microsomal lipid peroxidation in the liver was assayed in two systems: uninduced and Fe³⁺/ADP/NADPH-stimulated (enzymatic). The level of lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) (16). The level of glutathione was evaluated by the determination of non-protein sulfhydryl groups concentration in liver homogenate with Ellman's reagent (17).

Antioxidant enzymes were assayed in the liver cytosol. Glutathione peroxidase (GPx) activity was determined according to Mohandas et al. (18). Hydrogen peroxide was used as a substrate. The disappearance of NADPH was a measure of the enzyme activity. Glutathione reductase (GR) was assayed by measuring NADPH oxidation using oxidized glutathione as a substrate (18). Glutathione S-transferase (GST) activity measurement was based on the spectrophotometric determination of 1-chloro-2,4-dinitrobenzene (CDNB) conjugate formed in a GSH coupled reaction (18). Superoxide dismutase (SOD) assay was based on its ability to inhibit spontaneous epinephrine oxidation (19).

Catalase (CAT) activity was determined by monitoring the rate of hydrogen peroxide decomposition (19). 4-Nitrophenol hydroxylase (PNPH) activity was determined by the method described by Reinke and Moyer (20). The method relies on the formation of 4-nitrocatechol which can be measured spectrophotometrically.

Statistical analysis

The data were expressed as the means \pm SD. One way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons were used.

RESULTS

Our study showed statistically significant differences between rats in voluntary EtOH intake (Table 1). Ethanol-preferring animals (PRF) consumed significantly greater amount of EtOH than the corresponding NPF rats. Multiple AC administration resulted in a decrease in EtOH intake in PRF rats. Simultaneously, no effect of AC on EtOH intake in NPF animals was found. The differences in

the total fluid intake and in the body weight between all groups were statistically insignificant.

Unstimulated lipid peroxidation was moderately increased, by 33%, only in NPF rats. Enzymatically-driven lipid peroxidation was enhanced by ethanol in PRF and NPF rats by 67% and 82%, respectively, as compared to control rats. The drug tested did not affect this parameter in PRF rats. However, in NPF group acamprostate caused 36% increase in the level of stimulated lipid peroxidation (Table 2).

The concentration of hepatic GSH was not changed in any of the groups (data not shown).

Results of antioxidant enzymes activities determination are shown in Figure 1. To facilitate the evaluation of ethanol effect on enzymes tested, the results obtained from ethanol drinking rats (PRF and NPF groups) were compared with those from control group, and PRF rats were compared with NPF rats. Effect of acamprostate was assessed by comparison of ethanol alone drinking rats with rats exposed to ethanol and acamprostate together.

SOD activity was decreased in both groups of rats administered ethanol alone, by 30% in PRF rats

Table 1. Effect of acamprostate on ethanol intake in ethanol preferring and ethanol non-preferring rats.

Treatment	Body weight (g)	Total fluid intake (g/kg/day)	Ethanol intake (g/kg/day)
Control	525 \pm 44	90.7 \pm 4.9	-
NPF	516 \pm 39	89.9 \pm 8.8	1.2 \pm 0.2 ^B
NPF + AC	479 \pm 34	102.1 \pm 16.6	1.6 \pm 1.2
PRF	476 \pm 49	87.6 \pm 15.2	4.9 \pm 1.5 ^{AB}
PRF + AC	466 \pm 69	104.0 \pm 37.4	3.4 \pm 1.5 ^A

NPF = ethanol-non-preferring rats; AC = acamprostate; PRF = ethanol-preferring rats. Results are the mean of 6 rats \pm SD. Results with the same superscripts are significantly different, A = $p < 0.05$, B = $p < 0.01$.

Table 2. Microsomal lipid peroxidation in the liver of rats administered ethanol and acamprostate.

Treatment	Lipid peroxidation unstimulated (nmol/TBARS/mg protein)	Lipid peroxidation Fe ³⁺ /ADP/NADPH-stimulated (nmol/TBARS/mg protein)
Control	0.48 \pm 0.08 ^A	20.1 \pm 3.2 ^A
PRF	0.57 \pm 0.14	33.5 \pm 4.9 ^A
PRF + AC	0.63 \pm 0.02	40.2 \pm 5.2
NPF	0.64 \pm 0.08 ^A	36.5 \pm 2.9 ^{A,B}
NPF + AC	0.68 \pm 0.07	49.7 \pm 4.4 ^B

PRF = ethanol-preferring rats; NPF = ethanol-non-preferring rats; AC = acamprostate. Results are the mean of 6 rats \pm SD. A - PRF and NPF groups are compared with control group; B - groups fed alcohol alone (PRF and NPF) are compared with rats fed alcohol + acamprostate; values with the same superscripts are significantly different, $p < 0.01$.

and by 41% in NPF rats. Acamprosate did not affect the SOD activity in ethanol drinking rats.

CAT activity was diminished by 30% in PRF rats and raised by 26% in NPF rats. Administration of acamprosate had no effect on CAT activity in PRF groups, while in NPF animals acamprosate caused the reduction of enzyme activity by 47% as compared to that in rats receiving ethanol alone.

The activity of GPx was decreased in both groups of rats receiving ethanol, in PRF rats - by 64%, while in NPF rats the decrease was small and insignificant. Administration of acamprosate to PRF rats caused a weak insignificant increase in this enzyme activity as compared to that of the rats receiving

ethanol alone. Conversely, in rats with lower ethanol intake (NPF) a decrease in the GPx activity by 37% after acamprosate administration was observed.

The activity of glutathione reductase (GR) was decreased in PRF and NPF group by 51% and 30%, respectively, as compared to controls. No changes in GR activity in PRF rats administered acamprosate were found. In NPF group the activity of the enzyme was lowered by 33% after acamprosate administration.

The activity of GST in PRF group was decreased by 31% in comparison with controls. In NPF rats the decrease in GST activity was statistically insignificant. Acamprosate administration to

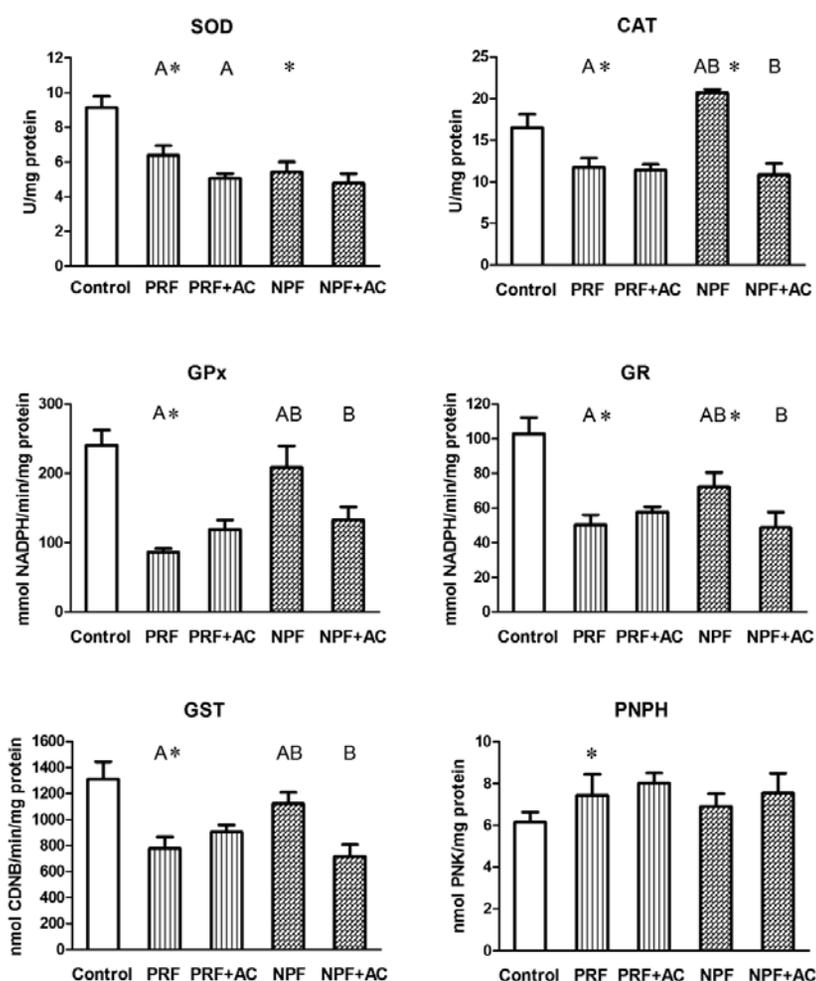


Figure 1. Activity of antioxidant enzymes and p-nitrophenol hydroxylase in the liver of rats administered ethanol and acamprosate. PRF = ethanol-preferring rats; NPF = ethanol-non-preferring rats; AC = acamprosate; SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; GR = glutathione reductase; GST = glutathione S-transferase; PNP = p-nitrophenol hydroxylase. Results are the mean of 6 rats \pm SD. * PRF and NPF groups are compared with control group (without AC treatment); A) PRF group is compared with NPF and with PRF + AC groups; B) NPF group is compared with NPF+AC group. Values with the same superscripts are significantly different, $p < 0.01$.

NPF rats caused further reduction of GST activity by 37%.

4-Nitrophenol hydroxylase activity was slightly increased, by 20%, only in PRF rats receiving ethanol alone. Acamprostate did not affect this enzyme activity in rats drinking ethanol (Figure 1).

DISCUSSION

Two groups of rats (PRF and NPF) differing in voluntary ethanol intake were used in this study and the observed differences in alcohol drinking were in line with our previous report (13). The ethanol drinking pattern observed in the PRF group (> 4.0 g/kg/day) was in agreement with the suggestion, that such level of drinking may be considered as "preference" (21). The dose of AC (500 mg/kg, *p.o.*) was chosen on the basis of the previous findings (12). AC treatment led to a decrease in EtOH intake in PRF rats, what was in accordance with antialcoholic activity of acamprostate.

In the majority of chronic experiments with ethanol feeding an increase in hepatic lipid peroxidation in rodents was observed (22, 23). However, numerous studies using ethanol-feeding models failed to show the increase in lipid peroxidation (24, 25). It was demonstrated that enhanced liver lipid peroxidation was not a constant feature after long term ethanol consumption. It may be due to adaptive processes that result in an enhanced antioxidant defense (24).

In our experiment, enzymatically-driven microsomal lipid peroxidation level in the liver of rats fed ethanol alone was increased to a greater extent than non-stimulated lipid peroxidation level. The assay using enzymatic induction of lipid peroxidation aimed at assessment of the resistance of microsomes of rats fed ethanol to oxidative damage. Our results demonstrated that the liver microsomes of NPF rats exposed to the lower dose of ethanol were less resistant because the level of lipid peroxidation was higher in this group of rats. Thus, it could be suggested that in ethanol-preferring rats some adaptive mechanisms counteracting ethanol-related increase in ROS level started earlier or were more efficient than in NPF rats.

There was a difference in the TBARS level in PRF and NPF rats administered acamprostate. The increase in the level of enzymatically-driven lipid peroxidation was observed only in livers of NPF rats administered acamprostate. It should be emphasized that this increase was consistent with the reduced activity of hepatic antioxidant enzymes in the same group.

Many authors reported a decrease in GSH level in acute intoxication with ethanol (24). It is suggested that GSH depletion is caused by the enhancement of oxidative process mediated by Fe²⁺ ions liberated from ferritin and hemosiderin by ethanol. Similarly to that referring to antioxidant enzymes, the data on liver GSH in animals chronically treated with ethanol are inconsistent. In several experiments the increased GSH concentration in livers of rats fed ethanol was found (25, 26), some authors reported that GSH levels remain unchanged (27). The present studies confirmed the latter findings. According to Oh et al. (25) these discrepancies in the GSH levels might have originated from differences in the strain of rodents used and the dose as well as duration of ethanol administration.

The results of this study show that the activity of hepatic antioxidant enzymes was decreased in rats treated with ethanol alone as compared to that in control group, except for catalase which activity was raised in NPF rats. However, this decrease was greater in ethanol-preferring rats (difference insignificant only for SOD). This is consistent with the free radical theory of ethanol toxicity - the greater the dose of ethanol the greater generation of ROS and inactivation of antioxidant enzymes.

Generally, it was evidenced that an acute ethanol load elicits decreased efficiency of antioxidant enzymes (24). However, there are some controversies regarding the changes in antioxidant enzymes activity in animals chronically exposed to ethanol. It is suggested that chronic intake of ethanol induces CYP2E1 and triggers some adoptive mechanisms counteracting the impairment of cell antioxidant defense system including antioxidant enzymes (1). We have found that in PRF rats GPx activity was markedly reduced as compared to that in control group. In rats with lower intake of ethanol (NPF) the decrease in GPx activity was not statistically significant. In several experiments with a similar protocol (i.e., 4 week ethanol drinking, 6-16 g/kg b.w./day) a decrease in this enzyme activity was also found (25, 26). In two other reports the activity of GPx was not changed (28, 29). On the contrary, the increase in this enzyme activity was observed in rats receiving ethanol in a dose of 5.0 g/kg for 5 and 6 weeks (30). In our experiment GR activity was reduced to a greater extent in PRF than in NPF rats. Similar response of GR to ethanol was reported by Mallikarjuna et al. (31). However, some authors demonstrated an increase in GR activity in rats fed ethanol for several weeks (25, 26). It was hypothesized that the explanation might be the enhancement of gene expression mediated by antioxidant respon-

sive element (ARE) as a response to free radicals generated by ethanol (32). The decrease in GST activity in rats fed ethanol found in the present experiment was not confirmed by reports of other authors, who found that chronic treatment of rats with ethanol resulted in the raise in GST activity (25, 29, 31). It was suggested that the induction of GST by ethanol could be considered as an adaptive response to ethanol-induced oxidative stress. Enhanced GST activity facilitates conjugation of cytotoxic aldehydes, such as 4-hydroxynonenal, with GSH and reduction of lipid hydroperoxides, thus preventing lipid peroxidation (31).

The increase in CAT activity observed in our experiment in rats treated with the lower dose of ethanol, e.g., in NPF rats, was also reported by Oh et al. (25). It is consistent with the interpretation that in animals chronically intoxicated with ethanol some adaptive mechanisms are triggered. However, in PRF rats the activity of CAT was lower than in controls, which could be due to CAT inactivation by free radicals generated by the higher dose of ethanol. SOD activity was decreased in both PRF and NPF animals which was confirmed by other authors' findings (23, 29, 31). The changes in SOD, CAT and GPx in rats fed chronically ethanol reported in literature appear highly contradictory (24). It could be suggested that the response of antioxidant enzymes to chronic ethanol feeding depends on both, the dose and the time of exposure.

In PRF rats acamprosate did not change the activity of antioxidant enzymes. However, the activity of the majority of enzymes in NPF group was decreased after acamprosate administration. SOD was the only enzyme whose activity was not affected by acamprosate in any group of rats fed ethanol. In the available literature we have found one report concerning the effect of an acamprosate analogue, taurine, on hepatic antioxidant enzymes in rats intoxicated with ethanol. Pushpakiran et al. (33) found about 50% decrease in the activity of SOD, CAT and GPx after ethanol administration in a dose of 6 g/kg/day and the recovery of all enzymes activity to the basal level in rats fed simultaneously taurine for 28 days.

The results of the current study differ from those presented by Pushpakiran et al. (33) and Balkan et al. (34), however, direct comparison of the data obtained in our experiment and those in the reports cited is not justified because we used taurine analogue, acamprosate, not taurine itself, which might affect antioxidant enzymes in a different way. In our experiment acamprosate, a potential source of taurine (7), caused a decrease in antioxidant

enzymes activity only in rats fed the lower dose of ethanol. The different response of antioxidant enzymes to acamprosate in PRF and NPF rats was apparently associated with the dose of ethanol, however, the mechanism of this relationship is not clear. It could be suggested that this difference might be due to some adaptive mechanism evoked by the long term exposure to ethanol. Probably this adaptive process appeared earlier in PRF rats consuming the higher dose of ethanol. Hence, hepatic antioxidant enzymes in this group were more resistant to changes caused by acamprosate.

Chronic ethanol consumption leads to an increase in the content of CYP2E1 in the liver and enhances its catalytic activity in the microsomal fraction. In numerous reports induction of CYP2E1 in alcohol-fed animals has been shown to increase the lipid peroxidation in hepatic microsomes (35). It is known that CYP2E1 plays an important role in the generation of hydroxyethyl radical during chronic feeding of ethanol and that ethanol-derived free radicals are major contributors to ethanol-induced oxidative stress and liver injury (1). In the current experiment, the activity of 4-nitrophenol hydroxylase (PNPH), which is known to be CYP2E1 dependent, was assayed (36). The activity of this enzyme was slightly increased only in PRF rats administered ethanol alone. In the other groups no changes in PNPH activity were observed. Similar results were reported by Kerai et al. (22), who did not observe any increase in PNPH activity in rats fed ethanol in liquid diet for 28 days. As in the current experiment, the increase in PNPH activity was noticed only in PRF rats receiving higher dose of ethanol, it could be suggested that the dose of ethanol consumed by NPF rats was too low to induce CYP2E1 activity. The lack of marked CYP2E1 induction observed in our experiment, is consistent with low level of unstimulated lipid peroxidation in the hepatic microsomes of rats chronically fed ethanol. Acamprosate did not affect the activity of CYP2E1 although it was shown that acamprosate analogue, taurine, can inactivate this isoform of cytochrome P450 (22).

Summing up, some consistency in the effects of acamprosate on the parameters of alcohol-induced oxidative stress in the liver was observed, namely, an increase in the level of lipid peroxidation and a decrease in the activity of antioxidant enzymes. However, these effects were restricted to rats non preferring alcohol. Hence, it could be expected that, by analogy to our findings, these adverse effects are not demonstrated in alcohol-dependent humans treated with acamprosate.

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