Effect of chronic exposure to aspartame on oxidative stress in the brain of albino rats

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This study was aimed at investigating the chronic effect of the artificial sweetener aspartame on oxidative stress in brain regions of Wistar strain albino rats. Many controversial reports are available on the use of aspartame as it releases methanol as one of its metabolite during metabolism. The present study proposed to investigate whether chronic aspartame (75 mg/kg) administration could release methanol and induce oxidative stress in the rat brain. To mimic the human methanol metabolism, methotrexate (MTX)-treated rats were included to study the aspartame effects. Wistar strain male albino rats were administered with aspartame orally and studied along with controls and MTX-treated controls. The blood methanol level was estimated, the animal was sacrificed and the free radical changes were observed in brain discrete regions by assessing the scavenging enzymes, reduced glutathione, lipid peroxidation (LPO) and protein thiol levels. It was observed that there was a significant increase in LPO levels, superoxide dismutase (SOD) activity, GPx levels and CAT activity with a significant decrease in GSH and protein thiol. Moreover, the increases in some of these enzymes were region specific. Chronic exposure of aspartame resulted in detectable methanol in blood. Methanol per se and its metabolites may be responsible for the generation of oxidative stress in brain regions.

1. Introduction

Aspartame (L-aspartyl-L-phenylalanine methyl ester), a low-calorie sweetener, is about 180 times sweeter than normal sugar and is used in over 5000 foods and beverages in today’s market. The use of aspartame by diabetic individuals is increasing; it is widely used in the weight loss regime, and approximately 200 million people consume aspartame worldwide (Shapiro 1988). In 1965 aspartame was discovered by James Schlatter of the G.D. Searle Company (Garriga and Metcalfe 1988). Aspartame has a biological effect even at the recommended daily dose (Gombos et al. 2007). Approximately 50% of the aspartame molecule is phenylalanine, 40% is aspartic acid and 10% is methanol (Newsome 1986). Aspartic acid is a metabolite of aspartame that is an excitatory amino acid and is normally found in high levels in the brain. These levels are controlled by the blood–brain barrier, which protects the brain from large fluctuations in plasma aspartate (Maher and Wurtman 1987). Phenylalanine is an amino acid essential to the production of monoamines in the brain and is found in nearly all protein foods (Fernstrom et al. 1983). On the other hand, due to the high levels of phenylalanine in blood, consumption of aspartame may cause brain damage (Haschemeyer and Haschemeyer 1973). Certain brain amino acid levels have been shown to be increased after aspartame consumption (Dailey et al. 1991; Diomede et al. 1991; Yokogoshi et al. 1984). Although methanol is released during aspartame digestion, it only accounts for 10% of the aspartame molecule (Stegink et al. 1983), and among the metabolites, methanol is a toxicant that causes systemic toxicity (Kruse 1992).

Various neurochemical effects due to aspartame consumption have been reported (Coulombe and Sharma 1986; Pan-Hou et al. 1990; Goerss et al. 2000). Their adverse

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neurological effects include headache (Johns 1986), insomnia and seizures after ingestion of aspartame, which are also accompanied by the alterations in regional concentrations of catecholamine (Coulombe and Sharma 1986). Previous findings have shown that aspartate may lead to neurotoxicity through sustained contact with the receptors, such as glutamate producing an excitotoxic effect (Olney et al. 1980).

Large doses of both aspartame as well as these individual metabolites have been tested in humans and other animals, with controversial reports. It has been reported that not only the metabolites of methanol, but methanol per se as well, is toxic to the brain (Jeganathan and Namasiyavay 1998). The primary metabolic fate of methanol is the direct oxidation to formaldehyde and then into formate. The toxic effects of methanol in humans are due to the accumulation of its metabolite formate (Tephly and McMartin 1984; Tephly 1991). The severity of clinical findings in methanol intoxication correlated better with formate levels (Osterloh et al. 1986). Formate is metabolized twice as fast in rat as in monkey (McMartin et al. 1978). The metabolism of formate is mediated through a tetra-hydro-folate-dependent pathway (Eells et al. 1982). Humans and non-human primates are uniquely sensitive to methanol poisoning because of their low liver folate content (Johlin et al. 1990). There are profound differences in the rate of formate oxidation in different species which determine their sensitivity to methanol (McMartin et al. 1978; Eells et al. 1983). In non-human primates and humans, alcohol dehydrogenase mediates this reaction (Makar et al. 1990). In rats and other non-primate species, this reaction is mediated by catalase. Microsomal oxidizing system is reported to be responsible for free radical generation. In addition to that, inhibition of cytochrome oxidase by formate leads to the generation of superoxide, peroxyl and hydroxyl radicals. Tephly (1991) also proved that a catalase system is also one of the major pathways of methanol oxidation in rat hepatocytes. Free methanol is created from aspartame when it is heated to above 86°F (30°C). This would occur when an aspartame-containing product is improperly stored or when it is heated (e.g. as part of a ‘food’ product such as Jello). Methanol is gradually released in the small intestine when the methyl group of aspartame encounters the enzyme chymotrypsin. Methanol breaks down into formic acid and formaldehyde in the body. Formaldehyde is a deadly neurotoxin (Lee et al. 1994; Eells et al. 2000). Rodents do not develop metabolic acidosis during methanol poisoning, owing to their high liver folate content, and in order to create similar results in human beings, only folate-deficient rodents are required to accumulate formate in order to develop acidosis in methanol poisoning (Lee et al. 1994; Eells et al. 2000).

Hence, in this study, in order to mimic the human situation, a folate deficiency status was induced by administering methotrexate (MTX). The focus of this study is to investigate whether the chronic oral administration of aspartame (75 mg/kg) can release methanol as a by-product after metabolism and whether it induces oxidative stress in the rat brain regions after aspartame administration.

2. Materials and methods

2.1 Animals

Wistar strain male albino rats (200–220 gm) were maintained under standard laboratory conditions with water and food. For the folate-deficient group, folate-deficient diet was provided for 45 days prior to the experiment and MTX was administered for a week before the experiment. The animals were handled according to the principles of laboratory care framed by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Prior to the experimentation, proper approval was obtained from the Institutional Animal Ethical Committee (No: 01/032/2010).

2.2 Chemicals

Aspartame, methotrexate and malondialdehyde were purchased from Sigma-Aldrich Co., St. Louis, USA. All the other chemicals were of analytical grade obtained from Sisco research laboratory, Mumbai, India.

2.3 Experimental design

The rats were divided in to three groups, namely, saline control, MTX-treated control, and MTX-treated aspartame-administered groups. Each group consisted of six animals. Aspartame mixed in sterile saline was administered orally (75 mg/kg body weight), based on the earlier report (Leon et al. 1989). However, early reports on aspartame of higher doses included two different authors, namely, Labra-Ruiz et al. (2007) and Leon et al. (1989), and the reports are controversial. This provided additional interest to use this dosage in our study. In order to confine within the human exposure limit, this dose was selected. A 1 L (approx. 1 quart) aspartame-sweetened beverage contains about 56 mg of methanol was used. Heavy users of aspartame-containing products consume as much as 250 mg of methanol daily, or 32 times the EPA limit.

MTX in sterile saline was administered (0.2 mg/kg/day) subcutaneously for 7 days to folate-deficient treated as well as to folate-treated aspartame groups (Gonzalez-Quevedo et al. 2002). One week after treatment with MTX, folate deficiency was confirmed by estimating the urinary excretion of formamino glutamic acid (FIGLU) (Tabor and Wyngarden 1962). From the eighth day, only the MTX-treated aspartame
group received the aspartame, whereas the other two groups received equivalent volumes of saline as an oral dose and all animals were handled similarly. The chronic dose of aspartame was given for 90 days and all the animals were fed folate-deficient diet except the control animals till 90 days.

2.4 Sample collections

The blood samples and isolation of brain was performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. Stress-free blood samples were collected as per the technique described by Feldman and Conforti (1980).

2.5 Brain dissection

The animals were sacrificed and the brain was immediately removed and washed with ice-cold phosphate buffered saline (PBS). To expose the brain, the tip of curved scissors was inserted into the foramen magnum and a single lateral cut was made into the skull extending forward on the left and right side. With a bone cutter, the dorsal portion of cranium was peeled off, and using a blunt forceps, the brain was dropped onto the ice-cold glass plate, leaving the olfactory bulbs behind. The whole process of removing brain took less than 2 min. After removing the brain, it was blotted and chilled. Further dissection was made on ice-cold glass plate. The discrete regions of brain (cerebral cortex, cerebellum, midbrain, pons medulla, hippocampus and hypothalamus) were dissected according the method given by Glowinski and Iverson (1996). The homogenate (10%w/v) of the individual regions were prepared in a Teflon-glass tissue homogenizer, using ice-cold Tris HCl (100 mm, pH 7.4) buffer and centrifuged separately in refrigerated centrifuge at 300 g for 15 min. The supernatant was used for analysing the parameters in this study.

2.6 Blood methanol measurement

100 mL plasma was deproteinized with equal volume of acetonitrile and centrifuged for 7 min at 4°C (Dorman et al. 1994). The supernatant (20 mL) was analysed for blood methanol and formate using a HPLC refractive index detector system (Shimadzu RID, Japan) (equipped with Rezex ROA-organic acid column 300 mm×7.5 mm I.D., Phenomenex) with the security guard cartridge (AJ0 4490 Phenomenex). Column oven was used to maintain the temperature at 60°C. The mobile phase was 0.026 N sulphuric acid (Pecina et al. 1984). By using methanol as an external standard, the recovery of methanol (HPLC grade) from blood was found to be 92–96%. Linearity for methanol was found to be 5–500 mg/100 mL. The detector sensitivity for methanol was found to be 5 mg/100 mL and reproducibility was >93%.

2.7 Plasma corticosterone

Plasma corticosterone level was estimated according to the method described earlier by (Mattingly 1962) using excitation at 470 nm and emission at 530 nm in fluorescence spectrophotometer.

2.8 Lipid peroxidation

15 gm trichloro acetic acid was added to 100 mL of 0.25 N hydrochloric acid. 15 mg of thio barbituric acid was dissolved in 4 mL of trichloro acetic acid in HCl mixture. To 0.1 mL of homogenate, 0.4 mL trichloro acetic acid–thio barbituric acid–hydrochloric acid mixture was added and kept in a boiling water bath for 20 min. Then, it was cooled to room temperature gradually, followed by addition of 1 mL of n-butanol, vortexed well and centrifuged for 10 min. The supernatant was taken and was read at 532 nm in spectrophotometer. The level of LPO was expressed as nanomoles of malondialdehyde (an intermediary product of lipid peroxidation, using thio barbituric acid/mg protein (Ohkawa et al. 1970).

2.9 Superoxide dismutase (EC 1.15.1.1) (SOD)

0.5 mL of homogenate was mixed with 0.5 mL of ethanol chloroform mixture; the content was shaken well for 15 min and the centrifuged for 10 min. To 0.5 mL of supernatant taken, 2 mL of Tris-HCl buffer (pH 8.2) was added. Finally, 0.5 mL of freshly prepared pyrogallol solution was added and read at 470 nm in 0, 1, 2 and 3 min intervals. The activity was expressed as Units/min/mg protein (Marklund and Marklund 1974).

2.10 Catalase (EC.1.11.1.6)(CAT)

Four sets of 0.1 mL of homogenate was mixed with 1 mL of phosphate buffer and 0.5 mL of H₂O₂, and this reaction was arrested by addition of 2 mL potassium dichromate acetic acid reagent at various intervals of 0, 15, 30 and 60 s. Then all the samples were kept in a boiling water bath for 10 min and were cooled gradually. The developed green colour was read at 610 nm. The activity was expressed amount of H₂O₂ utilized/min/mg protein (Sinha 1972).

2.11 Glutathione peroxidase (EC.1.11.1.9) (GPx)

To three sets of test tubes 0.4 mL of phosphate buffer, 0.1 mL of sodium azide, 0.2 mL of standard GSH solution
and 1 mL of H$_2$O were sequentially mixed. To this 0.1 mL of H$_2$O$_2$ was added and the reaction in individual set was arrested with 1 mL of 10% TCA at 0, 1.5, 3 min intervals and then centrifuged for 10 min. To the 0.2 mL of supernatant 1.8 mL of H$_2$O followed by 1 mL of 5% TCA were added and allowed to stand at room temperature for 20 min. Then 4 mL of phosphate solution was added, followed by 0.5 mL of DTNB. The optical density was taken at 412 nm within 5 min. The GPx activity is expressed as units/min/mg protein (Rotruck et al. 1973).

2.12 Reduced glutathione

To 0.2 mL of homogenate, 1.8 mL of H$_2$O, 1 mL of 5% TCA were sequentially added and kept at room temperature for 20 min. Then, 4 mL of 0.3 M phosphate solution followed by 0.5 mL of DTNB was added and mixed well. The optical density was taken at 412 nm within 5 min. from the addition of DTNB. Reduced glutathione (GSH) was measured by its reaction with 5.5- dithiobis 2 nitro benzoic acid (DTNB), to form a compound that absorbs at 412 nm (Moron et al. 1979). The level of GSH is expressed as μg of GSH/mg of protein.

2.13 Protein thiol

To 1 mL of homogenate was added 1.5 mL of Tris-hydrochloric acid buffer (pH 8.2) which contained 0.02 ethylene di-amine tetra acetic acid . This was followed by the addition of 0.1 mL of DTNB solution and 7.5 mL methanol. The contents were mixed well in a vortex mixture and than centrifuged at 3000 g for 10 min. The colour developed was read at 412 nm. The level of thiol was expressed as μg/mg protein. Tissues were analysed for protein and sulfhydryl concentration (Sedlack and Lindsay 1968).

2.14 Statistical analysis

Statistical analysis was carried out using the SPSS statistical package version 17.0. The results are expressed as mean ± STD and the data were analysed by the one-way analysis of variance (ANOVA) followed by Turkey’s multiple comparison tests when there was a significant ‘F’ test ratio. The level of significance was fixed at $p \leq 0.05$.

3. Results

The data from various groups for the individual parameters are presented as bar diagram with mean ± STD.

3.1 Blood methanol level and plasma corticosterone level

The data for the blood methanol level and plasma corticosterone level after chronic exposure to aspartame administration are given in figure 1. Even after chronic exposure, the aspartame-treated MTX animals showed marked increase in the blood methanol level (df 2, F=2789) and plasma corticosterone level (df 2, F=874.6) from controls as well as from the MTX-treated group.

3.2 Lipid peroxidation, superoxide dismutase and catalase levels

The results are given in figure 2. The LPO levels in the lipid peroxidation (LPO) MTX-treated animals did not differ from those in the controls. However, the aspartame-treated MTX animals showed a marked increase in the LPO level in the entire brain regions studied, which included cerebral cortex (df 2, F=182), cerebellum (df 2, F=125.8), midbrain (df 2, F=272.2), pons-medulla (df 2, F=206.5), hippocampus (df 2, F=187.4) and hypothalamus (df 2, F=235.7) from the control as well as from the MTX-treated animals.

The results are given in figure 3. The superoxide dismutase (SOD) activity in the MTX-treated animals did not differ from the controls. However, the aspartame-treated MTX animals showed a marked increase in the SOD activity as compared to control and MTX-treated animals, in the cerebral cortex (df 2, F=50.5), cerebellum (df 2, F=19.2), midbrain (df 2, F=46.5), pons-medulla (df 2, F=90.3) hippocampus (df 2, F=106) and hypothalamus(df 2, F=84.8) from. The results are given in figure 4. The CAT activity in the MTX-treated animals did not differ from the controls. However, the aspartame-treated MTX animals showed a marked increase in the CAT activity as compared

![Figure 1](image_url)

Figure 1. Methanol level (mM) in rat blood upon aspartame administration (75 mg/kg) using HPLC and plasma corticosterone level.

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to control and MTX treated animals, in the cerebral cortex (df 2, F=130.2), pons-medulla (df 2, F=51), hippocampus (df 2, F=169) and hypothalamus (df 2, F=289.8). In the midbrain (df 2, F=196.7) and cerebellum (df 2, F=126), only a marked increase in CAT activity as compared to control was seen and this remained similar to MTX-treated animals.

3.3 Glutathione peroxidase, reduced glutathione and protein thiol levels

The results are given in figure 5. The GPx activity in the MTX-treated animals did not differ from the controls. However, the aspartame-treated MTX animals showed a marked increase in the GPx activity as compared to control and MTX-treated animals, in the cerebral cortex (df 2, F=35.4) and midbrain (df 2, F=89.9). In the cerebellum (df 2, F=83.6), pons-medulla (df 2, F=67.7), hippocampus (df 2, F=81.7) and hypothalamus (df 2, F=136.9), a marked increase in GPx activity as compared control was seen, and this remained similar to MTX-treated animals. The results are given in figure 6. The GSH levels in the MTX-treated animals did not differ from the controls. However, the aspartame-treated MTX animals showed a marked decrease in the GSH levels as compared to control and MTX-treated animals, in the cerebral cortex (df 2, F=21.4), midbrain (df 2, F=28.7), pons-medulla (df 2, F=23.1) and hippocampus (df 2, F=14.62). However, in the hypothalamus (df 2, F=19.4) the GSH level showed marked decrease as compared to controls but not the MTX-treated group. Moreover, in the cerebellum, the GSH level remained similar to that in control and MTX-treated animals. The results are given in figure 7. The protein thiol levels in the MTX-treated animals did not differ from

![Figure 2. Effect of aspartame (75 mg/kg body weight) on LPO activity (mmol/mg tissue) in rat brain discrete regions.](image)

![Figure 3. Effect of aspartame (75 mg/kg body weight) on SOD activity (units/mg tissue) in rat brain discrete regions.](image)
those in the controls. However, the aspartame-treated MTX animals showed a marked decrease in the protein thiol levels as compared to control and MTX-treated animals, in the entire brain regions studied such as the cerebral cortex (df 2, F=114.7), cerebellum (df 2, F=158.9), midbrain (df 2, F= 83.7), pons-medulla (df 2, F=37.1), hippocampus (df 2, F= 68.6) and hypothalamus (df 2, F=119.3).

4. Discussion

This study deals with strengthening the toxic metabolite methanol released in the body after the consumption of aspartame. The alteration in the free-radical-scavenging enzymes in the aspartame-administered animals clearly indicates that free radical generation may be due to the methanol which is one of the metabolic products of aspartame. Even after chronic aspartame administration, there was detectable blood methanol in the aspartame-treated animals. Methanol is metabolized by three enzyme systems, namely, the alcohol dehydrogenase system, the catalase peroxidative pathway and the microsomal oxidizing systems. Among these, the microsomal oxidizing system is reported to be responsible for free radical generation (Goodman and Tephly 1968). Exposure to methanol causes oxidative stress by altering the oxidant/antioxidant balance in lymphoid organs of rat (Parthasarathy et al. 2006). The condition could also be associated with the abundance of redox active transition metal ions, and the relative death of antioxidant defence system (Samuel et al. 2005). In addition to that, oxidative stress may lead to the generation of superoxide, peroxyl and hydroxyl radicals (Keyhani and Keyhani 1980). The increase in free radicals could not be ignored as cells can be injured or killed when the ROS generation overwhelms the cellular antioxidant capacity (Oberly and Oberly 1986). Particularly, the brain is more vulnerable to oxidative

![Figure 4](image1.png)

**Figure 4.** Effect of aspartame (75 mg/kg body weight) catalase activity (units/mg tissue) in rat brain discrete regions.

![Figure 5](image2.png)

**Figure 5.** Effect of aspartame (75 mg/kg body weight) on GPx activity (units/mg tissue) in rat brain discrete regions.
damage due its high oxygen consumption and due to the presence of high levels of polyunsaturated fatty acids (Floyd and Carney 1992).

SOD converts superoxide anion ($O_2^{-}$) to hydrogen peroxide ($H_2O_2$) (Akyol et al. 2002), which are subsequently converted to water and molecular oxygen by glutathione peroxidase or catalase (Dringen 2000). GPx is a major antioxidant enzyme in many tissues and has been speculated to be a major antioxidative mechanism in the brain (Barlow-Walden et al. 1995). In this study there was a significant increase in the SOD activity after chronic aspartame ingestion with an associated increase in the catalase activity. As catalase is the main scavenger of $H_2O_2$ at high concentration (Kono and Fridrich 1982), that indicates the accumulation of $H_2O_2$ as a result of increased SOD activity. It has been found that consumption of methanol provokes changes in the activity of antioxidant enzymes with an increase in the activity of catalase (Skrzydlewska et al. 1998). Hence, the increase in this enzyme activity may also be due to the free radicals generated. According to Yu (1994), free radicals may also induce the expression of antioxidant enzymes, thereby enhancing the neuronal resistance to subsequent oxidative challenges.

LPO is a free-radical-mediated process. In the entire rat brain regions after aspartame consumption, a marked increase in LPO was noted, which also supports the generation of free radicals. Generally, when the generation of reactive free radicals overwhelms the antioxidant defense, LPO of the cell membrane occurs. In spite of the increase in the SOD, CAT and GPx enzyme system in order to prevent the accumulation of free radical, a marked increase in the LPO in the entire brain regions indicated this result and possible loss of membrane integrity. Moreover, reactive-oxygen-mediated damage could not be overlooked particularly to proteins, which has been shown to deleteriously affect cellular functions (Sohal et al. 2002).

GSH is considered to be one of the most important components of the antioxidant defense of living cells. The reduced tri-peptide GSH is a hydroxyl radical and singlet oxygen scavenger, and participates in a wide range of cellular functions. Since GSH is present in high intracellular concentrations, there is a high probability that reactive oxygen species (such as superoxide, singlet oxygen and hydrogen peroxide) is to be quenched by reaction with GSH before they can initiate their chain reaction-damaging effects (Jones et al. 1981). The depletion of

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**Figure 6.** Effect of aspartame (75 mg/kg body weight) on GSH concentration (mmol/mg tissue) in rat brain discrete regions.

**Figure 7.** Effect of aspartame (75 mg/kg body weight) on protein thiol levels (mmol/mg tissue) in rat brain discrete regions. CC, cerebral cortex; CB, cerebellum; MB, mid brain; PM, pons medulla; HI, hippocampus; HY, hypothalamus. The level of significance was fixed at $p \leq 0.05$ ($n= 6$).
GSH may be one of the reasons for the increase in the vulnerability added to free-radical-induced damage. In addition, the decrease in GSH concentration is quite possible because the methanol metabolism also depends upon GSH (Pankow and Jagielki 1993).

The significant decrease in the protein thiol observed in this study may be due to the oxidation of proteins in oxidative process, and it is also justified by the decreased level of one of the major thiol substance GSH. Similarly decreased protein thiol in brain due to oxidative damage was reported by Patsoukis et al. (2004), and Nikolaos et al. (2004) support the present findings.

From the foregoing it is clear that the methanol, which is a by-product of aspartame, may be responsible for the alteration observed in the free-radical-scavenging system. Since methanol is freely permeable through membranes and lipids, it also gets distributed in the brain tissues and may cause the damage. Increased production of free radicals and increased oxidative damage to proteins in distinct brain regions, retina and optic nerve after methanol administration (Rajamani et al. 2006) was also reported earlier lending support to the present findings. The scientific reports on aspartame also revealed that aspartame consumption affects the brain. The aspartame intake has been reported to be responsible for neurological and behavioural disturbances in sensitive individuals (Johns 1986). Moreover, Mourad and Noor (2011) have observed that the daily ingestion of aspartame for 4 weeks induces significant increase in the LPO levels in the cerebral cortex, which is accompanied by significant decrease in GSH content and a significant increase in SOD activity, which is also in agreement with this study. According to them, the decrease in GSH content and increase in SOD activity persisted until after 6 weeks of aspartame treatment. Moreover, they add that aspartame-induced oxidative stress may depend on the duration of aspartame administration even within the acceptable daily intake dose. The present study reveals that aspartame administration in the body system persists for longer duration, which indicates the possible accumulation of methanol and its metabolite. Since it is consumed more by diabetic people whose metabolism is already altered, it is essential to do more work on these lines and create awareness regarding the usage of this artificial sweetener.

5. Conclusion

After chronic exposure of aspartame, detectable methanol continues to circulate in the blood; methanol per se and its metabolites may be responsible for the generation of oxidative stress in brain regions. This study confirms the presence of toxic metabolite after aspartame administration and it emphasises the need to caution the people who are using aspartame routinely.

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