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ORIGINAL ARTICLE

Hepatoprotective effects of whey protein isolate against acute liver toxicity induced by dimethylnitrosamine in rat

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Abstract In order to investigate the hepatoprotective effects of whey protein isolate against acute liver toxicity induced by dimethylnitrosamine (DMN), a randomized experimental study was conducted. Forty-eight Sprague Dawley rats were randomly divided into three groups. Groups A and B consumed a diet containing casein, and group C received a diet containing whey protein isolate for 18 days. Group A was then given an intraperitoneal saline injection. It continued on the casein diet for another 4 days before being sacrificed. Each animal in groups B and C was given a single intraperitoneal injection of DMN (30 mg/kg) on the 18th day of the study. All groups continued their diets for 4 days before their euthanasia. The supply of whey protein diet resulted in a decrease in aspartate amino transferase, alanine amino transferase, alkaline phosphatase, total bilirubin, and malondialdehyde (MDA). Morphological and biochemical data suggested that a diet containing whey protein isolate decreased DMN-induced liver damage and, therefore, had beneficial effects on hepatic failure.

Keywords Whey protein · Acute liver injury · Dimethylnitrosamine · Rat

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Introduction

Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species (ROS) that cause oxidative stress (Pihlanto 2006). It is stated that overproduction of ROS is a unifying mechanism of injury that occurs in many clinical disease processes, such as liver failure, cancer, and aging (Mostafavipour et al. 2008). Damage in the liver as a major health organ which has central, critical, and biochemical roles in the metabolisms, detoxification, and elimination of substances induces excessive stress to the physiological activities of other organs of the body. Diseases increase stress in different body organs and cause overproduction of free radicals and thereby the condition is aggravated (Fridrich 1978; Actis-Goretta et al. 2004).

Dimethylnitrosamine, a potent hepatotoxin, carcinogen, and mutagen, induces hepatic damage and hence increases oxidative stress (Kim et al. 2007; Lukivskaya et al. 2004). A high dose of dimethylnitrosamine (DMN) administered by a single injection in experimental animals causes central necrosis and hemorrhage and necrosis at its acute phase similar to human fulminant hepatitis (Jin et al. 2003) and induces liver fibrosis in a highly reproducible manner by establishing micronodular fibrosis and cirrhosis after 3 weeks of administration (Fehér et al. 1989).

In circumstances of overproduction of free radicals, the body's antioxidant defense system is not able to inhibit the damages caused by oxidants. Therefore, consuming foods containing antioxidant may correct this imbalance and revive the anatomy and physiology of the injured organ almost to its normal state (Kullisaar et al. 2003; Zommara et al. 1996). Many plant and animal proteins are known to possess significant antioxidant properties (Peña-Ramos et al. 2004; Okada and Okada 1998; Chang et al. 2007). The potential for whey protein used as a natural antioxidant was demonstrated in a study by Sukkar and Bounous (2004). It belongs to a class of protein that comprises approximately 20% of the total milk proteins (Hakkak et al. 2000; Hoffman and Falvo 2004; Eigel et al. 1984). All of the constituents of whey protein provide high levels of essential and branched chain amino acids. This protein possesses many beneficial bioactive properties as well (Hoffman and Falvo 2004). Beside this, it has the ability to act as an agent with beneficial properties such as antioxidant, antihypertensive, anticarcinogen, hypolipidemic, antiviral, antibacterial, and chelating agent (Hara et al. 2002; Marshall 2004). Whey protein concentrates and isolates are considered as functional food ingredients of important nutritional and health effects (Saleh et al. 2007).

While early studies relied on indirect measurement or showed an anabolic effect of protein or amino acid supplementation, more recent studies directly demonstrate that whey protein and its constituent amino acids efficiently promote protein synthesis (Bos et al. 1999; Tipton et al. 1999; Fouillet et al. 2002). Because of its high protein quality score and containing a relatively high proportion of branched chain amino acids, whey protein affects muscle metabolism and regeneration (Ha and Zemel 2003). As stated earlier, its antioxidant property is another health benefit of whey protein. It is stated that the main mechanism by which whey protein exerts this effect is conversion of the amino acid cysteine to glutathione (GSH), i.e., this protein is thought to be attributable to relatively high levels of *g*-glutamylcysteine groups which serve as substrate for glutathione synthetase, which is in turn one of the main intracellular antioxidants in the body (Marshall 2004; Hakkak et al. 2001). GSH participates directly in the destruction of reactive oxygen species (Tseng et al. 2006).

Despite current advances in medical management, no definite therapy for acute liver failure exists, and this abnormality remains a major public health concern. Therefore, prevention of the disease and supporting the body against such disorders should strongly be taken into consideration. Some aspects of the hepatoprotective effects of whey protein on chronic liver injuries have previously been reported (Kume et al. 2006); however, the mechanism of action of this product on protecting the hepatocytes from injury and enhancing tissue regeneration is unclear. The effect of this protein on acute liver injury is also not clear. Therefore, the present investigation was undertaken to evaluate the antioxidant and protective effect of whey protein isolate in acute liver damage caused by DMN in Sprague Dawley rats considering liver function test and liver tissue morphology.

Materials and methods

Animals and diets

Forty-eight male Sprague Dawley rats aged approximately 8 months and weighing 200±10 g (Razi institute, Tehran, Iran) were used in this experiment. They were kept according to experimental design in individual steel cages of 22×30×25 cm dimension, at 22±2°C temperature and 50±10% humidity with (12/12 h) light/dark cycle and food and water ad libitum. Groups A and B received a diet containing casein protein in addition to all other components, whereas the rats of group C received the same diet as the other two groups except for the protein content which was whey protein isolate (WPI).

The composition of the purified diet for the experimental rodents used during adult maintenance was as follows: casein or whey protein isolate 20/100 g (Dor Shimi Marjan, Tehran, Iran; Arla food Ingredients amba Denmark respectively), corn starch 56.07/100 g, sucrose 10/100 g, corn oil 4/100 g, cellulose 5/100 g, mineral mixture (AIN-93) 3.5/100 g and vitamin mixture (AIN-93) 1/100 g (Razi and Osveh companies Tehran, Iran), L-Cystine 0.18/100 g.

Food efficiency ratio (FER) of the different diets was calculated as body weight gain (gram) per food intake (gram; Smith and Circle 1971), and protein efficiency ratio (PER) was calculated as weight gained (gram) per protein intake (gram) in the period of the experiment (Pellet and Young 1980).

The study protocol and ethical aspects were approved by the ethics committee of the Research Council of the Dean of Research Affairs of Shiraz University of Medical Sciences.

Treatment

The rats were randomly divided into three equal groups, each having 16 rats. Prior to the experiment, the rats were allowed to adapt to the laboratory environment for 3 days. Experiments were performed for 18 days on the groups of rats as follows: The rats of group A were fed casein and treated with saline on day 18. The animals of group B (CAS+DMN) were fed a diet containing casein and then treated with 30 mg/kg DMN (Sigma Chemical, St. Louis,

MO, USA) on day 18. The rats of group C (WPI+DMN) were fed a diet containing WPI and then treated with DMN on day 18. All groups continued their diets for 4 days before their sacrifice.

The animals were weighed at the beginning of the study and every 4 days thereafter for the entire experimental period. Food intake was also determined every 4 days.

Biochemical and histopathological assessments

To assess the hepatic function, blood samples were collected before the animals were euthanized. Biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total protein, albumin, total bilirubin, and malondialdehyde (MDA) of the sera were measured using commercially available kits (Zist Chemi and Pars Azemoon, Tehran, Iran). ALT and AST were estimated by colorimetric methods according to Reitman and Frankel (1957), and ALP according to Belfield and Goldberg (1971). MDA, an indirect index of lipid peroxidation, was assayed as thiobarbituric acid reactive substances (TBARS) using colorimetric method. Briefly, 0.5 ml serum was added to 2 ml thiobarbituric acid (TBA) reagent containing 0.375% TBA (Sigma Chemical, St. Louis, MO, USA), 15% trichloroacetic acid, and 0.25 mol/l HCl. The mixture was boiled for 15 min, cooled, and centrifuged at 1,700 g for 15 min at 4°C. The absorbance of the supernatant was measured at 532 nm. The TBARS concentration was calculated using 1,1,3,3-tetraethoxy propane (Sigma Chemical, St Louis, Mo, USA) as a standard. Results are expressed as nanomole/milliliter (Mostafavipour et al. 2008).

On the 22nd day, all rats in each group were sacrificed under anesthesia with diethylether. The rats were then euthanized. The abdomen was opened immediately by a midline incision, and the liver was removed for histopathological analysis. The whole liver was weighed, and a small portion of the liver, approximately 1 cm^3, was isolated immediately and rinsed in cold saline before being placed in 10% neutral buffered formalin. The fixed tissue was then dehydrated, cleared, and embedded in paraffin wax. Sections of 5 μm in thickness were stained with H&E and studied by a routine light microscope.

Statistical analysis

The normality of distributions was checked for all variables. Differences between the groups were tested using analysis of variance (ANOVA) followed by Scheffe or Tamhane's post hoc comparisons. Data were expressed as mean and standard deviation (SD), and statistical significance is defined as $P<0.05$. All statistical analyses were computed using SPSS version 13 for Windows (SPSS Inc., Chicago, IL, USA).

Results

As shown in Table 1, although the food and macronutrient intake (protein, fat, and carbohydrate) of rats in the CAS+DMN (group B) and WPI+DMN (group C) were significantly lower than the control group (group A; $P<0.05$), weight gain and final weight did not significantly differ between the three groups. In addition, no significant differences exist in the FER, PER, and relative hepatic weight between the three groups.

Significant elevations in serum AST, ALP, and total bilirubin levels were recorded in the groups treated with DMN (Table 2). WPI ameliorated this increase significantly for AST and ALT; however, this improvement was not significant for total bilirubin. A significant decrease in the serum albumin level of the rats treated with DMN ($P<0.05$) was observed but that was significantly corrected by the WPI diet ($P<0.05$). DMN induced a 4.2-fold increase in the serum ALT levels in those rats that were fed the CAS diet as compared with that of the controls. However, the amount of serum ALT level in the rats that fed the WPI diet was even lower than that of the control group. DMN did not significantly affect the serum total protein and MDA.

Histopathological findings

Livers of the rats of the control group were grossly and histopathologically normal. The liver of all 13 rats of the CAS+DMN group showed different ranges of hepatocyte necrosis, congestion, and intravascular coagulation of the central vein, and hemorrhages. Nine rats of this group showed severe necrosis, massive hemorrhages, and disruption of tissue architecture (Figs. 1 and 2), while the livers of the rest were mildly to moderately necrotic. Congestion and hemorrhages were commonly seen in areas of most intense necrosis, especially around the central veins and in midzonal areas. Most of the hepatocytes in the centrilobular region were lysed and disappeared, and the nuclei of the remaining cells in this area and to a lesser extent the midzonal region were either piknotic, fragmented, or karyolyzed. Lymphocytes, plasma cells, and macrophages were mildly to moderately infiltrated in the portal areas of the liver of some of the rats of this group. The remaining hepatocytes in the centrilobular, midzonal, and periportal areas of the liver of seven rats of this group were swallowed and showed severe fatty degeneration and ballooning or eosinophilic changes of the cytoplasm and exhibited coarse chromatin granules. Disseminated intravascular coagulation was observed in the liver of seven (Figs. 1 and 2), and

Table 1 Food and macronutrient intake, final weight, weight gain, food efficiency ratio (FER), protein efficiency ratio (PER), hepatic weight, and relative hepatic weight of the rats of all three groups

Values in parenthesis indicate numbers of rats at the end of the study. Data are expressed as the mean ± SD. In each row, values with different letters are significantly different at $P<0.05$ (one-way ANOVA test)

	Control (n=16)	CAS+DMN (n=13)	WPI+DMN (n=16)
Food intake (g/day)	13.02±1.20 b	10.33±1.52 a	10.15±0.76 a
Protein intake (g/day)	2.60±0.24 b	2.07±0.30 a	2.03±0.15 a
Fat intake (g/day)	0.52±0.05 b	0.41±0.06 a	0.40±0.03 a
Carbohydrate intake (g/day)	9.25±0.86 b	7.34±1.08 a	7.21±0.54 a
Final weight (g)	243.56±18.69	233.92±12.38	234.19±18.61
Weight gain (g)	43.50±14.09	31.77±13.64	34.09±16.61
Food efficiency ratio	0.15±0.04	0.13±0.05	0.15±0.07
Protein efficiency ratio	0.75±0.19	0.65±0.27	0.75±0.34
Hepatic weight (g)	11.49±1.58	12.10±1.30	11.56±1.43
Relative hepatic weight	4.752±0.64	5.18±0.60	4.95±0.59

telangiectasis was evident in the liver section of four rats of this group. No mitotic figures or regeneration of the hepatocytes was evident in the liver of the animals of this group.

Whey protein significantly suppressed the DMN-induced histopathological changes such as hemorrhages, necrosis, and endothelial cell damage and inflammatory cell infiltration in the liver parenchyma. The hepatocytes of nine rats of the whey protein diet group showed mild coagulative necrosis, either as isolated cells in the centrilobular area or at most, one to three rows of the cells restricted around the central vein with hyperemia of the capillaries in three and telangiectasis in two rats (Figs. 3 and 4). The liver from one rat of this group showed severe necrosis and telangiectasis. Hyperemia of the sinusoids was present in one rat, and regeneration of the hepatocytes was seen in the liver of four animals of this group. The livers of the five remaining animals of this group were almost normal at histopathological level.

Discussion

It is possible that the pain due to acute liver injury induced by DMN administration affected the appetite and a concomitant decrease in food intake, thus resulted in a minor reduction in the body weight of the rats. However, due to the short period of this study, this loss of appetite did not end in a significant weight reduction. Nonsignificant increase in the weight gain of the control rats might be due to nonlinear relation between the rate of food intake and weight gain. This is in line with the findings of Kusunose et al. (2002) that noted an initial 5% decrease in body weight of rats administered with DMN. In addition, Kume et al. (2006) found that food intake and body weight gain were almost the same between the diets containing casein and whey proteins in D-galactosamine-induced hepatitis and liver fibrosis in Sprague Dawley rats. Despite a slight decrease in PER and FER in CAS+DMN, these factors did not show a significant difference between the three groups of the present experiment. In addition, the relative liver weights of the rats of the three groups were not significantly different. This finding is in contrast with those of Saleh et al. (2007) which reported a higher growth rate and FER in rats fed a diet containing WPI as compared with rats fed a casein-based diet.

DMN-treated animal model is known to be a major cause of hepatocellular damage and fibrosis (Kim et al. 2007). Damage and consequent cytolysis of hepatic cells caused by DMN induce an increase in serum AST, ALT, and ALP levels (George and Chandrakasan 2000; George et al. 2001). After DMN injection, remarkable increases in serum AST, ALT, ALP, and total bilirubin levels were observed. In acute injury of hepatocytes leading to acute hepatitis, a rapid rise in cytosolic enzymes such as aminotransferases (AST, ALT), ALP, and bilirubin is recognized. Elevation in either AST or ALT suggests the possibility of toxic or ischemic liver injury (Hasan and Owyed 2003;

Table 2 Serum biochemical parameters of the experimental and control rats

Values in parenthesis indicate numbers of rats at the end of the study. Data are expressed as the mean ± SD. In each row, values with different letters are significantly different at $P<0.05$ (one-way ANOVA test)

	Control (n=16)	CAS+DMN (n=13)	WPI+DMN (n=16)
AST (IU/l)	134.82±73.01 b	469.99±343.33 a	429.64±263.42 a
ALT (IU/l)	194.82±52.56 a	826.70±766.22 b	156.84±213.09 a
ALP (IU/l)	159.12±71.12 b	339.08±103.88 a	383.94±109.02 a
T-protein (g/dl)	6.79±0.72	7.34±0.63	6.84±0.61
Albumin (g/dl)	5.16±1.05 a	3.37±0.75 b	4.56±0.45 a
T-bilirubin (mg/dl)	1.65±0.62 b	3.51±1.58 a	2.55±1.23 a
MDA (nmol/ml)	6.66±1.01	6.57±1.24	6.77±0.82

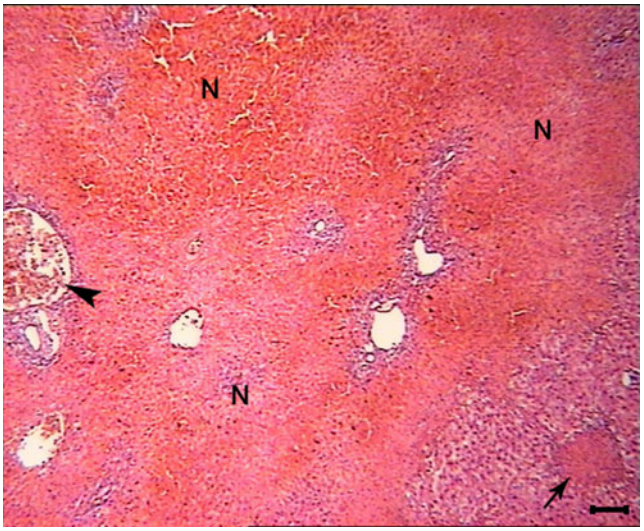

Fig. 1 Section from the liver of a DMN+casein-treated rat shows severe necrosis (*N*) and hemorrhages. One dilated central vein (*arrow head*) and a venule containing disseminated intravascular coagulation (*arrow*) are seen. Hemosiderin granules are seen in the areas of necrosis and hemorrhages (H&E, *scale bar*=210 μm)

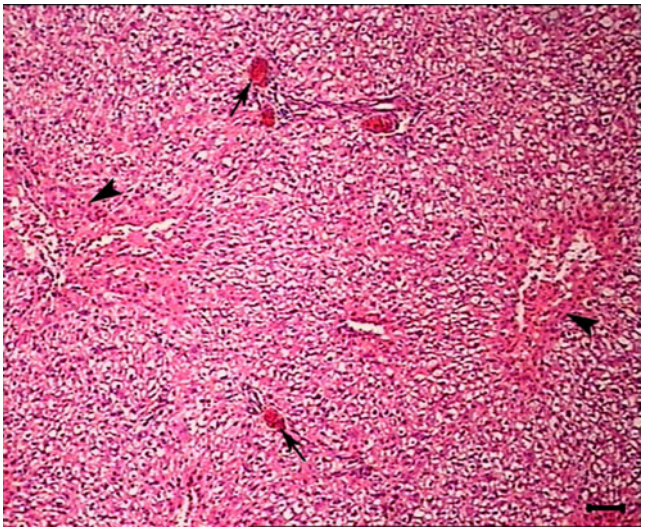

Fig. 3 Section from the liver of a DMN+whey-treated rat. Few cells around the central vein (*arrow heads*) show necrosis, and the rest of the hepatocytes show mild fatty changes. The arterioles are hyperemic (H&E, *scale bar*=210 μm)

Burtis et al. 2007). These findings are in agreement with those of Kim et al. (2007) and Kusunose et al. (2002) who indicated significant increases in serum AST and ALT levels, as well as with the findings of Lukivskaya et al. (2004) and Shin et al. (2006), which showed a significant rise of ALP level after DMN therapy. ALT and total bilirubin were insignificantly decreased by WPI. This protein significantly increased the serum albumin level and, in addition, resulted in a nonsignificant decline in the serum AST levels. Marshal (2004) showed that whey protein reduced the ALT level of the sera of the patients infected with hepatitis B virus, and Kume et al. (2006) noted that whey protein could decrease increased levels of AST and ALT in D-galactosamine-induced hepatitis in rats.

Severe massive necrosis and hemorrhages with inflammatory cell infiltration with no signs of hepatocytes regeneration at histopathological level in the rats of the CAS+DMN groups of the present study resulted in an

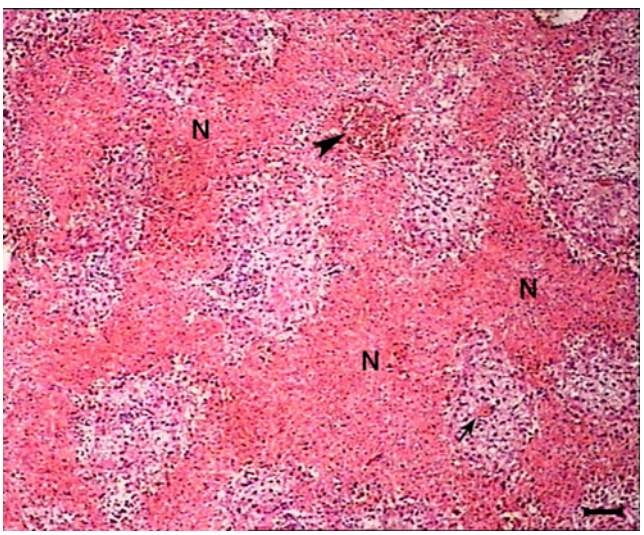

Fig. 2 Section from the liver of a DMN+casein-treated rat shows massive necrosis (*N*) with hemorrhages and hyperemia (*arrow head*). The cells around the central veins and midzonal areas are heavily injured, while those close to the portal area are almost normal. A microthrombi (*arrow*) is seen in one of the portal venules (H&E, *scale bar*=210 μm)

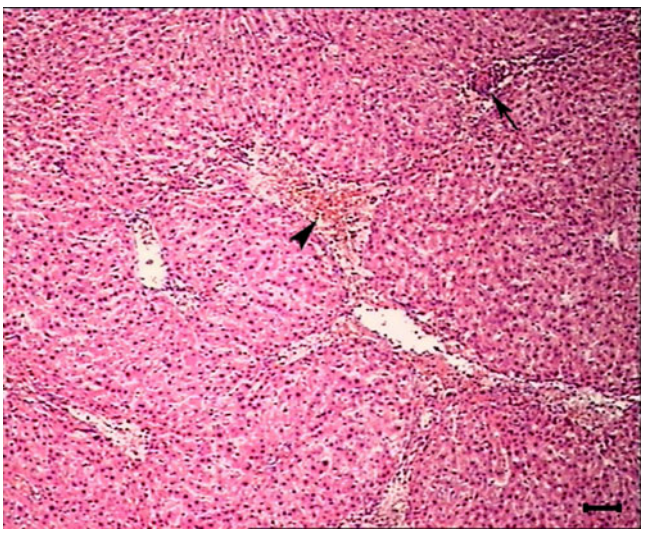

Fig. 4 Section from the liver of a DMNA+whey-treated rat. The hepatocytes and the liver architecture are almost normal. However, mild telangiectasis (*arrow head*) with congestion of one of the portal arterioles (*arrow*) is seen in the section (H&E, whey protein *scale bar*=210 μm)

increase in the AST, ALT, and ALP levels. Decrease of AST and ALT levels in the sera of the rats with the WPI diet showed that whey efficiently prevented necrotic and inflammatory processes and protected the hepatocytes from further degradation. However, the insignificant decrease of AST and ALP in rats fed the WPI diet might be due to the short period of the study and also because of the secretion of these enzymes from other organs in the body such as the heart, skeletal muscles, kidney, brain, and pancreas for AST and bones, placenta and small intestine for ALP.

A lower serum albumin level was noted in the rats of the CAS+DMN group. The serum albumin levels of the rats fed the WPI diet was somewhat between the control and the CAS+DMN group. This indicated that WPI protected the hepatocytes from further damage caused by DMN. This was also true for the total bilirubin level of the sera that was reduced after WPI consumption.

How the development of acute hepatitis and tissue necrosis in the rats fed a diet containing whey protein are suppressed is not clear, but the findings of the present experiment shows that whey protein isolate can aid in recovery from necrosis and hepatitis and/or suppress their development after DMN therapy. It is more likely that after DMN therapy, internal endotoxins such as lipopolysaccharide might induce cytokine production such as TNF-α and IL-6 from macrophages and histiocytes including Kupffer cells. Whey protein might inhibit such production, and as a result, the liver would be protected from necrosis and hepatitis. Further experiments are needed to elucidate how whey protein inhibits this cytokine production.

Our results showed that after DMN injection, marked increases in serum AST, ALT, ALP, and total bilirubin levels were observed. Whey protein isolate prevented acute liver injury and improved liver function by inhibiting further tissue necrosis and inflammatory cell infiltration due to DMN treatment and consequently, normalized the enzymes and other serum biochemical markers that were affected due to the liver injury. Furthermore, whey protein also accelerated hepatocytes regeneration.

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