High-Salt Diet Induces Gastric Epithelial Hyperplasia and Parietal Cell Loss, and Enhances Helicobacter pylori Colonization in C57BL/6 Mice

James G. Fox, Charles A. Dangler, Nancy S. Taylor, Amy King, Theodore J. Koh, and Timothy C. Wang

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [J. G. F., C. A. D., N. S. T., A. K.], and Gastrointestinal Unit, Massachusetts General Hospital, Boston, Massachusetts 02144 [T. J. K., T. C. W.]

ABSTRACT

A high-salt diet in humans and experimental animals is known to cause gastritis, has been associated with a high risk of atrophic gastritis, and is considered a gastric tumor promoter. In laboratory rodents, salt is known to cause gastritis, and when administered, it promotes the carcinogenic effects of known gastric carcinogens. Because Helicobacter pylori has been associated with a progression from gastritis to gastric cancer, we designed a study to determine whether excessive dietary NaCl would have an effect on colonization and gastritis in the mouse model of H. pylori infection. Seventy-two, 8-week-old female C57BL/6 mice were infected with H. pylori strain Sydney, and 36 control mice were dosed with vehicle only. One-half of the infected and control mice were fed a high-salt diet (7.5% versus 0.25%) for 2 weeks prior to dosing and throughout the entire experiment. Twelve infected and 6 control animals from the high-salt and normal diet groups were euthanized at 4, 8, and 16 weeks. At 8 and 16 weeks postinfection (WPI), the colony-forming units per gram of tissue were significantly higher (P < 0.05) in the corpus and antrum of animals in the high-salt diet group compared with those on the normal diet. Quantitative urease was significantly higher (P < 0.05) at 4 and 8 WPI in the corpus and antrum of animals on the high-salt diet when compared with controls. At 16 WPI, mice in both the normal and high-salt diet groups developed moderate to marked atrophic gastritis of the corpus in response to H. pylori infection. However, the gastric pits of the corpus mucosa in mice on the high-salt diet were elongated and colonized by H. pylori more frequently than those in mice on the normal diet. The high-salt diet was also associated with a significant increase in proliferation in the proximal corpus and antrum and a multifocal reduction in parietal cell numbers in the proximal corpus, resulting in the elongation of gastric pits. We conclude that excessive NaCl intake enhances H. pylori colonization in mice and in humans and that chronic salt intake may exacerbate gastritis by increasing H. pylori colonization. Furthermore, elevated salt intake may potentiate H. pylori-associated carcinogenesis by inducing proliferation, pit cell hyperplasia, and glandular atrophy.

INTRODUCTION

Salt and its role in the development of gastric cancer has been debated for decades (1–4). Evidence of its deleterious effects is based on epidemiological studies, biochemical analyses, and in vivo experimentation (5, 6). Studies have associated an increased risk of gastric cancer with the ingestion of preserved, often salty food (7) or a preference for salty foods (2, 8). This is further supported by the analysis of study data that incorporate the measurement of salt consumption (9–12). Given the late onset of gastric cancer in human populations, it is probable that both tumor initiators as well as tumor promoters play an important role in gastric carcinogenesis. In experiments with rats dosed with MNNG, NaCl has been shown to have a promoting effect on induction of gastric adenocarcinoma when salt is given intragastrically weekly or in the diet during 12–20 weeks of exposure to MNNG in drinking water (13–15). It was initially established that ingestion of salt by rats led to chronic injury to gastric surface epithelium, followed by the proliferation of gastric epithelium (16–19). Studies also showed that 80% of rats given salt in the diet and MNNG in drinking water developed adenocarcinomas in the antrum but not in the corpus (14). In addition, ACI rats given a single initiation dose of MNNG [5 g/L MNNG in water, 0.25 ml/10 g body weight] by intubation and fed a 10% NaCl diet had a significantly increased number of tumors in both forestomach and glandular stomach after being on the salt diet for 1 year (20). Thus, studies in rats suggest that a variety of factors probably contribute to the cocarcinogenic effect of salt in gastric cancer.

Mice have also been used on a limited basis in studies addressing gastric damage due to salt intake. Mice fed a rice diet containing highly salted food developed acute gastric mucosal damage (21). In later studies, Swiss/ICR mice fed salted [10% NaCl (w/w)] rice diets for 3–12 months developed hypertrophy of the forestomach and atrophy (considered a marker of premalignancy in humans) of the glandular stomach (22). The authors emphasized that a reduction in parietal cell mass accounted for the atrophy observed in the corpus of the mice (22). Overall, these studies in rodents support the hypothesis that salt can contribute to atrophy and function as a carcinogen. However, the precise role of these models in relationship to Helicobacter-associated gastric disease has not been addressed.

It is now known that there is a strong association between Helicobacter pylori and chronic atrophic gastritis (23). This suggests that Helicobacter infection may also play a role in the development of gastric cancer. Evidence supporting this observation includes numerous studies documenting that H. pylori infection causes chronic atrophic gastritis. In some cases (particularly in certain populations), chronic gastritis progresses to atrophy, intestinal metaplasia, and dysplasia, features that are consistent with Correa’s model of progression to gastric cancer (24, 25). This hypothesis has been supported by an increasing number of epidemiological studies reported in the literature beginning in the late 1980s to the present, of which almost all have concluded that H. pylori is the missing environmental factor in the multifactorial pathogenesis of gastric cancer (24–26). Importantly, mouse models of H. felis (27, 28) and H. pylori (29) have shown that chronic Helicobacter infection of inbred mouse strains can lead to atrophy, metaplasia, and preneoplastic lesions. However, although H. pylori infection is now accepted as the preeminent environmental factor in gastric cancer, the possible interactions between H. pylori and dietary factors such as salt have not been studied.

To investigate these possible interactions, we designed an experiment in which mice infected with H. pylori were fed a high-salt diet to ascertain whether both gastric infection and elevated dietary salt increased the severity of gastric lesions and affected levels of H. pylori colonization.
Materials and Methods

Animals. One hundred eight, 8-week-old female C57BL mice that were free of Helicobacter spp, Citrobacter rodentium, Salmonella spp, endoparasites, and antibodies to viral pathogens were obtained from Taconic Farms ( Germantown, NY). The mice were housed in microisolator cages within an AAALAC-accredited facility and animal use was approved by the MIT Animal Care and Use Committee.

Bacteria. H. pylori Sydney strain was used for oral inoculation as described previously (29). The organism was grown for 48 h at 37°C under microaerobic conditions on 5% lysed horse blood agar. The bacteria were harvested after 48 h of growth; resuspended in PBS; assessed by Gram stain and phase microscopy for purity, morphology, and motility; and tested for urease, catalase, and oxidase activity.

Experimental Infection. Seventy-two C57BL p.o. infected with 10^8 CFU H. pylori Sydney strain in 0.3 ml of PBS given three times every other day. Thirty-six control mice were dosed with PBS only. One-half of the infected (n = 36) and one-half of the control mice (n = 18) were fed a high-salt diet (7.5% versus 0.75% Purina Labs Special Formulation, Richmond, IN) for 2 weeks prior to the dosing with H. pylori and throughout the experiment. At 4, 8, and 16 weeks post challenge, 12 infected and 6 uninfected mice from each diet group were euthanized with CO2. Gastric tissues were collected from the corpus and antrum and used for quantitative urease activity, quantitative H. pylori culture, and histopathological evaluation.

Quantitative Urease Activity Assay. Gastric samples (1–2 mm^2) were excised from the midportion of the corpus and the antrum. A quantitative urease assay was performed as described elsewhere. In brief, the tissues were incubated in 1 ml of urea broth for 4 h and centrifuged, and duplicate aliquots (200 μl) of urea broth from each gastric tissue were placed in microtitre plates. The extent of color change was recorded in an automated ELISA reader at 550 nm (30).

Quantitative Culture. The mass of the tissue was determined by subtracting the mass of the tube containing media from the mass after tissue was added. The tissue was homogenized with glass tissue grinders, and the homogenate was diluted 100- and 1000-fold in Brucella broth containing FCS. One hundred μl each of the dilution were spread on selective medium: Blood Agar Base no. 2 (DIFCO Laboratories, Detroit, MI) supplemented with 5% horse blood (Remel, Lenexa, KS), 50 μg/ml amphotericin B, 100 μg/ml vancomycin, 3.3 μg/ml polymyxin B, 200 μg/ml bacitracin, and 10.7 μg/ml nalidixic acid (Sigma Chemical Company, St. Louis, MO). Plates were incubated microaerobically at 37°C for 3–5 days. After verification by Gram stain and urease, catalase, and oxidase reactions, the H. pylori colonies were counted and the CFU per gram of tissue calculated. Comparisons between groups were based on the log concentrations of bacteria.

Histological Evaluation. The tissue examined consisted of a section of stomach taken from the greater curvature beginning at the squamocolumnar junction and ending at the gastroduodenal junction. Stomach tissues were fixed in neutral buffered 10% formalin, processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with H&E and Warthin-Starry. The glandular mucosa of the corpus and antrum were examined histologically for inflammatory and epithelial changes and for the presence of H. pylori. Inflammation was distinguished histologically into chronic (lymphohistiocytic) and active (granulocytic) components. The contributions of both were graded on an ascending scale ranging from 0 to 4, based on the intensity, distribution, and confluence of inflammatory infiltrates. Group data were compared using the Mann-Whitney analysis of nonparametric data. At 16 WPI, the extent of multifocal glandular atrophy in the proximal mucosa was measured in the high-salt diet group and compared with the corresponding glandular region in the normal diet group. The length of the glandular zone, primarily composed of parietal cells, was measured as a proportion of total mucosal thickness in the proximal corpus with a 10 x 10 ocular reticle grid. Similar measurements were not useful in the H. pylori-infected groups because the marked glandular atrophy induced by H. pylori and other gastric Helicobacters in C57BL mice (27, 29) obviated the observation of a further contribution due to the high-salt diet.

BrdUrd Immunocytochemistry. Animals received a single i.p. injection of BrdUrd (50 mg/kg) from a freshly made stock solution (5 mg/ml) dissolved in PBS according to our previously published protocol (27). The mice were euthanized 1 h later. At necropsy, a longitudinal section of stomach was taken from the greater curvature extending from the squamocolumnar junction to the gastroduodenal junction. Samples were placed immediately in cassettes, fixed in 10% neutral buffered formalin, and embedded in paraffin wax. Immunohistochemical detection of BrdUrd incorporation was performed on 5-μm sections and visualized with a modified avidin-biotin monoclonal antibody immunohistochemical technique, which eliminated background signals due to binding of the secondary antibody to mouse immunoglobulins in the tissue sections. The day prior to the BrdUrd immunodetection, the biotinylated secondary antibody (rabbit antimouse IgG) was conjugated to the primary antibody (mouse monoclonal anti-BrdUrd; Dako Corp., Carpinteria, CA) in solution. The primary and secondary antibodies were mixed together in TBS at 1:40 and 1:200 dilutions, respectively, and incubated at 4°C overnight with gentle agitation. Prior to using the following day, a 1:20 volume of normal mouse serum was added to the conjugate solution and incubated at 4°C for 2 h with gentle agitation to quench unbound sites on the secondary antibody. After deparaffinization in xylene and graded ethanol series, the tissue sections were hydrated with PBS and treated with 20 μg/ml proteinase K at 37°C for 5 min. Endogenous peroxidase activity in the tissue section was blocked by immersing the slides in 1% hydrogen peroxide in methanol. The slides were then washed in tap water. The BrdUrd monoclonal antibody identifies only single-stranded DNA. Denaturation of the tissue DNA was achieved by incubation in 1 M HCl at 60°C for 8 min. The slides were washed in tap water and then TBS and incubated with 5% normal rabbit serum to block nonspecific binding of the secondary antibody. The tissue was then incubated with the conjugate of mouse monoclonal anti-BrdUrd and biotinylated rabbit antimouse IgG for 4 h at room temperature. After washing in TBS, slides were incubated with peroxidase-conjugated streptavidin (1:400 in TBS) and washed in TBS; the labeled cells were then visualized by the diaminobenzidine reaction. Sections were lightly counterstained with hematoxylin. The nuclei of cells at S-phase of the cell cycle during the in vivo BrdUrd incorporation phase were stained brown.

Quantitation of BrdUrd Incorporation. Quantitation of epithelial proliferation based on BrdUrd incorporation was focused on the corpus mucosa adjacent to the squamocolumnar junction (i.e., limiting ridge) and the antrum. The proximal corpus in C57BL mice has been shown to be a target zone for H. pylori-induced gastric lesions. In contrast, Helicobacter-associated lesions in the antrum are usually limited, although this site is colonized more densely than the corpus. Regions within the proximal corpus and antrum, in which the gastric pits and proliferative zones were aligned in the plane of section, were identified histologically. Some samples were eliminated from this test because of the absence of appropriate tissue representation in the sections evaluated. Positively (i.e., brown nuclei) stained epithelial cells were counted in 5–17 (mean, 11.3) and 8–31 (mean, 15.1) contiguous glands in the proximal corpus and antrum, respectively. Assessment of epithelial proliferation was based on the density of BrdUrd-positive epithelial cells per gastric pit. Groups were compared using the two-tailed Student’s t test for unpaired data; P < 0.05 was considered statistically significant.

Gastrin RIA. Plasma gastrin levels (gastrin amidated at the COOH terminus) were determined by RIA using rabbit antiserum L2, which reacts similarly with G17 and G34 (31).

Results

Quantitative Urease. The mean urease activity (absorbance per gram of tissue) at 4 and 8 WPI was significantly increased (P < 0.05) in the corpus and antrum of H. pylori-infected mice fed the high-salt diet compared with their H. pylori-infected counterparts on the normal diet (Table 1). The mean concentration of urease remained elevated in the high-salt group at 16 WPI.

Quantitative H. pylori Culture. The mean density of H. pylori (CFU/g tissue) at 8 and 16 WPI was increased significantly (P < 0.05) in mice fed the high-salt diet compared with their counterparts on the normal diet. This effect was evident in samples from the corpus (Fig. 1) and antrum (Fig. 2). A gradual decline in the mean density of H. pylori was present in mice on the normal diet. In contrast, the mean density was relatively stable in the corpus of mice on the high-salt diet.
Histopathological Observations. At 16 WPI, mice fed the high-salt diet had multifocal elongation of the gastric pits and reduction in the parietal cell zone; this change was observed independently of H. pylori infection (Fig. 3). The apparent increase in gastric pit length was not associated with an increase in total mucosal thickness, indicating the increased length of the gastric pits resulted from diminution of the glandular zone, specifically the parietal cell component. Chief cells at the base of the glands were not notably affected. Within the high-salt diet group, the length of glandular zones in atrophic foci ranged from 34 to 82% (mean, 64%), a significant decrease \((P < 0.05)\) compared with the comparable region in the normal diet group, which ranged from 75 to 87% (mean, 82%). No statistically significant increases in inflammation scores were observed in uninfected mice receiving the high-salt diet, compared with their counterparts on the low-salt diet. Therefore, the effects associated with the high-salt diet on the gastric pits and parietal cell numbers occurred independent of local inflammation in the uninfected mice.

Mice infected with H. pylori typically developed moderate to marked atrophic gastritis of the corpus at 16 WPI, characterized by chronic active inflammation composed largely of lymphocytes and granulocytes. The corpus mucosa was characterized by marked loss of parietal cells and replacement of the normal oxyntic epithelium by hypertrophy and hyperplasia of the mucus epithelium (Fig. 3). Statistically significant increases in granulocyte infiltration \((P < 0.02)\) and chronic inflammation \((P < 0.005)\) were observed in H. pylori-infected mice of both diet groups compared with their uninfected counterparts. Among H. pylori-infected mice, no significant exacerbation of inflammation was associated with high salt intake.

BrdUrd Analysis. At 16 WPI, BrdUrd incorporation in the proximal corpus and antrum was significantly increased \((P < 0.005)\) in uninfected mice fed the high-salt diet compared with those on the normal diet (Table 2 and Fig. 4). Among H. pylori-infected mice, a significant increase \((P < 0.05)\) in antral BrdUrd labeling was also associated with the high-salt diet. In the proximal corpus, BrdUrd incorporation was also increased significantly \((P < 0.05)\) in association with H. pylori infection in the normal diet group. H. pylori infection in the high-salt diet group was associated with the two highest BrdUrd labeling densities of the proximal corpus as well as a mean increase in BrdUrd incorporation, compared with uninfected mice on the same diet. However, because of the higher variance associated with H. pylori infection, no statistical significance was associated with high salt among infected mice. Likewise, H. pylori infection with high salt did not induce a significant increase above high-salt diet alone. As suggested by the minimal increase of H. pylori-associated antral lesions in the C57BL mice, no significant increase in labeling was observed in the antrum of H. pylori-infected mice in either diet group, compared with their uninfected counterparts.

Gastrin Analysis. Serum gastrin concentrations were significantly increased \((P < 0.05)\) in association with H. pylori infection among mice on the normal diet (Table 3). Although H. pylori infection in the high-salt diet group was associated with a mean increase in serum gastrin compared with uninfected mice on the same diet, statistical significance was not achieved. Exposure to the high-salt diet among uninfected mice resulted in a mean increase compared with uninfected mice on the normal diet that did not achieve significance. No substantial differences were apparent between the diet groups among H. pylori-infected mice. Serum gastrin concentrations had no correlation with epithelial proliferation as assessed by BrdUrd incorporation.

### DISCUSSION

Dietary salt has been linked epidemiologically to gastric cancer risk. Morbidity and mortality rates of gastric cancer in Asia remain the highest in the world (32). A dietary component that stands out as an important factor that contributes to Japanese stomach cancer is the intake of highly salted food (21). The incidence of gastric cancer is clearly higher in countries where diets rich in salt [dried fish preserved in salt (3–20%) and soy sauce (19% salt), and pickled foods (13–25% salt)] are consumed in large amounts (12, 33). Daily consumption of a hot rice gruel seasoned with soybean sauce (salt content, 18%) has been related to high gastric cancer incidence in Japan (33). One study reported that humans in areas where salted fish was a dietary staple excreted as much as 50 g of salt daily in the urine (12). Geographic comparisons within Japan indicated that the incidence of gastric cancer paralleled the amount of salted foods consumed (12). These earlier epidemiological data are supported by recent results from both the Intersalt Study and a Japanese study (3, 4). Indeed, the Japanese study suggested that the relationship between H. pylori infection and...
gastric cancer may be confounded by salt consumption (34). This observation, if substantiated, is particularly important in Japan, where 70% of men 25–34 years of age and 90% of men 55–64 years of age have H. pylori infection (35). In addition, DeKoster et al. (36) have shown that although the in vivo gastric mucosal cell proliferation rate does indeed correlate with salt intake, it only does so in H. pylori-positive patients. In contrast, our present findings in salt-fed mice indicate that dietary salt may increase gastric epithelial proliferation independent of H. pylori infection.

The role of salt consumption has also been implicated by experimental animal models in which animals fed high-salt diets developed gastric mucosal changes. Most of the salt-associated carcinogenesis studies have been performed in rats and mice, in which the concentrations of sodium salt varied from 0.7 to 20% in drinking water or diet. For example, mice fed a diet of dried cod containing 7% NaCl developed both acute and chronic gastritis (21). Another group of investigators fed excessively salted rice to mice over a period of time and induced significant glandular atrophy (22). It has been demonstrated that a sodium-deficient diet restricted tumor growth and that the antineoplastic activity of certain anticancer agents decreased in mice when given in salted vehicle (37, 38). In rodent models, a concentrated salt diet caused excessive cell replication in the gastric mucosa, an effect that possibly increases the incidence of endogenous mutations and potentiates the actions of other carcinogens (13, 39). High salt intake in rodents has been shown to increase the absorption of known gastric carcinogens, such as polycyclic aromatic hydrocarbons (40). Several independent studies have shown that NaCl solution increased the rate of MNNG-induced gastric adenocarcinomas in rats (13, 15, 41).

The availability of the H. pylori mouse model offers an ideal opportunity to directly examine the interaction between dietary salt and H. pylori infection in accelerating gastric injury and promoting the development of premalignant gastric lesions. The histopathological observations of this study at 16 WPI are consistent with and extend previous observations of the effect of high salt intake on the gastric mucosa (16–19, 22). Persistent infection with H. pylori Sydney strain was achieved in both the high- and low-dose salt diet-treated C57BL mice for the 16-week duration of the study. In the mouse model, high dietary salt statistically increased the level of H. pylori colonization in the body mucosa at all time points evaluated in the study (i.e., 4, 8, and 16 WPI) based on either quantitative urease assay or culture. The increase in H. pylori gastric colonization associated with the high-salt diet implicates a unique and potentially synergistic mechanism. High salt intake may potentiate carcinogenesis by facilitating colonization and thereby increase the impact of chronic H. pylori gastritis.

The increased colonization density of H. pylori could be potentiated through several mechanisms. Interestingly, a preliminary report has indicated that gastrin appears to be a H. pylori-specific growth factor in vitro. Human gastrin stimulated the growth of eight different H. pylori isolates in a specific, dose-dependent manner (42). In the present study, terminal serum gastrin concentrations were statistically increased in H. pylori-infected mice and elevated in mice fed high-salt diets. At present, the serum gastrin concentrations do not wholly parallel the colonization data; however, single sample values from serum offer only an indirect assessment of tissue concentrations. The results may also indicate that the observed salt-associated effects

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**Table 2** Effect of high salt and H. pylori infection on gastric pit BrdUrd incorporation* in the proximal corpus and antrum

<table>
<thead>
<tr>
<th>Proximal corpus</th>
<th>Antrum</th>
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<tbody>
<tr>
<td></td>
<td>Normal diet</td>
</tr>
<tr>
<td>Sham infected</td>
<td>2.7 (1.2)</td>
</tr>
<tr>
<td>H. pylori infected</td>
<td>6.0 (2.7)</td>
</tr>
</tbody>
</table>

*Mean number of BrdUrd-positive cells per gastric pit (SD).

aSignificant effect associated with high salt.

bSignificant effect associated with H. pylori infection.
occur through an event downstream from gastrin stimulation or through a gastrin-independent process.

Another factor in the increase of *H. pylori* colonization is the induction of foveolar hyperplasia in mice fed high-salt diets. The gastric foveolae, or pits, represent the primary niche and site of attachment for *H. pylori* organisms (43). High-salt diets may synergize with gastric *Helicobacter* infections through expansion of immunopositive cells to the base of the glands (arrow). The effacement of the normal glandular zone by the hyperplastic mucous epithelium results in spread of immunopositive cells to the base of the glands (arrow). Although no statistical difference was observed between the two infected groups, the highest density of BrdUrd labeling was observed in infected mice on the high-salt diet. Streptavidin-horseradish peroxidase, 3,3'-diaminobenzidine with hematoxylin counterstain. Bar, 100 μm.

Table 3: Effect of high salt and *H. pylori* infection on serum gastrin

<table>
<thead>
<tr>
<th></th>
<th>Normal diet</th>
<th>High-salt diet</th>
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<tbody>
<tr>
<td>Sham infected</td>
<td>23.7 (9.7)</td>
<td>30.7 (8.5)</td>
</tr>
<tr>
<td><em>n</em> = 6</td>
<td><em>n</em> = 7</td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> infected</td>
<td>51.8 (29)</td>
<td>49.7 (42)</td>
</tr>
<tr>
<td><em>n</em> = 12</td>
<td><em>n</em> = 11</td>
<td></td>
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*Mean concentration [pM] of serum gastrin (SD).*

*Significant effect associated with *H. pylori* infection, *P* < 0.05.*
salt-associated effect on epithelial proliferation in the antral mucosa of infected mice. Because local inflammation may confound or exacerbate the long-term progression of high-salt-associated lesions, in future studies it would be useful to examine other mouse strains (e.g., BALB/c) that develop less severe *H. pylori*-associated gastritis in parallel with C57BL/6 mice.

In conclusion, our study suggests that high-salt diets contribute to gastric atrophy and synergize with *Helicobacter* infections through foveolar hyperplasia and expansion of *H. pylori* colonization. Whether the effects on gastric differentiation and increased *H. pylori* colonization lead to accelerated progression to gastric cancer is unknown at present but should be addressed in future studies.

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REFERENCES


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