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Dipeptiven® improves kidney pathology in a rat model of chronic kidney disease

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SUMMARY

Background & aims: Administration of glutamine in patients with renal dysfunction is considered to be potentially adverse. In a rat model of moderate kidney dysfunction dose-dependent effects of intravenous alanyl-glutamine infusion on possible biochemical and histological signs of toxicity were investigated.
Methods: Rats with renal dysfunction resulting from 5/6 nephrectomy received a 9 days continuous intravenous infusion of either saline or 0.5 g/kg/day or 3.0 g/kg/day alanyl-glutamine (Dipeptiven®) or 3.0 g/kg/day alanine. Dose-dependent effects on kidney and other organs were assessed by analyzing blood levels of creatinine, ammonia, urea, ALT, AST, ALP, pH, pO_2, pCO_2, glutamine, and histopathology.
Results: Continuous intravenous infusion of 3.0 g/kg/day alanyl-glutamine increased plasma glutamine concentrations up to 60% without aggravating the underlying kidney injury. In contrast, the morphology of the kidneys was improved due to reduced glomerulosclerosis and tubular proteinaceous casts. An increase in plasma urea concentrations observed in the 3.0 g/kg/day alanyl-glutamine group only was not associated with worsening of the phenotype.
Conclusions: Continuous intravenous infusion of alanyl-glutamine at 0.5 and 3.0 g/kg/day up to 9 consecutive days is safe in a rat

Abbreviations: Ala-Gln, alanyl-glutamine; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPN, chronic progressive nephropathy; eGFR, estimated glomerular filtration rate; HCO_3^-, hydrogen carbonate; pO_2, partial pressure of oxygen; pCO_2, partial pressure of carbon dioxide; tCO_2, total carbon dioxide; sO_2, oxygen saturation.

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model of chronic moderate kidney dysfunction and improved the renal morphology by reducing glomerulosclerosis and tubular proteinaceous casts. In these animals a decreased incidence and severity of chronic progressive nephropathy was observed compared to the saline and alanine treated animals.

1. Introduction

Glutamine, the most abundant amino acid in the body, is considered conditionally essential in the catabolic state occurring among others in critical illness, trauma, and infection [1]. Administration of high doses of glutamine in critically ill patients, however, was associated with increased mortality in a large multi-center trial [2]. A post-hoc analysis of this trial revealed that high dosages of glutamine were especially not tolerated by patients with renal dysfunction at baseline [3], while no harm was observed in patients without renal dysfunction at baseline. This raised concerns regarding the general safety of glutamine administration in patients with kidney dysfunction. However, within the group of patients with renal dysfunction at baseline, acute and acute-on-chronic renal failure are summarized, not allowing important differentiation. In their post-hoc analysis, Heyland and coworkers defined renal dysfunction at baseline as serum creatinine $\geq$171 μmol/L or a urine output of <500 mL/past 24 h in patients without known renal dysfunction (acute injury), while in patients with acute-on-chronic renal failure (predialysis), an absolute increase of $\geq$80 μmol/L from baseline or a urine output of <500 mL/past 24 h was considered.

This definition, however, does not reflect estimated glomerular filtration rate (eGFR) or creatinine clearance, thereby not allowing to differentiate different stages of renal dysfunction. Importantly the serum creatinine based “renal dysfunction” group may among others include patients with a creatinine clearance of less than 25 ml/min. In such patients, administration of alanyl-glutamine is contra-indicated [4]. Assuming a normal creatinine clearance of more than approximately 90 ml/min [5] in humans, a creatinine clearance of 25 ml/min represents 30% of the normal minimal creatinine clearance. We hypothesized that administration of alanyl-glutamine during conditions of renal dysfunction is not associated with harmful effects at a creatinine clearance of more than 30% of the normal creatinine clearance in rats.

To test this hypothesis we intravenously infused alanyl-glutamine to rats with renal dysfunction achieved by 5/6 nephrectomy. In this model, rats show a reduction in creatinine clearance of approximately 50%. Thus, administration of alanyl-glutamine would not be contraindicated at this level of renal dysfunction. Here we present the results of repeated intravenous infusion of alanyl-glutamine in rats with moderate chronic renal dysfunction.

2. Materials and methods

2.1. Chemicals and drugs

Dipeptiven® (batch 16HM0212) was provided by Fresenius Kabi Austria (Graz, Austria), L-alanine (batch 341014) was obtained from Gerbu Biotechnik GmbH (Heidelberg, Germany), and saline (batch 035752) was obtained from Lavoisier (Paris, France).

2.2. Animals

Fourty male nephrectomized Wistar: Crl: WI (Han) rats and ten sham operated rats were obtained from Charles River Laboratories (Domain des Oncins, France). The nephrectomized animals underwent

a 5/6 kidney nephrectomy according to a two-stage surgery with 5–7 days between surgeries. The first surgery removes the 2/3 of one kidney while the second surgery removes the entire other kidney. The second surgery was performed 5–6 days before the transfer of the animals to the experimental facility where the study took place. There the rats had an acclimatization period of at least seven days before start of the experiments at the age of 12 weeks.

During the acclimatization period a polyurethane catheter was implanted into the posterior vena cava via the left femoral vein. Following implantation, the animals were maintained on continuous infusion with physiological saline prior to the start of treatment. All animals were housed singly in a temperature controlled room with a 12 h light/dark cycle. The room temperature and relative humidity were maintained in the ranges of 22 ± 3 °C and >35%. All animals had access to food and water *ad libitum*. All animals received diet C 1003 from Altromin (Lage, Germany) modified to contain 12% protein except for the group receiving 3.0 g/kg/day Ala-Gln or 3.0 g/kg/day alanine. To account for the higher intravenous amino acid administration from the start of the infusion the amount of protein in the diet was reduced to 9% protein in these groups. The body weights of the nephrectomized animals at the start of infusion ranged from 273 to 346 g. The study was approved by the ethical committee of Charles River and authorized by the French authorities.

2.3. Experimental design

Animals were divided into 5 groups and continuous intravenous infusion was performed as follows: Group 1 (sham operated group) received saline infusion. Group 2 (nephrectomized) received a saline infusion, group 3 (nephrectomized) received 0.5 g/kg/day alanyl-glutamine (human dose) and group 4 (nephrectomized) received 3.0 g/kg/day alanyl-glutamine (rat adapted human dose [6]). Group 5 (nephrectomized) received 3.0 g/kg/day alanine and was used as a control for the effects of the protein reduction in the diet in group 4. The continuous intravenous infusion was performed for 9 days in total. The experimental design is depicted in Fig. 1.

2.4. Blood collection

Blood was drawn on days 3 and 6 from the tail vein for ammonia determination and from the retroorbital sinus under isflurane anesthesia for urea determination. In addition, it was taken on days 3 and 6 from the retroorbital sinus under isoflurane anesthesia for clinical chemistry parameters and on days −1, 4, and 7 for glutamine from 5 animals of each group. Another 5 animals of each group were sampled on days 3 and 6 from the tail artery for blood gas analysis.

2.5. Analysis of clinical chemistry and ammonia

ALP, ALT, AST, and urea were determined using the AU640 Beckmann coulter system (Roissy, France). Ammonia was measured in full blood immediately after withdrawal with the PocketChem BA PA-4140 obtained from Axonlab (Stuttgart, Germany) according to the manufacturer's instruction. Base excess (BE), pH, pO_2, pCO_2, tCO_2, sO_2, HCO_3^- were analyzed using the G3+ cartridges in the Abbott i-

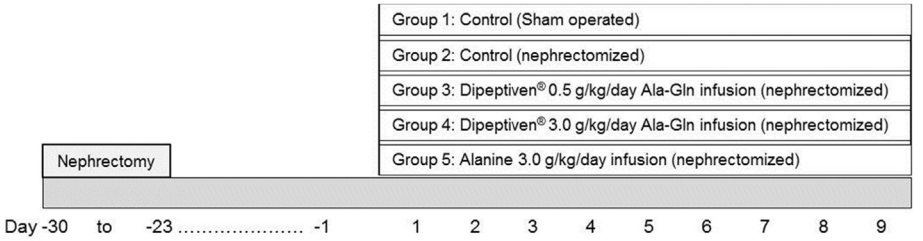

Fig. 1. Three weeks after 5/6 nephrectomy the rats received a continuous intravenous infusion of either alanyl-glutamine (Dipeptiven®), alanine or saline. Ala-Gln = alanyl-glutamine.

STAT 1 analyzer obtained from Axonlab (Stuttgart, Germany) according to the manufacturer's instruction.

2.6. Analysis of plasma glutamine

Free amino acid (AA) concentrations in plasma samples were determined using fully automated reversed phase high performance liquid chromatography as previously described [7]. Proteins were precipitated by sulphosalicylic acid (SSA, 30%), containing norvaline as internal standard. The free AA in protein-free supernatants were initially derivatized using ortho-phthaldialdehyde and 3-mercaptopropionic acid (3-MPA) and subsequently separated in a RP-C18 column by gradient elution. Fluorescent AA adducts are detected at excitation wavelength of 330 nm and emission wavelength of 450 nm. Quantification was based on repeated analysis of AA standards using norvaline as internal standard.

2.7. Histopathological analysis

At the end of the continuous intravenous infusion animals were euthanized by carbon dioxide inhalation and exsanguination and submitted to necropsy. A complete full set of organs of every animal consisting of brain, stomach, intestine, kidneys, liver, lung, injection site and any other organ with macroscopic lesions was fixed in 10% neutral formalin, trimmed, embedded in paraffin blocks, sectioned at nominally 4 μm and hematoxylin & eosin stained slides were analyzed histopathologically.

2.8. Statistical analysis

Data were reported as individual measurement points. Differences between control and treatment groups were statistically evaluated using one way ANOVA followed by Dunnett's multiple comparisons test for each time point individually. The significance level was set at alpha = 5%. Data analysis was performed with GraphPad Prism 7 (GraphPad Software, Inc., California, US). Values of the sham group were used as healthy control reference ranges. Grubb's test was performed non-recurrent for outliers in every group for every single day and significant outliers ($p < 0.05$) were excluded from analysis.

3. Results

3.1. Mortality

No mortality related to alanyl-glutamine infusion was observed in this study.

3.2. Body weight

There was no statistically significant reduction in body weight gain comparing the sham operated to the nephrectomized control animals (2.95 ± 1.12% in sham operated rats (group 1) versus 2.77 ± 2.08% in nephrectomized rats (group 2)). The body weight gain was reduced without reaching statistical significance in the groups receiving alanyl-glutamine infusion compared to the nephrectomized animals receiving saline (1.99 ± 1.92% in group 3 receiving 0.5 g/kg/day alanyl-glutamine and 1.17 ± 2.82% in group 4 receiving 3.0 g/kg/day alanyl-glutamine).

3.3. Plasma glutamine concentrations

Plasma glutamine concentrations (Fig. 2) were not statistically significantly different in sham operated and nephrectomized animals receiving saline infusion throughout the duration of the study ($p = 0.1780$). Concentrations in nephrectomized control animals receiving saline infusion were 821.00 ± 170.22 μmol/L on day 4 and 766.46 ± 64.43 μmol/L on day 7. Infusion of 0.5 g/kg/day alanyl-glutamine resulted in plasma glutamine concentrations of 885.56 ± 96.65 μmol/L on day 4 and 829.28 ± 60.26 μmol/l on day 7. Infusion of 3.0 g/kg/day alanyl-glutamine led to mean plasma

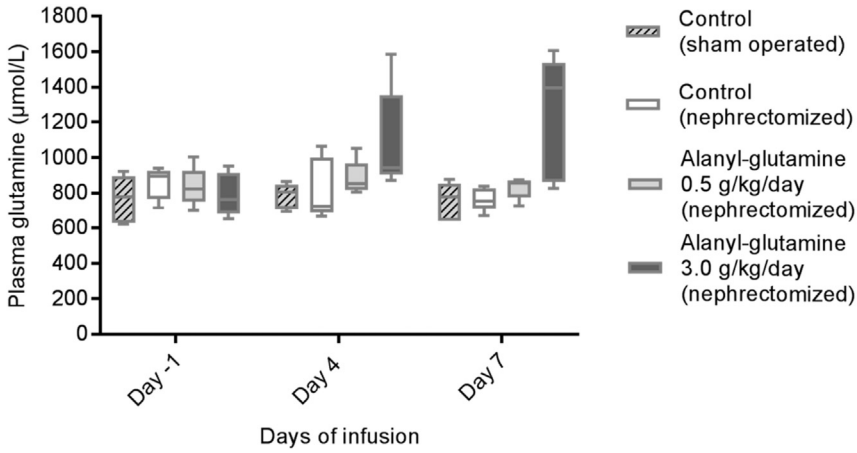

Fig. 2. Ala-Gln infusion of 3.0 g/kg/day, but not 0.5 g/kg/day, increased the plasma glutamine concentrations on infusion days 4 and 7. Data reported as mean and interquartile range (IQR).

glutamine concentrations of 1091.86 ± 291.69 µmol/L on day 4 and 1239.14 ± 354.08 µmol/L on day 7. None of these levels reached statistical significance when compared to nephrectomized controls receiving saline.

3.4. Serum clinical chemistry

Plasma urea concentrations (Fig. 3a) were increased in all nephrectomized animals (group 2–4) compared to sham operated controls (group 1). While infusion of 0.5 g/kg/day alanyl-glutamine did not further increase the plasma urea concentrations, infusion of 3.0 g/kg/day alanyl-glutamine led to higher levels of urea compared to nephrectomized controls receiving saline (13.78 ± 1.92 mmol/L in group 4 versus 11.90 ± 3.99 mmol/L in group 2 on day 3 and 14.25** ± 1.62 mmol/L in group 4 versus 10.66 ± 2.29 mmol/L in group 2 on day 6 (**$p < 0.01$)).

Blood ammonia levels (Fig. 3b) were not increased in nephrectomized animals compared to sham operated controls. Infusion of neither alanyl-glutamine dose led to elevation of blood ammonia.

Plasma creatinine levels (Fig. 3c) were increased in all nephrectomized animals. Administration of alanyl-glutamine did not further elevate the plasma creatinine levels.

ALP, ALT, AST, BE, pH, pO_2, pCO_2, tCO_2, sO_2, and HCO_3^- neither differed between nephrectomized and sham operated nor between animals receiving saline or alanyl-glutamine infusion.

3.5. Histopathology

In the kidneys, histopathological analysis revealed a decreased incidence of chronic progressive nephropathy (CPN) in animals treated with alanyl-glutamine (Fig. 4a and b). CPN was observed in 3/5 animals in group 2 receiving saline (Fig. 4a), 2/5 animals in group 3 receiving 0.5 g/kg/day alanyl-glutamine, 0/5 animals in group 4 receiving 3.0 g/kg/day alanyl-glutamine (Fig. 4b) and in 3/5 animals in group 5 receiving 3.0 g/kg/day alanine. No further changes were observed in any of the organs examined.

4. Discussion

The main finding of this study is that Dipetiven® is not only safe but even beneficial in a rat model of moderate renal dysfunction. In our study, continuous intravenous infusion of alanyl-glutamine for 9 consecutive days reduced the incidence of chronic progressive nephropathy (CPN). CPN is a

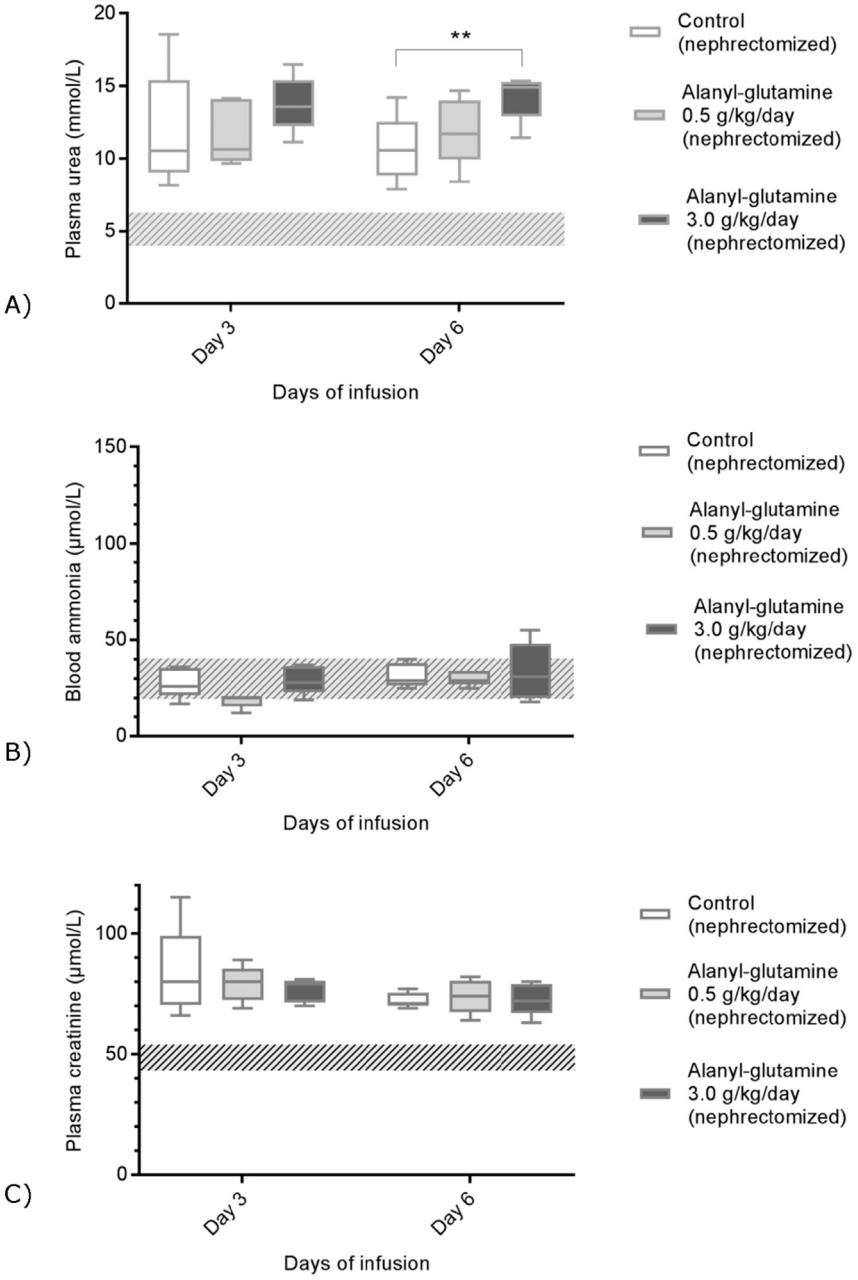

Fig. 3. Plasma urea (A) and plasma creatinine (C) levels are increased in the nephrectomized animals compared to sham controls (grey shaded horizontal bar), while ammonia levels (B) are not. Ala-Gln infusion did not increase creatinine and ammonia levels, but increased urea levels were observed compared to nephrectomized controls in the animals receiving 3.0 g/kg/day infusion. Statistical significance was reached for this observation on day 6. ** = $p < 0.01$, data reported as mean and IQR.

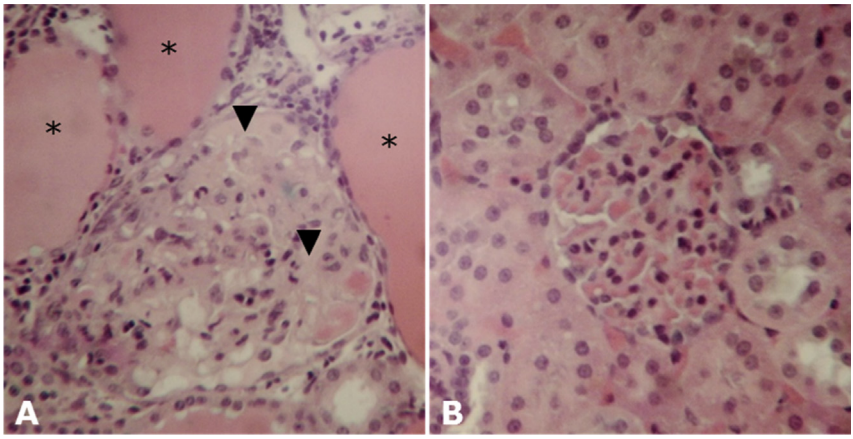

Fig. 4. (A) Representative picture of saline receiving nephrectomized animals showing chronic progressive nephropathy with global glomerular sclerosis (arrow heads) and tubular proteinaceous casts (asterisks). (B) Glomerular sclerosis and tubular proteinaceous casts were absent in animals receiving 3.0 g/kg/day alanyl-glutamine intravenously for 9 consecutive days.

spontaneous renal disease in common rats strains, predominantly in males and in this study its onset may have been accelerated by the reduction of nephrons due to the partial nephrectomy [8]. Histological hallmarks of the disease are a thickened tubular basement membrane, hyaline cast formation and glomerulosclerosis [9], which were dose-dependently reduced or absent in animals treated with Dipeptiven®. Of note, protein restriction in the diet is known to ameliorate the progression of this disease [8]. In our study, the amount of protein in the diet was reduced in the group of animals receiving 3.0 g/kg/day alanyl-glutamine to allow for approximately equal protein or amino acid uptake in the groups. However, the impact of this oral protein restriction on the improvement of the kidney morphology was considered virtually absent as the infusion of alanine in rats receiving the same protein restricted diet did not reduce the incidence of CPN.

The improved morphology of the kidney did not result in reduced serum creatinine levels in our study. Serum creatinine is a rather insensitive parameter. The analysis of more sensitive parameters like tissue inhibitor of metalloproteinase 2 (TIMP-2) or a treatment duration of more than 9 days might have revealed functional impact of this morphological alteration.

While serum creatinine and ammonia were unchanged during the course of our study, plasma urea increased in the animals receiving 3.0 g/kg/day alanyl-glutamine. Due to the renal dysfunction of nephrectomized animals, plasma urea concentrations were approximately 10 mmol/L from the start of infusion, which is twice the normal value. And the levels in the rats further increased to 15 mmol/L when high dosages of alanyl-glutamine were administered. An even higher increase in plasma urea concentration (>50 mmol/L) was observed in human patients receiving even higher dosages of alanyl-glutamine in the study of Heyland and coworkers [3]. Glutamine gives rise to urea in the liver and this elevated concentration may simply reflect a higher glutamine turnover in the animals of our study.

One of the major discrepancies of our animal study and the human trial performed by Heyland and coworkers is the nature of kidney injury. The study of Heyland and coworkers was performed in acute critically ill patients and thus in their post-hoc analysis they had to focus on patients developing acute kidney insufficiency, while we test the effects of alanyl-glutamine in rats with chronic kidney disease. There is, however, abundant animal data that glutamine administration is rather beneficial than harmful in models of acute kidney injury [10–12]. In these animal studies, glutamine was administered as a single bolus of 0.5 g alanyl-glutamine/kg/day. Heyland and coworkers provided the human patients with 0.5 g alanyl-glutamine as a continuous intravenous infusion. When they combined this intravenous dose with an additional amount of 30 g oral glutamine [3] they observed harmful effects, which may result from the relative high total dose of glutamine.

A possible negative impact of exogenously or endogenously elevated plasma glutamine levels on renal function is currently unclear [13]. Associations of low and elevated plasma glutamine levels with worse outcome may rather reflect the impact of organ failure with elevated plasma glutamine being a surrogate marker of impaired hepatic and renal function. According to the literature plasma glutamine concentration is normal in patients with chronic kidney disease [14–16]. This is also reflected in our animal model, as the animals show plasma glutamine concentrations in the normal range. Patients with acute renal failure may rather show elevated plasma glutamine concentrations [17] and thus may be more sensitive to high dose glutamine administration.

In summary our data show that administration of up to 3.0 g/kg/day alanyl-glutamine to rats in a model of chronic renal dysfunction represented by a reduction in creatinine clearance of 50% (measured in a pilot study) did not result in adverse effects. In contrast, alanyl-glutamine infusion improved the kidney morphology, warranting further investigations in different kinds of kidney injury like acute or acute-on-chronic injury as well.

Statement of authorship

MKB, HT, RA, JFS, JH and MW designed the study, and interpreted the data. BA and PS analyzed the plasma glutamine concentrations and DB interpreted the data. MKB drafted the article and DB, HT, RA, JFS, BA, PS, JH, and MW revised it critically and finally approved the version to be submitted.

Conflicts of interest

MKB, DB, HT, RA, JFS, JH, and MW are employees of Fresenius Kabi Deutschland GmbH, the sponsor of this study.

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