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High affinity uptake by isolated rat hepatocytes of a linear pseudo-hexapeptide, ditekiren

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Abstract

The hepatic elimination of many oligopeptides is both rapid and extensive, and often limits their potential as therapeutic agents. The linear, hydrophobic pseudo-hexapeptide ditekiren, a renin inhibitor, is one such example. The mechanism(s) involved in its hepatic clearance are largely unknown; accordingly, the characteristics of ditekiren's transport into isolated rat hepatocytes was investigated. In addition to a concentration-independent, linear process, uptake also involved a carrier-mediated component ($K_m = 0.2 \pm 0.05$ μM; $V_{max} = 11.6 \pm 0.6$ pmol (mg protein)$^{-1}$ min^{-1}). Phenobarbital pretreatment in vivo resulted in marked induction of such transport. Negative results from *cis*-inhibition studies with substrates and/or inhibitors of well-established hepatic transport systems, e.g., sodium-dependent bile acid, sodium-independent multispecific bile acid and cation carriers, ruled out their involvement in ditekiren's uptake. By contrast, a number of cyclic and linear oligopeptides inhibited the uptake process to varying extents and in the case of EMD-59121, the most inhibitory compound, the interaction was competitive in nature. Collectively, these data suggest the presence of a novel high affinity, low capacity transporter in rat hepatocytes with specific affinity for ditekiren and possibly other oligopeptides. © 1997 Elsevier Science B.V.

Keywords: Ditekiren; Oligopeptide; Hepatocyte; Liver uptake; Hepatic transporter

1. Introduction

The therapeutic potential of biologically active oligopeptides (4–10 amino acids) is often limited by problems associated with drug delivery, especially after oral administration. But, even if absorption difficulties by this route can be overcome, there remains the further possibility of a substantial first-pass effect by the liver and subsequent rapid hepatic elimination from the systemic circulation. Previous studies with, for example, an orally active renin inhibitor, ditekiren, exemplify these problems. Even though this linear pseudo-hexapeptide is metabolically stable, its hepatic first-pass elimination in the rat is about 70% [1] and removal from the body is extremely rapid [2,3] because of efficient and extensive biliary excretion

Abbreviations: CCK-8, cholecystokinin octapeptide; DIDS, 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Ntcp, sodium-taurocholate cotransporting polypeptide; Oatp1, organic anion transporting polypeptide

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[1,3]. Such elimination involves an initial hepatocellular uptake process followed by secretion across the canalicular membrane, both of which have characteristics consistent with carrier-mediated transport [1]. An ATP-dependent, P-glycoprotein-type transporter appears to be responsible for translocating intracellular ditekiren into the bile canaliculus [4], however, the nature and characteristics involved in the hepatic uptake process for this and oligopeptides, in general, are largely unknown.

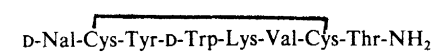
A number of distinct carrier-mediated systems at the hepatic basolateral (sinusoidal) membrane are involved in the uptake of various endogenous and exogenous compounds. These have mainly been defined by their functional characteristics in hepatocytes [5,6] but, more recently, individual transporter proteins have been cloned and heterologously expressed [7,8]. Two different mechanisms have been described for the uptake of organic anions, i.e., Na^+-independent and Na^+-dependent systems. The latter is driven by a Na^+,K^+-ATPase derived sodium gradient, and a recently cloned transporter, Ntcp, exhibits many of the properties of this system, including the high affinity transport of conjugated bile acids such as taurocholate [8]. By contrast, non-conjugated bile acids (e.g., cholate) and other organic anions like bilirubin, bromosulfophthalein and a number of drugs, e.g., pravastatin, are substrates for one or more Na^+-independent uptake systems. The cloned organic anion transporting polypeptide (Oatp1) appears to be one protein involved in such uptake but other transporters probably exist in order to account for previously observed functional characteristics, e.g., the multispecific bile acid carrier system [5,6]. Similarly, multiple mechanisms appear to be present for the hepatic uptake of organic cations [7].

Several studies have investigated the role of these functionally characterized transporter systems in the hepatocellular uptake of oligopeptides. Findings with both hydrophobic cyclic and linear peptides [9–14] have, in general, indicated the involvement of a Na^+-independent process(es). However, a Na^+-dependent component has been observed for other cyclic oligopeptides including octreotide [15,16] and a cationic transporter has been demonstrated to be involved in the uptake of certain hydrophilic oligopeptides [17]. The present study was, therefore, undertaken to obtain further information on the characteristics of hepatic uptake of oligopeptides using ditekiren as a model probe.

2. Materials and methods

2.1. Materials

Unlabeled and radiolabeled [*prolyl*-^{3}H]ditekiren (Boc-Pro-Phe-*N*-MeHis-Leuψ[CHOHCH$_2$]-Val-Ile-(aminomethylpyridine)), U-77436 (Tham-Pro-Phe-*N*-MeHis-Leuψ[CHOHCH$_2$]-Val-Ile-(aminomethylpyridine-*N*-oxide) and U-71013 (Boc-Pro-Phe-*N*-MeHis-Leuψ[CHOHCH$_2$]-Ile-(aminomethylpyridine)) were obtained from The Upjohn Co. (Kalamazoo, MI, USA). The radiopurity of the labeled peptide was greater than 98% by thin-layer chromatography and its specific activity was 33 mCi/μmol. The renin inhibitors EMD-51921 (Boc-Phe-Gly-(amino-5-cyclohexyl-3-hydroxypentanoyl)-Ile-(*N*-4-amino-2-methyl-5-pyrimidinylmethylamide), EMD-55068 (6-aminohexanonyl-Phe-Gly-(4-amino-5-cyclohexyl-3-hydroxypentanoyl)-Ile-(*N*-2-amino-5,6-dimethyl-3-pyrazinylmethylamide) were supplied by E. Merck (Darmstadt, Germany) whereas angiopeptin (lantreotide),



a somatostatin analog, was provided by the Henri Beaufour Institute (Washington, DC, USA). Procainamide ethobromide was obtained from Bristol-Meyers Squibb (Princeton, NJ, USA) and collagenase D was purchased from Boehringer Mannheim (Indianapolis, IN, USA). All other chemicals were of reagent grade and obtained from either Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Hepatocyte preparation

Hepatocytes were prepared from 225–275 g male Wistar rats (Harlan Industries, Indianapolis, IN, USA) who were fed ad libitum and had free access to drinking water. In a number of studies, rats were pretreated for 5 days with phenobarbital (1 mg/ml in drinking water). A total cell suspension was prepared using a modified collagenase perfusion method [18] and, after isolation, the cells were suspended (2×10^6

cells/ml) in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$ 5.5 mM glucose, 0.42 mM NaH_2PO4, pH 7.4) at 37°C into which 95% O_2–5% CO_2 was continuously bubbled. Cell viability was determined by trypan blue exclusion; generally, cell damage was less than 5% and preparations were not used if this value exceeded 15%.

2.3. Uptake studies

Transport studies were performed in a total volume of 2 ml consisting of 1.45 ml hepatocyte suspension, 0.5 ml Tyrode buffer solution containing unlabelled ditekiren, and 50 μl [^{3}H]ditekiren in ethanol (final concentration 0.24 μM) and/or various amounts of putative inhibitors of uptake. Uptake was terminated by rapidly transferring 0.2 ml of the incubation mixture into 5 ml ice-cold, stop-solution (30 μM ditekiren and 200 μM phloretin in Tyrode buffer). This step was immediately followed by rapid filtration through pre-wetted, glass fiber filters (Whatman 1.2 μm diameter, Fisher Scientific) and a subsequent wash with 5 ml ice-cold, stop-solution. Filters were dissolved in 5 ml of liquid scintillation fluid (BCS, Amersham, Arlington Heights, IL, USA) and the radioactivity determined by liquid scintillation spectrometry (Model 1219, Rackbeta®, LKB Instruments Inc., Gaithersburg, MD, USA). The measured uptake was corrected for non-specific binding to the filter by subtracting the amount of radioactivity present in an aliquot taken immediately after addition of [^{3}H]ditekiren. The rate of initial uptake was estimated by linear regression of data obtained at 10, 20, 30, 45 and 60 s after addition of labeled ditekiren. All studies were routinely performed in duplicate/triplicate and were confirmed in at least three separate hepatocyte preparations.

To define a saturable uptake process with respect to ditekiren concentration, the initial rates of uptake measured during incubation at 4°C were subtracted from similar values obtained following incubation at 37°C. The resulting curve was subsequently analyzed (Fig. Perfect, Biosoft, Miltown, NJ) according to simple Michaelis-Menten type, non-linear kinetics. An unpaired Student's *t*-test was used for statistical comparisons and $P < 0.05$ was taken as the minimum level of statistical significance.

3. Results

3.1. Uptake kinetics of [^{3}H]ditekiren and determinants

[H^3]Ditekiren uptake into hepatocytes was rapid with equilibrium being reached by about 20 min; for the first 90 s, uptake was linear over the concentration range studied, thus, allowing determination of an initial uptake rate over the first 60 s (Fig. 1A). The relationship between this rate and ditekiren concentration was curvilinear when determined at 37°C but linear at 4°C (Fig. 1B). The latter was interpreted to reflect passive diffusion and/or non-specific binding and subtraction of this component revealed a saturable transport process characterized by Michaelis-Menten kinetics, with a K_m value of 0.20 ± 0.05 μM and a V_{max} value of 11.6 ± 0.6 pmol (mg protein)$^{-1}$ min^{-1}.

The dependency of ditekiren uptake on cellular metabolic status was assessed by replacing oxygen with nitrogen in the gas mixture bubbled through the hepatocyte suspension. Such pretreatment for 30 min resulted in a 41 ± 7% reduction in ditekiren's initial uptake rate which was fully reversed by subsequent oxygenation for 30 min. In addition, pre-incubation for 30 min with a number of known metabolic inhibitors or ATP-depleting agents also resulted in a reduction in uptake: 10 mM iodoacetamide (23 ± 5%), 10 mM FCCP (21 ± 8%), 1 mM ouabain (37 ± 9), 1 mM dinitrophenol (48 ± 8), 10 mM sodium azide (44 ± 15%), and 30 μM rotenone (63 ± 2%).

By contrast, replacement of Na^+ by K^+, Li^+, or choline in the buffer solution in which the hepatocytes were suspended did not alter ditekiren's uptake. Similarly, Cl^- replacement with either NO_3^-, SO_4^{2-} or gluconate was without effect. Valinomycin (90 nM), which induces an inwardly directed negative potential [14], also did not affect ditekiren uptake, when added to the buffer 10 min prior to addition of labeled ditekiren. However, the electroneutral Na^+/H^+ exchanger, monensin (144 nM), resulted in a 58 ± 10% reduction.

3.2. Effect of inhibitors on [^{3}H]ditekiren uptake

cis-Inhibition studies were performed by the addition of putative inhibitors to the hepatocyte suspen-

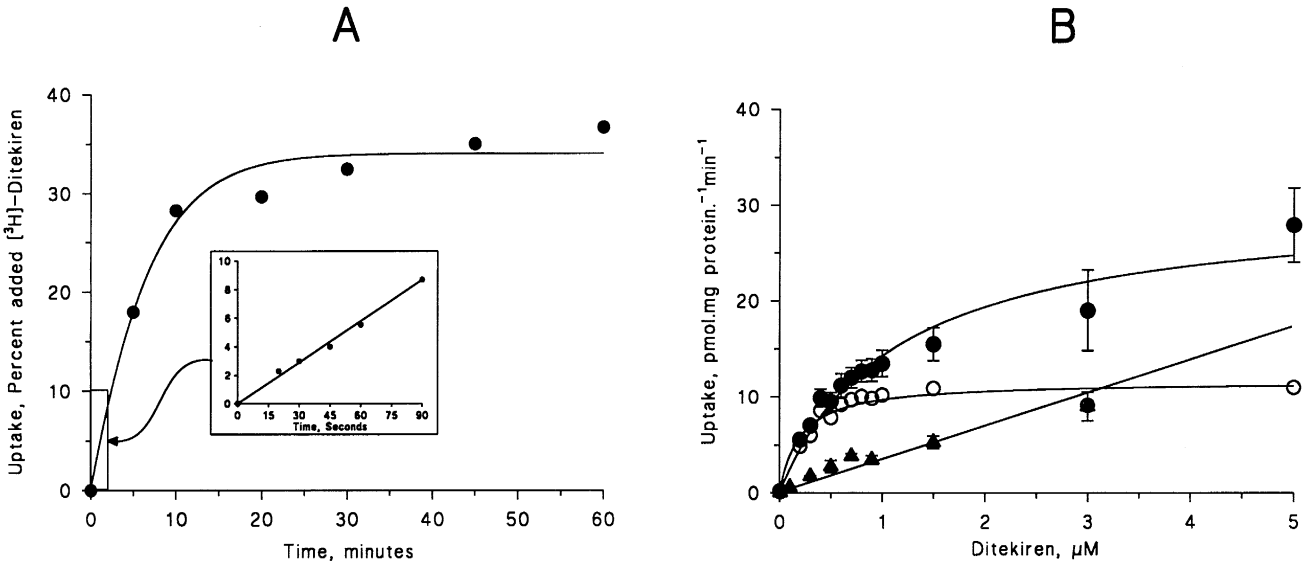


Fig. 1. Time course of [^{3}H]ditekiren (0.24 μM) uptake into rat hepatocytes (Panel A) and the effect of ditekiren concentration on its initial uptake rate (Panel B). Mean data ± S.E. of total uptake at 37°C (●) and 4°C (▲) and carrier-mediated uptaken (○).

sion 30 s prior to the addition of [^{3}H]ditekiren. No statistically significant change in the initial uptake rate of ditekiren was observed with taurocholate (100 μM), cholate (100 μM), DIDS (1 mM), procainamide ethobromide (100 μM), *N*-methylnicotinamide (100 μM), and oleate (30 μM) (Table 1). On the other hand, bromosulfophthalein (100 μM) caused a $57 \pm 6\%$ ($P < 0.05$) reduction in uptake, and inhibition ranging from 0 to over 90% was also noted with a number of cyclic and linear oligopeptides with varying structural similarity to ditekiren (Table 1, Fig. 2). In the case of EMD-51921, such inhibition was found to be competitive in nature with a K_i value of 1.2 μM (Fig. 3); other inhibitory peptides were not investigated in this regard.

Table 1
Inhibition of ditekiren transport by various compounds

Inhibitor	Percentage reduction in uptake
Taurocholate (100 μM)	2 ± 5
Cholate (100 μM)	3 ± 5
DIDS (1 mM)	10 ± 7
BSP (100 μM)	57 ± 6 [a]
Procainamide ethobromide (100 μM)	12 ± 9
N-Methylnicotinamide (100 μM)	0 ± 4
Oleate (30 mM)	8 ± 8
Phalloidin (100 μM)	31 ± 4 [a]
Angiopeptin (100 μM)	82 ± 1 [a]
CCK-8 (10 μM)	0 ± 6
U-77436 (100 μM)	45 ± 7 [a]
U-71013 (100 μM)	76 ± 5 [a]
EMD-55068 (100 μM)	80 ± 1 [a]
EMD-51921 (100 μM)	93 ± 1 [a]

[a] $P < 0.05$

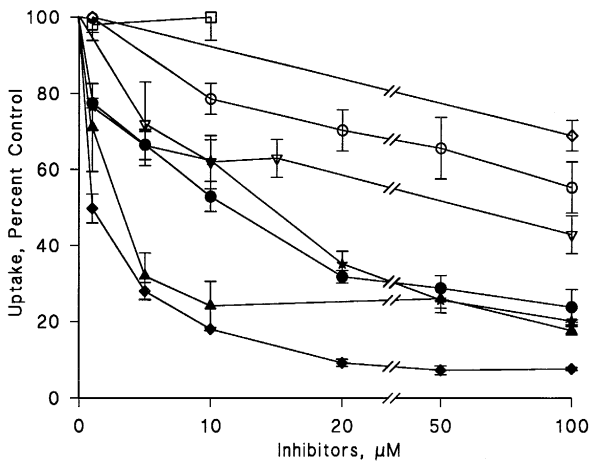


Fig. 2. *cis*-Inhibition of ditekiren's initial uptake rate at 37°C by BSP (▽) and various oligopeptides: □ CCK-8, ◇ phalloidin, ○ U77436, ● U71013, ▲ angiopeptin, ★EMD 55068, ◆ EMD 51921.

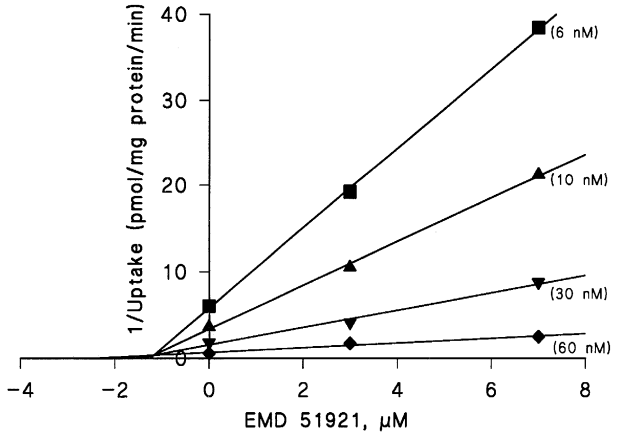


Fig. 3. Dixon plot analysis of ditekiren transport inhibiton by EMD51921.

3.3. Induction of [^{3}H]ditekiren uptake by phenobarbital

Pretreatment of rats with phenobarbital for 5 days prior to hepatocyte preparation markedly increased ditekiren's initial uptake rate (Fig. 4). Kinetic analysis showed that this was associated with marked enhancement (10-fold) of the parameters associated with the saturable, carrier-mediated process ($K_m = 2.3 \pm 0.2$ μM, $V_{max} = 101 \pm 5$ pmol (mg protein)$^{-1}$ min^{-1}) rather than the linear passive diffusion component, which was similar to that determined in untreated animals.

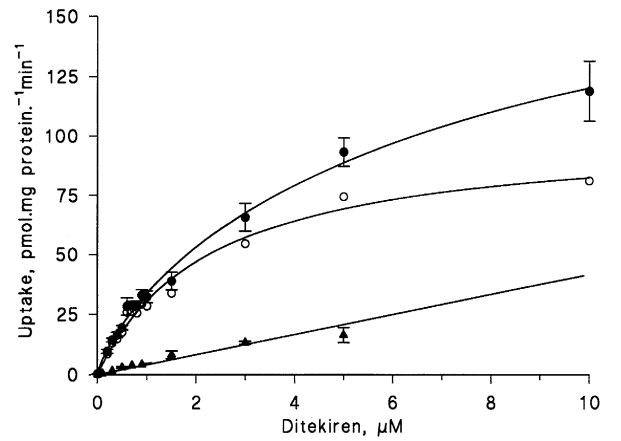


Fig. 4. Induction of ditekiren uptake in hepatocytes derived from rats pretreated with phenobarbital. Mean data ± S.E. of total uptake at 37°C (●) and 4°C (▲) and carrier-mediated uptake (○).

4. Discussion

A limiting factor in the development of potentially useful therapeutic oligopeptides has been their rapid hepatic elimination, which often involves biliary excretion. Such clearance requires vectorial transport from the blood into the hepatocyte and subsequent translocation into the bile canaliculus. Accordingly, uptake of such oligopeptides across the basolateral (sinusoidal) membrane into the hepatocyte is the critical first step. The results of this study demonstrate that two different mechanisms are involved in this initial process with respect to the pseudo-hexapeptide ditekiren. First, a linear, concentration-independent process was present consistent with passive diffusion of this very hydrophobic oligopeptide – the logarithm of its octanol/water partition coefficient is greater than 4 [19]. In addition, a saturable, concentration-dependent uptake process exhibiting several further characteristics of a carrier-mediated process was definable. For example, uptake was temperature-dependent as well as being determined by the metabolic status of the cell, moreover, transport was inhibited by several other oligopeptides but not by non-peptide organic anions and cations, with the exception of bromosulfophthalein. Kinetic analysis indicated that this transport system had a low capacity but a surprisingly high affinity for ditekiren as indicated by a K_m value of 0.2 μM, which is considerably smaller than the typical K_m values of substrates for other characterized hepatic basolateral membrane transporters [5,6]. In addition, the carrier-mediated process was markedly induced by pretreatment with phenobarbital; however, the 10-fold increase in K_m suggests that such induction involved the expression of other transporters with different kinetic characteristics to the constitutive protein. Such inductive effects to phenobarbital have been observed with respect to the uptake of certain organic anions [20,21]; for example, a 6-fold induction in BSP uptake which also involved changes in both V_{max} and K_m [21].

Recently, there has been increased interest in the carrier-mediated transport of both endogenous and exogenous compounds in the liver [5,6], primarily as a result of the successful cloning of a number of individual transporters at both the basolateral and canalicular membranes [7,8]. The question, therefore,

arises as to whether any of these well-characterized processes are involved in the uptake of oligopeptides. For example, the cyclic octapeptide and somatostatin analog octreotide appears to be transported into rat hepatocytes by an Na^+-dependent process suggesting that a bile acid transporter such as Ntcp may be involved [15]. By contrast, the hepatocellular uptake of several other, and structurally divergent oligopeptides appears to be Na^+-independent; for example, several hydrophobic cyclic oligopeptides such as the amatoxins, like phalloidin [9] and antamanide [10], cyclosomatostatins [11,12], and linear renin inhibiting peptides, exemplified by EMD-51921 [13,14]. Because of the broad substrate specificity of the involved process, it has generally been referred to as the multispecific bile acid system [5] to distinguish it from Na^+-dependent bile acid transport. Currently, it is not clear whether this functionally defined system corresponds to the recently cloned Oatp1 transporter [7,8] and the ability of the Oatp1 to transport oligopeptides has not been demonstrated. On the other hand, the hepatocellular uptake of a hydrophobic, linear renin inhibitor (EMD-56133) appears to involve a Na^+-independent transport system for bivalent cations or uncharged compounds like ouabain [15]. Also, several different transporters may be involved in the uptake of some oligopeptides [17]. Such differences in carrier-mediated transport are not surprising given the diversity of oligopeptides with respect to such putative determinants as size, structure, lipophilicity and charge. The findings with ditekiren suggest, however, the presence of an additional and previously undescribed transporter process for oligopeptides.

The fact that ditekiren's uptake was Na^+-independent and also that taurocholate did not inhibit such transport indicate a lack of involvement of Ntcp. Moreover, the inability of ditekiren to inhibit taurocholate transport by human NTCP as well as not being a substrate has recently been directly demonstrated [22]. Inhibition of uptake by bromosulfophthalein and also monensin would be consistent with a role for the multispecific bile acid transporter [5], however, several other observations do not support this interpretation. For example, the hepatocellular uptake of many substrates for this transporter is Cl^--sensitive and modulated by NO_3^-, SO_4^{2-}; additionally, both cholate and DIDS are effective inhibitors [5]. None of these characteristics were exhibited by the ditekiren carrier-mediated process; moreover, the oligopeptide does not appear to be a substrate for Oatp1 in a recombinant expression system (Kim, R.B. and Wilkinson, G.R., unpublished data). A role for cationic hepatic transporters [5,6] would also appear to be precluded because of the lack of inhibition produced by procainamide ethobromide and *N*-methylnicotinamide. By contrast, a number of other oligopeptides, but not all, reduced ditekiren's hepatocellular uptake suggesting a degree of specificity in the interaction. However, these findings do not necessarily indicate that such oligopeptides are substrates for the involved transporter. EMD-51921, for example, was the most potent inhibitor and this occurred in a competitive fashion; however, previous studies have shown that this renin inhibitor is transported into hepatocytes by the multispecific bile acid transporter with completely different functional characteristics to that of ditekiren [13,14].

The overall findings of this study, therefore, collectively suggest the presence of a transport system for ditekiren, likely in the basolateral membrane of hepatocytes, with characteristics unlike those previously reported for other oligopeptides. Moreover, the high affinity of this carrier system ($K_m = 0.2\ \mu M$) might indicate a physiological role for endogenous oligopeptides. Molecular cloning of such a carrier system will be required for further characterization and comparison with other oligopeptide transporters.

Acknowledgements

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