Prevention of Thrombosis and Enhancement of Thrombolysis in Rabbits by SR 121787, a Glycoprotein II/III Antagonist

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ABSTRACT
SR 121566, a novel nonpeptide antiplatelet agent with high affinity and specificity for the GP IIb/IIIa complex, exhibits potent in vitro antiaggregating activity in rabbit platelets. This paper reports results from a study in rabbits about the efficacy and tolerability of SR 121787, the prodrug of SR 121566. After p.o. pretreatment with SR 121787, ADP-, arachidonic acid- and collagen-induced rabbit platelet aggregation was inhibited ex vivo in a dose-dependent manner (ED50 between 2.3 and 6.1 mg/kg). Collagen-induced thrombocytopenia was totally abolished by SR 121787 at 20 mg/kg p.o. In a carotid artery lesion model of arterial thrombosis, p.o. administration of SR 121787 resulted in a dose-dependent inhibition of thrombosis with a maximum effect of 68% (ED50 = 16.0 ± 0.3 mg/kg). Recombinant tissue plasminogen activator-induced thrombolysis of a preformed thrombus in the jugular vein was potentiated by SR 121787 at doses between 1 and 6 mg/kg i.v. In an ear incision bleeding model, SR 121787 at doses up to 15 mg/kg p.o. did not cause an increase in blood loss. These results demonstrate that SR 121787 exerts oral antiplatelet, antithrombotic and thrombolysis-enhancing efficacy in rabbits. SR 121787 appears to be a promising compound for evaluation under clinical conditions in the therapy of acute coronary syndromes.

The activation of platelets and the resultant aggregation have been shown to play an important role in the pathogenesis of cardiovascular, cerebrovascular and peripheral vascular diseases and in the therapy of acute coronary syndromes (Hennekens et al., 1989). Hence platelet adhesion and aggregation have been identified as promising targets for the development of antithrombotic drugs. GP IIb/IIIa antagonism exerts a strong antiplatelet effect, because this interference inhibits the final common pathway of platelet aggregation and is not dependent on a single activation pathway. In vivo studies in several thrombosis models have provided evidence that the blockade of GP IIb/IIIa receptors could protect animals from acute thrombosis induced by vascular injury and facilitate the lysis of preformed thrombi by thrombolytic agents (Coller, 1997). Clinical trials demonstrated benefits of GPIIb/IIIa receptor inhibitor therapy with different agents and in different clinical indications of acute coronary syndromes (Topol, 1997).

Despite the extensive testing of GP IIb/IIIa antagonists, it remains to be determined whether effective antithrombotic activity can be achieved without inducing unwanted hemorrhagic side effects. Moreover, comparative studies on the efficacy and tolerability of GP IIb/IIIa antagonists and other antiplatelet agents are lacking. In the clinical trials utilizing GP IIb/IIIa inhibitors, ASA was used in the control group. The effects of GP IIb/IIIa inhibitors were therefore additional to the effects of ASA. Clopidogrel, a ticlopidine-related thienopyridine, has also been shown to be more efficacious than, and at least as safe as, ASA in preventing the combined outcome cluster of myocardial infarction, ischemic stroke and peripheral arterial disease in patients with symptomatic atherosclerotic disease (Caprie, 1996). Clinical studies that compare the efficacy of GP IIb/IIIa antagonists and thienopyridines have not yet been done.

Species differences in the platelet inhibitory activity of GP IIb/IIIa antagonists have complicated their in vivo evaluation in experimental models of thrombosis. It is generally considered that rats and rabbits, in which most thrombosis models are developed, are not appropriate for testing GP IIb/IIIa antagonists designed for human use, because GP IIb/IIIa antagonism results in only a weak inhibition of platelet aggregation in these species, compared with the inhibitory effects on human platelets (Harfenist et al., 1988; Verhallen and Barth, 1991). Comparative studies in dogs and in guinea pigs revealed that GP IIb/IIIa antagonists possess a higher antithrombotic efficacy than ASA (Frederick et al., 1996; Nishiyama et al., 1995).

SR 121566, a novel nonpeptide antiplatelet agent with high affinity and specificity for the GP IIb/IIIa complex, exhibited potent in vitro antiaggregating activity in rabbit platelets (Savi et al., 1998; Hoffmann et al., 1997). We sub-

ABBREVIATIONS: ASA, acetylsalicylic acid; AA, arachidonic acid; rt-PA, recombinant tissue plasminogen activator; SR 121787, (3-[[4-[4-(amino-ethoxycarbonylimino-methyl)-phenyl]-1,3-thiazol-2-yl]-[1-ethoxycarbonylmethyl-piperidin-4-yl]amino]-propionic acid ethylester.
sequently observed that SR 121566 and its prodrug SR 121787 exhibited antithrombotic efficacy in an arteriovenous shunt and in a venous stasis thrombosis model in rabbits (Hérault et al., 1998; Hoffmann et al., 1997). The present study was designed to characterize in more detail the effects of SR 121787 on acute arterial thrombosis in rabbits and to compare its efficacy and tolerability with that of ASA and clopidogrel. The *ex vivo* and *in vivo* antiplatelet effects after p.o. administration of SR 121787, ASA and clopidogrel were investigated. In addition, antithrombotic and thrombolysis-enhancing actions of these three compounds were compared, and bleeding side effects were examined.

**Materials and Methods**

**Drugs and materials.** The following drugs and chemicals were used in this study: ADP (Boehringer Mannheim, Mannheim, Germany), collagen (type I) (Sigma Chemical Co., St. Louis, MO), AA (Nu-Check Prep Inc., Elusian, MN), ASA (Synthelabo, Meudon-la-Forêt, France), rt-PA (Boehringer Ingelheim, Karl Thomaes, Biberach, Germany), heparin (Sigma, Saint-Quentin-Fallavier, France) and sodium pentobarbital, SR 121787 and clopidogrel (Sanofi Recherche, Toulouse, France). The dosages of clopidogrel and ASA that we used have been shown to be optimal in rabbits (Bernat et al., 1993; Herbert et al., 1993; Hoffmann et al., 1997).

**Animals.** Male New Zealand rabbits (3.0–3.5 kg) were obtained from Lago (Vonnas, France). The protocol of this study was approved by the Animal Care and Use Committee of Sanofi Recherche.

**Ex vivo platelet aggregation.** Rabbıts were pretreated by gavage with SR 121787, clopidogrel, ASA or water. SR 121787 was dissolved in 0.1 N HCl and diluted with purified water. Clopidogrel was dissolved in water, and ASA was dissolved in carboxymethyl cellulose (0.6%). Two hours after p.o. pretreatment, a time-point that has been shown to be optimal for the three compounds (Herbert et al., 1993; Hoffmann et al., 1997), blood samples obtained by venipuncture were collected into a 3.8% trisodium citrate solution (9/1 v/v). Platelet-rich plasma was obtained by centrifuging the blood sample at 80 × g for 20 min. Platelet aggregation was determined according to the method of Born (1961) on a dual-channel Chrono-Log aggregometer. Aggregation was induced by the addition of ADP, AA or collagen (final concentrations: 2.5 μM, 250 μM and 12 μg/ml, respectively). The extent of aggregation was estimated quantitatively by measuring the maximal curve height above the baseline level (ADP and AA) or the slope of the aggregation curve (collagen).

**Collagen-induced thrombocytopenia.** Collagen (4 mg/kg) or saline was infused for 3 min into the marginal ear veins of pentobarbital-anesthetized (30 mg/kg i.v.) rabbits 2 h after water or SR 121787, clopidogrel, ASA or AA treatment. At the indicated time-points, carotid artery blood was sampled into a 3.8% trisodium citrate solution (9/1 v/v), and the platelet count was determined immediately with a BAKER instrument hematogy counter.

**Thrombus formation in the carotid artery.** SR 121787, clopidogrel, ASA or water was administered p.o. 2 h before thrombosis induction, whereas heparin was injected i.v. 5 min before thrombosis induction. Thrombus formation was induced after creation of an endothelial lesion by electrical stimulation of the carotid artery according to a modified method of Hladovec (1971) as recently described (Herbert et al., 1996). Rabbıts were anesthetized with sodium pentobarbital (30 mg/kg i.v.). A segment of the left carotid artery (about 10 mm long) was exposed and dissected free of surrounding tissue. A small piece of insulating film (Parafilm M) and two stainless steel electrodes were positioned under the artery. Using a constant d.c. power supply (Apelix 3500), we stimulated the artery at 2.5 mA for 3 min. Blood flow was measured at a point distal to the site of the electrical stimulation with an electromagnetic flowmeter (Narco- matic, Roucaire, Velizy, France) at 5-min intervals over a 45-min period. After 45 min, the carotid arteries were opened longitudinally, and the thrombus, if apparent, was removed, blotted on filter paper and weighed.

**Lysis of a thrombus in the jugular vein.** The effect of SR 121787, clopidogrel and ASA on rt-PA-induced thrombolysis of a standard-sized, preformed thrombus in the external jugular vein of rabbits was investigated according to Collen et al. (1983) as recently described (Herbert et al., 1996). The jugular vein of pentobarbital-anesthetized rabbits (30 mg/kg i.v.) was isolated, and all tributaries at a distance of 4 cm from the main bifurcation of the external jugular and facial veins were ligated. A silk thread was then inserted through the vessel to anchor the thrombus and avoid embolization. Thrombus formation was induced by ligating the vein with two vessel clamps. After 30 min, the clamps were removed and the blood flow was restored. Thrombolysis was performed by infusion of rt-PA at 0.5 mg/kg for 4 h with a 10% bolus via the contralateral marginal ear vein. SR 121787, clopidogrel, ASA or saline was given as an i.v. bolus injection at the start of the infusion. At the end of the rt-PA infusion period, the thrombus was carefully removed from the vein and weighed.

**Bleeding.** Rabbits were anesthetized with sodium pentobarbital (30 mg/kg i.v.), and five standardized incisions were made through the ear with a scalpel blade (N°21, Swann-Norton) as described recently (Herbert et al., 1996). Care was taken to avoid any macroscopically visible vessel. The ear was immersed in a 500-ml saline bath at 37°C under continuous stirring. Blood loss was determined 10 min later by measurement of the hemoglobin content, using a spectrophotometric method, after the addition of a hemolyzing reagent (Zapoglobin, Coultronics, France). The antplatelet drug or vehicle was administered by gavage 2 h before ear incision.

**Statistical analysis.** The results shown are mean ± S.E.M. Data were statistically analyzed with ANOVA followed by Dunnett’s test. For the blood flow data, two-way ANOVA with a subsequent contrast analysis was used. Statistical significance was accepted at *P* < .05. ED<sub>50</sub> values ± 95% confidence intervals were calculated by fitting the logistic equation to the data by means of nonlinear regression.

**Results**

**Antiaggregating effects.** *Ex vivo* platelet aggregation induced by ADP, AA or collagen in a dose-dependent manner 2 h after the p.o. SR 121787 treatment of rabbits (fig. 1). The ED<sub>50</sub> values for ADP, AA- and collagen-induced aggregation were 2.3 ± 0.3, 6.1 ± 0.9 and <2.5 μg/ml, respectively. Single doses of SR 121787 (black columns), clopidogrel (hatched columns) or ASA (white columns) were administered by gavage. Ex *vivo* platelet aggregation was induced 2 h later by 2.5 μM ADP, 250 μM AA or 12 μg/ml collagen (final concentrations) as described under “Materials and Methods.” Each point represents the mean ± S.E.M. of five animals (*P* < .05 vs. control).

*Fig. 1.* Effects of SR 121787, clopidogrel and ASA on platelet aggregation *ex vivo*. Single doses of SR 121787 (black columns), clopidogrel (hatched columns) or ASA (white columns) were administered by gavage. Ex *vivo* platelet aggregation was induced 2 h later by 2.5 μM ADP, 250 μM AA or 12 μg/ml collagen (final concentrations) as described under “Materials and Methods.” Each point represents the mean ± S.E.M. of five animals (*P* < .05 vs. control).
mg/kg, respectively. Oral pretreatment with 100 mg/kg clopidogrel inhibited ADP- and collagen-induced aggregation by 68% and 80%, respectively, whereas AA-induced aggregation was not significantly influenced. Oral ASA pretreatment at 100 mg/kg inhibited the aggregating effects of AA and collagen (93% and 97%, respectively) without any influence on ADP-induced platelet aggregation.

**Inhibition of collagen-induced thrombocytopenia.** Infusion of saline into a marginal ear vein for 3 min did not significantly change the number of circulating platelets (fig. 2). Infusion of collagen (4 mg/kg) induced a drop in the circulating platelet count within 5 min, and platelet count remained at this low level during the 30-min observation period. Oral pretreatment with SR 121787 at a dose of 10 mg/kg 2 h before collagen infusion inhibited the thrombocytopenic effect of collagen slightly but nonsignificantly; a complete elimination of the collagen effect was observed after pretreatment with SR 121787 at 20 mg/kg (fig. 2A). Clopidogrel pretreatment at 100 mg/kg exhibited an inhibitory action on the drop in platelet count after collagen infusion that attained significance level at 20 and 30 min after collagen infusion, whereas pretreatment with ASA at 100 mg/kg did not influence collagen-induced thrombocytopenia (fig. 2B).

**Antithrombotic efficacy in a carotid artery lesion model.** Under control conditions, the thrombogenic subendothelial surface that was exposed after electrical stimulation of the carotid artery initiated the formation of an occlusive thrombus within 23.3 ± 2.4 min, i.e., blood flow was 100% inhibited (fig. 3A). Oral SR 121787 pretreatment at 5 mg/kg 2 h before electrical stimulation did not affect the reduction in blood flow. However, blood flow reduction was inhibited in a dose-dependent manner by pretreatment with SR 121787 at 10 or 20 mg/kg p.o. (fig. 3A). At the highest dose (20 mg/kg), initial blood flow was reduced by 32% at 45 min. The ED50 value was 16.0 ± 0.3 mg/kg.

SR 121787-induced inhibition of reduction in carotid blood flow correlated with the morphologic findings observed upon opening the arteries after the 45-min blood flow measurement period (table 1). Under control conditions, a large, red occlusive thrombus with an average wet weight of 4.8 ± 0.6 mg was found. After treatment with SR 121787 at 5 mg/kg, thrombus growth was not influenced. Pretreatment with 10 mg/kg SR 121787 resulted in a reduction of wet thrombus weight to 2.7 ± 0.6 mg. In animals treated with 20 mg/kg SR 121787, no measurable thrombus formation was found.

After pretreatment with clopidogrel at the dose of 100 mg/kg p.o., occlusion of the carotid artery was prevented. An inhibition of blood flow reduction by 24% (fig. 3B) and a 65% reduction in thrombus weight (table 1) was observed at 45 min. The antithrombotic effect of ASA at a dose of 100 mg/kg p.o. was weak. Ten percent inhibition of blood flow reduction was observed at 45 min (fig. 3B). Thrombus weight was nonsignificantly reduced by 17% (table 1). The inhibitory effects of clopidogrel and ASA on blood flow reduction and thrombus formation were smaller than the effects of SR 121787 at 20 mg/kg (P < .05).

**Lysis of a preformed thrombus in the jugular vein.** Infusion of rt-PA at the threshold dose of 0.5 mg/kg did not significantly reduce the weight of the preformed thrombus in

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**Fig. 2.** A) Inhibition of collagen-induced thrombocytopenia by SR 121787. Animals were pretreated by gavage with SR 121787 (10 or 20 mg/kg) or saline. Two hours later, collagen (4 mg/kg) was injected into a marginal ear vein. A second control group was not pretreated and was injected with saline. Each point represents the mean ± S.E.M. of 5 to 7 animals (* P < .05 vs. collagen-treated controls). B) Effects of clopidogrel and ASA on collagen-induced thrombocytopenia. Animals were pretreated by gavage with clopidogrel (100 mg/kg), ASA (100 mg/kg) or saline. Two hours later, collagen (4 mg/kg) was injected into a marginal ear vein. A second control group was not pretreated and was injected with saline. Each point represents the mean ± S.E.M. of 5 to 7 animals (* P < .05 vs. collagen-treated controls).
the jugular vein, compared with thrombi from control animals that were not treated with rt-PA (fig. 4). SR 121787 potentiated rt-PA thrombolysis (0.5 mg/kg) in a dose-dependent manner at a dose range between 1 and 6 mg/kg. The rt-PA thrombolysis-enhancing effects of clopidogrel and ASA (100 mg/kg) in this rabbit model were marginal and did not attain statistical significance (fig. 4). SR 121787, clopidogrel and ASA per se exhibited no thrombolytic activity.

Hemostasis. The effects of p.o. pretreatment with SR 121787, clopidogrel and ASA on blood loss from the incised rabbit ear are summarized in table 2. SR 121787, at p.o. doses up to 15 mg/kg administered 2 h before measurement of bleeding, did not cause a significant increase in blood loss.

A significant bleeding effect was found at 20 mg/kg SR 121787 (16-fold increase in blood loss). For each dose tested, bleeding was not observed at sites other than the confined site of measurements of bleeding time. Clopidogrel and ASA, at 100 mg/kg administered 2 h before the bleeding test, did not influence the blood loss from the incised ear.

Discussion

Main findings. The principal findings of this study are 1) that SR 121787 demonstrated antiplatelet, antithrombotic and thrombolysis-enhancing actions after p.o. administration to rabbits, 2) that SR 121787 exerted these actions with a high potency and efficacy compared with clopidogrel or ASA and 3) that no significant bleeding effect of SR 121787 was observed at a dose that resembled the ED50 in the arterial thrombosis model.

Antiplatelet effects. It is generally considered that rats and rabbits, in which most thrombosis models are developed, are not appropriate for testing GP IIb/IIIa antagonists designed for human use, because GP IIb/IIIa antagonism results in only a weak inhibition of platelet aggregation in these species (Harfenist et al., 1988; Verhallen and Barth, 1991). It has been shown, however, that SR 121566, the active moiety of the orally active prodrug SR 121787, exhibited a potent in vitro antiaggregating effect on rabbit platelets (Hoffman et al., 1997). Thienopyridines, on the other hand, exhibit a lower antiplatelet efficacy in rabbits than in rats, because ADP plays a less important role in thrombogenesis in rabbits (Defreyn et al., 1991). However, rats cannot be used for testing GP IIb/IIIa antagonists. Therefore, the rabbit seems an acceptable compromise, especially if one takes into account that the rabbit is one of the most frequently used species in thrombosis research.

In the present study, SR 121787 inhibited ex vivo platelet aggregation. It was equally effective against the three stim-
ul ADP, AA and collagen. The high potency and efficacy of SR 121787 against all three inducers of platelet aggregation, compared with the actions of clopidogrel and ASA, is consistent with the concept that inhibition of the final common pathway, the GP IIb/IIIa complex on the activated platelet’s membrane, is a more efficacious antiplatelet principle than inhibition of any single activation pathway. This view is also supported by the observation that SR 121787 revealed a higher efficacy than clopidogrel and ASA in the collagen-induced thrombocytopenia model. Efficacious doses of SR 121787 in this in vivo model, however, were higher than for inhibition of ex vivo platelet aggregation.

**Antithrombotic actions.** An endothelial injury model was used to examine the antithrombotic efficacy of SR 121787 in arterial-type acute thrombosis. Endothelial injury initiates platelet activation via subendothelial connective tissue structures and stimulates tissue factor-dependent generation of thrombin via proteolytic activation of the serine proteases that comprise the coagulation cascade (Harker, 1997). In this study, it was shown that SR 121787 inhibited thrombus formation in a dose-dependent manner, as evidenced by an inhibition of reduction in blood flow and by a decrease in thrombus weight. The antithrombotic ED$_{50}$ was comparable to effective doses in the collagen-induced thrombocytopenia model.

The maximal antithrombotic effect of SR 121787 in this carotid artery injury model was about 68%, although ex vivo platelet aggregation was inhibited by about 90% at this dose. The restricted antithrombotic efficacy of SR 121787 may be due to a contribution of the clotting system in the formation of the thrombus at the site of the endothelial lesion.

We previously reported that SR 121787 exhibited potent antithrombotic efficacy in an arteriovenous shunt model in rabbits (Hoffmann et al., 1997). Present results in the vascular injury model confirm the potent p.o. antithrombotic efficacy of SR 121787, with slightly higher antithrombotic ED$_{50}$ values (16.0 ± 0.3 vs. 10.4 ± 0.8 mg/kg in the arteriovenous shunt model).

Clopidogrel clearly demonstrated in vivo and ex vivo antiplatelet activity, but in the present vascular injury thrombosis model, it had a lower antithrombotic efficacy than SR 121787. Other studies compared the antithrombotic efficacy of the GP IIb/IIIa antagonists TAK-029 and DMP 728 with the effects of clopidogrel and ticlopidine in guinea pig thrombosis models (Mousa et al., 1994a; Kawamura et al., 1996). However, thienopyridines possess weak antiplatelet effects in this species (Panak et al., 1983). It appears, therefore, that available data on comparative antithrombotic efficacy between clopidogrel/ticlopidine and GP IIb/IIIa antagonists in guinea pigs (Mousa et al., 1994a; Kawamura et al., 1996) and rabbits (Hoffmann et al., 1997; this study) may underestimate the antithrombotic efficacy of clopidogrel compared with the effects of clopidogrel in other species such as rats and humans (Herbert et al., 1993).

ASA inhibited AA- and collagen-induced platelet aggregation ex vivo, but it failed to inhibit collagen-induced thrombocytopenia and exhibited a weak effect on thrombus formation in the injured carotid artery. This confirms recent data showing that GP IIb/IIIa antagonists are more effective than ASA in the Folts coronary artery model in dogs and in a guinea pig model with photochemically induced thrombosis in the femoral artery (Frederick et al., 1996; Nishiyama et al., 1995).

**Thrombolysis-enhancing activity.** Thrombolysis has become a standard treatment for patients with acute myocardial infarction. However, post-thrombotic reocclusion represents a significant problem. Experimental and clinical data implicate platelet-rich thrombus formation as a major factor in the failure to achieve successful thrombolysis and in abrupt closure of luminal patency (Collen, 1990). In the present rabbit rt-PA thrombosis model, SR 121787 showed a dose-dependent effect with a maximal efficacy of 54% in enhancing rt-PA thrombolysis. Comparable data have been published for the GP IIb/IIIa antagonist DMP728 in a canine model of femoral artery thrombosis, in which DMP728 enhanced the thrombolytic effect of streptokinase (Mousa et al., 1994b). In vitro, DMP728 dispersed a preformed platelet-rich clot and potentiated the effects of different thrombolytic drugs (Mousa et al., 1994b). In the current rabbit rt-PA thrombolysis model, clopidogrel and ASA revealed lower efficacy. The potential of clopidogrel to enhance rt-PA- and streptokinase-induced thrombolysis was previously described in dogs (Yao et al., 1994) and rabbits (Bernat et al., 1999). In the canine coronary artery thrombosis model, clopidogrel was more effective than aspirin in enhancing rt-PA thrombolysis.

**Hemorrhagic side effects.** Because of the key role of the GP IIb/IIIa complex in hemostasis, it remains to be determined whether effective antiaggregatory and antithrombotic activity in humans can be achieved by blocking this receptor without causing hemorrhagic problems (Raddatz and Gante, 1995). In the present study in rabbits, SR 121787 caused a slight but non-significant prolongation of bleeding time (1.7-fold) at a dose (15 mg/kg) that resembles the antithrombotic ED$_{50}$. However, there is considerable controversy about the correlation between bleeding models and blood loss in a clinical situation (Lind, 1991), so the relevance of the present observations for the clinical experience remains to be established.

In conclusion, SR 121787 is a GPIIb/IIIa antagonist with oral antiplatelet, antithrombotic and rt-PA thrombolysis-enhancing activity in rabbits. It appears to be a promising compound for evaluation in cardiovascular, cerebrovascular and peripheral vascular diseases and in the therapy of acute coronary syndromes.

### TABLE 2

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Control</th>
<th>SR 122787</th>
<th>Clopidogrel</th>
<th>ASA</th>
</tr>
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<tbody>
<tr>
<td>Blood loss (µl/10 min)</td>
<td>192 ± 66</td>
<td>96 ± 31</td>
<td>64 ± 24</td>
<td>335 ± 103</td>
</tr>
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*P < .05 vs. control.
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References


Fibrinolysis


Fibrinolysis

Acknowledgments


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