Inhibition of the amplification reactions of blood coagulation by site-specific inhibitors of α -thrombin

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Hirudin and hirulog-1 {D-Phe-Pro-Arg-Pro-[Gly]₄-desulphohirudin-(54–65)} abrogate the enzyme activities of α -thrombin by binding the enzyme simultaneously at its catalytic centre and fibrin(ogen)-recognition exosite. In contrast, hirugen [hirudin-(54–65)] binds α -thrombin solely at the fibrin(ogen)-recognition exosite, and competitively inhibits fibrinopeptide A release. To investigate the extent to which the fibrin(ogen)-recognition exosite is involved when α -thrombin catalyses the amplification reactions of coagulation, we compared the abilities of hirudin, hirulog-1 and hirugen to inhibit simultaneously Factor X, Factor V and prothrombin activation. Whereas 0.1 μ M-hirudin and 0.1 μ M-hirulog-1 (i.e. less than 10% of the concentration of prothrombin in plasma) inhibited Factor X, Factor V and prothrombin activation, 10 μ M was the minimum concentration of hirugen to achieve a similar anticoagulant action. Concentrations of hirudin and hirulog-1 equimolar to and 5 times greater than those of α -thrombin respectively abrogated Factor V activation by exogenous α -thrombin. In contrast, a 500-fold molar excess of hirugen could not. The inability of hirugen to inhibit the activation of the three clotting factors effectively suggests that the fibrin(ogen)-recognition exosite does not play a mandatory role when thrombin activates Factor V.

INTRODUCTION

α -Thrombin accelerates its own generation by activating Factor V and Factor VIII. For this reason, agents able to effect the inhibition of thrombin suppress prothrombin activation in plasma. For example, submicromolar concentrations of hirudin and heparin inhibit prothrombin activation by inhibiting the activation of Factor VIII and Factor V normally catalysed by endogenous α -thrombin [1–4]. α -Thrombin consists of a smaller A chain linked by a disulphide bond to the B chain [5]. The B chain has the catalytic triad and the fibrin(ogen)-recognition exosite important for the high specificity of α -thrombin towards fibrinogen [6–8]. Hirudin abrogates all enzymic functions of α -thrombin by forming a stable (K_i approx. 10 fM) but non-covalent complex with the enzyme [9–12]. The three-dimensional structures of r-hirudin variant 2-Lys-47 and r-hirudin variant-1 complexed to α -thrombin demonstrate that the apolar compact N-terminal head domain of hirudin binds α -thrombin in a manner that blocks access to the active-site cleft of the enzyme [13,14]. The C-terminal tail domain of hirudin occupies the fibrin(ogen) exosite, which is adjacent to the catalytic cleft of the enzyme, where it makes numerous electrostatic and hydrophobic contacts with the exosite [13,14].

Complexing of hirudin to α -thrombin is postulated to begin at the C-terminal tail of the inhibitor [13], and tail fragments of hirudin competitively inhibit the clotting of fibrinogen by α -thrombin [15–19]. Thus, with respect to fibrinogen, hirugen transiently converts α -thrombin into a γ -thrombin-like enzyme. The fibrin(ogen)-recognition exosite is essentially deleted in γ -thrombin, and this enzyme has less than 0.5% of the clotting activity of α -thrombin [8]. Hirulog-1 is a bivalent derivative of hirudin with the structure D-Phe-Pro-Arg-Pro-[Gly]₄-desulphohirudin-(54–65) [16]. It combines the high affinity of D-Phe-Pro-

Arg-CH₂Cl for the catalytic centre of α -thrombin [20] with the affinity of hirudin-(54–65) for the fibrin(ogen)-recognition exosite [16–18]. Hirulog-1 inhibits fibrinopeptide A release and the amidolytic activity of thrombin with K_i of 144 and approx. 2 nM respectively [15,16], whereas natural and recombinant hirudins inhibit fibrinopeptide A release and the amidolytic activity of thrombin with K_i in the femtomolar to picomolar range [9–12]. Although hirudin and hirulog-1 effect a dose-dependent prolongation of the activated partial thromboplastin time, prolongation of this test by hirugen is saturable [16,18,19]. During the activated partial thromboplastin time test, prothrombin is converted into α -thrombin, which then clots fibrinogen. The saturable prolongation of this test by hirugen raises the possibility that, unlike hirudin [4] and hirulog-1, hirugen cannot significantly inhibit prothrombin activation. The present study investigated the validity of this hypothesis by determining the extents to which hirudin, hirulog-1 and hirugen could inhibit Factor X, Factor V and prothrombin activation. Factor X and Factor V activation are requisite events for efficient prothrombin activation in plasma.

MATERIALS AND METHODS

Materials

Recombinant hirudin was a gift from Dr. R. B. Wallis, CIBA-GEIGY, Horsham, Sussex, U.K. Hirugen and hirulog-1 were synthesized and characterized by using previously described procedures [12,14]. Purified human Factor V was obtained from Haematologic Technologies, Essex Junction, VT, U.S.A., and labelled with Na¹²⁵I to a specific radioactivity of 20 μ Ci/mg, by procedures previously employed to iodinate prothrombin and Factor V [17,19]. Rabbit anti-(human prothrombin) serum used as the second antibody in the e.l.i.s.a. to quantify prothrombin

Abbreviation used: APTT, activated partial thromboplastin time.

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consumption [20] was obtained from Behring Diagnostics, Montreal, Quebec, Canada. D-Phe-Pro-Arg-CH₂Cl and dansyl-Glu-Gly-Arg-CH₂Cl, chloromethane inhibitors of α -thrombin and Factor Xa respectively [20,21], were obtained from Terachem, Toronto, Ontario, Canada. A mouse monoclonal antibody to Factor X was obtained from American Diagnostica, Greenwich, CT, U.S.A. Reagents and materials for PAGE were obtained from Bio-Rad Laboratories, Mississauga, Ontario, Canada. Activated partial thromboplastin time (APTT) reagent was obtained from Organon Technika, Toronto, Ontario, Canada. Rabbit brain tissue factor was a gift from Dr. Leon Poller, National Centre for Anticoagulant Reagents and Control, Manchester, U.K. Fatty acid-free BSA, other reagents and chemicals were obtained from Sigma Chemical Co., Mississauga, Ontario, Canada.

Prothrombin and Factor X activation and their inhibition by hirudin, hirulog-1 and hirugen

Activation of prothrombin in defibrinated plasma was initiated either by contact activation (intrinsic activation) or by rabbit brain tissue factor (extrinsic activation). Contact activation was effected by adding 1 vol. of APTT reagent to 2 vol. of plasma. After a 5 min incubation at 37 °C, 1 vol. of 40 mM-CaCl₂ (prewarmed to 37 °C) was added to initiate intrinsic coagulation. Samples were withdrawn at 15 s intervals into 4 vol. of a buffer containing 145 mM-NaCl, 36 mM-sodium barbiturate, 36 mM-sodium acetate, 10 mM-EDTA, 1 μ M recombinant hirudin, 5 units of heparin/ml and 0.1 mg of fatty acid-free BSA/ml at pH 7.4. This buffer served three functions. By chelating Ca²⁺, the EDTA stopped further Factor X and prothrombin activation. Hirudin inactivated any free thrombin, thus eliminating competition between thrombin and Factor Xa for antithrombin III. Heparin accelerated the inhibition of Factor Xa by antithrombin III [22]. After a 10 min incubation at 37 °C, the samples were frozen at -50 °C until the concentrations of prothrombin consumed and total Factor Xa-antithrombin-III generated were assayed. When Factor X and prothrombin activation were initiated by adding tissue factor and CaCl₂ to plasma, undiluted rabbit brain tissue factor replaced the APTT reagent. The e.l.i.s.a.s used to quantify prothrombin consumption and total Factor Xa-antithrombin III have been described previously [4,22]. However, the first antibody used in this study to bind Factor Xa-antithrombin III to the micro-titre plates was a monoclonal antibody to Factor X. When coated to micro-titre plates at a concentration of 300 ng/well, this antibody was found to react equally well with Factor X, Factor Xa and Factor Xa-antithrombin III. The effects of hirudin (0.1 μ M and 1 μ M), hirulog-1 (0.1 μ M, 1 μ M and 10 μ M) and hirugen (0.1 μ M, 1 μ M and 10 μ M) on prothrombin and Factor X activation were also investigated.

Factor V activation and its inhibition by hirudin, hirulog-1 and hirugen

Proteolysis of ¹²⁵I-labelled Factor V resulting from CaCl₂ addition to contact-activated plasma, and that resulting from tissue factor and CaCl₂ addition to plasma, were determined. ¹²⁵I-labelled Factor V was added to plasma to a final concentration of 10 ng/ml. Samples of the recalcified plasmas were added to equal volumes of 5 mM-EDTA, 1 μ M-D-Phe-Pro-Arg-CH₂Cl and 1 μ M-dansyl-Glu-Gly-Arg-CH₂Cl in 36 mM-sodium barbiturate/36 mM-sodium acetate buffer, pH 7.4, to stop further Factor V proteolysis. Electrophoretic buffer (80 μ l) was then added to 20 μ l of each sample, and a 50 μ l sample was subjected to electrophoresis in 5–15% gradient polyacrylamide gels containing 0.1% SDS [16]. The gels were dried under vacuum and

¹²⁵I-labelled Factor V and its proteolytic derivatives were detected by exposing the gels to X-ray films for up to 7 days [16].

RESULTS

Inhibition of Factor X activation by hirudin, hirulog-1 and hirugen

A coagulant surface, Factor Xa and Factor Va are required for optimizing prothrombin activation. We therefore quantified Factor X activation and the effects of hirudin, hirulog-1 and hirugen on Factor X activation. As none of the three inhibitors of α -thrombin inactivates Factor Xa, suppression of Factor Xa-antithrombin III formation by each agent directly reflected the extent to which it could inhibit Factor X activation. Approx. 1 nM-Factor Xa was generated 30 s after CaCl₂ was added again to control contact-activated plasma (Fig. 1), and this value had increased to 185 nM 60 s later (Fig. 1). A minimum incubation of 75 s and 90 s were required before Factor X activation could be

Fig. 1. Inhibition of intrinsic Factor X activation by hirudin (a) hirulog-1 (b) and hirugen (c).

Intrinsic Factor X activation was initiated by adding CaCl₂ to contact-activated plasma. Free Factor Xa in each sample was converted into Factor Xa-antithrombin III by adding 5 units of heparin/ml. The concentration of total Factor Xa-antithrombin III in each sample (i.e. endogenous Factor Xa-antithrombin III plus free Factor Xa) was measured by an e.l.i.s.a. for Factor Xa-antithrombin III. ○, Control contact-activated plasma; ●, 0.1 μ M-inhibitor; □, 1.0 μ M-inhibitor; ■, 10 μ M-inhibitor.

Fig. 2. Inhibition of extrinsic Factor X activation by hirudin (a), hirulog-1 (b) and hirugen (c).

Extrinsic Factor X activation was initiated by adding rabbit brain tissue factor and CaCl₂ to plasma. Free factor Xa in each sample was converted into Factor Xa-antithrombin III by adding heparin (5 units/ml) to each sample. The concentration of total Factor Xa-antithrombin III was measured by e.l.i.s.a. ○, Control plasma; ●, 0.1 μ M-inhibitor; □, 1.0 μ M-inhibitor; ■, 10 μ M-inhibitor.

Fig. 3. Intrinsic activation of Factor V in control plasma (a), and in plasma containing 1.0 μM -hirudin (b), 1.0 μM -hirulog-1 (c) and 10 μM -hirugen (d)

Intrinsic pathway activation of ^{125}I -labelled Factor V was initiated by adding CaCl_2 to contact-activated plasma. After various incubation times, the resulting proteolysis of ^{125}I -labelled Factor V was estimated by SDS/PAGE and autoradiography. (a) The recalcification times were as follows: lane 1, 0 s; lane 3, 30 s; lane 4, 45 s; lane 5, 60 s. M_r markers were applied in lane 2. (b) Plasma containing 1 μM -hirudin was recalcified as follows: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. (c) Plasma containing 1 μM -hirulog-1 was recalcified as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 4, 0 s. M_r markers were applied in lane 5. (d) Plasma containing 10 μM -hirugen was recalcified for the following incubation times: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. Key: V, Factor V; A, Factor V activation peptide; H and L represent the Factor Va heavy and light chains respectively.

demonstrated when contact-activated plasma also contained 0.1 μM - and 1.0 μM -hirudin respectively. The higher concentration of hirudin decreased the concentration of Factor Xa generated by approx. 98% over 90 s (Fig. 1a). Hirulog-1 inhibited Factor X activation less effectively than hirudin, and 0.1 μM -hirulog-1 delayed the onset of Factor X activation by 30 s (Fig. 1b) compared with 45 s for 0.1 μM -hirudin (Fig. 1a). Even at 10 μM , hirugen could not delay the onset of intrinsic Factor X activation by more than 15 s (Fig. 1c). Extrinsic Factor X activation was first detected 30 s after tissue factor and CaCl_2 had been added to control plasma and more than 200 nM-Factor Xa had been generated 30 s later (Fig. 2). The lowest concentrations of hirudin and hirulog-1 (0.1 μM) could significantly inhibit extrinsic Factor X activation (Figs. 2a and b). During the first 50 s of incubation,

Fig. 4. Inhibition of extrinsic ^{125}I -labelled Factor V activation in control plasma (a), and in plasma containing 0.1 μM -hirudin (b), 1.0 μM -hirulog-1 (c) and 10 μM -hirugen (d)

A suspension of rabbit brain tissue factor and CaCl_2 (at 37 $^{\circ}\text{C}$) was added to plasma preincubated to 37 $^{\circ}\text{C}$. After various incubation times at 37 $^{\circ}\text{C}$, the proteolysis of ^{125}I -labelled Factor V was assessed by SDS/PAGE and autoradiography. (a) The recalcification times of control plasma were as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 4, 0 s. Radioactive M_r markers were applied in lane 5. (b) Plasma containing 0.1 μM -hirudin was recalcified as follows: lane 1, 45 s; lane 2, 90 s; lane 3, 135 s. (c) Plasma containing 1 μM -hirulog-1 was recalcified as follows: lane 1, 0 s; lane 3, 45 s; lane 4, 90 s; lane 5, 135 s. M_r markers were applied in lane 2. (d) Plasma containing 10 μM -hirugen was recalcified as follows: lane 1, 45 s; lane 2, 60 s; lane 3, 90 s. V, A, L and H are as defined in Fig. 3 legend.

10 μM -hirugen inhibited extrinsic Factor X activation as effectively as 0.1 μM -hirudin or 0.1 μM -hirulog-1 (Fig. 2c).

Intrinsic Factor V activation: inhibition by hirudin, hirulog-1 and hirugen

Before the addition of CaCl_2 to plasma, ^{125}I -labelled Factor V (approx. M_r 330 000) and several derivatives (all with apparent M_r > 130 000) were detected in plasma (Figs. 3 and 4). Proteolysis of ^{125}I -labelled Factor V resulting in production of Factor Va heavy (H) and light (L) chains and Factor V activation peptide (A) became evident 30 s after CaCl_2 was added to contact-activated plasma (Fig. 3a, lane 3). Subsequently, the largest Factor V fragment detectable was the activation peptide, M_r approx. 150 000 (Fig. 3a, lanes 4 and 5). Hirudin (1 μM) delayed the onset of intrinsic Factor V activation and proteolysis of Factor V had not begun (Fig. 3b, lanes 1–3). At 1.0 μM , hirulog-

Fig. 5. Inhibition of intrinsic prothrombin activation by hirudin (a), hirulog-1 (b) and hirugen (c).

Intrinsic prothrombin activation was initiated by adding CaCl_2 to contact-activated plasma. Prothrombin activation was measured by the disappearance of prothrombin, measured by e.l.i.s.a. ○, Control contact-activated plasma; ●, 0.1 μM-inhibitor; □, 1.0 μM-inhibitor; ■, 10 μM-inhibitor.

Fig. 6. Inhibition of extrinsic prothrombin activation by hirudin (a), hirulog-1 (b) and hirugen (c).

Extrinsic prothrombin activation was initiated by adding rabbit brain tissue factor and CaCl_2 to plasma. Prothrombin activation was measured by estimating the disappearance of prothrombin, measured by e.l.i.s.a. ○, Control plasma; ●, 0.1 μM-inhibitor; □, 1.0 μM-inhibitor; ■, 10 μM-inhibitor.

1 delayed the onset of intrinsic Factor V activation by 60 s (Fig. 3c, lanes 1–3), suggesting that 1 μM-hirulog-1 could suppress intrinsic Factor V activation nearly as efficiently as 1 μM-hirudin. The minimum concentration of hirugen to delay the onset of intrinsic Factor V activation for at least 15 s was 10 μM (Fig. 3d, lanes 1–3).

Extrinsic Factor V activation: inhibition by hirudin, hirulog and hirugen

Factor Va heavy chain was first detected 30 s after tissue factor and CaCl_2 were added to plasma, with Factor Va light chain and the 150 000- M_r Factor V activation peptide being generated 10 s later (Fig. 4a, lane 2). When plasma contained 0.1 μM-hirudin, both the approx. 260 000- M_r and approx. 230 000- M_r Factor V intermediates could still be detected in the 45 s sample (Fig. 4b, lane 1). The 230 000- M_r Factor V fragment was barely detectable in the comparable sample of control plasma (Fig. 4a, lane 3). Hirulog-1 at 0.1 μM inhibited extrinsic Factor V activation to a similar extent to 0.1 μM-hirudin (results not shown). Whereas

1 μM-hirulog-1 could abrogate extrinsic proteolysis of Factor V for 45 s (Fig. 4c, lane 3), 1 μM-hirudin could do so for at least 60 s (results not shown). The minimum concentration of hirugen to delay the onset of extrinsic Factor V activation by 10 s was 10 μM (Fig. 4d).

Activation of plasma Factor V by exogenous thrombins: inhibition by hirudin, hirulog-1 and hirugen

α-Thrombin (20 nM) was added to plasma in a suspension that also contained coagulant phospholipids and CaCl_2 . Within 30 s of the enzyme addition, Factor V had been proteolysed into Factor Va heavy and light chains, Factor V activation peptide, a 260 000- M_r fragment and a 230 000- M_r fragment. An equimolar concentration of hirudin abrogated Factor V activation by exogenous α-thrombin (results not shown), whereas 100 nM-hirulog-1 was the minimum concentration to do so (results not shown). In contrast, up to 10 μM-hirugen could not abrogate Factor V activation by α-thrombin (results not shown). γ-Thrombin could also proteolyse plasma Factor V except that approx. 100 nM was the minimum concentration of γ-thrombin required to cleave plasma Factor V to the same extent as 20 nM-α-thrombin (results not shown).

Inhibition of prothrombin activation by hirudin, hirulog-1 and hirugen

Prothrombin activation began 30 s after CaCl_2 was added to control contact-activated plasma, and was essentially complete 60 s later (Fig. 5). The presence of 0.1 μM and 1.0 μM-hirudin delayed the onset of prothrombin consumption by 30 s and 60 s respectively (Fig. 5a). As summarized in Fig. 5(b), 1.0 μM-hirulog-1 delayed the onset of intrinsic prothrombin activation by 30 s, and thus was as effective (Fig. 5a) as 0.1 μM-hirudin. Hirugen was less effective than hirudin or hirulog-1, as 10 μM-hirugen could delay the onset of prothrombin activation by only 15 s (Fig. 5c). Prothrombin consumption by the extrinsic pathway was complete 60 s after its initiation (Fig. 6). Hirudin inhibited extrinsic prothrombin activation less effectively than it could inhibit intrinsic prothrombin activation (Fig. 6a). The minimum concentrations of hirulog-1 and hirugen able to delay the onset of extrinsic prothrombin activation by more than 10 s were 1.0 μM and 10 μM respectively (Figs. 6b and 6c).

DISCUSSION

The antithrombins D-Phe-Pro-Arg- CH_2Cl and hirudin delay the onset of prothrombin activation by inhibiting the amplification reactions of coagulation normally catalysed by α-thrombin generated *in situ* [1,3,4]. D-Phe-Pro-Arg- CH_2Cl inactivates thrombin by alkylating the histidine member of the catalytic triad of the enzyme [20,23]. Recombinant hirudins inactivate α-thrombin by forming a tight but non-covalent complex with the enzyme. The N-terminal apolar head domain of hirudin blocks access to the catalytic centre, whereas the anionic tail of hirudin binds the enzyme at the exosite required for fibrin(ogen) recognition [13,14]. Beginning with hirudin-(54–65) (hirugen), tail fragments of hirudin bind α-thrombin at the fibrin(ogen)-recognition exosite and act as competitive inhibitions of fibrinopeptide A release [15–19].

The present study determined whether the fibrin(ogen)-recognition exosite is essential when α-thrombin activates Factor V. We found that 20 nM-exogenous α-thrombin activated Factor V in plasma within 15–30 s. Concentrations of r-hirudin equimolar with α-thrombin, and a 5-fold molar excess of hirulog-1 over α-thrombin abrogated Factor V proteolysis by exogenous α-thrombin. In contrast, 10 μM-hirugen did not. Hirulog-1 is the bivalent thrombin inhibitor with the structure D-Phe-Pro-Arg-

Pro-[Gly]₄-desulphohirudin-(54–65) [16]. The first three residues have a high affinity for the catalytic site of α -thrombin [20,23–25]. The fourth residue, proline, was added to retard cleavage of the affinity ligand by α -thrombin [26]. The [Gly]₄ spacer arm provides the minimum distance to optimize the simultaneous binding of hirulog-1 to the enzyme at the catalytic centre and the fibrin(ogen)-recognition exosite [27]. On the basis of its binding affinity, more than 99.9% of the exogenous α -thrombin would be bound to r-hirudin at equimolar concentrations of enzyme and inhibitor. Similarly, a 5-fold molar excess of hirulog-1 over the enzyme would result in a 97.7% complexing of the enzyme to hirulog-1. At a 500-fold molar excess of hirugen over α -thrombin, 94.9% of the enzyme would be bound to the inhibitor. Our results therefore suggest that the exosite mandatory for fibrinogen cleavage is not crucial when α -thrombin activates Factor V. Moreover, this suggestion was verified by the finding that γ -thrombin was approx. 5-fold less active than α -thrombin in effecting plasma Factor V proteolysis.

Either 0.1 μ M-r-hirudin or 0.1 μ M-hirulog-1 delayed the onset of Factor V and Factor X activation by about 30 s compared with 10 μ M-hirugen required to achieve a similar effect. The reason why the effect of 10 μ M-hirugen on extrinsic activation of Factor X is more marked than its effect on the intrinsic activation of this zymogen is not known. The inability of hirugen to inhibit Factor X activation as effectively as hirudin and hirulog-1 suggests that the fibrin(ogen)-recognition exosite of α -thrombin is not crucial for thrombin to activate Factor VIII. Consistent with their effects on Factor V and Factor X activation, all three concentrations of r-hirudin or hirulog-1 inhibited prothrombin activation, whereas 10 μ M-hirugen primarily delayed the onset of prothrombin activation. The action of hirugen on prothrombin activation is consistent with the saturable prolongation of both intrinsic and extrinsic coagulation by hirugen [19]. The present data thus indicate that activation of Factor V by α -thrombin to the level required for optimizing prothrombin activation can occur even when the fibrin(ogen)-recognition exosite of α -thrombin is essentially occupied. Nonetheless, effective occupancy of this site (by saturating concentrations of hirugen) does lengthen the time required for saturating concentrations of Factor Va to become available *in situ*. The fibrin(ogen)-recognition exosite is not necessary when antithrombin III inactivates thrombin [28]. Thus γ -thrombin and α -thrombin are inactivated by antithrombin III at similar rates [29].

There is evidence also consistent with the idea that the site for the initial interactions of Factor V with α -thrombin is not the fibrin(ogen)-recognition exosite. The fifth epidermal growth factor domain of thrombomodulin binds α -thrombin at the fibrin(ogen) exosite [28]. Di-isopropylphosphonated (DIP) α -thrombin bound to cell surface thrombomodulin is displaced by hirudin, hirugen and fibrinogen [28]. In contrast, a synthetic peptide based on a sequence of Factor Va analogous to hirugen cannot displace DIP- α -thrombin from thrombomodulin [28]. These fundamental differences in recognition mechanisms for its various substrates may explain why α -thrombin can activate Factor V at concentrations lower than that required for clotting.

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