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12-LIPOXYGENASE FROM BOVINE POLYMORPHONUCLEAR LEUKOCYTES, AN ENZYME WITH LEUKOTRIENE A_4-SYNTHASE ACTIVITY

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Bovine polymorphonuclear leukocytes exhibit a 12-lipoxygenase activity upon sonication. In contrast to bovine platelet 12-lipoxygenase and other 12-lipoxygenases, this enzyme is unable to convert 5(S)-HETE (5(S)-hydroxy,6-trans-8,11,14-cis-eicosatetraenoic acid) or 5(S)-HPETE (5(S)-hydroperoxy,6-trans-8,11,14-cis-eicosatetraenoic acid) into 5(S),12(S)-dihydroxy-6,10-trans,8,14-cis-eicosatetraenoic acid. Surprisingly, the formation of leukotriene A_4-derived products namely leukotriene B_4 and the leukotriene B_4-isomers 12-epi,6-trans- leukotriene B_4 and 6-trans-leukotriene B_4, was observed upon incubation of this enzyme with 5(S)-HPETE. Hence, the 12-lipoxygenase from bovine polymorphonuclear leukocytes possesses leukotriene A_4-synthase activity.

The leukotrienes constitute a group of arachidonic acid metabolites with profound biological activities. There is much evidence that they play important roles in inflammatory and allergic diseases e.g. asthma (cf. 1 and 2). The initial step in the formation of the 5-series leukotrienes is a dioxygenation of arachidonic acid, giving rise to 5(S)-HPETE. This reaction is catalyzed by a 5-lipoxygenase (E.C. 1.13.11.12). Beside 5-lipoxygenases, also 12- and 15-lipoxygenases exist.

We have already shown that bovine PMN's possess a 5-lipoxygenase since they are capable of synthesizing large amounts of the spasmogenic LTC_4 next to LTB_4 and LTB_4-isomers [3]. Recently, we have also demonstrated a 12-lipoxygenase in bovine PMN's which becomes apparent upon sonication of the cells [4]. This PMN 12-lipoxygenase seems to resemble 5-lipoxygenases [5] by showing a preference for the site of hydrogen abstraction determined by the distance to the carboxylic end of the fatty acid substrate [4]. This is in contrast with platelet 12-lipoxygenase [6] and several other lipoxygenases like soybean lipoxygenase-1 [7] and reticulocyte 15-lipoxygenase [8], which show a preference for hydrogen abstraction at a position determined by the distance to the methyl end of the molecule.

Abbreviations: PBS: phosphate-buffered saline (0.9% (w/v) NaCl in 8.6 mM phosphatebuffer, pH 7.4), PMN: polymorphonuclear leukocyte, 5-H(P)ETE: 5-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid, 5,12-diHETE: 5,12-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid, LTA_4: 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, LTB_4: 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid , LTC_4 : 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14- *cis*-eicosatetraenoic acid

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It has been shown that most 5-lipoxygenases are not only capable of forming 5(S)-HPETE from arachidonic acid, but can also catalyze the formation of LTA_4 from 5(S)-HPETE involving a second hydrogen abstraction from a 1,4-*cis,cis*-pentadiene system [9-13]. Both the formation of LTA_4 from 5(S)-HPETE by 5-lipoxygenase [14-16] and that of 12-H(P)ETE from arachidonic acid by bovine PMN 12-lipoxygenase, require the abstraction of hydrogen at C10. Therefore, we investigated the possible LTA_4-synthase activity of this enzyme.

MATERIALS AND METHODS

Materials

Arachidonic acid (>99%) was purchased from Fluka AG (Buchs, Switzerland). Calcium ionophore A23187, prostaglandin B_2 and the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were obtained from Sigma (St. Louis, MO, U.S.A.). Ficoll-Paque was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Sample filters (pore size 0.45 μm) were from Nihon Millipore Kogyo K.K. (Yonezawa, Japan). Methanol and water (both HPLC quality) were purchased from Merck (Darmstadt, F.R.G.). Tetrahydrofuran (HPLC quality) and octadecyl solid-phase extraction columns (6 ml) were obtained from Baker (Deventer, The Netherlands). Synthetic LTB_4 and LTC_4 were a kind gift of Dr. J. Rokach (Merck-Frosst Laboratories, Pointe Claire/Dorval, Quebec, Canada). 5(S),12(S)-diHETE, more accurately designated as 12-*epi*,6-*trans*,8-*cis*-leukotriene B_4, was biosynthesized from 12-HETE (Dr. J. Verhagen, unpublished data). Racemic 5-HETE was generously donated by Mr. G.A.A. Kivits (Unilever Research Laboratory, Vlaardingen, The Netherlands). 5(S)-HPETE was from Cayman Chemical (Ann Arbor, MI, U.S.A.).

Purification of PMN's and platelets

PMN's and platelets were isolated from bovine blood obtained from a local slaughterhouse. PMN's were purified as described previously [3]. The yield was (1.8 ± 0.2) x 10^9 cells from 0.7 l bovine blood with a PMN to platelet ratio of 0.2 ± 0.1 (mean value ± S.E.M., n = 5). Platelets were isolated as in [4]. The yield was 27 x 10^9 cells from 0.7 l blood.

Preparation of the cell-free system

PMN's (4.0 x 10^7/ml) or platelets (1.0 x 10^9/ml) were suspended in PBS (0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4) containing 0.01% (w/v) sodium-deoxycholate. The cells were sonicated twice for 10 s at 60 Watt with a Branson sonifier B-12 at 0°C. The suspension was centrifuged at 12000 x g for 20 min at 4°C and the supernatant was used as the cell-free system.

Incubation procedure and sample preparation

PMN's were suspended in PBS at a final concentration of 4.0 x 10^7 cells/ml. Purified platelets were suspended in PBS at a final concentration of 1.0 x 10^9 cells/ml. The cell suspensions or cell-free systems were preincubated at 37°C for approx. 10 min. Incubation was for 10 min at 37°C and pH 7.4 in the presence of 20 μM of the calciumionophore A23187 and 2 mM Ca^{2+}. Substrate was added dissolved in ethanol, concentrations were as indicated. The final ethanol concentration never exceeded 0.1% (v/v). 5-HPETE and 5-HETE solutions were freshly prepared prior to incubation and the concentrations were determined spectrophotometrically, using a molar absorption coefficient of 29500 $M^{-1}cm^{-1}$ at 237 nm [17]. Reactions were stopped by the addition of ice and the incubation mixture was centrifuged at 12000 x g for 20 min at 4°C. Eicosanoids were extracted with octadecyl solid-phase extraction columns as described previously [18]. Prostaglandin B_2 was added as an internal standard. The methanol eluates were stored under nitrogen at -20°C after the addition of 0.6 μg/ml of the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical. Before HPLC analysis, the eluates were concentrated and filtered through sample filters (0.45 μm).

Reversed-phase HPLC analysis

A CP™ Spher 8-C18 column (250 x 4.6 mm, Chrompack, Middelburg, The Netherlands) was attached either to a Kratos 783 detector combined with a Kratos 400 pump or to an HP 1040A diode-array detector combined with an HP 1090 solvent-delivery system.

Isocratic elution was carried out using tetrahydrofuran/methanol/water/acetic acid 25:30:45:0.1 (v/v) which had been brought to pH 5.5 with ammonia [19]. The aqueous phase contained 0.1% (w/v) EDTA, which improves the recovery of the sulfidopeptide leukotrienes [20]. A flow-rate of 0.9 ml/min was used and the detection was at 280 nm or at 237 nm (Kratos system) or from 220 to 350 nm (HP 1040A). Data were processed by either a Shimadzu C-R3A integrator or a HP 310 SPU workstation. Leukotrienes were quantified by relating peak areas to that of the internal standard prostaglandin B_2. Molar absorption coefficients used are: 28650 $M^{-1}cm^{-1}$ at 280 nm for prostaglandin B_2, 40000 $M^{-1}cm^{-1}$ at 280 nm for the leukotrienes and 29500 $M^{-1}cm^{-1}$ at 237 nm for the H(P)ETE's.

RESULTS

Incubation of bovine platelets with racemic 5-HETE or 5(S)-HPETE resulted in the formation of only 5,12(S)-diHETE or 5(S),12(S)-diHETE respectively (Fig. 1A and 1B). In both cases only one peak is observed because, in our reversed-phase HPLC system, 5(S),12(S)-diHETE and 5(R),12(S)-diHETE coelute. LTA_4-derived products could not be detected. Such features have also been reported for several other 12-lipoxygenases [15,21,22].

5,12(S)-diHETE was also the only leukotriene formed when either intact or sonicated bovine PMN's were incubated with 5-HETE (Fig. 1C and 1D), in amounts that could be attributed to the platelet contamination. The quantity formed by the supernatant of sonicated bovine PMN's was much smaller than that formed by intact PMN's being 0.5 ± 0.3 nmol per ml compared to 4 ± 1 nmol per ml (mean value ± S.E.M., n = 5), respectively. Since we have shown that upon sonication of the cells, platelet 12-lipoxygenase activity is decreased while PMN 12-lipoxygenase activity is enhanced [4], this demonstrates that the 12-lipoxygenase that converts 5-HETE into 5,12(S)-diHETE can indeed be attributed to the contaminating platelets and not to the PMN's.

When intact bovine PMN's were incubated with 5(S)-HPETE, LTB_4, 6-*trans*-LTB_4,12-*epi*,6-*trans*-LTB_4 and 5(S),12(S)-diHETE were formed (Fig. 1E). The last compound was formed in comparable amounts to 5,12(S)-diHETE when 5-HETE was used as a substrate. Because LTA_4 is very unstable and is converted directly either enzymatically into LTB_4 and LTC_4 or non-enzymatically into 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4, the synthesis of LTA_4 can, in such systems, only be demonstrated by the formation of LTA_4-derived products. Therefore, from the formation of LTB_4, 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4 it is concluded that bovine PMN's are capable of forming LTA_4 from 5(S)-HPETE.

The cell-free system from bovine PMN's was also able to synthesize LTA_4 from 5(S)-HPETE which is shown by the formation of LTB_4, 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4 (Fig. 1F).

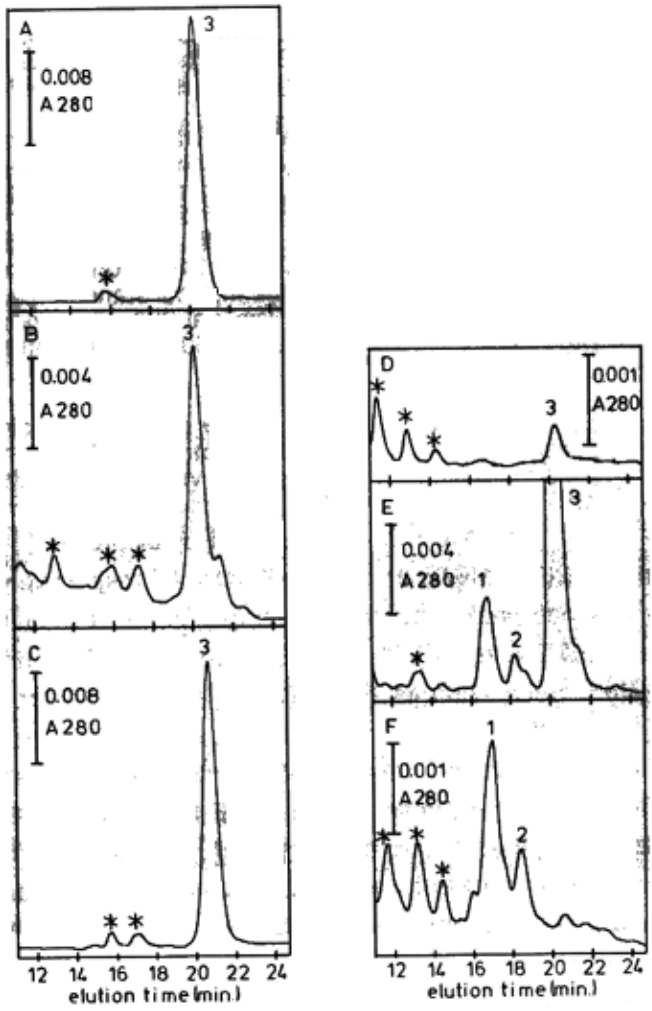

Fig. 1. Reversed-phase HPLC profiles of the leukotrienes formed by intact platelets (A and B), intact PMN's (C and E) and sonicated PMN's (D and F). 1 x 10^9 platelets, 4 x 10^7 PMN's or the supernatant of 4 x 10^7 PMN's were brought to 1 ml. Incubation was in the presence of 20 µM calcium ionophore A23187, 2 mM Ca^{2+} and 20 µM 5-HETE (A) or 11 µM 5(S)-HPETE (B) or 65.4 µM 5-HETE (C and D) or 65.4 µM 5(S)-HPETE (E and F) in PBS pH 7.4 for 10 min. Solvent system was tetrahydrofuran/methanol/water/ acetic acid (25:30:45:0.1, by vol.), pH 5.5, with a flow-rate of 0.9 ml/min. Detection was at 280 nm using prostaglandin B_2 as an internal standard. Peaks were identified by HPLC behaviour, coelution with synthetic standards and ultraviolet spectra. Peak 1: 12-*epi*,6-*trans*- LTB_4 and 6-*trans*-LTB_4; peak 2: LTB_4; peak 3: 5,12(S)- diHETE. Peaks marked with * did not have a leukotriene ultraviolet spectrum.

From Fig. 2 it is clear that the LTA_4-synthase activity of the cell-free system from bovine PMN's can only be demonstrated if 5(S)-HPETE is used as a substrate. No 5-lipoxygenase activity was present in the cell-free system since incubation with arachidonic acid resulted in the formation of 12-HETE only. A survey of the products formed by incubation of intact and sonicated PMN's and platelets with arachidonic acid, 5-HETE and 5(S)-HPETE as a substrate is given in Table 1.

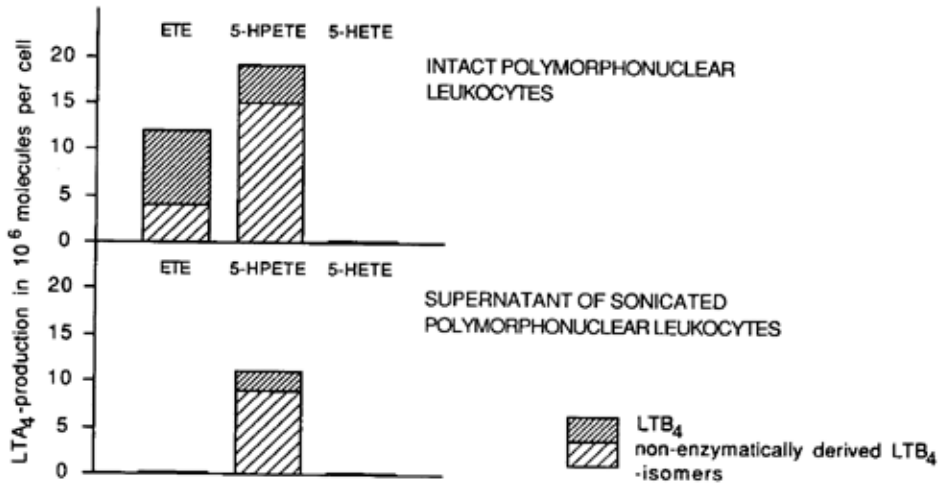

Fig. 2. The formation of LTA_4 by intact and sonicated PMN's. LTA_4 is defined as the sum of LTB_4 and the non-enzymatically from LTA_4 derived LTB_4-isomers 12-*epi*,6-*trans*-LTB_4 and 6-*trans*-LTB_4. 4 x 10^7 PMN's or the supernatant of 4 x 10^7 PMN's were brought to 1 ml. Incubation was in the presence of 20 µM calcium ionophore A23187, 2 mM Ca^{2+} and 70 µM of the indicated substrate in PBS pH 7.4 for 10 min. Results are given as 10^6 molecules of LTA_4 formed per cell (n = 2). ETE : arachidonic acid.

DISCUSSION

We have demonstrated that bovine PMN's exhibit a 12-lipoxygenase activity upon sonication of the cells [4]. Until now, all 12-lipoxygenases have been reported to be capable of converting 5-HETE and 5-HPETE into 5,12(S)-diHETE [15,21-24]. However, the 12-lipoxygenase from bovine PMN's was found to be unable to catalyze this conversion. Surprisingly, the formation of LTA_4-derived products was observed when the supernatant of sonicated bovine PMN's was incubated with 5(S)-HPETE,

Table 1. Formation of leukotrienes and 12-HETE by intact and sonicated bovine PMN's and platelets with arachidonic acid (ETE), 5(S)-HPETE and 5-HETE as a substrate

LTA_4 is defined as LTB_4 + 6-*trans*-LTB_4 + 12-*epi*, 6-*trans*-LTB_4.

cell-preparation	ETE	5(S)-HPETE	5-HETE
S-PMN	12-HETE	LTA_4 + 5(S),12(S)-diHETE*	5,12(S)-diHETE*
I-PMN	LTA_4 + 5(S),12(S)-diHETE*	LTA_4 + 5(S),12(S)-diHETE*	5,12(S)-diHETE*
I-PLT + S-PLT	12-HETE	5(S),12(S)-diHETE	5,12(S)-diHETE

* derived from the platelet contamination

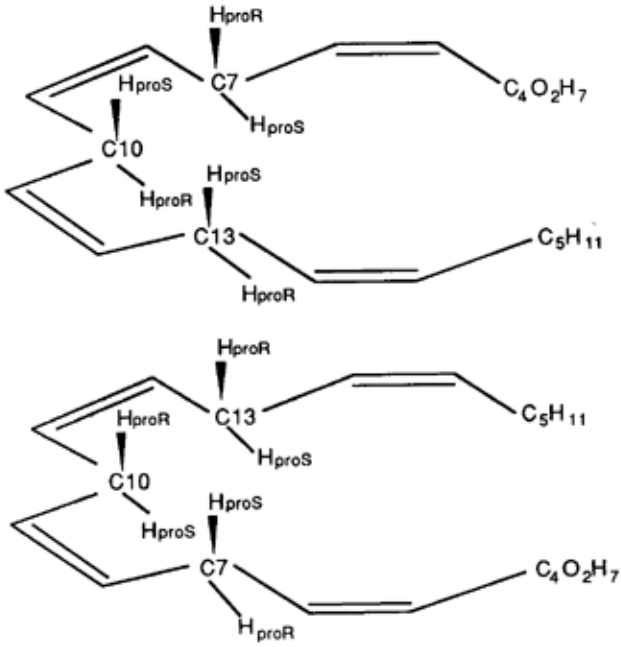

Fig. 3. Three-dimensional depiction of arachidonic acid on the enzyme. A. In the normal position. B. In the inverted position.

showing that this 12-lipoxygenase has LTA_4-synthase activity. Both the formation of 12(S)-HETE from arachidonic acid and the formation of LTA_4 from 5(S)-HPETE [14] proceed via a radical mechanism, and both require the abstraction of hydrogen at C10. Assuming an antarafacial relationship between hydrogen abstraction and oxygen insertion, which is the generally accepted mechanism for lipoxygenase-catalyzed dioxygenations, the H_{proS} (for the definition of pro S and pro R, the reader is referred to 25) at C10 has to be abstracted in order to form 12(S)-HPETE. For the formation of LTA_4 from 5(S)-HPETE, it was shown that the H_{proR} at C10 is abstracted [14-16]. Hence, the formation of 12(S)-HETE by the bovine PMN 12-lipoxygenase seems to be in contradiction with the observation that this enzyme possesses LTA_4-synthase activity. However, this can be explained adequately, by assuming an inverted position of arachidonic acid on the enzyme in the cell-free system. For the formation of LTA_4 from 5(S)-HPETE the H_{proR} at C10 has to be abstracted, which is on the lower side of the molecule (Fig. 3A). Radical migration proceeds towards the carboxylic end. However, in order to produce 12(S)-HPETE, H_{proS} at C10 has to be abstracted which is located on the opposite (upper) side of the molecule and radical migration proceeds towards the methyl end. We therefore postulate that, in the cell-free system, arachidonic acid is in the inverted position on the enzyme. That is, upside down with the methyl group in the position of the carboxylic group (Fig. 3B) [cf. 26]. The H_{proS} at C10 is now on the lower side of the molecule and radical migration will still proceed towards the methyl end which is, with respect to the enzyme, now in the same direction as for the formation of LTA_4.

Such an inverted orientation of the substrate might be a more general feature of lipoxygenase-catalyzed reactions, for instance in the double dioxygenation of arachidonic acid by soybean lipoxygenase-1. For the formation of 15(S)-HPETE from arachidonic acid by this enzyme, the H_{proS} at C13, on the upper side of the molecule in Fig. 3A is abstracted [28] and the radical migrates towards the methyl end. The second dioxygenation, by soybean lipoxygenase-1, results in the formation of 8(S),15(S)-dihydroxy-5,11-*cis*,9,13-*trans*-eicosatetraenoic acid and 5(S),15(S)-dihydroxy-6,13-*trans*,8,11-*cis*-eicosatetraenoic acid [17]. Assuming an antarafacial relationship between hydrogen abstraction and oxygen insertion, this means that the H_{proR} at C10 and the H_{proS} at C7 are abstracted respectively*. If the substrate is bound in the same way as in the first dioxygenation, the H_{proR} at C10 and the H_{proS} at C7 are both on the opposite (lower) side of the molecule (Fig. 3A) as compared to the situation in the first dioxygenation. Therefore, we suggest that in the case of the second dioxygenation of arachidonic acid by soybean lipoxygenase-1 the substrate is in an inverted position (Fig. 3B) on the enzyme because in that case hydrogen abstraction occurs at the same side of the molecule and radical migration is, with respect to the enzyme, in the same direction as in the first dioxygenation. This proposal is supported by the observations of Van Os et al. [17] that the double dioxygenation of arachidonic acid by soybean lipoxygenase-1 is a two step reaction, with K_m's of 8.5 μM (arachidonic acid) and 440 μM (15-HPETE), respectively. This means that 15-HPETE is released from the enzyme after the first dioxygenation and serves as a substrate for the second dioxygenation, which has a much larger K_m.

Recently, a similar mechanism has been suggested by Kühn et al. [27] for the double dioxygenation of arachidonic acid by reticulocyte 15-lipoxygenase. Their finding that derivatives of 15-HETE with increased hydrophobicity of the carboxylgroup such as the methylester, exhibit a much higher oxygenation rate than the free hydroxy acid, is in agreement with the hypothesis of inverted binding.

In the literature [10-13], LTA_4-synthase activity has been reported to be an intrinsic property of 5-lipoxygenases. If the bovine PMN 5-lipoxygenase also has these two activities, the 12-lipoxygenase activity which becomes apparent upon sonication of the cells, could represent the remaining LTA_4-synthase activity of the 5-lipoxygenase. When the dioxygenase activity is destroyed upon sonication, the second activity, the ability to abstract hydrogen at C10, will result in the formation of 12-HETE upon incubation of this enzyme with arachidonic acid. This hypothesis is in agreement with our earlier observations that the appearance of bovine PMN 12-lipoxygenase activity upon sonication of the cells, occurs concomitantly with the disappearance of the 5-lipoxygenase activity [4].

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* It should be noted that in 15-HPETE, the designation of the hydrogens at C10 and C7 according to [24] is reversed because of a change in the priorities due to the introduction of the hydroperoxy group at C15. However, to avoid confusion, we will use the notation based on arachidonic acid throughout.

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