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Enzyme Immunoassay for the Determination of Des-Gly[10]-NH_2-LH-RH-ethylamide (Fertirelin) in Bovine Plasma

JUNYA OKADA* and SADAO KONDO

Research and Development, Animal Health Products Division, Takeda Chemical Industries Ltd., 17–85 Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan

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A double-antibody solid-phase enzyme immunoassay for determining des-Gly[10]-NH_2-LH-RH-ethylamide (fertirelin) in bovine plasma was developed. Antiserum was raised against fertirelin-BSA conjugate, and enzyme-labeled antigens were prepared by coupling fertirelin analogues bearing an N-terminal amino group with *β*-D-galactosidase using *N*-(*m*-maleimidobenzoyloxy)-succinimide (MBS). The antiserum cross-reacted hardly at all with LH-RH and only slightly with peptides bearing a different C-terminal alkyl substituent, but it was less specific for the N-terminus.

Plasma specimens were extracted with antibody-immobilized cellulose in order to minimize substances interfering with the assay. This assay system could detect as little as 0.2 ng/ml of fertirelin in the absence of plasma extract and 1.0 ng/ml in the presence of the extract. The mean recovery of fertirelin added to plasma was 80.9% and the coefficients of variation were 14.4% (within assay) and 17.5% (between assay).

Keywords——enzyme immunoassay; double-antibody solid-phase; des-Gly[10]-NH_2-LH-RH-ethylamide; fertirelin; *β*-D-galactosidase; bovine plasma; antibody-immobilized cellulose extraction

Des-Gly[10]-NH_2-LH-RH-ethylamide (fertirelin,[1] I), synthesized by Fujino *et al.*,[2] is one of the most potent synthetic analogues of hypothalamic luteinizing hormone-releasing hormone (LH-RH), exhibiting approximately five times greater activity for the induction of ovulation than LH-RH in rats, and being over 2.5 times as active as LH-RH in the release of both LH and follicle-stimulating hormone (FSH) *in vitro*.[2] The intramuscular injection of the nonapeptide into cattle at the dosage of 100 to 200 μg per head was approved in 1981 in Japan for the treatment of ovarian follicular cysts, ovulation failure, ovarian quiescence and for improvement of ovulation.

As judged from the plasma level of LH-RH in man,[3] dog,[3] and sheep[4] after intravenous injection of LH-RH, the plasma level of I injected in the range of the approved dosage is presumed to be as low as several ng per ml even at the peak. Only immunochemical assays appear to be able to detect such a small amount of the peptide. In this report, we describe an enzyme immunoassay (EIA) of fertirelin developed in order to obtain pharmacokinetic information on I when it is injected into cattle.

Materials and Methods

Materials——Fertirelin and its related peptides II through XXIII shown in Table II were synthesized by the conventional solution method in the Chemistry Laboratories of the Central Research Division of our company. *β*-D-Galactosidase (EC 3.2.1.23) from *E. coli* (30 units per mg protein) and 4-methylumbelliferyl-*β*-D-galactopyranoside (4-MUG) were purchased from Boehringer Mannheim (West Germany) and P-L Biochemical Inc. (Wis., U.S.A.), respectively. Goat anti-rabbit immunoglobulin G (IgG) antiserum (IgG fraction) was obtained from Miles Laboratories Inc. (Kankakee, Ill.). Microcrystalline cellulose (Avicel SF) was from Funakoshi Ltd. (Tokyo). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI) was from Fluka AG (Buchs SG, Switzerland). Sephadex G-100 was

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from Pharmacia Fine Chemicals (Sweden), Visking tubing was from Shiraimatsu & Co., Ltd. (Osaka), and Freund's complete adjuvant was from Difco Laboratories Inc. (Detroit, Mich.). *N*-(*m*-Maleimidobenzoyloxy)succinimide (MBS) was prepared according to the methods previously reported.[5] Other reagents were of reagent grade, purchased from Wako Pure Chemical Industries (Osaka).

Preparation of Fertirelin-Specific Antibody——A mixture of I (20 mg), BSA (20 mg), and ECDI (200 mg) in water (1 ml) was stirred overnight at room temperature.[6] After dialysis against water and lyophilization, the conjugate obtained was dissolved in water at the protein concentration of 1 mg/ml and emulsified with an equal volume of Freund's complete adjuvant. A 1 ml aliquot of the emulsion was injected at multiple intradermal sites on the back of 3 male white New Zealand rabbits (weighing about 3.5 kg) 4 times at 1-month intervals. Whole blood was taken from the heart of the rabbits 10 d after the final injection, allowed to clot overnight at 4 °C and centrifuged to separate the serum.

Preparation of Peptide–Enzyme Conjugate——Peptide-β-D-galactosidase conjugates for peptides without a free amino group were prepared in the same manner as used for the preparation of the immunogen with ECDI as a coupling agent. The peptides bearing a free amino group were coupled to β-D-galactosidase with MBS by the method of Kitagawa and Aikawa.[5] A slight excess of 1% triethylamine, and 1 ml of 0.02 M phosphate buffer (pH 7.0) were added to 1 μmol (1.3—1.6 mg) of II, III, or XXIII. The solution was stirred with MBS (0.5 mg) in tetrahydrofuran (0.5 ml) for 30 min at room temperature, then THF was removed by rotary evaporation, and the solution was lyophilized. The product was washed several times with a mixture of dichloromethane and ethyl ether (1 : 1) to remove remaining MBS, and then dried *in vacuo*. The MBS-acylated peptide thus obtained was reconstituted in the phosphate buffer (1 ml) and incubated with commercial β-D-galactosidase suspension (100 μl) for 2 h at room temperature. The conjugate thus obtained was purified by Sephadex G-100 column (1.5 × 25 cm) chromatography using 0.02 M phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3 (buffer A) as an eluent. The fraction (1 ml) containing the peak of enzyme activity was stored at 4 °C and used for EIA.

Preparation of Immobilized Goat Anti-rabbit IgG (RIgG)——IgG fraction (5.6 mg) of goat anti-RIgG antiserum was coupled to microcrystalline cellulose (5 g) oxidized with sodium *m*-periodate by the method of Ferrua *et al.*[7] The immunosorbent was suspended in buffer A at a concentration of 5% for EIA.

Preparation of Immobilized Anti-fertirelin IgG——IgG fraction (S1G) of rabbit anti-fertirelin antiserum (S1C) was prepared by an Na_2SO_4 precipitation method.[8] After dialysis against 0.02 M borate buffer (pH 8.0), immunoglobulin equivalent to 2.5 ml antiserum was coupled to microcrystalline cellulose (5 g) in the same manner as in the preparation of the immobilized goat anti-RIgG. The immobilized antibody was suspended in 0.05 M Tris–HCl buffer (pH 7.5) at a concentration of 5% for the extraction of plasma samples.

Extraction of Fertirelin from Bovine Plasma with Anti-fertirelin IgG Immobilized Cellulose——A suspension (0.5 ml) of the antibody-immobilized cellulose was added to a plasma specimen (0.5 ml or less) in a polystyrene tube. Plasma samples of less than 0.5 ml were made up to 0.5 ml with 0.9% saline. The mixtures were shaken overnight at 4 °C. The tubes were centrifuged and the pellets of cellulose were washed twice with water. Fertirelin bound to the solid antibody was eluted by shaking with two 1 ml aliquots of 0.05 M acetic acid for 30 min each at room temperature. Acetic acid extract was collected through a glass pipette plugged with cotton. The combined extracts were dried under a stream of nitrogen in a water bath below 40 °C. The residues were dissolved in buffer A for the assay.

Assay Procedures——Enzyme immunoassay of fertirelin was performed by the double-antibody solid-phase method.[9] One hundred μl of a sample solution was mixed with 100 μl of 0.02 M phosphate-buffered saline (PBS, pH 7.0), 100 μl of the antibody (S1G), and 100 μl of the peptide–enzyme conjugate containing 0.5 μU of β-D-galactosidase activity. The antiserum was diluted with buffer A so that it might bind approximately 30% of the enzyme conjugate added. For the preparation of a standard curve, various amounts of fertirelin were dissolved in 100 μl of PBS, and 100 μl of buffer A was added in place of the sample solutions. The mixture was allowed to stand at 4 °C for more than 16 h. Then, 100 μl of the anti-RIgG immobilized cellulose suspension was added and the whole was kept at 30 °C for 4 h with vigorous shaking once every hour. After two washings with saline and one washing with buffer A, the pellet was suspended in 500 μl of the solution containing 10 μg of 4-MUG as a substrate in buffer A and incubated overnight at room temperature to determine the enzyme activity. The reaction was terminated by adding 3.5 ml of 0.1 M carbonate buffer (pH 10.5) and the suspension was centrifuged. The fluorescence intensity of the supernatant was measured with a fluorometer at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Results

Immunization

The fertirelin–BSA conjugate was found to contain about 10 molecules of fertirelin per molecule of BSA when determined by the method of Goodfriend *et al.*[10] Sera from all 3 rabbits immunized with this immunogen bound to a considerable extent to most of the peptide–galactosidase conjugates prepared with both ECDI and MBS even after the first

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TABLE I. Midpoints for Fertirelin, Its Analogues, and LH-RH–Ovalbumin Conjugate (in ng/ml) in the Enzyme Immunoassay Using Various Peptide–Enzyme Conjugates

Enzyme conjugate		Maximum binding[b] (%)	Peptide or peptide–protein conjugate				
Peptide[a]	Coupling agent		I	II	III	IV (LH-RH)	LH-RH-OVA
I	ECDI	85.9	>10000	>10000	>10000	—	—
II	MBS	70.2	0.27	0.29	0.73	3000	—
III	ECDI	51.0	>10000	—	—	—	—
III	MBS	50.6	0.35	0.39	0.73	3900	—
IX	ECDI	74.3	NI	NI	NI	NI	550
XXII	ECDI	49.1	NI	NI	NI	NI	700
XXIII	MBS	6.2	—	—	—	—	—
XXIII	ECDI	70.8	NI	NI	NI	NI	1000

a) See Table II. *b*) Percentage of peptide–enzyme conjugate which bound to the antibody. —, Not tested. NI, No inhibition was observed up to a peptide concentration of 10 μg/ml.

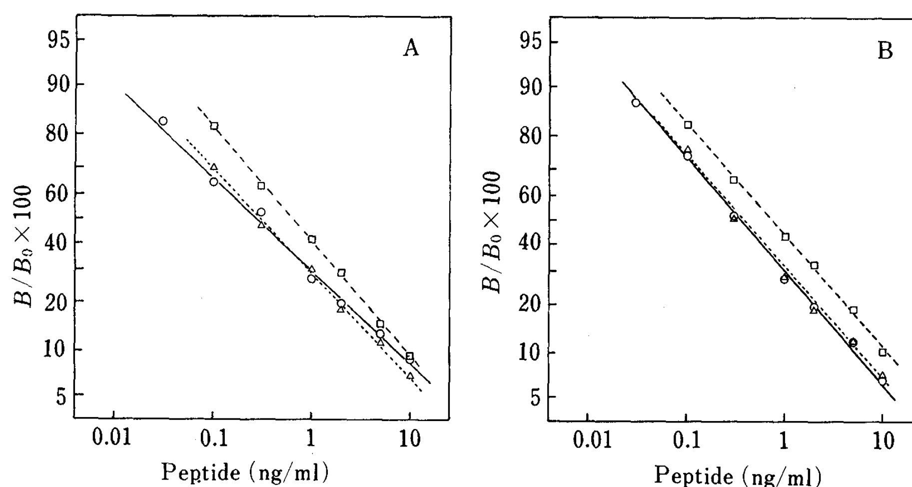

Fig. 1. Inhibition-Binding Curves of Enzyme-Labeled Haptens with Fertirelin and Related Peptides

Peptides II and III were used as enzyme-labeled hapten in A and B, respectively. —○—, fertirelin; ---△---, peptide II; --□--, peptide III.

booster injection. The antibody titers increased as the number of injections increased and sufficient titers for EIA were obtained after the third boost. The IgG fraction (S1G) of the serum (S1C) showing the highest titer from rabbit No. 1 was used throughout this study.

Effect of Peptide–Enzyme Conjugates on the Sensitivity of the Assay

All the conjugates prepared using ECDI were bound to the antibody at high percentages. Those binding, however, could not be inhibited by any of the free peptides, though they were inhibited by LH-RH–ovalbumin conjugate prepared using ECDI (Table I). As shown in Fig. 1, a linear relationship was obtained between the logarithms of the concentration of fertirelin and the logit (B/B_0)[11] in the assay systems in which peptides II and III, and MBS were used as enzyme-labeled antigen and coupling agent, respectively. The sensitivities of both systems were much the same, and the detection limit, defined as the concentration of antigen exhibiting 90% B/B_0, was about 200 pg/ml.

Specificity of the Assay Systems

The immunological specificity of the antibody (S1G) was investigated by determining the

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TABLE II. Cross-Reactivity of Fertirelin Analogues (%)

Peptide	Structure	Peptide in enzyme conjugate	
		II	III
	1 2 3 4 5 6 7 8 9 10		
I	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–ethylamide (fertirelin)	100	100
II	Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–ethylamide	93.1	88.8
III	H–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–ethylamide	36.8	48.2
IV	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–RH)	<0.01	<0.01
V	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–methylamide	5.7	1.7
VI	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–propylamide	42.9	29.3
VII	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–isopropylamide	3.5	3.2
VIII	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–*n*-butylamide	0.6	0.6
IX	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–diethylamide	3.9	7.5
X	pGlu–His–Trp–Ser–Tyr–Gly–OH	<0.01	<0.01
XI	pGlu–His–Trp–Ser–Tyr–OH	<0.01	<0.01
XII	pGlu–His–Trp–OH	<0.01	<0.01
XIII	pGlu–His–OH	<0.01	<0.01
XIV	pGlu–His–Pro–NH_2 (TRH)	<0.01	<0.01
XV	pGlu–D-His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–ethylamide	131.7	156.5
XVI	pGlu–Phe–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–ethylamide	5.6	6.1
XVII	pGlu–His–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–ethylamide	22.1	51.3
XVIII	pGlu–His–Trp–D-Ser–Tyr–Gly–Leu–Arg–Pro–ethylamide	123.4	98.9
XIX	pGlu–His–Trp–D-Ser–Tyr–D-Ala–Leu–Arg–Pro–ethylamide	33.5	61.7
XX	pGlu–His–Trp–Ser–Tyr–Leu–Leu–Arg–Pro–ethylamide	2.8	6.2
XXI	pGlu–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–ethylamide	7.0	6.4
XXII	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–OH	<0.01	<0.01
XXIII	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Lys–Pro–Gly–NH_2	<0.01	<0.01

ability of fertirelin analogues to displace the binding of the enzyme conjugate with the antibody. Percent cross-reactivities of the analogues are shown in Table II. No significant difference was observed in specificity between the two EIA systems using peptides II and III as an enzyme-labeled peptide and MBS as a coupling agent. Among the analogues possessing different Pro^9-alkylamides, Pro^9-propylamide analogue corss-reacted 29 to 43%, whereas the other amides showed cross-reactivities of less than 8%. LH-RH was discriminated more clearly than these peptides. Peptide II displaced the conjugate as effectively as fertirelin itself and peptide III cross-reacted 37 to 48%. These results indicate that this anitbody is less specific for the N-terminus than for the C-terminus. Changing the L-amino acid in the 2 or 4 position of fertirelin to the corresponding D-amino acid enhanced the cross-reactivity, whereas single or double substitution in the 2, 4 or 6 position(s) reduced the cross-reactivity. The N-terminal hexapeptide and smaller peptides (as well as TRH) showed no cross-reactivity. LH and FSH did not cross-react at all.

Extraction and Purification of Fertirelin from Bovine Plasma

A combination of deproteination with methanol and deffating with petroleum ether[12] removed interfering substances to a great extent, but those still remaining interfered with the coincidence of the standard curves in the plasma extract and the buffer; as a result, excessively large apparent recoveries ($216 \pm 53\%$, mean$\pm$S.D., $n=14$) were obtained (Fig. 2). On the other hand, the extraction method using antibody-immobilized cellulose afforded two advantages: it gave acceptable blank values (Table III), and little discrepancy was found between the standard curves in the plasma extracts and buffer A.

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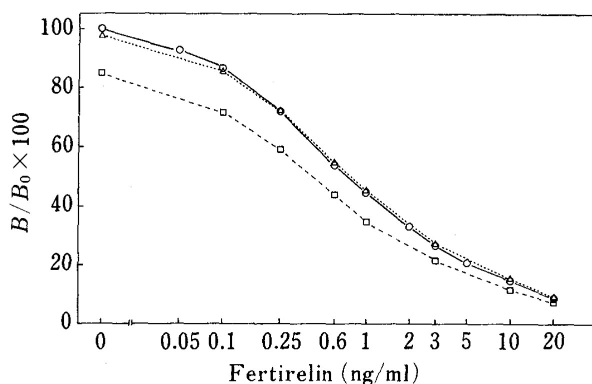

Fig. 2. Comparison of Standard Curves for Fertirelin

—○—, in the absence of plasma extract; ---△---, in the presence of antibody-immobilized cellulose extract of bovine control plasma; --□--, in the presence of methanol extract.

TABLE III. Comparison of Plasma Blank Values Obtained by Antibody-Immobilized Cellulose and Methanol Extraction Methods[a]

Blank plasma specimen	Antibody-immobilized cellulose extraction (ng/ml)	Methanol extraction[b] (ng/ml)
1	<0.1	0.15
	<0.25	0.6
2	<0.1	0.14
3	<0.1	0.23
	0.11	0.13
	0[c]	0.2
4	<0.25	0.7
5	<0.1	0.2
	<0.1	0.31
6	<0.1	0.17
7	0[c]	0.15
8	0.62	1.9
9	0.22	0.66
10	0.37	0.87
11	0.37	0.53
12	0.23	1.02

a) Comparison was made on the same day. *b*) The plasma specimen was extracted with 3 parts of MeOH. The extract was evaporated to dryness, then the residue was defatted with petroleum ether and reconstituted in buffer A. *c*) The fluorescence intensity of the blank sample (B) was larger than that of the antigen-free standard sample (B_0).

TABLE IV. Recovery of Fertirelin Added to Bovine Plasma

Fortification level (ng/ml)	Recovery (%) (Mean ± S.D.)	n
0.5	75.0 ± 11.4	4
1.0	88.0 ± 15.9	8
2.0	71.8 ± 18.2	18
5.0	85.7 ± 20.2	10
10.0	92.0 ± 19.3	12
20.0	78.4 ± 21.9	12
50.0	77.8 ± 21.5	6

Recovery of Fertirelin Added to Plasma and Coefficients of Variation

Various amounts of fertirelin were added to plasma obtained from cattle which had never been injected with fertirelin and the mixtures were assayed for fertirelin as described above.

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The mean recoveries ranged from 75.0 to 92.0% for the fortification levels from 0.5 to 50.0 ng/ml (Table IV). The coefficients of variation calculated from recovery data were 14.4% (within assay) and 17.5% (between assay).

Discussion

Three types of hapten–protein conjugates have been reported for preparing immunogen to obtain anti-LH-RH antibodies: first, LH-RH was coupled to protein at the C-terminus,[4,13] second, the hapten was coupled to protein in the central part of the molecule,[3,4,6,13,14] and third, protein was coupled to LH-RH analogues possessing a free amino group at the N-terminus.[15] Among these, the conjugation method with ECDI, which was reported to couple protein with LH-RH at the tyrosine residue at position 5 of the molecule,[6b] was adopted to prepare fertirelin–BSA conjugate because it was expected to produce antibodies recognizing both the carboxy and amino termini of fertirelin. The antibodies thus obtained most clearly discriminated LH-RH and to some extent analogues possessing different proline alkylamide groups, but showed low specificity for the N-terminus as reported in the RIA of LH-RH.[13]

In order to establish the EIA system, several peptide–enzyme conjugates were prepared as shown in Table I. The conjugates prepared by using ECDI were bound to the antibody but were not displaced by fertirelin or its analogues, and so EIA was unsuccessful. However, peptide–protein conjugate such as LH-RH–ovalbumin could displace them. This phenomenon may be reasonably explained by the assumption that the antibody binds too strongly to the linking part between hapten and enzyme, because the same coupling procedure as in the preparation of the immunogen was adopted.[16] On the other hand, the enzyme coupled to peptide XXIII bearing a free amino group at position 8 by using MBS was not bound by the antibody. Thus, it may be concluded that the N-terminus of fertirelin is not included in the antigenic determinant, and this is the reason why the antibody is less specific for the N-terminus. Then we prepared enzyme conjugates by coupling the enzyme to the amino moieties of peptides II and III with MBS. Both conjugates thus obtained could be used successfully in EIA systems for fertirelin; they had similar sensitivity to fertirelin and similar specificity to related peptides.

For preparing anti-RIgG immobilized cellulose, the *m*-periodate method was very useful rather than the conventional BrCN method. While anti-RIgG immobilized cellulose prepared by the BrCN method non-specifically bound about 10% of the enzyme conjugate added and lowered the precision of the assay, the cellulose prepared by the *m*-periodate method showed almost no non-specific binding and accordingly improved the precision of the assay.

Although the sensitivity of the EIA was sufficient for determining fertirelin in plasma, it was impossible to assay plasma directly owing to the presence of interfering substances, even if 10 μl was used for assay. Attempts were made to remove such substances, by methanol extraction,[14,17] methanol extraction and defatting with petroleum ether,[12] extraction with ethanol containing acetic acid,[17] Sep-Pak® C_{18} treatment, and extractions with Florisil,[18] Fuller's earth,[19] and antibody-immobilized Sepharose 4B.[20] Among these treatments, only the antibody-Sepharose 4B treatment gave a satisfactory result. However, cellulose was better than Sepharose 4B because the latter tends to float when centrifuged, mixing with the sample extract and consequently causing unreliable determination. In fact, cellulose is more densely packed at the bottom of the tube than Sepharose 4B, making pipetting easier. Standard curves prepared with control plasma extract obtained by using antibody-immobilized cellulose were consistent with those in buffer A over approximately 0.25 ng/ml. This means that determination can be done with a standard curve prepared with the buffer free from control plasma extract. None of the blank values exceeded 1 ng/ml and recoveries of fertirelin added to plasma at 1.0 ng/ml and higher ranged from 72 to 92%; hence values over 1.0 ng/ml

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obtained by this EIA may be reliable. Coefficients of variation seem slightly larger than those of usual plasma direct immunoassays reported by other researchers. However, these values are adequate for EIA such as the present method, which includes an extraction using antibody-immobilized cellulose.

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