HIGHER MOLECULAR WEIGHT IMMUNOREACTIVE SPECIES OF LUTEINIZING HORMONE RELEASING HORMONE : POSSIBLE PRECURSORS OF THE HORMONE

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SUMMARY. Separation of extracts of sheep hypothalami on Sephadex G-25 gave three peaks exhibiting luteinizing hormone releasing hormone immunoreactivity. One peak corresponded in elution volume with luteinizing hormone releasing hormone but the others (I and II) eluted earlier, indicating that they are of higher molecular weight. Elution volumes were unaffected by 8 M urea treatment. Incubation of I and II with hypothalamic peptidases produced a small quantity of immunoreactive material eluting in the luteinizing hormone releasing hormone region. Digestion of I with trypsin resulted in a marked increase in total immunoreactivity and the production of material with the same elution volume as II. Tryptic digestion of II gave rise to a small quantity of immunoreactive peptide eluting in the luteinizing hormone releasing hormone region. The amount of I and II relative to luteinizing hormone releasing hormone was lower in the median eminence than in the supra optic chiasmatic and basal hypothalamic regions.

INTRODUCTION

The isolation, structural characterisation (1,2,3), and synthesis (4,5) of the hypothalamic decapeptide, luteinizing hormone releasing hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) has led to rapid advances in our knowledge of the physiological effects of the hormone(6). However, little is known about the mechanisms regulating the production and secretion of LH-RH. Two possible mechanisms of biosynthesis might be considered. In view of the fact that many biologically active small peptides such as glutathione (7) and several antibiotic peptides (8) are assembled by specific enzymes in the absence of an mRNA template, the majority of studies have sought to demonstrate a non-ribosomal biosynthesis of the small hypothalamic peptide hormones (9,10,11,12). Alternatively, the hypothalamic peptides could be synthesized conventionally on ribosomes as larger precursor peptides (prohormones) which are cleaved by peptidases to form the biologically active hormones. Higher molecular weight immunoreactive species of LH-RH and somatostatin have been reported (13,14) and confirmed in this laboratory (15). In the present study additional evidence in

Abbreviation : LH-RH, luteinizing hormone releasing hormone.

support of the existence of higher molecular weight species of LH-RH is presented.

METHODS

Extraction and separation procedures: The hypothalamic region encompassing the area between the optic chiasma and mammillary bodies was dissected to a depth of 5 mm from sheep brains within 15 min of death and immediately frozen on solid CO_2. Batches of 50 hypothalami were lyophilized and defatted with petroleum ether (40-60°C) and acetone, homogenized in 2N acetic acid and centrifuged at 18,000 g for 30 min. The supernatant was filtered through an Amicon PM30 filter and the filtrate concentrated and chromatographed on Sephadex G-25 with 0.2N acetic acid. Fractions were lyophilized, taken up in EDTA phosphate buffer and assayed for LH-RH immunoreactivity using a specific antiserum to LH-RH, ^{125}I LH-RH and synthetic LH-RH standards (16). Three peaks of immunoreactivity were concentrated separately and rechromatographed before and after treatment with 8M urea.

Digestion with Hypothalamic Peptidases : Crude extracts of hypothalamic peptidases were obtained by homogenizing 2 sheep or 30 rat hypothalami in 3 ml of 0.02 M Tris buffer (pH 7.2) and centrifuging at 18,000 g for 30 min. The supernatant was decanted, 100 µl of a 10 nM solution of chlorides of Cu Co Ca Zn Mn and Mg added and the mixture incubated for 30 min at 37°C to allow catabolism of LH-RH already present in the extract. Higher molecular weight species of LH-RH were incubated with this LH-RH free preincubated enzyme extract as described in the results. To prevent excessive catabolism of any LH-RH produced during incubation of higher molecular weight LH-RH species, the activity of endopeptidases and arylamidases known to rapidly catabolise LH-RH (17,18,19,20) was reduced by addition of 10 µg of thyrotropin releasing hormone, L-leucyl-β-napthylamide, leucinamide, L-alanyl-β-napthylamide and L-tryptophan-β-napthylamide. Digested extracts were rechromatographed and LH-RH immunoreactivity assayed.

Trypsin digestion of peptides: Higher molecular weight immunoreactive peptides were incubated with 0.1 mg trypsin (BDH) in 2 ml 0.04 M phosphate buffer (pH 7.0) at 37°C and the reaction terminated at 0,5,15,60, and 180 min by transferring samples to a boiling water bath for 10 min. Total immunoreactivity was assayed in aliquots before and after each digestion. Samples were centrifuged to remove any precipitated protein and the supernatant chromatographed on Sephadex G-25 and Bio-Gel P2.

RESULTS AND DISCUSSION .

Three distinct peaks of LH-RH immunoreactivity were separated by Sephadex G-25 gel filtration (Fig. 1). The last emerging peak (III) corresponded with synthetic LH-RH in elution volume (K_{av} = 0.84). A small rider peak was also detected on the ascending shoulder in this region. Another immunoreactive peak (I) emerged in the void volume indicating molecular weight exceeds 5,000 daltons while the position of the third peak (II) was intermediate between I and LH-RH (Kav = 0.58) in elution volume. In twelve separate extractions of batches of 50 sheep hypothalami the three immunoreactive materials were separated and eluted with their characteristic elution volumes. The higher molecular species are clearly not oligomers of LH-RH since synthetic LH-RH always eluted as a single peak. On rechromatography, before and after treatment with 8 M urea the peptides retained their characteristic elution

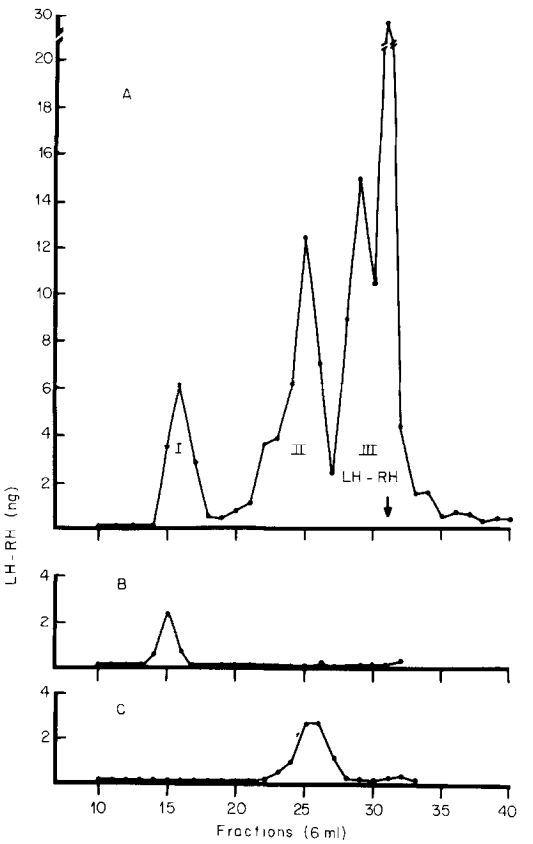

Fig. 1. (A) Separation of LH-RH immunoreactive peptides on a Sephadex G-25 column (1.6 cm x 90 cm). (B) and (C) Rechromatography of peptides I and II after 14 h treatment with 8 M urea. Elution volume of LH-RH arrowed.

volumes (Fig.1.). This suggests that these higher molecular weight species are not merely LH-RH bound to other peptides, although it is conceivable that recombination might occur on the column since urea could not be included in the 0.2 N acetic acid eluant. The latter possibility seems unlikely, however, as the separate peaks were still present even when 2 N acetic acid eluant was used; conditions which dissociate peptides from antibodies or binding peptides such as neurophysin (21).

The possibility that these higher molecular weight immunoreactive forms of LH-RH could be cleaved to smaller immunoreactive products was investigated by incubation with crude hypothalamic peptidase extracts. However, it was first

TABLE 1. CATABOLISM OF LH-RH BY HYPOTHALAMIC EXTRACTS.

PREPARATION	LH-RH (pg/100 µl)	
	Before digestion	30 min digestion
Sheep hypothalamus	505	390
Rat hypothalamus	1780	185
Rat hypothalamus + LH-RH + "Inhibitors"	735	330

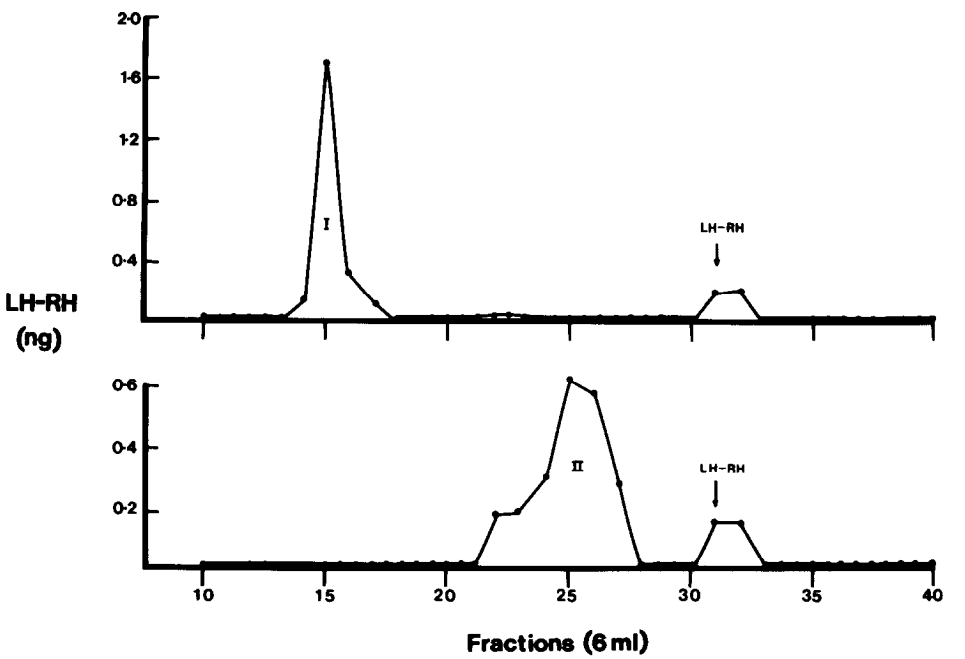

Fig. 2. Rat hypothalamic enzyme extract (200 µl) digestion of 4 ng peptide I for 20 min at 37°C (upper panel) and digestion of 8.9 ng peptide II for 40 min (lower panel). No LH-RH immunoreactivity was detectable in any fractions when hypothalamic enzyme extract alone was incubated and chromatographed.

necessary to remove the endogenous LH-RH contained in the enzyme extracts themselves. This was achieved by carrying out an initial preincubation during which hypothalamic peptidases known to catabolise LH-RH (17, 18, 19, 20) would reduce the

TABLE 2. TRYPSIN DIGESTION OF LH-RH IMMUNOREACTIVE PEPTIDES

	Incubation time	LH-RH Immunoreactivity (ng)
PEPTIDE I	0 min	6.4
	5 min	34.0
	60 min	>100.0
	180 min	8.8
PEPTIDE II	0 min	4.6
	30 min	0.88
LH-RH	0 min	4.2
	30 min	0.54

hormone to undetectable levels. Extracts of sheep hypothalamic peptidases were found to catabolise LH-RH to low levels after 30 min incubation (Table 1). Having removed endogenous LH-RH from the extracts it was then necessary to minimise catabolism of LH-RH or LH-RH like peptides which might be produced from I or II by addition of an excess of alternative substrates. Carboxypeptidases, arylamidases and an endopeptidase cleaving at Gly^6 in LH-RH have been implicated (18, 19). Addition of thyrotropin releasing hormone, L-leucyl-β-napthylamide, leucinamide, L-alanyl-β-napthylamide and L-tryptophan-β-napthylamide reduced the rate of LH-RH catabolism. Digestion of both I and II with the hypothalamic enzyme prepration caused a decline in total immunoreactivity and after separation on Sephadex G-25 only small quantities of immunoreactive material could be detected in the LH-RH region (Fig.2). Interestingly, although I gave rise to some immunoreactive material in the LH-RH region there was a complete absence of material eluting in region II. Thus if peptide II is intermediate in the conversion of I to material eluting in the LH-RH region it must be rapidly converted. This is contrary to the effects of digestion with trypsin (see below). Hypothalamic enzyme preparation incubated alone and chromatographed on Sephadex G-25 yielded no immunoreactive material in any fractions.

In studies on the effects of tryptic digestion of I, an increase in total immunoreactivity occurred after 5 and 60 min followed by a decline after 180 min digestion (Table 2). In Fig.3, a relative decrease in I with concomitant increase in II is apparent as time of incubation with trypsin is increased. Very little material

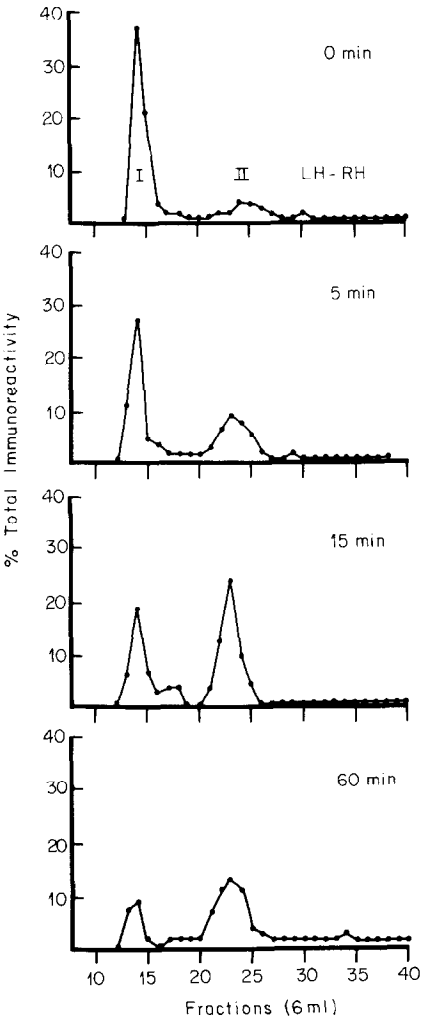

Fig. 3. Elution of immunoreactive peptide on Sephadex G-25 after incubation of peptide I with trypsin.

eluting in the LH-RH region could be detected. Failure to accumulate material eluting in the LH-RH region is due to the rapid catabolism of LH-RH by this trypsin preparation (Table 2). Furthermore, digestion of II with trypsin gave rise to a marked decrease in total immunoreactivity (Table 2) with formation of very little material eluting in the region of LH-RH (Fig.4). In this study a Bio-Gel P-2 column was used for enhanced separation of II from LH-RH.

Tryptic cleavage of LH-RH is unexpected as the Arg-Pro peptide bond is not

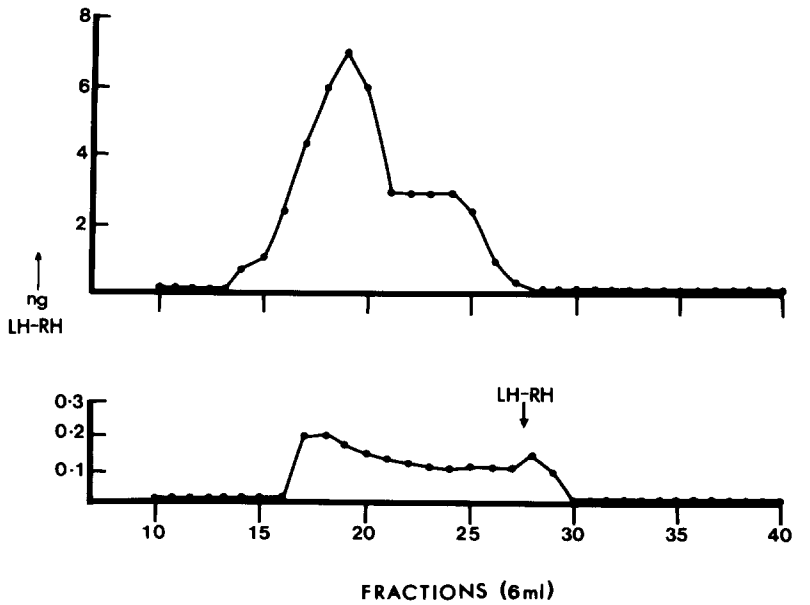

Fig. 4. Elution profile of peptide II on a Biogel P-2 column (50 cm x 2.6 cm) before (upper panel) and after (lower panel) incubation with trypsin for 30 min at 37°C.

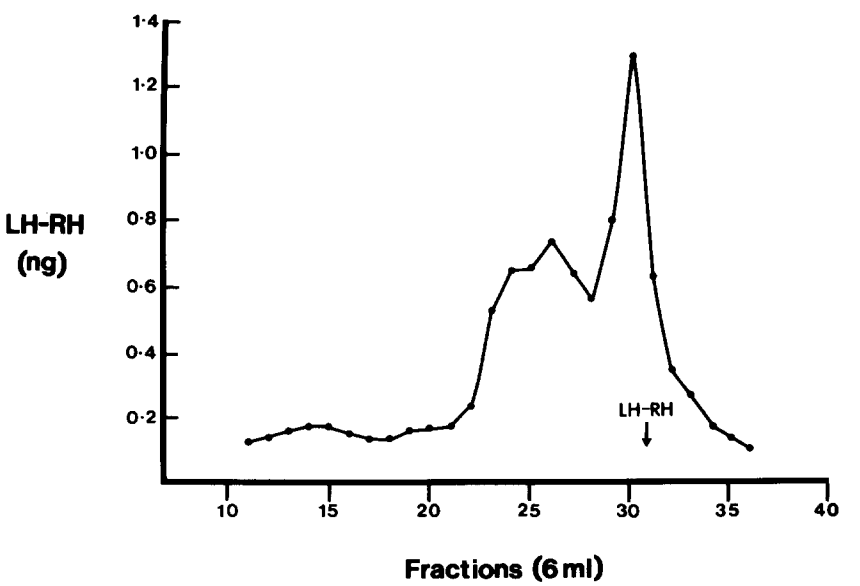

Fig. 5. Elution of LH-RH immunoreactive material after 180 min digestion of peptide I with Difco trypsin.

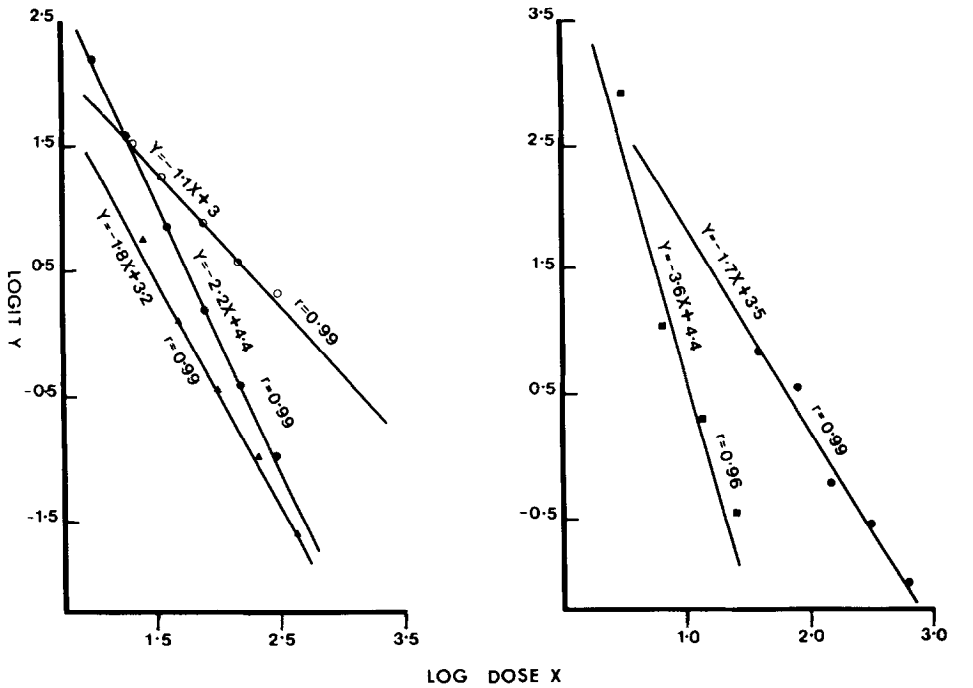

Fig. 6. Displacement of ^{125}I LH-RH from antibody by LH-RH (●), peptide I (■), II (▲) and II_1 (o).

usually cleaved by trypsin (22). Contamination of this particular trypsin preparation with other peptidases might account for degradation of LH-RH. In another preliminary study, incubation of I with Difco trypsin for 180 min gave rise to an accumulation of immunoreactive material eluting in the region of II and LH-RH (Fig 5) and we are presently extending these studies.

Overall, these findings demonstrate that the higher molecular weight material I increases in immunoreactivity after limited tryptic digestion and is suggestive that it may be a precursor of LH-RH. Increased immunoreactivity after limited tryptic digestion is most likely due to an increased accessibility of LH-RH immunogenic sites (mainly amino acids 6-8 (16))after removal of peptide fragments obscuring the site and/or altering the conformation of the peptide. The possibility that tryptic production of lower molecular weight immunoreactive material is artifactually produced by cleavage of a peptide binding LH-RH seems unlikely since urea and 2N acetic acid did not affect the elution volumes of I or II.

TABLE 3. CONTENT OF LH-RH IN REGIONS OF SHEEP BRAIN

Region	ng LH-RH/g wet weight tissue
Median eminence	286.0
Basal hypothalamus	30.0
Posterior pituitary	14.2
Supra-optic-chiasmatic	9.5
Pineal	6.2
Optic chiasma	3.5
Cerebellum	0.57
Thalamus	0.26
Anterior pituitary	0.23
Cerebral cortex	0.17
Medulla oblongata	0.13

Studies on the comparative displacement of ^{125}I LH-RH from antibody by I, II and material formed from I and eluting in the region of II (designated II_I) are shown in Fig.6. The linearised (logit transformed) displacement curve of I is clearly not parallel to that of LH-RH or II thus emphasizing its structural differences from these peptides. By contrast the similarity of II and LH-RH is suggested by the close parallelism of their displacement curves. Although II_I, (the tryptic product of I) elutes in the same region on G-25 as II and is most likely of similar size to II_I the slope of the displacement curve is less than that of II or LH-RH. Thus II_I and II are clearly structurally dissimilar.

As has been proposed for oxytocin (23), LH-RH (or precursors) might be synthesised in cell bodies remote from the median eminence and pass down axons to be secreted at the nerve termini in the stalk median eminence. Such a postulate finds support in the report that lesions in the supra optic chiasmatic region lead to diminished production of gonadotrophin releasing hormone (24) and the recent demonstration that surgical isolation of the hypothalamic region results in a rapid decline in LH-RH content (25). In preliminary studies we observed that LH-RH is detectable in both the supra optic chiasmatic region and basal hypothalamic region (Table 3). LH-RH and associated peptides were then simultaneously extracted from

TABLE 4. RELATIVE OCCURRENCE OF LH-RH IMMUNOREACTIVE PEPTIDES IN HYPOTHALAMIC REGIONS.

REGION	IMMUNOREACTIVITY (ng LH-RH)* I	II	LH-RH	LH-RH/I	LH-RH/II
Median eminence	2.1	113.9	415.0	200	3.6
Basal & optic	1.6	2.3	6.0	3.8	2.6
Median eminence	2.0	98.4	378.8	189.8	3.6
Basal	3.8	33.9	53.5	14.1	1.6
Optic	0.8	11.2	25.7	32.1	2.3
Median eminence	7.7	63.2	908.7	118	14.4
Basal	1.2	4.3	26.2	22.0	6.0
Optic	1.4	5.1	25.3	18.5	4.9
Pineal	0	0	38.0	-	-

*Values indicate total immunoreactivity in extracts of regions from 50 sheep brains

the median eminence, basal hypothalamus and supra optic chiasmatic region in batches of 50 sheep brains and separated on Sephadex G-25 columns. When compared with the stalk median eminence, both the supra optic and basal hypothalamic regions had much higher ratios of immunoreactive material in regions I and II relative to the LH-RH region (Table 4). Moreover, separation of extracts of 50 sheep pineal glands, previously reported to contain LH-RH (20) demonstrated that all immunoreactivity eluted in the LH-RH region while there was a complete absence of material in regions I or II (Table 4).

The results of this study demonstrate that higher molecular weight immunoreactive species of LH-RH occur in hypothalamic tissue. The increase in total immunoreactivity of peptide I on limited trypsin digestion supports the concept that it may be a precursor (or prohormone) of LH-RH. However, it was not possible to firmly establish that the higher molecular forms of LH-RH give rise to LH-RH per se. This, and the categorical conclusion that LH-RH is formed from larger peptides assembled conventionally on ribosomes awaits further investigation.

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