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SERUM AMINOPEPTIDASES, "ANGIOTENSINASE," AND HYPERTENSION—II.

AMINO ACID β-NAPTHYLAMIDE HYDROLYSIS BY NORMAL AND HYPERTENSIVE SERUM

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Abstract—Comparative experimental data indicate that the release of aspartic acid from both angiotensin II and α-L-aspartyl β-naphthylamide is mediated by an enzyme(s) in human serum having characteristics similar to aminopeptidase A. A colorimetric assay employing α-L-aspartyl β-naphthylamide has been used as a measure of the initial N.terminal degradation of angiotensin II by human serum.

The application of this enzyme assay method to sera from a variety of clinical states indicates no variation from normal in hypertensive patients. A reduction of enzymic activity, however, was found in sera from hypertensive patients with secondary hyperaldosteronism. In addition, a significant elevation of enzymic activity was observed in sera during the third trimester of pregnancy.

OUR previous studies[1] indicated that the hydrolysis of the N-terminal amino acid residue from angiotensin II and angiotensin II amide is mediated by different enzymes in human serum and showed that the release of aspartic acid[1] from angiotensin II was mediated by an enzyme(s) the properties of which resemble those of aminopeptidase A[2], e.g. calcium activation and EDTA inhibition. In addition, angiotensin II competitively inhibited the hydrolysis of two chromogenic substrates, α-L-aspartyl β-naphthylamide and α-L-glutamyl β-naphthylamide, which were originally employed in the characterization of mammalian aminopeptidase A.

The data presented in this communication support a suggestion that the release of aspartic acid from angiotensin II and α-L-aspartyl β-naphthylamide is catalyzed by the same enzyme. These data also show that α-L-aspartyl β-naphthylamide may be employed in a colorimetric assay procedure to measure the activity of the enzyme(s) that causes the initial aminopeptidase-catalyzed breakdown of angiotensin II. There is evidence that the hydrolysis of α-L-glutamyl β-naphthylamide, however, is catalyzed by more than one enzyme.

METHODS

The chromogenic β-naphthylamide derivatives of α-L-aspartic acid (α-ANA),† α-L-glutamic acid (α-GNA),† α-L-asparagine, and α-L-glutamine, as well as the dipeptide

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† Now commercially available from Cyclo Chemical Co., Los Angeles, Calif.

α-L-glutamyl-L-phenylalanine were prepared in our laboratories.[3] The L-leucyl β-naphthylamide (LNA) was obtained from Mann Research Laboratories, New York, N.Y., and aldosterone from New England Nuclear Corp., Boston, Mass. Aldosterone secretion rates were determined by the method of Peterson.[4] Sera were obtained from 39 normal human volunteers and 51 patients having a variety of disease states. Fasting blood samples were collected from all subjects in the early morning and immediately refrigerated at 4°.

The incubation solution for the standard assay was composed of 0·4 ml, 0·3 ml, or 0·1 ml of serum with α-ANA, α-GNA, or LNA as substrate respectively; 1·5 ml of 0·2 M Tris–maleate buffer, pH 7·0; 1·5 μmoles substrate, 30 μmoles calcium chloride dihydrate, made up to a total of 3·0 ml with distilled water. Incubation was carried out for 30 min at 37° with gentle shaking. The reaction was stopped by the addition of 1·0 ml of 10% "Tween-20" in 1 M acetate buffer, pH 4·2, containing 0·5 mg of the stabilized diazonium salt Fast Garnet GBC*. Absorbance was read after 30 min at 525 mμ. A confirmatory fluorometric assay procedure[5] was also employed. When inhibitors or activators were studied, these replaced calcium chloride in the incubation solution. Other buffers used in this study were Tris–HCl and Tris–acetate, 0·1 M, pH 7·0. Threefold-concentrated dialyzed and lyophilized serum[1] was also assayed for the relative hydrolysis rates of the above substrates and compared to untreated serum. In view of the marked aqueous insolubility of α-L-asparaginyl β-naphthylamide, comparative studies of the hydrolysis of the entire series of β-naphthylamide substrates were carried out in the standard incubation solution in the presence of N,N-dimethylformamide (3%). The reproducibility of the method in single patients was checked by assaying five samples of sera obtained on different days from both a normal and hypertensive subject.

RESULTS

Substrate hydrolysis characterization

The properties of enzymes in human serum that catalyze the hydrolysis of α-ANA, α-GNA, and LNA were studied in the presence of calcium at a concentration which had been found optimal for activating α-ANA hydrolysis, 0·01 M. The time course of the hydrolysis of these substrates in the presence of calcium was linear for 2 hr with α-ANA and for 30 min with α-GNA and LNA. The hydrolysis rates of these substrates were proportional to serum concentration and were independent of substrate concentration above 0·5 mM at a constant enzyme concentration. With the above substrates in the presence of normal (unconcentrated) and threefold-concentrated, dialyzed, and lyophilized serum the ratios of activity remained essentially proportional to serum concentration. The rates of hydrolysis in normal serum in the presence of 0·01 M calcium chloride in international units (IU) (μmoles substrate hydrolyzed/min/liter of serum at 37°) were 0·88 ± 0·17 for α-ANA, 7·04 ± 1·12 for α-GNA, and 29·20 ± 8·31 for LNA. Five consecutive daily serum samples from both a normal and a hypertensive patient gave mean values of 0·75 ± 0·11 and 0·87 ± 0·06 IU for α-ANA respectively.

When a single serum sample was employed, the values for the maximal velocity of hydrolysis were 0·98, 6·70, and 34·00 for α-ANA, α-GNA, and LNA respectively. When mixtures of equimolar concentrations of α-ANA and α-GNA were used, the

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maximal velocity was 6·66; of α-ANA and LNA, 34·74; and of α-GNA and LNA, 37·50.

The effect of potential activators and inhibitors on the hydrolysis in normal serum of α-ANA, α-GNA, and LNA are presented in Table 1. Variations in affector characteristics depended in some cases on the buffer used. For example, in Tris–HCl buffer,

TABLE 1. EFFECT OF REAGENTS ON SERUM HYDROLYSIS OF α-ANA, α-GNA, AND LNA

Affector		α-ANA (%)	α-GNA (%)	LNA (%)
None		100 (0·27)	100 (5·85)	100 (32·20)
Ca^{++}	10·0 mM	333	120	91
Ni^{++}	2·0 mM	0	38	108
Mg^{++}	2·0 mM	91	96	97
Mn^{++}	2·0 mM	85	88	97
Zn^{++}	2·0 mM	30	30	70
Cd^{++}	2·0 mM	0	0	29
Co^{++}	2·0 mM	103	97	107
CN^-	0·5 mM	109	102	105
CN^-	10·0 mM	111	91	78
EDTA	10·0 mM	0	0	14
α-GPA	1·0 mM	62	69	92

Effect expressed in per cent of normal activity of human serum. All cations and α-L-glutamyl-L-phenylalanine (α-GPA) incorporated with substrate into the standard incubation medium as described in Methods. Ethylenediamine tetraacetate (EDTA) was preincubated with serum for 1 hr at 25° prior to addition of substrate. Values in parentheses, substrate hydrolysis in μmoles/min/liter of normal serum. Reaction for 30 min at 37°.

TABLE 2. AFFECTOR CHARACTERISTICS OF SERUM HYDROLYSIS OF AMINO ACID β-NAPHTHYLAMIDES

	Incubating solution with 3% N,N-dimethylformamide					Standard incubation solution		
Affector	α-ANA (%)	Asparaginyl β-NA (%)	α-GNA (%)	Glutaminyl β-NA (%)	LNA (%)	α-ANA (%)	α-GNA (%)	LNA (%)
None	100 (0·02)	100 (0·28)	100 (4·01)	100 (10·85)	100 (19·60)	100 (0·27)	100 (5·85)	100 (32·20)
Ca^{++} 0·01 M	2350	45	140	82	90	333	120	91
Ni^{++} 0·002 M		25		80		0	38	108
α-GPA 0·001 M		46		99		62	69	92
EDTA 0·01 M		5		36		0	0	14

Effect in per cent of normal serum activity in the presence and absence of 3% N,N-dimethylformamide. Reaction conditions the same as in Table 1. Values in parentheses, substrate hydrolysis in μmoles/min/liter of normal serum. Reaction for 30 min at 37°.

cobaltous ions activated LNA hydrolysis 11% and inhibited α-ANA and α-GNA 66% and 10% respectively; in Tris–acetate buffer calcium ions activated α-ANA and α-GNA hydrolysis 104% and 13% respectively, but inhibited LNA 21%. The effects of a variety of reagents on the hydrolysis of the series of β-naphthylamide substrates are recorded in Table 2. Inhibition by α-L-glutamyl-L-phenylalanine (α-GPA) of the

hydrolysis of α-ANA (K_m, 1×10^{-2} M) and α-GNA (K_m, 5×10^{-4} M) was competitive with a K_i of $3{\cdot}76 \times 10^{-4}$ M and $3{\cdot}57 \times 10^{-4}$ M respectively. No inhibition of LNA hydrolysis was observed.

Sharp pH maxima for α-ANA and LNA hydrolysis were found at pH 6·7. A broad pH maximum ranging from pH 7·0 to 7·5 was observed with α-GNA. Hydrolysis rates of α-ANA vs. α-GNA by human sera from normal and disease states are plotted in Fig. 1 and reveal a positive correlation (r = 0·635). There was, however, no such correlation when the hydrolysis rates of either α-ANA or α-GNA were plotted against those of LNA (r = 0·304 in the case of α-ANA).

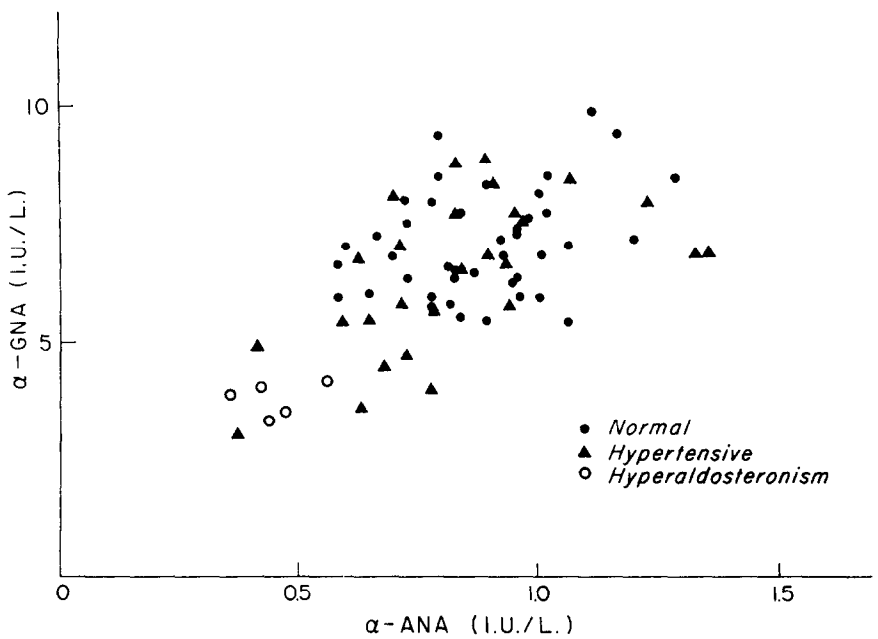

FIG. 1. Relationship between hydrolysis of α-ANA and α-GNA in a variety of human sera.

Clinical data

Fasting blood samples were obtained from the following subjects: 39 normal patients; 27 hypertensive patients, 4 of whom had renal vascular disease as the etiology of their hypertension, the remaining 23 having no demonstrable cause for the blood pressure elevation; 14 females in the third trimester of an uncomplicated pregnancy; 6 patients with hypertension and documented hyperaldosteronism, 4 of whom underwent subtotal adrenalectomy; 1 patient with Addison's disease; 1 patient with pheochromocytoma; 2 patients with coarctation of the aorts and hypertension. Postoperative blood samples were obtained from 2 weeks to 4 months after surgery in 2 hypertensive patients undergoing nephrectomy, 1 patient after correction of a coarctation of the aorta, and 1 patient after removal of a pheochromocytoma. In only the last instance was there a significant alteration from preoperative levels of α-ANA hydrolysis after surgery. In this case, initial α-ANA hydrolysis was 1·03 IU, and 2 weeks after surgery the enzyme activity was 0·61 IU. Those patients classified as having

primary hypertension or renal vascular hypertension had neither laboratory nor clinical evidence of hyperaldosteronism.

The mean values ±S.D. for α-ANA hydrolysis for the normal, hypertensive, third-trimester pregnancy and hyperaldosteronism patients are listed in Table 3, as are the

TABLE 3. α-ANA HYDROLYSIS AND ALDOSTERONE SECRETORY RATES

Diagnosis (no of patients)	α-ANA Hydrolysis (IU)	Aldosterone secretory rate (μg/24 hr)*
Normals (39)	0·88 ± 0·17†	<250
Hypertension (27)	0·85 ± 0·21	
Pregnancy, 3rd trimester (14)	1·86 ± 0·57	
Hyperaldosteronism (6)	0·51 ± 0·11	
A.B.	0·56	1,400
P.B.	0·36	270
G.B.	0·42	366
H.R.	0·48	291
R.B.	0·55	350
C.M.	0·67	300

* Upper limits of normal: 250 μg/24 hr.
† Mean ± 1 S.D.

individual aldosterone secretion rates of each of the last group. No significant difference in serum enzyme activity is demonstrable between the normals and those patients with hypertension (including 4 cases of reversible hypertension after surgery). However, the values obtained from the sera of the patients with hyperaldosteronism are significantly lower than the normals ($P < 0·0005$). The values obtained from the sera of third-trimester pregnant females are significantly higher than those of normals ($P < 0·004$). These data are graphically represented in Fig. 2. Comparison of the L-leucyl β-naphthylamide-hydrolyzing activity of sera from normals and from the third-trimester pregnancy patients revealed markedly increased hydrolytic activity in the sera of the pregnant group. The values for the normals were 29·2 ± 8·3 (1 S.D.) IU, and for the pregnant women 75·6 ± 11·8 (1 S.D.) IU ($P < 0·0005$). The α-ANA-hydrolyzing activity of sera from the patient with Addison's disease, the two with coarctation of the aorta, and the one with pheochromocytoma was within the range of values obtained from normals and patients with uncomplicated hypertension. The elevated aldosterone secretion rates are also listed in Table 3 and range from 270 to 1,400 μg/24 hr. There is no correlation between α-ANA hydrolysis levels in these patients and their degree of elevation of aldosterone secretion.

DISCUSSION

In order to estimate the value of α-L-aspartyl or α-L-glutamyl β-naphthylamides as chromogenic indicators of the release by human serum of the N-terminal aspartic acid residue from angiotensin II, it was first necessary to determine that the hydrolytic characteristics of these β-naphthylamide substrates in human serum were similar to those mediating aspartyl[1] release from angiotensin II. Hydrolysis of both dicarboxylic amino acid β-naphthylamides was competitively inhibited by angiotensin II, was activated by calcium ions, and was inhibited significantly by EDTA and nickel ions. These findings indicate that the hydrolysis of both chromogenic substrates was

afforded by enzymes in human serum similar to or identical with those effecting the initial breakdown of angiotensin II. The hydrolytic characteristics in human serum of a variety of other β-naphthylamide substrates, including the α-L-asparaginyl, α-L-glutaminyl, and L-leucyl derivatives, failed to show a similar correspondence.

It appears from this evidence that both the α-L-aspartyl and α-L-glutamyl β-naphthylamides can be used as chromogenic indicators of the initial aminopeptidase degradation

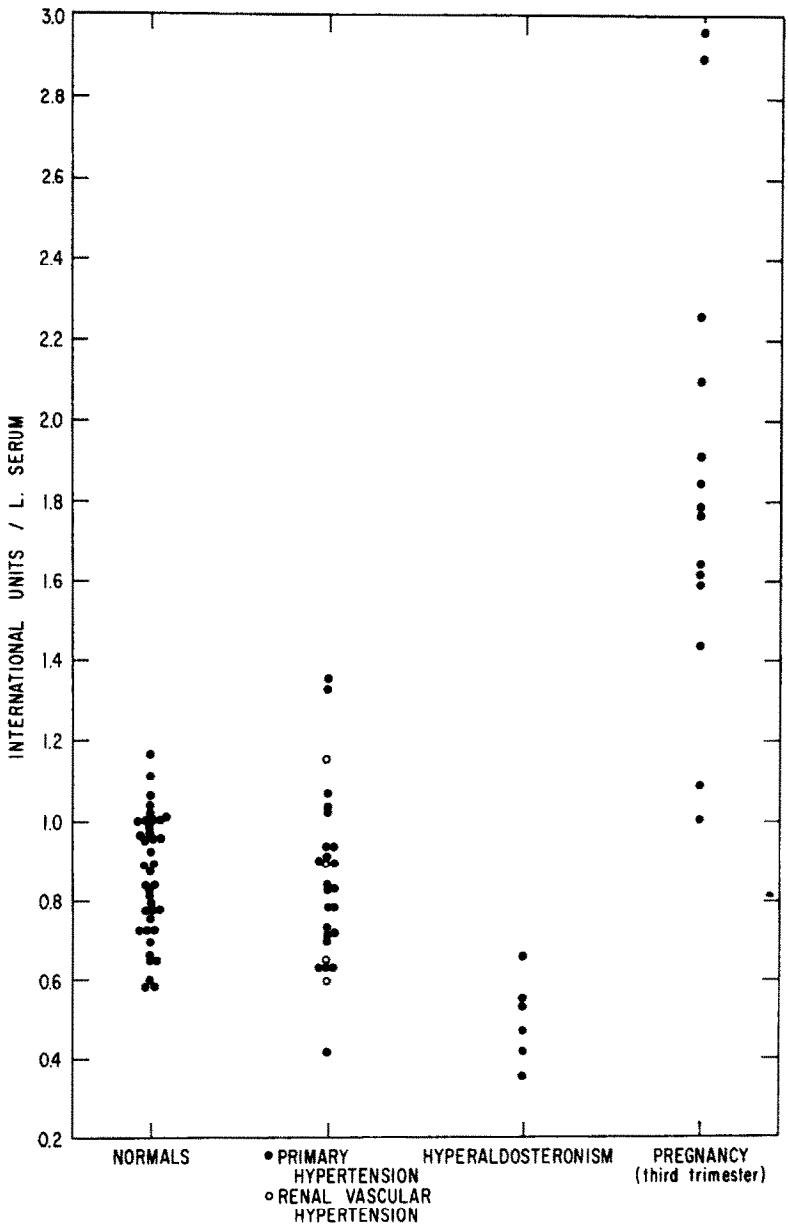

FIG. 2. The hydrolysis of α-ANA by human sera.

of angiotensin II. The relationship of the rates of hydrolysis of these two substrates was also investigated. A scattergram of the hydrolytic rates of both substrates in normal sera and sera from a variety of disease states showed a significant correlation. Both substrates were competitively inhibited by α-L-glutamyl-L-phenylalanine with essentially identical K_i values. When the mixed maximal velocity V_{max} of the chromogenic aspartyl and glutamyl substrates was determined, this value was approximately equal to that of the highest individual velocity. However, though the mixed V_{max} of the aspartyl and leucyl derivatives was approximately equal to the sum of the individual velocities, the mixed V_{max} for the glutamyl and leucyl derivatives was less than their sum but greater than the highest individual velocity. The possibility, therefore, exists that the glutamyl derivatives may be acted upon to some extent by enzymes that do not mediate the hydrolysis of the aspartyl compound. It is therefore suggested that α-L-aspartyl β-naphthylamide is the more specific indicator of the initial aminopeptidase hydrolysis of angiotensin II.

Classic hog kidney leucine aminopeptidase[6] has been shown to produce a 90% destruction of angiotensin II amide, but to have no significant effect under similar conditions on angiotensin II.[7] It has now been adequately demonstrated that L-leucyl β-naphthylamide is hydrolyzed in human serum and in a variety of tissues by enzymes other than classic leucine aminopeptidase.[8–13] During the present studies, a similarity was noted in the characteristics of hydrolysis by serum of asparagine[1] from angiotensin II amide[1] and in the hydrolysis of L-leucyl β-naphthylamide. The same relationship had also been noted by Klaus *et al.*,[14] who found that the hydrolysis by serum of angiotensin II amide and L-leucyl β-naphthylamide had many properties in common. Klaus and co-workers also observed a significant correlation between the hydrolytic rates of both compounds in normal sera and elevation of both rates in hepatic disease.[15, 16] These authors found, however, that though the serum hydrolysis of L-leucyl β-naphthylamide was increased in the third trimester of pregnancy (as shown in this study also), that of angiotensin II amide was not.[16] They therefore concluded that different enzymes mediated the hydrolysis of angiotensin II amide and L-leucyl β-naphthylamide in human serum. Recent evidence, however, indicates that several enzymic fractions hydrolyzing L-leucy β-naphthylamide in human serum can be separated by column chromatography[17, 18] and paper electrophoresis.[19–21] The most active of these components, present in the α_1-globulin fraction, is elevated in biliary obstruction.[20, 21] Another component present in the α_2-globulin fraction has been shown to be elevated only in the third trimester of pregnancy.[20, 21] It is therefore possible that some, but not all, of the serum fractions mediating L-leucyl β-naphthylamide hydrolysis act on angiotensin II amide. It is also possible that L-leucyl β-naphthylamide is not hydrolyzed by all serum components mediating angiotensin II amide degradation.[9]

If the premise is correct that a serum enzyme that causes the hydrolysis of α-L-aspartyl β-naphthylamide is specific for the initial degradation of the naturally occurring angiotensin II, then it is important to investigate various clinical conditions for altered levels of α-L-aspartyl β-naphthylamide hydrolysis. Our results have demonstrated no significant difference between the serum enzyme levels of normal subjects and patients with hypertension of various etiologies with one notable exception. Those patients with increased aldosterone secretory rate had significantly reduced serum enzyme levels (Fig. 2), but clinical conclusions are not afforded by these

preliminary data. However, that there is no difference in the level of the enzyme(s) acting upon naturally occurring angiotensin II between normal subjects and uncomplicated hypertensive patients is of interest. Hickler *et al.*[22] demonstrated in hypertensives increased serum enzyme activity as measured by the rate of degradation of synthetic angiotensin II amide and explained their results as an adaptive increase in enzyme in the probable presence of increased production of angiotensin. In light of the present data such conclusions may require revision if indeed different enzymes are responsible for the hydrolysis of the chemically different synthetic and natural pressor peptides. Why reduced levels of specific serum enzyme activity are found in the presence of increased aldosterone secretory rates is not clear. It may be that such a reduction of enzyme concentration might lead to increases in substrate levels (i.e. angiotensin), which in turn could serve as the stimulus for increased aldosterone production. Another possibility is that the reduced levels of this hydrolytic enzyme may either reflect or result in an altered degradation pathway of angiotensin II, bringing about the formation of smaller peptides possessing hormonal functions themselves.

A reduced vascular response to infused angiotensin II amide has been demonstrated in cirrhotics with increased aldosterone secretion by Laragh[23] and in patients with hyperaldosteronism associated with cirrhosis, nephrotic syndrome, and resistant congestive heart failure by Johnston and Jose.[24] Increased serum levels of one or more of the several enzyme fractions mediating the hydrolysis of angiotensin II amide and L-leucyl β-naphthylamide (see above) may explain the reduced pressor responses in these patients. To extend our present studies to patients with these diseases which cause secondary hyperaldosteronism will be of considerable interest. As already stated, the hydrolysis of L-leucyl β-naphthylamide is increased by the sera from patients in the third trimester of uncomplicated pregnancies, but that of angiotensin II amide is not.[16] In this study we have demonstrated not only increased rates of L-leucyl β-naphthylamide hydrolysis by sera from pregnant women in the third trimester, but also increased hydrolytic rates of α-L-aspartyl β-naphthylamide. What significance the increased levels of a serum enzyme implicated in the degradation of the naturally occurring angiotensin II may have during the final months of normal pregnancy is not known. Perhaps some insights into this question might be gained from measuring the α-L-aspartyl β-naphthylamide in patients with toxemia of pregnancy. To determine the true physiologic significance of wide variations in hydrolysis rates of α-L-aspartyl β-naphthylamide in human serum, it would be desirable to compare the data reported here on the hydrolysis of this chromogenic substrate with the results of bioassay on the destruction of angiotensin II by sera from patients with secondary hyperaldosteronism and those in the third trimester of pregnancy. Data on the biologic destruction of angiotensin II in these conditions are not at present available.

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