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Cleavage of the ARG[1]–PRO[2] Bond of Bradykinin by a Human Lung Peptidase: Isolation, Characterization, and Inhibition by Several β-Lactam Antibiotics[1] (41828)

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Abstract. An aminopeptidase P (EC 3.4.11.9) that cleaves the Arg[1]–Pro[2] bond of bradykinin has been isolated for the first time from human lung and purified 473-fold. The enzyme also catalyzes the cleavage of arginine from *des*-[Arg[9]]-bradykinin and the hydrolysis of several X-proline dipeptides including L-arginyl-L-proline, L-leucyl-L-proline, and L-alanyl-L-proline. Purified enzyme was routinely assayed (after initial identification with *des*-[Arg[9]]-bradykinin) with L-leucyl-L-proline. The molecular weight, in nondenaturing buffers, is 188,000 ± 8500 Da. The pH optimum was 8.0 with arginyl-proline, and was 6.8 with leucyl-proline. Chelating agents do not inactivate the enzyme, but rather only remove loosely bound cations that stimulate the enzyme. Manganese is the principal cation that stimulates the enzyme. The enzyme is inhibited by several β-lactam antibiotics, cephalexin and oxacillin being the most effective of those tested. The antibiotic inhibition is time and temperature dependent, and it is not fully reversible by exhaustive dialysis of the antibiotic-treated enzyme.

The mechanisms for bradykinin catabolism include the previously fully described arginine carboxypeptidase (carboxypeptidase N or Kinase-I, EC 3.4.17.3) and dipeptidyl peptidase II (Kinase-II, EC 3.4.15.1) (1, 2). Other proposed cleavages include Ser[6]–Pro[7] cleavage (3) and Arg[1]–Pro[2] cleavage (3, 4). Two bradykinin cleavages have been suggested to be of primary importance, namely, Ser[6]–Pro[7] and Arg[1]–Pro[2] cleavage (3). The latter of these two involves the action of an aminopeptidase that removes the N-terminal arginyl residue from bradykinin in which case the penultimate N-terminal residue, proline, does not block this particular aminopeptidase. The existence of such an aminopeptidase in lung has been proposed by Ryan *et al.* (3); other workers have referred to this enzyme as prolidase (4) or aminopeptidase P (EC 3.4.11.9) (5). Recently workers in our laboratory have isolated two enzymes from bovine lung; one cleaves the Arg[1]–Pro[2] bond of *des*-[Arg[9]]-bradykinin and a variety of X-proline dipeptides, and the second (with kinin converting enzyme activity) cleaves methionine and lysine, in a stepwise manner, from methionyl-lysyl-bradykinin to yield bradykinin (6, 7).

The occurence of an enzyme in lung that cleaves the Arg[1]–Pro[2] bond of bradykinin raises the possibility of additional pathway(s) for kinin catabolism. To investigate this we have now focused on human lung, from which we have isolated and partially characterized an enzyme that cleaves the N-terminal arginyl residue from bradykinin. This report will be concerned with human lung aminopeptidase P.

Methods. *Tissue.* Human lung tissue was obtained at autopsy and was taken only from those cases wherein there was no evidence of pulmonary diseases. In almost all cases, the cause of death was traumatic without involve-

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ment of the thorax. Tissue was taken within 3 or 4 hr after death. Lung tissue was frozen at −20°C until used for enzyme preparation.

Enzyme units. The unit of enzyme activity was defined as that amount of enzyme required to convert 1 μmole of substrate to 1 μmole of product at 37°C under the conditions specified for each enzyme assay.

Aminopeptidase P assay. The confirmation of aminopeptidase P activity involved the liberation of arginine from *des*-[Arg^9]-bradykinin, as described below under *Paper chromatography.* Once such activity was confirmed, it was routinely assayed by a more convenient method involving the liberation of arginine from L-arginyl-L-proline (or other amino acids from X-prolyl dipeptides). In this simplified and modified procedure 2.0 μmole of substrate were combined with 1.0 ml of 0.05 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.0, and after addition of an appropriate amount of enzyme, the mixture was incubated at 37°C for a period of time up to 60 min. The mixtures were quickly brought to 100°C, and maintained at that temperature for 5 min, and then chilled in an ice-water bath. Following this the amino acid oxidase method was utilized to determine the free arginine as follows.

The amino acid oxidase reagent (2 mg L-amino acid oxidase, 0.4 mg horseradish peroxidase, and 2 mg of ortho-dianisidine per 10 ml of 0.05 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.0) in an amount of 1.5 ml was added to each incubation mixture. After another incubation at 37°C for 30 min, 1.0 ml of 6 *N* H_2SO_4 was added to each tube, and after mixing the absorbance was measured at 530 nm. Arginine concentration was calculated from a standard curve. Proline yielded no color and did not interfere with the amount of color produced by arginine. Appropriate substrate and enzyme blanks were used. Arginine liberation was linear as function of time and enzyme concentration. Velocities were measured at substrate consumptions of less than 20%; the minimum readily detectable arginine concentration was 5 n*M*.

Paper chromatography. To establish whether or not the Arg^1–Pro^2 bond of bradykinin is cleaved by a given enzyme, *des*-[Arg^9]-bradykinin was incubated with that enzyme, after which paper chromatography was employed to determine whether arginine appeared in the reaction mixture as an early reaction product. The amino acid composition of incubation mixtures was determined by paper chromatography. Aliquots of incubation mixtures were applied to Whatman No. 3 filter paper sheets (26 × 36 cm) along a line 2.5 cm from the shorter edge at points 4 cm apart. After drying, the sheets were formed into cylinders along the long axis by means of paper staples. The filter paper cylinders were then placed in chromatography jars and developed in the ascending mode with an *n*-butanol/acetate acid/water solvent as described by Berry *et al.* (8) until the solvent front was within 3–5 cm from the top of the paper. After drying, the cylinders were opened, flattened, and sprayed with a ninhydrin as described by Berry *et al.* (8). Arginine and other amino acids were identified by the use of appropriate standards that were chromatographed as were the incubation mixture aliquots.

Kinin converting enzyme assay. The assay of kinin converting enzyme activity was carried out by two methods, one involving the release of methionine from methionyl-lysyl-bradykinin, and the second method involving the release of β-naphthylamine from methionyl-β-naphthylamide, as described by Starnes *et al.* (9).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Starnes and Běhal (10).

Protein determination. Protein was determined by measuring the absorbance at 280 and 260 nm in a Beckman Acta 111 recording UV/Vis Spectrophotometer.

Molecular weight determination. Molecular weight determinations were carried out by gel filtration. Agarose beads (Bio-Gel A5m, Bio-Rad Laboratories) were used in conjunction with a 1.5 × 54-cm column (Glenco Scientific Co.). The agarose packed in the column and the samples were equilibrated with 0.05 *M* borate buffer, pH 8.0, which was also 0.5 *M* with respect to NaCl. The reference proteins with known molecular weights were ox liver catalase (EC 1.11.1.6, molecular weight = 250,000 (11)), *Escherichia coli* tryptophanse (EC 4.1.99.1, molecular weight = 208,000 (12)), yeast aldehyde dehydrogenase (EC

1.2.1.5, molecular weight = 205,000 (13)), yeast alcohol dehydrogenase (EC 1.1.1.1, molecular weight = 150,000 (14)), and yeast hexokinase (EC 2.7.1.1, molecular weight = 103,000 (15)). All of these standards were obtained from Sigma Chemical Company; the standards were treated in the same way as described above before application to an Agarose column which was eluted at 0.15–0.25 ml/min at 4°C. A standard curve was constructed with the elution volume on the horizontal axis and with $\log_{10}$ mol wt on the vertical axis. A straight line was passed through the points corresponding to the various standards by the least-squares linear regression analysis method.

Tissue homogenization. Tissue was homogenized for 20 min in a Sorvall Omni-Mixer Homogenizer at 4°C after which the tissue homogenate was further disrupted with a Tekmar SDT disrupter for 20 min. During these two homogenization processes care was used to ensure that the sample was not heated. This was accomplished by operating the homogenizer for 1 min at full speed and then operating the homogenizer at low speed for 2 min to allow any heat to be dissipated into the ice-water bath surrounding the homogenization chamber. Thus to obtain 20 min of homogenization, 20 cycles of 1 min full speed and 2 min low speed were carried out. Finally the homogenate was even further disrupted by treatment with a Branson S-125 Sonifier for 20 min at 4°C.

Results. Human lung aminopeptidase P was prepared as follows from 120 g of tissue. The tissue was thawed and quickly cut into small pieces (about 5 g each) which were immediately put in 350 ml of 0.05 *M* phosphate buffer, pH 7.4, which was also 10^{-5} *M* with respect to L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) and *p*-tosyl-L-lysine chloromethylketone (TLCK). The TPCK- and TLCK-containing buffer was chilled to 4°C prior to use and it was maintained at that temperature during the tissue dissection. The tissue was then homogenized as described above. The resulting homogenate was clarified by centrifugation at 30,000*g* to yield an extract of 300 ml which contained 58 units of aminopeptidase P activity. The extract was then concentrated to 250 ml and then dialyzed against 20 vol of 0.05 *M* borate buffer, pH 8.0, which was also 0.5 *M* with respect to sodium chloride. This preparation was then applied to a 5.0 × 80-cm column (with upward flow) containing agarose beads (Bio-Gel A5m, Bio-Rad Laboratories, Richmond, Calif.) that had previously been equilibrated with the borate/sodium chloride buffer described above. The column was then eluted with this same buffer at 20 ml/hr in the ascending mode with an LKB Varioperpex II peristaltic pump. Fractions of 20 ml each were collected until 100 fractions were obtained; the aminopeptidase P activity was contained in fractions 114–130 which were then concentrated to 60 ml which contained 57 units of aminopeptidase P activity. This sample was then dialyzed against 20 vol of 0.005 *M* phosphate buffer, pH 8.6, for 16 hr at 4°C. This preparation was then applied to a 2.5 × 60-cm column containing diethylaminoethyl agarose (DEAE Bio-Gel A, Bio-Rad Laboratories), which had previously been equilibrated with 0.005 *M* phosphate buffer, pH 8.6, at 4°C. The DEAE column was then eluted with 1500 ml of a phosphate buffer/sodium chloride gradient solution for which the initial composition was 0.005 *M* phosphate, pH 8.6, and sodium chloride, 0.04 *M*; the limit composition was phosphate, 0.01 *M*, pH 6.8, and sodium chloride, 0.09 *M*. The gradient was linear. The elution rate was 20 ml/hr; 100 fractions was collected and the activity in fractions 52–62 was 46 units. These fractions were combined, concentrated, and applied to a second DEAE column identical to the first one except that the limit NaCl concentration was 0.07 *M;* 120 fractions were collected and 26 units of aminopeptidase P were recovered in fractions 63–70 (Table I). The preparation (32 μg) gave a single band upon electrophoresis (Fig. 1). The preparation (50 μg) was incubated with 2 mole of *des*-[Arg^9]-bradykinin in 2 ml of pH 8.0 buffer; aliquots were taken from the reaction mixture, for paper chromatographic analysis, at 10-min intervals. Arginine was the earliest appearing ninhydrin-positive product.

Molecular weight. The molecular weight of human lung aminopeptidase P was estimated by means of gel filtration as described under Methods. A value of 188,000 ± 8500 Da (N = 7) was obtained; the individual values

TABLE I. PURIFICATION OF HUMAN LUNG AMINOPEPTIDASE P

Step	Units[a]	Recovery (%)	Protein (mg)	Specific[b] activity	Purification
Homogenate supernatant from 100 g tissue	58	100	7400	0.00784	—
Gel filtration	57	98	2600	0.0219	2.79
Ion exchange chromatography					
Gradient No. 1	46	79	348	0.132	16.9
Gradient No. 2	26	44	7	3.71	473[c,d]

[a] For this purification, the substrate for routine assay was L-arginyl-L-proline; the assay procedure was described under Methods (Aminopeptidase P assay). The unit of activity was defined as that amount of enzyme required to convert 1 μmole of substrate to product(s) at 37°C under the condition of this assay as described under Methods.

[b] Specific activity is defined as units of activity per milligram of protein.

[c] This preparation, when incubated with *des*-[Arg^9]-bradykinin, cleaved the Arg^1–Pro^2 bond of that compond; see purification data under Results for details.

[d] This preparation, and the material from the preceding step, had activity ratios of 4:1, when assayed with *des*-[Arg^9]-bradykinin as compared to assay with L-arginyl-L-proline. In the gel filtration step this ratio was 2.1:1.

ranged from 174,000 to 199,000 Da. This value is significantly higher than the value for erythrocyte aminopeptidase P, 155,000 ± 7000 Da, which we have determined for comparative purposes.

pH Optimum. Aliquots of enzyme were adjusted to various pH values and maintained at those pH values for 1 hr at 37°C after which the pH was readjusted to 8.0. Under these conditions there was no alteration of enzyme activity as a result of exposure to the various hydrogen ion concentrations before assay at pH 8.0. Then aminopeptidase P assay was performed to determine the pH optimum for the reaction itself. For arginyl–proline hydrolysis the pH optimum was 8.0 and for leucyl–proline hydrolysis the pH optimum was 6.8. See Fig. 2.

Effect of chelating agents. A 2.5-ml aliquot of enzyme was adjusted to 0.001 *M* with respect to ethylenediaminetetraacetic acid (EDTA) at pH 8.0 and 4°C and was maintained under these conditions for 4 hr. Afterward the sample was placed in a dialysis membrane and dialyzed against 500 vol of 0.05 *M* Tris–HCl buffer, pH 8.0, at 4°C for

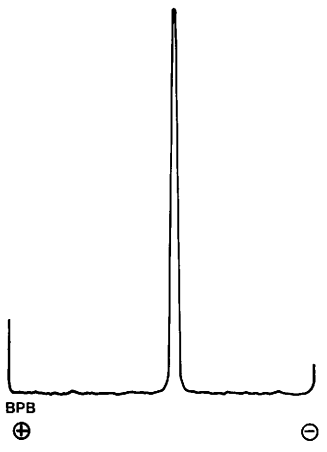

FIG. 1. Spectrophotometric scan of polyacrylamide gel electrophoresis of human lung aminopeptidase P. The sample applied to the gel contained 32 μg of protein; the gel was stained with Coomassie brilliant blue and the scan wavelength was 650 nm.

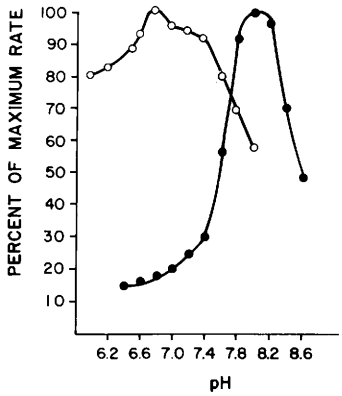

FIG. 2. Plots of the activity of aminopeptidase P as function of pH for the substrate L-arginyl-L-proline (●), and the substrate L-leucyl-L-proline (○). In each case the pH value giving the highest velocity was designated 100%; the curves are not comparable, but rather they were constructed independently with 100% being arbitrarily assigned to the highest rate for the leucyl-proline data, and to the highest rate for the arginyl-proline data.

16 hr. The EDTA did not inactivate the enzyme per se but rather only reduced the activity of a given preparation to a "divalent-cation-free" baseline level, with the extent of the inhibition being a function of the ion concentration, initially, in the preparation. Thus human lung aminopeptidase P does not have an absolute requirement for divalent cations.

Effect of metal ions. The EDTA-treated enzyme described above was used to determine which of the common divalent metal ions would stimulate the enzyme. The effects of divalent zinc, magnesium, manganese, and cobalt were determined over a range of concentrations from 10^{-6} to 10^{-3} *M*. Manganous ions stimulated the enzyme; the maximum stimulation was reached at 4×10^{-4} *M*. Zinc was weakly inhibitory; Mn^{2+} and Co^{2+} were without influence at these concentrations. With respect to other potential enzyme activators or cofactors, we repeatedly noted that there was an apparent enzyme activity loss associated with concentration of the enzyme. No activity was recovered in the filtrate from the concentration device (Amicon Model 402 with XM-50 membrane). Upon dilution of the concentrated enzyme preparation, with the filtrate *or with deionized water* to a point comparable to that before concentration, the original activity was recovered. Such activity recovery was routinely obtained with several aminopeptidase P preparations. Therefore the loss of some "activator" other than manganese was ruled out.

Activity on des-[*Arg*^{9}]-*bradykinin and X-proline dipeptides.* The rates of liberation of arginine from *des*-[Arg^9]-bradykinin and from arginyl–proline, alanyl–proline, and leucyl–proline were determined. These determinations were made initially at pH 8.0 in the presence of 4×10^{-4} *M* manganous ions; in all cases substrate concentration was 0.1 m*M*. Subsequent experiments showed that the pH response for the indicator reaction, that is, L-amino acid oxidase + peroxidase, was very small over the pH range, 6.8 to 8.0. Next comparisons of the rates of cleavage of these four compounds were made at pH 7.4. Since pH 7.4 is the physiological value, we chose to use the data obtained at pH 7.4 to obtain the factors that would relate *des*-[Arg^9]-bradykinin cleavage (Arg^1–Pro^2 bond) to the X-proline dipeptide cleavages. The mean ($N = 3$) values are *des*-[Arg^9]-bradykinin, 100; arginyl–proline, 23; leucyl–proline, 430; and alanyl–proline, 12. Thus with the purified enzyme leucyl–(or arginyl–) proline could be used as substrate, and related to the rate of cleavage of *des*-[Arg^9]-bradykinin.

Inhibition of aminopeptidase P by β-lactam antibiotics. Several β-lactam antibiotics have been screened for aminopeptidase P inhibition; these are listed on Table II where the relative inhibitory potencies of these antibiotics are given at four different concentrations. Cephalexin was the most effective inhibitor. Some preliminary experiments had indicated that preincubation of antibiotic with enzyme enhanced the degree of inhibition produced by the antibiotic. Thus in the experiments described on Table II there was a 2-hr preincubation of cephalexin (or other antibiotic) with aminopeptidase P prior to carrying out the assay itself.

The nature of the time dependency of the antibiotic–enzyme interaction was studied next; the extent of cephalexin inhibition was determined as a function of length of time for the preincubation of antibiotic with enzyme prior to aminopeptidase P activity assay. These

TABLE II. AMINOPEPTIDASE P INHIBITION BY VARIOUS β-LACTAM AND OTHER ANTIBIOTICS

Antibiotic	Percentage inhibition[a]			
	5 m*M*	10 m*M*	25 m*M*	50 m*M*
Cephalexin	72	99	100	100
Methicillin	26	46	100	100
Cloxacillin	15	20	40	66
Oxacillin	26	34	41	55
Neomycin	0	22	40	63
Carbenicillin	8	30	54	92
Ampicillin	13	11	20	27
Penicillin G	0	5	16	70
Gentamycin	0	0	9	37
Chloramphenicol	0	0	0	14
Kanamycin	0	0	0	0

[a] In this experiment 0.02 units of enzyme were incubated with 100 μl of buffered (pH = 7.4) antibiotic containing solutions at the various concentrations indicated in the heading of this table. After 2 hr incubation of enzyme with antibiotic, the aminopeptidase P assay was performed in order to determine the residual activity, from which the percentage inhibition values were calculated. The substrate in this experiment was L-leucyl–L-proline. Appropriate antibiotic controls were assayed to correct for any color that might be derived from the antibiotics themselves, and to ensure that the antibiotics did not influence the amino acid oxidase reaction.

results are shown on Table III. The temperature dependency of the antibiotic–enzyme interaction was also studied; the inhibition did not occur when the preincubation was carried out at 0°C, whereas the inhibition did occur when the preincubation was carried out at 37°C. The reversibility of the antibiotic–enzyme interaction was studied next; after preincubation of cephalexin with aminopeptidase P, the antibiotic-treated enzyme was dialyzed against buffer for 4 hr after which the residual activity was determined; only 50% of original activity could be recovered.

Discussion. Two enzyme pathways for kinin catabolism, carboxypeptidase N and Kinase II, have been thoroughly investigated. Others have been suggested as functional in kinin catabolism, but thus far no direct enzymologic evidence has been provided as to the actual presence of these enzymes in human tissues, especially lung. We now report the isolation of a human lung aminopeptidase P (EC 3.4.11.9), which cleaves the Arg^1–Pro^2 bond of bradykinin.

The molecular weight of this human lung enzyme (188,000 ± 8500) is very near the molecular weight of the *E. coli* aminopeptidase P described by Yaron and Berger (16). The possibility that the lung enzyme might have been in actuality an aminopeptidase derived from trapped erythrocytes has been eliminated since the molecular weights of the human lung aminopeptidase and the human erythrocyte aminopeptidase have differing molecular weights, 188,000 and 155,000 Da, respectively. The human lung enzyme and the *E. coli* enzyme have pH optima at 8.0 and 8.6, respectively. This applies when the substrate for the human enzyme is L-arginyl-L-proline; the pH optimum for the human enzyme decreases to 7.0 for substrates containing N-terminal neutral amino acids. This could be explained by postulating that the human enzyme is more active when the substrate does not have a charge on the N-terminal residue; the higher pH optimum for L-arginyl-L-proline would reflect a decreased charge on arginine at 8.0 as compared to 7.0. The human enzyme is not completely inactivated by EDTA treatment; only the stimulatory effect of any divalent cations occurring in a given preparation is abolished. By contrast the *E. coli* enzyme is completely inactivated by EDTA. Manganous ions stimulate both enzymes. As with the bacterial enzyme, we noted lower cleavage rates with substrates such as L-alanyl-L-proline, but higher cleavage rates with *des*-[Arg^9]-bradykinin and L-leucyl-L-proline. No other cofactors could be demonstrated. The cumulative effects of giving cephalexin in large doses over several days could inhibit a very significant portion of the lung aminopeptidase P. In patients with renal function compromise, the blood cephalexin concentrations would be even higher, thereby enhancing the degree of inhibition.

Lung has been referred to as a paraendocrine organ, and its endothelial cells are potent metabolic sites for "clearing" a number of circulating substances, e.g., kinins and prostaglandins (17). It has been proposed that an enzyme that cleaves the Arg^1–Pro^2 bond of bradykinin is located in the alveolar–capillary membrane (3); furthermore it has been suggested that these enzymes are firmly embedded in these membranes on the basis of the failure to obtain any kinase activity in the perfusion medium following perfusion of isolated rat lung (3). Plasma itself has been reported to be lacking in aminopeptidase P activity (18), but it does occur in erythrocytes (4). The bradykinin analog, *des*-[Arg^9]-bradykinin, is a strong B receptor stimulant, and it exists in the proper

TABLE III. TIME DEPENDENCY OF THE EXTENT OF AMINOPEPTIDASE P INHIBITION BY CEPHALEXIN

Duration of preincubation (min)	Percentage inhibition[a] (%)
None	40
5	53
15	60
30	64
60	75
120	78
240	80

[a] In this experiment 0.02 units of enzyme were incubated with 2.5 m*M* cephalexin at pH 8.0 at 37°C. for the various times indicated, after which the residual aminopeptidase P activity was assayed. Control values for each data point were obtained by omitting the cephalexin from enzyme preparations treated exactly as above. Appropriate antibiotic controls were also assayed to correct, if necessary, for any color that might be derived from the antibiotics themselves, and to ensure that the antibiotics did not influence the amino acid oxidase reaction.

solution conformation for biological activity (18, 19).

This study on human lung aminopeptidase P is a part of our overall study on the contributions of various kinin catabolizing enzymes to the total rate of kinin catabolism in lung; the other enzymes include (a) postproline endopeptidase (cleaving enzyme) which we have detected in human lung and which has been shown by Koida and Walter (20) to cleave bradykinin, (b) dipeptidyl-peptidase IV (which will cleave the dipeptide, Pro–Pro, from *des*-[Arg^1]-bradykinin, the product of aminopeptidase P action) which we have recently purified from human kidney and human lung (21, 22), (c) two endopeptidases, and of course (d) carboxypeptidase N and Kinase II. Our results provide enzymologic evidence for a kinin cleaving enzyme, other than carboxypeptidase N and Kinase II, in human lung. The enzyme we have described cleaves the Arg^1–Pro^2 bond of bradykinin and is consistent with the earlier proposal by Ryan *et al.* (3) that such a cleavage does take place in lung.

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