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Effect of buffer pH, buffer concentration and skin with or without enzyme inhibitors on the stability of [Arg^8]-vasopressin

Mingda Bi, Jagdish Singh *

Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State University, Fargo, ND 58105, USA

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Abstract

The stability of [Arg^8]-vasopressin (AVP) as a function of buffer pH, buffer concentration, salt concentration, temperature, and skin with and without enzyme inhibitors was investigated. AVP was analyzed by reverse-phase high-performance liquid chromatography. The results indicated that the buffer's pH affected the degradation rate of AVP. Buffer ions ($H_2PO_4^-$ and HPO_4^{2-}) and salt concentrations had no effect on the degradation of AVP. Maximum stability was achieved at pH 3.35 among pH values tested. The activation energy for the overall reaction was 21.5 kcal mol^{-1} at pH 3.35. From the Arrhenius equation, the shelf-life of AVP at 25°C and pH 3.35 was calculated to be 1.38 years. The degradation rate of AVP in the skin (area: 9 cm^2, thickness: 0.5 mm) was 0.22 h^{-1}. Bestatin (an aminopeptidase inhibitor) had the best stabilizing effect on the degradation of AVP by skin among the three enzyme inhibitors (i.e. aprotinin, bestatin, and leupeptin) studied. The degradation rate of AVP in the skin was reduced to 0.059 h^{-1} in the presence of bestatin in comparison with no inhibitor (0.22 h^{-1}).

Keywords: Stability; Vasopressin; pH; Buffer concentration; Skin; Enzyme inhibitors

1. Introduction

Advances in recombinant DNA technology have resulted in the availability of several therapeutic peptide and protein drugs. Currently, there are many recombinant proteins in phase I clinical trials or beyond, and almost a dozen have received FDA approval (Zhou and Li Wan Po, 1991). Peptides and proteins are inherently unstable molecules (Schmid, 1979), which presents unique difficulties in their formulation, storage, and delivery. They possess multiple functional groups in addition to their three-dimensional structure, which results in degradation via both chemical and physical pathways. Therefore, the physicochemical stability of peptides and proteins in formulations and delivery systems should be considered carefully in order to improve their systemic bioavailability.

* Corresponding author. Tel.: +1-701-2317943; fax: +1-701-2317606.

E-mail address: jsingh@plains.nodak.edu (J. Singh)

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Several factors (e.g. buffer pH, buffer concentration, type of the buffer, ionic strength, and temperature) have been shown to affect the stability of proteins and peptides (Helm and Muller, 1991; Brange et al., 1992; Lee et al., 1992). The kinetics of diketopeperazine (DPK) formation using model peptides was studied. The rate of DPK formation increased with increasing buffer concentration and temperature (Chimanlall and Borchardt, 1998). The effect of buffer concentrations and ionic strengths was evaluated on the stability of luteinizing hormone releasing hormone (LHRH) (Hoitink et al., 1996). It was found that phosphate buffer ions had no effect on the degradation of LHRH. There was no relationship between the degradation rate of LHRH and ionic strength of phosphate buffer at pH 9; however, the degradation of LHRH decreased with increasing ionic strengths at pH 2.

Skin contains fewer proteolytic enzymes than other mucosa (Lee, 1988) and drugs absorbed through it can escape first-pass metabolism. However, the stability of proteins and peptides in the skin remains problematic (Martin et al., 1987; Banga et al., 1995). Recently, several reports introduced the use of proteolytic enzyme-inhibitors to stabilize proteins and peptides during their transmucosal delivery (Morimoto et al., 1995; Yamamoto et al., 1996). The results indicated that the bioavailability of these drugs was significantly improved in the presence of enzyme inhibitors.

[Arg^8]-vasopressin (AVP) is synthesized in the hypothalamus and transported to the posterior pituitary for storage. It is released in response to hyperosmolality, hypovolemia, hypotension, emotional stress, posture, temperature and many pharmacological agents (Davis et al., 1977). The primary structure of AVP is Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Arg–Gly–NH_2 [disulfide bridge (1-6)]. It can be used to treat diabetes insipidus as well as a number of other diseases (Shimizu and Hoshino, 1978; Clarke et al., 1979). In this study, we investigated the stability of AVP as a function of buffer pH, temperature, buffer concentration, salt concentration, and skin with and without enzyme inhibitors.

2. Materials and methods

2.1. Materials

Synthetic [Arg^8]-vasopressin, bestatin (hydrochloride), leupeptin (trifluoroacetic salt), and aprotinin (bovine lung, 3–7 TIU mg^{-1}) were obtained from Sigma (St Louis, MO). HPLC grade methanol and trifluoroacetic acid were obtained from Fisher (Los Angeles, CA). A Brownlee Spheri-ODS column (25 cm × 4.6 mm, 5 μm, 100 Å) was obtained from Alltech Associates (Deerfield, IL). All solutions and buffers were prepared using deionized distilled water. All other chemicals used were of analytical grade.

2.2. Degradation of AVP as a function of buffer pH, temperature, buffer concentration, and salt concentration

A stock solution containing 2.5 mg AVP in 1 ml of deionized distilled water was prepared. Test solutions were prepared by adding 6 μl of stock AVP solution to reaction vials (12 × 32 mm) containing 0.994 ml of buffer solutions. The effect of various pHs (2.6–8.5) of 0.1 M phosphate buffer containing 0.3 M NaCl was studied at 50°C. Phosphate buffer concentrations (i.e. 0.05, 0.1 and 0.15 M) and salt concentrations (0.26, 0.4 and 0.6 M) were also studied at 50°C. The stability of AVP in a phosphate buffer (0.1 M) and salt concentration (0.3 M) at pH 3.35 was studied at various temperatures (50 ± 0.1, 75 ± 0.1, 80 ± 0.1, and 90 ± 0.1°C). The reaction vials, sealed with rubber septa, were maintained at constant temperatures in ovens (DK-63, Scientific Products, Hewlett Packard, St, Paul, MN). The final pH values of the phosphate buffers at reaction temperatures were also determined. Samples were periodically removed from the ovens and the concentration of AVP was determined by the stability-indicating HPLC method (Bi and Singh, 1999). Briefly, a 220 nm UV detection, Brownlee Spheri-5 ODS column (250 × 4.6 mm, 5 μm, 100 Å), mobile phase (menthol/0.1% aqueous trifluoro acetic acid: 3:7) and 1.5 ml min^{-1} flow rate were used in the analysis of AVP by a Hewlett Packard series 1050 liquid chromatograph (Hewlett Packard, Germany).

2.3. Degradation of AVP in skin with and without inhibitors

Fresh and viable pig ear skin was obtained from the Department of Livestock, North Dakota State University. The skin was dermatomed to a 0.5 mm thickness (i.e. epidermis and part of dermis) using a dermatome (Padgett Instrument, Kansas City, MO) and was cut into 9 cm^2 pieces. The skin used was from the same ear of the pig. The skin pieces were stored in ice-cold physiological saline solution for 7 h and thereafter at −80°C. The test tubes, containing 25 μg ml^{-1} AVP in physiological saline, were maintained at 37 ± 0.5°C in a shaking water bath during the experiments. After a 20-min temperature equilibration, one piece of the skin was added into each test tube. The concentration of enzyme inhibitors used was 0.3 mg ml^{-1} (Morimoto et al., 1995). The total volume of solution in each test tube was 5 ml. At specific time intervals, 0.2-ml samples were withdrawn from the test tubes for analysis. The metabolic degradation reaction of AVP due to peptidases was halted by heating the samples at 100°C for 6 min (Advis et al., 1982; Bi and Singh, 1998). AVP solution incubated with the above preboiled (100°C for 6 min) skin was used as the control. The concentration of AVP was determined by the HPLC (Bi and Singh, 1999).

2.4. Data analysis

The degradation rate constant (k) was obtained from the slope of the semilogarithmic plot of the residual amount of AVP versus time profile by statistical regression analysis. An Arrhenius plot was generated from the observed k values at different temperatures and pH 3.35 based on the equation: $\log k = \log A - E_a/2.303RT$ (k, rate constant; A, frequency factor; E_a, energy of activation; R, gas constant, and T, absolute temperature). From the Arrhenius plot, we calculated the E_a at pH 3.35. The shelf-life of AVP at 25°C was then estimated based on $t_{90} = 0.105/k$ (k, degradation rate constant of AVP at 25°C).

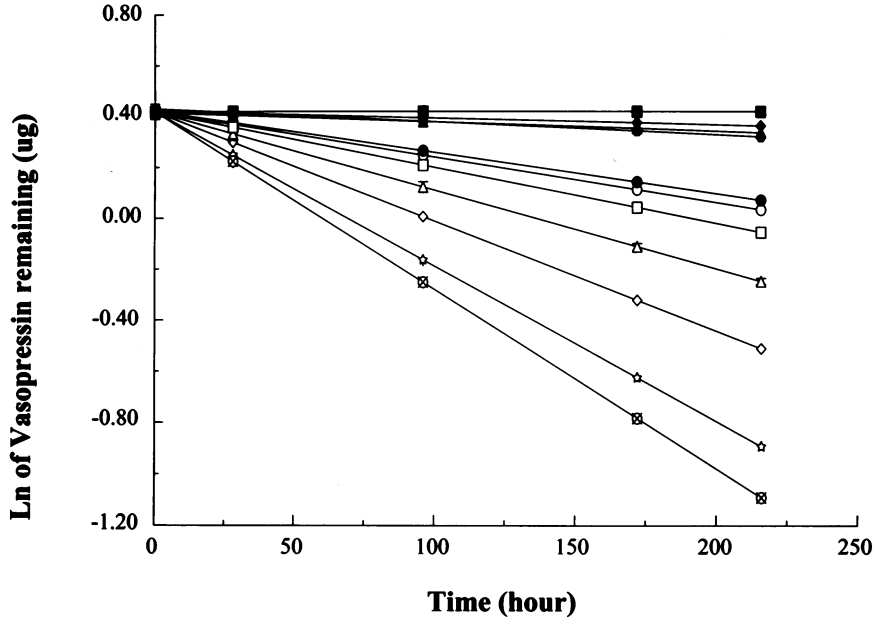

Fig. 1. First-order plot of the degradation of AVP stored in phosphate buffer at different pH values and 50°C. Key: closed square, pH 3.35; closed diamond, pH 3.66; closed triangle, pH 4.07; closed hexagon, pH 4.09; closed circle, pH 2.6; open circle, pH 5.1; open square, pH 5.3; open triangle, pH 5.44; open diamond, pH 6.06; open star, pH 6.9; cross in circle, pH 8.5.

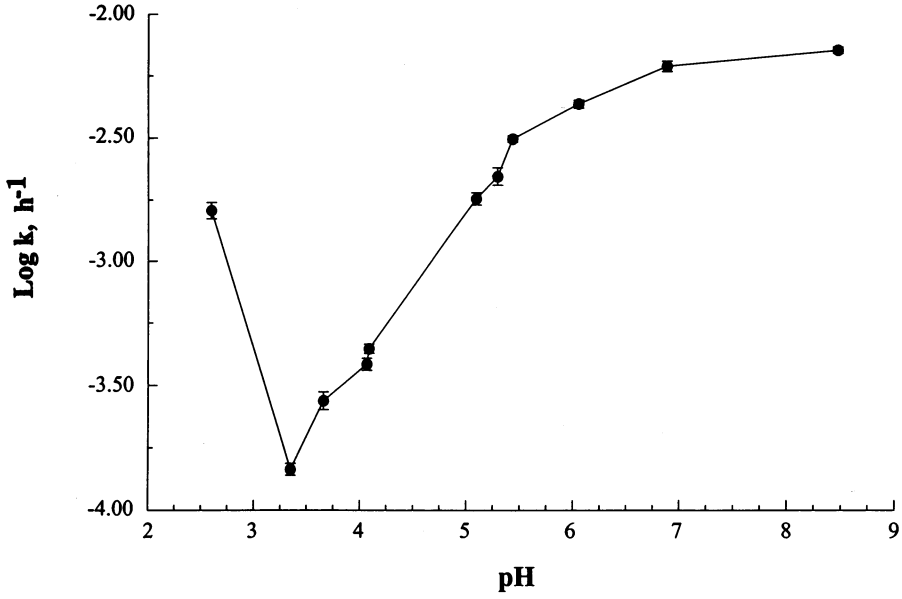

Fig. 2. pH-rate profile for the degradation of AVP at 50°C.

3. Results and discussion

Fig. 1 shows a semilogarithmic plot of the residual amounts of AVP versus time in phosphate buffer at 50°C. It was found that pH affected the degradation rate of AVP following apparent first-order kinetics. Fig. 2 indicates that AVP is most stable at pH 3.35 among pH values tested and its degradation rate is highly pH-dependent. Table 1 illustrates the effect of buffer and salt concentrations on the degradation of AVP. The results showed that the AVP degradation rate did not depend on the buffer and salt concentrations at pH 7.5. Buffer concentrations did not influence the degradation of AVP and, therefore, buffer ions ($H_2PO_4^-$ and HPO_4^{2-}) had no catalytic effect on AVP.

Fig. 3 shows the effect of temperature on the degradation of AVP. The result indicates that AVP degradation shows a marked dependence on temperature. The speed of many reactions increases with an increase in temperature. This can simply be explained by the Arrhenius equation. Classic collision theory and transition state theory also indicates that reaction velocity is highly temperature-dependent.

The Arrhenius plot of log k against $1/T$ for AVP at pH 3.35 was linear (Fig. 3) with a correlation coefficient greater than 0.99 which indicated that the reaction mechanism did not change within the range of temperatures used. The HPLC chromatograms (not included) showed the same degradation pattern at 50, 75, 80 and 90°C, which further confirmed that the same reaction mechanism took place. Therefore, it should be reliable to predict the shelf-life at pH 3.35 and room temperature based on the calculated activation energy. The slope of the

Table 1
Effect of phosphate buffers and salt concentrations on the observed degradation rate of AVP at pH 7.5 and 50°C

Phosphate buffer concentration (M)	Salt (NaCl) concentration (M)	$k_{obs} \times 10^3$ (h^{-1})[a]
0.05	0.40	6.38 ± 0.03
0.10	0.40	6.41 ± 0.01
0.15	0.40	6.40 ± 0.02
0.10	0.26	6.42 ± 0.01
0.10	0.60	6.39 ± 0.02

[a] Values are mean $\pm$ S.E., $n = 3$.

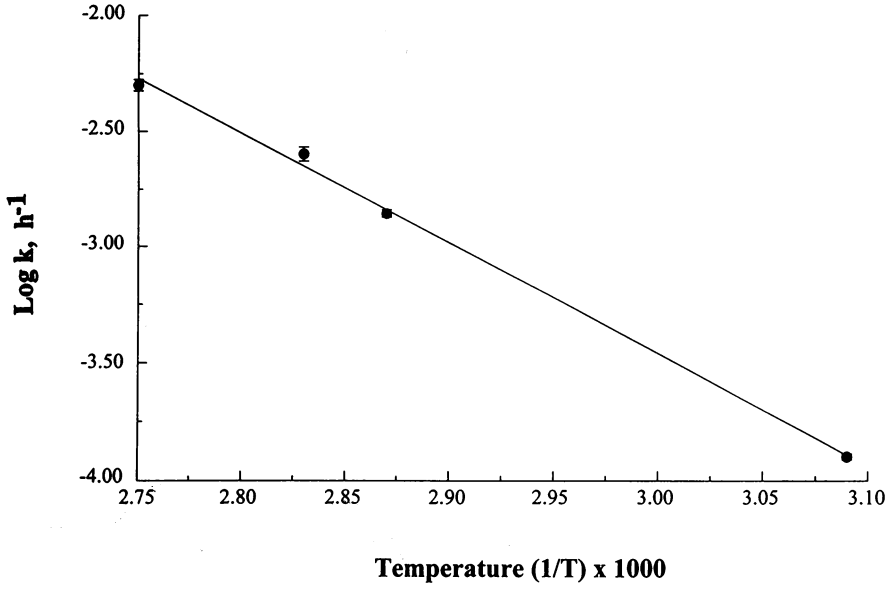

Fig. 3. Arrhenius plot of log rate constant versus $1/T$ for the degradation of AVP at pH 3.35 in 0.1 M phosphate buffer; k is the degradation rate constant and T is the absolute temperature.

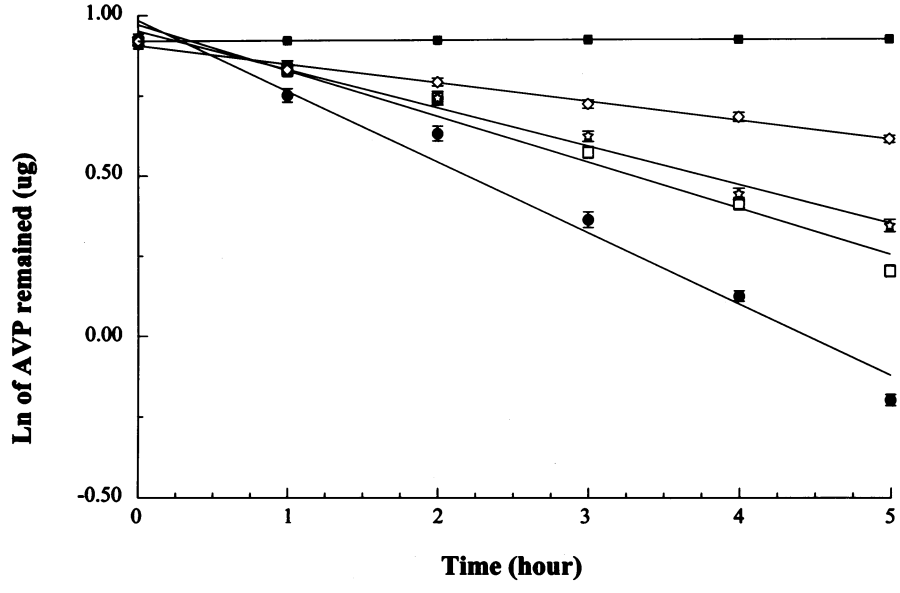

Fig. 4. First-order plot for the degradation of AVP by the skin with or without enzyme inhibitors. Key: closed square, control; closed circle, AVP incubated with skin; open square, AVP incubated with skin and aprotinin; open star, AVP incubated with skin and leupeptin; open diamond, AVP incubated with skin and bestatin.

Arrhenius plot obtained was equal to $-E_a/2.303R$ from which the activation energy was calculated. The activation energy was found to be 21.5 kcal mol^{-1}. Based on the Arrhenius equation, shelf-life of AVP at 25°C and pH 3.35 was found to be 1.38 years.

Fig. 4 depicts the degradation of AVP in the pigs' skin with and without enzyme inhibitors. A first-order kinetic model could approximate the degradation of AVP. The correlation coefficients were 0.99, 0.99, 0.98, and 0.98 for bestatin, leupeptin, aprotinin, and skin without enzyme inhibitor, respectively. The degradation rate without enzyme inhibitors calculated from the concentration–time profile was 0.22 h^{-1} (Fig. 4). AVP degraded 65% in 5 h. However, there was no degradation in the control. Bestatin had the best stabilizing effect on the AVP degradation by the skin among the three enzyme inhibitors. Degradation rates were 0.059, 0.144, and 0.121 h^{-1} for bestatin, aprotinin and leupeptin, respectively. Fig. 5 shows the HPLC chromatograms of AVP incubated in skin at 37°C for 5 h. AVP was readily metabolized in the skin as indicated by the additional peaks of metabolites in the chromatograms. The absence of additional peaks in the chromatogram of the control indicates that these peaks are not due to skin and physiological saline. Fig. 5 also shows that bestatin has the best stabilizing effect on the degradation of AVP by the skin.

There are numerous enzymes in the skin (e.g. chymotrypsin-like enzyme, aminopeptidase, leucine aminopeptidase, trypsin-like enzyme, cathepsin B, cathepsin D and others), which are responsible for the AVP degradation (Zaets et al., 1977; Wintroub et al., 1984; Morimoto et al., 1992). The chemical structure reveals that the AVP molecule with its unblocked *N*-terminus (Cys) and Arg followed by glycinamide is susceptible to cleavage by aminopeptidase and trypsin. Therefore, aminopeptidase and trypsin inhibitors should have stabilizing effects on the degradation of AVP by the skin. Our results showed that

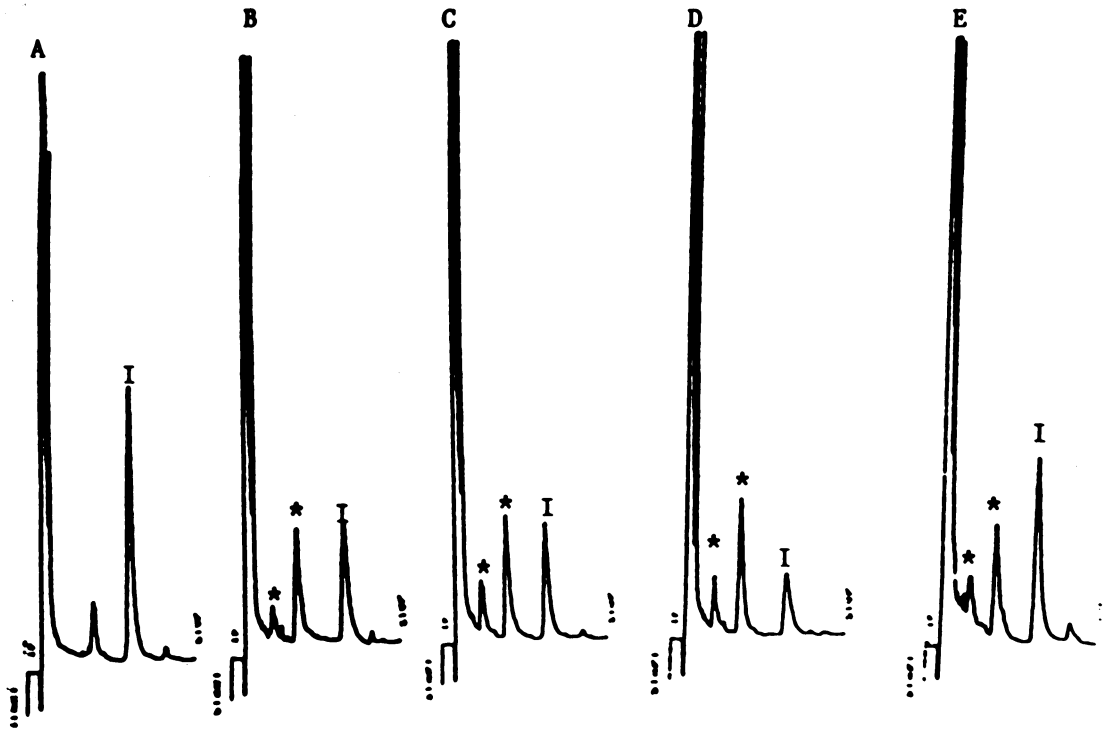

Fig. 5. HPLC chromatograms of AVP incubated with skin and with or without enzyme inhibitors at 37°C for 5 h. Key: A, control chromatogram of skin incubated in physiological saline; B, chromatogram of AVP incubated with skin and leupeptin; C, chromatogram of AVP incubated with skin and aprotinin; D, chromatogram of AVP incubated with skin; E, chromatogram of AVP incubated with skin and bestatin; I, intact AVP peaks; closed star, AVP degradation peaks.

bestatin (aminopeptidase inhibitor), aprotinin (trypsin inhibitor) and leupeptin (trypsin and cathepsin B inhibitor) had an inhibiting effect on the degradation of AVP by the skin, which is consistent with the theory. However, bestatin had a better stabilizing effect than the other two inhibitors used in this study. This showed that aminopeptidase, which cleaves AVP, has greater activity in the skin.

Morimoto et al. (1992) investigated the peptidase activities in the skin and concluded that the enzyme activities were in the following order: aminopeptidase > cathepsin-B > trypsin. Our results were consistent with their conclusions.

In conclusion, AVP was chemically stable and its shelf-life in 0.1 M phosphate buffer at pH 3.35 and 25°C was found to be 1.38 years. Skin degraded 65% of AVP in 5 h. An aminopeptidase inhibitor (bestatin) had the best stabilizing effect on the degradation of AVP by the skin, which could reduce AVP degradation from 65 to 25% in 5 h. Based upon the above findings, it is reasonable to speculate that a stable AVP formulation at pH 3.35 and in the presence of bestatin can be developed and optimized for delivering the peptide in the biologically active form into circulation by transdermal route.

Acknowledgements

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