Journal of Chromatography, 168 (1979) 385–393

CHROM. 11,357

SEPARATION OF UNCONJUGATED PTERIDINES BY HIGH-PRESSURE CATION-EXCHANGE LIQUID CHROMATOGRAPHY

BALDASSARRE STEA, RICHARD M. HALPERN and ROBERTS A. SMITH*
Department of Chemistry and the Molecular Biology Institute, University of California, Los Angeles, Calif. 90024 (U.S.A.)
(Received August 2nd, 1978)

SUMMARY

In the course of determining the levels of unconjugated pteridines occurring in various biological fluids, such as urines, plasma and tissue culture media, a method has been developed for the separation and quantitative determination in the picomole range of ten 2-amino-4-hydroxy substituted pteridines. This method involves separation by high-pressure cation-exchange liquid chromatography and fluorescence detection of the eluted compounds at 450 nm. Optimal separation was obtained by isocratic elution with 3 m*M* phosphoric acid–7% methanol–1% acetonitrile at a flow-rate of 2 ml/min or with 1 m*M* ammonium dihydrogenphosphate pH 2.8–7% methanol–5% acetonitrile at a flow-rate of 1.5 ml/min. With either solvent, the order of elution of the compounds is: isoxanthopterin, pterin-6-carboxylic acid, xanthopterin, pterin-6-carboxaldehyde, D-*erythro*-neopterin, L-*threo*-neopterin, biopterin, 6-hydroxymethylpterin, pterin, 6-methylpterin. In addition, a systematic investigation of the effects of ammonium ion concentration and pH of the solvent as well as column temperature on the separation of these compounds was also conducted.

INTRODUCTION

Separation of pteridines has been accomplished by a variety of chromatographic techniques: paper chromatography and monodimensional thin-layer chromatography (TLC)[1,2] have widely been used, but two-dimensional TLC[3], ion-exchange chromatography[4], gas chromatography–mass spectrometry[5], and finally, high-pressure liquid chromatography (HPLC)[6] have also found application in this field. The quantitative determination of pteridines in biological materials, however, has usually been accomplished by laborious and time-consuming techniques involving a combination of ion-exchange chromatography and paper or TLC[3,4,7–9]. Only recently, rapid methods for such determinations have been developed using gas chromatography–mass spectrometry[10] and a combination of HPLC and gas chromatography[11]. These methods, however, were not chosen for determination of all the pteridines known to occur in biological materials, but only applied to the quantitation of two of them, biopterin and neopterin.

* To whom correspondence should be addressed.

During our attempts to determine the levels of unconjugated pterins (2-amino-4-hydroxy substituted pteridines) occurring in urines of normal individuals as well as in urines of cancer patients[12], it became necessary to adopt a chromatographic system that would separate and quantitate all the pterins that were likely to be found in biological fluids. HPLC became the method of choice when speed, reproducibility and cost were considered.

In the present communication, we report the results of a systematic investigation on the separation of most of the biologically occurring pterins by cation-exchange HPLC. The aims of our study are two-fold: (a) The method should separate closely related pterins and have a wide applicability, *i.e.*, it should be useful for the separation of pterins from a wide variety of biological sources. (b) By making use of their natural fluorescence, the method should lend itself to the quantitation of pterins in the picomole range.

MATERIALS AND METHODS

Isoxanthopterin, xanthopterin, pterin and pterin-6-carboxylic acid (Pt-6-COOH) were purchased from Sigma (St. Louis, Mo., U.S.A.); biopterin was obtained from Calbiochem (San Diego, Calif., U.S.A.); pterin-6-carboxaldehyde (Pt-6-CHO) and 6-hydroxymethylpterin (Pt-6-CH_2OH) were prepared by the method of Thijssen[13] with minor modifications as described previously[14]. Neopterin was prepared by the method of Rembold and Eder[15] and 6-methylpterin (Pt-6-CH_3) by reductive cleavage of folic acid[16]. Organic solvents used to prepare buffers for the chromatographic separations were of the "liquid chromatography" grade. HPLC was performed with an Altex Model 100 pump equipped with an Altex model 905-42 sample injector (Altex Scientific, Berkeley, Calif., U.S.A.). A 25 cm × 4.6 mm Partisil-10 SCX column (Whatman, Clifton, N.J., U.S.A.) equipped with a 5 cm × 4.6 mm precolumn filled with the same kind of packing as the analytical column was used for all the separations described. Detection of the eluant was performed with a Farrand A_4 fluorimeter (Farrand Optical, Valhalla, N.Y., U.S.A.) equipped with a 7-60 excitation filter (band center at 360 nm) and a narrow band interference filter with peak transmittance at 450 nm. The aperture of the fluorimeter was set at 5 and the range at 1. In some experiments, the column temperature was varied by means of a water bath. The compounds were injected in various volumes; varying the injection volume between 5 and 200 μl did not significantly affect peak shapes nor retention times. The solvents used throughout were filtered with a 0.4-μm millipore filter and degassed under vacuum just prior to use. When solutions of ammonium dihydrogenphosphate and organic solvents were prepared, we first adjusted the pH to the required end point, and then we mixed the salt solution with the required volume of organic solvent.

RESULTS

In our continuing study of folates and pterins metabolism, we became interested in the separation and quantitation of pterins present in biological samples such as body fluids, enzymatic reactions, tissue culture media, etc. In previous investigations[12,14], we have made use of thin-layer and paper chromatography, but these techniques do not have enough resolving power and are not easily amenable to quantitation.

Thus, we became interested in ion-exchange HPLC. Although HPLC systems have been developed for the separation of many diverse pteridine derivatives[6], these systems could not separate the pterins in which we are interested, such as the pterins known to occur in urines.

Our approach to the separation of such pterins is based on the consideration that the pK for many of these compounds ranges between the limits of pH 1–3 (ref. 17); thus, we tested various concentrations of phosphoric acid for their ability to separate the compounds of interest on a strong cation-exchange column. We found that solutions of 1–10 mM phosphoric acid were able to separate most of the pterins studied except for xanthopterin and Pt-6-CHO which cochromatographed under those conditions. However, the simple addition of 5–10% methanol to the phosphoric acid buffer made this separation possible. Fig. 1 shows the separation of nine pterins on a Partisil-10 SCX column when eluted isocratically with 3 mM phosphoric acid–7% methanol–1% acetonitrile. The addition of 1% acetonitrile was rendered necessary by the very long elution time of Pt-6-CH_3 when the 3 mM phosphoric acid–7% methanol system was used. Addition of a small amount of acetonitrile probably helps to overcome hydrophobic interactions of the methyl group on the pteridine with the column backbone. This explanation is supported by the observation that while 6-methylpterin can be eluted with a solvent containing only 1% acetonitrile, 6,7-dimethylpterin requires a greater proportion of acetonitrile in the solvent in order to be eluted from the same column (results not shown).

Although the 3 mM phosphoric acid–7% methanol–1% acetonitrile system gives reproducible retention times, it is very sensitive to the presence of cations in the

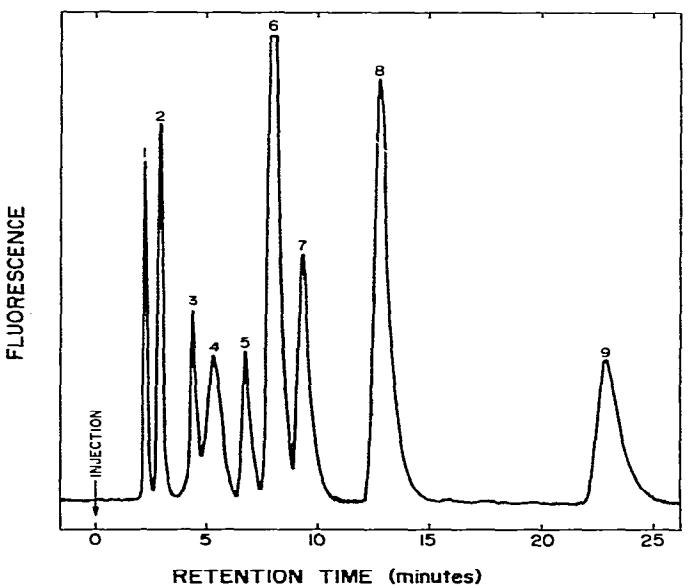

Fig. 1. Separation of pterins on a Partisil-10 SCX column. Solvent, 3 mM phosphoric acid–7% methanol–1% acetonitrile; flow-rate, 2 ml/min; column temperature, 20°. Peaks: 1 = isoxanthopterin; 2 = Pt-6-COOH; 3 = xanthopterin; 4 = Pt-6-CHO; 5, D-*erythro*-neopterin; 6 = biopterin; 7 = Pt-6-CH_2OH; 8 = pterin; 9 = Pt-6-CH_3.

solutions injected. Thus, if, for instance, a solution of pterins in ammonium hydroxide is injected, retention times will be somewhat changed depending on the injection volume and concentration of the cation. For this reason, we studied the chromatographic behavior of some pterins when eluted from a cation-exchange column with ammonium dihydrogen phosphate solutions rather than phosphoric acid. Fig. 2 shows how the mobility of some pterins changes as a function of the NH_4^+ concentration in the eluting buffer, while the pH was kept constant to a value of 2.6 with phosphoric acid. Clearly, the higher the NH_4^+ concentration, the poorer the separation obtained. A good compromise between buffering capacity and separation was chosen at a value of 1 m*M* NH_4^+. We also studied the mobility of pterins as a function of the pH of the eluting buffer (1 m*M* ammonium dihydrogenphosphate). The pH was adjusted to the indicated values (Fig. 3) by additions of phosphoric acid. A pH optimum for separation was obtained at a value between 2.6 and 2.8, thus indicating that separation was probably being achieved on the the basis of the basic p*K* of these compounds. Separation decreases on either side of the pH optimum, probably because the positive charge on the pterin is lost at higher pH values and because at lower pH values the exchanger groups start to get protonated. It is interesting to note that

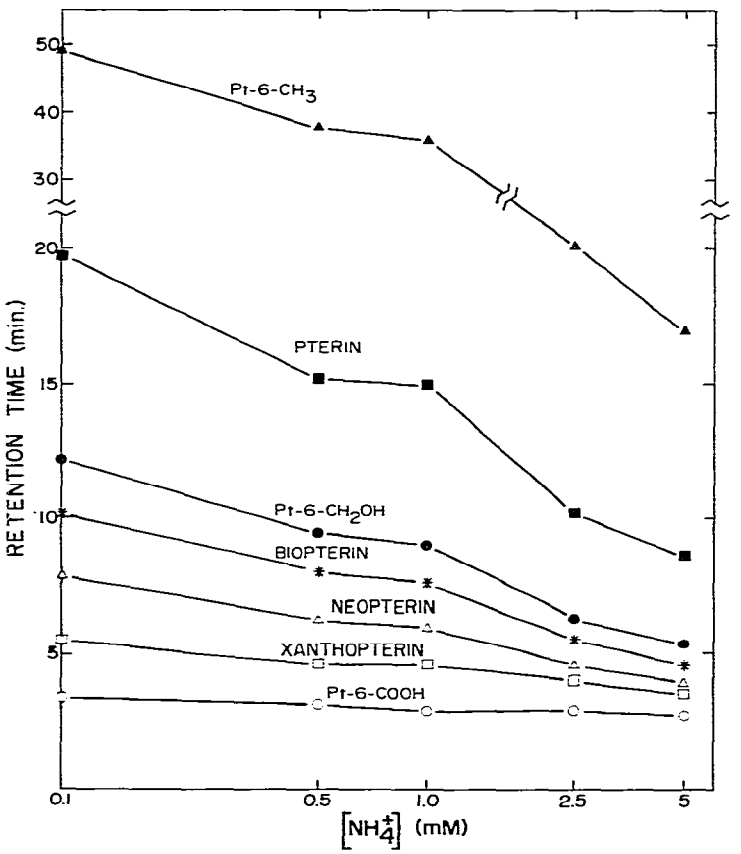

Fig. 2. Effect of NH_4^+ concentration of the solvent on retention times of pterins on Partisil-10 SCX. Solvent, different concentrations of ammonium dihydrogenphosphate adjusted to pH 2.6 with phosphoric acid; flow-rate, 2 ml/min; column temperature, 23°.

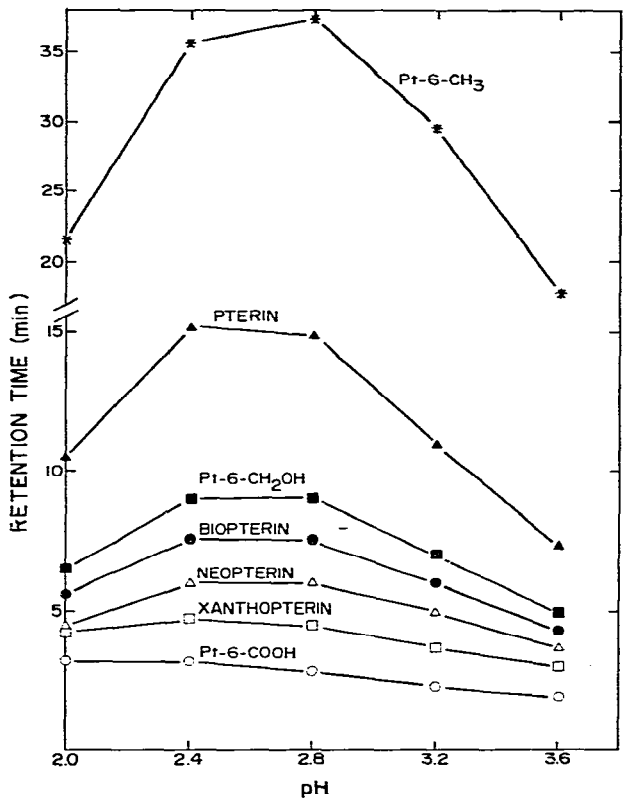

Fig. 3. Effect of pH of the solvent on retention times of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogenphosphate adjusted to the indicated pH with phosphoric acid; flow-rate, 2 ml/min; column temperature, 23°.

between the pH values tested (2.0–3.6), the pterins studied retain the same relative position with respect to each other.

Mobility and therefore resolution is also affected by the column temperature. Fig. 4 shows the change in retention times as a function of the column temperature for the same compounds tested in the studies of Fig. 2 and 3. The pterin most affected by the temperature change is Pt-6-CH_3, which shows a dramatic decrease in retention time as the column temperature is increased. Peak shapes are also affected by the temperature; that is, in general, the peaks get sharper with increasing temperature. This phenomenon can be attributed to the earlier elution, as it is known that the number of theoretical plates (a measure of column efficiency) is directly proportional to the square of the retention time. When the parameter N (number of theoretical plates) calculated for Pt-6-CH_3 is plotted against the elution temperature, an optimum temperature of elution for Pt-6-CH_3 is reached around 45° (results not shown). However, for maximum resolution, zone separation must be considered in addition to zone width. An interesting phenomenon occurs only with the parent compound, pterin, at a temperature of 30°. At this specific temperature pterin chromatographs as a double peak, that is, as a peak with a shoulder, but at any other temperature it gives only a single peak (Fig. 5). One possible explanation for this phenomenon is offered by the

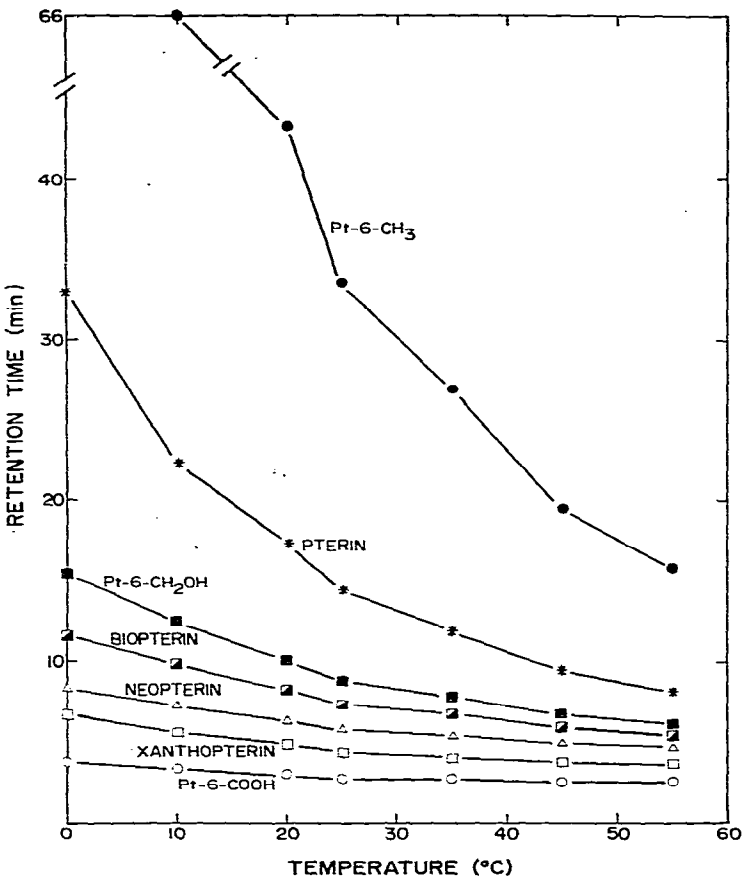

Fig. 4. Effect of column temperature on retention times of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogen phosphate, pH 2.6; flow-rate, 2 ml/min.

existence of an hydrated form of the compound in rapid equilibrium with the non-hydrated form[17]. It is possible that during chromatography at 30° the conditions are not favorable for the equilibration of the two forms, thus resulting in the appearance of a new entity.

Thus, column temperature, pH and counter-ion concentration of the solvent as well as flow rate, can all be optimized to obtain the best resolution. However, separation between Pt-6-CHO and xanthopterin can only be achieved by addition of 7% methanol to the salt solution, as in the case of the phosphoric acid solution. Fig. 6 shows the separation of ten closely related pterins obtained with a 1 m*M* ammonium dihydrogenphosphate, pH 2.8–7% methanol–5% acetonitrile system at 25°. In this case, the small amount of acetonitrile is needed to decrease the "tailing" effect observed with the ammonium dihydrogenphosphate-containing buffers, as well as to decrease the elution time of Pt-6-CH_3. The resolving power of this technique is well evidenced by the ability to separate two optical isomers of neopterin: the D-*erythro*- from the L-*threo*-neopterin, both of which have been reported to occur in human

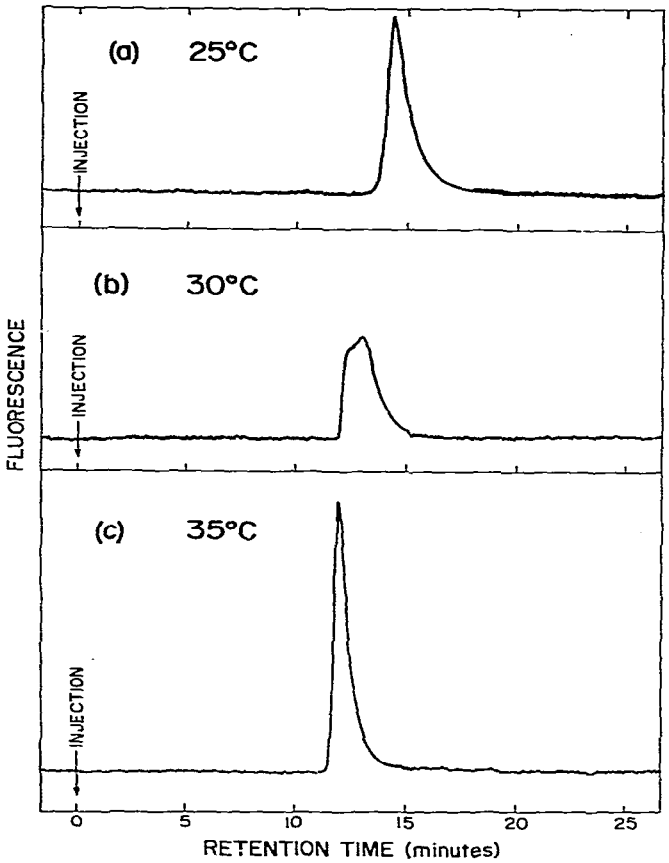

Fig. 5. Effect of column temperature on the shape of the pterin peak. Solvent, 1 m*M* ammonium dihydrogenphosphate, pH 2.6; flow-rate = 2 ml/min; column temperature, (a) 25°; (b) 30°; (c) 35°.

urine[9]. The method is also extremely sensitive: the peaks reproduced in Fig. 6 represent 20 pmoles of isoxanthopterin and Pt-6-COOH, and 100 pmoles of all the other compounds. The sensitivity of the fluorimeter can still be increased by a factor of four with a good signal-to-noise ratio, thus it is possible to detect sub-picomole amounts of isoxanthopterin and Pt-6-COOH and at least 5–10 pmoles of other pterins.

DISCUSSION

The method that we have developed allows a very rapid and sensitive determination of many biologically occurring pterins in a single chromatographic run, which can be accomplished in a time-span of about 40 min. We included Pt-6-CHO and Pt-6-CH_2OH among the pterins that we wanted to separate, because the former has been reported to occur in urines from cancer patients[12] when they were chromatographed by TLC, and the latter in tissue culture media of malignant cells[14]. Pt-6-CH_3, though it has not been found in biological materials, was included because of its potential use as

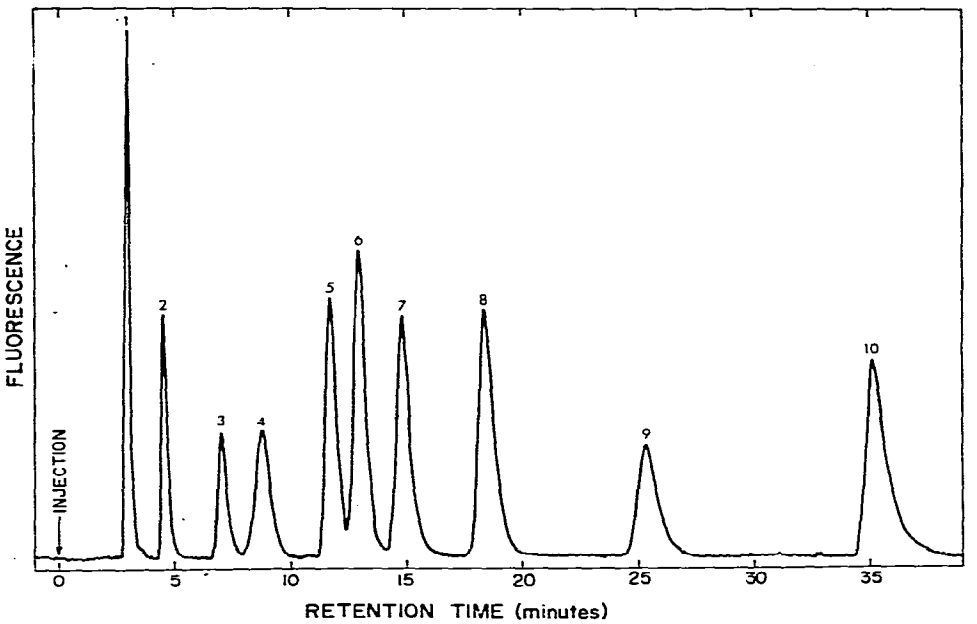

Fig. 6. Separation of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogenphosphate, pH 2.8–7% methanol–5% acetonitrile; flow-rate, 1.5 ml/min; column temperature, 25°. Peaks: 1 = isoxanthopterin; 2 = Pt-6-COOH; 3 = xanthopterin; 4 = Pt-6-CHO; 5 = D-*erythro*-neopterin; 6 = L-*threo*-neopterin; 7 = biopterin; 8 = Pt-6-CH_2OH; 9 = pterin; 10 = Pt-6-CH_3.

internal standard, as in many cases a preliminary purification of the biologically occurring pterins is necessary before separation and quantitation by HPLC can be accomplished[10,11]. We have successfully applied this technique in (i) the quantitation of the metabolic products of Pt-6-CHO by normal and malignant cells grown in culture[15]; (ii) the measurement of the levels of enzyme(s) involved in the metabolism of Pt-6-CHO[18]; (iii) monitoring purification of the 6-isomer from the 7-isomer produced during synthesis of some of the pterins utilized in this study (results not shown); and finally, (iv) the quantitative determination of pterins in human urine[19]. Because of its versatility, reproducibility and sensitivity, this procedure lends itself to a wide range of applications.

ACKNOWLEDGEMENTS

This work was supported by Grant No. Ca 17332 from the United States Public Health Service, by the Cellular and Molecular Biology Training Grant No. GM 07185-04, and the Julius and Dorothy Fried Research Foundation.

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