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COLUMN CHROMATOGRAPHY OF 5-FLUOROURACIL ON SEPHADEX G-10

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SUMMARY

The influence of pH, ionic strength (I) and flow-rate of eluent on the chromatographic parameters of 5-fluorouracil (5-FU) on Sephadex G-10 columns was studied. Using an optimized phosphate buffer (pH 7.0, I 0.12), 5-FU showed reversible adsorption on the gel matrix and can be chromatographed in the submicrogram range without losses.

INTRODUCTION

5-Fluorouracil (5-FU) is a chemotherapeutic agent used in many types of cancer treatment. With a pyrimidinedione structure, this compound has a high solubility in water (hydrogen bridge formation) and cannot be isolated satisfactorily from aqueous biological materials by the usual two-phase extraction with a water-immiscible organic solvent.

Windheuser *et al.*[1] isolated 5-FU from blood samples with the aid of dialysis. Although a good recovery was reported, the method is time consuming and affords relatively impure extracts.

The present study was carried out with the aim of developing the chromatographic isolation of 5-FU on Sephadex G-10 columns. The optimal column chromatographic parameters, *i.e.* composition, pH, ionic strength and flow characteristics of the eluent, as well as some column dimensions for the later purpose of quantitative isolation and purification of 5-FU from naturally occurring compounds present in the blood of treated cancer patients, were determined.

MATERIALS AND METHODS

Solutions for pH and ionic strength experiments

Sample stock solutions. Three stock solutions, A, B and C, were prepared: A, 20 mg of 5-FU were dissolved in 20 ml of water; B, 100 mg of Blue Dextran 2000

(mol. wt. 2×10^6; Pharmacia, Uppsala, Sweden) were dissolved in 10 ml of water; C, 20 ml of acetone were diluted to 100 ml with water.

Two further stock solutions, A′ and B′, were prepared; A′, 40 mg of 5-FU were dissolved in 10 ml of water; B′, 200 mg of Blue Dextran 2000 were dissolved in 10 ml of water.

Sample working solutions. Three working solutions, a, b and c, were prepared by combining aliquots of stock solutions A, B and C and diluting with each buffer solution: a, 0.2 ml of A, 0.2 ml of B and 4.6 ml of buffer solution; b, 0.2 ml of B, 0.2 ml of C and 4.6 ml of buffer solution; c, 0.2 ml of A, 0.2 ml of B, 0.2 ml of C and 4.4 ml of buffer solution.

Three further working solutions, a′, b′ and c′, were prepared by combining aliquots of stock solutions A′, B′, C and diluting with buffer solution: a′, 0.4 ml of A′, 0.4 ml of C and 4.2 ml of buffer solution; b′, 0.4 ml of B′, 0.4 ml of C and 4.2 ml of buffer solution; c′, 0.4 ml of A′, 0.4 ml of B′, 0.4 ml of C and 3.8 ml of buffer solution.

Buffer solutions. Six buffer solutions covering a pH range from 6.00 to 10.00 were prepared according to the literature[2]:

pH 6.00: 5.6 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4
pH 7.00: 29.1 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4
pH 8.00: 46.7 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4
pH 8.20: 18.8 ml of 0.1 *M* HCl + 50.0 ml of 0.025 *M* $Na_2B_4O_7 \cdot 10H_2O$
pH 9.00: 4.6 ml of 0.1 *M* HCl + 50.0 ml of 0.025 *M* $Na_2B_4O_7 \cdot 10H_2O$
pH 10.00: 18.3 ml of 0.1 *M* NaOH + 50.0 ml of 0.025 *M* $Na_2B_4O_7 \cdot 10H_2O$

From each buffer solution, volumes of 30.0 ml and 65.0 ml were further diluted with water to 100.0 ml. Overall, three series of buffer solutions of given pH but different ionic strength, *I*, were used, as indicated below:

pH	*I*	*pH*	*I*
6.05	0.03	8.33	0.01
6.02	0.07	8.29	0.02
6.02	0.10	8.25	0.04
7.10	0.04	8.69	0.01
7.02	0.08	8.67	0.02
7.10	0.12	8.97	0.04
7.78	0.04	9.70	0.02
7.82	0.09	9.78	0.04
7.90	0.14	9.92	0.06

For preventing microbial growth, an amount of trichlorobutanol was added to all solutions so as to give a final concentration of 0.1 g per 100 ml.

Solutions for flow experiments

Sample stock solutions. A stock solution, A″, was prepared by dissolving 20 mg of 5-FU in 10 ml of water; stock solutions B and C were also used in this experiment.

Sample working solution. One working solution, c″, was prepared by combin-

ing aliquots of stock solutions A'', B and C and diluting with buffer solution of pH 7.0 and *I* 0.12: 0.8 ml of A'', 0.8 ml of B, 0.8 ml of C and 7.6 ml of buffer solution (29.1 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4 + 0.2% trichlorobutanol).

Solutions for recovery experiments as determined by UV detection

Sample stock solution. A solution containing 40 mg of 5-FU in 10 ml of water was prepared (same as A').

Sample working solutions. Five working solutions were prepared by pipetting 0.20, 0.30, 0.40, 0.50 and 0.60 ml of stock solution A' into flasks and diluting to 5.00 ml with buffer solution of pH 7.0 and *I* 0.12.

UV reference solutions. A volume of 0.25 ml of each sample working solution was diluted to 10.00 ml with buffer solution of pH 7.0 and *I* 0.12.

Solutions for recovery experiments as determined by radioactivity measurements

Sample stock solution. A solution containing 20 mg of 5-FU in 100 ml of buffer solution of pH 7.0 and *I* 0.12 was prepared and 1.0 ml of this solution was mixed with 98.3 μl of [6-^{3}H]-5-FU (specific activity 876 Ci/mole; Radiochemical Centre, Amersham, Great Britain) and diluted with citrate plasma of human origin to a final volume of 10.0 ml. This corresponds to 2 μg of 5-FU and 26 ng of [6-^{3}H]-5-FU per millilitre.

Sample working solutions. Five working solutions were prepared by pipetting 0.40, 0.80, 1.20, 1.60 and 2.00 ml of stock solution into flasks and diluting to 2.00 ml with the same citrate plasma as used previously.

Column preparation

Three columns were used: K 9/15, K 9/30 and K 9/60 (each with an I.D. of 9 mm and lengths of 15, 30 and 60 cm, respectively), as commercially available from Pharmacia (Uppsala, Sweden).

All columns were packed with Sephadex G-10. An appropriate amount was swollen in an excess of boiling water, after 45 min the finest particles were decanted off, more water was added, with boiling for another 15 min, and finally the mixture was cooled. The suspension was poured into a column, allowed to settle for 30 min, and eluted first with supernatant fluid and then with about four times the column volume of buffer solution. When starting a new experiment, a used column was also pre-eluted with about four times the column volume of the appropriate buffer solution.

Apparatus

pH measurements were performed with the aid of a titrator (Type TTT 1C, Radiometer, Copenhagen, Denmark) equipped with a glass electrode (Radiometer, Type G 202 B) and a standard calomel electrode (Radiometer, Type K 401).

A Perpex peristaltic pump (10200A-2, LKB, Stockholm, Sweden) was used to produce constant but adjustable (4.5–180 ml/h) eluent flow-rates.

An Ultra-Rac fraction collector (LKB, 7000A) working on a drop number or a time basis was used in the recovery experiments.

A Uvicord I (LKB, 4700A-2) or a Uvicord III (LKB, 2089) was used as detector for the compounds eluted. The former was connected to a one-channel re-

corder (LKB, 6520A-2) used at a chart paper speed of 60 mm/h to measure continuously the percentage transmittance (T) at 254 nm. The latter was equipped with a six-channel chopper bar recorder (LKB, 6520-7/8) also used at a chart paper speed of 60 mm/h and enabled the elution pattern to be followed automatically by measuring the percentage transmittance (T) or absorbance ($A = -\log T$) at 254 and 280 nm *versus* a buffer blank (double-beam measurement at two wavelengths).

All tubing in the chromatographic system was made of PTFE and had an I.D. of 1.2 mm.

UV spectra were scanned automatically using a double-beam UV–visible range spectrophotometer (ACTA V, Beckman, Fullerton, Calif., U.S.A.). Radioactivities were measured in a Packard (Downers Grove, Ill., U.S.A.) Tri-Carb Model 3380 liquid scintillation spectrometer.

Chromatography

For each chromatographic run, a 0.25 or 0.50-ml (for radioactivity experiments) sample working solution was applied on top of the column with a disposable pipette. After the sample had penetrated into the gel matrix, elution was started with buffer solution at suitable flow-rate. The compounds eluted were detected continuously by means of the above UV detectors.

Recovery experiments

In the recovery experiments from pure solutions, the eluent was collected in 0.5-ml fractions. All fractions containing 5-FU were combined and diluted with buffer solution to a final volume of 10.0 ml, and an aliquot of the latter was used for running a UV spectrum in the wavelength range 190–400 nm to be compared with the UV spectrum of a 5-FU reference solution (0.25 ml of sample working solution per 10.0 ml of buffer solution and no chromatography).

For experiments on the recovery of 5-FU from plasma samples, the eluent was collected in 1.0-ml fractions that were transferred to polyethylene counting vials and mixed with 10.0 ml of Corusolve scintillation cocktail (ICN-Tracerlab, Cleveland, Ohio, U.S.A.). Controls (no chromatography) consisting of 0.5-ml sample working solutions were mixed with 0.5 ml of buffer solution of pH 7.0 and I 0.12 and 10.0 ml of Corusolve scintillation cocktail. All samples were counted three times for a period of 5 min and the results with the least quenching were chosen for calculation. Sufficient counts were added in order to obtain a probable counting error with a standard deviation of less than 1.5%. Quenching was corrected using the external standard ratio method (AE.S. ratio, Packard) with a compound source (^{241}Am–^{226}Ra).

Calculations

The gel chromatographic elution parameters K_d and K_{av} were calculated by following equations as described in the literature[3–5]:

$$K_d = \frac{V_e - V_0}{V_t}$$

where

K_d = distribution coefficient of the component (5-FU) between the mobile phase and the internal volume of the stationary phase actually available;

V_e = elution volume of the component (5-FU) (ml);

V_0 = void volume or volume outside the gel beads as determined by the elution volume of an excluded substance (Blue Dextran 2000) (ml);
V_i = inner volume or volume of liquid within the gel accessible to small molecules as determined by the elution volume of a model compound (acetone) minus the void volume (ml).

and

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where

K_{av} = distribution coefficient of the component (5-FU) between the mobile phase and the total gel phase;
V_t = total volume of the packed gel bed (ml).

Column efficiencies were calculated as the number of theoretical plates (N) or height equivalent to a theoretical plate (HETP, mm)[5] using the equations used in gas–liquid chromatography:

$$N = 5.53 \left(\frac{V_e}{w_{0.5h}}\right)^2$$

where

V_e = elution volume of the component (5-FU) measured on the chromatogram as the distance in centimetres from the point of sample application to the peak maximum.
$w_{0.5h}$ = peak width at half-height in centimetres.

and

$$\text{HETP} = l_{gel}/N$$

where

l_{gel} = length of packed gel bed (mm).

RESULTS AND DISCUSSION

A study was made of the influence of pH and ionic strength, I, of the eluent buffer on the chromatographic behaviour of 5-FU on Sephadex G-10 columns. Results for elution constants, K_d and K_{av}, and column efficiencies, N and HETP, were obtained on a K 9/30 column operated at an eluent flow-rate in the range 5.5–17.1 ml/h and are given in Table I. For measuring these parameters, we used Blue Dextran 2000 as a completely excluded substance ($K_d = 0$) and acetone as a marker substance ($K_d = 1$), both compounds having good UV absorptivities.

As can be seen from Fig. 1, the K_d *versus* pH and K_{av} *versus* pH plots show a very small slope on the acidic side ($6.0 < \text{pH} < 7.5$, 5-FU existing as a non-ionic species), a very steep change around pH 8.0 (pK_a of 5-FU) and a downward slope on the basic side ($8.6 < \text{pH} < 10.0$, 5-FU occurring as a mono-anionic species). This overall correlation follows the general appearance of K_d *versus* pH plots for organic acids as previously reported[6].

At pH 7, where 5-FU exists mainly as non-ionic species, three definite ad-

TABLE I

INFLUENCE OF pH AND IONIC STRENGTH, *I*, OF THE ELUENT ON ELUTION CHARACTERISTICS AND EFFICIENCY OF SEPHADEX G-10 COLUMN CHROMATOGRAPHY OF 5-FU

K 9/30 column; packed bed, 29.1 ± 0.2 cm length. Eluent flow-rates, 5.5–6.0 ml/h.

pH	*Ionic strength (I)*	*Elution characteristics*		*Column efficiency*	
		K_d	K_{av}	*N*	*HETP (mm)*
6.05	0.03	2.37	1.02	408	0.71
6.02	0.07	2.34	1.02	427	0.68
6.02	0.10	2.31	0.99	510	0.57
7.10	0.04	2.36	0.99	448	0.65
7.02	0.08	2.28	1.00	500	0.58
7.10	0.12	2.31	0.99	475	0.62
7.78	0.04	2.16	0.90	404	0.72
7.82	0.09	2.21	0.95	523	0.56
7.90	0.14	2.28	0.97	427	0.68
8.33*	0.01	1.06	0.51	285	1.03
8.29*	0.02	1.15	0.56	292	1.00
8.25*	0.04	1.26	0.60	331	0.88
8.69*	0.01	0.71	0.35	315	0.93
8.67*	0.02	0.83	0.39	314	0.93
8.97*	0.04	0.69	0.29	391	0.75
9.70	0.02	0.39	0.17	362	0.81
9.78	0.04	0.44	0.19	308	0.95
9.92	0.06	0.47	0.20	328	0.89

* Eluent flow rates, 12.0–17.1 ml/h.

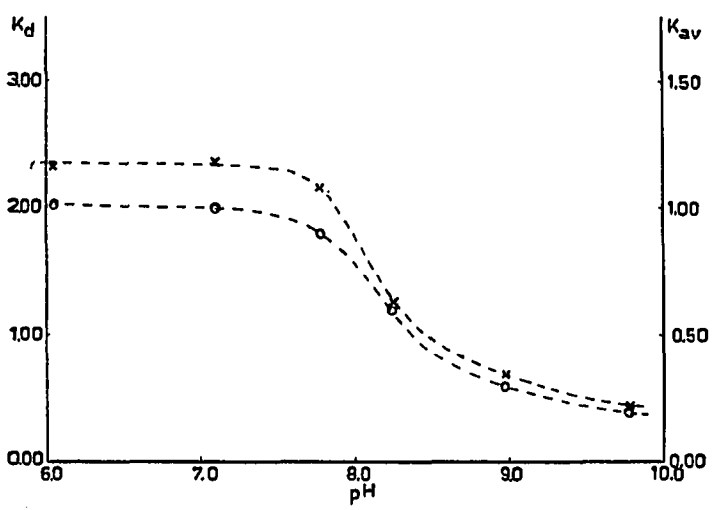

Fig. 1. K_d *versus* pH (×) and K_{av} *versus* pH (○) plots for 5-FU as determined by Sephadex G-10 column chromatography. Operating conditions: K 9/30 column (packed bed, 29.1 ± 0.2 cm length); eluent, same ionic strength, *I*, 0.04 (except for pH 6.0, when $I = 0.03$); flow-rates, 5.5–17.1 ml/h.

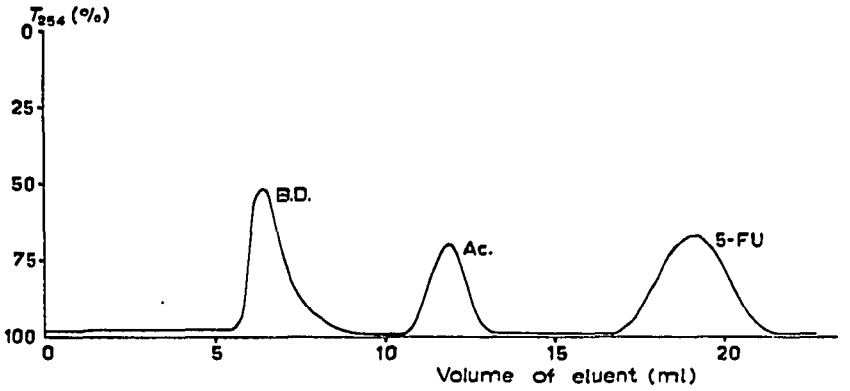

Fig. 2. Chromatogram of 5-FU on a Sephadex G-10 column. B.D. = Blue Dextran 2000 (400 μg); Ac. = acetone (4 μl); 5-FU = 5-fluorouracil (40 μg). Operating conditions: K 9/30 column (packed bed, 28.9 cm length); eluent, phosphate buffer, pH 7.0 and I 0.12; flow-rate, 5.5 ml/h; Uvicord I (LKB 4700A-2) and one-channel recorder (LKB 6520A-2) at 60 mm/h chart paper speed.

vantages with regard to the reproducible isolation from blood samples are noteworthy: only minor changes in elution behaviour of 5-FU with larger pH fluctuations occur, the highest K_d or K_{av} values are obtained (only a few substances are expected to elute in that region) and blood proteins are less denatured. The increased elution constants noticeable at pH 7 indicate selective retention or reversible adsorption[7] of 5-FU at the dextran gel matrix. Undoubtedly, the pyrimidinedione structure, which

TABLE II

INFLUENCE OF ELUENT FLOW-RATE ON ELUTION CHARACTERISTICS AND EFFICIENCY OF THE SEPHADEX G-10 COLUMN CHROMATOGRAPHY OF 5-FU

Phosphate buffer, pH 7.0 and I 0.12 (29.1 ml of 0.1 M NaOH + 50.0 ml of 0.1 M KH_2PO_4 + 0.2% trichlorobutanol), as eluent.

*Column type**	*Eluent flow-rate (ml/h)*	*Elution characteristics*		*Column efficiency*	
		K_d	K_{av}	N	*HETP (mm)*
K 9/15	5.5	2.33	1.37	259	0.57
	10.7	2.44	1.29	206	0.71
	16.1	2.34	1.26	181	0.81
	20.5	2.33	1.25	150	0.98
K 9/30	5.5	2.36	1.07	391	0.74
	9.6	2.35	1.12	400	0.72
	14.4	2.36	1.06	416	0.69
	19.9	2.24	1.08	277	1.04
	31.3	2.33	1.11	288	1.00
K 9/60	5.5	2.34	1.09	791	0.74
	9.6	2.40	1.08	686	0.85
	14.4	2.38	1.03	855	0.68

* Length of packed bed: K 9/15 column, 14.7 cm; K 9/30 column, 28.8 cm; K 9/60 column, 58.5 cm.

is capable of hydrogen bridge formation and π-bonding[8–10] is responsible for this effect. Notwithstanding a somewhat longer analysis time, the combination of gel permeation and adsorption phenomena seems very promising as regards the selectivity of this chromatographic process, for which an example is given in Fig. 2.

The ionic strength, *I*, is a parameter known from theoretical considerations to be limited to low values (*ca.* 0.01)[11]. As can be seen from Table I, only slight influences for the anionic species were found.

Results on the elution of 5-FU on Sephadex G-10 columns at different eluent flow-rates, obtained on three different columns (K 9/15, K 9/30 and K 9/60) and by use of a single phosphate buffer of pH 7.0 and *I* 0.12, are presented in Table II. An optimum flow-rate as a function of column length was not noticed. Small changes in eluent flow-rate do not result in a dramatic loss of column efficiency and even two- to three-fold increases are justified in order to shorten the analysis time.

The recovery of 5-FU from pure solutions, when applied in amounts of 40–120 μg per 0.25 ml, was examined on K 9/15 and K 9/30 Sephadex G-10 columns using phosphate buffer of pH 7.0 and *I* 0.12. Linearities, as checked by continuously monitoring the column effluent with Uvicord detectors, were excellent. Quantitative results obtained by fraction collection in combination with scanning of UV spectra are given in Table III and shown graphically in Fig. 3. The recoveries in the microgram range were 102.5 (K 9/15) and 99.6% (K 9/30) with standard deviations of 3.8 and 1.8%, respectively.

TABLE III

RECOVERY OF 5-FU FROM PURE SOLUTIONS CHROMATOGRAPHED ON SEPHADEX G-10 COLUMNS AS DETERMINED BY FRACTION COLLECTION AND SCANNING OF UV SPECTRA

Phosphate buffer, pH 7.00 and *I* 0.12 (29.1 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4 + 0.2% trichlorobutanol), as eluent. Eluent flow-rates: 11.7 ml/h on K 9/15 column (packed bed, 13.6 cm length) and 5.06 ml/h on K 9/30 column (packed bed, 28.5 cm length).

Amount of 5-FU applied on top of column (µg)	*Absorbance at 266 nm*			*Recovery (%)*	
	Peak eluted from K 9/15 column	*Peak eluted from K 9/30 column*	*Standard solution**	*K 9/15 column*	*K 9/30 column*
40	0.220	0.209	0.205	107.3	102.0
60	0.320	0.314	0.314	101.9	100.0
80	0.453	0.453	0.467	97.0	97.0
100	0.551	0.552	0.527	104.6	99.1
120	0.677	0.664	0.664	101.9	100.0
			Mean	102.5	99.6
			Standard deviation	3.8	1.8

* 0.25 ml of sample working solution diluted to 10.00 ml with buffer solution of pH 7.00 and *I* 0.12 (without chromatography).

The recovery of 5-FU from plasma samples, when applied in amounts of 205–1025 ng per 0.5 ml, was checked on a K 9/30 Sephadex G-10 column using phosphate

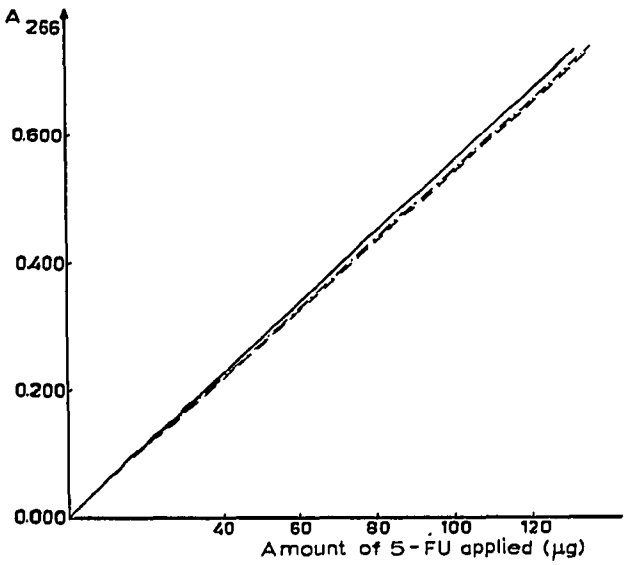

Fig. 3. Recovery of 5-FU chromatographed in microgram amounts on Sephadex G-10 columns. ———, K 9/15 column; — — —, K 9/30 column; –·–·–, reference solution.

buffer of pH 7.0 and *I* 0.12. Quantitative results obtained by fraction collection in combination with liquid scintillation counting are given in Table IV; the recovery in the submicrogram range was 99.2% with a standard deviation of 0.4%.

All of the results indicate that, within the limits of experimental error, the recovery of 5-FU chromatographed on Sephadex G-10 columns in the micro- and submicrogram range is essentially quantitative.

TABLE IV

RECOVERY OF 5-FU FROM PLASMA SAMPLES CHROMATOGRAPHED ON SEPHADEX G-10 AS DETERMINED BY FRACTION COLLECTION AND LIQUID SCINTILLATION COUNTING

Phosphate buffer, pH 7.00 and *I* 0.12 (29.1 ml of 0.1 *M* NaOH + 50.0 ml of 0.1 *M* KH_2PO_4), as eluent. Eluent flow-rate: 15.0 ml/h on K 9/30 column (packed bed, 28.5 cm length).

Amount applied on top of column (ng)			*Recovery (%)*
5-FU	*[6-^{3}H]-5-FU*		
202	2.6		98.7
405	5.2		98.9
607	7.8		99.1
810	10.4		99.6
1012	13.0		99.5
		Mean	99.2
		Standard deviation	0.4

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