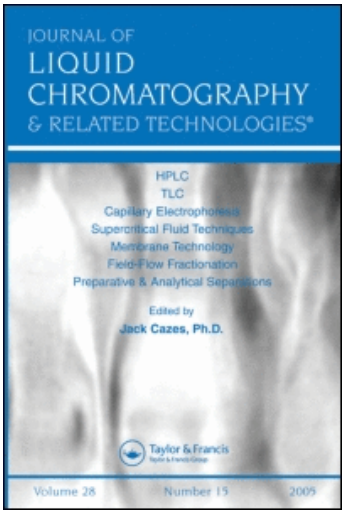

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CROSS-AXIS SYNCHRONOUS FLOW-THROUGH COIL PLANET CENTRIFUGE (TYPE XLL) III. PERFORMANCE OF MULTILAYER COILS IN PREPARATIVE SEPARATION OF DIPEPTIDES

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ABSTRACT

Performance of the type-XLL cross-axis coil planet centrifuge with three different size of the multilayer coils was evaluated by separating dipeptide samples on a polar biphasic solvent composed of *n*-butanol/acetic acid/water (4:1:5, v/v/v). Best results were obtained from the large helical diameter coils with β values of 1.00-1.20 [1] by eluting with the upper nonaqueous phase at 120 ml/h. The four dipeptides, L-tryptophyl-L-tyrosine, L-leucyl-L-tyrosine, L-valyl-L-tyrosine and L-tyrosylglycine, were completely resolved in 9 hours with high peak resolution ($Rs > 1.5$).

INTRODUCTION

As described in Part I [1], the type-XLL cross-axis synchronous flow-through coil planet centrifuge (XLL CPC) has a unique capability of retaining a large amount of the stationary phase in the column for hydrophilic two-phase solvent systems which can be efficiently applied for separation of polar compounds.

In the present paper, the capability of the XLL CPC has been evaluated in separation of a set of dipeptide samples in a commonly used two-phase solvent system composed of *n*-butanol, acetic acid and water at a volume ratio of 4:1:5. The experiments were performed with the separation columns consisting of a pair of multilayer coils coaxially mounted around the column holders. The performance of three columns, each mounted on the holder measuring 3.8, 7.6 or 15.2 cm in hub diameter, was compared in terms of theoretical plate number, peak resolution, stationary phase retention, *etc.*

APPARATUS

The design of the apparatus has been described in detail in Part I [1] and, therefore, is briefly given here. The apparatus holds a pair of horizontal rotary shaft on the rotary frame symmetrically at a distance (R) of 7.6 cm from the central axis of the centrifuge. As indicated by its name, each rotary shaft forms a cross to the vertical axis of the centrifuge. A spool-shaped coil holder is mounted on each rotary shaft a lateral position (L) 15 cm away from its middle point. The synchronous planetary motion of the holder is provided as follows: A pair of countershafts is radially mounted at the bottom plate of the rotary frame through a pair of ball bearings. Each countershaft is equipped with a plastic miter gear (45°) at its proximal end which is interlocked to an identical stationary gear mounted at the bottom plate of the centrifuge around the central

shaft. This gear arrangement produces synchronous rotation of the countershaft on the rotating rotary frame. This motion is further conveyed to the column holder by coupling the toothed pulley mounted on the distal end of the countershaft with a toothed belt to an identical pulley coaxially fixed to the column holder. The coil holder can be removed from the rotary frame by loosening a pair of screws on each bearing block and four set screws on the rotary shaft connector.

In the present study, we have used three pairs of coil holders with different hub diameters each measuring 3.8, 7.6 and 15.2 cm all with 5 cm width between the pair of flanges. Each multilayer coil was mounted on the holder by winding a 2.6 mm ID PTFE (polytetrafluoroethylene) tube (Zeus Industrial Products, Raritan, NJ, U.S.A.) directly onto the holder hub making the desired number of tight layers of left-handed coils. In the 3.8 and 7.6 cm diameter holders, these coiled layers were connected in series by bridging the neighboring layers with a piece of small-bore PTFE transfer tubing (0.7 mm ID) across the width of the column. Leak-proof connection was made by inserting each end of the transfer tube into the column terminal by using a short sheath of intermediate-size PTFE tubing (1.6 mm ID) as a spacer. The junction was further reinforced by winding a piece of copper wire over it and twisting the joined ends tightly together. When tested, the junction withstood several hundred psi of the applied back pressure. In the 15.2 cm diameter holder, the left-handed multilayer coil was prepared from a single piece of 2.6 mm ID PTFE tubing by winding it onto the holder from one side to the other and then immediately returning the starting position to continue winding the next layer. In both types of columns each coiled layer was wrapped with a few pieces of fiberglass-reinforced adhesive tape. In order to prevent dislocation of the column on the holder, short pieces of the same adhesive tape were occasionally applied across the width of the column by anchoring the ends onto the flanges.

A pair of multilayer coils mounted one on each side of the rotary frame was connected in series with flow tubes (0.7 mm ID)

and both inlet and outlet flow tubes were tightly supported with a silicon-rubber padded clamp at the top of the centrifuge. As mentioned earlier [1], these flow tubes can rotate with the rotary frame without twisting, thus permitting continuous elution through the rotating column without the conventional rotary seal device which would become a source of leakage and contamination.

The dimensions of each multilayer coil including the number of layers, total capacity, range of parameter β, *etc.* are summarized in Table I.

EXPERIMENTAL

Reagents

HPLC-grade glass-distilled *n*-butanol and methanol were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI, U.S.A. and reagent grade glacial acetic acid from Mallinckrodt, Inc., Paris, KY, U.S.A. Dipeptide samples, including L-tyrosylglycine (tyr-gly), L-valyl-L-tyrosine (val-tyr), L-leucyl-L-tyrosine (leu-tyr) and L-tryptophyl-L-tyrosine (trp-tyr), were all obtained from Sigma Chemical Company, St. Louis, MO, U.S.A.

Preparation of two-phase solvent system and sample solution

In the present study, a two-phase solvent system composed of *n*-butanol, acetic acid and water (4:1:5, v/v/v) was used for separation of dipeptides. The solvent mixture was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking and degassing and the two phases were separated shortly before use.

The sample solution was prepared by dissolving 30 mg each of tyr-gly, val-tyr, leu-tyr and 10 mg of trp-tyr in 8.0 ml of the above solvent mixture consisting of equal volumes of the upper and the lower phases.

Separation procedure

The experiments were performed according to the standard procedure used in the previous studies [2]. In each separation, the

TABLE I
DIMENSIONS OF THREE DIFFERENT COAXIAL MULTILAYER COLUMNS USED FOR DIPEPTIDE SEPARATION

Coil Holder			Column			
Diameter (cm)	Width (cm)	Coil Layers (β value)	Tube I.D. (mm)	Length (m)	Turns	Capacity (ml)
3.8	5.1	8 layers (0.25-0.60)	2.6	47	208	250
7.6	5.1	6 layers (0.50-0.80)	2.6	53	156	280
15.2	5.1	4 layers (1.00-1.20)	2.6	85	104	450

column was entirely filled with the stationary phase, either upper nonaqueous or lower aqueous phase, followed by injection of sample solution through the sample port. Then, the apparatus was rotated at the desired rpm while the mobile phase was pumped into the column in a proper elution mode given by a combination of the planetary motion (P_I or P_{II}), head-tail orientation and inward-outward direction. The effluent from the outlet of the column was continuously monitored with a UV monitor (LKB Uvicord S, LKB Instruments, Bromma, Stockholm, Sweden) at 280 nm and collected in 3.0 ml fractions with an LKB fraction collector (Ultrorac, LKB Instruments). During the separation, temperature of the centrifuge was kept fairly constant (23-26°C) by placing an ice bag over the top plate of the centrifuge. After all peaks were eluted from the column, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the column inlet to a pressured N_2 line (*ca.* 80 psi) while the apparatus was slowly rotated in a tail-to-head elution mode. From the collected stationary phase volume as well as the total volume of the column contents collected, the retention of the stationary phase was expressed in percentage relative to the total column capacity. The column was then flashed with methanol before starting the next experiment.

Analysis of fractions

An aliquot of each fraction, collected after the solvent front was emerged, was mixed with methanol and the absorbance was determined at 280 nm using a Zeiss PM6 spectrophotometer.

Evaluation of separation efficiency

From the obtained chromatograms, the partition efficiency was measured and expressed in terms of theoretical plate number according to the gas chromatographic formula:

$$N = 5.54\ (R/W_{h/2})^2 \qquad (1)$$

where N is the theoretical plate number, R, the retention time or

volume of the peak maximum and $W_{h/2}$, the width of the peak at its half height expressed in the same unit as R. The efficiency in separation was also expressed in peak resolution, Rs, using the conventional equation:

$$Rs = 2\,(R_2 - R_1)/(W_1 + W_2) \qquad (2)$$

where R_1 and R_2 are retention time or volume of two adjacent peaks and W_1 and W_2, the peak width of the same peaks expressed in the same unit as R_1 and R_2.

RESULTS AND DISCUSSION

Stationary phase retention in three different columns

It has been reported that the XLL CPC has a unique capability of retaining a large amount of stationary phase for viscous, low interfacial tension solvent systems. Further, the small diameter column with a small β value shows excellent stationary phase retention of the hydrophilic solvent systems such as butanol systems [1].

In the present study, the effects of the flow-rate of the mobile phase on the retention of the stationary phase were investigated at a revolution speed of 750 rpm using the solvent system composed of *n*-butanol, acetic acid and water (4:1:5, v/v/v). The results of the experiments are illustrated in Fig. 1 where the volume of the retained stationary upper or lower phase relative to the total column capacity are plotted against the applied flow-rate. The open symbols indicate the retention of the upper organic stationary phases and the solid symbols, those of the aqueous lower phases. The retention of the upper phase is always greater than those of the lower phase, except for the large column with large β values at slow flow rates of 60-120 ml/h. The retention of the stationary upper and lower phases in the small column with small β values are greater than in the middle and large size columns. Even at a high flow rate of 120

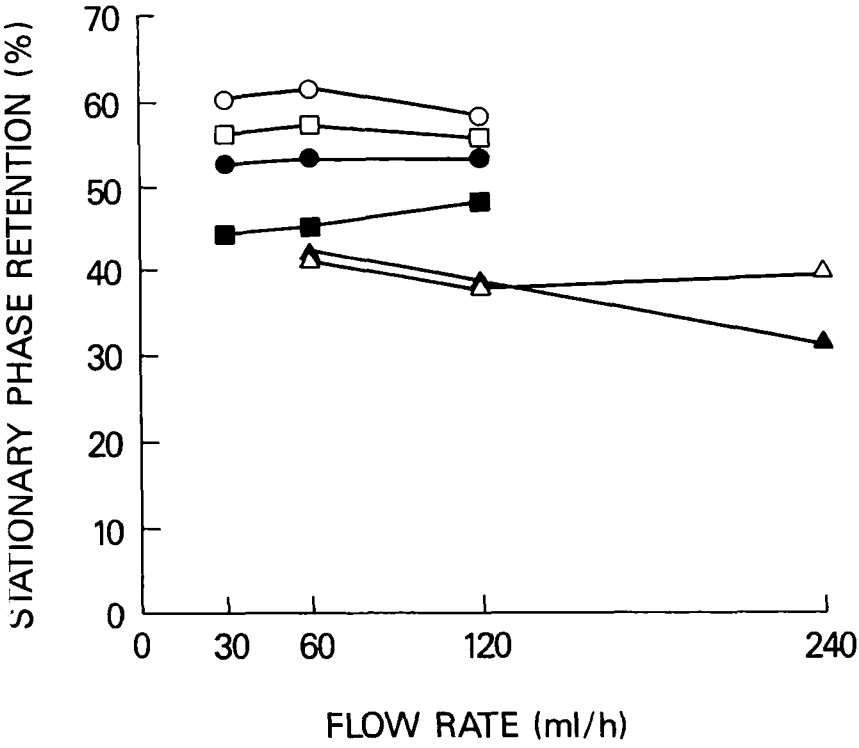

Fig. 1. Effect of the flow rate on the retention of the upper (○ □ △) and lower (● ■ ▲) stationary phases in small (○ ●), middle (□ ■) and large (△ ▲) columns.

ml/h, the small column can retain the upper stationary phase near 60% of the total column capacity. The results indicate that the retention of the stationary phase greatly depends on the β values of the multilayer coil.

Separation and evaluation of partition efficiency

In countercurrent chromatography, the retention time of the compounds widely depends on the partition coefficient of the solute which can be easily determined by a simple test tube procedure [3]. In the previous paper, the partition coefficients (K) of four

dipeptides were determined in a two-phase solvent system composed of *n*-butanol/acetic acid/water (4:1:5, v/v/v) [4]. Each K value is expressed in two different ways, $K(C_L/C_U)$ or $K(C_U/C_L)$, where C_L indicates the solute concentration in the lower phase and C_U that in the lower phase. In the above solvent system, $K(C_L/C_U)$ of tyr-gly, val-tyr, leu-tyr and trp-tyr are 3.23, 1.89, 1.00 and 0.45, while $K(C_U/C_L)$ are 0.31, 0.53, 1.00 and 2.20, respectively. The eluting order of these dipeptides is tyr-gly, val-tyr, leu-tyr and trp-tyr when the lower phase is used as the mobile phase. This order is reversed when the upper phase is used as the mobile phase.

The peak resolution (Rs) values between tyr-gly and val-tyr, val-tyr and leu-tyr, and leu-tyr and trp-tyr are listed in Table II at various flow-rates ranging from 30 ml/h to 240 ml/h in three different columns. The Rs values of 1.0 and 1.5 corresponds to a 4δ (98% pure) and a baseline separation (6δ, 99.7% pure), respectively. In Table II, P_I and P_{II} indicate the directions of the planetary motion; H, the head-tail elution mode; and I and O, inward and outward elution modes, respectively. In order to obtain a satisfactory retention of the stationary phase, the lower phase should be eluted outwardly along the direction of the lateral force field and the upper phase in the opposite direction. The Rs values between adjacent dipeptides obtained by using the upper phase as the mobile phase are always greater than those obtained by using the lower phase as the mobile phase, except for the Rs values between leu-tyr and trp-tyr with the middle-size column (280 ml capacity) at 30 ml/h and 120 ml/h flow-rates. Except for a few instances, the Rs values between these four dipeptides increase at the decreased flow rates of the mobile phase in all columns. Rs values over 1.5 (99.7% pure) between each adjacent peaks was achieved by the large column (β=1.00-1.20) at a 120 ml/h flow-rate using upper phase as the mobile phase.

Fig. 2 shows a chromatogram of the four dipeptide samples obtained by the XLL CPC equipped with a pair of multilayer coils of helical diameters (β=1.00-1.20). The separation was performed with a solvent system composed of *n*-butanol/acetic acid/water (4:1:5,

TABLE II
PEAK RESOLUTION (Rs) OF DIPEPTIDES OBTAINED BY X-AXIS COIL PLANET CENTRIFUGE WITH THREE DIFFERENT COLUMNS

Column Capacity (β value)	Flow rate (ml/h)	Mobile Phase	Elution Mode	Rs			SR* (%)
				try-gly/val-tyr	val-tyr/leu-try	leu-try/trp-tyr	
250 ml (0.25-0.60)	30	Lower	P_IHO	1.24	0.76	2.05	60.0
	60	Lower	P_IHO	0.99	0.89	1.82	61.5
	120	Lower	P_IHO	0.84	0.73	1.57	58.2
	30	Upper	$P_{II}HI$	1.88	1.48	2.30	52.6
	60	Upper	$P_{II}HI$	1.89	1.45	2.06	53.1
	120	Upper	$P_{II}HI$	1.86	1.18	1.83	53.2
280 ml (0.50-0.80)	30	Lower	P_IHO	1.13	0.94	1.89	56.4
	60	Lower	P_IHO	0.93	0.89	1.95	57.4
	120	Lower	P_IHO	0.91	0.85	1.87	55.8
	30	Upper	$P_{II}HI$	2.61	1.34	2.19	44.4
	60	Upper	$P_{II}HI$	2.11	1.24	1.59	45.2
	120	Upper	$P_{II}HI$	1.57	1.00	1.67	48.9
450 ml (1.00-1.20)	60	Lower	P_IHO	1.37	1.10	1.62	41.0
	120	Lower	P_IHO	1.14	0.97	1.95	37.8
	240	Lower	P_IHO	1.08	0.71	1.48	40.0
	60	Upper	$P_{II}HI$	2.15	1.45	2.11	41.8
	120	Upper	$P_{II}HI$	1.60	1.92	1.95	38.5
	240	Upper	$P_{II}HI$	1.46	0.91	1.62	31.3

***SR ; Stationary phase retention. Revolution speed ; 750 rpm.**

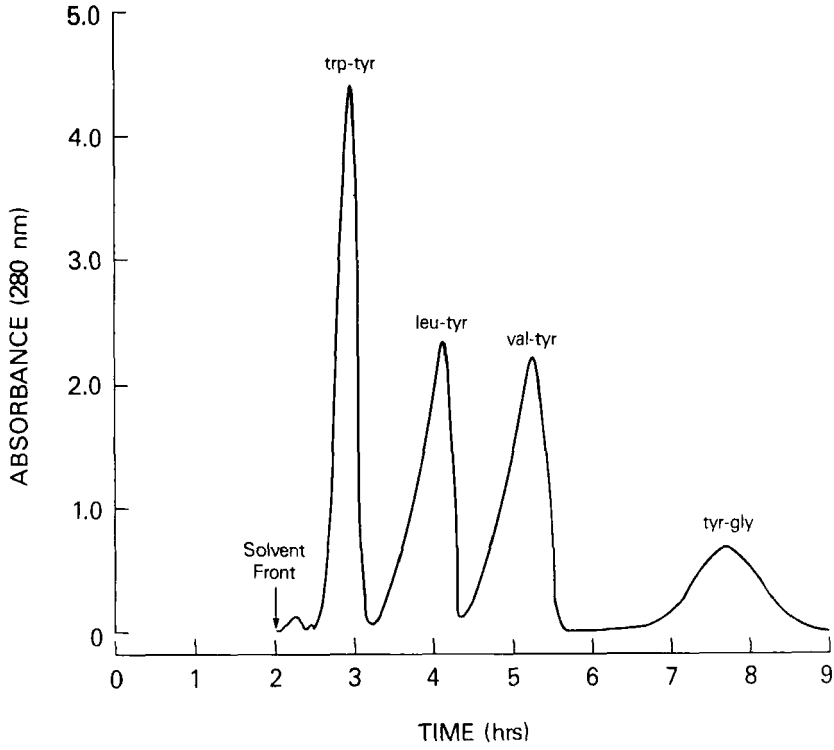

Fig. 2. Separation of dipeptides by the XLL CPC with the upper organic phase as a mobile phase.
Experimental conditions are as follows: Apparatus: XLL CPC with a 7.5 cm revolution radius. Columns: A set of multilayer coils connected in series, 2.6 mm and 450 ml capacity (β=1.00-1.20); Solvent system: *n*-butanol/acetic acid/water (4:1:5, v/v/v); Mobile phase : upper phase; Elution mode: $P_{II}HI$; Flow rate: 2.0 ml/min; Revolution speed: 750rpm; Sample: Dipeptide mixture consisting of 30 mg each of try-gly, val-tyr, leu-tyr and 10 mg of trp-tyr dissolved in 8.0 ml of the above solvent mixture.

v/v/v) eluting with the upper organic phase at a flow rate of 120 ml/h in a $P_{II}HI$ elution mode under a revolution speed of 750 rpm. All dipeptides were well resolved within 9 hours.

In order to compare the partition efficiency obtained from three columns different in length and capacity, the following expressions are used: $Rs/\sqrt{L}$ (peak resolution produced by the unit length of the column; N/turn (number of theoretical plates produced by one helical turn of the column); L/N (a length of the column required to produce one theoretical plate, which is somewhat equivalent to HETP in HPLC column); T/N (time required to produce one theoretical plate and this is obtained by dividing the retention time of the solvent front with N), where, L is the length of the column, N is the number of the theoretical plate in each peak and T is a retention time of the solvent front.

The N/turn and the $Rs/\sqrt{L}$ values obtained from three kinds of columns with different β values at a high flow-rate of 120 ml/h are listed in Table III. The N/turn values of the dipeptides increase with the increased β values when the lower phase is used as the mobile phase. This may indicate that the high column efficiency (N) is obtained from a large helical diameter column, probably due to a longer length of tubing per one helical turn compared with the smaller columns. The similar results were obtained at the first (trp-tyr) and third peaks (val-tyr) by using the upper phase as the mobile phase.

The $Rs/\sqrt{L}$ values, which indicate the peak resolution by the unit length of the column, are essentially the same in three different size columns, if the lower phase is used as a mobile phase. However, when the upper nonaqueous phase is used as the mobile phase, the $Rs/\sqrt{L}$ values between peak 1 (trp-tyr) and peak 2 (leu-tyr), and peak 3 (val-tyr) and peak 4 (try-gly) become always greater in the smaller coils

The L/N and the T/N values obtained by the three different columns at a flow rate of 120 ml/h are also listed in the Table IV. The smaller L/N and T/N values of the dipeptides peaks indicate better partition efficiencies. The L/N values, which are equivalent to

TABLE III
COMPARISON OF PARTITION EFFICIENCIES OBTAINED BY THREE DIFFERENT COLUMNS

Elution Mode (Mobile Phase) Dipeptides	N/Turn*			Rs/$\sqrt{L}$ (m^{-2})*		
	Small**	Middle**	Large**	Small	Middle	Large
P_IHO (LP***)						
tyr-gly	2.12	3.40	13.08	0.12	0.12	0.12
val-tyr	1.30	2.56	7.02	0.11	0.12	0.11
leu-tyr	1.11	1.92	5.19	0.23	0.26	0.21
trp-tyr	0.63	0.96	3.37			
P_{II}HI (UP***)						
trp-tyr	1.97	2.50	6.83	0.27	0.23	0.21
leu-tyr	1.88	1.86	4.52	0.17	0.14	0.21
val-tyr	1.73	2.05	5.87	0.36	0.22	0.17
tyr-gly	2.02	1.41	4.33			

*** N ; number of theoretical plate, Rs ; column efficiency, L ; column length.**

**** Small ; β=0.25-0.60, middle ; β=0.50-0.80, large ; β=1.00-1.20.**

***** LP=lower phase, UP=upper phase. Flow rate ; 120 ml/h. Revolution speed ; 750 rpm.**

TABLE IV
COMPARISON OF PARTITION EFFICIENCIES OBTAINED BY THREE DIFFERENT COLUMNS

Elution Mode (Mobile Phase) Dipeptides	L/N* (mm)			T/N* (min)		
	Small**	Middle**	Large**	Small	Middle	Large
P_IHO (LP***)						
tyr-gly	107	100	63	0.12	0.12	0.07
val-tyr	174	133	116	0.20	0.16	0.13
leu-tyr	204	177	157	0.23	0.21	0.17
trp-tyr	362	353	243	0.42	0.41	0.27
P_{II}HI (UP***)						
trp-tyr	115	136	120	0.15	0.18	0.17
leu-tyr	121	183	181	0.15	0.25	0.26
val-tyr	131	166	139	0.17	0.23	0.20
tyr-gly	112	241	189	0.14	0.16	0.27

*** L ; column length, T ; retention time of solvent front, N ; number of theoretical plate.**

**** Small ; β=0.25-0.60, middle ; β=0.50-1.00, large ; β=1.00-1.20.**

***** LP ; lower phase, UP ; upper phase. Flow rate ; 120 ml/h. Revolution speed ; 750 rpm.**

HETP in HPLC decrease as the β value of the column increases if the lower aqueous phase is used as the mobile phase. In contrast, if the upper phase is used as the mobile phase, the smallest *L/N* value of each peak is obtained from the column with the smallest β value.

The *T/N* values, which indicate the time required to produce one theoretical plate number are almost the same, in the small and middle size columns. In the largest column, the smallest *T/N* value of each peak is obtained by eluting with the lower aqueous phase, whereas in the small column, the smallest *T/N* value of each peak is obtained by eluting the dipeptides with the upper organic phase.

The overall results of the present study indicate that the multilayer coils with large helical diameters (β=1.00-1.20) yield the high partition efficiencies if the lower aqueous phase is used as the mobile phase, while the small helical diameter coils (β=0.25-0.60) yield better efficiencies if the upper phase is used as the mobile phase. The capability of the XLL CPC may be extended to separation and purification of various peptides in large scale.

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