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SILICA GEL LIQUID–LIQUID CHROMATOGRAPHY USING AQUEOUS BINARY PHASE SYSTEMS

HIGH-EFFICIENCY EXTRACTION AND RESOLUTION OF PHENOLS AND CARBOXYLIC ACIDS

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SUMMARY

An investigation was made of silica gel liquid–liquid partition chromatographic systems incorporating aqueous phases in order to provide a highly efficient solvent extraction procedure. By employing a simple aqueous binary solvent system containing diethyl ether and/or *n*-hexane and a droplet current pre-equilibration device, an instrumental aqueous two-phase distribution chromatographic system was constructed. Hydrophilic solutes such as polyhydroxybenzene derivatives and free carboxylic acids were separated by using silica gel columns treated with mineral acids. The capacity ratios of the solutes were found to be proportional to the distribution ratios of the samples, and therefore the resolution mechanism of this system can be assumed to be a liquid–liquid distribution.

INTRODUCTION

The use of organic solvents for the extraction of solutes in aqueous solutions has been widely applied in preparative processes in analytical and synthetic chemistry. Although manual batch experiments are commonly carried out in these fields, mechanical multi-step methods such as counter-current distribution and liquid–liquid partition chromatography (LLC) are often adopted in order to improve the efficiency of the separation process as alternative techniques.

LLC separation involving an aqueous stationary phase was achieved by employing cellulose or silica gel as a support material. Hydrophilic organic substances such as α-amino acid derivatives and carbohydrates were resolved by applying the classical LLC system from which the concepts and technology of today's chromatography were originally developed[1].

Several contributions by Huber's and Karger's groups incorporating modern technology showed the usueful selectivities and the high separation power of the LLC system[2–5]; however, high-performance aqueous-phase LLC has not been accepted for routine analysis, probably because the selection procedure for the phase system is always troublesome and the pre-conditioning of LLC columns is time consuming. For

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instance, phase liquids are commonly prepared by using ternary solvents obtained by the addition of a third solvent that is miscible with both of the components of the two-phase system. Therefore, in recent years, reversed-phase chromatography incorporating chemically bonded lipophilic phases and aqueous binary solvents has been commonly adopted for the routine analysis of water-soluble substances. LLC involving two aqueous phases has been replaced by more stable and convenient techniques for these practical reasons.

Highly efficient solvent extraction procedures were required in our laboratory for the development of a "programmed flow preparation" system[6] which permits continuous fractionation and separation. Such a process would contribute to the improvement of clean-up techniques used in the analysis of biological fluids. Therefore, the aqueous-phase LLC system was re-examined as an organic solvent multi-extraction process having high efficiency. In order to construct the aqueous-phase LLC system, we first tried to simplify the phase systems by removing the third solvent that is miscible with water and to use solvents that are commonly applied for the extraction procedure. Second, we designed dynamic pre-conditioning equipment for the carrier to accomplish the equilibration of the system and to obtain the stable analytical column rapidly. Lacking a pre-column, which is commonly required in LLC, the system was instrumentalized by using a UV detector.

Silica gel columns having a high theoretical plate number were incorporated to extract and separate water-soluble solutes, such as polyhydroxybenzene derivatives and free carboxylic acids, which were selected as model compounds for our basic study. Even water-coated silica gel showed an adsorption activity for polar substances such as hydroxycarboxylic acids; however, it was found that deactivation of the silica gel was achieved by treating the surface with mineral acids. In this paper, high-efficiency extraction and resolution of hydrophilic solutes in an aqueous two-phase partition system were accomplished by using acid-treated silica gel columns.

EXPERIMENTAL

Apparatus

The chromatographic apparatus is shown in Fig. 1. The droplet current equilibration system consisted of three stainless-steel tubes (250 × 10 mm I.D.). Each tube contained 16 ml of water saturated with a carrier solvent. The equilibration between the solvent and water was achieved during passage of droplets of a carrier solvent through the three tubes.

Packing material and column

Spherical silica gel with a pore size of 95 Å, particle size 10 μm and specific surface area 380 m^2/g (Kusano Scientific Co., Tokyo, Japan) was packed into stainless-steel tubes (200 × 4.6 mm I.D.) by the high-pressure slurry procedure.

Preparation of liquid–liquid partition column

Treatment with mineral acid. The packed column was washed with 20 ml of methanol and 100 ml of distilled water, and then treated with 20 ml of 20% sulphuric acid. The acid-treated column was washed with distilled water until the effluent gave no precipitate of barium sulphate on addition of saturated barium nitrate solution. The column was then further washed with 60 ml of distilled water.

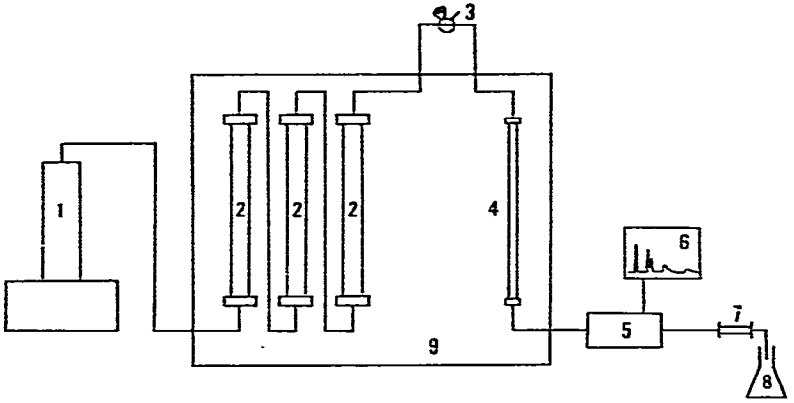

Fig. 1. Schematic diagram of the apparatus. 1 = High-pressure syringe-type pump (LCP-150; Japan Spectroscopic Co., Tokyo, Japan); 2 = droplet current equilibration system (250 × 10 mm I.D.); 3 = loop injector (Rheodyne Model 7125); 4 = separation column (200 × 4.6 mm I.D.); 5 = UV detector (UVIDEC 100-II; Japan Spectroscopic Co.); 6 = recorder; 7 = back-pressure column; 8 = waste reservoir; 9 = column oven (TU-100; Japan Spectroscopic Co.).

Column conditioning. The resulting column was connected to the chromatographic apparatus shown in Fig. 1 and purged with diethyl ether saturated with water at a flow-rate of 3 ml/min until a stable baseline was obtained.

Samples

All samples except Bz-DL-serine were commercially available (Wako, Osaka, Japan). Bz-DL-serine was prepared by the usual Schotten–Baumann procedure, and identified from IR spectral data. All samples and chromatographic solvents were of reagent grade (Wako).

Chromatographic procedure

The flow-rate of the eluent was 1 ml/min. The hold-up volume, V_0, was measured by injecting benzene as a standard sample. The ratio of stationary phase to mobile phase was calculated with the equation

$$V_s/V_m = (V_{oi} - V_{oc})/V_{oc}$$

where V_s is the volume of stationary phase, V_m is the volume of mobile phase, V_{oi} is the hold-up volume of the column containing no liquid stationary phase and V_{oc} is the hold-up volume of the column containing liquid stationary phase.

The droplet current equilibration system and separation column were maintained at 20°C. The capacity ratio, k', was calculated by the equation $k' = (V_r - V_0)/V_0$, where V_r is the retention volume.

The sample (0.1–1 μg) in diethyl ether (*ca.* 5 μl) was injected. The sample eluate was detected by UV absorbance at 254 nm.

Measurement of distribution ratio

The distribution ratios of solutes between water and diethyl ether were calcu-

lated with the equation $D = C_s/C_m$, where, D is the distribution ratio, C_s is the concentration of solute in the aqueous phase (stationary phase) and C_m is the concentration of solute in the diethyl ether phase (mobile phase).

C_s and C_m were obtained by the usual batch procedure in which the distribution process was carried out at 20°C and concentrations of solutes were determined by calibration graphs using UV absorbance.

RESULTS AND DISCUSSION

Several problems were encountered in an attempt to improve the performance and the instrumentalization of aqueous-phase LLC systems. These problems included the selection of a fine porous support material for coating the aqueous stationary phase, elimination of the adsorption activity for the packing surface and the preparation of high-efficiency columns supporting the aqueous stationary phase having sufficient stability for monitoring the solute elution. For realization of the system, we examined a silica gel column prepared by the common slurry packing procedure to support the aqueous stationary phase. As an alternative to the pre-column, which is normally required to obtain a stable analytical system for LLC, droplet current liquid columns were used.

Equipment for pre-equilibrating the carrier by passing water as droplets was constructed from empty HPLC tubes made of glass or stainless steel. Several tubes containing water were connected in a series using a pump. The flow-chart of this system is illustrated in Fig. 1. The droplet current conditioning device provided an efficient dynamic equilibration on the phase system without a pre-column and also avoided the preparation of excessive amounts of mixed solvents as carriers. This

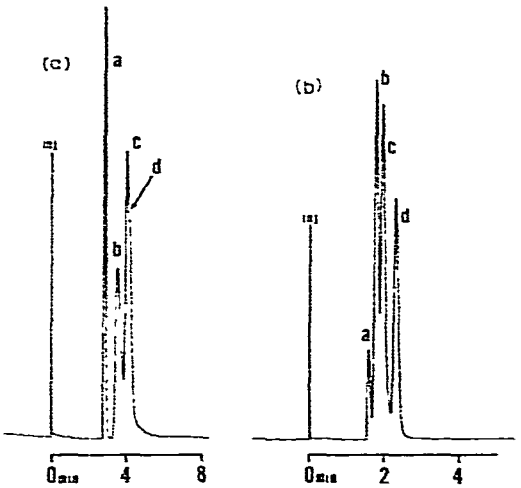

Fig. 2. (a) Separation of dihydroxybenzene isomers by adsorption chromatography. (a) Benzene; (b) catechol; (c) resorcinol; (d) hydroquinone. Packing: spherical silica gel (pore size 95 Å, particle size 10 μm). Eluent: diethyl ether. Flow-rate: 1 ml/min. Column temperature: 20°C. Detection: UV absorbance at 254 nm. (b) Separation of dihydroxybenzene isomers by LLC using an aqueous binary phase system. (a) Benzene; (b) catechol; (c) resorcinol; (d) hydroquinone. Support: spherical silica gel (pore size 95 Å, particle size 10 μm) (not treated with acid). Phase system: diethyl ether–water. Phase ratio (V_s/V_m): 0.70. Flow-rate: 1 ml/min. Column temperature; 20°C. Detection: UV absorbance at 254 nm.

system and separation column were allowed to equilibrate in a temperature-controlled chamber. A stable analytical system was constructed using a UV detector.

Diethyl ether, which is widely utilized for the extraction of solutes in aqueous solution, was selected as an organic solvent. *n*-Hexane was additionally employed to control the retention of the solutes. Hydrophilic samples such as phenols and carboxylic acids were used as samples. The retention behaviour of homologous compounds were examined in this phase system.

Before testing water-coated silica gel columns, we first examined the retention characteristics of three isomeric dihydroxybenzene derivatives in adsorption chromatography by using diethyl ether as the eluent on a bare silica gel (pore size 95 Å) column. The chromatogram is shown in Fig. 2a. Three peaks appeared; however, the resolution of resorcinol and hydroquinone was incomplete.

Next, the column was coated with water. Although three peaks were also obtained with the same elution order, the resolution of the first two peaks was incomplete. The result is shown in Fig. 2b. The decrease of the hold-up volume for the water-coated column clearly suggested that the aqueous phase was tightly retained on the surface of the packing material. To increase the retention of the solutes, *n*-hexane was added to the carrier.

The retention of the solutes was examined using *n*-hexane–diethyl ether (1:1) as the eluent. The resulting adsorption chromatogram is depicted in Fig. 3a. The resolution of the three isomers was not improved by tailing phenomena. On the other hand, a partition chromatogram (Fig. 3b) indicated that the LLC mode is very suitable for the resolution of the given sample mixture.

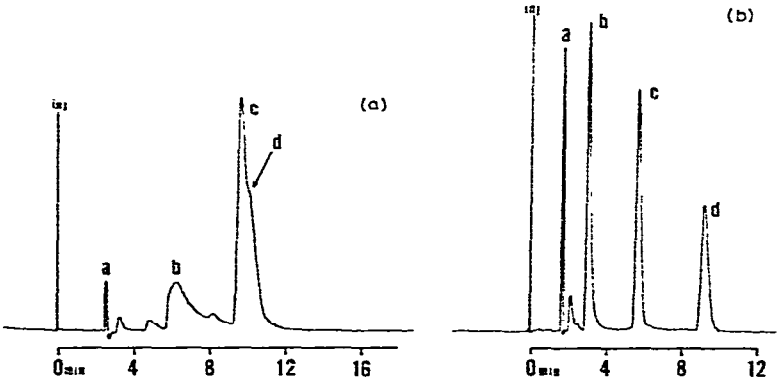

Fig. 3. (a) Separation of dihydroxybenzene isomers by adsorption chromatography. (a) Benzene; (b) catechol; (c) resorcinol; (d) hydroquinone. Separation conditions as in Fig. 2a except for the eluent: *n*-hexane–diethyl ether (1:1). (b) Separation of dihydroxybenzene isomers by LLC using *n*-hexane-diethyl ether/aqueous phase system. (a) Benzene; (b) catechol; (c) resorcinol; (d) hydroquinone. Separation conditions as in Fig. 2b except for the phase system: *n*-hexane–diethyl ether (1:1)/water. Phase ratio (V_s/V_m): 0.60.

Trihydroxybenzene and carboxylic acids that are more soluble in water were then injected into the aqueous-phase LLC columns. These solutes were eluted using diethyl ether as the eluent; however, some polar compounds such as gallic acid and vanilylmandelic acid gave tailing peaks. This phenomenon was interpreted as a stronger interaction between polar functional groups in the solutes and the active sites

of the silica gel surface. Elimination of such interactions required the elimination of the adsorption activity on the silica gel surface. Various methods for treating the silica gel were examined and washing with mineral acids such as 20% hydrochloric or sulphuric acids was found to be a suitable means of deactivating the sites. To eliminate excess of acids, the acid-treated columns were washed with water until the carrier was free of acid. A trace amount of acid was localized on the active sites of the support; however, peak tailing decreased and the resolution of the polar solute mix-

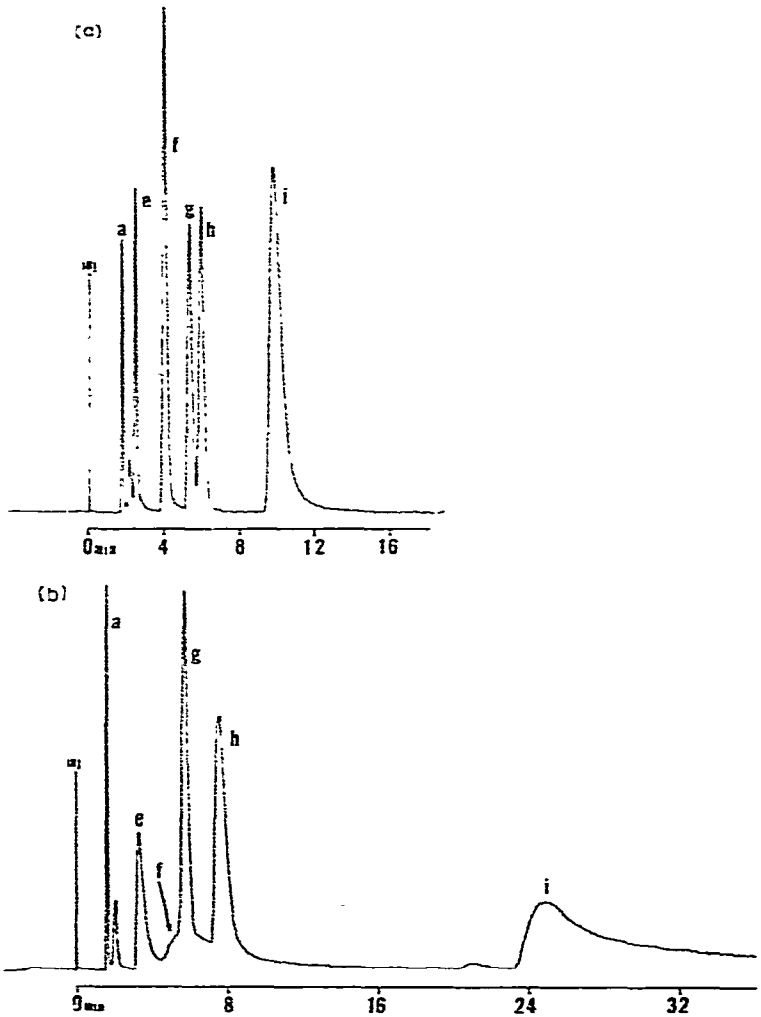

Fig. 4. (a) Separation of a mixture of trihydroxybenzenes and a group of carboxylic acids by LLC using an acid-treated support. (a) Benzene; (e) fumaric acid; (f) gallic acid; (g) phloroglucinol; (h) hippuric acid; (i) 4-hydroxy-3-methoxymandelic acid (VMA). Support: acid-treated spherical silica gel (pore size 95 Å, particle size 10 μm). Phase system: diethyl ether–water. Phase ratio (V_s/V_m): 0.60. Flow-rate: 1 ml/min. Column temperature: 20°C. Detection: UV absorbance at 254 nm. (b) Separation of a mixture of trihydroxybenzenes and a group of carboxylic acids in LLC using a support not treated with acid. (a) Benzene; (e) fumaric acid; (f) gallic acid; (g) phloroglucinol; (h) hippuric acid; (i) 4-hydroxy-3-methoxymandelic acid (VMA). Separation conditions as in Fig. 2b.

ture was improved. Continuous analysis over a period of several weeks was possible by employing the acid-treated columns. If the treated surface of the support in the column showed deterioration, for example if the carrier component or carrier composition changed considerably, the columns were regenerated by washing successively with methanol and acid *in situ*.

A mixture of trihydroxybenzene and a group of carboxylic acids were separated by using acid-treated columns and aqueous diethyl ether as the eluent. Chromatograms are illustrated in Fig. 4a. Fig. 4b shows a chromatogram obtained by using a non-acid-treated column, long tailing peaks being observed for gallic and vanilylmandelic acid.

Salicylic acid, gentisic acid and salicyluric acid are known as the main metabolites of aspirin[7,8]. A mixture of these metabolites was resolved by using an acid-treated column and *n*-hexane–diethyl ether (1:1)/aqueous phase system. The chromatogram is depicted in Fig. 5. The capacity ratios of phenols and carboxylic acid derivatives in *n*-hexane–diethyl ether or diethyl ether/aqueous phase systems are summarized in Table I. In these separations, an aqueous phase-supported silica gel column showed approximately 10,000 plates/m.

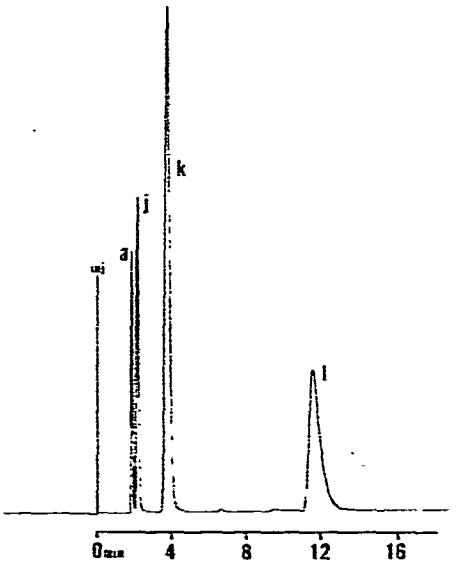

Fig. 5. Separation of the main metabolites of asparin by LLC. (a) Benzene (internal standard for V_o); (j) salicylic acid; (k) gentisic acid; (l) salicyluric acid. Separation conditions as in Fig. 4a except for the phase system: *n*-hexane–diethyl ether (1:1). Phase ratio (V_s/V_m): 0.53.

To confirm the separation mechanism of the column, the capacity ratios of the solutes obtained by using an acid-treated column in the present study were compared with the distribution ratios of the same samples in the phase system of diethyl ether and pure water, which were measured by a common batch procedure. Later ratios were determined by using the UV absorbance of solutes in the two equilibrated phases. If the retention mechanism is attributed to liquid–liquid distribution, the

TABLE I

CAPACITY RATIOS (k') OF PHENOLS AND CARBOXYLIC ACIDS IN VARIOUS PHASE SYSTEMS USING AN ACID-TREATED SUPPORT

Sample	*Phase system**			
	1	*2*	*3*	*4*
Catechol	0.13	0.32	0.89	2.73
Resorcinol	0.25	0.74	2.54	10.42
Hydroquinone	0.42	1.22	4.12	16.41
Pyrogallol	0.36	–	–	–
Phloroglucinol	2.11	–	–	–
Salicylic acid	–	0.06	0.15	0.33
Gentisic acid	–	0.32	0.97	3.98
Salicyluric acid	–	1.34	5.19	–
p-Hydroxybenzoic acid	0.18	0.52	1.76	7.35
Protocatechuic acid	0.41	1.56	7.42	–
Fumaric acid	0.43	1.48	5.50	–
Gallic acid	1.35	–	–	–
Hippuric acid	2.46	–	–	–
4-Hydroxy-3-methoxymandelic acid	4.70	–	–	–
N-Benzoylserine	8.47	–	–	–
3,4-Dihydroxymandelic acid	11.04	–	–	–
Maleic acid	13.54	–	–	–

* Phase systems: 1, diethyl ether–water (phase ratio, $V_o/V_m = 0.58$); 2, *n*-hexane–diethyl ether (1:3) water ($V_o/V_m = 0.58$); 3, *n*-hexane–diethyl ether (1:1), water ($V_o/V_m = 0.54$); 4, *n*-hexane–diethyl ether (7:3), water ($V_o/V_m = 0.52$).

following relationship should hold:

$$k' = DV_s/V_m$$

where D is the distribution ratio, k' is the capacity ratio, V_s is the volume of the stationary phase and V_m is the volume of the mobile phase.

The correlation between the capacity ratios and distribution ratios of a group of solutes is shown in Fig. 6. The linear relationship was indicated with a correlation coefficient of 0.978 and a slope of 0.69. This slope represents the phase ratio (V_s/V_m) of the column. The phase ratio can also be calculated from the following equation:

$$V_s/V_m = (V_{oi} - V_{oc})/V_{oc}$$

where V_{oi} is the hold-up volume of columns containing no stationary phase and V_{oc} is the hold-up volume of columns containing an aqueous stationary phase.

The phase ratio obtained from the above equation is 0.60 and is consistent with the slope of the k' *versus* D plot. These results suggest that the retention mechanism of this system depends on a liquid–liquid distribution and that the acid on the surface of the support doses not affect the distribution process.

From the value of V_s/V_m, the hold-up volume of the column, V_o, and the

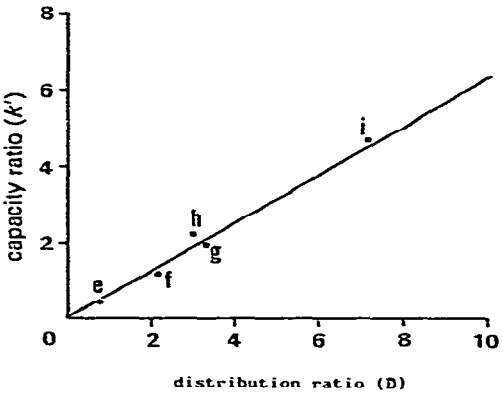

Fig. 6. Correlation of distribution ratios (D) and capacity ratios (k'). The correlation coefficient is 0.978 and the slope is 0.69 (this represents the phase ratio, V_s/V_m). The phase ratio calculated from V_0 is 0.6. Phase system: diethyl ether–water. Capacity ratios were obtained by using an acid-treated column. Samples: (e) fumaric acid; (f) gallic acid; (g) phloroglucinol; (h) hippuric acid; (i) 4-hydroxy-3-methoxymandelic acid (VMA).

specific surface area of support, it is assumed that about ten water molecules are layered on the support surface.

CONCLUSION

Aqueous binary-phase LLC systems using silica gel columns and acid-treated silica gel columns proved to be useful for the simultaneous extraction of water-soluble solutes such as phenols and free carboxylic acids and possibly other water-soluble materials from aqueous solutions and the resolution of the mixture. High-efficiency columns and pre-equilibration systems allowed the construction of an instrumentalized aqueous-phase HPLC system. In this paper preliminary results have been described; however, it must be ensured that the system has a high resolution power permitting to be applied to wide range of analytical and preparative chemistry research problems.

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