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CHARACTERISATION OF CROSS-LINKED POLY(ACRYLOYLMORPHOLINES) AS MATRICES FOR AQUEOUS GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

The characterisation of two cross-linked poly(acryloylmorpholines), Enzacry Gel K1 and Enzacryl Gel K2, as matrices for aqueous gel permeation chromatography is described. Near ideal plots of logarithm molecular weight *versus* distribution coefficient, K_d, are obtained for polyethylene glycols and linear, oligomeric α,ω-diols, approximate molecular weight ($\overline{M}_n$) fractionation ranges being 0–4000 and 0–20,000 for Enzacryl Gel K1 and Enzacryl Gel K2, respectively. Anomalous retardation of the Schardinger dextrins, cyclomaltohexose and cyclomaltoheptose, is observed although linear maltosaccharides behave normally. The internal gel volumes, calculated from column elution data in water, are significantly larger than the volumes of solvent imbibed by the dry column packings on constituting the gel. Internal gel volumes and solvent imbibition volumes in water are compared with the corresponding values obtained in chloroform and tetrahydrofuran. The two parameters are discussed, in the case of Enzacryl Gel K2 in water and chloroform, in the light of plots of logarithm viscometric hydrodynamic volume *versus* K_d for polyethylene glycols.

INTRODUCTION

Aqueous gel permeation chromatography (GPC) of small organic solutes is usually performed using xerogels derived from cross-linked dextran (Sephadex)[1,2] or cross-linked polyacrylamide (Bio-Gel P)[3,4]. These materials are soft gels of high capacity ratio. Agarose gel[5], the matrix of choice for the fractionation of water-soluble macromolecules, is too porous to be effective in separating small solutes. This is also true of semi-rigid, lightly sulphonated, cross-linked polystyrene[6] and the inorganic aerogels, porous silica[7] and porous "silica-rich" glass[8]. Other hydrophilic column packings for GPC are those based on cross-linked poly(2-hydroxyethyl methacrylate)[9]. The latter may be prepared either as simple, xerogel-forming, materials or with a macroporous structure and hold promise for the fractionation of both low- and high-molecular-weight solutes.

Recently, we have described the preparation of two new column packings for GPC. These are both bead polymerised, cross-linked poly(acryloylmorpholines) (Enzacryl Gel K1 and Enzacryl Gel K2). Organic xerogels, derived by swelling

these materials in chloroform or tetrahydrofuran, have proved effective for the GPC of small molecules[10]. It seemed probable, from swelling measurements, that xerogels derived from cross-linked poly(acryloylmorpholines) by imbibition of water would also be suitable in this application. The present studies have proved this to be the case, classical GPC behaviour being observed on aqueous chromatography of saccharides, polyethylene glycols and oligomeric α,ω-diols.

Insofar as Enzacryl Gel is applicable in both aqueous and organic solvents it is a "universal" GPC matrix. In this respect it is similar to the hydroxypropyl derivative of cross-linked dextran (Sephadex LH-20)[11] and the simple poly(2-hydroxyethyl methacrylates). However, hydrated Enzacryl Gel differs from aqueous xerogels based on dextrans, poly(2-hydroxyethyl methacrylates) and polyacrylamides in one important respect.The network of cross-linked poly(acryloylmorpholine) chains comprising the Enzacryl Gel matrix has relatively few labile protons to exchange with the eluting solvent or its deuterated equivalent. Consequently it should be possible to determine internal gel volumes for each column precisely and without further correction by subtracting the elution volume for Blue Dextran (the void volume) from that recorded on elution of a sample of deuterium oxide. Reliable column calibration should, in turn, facilitate accurate determination of Wheaton and Baumann[12] distribution coefficients. Rarely has this been achieved for aqueous xerogel packings effective in fractionating low-molecular-weight solutes.

EXPERIMENTAL

Commercial materials

Enzacryl Gel K1 and Enzacryl Gel K2, 72–240 dry mesh, were supplied by Koch-Light, Colnbrook, Great Britain. Dextrans T 10, T 20 and T 40 ($\overline{M}_n$= 5700, 15,000 and 25,700) and Blue Dextran ($\overline{M}_w=2\cdot10^6$) were purchased from Pharmacia, London, Great Britain. Polyethylene glycols were obtained from Phase Separations, Queensferry, Great Britain ($\overline{M}_n$=200, 400, 600, 750, 1000, 1500 and 4000), from BDH, Poole, Great Britain ($\overline{M}_n$=6000) and from Micro-Bio Labs., London, Great Britain ($\overline{M}_n$=18,500 and 37,500).

Packing and elution of columns

Glass GPC columns of 2.5×45 cm were obtained from Pharmacia. Dry Enzacryl Gel beads were dispersed in distilled water and allowed to swell overnight to attain equilibrium. The stirred slurries were then "degassed" and poured into the chromatographic columns. Packing was completed under flow at 15 $ml\cdot h^{-1}\cdot cm^{-2}$.

For GPC, columns were connected to the proportionating pump of a Technicon AutoAnalyzer and eluted at a flow-rate of 2 $ml\cdot h^{-1}\cdot cm^{-2}$ at ambient temperature. Samples to be chromatographed were injected directly into the solvent inlet line. Sample volumes injected did not exceed 1 ml and solute concentrations were generally less than 1%.

Determination of column and gel parameters

The void volume, V_0, was estimated from the elution volume of the totally excluded solute, Blue Dextran. It was assumed that the elution volume, V_s, of the mobile phase corresponded to that obtained on elution of a sample of deuterium

oxide. The permeation behaviour of each partially included solute was characterised by measuring their elution volume, V_e, and calculating the Wheaton and Baumann[12] distribution coefficient, K_d, using the relationship

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_0}{V_s - V_0}$$

where V_i is the internal volume of the gel packing available to a totally included solute.

In order to quantify column efficiency the number of theoretical plates per metre, n, for glucose was calculated by applying the equation[13]

$$n = \frac{16}{L}\left(\frac{V_e}{w}\right)^2$$

where L is the column length and w the peak width. For a given peak w was determined by producing tangents through the points of inflection and measuring the distance between the points at which the tangents intersected the baseline.

Analysis of column effluents

Deuterium oxide was estimated by mass spectrometry. Peak height for the molecular ion was related to deuterium oxide concentration by means of a linear calibration graph obtained on assay of standard water–deuterium oxide mixtures. Blue Dextran was monitored spectrophotometrically at 260 nm.

Polyethylene glycols and saccharides were routinely monitored by automated procedures using Technicon modular equipment. In the case of polyethylene glycols, the automated method was similar to that described by Johnson and Samuelson[14], for uronic acids. Thus column effluents were sampled continuously in the Auto-Analyzer and the samples diluted five fold with chromic acid reagent. The resulting mixture was heated for 3 min at 95°, cooled, "debubbled" and the absorbance recorded at 440 nm. Routine monitoring of saccharides was achieved by continually assaying samples of column effluent for total hexose by the automated cysteine–sulphuric acid assay as described by Barker *et al.*[15].

When, as in the case of dextrans, it was desired to determine the average molecular weight, $\overline{M}_n$, of polysaccharide emerging from the column, part of the eluate was monitored continuously by automated hexose assay and the remainder collected in fractions. These were subjected to manual assay with ferricyanide reagent to determine reducing end groups[16]. Since each dextran molecule normally contains only a single reducing end group it was possible, knowing the total hexose content of the sample, to calculate the number of hexose residues per molecule and hence $\overline{M}_n$.

RESULTS AND DISCUSSION

Aqueous xerogels derived from Enzacryl Gel K1 and Enzacryl Gel K2 behaved as typical GPC packings in that, in both cases, solutes were eluted from the gel bed in a sequence of decreasing molecular weights. For all solutes, characteristic Gaussian distribution curves were obtained and in no instance was there any

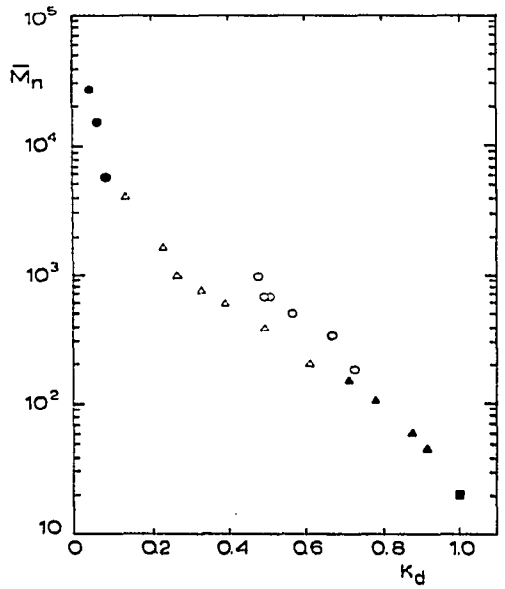
evidence of "tailing". Column efficiencies, expressed in terms of the number of theoretical plates per metre for the solute glucose (Table I), were comparable to those obtainable with other aqueous xerogel packings[17].

TABLE I

COLUMN PARAMETERS AND EFFICIENCIES

Column packing	*Column parameters*				*Theoretical plates (m^{-1}) for glucose*
	V_0 (*ml*)	V_s (*ml*)	V_t (*ml*)	W_g (*g*)	
Enzacryl Gel K1	69.6	161.1	188.1	45.14	2690
Enzacryl Gel K2	51.5	167.75	189.1	33.75	1780

Near ideal plots of logarithm molecular weight *versus* K_d were obtained for polyethylene glycols and linear, oligomeric α,ω-diols (Figs. 1 and 2). As anticipated, oligosaccharides, being more compact molecules, had relatively higher K_d values (Table II) and were best fractionated on Enzacryl Gel K1. Polydisperse solutes traversed the columns with significant band broadening, which was ascribed to fractionation into zones of diminished dispersity and progressively decreasing



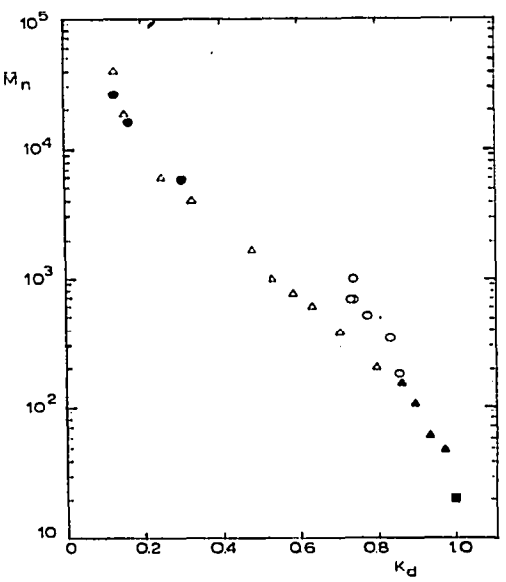


Fig. 1. Relationship between logarithm molecular weight and distribution coefficient for solutes chromatographed on Enzacryl Gel K1: (●) dextrans; (△) polyethylene glycols; (○) oligosaccharides; (▲) small α,ω-diols; (■) deuterium oxide.

Fig. 2. Relationship between logarithm molecular weight and distribution coefficient for solutes chromatographed on Enzacryl Gel K2: (●) dextrans; (△) polyethylene glycols; (○) oligosaccharides; (▲) small α, ω-diols; (■) deuterium oxide.

TABLE II

K_d VALUES FOR VARIOUS SOLUTES IN GPC ON ENZACRYL GEL

Solute	*Enzacryl Gel K1*	*Enzacryl Gel K2*	*Solute*	*Enzacryl Gel K1*	*Enzacryl Gel K2*
Formaldehyde	0.92	0.96	Glucose	0.73	0.86
Ethylene glycol	0.87	0.93	Maltose	0.67	0.83
Diethylene glycol	0.78	0.89	Raffinose	0.57	0.77
Triethylene glycol	0.71	0.86	Stachyose	0.51	0.74
Polyethylene glycol 200	0.61	0.80	Isomaltotetrose	0.49	0.74
Polyethylene glycol 400	0.50	0.71	Maltohexose	0.48	0.74
Polyethylene glycol 600	0.39	0.64			
Polyethylene glycol 750	0.32	0.59	Dextran T 10	0.09	0.30
Polyethylene glycol 1000	0.26	0.53	Dextran T 20	0.06	0.17
Polyethylene glycol 1500	0.22	0.48	Dextran T 40	0.04	0.14
Polyethylene glycol 4000	0.14	0.33			
Polyethylene glycol 6000	–	0.26	Schardinger α dextrin	0.93	1.03
Polyethylene glycol 20,000	–	0.16	Schardinger β dextrin	2.20	1.88
Polyethylene glycol 40,000	–	0.13			

molecular weight. This was confirmed, on chromatography of Dextran T 10 on Enzacryl Gel K2, by assaying successive fractions of eluant containing the emerging solute band both for total hexose[15] and reducing end groups[16]. This enabled the average molecular weight of the polysaccharide in each fraction to be calculated. A plot of logarithm average molecular weight *versus* K_d gave a near linear relationship (Fig. 3).

Columns packed with xerogels derived from cross-linked poly(acryloylmorpholines) are amenable to particularly accurate calibration, since the precise elution volume, V_s, of a cross-section of the mobile phase available to a totally included solute may be determined directly by eluting a sample of isotopically

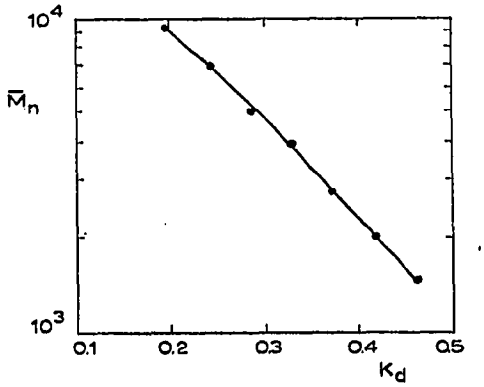


Fig. 3. Plot of logarithm molecular weight *versus* distribution coefficient for successive fractions of polysaccharide obtained on chromatography of a sample of Dextran T 10 on Enzacryl Gel K2.

labelled solvent. This calibration technique was first used by Ackers[17] for the calibration of GPC columns packed with aqueous gels derived from cross-linked dextran (Sephadex). In the case of Sephadex gels and gels derived from cross-linked polyacrylamide (Bio-Gel P), the polymer matrix contains a large number of labile protons in dynamic equilibrium with the eluant (water) or its isotopically labelled equivalent. This presents a serious complication in column calibration because, to a deuterated or tritiated water molecule, the volume occupied by the labile protons of the polymer matrix behaves as an extension of the mobile phase. For example, in the case of Sephadex G-25 Marsden[18] found that the internal volume of the gel permeable to tritium oxide was 6% greater than that of the volume of water imbibed on swelling the dry matrix. Due correction had to be made when tritium oxide was used to determine V_s. In the present studies V_s was determined as the elution volume of a sample of deuterium oxide. This was estimated in column effluents by a quantitative mass spectrometric technique[10]. No correction for isotope exchange was made since the linear poly(acryloylmorpholine) chains comprising the gel matrix do not contain labile protons. Exchange with the secondary amide protons of the cross-links was neglected since their gross contribution to the gel matrix is very small. Simple, direct measurement of V_s is useful because it facilitates determination of the internal volume, V_i ($=V_s-V_0$), of the gel.

Good evidence for the accuracy of internal gel volumes calculated from isotope elution data is provided by the plots of logarithm molecular weight *versus* K_d ($=\overline{V_e-V_0}/V_i$) (Figs. 1 and 2). Both plots extrapolate precisely to the coordinates occupied by ^2H_2O, the K_d value of which must be unity. This implies that the whole of the water within the gel contributes to the internal gel volume available to a totally included solute.

In Table III internal gel volumes obtained from elution data are compared with the volumes of solvent imbibed by corresponding amounts of dry Enzacryl Gel on swelling to equilibrium. It is apparent that the internal volume obtained from elution data, the "dynamic internal volume", may differ considerably from the volume of solvent imbibed. This is the case, not only for water, but for other solvents, notably chloroform and tetrahydrofuran, in which xerogels derived from Enzacryl Gel may also be used for GPC[10]. These observations are important

TABLE III

COMPARISON OF INTERNAL GEL VOLUMES AND SOLVENT IMBIBITION VALUES

Column packing	*Solvent*	*Internal volume* V_i ($ml \cdot g^{-1}$)	*Imbibition value* S ($ml \cdot g^{-1}$)
Enzacryl Gel K1	Water	2.03	1.74
Enzacryl Gel K2	Water	3.45	2.43
Enzacryl Gel K1	Chloroform	1.85	1.86
Enzacryl Gel K2	Chloroform	3.05	2.61
Enzacryl Gel K1	Tetrahydrofuran	0.79	1.30
Enzacryl Gel K2	Tetrahydrofuran	1.07	1.77

because, traditionally, the solvent imbibition value for the total xerogel packing in a given column has been assumed to correspond to V_t[19–21]. This general assumption, which is clearly invalid for cross-linked poly(acryloylmorpholine) gels, still persists[22–24].

Regardless of the GPC solvent or the organic polymer matrix involved, xerogel formation is a process of dissolution resulting in a constrained solution of cross-linked polymer chains. The parts of this solution which correspond to the "dynamic internal volume", V_i, and the volume occupied by the constrained poly(acryloylmorpholine) matrix, V_m, are both problematical and will vary from solvent to solvent. A simple relationship between these parameters and the volumes occupied by the solvent component and dry polymer matrix in isolation seems unlikely. It is clear from Table III that, for Enzacryl Gel, V_i may be greater or less than the volume of solvent imbibed in constituting a given xerogel.

Discrepancy between dynamic internal volumes and solvent imbibition values has often been recorded for aqueous xerogels derived from the less porous cross-linked dextrans (Sephadex G-10, G-15 and G-25)[18,21,24]. In contrast to our findings with aqueous xerogels derived from cross-linked poly(acryloylmorpholines), the dynamic internal volumes of dextran gels, though difficult to measure precisely, are much smaller than the corresponding solvent imbibition volumes. It has been common practice to assume that the solvent imbibition value for cross-linked dextran gives a true measure of V_t and that the discrepancy arises owing to "water of hydration" which is tightly bound to the gel and impermeable to the molecular species undergoing GPC. Unfortunately, it is difficult to obtain unambiguous evidence for or against "water of hydration" in dextran gels. However, in the case of cross-linked poly(acryloylmorpholines), it is clear that "water of hydration", if indeed it exists, has no influence on GPC elution volumes. The relatively greater "dynamic internal volume" of poly(acryloylmorpholine) gels implies a superior capacity ratio to dextran gels derived from comparable amounts of polymer. This is important in the case of xerogels designed to fractionate small molecules.

In the case of Enzacryl Gel K2 and polyethylene glycols, it is of interest to compare K_d values obtained in water with those which we have reported previously for GPC in chloroform[10]. Such comparison is not possible in the case of Enzacryl Gel K1 because, in organic solvents, the terminal hydroxyl groups of the necessarily small polyethylene glycols lead to sorption. Since the dynamic internal volume, V_i, of Enzacryl Gel K2 is greater in water than in chloroform (Table III) then, assuming pore size to be proportional to V_i, K_d values for molecules of similar size should be larger for aqueous GPC. In practice, polyethylene glycols of higher molecular weight have relatively greater K_d values in water, and those of lower molecular weight have greater K_d values in chloroform (Table IV). This generalisation holds when due allowance is made for the difference in hydrodynamic volume of polyethylene glycols in the two solvents. This is apparent from plots of the logarithm of the solute viscometric hydrodynamic volume, $[\eta]\overline{M}_n$, where η is the limiting viscosity index, against K_d (Fig. 4). These results indicate that the pore size distribution of xerogels derived from Enzacryl Gel may vary depending on the solvent component. This adds weight to our earlier hypothesis, proposed on the basis of swelling measurements in a range of solvents, that cross-linked poly(acryloylmorpholines) undergo different gelation mechanisms in aqueous and non-aqueous media[10].

TABLE IV

K_d VALUES OBTAINED ON GPC OF POLYETHYLENE GLYCOLS ON ENZACRYL GEL IN WATER AND CHLOROFORM AND CORRESPONDING SOLUTE VISCOMETRIC HYDRODYNAMIC VOLUMES

Molecular weight of polyethylene glycol, $\overline{M}_n$	*Distribution coefficient,* K_d, *in* H_2O	*Hydrodynamic volume,* $[\eta]\overline{M}_n$, *in* H_2O	*Distribution coefficient,* K_d, *in* $CHCl_3$	*Hydrodynamic volume,* $[\eta]\overline{M}_n$, *in* $CHCl_3$
40,000	0.13	23,000	0.05	34,000
20,000	0.16	7,660	0.12	11,000
4000	0.33	492	0.38	680
1500	0.48	—	0.54	105

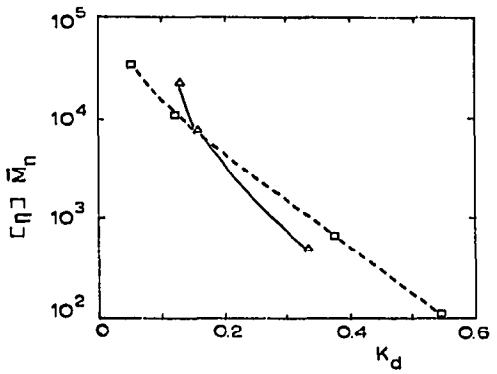


Fig. 4. Plots of logarithm intrinsic viscosity *versus* distribution coefficient for polyethylene glycols chromatographed on Enzacryl Gel K2 in water (△) and in chloroform (□).

Of all the solutes subjected to aqueous GPC on Enzacryl Gel, anomalous elution behaviour was observed only in the case of the Schardinger dextrins, cyclomaltohexose and cyclomaltoheptose. The elution profile recorded on Enzacryl Gel K1 (Fig. 5) bore a striking resemblance to that obtained by Carter and Lee[25] on chromatographing these solutes on Sephadex G-15. It is of interest to compare the retention volume, $V_e - V_0$, for each Schardinger dextrin relative to that of glucose in the two experiments. Surprisingly, the relative retention volumes of cyclomaltohexose on the two column packings were identical, both being 1.2 times that of glucose. For cyclomaltoheptose, the retention volumes relative to glucose were 2.8 and 2.0 for Enzacryl Gel and Sephadex G-15, respectively. It is relevant that normal GPC behaviour has been observed[25] on chromatography of Schardinger dextrins on the tightly cross-linked polyacrylamide, Bio-Gel P-2.

Since Schardinger dextrins are known to form molecular inclusion complexes with molecules of similar size to the pendant morpholino groups on Enzacryl Gel[26, 27], it is tempting to speculate that anomalous retardation is caused by a specific inclusion mechanism. However, such an interpretation may be naive in view of the similarity of the elution profiles obtained on Enzacryl Gel K1 and Sephadex G-15,

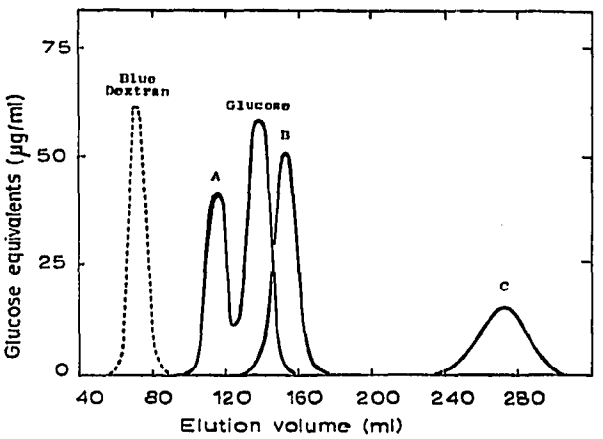


Fig. 5. Chromatography of a mixture of Blue Dextran, maltohexose (peak A) and glucose and of a mixture of cyclomaltohexose (peak B) and cyclomaltoheptose (peak C) on Enzacryl Gel K1. Column dimensions 2.5 × 33 cm.

which consists essentially of a cross-linked space network of dextran chains. The latter are comprised of (α-1→6) linked glucose residues which, if sorption is a specific inclusion process, must be able to penetrate the cyclodextrin ring. Even assuming that the dextran chain is in the most favourable conformation for this to occur, steric effects preclude more than minimal penetration. Possibly, in the sorptive retardation of cyclomaltohexose, neither the morpholino group nor the accessible part of the intercatenate glucose rings can fully penetrate the Schardinger helix. Facile penetration of the cyclomaltoheptose ring by the morpholino groups and restricted penetration by the intercatenate glucose residues might explain the differing retardation of the oligosaccharide on the two polymers.

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