STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. X. THE DEPENDENCE OF THE EQUILIBRIUM DISTRIBUTION COEFFICIENT OF A SOLUTE IN POLARIZED WATER ON THE MOLECULAR WEIGHTS OF THE SOLUTE: EXPERIMENTAL CONFIRMATION OF THE "SIZE RULE" IN MODEL STUDIES

G. N. LING and W. HU
Department of Molecular Biology, Pennsylvania Hospital, Eighth and Spruce Streets, Philadelphia, PA *19107.*

The equilibrium distribution of I4 sugars, sugar alcohols, and other nonelectrolytes in solutions of polyethylene oxide (PEO) and of native and alkali-denatured bovine hemoglobin were studied over wide concentration ranges. The results show that the equilibrium concentrations of all the solutes studied are rectilinearly related to their external concentrations. This straight-line relationship demonstrates the existence of these solutes entirely or almost entirely in the aqueous phase of these systems. Therefore the slope of each of these straight lines equals the equilibrium distribution coefficient or q-value of the solute involved. In general, the q-values decrease with increasing molecular weights (M. W.) of the solutes in 15% solutions of PEO, 20% solutions of alkali-denatured hemoglobin (and in 18% gelatin) but not in 39% solution of native hemoglobin. In solutions of PEO, of alkali-denatured hemoglobin studied (and of gelatin) a fraction of the water (20% to 30%) appears to have solvency similar to that of normal liquid water. The experimental findings of M. W.-dependent solute exclusion were discussed in the light of four alternative theories that have been offered to explain this type of phenomena. Among these four theories only thepolarized multilayer theory agrees with most, if not all the facts known.

INTRODUCTION

According to the polarized multilayer (PM) theory of cell water, all or virtually all water in resting living cells exists in the state of polarized multilayers as a result of interaction with the exposed NH and CO groups of a matrix of fully extended polypeptide chains, pervasively present in all living cells. Investigations in this laboratory and elsewhere have shown that various physiological manifestations of the living cell that have remained thus far unexplained or have been attributed to other causes are, in fact, expressions of the unusual physico-chemical properties of cell water existing in the state of polarized multilayers.

Among the physiological manifestations of the living cells that have remained unexplained hithertofore but are now explained in terms of the PM theory is the essential constancy of the water content of each type of adult normal living cells under physiological conditions (Ling and Hu, 1987). Osmotic activity of the living cells offers one example of physiological phenomenon once attributed to wrong causes, i.e., free K^+ (and free anions) in cells now shown to be adsorbed and thus osmotically inactive, but is readily explained by the reduction of water activity due to its multilayer polarization (Ling, 1983). Theoretically, a second set of examples is the phenomenon of the equal distribution of urea and ethylene glycol between intra- and extracellu-

lar water, once believed to indicate normalcy of the bulk of cell water (Hill, 1930; McLeod and Ponder, 1936), and the maintained low levels of Na^+ and other solutes in living cells attributed to the continual operation of postulated membrane pumps that have been shown to be untenable from energy and other considerations (Ling, 1962; 1984). Indeed it was the difficulty with solute distribution problem that had led to the development of the association-induction (AI) hypothesis. Its subsidiary, the polarized multilayer (PM) theory of cell water offers at once an explanation for the equal distribution of urea, ethylene glycol side by side with the unequal distribution of Nd, sugars, and free amino acids in terms of what is now known as the *"size rule."*

In the PM theory, solutes are found in the polarized cell water in a size-dependent manner. Solute molecules that are small and/or can tit into the dynamic water structure like urea or ethylene glycol may exist at equilibrium in the same or even higher concentration in the polarized water than that in normal liquid water with an equilibrium distribution coefficient (q) equal to or exceeding 1; solute molecules that are large and complex like hydrated Na^+, sugars, and free amino acids are, as a rule, found in lower concentration; $q < 1$. This size dependency in solute distribution or size rule is theoretically the consequence of the two-fold molecular mechanisms for solute exclusion: it costs more energy (or enthalpy) to excavate a (larger) hole in the polarized water to accommodate a (larger) solute molecule than the energy gained in filling the hole left behind in normal liquid water; a larger solute also loses more motional-but especially rotational motional-freedom when placed in "stickier" and less mobile, polarized water with a consequent loss of entropy (Ling, 1972).

The size rule has already received verification in living cells as well as in model systems (see Ling, 1984, p. 272 to 278) but the verification was sketchy and not definitive because more often the distribution data were in the form of the "apparent equilibrium distribution coefficients" (p-value) which may or may not equal the true equilibrium coefficient due to possible inclusion of unknown amount adsorbed solute in the cell or model system. The present communication, the first of a succession of papers devoted primarily to a more rigorous and comprehensive testing of the size rule on living cells and model systems, describes results of experimental testing on various inanimate model systems of the PM theory in general and the size rule in particular.

MATERIALS AND METHODS

The basic method used in these studies is equilibrium dialysis. Spectra Por 2 tubing (diameter 6.4 mm) with molecular weight cutoff at 12,000 to 14,000 was used. With the aid of 1 ml. syringe, a concentrated protein or polymer solution was introduced into the dialysis tubing which was tightly closed at one end with ligatures of nylon monofilaments. The protein solution introduced is squeezed to the closed end of the sac as tightly as possible with a pair of rubber tipped hemostats before the second set of ligatures was made. Three or four sacs were placed in each 30 ml screw cap Pyrex culture tube, which held 20 ml. of the experimental solution containing various probe molecules under study with or without radioactive labels. Laid horizontally, the tubes were shaken on a New Brunswick rotary shaker at a rate of 60 cycles per minute in a constant temperature room (25" $\pm$ 1°C) usually for five days but sometimes longer.

After incubation, the sacs were blotted dry and the content of each sac removed and divided into two portions. One portion was transferred to a preweighed 5 ml polystyrene cup (Fisher Scientific, Disposable microbeakers, Cat. No. 2-544-30), weighed, dried in a

vacuum oven (80°C, 20 mm Hg vacuum, usually 48 hours), and weighed again to determine the water content, The other portion was transferred to the bottom of a preweighed 15 ml Nalgene centrifuge tube and the assembly weighed to determine the weight of the sample. 2 ml of 0.3 M LiOH solution was then added. Dissolution was complete after overnight incubation, after which 2 ml of 0.6 M solution of trichloroacetic acid was added and mixed. After centrifugation, 0.5 ml of the clear supernatant was taken out and either mixed with Bray's scintillation fluid (Bray, 1960) for radioactivity assay in a β-scintillation counter, or chemically analyzed (see below). Samples of the initial and final supernatant solutions were also diluted with LiOH and TCA solution to produce the same final concentrations of Li^+ and TCA in the samples and in the diluted supernatant solution.

To assay accurately the concentration of radioactively labeled solutes we found it necessary to determine the quenching factor of each individual sample by counting all samples a second time after the addition of an exactly measured small volume of a solution (with a Hamilton micropipettor) containing the same radioactive isotope (^{14}C or ^{3}H as the case may be) but at a concentration at least five times higher than those of the samples measured for the first time. Extra counts obtained allow accurate correction for errors due to unavoidable differences in the chemical composition of the samples and the supernatant solutions.

The following chemical assay methods were used on tissue extracts and bathing solutions described above: polyols (Burton, 1957); pentoses (Roe and Rice, 1948); D-glucose (Hyvarinen and Kikkila, 1962); inulin (Roe et al., 1949).

The following chemicals used were from Sigma Chemical Co., St. Louis, MO: bovine hemoglobin (about 75% methemoglobin and 25% oxyhemoglobin) (Lot 112F-9300); erythritol (Lot 57C-0058); xylitol (Lot 59C-00401); D-sorbitol (Lot 34F-0016); D-mannitol (Lot 81F-0517); D-xylose (Lot 23C-1640); D-arabinose (Lot 87C-0440); D-trehalose (Lot 57C-3875); polyethylene glycol, PEG-3350 (Lot 16F-0477). Other chemicals and radiochemicals were from following sources: polyethylene oxide, WSR-205, Lot 2204 (gift from Union Carbide); ethylene glycol, Lot 018341 (Baker); glycerol, Lot 735506 and sucrose, Lot 851322 (Fisher Scientific); inulin (Pfanstiehl); D-raffinose, Lot 00302BM (Aldrich); ?-ethylene glycol (Lot 1072-288); ^{14}C-inulin (Lot 1141-229) and ^{14}C-glycerol (Lot 1258-206) (New England Nuclear); ^{3}H-mannitol (ICN); ^{14}C-D-xylose, Batch 29 (Amersham); ^{14}C-sucrose, Lot 2063-l 18, and ^{3}H-PEG-4000, Lot 2109-295, (Du Pont).

RESULTS

Time Course of Sucrose Distribution in Dialysis Sacs Containing Hemoglobin.

Figure 1 shows the time course of equilibration of labeled sucrose between 39% hemoglobin solutions within the dialysis sacs and the external bathing medium containing 0.4 M NaCl (A) or NaOH (B). For native hemo-

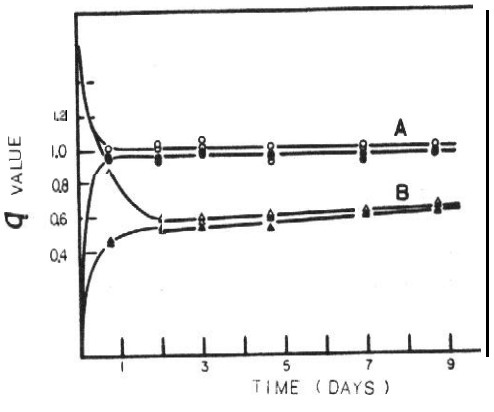

FIGURE 1. Dual time course of equilibration of sucrose in a solution containing 3% hemoglobin, 50 mM sucrose, and 0.4 NaCl (0, 0) or 0.4 NaOH (A, Δ) (25°C). For each set of experiments, in one group the labeled sucrose was added inside the dialysis bag with hem&-bin; in the other it was added outside.

globin solution at neutral pH (A), equilibrium in 0.4 M NaCl was reached at one day or earlier and remained essentially unchanging for eight days following. However, when the labeled sucrose was added to the inside of the sacs (open circles) the equilibrium level of sucrose reached stayed slightly but consistently above the equilibrium level reached in the companion experiment in which the labeled sucrose was added to the outside solution. The same disparity was seen also for the alkaline hemoglobin solution dialyzing in 0.4 M NaOH (Figure 1B). Only here, it took two days to reach equilibrium and the q-value was much lower than 1.0; after two days of incubation, the q-value of sucrose slowly but steadily increased from below 0.5 to above *0.6.*

The constancy of the q-value for sucrose in neutral hemoglobin solution was paralleled by a constancy in the water content of the sacs. In contrast, the steadily rising q-value of sucrose in alkaline hemoglobin solution was paralleled by a steady increase of the water contents of the sacs from 73.6 ± 0.51 (S.D.) to *79.7 ± 0.34.* Since an increase of water content as a rule causes an increase of q-value for probe molecules (Ling et al., 1980a; Liig and Ochsenfeld, 1983), the water-content change may offer at least a partial explanation of the gradual rise of q-value for sucrose in solutions of alkaline hemoglobin immersed in solution of Na citrate had been previously reported (Ling and Ochsenfeld, 1977, Figures 11, 12).

Now very high pH is known to cause hemoglobin to split into its subunits (Hasselrodt and Vinograd, 1959). An increase of osmotic activity and swelling of in the sac may thus occur as a result. While subunit separation might have played a role in the continued increase of water content (and q-value rise for sucrose), it is not likely the sole cause of the observed swelling. Thus extremely *low* pH also causes subunit separation (Reichmann and Colvin, 1956) but does not cause continued rise of water contents (Ling and Hu, unpublished).

Sucrose is known to pass very slowly through inverted frog skin and models of activated cellulose acetate membranes (Ling, 1973). Similarly, sucrose in dialysis sacs containing urea-denatured proteins also took fully 3 days to reach diffusion equilibrium (Ling and Ochsenfeld, 1988). For the sake of safety, we adopted a standard five-day incubation period for all solutes studied and described in this communication, with the radioactively labelled probe always added to the external bathing solution. It may be mentioned that even the largest solute molecule investigated (polyethylene glycol with a molecular weight of 4000, PEG-4000) reached diffusion equilibration in 39% of native hemoglobin (at neutral pH) in five days. At acidic and alkaline pH, however, the diffusion equilibration was less complete even after five days of incubation but the error introduced was estimated at no more than 10% on the low side.

The Equilibrium Distribution of Nonelectrolytes in Solutions of Alkali-denatured and of Native Bovine Hemoglobin.

Figure 2 shows the equilibrium distribution profiles of 9 nonelectrolytes in alkaline hemoglobin solution; each point is the average of at least 3 determinations. The standard deviations of each point, not shown in Figure 2, are given in Table I, which also includes two other nonelectrolytes studied, xylitol and sorbitol, (also not shown in Figure 2 in order to avoid overcrowding). That the distribution curves of all 11 electrolytes are indeed rectilinear is supported by the high positive linear correlation coefficient (T) between the equilibrium concentration of the solute in the bag. $[S]_{in}$ and outside the bag, $[S]_{ex}$; the overall average and standard deviations (S.D.) of the r's for all 11 sets of data are i-O.994 ± 0.007. The rectilinearity of the distribution curves affirms that the slopes of each straight

line curve represents the true equilibrium distribution coefficient or q-value for that solute. In the data presented, the q-values range from near unity (0.998) for ethylene glycol to 0.258 for polyethylene glycol, (PEG-4000). Although the initial concentration of the hemoglobin solution was 39%, its fmal concentration was on the average about 2%. That is, considerable swelling occurred in consequence of the exposure of the hemoglobin to the strongly alkaline solution.

Figure 3 shows a parallel study of the distribution of 10 nonelectrolytes in a neutral solution of native hemoglobin, also at an initial hemoglobin concentration of 39%. Here the sacs did not swell; instead, the hemoglobin in these sacs remained at essentially the same concentration at the end of the incubation. All 10 nonelectrolytes also show perfectly rectilinear distribution as demonstrated by the positive linear correlation coefficients (r) between $[S]_{in}$ and $[S]_{ex}$ (Table II). The average and S.D. of the r values of all 10 sets of data are $(+0.996) \pm (+0.002)$.

In Figure 4 the q-values of the 11 nonelectrolytes in alkaline solutions of hemoglobin (open circles) and of the 10 nonelectrolytes in neutral solutions of native hemoglobin (half-tilled circles) are plotted against the respective molecular weights of the nonelectrolytes. Whereas in native hemoglobin solution, the q-values of the different nonelectrolytes all hover around the value of unity with no conspicuous trend of changes as the molecu-

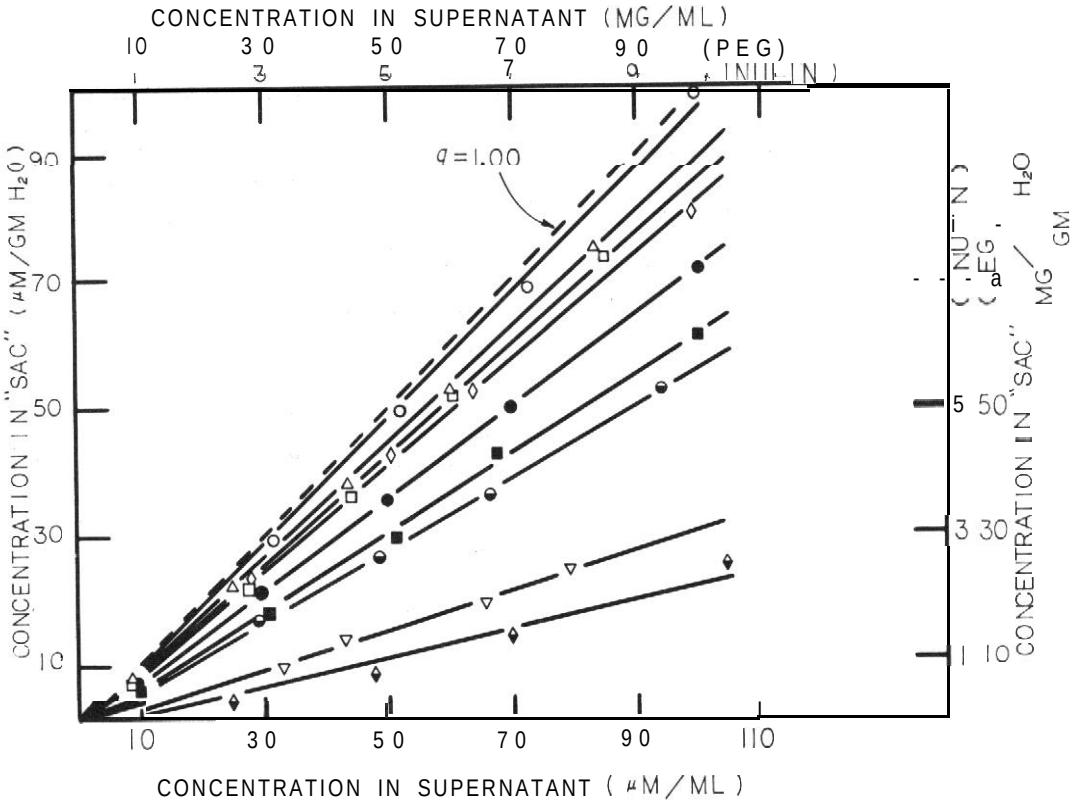

FIGURE 2. Equilibrium distribution of various solutes in an alkaline solution containing 2% bovine hemoglobin and 0.4 M NaOH. Incubation time was 5 days (25°C). Ethylene glycol 0, Glycerol A, Erythritol □, Mannitol ◊, Sucrose ●, Trehalose ■, Raffinose ◒, Inulin ▽, PEG V

lar weights increases*; in the alkali-denatured hemoglobin solution, there is a pronounced decline of the q-values of the nonelectrolytes as their molecular weights increases. Thus alkali-denatured hemoglobin at a final concentration of 20% exercises strong influence on the solvency of the bulk phase water, whereas native hemoglobin at a final concentration nearly twice as high (i.e., 39%) exercises much less or no influence on the solvency of the bulk phase water for solutes ranging in molecular weight from 62.1 (ethylene glycol) to 504.5 (raffinose). However, this dependency of the q-value on the molecular weight in alkali-denatured hemoglobin did not continue steadily with increasing molecular weight. Rather as the M.W. increases, the q-value begins to flatten out when the molecular weights reaches about 1000.

The Equilibrium Distribution of Nonelectrolytes *in Solutions of Polyethylene Oxide (PEO) and in Gelatin.*

We studied the equilibrium distribution of another set of 11 nonelectrolytes in 3% PEO solution. The final concentration of PEO at the conclusion of the five-day incubation was only 15%. The results are fully presented in Table III; again only nine of these 11 sets of the data are presented in Figure 5 to prevent confusion due to overcrowding. The two sets

*For trends of much more modest q-value changes seen in native hemoglobin solutions see Gary-Bobo, 1967.

TABLE I. The equilibrium distribution of 11 sugars, sugar alcohols, and other nonelectrolytes in an alkaline solution of alkali-denatured bovine hemoglobin initially containing 0.4 M NaOH (25°C). $[S]_{in}$ and $[S]_{ex}$ represent the equilibrium solute concentration inside and outside the dialysis bags respectively. Number given under $[S]_{in}$ represents mean ± standard deviations for at least three individual, independent determinations. q is the (true) equilibrium distribution coefficient obtained by method of least squares for all the data points of each series. r is the linear correlation coefficient for data points whose numbers are shown in brackets following the value of r. While most solute concentrations are given in millimolarity, concentration of inulin and PEG (marked with asterisks) are in units of mg/ml. Initial concentration of hemoglobin was 39%; its final concentration was 20%.

	M.W.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q	r
Ethylene glycol	62.1	31.6	29.6 ± 0.25	0.998	+0.999(13)
		52.0	49.4 ± 0.15		
		72.8	68.6 ± 0.72		
		99.6	99.0 ± 0.19		
Glycerol	92.1	8.68	9.31 ± 0.79	0.887	+0.999(15)
		24.1	22.1 ± 0.18		
		43.3	38.1 ± 0.45		
		60.0	52.7 ± 1.05		
		83.3	74.8 f0.84		
Erythritol	122.1	8.78	7.34 ± 0.18	0.856	+0.997(16)
		27.4	22.0 f0.24		
		43.9	36.3 ± 1.20		
		60.4	51.9 ± 1.72		
		84.9	73.6 f0.32		
Xylitol	152.1	28.4	26.4 ± 0.98	0.837	+0.999(13)
		48.9	42.5 ± 1.20		
		66.0	55.9 f0.28		
		100.1	85.5 ± 1.02		

of data not shown are those of D-arabinose and D-glucose. As in the data already shown above, all 11 distribution curves are rectilinear, the linear correlation coefficients, r, of the 11 sets of data being above +0.99; their average and standard deviations are (i-0.997) ± (+0.002). The q-values which equal the slopes of these rectilinear curves, range from 0.949 for ethylene glycol to 0.257 for polyethylene glycol (PEG-4000). Like alkalidenatured hemoglobin, PEO also exercises strong influence on the solvency of the bulk phase water. The q-values follow the size rule as shown in the plot of the q-values against the molecular weights (half-filled circles) and labeled A to K in Figure 6. As in the case of alkalidenatured hemoglobin, the q-values continually decreases with continually increasing molecular weights until it levels off after the molecular weights reaches about 1000.

In Figure 6 we also plotted data calculated from the equilibrium distribution levels of 17 nonelectrolytes in 18% gelatin from the work of Gary-Bobo and Lindenberg (1969). However, since the distribution ratios were estimated at a single concentration, there is some uncertainty that they indeed represent the q-values. Nevertheless, for most of their data points the assumption that they represent true q-values are probably not far from the truth. This surmise is supported firstly by their overall agreement with our PEO data which for reasons discussed above, are q-

Sorbitol	182.2	29.5	23.3 ± 2.79		
		48.1	39.9 ± 2.78	0.840	+0.991(9)
		61.6	51.1 ± 1.66		
D-mannitol	182.2	27.9	23.7 ± 1.64		
		so.2	42.7 f3.67	0.802	+0.989(11)
		63.6	52.6 ± 5.41		
		99.2	80.1 ± 1.46		
Trehalose	342	9.61	7.29 ± 0.29		
		29.3	21.6 ± 0.38		
		49.9	35.9 f0.59	0.713	+0.999(15)
		69.7	50.1 ± 0.24		
		Loo	71.7 ± l.M		
Sucrose	342	10.1	6.20 ± 0.16		
		30.7	18.4 f0.23		
		51.2	29.8 ± 0.28	0.627	+0.996(15)
		67.4	43.0 ± 1.13		
		100	61.2 ± 0.59		
Raffinose	504.5	29.0	17.2 ± 0.23		
		48.6	26.7 ± 0.40	0.552	+0.997(13)
		66.4	36.5 ± 1.10		
		94 0	5.28 ± 1.79		
Inulin*	900	3.29	0.99 ± 0.24		
		4.29	1.41 ± 0.15	0.308	+0.975(12)
		6.56	1.96 ± 0.15		
		7.91	2.49 ± 0.02		
PEG*	4000	24.9	4.59 ± 0.18		
		47.6	8.60 ± 0.25	0.258	+0.988(13)
		69.7	14.5 ± 0.21		
		lo4.L	25.9 ± 0.41		

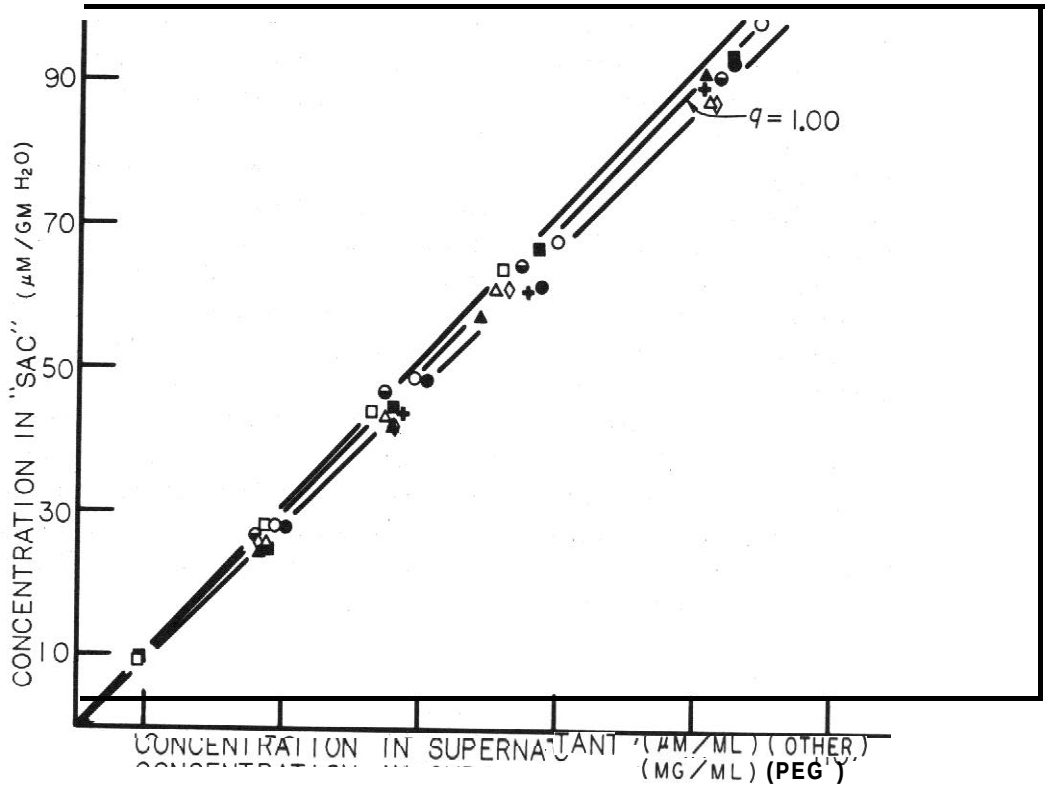

FIGURE 3. Equilibrium distribution of various solutes in a neutral solution of bovine hemoglobin (39% ± 1%) after 5 *days* of incubation at 25°C. Solution contained 0.4 M NaCl. Ethylene glycol ● . Glycerol ▲, Erythritol □, D-xylose ✚, Sorbitol A, Mannitol ◊ , Trehalose ■, Raffinose ◒, PEG-4000 ◆ .

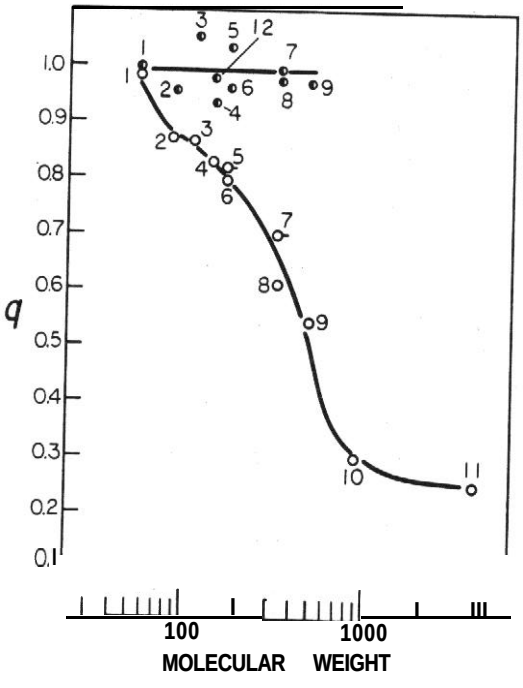

FIGURE 4. The relation between the equilibrium distribution coefficients (q) of various solutes in solutions of native hemoglobin at neutral pH (A), half-filled circles, and in solutions of alkali-denatured hemoglobin at alkaline pH (B), empty circles. 1, Ethylene glycol; 2, Glycerol; 3, Erythritol; 4, Xylitol; 5, Sorbitol; 6, D-mannitol; 7, Sucrose; 8, Trehalose; 9, Raffinose; 10, Inulin; 11, PEG-4000; 12, D-xylose. Data in A are the same as shorn in Figure 3 and Table I: data in B are the same as shown in Figure 2 and Table II.

TABLE I,. The equilibrium distribution of 10 sugars, sugar alcohols, and other nonelectrolytes in a neutral solution of native bovine hemoglobin solution initially containing 0.4 M NaCl (25°). Initial and final concentration of hemoglobin was 39%. For other information see legend of Table I.

	M.W.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q	r
Ethylene glycol	62.1	28.7	28.3 ± 0.31		
		49.0	48.8 ± 0.57	0.998	+0.999(13)
		69.6	68.1 ± 0.16		
		99.1	99.0 ± 1.03		
Glycerol	92.1	27.5	26.0 ± 1.07		
		44.8	43.4 f0.91	0.958	+0.995(11)
		60.7	61.3 ± 1.76		
		91.9	87.5 ± 3.86		
Erythritol	122.1	8.89	9.60 ± 0.17		
		27.3	28.4 f0.23		
		42.8	49.2 ± 1.46	1.053	+0.999(16)
		61.7	64.2 ± 0.25		
		103.3	108.9 ± 2.16		
D-xylose	150.1	26.5	25.7 f0.33		
		47.1	44.0 ± 3.13	0.980	+0.995(13)
		65.5	60.7 il.75		
		90.8	90.0 f2.65		
Xylitol	152.1	25.3	30.2 f0.4		
		51.2	47.8 ± 1.41	0.936	+0.991(13)
		61.2	63.2 ± 1.13		
		91.6	91.2 ±2.51		
Sorbitol	182.2	25.7	22.5; 23.0		
		47.6	42.1 f2.03	1.035	+0.994(12)
		58.5	52.3 ± 2.18		
		90.9	91.5 ± 4.18		
D-mannitol	182.2	26.5	24.5 ± 0.72		
		45.8	41.7 ± 0.57	0.961	+0.995(13)
		62.6	61.4 ± 1.29		
		92.6	89.5 ± 1.62		
Trehalose	342.3	8.30	9.03 ± 0.28		
		27.6	25.1 ± 1.03		
		45 9	44.8 ± 1.03	0.997	+0.998(16)
		67.0	67.4 ±2.00		
		94.9	94.1 i3.57		
Sucrose	342.3	30.5	27.1 ± 0.21		
		50.9	48.5 ±1.28	0.976	+0.998
		67.4	61.6 ±1.49		
		95.2	92.9 f0.95		
Raffinose	504.5	75.9	26.9 ±1.13		
		44.7	46.8 f0.4,		
		64.5	64.2 f0.79	0.971	+0.998(13)
		92.0	9.07 f2.56		

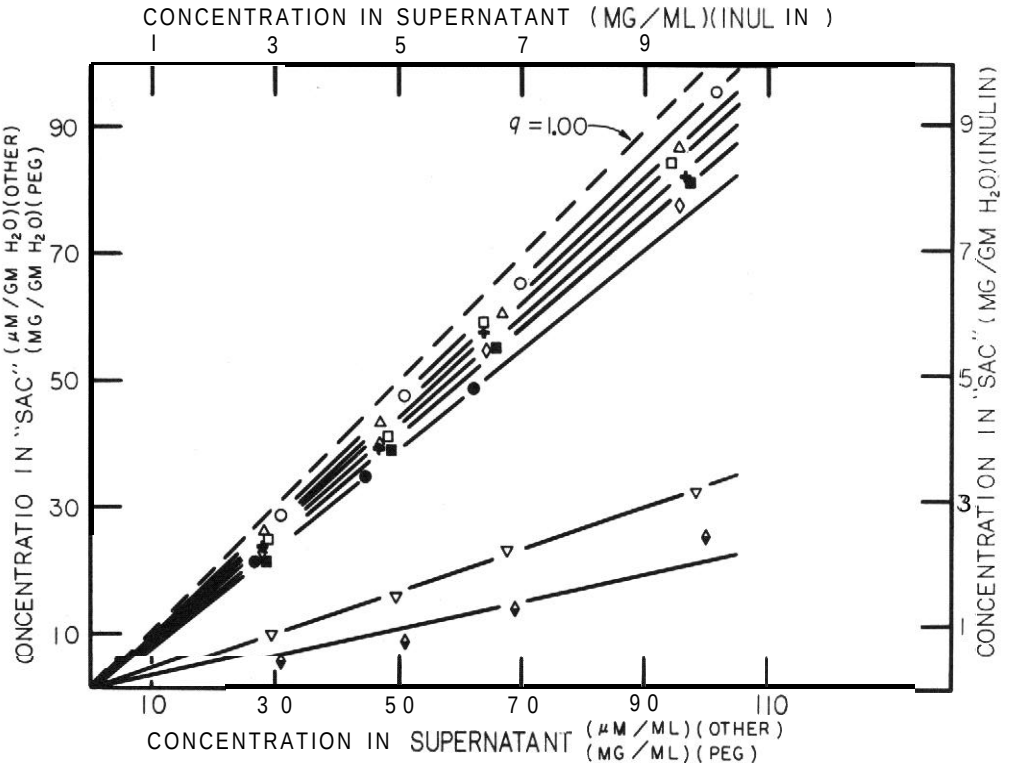

FIGURE 5. The equilibrium distribution of various solutes in a solution of polyethylene oxide (PEG). Final polymer concentration was 15%. Solution also contains NaCl(0.4 M). Ethylene glycol O, Glycerol A, Erythritol □, D-xylose â, Mannitol b, Sucrose ●, Trehalose ■, Inulin ▽, PEG-4000. ♦

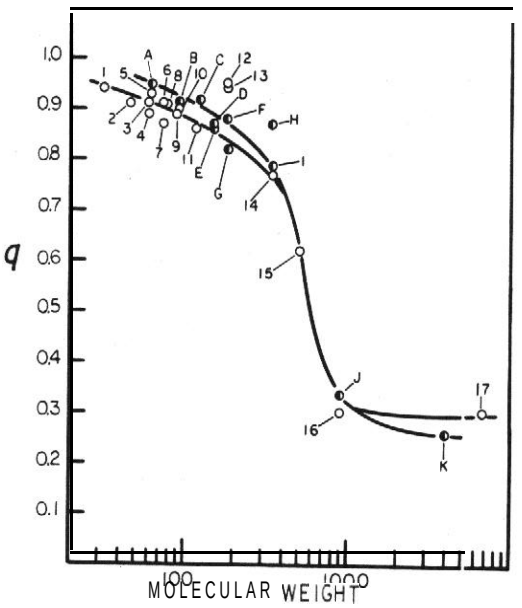

FIGURE 6. The relationship between the equilibrium distribution coefficient (q) of various nonelectrolytes in solutions of polyethylene glycol (PEO) (half-filled circles) and in gelatin (empty circles) and the M.W. of the non-electrolytes. PEO data are the same as shown in Figure 5 and Table III. The gelatin data are those of Gary-Bobo and Lindenberg (1969) on the assumption that the distribution coefficients calculated from their data are true equilibrium distribution data (see text). A, Ethylene lycol; B, Glycerol; C, Erythritol; D, D-xylose; E, D. arabinose; F, D-glucose; G, D-mannitol; H, Trehalose; I, Sucrose; J, Inulin; K. PEG-4000. I, Methanol; 2, Ethanol; 3, n-propanol (m.w. 60.1); 4, sec.-propanol (60.1); 5, Ethylene glycol (62.1); 6, n-butanol (74.1); 7, tert-butanol (74.1); 8, 1,2-propanediol (76.09); 9, Butanediol (90.1); 10, Glycerol (92.1); 11, Pinacol(ll8.2); 12, D-glucose (180.2); 13, Fructose (180.2); 14, Sucrose (342.3); 15, Raffinose (504.5); 16, Inulin (900); 17, Hemoglobin (67.000).

TABLE III. The equilibrium distribution of 11 sugars, sugar alcohols, and other nonelectrolytes in a neutral polyethylene oxide solution, initially containing 0.4 M NaCl (25°C). The initial concentration of PEG was 30%; its final concentration was 15%. For other information (e.g., *) see legend of Table I.

	M.W.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q	r
Ethylene glycol	62.1	30.9	29.1 ± 0.48		
		51.0	47.9 ± 1.72		
		69.9	65.5 ± 1.53	0.949	+0.999(13)
		101.5	96.2 ± 1.83		
Glycerol	92.1	28.1	26.3 ± 0.44		
		47.1	43.7 ± 0.28		
		66.9	60.9 ± 0.41	0.909	+0.999(13)
		95.3	87.3 f0.74		
Erythritol	122.1	28.8	25.0 f0.06		
		48.3	41.4 ± 0.004	0.920	+0.998(13)
		63.5	59.7 ± 0.55		
		94.1	84.9 ± I.69		
D-arabinose	150.1	29.8	25.1 ± 0.34		
		47.8	41.5 ± 1.06	0.861	+0.998(13)
		67.7	59.0 ± 0.29		
		95.4	81.6 ± I.82		
D-xylose	150.1	28.2	24.7 ± 0.65		
		47.4	40.7 ± 0.31	0.864	+0.997(13)
		63.7	58.1 ± 0.18		
		96.4	82.5 ± 1.11		
D-glucose	180.2	26.1	21.9 ± 0.09		
		43.2	37.7 ± 0.28	0.879	+0.998(13)
		60.3	52.9 f0.73		
		84.4	74.5 ± 1.53		
D-mannitol	182.2	27.9	23.1 ± 0.20		
		41.4	39.8 f0.54	0.820	+0.997(13)
		64.3	55.1 ± 1.05		
		95.6	78.2 ± 0.49		
Trehalose	342.3	28.6	21.4 ± 0.29		
		48.8	39.3 ± 2.23	0.870	+0.997(13)
		65.7	55.8 ± 0.47		
		96.8	81.8 ± I.54		
Sucrose	342.3	26.7	21.8 ± 0.37		
		44.5	35.0 ± 0.51	0.786	+0.998(13)
		62.2	49.3 ± 1.21		
Inulin*	900	2.95	1.01 ± 0.06		
		4.96	1.61 ± 0.17	0.332	+0.992(13)
		6.76	2.34 ± 0.09		
		9.85	3.26 ± 0.14		
PEG'	4000	31.0	4.32 ± 0.10		
		51.1	8.89 ± 0.07	0.257	+0.992(13)
		69.2	14.3 ± 0.69		
		100.0	25.7 f0.67		

values. Secondly, given the close similarity of the two sets of data, that most of their data points fall *below our* PEO q-value data is also significant. If considerable adsorbed solute exists beyond that dissolved in the gelatin water, one might expect the q vs. M.W. plot to be above the PEO plot.

Minor differences notwithstanding, the agreement between the gelatin data and our own PEO as well as alkali-denatured hemoglobin data (Figure 3) are striking. All three sets of data demonstrate similar dependence of q-values on the molecular weights of the solutes. Once the molecular weight reaches about 1000, however, the q-value begins to flatten out at between 0.2 and 0.3.

DISCUSSION

The most important result described in this communication is the confirmation of the size rule in model systems that satisfies the theoretical requirements of NP-NP-NP system (i.e., gelatin, alkali-denatured hemoglobin), or NO-NO-NO system (i.e., PEO) but not in solution of native hemoglobin with its NH and CO groups locked in internal I-I bonds, also in agreement with the PM theory. Before further discussion of the implications of these findings in our understanding of water in living cells, it seems appropriate to examine several other theoretical mechanisms that have been proposed to explain similar size-dependent solute exclusion phenomena observed in other inanimate systems.

Other Interpretations of Size-dependent Solute Exclusion Phenomena.

In rigid water-containing systems such as cross-linked ion exchange resins (Helfferich, 1962, p. 160) and tightly cross-linked dextran gel (Marsden, 1965), equilibrium distribution of various solutes have been shown to vary with the molecular sizes of the solutes. A popular interpretation was based on the molecular sieve (or steric exclusion) mechanism. The exchange resin or tightly cross-linked dextran gel contains pores of diverse size. Only small solute molecules can have access to all pores big or small, while larger ones can only enter the limited number of pores that are large ones. As pointed out by Marsden (1965), this sieve concept does not agree with the generally lower level of sugar and sugar alcohols found in exchange resin in the *more swollen* Li^+ form than in the less *swollen* NH_4^+ form (Ginzburg and Cohen, 1964). A similar conclusion in regard to the sieve concept was reached by Ling and Sobel (1975) and by Ling (1987) when they demonstrated that in sulfonate ion exchange resin in the least hydrated Cs^+ form, and hence minimal average pore size, there was no exclusion for D-arabinose at all. However, the *degree of exclusion increased* rather than decreased as the degree of hydration, and hence average pore sizes, progressively *increased* when Cs^+ was replaced by Rb^+, K^+, Nd, and Li' in that order. This type of observations led Ginzburg and Cohen to attribute the higher degree of exclusion of sugar and sugar alcohols in the more swollen Li^+ resin not to heterogeneous pore sizes but to an "internal pressure" generated by the elastic network of the solid component of the cross-linked polymers.

For the sieve mechanism as well as internal pressure mechanism to operate, the existence of a rigid three-dimensional network is essential. Gary-Bobo and Lindenberg referred to both of these mechanisms in discussing their data on gelatin, which exists as a three-dimensional network of semisolid gel. However, we believe that while the internal pressure concept may be argued for cross-linked ion exchange resin with its extremely high density of fixed charges and counterions (e.g., 2 M or more); its applicability to gelatin or cross-linked blue dextran is doubtful because neither contains high density fixed charges and counterions to create the osmotic force and hence internal pressure. Indeed, dextran, a D-glucose polymer, is electrically neutral.

A 20% commercial high quality gelatin contains only about a total of 10 mM of Ca^{2+}, Na^{+}, and other ions, mostly bound and thus osmotically inactive. The internal pressure concept is even less applicable to PEO and alkali-denatured hemoglobin because they are free-flowing viscous solutions. There is no way for these solutions to sustain rigid pores of finite sizes or an internal pressure. Therefore we conclude that neither the molecular sieve, nor the internal pressure concept can be applied to the PEO and alkali-denatured hemoglobin data. By analogy, one also doubts that it is a large enough internal pressure that gave rise to the solute exclusion properties seen in gelatin or blue dextran.

There is a third concept that has been suggested to explain solute exclusion phenomenon from protein solutions, in particular hemoglobin solution (Borst-Pauwels and Goldstein, 1970). In this "geometrical exclusion volume" concept, there is a space surrounding each protein molecule which in thickness equals that of the radius, r, of the probe molecule under study that is "not available" to this probe molecule. Since larger probe molecule has larger r and hence larger "geometrical exclusion volume", one might thus expect a dependency of the q-value on the molecular size. A full discussion of various evidence against this concept will be given in a following article. Suffice to mention here that the "geometrical exclusion volume" concept is not applicable here. Were it otherwise, one cannot see how in a solution of 3% native proteins with corresponding large surface area, there should be no exclusion at all for all solutes studied including raffinose with a molecular weight of 504, while in the much less concentrated alkali-denatured hemoglobin, there is a distinct exclusion for solute 1/5 the size of raffinose (see Figure 3).

In conclusion, the size-dependent solute exclusion phenomena seen in solutions of PEO, gelatin, and alkali-denatured hemoglobin cannot be explained in terms of the molecular sieve (or steric exclusion) mechanisms, the internal pressure mechanism, nor "geometrical exclusion volume" mechanism. A theoretical mechanism that *can* account for all known facts is the PM theory for solute exclusion.

Further Discussion of Observed Size-dependent Solute Exclusion in PEO, Gelatin, and Alkali-denatured Protein Solution.

There is a similarity in the size-dependent solute distribution in solution of PEO, gelatin, and alkalidenatured hemoglobin. There is also a dissimilarity of these three systems as a group from solutions of native hemoglobin- which demonstrated no size-dependent solute exclusion for solutes as large as raffinose. Considered together, these findings add yet one more set of physico-chemical properties of the bulk phase water affected by the fully extended NP-NP-NP chains of gelatin, and (presumably) of alkali-denatured hemoglobin, and of the NO-NO-NO chains of PEO; but not affected or affected much less by solution of native hemoglobin — in which case most of the water-polarizing CO and NH groups are locked in inter- and intramacromolecular H bonds (for definition of NP-NP-NP systerns etc. and physicochemical properties affected by these and other systems, see Ling and Hu, 1987, Table VII and p. 264).

That gelatin exists at least to a large extent in the fully extended conformation can be attributed to the preponderance of nonhelix forming amino acid residues (proline, hydroxyproline, and glycine) in its amino acid composition (Ling, 1984, p. 175); and that PEO exists in the fully extended conformation can be attributed to the lack of proton-donating groups on pyrrole nitrogen atoms are by now well established. This is not the case for solutions of alkali-denatured proteins, for which in a prior publication Ling and Ochsenfeld (1987) have demonstrated swelling and shrinkage behavior very similar to solutions of PEO and other NO-NO-NO

polymers. Why alkali treatment converts a non-water-polarizing native protein to one strongly polarizing water will be the subject of a future publication.

Is There a Fraction of Water in the Solutions of PEO, Alkali-denatured Hemoglobin and Gelatin Which in Solvency Strongly Resembles Normal Liquid Water?

If the water polarizing polymers or proteins are distributed in a solution in an entirely uniform manner, one may anticipate that all the water in the system has similar properties. However, if the polymer or protein are low in concentration and/ or unevenly distributed, part of the bulk phase water may be out of the reach of the polarizing influence of the nearest polymer or protein chains and exhibit properties essentially that of normal liquid water. The data of Figures 4 and 6 suggest that a significant fraction of the water in the alkali-denatured hemoglobin, PEO, and gelatin is indeed of this nature and will be referred to as "apparently normal liquid water" (ANLIW). The evidence for the existence of ANLIW is that in each case a 20 to 30% of the water demonstrates size-independent q-values. Thus in both alkali-denatured hemoglobin and PEO solution, the q-value levels off after the M.W. reaches about 1000. Particularly significant is the point marked of Figure 6 (in the gelatin data from Gary-Bobo and Lindenberg) corresponding to the very large probe solute, hemoglobin with a M.W. of 67,000, which exhibits the same q-value as that of inulin with a M.W. of only 900. Other unpublished data show that there was no unusual significance to the figure of 20-30% of ANLIW: when the polymer or protein concentration decreases, ANLIW rises to a higher percentage; when the concentrations of polymer or protein increase, the percentage of ANLIW falls to a lower percentage.

The Historical Significance of the High q-value for Ethylene Glycol and Low q-value for Sucrose in Cell Physiology.

In the 1920's and 1930's, scientists who believed that part of cell water exists as "bound water" supported their argument with experimental demonstration of "non-solvent water" often using as their molecular probe, sucrose (Jones and Gortner, 1932). A severe blow to this earlier colloidal approach to living phenomena came from the demonstration that urea (Hill, 1930) and ethylene glycol (McLeod and Ponder, 1936) distribute themselves equally between water in living cells and their surrounding media. These findings clearly established the absence of large amounts of *categorically* non-solvent water in living cells. Nonetheless, the ability of water associated with gelatin and other materials to exclude sucrose remains as valid as ever.

The situation has changed profoundly with the introduction of the PM theory, according to which there is no categorically non-solvent water. Rather, water existing in the state of polarized multilayers excludes or does not exclude a specific solute, depending on its molecular size (and to a lesser degree on the steric conformation which can fit or not fit into the dynamic structure of the polarized water).

In preceding publications, Ling and coworkers have already demonstrated that while a urea-denatured protein, solution excludes sucrose, it does not exclude urea itself (Liig et al., 1980); it is the present paper that has completed the argument in inanimate models by showing equal or nearly equal distribution of ethylene glycol (as well as urea) in solution of alkali-denatured protein while excluding sucrose (Tables I and III). Thus water in living cells and model systems is not "non-solvent" to all solutes. But under the same experimental condition, this water is solvent or nonsolvent, depending mostly on the molecular weights of the probe solutes under study. In a following paper evidence for closely similar size-dependent solute exclusion in living cells

will be presented (Ling, Niu and Hu, to be published).

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