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Gel Permeation Analysis of Macromolecular Association by an Equilibrium Method*

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Summary

An equilibrium method for determining macromolecular association by gel permeation is described. A thermodynamic description of the simplest case, dimerization, is presented in terms of the equilibrium constant for association, and equilibrium partition coefficients for species present. The theoretical analysis yields an equation relating the equivalents of monomer in the external phase to total equivalents, from which the equilibrium constant for association may be obtained.

Those parameters critical in experimental design, gel swelling time, and centrifugal collapse point are determined for Sephadex gels of porosity G-25 to G-200.

Application of the method to human hemoglobin gives a value of $K'_a = 5 \times 10^{-6}$ for dissociation of oxyhemoglobin at pH 7.0 in 0.1 M buffer.

INTRODUCTION

A common feature of many biopolymers, such as enzymes or other protein molecules, is the presence of quaternary structure, i.e.,

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the native molecule is an assembly of subunits. The hemoglobin molecule is a classic example, consisting of a tetramer formed from two pairs of similar polypeptide chains (*1*). Numerous other enzymes have been shown to be *n*-mers of identical or closely similar units (*2*).

It is also well established in many cases that the molecule is in rapid association-dissociation equilibrium (*3*), and that this dynamic subunit structure may be of utmost importance in an analysis of reactivity and structure (*4*). Hence, measurement of this equilibrium is of basic importance.

Similar equilibria are also encountered in nonbiological systems; thus considerations presented here are of general application.

The simplest case to consider is that of a monomer-dimer equilibrium in which rates of association and dissociation are rapid with respect to the technique used for measurement.

$$2\mathrm{M} \overset{K}{\rightleftharpoons} \mathrm{D}$$

where $K = [\mathrm{D}]/[\mathrm{M}]^2$.

Let χ be the fraction monomer, $\chi = [\mathrm{M}]/([\mathrm{M}] + [\mathrm{D}])$ where $[\mathrm{M}] + [\mathrm{D}] = C$ (total concentration); then

$$KC = \frac{1 - \chi}{\chi^2}$$

and a plot of $f(\chi)$ vs. C may be analyzed to obtain the value of K. It is possible to obtain χ experimentally, since it is a direct function of apparent molecular weight. Such considerations have been applied by several investigators to the analysis of ultracentrifugation of associating molecular systems (*5–7*).

It is well established that gel permeation, or molecular sieve, chromatography can also determine an apparent molecular weight (*8*). Application of this experimental method to associating systems has also been analyzed, especially in a series of papers by Ackers and coworkers (*9*). Analysis of the chromatographic system is difficult however, since it is a transport phenomenon, and a suitable theoretical model must be chosen. Also, there is a concentration profile within the column, and the relationship of elution volume to initial concentration is complex.

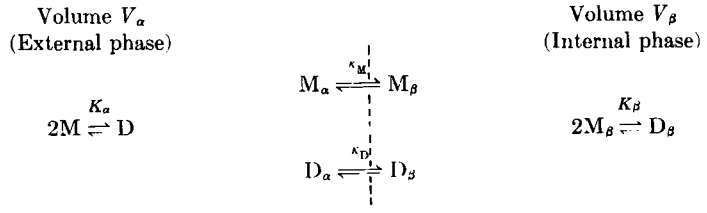
An alternate method to employ gel permeation materials is in an equilibrium system (*10*). All relevant concentrations are now well defined and experimentally accessible. Dry gel is added to the solution containing the macromolecule and, as the gel takes up solvent, the

macromolecular species are distributed between internal and external solvent spaces. The gel volume penetrated is a function of the molecular weight of the solute species. Measurement of concentration in the supernatant (external phase) and knowledge of the initial total amount allows the internal available volume to be calculated.

THEORY

Consider again the simplest case as described above, a monomer-dimer equilibrium under conditions of gel permeation equilibrium partition.

The system is essentially:



where the external phase (α) has volume V_α and the internal phase (β) volume V_β.

The relevant equilibrium constants are defined in terms of amounts of the various species as

$$K_\alpha = \frac{[D_\alpha]}{[M_\alpha]^2} = \frac{D_\alpha V_\alpha}{M_\alpha^2} \tag{1a}$$

$$\kappa_M = \frac{[M_\beta]}{[M_\alpha]} = \frac{M_\beta V_\alpha}{M_\alpha V_\beta} \tag{1b}$$

$$\kappa_D = \frac{[D_\beta]}{[D_\alpha]} = \frac{D_\beta V_\alpha}{D_\alpha V_\beta} \tag{1c}$$

Defining N as equivalents of monomer, N_0 = total equivalents; then:

$$N_0 = N_\alpha + N_\beta = M_\alpha + 2D_\alpha + M_\beta + 2D_\beta \tag{2}$$

Substituting for M_β and D_β from Eqs. (1b) and (1c), and defining

$$\mu = 1 + \frac{\kappa_M V_\beta}{V_\alpha} \tag{3a}$$

$$\delta = 1 + \frac{\kappa_D V_\beta}{V_\alpha} \tag{3b}$$

then

$$N_0 = \mu M_\alpha + 2\delta D_\alpha \tag{4}$$

Combining Eqs. (1a) and (4) and rearranging

$$\frac{2\delta K_\alpha}{V_\alpha} M_\alpha^2 + \mu M_\alpha - N_0 = 0 \tag{5}$$

The solution of the quadratic yields, after rearrangement

$$M_\alpha = -\frac{\mu V_\alpha}{4\delta K_\alpha} + \sqrt{\frac{\mu^2 V_\alpha^2}{16\delta^2 K_\alpha^2} + \frac{V_\alpha N_0}{2\delta K_\alpha}} \tag{6}$$

The positive sign of the radical is chosen since M_α must be >0.

Now defining

$$\mathbf{D} = \mu/\delta \tag{7a}$$

$$\mathbf{K} = V_\alpha/2K_\alpha \tag{7b}$$

and substituting into Eq. (6)

$$M_\alpha = -\frac{\mathbf{KD}}{2} + \frac{1}{2}\sqrt{\mathbf{K}^2\mathbf{D}^2 + \frac{4\mathbf{K}N_0}{\delta}} \tag{8}$$

Consider now the external phase α, on which experimental measurements are made:

$$N_\alpha = M_\alpha + 2D_\alpha \tag{9}$$

From Eq. (1a) and the definition of **K** in Eq. (7b)

$$N_\alpha = M_\alpha + \frac{1}{\mathbf{K}} M_\alpha^2 \tag{10}$$

Combining Eqs. (8) and (10) and simplifying

$$\frac{N_\alpha}{N_0} = \frac{1}{\delta} + \frac{1}{2}\left(\frac{\mathbf{D} - 1}{N_0}\right)\left(\mathbf{KD} - \sqrt{(\mathbf{KD})^2 + \frac{4\mathbf{K}N_0}{\delta}}\right) \tag{11}$$

This final equation relates N_α, equivalents in the external phase at equilibrium, to the total equivalents added, N_0. That this expression correctly describes the physical situation can be shown by examining the behavior of this function under specific limiting conditions.

For example, from Eqs. (7b) and (11)

$$\lim_{K_\alpha \to \infty}\left[\frac{N_\alpha}{N_0}\right] = \lim_{\mathbf{K}\to 0}\left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\delta} = \frac{V_\alpha}{V_\alpha + \kappa_D V_\beta}$$

This is as expected, since for $K_\alpha = \infty$ all material is present as the dimer, and hence N_α is related to N_0 only by the distribution parameter δ, referring to this species.

Similarly

$$\lim_{K_\alpha \to 0} \left[\frac{N_\alpha}{N_0}\right] = \lim_{\mathbf{K} \to \infty} \left[\frac{N_\alpha}{N_0}\right]$$

This limit is indeterminate and must be evaluated by L'Hospital's rule. Taking part of the right-hand side of Eq. (11) and substituting for **K** from Eq. (7b)

$$\mathbf{KD} - \sqrt{\mathbf{K}^2\mathbf{D}^2 + \frac{4\mathbf{K}N_0}{\delta}} = \frac{\mu - \sqrt{\mu^2 + \dfrac{8K_\alpha \delta N_0}{V_\alpha}}}{\dfrac{2K_\alpha \delta}{V_\alpha}} \tag{12}$$

$$\lim_{K_\alpha \to 0} (\text{LHS})^* = \lim_{K_\alpha \to 0} \left[\frac{-\dfrac{1}{2}\left(\mu^2 + \dfrac{8K_\alpha \delta N_0}{V_\alpha}\right)^{-\frac{1}{2}} \left(\dfrac{8\delta N_0}{V_\alpha}\right)}{\dfrac{2\delta}{V_\alpha}}\right] \tag{13}$$

$$\lim_{K_\alpha \to 0} (\text{LHS}) = \frac{-2N_0}{\mu} \tag{14}$$

Combining Eqs. (7a), (11), and (14)

$$\lim_{K_\alpha \to 0} \left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\delta} + \left(\frac{\mu}{\delta} - 1\right)\left(-\frac{1}{\mu}\right) \tag{15a}$$

$$\lim_{K_\alpha \to 0} \left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\mu} = \frac{V_\alpha}{V_\alpha + \kappa_M V_\beta} \tag{15b}$$

This expression relates N_α to N_0 only by the distribution parameter, μ, for the monomer and agrees with the physical situation since for $K_\alpha = 0$ all material is present as the monomer.

It is apparent that the equilibrium constant K_α may be calculated from the variation of N_α/N_0 with N_0. All other quantities in Eq. (11) may be obtained independently; for example, V_α may be measured by using as a marker a high MW molecule which cannot penetrate the gel matrix, $(V_\alpha + V_\beta)$ is the total initial volume, and V_β may be obtained by difference. Some investigators have equilibrated the macromolecular solution against preswollen gel (*11*, *12*). In this case $(V_\alpha + V_\beta)$ may be measured using a low MW completely penetrant small molecule. The values of κ_M and κ_D may be obtained from the distribution at low and high total concentration, respectively, provided that those regions may be reached at which essentially pure monomeric

* Left-hand side.

or dimeric species exist; alternatively they may be obtained by analysis of the experimental plot.

Note that, from Eq. (11), the limit approached by N_α/N_0 as N_0 approaches either 0 or ∞ is indeterminate. Again, application of L'Hospital's rule is essential.

$$\lim \left[\frac{(\mathbf{D}-1)\left(\mathbf{KD} - \sqrt{(\mathbf{KD})^2 + \frac{4\mathbf{K}N_0}{\delta}}\right)}{2N_0} \right] = \lim \left[\frac{-(\mathbf{D}-1)\mathbf{K}}{\delta\sqrt{(\mathbf{KD})^2 + \frac{4\mathbf{K}N_0}{\delta}}} \right] \quad (16)$$

and therefore

$$\lim_{N_0 \to \infty} \left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\delta} \quad (17a)$$

and

$$\lim_{N_0 \to 0} \left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\delta} - \frac{\mathbf{D}-1}{\delta\mathbf{D}} \quad (17b)$$

Combining Eqs. (7a) and (17b) and simplifying

$$\lim_{N_0 \to 0} \left[\frac{N_\alpha}{N_0}\right] = \frac{1}{\delta} - \frac{\mu - \delta}{\delta\mu} = \frac{1}{\mu} \quad (18)$$

An example is given in Fig. 1 of a calculated curve for a system with an association constant K_α of 10^5 for both monomer and dimer penetrant.

This thermodynamic analysis of the equilibrium gel permeation system does not require a postulate as to mechanism of the interaction of the gel with the associating macromolecular system (restricted penetration, adsorption, partition, etc.). The form of the equations is similar to those derived for analysis of potentiometric or colligative property data (*13*, *14*) with the gel acting as a ligand. The only requirement for variation of N_α with N_0 is that κ_M and κ_D differ; these may represent distribution coefficients, binding constants, or some multiplicative combination of individual constants.

EXPERIMENTAL

Examination of the basic equation, Eq. (11), relating N_α/N_0 to N_0 indicates that for experimental discrimination in evaluating K_α several conditions must be optimized. First, κ_M and κ_D must differ significantly [if $\kappa_M = \kappa_D$, then, from Eqs. (3a), (3b), and (7a), $\mathbf{D} = 1$, and N_α/N_0 is not a function of K_α]. This implies choosing a

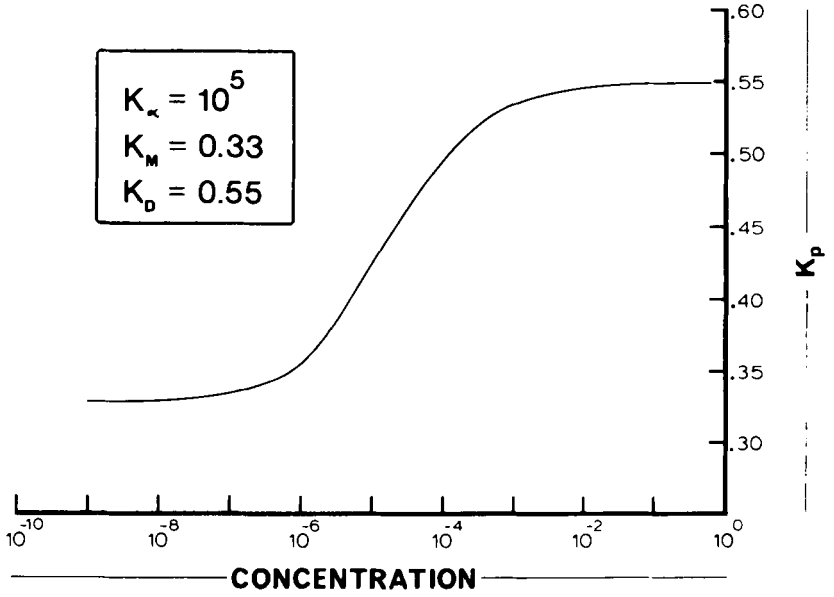


FIG. 1. Variation of equilibrium distribution with total concentration of a monomer-dimer system, theoretical curve. In analogy to gel permeation chromatography, the quantity K_p is plotted rather than N_α/N_0 where $K_p = [1 - (N_\beta V_\alpha/N_\alpha V_\beta)]$. Thus K_p varies from 1 for a completely excluded molecule to 0 for a completely penetrant molecule. In the symbolism usual for Sephadex chromatography (*15*), $K_p = (1 - \text{"}K_D\text{"})$, where $\text{"}K_D\text{"} = V_p/V_\beta$; V_p is defined as that fraction of the gel volume V_β which is penetrated by the molecular species considered. Thus $V_P[N_\beta] = N_\beta$ or $V_P N_\alpha/V_\alpha = N_\beta$ and $V_P = N_\beta V_\alpha/N_\alpha$. Also, the variables K_M and K_D are related to those in Sephadex chromatography; $\mathbf{K}_M = M_\alpha/(M_\alpha + M_\beta) = 1/\mu$ and similarly $\mathbf{K}_D = 1/\delta$.

type of gel permeation material with suitable exclusion limits based on molecular weights of monomer and dimer.

The ratio of V_α to V_β should preferably be small (as V_α becomes large, $V_\alpha/V_{\text{total}}$ approaches 1 and N_α approaches N_0). If the amount of material is limiting, total volumes must be small.

For aqueous systems, the gel permeation materials available swell in the solvent. It is critical that complete swelling occur before measurements are made. An appreciable proportion of the volume V_α is within the swollen gel but external to the gel grains, and it may become necessary to use suction or pressure to recover sufficient supernatant for measurement. If this is the case, the mechanical properties of the gel must be considered in experimental design, since those grades with higher exclusion limits are soft, and may collapse under pressure,

giving false low values for the internal volume. The collapse point may be as low as $30g$; this feature has been ignored in some published studies of equilibrium swelling (*16*).

Materials and Methods

Gel filtration materials used were the Sephadex series of dextran gels of porosity grades from G-25 to G-200 (MW exclusion limit for proteins of 5,000 and 800,000 respectively). Both powder and bead polymerized Sephadex were used without significant differences.

Blue Dextran 2000, an artificially colored high MW dextran, was procured from Pharmacia Fine Chemicals.

Gel swelling was carried out in 50 ml capped flasks in a cold room with continual agitation on an Eberbach reciprocating shaker.

Centrifugal filtration was done at 5°C in an International PR-2 refrigerated centrifuge, using the Millipore Filterfuge tube and 5 μ pore diameter Millipore filters.

Spectrophotometric analysis of the supernatant was on a Zeiss PMQ-II spectrophotometer at 620 nm, the absorption maximum of Blue Dextran 2000. Measurement for hemoglobin was at 540 nm, the maximum of cyanoferrihemoglobin.

Hemoglobin was prepared from human blood by standard methods (*17*).

Swelling Time Experiments

A weighed amount of Sephadex, 500 mg, was added to a 10–20-ml aliquot of 0.05% Blue Dextran in 0.1 M NaCl (volume depending on gel porosity), and allowed to swell for varying times at 5° with shaking. The supernatant was collected by gravity filtration. Recovery was low but enough for analysis; centrifugal or pressure filtration was not used for initial experiments. After gel collapse points were determined (see below), time-of-swelling experiments were repeated using centrifugal filtration at appropriate speeds, with experimentally indistinguishable results.

Results are summarized on Fig. 2. As swelling proceeds, the completely nonpenetrant Blue Dextran is more concentrated in the external volume until a plateau is reached at complete swelling. The time required ranges from 1 hr for G-25 to 48 hr for G-200 (at 5° with shaking).

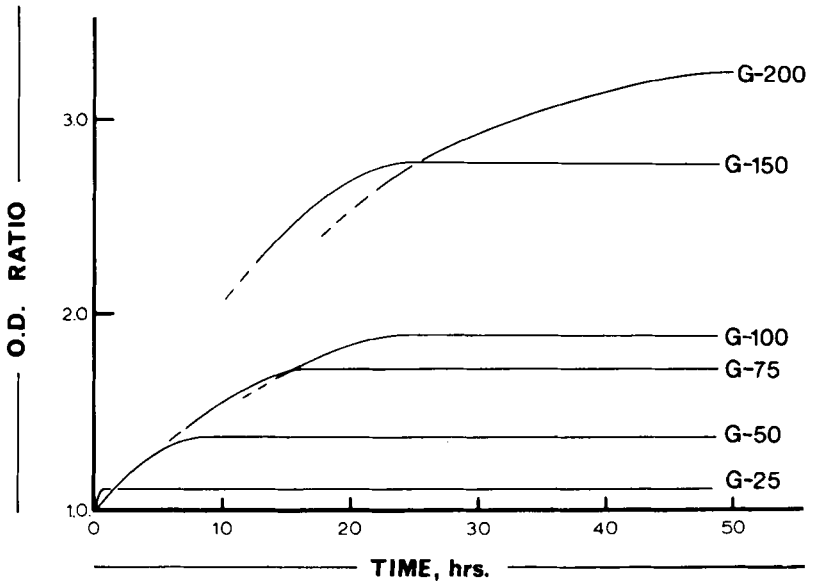


FIG. 2. Swelling time curves for Sephadex gels of varying porosity. The ordinate plotted is the ratio of optical density in V_a to optical density of the Blue Dextran stock solution.

Gel Collapse Experiments

A weighed amount of Sephadex was added to an aliquot of Blue Dextran and allowed to swell to equilibrium (see above). The supernatant was collected by centrifugal filtration at various speeds. In this case, the gel collapse point is determined by that force at which relative dilution of the supernatant occurs due to expression of solvent from the swollen gel matrix.

Results are shown in Fig. 3. Gel collapse points range from greater than 1000g for G-25 to approximately 30g for G-200.

Macromolecular Dissociation

A series of experiments was carried out on a hemoglobin model system to evaluate the utility of the gel filtration equilibrium technique in analysis of protein dissociation.

Human oxyhemoglobin, at concentrations between 5×10^{-7} and

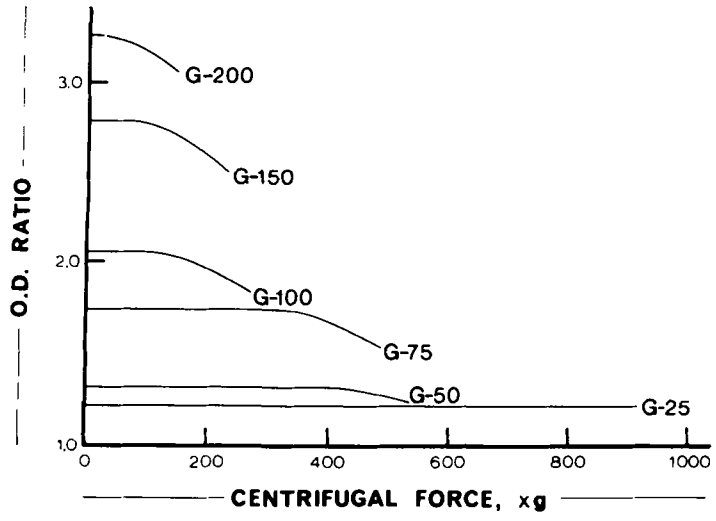


FIG. 3. Centrifugal collapse point curves for Sephadex gels of varying porosity. The ordinate variable is the same as in Fig. 2.

$5 \times 10^{-3} M$ in heme iron, was equilibrated in 0.1 M Na-phosphate buffer pH 7.0 with dry Sephadex powder. The gel was allowed to swell for 48 hr at 5° with shaking, and an aliquot of the supernatant, V_a, was collected by centrifugal filtration at a force just below the gel collapse point.

The results are shown in Fig. 4. For a Sephadex grade which excludes the protein, there is no variation of distribution with total concentration. However, on G-200 which allows hemoglobin (MW 67,000) to penetrate, the distribution coefficient K_p (defined in the legend to Fig. 1) falls with dilution. Theoretically, at complete protein dissociation, K_p should reach another plateau characteristic of the distribution of the half-molecule; in the experiments shown this level of dilution was not reached.

The dissociation constant for human hemoglobin, conventionally expressed as

$$\alpha_2\beta_2 \overset{K'_a}{\rightleftharpoons} 2\alpha\beta; \qquad K'_a = 5 \times 10^{-6}\ M$$

may be obtained from Fig. 4.

Lacking data for low concentrations, this value is subject to an uncertainty of about ±50%. It agrees well with estimates of this equilibrium constant obtained by restricted diffusion (*9*), molecular sieve

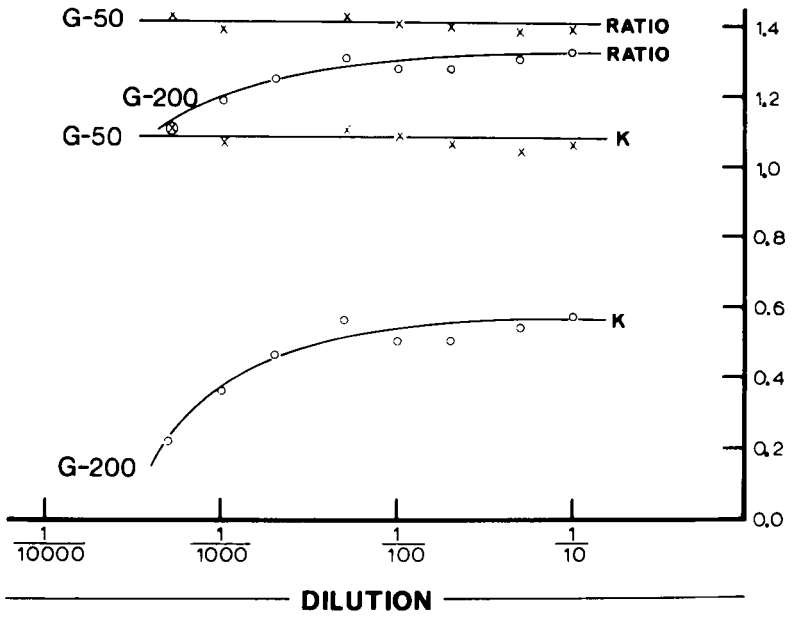


FIG. 4. Equilibrium gel partition on Sephadex G-50 and G-200 of human oxyhemoglobin, stock solution $5 \times 10^{-3} M$ in heme iron. The quantity K is identical to K_p as defined in the legend to Fig. 1, RATIO is the O. D. ratio defined in the legend to Fig. 2 under the specific experimental conditions employed.

chromatography (*18*), osmotic pressure (*3*), or ultracentrifugation (*19*).

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