British Columbia (*3, 4*); Vema Fracture Zone (*4*); Santa Barbara Basin, California (*5*); Lake Biwa, Japan (*6*); Lake Washington, Washington (*7*); and the Amazon River (*8*). It is thought that perylene in these sediments results from the diagenesis of terrestrial pigments which have been rapidly deposited into a reducing sediment. This idea also seems to account for the presence of perylene in this sewage lagoon. We feel it is important not to perpetuate Rose and Harshbarger's suggestion that perylene in this lagoon results from the activity of jet aircraft when a natural source seems more likely.

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Windsor *et al.* offer another possible source of perylene for the sewage lagoon at Reese Air Force Base and suggest that the diagenesis of terrestrial pigments seems more likely. We offered four possibilities: a fuel spill, dumping (and subsequent removal) of asphalt into the lake, diesel fuel used as a mosquitocide, and jet exhaust. Recent conversations with the base entomologist confirmed what we had expected, that diesel fuel was used as a mosquitocide through 1976. The rate of application was 56 liters per acre. Since the lake is about 30 acres in area, the input is 1680 liters times two to five sprayings per year, or 3360 to 8400 liters per year. Agreed, this does not account for the disproportionately high level of perylene; however, recent evidence indicates that while perylene is high, other PAH's [notably benz(*a*)pyrene] are higher than originally reported. The absence of tumorous animals in other sewage lagoons not associated with the base (but not eliminated from the diagenesis of terrestrial pigments) further substantiates the view that the high lesion rate is base-related.

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Potassium Accumulation in Frog Muscle: The Association-Induction Hypothesis versus the Membrane Theory

Palmer and Gulati (*1*) demonstrated that frog muscle cells could accumulate K^+ up to a concentration of 580 m*M*, while accommodating Na^+ to a steady concentration of no more than 20 to 30 percent of that in the external medium. Since according to their calculations the muscle cells have less than 580 m*M* anionic sites, they concluded that (i) (intracellular) K^+ is free under all conditions; (ii) at most 20 percent of the cell water is bound, in the sense that it excludes electrolytes; and (iii) the data support the membrane theory, in which the cell is thought to represent a simple Donnan equilibrium, but refute the basic tenet of the association-induction hypothesis.

I criticize the report of Palmer and Gulati for two reasons. First, the version of the association-induction hypothesis which they present is incorrect, and hence their conclusions concerning it are invalid. Solute distribution in living cells has been described in a general equation (*2, 3*) which, as applied to the intracellular K^+ concentration in moles per liter of cell water, $[K^+]_{cw}$, may be written as

$$[K^+]_{cw} = q_{K(Cl)}[K^+]_{ex} + [K^+]^{I}_{ad} + [K^+]^{II}_{ad} + [K^+]^{III}_{ad} \quad (1)$$

where $q_{K(Cl)}$ is the equilibrium distribution coefficient of K^+ (as chloride) between the cell water and the external medium (*4, 5*); $[K^+]_{ex}$ is the equilibrium external K^+ concentration; and the last three terms refer to K^+ adsorbed on three different types of adsorbing sites. Equation 1 hypothesizes a cell K^+ fraction, indicated by the first term on the right-hand side, which increases linearly with increases of external K^+ and is thus unsaturable. Therefore, cell K^+ cannot be a saturable function of external K^+. Yet Palmer and Gulati's argument against the association-induction hypothesis rests on their statement that it is a crucial prediction of the hypothesis "that the K content of the cell should be a saturable function of external K" (*1*).

Second, Palmer and Gulati ignored relevant experimental findings, including their own. The evidence they ignored includes (i) the finding that the degree of displacement of an accumulated cation such as K^+ depends on the nature and not merely on the valence of the displacing cation, in agreement with the association-induction hypothesis and not with the Donnan equilibrium theory (*6*), and (ii) the long-established finding that at external K^+ concentrations below 2.5 m*M* the cell undergoes a cooperative transition, shifting toward and approaching total displacement of cell K^+ by Na^+ at zero external K^+ (*2, 7–11*). In (*1*) they presented only the range of experimental data which indicates that at very low external K^+ concentrations the amount of cell K+ does not approach zero but instead levels off at a constant high value of 150 m*M*, as demanded by the Donnan membrane theory.

I will now demonstrate that the data presented by Palmer and Gulati (*1*), the data presented earlier by Gulati and Reisin (*10*), and our experimental data together confirm the general equation for solute distribution presented as part of the association-induction hypothesis (*2*).

Potassium in cell water. According to the association-induction hypothesis, cell water existing as polarized multilayers on certain extended polypeptide chains is not nonsolvent water in the sense that it does not dissolve any solute. Instead, different solutes have various solubilities in it because they have different standard free energies of distribution between cell water and water in the surrounding medium (*4, 5*). So far, there has been little direct experimental investigation of $q_{K(Cl)}$ in cell water. The q value of KCl in water in a silica gel is 0.77, whereas that for NaCl is only 0.51 (*12*). Similar values were obtained for the nitrate salts (*13*). Both sets of data show that the q value for K^+ in this model system tends to be significantly higher than that for Na^+.

The concentration of Na^+ in the cell water in Palmer and Gulati's experiment was about 20 m*M* at an external NaCl concentration of 91 m*M*, giving $q_{Na(Cl)} = 20/91 = 0.22$. In our experiments, the somewhat higher value of 0.29 was obtained. Thus, $q_{K(Cl)} = 0.5$ should be a reasonable value under the conditions of Palmer and Gulati's experiment. This value yielded the first component of the theoretical curve shown in Fig. 1 as the straight line labeled C.

Adsorbed potassium. According to the association-induction hypothesis, fixed anionic sites on cell proteins (for example, β- and γ-carboxyl groups) in normal cells not only provide preferential adsorption sites (type I sites) for K^+ but also help to maintain cell shape and volume by forming salt linkages with oppositely charged sites (such as imidazole, ϵ-amino, and guanidyl groups) on neighboring proteins within the cells (*14*). Salt

linkages that form between fixed anion (F^-) and fixed cation (F^+) groups can be dissociated by high concentrations of salts such as KCl:

$$F^+F^- + K^+ + Cl^- \rightleftarrows F^+Cl^- + F^-K^+ \quad (2)$$

resulting in new anionic adsorption sites specific for both K^+ (type II and type III sites) and Cl^-.

Type I sites, according to the hypothesis, adsorb most of the K^+ in frog muscle cells in vivo. The predicted cooperative shift to Na^+ adsorption at low K^+ concentrations has been confirmed repeatedly in frog muscle and other tissue (*2*, *7–9*). The characteristic constants of this type of adsorption sites described previously are used to construct the second component of the theoretical curve depicted as curve D in Fig. 1.

When frog muscles are immersed in an isotonic KCl solution, swelling of the cells occurs. In terms of the association-induction hypothesis, the high concentration of KCl dissociates the restraining salt linkages. More water can then move into the cell to compensate for the "loss" of water activity through multilayer adsorption on the protein backbones (*15*). Muscle cells that become swollen in isotonic KCl do not show a pronounced gain in the intracellular K^+ concentration because water accumulation accompanies the increased adsorption and thus dilutes the K^+ gained. This dilution effect can be inhibited by including in the KCl solution an "isotonic" concentration of NaCl, as was the case in the experiment of Palmer and Gulati. With little or no inward movement of water, the net gains of adsorbed K^+, through salt-linkage dissociation, then produce a significant increase in the concentration of cell K^+.

With a low external NaCl concentration, increasing the external KCl concentration produces marked swelling in two steps, as shown in curve B of Fig. 1. A high external concentration of NaCl (91 m*M*) increases the concentration of Cl^- in the system, driving the reaction in Eq. 2 farther to the right. Therefore, we would expect both salt-linkage dissociation steps to occur at somewhat lower KCl concentrations. The consequent unmasking of type II and type III sites with increasing external KCl concentrations is described by theoretical curves E and F in Fig. 1. Adding curves C, D, E, and F, we obtain curve A, the theoretical curve based on the general form of Eq. 1 (*3*) for the total intracellular K^+ concentration. I have obtained new experimental data confirming those presented in (*1*) and, as shown in Fig. 1, curve A goes through most of the experimental points. Similarly, with somewhat different parameters, theoretical curves have been derived that fit the data of Palmer and Gulati at high external K^+ concentrations and those of Gulati and Reisin at low external K^+ concentrations (Fig. 2).

The theoretical Cl^- distribution curve in Fig. 2 is a composite of curves similar

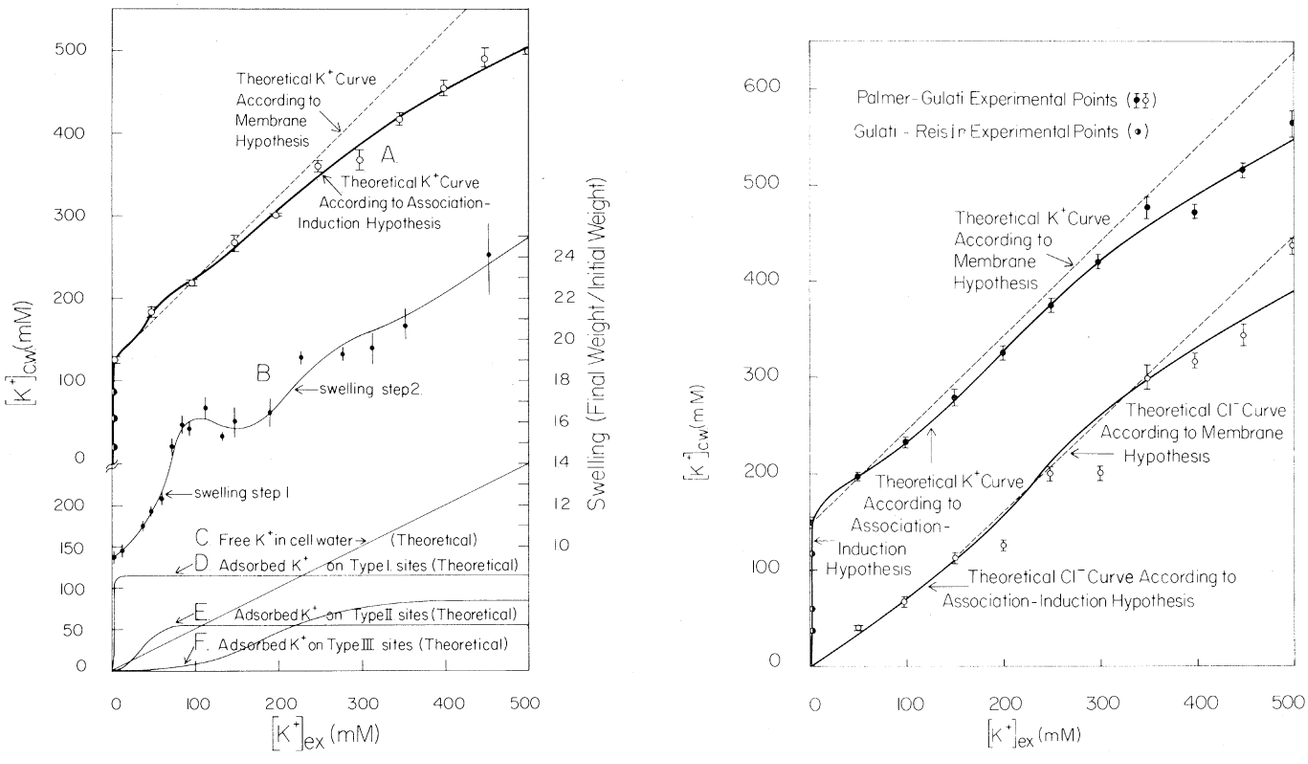

Fig. 1 (left). Potassium concentration in frog muscle cells in the presence of 91 m*M* external NaCl. (○) New data on K^+ accumulation confirming those of Palmer and Gulati (*1*); (●) new data on muscle swelling; and (◐) old data of Ling and Bohr (*8*) on K^+ accumulation. Curve A is a theoretical curve derived from the explicit form of Eq. 1 (*3*), which is resolvable into components shown as curve C [free K(Cl)], curve D (type I adsorption), curve E (type II adsorption), and curve F (type III adsorption). The contribution of type I sites was determined from the results of previous studies (*7–9*); those of type II and type III sites were estimated from curve B, which records the two-step swelling of frog muscles under conditions similar to those of curve A, except that a low external NaCl concentration of 30 m*M* was used (*19*). The q value used to obtain curve C was 0.5. Other numerical values used to obtain curves D, E, and F, respectively, were $[F]_L$ = 122, 55, and 85 m*M*; K_L = 1.35, 35, and 185 m*M*; and $-\gamma/2$ = 0.54, 1.36, and 0.91 kcal/mole. For all data points the lengths of the error bars represent twice the standard error based on four or more determinations. The dashed straight line, predicted on the basis of the membrane theory as given by Palmer and Gulati (*1*), intercepts the ordinate at about 150 m*M*. Fig. 2 (right). Potassium and chloride in frog muscle cells. The experimental points are from Palmer and Gulati (*1*) and Gulati and Reisin (*10*) as indicated. Solid curves were derived from the explicit form of Eq. 1 (*3*). Dashed lines were derived on the basis of the membrane theory. The numerical values used to obtain the theoretical curves for K^+ were q = 0.5 for curve C and, for curves D, E, and F, respectively, $[F]_L$ = 150, 12, and 120 m*M*; K_L = 1.0, 28, and 210 m*M*; and $-\gamma/2$ = 0.60, 1.36, and 0.91 kcal/mole. The theoretical curve of Cl^- accumulation is equal to that for K^+ accumulation minus type I adsorption.

to C, E, and F of Fig. 1; in this case curve D is omitted because normal resting muscle contains an insignificant number of Cl^- adsorption sites. As shown in Eq. 2, the adsorption of Cl^- on type II and type III sites is quantitatively equal to that of K^+. Similarly, the q value for Cl^- is equal to that for K^+ when they are added together as KCl.

The total concentration of anionic sites in cell water is 250 mM for the theoretical curve describing the data shown in Fig. 1 and 282 mM for the Palmer-Gulati data shown in Fig. 2. The total concentration of anionic protein sites in frog muscle cells is 288 μmole per gram fresh weight (*6*). Converted to concentration in cell water, this corresponds to 406 mM, which is more than adequate for adsorption (*16*).

Palmer and Gulati's theoretical curves based on the membrane hypothesis are shown as dashed lines in Figs. 1 and 2 for comparison.

In summary, the association-induction hypothesis can quantitatively explain both the data of Palmer and Gulati (Fig. 2) and our confirmatory data (Fig. 1). It can also explain (i) the data on cell K^+ concentrations when the external K^+ concentration is below 2.5 mM in both sets of data, (ii) the clear-cut demonstration of specificity in the effectiveness of competing monovalent cations in displacing cell K^+, and (iii) the exclusion of permeant Na^+ from the cell water (*2, 14, 17, 18*). To the best of my knowledge, the membrane theory cannot do the same.

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15. In support of this hypothesis, it was shown that swelling in concentrated KCl and in acid or alkaline solutions is indifferent to the presence of an intact membrane [G. N. Ling and C. L. Walton, *Science* **191**, 293 (1976)] and that artificially formed salt linkages between positively and negatively charged polyelectrolytes can be similarly dissociated in a cooperative manner by high concentrations of salts and extremes of pH (*14*).
16. I have assumed that both type II and type III sites are unmasked salt linkages. The data indicate that there are more than enough β- and γ-carboxyl sites in muscle cells to achieve this. However, there is now increasing evidence that carbonyl oxygen on the protein backbone can form complexes with alkali metal ions [C. B. Baddiel, D. Chandure, B. C. Stace, *Biopolymers* **10**, 1169 (1971); D. Balasurbramanian and R. Schaikh, *ibid.* **12**, 1639 (1973)]. Thus, it is not impossible that hydrogen-bonded $-NH\ldots OC-$ pairs may also be dissociated by salts, providing different types of adsorption sites.
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20. Supported in part by NIH grants 1-R01-CA16301-02 and 2 R01-GM11422-12 and ONR contract 105-326. I thank M. DeFeo for much help.

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The discussion here concerns a common property of most cells, the maintenance of an asymmetric distribution of solutes between the cell and its environment. In the case of electrolytes, the potassium concentration is high in the cell and low outside, while the sodium and chloride concentrations are low in the cell and high in the external solution. Two major classes of mechanisms, the membrane theory and the binding hypothesis, have been proposed to explain these properties of the living cell. According to the membrane theory, solutes are freely dissolved in the cell water, implying that some solutes (such as Na^+) are maintained at concentrations far from those of electrochemical equilibrium. In contrast, the binding hypothesis postulates that ions are at equilibrium between the cell and its external environment. Thus K^+ is concentrated in the cell because of its selective binding to fixed anionic sites, while the Na^+ concentration is low because of exclusion by a tightly structured cell water.

Our recent experimental findings (*1*), confirmed by Ling above, showed that frog muscles can be made to survive in solutions containing as much as 0.5M KCl. These cells accumulated K^+ to a level that exceeded the available anionic sites. We therefore consider our findings inconsistent with the binding hypothesis. In contrast, the membrane theory gives a straightforward explanation of the data. It indicates that the bulk of K^+ in the cell is free and that at most 20 percent of the cell water may be inaccessible to the solutes.

The main issue raised by Ling is that of the equilibrium distribution coefficient (q_K) for free K^+ ions between the cell and the external water. Proponents of the membrane theory usually assume that this parameter is approximately equal to one. Ling and Ochsenfeld (*2*) used a value of zero in 1966. In 1973 Ling *et al.* (*3*) suggested a value of about 0.1 for both K^+ and Na^+. Now to fit the new data (*1*), Ling postulates a distribution coefficient of 0.5 for K^+. He does this in order to selectively attribute large fractions of accumulated K^+ near and at the highest values of K(ex) in our experiments to those dissolved in cell water. We argue for negligibly small distribution coefficients for both K^+ and Na^+ in the binding theory. These small q's are due to the important influence of cellular electrical potentials, which Ling ignores.

Ling's theory contends that Na^+ is excluded from the cell water at equilibrium because of an increase in the standard free energy ($\Delta\mu_i^0$) of the ions in the cell over those in the external medium. For nonelectrolytes, or for electrolytes in the absence of electrical potential gradients, q_i of a solute i is related to $\Delta\mu_i^0$ by the expression (*4*)

$$q_i = e\Delta\mu_i^{0/RT} \quad (1)$$

where R is the gas constant, T is the absolute temperature, and

$$\Delta\mu_i^0 = \mu_i^0(\text{ex}) - \mu_i^0(\text{cell})$$

Equilibrium distributions of charged species in living cells are complicated by the presence of electrical potential gradients. For the frog muscle, under physiological conditions, we can use the general relation

$$\mu_i^0(\text{cell}) + RT \ln a_i(\text{cell}) + F\chi(\text{cell}) = \mu_i^0(\text{ex}) + RT \ln a_i(\text{ex}) + F\chi(\text{ex}) \quad (2)$$

where a_i is the activity of species i, χ is the electrical potential, F is the Faraday constant, and (cell) and (ex) refer to the cellular and the extracellular compartments, respectively. It is assumed that the potential gradient $\Delta\chi$ exists only across the cell membrane or surface and that the points along the cytoplasm are all equipotential.

Rearranging and replacing activities with concentrations

$$C_i(\text{cell}) = C_i(\text{ex}) \times e^{\Delta\mu_i^0/RT} \times e^{F\Delta\chi/RT} \equiv C_i(\text{ex}) \times q_i \times \rho \quad (3)$$

where ρ is substituted for $e^{F\Delta\chi/RT}$.

For Na^+ in normal Ringer's solution, $C_{Na}(\text{cell}) = 30$ mM and $C_{Na}(\text{ex}) = 113$

mM (*1*). Using $\Delta\chi = 100$ mv, we calculate at 25°C $\rho = 50$ and $q_{Na} = 0.0055$. This is probably an overestimate of q_{Na}, since many studies have indicated that only part of the total Na^+ is free cytoplasmic ion (*5*). Subtracting the bound fraction of Na^+ from the measured value yields $q_{Na} \simeq 0.0028$. In contrast, the new value calculated above by Ling is too high since it includes neither the ρ term nor the contribution of bound sodium.

The K^+ distribution ratio can be as much as twice that for Na^+ in a silica gel model system (*6*), and Ling postulates that a similar correlation between q_K and q_{Na} exists in the living cell. If this is correct, it may be reasonable to assume an upper limit of 0.01 for q_K in the muscle cells. Thus, under physiological conditions, the concentration of free K^+ according to the equilibrium model is $2.5 \times \rho \times q = 1.25$ mM. At the highest external K^+ concentration we used (500 mM) (*1*) the cell is most likely depolarized, so that $\Delta\chi \simeq 0$ and $\rho = 1$, and the concentration of free K^+ calculated with Eq. 3 is at most 5 mM, a negligible amount. According to the association-induction hypothesis or any other equilibrium binding model, essentially all the accumulated K^+ must therefore be treated as if bound. And for the binding hypothesis to be correct, our original statement should hold—within experimental error the K^+ uptake must be a saturable function of K(ex). This is clearly not the case [see also reference 19 in (*1*)].

It is possible to postulate that the distribution coefficient for K^+ is two orders of magnitude larger than that for Na^+. As far as we know, no one has considered this possibility, nor does it have any theoretical or experimental basis. An interesting modification of the binding theory is to postulate that q increases as K(ex) is increased. In this case, q could be low (as calculated above) under physiological conditions and approach 0.3 (for q_{Na}) and 0.5 (for q_K) at high K^+ concentrations. However, our measurements of the sucrose distribution at various KCl concentrations (*1*) argue against this possibility. According to Ling's ideas, sucrose can permeate the muscle cell but distributes mainly in the extracellular space because of its low partition coefficient (*3*). Since the sucrose space did not increase with increasing K(ex), we assume that the putative water structure did not change.

The sodium measurements showed a drop in cell Na^+ as the K(ex) was increased over 2.5 mM [see figure 1 in (*1*)]. This is additional evidence that q in the binding theory is not influenced by variations in the K^+ concentration (*7*). Since the cell water structure is considered the main mechanism for the exclusion of solutes in this theory, the observed decrease in cell Na^+ at high KCl indicates also that the q values for the monovalent cations must be low—of the order of magnitude calculated from Eq. 3. Assumptions of high values for q fail to explain the drop in cell Na^+ (*7*).

Type II and type III adsorptions are postulated by Ling to account for the intracellular K^+ accumulated after the type I or high-affinity sites have been saturated. The amounts of these secondary adsorptions are estimated by arbitrarily selecting 30 mM NaCl in the external solution, causing cell swelling. The assumption is made that the new sites become unmasked in the swollen muscle. In our experiments, the NaCl concentration was controlled strictly in accordance with the theoretical considerations to keep the cell volume constant (*8*); thus, even if these secondary sites existed, they would have remained masked.

Finally, our use of the Donnan theory after Boyle and Conway (*9*) was based on the idea that under our experimental conditions Na^+ ions are effectively nonpermeating. Since Na^+ can penetrate the cell water, an active transport system must be invoked to keep the intracellular Na^+ concentration low (*10*). This mechanism is commonly thought to involve a membrane-bound adenosine triphosphatase which requires the presence of external K^+ for enzyme activity (*11*). The observed decrease in cell Na^+ with increased KCl (*1*, *7*) implies, according to the membrane theory, (i) that the pump operates with greater efficiency and/or (ii) that leakage of Na^+ into the cell is decreased under these conditions. At low external K^+ concentrations (C near 2.5 mM and below), the condition of effective nonpermeation appears to break down, which also explains the well-known deviations (*12*) from the Donnan theory under those conditions.

In summary, it should be noted that in addition to (i) using values for q that are too high and (ii) making the ad hoc assumption regarding the presence of three different types of binding sites, Ling requires nine arbitrary parameters to explain our findings by the binding theory (the values of $[F]_L$, K_L, and $-\gamma/2$ are separately selected for each of the three types of postulated adsorption sites to do the curve fitting; see the legends of Figs. 1 and 2 in Ling's comment above). Even a linear plot of K(cell) against K(ex) could be accounted for by assuming a large number of low-affinity sites. On the other hand, assuming only that the Donnan principles apply, the membrane theory gave the simplest explanation of the data (*1*). In conclusion, even though the ideas contained in the association-induction hypothesis have, in the past, provided a stimulating challenge to the membrane theory, the new findings show that the binding hypothesis is no longer a useful alternative.

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