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Translocation pathway in the catalysis of active transport

(bioenergetics/chemiosmotic processes/membrane channels)

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ABSTRACT **Possible pathways for translocation across the membrane in active transport processes are examined theoretically. Thermodynamic and kinetic requirements are readily satisfied by an alternating-access mechanism of the kind that has been proposed in the past by several investigators. The essential features of this mechanism (for transport of a single species) are shown to be defined by four explicit conditions. (*i*) The transport protein must have at least two distinct conformational states, each accessible from only one side of the membrane. (*ii*) Binding affinity for the transported species is high in the state accessible from the uptake side of the membrane and much lower in the state accessible from the discharge side. (*iii*) The change from one conformation to the other involves movement of the binding site itself (with the transported species remaining attached) or rearrangement within the site that is topologically equivalent to such movement. (*iv*) Return to the original conformation occurs with unoccupied binding sites. The analysis demonstrates that a passage through the membrane that is simultaneously accessible from both sides cannot be used for active transport regardless of what the energetics of opening or closing of the passage may be. Even movement from one fixed site to another within the protein, without access to the outside, is virtually excluded as a possible element of the central mechanism. A ligand conduction mechanism for ATP-linked ion transport is in principle conceivable but is subject to restrictions that make it improbable.**

The device used by transport proteins to allow a transported species to traverse an otherwise essentially impermeable membrane must be different in active and passive transport systems. In active transport, translocation is thermodynamically uphill, requiring some kind of boost from the protein. In passive transport, translocation is thermodynamically downhill, and nothing more is needed than a simple pathway across the membrane, simultaneously accessible from both sides. Many investigators have recognized this difference, and the most frequently proposed model for active transport involves two conformational states of the protein, with alternate access from the two sides of the membrane, permitting the requisite boost to the transported species to take place in the transition from one conformational state to the other (1–8). However, some investigators still view the translocation domain of an active transport protein as resembling a channel or pore of the type that is used to facilitate passive transport (9, 10). Yet another proposal is provided by the ligand conduction model of Mitchell (11) in which the uphill boost to the transported species is provided by direct chemical interaction with the free energy-donor species, instead of through the protein. The existence of these contrary views has prompted the theoretical investigation described in this paper.

Relative contributions of osmotic and electrical components to the work of transport are not relevant to the analysis of this paper, and the formal treatment will assume that only osmotic work is involved. Most of the analysis will refer to a single unspecified transported species (L), which can be considered to be either an uncharged molecule or an ion traversing a membrane with zero transmembrane potential. The two sides of the membrane are designated as "in" and "out," with active transport in the outward direction so as to maintain [L(out)] >> [L(in)] at the physiological steady state.

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Procedure

The theoretical approach is similar to the approach used in a previous analysis of limitations on kinetic and thermodynamic parameters for active transport processes (12). The principle involved is that thermodynamic considerations (enforced coupling of transport to a source of free energy) do not by themselves constitute an adequate criterion for evaluating a transport mechanism. Kinetic requirements are equally important. The catalytic process must occur at an adequate rate and must respond to changes in the concentrations of the transported species on the two sides of the membrane in a physiologically useful way. Moreover, both these goals must be accomplished with physically reasonable values of rate constants for individual steps of the reaction mechanism. Second-order rate constants for binding of ligands to proteins have an upper limit of about 10^8 $M^{-1}sec^{-1}$, and first-order rate constants for protein conformational change or for processes involving changes in covalent bonding of bound substrates are limited to about 10^4 sec^{-1} (13).

Application of these kinetic criteria depends in part on numerical calculations for suitable model reaction sequences. For example, it may be self-evident that a given mechanism requires that a particular step be fast, but it is not usually self-evident whether it needs to be so fast as to exceed acceptable physical limits. Moreover, just how fast a particular step needs to be usually depends on the rates of other steps in the reaction sequence. In practice, therefore, it becomes necessary to vary a large number of kinetic parameters to establish whether or not a given mechanism is kinetically feasible. Determination of the effect of these variations on the overall rate of transport and response to changes in external variables is greatly facilitated by the methods devised by Hill (14) to extend the King–Altman diagram method (15) for derivation of steady-state rate equations.

To ensure that no significant variations within a given reaction sequence are neglected (e.g., designation of relatively slow and fast steps) typically requires generation of 100,000 or more different sets of parameters. The results are analyzed with the aid of a computer to retain those combinations that satisfy functional requirements. In addition to the requirements for an adequate overall rate and physiological response as described (12), the numerical requirements include realistic maximal turnover numbers under laboratory conditions (50–100 sec^{-1} in

the direction of uphill transport and at least 5 sec^{-1} in the reverse direction) and, of course, adherence to the upper bounds for rate constants mentioned above.

Alternating-access model

An alternating-access model is the most commonly proposed model for translocation across the membrane in active transport. The version of the model proposed here is more explicit than previous versions and defines the model in terms of four mechanistic conditions.

(*i*) At least two conformational states of the transport protein are necessary. Binding sites for the transported species are accessible to one side of the membrane in one conformation and accessible to the opposite side in the other conformation. The same site or connected sites are never accessible from both sides of the membrane simultaneously. (It is not implied that two conformational states will necessarily prove to be sufficient when aspects of active transport other than translocation *per se* are considered.)

(*ii*) The two conformations have different thermodynamic affinities (equilibrium binding constants) for the transported species. The affinity is high on the uptake side, where the species has to be bound to the protein from a medium of low thermodynamic activity, and the affinity is much lower on the discharge side to facilitate release of the transported species into a medium of relatively high thermodynamic activity. An important consequence is that most of the work of transport is done while the transported species L is bound to the protein (16). In terms of the standard chemical potential of bound *L* ($\mu^0_{L,b}$),

$$\mu^0_{L,b}(\text{discharge site}) - \mu^0_{L,b}(\text{uptake site}) \simeq \Delta\mu_{L,aq} \quad [1]$$

where $\Delta\mu_{L,aq}$ is the difference in chemical potential of L between the aqueous solutions on the two sides of the membrane.

(*iii*) The transition from one conformation to the other involves *movement of the binding site itself* (with L remaining bound to the site) or rearrangement of the protein structure around the site that is topologically equivalent to movement, as suggested in a previous speculative model (8). Movement of L from one fixed site to another (without conformational change) is explicitly excluded in the present formulation of the model except as part of trivial steps of the overall transport cycle that are not directly involved in changing $\mu^0_{L,b}$. (Because movement of the site involves a change in $\mu^0_{L,b}$, it is necessarily coupled within the protein to the processing of the free energy donor that provides the driving force for active transport. There is no intent here to suggest a parallel to energetically inert movement of bound ligands across the membranes, such as occurs in carrier-mediated passive transport.)

(*iv*) The transport protein must return to its original state at the end of each reaction cycle. Therefore, the conformational transition has to occur twice within each cycle, once when L is bound to the binding site (or sites) and once when the site is unoccupied. (Where two different species are transported in opposite directions, as in Na^+,K^+ pumps, it is possible or even likely that one species is bound when the conformational transition takes place in one direction and that the second species is bound on the return trip.)

Many different model reaction sequences that incorporate these defining characteristics can yield realistic kinetic behavior for active transport systems. The four defining statements are also consistent with experimentally deduced reaction sequences for several ATP-driven ion pumps (5, 6, 17, 18). The purpose of the present paper is to show that all parts of the definition are virtually *essential* for a kinetically feasible active transport process. The validity of condition *ii* has been demonstrated previously (12), and the analysis in this paper focuses on conditions *i* and *iii*.

Condition *iv* is seemingly trivial and does not enter explicitly into the analysis. It actually raises some questions at a deeper level of enquiry into the mechanism of active transport and will be considered briefly in that context in the *Discussion*.

Channels with simultaneous access from both sides of the membrane

A channel with simultaneous access from both sides of the membrane facilitates passive exchange of a transported species L between the two sides of a membrane, thermodynamically downhill, aspiring to an equilibrium state in which the electrochemical potential is equal on the two sides. In the present context (electrical effects assumed absent), the equilibrium state would be [L(in)] = [L(out)]. This fact is independent of the thermodynamic and kinetic parameters that govern translocation through the channel. It remains true even when the binding constants of L to the sites at the end of the channel are very different on the two sides of the membrane.

The same principle applies when a channel is normally closed, and free energy is required to open it, as illustrated by Fig. 1. The upper and lower parts of this reaction scheme do not share a reaction step and are incapable of being coupled to each other (see figure 2.4 of ref. 14). The upper part catalyzes only the reaction S $\rightleftharpoons$ P, the lower part catalyzes only the downhill translocation of L. The two processes compete for the same species (state *a* of Fig. 1), and relative rate constants will affect the relative rates of the two processes, but there is no way either process can force the other to proceed in a thermodynamically uphill direction.

Thermodynamic coupling between translocation of L and the reaction S $\rightleftharpoons$ P can occur only if the two processes share at least one reaction step. If a transmembrane channel is to be incorporated into an active transport system, the translocation step (*b* $\rightleftharpoons$ *c* in Fig. 1) is the step that needs to be shared, as illustrated by Fig. 2. It is immediately evident that two conformational states of the protein, here designated E and E′, are required to achieve this. State E must have L bound from the

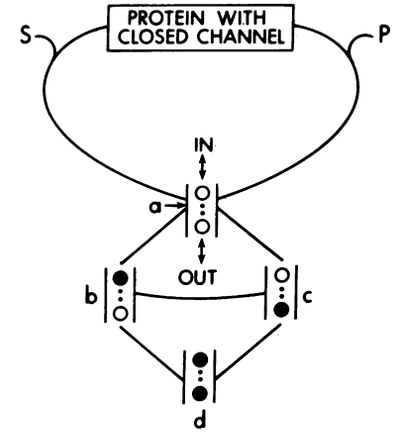

FIG. 1. Gated channel coupled to a free energy-donating reaction S $\rightleftharpoons$ P. The channel is defined by a four-state model (states *a–d*) with simultaneous access to both sides of the membrane. Circles represent binding sites in contact with the solutions on both sides of the membrane. ○, Unfilled sites; ●, sites occupied by the transported species. Translocation occurs by means of step *b* $\rightleftharpoons$ *c*. Intermediate channel sites between the designated binding sites would not affect the conclusions based on this model.

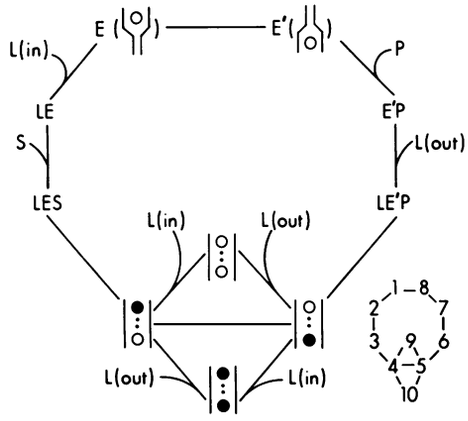

FIG. 2. Alternating-access model with transiently open channel. Numbering system for the states is shown at the lower right. L is the transported species, and E and E′ are distinct conformational states of the protein. In E, only the upper half of the channel is open, providing access from the inside. In E′, only the lower half is open, providing access from the outside. The channel is open to both sides in states 4, 5, 9, and 10. In the text, k_{ij} refers to the rate constant for the step $i \rightarrow j$. Rate constants for binding steps (k_{12}, k_{87}, k_{94}, etc.) are second-order rate constants. For numerical calculations, the free energy available from S→ P was set at 5.7 kcal/mol, corresponding to the ability to maintain [L(out)]/[L(in)] at a maximal value of 10^4 for 100% coupling efficiency. [L(out)] was fixed at 10×10^{-3} M. ○, Unfilled sites; ●, sites occupied by the transported species.

inside only, so as to be convertible to state 4 (Fig. 2), and state E′ must have L bound from the opposite side so as to be interconvertible with state 5. The need for an alternating-access mechanism is thus demonstrated.

Fig. 2 demonstrates the need for an alternating-access mechanism but does not by itself eliminate the possibility that a transmembrane channel, transiently open to both sides of the membrane, might serve as the translocation pathway. The reaction scheme in Fig. 2 would in fact represent this possibility if E and E′ are visualized as states of the protein in each of which half of a channel is open and the other half is blocked. However, kinetic considerations lead to rejection of this possibility. Reasonably efficient coupling between transport and the reaction S ⇌ P can take place only if the rate constants k_{45} and k_{56} are fast compared to the rates of binding and dissociation of L in steps 4 ⇌ 9, 5 ⇌ 9, etc. One of these rate constants (k_{45}) can indeed be assumed to be very large, but k_{56} refers to a reaction step that has to be inherently slow, with an upper limit of about 10^4 sec^{-1} because it involves a conformational change of the protein. This means that the condition for reasonably efficient coupling can be met only by correspondingly slow rates of binding and dissociation of L to or from the sites on the supposedly open channel of state 9 of Fig. 2. Numerical calculations demonstrate that these rates must be at least 1 or 2 orders of magnitude smaller than the rates of binding or dissociation in steps 1 ⇌ 2 and 6 ⇌ 7, a result that is incompatible with the notion that the access sites in E and E′ represent halves of a channel that is fully open in state 9.

Calculations were made with the assumption that the rate of step 4 ⇌ 5 is very fast. Rates of binding or dissociation of *L* to or from states 4 and 5 were initially assumed to be zero, and even then only a small number of combinations of kinetic parameters led to kinetically acceptable results, for reasons explained in the following section. Rate constants for the steps 4 ⇌ 9, etc., were then progressively increased, imposing the condition that the binding constants of L to the channel sites are the same as the binding constants to states E or E′ (i.e., $k_{94}/k_{49} = k_{5,10}/k_{10,5} = k_{12}/k_{21}$ for binding from the inside and similar relations for the opposite side). Reasonably efficient coupling was defined as the ability to utilize 75% of the free energy of S → P for generation of a steady-state concentration gradient in the absence of any extraneous leak mechanism. It was found that the second-order rate constants for binding to channel sites were limited to values well below 1×10^6 $M^{-1}sec^{-1}$, whereas most acceptable combinations of parameters for the overall process required $k_{12} = 1 \times 10^8$ $M^{-1}sec^{-1}$ for an adequate overall rate.

Another consequence of the mechanism of Fig. 2 would be easily detectable variation in transport stoichiometry in laboratory experiments in which L is pumped into initially empty vesicles. Spontaneous transport through the channel would initially be in the same direction as coupled transport, but it would oppose coupled transport when the intravesicular concentration of L rises above the external concentration. The molar ratio (L transported)/(S converted to P) would change from >1 to ≪1 in the course of the experiment.

Movement between fixed sites within the protein

Movement of L through the transport protein is accompanied by an increase in the standard chemical potential $\mu^0_{L,b}$ (Eq. 1). Because movement of a ligand from one fixed site to another occurs spontaneously in the direction of negative $\Delta\mu^0_{L,b}$, it obviously will be difficult to incorporate any kind of transfer between fixed sites into an active transport process, except in energetically trivial steps ($\Delta\mu^0_{L,b} \simeq 0$) that do not contribute to the free energy-transduction mechanism.

Consider, for example, the situation illustrated by Fig. 3, in which step 4 ⇌ 5 represents movement of L between fixed sites within the protein, in a space inaccessible from either side of the membrane. Such movement, in the absence of conformational change, would not be expected to be a slow step in the reaction cycle, so that states 4 and 5 can without loss of generality be considered to be in equilibrium with each other, regardless of the state of the overall reaction. The equilibrium ratio of the concentrations of the two states is identical to the relative probability of occupancy of the two sites. Where p_i represents concentration, $p_5/p_4 = \exp(-\Delta\mu^0_{L,b}/RT)$, in which $\Delta\mu^0_{L,b}$ is the change in $\mu^0_{L,b}$ in the direction 4 → 5. The value of p_5/p_4 limits the maximal forward turnover rate of the pump cycle, which cannot exceed the maximal forward transition rate in any single step of the mechanism. For step 5 → 6, the maximal forward transition rate is $k_{56}p_5/(p_4 + p_5)$. Because there

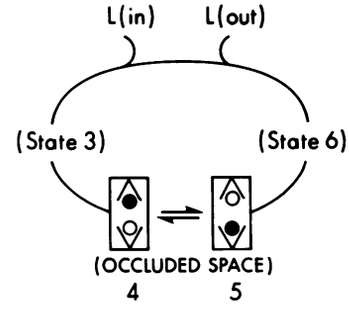

FIG. 3. Alternating-access model with movement of L between fixed sites within the protein molecule that are not directly accessible from either side of the membrane. The states are numbered to correspond to the reaction scheme of Fig. 2, but a similar sequence could occur in a variety of overall reaction schemes. Movement between states 4 and 5 could represent movement within an occluded solvent-filled space or along a pathway of the type suggested by the "proton wire" of Nagle and Morowitz (19). ○, Unfilled site; ●, site occupied by transported species.

is a conformational change in this step, k_{56} is in practice limited to a value of about 10^4 sec^{-1}; therefore, achievement of the physiologically obligate maximal turnover rate of 50–100 sec^{-1} requires $p_5/p_4 > 5 \times 10^{-3}$ or $\Delta\mu^0_{L,b} < 3.5$ kcal/mol.

The considerations of the preceding section, based on the model of Fig. 2, showed that it is formally possible to include transfer between fixed sites as part of an active transport mechanism, provided that the fixed sites are difficult to access from the aqueous solutions on either side of the membrane. It needs to be pointed out, however, that Fig. 2 represents an atypical transport system, with a low work load. Free energy transduction amounts to only 5.7 kcal/mol per reaction cycle, and only one molecule of L is transported in each cycle. A consequence of the 1:1 stoichiometry is that it permits a somewhat broader range of equilibrium binding constants for L to E or E′ than would be possible for systems in which more than one molecule is transported per cycle (12), and this in turn increases the magnitude by which $\Delta\mu^0_{L,b}$ can differ from $\Delta\mu_{L,aq}$ (Eq. 1). With $\Delta\mu_{L,aq}$ itself at a relatively low value, this makes a transfer of the type envisaged by Fig. 3 marginally possible. It should be recalled that only a small number of combinations of kinetic parameters were found to yield acceptable overall rates in the calculations based on Fig. 2 with states 9 and 10 treated as inaccessible. Even these limited results almost all required use of maximal rate constants (1–2 $\times$ 10^4 sec^{-1}) for inherently slow steps in the forward direction (k_{34}, k_{56}, k_{81}) to pull L through the difficult site transfer step.

In the best-known active transport proteins, ATP-driven ion pumps, there are usually two or more ions transported per reaction cycle, and the free energy exchanged between the chemical donor and the transported ions is 11–14 kcal/mol per cycle (20). Under these conditions it becomes essentially impossible to have most of the requisite change in $\mu^0_{L,b}$ take place in steps in which the ions move between fixed sites within the protein molecule. Numerical calculations were made for an ATP-driven Ca^{2+} pump, based on a slightly modified version of the reaction cycle used for previous calculations (12), with a constant stoichiometry of two Ca^{2+} ions transported per ATP hydrolyzed. The constant stoichiometry requires that site-to-site transfer as represented by Fig. 3 has to involve both Ca^{2+} ions, and it makes no difference whether the two ions move simultaneously or sequentially: in either case a barrier of the order of 12 kcal/mol is created between the states in the Ca^{2+} pump cycle that are analogous to states 4 and 5 of Fig. 3. The calculations showed that this barrier could be overcome only if unrealistically high values were assigned to other rate constants in the cycle. One possibility was to assign an extreme value of 5 $\times$ 10^8 $M^{-1}sec^{-1}$ to the rate constants for binding of Ca^{2+} to each of the two binding sites in the state E and values of 5 $\times$ 10^5 sec^{-1} to *all three* of the inherently slow isomerization steps that follow the site-transfer step (analogous to k_{56}, k_{78}, and k_{81} in Fig. 2). When the binding rate constants were assigned the more reasonable value of 1 $\times$ 10^8 $M^{-1}sec^{-1}$, an adequate rate profile could be achieved only when k_{ij} values for isomerization steps were permitted to be as large as 5 $\times$ 10^6 sec^{-1}.

These considerations demonstrate the validity of statement *iii* of the definition used in this paper for defining an alternating-access model. Site-to-site movement, in a fixed conformational state of the protein, cannot be regarded as a realistic possibility for free energy-transducing steps of an active transport mechanism.

Access channels

There are of course no restrictions to exclude movement of the transported species between fixed sites if the $\mu^0_{L,b}$ values at the two sites are approximately equal. For example, the alternating access sites that change in the E-to-E′ transition need not be directly accessible to the adjacent aqueous media. It is possible that they can be reached only through access channels containing sites that the transported species would have to occupy sequentially on its way to or from the bulk solutions. If no significant free energy barriers are involved, little effect on the overall kinetics would result.

Reversal of active transport (ATP synthesis)

The foregoing objections to movement between fixed sites apply equally to pumps working in the reverse direction, e.g., to systems synthesizing ATP by use of free energy released in downhill transport. Step 5 $\rightleftharpoons$ 4 in Fig. 4 would now occur spontaneously in the desired direction, with a free-energy decrease equal to $\Delta\mu^0_{L,b}$. However, the free energy released would be dissipated (used for rate acceleration) and would not be available for its subsequent synthetic task.

Ligand conduction

Movement of the binding site with L attached to it is not the only conceivable way to avoid the free energy barrier inherent to uphill movement of L from one fixed site to another or to avoid the free energy dissipation that would accompany spontaneous downhill movement in the reverse direction. If the free energy-donor molecule, in the course of the reaction S $\rightleftharpoons$ P by which its latent free energy is released, were to be translocated across the membrane in the same direction as *L*, along all parts of the pathway that involve a change in $\mu^0_{L,b}$, then L could in principle be carried along with it. Binding of L to the donor molecule would be kinetically equivalent to binding to a site on the protein. To complete the analogy to an alternating-access model, the affinity of L for P would have to be much less than the affinity of L for S.

This is essentially the idea proposed by Mitchell (11) and given the name "ligand conduction." The idea has been formulated most explicitly for ATP synthesis driven by a gradient in the electrochemical potential of H^+, in which it is proposed (at least implicitly) that H^+ binds to inorganic phosphate on one side of the membrane and that ATP and energetically debased H^+ are produced on the opposite side. However, applicability to ATP-driven uphill transport of other inorganic ions has also been suggested (11). The important aspect of the proposal in the context of the present paper is the unavoidable requirement for movement of phosphate across the membrane in intimate association with the translocated ion. This requirement appears to be inconsistent with available experimental data (e.g., ref. 21); for this reason, other possible difficulties with this mechanism were not investigated. It is worth noting that a ligand conduction mechanism would complicate analysis of the energetics of transport because the difference in the electrochemical potential of phosphate on the two sides of the membranes would have to be taken into account in formulation of the overall free energy balance.

Discussion

This paper has demonstrated that an alternating-access model is the only plausible model for translocation of the transported species in an active transport system and has provided four operational conditions that in effect define the model. The results apply most forcefully to ATP-driven ion pumps, where exchange of free energy between chemical donor and transported ions is typically 11–14 kcal/mol per reaction cycle. Alternative

pathways across the membrane cannot be rejected with equal confidence when the free energy exchange per reaction cycle is small, say 5 kcal/mol or less. The procedure used for the analysis rests heavily on assignment of upper limits to rate constants for individual steps of the reaction cycle. These limits are in part dictated by experience rather than by rigorous theoretical treatment, which suggests that the conclusions should be viewed as statements of high probability rather than absolute certainty.

The reaction schemes used here and in my previous analysis of an ATP-driven Ca^{2+} pump (12), and most reaction schemes proposed in the literature, show only the sequential steps that are needed to produce an appropriately coupled overall reaction. An equally important aspect of the mechanism of active transport is the need to avoid reaction steps that would lead to catalysis of uncoupled partial reactions, as has been stressed by Jencks (4) and Hammes (22). In relation to translocation, the need to avoid a transmembrane channel open to both sides is a restriction of this kind. An additional restriction becomes necessary when the transport stoichiometry is >1 (i.e., more than one copy of L is transported per reaction cycle). In a model invoking only two principal conformational states of the protein, all copies of L have to be translocated together when the E-to-E′ conformational change occurs with L bound to the protein. At the same time, condition *iv* of the definition of the model still applies: return to the original conformational state (for transport of a single species) must occur with all binding sites unoccupied. This situation clearly creates a new restriction on an alternating-access model. The transition from one conformation to the other may occur with all sites occupied or all sites empty but not in the intermediate situation where both filled and empty sites are present. How this might be accomplished is still an unsolved problem, and extension or modification of the basic alternating-access model may become necessary to take this additional restriction into account.

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