Journal of Supramolecular Structure 6:169–177 (1977)
Molecular Aspects of Membrane Transport 95–103

Transport in Halobacterium Halobium: Light-Induced Cation-Gradients, Amino Acid Transport Kinetics, and Properties of Transport Carriers

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Cell envelope vesicles prepared from H. halobium contain bacteriorhodopsin and upon illumination protons are ejected. Coupled to the proton motive force is the efflux of Na^+. Measurements of ^{22}Na flux, exterior pH change, and membrane potential, $\Delta\psi$ (with the dye 3,3′-dipentyloxadicarbocyanine) indicate that the means of Na^+ transport is sodium/proton exchange. The kinetics of the pH changes and other evidence suggests that the antiport is electrogenic ($H^+/Na^+ > 1$). The resulting large chemical gradient for Na^+ (outside $\gg$ inside), as well as the membrane potential, will drive the transport of 18 amino acids. The 19th, glutamate, is unique in that its accumulation is indifferent to $\Delta\psi$: this amino acid is transported only when a chemical gradient for Na^+ is present. Thus, when more and more NaCl is included in the vesicles glutamate transport proceeds with longer and longer lags. After illumination the gradient of H^+ collapses within 1 min, while the large Na^+ gradient and glutamate transporting activity persists for 10–15 min, indicating that proton motive force is not necessary for transport. A chemical gradient of Na^+, arranged by suspending vesicles loaded with KCl in NaCl, drives glutamate transport in the dark without other sources of energy, with V_{max} and K_m comparable to light-induced transport. These and other lines of evidence suggest that the transport of glutamate is facilitated by symport with Na^+, in an electrically neutral fashion, so that only the chemical component of the Na^+ gradient is a driving force.

The transport of all amino acids but glutamate is bidirectional. Actively driven efflux can be obtained with reversed Na^+ gradients (inside $>$ outside), and passive efflux is considerably enhanced by intravesicle Na^+. These results suggest that the transport carriers are functionally symmetrical. On the other hand, noncompetitive inhibition of transport by cysteine (a specific inhibitor of several of the carriers) is only obtained from the vesicle exterior and only for influx: these results suggest that in some respects the carriers are asymmetrical.

A protein fraction which binds glutamate has been found in cholate-solubilized H. halobium membranes, with an apparent molecular weight of 50,000. When this fraction (but not the others eluted from an Agarose column) is reconstituted with soybean lipids to yield lipoprotein vesicles, facilitated transport activity is regained. Neither binding nor reconstituted transport depend on the presence of Na^+. The kinetics of the transport and of the competitive inhibition by glutamate analogs suggest that the protein fraction responsible is derived from the intact transport system.

Key words: Halobacterium halobium, amino acid transport, sodium-proton exchange, asymmetry of transport system, reconstitution of glutamate transport

Received March 14, 1977; accepted April 26, 1977

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INTRODUCTION

In intact bacteria the active transport of various amino acids and sugars is energized either by terminal oxidation or by hydrolysis of ATP (1, 2). When the source of energy is terminal oxidation the means of energy coupling has been shown to be the H^+ gradient generated. In such transport it is assumed that the translocation of the substrates is coupled to the movement of H^+ down its electrochemical gradient – symport (3). Transport of this type is obtained also in cell envelope vesicles (4, 5), which are devoid of soluble components. The transport of those substrates which depend on ATP hydrolysis but not proton motive force is more complex. In such transport soluble factors have also been implicated, the "shockable proteins" (2), many of which have been isolated and show binding of the substrates of transport.

The energetics of transport in the first category has occupied many students of transport, and the relationship of various cation gradients to substrate accumulation is still under intense scrutiny. Another, more recent approach to the study of transport is the isolation and characterization of membrane proteins which accomplish the translocation of substrates across the membranes. Such studies (6–10) will be undoubtedly followed in the future by attempts to describe the molecular details of the mechanisms responsible for the movement of the substrate and for the coupling between the substrate and cation fluxes.

With the discovery of bacteriorhodopsin (11, 12) Halobacterium halobium became a prime candidate for transport studies, since the function of this pigment is to generate a gradient for H^+ across the cell membrane upon illumination (12, 13). Membranes of this organism are thus easily energized by illumination and deenergized by witholding illumination. Cell envelope vesicles from H. halobium, used for these studies, have inside-in membrane orientation (13–15). These preparations are inert unless ion gradients are established, either by preloading with the appropriate salts, or by illumination or substrate oxidation. Since the envelopes contain both the purple membrane and the rest of the cytoplasmic membrane, the components which couple proton motive force to energy-utilizing processes are present. The secondary energized events which follow H^+ extrusion include the transport of Na^+, K^+, and amino acids. The relationship of these to one another and to the gradient of H^+ has been examined in detail.

Although the nature and mechanism of the membrane components which catalyze the translocation of the amino acids remain obscure, the existence of a protein fraction which binds glutamate and which can be reconstituted with soybean lipids to yield specific transport for this amino acid has been demonstrated.

LIGHT-INDUCED GRADIENTS FOR H^+ AND Na^+

In H. halobium envelope vesicles light-induced proton release can be conveniently followed by pH measurements in the extravesicle medium (14, 16–18). The existence of a gradient of protons is inferred from such measurements on the basis of the fact that the number of protons which appear in the medium is many times the number of pigment molecules (18). In addition, heavily buffered vesicles release 2–3 times more H^+ during illumination (18), suggesting that H^+ release is normally accompanied by a pH rise inside the vesicles. Within a minute after the beginning of the illumination an equilibrium is reached, where the H^+ extrusion is equal to H^+ influx, due to various dissipative mechanisms. The number of protons translocated at equilibrium is greatly dependent on pH; optimal H^+ release is near pH 3.4–4, and is greatly diminished at and above pH 6

(19). The pH dependence may be caused by direct pH effects on bacteriorhodopsin, by mass-action effect of H^+ concentration inside the vesicles, or by possible lack of counter-ion permeability at higher pH, which would allow the development of higher electrical potentials but lower pH gradients. The last does not seem to be the case since the electrical potentials are also less at higher pH (17), and passive cation permeability rises rather than falls with pH (20). Drachev et al. (21) reported that in a planar membrane containing bacteriorhodopsin the photopotentials were diminished when H^+ translocation was against a pH gradient.

The electrical potentials which arise during illumination have been measured using the fluorescent dye 3,3′-dipentyloxadicarbocyanine (di-0-C_5) (16–18). As described earlier by Sims et al. (22), membrane potential (negative inside) will cause this cationic dye to accumulate in the vesicle interior resulting in aggregation and fluorescence quenching. Diffusion potentials for K^+ in the presence of valinomycin were used to calibrate the fluorescence changes, and yielded a slope of –0.33 percent/mV up to about –110 mV (17).

The pH changes and electrical potential during illumination depend greatly on the presence of Na^+ inside the envelope vesicles, while the composition of the exterior solution has much less influence. When the vesicles contain only KCl the pH difference which develops across the membrane amounts to about 2 pH-units and the potential, $\Delta\psi$, is low, about –30 mV (17). When the vesicles contain only NaCl, ΔpH is smaller and $\Delta\psi$ is increased to as much as –100 mV (17). Vesicles containing both NaCl and KCl show complex time-dependent changes during illumination for both ΔpH and $\Delta\psi$ (18, 23). Dependent on the amount of Na^+ inside the vesicles, there is an initial period of smaller pH change and larger potential, similar to the results obtained with NaCl-loaded vesicles. After this initial period ΔpH increases and $\Delta\psi$ decreases until the gradients resemble those obtained with KCl-loaded vesicles. The lengths of the initial periods are roughly proportional to the concentration of NaCl in the vesicles.

The significance of the above observations became clear once it was discovered that during illumination Na^+ is extruded against its electrochemical potential (16, 18). Light-induced Na^+ efflux is thus accompanied by decreased ΔpH and increased $\Delta\psi$, until ^{22}Na is depleted from the vesicles. Such a result is consistent with H^+/Na^+ exchange, or in Mitchell's terminology, antiport (3). Protons returned by the antiporter are reejected by bacteriorhodopsin, and the net result is the removal of Na^+ (and positive charges), leading to the increased membrane potential observed.

If the stoichiometry of H^+ to Na^+ exchanged were 1:1, there would be no net transport of charges and the antiport would be driven only by ΔpH. However, when the light-induced pH changes in NaCl-containing vesicles are measured at a higher pH, at pH 7–7.5, the initial H^+ extrusion is seen to be followed by H^+ influx, leading to a transient reversal of ΔpH (18). This reversal suggests that the H^+/Na^+ antiport cannot proceed with a stoichiometry of 1:1, because such a process would be abolished as ΔpH approaches zero. On the other hand, at higher H^+/Na^+ stoichiometries the exchange would be electrogenic and would be driven also by the electrical potential. Potential in this model would serve to balance the system at a reversed pH gradient. The electrogenicity of the H^+/Na^+ exchange is suggested also by results from a more direct approach (MacDonald and Lanyi, unpublished experiments), where Na^+-gradient (outside > inside) dependent [^{3}H] triphenylmethyl phosphonium ion uptake was observed in KCl-loaded, nonenergized vesicles.

The characteristic pH changes during illumination, which reveal the operation of H^+/Na^+ antiport, are not seen during a second illumination unless an interval of several hours is allowed between the 2 illuminations (18). The slowness of the return of the effect with the incubation in the dark is consistent with the depletion of the vesicles of Na^+ during the first illumination and the slowness of the exchange of K^+ and Na^+ in the dark (20).

The efflux of Na^+ could not proceed extensively unless the inward movement of another ion decreased the magnitude of the electrical potential which develops. Earlier results by Kanner and Racker (19) indicate that the membranes are somewhat permeable to K^+, and illumination will drive the uptake of K^+ even in the absence of valinomycin. It seems reasonable that the influx of K^+ would compensate for the efflux of Na^+ in these vesicles. The scheme of ionic fluxes in the model proposed above is given in Fig. 1.

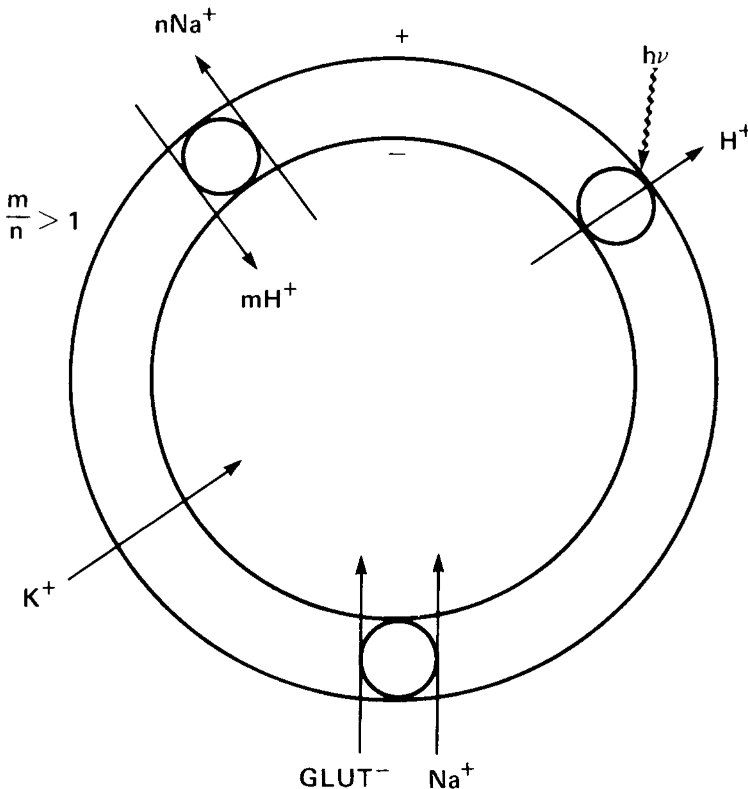

Fig. 1. Scheme of energy transduction in H. halobium cell envelope vesicles. As explained in the text, 3 active components are proposed: bacteriorhodopsin, activated by light, the proton-sodium antiporter, and the glutamate carrier, which functions as a symporter for glutamate and Na^+. Unlike the transport of glutamate, the transport of other amino acids responds to membrane potential, in addition to a chemical gradient for Na^+, implying that those translocations involve net transport of charges. The accumulation of K^+ may be facilitated by a specific ionophore, but such has not been found yet.

AMINO ACID TRANSPORT KINETICS

According to the principle of chemiosmotic energy coupling the ion gradients, which arise as a result of metabolic activity, will drive the accumulation of transported substrates (3). The relationship of the cation gradients in H. halobium cell envelopes to substrate transport is therefore of interest. Findings which identify such gradients as driving forces for transport should be considered indirect evidence for the existence of specific membrane components which couple ion and substrate movements to one another.

Cell envelopes from H. halobium actively transport 19 amino acids when illuminated (14–18, 24–26). The energetics of such transport have been described in some detail, and the results suggest that the driving force for most amino acids includes both chemical (ΔNa^+) and electrical ($\Delta\psi$) components of the Na^+ gradient which arises during illumination. Glutamate transport, which is somewhat different in this respect from the others, has proved particularly interesting. The transport of this amino acid can be driven either by illumination (16, 24) or by substrate oxidation (27). The results in both cases are similar: the transport kinetics are dependent on the concentration of Na^+ inside the vesicles. Analogously to the light-induced pH changes, described in the previous section, and on a similar time scale, glutamate transport during illumination shows lags whose duration is determined by the intravesicle Na^+ concentration. Lags are also observed when the light intensity is lowered. On the other hand, when the intravesicle concentration of Na^+ is set at a very low value and the external medium contains Na^+, transport of glutamate occurs even in the dark. At a very high initial Na^+-concentration difference (outside/inside $\geqslant 500$) the rates of transport (and the K_m as well, $\sim 1.3 \times 10^{-7}$M) were very similar to transport induced by illumination. These results strongly suggest that the sodium gradient developed during illumination is a driving force for glutamate transport, and imply that the mechanism involves glutamate-Na^+ symport.

Lack of glutamate transport during the illumination of the vesicles, until a large chemical gradient for Na^+ is developed, argues that ΔNa^+ is an obligatory ingredient of the driving force, particularly since under these conditions large electrical potentials are observed (18). The fact that transport of glutamate can be induced with an artificially set Na^+ gradient argues that the Na^+ gradient alone is sufficient as a driving force. The latter question was approached also by measuring transport activity following illumination, after various lengths of incubation in the dark. Both components of the proton motive force (ΔpH and $\Delta\psi$) decay within 1 min under these conditions (17, 18), but ΔNa^+ and glutamate transport persists for 10–15 min. It may be concluded that a chemical gradient for Na^+ alone will drive glutamate transport.

Agents which discharge one or both components of the proton motive force inhibit light-induced glutamate transport but have much less effect on transport driven in the dark by a chemical Na^+ gradient in KCl-loaded vesicles. It has been concluded from such results that proton motive force is also a driving force for glutamate uptake, but only insofar as it produces a chemical gradient for Na^+ (25). Thus, as indicated in Fig. 1, energy conservation in this system involves first the photochemical reactions of bacteriorhodopsin, second a gradient for protons, produced by the light-induced H^+ translocation, third a gradient for Na^+, generated by proton/sodium antiport, and fourth, gradients for amino acids, such as glutamate, achieved by symport with Na^+.

As noted above, all the amino acids can be transported in H. halobium with a Na^+ gradient. This is probably a consequence of the fact that the organisms have adapted

to living at high concentrations of NaCl (28–30). There exist Na^+-dependent transport systems in other microorganisms, however, and recent results indicate that some of these, e.g., proline transport in Mycobacterium phlei and glutamate transport in Escherichia coli B, are driven by a sodium gradient generated by respiratory activity (MacDonald and Lanyi, manuscripts in preparation). Thus, it is likely that parts of the scheme presented in Fig. 1 will apply to nonhalophilic bacteria as well.

SYMMETRY OF AMINO ACID TRANSPORT CARRIERS

Many different lines of evidence point to the existence of proteins which are responsible for substrate translocation. One of the questions relating to the molecular details of such transport carriers, which can be studied with a kinetic approach, is the symmetry of the protein involved with respect to transport in the 2 directions. In H. halobium envelope vesicles all the amino acids but glutamate show efflux when illumination is stopped or when excess unlabeled amino acid is added (14, 25, MacDonald, unpublished experiments). Such efflux, which has been studied also in vesicles preloaded with radioactive amino acids, has a large Na^+-dependent component, suggesting that the translocation involves the carrier in this direction also. Half-saturating concentration for Na^+ is about 150 mM for exchange diffusion of a number of amino acids in either direction (MacDonald, unpublished experiments). Moreover, prearranged Na^+ gradients in the reverse direction (inside > outside) accelerate amino acid efflux, indicating that both active and passive transport are bidirectional. These results are consistent with either symmetrical carriers for the amino acids or carriers with mixed orientation in the membranes. Investigations of the noncompetitive inhibition of transport by cysteine have resolved this question. Cysteine, which seems to inactivate some of the transport carriers in H. halobium (but not via a redox reaction), acts in an asymmetrical manner: both passive and active influx of methionine are inhibited when cysteine is added on the outside of the vesicles, but remain unaffected when the inhibitor is added to the inside only (Helgerson and Lanyi, manuscript in preparation). On the other hand, efflux of methionine, passive or actively driven, is not inhibited by cysteine when present on either (or both) side of the membrane. Thus, the transport carrier for methionine a) must be uniformly oriented in the envelope vesicles and b) appears as symmetrical in transport functions but asymmetrical in the cysteine effect.

The glutamate transport system is clearly different from the others in that the efflux of glutamate does not occur upon adding unlabeled glutamate to either energized or nonenergized envelopes (24). Since glutamate is chemically unchanged during transport, the irreversibility observed must reflect the properties of the transport carrier. Although glutamate would not exit passively, it can be driven from preloaded vesicles by a reverse Na^+ gradient (Na^+ inside, K^+ outside) (24). The functional asymmetry of this carrier may therefore reflect specific requirements for different cations on either side of the membrane. At this time it is difficult to visualize a molecular mechanism which would generate such properties.

RECONSTITUTION OF TRANSPORT

Ultimately, the questions relating to the functioning of transport carriers will be solved only after the proteins involved are isolated in a functional form. Since these proteins are membrane-bound and their assay requires the measurement of substrate

fluxes across membranes, attempts to isolate them have succeeded only recently, after techniques to solubilize and reconstitute membrane proteins became available (31–33).

When H. halobium cell membranes are disrupted with cholate, the soluble fraction binds [^{3}H]glutamate. Figure 2 shows a Scatchard plot of the binding data, indicating a single kind of binding site, with the dissociation constant $K_{diss} = 6 \times 10^{-8}$M (Lanyi, Yearwood-Drayton, and MacDonald, manuscript in preparation). The proteins(s) involved in the binding elute from an Agarose column, equilibrated with detergent-buffer, at an approximate molecular weight of 50,000. This fraction, but not the others eluted, can be reconstituted with soybean lipids to yield lipoprotein vesicles permeable to glutamate. Reconstituted transport is shown in Fig. 3. No uptake of aspartate is observed, as expected, since in H. halobium this amino acid does not share its carrier with glutamate (25). The transport of glutamate in the liposomes appears to be facilitated equilibration

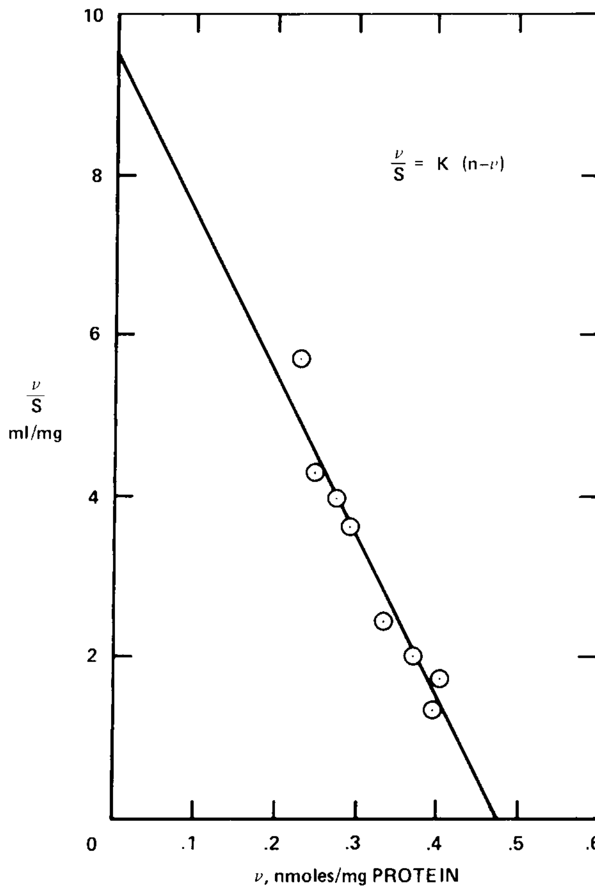

Fig. 2. Glutamate binding by cholate-solubilized membrane proteins from H. halobium. The binding data (Lanyi, Yearwood-Drayton, and MacDonald, manuscript in preparation) are plotted according to the modified Scatchard equation shown: υ) bound [^{3}H]glutamate, nmol/mg protein; S) total glutamate concentration (essentially equal to free glutamate), nmol/ml; and n) specific glutamate binding activity, nmol/mg protein. From the intercept on the ordinate a dissociation constant of 6×10^{-8} is calculated.

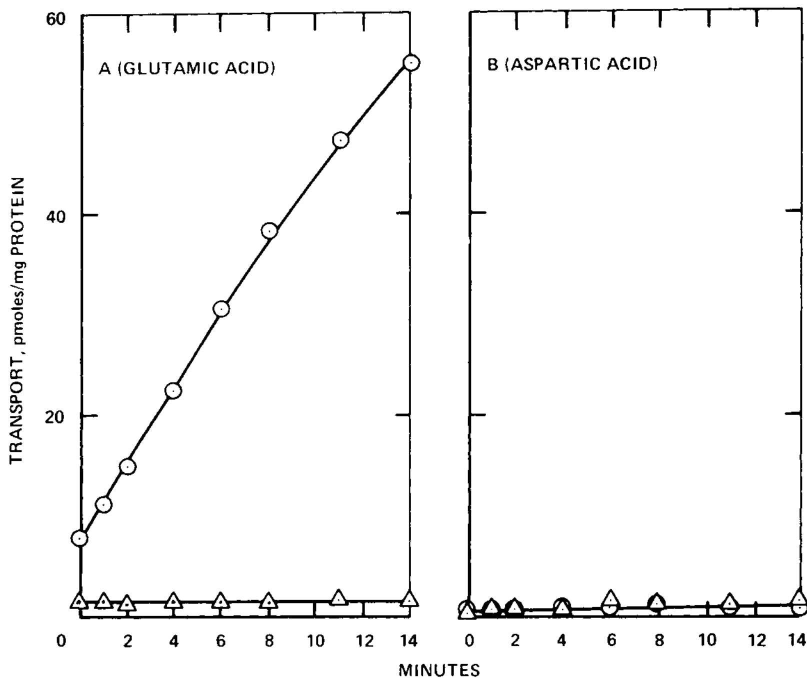

Fig. 3. Transport of glutamate and aspartate in liposomes reconstituted with a membrane protein fraction from H. halobium. Reconstitution by the cholate dialysis method (31). At zero time the lipoprotein vesicles were added to buffer containing [^{3}H]-labeled amino acid and at the indicated times samples were withdrawn. Retained radioactivity was determined by rapid elution of the liposomes from small gel columns (Lanyi, Yearwood-Drayton, and MacDonald, manuscript in preparation). ⊙) Complete system; △) protein omitted. No transport was seen when the lipids were omitted.

rather than active accumulation. Neither binding nor reconstituted transport requires Na^+.

Specificity of reconstituted transport is often ascertained by the use of inhibitors (6, 8–10). In the glutamate transport system of H. halobium only structural analogs of glutamic acid are available as inhibitors. Results with these show that the kinetic constants for competitive inhibition by kainic acid, α-methylglutamic acid, and N-methylglutamic acid form a consistent pattern in 3 systems: transport in intact envelopes, binding in cholate-buffer, and transport in the reconstituted liposomes. It was concluded from these studies (Lanyi, Yearwood-Drayton, and MacDonald, manuscript in preparation) that the recognition site for glutamate is the same in the 3 measurements. The protein fraction isolated thus appears to be derived from the intact transport system, but has lost the ability to couple the translocation of glutamate with that of Na^+. Binding and transport of glutamate are therefore not obligatorily linked to the coupling function. The protein fraction is presently about 12- to 15-fold purified. Future studies with further purified material may reveal more properties of the protein(s) involved and shed some light on the mechanism of the substrate translocation.

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