Forschung, Wissenschaft

Change in Aggregation of Lecithin Due to Valinomycin-Lipid Interaction in Vesicle Membranes *

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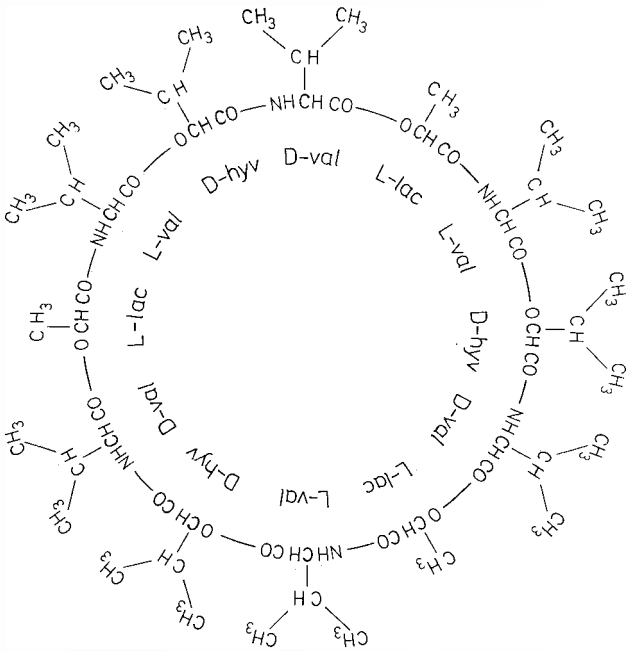
Abstract

Vesicles obtained by sonication of chlorophyll-lecithin mixtures dispersed in an aqueous medium are bounded by a single bilayer membrane with incorporated chlorophyll *a* or *b* in monomeric form. Valinomycin added to a suspension of such vesicles induces slight changes in the absorption spectrum of the chlorophylls. This effect is neither associated with the ionophoric properties of valinomycin nor due to a direct interaction of this agent with chlorophyll, but indicates an interaction between lecithin and valinomycin. This interaction alters the aggregational state of about 35 lipids around each valinomycin molecule, and the chlorophylls act as sensors for this phenomenon. The sensor ability stems predominantly from different electrostatic interactions to which the chromophores are subjected in different lipid aggregations.

Valinomycin is one of the well-known antibiotics which display ionophoric properties, i.e., it mediates the transport of alkali ions through membranes by complexing the ions at the water/membrane interfaces and carrying them across the membrane's hydrophobic core [1]. This ability arises from a considerable conformational flexibility of the cyclic depsipeptide; the molecule can adapt itself to a hydrophylic environment by exposing the carbonyls of the ester and amid groups to the surroundings, as well as to a hydrophobic phase by refolding the backbone which causes the aliphatic side chains to form the molecular surface. The latter conformation has all carbonyl groups oriented toward the interior of the molecule, with some of them stabilizing the conformation by means of hydrogen bonds while the others form the coordination sites for the alkali ions in the molecular cavity [2].

Due to this adaption to the environmental polarity, valinomycin easily penetrates into lipid bilayer membranes. The polarity of such a membrane ranges from an almost aqueous domain, represented by the hydrated polar head groups of the lipids at the membrane/water interfaces, to a highly apolar domain constituted by the aliphatic fatty acid chains of the lipids. Hydrophobic interactions between these chains hold the two monolayers of lipids together thus forming the bilayer membrane, and give rise to the tendency of such membranes to form closed, vesicular structures when suspended in an aqueous medium. In view of these features of a lipid membrane, it is conceivable that other, non-lipid but amphophilic molecular species can be incorporated into the bilayer as exemplified by chlorophyll *a* or *b*; the porphyrin ring with the complexed magnesium ion represents the polar and hydrophylic head while the phythyl side chain forms the apolar and hydrophobic moiety of the molecule. One should expect, however, that the incorporation of a non-lipid component into the membrane has an influence on the lipid matrix particularly when the component, as in the case of valinomycin, moves preferentially from the aqueous into the membraneous phase which indicates a strong interaction between the component and the lipids. This report then presents evidence for an alteration of the lipid matrix in lecithin vesicles due to a valinomycin-lipid interaction, deduced from the spectroscopic behaviour of chlorophyll in the membrane which acts as a sensor for the lipids' aggregational state.



Valinomycin (val = valine, lac = lactic acid, hyv = α-hydroxyisovaleric acid)

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Chlorophyll containing lecithin vesicles

Single-shelled vesicles consist of one spherical bilayer membrane which encapsulates a small volume of an aqueous phase whose composition is equal to or different from that of the suspending aqueous medium. They are prepared by dispersing dry lipids in an aqueous buffer solution and exposing this dispersion to ultrasonic irradiation for 2.5 hours at 0 °C under nitrogen. After the removal of larger lipid aggregates by centrifugation at 105000 xg for 1 hour, the vesicle suspension is subjected to sieve chromatography on Sepharose 4B which separates the larger, multi-shelled liposomes from the single-shelled vesicles [3].

Chlorophyll containing lecithin vesicles can be prepared by the same procedure but starting from a molecular dispers, dry mixture of chlorophyll and lecithin [4]. Fig. 1 then shows, by way of example, the elution pattern of chlorophyll *a*-lecithin vesicles from the Sepharose column. The absorbance at 254 nm, which arises predominantly from light scattered by the vesicles and to a lesser extent from the absorption of lecithin and chlorophyll *a*, is paralleled by the absorbance at 668 nm which is essentially due to chlorophyll *a* (see Fig. 3) thus indicating that the content of the non-lipid component is about constant for the liposome and the vesicle fraction. Moreover, the symmetry of the bell-shaped elution curve suggests an almost monodispers vesicle population whose average Stokes' radius can be estimated by means of the molecular sieve coefficient σ introduced by *Ackers* [5]. The value thus determined (102 ± 5 Å) is about 15 Å smaller than that estimated for pure lecithin vesicles [3,4] which reflects the interaction of the non-lipid component with lecithin and probably an alteration of the lipid matrix. The spherical shape of the vesicles is evident from the electronmicrograph shown in Fig. 2. The shadowing of the spheres with platinum allows for an independent measurement of the particle radii and the range of 80–120 Å thus found confirms the low polydispersity [4].

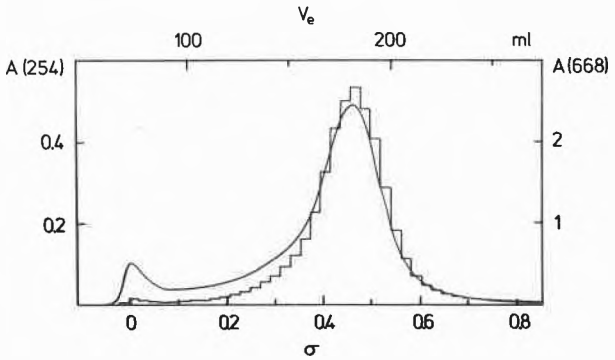

Fig. 1: Elution of chlorophyll *a* containing lecithin vesicles from a Sepharose 4B column. The absorbance of the effluent was monitored continuously at 254 nm in a circular cell of 3 mm diameter (curve) and measured for each fraction at 668 nm in a 1 cm rectangular cuvette (histogram). The elution volume, V_e, is given by the scale on the top; the scale on the bottom indicates the molecular sieve coefficient, σ, according to Ackers [5].

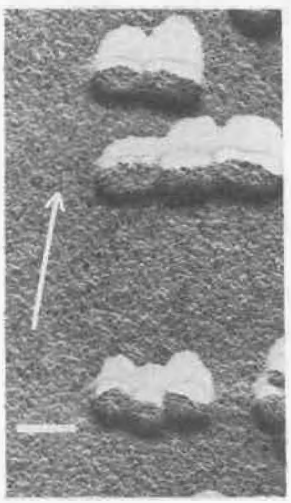

Fig. 2: Vesicles shadowed with platinum in the direction indicated by the arrow at an angle of 25°. The bar is 500 Å × 150000.

The highest molar ratio of chlorophyll to lecithin which could be achieved was 0.018 corresponding to an average of 42 pigment molecules per vesicle. The absorbance spectra of chlorophyll *a* (Fig. 3) as well as *b* were essentially the same within the whole range of solubility of chlorophylls in lecithin membranes, though minor changes were observed from one vesicle preparation to another [6]. In any case, however, the spectra were typical for the pigments in the monomeric form [7,8], hence phase separations between chlorophylls and lecithins with a concomitant aggregation of pigment molecules as observed under certain conditions [9,10] did not occur.

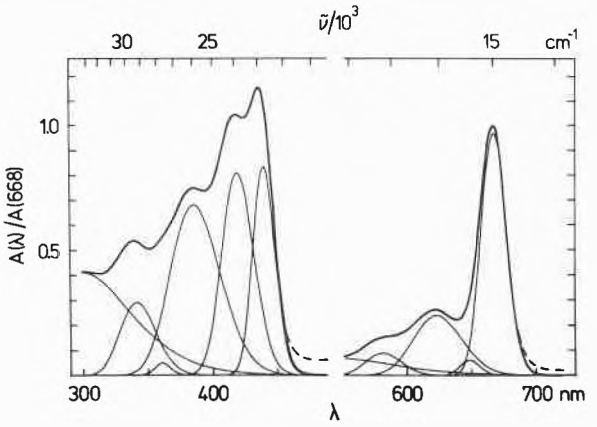

Fig. 3: Absorption spectrum of chlorophyll *a* incorporated into membranes of lecithin vesicles, A (λ), normalized by the absorbance at the main red peak, A (668). Thin lines represent Gaussian curves which were obtained by the deconvolution of the spectrum. The heavy line indicates the sum of these curves and coincides with the measured spectrum (broken line) except for those wavelength ranges where Gaussian curves were neglected. The spectrum in the omitted range between 490 and 550 nm runs almost parallel to the abscissa as a slightly wavy line. The upper scale indicates the wavenumber, $\bar{\nu}$.

Valinomycin-induced changes in absorbance of chlorophylls

Two optically matched cuvettes filled with an equal volume (3 ml) of the same vesicle suspension obviously have identical absorbances at all wavelengths. However, when a small volume (5 μl) of a methanolic valinomycin solution (1 mM) and the same amount of methanol were added to the sample and the reference cuvette, respectively, clear-cut absorbance differences could be detected between 300 nm and 700 nm. It should be mentioned that the absorption of valinomycin in this wavelength range is vanishingly small. Adding additionally the same amounts of valinomycin solution and methanol but now to the reference and the sample cuvette, respectively, establishes again identical conditions in both cuvettes and the absorbance differences indeed vanished almost completely [6]. Fig. 4 then shows a typical difference spectrum for chlorophyll *a*, normalized to the amount of pigment present as expressed by the absorbance of the vesicle suspension at the highest peak of the red band of chlorophyll *a* (see Fig. 3). The absorbance differences are clearly related to chlorophyll *a* since only detectable in those wavelength ranges where the main absorption bands of chlorophyll *a* are located, viz. below 470 nm (Soret band) and from 550 nm to 700 nm (red band).
During the preparation of the vesicles as well as when diluting the vesicle stock solutions for spectroscopic measurements, care was taken to have the same concentrations of alkali ions present in the system inside the vesicles and in the suspending medium. Therefore, the addition of the ionophore valinomycin could not give rise to the formation of a diffusion potential across (or an electric field within) the membrane which, due to the electrochromic response of chlorophylls [11, 12], would change the spectral parameters of the pigment molecules in the membrane. However, one could think of local electric fields associated with the positively charged valinomycin-alkali ion complexes in the membrane; the absorbance differences should then depend on the concentration and, even more so, on the species of alkali ions present since valinomycin complexes preferentially potassium and rubidium ions [2]. Neither concentration nor ion species had any detectable effects on the absorbance differences, and charged complexes in the membrane can therefore not explain the spectral response of chlorophylls to the presence of valinomycin. Another explanation assumes complexing of the nucleophilic carbonyl groups of valinomycin to one of the free coordination sites of the magnesium ion in chlorophyll. It is supported by the extensive investigations of Katz and co-workers showing appreciable spectral changes of chlorophyll upon complexation with nucleophiles [13]. The wavelength dependence of the absorbance changes should then be identical for all conditions but their intensity should vary with the respective concentrations of chlorophyll and valinomycin in the membrane. Using different vesicle preparations, whose chlorophyll content ranged between 1 and 15 pigments per 1000 lecithins, and by variation of the molar ratios of valinomycin to chlorophyll in the samples, the first postulate could be verified [6]. The experimental data, however, did not comply with the relations indicating the dependence of the intensity of the absorbance changes on the relevant parameters as derived from the complex formation scheme [6], and the latter can therefore be dis-

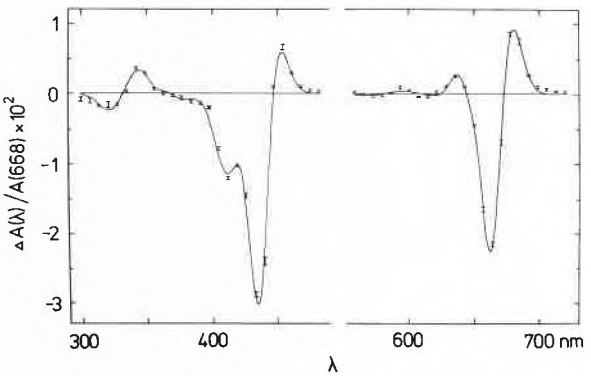

Fig. 4: Absorbance differences, ΔA (λ), induced by valinomycin in the spectrum of chlorophyll *a*, normalized by the absorbance of the vesicle suspension at 668 nm, A (668). The experimental data, which were measured and evaluated as described in ref. 6, are represented by a bar indicating ± standard deviation. The curve was calculated with the estimated solvatochromic coefficients and the Gaussian components of the spectrum (see Fig. 3).

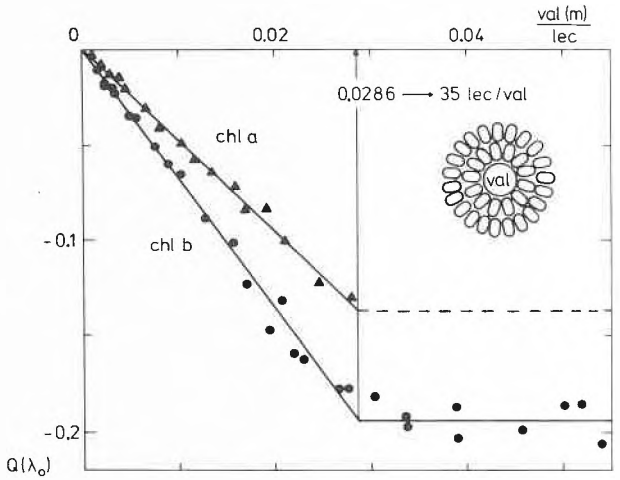

Fig. 5: Dependence of the intensity of the difference spectra on the molar ratio of valinomycin dissolved in the membrane to lecithin, val (m)/lec. The intensity is represented by the quantity Q (λ_0) which is estimated form the whole difference spectra but expressed for a selected wavelength, λ_0 (435 nm for chlorophyll *a* and 461 nm for chlorophyll *b*, see ref. 6). Experimental conditions limit the range of val (m)/lec-values for chlorophyll *a* containing vesicles at 0.028 [6]; for chlorophyll *b* containing vesicles, however, this limit is higher due to about three fold larger molar extinction coefficients of the Soret band. The inset shows how 35 lecithin molecules (surface area in the membrane 70 Å^2) just fit into two circular layers around a valinomycin molecule.

missed. Similarly, the experimental data did not conform to relations derived from a less restrictive scheme, assuming direct but not specified interactions between chlorophyll and valinomycin [6], hence such interactions can definitely be excluded.

What remains then is an interaction between lecithin and valinomycin with chlorophylls acting as sensors for this interaction, an interpretation which is corroborated by the agreement of the experimental data with the pertinent relations derived from this scheme [6]. Moreover, these relations allow for an estimate of the partition coefficient of valinomycin for the vesicle membrane/water two-phase system and, by means of this quantity found to be about 20000, the molar ratio of valinomycin dissolved in the membrane to lecithin can be calculated for each experiment. As shown in Fig. 5, the intensity of the difference spectra, which is expressed by the quantity Q (λ_0) [6], increases proportionally to this ratio but levels off to a constant value at a critical ratio of 0.0286. It thus appears that the solubility of valinomycin in lecithin membranes reaches its limit at this ratio and, in a valinomycin-saturated membrane, we then find an average of 35 lecithins per valinomycin which can be arranged in two circular layers around the antibiotic (see inset of Fig. 5).

Chlorophylls as sensors for aggregational states of lipids

Since a direct interaction between valinomycin and chlorophyll could be excluded, the observed changes of the chlorophylls' absorbance upon incorporation of valinomycin into the membranes can only arise from an alteration of the pigment's environment represented by the lipid matrix. Hence, the state of aggregation of lecithins surrounding a valinomycin molecule should be different from that of lipids in a domain without valinomycin in the sense that the two states represent two slightly different solvents for chlorophyll. The spectral changes can then be understood in terms of a solvatochromism, i.e., the influence of the solvent on the absorption spectrum of the solute, which is a well-known phenomenon for chlorophylls [14,15].

Solvatochromism arises from dispersion interaction between solvent and solute as well as electrostatic interaction due to permanent or induced electric fields acting on the chromophore. Both interactions cause changes in the relative positions of the energy levels in the pigment molecule and affect the transition moments of electronic transitions [16,17]. In terms of an absorption band of the pigment, a change in transition moment expresses itself as an increase (or a decrease) of the band's intensity which is proportional to this intensity, while changes in energy levels create a band-shift and a band-broadening (or band-sharpening) which are proportional, respectively, to the first and the second derivative of the band with respect to the wavenumber [18]. Although these features apply to all absorption bands pertinent to the electronic transitions which occur on excitation with light, the transitions usually respond differently to the pigment's interaction with the environment, and the extents of the forementioned changes vary considerably from one transition to another.

Both, the red and the *Soret* band of the chlorophylls originate from at least two electronic transitions, and the analysis of the difference spectra in terms of solvatochromism thus requires the resolution of the spectra into bands pertinent to these transitions. Unfortunately, an unambiguous resolution is not yet practicable, however, at least part of the bands can be approximated by Gaussian curves [7]. For each of the Gaussian components, which are obtained by the deconvolution of the chlorophyll spectra (see Fig. 3), a set of 3 solvatochromic coefficients can be determined which indicate the change in transition moment as well as the band-shift and the band-broadening effect [18]. In order to reduce the possible ambiguity inherent in an estimation of a rather large number of coefficients, the proportionality of the absorbance differences to the molar ratio of valinomycin dissolved in the membrane to lecithin was used as a selection criterion [18], and the solvatochromic coefficients are then estimated from the slopes of the straight lines in such plots as shown, by way of example, for the red band of chlorophyll *a* on the left-hand side of Fig. 6. The contributions of the first two Gaussian components of this band, which essentially determine the absorbance differences in this wavelength range, are depicted on the right-hand side of Fig. 6, and Fig. 4 then demostrates that difference spectra which are calculated by means of the estimated solvatochromic coefficients and the pertinent Gaussian curves coincide almost perfectly with the experimental data.

Comparing the solvatochromic coefficients with data for chlorophyll in different solvents [14,15] reveals that a difference in electrostatic interaction is the main reason for the absorbance differences, with dispersion interaction yielding only a minor contribution. Moreover, from data obtained by electro-optical measurements for different chromophores (compiled by *Liptay* [16] and *Labhart* [17]), it can be inferred that the electric field strength effective for chlorophyll in the valinomycin-induced state of lipid aggregation differs by the order of 10^7 V/cm from that in the unaltered state. A model calculation has shown that the ordered layers of dipoles on the surfaces of a lecithin membrane indeed give rise to such tremendous field strengths at least in the domain where the glycerol-ester moieties of the lipids are located, and the estimated difference in electric field strength can then be rationalized by different orientations of the anisotropic chromophore (i.e., the porphyrin ring) and possibly also of the polar head groups [18].

Conclusions

The altered lipid aggregation around a valinomycin molecule in lecithin membranes is obviously the two-dimensional analogue to the well-known phenomena which occur three-dimensionally in bulk solvents and are known as solvation of a solute. The lipids around valinomycin may thus be compared to water in the hydration sphere of an ion, although the kind of interaction which causes the altered state of aggregation for solutes in the solvation shell is electrostatic in the latter case and most likely hydrophobic in the former case. The experimental data reported here allow us to sketch the scheme shown in the upper part of Fig. 7. From the limit of solubility of valinomycin in lecithin membranes, which is presumably reached when the aggregational state of all lipids is altered, we can conclude that the solvation shell extends over two circular layers of lipids. Chlorophyll which is present in such a shell adapts to the altered lipid environment by a reorientation of the porphyrin ring and, as a consequence of the concomitantly changed electrostatic interaction with the lipids, has an absorption spectrum which slightly differs from that of chlorophyll outside the shell.

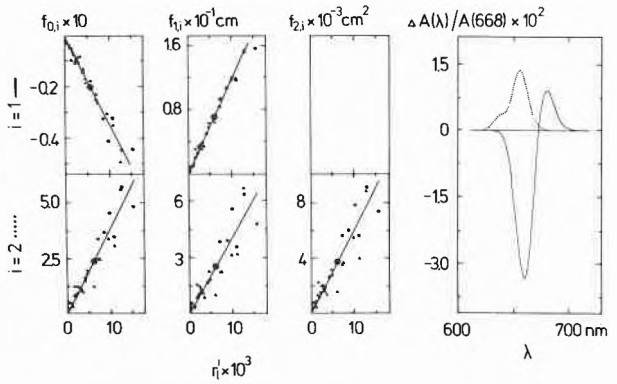

Fig. 6: On the left-hand side, the parameters $f_{j,i}$, which express the change in intensity (j = 0), the band-shift (j = 1) and the band-broadening (j = 2) of the *i* the Gaussian component are plotted versus the molar ratio of valinomycin dissolved in the membrane to lecithin, r_1', for the first two Gaussian curves in the red band of chlorophyll *a*. The solvatochromic coefficients are determined by the slopes of the lines, and an empty box then means that the corresponding coefficient is zero. The right-hand side shows the contributions of the two Gaussian components to the difference spectrum plotted in Fig. 4.

In more general terms, we could say that the lipid matrix establishes an indirect interaction between valinomycin and chlorophyll as indicated by the broken arrow in Fig. 7. It would be rather surprising, however, when such an interaction were restricted to chlorophyll, hence we should expect similar phenomena for other components present in a membrane. The scheme in the lower part of Fig. 7 then illustrates how valinomycin could interact indirectly with a protein in a biological membrane and thus influence the protein's conformational state and functioning. There are indeed

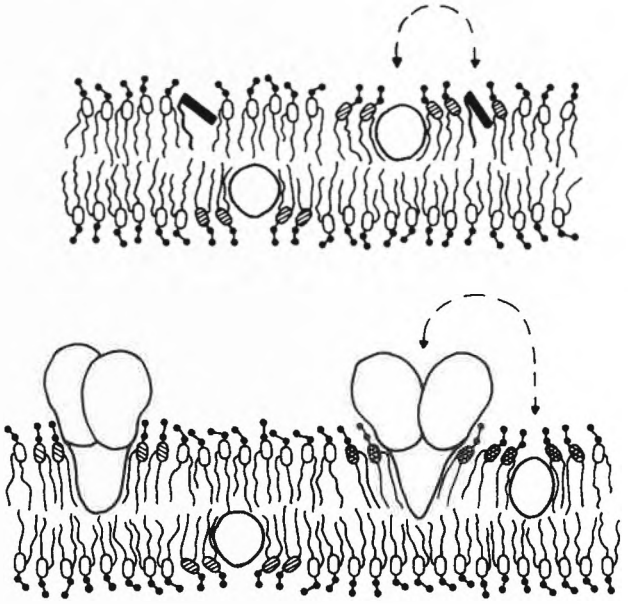

Fig. 7: Schematic representation of a membrane containing chlorophyll (symbolized by the heavy bar with tail, upper part), or a protein (lower part). Valinomycin dissolved in the membrane (indicated by the circles) changes the aggregational state of two layers of lipids surrounding it and thus influences chlorophyll or the protein. Note that a similar solvation shell of lipids has to be expected for the membrane bound protein, a phenomenon known as boundary lipid formation in membrane biology.

several reports in the literature that valinomycin produces effects in certain biological systems which are not related to its ionophoric properties. The phenomena observed range from phase shifts of circadian rythms in plants to changes in electrical excitability of neuroblastoma cells and inhibition of energy conversion in chloroplasts; as discussed elsewhere [19], they may be considered as illustrating examples to the still speculative scheme shown in the lower part of Fig. 7.

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