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(9) The yield was determined by VPC and represents the fraction of the total pyrolysis mixture (84% mass balance). In addition to **10** and starting material (81%), 3% of an as yet unidentified material is also formed. Bridgehead diene can be isolated in 11% overall yield based upon starting triene. The yield of **10** is not an equilibrium value. When **10** is subjected to the reaction conditions, triene **9** was formed in 51% yield. We are currently attempting to obtain equilibrium values for this system to establish the thermodynamic relationship between reactants and products.
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A New Fluorescence Technique to Measure the Permeation of Water Molecules across Bilayer Membranes

Sir:

The influence of proteins, lipid composition, and various additives on the permeability of water molecules across biological membranes is still not understood in great detail. This is partly due to a lack of versatile techniques allowing routine measurements. The existing methods (NMR,[1-5] osmotic gradient,[6-13] and measurements of the permeating tritium-labeled water across cellular membranes in a fast-flow tube[8,12,14,15]) have both merits and disadvantages. The new method to be described is especially applicable to phospholipid vesicles, and uses only minimal quantities of material both in volume and concentration so that routine measurements should be possible. The method is based on the solvent-isotope effect of the fluorescence quantum yield of indole chromophores.[16-20] The physical reason why, e.g., indole chromophores, have higher quantum yields in D_2O than in H_2O is still open for discussion[16-19] and will not be persued here. With few exceptions[20,21] the solvent-isotope effect of the fluorescence quantum yield has been overlooked by most molecular biologists. When combined with fast mixing devices, this effect offers a broad field of application; the permeation of water molecules across biological membranes and the accessibility of endogeneous tryptophan residues to water molecules[20] under various experimental conditions are just two examples.

The adaption of this isotope effect to measurements of water permeation across vesicular bilayers includes the following steps: (i) the preparation of an aqueous (H_2O) vesicle solution containing, e.g., tryptamine (3-(2-aminoethyl)indole), both intra- and extravesicularly, (ii) the removal of the extravesicular tryptamine either by dialysis or by chromatographic methods, and (iii) the fast mixing of this H_2O vesicle solution with an equivalent D_2O solvent while the tryptamine fluorescence is monitored. As a consequence of the fast mixing of these solutions the observed fluorescence intensity will first drop within the short mixing time owing to the dilution of the chromophore containing vesicle solution. With increasing exchange of the intravesicular H_2O by the extravesicularly offered D_2O molecules, the fluorescence intensity will then increase again until it reaches a plateau reflecting a statistical distribution of the H_2O and D_2O molecules around the chromophore. Thus, the time course of this increase in the fluorescence intensity becomes a direct measure of the permeating D_2O (or HDO) molecules.

The above-outlined idea has been realized by the following preliminary experiments. Dipalmitoylphosphatidylcholine (~10 mg/mL) together with 0.3 M tryptamine HCl were sonified in aqueous (H_2O) 20 mM $CaCl_2$ at neutral pH above the crystalline to liquid-crystalline phase transition temperature (T_c). Tryptamine HCl was chosen instead of the amino acid tryptophan because of the higher water solubility of tryptamine. As result of the sonication above T_c, usually small and unilamellar vesicles are formed which are almost impermeable to ions at moderate temperatures.[22,23] Subsequently the extravesicular tryptamine was removed either by dialysis (24–48 h at $T < T_c$) against 20 mM $CaCl_2$ or the vesicle solution was passed through a preequilibrated sepharose 4B column[24] and eluted with 20 mM $CaCl_2$ also below T_c, or both methods were employed. Except for a small leakage the extravesicular tryptamine molecules were almost quantitatively removed. This vesicle solution was diluted (1:5 to 1:20) and then put into syringe 1 of a stopped-flow apparatus. Syringe 2 contained 20 mM $CaCl_2$ in D_2O. After thermal equilibration the two solutions were rapidly mixed within 1 ms while the time-dependent fluorescence intensity was recorded (λ_{ex} 280, 295 nm, λ_{em} >340 nm by cut-off filter, or 340 ≤ λ_{em} ≤ 400 nm by band-pass filter). A typical record (using a transient recorder) of these experiments together with the experimental setup is shown in Figure 1. Additionally, Figure 1 contains a plot of the logarithm of the fluorescence intensity vs. time, starting after the mixing period. The latter curve reveals that the fluorescence intensity reaches its new equilibrium value in an almost single-exponential manner, so that the rate constant k describing the permeation of D_2O (HDO) can easily be deduced. Electron micrographs (by staining methods) show rather large vesicles with a radius r of ~5000 Å; thus, a permeability coefficient P_d (according to $P_d = r(k/3)$) of 1.4×10^{-4} cm s^{-1} is calculated which corresponds well with the data measured by the NMR relaxation technique on unilamellar vesicles.[2]

The validity of the above method and the interpretation of the resultant data in terms of a water (here D_2O, HDO) permeation are based on the following assumptions.

(i) The tryptamine molecules are entrapped within the aqueous phase of the intravesicular compartment. This point raises the critical question about the location and permeability of the probe molecule. The experiments were carried out at neutral pH where tryptamine is positively charged and the lipids are zwitterionic. As the pK values are far from neutral,

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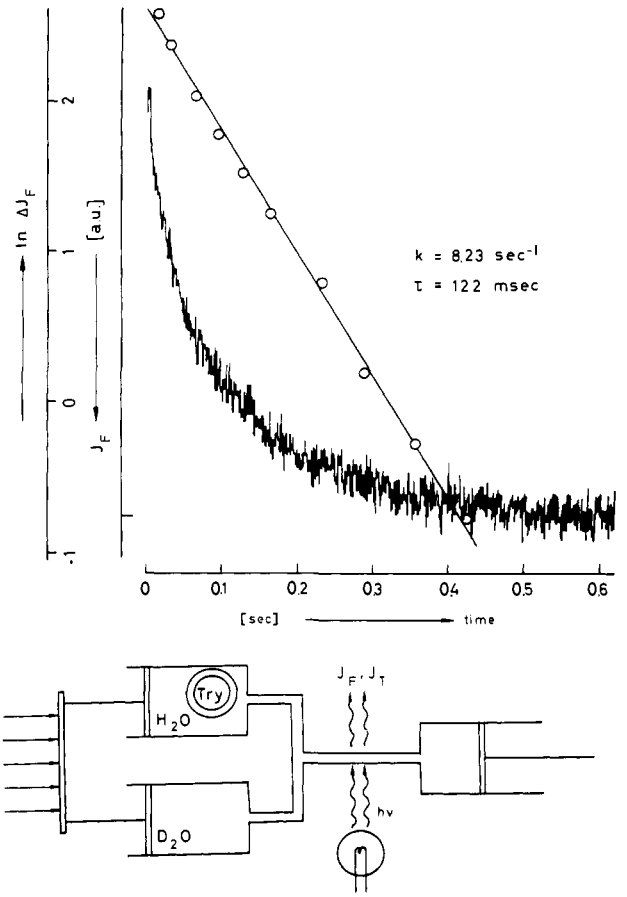

Figure 1. Experimental setup, time response of the tryptamine fluorescence intensity I_F (λ_{ex} 280 nm, λ_{em} >340 nm), due to the permeation of D_2O molecules across the vesicular lipid bilayer, and $\ln [I_\infty - I_F(t)] = \ln \Delta I_F$ as function of time t starting after the short mixing period. I_∞ is the fluorescence intensity at long times for a 1:1 distribution of D_2O and H_2O around the intravesicular tryptamine molecules. The two stopped-flow syringes contained (1) lecithin vesicles with the intravesicular tryptamine (50 mg of dipalmitoylphosphatidylcholine was sonified in 2 mL of aqueous (H_2O), 0.3 M tryptamine HCl, 20 mM $CaCl_2$ at neutral pH above T_c, extensively dialyzed below T_c, and diluted 1:10 with aqueous (H_2O) 20 mM $CaCl_2$); (2) 20 mM $CaCl_2$ in D_2O, T = 39.4 °C.

small pH changes should not influence the ionic states of the molecules in question. The existence of the described solvent-isotope effect (~10-20% of the total fluorescence intensity under the current experimental conditions) implies that the probe molecules are accessable to the aqueous phase and are thus not buried inside the lipid bilayer. The fact that a measurable quantity of tryptamine molecules is retained within the intravesicular compartment even after extended dialysis times led us to assume that the partition coefficient between the lipid and the water phase as well as the permeability coefficient should be rather low for our tryptamine molecules below T_c.

It is further known that most indole derivatives show a blue shift in the fluorescence emission spectra by going from polar to nonpolar solvents.[19,25] Such a blue shift was neither detected in the vesicular systems nor would any blue-shifted part of the emission spectra interfere with the resultant data according to our instrumental setting. In addition, the shape of the crystalline to liquid-crystalline phase transition of the lipid bilayer (measured by temperature scans of the light scattering at 600 nm) is not affected for the tryptamine-containing vesicles. As the temperature profile of the phase transition is a sensitive measure of any incorporation, the insertion of the tryptamine molecules into the bilayer part of the vesicles can be excluded at least up to a measurable degree. Also the temperature dependence of the tryptamine fluorescence intensity does not reveal a break at the phase transition. However, it should be noted that the phase transition temperature itself is shifted from 41-42 to 43-44 °C for the tryptamine-containing vesicles; here weak interactions of the positively charged tryptamine molecules with the polar head groups of the lipid bilayer are conceivable which might explain the small shift in T_c. Similar trends were observed by the interaction of bi- and trivalent cations with lipid bilayers.[26-28] A possible competition between the efflux of tryptamine HCl and the influx of D_2O during the stopped-flow experiment can be neglected because the respective permeability coefficients differ in orders of magnitude, i.e., $P_d(H_2O) \gg P_d$(tryptamine), even for bilayers composed of natural lipids.[29] All of these problems which are concerned with the location and permeability or residual leakage of the probe molecules can easily be minimized by the use of indole derivatives having an even smaller partition coefficient or by the use of water-soluble and nonpenetrating proteins, or polymers with high tryptophan content. Indeed, preliminary studies where pepsin was incorporated into the intravesicular compartment showed the same solvent-isotope behavior, although the signal to noise ratio was rather low. In conclusion, the experimental evidence support the assumption that the tryptamine molecules are entrapped within or face the aqueous milieu of the intravesicular compartment and have a rather small permeability coefficient across the vesicular bilayer below T_c. Up to measurable degree the probe molecules do not alter the intrinsic bilayer qualities.

(ii) The vesicles stay intact during the stopped-flow experiment. This fact was checked by a redialysis (to remove D_2O) of the already used solutions and a subsequent repetition of the stopped-flow experiment. In addition, electron micrographs show that the vesicles are of similar size before and after the stopped-flow experiment. Control tests, where syringe 2 also contained an aqueous (H_2O) 20 mM $CaCl_2$ solution, neither revealed the time-dependent increase of the fluorescence intensity after the mixing period nor showed a change in the light scattering which would reflect changes in size of the vesicular system.[30]

(iii) There are several pieces of evidence showing that the permeation of the D_2O molecules is the rate-limiting step. In contrast to the exchange reaction of the indole-ring N-H proton (measured by UV absorption spectroscopy[31]), the change of the fluorescence quantum yields due to the isotopic solvent substitution is much faster and not detectable in pure tryptamine-containing solutions by stopped-flow methods. Additional hints in that direction come from Stern-Volmer evaluation of H_2O-D_2O titration data. Under the assumption of collisional quenching processes and fluorescence lifetimes for tryptamine molecules of ~4 ns,[18] a bimolecular rate constant for the H_2O quenching in the range of 2.5 10^6 L/mol·s can be calculated which also excludes the pure solvent isotope effect as the rate-limiting factor. This fact is further supported by temperature-dependent measurements and experiments with mixtures of dipalmitoylphosphatidylcholine and cholesterol. As may be anticipated, the permeation of water becomes faster as the phase transition temperature is approached, and the incorporation of cholesterol into the lecithin matrix leads to a faster permeation rate below the phase transition temperature compared with that of pure lecithin bilayers. Both results are in accord with literature data[2,11,13] and reflect the characteristic behavior of phospholipid vesicles below the phase transition temperature.

The initial high tryptamine concentration together with spurious impurities can possibly influence the bilayer dynamics with respect to vesicle fusion and lipid transfer[30] and thus explain the large vesicle sizes. Although large vesicles are an inherent advantage of the described method (the exchange relaxation time is a function of the vesicle size), these problems can be reduced by lowering the tryptamine concentration and

using more efficient optical and electronic devices. Any osmotic gradients during the sample handling can be avoided by dialysis against or chromatography with aqueous solutions containing nonfluorescent and noninteracting additives of the same concentration as the indole derivatives. It is further hoped that in natural vesicle-like particles or cells the endogeneous tryptophan-containing (e.g., cytoplasmatic) proteins can play the same role as the so far artificially introduced tryptamine molecules.

So far, measurements above the phase transition temperature are still poor because of technical and experimental reasons. Evidently tryptamine seems to leak faster out of the intravesicular compartment at temperatures above T_c so that the response signal becomes rather small. In principle any extravesicular tryptamine molecules do not interfere with the discussed effects (the time response for these molecules is beyond the stopped-flow limits); however, the extravesicular tryptamine increases the fluorescence background and makes the detection of the signal response due to the intravesicularly remaining tryptamine molecules more difficult. Above T_c the time course due the permeation of D_2O becomes rather fast but still measurable by stopped-flow techniques especially for large vesicles.

In conclusion an easy method has been described to measure the permeation of water (D_2O or alternatively H_2O) across vesicular lipid bilayers and—possibly under favorable conditions—also across intact biological membranes. The method is based on a solvent-isotope effect of fluorescence quantum yields of indole chromophores. Various factors involved have been discussed. Preliminary experiments using phospholipid vesicles where tryptamine resides almost only within the intravesicular compartment show that the new methods gives reliable results. Experimental details together with further results will be reported later.

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Cyanoketenes. Mechanism of *tert*-Butylcyanoketene Cycloaddition to Methyl- and Dimethylketene

Sir:

Ketene dimerizations are fraught with ambiguities regarding their operative mechanisms. In certain cases it appears that the mechanism is a concerted $[_\pi 2_s + _\pi 2_a]$ process. For example, dimethylketene dimerization shows a high negative entropy of activation and little solvent polarity dependence.[1] Also, from a concerted dimerization of unsymmetrical ketoketenes, the resulting cyclobutane-1,3-diones would be expected to show predominantly cis stereochemistry for the bulkier substituent, and such has been observed for the homodimerizations of benzylphenyl and benzylmethylketenes.[2] In other cases data appear more consistent with a nonconcerted, stepwise mechanism. For example, when methylchloro- and *tert*-butylchloroketene, generated in situ from the corresponding acid chlorides, cycloadd to methyl-*n*-propyl- and methylisopropylketene, equal amounts of isomeric *cis*- and *trans*-cyclobutane-1,3-diones result.[3] Another anomolous observation for a concerted mechanism is the formation of 2-oxetanone products which are often observed in many homodimerizations of aldoketenes.[4] Examples are the dimerizations of ketene itself,[5] methylketene,[6] butylketene,[7] and phenylketene.[8] Mixed dimerizations of ketoketones and aldoketenes also often yield 2-oxetanone products.[3] In addition, bis(trifluoromethyl)ketene cycloadds to dimethylketene to give a mixture of the corresponding cyclobutane-1,3-dione and 2-oxetanone.[9] Clearly, the above results suggest that both concerted and stepwise mechanisms are possible, but little unambiguous mechanistic data has appeared.

Our objective regarding the above general problem was to gain mechanistic information concerning the cycloaddition of an electron-deficient ketene to electron-rich aldo- and ketoketene analogues. Specifically, we report here a mechanistic study of the cycloaddition of *tert*-butylcyanoketene (**1**) to methyl-and dimethylketene which unambiguously establishes these cycloadditions to be nonconcerted dipolar processes. *The most unique observation of this study is that the zwitterionic intermediate proposed in a ketene to ketene cycloaddition has, for the first time, been independently generated, trapped, and shown to give the same products as observed in the cycloadditions themselves.* The genesis of this mechanistic probe stems from our earlier reports that zwitterionic intermediates are readily generated from the thermolysis of appropriately substituted cyclic vinyl azides,[10,11] and the results here further document this to be a most powerful tool for the study of cyanoketene cycloadditions.

Methylketene was generated and condensed at liquid nitrogen temperature.[12] It was then treated with a toluene solution of *tert*-butylcyanoketene[13] and the reaction solution allowed to warm to ambient temperature. After 30 min the solvent was removed in vacuo and the crude oil purified to give the 2-oxetanone (**2**, Scheme I) as a colorless oil in 49% purified

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