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Kinetic Study of the Diffusion of Ribonuclease across a Liquid–Liquid Interface*

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ABSTRACT: The purpose of this work was to explore the possibility of observing the rate of diffusion of a protein through the undisturbed interface separating two immiscible liquid phases. Since ribonuclease is known to have appreciable solubility in both of the immiscible phases obtainable from a mixture of water, 2-ethoxyethanol (Cellosolve), and ammonium sulfate, it was anticipated that this combination of protein and solvent system would be suitable for the desired study. By choosing a composition close to the critical (plait) point, a liquid–liquid interface was obtained which was sufficiently permeable to diffusing ribonuclease molecules, without the use of shaking, to permit a kinetic investigation. The diffusion was observed with ultraviolet absorption optics in the analytical ultracentrifuge operating at minimal speed after the less dense phase of the two-phase system had been layered over the denser phase containing ribonuclease. The kinetic study was based on the densitometer traces of the photographs which indicate protein concentration *vs.* position throughout the system at successive times during the approach to partition equilibrium. Mathematical analyses of the traces for the early (free diffusion) and late (restricted diffusion) stages of the experiment indicate that the free energies of activation for penetration of the interface by ribonuclease in the two directions were at most of the order of 12 kcal/mole.

Liquid–liquid interfaces which are permeable to proteins are not common. The requirement that two liquids be immiscible, in order that an interface be formed, is almost incompatible with the requirement that protein have appreciable solubility in each. Nevertheless, such immiscible solvents have been devised and form the basis for the extraction techniques of countercurrent distribution (Craig and Craig, 1956; von Tavel and Signer, 1956) and partition chromatography (Martin and Porter, 1951) used for the fractionation of protein mixtures. The developers of these techniques have determined protein distribution coefficients between phases at equilibrium but a kinetic study of the rate of diffusion of a protein through an undisturbed liquid–liquid interface has not been previously reported. We have initiated such studies using the analytical ultracentrifuge as a diffusion apparatus to observe RNase[1] in the water–Cellosolve–ammonium sulfate system of Martin and Porter (1951).

Experimental Procedures

Water and Cellosolve are miscible in all proportions at room temperature but two liquid phases separate when sufficient ammonium sulfate is added. We chose a weight composition (59.5% water, 18.2% Cellosolve, and 22.3% ammonium sulfate) close to the plait point on the phase diagram of the system (Martin and Porter, 1951) in order that the two phases obtained would be nearly identical thereby minimizing the interfacial resistance to protein transfer. The method of preparation was to dissolve the salt in the water, add the Cellosolve, shake the system, and allow the phases to separate. The bottom phase B (density = 1.151 g/cm^3) contains more salt than Cellosolve while the opposite is true of the top phase T (density = 1.024 g/cm^3). The pH of both phases was 5.2. We used chromatoquality Cellosolve (Matheson Coleman & Bell) which we triple distilled at atmospheric pressure to remove impurities absorbing at 265 mμ, the

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[1] Abbreviation used is: RNase, bovine pancreatic ribonuclease.

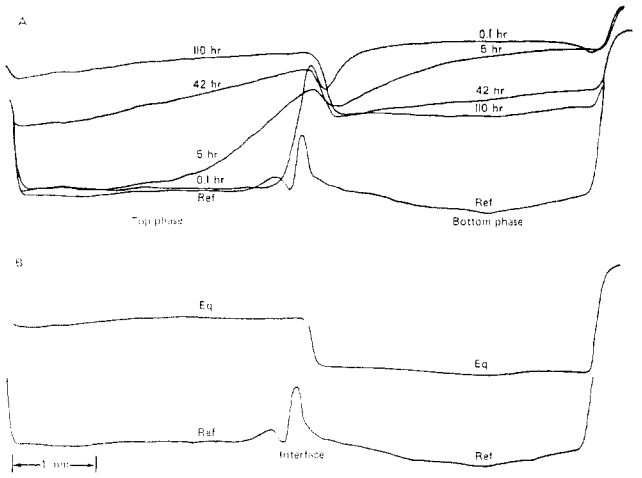

FIGURE 1: Inverted densitometer traces of ultraviolet photographs with ordinates proportional to protein concentration. (A) The distribution of protein (RNase) between the two phases of the water–Cellosolve–ammonium sulfate system at various times during the diffusion experiment. The sample was prepared by layering pure top phase over protein-containing bottom phase. The reference trace (Ref) corresponds to the two-phase system without protein. (B) The trace (Eq) obtained for a second sample which was equilibrated immediately by shaking.

wavelength used for the detection of protein in the Spinco Model E analytical ultracentrifuge. To observe rate of transfer across the interface, we wished to start with a solution of RNase in B and place T in contact with it. However, RNase would not dissolve when placed directly in B. To prepare the solution, the protein (17.3 mg of crystalline RNase, Worthington, code:R) was first dissolved in water (0.15 ml). Then B (5 ml) and T (2.5 ml) were added, the system was shaken and the phases were allowed to separate. For the diffusion experiment in the ultracentrifuge, 0.20 ml of the bottom protein solution was placed in a 12-mm aluminum, 4-deg sector cell. A protein-free upper phase which would be in equilibrium with the *solvent* in this bottom solution was obtained by repeating the above procedure but without protein, *i.e.*, by combining 0.15 ml of water, 5 ml of B, and 2.5 ml of T, shaking, and allowing the phases to separate. From the top phase, 0.20 ml was taken and gently layered over the 0.20 ml of protein-containing bottom phase already in the cell. The cell was then placed in the rotor and brought to a speed of about 2000 rpm at 21.3° (room temperature) whereupon ultraviolet photographs were taken on Kodak commercial film to follow the protein diffusion. There was an 8-min delay after putting the phases in contact before the first photograph could be taken. The optical density of the developed film as function of position was measured using a Beckman analytrol with densitometer attachment. The film optical densities were then inverted to provide traces whose ordinates are proportional to solution optical density and hence protein concentration.

Results

Some of the inverted traces showing the distribution of RNase between phases at various times are presented in Figure 1A. A reference trace (Ref) corresponding to the two-phase system without protein is included. The 110-hr trace in Figure 1A showing the "final" distribution arrived at by diffusion in the static system is in good agreement with the trace (Eq) in Figure 1B for a second sample which was equilibrated directly by shaking. Spectrophotometric measurement gave a value of 3.0 for the distribution coefficient, K, defined as the ratio of the RNase concentration in the top phase to that in the bottom phase at equilibrium. With $K > 1$, and all of the RNase originally in B, transport of protein from B to T eventually had to proceed *against* a concentration gradient across the phase boundary in order for equilibrium to be achieved.

The large gradients built up within the phases, as seen for example in the 5-hr trace, show that diffusional resistance in the bulk phases was affecting the rate of approach to equilibrium. However, interfacial resistance was not altogether absent as evidenced by the delay of more than 20 hr before the ratio of the RNase concentrations immediately on each side of the liquid–liquid boundary, C_T^i/C_B^i, was within 25% of its equilibrium value. Although this first experiment was intended to be a qualitative exploration of the feasibility of observing protein transport across interfaces in static systems, we have gone a step further and attempted to draw some quantitative conclusions concerning the free-energy barriers, at least to the extent possible with the data at hand. Working within the rectangular approximation to the centrifuge cell (Yphantis and Waugh, 1956; Nazarian, 1958), we have applied two forms of diffusion theory to the observations, one for the early stages and the other for the late stages of the experiment.

For a description of the early stages, theory gives a simple result if both liquids are considered to be infinite in extent. Then for the hypothetical case of zero interfacial resistance, where $C_T^i/C_B^i = K$ throughout the experiment, diffusion theory predicts the amount of solute transferred from one phase to the other will be proportional to the square root of the elapsed time (Crank, 1956; Davies and Rideal, 1963). Taking the area S under the inverted densitometer trace of the top phase at a given time to be a measure of the net amount of protein which has permeated the interface up to that time, and introducing quantities which pertain to the finite system, the result can be written as

$$(S/S_{Eq})^0 = At^{1/2} \qquad (1)$$

where $A = (2/L)[(1 + K)/(1 + \beta K)](D_T/\pi)^{1/2}$, $\beta = (D_T/D_B)^{1/2}$, S_{Eq} is the area under the equilibrium trace of the top phase, D_T and D_B are diffusion coefficients of the protein in the two phases, and L is the length of the liquid column of each phase. To convert the diffusion coefficient D_w of RNase in water having viscosity η_w into the corresponding value D in a medium of viscosity η, we can use $D = D_w(\eta_w/\eta)$ which follows from the Stokes–Einstein relation assuming no change in the protein. At 20°, $D_w = 1.1 \times 10^{-6}$ cm²/sec (Creeth, 1958; Van Holde and Baldwin, 1958) and our measurements with an Ostwald viscometer at 22.2° gave $\eta_w/\eta_T = 0.2576$ and $\eta_w/\eta_B = 0.3433$. Using these values and correcting to the temperature of the experiment, 21.3°, we find $D_T = 2.9 \times 10^{-7}$ cm²/sec and $D_B = 3.9 \times 10^{-7}$ cm²/sec. Introducing these estimates, along with $K = 3.0$ and $L = 0.35$ cm in eq 1, we obtain the theoretical line for zero interfacial resistance

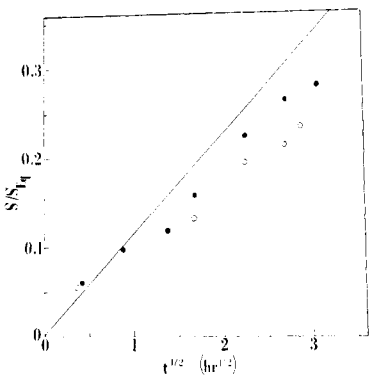

FIGURE 2: Net transfer of protein into the top phase during the early stages of the diffusion experiment. The points represent two sets of results obtained from the densitometer traces of the early ultraviolet photographs. The line represents the behavior predicted theoretically (eq 1) for the case of zero interfacial resistance.

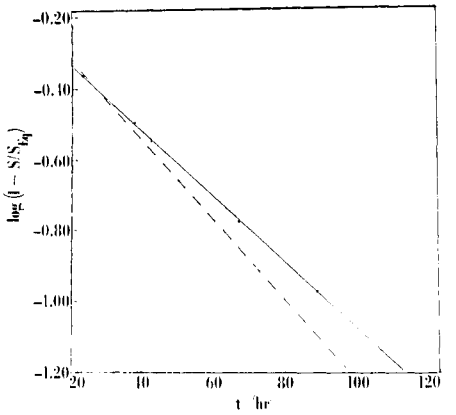

FIGURE 3: Approach to equilibrium in the diffusion experiment. Data from the later ultraviolet photographs are plotted as suggested by eq 4 for the determination of interfacial permeability. The dashed line is the theoretical result expected for infinite permeability, *i.e.*, zero interfacial resistance.

shown in Figure 2. The data obtained during the first 9 hr of the experiment (concentration changes became noticeable at the boundaries after about 13 hr) are also shown in Figure 2. The observations lie only slightly below the zero resistance line, indicating that the rate of approach to equilibrium was close to that expected on the basis of bulk diffusion alone. Interfacial resistance cannot be ruled out, however, and an indication as to the possible heights of the free-energy barriers in this system can be obtained by comparing the data with the modified theory involving permeabilities k_{BT} and k_{TB}, which are the reciprocals of the resistances, defined such that the difference $k_{BT}C_B^1 - k_{TB}C_T^1$ gives the net transfer across unit area of interface in unit time from phase B to phase T. From the equilibrium condition, we have $k_{BT}/k_{TB} = K$. When these permeabilities are included, the theoretical result can be expressed as follows (Davies and Wiggill, 1960)

$$S/S_{Eq} = A(t^{1/2} - 1/\gamma + 2/\pi\gamma^2 t^{1/2} - \ldots\ldots) \quad (2)$$

where $\gamma = 2(k_{BT}/D_B^{1/2} + k_{TB}/D_T^{1/2})/\pi^{1/2}$. For large permeabilities, eq 2 indicates that the graph of S/S_{Eq} *vs.* $t^{1/2}$ soon becomes a straight line parallel to the zero resistance line of eq 1 with an intercept $1/\gamma$ on the abscissa. From the data in Figure 2 we obtain $1/\gamma = 0.54$ hr$^{1/2}$ which yields $k_{BT} = 1.2 \times 10^{-5}$ cm/sec. This can be introduced into the expression relating free energy of activation $\Delta F^{\ddagger}$ to permeability (Davies, 1950)

$$k_{BT} = (RT/2\pi M)^{1/2} e^{-\Delta F_{BT}^{\ddagger}/RT} \quad (3)$$

where R is the molar gas constant, T is absolute temperature, and M is molecular weight of the solute, to give $\Delta F_{BT}^{\ddagger} = 10.3$ kcal/mole. For the reverse process, we have $\Delta F_{TB}^{\ddagger} = 10.9$ kcal/mole since $K = \exp[(\Delta F_{TB}^{\ddagger} - \Delta F_{BT}^{\ddagger})/RT] = 3.0$. We emphasize that these activation energies for penetration of the interface by RNase are really upper bounds. Values lower than these by more than about 1 kcal/mole are experimentally undetectable in this system since the associated delays would be negligible compared with the delay due to diffusional resistance in the bulk liquids.

Analysis of the data obtained during later stages of the experiment can be based on the theoretical solution derived for a system of finite extent (Scott *et al.*, 1951; Tung and Drickamer, 1952). This solution eventually takes the form

$$1 - (S/S_{Eq}) = ae^{-b^2 t} \quad (4)$$

where a and b are constants related to the system parameters. In the case of b, the specific relation is

$$k_{BT} = \frac{bKD_T^{1/2}}{\mathrm{ctn}(bL/D_T^{1/2}) + \beta K \,\mathrm{ctn}(bL/D_B^{1/2})} \quad (5)$$

According to eq 4, a graph of log $(1 - S/S_{Eq})$ *vs.* t will give a straight line for the latter part of the experiment and b can be calculated from the slope. Then the permeability can be calculated from eq 5. From the plot of our data in Figure 3, we find $k_{BT} = 7.5 \times 10^{-6}$ cm/sec which corresponds to $\Delta F_{BT}^{\ddagger} = 10.6$ kcal/mole and $\Delta F_{TB}^{\ddagger} = 11.2$ kcal/mole in good agreement with the result obtained from the early part of the experiment. Included in Figure 3 is the theoretical line for the case of no interfacial resistance using values of a and b appropriate to the limit $k_{BT} \to \infty$. The results obtained for the free energies of activation are relatively insensitive to an increase in the values used for D_T and D_B. On the other hand, the results are extremely sensitive to a shift of the diffusion coefficients to lower values, a 20% reduction being sufficient to explain the data entirely on the basis of bulk diffusion. However, independent estimates obtained by direct application of the diffusion equation to regions within each of the phases indicate that our values $D_T = 2.9 \times 10^{-7}$ cm^2/sec and $D_B = 3.9 \times 10^{-7}$ cm^2/sec are not too high. At any point x_1 in the top phase $(-L < x < 0)$, the diffusion equation is $D_T(\partial C_T/\partial x)_{x_1} = \mathrm{d}\left(\int_{-L}^{x_1} C_T \mathrm{d}x\right)\Big/\mathrm{d}t$ from which D_T can be calculated if the other quantities can be measured. A similar expression is applicable to the bottom phase. Allowing for the considerable errors which enter into the evaluation of these quantities from the sequence of densitometer traces, this approach suggests that D_T is between 3×10^{-7} and 5×10^{-7}

cm^2 per sec and that D_B is between 4×10^{-7} and 7×10^{-7} cm^2 per sec.

The order of magnitude of these diffusion coefficients is consistent with that expected for a molecule having the size of RNase. Nevertheless, to obtain stronger evidence as to whether it was indeed the intact RNase molecule which was being observed in our distribution experiment, we performed a molecular weight determination by means of a sedimentation equilibrium experiment on a solution prepared by dissolving RNase in the top phase T. The trace of the ultraviolet absorption photograph taken at equilibrium was analyzed directly by the method of finite differences originally devised for the analysis of interference photographs (Nazarian, 1968) without the need for absolute calibration in terms of concentration. If we assume the partial specific volume of RNase in T is the same as in water, 0.703 ml/g (Cox and Schumaker, 1961), we find $M = 1.32 \times 10^4$ in satisfactory agreement with the known value 1.37×10^4 (Hirs *et al.*, 1956).

Discussion

It has been found that RNase can be readily partitioned between a suitable pair of immiscible liquids under static conditions, *i.e.*, without the assistance of shaking or any other disturbance. To make this possible, we took the system water–Cellosolve–ammonium sulfate which was known to give two phases having appreciable solvent power for RNase and chose a composition close to the plait point so that the phases obtained would be very similar. As a result, the resistance to diffusion through the interface was down to a level where it was comparable with diffusional resistance in the bulk phases. In future experiments, one can gradually vary the system composition away from the plait point to obtain increasingly dissimilar pairs of phases with increasing interfacial resistance. For each of these, the corresponding free energies of activation for the processes which change the state of solvation of the protein molecule as it passes across the phase boundary can be determined. Such measurements should lead to a better understanding of solvation processes in proteins.

In the past, a parallelism has been shown to exist between the rates of penetration of *small* molecules into living cells and their lipid–water distribution coefficients (Bull, 1951). Thus the diffusion studies along the lines suggested above might reveal that for protein molecules a similar parallelism exists between rates of penetration of liquid–liquid interfaces and equilibrium distribution coefficients. Such a finding could have some bearing on the curious fact that certain proteins such as the inactive forms of the digestive enzymes are able to pass out of the cells in which they are manufactured even though living cell membranes generally are supposed to be impermeable to macromolecules. Instead of a model in which the membrane is considered to be a more or less rigid structure with small pores to give it the ability to keep large molecules inside, the diffusion postulate would make it a fluid region and attribute this ability to an unfavorable interfacial activation energy and/or distribution coefficient for the particular protein. A protein having favorable values of these parameters, on the other hand, could still pass through.

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