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ROLE OF DIFFUSION POTENTIAL ON THE FLOW OF ANGIOTENSIN II, BRADYKININ AND RELATED MOLECULES THROUGH CELLOPHANE MEMBRANES

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The diffusion of electrically charged peptides (angiotensin II, bradykinin and [Suc^1]angiotensin II) across tight cellophane membranes, obtained by different degrees of acetylation, shows a kinetic behaviour which was interpreted in the literature as indicative of the existence of different molecular conformations presenting slow interconversion velocities and different permeabilities across the membrane. A diffusion potential ($\Delta\psi$) was found to be present across the membrane along diffusion experiments performed in low ionic strength. Upon annihilation of $\Delta\psi$ by chemical voltage clamping (by equally increasing the ionic strength on both bathing solutions) the diffusion rate was decreased and the flow followed first order kinetics, indicating a major role of $\Delta\psi$ in the process. As the ionic strength increase could also affect molecular conformation, the role of $\Delta\psi$ on the diffusion of those molecules was tested by fitting flux and $\Delta\psi$ experimental results by an integrated form of Nernst-Planck flux equation. It is concluded that the deviation from first order diffusion kinetics, observed in low ionic strength, is solely due to the diffusion potential, and not to the existence of more than one molecular conformation in aqueous solution. This study was extended to amino acids and other related charged molecules.

Introduction

The analysis of the diffusion process across cellophane membranes has been used to obtain information regarding size and shape of permeant molecules [1–4]. The 'thin-film dialysis method' described by Craig et al. (1975) [5] has been extensively employed in a systematic study of several proteins [25], polypeptides [6,7], peptides [8–12], sugars [13] and amino acids [14].

The thin-film method implies that the net rate of passage of solute molecules across the membrane, from a small volume compartment (where the molecules are concentrated) to a large compartment (where the concentration is close to zero) depends only on solute concentration, so that the concentration decrease in the small compartment follows first order kinetics. With several pure solutes Craig and his co-workers were able to fit experimental results to the predicted behaviour. However, in some cases, the diffusion velocity constant changes during the dialysis and this was interpreted as due to slowly interconverting conformations of a single solute [5,7,15].

Craig and Ansevin (1963) [14] measuring diffusion potentials of NaCl and of positively and negatively charged amino acids, showed that cellophane membranes bear fixed negative charges. However, Craig et al. (1957) [5] observed that the velocities of diffusion of proteins were a function of molecular size but could be regarded as independent of their net electrical charge. For amino acids, Craig and Ansevin (1963) [14] obtained similar results. Changes in the diffusion velocity

constants of peptides and proteins, induced by alterations in ionic strength and pH, were explained as due to conformational changes inducing modifications in the effective molecular diffusional size [3,5,8,9,16,17].

However, Craig et al. (1975) [18] observed that in low ionic strength an electrical potential difference contributed to the kinetics of dialysis of peptides across cellophane membranes. This observation indicated the need for a detailed and more rigorous study of the relative importance of the electrical and chemical potential differences on the diffusion rates of charged peptide molecules across cellophane membranes, in order to re-evaluate the use of dialysis data for conformational analysis.

The present work deals with a detailed study of the diffusion of peptides (bradykinin, angiotensin II and [Suc^1]angiotensin II), amino acids (L-tyrosine and L-phenylalanine) and other charged solute (picric acid) in order to determine the relevance of the electrical potential difference that develops across cellophane membranes when those molecules diffuse through them. We were able to show that previously described deviations from first order kinetics [16] may be simply explained on the grounds of membrane electrical potential differences when the flux of substances is fitted by the integrated form of the Nernst-Planck equation [19].

We have also investigated the importance of the stirring rate of the two solutions bathing the membrane, which has been neglected in most of the previous studies, since Craig (1960) [1] and Craig and Konigsberg (1961) [2] reported that by increasing the stirring rate of the dilute solution (with no stirring of the concentrated one) the diffusion velocity increased without, however, affecting the dialysis kinetic constant.

Materials and Methods

Cellophane membranes (dialyser tubing, Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) with average pore diameter of 4.8 nm were submitted to different degrees of acetylation by treatment with pyridine/acetic acid [2] for times varying from 1.5 to 2.5 h in order to obtain membranes of different permeabilities. The acetylated membranes were kept in distilled water containing 1% formaldehyde to prevent microorganism growth.

The apparatus used in the present study (Fig. 1) consisted of an upper chamber made of polyethylene tubing (30 mm long and 5 mm internal diameter) closed at the lower end by the cellophane membrane held in place by an internal polyethylene ring of 4 mm internal diameter. The effective area of membrane was 12.6 mm^2. The upper chamber, with the membrane in place, was checked for leakage and kept in formaldehyde solution as described above. For the experiments, the upper chamber, with membrane in place, was inserted and tightly fitted to the top of a square photometer cell (lower chamber) of 10 mm light path, with fused silica windows. Stirring of the bathing solutions was done by a small magnetic stirring bar in the lower chamber and by a small polyethylene propeller driven by an electrical motor, in the upper chamber. At regular time intervals, stirring was interrupted for 20 to 30 s for measuring the absorbance or the electrical potential difference. The absorbance of the lower bathing solution was read against a blank of pure solvent in a Beckman Acta V double monochromator spectrophotometer at the appropriated wave length according to the substance in study. The electrical potential difference was measured by means of an electrometer (Keithley model 602) connected to the bathing

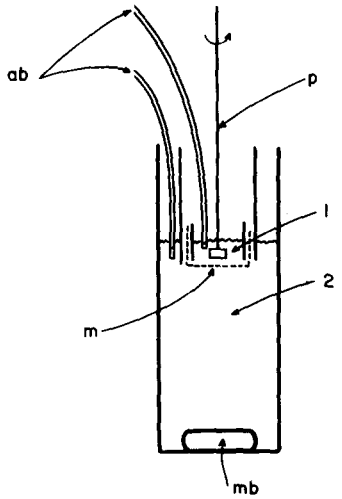

Fig. 1. Diagram of the diffusion cell used for kinetic experiments and voltage measurements. Upper (1) and lower (2) hemi-chambers; m, membrane; p, propeller; mb, magnetic stirring bar; ab, agar bridges.

solutions through calomel half-cells and 1 M KCl agar bridges. The agar bridges were contained in polyethylene tubes (1 mm inner diameter) whose tips in contact with the membrane bathing solutions had been completely closed by heating, clamping and then perforated by a small pinhole. To further minimize KCl leakage, the bridges were left in contact with the bathing solutions only during the few seconds necessary to obtain stable readings.

The volumes of the bathing solutions were 50 μl in the upper and 2.2 ml in the lower compartments. The position of the upper chamber in the photometer cell was carefully adjusted so that the liquid levels were the same in both compartments. The kinetics of dialysis were tested according to a first order process by the following relationship:

$$\log(A_\infty - A) = \log A_\infty - k_1 Bt \qquad (1)$$

where A is the absorbance in the lower chamber (which is directly proportional to solute concentration) at time t, A_∞ is the absorbance at equilibrium, k_1 is the velocity constant of the process and B is a geometric constant that is a function of the volume of the bathing solutions.

The solute flux (J) across the membrane in the case of a simple diffusion is given by:

$$J = P(C_1 - C_2) \qquad (2)$$

where P is the membrane permeability, C is the solute concentration and the subscripts 1 and 2 refer to the upper and lower chambers, respectively.

To test whether deviations from first order kinetics could be ascribed to electrical potential differences across the membrane, the solute flux, J, in these cases, was assumed to be a function of C_1, C_2 and of the electrical potential difference across the membrane ($\Delta\psi = \psi_1 - \psi_2$), and could be expressed by the integrated form of Nernst-Planck equation assuming approximately equal total ion concentrations in the solutions bathing the membrane [19,20] and low membrane charge density, since the experiments were performed at low pH (pH 3.3 to 4.3) and the carboxyl groups of cellulose have a pK between 4 and 5 [21,22].

$$\frac{J\text{calc}}{P} = z\frac{F\Delta\psi}{RT} \cdot \frac{C_2 \exp(-zF\Delta\psi/RT) - C_1}{\exp(-zF\Delta\psi/RT) - 1} \qquad (3)$$

where z is the valence of the solute, F the Faraday's constant, R the gas constant and T the absolute temperature. A program written in Basic for a Varian model 620/L-100 computer was used to calculate J values from the rate of appearance of solute in the lower compartment and J_{calc}/P from the concentration and electrical potential difference values.

In a few experiments, Ussing-Zerahn chambers [23] were used to test the effect of voltage clamping upon the rate of diffusion of picric acid across cellophane membranes. The hemi-chambers were connected to the voltage clamp apparatus (DVC-100 Dual Voltage Clamp Apparatus, Yale University Department of Physiology), through saturated KCl agar bridges and calomel half-cells, for voltage measurements, and Cu-$CuSO_4$ for current passing. Stirring was done by an air-lift pump. The total capacity of each compartment was 50 ml. The flow of solute was calculated by measuring photometrically its concentration in the bathing solutions by taking 50 μl samples at regular time intervals.

The peptides studied were: [Ile^5]angiotensin II (angiotensin II), its analog lacking the N-terminal amino group ([Suc^1]angiotensin II) and bradykinin, as acetate salts. They were synthesized by the solid-phase method [24] and shown to be homogeneous by paper electrophoresis in three buffers (pH 2.8, 4.9 and 9.9), by thin-layer chromatography on silica gel with three different solvent systems, and by amino acid analysis of the acid hydrolyzates.

Amino acids, as chloride salts, were chromatographically pure products from Sigma Chemical Co. or Aldrich Chemical Co. The picric acid was from E. Merck, Darmstadt. The experiments were performed at room temperature (18–28°C).

Straight lines were fitted by the least squares method. Molecular charge density was calculated from the pK values of ionizable groups, obtained by electrometric titration [25–27]. In most of the experiments the solutes were dissolved either in 0.015 M acetic acid or 0.015 M acetic acid containing 0.1 M KCl.

Results and Discussion

The flow of angiotensin II across acetylated cellophane membranes in low ionic strength and

acid pH does not conform with Eqn. 1, as is shown in Fig. 2. Deviation from linearity is evident for values obtained in the first two hours of experiment while latter values could be fitted reasonably well by a straight line, that is parallel to the one fitted to all experiment points obtained in the flow of angiotensin II at high ionic strength. Similar observations have been thought to be strong evidence in favour of the existence of more than one molecular conformation of angiotensin II in solution, with slow interconvertion velocities [5,6,16].

Electrical measurements during angiotensin II diffusion across acetylated cellophane membrane (Fig. 3) showed a potential difference with the upper compartment positive, which declined exponentially with time. This electrical potential difference is due to a higher mobility of the counterion (acetate) as compared to that of angiotensin II. It is reasonable to assume that, in the presence of a greater electrical potential difference, the rate of passage of angiotensin II molecules across the membrane would be higher. This assumption is supported by measurements of angiotensin II diffusion at high ionic strength obtained by the presence of KCl in equal concentrations (0.1 M), in the bathing solutions on both sides of the membrane. In this condition, which short-circuits the membrane potential, the rate of angiotensin II flow across the membrane was reduced and conformed to Eqn. 1 as is shown in Fig. 2. Its kinetic constant, k_1, has the same value ($4.1 \cdot 10^{-3}$ min^{-1}) as that obtained from the straight line fitted to the four last experimental values of the diffusion of angiotensin II across acetylated cellophane membrane at low ionic strength, indicating that the kinetic constant in this last condition, decreases with time and approaches that at high ionic strength. This is a reflection of a decrease in importance of the membrane electrical difference due to its reduction with time as shown in Fig. 3.

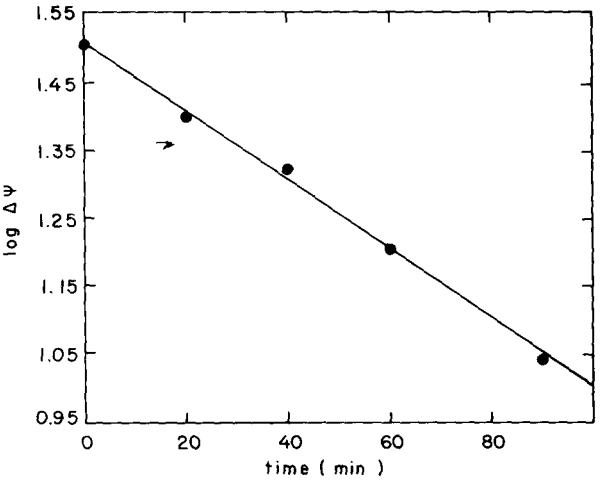

Fig. 3. Time-dependence of membrane electrical potential difference ($\Delta\psi = \psi_1 - \psi_2$) during angiotensin II diffusion across acetylated cellophane membrane at ionic strength <0.001, pH 4.2. Same experimental conditions as in Fig. 2.

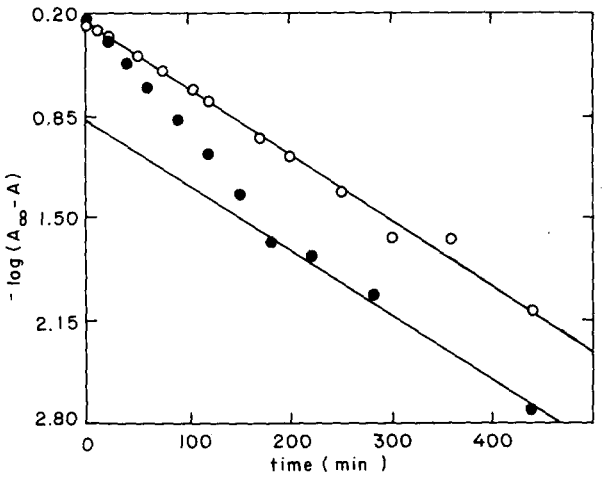

Fig. 2. Effect of ionic strength on the kinetics of dialysis of angiotensin II. Initial concentration in the upper chamber $3.8 \cdot 10^{-3}$ M. ●, in 0.015 M acetic acid, ionic strength <0.001, pH 4.2, ○, in 0.015 M acetic acid containing 0.1 M KCl, ionic strength=0.1, pH 4.2. The straight lines were fitted by the least-square method to all the experimental points at high ionic strength, and to the four last experimental values at low ionic strength. $k_1 = 4.1 \cdot 10^{-3}$ min^{-1} for both straight lines.

Although these findings indicate the importance of the electrical potential difference on angiotensin II flow, possible effects of ionic strength on conformational behaviour of angiotensin II cannot be discarded. In order to be able to decide between these two possibilities, experiments were carried out at low ionic strength, without nullifying the electrical potential difference, and the role of $\Delta\psi$ was tested by means of Eqn. 3.

Fig. 4 shows the relationship obtained when the experimental values for the flux of angiotensin II across the membrane are plotted against J_{calc}/P (a calculated value) obtained from the integrated form of Nernst-Planck equation, that takes into account

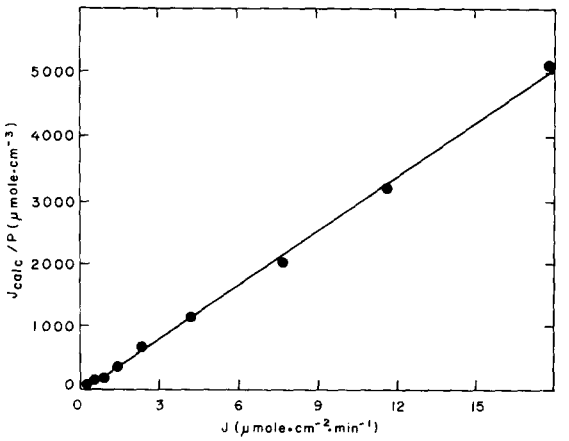

Fig. 4. Relationship between the flow of angiotensin II (J) obtained from the data of Fig. 2 (low ionic strength) and the calculated flow (J_{calc}) according to Eqn. 3 using the data of Fig. 2 and 3.

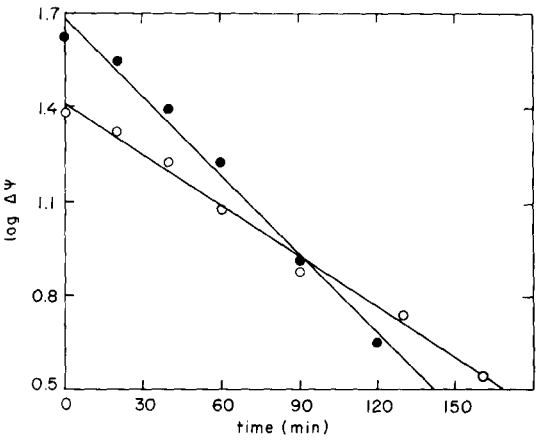

Fig. 5. Time-dependence of membrane electrical potential difference during bradykinin (●) and [Suc^1]angiotensin II (○) diffusion across acetylated cellophane membranes.

the concentrations and the electrical potential difference across the membrane. The fact that a linear dependence is observed between J_{calc}/P and J strongly supports the notion that the electrical potential difference plays a significant role in the kinetics of dialysis of angiotensin II across cellophane membrane. These results consequently render unnecessary the interpretation of two molecular conformations being responsible for the non-first order behaviour shown in Fig. 2.

We have extended this study to other peptides with molecular charge density lower ([Suc^1]angiotensin II) or higher (bradykinin) than angiotensin II at acid pH, where they are positively charged, with z equal to $+0.65$, $+2.08$ and $+1.23$, respectively. Results similar to those of angiotensin II were obtained for those molecules, as shown in Figs. 5, 6a and 6b.

The role of unstirred layers adjacent to membrane surfaces was studied in experiments with no stirring in the upper chamber (concentrated side) and mild stirring rate in the lower compartment. In these conditions, the kinetics of dialysis of angiotensin II, bradykinin and [Suc^1]angiotensin II, in low ionic strength, could be fitted by Eqn. 1, particularly when very permeable membranes were used. Measurements of the initial electrical potential difference across the membranes in these cases, yielded values that were similar to those shown in Figs. 3 and 5. These results are a clear indication that in these experimental conditions, despite the existence of an electrical potential difference across the membrane, the main barrier to flow are the unstirred layers. The importance of unstirred layers was also clearly shown in experiments with picric acid, in which the membrane plays a much less important role as a barrier, due to the smaller size of the picrate ion, as compared to the peptides mentioned above. The diffusion of picric acid, despite generating an electrical potential difference across the membrane, having H^+ as counter-ion ($\Delta\psi = -49$ mV for a concentration ratio of 66.5; $C_1 = 4.0 \cdot 10^{-3}$ M), was neither altered by chemical voltage clamp (obtained by addition of 0.1 M KCl to both bathing solutions) nor sensitive to electrical voltage clamp, obtained by fixing the membrane potential to -100 mV and $+100$ mV, as shown in Fig. 7.

With amino acids, like L-tyrosine and L-phenylalanine, having Cl^- as counter-ion, a behaviour similar to that described for picric acid was observed. An electrical potential was generated during diffusion across acetylated cellophane membranes. With a tyrosine concentration ratio of 70, $C_1 = 2.5 \cdot 10^{-3}$ M, $z = +0.03$ at pH 3.7, a potential difference of $+12$ mV was measured. With phenylalanine concentration ratio of 400, $C_1 = 6.7 \cdot 10^{-2}$ M, $z = +0.005$ at pH 4.15 a potential difference of $+25$ mV was found. Nevertheless, for both amino acids, the flow conformed with Eqn. 1 and was not affected by pH or ionic

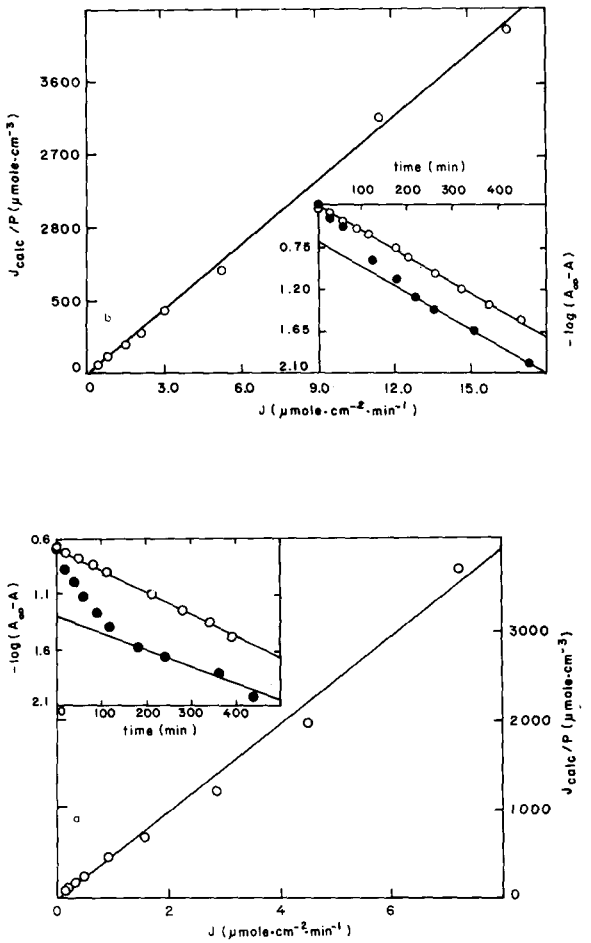

Fig. 6. Relationship between the flow of bradykinin (a) and of [Suc^1]angiotensin II (b), J, obtained from their respective data at low ionic strength, and the calculated flow, J_{calc}, according to Eqn. 3 using data of Fig. 5 and of this figure. The inserts show the effect of ionic strength on the kinetics of dialysis of bradykinin (a) and [Suc^1]angiotensin II (b). ●, 0.015 M acetic acid, ionic strength <0.001, pH 4.3, ○, 0.015 M acetic acid containing 0.1 M KCl, ionic strength 0.1, pH 4.3. The straight lines were fitted to all experimental points at high ionic strength and to the last four points at low ionic strength by the least square method. Initial concentrations of bradykinin and [Suc^1]angiotensin II in the upper chamber are respectively $1.7 \cdot 10^{-3}$ M and $4.1 \cdot 10^{-3}$ M. $k_1 = 1.7 \cdot 10^{-3}$ min^{-1} at low ionic strength and $2.0 \cdot 10^{-3}$ min^{-1} at high ionic strength with bradykinin. $k_1 = 3.9 \cdot 10^{-3}$ min^{-1} for both straight lines in the case of [Suc^1]angiotensin II.

strength (Fig. 8). These results are consistent with unstirred layers playing the major role as a barrier to amino acid flow across these membranes.

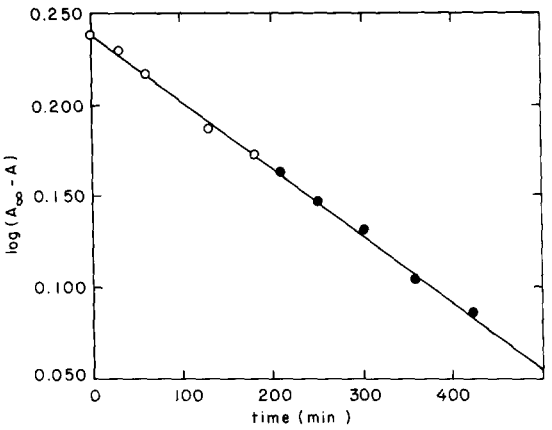

Fig. 7. Kinetics of dialysis of picric acid and the absence of effect of voltage-clamp-imposed membrane electrical potential difference ($\Delta\psi = \psi_1 - \psi_2$). ●, $\Delta\psi = +100$ mV; ○, $\Delta\psi = -100$ mV. Initial concentration of picric acid in one hemi-chamber was $4.4 \cdot 10^{-3}$ M, in 0.015 acetic acid, pH 2.6. It diffused across acetylated cellophane membrane to the other hemi-chamber containing 0.015 M acetic acid, pH 3.3, with a kinetic constant $k_1 = 3.7 \cdot 10^{-4}$ min^{-1}.

Conclusions

Our results clearly show that the diffusion of cationic peptides (angiotensin II, [Suc^1]angiotensin II and bradykinin) through cellophane membranes is significantly affected by the electrical potential difference that develops across the membrane during the process. The fluxes of these peptides across

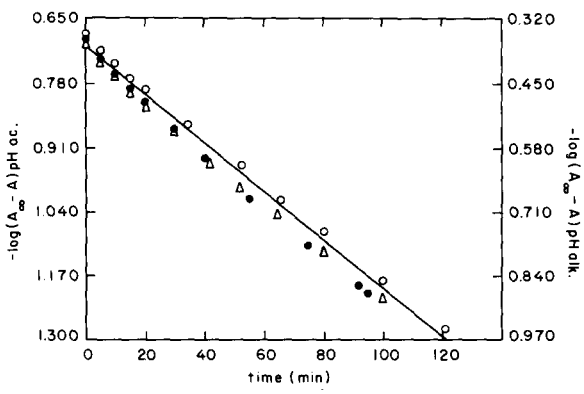

Fig. 8. Kinetics of dialysis of L-tyrosine at different pH values and ionic strengths: ●, in 0.2 M Tris buffer pH 8.2, ionic strength 0.2; ○, in 0.015 M acetic acid, pH 3.3, ionic strength <0.001; △, in 0.015 M acetic acid containing 0.3 M KCl, pH 3.3, ionic strength 0.3. Initial concentration of L-tyrosine in the upper chamber $4.4 \cdot 10^{-3}$ M. $k_1 = 4.9 \cdot 10^{-3}$ min^{-1}.

the membrane is a function of the concentrations in the bathing solutions and of the electrical potential difference present across the membrane, and are well fitted by an integrated form of the Nernst-Planck equation. In view of these results, previous interpretations of dialysis kinetic results in terms of changes in solute conformation, should be revised to take into account possible changes in membrane potential during the measurements.

For smaller molecules, like amino acids and picric acid the flow across acetylated cellophane membranes depends only on the concentration difference across the membrane in spite of an existing diffusion potential difference. This strongly suggests that, for these molecules, the main barriers to diffusion across the membrane are the unstirred layers adjacent to membrane surfaces.

Acknowledgement

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