Dynamic Dialysis as a Method for Studying Protein Binding I: Factors Affecting the Kinetics of Dialysis Through a Cellophane Membrane

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Abstract □ A potentially useful method for determining the extent to which binding occurs in a protein-small molecule system consists of studying the kinetics of dialysis of the small molecule in the absence and presence of protein. The kinetics of dialysis of a number of compounds, in the absence of protein, was studied in some detail in order to characterize the nature of the dialytic process and its dependency on experimental variables. It was shown that the rate of escape of the small molecule from a dialysis cell was a first-order process provided that sink conditions were maintained. The influence of such variables as cell size, solution volume, stirring rates, temperature, pH, ionic strength, and viscosity on the rate process was considered and investigated. It was found that stirring rate (above a minimum), ionic strength, and viscosity did not markedly affect the kinetic picture. Temperature, pH (for one compound), the size of the cell, and the volume of solution contained by the cell did affect the rate of escape. Binding by the cellophane membrane was not encountered as a problem with the compounds studied. Experimental variables could be readily controlled to yield reproducible and dependable results.

Keyphrases □ Dialysis, dynamic—protein binding □ Protein binding determination—cellophane membrane, dialysis □ Permeation half-lives, apparent—small molecules □ Temperature, stirring, pH-buffer concentration effect—dialysis rate □ Viscosity effect—dialytic rate □ Ultrafiltration—phenol red-methylcellulose interaction determination □ UV spectrophotometry—analysis

A previous report (1) described a potentially useful method for studying protein-small molecule interactions. The technique consisted of determining, as a function of time, the escape of the small molecule from a dialysis cell, in the presence and absence of protein. The report indicated the promise of this approach for the rapid and convenient determination of fundamental binding parameters.

The present communication describes studies which were conducted to evaluate the influences of experimental variables which might be encountered in utilizing this technique. The effect of temperature, pH, viscosity, stirring rate, membrane area, liquid volume of the system, and binding of the small molecule by the dialysis membrane were considered.

EXPERIMENTAL

Materials—Bovine serum albumin (BSA), Fraction V (Calbiochem Laboratories) was used in this study. The 8-nitrotheophylline was synthesized according to the procedure reported by Morozowich (2). Other xanthine derivatives, phenol red, and methyl orange were obtained from commercial sources. Regenerated cellulose dialysis tubing [Union Carbide No. 20, 2.50 cm. (0.984 in.) flat width, with an average pore size of 24 Å] was conditioned prior to use by rapidly running distilled water through the tubing for several hours, with the tubing immersed in distilled water. The tubing was stored in distilled water at 2°.

Dynamic Dialysis Studies—The experimental system, the general protocol, and the treatment of data were described previously(1). Generally the systems were prepared to contain 7 ml. of a solution of small molecule in 0.04 *M*, pH 7.3, phosphate buffer, inside a 7-cm. long sac prepared from the dialysis tubing. The sac was immersed in 200 ml. of 0.04 *M*, pH 7.3, phosphate buffer contained in a water-jacketed beaker, in the manner previously described (1). The systems were maintained at 25 ± 0.2°. The contents of the sac were stirred with a twisted glass rod rotated at 125 r.p.m. Stirring of the external solution was achieved with a magnetic stirring bar. At time intervals, 100 ml. of the external solution was removed and immediately replaced with 100 ml. of fresh buffer. The concentration of small molecule in the removed sample was determined spectrophotometrically and the concentration of small molecule in the protein compartment was calculated from a knowledge of the initial concentration and the total amount of small molecule which had appeared in the external solution. During the investigation of a particular experimental variable, the remaining variables were held constant, as described in the conditions for the general system. Whenever possible, the order of experiments concerned with a particular variable, was selected to evaluate any progressive or irreversible effect.

The influence of temperature on the rate of dialysis of phenol red was studied at 25, 10, and 40 ± 0.2°, in that order. Excellent temperature stability was attained through the use of the water-jacketed beakers in combination with a constant-temperature water bath and circulator.

In order to test the effect of stirring the system, the dialysis of phenol red was followed under four different conditions of stirring. The same dialysis sac was used for the four experiments. At the termination of each run the dialysis sac was thoroughly rinsed with distilled water. In the initial study, a dialysis sac containing the dye was suspended in the external buffer solution. The twisted glass rod used to agitate the sac contents, and the magnetic stir bar used to stir the external buffer bathing solution, were in place, but motionless. In a second study, only the magnetic stir bar was rotated. The third and fourth studies had external stirring *via* the magnetic stir bar, and internal stirring at either 125 or 500 r.p.m. The internal rate of stirring was controlled with a Servodyne rotator.

The influence of viscosity on the rate of dialysis was studied using methylcellulose (Fisher-100 cps.) as the viscosity inducing agent. Phenol red solutions were prepared at pH 7.3, to contain 0, 0.032 or 0.064% methylcellulose. The relative viscosities of the various phenol red-methylcellulose solutions or phenol red-BSA solutions, at pH 7.3 and 25°, were determined using a Gilmont falling-ball viscometer.

The effect of pH on dialytic rate was evaluated over a pH range of 3–11 for 8-nitrotheophylline, caffeine, 8-chlorotheophylline, and phenol red, in 0.06 *M* phosphate buffer. In addition, the dialysis of 8-chlorotheophylline was studied at pH 3 in 0.006 *M* phosphate buffer. The same dialysis sac was used for each experimental series involving a given small molecule. The pH of each system was maintained by preparing the internal and external solutions at the same pH and buffer concentration. The pH values were measured before and after each dialysis run and no variation in pH was noted during the course of an experiment.

The influence of the size of the dialysis sac, *i.e.*, the membrane area exposed to the external buffer solution, and the influence of the volume of the solution both inside the sac and external to the sac, were studied in a series of experiments conducted with phenol red in 0.04 *M*, pH 7.3, phosphate buffer, at 25°. First, three control runs were made in which 7 ml. of phenol red solution was placed into each of three, 7-cm. long, dialysis sacs. The sacs were placed into 200 ml. of pH 7.3 phosphate buffer in the usual manner. The loss of phenol red from each of these sacs was followed through about three half-lives. For the second portion of this study the sacs were rinsed and 6, 5, or 4 ml. of dye solution, of the same concentration as the initial control run, was placed into the three sacs and dialyzed. The third experimental series involved rinsing the sacs from the previous study, placing 7 ml. of phenol red solution into two of the sacs, and placing the two sacs into 350 or 500 ml. of pH 7.3 buffer

Table I—Summary of Apparent Permeation Half-Lives for Various Small Molecules

Compound	Mol. Wt.	Apparent Permeation Half-Life, min.[a]	No. of Trials
Trypan blue	960.83	No diffusion after 2 hr.	(2)
Phenol red	354.37	77.1 ± 5.6	(9)
Methyl orange	327.34	51.0 ± 4.0	(5)
Warfarin	308.32	67.5 ± 6.9	(5)
8-Nitro-theophylline	225.17	33.2 ± 2.7	(15)
8-Chloro-theophylline	214.62	38.2 ± 2.1	(6)
Caffeine	194.19	31.9 ± 3.1	(15)
Salicylic acid	138.13	24.0	(1)

[a] Values reported are average values ±1 *SD*.

Table II—Summary of Viscosity Determinations for Various BSA and Methylcellulose Solutions

Methylcellulose, %	BSA, %	Relative Viscosity[a]	Literature (8) Rel. Viscosity for BSA[b]
0.0	0.0	1.000	—
—	1.52	1.090	1.06
0.032	—	1.127	—
—	3.07	1.170	1.13
0.064	5.18	1.290	1.23

[a] BSA and/or methylcellulose solutions in pH 7.3 phosphate buffer at 25°. [b] BSA in pH 6.9 phosphate buffer at 25°.

solution. The loss of phenol red was again followed. The fourth experimental series was conducted to ensure that the sacs had not suffered any damage or alteration in dialytic characteristics during the course of the first three studies. This study involved repeating the control runs in which 7 ml. of phenol red solution was placed into the dialysis sacs and the sacs were immersed in 200 ml. of buffer. Finally, a portion of each sac was cut off so that instead of each being 7 cm. long they were reduced to 6, 5, or 4 cm. The sacs were then placed into the three jacketed beakers containing 200 ml. of pH 7.3 buffer. A sufficient volume of the phenol red solution was added to the sacs to equalize the internal and external liquid levels. The final volumes inside the sacs were 5.7, 3.6, and 2.1 ml., respectively, for the 6-, 5-, and 4-cm. sacs. In addition to these studies, a system was also run in which the level of the liquid inside the sac exceeded the external liquid level. For this system 10 ml. of the phenol red solution was placed into a 10-cm. long dialysis sac and the sac was immersed in 425 ml. of buffer. The height of the internal liquid extended about 1.5 cm. above the height of the external liquid level. Data from this system were compared with data obtained with the same sac containing only 7 ml. of phenol red solution. In the latter system the internal and external liquid levels were the same. A final study determined the effect of variation in internal liquid volume under conditions of a progressively decreasing internal volume during the course of the dialysis. Initially 10 ml. of 0.44 mg./ml. phenol red-buffer solution, at pH 7.3, was placed into a 9-cm. long dialysis sac, and the sac was placed into 500 ml. of pH 7.3 buffer. The dialysis was allowed to proceed with sampling of the external solution as usual. After approximately 2 hr., 2 ml. of the internal solution was withdrawn without replacement. Sampling of the external solution proceeded for an additional 2 hr., at which time 2 ml. was again withdrawn from the internal solution. After an additional 2 hr. the sac was removed and washed with distilled water. Ten milliliters of a 0.28 mg./ml. phenol red solution was then placed into the sac, the sac was immersed in 500 ml. of fresh buffer, and the rate of phenol red dialysis was again determined for the 10-ml. internal volume system. Thus in these studies the internal volume was decreased from 10 to 8 to 6 ml. and then restored to 10 ml.

The influence of the volume of solution in the sac on the rate of dialysis of 8-chlorotheophylline was also studied. The investigation was conducted by initially placing 10 ml. of 8-chlorotheophylline in pH 11.0 buffer solution into a 9-cm. sac, and suspending the sac in 500 ml. of pH 11.0 buffer. After the dialysis had been followed for a period of 1–1.5 hr., 4 ml. of the 8-chlorotheophylline solution inside the sac was withdrawn without replacement. The dialysis was allowed to proceed for an additional period of time. Thus the positioning of the membrane remained undisturbed throughout the course of the dialysis. This procedure was repeated at pH 3.0 using the same membrane.

The potential effect of membrane binding of the small molecule on the dialytic rate was illustrated by simulating strong membrane binding using activated charcoal (Merck, NF Powder). In these studies the dialysis of methyl orange in pH 7.3, 0.04 *M*, phosphate buffer, at 25°, was followed in the presence of 7.45 mg. of charcoal and in the absence of charcoal. The same dialysis sac was used for both experiments.

Ultrafiltration Studies—Ultrafiltration was employed to measure the interaction between phenol red and methylcellulose. Phenol red solutions were prepared, in pH 7.3, 0.04 *M*, phosphate buffer, to contain from 0.0–0.09% methylcellulose. Ten milliliters of each

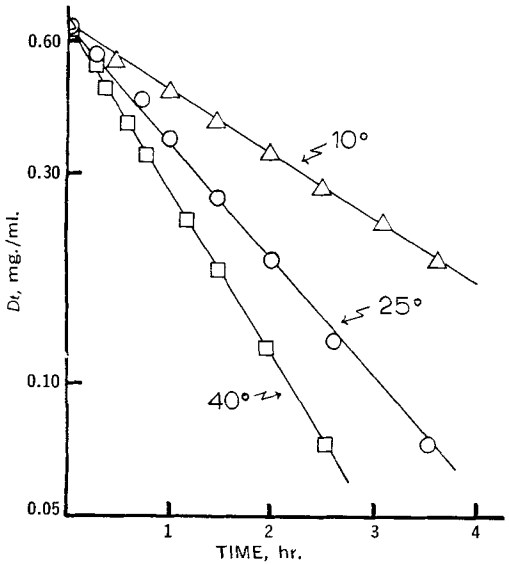

Figure 1—*The influence of temperature on the apparent first-order escape of phenol red from the dialysis cell, at pH 7.3.*

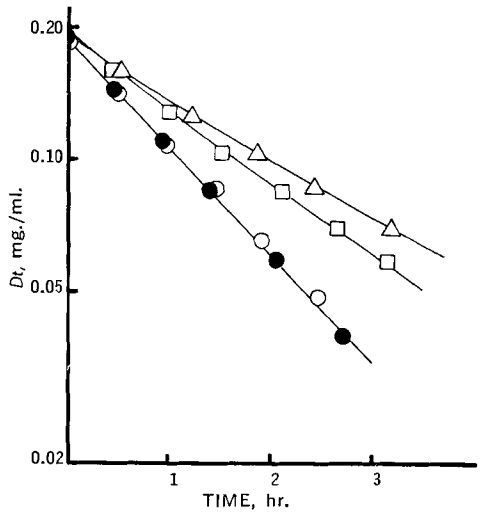

Figure 2—*The influence of stirring on the apparent first-order escape of phenol red from the dialysis cell, at 25°. △, no stirring; □, external stirring only; ○, internal stirring at 125 r.p.m.; ●, internal stirring at 500 r.p.m.*

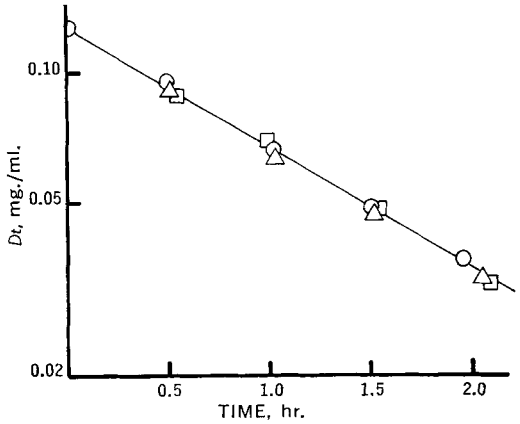

Figure 3—*The effect of viscosity on the apparent first-order escape of phenol red in the presence and absence of methylcellulose at pH 7.3 and 25°. Key: △, 0% methylcellulose; ○, 0.032% methylcellulose; □, 0.064% methylcellulose.*

solution was placed into 12.5-cm. long, double-walled dialysis sacs. The sacs were prepared by partially everting a 25-cm. segment of hydrated cellulose dialysis tubing. The sacs were secured in glass stoppered centrifuge tubes by means of the excess tubing extending out of the mouth of the centrifuge tube. The tubes were centrifuged at 2000 r.p.m. for about 5 min. The filtrate, approximately 0.2 ml., was discarded to minimize error due to moisture initially associated with the membrane. The centrifugation was then continued for approximately 25 min. to collect an additional 0.5 ml. The filtrate and solution inside the sac were analyzed spectrophotometrically to obtain the free and total dye concentration, respectively. There was no evidence of leakage of the methylcellulose from the sac, as determined by the colorimetric test of Kanzaki and Berger (3), or of significant membrane binding of the phenol red.

Analytical Methods—The compounds studied were assayed spectrophotometrically with a spectrophotometer (Beckman DU) equipped with a power source and digital, absorbance read-out (Gilford). Concentrations were determined from Beer's law plots prepared at appropriate wavelengths and pH's.

RESULTS AND DISCUSSION

There are at least three mechanisms which have been suggested to explain the selective permeability of dialysis membranes (4).

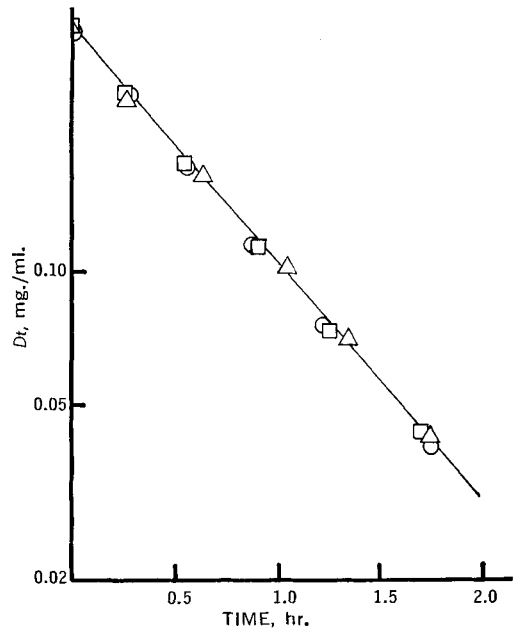

Figure 4—*The effect of pH on the apparent first-order escape of 8-nitrotheophylline at 25°. Key: □, pH 3.0; ○, pH 7.0; △, pH 11.0.*

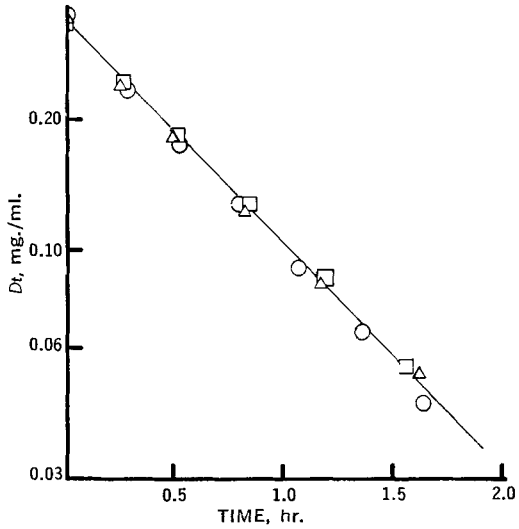

Figure 5—*The effect of pH and buffer strength on the apparent first-order escape of 8-chlorotheophylline at 25°. Key: ○, pH 3.0, 0.06 M buffer; △, pH 3.0, 0.006 M buffer; □, pH 11.0, 0.06 M buffer.*

Cellophane membranes are generally thought to function on the basis of diffusion through pores (5), rather than by dissolution in the membranes, or some other mechanism.

Table I indicates the relationships observed between molecular weight and the apparent permeation half-lives for the dialysis of a variety of small molecules, as determined in the system employed at 25°. The permeation half-life is defined as the time required for the total small molecule concentration within the dialysis sac to decrease by 50%. The half-lives were determined from studies conducted at pH 7.0–7.3, with the exception of studies involving the xanthines. The dialytic rates of the xanthines studied were determined, in separate experiments, to be independent of pH. As a result, average half-lives for these compounds include data for studies conducted over a pH range of 3–11.

The data in Table I show that the bulky, high molecular weight compounds diffuse through the membrane with the most difficulty, and the smaller, lower molecular weight xanthines diffuse more rapidly, and at similar rates. Some of the data in Table I conformed quite well to the direct relationship between the permeability of small molecules through membrane pores and the square root of the molecular weight, as suggested by Danielli (6). Trypan blue, phenol red, and warfarin, however, permeated the membrane at rates which were considerably slower than would be predicted on this basis. These relatively bulky compounds illustrate the)in-

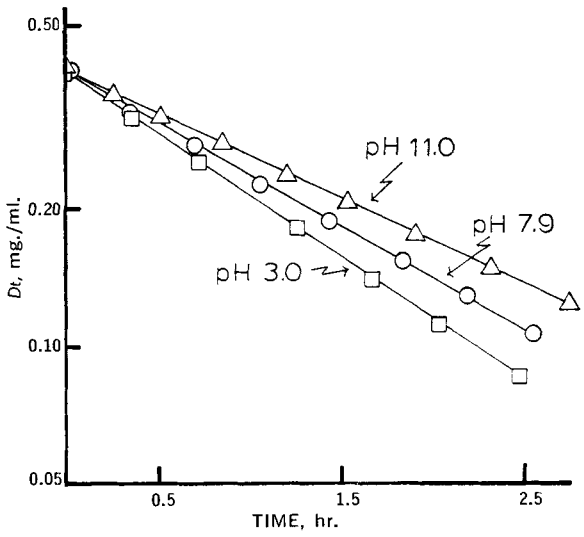

Figure 6—*The effect of pH on the apparent first-order escape of phenol red at 25°.*

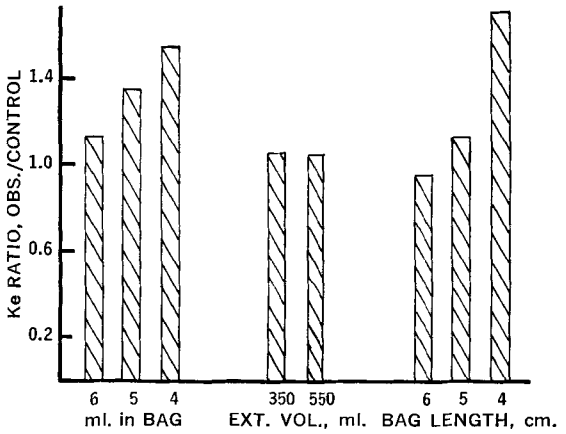

Figure 7—*Effect of liquid volume and membrane area on the apparent permeability constant for the dialysis of phenol red at pH 7.3 and 25°.*

fluence of molecular volume on membrane permeability, which has also been discussed by Danielli (6).

Osmotic Effect of the Protein—It was not anticipated that there would be any dilution of the contents of the dialysis sac due to the presence of protein because the protein concentrations were at least 100 times less than the buffer concentration of 0.04 *M* which was present both inside the dialysis sac and external to it. The lack of dilution was verified by determining that solutions of 1.7% BSA prepared in pH 7.3, 0.04 *M*, phosphate buffer, and placed into dialysis sacs, showed no change in BSA concentration after several hours, when the sacs were placed into the external buffer bathing solution.

The Effect of Temperature on Rate of Dialysis—Figure 1 illustrates the effect of temperature on the rate of dialysis of phenol red, at pH 7.3. The apparent half-life for escape of phenol red from the dialysis cell was calculated to be 122.4, 67.8, and 48.6 min. for the 10, 25, and 40° systems, respectively. The data were further treated utilizing the Arrhenius expression, and the energy of activation for the dialysis of phenol red through the cellophane membrane was graphically determined to be 5.5 kcal./mole/deg. The results of these studies clearly indicate the importance of adequate temperature control, in order to preclude any temperature effect on the intrinsic dialytic rate of the small molecule through the membrane.

Influence of Stirring on Rate of Dialysis—The effect of agitation on the rate of dialysis through a membrane has been discussed by Carr (7). At the initiation of dialysis a concentration gradient for the diffusing species exists across the membrane, from a region of uniform high diffusate concentration on one side, to a region of zero concentration on the other. As the dialysis proceeds there is an accumulation of diffusate on one side and a depletion on the other, with a resulting marked diminution of the concentration gradient across the membrane. Therefore agitation of the solution on each side of the membrane tends to homogenize the concentrations of the solutions and results in an increase in the rate of dialysis, which is proportional to the concentration gradient.

The dynamic dialysis system allows stirring, both internally *via* a twisted glass rod attached to a variable speed motor, and externally by means of a magnetic stir bar. Figure 2 illustrates the effect of stirring on the rate of dialysis of phenol red. There was no apparent difference between an internal stirring rate of 125 and 500 r.p.m., indicating that no advantage is to be gained by stirring the internal solution at other than a moderate sitrring rate. It was interesting to observe that for the unstirred systems, there was an initial rapid rate of dialysis, followed by a significantly slower rate which was apparent after approximately 1 hr. This was undoubtedly a result of the accumulation and depletion phenomena discusssed with respect to the diffusion gradient existing across the membrane. In the totally unstirred system it was observed that a visible diffusion layer developed on the outside of the membrane.

In view of these results, subsequent dynamic dialysis studies were conducted under stirred conditions. The external solution was agitated at a relatively constant rate with a magnetic stir bar. The contents of the dialysis sac were stirred with a twisted glass rod rotated at approximately 125 r.p.m.

Influence of Viscosity on Rates of Dialysis—The dynamic dialysis method for measuring protein-small molecule interactions is dependent on the assumption that the free, unbound diffusing species will dialyze with an identical rate in the presence or absence of protein. It was therefore necessary to establish that increased viscosity of the internal solution, due to the presence of protein, did not affect the rate of dialysis. In other words, it was necessary to show that in the presence of protein, diffusion through the membrane rather than diffusion to the membrane was still the rate-limiting step. To study the potential effect of viscosity, an agent was required which would simulate the viscosity of a protein solution, would not bind the diffusing species, and would not diffuse through the dialysis membrane. Phenol red was utilized as the small molecule and methylcellulose was employed in these studies to simulate the viscosity of solutions containing protein.

The relative viscosities of a series of protein-phenol red solutions of various BSA concentrations were measured with a Gilmont falling-ball viscometer. Other experiments showed that phenol red solutions, at 25°, containing 0.01–0.07% methylcellulose, in pH 7.3 buffer, had relative viscosities comparable to those of 1–5% BSA-phenol red solutions, also at pH 7.3 and 25°. The relative viscosities were calculated as the observed viscosity divided by the viscosity of water at the experimental temperature. Table II illustrates the relative viscosities obtained for solutions of BSA and methylcellulose at various concentrations. In addition, data obtained by Shikama (8), using an Ostwald-type coil viscometer, are given for comparison.

It was also necessary to show that methylcellulose did not interact, to a significant degree, with phenol red. The lack of interaction was established through ultrafiltration experiments. The results of these studies indicated that a maximum of approximately 4 % of the phenol red was bound to the methylcellulose, over the concentration range studied. This amount was too small to significantly affect the rate of phenol red dialysis in the dynamic dialysis system.

The results of the dialysis studies of phenol red at pH 7.3 and 25°, in the presence and absence of methylcellulose, is given in Fig. 3. It is apparent that the increased viscosity did not affect the rate of dialysis. Thus it may be concluded that increases in viscosity due to the presence of at least 5.2 % BSA, will not influence the rate of dialysis.

Influence of pH and Buffer Concentration on Rates of Dialysis—The effect of pH on dialytic rate was studied to observe whether or not the degree of ionization of a diffusing species affected the observed rate of dialysis. In addition, it was of interest to investigate the possibility that ionizable groups in the cellophane membrane could influence the rate of dialysis of a charged species. It should be noted, however, that there is some disagreement in the literature (5, 9) as to whether charged groups, primarily carboxyl groups, are actually present in cellophane.

The dialysis of 8-nitrotheophylline, which has a pKa of 2.11, was studied at pH 7.0, 3.0, and 11.0, in that order, with the same dialysis sac utilized for all three experiments. The results are illustrated

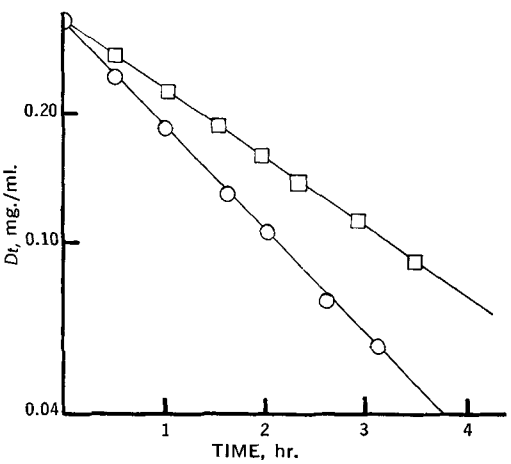

Figure 8—*The effect of a pressure head on the apparent first-order escape of phenol red, at pH 7.3 and 25°. Key: □, 10 ml. of solution inside sac; ○, 7 ml. of solution inside sac.*

in Fig. 4. It is apparent that the rate of dialysis was constant over the wide pH range. In addition to the lack of effect of pH, Fig. 4 indicates the excellent reproducibility for various experiments conducted with the same dialysis sac.

As a further test of the effect of pH on the rate of dialysis, the behavior of 8-chlorotheophylline, which has a pKa of 5.28, was studied. The investigations were conducted at pH 3.0 and 11.0 in 0.06 *M* phosphate buffer, and at pH 3.0 in 0.006 *M* phosphate buffer, using the same dialysis sac. The results of these studies are shown in Fig. 5 and indicated that the state of ionization of the 8-chlorotheophylline and the buffer concentration, within the concentration range utilized, did not influence the rate of dialysis.

The dialysis of caffeine, which is nonionized over the pH range of interest, was studied at pH 3.0 and 11.0. The results were identical to those obtained for 8-nitrotheophylline and 8-chlorotheophylline and showed that the rate of caffeine dialysis was independent of pH and highly reproducible for a given membrane.

Finally, the dialysis of phenol red, at several pH's, was studied using the same sac for each pH. This dye was selected because it is a dibasic acid with a $pK_1 < 1$ and a $pK_2 = 7.9$. The dialysis studies were conducted at pH 11.0, 3.0, and 7.9. The order of the experiments was again chosen to enable the determination of reversibility of any observed effect of pH. The results of these studies are shown in Fig. 6, and illustrate a definite pH effect. The observed results may be rationalized on the basis of an increased effective molecular size due to an increased state of hydration of the phenol red as it changed from a singly charged to a doubly charged anion. This increase in effective size may retard passage of the ion either through the tortuous pores of the membrane or through a diffusion layer. The possibility also exists that the pH affected the ionization of groups of the membrane and that these groups are only important in retarding the dialysis of a relatively large anionic species such as phenol red.

The primary conclusion to be obtained from these experiments is that if protein binding studies are to be conducted at several pH's, using the dynamic dialysis technique, it may be necessary to run control experiments in the absence of protein at each pH, to establish the value for the apparent permeability constant for the small molecule.

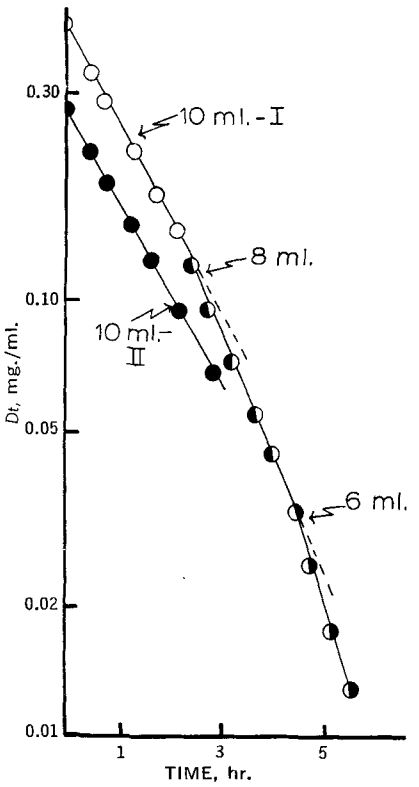

Figure 9—*The influence of changing solution volume, within the dialysis sac, during the course of phenol red dialysis at pH 7.3 and 25°. (I), initial 10-ml. volume of the dialysis cell decreased by 2 ml. at approximately 2-hr. intervals; (II), volume of the dialysis cell restored to 10 ml.*

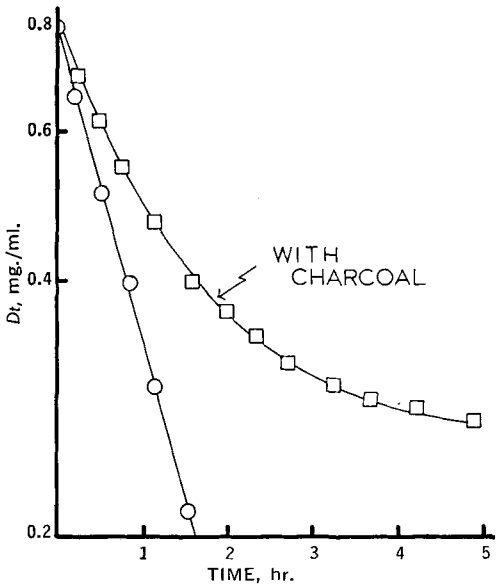

Figure 10—*The effect of 7.45 mg. of charcoal on the apparent first-order escape of methyl orange at pH 7.3 and 25°.*

Influence of Dialysis Sac Size and Solution Volume on Rates of Dialysis—The rate of dialysis would be expected to depend on the area of the membrane exposed to the solution of diffusing species. It was, therefore, considered important to evaluate the extent to which sac size influenced rate of dialysis. In addition, experiments were conducted to determine if the volume of external bathing solution could affect the observed rate of dialysis.

The first experimental series followed phenol red dialysis from systems in which the internal volume was 6, 5, or 4 ml. The next series utilized an external volume of 350 or 500 ml. of buffer bathing solution. Finally, the length of the sacs was decreased and the phenol red dialysis was followed from sacs which were 6, 5, or 4 cm. long. In order to evaluate the effects of volume and sac size variation, the apparent permeability rate constants obtained from the various experiments were divided by the appropriate apparent rate constant obtained with the same sac under control conditions. This was done to normalize the data so as to correct for variation in the intrinsic permeability of each of the three dialysis sacs employed in these studies. Control runs were conducted with 7-cm. long sacs containing 7 ml. of phenol red solution. The control sacs were suspended in 200 ml. of external buffer bathing solution.

The results of these investigations are illustrated in Fig. 7, where the Ke ratio is the apparent permeability constant obtained for a given experimental condition, divided by the constant obtained for the given membrane in the control study. It is apparent from this figure that a decrease in the volume of the liquid inside the sac, or decreasing the length of the sac, with a concomitant decrease in volume of the internal solution, caused a progressive increase in the apparent permeability rate constant, and therefore a progressive decrease in the apparent half-life for dialysis. An analysis of these results showed that as the ratio of membrane surface area to the volume of solution in the sac increased, the apparent permeability constant also increased.

In addition to these studies, a system was also run in which the level of the liquid inside the sac exceeded the external liquid level, *i.e.*, a pressure head was present inside the sac. The results of this study are shown in Fig. 8 and indicate an increase in the apparent half-life of dialysis of approximately 50% for the system with the smaller surface area-to-liquid volume ratio.

As a final illustration of the effect of varying the internal liquid volume, a system was investigated which involved progressively decreasing the internal volume of phenol red solution throughout the course of the run. After sampling of the internal solution, without replacement, the internal and external liquid levels immediately equalized. Thus the area of the membrane exposed to both solutions remained relatively constant. The results of this study are illus-

trated in Fig. 9 and clearly demonstrate the previously observed increase in rate of dialysis with decrease in internal liquid volume. It should be noted that the influence of volume of internal liquid was entirely reversible as evidenced by the close agreement between the two runs, with 10 ml. of liquid inside the sac, conducted before and after the systems involving 8 and 6 ml. of internal liquid. Results, analogous to these, were obtained when 8-chlorotheophylline was employed as the diffusing species at both pH 3 and 11.

Several explanations may be offered to account for the observed behavior. It was noted that as the volume of the liquid inside the sac was decreased the sac assumed a semicollapsed state. It is possible, therefore, that as the size of the sac was increased for a given internal volume, or the volume was decreased for a given size sac, the mean distance from the center of the sac to the membrane decreased, and this decrease resulted in an increased rate. However, this would suggest that diffusion to the membrane was the rate limiting step, and since the solutions were stirred inside and outside, this explanation seemed unlikely in view of the stirring rate experiments. It is possible, however that as the distance between the stirring rod and the membrane decreased, more efficient stirring resulted and the thickness of the diffusion layer, which may still be present under moderate stirring conditions, was reduced and led to an enhancement of the diffusion rate. Alternatively, it is possible that as the surface-to-volume ratio increased, *i.e.*, as the bag was able to assume a somewhat collapsed state, the decrease in tension on the sac caused an alteration in the pore structure to one more favorable for the passage of the phenol red molecule. This may be visualized as either a decrease in the tortuosity of the pore, or perhaps a realignment of charged groups, which may be present in the membrane, in the vicinity of the pores. Other workers (5) have observed that changes in the tension on a membrane can have an effect on the permeability characteristics of a membrane. While the changes in the tension of the membrane, due to the changes in volume of the internal solution, are admittedly relatively small, it is conceivable that they are sufficiently great to cause an alteration in the tortuosity or size of the pore. This is more convincing when one examines the schematic concept which Mosse (10) has presented of the complex and tortuous nature of the pore structure of a cellulose derivative membrane. As further evidence for this hypothesis of variable pore structure, the work of Craig (5) may be cited. He studied the rate of diffusion of ribonuclease through cellophane casing under various conditions of membrane stretching. A threefold increase, in the apparent half-life observed for ribonuclease diffusion with linear stretching of the membrane, was attributed to deformation of the pores. The degree of stretching was much greater in these studies than in the present investigation. However, the results do give some insight into the potential effect of alteration of pore structure.

As a result of these experiments it was concluded that the volume of the solution external to the dialysis sac does not affect the rate of dialysis of a small molecule from within the sac. However the size of the sac and the volume of the diffusate solution within the sac can have a significant influence on the rate of dialysis. Fortunately, when sac size and volume are maintained constant, excellent run to run reproducibility can be achieved.

Binding of Small Molecules by the Dialysis Membrane—Some degree of membrane binding of a compound is a frequently observed occurrence in dialysis studies. If it is extensive, then a correction must be applied to account for the amount of small molecule which has been bound in this manner.

If this type of interaction is essentially an adsorption phenomenon, then it should be reversible. Ideally the membrane may be considered as another binding species and for systems containing protein, the membrane may be treated mathematically as though a second protein species were present. If the preceding concepts of membrane binding are correct, then in the absence of protein, a small molecule which strongly interacts with the membrane should yield curvature in the semilog plot of total drug remaining in a dialysis sac *versus* time. Thus the possibility of sac binding must be recognized in using the dynamic dialysis technique or erroneous characterization of protein binding behavior may result.

If membrane binding is acknowledged to be essentially an adsorption phenomenon, then very strong sac binding should be simulated by the presence of an adsorbent such as charcoal. Experiments were, therefore, conducted to measure the diffusion of methyl orange from a dialysis sac, in the presence and absence of 7.45 mg. of charcoal. The results of these studies are shown in Fig. 10 and illustrate the expected result that the presence of the adsorptive agent resulted in a nonexponential escape of the methyl orange from the dialysis sac.

Most studies in the literature, involving diffusion of small molecules through cellulose dialysis tubing, have reported less than 10%, and usually negligible membrane binding. Since a reasonably strong association between the small molecule and membrane would be necessary for any significant effect on the kinetics of dialysis, it is likely that only in the event of very strong sac binding would the dynamic dialysis method be affected. One means of detecting any effect of sac binding would be to observe for curvature in the semilog plot of total drug concentration remaining in the sac *versus* time, in the absence of protein. Such curvature was never observed for any of the small molecules employed during the course of these investigations.

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