RESTRICTED DIFFUSION

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CHAPTER I

INTRODUCTION

Diffusion through a membrane that has very fine pores may be used to investigate the fractionation and identification of proteins. The process will be called "restricted diffusion." Membranes of specified pore sizes are now commercially available which can be used for this purpose. Another material which may be employed in this connection is Sephadex gel. In either case, the rate of diffusion is limited by the particle size on the one hand and the structure of the barrier on the other.

Chapter III presents the rates of diffusion of various substances through "Millipore" membranes of different pore sizes. The results obtained showed a rather wide variation and it was therefore considered desirable to measure the porosity of the membranes by an independent method. Chapter IV describes a novel and simple technique to determine the pore size of membranes.

Recently, considerable attention has been focused on the association and dissociation processes which occur in protein solutions. It was thought that diffusion through membranes and Sephadex dialysis might be useful in the study of these phenomena. In Chapter V are discussed the results that were obtained with the enzyme urease which exhibits an interesting association - dissociation behavior.

Chapters III, IV, V are written in a form suitable for publication

in a journal.

Chapter II presents reviews of the pertinent literature.

CHAPTER II

Literature Review

This chapter is divided into three sections. The first section deals with free diffusion in solution and the diffusion of solute molecules through membranes or other barriers. Section two gives consideration to the determination of pore size in membranes. Part three deals with the characteristics of "Sephadex", a synthetic material that is used to separate macromolecules from materials of lower molecular weight.

FREE DIFFUSION IN LIQUIDS

This topic has been extensively discussed by Tuwiner (1), by Gosting (2) and by others (3-5). Diffusion may be defined as the movement of solute particles towards the regions of lower concentrations due to their thermal energy. Einstein (6) pointed out that the diffusion of macromolecules occurs by Brownian movement. This spontaneous process eventually results in the establishment of thermodynamic equilibrium with a concomitant net increase in the entropy of the system.

In 1850 Fick formulated the fundamental phenomenological equation to describe quantitatively the diffusion process,

$$J = -D \frac{\partial c}{\partial x} \qquad (1)$$

where $\underline{J}$ is the flux of solute; $(\frac{\partial c}{\partial x})$ represents solute concentration

gradient and D is the diffusion coefficient. This is called Fick's First Law. It can be derived on the assumption that the driving force f responsible for diffusion is equal to chemical potential gradient $\frac{\partial u}{\partial x}$, i.e.,

$$f = \frac{\partial u}{\partial x} \tag{2}$$

This driving force f is what causes the molecules to move against the resistance offered by the medium. The mean velocity v of the molecules diffusing through the medium is directly proportional to the acting force f. Introducing a proportionality constant K, the following equation is obtained

$$v = Kf \tag{3}$$

Combining (2) & (3), we get,

$$v = K \frac{\partial u}{\partial x}$$

or

$$v = \frac{RT}{a} K \frac{\partial a}{\partial x} \tag{4}$$

since

$$\frac{\partial u}{\partial x} = \frac{RT}{a} \frac{\partial a}{\partial x}$$

where a is the activity of the diffusing component; T and R are the temperature in absolute degrees and the gas constant, respectively.

Since most diffusion experiments are done in dilute solutions, the concentrations can replace the activity in equation (4); we then have

$$v = - \frac{RT}{c} K \frac{\partial c}{\partial x} \tag{5}$$

which on rearrangement gives:

$$cv = - RTK \frac{\partial c}{\partial x} \tag{6}$$

If we replace the constants RTK by D, and cv by J, we get equation (1) - Fick's First Law:

$$J = -D \frac{\partial c}{\partial x} \tag{1}$$

When we differentiate the above equation with respect to x, we get

$$\frac{\partial J}{\partial x} = -D \frac{\partial^2 c}{\partial x^2}$$

which gives:

$$\frac{\partial c}{\partial t} = -D \frac{\partial^2 c}{\partial x^2} \tag{7}$$

This is known as Fick's Second Law. The term $(\frac{\partial c}{\partial t})$ is an experimentally measureable quantity; and therefore Fick's Second Law provides a direct and easy way for the determination of D - the diffusion coefficient. In equation (1) or Fick's First law, on the other hand, the flux J comprises the velocity v of the diffusion species which cannot be measured directly; the value of D therefore cannot be determined directly from equation (1). Both laws are otherwise basically identical.

D can also be determined in a diaphragm cell which is represented schematically in Fig. 1. Two compartments A & B of capacity V_A & V_B are interconnected by a boundary G of length h and cross-sectional area a. The diffusing species traverse this region (shaded area) in going from one compartment to the other. For simplicity, the volumes of the two compartments may be made equal i.e. $V_A = V_B = V$. C_A & C_B are the concen-

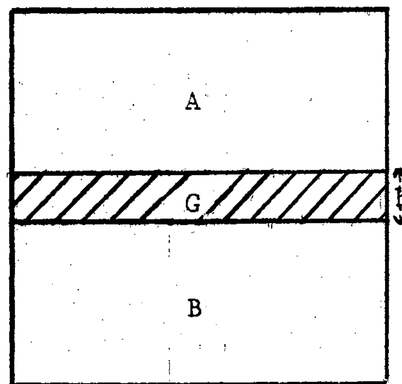


Fig. 1

trations of the solute in compartment A & B respectively, so that C_A is greater than C_B. If the diffusion of the solute is allowed to take place for a sufficient length of time, a concentration gradient equal to $(C_A - C_B)/h$ will be established throughout the region G, and this will remain valid provided D is independent of concentration.

When $(\frac{\partial c}{\partial x})$ is replaced by $(C_A - C_B)/h$, equation (1) becomes:

$$J = -D\left(\frac{C_A - C_B}{h}\right) \tag{8}$$

let dq be the amount of solute which diffuses through an area of cross-section a perpendicular to the direction of the flow of solute, in an interval of time, dt. The flux of solute J therefore is:

$$J = \frac{dq}{a\,dt} \tag{9}$$

Combining equations (8) & (9) and on rearrangement we get:

$$dq = -\frac{Da}{h}(C_A - C_B)\,dt \tag{10}$$

Since $dq = -dq_A = dq_B$ and also $dq = Vdc$; equation (10) therefore takes the following form for each of the compartments A & B;

$$V_A\,dC_A = -\frac{Da}{h}(C_A - C_B)\,dt \tag{11a}$$

$$V_B\,dC_B = +\frac{Da}{h}(C_A - C_B)\,dt \tag{11b}$$

From equations (11a) & (11b), we get

$$V_A\,dC_A - V_B\,dC_B = -\frac{2aD}{h}(C_A - C_B)\,dt \tag{12}$$

Now $$V_A = V_B = V$$

Equation (12) becomes:

$$V(dC_A - dC_B) = \frac{-2aD}{h}(C_A - C_B)\,dt$$

or

$$\frac{dC_A - dC_B}{C_A - C_B} = -\frac{2aD}{hv}\, dt$$

or

$$\frac{d(C_A - C_B)}{C_A - C_B} = -\frac{2aD}{hv}\, dt \qquad (13)$$

Equation (13) on integration, from the start of the experiment when the concentrations are C_A° & C_B° to the time t, when the concentrations are C_A & C_B respectively, gives:

$$\ln\left[\frac{C_A' - C_B}{C_A^\circ - C_B^\circ}\right] = -\frac{2aD}{hv}\, t \qquad (14)$$

or on rearrangement, we have the following equation:

$$D = \frac{1}{\beta t} \ln\left[\frac{C_A^\circ - C_B^\circ}{C_A' - C_B}\right] \qquad (15)$$

The constant β replaces $(\frac{2a}{hv})$ and is called the cell constant. In practice β is determined by using a substance of known diffusion coefficient D.

The validity of equation (15) depends upon some assumptions which were made in its derivation. One assumption is that a linear gradient exists across the region G during the whole course of diffusion. Barnes (7) pointed out that this assumption will not introduce an appreciable error unless the time of diffusion is very short and/or λ, the ratio of the volume of the membrane to that of the sample volume taken in the cell compartment, is greater than 0.1. An appreciable error will however be introduced, even with λ less than 0.02, if the preliminary treatment of the membrane fails to provide an initial linear gradient across it. Another assumption is that diffusion

coefficient D remains constant. This may not be true because one of the terms K - mobility of the diffusing species [D replaces RTK in equation 6] may be influenced by the concentration of the system. D therefore may be concentration dependent.

CHARACTERISTICS OF MEMBRANES

Free diffusion is not very effective in separating molecules of different molecular weights. For example less than a four-fold difference is observed in the values of the diffusion coefficient D for ribonuclease (13,500 molecular weight) and urease (480,000 molecular weight) (2), since D is inversely proportional to the cube root of the molecular weight. Much greater discrimination between molecules can be realized, if they are allowed to diffuse through a barrier with fine capillaries. This section of the thesis presents a review of the characteristics of such barriers.

Graham (8) observed in 1855 that egg albumin diffuses very slowly as compared to sodium chloride; and six years later he succeeded in separating the two by using parchment paper as a barrier to the passage of albumin. Animal membranes such as pig or fish bladders, intestines, etc., were then used for some time, for dialysis.

In 1896 Martin (9) separated colloids from crystalloids using a bacteriological candle impregnated with gelatin. Later collodion membranes were introduced (10). Bechhold prepared membranes of this material with pore sizes ranging from one micron down to molecular size. Various other types of membranes--inorganic as well as organic, were introduced and various methods were devised to grade them (11-14). Satisfactory graded collodion membranes were prepared for the first

time by Elford in 1933 (15). He controlled the pore size by controlling the evaporation rate of the solvent.

Craig and his coworkers (16) used commercially available Visking dialysis tubing for fractionating various protein molecules. They found that by controlling the pore radii (which they did chemically as well as mechanically), the rate of diffusion of a particle can be regulated. Ackers & Steere (17) prepared agar gel membranes and controlled their pore size by varying the agar gel concentration. At the present time, membranes of 'specified' pore size are available commercially (18-19). One of them was used in the present study (18).

Structure of Membranes

Various models have been proposed for the structure of membranes. The simplest one is in which the membrane is considered a sheet pierced by circular cylinders (10). According to Manegold (20), a membrane can have either a canal type structure (pores, cracks, etc.) in which the solid phase is continuous, or a branching type structure (packed spheres, rods, etc.) in which the solid phase is discontinuous. In some membranes the amount of free space is too large to be compatible with a closely-packed sphere structure (21).

Some of the methods employed for studying the membrane structure are discussed below; one that employs the rate of flow of water is discussed separately in the next section.

a) Microscopic Analysis:

Elford (22) in 1930 investigated collodion filters formed from acetic acid and ether-alcohol solvent with the ultramicroscope and

found two different types of structures: (i) a highly irregular structure with different pore sizes; and (ii) a very fine uniform granular structure. The experimental conditions were found to influence the particular type of structure.

Riley et al (23) took electronmicrographs of cellulose acetate filters and found that they have a dense surface layer which is devoid of any structural characteristics. The sub-structure of these membranes was found to be ill-defined.

b) Determination of Specific Water Content:

The proportion of empty space in a membrane can be indirectly determined in terms of the 'specific water content', SWC (24). It was found that ether-alcohol collodion membranes have high SWC values-- 80 to 90%. This value was found to remain constant for membranes above 20 millimicron pore size. Manegold et al (25) found that their membranes, with pore sizes varying from 25 to 60 millimicron, had constant SWC values. None of these results seem to indicate the presence of a continuous solid structure.

c) Air-Bubble Method:

Bartell and Carpenter (26) determined filter pore sizes by forcing air through a wet collodion membrane. Later on Hitchcock (27) using the same membranes found that his data, obtained by simply letting the water flow through them, gives 40 to 130 times smaller values as compared to Bartell & Carpenter's. The higher values obtained could be the result of structural changes brought by the pressure which was used for forcing air through the membranes; such a

possibility was ignored by these authors however.

d) Mercury-Intrusion Technique:

Mercury which does not wet the membranes, is forced into the pores by applying some external pressure. The following expression is given relating this pressure P, with the surface tension σ, which must be overcome to force mercury into the pores:

$$P r = -2\sigma \cos \theta \qquad (16)$$

θ is the contact angle and r is the pore radius of the membrane. Harold and Skau (28) determined the pore size of 'Millipore' filters by using this method. It was found that the filters which could retain particles of 0.3 to 0.5 micron size, have pore diameters in the range 0.1 to 0.7 micron with median ~ 0.6 micron.

This method has the same weakness which is inherent in air-bubble technique.

e) Filtration of Particles of Known Size:

Particles of known sizes are filtered through the membrane. The filtrate is then analyzed for the largest particle which has filtered through. From this a rough estimate regarding pore size of the filter can be made. The conclusion drawn this way could be erroneous however, since filters are known which retain particles of diameter much smaller than their pore size (18-19).

Water Flow Rate Measurements

Guérout (12) in 1872 suggested that the porosity of a membrane could be determined from the rate of flow of water. Hitchcock (27)

checked the pore size of Bartell and Carpenter's membranes by this method and obtained reasonable data. A more detailed discussion of this method is given in this section because it was used for checking the pore size of "Millipore" filters in the present study.

The following three assumptions have been made when this technique is used for the determination of membrane's pore size:

a) the capillaries are parallel, cylindrical and perpendicular to the surface of the membranes;

b) the flow rate follows Poiseuille's law;

c) the total volume of the pores, as given by specific water content represents the total volume effective in filtration. In other words there are no "blind" channels or pores and there is no appreciable immobilized water layer, lining the pore walls.

Ferry (21) related the pore radius r with the rate of flow of water, (v/t), through the membrane by the equation:

$$r = 2\left[\frac{2V\eta l}{SAPt}\right]^{\frac{1}{2}} \qquad (17)$$

where l is the pore length; η the viscosity of solvent; P pressure on the system causing the flow through membrane; A is the membrane area exposed to water and S the specific water content.

The validity of the aforementioned assumptions is discussed below. The first assumption is rather arbitrary. Bechhold (29) suggested that the actual pore length is three to four times the thickness of membrane whereas Elford and Ferry (30) put this value as twice the membrane thickness. Millipore membranes (18) supposedly permit a flow rate 4 to 5 times faster than that of other membranes; if so, it might be concluded that their pore length is not very different from their

thickness.

Duclaux and Errera (31) suggested that Poiseuille's law is applicable in general; however, Elford (15) latter commented that it is not valid for pores less than 10 millimicrons because of electrokinetic and/or steric effects. In 1966, Longuet-Higgins and Austin (32) concluded from statistical calculations that Poiseuille's law is applicable to pores as small as 4.5 Å (radius) when water is allowed to flow through under hydrostatic pressure. The failure of Poiseuille's law or of equation (17) below this limit is due to the predominance of diffusional mechanism over streamline flow.

The calculated average pore radii will be too small, if the third assumption does not hold. In other words the membranes might show high water content and still lack high porosity. Elford and Ferry (30) stated, however, that for membranes with SWC above 80%, the error involved in the calculations for $\underline{r}$ according to equation (17) is not greater than 25% from this assumption. In the case of "Millipore" filters, a comparitively high flow rate was found which indicates that there are not too many blind channels in these membranes.

GEL FILTRATION

A comparitively new technique, Gel Filtration, has recently been developed for the purpose of separating molecules according to their size. In this technique a column is filled with a gel that has been swollen with solvent. A sample of the mixture to be separated is placed over the top of the column so that the gel bed is not disturbed. After some time, the solution layer passes into the gel, and the column is then washed with solvent. The components of the mixture

migrate at different speeds and ideally appear in the eluant in order of decreasing molecular weights. The liquid is allowed to flow through the column under the influence of gravity to avoid compression of the gel granules.

Gel filtration differs from ordinary filtration in the sense that smaller molecules are retained by the gel in preference to the bigger ones. Gel filtration has been compared with dialysis. The gel particles behave both as membranes and as receivers for the dialysate and passage through the column may be considered a multiple-stage dialysis. Kisluk (33) compared gel filtration and dialysis in the separation of some enzymes and co-factors. The former method was found to be as effective as the later in identifying the various mixture components and had in addition the following advantages: speed, complete removal of cofactors in one step and elution of the components in relatively small volumes.

It may be pertinent to mention at this point that a 'good' gel must be mechanically as well as chemically stable. The gel particles should be rigid spheres and not flexible solid masses, and should not bind irreversibly with the solute under investigation.

Historical Development:

Duel et al (34) used Amberlite IR-4B for some fractionations. They found that the degree of retention of clupein increases with the degree of swelling of the resin, whereas amino acids are readily retained even if there is little swelling of the resin. Duel and Neukom (35) in 1954 reported the cross-linkage of locust bean gum with epichlorohydrin in alkaline solution. Gels were obtained which,

after swelling in water, could be used for separation purposes. They pointed out that the retention volume of a substance will depend upon its molecular size.

Clark (36) fractionated a mixture of polyhydric alcohols over Dowex 50 x 12 ion exchanger. The main draw-back with these ion-exchangers is that losses due to sorption are great (37). It may be possible to identify qualitatively the number of components in an unknown mixture, but the quantitative significance of molecular weights or sizes determined this way will be very much questionable.

To avoid sorption, it is desirable to use an inert substance for gel filtration. Various types of inert gels, synthetic as well as natural have replaced the ion-exchangers (37-40,42-48). To mention a few, starch gel has been used for separating amino acid mixtures. Lathe and Ruthven (39) found that maize starch granules swollen at 61 - 68°C in water, efficiently fractionated a mixture of substances with a molecular weight range of 60 to 1×10^6, but the cellulose columns could not differentiate between globin and urea. Both of them were eluted at the same time.

Polson (40) found that the penetration of protein molecules into agar gels is limited by their size. The spacing within the solid phase, was found to decrease as the gel concentration was increased (41).

Rubber pieces (48) have also been used in the columns, but these pieces had the draw-back of being flexible.

Porath and Flodin (42) in 1959, prepared dextran gels by cross-linking dextran with epichlorohydrin in alkaline medium. Dextran was synthesized microbiologically by the action of Leuconostoc Mesenter-

oides strain NRRL B-512 on sucrose. These gels, with different degrees of cross-linkage, are now commercially available under the name of 'Sephadex'.

Sephadex gels were used in the present study and will therefore be discussed in some details. These water swollen gels are inert and offer a sort of molecular sieve to solute particles. The presence of large numbers of hydroxyl groups from the polysaccharides are responsible for their great affinity for water. The gel properties have been modified by causing substitution at some of the hydroxyl groups.

Solvation, on treatment of the gel with water, begins at the outermost layer of the gel particle, which expands exposing the next inner layer to the solvent. This process continues till completed. Swelling of gels can therefore be compared to the 'popping' of corn. Expansion of gel granules is actually the result of the tendency of the glucose residues to disperse and seperate as far as possible; this movement is prevented by crosslinkage. The degree of swelling therefore depends upon the amount of crosslinkage and the solvent power of the solvent used. Water, glycerol, formamide, etc., are found to be good solvents, whereas less polar solvents like methyl-alcohol, acetone, etc., that are miscible with water, dehydrate these water-swollen gels.

A simple method for the determination of the water regain of dry gel is discussed in Chapter V.

Various explanations have been offered to interpret the retention of solute particles in the gel columns. Wheaton and Bauman (37) attributed the fractionation of amino acids to a definite "pore diameter" in the starch granules. Lathe and Ruthven (39) argued that

the retardation of various molecules is due neither to surface phenomena on the gel phase, nor to partition of the molecules between the mobile and gel phases. In the case of crosslinked gels, the retardation is attributed to the degree of penetration into the gel structure, depending upon the particle size and the gel cavity size. Flodin (49) introduced the term of "forbidden region" to interpret this process. As a molecule diffuses through gel, it encounters regions so densely populated with dextran chains that its passage is blocked. Around each crosslinking site, there is a forbidden region, the size of which depends upon the size of the diffusing molecule. In all other parts of the gel, the solute can diffuse freely. In a limiting case when the molecule is too big to penetrate, the whole region is forbidden to it.

Molecular Weight Determination:

The ability of this gel filtration method to fractionate particles resulted in an interest to find a relationship between the molecular weights and the elution volume of the particles. The first attempt to relate these was made on antigens. Their effective size were estimated within ± 30% (41).

Andrews (50) attempted to correlate the 'peak' volume with the logarithm of the molecular weight for various proteins using agar gel columns. A linear relationship is not shown by his curves. It could be the result of adsorption of the molecules due to the presence of ionised groups in the gel granules.

Whitaker (51) observed a linear relationship between the logarithm of the molecular weight and (V_e/V_o), the ratio of the elution volume and the "void" volume, which is the volume in the gel

column which is outside the gel phase.

These relationships between the molecular weights and the behavior of the particles in the gel columns were empirical.

Porath (52) and Squire (53) independently derived a mathematical relationship between the behavior of the molecules in the gel column and their molecular weight.

Porath's equation is:

$$K_d = K\,[1 - K_1\, M^{\frac{1}{2}}/(S_r-\alpha)^{\frac{1}{3}}]^3 \qquad (18)$$

where K_d is the distribution constant for the particle between the gel structure and the excluded solvent; K_1 & K are the experimentally determined proportionality constants; M is molecular weight of the molecule; S_r is volume of the solvent in the gel, called "solvent regain"; and α represents the correction term introduced to account for the solvent which is bound to the gel matrix and is not therefore exchangeable for the solutes. The elution volume V_e is given by

$$V_e = V_o + K_d\, V_i \qquad (19)$$

where V_o is void volume and V_i is the volume of the solvent in the gel which is exchangeable for the solutes. From these equations, the following expression is obtained:

$$\Delta V = V_e - V_o = K\, V_i\, [1 - K_1\, M^{\frac{1}{2}}/(S_r-\alpha)^{\frac{1}{3}}]^3 \qquad (20)$$

Squire's equation is:

$$\frac{V_e}{V_o} = [1 + g\,(1 - \frac{M^{\frac{1}{3}}}{C^{\frac{1}{3}}})]^3 \qquad (21)$$

where g is an experimentally determined constant; and C is the molecular weight of the smallest protein which is excluded from the gel cavity. V_o, V_e, & M are the same as defined earlier.

Both these equations consider the cavities of the gel structure as conical, cylindrical & crevical in shape; and both are not applicable when $V_e = V_o$ i.e. when the molecule is completely excluded from the gel.

From these above mentioned equations, a linear plot for $(\Delta V)^{1/3}$ or $(K_d)^{1/3}$ VS. $(M)^{1/2}$ and (V_e/V_o) VS M is expected and this is found to be the case within certain limits (52,53,54). Using proteins of known molecular weights, a standard curve can be drawn which is then used for the determination of molecular weights of the unknown particles. There are, however, complicating factors which impair the validity of results obtained from these standard curves.

Gelotte (55) and Porath (56) found that basic proteins and those compounds which have aromatic or heterocyclic rings, are absorbed on the G-200 column. The elution volume for lysozyme indicated the presence of a 6,600 molecular weight unit; even though the molecule is known to be twice that much. Similarly ovomucoid exhibited more than expected retention on the column.

Shape factors can also influence the elution volume of the molecules passing through the column. Siegel and Monty (57) found that fibrinogen (molecular weight 360,000) preceded ferritin (molecular weight 1.3×10^6) on G-200. Similarly urease (molecular weight 480,000) was retained in preference to fibrinogen. They pointed out that their data indicated a linear relationship between $(K_d)^{1/3}$ and the Stokes radius of the particle , i.e. it is not the molecular weight but the Stokes radius which plays a determining role during the gel filtration process.

From the above discussion, it does not seem possible to predict whether the behavior of the unknown particle is going to be typical of

its molecular weight or it would be abnormal. It is therefore suggested that for the determination of the "true" molecular weight of the unknown particle some other physico-chemical method where the above mentioned limitations do not exist, should be employed. The gel filtration technique can however serve to give some idea about the molecular weight of an unknown particle.

Association - Dissociation System:

Gel filtration provides a means of studying systems that undergo association - dissociation. If the rate of equilibrium is slow as compared with that of gel filtration, separation may be possible without complications. The problem becomes intriguing, however, if the equilibrium is established instantaneously or if the system has a very weak tendency to associate or dissociate.

Gilbert (58) made a theoretical analysis of the problem and concluded that gel filtration could be used provided: (a) that only the smallest unit exists below a certain concentration and similarly the largest unit exists above a certain concentration; and (b) one of the units can be 'arrested'. Winzor and Scheraga (59) tested this theory with α-chymotrypsin on a Sephadex G-100 column; according to these authors the substance undergoes a monomer-dimer reaction and the two species were identified.

Gilbert's theory will not provide reliable data for a system which associates or dissociates slowly. He pointed out that even theoretically the sedimentation constant S will not change significantly over a wide concentration range. [ln one typical calculation, S changes from 2.5 to 2.62 when concentration of the system changes from zero to

15 mg/ml].

Kikuichi et al (60) investigated the behavior of B - α Amylase on a Sephadex G-100 column. It was found that, as the elution rate was reduced, the peak height corresponding to the monomer increased. In the opinion of the author, the low rate of elution results in an increased contact time between the gel and the migrating species, which results in the increased retention.

In some cases in which column chromatography was not successful, employment of Sephadex in a 'batch wise' manner affected the desired separation. For example Richterich et al (61) could not separate LDH isoenzymes on a gel column, but succeeded in fractionating the mixture by mixing it with a 2% suspension of DEAE - Sephadex A-50. The mixture was centrifuged and the supernatant was assayed; then the residue was again mixed with fresh solvent. This resulted in the separation of α_1; α_2; β and γ- isoenzymes. Baumstark et al (62) separated γ - globulins from human serum by this 'batch wise' method.

The advantage of this method is that comparatively little Sephadex and sample are needed. Also, since the solute particles are in contact with the gel structure for a relatively longer period, they have more chance of penetrating into the gel cavity. This step is very significant in systems which associate or dissociate slowly.

CHAPTER III

HINDERED DIFFUSION OF MACROMOLECULES THROUGH MEMBRANE FILTERS

(This paper is written in a form suitable for publication in a journal).

(ABSTRACT)

An apparatus and procedure are described for measuring rates of diffusion through membranes. The diffusion of the following materials through "Millipore" membranes of various nominal pore-sizes was measured: p-aminobenzoic acid through 10, 50, 100, and 450-mμ membranes; lysozyme, β-lactoglobulin, and bovine serum albumin (BSA) through 10-mμ membranes; 88-mμ polystyrene latex through 100, 450, and 800-mμ membranes. Also, the diffusion of BSA through pieces of "Visking" dialysis tubing was measured. The area available for diffusion of p-aminobenzoic acid changes little with pore size. The area available for diffusion of proteins through 10-mμ membranes decreases with increasing molecular size, but all the proteins studied pass at an appreciable rate. BSA does not pass through dialysis tubing, and the polystyrene latex does not pass through 100-mμ membranes. It is concluded that some information concerning the shape and size of molecules can be obtained by measurements of this type but that the usefulness of the method is rather limited; it has, however, other potential applications.

INTRODUCTION

Study of the diffusion of proteins in solution is one means of obtaining information about their size and shape. But the rate of diffusion is not very sensitive to size. Most globular proteins are nearly spherical and for spheres the diffusion coefficient varies as the inverse cube root of the volume. Thus one finds less than a fourfold difference between the experimental diffusion coefficients of, say, ribonuclease, M. W. 13,500, and urease, M. W. 480,000 (2).

Much greater differences can be realized if diffusion is allowed to take place through a barrier which has very fine channels, of the same order of magnitude as the molecules passing through (16). This process will be called "hindered diffusion". An extreme kind of hindered diffusion is very commonly practiced in separating proteins from subtances of low molecular weights by dialysis. For this purpose, membranes of very small pore size are used; the "Visking" dialysis tubing which is most commonly employed at the present time has pores of 4-5 mμ diameter (63). With this type of barrier, however, all but the smallest proteins are completely excluded, and nothing can be learned about their shape and size except possibly a lower limit.

Recently, membrane filters have become commercially available that are produced with pores of graded sizes, varying from about 5-10 mμ upward; it seemed of interest to study the properties of these membranes with respect to their ability to pass proteins of various sizes. A preliminary report on this work has been made (64). The present paper reports new measurements, made with some improvements in apparatus and technique, on lysozyme, β-lactoglobulin and bovine serum albumin (BSA). The new measurements with BSA do not agree with those reported earlier

and this problem will be discussed. Also, diffusion measurements have been made on p-aminobenzoic acid and 88-mμ polystyrene latex.

It is not possible to review the pertinent literature in this paper. There is a voluminous literature on osmosis and dialysis (65) and much work has been done specifically on the properties of membranes and their permeability to various subtances (1). Considerable work has also been done on ultrafiltration, a closely related technique that in many cases gives pertinent information (15,21). In the limited space available here, reference will be made only to work which has appeared in the last five years and deals specifically with the relation between diffusion through membranes and molecular size. Craig and co-workers have made several contributions, which will not be mentioned individually since they have been covered in a recent review (16). These investigators made use of a "thin-film" apparatus which gives comparatively fast escape rates and minimizes back diffusion. Dialysis tubing was used as the barrier material; for use in the 10^4 - 10^5 molecular-weight range, mechanical or chemical modification of the tubing was found necessary to enlarge the pore size. At the other extreme stands the work of Hoch and Turner (66) who used membranes of 250 - 450 mμ pore size in a study of the diffusion of blood proteins; with such large pore sizes the amount of hindered diffusion should be small and the results therefore comparable to those obtainable with free diffusion. In order to provide a continuous range of pore sizes, Ackers and Steere (17) experimented with agar-gel membranes, in which the pore size could be varied by changing the concentration of agar. The relation of the present work to these investigations will be considered in the last section of this paper.

EXPERIMENTAL

(The work to be described comprises two series of experiments that were done at different times with certain differences in apparatus and technique; when necessary to distinguish between them, they will be referred to as Study I and Study II).

Materials:

Lysozyme was obtained from Calbiochem, Los Angeles; β-lactoglobulin from Koch-Light, Colnbrook, England; BSA from Pentex, Kankakee, Ill. Polystyrene was a gift from the Dow Chemical Company, Midland, Michigan, obtained through the courtesy of Dr. J. W. Vanderhoff (Run #LS-040-A). All other chemicals were of A.C.S.-reagent grade. "Millipore" membranes were purchased from the Millipore Filter Corp., Bedford, Massachusetts. Dialysis tubing was purchased from the Visking Division, Union Carbide Corp., Chicago.

Diffusion Apparatus:

Fig. 2 shows a schematic diagram of the apparatus and cells used in Study II. The motor driving the stirrers was a "Hurst" (Princeton, Indiana) synchronous motor, Type DA, speed 600 rpm. The cells were constructed of stainless steel, Type 316; they had the following "window" areas and approximate volumes: A, 2.390 $cm.^2$, 10 ml.; B, 0.8495 $cm.^2$, 2 ml.; C, 0.5026 $cm.^2$, 1 ml. During the measurements the cells were immersed in a constant-temperature bath and the joint in the middle of the cell was kept covered with waterproof plastic friction-tape.

In Study I, cell D was used, a diagram of which was shown in the

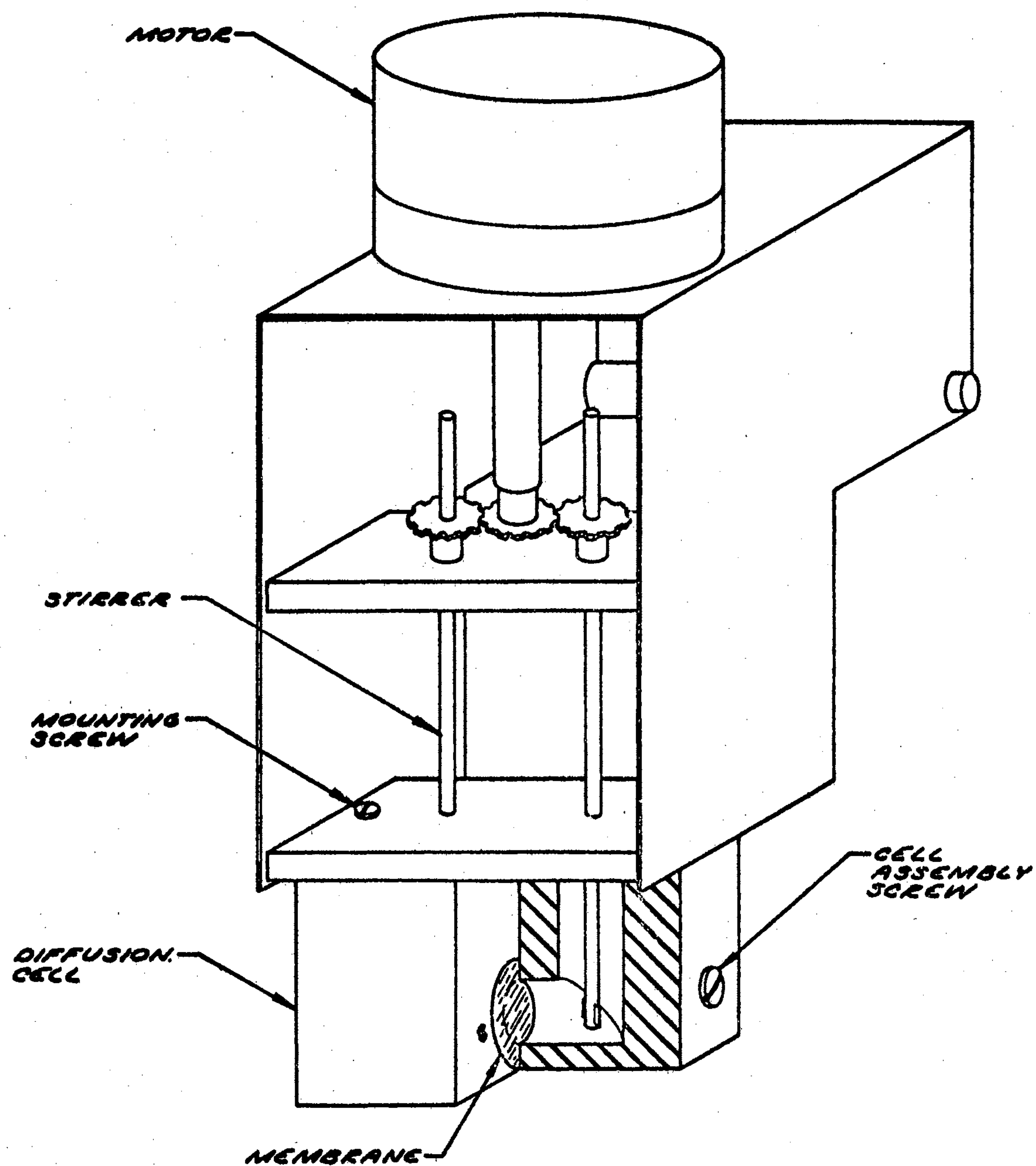


Figure 2. Schematic Diagram of
Diffusion Cell and Stirring Assembly

earlier paper (64); the cross sectional area and approximate volume were 2.56 $cm.^2$ and 9 ml., respectively. In that study, the stirrers were actuated by a friction drive instead of gears.

Diffusion Measurements on p-Aminobenzoic Acid. Procedure I:

In Study I the membranes were initially dry. p-Aminobenzoic acid was dissolved in water to the concentration 4.00 g./liter. A portion of the solution, 8.50 ml. was pipetted into compartment 1 of the cell and one minute was allowed to elapse before an equal volume of water was added to compartment 2. Time zero was taken when the water compartment had been filled. Aliquot samples, 100 λ, were withdrawn from compartment 2 at 5-min. intervals, diluted to 5.00 ml., and the absorption was measured with a Beckman DU spectrophotometer; an equal aliquot was withdrawn from compartment 1 to keep the volumes and levels the same. The temperature was in the range 20-30° and was not controlled; in any one experiment the change was no greater than 1°. A temperature correction was applied, as explained in the next section, to reduce the results to a common basis. Whenever multiple determinations were made with a single membrane, it and the cell were rinsed with water, the water was removed without allowing the membrane to dry, and a new solution was then placed in the cell.

Diffusion of p-Aminobenzoic Acid. Procedure II:

Phosphate buffer, 0.16M and of pH 7.0, was the medium used in these experiments; the solutions of p-aminobenzoic acid contained 4.00 g./liter. The membrane was put in place and soaked in water for about 3 hours before making measurements. Then, the water was poured out

and the last traces of it were removed, within 1 min., with the aid of a polyethylene tube which was closed at one end and had a few pin holes near the closed end; this end was passed along the walls while suction was applied at the other end. Solution and buffer were added to the respective compartments at the same time. Stirring was started within 30 sec. The cell was kept in the water bath at 25 ± 0.1°. After about 10% diffusion had taken place the cell contents were removed and their absorbances measured after appropriate dilution.

Diffusion of Proteins:

The protein solutions were 1% in phosphate buffer. First, a measurement of diffusion was made with p-aminobenzoic acid by Procedure II. Then the cell and membrane were rinsed and a measurement made on the protein solution. In several experiments, a determination was made of the volume present in each compartment at the end.

Diffusion of Polystyrene Latex:

Equal volumes of distilled water were placed in each of the two compartments and the cell was put in place. After some time, 5 or 10 λ of latex was added to compartment 1 and stirring was started. After 2 min. a 100-λ aliquot was withdrawn from this compartment, diluted to 100 ml., and its absorbance measured at 265 mμ; this was taken to be the original concentration. Samples were then withdrawn from compartment 2 at appropriate intervals.

CALCULATIONS

Diffusion Measurements:

Gosting (2) gives the basic equation for determining diffusion coefficients in a diaphragm cell; the diffusion coefficient D is given by:

$$D = \frac{1}{\beta t} \ln \left[\frac{C_1^o - C_2^o}{C_1 - C_2}\right] \tag{1}$$

where C_1^o and C_2^o are the concentrations in the two compartments at the starting time, taken after steady-state diffusion has been established in the diaphragm; C_1 and C_2 are the concentrations at time t afterwards, and β is the cell constant. Assuming that steady-state diffusion is established instantaneously in the membrane diaphragm (an assumption which will be discussed in the next section) one has $C_2^o = 0$ and, if the volumes are equal, $C_1 = C_1^o - C_2$, the equation then reduces to

$$D = \frac{1}{\beta t} \ln \left[\frac{C_1^o}{C_1^o - 2C_2}\right] \tag{2}$$

The area available for diffusion, a, was obtained in the following way. For an idealized cell in which diffusion takes place between compartments, each of volume V, through a straight tube of cross section a and length h, the cell constant is given by:

$$\beta = 2a/hV \tag{3}$$

If h is set equal to the thickness of the membrane and D is taken from free diffusion data, a is given by:

$$a = \frac{2.303\ hV}{2\ Dt} \log \left[\frac{C_1^o}{C_1^o - 2C_2}\right] \tag{4}$$

V is the volume in each compartment and it is assumed its value does

not change in the experiment; this point will be discussed in the next section. The factor F is defined as the ratio of the effective area and the geometric area A of the membrane exposed to diffusion: $F = a/A$.

In Procedure I, the course of diffusion was followed by analyzing aliquots portions of the cell contents at intervals. The values of C_2 were plotted against time, the best straight line was drawn through the points, and its slope was determined. This can be related to the equation given above by the following considerations. For small values of $(2C_2/C_1^o)$ one can use the approximation $\ln(1 + x) \sim x$; to this approximation, equation (2) is transformed to

$$C_2 = \beta D C_1^o t/2 \qquad (5)$$

For values of $(2C_2/C_1^o) = 0.20$, i.e., 10% diffusion, the error caused by the approximation is about 10%. An appreciable portion of the solutions was withdrawn for sampling, 0.50 ml. from a total of 8.5 ml., and if no correction is made for this, up to 6% error is made. Since the two errors are in opposite directions, they cancel to some extent and it can be calculated that making no corrections will cause the slope to be 1.04 times greater than it should be. Accordingly, the slopes obtained by Procedure I were decreased by the factor 1.04 and then used to calculate a.

The temperature correction was made to 25° by means of the Stokes-Einstein equation (2), taking the viscosities of the solutions to be proportional to those of water. At 25°, $\eta = 0.8937$ centipoise, and

$$D_{25} = D_T(298/T)\,(\eta_T/\eta_{25}) = 333.4\, D_T(\eta_T/T).$$

where T is the temperature of the measurement in °K and η_T the viscosity of water at that temperature.

Calculations of the Time Required to Attain Steady-State Diffusion:

This quantity, t_{ss}, will be used in the discussion that follows. According to Longworth (67), it can be calculated approximately from the expression:

$$t_{ss} = 1.2\ h^2/D \qquad (6)$$

For p-aminobenzoic acid $D = 8.4 \times 10^{-6}$ cm.^{2}sec.$^{-1}$; if h is 0.0130 cm., t_{ss} is then 240 sec.

RESULTS AND DISCUSSION

Membrane Properties and Their Reproducibility:

The "Millipore" membranes used in this work presented a very uniform, smooth and clean appearance. Although it cannot be expected that they would all be exactly the same in internal structure, it was hopefully anticipated at the beginning of this work that microscopic differences would be effaced by averaging so that different membranes would give results reproducible within a range of, say, ± 5%.

The results obtained at the beginning of this work with a limited number of membranes seemed to conform with this expectation, although occasional exceptions were noted. These exceptions were at that time ascribed to uncontrolled experimental errors, and changes were then made in the apparatus and procedure to minimize such errors -- a more reliable stirring apparatus was devised, the cells were kept in a thermostat, sampling and measuring procedures were improved to less than 2% uncertainty. However, these changes gave no systematic improvement in the reproducibility of the experimental results; on the contrary, as a larger number of membranes was tested, even greater deviations from

the average were occasionally found. This led to the conclusion that differences in the membranes themselves were the major cause of the observed variation.

Table I summarizes the results obtained with fifty membranes, nominally of 10-mμ pore size. The results are expressed as the ratio $\underline{F}$ between the rate of diffusion observed and that calculated for free diffusion through a cylinder of cross section equal to the area of the membrane and of length equal to its thickness. The membranes are divided in groups of about 10 each, and the groups with the same Roman numeral were taken from the same box of filters as purchased from the manufacturer. It can be seen that the average deviation was 3-5%, but that the extreme difference within a group could be as great as 30%. Differences of essentially the same order of magnitude were found when a <u>single</u> membrane was used for repeated trials, usually 3. The results of 75 trials with 25 membranes were as follows: the average deviation of <u>each</u> trial from the <u>average value for the particular membrane</u>, was 5%, and the maximum deviation, the greatest difference found in the entire set as percent of the average value, was 40%. At first, the fact that the same membrane gave different values was taken as <u>prima facie</u> evidence that other uncontrolled sources of errors were operative. However, it will be naive to consider the membrane as having a uniform structure throughout; furthermore, it cannot be taken for granted that the structure of the membrane will remain unchanged with time (68).

Nearly all the data reported above were obtained with membranes that had been soaked for some time before the measurements. Some work also was done with initially dry membranes (see Experimental section, Procedure I). In these experiments, the absorbances due to diffused

TABLE I

VALUES OF "F" FOR 10-mμ MEMBRANES

Group	Number Tested	Cell	$F^* = a/A$ Max.	Min.	Avg. ± Avg. dev.	Max. difference, % of avg.
I-1A	10	A	0.264	0.230	0.248 ± 0.010	14
I-1B	10	B	0.234	0.205	0.220 ± 0.0083	14
I-1C	10	C	0.233	0.165	0.210 ± 0.0094	33
I-2C	10	C	0.207	0.164	0.192 ± 0.014	22
II-1B	10	B	0.190	0.165	0.177 ± 0.005	14
III-1D[b]	8	D	0.243	0.210	0.23 ± 0.011	14

*See Calculations section

[b]By Procedure I

p-aminobenzoic acid were determined at short intervals after the cell had been filled in the manner described. The results, when plotted as a function of time, extrapolated to zero concentration at zero time within the experimental uncertainty (which, admittedly, is rather large near the origin); this result is in conformance with equation (2) (see Calculation section). There are some advantages to starting with dry membranes, but this of course could be done only once. Since in the studies of protein diffusion it was thought desirable to do two measurements with the same membrane (vide infra), the applicability of Procedure I was not investigated beyond the points just discussed.

Area Available for Diffusion; Relation of Diffusion Measurements to Pore Size:

Table II summarizes the results obtained from measurements of the diffusion of p-aminobenzoic acid through 10, 50, and 100-mμ membranes. For the first three grades, about the same degree of variability was found, while the 450-mμ membranes had a larger average deviation.

It is very interesting to note that *F* has nearly the same value, 0.2, for 10, 50, and 100-mμ membranes, i.e., the area effectively available for diffusion of p-aminobenzoic acid molecules is nearly the same despite the difference in pore size. This finding supports the manufacturer's assertion that the membranes contain essentially the same proportion of free-space (70-74%) (73).

Diffusion of Proteins Through '10-mμ' Membranes:

In these experiments, the rate of diffusion of p-aminobenzoic acid through a particular membrane was measured and the value of *F* was

TABLE II

DIFFUSION OF p-AMINOBENZOIC ACID THROUGH MEMBRANES

Nominal Pore size, mμ	Cell	$F^* = a/A$		
		Max.	Min.	Average ± Avg. deviation
10	B	0.234	0.205	0.212 ± 0.008
50	B	0.256	0.228	0.235 ± 0.0120
100	B	0.219	0.180	0.209 ± 0.0094
450	B	0.428	0.222	0.321 ± 0.059

*20 determinations for each pore size; $\underline{h}$ = 0.0130 cm. for 10, 50, 100-mμ; 0.0150 for 450-mμ membranes (18).

determined as already explained. The procedure was then repeated for the protein solution, using the same membrane. The results are reported as the ratio $\underline{G}$ between the two $\underline{F}$ values

$$G = F_{protein}/F_{PAB} = a_{protein}/a_{PAB}$$

In the calculations, no correction was applied for the change in $\underline{V}$ consequent upon the osmotic transfer of water into the protein compartment (compartment 1), even though this was appreciable, ca. 5% in 10 hours; the correction is not sufficiently large to make a significant difference in the results. Table III summarizes the results obtained with lysozyme, β-lactoglobulin and BSA.

It is seen that, unfortunately, the precision of the results is not high. The average deviation is about 10% and the extreme differences found in a group of ten membranes was as great as 20%. Since initial differences in the area available for diffusion were corrected for on the basis of the p-aminobenzoic acid measurements, this variation reflects further differences in the membranes, e.g., changes in the structure that may take place during the measurements and differences in the distribution of pore sizes. However, the differences obtained by averaging the several observations clearly are significant; the area available to diffusion decreases as the molecular weight (and size) increases, from ~78% for lysozyme to 50% for BSA).

The last column of Table III gives values of $\underline{T}_{0.1}$, the time required for 10% diffusion, which can be compared with the results of the earlier investigation (64). The value for lysozyme compares with that reported earlier. However, the results for BSA is quite different; in the earlier experiments, less than 1% diffusion was found in 24 hr. To

TABLE III

DIFFUSION OF PROTEIN THROUGH MEMBRANES

Protein	M.W. $\times 10^{-3}$	$D \times 10^{7*}$ $cm.^2 sec.^{-1}$	No. of membranes	$G = F_{protein}/F_{PAB}$ Max.	Min.	Avg. ±Avg. deviation	$T_{0.1}$ hrs.	Cell
Lysozyme	14.4	10.7	20	0.858	0.693	0.775 ± 0.089	5.6	B
β-lacto-globulin	37.7	8.7	10	0.643	0.533	0.587 ± 0.065	7.25	B
Bovine Serum Albumin	69	6.8	10	0.515	0.481	0.503 ± 0.017	12.5	B

*From Reference (2) adjusted to 25° if necessary.

preclude the possibility that the present results might be due to the presence of a low molecular weight impurity, measurements were made with a different sample of BSA; although the results were less precise they agreed with those of the first sample. Also, diffusion was allowed to proceed to the extent of 20% and the rate for the second 10% was found to be comparable to that of the first. Thus considerable confidence can be placed in the present results. We can offer no explanation for the difference from the results reported earlier; either a mistake was made or the pores of the membranes used in the earlier work were substantially smaller. With reference to this possibility it might be added that the measurement of flow rate through '10-mμ' membranes indicate an effective pore size of some 72 mμ (Chap. IV). If this were even approximately correct, there would be no reason to expect that the pores would prevent the passage of BSA molecules; on the other hand, BSA molecules (4.04 mμ radius) would be completely barred from diffusion through '10-mμ' pores.

In some instances two or more measurements were done with a single membrane. No better agreement between measurements was achieved, but it is worthy of note that the variations were random and not consistently downward, such as would be caused by a gradual clogging of the membrane pores. The limited precision of the data do not permit a precise evaluation of this question, but it can be stated that clogging, if it occurs, has no more effect than other sources of variation in the membranes.

Attempted Diffusion of BSA Through Dialysis Membranes:

It seemed of interest to test, with the present apparatus and

technique, "Visking" dialysis membrane, which has much finer pores than the "10-mμ Millipore" membranes. A set of measurements accordingly was made with ten pieces of membrane that were cut from a single piece of tubing. The thickness of the membrane when dry was measured with a micrometer and found to be 0.00216 cm. Taking this as the value of $\underline{h}$ in equation (4), the experimental data gives for p-aminobenzoic acid a value of F = 0.022. This is much smaller than that for the "Millipore" membranes, i.e., the dialysis membrane is much "denser". However, it is also thinner, so that the times effectively required for diffusion are not proportionally greater; $\underline{T}_{0.1}$ is 45 min. as compared for 32 min. for the "Millipore" membrane. It is noteworthy that the average deviation found with the tubing was only about 2%, much less than for the "Millipore" membranes; the dialysis membrane seems to be a much "harder" material and this may be an important factor in determining the reproducibility of pore sizes.

When BSA was put in diffusion cell B with "Visking" membrane as diaphragm, less than 1% diffusion was found in 24 hours. This is in accordance with expectations, but serves as a check on the validity of the method.

Diffusion of Polystyrene Latex Through "Millipore" Membranes:

It also seemed desirable to determine whether "Millipore" membranes would prevent the passage of particles larger than those of BSA. The material investigated was polystyrene latex, which contains spherical particles of 88-mμ diameter, with a standard deviation of ± 8-mμ; these dimensions have been determined by electron microscopy. The first trial was made with membranes of 100-mμ nominal pore size and it

was found that less than 1% diffusion took place in 24 hrs. Measurements were then made with membranes of 450 and 800-mμ nominal pore size. The average of the ten measurements with 450-mμ membranes gave an F value of 0.128 and a G value of 0.389; with 800-mμ membranes the F value was 0.208 (the diffusion of p-aminobenzoic acid through these membranes was not measured and therefore no G value can be quoted).

A noteworthy point of difference between these experiments and those with proteins was that, when repeated diffusion measurements were made with the same membranes, a systematic decrease in the value of G was noted, which might result from clogging of the membrane. Confirmatory evidence for such an effect was afforded by the fact that the sum of the absorbances in the two compartments at the end of the experiment was less than that orginally taken; it should be added that the cencentrations of latex used in these experiments was only 0.1%, and that sequestration of a fraction of the latex from solution would accordingly be more noticeable than in the protein experiments.

CONCLUSIONS

A comparatively simple apparatus and technique have been presented, for studying the process of diffusion through porous barriers. A cell has been described in which an accurately defined and controllable area of the barrier material is exposed; this facilitates comparisons and makes possible a more searching interpretation of the results.

With "Millipore" membranes as diaphragm, the time required for diffusion measurements is greatly shortened in comparison to cells of conventional design, in which diffusion occurs through a thick, fixed diaphragm. Craig's "thin-film" technique (16) affords even faster

diffusion rates, but the apparatus does not lend itself as readily to a study and evaluation of the membrane material.

The following characteristics of "Millipore" membranes have been ascertained in this work:

(a) membranes of various grades, from 10 to 100-mμ nominal pore size, have about the same proportion of free space available for the diffusion of small molecules; diffusion is only about one-fourth slower than <u>free</u> diffusion through a layer of the same area and thickness;

(b) individual membranes show random differences as great as 30% and averaging 5%, which limits their effective application to <u>quantitative</u> studies of diffusion, at least for molecules of molecular weight 10^4 - 10^5; however, significant results can be obtained by averaging a sufficient number of observations.

With respect to the characteristics of the diffusing particles, it has been possible to demonstrate that 100-mμ "Millipore" membranes effectively prevent the passage of 88-mμ polystyrene latex, and that Visking dialysis tubing prevents the passage of BSA. As the particles become smaller with respect to the pore size, there is a <u>gradual</u> increase in the area available for diffusion; this leads one to expect that there will not be a sharp "cut-off" of diffusion at a particular molecular size.

The present work was originally motivated by the desire to develop a novel method of investigating molecular sizes and shapes. It has not so far been possible to obtain precise information about these matters by studies of hindered diffusion, and at the present time it seems doubtful that the method could be perfected to give results of truly satisfactory precision. However, it is hoped that the results

described will serve as a basis for (i) further studies of membrane materials; (ii) "rough-but-ready" determinations of molecular size; (iii) analytical and preparative separation of molecules of widely different sizes. Investigations along these lines are in progress.

CHAPTER IV

PORE SIZE OF "MILLIPORE" FILTERS

(This paper has been written in a form suitable for publication in a journal).

The rate of flow of water through membranes has been employed to obtain information about the size of the pores in the membranes and about variations in the membranes characteristics.

The suggestion that the porosity of a membrane might be determined by measuring the rate of flow of liquid through it was first made in 1872 by Guérout (12). Since then this method has been used frequently to determine the porosities of various types of beds as well as membranes (13,14,27,68,69). Other techniques have since been devised for the same purpose: mercury intrusion (28); capillary rise (10,26,70); vapour pressure (71) etc. The flow-rate method is the simplest and easier to apply, however.

This chapter describes the application of the method to "Millipore" filters. A very simple apparatus is described to determine this flow rate. 'Millipore' filters of pore sizes 10, 50 and 100 millimicron (mμ) were examined. The rates of flow of water through these filters were found to vary with an average deviation of 10-14% and an extreme deviation of about 40%. The results of the present work indicate that the pore size of "10 mμ" filters is actually 72 mμ; that in 50 -, and 100 - mμ filters, is about twice the nominal value. It is found that

the two sides of the '10 mμ' membrane, bright and dull, are different with respect to the change of flow rate of water with time.

Experimental

"Millipore" filters were purchased from the 'Millipore' Filter Corporation, Bedford, Massachusetts.

The stainless steel cell with 'window' area 2.390 cm^2 (described in Chapter III) was used in this study with one modification: A lucite disc was made which covered the top of one compartment of the cell, making a water- and air-tight seal. A glass tube, with inner diameter 0.20 cm, was sealed vertically through the cover so that it protruded 3 cm below and about 16 cm above the cover. Two marks, on the glass tube, at 13.75 and 15 cm above the cover, were marked with glass-marking pencil.

The two compartments of the cell were assembled so that the filter was sandwiched between them. Both the compartments were filled with water to the brim and the assembly was then placed in a water bath with the glass tube vertical. The temperature of the bath was maintained at 25 ± 0.1°C. After about 15 minutes, some water was withdrawn from the open compartment with a syringe and added to the other compartment through the top of the tube until the meniscus of the water column was above the top mark. The time required for the meniscus to cross the distance between the two marks was then determined.

Calculations

The flow φ across the membrane is the volume v passing in time t. This volume can be calculated from the decrease in the height of the

water column, Δm, that supplies the hydrostatic pressure, and from the radius of column, r. It is assumed that the flow is proportional to the pressure P, and since Δm is small, it is sufficiently accurate to consider the pressure constant. The flow takes place across the area A of the cell "window"; and the flow per unit area is therefore given by

$$\frac{\varphi}{A} = \frac{V}{At} = \frac{\pi r^2 \Delta m}{At} = KP. \qquad (1)$$

The proportionality constant K is the volume of the liquid which flows per unit area in unit time under unit pressure. A representative set of data obtained with a "10-mμ" membrane was as follows: $\Delta m = 1.25$ cm; $r = 0.10$ cm; $A = 2.39$ cm^2 and $P = 14.4$ cm of water or 10.6 torr; the average time was 1.93 minutes and

$$K = \frac{\pi r^2 \Delta m}{APt} \qquad (2)$$

$$= 0.000803 \text{ ml cm}^{-2} \text{ min}^{-1} \text{ torr}^{-1}$$

The flow under 70.0 cm of mercury pressure would then be 0.562.

Results and Discussion

The membranes used in this work were taken from the same lot which had been used earlier (Chapter III). They were smooth, uniform and clean in appearance. A comparison of different membranes could be made very simply by timing the passage of a certain volume of water through the filter, which in turn corresponded to a specified decrease in the height of the hydrostatic head. Repeated measurements could be taken within a short period of time, and the reproducibility was of the order of 2%. Table IV summarizes the results obtained with twenty "10-mμ" membranes, which were all taken from the same box. It can be

TABLE IV

RATE OF WATER FLOW THROUGH MEMBRANES

		Nominal pore size of membranes		
		10-mμ	50-mμ	100-mμ
(1)	No. of membranes examined	20	20	20
(2)	Flow times, sec.			
	(a) maximum	150	54	25
	(b) minimum	97	30	21
	(c) average ± avg. deviation	116 ± 14	40.7 ± 8.5	22.0 ± 1.2
	(d) maximum difference, % of average	46	60	18
(3)	Rate of flow			
	(a) k from eq. (2) x 10^4, ml.cm.$^{-2}$min^{-1} (torr)$^{-1}$	8.0	22.9	42
	(b) ml.cm.$^{-2}$ min.$^{-1}$ under 700 torr pressure	0.56	1.59	2.93
	(c) Approx. manufacturer spec.	0.5	1.5	3.0

seen that the average deviation and the difference between the extreme are even greater than the corresponding values found in the diffusion measurements. This provides confirmatory evidence that differences in the membranes themselves are the principal cause for the variation in the results. Illani (72) also reported deviations of the order of 25% in his determinations of the potassium ion flux through bromobenzene-saturated "Millipore" filters.

It should be noted that the average value for the rate of flow of water found in the present work corresponds quite closely with the approximate specifications given by the manufacturer (73).

Measurements made at intervals of time confirmed the suspicion that the structure of the membrane might change on prolonged contact with water. Measurements were made on a membrane 15 minutes after it had first been soaked, and at 1- or 2-hour intervals thereafter, for 8 to 10 hours. The changes found with different membranes were variable; but always appreciable--typically 20% of the original value in 8 hours. These variations are in line with those observed by Bartell and Osterhoff (68) with their membranes. Bartell (74) attributed this decrease in permeability to mechanical clogging of the pores by very fine particles. Whereas this possibility cannot be excluded in our case, it may also be that hydration of the cellulose material causes structural alterations.

An interesting finding was made in these experiments, namely that when the shiny side of the membrane faced the pressure head, the flow rate changed considerably faster than when the dull side was facing the pressure head. In the protein measurements, no appreciable difference was found between the shiny and dull sides. These experiments indicate

that the passage of water and proteins do not depend on the same membrane characteristics; but there can be little doubt that both processes depend on the structure of the membrane, and are affected by the changes in it.

Relationship Between Pore Size and the Rate of Flow of Water:

Ferry (21) has given an equation relating the flow of water through membranes having very fine capillaries of radius $\bar{\rho}$, based on the following assumptions:

(a) Poiseuille's law governs the flow rate through capillaries;

(b) The capillaries are parallel to one another and perpendicular to the membrane structure;

(c) The total volume of pores represents the total effective volume in filtration i.e. (i) there are no blind channels; (ii) there is no appreciable immobilized layer of water lining the pore walls. The equation is:

$$\bar{\rho} = \left(\frac{8v\eta h}{SAPt}\right)^{\frac{1}{2}} \qquad (3)$$

where η is the viscosity of water and S is the specific water content of the membrane, and h is the length of the capillary.

Combining equations (1) and (3), we get

$$\bar{\rho} = \left(\frac{8\eta\, hK}{S}\right)^{\frac{1}{2}} \qquad (4)$$

The results obtained by setting h equal to the thickness of the membrane, an assumption which will be discussed later, and S to the porosity of the membrane as given in the manufacturer's specifications (73), are $\bar{\rho}$ = 36 mμ for "10-mμ" filters; 60 mμ for "50-mμ" and 90 mμ for "100-mμ" filters. (Note that pore size is $2\bar{\rho}$).

The validity of equation (4) i.e. Poiseuille's law, for determining

the pore size of the membrane is subject to the value of viscosity of water inside the membrane structure and the actual pore length.

Bartell and Osterhoff (68) compared the results of Poiseuille's and capillary rise technique, obtained in carbon beds of approximate pore size 90 mμ. The results were in fair agreement. They estimated that 12-15 mμ would be the lower limit at which Poiseuille's law could be applicable, assuming that the viscosity of water inside the pores is the same as in the bulk volume.

Terzaghi (75) estimated that the viscosity of water η' inside a pore would increase according to the equation:

$$\eta' = \eta \left(1 + \frac{6.02 \times 10^{-42}}{\rho^8}\right) \text{ to } \eta \left(1 + \frac{2.42 \times 10^{-43}}{\rho^8}\right)$$

where η is the normal viscosity of water and ρ is the radius of the pore. According to this equation, water would have such a high viscosity inside a 10-mμ pore, that the flow would be much smaller than is observed. On the other hand, essentially normal values of the viscosity would be obtained if ρ was 100-mμ or greater.

Recently Longuet-Higgins and Austin (32) deduced from statistical mechanics that the flow of water through pores as small as 9.0Å diameter would follow Poiseuille's law.

Guérout (12) investigated beds of sand and of asbestos filaments. The pore radius ρ for the latter, as determined by microscopic examination, was found to be in fair agreement with that calculated from rate of water flow (0.00283 cm and 0.00276 cm respectively). The flow rate through sand beds gave ρ as 0.71 times the value obtained from microscopic data (0.000795 cm and 0.00112 cm respectively). Bartell and Osterhoff (68) assumed the pore length of their carbon and silica beds

to be ($\pi/2$) times the bed thickness, but stated that the pore length of collodion type membranes is equal to their thickness. On the contrary Elford and Ferry (30) suggested that the actual pore length of these membranes is a little less than twice their thickness; whereas Bechhold (29) believed that his collodion membranes had pore length three to four times the thickness.

Carman (69) reviewed this problem for granular beds and concluded that the actual pore length is some multiple, ($\pi/2$ or $\sqrt{\pi}$), depending upon the bed type, of their thickness. A similar theoretical analysis has not been made for cellulose filters. For membranes of pore size below 20-mμ, the actual pore length is likely to be larger than their thickness (21). For a given flow rate, therefore, substitution of membrane thickness for h in equation (4) will give a smaller value for $\bar{\rho}$. In case of "Millipore" filters of nominal pore size '10-mμ', when the pore length, h, is considered to be equal to the membrane thickness, the value of $\bar{\rho}$ calculated from equation (4), comes out to be six to seven times the manufacturer's value.

This indicates that the model used as the basis of equation (4) is probably inadequate, and one cannot draw definite conclusions concerning the size of the pores in the membranes. However, they are undoubtedly larger than nominal size. Evidence of this is provided by the fact that bovine serum albumin molecules (4.04 mμ radius) (17) diffuse reasonably rapidly through the so called 10-mμ "Millipore" filters, whereas they would be all but completely barred from passing through 10-mμ pores as is indicated by the following equation given by Renkin (76) to account for steric and frictional hindrance to diffusion:

$$A/A_o = (1 - \frac{a}{r})^2 \left[1 - 2.104 \left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^2 - 0.95 \left(\frac{a}{r}\right)^5\right] \qquad (5)$$

where a is the radius of the particle diffusing through a pore of radius r; A_o and A are the apparent and actual membrane area available for diffusion. Substituting for a = 4.04 mμ and r = 5-mμ, we get $\frac{A}{A_o} \cong 0.034$ i.e. bovine serum albumin will be severely restricted by 10-mμ pores.

CHAPTER V

STUDIES ON THE DISSOCIATION OF UREASE BY HINDERED DIFFUSION THROUGH MEMBRANES AND INTO SEPHADEX GEL

(This paper is written in a form suitable for publication in a journal).

SUMMARY

Urease at 0.05 mg/ml concentration can penetrate Sephadex gel G-100 while Sephadex G-75 completely excludes it. It is concluded that urease is dissociated to some extent into a subunit of molecular weight about 80,000; for reasons that will be discussed, the extent of dissociation cannot be deduced. The diffusion of urease through 10-mμ Millipore membranes was measured also and the results confirm the conclusions of the gel-diffusion experiments. For comparison, measurements were also made with serum albumin, some amino acids, and salt solutions.

INTRODUCTION

Passage through columns of Sephadex gel has been used to separate simple mixtures of components of different molecular weights and size, that do not interact with one another. If rapid association-dissociation occurs, the phenomena observed are more complicated. Gilbert (58) has discussed the possibility of studying such a system. Winzor and Scheraga (59) have applied the method of gel filtration to study α-chymotrypsin.

In the present study, passing urease through Sephadex gel column gave equivocal results, possibly due to the occurance of absorption. In order to minimize this possibility, experiments have been done by mixing the solution with an appropriate amount of dry Sephadex and separating the gel phase from the supernatant after some time. This procedure will be called Sephadex dialysis. It has the additional advantage that the contact time can be lengthened at will, thus avoiding the problem that might arise in flow experiments because of failure to establish equilibrium.

Urease was also investigated with respect to its ability to pass through Millipore membranes, of 10-mμ nominal pore size. The results of these hindered diffusion studies are in qualitative agreement with those of the Sephadex dialysis.

MATERIAL AND METHODS

Materials:

Urea; p-aminobenzoic acid (Fisher Scientific Co. N.J.); L-tyrosine; DL-tryptophan (California Corp. for Biochem. Research, L.A.); bovine serum albumin (Pentex Corporation; Kankakee, Ill.); $CuSO_4$; $CuCl_2 \cdot 2H_2O$; $CrCl_3 \cdot 6H_2O$ and K_2CrO_4 were used without any further purification. Urease was prepared from Jack beans by the method of Gorin et al (78).

Millipore membranes of 10 ±2mμ nominal pore size were purchased from the Millipore Filter Corporation, Mass; and Sephadex G-100 (lot no. JO 5967; particle size 40-120μ) and G-75 (lot no. JO 2393; particle size 100-270 mesh) were obtained from Pharmacia, Uppsala, Sweden.

Experimental:

a) Determination of Water Regain by Sephadex:

Dry Sephadex, 50 mg, was weighed into a test tube, 0.84 x 2.82 cm, and 1.30 ml of water was added. The contents were shaken and the tube was placed in a water bath at 25 ± 0.1 C°. The sample was shaken after every 30 minutes, except during the night. After 72 hrs, the contents were transfered to a filter-centrifuge tube (no. 1199 International Equipment Co.) This tube consists of two compartments separated by a sieve, on which is placed an appropriate filter. In the present study, Millipore membranes of pore size 100-mμ were used. The tube and contents were centrifuged for about 10 minutes at 3000 rpm. Water was filtered through the membrane into the lower compartment, leaving a 'wet' non-sticking Sephadex bead on top of the filter. This could be then transfered directly to the balance pan and weighed. The water regain was determined by difference.

b) Sephadex Dialysis Technique:

Dry Sephadex, 50 mg, was weighed into a vial of 1.5 ml capacity; and 1.3 ml of test solution was added. The vial was capped; and placed in a bath at 25 ± 0.1 C°. The vials were shaken at 15 min. intervals for 5-6 hours. No significant difference was found for identically prepared samples when equilibration was allowed to proceed for 5, 10, 24 hours. In subsequent experiments, therefore, equilibration time was limited to 5 to 6 hours. The contents were centrifuged as described earlier. The filtrate was assayed spectrophotometrically: p-aminobenzoic acid at 273 mμ; DL-tryptophan at 278 mμ; bovine serum albumin and L-tyrosine at 275 mμ; and Methyl red at 450 mμ. Dextrose was determined by the Nelson test (77). Urease in both the filtrate

and the Sephadex phase was assayed according to Gorin et al (78).

c) Hindered Diffusion Through Membranes:

Three lucite micro-cells were constructed. Figure 3 shows an exploded view of them. Each compartment held about 0.5 ml of solution or solvent. A piece of '10-mμ' membrane was sandwiched between the two compartments. Silicone rubber 'clear seal' (General Electric, N.Y.) was applied over the joints outside the cell, and left to dry overnight.

The two sides were filled with 0.02M phosphate buffer pH7.0, containing 1.0×10^{-3} M EDTA, with the help of hypodermic syringe, and left for 3 to 4 hours. The buffer was taken out from one side with the syringe. This side was marked as solution side; and was rinsed with the test solution. It was then filled with the test solution. This whole procedure did not take more than 60 seconds. The other two cells were filled likewise; and all the three cells were then suspended for an appropriate time in a water bath, at 25 ± 0.1 C°.

With each membrane piece an experiment was first conducted with 0.4% p-aminobenzoic acid; then a determination was made with urease at the desired concentration, 0.16 to 1.9 mg/ml. In certain cases, 0.1% bovine serum albumin was diffused afterwards and an additional p-aminobenzoic acid run was done at the end. The two compartments were rinsed with solvent after every run.

CALCULATIONS

Since the solute distributes itself between the Sephadex and the solvent phases, we have therefore the following conservation equation:

$$S = S_i + S_e \qquad (1)$$

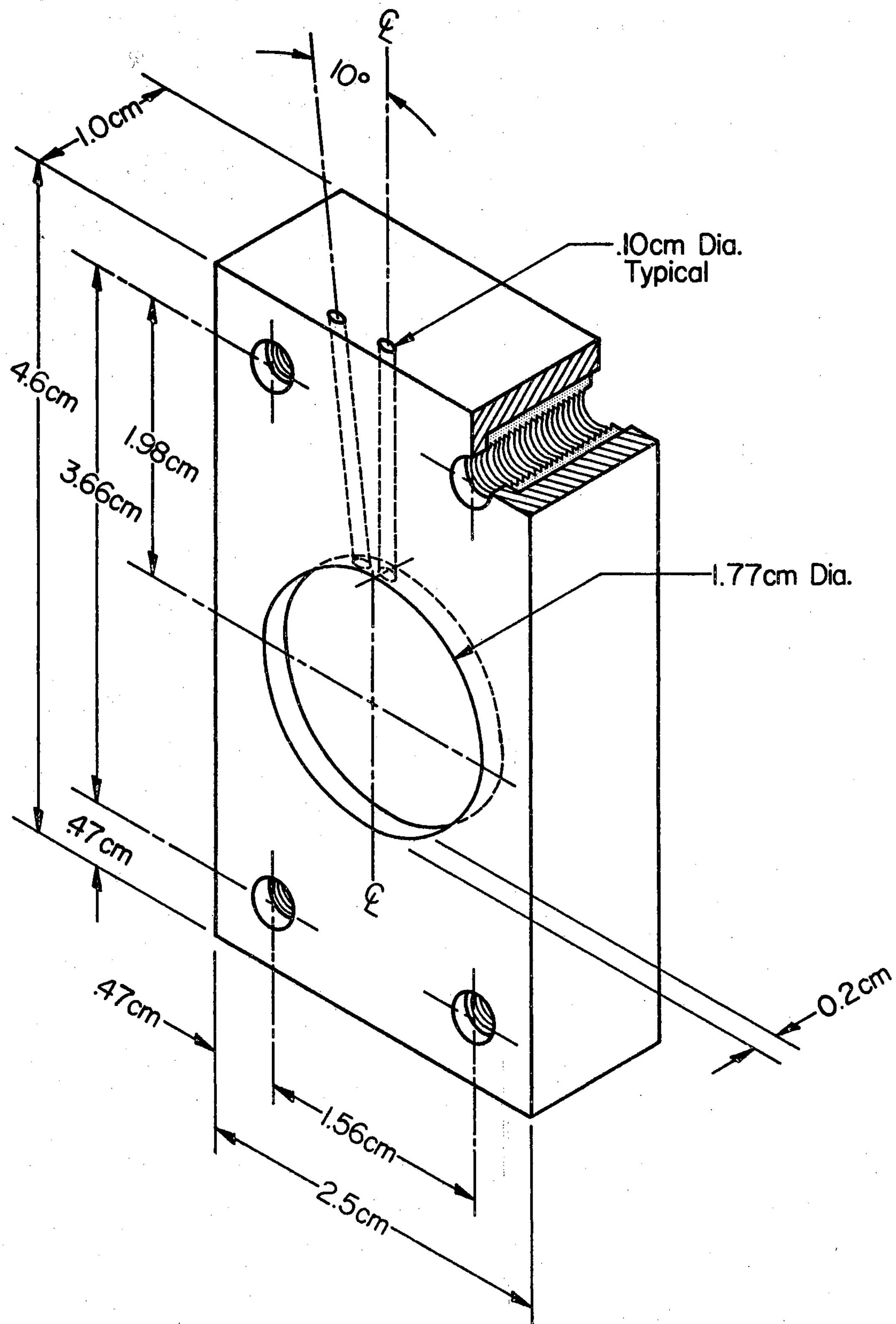


Figure 3. Schematic Diagram of Micro-Diffusion Cell

where $\underline{S}$ is the total amount of solute in the system; $\underline{S_i}$ and $\underline{S_e}$ represent the total amounts of the solute in the Sephadex (internal) and the solvent (external) phases respectively. It is further defined that

$$[S_i] = S_i/V_i \qquad (2a)$$

and

$$[S_e] = S_e/V_e \qquad (2b)$$

where $\underline{V_i}$ and $\underline{V_e}$ represent the volumes of solvent inside and outside the Sephadex phase. $[S_i]$ and $[S_e]$ are the concentrations in the respective phases. $\underline{S}$ and $[S_e]$ can be measured directly, and $[S_i]$ is determined by difference.

Ackers (79) has given the following equation which describes an equilibrium state between the two phases:

$$K_d = \frac{S - V_e\,[S_e]}{[S_i]\,V_i} \qquad (3)$$

where $\underline{K_d}$ is defined as the fraction of the interior volume of Sephadex available for distribution of the solute. $\underline{K_d}$ in equation (3) may be regarded as a distribution coefficient for the solute and equation (3) is rewritten as:

$$K_d = \frac{[S_i]}{[S_e]} \qquad (4)$$

The value of $\underline{K_d}$ will be $\underline{0}$ when the molecule is completely excluded from the Sephadex phase, and $\underline{1}$ in the case it diffuses freely with no restriction. It may be greater than one if there is absorption and/or some additional reaction such as an association-dissociation process.

In the case of association-dissociation i.e. when the molecular species in the two phases is different, equation (4) may be written in a modified form. Say a substance $\underline{A_n}$ undergoes molecular change inside the Sephadex phase and gives $\underline{A_m}$ i.e.

$$\underset{\text{aqueous}}{A_n} \overset{K}{\rightleftharpoons} \frac{n}{m}\underset{\text{Sephadex}}{A_m} \qquad (5)$$

The equilibrium constant

$$K = \frac{[A_m]^{n/m}}{[A_n]} \qquad (6)$$

If we assume that a specific property, such as absorbance or activity, of the species remains unchanged irrespective of the transformations, the terms $[A_m]$ and $[A_n]$ can be related with $[S_i]$ and $[S_e]$ by

$$[A_m] = \frac{n}{m}[S_i] \qquad (7a)$$

$$[A_n] = [S_e] \qquad (7b)$$

combining equations (6), (7a) and (7b), we get

$$K = \frac{\left(\frac{n}{m}[S_i]\right)^{n/m}}{[S_e]} \qquad (8)$$

Taking logarithm of both sides and rearranging the terms, we get

$$\log [S_i] = \frac{m}{n} \log K - \log \left(\frac{n}{m}\right) + \frac{m}{n} \log [S_e] \qquad (9)$$

The slope of the plot of $\log [S_i]$ vs $\log [S_e]$ will give the value of $\frac{m}{n}$, and from the intercept of the plot the value of K can be calculated.

RESULTS

Gel Dialysis:

The water regain for Sephadex G-100 and G-75 was found to be 10 ± 0.5 and 8 ± 0.2 ml per gram of dry gel, respectively.

It was found that urease does not penetrate G-75 gel, whereas it is retained to a considerable extent by the more sparsely crosslinked G-100 structure. According to Fig. (4), the degree of penetration for the enzyme increases up to 1.65×10^{-6} M which seems to be the saturation limit; the amount of enzyme retained by the gel structure at higher concentrations remains constant.

It was also found that urease at 1.73×10^{-7} M concentration and containing 0.001 M EDTA, had lost 38% activity during two hours; but

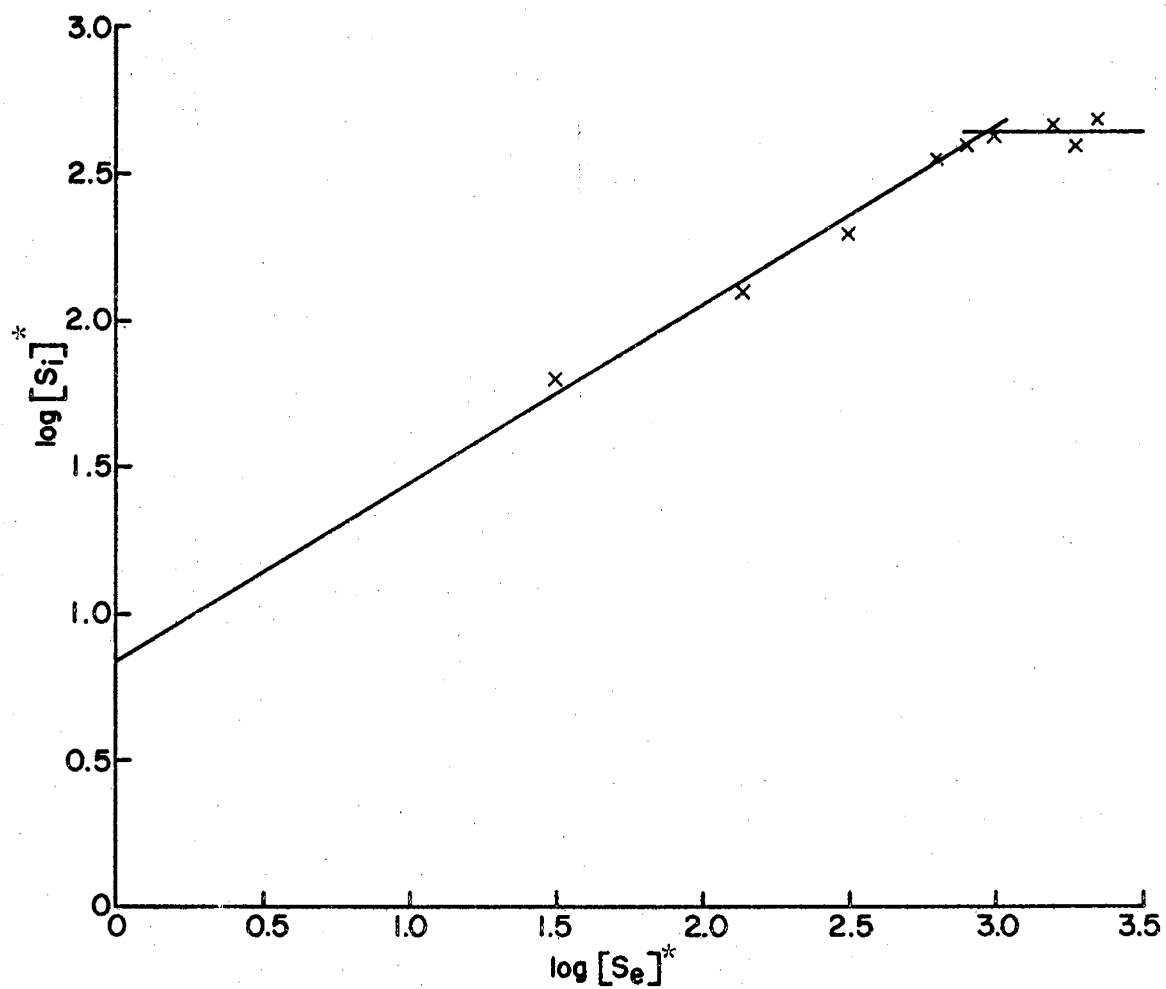


Figure 4. Log $[S_e]$ vs. log $[S_i]$ Plot for Diffusion of Urease into Sephadex gel G-100

*Concentration in (mg/ml) x 10^3

when EDTA content was raised to 0.003 M, full urease activity was restored.

Dextrose and p-Aminobenzoic acid distribute evenly between the two phases; also the inorganic salts tested exhibited normal distribution; on the other hand, L-tyrosine, DL-tryptophan and methyl red entered Sephadex to an extent greater than normal as indicated by figs. (5), (6) and (7). The values of $\frac{m}{n}$, table V, indicate that L-tyrosine, DL-tryptophan and urease may undergo molecular changes in Sephadex phase.

An apparent increase in the concentration of solute in the aqueous phase occured, for inorganic salts as well as amino acids, at very low concentrations. This may be attributed to the presence of impurities introduced during the manipulation; at moderate or higher concentrations, the same impurities have a negligible effect on the results.

Diffusion Through Membranes:

p-Aminobenzoic acid diffused to the extent of 25% in 15 minutes and bovine serum albumin diffused to the extent of 21-24% in 2 hours.

Urease samples exhibited an increase in the extent of diffusion with increasing dilution. Extrapolation of the plot for log (percentage diffusion) vs concentration, fig. (8) gives a value of 26%, which is comparable to values found for bovine serum albumin.

DISCUSSION

Table V shows that many substances show abnormal behaviour in the Sephadex-water system; methyl red distributes itself preferentially in Sephadex phase but still retains its molecular entity, whereas DL-tryptophan, L-tyrosine and urease show molecular changes.

L-tyrosine and DL-trytophan seem to undergo dimerisation in the Sephadex cavities. This might involve hydrogen bonding:

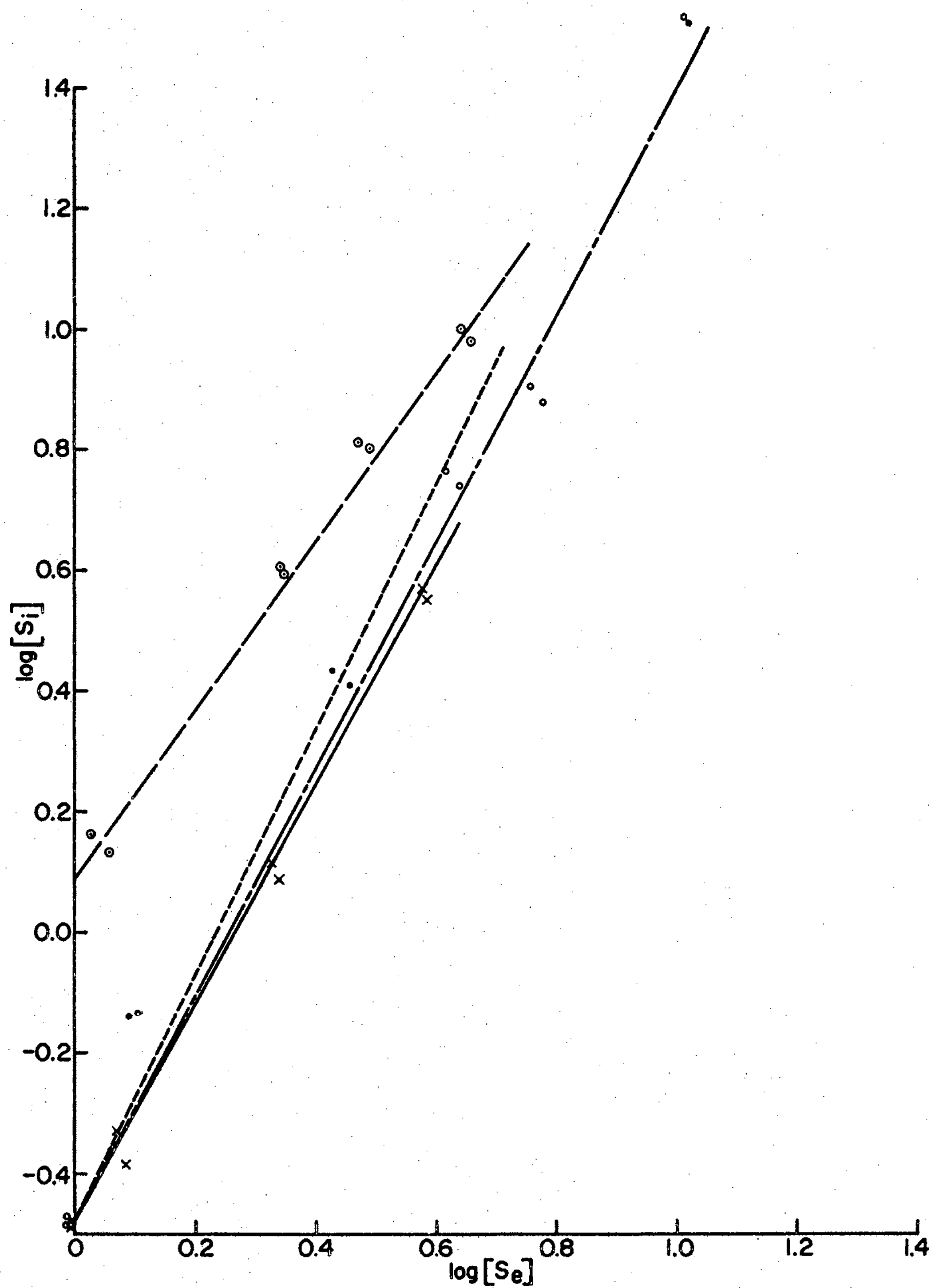


Figure 5. log $[S_e]$ vs. log $[S_i]$ Plot for Diffusion of xx - L-Tyrosine (0.1 M NaCl), .. - L-Tyrosine (0.0M NaCl), ⊙⊙⊙⊙ - DL-Tryptophan (0.1 M NaCl), ooo- DL-Tryptophan (0.0 M NaCl) into Sephadex gel G-100.

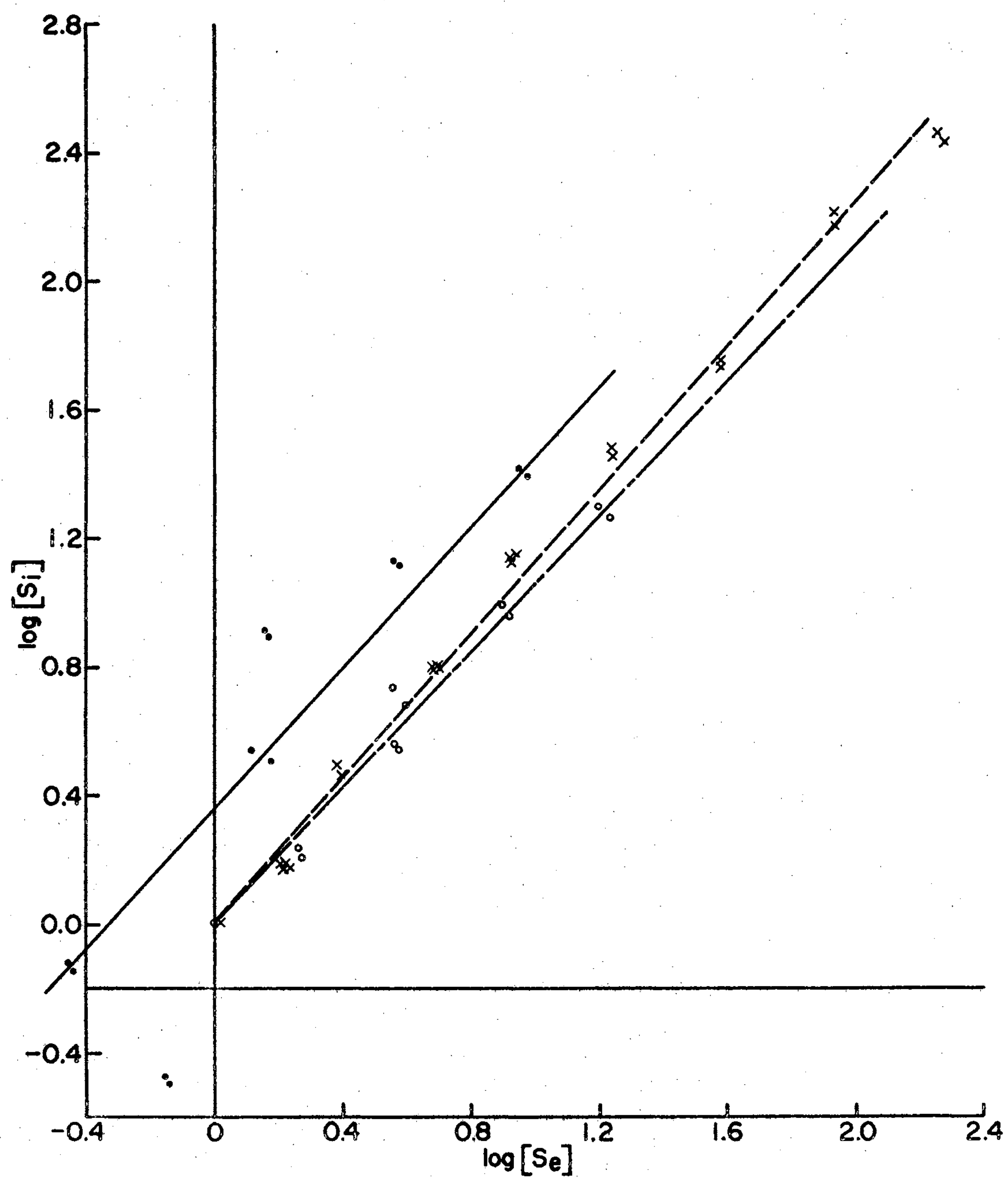


Figure 6. Log $[S_e]$ vs. log $[S_i]$ Plot for Diffusion of xx - p-aminobenzoic acid, . . . - Methyl red, ooo - dextrose into Sephadex gel G-100.

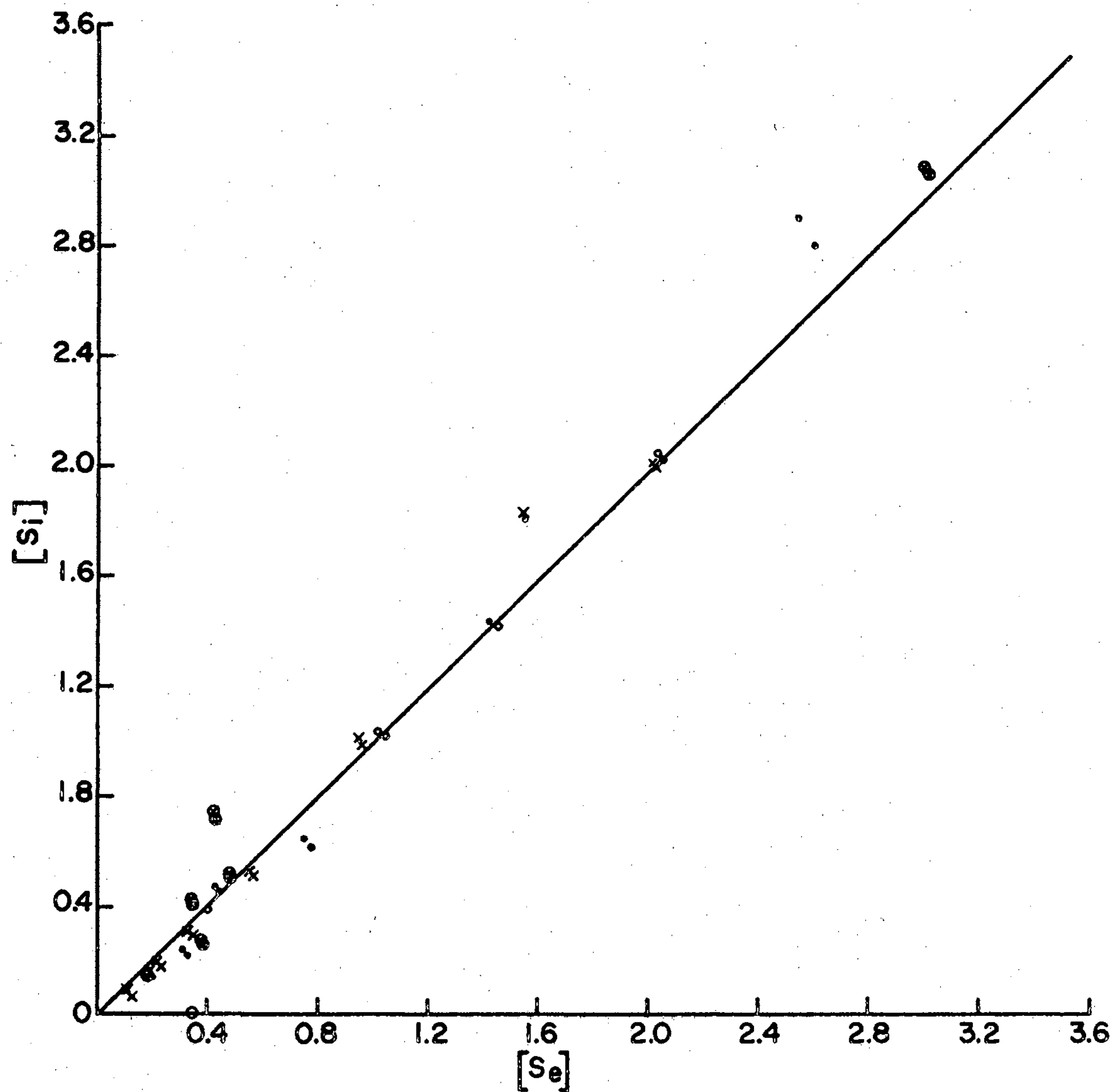


Figure 7. [S_e] vs. [S_i] Plot for Diffusion of xx - $CuSO_4$, ⊗⊗⊗ - $CrCl_3 \cdot 6H_2O$, ooo - K_2CrO_4, ····- $CuCl_2 \cdot 2H_2O$ into Sephadex gel G-100.

TABLE V

SEPHADEX DIALYSIS DATA*

Compound	K	m/n	Molecular Weight in Liquid Phase	Molecular Weight in Sephadex Phase
p-aminobenzoic acid	1	1	137	137
Dextrose	1	1	180	180
Methyl red	2.3	1	269	269
L-tyrosine, 0.1M NaCl	0.1	~2	181	362
L-tyrosine, no NaCl	0.1	2	181	362
DL-tryptophan, 0.1M NaCl	0.31	2	204	408
DL-tryptophan, no NaCl	0.1	2	204	408
Urease	12	0.6	480,000	240,000

*According to Equation (7) for Sephadex gel G-100.

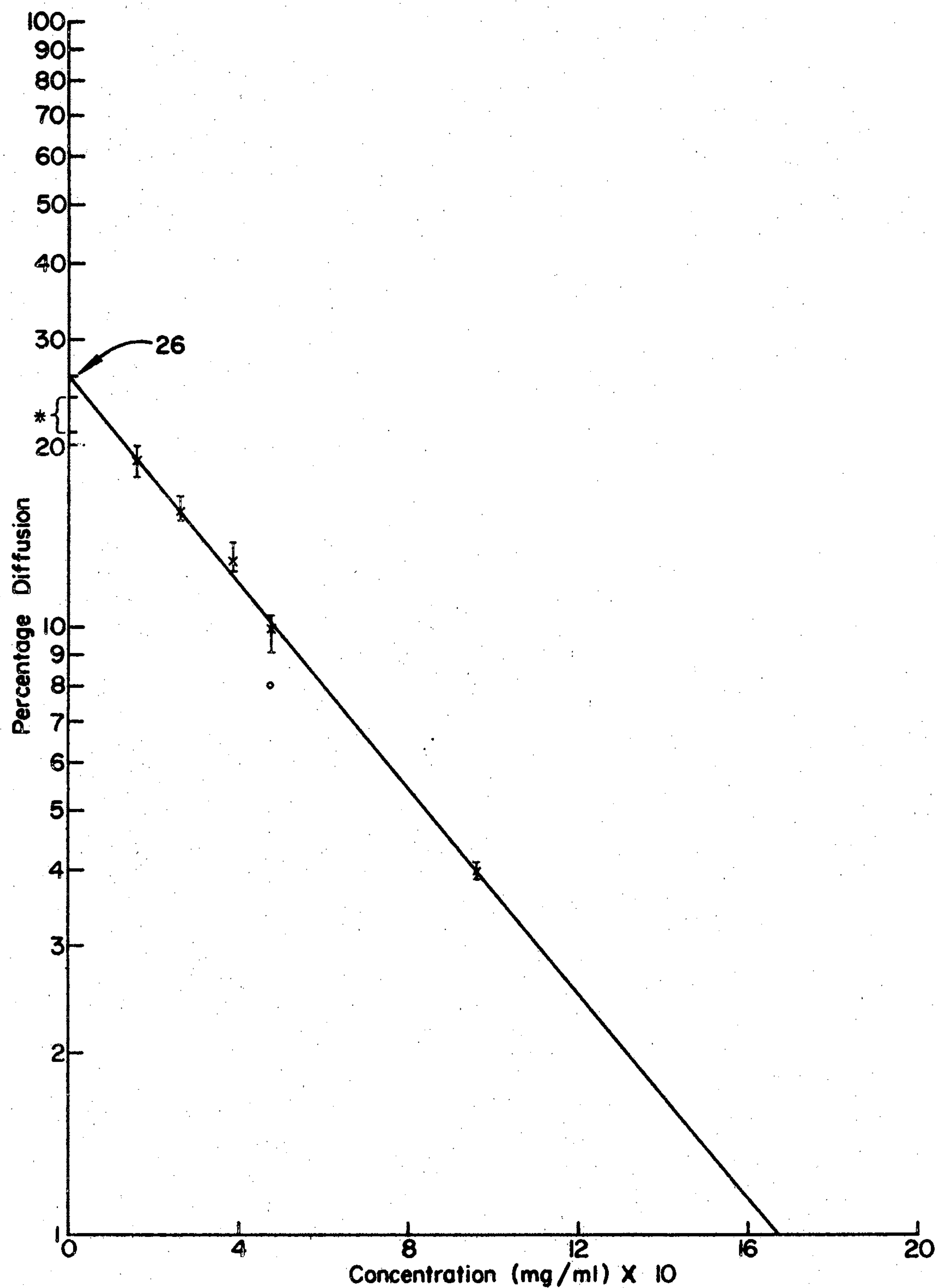
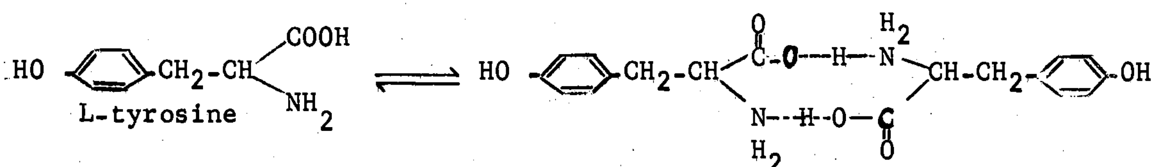
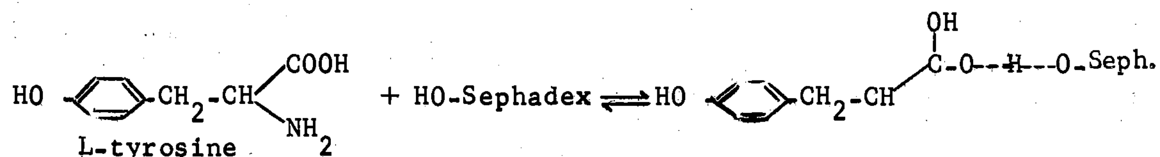


Figure 8. Concentration vs. log (percentage diffusion) Plot for Diffusion of Urease Through 10-mμ Millipore Membranes.

*Range for bovine serum albumin runs through 10-mμ Millipore Membrane.



Alternately, the abnormal distribution might be due to interaction with the Sephadex:



Similar equations can be written for DL-tryptophan.

The enzyme urease, molecular weight 480,000, is excluded completely from G-75 (exclusion limit 75,000), but it is retained to a certain degree, which depends upon the sample concentration up to 1.65 x 10^{-6} mM/ml, by G-100 gel. The cavities of this gel have an exclusion limit of 150,000. This indicates that a subunit with molecular weight larger than 75,000 but smaller than 150,000 is present in the test solutions. If one plots this data according to equation (9), one obtains $\frac{m}{n}$= 0.61, and this indicates the presence of a species, with 240,000 molecular weight, inside the G-100 cavities.

Equation (9) would not be applicable if absorption process occurs and this cannot be ruled out at the present time. This would change the quantitative results. However, it would not alter the qualitative conclusion that subunit with an approximate molecular weight of 80,000 is present in the solutions of urease tested.

Diffusion Through Membranes:

Diffusion through a barrier has been used by various workers for the identification of macromolecules (80,81). This technique, used in

conjuction with barriers that have pores of molecular size, may prove of some use in the study of association-dissociation processes.

We observed earlier (Chapter III), that Millipore membranes of '10-mμ' nominal pore size, by restricting the diffusion rate of 'heavy' molecules, can separate them from low molecular weight species. Figure (8) shows that the amount of urease, which diffused through these membranes increased with decreasing enzyme concentration. Extrapolation of the values obtained after 2 hours diffusion give the value 26%; This is comparable with the diffusion of bovine serum albumin (molecular weight 69,000), 21 to 24%.

These results indicate that i) a low molecular weight unit is present in the solutions; ii) more of this subunit is formed with decreasing urease concentration; and iii) the subunit has an approximate molecular weight of 80,000.

General Remarks:

Creeth and Nichol (82) reported the presence of urease species with sedimentation constants 19, 28 and 36 from their ultra centrifuge data; which were latter identified by Siegel and Monty (83) by means of Sephadex gel column. Sheppard (84) mentioned the presence of 6.2S subunit. Huber (85) and Setlow (86) observed a 100,000 molecular weight unit by means of electron and deutron bombardment techniques. Reithel et al (87) dissociated the native enzyme into 6 units by 6M guanidine hydrochloride; whereas Chen (88) showed the presence of a 4.5S subunit, corresponding to 83,000 molecular weight, in 0.1M acetate buffer of pH 3.1.

BIBLIOGRAPHY

(1) Tuwiner, S. B., "Diffusion and Membrane Technology", Rinehold Publishing Corporation, New York 1962.

(2) Gosting, L. J., Adv. Protein Chem. 11, 429 (1955).

(3) Jost, W., "Diffusion in Solids, Liquids, Gases", Academic Press, Inc., Publishers, New York 1960.

(4) Crank, J., "The Mathematics of Diffusion", Oxford Univ. Press. London 1957.

(5) Barrer, R. M., "Diffusion in and Through Solids", Cambridge Univ. Press, 1941.

(6) Einstein, A., Ann. Physik (4), 17, 549 (1905); 19, 371 (1906).

(7) Barnes, C., Physics 5, 4 (1934).

(8) Graham, T., Trans. Faraday Soc. London, 151, 183 (1861).

(9) Martin, C. J., J. Physiol. 20, 364 (1896).

(10) Bechhold, H., Z. Physik. Chem. 60, 257 (1907); 64, 328 (1908).

(11) Pfeffer, Osmotische Untersuchungen Leipzig 1877.

(12) Guérout, Comp. Rend. 75, 1809 (1872).

(13) Bigelow, S. L., and Gemberling, A., J. Am. Chem. Soc. 29, 1576 (1907).

(14) Bigelow, S. L., J. Am. Chem. Soc. 29, 1675 (1907).

(15) Elford, W. J., Proc. Royal Soc. London 112B, 384 (1933).

(16) Craig, L. C., Science 144, 1093 (1964).

(17) Ackers, G, K., and Steere, R. L., Biochim. Biophys. Acta. 59, 137 (1962).

(18) Millipore Filter Corporation, Bedford, Mass.

(19) S & S Filters, Carl Schleicher & Schuell Co. Keene, N. H.

(20) Manegold, E., Kolloid Z. 49, 372 (1929); 61, 140 (1932).

(21) Ferry, J. D., Chem. Review 18, 373 (1936).

(22) Elford, W. J., Proc. Roy. Soc. London 106B, 216 (1930).

(23) Riley, R., Gardner, J. O., and Merten, U., Science 143, 801 (1964).

(24) Walpole, G. S., Biochem. J. 9, 284 (1915).

(25) Manegold, E., and Hofmann, R., Kolloid Z. 50, 22 (1930).

(26) Bartell, F. E., and Carpenter, D. C., J. Phys. Chem. 27, 252 (1923).

(27) Hitchcock, D. I., J. Gen. Physiol. 9, 755 (1926).

(28) Honold, E., and Skau, E. L., Science 120, 805 (1954).

(29) Bechhold, H., Kolloid Z. 66, 329 (1934); 67, 66 (1934).

(30) Elford, W. J., and Ferry, J. D., British J. Exptl. Path. 16, 1 (1935).

(31) Duclaux, J., and Errera, J., Kolloid Z. 38, 54 (1926).

(32) Longuet-Higgins, H. N., and Austin, G., Biophys.J.6, 217 (1966).

(33) Kisluk, R. L., Biochim. Biophys. Acta. 40, 531 (1960).

(34) Duel, H., Solms, J., and Anyas-Weisz, L., Helv. Chim. Acta. 33, 2171 (1950).

(35) Duel, H., and Neukom, H., Adv. in Chem. 11, P.51 (1954).

(36) Clark, R. T., Anal. Chem. 30, 1676 (1958).

(37) Wheaton, R. M., and Bauman, W. C., Ann. N.Y. Acad. Sci. 57, 159 (1953).

(38) Lindqvist, B., and Storgards, T., Nature 175, 511 (1955).

(39) Lathe, G. H., and Ruthven, C. R. J., Biochem. J. 60, XXXIV (1955); 62, 665 (1956).

(40) Polson, A., Biochim. Biophys. Acta. 19, 53 (1956).

(41) Allison, A. C., and Humphrey, J. H., Nature 183, 1590 (1959).

(42) Porath, J., and Flodin, P., Nature 183, 1657 (1959).

(43) Dextran Gels; Pharmacia, Uppsala, Sweden.

(44) Lea, D. J., and Sehon, A. H., Can. J. Chem. 40, 159 (1962).

(45) Ogston, A. G., and Phelps, C. F., Biochem. J. 78, 827 (1961).

(46) Vaughan, M. F., Nature 188, 55 (1960).

(47) Curtis-Jones, B., Nature 191, 272 (1961).

(48) Brewer, P. J., Nature 188, 934 (1960); 190, 625 (1961).

(49) Flodin, P., "Dextran gels & their application in gel filtration", Uppsala, Sweden, 1962.

(50) Andrews, P., Nature 196, 36 (1962).

(51) Whitaker, J. R., Anal. Chem. 35, 1950 (1963).

(52) Porath, J., Pure App. Chem. 6, 233 (1963).

(53) Squire, P. G., Archiv. Biochem. Biophys. 107, 471 (1964).

(54) Determann, H., Angew. Chem. Int. Ed. 3, 608 (1964).

(55) Gelotte, B., J. Chromat. 3, 330 (1960).

(56) Porath, J., Biochim. Biophys. Acta. 39, 193 (1960).

(57) Siegel, L. M., and Monty, K. J., Biochim. Biophys. Acta 112, 346 (1966).

(58) Gilbert, G. A., Discussions Faraday Soc. 13, 159 (1953); 20, 68 (1956).

(59) Winzor, D. J., and Scheraga, H. A., Biochem. 2, 1263 (1963).

(60) Kikuichi, K., Kato, S., Imanishi, A., and Isemura, T., J. Biochem. 55, 102 (1964).

(61) Richterich, R., Schafroth, P., and Aebi, H., Clin. Chim. Acta. 8, 178 (1963).

(62) Baumstark, J. S., Laffin, R. J., and Bardawil, W. A., Arch. Biochem. Biophys. 108, 514 (1964).

(63) Union Carbide Corp., Visking Division, "'Visking' Dialysis Tubing", Technical Information.

(64) Fuchs, E., and Gorin, G., Biochem. Biophys. Res. Commun. 5, 196 (1961).

(65) Stauffer, R. E., in "Technique of Organic Chemistry", (A. Weissberger, ed.), 2nd ed., Vol. III, part I ("Separation and Purification"), Interscience, New York, 1956.

(66) Hoch, H., and Turner, M. E., Biochim. Biophys. Acta. 38, 410 (1959).

(67) Longworth, L. J., J. Am. Chem. Soc. 75, 5705 (1953).

(68) Bartell, F. E., and Osterhoff, H. J., J. Phys. Chem. 32, 1553 (1928).

(69) Carman, P. C., Trans. Inst. Chem. Eng. 15, 150 (1937).

(70) Bigelow, S. L., and Bartell, F. E., J. Am. Chem. Soc. 31, 1194 (1909).

(71) Anderson; Z. Physik Chem. 88, 191 (1914).

(72) Illani, A., Biochim. Biophys. Acta, 94, 405 (1965).

(73) Millipore Filter Corporation; "Catalog M.F.-64" p.7 (1964).

(74) Bartell, F. E., J. Phys. Chem. 15, 665 (1911).

(75) Terzaghi, Eng. News. Rec. 95, 832 (1925).

(76) Renken, E. M., J. Gen. Physiol., 38, 225 (1954).

(77) Nelson, N., J. Biol. Chem. 153, 375 (1944).

(78) Gorin, G., Fuchs, E., Butler, L. G., Chopra, S. L., and Hersch, R. T., Biochemistry; 1, 911 (1962).

(79) Ackers, G. K., Biochem. 3, 723 (1964).

(80) Craig, L. C., and King, T. P., J. Am. Chem. Soc. 77, 6620 (1955); 78, 4171 (1956).

(81) Kamateka, M., and Hatakayaura, K., Tohaku. J. Agr. Research, 3, 101 (1952).

(82) Creeth, I. M., and Nichol, L. W.; Biochem. J. 77, 230 (1960).

(83) Seigel, L. M., and Monty, K. J., Biochem. Biophys. Res. Comm. 19, 494 (1965).

(84) Sheppard, E., Ph.D. Thesis. Polytech. Inst. of Brooklyn (1951).

(85) Huber, W., Naturwissenschaften 38, 21 (1951).

(86) Setlow, R. B., Arch. Biochem. Biophys. 36, 328 (1952).

(87) Reithel, F. J., Robbins, J. E., and Gorin, G., Arch. Biochem. Biophys. 108, 409 (1964).

(88) Chin, C. C., Ph.D. Thesis, Oklahoma State Univ., (1967).

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