STUDIES OF RATES OF ESCAPE OF PROTEINS THROUGH MEMBRANES

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SUMMARY

Rate coefficients of escape of BPA and Fraction II human γ-globulin through large pore size nitro-cellulose membranes were shown to be constant up to 1 g/100 ml.

A general scheme of statistical analysis of diffusion data for a two-component system is presented. One particular application consisted in the calculation of the original composition of a known mixture of BPA and Fraction II from the shape of the escape rate curve. The calculated concentrations agreed to within 5 % with those directly measured.

Prolonged diffusion of a concentrated solution of Fraction V human albumin during which 99.16 % had escaped, showed that all the large molecular weight impurities of Fraction V which remained "inside" had become insoluble.

INTRODUCTION

Escape* rate measurements have been shown to serve as a test for heterogeneity of small molecules[1-3]. Heterogeneity tests of this type might be of considerable value for large molecular weight substances in supplementing those used in ultracentrifugation, electrophoresis and diffusion, by virtue that, in diffusion through a membrane, small differences in size, shape and charge may be more readily demonstrable. Escape rate measurements are also likely to provide a convenient way for detecting interaction between proteins, or proteins and haptens. In the present paper the theory of the two-component problem is discussed and in part illustrated by a statistical analysis of data obtained with solutions of human plasma albumin, bovine plasma albumin, human γ-globulin and a mixture of the last two.

The use of large pore size membranes with an open cell necessitated precautions to avoid hydrostatic flow. A microtechnique for work with volumes between 0.3 and 0.5 ml is described.

MATERIALS AND METHODS

Crystalline bovine plasma albumin (BPA), a sample from the batch No. P 67403 made by Armour Co., and human albumin Fraction V and human γ-globulin Fraction II

Abbreviation: BPA, bovine plasma albumin.

* This term is used by CRAIG *et al.*[1] apparently in distinction to "dialysis" which refers to small molecules in the presence of large ones to which the membrane is impermeable.

were used. The latter two were given to us by the American Red Cross Blood Program. The buffer solution used contained 0.1 mole KCl, 0.01 mole each of sodium acetate and acetic acid and 0.0015 mole NaN_3 (= 0.1 g) per l and had a pH of 4.7. The proteins were dissolved in the buffer solution and centrifuged. Fraction II was stabilized with 1/20 of its weight of BPA.

The apparatus was the same as described earlier[3] except for the cell (Fig. 1) which had been modified for use with large pore size membranes and for much smaller volumes. The area of the exposed membrane surface was 0.754 cm^2. A Schleicher and Schuell medium Membrane Filter of 0.25 micron average and 0.5 μ maximum pore size served as membrane. Three pointed lucite pins (Fig. 1, P) were used as level indicators in the procedure of adjusting the central shaft vertical and the membrane parallel to the "outside" fluid surface. The level of the 1500 ml outside fluid was adjusted by means of a vertical glass rod, 9.1 mm in diameter, which was lowered until the surface of the fluid touched one levelling pin. This adjustment was reproducible to within 1 or 2 mm on the rod, that is, to better than 0.01 mm of the fluid level in the container, the free surface area being 164 cm^2. The water lost by evaporation was replaced at half hourly (1–2 ml) and the acetic acid at daily intervals (0.05–0.1 ml glacial acetic acid). All experiments were carried out at 25.0° with fluctuations usually of less than 0.1°. The "inside" surface of the membrane was swabbed with a small cylindrical mop of tissue paper held by a polyethylene clamp (Fig. 1, T). The sample was introduced and withdrawn from the cell by means of a capillary point polyethylene pipette. At the end of every run the weights were

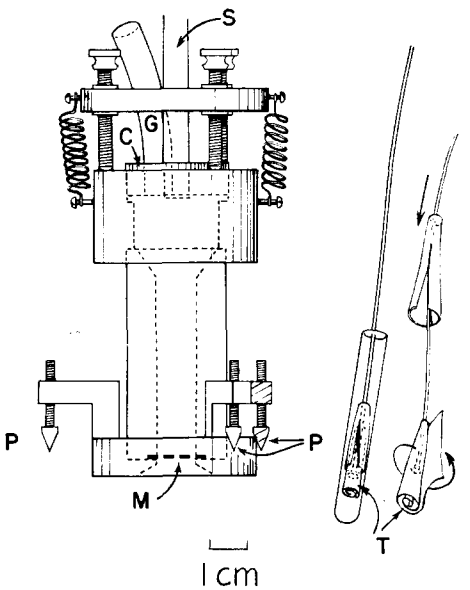

Fig. 1. Diffusion cell. M = membrane, P = levelling pins, G = polyethylene guiding tube for pipette, S = shaft, C = cork, T = tissue paper mop. The cylindrical center section has shallow concentric groves by which the membrane tightly seals the chamber. It is attached to the end pieces by three 3/16 stainless steel bolts (not drawn in the figure) the ends of which at the bottom are sealed in by a plug of lucite. Paddles for stirring the outside fluid, as shown in the earlier paper[3], are used also here, but for clarity have been omitted from the drawing. They are shaped so that on every stroke of the cell the bottom surface of the membrane is flushed.

taken of (a) the solution after transfer into a small serological test tube (50 × 6 mm), (b) the pipette before and after use and (c) the mop before and after use in order to obtain the total amount of solution at the end of the run. Preliminary tests with buffer instead of protein solution were made to determine the volume for which the hydrostatic flow was zero, that is, the volume of that amount of fluid which was recovered unchanged in weight. The same volume of protein solution was then used; this was 0.475 ml within a range of ± 0.5 μl and a precision of 0.1 or 0.2 μl. The volume changes ranged from —6.8 μl (—1.4 %) to + 8.9 μl (1.9 %); 70 % of the volume changes were within the range —4.2 to + 6.2 μl (—0.9 % and + 1.3 %). Whenever positive changes in volume occurred the values for the absorbancy (A) were corrected to refer to the original volume. Negative values for volume changes occurred in half the number of runs and were disregarded since they would not affect the protein concentration except secondarily by slightly raising the value for the rate coefficient. The outside concentration of protein never exceeded 0.2 % of that inside and was disregarded.

The concentrations of the proteins were determined by measuring the absorbancy at 278 mμ (half intensity band width 1 mμ) in a Beckman DU spectrophotometer with a photomultiplier attachment. The instrument was stabilized by two Sola constant voltage transformers, one before the powerpack and the other in series with the trickle charger and a variable resistor, adjusted so as to obviate the discharge of the battery. The micro-cells of 0.55 ml capacity were those manufactured by the

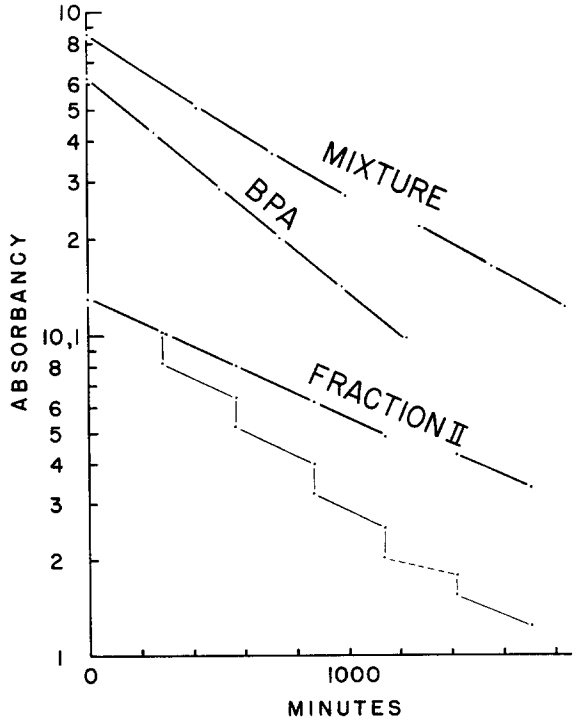

Fig. 2. Diffusion analysis of a mixture. The three upper lines were calculated to represent the amount of protein remaining inside as a function of time. The lowest line represents the actual initial and final absorbancies for each run in the Fraction II set.

Pyrocell Company, New York. As a safeguard against accidental error from light scattering of suspended particles, the solutions were routinely centrifuged for at least 30 min at 1000 × *g*. Three pipettes of the Pregl type, 0.0658, 0.1129 and 0.2615 ml, were employed for all dilution procedures. To the more concentrated solutions, recovered at the end of each run in the initial stages of a set of runs, 0.1129 ml outside buffer was added; 0.0658 ml of the mixture was used for analysis and the remainder for the succeeding run. The 0.0658 ml was diluted either with once or with twice 0.2615 ml outside buffer, corresponding to approximately 5 and 9 fold dilutions. To the more dilute solutions, toward the end of the set of runs, 0.0658 ml buffer was added, in order that after the absorbancy measurements sufficient solution should be available for the succeeding run. The outside buffer as reference and dilution fluid was preferred to unused buffer because it was found that the absorbancy of the buffer solution at 278 mμ, mostly contributed by NaN_3, decreased from 0.02 to about 0.012 in 5 h, to 0.008 in 9 h and then possibly somewhat further, in the presence of traces of albumin or of Fraction II. With the majority of the values for A ranging between 0.4 and 1.5 the uncertainty in the blank value did not seem to lead to serious error. Precautions were taken to minimize errors caused by vaporization of water from small samples. Further, by weighing all pipetted quantities to 0.1 mg precision, the pipetting error was reduced; for example, that of the smallest pipette was reduced to about 0.2 %. The uncertainty for the larger pipettes was correspondingly less, percentagewise, but it depended also on the values assumed for the density increments of the proteins (0.003 for a 1 % solution). Using 0.15 to 0.2 ml for each absorbancy measurement it was possible to measure all solutions in duplicate or triplicate except for the 5-fold dilutions (which occurred once in every set of runs).

Statistical inference in the two component system

In setting up the model for use in the statistical analysis, the activity coefficients of the proteins were taken to be constant and the outside concentration of protein as negligibly small. Thus it was assumed that the exponential law is obeyed[3]

$$A' = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \tag{I}$$

where A' is the absorbancy of the solution at time t, a_1, and a_2 are the absorbancies at time zero contributed by components 1 and 2, and k_1 and k_2 are the rate coefficients and include the activity coefficients of the proteins as factors. There are four parameters, a_1, a_2, k_1, and k_2, about which it is possible to draw inferences. We can imagine several different situations resulting from experimental circumstances. We suppose in each case that measurements of A' are made at various times t.

Now we do not measure A' but instead an approximate value A where

$$A = A' + A'\varepsilon \tag{II}$$

and ε we take to be normally distributed, with a mean of zero and constant variance σ^2. To a first approximation we can replace (II) by

$$A = A' + A\varepsilon \tag{III}$$

Thus

$$A = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} + A\varepsilon \tag{IV}$$

is an ordinary regression model with weights

$$w = 1/A^2 \tag{V}$$

Now (IV) can be written approximately

$$A \doteqdot a_1 e^{-k_{10}t} + a_2 e^{-k_{20}t} - a_1(k_1 - k_{10})te^{-k_{10}t} - a_2(k_2 - k_{20})te^{-k_{20}t} + A\varepsilon \qquad \text{(VI)}$$

where k_{10} and k_{20} are either known or trial values of k_1 and k_2. Let

$$\begin{array}{lll} x_1 = e^{-k_{10}t} & x_3 = te^{-k_{10}t} & a_3 = -a_1(k_1 - k_{10}) \\ x_2 = e^{-k_{20}t} & x_4 = te^{-k_{20}t} & a_4 = -a_2(k_2 - k_{20}) \end{array} \qquad \text{(VII)}$$

Then (VI) becomes

$$A \doteqdot a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + A\varepsilon \qquad \text{(VIII)}$$

Estimation

Maximum likelihood estimates for the four unknown parameters a_1, a_2, k_1, and k_2 are found by applying the method of least squares to the linearized model (VIII) in successive iterations. If the k's are both known $k_{10} = k_1$ and $k_{20} = k_2$ and hence $a_3 = a_4 = 0$. In this case a single cycle of iteration provides final estimates of a_1 and a_2. On the other hand if at least one k is unknown (*i.e.*, must be estimated) then preliminary estimates of a_1, a_2, a_3, and a_4 are found which also by (VII) provide improved values for k_1 and k_2. These replace k_{10} and k_{20} in the determination of the x's, using eqn. (VII), and a 2nd cycle is performed. Additional cycles are performed until stable estimates of a_1, a_2, k_1, and k_2 are obtained. These are then the maximum likelihood estimates.

In order to present a unified method of estimation for the various cases of *a priori* knowledge we define an "adjusted absorbancy" A^* as follows:

a_1, k_1, and k_2 known	$A^* = A - a_1x_1$
k_1 and k_2 known	$A^* = A$
a_1, k_1 and a_2 known	$A^* = A - a_1x_1 - a_2x_2$
a_1 and a_2 known	$A^* = A - a_1x_1 - a_2x_2$
k_1 known	$A^* = A$
a_1 known	$A^* = A - a_1x_1$
none of the parameters known	$A^* = A$
a_1 and k_1 known	$A^* = A - a_1x_1$

The method of least squares gives rise to the following "normal equations":

$$\begin{array}{llll} \Sigma wx_1^2 \cdot a_1 & + \Sigma wx_1x_2 \cdot a_2 + \Sigma wx_1x_3 \cdot a_3 + \Sigma wx_1x_4 \cdot a_4 = \Sigma wx_1A^* \\ \Sigma wx_1x_2 \cdot a_1 + \Sigma wx_2^2 \cdot a_2 & + \Sigma wx_2x_3 \cdot a_3 + \Sigma wx_2x_4 \cdot a_4 = \Sigma wx_2A^* \\ \Sigma wx_1x_3 \cdot a_1 + \Sigma wx_2x_3 \cdot a_2 + \Sigma wx_3^2 \cdot a_3 & + \Sigma wx_3x_4 \cdot a_4 = \Sigma wx_3A^* \\ \Sigma wx_1x_4 \cdot a_1 + \Sigma wx_2x_4 \cdot a_2 + \Sigma wx_3x_4 \cdot a_3 & + \Sigma wx_4^2 \cdot a_4 = \Sigma wx_4A^* \end{array} \qquad \text{(IX)}$$

where the weights w are given by (V), the x's are given by (VII) and the A^*'s are the adjusted observed absorbancies. The numerical solution of the linear eqns. (IX) yields estimates of the a's which are then iterated as indicated above. Equations and terms corresponding to known parameters are omitted. For example, in the case that a_1 and k_1 are known *a priori*, the first and third equations are omitted as are the first and third terms of the remaining equations.

Confidence limits

Where possible, it is desirable to obtain a range of values within which an estimated parameter is deemed to lie. Such a range is called a confidence region and the end points of the interval are known as confidence limits. In some cases, if one

states that the parameter lies within a region, an exact probability that the statement is in error can be found. Then the region is said to be an exact confidence region. Under certain restricted conditions an exact confidence region can be found that utilizes all of the information in the data concerning the parameter. This exact confidence region is termed a fiducial region and the bounds are fiducial limits. Only the fiducial limits truly represent the amount of information about the parameter inherent in the data. In still other cases no exact confidence region is easily found but instead exact limiting results for very large samples may be obtained. Limiting regions of this kind are termed asymptotic confidence regions and may be approximately correct for moderately large samples of data. Asymptotic confidence regions can be quite inaccurate for small amounts of data. The amount of data required for sufficient accuracy is not always easily determined and depends upon, among other things, the true values of the parameters.

Asymptotic results may in principle always be obtained for maximum likelihood estimators. Hence these are available for all of the estimators described in the previous section. Since it is expected that application of the techniques herein discussed will be mainly confined to relatively small samples, details of computation of asymptotic confidence regions will not be given. For these details see DEMING[4]. Exact joint confidence regions for the exponential parameters can be obtained by methods discussed by TURNER[5]. The discussion here will be limited to those cases in which the exponential parameters k_1 and k_2 are known *a priori*. In these cases fiducial regions are available and easily obtained.

The computations are carried out in stepwise fashion. First eqns. (IX) are modified after eliminating 3rd and 4th equations and terms, to

$$\begin{aligned} \Sigma w x_1^2 \cdot c_{11,2} \quad &+ \Sigma w x_1 x_2 \cdot c_{21,2} = 1, 0 \\ \Sigma w x_1 x_2 \cdot c_{11,2} &+ \Sigma w x_2^2 \cdot c_{21,2} \quad = 0, 1 \end{aligned} \tag{X}$$

The first set of numbers (1 and 0) on the right are used to obtain c_{11} and c_{21} and then the second set (0 and 1) are used to obtain c_{12} and c_{22}. Next we calculate an estimate of the error variance σ^2. This is given by

$$s^2 = \frac{1}{n-2}\left\{\Sigma w(A^*)^2 - \hat{a}_1 \Sigma w x_1 A^* - \hat{a}_2 \Sigma w x_2 A^*\right\} \tag{XI}$$

where $\hat{a}_1$ and $\hat{a}_2$ are the maximum likelihood estimates and n is the number of observations. A value of STUDENT's t with n-1 degrees of freedom is chosen at the desired confidence level (*e.g.*, 95) and confidence regions are defined by

$$\begin{aligned} \hat{a}_1 - t\sqrt{c_{11}s^2} \leqslant a_1 \leqslant \hat{a}_1 + t\sqrt{c_{11}s^2} \\ \hat{a}_2 - t\sqrt{c_{22}s^2} \leqslant a_2 \leqslant \hat{a}_2 + t\sqrt{c_{22}s^2} \end{aligned} \tag{XII}$$

Thus we state that a_1 and a_2 fall within specified intervals and these intervals have been chosen so as to coincide with a specified risk of error in making such statements.

If a_1 is known in addition to k_1 and k_2 then (X) is replaced (by proper omissions) by

$$c_{22} = 1/\Sigma w x_2^2 \tag{XIII}$$

and the divisor in (XI) becomes n-1 as does the degrees of freedom for STUDENT's t.

RESULTS

Known mixture

Table I and Fig. 1 show the results of experiments with BPA, Fraction II and a mixture of the two. The data for BPA were analyzed on the basis of eqn. (XIV)

$$A' = a_1 e^{-k_1 t} \quad \text{(XIV)}$$

The curves for Fraction II and for the mixture of BPA and Fraction II show a discontinuity which was attributed to the instability of Fraction II when diluted with buffer solution. This dilution was necessary for the measurement of absorbancy. After

TABLE I

RATES OF ESCAPE OF PROTEINS THROUGH MEMBRANES

Solution 1 10 mg/ml BPA			*Solution 2* 10 mg/ml fraction II and 0.5 mg/ml BPA			*Mixture* 2 ml solution 1 and 1 ml solution 2		
Time (min)	*Absorbancy**		*Time (min)*	*Absorbancy**		*Time (min)*	*Absorbancy**	
	Obs.	*Calc.*		*Obs.*	*Calc.*		*Obs.*	*Calc.*
0.0	6.224	6.1660	0.0	12.95	13.0905	0.0	8.503	8.4304
255.0	4.219	4.1901	286.0	10.262	10.2106	422.0	5.065	5.1319
505.0	2.834	2.8690	564.5	8.0754	8.0219	718.0	3.653	3.6643
743.5	1.999_3	1.9990	867.0	6.183	6.1762	998.0	2.708	2.6870
978.5	1.416_3	1.4002	1142.5	4.833	4.8696	*Discontinuity*		
1227.5	0.981_1	0.9602	*Discontinuity*			1284.0	2.174	2.1709
			1420.0	4.245	4.2582	1560.0	1.629_9	1.6319
			1704.0	3.346	3.3357	1843.5	1.225_5	1.2253
			0.0	—	—			
			297.0	10.176	10.1140			

* Corrected to refer to the original volume, 0.475 ml.

the point of discontinuity the solutions were measured almost undiluted (1 in 1.14) and Fraction II was then assumed to be stable under the conditions of handling the samples and to obey eqn. (I). However, before the discontinuity we have postulated that, on account of the losses of Fraction II by precipitation, eqn. (I) be modified to

$$A' = a_1 e^{-k_1 t} + a_2[f(e^{-k_1 t}, e^{-k_2 t})]\, e^{-k_3 t} \quad \text{(XV)}$$

Now $f\,(e^{-k_1 t}, e^{-k_2 t})$ is unknown but can be replaced approximately by a truncated TAYLOR'S series. The crudest approximation is found by taking only the constant term. Thus (XV) becomes

$$A' \text{ (before discontinuity)} = a_1 e^{-k_1 t} + a_2 \beta_0 e^{-k_3 t} \quad \text{(XVI)}$$

The value for a_1, which is here the absorbancy corresponding to the concentration of BPA in the "stabilized" Fraction II solution, was calculated to be equal to 0.3085. Better approximations are given of course, by including higher powers, but when only a few data-points are available a condition of relative indeterminacy arises. This means that although the model will provide a good description of the data, the determination of individual parameters will be unreliable. Eqn. (XVI) was considered

appropriate* in the analysis of the experiment with the stabilized Fraction II and the mixture by assuming (a) and equal degree of instability for a 1:9 and a 1:5 dilution and (b) no precipitation by the slight dilution at the two final points in the set. The eqn. (XVI) neglects the variation in the stabilizing action of BPA, although assumption (a) implies a more stable solution in the beginning when 1:9 dilutions were used and relatively more BPA was present, than at later stages when 1:5 dilutions were used.

The magnitudes of the errors which were known to occur in the analysis were calculated on the basis of an assumed $\sigma_A = 0.001$ each in the absorbancies below 0.5 and the blank measurement, 0.002 at 0.7, 0.004 at 1.1, $\sigma_w = 0.1$ mg for each weighing in the dilution procedure and a multiplicative error caused by neglecting the effect of the volume change of the solution during the run on k, which was less than 0.5 %. From the values for the calculated errors at different stages in the experiment with BPA, shown in Table II, it seemed appropriate to postulate a multiplicative error, as was expressed in eqns. (II) and (III), rather than an additive one, in the values for the absorbancies.

TABLE II

CALCULATED MULTIPLICATIVE ERRORS AT DIFFERENT STAGES IN THE BPA SET

	Initial	*At end of run*				
		No. 1	*No. 2*	*No. 3*	*No. 4*	*No. 5*
In A, %	0.2	0.4	0.4	0.3*	0.3	0.3
In dilution factor, %	0.2	0.2	0.2	0.1	0.1	0.1

* Principally error of reading the scale.

The methods discussed in the section on estimation were extended to obtain maximum likelihood estimates for the model corresponding to eqn. (XVI). Estimates of k_1 and k_2 were determined from experiments with BPA and stabilized Fraction II alone. Since the sampling variations for these estimates are quite small, they were taken to be known in the analysis of the mixture experiment, the aim of which was the determination of the composition of the mixture. The values of the estimates obtained are given in Table IIIa. Those referring to the mixture may be compared with the values calculated from the amounts used in preparing the mixture (Table IIIb).

Heterogeneity

118 mg of Fraction V were added to 0.5 ml of buffer solution and placed in the dialysis cell over a medium membrane. After 65 h in contact with several changes of buffer solution 99.16 % of the protein (in terms of the absorbancy 278 mμ) had escaped. The residual solution which was turbid, was centrifuged and the clear

* That no other "Accident", for example inadvertent loss of solution at the discontinuity of the mixture experiment, has introduced an error in the values after the discontinuity was shown by fitting

$$A' \text{ (before discontinuity)} = a_1 e^{-k_1 t} + a_2 \beta_0 e^{-k_2 t}$$
$$A' \text{ (after discontinuity)} = (a_1 e^{-k_1 t} + a_2 e^{-k_2 t}) r \qquad \text{(XVII)}$$

to the data for varying values of r. The sum of squares was at a minimum when r was close to unity ($\hat{r} = 1.02$).

TABLE III (a)

MAXIMUM LIKELIHOOD ESTIMATES OF THE ESCAPE RATE COEFFICIENTS AND INITIAL CONCENTRATIONS

BPA		*Fraction II*		*Mixture of solutions 1 and 2*	
Parameter	*Estimate*	*Parameter*	*Estimate*	*Parameter*	*Estimate*
k_1	0.001505	k_2	0.00085	a_1	4.4897
a_1	6.166	a_2	14.209	a_2	4.5296
		β_0	0.90	β_0	0.87
		$0.3085 + a_2\beta_0 = 13.0905$		$a_1 + a_2\beta_0 = 8.4304$	

(b)

ABSORBANCIES IN MIXTURE CALCULATED FROM INITIAL CONCENTRATIONS OF SOLUTIONS 1 AND 2

	From best fit curve	*From measured values*
BPA	4.2135	4.2522
Fr. II	4.7363	4.6841
Total	8.4743	8.4660
Total observed	8.503	

supernate was tested with the same membrane as used for the experiments with BPA and Fraction II. At the end of the first run the solution showed opalescence which was removed by centrifugation. After measurement of absorbancy the solution was re-run twice, each time supplemented with just sufficient initial supernate to give the required 0.5 ml for the next run. A run with a fresh solution of BPA was included in the set for comparison. The results are given in Table IV.

It was suprising to find so little difference in the values for k between the soluble residue and the original Fraction V since even 1 or 2 % of material of molecular weight larger than γ-globulin would have given a considerably lower k-value, had this material remained soluble. The conclusion that can be made from these experiments is that Fraction V contains as contaminant principally material which is easily denatured and that the residual soluble material shows the same escape rate as albumin.

TABLE IV

COMPARISON OF A DILUTE SOLUTION OF FRACTION V WITH THE RESIDUAL 0.84 % FROM A CONCENTRATED SOLUTION OF FRACTION V AFTER DIFFUSION AT pH 4.9

	Fraction V 11.3 mg/10 ml	*BPA* 9.4 mg/10 ml	*Fraction V* 11.3 mg/10 ml	*Fraction V residue*		
				Run 1	*Run 2*	*Run 3*
Day	0	4	5	6	7	8
Time (min)	300	300	300	343	360	333
k	0.00143	0.00144	0.00141	0.00118	0.00132	0.00142

DISCUSSION

Several factors are expected to have an influence on the escape rate coefficients of proteins as calculated on the basis of the observed concentrations. At high concentrations one would expect larger escape rate coefficients for the same reason one finds

positive deviations from VAN 'T HOFF's law of osmotic pressure. Such a trend has not been found by the present technique at the low concentrations of protein. A change in pH away from the isoelectric point will be effective because the value for the coefficient of (free) diffusion increases through the accelerating action of the small gegenions, as also because the charge on the membrane will come into play. If protein becomes insoluble during the run, as actually occurred to a small extent with a human albumin preparation (Fraction V) after 99.16 % had escaped through a membrane, the escape rate coefficient calculated from absorbancies of centrifuged solutions must be too large.

MAURO[6] has discussed solvent transfer in diffusion and ROBBINS AND MAURO[7] have reported experiments on non-diffusional transfer through membranes using closed cells. In an open cell, as used in the present experiments, such non-diffusional transfer of solute is expected to be very much smaller, since it depends on the rate at which the hydrostatic pressure, equivalent to the osmotic pressure, is built up. Actually, with the protein concentrations used, hydrostatic flow appeared to be negligible. For a closed cell non-diffusional transfer would be expected to begin at the moment of contact between solution and solvent.

The feasibility of a mathematical analysis of the escape rate curve for a mixture of two known substances does not depend on whether the substances when by themselves obey the linear log (concentration) — time relationship, since this can be allowed for by introducing higher powers in the exponents of eqn. (XVI). It is, however, necessary to postulate that the two substances do not interact with each other, *i.e.* that they behave in the presence of each other as they behave singly. On the other hand, deviation of the experimental escape rate curve for a mixture from that calculated (known are the initial concentrations and the escape rate curves for the components) indicates interaction. Statistical tests can be constructed to quantity this deviation.

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