ACKNOWLEDGMENTS AND ADDRESSES

Received October 15, 1974, from the *Division of Clinical Oncology, University of Wisconsin Medical School, Madison, WI 53706*

Accepted for publication November 14, 1974.

Supported in part by Grant CA 13290 from the National Cancer Institute, U.S. Public Health Service.

The authors thank Dr. R. Scott of Hoffmann-La Roche, Inc., Nutley, N.J., and Dr. F. J. Ansfield of the University of Wisconsin Medical School, Madison, Wis., for gifts of fluorouracil and floxuridine. They also acknowledge the technical advice and tumor samples supplied by Professors G. T. Bryan, C. Heidelberger, and K. Clifton of the University of Wisconsin Medical School.

* Present address: University Centre for Health Sciences, B.P. 1364, Yaounde, Cameroon.

[x] To whom inquiries should be directed.

Evaluation of a Dynamic Permeation Technique for Studying Drug–Macromolecule Interactions

F. BOTTARI, G. Di COLO, E. NANNIPIERI [x], M. F. SAETTONE, and M. F. SERAFINI

Abstract □ The applicability of a permeation rate technique to the determination of drug–macromolecule interactions was tested by measuring the extent of interaction of methylparaben with polyvinylpyrrolidone and polysorbate 80. Results were in agreement with literature data obtained by other techniques. The present method, although restricted to permeant molecules that diffuse readily through nonporous nylon membranes, is of potential value for investigations of drug binding by macromolecules not retained by porous dialysis membranes.

Keyphrases □ Permeation rate technique for determining drug–macromolecule interactions—tested using methylparaben with polyvinylpyrrolidone and polysorbate 80 □ Drug–macromolecule interactions—determination by permeation rate technique, compared to literature dialysis data

The phenomena of drug–macromolecule interactions have received wide attention in recent years in view of their relevance to drug absorption, transport, and overall availability. Dynamic dialysis[1] techniques have been successfully applied to the study of such interactions (1–3). These methods consist of the measurement of the rate of disappearance of a small molecule from a macromolecule-containing compartment under quasi-steady-state conditions. Their application purportedly offers some advantages (rapidity, economy, simplicity, *etc.*) over other time-honored techniques but is limited by the fact that porous dialysis membranes are pervious to many macromolecules of pharmaceutical interest.

Analogous techniques involving nonporous, lipid-like membranes have been proposed. Permeation rate methods (using nylon and dimethylpolysiloxane membranes) were applied to the study of complex formation between small molecules (4, 5). Nakano (6) investigated the interaction of chlorpromazine with several macromolecules, using a permeation rate method with dimethylpolysiloxane membranes, but his experimental system did not allow quantitative estimates of free and bound drug.

Nylon, whose permeability characteristics have been described (7), appeared to be an interesting membrane material for the study of drug–macromolecule interactions by a quasi-steady-state permeation rate technique. The purposes of this preliminary investigation were to evaluate the technique and to compare the results with literature data obtained by equilibrium dialysis.

EXPERIMENTAL

Materials—Methyl *p*-hydroxybenzoate[2] was recrystallized from methanol to a constant melting point of 127–128°. Polysorbate 80[3] and polyvinylpyrrolidone[4] were used as received. Nylon 6 (polycaprolactam) film from a single roll[5], in a labeled thickness of 0.5 mil (0.00127 cm), was used.

Apparatus—The permeation rate experiments were run with a specially designed cell (Fig. 1). The cell interior could be easily cleaned between experiments by removing the upper part (A) without disturbing the membrane. The nylon membrane was securely kept in place by a circular metal plate (C). Fluid tightness was ensured by two O-ring gaskets fitted in machined dies in the upper and lower part of the cell body (B). All parts in contact with the solution were either stainless steel or polytetrafluoroethylene[6] to avoid absorption of the diffusant by the cell material.

The approximate internal volume was 30 ml, and the diameter of the area available for diffusion was 6.0 cm. For use, the cell was placed in a jacketed beaker (internal height of 10.0 cm, internal diameter of 12.0 cm) connected to a thermostatted (30 ± 0.1°) water bath and circulator. Both the "internal" (cell) and "external" (beaker) solutions were stirred by synchronous motors[7]. One motor (60 rpm) was connected to the cell stirrer; the other (500 rpm) operated a magnetic stirrer.

To obtain reproducible results, newly cut membranes were soaked for at least 3 days in several changes of distilled water at 35°. Soaking at lower temperatures or for shorter times resulted in progressively decreasing permeation rates until an apparent stabilization occurred. This phenomenon resulted probably from insuf-

[1] The term dialysis is reserved here for a process involving diffusion through porous membranes, while permeation refers to diffusion through nonporous membranes.

[2] Carlo Erba, Milano, Italy.
[3] Tween 80, Atlas Europol SpA, Varese, Italy.
[4] Plasdone C, GAF Corp., New York, N.Y.
[5] Capran 77 C, Lot G/705283, Allied Chemical Corp., Morristown, N.J.
[6] Teflon, E.I. du Pont de Nemours & Co., Wilmington, Del.
[7] Crouzet S.A., Paris, France.

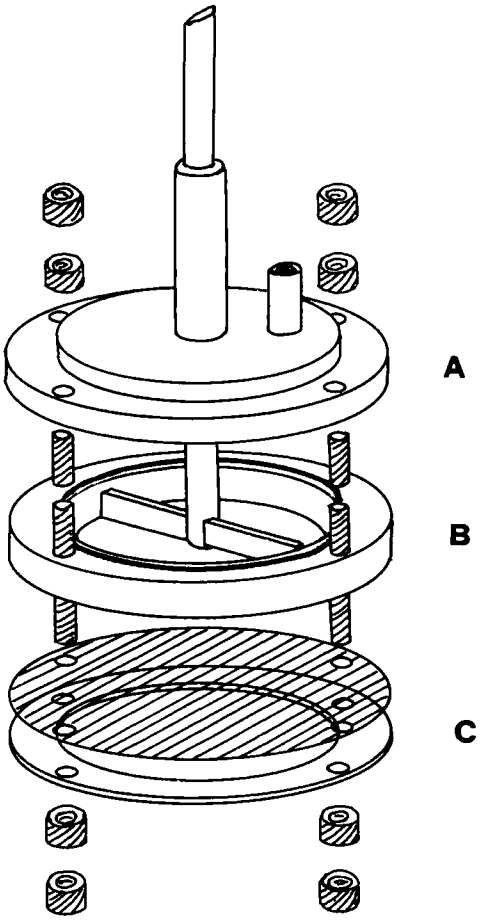

Figure 1—*Cell used for the permeation experiments; see text for details.*

ficient hydration and/or removal of leachable constituents from the membrane. An increase in the density of polycaprolactam fibers on washing, due to removal of low molecular weight components, was reported previously (8).

Permeation Rate Studies—The cell was positioned in the beaker containing 200 ml of distilled water. After equilibration, at t = 0, a 15-ml portion of prewarmed (30°) diffusing solution was introduced into the cell and stirring was initiated. A 100-ml portion of the external solution was periodically removed and immediately replaced with an equal amount of prewarmed water. To approximate sink conditions, care was taken that the external concentration never exceeded one-tenth of the concentration of the diffusant inside the cell.

The removed samples were analyzed spectrophotometrically[8] (255 nm) for methylparaben. The concentration of the diffusing solution (C_i) at different times was calculated by difference from a knowledge of the initial concentration (C_{i0}) and of the cumulative amount that had appeared in the receiving solution. In the present case, analysis of the diffusing solution at the end of several preliminary runs proved that absorption of the diffusant by the cell–membrane complex was minimal or at least within the range of experimental error. It was calculated, using data of Patel and Nagabushan (9) for the interaction of methylparaben with nylon film, that the amount of permeant sorbed by the membrane under the present experimental conditions should not exceed 2.6% of the total.

Data Treatment—Each experiment was repeated at least four times. In the case of linear plots, the data were fed to a desk electronic computer[9] fitted with a linear regression program for computation of regression parameters and statistics. All linear regressions were highly significant ($p < 0.005$), and deviations from linearity were not significant. The coefficient of variation for the slopes of replicate permeation experiments, carried out on consecutive days with the same membrane, was in all cases within the range of 4–11%, thus indicating a satisfactory reproducibility.

For nonlinear plots, the C_i *versus* time data were fitted to a four-parameter biexponential equation (*e.g.*, $C_i = ae^{-bt} + ce^{-dt}$) with the aid of a digital computer[10]. Differentiation of the equation allowed calculation of the concentration of unbound diffusant (C_f) at various values of C_i (*cf.*, Eq. 3). The procedure is essentially similar to that described by Meyer and Guttman (1).

RESULTS AND DISCUSSION

Figure 2 shows the results of permeation rate experiments in which methylparaben (initial concentration of 400 mg/liter, about 2.63×10^{-3} mole/liter) alone or in the presence of polyvinylpyrrolidone [concentration range of 1–4% (w/v)] was allowed to escape through nylon film. The plots are expressed as log C_i/C_{i0} *versus* time, C_i being the concentration of diffusant at time = t, and C_{i0} being the concentration at $t = 0$. A linear relationship between log C_i/C_{i0} and time was observed in all cases.

In the absence of the macromolecule, the following expression of Fick's law applied to the transfer of a solute through a nonporous membrane, under quasi-steady-state conditions, may be considered valid:

$$\ln (C_i/C_{i0}) = \frac{D'At}{V_iX} \qquad \text{(Eq. 1)}$$

where D', the permeability coefficient, is a function of the diffusion coefficient of the solute within the membrane and of the partition coefficient of the solute between the membrane and the solution; A and X are the area and thickness of membrane, respectively; and V_i is the volume of solution inside the cell.

The value of the permeability coefficient (D') of methylparaben through nylon film at 30°, calculated from the permeation data in the absence of a macromolecule using Eq. 1, is 37.4×10^{-9} cm²/sec, which is in good agreement with values reported by Kostenbauder *et al.* (7), $36.8–38.8 \times 10^{-9}$ cm²/sec.

As shown by the graphs in Fig. 2, in the presence of increasing concentrations of polyvinylpyrrolidone, a linear relationship between log (C_i/C_{i0}) and time was always observed; the lines showed progressively decreasing slopes with an increasing macromolecule concentration. Similar graphs, showing the effect of an increasing

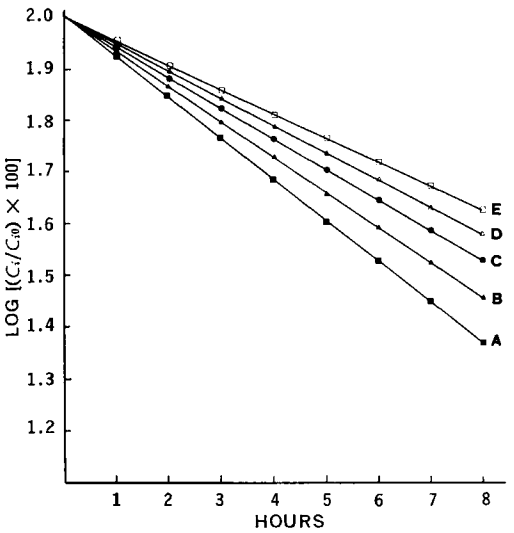

Figure 2—*Plots for the rate of permeation of methylparaben alone (A) and in the presence of 1.0% (w/v) (B), 2.0% (w/v) (C), 3.0% (w/v) (D), and 4.0% (w/v) (E) polyvinylpyrrolidone. The initial concentration of methylparaben was 2.63 × 10^{-3} mole/liter.*

[8] Beckman DU spectrophotometer.

[9] Olivetti Programma 101.

[10] IBM 370/158; nonlinear least-squares regression program BMDX85 (W. J. Dixon, "Biomedical Computer Program, X-Series Supplement," University of California Press, Berkeley, Calif., 1970).

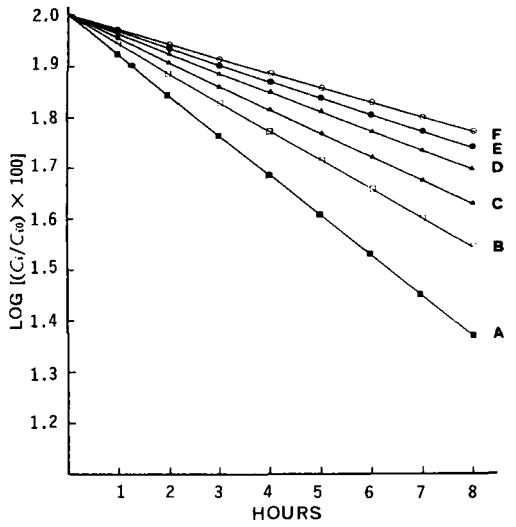

Figure 3—*Plots for the rate of permeation of methylparaben alone (A) and in the presence of 0.5% (w/v) (B), 1.0% (w/v) (C), 1.5% (w/v) (D), 2.0% (w/v) (E), and 2.5% (w/v) (F) polysorbate 80. The initial concentration of methylparaben was 2.63 × 10⁻³ mole/liter.*

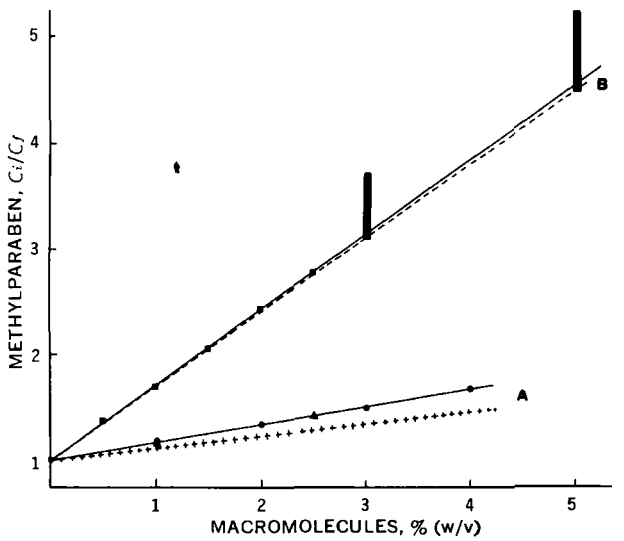

Figure 4—*Plots of the* C_i/C_f *ratio of methylparaben* versus *polyvinylpyrrolidone (A) and polysorbate 80 (B) concentrations. Key: +++, data of Jurgensen Eide and Speiser (11); ▲, data of Miyawaki* et al. *(10); ●, present experimental data; ----, data of Patel and Kostenbauder (12); and ■ and the vertical lines, present experimental data.*

polysorbate 80 concentration [0.5–2.5% (w/v)] on the permeation rate of methylparaben, at the same initial concentration (400 mg/liter), are presented in Fig. 3. The decreased slopes observed in the presence of macromolecules provide evidence of an interaction of the diffusant with the macromolecules. Indeed, the permeation rate in the absence of the macromolecule can be described by the following equation:

$$-dC_i/dt = K_eC_i \qquad \text{(Eq. 2)}$$

where $K_e = D'A/V_iX$ (Eq. 1), and C_i is the total concentration of methylparaben in the diffusing solution. In the presence of the macromolecule, Eq. 2 becomes:

$$-dC_i/dt = K_eC_f \qquad \text{(Eq. 3)}$$

where C_f is the concentration of unbound diffusant inside the cell. The experimental results in the presence of the macromolecule can be described by an equation of the type:

$$-dC_i/dt = K_e'C_i \qquad \text{(Eq. 4)}$$

where K_e' is the experimentally observed slope. Combination of Eqs. 3 and 4 gives the following:

$$C_f/C_i = K_e'/K_e \qquad \text{(Eq. 5)}$$

which shows the relationship existing between the relative amount of unbound diffusant (C_f/C_i) and the ratio (R) of the slope observed in the presence of the macromolecule to the slope observed in the absence of the macromolecule. The ratio of the concentrations of total to free *p*-hydroxybenzoate (C_i/C_f), calculated from the experimental data using Eq. 5, is plotted in Fig. 4 as a function of the concentration of polyvinylpyrrolidone and of polysorbate 80. Data obtained for polyvinylpyrrolidone (10, 11) and for polysorbate 80 (12) are also reported for comparison. The agreement between the present data and the previously reported data, obtained by equilibrium dialysis techniques, appears quite good. The somewhat lower binding values reported by Jurgensen Eide and Speiser (11) are probably due to the lower temperature (22°) of their experiments. The same investigators (11) observed an increasing binding tendency of propylparaben with polyvinylpyrrolidone with rising temperature.

The following expression, which describes the rate of disappearance of diffusant from the cell as a function of its total (C_i), bound (C_b), and free (C_f) concentrations can be obtained from Eq. 3, considering that $C_i = C_f + C_b$:

$$-dC_i/dt = \frac{K_eC_i}{1 + C_b/C_f} \qquad \text{(Eq. 6)}$$

As shown by Eq. 6, the permeation process in the presence of a binding macromolecule follows first-order kinetics only if C_b/C_f, the ratio of concentrations of bound to free permeant, remains constant during each experiment. The linearity of the graphs in Figs. 2 and 3 points, in fact, to a constant C_b/C_f ratio. In the case of the interaction of methylparaben with polyvinylpyrrolidone, this finding appears to agree with previous data (10, 11). Data for the interaction of methylparaben with polysorbate 80 (12), however, indicate a constant C_b/C_f ratio only at a low total permeant concentration (up to about 6.0×10^{-3} mole/liter). The observed constancy of this ratio in the present experiments might, therefore, depend on the low total concentration range of methylparaben explored in each permeation run.

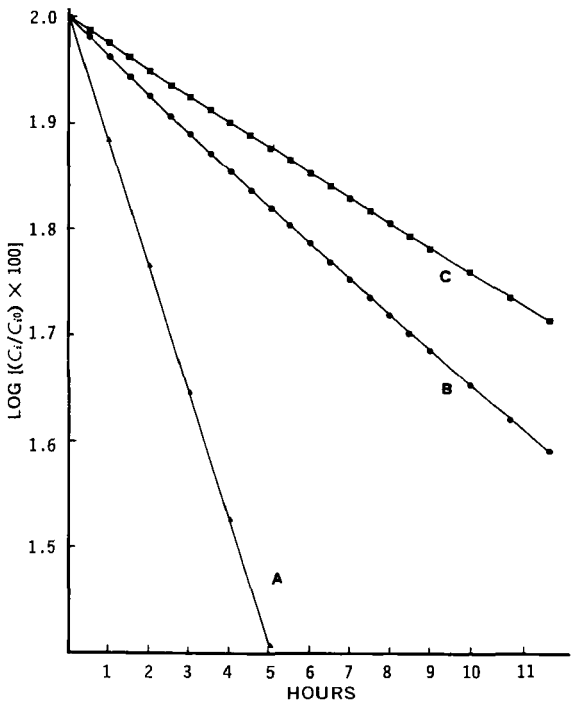

Figure 5—*Plots for the rate of permeation of methylparaben alone (A, initial concentration of 1.0 × 10⁻² mole/liter) and in the presence of 3.0% (w/v) (B) and 5.0% (w/v) (C) polysorbate 80. In the latter two cases, the initial concentration was 2.96 × 10⁻² mole/liter.*

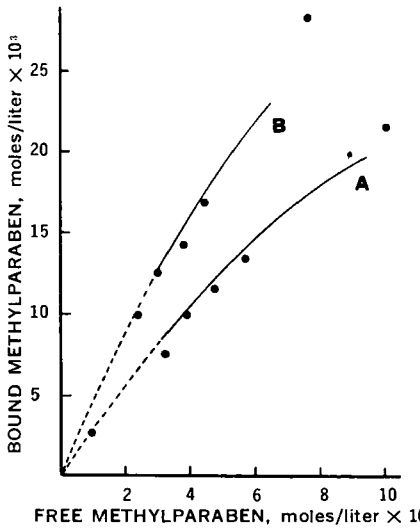

Figure 6—*A* C_b versus C_f *plot for methylparaben in the presence of 3.0% (w/v) (A) and 5.0% (w/v) (B) polysorbate 80. Key:* ●, *data of Patel and Kostenbauder (12); and* —, *present experimental data* (cf., *plots of Fig. 5*).

The results of permeation experiments, in which a higher initial concentration of methylparaben (1.0×10^{-2} mole/liter in the absence and 2.96×10^{-2} mole/liter in the presence of the macromolecule) and higher concentrations of polysorbate [3.0 and 5.0% (w/v)] were used, are illustrated in Fig. 5. As shown by the graphs, under these conditions a linear relationship between log (C_i/C_{i0}) and time was no longer observed. On the basis of Eq. 6, this finding indicates a variation of the C_b/C_f ratio in the course of the permeation experiments. The values of C_b and C_f corresponding to different values of C_i were calculated in this case by treating the data as indicated in the *Experimental* section. The values obtained are reported in Fig. 6 as a C_b *versus* C_f plot, together with the corresponding data from Patel and Kostenbauder (12). The agreement appears satisfactory.

The graphs in Fig. 6 show, for each permeation experiment in the presence of the macromolecule, a C_i/C_f range rather than the constant value observed in the experiments at lower permeant concentration. Such C_i/C_f ranges are indicated in Fig. 4 as vertical lines. It is quite possible that the C_i/C_f ratio might vary within a small range also at low methylparaben concentrations; the present experimental technique, however, could not detect such variation, as shown by the apparent linearity of the graphs in Figs. 2 and 3.

Graphs of the type illustrated in Fig. 4 have been proposed to facilitate the determination of the quantity of preservative to be added to systems containing known concentrations of surfactants. Appropriate use of such plots is made if the user recognizes that the C_i/C_f ratio is a linear function of the macromolecule concentration only at that fixed concentration of free preservative (C_f) that corresponds to the minimum effective concentration required for the desired biological activity. As shown, an apparent linear relationship between the C_i/C_f ratio and S, the surfactant concentration, may be observed only when the C_b/C_f ratio is practically constant for each given S value over a range of total preservative concentrations (C_i). This may occur either at low C_i values (as in the present case) or at constant C_f. Full discussions of the problems involved and the methods for expressing the preservative–surfactant interactions can be found in Refs. 13 and 14.

In conclusion, the present data point to the utility of quasi-steady-state permeation rate techniques for reasonably fast and accurate quantitative determinations of binding of drugs by macromolecules. These techniques, although restricted to permeants that diffuse readily through nonporous membranes, can be useful for studies of binding by macromolecules not retained by porous dialysis membranes. Further work, aimed at a fuller evaluation of the scope and limitations of this experimental approach, is now underway.

REFERENCES

(1) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 1627(1968).

(2) *Ibid.*, **58**, 33(1969).

(3) *Ibid.*, **59**, 39(1970).

(4) R. H. Reuning and G. Levy, *J. Pharm. Sci.*, **57**, 1556(1968).

(5) M. Nakano and N. K. Patel, *ibid.*, **59**, 77(1970).

(6) M. Nakano, *ibid.*, **60**, 571(1971).

(7) H. B. Kostenbauder, H. G. Boxenbaum, and P. P. Deluca, *ibid.*, **58**, 753(1969).

(8) V. K. Smirnov and V. A. Berestnev, *Tekst. Prom. (Moscow)*, **29**, 58(1969); through *Chem. Abstr.*, **72**, 56544(1970).

(9) N. K. Patel and N. Nagabushan, *J. Pharm. Sci.*, **59**, 264(1970).

(10) G. M. Miyawaki, N. K. Patel, and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 315(1959).

(11) G. Jurgensen Eide and P. Speiser, *Acta Pharm. Suecica*, **4**, 185(1967).

(12) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 289(1958).

(13) S. J. A. Kazmi and A. G. Mitchell, *J. Pharm. Pharmacol.*, **23**, 482(1971).

(14) P. Mukerjee, *J. Pharm. Sci.*, **60**, 1531(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 5, 1974, from the *Pharmaceutical Technology Laboratory, Pharmaceutical Chemistry Institute, University of Pisa, 56100 Pisa, Italy.*

Accepted for publication November 12, 1974.

Supported in part by a grant from the Consiglio Nazionale delle Ricerche.

* To whom inquiries should be directed.