Improved Transport Apparatus for Examining Diffusion of Drugs across Isolated Tissues and Synthetic Membranes

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Abstract □ A three-compartment diffusion apparatus that can accommodate synthetic as well as small isolated biological membranes such as rat intestine was developed and tested. The diffusion of drug from two outer donor cells into a central recipient cell allows for more membrane surface exposure and greater sensitivity in assaying as compared with previously published techniques. Reproducibility values for the synthetic membrane were equivalent to those obtained in more elaborate designs, while the reproducibility for biological membranes compared favorably with everted gut preparations and a similar diffusion apparatus. The clearance, per square centimeter of membrane, of salicylamide at pH 7.4 and 37° was 0.288 ml/hr/cm^2, which agreed with previous literature reports of different techniques. Although the diffusion through a synthetic membrane was more rapid, introduction of a nonionic surfactant enhanced the biological perfusion rate while having no significant effect on the synthetic barrier. The enhancement of the diffusion rate effected by 0.1 and 0.01% (w/v) surfactant was about twofold, rapid in onset, and not progressive, giving clearance values per square centimeter of 0.606 and 0.537 ml/hr/cm^2, respectively.

Keyphrases □ Diffusion—three-compartment apparatus developed, evaluated, and compared to other methods, synthetic membranes and isolated biological tissues studied using salicylamide □ Salicylamide—diffusion across synthetic membranes and isolated biological tissues, three-compartment apparatus developed, evaluated, and compared to other methods

Numerous methods have been devised to study the *in vitro* diffusion kinetics of drugs across biological and synthetic membranes. The most common of the biological studies involved some variation of the everted gut technique (1–6), while other systems were designed to accommodate one particular membrane (7–9). Not limited by the viability and size of living tissue, diffusion studies incorporating synthetic membranes have had more freedom in apparatus design (10–13).

Recently, Mayersohn and Suryasaputra (7) initiated studies of living tissue diffusion in an apparatus that can also accommodate synthetic membranes. Thus, both living and nonliving drug barriers can be studied under more identical conditions, the geometry of membrane placement and the mechanics of stirring being duplicated in both systems. Stirring of the serosal fluid, which cannot be readily achieved in everted guts, lends to a more uniform sampling, and elimination of eversion precludes any unnecessary damage to the tissue.

Ho (14) recently demonstrated that results obtained by the use of an apparatus such as that described by Doluisio *et al.* are, in part, dependent upon the sampling interval employed. Since the serosal compartment is not normally adequately stirred throughout the experiment, the sampling procedure in itself introduces additional stirring and enhances the diffusion. Thus, more frequently sampled experiments yield larger apparent diffusion rates. However, as mentioned previously (7, 10), the low diffusion area to recipient volume ratio is a major drawback to the diffusion cell method, the assay sample concentration being directly dependent upon this ratio.

This study concerns a modified model of the diffusion apparatus of Mayersohn and Suryasaputra (7), which allows for greater assay sensitivity (Fig. 1). The sandwiching in of the recipient cell, containing two membrane sections, with two donor cells obviously yields at least a twofold increase in diffusion area. In addition, it was possible to increase both the length and width of the diffusion window, thus exposing an even greater area to diffusion. The modified model allows for an apparent diffusion area of 5.9 cm^2, as compared to the 1.8 cm^2 achieved previously (Table I).

Since 20 ml of recipient solution was also used in this study, there was more than a threefold increase in the diffusion area–recipient volume ratio. In addition, the donor volume–diffusion area ratio was greatly increased, thus assuring a steady-state concentration gradient across the membrane. Donor solution concentrations decreased only 5.9 and 3.4% after 2-hr runs (salicylamide, 2 mg/ml) with and without surfactant, respectively.

EXPERIMENTAL

Materials—Figure 1 shows the three-compartment diffusion apparatus. The two outer donor compartments contain the appropriate mucosal solution, while the inner recipient compartment contains the serosal solution. The entire apparatus, with the exception of the securing bolts, wingnuts, and pins, was fabricated out of clear 95-mm (0.38-in.) plastic[1]. The water seal was facilitated by milling out a gasket around all four diffusion windows to give a plateau of minimal surface area.

A propeller stirrer[2] was used to agitate the recipient compartment; immersible magnetic stirrers[3], with 1.27-cm (0.5-in.) stirring bars[4], were used to stir the outer donor compartments. The removable recipient compartment cover has a silicone[5] gasket, giving a water seal when secured with small screws. The 48-mm (0.18-in.) securing bolts were set within the length of the diffusion windows so that the pressure could be distributed effectively to the mounting plateau where it is needed. [The bolts thus passed through the donor chambers *via* 63-mm (0.25-in.) diameter plastic tubing.]

The following reagent grade chemicals were employed: sodium chloride, potassium chloride, sodium bicarbonate, monobasic potassium phosphate, mercuric chloride, and ferric nitrate[6]. Salicylamide[7] (mp 140–142°) and the nonionic surfactant[8] polyoxy-

[1] Plexiglas, Rohm and Haas.
[2] Model 102, Talboy Engineering Corp.
[3] VWR Catalog No. 58947-055 (1972), Bronwill Scientific.
[4] Coated with Teflon (duPont).
[5] General Electric.
[6] Baker.
[7] Lot 711-1, Eastman Kodak.
[8] Brij 35, Lot 518, ICI American, Inc. (formerly Atlas).

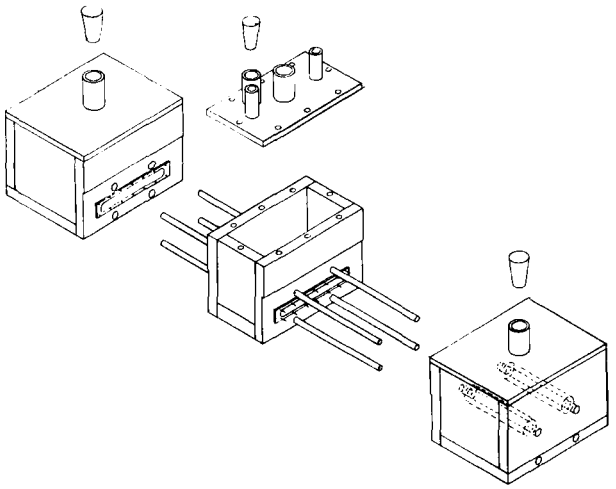

Figure 1—*Three-compartment diffusion apparatus. Inside dimensions are: outer (donor) cells, 50 × 72 × 50 mm; inner (recipient) cell, 32 × 72 × 50 mm; and diffusion windows, 5 × 70 mm.*

ethylene (23) lauryl ether (solid at room temperature) were the chemicals studied for diffusion characteristics. The gas mixtures were oxygen–carbon dioxide (95:5) and nitrogen–carbon dioxide (95:5). A regenerated cellulose[9] membrane, with a dry thickness of 2.5×10^{-3} cm (0.001 in.), was used as the synthetic membrane.

Procedure—*Living Membrane*—Male Sprague–Dawley rats (303–412 g), starved for 24 hr before use, were sacrificed in ether, and a midline abdominal incision was made to remove the small intestine at the pyloric sphincter. The intestine was immediately placed in Kreb's bicarbonate buffer, pH 7.4 (3, 5), at 10–15°; the buffer had been perfused previously with oxygen–carbon dioxide (95:5) for 20 min.

The initial 15 cm distal to the sphincter was discarded, and the first and second 10-cm sections thereafter were used for the diffusion runs. A 7-cm portion of each 10-cm section was sleeved on a rounded-end capillary tube, rinsed with buffer solution, cut lengthwise, and stretched over and secured by pins circumscribing the diffusion windows on the recipient compartment. A silicone[5] bung in the shape of the window was inserted to prevent the tissue from falling through during the mounting procedure.

After mounting the first segment with the mucosal surface facing the donor compartment, the appropriate donor cell was attached by guiding it on the bolts of the recipient compartment and securing it with wingnuts. The donor chamber was then filled with chilled buffer to bathe the tissue during the mounting of the second segment, which was treated similarly. The apparatus was then observed for 5 min to detect any mechanical or physiological leaks between compartments.

Once a good water seal was obtained, the donor compartments were rinsed and filled with buffered salicylamide solution, 2 mg/ml, previously perfused with oxygen–carbon dioxide. The compartments were then placed in a water bath maintained at 36.5 ± 1° throughout the experiment. *Via* thin polyethylene tubing[10], each compartment was constantly perfused with oxygen–carbon dioxide (washed through buffer solution) to maintain the pH at 7.4 ± 0.05. The run was commenced upon the addition of 20 ml of buffer solution to the recipient cell.

Synthetic Membrane—Regenerated cellulose, with a wet thickness of 34.7 ± 2.2 μm, was used as a comparison for the living tissue. Due to its thinness, it was necessary to use gaskets made out of plastic[11] to ensure a tight seal. Other than the use of a nitrogen–carbon dioxide (95:5) gas mixture instead of oxygen–carbon dioxide to maintain the buffer pH at 7.4, the synthetic runs were conducted in a similar fashion to the intestinal runs.

Studies on the effect of the stirring rate on the diffusion rate were conducted to optimize this important parameter in both donor and recipient compartments. An increase in the stirring rate of the recipient compartment to over 200 rpm caused an insignificant effect on the rate of drug diffusion. However, the rate was kept at about 250 rpm to ensure a homogeneous solution. Although the diffusion rate did not reach a maximum plateau value at 500 rpm in the donor compartments, the increase in diffusion was not significant enough to warrant such a high rate of stirring. A rate of 250 rpm was sufficient to minimize any stirring variance due to a fluctuation in the water pressure driving the immersible stirrers.

Table I—Comparison of Synthetic Membrane Diffusion Studies

Reference	Number of Determinations	Membrane	*RSD*, %	Temperature	Time of Experiment
10	23	Dimethyl	±6.29	37 ± 0.01°	—
12	5	Dimethyl	±7.0	37.3 ± 0.1°	8 hr
This study	12	Cellulose	±6.3	36.5 ± 1.0°	30 min

Assay—Ten-milliliter samples were taken from the recipient (serosal) compartment at 5-min intervals during the 1st hr of the run and every 10 min during the 2nd hr; the sample was replaced with 10 ml of fresh bicarbonate buffer. In calculation of transport rates, allowance was made for drug removed by sampling.

The salicylamide was assayed spectrophotometrically[12] at 540 nm and pH 1.5 using a modification of the Trinder method (15). The 10-ml samples were diluted to 25 ml with hydrochloric acid–potassium chloride buffer (pH 1.1) after the addition of 5 ml of Trinder's reagent. The samples from the intestinal studies were run through a 0.45-μm filter[13] to remove any tissue precipitate before a final adjustment to pH 1.5 was made.

A simple shake-foam test was used to detect the presence of surfactant in the serosal compartment. At the end of each diffusion study, a 5-ml serosal sample was shaken vigorously in a test tube for 5 sec and then observed; the time required for the foam to subside was noted. The results were compared with a calibration curve prepared from a range of known surfactant concentrations [1×10^{-1}–1×10^{-6}% (w/w)] in Kreb's bicarbonate buffer containing 0.2 mg/ml of salicylamide.

The foam time increased significantly for each 10-fold increment in surfactant concentration and was sensitive to 1×10^{-4}% (w/w). The foam time for a blank sample (no surfactant) was 124.6 ± 18 sec, as compared to a foam time of 4498 ± 322 sec for 0.0001% (w/v) surfactant. This difference was significant at the 99% level of confidence ($p < 0.01$).

RESULTS AND DISCUSSION

As can be seen from the data in Table I, the reproducibility of the synthetic membrane runs, with no surfactant, was equivalent to other diffusion apparatus. The reproducibility of seven intestinal runs with no surfactant compared favorably with previously reported data (Table II). Although the relative standard deviation was higher than that of everted gut studies, it compared well with the monodonor cell. The larger relative standard deviation can be explained in part by the greater age and weight variance of the rats. Rats used for the surfactant studies were younger (weighed less) and had a narrow weight range with a subsequent lower relative standard deviation, comparable with the everted intestinal studies (Table III).

As before (4, 6), there was no significant difference between the clearance rates for the 1st and 2nd hr of the intestinal runs. Thus, they were combined and included in Table III. Kreb's bicarbonate buffer was partly responsible for this effect; it has been shown to maintain biological material in a more constant and viable state than other buffer systems (5).

The surfactant's effect on the biological membrane was of rapid onset and was not progressive for at least 2 hr since the diffusion rate remained constant throughout the experiment. Current work

[9] Catalog No. 25225-260 (1972), VWR Scientific.
[10] Intramedic, Clay Adams.
[11] Parafilm, American Can Co.
[12] Perkin-Elmer 139.
[13] Millipore Corp.

Table II—Comparison of *In Vitro* Diffusion Studies Using Rat Intestine

Technique	pH of Kreb's Bicarbonate Buffer	Mucosal Surface Area, cm^2	Volume, ml: Mucosal Solution (Donor)	Volume, ml: Serosal Solution (Recipient)	Ratio of Diffusion Area to Recipient Volume	Ratio of Donor Volume to Diffusion Area	Donor Salicylamide Concentration, mg/ml	Number of Experiments	Clearance, ml/hr	Clearance, $ml/hr/cm^2$ ± *RSD*
Everted gut (3)	7.4	13.0 (7)	100	2	6.50	7.7	1.0	5	3.42	0.263 ± 4%
Single-donor cell apparatus (7)	7.4	1.79[a]	20	20	0.0894	11.2	4.0	7	0.287	0.160 ± 12%
Three-compartment apparatus (this study)	7.4 ± 0.05	5.9[a]	340	20	0.295	57.6	2.0	7	1.63	0–60 min, 0.276 ± 14.9% 60–120 min, 0.296 ± 19.3%

[a] Based on diffusion window dimensions.

Table III—Summary of Diffusion Studies Using Three-Compartment Apparatus

Membrane	Number of Experiments	Range of Rat Weights, g	Surfactant, % (w/v)	Clearance per Unit Area, $ml/hr/cm^2$: 0–60 min	60–120 min	0–120 min	Range[a]
Cellulose	12	—	0.0	1.114 ± 6.3	—	—	—
Cellulose	3	—	0.1	1.167 ± 5.2	—	—	—
Intestine	7	320–412	0.0	0.276 ± 14.9	0.296 ± 19.3	0.288 ± 16.75	—
Intestine	3	305–332	0.1	0.582 ± 7.7	0.630	0.606	0.533–0.686
Intestine	2	303–326	0.01	0.532 ± 7.2	0.538	0.537	0.505–0.553

[a] The 1st and 2nd hr are taken separately.

is being focused on the reversibility of this effect.

Wolfe *et al.* (4) showed, by removing the intestinal serosa and muscularis externa, that these tissues were the rate-limiting barriers in transport studies conducted on unstripped rat intestine. The increased diffusivity effected by the surfactant could be rationalized by either one or both of the following:

1. The surfactant increased the permeability of the muscularis externa and serosa.

2. The surfactant increased the partitioning of the drug into the inner, mucosal-side, tissue layers of the intestine, thus creating a greater concentration gradient across the muscularis externa and serosa.

The fact that the shake-foam test of recipient cell solutions showed no evidence of the surfactant's ability to cross the intestinal barrier makes the second explanation more probable.

Table II summarizes the results and pertinent apparatus parameters for the everted gut, single-donor cell, and our modified model. This study's clearance per square centimeter is more in agreement with the eversion technique than the Mayersohn cell. As mentioned previously (7), the possible distention of the everted gut would expose more of the convoluted surface to the muscosal solution, thereby increasing the clearance. Since the diffusion window was wider than in the previous cells, it was necessary to stretch the segment of intestine more during the mounting procedure, thus duplicating this distention. It was felt that stretching the tissue provided a more reproducible means of mounting, whereas it would be difficult to judge how much tissue was actually exposed in the window of an unstretched segment.

Preliminary studies were conducted on the effect of differences between the height of solutions in the donor and recipient cell on transport rates. Data obtained indicate that major effects are unlikely to be produced by variation in hydrostatic pressure. However, unless there is specific interest in investigating possible hydrostatic involvement in transport, it may be advantageous to use this apparatus with equal heights of solutions in all three compartments.

In conclusion, the economical and reproducible diffusion apparatus discussed here shows great potential for correlating *in vitro* drug diffusion between living and nonliving membranes. The increased surface area available for perfusion would make studies involving less viable tissue and/or slower diffusing drugs more amenable to current assay sensitivities. Further work is planned for other surfactant systems and drugs.

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