Topical Vaginal Drug Delivery I: Effect of the Estrous Cycle on Vaginal Membrane Permeability and Diffusivity of Vidarabine in Mice

C. C. HSU, J. Y. PARK, N. F. H. HO, W. I. HIGUCHI ˣ, and J. L. FOX

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Abstract □ Preliminary studies showed that the vaginal membrane permeability coefficients for vidarabine (9-β-D-arabinofuranosyladenine) varied widely within a group of mice of the same species and age. This finding prompted an investigation of the influence of the female mouse sexual cycle on the vidarabine permeability. By means of a vaginal smear technique, the sexual cycle, which was ~5 days in duration, was divided into five phases. The vaginal membrane permeability of vidarabine was determined during each phase. The results revealed that the permeability coefficients for vidarabine during the diestrus phase (3×10^{-6}–4×10^{-5} cm/sec) were 10–100 times higher than those obtained at the early estrus or estrus phases ($1–3 \times 10^{-7}$ cm/sec). Further permeation studies on membranes at early estrus and estrus were performed by separating the cornified layer from the noncornified portion of the membrane. The low permeability coefficient of vidarabine across the cornified layer (4×10^{-7} cm/sec) suggests that this layer may be the major diffusion barrier for vidarabine when the drug is topically applied. Collectively, the data also suggest that during estrus a three-layer diffusion model is appropriate, that during early diestrus a single-layer diffusion model may apply, and that during proestrus and postestrus the situations are intermediate and more complicated.

Keyphrases □ Vidarabine—vaginal membrane permeability in mice, diffusion barriers, effect of the estrous cycle □ Permeability—vaginal membrane to vidarabine, determination of coefficients, effect of the estrous cycle □ Estrous cycle—effect of vaginal membrane permeability to vidarabine in mice

A physical model of simultaneous transport and bioconversion of vidarabine (9-β-D-arabinofuranosyladenine, I) in the hairless mouse skin has been reported, (1, 2). It was concluded that the low efficacy of I in topical therapy involving skin may be due to the extremely low permeability of I through the stratum corneum and the rapid enzymatic decomposition of I to 9-β-D-arabinofuranosylhypoxanthine (II). In more recent reports (3, 4), the model was explored in detail, and a prodrug of I, vidarabine-5′-valerate (III), was investigated for its ability to overcome both the low permeability and enzymatic decomposition problems.

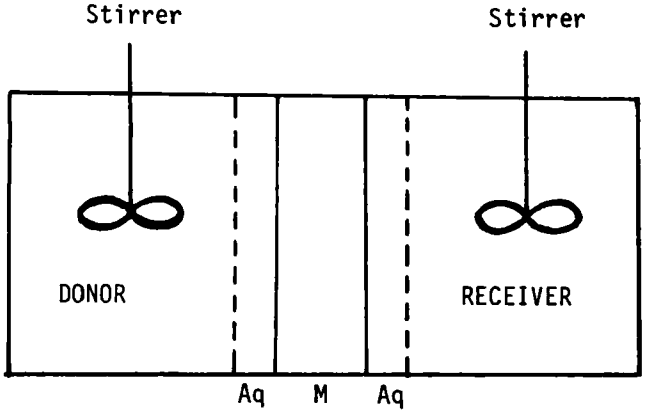

Figure 1—*Diffusion cell for the membrane permeability coefficient determinations. Key: (M) membrane; (Aq) aqueous diffusion layer.*

Since infections of the human genital tract with Herpes simplex virus (HSV types 1 and 2) have become increasingly important (5–7), it is desirable to investigate the deliverability of I, an antiviral drug, in the genital tract using an animal model. In the present report the permeability and diffusivity parameters of I were studied on excised vaginal membranes of the female mouse, with particular emphasis on how the estrous cycle may affect these transport parameters.

THEORETICAL

Aqueous Diffusion Layer Permeability Coefficient—Often the aqueous diffusion layer is unimportant in influencing overall transport in membrane diffusion experiments. However, in instances where the aqueous diffusion layer thickness is relatively large, its influences on both the steady-state transport and the lag time may be appreciable. The aqueous diffusion layer permeability (P_{aq}) of I may be calculated from:

$$P_{aq} = \frac{D_a}{h_{aq}} \qquad \text{(Eq. 1)}$$

where D_a is the aqueous diffusivity of I and h_{aq} is the aqueous diffusion layer thickness. The aqueous diffusion layer thickness has been determined (4) from initial dissolution rate data obtained using benzoic acid disks placed in the same configuration as the membrane in the diffusion cell by means of:

$$\frac{R}{A} = \left(\frac{D}{h_{aq}}\right)(C_s) \qquad \text{(Eq. 2)}$$

where R is the initial dissolution rate of benzoic acid, A is the effective diffusional area, C_s is the aqueous solubility of benzoic acid, and D is the aqueous diffusivity of benzoic acid. The initial dissolution rate of benzoic acid is the initial linear slope obtained by plotting the amount of benzoic acid dissolved in the diffusion cell against time. The aqueous diffusivity of I can be estimated from:

$$D_a = \left(\frac{M}{M_a}\right)^{1/3} D \qquad \text{(Eq. 3)}$$

where M_a is the molecular weight of I and M is the molecular weight of benzoic acid.

Vaginal Membrane Permeability Coefficient—The vaginal membrane permeability coefficient (P) of I can be determined by conducting a permeation experiment in a diffusion cell (Fig. 1). In such an experiment the steady-state flux of I across the membrane is equated to the product of the apparent permeability coefficient and the concentration gradient:

$$\left(\frac{dC_t}{dt}\right)\left(\frac{V}{A}\right) = \frac{1}{\frac{1}{P} + \frac{1}{2P_{aq}}}(C_t^d - C_t) \qquad \text{(Eq. 4)}$$

Where V is the volume of solution in the receiver cell, C_t^d is the donor side concentration of I at time t, and C_t is the concentration of I in the receiver cell at time t. When only initial steady-state flux is used, C_t is much smaller than C_t^d, and $C_t^d - C_t \approx C_0$, where C_0 is the initial donor side concentration of I. Equation 4 may be simplified and solved to give:

$$C_t = \left(\frac{A}{V}\right) C_0 \left(\frac{1}{\frac{1}{P} + \frac{1}{2P_{aq}}}\right)(t - L) \qquad \text{(Eq. 5)}$$

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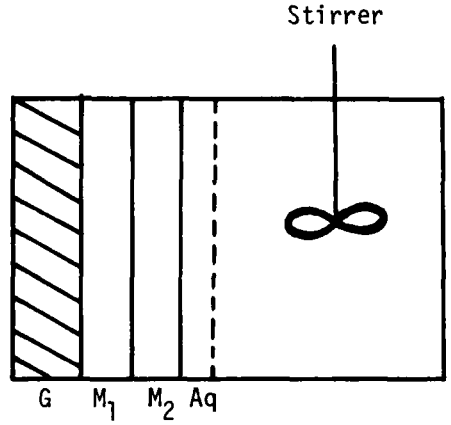

Figure 2—*Diffusion chamber modified for a bilayer membrane desorption experiment. Key: (M_1) membrane sublayer 1; (M_2) membrane sublayer 2; (G) glass block; (Aq) aqueous diffusion layer.*

where L is the lag time and may be obtained from experimental data as the intercept on the time-axis when the steady-state C_t values are plotted against time. The membrane permeability coefficient of I can thus be calculated from the initial linear slope of the concentration–time plot.

Calculation of the Membrane Thickness from the Lag Time—If the vaginal membrane can be viewed as mono- or multi-ply laminate-like skin, it is possible to calculate the theoretical membrane thickness from the permeation lag time. The general relationship between the lag time and the layer thickness for an n-layer laminate (8) is:

$$L = \left[\sum_{i=1}^{n}\left(\frac{h_i}{D_i}\prod_{j=0}^{i-1}k_j\right)\right]^{-1}\left[\sum_{i=1}^{n}\left\{\frac{h_i^2}{2D_i}\sum_{i=i}^{n}\left(\frac{h_i}{D_i}\prod_{j=0}^{i-1}k_j\right)-\frac{h_i^3}{3D_i}\prod_{j=0}^{i-1}k_j\right\}\right.$$

$$\left.+\sum_{i=1}^{n}\left\{\frac{h_i}{D_i}\left(\prod_{j=0}^{i-1}k_j\right)\sum_{\beta=i+1}^{n}\left[\frac{h_\beta}{\prod_{j=0}^{\beta-1}k_j}\sum_{\beta=\beta}^{n}\left(\frac{h_\beta}{D_\beta}\prod_{j=0}^{\beta-1}k_j\right)-\frac{h_\beta^2}{2D_\beta}\right]\right\}\right] \quad \text{(Eq. 6)}$$

where L is the lag time, k_i is the partition coefficient between ith and (i + 1)th layers, D_i is the diffusivity of ith layer, and h_i is the thickness of ith layer.

In the case of a homogeneous membrane, the total number of layers in Eq. 6 is 3 (n = 3), because there exist two identical aqueous diffusion layers, one on each side of the membrane. Also, if it is assumed that the partition coefficient of I between viable tissue and water is unity [which is a good assumption for the solute in this study (4)], then:

$$P_i = \frac{D_i}{h_i} \quad \text{(Eq. 7)}$$

where P_i is the permeability coefficient of the ith layer. Equation 6 can be rewritten as:

$$L = \left(\frac{2}{P_{aq}}+\frac{1}{P}\right)^{-1}\left[\frac{h_{aq}}{P_{aq}}\left(\frac{4}{3P_{aq}}+\frac{1}{P}\right)+\frac{h}{P}\left(\frac{1}{P_{aq}}+\frac{1}{6P}\right)+\frac{h}{P_{aq}^2}\right] \quad \text{(Eq. 8)}$$

where p is the permeability of homogeneous membrane and h is the membrane thickness.

Membrane Diffusivity from a Desorption Experiment—To confirm the diffusivity values obtained from the permeation experiments, an alternative experiment, called the desorption experiment, may be conducted which gives a direct estimate of the membrane diffusivity. In this method, the initial desorption rate of a membrane that has been loaded with a known concentration of drug is measured. The membrane diffusivity is then determined from:

$$\frac{dC_t}{dt}\frac{V}{A} = C_0\sqrt{\frac{D}{\pi t}} \quad \text{(Eq. 9)}$$

and

$$C_t = \left(\sqrt{\frac{4C_0^2A^2D}{V^2\pi}}\right)\sqrt{t} \quad \text{(Eq. 10)}$$

where C_t is the solution concentration at time t, V is the volume of the diffusion cell solution, A is the effective diffusional area, C_0 is the initial drug concentration in the membrane, and D is the diffusivity of the membrane. Equation 9 is solved to give Eq. 10. By plotting the C_t *versus* $\sqrt{t}$, the diffusivity of the membrane can be calculated easily from the initial slope.

If the membrane is effectively multilayered, only the diffusivity of the sublayer facing the diffusion chamber is determined from the initial desorption rate. In the simplest case of a bilayer membrane, the diffusivity of each layer may be measured independently with this method (Fig. 2).

Table I—Microscopic Characteristics of the Vaginal Smear as a Function of the Sexual Cycle

Phase	Duration, days	Microscopic Characteristics of Vaginal Smears
Diestrus	1–3	Exclusively leukocytes
Proestrus	~1	Leukocytes and nucleated epithelial cells
Early estrus	0.5–1	Epithelial cells, may have some cornified cells
Estrus	0.5–1	Exclusively cornified cells
Postestrus	~1	Leukocytes and cornified cells

Table II—Aqueous Diffusion Layer Permeability Coefficients of I

Experiment	$h_{aq} \times 10^2$, cm	$P_{aq} \times 10^3$, cm/sec
1	1.07	1.01
2	1.00	1.08
3	1.02	1.06
Average	1.03	1.05

EXPERIMENTAL

Materials—[^{3}H]-2-I[1] was used after being purified by high-performance liquid chromatography (HPLC), and the purity was further checked by TLC before experiments were started. [^{3}H]-2-II was prepared from [^{3}H]-2-I by treating the latter with adenosine deaminase[2] and TLC purification. Female Swiss Webster mice[3], 10–14 weeks old, were used for the experiments. Liquid scintillation counter cocktail[4], normal saline solution[5], 1% methylene blue in methanol solution, cellulose membrane[6], sulfuric acid[7], and sodium sulfite[7] were also used in the experiments.

Diffusion Cells—Because of the small available area of the mouse vaginal membrane (~0.8 cm × 1.0 cm), specially designed diffusion cells were used in these experiments. The outside dimensions of the glass diffusion cell are shown in Fig. 3. It has two ports, one for the stirrer and the other for sampling access. The stirrer port was 2.2 cm in length and the sampling port was 1.0 cm in length. The diameter of the cell opening was 0.6 cm, which provides an effective diffusional area of 0.28 cm^2 for a cell volume of 1 ml. The stirrer was made of polytetrafluoroethylene[8] and was operated by a constant-speed motor[9].

Vaginal Membrane Preparation—The female mouse was sacrificed by snapping the spinal cord at the neck. The lower abdomen was cut open, and the fat tissue was removed to reveal the Y-shaped reproductive system. A pair of hemostatic forceps were clamped on the uterus ~0.5 cm above the cervix to fix the position while the pubic bone was cut open, and the connection between the system and the surrounding tissue was carefully removed. The vagina, which is located between the cervix and the orifice, was then separated from the uterus. The tube-shaped vagina was carefully checked to free any tissue debris from the surface prior to cutting it open and using it for the experiment.

In cases where only the noncornified part of the membrane was to be used for an experiment, the vagina of the mouse at estrus was obtained by the aforementioned method. After cutting open the membrane, the cornified layer, which is on the mucosal side, was completely removed from the rest of the membrane with a pair of tweezers.

[1] Courtesy of Dr. T. H. Haskell, Warner-Lambert Labs., Ann Arbor, MI 48105.
[2] Sigma Chemical Co., St. Louis, MO 63178.
[3] Charles River Breeding Labs., Inc., Wilmington, Mass.
[4] Amersham Corp., Arlington Hts., IL 60005.
[5] 0.9% NaCl irrigation USP, Abbott Labs., N. Chicago, IL 60060.
[6] Spectrapor #1, Spectrum Medical Industries, Inc., Los Angeles, CA 90054.
[7] Fisher Scientific Co., Fair Lawn, NJ 07410.
[8] Teflon; registered trademark of E.I. du Pont de Nemours & Co., Inc., Wilmington, Del.
[9] Hurst Mfg. Co., Princeton, Ind.

Table III—Vaginal Membrane Stability with Respect to the Permeability of I

Cycle Phase	Mouse	Permeability Coefficient × 10^6, cm/sec					
		Fresh	t = 4 hr	t = 8 hr	t = 16 hr	t = 20 hr	t = 27 hr
Diestrus	79013	30.8	29.6	30.7	74.0	—[b]	97.1
	79016	41.1	35.2	39.5	50.5	—	63.4
	79018	35.1	33.8	34.4	56.5	—	71.2
	79023	30.3	38.9	26.6	50.4	—	71.5
	79051	16.2	14.5	21.1	56.4	67.7	—
	79030	08.5	07.8	—	—	—	—
	79020	03.4	03.2	—	—	—	—
	79045	06.5	02.7	01.8	—	—	—
	79044	05.9	03.9	03.8	12.6	16.5	—
	79052	04.7	01.1	01.8	04.9	06.3	—
	79053	05.1	05.6	16.9	53.5	58.6	—
Estrus[a]	79043	0.98	0.65	0.55	—	—	—
	79009	0.93	0.74	—	—	—	—
	79067	1.25	—	0.90	—	—	—

[a] Only the noncornified part of the estrus membrane was used in this experiment. [b] — Not determined.

Vaginal Membrane Thickness Measurement—A micrometer[10] was used to obtain the membrane thickness. The vaginal membrane, after being removed from the body and cut open, was sandwiched between two microscope cover glasses. The total thickness was then measured with the micrometer. The membrane thickness was obtained by subtracting the thicknesses of the cover glasses from the total thickness.

Mouse Estrous Cycle Determination—The female mouse estrous cycle could be determined easily by microscopic examination of the vaginal smears (9). The vaginal smears were obtained by means of an ordinary pipet, the tip of which had been flamed to a smooth, reduced aperture. A few drops of normal saline solution were drawn into the pipet, introduced into the vagina, and then retracted into the pipet. The fluid was transferred to a microscope slide and stained with a drop of methylene blue to add contrast and bring out the nuclei. Examination for cell type was carried out under low- and high-power magnification. Three major types of cells were found in the vaginal smears: leukocytes, epithelial cells, and cornified epithelial cells. The estrous cycle phase was determined by the types of cells present in the vaginal smears (Table I).

Aqueous Permeability Coefficient Determination—Benzoic acid pellets were made by directly compressing 100 mg of the material in a die (1.3-cm i.d.) under a force of 1362 kg (3000 lb) using a laboratory press[11]. The pellet was mounted between one chamber of a diffusion cell and a same-size glass plate. The whole setup was coated with wax and immersed in a 37° water bath. One milliliter of 0.01 *N* HCl, pre-equilibrated at 37°, was introduced into the cell at time zero. The benzoic acid concentration in the solution was determined by UV absorption at 227 nm against a standard curve.

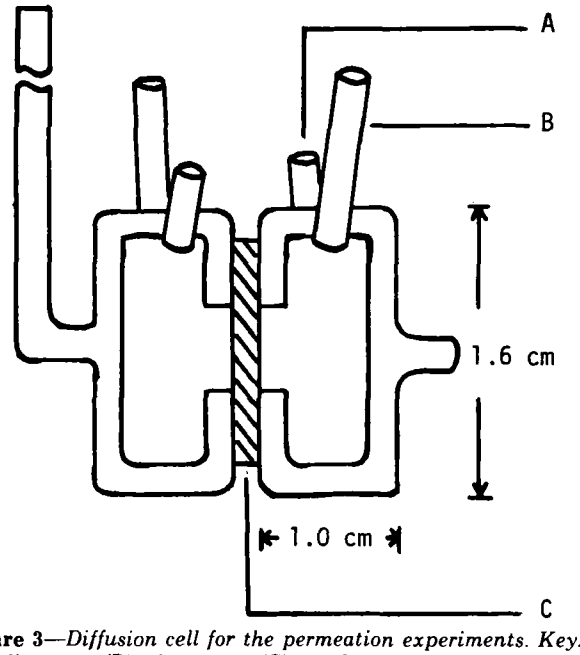

Figure 3—*Diffusion cell for the permeation experiments. Key: (A) sampling port; (B) stirrer port; (C) membrane.*

Vaginal Membrane Permeability Coefficient Determination—As soon as the vaginal membrane was sandwiched between two diffusion cells, 0.9 ml of the normal saline solution, pre-equilibrated at 37°, was introduced into each chamber. The diffusion cell setup was then immersed in a 37° water bath and was allowed to stand for 5 min to reach thermal equilibrium. Normal saline solution (100 μl) was added to the receiver chamber at the end of this 5-min period, and [^{3}H]-2-I or [^{3}H]-2-II in 100 μl of normal saline solution was added to the donor chamber by pipet to start the experiment. Aliquots (100 μl) from the receiver solution were drawn with a micropipet at given time intervals and were transferred immediately into a scintillation vial containing 10 ml of scintillation counter cocktail. An equal volume of normal saline solution was added to the receiver chamber after each draw to replace the removed aliquot. The donor solution concentrations at the first half-minute and at the end of the experiment also were determined in the same way to obtain the initial concentration and to check the mass balance.

Preparation of Cellulose Membrane—The cellulose membrane was cut to a suitable size before it was soaked in running water for 3–4 hr to remove the glycerol. The membrane was then treated with 0.3% sodium sulfite solution, preheated to 80°, for 1 min followed by washing with 60° distilled water for 2 min. The washed membrane was acidified with 0.2% sulfuric acid and then cleaned with distilled water. The purpose of this procedure was to remove sulfides on the membrane.

Cellulose Membrane Permeability Coefficients—Experiments to determine the cellulose membrane permeability coefficients were performed in the same manner as the determination of vaginal membrane permeability coefficients, except that the cellulose membrane was mounted in the diffusion cell.

Stability of the Vaginal Membrane—The vaginal membrane stability under the experimental conditions was studied by performing consecutive permeation experiments on the same membrane. The membrane was washed several times with normal saline solution for 2 hr between the two experiments. The washing solution from both chambers was checked to make certain that the membrane was free of any residue from the previous run. When the cellulose membrane was used to increase the vaginal membrane stability, the excised vaginal membrane was sandwiched between two pieces of cellulose membrane

Table IV—Stability of Cellulose Membrane-Protected Vaginal Membrane with Respect to the Permeability of I[a]

Cycle Phase	Mouse	Permeability Coefficient × 10^6, cm/sec				
		Fresh	t = 2 hr	t = 4 hr	t = 8 hr	t = 24 hr
Diestrus	80113	4.6	5.0	5.2	4.5	7.8
	80139	3.8	3.6	3.2	3.6	3.5
Estrus[b]	80146	1.10	—[d]	1.02	1.08	—
	80160	1.17	—	1.21	1.15	—
Cellulose membrane[c]	—	90.0	—	91.0	—	89.5

[a] The membrane was sandwiched between two pieces of cellulose membrane before being mounted in the diffusion cells. Each permeability measurement was conducted for 2 hr. The membrane was washed with normal saline between runs. [b] Noncornified part of the membrane. [c] The reported values have been corrected for the presence of aqueous diffusion layers. [d] — Not determined.

[10] L. S. Starrett Co., Athol, MA 01331.
[11] Model B, Fred Carver, Inc., Summit, N.J.

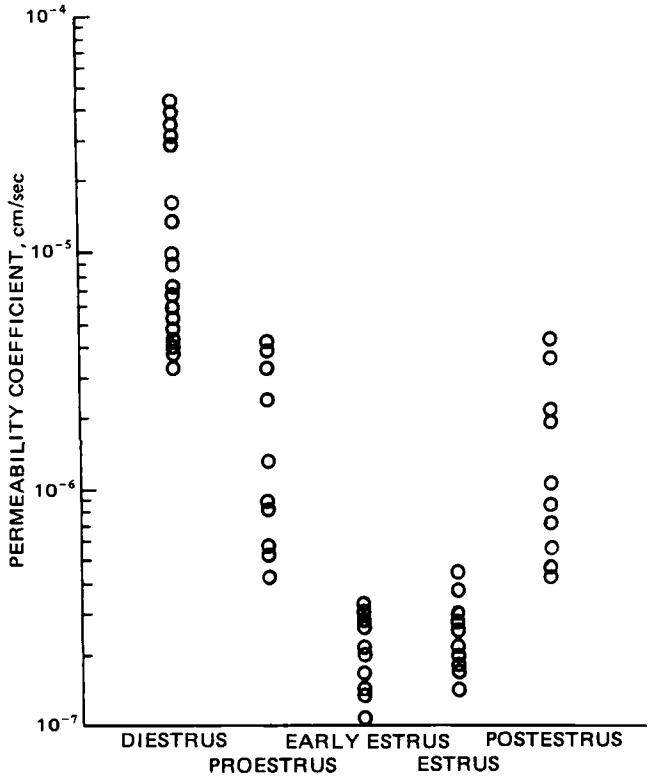

Figure 4—*Vaginal membrane permeability coefficients of I during different estrous cycle phases.*

before being mounted in the diffusion cell. This procedure was carried out very carefully to prevent any air bubbles from being trapped between the vaginal and cellulose membranes.

Desorption Experiment—The prepared vaginal membrane was soaked for 3 hr in 0.5 ml of normal saline with a known amount of compound I in the solution. It was then mounted between a diffusion cell and a glass block. One milliliter of normal saline, pre-equilibrated at 37°, was added to the diffusion chamber at time zero. The concentration in the diffusion cell was determined in the same manner used in the membrane permeability experiment.

RESULTS AND DISCUSSION

Aqueous Diffusion Layer Permeability—Three benzoic acid dissolution experiments were conducted which gave an average aqueous

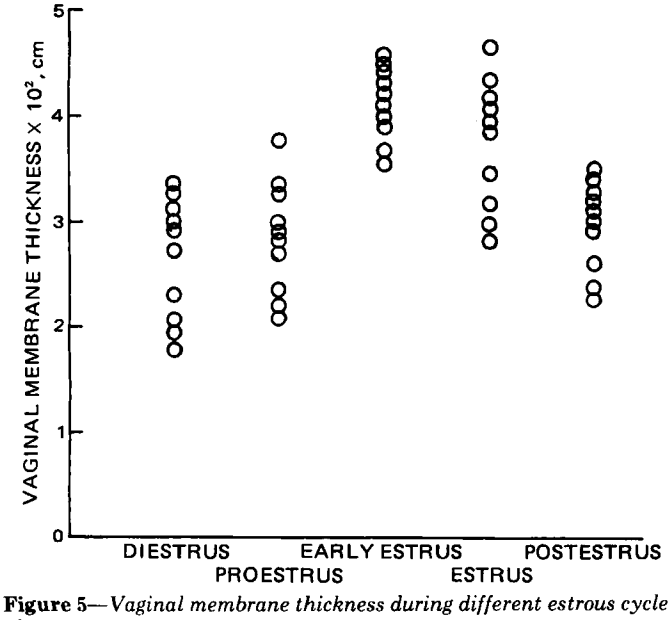

Figure 5—*Vaginal membrane thickness during different estrous cycle phases.*

Table V—Comparison of Vaginal Membrane Permeability Coefficients of I and II[a]

Cycle Phase	Mouse	Permeability Coefficient[b] × 10^6, cm/sec I	II
Diestrus	80157	9.24 (1)[c]	9.37 (2)
	80233	5.28 (2)	5.40 (1)
Estrus[d]	80229	1.34 (1)	1.21 (2)
	80228	1.01 (2)	1.08 (1)

[a] Membranes were protected with cellulose membrane to increase stability. [b] Covidarabine, an adenosine deaminase inhibitor, was used in the permeability coefficient measurement of I to inhibit the conversion of I to II. [c] The number in the parentheses indicates the run order. [d] Noncornified part of the membrane.

Table VI—Statistical Comparison of Vaginal Membrane Permeability Coefficients of I During Different Cycle Phases[a]

	Significance[b]				
	Diestrus	Proestrus	Early estrus	Estrus	Postestrus
Diestrus	—	0.0000	0.0000	0.0000	0.0000
Proestrus	0.0000	—	0.0002	0.0002	0.6501
Early estrus	0.0000	0.0002	—	0.5706	0.0002
Estrus	0.0000	0.0002	0.5706	—	0.0002
Postestrus	0.0000	0.6501	0.0002	0.0002	—

[a] Nonparametric Mann-Whitney method was used in this statistical comparison. [b] Significance value smaller than 0.05 was considered as an indication of significant difference between phases.

Table VII—Statistical Comparison of Vaginal Membrane Thicknesses During Different Cycle Phases[a]

	Significance[b]				
	Diestrus	Proestrus	Early estrus	Estrus	Postestrus
Diestrus	—	0.5437	0.0002	0.0035	0.2879
Proestrus	0.5437	—	0.0003	0.0040	0.6493
Early estrus	0.0002	0.0003	—	0.3047	0.0002
Estrus	0.0035	0.0040	0.3047	—	0.0100
Postestrus	0.2879	0.6493	0.0002	0.0100	—

[a] Nonparametric Mann-Whitney method was used in this statistical comparison. [b] Significance value smaller than 0.05 was considered as an indication of significant difference between phases.

Table VIII—Permeability Coefficients of I in the Noncornified Part of the Vaginal Membrane at the Estrus Phase

Mouse	Permeability Coefficient × 10^6, cm/sec
79009	0.93
79043	0.98
79067	1.25
80109	1.32
80122	1.32
80127	1.00
Average	1.13 ± 0.07

diffusion layer thickness of 1.03×10^{-2} cm. The aqueous diffusivity of I was estimated from Eq. 3 using a reported value (10) of 1.4×10^{-5} cm^2/sec for the aqueous diffusivity of benzoic acid. The aqueous diffusion layer permeability coefficient was then calculated from Eq. 1 (Table II).

Stability of the Vaginal Membrane—The vaginal membrane stability was checked at two different cycle phases (Table III). The membranes obtained during the diestrus phase could be divided roughly into two groups. Those with lower permeability coefficients ($4–9 \times 10^{-6}$ cm/sec) showed different degrees of drop in the P-values for the first 4–8 hr followed by a slow increase in P afterward. On the other hand, the membranes with high P-valves ($3–5 \times 10^{-5}$ cm/sec) were fairly stable up to 8 hr. The membranes obtained at estrus failed to show good stability.

It was found that membranes protected with the cellulose membrane had increased stability (Table IV). The permeability coefficients were essentially constant up to 8 hr for the protected vaginal membranes. This technique was of great value for those situations in which a direct comparison of P-values was desired using the same vaginal membrane. The

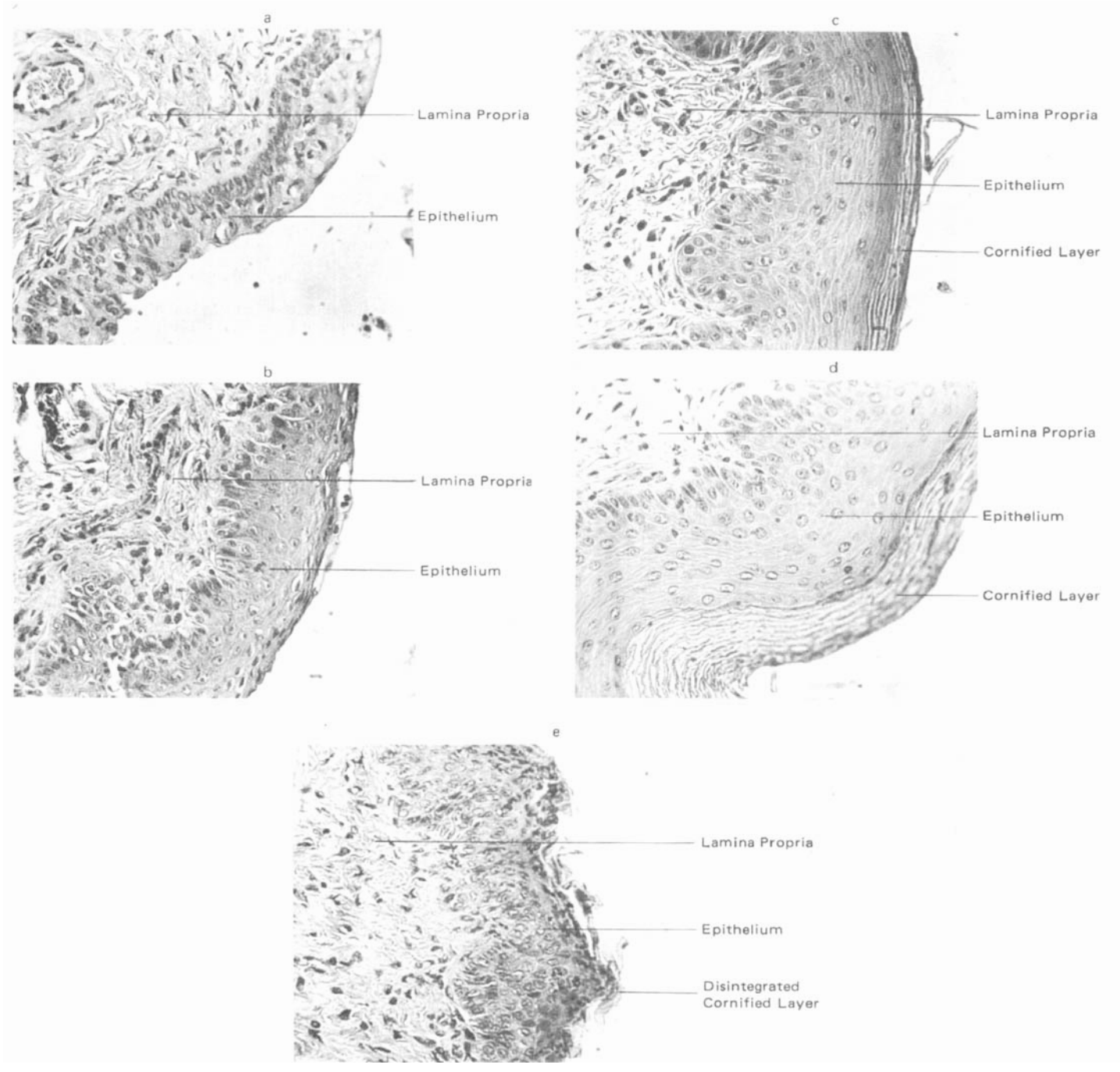

Figure 6—*Cross sections of mice vaginal membrane during different estrus cycle phases. Key: (a) diestrus phase; (b) proestrus phase; (c) early estrus phase; (d) estrus phase; (e) postestrus phase.*

cellulose membrane permeability coefficients of I are also listed in Table IV.

Permeability Coefficients of I and II—Since an adenosine deaminase inhibitor was not used in the experiments, it was necessary to prove that I and its metabolite, II, possessed the same permeability coefficient in the vaginal membrane. The permeability coefficients for both I and II were obtained on the same piece of vaginal membrane using the aforementioned membrane protection technique. As expected, the vaginal membrane permeability coefficients of I and II were essentially identical regardless of the cycle phase (Table V).

Table IX—Permeability Coefficient of Cornified, Noncornified, and Full-Thickness Membrane During the Estrus Phase

Membrane Preparation	Thickness[a] × 10^4, cm	Permeability Coefficient × 10^7, cm/sec
Cornified layer	40	2.98[b]
Noncornified layer	320	11.3 ± 0.07[c]
Full-thickness membrane	410	2.36 ± 0.07

[a] An estimate from direct measurement of the separated cornified layer and from microscopic measurement of the membrane cross section. [b] Calculated from permeability coefficients of the noncornified layer and full-thickness membrane using Eq. 12. [c] Average value from Table VIII.

Effect of the Estrous Cycle on the Vaginal Membrane Thickness and Permeability Coefficient—The permeability coefficient data collected from 58 permeability experiments were grouped according to the different phases of the cycle (Fig. 4). Statistical comparison of these five groups of data was done with the nonparametric Mann-Whitney method[12] and the results are reported in Table VI. It was clear that the differences between the permeability coefficients at the diestrus phase and those at any other phase were highly significant. On the other hand, no significant difference in the permeability coefficients between the early estrus and estrus phases was shown, although these two phases were

[12] MIDAS program; Michigan Terminal System, University of Michigan, Ann Arbor, Mich.

Table X—Calculation of Theoretical Membrane Thickness from Permeation Lag Time

Cycle Phase	Mouse	Permeability Coefficient × 10^6, cm/sec	Membrane Thickness × 10^4, cm		Diffusivity[b] × 10^6, cm^2/sec
			Calculated[a]	Measured	
	65799	0.93	63	290	
Estrus[c]	47943	0.98	71	320	
	79067	1.25	66	310	
	80109	1.35	66	300	
	80122	1.32	70	330	
	80127	1.00	74	330	
Average		1.13 ± 0.07	68.3 ± 3.7	313 ± 7	
	79015	30.8	284	300	0.88
	79016	41.1	310	270	1.27
Diestrus[d]	79018	35.1	317	340	1.11
	79023	30.3	368	280	1.12
	79060	37.8	305	320	1.15
Average		35.0 ± 2.0	317 ± 14	302 ± 13	1.10 ± 0.06

[a] Calculated from the permeation lag time using Eq. 8, when $P_{aq} = 1.05 \times 10^{-3}$ cm/sec and $haq = 1.03 \times 10^{-2}$ cm. [b] Calculated (for diestrus only) from the permeability coefficient and measured membrane thickness using Eq. 13. [c] Noncornified part of the membrane only. [d] Early stage of the diestrous phase.

distinguishable with the vaginal smear technique. Since the permeability coefficient was also a function of membrane thickness, the estrous cycle effect on vaginal membrane thickness was estimated; such data are shown in Fig. 5. Although statistical treatment of the data (Table VII) indicated that the membrane thickness at the diestrus phase was different from the thickness at the early estrus or estrus phase, the difference was only about twofold. In contrast, the permeability coefficients of I at estrus or early estrus were at least 10 and up to 100 times lower than those at diestrus. This suggested that the change of the permeability coefficients for I during the estrous cycle probably was due mainly to the physiological changes in the membrane cells rather than simply the increase of membrane thickness. A cross section of the membrane at each phase was examined under a microscope (Fig. 6). It showed that the membrane consisted of either two or three layers, *i.e.*, a loose cornified epithelial layer, a regular epithelial layer, and a combined lamina propria and muscular layer. The cornified epithelial layer, which appeared only at estrus and early estrus, was suspected as the major barrier for the penetration of I into the membrane. The permeability data also showed, however, that the mean permeability coefficient of I for the membrane at proestrus (for which there is not a cornified layer) already was reduced to 1.8×10^{-6} cm/sec. This value was about 10-fold lower than the corresponding value at diestrus. Another point is the relatively wide variation in permeability coefficients of I observed during diestrus. All these suggest that although the cornified epithelial layer could be a primary barrier for the transport of I through the membrane, it was not the sole barrier. A decrease in permeability could be caused also by the changes in the cell properties during proestrus.

Further permeation studies on vaginal membrane at estrus were performed by separating the cornified epithelial layer from the noncornified part of the membrane, the latter consisting of an epithelial layer and the combined lamina propria and muscular layer. Since it was very difficult to obtain intact cornified epithelial layers, only the permeability coefficient of the noncornified part of the membrane was measured (Table VIII). The permeability coefficient of the cornified epithelial layer (P_1) could then be estimated indirectly from:

$$\frac{1}{P_1} = \frac{1}{P_{1+2}} - \frac{1}{P_2} \qquad \text{(Eq. 11)}$$

where the permeability coefficients for the full-thickness membrane at estrus (P_{1+2}) and the permeability coefficients of the noncornified part of the membrane (P_2) were determined experimentally. The results are reported in Table IX. Statistical comparison showed that the permeability coefficients of the noncornified part of the membrane at estrus were significantly lower than those values obtained for the membrane at diestrus. They were, in fact, comparable to the permeability coefficients of the membrane at proestrus. These findings support the previous suggestion that there is a cycle-related low permeability layer in the vaginal membrane in addition to the cornified layer.

Estimation of the Permeability Coefficients of Membrane Sublayers—To investigate the homogeneity of the membranes with respect to diffusivity, theoretical membrane thickness was calculated from permeation lag times using Eq. 8. Two special cases are discussed here. The first is the results obtained using the noncornified part of the membrane obtained at estrus. These results show that the calculated membrane thicknesses are much smaller than those obtained from direct measurement (Table X). The second case involves membranes obtained during the early stage of the diestrus phase. These were the membranes with very large P-values for I. The calculated membrane thicknesses in this case showed no statistically significant differences from measured membrane thickness (Table X). The results from the second case suggest that, although there were two distinct physiological layers in the membrane at this stage in the estrous cycle, the differences between the diffusivities of I in these two layers must be either small or negligible. Therefore, a homogeneous membrane model should be a proper approximation for the membrane at this stage as far as diffusivity is concerned. The diffusivity for I for this homogeneous membrane may be calculated (assuming $k_P = 1$) from:

$$D = P \cdot h \qquad \text{(Eq. 12)}$$

The calculated diffusivity is shown in Table X.

The inconsistency of the calculated and measured membrane thickness in the first case strongly indicates that the noncornified part of the membrane at the estrus phase was not homogeneous. Since it is believed that membrane changes during the estrous cycle involve mainly the epithelial cells (9), the diffusivity of I in the combined lamina propria and muscular layer may be viewed as essentially constant throughout the cycle. Accordingly, the average diffusivity of I in membrane during the early stage of the diestrus phase (Table X) could be used as the diffusivity in the combined lamina propria and muscular layer at estrus. By microscopic estimation, of the thickness of this layer was $\sim 2.5 \times 10^{-2}$ cm; therefore, the estimated permeability coefficient was 4.4×10^{-5} cm/sec. This high permeability coefficient value shows that the combined lamina propria and muscular layer has little influence on the total permeability of I at estrus. As a result, the average permeability coefficient of 1.13×10^{-6} cm/sec obtained from the noncornified part of the membrane at estrus could be treated as the permeability coefficient of the epithelial layer at this phase without causing any serious error.

Direct Measurement of Membrane Diffusivity from Desorption Experiment—The diffusivity data obtained from desorption experiments for both layers of the membrane during the early stage of the diestrus phase of the estrous cycle are given in Table XI. Although the diffusivity of I in the epithelial layer was lower than that of the combined

Table XI—Direct Measurement of the Diffusivity of I on Mouse Vaginal Membrane from the Desorption Experiments

Cycle Phase	Mouse	Diffusivity[a] × 10^7, cm^2/sec	
		Epithelial Layer	Combined Lamina Propria and Muscular Layer
	80099	8.91	09.6
Diestrus[b]	80111	8.50	13.0
	80102	8.63	08.3
	80126	9.41	12.6
Average		8.9 ± 0.2	10.9 ± 1.2
	80110	0.080	15.6
Estrus[c]	80149	0.108	17.1
	80136	0.089	14.2
	80141	0.097	17.5
Average		0.094 ± 0.006	16.1 ± 0.8

[a] Calculated from Eq. 10, where $k_P = 1$, $A = 0.28$ cm^2, and $V = 1$ cm^3. [b] Early stage of the diestrus phase. [c] Noncornified part of the membrane.

Table XII—Estimation of the Epithelial Layer Thickness from Permeability and Desorption Experiments[a]

Mouse	Measured Membrane Thickness $\times 10^4$, cm	Permeability Coefficient[a] $\times 10^6$, cm/sec	Diffusivity of Epithelial Layer[b] $\times 10^9$, cm^2/sec	Diffusivity of Combined Lamina Propria and Muscular Layer[b] $\times 10^6$, cm^2/sec	Epithelial Layer Thickness[c] $\times 10^4$, cm
80127	330	1.00	8.49	1.60	87.7
80122	320	1.32	9.53	1.74	70.8
80109	300	1.35	11.4	1.46	83.0
Average:					78.8

[a] Noncornified part of the membrane during the estrus phase. [b] Determined from the permeability experiments. [c] Determined from the desorption experiments. [d] Calculated from Eq. 13.

lamina propria and muscular layer, the differences were small. Thus, the homogeneous membrane model still could be considered a good approximation for the membrane at the early stage of diestrus; this is in agreement with the previous discussion. The average diffusivities (as listed in the last row of Table XI) also were comparable to the diffusivity data calculated from the permeability coefficient (Table X).

In the case of the noncornified part of the membrane obtained at estrus, the results showed a great difference between the diffusivities of I in the two layers (Table XI). The average diffusivity value in the combined lamina propria and muscular layer, 1.61×10^{-6} cm^2/sec, was only slightly higher than the corresponding value, 1.09×10^{-6} cm^2/sec, obtained during diestrus. These results support the concept that the cycle effect is confined to the epithelial layer, as mentioned previously.

Calculation of Epithelial Layer Thickness—By performing the permeability and desorption experiments on the same piece of membrane, the thickness of the epithelial layer for the membrane during estrus could be calculated with better accuracy, using:

$$\frac{1}{P_2} = \frac{h_1}{D_1} + \frac{h_2 - h_1}{D_L} \qquad \text{(Eq. 13)}$$

where P_2 is the permeability coefficient of the noncornified part of the membrane, h_1 is the thickness of the epithelial layer, h_2 is the thickness of the noncornified part of the membrane, D_1 is the diffusivity of I in the epithelial layer, and D_L is the diffusivity of I in combined lamina propria and muscular layer. P_2 could be obtained from the permeability experiment; D_1 and D_L could be obtained from the desorption experiment; h_2 is estimated from direct measurement. The only unknown left is h_1, which then could be calculated easily. The results are listed in Table XII. The average thickness of the epithelial layer, 78.8×10^{-4} cm, is in good agreement with the thickness estimated from the microscopic membrane cross section ($70–90 \times 10^{-4}$ cm).

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