tively short time (20 min) because of the instability of the enzyme to these conditions even in the absence of the inhibitors.

Two substrate concentrations (31 and 172 μM) were used in an attempt to detect any irreversible inhibition. In experiments utilizing 31 μM, the same degree of inhibition was observed as that previously found without prior exposure of the enzyme to the inhibitor (Table III). In experiments utilizing 172 μM, no inhibition was found. This substrate concentration was sufficient to eliminate all of the reversible inhibition exhibited by VIIa. Thus, it appears that the epoxy acid esters are inactive as irreversible inhibitors of the reductase.

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Continuous-Flow System for Determination of Diffusion Coefficients: Use of a Natural Membrane

GARNET G. SMITH * and GARNET E. PECK ˣ

Abstract □ A natural membrane was employed in an automated diffusion system. A mature male Mongolian gerbil sebaceous gland pad was excised and mounted into a suitable retainer so that the external surface was oriented toward the concentrated aqueous drug solution. Aqueous solutions of benzoic acid and the three commonly used parabens were studied. The gerbil sebaceous pad effectively prevented any diffusion of these drug solutions within 15 hr. Water by itself, however, was transported through the skin even against a pressure gradient. Although no apparent diffusion of these compounds occurred, a significant amount of drug was retained by the sebaceous pad. An expression for membrane–water partition coefficients could be calculated. Based upon thicknesses of natural and synthetic membranes, theoretical approximations of diffusion rates were found using lag time calculations.

Keyphrases □ Diffusion coefficients—determined in automated continuous-flow system, gerbil sebaceous pad □ Partition coefficients—determined in automated continuous-flow system, gerbil sebaceous pad □ Membranes, natural—gerbil sebaceous pad in automated continuous-flow system, diffusion coefficients

The outer surface of the skin is an unspecific, rugged, impermeable membrane, allowing few molecules to penetrate readily. This nonspecific barrier of the skin slows the transfer of all substances; severe chemical treatment may be required to increase the rate of penetration. When the epidermis is separated from the dermis, the epidermis retains all of the resistance to diffusion through skin.

Skin permeability has been studied for more than 50 years, and an enormous quantity of qualitative data has been generated. However, few reports have appeared on the methods or experiments for routine *in vitro* evaluation of the diffusion process in natural skin membranes.

An improvement in quantifying penetration rates came with the use of excised skin in diffusion cells (1). Diffusion cells employing excised skin are capable of producing rapid and reproducible results. Their use depends upon three assumptions.

1. Dead skin has the same permeability properties as living membranes, a relationship that has been shown to hold for several compounds (1).
2. In the diffusion cell, the penetrant must pass through the full thickness of the dermis; whereas in the natural state, the capillary network between the dermis and epidermis can remove the drug (2).
3. The excised skin surface properties remain constant for a reasonable length of time.

The chief advantage of the diffusion cell over the intact animal is the ease in measurement of the penetrant, which is not diluted or altered by the body (3).

EXPERIMENTAL

Chemicals—The drugs whose diffusion constants were to be

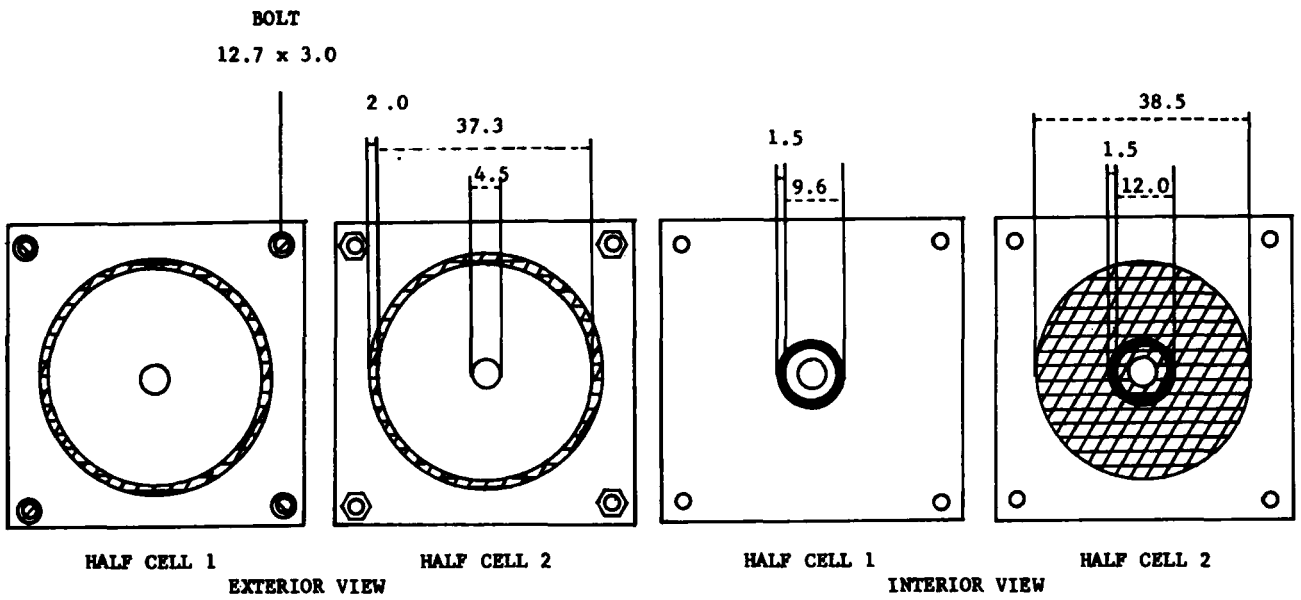

Figure 1—*Schematic diagram of the natural membrane retainer (dimensions in millimeters). Key: ▨, recessed region; and ■, elevated region.*

determined included reagent grade benzoic acid[1], methylparaben[2], propylparaben[3], and butylparaben[4].

Natural Membrane—The natural membrane used was the excised sebaceous gland pad from the mature male Mongolian gerbil. Glenn and Gray (4) described the biological and histological characteristics of the abdominal sebaceous gland pad of the gerbil (*Meriones unguiculatus*). The yellowish-brown, wax-coated secretory surface of the male pad extends up to 25 mm in length and 5 mm in width. The female pad is smaller and sometimes absent. The mature gerbil pad is easily detected in the midline of the abdomen. The sebaceous pad was selected because of its high concentration of sebum.

The animal was sacrificed, and the abdominal hair was depilated with standard animal shears. The sebaceous gland pad, with approximately 12 mm of surrounding skin, was removed using surgical scissors. Care was taken to remove only the skin and not connective tissue or peritoneal wall.

Diffusion Cell—The diffusion cell was a modification of one described previously (5–7). The sink side of the cell had two small side arms, 4 cm long and 0.635 cm i.d., placed near the bottom of the flask; one was directly opposite the large side arm, and the other was at right angles to it. In the center of the two washers[5] used to hold the membrane was located an opening to expose 4.42×10^{-1} cm^2 of the membrane. Stirring in both flasks was achieved by magnetic stirring bars rotating at 300 rpm.

Figure 1 shows a schematic diagram of the tissue holder. It was fabricated of 5-mm thick sheeting[5] and contained a hole in the center with a diameter of 4.5 mm to expose an area of 1.59×10^{-1} cm^2. This size opening corresponded to the width of the gerbil pad. The tissue holder design allowed the mounting of thick skin without leaking.

Automated Analysis System—Figure 2 is a schematic diagram of the experimental apparatus system. The diffusion cell was placed in a water bath maintained at 37.5°. To the two small side arms of the sink side was attached 1.0-cm o.d. Tygon tubing, which allowed the sink solution to pass through a flowcell[6] placed inside a spectrophotometer[7]. This arrangement was accomplished by circulating the fluid at a fixed flow rate using a peristaltic pump[8].

Initially, the action of the pump created sympathetic vibrations in the diffusion cell membrane, which exerted a force in addition to the concentration gradient on the diffusion process. To overcome this effect, two aspirator bottles were used as pressure release reservoirs. A 250-ml aspirator bottle was placed in line before the peristaltic pump, and a 500-ml aspirator bottle was placed after it. These reservoir tanks were partially filled with fluid and closed to the atmosphere so that the air over the fluid was compressed by the pumping action instead of the fluid, thus eliminating vibrations in the membrane.

A recent study described a slightly different diffusional system, used in the evaluation of a series of *p*-aminobenzoates (8). The cell used in that study had a diffusional area of 10 cm^2 and was made to utilize dimethyl polysiloxane membranes (9).

The spectrophotometer was connected to a recorder, which plotted the absorbance values as a function of time at a single wavelength.

Partition Coefficients—Equilibrium partition coefficients were determined at 37.5°. Equal aliquots of chloroform and aqueous drug solutions of known concentration were stirred together until equilibrium was attained. The aqueous phase was assayed spectrophotometrically.

RESULTS AND DISCUSSION

Water Transfer through Pad—The natural membrane, excised male gerbil sebaceous pad, was mounted into the automated diffusion system. The exterior surface of the skin was oriented toward the concentrated side of the diffusion cell. With the Y-tube stopcocks open, it was impossible to attain fluid equilibrium in the system. Even raising the pressure release reservoirs [Fig. 2, 45.7-cm (18-in.) pressure head] to excessive heights did not prevent water from being transferred out of the concentrated side of the cell. It appeared that water moved across the skin, even against a pressure gradient.

Fluid disappeared at the rate of approximately 0.14 ml/hr. By reversing the skin so that the interior surface of the membrane was exposed to the concentrated side of the diffusion cell, the fluid level on this side increased at the rate of approximately 0.07 ml/hr. These results indicated the possibility of facilitated transport of water through gerbil sebaceous pad since the water moved against a pressure gradient. Verification of this theory could be done by monitoring a substance, such as tritiated water, and following this movement through the sebaceous pad by another technique.

Bathing Solution of Pad—To preserve the integrity of the skin, lactated Ringer's solution replaced the water as solvent in the automated diffusion system and the stock solutions. A visible turbidity appeared in the sink side of the diffusion cell after a few hours (usually 6). The turbidity resulted in excessive "noise" on the recorder, making any spectral measurements impossible. By returning to the use of sterile water for injection, this turbidity did

[1] J. T. Baker Chemical Co., Phillipsburg, N.J.
[2] Heyden Newport Chemical Corp., New York, N.Y.
[3] Heyden Chemical Corp., New York, N.Y.
[4] Merck and Co., Inc., Rahway, N.J.
[5] Coated with Teflon (du Pont).
[6] One-milliliter volume cell, Scientific Glass Apparatus Co., Bloomfield, N.J.
[7] Beckman DB, Beckman Instruments, Fullerton, Calif.
[8] Zero-Max Co., Minneapolis, Minn.

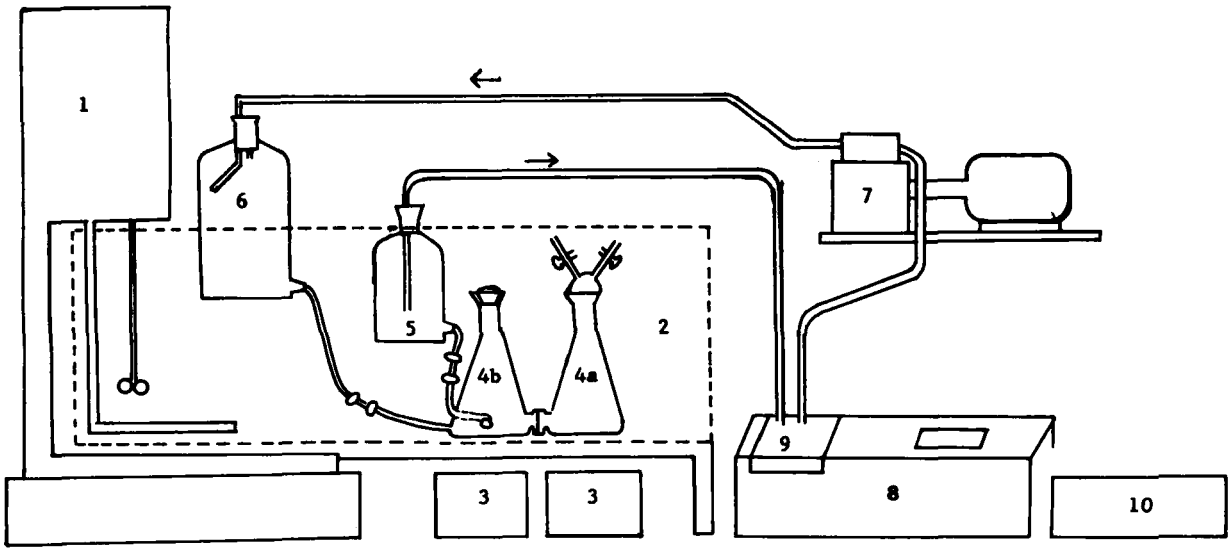

Figure 2—*Schematic diagram of the automated diffusion system. Key: 1, heater; 2, aquarium; 3, magnetic stirrer; 4a, diffusion cell, concentrated side; 4b, diffusion cell, sink side; 5, reservoir outflow; 6, reservoir inflow; 7, peristaltic pump; 8, spectrophotometer; 9, flowcell; and 10, recorder.*

not occur and constant monitoring of the UV absorbance was possible. Complex formation between the lactic acid of the Ringer's solution and some material leached from the sebaceous pad apparently produced this turbidity. This turbidity was seen only when Ringer's solution was used. Because of this reaction, distilled water only was used.

When one natural membrane was used for all experiments, severe decomposition of the membrane occurred after 3–5 days of determinations. Breakdown of the membrane was quickly noted by excessive noise on the recorder. These erratic absorbance values seemed to be due to a viscous decomposition product from the pad, which would coat the entire sink side of the diffusion system. Therefore, a new sebaceous pad was used for each experimental run and the absorbance values could be recorded over 15 hr.

Composition of Sebum—Sebum is a complex mixture of lipid substances whose detailed chemical composition is unknown (10). Sebum comprises the main portion of skin surface lipids (11); as much as 30% of this lipid material may consist of free fatty acids, more than half of which are saturated and unsaturated C_{16} and C_{18} acids with a wide range of branched and unbranched C_{14}, C_{15}, C_{17}, and some shorter carbon chain acids. The remaining materials are esterified acids, wax alcohols, squalene, sterols, and a small quantity of paraffin hydrocarbons (10, 11). There is no free cholesterol present in the gerbil sebum; it is apparently all esterified (12).

Sebum is essentially a hydrophobic oil; water will not spread over sebum but will immediately form droplets (13). Old sebum is chemically different from recently formed sebum and from the lipid droplets in the sebaceous cells (14). Kile *et al.* (15) studied the UV and IR spectra of sebum but found the interpretation of results difficult.

Drug Diffusion Studies—To separate the absorbance values for the particular diffusing drug from absorbances due to cellular components released from the skin, blank runs were made. Several blank experiments (at least five), in which no drug was placed in the concentrated side of the diffusion cell, were measured. Absorbance values for the blank were subtracted from the absorbance values obtained when a drug solution was present in the concentrated side of the diffusion cell.

Absorbance values, an average of five determinations using five different sebaceous pads, were found at two wavelengths of maximum absorbance, 227.5 and 256 nm, corresponding to those for benzoic acid and the parabens series, respectively. The absorbance units at 227.5 nm varied from 0.085 ± 0.015 at time zero to 0.096 ± 0.015 after 15 hr. At 256 nm, the same readings were 0.029 ± 0.015 at time zero to 0.032 ± 0.017 at 15 hr. The absorbance of the water in the concentrated side of the diffusion cell was found to be effectively zero at the end of each experiment.

Table I illustrates the average results of four determinations of the UV scan for the decomposition products of the sebaceous gland pad. The decomposition products from the inner surface of the gerbil sebaceous pad absorbed light throughout the entire UV spectrum, with maximum values in the 220–230-nm range. These absorbing materials apparently started leaching from the pad as soon as the pad was mounted.

The change in absorbance values with time was measured when 0.01 *M* benzoic acid solution was placed into the concentrated side of the diffusion cell. The absorbance units varied from 0.079 ± 0.016 at time zero to 0.093 ± 0.019 at 15 hr. By comparison with the blank values at 227.5 nm, there was little or no difference between these values at the same respective times. This finding indicates that apparently no net benzoic acid diffused across the entire thickness of the gerbil sebaceous gland pad.

The change in absorbance values with time was measured when the paraben solutions were placed into the concentrated side of the diffusion cell. The absorbance units varied from 0.029 ± 0.005 to 0.032 ± 0.007 for 0.01 *M* methylparaben solution, from 0.030 ± 0.002 to 0.031 ± 0.001 for 0.00248 *M* propylparaben solution, and from 0.027 ± 0.007 to 0.029 ± 0.009 for 0.00111 *M* butylparaben solution at times zero and 15 hr, respectively. Each absorbance value was an average of five individual values using five different sebaceous pads. A comparison of these absorbance values with those of the blank at 256 nm showed that no net transfer of the parabens through this natural membrane was observed.

Membrane–Water Partition Coefficients—At the termination of each individual experiment, the drug solution in the concentrated side of the diffusion cell was assayed spectrophotometrically. By subtracting this value from the known concentration placed there initially, a measure of the quantity of drug held by

Table I—UV Scan of a Sample of Gerbil Sebaceous Pad after 15 hr in Distilled Water

Wavelength, nm	Absorbance Units[a]	*SD*[b]
220	0.089	0.019
224	0.091	0.018
227.5	0.089	0.018
232	0.078	0.018
240	0.044	0.015
248	0.030	0.010
256	0.027	0.009
264	0.027	0.008
272	0.025	0.009
280	0.021	0.008
288	0.017	0.007
296	0.016	0.007
304	0.014	0.005
312	0.013	0.005
320	0.010	0.005

[a] Average of four individual values. [b] Determination from these four values.

Table II—Membrane–Water Partition Coefficients for the Gerbil Sebaceous Pad at 37.5°[a]

Drug	Trial Number	Amount of Drug Retained by Membrane, mg	Partition Coefficient, Membrane–Water, $\times 10^3$
Benzoic acid	1	1.45	3.93
	2	2.90	7.91
	3	2.22	6.40
	4	4.35	11.76
	5	2.18	5.92
	6	4.71	12.72
Mean		2.97	6.20
SD		1.19	3.16
Methylparaben	1	6.53	14.98
	2	2.18	4.98
	3	2.18	4.98
	4	5.16	11.53
	5	6.64	14.88
Mean		4.54	10.27
SD		2.00	4.49
Propylparaben	1	8.56	78.15
	2	3.19	27.78
	3	3.19	27.78
	4	3.77	33.08
	5	2.61	22.67
Mean		4.26	37.89
SD		2.18	20.40
Butylparaben	1	5.37	83.71
	2	3.05	45.85
	3	4.06	59.83
	4	2.47	35.49
	5	6.38	117.02
Mean		4.26	68.38
SD		1.45	29.20

[a] After a 15-hr exposure to water.

the skin was obtained. Table II shows the effective partition coefficient for the membrane to the water phase at 37.5°. As the polarity of the compound decreased (a higher membrane–water partition coefficient), more drug adhered to the sebaceous pad.

It is feasible that the natural membrane–water partition coefficient could be related to the chloroform–water or the octanol–water ratios. Table III summarizes these values. Figures 3–5 show semilogarithmic plots of the partition coefficient as a function of the number of carbons in the ester moiety of the solute. The slope in the case of the natural membrane–water partition coefficient (Fig. 3) is 0.26. This slope differs from that of chloroform–water (0.49) (Fig. 4) or octanol–water (0.54) (Fig. 5). This finding implies that previously reported assumptions (16, 17) that an organic phase can simulate the *in vivo* state in drug distribution are valid.

Considering the differences among Figs. 3–5, the retention of nonpolar compounds by the sebaceous gland pad must require the membrane to have some degree of polarity. Although the principal component of this skin is the hydrophobic material sebum, other materials must be present to impart some polarity to the membrane. The use of a purely nonpolar liquid as a model for the sebaceous pad would appear not to be valid. Levy and Mroszczak (18) verified this point by showing that a dye complexed with caffeine to make it more lipid soluble diffused at different rates through artificial barriers than across biological membranes. They concluded that an *in vitro* system of a lipid barrier separating two aqueous

Table III—Apparent Partition Coefficients of Aromatic Drugs

Drug	Partition Coefficient: Chloroform–Water	Partition Coefficient: Octanol–Water[a]
Benzoic acid	4.11	74.2
Methylparaben	4.77	91.2
Propylparaben	73.19	1096.0
Butylparaben	165.46	3715.0

[a] Values abstracted from L. Albert, C. Hansch, and D. Elkins, *Chem. Rev.*, 71, 525(1971).

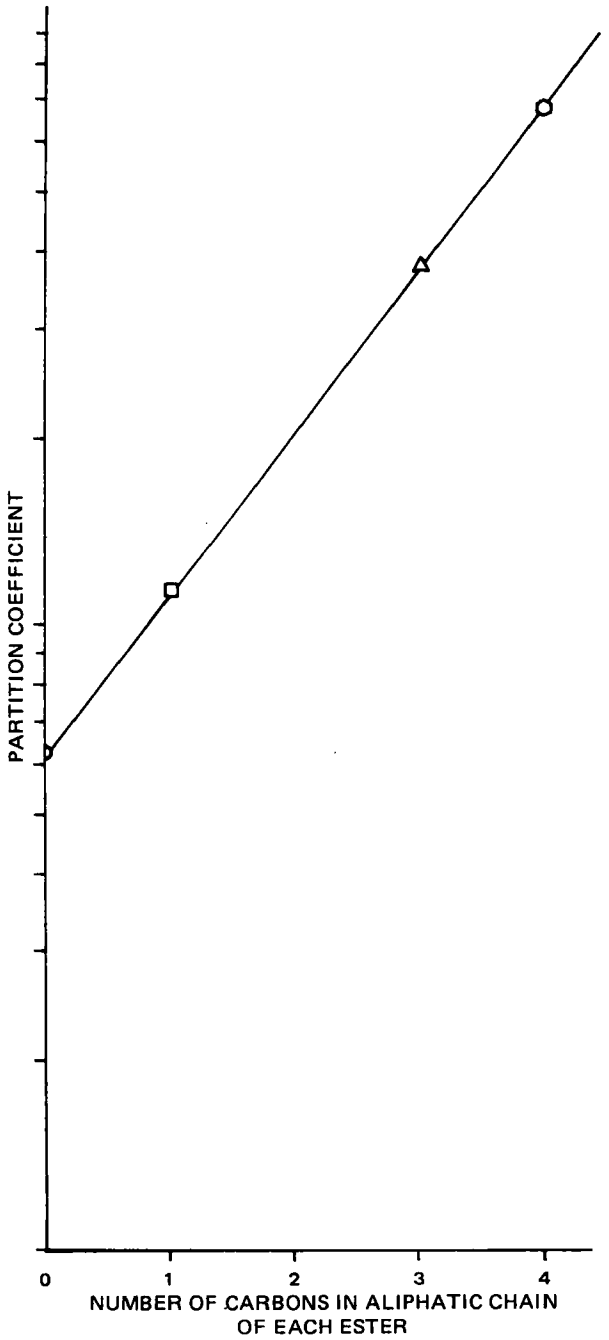

Figure 3—*Relationship between the natural membrane–water partition coefficient and the number of carbons in the aliphatic chain. Key:* ○, *benzoic acid;* □, *methylparaben;* △, *propylparaben; and* ○, *butylparaben.*

regions did not adequately reflect the *in vivo* situation for this complex. Treherne (2) showed that nonelectrolytes diffused across whole excised rabbit skin at rates proportional to their ether–water partition coefficients. The dielectric constant of ether is similar in value to that of chloroform.

All of the parabens are capable of sensitizing the skin; and since nonpolar compounds are retained to a greater extent by the gerbil sebaceous pad, this could explain some of their adverse effects (19). The best antifungal property is associated with butylparaben and could be directly related to its membrane–water partition coefficient being larger than that of the lower molecular weight parabens.

The significance of the thickness of a natural membrane in comparison to the synthetic membrane can be considered from a discussion of lag times. The lag time, t_L, by definition is the time interval before steady state is reached due to a finite diffusion veloc-

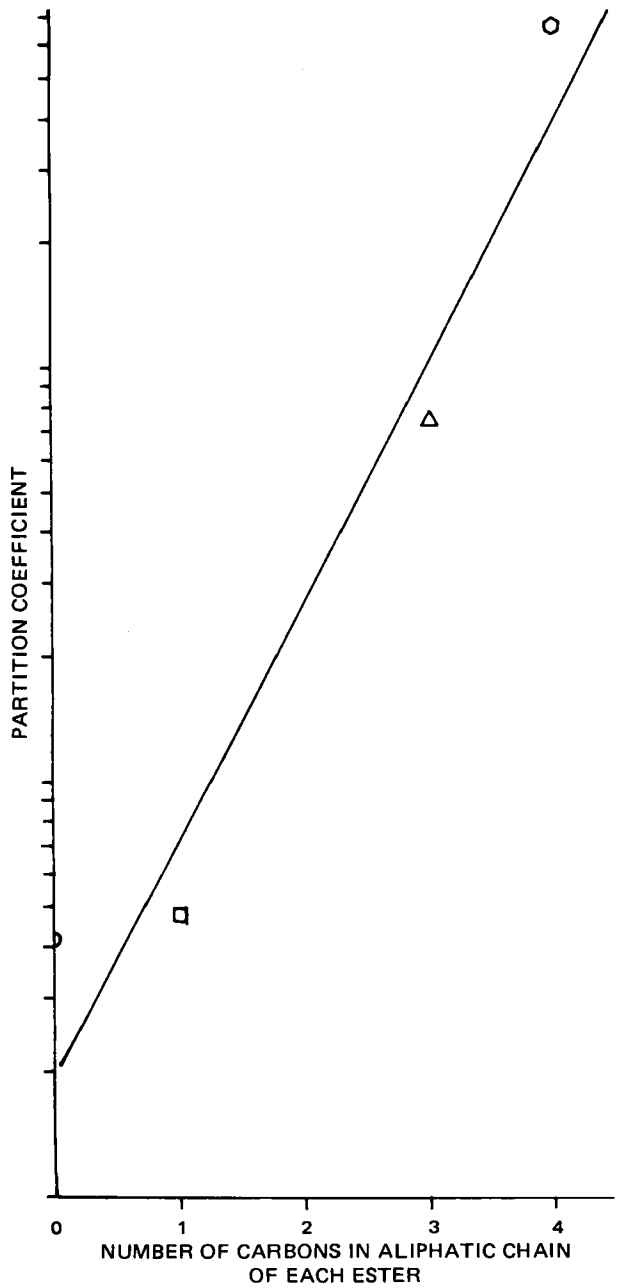

Figure 4—*Relationship between the chloroform–water partition coefficient and the number of carbons in the aliphatic chain. Key:* ○, *benzoic acid;* □, *methylparaben;* △, *propylparaben; and* ⬡, *butylparaben.*

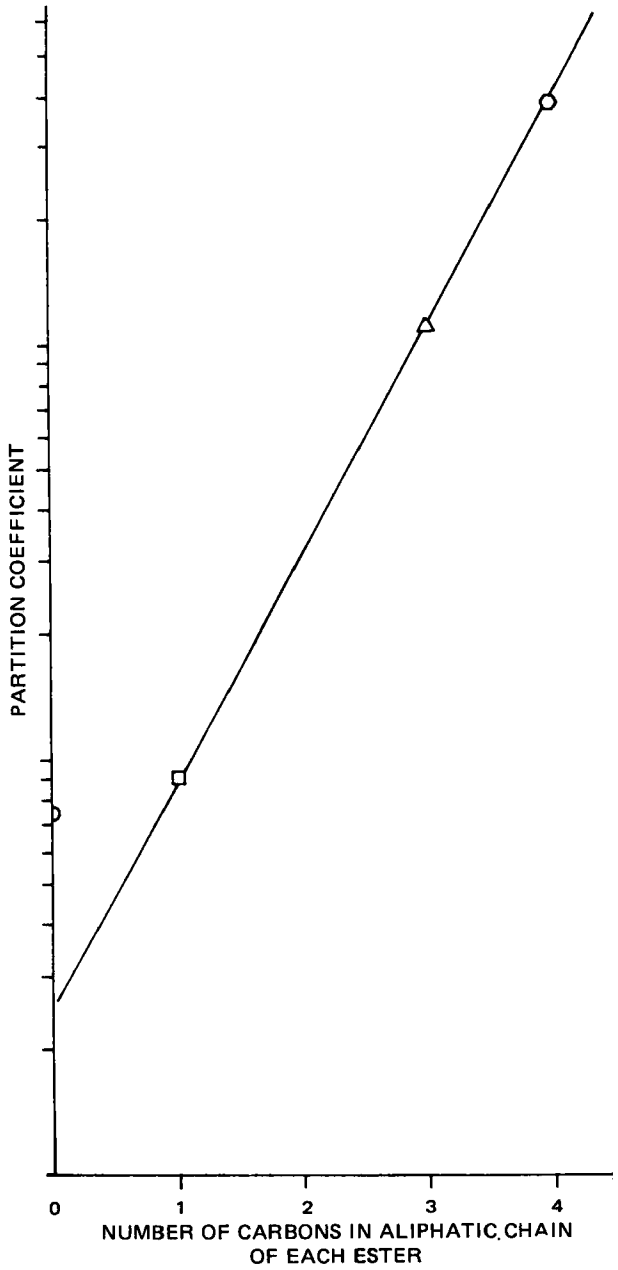

Figure 5—*Relationship between the octanol–water partition coefficient and the number of carbons in the aliphatic chain. Key:* ○, *benzoic acid;* □, *methylparaben;* △, *propylparaben; and* ⬡, *butylparaben.*

ity of the drug within the membrane. When the amount of drug diffusing is plotted against time, the resulting curve emerges from the origin with a very small slope and extends over a finite time period. The slope of the curve gradually increases with time to become a straight line (steady state). The intercept t_L on the time axis made by the asymptotic curve is:

$$t_L = \frac{h^2}{6D} \qquad \text{(Eq. 1)}$$

where h is the membrane thickness, and D is the diffusion coefficient. This equation is a simplified form of the more general expression (20). This relationship can be considered with respect to the diffusion coefficient for benzoic acid ($D = 1 \times 10^{-5}$ cm^2/sec approximately) through a microweb membrane (h = 0.013 cm) as compared to the gerbil skin (h = 0.08 cm before, or h = 0.18 cm after, swelling). The diffusion process through a microporous structure such as this synthetic cellulosic filter is assumed to be the same as through the skin. Although this assumption is false, such a situation would result in a diffusion coefficient of maximum value. By using Eq. 1, an equivalent thickness of 0.08 cm for a microweb membrane would yield a diffusion coefficient for benzoic acid approximately 38 times smaller than the same constant through the 0.013-cm synthetic membrane. Similarly, for the hydrated membrane, the diffusion rate would be 192 times slower than through the synthetic membrane.

By inserting these approximations back into Eq. 1, a comparison of lag times is possible for the three thicknesses of membrane (Table IV). From these approximations, it can be concluded that if the diffusion process in the gerbil sebaceous pad were the same as that through a cellulosic membrane, it would take about 29 hr before any measureable steady-state concentrations could be detected. This fact could possibly imply that the preparation of a synthetic membrane model to approximate the diffusion process through whole skin may be an exceedingly difficult task since the excised tissue could decompose before any drug passed into the sink. Zwolinski *et al.* (21) indicated that diffusion constants for

Table IV—Comparison of Theoretical Lag Times Based on Three Different Membrane Thicknesses

Membranes Thickness h, cm	Diffusion Coefficient D, cm^2/sec	Lag Time t_L, sec
Synthetic = 0.013	1×10^{-5}	2.8
Natural = 0.08	2.6×10^{-7}	4.1×10^{3}
Natural, hydrated = 0.18	5.2×10^{-8}	1.04×10^{5}

nonelectrolytes in natural membranes are 10^4–10^5 times smaller than their values in aqueous solutions, primarily due to higher activation energies.

SUMMARY

The following conclusions were found relative to this study:

1. The natural membrane used in an automated diffusion system was the excised sebaceous gland pad of the mature male Mongolian gerbil. To accommodate a membrane of such thickness, the fabrication of a new tissue holder was required.

2. Mounting of the sebaceous pad in an aqueous environment produced minimal decomposition of the skin over 15 hr. The use of lactated Ringer's solution to bathe the tissue resulted in a visible turbidity of the sink side of the diffusion cell. This turbidity apparently was caused by some complex formation within the gerbil pad and was not seen when high purity water was used.

3. Water was transferred across the skin against a fluid pressure gradient, indicating the possibility of facilitated transport.

4. No transfer of benzoic acid, methylparaben, propylparaben, and butylparaben was observed through the gerbil pad as indicated by a lack of change in drug concentration on the sink side of the cell after 15 hr of exposure in the cell.

5. Significant quantities of the drugs were retained by this natural membrane after 15 hr of exposure at 37.5° in the test cell. From these amounts retained, a membrane–water partition coefficient was calculated. It increased in value as the polarity of the diffusing substance decreased.

6. Plots were made relating the membrane–water partition coefficients for the drugs studied to those obtained for chloroform–water and octanol–water systems. Because of the differences in physicochemical properties between chloroform and octanol and the natural membrane, an *in vitro* organic phase can only approximate as a comparative model to the sebaceous pad.

7. The natural membrane incorporated sufficient water in the hydrated state in the automated diffusion system to increase its thickness by more than twice its original size. This observation was used to consider theoretical diffusion lag times based on thickness differences. This concept may require further consideration when designing membrane systems to approximate the natural membrane being proposed for a particular study.

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