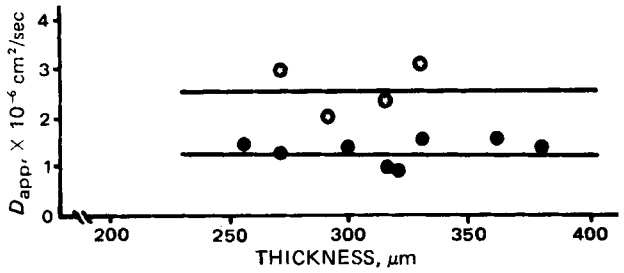

Figure 2—*Plot of the apparent diffusivities* versus *the thickness of the dermis. Key: see Fig. 1.*

the stratum corneum were calculated from the permeation data on whole skin and stripped skin from:

$$\frac{1}{P_{sc}} = \frac{1}{P_{ws}} - \frac{1}{P_{ss}} \qquad \text{(Eq. 2)}$$

where P_{sc}, P_{ws}, and P_{ss} are the permeability coefficients for the stratum corneum, the whole skin, and the stripped skin, respectively. Similarly, the permeability coefficients for the epidermis may be calculated from:

$$\frac{1}{P_e} = \frac{1}{P_{ss}} - \frac{1}{P_d} \qquad \text{(Eq. 3)}$$

where P_e and P_d are the permeability coefficients for the epidermis and dermis, respectively.

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Physical Model Evaluation of Topical Prodrug Delivery—Simultaneous Transport and Bioconversion of Vidarabine-5′-valerate IV: Distribution of Esterase and Deaminase Enzymes in Hairless Mouse Skin

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Received August 2, 1979, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.* Accepted for publication December 13, 1979. *Present address: Syntex Research, Stanford Industrial Park, Palo Alto, CA 94304.

Abstract □ A semiquantitative assessment of esterase and deaminase distributions in hairless mouse skin was performed *in vitro*. The enzyme activities were quantified using ^{3}H-vidarabine and its 5′-valerate as the substrates. Full-thickness skin of the hairless mouse was cut into two halves, and each half was homogenized in pH 7.4 buffer. Both the supernate and the residue of the homogenate were assayed for esterase and deaminase activities. Results show that the outer half-thickness of the skin contained more esterase but slightly less deaminase than the other half. The characteristics of the esterase and the deaminase reactions also were studied employing the crude enzyme extract; these reactions were essentially irreversible. The deaminase reaction was in the linear region of Michaelis–Menten kinetics for substrate concentrations up to 4.5×10^{-5} *M*.

Keyphrases □ Vidarabine valerate prodrug—topical dosage forms, distribution of adenosine deaminase and esterase enzymes in various mouse skin strata □ Prodrugs, topical—simultaneous transport and bioconversion of vidarabine, distribution of adenosine deaminase and esterase enzymes in mouse skin, pharmacokinetics □ Enzyme distribution studies—location and nature of adenosine deaminase and esterase in mouse skin, pharmacokinetic parameters of deaminase and esterase □ Models, physical—based on simultaneous transport and metabolism, effect of nonhomogeneous distribution on prodrug evaluation

A physical model for the evaluation of a topical prodrug of vidarabine (9-β-D-arabinofuranosyladenine, I) was presented previously (1). The activity of the esterase that converts vidarabine-5′-valerate (III) to I and the activity of the deaminase that converts I to 9-β-D-arabinofuranosylhypoxanthine (II) were potentially capable of influencing the efficacy of the prodrug according to the model. For mathematical simplicity, the model assumed that the enzyme reactions were irreversible and within the linear range of Michaelis–Menten kinetics and that the distributions of both enzymes were homogeneous throughout the viable cutaneous tissue.

Subsequent experiments and data analysis using the model revealed some inconsistencies. Experiments conducted under two different boundary conditions yielded significantly different *in situ* enzyme rate constants. This result raised questions concerning the validity of the homogeneous enzyme distribution assumption employed in the calculations and the need for a more detailed study of the enzyme distribution in the membrane. Thus, the purpose of the present investigation was to study the distributions of the two enzymes in hairless mouse skin as well as the nature of the enzyme reactions.

EXPERIMENTAL

Materials—All materials used were described previously (2), except

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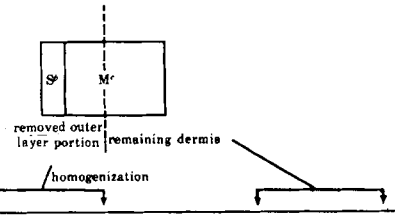
Table I—Distributions of the Esterase and the Deaminase in Hairless Mouse Skin [a]



Enzyme	Extract	Residue	Extract	Residue
Deaminase	1.95×10^{-2}	0.10×10^{-2}	3.59×10^{-2}	0.18×10^{-2}
Esterase[d]	21.7×10^{-3}	7.93×10^{-3}	6.46×10^{-3}	0

[a] Values are in reciprocal seconds. [b] S is the stratum corneum. [c] M is the cutaneous tissue. [d] Raw data are shown in Figs. 1 and 2.

for a 0.01 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid[1] buffer solution (adjusted with 10 *N* NaOH to pH 7.4).

Enzyme Distribution Studies—Although extraction of enzymes from tissue homogenates may not reach 100%, useful data for comparing the relative enzyme content of tissue of the same mass may be obtained. Full-thickness skin may be planed into two halves, and each half may be extracted for its enzyme content. A comparison of enzyme activities in the extracts then may provide some measure of the relative enzyme distribution in the whole skin.

Hairless mice were sacrificed by snapping the neck. After sacrifice, the abdominal skin was planed with a dermatome[2] to remove a top layer (including the stratum corneum, the epidermis, and part of the dermis) about 150–200 μm thick. The remaining dermis then was excised from the animal. The two pieces, after weighing, were homogenized separately in a centrifuge-type tissue grinder[3] with 1 ml each of the buffer. The tissue grinder was immersed in an ice–water mixture during the grinding.

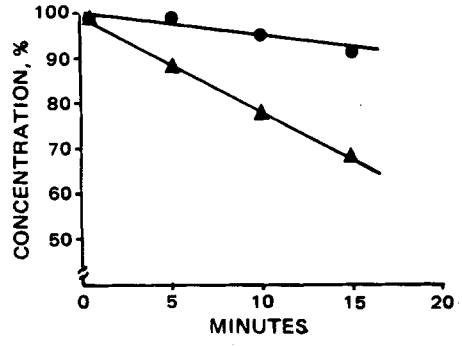


Figure 1—*Esterase activities in the supernates of the homogenates. Key: ●, supernate of the remaining dermis; and ▲, supernate of the dermatomed outer layer of the skin.*

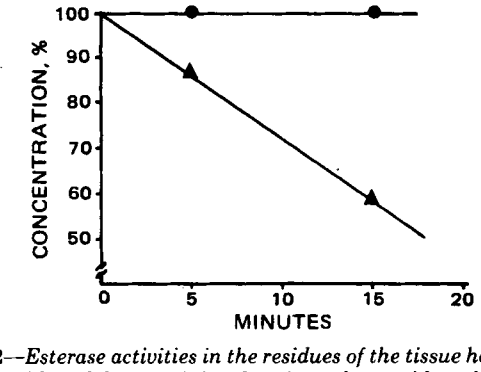
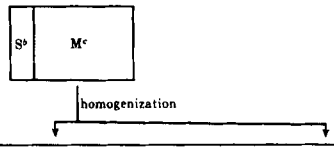


Figure 2—*Esterase activities in the residues of the tissue homogenate. Key: ●, residue of the remaining dermis; and ▲, residue of the dermatomed outer layer of the skin.*

[1] Calbiochem, San Diego, Calif.
[2] Castroviejo, a small electrical dermatome, Storz Instrument Co., St. Louis, MO 63110.
[3] Bellco Glass Inc., Vineland, NJ 08360.

Table II—Esterase and Deaminase Activities in Tissue Homogenate of Hairless Mouse Skin [a]



Enzyme	Extract	Residue
Deaminase	2.28×10^{-2}	0.15×10^{-2}
Esterase	5.78×10^{-3}	1.9×10^{-3}

[a] Values are in reciprocal seconds. [b] S is the stratum corneum. [c] M is the cutaneous tissue.

For each homogenate, the supernate was separated from the residue by centrifugation at 3000 rpm for 5 min. A 0.5-ml aliquot of the supernate then was divided into two aliquots. One aliquot was incubated with 5 μCi (specific activity of 41.7 μCi/μg) of ^{3}H-2-I and the other with ^{3}H-2-III at 37°. The residues were redispersed in 1 ml of buffer after washing several times by redispersion and centrifugation. The redispersed residues were divided into aliquots and incubated with ^{3}H-2-I and ^{3}H-2-III, as were the supernates. Samples were analyzed by TLC and scintillation counting as described previously (2). The initial rates were obtained from the substrate concentration–time curve.

Studies of Irreversible Nature of Enzyme Reactions—Two methods were employed to study the irreversibility of the esterase and deaminase enzyme reactions. One method involved the crude extracts; the other was based on the in-and-out method with intact full-thickness skin. The procedure for the in-and-out experiments was described previously (2), and the homogenization procedure for obtaining the enzyme extracts was the same as that given under *Enzyme Distribution Studies*, except that full-thickness abdominal skin (0.334 g) was homogenized without dermatome planing.

Determination of Linear Region of Enzyme Reaction—The spectrophotometric assay of Kalckar (3–5) for adenosine deaminase was employed. The skin homogenate (1.6 g from three mice of the same age in 5 ml of buffer) was obtained as described. After centrifugation, 0.05 ml of the supernate was diluted to 20 ml with buffer. A different enzyme concentration was prepared by diluting 0.5 ml of the supernate to 40 ml with the buffer.

To determine the reaction rate for a given substrate concentration, 2 ml of one enzyme solution was transferred into a cell of which 50 μl then was replaced with the appropriate solution of I at zero time. The change in absorbance at 265 μm (ΔA_{265}) was measured with a spectrophotometer[4] for 1 hr at 5-min intervals. A background reading was taken before

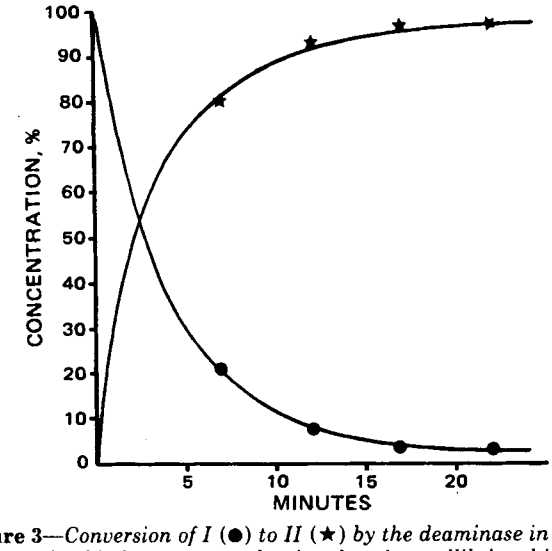


Figure 3—*Conversion of I (●) to II (★) by the deaminase in the supernate of a skin homogenate, showing that the equilibrium highly favors the formation of II.*

[4] Beckman DB, Beckman Instruments, Fullerton, Calif.

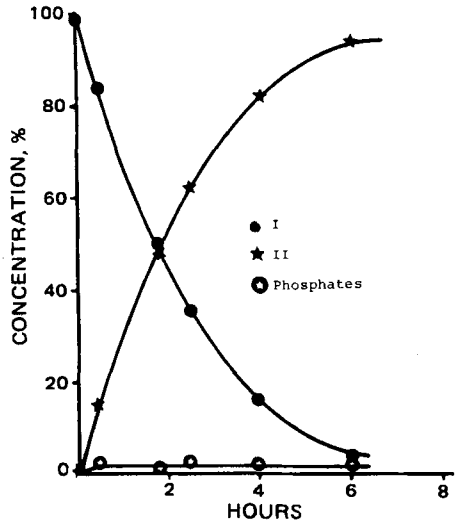


Figure 4—*Deamination of I by the deaminase in an in-and-out experiment, showing that the equilibrium highly favors the formation of II. Corresponding nucleotides formed from I and II are assayed as total phosphates.*

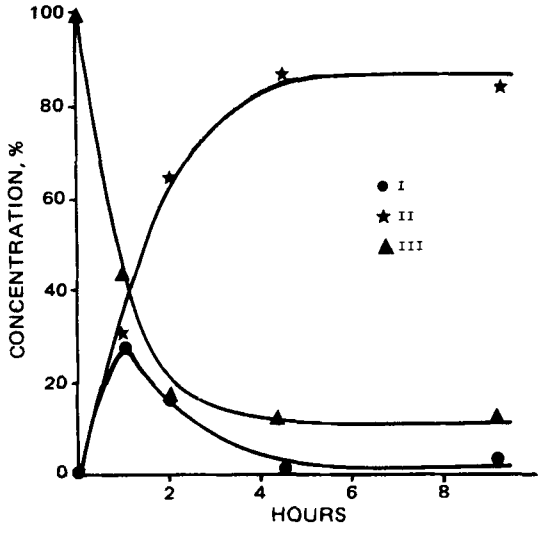


Figure 5—*Bioconversions of III to I and further to II by the esterase and the deaminase in an in-and-out experiment, showing that the equilibria highly favor formation of the products.*

introduction of the substrate, and the zero-time reading was taken 20 sec after the substrate was mixed well with enzyme solution in the cell.

RESULTS AND DISCUSSION

Esterase and Deaminase Distributions in Hairless Mouse Skin—Figures 1 and 2 show the esterase activities in the supernate and the residues of the homogenates prepared from the outer layer of skin and from the remaining dermis. The initial rates of the esterase and deaminase reactions obtained from these substrate concentration–time curves are tabulated in Table I. The esterase activity was much higher in the outer layer half than in the other half of the skin, whereas the deaminase distribution was in the reverse order but with less difference between the two halves. Table II shows the results of similar studies with a whole skin sample. These results have significant consequences in the physical model analysis of the transport and metabolism problem.

Because of the nonhomogeneous distribution of these enzymes, the analytical solutions describing the simultaneous diffusion and metabolism (1) based on the homogeneous enzyme distribution assumption may serve only as a first approximation in the evaluation of a prodrug. A more rigorous analysis that considers the effects of nonhomogeneous enzyme distributions may be carried out with a numerical computational procedure. A computer program for handling any possible enzyme distri-

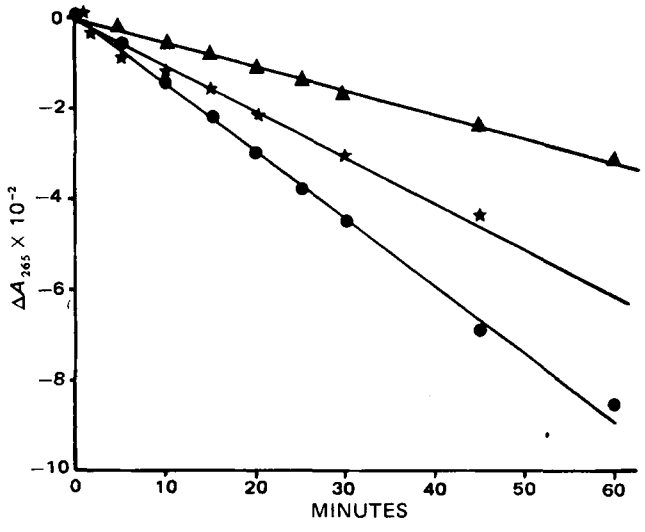


Figure 6—*Time curves showing the decrease in absorbance (ΔA_{265}) resulting from incubation of various concentrations of I with a crude extract of skin deaminase. Key: ▲, 3.1 μg/ml; ★, 6.5 μg/ml; and ●, 12.0 μg/ml.*

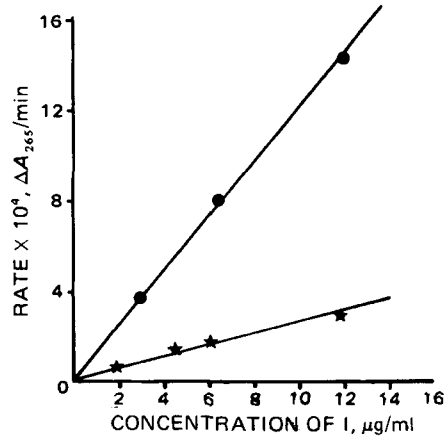


Figure 7—*Linear range of the deaminase activity, showing that the first-order enzyme kinetics hold up to a substrate concentration of 12 μg/ml (or 4.5 × 10^{-5} M). Key: ●, high enzyme concentration; and ★, low enzyme concentration.*

bution was developed by Fox *et al.* (6), and the rigorous evaluation of III as a potential prodrug using this approach will be presented later.

Irreversibility of Enzyme Reactions—Figure 3 shows the conversion of I to II by the deaminase in the supernate of a skin homogenate. The reaction is essentially complete in 20 min. Thus, the reaction is essentially irreversible from a practical standpoint. Similar results were obtained for the deaminase reaction in the intact skin with the in-and-out experiment. Figure 4 shows that the conversion of I to II by the deaminase in the intact skin experiment was almost complete in 6 hr. In the in-and-out experiment involving an intact skin incubated with ^{3}H-2-III, a typical A → B → C reaction pattern was observed (Fig. 5).

Linear Region of Michaelis–Menten Kinetics for Deaminase Reaction—Figure 6 shows the ΔA_{265} *versus* time curve for three different concentrations of I incubated at room temperature with the enzyme solution. The initial rates obtained from the slopes were plotted *versus* the substrate concentration (Fig. 7). Results of similar experiments with a different enzyme concentration also were plotted (Fig. 7). The deaminase enzyme reaction was in the linear region of Michaelis–Menten kinetics for substrate concentrations up to 12 μg/ml or 4.5 × 10^{-5} *M* for I. This result is in good agreement with reported K_m values (7–9) for purified adenosine deaminase (using I as the substrate) obtained from calf intestine mucosa, which range from 7 to 14 × 10^{-5} *M*.

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Physical Model Evaluation of Topical Prodrug Delivery—Simultaneous Transport and Bioconversion of Vidarabine-5′-valerate V: Mechanistic Analysis of Influence of Nonhomogeneous Enzyme Distributions in Hairless Mouse Skin

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Received August 2, 1979, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.* Accepted for publication December 13, 1979. *Present address: Syntex Research, Stanford Industrial Park, Palo Alto, CA 94304.

Abstract □ The mathematical problem of simultaneous transport and metabolism in the case of nonuniform enzyme distributions in the skin was solved, and the solutions were used for analyzing experimental data. Experimental data were obtained from permeation experiments with ^{3}H-vidarabine and its 5′-valerate using cellophane tape-stripped hairless mouse skin. Results of the analyses revealed that the esterase activity was four to nine times higher in the epidermis than in the dermis, whereas the deaminase activity was about the same in the two strata. These results were in good agreement with independent experiments using tissue homogenates. The enzyme distributions and the previously reported diffusivities were employed in generating concentration profiles for the prodrug and the drug in the skin. These results may be used in predicting the possible therapeutic effect of the prodrug when it is topically applied.

Keyphrases □ Vidarabine valerate prodrug—topical dosage forms, physical models employing homogeneous and nonhomogeneous enzyme distributions, various mouse skin strata, mathematical analysis □ Prodrugs, topical—simultaneous transport and bioconversion of vidarabine valerate, effect of enzyme distribution and diffusivities on bioavailability, various mouse skin strata, mathematical analysis □ Models, physical—homogeneous and nonhomogeneous enzyme distributions, various mouse skin strata, vidarabine valerate prodrug, topical dosage forms, mathematical analysis

A physicochemical method was developed to determine the *in situ* enzyme rate constants in hairless mouse skin (1, 2). Because of the lack of specific information, preliminary analyses of the experimental data were conducted based on the assumptions of uniform enzyme distribution and constant membrane diffusivities for the substrate and metabolite. It was believed then that when appropriate data became available, a more comprehensive analysis should be conducted based on the possible nonuniform enzyme distributions and the possible variations in diffusivity with respect to position in the membrane.

Subsequent studies were conducted to assess the possible variations in diffusivities and enzyme rate constants. Permeability coefficients of vidarabine (9-β-D-arabinofuranosyladenine, I) in various components of hairless mouse skin were factored out, and the dermis permeability of I was found to be constant throughout the whole stratum (3). Experiments using tissue homogenates revealed (4) that the outer half-thickness of the skin (*i.e.*, the stratum corneum, the epidermis, and part of the dermis) contained more esterase but slightly less deaminase than the remainder of the skin. These data now form the basis for a more comprehensive analysis.

The purposes of the present study were to solve the mathematical problem involved in the simultaneous diffusion and metabolism in the case of the nonuniform enzyme distributions and to analyze the experimental data using these mathematical solutions. The enzyme distributions together with the diffusivities then were employed to generate concentration profiles for the prodrug and the drug in the skin from which the behavior, including the efficacy, of the prodrug may be predicted.

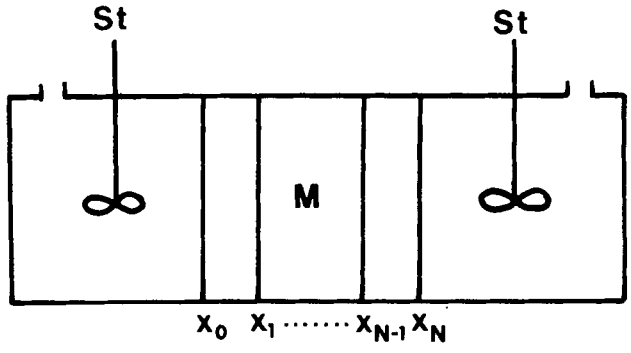

Figure 1—*Multilayer model for simultaneous diffusion and metabolism. Key: M, cutaneous membrane that consists of* N *layers; and St, stirrer.*

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