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Research Article

Transdermal Drug Transport and Metabolism. II. The Role of Competing Kinetic Events

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The steady-state flux and skin tissue distribution of a topically applied diester of salicylic acid was measured *in vitro* in the presence and absence of an esterase inhibitor. When compared with data obtained previously under *in vivo* conditions, the results presented here provide insight into the role of competing diffusional and hydrolytic events in the delivery and distribution of topically applied drugs. Furthermore, these results, when combined with a theoretical analysis of topical drug delivery and metabolism, suggest that the differences in delivery and drug distribution seen under *in vitro* conditions are related to both altered drug removal and altered hydrolysis rates.

KEY WORDS: transport and metabolism; epidermis; esterase.

INTRODUCTION

As shown by the results of a previous study (1), there are major differences in the metabolism of a salicylate diester in human skin under *in vitro* vs *in vivo* conditions. In order better to understand these differences and their influence on drug transport, the various thermodynamic and kinetic steps controlling the delivery and metabolism of topically applied drugs and prodrugs need to be considered. These steps are (a) the partitioning of the drug between the vehicle and the stratum corneum barrier, (b) the drug activity gradient across the barrier, (c) the diffusivity of the drug through the barrier, (d) the enzymatic hydrolysis of the drug, and (e) the ultimate removal of the drug and/or its metabolites by the dermal capillary network. By appropriate experimental design (e.g., the use of human skin and the same drug/vehicle throughout), the contribution of thermodynamic terms (partitioning and activity) can be standardized and the role of kinetic effects (diffusion, hydrolysis, and elimination) evaluated. Furthermore, since metabolism occurs primarily below the stratum corneum, it can be safely assumed that permeation of the intact drug through this barrier layer will be similar under *in vivo* and *in vitro* conditions. Enzymatic hydrolysis and removal of drug and metabolites, however, may differ between *in vivo* and *in vitro* conditions. In this investigation we present experimental and theoretical results comparing the role of these two competing kinetic events in the transdermal transport and metabolism of a salicylate diester.

METHODS

The synthesis of salicylate esters, high-performance liquid chromatographic (HPLC) procedures, and techniques for measurement of drug distribution are identical to those described previously (1). The structures of the salicylate diester [methyl 2-(ethoxycarbonyloxy)benzoate], monoester (methyl salicylate), and salicylate are also described in that publication.

In Vitro Flux Measurements

Human facial skin, dermatomed to 300-μm thickness, was mounted stratum corneum side up in an open-top diffusion chamber of 1.8-cm^2 cross-sectional area. The receiver phase contained 5 ml of phosphate-buffered saline (PBS), pH 7.2, which was mixed with a magnetic stir bar. About 50 mg of diester in the same water/oil vehicle as previously used (1) was spread over the surface of the skin to initiate the flux experiment. The formulation contained 25% (w/w) ^{14}C-labeled diester at a specific activity of 220 dpm/nmol. At periodic time intervals, 100 μl of buffer was removed from the receiver phase and analyzed via HPLC and liquid scintillation techniques as described previously (1). The steady-state flux was calculated from the slope of the linear portion of a plot of the cumulative disintegrations per minute (dpm) vs time. In separate experiments, the receiver phase contained 1 m*M* diisopropylfluorophosphate (DFP). In these studies the skin samples (dermis side) were held in contact with this esterase inhibitor for 4 hr prior to application of the radiolabeled drug to the stratum corneum. Drug distribution results were obtained as described previously (1). In brief, samples of skin were frozen and cut into 40-μm sections, parallel to the skin's surface. The diester and its metabolites were then extracted from each section and subjected to HPLC analysis. All flux and drug distribution experiments were performed in duplicate with facial skin samples from different subjects.

RESULTS

In order to assess the role of drug removal from the

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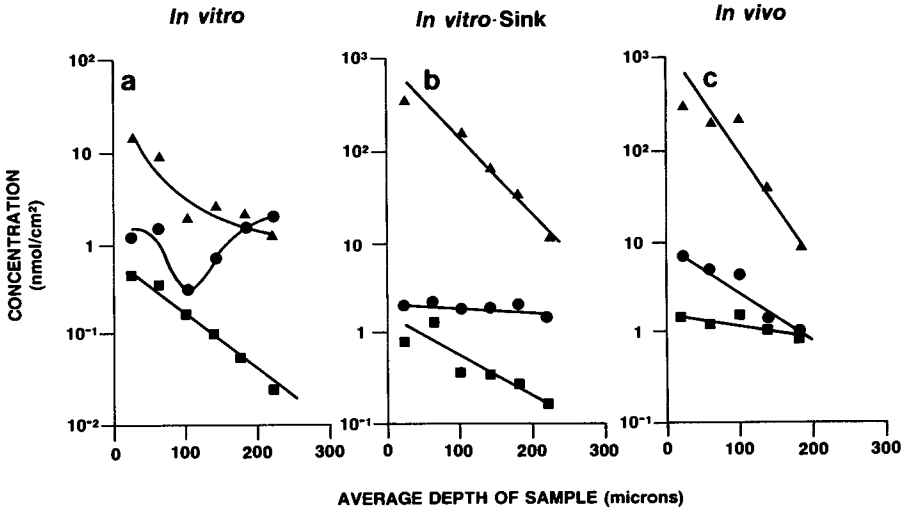

Fig. 1. The distribution of intact salicylate diester (▲), monoester (■), and salicylic acid (●) as a function of depth in human skin. Experiments were performed *in vitro* lacking sink conditions (a), *in vitro* with sink conditions in the receiver chamber (b), and *in vivo* using human skin grafted to athymic mice (c). See text for details.

skin, *in vitro* experiments were performed where the dermal side of the sample was placed in continuous contact with a large volume of stirred aqueous buffer (referred to henceforth as *in vitro*-sink conditions). Figure 1b shows the distribution of the diester and its metabolites obtained after 6 hr of topical application under *in vitro*-sink conditions. For comparison, results obtained previously (1) with human skin and the same drug formulation under *in vitro* (in the absence of sink conditions) and *in vivo* conditions are also shown in Figs. 1a and c, respectively. These results show that concentration profiles obtained under *in vivo* (Fig. 1c) and *in vitro*-sink (Fig. 1b) conditions were very similar for all three compounds. Furthermore, an approximate 100:1 ratio of the concentration of the diester to the sum of the monoester and salicylate was detected in the outermost layer, which decreased to about 10:1 at several hundred microns beneath the surface. The distribution obtained *in vitro* without sink conditions (Fig. 1a), however, shows that this ratio was lower throughout the sample and decreased from about 10:1 near the surface to near-unity in deeper sections. In addition, the salicylate concentration actually increased at depths greater than 100 μm in experiments lacking sink conditions, while a monotonic decrease was noted in other experiments. Thus, when the skin was in contact with excess aqueous buffer on the dermis side, *in vitro* concentration profiles were very similar to those obtained *in vivo*. In marked contrast, the lack of sink conditions resulted in a concentration buildup of the ultimate metabolite, salicylate, at lower levels of the skin and a lower ratio of the diester concentration to its metabolites.

The effect of enzymatic hydrolysis on delivery was studied by performing flux and distribution experiments in the presence and absence of an esterase inhibitor. The results in Fig. 2 show the appearance of ^{14}C-labeled material in the receiver chamber after topical application of the diester. Results obtained in the presence of an esterase inhibitor (Fig. 2a) show that delivery was characterized by a lag time of 9.1 hr and a steady-state flux of 0.83 nmol/cm^2/hr. Furthermore, results of HPLC analysis showed that all of the radiolabeled material appearing in the receiver chamber was unhydrolyzed diester. In the absence of an inhibitor (Fig. 2b), the lag time was 1.6 min, and the steady-state flux was 18 nmol/cm^2/hr, and only salicylate was found in the receiver chamber. Thus, in the presence of an esterase inhibitor, only intact diester was transported through human skin *in vitro*, and then the delivery was significantly retarded relative to the rapid transport of salicylate seen in the absence of the inhibitor.

The distribution of the diester and its metabolites within the skin in the presence of an esterase inhibitor is shown in Fig. 3a. These data show the average concentration plus the standard error at each depth for duplicate samples from two individuals. These results, obtained after about 30 hr under *in vitro*-sink conditions, show a monotonic decrease in all three compounds with increasing depth. In addition, about 90% of the total concentration found at all three levels examined was due to unhydrolyzed diester. In contrast, data obtained previously using human skin grafted to an athymic mouse and the same drug formulation (1) showed that after 24 hr of treatment *in vivo* (Fig. 3b), much of the diester was converted to monoester and salicylate at the lower levels studied. Note that the standard errors, shown for the sake of clarity only for the first points in Fig. 3b, were of a similar magnitude in all experiments performed. Thus, a comparison of these data show that the presence of an esterase inhibitor increased the concentration ratio of the diester to its metabolites, relative to that seen *in vivo*, especially at greater depths within the sample.

DISCUSSION

The results in Figs. 1a–c show that while *in vivo* and *in vitro*-sink results were very similar, the lack of sink conditions *in vitro* resulted in substantial changes in the dermal drug profiles. In particular, the lack of sink conditions prevented efficient removal of the ultimate metabolite salicy-

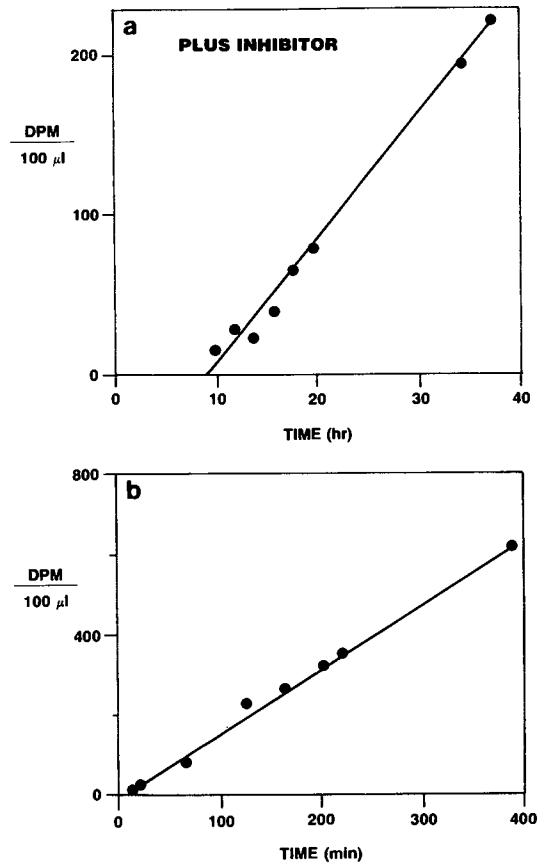

Fig. 2. The appearance of ^{14}C-radiolabeled material in the receiver chamber of an *in vitro* diffusion experiment as a function of the time after application of drug to the surface of the skin. The experiments were performed in the presence (a) or absence (b) of 1 m*M* DFP in the receiver chamber buffer.

late, and as a result, the salicylate concentration increased at depths approaching the bottom of the 300-μm section of skin (Fig. 1a). Similar increases in drug concentration with increasing depths have been noted by others and attributed to inefficient removal of drug under *in vitro* conditions (2–4). Alternatively, the increase in salicylate concentration near the bottom of the sample could have resulted from hydrolysis of intact diester in the receiver chamber, followed by back diffusion. This seems unlikely, however, since an increase in the monoester concentration in the lower skin sections should similarly occur from back diffusion yet was not observed. Thus, it seems most likely that sink conditions at the dermal interface allowed for efficient exchange of drug from the skin, preventing drug accumulation (Fig. 1b) and resulting in concentration profiles nearly identical to those obtained *in vivo* (Fig. 1c).

The ratio of the concentration of the diester to that of its metabolites inversely reflects the apparent extent of hydrolysis in the tissue. From inspection of Figs. 1a–c, it can be seen that this ratio decreased with increasing depth in all three experiments, suggesting that hydrolysis increased with the depths into and/or time within the tissue. The ratio obtained *in vitro* without sink conditions, however, was lower than that obtained with sink conditions or *in vivo*. Thus, the lack of sink conditions increased the apparent extent of ester

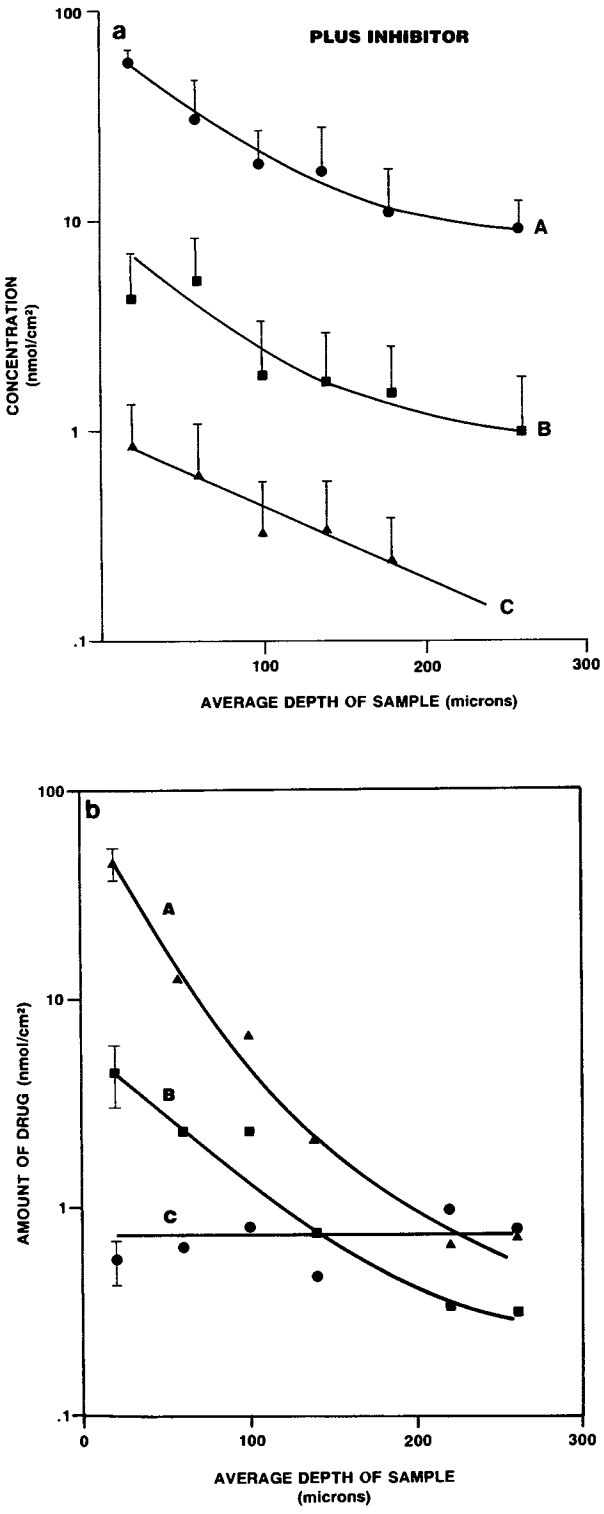

Fig. 3. (a) The distribution of salicylate diester (A), monoester (B), and salicylic acid (C) as a function of the depth in human skin in contact (dermis side) with 1 m*M* DFP. Each point represents the average and standard error of duplicate samples from two individuals (N = 4). (b) The distribution of the salicylate diester (A), monoester (B), and salicylate (C) as a function of the depth in human skin obtained *in vivo* using skin grafted to athymic mice. Each point represents the average of duplicate samples from two individuals. For the sake of clarity the standard error is shown only for the first points. Similar variation was seen in all data.

hydrolysis relative to the other experimental conditions studied. Increased apparent hydrolysis could result from either increased enzymatic activity or a longer residence time of the diester in the presence of the enzymes.

As shown by the results in Figs. 2 and 3, inhibition of esterase activity within the skin dramatically altered drug delivery and dermal distribution. Flux results showed that in the absence of an esterase inhibitor, salicylate rapidly accumulated in the receiver chamber (Fig. 2b), while the presence of an inhibitor substantially slowed delivery and only intact drug appeared in the receiver chamber (Fig. 2a). The dramatic differences in delivery and distribution in the presence and absence of inhibitor demonstrate that inhibition occurs within the skin. If the inhibitor remained only in the aqueous receiver phase, the same molecular species would be transported across the skin, with unaltered kinetics and skin drug distribution. On the other hand, one could argue that the presence of the inhibitor in the skin could alter the stratum corneum barrier properties and, hence, delivery; however, this seems unlikely for several reasons. First, the inhibitor was applied to the dermis side of the skin so that concentrations in the stratum corneum were minimized. Second, incubation of human stratum corneum in DFP resulted in no changes in lipid or protein thermal transitions as measured by differential scanning calorimetry. Since extensive data from our laboratory (5) and others (6) have shown a strong correlation between barrier alteration and changes in these thermal transitions, barrier alteration by DFP seems unlikely.

Similar results were obtained in a study of hydrocortisone esters (7). In particular, when hydrocortisone 21-heptanoate was topically applied to hairless mouse skin *in vitro,* only hydrocortisone was detected in the receiver chamber. When an esterase inhibitor was coapplied to the stratum corneum side of the skin, however, only intact ester was detected in the receiver phase, and the steady-state flux was about one-tenth the hydrocortisone flux observed in the absence of the inhibitor. These results, combined with those presented here, show that enzymatic hydrolysis of ester derivatives is important to transport through skin.

The results presented in Figs. 2a and b, along with HPLC analysis of the contents of the receiver phase, show that enzymatic hydrolysis results in a polar, water-soluble compound, which is rapidly taken into the underlying aqueous phase. When hydrolysis is inhibited, however, the lipophilic diester remains unhydrolyzed and is retained in the more lipophilic environment of the dermis. Similar results have been obtained with mouse skin *in vitro,* where metabolism of topically applied xenobiotics resulted in polar compounds which were preferentially removed to the aqueous buffer (8). Furthermore, when enzymatic activity was destroyed by freezing and thawing the skin, reduced flux and a decreased ratio of polar to nonpolar compounds in the receiver phase were observed. Those results combined with the metabolic inhibition results presented here and elsewhere (7) strongly support a correlation between transdermal flux and metabolism. Hence, like the liver, the skin has metabolic activity capable of converting highly lipophilic drugs into more polar metabolites which can be more efficiently removed via the underlying vasculature. In further analogy with hepatic metabolism, the formation of polar metabolites may be important in chemical detoxification by the skin.

Altered *in vitro* drug distribution (Fig. 1a vs Fig. 1b) cannot be explained completely by changes in drug removal. Changes in enzymatic activity must also occur. The relative effects of removal and enzymatic events can best be demonstrated by simple model calculations. If one assumes a uniform enzyme distribution, a drug removal rate that is proportional to the drug concentration at the skin/aqueous interface, and a rate of enzymatic hydrolysis that is proportional to the substrate (drug) concentration (9), then the steady-state concentration of drug as a function of depth in the skin can be described (see Appendix for derivation). Yu *et al.* have derived a more complex model of transport and metabolism which assumes different diffusion and partition coefficients and enzymatic rate constants in the stratum corneum, epidermis, and dermis (10). Using experimentally derived values for these parameters, they calculated steady-state concentration profiles for a prodrug (Ara-A) and its metabolites. No experimental drug profile data, however, were presented for comparison. The simpler model presented here is used to describe qualitatively changes in drug profiles due to altered hydrolysis and removal rates in an effort to point out the importance of each of these kinetic parameters to topical drug delivery.

Plots of the relative change in concentration vs depth based on this model are shown in Figs. 4a and b. In Fig. 4a the effect of altered enzyme activity is demonstrated by plotting the concentration profiles resulting from changes in the hydrolysis rate constant, while the removal rate constant remains fixed. Figure 4b shows the effect of altered removal rates at a constant hydrolytic rate. These illustrations show that changes in removal rate affect primarily the deeper regions of the skin, with little relative change in surface concentrations. In contrast, relatively small changes in the hydrolysis rate can dramatically alter the drug profile throughout the tissue. Thus, according to this model, drug distribution, especially near the surface of the skin, is highly sensitive to changes in the rate of enzymatic hydrolysis.

To demonstrate the effect of changes in enzyme activity on drug concentration profiles, the diester data in Figs. 3a and b for the intact drug are redrawn in Fig. 5. These results show that at each depth measured, the diester concentration increased in the presence of the esterase inhibitor relative to the values obtained *in vivo*. A comparison of the data in Fig. 5 with the model profiles drawn in Fig. 4a shows a correlation between theoretical expectations and experimental values, namely, reduce enzymatic activity (i.e., the presence of an inhibitor) increases the concentration of unmetabolized drug throughout the skin.

Figure 6 shows the diester concentration profiles redrawn from Figs. 1a–c. These results show that the profile obtained under *in vivo* and *in vitro*-sink conditions were indistinguishable (upper trace), while substantially less diester was found at all levels in experiments lacking sink conditions. As discussed above, these results cannot be explained entirely by decreased removal of drug due to the lack of clearance, since such changes should affect primarily the lower layers. As shown by both theoretical and experimental results, an increase in the rate of enzymatic hydrolysis can dramatically decrease the concentration of the intact drug,

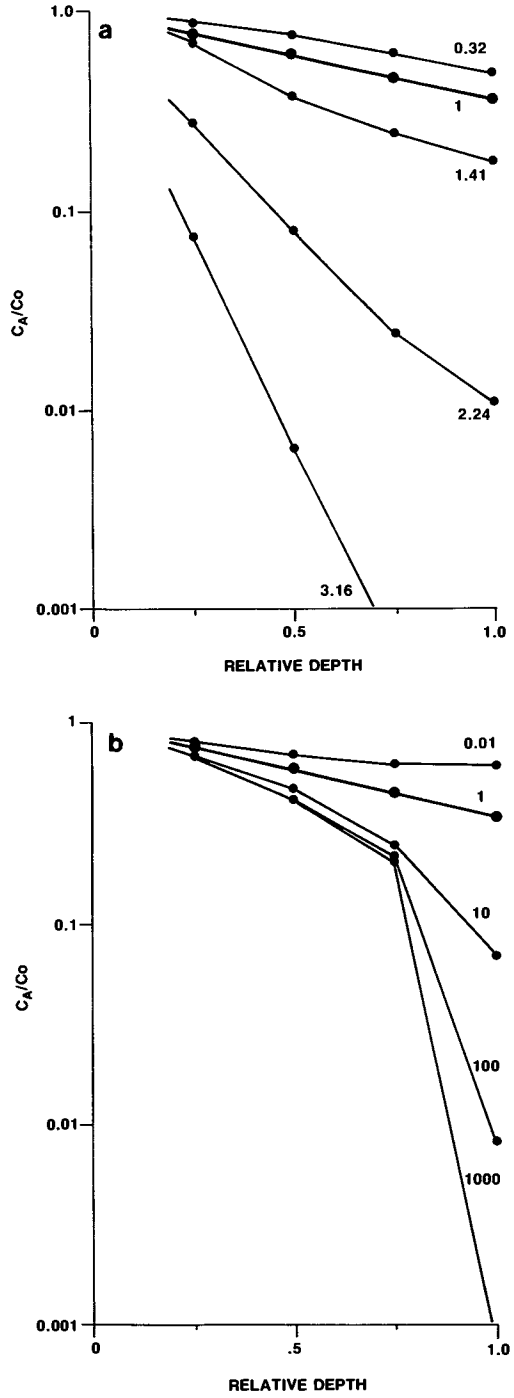

Fig. 4. The relative distribution of drug vs the relative depth within the sample calculated according to the model described in the Appendix. The results in (a) show the change in distribution due to a 10-fold increase in the rate of enzymatic hydrolysis with no change in the removal rate. The results in (b) show the change in the distribution due to a 10,000-fold change in the removal rate but no change in hydrolysis.

even at levels near the surface. Alternatively, one could postulate that differences in the drug profile due to the absence of sink conditions reflect simply a longer drug residence time in the skin. Hence, rather than increased enzymatic activity, longer incubation of the drug with enzyme could be respon-

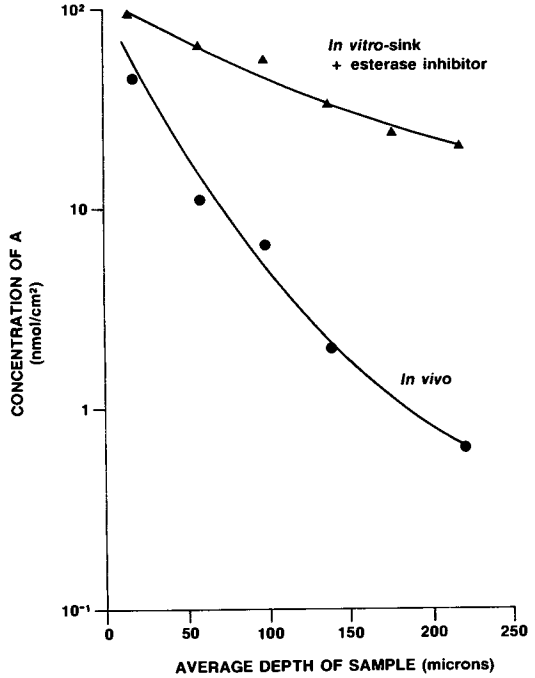

Fig. 5. The distribution of diester redrawn from the data in Figs. 3a and b.

sible for the differences observed. Under the steady-state conditions described here, however, increased residence time would increase the concentration at all levels within the skin, offsetting decreases in concentration due to prolonged hydrolysis. Thus, increased enzymatic activity in *in vitro* experiments lacking sink conditions is a much more reasonable explanation of these results.

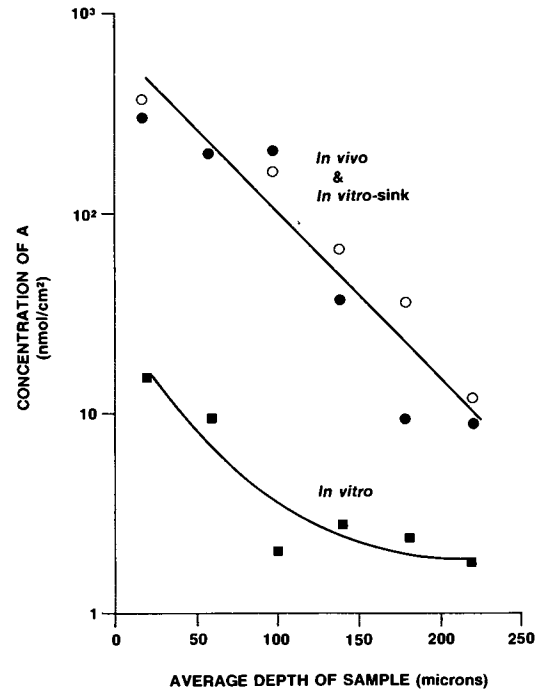

Fig. 6. The distribution of diester redrawn from the data in Figs. 1a–c.

This rather surprising conclusion of greater enzymatic hydrolysis *in vitro* (lacking sink conditions) than *in vivo* is supported by the results of several other studies. In particular, Kao and co-workers showed that numerous mitochondrial and cytoplasmic enzymes were released from freshly excised mouse skin (11). In fact, the amount of activity released during a 16-hr incubation of skin in culture medium was greater than the subsequent release induced by freeze–thaw treatments. Similarly, other investigators have detected enzymatic activity in the receiver phase of *in vitro* flux experiments, due to the leaching of enzymes from freshly excised skin (12). Thus, it seems clear that the surgical removal of skin results in the liberation of enzymes which can be removed by exchange with aqueous media. In the absence of sink conditions, however, liberated enzymes remain in the skin, resulting in a greater degree of drug hydrolysis. In addition, as the tissue viability decreases *in vitro,* lysosomal enzymes may be released, also increasing the esterase activity in the skin. Thus, surgical removal and subsequent loss of tissue viability may liberate enzymes during *in vitro* experiments. As a result, the flux and skin distribution of metabolizable drugs may be altered.

In conclusion, the results presented here suggest that enzymatic hydrolysis and drug removal are competing kinetic events, each important in transdermal drug delivery and metabolism. Furthermore, these results suggest that care must be taken in experimental design and interpretation with *in vitro* studies. While sink conditions produced *in vitro* results similar to those obtained *in vivo,* this may not be true for other drugs or experimental conditions. Perhaps the definitive answers can come only from *in vivo* techniques such as those described in the previous paper (1) or by Kreuger and co-workers (13). In addition, these results support the idea that the skin is an important site of extrahepatic drug metabolism, capable of its own "first-pass" effect.

APPENDIX: DERIVATION OF EQUATIONS DESCRIBING DRUG DISTRIBUTION

The derivation is based upon a model that makes the following assumptions (9).

(1) The skin is of finite thickness l.

(2) Within this thickness the enzyme activity is uniformly distributed. In addition, the rate of enzymatic hydrolysis is assumed to be proportional to the concentration of the drug A (rate = $K_{hyd} * C_A$). This assumption is true if the substrate concentration is well below enzyme saturating levels.

(3) The removal of drug A at the skin/aqueous phase interface is proportional to the concentration at that depth.

At steady state, diffusional, hydrolytic, and removal rates are in competition, and thus,

$$dC_A/dt = D_a * d^2C_A/dx^2 - K_{hyd} * C_A = 0 \quad (1)$$

The general solution of this differential equation is of the form

$$C_A = M_1 * \exp(-A * x) + M_2 * \exp(A * x) \quad (2)$$

The boundary conditions are as follows.

(1) The concentration at the outer surface is C_o.

$$C_A(0) = C_o \quad (3)$$

(2) The removal is proportional to the concentration of drug A at depth l.

$$(dC_A/dx)_l = \{-K_r * l^2/D_A\} * C_A(l) \quad (4)$$

where K_r is the removal rate constant, D_A is the diffusion constant for drug A, and $C_A(l)$ is the concentration of A at depth l. Using these boundary conditions, the values of M_1 and M_2 are

$$M_1 = \frac{C_o * \exp(A * l) * (A + B)}{(A+B)\exp(A * l) + (A - B)\exp(-A * l)} \quad (5)$$

and

$$M_2 = \frac{C_o\exp(-A * l) * (A - B)}{(A + B)\exp(A * l) + (A - B)\exp(-A * l)} \quad (6)$$

where

$$A = (K_{hyd}/D_A)^{1/2} \quad (7)$$

$$B = (K_r * l^2)/D_A \quad (8)$$

By substituting Eqs. (5) and (6) into Eq. (2), the relative concentration (C_A/C_o) can be calculated as a function of the relative depth (x/l) within the tissue.These results are shown in Figs. 4a and b for relative changes in K_{hyd} and K_r.

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