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Report

Dose-Dependent Enhancement Effects of Azone on Skin Permeability

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In vitro permeability experiments have been combined with differential scanning calorimetry (DSC) studies in an attempt to address the dose-dependent influence of Azone on the permeability coefficients of solutes for hairless mouse stratum corneum. A spray technique was developed to deliver uniformly and quantitatively small amounts of Azone to the stratum corneum. Permeability data obtained for several model solutes of varying lipophilicity suggest lipid fluidization and polar route enhancement as the mechanisms of action for Azone. Alkanols and steroids, both of which are enhanced primarily by lipid fluidization, had different degrees of relative enhancement. This provides evidence that the stratum corneum barrier is heterogeneous, rather than a homogeneous slab barrier. Two effects of Azone on the stratum corneum were detected by DSC. A decrease in the area and a shift to lower temperatures were noted for the lipid endotherms with increasing doses of Azone. A lipid fluidizing effect would qualitatively account for the increases in the permeability coefficients noted for more lipophilic solutes. The stratum corneum keratin endotherm also appears to be altered in the presence of Azone. It is possible that alteration of the keratin structure could lead to the development of polar routes in the stratum corneum.

KEY WORDS: penetration enhancer; Azone; permeability; skin; transdermal; differential scanning calorimetry; stratum corneum.

INTRODUCTION

The stratum corneum is a barrier to all but a small number of compounds with favorable physical–chemical properties. Therefore, there is great interest in agents that can enhance the absorption of compounds across the stratum corneum. The penetration enhancer 1-dodecylazacycloheptan-2-one (Azone) has been shown to be effective in enhancing the permeability of many compounds through the stratum corneum (1). In general, lipid fluidization (2–5) and alteration of protein conformation (6–14) are the most common hypotheses for the mechanism of action of penetration enhancers. Only a few investigators have attempted to address the possible mechanism of action of Azone (15,16), although these studies did not include dose-dependent enhancement effects. An understanding of the dose-dependent mechanisms of action for Azone is of practical importance from delivery, formulation, and regulatory standpoints.

Permeability experiments using solutes of varying lipophilicity have the potential of providing macroscopic information on the effect of Azone on the permeation pathways. Complementary techniques such as differential scanning calorimetry (DSC) may provide more molecular level information on the possible effects of Azone on the lipid and protein components of the stratum corneum. Finally, for the study to be quantitative, it is essential that the delivery of the enhancer be measured.

In the present study, a spray method was developed for the uniform and quantitative delivery of Azone in order to address possible dose dependent enhancement effects. This method has the additional advantage of avoiding artifacts due to the use of cosolvents or surfactants which are commonly used in solubilizing Azone. The steady-state permeabilities of several model solutes through hairless mouse skin have been studied as a function of the amount of Azone applied per unit area of stratum corneum. The solutes ranged from ionic to semipolar compounds in order to probe both lipophilic and hydrophilic stratum corneum permeation pathways. Complementary DSC studies have also been used to investigate the effect of Azone on the lipid and protein transitions.

EXPERIMENTAL

Chemicals

[^{14}C]*n*-Butanol (NEC 130, New England Nuclear, Boston), corticosterone (Lot 35F-0535, Sigma Chemical Co., St. Louis, Mo.), [^{3}H]corticosterone (NET-182, New England Nuclear), [^{14}C]ethanol (NEC-029, New England Nuclear), hydrocortisone (Lot 102F-0586, Sigma Chemical Co.), [^{3}H]hydrocortisone (NET 185, New England Nuclear), [^{14}C]tetraethylammonium bromide (TEAB) (NEC 298, New

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England Nuclear), and vidarabine (Ara-A) and [^{3}H]vidarabine (D. C. Baker, Department of Chemistry, University of Alabama, University) were all used as received. Porcine trypsin (type IX) was obtained from the Sigma Chemical Co. 1-Dodecylazacycloheptan-2-one (Azone) was received through the courtesy of Nelson Research, Irvine, Calif.

Tissue Preparation

Abdominal skin excised from male hairless mice (SKH-HR-1, Skin Cancer Hospital, Temple University, Philadelphia, Pa.) aged 56 to 200 days was used in all studies. Stratum corneum for DSC analysis was separated from epidermis by placing the full-thickness skin, dermis side down, on filter paper saturated with 1% trypsin in 0.0666 *M*, pH 8, phosphate-buffered saline (PBS) for 4 hr at 37°C. The stratum corneum sheets were vortexed in deionized water several times before vacuum (10^{-4} Torr) drying.

Spray Technique

Azone was sprayed from a capillary tube in an EFFA Spray Mounter (E. F. Fullam, Inc., Latham, N.Y.) placed at a distance of 39 cm from the target. An inlet pressure of 30 psi was used. The apparatus and target area were assembled within a plexiglass box to eliminate air currents.

Uniformity of the spray over the desired radius was determined by weighing (Mettler M3 or AE163 balances, Mettler Instrument Corp., Hightstown, N.J.) filter-paper targets before and after spraying. Figure 1A depicts the target area used in the uniformity test, a series of squares up to 5 × 5 cm cut out of a 9-cm diameter circle.

Alignment was validated by spraying a 4 × 4-cm target made up of four 2 × 2-cm filter-paper squares (Fig. 1B). For Azone pretreatment of skin, a 2 × 2 cm of freshly excised skin was placed in square 2 of the alignment target. Thus, the amount of Azone delivered to the skin could be estimated by the amount delivered to the adjacent filter paper targets.

Permeation Experiments

The experimental procedures have been previously described (17) and are described only briefly here. Excised abdominal skin with adhering fat removed was mounted in the spray apparatus. Azone pretreated skin and controls were put on PBS-wetted filter paper (dermis side down) for 1 hr after spraying. A pretreatment time of 1 hr appears to be sufficient since all compounds plateau to maximum *P* values with 1 hr of pretreatment over the 0- to 7-mg/cm^2 dosage range. After the pretreatment, the skin was mounted in a two-chamber diffusion cell, and the cell was then immersed in a constant-temperature (37°C) water bath. Donor, receiver, and spike solutions were made from pH 7.3 isotonic 0.0666 *M* PBS. A ^{14}C- and a ^{3}H-labeled compound were "paired" in all diffusion cells to allow direct comparison of increases in permeability for a given dose of Azone. Dual-label liquid scintillation counting (LSC) was used to determine the amount of solute permeated as a function of time. All samples were put in 10 ml of scintillation cocktail (ACS, Amersham Corp., Arlington Heights, Ill.) for radiochemical assay (Liquid Scintillation Counter Model 1801 or 7500, Beckman Instruments, Fullerton, Calif.).

To ensure that the major permeant was not a trace impurity, thin-layer chromatography (TLC) was used to assay receiver sides of key Ara-A, corticosterone, and hydrocortisone experiments. The developing solvent for Ara-A was chloroform:methanol (2:8), while toluene:acetone (1:1) was used for corticosterone and hydrocortisone. Silica gel plates (13181, Eastman Kodak Co., Rochester, N.Y.) were used for all compounds. Unlabeled drug was also developed to allow UV detection. After development, the plates were cut into sections and put into vials with 1 ml of 0.1 *N* HCl. After at least 5 hr, 10 ml of scintillation cocktail was added, and the vials were assayed by LSC.

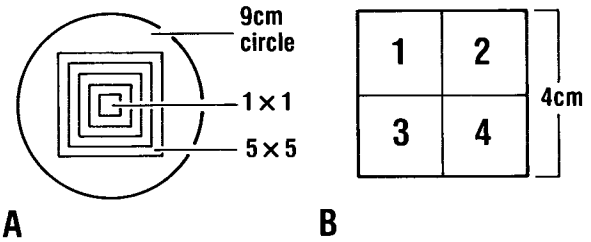


Fig. 1. Schematic of target used in (A) spray uniformity tests and (B) spray alignment tests and application to skin samples.

Differential Scanning Calorimetry

Desiccated stratum corneum was mounted in the spray apparatus and sprayed with Azone as described above. Stratum corneum was allowed 1 hr pretreatment time before the start of DSC experiments. Sample weights varied from approximately 4 to 6 mg. All thermograms were normalized by the weight of the sample. Samples were placed in aluminum pans (Perkin Elmer, Norwalk, Conn., 219-0041 or 219-0062) and heated at a rate of 10°C/min in a Perkin Elmer DSC-4 with a thermal analysis data station.

RESULTS AND DISCUSSION

Spray Technique

The EFFA spray mounter was chosen for the present study since it is commonly used to deposit microdroplets on microscopy samples. In order to validate the spray technique, it is necessary to show that the spray is both uniform and quantitative over the 4 × 4-cm target area. If the spray pattern is assumed to have a Gaussian distribution, then a uniform spray over the target area of interest can be obtained by varying the sprayer to target distance. Initial studies used a sprayer to target distance of 10 cm. In those studies, the amount of Azone per unit area dropped off by nearly 50% outside a 2 × 2-cm target area. Figure 2 shows the amount of Azone per unit area (normalized to the total amount recovered on the target) sprayed on a target (illustrated in Fig. 1A) from a distance of 39 cm. As can be seen, the spray appears to be uniform over a 5 × 5-cm target area.

It was not possible to obtain accurate weights of hydrated full-thickness skin due to water evaporation. Therefore, the quantity of Azone delivered to the skin was estimated from the weight of Azone delivered to squares of filter paper adjacent to a square of skin (square 2 in Fig. 1B).

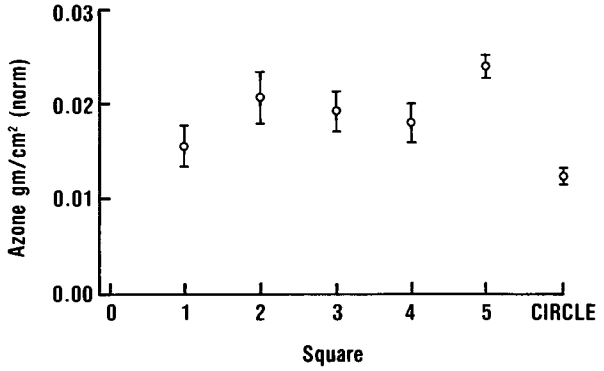


Fig. 2. Uniformity test of Azone spray from a distance of 39 cm normalized to the amount of Azone recovered. The standard deviation is indicated ($N = 5$).

Three different methods of estimating the actual amount of Azone delivered were developed. One estimate used the amount delivered to an adjacent square (square 1), another used the mean of the other three targets, and the third method used an adjacent square corrected by the ratio of the final two targets (the amount in square 1 times the amount in square 4 divided by the amount in square 3). Estimates of the amount delivered to square 2 versus the actual amount (filter paper target) are shown in Fig. 3. All three methods gave similar results; however, the third estimate gave a slope closest to unity. This is probably due to the fact that the center of the spray can never be perfectly centered, and the correction using ratios attempts to take this into account. The slope for the third estimate was 0.991, with a correlation coefficient of 0.995 and a 95% confidence interval of 0.111. The third method was used for estimating the amount of Azone delivered to all skin samples.

The above analysis demonstrates that the spray technique delivers a uniform dose of Azone on a macroscopic scale. It is a possibility, however, that the droplets are not uniformly distributed on the stratum corneum surface on a microscopic scale. For the present study, it was assumed that a homogeneous distribution of Azone exists in the stratum corneum following pretreatment. During the pretreatment time, Azone presumably diffuses in directions parallel and perpendicular to the surface. The translational diffusion of solutes in lipid bilayers is thought to be rapid (18) and would, therefore, tend to remove any initial nonhomogeneous distribution.

To determine if the force of the spray damages the stratum corneum, approximately 0.75 ml of PBS was sprayed on full-thickness skin as a control. The permeabilities of butanol and hydrocortisone were not affected by the spraying of PBS onto the stratum corneum.

Permeation Experiments

In order to probe the possible mechanisms of action of Azone, the effect of Azone on the permeation of a series of hydrophilic and lipophic solutes has been studied. Polar and ionized solutes are expected to be more sensitive to the enhancement of a polar route. The permeation of lipophilic solutes would be increased with lipid fluidization.

Ara-A (vidarabine) and tetraethylammonium bromide (TEAB) permeabilities were determined in "paired" experiments in order to ascertain if increases in the permeability coefficients of Ara-A are due to lipid fluidization or polar route formation. The results show dramatic increases in permeability for both solutes (Figure 4). TEAB, a quaternary ammonium salt, presumably permeates through only polar routes. Thus, any increase in permeability for TEAB should be due to polar route enhancement. TEAB and Ara-A permeability coefficients appear to plateau prior to reaching stripped skin values. This suggests either that the stratum corneum becomes saturated with Azone or that maximal polar route enhancement was achieved.

The permeability coefficients of Ara-A are slightly larger than those of TEAB following Azone pretreatment ($P < 0.05$). There are a number of possible explanations for the greater increase observed for Ara-A. There may be a small

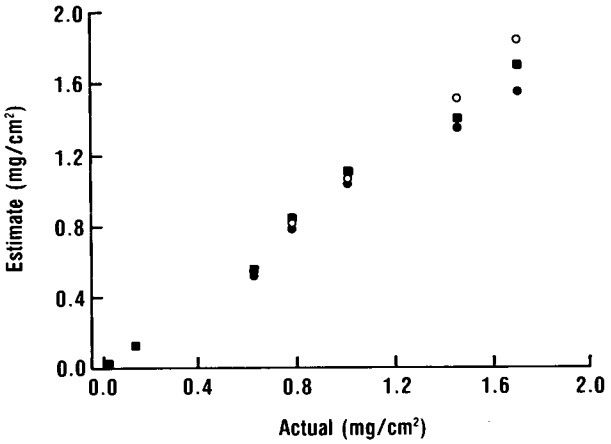


Fig. 3. Estimation of amount of Azone per unit area delivered to square 2 versus actual amount per unit area. Estimate equals (●) amount delivered to adjacent square; (○) mean amount of adjacent three targets; (■) amount delivered to adjacent square corrected by the ratio of the final two targets.

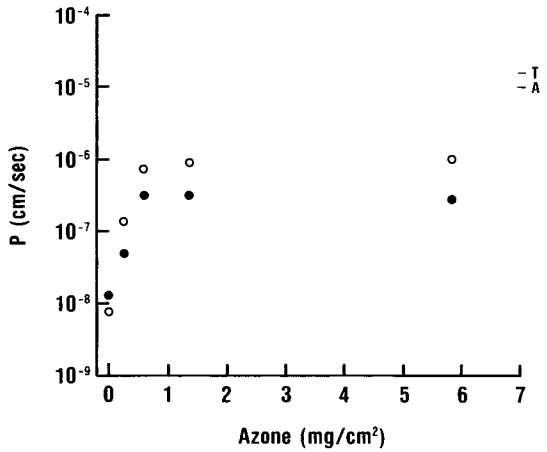


Fig. 4. Permeability at 37°C of Ara-A (○) and TEAB (●) as a function of amount of Azone per unit area. Stripped skin permeability coefficients are indicated by "−A" for Ara-A and "−T" for TEAB.

amount of an impurity present that has a higher permeability coefficient than Ara-A, such as tritiated water. However, TLC analysis of the receiver compartment at the end of an Ara-A experiment verified that the major permeant was either Ara-A or its metabolite, Ara-H. Ara-H has been shown to have a similar permeability coefficient to Ara-A (within 38.5%), allowing metabolism to be ignored (19). A second possibility is that the partition coefficient (polar route/aqueous) is lower for TEAB than for Ara-A. Due to the heterogeneous nature of the stratum corneum, this is a difficult hypothesis to test. The probable explanation is that Ara-A is able to permeate the lipid as well as the newly formed polar route. Ara-A will be sensitive to the lipid fluidizing effect of Azone, whereas TEAB, being ionized, will be less sensitive to lipid fluidization.

The dose dependencies of Azone on the hairless mouse skin permeabilities of Ara-A, butanol, and hydrocortisone are shown in Fig. 5. Hydrocortisone and butanol permeability coefficients appear to plateau to stripped skin values above 1-mg/cm^2 applications of Azone.

Mechanistic information can be gleaned from the region below the plateaued permeability coefficients (Fig. 5). If pore formation was the primary mechanism of action, the absolute increases in permeability would be the same for all permeants of similar molecular radius. This has been suggested as a possibility following long-term hydration (20). Pore formation does not represent a major mechanism of action for Azone at low doses for hydrocortisone and butanol because hydrocortisone, butanol, and Ara-A have different absolute increases in permeability.

Lipid fluidization has been proposed as a possible mechanism of action of Azone (15,16). For a *homogeneous lipid barrier,* the relative increase in permeability would be expected to be the same for all solutes, assuming that the increase in permeability is related to an increase in diffusivity and not in the partition coefficient. This assumption is considered valid since the enhancement effects are seen at extremely low doses of Azone. These low doses probably do not significantly alter the chemical nature of the stratum corneum lipids. This assumption is difficult to test since the stratum corneum/aqueous partition coefficient for a particular solute may not determine the partition coefficient of the rate limiting portion of the pathway for the solute.

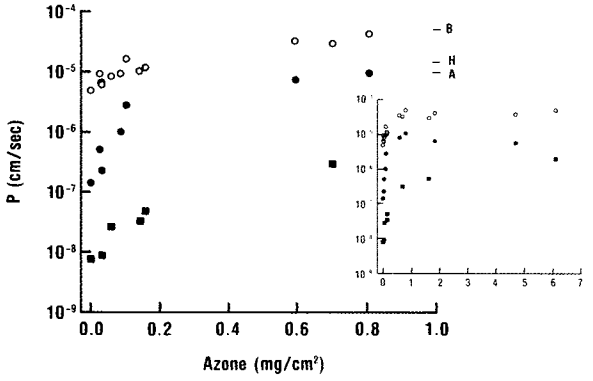
Azone doses from 0 to 0.2 mg/cm^2 result in approximately 6-, 20-, and 3-fold increases in permeability for Ara-A, hydrocortisone, and butanol, respectively. Ara-A is believed to permeate primarily through a polar route in the absence of Azone (21). Therefore, Ara-A would not be expected to be as sensitive to lipid fluidity changes as more lipophilic solutes, such as butanol or hydrocortisone.

The difference in the relative increase in permeability coefficients for hydrocortisone and butanol is more complex. Butanol is well below stripped skin P values (approximately fivefold less than stripped skin P values at a dose of 0.1 mg/cm^2 of Azone), so this cannot explain the lower enhancement experienced by butanol as compared to hydrocortisone. It is possible that hydrocortisone is sensitive to polar route enhancement, while butanol is not. To study this possibility, let ΔP_{HC}^{corr} be defined as follows:

$$\Delta P_{HC}^{corr} = P_{HC} - (P_{But}/P_{But,0})P_{HC,0}.$$

P_{But} and P_{HC} are the permeabilities of butanol and hydrocortisone at a specific Azone dose, and $P_{But,0}$ and $P_{HC,0}$ are the permeabilities of butanol and hydrocortisone with no Azone present. ΔP_{HC}^{corr} should be equal to zero for a homogeneous barrier. ΔP_{HC}^{corr} not only is greater than zero but also is much greater than the absolute increase in permeability experienced by Ara-A for a similar Azone dose. Ara-A is expected to be more sensitive to polar route enhancement than hydrocortisone. Thus, polar route formation in addition to increases in lipid fluidity does not account for the large relative increase in permeability noted for hydrocortisone over butanol.

Ethanol and corticosterone were used to investigate further the nonuniform enhancement characteristics of Azone. Azone doses from 0 to 0.2 mg/cm^2 result in approximately 2- and 25-fold increases in permeability (Fig. 6) for ethanol and corticosterone, respectively. Hydrocortisone and corticosterone permeabilities were also determined in a "paired" experiment using TLC to measure the respective fluxes. The



g. 5. Permeability at 37°C of butanol (○), hydrocortisone (●), and a-A (■) as a function of the amount of Azone per unit area (below) mg/cm^2). Stripped skin permeability coefficients for butanol, drocortisone, and Ara-A are indicated by "−B," "−H," and A," respectively. Inset shows data up to 7 mg/cm^2.

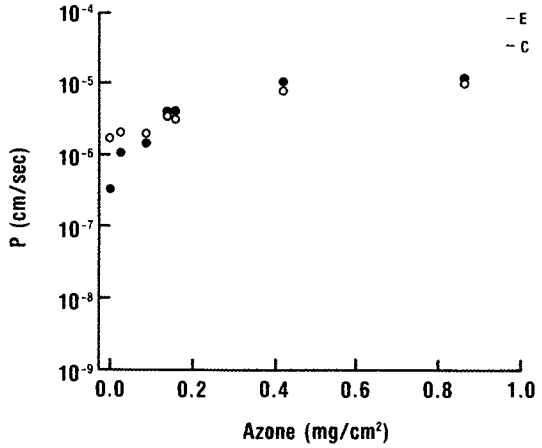


Fig. 6. Permeability at 37°C of corticosterone (●) and ethanol (○) as a function of the amount of Azone per unit area. Stripped skin permeability coefficients for ethanol and corticosterone are indicated by "−E" and "−C," respectively.

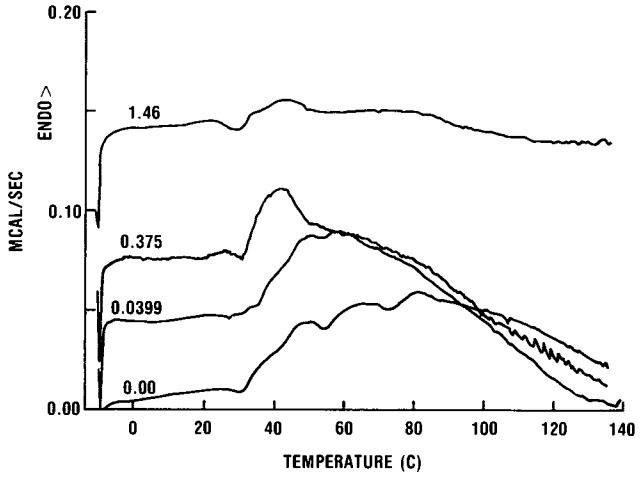


Fig. 7. Thermograms of stratum corneum samples pretreated with varying doses (mg/cm^2) of Azone.

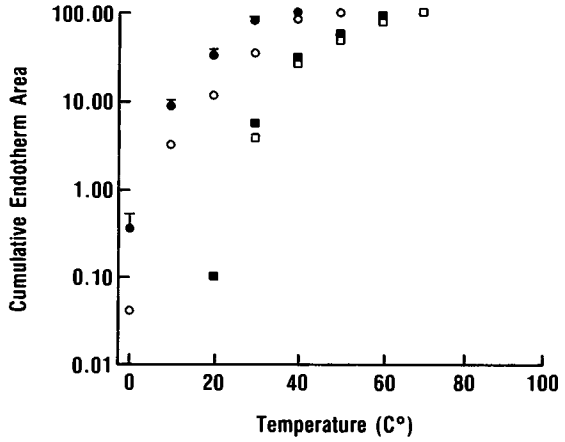


Fig. 9. Cumulative percentage endotherm area (and SD; $N = 2$) as a function of temperature for the second scan of stratum corneum samples pretreated with Azone. (□) Untreated; (■) 0.0399 mg/cm^2; (○) 0.375 mg/cm^2; (●) 1.46 mg/cm^2.

relative increases in permeability are similar for corticosterone and hydrocortisone.

The steroids are enhanced to a greater extent than the alkanols by Azone. It appears that the assumption of a homogeneous lipid model will not adequately describe the present data. High-resolution electron microscopy has demonstrated the existence of lipid bilayers in the intercellular space of stratum corneum (22,23). Gradients in fluidity and polarity have been demonstrated in model lipid bilayers (24,25). Therefore, bilayers are nonhomogenous barriers, whether diffusion pathways are perpendicular or parallel to the plane of the bilayer. The segregation of lipid classes within the bilayer could add another dimension to the concept of a nonhomogeneous barrier.

Solutes with smaller molecular radii may permeate more readily through structured lipids than larger solutes and would be less sensitive to enhancement by Azone. However, ethanol appears to be affected by Azone to the same degree as butanol, even though the molecular weight of ethanol is nearly 40% lower than the molecular weight of butanol. An alternate explanation for differential enhancement may be the fluidization of specific pools of lipids or specific portions of lipids within the bilayers. Permeants that are rate limited by a specific pool or bilayer portion would then be preferentially enhanced by their fluidization. Temperature-induced fluidization of lipid bilayers is expected to increase the diffusivity and partitioning of solutes in the lipid pathway (24). Fluidization of the lipid bilayers by Azone is also expected to affect partitioning and diffusivity.

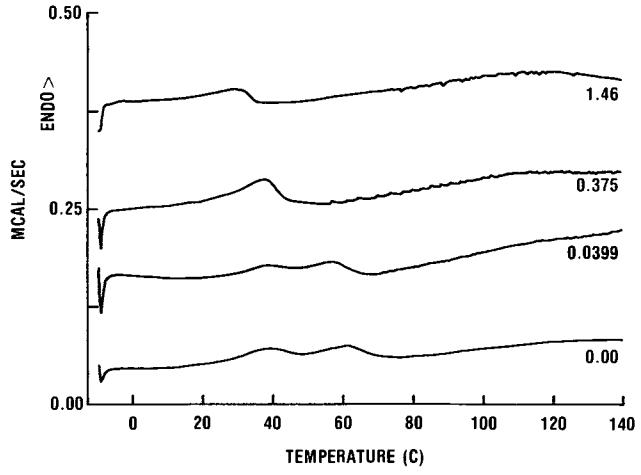


Fig. 8. Thermograms of stratum corneum samples pretreated with varying doses (mg/cm^2) of Azone following an initial scan to 140°C.

Differential Scanning Calorimetry

Previous DSC studies have demonstrated a broad endotherm from approximately 30 to 140°C which has been attributed to keratin (26). A number of lipid "melting" peaks were also observed from approximately 0 to 96°C. Two effects of Azone are evident by DSC. First, the underlying broad protein endotherm of stratum corneum appears to be altered with increasing amounts of applied Azone (Fig. 7). It is possible that alteration of the keratin structure could lead to the development of polar routes in the stratum corneum. This mechanism has been proposed for alkyl sulfoxides by Cooper (14). However, no quantitative correlation between this endotherm and permeability was found.

Increasing Azone doses also appear to alter the shape and area of the lipid endotherms (Fig. 7). This effect is more easily observed by temperature cycling. Scanning to 140°C has been shown to eliminate irreversibly the keratin endotherm (26). On the second scan of Azone-pretreated samples, the remaining lipid endotherms are noticeably altered by the presence of Azone (Fig. 8). The cumulative percent of endotherm area as a function of temperature is shown in Fig. 9. Increasing Azone doses shift the lipid transitions seen on the rescan to increasingly lower temperatures. This is good evidence of the lipid fluidizing effect of Azone on stratum corneum lipids.

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