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UNIVERSITY OF CALIFORNIA, IRVINE
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DAYTON, OHIO 45431

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AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

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TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-84-002

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

PAPER NO. 22

A DIRECT IN VIVO METHOD FOR STUDYING THE PERCUTANEOUS ABSORPTION OF VOLATILE CHEMICALS

A. S. Susten, Ph.D., B. Dames, B. S.
and R. W. Niemeier, Ph.D.

Division of Biomedical and Behavioral Science
Experimental Toxicology Branch
National Institute for Occupational Safety and Health
Cincinnati, Ohio

INTRODUCTION

Two general methods are commonly used to evaluate the in vivo, percutaneous absorption of chemicals. These are the indirect excretion analysis method originally described by Feldman and Maibach (1965, 1970) and the more direct method described and used by a number of investigators (Shah and Guthrie, 1983; Hofer and Hruby 1983; and Nishiyama et al., 1983). Direct methods provide better estimates of absorption for chemicals which tend to be very slowly eliminated from the body (Shah and Guthrie, 1983; Franz, 1975) and permit a more accurate indication of absorption during the periods immediately following dermal application of a test substance (Shah and Guthrie, 1983).

The purpose of this paper is to describe a direct method used in our laboratory to study the in vivo percutaneous absorption of volatile materials. Hairless mice were chosen as the animal model for these studies for a number of reasons. These include: (a) prior removal of hair is unnecessary; (b) the size and density of their hair follicles are more like humans when compared to heavily haired animals (Bronaugh et al., 1982); (c) skin permeabilities to a variety of chemicals have been shown in in vitro studies to be similar (within an order of magnitude) to those of human skin (Bronaugh et al., 1982; Stoughton, 1975); (d) the relatively inexpensive cost and ease of handling of mice compared to larger animals such as the pig and monkey; and (e) the ability to use larger numbers of animals to increase the power of the experiments.

A direct approach to studying dermal absorption of volatile materials was made possible by development of a "skin-depot" designed to capture the portion of test substances which would

normally be lost by evaporation. This feature allowed the use of metabolism cages for the capture of expired breath, as well as urine and feces. The paper will describe and discuss the following: the skin-depot design and its application; the general experimental procedures; tests performed to determine the utility of the skin-depot; and a comparison of benzene and toluene data obtained using different methods.

MATERIALS AND METHODS

Description of Skin-Depot

The skin-depot consists of 3 components and a guide needle which are illustrated in Figure 1.

The stainless steel casing is fabricated from "316 stainless steel stock." The bottom of the casing has a 3 mm wide flanged rim which surrounds an opening 1 cm in diameter. The flange serves to increase the area of skin contact and thus the attachment of the skin-depot. When glued to the skin using cyanoacrylate adhesive (Duro Super Glue, Loctite Corporation, Cleveland, Ohio), the skin-depot circumscribes a circular treatment area of approximately 0.8 cm^2.

Fitting snugly into the casing is a stainless steel wire mesh basket. The basket is fabricated by cutting and removing the bottom portion of a wire mesh filter element (Nupro, Catalog No. SS-4f-P4-230, Willoughby, Ohio). When placed inside of the casing, the basket holds approximately 100 mg of 20/40 mesh coconut derived activated charcoal, or a similar volume of other sorbent. A ridge, machined into the inner surface of the casing, supports the basket and insures that the basket will not slip onto the treatment site. The basket with its sorbent serves to capture volatilized test material for quantification and prevents contamination of expired air.

A teflon snap cap containing a 1/32-inch centered bore hole is used to close the skin-depot. Although not an integral part of the skin-depot, a 21-gauge disposable guide needle (Becton-Dickinson, Rutherford, New Jersey) is also shown in Figure 1 since it serves to facilitate the administration of the test material. Prior to the cell being affixed to the mouse, the guide needle is inserted through the hole in the snap cap and forced through the sorbent and wire mesh basket so that the tip of the needle is positioned just below the bottom edge of the basket (Figure 1). When fully assembled a skin-depot weighs about 3 g.

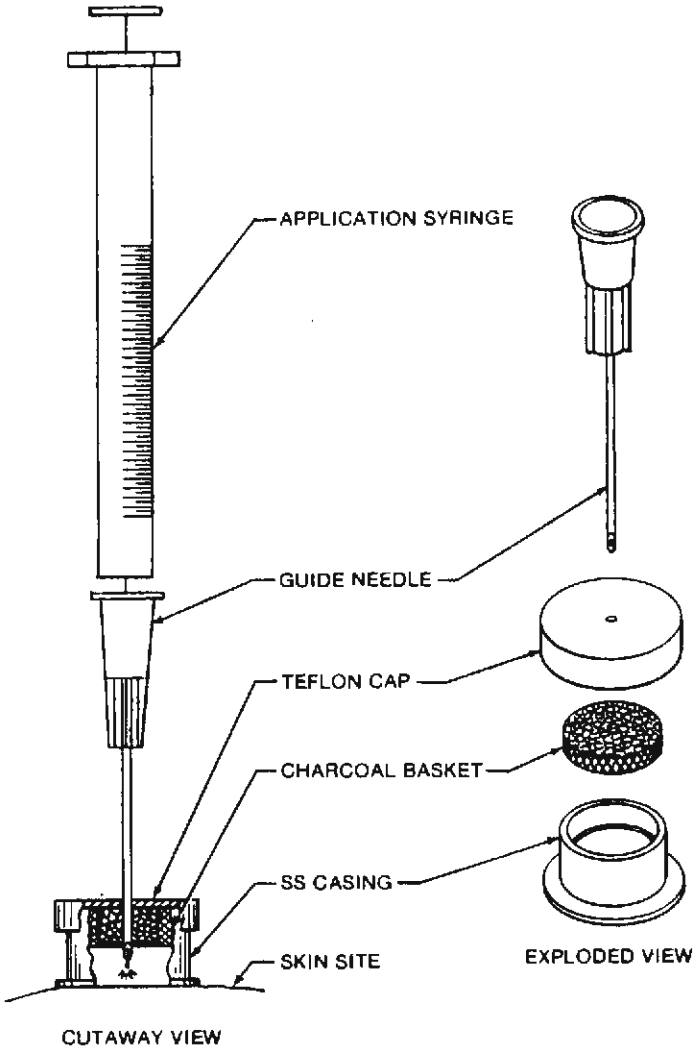

Figure 1. Diagram of skin-depot showing components (exploded view) and method for administering test substance to treatment site (cutaway view).

General Experimental Procedures

Attachment of the Skin-Depot

Affixing the skin-depot (with guide needle) to hairless mouse skin is best accomplished while the mice are anesthetized or sedated using a mixture of air and carbon dioxide (50:50 by volume). This allows the mice to recover from the anesthesia quickly and avoids the hyperexcitable phase common to barbiturate anesthesia. Attachment of the depot to the skin is normally complete within 1 to 2 minutes. However, to insure the integrity

of the attachment, the mice are kept sedated for an additional 3-5 minutes to visually inspect the attachment for gaps and, if necessary, add glue at the interface. Figure 2 shows the skin-depot mounted on a hairless mouse prior to the dermal application of the test chemical.

Figure 2. Photograph of skin-depot (including guide needle) attached to hairless mouse prior to the dermal application of test material.

Treatment Procedure

Administration of test substance is accomplished by inserting a 10 µL GC syringe (Hamilton Model No. 701) containing 5 µL of ^{14}C-labeled test substance through the guide needle until the blunted tip of the syringe needle is located slightly above the skin (Figure 1). After the test dose is applied, the syringe and guide needle are withdrawn from the skin-depot, which is lightly tapped to close any gaps in the sorbent. The mouse is lifted carefully to avoid dislodging the skin-depot and placed into a glass metabolism cage. Total elapsed time between the application of the test material and placement of an animal into a metabolism cage is less than 5 seconds.

Radioactivity delivered for each experiment is determined by injecting 5 µL of the test chemical into each of three 10 mL volumetric flasks containing an appropriate solvent, usually toluene. Triplicate 0.1 mL aliquots are taken from each flask to determine the average total ^{14}C in each flask. The mean for the three flasks is determined and used as the applied radioactive dose.

Metabolism Cage System

In the studies, two glass metabolism cage systems are connected to a vacuum system. Each cage system consists of: (1) a glass metabolism cage; (2) two air sampling tubes arranged in parallel to facilitate sampling during the experiment; and (3) two ethanolamine-containing towers which are also arranged in parallel. Flow meters control the air flow through each system to approximately 200-225 mL/minute; negative pressures within the cages are monitored and do not exceed 2 inches of water. Conditions are controlled so that flow and pressure are the same for each cage system. The cages permit collection of excreta (urine and feces) and expired air. Organics in expired air are trapped on appropriate air sampling tubes (charcoal, silica gel, etc); carbon dioxide is captured in ethanolamine-containing towers.

While in the metabolism cages, the mice are awake and unrestrained, but food or water are not provided due to the short duration of the study (4 hours or less). Access to the food chamber is blocked to prevent the mouse from dislodging the skin-depot. Two mice (1 per cage) are used for each experiment and three to six replicates are performed.

Sample Collection and Analysis

At the termination of an experiment, the mice are killed with carbon dioxide, the skin-depots are removed, and the radioactivity remaining in the depots are desorbed using 25 mL of an appropriate solvent. Aliquots of the solvent are taken for liquid scintillation counting. Thus a measure of the percentage of the dose which evaporates from the skin can be determined directly. Selection of the desorbing solvent as well as the absorbent (charcoal, silica gel, Tenax, etc.) used in the skin depot and air sampling tubes, is based initially on procedures for the specific test compound which are outlined in the NIOSH Manual of Analytical Methods (Eller, 1984). Desorption efficiencies for the solvent and adsorption capacity of the solid sorbent used for the test material are determined in preliminary

tests. Examples of the desorption efficiencies of solvents used during percutaneous absorption studies of benzene, carbon disulfide and toluene are shown in Table 1. These data also indicate the ability of the charcoal to adsorb organic solvents.

TABLE 1. DESORPTION EFFICIENCY OF SOLVENTS USED TO DESORB RADIOLABELED TEST SUBSTANCE FROM ACTIVATED CHARCOAL

Test Compound	Desorbing Solvent	Desorption Efficiency (%) of Dose
benzene	benzene	>98
toluene	toluene	>98
carbon disulfide	benzene	>98

* Desorption efficiencies were determined using a modification of the methods outlined in NIOSH's Manual of Analytical Methods (Eller, 1984). Briefly 2-3 air sampling tubes are used for each determination. The back end of a two compartment sampling tube (charcoal--100/50 mg, front and back respectively, SKC, Inc., Eighty-four, PA Catalog No. 226-01) was opened. The needle of a 10 µL GC syringe (Hamilton Model No. 701) was inserted into the opened sampling tube and forced through the back and front sections of the charcoal. Using a solvent flush technique, 5 µL of the ^{14}C-labeled test material was expelled into the front of the sampling tube below the level of the charcoal. The syringe was withdrawn from the sampling tube and the opened end capped. The charcoal tube was then placed in a vertical position with the front end positioned toward the top of the lab bench. The tubes remained in this position for 24 hours, although generally no liquid was visible in the tip of the tube after about 1 hour. The charcoal contained in the back and front sections was placed into separate scintillation vials, screw top lids were tightened, and the charcoal was desorbed for at least 1 hour in 5 mL of the appropriate desorbing fluid. The scintillation vials were occasionally agitated. Duplicate or triplicate 1 mL aliquots were taken for liquid scintillation counting. Values reported above represent the fraction of the dose found in the front section of the tubes since the skin-depot contains about 100 mg charcoal.

Skin application sites dissected from the carcass and the carcasses are placed individually into gas washing bottles, containing known volumes of 1N sodium hydroxide. Digestion

occurs with constant stirring under vacuum, at 55°C, for approximately 18 hours. Any volatile labeled materials released during the digestion process are trapped using in-line air sampling tubes, which are subsequently desorbed and quantified.

Fecal pellets and weighed aliquots of the digested application sites and carcass are oxidized in a Packard Tricarb Oxidizer using Carbosorb (Packard Instrument Company) as a trapping agent. Urine is diluted to 5 mL with a 50/50 methanol/water mixture. To determine the residual radioactivity remaining on the wire-mesh screen and the urine/feces separator, a small quantity (4-8 mL) of the methanol/water mixture is used to rinse the cages. This wash is collected and the volume adjusted to 10 mL with methanol/water mixture. Triplicate 1 mL aliquots of diluted urine and cage washings are pipetted directly into scintillation vials containing 10 mL of Permafluor V and 3 mL of Carbosorb.

Radioactivity is measured by liquid scintillation spectrometry. Samples are corrected for quenching, using an automatic external standardization procedure based on the H number concept (Horrocks, 1977).

Data Analysis

For each animal, the percent of the applied dose recovered is determined for the skin-depot, the guide needle, and the fraction that constituted the absorbed dose (excreta, expired breath, skin application site, and the carcass); total recoveries are based on these values. Group means (± standard error) and ranges, as well as distribution of the radiolabel are calculated. The percent of available dose absorbed is calculated and takes into consideration the variable amounts of test solvent which may adhere to the guide needle and are not available for absorption. Percent of available dose is defined as:

$$\frac{\text{Percent of Applied Dose Absorbed}}{\text{(Percent of Applied Dose Absorbed)-(Percent of Applied Dose in Guide Needle)}} \times 100$$

RESULTS AND DISCUSSION

Representative data from a series of experiments with benzene and toluene are shown in Tables 2 and 3. Total recovery for these volatile substances was above 90% with most of the recovered dose appearing in the skin-depot. The average percentages of the applied dose absorbed were: 0.89 and 2.06 for

benzene and toluene, respectively. Correction of the absorption data to account for the percentage of the applied dose that remained in the guide needle indicated that the percentage of the available dose absorbed was 0.98 and 2.2 for benzene and toluene, respectively.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY INTO VARIOUS SAMPLING COMPARTMENTS FOLLOWING THE DERMAL APPLICATION OF ^{14}C-BENZENE AND TOLUENE

	Means of Percents of Applied Dose Recovered[a]			
Test Materials	Absorbed[a]	Mean ± SD (Range) Skin Depot	Guide Needle	Total Recovered
Benzene (N=7)	0.89 ± 0.7 (0.13 - 2.12)	79.5 ± 9.5 (66.6 - 96.3)	10.4 ± 6.5 (0.06 - 19.1)	90.8 ± 4.9 (82.3 - 98.4)
Toluene (N=12)	2.06 ± 1.6 (0.3 - 4.8)	83.8 ± 6.0 (74.5 - 97.0)	9.1 ± 5.1 (0 - 14.8)	95.0 ± 3.1 (89.3 - 100.6)

[a] Absorbed = percent of dose applied which was recovered in excreta, carcass, skin application site, and expired breath.

Adherence of the depot was a concern since others have noted that attachments of skin devices to hairless skin is poor as compared to haired skin (Wilson et al., 1982, Bronaugh, personal communication). Proper attachment of the depot is essential to prevent falsely high estimates of dermal absorption. The ability of the depot to adhere to hairless mouse skin and to restrict the contact area of the test solvent to the circumscribed skin site was evaluated under conditions in which the mice were free to move about after treatment. Five to 20 µL of methylene blue-colored acetone, a solvent for the adhesive, were injected into the skin-depot. After approximately 15 minutes, the depots were removed and the circumscribed treatment area was observed. Well defined, circular areas of blue color were usually found with the outer borders of the circles sharply delineated at the interface of the inner perimeter of the skin-depot flange and the skin treatment site. Only when 20 µL volumes of colored acetone were administered did the blue coloration extend beyond the inner perimeter of the skin depot. In no case did the skin-depots become detached. Thus good adherence of the depot to the skin of hairless mice was confirmed for a time period of 15 minutes.

It should be noted, however, that during actual studies breaks in attachment have occurred before the 4-hour end point of our experiments. The initial sign of a deteriorating attachment

is a peeling back of the skin from the outer flange border. If peeling occurs, it usually begins 1 to 2 hours after the mice are placed into the metabolism cages. In some instances the break proceeds as far as the inner perimeter of the skin-depot.

TABLE 3. PERCENTAGE DISTRIBUTION OF THE ABSORBED DOSE (^{14}C) INTO VARIOUS FRACTIONS FOLLOWING THE DERMAL APPLICATION OF ^{14}C-BENZENE AND TOLUENE

	Percentage of Absorbed Dose in Various Fractions[a]			
Test Materials	Carcass	Mean ± SD (Range) Application Site	Expired Air	Excreta[a]
Benzene (N=7)	22.6 ± 17.0 (3.9 - 45.8)	4.7 ± 4.6 (0.28 - 13.3)	40.1 ± 28.0 (14.8 - 85.2)	32.7 ± 17.1 (9.6 - 64.6)
Toluene (N=12)	15.4 ± 7.89 (5.0 - 26.1)	11.0 ± 10.8 (1.6 - 41.9)	20.5 ± 17.5 (3.2 - 52.5)	53.1 ± 19.7 (11.5 - 80.1)

[a] Excreta = the sums of the means of the percentage absorbed for urine, cage washings and feces.

How this loosening of the skin-depot attachment affects the results was evaluated in two ways with benzene. First, data from a series of absorption studies with ^{14}C-benzene were carefully scrutinized for deviations in patterns of total absorbed and for percentages of radioactivity found in the urine, feces, and expired breath (Susten et al., 1984b). No consistent or significant differences were found in those values in mice with intact seals compared to those in which the seals were suspect or became incomplete over the course of the experiment. Second, a separate series of tests was conducted to determine the time required for the skin-depot to adsorb the volatilized portion of the test dose and to determine whether or not the level of radioactivity in the depot changed with time. Depots were glued to the backs of 6 hairless mice anesthetized with sodium pentobarbital (100 mg/kg, i.p.). At various times after the dermal application of 5 µL of labeled benzene, the skin-depots were pulled from the attachment sites and the radioactivity in the depots was determined. The data indicate that adsorption onto the skin-depot charcoal was maximal by 1.5 minutes and remained unchanged for at least 2.5 hours (Figure 3.). Thus it appeared that if the skin-depot attachments begin to fail after 5 minutes, loss of solvent from the skin site into the expired breath fraction is unlikely.

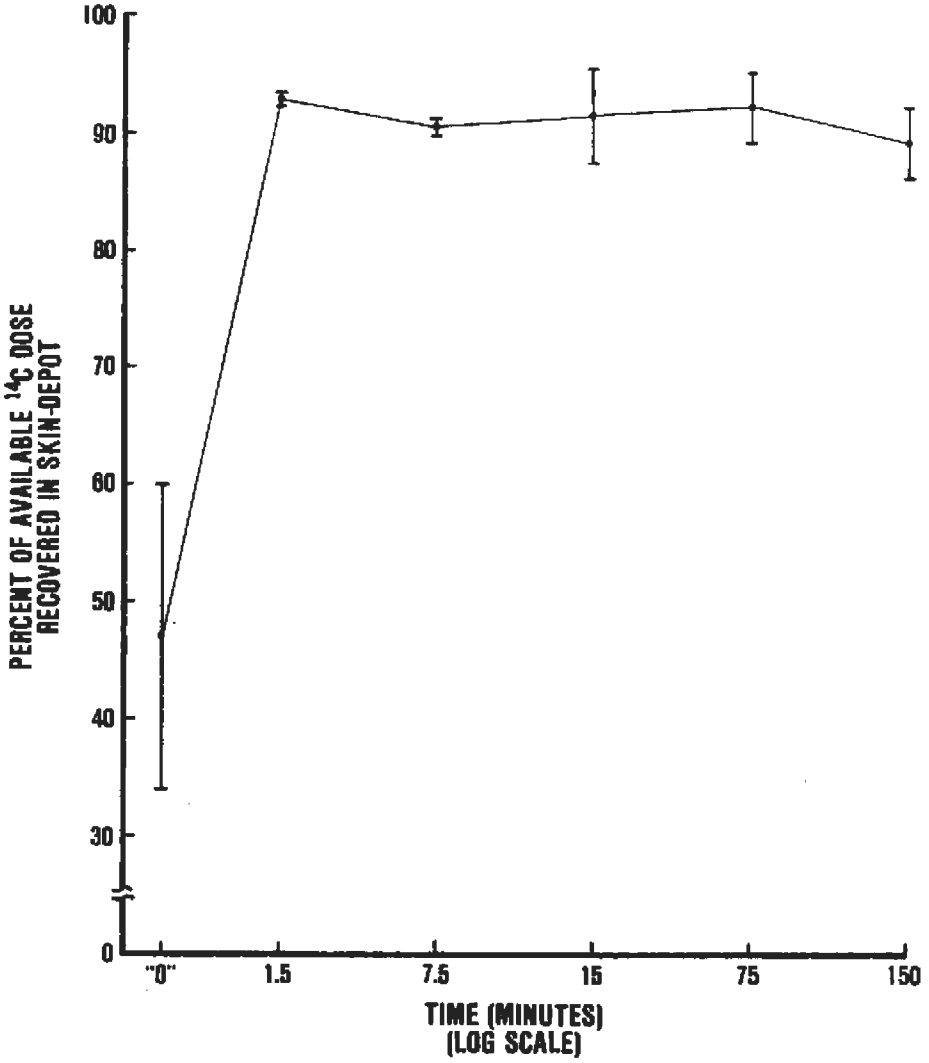

Figure 3. Percent of available dose (^{14}C) recovered in the skin-depot at selected times after the dermal application of ^{14}C-benzene to the skin of hairless mice. Each point represents the mean (± SE) of 3 replicates; all points were significantly different ($p<0.05$) from the "0" time period (time immediately past injection) but were not different from each other (Tukey's studentized range test for pairwise comparisons).

We believe that factors which tend to minimize the effects of breaks in attachment are small volume of test material, affinity of the sorbent for the test substance, and rapid absorption of test substances (Susten et al., 1984a,b).

To determine if there were leaks of test substance from the skin-depot by migration through the charcoal, the capacity of the skin-depot to capture and hold 5 µL of volatile test material was tested under simulated experimental conditions using labeled ^{14}C-benzene. In these studies, the attachment of the depots and treatment of the mice were the same as described under Materials and Methods, except that the mice were killed with an excess of carbon dioxide just prior to the administration of a test dose of labeled benzene. The dead mice were placed into metabolism cages and remained there for 4 hours. The charcoal tubes which would normally be used to capture expired breath were taken and processed for radioactivity. No radioactivity was found in the charcoal tubes indicating that the skin-depots, at least under these conditions, were capable of adsorbing all volatilized test material. These results were not surprising given the demonstrated adsorptive capacity of the charcoal, about 7 mg of benzene when used for air sampling at a rate of 0.2 L/min (Eller, 1984). The 7 mg capacity is in excess of the 4.4 mg (5 µL) dose of benzene used in these experiments.

Finally, to evaluate the utility of the method we compared absorption data, expressed as percent of dose absorbed, for several compounds with data obtained in man or in other animal models. Although there are problems in comparing data reported as percent of dose absorbed due to differences in specific dose (mg/cm^2), radioactive dose (µCi/kg), percent of the total body surface area exposed, and time of exposure, comparisons are still useful. Table 4 presents the results of our studies with benzene and toluene and of several recent studies reported in the literature using the indirect excretion analysis procedure. On the basis of percent of applied benzene dose absorbed, our data with the hairless mouse suggest that dermal absorption of benzene and toluene may be some 5-7 times greater than has been estimated in monkeys, and for benzene, 10-18 times greater than has been estimated in the minipig and human, respectively.

The present model can also be evaluated by comparing the ratios of absorption between compounds under similar methods of study. Franz (1980), using excretion analysis procedures in monkeys, presented data indicating that the absorption of toluene was about twice (2.2) that reported for benzene (0.44 vs. 0.20% of applied dose, respectively) (Table 5). Approximately the same ratio (2.3) was calculated for the absorption of toluene in the hairless mouse as compared to the absorption of benzene in the same species (Table 5). Similarly, if one compares the absorption of benzene and toluene across species (i.e., hairless mouse vs. monkey), ratios of 4.5 and 4.7 are calculated for

benzene and toluene, respectively (Table 5). Thus, similar patterns of absorption are observed in these species regardless of the experimental approach.

TABLE 4. COMPARISON OF IN VIVO DERMAL ABSORPTION OF LIQUID BENZENE AND TOLUENE (% APPLIED DOSE)

Species	Radioactive[a] Dose (μCi/kg)	Absorbed % of Applied Dose Mean ± SD	Source
BENZENE			
Hairless Mouse (back)	100	0.89 + 0.65 (7)[b]	Susten et al. 1984b
Monkey (forearm)	0.6	0.17 ± 0.14 (3)	Maibach and Anjo, 1981
Monkey (back)	13.3	0.14 ± 0.08 (6)	Franz, 1983
Human (palm)	0.1	0.13 ± 0.04 (4)	Maibach, 1980b
Mini-pig (back)	10	0.09 ± 0.04 (2)	Franz, 1983
Human (forearm)	0.06	0.07 ± 0.04 (4)	Maibach, 1980a
Human (back)	1.3	0.05 ± 0.05 (4)	Franz, 1983
TOLUENE			
Hairless Mouse (back)	20	2.06 ± 1.6 (12)	Susten et al. 1984a
Monkey (back)	13.3[c]	0.44 ± 0.07 (4)	Franz, 1980

[a] Weights (kg) were assumed to be as follows: hairless mouse (0.025); Rhesus monkey (7.5); mini-pig (10); human (80). Data calculated on the basis of information provided by the original reports.
[b] Number of subjects.
[c] Radioactive dose not given; assumed to be 100 μCi.

Some of the quantitative differences noted above may be due to the experimental approaches. The indirect excretion analysis method of Feldman and Maibach (1965; 1970) measures total radioactivity appearing in excreta (urine and feces), usually over a 5-day period following dermal exposure. To calculate total amount of labeled compound absorbed, the method requires the use of a correction factor based on the amount of radioactivity appearing in the excreta following a parenteral administration of the test compound. However, the assumption required, i.e., that the pharmacokinetics of parenterally administered compound is the same as dermally absorbed compound, may not always be valid, especially for compounds which are very slowly eliminated (Franz, 1983; Shah and Guthrie, 1983). The excretion analysis method, as generally performed, does not provide data on absorption and elimination by other routes at time periods immediately following dermal application. This is especially critical for volatile liquids such as benzene and toluene which are apparently rapidly absorbed (Franz, 1983; Susten et al., 1984a,b) and eliminated to some extent in expired

breath (Lauwerys, 1980). The direct method described in this paper does not require any correction factor and thus dermal absorption can be summed directly from levels of radioactivity in the carcass and expired breath as well as the excreta.

TABLE 5. RATIOS OF DERMAL ABSORPTION FOR BENZENE AND TOLUENE

Species	Percent of Dose Absorbed		Ratio
	Benzene	Toluene	
	(A)	(B)	(B/A)
Monkey[a]	0.20	0.44	2.2
	(C)	(D)	(D/C)
Hairless[b] Mouse	0.89	2.06	2.3
Ratio	(C/A) = 4.5	(D/B) = 4.7	

[a] Franz (1980), indirect method (excretion analysis).
[b] Susten (1984 a,b), direct method (measurement of expired breath, carcass, and excreta).

Two additional differences in methodology should also be noted. First, in experiments conducted in our laboratories, the exposure site was covered which may delay evaporation and increase absorption; sites were not covered in the studies reported by Maibach and Anjo (1981) and Franz (1980; 1983). Occlusion has been reported to increase absorption as much as 10 times (Feldman and Maibach, 1965). Second, the radioactive dose (μCi/kg) used by us was in some cases more than 100 times greater than those used by the other investigators (Table 4), thus enabling an increase in accuracy and sensitivity. The radioactive dose may be an important factor particularly in excretion analysis studies where rather small percentages of the dose are absorbed and even smaller amounts are excreted via the urine. The consequences of the latter would be very low count rates in the urine, often approximating the limits of detection which could compromise the accuracy of the excretion data; however, the system we devised would not approach occlusion conditions and was designed specifically to approach uncovered conditions.

In summary, we believe that the experimental model described in this paper is useful for directly evaluating the in vivo dermal absorption of volatile compounds.

DISCLAIMER

Mention of trade names does not constitute an endorsement by the National Institute for Occupational Safety and Health.

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Dr. Suskind (University of Cincinnati): Dr. Reifenrath told us that there was a difference in the dermal penetration of certain substances between the human/mouse skin preparations and nude mouse skin and explained that you had to excavate some of the dermis before you were able to do the human skin transplant and stated that the removal of part of the dermis constituted removal of a barrier. First of all, I am not convinced that the dermis is a barrier. I would like to suggest, however, that the increase in rate of penetration is due to higher blood flow rates in the mouse compared to man. Metabolic rate is much greater in the mouse than in man. I would think that the removal of the dermis also means removal of blood vessel access for the penetrant and that would result in a decrease of penetration in the man/mouse model instead of an increase. Had you considered that possibility before accepting the idea that differences were due to removal of the dermis?

Dr. Reifenrath (Letterman Army Institute of Research): One of the difficulties in trying to answer that issue is that we were only able to graft a split thickness of skin to the animal. It would have been nice to compare grafts of full and variable thicknesses. Perhaps with the nude rat, where it is possible to graft thicker pieces of skin, we will be able to examine that question. The explanation that we put forward is consistent with the data. There could be alternative explanations for those findings but they will have to await some further experimentation. In experiments which were done in vivo on pigs where we actually looked at residues in the dermis and subcutaneous fat and compared those residues to the octanol:water partition coefficients and the fact that we don't see the buildup of compounds in the dermis in the grafted nude mouse suggests that we removed some barrier.

Dr. Wester (University of California, San Francisco): I would like to add something to that. Dr. Suskind assumed that percutaneous absorption is correlated with blood flow and the fact is we do not know that. No one has ever done a definitive study to show that percutaneous absorption is dependent on blood flow. We used to assume and teach our students that when material got into the dermis it was immediately picked up and distributed through the body and whenever we discussed over the counter preparations where you rub the material on the skin and expect it to get into the muscle to relieve the soreness and we as pharmacists in the School of Pharmacy assumed that that couldn't happen. We now know that material rubbed on the skin goes down into the muscle,

bypassing the capillary system. I think that you have to be very careful when you make the assumption that percutaneous absorption and blood flow correlate.

Dr. Spencer (S. C. Johnson and Son, Inc.): In the nude mouse model of grafted human skin, were histology studies made to demonstrate whether or not capillaries or blood vessels move into the graft to give competent capillary flow?

Dr. Reifenrath: I have not done those examinations but from histology of the grafts and the surrounding tissue the host blood vessels grow right up to the graft vessels and sort of join or merge at that point. You don't see the host blood vessels invading into the graft vessels. They appear to join.

Dr. Slonim (Wright Patterson Air Force Base): Dr. Wester, what would you do the instant lindane spilled on your arm?

Dr. Wester: What would I do? I would wash it with soap and water and I wouldn't touch it again. I think it is very interesting that as you noticed I was knocking soap and water treatment because it wasn't doing what we thought it was, but if you start removing chemicals from skin with things like rubbing alcohol or acetone you only make things worse. People ask what I would do. If it happened to me or happened to a patient I would wash with soap and water and then I would see that the patient had some type of follow up attention. The follow up is important in case there was serious contamination. I think the important thing is to wash with soap and water because we don't have anything better, and then follow that individual to be sure that nothing worse develops.

Dr. Dost (Oregon State University): I have a very pragmatic interest in this problem because we deal with pesticide applicators and workers who are exposed to occupational levels of materials, and sometimes catastrophic exposures occur such as crop dusting pilots who fly into hillsides and that happens every once in awhile. What is the influence of the carrier solvent on experimental results? Feldman and Maibach used an acetone carrier in those experiments quoted here and also in their work with other pesticides. What has been done with actual formulations of various pesticides?

Dr. Reifenrath: I can add a few comments about the use of vehicles such as acetone or ethanol compared with neat application of materials. We studied diethylmalinate which has similar physical properties to some of the organophosphorus compounds, and we also studied DFP. Skin penetration of these compounds is

slightly increased when either acetone or ethanol are used as vehicles. The vehicle may disperse the material over a larger surface area of the skin as opposed to neat application of solid materials. With in vivo application of diethylmalinate, when we decontaminated the skin 48 hours posttreatment with ethanol we saw some increase in the radiolabel appearing in the animals' excreta. This was similar to what Dr. Wester described as the wash effect. From the experiments that we have done these vehicles have an effect on penetration of compounds and seem to increase it.

Dr. Bronaugh (Food and Drug Administration): One of the toughest questions that we face is what vehicle should be used in testing some of these compounds. It is probably always best if you use the formulation that the material is present in. If you are going to use only one vehicle to represent the absorption of a compound that may be found in a number of different vehicles, then I am of the opinion that acetone is a good choice, because you remove the effect of the vehicle on permeation, although you don't know what the penetration would be in every formulation. There has been much concern about the effect of acetone or other vehicles on damaging the barrier properties of the skin. We have conducted some unpublished studies that show that if we pretreat an area of skin with small amounts of acetone, 5 microliters per square centimeter, it evaporates within a minute and does not alter the permeability of skin to compounds applied to the pretreated skin. I would not be concerned about vehicle damage to the skin and I would say as guidance that if you are going to use a vehicle, acetone is not a bad choice. That could differ with some formulations.

Dr. Wester: I can't cite the reference but someone has looked at what happens to the solvent that materials are applied in. This individual used radiolabeled ethanol and within a few minutes 95% of the ethanol that was applied had evaporated and really had no effect on the skin. Essentially what you get is a net deposit of the neat chemical using a carrier which then evaporates and goes away. I agree that there probably is not any real solvent effect when we do studies in an open air environment.

Dr. Susten (NIOSH): Dealing with volatile compounds may be a somewhat different story. Franz did a series of experiments for the American Petroleum Institute and showed that he could increase the absorption of benzene as percent of applied dose if it was applied in P6 gasoline. There are some data that show a very large increase in the absorption of benzene in the in vitro system when benzene was applied in toluene. Comparisons are difficult to make with volatile compounds where you can see some

increased absorption. We ran some studies with a rubber solvent and one thing we did that had not been done before was to give equal doses of radiolabeled material. We gave benzene as a 100 microcuries per kilogram dose. The reason that we gave 100 microcuries per kilogram as a neat compound was that you get less than 1% absorption and you excrete it. When we tested benzene in a rubber solvent with a benzene content of 0.5% and gave it at equal radiolabeled concentration, 100 microcuries per kilogram, we saw no differences as percent of applied dose.

Dr. McDougal (AFAMRL/THB): I think there is another way that we can look at solvent vehicles. It's best to try to avoid them and basically I think that's what we do when we use a very small quantity of acetone. I think that you can also utilize a vehicle if you understand what the vehicle is doing to the penetration rate of the chemical and the vehicle itself doesn't have an effect on the surface barrier and if you know the partition coefficient of the chemical that you are looking at in that vehicle. Its main effect can be the alteration of the effective concentration of the chemical that's presented to the skin. We have done some preliminary studies with dihalomethanes that show we can calculate the same permeability constant in centimeters per hour by compensating for the exposure concentration depending on the vehicle. We have looked at a water vehicle, mineral oil, and at what we call "no vehicle". If we consider the partition coefficient of dibromomethane in that vehicle and reduce the exposure concentration for that effect; then we get a very similar permeability constant.

Dr. Miller (Ohio State University): Dr. Reifenrath, have you compared the nude mouse with nude mouse skin grafted on another nude mouse?

Dr. Reifenrath: We have not made that comparison.

Dr. Miller: In your presentation you showed a pig in a metabolism cage and I thought I could see wire mesh holding the bandage away from the skin. Was my interpretation of the slide correct?

Dr. Reifenrath: That's correct.

Dr. Miller: Was it open at the ends so that air could circulate or were the ends covered with gauze?

Dr. Reifenrath: The patch is shaped like a foam donut and has a hollow area in the middle, peripheral to the application area, and then a layer of nylon window screen is glued to the top of

the opening with gauze layered on top of that. In our experiments we see a decrease in total recovery of the compound as volatility increases, and in that test system the patch does not prevent compounds from evaporating from the skin. Some material is caught in the bandage, but it is not designed as a trap. It's designed to be a nonocclusive covering.

Dr. Crocker (University of California, Irvine): I want to thank Dr. McDougal and his associates for an elegant piece of work which I think has modified the way we will look at whole body exposures in the future, especially to volatile materials that may be absorbed across the skin. I am more concerned, at the moment, with the problems of in vitro penetration studies because of the in vitro work we have been trying to do with whole trachea, where much the same problem occurs because there is metabolic function of the organ as a membrane that has secretory and absorbtive power. Most of these phenomena are dependent upon maintaining the organ in an intact vital state. I would expect that the in vitro preparations discussed here used dead skin and therefore there was no metabolic potentiation. Essentially, we have been discussing the treatment of thin sheets of leather in terms of what passes across them. I feel that we have little to learn from such data unless we can validate that the tissue is living. I am sure that there are some answers to this.

Dr. Bronaugh: I wouldn't be too sympathetic. History has shown numerous in vivo and in vitro comparisons where good agreement is obtained even though dead skin or, as you say, leather was used. It's a fact that the barrier in the skin is different from the barrier in the trachea or the gut. This is an area of controversy and I guess we're here to discuss areas of controversy. Toxicologists are concerned about the viability of skin and rightly so. The speakers here have helped increase the awareness of the possibility of skin metabolism affecting the rate of penetration. I'm talking about the rate of penetration into the skin and not what happens after it gets there. We know compounds are metabolized in the skin and that affects the rate of penetration. Some elegant work has been done with benzpyrene to show that it is metabolized in the skin and that it is necessary to compare its in vivo and in vitro penetration and not just do a study where benzpyrene is applied to viable skin and then look for polar metabolites in the receptor fluid where it cannot enter. We measured the in vivo absorption of benzpyrene in rats and found that we got close to 50% absorption and when we used the leather from rats we got 50% penetration over the course of 8 days. There is no significant difference in absorption of benzpyrene through rat skin in vitro if we put non-ionic surfactant solution in the receptor fluid to permit the benzpyrene to

enter the receptor fluid. We know that many compounds are metabolized in the skin but the question is how much of these compounds are metabolized. Is 1/10 of a percent of what gets into the skin metabolized or is it 50 to 100%? I think that what we are going to find in the long run is that the metabolism that takes place in the skin does not significantly alter the rate of entry of material into the skin. Metabolism affects the pharmacokinetic and pharmacologic action of the material after it penetrates.

Dr. Kao (Martin Marietta Energy Systems, Inc.): We may be partly responsible for some of this controversy because of the in vitro system that we developed where we maintained viability of the skin. If you compare skin from animals that have been pretreated with something like TCDD or 3-methlylcholanthrene, you indeed get a 3 to 4 fold increase in the rate of penetration of benzpyrene. We have extended that study to look at testosterone where we compared its effect in viable versus non-viable skin in the mouse and we did not see any differences in the rate of penetration. However, when we took the receptor fluid and examined it we found that 50% of the testosterone was metabolized, suggesting that in viable tissue there is first pass metabolism of something that you may apply topically. I think that we ought to put more effort into looking at what extent metabolism plays a role in percutaneous absorption.

Dr. Reifenrath: We have tried to maintain viability of the tissue in our in vitro test system and we maintained that pig skin under tissue culture conditions similar to those described here today and were able to show that the skin can then be grafted to the nude mouse and survive. We have looked at radiolabeled glucose turnover and after 48 hours we still maintained 75% of control values. We have been able to show from some in vitro experiments that were done in parallel with the in vivo experiments that similar metabolic conversions happen. In our experiments we may be using doses of compounds that overwhelm the capacity of the skin to metabolize and it may not appear that metabolism is doing anything to the material. When lower doses of materials are used, the ability of the skin to metabolize the compounds becomes more evident. We have taken a number of compounds and exposed skin, using the method I described and then took the skin and put it in a jar of ethylene oxide vapor for 8 hours. We are not sure we have killed the skin but we think we might have done something bad to it and we see no difference in the disposition of the radiolabel. We may have overwhelmed the effect that we were looking for by using large doses. For many compounds the feeble metabolic activity of skin is not something

that is readily seen in these experiments and I am sure there are going to be exceptions or variations from one skin to the next. So with that I'll be quiet.

Dr. Spencer: I want to comment on the statement that was made about skin metabolism equating stratum corneum to leather. Those of us who work with skin penetration understand that the stratum corneum is not leather and we should be very explicit about this. Leather is a tanned collagen material that is not only dead to start with but is further tanned to make it tough. Stratum corneum is a fully differentiated membrane on the surface of the skin made up of biologically dead cells that are basically keratinaceous in structure put together with a series of lipid layers. That structure is definitely not equatable to leather and I don't want anyone to go away with that misconception. There are processes going on in the stratum corneum because the entire membrane is undergoing desquamation. We don't know exactly what causes that process but conceivably there could be enzymes still active within the stratum corneum which is presumably dead. Getting back to the question of decontamination, I want to make another comment. In a pragmatic sense we are talking about washing the skin with soap and water and one thing that we shouldn't forget is to remove the reservoir source of contaminant from the skin. Clothing may be wet and we should get the person out of the area if there is a major spill. The other thing about decontamination which I would like to hear comment on is the question of actual concentration on the skin surface. Percent of applied dose is good for expressing experimental data but if you've got a material applied at a high concentration to the skin surface, the toxicologic significance to the individual is much greater. I would presume that if you've got just a few percent of toxic material the net effect on the organ would be different. I would be interested to hear others elaborate on that point.

Dr. Wester: First, I think we should realize that metabolism is important and most of the penetration rates measured either in vitro or in vivo are done with radiolabeled materials. You are really measuring penetration rates of carbon 14 and once you get to the actual compound like we saw today there may be a different story. Second, those of us involved in transdermal delivery studies are very concerned about first pass percutaneous metabolism because we want to deliver the drug unchanged. We know that with the new nitroglycerin transdermal systems, 25% of the nitroglycerin is destroyed by the skin as it passes through and as our techniques improves we will know more about the effects of first pass percutaneous metabolism. Dr. Spencer brought up a very good point that reservoir sources such as clothing are a key

to the toxic effect of a compound. What happens in the field is that workers will go out and not only get a chemical on their hands but they will also get it into their clothing and then the clothing becomes a reservoir that continues to supply material to the skin and into the body. When we see acute poisoning it is usually from some type of accident that involves a reservoir. For instance, a back pack container of pesticide that is being sprayed leaks and gives a continual supply of material to both the clothing and the back. There are case reports of people who have sprayed pesticides and gotten material inside their boots that they continued to wear for four hours or more and the boots became a reservoir of the material for them. I think reservoir sources are extremely important.

Dr. Suskind: We have spent a lot of time talking about practical things and that is really very appropriate, but I think we ought to spend a little more time discussing the last paper. That presentation introduced a new, very sophisticated, and very carefully designed toxicologic method. A method which I hope can not only be used to assess penetration or reabsorption of a radiolabeled material but I would hope you can actually use that method to determine the metabolites of the penetrant. It has the potential to do that, not only for the very volatile materials but perhaps even less volatile materials. It's a very unique method and I'd like to ask Dr. Susten whether he has any idea as to why his results are so different from results obtained using the indirect method in human skin?

Dr. Susten: The first obvious point is that we used a different skin but that only explains part of it. There are definite methodological differences. Our skin depot was designed with a hole in the top to represent an uncovered skin. It is not occluded because the volatile material is absorbed and leaves the site of application. We have reported our data in percent of absorbed dose. Unfortunately the specific dose, reported as milligrams per centimeter squared was not the same. Because the volume applied per square centimeter is different, the percent of absorbed radioactive dose is going to be different. This becomes exceedingly important where you have materials that are volatilized from the skin. We know that benzene does not penetrate very well. If you do excretion analysis procedures and measure the benzene in urine, feces, and expired breath you will find that only a very small amount was absorbed percutaneously, and an even smaller fraction of that appeared in the urine. Unless your radioactive dose is very high you are going to be down around background levels of radioactivity. I am not sure of the accuracy of that type of data. We decided to use 100 microcuries of material based on an estimate of how much would appear in the

digest. We wanted to get at least twice the background value. The excretion analysis procedure makes the assumption that what you apply as a bolus dose intraperitoneally has the same pharmacokinetic action as a dermally applied material. In answer to your question, we used a covered area which has been shown to increase absorption.

Dr. Garvin (Standard Oil Company, Indiana): Dr. Bronaugh, you showed that penetration of hydrophobic compounds is enhanced with the oleth 20 receptor fluid. What is the relationship of this observation to an in vivo situation? How do the data for in vitro penetration into the oleth 20 correlate with in vivo penetration?

Dr. Bronaugh: I gave an example, this morning, comparing our results to in vivo data. It certainly agrees more closely when we use the non-ionic surfactant. Are you concerned about damage to the skin?

Dr. Garvin: No. We are concerned about petroleum products that have little or no solubility in aqueous media and in the in vitro situation it is difficult to find a suitable receptor fluid which would mimic a physiologic situation and obtain meaningful data.

Dr. Bronaugh: You need to have some idea of what the in vivo absorption of your compound is and then see if a non-ionic surfactant will work. We are using it now only as a temporary way to enhance partitioning of compounds from the skin into the receptor fluid. I think that each compound will have different effects. The more hydrophobic a compound is the greater help you will get with a non-ionic surfactant, but you may still be further away from in vivo results unless you use the thin sections of skin that I talked about earlier today. If you have too much dermis it makes it harder to extract the material from the dermis into the receptor solution and this is a problem that we have just started to look at.

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Papers covered factors influencing lung toxicity including preexisting fibrosis of emphysema, mixtures, exercise, and age. Discussions of issue involved in carcinogenesis testing included short-term, subchronic, and chronic tests. In discussions of in vitro toxicity testing, systems using cell death and cellular communication effects as end points were considered along with use of a set of cell culture assays. The requirements of in vitro methods with regard to correspondence to in vivo systems were treated. The parameters of using cultures of hepatocytes and airway epithelial cells were analyzed and mechanisms of action of hepatoxic chemicals discussed. Papers were presented on interactions of chemicals with skin percutaneous penetration of liquids and vapors and dihalomethane vapor absorption.

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