EVAPORATION AND SKIN PENETRATION CHARACTERISTICS OF MOSQUITO REPELLENT FORMULATIONS[1, 2, 3, 4]

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ABSTRACT. Formulations of the mosquito repellent N,N-diethyl-3-methylbenzamide (deet) in combination with a variety of additives were developed to control repellent evaporation and percutaneous penetration. Deet was also formulated with the repellent dimethyl phthalate to study the interaction of the two compounds on the skin. The evaporation and penetration processes were evaluated on whole and split-thickness pig skin using radiolabeled repellents with an in vitro apparatus. Under essentially still air and air flow conditions, one of the deet formulations resulted in significantly reduced total evaporation and percutaneous penetration of deet as compared to unformulated repellent. When deet and dimethyl phthalate were combined, neither repellent affected the total amount of evaporation and penetration of the other compound. However, initial percutaneous penetration and evaporation rates were slightly less and decayed less rapidly than when both chemicals were tested separately at the same dose. These results indicated a degree of competition of the two compounds for the same avenues of loss.

INTRODUCTION

A variety of chemical structures possess mosquito repellent activity. However, an examination of an homologous series of repellent compounds revealed a maximum of protection time within a certain range of boiling point (Skinner and Johnson 1980). Compounds with low boiling points are lost rapidly by evaporation from the skin, where they must be retained to repel insects. The short duration of compounds with high boiling points suggests that a minimum evaporation rate must occur to repel mosquitoes. Consistent with this hypothesis were the findings that repellent activity could be extinguished by removing certain receptors from mosquito antenna, which do not come in direct contact with the repellent treated skin of man, but would be exposed to the vapor (Stewart and Atwood 1963). Other studies have shown that duration of repellent efficacy on man correlated with the time that vapor levels exceeded the minimum effective evaporation rates in vitro (Reifenrath and Robinson 1982).

The duration of protection afforded by a repellent can be increased by controlling the skin surface reservoir in order to extend the time of minimum effective evaporation from the skin. The surface reservoir may be controlled by reducing penetration into the skin and preventing excessive evaporation from the skin. A large number of mosquito repellent formulations used today contain deet as the active ingredient (United States Environmental Protection Agency 1980). Formulation of deet with polymers or other inert additives has been one approach to control the disposition of the repellent on the skin. In this report, an in vitro skin evaporation-penetration apparatus was used to characterize the disposition of radiolabeled deet in several formulations on pig skin. The purpose of this study was the evaluation of selected mosquito repellent formulations for the characteristics of evaporation and skin penetration.

MATERIALS AND METHODS

To evaluate the effects of several additives, N,N-diethyl-3-methylbenzamide (deet, Aldrich Chemical Co., Milwaukee, WI) was formulated with a silicone polymer (200 fluid, 1000 centistoke, Dow Corning Corporation, Midland, MI), formulation A; an acrylate polymer (Carboset 515, B. F. Goodrich Chemical Co., Cleveland, OH), formulation B; a high molecular weight fatty acid (1010 Dimer acid, Emery Industries, Cincinnati, OH), formulation C; a proprietary film forming polymer, formulation D; and dimethyl phthalate (DMP), formulation E. The composition of the formulations are given in Table 1 and the details of their preparation follow. Formulations A, B and C were prepared

[1] In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

[2] The volunteers participating in this study gave free and informed voluntary consent, and the investigators adhered to Army regulation 70-25 and Army Medical Research and Development Command regulation 70-25 governing the use of volunteers in research.

[3] Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

[4] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting views of the Department of the Army or the Department of Defense (AR 360-5).

Table 1. Composition of mosquito repellent formulations.

Formulation	Deet dose ($\mu g/cm^2$)	Vehicle and dose ($\mu l/cm^2$)	Formulation additive and dose ($\mu g/cm^2$)
A	320	Ethanol (8.3)	Silicone polymer (167)
B	320	Ethanol (8.3)	Acrylate polymer (167)
C	320	Ethanol (8.3)	Fatty acid (167)
D	360	Isopropanol (12.5)	Polymer[a] (830)
D′	360	Isopropanol (12.5)	Polymer[a] (415)
E	320	Ethanol (6.3)	Dimethylphthalate (320)

[a] Proprietary polymer.

by adding 195 mg of deet and 100 mg of additive to ethanol so that a final volume of 5 ml was obtained. Radiolabeled deet (carbonyl-^{14}C)-N,N-diethyl-3-methylbenzamide (specific activity 8.3 mCi/mM, reported radiochemical purity of 98%, New England Nuclear, Boston, MA) was added to each of the formulations so that application of 10 μl of formulation on a 1.2 cm^2 area of skin resulted in a chemical dose of deet of 320 $\mu g/cm^2$ and a radioactive dose of approximately 0.05 μCi. A chemical dose of 300 ± 200 $\mu g/cm^2$ was obtained when volunteers applied a 25% alcoholic solution of deet to their forearms (W.G. Reifenrath, unpublished data). This concentration was similar to that in several commercial insect repellent solutions (United States Environmental Protection Agency 1980). Formulations A, B and C were applied to the outer surface of freshly excised whole pig skin mounted in evaporation-penetration cells. The penetration cells were modified Franz cells, which allowed continuous flow of Tyrodes solution. Details of the design of these cells have been reported (Hawkins and Reifenrath 1984). Profiles of the evaporation and percutaneous penetration of the radiolabel over a 50-hr period were determined for the formulations and compared to those of unformulated deet at the same dose. Detailed procedures for making these determinations have been published elsewhere (Hawkins and Reifenrath 1984). Formulation D was prepared by adding radiolabeled deet to an isopropanol solution of a proprietary polymer. An application of 10 μl of formulation D to a skin area of 0.8 cm^2 resulted in a chemical dose of deet of 360 $\mu g/cm^2$ and a radioactive dose of approximately 0.05 μCi. Two variations of formulation D were prepared: one gave a dose of solids from the polymer solution of either 415 $\mu g/cm^2$ (formulation D′), and the second gave a dose of 830 $\mu g/cm^2$ (formulation D). Formulation E was prepared by adding tritium labeled dimethyl phthalate (dimethyl (ring-^{3}H)phthalate, specific activity 9.1 mCi/mM, reported radiochemical purity of 99%, New England Nuclear) to ^{14}C-radiolabeled deet in ethanol. An application of 5 μl of formulation E to a skin area of 0.8 cm^2 resulted in a chemical dose of 320 $\mu g/cm^2$ of each labeled compound (0.05 μCi of each isotope).

Formulation D and E and unformulated deet and DMP were applied to the outer surface of either excised pig skin or human skin. Skin samples were used immediately after excision. Human samples were from breast or abdominal tissue removed in the course of medical treatment of donors who gave informed consent. Samples were prepared by removing a portion of the dermis (split thickness) with a dermatome (Brown model 901, Zimmer USA, Warsaw, IN) to leave a final thickness of approximately 1 mm. Split thickness skin samples were used in these determinations after other studies (Hawkins and Reifenrath 1986) found it a better model in general than full thickness skin. Skin samples were mounted on commercially available evaporation-penetration cells (LG-1083-C, Laboratory Glass Apparatus, Berkeley, CA). The evaporation cells had air (24°C and approximately 40% relative humidity) flowing at a rate of either 60 or 600 ml/min. The penetration cells were perfused with oxygenated (95% O_2, 5% CO_2) tissue culture medium (Rose Park Memorial Institute media 1640, formula 78-5117, Gibco, Grand Island, NY) at a flow rate of 5 ml/hr. This medium allowed skin to remain viable under the test conditions (Hawkins and Reifenrath 1986). Gentamicin sulfate (5 mg/liter) was present in the medium. Profiles of evaporation and penetration of the radiolabeled compounds over a 50-hr period were determined for the formulations and were compared to those of unformulated deet or DMP. Procedures for making these determinations were similar to those previously published (Hawkins and Reifenrath 1984); however, an additional step was taken at the end of the experiment to isolate the application area from the surrounding skin and section it into two layers – an upper layer (approximately 100 μm) containing the epidermis with some dermis and a lower layer consisting of dermis. The skin surface was not washed prior to sectioning. The sectioning was accomplished by using a microtome (model 880, American Optical Corp., Buffalo, NY) with a CO_2 freezing attachment. Formulation D was also evaluated

on split-thickness pig skin which had been kept frozen (−20°C for 8 hr) and subsequently exposed to a saturated atmosphere of ethylene oxide (HW Anderson Products, Oyster Bay, NY) at 20°C.

An ethanolic solution of radiolabeled deet was also prepared to deliver a chemical dose of 10 $\mu g/cm^2$ and a radioactive dose of approximately 0.05 μCi. This dose corresponded to the ED95, the dose of deet required to repel 95% of the mosquitoes (R. A. Wirtz, unpublished data). The evaporation rate of deet was determined from fresh split-thickness human or pig skin by using equipment and procedures as described for the evaluation of formulation D and E.

RESULTS AND DISCUSSION

For formulations A, B and C, no statistically significant differences in total percutaneous penetration or evaporation were found between unformulated and formulated deet over a 50 hr period following topical application (experiment 1, Table 2). Also, profiles of evaporation vs. time were similar for these substances. In independent tests against mosquitoes, no consistent differences in duration of protection between formulation A or B and deet were observed (Reifenrath and Rutledge 1983). Formulation C was not tested against mosquitoes.

Combination of deet with a proprietary polymer (formulation D) produced an effect on the in vitro evaporation and penetration of deet. Plots of evaporation rates vs. time (Fig. 1), and least squares linear regression revealed reduced initial evaporation rate of deet and a more constant evaporation rate over time as noted by the smaller slope for formulation D.

In addition to its effects on evaporation, the polymer in formulation D significantly reduced total percutaneous penetration over unformulated deet (experiment 2, Table 2 and Fig. 2). With formulation D, substantially more deet was found to remain with the skin, since total evaporation was reduced approximately 50 percent. The majority of the radioactivity was recovered in the upper 100 μm skin layer (experiment 2, Table 2). Together, these results indicated that formulation D maintained the repellent in a surface reservoir and was able to control its evaporation and penetration rates. Reducing the ratio of polymer to deet in the formulation gave results intermediate between the original formulation and unformulated deet (experiment 3, Table 2 and Fig. 1).

The percutaneous penetration and evaporation of deet (unformulated and formulation D) were determined on pig skin that had been frozen and exposed to ethylene oxide, conditions which should inhibit the metabolic activity of the skin (Perkins 1969, Kao et al. 1985). No significant differences were found between determinations on fresh skin (experiment 2, Table 2) and treated skin (experiment 4, Table 2), although the variability of the determinations

Table 2. Disposition of radioactivity following topical application of unformulated (control) and formulated N,N-diethyl-3-methylbenzamide (deet) to pig skin.

			Percent of applied radioactive dose[a]				
Experiment	n	Formulation	Evaporation	Penetration	Upper skin layer	Dermis	Total recovery
1[b]	5–6	A	40 ± 7	23 ± 4	ND[c]	ND	89 ± 3
		B	39 ± 6	22 ± 4	ND	ND	92 ± 5
		C	33 ± 4	23 ± 5	ND	ND	92 ± 3
		Control	38 ± 8	22 ± 2	ND	ND	85 ± 7
2	3	D	34 ± 2	4 ± 1	39 ± 7	3 ± 3	87 ± 6
		Control	66 ± 7	19 ± 3	4 ± 1	0.9 ± 0.5	93 ± 8
3	7–8	D′	49 ± 5	15 ± 5	25 ± 4	2 ± 1	95 ± 3
		Control	60 ± 8	26 ± 3	6 ± 2	1.4 ± 0.6	96 ± 2
4[d]	3	D	36 ± 5	7 ± 4	41 ± 4	3 ± 3	90 ± 3
		Control	52 ± 5	31 ± 10	6 ± 3	1 ± 1	93 ± 3
5[e]	9	D	51 ± 5	3 ± 1	ND	ND	94 ± 3
		Control	73 ± 4	13 ± 2	ND	ND	93 ± 1

[a] Values are mean ± SD of values 50 hr after application of deet to split thickness pig skin at a chemical dose of 360 $\mu g/cm^2$, except as noted. Air flow through the evaporation cell was 60 ml/min, except in experiment 5 (600 ml/min).

[b] Deet was applied to whole pig skin at a dose of 320 $\mu g/cm^2$. Approximately 30% of the applied dose was recovered from skin surface rinse and by oxidation of the skin sample.

[c] Not determined. The skin surface was rinsed with ethanol and the skin sample oxidized.

[d] Skin was frozen and exposed to ethylene oxide prior to application of formulation D and control.

[e] Disposition of radioactivity 25 hr after application of radiolabeled deet. For formulation D, approximately 30% of the applied dose was recovered by skin surface rinse.

increased with the treated skin. These results suggested that deet was not significantly metabolized by pig skin at the doses studied. Indeed, when measurements of in vitro evaporation and percutaneous penetration were conducted using a gas chromatographic assay for deet, essentially all the radioactivity recovered could be accounted for as deet (W. G. Reifenrath, unpublished data). For both fresh and treated skin, percutaneous penetration of deet was lower, and the evaporation rate of deet was more constant with formulation D as compared to unformulated deet (Figs. 3 and 4).

The use of a different evaporation-penetration cell design may account in part for differences in control values of evaporation between experiment 1 and experiments 2–4 (Table 2). Biological variation between skin samples is also a factor in variation.

During the course of this study, other work (Reifenrath and Hawkins 1986) had shown that

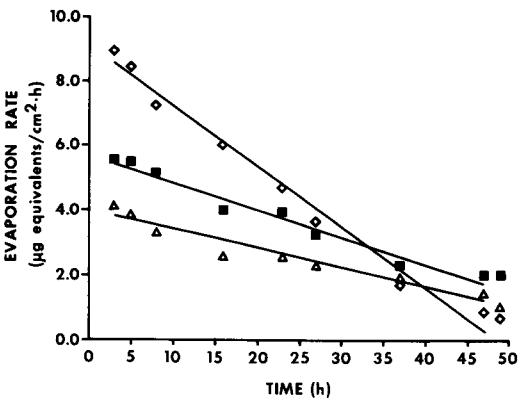

Fig. 1. Evaporation of N,N-diethyl-3-methylbenzamide at 360 $\mu g/cm^2$ on pig skin unformulated (◇) versus formulation D (△) and formulation D′ (□).

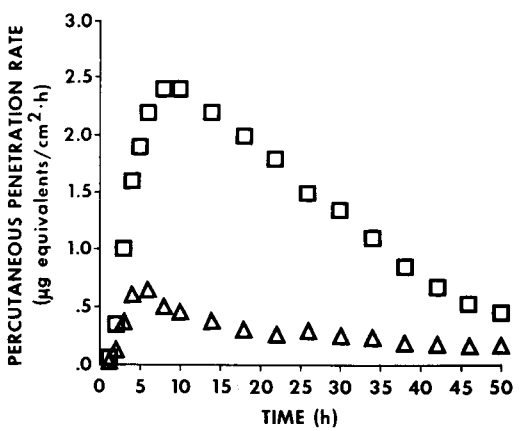

Fig. 2. Percutaneous penetration of N,N-diethyl-3-methylbenzamide on pig skin unformulated (□) versus formulation D (△).

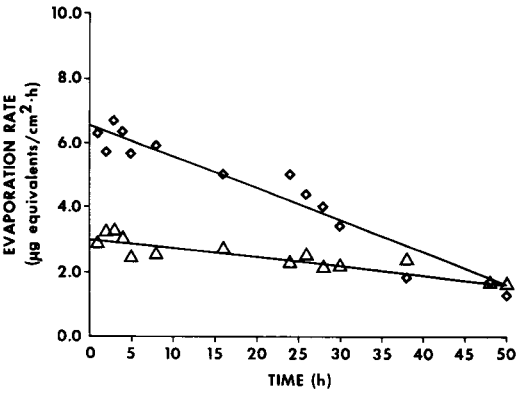

Fig. 3. Evaporation of N,N-diethyl-3-methylbenzamide at 360 $\mu g/cm^2$ on frozen pig skin exposed to ethylene oxide unformulated (◇) versus formulation D (△).

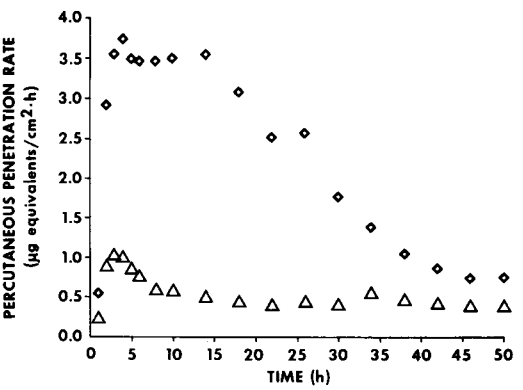

Fig. 4. Percutaneous penetration of N,N-diethyl-3-methylbenzamide at 360 $\mu g/cm^2$ on frozen skin exposed to ethylene oxide unformulated (◇) versus formulation D (△).

an air flow of 60 ml/min through the evaporation cell underestimated evaporative loss and resulted in greater skin penetration than observed in vivo. To model evaporative loss which occurs under actual conditions of use, it was necessary to use an air flow of 600 ml/min. This resulted in an increase in total evaporative loss and a slight decrease in penetration of deet in both formulation D and unformulated deet (compare experiments 2 and 5, Table 2). However, a greater effect of the increased air flow was noted on the profiles of evaporation rate vs. time (compare Fig. 5 to Fig. 1). At the higher air flow rate, formulation D continued to provide a measurable evaporation rate of deet at 25 hr after application, while unformulated deet was depleted from the skin surface (essentially zero evaporation rate at this time point). In Fig. 6, profiles of penetration of deet in formulation D and unformulated deet are shown at the higher air flow rate. Under conditions where the surface

dose remained relatively constant (e.g., high dose, low air flow), a zero order evaporation rate vs. time was expected. Higher air flows or a lower surface dose would favor surface reservoir depletion and an exponential (first order) decay of evaporation rate vs. time (Fig. 5).

Previous studies have shown that the duration of mosquito repellent activity on persons could be correlated with the time that vapor levels for the repellent exceeded its minimum effective evaporation rate in vitro (Reifenrath and Robinson 1982). To relate the in vitro evaporation rates of deet in formulation D to in vivo results, the minimum effective evaporation rate of deet was determined. In the rabbit test system (R. A. Wirtz, unpublished data), the ED95 of deet was approximately 10 $\mu g/cm^2$. A dose of 10 $\mu g/cm^2$ of deet was applied to pig and human skin in vitro and the evaporation rate measured during a 15–30-min interval following application. This interval was chosen to reflect the delay between application and challenge by mosquitoes. A value of approximately 5 $\mu g/cm^2/hr$ was obtained from these tests by using an air flow of 600 ml/min (Table 3). This value, an estimate of the minimum effective evaporation rate for the repellent, is higher than the previously reported (Reifenrath and Robinson 1982) value (approximately 1 $\mu g/cm^2/hr$) which was obtained under essentially still air conditions (air flow of 30 ml/min through the evaporation cell). Although the evaporation rate of deet from formulation D at 24 hr falls slightly below the 5 $\mu g/cm^2/hr$ level (Fig. 5), a higher evaporation rate was obtained for formulated vs. unformulated deet. These results agreed with in vivo efficacy data, as formulation D was found more effective at 24 hr than unformulated deet in tests against mosquitoes on the rabbit (Mehr et al. 1985).

Because a single active ingredient in an insect repellent formulation may not provide the desired spectrum of activity, an improved formu-

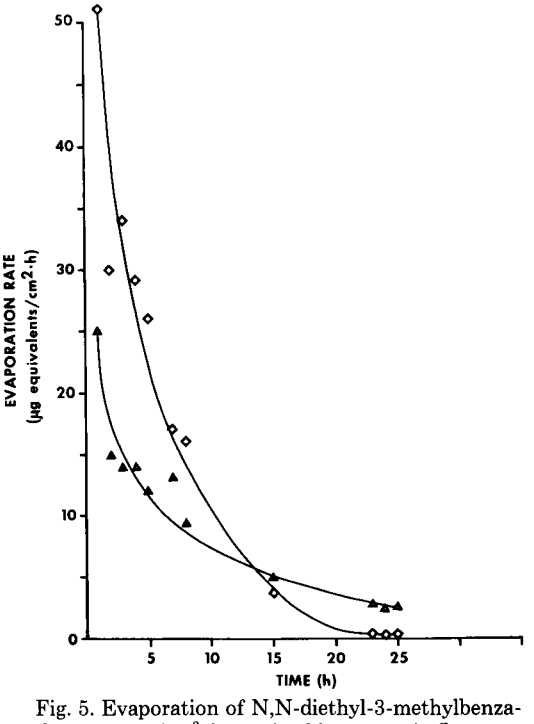

Fig. 5. Evaporation of N,N-diethyl-3-methylbenzamide at 360 $\mu g/cm^2$ from pig skin at an air flow rate of 600 ml/min unformulated (◇) versus formulation D (△).

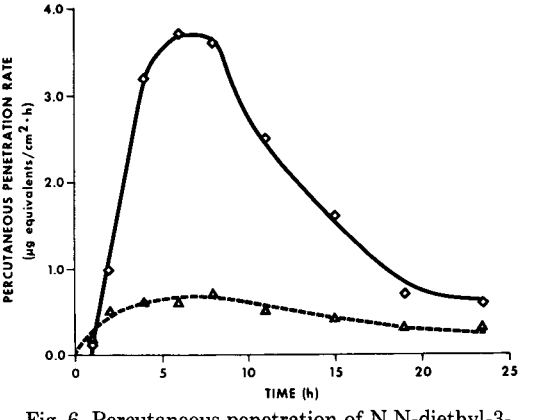

Fig. 6. Percutaneous penetration of N,N-diethyl-3-methylbenzamide at 360 $\mu g/cm^2$ on pig skin at an air flow rate of 600 ml/min unformulated (◇) versus formulation D (△).

Table 3. Evaporation rate of N,N-diethyl-3-methylbenzamide (deet) following topical application to excised pig and human skin at a chemical dose of 10 $\mu g/cm^2$.

Skin	Run[b]	Evaporation rate ($\mu g/cm^2/hr$)[a] 0–15 min	15–30 min	30–45 min	45–60 min	n
Pig	1	10 ± 3	4 ± 2	5 ± 3	2 ± 1	6
Pig	2	11 ± 3	6 ± 2	3 ± 2	1 ± 1	3
Human	1	7 ± 3	7 ± 2	5 ± 2	3 ± 1	6
Human	2	8 ± 1	5 ± 1	5 ± 3	2 ± 2	3

[a] Microgram equivalents per unit area per 1 hr at an air flow of 600 ml/min through the evaporation cell. Values are mean ± SD.

[b] Each run was conducted with skin from a different source.

Table 4. Interaction of radiolabeled N,N-diethyl-3-methylbenzamide (deet) and dimethyl phthalate (DMP) on pig skin in vitro.

Material applied	Deet disposition[a]		DMP disposition[a]	
	% Evap	% Pen	% Evap	% Pen
Deet (0.32 mg/cm^2)	40 ± 8	21 ± 6	—	—
DMP (0.32 mg/cm^2)	—	—	49 ± 7	18 ± 7
Deet (0.32 mg/cm^2) plus DMP (0.32 mg/cm^2) (Formulation E)	38 ± 5	20 ± 4	46 ± 7	21 ± 5

[a] Percent of applied radioactive dose resulting in evaporation (Evap) and percutaneous penetration (Pen) over a 50-hr period following topical application. Results are mean ± SD with 6–9 replicates.

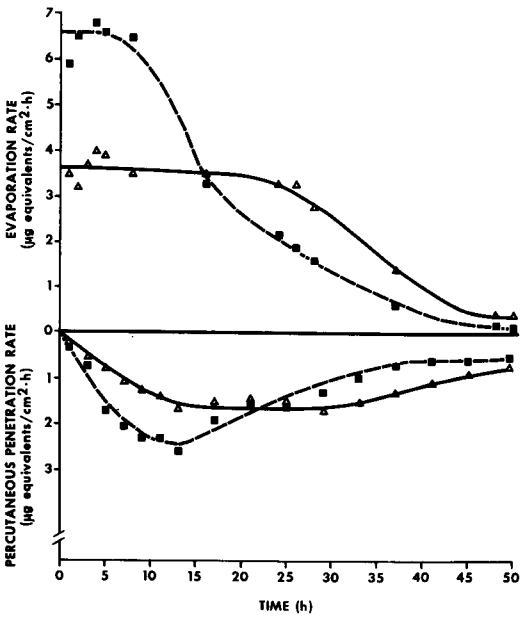

Fig. 7. Evaporation and percutaneous penetration of N,N-diethyl-3-methylbenzamide at 320 µg/cm^2 on pig skin alone (□) and in combination with dimethyl phthalate (Δ) at a dose of 320 µg/cm^2.

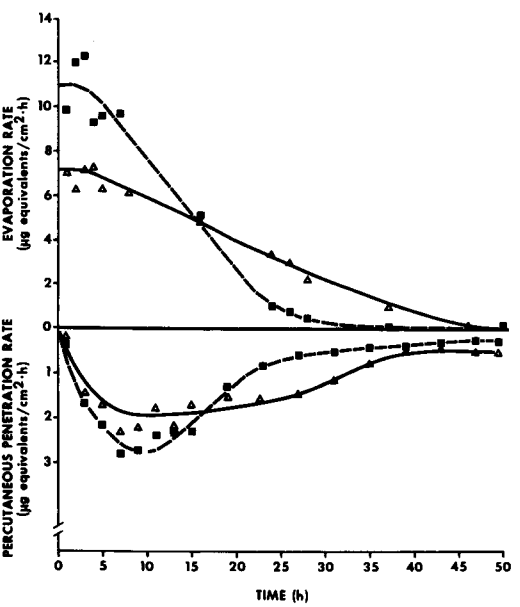

Fig. 8. Evaporation and percutaneous penetration of dimethyl phthalate at 320 µg/cm^2 on pig skin alone (□) and in combination with N,N-diethyl-3-methylbenzamide (Δ) at a dose of 320 µg/cm^2.

lation may require two repellent chemicals. Therefore, the interaction of two repellents on the skin was studied using the in vitro skin penetration-evaporation cells with pig skin. The disposition of topically applied carbon 14 labeled deet and tritium labeled DMP were determined separately and in a combined formulation (formulation E). Total evaporation and percutaneous penetration of each repellent did not change when the two were combined (Table 4). However, the maximum rate of evaporation and penetration was lower and extended over a longer period of time when the two repellents were tested in combination, as opposed to using separate determinations (Figs. 7 and 8). These results indicated that the two repellents competed in part for the same routes of loss; the presence of one repellent did not affect the total evaporation or penetration of the other repellent, it only delayed the rate at which these events took place.

In summary, a more accurate estimate of the minimum effective evaporation rate of deet to repel mosquitoes under laboratory conditions was obtained. Formulation of certain polymers with deet was found to retard its percutaneous penetration and promote a more constant rate of evaporation of deet. When deet was formulated with a second insect repellent (dimethyl phthalate) in ethanol, neither repellent affected the total evaporation or penetration of the other repellent.

REFERENCES CITED

Hawkins, G. S. and W. G. Reifenrath. 1984. Development of an in vitro model for determining the fate

of chemicals applied to skin. Fundam. Appl. Toxicol. 4:S133–S144.

Hawkins, G. S. and W. G. Reifenrath. 1986. Influence of skin source, penetration cell fluid, and partition coefficient on in vitro skin penetration. J. Pharm. Sci. 75:378–381.

Kao, J., F. K. Patterson and J. Hall. 1985. Skin penetration and metabolism of topically applied chemicals in six mammalian species, including man: an in vitro study with benzo(a)pyrene and testosterone. Toxicol. Appl. Pharmacol. 81:502–516.

Mehr, Z. A., L. C. Rutledge, E. L. Morales, V. E. Meixsell and D. W. Korte. 1985. Laboratory evaluation of controlled-release insect repellent formulation. J. Am. Mosq. Control Assoc. 1:143–147.

Perkins, J. J. 1969. Principles and methods of sterilization in Health Sciences. pp. 501–503. Charles C. Thomas, Springfield.

Reifenrath, W. G. and G. S. Hawkins, 1986. pp. 673–680. *In*: M. E. Tumbleson (ed.), Swine in biomedical research. Plenum, New York.

Reifenrath, W. G. and P. B. Robinson. 1982. In vitro skin evaporation and penetration characteristics of mosquito repellents. J. Pharm. Sci. 71:1014–1018.

Reifenrath, W. G. and L. C. Rutledge. 1983. Evaluation of mosquito repellent formulations. J. Pharm. Sci. 72:169–173.

Skinner, W. A. and H. L. Johnson. 1980. The design of insect repellents. pp. 277–305 *In*: E. J. Ariens (ed.), Drug design, vol 10. Academic Press, New York.

Steward, C. C. and C. E. Atwood. 1963. The sensory organs of the mosquito antenna. Can. J. Zool. 41:577–594.

United States Environmental Protection Agency. 1980. Registrants with pesticide products containing the active ingredient N,N-diethyl-m-toluamide and other isomers. Washington, D.C. 146 p.