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the Gulf War

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PERCUTANEOUS ABSORPTION OF CHEMICAL MIXTURES RELEVANT TO THE GULF WAR

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Co-PI: Dr. Nancy A. Monteiro
Co-I: Dr. Ronald E. Baynes

5. INTRODUCTION

The purpose of this research is to quantitate the dermal absorption and cutaneous toxicity of chemical mixtures that veterans may have been exposed to during the Persian Gulf War. Diethyl-m-toluamide (DEET) was used extensively in the Persian Gulf War as a repellent against insects and it is implicated in the cause of the Gulf War Syndrome in Gulf War veterans. Data from recent studies suggest that this repellent can modulate dermal absorption of pesticides. The primary focus in the first year of this proposal has been on chemical mixtures composed of the insect repellent DEET and the insecticide permethrin exposed in various vehicles. Analytical methods for these penetrants have been developed. These studies were conducted using three *in vitro* systems of increasing biological complexity: inert silastic and dermatomed pig skin flow-through diffusion cells as well as the isolated perfused porcine skin flap (IPPSF). This allows physical chemical interactions to be separated from those involving the stratum corneum lipid barrier and from true physiological and metabolic effects. Secondly, assays for a number of cytokines (IL-8, TNF α , PGE-2) have been developed and utilized as biomarkers of direct toxic effects of these mixtures. These experiments have provided the basis for conducting experiments in the second year of this proposal designed to assess the effect of systemic pyridostigmine on DEET and permethrin absorption, and finally to assess the effects of other organophosphates, jet fuel or sulfur mustard on chemical absorption. Conducting these studies in all three-model systems will allow the mechanisms of these interactions to be identified, and extrapolated to additional chemical mixture exposure scenarios.

6. BODY

The research in the first year of this project addresses Technical Objectives I, II, III and V of the approved Statement of Work of this proposal. However, as discussed with our COR Colonel David Danley at a site visit on November 11, 1999; we have changed the *order* of experiment to better utilize resources and optimize experimental design. The basic change is that we have analyzed simple mixtures of DEET, permethrin, and vehicles in all three-model systems to better define interactions. Secondly, we have had to spend additional effort in working out biomarker assays in all three of our systems. These developmental studies are now complete. Finally, as discussed below, we have found significant vehicle effects (water, ethanol) that require study before complex mixtures can be approached. These pharmaceutical studies (see below) have now been completed. When these simple mixtures are completed, we will proceed to assess the effects of systemic pyridostigmine and other mixture components. Thus, the *scope* of the project is *unchanged*, only the sequence of experiments are altered.

We have had to redirect some resources to the biomarker and pharmaceutical interaction studies. We have accomplished this by decreasing Dr. Baynes' effort and increasing budgeted services to cover

the costs of these experiments. We do not feel that these will significantly affect the overall progress on this work and keep the experiments well within budget.

We have elected to present these data by the experiments conducted, which cut across the technical objectives, since the latter were designed to assess phenomenon across all model systems. Since this is only the first year of this proposal, we are still in the data gathering and experimental phase, and are not ready to make conclusions. The nature of this research will delay such integration until all experimental studies have been conducted. A significant portion of this work will serve as the dissertation project of doctoral student Ms. Kristina Powers. However, as can be appreciated from the data presented below, significant progress has been made and we have now developed experimental systems to assess mixture interactions at three levels of biological complexity.

HPLC METHOD DEVELOPMENT FOR PERMETHRIN AND DEET

HPLC methods were developed to assay for DEET and permethrin in perfusate samples from skin perfused with bovine serum albumen media.

MATERIALS & METHODS FOR DEET

Reagents and Chemicals: *N,N*-Diethyl-*m*-toluamide (DEET) was purchased from Chem Service (West Chester, PA). HPLC grade acetonitrile and ammonium acetate was purchased from Fisher Scientific (Fair Lawn, NJ). All water was purified with an ultra high purity water filtration system (Dracor Water Systems, Durham, NC). Reagent grade, glacial acetic acid was also purchased from Fisher Scientific (Fair Lawn, NJ)

Extraction procedure: A solid phase extraction method originally developed by Qiu and Jun (1996) was used to extract DEET from the sample media. One milliliter of each sample was placed in 16 x 125-mm glass culture tubes. Each sample was then diluted with three milliliters of HPLC grade water. The solid phase extraction cartridges (Ansyl SPEC PLUS 3ml C18 15mg) were preconditioned with 500 µl of acetonitrile followed by 500 µl of water. Care was taken not to allow the cartridges to dry out. No vacuum was used during preconditioning or sample loading. The samples were loaded onto the cartridges using glass disposable Pasteur Pipettes. Care was taken to minimize bubble formation. The samples were allowed to drain through the cartridges very slowly (about 0.5 ml/min.) using only gravity. When the sample meniscus reached the top of the cartridge bed, 500 µl of the wash solvent (10:90 Acetonitrile: pH 4.5 ammonium acetate buffer 0.03M) was added to the cartridges. Once the sample wash drained through the cartridge bed, a strong vacuum (15 in. Hg) was pulled to dry out the beds before the final elution step. New clean glass tubes were placed in the vacuum manifold. The samples were then eluted with 1 ml of the elution solvent (40:60 acetonitrile: pH 4.5 ammonium acetate buffer 0.03M) and placed directly into HPLC vials for analysis.

HPLC conditions: The Waters HPLC system was equipped with a 996 PDA detector, 717plus autosampler, 600 controller, temperature control module, and model 60F solvent pumping system. All data were collected on a Gateway E3110 computer utilizing Waters Millennium version 32 software. A Waters SymmetryShield RP18 (3.5-micron, 4.6x150mm) column was used for the separations. The mobile phase consisted of 36% acetonitrile and 64% pH 4.5 ammonium acetate

buffer, 0.03M. The column temperature was 35°C. The detector wavelength was 220 nm. The flow rate and injection volume was 1.0 ml/min. and 10µL respectively.

MATERIALS & METHODS FOR PERMETHRIN

Reagents and Chemicals: Permethrin was purchased from Chem Service (West Chester, PA). HPLC grade acetonitrile and isooctane was purchased from Fisher Scientific (Fair Lawn, NJ). All water was purified with an ultra high purity water filtration system (Dracor Water Systems, Durham, NC). Ethanol, 200 proof, was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY).

Extraction procedure: One milliliter of each sample was placed in a 16 x 125 mm borosilicate screw-top glass culture tube. Two milliliters of 200 proof ethanol was added to each tube and vortexed for 15 seconds. Eight milliliters of isooctane was added to each tube. The samples were then placed on a shaker and rocked gently for 15 minutes. The samples were removed from the shaker and placed in a centrifuge for 15 minutes at 25°C and 4000 RPM. The top isooctane layer was removed with disposable Pasteur pipettes and placed into new clean tubes. The tubes were then placed in a Zymark TurboVap at 40°C for approximately 40-45 minutes to be evaporated down to dryness under nitrogen. The samples were reconstituted in one milliliter of acetonitrile before analysis by HPLC.

HPLC conditions. The Waters HPLC system was equipped with a 996 PDA detector, 717plus autosampler, 600 controller, temperature control module, and model 60F solvent pumping system. All data were collected on a Gateway E3110 computer utilizing Waters Millennium version 32 software. A Waters SymmetryShield RP18 (3.5-micron, 4.6x150mm) column was used for the separations. The mobile phase consisted of 70% acetonitrile and 30% water. The column temperature was 35°C. The detector wavelength was 200 nm. The flow rate and injection volume was 1.5 ml/min. and 50µL respectively.

RESULTS

Extraction Efficiency for DEET: Recoveries and blanks were run with every batch of samples. The average percent recoveries obtained thus far were 97% (n=9, CV=64%); 103% (n=9, CV=7%); 100% (n=9, CV=4%); and 105% (n=8, CV=6%) for sample media spiked with 0.1, 0.5, 1.0 and 5.0 ppm DEET, respectively (Figure 1a). This method was very reproducible for sample media spiked with DEET at 0.5ppm and higher. The standard curves, prepared by spiking the same volumes of mobile phase with the same amounts of DEET used for spiking the media, revealed a minimum R^2 of 0.999.

Extraction Efficiency for Permethrin: The following table 1 shows the results of the recoveries obtained from sample media spiked with permethrin (62% cis and 37% trans) (Figure 1b)

HPLC chromatogram of N,N-diethyl-m-toluidide (DEET) following solid-phase extraction of 1.0 ppm DEET from BSA perfusate

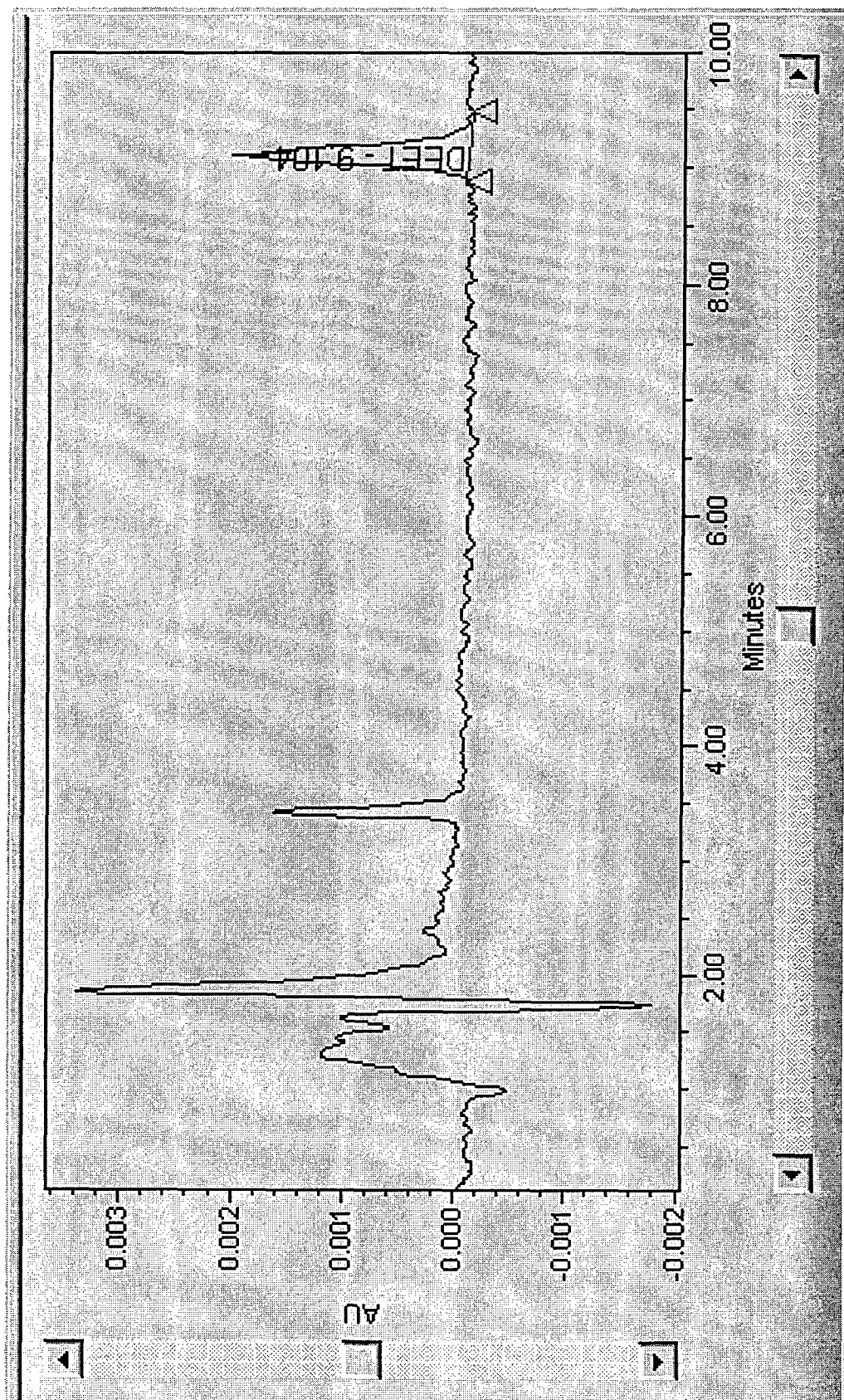


Figure 1b

HPLC chromatogram of cis and trans isomers of permethrin following liquid-liquid extraction of 1.0 ppm permethrin from BSA perfusate

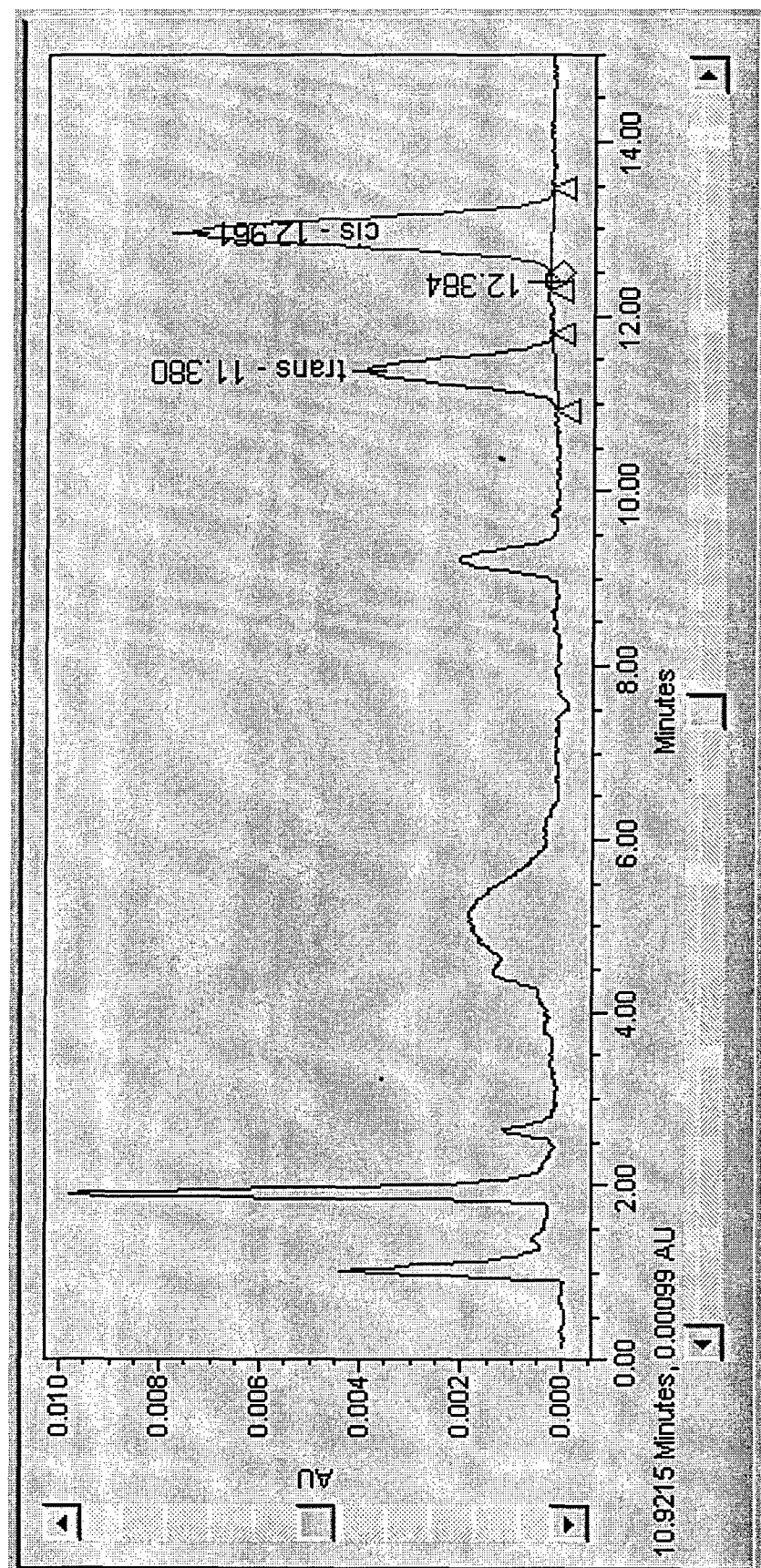


Table I. Average percent recoveries and coefficients of variation (%) for trans- and cis-permethrin. (37% trans and 62% cis).

Total Permethrin, ppm	trans-permethrin, ppm	Avg. % rec., trans-	CV(%), trans-	Cis-Permethrin, Ppm	Avg. % rec., cis-	CV(%), cis-
0.1	0.04	93	29	0.06	83	0.9
0.5	0.2	94	4	0.3	92	2
1.0	0.4	96	4	0.6	95	3
5.0	1.8	76	3	3.1	75	3

The standard curve, prepared by spiking the same volumes of mobile phase with the same amounts of permethrin used for spiking the media, revealed an R^2 of 0.9999 for both trans and cis-permethrin

SUMMARY

Sensitive and specific HPLC assays for both permethrin and DEET were developed and were utilized for the experimental protocols discussed below.

DIFFUSION CHARACTERISTICS OF PERMETHRIN AND DEET IN MIXTURES APPLIED TO SILASTIC MEMBRANES AND PORCINE SKIN SECTIONS

INTRODUCTION

Previous studies had demonstrated that DEET can influence the dermal disposition of pesticides and drugs (Windheuser et al., 1982; Baynes et al., 1997; Baynes and Riviere, 1998). Data from these later studies suggest that DEET may inhibit permethrin and carbaryl absorption in porcine skin. The aim of the current studies is to assess with *in vitro* flow through diffusion cells whether these interactions will occur in skin exposed to DEET concentrations relevant to the Gulf War scenario and whether skin hydration (presence of water) will influence the dermal disposition *in vitro*. These studies will also assess whether hydration will influence DEET absorption. In order to assess how chemical interactions influence chemical diffusion independent of biological interactions in skin, these mixtures were also applied to silastic membranes. Unlike skin, silastic is an inert membrane, however, by using such a membrane with thickness similar to that of skin, diffusion properties independent of biological interactions can be adequately assessed.

MATERIALS & METHODS

Chemicals: Preliminary diffusion studies demonstrated that very little permethrin was detectable in the perfusate using HPLC-UV. The decision was therefore made to use radiolabelled permethrin to better detect interactions in the skin as well as the perfusate which was the primary focus of this project. C14-permethrin, composed of 74% cis isomer and 25% trans isomer and 98% pure N,N Diethyl-m-toluamide (DEET) were purchased from Chem Service Company, West Chester, PA.

Dosing solutions, were formulated to contain $40\mu\text{g}/\text{cm}^2$ for C14-permethrin mixture experiments. The ethanol/water ratio in these mixtures was always 1.5:1. The following 6 mixtures were prepared and topically applied to porcine skin and silastic membranes using flow-through diffusion cells.

Ethanol+Permethrin
25%Ethanol+75%DEET
Ethanol+Permethrin+75%DEET

Ethanol+Permethrin+Water
Ethanol+75%DEET+Water
Ethanol+Permethrin+75%DEET+Water

Flow-through Diffusion Cell System: The flow-through diffusion cell system (Bronaugh and Stewart, 1985) was enclosed in an environmentally controlled chamber previously described by Chang and Riviere (1991). In the case of the pig skin, the hair on the dorsal area of weanling female Yorkshire pigs was clipped 48 hr before harvest and the skin was dermatomed to a thickness of 200 - 300 μ m with a Padgett Dermatome, (Padgett Instruments Inc, Kansas City, MO). Silastic membranes (250 μ m) were obtained from Dow Corning. Circular skin and silastic sections were punched to provide a dosing surface area of 0.64 cm² and then placed into a two-compartment Teflon flow-through diffusion cell. Skin and silastic discs were perfused using Krebs-Ringer bicarbonate buffer spiked with dextrose and bovine serum albumin. The temperature of the perfusate and flow-through cell was maintained at 37°C using a Brinkmann constant-temperature circulator (Brinkmann Inc., Westbury, NY). The pH was maintained between 7.4 and 7.5 and relative humidity between 50 and 60%. Perfusate flow rate was 4.0 mL/hr and perfusate samples were collected hourly for 8 hr.

Chemical Assay: For determination of DEET, 1mL of each perfusate sample was extracted using solid phase extraction method and HPLC analysis as described in the previous section of this report. For determination of ¹⁴C-permethrin, perfusate samples were combusted in a Packard Model 306 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL) and then analyzed by Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL) for total ¹⁴C determination. *Future HPLC analysis with fraction collection using methods described in the previous section of this report will be used to determine what proportion of cis- and trans-isomers of permethrin is readily absorbed in these mixtures.* This is a unique area of dermal toxicology that has received little attention in the past.

Statistics and Calculations: Standard curves obtained by our laboratory for DEET and permethrin were used to calculate the amount of each compound in the samples. Absorption was defined as the total percentage of initial dose detected in the perfusate for the entire 8-hr perfusion period. Standard errors were determined for all data sets. For analysis of total absorption data, multiple comparison tests were performed using the LSD method with significance level 0.05. All analyses were carried out using SAS software (SAS Institute Inc., Cary, NC).

RESULTS and DISCUSSION

Please refer to Figure 2a for comparison of flux profiles and Figure 2b for skin deposition of permethrin in aqueous mixtures of ethanol and ethanol+DEET, respectively. Although the percent absorption of permethrin was similar over 8 hours for both mixtures, deposition into skin and stratum corneum (SC) was greater when DEET was absent from the mixture. Permethrin absorption was significantly greater in silastic membranes (Figure 2c and 2d) than in skin, but DEET inhibited permethrin absorption by as much as 40 - 100%. The presence of water appears to increase peak time from 2 hours to 4 hours and also further reduce permethrin absorption in the presence of DEET. These results are similar to preliminary data previously reported by this group (Baynes et al., 1997). DEET absorption was 0.37% Dose for DEET+ethanol mixtures, and was continuing to increase beyond the 8-hour perfusion period especially with non-aqueous mixtures (Figure 2e).

Figure 2a

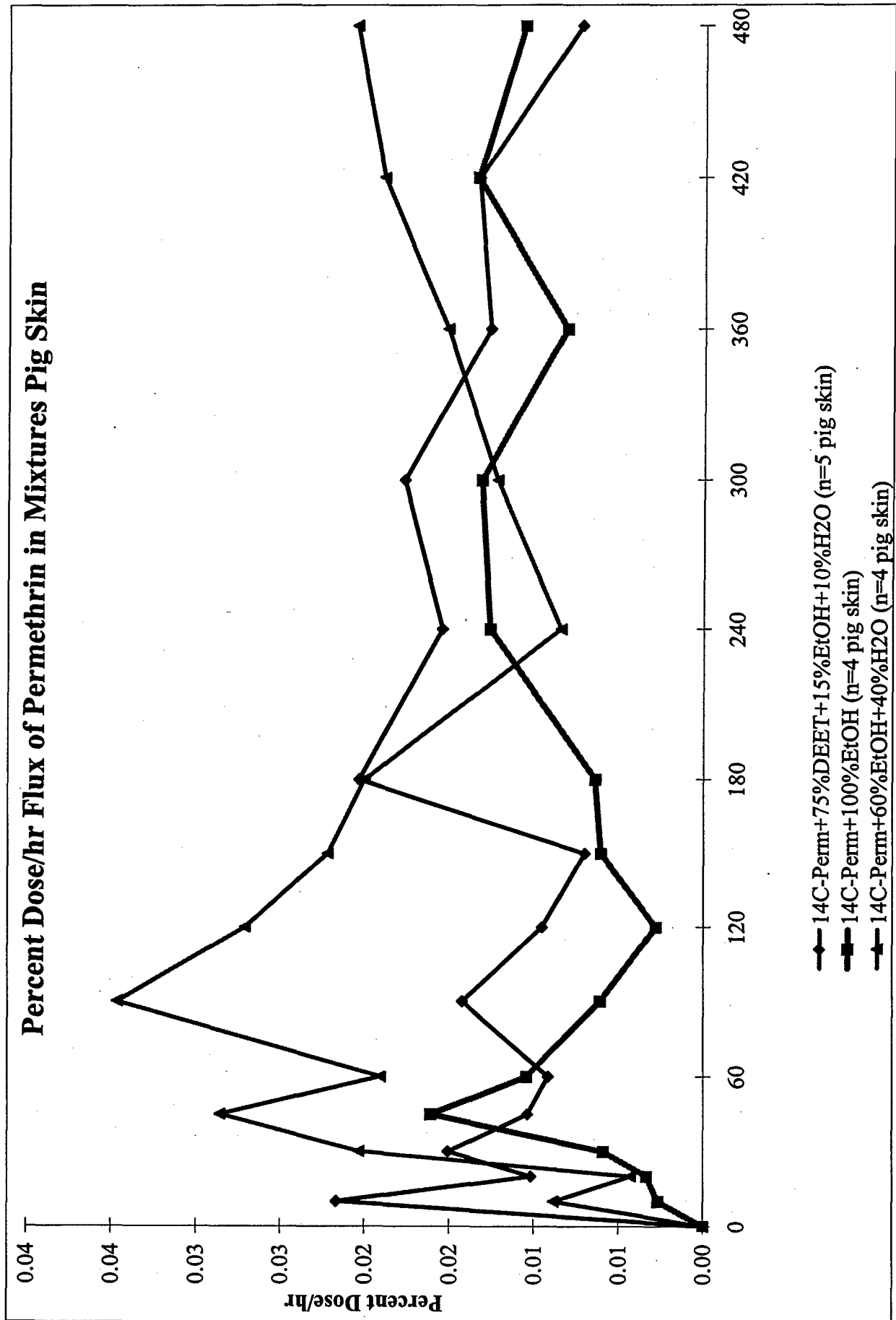
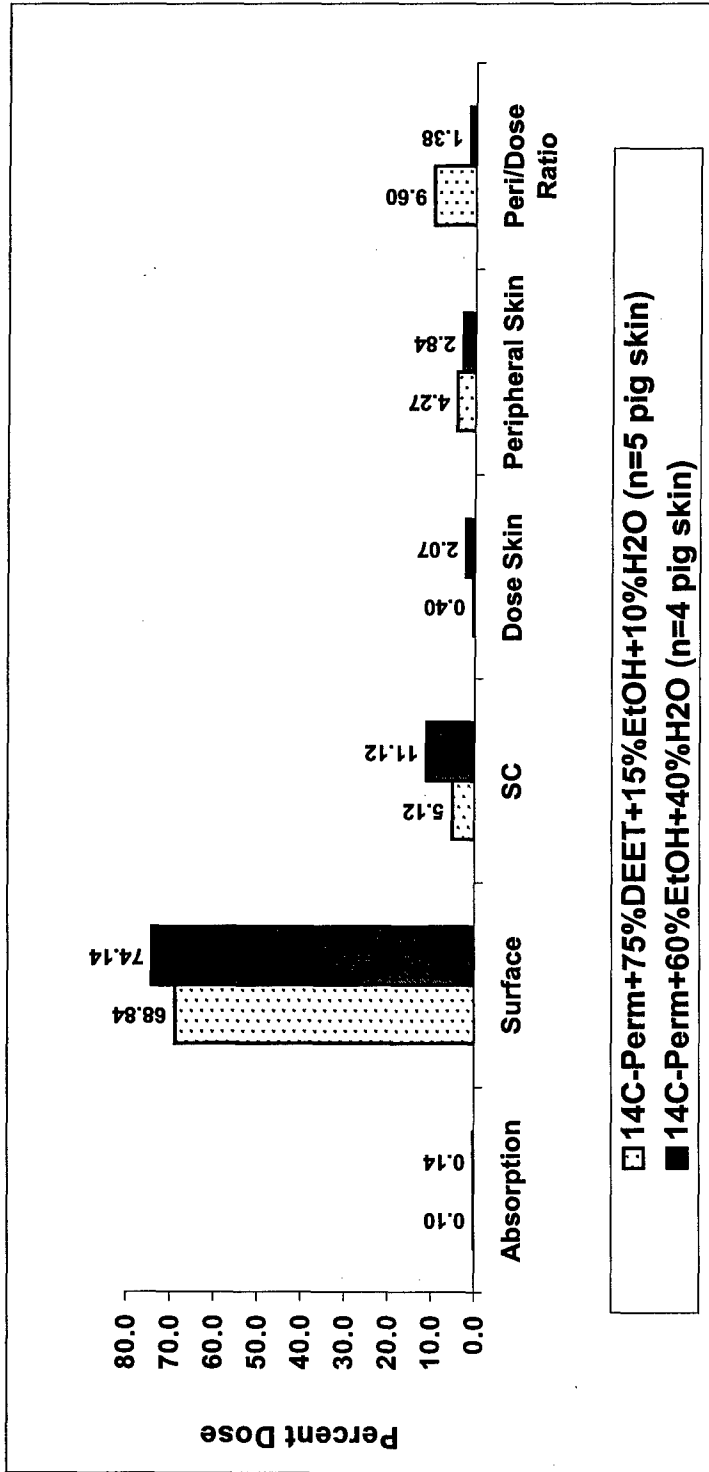


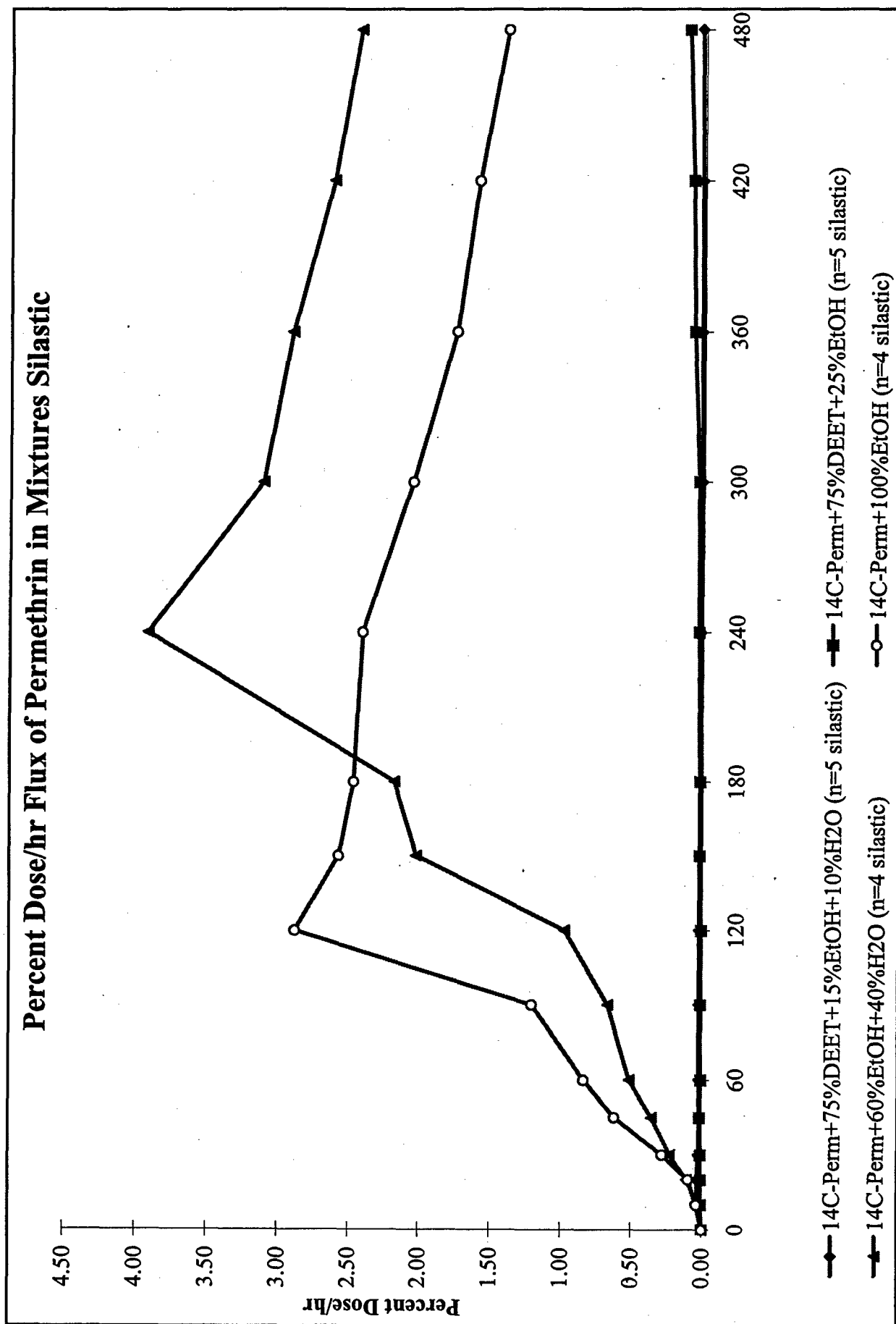
Figure 2b



Jim, all I need to add to this figure is the info for 14C-Permethrin+100%EtOH (n=4 pig skin) from FT(The result is comparison of 3 mixtures on the same figure

This is from figure 2 in Z:\Baynes\GWFT

Figure 2c



Mean percent dose of C14-permethrin absorbed in silastic membrane and remaining on the surface and in the membrane

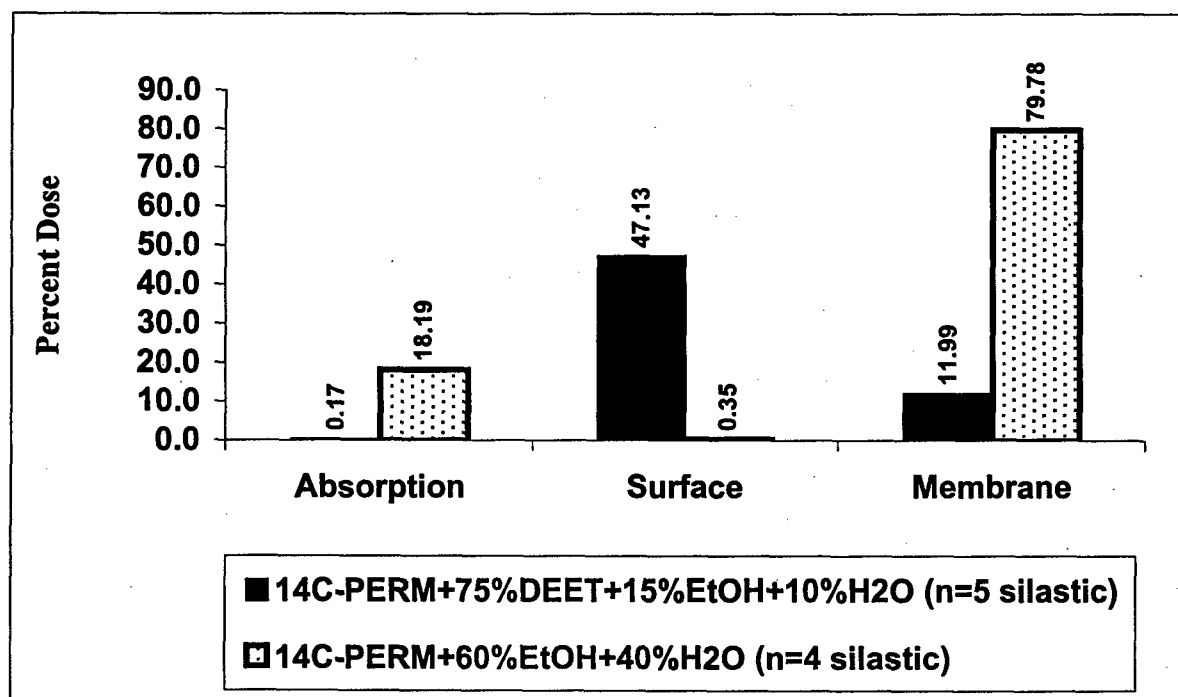
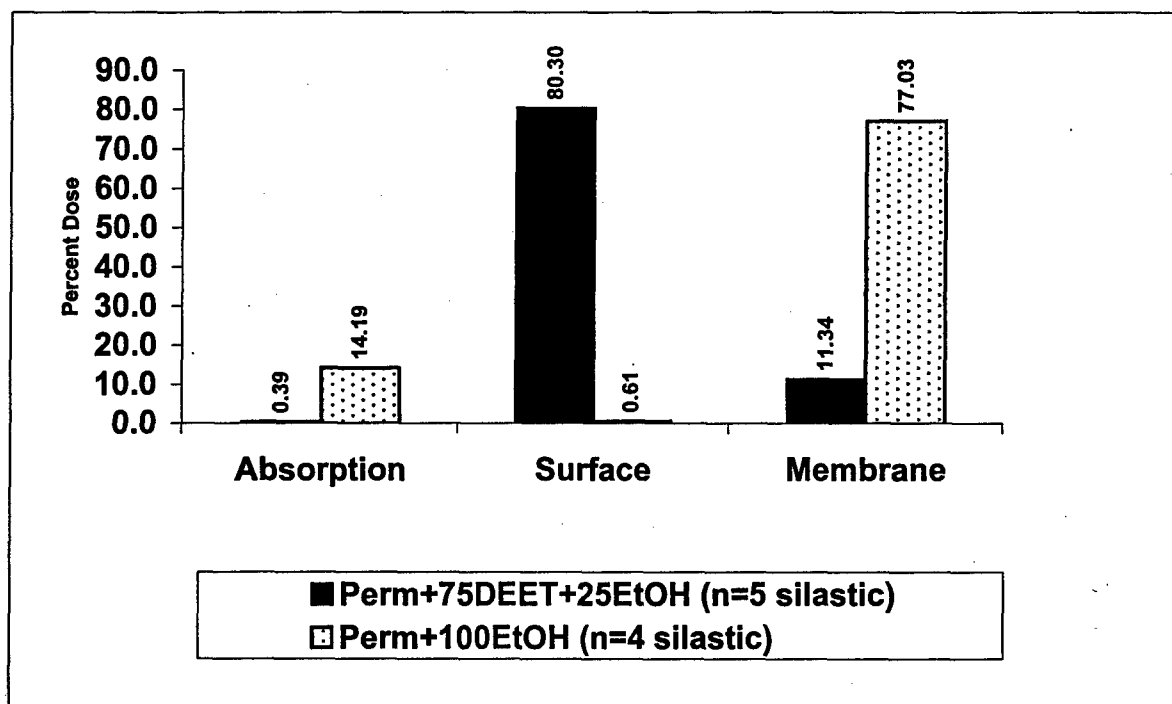
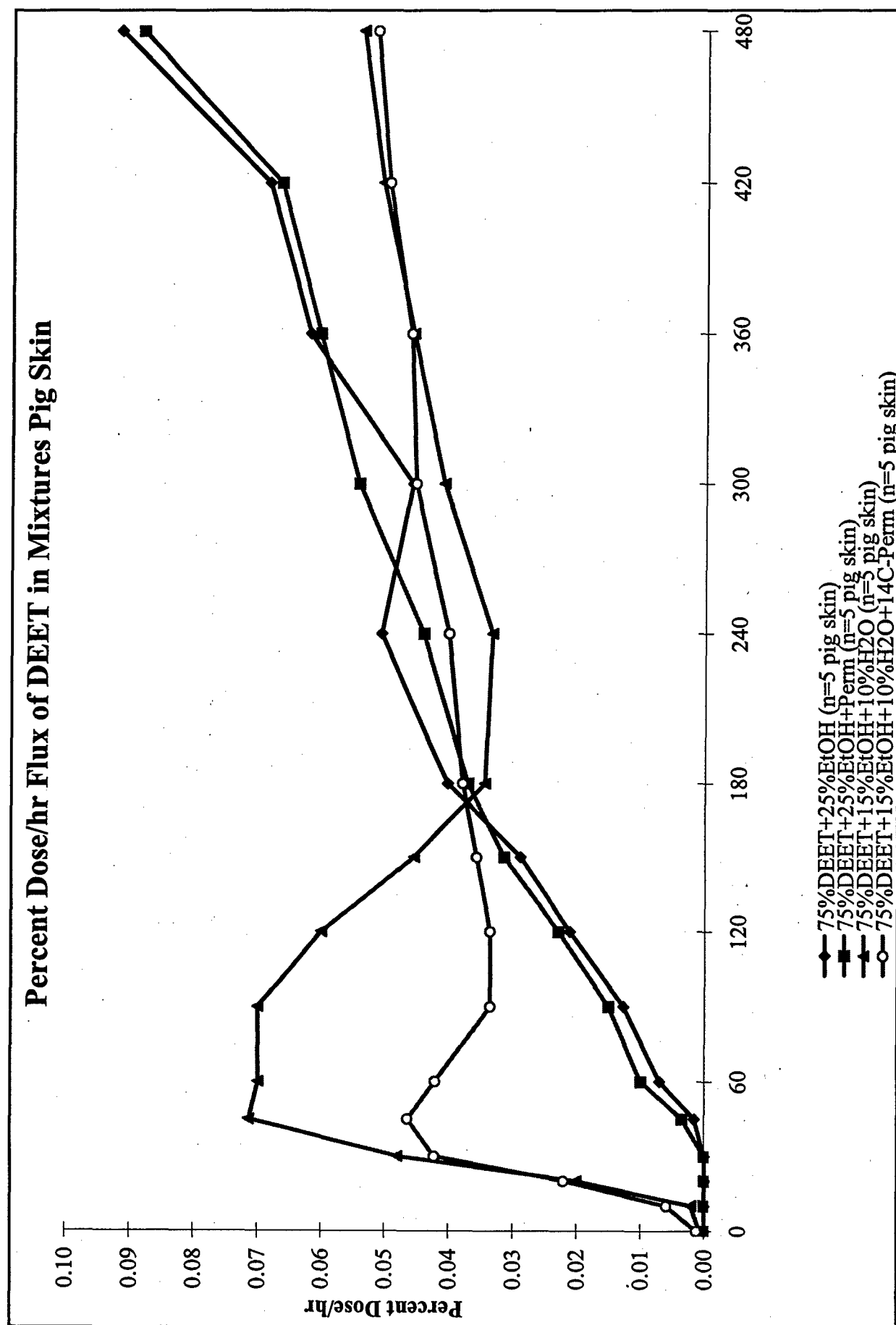


Figure 2e



DERMAL ABSORPTION OF PERMETHRIN AND DEET MIXTURES IN ISOLATED PERFUSED PORCINE SKIN FLAPS

INTRODUCTION:

The isolated perfused porcine skin flap is an intact skin model and therefore more biologically complex than the silastic membrane and the porcine skin sections (Riviere et al., 1986). Changes seen in IPPSF profiles will reflect the inert chemical-chemical interactions seen in silastic systems, the chemical to stratum corneum lipid interactions seen in porcine skin diffusion cell studies, as well as metabolic, inflammatory and vascular changes seen in the IPPSF. The aim of these studies is to probe interactions observed in the diffusion cells studies in this biologically intact model system.

METHODS:

Chemicals and Chemical Mixtures: The following mixtures were prepared, with one exception * have been topically applied to IPPSFs as of the writing of this report:

Ethanol+Permethrin	Ethanol+Permethrin+Water
25%Ethanol+75%DEET	Ethanol+75%DEET+Water
*Ethanol+Permethrin+75%DEET	Ethanol+Permethrin+75%DEET+Water

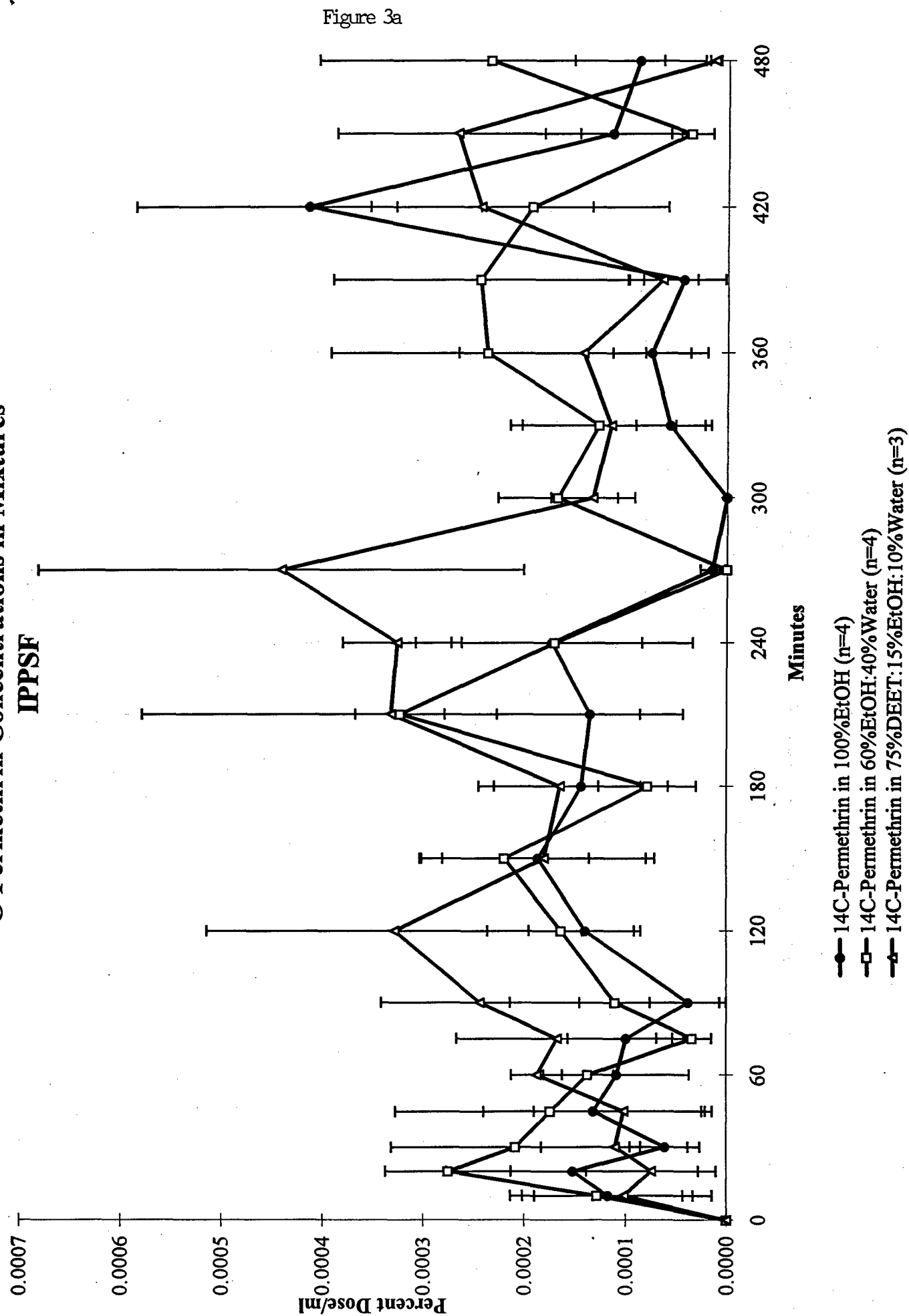
Isolated Perfused Porcine Skin Flaps (IPPSFs): IPPSFs were prepared according to procedures previously reported in the literature (Riviere et al., 1986; Monteiro-Riviere, 1990; Bowman et al., 1991). Skin flaps were perfused in a non-recirculating system with oxygenated (95% O₂ and 5% CO₂) Krebs-Ringer bicarbonate buffer spiked with glucose and bovine serum albumin. The perfusion chambers were maintained at 37°C and a relative humidity of 50-60%. After perfusing the skin flaps for 1 hour, a flexible template measuring 1.0 x 5.0 cm (Stomadhesive, ConvaTec-Squibb, Princeton, NJ) was affixed to the skin surface with Skin Bond (Pfizer Hospital Products, Inc., Largo, FL) to provide a surface area of 5.0 cm² for applying dosing solutions. One hundred microliter doses were applied to each dose site providing an applied surface concentration of 40 µg/cm² C14-permethrin. Venous perfusate samples were collected at 30 minute intervals for the first 2 hours post-dosing and then at 1 hour intervals for a total of 8 hours. Monitoring vascular resistance (VR) (perfusate pressure/flow) and cumulative glucose utilization (CGU) assessed skin flap viability. Arterial perfusate samples were collected hourly and compared with venous samples to determine CGU, while VR was defined as the ratio of arterial pressure to perfusate flow rate.

Chemical Analysis: DEET content in perfusate was determined according to HPLC methods developed and previously described in this report. To date (02/23/00), not all of the perfusate samples have been analyzed for DEET, but we anticipate completion of these analyses by the end of April. For determination of ¹⁴C-permethrin, perfusate samples were combusted in a Packard Model 306 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL) and then analyzed by Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL) for total ¹⁴C determination.

RESULTS and DISCUSSION:

Figure 3a shows the percent dose absorption of permethrin in flaps dosed with permethrin/DEET mixtures in ethanol while Figure 3b illustrates the skin deposition. Figure 3c demonstrates the percent dose absorption of DEET in flaps dosed with permethrin/DEET mixtures in ethanol. Since these studies are still in progress, it is premature to discuss their relevance, which only can be interpreted by integrating the results of the simpler model systems.

¹⁴C-Permethrin Concentrations in Mixtures IPPSF



Mean ¹⁴C-Permethrin Residues in Mixtures IPPSF

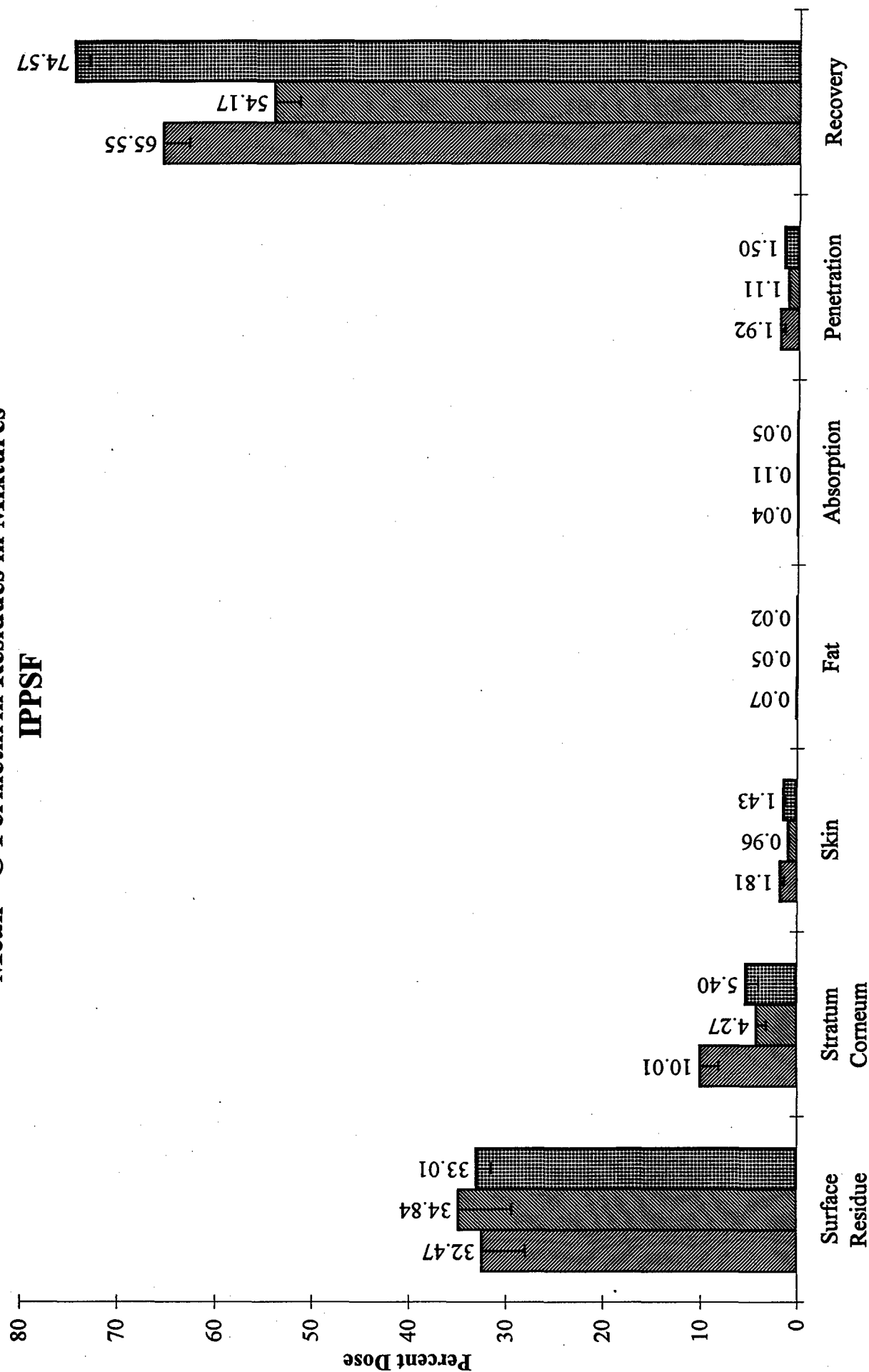
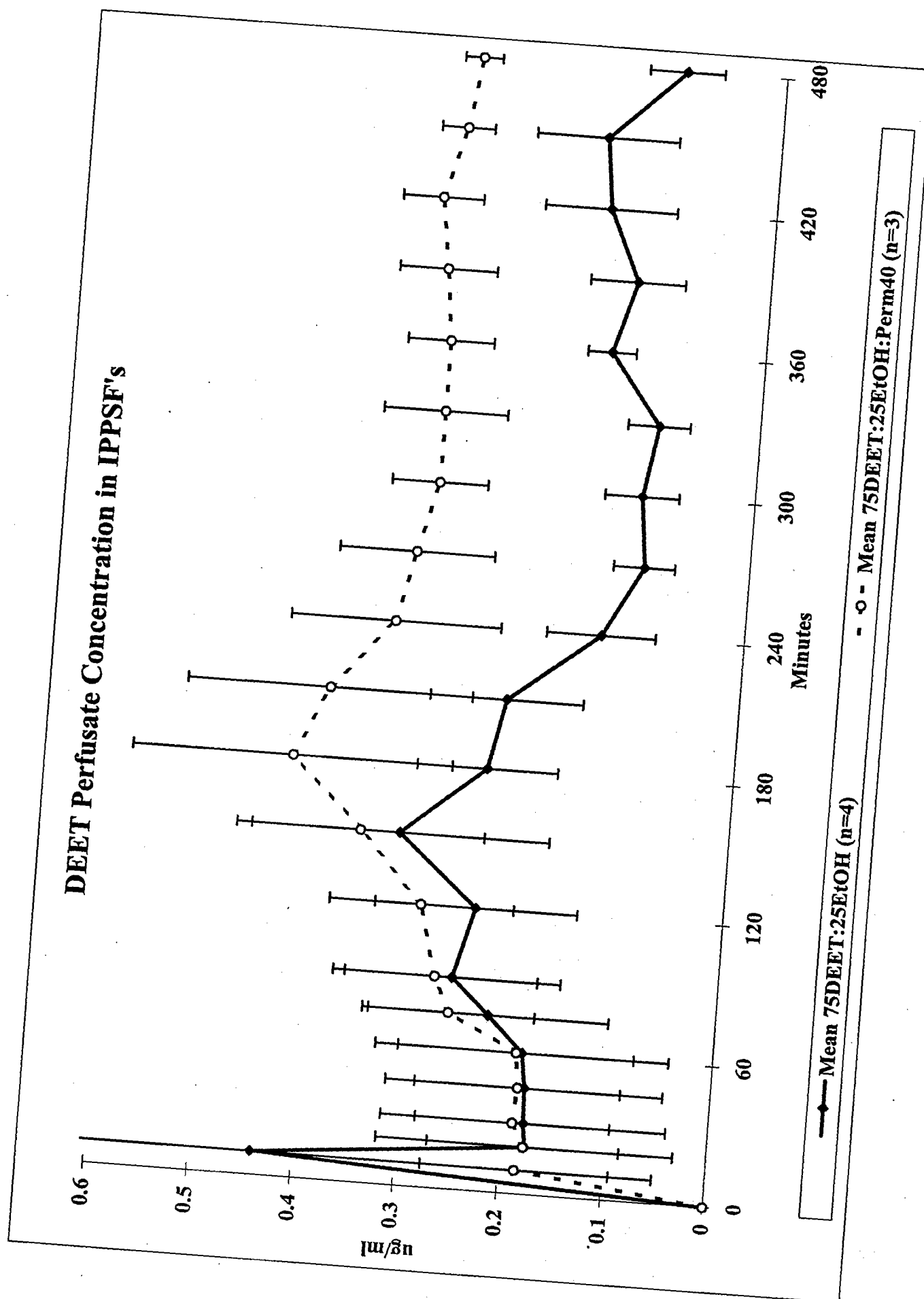


Figure 3b

Figure 3c



INFLUENCE OF DEET ON THE PERMEABILITY OF PERMETHRIN

INTRODUCTION:

The purpose of these pharmaceutical studies is to better understand how DEET interacts chemically with permethrin and biologically with stratum corneum and skin to influence the dermal permeability of permethrin. The first series of experiments were designed to study how different concentrations of DEET (0, 1, 3, 7, and 15%) and the presence of water influenced permethrin partitioning into the stratum corneum. Future studies will be aimed at assessing partitioning characteristics in the viable epidermis and dermis of porcine skin and inert silastic membranes and determine whether this partitioning behavior is consistent for both cis- and trans-isomers of permethrin. This report focuses only on the initial partition coefficient experiments using porcine stratum corneum. These studies, not initially proposed in the original grant application, are critical to interpret all absorption studies using these compounds.

MATERIALS AND METHODS:

Dissected skin from the abdomen of a female weanling Yorkshire pig was cut into pieces to fit between preheated aluminum blocks and placed in an oven at 60° for 6 to 8 minutes. The SC/epidermis was removed using dissection forceps and placed dermis side down into petri dishes lined with filter paper containing 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) to dissolve the epidermis. After 24 hours incubation in an oven at 70°C, trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) was added to neutralize trypsin and SC was washed with distilled water, dried at room temperature for approximately 24 hours, weighed (5-8mg sample) and placed in vials. 500µl of each mixture was added to the SC sample vial (n=4), capped, sealed and allowed to remain undisturbed at room temperature for 24 hours. After the vials were uncapped, 10µl of the vehicle was removed and 15ml of Ecolume (ICN Costa Mesa, CA) was added to each sample. The SC sample was removed, gently blotted on a Kimwipe (Kimberly-Clark Co., Roswell, GA) to remove excess solution then burned in an open flame tissue oxidizer (Model 306; Packard Instrument, Downers Grove, IL). The radioactivity of the recovered vehicle and SC samples was counted by a liquid scintillation analyzer (Model 1900 TR Tri-Carb, Packard Instrument, Downers Grove, IL).

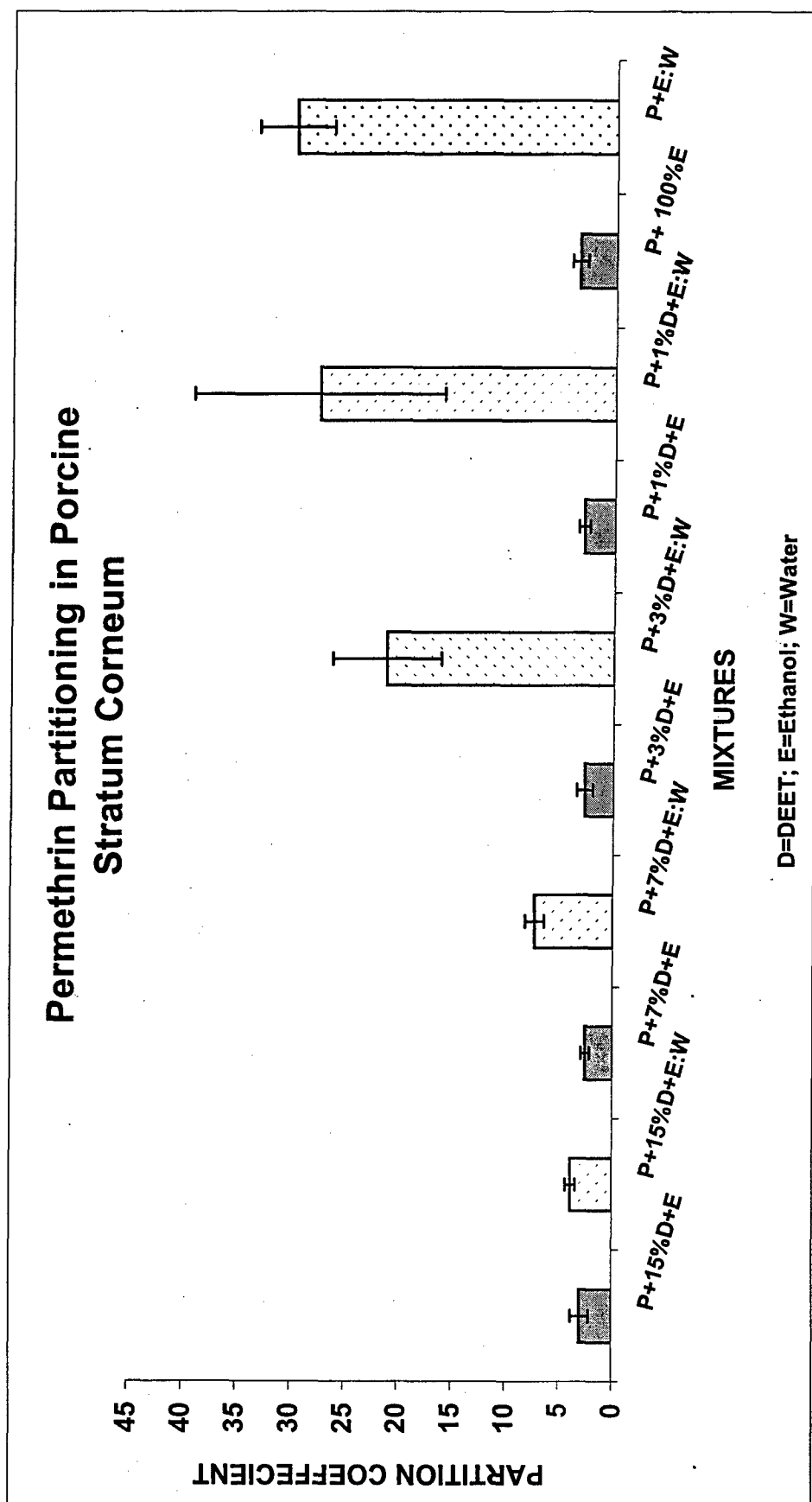
SC/Vehicle Partition Coefficient Determination: To determine the effects that various mixtures have on the partitioning of permethrin; 10 mixtures were formulated with 0%, 1%, 3%, 7% and 10% DEET in either ethanol alone or in equal parts of ethanol and distilled water, 10µl of [¹⁴C]permethrin was added to each mixture. After the radioactivity content of the vehicle and SC was determined, these values were normalized to 1000mg SC (C_{sc}) and 1000mg vehicle (C_v) for comparison. The SC/vehicle partition coefficient was determined by the equation: $PC = C_{sc}/C_v$.

Compounds: DEET (N,N-diethyl-m-toluamide) was obtained from Chem Service, Inc., West Chester, PA and had a purity of 98%; [¹⁴C]permethrin from Sigma St. Louis, MO with specific activity of 10.9mCi/mmol; and ethanol from Aldrich Chemical Co., Milwaukee WI

RESULTS and DISCUSSION

Please see Figure 4 below for a summary of observed partitioning behavior of permethrin in the presence of DEET in porcine stratum corneum. The important finding which can be seen from data is the increased partitioning of permethrin into stratum corneum seen from vehicles containing low concentrations of DEET in an ethanol/water vehicle.

Figure 4



BIOMARKER (IL-8, TNF- α , and PGE₂) RELEASE FROM SKIN FLAPS (IPPSF) EXPOSED TO GULF WAR MIXTURES

INTRODUCTION

The bulk of the experiments discussed above were designed to assess chemical-chemical and chemical-biological interactions such as altered solubility, partitioning, permeability or pharmacology. However, in intact organisms or models such as the IPPSF, chemicals may also induce subtle toxicologic effects which alter physiology and thus modulate absorption. This could be the primary reason why effects seen in diffusion cells would not predict those seen in the IPPSF or *in vivo*. To assess this level of interaction, biomarkers were monitored in exposed IPPSFs. Based on mechanisms of action and previous studies, IL-8, TNF- α , and PGE₂ were selected.

METHODS

IPPSFs were prepared and dosed topically with DEET and DEET/Permethrin mixtures as described in the previous section of this report. Perfusate samples were collected and assayed for IL-8, TNF- α , and PGE₂.

Enzyme-linked Immunosorbent Assay (ELISA): To determine secreted protein from IPPSFs in response to DEET and DEET+Permethrin exposure, a sandwich ELISA assay was used for both IL-8 and TNF- α . Perfusate samples were collected at 0, 0.5, 1.0, 2.0, 4.0, and 8.0 hours post-dose and frozen at -80°C for later assay. Plates (96-well) coated with anti-IL-8 or anti-TNF- α were obtained from a commercial source (Biosource). Perfusate diluted with the Standard Diluent Buffer was added to each well and incubated for 2hr at room temperature. Following extensive washing, biotinylated anti-IgG antibody (IL-8 or TNF- α) was added to each well and incubated for 1hr at room temperature. A streptavidin-peroxidase conjugate was then added to each well to bind to the biotin molecules. After adding a stabilized chromagen followed by the Stop Solution, the plates were read at 450nm on a Labsystems RC plate reader. For both ELISA assays, a recombinant human IL-8 or TNF- α was diluted to obtain a standard reference curve. To ensure that neither the perfusate or the dosed chemicals adversely affected the efficiency of the ELISA, a sample from each treatment group was spiked with a known concentration of the recombinant proteins and measured for accuracy.

PGE₂ was measured in the perfusate samples with a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The perfusate samples were collected in pyrogen- and endotoxin-free tubes, quickly frozen in 500 μ l aliquot samples, and stored at -80°C. To purify the perfusate, 250 μ l of each sample was added to 8ml of 0.1M potassium phosphate monobasic (pH 5.0) and extracted in a C18 column (MetaChem, Torrance, CA). To determine recovery, perfusate samples were spiked with PGE₂. Each column was then eluted with 8ml of ethyl acetate to remove unbound PGE₂ from the C18 bed. The samples were evaporated until dry on a TurboVap LV Evaporator (Zymark) with dry nitrogen. The samples were reconstituted with 1000 μ l of EIA buffer and incubated (triplicate) along with the Standards (duplicate) on a 96 well plate at 4°C. The wells were developed in Ellman's Reagent and the absorbance read at 405nm using the Labsystems Multiskan RC (Fisher Scientific). The absorbance of each Standard was plotted against the concentration and an exponential best fit line determined. The concentration of each sample was then determined. The results were corrected for dilution and recovery from the perfusate.

RESULTS and DISCUSSION:

Please see Figures 5a – 5c below for biomarker release from skin flaps exposed to DEET/permethrin mixtures. The first observation is that there are definite time factors seen for each cytokine, with TNF- α showing the earliest response compared to IL-8 or PGE₂. Permethrin in ethanol/water/DEET had the greatest increase in TNF- α and PGE₂ release. It was also one of the treatment groups with the highest IL-8 release. Further interpretation of this data must await complete statistical analysis and integration with the absorption data presented earlier.

Figure 5a

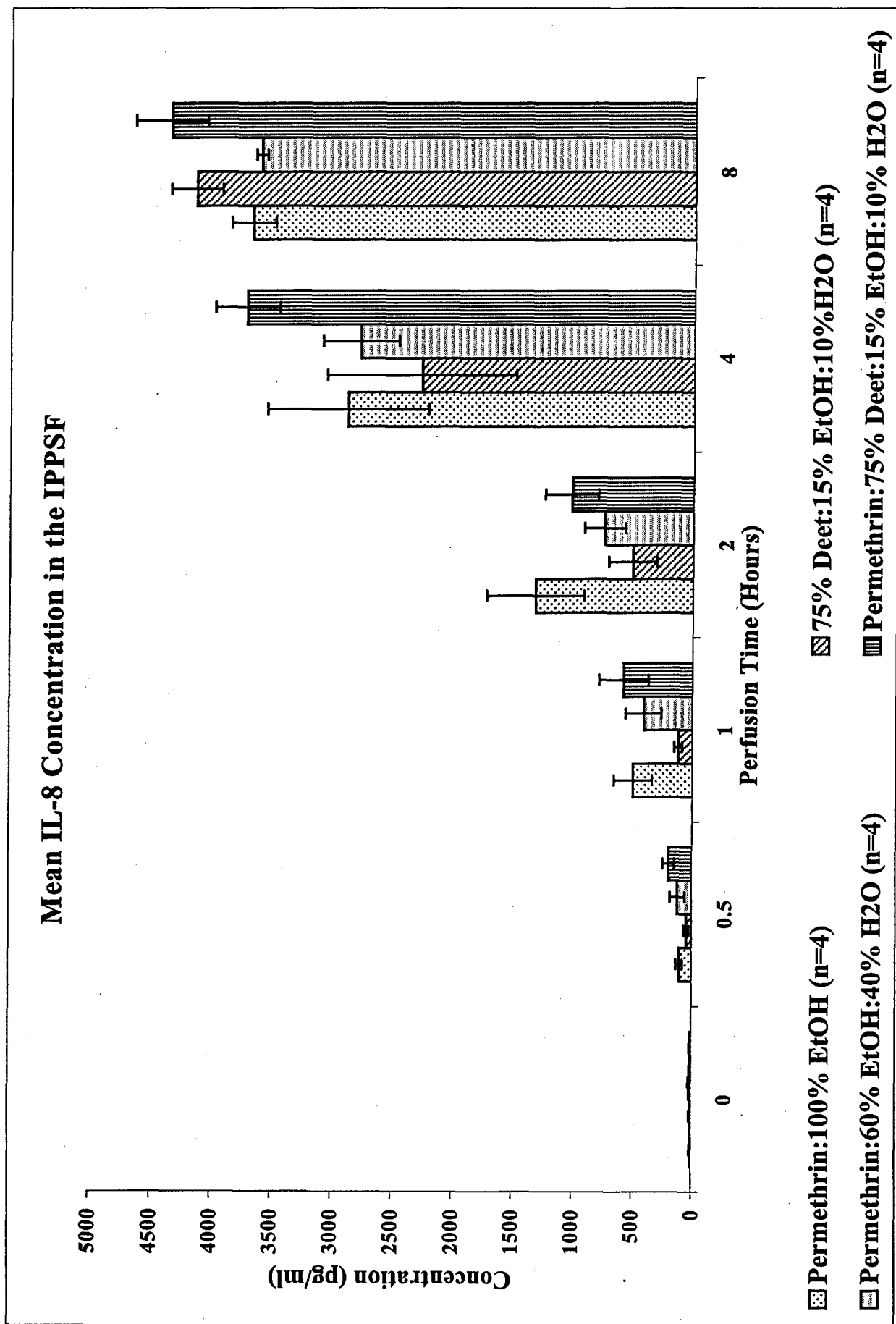


Figure 5b

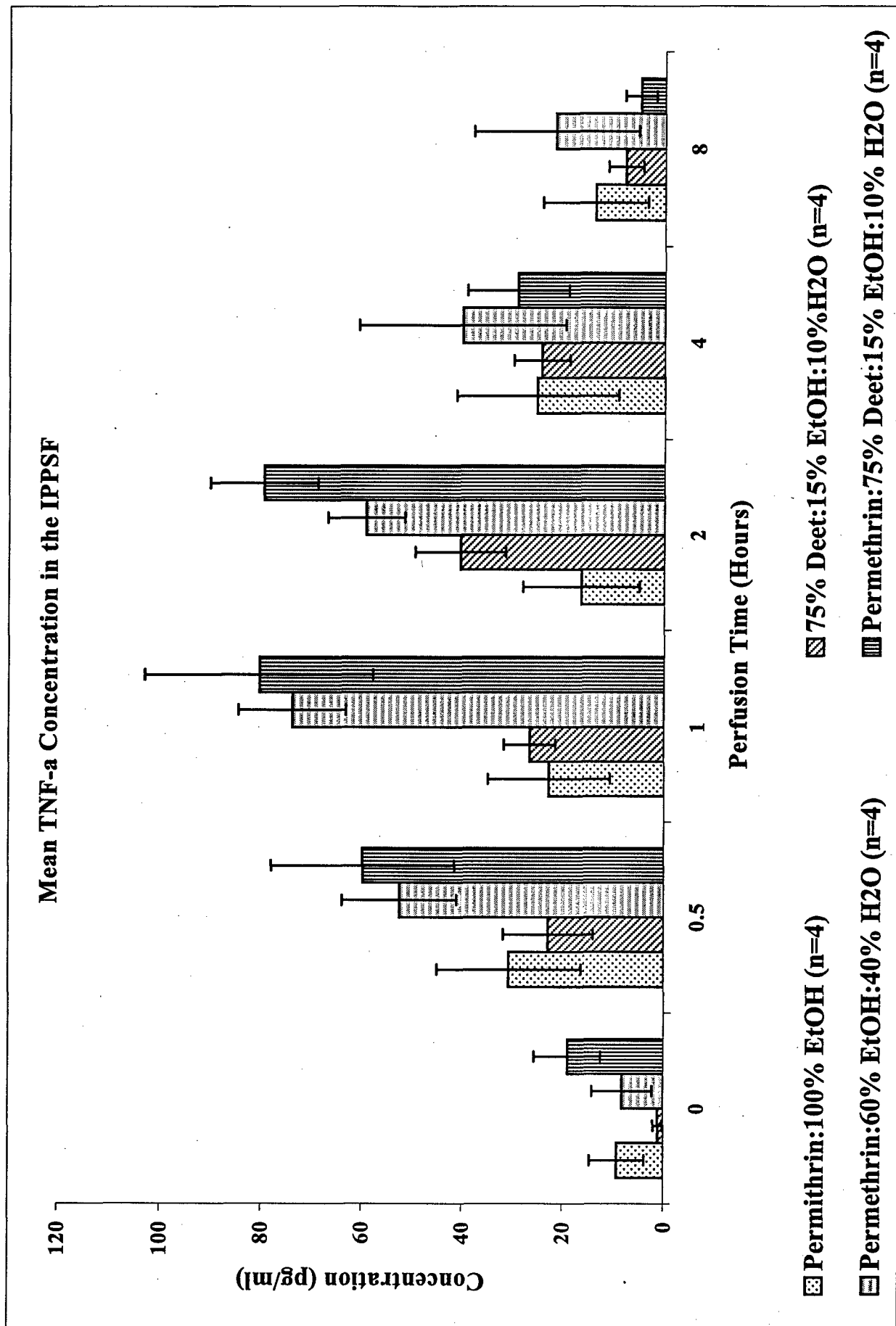
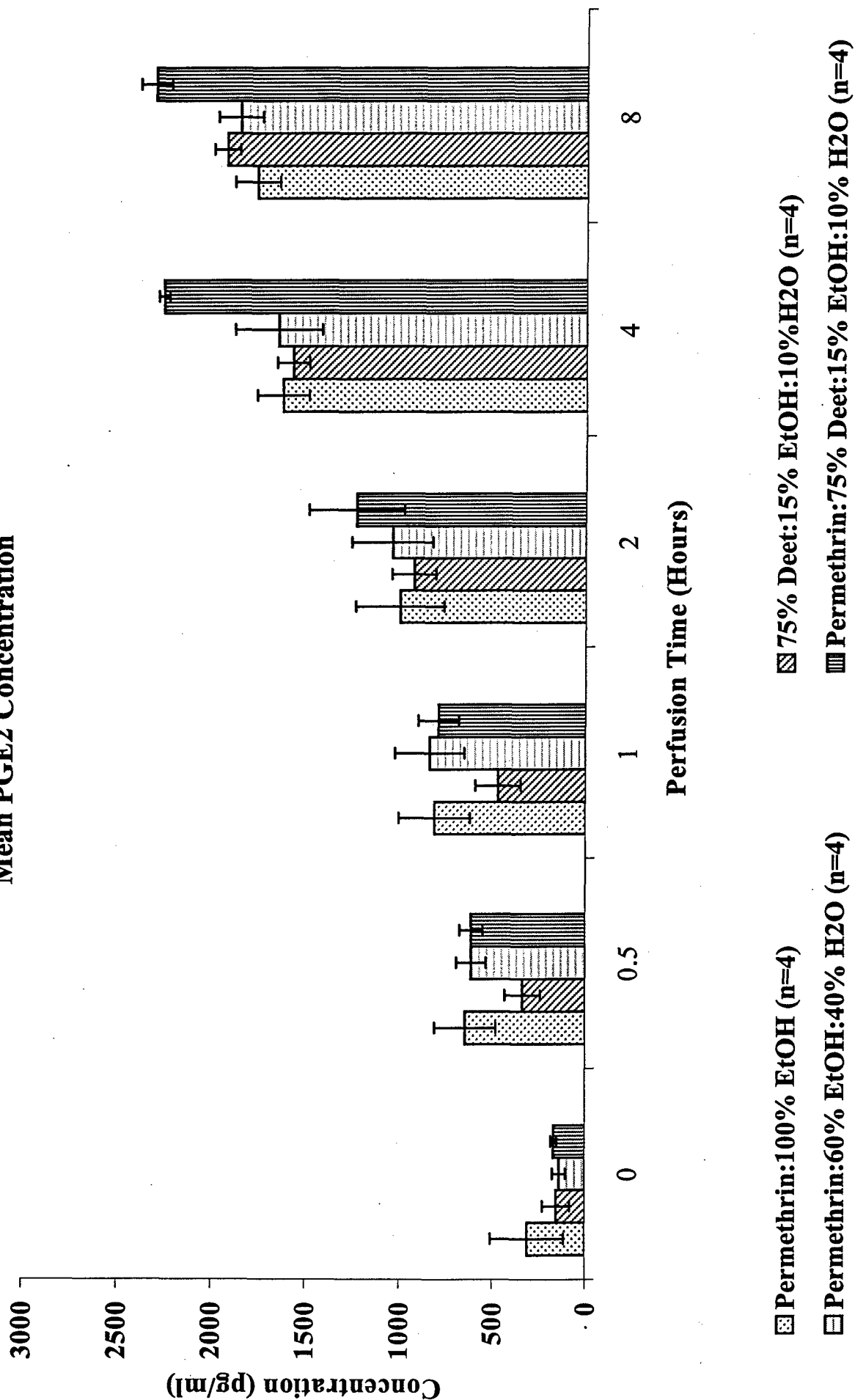


Figure 5c

Mean PGE2 Concentration



7. KEY RESEARCH ACCOMPLISHMENTS

- Development of sensitive analytical methods for detection of DEET and permethrin.
- Adaptation of assays for TNF- α , IL-8 and PGE₂ in experimental systems used in this proposal.
- Identified mixture-specific interactions on partitioning characteristics of permethrin in water/alcohol/DEET mixtures.
- Identified mixture-specific interactions on absorption, and skin deposition of permethrin and DEET in all three model systems studied.
- Identified release of inflammatory biomarkers TNF- α , IL-8 and PGE₂ after exposure to topical mixtures containing DEET and permethrin.
- Developed battery of *in vitro* skin absorption models to assess dermal absorption of components of a chemical mixture.

8. REPORTABLE OUTCOMES

- Presentation: KL Powers, RE Baynes, JD Brooks, JE Riviere: Influence of DEET on the permeability of permethrin. NCSU Department of Toxicology Research Symposium, 3/2000.
- This grant supports the PhD dissertation research of Ms. Kristina Powers.
- The work completed in year one of this research has resulted in the development of a four-phase integrated *in vitro* experimental battery to evaluate the mechanisms of interactions seen after topical exposure of complex chemical mixtures. This includes 1.) assessment of mixture effects on partitioning behavior, 2.) assessment of absorption through inert silastic membranes, 3.) assessment of potential lipid interaction in *in vitro* porcine diffusion cells, and 4.) assessment of integrated effects in the isolated perfused porcine skin flap.

9. CONCLUSIONS

The preliminary results of the first year of this research proposal suggest that there are significant and complex interactions between DEET and permethrin which modulate the percutaneous absorption of these compounds. It is too early in the analysis of these partially completed studies to make general conclusions about these mixtures effects. As mentioned in the proposal, the experimental design of these studies are both partial- and complete-factorial analyses which require all blocks to be finished before an analysis can be performed. At this stage in these experiments, we have only validated that individual blocks have been properly conducted and have only glimpsed at possible comparisons. Some blocks will require additional replicates. As expected, responses are different in the model systems evaluated. *We cannot make firm comparisons until all blocks have been complete and results analyzed across all models.*

Logistics: The work this year has allowed our group to optimize the experimental protocols for assessing mixture interactions in three model systems of varying levels of biological complexity. We have added experiments to detect chemical effects on partitioning phenomenon separate from penetration detected in diffusion cell experiments. This level of integrated assessment of chemical absorption has *never* been reported in the literature. This work was funded by decreasing the percent effort of the co-investigator Dr. Baynes and using these funds to support Center services to accomplish these tasks. Additionally, we have also incorporated analysis for inflammatory cytokines

in these systems which will allow us to separate purely physical-chemical interactions from chemical-induced irritation which also may modulate disposition in intact biological systems.

Based on these data, we have modified the approach to assess additional interactions from that originally proposed. As mentioned in the introduction, this does not alter the scope of the proposed research, but only the sequence in which the interactions are assessed. In year two of this work, we plan on assessing the effects of systemic pyridostigmine on DEET and permethrin absorption in all model systems. We additionally plan on starting the assessment of concomitant exposure to chemical warfare agents (sulfur mustard, organophosphate agent simulant DFP) and jet fuel on chemical deposition. We have recently gained considerable experience in assessing jet-fuel effects on compound absorption (Riviere et al., 1999) through the auspices of a USAFOSR research agreement and will integrate this knowledge into the jet-fuel experiments planned for next year. Thus, *all approved* Specific Aims are simultaneously being addressed in the experiments planned for next year.

Proposal for Additional Studies: These data have also suggested that additional interactions, not originally proposed in the grant application, may also significantly affect chemical absorption. Based on the 1996 NAS/IOM report on *"Interactions of Drugs, Biologics, and Chemicals in U.S. Military Forces,"* it is clear that additional substances used by US forces could further alter absorption of topical chemicals and pesticides and thus potentiate adverse effects. These include systemic non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen; anti-malarial drugs such as mefloquine, chloroquine, or primaquine; loperamide; additional analgesics and antipyretics; as well as topically applied steroids and OTC preparations such as "Skin-So-Soft®." For example, would the interactions detected above with DEET and permethrin still occur if skin were pre-treated with topical steroids or creams? Would the increase in inflammatory cytokines still occur if effective levels of ibuprofen (e.g. Motrim®, Advil®) were present? These would optimally be investigated using the experimental data generated in year two of the present proposal as the existing 2- and 3-component mixtures could be used as controls. *We propose to file for a Supplement and Extension of the present proposal to incorporate these additional chemicals into the existing experimental framework.*

"So-What": The preliminary results presented above indicate that the dermal absorption of permethrin and DEET are significantly modulated by the composition of the topical mixture they are exposed in. Secondly, the nature of these interactions are complex as reflected by the different responses seen in experimental systems of differing levels of biological complexity. These studies provide a rigorous experimental and conceptual framework to identify the nature of interactions which would significantly alter absorption of these potentially toxic chemicals.

10. REFERENCES

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REPLY TO
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