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14. ABSTRACT

The focus of this research was to assess the dermal absorption and skin toxicity of topically applied jet fuels [Jet A, JP-8, JP-8(100)] using pigs, *in vitro* porcine skin and inert membrane models as well as human keratinocyte cell cultures. Our working hypothesis was that jet fuel dermal toxicity was secondary to its hydrocarbon components with differences between fuel types due to additive modulation of hydrocarbon deposition. Consistent with previous JP-8 additive studies, individual JP-8(100) additives modulated hydrocarbon disposition. Combinations of multiple additives were not predictable from simpler mixtures. An *in vitro* porcine skin dose escalation study demonstrated dose-related dermal absorption of aromatics, but saturation of aliphatic components. Previous exposure to JP-8 constituents resulted in skin depot formation. Cell culture studies demonstrated divergent relations between structure and resulting cytotoxicity and irritation (Il-8 release). Across 8 aliphatic and 6 aromatic hydrocarbons studied *in vivo*, tridecane, tetradecane and pentadecane produced gross and microscopic lesions similar to JP-8, suggesting they may be responsible for JP-8 irritation. Ultrastructural changes were characterized by alterations in the stratum corneum lipid bilayers. Finally, a novel membrane coated fiber technique was developed that was capable of rapidly determining physical chemical properties of all jet fuel hydrocarbon constituents as well as assessing additive and solvent effects on membrane diffusion and partitioning.

15. SUBJECT TERMS

Jet Fuel, skin, percutaneous absorption, dermal toxicity, kinetics, toxicology, partition coefficient, ultrastructure, JP-8, Jet A, JP8+100, MDA, BHT, 8Q405, naphthalene, dodecane

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Executive Summary

The research conducted under the auspices of the present grant was a continuation of ongoing studies on the dermal absorption and potential skin toxicity of topically applied jet fuels (Jet A, JP-8 and JP-8(100). A great deal of progress was made on characterizing the absorption and dermal toxicity of individual hydrocarbons and assessing how performance additives that comprise the JP-8 fuel series modulate absorption of these components. A novel technique was also developed which should have significant utility in quantitatively assessing the nature of interactions that occur in jet fuel mixtures as they interface the dermal barrier.

The working hypothesis for this research is that jet fuel dermal toxicity is secondary to its hydrocarbon components with differences between fuel types being a result of additive modulation of hydrocarbon deposition. Data collected in our studies support this hypothesis and begin to define the nature of complex chemical mixture risk assessment where components modify mixture toxicity by modulating their disposition. The following are the major research accomplishments over the last three years. The cited publications listed at the end of this report should be consulted for a complete presentation of these data and analyses.

- Paralleling an earlier study on the effects of JP-8 additives on naphthalene and dodecane dermal disposition, we conducted a complete factorial study on the effects of the three JP-8 (100) additives (MDA, BHT, 8Q405) on hydrocarbon marker disposition. As with the JP-8 studies, individual additives modulated disposition, but combination effects were not predictable from simpler scenarios.
- A dose escalation study of seven JP-8 hydrocarbons was conducted using an *in vitro* porcine skin model that demonstrated dose-related dermal absorption of aromatics, but saturation of aliphatic components. This divergent kinetic behavior (first versus zero order absorption) must be taken into account when constructing multicomponent pharmacokinetic models as well as when jet fuel exposure risk assessments are conducted since the link between dose and effect is component specific. Since our data suggests that additives modify component deposition, the final effect seen after exposure to a complete fuel will be very dependent upon it composition.
- Repeated exposure to individual hydrocarbon components (8 aliphatics, 6 aromatics) demonstrated enhanced absorption from JP-8 pre-treated compared to naive skin. Previously absorbed hydrocarbons were released after repeated exposure and flux of the

individual components was enhanced. Fourier Transform Infrared Spectroscopic (FTIR) analysis of treated sections demonstrated two different phenomena depending on the individual hydrocarbon being studied; lipid extraction or hydrocarbon deposition and binding to stratum corneum.

- Human epidermal keratinocyte cell cultures were used to assess the potential for individual hydrocarbons to cause cytotoxicity or irritation (IL-8 release). These endpoints diverged across series of aromatic and aliphatic compounds. For aliphatics, toxicity was highest with the short chain congeners, while IL-8 release peaked at C-9 through C-13. Aromatics had more complex structure-toxicity relationships, and in some cases actually decreased IL-8 release. The response seen after complete fuel exposure is thus a composite of these individual responses.
- *In vivo* hydrocarbon exposure studies indicate that consistent with the cell culture data, tridecane, tetradecane and pentadecane produce gross and microscopic lesions similar to JP-8, suggesting that these three aliphatic components may be the principal candidates for jet fuel induced irritation. Aromatic compounds had minimal effects under the conditions of these exposures.
- Ultrastructural analysis of *in vivo* skin after jet fuel exposure indicated abnormalities in stratum corneum lipid bilayers and changes in the lamellar body secretory system, two changes that would be expected to modify epidermal barrier function.
- A novel *in vitro* model, the membrane coated fiber (MCF), was developed and used to rapidly determine partition coefficients and diffusion metrics for a number of jet fuel hydrocarbons. This approach would offer the promise of generating diffusion parameters under varied exposure conditions without doing complex diffusion cell studies. The advantage of this system is its ability to rapidly determine absorption parameters for all fuel constituents, including very hydrophobic compounds. The system is also compatible with high throughput screening approaches. Multiple membrane MCF systems would be an ideal approach to cluster constituent hydrocarbon components of jet fuels for interpreting PBPK models as well as quantitatively assessing mixture interactions (e.g. jet fuel additives) that may modify dermal absorption and systemic exposure.

Introduction: The focus of this research was to assess the percutaneous absorption and cutaneous toxicity of jet fuel hydrocarbons and performance additives that make up Jet-A, JP-8 and JP-8 +100. Porcine skin is the animal model utilized due to its documented similarity to human skin relative to chemical and drug absorption. A number of the studies supported by this grant have been published in the literature (See List at End of Report on pg. 31). They form the basis of this report. These references (underlined citations) should be consulted for full details.

*The **working hypothesis** of this research is that the chemical components of a mixture (e.g. jet fuel performance additives) may modulate the percutaneous absorption of other components (e.g. hydrocarbons) in jet fuel. If these modulated components are the agents responsible for toxicity, then an additive that alters their absorption, but by itself is not toxic, would potentiate the toxicity of a mixture containing the additive. If skin deposition is favored, this mixture might be expected to induce dermal toxicity. In contrast, if percutaneous absorption is facilitated then systemic toxicity may be potentiated. If the toxicity of the additive were assessed alone, this potential to enhance the toxicity of a mixture of which it is a component, would not be detected. If the component were directly toxic, the toxicity of the mixture might even be exaggerated.*

The fundamental scientific concept being investigated in these studies is the ability to extrapolate toxicity and disposition of a single chemical to more complex mixtures, and the behavior of a simple defined mixture to a complex mixture. Work under the auspices of the previous AFOSR grant (F492620-98-1-0105) defined the general parameters upon which the present research was conducted. These included studies that led to the above hypothesis of additives modulating the absorption of hydrocarbons that elicited the toxic responses characteristic of the complete fuel. This work was originally conducted using JP-8 additives and was extended in the present grant to assess the effects of the JP-8 (100) additive package on two markers of jet fuel hydrocarbon absorption: naphthalene and dodecane. Extensive cell culture studies were performed to assess the innate toxicity of a wide range of individual aliphatic and aromatic hydrocarbon markers for their ability to induce irritation and overt cellular toxicity. *In vivo* pig experiments were then conducted to validate the *in vitro* findings. Finally, in the fuel absorption studies, it became very clear that the number of potential interactions between constituent fuel hydrocarbons themselves and biological barrier membranes is enormous and beyond the ability of current *in vitro* or *in vivo* models to deal with. To assess this issue of

complex chemical mixture interactions, we developed a novel *in vitro* approach, the membrane coated fiber (MCF) model. These studies will be reviewed below.

Dermal Absorption Studies:

Effects of JP-8(100) Additives: Based on the effects of JP-8 additives on naphthalene and dodecane absorption previously reported (*Baynes RE et al. Toxicol. Appl. Pharmacol. 175: 269-281, 2001*), we conducted a similar complete-factorial analysis (Table One) on the effects of three JP-8 (100) additives [MDA, BHT, 8Q405] on these marker hydrocarbon absorption and dermal penetration using *in vitro* diffusion cells with silastic membranes or split-thickness porcine skin, as well as in the isolated perfused porcine skin flap (IPPSF) model developed by our group (Muhammad, Brooks and Riviere, 2004).

TABLE ONE: Jet Fuel Mixtures Studied

No additive	Single additive	Two additives	Three additives
JP-8	JP-8 + MDA	JP-8 + MDA + BHT	JP-8 (100)
	JP-8 + BHT	JP-8 + MDA + 8Q405	
	JP-8 + 8Q405	JP-8 + BHT + 8Q405	

The IPPSF is illustrated in Figure One below. A two-stage surgical procedure is employed which results in a single pedicle axial pattern tubed skin flap supplied by a cannulated artery that is then perfused in an isolated organ perfusion chamber (Figure Two) (*Riviere et al. Fundamental and Applied Toxicology 7: 444-453, 1986*). Test substances are placed on the surface of the flap and percutaneous absorption is tracked by assaying chemical flux in the venous effluent. Absorption of compounds in the IPPSF has been shown to be predictive of *in vivo* human absorption (*Wester et al. Toxicology and Applied Pharmacology 151: 159-165, 1998*). At the end of an experiment, the skin is biopsied for histopathology and assessment of cutaneous deposition of the applied drug. Venous effluent may simultaneously be assayed for pro-inflammatory cytokine release due to skin irritation from the applied chemicals.

FIGURE ONE: Two-stage IPPSF surgery

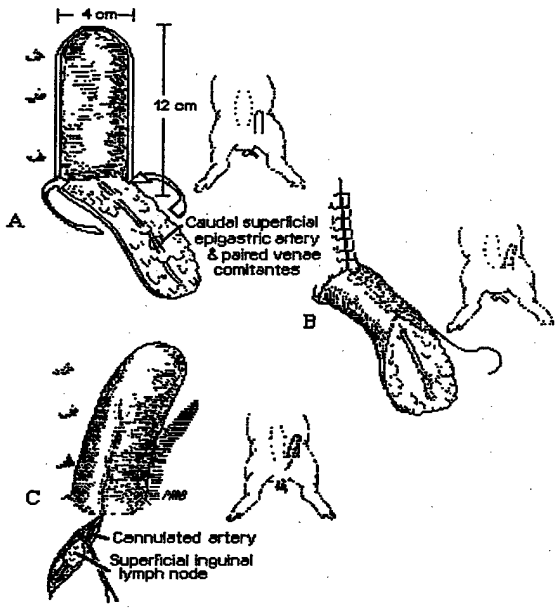
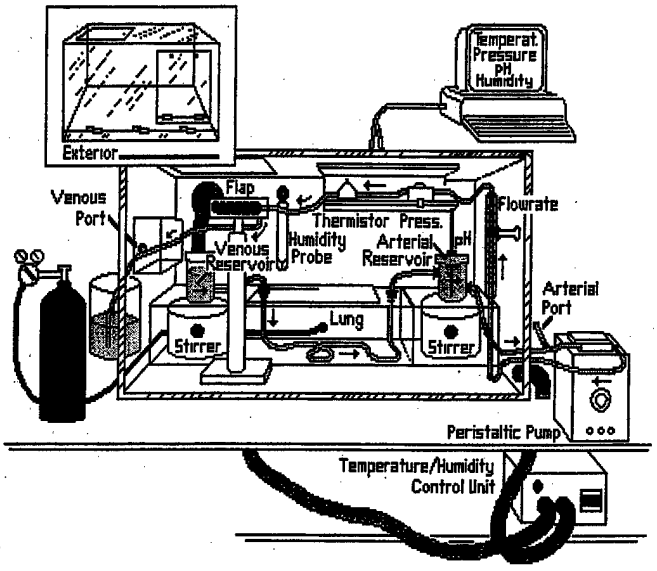


FIGURE TWO: IPPSF perfusion chamber



Influence of JP-8 (100) additives on the dermal kinetics of ^{14}C-naphthalene and ^{3}H-dodecane as markers of hydrocarbon absorption, were statistically evaluated using analysis of means (ANOM) and analysis of variance (ANOVA). These data are tabulated in Table Two below. This study indicated that the naphthalene absorption through silastic membrane was significantly different with JP-8 plus individual additives as compared to controls i.e. JP-8 and JP-8(100). The porcine skin data indicated that neither individual nor combinations of additives affected naphthalene absorption. All individual and combinations of two additives with JP-8 affected naphthalene and dodecane surface retention in silastic membrane. The 8Q405 significantly reduced naphthalene contents in dosed silastic and skin indicating a direct interaction between additive and marker hydrocarbons. The IPPSF (Figure Three) showed that only MDA and BHT altered naphthalene absorption, with MDA significantly suppressing and BHT significantly enhancing naphthalene absorption. MDA significantly decreased dodecane absorption in skin flaps. When both were present, the combination resembled the JP-8 and JP-8 + MDA profiles (Figure Four). Only 8Q405 was a significant modulator of surface retention for both marker hydrocarbons in the IPPSF. The MDA and BHT, which significantly retained naphthalene in the stratum corneum of porcine skin individually, led to a statistical decrease in its retention in the stratum corneum when in combination (MDA + BHT) suggesting a potential biological interaction. These observations demonstrate that the single membrane system may not

be suitable for the final prediction of complex additive interactions in jet fuels. Rather a combination of different membrane systems may provide the insight to elucidate the possible mechanism for additive interactions. Finally, it is important to assess all components of a chemical mixture since the effects of single components administered alone or as pairs may be confounded when all are present in the complete mixture.

TABLE TWO: Mean (SEM)* Steady State Flux, Permeability and Diffusivity Following Topical Doses of Naphthalene and Dodecane in Jet Fuel Mixtures in Silastic and Porcine Skin.

Naphthalene (Silastic membrane)	Flux ($\mu g/cm^2/hr$)	Permeability ($cm/hr \times 10^{-3}$)	Diffusivity ($cm^2/hr \times 10^{-6}$)
JP-8 (n=5)	19.29 (0.60) ^{b}	1.78 (0.06) ^{d}	1,690 (490) ^{b}
JP-8+MDA (n=5)	28.23 (1.62) ^{a}	2.30 (0.13) ^{a}	16,775 (5,672) ^{a}
JP-8+BHT (n=5)	27.17 (0.84) ^{a}	2.22 (0.07) ^{ab}	3,855 (907) ^{b}
JP-8+8Q405 (n=4)	26.86 (0.71) ^{a}	2.19 (0.06) ^{ab}	2,164 (403) ^{b}
JP-8+MDA+BHT (n=5)	21.22 (0.52) ^{b}	1.99 (0.05) ^{bcd}	1,408 (411) ^{b}
JP-8+MDA+8Q405 (n=4)	21.65 (0.62) ^{b}	2.03 (0.06) ^{bc}	1,469 (790) ^{b}
JP-8+BHT+8Q405 (n=4)	21.75 (0.73) ^{b}	2.04 (0.07) ^{bc}	251 (23) ^{b}
JP-8(100) (n=5)	21.01 (0.86) ^{b}	1.93 (0.08) ^{cd}	6,823 (5,836) ^{b}
Dodecane (Silastic membrane)			
JP-8 (n=5)	1.46 (0.08) ^{a}	0.041 (0.002) ^{a}	3,577 (1,956) ^{ab} (n=3)
JP-8+MDA (n=5)	0.70 (0.03) ^{b}	0.020 (0.001) ^{b}	9,819 (4,607) ^{a}
JP-8+BHT (n=5)	0.90 (0.07) ^{b}	0.026 (0.002) ^{b}	2,187 (628) ^{b}
JP-8+8Q405 (n=4)	0.75 (0.04) ^{b}	0.021 (0.001) ^{b}	1,805 (336) ^{b}
JP-8+MDA+BHT (n=5)	0.89 (0.15) ^{b}	0.025 (0.004) ^{b}	3,741 (1,313) ^{ab} (n=4)
JP-8+MDA+8Q405 (n=4)	0.84 (0.08) ^{b}	0.024 (0.002) ^{b}	3,601 (1,822) ^{ab}
JP-8+BHT+8Q405 (n=4)	0.74 (0.24) ^{b}	0.021 (0.007) ^{b}	2,972 (1,247) ^{ab}
JP-8(100) (n=5)	1.34 (0.05) ^{a}	0.038 (0.001) ^{a}	2,484 (873) ^{b} (n=3)
Naphthalene (Pig Skin)			
JP-8 (n=5)	2.21 (0.27) ^{a}	0.21 (0.03) ^{a}	455 (49) ^{a}
JP-8+MDA (n=5)	2.63 (0.09) ^{a}	0.20 (0.01) ^{a}	337 (23) ^{b}
JP-8+BHT (n=5)	2.48 (0.28) ^{a}	0.19 (0.02) ^{a}	324 (39) ^{b}
JP-8+8Q405 (n=4)	2.17 (0.16) ^{a}	0.17 (0.01) ^{a}	330 (42) ^{b}
JP-8+MDA+BHT (n=5)	2.88 (0.35) ^{a}	0.23 (0.03) ^{a}	176 (15) ^{c}
JP-8+MDA+8Q405 (n=5)	2.64 (0.47) ^{a}	0.21 (0.04) ^{a}	158 (9) ^{c}
JP-8+BHT+8Q405 (n=4)	2.70 (0.48) ^{a}	0.21 (0.04) ^{a}	161 (8) ^{c}
JP-8(100) (n=5)	2.24 (0.25) ^{a}	0.21 (0.02) ^{a}	402 (37) ^{ab}
Dodecane (Pig Skin)			
JP-8 (n=5)	0.090 (0.01) ^{b}	0.0025 (0.00) ^{b}	1,179 (331) ^{bc}
JP-8+MDA (n=4)	0.164 (0.03) ^{a}	0.0047 (0.00) ^{a}	352 (111) ^{c}
JP-8+BHT (n=5)	0.123 (0.01) ^{ab}	0.0035 (0.00) ^{ab}	455 (33) ^{c}
JP-8+8Q405 (n=4)	0.171 (0.05) ^{a}	0.0049 (0.00) ^{a}	271 (47) ^{c}
JP-8+MDA+BHT (n=5)	0.077 (0.01) ^{b}	0.0022 (0.00) ^{b}	2,565 (778) ^{a}
JP-8+MDA+8Q405 (n=4)	0.097 (0.01) ^{b}	0.0028 (0.00) ^{b}	1,147 (330) ^{bc}
JP-8+BHT+8Q405 (n=4)	0.079 (0.01) ^{b}	0.0022 (0.00) ^{b}	1,652 (435) ^{ab}
JP-8(100) (n=5)	0.094 (0.02) ^{b}	0.0027 (0.00) ^{b}	1,174 (384) ^{bc}

*Superscripts represent significant differences among treatments within a parameter ($p<0.05$). Means with the same letter are not significantly different.

FIGURE THREE: Absorption (µg/hr) profiles of naphthalene and dodecane after IPPSF dosing with JP-8, JP-8+MDA and JP-8+BHT.

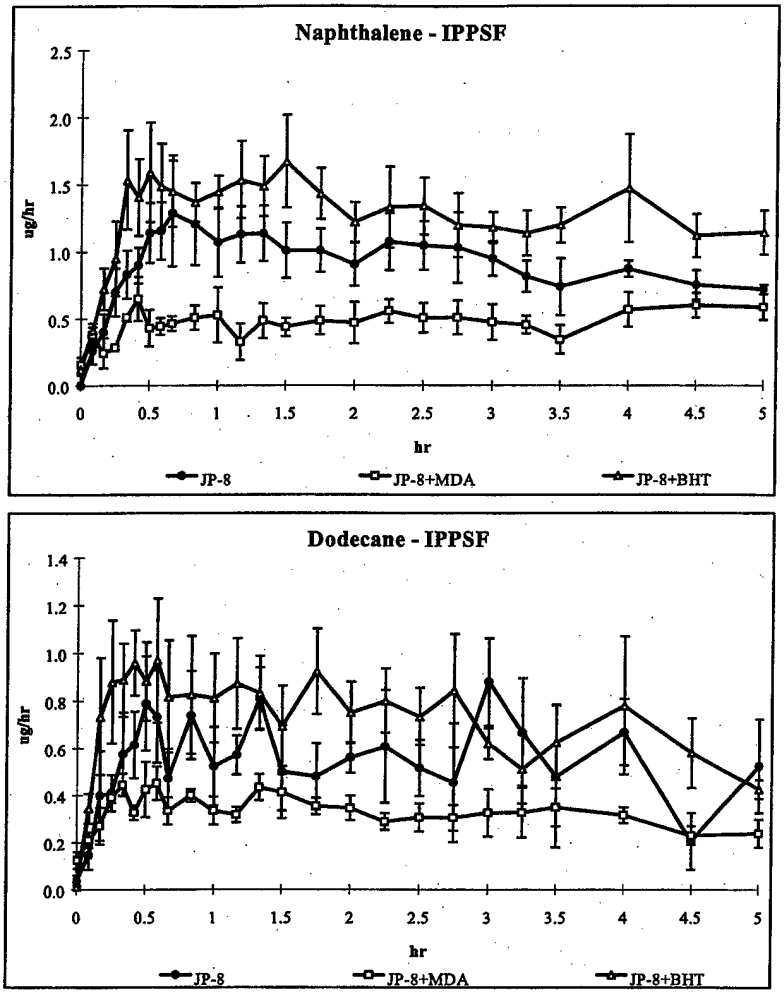
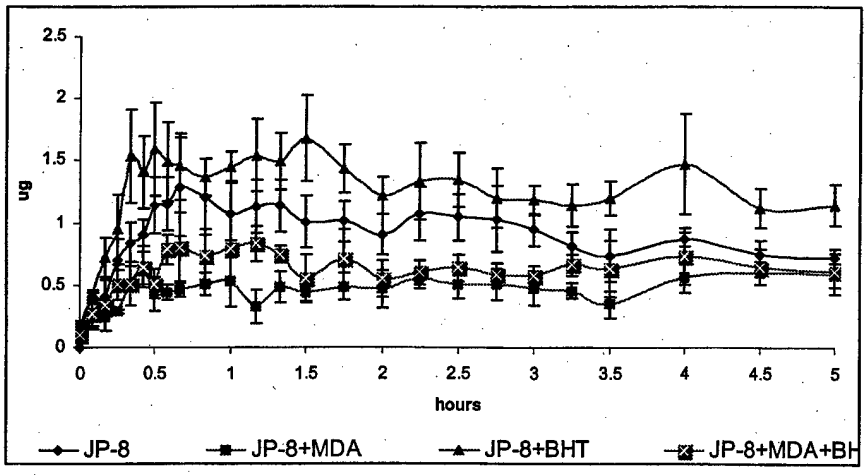


FIGURE FOUR: Absorption (µg/hr) profiles of naphthalene after IPPSF dosing with JP-8, JP-8+MDA and JP-8+BHT, as well as JP-8 + MDA + BHT.



This observed additive modulation of fuel component absorption, conceptually very similar to that seen with JP-8 additives, suggests that predicting the toxicity of exposure to different additive factors is problematic. Additionally, one must consider what the toxicological implications of the presence of opposing additives (MDA and BHT) are on interpreting equivalent naphthalene fluxes in mixtures containing both additives compared to those without any additives. Do these opposing mechanisms, which cancel out individual modulating effects of each additive, also modify the potential for direct skin toxicity compared to naphthalene alone?

It may be a mistake to assume that these opposite effects simply cancel one another out and that the flux of chemical is now equivalent to it being applied alone. The mechanisms behind the similarity in fluxes are different. Fick's First Law of Diffusion can be used to illustrate this. In the base situation (∅), compound flux (e.g. naphthalene or dodecane) would equal:

$$\text{Flux}_{\varnothing} = (K_p)\,(\Delta C)$$

where K_p is the permeability coefficient and ΔC is the concentration gradient driving the absorption process. We will consider ΔC the effective dermal dose since increasing concentration on the surface of skin effectively increases ΔC. In the presence of additives, we had two scenarios where additive A decreased absorption by retaining chemical on the surface, effectively reducing ΔC:

$$\downarrow \text{Flux}_{A} = (K_p)\,(\downarrow \Delta C)$$

and scenario B where flux increased due to an increased K_p:

$$\uparrow \text{Flux}_{B} = (\uparrow K_p)\,(\Delta C)$$

When both A and B are present (e.g. MDA + BHT), the flux is now back to baseline levels, but is governed by a fundamentally different set of diffusion parameters:

$$\text{Flux}_{A+B} \cong \text{Flux}_{\varnothing} = (\uparrow K_p)\,(\downarrow \Delta C)$$

Different factors that interact with these altered parameters could drastically change dermal flux compared to the baseline scenario. This dimension of complex chemical mixture toxicology, nicely illustrated with these JP-8 additives on naphthalene and dodecane, has not been adequately addressed nor understood. If this type of emergent behavior occurs with two component mixtures, what happens when higher-order mixtures are encountered?

In conclusion, this study indicated that MDA is a significant antagonist of both naphthalene and dodecane absorption while BHT is a potent synergist of naphthalene absorption in IPPSF. Porcine skin did not depict any significant effect of additives on marker absorption.

The 8Q405 has no effect on marker absorption, but significantly retained it on the surface of membranes. 8Q405 alone, and in combination with BHT, significantly reduced naphthalene contents in porcine skin but not in IPPSF; which suggest as additional biological effect of this additive. These observations demonstrate that a single membrane system may not be suitable for the final prediction of complex additive interactions in jet fuels. Rather a combination of different membrane systems may provide the insight for possible mechanism for additive interactions. A membrane system with intact microvasculature such as the IPPSF that closely mimics *in vivo*, should probably be used for predictive purposes.

Dose Related Dermal Absorption of Hydrocarbon Jet Fuel Constituents: Based upon our working hypothesis that it is the individual hydrocarbons that are responsible for JP-8 toxicity, we conducted a series of studies exploring the dermal absorption of individual hydrocarbons using the *in vitro* flow-through diffusion cell model (Muhammad, Baynes, Monteiro-Riviere, Xia and Riviere, 2004). The first treatment (1X) was comprised of mixtures containing undecane (4.1%), dodecane (4.7%), tridecane (4.4%), tetradecane (3%), pentadecane (1.6%), naphthalene (1.1%) and dimethyl naphthalene (1.3% of jet fuels) in hexadecane solvent applied to porcine skin in flow through diffusion cells. Other treatments (n=4 cells) were 2X and 5X component concentrations. Perfusate samples were analyzed with gas chromatography-flame ionization detector (GC-FID) using head space solid phase micro-extraction fibers. We have standardized the assay to have excellent linear correlation for all the tested components in media standards.

Absorption parameters including diffusivity, permeability, steady state flux and percent dose absorbed were estimated for all the tested hydrocarbons (Table Three). These data as well as the flux profiles in Figure Five below, demonstrated a dose related increase in dermal absorption of naphthalene and dimethylnaphthalene in porcine skin. A different scenario was seen with the aliphatic markers (Figure Six). All calculated parameters for undecane are statistically similar for the three doses. The concentration versus time profiles for dodecane are similar to undecane, but with a reduced perfusate concentration of about 3.4ng/ml with the 5X dose. The maximum concentration achieved by tridecane in perfusate was only 1ng/ml with the 5X dose. Also, there was no dose dependent trend evident in the profiles that were similar to that of undecane in Figure Five. There were no significant differences among 1, 2 and 5X doses with regards to flux, permeability, diffusivity nor the percent dose absorbed of dodecane and tridecane. This can be anticipated since the size of the hydrocarbon is increased, the percutaneous absorption profiles are decreased correspondingly. The high molecular weight

tetradecane was not detected in the perfusate samples at the low doses, while high background values for pentadecane confounded the results. The data for these two hydrocarbons were excluded from further analysis.

TABLE THREE: Mean ± SEM* Hydrocarbon Flux, Permeability, Diffusivity And Percent Dose Absorbed In Porcine Skin Sections Exposed To Three Dosing Mixtures For 5 Hrs.

Hydrocarbon	Flux (µg/cm^2/h)	Permeability (cm/hr*1,000)	Diffusivity (cm^2/h*1,000,000)	Percent Dose
Naphthalene				
IX dose (n=4)	0.43 ± 0.12^{bA}	0.0333 ± 0.0090^{aA}	244 ± 40^{aABC}	0.3555 ± 0.120^{aA}
2X dose (n=4)	1.24 ± 0.26^{bA}	0.0485 ± 0.0101^{aA}	211 ± 17^{aBC}	0.4653 ± 0.101^{aA}
5X dose (n=5)	3.63 ± 0.24^{aA}	0.0569 ± 0.0066^{aA}	213 ± 14^{aAB}	0.5424 ± 0.066^{aA}
Dimethyl-	**Naphthalene**			
IX dose (n=4)	0.13 ± 0.01^{bB}	0.0095 ± 0.0007^{aB}	153 ± 9^{aC}	0.0700 ± 0.007^{aB}
2X dose (n=4)	0.23 ± 0.05^{bB}	0.0088 ± 0.0020^{aB}	151 ± 4^{aC}	0.0650 ± 0.017^{aB}
5X dose (n=5)	0.58 ± 0.09^{aB}	0.0088 ± 0.0014^{aB}	153 ± 3^{aB}	0.0644 ± 0.011^{aB}
Undecane				
IX dose (n=3)	0.03 ± 0.00^{aC}	0.0002 ± 0.0000^{aC}	349 ± 58^{aA}	0.0066 ± 0.001^{aC}
2X dose (n=4)	0.03 ± 0.00^{aC}	0.0002 ± 0.0000^{aC}	420 ± 24^{aA}	0.0050 ± 0.001^{aC}
5X dose (n=5)	0.04 ± 0.01^{aC}	0.0003 ± 0.0000^{aC}	311 ± 53^{aA}	0.0046 ± 0.001^{aC}
Dodecane				
IX dose (n=4)	0.01 ± 0.00^{aCD}	0.0003 ± 0.0001^{aC}	293 ± 37^{aAB}	0.0033 ± 0.001^{aC}
2X dose (n=4)	0.02 ± 0.00^{aC}	0.0002 ± 0.0001^{aC}	244 ± 36^{aB}	0.0020 ± 0.001^{aC}
5X dose (n=5)	0.03 ± 0.01^{aC}	0.0001 ± 0.0000^{aC}	293 ± 63^{aA}	0.0014 ± 0.000^{aC}
Tridecane				
IX dose (n=3)	0.004 ± 0.00^{aD}	0.0001 ± 0.0000^{aC}	195 ± 25^{aBC}	0.0015 ± 0.000^{aC}
2X dose (n=4)	0.006 ± 0.00^{aC}	0.0001 ± 0.0000^{aC}	214 ± 18^{aBC}	0.0015 ± 0.001^{aC}
5X dose (n=4)	0.008 ± 0.00^{aC}	0.0001 ± 0.0000^{aC}	172 ± 7^{aB}	0.0014 ± 0.000^{aC}

*Lower case superscripts represent significant differences between treatments within a parameter. Upper case represent significant differences among hydrocarbons for a specific treatment ($P<0.05$). Means with same letter are not significantly different.

FIGURE FIVE. Perfusate concentrations (ng/ml) of naphthalene (left panel) and dimethyl naphthalene (right panel) after 1X (-Δ-, n=4), 2X (-◊-, n=4) and 5X dosing (-□-, n=5). *indicates the first time point at which the 5X dose profile becomes statistically different from 2X and 1X doses.

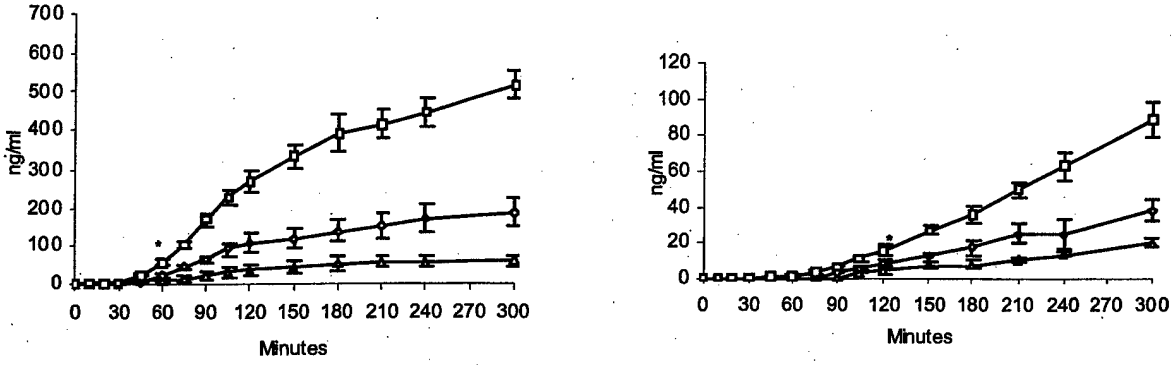
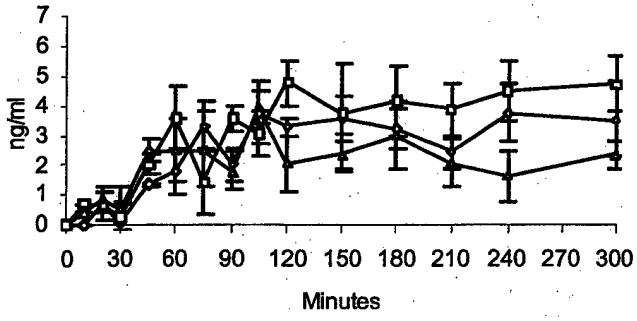


FIGURE SIX . Perfusate concentrations (ng/ml) of undecane after dosing with 1X dosing mixture (-Δ-, n=4), 2X dosing mixture (-◊-, n=4) and 5X dosing mixture (-□-, n=5).

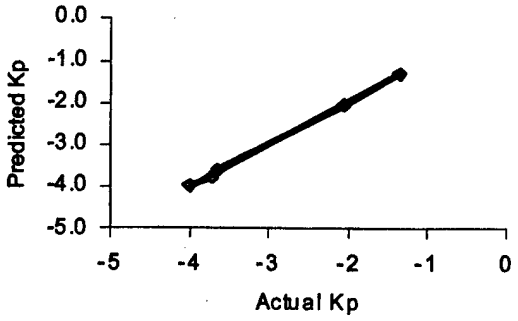


In order to study the relationship of permeability of these hydrocarbons to their physicochemical properties [molecular weight, logarithm of octanol- water partition coefficient (log Ko/w) and water solubility], a multiple regression analysis was performed with level of significance ($\alpha = 0.05$) (R^2=0.9985). This high correlation coefficient may be due to the fact that for this small set of studied hydrocarbons (naphthalene, DMN, undecane, dodecane and tridecane), the available physicochemical properties in literature varies in the same order of magnitude as permeability. This can be anticipated from coefficients in the equation below where permeability (Kp) is inversely related to molecular weight and log Ko/w, but directly related to water solubility.

$$\text{Log } Kp = C + \alpha MW + \beta \text{Log } Ko/w + \gamma H_2O \text{ solubility}$$

were: Intercept (C = 0.3908), molecular weight coefficient (α = - 0.0004), log Ko/w coefficient (β = - 0.565), and water solubility coefficient (γ = 0.0073). A plot of actual and predicted values of log Kp is shown in Figure Seven.

FIGURE SEVEN. Relationship between actual versus model predicted Kp (-◊-) values.



These data clearly indicate that at concentrations found in jet fuels, the two aromatic markers demonstrated a dose related increase in absorption. As will be discussed next, this is the same relationship seen for thee compound's toxicity to cultured keratinocytes. This also suggests that as exposure increases in the occupational setting, absorption and thus systemic exposure to aromatic hydrocarbons such as naphthalene increase. However, this is not the scenario operative after exposure to aliphatic hydrocarbons. In this case, increased exposure does not result in increased transdermal flux. Saturation is evident. The quantitative structure permeability relationships (QSPR) relationship suggests that as lipophilicity increases, transdermal flux decreases, most probably secondary to depot formation as previously seen for dodecane and hexadecane in our original jet fuel IPPSF studies (*Riviere et al., Toxicol. App. Pharmacol 160: 60-75, 1999*). This finding would suggest that exposure to aliphatics from jet fuel may have a greater impact on local skin toxicity rather than systemic toxicity secondary to transdermal absorption. As discussed below, aliphatics also behave differently relative to their propensity to cause overt cytotoxicity versus irritation (Il-8 release).

Repeated Exposure: The absorption studies conducted to date deal with single dose exposure. Repeated daily exposure is the occupationally relevant situation. Almost no information is available on percutaneous absorption of jet fuel components after repeated or pre-exposure scenarios. We tested the hypothesis that "repeated/pre-exposures of skin to JP8 jet fuel cause disruption in skin barrier functions by extracting/altering the lipids in the stratum corneum leading to increased dermal absorption of the hydrocarbons on subsequent jet fuel exposures."

In this study we exposed pigs to JP-8 jet fuel for 1 and 4 days (with repeated daily exposure) using cotton fabric to mimic the occupational scenario. The skin from a JP-8 pre-exposed pig was dermatomed for *in vitro* diffusion flow through cell experiments. The dosing mixture was composed of 8 neat aliphatic (nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadeane) and 6 aromatic (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, dimethyl naphthalene) hydrocarbons using water + ethanol (50:50) as a solvent. The samples were analyzed as in the dose escalation study above. The results supported our proposed hypothesis. We observed an increase in absorption of both aliphatic and aromatic hydrocarbons upon repeated JP-8 exposure to pig skin. The amount of aliphatic component absorption was directly proportional to the length of exposure, showing 2-3-fold increase after 1 day and 3-4 fold increase after 4-days through JP-8 pre-exposed skin. Similarly, aromatic hydrocarbons like ethyl benzene, o-xylene and trimethyl benzene absorbed 2 and 4 times more after 1 and 4 days pre-exposures, respectively (Table Four). As seen with the single dose exposures, very long chain aliphatics were not detected in the perfusate.

TABLE FOUR: Comparative Absorption Ratios (exposed/control) of Different Hydrocarbons in 1 and 4 Days JP-8 Pre-exposed Porcine Skin

Hydrocarbons	Absorption (ng)	Absorption (ng)
	1-day pre-exposure	4-day pre-exposure
Naphthalene	1.73	1.65
Dimethyl naphthalene	1.35	1.74
Ethyl Benzene	1.98	4.07
Trimethyl Benzene	1.93	4.56
Cyclohexyl Benzene	3.02	1.81
O-xylene	1.88	4.21
Nonane	3.03	2.23
Undecane	2.25	1.95
Dodecane	1.65	3.50
Tridecane	0.74	4.90

In order to gain insight into the mechanism for this increased absorption through JP-8 pre-exposed skin, we performed studies with Fourier Transform Infra Red (FTIR) spectroscopy. We observed stratum corneum lipid extraction with most of the aromatic and short chain aliphatic hydrocarbons in this study. As mentioned above, very long chain aliphatic hydrocarbons were not detected in our perfusate samples. One possible reason was that these highly hydrophobic compounds (log Ko/w > 5) could not partition out of stratum corneum lipids and thus might not be absorbed through the skin. FTIR analysis revealed that these long chain

aliphatics could bind with the stratum corneum lipids. An important finding was the formation of dermal depots with certain hydrocarbons (naphthalene, dimethyl naphthalene and cyclohexyl benzene) as a result of repeated skin exposures to JP-8. These compounds could be detected in perfusate from JP-8 pre-exposed skin by topically dosing with only diluent, suggesting that prior exposure created a mobile depot. These results suggest that single dose application data for jet fuel marker components cannot be used to predict the toxic potential for repeated exposures.

Keratinocyte Cell Culture Studies:

In order to probe the mechanism of jet fuel toxicity independent of absorption parameters, we continued studies using human epidermal keratinocyte (HEK) cell cultures.

Toxicity of Individual Hydrocarbons: Based on the hydrocarbon disposition studies, we conducted a number of investigations on the inherent toxicity of aliphatic and aromatic hydrocarbons using HEK. (Chou, Riviere, Monteiro-Riviere, 2002, 2003). Lethality and cytokine release were employed as biomarkers of toxicity and irritation. These studies were designed to assess if all hydrocarbon components of fuel are equi-toxic relative to their ability to induce cytokine release (IL-8), and furthermore if there was a relationship between direct cytotoxicity and cytokine release. Theoretically, IL-8 release should correlate to *in vivo* dermal irritation since it is an early cytokine in the dermal inflammation cascade. Using the QSAR JP-8 cluster analysis provided by Dr. Basak, 10 aliphatic and 9 aromatic hydrocarbons were tested in human keratinocyte cultures. For the aliphatics (C6-C16), increased cytotoxicity was associated with decreased chain length (i.e. octane, nonane, cyclohexane). In contrast, maximum Il-8 release peaked at chain lengths of C-9 to C-13 (i.e. nonane, decane, undecane, dodecane, tridecane). Responses were significantly more complex with the aromatic hydrocarbons. Rank order of cytotoxicity was cyclohexylbenzene > trimethylbenzene $\geq$ xylene > dimethylnaphthalene > ethylbenzene > naphthalene > toluene > benzene. Methylnaphthalene, dimethylnaphthalene and naphthalene showed an increase in IL-8 release. In contrast, many of these aromatic compounds significantly decreased Il-8 release at non-cytotoxic (< 5% lethality) doses. This suggests that the *in vivo* irritation seen must be related to the irritating aromatic or aliphatic components and that this response overwhelms any protective effect conferred by inhibitory aromatics. The aliphatic data are illustrated for cytotoxicity (Figure Eight) and IL-8 release (Figure Nine) below.

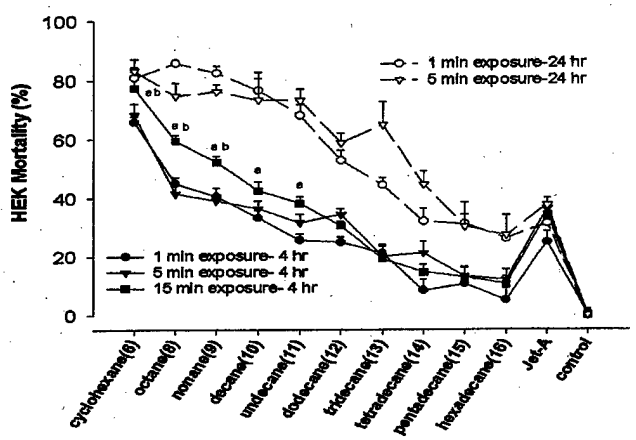


FIGURE EIGHT: Cytotoxicity of alkanes to human keratinocytes

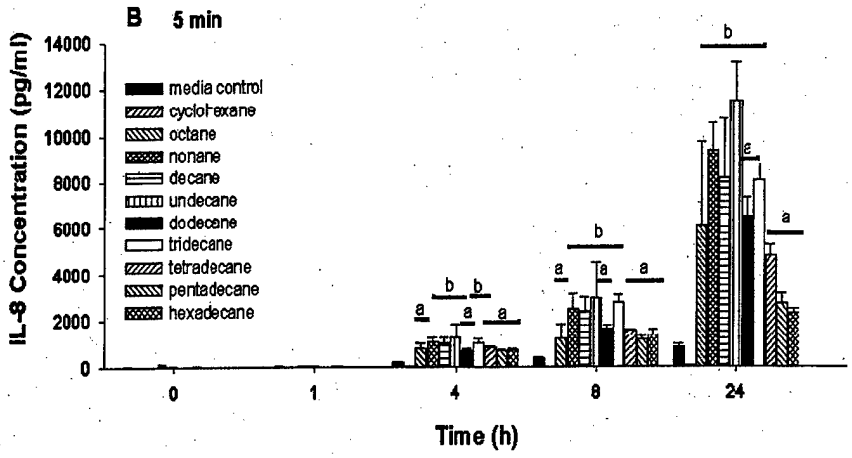


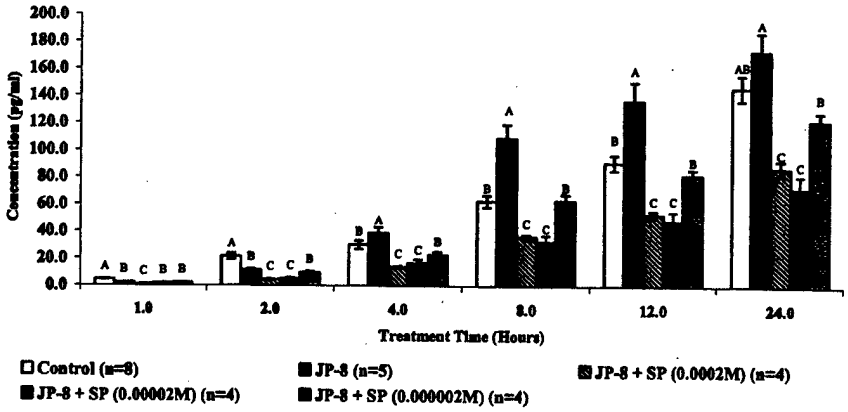
FIGURE NINE: IL-8 Release from human keratinocytes after alkane exposure.

Earlier research demonstrated that Jet-A, JP-8 and JP-8(100) were equitoxic to HEK in culture (cytotoxicity, IL-8, TNFα endpoints), suggesting that additives were not synergistically toxic to keratinocytes (*Allen et al., J. Biochem. Molec. Toxicol. 14: 231-237, 2000; AFOSR GF 49620-98-1-0105 final report*). However, different fuels applied to skin *in vivo* (*Monteiro-Riviere et al., J. Appl. Toxicol. 21: 485-494, 2001)*, showed graded toxic responses with JP-8 (100) tending to be most severe. JP-8 (100) also caused the most modulation of enzyme histochemistry activity *in vivo* (Rhyne, Pirone, Riviere, Monteiro-Riviere, 2002). These findings are consistent with our working hypothesis that additives are not inherently toxic to keratinocytes, but rather modulate

the dermal delivery of toxic hydrocarbons to keratinocytes by modulating their absorption across the dermal barrier. Both these data sets strongly suggest that cytotoxicity and the ability to cause Il-8 release are independent toxicological effects.

Effects of Substance P (SP) on Modulating JP-8 HEK Toxicity: Studies were also completed which probed the effects of SP on modulating HEK toxicity. SP has been previously implicated as being capable of blocking JP-8 and tetradecane pulmonary toxicity (*Robledo et al., Am. J. Physiol. 276: L229-L238, 1999*) via modulation of the neurokinin receptor NK_1. The same substance P used in the inhalational studies {Sar^9, $Met(O_2)^{11}$ substance P} was co-exposed with JP-8 to human epidermal keratinocytes in cell culture. Figure Ten below illustrates that three concentrations of SP statistically reduced the IL-8 release normally seen after JP-8 exposure in human epidermal keratinocytes. Additional experiments also showed no effect of SP on IL-8 when dosed in the media alone. These findings suggest that there may be a linkage between the mechanism of JP-8 toxicity (and or the hydrocarbons specifically responsible for IL-8 release) in the skin and lung. (Monteiro-Riviere, Inman, Riviere, 2004).

FIGURE TEN: Substance P ((Sar9, Met (O2)11) modulation of IL-8 release in HEK



In Vivo Toxicity:

An extensive *in vivo* jet fuel exposure study was the subject of the previous final report (*AFOSR GF 49620-98-1-0105)* as well as an earlier publication (*Monteiro-Riviere et al., J. Appl. Toxicol. 21: 485-494, 2001).* These studies clearly demonstrated significant dermal irritation (erythema, edema, epidermal thickening) after exposure to all three jet fuels. The studies

conducted under the auspices of the present grant extended these observations using electron microscopy (Monteiro-Riviere, Inman, Riviere, 2004). An additional study, for which only preliminary data is available, assessed exposure to individual hydrocarbon components of jet fuel to correlate *in vivo* response to the *in vitro* toxicity and absorption data reviewed above.

Ultrastructural Effects after Jet Fuel Exposure: The primary change seen after exposure to all fuels was low-level inflammation accompanied by formation of lipid droplets in various skin layers, mitochondrial and nucleolar changes, as well as disorganization in the stratum granulosum – stratum corneum interface. All three jet fuels, especially JP-8 (100), induced cleft formations or expansions within the intercellular lipid lamellar bilayers of the stratum corneum. The pertinent changes are illustrated in Figures Eleven and Twelve below.

These ultrastructural changes seen after topical exposure to jet fuels relate to specific morphological effects in the lipid bilayers of the skin that would be expected to affect the epidermal-dermal barrier. Similar changes in skin were previously noted after exposure to drugs such as lovastatin, a HMG CoA reductase inhibitor of cholesterol systhesis. The structural abnormalities seen in the lamellar body secretory system would greatly affect barrier function, and may be a primary event in jet fuel toxicity to the skin. Similar changes have been previously reported after exposure to kerosene, again supporting the hypothesis that constituent hydrocarbons are the primary toxic entity.

FIGURE ELEVEN. Transmission electron micrograph depicting intercellular edema (arrows), lipid droplets (*) at the basal pole of the stratum basale (SB), and stratum spinosum (SS) cells. (D), dermis. Jet A, 24 h; X 7,400.

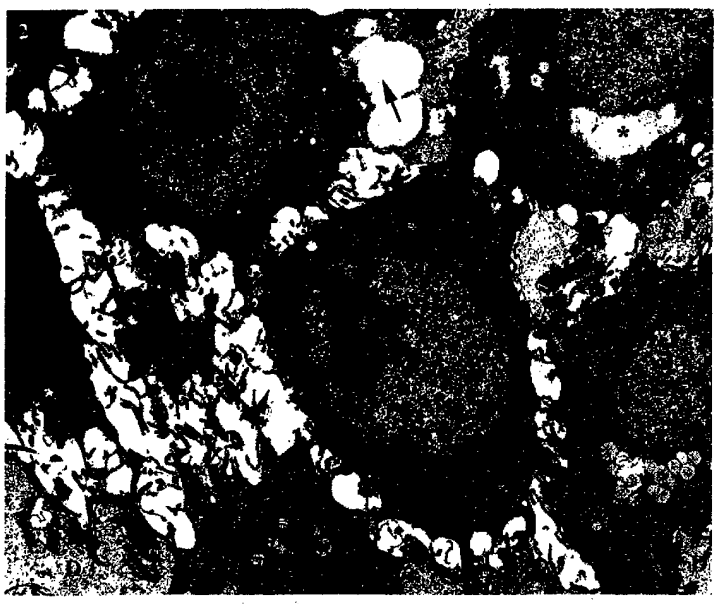


FIGURE TWELVE. Jet fuel treated skin depicting degradation of desmosomes and expanded intercellular space. The electron-dense desmosomes have separated from the central core (arrows) leaving a space within the desmosomes. Note expansion of the intercellular space (*) where the intercellular lipid lamellae appeared extracted. JP-8+100. X 117,000.

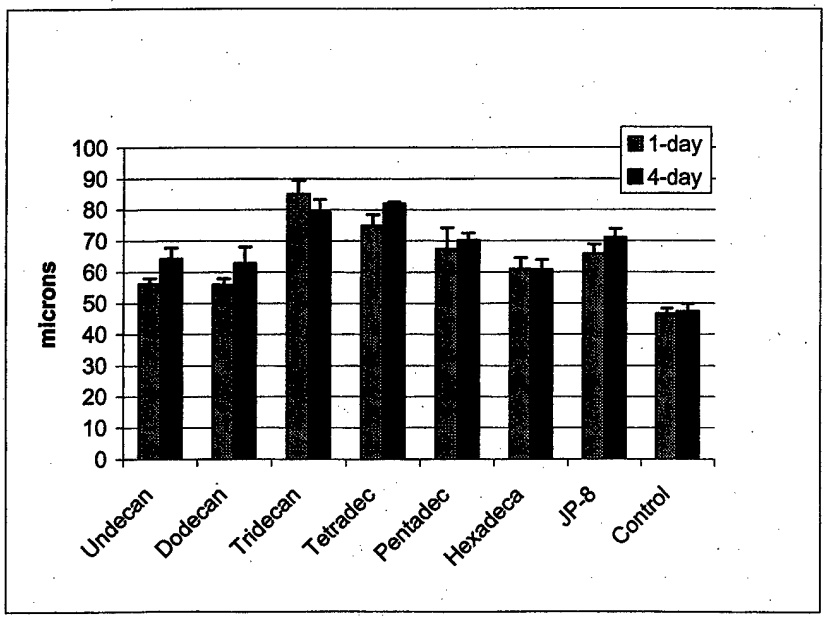
Differences between fuels relative to dermal effects are most probably secondary to additive modulation of hydrocarbon absorption and delivery to target keratinocytes. Stratum corneum changes would be expected to further increase jet fuel absorption in chronic occupational exposure scenarios. Barrier disruption alone has been associated with increased cytokine release. Changes in nucleolar structure suggest abnormalities in DNA signal transduction that might be related to other toxicological manifestations.

These observations confirm the toxicological effects of repeated jet fuel exposure to *in vivo* pig skin and are consistent with the nature of *in vitro* biomarkers employed in our studies. A large component of jet fuel toxicity to skin seems primarily related to hydrophobic hydrocarbons interacting through physical chemical mechanisms with the lipid constituents of the stratum corneum, which results in barrier malfunction and disease, coupled with direct toxicological interactions to keratinocytes resulting in either cytotoxicity or irritant release.

Individual Hydrocarbon Exposure *In Vivo* to Pigs. Parallel to the hydrocarbon component absorption studies discussed above, we also assessed the *in vivo* response to application of individual jet fuel hydrocarbons. As in our previous *in vivo* studies, these experiments were

conducted to mimic complete saturation of cotton clothing in workers (e.g. soaked fabric) and assess their effects after 1 day (n=4) and 4 day repeated exposures (n=4) with evaluation on day 5. The cotton fabric strips were fixed on the back of pigs and saturated with the same 8 aliphatic and 6 aromatic hydrocarbons whose absorption was individually assessed. Non-treated fabric and fabric soaked with JP-8 served as the controls. Gross erythema was observed with tridecane, tetradecane and pentadecane in 1-day exposures. Intense dark brown spots (crusts) were observed with these three components similar to that seen after 4-day JP-8 exposures. The aromatic hydrocarbons did not produce gross lesions in 1 and 4- days *in vivo* exposures. Microscopically, corneal vesicles/abscesses composed predominantly of neutrophils were observed with JP-8 as well as tridecane, tetradecane, and pentadecane 4-day exposure. Maximum epidermal thicknening was observed with these three hydrocarbons and was similar to that seen with JP-8 (Figure Thirteen). The epidermal thickness values for JP-8 versus control in the present study are in accordance to our previous *in vivo* exposures (*Monteiro-Riviere et al., J. Appl. Toxicol. 21: 485-494, 200)* supporting the reproducibility of our findings. The maximum irritation produced by tridecane, tetradecane and pentadecane is also in accordance to our *in vitro* HEK cell culture studies reviewed above, suggesting that these components (or similar congeners) may be the principal entities responsible for jet fuel irritation after topical exposure.

FIGURE THIRTEEN: Epidermal Thickness of Skin Exposed to Jet Fuel Hydrocarbons for One and Four Days.



Membrane Coated Fiber Model:

The research outlined above clearly indicates that 1.) physical chemical properties of hydrocarbon constituents are critical in determining transdermal flux and skin deposition after topical jet fuel exposure, and 2.) additive modulation of hydrocarbon disposition is an important factor in predicting toxicity after exposure to different fuel formulations. Jet fuel consists of hundreds of hydrocarbon components, only a few of which have been studied to any degree of detail, especially to the level reported above. *There are no reasonable theoretical nor experimental approaches to assess how intermolecular interactions between multiple fuel constituents (hydrocarbons and additives) would modify their subsequent absorption through skin.* In order to address this limitation, we developed a novel *in vitro* technique termed the Membrane Coated Fiber (MCF) that allows partitioning phenomenon, the rate-limiting step for Fickian diffusion through lipid membranes such as the stratum corneum, to be rapidly determined for individual compounds (Xia, Baynes, Monteiro-Riviere, Leidy, Shea, Riviere, 2003; Xia Baynes, Monteiro-Riviere, Riviere, 2004a, 2004b). This approach models chemical uptake from a stirred solution into a MCF that allows for direct injection into a GC/MS sampling port. It represents an efficient experimental system for determining solvatochromatic parameters important in governing free energy related phenomena such as partitioning, solubility and diffusion, parameters critical for estimating dermal absorption as well as many disposition processes quantitated in systemic physiological-based pharmacokinetic (PBPK) models.

There have been numerous and well documented approaches to quantitate the rate and extent of percutaneous absorption using (QSPR) for dermal chemical absorption. The classical analysis (*Potts and Guy, Pharm. Res. 9: 663-669, 1992*) quantified permeability in a QSPR equation:

$$\text{Log } K_p = 0.71 \text{ log } K_{o/w} - 0.0061 \text{ MW} - 6.3 \qquad (R^2 = 0.67)$$

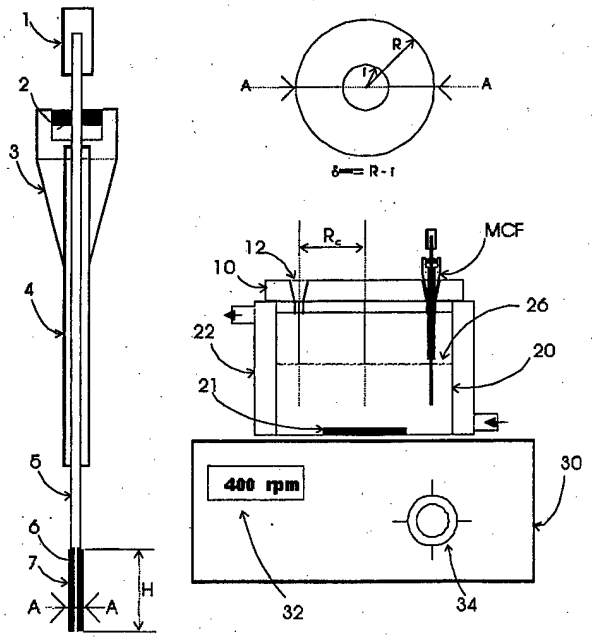
This early work has now become the basis of the EPA's estimate of dermal permeability. In one manifestation of MCF applications, log $K_{o/w}$ estimated as a MCF partition coefficient could classify jet fuel hydrocarbons based on this pivotal parameter. More importantly, these could also be determined in mixtures consisting of fuel +/- additives to assess how components modify this critical parameter. Abrahams and co-workers (*J. Pharm. Pharmacol. 47: 8-16, 1995; Pesticide Sci. 55: 78-88, 1999*) attempted to generalize these solvatochromatic interactions for permeability through any biological membrane, including skin, in the context of linear free energy (LFE) relationships. It is this generalization of Abraham that would allow MCF

parameters, which can be described using LFE parameters, to be used to predict dermal absorption as well as correlate to general PBPK parameters that are not measurable *in vivo* for a mixture as complex as JP-8. Categorizing individual hydrocarbon MCF partition coefficients in multiple membrane systems would allow for clustering or lumping hydrocarbons into groups that have similar solvatochromatic properties that are reflected in their MCF partition coefficients. This clustering based on MCF data should parallel similar clusters of hydrocarbon components based on blood/tissue partitioning coefficients relevant to a PBPK model.

An MCF experiment is conducted by adding test compounds to the stirred reservoir (donor solution) into which a MCF is immersed. The apparatus for holding coated-fibers is depicted in Figure Fourteen below. When absorption equilibrium is reached, the MCF is removed from the solution and directly transferred into a GC injection port to desorb the partitioned chemicals for qualitative and quantitative analyses. If the initial concentration of a given chemical is C_o, the equilibrium absorption amount measured with the MCF is n°, the volume of the donor solution is V_d, and that of the membrane is V_m, the equilibrium concentration, C_{de}, in the donor solution will be ($C_{de} = C_o - n°/V_d$), and in the membrane D_{me} will be ($C_{me} = n°/V_m$). Thus, the partition coefficient log $K_{m/s}$ between the membrane (m) and the donor solution (s) is:

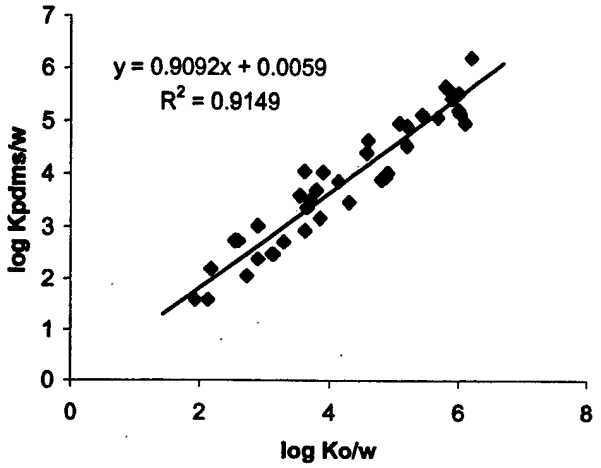
$$\log K_{m/s} = \frac{C_{me}}{C_{de}} = \frac{n^{\circ} V_d}{V_m (V_d C_o - n^{\circ})}$$

FIGURE FOURTEEN: MCF Fiber (left side) and Experimental Apparatus (right side)



Determination of Partition Coefficients: Partitioning into a silicone membrane (PDMS) coated fiber (log $K_{PDMS/W}$) is highly correlated (R^2 = 0.92) to published log $K_{o/w}$ for a series of 30 pesticides and 9 aromatic JP-8 hydrocarbons as seen in Figure Fifteen below.

FIGURE FIFTEEN: Correlation between log $K_{o/w}$ and MCF log $K_{PDMS/W}$

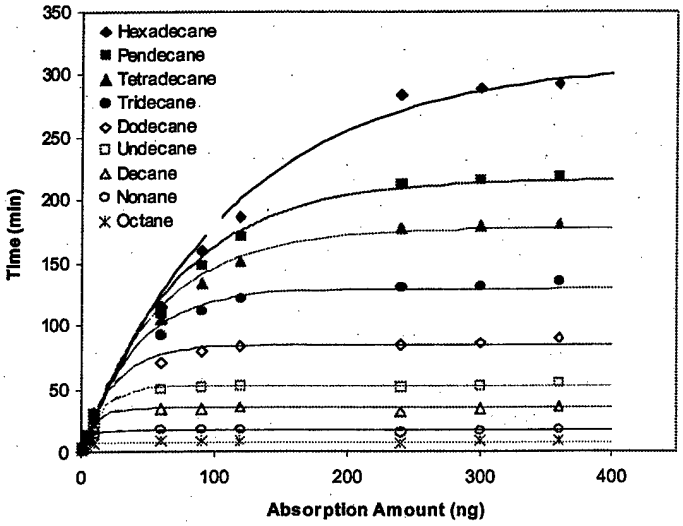


This experimental approach has now been modified to insure that sparingly water-soluble hydrophobic chemicals, such as long chain aliphatics found in jet fuels, can also be studied with this technique. It is a challenging task to study absorption kinetics of very hydrophobic compounds, such as aliphatic components in jet fuel, because of their very low solubility in water. Any dosing concentration may lead to formation of a layer of fuel-film on the surface of the aqueous solution, which may provided misleading results for absorption kinetics in membrane/aqueous systems. The solid-phase microextraction (SPME) method was previously reported as not being suitable for study of very hydrophobic compounds. With our MCF technique in the ethanol/water system, the absorption kinetics of very hydrophobic compounds can be studied. This technique is facilitated further by the use of a constantly replenished reservoir fed by a buffer containing a saturated solution of the aliphatic hydrocarbon being studied. In this manner, C_o is now constant and not affected by uptake into the MCF, a process that could deplete a minimally soluble hydrocarbon from a static exposure reservoir. The partition coefficient can now be calculated simply as log $K_{m/s} = n^o / (V_m C_o)$. This experimental approach for determining partition coefficients for sparingly water-soluble compounds such as

long chain aliphatic hydrocarbons is a technical advance over classic methods which often are plagued by problems such as emulsion formation and prolonged, multi-day experiments.

Figure Sixteen shows the absorption kinetics of the aliphatic components in jet fuel in 50% (v/v) ethanol/water system. The scattered points are experimental data and the solid lines are regression curves generated by the mathematical model of the MCF technique. The partition coefficients and kinetic parameters for each compound can easily be obtained. It is noted that the initial absorption rates of all of the aliphatic compounds are about the same, while the absorption amounts are significantly different. This phenomenon is very difficult to observe by other techniques. It may provide new evidence on how the jet fuel components are absorbed by a lipophilic membrane such as skin. This approach can be further extended to study absorption kinetics of jet fuel components from hydrocarbon solvents such as benzene, hexane, and naphthalene. These approaches would provide a direct mechanism of quantitating kinetic parameters for jet fuel components.

FIGURE SIXTEEN: Absorption Kinetics of Aliphatic Components in Jet Fuel by the MCF Technique in a 50% Ethanol/water System



Determination of Kinetic Parameters: A mathematical model of chemical uptake into the MCF from the vehicle was also developed (Xia et al., 2004b) which allows estimation of the rate of compound uptake [a] and its diffusivity [$\log(D_M)$] through the membrane. These parameters would provide more insight into how hydrocarbon components of JP-8 react with membrane barriers, and the time frame of this interaction. This toxicodynamic data is especially important

since for topically applied fuel, uptake into skin keratinocytes that may result in toxicity, competes with both evaporation from the dosed site and transdermal absorption into the systemic circulation. The balance determines the toxicological effect seen. Again, the unique aspect of the MCF system is that these data could also be collected in the presence of fuel additives to specifically determine their effects on partition coefficients that modulate membrane diffusion.

This system is an efficient and cost-effective method to experimentally determine partition coefficients for a large number of the hydrocarbon components of jet fuel. It would provide experimental data that could be used to validate theoretical QSAR models. Use of membrane materials other than silicone would reflect non-hydrophobic interactions such as hydrogen-bonding. These interactions, reflective of partitioning into different membrane types, would provide data to more fully characterize how individual hydrocarbons are physiochemically related to one another, how they might partition and diffuse though different biological tissue barriers, and how mixture components would modify these properties. These data would be invaluable in extending PBPK models to jet fuel hydrocarbons not specifically included in ongoing modeling efforts.

Studies on aromatic hydrocarbons illustrate the utility of this approach. Table Five depicts the four parameters calculated from an MCF experiment. Diffusivity as well as the rate parameter "a" did not parallel log $K_{m/w}$, suggesting that these kinetic parameters are more sensitive to the structural difference seen between the substituted aromatics.

TABLE FIVE: Parameters Obtained with the MCF Technique

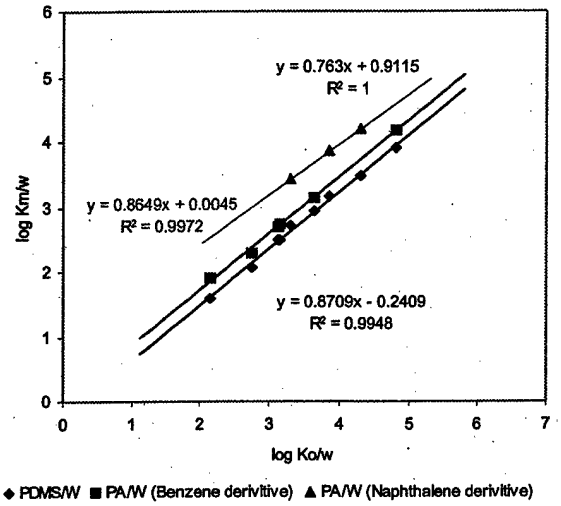
Compound	a (1/min)	n^o (ng)	$\log(K_{m/w})$	$\log(D_m)$ (cm^2/s)
Ethylbenzene	0.0999	27.7	3.09	-4.08
o-Xylene	0.1977	31.6	3.15	-3.79
Trimethylbenzene	0.1115	63.2	3.49	-4.07
Naphthalene	0.2025	75.2	3.58	-3.83
Methylnaphthalene	0.1230	137.6	3.93	-4.14
Cyclohexylbenzene	0.0208	222.1	4.31	-5.08
Dimethylnaphthalene	0.0558	256.8	4.47	-4.74

$K=(n^oV_d)/(V_m(V_dC_o-n^o))$, V_d=10ml, V_m=0.612ul, C_o=40ng/ml

$D_m=aV_d\delta_m/2A(K_{m/w}V_m+V_d)$, $k_p=K_{m/w}D_m/\delta_m$, δ_m=0.0100cm, A=0.0926cm^2.

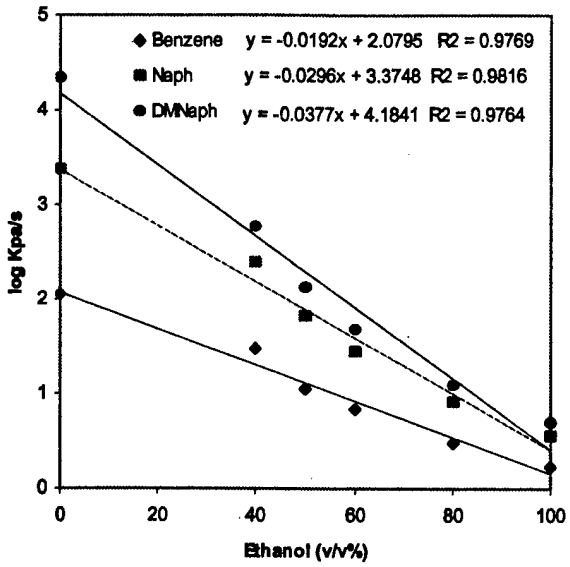
In addition to employing kinetic parameters to discriminate between different molecular properties of study hydrocarbons, polyacrylate (PA) was also used as a MCF membrane for studying nine JP-8 aromatic constituents. Figure Seventeen depicts the relation of log $K_{m/w}$ for PDMS and PA versus log $K_{o/w}$. In this case, naphthalene derivatives have different behavior in the PA system, while benzene derivatives parallel that seen with PDMS. These data confirm that the MCF technique is sensitive enough to detect intermolecular interaction differences between membrane-naphthalene derivatives and membrane-benzene derivatives.

FIGURE SEVENTEEN: Relationship between log $K_{o/w}$ and MCF log $K_{PDMS/W}$ or log $K_{PA/W}$ for naphthalene versus benzene fuel constituents



Solvent Effects: Jet fuels essentially are complex solvents. Solvent effects may play an important role in the absorption of its components. To assess the ability of MCFs to detect solvent effects on hydrocarbon partitioning, we conducted studies in an ethanol/water system to illustrate the full power of this technique to model solvent effects on MCF partitioning. Figure Eighteen depicts log $K_{PA/Ethanol}$ for benzene, naphthalene and dimethylnaphthalene in a graded series of water/alcohol mixtures. As expected, partition coefficients for these compounds were reduced with increasing ethanol ratio. However, the reduction in slope was different for different compounds, a finding that is not easily predictable. The unique advantage of the MCF approach was that the slopes representing this relationship could be precisely defined. An understanding of such solvent effects is critical to predict vehicle modulation compound partitioning in mixtures.

FIGURE EIGHTEEN. Relation of Log $K_{pdms/w}$ versus % Ethanol



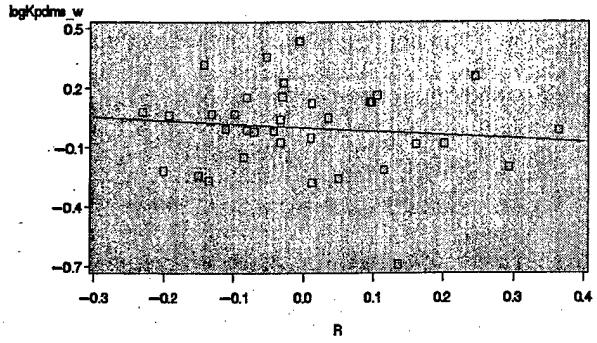
Molecular Descriptors: The MCF technique integrates the membrane absorption and quantitative analysis into one step, which offers high sensitivity, high throughput and improved accuracy. It can also be used to study intermolecular interactions by determination of the solute descriptors using a linear free energy relationship (LFER) approach. In this novel molecular descriptor array approach, the strength of a chemical's molecular interaction is described by five molecular descriptors representing the relative strength of five basic molecular forces: lone-pair electrons, dipolarity/dipolarizability, hydrogen bonding interactions and London dispersion. Partitioning into skin or a cellular membrane is also described by five corresponding descriptors, called system constants. Once multiple membrane/solvent system are calibrated with a set of reference compounds with know molecular descriptors, these data can be used to determine molecular descriptors for any compound of interest, including all of the jet fuel components. The molecular descriptors determined for the jet fuel components could be valuable parameters in the QSAR model development since the molecular interactions governs the behavior of the chemicals in the biological system. We have determined the system constants of the PDMS membrane with 36 reference compounds. The contributions of five types of intermolecular interaction forces to the membrane/water partition coefficients are shown in Figure Nineteen. The system constants of the PDMS/water are given in the following LFER equation:

$$\text{Log } K_{PDMS/Water} = -0.35 - 0.18R - 0.21\pi - 2.28\alpha - 3.81\beta + 3.34V$$

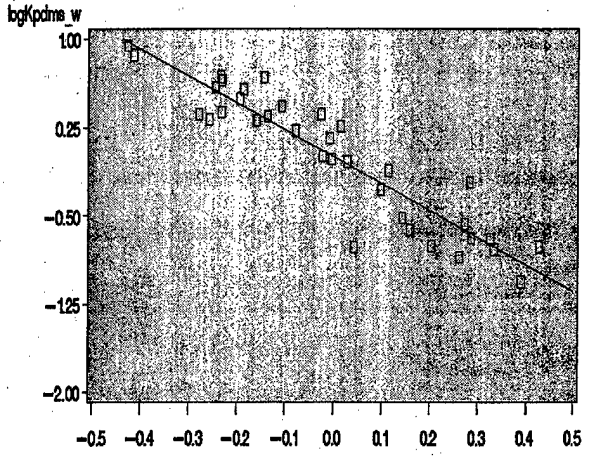
where R is an excess molar refraction representing the molecular force of lone-pair electrons, which can be experimentally determined or calculated from refractive index; π is the effective solute dipolarity and polarizability; α is the effective solute H-bond acidity, a summation of acidity from all H-bonds of the solute; β is the effective solute H-bond basicity, a summation of basicity from all H-bonds of the solute; and V is the McGowan characteristic volume that represents London dispersion.

FIGURE NINETEEN: Contribution of five molecular forces to log $K_{PDMS/WATER}$

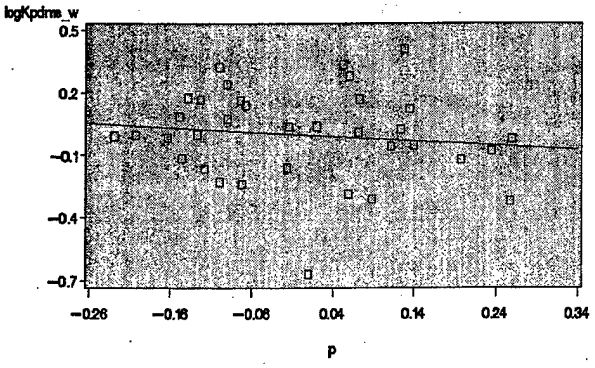
Lone-pair electrons



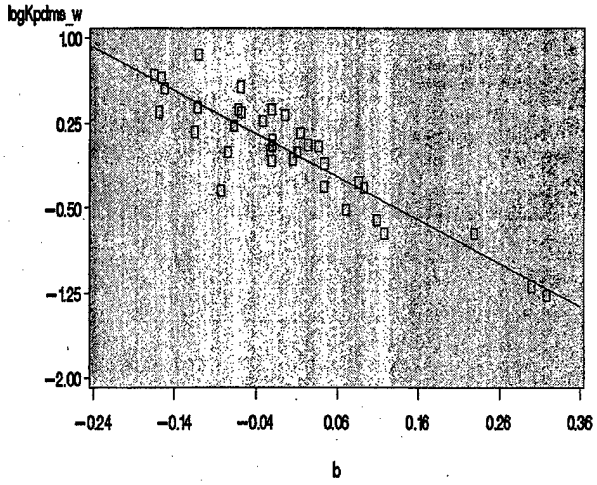
Hydrogen-bond acidity



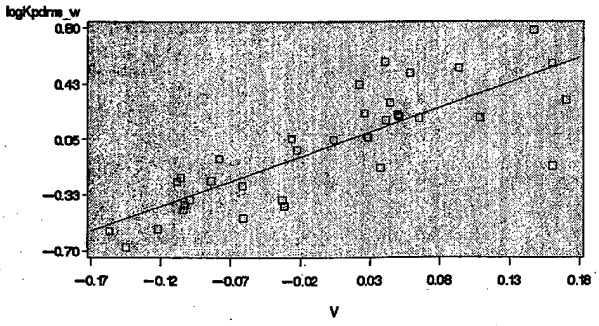
Dipolarity/diplorizability



Hydrogen-bond basicity



London dispersion



These data illustrate the potential for the MCF technique to generate a number of quantitative parameters that could differentiate jet fuel hydrocarbons in respect to partition behavior or diffusivity. The MCF technique can utilize multiple membranes and solvent systems (e.g. different jet fuels) to analyze a large subset of aliphatic and aromatic hydrocarbons to generate MCF parameters (log K, log D_M, a, LEF coefficients) for detailed QSAR analysis, as well as to provide clustering endpoints for PBPK models. The system provides a unique experimental approach for assessing the behavior of all jet fuel hydrocarbons under defined conditions.

Discussion:

The studies completed under the auspices of the present proposal have increased our understanding of a number of aspects of jet fuel chemistry, dermal absorption and toxicity. Our working hypothesis that fuel performance additives modulate the absorption, and thus toxicity, of constituent hydrocarbons has been supported. Based on these studies, it appears that mid-chain length aliphatic hydrocarbons or aromatics such as naphthalene may be responsible for the toxicological pattern observed. The data suggesting greater severity with JP-8 (100) is best explained by its additives modulating delivery of toxic hydrocarbons. The cell culture studies clearly showed that irritation and cytotoxicity of hydrocarbons match exposure to complete fuels, as well as that the relationship between a compounds potential to induce irritation is not the same as its potential to cause cytotoxicity. Cytotoxicity was worse with short chain aliphatics and increased with chain length. Irritation peaked with mid-chain hydrocarbons (nonane through tridecane), a finding consistent with the *in vivo* data. Irritation and cytotoxicity was not consistent across the aromatic hydrocarbons studied (naphthalenes increased Il-8 release, other inhibited). Absorption studies suggest that longer chain aliphatics show saturated absorption kinetics across skin with increasing dose. This behavior would minimize dose related systemic exposure of aliphatics compared to aromatics such as naphthalene, but could potentiate dermal reservoir formation and thus local toxicity. Repeated exposure studies confirmed the existence of dermal depots. Previous IPPSF studies had shown the tendency of aliphatics such as dodecane to persist in dermal tissue. The mechanism of jet fuel toxicity thus seems to be hydrocarbon mediated irritation or outward cytotoxicity of keratinocytes, coupled *in vivo* with direct alteration of the lipoidal stratum corneum barrier as evidenced by transmission electron microscopy. The molecular pathogenesis is complex, but may share common pathways with other tissues as evidenced by the inhibitory effects of Substance P. The MCF model developed in this work

appears to be a powerful technique to probe the physical chemical properties of individual hydrocarbons and their partitioning behavior in complex mixture or solvent systems.

Transition / Technology Transfers:

- The MCF fiber described above has been used as the basis of a number of new grant applications to NIH and EPA.
- U.S. Patent Application # 60/361,926, filed 3/5/2002. Method and Apparatus for Determining a Molecular Descriptor of Absorption for a Candidate Compound. (J. Riviere, X Xia, R. Baynes, N. Monteiro-Riviere); NCSU File 02-82.

Honors / Awards / Highlights:

- Dr. Riviere was elected to the National Academies Institute of Medicine in October 2003 partially based on his AFOSR-supported jet fuel research.
- The National Research Council Committee on Toxicology's Subcommittee on Jet-Propulsion Fuel 8 published a report on Toxicological Assessment of Jet-Propulsion Fuel 8 which quoted our JP-8 dermal research.

Publications Supported By This Grant Which Formed The Basis Of This Review.

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2. Chou CC, Riviere JE, Monteiro-Riviere NA. Differential relationship between the carbon chain-length of jet fuel aliphatic hydrocarbons and their ability to induce cytotoxicity versus interleukin-8 release in human epidermal keratinocytes. Toxicol. Sci. 69: 226-233, 2002.

3. Xia XR, Baynes RE, Monteiro-Riviere NA, Leidy RB, Shea D, Riviere JE. A novel in vitro technique for studying percutaneous permeation with a membrane coated fiber and gas chromatography / mass spectrometry. I. Performance of the technique and determination of the permeation rates and partition coefficients of chemical mixtures . Pharm Res. 20: 275-282, 2003.

4. Chou CC, Riviere JE, Monteiro-Riviere NA: The cytotoxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. Arch. Toxicol. 77: 384-391, 2003.

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6. Monteiro-Riviere NA, Inman AO, Riviere JE. Skin toxicity of jet fuels: Ultrastructural studies and the effects of substance P. Toxicol. Appl. Pharmacol. 2004 (In Press).

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8. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE. Characterization of polyacrylate membrane coated fibers used in chemical absorption studies with programmed thermal treatment and FTIR microscopy. Anal. Chem. 2004a (Accepted Pending Revision).

9. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE. Permeation kinetics of a membrane-coated fiber (MCF) technique for studying membrane permeation with MCF and gas chromatography / mass spectrometry. J. Pharm. Sci. 2004b (Accepted pending revision).

10. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE. Uptake kinetics of jet fuel aromatic hydrocarbons from aqueous solutions studied by a membrane coated fiber technique. (Submitted).

11. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE. Determination of the partition coefficient and absorption kinetic parameters of chemicals in a lipophiic membrane/water system by using a membrane coated fiber technique. (Submitted).

Published Abstracts Based on This Grant

1. Riviere JE, Baynes RE, Monetiro-Riviere NA, Allen DG, Budsaba K, Smith CE: Dermal absorption and toxicity of topically applied jet fuels: In vitro and in vivo studies. AFOSR JP-8 Jet Fuel Toxicology Workshop. Tucson, AZ, January, 2001 (www.JP8.org).

2. Basak SC, Gute BD, Grumald GD, Riviere JE. On clustering of JP-8 chemicals. Joint Meeting Soc. Environ. Toxicol. Chem, and Soc. Toxicology, Duluth, MN, April, 2002.

3. Riviere JE, Brooks JD, Baynes RE, Monteiro-Riviere NA: Effects of sulfur mustard and JP-8 jet fuel on percutaneous absorption of simultaneously administered topical permethrin and DEET. Toxicological Sci. 66 (1S): 162-163, 2002.

4. Chou CC, Monteiro-Riviere NA, Baynes RE, Riviere JE. Assessment of cytotoxicity to human epidermal keratinocytes after exposure to aliphatic and aromatic hydrocarbons. Toxicological Sci. 66(1S): 163, 2002.

5. Xia XR, Baynes RE, Monteiro-Riviere NA, Leidy RB, Riviere JE: A novel technique to study percutaneous absorption by using a silastic membrane coated fiber. Toxicological Sci. 66(1S): 163, 2002 (*Winner of SOT In Vitro Specialty Section poster award*).

6. Riviere JE, Monetiro-Riviere NA, Chou CC: Dermal absorption and toxicity of jet fuel and its components. AFOSR JP-8 Jet Fuel Toxicology Workshop. Tucson, AZ, May, 2002.

7. Monteiro-Riviere NA, Chou CC, Riviere JE. The cytotooxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. Toxicological Sci. 72 (S1): 383, 2003.

8. Xia XR, Baynes RE, Monteiro-Riviere NA, Riviere JE: A mathematical model of permeation kinetics of the membrane-coated fiber technique accounting for partition, diffusion and boundary layer factors. Toxicological Sci. 72 (S1): 379, 2003.

9. Muhammad F, Baynes RE, Monteiro-Riviere NA, Xia XR, Riviere JE: Absorption through porcine skin exposed to various doses of jet fuel marker components determined with GC-FID using Head Space SPME fiber. Toxicological Sci. 72 (S1): 383, 2003 (*Winner of SOT Dermatotoxicology Specialty Section poster awa*rd).

10. Riviere JE, Monteiro-Riviere NA, Xia XR, Muhammad F. JP-8 Interactions with the Skin: Absorption of Hydrocarbon Components, A Novel Membrane Coated Fiber (MCF) Technique for Fuel Component Clustering / QSAR Analysis, and the Effects of Inhibitors (Including Substance P) on Dermal Toxicity. AFOSR JP-8 Jet Fuel Toxicology Workshop. Tucson, AZ, May, 2003.

11. Basak SC, Gute BD, Xia XR, Riviere JE. Predicting percutaneous permeation of chemicals using HiQSAR. AFOSR JP-8 Jet Fuel Toxicology Workshop. Tucson, AZ, May, 2003.

12. Monteiro-Riviere NA, Chou CC, Riviere JE: Dose-related interleukin-8 release from epidermal keratinocytes after exposure to jet fuel aromatic hydrocarbons. J. Vet. Pharmacol. Therap. 26 (S1): 162, 2003.