Environmental Health Perspectives
Vol., 38, pp. 149-155, 1981

Relative Potency Estimation for Synthetic Petroleum Skin Carcinogens

by J. M. Holland,* D. A. Wolf,† and B. R. Clark‡

A procedure for quantitative analysis of skin carcinogenesis data, for the purpose of establishing carcinogenic potency, has been applied to observations obtained from C3H mice exposed continuously to synthetic and natural petroleums. The importance of total polynuclear aromatic (PNA) content to the skin carcinogenic activity of the crude materials was also examined.

Of three synthetic petroleums evaluated, all were shown capable of inducing skin neoplasms within a two-year exposure period. Under comparable exposure conditions a composite sample of five natural petroleums was noncarcinogenic. Comparison of the distributions of times to initial skin neoplasm versus dose rate, for groups exposed to synthetic fossil liquids and the reference skin carcinogen, benzo(a)pyrene, provided estimates of relative carcinotenic potency for the synthetic petroleums ranging from 1/500 to 1/1400 the potency of benzo(a)pyrene. The carcinogenic activity of a chemically isolated PNA fraction versus the crude from which it was derived suggested that this fraction was responsible for the carcinogenic activity of these synthetic petroleums in mouse skin.

Introduction

The number of chemical and physical agents that are capable of evoking skin neoplasms is, for all practical purposes, limitless. Since many of these known or potential oncogens are valuable commodity chemicals, it is essential that methods be developed to rank them in order of carcinogenic potency. While we are in no position to judge the level of potency at which risk to human beings becomes unacceptable, we feel that an accurate means for estimating relative potency is an essential first step in this process.

This paper illustrates the approach we are using to obtain relative potency estimates for various synthetic and natural petroleum products applied to the skin. Groups of genetically homogeneous inbred mice are exposed repetitively to serial dilutions of the test material. In the simplest case, observation establishes time to appearance of a clinically typical epidermal neoplasm or to death of the animal before it develops a tumor. A Weibull distribution is fit to the time to tumor appearance for each material-dose combination. Tumorigenic efficiency of a combination is then measured by the logarithm of the estimated Weibull scale parameter. The Weibull model is preferred over other alternatives because it has been shown to fit a wide variety of time to tumor data (*1-4*).

To relate skin tumorigenic potencies for different materials and mixtures tested at the same or different times, we include a reference carcinogen standard with each series of unknowns; this choice is arbitrary. For our purposes we have selected benzo(a)pyrene (BP), which is commercially available in high purity, chemically stable, and a highly efficient mouse skin carcinogen.

For estimation of relative potencies, the Weibull scale parameters are assumed to be a linear function of dose for each material on a log-log scale; dose responses for materials in the same strain are also assumed to be parallel. These assumptions appear to be reasonable in this and in previous experiments (*2, 5*). The potency of an unknown material

*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.
†Computer Sciences Division, Union Carbide Corporation, Oak Ridge, Tennessee 37830.
‡Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

Table 1. Materials tested for relative skin carcinogenicity.

Material	Treatment group	Weekly dose,[a] mg
Synthoil crude	A	3.0,0.6,0.12,0.9
Shale oil crude	B	7.5,1.5,0.3,0.9
COED syncrude	C	2.5,0.5,0.1,0.9
Natural petroleum blend	D	6.0,1.2,0.24,0.9
Synthoil PNA isolate	AN	0.15,0.03,0.006
Shale oil PNA isolate	BN	0.15,0.03,0.006
COED PNA fraction	CN	0.15,0.03,0.006
Petroleum PNA fraction	DN	0.15,0.03,0.006
Benzo(a)pyrene	BP	0.15,0.03,0.006
Acetone–cyclohexane solvent control	S	0.15

[a] Applied in three increments on Monday, Wednesday, and Friday.

relative to the reference carcinogen is estimated by the ratio of dosages required to elicit comparable oncogenic effects.

Materials and Methods

Animal Exposure

Groups consisting of 25 male and 25 female C3Hf/Bd mice were exposed to graded dosages of synthetic petroleums, their respective polynuclear aromatic (PNA) fractions, reference standard BP, and a vehicle control consisting of 70% acetone and 30% cyclohexane by volume. Exposures were to the shaved dorsal skin in a volume of 50 μl commencing at 10 weeks of age and continuing for 24 months or until death. The materials used and absolute amounts applied are given in Table 1. To simplify presentation of the data a letter code for each treatment group is also given in Table 1. Details concerning

Table 2. Characteristics of materials tested for skin carcinogenicity.

Material	Type	Specific gravity, g/cm^3	Viscosity, sec at 100°F	Pour point, °F	Color	Sulfur, wt-%	Nitrogen, wt-%
Synthoil crude[a]	Centrifuged crude product	1.136	80(180°F)	–	Brownish-black	0.52	1.30
Shale oil crude[b]	Centrifuged crude product	0.909	66	30	Brownish-black	0.93	1.14
COED syncrude[c]	Hydrotreated product oil	0.940	48	43	Pale yellow	0.05	0.05
Natural petroleum sample							
Wilmington, California[d] (10%)	Natural	0.938	470	< 5	Brownish-black	1.59	0.631
South Swan Hills, Alberta, Canada[d] (20%)	Natural	0.826	37	< 5	Brownish-green	0.11	0.056
Prudhoe Bay, Alaska[d] (20%)	Natural	0.893	84	15	Brownish-black	0.82	0.230
Gach Saran, Iran[d] (20%)	Natural	0.880	72	< 5	Brownish-black	1.57	0.226
Louisiana-Mississippi sweet[e] (10%)	Natural	0.825	50	< 5	Brownish-green	0.17	0.067
Arabian light[e] (20%)	Natural	0.858	46	<	Brownish-black	1.80	0.1–0.2

[a] Pittsburgh Energy Technology Center. From Run FB46 of western Virginia coal, Pittsburgh seam, Ireland mine; 1/2 ton/day, 24.5-ft catalytic reactor (450°C, 4000 psi), feed rate 25 lb/hr of 35% solid slurry in equilibrium coal-derived oil. Data provided by Nestor Mazzoco, Pittsburgh Energy Technology Center.

[b] Laramine Energy Technology Center. Run No. 14, Colorado shale, Rifle, Colorado; Fisher assay 24.4 gal/ton from a 150-ton above-ground simulated *in situ* retort. Data provided by John McKay, Laramine Energy Technology Center.

[c] FMC Corporation, Princeton, New Jersey, from western Kentucky coal. Analysis is not based upon this specific sample but is valid for generically similar material; therefore, the data are presented for comparison purposes only. Data provided by Dr. C. A. Hochwalt, Jr., Cogas Development Co., Princeton, New Jersey.

[d] Bureau of Mines routine crude oil analysis. Data provided by J. Dooley, Bartelsville Energy Technology Center, Bartlesville, Oklahoma. Numbers in parentheses refer to the volume percent of each component in the composite sample.

[e] Analysis is not based on these specific samples, therefore the data are approximate and given for comparison purposes only; data provided by J. Dooley. Numbers in parentheses as in footnote d.

the source and history of the crude materials are given in Table 2. The natural petroleum sample consisted of six separate components, present in the percentage listed in Table 2.

Mice were treated three times weekly, and at each exposure were examined for evidence of skin neoplasia arising in the area directly exposed to the test material. When grossly typical skin neoplasms were observed, the exact date of initial observation was recorded. Only animals in which the initial tumor grew progressively were included in the analysis. When an animal died or was killed at the end of an experiment, the skin tumors were excised and the animal was subjected to gross necropsy for detection of internal lesions and evidence of regional metastasis of skin neoplasms.

The observations, consisting of an animal identification number, birth date, date of initial treatment, and observation of initial skin neoplasm (if any), and death or sacrifice date, were coded and verified. Two computer programs provided by the National Cancer Institute were used to estimate the statistical distribution of time to tumor observation for each material-dose combination (*6*). One program fits the Weibull distribution (D. G. Thomas, personal communication), and the other obtains the Kaplan-Meier nonparametric estimate of the distribution. Each program utilizes the information on animals that die or are killed before a treatment-related tumor is observed.

Dosage Selection

These experiments were designed to evaluate the relative contributions of PNA components of a fossil liquid as determining factors in skin tumor induction. If additivity of the effect of active components is assumed, it should be possible to correlate skin carcinogenicity with the amount and composition of the PNA fraction.

To accomplish this we determined the weight percent of the crude contributed by the PNA fraction. Details concerning the methods used and results for the remaining chemical class fractions of these materials can be found elsewhere (*7-9*). Gravimetrically determined percent PNA materials were 5.1, 2.0, 6.0, and 2.6% for materials A, B, C, and D, respectively. Under the assumption that the PNA fraction of each crude was equivalent in potency to BP, we set the weekly exposure for each whole crude at a level equivalent to 0.15, 0.03, and 0.006 mg of BP. Since each crude differed in the amount of material recovered as the PNA fraction, the doses differed accordingly. To compare the oncogenic potency of the whole material with that of its respective PNA isolate and to compare isolates with each other, we treated separate groups with equal quantities of each isolate.

Results and Discussion

Skin Tumor Dose Responses

The skin tumor data were analyzed separately for each sex; however, significant ($p < 0.05$) differences were not observed, thus estimations and comparisons were made after the data were pooled. Skin tumors were induced in groups exposed to A, B, C, AN, BN, and BP (Table 3). At the very low rates used in this study, skin tumors were not induced in mice exposed to natural petroleum or its PNA fraction. Neither were skin tumors observed in mice exposed to the PNA isolate derived from the COED process hydrotreated coal liquid. On this basis we conclude that the PNA compositions of these materials differ considerably, a conclusion amply illustrated by chemical analysis (*7-9*). Mean time to tumor is also given in Table 3, but very little can be concluded from this summary, primarily because so few responses were obtained in many groups. Pulmonary metastasis of syncrude induced squamous carcinomas was confirmed histologically in animals exposed to synthoil, shale oil and benzo(a)pyrene. The frequency of metastasis paralled the frequency of skin tumor induction. The true metastatic potential of the skin tumors induced at the two highest B(a)P concentrations could not be determined because these groups were killed early in the study, after nearly 100% of the animals had developed a tumor.

Mortality

Over the 24-month duration of the experiment, differences in mortality rate between treated and vehicle control mice were also noted in mice exposed to whole crudes. A chi-square and exact p value (Cox's test) were used for the comparison of treated with vehicle control mice. The results are given in Table 4. Mortality significantly greater than in controls ($p < 0.05$) occurred in groups A and B at the highest dosage and in group C at next to the lowest dosage. Mortality in BP-treated mice is reported at the lowest dosage only, since the two higher dosage groups were killed at 250 and 360 days of exposure, respectively. With the exception of the 0.5-mg dose of C, mortality significantly greater than in controls occurred in groups with a high incidence of skin tumor. The most likely interpretation of this observation is that skin tumors were lethal, as a result of either metastasis,

Table 3. Summary of the skin tumor response obtained in mice exposed repetitively to synthetic fossil liquids.

Material	Group	Weekly dose, mg	No. mice skin tumors	No. mice with pulmonary metastases	Mean time to tumor, days (± SE)
Synthoil crude	A	3.0	46	9	490 (12.7)
	D	0.9	13	2	569 (17.8)
	B	0.6	1	0	596
Shale oil crude	A	7.5	45	7	482 (14.8)
	B	1.5	1	0	315
COED crude	A	2.4	4	0	549 (45.8)
	D	0.9	1	0	494
	B	0.5	1	0	658
Synthoil PNA-isolate	A	0.15	4	0	573 (38.9)
Shale oil PNA isolate	A	0.15	2	0	541 (55.5)
	B	0.03	1	0	485
	C	0.006	1	0	392
Benzo(a)pyrene	A	0.15	48	n.d.	146 (2.6)
	B	0.03	49	2	216 (5.2)
	C	0.006	43	7	513 (14.3)
Solvent control		0.15	0	-	-

toxemia, or both. Other than mortality associated with the presence of a skin tumor there was no evidence of systemic toxicity as reflected by dose dependent differences in mortality rate in treated versus vehicle control groups. Microscopic effects were restricted to the skin and consisted of hyperkeratosis, epiliation and occasional ulceration due to the non-specific irritant properties of the various compounds.

Estimation of Time-to-Tumor Distributions

The time-to-tumor distributions provide a clear picture of the tumorigenic effects of the various treatments by illustrating the patterns of tumor occurrences. Furthermore, if a particular parametric form for the distribution is assumed, relative potency estimates can be calculated. The distribution of the time to tumor is estimated parametrically and nonparametrically by methods that fully utilize the information in both the uncensored and censored observations ("censored observations" represent mice that die from natural or accidental causes or are killed at the end of the study before a treatment-related skin tumor is induced).

The nonparametric estimates are calculated by the Kaplan-Meier method (*10*). With unique observation times and no censoring, the Kaplan-Meier estimate is a step function starting at 1 at the first exposure time and decreasing at each of the ordered times to tumor by increments of the recipro-

Table 4. Differences in survival rate between mice treated continuously with either fossil liquids or vehicle alone

Material	Weekly dose, mg	No. of deaths	Chi square	Exact p (Cox's test)
Synthoild crude	3.0	38	24.6	< 0.01
	0.9	19	0.8	0.36
	0.6	18	0.8	0.37
	0.12	13	0.04	0.85
Shale oil crude	7.5	32	13.1	<0.01
	1.5	20	1.2	0.26
	0.9	20	1.7	0.20
	0.3	11	0.08	0.77
COED syncrude	2.5	22	2.4	0.12
	0.9	18	1.0	0.32
	0.5	23	3.8	0.05
	0.1	22	3.0	0.08
Petroleum blend	6.0	10	0.2	0.68
	1.2	14	0.02	0.89
	0.9	11	0.4	0.50
	0.24	15	0.02	0.89
Benzo(a)pyrene	0.006	28	9.1	<0.01
Solvent control	0.15	14		

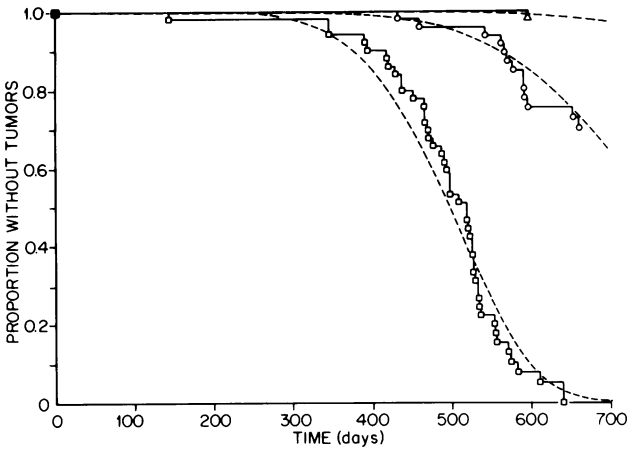

FIGURE 1. Proportions of mice without tumor associated with continuous exposure to synthoil crude at various dosages: (□) 3.0 mg/week, (O) 0.9 mg/week, (Δ) 0.6 mg/week; (—) Kaplan-Meier estimates; (- -) (the Weibull fitted curve.

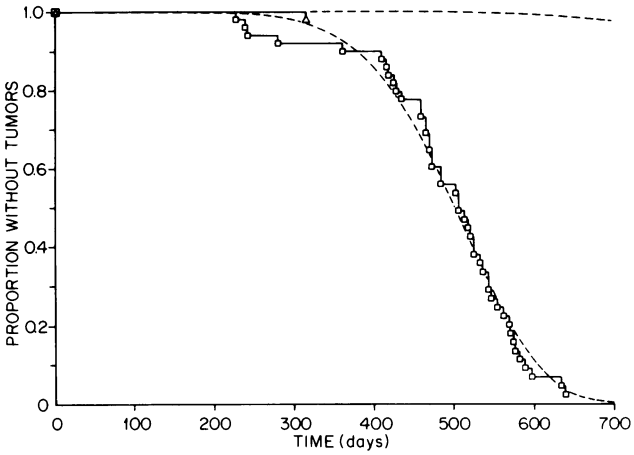

FIGURE 2. Proportions of mice without tumor associated with continuous exposure to shale oil at approximately (□) 7.5 mg/week and (Δ) 1.5 mg/week; (——) Kaplan-Meier; (- -) fitted Weibull.

cal of the number of animals in the group. When the observation times are not unique and there is censoring, the step size is modified appropriately, so that steps occur only when new skin-tumor-bearing animals are observed.

The Kaplan-Meier estimates were used to visually compare dose groups for each material without assumption of a distribution for the data. These curves are represented in Figures 1-4 by solid lines. To assess the explicit dose-response relationship and, subsequently, to compare these estimates for purposes of establishing relative potency, we have assumed a parametric form for the distribution. The model we have chosen is the three-parameter Weibull model, under which the distribution function is $1 - \exp[-b(t - w)^k]$, where $t > w$, $b > 0$, $k > 0$. The parameters k, w, and b are the shape, location, and scale parameters, respectively, and t is the time (days) to initial observation of a skin tumor. The survivor function gives the probability that the time to tumor exceeds t; w being a minimum latency period before which no tumor can occur. Pike (*1*) suggested that for a particular pure strain of animal the k and w would be independent of the treatment. Maximum likelihood estimates of a common k and a common w for all groups in which two or more skin tumors were observed gave values of 5.97 for k and 43.4 for w. On using these values for k and w, it was

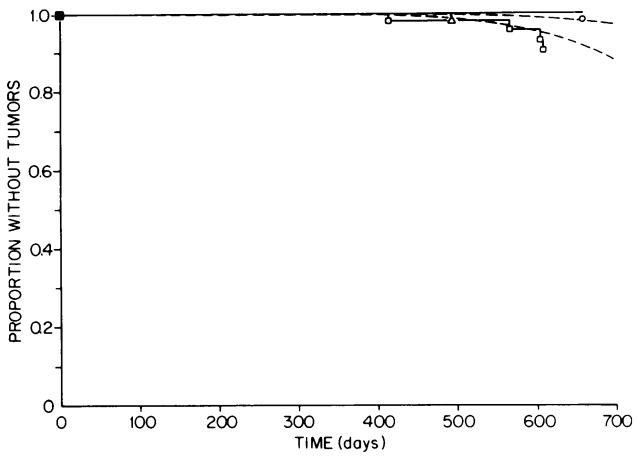

FIGURE 3. Proportions of mice without tumor associated with continuous exposure to COED process coal liquid at approximately (□) 2.4 mg/week and (O) 0.9 mg/week; (—) Kaplan-Meier; (- -) fitted Weibull.

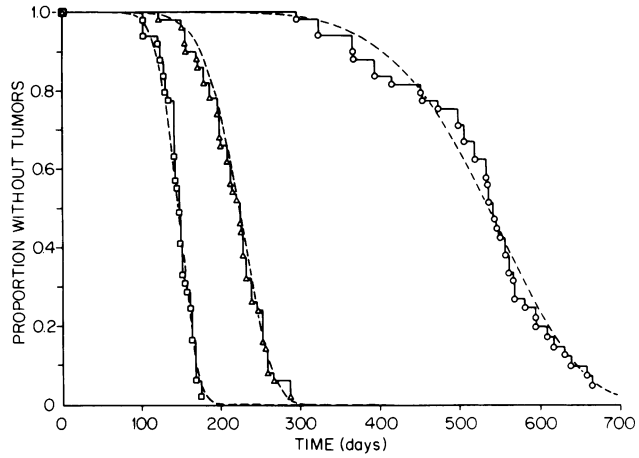

FIGURE 4. Proportions of mice without tumor associated with continuous exposure to BP at (□) 0.15 mg/week, (Δ) 0.03 mg/week, (O) 0.006 mg/week; (—) Kaplan-Meier; (- -) fitted Weibull.

possible to estimate the parameter b for each material-dose combination. The estimated Weibull time distributions for skin tumors are also shown in Figures 1-4 by dashed lines superimposed over the corresponding Kaplan-Meier nonparametric estimates. The observed agreement between these two methods is remarkable given the wide variation in tumor frequencies and temporal distributions across the different treatment groups.

Estimation of Relative Potency

The calculated scale parameter b, derived from the Weibull fit to each material-dose combination, is used to obtain estimates of relative carcinogenic potency for materials tested in a common inbred mouse strain under reasonably stable and reproducible environmental conditions. A weighted linear regression of the logarithm of b on the logarithm of dose in milligrams per week is performed for each material for which responses were obtained in two or more dose groups. Weighting for each data point was the reciprocal of the number of tumors for that material-dose combination. The additional constraint that the regression lines be parallel for all test materials and the reference carcinogen, an assumption that appears to be reasonable when compounds are compared in the same strain, makes it possible to obtain estimates of relative potency, even when responses are limited to occurrences in one group or are low in frequency. Only data sets for materials with responses in two or more dose groups contribute to the common slope of the regression lines.

The dose-response lines for these materials and their respective tumor-inducing PNA fractions are shown graphically in Figure 5. Open circles correspond to the logarithms of the estimated b values, and squares are the corresponding values on the regression lines. The potency of a syncrude relative to BP is the antilog of the absolute difference between the synthetic petroleum's log b intercept and the BP's log b intercept divided by the common slope, these values coming from the fitted regression lines shown in Figure 5. Approximate 95% confidence limits on the relative potencies were calculated by use of Fieller's theorem (*11*). For comparisons in which the unknown material is unlikely to be as potent as BP, only upper 95% confidence limits are provided. However, when there is no justification for this both the upper and lower limits are provided. By these procedures the carcinogenic potencies of the three syncrudes and their respective PNA isolates were determined relative to BP. The results are summarized in Table 5.

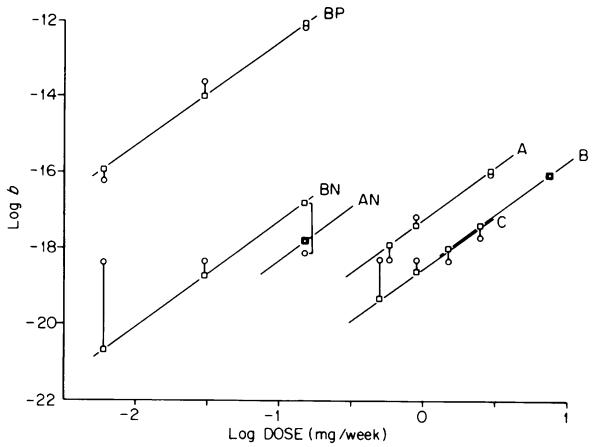

FIGURE 5. The relationship between log b, a scale parameter obtained from the Weibull fits with a constant k and w for all comparisons, and the log dose rate: (O) log b from Weibull; (□) weighted, linear-parallel, least-squares fitted values at each data point. Parallel lines drawn through the latter points are the basis for the calculation of relative potency. Treatment groups are identified by the letter codes in Table 1.

In the context of the original objectives of this experiment, these observations permit several conclusions. (1) Diluting the crude materials so that dosages of total PNA's were approximately equal failed to equalize the skin tumorigenic potency of the syncrudes; therefore factors other than PNA's were contributing to the response, or qualitative and quantitative differences in the composition of the PNA class affected the response. (2) None of the crudes was more than 1/500 as active as BP, and the single coal liquid that had been upgraded by hydrotreatment (COED syncrude) was 1/1400 as potent a skin carcinogen as an equivalent amount of BP. (3) The composite sample of natural petroleums, while containing materials recoverable in a PNA fraction, failed to elicit a measurable response under the conditions of this bioassay. (4) A threefold difference in the skin carcinogenic potency of this set of three active syncrudes was demonstrated. (5) The activity of PNA isolates was substantially greater than that of the parent mixture. This is

Table 5. Skin carcinogenic potency of synthetic petroleums and PNA fractions relative to BP.

Material	Relative potency	Upper 95% confidence limit
Synthoil crude	1/503	1/128
Shale oil crude	1/1380	1/275
COED syncrude	1/1440	1/263
Synthoil PNA isolate	1/119	1/27
Shale oil PNA isolate	1/51	1/13

consistent with the view that skin carcinogenic principles present within these complex materials are concentrated within this chemical class.

Comparison of the potency of the parent mixture with that of the respective PNA isolate will reveal the likelihood that components present within this fraction determine the mixture's activity. Table 6 gives these comparisons for both synthoil and shale oil versus their respective PNA isolates. In this instance, the relative potency of the synthoil crude is 1/4 that of its isolate, and shale oil is 1/27 as potent as its isolate. This suggests that components present within the PNA fraction are accountable for most of the skin carcinogenicity of the crude and that differences that persist after normalization reflect qualitative or quantitative differences in the composition of this fraction.

The question of whether differences in the composition of the PNA isolates were responsible for differences in the activity of the whole mixture could be addressed by comparing the potency of the two isolates. In this case the shale oil PNA fraction was observed to be 2.3 times more potent than the synthoil PNA fraction but the 95% confidence limits on this estimate were 0.3-22.3. Thus the data were inadequate to establish a statistically significant difference between these fractions.

This study provides a basis for comparison of other mixtures and chemical fractions of these mixtures with the current materials. We anticipate that as our quantitative data base expands for a wide variety of contemporary synthetic fossil liquids, it will become feasible to search for short-term *in vitro* and *in vivo* biochemical correlates of both exposure and effect. If these efforts are successful, then an approach toward direct estimation of human exposure risk may be developed with organ-cultured human skin or human skin grafts maintained on athymic nude mice.

We thank L. C. Gipson, M. S. Whitaker, and T. J. Stephens for animal exposure and observation; D. G. Gosslee for assistance with statistical analysis; J. S. Trent for data coding; and C. S. Rains for secretarial support.

This research was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

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