Lung Tumorigenic Interactions in Strain A/J Mice of Five Environmental Polycyclic Aromatic Hydrocarbons

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The binary, ternary, quaternary, and quintary interactions of a five-component mixture of carcinogenic environmental polycyclic aromatic hydrocarbons (PAHs) using response surface analyses are described. Initially, lung tumor dose–response curves in strain A/J mice for each of the individual PAHs benzo[a]pyrene (BaP), benzo[b]fluoranthene (B[b]F), dibenz[a,h]anthracene (DBA), 5-methylchrysene (5MC), and cyclopenta(c,d)pyrene (CPP) were obtained. From these data, doses were selected for the quintary mixture study based on toxicity, survival, range of response, and predicted tumor yields. The ratios of doses among PAHs were designed to simulate PAH ratios found in environmental air and combustion samples. Quintary mixtures of B[a]P, B[b]F, DBA, 5MC, and CPP were administered to male strain A/J mice in a 25 factorial 32-dose group dosing scheme (combinations of five PAHs each at either high or low doses) and lung adenomas were scored. Comparison of observed lung adenoma formation with that expected from additivity identified both greater than additive and less than additive interactions that were dose related i.e., greater than additive at lower doses and less than additive at higher doses. To identify specific interactions, a response surface analysis using response addition was applied to the tumor data. This response surface model contained five dose, ten binary, ten ternary, five quaternary, and one quintary parameter. This analysis produced statistically significant values for 16 parameters. The model and model parameters were evaluated by estimating the dose–response relationships for each of the five PAHs. The predicted dose–response curves for all five PAHs indicated a good estimation. The binary interaction functions were dominated for the most part by DBA and were inhibitory. The response surface model predicted, to a significant degree, the observed lung tumorigenic responses of the quintary mixtures. These data suggest that although interactions between PAHs do occur, they are limited in extent. — Environ Health Perspect 106(Suppl 6):1337-1346 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1337-1346nesnow/abstract.html

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The interactions of mixtures of chemicals in biologic systems have been studied extensively in pharmacology (1,2) and toxicology (3). In general, these interactions can be classified as enhancing (greater than additive), inhibitory (less than additive), or no interaction (additive). When administered to experimental animals, chemical carcinogens exhibit all three of these effects depending on the chemicals, route of administration, sex, species, and target organ. The majority of these interaction studies have been performed using only two administered agents and a database of binary carcinogen interactions has been reported (4,5).

Polycyclic aromatic hydrocarbons (PAHs) are a pervasive class of environmental pollutant formed by the incomplete combustion of organic materials. Humans are exposed to PAHs from cigarette smoke, combustion products from gasoline, diesel fuel, coal, and oil, as well as from broiled and smoked foods (6). Many PAHs are carcinogenic in experimental animals and several PAH-containing mixtures (i.e., coke oven emissions, cigarette smoke, and coal tars) are human carcinogens (7–9). Although there have been a number of studies of interactions of PAHs within binary mixtures of PAHs, little work has focused on larger component mixtures and using lung tissues as tumor targets.

We selected five environmental PAHs to construct quintary (five-component) mixtures to examine tumorigenic interactions among PAHs. The PAHs benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), dibenz[a,h]anthracene (DBA), 5-methylchrysene (5MC), and cyclopenta[c,d]pyrene (CPP) were selected for the extent and pervasiveness of their environmental occurrence, structural diversity, metabolic diversity, and range of tumorigenic potency. Using the strain A/J mouse lung as a target organ, we sought to answer the following questions: Are the mouse lung tumorigenic activities of these five PAHs additive? What is the extent of the deviation from additivity? Can specific interaction parameters be calculated? What is the effect of a nontumorigenic PAH on the tumorigenic activity of a quintary mixture of PAHs?

Methods for analysis of interactions have been reviewed including isobolographic analyses (10), interaction indexes (11), and response surface approaches (12). Response surface approaches have found use in identifying and quantitating chemical and drug interactions (13–16). Response surface methods use regression techniques that are both descriptive and predictive and are not limited to number of independent variables or same mechanisms of action or dose–response slopes. We utilized a response surface methodology,
using response addition, to both design and analyze a study that sought to identify and quantitate the interactions among five environmental PAH lung tumorigens. Using a 2\(^5\) factorial experimental design (five PAHs at two doses each) strain A/J mice were treated with a series of quinary PAH mixtures and lung adenomas were enumerated after 8 months.

We report that PAH-induced lung adenoma formation in strain A/J mice can exhibit both greater than additive and less than additive interactions that are dose related. Interaction analyses by maximum likelihood methods using a response surface model identified statistically significant binary, ternary, and quaternary interactions.

**Materials and Methods**

**Chemicals**

B(\(\beta\))F (99%), DBA (97%), urethane (99%), and pyrene were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin) and B(\(a\))P (≥98%) from Sigma Chemical Co. (St. Louis, Missouri). CPP (99%) was obtained from A. Gold (University of North Carolina, Chapel Hill, North Carolina) and 5MC (99%) from S. Amin (American Health Foundation, Valhalla, New York). Tricaprylin was purchased from Eastman Kodak (Rochester, New York). The crude pyrene was purified by column chromatography in hexane using silica gel, recrystallized from hexane, and sublimed in vacuo at 170°C to give a melting point of 149 to 151°C. The reported melting point was 149.6 ± 150.3°C (17). Liquid chromatography–mass spectrometry indicated this product was 99.7% pure.

**Tumor Studies**

Male strain A/J mice 6 to 8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in laminar flow rooms in groups of four in polycarbonate cages. Mice were maintained under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hr light/dark cycle) and received food and water ad libitum. In the first study, individual PAHs were administered to male strain A/J mice at several doses. On the day of treatment, PAHs were sonicated in tricaprylin until complete solution was achieved, then mice were injected ip (0.2 ml/mouse). Urethane- and tricaprylin-treated mice served as positive and negative controls, respectively. After 8 months, all mice were sacrificed by cervical dislocation, the lungs removed, fixed in 10% neutral buffered formalin, and the surface tumors counted. No detailed histopathology was performed, as previous studies have identified these lesions as adenomas (18). In the second study (mixture study), a 2\(^5\) factorial experimental design (five agents each at two doses) was used. Thirty-two groups of mice were randomized (20 per group) and dosing was performed in the order of the randomized group number. PAH mixtures were prepared by transferring individually weighed PAHs into each of 32 vials to construct the 32 dosing vials of quinary mixtures of PAHs. Quality assurance analysis by HPLC verified that the target doses were achieved (data not shown). Animals were treated and tumors were scored as described previously. In the second study smaller numbers of animals (10 mice per dose) were treated with the high dose of each PAH as indicator controls. Animal care and treatment were conducted in accordance with the guidelines established in the *Guide for the Care and Use of Laboratory Animals* (19). All animals were treated humanely with due consideration to the alleviation of distress and discomfort.

**Statistics**

Normality was examined by the Kolmogorov-Smirnov test, multiple comparisons by the Bonferroni multiple comparison test or Students-Newman-Kuels multiple comparison test (SigmaStat, Jandel Scientific, San Rafael, California), log likelihood analysis by SAS (SAS Institute, Cary, North Carolina), and multiple linear regression analysis using Mathematica (Wolfram Research, Champaign, Illinois).

**Results**

**Dose Responses of Individual Polycyclic Aromatic Hydrocarbons**

Dose–response studies were performed with each of the five PAHs at dose ranges that resulted in a survival of 75 to 100% (Table 1). Statistical analysis indicated that 15 of the 22 groups were significantly different (\(p < 0.01\)) from the tricaprylin control group using a Bonferroni multiple comparison test on the square root transformed tumor response data. Both positive (urethane) and vehicle (tricaprylin) controls for lung adenoma response were in agreement with historical data (18).

**Selecting Dose Levels for Quinary Mixtures of Polycyclic Aromatic Hydrocarbons**

Dose levels were selected that would satisfy the following: <25% mortality; <10% reduction in weight gain at the end of the study; a predicted range of tumor response between 2 and 100 lung adenomas per mouse; and the ability to observe an overall 2-fold greater than additive and a 4-fold less than additive tumor response. In addition, the PAH dose levels were prepared in ratios similar to those found in environmental air and combustion samples. In cigarette smoke (9), coal gasification emissions (21), ambient air (22), coke oven emissions (23), gasoline exhaust, and diesel exhaust (24), B(\(\beta\))F and B(\(a\))P are found in approximately equal amounts. In ambient air (22), diesel exhaust (24), and coke oven emissions (25), DBA is found in amounts that are approximately 5 to 40% that of B(\(\beta\))F or B(\(a\))P. CPP environmental levels range from 250 to 2000% of that of B(\(\beta\))F or B(\(a\))P in ambient air (22), gasoline exhaust, and diesel exhaust (24). 5MC has been detected in gasoline exhaust at approximately 6 to 10% that of B(\(\beta\))F or B(\(a\))P (24). Therefore, based on the dose–response data in Table 1 and the environmental levels discussed previously, the following dose levels in milligrams per kilogram were selected (high dose/low dose): B(\(a\))P, 75/30; B(\(\beta\))F, 75/30; DBA, 10/2.5; 5MC, 30/10; and CPP 100/30. The dosing groups were selected according to a 2\(^{5}\) factorial dosing scheme (12,13). This resulted in 32 PAH mixture groups representing combinations of five PAHs at either high or low dose (Table 2). This dose scheme would allow the calculation of five PAH dose parameters, ten binary interaction parameters, ten ternary interaction parameters, five quaternary interaction parameters, and one quinary parameter.

**Analyses of Polycyclic Aromatic Hydrocarbon Mixture Tumor Data**

Survival of mice in the 32 PAH mixture groups ranged from 70 to 100%, with a median of 85% and a mean (± SD) of 84.8 ± 9.1 (Table 2). No dose dependency could be established between survival and doses of PAHs with the dose range tested. The mean body weights for the highest dosed group compared to tricaprylin control indicated a significant loss of weight at day 7 with a recovery to control values on day 14 and beyond (data not available).
LUNG TUMORIGENIC INTERACTIONS OF PAHs

Table 1. Lung tumorogenic responses of male strain A/J mice exposed to individual PAHs.

| Dose, mg/kg | No. mice treated/ mice scored | Mice surviving, % | Mean observed lung adenomas per mouse | Variance |
|------------|--------------------------------|-------------------|--------------------------------------|----------|
|            |                                |                   |                                      |          |
| B(a)P      | 5                               | 20/20             | 100                                  | 0.45     | 0.682 |
|            | 10                              | 20/17             | 95                                   | 0.529    | 0.640 |
|            | 50                              | 20/19             | 90                                   | 4.37**   | 7.91  |
|            | 100                             | 20/24             | 90                                   | 12.5**   | 19.6  |
|            | 200                             | 20/18             | 90                                   | 33.0**   | 109   |
|            | 500                             | 20/20             | 90                                   | 2.00*    | 3.47  |
|            | 1000                            | 20/20             | 90                                   | 5.30**   | 10.9  |
|            | 2000                            | 20/20             | 90                                   | 6.35**   | 13.1  |
|            | 5000                            | 20/20             | 90                                   | 13.1**   | 37.7  |
|            | 10000                           | 20/20             | 90                                   | 32.1**   | 123   |
|            | 20000                           | 20/20             | 90                                   | 39.0**   | 187   |
|            | 50000                           | 20/20             | 90                                   | 53.1**   | 399   |
|            | 100000                          | 20/20             | 90                                   | 72.1**   | 472   |
|            | 200000                          | 20/20             | 90                                   | 32.5**   | 242   |
|            | 500000                          | 20/20             | 90                                   | 103**    | 350   |
|            | 1000000                         | 20/20             | 90                                   | 28.1**   | 25.9  |

* Mice were treated with each PAH dissolved in tricaprylin by single ip injection (0.2 ml) and tumors scored 8 months later. These data have been reported in earlier publications and are reproduced for comparison to the response surface model results [20] and references therein. * This group exhibited a 100% tumor incidence. * Tricaprylin vehicle control. * Significantly different at p<0.01 from the tricaprylin control by the Bonferroni multiple comparison test after a square root transformation of each data point. Values in bold text are statistically significant.

shown). The mean body weights for each group at sacrifice were indistinguishable from that of the tricaprylin control group (data not shown).

The 32 PAH mixture groups gave tumor responses that ranged from 16.8 to 63.8 lung adenomas per mouse (Table 2). Statistical analysis using the Kolmogorov-Smirnov test on the numbers of tumors per mouse for each group indicated that many groups were not normally distributed, a conclusion supported by the observation that the variances exceeded the means for many groups. Statistical comparison indicated, however, that each group was significantly different (p<0.01) from the tricaprylin control group using a Bonferroni multiple comparison test on the square root transformed tumor response data.

**Comparison of Observed Responses to those Predicted Based on Additive Responses from Individual Polycyclic Aromatic Hydrocarbon Dose–Response Data**

The expected additive responses for each PAH mixture were calculated by summing the responses from individual PAHs that comprised each mixture, using data from Table 1. Tumor responses for intermediate doses not specifically tested were obtained by estimates derived using curve-fitting procedures from a power equation as described in Nesnow et al. [20]. The relationship between the observed lung adenomas per mouse and the expected lung adenomas per mouse based on additive responses for each of the 32 PAH mixture groups indicated that many of the observed data points deviated from the expected based on additivity (Figure 1). Two groups exhibited a statistically significant increase in expected tumor responses from those expected based on additivity (Figure 1). Two groups exhibited a statistically significant increase in expected tumor responses and 13 groups exhibited a significant decrease (p<0.05) by the Students-Newman-Keuls multiple comparison test on the square root transformed data. Regressing the data to a linear function (R²= 0.679) gave major deviations from the expected slope of unity (calculated slope = 1.70) and the expected y-intercept of zero (calculated y-intercept = -13.53). Calculation of the percent increase or decrease of observed tumor responses over the expected tumor responses based on additivity (100 x ([observed additivity]/additivity)) gave a range of 97.4% superadditivity to 55% supraadditivity.

**Estimation of the Response Surface Model**

A generalized linear model (26) was fit to the data parameterized according to Equation 1 (Appendix). The variance of the response was assumed to be of the form Φₙ (i.e., a constant times the mean). The method of maximum likelihood was used to estimate the unknown model parameters using a ridge-stabilized Newton-Raphson algorithm (PROC GENMOD, SAS version 6.09). A power link function was used for g(μ) in Equation 1 with the best power estimate determined from a plot of the log likelihood versus the power parameter (Figure 2). The peak of this plot was approximately 0.5. Therefore, the square root transformation of the response data (lung adenomas per mouse) was used in the subsequent analysis of the data. To determine the shapes of the dose–response curves using the square root transformation for each individual PAH, the tumor data in Table 1 was square root transformed and plotted against dose (data not shown). Each PAH yielded a linear relationship with correlation coefficients > 0.90, adding support for the use of a square root-linear equation to describe the mixture data in the response surface analyses.

**Response Surface Analyses**

A square root-linear equation (Equation 1) was used to describe the 32-group quintary PAH mixture data. The equation contained 5 dose parameters and 26 interaction parameters: 10 binary, 10 ternary, 5 quaternary, and 1 quintary. A regression
The matrix of 1001 equations was constructed representing the dose and response data from each of the 1001 mice tested. This regression matrix included data from the 32 quintary mixture groups, the individual PAH dose—response data, and the indicator controls. Multiple linear regression techniques solved this matrix of equations and gave estimated parameters, 95% confidence intervals, and their associated $p$ values (Table 3). The estimated scale parameter, $\phi$, was 2.13. The goodness of fit of the model was assessed via the scaled deviance compared to a chi-square distribution and was determined to be adequate ($p < 0.05$). The test for the significance of each model parameter was based on a comparison of the likelihood values with the full model and the model excluding the parameter under study. The $p$ value is derived from comparing the likelihood ratio test to a chi-square distribution with one degree of freedom.

This model produced statistically significant values for 16 parameters (Table 3). All of the linear terms were highly significant ($p < 0.001$). Furthermore, 10 of the interaction terms were significantly different from zero at the 5% significance level. The five significant binary interaction terms were all negative, giving less than additive interactions: $\beta_{13}$ (B[a]P–DBA), $\beta_{15}$ (B[a]P–CPP), $\beta_{23}$ (B[b]F–DBA), $\beta_{24}$ (B[b]F–DBA–5MC), and $\beta_{445}$ (DBA–5MC–CPP). One quaternary term was statistically significant and negative: $\beta_{345}$ (B[a]P–DBA–5MC–CPP).

### Additivity Calculated from the Response Surface Model

Following the definition of additivity as given by Berenbaum, the logic of Carter et al. (12), the model in Equation 1 was reparameterized under the hypothesis of additivity (i.e., all of the interaction terms were removed) by including only the intercept and linear terms (i.e., $\beta_0$, $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$). A likelihood ratio test was used to test the hypothesis of additivity by comparing the likelihood of the constrained model (no interaction parameters) to the full model, a global test

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**Table 2.** Lung tumorigenic responses of male strain A/J mice exposed to quintary mixtures of polycyclic aromatic hydrocarbons.a

| Dose, mg/kg | B[a]P | B[b]F | DBA | SMC | CPP | No. mice treated/mice scored | Mice surviving, % | Mean observed lung adenomas per mouse | Variance |
|------------|-------|-------|-----|-----|-----|-----------------------------|------------------|--------------------------------------|---------|
| 30 30 30 10 30 30 | 20/15 | 75 | 18.7* | 158 |
| 30 30 2.5 10 30 30 | 20/17 | 85 | 26.7* | 163 |
| 30 30 2.5 10 30 30 | 20/20 | 100 | 16.8* | 82.0 |
| 30 30 2.5 10 30 30 | 20/18 | 90 | 26.2* | 107 |
| 30 30 2.5 10 30 30 | 20/25 | 100 | 23.8* | 75.9 |
| 30 30 2.5 10 100 | 20/16 | 80 | 31.8* | 242 |
| 75 75 10 10 10 30 | 20/18 | 90 | 20.6* | 50.4 |
| 75 30 10 10 10 30 | 20/19 | 95 | 34.2* | 206 |
| 75 30 2.5 30 30 30 | 20/16 | 80 | 30.1* | 175 |
| 30 75 2.5 10 10 100 | 20/20 | 100 | 25.9* | 78.8 |
| 30 75 2.5 10 10 100 | 20/20 | 100 | 20.9* | 121 |
| 30 75 2.5 10 100 30 | 20/16 | 80 | 37.7* | 298 |
| 30 75 2.5 10 30 30 | 20/17 | 85 | 42.4* | 258 |
| 75 75 10 10 10 30 | 20/16 | 85 | 47.1* | 209 |
| 75 75 2.5 30 30 30 | 20/20 | 100 | 29.4* | 114 |
| 75 75 2.5 10 100 30 | 20/14 | 70 | 31.8* | 95.8 |
| 75 30 10 10 30 30 | 20/18 | 90 | 50.4* | 452 |
| 75 30 10 10 100 30 | 20/17 | 85 | 44.3* | 282 |
| 30 75 10 10 30 30 | 20/19 | 95 | 39.0* | 256 |
| 30 75 10 10 100 30 | 20/18 | 90 | 32.3* | 75.0 |
| 30 75 10 10 30 100 | 20/14 | 70 | 57.8* | 363 |
| 30 75 2.5 30 30 30 | 20/17 | 85 | 45.4* | 226 |
| 30 75 2.5 30 30 100 | 20/19 | 95 | 39.2* | 176 |
| 75 75 10 10 30 30 | 20/14 | 70 | 45.7* | 110 |
| 75 75 10 10 100 30 | 20/18 | 90 | 36.6* | 102 |
| 75 30 10 10 30 100 | 20/17 | 85 | 48.9* | 461 |
| 30 75 2.5 30 30 100 | 20/16 | 80 | 47.8* | 161 |
| 75 75 10 30 30 100 | 20/17 | 85 | 44.8* | 341 |
| 75 75 10 30 100 30 | 20/14 | 70 | 63.8* | 393 |
| 0 0 0 0 0 0 | 10/7 | 70 | 7.1* | 12.9 |
| 0 75 20 0 0 0 | 10/10 | 100 | 41.1* | 121 |
| 0 0 0 10 0 0 | 11/11 | 100 | 22.9* | 70.6 |
| 0 0 0 30 0 0 | 10/10 | 100 | 13.5* | 49 |
| 0 0 0 0 100 0 | 10/9 | 90 | 23* | 106 |
| 0 0 0 0 0 0 | 20/19 | 95 | 0.32 | 0.36 |

* Mice were treated with each mixture dissolved in tricaprylin by single ip injection (0.2 ml) and tumors scored 8 months later. Each group except the tricaprylin control exhibited a 100% tumor incidence. a Tricaprylin vehicle control. b Significantly different at $p < 0.01$ from the tricaprylin control by the Bonferroni multiple comparison test after a square root transformation of each data point.
for additivity. Departure from additivity was found ($p < 0.001$). A comparison of the additive responses derived from the constrained model (no interaction parameters) indicated close agreement with the additive responses obtained from the sum of the individual PAH dose responses from Table 1 (data not shown).

### Prediction of Individual Dose–Response Curves

To test the model and parameter predictions, predictions for the dose–response relationships for each of the five PAHs were calculated. Individual dose–response curves were generated with the model parameters ($\beta_0$, $\beta_1$, $\beta_2$, $\beta_3$$\beta_4$) found in Table 3 and compared to the observed data found in Table 1 (Figure 3). There was excellent agreement between the two data sets. The dose–response data for each of the individual PAHs (Table 1), although used in combination with the mixture tumor data to derive the model parameters, represent less than 15% of the total data set and therefore were not expected to dominate these predictions.

### Prediction by the Response Surface Model of the Tumorigenicity of the Quintary Mixtures

Using Equation 1 and the parameters found in Table 3, the predicted response for each quintary mixture was estimated and compared to the observed responses (Table 2) for each of the 32 quintary mixture groups (Figure 4). Even though only 10 of the possible 26 interaction parameters were significant ($p < 0.05$), the model and model parameters predicted the observed responses to a high degree. The correlation coefficient $R^2$ was 0.979, the slope of the line was 0.996, and the $y$-intercept was 0.167. The 95% prediction intervals encompassed almost all of the data points.

### Effect of Pyrene on One Quintary Mixture

An additional group of mice was treated with a mixture containing the following PAHs (milligrams/kilogram): B[a]P, 30; B[b]F, 30; DBA, 2.5; 5MC, 30; CPP, 100; and pyrene, 100, using the same experimental protocol as described for the 32 quintary PAH groups. Pyrene was not

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### Table 3. Estimated model parameters for the square root linear response surface model based on Equation 1.

| Parameter | Estimate | 95% CI | $p$ value |
|-----------|----------|--------|-----------|
| $\beta_0$ | 0.437 | 0.276, 0.597 | $< 0.001$ |
| $\beta_1$ | 0.0721 | 0.0049, 0.0294 | $< 0.001$ |
| $\beta_2$ | 0.0125 | 0.0017, 0.0149 | $< 0.001$ |
| $\beta_3$ | 0.497 | 0.456, 0.538 | $< 0.001$ |
| $\beta_4$ | 0.0968 | 0.0815, 0.102 | $< 0.001$ |
| $\hat{\beta}_5$ | 0.0473 | 0.0449, 0.0497 | $< 0.001$ |
| $\hat{\beta}_{12}$ | 0.317 x 10^{-4} | — | 0.809 |
| $\hat{\beta}_{13}$ | -30.3 x 10^{-4} | -58.1 x 10^{-4}, -2.53 x 10^{-4} | 0.032 |
| $\hat{\beta}_{14}$ | 1.88 x 10^{-4} | 0.587 |
| $\hat{\beta}_{15}$ | -4.67 x 10^{-4} | -7.20 x 10^{-4}, -2.14 x 10^{-4} | 0.001 |
| $\hat{\beta}_{16}$ | -0.9 x 10^{-4} | 0.0046 |
| $\hat{\beta}_{17}$ | 1.6 x 10^{-4} | — | 0.857 |
| $\hat{\beta}_{18}$ | 0.009 x 10^{-4} | — | 0.943 |
| $\hat{\beta}_{19}$ | -179 x 10^{-4} | -260 x 10^{-4}, -98.8 x 10^{-4} | 0.001 |
| $\hat{\beta}_{20}$ | -54.3 x 10^{-4} | -78.6 x 10^{-4}, -20.9 x 10^{-4} | 0.001 |
| $\hat{\beta}_{21}$ | -3.94 x 10^{-4} | 0.290 |
| $\hat{\beta}_{22}$ | 22.7 x 10^{-6} | — | 0.531 |
| $\hat{\beta}_{23}$ | -18.6 x 10^{-6} | — | 0.064 |
| $\hat{\beta}_{24}$ | -0.04 x 10^{-6} | — | 0.991 |
| $\hat{\beta}_{25}$ | 196 x 10^{-6} | 3.25 x 10^{-6}, 3.89 x 10^{-6} | 0.046 |
| $\hat{\beta}_{26}$ | 86.0 x 10^{-6} | 24.8 x 10^{-6}, 147 x 10^{-6} | 0.008 |
| $\hat{\beta}_{27}$ | 3.17 x 10^{-6} | 0.732 |
| $\hat{\beta}_{28}$ | 202 x 10^{-6} | 128 x 10^{-6}, 391 x 10^{-6} | 0.036 |
| $\hat{\beta}_{29}$ | 37.5 x 10^{-6} | — | 0.220 |
| $\hat{\beta}_{30}$ | -11.1 x 10^{-6} | — | 0.220 |
| $\hat{\beta}_{31}$ | 285 x 10^{-6} | 113 x 10^{-6}, 458 x 10^{-6} | 0.001 |
| $\hat{\beta}_{32}$ | -14.1 x 10^{-7} | — | 0.511 |
| $\hat{\beta}_{33}$ | -6.22 x 10^{-7} | — | 0.218 |
| $\hat{\beta}_{34}$ | 2.38 x 10^{-7} | — | 0.234 |
| $\hat{\beta}_{35}$ | -52.5 x 10^{-7} | -87.8 x 10^{-7}, -17.4 x 10^{-7} | 0.003 |
| $\hat{\beta}_{36}$ | -31.7 x 10^{-7} | — | 0.074 |
| $\hat{\beta}_{37}$ | 6.1 x 10^{-8} | — | 0.082 |

*The estimate for $\Phi$ is 2.13. Values in bold text are statistically significant.*
control described data. The subset of the PAHs, the five PAHs, were used to fit a subset of the dose-response curves for each polycyclic aromatic hydrocarbon generated by the response surface model. The curves are from Equation 1 using the dose parameters from Table 3 (α, β, β, β, β, β). The data points represent means ± SD of observed lung adenomas/mouse from Table 1.

Table 4. Influence of pyrene on lung tumorigenicity of a quintary mixture of polycyclic aromatic hydrocarbons in male strain A/J mice.

| Treatment                  | Dose, mg/kg | No. mice treated/scored | Mice with lung adenomas, | Lung adenomas per mouse, mean ± SD |
|---------------------------|-------------|--------------------------|--------------------------|-----------------------------------|
| Pyrene                    | 200         | 20/20                    | 40                       | 0.6 ± 0.8*                        |
|                           | 100         | 20/20                    | 30                       | 0.4 ± 0.9*                        |
|                           | 50          | 20/20                    | 15                       | 0.3 ± 0.8*                        |
|                           | 10          | 20/20                    | 30                       | 0.5 ± 0.9*                        |
| Five-PAH mixture           | 20/20       | 100                      | 47.1 ± 15.6**            |
| Five-PAH mixture + pyrene  | 20/15       | 100                      | 30.5 ± 14**              |
| Tricaprylin                | 20/19       | 25                       | 0.32 ± 0.57              |

* Mice were treated with each mixture dissolved in tricaprylin by single ip injection (0.2 ml) and tumors scored 8 months later. † The doses (milligrams/kilogram) of the PAHs were B[a]P, 30; B[b]F, 30; DBA, 2.5; SMC, 30; and CPP, 100. ‡ The doses (mg/kg) of the PAHs were B[a]P, 30; B[b]F, 30; DBA, 2.5; SMC, 30; CPP, 100; and pyrene, 100. * Not statistically different from tricaprylin control (p=0.614). ** Statistically different from tricaprylin control (p<0.001). *** Statistically different from the five-PAH mixture (p=0.007). Values in bold text are statistically significant.

Figure 3. Observed male strain A/J mouse lung adenoma data for (A) B[a]P and B[b]F, (B) DBA, (C) CPP, and (D) SMC compared to dose–response curves for each polycyclic aromatic hydrocarbon generated by the response surface model. The data points represent the mean responses for each of the 32 quintary mixtures. R² = 0.979; slope = 0.996; y-intercept = 0.167. The dashed lines represent 95% prediction intervals. The predicted responses were derived using Equation 1 and the parameters found in Table 3. Sixteen of the 32 parameters used in these calculations were significant at p < 0.05.

Figure 4. Correlation between the observed tumorigenic responses of male strain A/J mice treated with quintary mixtures of polycyclic aromatic hydrocarbons and the predicted responses from the response surface model. The data points represent the mean responses for each of the 32 quintary mixtures. R² = 0.979; slope = 0.996; y-intercept = 0.167. The dashed lines represent 95% prediction intervals. The predicted responses were derived using Equation 1 and the parameters found in Table 3. Sixteen of the 32 parameters used in these calculations were significant at p < 0.05.

Discussion

Polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants found in the air, soil, and water, and in hazardous waste sites. Since their discovery as carcinogens in 1915 (27), their toxicologic effects have been studied intensively. Many PAHs are carcinogenic in multiple species (28) and are suspected carcinogens in humans (29), which also makes them an important chemical class from a public health standpoint. The epidemiologic data on PAH-containing mixtures strongly suggests that they are human respiratory carcinogens. The experimental animal data also point to lung, subcutaneous tissue, mammary tissue, and liver (in newborn and juvenile rodents) as targets of PAHs by various routes of administration (ip, intramammary, intrapleural, oral, inhalation, dermal, and iv). Because humans are exposed to mixtures of PAHs, it is important to understand the interactions among PAHs in these mixtures to assess their risk to humans. Statistical methodology is available that allows the determination of specific interactions between groups of toxicologic and pharmacologic agents using response surface methods (12,30,31).
To determine some of these potential PAH interactions, a study was constructed using five environmentally relevant PAHs administered as quintary mixtures to strain A/J mice with lung adenoma formation as the toxicologic outcome. The PAHs selected were B[a]P, B[β]F, DBA, 5MC, and CPP, based on their environmental occurrence, range of tumorigenic activities (32–37), structural features (methylated vs nonmethylated, condensed vs linear, alternating vs nonalternating), and a diversity of routes of metabolic activation (38–46).

The strain A/J mouse system is a medium-term tumorigenesis bioassay where tumors can be detected and quantitated 8 months after treatment. This mouse carries a lung cancer susceptibility gene or genes that have not yet been identified (18). Studies have shown that lung adenomas in this mouse will progress to adenocarcinomas after 18 to 24 months, with some metastasis. In addition, alveogenic carcinomas in humans are similar in morphology to adenocarcinomas in mouse lung (18). Finally, studies have shown that lung tumors in strain A/J mice produced by PAHs exhibit high proportions of Ki-ras mutations with mutation spectra different from the spontaneous controls (47–50). From these facts, we conclude that tumor formation in the strain A/J mouse has some relevance in the study of human lung cancer.

The results of these investigations indicate significant deviations from additivity that were both greater than additive and less than additive. The extent of these deviations ranged from +97.4 to −55% of that expected from additivity. Significant deviations were observed that were dose related i.e., lower doses, greater than additive; higher doses, less than additive. However, less than additive interactions dominated under most mixture conditions. Response surface modeling using multilinear regression techniques identified 6 statistically significant dose parameters and 10 significant interaction parameters of 32 possible parameters. The model and the estimated model parameters predicted the observed responses as well as the individual PAH dose–response curves. In additional studies we examined the need of all of the interaction parameters and found that significant fits to the data could not be obtained with fewer than the full complement of 26 interaction parameters (data not shown).

An analysis of the binary carcinogen interaction literature that encompasses multiple species, organs, and routes of administration has identified both greater than additive and less than additive effects for PAH–PAH interactions depending on target tissue species and route of administration (4). In single subcutaneous injection studies in female NMRI mice, Pfeiffer (37) reported dose–response sarcoma data with B[a]P, DBA, and mixtures of B[a]P and DBA at 114 weeks. The authors found the dose–response curves of the DBA group and the binary mixtures of B[a]P and DBA were not statistically different. This suggested a less than additive interaction between B[a]P and DBA. Schmähl et al. (51) summarized lifetime dermal application studies (two applications/week) of B[a]P and mixtures of PAHs, including B[a]P, B[β]F, and DBA in NMRI mice. Significant less than additive responses from the B[a]P dose response were noted in the four carcinogenic PAH mixture group receiving B[a]P, B[β]F, DBA, and benz[a]anthracene. Therefore, the studies by Pfeiffer (37) and Schmähl et al. (51) on the inhibitory tumorigenic activity of DBA in mouse skin and subcutaneous tissues are consistent with the inhibitory lung tumorigenic activity of DBA with other PAHs, possibly suggesting competitive inhibition of the enzymes involved in the metabolic activation of these PAHs.

There are a number of seemingly conflicting reports on the interactive effects of PAH, either in binary mixtures or in combination with complex mixtures containing PAHs. For example, B[a]P and CPP exert a greater than additive effect toward the induction of mouse skin papillomas (4), whereas this mouse lung study identified a less than additive interaction. Similarly, B[a]P and pyrene induce a greater than additive effect in papilloma formation in mouse skin tumor initiation studies (36), whereas pyrene, which induces neither mouse skin tumors (36) nor mouse lung tumors in strain A/J mice, significantly inhibits the lung adenoma formation of a quintary mixture of B[a]P, B[β]F, DBA, 5MC, and CPP. Finally, in mouse skin tumor coinitiation studies, cotreatment (initiation) of mice with B[a]P and cigarette smoke condensate followed by 12-O-tetradecanoylphorbol-13-acetate promotion produced a greater than additive effect, whereas similar studies with B[a]P and diesel exhaust particulate extracts produced a less than additive effect (36). Certainly, mixture composition, target tissues, species, strain, sex, and route of administration must play a role in the tumor outcome. Moreover, carcinogenesis is a multistage process that can involve absorption, distribution, metabolism, detoxification, elimination, macromolecular damage, mutation and/or chromosomal damage, DNA repair, altered gene expression, cytotoxicity, tissue injury, cell proliferation, and apoptosis. Many of these processes can be altered by enzyme induction and inhibition (3). Gibb and Chen (52) suggested that in the multistage model, a multiplicative effect of two or more carcinogens is consistent where each carcinogen acts on a different stage, whereas additivity occurs when each carcinogen acts on the same stage. Synergism has also been defined as occurring when the rate-limiting step in the generation of a single type of tumor differs for each of the two interacting carcinogens (53).

One approach to teasing out the dominant factors is to examine each separately. Future studies include examining quantitative DNA adduct formation, persistence, and repair over time and comparing the extent of DNA adducts formed by the quintary mixtures with the levels of adducts expected from additivity for each of the PAHs. Mixtures of PAHs enhance and inhibit covalent DNA binding (54). These studies are currently in progress.

In conclusion, a response surface model has identified a number of PAH–PAH interactions in a quintary mixture that accurately accounted for all of the observed responses. The observation of greater than additive responses from lower exposures is significant. However, because the magnitudes of all of the interactions were relatively small, these data suggest that although interactions of PAHs do occur, they are limited in extent.

**DISCLAIMER:** This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. EPA, and approved for publication. Mention of trade names or commercial products should not be construed as endorsement or recommendation for use.
Appendix

\[
g(\mu) = x^\beta
\]

where: 
\( \mu \) is the number of lung adenomas for the \( n \)th mouse
\( g(\cdot) \) is a specified monotone function of the mean

\[
x^\beta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5
\]

\[
+ \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{23} x_2 x_3
\]

\[
+ \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5
\]

\[
+ \beta_{123} x_1 x_2 x_3 + \beta_{124} x_1 x_2 x_4 + \beta_{125} x_1 x_2 x_5
\]

\[
+ \beta_{134} x_1 x_3 x_4 + \beta_{135} x_1 x_3 x_5 + \beta_{145} x_1 x_4 x_5 + \beta_{234} x_2 x_3 x_4 + \beta_{235} x_2 x_3 x_5
\]

\[
+ \beta_{245} x_2 x_4 x_5 + \beta_{345} x_3 x_4 x_5 + \beta_{1234} x_1 x_2 x_3 x_4 + \beta_{1235} x_1 x_2 x_3 x_5
\]

\[
+ \beta_{1245} x_1 x_2 x_4 x_5 + \beta_{1345} x_1 x_3 x_4 x_5 + \beta_{2345} x_2 x_3 x_4 x_5 + \beta_{12345} x_1 x_2 x_3 x_4 x_5
\]

\[
x_1 = \text{dose of } B[a]P \text{ (mg/kg)}
\]

\[
x_2 = \text{dose of } B[\beta]F \text{ (mg/kg)}
\]

\[
x_3 = \text{dose of } \text{DBA (mg/kg)}
\]

\[
x_4 = \text{dose of } \text{5MC (mg/kg)}
\]

\[
x_5 = \text{dose of } \text{CPP (mg/kg)}
\]

\( \beta_0 \) = unknown parameter associated with the background number of tumors

\( \beta_1 \) = unknown parameter associated with the effect of \( B[a]P \) on the number of tumors

\( \beta_2 \) = unknown parameter associated with the effect of \( B[\beta]F \) on the number of tumors

\( \beta_3 \) = unknown parameter associated with the effect of \( \text{DBA} \) on the number of tumors

\( \beta_4 \) = unknown parameter associated with the effect of \( \text{5MC} \) on the number of tumors

\( \beta_5 \) = unknown parameter associated with the effect of \( \text{CPP} \) on the number of tumors

\( \beta_{12} \) = unknown parameter associated with the interaction of \( B[a]P \) and \( B[\beta]F \) on the number of tumors

\( \beta_{13} \) = unknown parameter associated with the interaction of \( B[a]P \) and \( \text{DBA} \) on the number of tumors

\( \beta_{14} \) = unknown parameter associated with the interaction of \( B[a]P \) and \( \text{5MC} \) on the number of tumors

\( \beta_{15} \) = unknown parameter associated with the interaction of \( B[a]P \) and \( \text{CPP} \) on the number of tumors

\( \beta_{23} \) = unknown parameter associated with the interaction of \( B[\beta]F \) and \( \text{DBA} \) on the number of tumors

\( \beta_{24} \) = unknown parameter associated with the interaction of \( B[\beta]F \) and \( \text{5MC} \) on the number of tumors

\( \beta_{25} \) = unknown parameter associated with the interaction of \( B[\beta]F \) and \( \text{CPP} \) on the number of tumors

\( \beta_{34} \) = unknown parameter associated with the interaction of \( \text{DBA} \) and \( \text{5MC} \) on the number of tumors

\( \beta_{35} \) = unknown parameter associated with the interaction of \( \text{DBA} \) and \( \text{CPP} \) on the number of tumors

\( \beta_{45} \) = unknown parameter associated with the interaction of \( \text{5MC} \) and \( \text{CPP} \) on the number of tumors

\( \beta_{123} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F \), and \( \text{DBA} \) on the number of tumors

\( \beta_{124} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F \), and \( \text{5MC} \) on the number of tumors

\( \beta_{125} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F \), and \( \text{CPP} \) on the number of tumors

\( \beta_{134} \) = unknown parameter associated with the interaction of \( B[a]P, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{135} \) = unknown parameter associated with the interaction of \( B[a]P, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{145} \) = unknown parameter associated with the interaction of \( B[a]P, 5MC \), and \( \text{5MC} \) on the number of tumors

\( \beta_{234} \) = unknown parameter associated with the interaction of \( B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{235} \) = unknown parameter associated with the interaction of \( B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{245} \) = unknown parameter associated with the interaction of \( B[\beta]F, 5MC \), and \( \text{5MC} \) on the number of tumors

\( \beta_{345} \) = unknown parameter associated with the interaction of \( \text{DBA}, 5MC \), and \( \text{5MC} \) on the number of tumors

\( \beta_{1234} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{1235} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{1245} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

\( \beta_{1345} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F, \text{5MC}, \text{5MC} \) on the number of tumors

\( \beta_{1354} \) = unknown parameter associated with the interaction of \( B[a]P, \text{DBA}, \text{5MC}, \text{5MC} \) on the number of tumors

\( \beta_{1435} \) = unknown parameter associated with the interaction of \( B[a]P, 5MC, \text{5MC} \) on the number of tumors

\( \beta_{2345} \) = unknown parameter associated with the interaction of \( B[\beta]F, \text{DBA}, \text{5MC}, \text{5MC} \) on the number of tumors

\( \beta_{2354} \) = unknown parameter associated with the interaction of \( B[\beta]F, \text{DBA}, 5MC, \text{5MC} \) on the number of tumors

\( \beta_{2435} \) = unknown parameter associated with the interaction of \( B[\beta]F, \text{DBA}, 5MC, \text{5MC} \) on the number of tumors

\( \beta_{345} \) = unknown parameter associated with the interaction of \( \text{DBA}, 5MC, \text{5MC} \) on the number of tumors

\( \beta_{12345} \) = unknown parameter associated with the interaction of \( B[a]P, B[\beta]F, \text{DBA}, \text{5MC} \) on the number of tumors

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