THE EFFECTS OF 3-METHYLCHOLANTHRENE, BENZ(A)ANTHRACENE, AND ANTHRACENE ON FRIEND VIRUS LEUKEMIA

by

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

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Professor of Microbiology

Date
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To Mr. Bart Cross and Computer Center personnel for their assistance with the statistical analysis.
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ABSTRACT

The effects of 3-methylcholanthrene, benz(a)-anthracene and anthracene on Friend virus leukemia in BALB/c mice were studied. White blood cell count, hematocrit, spleen weight/total body weight ratio, liver weight/total body weight ratio were the parameters used to determine the effects of the chemicals. By analyses of variance it was determined that (1) anthracene slightly affected the leukemia, (2) 3-methylcholanthrene was slightly toxic to untreated mice, and (3) benz(a)anthracene had no effect on the leukemia.
INTRODUCTION

Since both chemicals and viruses cause carcinogenesis in experimental animals, the interactions of these two carcinogenic agents have been widely studied. Prior to 1967 all studies had shown an enhancement of the virus-induced neoplasms by the chemicals for the following oncogenic viruses: Shope papilloma, Shope fibroma, Rous sarcoma, polyoma, and Graffi's leukemogenic. (The literature is summarized by Duran-Reynals, 1963, and Salaman and Roe, 1964.)

From 1936 to 1944 Rous and his colleagues studied the effect of the combined treatment of Shope papilloma virus (SPV) and various carcinogens (tar, 3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene) and non-carcinogens (turpentine, benzene, chloroform) on rabbit skin. Both types of chemicals had a similar effect on the early stage of infection. If they were painted on the rabbit ears a few days before inoculation of the same site with SPV, the titer of the virus increased between tenfold and one hundredfold, and the latent period of papilloma production was shortened. However, only the carcinogenic agents increased the rate of the malignant transformation of the SPV-induced papillomas.
The result was different when SPV was injected intravenously into rabbits already bearing chemically induced papillomas. The growth rate of these papillomas was increased, and malignant changes occurred within these papillomas and also in skin where tumors were not visible before injection of SPV.

In 1938 Andrewes and Ahlstrom inoculated rabbits intramuscularly with tar, 3-methylcholanthrene (3-MC), or benzo(a)pyrene, in amounts insufficient to produce tumors by themselves, and with Shope fibroma virus (SFV). In the tarred rabbits the virus induced large tumors which progressed until the death of the host. In rabbits not inoculated with any carcinogen, the virus induced skin fibromas which regressed in a few weeks leaving the animal immune to further infection by SFV. In general the disease in the domestic rabbit injected with tar and SFV resembled that in the more susceptible cottontail rabbit injected with virus alone or that in the domestic rabbit injected with the related, but more virulent, myxoma virus alone.

Carr, in 1942, inoculated inbred chickens into the breast with Rous sarcoma virus (RSV) and later gave the same chickens an injection of 3-MC into the leg. These birds were only slightly susceptible to the virus because they developed small, slowly growing tumors. In three weeks, the tumors of the breast regressed, while at the same time
swelling developed in the 3-MC injected leg, subsiding as lumps developed in different sites in the leg. Tumors removed from the leg were histologically indistinguishable from those induced by RSV. In chickens which had not received RSV but were inoculated with 3-MC in the leg, there was no reaction to the carcinogen.

In 1961, Rawson studied the combined effects on mice of polyoma virus and various chemical agents. The virus was injected into neonatal mice, and from the seventh week on these mice received (a) one painting with 7, 12-dimethylbenz(a)anthracene (DMBA) alone, or (b) one painting with DMBA followed by 15 paintings with croton oil, or (c) 15 paintings with croton oil alone, or (d) 40 paintings with benzo(a)pyrene alone. Comparable control groups received virus or chemical treatments alone. The incidence of polyoma-induced tumors was significantly higher in all infected mice painted with different chemicals than in the control mice.

The combined effect of urethane and Graffi's leukemogenic virus on the incidence of leukemia in mice was studied in 1963. When the Graffi virus, followed by urethane, was injected into adult C57BL mice, the incidence of leukemia was significantly greater than the incidence in control mice given each agent alone.
The interaction between oncogenic virus and chemical carcinogen can be summarized briefly as follows:

1. Tumors appear earlier and are more extensive than when either agent is used alone.
2. Virus-induced tumors are localized at the site of carcinogen application.
3. The combination of virus and chemical may produce tumors when neither agent does so by itself.
4. The tumors are usually of the type characteristic of the effect of the virus rather than the chemical.
5. Generally, more tumors are malignant and therefore metastasize more commonly than after virus infection alone.
6. The whole course of viral oncogenesis may resemble that induced by a more virulent virus in a similar host or by the same virus in a more susceptible host.

The following possible mechanisms of the viral and chemical agents in the production of a cocarcinogenic effect have been postulated (Boyland, 1964; Hughes, 1965; Martin, 1964; Salaman and Roe, 1964):

1. The carcinogenic polycyclic aromatic hydrocarbons (PAH) may complex with the nucleic acids of the cell. Their molecules inserted into the three-dimensional model of the DNA molecule would stretch
and distort it. This insertion and subsequent disturbance might induce heritable somatic mutations and account for the carcinogenicity of the molecule.

2. The chemical may cause carcinogenesis by derepression.

(a) It may inactivate the repressors which normally control the activity and proliferation of a virus. 3-MC inhibits the production of interferon in rat cells. As interferon prevents the action of viruses, it is possible that the induction of some tumors by PAH's might be due to the inhibition of interferon so that the otherwise latent virus becomes active.

(b) It may interfere with repressor proteins which control the synthesis of enzymes since there is markedly increased enzyme activity in animals treated with carcinogens.

(c) It may interfere with repressors controlling mitosis. This would allow the cells to reproduce at an abnormal rate resulting in tumor production.

3. The chemical agent may also act on the immune mechanism of the host.

(a) It might reduce the immune reaction to the virus allowing it to proliferate and invade more host cells.
(b) It might reduce the immune reaction to a new antigen in the neoplastic cell allowing this cell to readily reproduce.

In 1957 Fiscus, Schloss, and Wertman reported that the two strong carcinogens DMBA and 3-MC inhibited the splenomegaly associated with Friend virus (FV) and Rauscher virus (RV) murine leukemias. When these two carcinogens were applied to the skin of the back of FV-infected or RV-infected mice, the latent period of the leukemia and the survival time of the mice were prolonged, and the spleen weights were small. The causes of the inhibition of splenomegaly by DMBA and 3-MC were (a) inhibition of hemorrhagic necrosis and (b) lack of erythroblastosis.

After this report, it was of interest to know whether this effect was due to the carcinogenicity of a PAH, or if it could also be produced by related non-carcinogens or only mildly carcinogenic compounds.

To answer this question, three PAH's were tested on FV-infected mice: 3-MC, a powerful carcinogen; benz(a)anthracene, a weak carcinogen; and anthracene, a non-carcinogen.

The carcinogenicity of PAH's has been extensively tested in studies of tumor production and protein binding. The development of tumors after application of the chemicals to mice, by skin painting or subcutaneous injection, is a
criterion of carcinogenicity. Using fluorescent derivatives and the sensitive radioactive tracer technique, a causal association has been found between carcinogenesis and protein binding of the chemical to the epidermal protein of the mouse (Miller, 1951; Heidelberger and Weiss, 1951; Wiest and Heidelberger, 1953; Heidelberger and Moldenhauer, 1955).

The data accumulated for the three compounds used in the present study are as follows:

In experiments with skin painting or subcutaneous injection, the unsubstituted tricyclic aromatic hydrocarbon anthracene is considered completely non-carcinogenic (Hueper and Conway, 1964). Twenty-four hours after application to mouse skin, the protein binding of anthracene-9, 10-C¹⁴ has been shown to be much lower than the protein binding of DMBA or benz(a)anthracene. However, neither Somerville and Heidelberger (1961) nor Moodie, Reed, and Wallick (1954) have demonstrated any protein binding of anthracene.

Benz(a)anthracene is considered a weak carcinogen (Hueper and Conway, 1964). Thirteen investigations of its carcinogenic effect have been summarized by Steiner and Falk (1951). Nine workers found it inactive; four workers found it produced an insignificant number of tumors after skin painting. Steiner and Edgecomb (1952) reported benz(a)anthracene to be a carcinogen of moderate potency as tested by sarcoma induction in C57BL mice following
subcutaneous injection. Heidelberger and Moldenhauer (1955, 1956) found a small amount of radioactivity bound to the skin of mice after applying C\textsuperscript{14}\textsuperscript{-labeled benz(a)anthracene.

Three-methylcholanthrene, a derivative of benz(a)anthracene, is one of the most potent chemical carcinogens and has induced tumors in several species (Hueper and Conway, 1964). It is highly significant in the production of tumors in the mouse by both skin painting and subcutaneous injection. It has been shown that 3-MC is bound to the skin of mice to a great extent and approximately equals the protein binding of DMBA (Heidelberger and Moldenhauer, 1956).
OBJECTIVE

To determine the enhancement or suppression, if any, of Friend virus leukemia in BALB/c mice by 3-methylcholanthrene, benz(a)anthracene, and anthracene.
EXPERIMENTAL DESIGN

Description of Treatments

The design of the experiment required eight different virus-chemical combinations to be tested on ten mice per week for 12 weeks. The eight combinations were:

1. no virus + no chemical
   These untreated controls were used as a reference of "normal" murine development over a 12-week period.

2. PV + no chemical
   These mice were infected with PV only in order to follow the development of PV leukemia.

3. PV + 3-MC
   The mice in this group were infected with PV and treated with 200 μg 3-MC weekly.

4. no virus + 3-MC
   These mice were treated only with 200 μg 3-MC weekly.

5. PV + benz(a)anthracene
   These mice were infected with PV and treated with 800 μg benz(a)anthracene weekly.

6. no virus + benz(a)anthracene
   The mice in this group were treated weekly with 800 μg benz(a)anthracene.
7. FV + anthracene

These mice were infected with FV and treated with 800 µg anthracene weekly.

8. no virus + anthracene

The mice in this group were treated weekly with 800 µg anthracene.

**Statement of Hypotheses**

In order to determine the stated objective, it was necessary to test two null hypotheses.

**Hypothesis I:**

For a given week those mice receiving a given chemical and no virus are of the same population as those mice receiving neither chemical nor virus. If this null hypothesis is accepted, the chemical was not toxic to the mice as indicated by the parameters measured. If the null hypothesis is rejected, the chemical was toxic to the animals.

**Hypothesis II:**

For a given week those mice receiving a given chemical and FV are of the same population as those mice receiving FV only.

If this null hypothesis is accepted, the chemical had no effect on the FV leukemia; if the null hypothesis is rejected, the chemical affected the development of the leukemia.
The logic algorithm for the conclusions which can be drawn in this problem is shown in Figure 1.

The four conclusions which can be made from the algorithm are as follows:

Conclusion A:

If the chemical was not toxic to the mice as indicated by the measured parameters, and if the chemical had no effect on the development of the leukemia, then the chemical was ineffective on the measured parameters.

Conclusion B:

If the chemical was not toxic to the mice but did affect the development of the leukemia, then the chemical enhanced or repressed the leukemia without the compounding factor of toxicity.

Conclusion C:

If the chemical was toxic to the mice but did not affect the leukemia, then only the effect of toxicity existed.

Conclusion D:

If the chemical was toxic to the mice and also affected the leukemia, then the chemical enhanced or suppressed the leukemia with the compounding factor of toxicity.
Figure 1. Logic algorithm for hypotheses tested by the reported experiment.
Method of Testing Hypotheses

The parameters used as an indication of chemical toxicity and leukemic development were:

1. total body weight
2. spleen weight
3. liver weight
4. white blood cell count (WBC)
5. per cent red blood cell volume (hematocrit)

To test the two null hypotheses, analyses of variance were made. One-way analyses for each individual parameter were done using the ANOV 44 library computer program, and multivariate analyses of variance were done with a program prepared for this problem. (See Appendix A for the computational procedure.) The University of Arizona's IBM 7072 and CDC 6400 were used to process the programs.

An assumption of each null hypothesis is that the means of the two populations are the same. This assumption is proved or disproved by the analysis of variance which gives a measure of:

(a) the effects of the different treatments on the two samples
(b) the variance within one and the same sample.

The F-test answers the question whether the variance between samples is significantly different from the variance
within the samples. $F$ is defined as

$$F = \frac{\text{between sample variance}}{\text{within sample variance}}.$$ 

To test Hypothesis I a week-by-week analysis of variance was made using the measured parameters to compare:

1. no virus + no chemical vs. no virus + 3-MC
2. no virus + no chemical vs. no virus + benz(a)anthracene
3. no virus + no chemical vs. no virus + anthracene

The $F$-values obtained were compared to the values in an $F$-table at the 95% confidence level for the computed number of degrees of freedom. Those $F$-values equal to or greater than the given $F$-value were considered significant, meaning the mice treated with a given chemical were not of the same population as the untreated mice. $F$-values less than the given $F$-value were considered non-significant, meaning the mice treated with a given chemical were of the same population as the untreated mice.

To test Hypothesis II a week-by-week analysis of variance was made to compare:

1. $FV$ + no chemical vs. $FV$ + 3-MC
2. $FV$ + no chemical vs. $FV$ + benz(a)anthracene
3. $FV$ + no chemical vs. $FV$ + anthracene
The F-values obtained were treated in the same manner as those for testing Hypothesis I.

In addition to the five parameters listed above, leukemic development was also measured by (1) the latent period of the leukemia and (2) the survival time of the mice infected with FV. To test the effect of the chemicals on the latent period of the leukemia, an analysis of variance was done. A similar analysis was done on the time of death for those mice which were not sacrificed.

The F-values obtained from these analyses were decided to be significant or non-significant by referring to the F-table at 95% confidence level for the computed degrees of freedom.
MATERIALS AND METHODS

Host

Inbred BALB/c mice were used as the host because of their low incidence of spontaneous leukemia and because they are considered 100 per cent responsive to Friend virus (Fieldsteel, Dawson, Bostick, 1961). The mice were bred and raised at The University of Arizona. Both male and female mice, 3-8 weeks old, were used throughout the study. Their weight at the beginning of the experiment ranged from 9.4 g to 24.6 g.

The mice were kept ten to a cage, fed Lab-Blox pellets, and given water ad libitum.

Stock Virus

The initial Friend virus stock was obtained from A. Howard Fieldsteel, Ph.D., Stanford Research Institute, Menlo Park, California. At that time it had been passaged eight times in BALB/c mice. A stock virus pool was prepared from infected spleen tissue after the twelfth passage.

Fifty infected mice were sacrificed by cervical fracture 24 days after Friend virus inoculation. The enlarged spleens were aseptically removed, pooled, and weighed. A 20 per cent by weight suspension was made in
sterile sucrose stabilizer (Bovarnick, Miller, Snyder, 1950) 
as modified by Fieldsteel (1964).

Formula:

<table>
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<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>KOH</td>
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</tr>
<tr>
<td>L-glutamic acid</td>
<td>1.44 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.508 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.034 g</td>
</tr>
<tr>
<td>sucrose</td>
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</tr>
<tr>
<td>H₂O</td>
<td>1000.0 ml</td>
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</table>

The whole spleens suspended in the sucrose stabilizer were homogenized in an ice bath at maximum speed for two minutes in a Virtis Homogenizer. Homogenization was stopped for two minutes, then the suspension was re-homogenized for two minutes.

The homogenate was dispensed in 3 ml and 15 ml amounts and designated as pool No. 3. It was quickly frozen in an alcohol-dry ice bath and stored at -70°C until used.

The titer of the homogenized pool was 10⁵.2 ID₅₀/ml as calculated by the method of Reed and Muench (1938).

**Virus Inoculation**

The stock virus homogenate was thawed in a 37°C water bath by gentle swirling and centrifuged at 1500 rpm for 10 minutes in a refrigerated angle-head centrifuge to remove the particulate matter. The supernatant fluid was
removed, and a $10^{-3}$ dilution was made using cold, sterile, sucrose stabilizer. Two-tenths ml of the dilution was injected intraperitoneally into each mouse.

**Chemicals**

The three polycyclic aromatic hydrocarbons used were:

1. anthracene

![Anthracene](image)

2. benz(a)anthracene

![Benz(a)anthracene](image)

3. 3-methylcholanthrene

![3-Methylcholanthrene](image)

The chemicals were prepared weekly in a concentration of 200 µg dissolved in 0.05 ml reagent grade acetone (Terracini, Subik, Della Porta, 1960) for 3-MC and in a concentration of 800 µg in 0.05 ml for benz(a)anthracene and anthracene.
Skin Painting

The hair was removed weekly from the back of each mouse with a commercial depilatory, "Nair." The carcinogen-acetone solution was applied weekly to the skin beginning 24 hours after virus inoculation. A one milliliter syringe with an 18 gauge needle was used to apply the solution. The needle was placed bevel up on the mouse skin, and 0.05 ml of the solution was applied. The procedure was carried out under a stream of air to hasten the evaporation of the acetone and to prevent the solution from running off the skin.

Measurement Techniques

The parameters used to measure the effects of the three chemicals on Friend virus leukemia were white blood cell count, hematocrit, animal weight, spleen weight, and liver weight. Each parameter was measured once a week for 12 weeks.

An example of the manner in which the raw data were collected is shown in Table 1.

The latent period of the leukemia and the survival time of the mice receiving the virus only or the virus-chemical combinations were also determined.

Blood was obtained from each mouse by means of a capillary tube inserted into the orbital sinus of one eye. For the determination of the hematocrit, the capillary tube was filled with blood, plugged at one end with "Critoseal,"
Table 1. A sample of the form in which the raw data were collected

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<td>19.2</td>
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and centrifuged in an International Clinical centrifuge (Model CL, Ser. 80899M-1) for ten minutes. The per cent red blood cell volume was determined using a "Critocap" microhematocrit tube reader.

For the WBC count, 0.02 ml blood was drawn into a Sahli pipette and immediately transferred to 10 ml of a 0.15M NaCl plus 0.04% formalin solution. One-tenth ml of a 2% saponin solution was added to each tube. After complete lysis of the RBC's (2-3 minutes), the WBC count was determined with a Coulter Counter (aperture setting = 0.707, amplification = 1, lower threshold = 20, no upper threshold, background count < 100).

The mice were sacrificed by cervical fracture and then weighed. The liver and spleen of each mouse were removed and weighed.

The ten mice to be sacrificed at the twelfth week were palpated every other day starting the second day after virus inoculation to determine the latent period of the leukemia. The result was considered positive when the tip of the spleen crossed the breast line.

All cages were checked twice a day for dead mice. If a mouse died before the pre-determined day of sacrifice, the day of death was recorded, the mouse was weighed, and the liver and spleen were removed and weighed.
RESULTS

The results for the one-way analyses of variance for each individual parameter are shown in Tables 2-7. (The values for each parameter are normally distributed.) During the course of these analyses, the mice were found to be of different populations by weight. This difference in weight consequently made the spleens and livers of different populations. In order to correctly determine the effects of the chemicals, it was necessary to normalize this relation.

To do this, the ratios \( \frac{\text{liver weight}}{\text{total body weight}} \left( \frac{\text{LW}}{\text{BW}} \right) \) and \( \frac{\text{spleen weight}}{\text{total body weight}} \left( \frac{\text{SW}}{\text{BW}} \right) \) were used in the final analyses of variance. Total body weight was not used alone as a measure of chemical carcinogenicity or toxicity.

The results of the multivariate analyses of variance are also shown in Tables 2-7. One multivariate analysis was done using all five parameters; (WBC, hematocrit, spleen weight, liver weight, and total body weight); the other was done using \( \frac{\text{SW}}{\text{BW}} \) and \( \frac{\text{LW}}{\text{BW}} \) and omitting total body weight.

The results of the analyses of variance for the latent period of FV leukemia and the survival time of the non-sacrificed mice are shown in Tables 8a-8b.
Table 2. Table comparing calculated F-values to significant F-values at the 95% confidence level for no virus + no chemical vs. no virus + anthracene.

The upper values in each block are the calculated F-values compared to the significant F-value at the 95% confidence level, i.e., \( F_{calculated} < 0.95 \).

The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., mean control - mean treated (only shown if F-value significant).

\*

\* indicates a significant F-value meaning the treated mice were of a different population than the untreated mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>week</th>
<th>1</th>
<th>2</th>
<th>3</th>
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Table 3. Table comparing calculated F-values to significant F-values at the 95% confidence level for Friend virus + no chemical vs. Friend virus + anthracene.

The upper values in each block are the calculated F-values compared to the significant F-values at the 95% confidence level, i.e., $F_{calculated}/F_{95\%}$. The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., $mean_{control}/mean_{treated}$ (only shown if F-value significant).

<table>
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<tr>
<th>parameter</th>
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</tbody>
</table>

**E** indicates an enhancement of the leukemia by the chemical.

**R** indicates a repression of the leukemia by the chemical.
Table 4. Table comparing calculated F-values to significant F-values at the 95% confidence level for no virus + no chemical vs. no virus + benz(a)-anthracene.

The upper values in each block are the calculated F-values compared to the significant F-values at the 95% c. 1., i.e., \( F_{\text{calculated}} < F_{\text{0.95}} \).

The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., mean_{control} - mean_{treated} (only shown if F-value significant).

\[ \begin{array}{cccccccccccccc}
\text{parameter} & \text{week} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 \\
\hline
\text{white blood cell count} & & 13.26 & 4.49 & 4.97 & 7.700 < \text{15,100} & 1.73 & 3.13 & 9.63 & 4.49 & 0.99 & 1.57 & 10.03 & 8.49 & 8.84 & 4.38 \\
\text{hematocrit} & & 12.07 & 4.49 & 3.96 & 2.97 & 1.85 & 3.60 & 2.17 & 3.05 & 0.17 & 1.02 & 16.05 & 4.49 & 4.89 & 8.84 \\
\text{spleen weight} & & 2.85 & 4.49 & 0.43 & 3.35 & 2.01 & 2.15 & 2.25 & 4.04 & 0.41 & 1.01 & 2.00 & 0.004 < \text{0.007} & 0.004 < \text{0.005} \\
\text{liver weight} & & 1.84 & 4.49 & 0.12 & 0.19 & 2.19 & 1.86 & 0.81 & 2.13 & 0.41 & 0.81 & 2.00 & 0.004 < \text{0.005} & 0.004 < \text{0.005} \\
\text{multivariate with four parameters} & & 10.78 & 2.65 & 1.28 & 1.38 & 1.13 & 11.89 & 0.40 & 2.20 & 1.48 & 1.98 & 1.67 & 1.36 & 6.65 \\
\text{body weight} & & 10.44 & 2.65 & 4.71 & 4.49 & 9.96 & 14.9 < \text{17.7} & 20.95 & 4.49 & 3.29 & 4.89 & 0.000 < \text{0.001} & 2.52 & 2.69 & 2.69 \\
\text{multivariate with five parameters} & & 8.40 & 2.53 & 4.71 & 2.68 & 2.88 & 7.71 & 0.73 & 2.59 & 3.63 & 2.52 & 1.05 & 1.47 & 6.96 \\
\end{array} \]
Table 5. Table comparing calculated F-values to significant F-values at the 95% confidence level for Friend virus + no chemical vs. Friend virus + benz(a)anthracene.

The upper values in each block are the calculated F-values compared to the significant F-values at the 95% confidence level, i.e., \( F_{calculated} \) vs. \( F_{significant} \).

The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., mean control vs. mean treated (only shown if F-value significant).

\( \text{E} \) indicates an enhancement of the leukemia by the chemical.

\( \text{R} \) indicates a repression of the leukemia by the chemical.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>0.23</td>
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<tr>
<td>Hematocrit</td>
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<td>3.75</td>
<td>3.75</td>
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<tr>
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<td>0.05</td>
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<td>2.69</td>
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<tr>
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</tr>
</tbody>
</table>
Table 6. Table comparing calculated F-values to significant F-values at the 95% confidence level for no virus + no chemical vs. no virus + 3-methylcholanthrene.

The upper values in each block are the calculated F-values compared to the significant F-values at the 95% confidence level, i.e., F_{calculated} / F_{0.95}.

The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., mean_{control} / mean_{treated} (only shown if F-value significant).

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<th>3</th>
<th>4</th>
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<td>30.74</td>
<td>47.90</td>
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<td>81.64</td>
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</tbody>
</table>

indicates a significant F-value.
Table 7. Table comparing calculated F-values to significant F-values at the 95% confidence level for Friend virus + no chemical vs. Friend virus + 3-methylcholanthrene.

The upper values in each block are the calculated F-values compared to the significant F-values at the 95% confidence level, i.e., $F_{calculated} > F_{0.95}$.

The lower values in each block are the mean of the control group compared to the mean of the treated group, i.e., $\text{mean}_{control} < \text{mean}_{treated}$ (only shown if F-value significant).

::: indicates a significant F-value.

E indicates enhancement of the leukemia by the chemical.

R indicates repression of the leukemia by the chemical.

<table>
<thead>
<tr>
<th>week</th>
<th>1</th>
<th>2</th>
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Table 8a. Table comparing calculated F-values to significant F-values at the 95% confidence level for the latent period of Friend virus leukemia.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>F&lt;sub&gt;calculated&lt;/sub&gt;</th>
<th>Mean&lt;sub&gt;control&lt;/sub&gt;</th>
<th>Mean&lt;sub&gt;treated&lt;/sub&gt;</th>
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<tr>
<td>3-methylcholanthrene</td>
<td>1.18/4.45</td>
<td>20</td>
<td>18</td>
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<tr>
<td>benz(a)anthracene</td>
<td>0.04/4.41</td>
<td>20</td>
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<tr>
<td>anthracene</td>
<td>1.67/4.45</td>
<td>20</td>
<td>18</td>
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</table>

Table 8b. Table comparing calculated F-values to significant F-values at the 95% confidence level for the survival time of non-sacrificed mice.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>F&lt;sub&gt;calculated&lt;/sub&gt;</th>
<th>Mean&lt;sub&gt;control&lt;/sub&gt;</th>
<th>Mean&lt;sub&gt;treated&lt;/sub&gt;</th>
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<tr>
<td>3-methylcholanthrene</td>
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<td>43</td>
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<td>benz(a)anthracene</td>
<td>2.64/4.00</td>
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<td>anthracene</td>
<td>1.52/4.08</td>
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<td>45</td>
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</table>
DISCUSSION

In the one-way analyses of variance, although each parameter was analyzed, WBC and hematocrit were judged to be the two parameters most indicative of chemical toxicity. Although total body weight is indicative of toxicity, it was not used because often male mice were compared to female mice. The weight difference between the sexes was enough to make them of different populations without treatment. So, more consideration was given to the F-values of WBC and hematocrit when it was decided whether a chemical was toxic. White blood cell count and spleen weight were considered more sensitive measurements of leukemic development than the other parameters. Therefore, more consideration was given to these F-values when the effect of the chemicals on FV leukemia was determined.

Table 2. Table comparing calculated F-values to significant F-values at the 95% confidence level for no virus + no chemical vs. no virus + anthracene.

Using WBC and hematocrit as measurements of toxicity, from the one-way analyses of variance anthracene was not toxic to untreated mice because of the lack and randomness of significant F-values for these two parameters. The multivariate analysis using four parameters (WBC, HMT, \( \frac{SW}{BW} \), \( \frac{LW}{BW} \)) substantiates this conclusion since six out of 12
F-values are non-significant meaning the control mice and the mice receiving only anthracene are of the same population, i.e., anthracene is not toxic. The multivariate analysis using five parameters (WBC, HMT, SW, LW, BW) seems to indicate that anthracene was toxic to the mice since nine out of 12 F-values are significant. However, the power total body weight has in determining significance is demonstrated here. With one exception (week four), for every week body weight is significant, the multivariate is also significant; if the body weight is not significant neither is the multivariate. So, it is difficult to draw any conclusion from the multivariate using body weight because of the overshadowing influence it has on the other parameters.

Table 3. Table comparing calculated F-values to significant F-values at the 95% confidence level for Friend virus + no chemical vs. Friend virus + anthracene.

Because of the many significant F-values for WBC and in the univariate analyses, anthracene appears to have a slight effect on FV leukemia. But the significant F-values do not have a definite pattern, so the effect cannot be determined. Also, the reversal from enhancement to suppression of the leukemia is confusing. The multivariate with four parameters indicates anthracene may have affected the leukemia since six F-values are significant; these values show no pattern but do coincide with significant WBC or F-values. It is interesting to note that none of these
significant values occur in the last three weeks of the experiment, whereas it would be logical to assume that more significant F-values would occur in these weeks because of the build-up effect of the chemical.

The five-parameter multivariate is again significant only if body weight is significant with the exception of week six. For week 11, body weight is the only significant parameter, and this is reflected in the significant multivariate F-value. The obvious conclusion to draw from the multivariate is that anthracene affected the leukemia, but because of the influence of body weight, I hesitate to make this conclusion.

For anthracene, Hypothesis I is accepted and Hypothesis II is rejected: anthracene was not toxic for untreated mice and it slightly affected the development of FV leukemia.

Table 4. Table comparing calculated F-values to significant F-values at the 95% confidence level for no virus + no chemical vs. no virus + benz(a)anthracene.

For WBC and hematocrit of the univariate analyses, very few of the F-values are significant; those that are significant are randomly distributed and have no pattern with the exception of week one. In week one both the WBC and hematocrit were significant, but the mice themselves were of different populations as indicated by the significant body weight F-values. This may explain why the WBC's
and hematocrits were of different populations. The four-parameter multivariate indicates benz(a)anthracene was toxic to untreated mice because seven out of 12 F-values are significant.

Table 5. Table comparing calculated F-values to significant F-values at the 95% confidence level for Friend virus + no chemical vs. Friend virus + benz(a)-anthracene.

Again, there are few significant F-values for WBC and SW/BW and also for the other parameters in the one-way analyses. The significant F-values show no pattern of distribution except for weeks two and three. The four-way multivariate with seven out of 12 non-significant F-values also indicates benz(a)anthracene did not affect the leukemia. These non-significant F-values show a pattern in weeks 10-12. Why there are not more significant parameters in the last weeks of the leukemia cannot be explained.

The five-parameter multivariate also seems to indicate no effect, but, again, because of the power of body weight (week five for example), it is difficult to make any conclusions.

For benz(a)anthracene Hypothesis I is accepted and Hypothesis II is accepted: benz(a)anthracene is not toxic to untreated mice and has no effect on FV leukemia.
For WBC and hematocrit in the one-way analyses, 3-MC appears to be toxic to untreated mice as indicated by the significance and pattern of the F-values for both parameters for a majority of weeks. Enhancement of both parameters is indicated for weeks 7-12 with the exception of week 10. Why week 10 did not also show an enhancement cannot be explained. (Eight mice of the no virus + no chemical group were compared with 10 mice of the no virus + 3-MC group, so the sample size was sufficiently large to detect different populations.) Both multivariate analyses show 12 out of 12 significant F-values indicating the chemical affected the mice. Therefore, 3-MC is toxic to untreated mice.

White blood cell count and $\frac{SW}{BW}$ were used as the parameters most indicative of development of leukemia in the one-way analyses. Because of the paucity of significant F-values for these two parameters in particular and the other parameters in general, I conclude that 3-MC does not enhance or repress FV leukemia. Both multivariate analyses show five out of 12 significant F-values, and each has two F-values that are barely significant. This is ambiguous but,
in the light of the results of the one-way analyses, was interpreted to mean that 3-MC did not affect the leukemia.

Fiscus, Schloss, and Wertman (1967) reported that the average spleen weights of mice infected with FV and treated with 3-MC were significantly lower at the 95% confidence level than the spleen weights of infected untreated control mice and not significantly different from the spleen weights of normal control mice. This was true for both subcutaneous injection and skin painting of 3-MC, but the spleen weights of the subcutaneously injected mice were smaller than those mice painted with 3-MC.

The results of the reported study differ from those of Fiscus, Schloss, and Wertman (1967). Table 7 shows only four out of 12 weeks with the $\frac{SW}{BW}$ ratio significant meaning 3-MC affected the splenic development; two of these four significant weeks show a suppression of the $\frac{SW}{BW}$, and two show an enhancement. The logical conclusion seems to be that 3-MC did not significantly affect the leukemia.

For 3-MC, then, Hypothesis I is rejected and Hypothesis II is accepted: 3-MC is toxic to mice but does not affect FV leukemia.

Table 8a. Table comparing calculated F-values to significant F-values at the 95% confidence level for the latent period of Friend virus leukemia.

The latent period of the leukemia was not affected by any of the chemicals as indicated by the non-significant
F-values obtained when the latent period of the leukemia in those mice receiving only FV was compared to the latent period of mice receiving FV + chemical. Fiscus, Schloss, and Wertman (1967) were unable to show any significant differences at the 95% confidence level between infected, treated mice and infected mice, although they concluded the latent periods seemed to be longer in the treated mice.

I can only conclude that none of the chemicals affected the latent period of the leukemia.

Table 8b. Table comparing calculated F-values to significant F-values at the 95% confidence level for the survival time of non-sacrificed mice.

The survival time of those mice receiving FV + chemical was not significantly different from the mice receiving only FV as indicated by the non-significant F-values. Therefore, none of the chemicals affected the survival time of those mice receiving FV.
CONCLUSIONS

(1) Anthracene slightly affects FV leukemia in BALB/c mice.
(2) Benz(a)anthracene and 3-MC have no effect on FV leukemia in BALB/c mice.
(3) Three-methylcholanthrene is slightly toxic to BALB/c mice.
(4) Benz(a)anthracene and anthracene are not toxic to BALB/c mice.
APPENDIX A

Computational procedure for data from sacrificed animals:

Consider a given week and animals without virus.
Let $B$, $S$, $L$, $W$, $H$ denote, respectively, body weight, spleen weight, liver weight, white blood cell count, and hematocrit. Let treatment numbers 1, 2, 3, 4 mean respectively, untreated control, chemical A, chemical B, chemical C.

Thus $B_{ij}$ means the body weight of the $j$th mouse having the $i$th treatment. $S_{ij}$, ..., $H_{ij}$ have similar meanings.

Let $Y_{ij}$ denote the vector

$$\begin{bmatrix}
B_{ij} \\
S_{ij} \\
L_{ij} \\
W_{ij} \\
H_{ij}
\end{bmatrix}$$

Let $N_i$ = the number of mice receiving the treatment $i$.

Let $\bar{Y}_i = \frac{1}{N_i} \sum_{j=1}^{N_i} Y_{ij}$

$$\bar{Y} = \frac{1}{N} \sum_{i=1}^{4} \sum_{j=1}^{N_i} Y_{ij} = \frac{1}{N} \sum_{i=1}^{4} N_i \bar{Y}_i$$

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Note that both $\bar{Y}_1$ and $\bar{Y}$ are vectors having 5 elements.

In the following formula a prime denotes transpose of a matrix.

Let $A = \sum_{i=1}^{4} \sum_{j=1}^{N_i} (Y_{ij} - \bar{Y}_1)(Y_{ij} - \bar{Y}_1)'$

$$b_{12} = \bar{Y}_1 - \bar{Y}_2$$
$$b_{13} = \bar{Y}_1 - \bar{Y}_3$$
$$b_{14} = \bar{Y}_1 - \bar{Y}_4$$
$$\nu = N_1 + N_2 + N_3 + N_4 - 4$$

$A$ is a $5 \times 5$ matrix; $b_{12}$, $b_{13}$, and $b_{14}$ are 5-dimensional column vectors; $b_{12}'$, $b_{13}'$, $b_{14}'$ are the corresponding row vectors.

Compute

$$V_{12} = \frac{N_1 N_2}{N_1 + N_2} \cdot \frac{\nu - 4}{5} \cdot b_{12}' A^{-1} b_{12}$$

$$V_{13} = \frac{N_1 N_3}{N_1 + N_3} \cdot \frac{\nu - 4}{5} \cdot b_{13}' A^{-1} b_{13}$$

$$V_{14} = \frac{N_1 N_4}{N_1 + N_4} \cdot \frac{\nu - 4}{5} \cdot b_{14}' A^{-1} b_{14}$$

Significance test for each chemical against the control:

If $V_{12}$ exceeds the upper 5 per cent point of $F$-distribution with numerator degrees of freedom 5 and
denominator degrees of freedom $v-4$, then the chemical A has a significant effect. Otherwise, the effect of chemical A is insignificant. In exactly the same way, $V_{13}$ will be used to compare chemical B with the control, and $V_{14}$ will be used to compare chemical C with the control.

The above procedure can be repeated for all weeks and also for those animals that received virus.


