Summation and Inhibition Effects of Weak and Strong Carcinogenic Hydrocarbons: 1:2-Benzanthracene, Chrysene, 1:2:5:6-Dibenzanthracene, and 20-Methylcholanthrene*

PAUL E. STEINER AND HANS L. FALK

(From the Department of Pathology, University of Chicago, Chicago 37, Ill.)

INTRODUCTION

The summation or additive effects of carcinogenic agents have received much attention in efforts made to understand the action of carcinogens and the events that occur in carcinogenesis (5, 21). Chemical carcinogens, tumor-producing viruses, radiant energy, and trauma have been tried in various combinations (46). Some agents showed summation, others did not, and a third group showed inhibition. The present report is concerned with combinations of chemical agents only.

The earliest work with summation of chemical carcinogens was done with tars (5) or other mixtures. After the carcinogenic hydrocarbons were discovered in 1931, the first summation experiment with these compounds was made by Hieger (24). He found that one strong carcinogen (1:2:5:6-dibenzanthracene) could complete the carcinogenic action on skin begun by another (3:4-benzpyrene). Lavik et al. reported that methylcholanthrene, benzpyrene, and dibenzanthracene administered in subcarcinogenic doses could elicit tumors following methylcholanthrene preparation (35). Rusch et al. observed that combinations of chemicals were additive in carcinogenesis on mouse skin but that chemicals and ultraviolet light, Shope virus and ultraviolet light, and Shope virus and x-ray irradiation did not summate (46). No addition was observed by Jaffé when two carcinogens of a different chemical nature were used (27, 28). Thus, neither urethan nor azo dyes were additive to methylcholanthrene. Stasney et al. observed augmentation of liver tumors in rats fed 2-acetaminofluorene if estradiol or pregnant mare serum gonadotrophin were also administered (49). It is assumed that in those experiments where summation did not occur its absence was due to failure of the agents to act on the same intracellular receptors.

Inhibition of one chemical carcinogen by another chemical has also been demonstrated a number of times. Experiments in which the exact chemical nature of either agent was unknown are omitted from the present discussion. For experiments where both agents were pure chemicals—using 20-methylcholanthrene, 3:4-benzpyrene and 1:2:5:6-dibenzanthracene as inductors—the inhibitors can be divided into four classes; namely, hydrochloric acid–liberating compounds (6, 15), aliphatic aldehydes (18, 16, 44, 45), unsaturated dibasic acids (17), and aromatic compounds (18, 19, 34, 53). All four classes seem to interfere with the sulfur metabolism of the cell. Some of the inhibiting compounds of the aromatic series are closely related to actual carcinogens, suggesting that the mode of action is a competition for available SH groups.

The present report concerns summation and inhibition effects by new chemical combinations. The experiments were planned to cover the interactions of extremes in potency; two weak, two strong, and a weak plus a strong chemical carcinogen were used. The strong carcinogens (1:2:5:6-dibenzanthracene and 20-methylcholanthrene) were chosen because of the large amount of careful work done by others on titrating the 50 per cent tumor dose (TD50). The weak carcinogens (chrysene and 1:2-benzanthracene) were selected because they represent two basic ring structures from which nearly all carcinogenic hydrocarbons are derived and because they have often been reported to be carcinogenically inert.

Thirteen tests of the carcinogenicity of 1:2-benzanthracene have been reported. Most workers have found it to be inactive (1, 8, 10, 24, 36, 39, 40, 48, 55). However, Kennaway (30) produced one transitory papilloma in fifty mice in an unspecified time. Cook, in 1933 (14), obtained one epithelioma in eighty mice, also in an unspecified...
time. Barry et al. (4) found one epithelioma among thirty mice painted for periods up to 584 days. White and Eschenbrenner (54) fed this chemical to six rats for 14 months and observed two hepatomas. From the collected experiments, it can be concluded that weak cancer-producing activity has been shown for this compound.

Much to our surprise, 1:2-benzanthracene proved to be a fairly potent carcinogen. The specimen used was a commercial product. Spectrophotometric examination of fractions obtained by chromatography showed the ultraviolet absorption spectrum of 1:2-benzanthracene, and not that of any other known carcinogen. The discovery that 1:2-benzanthracene was definitely carcinogenic appeared of such great importance for our work, and in explaining hitherto contradictory results observed by others in collateral lines of research (see "Discussion"), that the experiments were immediately repeated and extended. The final results of these new tests are not yet available, but they already confirm on a large scale the carcinogenicity of 1:2-benzanthracene; they will be reported on completion. For present purposes it should be emphasized that our confidence in the carcinogenicity of 1:2-benzanthracene is based on far more extensive data than are presented in this paper.

Chrysene has been tested for carcinogenicity many times. Most investigators have reported it to be inactive (10, 23, 31-33, 37-39, 40-42, 47, 48). A few, however, found it to possess mild activity. Twort and Fulton, in 1930 (51), induced three tumors in 70-85 weeks in an unstated number of surviving mice by skin-painting chrysene in liquid paraffin or in oleic acid. Cook (14) found three sarcomas in ten rats injected with chrysene in a fatty medium. Bottomley and Twort (9) skin-painted mice with chrysene in various solvents for periods of 50-78 weeks and obtained thirteen tumors, of which nine were found in tests with oleic acid as the solvent. Barry and Cook (8) tested chrysene by subcutaneous injection and found four malignant tumors in ten rats within 20 months. That sample of chrysene may have been impure. Barry et al. (4) painted the skins of 300 mice for periods lasting from 440 to 797 days and observed one epithelioma and three papillomas. Bachmann et al. (2) saw one papilloma on a mouse on the 85th day. Twort and Twort (52) painted mice with chrysene in oleic acid or chloroform and obtained tumors with the former. The substance was regarded as one-tenth as potent as 1:2:5:6-dibenzanthracene in oleic acid.

The strong carcinogenic potencies of 20-methylcholanthrene and of 1:2:5:6-dibenzanthracene are well known. An attempt was made to find and use the amount that would produce tumors in 50 per cent of the animals, so that either summation or inhibition effects could be recognized (11, 12).

METHODS

Fifty C57 black mice, the second and third generation descendants of breeding stock obtained from the Roscoe B. Jackson Memorial Laboratory, 3-4 months old and about equally divided as to sex, were used in each experiment. They were fed laboratory chow pellets and water and were housed with ten to fifteen in a cage. Weights were taken at monthly intervals. The observed differences in tumor yield are not explained by inequalities in animal weight or by sex, so that these two factors are not mentioned again.

The mice received subcutaneous injections in the interscapular region with the designated amount of chemical in 0.5 cc. tricaprylin. The mice were observed weekly for a month, to record the amount of loss by early ulceration; thereafter, they were observed monthly until tumors appeared, after which period weekly observations were resumed until the experiments were terminated in the 22nd month. About 5 per cent of the mice in each experiment, including the tricaprylin controls, lost their chemical soon after injection, as indicated by sloughs and by failure to show a residual deposit at necropsy. The loss appeared to be about equal in all groups. All mice were examined at autopsy, and all suspicious lesions and tumors were examined on microscopic sections. Only sarcomas at the site of injection were counted as induced tumors. In many instances, the residual oil and chemical mass was saved at necropsy for spectrophotometric analysis, and many injection masses were saved for histological study of the cellular reaction. Spectrophotometric examination of residues of injected materials was made in fifteen mice. In fourteen, the presence of the expected chemical was demonstrated; in one mouse there was a 1-mm. deposit of clear oil, but no chemical could be detected. Most of these analyses were done at or near the end of the experiments.

Three of the chemicals (1:2-benzanthracene, 20-methylcholanthrene, and 1:2:5:6-dibenzanthracene) were commercial products obtained from the Eastman Kodak Company. Chrysene was obtained from the Reilly Tar and Chemical Corporation. The purity of the 1:2-benzanthracene has already been described. The other three chemicals were not purified, but previous studies by spectrophotometry following chromatography had shown absence of absorption spectra of other carcinogenic hydrocarbons.
Because, according to the reports in the literature, chrysene and 1:2-benzanthracene were questionably carcinogenic, the large dose of 5.0 mg. per mouse was selected to assure a saturation or excessive dose of the compounds. In the case of the strong carcinogens, where addition effects, if they existed, were sought, the median tumor dose (TD<sub>50</sub>) was used. The results of the tests, therefore, are not directly comparable, because the quantity of the injected chemicals varied from TD<sub>50</sub> to TD<sub>100</sub>. For the same reason the carcinogenic index of Ibáll (25) and the carcinogenic grade of Berenblum (7) are not directly determinable, from the data, for all chemicals.

The results are given in Table 1. Table 2 is also provided to supply these data, because deaths from intercurrent infections were not equal in all experiments. This table shows the number of survivors and the deaths from tumors for each month. The difference between these figures represents death from other causes. Survival was poorest in the 1:2-benzanthracene experiment.

**Controls.**—No sarcomas were found in the un.injected controls. In our experience, spontaneous fibrosarcomas occur very rarely in the C57 black strain—and then only in old mice and usually in the extremities rather than on the trunk, where tumors are induced.

Three sarcomas were found in the tricaprylin controls, constituting an incidence of about 1 per

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**TABLE 1**

**CARCINOGENICITY AND COMBINATION EFFECTS OF HYDROCARBONS**

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Amt. of chemical</th>
<th>No. of mice</th>
<th>Effective total mice*</th>
<th>Effective total mice†</th>
<th>No. of induced sarcomas</th>
<th>Tumor yield‡ (per cent)</th>
<th>Tumor yield‡ (per cent)</th>
<th>Mini. induction time (days)</th>
<th>Av. induction time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysene</td>
<td>5.0 mg.</td>
<td>50</td>
<td>39 24 4</td>
<td>10.3</td>
<td>16.6</td>
<td>549</td>
<td>549</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>1:2-Benzanthracene</td>
<td>5.0 mg.</td>
<td>50</td>
<td>46 44 8</td>
<td>17.4</td>
<td>18.2</td>
<td>161</td>
<td>161</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>1:2:5:6-Dibenzanthracene</td>
<td>0.02 mg.</td>
<td>50</td>
<td>45 43 25</td>
<td>55.5</td>
<td>58.1</td>
<td>153</td>
<td>153</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>20-Methylcholanthrene</td>
<td>0.02 mg.</td>
<td>50</td>
<td>45 43 25</td>
<td>55.5</td>
<td>58.1</td>
<td>153</td>
<td>153</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>Chrysene+1:2-benzanthracene</td>
<td>2.5 mg. of each</td>
<td>50</td>
<td>41 34 15</td>
<td>56.6</td>
<td>44.1</td>
<td>250</td>
<td>250</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>Tricaprylin controls</td>
<td>0.02 mg. of each</td>
<td>50</td>
<td>42 42 33</td>
<td>78.5</td>
<td>78.5</td>
<td>120</td>
<td>120</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>Uninjected controls</td>
<td>0.02 cc to 2.0 cc</td>
<td>304</td>
<td>280 223</td>
<td>3</td>
<td>1.1</td>
<td>928</td>
<td>928</td>
<td>30</td>
<td>108</td>
</tr>
</tbody>
</table>

* Number of survivors at 4 months, when the first tumor appeared among all compounds.
† Number of mice surviving when the first tumor occurred with that particular compound.
‡ Based on the effective total surviving when the first tumor occurred with that compound.

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**RESULTS**

**Single carcinogens.**—Chrysene and 1:2-benzanthracene were definitely carcinogenic (Table 1). The latter was stronger than the former, as indicated by a higher tumor yield and shorter minimum and average induction periods. Chrysene was a slow carcinogen (Fig. 1). Its carcinogenic index (Iball) was low, being 4.1, in contrast with that of 1:2-benzanthracene, which was 6.4.

The quantity of 1:2:5:6-dibenzanthracene and of 20-methylcholanthrene injected (0.02 mg.) proved, as anticipated, to be near the TD<sub>50</sub>. Although the eventual tumor yields were the same, the former was a slower carcinogen than the latter (Fig. 1). The induction times of both were shorter than those of the weaker carcinogens.

Neither the data in Table 1 nor those in Figure 1 give adequate information on the rate of non-tumor deaths that occurred after the minimum induction time had passed. Table 2 is provided to supply these data, because deaths from intercurrent infections were not equal in all experiments. This table shows the number of survivors and the deaths from tumors for each month. The difference between these figures represents death from other causes. Survival was poorest in the 1:2-benzanthracene experiment.

**Controls.**—No sarcomas were found in the uninjected controls. In our experience, spontaneous fibrosarcomas occur very rarely in the C57 black strain—and then only in old mice and usually in the extremities rather than on the trunk, where tumors are induced.

Three sarcomas were found in the tricaprylin controls, constituting an incidence of about 1 per
amount of chemicals, the result was equal to summation of their individual responses at full doses. Two interpretations are possible: (a) the dosage of 5 mg. of these compounds was above the saturation dose level for producing the maximum number of tumors. In this case half the amount, or 2.5 mg., would have elicited the same number of tumors. If this explanation is correct, the two chemicals were additive and without mutual interference.

(b) The result observed was not that of mere summation but of synergism, i.e., an effect greater in combination than the sum of the individual effects. Sufficient data are not at hand to decide whether the observed results were those of summation or of synergism. Further experiments are in progress to clarify these problems and to determine the maximum and minimum dose levels of 1:2-benzanthracene.

![Graph showing cumulative mortality from induced tumors](image)

**Fig. 1.**—Cumulative mortality from induced tumors. Shows cumulative percentage of the mice injected with each compound dying of tumor in each month. For calculating these figures the effective total mice in each experiment was the number surviving at 4 months.
When a weak and a strong carcinogen were injected together (1:2-benzanthracene plus 1:2:5:6-dibenzanthracene), the result was not summation, but apparent inhibition. The tumor yield was about half the sum of their individual tumor yields. This interesting inhibition phenomenon is being further investigated. It has its counterpart in principle (inhibition of a strong by a weak chemical carcinogen of similar molecular configuration), although not with the same compounds and to a smaller degree, in the inhibition of 20-methylcholanthrene by dibenzofluorene, of 1:2:5:6-dibenzanthracene by 1:2:5:6-dibenzoacridine, and of 20-methylcholanthrene by chrysene (34, 53). The inhibited theories of molecular structure in relation to carcinogenic activity, which had been based on the supposition that this chemical was inactive. For example, Pullman calculated the critical electron density in the K-region of aromatic hydrocarbons and concluded that any value below 1.29e would indicate inactivity (48). The value for 1:2-benzanthracene is 1.288e. The critical level, therefore, must now be revised downward. In addition, the possibility arises that other chemicals within this range might be active and that they should be retested.

The correlation of the absorption spectra of 1:2-benzanthracene and its derivatives with car-

### Table 2

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Time in months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0   4   5   6   7   8   9   10   11   12   13   14   15   16   17   18   19   20   21</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>1:2-Benzanthracene</td>
<td>50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50</td>
</tr>
<tr>
<td>1:2:5:6-Dibenzoanthracene</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>20-Methylcholanthrene</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>Chrysene+1:2-Benzanthracene</td>
<td>50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50   50</td>
</tr>
<tr>
<td>1:2-Benzanthracene+1:2:5:6-</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>dibenzanthracene</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>20-Methylcholanthrene+1:2:5:6-</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>dibenzanthracene</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>Tricaprylin controls</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
<tr>
<td>Uninjected controls</td>
<td>0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0    0</td>
</tr>
</tbody>
</table>

*Non-tumor deaths can be calculated by subtracting the surviving mice and deaths due to tumors from the survivors in the preceding month.
†The experiments were terminated early in the SD month, except for some of the tricaprylin controls.
‡The sarcomas represent time of death and not induction time.

hobition was observed on skin-painting by the latter workers, whereas the inhibition observed in the present experiment follows subcutaneous injection.

When the two strong carcinogens were injected together, each in approximately TD<sub>50</sub>, addition of effects was noted, but not to a full 100 per cent tumor yield. Nevertheless, because the latent period was shortened, the carcinogenic index for the combination equals the sum of the two individual indices. The possible reason for imperfect addition of effects is perhaps found in Table 2, where it is seen that the induction time for these two compounds is different. 1:2:5:6-Dibenzoacridine is slower, so the maximum effects of each on the exposed cells may not have been exerted at the same time.

**COMMENTS**

The demonstration that 1:2-benzanthracene is carcinogenic requires modifications of several previously公

bons on growth of transplanted tumors. They found that known carcinogens inhibited tumor growth rate but that noncarcinogens did not. They formed a separate category for 1:2-benzanthracene and chrysene, which inhibited growth rate but were thought to belong with the very weak carcinogens. This problem seems to be solved by the results here reported. Demerec (20) observed that 1:2-benzanthracene increased mutation rate in Drosophila, as did three strong carcinogens, whereas other noncarcinogens did not. The inconsistency for 1:2-benzanthracene which he found is eliminated by reclassifying it as a carcinogen according to present results.

1:2-Benzanthracene and chrysene were found to be definite carcinogens, contrary to many reports in the literature. These differences may be explained largely by two factors; namely, the use of a greater number of animals and a longer duration of test in the present work. The minimum induction time for 1:2-benzanthracene in mice was 161 days. Many tests reported in the literature were shorter than that (1, 8, 36, 39, 40, 55), and they may have been negative for that reason. In a few reports, the number of animals tested was probably inadequate as a basis from which to draw conclusions (1, 55). In some reports, the data submitted on survival are inadequate for judging carcinogenicity (24). Only two papers giving negative results appear to be based on reasonably adequate testing. Thus, Boyland and Burrows (10), after subcutaneous injection of aqueous colloidal solutions, had only seven rats alive at 6 months and one at 12 months, all without tumors. Shear and Leiter (48) made subcutaneous injections in mice, ten of which survived for 15 months without tumor. The few tumors previously reported have already been mentioned in the introduction. It can be concluded that the widespread impression that 1:2-benzanthracene is inactive is based on inadequate testing.

The minimum induction time for chrysene in our experiments was 849 days. Many of the reported experiments yielding negative results with chrysene were of much shorter duration than that (23, 31, 32, 37–42), and some of the tests were made on insufficient numbers of animals (33, 57–59). Among those performing more adequate tests were Boyland and Burrows (10), who found no tumors in four rats surviving at 12 months, and Shear and Leiter (48), who had thirteen mice alive without tumor at 12 months. Both these groups had attained the same induction time which we are reporting. The few positive results have already been mentioned in the introduction.

If the present claims for the carcinogenicity of chrysene are conceded, the finding of Haddow and Robinson of the tumor growth-inhibiting action of this compound, like that of strong carcinogens, is clarified (22).

Although 1:2-benzanthracene was found in these experiments to be a more potent tumor-inducing agent than chrysene, this should not be accepted as their final relative position, because they were tested in only one solvent. Chrysene had a lower solubility in triacrylin than did 1:2-benzanthracene. Much of the 5.0 mg. injected was not in solution. It is possible that fewer molecules of the former than of the latter were exerting their effects on the surrounding cells at any one time. The great importance of the solvent on the carcinogenic response is well known. In some other solvent the potency of these two chemicals might be different.

These experiments appear to be the first demonstration of additive or inhibitory carcinogenic effects of chemicals by the parenteral injection method. The previous work had been done by skin-painting or by feeding. The method appears
to open up new and quantitative ways of studying the dose:response relationships and the mechanisms of action.

Neither additive nor synergistic effects of two weak chemical carcinogens have been previously reported. The explanation is difficult, regardless of which phenomenon is accepted as explaining present results. Each chemical presumably was injected in an excessive or supersaturation dose. Just why the tumor yield should have been more than doubled when the dose of chemical was halved is not clear at the moment, although the possibilities were discussed.

The successful summation of the effects of two strong carcinogens was as anticipated and confirms previous results of others by skin-painting.

The inhibition of a strong by a weak carcinogen confirms the work of Lacassagne et al. (34) and of Wartman et al. (55). Such inhibition has been explained by Crabtree as the competition for the available receptors, presumably the SH groups. This theory might explain the inhibition of 1:2:5:6-dibenzanthracene by 1:2-benzanthracene, but the theory breaks down in the case of the two weaker carcinogens, chrysene and 1:2-benzanthracene, where summation was observed. Further experiments to elucidate this problem are under way.

**SUMMARY**

1. 1:2-Benzanthracene and chrysene were carcinogenic, inducing sarcoma on subcutaneous injection. The former was stronger than the latter, having both a shorter induction time and a higher tumor yield. The significance of these results with regard to the relation of chemical-physical characteristics of hydrocarbons to their carcinogenicity is discussed.

2. 1:2-Benzanthracene and chrysene injected together showed definite summation of carcinogenic effects if not actually a synergistic action. The tumor yield was greater than the sum of their individual tumor yields, despite the halving of the dose of each compound injected. This observation is significant, because it cannot be explained by the existing theories of inhibition or summation effects of carcinogenic compounds.

3. The carcinogenic effects of 1:2-benzanthracene and 1:2:5:6-dibenzanthracene injected together were not those of summation but apparently of inhibition.

4. The carcinogenic effects of 1:2:5:6-dibenzanthracene and 20-methylcholanthrene injected together were additive, although imperfectly so.

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