Susceptibility of Strain B6AF1/J Hybrid Infant Mice to Tumorigenesis with 1,2-Benzanthracene, Deoxycholic Acid, and 3-Methylcholanthrene*

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SUMMARY

Male mice belonging to strain B6AF1/J were given fifteen injections by stomach tube of 1,2-benzanthracene, deoxycholic acid, or 3-methylcholanthrene, each suspended in methocel-Aerosol O.T. Injections were started on the 7th-8th day post partum. Control mice received no treatment, or methocel-Aerosol O.T. alone. Some of the mice were killed at median ages of from 340 to 444 days, others at median ages of 547—600 days.

Many of the mice exposed to benzanthracene had pulmonary adenomas and hepatomas at autopsy. On the other hand, the mice treated with benzanthracene and killed at a median age of 547 days all bore hepatomas. In addition, almost all had pulmonary adenomas. This demonstrates that benzanthracene was highly carcinogenic when treatment with this agent was instituted during infancy. This conclusion is reinforced by the observation of numerous tumors in the lungs and livers of mice that received only two doses of benzanthracene during infancy. Although occasional pulmonary adenomas and hepatomas were found among the mice exposed repeatedly to deoxycholic acid, a comparison of tumor yields between these mice and the untreated controls, or the mice treated with methocel-Aerosol O.T. only, indicated a lack of carcinogenic activity for deoxycholic acid. Many pulmonary adenomas, hepatomas, and forestomach papillomas were noted in the mice exposed to methylcholanthrene. Several of the mice bore lymphocytic neoplasms, and others developed adenocarcinoma of the large intestine. A more potent carcinogenic effect was produced by repeated treatment with methylcholanthrene than with benzanthracene.

Although the hydrocarbon 1,2-benzanthracene is considered generally to be noncarcinogenic (6, 9, 10, 17—20), or weakly so (2, 6, 8), several investigators have observed this compound to be active in initiating skin tumorigenesis in the mouse (4, 5, 18). White and Eschenbrenner (24) added 1,2-benzanthracene to the diet and observed that two out of six rats developed multiple hepatomas which were considered to have been induced. In a series of experiments, Steiner and co-workers (21—23) observed that subcutaneous injection of this compound alone was followed by the development of sarcomas in as many as 55 per cent of the mice. Klein (12) injected 1,2-benzanthracene and croton oil into mice intramuscularly and obtained several hemangioendotheliomas and one fibrosarcoma at the injection sites. He concluded that these tumors were probably induced. In a series of experiments with different chemical agents including 3-methylcholanthrene, N-fluorenylacetic acid, and urethan, each administered orally to infant mice, Klein (13—15) demonstrated that the young mouse was especially susceptible to carcinogenesis. Advantage was taken of the increased sensitivity offered by this technic by feeding the questionable carcinogen benzanthracene to young mice in the present investigation. Under these cir-

* Supported by research grant CA-04097-05 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.
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Received for publication July 15, 1963.
cumstances, a potent tumorigenic influence was demonstrated in both the lungs and liver.

A number of investigators have exposed mice or rats to deoxycholic acid and found this agent to be noncancinogenic or weakly carcinogenic (6, 12). In two experiments, however, a significant increase in incidence of spindle-celled sarcomas (1) and lung tumors (16) was obtained following injection of deoxycholic acid into mice. It is known that the potent carcinogen 3-methylcholanthrene may be produced by an in vitro pyrolysis of deoxycholic acid followed by hydrogenation (25), although it would appear from the general lack of tumors reported for the latter compound that such a conversion either does not occur in vivo or does so infrequently. The occasional report of a tumorigenic effect produced by deoxycholic acid suggested that a similar effect might be obtained more readily by using an especially sensitive testing procedure such as that provided by instituting treatment during infancy. Thus, this agent was administered repeatedly to young mice in the present investigation. However, no enhancement in tumorigenesis was found even after a prolonged period of observation.

The carcinogenicity of methylcholanthrene in mice and in other species is well known (6, 19). In addition, methylcholanthrene has been shown to be active in hepatocarcinogenesis when administered to mice beginning in infancy (18) or as newborns (11). In the present study, suckling mice were again given oral injections of methylcholanthrene. This was done to provide a reference for evaluating the carcinogenic potency of orally administered benzanthracene or deoxycholic acid with that of a known potent carcinogen such as methylcholanthrene, and to confirm the hepatocarcinogenic action of the latter compound in a different strain of mouse. Many tumors were noted among the mice treated with methylcholanthrene. The details of this experiment and those referred to earlier are reported in the present communication.

MATERIALS AND METHODS

The investigation consisted of two related experiments, run concurrently, both using strain B6AF1/J infant male hybrid mice obtained from a cross between C57BL/6J females and A/J males. The litters derived from these matings were assigned at random to different groups. Experiment I consisted of five groups of 40 mice each and Experiment II of five groups of twenty mice each at the start of treatment. A major difference between the two experiments was the median age of the mice at death. Those in Experiment I were killed at median ages of from 340 to 444 days. Since some of the agents employed in this investigation—in particular, 1,2-benzanthracene and deoxycholic acid—are considered by many investigators to be weak carcinogens, at best, the mice in Experiment II were kept under observation longer (median age at death, 547–600 days), since it was considered probable that more time would be required to demonstrate carcinogenic activity for these agents.

Beginning with the 7th–8th day post partum, the mice were treated repeatedly by stomach tube with one of the following chemical agents: 1,2-benzanthracene, deoxycholic acid, or 3-methylcholanthrene. These were suspended in 0.1 per cent methocel-Aerosol O.T. (dioctyl ester of sodium sulfo-succinic acid) and administered 3 times weekly, 0.05 ml/dose. In one group the mice received a total of only two doses of benzanthracene given on alternate days. For controls, some of the mice were treated with 0.1 per cent methocel-Aerosol O.T. alone, whereas others were given no treatment. Treated mice were returned to their respective mothers immediately following each exposure to agent. The mice were weaned at about 5 weeks of age.

The specific treatments scheduled for the different groups in each experiment follow. The mice received a total of fifteen doses of agent in all groups under treatment except group 10, in which they received only two doses.

**Experiment I**

Group 1, no treatment.

Group 2, 0.1 per cent methocel-Aerosol O.T. only.

Group 3, 3 per cent 1,2-benzanthracene in methocel-Aerosol O.T.

Group 4, 3 per cent deoxycholic acid in methocel-Aerosol O.T.

Group 5, 3 per cent 3-methylcholanthrene in methocel-Aerosol O.T.

**Experiment II**

Group 6, no treatment.

Group 7, 0.1 per cent methocel-Aerosol O.T.

Group 8, 3 per cent benzanthracene in methocel-Aerosol O.T.

Group 9, 3 per cent deoxycholic acid in methocel-Aerosol O.T.

Group 10, 3 per cent benzanthracene in methocel-Aerosol O.T.

1 Obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

2 Obtained from Eastman Organic Chemicals, Rochester 8, N.Y. The sample used in this investigation, on analysis, showed a melting point of 161°–162° C.

3 Obtained from Matheson-Coleman & Bell Divn., The Matheson Co., East Rutherford, N.J.
In view of the similarity between these two experiments, the data from each were combined under the same headings in Table 1.

The animals were kept in stainless-steel, hanging cages in an air-conditioned room and fed Purina Laboratory Chow and tap water ad libitum. Mice were checked daily, and cachexic animals were killed by cervical dislocation, as were the survivors subsequently. All mice were autopsied, and grossly visible tumors and other macroscopic pathology were recorded. An occasional animal was lost because of cannibalism or advanced post-mortem changes. These mice are not included in the totals in Table 1. Organs containing tumors or masses suspected of being tumors were excised, fixed in Tellyesniczky’s fluid, and the tissues prepared for subsequent staining with hematoxylin and eosin. All tumor diagnoses were confirmed histologically.

RESULTS

**Experiment I.—** Carcinogenicity of benzanthracene, deoxycholic acid, and methylcholanthrene in B6AF1/J male mice autopsied at a median age of 340 or 437 days (Table 1).

The present experiment is part of a continuing study on chemical carcinogenesis with the use of infant male mice of the B6AF1/J hybrid strain. A 10 per cent incidence of pulmonary adenomas was observed among untreated male mice of this strain when these were killed at a median age of 441 days (15). In the same study, another group of infant males was treated with the solvent methocel-Aerosol O.T. alone, and a pulmonary tumor incidence of 26 per cent was recorded during a comparable observation period. These lung tumors and those in the untreated controls, in most instances, occurred as small, solitary nodules. Aside from one additional tumor (a reticulum-cell neoplasm, type A, observed in the small intestine of a mouse treated with methocel-Aerosol), no other neoplasms were noted among the mice described above. These data are listed in Table 1 under groups 1 and 2.

The mice in group 3 were exposed repeatedly to benzanthracene beginning with infancy and were autopsied at a median age of 437 days. The incidence of pulmonary adenomas for these mice rose to 95 per cent. Thirty-six of the 39 mice in this group developed hepatomas, an average of 2.1 per tumor-bearer. Two other mice in the group were observed with solitary forestomach papillomas, and one with a reticulum-cell neoplasm, type A, found in

### Table 1

Susceptibility of Infant B6AF1/J Hybrid Male Mice to Tumor Induction Following Repeated Oral Administration of 1,2-Benzanthracene (BA), Deoxycholic Acid (DOCA), and 3-Methylcholanthrene (MCA) Each Suspended in Methocel-Aerosol O.T. (MA)

<table>
<thead>
<tr>
<th>Exp. NO.</th>
<th>Group no.</th>
<th>Total No. Mice*</th>
<th>Treatment†</th>
<th>Hepatomas</th>
<th>Pulmonary adenomas</th>
<th>Lymphocytic neoplasms</th>
<th>Reticulum-cell neoplasms type A</th>
<th>Fore-stomach papillomas</th>
<th>Large intestine adenocarcinomas</th>
<th>Median age at death all mice (days)</th>
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<tr>
<td>I</td>
<td>1†</td>
<td>59</td>
<td>None</td>
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<td>0</td>
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<td>2†</td>
<td>58</td>
<td>0.1% MA only</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>444</td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>20</td>
<td>3% BA in MA</td>
<td>18</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>492</td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>20</td>
<td>3% DOCA in MA</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>497</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>35</td>
<td>3% MCA in MA</td>
<td>40</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>540</td>
</tr>
<tr>
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<td>6</td>
<td>20</td>
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<td>0</td>
<td>600</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>20</td>
<td>0.1% MA only</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>547</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>20</td>
<td>3% BA in MA</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>547</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>20</td>
<td>3% DOCA in MA</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>547</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>20</td>
<td>3% BA in MA</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>568</td>
</tr>
</tbody>
</table>

* Survivors at time first tumor was observed in the group.
† The treated mice received a total of fifteen doses each, except in group 10, where only two doses were administered (0.05 ml/dose).
‡ Data for these two groups were obtained from a previous report (15).
an inguinal lymph node. Similar tumors have been described in detail by Dunn (3).

Few tumors were observed among the mice treated with deoxycholic acid and killed at a median age of 437 days (group 4). These tumors were all pulmonary adenomas, a total of five being noted in the four tumor-bearing mice in the group.

The mice in group 5 were exposed repeatedly to methylcholanthrene. Many of these animals died relatively early in the experiment, the median age at death for all the mice being 340 days. Some of the animals appeared cachexic prior to death, and at autopsy the lungs of these mice were found to contain numerous tumor nodules. A potent tumorigenic effect was observed for methylcholanthrene, neoplasms appearing in the lungs, liver, forestomach, thymus, and large intestine. All 35 of the mice developed multiple lung tumors that were diagnosed histologically as pulmonary adenomas. Frequently, the lungs were crowded with tumor nodules 1–2 mm. in diameter and larger. Seventeen of the mice (49 per cent) bore hepatomas, an average of 2.6 per tumor-bearing. Papillomas of the forestomach were noted in 63 per cent of the mice. These tumors were multiple in fifteen of the mice (49 per cent) bore hepatomas, an average of 2.6 per tumor-bearer. Papillomas of the forestomach were noted in 63 per cent of the mice. Upon histologic examination, it was noted that the thymus and surrounding tissues were completely replaced by a large, compact mass of small lymphocytic cells. Tumors of the thymus and surrounding tissues were completely replaced by a large, compact mass of small lymphocytic cells. Tumors of the large intestine in the region of the caecum were noted in 63 per cent of the mice. All 35 of the mice developed multiple lung tumors that were diagnosed histologically as pulmonary adenomas. The median age at death was the same for this group as for the methocel-Aerosol-treated controls in group 7. Ninety per cent of the mice with hepatomas had more than one such neoplasm per liver, the average being 3.2 tumors per mouse. All nineteen of the mice with pulmonary adenomas had multiple tumors, an average of 3.5 per mouse. Aside from these tumors and the hepatomas, no other neoplasms were noted.

Few tumors were observed in the mice treated with deoxycholic acid and autopsied at a median age of 547 days (group 9). Only three mice out of nineteen bore hepatomas, all solitary tumors, whereas seven bore pulmonary adenomas, an average of 1.3 per tumor-bearer.

The mice in group 10 received two doses of benzanthracene as compared with fifteen doses in group 8. The median age at death was slightly longer for the former mice (568 days, as compared with 547 days). Eighty per cent bore hepatomas, an average of 1.8 per tumor-bearer, whereas all three of the mice (85 per cent) bore pulmonary adenomas, an average of 3.1 per mouse. No other neoplasms were noted in this group.

Purity of benz[a]anthracene.—The marked carcinogenic activity demonstrated for 1,2-benz[a]anthracene in this investigation contrasted sharply with the striking lack of carcinogenic influence noted by others. The possibility was considered that perhaps some impurity in the sample administered rather than the 1,2-benz[a]anthracene was responsible for the tumorigenic effect. An extensive chemical analysis of the original sample was therefore conducted with the following results:

Thin-layer chromatography, with a solvent system of Skelly F: diethyl ether: glacial acetic acid in the ratio of 50:50:1 and with a supporting medium of silica gel G, showed one spot when the sample was dissolved in hexane. The Rf value was 0.79. One peak, slightly unsymmetrical, was observed with gas chromatography. The sample was dissolved in chloroform and the column used was Silicone DOW II on Chromasorb W. The ultraviolet spectrum was identical with the published spectrum of 1,2-benz[a]anthracene. In the latter

instance, the sample was dissolved in hexane. With reverse-phase ascending paper chromatography on mineral oil-embedded filter paper and with a solvent system of ethanol and methanol, 1:1, a trace contaminant that fluoresced slightly under ultraviolet was located below the highly fluorescent major component benz[a]anthracene. The sample itself was dissolved in hexane. The Rf value for the major component, benz[a]anthracene, was 0.44, and for the minor component the value was 0.18. It appeared that the impurity had about 1 per cent of the fluorescence of the major spot. Recrystallization, 3X, of the commercial sample of benz[a]anthracene from hot ethanol followed by reverse-phase chromatographic analysis of the purified compound showed the same trace spot with about the same relative degree of fluorescence as compared with the major spot. This trace compound is not 1,2-benz[a]anthracene-7,12-dione, the starting material used in the synthesis of 1,2-benz[a]anthracene by Eastman Kodak, or naphthacene, a by-product that occasionally forms during the course of the reaction. Both these compounds migrated differently from the impurity with the reverse-phase chromatographic system in use.

**DISCUSSION**

Many tumors, especially pulmonary adenomas and hepatomas, were found among the mice after repeated treatment with 1,2-benzanthracene. The incidence of lung tumors remained at 95 per cent for the mice killed at a median age of 437 or 547 days (groups 3, 8). However, incidence of hepatomas increased with age from 46 to 100 per cent in these two groups, respectively. It is apparent from these data that administration of 1,2-benzanthracene, when instituted during infancy, produced a potent carcinogenic effect on both the lungs and liver of the host mice. Reduction of the total administered dose of hydrocarbon from 22.5 mg. (groups 3, 8) to 3 mg. (group 10) still resulted in a high yield of pulmonary adenomas and hepatomas, providing additional evidence of the carcinogenic potency of 1,2-benzanthracene under the conditions of testing employed. These findings support the data of Steiner and co-workers (21--23), who showed that this compound was tumorigenic when injected subcutaneously into young adult mice. Contrary to these results, a number of investigators (2, 6, 8--10, 17--20) have concluded that benzanthracene possesses little or no carcinogenic activity. Steiner and Falk (23) suggested two reasons for the failure of some of these investigators to obtain tumors with benzanthracene: (a) the use of too few animals and (b) too short a period of observation. To this may now be added a third—namely, the treatment of older rather than young animals such as were used in the present study. The fact that a compound considered to be noncarcinogenic under one set of conditions may demonstrate some or appreciable carcinogenic activity under other conditions is not surprising. However, this emphasizes the need for a comprehensive testing program when agents suspected of being carcinogens or related chemically to known carcinogens are to be evaluated. Such a program certainly ought to include long as well as shorter periods of observation, sufficiently large groups of animals, different routes of administration of agents, different species, including, perhaps, some not now commonly employed in the laboratory, and infants (or newborns), as well as older animals. It is possible that some compounds now considered to be noncarcinogenic, or questionably so, will show a moderate or high degree of carcinogenic activity when these are re-examined along the line suggested above.

The relationship of purity of 1,2-benz[a]anthracene to the findings in the present investigation was discussed with Dr. Falk of the Carcinogenesis Studies Branch, National Cancer Institute. The following comments are derived from this discussion.

1. Steiner and co-workers considered the possibility that highly potent impurities might perhaps account for the occasional tumorigenic activity reported for benz[a]anthracene. Thus the same commercial product but from a different batch was tested for carcinogenicity, as was another obtained from England, and a synthetic sample prepared in Dr. Falk’s laboratory. All the samples possessed carcinogenic activity. The present findings with commercial benz[a]anthracene thus confirm this carcinogenic activity.

2. The compounds suspected as impurities include 1,2-benz[a]anthracene-7,12-dione, 1,2-benz[a]anthracene-7,12-diol, and naphthacene. Of the three, naphthacene already has been tested and reported to be noncarcinogenic (6). Although published data on the carcinogenicity of the other two compounds appear to be lacking, it is probable that these two derivatives of 1,2-benz[a]anthracene possess little, if any, carcinogenic activity. It may be pertinent here to cite the recent findings of Heidelberger et al. (7) on the carcinogenic potency of different derivatives of 1,2,5,6-dibenzanthracene. The latter investigators found that the 3-4 and 9-10 anthraquinones were noncarcinogenic when applied repeatedly to the skin of mice or when injected subcutaneously.

*H. L. Falk, personal communication.
Under these circumstances, it appears justifiable to attribute the carcinogenic influence noted in the present study to benz(a)anthracene rather than to the trace impurity in the sample administered.

Deoxycholic acid, unlike benzanthracene, did not influence carcinogenesis when fed to young mice (Table 1, groups 4, 9). Aside from an occasional report ascribing tumorigenic activity to deoxycholic acid (1, 18), most investigators have found the latter compound to be noncarcinogenic or weakly so (6, 12). Kennaway tried unsuccessfully to confirm the marked carcinogenicity of deoxycholic acid reported earlier from his laboratory (1). Although experimental conditions were essentially the same in the repeat experiment, the deoxycholic acid had to be dissolved in a different sample of sesame oil, since the original solvent was completely used up. This suggested to Kennaway that the marked carcinogenic activity observed initially in his laboratory for deoxycholic acid was probably due to the presence of a co-carcinogen in the particular batch of sesame oil. Since the use of infant mice provides a sensitive test for carcinogenesis (13–15), failure to enhance tumorigenesis in the present investigation adds strong support to the view that deoxycholic acid is noncarcinogenic in this species.

Many of the mice treated with methylcholanthrene developed hepatomas, pulmonary adenomas, and forestomach papillomas (Table 1, group 5). In addition, some bore lymphocytic neoplasms and adenocarcinomas of the large intestine. The effectiveness of this hydrocarbon as a hepatocarcinogen in strain B6AF/1 confirms a similar observation made with mice related to strain A/He (13) and thus demonstrates that the action is not strain-specific. Occasional hepatomas have also been induced in mice belonging to strains C57BL and C3H when these were given one subcutaneous injection of methylcholanthrene as newborns (11).

Although essentially the same incidence of lung tumors was obtained for benzanthracene as for methylcholanthrene, other data demonstrate the latter compound to be a more potent carcinogen in the infant mouse. Thus, an average of three forestomach, skin with its stratified squamous epithelium has also been shown to be refractory to the tumorigenic action of benzanthracene alone (6, 18, 19). However, skin tumorigenesis has been initiated with this agent in the mouse (4, 5, 18). A similar finding has not yet been reported for the forestomach, but this is anticipated.

ACKNOWLEDGMENTS

The author is deeply indebted to Dr. Joseph A. Onisko and Mr. Weiss R. Moorehead of the Department of Biochemistry, University of Tennessee Medical Units, for their splendid cooperation and able efforts in completing the chemical analyses reported in this study. Also, the author is most grateful to Dr. Hans Folk, Chief, Carcinogenesis Studies Branch, National Cancer Institute, for his discussion relative to the chemical purity of benz(a)anthracene. Finally, many thanks are due Mrs. Sue Little and Miss Bettye Jennings, and to Mr. William E. Wright, dental student in the University of Tennessee College of Dentistry, for their able technical assistance.

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Michael Klein