In a previous publication responses of 3,4,9,10-dibenzpyrene-induced subcutaneous sarcomas and their transplants to various chemotherapeutic agents were described (5). This report deals with intrinsic factors such as sex, age, nutritional status, and connective-tissue reactivity which determine the rate of formation and subsequent behavior of such tumors in the untreated hosts, and with studies on the fate of the carcinogen, large amounts of which, in contrast to other polycyclic hydrocarbons, remain at the injection site and are demonstrable in the tumors.

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† The authors wish to acknowledge the cooperation of Dr. Agnes B. Russfield and her valuable contribution to the study of the histopathology of these tumors. The technical assistance of R. Kenney, H. Rys, C. M. Crooker, and Maureen F. O’Connell is also gratefully acknowledged.

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**MATERIALS AND METHODS**

The *carcinogen* used was 3,4,9,10-dibenzpyrene, produced by the A. D. Little Company, Cambridge, Mass., as sample number C-61947, with an infrared spectrum superimposable on that previously obtained with 3,4,9,10-dibenzpyrene furnished us by Lacassagne (6). Earlier experiences with this carcinogen have been reviewed (3), and dosage response curves have been published. A standard dose of 500 μg. per mouse was used, equivalent to five times the 100 per cent tumor dose previously established (4). This was suspended in peanut oil by stirring for 8 hours at 80°–100° C., or for 2–4 hours at 150°–175° C. These procedures did not alter the ultraviolet spectrum of the carcinogen. The carcinogen (500 μg. of 3,4,9,10-dibenzpyrene in 0.1 ml. of peanut oil) was injected into the left groin by means of a 22-gauge needle. Male and female *C57BL/6* Jax mice, 2–5 months of age, were used. They were housed, ten to twelve to a cage, on San-I-cell in air-conditioned and light-conditioned animal.
rooms (12-hour light-dark cycle) and were given tap water and Purina chow ad libitum. They received a single injection of the carcinogen in groups of 250–300 animals, one group being given injections each week.

Some of these animals were used in a mass chemotherapy screening program of the Sloan-Kettering Institute, and advantage was taken of the availability of 24,952 males and 6,701 females with induced tumors to obtain statistical information. The nutritional and biochemical studies reported here were done on additional smaller groups of similar animals. To keep conditions as uniform as possible, the relatively large dose of 5 times 100 per cent tumor dose (500 \( \mu g \)) was used, and all observations reported here are applicable only to animals treated by such doses of 3,4,9,10-dibenzpyrene.

Tumor incidence was studied by weekly palpation of the injection site, and, when the tumor had reached 1 cm. in diameter, the time elapsed since injection was recorded. Tumor incidence was corrected for deaths from nontumor causes. The variation in tumor incidence from one sample group to another was examined by means of a simulation model which served to establish the variations due to chance. The significance of differences between the slopes of tumor-incidence curves in males and females was ascertained by the usual "t"-test.\(^1\) The latent periods were calculated by the method described by W. R. Bryan and M. B. Shimkin (2).

"Tumor yield" was defined as the percentage of tumors arising from a selected group of approximately 1,000 animals 10–28 weeks after injection of carcinogen was begun, as described above. Body weights were measured in 77 males prior to injection of carcinogen and again upon appearance of the tumors. The effects of caloric restriction were studied by limiting the food available to each mouse to 2–2.5 gm. of Purina chow pellets per day for moderate, and to 0.5–1 gm. per day for severe caloric restriction, water being allowed ad libitum. In these studies the mice were kept in individual cages and weighed weekly at the same hour of the day. The effects of such restriction were studied during the induction period, the restriction beginning 4 or 5 weeks after injection of carcinogen and in other animals at the time when tumors had reached 1 cm. in diameter. Effects of age were studied by comparing tumor incidence and average latency of tumors in approximately 100 older animals given injections when 8–12 months old, with the data obtained in younger animals.

**Histopathogenesis of tumors.**—At approximately weekly intervals, from 2 to 16 weeks after injection, 5–10 males and 5–10 females were killed and the injection site was excised and fixed in 4 per cent formaldehyde for histologic and fluorescence microscopic study. Transplant characteristics were ascertained by trocar transplantation of numerous induced tumors into C57BL/6 and other strains and by comparison in hosts of both sexes of tumor growth rates in the resulting tumor lines. Tumors which had arisen 10–13 weeks after carcinogen injection were transplanted, and their growth rate was compared with that of transplants from tumors that had arisen 23–25 weeks after injection of the carcinogen. Transplantation results, with small versus large donor tumors, were compared.

**Growth rate of the induced tumors.**—Plastograms (1) were made and growth curves in males and females were obtained.

**Determination of the fate of the carcinogen.**—One thousand C57BL/6 males, 10 to a cage, were kept on a fine wire screen placed on top of a sheet of Whatman filter paper No. 3, which covered the bottom of the cage. Half these mice received 500 \( \mu g \) of 3,4,9,10-dibenzpyrene in 0.1 ml of peanut oil subcutaneously, and the other 500 mice received the same injections with 3 per cent cholesterol added to the peanut oil. The urine which was absorbed by the filter paper and the feces which remained on top of the screen were collected separately twice a week for 4 months and placed into 95 per cent ethanol. After evaporation of the alcohol, urine and feces were extracted with organic solvents and the extracts studied by ultraviolet spectroscopy. This was done in our own laboratory, as well as by others (7). Extracts were also made from fully established tumors and examined for 3,4,9,10-dibenzpyrene. The stability of the carcinogen in the presence of urine and feces and while exposed to light on filter paper was ascertained by placing pure 3,4,9,10-dibenzpyrene under these conditions for various periods of time, followed by re-extraction and ultraviolet spectrographic analysis.

Paraffin sections were made from tumors fixed for short periods of time in 4 per cent formalin for study by ultraviolet fluorescence microscopy. The bright yellow fluorescence of the carcinogen permits its localization.

**RESULTS**

The curve of the average tumor-appearance rate is shown in Chart 1 for males and females.
Tests of significance are extremely difficult to justify because of the high correlation from one point to the next on such a cumulative curve. Variations expected by chance were therefore simulated by actually carrying them out in the process of sampling, and a direct comparison for the observed data was thus created. Starting with the average curves in A as actual theoretical cumulative curves, a population was made up for both the male and the female groups having the characteristics of these given curves. Samples were then drawn artificially at random from these specific populations. The resulting curves indicate expected chance variations if the populations in A were as indicated. Within the males and females, respectively, variations were small and close to the theoretical curves based on expected chance variations alone. The variability of tumor incidence from group to group was negligible, and the rate of tumor development in such groups is predictable. There was a significant difference between the curve of tumor development in males as compared with that in females.

**Average time of latency.**—For the males the average time of latency was 14.5 weeks, with a standard error of the mean of 0.8; for the females, 18 weeks, with a standard error of the mean of 1.4. A value of "t" of 15.2 indicates that this difference has a probability less than 0.01 of occurring by chance, and therefore it can be considered as highly significant. The average weekly tumor yield in males after the 10th week following the beginning of injection of carcinogen was 12.9 per cent of the population examined, and in females it was 9.3 per cent. The difference between yield from males versus females is significant at a P of < 0.01 with \( t = 6.2 \).

**Body weight.**—At the time of injection body weight, within a range from 11.0 to 20.7 gm., had no effect on the rate of tumor development, and body weight gains during tumor development, ranging from 2.9 gm. to 15.4 gm., were in no way correlated with the rate of tumor formation. The lack of correlation between body weight at time of carcinogen injection and time of tumor appearance is shown in Table 1. This table also shows the

### TABLE 1

**Body Weight Changes During Tumor Development and Time of Tumor Appearance in 77 Males**

<table>
<thead>
<tr>
<th>No. WEEKS</th>
<th>No. ANIMALS</th>
<th>Initial Body Weight Range, Gm.</th>
<th>Body Weight Increase, Gm.</th>
<th>Weekly Body Weight Increment, Gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEFORE TUMOR DETECTION</td>
<td></td>
<td></td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>13</td>
<td>19</td>
<td>11.0–19.6</td>
<td>8.4</td>
<td>3.6–12.2</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>11.4–19.8</td>
<td>9.04</td>
<td>3.9–13.2</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>11.9–20.7</td>
<td>10.0</td>
<td>2.9–15.4</td>
</tr>
<tr>
<td>16</td>
<td>11</td>
<td>13.3–18.4</td>
<td>9.1</td>
<td>5.2–13.1</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>13.8–17.9</td>
<td>9.2</td>
<td>4.3–12.2</td>
</tr>
</tbody>
</table>

* Note absence of correlation between these two parameters.
lack of correlation between weight gain during the latent period and the length of the latent period. Moderate caloric restrictions in males, limiting weight gain to less than 10 per cent of body weight during the 23 weeks of tumor induction, resulted in a significant prolongation of the average time of latency to 20.3 weeks. Chart 2 shows the delay of tumor formation in animals on restricted caloric intake. This differs from the changes in the rate of tumor appearance brought about by chemotherapy, which delays by 6 weeks, and subsequently accelerates, tumor formation (cf. Ref. 5). The established tumor is no longer susceptible to inhibition by caloric restriction (Table 2). How-

**TABLE 2**

**EFFECT OF CALORIC RESTRICTION ON THE GROWTH RATE OF ESTABLISHED, INDUCED TUMORS**

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Sex</th>
<th>Period of caloric restriction</th>
<th>Body weight loss (–) or gain (+) in gm.</th>
<th>Tumor weight at end of restricted period (gm.)</th>
<th>Significance t (P&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>♂</td>
<td>23</td>
<td>–3.3</td>
<td>3.5</td>
<td>NS*</td>
</tr>
<tr>
<td>25</td>
<td>♂</td>
<td>Controls ad lib.</td>
<td>–1.3</td>
<td>3.05</td>
<td>NS</td>
</tr>
<tr>
<td>25</td>
<td>♀</td>
<td>23</td>
<td>–4.8</td>
<td>3.3</td>
<td>NS</td>
</tr>
<tr>
<td>17</td>
<td>♀</td>
<td>9</td>
<td>–1.4</td>
<td>3.9</td>
<td>NS</td>
</tr>
<tr>
<td>17</td>
<td>♂</td>
<td>Controls ad lib.</td>
<td>–0.33</td>
<td>0.8</td>
<td>2.3 B†</td>
</tr>
<tr>
<td>16</td>
<td>♂</td>
<td>7</td>
<td>–2.7</td>
<td>0.33</td>
<td>1.0 NS</td>
</tr>
<tr>
<td>15</td>
<td>♂</td>
<td>Controls ad lib.</td>
<td>+0.5</td>
<td>0.5</td>
<td>1.0 NS</td>
</tr>
<tr>
<td>8</td>
<td>♀</td>
<td>7</td>
<td>–3.2</td>
<td>0.2</td>
<td>2.0 NS</td>
</tr>
<tr>
<td>7</td>
<td>♀</td>
<td>Controls ad lib.</td>
<td>+0.9</td>
<td>0.5</td>
<td>2.0 NS</td>
</tr>
</tbody>
</table>

* Not significant. † Borderline.

**CHART 2.**—Showing the effect of caloric restriction on cumulative tumor incidence following subcutaneous injection of 3,4,9,10-dibenzapyrene. **Top figure:** Slightly delayed appearance of first tumors and the markedly depressed slope of the tumor-incidence curve. **Bottom figure:** The caloric restriction applied maintained relatively constant body weight.
ever, after the tumor has reached 1 cm., the survival time is prolonged from an average of 27 days to a significantly longer average of 36 days in the calorie-restricted animals.

Age at the time of carcinogen injection had only questionable effect on the average latent period in the males. In 100 young males the average latent period was 14.5 weeks, and in the older (8–12 months) males it was 16.2 weeks. The t value for the difference was 2.1, P 0.01–0.025, therefore of borderline significance. In 103 females more advanced age at the time of carcinogen injection (8–10 months) resulted in a significantly shorter time of latency, 15.3 weeks, as compared with 18 weeks with a t value of 2.9 and P < 0.01, and, therefore, age at the time of injection appears to affect the resulting tumors in females.

Histopathogenesis of tumors.—Two weeks after injection, large subcutaneous cysts and Langhans'-type giant cells were seen. These cysts divided into smaller ones at about 8 weeks, at which time the number of Langhans' cells reached its maximum.

Severe inflammation was found in about half the mice of both sexes at 2 weeks; in all males by 8 weeks; and in all females by 10–11 weeks. Hyper trophy and increasing atypicality of fibroblasts were seen (Figs. 1 and 2). This culminated in development of the first histologically unquestionable fibrosarcomas by 8 weeks in males and a little later in females (Figs. 3 and 4).

All tumors examined in approximately 200 mice of both sexes were fibrosarcomas, with occasionally bizarre giant cells, areas suggesting myxomatous degeneration and necrotic foci. Tumors in the males seemed to be surrounded by a more intense connective-tissue reaction than those in females. Tumors in females had a slightly greater tendency to invade skin and muscle.

Transplantability.—The transplantability of the induced tumors was ascertained by implanting them into C57BL/6 mice, where they grew with 100 per cent takes and led to death within 4 weeks. In other strains, C57BR/cd and Swiss mice, there was temporary growth followed by regression. The characteristics of transplants derived from these induced tumors are described elsewhere (5).

Transplants into C57BL/6 mice from tumors which had arisen 13 weeks after carcinogen injection weighed 1.6 gm. after 21 days, and corresponding transplants made from tumors that had arisen 25 weeks after carcinogen injection weighed 1.02 gm., the difference being significant. Thus the growth rate of the induced tumor, when transplanted, appears to be related to the time of latency of the initially induced tumor (short latency—rapid growth; long latency—slow growth). No such correlation exists for the growth rate and latency of the induced tumor within its original host. The size of the induced donor tumor had no clear-cut effect upon the growth rate of its transplants.

Growth rate of the established tumors.—Once the tumors had reached 1 cm. in diameter, they grew at a fairly uniform rate to approximately 2 sq. cm. in surface by the end of 1 week. At the end of the 2d week, however, tumor sizes varied from 2.5 to 6.5 sq. cm. Half the animals survived to the end of the 3rd week, with tumors then measuring from 3 to 5.5 cm. A few animals survived more than 4 weeks, and the largest tumors at that time reached 7 sq. cm. Based on studies of 60 males and females with tumors appearing within 16 weeks after carcinogen injection, and 50 males and females with tumors appearing later, the growth rate of the established tumor was not dependent on its latent time after carcinogen injection.

Fate of the carcinogen.—Extraction with organic solvents of urine and feces of mice having received subcutaneous injections of 3,4,9,10-dibenzpyrene in peanut oil containing 3 per cent cholesterol failed to reveal any polycyclic metabolites of the carcinogen. Some cholesterol was recovered from the feces. Extraction of fully developed tumors done in our own laboratory as well as by others yielded only unaltered 3,4,9,10-dibenzpyrene. The procedures used by us were the same as those of Unsereen and Fieser (7), and our observations confirmed these authors’ work and extended it to include extracts from urine excreted by several hundred mice for 30 days.

Ultraviolet fluorescence microscopy of tumors showed that during the early phases after injection, the fluorescent material is taken up by foreign-body giant cells. In fully developed tumors, fluorescent material remains in the center and at the periphery of the tumors, largely in foreign-body giant cells and scattered throughout the stroma.

In transplants derived from induced tumors, the fluorescent material remained visible in the first, second, and third transplant generations but not in subsequent transplants. Studies with the polarizing microscope revealed that in animals which had received cholesterol a good proportion of this material could be demonstrated by its birefringence in much the same tissue sites as the carcinogen.

*L. F. Fieser, personal communication.
DISCUSSION

Single subcutaneous injections of 500 μg. of 3,4,9,10-dibenzpyrene in peanut oil produced in C57BL/6 mice subcutaneous fibrosarcomas with great regularity. There are, however, wide individual variations in the time of latency. Some animals will have 1-cm. tumors 10 weeks after injection, while others develop tumors 24 weeks and later after injection. The distribution of this biologic variability among 24,952 males and 6,701 females in groups of 250 to 300 animals was random, and the expected tumor yield from a series of mice of an inbred line given single subcutaneous injections of 500 μg. of 3,4,9,10-dibenzpyrene was predictable. Variations of the growth rate of the established tumor were unrelated to the times of latency. Moderate caloric restriction depressed and postponed in time the curve of tumor appearance. This effect was readily distinguishable from that which chemotherapeutic agents given during induction exert upon the rate of tumor formation. The established (1-cm.) tumor is not affected by moderate or severe caloric restriction, which is of advantage for its use in chemotherapeutic studies. These induced neoplasms are transplantable within the strain of origin, and transplants from tumors which were rapidly induced grew faster than those of tumors with long times of latency. The size of the donor tumors did not affect the growth of transplants.

There are significant differences in rate of tumor formation (and consequently, tumor yield) between males and females which correspond closely with the sex difference of the initial inflammatory response to the injection of carcinogen. Fully developed tumors are surrounded by a more intensive connective-tissue reaction in males than in females. Age at the time of injection had no significant effect in males, whereas in females tumors appeared faster in older mice. In studies of these autochthonous tumors, these sex differences must be taken into consideration. The retention of the carcinogen within the tumor may have disadvantages for some types of studies, but it presents an interesting opportunity for cytologic localization by means of ultraviolet fluorescence microscopy.

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Host Factors Influencing the Behavior of Subcutaneous Sarcomas Induced by 3,4,9,10-Dibenzpyrene in C57BL/6 Mice

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