Factors Affecting the Polynuclear Hydrocarbon Level in Rat Mammary Glands*

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SUMMARY

The levels of 3-methylcholanthrene (MCA) in rat mammary and fat tissues after feedings were measured. After a single feeding, the amounts which were found in the mammary tissue and fat were proportional to the amount fed. In contrast, multiple feedings did not result in higher tissue levels. MCA levels after feeding were lower in pregnant rats than in normal rats, whereas those of either castrated or hypophysectomized rats were higher. There was little difference between results found for normal males and normal females.

The amount of hydrocarbon found in the mammary gland and fat after feeding depended upon the nature of the hydrocarbon fed. Very little, if any, phenanthrene was found in these tissues 4 hours after a phenanthrene meal. Benzo[a]pyrene was localized in the mammary tissue and fat better than was MCA, whereas 7,12-dimethylbenz[a]anthracene behaved similarly to MCA.

The data suggest that hydrocarbons reached the mammary glandular cells because of their intimate association with the mammary fat pads. This mechanism appears to account for much of the sensitivity of mammary glands to oral feeding of polycyclic hydrocarbons.

Several workers (1, 14, 21) have reported that oral administration of polycyclic carcinogens will produce mammary carcinomas in mice. The process has required many months, and, in some studies, feeding of carcinogens failed to cause mammary cancer altogether (2, 7, 22, 28). In contrast, Huggins et al. (17, 18) and Dao and Sunderland (12) have observed the rapid development of mammary adenocarcinomas in female rats after oral administration of 3-methylcholanthrene (MCA). Time for tumor induction was dramatically reduced if the rats were bred, the data (12) being suggestive of the cocarcinogenic relationship between croton oil and subcarcinogenic doses of 7,12-dimethylbenz[a]anthracene (DMBA) (3, 4).

It has been shown that, after intragastric administration, methylcholanthrene is found in the mammary tissue and milk of lactating rats (10, 25). The levels of unchanged hydrocarbon in both fat and mammary glands were many times higher than in any other tissue examined, and it was suggested that adipose tissue of the mammary gland served as a storage depot for the carcinogen. The high levels of methylcholanthrene in the breast explained at least part of the unique sensitivity of that organ.

Experimental conditions which affect rat mammary tumor induction are duration of treatment, sex, presence of the gonads and hypophysis, existing pregnancy at the time of exposure to the carcinogen, and the temporal relationship between treatment with carcinogens and any subsequent pregnancy (11). Most of these factors alter the breast tissue through endocrine mechanisms, and it is most probable that their effect on tumor development is by these means. Nevertheless, the similarity in structure between methylcholanthrene and the steroid hormones leaves room for speculation that hormonal states of the rat might affect the amount of methylcholanthrene available to the breast. In this way, alteration of hormonal states might alter tumor yields by changing the
concentration of the carcinogen in the target tissue rather than by affecting the target cells directly. The experiments reported here were undertaken to evaluate this possibility. To do so, direct analyses of mammary levels of carcinogens were carried out. Depot fat also was analyzed to show whether the hydrocarbons were handled differently by mammary tissue and by general body fat.

MATERIALS AND METHODS

The procedures used in this study have been reported in detail elsewhere (10). By means of stomach intubation, Sprague-Dawley rats were fed 1.0 ml. of sesame oil containing 10–50 mg. of hydrocarbon. After a variable period of time, the animals were sacrificed. In pregnant rats, the isolation of mammary tissues from the mammary fat presented no difficulty, since the mammary gland consisted almost entirely of glandular tissues and they were easily recognizable. The isolation of mammary tissue from the mammary fat was difficult in non-pregnant rats, since the amount of mammary fat greatly exceeded that of the glandular tissue. In a few experiments with nonpregnant rats, the superficial mammary fat pads lying above the residual glandular tissue were carefully dissected away under a dissecting microscope. The exact amount of fat in the mammary tissue so removed, however, was not determined in the present experiment. Mammary tissue, mammary fat, and perirenal fat samples were weighed, saponified, and extracted with benzene to collect the nonsaponifiable lipides. Quantitative assays of MCA, DMBA, benzo[a]pyrene (BP), and phenanthrene (Ph), were carried out directly on the crude benzene extract, with the use of an Aminco-Bowman spectrophotofluorometer. The wavelengths used for the analyses were selected to employ the maximum excitation peak for each compound and, for Ph, the maximum emission peak. For MCA, DMBA, and BP, less interference by natural fluorescent materials was encountered by measuring the second major emission peak.

Prior to use, the rats of several groups were treated in various ways to alter their hormonal state. The nature of these changes will be described in the appropriate parts of the next section.

RESULTS

Reliability of the assay method.—A comparison of the fluorescence spectrum of a milk extract from a MCA-fed rat with that of reference methylcholanthrene has been presented elsewhere (10). Methylcholanthrene and other derivatives having the four aromatic rings of benz[a]anthracene show three distinct peaks, with two shoulders in the excitation spectrum and two distinct peaks in the emission spectrum. These features make qualitative identification very dependable. The intensity of fluorescence is high, so that even with very narrow beam slits a sample containing 0.1 μg of MCA/ml can be read with accuracy. At low concentrations, the intensity of fluorescence is directly proportional to concentration.

Extracts of normal tissues from untreated rats were fluorescent but gave very low background readings (Table 1). Comparison of these readings with those found in the experimental series indicated that the background fluorescence did not seriously compromise the data obtained in the experiments. Recovery of known amounts of hydrocarbon from tissues was between 85 and 95 per cent.

Effect of the amount of hydrocarbon fed.—Higher levels of mammary MCA were obtained after a single feeding of 30 mg. of the hydrocarbon than when a 10-mg. dose was used (Table 2). In both cases, 1.0 ml. of sesame oil was used as the solvent. The average values found after 24 hours were 18.0 μg/gm and 6.6 μg/gm, respectively. Thus, the tissue concentration was proportional to the concentration of hydrocarbon in the vehicle. On the other hand, there was little difference between the tissue MCA levels of rats fed a single dose of MCA and those fed ten daily doses. Twenty-four hours after the last feeding, the average level in the mammary glands of rats fed one dose of 10 mg. was 6.6 μg/gm, whereas in rats fed ten daily doses of 10 mg. each was only 6.8 μg/gm. When rats were fed 0.1 ml. of sesame oil daily for 9 days prior to a single feeding of MCA, there was no significant effect on the uptake of the hydrocarbon by mammary gland and fat. Thus, the mammary level after a single feeding of 30 mg. of MCA was 18.0 μg/gm, without prior sesame oil, and 19.7 μg/gm after conditioning by 9 days of sesame oil feedings. The clearance appeared to be independent of the number of daily doses of hydrocarbon.

TABLE 1

<table>
<thead>
<tr>
<th>HYDROCARBON</th>
<th>APPARENT LEVEL (μG/GM) OF HYDROCARBON IN NORMAL RAT TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methylcholanthrene</td>
<td>.08 .47</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>.03 .26</td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene</td>
<td>.16 1.00</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>2.5 2.7</td>
</tr>
</tbody>
</table>

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Effect of the hormonal state of the animal.—Charts 1 and 2 show the levels of MCA in the mammary gland and fat of normal female rats, along with the tissue levels of (a) normal males, approximately 55 days old, (b) 55-day-old females, castrated 14 days before use, (c) 55-day-old females hypophysectomized 7 days before use, and (d) females in the early stages of pregnancy. Levels in both mammary glands and fat in pregnant rats were lower than in normal females, whereas those of either castrated or hypophysectomized rat tissue were higher than those of normal animals. There was little difference between the MCA levels of normal male and female rat tissues. It must be pointed out, however, that the amount of fat in the mammary gland varies with the change of the endocrine state of the animal. During pregnancy and lactation, the amount of mammary tissue greatly exceeds that of the fat. Following castration or hypophysectomy, both general body fat and the fat in the breast tissues are markedly increased. In the present experiment, no separate determination was made in breast fat pad and the remaining glandular tissue. Experiments are now under way to determine the fat contents of the mammary gland under different endocrine states.

**TABLE 2**

<table>
<thead>
<tr>
<th>DAYS AFTER LAST FEEDING</th>
<th>AT. MCA LEVEL (μg/30m) AFTER:</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single 10-mg. feeding</td>
<td>Ten daily 10-mg. feedings</td>
<td>Single 80-mg. feeding</td>
<td>Single 80-mg. feeding after 9 days of feeding of sesame oil alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. Mammary tissue</td>
<td>Fat</td>
<td>No. Mammary tissue</td>
<td>Fat</td>
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<tr>
<td>1</td>
<td>7</td>
<td>6.6</td>
<td>8.9</td>
<td>10</td>
<td>6.8</td>
<td>15.4</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>4.0</td>
<td>13.0</td>
<td>11</td>
<td>17.6</td>
<td>43.5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2.6</td>
<td>12.2</td>
<td>13</td>
<td>4.5</td>
<td>11.6</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1.1</td>
<td>4.1</td>
<td>20</td>
<td>1.1</td>
<td>4.0</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0.5</td>
<td>1.8</td>
<td>20</td>
<td>1.1</td>
<td>4.0</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.5</td>
<td>1.8</td>
<td>20</td>
<td>1.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**Chart 1.**—3-Methylcholanthrene levels of rat mammary gland after feeding. Each rat was given a single feeding of 30 mg of MCA in 1.0 ml of sesame oil. Each point on the curves for pregnant, normal, and hypophysectomized females represents an average of values obtained with six to twelve animals. Each point on the curves for normal males and castrated females represents an average of values obtained with five animals.

**Chart 2.**—3-Methylcholanthrene levels of rat fat after feeding. The rats were the same as those used for Chart 1.

These results are in contrast to those obtained earlier with lactating rats, in which mammary levels decreased rapidly as compared with controls, although the level in the fat was nearly the same for both normal and lactating animals (11). Location of the hydrocarbon in breast tissue.—In
a few experiments, the major part of the fat pad was dissected away from the glandular tissue of the breast. In every case, the concentration of hydrocarbons in the fat pad itself was greater than in the remaining glandular tissue (Table 3).

Effect of the molecular structure of the hydrocarbon.—Somewhat higher levels of hydrocarbon were found in the tissues after BP was administered than after feeding of either MCA or DMBA (Table 4). The average levels of hydrocarbon in the breast 24 hours after a 30-mg meal were found to be 31 \( \mu \text{g/gm} \) for BP, 23 \( \mu \text{g/gm} \) for DMBA, and 19 \( \mu \text{g/gm} \) for MCA. The difference between the levels for BP and MCA was significant at the 5 per cent level. When Ph was fed, the levels found in breast and fat after 24 hours were not significantly higher than "blank" values found in untreated normal tissues (Table 1).

**DISCUSSION**

Several experiments have demonstrated the special sensitivity of lung and mammary tissue to systemic administration of polycyclic hydrocarbons (26, 27). No doubt the sensitivity of these tissues is due, in part, to the localization of the carcinogen within them. In general, hydrocarbons are cleared from the body rapidly, appearing in the bile as oxygenated derivatives (19). In contrast, the lungs and adipose tissue (including the breast) retain unaltered hydrocarbons for long periods of time. In the lungs, the capillary bed filters colloidal hydrocarbon crystals from the blood stream (25). Time is required for the crystals to be dissolved and removed from the lung. In the breast, the fat pad appears to act as a carcinogen trap, retaining unaltered hydrocarbons within the adipose cells, from which it is released very slowly.

That the MCA of the mammary fat pad can be transferred to the glandular cells has been established by experiments where large quantities of the hydrocarbon were found in milk of lactating rats previously fed the carcinogen (11). This transfer was so rapid that the concentration of MCA in milk was nearly as great as in the breast from which it was elaborated. In treated rats, the epithelial cells lying within the mammary fat pad are exposed to substantial levels of MCA over a prolonged period of time.

In every case we studied, the concentration of MCA in the general body fat was greater than that in the whole breast. Likewise, the concentration in the fat pad itself was greater than that in the residual mammary tissue, which contained relatively less adipose tissue. These relationships were not altered by changes in the endocrine state of the rats. Accordingly, it appears that the exposure of glandular cells to carcinogen is a function of the localization of the carcinogen in the adipose cells.

Transport of hydrocarbon to the adipose tissue and incorporation into the lipide cells depends upon the nature of the vehicle and, to a lesser extent, upon the status of the rat. The need for a lipide vehicle is quite apparent from our earlier work, in which aqueous suspensions of MCA were employed (11). In that study, the 24-hour level of MCA in fat after a feeding of 100 mg in aqueous suspension was only about 6 per cent of that found after feeding only 30 mg in sesame oil. It has also been found that in mice the natural fats olive oil and saeame oil are superior to the synthetic fat tricaprylin. These observations probably explain the

**TABLE 3**

<table>
<thead>
<tr>
<th>HYDROCARBON</th>
<th>Dose (mg.)</th>
<th>CONCENTRATION OF HYDROCARBON* (( \mu \text{g/gm} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In fat</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>30†</td>
<td>21.6</td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene</td>
<td>30</td>
<td>31.8</td>
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<tr>
<td></td>
<td>10</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>37.6</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>30</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>58.5</td>
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<td>70.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.4</td>
</tr>
</tbody>
</table>

* In individual animals.
† Analyses conducted after only 2 hours.
reported failures of orally administered hydrocarbon carcinogens to induce mammary cancer in mice (2, 7, 22, 28). It is of great significance that in only one of these four studies, was an oil used as the vehicle. In studies on rats or mice (1, 12, 14, 17, 21, 24), where oral hydrocarbons induced breast tumors, oils were used as the vehicle.

In the present experiments the level of carcinogen achieved in the fat was dependent on the concentration of hydrocarbon in the test meal. On the other hand, in animals given daily doses of MCA, the optimum tissue level was not greater than in rats given only one dose. In view of the relatively slow clearance of MCA from the mammary tissue and fat, this result is paradoxical.

Three possible explanations are apparent. The effect could be due to (a) saturation of the cells by MCA, (b) saturation of the cells by the carrying vehicle, and (c) effects upon the cells by pre-existing levels of hydrocarbons. The first possibility is not consistent with the observation that an increase in the concentration of MCA in the vehicle was followed by a commensurate increase in the hydrocarbon level in the fat and breast. The second mechanism is not consistent with the observation made upon rats which had been fed sesame oil for 9 days prior to the feeding of the hydrocarbon. In this case, uptake of MCA by the fat was as good as in animals which had not been "pre-treated" by the vehicle feedings.

The present experiments do not test the third hypothesis. Brown et al. (6), however, found that feeding rats MCA caused an increase in the capacity of the liver to demethylate 3-methyl-4-monooethylaminobenzene. This increase was apparently due to induction of enzyme and was generally produced by a variety of carcinogenic hydrocarbons. Conney et al. (8) observed that, in a similar fashion, intraperitoneal injection of MCA and other hydrocarbons caused a many-fold increase in the BP hydroxylase activity of the rat liver. It is conceivable that a similar mechanism might affect the manner in which MCA itself is metabolized.

In addition to the effect of prior feeding, the endocrine state of the rats affected the uptake of MCA by the adipose tissues. These effects were small, and the mechanisms cannot be discerned from the present experiments.

The level of hydrocarbon found in tissue after parenteral administration depends upon the relative rates of its incorporation into the cells and its removal from (or metabolic destruction within) the cells. The differences in concentration of the four hydrocarbons used in these studies is a reflection of these rates. Only slight differences were found between MCA, BP, and DMBA, which might suggest similar behavior of these compounds in the system under investigation. Nevertheless, the very low level of Ph indicates either very low absorption into the adipose tissue or else very rapid removal or metabolism.

Although the present data are insufficient to make possible a selection among these possibilities, certain other information indicates that rapid metabolism may be involved. Crabtree (9) found that Ph caused a decrease in tissue sulfhydryl levels, whereas BP did not produce a significant change. Presumably, this effect was due to the participation of Ph in the metabolic patterns of the tissue. In the skin, uptake and retention of Ph are about 10 times as great as in the case of MCA (5).

Thus, it is clear that there is no absolute barrier to the penetration of Ph into cells in general. A final resolution of this problem may be provided by a study of the levels of Ph in adipose tissue during the first few hours after feeding. These determinations are being made and will be reported later.

The over-all behavior of MCA in our experiments was remarkably similar to that of Sudan III in studies made by Gage and Fish (16). The dye was observed to accumulate in adipose tissue, including the mammary gland. The concentration of dye in these tissues depended upon the nature of the vehicle employed, and an oil was superior to aqueous mediums. The parallel between MCA and the chemically dissimilar Sudan III suggests that the hydrocarbons are retained by the adipose tissues, not because of a particular chemical property, but rather because of the manner in which a fat meal is handled by the body. It is possible that some dietary MCA is transported from the gut to the tissues by chylomicra, by-passing the portal circulation into adipose tissues (15). Such a mechanism would explain the dependence of MCA localization on the nature of the vehicle.

The appearance of large amounts of MCA in the milk of rats is of interest from another point of view. Gage and Fish (16) found no Sudan III in the milk of cows, although it did appear in the milk of rats, a goat, and a cat after feeding in oil. Larionow (20) found little or no transfer of BP to the milk of a rabbit or a dog after intravenous administration, even though high levels were achieved in the adipose tissues. In contrast, poly-cyclic hydrocarbons have been found in the milk of treated mice and rats (10, 13, 25). In view of these findings, it seems probable that passage of lipide-soluble materials from the fat pad to the glandular breast is affected by species differences. The high sensitivity of rat breast toward feeding
of MCA is undoubtedly related to the ready availability of the hydrocarbon to the mammary epithelium of this species.

In our studies, the endocrine state of the rats had little effect on the concentration of MCA in either the general body fat or in the breast of the treated rats. Accordingly, the endocrine effects upon tumor formation appear to be due primarily to alteration of the target cells rather than to changes in the amount of carcinogen to which they are exposed. Alteration of the target cell could affect either absorption of the carcinogen by the epithelial cell from its fat pad, or metabolism of the carcinogen within the cell. The data currently available do not distinguish between these possibilities.

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