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DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF RATS AFTER ORAL ADMINISTRATION OF 7,12-DIMETHYLBENZ(A)ANTHRACENE-^{3}H*

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Abstract—Tritium-labeled 7,12-dimethylbenz(a)anthracene (DMBA), prepared by the method of acid-catalyzed exchange, was found to accumulate in adipose tissue of perirenal fat and mammary gland *in vivo* and is present mainly as the parent hydrocarbon. In liver, most of the hydrocarbon has been transformed into highly polar metabolites. From these observations, it appears that fatty tissues are able to take up and retain the parent hydrocarbon, thereby retarding its metabolism and prolonging its duration of action. Radioactivity present in protein and nucleic acid fractions apparently represents a bound form of the hydrocarbon to these cellular components. Pretreatment of animals with estradiol and progesterone alters the susceptibility of the mammary gland to tumor formation by DMBA without changing the amount of radioactivity present or the degree of binding to nucleic acids and proteins.

7,12-DIMETHYLBENZ(A)ANTHRACENE (DMBA)‡ is known to be one of the most potent carcinogens in the female Sprague–Dawley rat. A single 20 mg feeding invariably induces breast cancer in this animal.[1] It has not been established, however, whether the parent hydrocarbon or one or more of the metabolites of DMBA is the carcinogenic agent.

This study was undertaken to obtain more information regarding the distribution and metabolism of DMBA in several tissues, in an effort to clarify the relationship of metabolism to the complicated series of events leading to tumorigenesis.

METHODS

Preparation of 7,12-dimethylbenz(a)anthracene-^{3}H

Tritium-labeled DMBA was prepared by the method of acid-catalyzed tritium exchange. 7,12-Dimethylbenz(a)anthracene, m.p. 122–123° was purified by florisil chromatography, then by recrystallization from benzene–ethanol.

Tritiated trifluoroacetic acid§ (456 mg, 4·0 m-mole, 1·0 c) was transferred by distillation under reduced pressure to a small flask cooled in liquid nitrogen containing

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§ Purchased from New England Nuclear Corp., Boston, Mass.

DMBA (102 mg, 0·4 m-mole). After bringing the pressure in the flask to atmospheric pressure with gaseous nitrogen, the bath temperature was increased until refluxing began. Refluxing was maintained for 44 hr while stirring with a magnetic stirrer. The reaction flask was cooled in liquid nitrogen again and the trifluoroacetic acid removed under reduced pressure. The residue was dissolved in benzene and the solution evaporated to dryness under nitrogen on a steam bath, then redissolved in hexane (7 ml). The solution was placed on a florisil (60–100 mesh) column (7 × 250 mm) and developed with hexane. The product (77 mg) was homogeneous when examined by analytical paper chromatography in the dimethylformamide–hexane system.[2]

The product had an u.v. absorption spectrum characteristic of 7,12-dimethylbenz(a)anthracene, m.p. 122–123°, and a sp. act. of 3·4 mc/mg.

Assay of radioactivity

The assays of radioactivity in the various tissue extracts and residues were carried out as described by Jacobson *et al.*[3] or by the oxygen flask technique described previously.[4]

Efficiency of the counting system was determined by the channels ratio method.[5]

Processing of tissues

Female rats were obtained from Sprague–Dawley (Madison, Wis.) at 30–40 days of age. At 50 days of age the animals were given ^{3}H-DMBA by stomach tube in 1 ml sesame oil and after various time intervals the rats were decapitated and the desired tissue removed rapidly, blotted on filter paper, and placed immediately on solid carbon dioxide. For the determination of total nonvolatile radioactivity, the tissue was dried from the frozen state and the residue analyzed for tritium content by combustion. The volatile fraction was collected in a trap cooled in liquid nitrogen and the radioactivity assayed directly by adding a 50–100 μl aliquot to the phosphor solution and counting in a liquid scintillation spectrometer.

For fractionation into acetone-soluble, acetone-insoluble, ether-soluble, and water-soluble material the frozen tissues were weighed, homogenized in ice-cold 70% acetone (20 ml), and the homogenate centrifuged. The acetone-insoluble residue thus obtained was washed with 70% acetone (3 × 7 ml) and finally with 100% acetone (5 ml). The washes were combined with the acetone-soluble fraction and the acetone removed under reduced pressure. The remaining aqueous solution (5–10 ml) was partitioned between ether (4 × 10 ml) and water (10 ml) to yield ether-soluble and water-soluble fractions.

All fractions except the volatile fraction, which was counted directly, were analyzed for radioactivity by combustion.

Nucleic acids and proteins were isolated by the *p*-aminosalicylic acid–phenol method of Kirby.[6] The protein precipitate was washed with water and methanol and dried under high vacuum.

Chromatography of ether fraction

The ether-soluble fraction was chromatographed on Whatmann No. 1 paper strips by the system of Tarbell *et al.*[2] Reference compounds were chromatographed on control strips run in parallel with the radioactive strip. To assist in the recognition

of metabolites, carrier ^{14}C-DMBA* was added to the ether solution to be chromatographed. The added ^{14}C carrier compound served to detect and measure any transformation, such as photo-oxidation, of the carcinogen that might occur during the development of the chromatogram.

The chromatogram was dried in air, cut into 1·0-cm pieces, and each piece was placed in an individual counting bottle. Five ml of spectrograde dioxane was added to each bottle to elute the radioactivity from the paper. The sample was allowed to stand overnight; then 10 ml of 2,5-diphenyloxazole-toluene (4 g/l.) solution was added and the bottle, with the paper still in place, was counted in the Tri-Carb liquid scintillation spectrometer.

RESULTS

Distribution of radioactivity in tissues of rats after the administration of DMBA

^{3}H-DMBA (30 mg) was administered orally at two dose levels of radioactivity, 26 μc and 2600 μc. The rats were killed at 24 and 72 hr after administration of the carcinogen. The distribution of radioactivity (dpm/mg wet wt.) in the tissues analyzed is shown in Table 1.

TABLE 1. SPECIFIC RADIOACTIVITY OF NON-VOLATILE FRACTION OF RAT TISSUES AFTER ORAL ADMINISTRATION OF ^{3}H-DMBA (30 MG)

	Administered radioactivity			
	(26 μc)		(2600 μc)	
Tissue	Pair 1 (24 hr)	Pair 2 (72 hr)	Pair 3 (24 hr)	Pair 4 (72 hr)
Fat	93	30	6570	1600
Kidney	45	48	6000	3000
Volatile fraction	7	11	680	790
Liver	50	38	4340	1300
Volatile fraction	7	11	680	630
Mammary gland	44	11	3640	700
Adrenal	22	19	2200	1000
Uterus	8	8	620	300
Blood	10	9	1100	620

Results are expressed as specific radioactivity of fresh tissue (dpm/mg). Each time point represents 2 rats. Each tissue was analyzed in duplicate and the values are average values for 2 rats. The figures given for the volatile fraction are the radioactivity in the distillate from 1 mg of fresh tissue. The nonvolatile fraction is the tissue residue after freeze-drying of the tissue.

Of the tissues examined, perirenal fat had the greatest amount of radioactivity, which was equivalent to approximately 35–49 μg hydrocarbon/g fat (calculated from the sp. act. of DMBA) 24 hr after administration. The lowest amounts were in uterus and in blood, calculated from the sp. act. to be equivalent to 3–4 μg of DMBA/g uterus. Intermediate values were observed for kidney, liver, and mammary gland, and a somewhat lower value for adrenal gland (12 μg/g). The distribution of radio activity was not dependent on the sp. act. of ^{3}H-DMBA used. Thus, a 100-fold increase in

* Purchased from Nuclear-Chicago, Chicago, Ill.

the sp. act. administered resulted in a corresponding increase in specific radioactivity of each of the tissues.

DMBA was not detected in the volatile fraction (the distillate from the freeze-drying operation); therefore, this fraction probably originates from the metabolism of the compound, and was a significant fraction of the total radioactivity.

Time course of specific radioactivity of tissue fractions and the effect of prior treatment with estradiol and progesterone

The relative distribution in tissue fractions at various times after a single oral administration of 10 mg of ^{3}H-DMBA (1000 μc) was compared with the distribution obtained by pretreatment of animals with estradiol (20 μg) and progesterone (4 mg) daily for 14 days. This is shown in Figs. 1 and 2.

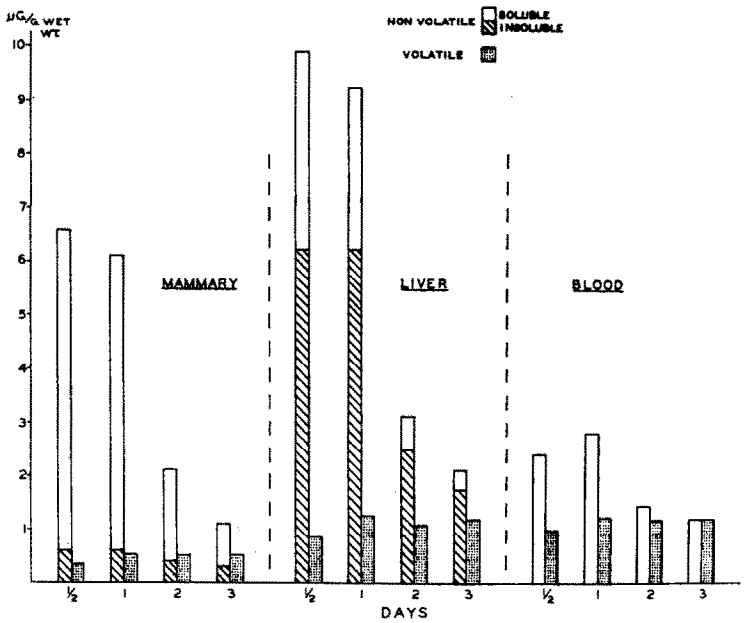

FIG. 1. Specific radioactivity of tissue fractions expressed as unchanged hydrocarbon (μg/g) after a single oral dose of 10 mg of ^{3}H-DMBA (1000 μc). Values shown are the mean of duplicate assays of tissue pooled from four animals. Soluble and insoluble refer respectively to the acetone-soluble and acetone-insoluble fractions of the nonvolatile tissue portion. The bar height for the nonvoltatile portion is the total of the individual soluble and insoluble bar height.

In mammary tissue, the majority of the radioactivity was in the acetone-soluble fraction. This fraction increased rapidly to a maximum at 12 hr of 6 μg/g fresh tissue and then declined to about 0·7 μg/g at day 3. Binding of hydrocarbon to the acetone-insoluble material was rapid and maximal at 12 hr. The concentration of acetone-insoluble residue was approximately 0·6 μg/g at 12 hr and 0·3 μg/g at 72 hr. Volatile activity remained about constant at a level of 0·5 μg/g throughout the observation period of 3 days. In the liver, the amount of radioactivity in the acetone-soluble fraction was 3·7 μg/g at 12 hr and 0·3 μg/g at 72 hr. On the other hand, the radioactivity of the acetone-insoluble residue was considerably higher in liver, being about 6 μg/g at 12 hr and 2 μg/g at 72 hr.

A study was conducted *in vitro* to determine the concentration of radioactivity in the acetone-insoluble fraction. When comparable amounts of tritium-labeled DMBA were added to the tissue during homogenization, the radioactivity recovered in the acetone-insoluble material was only about 5 per cent of what was obtained *in vivo*. This suggests that DMBA had undergone a metabolic transformation before becoming part of the acetone-insoluble fraction.

Treatment of the animals with estradiol and progesterone resulted in a decrease of radioactivity in the acetone-insoluble fraction from liver (Fig. 2). This suggests a

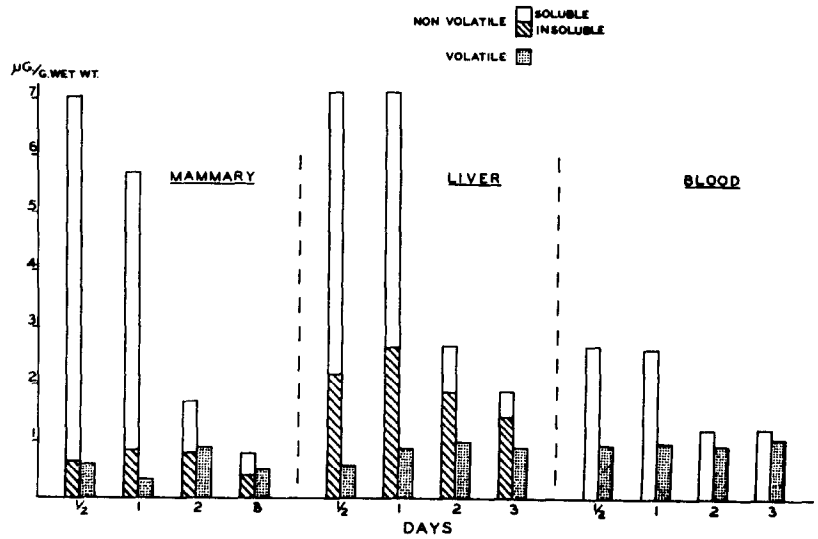

FIG. 2. Effect of estradiol and progesterone on specific radioactivity of tissue fractions expressed as unchanged hydrocarbon (μg/g) after a single dose of ^{3}H-DMBA (10 μg, 500 μc). Values are the mean of four animals on each time point. (Rats were given subcutaneous injection of estradiol-17β, 20 μg, in combination with 4 mg progesterone, daily for 14 days prior to feeding 10 mg ^{3}H-DMBA, 500 μc.) Each set of values represents the mean of four animals. Soluble and insoluble refer respectively to the acetone-soluble and acetone-insoluble fractions of the nonvolatile tissue portion.

competition for binding sites between the steroids and the hydrocarbon or its metabolites in the liver. Estradiol or progesterone might also decrease hydroxylation, thereby decreasing the respective amounts of acetone-insoluble matter. Unlike liver, no such effect of hormone treatment was observed in mammary gland.

Comparison of specific radioactivity of tissue fractions from mammary gland, perirenal fat, and liver

The specific radioactivity of tissue fractions (acetone-insoluble, ether-soluble, and water-soluble) from mammary gland, fat, and liver is shown in Fig. 3. In this experiment, six female rats, age 50 days, were fed 30 mg ^{3}H-DMBA (1000 μc) and sacrificed 24 hr later.

The major portion of radioactivity was present in the ether-soluble fractions from breast and fat. Only small amounts were recovered from the water-soluble and acetone-insoluble fraction. On the other hand, liver mostly contained water-soluble material and radioactivity in acetone-insoluble material.

The results of paper chromatography of the ether-soluble fractions derived from these tissues are presented in Figs. 4, 5, and 6. There are three peaks in the fraction from mammary gland. The major peak corresponds to DMBA and is indicated by the presence of the marker, ^{14}C-DMBA. The absence of ^{14}C in the minor peaks indicates that the radioactive compound probably arises by a metabolic transformation. The

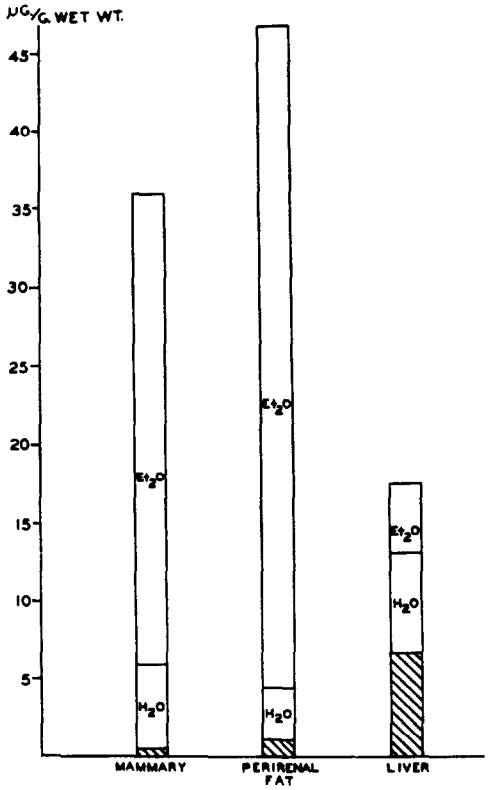

FIG. 3. Specific radioactivity of tissue fractions expressed as unchanged hydrocarbon (μg/g) 24 hr after a single oral dose of 30 mg of ^{3}H-DMBA (1000 μl). Values are the mean of triplicate assays of tissue pooled from 6 animals. The total height of each bar indicates the total radioactivity in the nonvolatile portion of the tissue. The cross-hatching represents the acetone-insoluble fraction. Et_2O and H_2O refer respectively to the ether-soluble and water-soluble fractions of the acetone extract.

minor peak with ^{14}C is the photo-oxide formed during manipulation of the sample. The radioactivity at the origin probably represents hydroxylated derivatives of DMBA. (4-Hydroxy-7,12-dimethylbenz(a)anthracene; 7-hydroxymethyl-12-methylbenz(a)-anthracene; 7,12-dihydroxymethylbenz(a)anthracene; and cis-5,6-dihydroxy 7,12-dimethylbenz(a)anthracene all have R_f values in this system of less than 0·05.)* The chromatogram of perirenal fat is similar to that observed for mammary gland. Very little DMBA could be identified in the ether fraction from liver. Most of the radio-activity is present close to the origin, suggesting transformation to highly polar hydroxylated derivatives.

* J. W. Flesher, unpublished data.

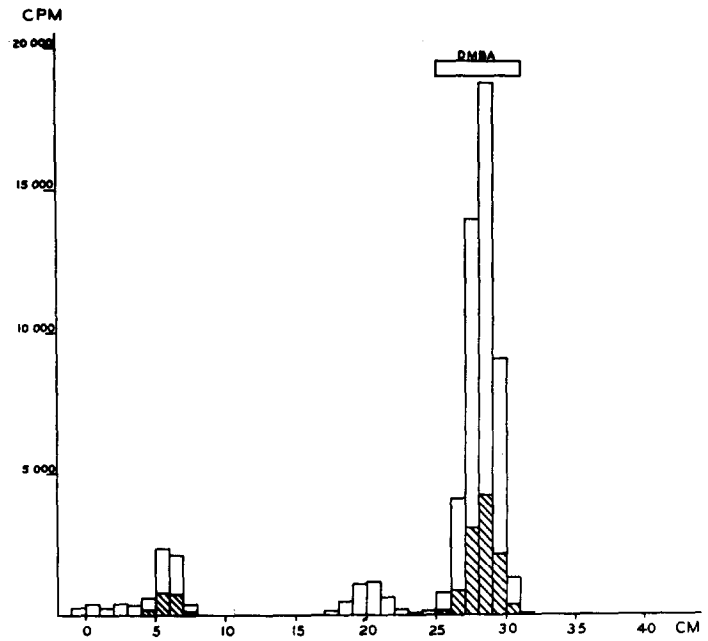

FIG. 4. Analytical paper chromatogram of ether fraction from mammary tissue. The shaded area represents added carrier ^{14}C-DMBA. See text for explanation.

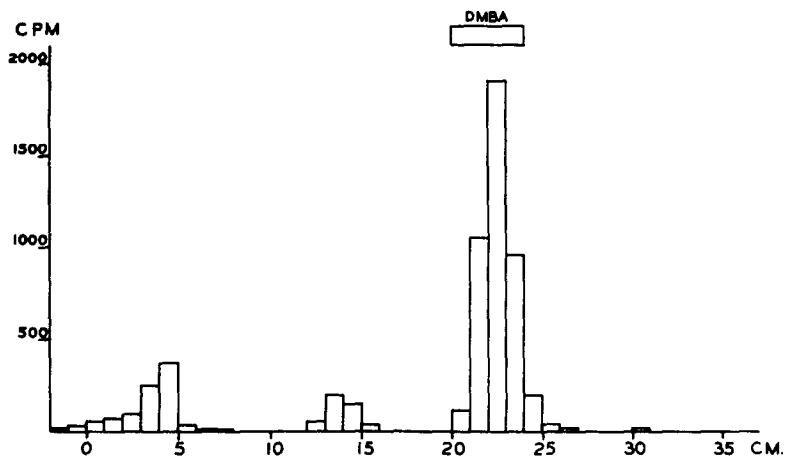

FIG. 5. Analytical paper chromatogram of ether fraction from perirenal fat. The major peak corresponds to 7,12-dimethylbenz(a)anthracene.

Effect of estradiol and progesterone on the binding of labeled hydrocarbon to mixed nucleic acids and proteins of mammary gland

The binding of labeled hydrocarbon to nucleic acids and proteins isolated from mammary gland was not affected by pretreatment with estradiol and progesterone (Table 2).

From 140–500 times as much hydrocarbon was bound to nucleic acids as was bound to protein, assuming an average molecular weight of 6·7 million for nucleic acids and 0·1 million for proteins.

When the nucleic acids were redissolved in 1% sodium acetate and reprecipitated by the addition of 2 vol. of ethanol, no change in sp. act. of the nucleic acids occurred, eliminating the possibility that the radioactivity was trapped in the sampling procedure.

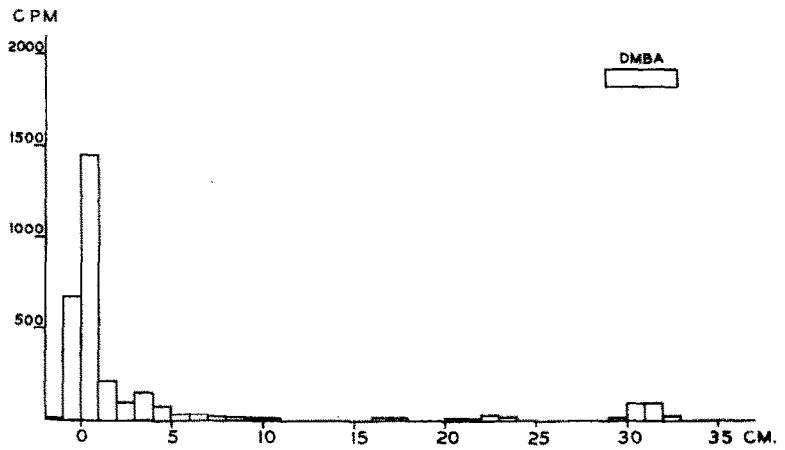

FIG. 6. Analytical paper chromatogram of ether fraction from liver.

TABLE 2. BINDING OF ^{3}H-DMBA TO MIXED NUCLEIC ACIDS AND PROTEINS OF MAMMARY GLAND 24 HR AFTER ORAL ADMINISTRATION OF ^{3}H-DMBA (20 MG, 1120 μC)

	Molecules of hydrocarbon bound to each molecule of nucleic acid or protein	
	Pretreatment with sesame oil	Pretreatment with estradiol and progesterone
Nucleic acids		
1st ppt.	0·034	0·045
2nd ppt.	0·040	0·042
Protein	0·00008	0·00010

TABLE 3. BINDING OF ^{3}H-HYDROCARBON TO MIXED NUCLEIC ACIDS AND PROTEINS OF MAMMARY GLAND AND LIVER 24 HR AFTER ORAL ADMINISTRATION OF ^{3}H-DMBA (20 MG, 550 μC)

	Molecules of hydrocarbon bound to each molecule of nucleic acid or protein	
Tissue fraction	Mammary gland	Liver
Nucleic acid	0·05	0·15
Protein	0·00035	0·0050

In liver, about 30 times as much hydrocarbon was bound to nucleic acids as was bound to proteins, assuming the above molecular weights (Table 3).

DISCUSSION

In the present study, evidence is presented that mammary gland accumulates the hydrocarbon and retains significant levels for at least 3 days; detectable levels are observed for as long as 1 week. In an earlier study with ^{3}H-3-methylcholanthrene (3-MC), Flesher and Sydnor[7] found radioactivity equivalent to 5–7 μg 3-MC/g mammary gland 24 hr after feeding a 10-mg dose of hydrocarbon.

The results of the present study are similar to those observed with 3-MC and DMBA by Bock and Dao.[8] These investigators found, by fluorescent techniques, a concentration of 23 μg/g DMBA/g breast tissue at 24 hr.

Gammal *et al.*[9] used gas-liquid chromatography and found that a 20-mg dose of DMBA resulted in concentrations of 16 μg/g, 14 μg/g, and 9 μg/g in mammary gland after 6, 12 and 24 hr, respectively.

Hamilton and Jacobson[10] have shown that, after administering 30 mg ^{3}H-DMBA, the radioactivity in plasma reaches a maximum 8–12 hr after a single feeding of 30 mg ^{3}H-DMBA in sesame oil, a sp. act. equivalent to 14 μg DMBA/ml and falling to 2 μg/ml at 72 hr.

There is general agreement among workers regarding the time course of the uptake and clearance of the carcinogen in tissues, although Lo-Sin-Mao[11] was unable to show any accumulation of ^{14}C-DMBA in mammary gland after a single i.v. dose, and Goodall *et al.*[12] failed to localize 3-MC by autoradiography in rat mammary tissue after a single 10-mg dose by stomach tube.

The highest concentrations of hydrocarbon occur in fatty tissues and no doubt this is due to its lipid-solubility. Adipose tissue is the predominant component of mammary gland.[13] The higher concentrations of DMBA in fatty tissue, coupled with the fact that only small amounts of metabolites are observed in these tissues, suggest that DMBA may be the active form of the carcinogen which interacts with the mammary epithelial cell. This is in accord with the observation of Boyland[14] that metabolites of polycyclic hydrocarbons examined for carcinogenic properties either have been inactive or are much less active than the parent compound. The evidence is not conclusive however, since it may be that active metabolites are difficult to detect because they react with the cell so readily that there is little chance of observing them in free form.

The recent findings of Boyland and Sims,[15] which demonstrate the conversion of DMBA to 7-hydroxymethyl-12-methylbenz(a)anthracene by rat liver homogenate, and the finding of Boyland, Sims and Huggins[16] that this metabolite has carcinogenic properties, suggest the possibility that a metabolite may be the active carcinogen.

In an interesting study, Jellinck and Goudy[17] showed that pretreatment of rats with 3-methylcholanthrene, which is known to increase liver microsomal hydroxylating enzymes, brings about an alteration in the pattern of metabolites from the formation of hydroxymethyl derivatives to ring-hydroxylated metabolites. These observations support the hypothesis that the protective action of polycyclic hydrocarbons against induction of adrenal necrosis by DMBA results from the increase in liver microsomal hydroxylating enzymes which rapidly degrade DMBA to inactive metabolites.

Huggins, Moon and Morii[18] showed that when estradiol (20 μg) and progesterone (4 mg) were injected daily between age 65 and 95 days, after a single feeding of DMBA (20 mg) at age 50 days, only 48 per cent of the animals developed mammary cancer, whereas DMBA (20 mg) alone produced mammary cancer in 100 per cent of the animals.

In the present study, pretreatment with estradiol and progesterone made no detectable difference in the accumulation of radioactivity in mammary gland or its binding to nucleic acids and proteins. Thus it seems that the carcinogen interacts with the cell in spite of unfavorable hormonal environment. Apparently, the susceptibility of mammary epithelial cells can be altered without a change in the capacity of the cellular components to bind the carcinogen. It has been shown that rats with mammary cancers show a decline in the number and size of the tumors when treated with estradiol and progesterone.[1]

Bock and Dao[19] also have shown that the endocrine state of the rat has little influence on the concentration of 3-MC in the mammary gland, a finding similar to the one in the present study with DMBA. If the initial carcinogenic event is the binding of carcinogen to DNA, then these data are consistent with the hypothesis that hormones may not influence the primary carcinogenic event, but may influence the subsequent development of the neoplasm.[18]

In a study of binding of tritium-labeled hydrocarbons to nucleic acids and proteins, Brookes and Lawley[20] found that binding reached a maximum approximately 24 hr after application of the hydrocarbon to skin of mice. These workers concluded that 0·01–0·02 molecules of hydrocarbon per molecule of protein and 1·4 molecules per molecule of DNA were bound.

The observations presented in this paper are in general agreement with those reported by Brookes and Lawley,[20] but since the experimental conditions are different, the lower binding values for nucleic acids and proteins cannot be evaluated on a comparative basis. Although it is conceivable that binding to nucleic acids may be the result of complex formation or other short-range forces, the constant specific activity of the nucleic acids after precipitation with ethanol followed by solubilization and reprecipitation indicates the firm nature of the binding.

It is to be noted that nucleic acids prepared by the method of Kirby contain several per cent protein and, since the molecular weights of protein are less than one-sixtieth the weight of nucleic acid, the protein contamination may contribute significant amounts of radioactivity.

^{3}H-DMBA was shown to be localized in mouse skin epidermis and it was concluded that the labeling of the cells was owing to binding of ^{3}H-DMBA to cellular proteins.[21, 22]

The significance of carcinogen binding to cellular constituents is not clear, but such binding is presumably an early event in the process of tumorigenesis.

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