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Comparative Metabolism of 7,12-Dimethylbenz(a)anthracene in Liver and Mammary Tissue[1]

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SUMMARY

The metabolism of 7,12-dimethylbenz(a)anthracene to hydroxymethyl derivatives differs in hepatic and mammary tissue homogenates. Countercurrent distribution and carrier recrystallization have been shown to be effective additional tools in the identification of 7-hydroxymethyl-12-methylbenz(a)anthracene, 12-hydroxymethyl-7-methylbenz(a)anthracene, and 7,12-dihydroxymethylbenz(a)anthracene. In the hepatic tissue, 7,12-dimethylbenz(a)anthracene is transformed into all three of these metabolites whereas the mammary gland formed only the monohydroxy compounds. Pretreatment of rats with 3-methylcholanthrene induces a marked increase in 7,12-dimethylbenz(a)anthracene-metabolizing enzymes in the liver causing further conversion of the 7-hydroxymethyl-12-methylbenz(a)anthracene, 12-hydroxymethyl-7-methylbenz(a)anthracene, and 7,12-dihydroxy-7,12-dimethylbenz(a)anthracene to more polar components. In contrast, mammary gland caused a diminution but still detectable level of the carcinogenic 7-hydroxymethyl-12-methylbenz(a)anthracene and a noticeable increase in the conversion to 12-hydroxymethyl-7-methylbenz(a)anthracene. The data illustrate, for the first time, the ability of the mammary tissue to metabolize 7,12-dimethylbenz(a)anthracene.

INTRODUCTION

The metabolism of DMBA[4] by rat liver homogenates has been investigated by Boyland and Sims (1). These authors reported isolation and identification of the 2 isomeric monohydroxymethyl derivatives as the major metabolic products. Boyland *et al.* (2) subsequently demonstrated that one of the isomeric monohydroxymethyl derivatives, 7-OHM-12-MBA, was both adrenolytic and carcinogenic. Jellinck *et al.* (8) later demonstrated differences both in the rate of metabolism and in the nature of the metabolites in the liver microsomes of mice and adult or immature rats. These authors showed that there was a complete lack of DMBA-metabolizing enzymes in the adrenals of mice and immature rats. Accordingly, it was suggested that the lack of adrenocorticolytic effect of DMBA in mice and immature rats was due to inability of the adrenal gland to metabolize DMBA to the active adrenocorticolytic agent, 7-OHM-12-MBA. Recent studies by Sims (10), however, showed that whereas the major metabolites of DMBA by liver homogenate were 7-OHM-12-MBA and 12-OHM-7-MBA, that of DMBA by the adrenal homogenate was 8,9-dihydro-8,9-dihydroxy-DMBA instead of the isomeric monohydroxymethyl derivatives. These results suggest that 7-OHM-12-MBA may not be the proximate agent responsible for adrenal necrosis.

It is now well established that the potent carcinogen DMBA, when given either p.o. or i.v. to an adult female rat, can induce mammary cancer (7). Conclusive evidence is lacking either to support the postulate that polycyclic aromatic hydrocarbons must necessarily be metabolically transformed for their ultimate carcinogenic action or to rule out entirely the possibility that metabolic transformation is not an obligatory step in polycyclic hydrocarbon carcinogenesis. It is suggested that 1 important approach to answer the question whether carcinogenic hydrocarbon metabolism is related to tumor induction is to study the metabolism of the carcinogen in the target tissue. Dao (4) showed earlier that induction of mammary cancer could occur after *in vitro* exposure of the target tissue to DMBA. Recently, Dao and Sinha (5) demonstrated conclusively the induction of cancer in mammary explants in organ culture. These experiments clearly suggest that if prior metabolism must occur in carcinogenesis induced in the mammary gland by DMBA, it must take place in the mammary gland since in these experiments, the carcinogen was not administered systemically. A comparative study of the metabolism of DMBA by hepatic and mammary tissue homogenates of the female rat is useful in elucidating the role of metabolism of carcinogens in the induction of cancer.

MATERIALS AND METHODS

Chemicals and Animals. Female Sprague-Dawley rats, 55 to 60 days old, (supplied by the Holtzman Co., Madison,

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[4] The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; 12-OHM-7-MBA, 12-hydroxymethyl-7-methylbenz(a)anthracene; 3-MC, 3-methylcholanthrene; TLC, thin-layer chromatography; 7,12-diOHMBA, 7,12-dihydroxymethylbenz(a)anthracene; CCD, counter-current distribution.

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Wis.) weighing 190 to 200 g, were used in the study. They were fed a Rockland diet, given water *ad libitum*, and housed in an air-conditioned room.

DMBA (m.p., 128°) and 3-MC (m.p., 180°) were purchased from the Eastman Organic Co., Inc., Rochester, N.Y., and were used without further purification. DMBA-^{3}H (specific activity, 500 μCi/μM), purchased from Amersham-Searle, Chicago, Ill., was purified by silica gel (100 to 200 mesh) column chromatography in a 100% benzene system followed by TLC in 2% ethylene glycol monomethyl ether in benzene.

Compounds used for standards, including 7-OHM-12-MBA (m.p. 162°), 12-OHM-7-MBA (m.p. 164°), and 7,12-diOHMBA (m.p. 221 to 223°), were generously provided by Dr. P. H. Jellinck (Queen's University, London, Ontario, Canada) and Dr. P. Sims (Chester Beatty Institute, London, England). Single spots were obtained by TLC of these compounds in 2% ethylene glycol monomethyl ether in benzene.

Metabolism of DMBA. Rats were sacrificed by cervical dislocation. Tissues were removed, weighed, and homogenized in cold 0.25 M sucrose: liver, 50 mg wet weight per ml; and mammary gland, 300 mg wet weight per ml. The low cellularity of the mammary gland requires that a greater wet weight of this tissue be used per ml sucrose. Protein determinations were done using the Biuret method: liver, 9.9 mg/ml; and normal mammary gland, 4.8 mg/ml. A Potter-Elvehjem homogenizer was used for liver tissue, and a Virtis homogenizer was used for mammary tissue. The homogenate was centrifuged at 0° and 1000 × *g* for 15 min in a Sorval refrigerated centrifuge. The supernatant was used for incubation. For reaction with 15 ml supernatant, the reaction mixture contained 75 μCi DMBA-^{3}H (500 mCi/mM, added in 0.2 ml ethanol); 9.0 ml 0.5 M potassium phosphate buffer, pH 7.4, made fresh for each experiment with K_2HPO_4 and KH_2PO_4; 1.8 ml 0.1 M glucose 6-phosphate; 1.8 ml 0.01 M NADP; 37 units glucose 6-phosphate dehydrogenase; and 32.4 ml H_2O.

Reaction mixture plus supernatant was oxygenated for 30 sec and immediately stoppered. The incubation was carried out at 37° for 20 min in a Dubnoff constant-temperature metabolic incubator. The reaction was stopped with 15 ml 1 N HCl and the incubation mixture was extracted 3 times with equal volumes (75 ml) peroxide-free ether. After drying over anhydrous Na_2SO_4 the ether fraction was evaporated to dryness with N_2 and then diluted to 1.0 ml with acetone. A 100-μl aliquot was chromatographed on a thin-layer chromatogram without fluorescent indicator in 2% ethylene glycol monomethyl ether. Ten chromatograms were done for the sample plus one for a 2- × 100-μl acetone rinse. A nonradioactive standard containing 7,12-diOHMBA, 7-OHM-12-MBA, 12-OHM-7-MBA, and DMBA was spotted over each sample and was used to determine the position of each of these metabolites. Each metabolite was located under UV and the appropriate area scraped directly onto a 0.5-cm silica column contained in a Pasteur pipet; the column was eluted with 3 × 1 ml ethanol. Individual metabolites from each chromatogram were pooled, taken to dryness under N_2, and further purified by 100-tube CCD in the appropriate system: 7,12-diOHMBA, 70% methanol: H_2O:40% $CHCl_3$; and 7-OHM-12-MBA and 12-OHM-7-MBA; 55% ethanol:100% heptane and 0.5% NaCl. An aliquot (1 ml of 10 ml) was taken from both the upper and lower phase of each tube and the radioactivity was determined. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Scintillation fluid consisted of 0.4% PPO (Amersham-Searle) and 0.1% dimethyl-POPOP (Nuclear-Chicago Corp., Des Plaines, Ill.) in ethanol:toluene (2:3). Identification of metabolites in appropriate peak tubes was determined by carrier crystallizations. A 15-mg sample of unlabeled standard was added to the pooled peak tubes containing the radioactive compounds. The solutions were evaporated to dryness and the compound repeatedly crystallized from acetone for 7,12-diOHMBA and from ethanol for 7-OHM-12-MBA and 12-OHM-7-MBA. All carrier crystallizations are reported as dpm/mg. All procedures were carried out in the absence of fluorescent lighting.

Fluorescence measurements used in partition coefficient and carrier crystallization determinations were done on an Aminco spectrophotofluorometer. It was necessary to determine the maximum excitation and emission wavelength for each compound for fluorometric determinations. Using excitation and emission spectra, the maximum wavelengths were found to be (excitation and emission, respectively, given in nm): 7,12-diOHMBA, 363 and 407; 7-OHM-12-MBA, 408; and 12-OHM-7-MBA, 360 and 412. All readings were done in ethanol.

Metabolism of DMBA in Rats Pretreated with 3-MC. In this experiment, rats were given a single dose of 0.5 mg 3-MC in 0.1 ml dimethyl sulfoxide by tail vein injection. In the controls, 0.1 ml of dimethyl sulfoxide was given by i.v. injection.

Animals were sacrificed by cervical dislocation 24 hr after injection. The tissue was removed (lymph nodes were excised from mammary glands) and placed in cold 0.25 M sucrose. Mammary tissue from 5 rats was pooled; liver tissue from 1 rat was sufficient. All solutions, dissecting instruments, and glassware were kept at 0°.

RESULTS

Application of CCD to the Separation of DMBA Metabolites. The selection of a system which would separate the 2 most closely related compounds, 7-OHM-12-MBA and 12-OHM-7-MBA, and the breaking up of emulsions, the formation of which was aggravated by tissue components, constituted 2 problems that required solution. The solvent systems consisting of 70% methanol:H_2O:40% $CHCl_3$:CCl_4 (System 1) for the 7,12-diOHMBA and 55% ethanol:H_2O:heptane (System 2) for the 7-OHM-12-MBA and 12-OHM-7-MBA were found to be adequate.

The emulsions formed by the introduction of tissue extracts can be successfully separated by simply raising the ionic strength of the aqueous layer by the introduction of 0.5% NaCl in System 2.

The partition coefficients in System 1 were 0.02, 0.06, 0.08, and 0.91 for DMBA, 12-OHM-7-MBA, 7-OHM-12-MBA, and 7,12-diOHMBA, respectively. In System 2,

the partition coefficients were 32.3, 1.17, 0.70, and 0.02 for DMBA, 12-OHM-7-MBA, 7-OHM-12-MBA, and 7, 12-diOHMBA, respectively.

Metabolism of DMBA in Normal Liver and in the Liver from Rats Pretreated with 3-MC. Following TLC, the zones corresponding to the hydroxymethyl derivatives of DMBA were subjected to CCD. Authentic standard derivatives showed peaks occurring at Tubes 41, 54, and 47 for 7-OHM-12-MBA, 12-OHM-7-MBA, and 7,12-diOHMBA, respectively. In the CCD of those TLC zones derived from tissue incubations, which are shown in Charts 1 to 3, the tubes in the general vicinity of these peaks were pooled. Thus Tubes 40 to 44, 51 to 55, and 46 to 51 for the normal liver and Tubes 39 to 43, 52 to 54, and 46 to 48 for the pretreated liver, which correspond to 7-OHM-12-MBA, 12-OHM-7-MBA, and 7,12-diOHMBA, respectively, were combined for carrier recrystallization with authentic standards.

The results shown in Table 1 confirm the presence of 7-OHM-12-MBA, 12-OHM-7-MBA, and 7,12-diOHMBA as metabolites of DMBA by normal rat liver homogenate. The percentage conversions of the DMBA to these hydroxymethyl derivatives are 0.27, 0.42, and 0.02 for 7-OHM-12-MBA, 12-OHM-7-MBA, and 7,12-diOHMBA, respectively. The percentage conversion of the hydrocarbon to hydroxymethyl derivatives was estimated by taking the sum of the dpm in the CCD tubes which were utilized for carrier recrystallization and dividing this number by the total dpm used in the incubation minus the dpm remaining in the aqueous phase.

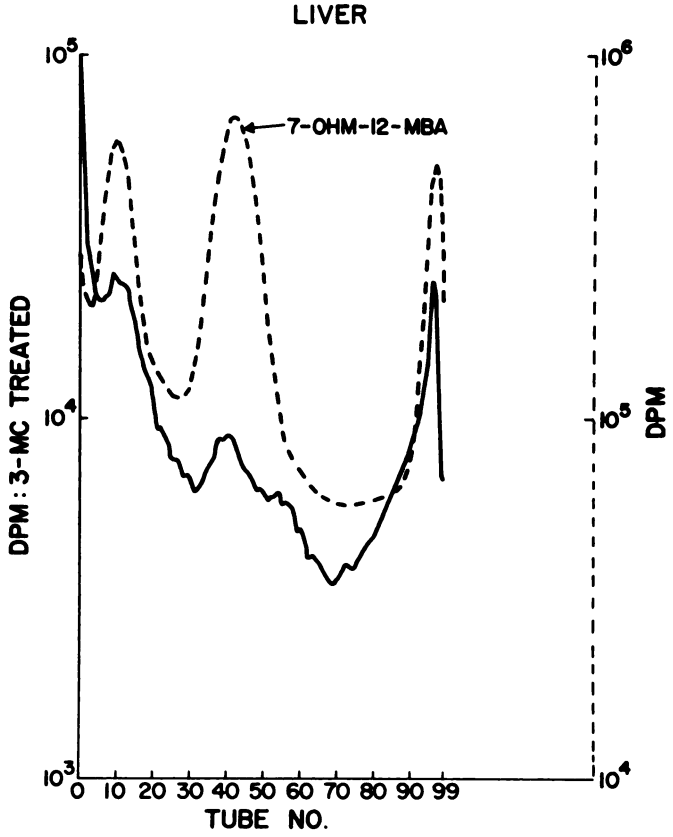

Chart 1. One hundred-transfer CCD in System 2 of the 7-OHM-12-MBA fraction. - - - -, normal liver; ——, liver from rats pretreated with 3-MC. Note the great reduction of radioactivity in the 7-OHM-12-MBA zone of the pretreated liver.

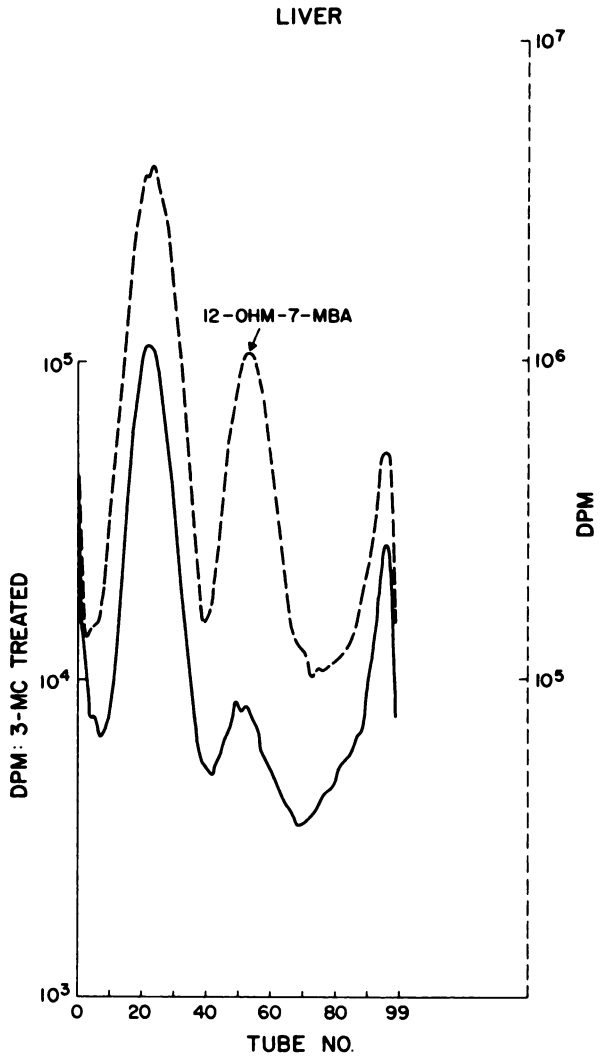

Chart 2. One hundred-transfer CCD in System 2 of the 12-OHM-7-MBA fraction. - - - -, normal liver; ——, liver from rats pretreated with 3-MC. Note the great reduction in radioactivity and loss of peak sharpness in the 12-OHM-7-MBA zone of the pretreated liver.

Pretreatment of rats with 3-MC results in the absence of 7- and 12-hydroxylated derivatives as is shown by the lack of any peaks in those CCD zones representative of these derivatives and by the consistent loss in specific activity of representative samples recrystallized with authentic standards.

Metabolism of DMBA in Normal Mammary Tissue and in the Mammary Gland from Rats Pretreated with 3-MC. Shown in Charts 4 and 5 are the CCD patterns of the TLC zones corresponding to the mono and dihydroxy derivatives of DMBA. There was no radioactivity in the area representative of the 7,12-dihydroxy compound. Tubes 40 to 43 representative of 7-OHM-12-MBA and Tubes 52 to 56 representative of 12-OHM-7-MBA were subjected to carrier recrystallization with authentic standards to constant specific activity, confirming the presence of these metabolites

(Table 2). The percentage conversions of DMBA to these derivatives are 0.27 and 0.36 for 7-OHM-12-MBA and 12-OHM-7-MBA, respectively.

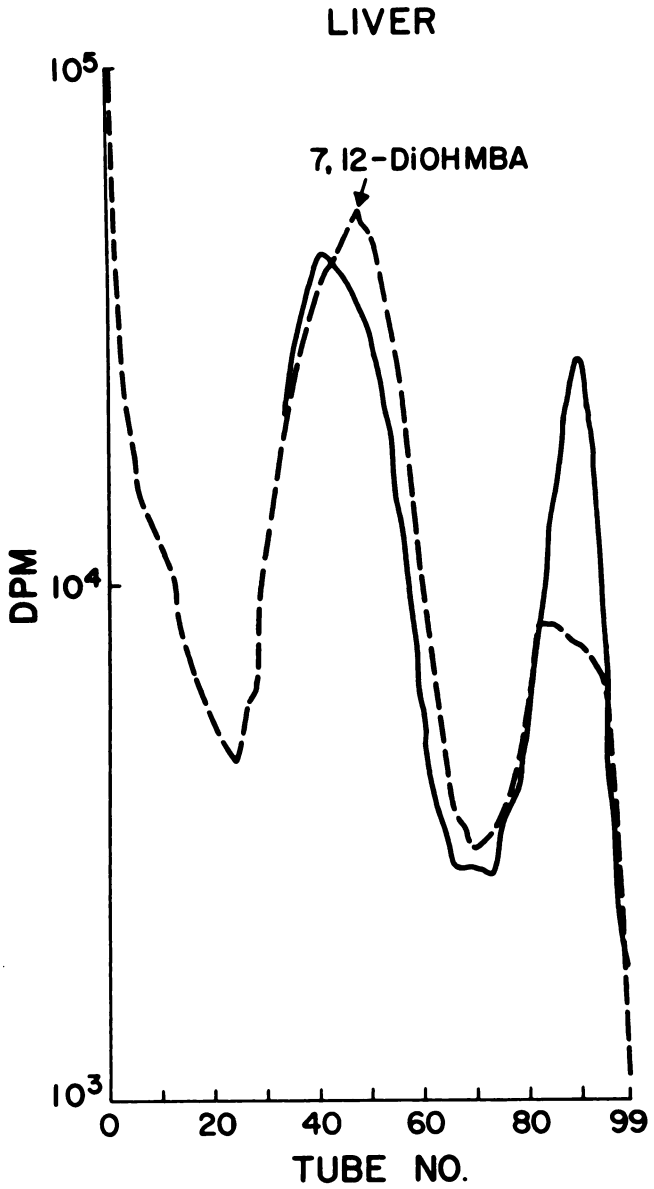

Chart 3. One hundred-transfer CCD in System 1 of the 7, 12-diOHMBA fraction. - - - -, normal liver; ——, liver from rats pretreated with 3-MC. Note the shift of the peak tube from 52 in the normal liver to 42 in the pretreated liver.

Metabolism of DMBA to hydroxylated derivatives by mammary gland homogenate from rats that had been pretreated with 3-MC was also investigated. TLC, CCD, and carrier recrystallization of Tubes 39 to 43 for 7-OHM-12-MBA and Tubes 52 to 56 for 12-OHM-7-MBA showed positive identifications for both of these derivatives in percentages of 0.28 and 0.10, respectively. The carrier recrystallization data are shown in Table 3.

Influence of 3-MC Pretreatment on the Formation of Polar Metabolites. An increase in the amounts of both polar and water-soluble metabolites was observed when the tissues under study were from rats pretreated with 3-MC. This observation is in agreement with previously reported studies (9). Thus, the yield of water-soluble derivatives increased from 12.7 to 34.8% in the liver and from 18.7 to 46.7% in the mammary gland.

The magnitude of conversion to water-soluble metabolites was estimated by calculating the dpms of the material remaining in the aqueous homogenate layer after ether extraction and dividing this number by the total radioactivity added to the incubation medium.

DISCUSSION

The successful application of CCD to the purification of the tissue extracts led to the acquisition of partition coefficients the magnitudes of which were sufficiently different to make possible the positive identification of these metabolites of DMBA in both liver and mammary tissue homogenates.

In the study of rat liver homogenates, Boyland and Sims (1) and Sims (10) identified only the 7- and 12-monohydroxylated products of DMBA using chromatographic techniques and UV absorption data. Also, Flesher *et al.* (6), by rechromatography of the polar zone, detected the 7, 12-dihydroxy derivative.

In the present study, the data obtainable solely from chromatography and rechromatography in different solvent systems were not considered unequivocal. The CCD patterns in Charts 1 to 5, which were obtained from narrow TLC bands, illustrate the complex nature of these zones. Carrier recrystallization of the TLC bands prior to CCD purification proved inconclusive for all 3 components studied. However, the purified peaks gave positive carrier recrystallization data from 7-OHM-12-MBA, 12-OHM-7-MBA, and 7, 12-diOHMBA, as exemplified by the mainte-

Table 1

Identification of DMBA metabolites in rat liver homogenate by carrier recrystallization

Recrystallization	Specific activity (dpm/mg) of metabolite					
	7, 12-diOHMBA		7-OHM-12-MBA		12-OHM-7-MBA	
	Crystals	Filtrates	Crystals	Filtrates	Crystals	Filtrates
Start	21,605		116,333		620,053	
1	11,358	29,198	117,262	156,545	570,696	776,181
2	11,663	12,943	112,423	137,362	543,817	631,053
3	11,357	12,083	108,540	118,625	519,714	635,771
4			107,641	118,933	540,673	548,953
5			104,312	106,894	500,380	506,346

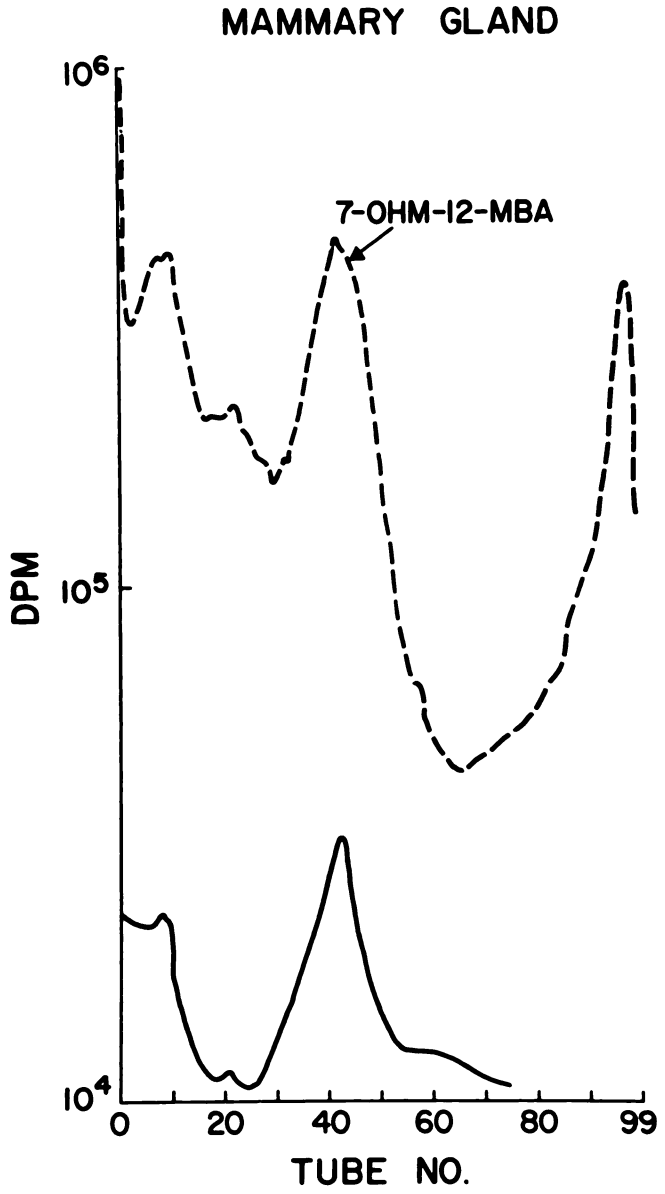

Chart 4. One hundred-transfer CCD in System 2 of the 7-OHM-12-MBA fraction. - - - -, normal mammary gland; ——, mammary gland from rats pretreated with 3-MC. Note the great reduction in the level of radioactivity in the peak zone.

nance of constant specific activity as shown in Table 1.

In the liver homogenates of 3-MC-treated rats, no metabolites corresponding to exclusively side chain hydroxylation were detected in the present study, which is attributable to a greater polycylic hydrocarbon hydroxylase activity. This amplification of hydroxylase activity by the pretreatment with 3-MC probably leads to polyhydroxy compounds including ring hydroxylation.

The elevated level of water-soluble metabolites also reflects an increase in aryl hydroxylase activity. Thus, incubation of tissue from pretreated rats showed 22 and 28% rises in water-soluble metabolites isolated from liver and mammary gland homogenates, respectively.

A metabolite, the identity of which is unknown but which probably represents a dihydroxy ring hydroxylated derivative of DMBA, with a partition coefficient almost identical to that of 7,12-diOHMBA, is evident in the CCD patterns. This partly accounts for the great difficulty experienced in the identification of this metabolite, since the 7,12-diOHMBA represents a shoulder on the side of the less polar but more plentiful contaminant. The absence of this shoulder in the liver of the 3-MC-treated animal and the negative carrier recrystallization data verified its absence as a metabolite of DMBA by this tissue.

Of great interest in the present study is the discovery that the mammary gland, which is the primary target tissue of the carcinogenic action of DMBA, is indeed able to metabolize this polycylic hydrocarbon to hydroxylated derivatives. Prominent peaks corresponding to 7-OHM-12-MBA and 12-OHM-7-MBA (Charts 4 and 5) were found by the CCD of representative TLC zones of these metabolites.

In contrast to the liver data, 7,12-diOHMBA was not found in the mammary gland. Also, mammary tissue from rats pretreated with 3-MC metabolized DMBA differently. Whereas pretreatment with 3-MC shifts metabolism from side chain to ring hydroxylation in the liver, the mammary

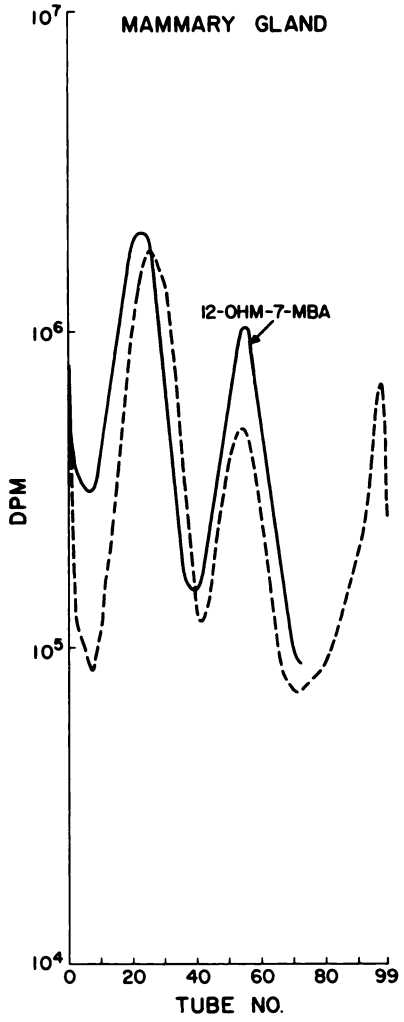

Chart 5. One hundred-transfer CCD in System 2 of the 12-OHM-7-MBA fraction. - - - -, normal mammary gland; ——, mammary gland from rats pretreated with 3-MC. Note the elevation in the level of radioactivity in the peak zone.

Table 2

Identification of DMBA metabolites in rat mammary gland homogenate by carrier recrystallization

Recrystallization	Specific activity (dpm/mg) of metabolite					
	7,12-diOHMBA		7-OHM-12-MBA		12-OHM-7-MBA	
	Crystals	Filtrates	Crystals	Filtrates	Crystals	Filtrates
Start			82,130		155,609	
1			74,063	109,760	116,574	276,238
2			77,531	84,035	114,617	148,852
3			73,120	79,478	114,140	119,229
4					112,804	110,664

Table 3

Identification by carrier recrystallization of DMBA metabolites in mammary gland homogenate from rats pretreated with 3-MC

Recrystallization	Specific activity (dpm/mg) of metabolites			
	7-OHM-12-MBA		12-OHM-7-MBA	
	Crystals	Filtrates	Crystals	Filtrates
Start	6021		3816	
1	6205	9074	2960	6766
2	5587	6867	3229	4154
3	5456	5607	2813	2772
4	5167	5000	3328	2535
5	5266	4848		

gland retains its ability to terminate the hydroxylation of DMBA at the monohydroxy stage. This ability to form the 7-hydroxy derivative by the mammary gland is markedly decreased as a result of 3-MC pretreatment. This finding agrees with the principle that pretreatment with 3-MC stimulates further metabolism to both polar and water-soluble components. However, this interpretation cannot explain the actual rise in the percentage conversion to the 12-hydroxy derivative.

We reported earlier that pretreatment of rats with 3-MC does not reduce the incidence of breast tumors caused by DMBA, which was a point in favor of DMBA per se as the active carcinogen (3). However, the continued ability of the mammary gland to form monohydroxy derivatives of DMBA illustrates that if metabolism to a carcinogenic agent occurs in this target tissue, this metabolism can continue to occur even in the presence of aryl hydroxylase stimulation.

It is still not known with certainty whether the carcinogenic action of DMBA is attributable to any of its derivatives. The present data illustrate for the 1st time that enzymes capable of transforming DMBA to the hydroxymethyl derivatives are present in the mammary tissue. This observation supports the earlier postulate that if conversion to a proximate carcinogen is a necessary intermediate step in the carcinogenesis of the mammary gland, it must occur in this target tissue (4).

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