Biochemical Pharmacology, Vol. 21, pp. 1603–1608. Pergamon Press, 1972 Printed in Great Britain.

N-ETHYLMALEIMIDE ADDUCT OF 7,12-DIMETHYLBENZANTHRACENE

PROPERTIES AND METABOLISM BY RAT LIVER MICROSOMES

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(*Received* 11 *October* 1971; *accepted* 17 *December* 1971)

Abstract—The endocyclic *N*-ethylmaleimide adduct of 7,12-dimethylbenz(a)anthracene (DMBA-NEM) was prepared, and its physical, chemical and biological properties were examined. DMBA-NEM was a poor inducer of aryl hydrocarbon hydroxylase but increased the conversion of estradiol to water-soluble products by rat liver microsomes. The DMBA-NEM adduct, like the parent hydrocarbon, was metabolized by the microsomal system to more polar products, but pretreatment of rats with polycyclic hydrocarbons did not influence the metabolism *in vitro* of the adduct. DMBA-NEM caused neither adrenal necrosis nor mammary tumors in rats, but it had some anti-adrenocorticolytic activity. The properties of DMBA-NEM in relation to its structure are discussed.

IT IS now well established[1,2] that DMBA* causes mammary cancer and necrosis of the adrenal glands in rats and that these animals can be protected by pretreatment with certain aromatic substances.[3,4] During experiments to investigate differences in the metabolism of DMBA by liver microsomes derived from normal and methylcholanthrene-treated rats,[5,6] a new product was shown to be formed when DMBA was incubated with *N*-ethylmaleimide. This paper describes some of the properties of this derivative of DMBA and also its metabolism by rat liver microsomes.

MATERIALS AND METHODS

Reagents. DMBA-12-^{14}C (6·5 mc/m-mole), estradiol-4-^{14}C (51·4 mc/m-mole) and *N*-ethylmaleimide-2,3-^{14}C (2·0 mc/m-mole), were obtained from Amersham/Searle, Toronto and stored as a stock solution at 4° in ethanol (1 mg/ml). DMBA-12-^{14}C was freed of radioactive decomposition products detectable by thin-layer chromatography and autoradiography by washing through a Silica gel column with benzene. The estradiol-4-^{14}C and *N*-ethylmaleimide-2,3-^{14}C were found to be virtually free of radioactive contaminants. DMBA and the sodium salts of NADP and glucose 6-phosphate were purchased from Sigma Chemical Company, St. Louis, Mo. *N*-ethylmaleimide and 2,4,7-trinitro-9-fluorenone were obtained from Eastman Kodak, Rochester, N.Y. All solvents were redistilled.

Animals and experimental procedure. Mature Sprague–Dawley or Holtzman rats weighing 180–200 g (females) or 250–380 g (males) were used. All animals had free access to food (Rockland diet) and water. The polycyclic hydrocarbons dissolved

* Abbreviations used: DMBA, 7,12-dimethylbenz(a)anthracene; NEM, *N*-ethylmaleimide; MC, 3-methylcholanthrene.

in sesame oil (1 ml) by gentle heating were administered by stomach tube, and unless otherwise indicated, the rats received 10 mg hydrocarbon in 1 ml oil. Control animals received oil only. Phenobarbital (30 mg/kg) was injected intraperitoneally in 0·9% saline (0·4 ml) for 4 days. In the studies to determine pathological effects, the animals were killed either 3 days after treatment with the hydrocarbons and the adrenals examined for gross hemorrhage or after 6 months if no mammary tumors had appeared by then.

Preparation of microsomal fraction and incubation. The animals were given an oral dose of hydrocarbon and killed 24 hr later by cervical dislocation after CO_2 anaesthesia. The liver was rapidly removed, washed with 0·25 M sucrose and weighed. A supernatant fraction (microsomes plus cell sap) was obtained at 8000 *g* from the liver homogenate (50 mg/ml) as described previously.[7] The liver preparation (1 ml) was shaken in O_2 at 37° with NADP (0·3 mM), glucose 6-phosphate (3mM) and either DMBA-12-^{14}C ($1{\cdot}5 \times 10^5$ dis./min in 2 μg), DMBA-12-^{14}C-NEM ($5{\cdot}5 \times 10^4$ dis./min in 10 μg) or estradiol-4-^{14}C ($2{\cdot}85 \times 10^5$ dis./min in 10 μg) in 0·1 M potassium phosphate buffer, pH 7·4, in a total volume of 4 ml. The incubation mixture was extracted three times with equal volumes of peroxide-free ether and the amount of radioactivity in the ether-soluble and residual aqueous fraction was determined in a Nuclear-Chicago (Des Plaines, Ill.) liquid scintillation counter as described previously.[7] Generally, 85–95 per cent of the added radioactivity could be accounted for and all incubations were repeated at least three times, with consistent results. The ether-soluble material was examined by thin-layer chromatography in benzene–ethanol (19:1) followed by autoradiography.[7]

Preparation of the N-*ethylmaleimide adduct of DMBA (DMBA-NEM).* DMBA-12-^{14}C (0·15 mc in 0·1 m-mole) was mixed with *N*-ethylmaleimide (0·2 m-mole) and dissolved by heating in benzene (0·2 ml) followed by the addition of ethanol (0·8 ml). The slightly turbid solution was centrifuged for 2 min at 1000 *g*, and the clear supernatant removed by Pasteur pipette. On standing, this solution yielded pale yellow needles which were washed twice with 0·5 ml of benzene–ethanol (1:4) and recrystallized from this solvent mixture. Yield: 16·4 mg, m.p. 188–191°, specific activity, $9{\cdot}1 \times 10^6$ dis./min/mg. DMBA-NEM-2,3-^{14}C, with the radioactivity in the maleimide portion of the adduct, and a larger amount (2·1 g) of non-radioactive DMBA-NEM were also prepared by this method. The evidence for the formation of a 1:1 adduct between DMBA and NEM and some properties of this compound are given under Results. The elemental analyses were carried out by Schwarzkopf Microanalytical Laboratory, Inc., Woodside, N.Y. Ultraviolet absorption spectra were determined in ethanol with a Unicam SP-800 recording spectrophotometer and the infrared spectra in chloroform with an Infracord model 137.

Visualization. After thin-layer chromatography on Silica gel, the polycyclic hydrocarbons and their metabolites were visualized by autoradiography, fluorescence at 254 nm in the presence or absence of NH_3 or by spraying with chromogenic reagents. These included conc. H_2SO_4, HCl (15 min at 100°), or 2,4,7-trinitrofluorenone (0·5 w/v in acetone) which forms a charge-transfer complex with aromatic hydrocarbons.[8]

RESULTS

Characterization of the DMBA-NEM adduct. By analogy with anthracene[9] it is probable that DMBA undergoes a Diels–Alder reaction with NEM at C-7 and C-12

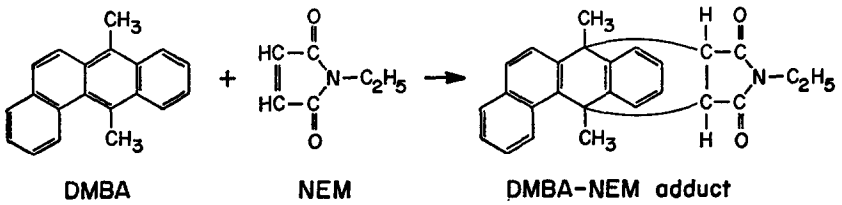

FIG. 1. Formation and proposed structure of the DMBA-NEM adduct.

corresponding to the reactive meso positions (Fig. 1), although a different type of addition cannot be completely excluded. The formation of a 1:1 adduct was supported by the specific activity of the ^{14}C-labelled product. *Elemental anal.* Calcd. for $C_{26}H_{23}O_2N$: C, 81·8; H, 6·1; N, 3·7. Found: C, 81·7; H, 6·0; N, 3·9. This endocyclic derivative of DMBA was found to be stable on boiling in organic solvents such as acetone, benzene, ethyl acetate, ethanol and heptane, and a NEM adduct was also formed with the 7- and 12-hydroxymethyl derivatives of DMBA. Additional evidence for interaction between DMBA and NEM was provided by obtaining the same ^{14}C-labeled product with DMBA-12-^{14}C and non-radioactive NEM as with NEM-2,3-^{14}C and non-radioactive DMBA. The ultraviolet and infrared spectra of DMBA-NEM are given in Figs. 2 and 3 and some of its other properties in Table 1.

Enzyme induction experiments. The effect of pretreatment by phenobarbital and polycyclic hydrocarbons including DMBA-NEM on the metabolism of ^{14}C-labeled DMBA, DMBA-NEM and estradiol by liver microsomes from male and female rats is shown in Table 2. As in the case of DMBA, there was only a slight sex difference in the metabolism of DMBA-NEM, but unlike the parent hydrocarbon, the adduct was not metabolized more rapidly by liver microsomes from rats treated with

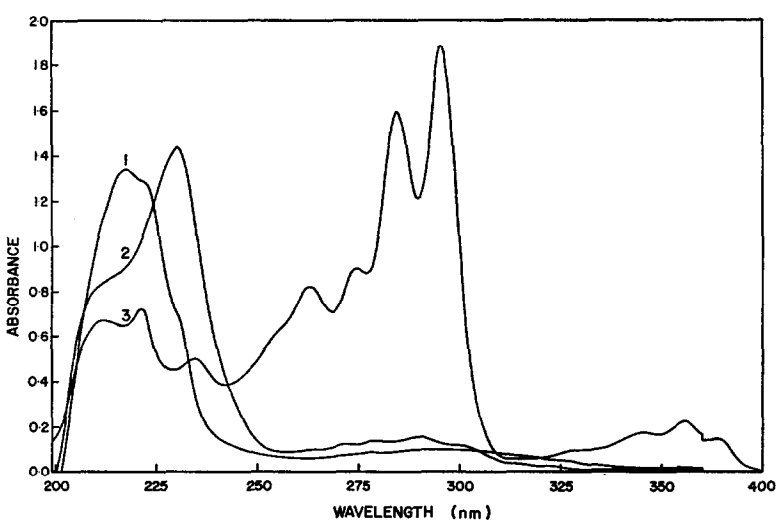

FIG. 2. Ultraviolet spectrum in ethanol of (1) NEM (12·5 μg/ml), (2) DMBA-NEM (9 μg/ml) and (3) DMBA (6·25 μg/ml).

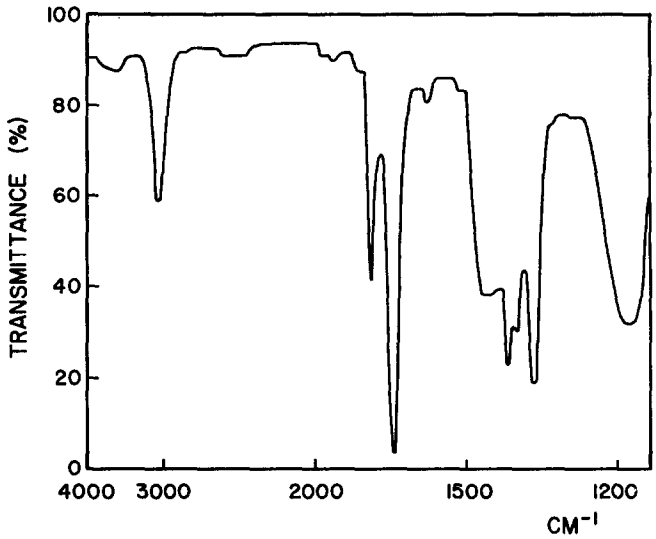

FIG. 3. Infrared spectrum of DMBA-NEM (25 mg/ml) in $CHCl_3$.

MC or DMBA. Phenobarbital, however, produced an increase in the conversion of both hydrocarbons, as well as of estradiol, to water-soluble products. DMBA-NEM was a poor inducer of aryl hydrocarbon-metabolizing enzymes but had some effect with estradiol as substrate. DMBA-12-^{14}C-NEM was shown by chromatography followed by autoradiography to be converted by rat liver microsomes to at leas

TABLE 1. PROPERTIES OF DMBA AND DMBA-NEM ON SILICA GEL THIN-LAYER CHROMATOGRAPHY

Property	DMBA	DMBA-NEM
R_f in benzene	0·96	0·27
R_f in benzene–ethanol (19:1)	0·93	0·46
R_f in light petroleum (60–80°)–ethanol (19:1)	0·58	0·25
Fluorescence in u.v. light (254 nm)	Violet	Pale violet
Fluorescence after exposure to NH_3	Violet	Violet
Color with conc. H_2SO_4*	Brown	No color
Color with HCl at 100°	Olive	No color
Color with trinitrofluorenone	Brown	Orange

* Tests were carried out as described in the text.

four ether-soluble radioactive metabolites more polar than the original adduct which, in the absence of standard reference compounds, were not identified. No metabolism of DMBA-NEM was observed with rat adrenal homogenates (10 mg/ml) fortified with NADP and glucose 6-phosphate, and virtually all the substrate could be recovered unchanged.

TABLE 2. EFFECT OF PRETREATMENT WITH POLYCYCLIC HYDROCARBONS AND PHENOBARBITAL ON THE CONVERSION OF ^{14}C-LABELLED DMBA, DMBA-NEM AND ESTRADIOL TO WATER-SOLUBLE PRODUCTS BY LIVER MICROSOMES FROM MALE AND FEMALE RATS*

Pretreatment	Per cent of added ^{14}C remaining in aqueous medium after extraction with ether					
	DMBA-12-^{14}C		DMBA-12-^{14}C-NEM		Estradiol-4-^{14}C	
	♂	♀	♂	♀	♂	♀
Control	22 ± 2·6	17 ± 2·5	27 ± 3·4	18 ± 2·8	52 ± 3·0	11 ± 2·3
MC	62	67	23	24	53	12
DMBA	46	58	29	24	51	15
DMBA-NEM	25	23	31	27	56	26
Phenobarbital	44	36	43	32	59	42

* The radioactive substrates were incubated with the 8000 *g* supernatant from 50 mg liver. Phenobarbital (30 mg/kg) was injected, i.p., in saline for 4 days; hydrocarbons (10 mg in oil) were given orally 24 hr before removing the liver. Each result is the mean of three or eight (standard error given) experiments. Other experimental conditions are described in text.

TABLE 3. INCIDENCE OF ADRENAL NECROSIS OR BREAST TUMORS IN RATS TREATED WITH DMBA AND DMBA-NEM

Group	Treatment*	Dose (mg)	No. of rats with adrenal necrosis	No. of rats with breast tumors
I	Oil control		0/6	0/6
II	DMBA	30	7/8	
III	DMBA-NEM	30	0/6	
IV	DMBA	15 + 10		7/7
V	DMBA-NEM	15 + 10		0/6

* Rats (♀) were given a single oral dose of DMBA or DMBA-NEM in oil (2·5 ml) or two doses at an interval of 1 week. Other conditions as described in text.

TABLE 4. INCIDENCE OF DMBA-INDUCED ADRENAL NECROSIS IN RATS PRETREATED WITH 3-METHYLCHOLANTHRENE OR DMBA-NEM

Group	Pretreatment*	Dose (mg)	No. of rats with adrenal necrosis
I	Oil control		7/8
II	MC	10	0/6
III	DMBA-NEM	10	6/13
IV	DMBA-NEM	20	1/6

* Rats (♀) were given an oral dose of MC or DMBA-NEM 24 hr before DMBA (30 mg). Other conditions as described in text. DMBA-NEM (30 mg) given at the same time as DMBA resulted in an adrenal necrosis incidence of 4/4.

Biological properties of the DMBA-NEM adduct. Unlike DMBA, the adduct produced neither necrosis of the adrenals nor mammary tumors (Table 3). However, a large dose of DMBA-NEM protected female rats against adrenal necrosis induced by DMBA administered 24 hr later but not if given at the same time (Table 4).

DISCUSSION

The diverse biological effects[10] of DMBA make derivatives of this polycyclic hydrocarbon ideal for the investigation of structure–activity relationships. Several such studies [10–12] varying the substituents on the aromatic rings of benz(a)anthracene have been carried out, but the NEM adduct presents a new type of derivative which retains some aromaticity but is yet nonplanar. It was therefore of interest to test whether DMBA-NEM was adrenocorticolytic or would cause mammary tumors in female rats and whether aromatic compounds, such as MC, which induce liver enzymes to hydroxylate DMBA[5,13] would have any effect on the NEM adduct.

Although DMBA-NEM was a poor inducer of aryl hydrocarbon hydroxylase, it produced some increase in the rate of hydroxylation of estradiol which possesses both aromatic and alicyclic characteristics.

The metabolism of endocyclic NEM adducts of polycyclic hydrocarbons, as far as is known, has not been studied previously. DMBA-NEM was converted to water-soluble metabolites more readily by liver microsomes from male than from female rats. The ether-soluble products which were all more polar than the substrate were, by analogy with DMBA,[14] presumed to be hydroxylated derivatives of DMBA-NEM.

It had been hoped that some dissociation into the component molecules would occur under physiological conditions so that any freed NEM would react with tissue sulfhydryl groups or glutathione with release of the hydrocarbon. There is evidence[15] that glutathione and other -SH compounds are involved in cell division; NEM adducts capable of dissociating could conceivably have influenced this process.

Acknowledgement—This work was supported by the National Cancer Institute of Canada.

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