Br. J. exp. Path. (1969) **50**, 78.

EFFECT OF *dl*-ETHIONINE AND NATURALLY OCCURRING AMINO ACIDS ON ADRENAL NECROSIS INDUCED BY 7,12-DIMETHYLBENZ[*a*]ANTHRACENE AND ITS 7-HYDROXYMETHYL DERIVATIVE IN FEMALE SPRAGUE-DAWLEY RATS

D. N. WHEATLEY

From the Department of Pathology, University Medical Buildings, Foresterhill, Aberdeen, Scotland

Received for publication August 8, 1968

SUMMARY.—*dl*-Ethionine at a dose of 125 mg. i.p. 3 days and, to a lesser extent, 4 days before i.v. treatment with 3 mg. 7,12-dimethylbenz[*a*]anthracene (DMBA) protects the adrenal glands from the adrenocorticolytic action of the polycyclic hydrocarbon in female Sprague-Dawley rats. Treatment of rats less than 3 days or more than 4 days before DMBA challenge has no protective effect. *dl*-Methionine at the same dose as ethionine is not protective when given 3 days before DMBA but shows some protective action as a 1-day pretreatment.

The protective action of ethionine seems unrelated to the suppression of protein synthesis or to the production of fatty liver. With both methionine, and ethionine, protection of adrenal glands is correlated with the marked vacuolation of the cytoplasm of hepatocytes.

Administration of methionine at the same dose as ethionine abolishes the protective action of the latter when they are given 3 days before DMBA.

Glycine, like methionine, also protects when given from about the same time as DMBA to 24 hr. beforehand. Its protective action can be prevented by concomitant administration of large doses of arginine.

None of the aforementioned protective treatments were effective against adrenal necrosis induced by 7-hydroxymethyl-12-methylbenz[*a*]anthracene.

ADRENAL glands of Sprague–Dawley female rats can be protected from the damaging action of 7,12-dimethylbenz[*a*]anthracene (9,10-dimethyl-1,2-benzanthracene, DMBA) by impairment of liver function (Wheatley, Kernohan and Currie, 1966b). CCl_4-induced centrilobular necrosis of the liver, or partial hepatectomy 24 hr. before an i.v. challenge of 3 mg. DMBA in lipid emulsion gave a high degree of protection. It was postulated that DMBA is metabolized by the liver to an active adrenocorticolytic derivative. CCl_4 treatment and partial hepatectomy are both relatively unrefined procedures which give little indication of the role played by the liver in the production of adrenal necrosis. It was considered possible that other hepatotoxins which affect the liver in different ways may help to elucidate the part played by the liver in the induction of adrenal necrosis by DMBA.

dl-Ethionine is a known hepatotoxin; in contrast to CCl_4 it induces an accumulation of fat in the periportal region of the liver lobules (Farber, Simpson and Tarver, 1950) but does not cause necrosis (Koch-Weser, Farber and Popper, 1951). Whilst peak fatty accumulation occurs between 36–50 hr. after treatment of female rats, ethionine inhibits protein synthesis in the liver within a few hr. of injection

(Simpson, Farber and Tarver, 1950) and this prevents the induction of microsomal enzyme systems in response to many drugs. Conney, Miller and Miller (1956, 1957), Neubert (1957), Gelboin, Miller and Miller (1959), Cramer, Miller and Miller (1960) amongst others report the ability of ethionine to suppress the induction of enzymes which metabolize polycyclic hydrocarbons, azo dyes and other compounds. An investigation of the effects of ethionine and several naturally-occurring amino acids on DMBA-induced adrenal necrosis was undertaken.

MATERIALS AND METHODS

Female rats of the Sprague–Dawley strain were obtained from Oxford Laboratory Animal Colonies, Bicester, Oxon. At appropriate times before DMBA administration they were treated with amino acids or placebo solutions so that rats received DMBA when 50 days old and weighing 140–170 g. They were fed modified Thompson rat cake (North-Eastern Agricultural Society Ltd., Aberdeen) and allowed water *ad libitum*.

Amino acids of analar quality (British Drug Houses, Poole, Dorset, England) were dissolved in sterile saline at 25 mg./ml. The solutions were warmed to body temperature for i.p. injection. After overnight starvation, each rat received a total of 125 mg. amino acid, given in 2 lots with an interval of 2 hr. between injections.

7,12-*dimethylbenz*[a]*anthracene* (*DMBA*) was injected i.v. at a dose of 3 mg. in 0·6 ml. of a 15 per cent lipid emulsion when the rats were 50 days old. Control rats received 0·6 ml. of the 15 per cent lipid emulsion free of DMBA.

7-*hydroxymethyl*-12-*methylbenz*[a]*anthracene* (7-*OHM-MBA*) was dissolved in olive oil at 15 mg./ml. and 1 ml. was given by stomach tube. Controls received 1 ml. olive oil.

Three days after DMBA injections, rats were killed by a blow over the head. Adrenal glands were excised, weighed and fixed in 4 per cent neutral buffered formaldehyde. One adrenal gland of each pair was frozen and cryostat sections were stained with oil red 0 for lipid. The other gland was embedded in paraffin wax and 5 μ sections were stained with haematoxylin and eosin. Livers were excised and weighed. Two pieces from each liver were fixed in 4 per cent neutral buffered formaldehyde, one piece being paraffin embedded, the other being frozen. Sections were stained with haematoxylin and eosin or oil red 0 as described above. For glycogen, the periodic acid-Schiff (PAS) procedure, with and without diastase treatment was carried out on liver sections.

RESULTS

Protection against DMBA-induced necrosis

Table I summarizes the experimental details and results of *dl*-ethionine, *dl*-methionine and saline pretreatments.

Pretreatment intervals of 1 day or less with ethionine had little effect on DMBA-induced adrenal necrosis. At 3 days after ethionine treatment only 1 of 20 rats developed adrenal necrosis. In protected rats, adrenal histology was normal. If the pretreatment interval was increased to 4 days, protection was achieved in the majority of the rats. With 5 or 6 day pretreatments with ethionine, little or no protection was observed.

dl-methionine at 3 and 6 days before DMBA had no protective effect. With a 1-day pretreatment fewer rats developed adrenal necrosis compared with a 1-day saline pretreatment, the difference being significant (Table I).

Effect of dl-*ethionine and* dl-*methionine on liver and adrenal glands*

To study the effects of ethionine and methionine on liver and adrenal glands, groups of 5 rats were treated as described before and killed at the time they would have received DMBA.

TABLE I.—*The Effect of* dl-*Ethionine and* dl-*Methionine on DMBA induced Adrenal Necrosis*

Pretreatment	Pretreatment interval	Challenge	No. rats with severe necrosis
dl-Ethionine 125 mg. i.p. in 2 × 2·5 ml. sterile saline	6 days	DMBA 3 mg. i.v.	6/10
	5 days	DMBA 3 mg. i.v.	6/10
	4 days	DMBA 3 mg. i.v.	2/10*
	3 days	DMBA 3 mg. i.v.	1/20†§¶
	2 days	DMBA 3 mg. i.v.	14/20
	1 day	DMBA 3 mg. i.v.	15/20
	8 hr.	DMBA 3 mg. i.v.	6/10
	2 hr.	DMBA 3 mg. i.v.	8/10
	3 days	Control emulsion	0/10
	1 day	Control emulsion	0/10
dl-Methionine 125 mg. i.p. in 2 × 2·5 ml. sterile saline	6 days	DMBA 3 mg. i.v.	7/10
	3 days	DMBA 3 mg. i.v.	7/10¶
	1 day	DMBA 3 mg. i.v.	3/10‡
	3 days	Control emulsion	0/10
	1 day	Control emulsion	0/10
dl-Ethionine 125 mg. + *dl*-methionine 125 mg. i.p.	3 days	DMBA 3 mg. i.v.	5/5§
Sterile saline 2 × 2·5 ml.	4 days	DMBA 3 mg. i.v.	9/10*
	3 days	DMBA 3 mg. i.v.	8/10†
	1 day	DMBA 3 mg. i.v.	9/10‡
None	—	DMBA 3 mg. i.v.	16/20

* $P = 0{\cdot}005$. † $P \ll 0{\cdot}0005$. ‡ $P < 0{\cdot}025$. § $P < 0{\cdot}0005$. ¶ $P < 0{\cdot}001$.

One day after ethionine treatment, liver histology and staining properties showed a loss of the characteristic granularity of the cytoplasm of hepatocytes (Fig. 1) associated with loss of PAS staining, indicative of glycogen depletion. Lipid staining was maximal 2 days after ethionine. At 3 days, liver showed vacuolation of the parenchymal cell cytoplasm (Fig. 2). There was intense PAS staining but little lipid staining. Liver 6 days after ethionine treatment appeared normal.

Severe vacuolation of liver parenchymal cells and some fatty accumulation occurred 1 day after methionine treatment (Fig. 3); these changes were not seen 3 days after methionine treatment when liver histology and staining properties were essentially normal (Fig. 4).

In the adrenal glands, ethionine and methionine injection caused some lipid depletion most noticeable 1 day after treatment but no other change was observed.

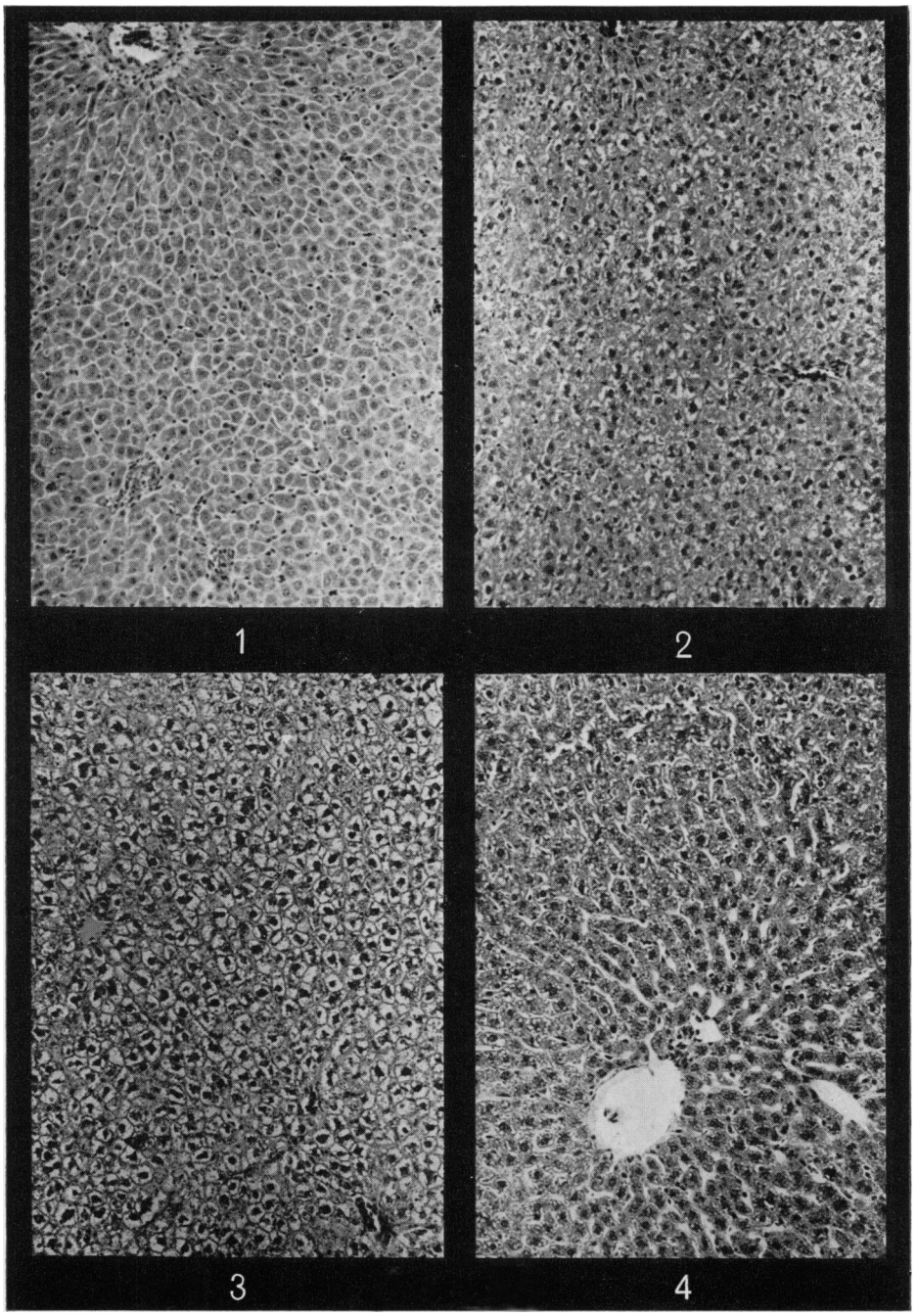
EXPLANATION OF PLATE

FIG. 1.—Liver of rat 1 day after i.p. injection of 125 mg. *dl*-ethionine, showing hyaline eosinophilic cytoplasm of the hepatocytes. H. and E. × 110.

FIG. 2.—Liver of rat 3 days after ethionine. Vacuolation of cytoplasm of hepatocytes. Compare with Fig. 1. H. and E. × 110.

FIG. 3.—Liver of rat 1 day after *dl*-methionine. Severe vacuolation of cytoplasm. H. and E. × 110.

FIG. 4.—Liver of rat 3 days after methionine. Histologically similar comparing closely to normal rat liver showing characteristically granular cytoplasm. H. and E. × 110.



Wheatley.

Abolition of the effect of dl-*ethionine on adrenal necrosis by* dl-*methionine*

Since many of the effects of ethionine can be counteracted by concomitant treatment with methionine (Simpson *et al.*, 1950; Farber *et al.*, 1950), rats were injected i.p. with 125 mg. *dl*-methionine dissolved in the solution containing 125 mg. *dl*-ethionine 3 days before 3 mg. DMBA i.v. Of 5 rats treated in this way (Table I), all developed severe adrenal necrosis ($P < 0{\cdot}0005$ compared with ethionine alone 3 days before DMBA).

Naturally occurring amino acids

Since earlier experiments had shown a protective action when glycine was given 1 day before DMBA and it has now been found that *dl*-methionine also gives significant protection at this same interval, a number of other amino acids were studied. The effects of 1- and 3-day pretreatments are shown in Table II. Only

TABLE II.—*Effect of Naturally Occurring Amino Acids, Given at a Dose of* 125 *mg. i.p.* 1 *or* 3 *Days Before* 3 *mg. DMBA, on Adrenal Necrosis in Sprague–Dawley Female Rats*

Amino acid	No. 1-day pretreated rats with severe adrenal necrosis	P	No. 3-day pretreated rats with severe adrenal necrosis	P
dl Methionine	3/10	$< 0{\cdot}025$	5/5	ns.
l-Methionine	3/10	$< 0{\cdot}025$	—	—
Glycine*	11/30	$= 0{\cdot}01$	4/5	ns.
l-Valine	9/10	ns.	5/5	ns.
l-Leucine	8/10	ns.	4/5	ns.
l-Arginine	8/10	ns.	5/5	ns.
dl-Phenylalanine	7/10	ns.	5/5	ns.
Saline	9/10	—	4/5	—

* Results from 3 separate experiments each showing similar protective capacity.

glycine and methionine (the *l*-isomer being as active as the racemic mixture in the latter case) gave significant protection whilst the other amino acids (*l*-valine, *l*-leucine, *l*-arginine and *dl*-phenylalanine) did not.

Glycine was further investigated by treating rats at different times in relation to the administration of DMBA. It gave the best protection as a 2-hr. pretreatment but good protection was obtained when it was given up to 24 hr. beforehand or simultaneously with DMBA administration (Table III).

It is well known that infusions of large amounts of glycine can result in the formation of ammonia (see Greenstein and Winitz, 1961) which may damage the

TABLE III.—*Effect of Varying the Interval Between Glycine Treatment of* 125 *mg. i.p. and DMBA Treatment on Adrenal Necrosis in Sprague–Dawley Female Rats*

Time of glycine administration in relation to DMBA	No. rats with severe adrenal necrosis
2 days before	7/10
1 day before	8/20
2 hr. before	3/20
Simultaneous	3/10
12 hr. after	6/10

liver. Since arginine given with glycine can offset this effect, probably by stimulating the formation of urea from ammonia (Najarian and Harper, 1956), we investigated the effect of arginine on glycine-induced protection. Arginine did not abolish the protective action of glycine when given simultaneously at 125 mg. but was effective when given at 250 mg. (Table IV).

TABLE IV.—*Glycine Protection: Effect of Treatment with Arginine at the Same Time as* 125 *mg. Glycine*, i.e. 2 *hr. Before DMBA, on Protection of the Adrenal from Necrosis*

Treatment	No. rats with severe adrenal necrosis
Glycine + saline . . .	1/10
Glycine + arginine (125 mg.) .	1/10
Glycine + arginine (250 mg.) .	10/10
Arginine (250 mg.) . . .	7/10

Lack of protection against 7-OHM-MBA-induced adrenal necrosis

The protective actions shown against DMBA-induced adrenal necrosis by ethionine, methionine and glycine were found to be without effect on 7-OHM-MBA-induced adrenal necrosis. This is similar to the results already reported (Wheatley, Hamilton, Currie, Boyland and Sims, 1966) that liver interference by CCl_4 or partial hepatectomy did not affect 7-OHM-MBA-induced adrenal necrosis.

DISCUSSION

DMBA probably produces adrenal necrosis only after it has been metabolized to an adrenocorticolytic derivative by the liver (Wheatley *et al.*, 1966*b*). Hydroxylation of the 7-methyl group results in a compound which is more potent than DMBA itself in producing adrenal necrosis (Boyland, Sims and Huggins, 1965; Wheatley *et al.*, 1966*a*). The metabolic conversion may require the induction of enzymes by the substrate, DMBA itself. Since ethionine can prevent the induction of drug metabolizing enzymes in response to polycyclic compounds (see Gelboin, 1967 for review), the lack of protective action in rats given ethionine 2, 8 or 24 hr. before DMBA challenge makes it improbable that an enzyme induction is necessary for the conversion of DMBA to its active derivative in sufficient quantity to damage the adrenal glands. Presumably there is sufficient pre-existing enzyme in the liver to carry out the conversion even in the presence of ethionine.

Since Farber *et al.* (1950) demonstrated that lipid accumulation in the liver reaches its peak about 2 days after ethionine treatment, it was considered that any protective action against DMBA might occur at this time. However, protection of adrenal glands against necrosis was better if ethionine was given 3 or even 4 days before DMBA. Sections of liver stained with oil red 0 showed lipid accumnlation to be far less intense at 3 and 4 days after ethionine than at 2 days. Protection is unlikely to have been due to sequestration of DMBA or its metabolites in accumulated lipid.

One consistent histological feature of hepatocytes in animals protected by amino acid pretreatment against DMBA-induced adrenal necrosis was the marked vacuolation of hepatocytes, particularly severe 1 day after methionine (Fig. 3) or glycine treatment but 3 days after ethionine treatment. It is possible that the metabolism of excessive amounts of some of the protective amino acids results in

either ammonia toxicity, *e.g.* glycine (Greenstein and Winitz, 1961) or the production of mercapturic acids (in the case of ethionine), which interferes with the metabolism of polycyclic hydrocarbons in the liver.

Ethionine protection does not seem to be related to either inhibition of enzyme induction or the production of fatty liver. Harris and Robinson (1961) have reported that ethionine impedes the uptake of chylomicra from the circulation. Since DMBA was injected i.v. in lipid emulsion, it is possible that ethionine sufficiently protracted DMBA uptake and metabolism by the liver so that a critical level of adrenocorticolytic derivative was not exceeded. We have compared the rates of clearance of DMBA-containing emulsion from the blood of rats given 3 day pretreatments with ethionine or saline. The 2 groups of rats showed no difference in the rate at which the emulsion was cleared from the blood-stream.

Apart from an early lipid depletion, ethionine itself caused no histological change in the adrenal cortex at the dose level used in the experiments, although Farber *et al.* (1950) have reported cortical haemorrhage with larger doses. The susceptibility of the adrenal gland to necrosis might, however, be due to the interference of ethionine with the normal functioning of this tissue but it is unlikely that this explains the protective mechanism since the cortex remains susceptible to 7-OHM-MBA-induced adrenal necrosis. It is more probable, therefore, that the protective action of ethionine against DMBA-induced adrenal necrosis is related to interference with the metabolism of the polycyclic hydrocarbon in the liver.

This work was supported by a grant to Professor A. R. Currie from the Scottish Hospital Endowments Research Trust. I wish to thank Professor Currie for his interest in this work. DMBA in lipid emulsion was given by Professor Charles B. Huggins and 7-OHM-MBA by Dr. Peter Sims to whom I am most grateful. The technical assistance of Mrs. M. Inglis, Mr. George Milne and Miss B. C. Cruden is acknowledged.

REFERENCES

BOYLAND, E., SIMS, P. AND HUGGINS, C.—(1965) *Nature, Lond.*, **207**, 816.
CONNEY, A. H., MILLER, E. C. AND MILLER J. A.—(1956) *Cancer Res.*, **16**, 450.—(1957) *J. biol. Chem.*, **228**, 753.
CRAMER, J. W., MILLER, J. A. AND MILLER, E. C.—(1960) *J. biol. Chem.* **235**, 250.
FARBER, E., SIMPSON, M. V. AND TARVER, H.—(1950) *J. biol. Chem.*, **182**, 91.
GELBOIN, H. V.—(1967) *Adv. Cancer Res.*, **10**, 1.
GELBOIN, H. V., MILLER, J. A. AND MILLER, E. C.—(1959) *Cancer Res.*, **19**, 975.
GREENSTEIN, J. P. AND WINITZ, M.—(1961) *In* ' Chemistry of the Amino Acids '. New York (Wiley), vol. 1, p. 286.
HARRIS, P. M. AND ROBINSON, D. S.—(1961) *Biochem. J.*, **80**, 352.
KOCH-WESER, D., FARBER, E. AND POPPER, H.—(1951) *Archs Path.*, **51**, 498.
NAJARIAN, J. S. AND HARPER, H. A.—(1956) *Proc. soc. Exp. Biol. Med.*, **92**, 560.
NEUBERT, D.—(1957) *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **352**, 235.
SIMPSON, M. V., FARBER, E. AND TARVER, H.—(1950) *J. biol. Chem.*, **182**, 81.
WHEATLEY, D. N., HAMILTON, A. G., CURRIE, A. R., BOYLAND, E. AND SIMS, P.—(1966*a*) *Nature, Lond.*, **211**, 1311.
WHEATLEY, D. N., KERNOHAN, I. R. AND CURRIE, A. R.—(1966*b*) *Nature, Lond.*, **211**, 387.