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Drug-induced porphyrin biosynthesis—I. The effect of porphyria-inducing drugs on *N*-demethylase activity of chick embryo liver

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THE ADMINISTRATION of phenobarbital and a variety of other drugs to experimental animals leads to an increase in activity of liver microsomal drug-oxidizing enzymes which is paralleled by an increase in the amount of microsomal cytochrome P-450.[1–3] This cytochrome plays an important role in drug oxidation, and it has been suggested that the increased activity of drug-oxidizing enzymes depends mainly on increased synthesis of this cytochrome.[1] Since phenobarbital and a variety of other drugs induce the formation of δ-aminolevulinic acid (δ-ALA) synthetase in liver mitochondria with a concomitant increase in porphyrin synthesis, Granick[4] suggested the following sequence of events in response to a porphyria-inducing drug. Derepression of δ-ALA synthetase in mitochondria leads to increased porphyrin and heme formation. The heme is utilized for the prosthetic group of microsomal cytochrome P-450 so that an increased amount of cytochrome is available and hence an increased level of drug-oxidizing activity. The objective of this study was to test the validity of this hypothesis. In view of the fact that Granick's studies were carried out in chick-embryo liver cells, which were responsive to porphyria-inducing drugs from the time the embryo was 9 days old, our first experiments were directed to demonstrating the presence and inducibility of drug-oxidizing enzymes in these livers. In our next series of experiments, a positive correlation was sought between the ability of several analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC; see Fig. 2b) to induce an increased level of drug-oxidizing enzymes in liver and their porphyria-inducing activity. As an approximate index of the level of drug-oxidizing activity in the liver, the oxidative demethylation of aminopyrine was measured.

MATERIALS AND METHODS

Fertilized eggs used were of a White Leghorn strain obtained from the University of Alberta Farm and stored at 10° for no longer than 7 days prior to incubation at 38°. The age of the embryo was taken as the number of days from the beginning of incubation.

Measurement of N-*demethylase activity.* The embryos were killed by decapitation and the livers immediately removed and homogenized in a Potter-Elvehjem apparatus with 5 vol. of 1·15% potassium chloride solution in the cold. The homogenate was centrifuged at 9000 *g* for 20 min at 4°. The demethylation of aminopyrine was estimated by measuring the amount of formaldehyde liberated according to the method of Nash,[5] as modified by Cochin and Axelrod.[6] The incubation mixture used was as follows: microsomes plus soluble fractions obtained from 0·4 g liver were incubated for 1 hr at 37° in air with 5·0 μmoles aminopyrine, 0·6 μmole NADP, 6·0 μmoles glucose 6-phosphate, 50 μmoles nicotinamide, 45 μmoles semicarbazide hydrochloride, 25 μmoles $MgCl_2$, and 3 ml of 0·1 M potassium phosphate buffer (pH 7·4) in a total volume of 6·0 ml.

N-demethylase activity at various stages of development of embryo. *N*-demethylase activity at various stages of development of the embryo are recorded in Fig. 1. At each stage of development, enough livers were pooled to provide sufficient material for the assay. Thus, at 11 days approximately sixty livers were pooled while three livers sufficed with 3-day-old chicks.

Effect of phenobarbital and allylisopropylacetamide (AIA) on N-demethylase activity of chick-embryo liver. Phenobarbital sodium (5 mg), dissolved in water (0·1 ml), was injected through the membrane

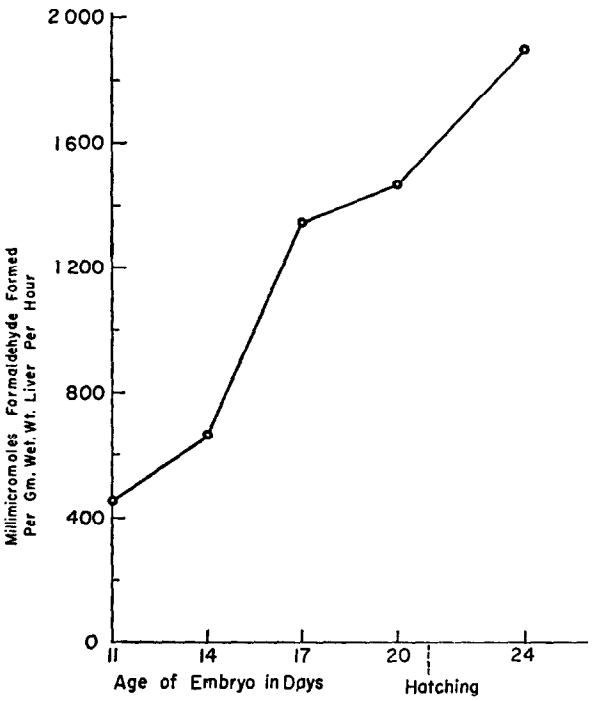


FIG. 1. Changes in *N*-demethylase activity at various stages of development of chick-embryo liver. The averages of the *N*-demethylase activity observed in at least three separate experiments are plotted at each time period.

TABLE 1. EFFECT OF PHENOBARBITAL AND AIA ON *N*-DEMETHYLASE ACTIVITY OF CHICK EMBRYO LIVER*

Compound injected in water (0·1 ml) once daily for 3 days	Daily dose (mg/egg)	Liver *N*-demethylase activity (mμmoles formaldehyde/g liver)	% Control
Control		1232	
Phenobarbital sodium	5	1824	148
Control		1465	
AIA	0·1	885	61
AIA	1·0	2650	180

* The embryos were injected on days 14, 15 and 16 of development and the enzyme activity was measured on day 17. Controls received the solvent alone. Livers of at least six embryos were pooled for each determination and the above data represent the average of two experiments.

of the air sack directly into the fluids surrounding the embryo on days 14, 15 and 16 of development. The embryo was sacrificed on day 17 and the liver *N*-demethylase activity was measured as described above. The above experiment was repeated using AIA (0·1 mg) in water (0·1 ml), and AIA (1 mg) in water (0·1 ml) in place of phenobarbital sodium. Controls were carried out in which the solvent alone was injected. The results of these experiments were recorded in Table 1.

Effect of DDC and analogues (Fig. 2) on levels of N-*demethylase activity after injection into the fluids surrounding the chick embryo.* DDC and its analogues (0·5 mg), dissolved in 95% ethanol (0·01 ml), were injected daily for 3 days into the fluids surrounding the chick embryo, as described above for phenobarbital, and the liver *N*-demethylase activity was estimated (Table 2). Controls were carried out in which the solvent alone was injected.

Effect of DDC and analogues on N-*demethylase activity of combined microsomal and soluble fraction.* DDC or its analogues, dissolved in 95% ethanol (0·01 ml), were added to the incubation mixture (6 ml) containing microsomes and soluble fraction, prepared as described above under measurement of *N*-demethylase activity. The effect of DDC and its analogues on *N*-demethylase activity is recorded in Table 3. Controls were carried out in which the solvent alone was added.

TABLE 2. EFFECTS OF DDC AND ANALOGUES ON LIVER *N*-DEMETHYLASE ACTIVITY AFTER INJECTION INTO THE FLUIDS SURROUNDING THE CHICK EMBRYO*

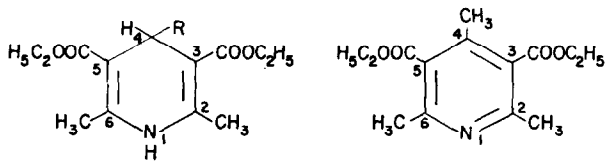
Compound injected in 95% ethanol (0·01 ml) daily for 3 days	Liver *N*-demethylase activity (mμmoles formaldehyde/g liver)	% Control	Porphyria-inducing activity† (μg porphyrin/g liver)
Control	785		
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 2a)‡	830	108	0·35 (0·32–0·38)
Control	1280		
3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine (Fig. 2e)	1240	97	0·70 (0·37–0·94)
Control	1270		
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (Fig. 2b)	307	24	136 (72–208)
Control	1515		
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (Fig. 2d)	125	8	Data not available§
Control	1435		
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 2c)	125	9	217 (201–247)

* The embryos were injected on days 14, 15 and 16 of development with 500 μg drug/day, and enzyme activity was measured on day 17. Livers of at least six embryos were pooled for each determination. The above data represent the average of two experiments, with the exception of DDC (Fig. 2b) which represents the average of three experiments.

† Drugs (4 mg) dissolved in dimethylsulfoxide were injected into the fluids surrounding the embryo. After incubation for 24 hr, the porphyrins were extracted from the liver and estimated fluorometrically. The data in this column are the averages of at least four determinations. The extreme values are given in parentheses. (Unpublished data of W. Racz of this laboratory.)

‡ This compound was dissolved in 0·02 ml of warm 95% ethanol due to its low solubility.

§ This compound was found to be toxic at the 4 mg dose level. However, data from cell culture studies[7] show this compound to have activity of a similar magnitude to 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 2c).



(a) (e)

(a); R = H

(b); R = CH_3

(c); R = CH_2CH_3

(d); R = $CH_2CH_2CH_3$

FIG. 2. Chemical structure of: (a) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine; (b) 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; (c) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; (d) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine; (e) 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine.

TABLE 3. EFFECT OF DDC AND ANALOGUES ON *N*-DEMETHYLASE ACTIVITY OF COMBINED MICROSOMAL AND SOLUBLE FRACTION*

Compound added in 95% ethanol (0·01 ml)†	Dose/ incubation tube (μg/6 ml)	Liver *N*-demethylase activity (mμmoles formaldehyde/g liver)	% Control	Porphyria-inducing activity‡
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 2a)	Control	1391		
	50	1325	96	1
	control	1350		
	500	975	72	
3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine (Fig. 2e)	control	1391		
	50	1167	84	2
	control	1580		
	500	466	30	
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Fig. 2b)	control	1391		
	50	825	59	3
	control	1580		
	500	275	14	
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (Fig. 2d)	control	1391		
	50	184	14	4
	control	1580		
	500	75	4	
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 2c)	control	1391		
	50	109	8	5
	control	1580		
	500	66	4	

* The drugs were added to the incubation mixture just prior to incubation. Data represent the average of two experiments at each dose level.

† 0·1 ml of 95% ethanol was required to dissolve 500 μg of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 2a).

‡ Compounds were ranked according to their threshold dose for inducing porphyrin accumulation in cell culture.[7] The most potent was ranked 5; the least potent was ranked 1, etc.

RESULTS AND DISCUSSION

Fouts and Adamson[8] were unable to demonstrate the presence of the enzyme systems which metabolize drugs in the livers of newborn rabbits. Moreover, it has been shown that the activity of these enzymes in rabbits cannot be stimulated before the last 4 days of fetal life.[9] In contrast to the above findings, Brodie and Maickel[10] have reported that drug-metabolizing enzyme systems can be demonstrated in the chick-embryo liver after 16 days of development. The results in Fig. 1 show the presence of considerable amounts of *N*-demethylase activity in chick-embryo liver as early as the eleventh day of development. Moreover, the amount increases steadily with the age of the embryo and continues to increase after hatching. It is of further interest that the amount of *N*-demethylase activity in the embryonic liver is increased after treatment with phenobarbital (Table 1). Thus the demonstration of the presence and inducibility of *N*-demethylase activity in these livers is consistent with Granick's interpretation of the sequence of events in response to a porphyria-inducing drug. The potent porphyria-inducing drug, AIA (1 mg/day for 3 days), behaved similarly to phenobarbital and caused an increased level of *N*-demethylase activity (Table 1). On the other hand, a smaller dose of AIA (0·1 mg/day for 3 days) caused a decreased level of *N*-demethylase activity (Table 1). The latter observation was surprising and is under further investigation. It is noteworthy that in preliminary experiments phenobarbital caused an increased level of *N*-demethylase activity at all doses tested (0·1, 1, 2, 5 and 10 mg).

In our next series of experiments, a positive correlation was sought between the porphyria-inducing activity of several DDC analogues and their ability to induce increased *N*-demethylase activity in the liver. The results (Table 2) show that, contrary to what was anticipated, the administration of DDC and its analogues resulted in decreased *N*-demethylase activity. Moreover, those drugs causing the most significant decrease in *N*-demethylase activity were the most potent in inducing porphyrin accumulation in the liver when injected into the fluids surrounding the chick embryo. It is of interest that Wada *et al.*[11] have recently demonstrated that administration of DDC to mice results in decreased levels of aminoazo dye *N*-demethylase activity in liver microsomes.

In further studies, it was shown (Table 3) that addition of DDC and its analogues to the combined microsomal and soluble fraction of chick-embryo liver resulted in inhibition of *N*-demethylase activity. Moreover, those drugs causing the most significant decreases in *N*-demethylase activity were the most potent in inducing porphyrin accumulation in chick-embryo liver cells grown on coverslips. It is necessary to point out that some DDC analogues, e.g. 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Fig. 2e), which induce porphyrin accumulation in chick-embryo liver cells grown on coverslips, do not do so when injected into the fluids surrounding the chick embryo. We have chosen to compare the effect of DDC and its analogues on the *N*-demethylase activity of the combined microsomal and soluble fraction (Table 3) with the porphyria-inducing activity of the drugs in chick-embryo iver cells grown in culture rather than with the results in the intact chick embryo. This procedure appeared reasonable, since porphyria-inducing activity observed in the intact chick embryo may be complicated by the inability of drugs to reach the site of action. It remains to be determined whether DDC and its analogues are inhibitors of the *N*-demethylase enzyme or are competitive substrates.

Since the administration of DDC and its analogues resulted in decreased *N*-demethylase activity instead of the anticipated increase, it is clear that the sequence of events suggested by Granick[4] to occur in response to porphyria-inducing drugs does not apply to these compounds. Further study is required to determine whether inhibition of the *N*-demethylase activity is related to the porphyria-inducing activity of these compounds.

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Effect of some *N N*-disubstituted dithiocarbamates on catecholamines level in rat brain

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DISULFIRAM, an *in vitro* and *in vivo* dopamine-β-hydroxylase inhibitor,[1] lowers the level of noradrenaline (NA) in various tissues, including the brain.[2] A similar effect is exerted by diethyldithiocarbamate (EE),[3] a compound regarded as the active metabolite of disulfiram.[4] Compounds with this type of action may serve as a tool in the investigations on functional role of NA in the central nervous system.

The mechanism of action of EE and of many other known dopamine-β-hydroxylase inhibitors depends upon their ability to form complexes with copper,[5] a metal playing the central role in the mechanism of oxidative hydroxylation of dopamine (DA).[6] *N,N*-disubstituted dithiocarbamates, analogs of EE, also form complexes with copper. It therefore seemed interesting to investigate their effect on the brain catecholamine (CA) level. In this paper the effects of four EE analogs on DA and NA levels in the whole brain of rats are presented. EE was also investigated as a reference compound.

MATERIALS AND METHODS

Sodium salts of the following dithiocarbamates were tested: *N,N*-dimethyl (.2H_2O) (MM), *N,N*-di-*n*-butyl (.H_2O) (BB), *N,N*-dicyclohexyl (CC), *N*-ethyl-*N*-phenyl (PhE, *N*-ethyldithiocarbanilate), and EE (.3H_2O). Male Wistar rats were injected i.p. with saline solutions of the investigated compounds. CC, owing to its poor solubility, was administered as a suspension in saline with Tween 80. Compounds were given at doses corresponding to approx. $\frac{1}{2}$ and $\frac{1}{4}$ of their LD_{50} values for mice.

Animals were killed by transcervical dislocation at various times after treatment, the brains were immediately removed and homogenized in ice-cold 0·4 N $HClO_4$. CA were absorbed on alumina[7] and determined spectrofluorometrically.[8]

RESULTS AND DISCUSSION

All the compounds investigated lowered the brain NA level (Table 1). MM was the most, and PhE the least potent in this respect. In most cases the maximum effect at the higher dose was not much greater than that of the lower. The maximum effect was observed in most cases after 2 hr.

The effect of the compounds on DA level varied. MM and PhE, similarly to EE, tended to enhance the brain DA level. BB and CC significantly decreased the DA brain level; the action of the latter depended upon the dose applied.

The results presented here show that, as expected, the *N,N*-disubstituted dithiocarbamates lower the NA brain level in rats. The dimethyl derivative was more potent in this respect than EE, and further investigations on this compound may prove to be rewarding. The results obtained with EE were similar to those reported by others.[3, 9] The effect of BB and CC on the DA brain content was unexpected, and the elucidation of the mechanism of this action needs further investigation. The