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Biochemical Pharmacology, Vol. 17, pp. 842–845. Pergamon Press. 1968. Printed in Great Britain

Inhibition of hexobarbital metabolism by diethylnitrosamine

(*Received* 11 *September* 1967; *accepted* 7 *December* 1967)

THE ACTIVITIES of various drug metabolising enzyme systems found in the microsomal fraction of liver homogenates can be greatly influenced by the pretreatment of animals with any of a considerable range of foreign chemicals.[1, 2] Inhibition may result from administration of compounds such as the diethylamino-ethanol ester of diphenylpropylacetic acid (SKF 525-A) while stimulatory agents include drugs like phenobarbital, insecticides of the chlorinated hydrocarbon type and certain polycyclic carcinogenic hydrocarbons.

Previously, it has been shown[3] that treatment of rats with the hepatocarcinogen diethylnitrosamine (DEN) increases the activity of liver UDP-glucuronyltransferase—the microsomal drug metabolising enzyme involved in the formation of glucuronides.[4] The present communication reports the effects of this treatment on the metabolism and duration of action of hexobarbital in rats.

MATERIALS AND METHODS

With the exception of the experiments described in Fig. 1, in which adult (200 g) rats were used, young male Wistar rats (80–100 g), purchased from Animal Suppliers Ltd., 685 High Road, London, N. 12., were used throughout. DEN was administered intraperitoneally in aqueous solution, equal numbers of treated and control (saline-injected) animals being used. The duration of hypnotic action and the *in vitro* metabolism of hexobarbital were determined simultaneously on separate groups of animals at varying intervals following administration of DEN.

The duration of sleep was assayed by determining the period for which rats lost the righting reflex following intraperitoneal administration of sodium hexobarbital (100 mg/kg).

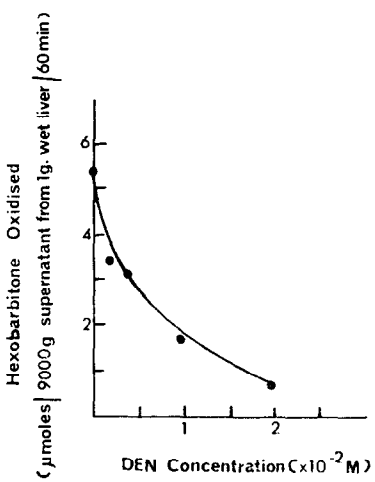

FIG. 1. *In vitro* effect of DEN on hexobarbital oxidation.
After incubation of hexobarbital with 9000 *g* liver supernatants from adult male rats, unchanged barbiturate was assayed by the TLC procedure described. The results shown are the mean of three separate experiments.

For *in vitro* metabolism experiments, rats were killed by stunning and subsequent cervical dislocation, the liver being rapidly excised and transferred to ice-cold isotonic KCl. 30% (w/v) liver homogenates were prepared (by homogenisation for 30 sec at 14,000 rpm) in an MSE homogeniser, then spun at 0–4° for 30 min at 9000 *g*. The resulting supernatant fraction was used for determination of enzyme activity, the metabolic pathway investigated being the side-chain oxidation of hexobarbital. The incubation mixture, total volume 2·0 ml, contained substrate and cofactors in the concentrations used by McLuen and Fouts,[5] together with 0·5 ml of the liver supernatant. The extent of hexobarbital metabolism after 60 min incubation was determined by measuring the amount of remaining substrate by the method of Cooper and Brodie.[6]

Estimation of hexobarbital using thin layer chromatography TLC

In experiments where DEN was added directly to the *in vitro* hexobarbital oxidation system, a different method for estimating hexobarbital was required, since DEN absorbs at 245 mμ and interferes in the final spectrophotometric estimation of the barbiturate by the Cooper and Brodie method. Separation of hexobarbital and DEN by solvent extraction proved difficult and an estimation procedure involving TLC was devised.

In this case, incubations were terminated by addition of 1·0 ml 0·15 M phosphate buffer pH 5·5 to the incubation tubes which were then immersed in boiling water for 2 min. After centrifuging, 2·0 ml of the supernatant was transferred to small glass-stoppered tubes containing 6·0 ml methylene dichloride and shaken for 15 min. 5·0 ml of the lower layer after centrifugation was evaporated to dryness at 50° under a stream of nitrogen, the residue being dissolved in 0·5 ml ethanol.

50 μl of this extract was spotted in duplicate on glass plates coated with silica gel G, (250 μ) then developed in a solvent system consisting of methanol/methylene dichloride (1·5:98·5, v/v). Under these conditions, DEN (detected by u.v. absorption) and hexobarbital (detected using the diphenylcarbazone[7] reagent) had R_f's of 0·70 and 0·35 respectively. For quantitative estimation of hexobarbital, a standard was run on each plate and detected as above; the corresponding untreated areas for each extract were scraped off into centrifuge tubes, eluted into pH 11 phosphate buffer[6] then read at 245 mμ. Hexobarbital in quantities greater than 5 μg could be estimated with approximately 5 per cent accuracy by this procedure.

RESULTS AND DISCUSSION

The results show that in contrast to its stimulatory effect on liver microsomal UDP-glucuronyltransferase, DEN treatment inhibited hexobarbital metabolism. DEN itself has no hypnotic properties but as is evident from Table 1, DEN pretreatment significantly increased the duration of sleep subsequently induced by hexobarbital. This was the case whether treatment consisted of three successive daily doses, with assays carried out during the following 2 weeks or of a single larger dose with sleeping time determinations 2 hr later. It is apparent too (Table 1) that this prolongation of

TABLE 1. HEXOBARBITAL METABOLISM AND SLEEPING TIMES IN CONTROL AND DEN-TREATED RATS

Experiment	Day of assay	Duration of sleep (min)*		Hexobarbital metabolised†	
		Control	Treated	Control	Treated
1	4	43 ± 14(9)	101 ± 21(9)	0·98 ± 0·24(3)	0·35 ± 0·04(3)
2	5	39 ± 6 (8)	86 ± 12(7)	0·71 ± 0·12(3)	0·18 ± 0·03(3)
3	7	48 ± 10(9)	90 ± 20(8)	0·83 ± 0·15(3)	0·25 ± 0·06(3)
4	9	50 ± 6(9)	92 ± 9(7)	1·16 ± 0·19(3)	0·44 ± 0·05(3)
5	16	35 ± 8(7)	69 ± 14(8)	0·71 ± 0·12(3)	0·27 ± 0·05(3)
6	—	59 ± 20(9)	113 ± 18(9)	0·87 ± 0·04(3)	0·44 ± 0·03(3)

The values given are means ± S.D. with the number of animals used in parentheses. Treated animals in experiments 1–5 were injected intraperitoneally with DEN (30 mg/kg) once daily on days 1, 2 and 3. In experiment 6, animals received DEN (100 mg/kg) 2 hr before the assays were carried out.

* In all sleeping time experiments, the results are significantly different at $P < 0·005$.

† Expressed as μmoles metabolised/supernatant from 1 g wt wt. liver/hr.

sleep results from reduced hexobarbital metabolism, since in each experiment the livers of treated animals had an appreciably reduced ability to metabolise hexobarbital *in vitro*. In this respect, the effects of administration of DEN resemble those following treatment with the commonly-studied inhibitor SKF 525-A and related compounds. However it is known[8] that after a single dose of SKF 525-A, the inhibition of microsomal drug metabolising enzymes is evident for less than 48 hr and may be followed by a period of stimulated drug metabolising enzyme activity. The inhibitory effects of DEN were much longer lasting, there being no obvious reduction in the degree of inhibition 13 days after the last injection of the carcinogen (Table 1). At no time was any stimulatory effect on hexobarbital metabolism observed.

The *in vitro* metabolism of hexobarbital involves oxidation by a microsomal enzyme system utilising $NADPH_2$ generated *in vitro*. The production of $NADPH_2$ by liver supernatants from DEN-treated rats was found to be unimpaired and the inhibited oxidation of hexobarbital must presumably result from some defect at the microsomal level. The results of acute DEN treatment on hepatic cell morphology have not yet been reported, but it is conceivable that this hepatocarcinogen might alter hepatic endoplasmic reticulum structure—the organelle with which many drug metabolising enzyme systems are associated.[9] Certainly, the closely related compound dimethylnitrosamine (DMN) is known to produce swelling and vacuolisation of the endoplasmic reticulum.[10] Interestingly, it has recently been shown that treatment of rats with DMN diminished the activity of the hepatic microsomal process responsible for demethylation of dimethylaniline.[11]

Using the thin layer estimation system described, DEN added directly to the *in vitro* incubation system was also found to inhibit hexobarbital oxidation. It is evident from Fig. 1 that relatively high concentrations were required, 50 per cent inhibition occurring in the presence of 5×10^{-3} M DEN. The possibility was considered that the reduced ability (Table 1) of livers from DEN-treated rats to oxidise hexobarbital *in vitro* might result from their content of unmetabolised DEN. However, estimation of DEN by a polarographic technique[12] showed the DEN content of livers from treated rats to be extremely low, being less than 5 μg DEN/g liver 2 days after the last of the three injections of the carcinogen. Since the concentration arising *in vitro* from this liver content is of the order of 10^{-6} M this possibility is extremely unlikely. Preliminary experiments, using short time incubations indicate that *in vitro* DEN itself and not a metabolite is the inhibitory species. This is again in contrast with the inhibitory agent SKF 525-A, whose inhibitory effects *in vitro* on drug metabolising enzymes appear to be due to a metabolite formed during incubation.[13]

DEN is obviously a compound which appreciably affects heptatic drug metabolising enzymes in the rat. Administration of this agent can apparently produce a stimulation of the microsomal drug metabolising enzyme UDP-glucuronyltransferase. Isolated liver microsomes from the same animals had a reduced capacity to oxidise hexobarbital however. The results indicate that this reduced hexobarbital oxidation is not due to a direct inhibition of the microsomal oxidase by DEN. It would appear more likely that other mechanisms such as repression of enzyme synthesis might be involved

Acknowledgements—We wish to thank the Medical Research Council for financial support. The technical assistance of Mrs. Anne Reekie is also gratefully acknowledged.

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Biochemical Pharmacology, Vol. 17, pp. 845–848. Pergamon Press. 1968. Printed in Great Britain

The effects of 1-(1-phenylcyclohexyl)piperidine·HCl (phencyclidine, Sernyl) on respiration and related reactions of liver mitochondria *in vitro**

(*Received* 31 *August* 1967; *accepted* 6 *November* 1967)

Previous work showed that 1-(1-phenylcyclohexyl)piperidine-HCl (phencyclidine, Sernyl) stimulated mitochondrial respiration and inhibited phosphorylation coupled to the oxidation of succinate,

* This work was supported by a grant from the Psychiatric Training and Research Fund of the Illinois Department of Mental Health.