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INHIBITION OF SOME RAT HEPATIC MICROSOMAL ENZYMES BY ETHOXYQUIN

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Abstract—Administration of single intragastric doses of ethoxyquin (500 mg/kg) to rats dramatically increased the hexobarbitone sleeping time. Conversely, dietary administration of ethoxyquin (0·5% w/w) for 14 days led to a 50 per cent decrease in the hexobarbitone sleeping time. Single doses of ethoxyquin had little effect on hepatic mixed function oxidase activity. Ethoxyquin was shown to be a potent competitive inhibitor of rat hepatic microsomal biphenyl 4-hydroxylase and ethylmorphine *N*-demethylase *in vitro* ($K_i = 4{\cdot}3 \times 10^{-6}$ M and $6{\cdot}0 \times 10^{-6}$ M respectively) and displayed a type 1 binding spectrum with cytochrome P-450 with a very high affinity ($K_s = 1{\cdot}06 \times 10^{-5}$ M). Ethoxyquin had no inhibitory effect on the activity of glucose-6-phosphatase *in vitro*.

ETHOXYQUIN causes induction of hepatic microsomal drug-metabolizing enzymes when administered to rats at a concentration of 0·5% (w/w) in the diet for 60 days post-weaning.[1] The induction in these rats was maximal after 7–30 days of exposure to dietary ethoxyquin. Single or multiple doses (oral or intraperitoneal) also cause induction of some drug-metabolizing enzymes 24–72 hr after the administration of ethoxyquin.[2] Single doses are rapidly metabolized *in vivo*,[3] and repeated dosing is necessary for maximal induction.[1] In this respect ethoxyquin resembles the short-acting barbiturates which undergo rapid metabolism and generally cause induction only after massive or repeated dosing, when the increased demand for microsomal metabolism is sustained.[4] This suggested that the enzyme induction observed with ethoxyquin might result from initial (competitive) inhibition leading to a demand for increased enzyme synthesis.

To test this hypothesis that ethoxyquin might be an initial inhibitor the effects of this antioxidant were investigated on (a) *in vitro* inhibition of biphenyl 4-hydroxylase, ethylmorphine *N*-demethylase, and glucose-6-phosphatase, (b) drug-metabolizing enzymes and certain other parameters at various intervals of time after single intragastric doses, and (c) *in vivo* inhibition of hexobarbitone metabolism after single intragastric doses and after chronic administration in the diet for 14 days. In addition, the spectral dissociation constant (k_s) of ethoxyquin was studied to see, if in the event of its being an inhibitor of microsomal enzymes, it could bind to cytochrome P-450 and act as a substrate for terminal mixed function oxidases.

MATERIALS AND METHODS

Chemicals. Ethoxyquin, 96% g.l.c. pure grade, was obtained from Koch Light Ltd. Hexobarbitone (B.P.C. 1959) was procured from May and Baker Ltd. and phenobarbitone sodium (BDH) was Laboratory Reagent Grade.

Mention has been made of other chemicals in the text where relevant.

Animals. Inbred male Wistar Albino rats were used and accommodated under controlled conditions of temperature (20–22°), relative humidity (50 per cent) and lighting (12 hr light, 12 hr dark).

Inhibition studies in vitro. Four rats were reared from weaning at 21 days of age on Spillers Small Animal diet, meal form, to which was added 2·5% v/w antioxidant-free arachis oil (Saladin Brand, Craigmiller Ltd). At 48 days of age the animals were killed by cervical dislocation, their livers pooled and a double-washed microsomal suspension prepared according to the method described by Basu *et al.*[5] The enzyme activities of this microsomal preparation were measured over a range of substrate concentrations and in the absence or presence of various concentrations of ethoxyquin.

Ethoxyquin was added as a 20 mM solution in ethanol–water (2:1 v/v) at a maximum volume of 6 μl per incubation mixture, and a similar volume of aqueous ethanol was added to controls without ethoxyquin.

Biphenyl 4-hydroxylase activity was assayed by the method of Creaven *et al.*,[6] as modified by Neale[7] except that the substrate concentration was varied between 0·6 and 1·2 mM and ethoxyquin was added at a concentration of 0–0·06 mM. Ethylmorphine *N*-demethylase activity was assayed by the method of Holtzman *et al.*,[8] at substrate concentrations of 2·08–4·17 mM and ethoxyquin concentrations of 0–0·066 mM. Glucose-6-phosphatase activity was assayed by the method of de Duve *et al.*[9] at substrate concentrations of 0·31–0·62 mM and ethoxyquin concentrations of 0–0·06 mM.

Single dose effect. Fifty-six weanling rats (21 days old) were dosed intragastrically with ethoxyquin at a level of 500 mg/kg. The ethoxyquin was administered as a 25% (w/v) solution in arachis oil. A second group of rats was used as controls and given the vehicle only. Immediately after dosing, eight rats dosed with ethoxyquin and 8 controls were killed by cervical dislocation and their livers removed, weighed and processed in groups of two animals. The 10,000 *g* supernatant was used to assay biphenyl 4-hydroxylase activity and ethylmorphine *N*-demethylase activity as before. The microsomal suspension was used to determine the concentrations of microsomal protein and cytochromes P-450 and b_5 by the methods of Lowry *et al.*,[10] Sladek and Mannering[11] and Schenkman *et al.*[12] respectively.

The remaining rats were given the control powdered diet and water *ad lib.* After 2, 4, 8, 12, 24 and 48 hr eight rats from each group were killed and examined as before.

Inhibition studies in vivo. The effects of ethoxyquin on hexobarbitone metabolism were studied *in vivo* in two experiments. In the first experiment 8–9-week-old rats reared on laboratory pellet diet (Spillers Ltd.) from weaning were given hexobarbitone (100 mg/kg) and its rate of metabolism studied by observing the sleeping-time at various intervals after a single intragastric dose of ethoxyquin (500 mg/kg). The ethoxyquin was administered as a 25% (w/v) solution in arachis oil and the hexobarbitone was dissolved in 2 N NaOH (100 mg/2 ml) adjusted to pH 11 and administered by intraperitoneal injection. The sleeping-time was taken as the time between loss and recovery of the righting reflex.

In the second experiment ethoxyquin at a level of 0·5% (w/w) in the diet was fed for 14 days to rats from weaning at 21 days of age. Their controls were pair-fed with

ethoxyquin-free powdered diet during the 14-day period. The hexobarbitone sleeping-time in these rats was studied after this pretreatment period.

Spectral dissociation constant (K_s) *of ethoxyquin.* A double-washed hepatic microsomal suspension (2 mg protein/ml in 0·1 M phosphate buffer pH 7·6) was prepared according to the method of Schenkman *et al.*[16] from the liver of weanling rats which had been pretreated with three daily intraperitoneal doses of phenobarbitone sodium (100 mg/kg).

The K_s of ethoxyquin was determined according to the method of Schenkman *et al.*,[16] modified as follows:

The difference spectrum at 380–425 nm was measured, on a Unicam SP1800 spectrophotometer, when ethoxyquin (0·004–0·04 mM) was added to 2·5 ml of microsomal suspension using split cuvettes to allow for the absorbance of ethoxyquin at these wavelengths. Both sample and reference cuvettes contained 2·5 ml microsomal suspension in the forward compartment and an equal volume of aqueous 65% (v/v) ethanol in the rear compartment. Ethoxyquin was added as a solution in aqueous 65% (v/v) ethanol (maximum volume 10 μl) to the microsomes in the sample cuvette and to the aqueous ethanol in the reference cuvette, while an equal volume of aqueous ethanol was added to the microsomes in the reference cuvette.

RESULTS

Inhibition studies in vitro. The results of the enzyme inhibition studies *in vitro* are shown as Dixon plots[13] in Figs. 1–3. The graphs in Figs. 1 and 2 are typical of competitive inhibition of both biphenyl 4-hydroxylase and ethylmorphine *N*-demethylase, the K_i values being respectively $4{\cdot}3 \times 10^{-6}$ M, and 6×10^{-6} M, ethoxyquin is thus a very powerful inhibitor of the activities of these enzymes. Figure 3, however, shows no inhibitory effect of ethoxyquin on the activity of glucose-6-phosphatase.

From double-reciprocal plots[14] of the results (Figs. 4–6) obtained over a range of substrate concentrations in the absence of ethoxyquin, the K_m of biphenyl 4-hydroxylase, ethylmorphine *N*-demethylase, and glucose-6-phosphatase was calculated, giving values of $3{\cdot}2 \times 10^{-4}$ M, $2{\cdot}2 \times 10^{-4}$ M and $6{\cdot}9 \times 10^{-4}$ M respectively. The values of biphenyl 4-hydroxylase and ethylmorphine *N*-demethylase correlated well with the theoretical values calculated from the relevant Dixon plots by using the formula $-K_i\,(s/K_m + 1) = -x$ where x is the intersection on the abscissa (see Figs. 1 and 2).

Effect of a single dose of ethoxyquin. The results of this investigation are given in Table 1. It can be seen that single doses of ethoxyquin had little effect on the concentration of microsomal protein and cytochromes P-450 and b_5. Similarly the specific activities of biphenyl 4-hydroxylase and of ethylmorphine *N*-demethylase, were also little affected.

Inhibition studies in vivo. The results of the hexobarbitone sleeping-time, after single intragastric dose of ethoxyquin, are shown in Fig. 7. It is observed from this figure that ethoxyquin is a potent inhibitor of hexobarbitone metabolism *in vivo*. When hexobarbitone was administered 1 hr after the ethoxyquin the sleeping-time was more than 8 hr compared with 16 min in controls, and after 24 hr the sleeping-time was still double that of controls. Recovery was not complete until 48 hr after the dose of ethoxyquin.

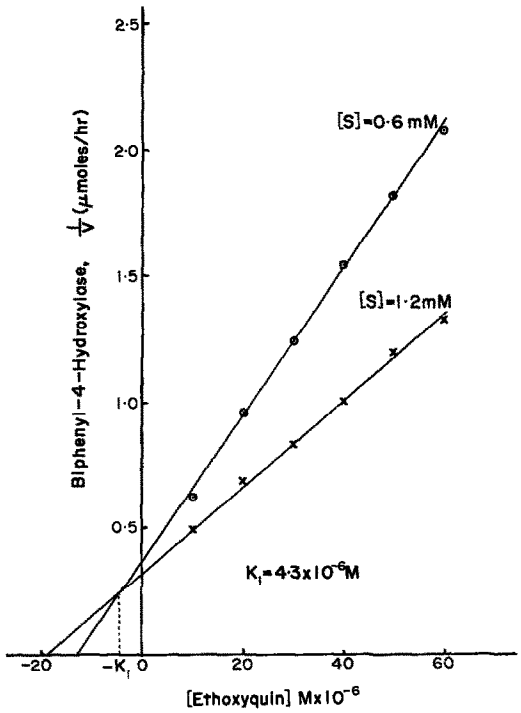

FIG. 1. Dixon plot showing competitive inhibition of biphenyl 4-hydroxylase activity by ethoxyquin. Biphenyl 4-hydroxylase activity (μmoles/hr/g liver) was assayed by the method of Creaven *et al.*,[6] as modified by Neale,[7] at substrate concentrations of 0·6 mM (○) and 1·2 mM (×) and ethoxyquin concentrations of 10–60 μM.

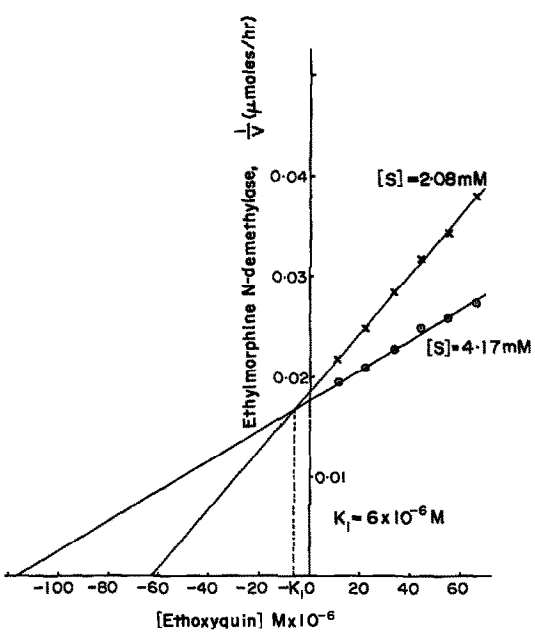

FIG. 2. Dixon plot showing competitive inhibition of ethylmorphine *N*-demethylase activity of ethoxyquin. Ethylmorphine *N*-demethylase activity (μmoles/hr/g liver) was assayed by the method of Holtzman *et al.*[8] at substrate concentrations of 2·08 mM (×) and 4·17 mM (○) and ethoxyquin concentrations of 11–66 μM.

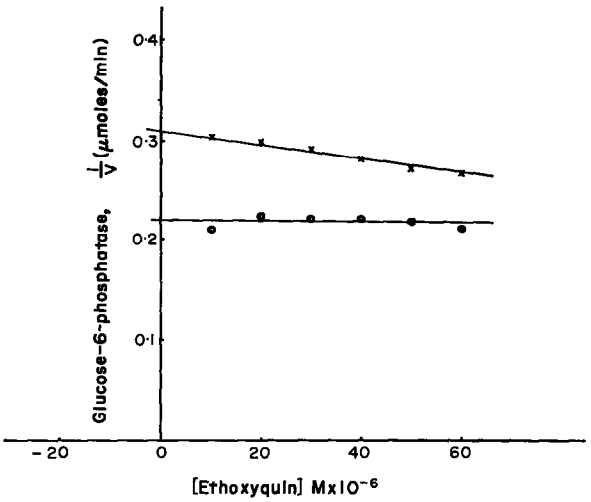

FIG. 3. Dixon plot showing effect of ethoxyquin on glucose-6-phosphatase activity. Glucose-6-phosphatase activity (μmoles/min/g liver) was assayed by the method of de Duve *et al.*[9] at substrate concentrations of 0·31 mM (×) and 0·62 mM (○) and ethoxyquin concentrations of 10–60 μM.

Table 2 contains the results of hexobarbitone sleeping-time after administration of ethoxyquin in the diet for 14 days. It is observed from this table that the sleeping-time was reduced in the pretreated rats to almost 50 per cent of the control value even 3 hr after the withdrawal of the diet containing ethoxyquin. This contrasts with the prolonged sleeping-time observed in rats after single intragastric dose of ethoxyquin.

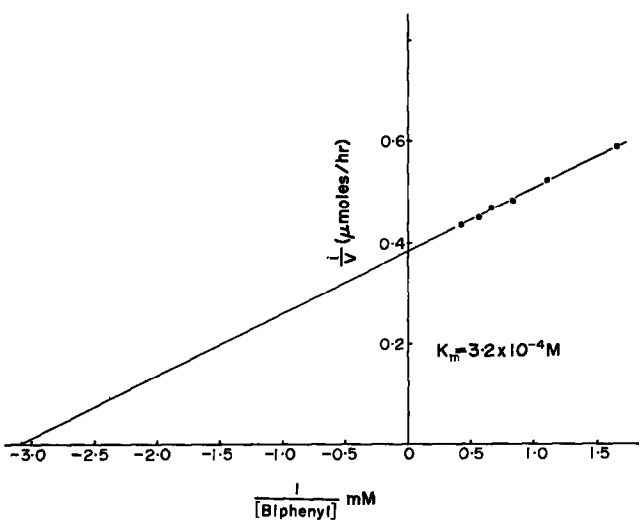

FIG. 4. Lineweaver–Burk reciprocal plot showing apparent Michaelis constant (K_m) for biphenyl 4-hydroxylase with respect to biphenyl. Biphenyl 4-hydroxylase activity (μmoles/hr/g liver) was assayed by the method of Creaven *et al.*,[6] as modified by Neale,[7] at substrate concentrations of 0·6–2·4 mM.

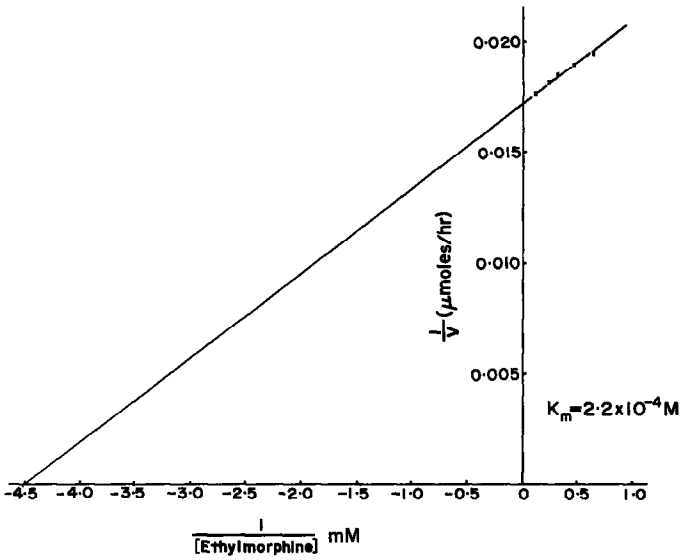

FIG. 5. Lineweaver–Burk reciprocal plot showing apparent Michaelis constant (K_m) for ethylmorphine *N*-demethylase with respect to ethylmorphine. Ethylmorphine *N*-demethylase activity (μmoles/hr/g liver) was assayed by the method of Holtzman *et al.*[8] at substrate concentrations of 1·04–8·33 mM.

Spectral dissociation constant of ethoxyquin. When added to microsomal suspensions ethoxyquin exhibited a type I difference spectrum with a peak at 385 nm and a trough at 420–422 nm. The magnitude of the difference (ΔE) between the absorbance at 385 nm and at 420 nm varied with ethoxyquin concentration and was taken to represent the extent of binding to cytochrome P-450. The results of this experiment are shown in Fig. 8 as a double-reciprocal plot of ΔE against ethoxyquin con-

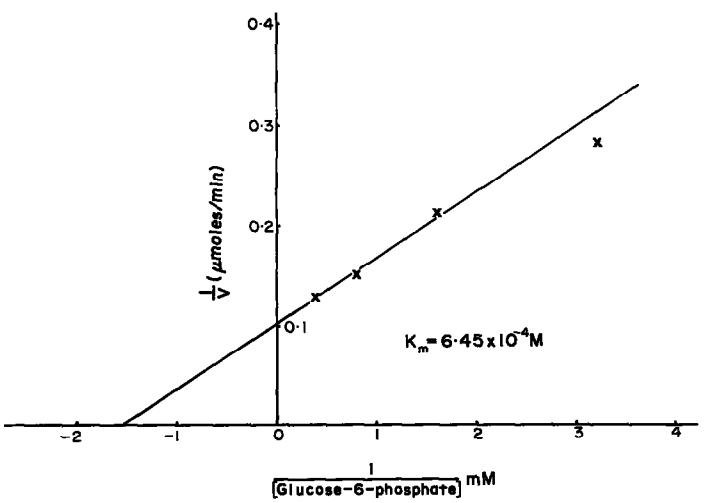

FIG. 6. Lineweaver–Burk reciprocal plot showing apparent Michaelis constant (K_m) for glucose-6-phosphatase with respect to glucose-6-phosphate. Glucose-6-phosphatase activity (μmoles/min/g liver) was assayed by the method of de Duve *et al.*[9] at substrate concentrations of 0·31–2·5 mM.

TABLE 1. EFFECT OF SINGLE INTRAGASTRIC DOSE OF ETHOXYQUIN (500 mg/kg) ON DRUG METABOLIZING ENZYMES AND CERTAIN OTHER PARAMETERS OF WEANLING MALE WISTAR ALBINO RATS

Parameters	Animals	Period after dose (hr) 0	2	4	8	12	24	48
Body wt (g)	Control	50 ± 0·4	49 ± 0·4	50 ± 0·4	50 ± 0·4	51 ± 0·5	58 ± 2	56 ± 2
	Test	50 ± 0·4	48 ± 0·4	49 ± 0·4	49 ± 0·4	50 ± 0·5	51 ± 2	53 ± 2
Liver wt (g)	Control	1·7 ± 0	1·8 ± 0·1	1·8 ± 0·1	1·8 ± 0·2	1·8 ± 0·1	2·1 ± 0·2	2·4 ± 0·2
	Test	1·7 ± 0	1·8 ± 0·2	1·8 ± 0·1	2·0 ± 0·2	2·1 ± 0·2	2·2 ± 0·2	2·5 ± 0·1
Relative liver wt (g/100 g body wt)	Control	3·4 ± 0·1	3·6 ± 0·2	3·6 ± 0·1	3·6 ± 0·2	3·6 ± 0·2	3·6 ± 0·3	4·2 ± 0·4
	Test	3·4 ± 0·1	3·7 ± 0·1	3·7 ± 0·1	4·0 ± 0·2	4·2 ± 0·3	4·4 ± 0·5	4·6 ± 0·3
Microsomal protein (mg/g liver)	Control	23·9 ± 0·6	24·8 ± 0·2	24·8 ± 0·2	25·0 ± 0·2	24·9 ± 0·3	24·8 ± 0·3	25·0 ± 0·4
	Test	24·0 ± 0·4	25·0 ± 0·3	24·6 ± 0·2	25·5 ± 0·3	26·1 ± 0·4	25·9 ± 0·2*	27·0 ± 0·5**
Cytochrome P-450 (nmoles/g liver)	Control	21·1 ± 0·5	21·1 ± 0·1	21·1 ± 0·3	21·2 ± 0·4	21·2 ± 0·4	21·0 ± 0·4	21·3 ± 0·4
	Test	21·0 ± 0·6	20·6 ± 0·2	20·8 ± 0·3	21·5 ± 0·4	22·5 ± 0·4	22·6 ± 0·5*	22·6 ± 0·4
Cytochrome b_5 (nmoles/g liver)	Control	13·1 ± 0·3	13·0 ± 0·1	13·1 ± 0·1	13·0 ± 0·1	13·1 ± 0·1	13·4 ± 0·1	13·8 ± 0·2
	Test	13·2 ± 0·3	12·9 ± 0·1	13·0 ± 0·1	13·0 ± 0·1	13·2 ± 0·1	13·2 ± 0·2	15·0 ± 0·1††
Biphenyl 4-hydroxylase activity (μmoles/hr/g liver)	Control	6·6 ± 0·3	6·3 ± 0·2	6·1 ± 0·1	6·2 ± 0·1	6·4 ± 0·1	6·3 ± 0·1	6·2 ± 0·6
	Test	6·6 ± 0·3	5·7 ± 0·2	5·9 ± 0·1	6·5 ± 0·1	6·9 ± 0·2	7·4 ± 0·2†	8·1 ± 0·5
Ethylmorphine *N*-demethylase activity (μmoles/hr/g liver)	Control	18·4 ± 0·9	20·1 ± 1·0	20·0 ± 0·5	19·0 ± 0·8	19·5 ± 0·9	20·0 ± 1·2	21·6 ± 1·1
	Test	18·5 ± 0·8	19·5 ± 0·8	20·1 ± 0·3	22·1 ± 1·0	23·2 ± 1·1*	27·2 ± 0·7†	24·9 ± 1·0

The ethoxyquin was administered as a 25% (w/v) solution in arachis oil. Controls and tests each consisted of eight animals. Values given are the means ± S.E.M. Significant effects are shown: *$P < 0·05$; **$P < 0·05$; †$P < 0·01$; ††$P < 0·001$.

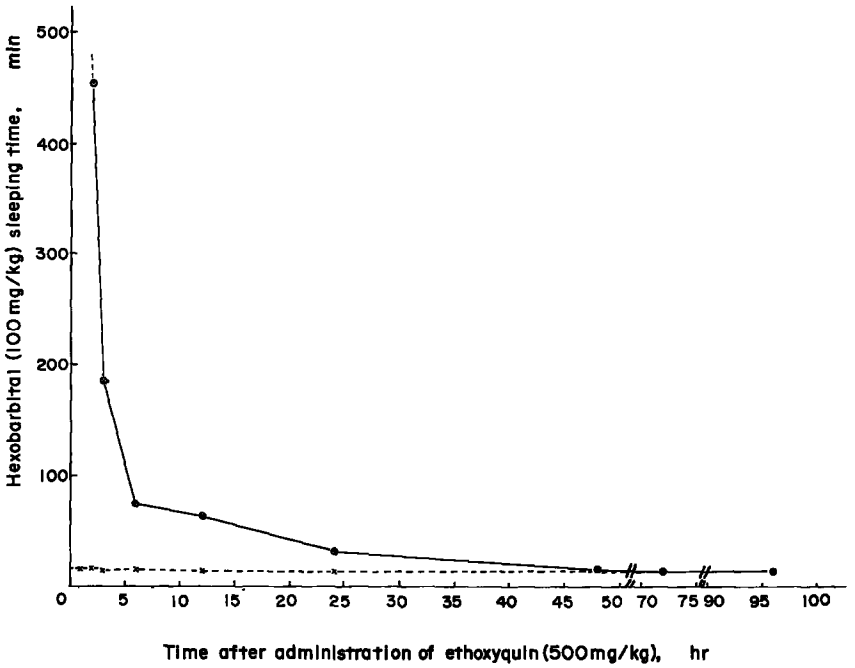

FIG. 7. Hexobarbitone (100 mg/kg, i.p.) sleeping-time of 8–9 week-old male Wistar albino rats after single dose of ethoxyquin (500 mg/kg, i.g.). Ethoxyquin was given as a 25% (w/v) solution in arachis oil. Hexobarbitone was administered as a 5% (w/v) solution in dilute aq. NaOH, pH 11.

centration. By extrapolating this plot to the abscissa the concentration of the ethoxyquin (substrate) required for half-maximal spectral change (or spectral dissocation constant, K_s) was determined and found to have a value of $1{\cdot}06 \times 10^{-5}$ M, indicating that ethoxyquin binds to cytochrome P-450 with a high affinity.

DISCUSSION

The *in vitro* studies reported here indicate that ethoxyquin binds strongly to cytochrome P-450 and is a potent competitive inhibitor of the drug-metabolizing activity of this enzyme. The induction of the the drug-metabolizing enzymes previously reported[1,2] would then appear to result from the increased metabolic demand in the presence of ethoxyquin and might thus be considered a physiological, adaptive re-

TABLE 2. HEXOBARBITONE (100 mg/kg) SLEEPING-TIME OF 36-DAY-OLD WISTAR MALE ALBINO RATS AFTER ADMINISTRATION OF ETHOXYQUIN AT A DIETARY LEVEL OF 0·5% (w/w) FOR 14 DAYS FROM WEANLING AT 21 DAYS OF AGE

Sl. No.	Time after withdrawal of diet containing ethoxyquin (min)	Sleeping-time (min)	
		Control	Ethoxyquin-fed rats
1	30	20·75 ± 0·5	9·25 ± 0·5*
2	180	26·0 ± 0·25	14·0 ± 0·25*

Hexobarbitone was administered as a 5% (w/v) solution in dilute aqueous NaOH, pH 11. Controls and tests each consisted of four animals. Values given are the means ± S.E.M. Significant effects are shown: *$P < 0{\cdot}001$.

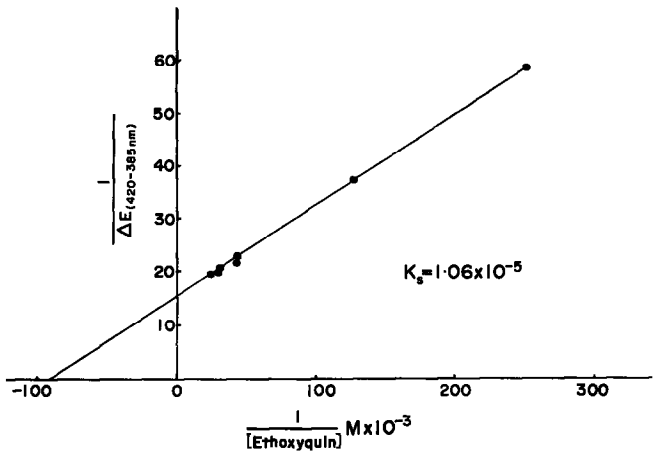

FIG. 8. Lineweaver–Burk reciprocal plot showing spectral dissociation constant (K_s) of ethoxyquin. Spectral dissociation constant of ethoxyquin was determined by the method of Schenkman *et al.*[12] from the Lineweaver–Burk plot made from the difference spectrum observed at 380–425 nm after adding aq. 65% ethanolic solution of ethoxyquin (0·004–0·04 mM in a max. vol. 10 μl) to a split cuvette containing 2·5 ml suspension of a double-washed rat liver microsomal preparation (2 mg protein/ml) in 0·1 M phosphate buffer, pH 7·6.

sponse. This view may be substantiated by the observation that, while single doses of ethoxyquin dramatically increased hexobarbitone sleeping-time repeated dosing had the effect of increasing hexobarbitone metabolism and decreasing the sleeping-time.

The induction of hexobarbitone oxidase by single and multiple doses of ethoxyquin has been reported previously by Cawthorne *et al.*[2] The apparent disagreement in the results after single doses may result from the fact that in the present work hexobarbitone sleeping-time was determined *in vivo* and inhibition was observed over a period of time during which residual ethoxyquin might be expected to be present in the liver.[3] On the other hand, Cawthorne *et al.*[2] examined hexobarbitone oxidase *in vitro* up to 72 hr and found induction from 24 hr and it is likely that ethoxyquin levels in the incubation mixture were lower than those occurring *in vivo* due to losses and dilution in the preparative procedures used. However, the results of *in vitro* assays after administering single doses of ethoxyquin reported here do not reveal any marked induction of microsomal mixed function oxidases which contrasts with the report of Cawthorne *et al.*[2] that aminopyrene demethylase and BHT oxidase were induced by single doses of ethoxyquin. It was previously found that repeated oral dosing with ethoxyquin caused a dramatic increase in microsomal mixed function oxidases and in this respect it resembles the short-acting barbiturates where repeated doses are necessary for maximal enzyme induction.

It was shown[15] that the *total* hepatic activity of glucose-6-phosphatase was not significantly affected by 14-days' pretreatment of rats with ethoxyquin and these *in vitro* studies revealed ethoxyquin to be without effect on the activity of this enzyme. This adds further weight to the view that the drop in *specific* activity of glucose-6-phosphatase in the liver microsomes of rats treated with ethoxyquin results from dilution with other enzyme proteins, the synthesis of which has been significantly induced.

REFERENCES

1. D. V. PARKE, A. RAHIM and R. WALKER, *Biochem. J.* **130,** 84P (1972).
2. M. A. CAWTHORNE, J. BUNYAN, M. V. SENNITT, J. GREEN and P. GRASSO, *Br. J. Nutr.* **24,** 357 (1970).
3. O. WISS, R. H. BUNNELL and U. GLOOR, *Vitams Horm.* **30,** 441 (1962).
4. C. IONNIDES and D. V. PARKE, *Biochem. Soc. Trans.* **1,** 716 (1973).
5. T. K. BASU, J. W. T. DICKERSON and D. V. W. PARKE, *Biochem. J.* **124,** 19 (1971).
6. P. J. CREAVEN, D. V. PARKE and R. T. WILLIAMS, *Biochem. J.* **96,** 879 (1965).
7. M. G. NEALE, Ph.D. Thesis, University of Surrey (1970).
8. J. L. HOLTZMAN, T. E. GRAM, P. L. GIGON and J. R. GILLETTE, *Biochem. J.* **110,** 407 (1968).
9. C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX and F. APPELMANS, *Biochem. J.* **60,** 604 (1955).
10. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
11. N. E. SLADEK and G. J. MANNERING, *Biochem. biophys. Res. Commun.* **24,** 668 (1966).
12. J. B. SCHENKMAN, I. FREY, H. REMMER and R. W. ESTABROOK, *Mol. Pharmac.* **3,** 516 (1967).
13. M. DIXON, *Biochem. J.* **55,** 170 (1953).
14. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56,** 658 (1934).
15. D. V. PARKE, A. RAHIM and R. WALKER, *Biochem. Pharmac.* **23,** 1871 (1974).
16. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Mol. Pharmac.* **3,** 113 (1967).