Biochemical Pharmacology, Vol. 22, pp. 3199–3207. Pergamon Press, 1973. Printed in Great Britain.

EFFECTS OF LIPID PEROXIDATION ON ACTIVITIES OF DRUG-METABOLIZING ENZYMES IN LIVER MICROSOMES OF RATS

TETSUYA KAMATAKI and HARUO KITAGAWA

Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Chiba, 1-33 Yayoi-cho, Chiba, Japan

(*Received* 2 *October* 1972; *accepted* 8 *June* 1973)

Abstract—A relationship was found between the formation of lipid peroxides and the activities of drug-metabolizing enzymes in liver microsomes of rats. Induction of lipid peroxidation by incubation with ferrous ion led to a sharp decline in the ability of the microsomal enzyme system to demethylate ethylmorphine. Inhibition of lipid peroxidation by EDTA increased the enzyme activity about two-fold. Addition of EDTA (0·1 mM) to the incubation mixture produced marked changes in the Michaelis constants of drug-metabolizing enzymes and in inhibition constants of SKF 525-A for drug-metabolizing enzymes.

FORMATION of lipid peroxides in liver microsomes has been found to produce degradation of phospholipids,[1,2] which are major components of microsomal membranes. Furthermore, Imai and Sato[3,4] demonstrated the requirement of a lipid-like factor to hydroxylate aniline. Recently, the role of phospholipids in the activities of drug-metabolizing enzymes and in cytochrome P-450 reduction has been reported.[5–12] Moreover, there have been reports on the effects of lipid peroxidation on activities of drug-metabolizing enzymes. Orrenius *et al.*[13] demonstrated that stimulation of lipid peroxidation only slightly affected the activities of aminopyrine *N*-demethylation and codeine demethylation. In addition, Wills[14] has shown that induction of lipid peroxidation in microsomes by preincubation with ascorbate or NADPH or by treatment with ionizing radiation leads to a sharp decline in the ability of microsomes to oxidize aminopyrine or to hydroxylate aniline. It was suggested that inactivation of drug-metabolizing enzymes was associated with disintegration of microsomal membranes but not with inhibition by toxic products formed by lipid peroxidation, because addition of a product of lipid peroxidation, malonaldehyde, did not cause any alterations in the capacity of fresh microsomes to oxidize aminopyrine. It has been hypothesized by several investigators[14–16] that apparent activation of drug-metabolizing enzymes should occur if lipid peroxidation is inhibited, since inhibition of lipid peroxidation prevents disintegration of microsomal membranes which occurs during incubation. However, successful results have not yet been shown except with an aldrin epoxidase.[17] This investigation was initiated to clarify the effects of lipid peroxidation on the activities of drug-metabolizing enzymes.

MATERIALS AND METHODS

Commercial aniline was redistilled under vacuum and the distillate was stored at about −10° under an atmosphere of nitrogen. Other substrates were purchased from

commercial sources and used without further purification. Thiobarbituric acid was purchased from Daiichi Pure Chemicals Co., Tokyo, Japan, and a 0·67 per cent solution was prepared by the addition of a minimal amount of 1 N NaOH to solubilize the thiobarbituric acid just before experiments. The absorption spectrum of this solution showed no peak at about 452 nm such as is seen on storage of sodium thiobarbiturate solution. NADP, glucose 6-phosphate-Na and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I, Kontroll-Nr. 7291111) were purchased from Boehringer Mannheim (Japan) Co., Ltd.

Male rats of Wistar strain, which were maintained on commercial rat chow, CE-2, Nippon Crea Co. Ltd., Japan, were starved for about 18 hr prior to sacrifice. Rats were killed by decapitation, and the livers were perfused with 1·15% KCl solution *in situ* to remove blood. The livers were removed and immediately placed in ice-cold 1·15% KCl solution. All subsequent procedures were performed below 4°. The livers were weighed and minced with scissors. The liver-mince was then homogenized with 3 vol. of the KCl solution in a motor-driven Potter homogenizer using a Teflon pestle. The homogenate was then centrifuged at 9000 *g* for 20 min and the resultant supernatant fraction without fatty layer at the top of the centrifuge tube was recentrifuged at 105,000 *g* for 1 hr in a Hitachi preparative ultracentrifuge, model 65 P, to prepare microsomal pellets. The microsomal pellet was resuspended in the KCl solution. The resultant microsomal suspension was employed as the enzyme preparation. Microsomal protein was determined according to the method of Lowry *et al.*[18] Aniline hydroxylase activity was measured by determining the amount of *p*-aminophenol formed by the method of Brodie and Axelrod[19] as modified by Kato and Gillette.[20] Oxidative demethylase activities were assayed with aminopyrine, codeine and ethylmorphine as substrates, and the amount of formaldehyde formed was measured by the Nash reaction.[21] Description for the preparation of the incubation system is given in the figures and tables. Lipid peroxide was determined by the thiobarbituric acid method essentially as described by Wills.[22] To a portion (0·5 ml) of the incubation mixture, 0·5 ml of trichloroacetic acid (10%) was added and protein was removed by centrifugation. The resultant supernatant fluid (0·5 ml) was transferred to a tube containing 3·5 ml of the thiobarbituric acid solution, mixed and heated at 100° for 10 min. The color of the solution was measured at 532 nm. The formation of lipid peroxide was represented as TBA values which were obtained by calculating $O.D._{532nm}$ per mg of microsomal protein added to the incubation mixture.

RESULTS

Figure 1 shows the effects of ferrous ion on lipid peroxidation and ethylmorphine *N*-demethylation. Ferrous ion has been shown to be a potent stimulator of lipid peroxidation,[23–26] so it was postulated that stimulation of lipid peroxidation by ferrous ion would result in a decrease in drug-oxidizing ability. As expected, increments of ferrous ion in the incubation mixture produced a parallel increase in lipid peroxidation and a concomitant decrease in ethylmorphine *N*-demethylating activity. The inhibition of ethylmorphine *N*-demethylation by ferrous ion may be a finding similar to that reported by Peters and Fouts.[27] It has been reported that formation of lipid peroxide is inhibited by various agents such as EDTA, drugs and SKF 525-A (*β*-diethylaminoethyl diphenyl-*n*-propylacetate), an inhibitor of drug-metabolizing enzymes.[14,23,28] Previously, Wills[14] demonstrated that there was no detectable

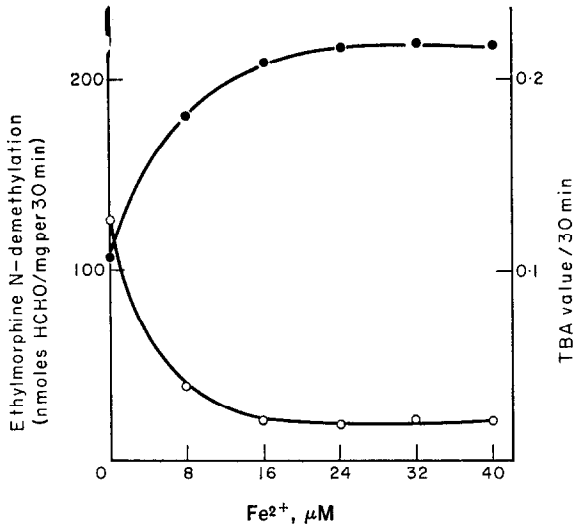

FIG. 1. Effects of ferrous ion on lipid peroxidation and ethylmorphine *N*-demethylation by liver microsomes of rats. Rats weighing about 190 g were used. The incubation mixture consisted of ethylmorphine (1 mM), microsomes (3·70 mg of protein), ferrous ion [as $Fe(NH_4)_2$-$(SO_4)_2$-6 H_2O], NADPH-generating system (0·33 mM NADP, 8 mM glucose 6-phosphate, 15 μmoles $MgCl_2$ and 0·113 unit glucose 6-phosphate dehydrogenase) and 0·8 ml of 0·2 M Na-K phosphate buffer, pH 7·4, in a final volume of 2·5 ml. Incubations were carried out at 37° for 30 min aerobically. ○——○, Ethylmorphine *N*-demethylation; ●——●, lipid peroxidation.

increase in the rate of aminopyrine *N*-demethylation when 0·5 to 1·0 mM of EDTA was added to the incubation mixture. We felt, however, that EDTA should increase apparent activities of drug-metabolizing enzymes because lipid peroxidation occurs in the incubation mixture containing rat liver microsomes and because formation of lipid peroxide results in inactivation of drug-metabolizing enzymes as reported by

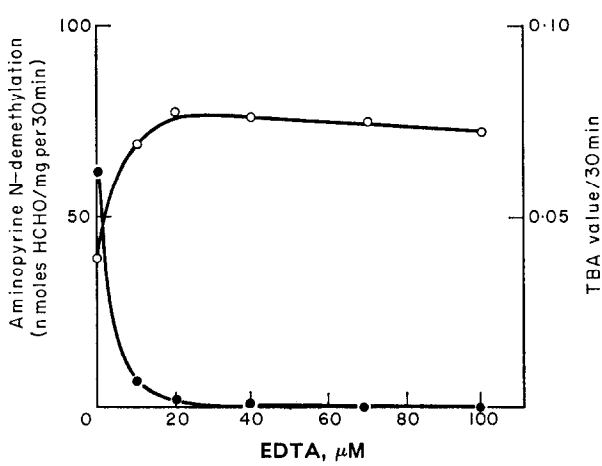

FIG. 2. Effects of EDTA on lipid peroxidation and aminopyrine *N*-demethylation by liver microsomes of rats. Rats weighing about 130 g were used. Experimental details on the incubation mixture were as described in Fig. 1 except that aminopyrine (1 mM) was used as a substrate, EDTA was added instead of ferrous ion and microsomal protein added was 3·66 mg. ○——○, Aminopyrine *N*-demethylation; ●——●, lipid peroxidation.

Wills[14,29,30] and as presented in Fig. 1 of this paper. In order to test this hypothesis, the effects of EDTA on *N*-demethylating activities of ethylmorphine and aminopyrine were examined with varying concentrations of EDTA. As expected, EDTA stimulated apparent activity of aminopyrine *N*-demethylation up to about 2-fold at 0·02 mM, and the activity gradually declined with increasing concentrations of EDTA (Fig. 2). Moreover, the stimulation of aminopyrine *N*-demethylation correlated closely with the decrease in formation of lipid peroxide. Similar effects of EDTA on drug-metabolizing activity, lipid peroxidation and correlation between them were found using ethylmorphine as a substrate (Fig. 3). To establish an inverse relationship

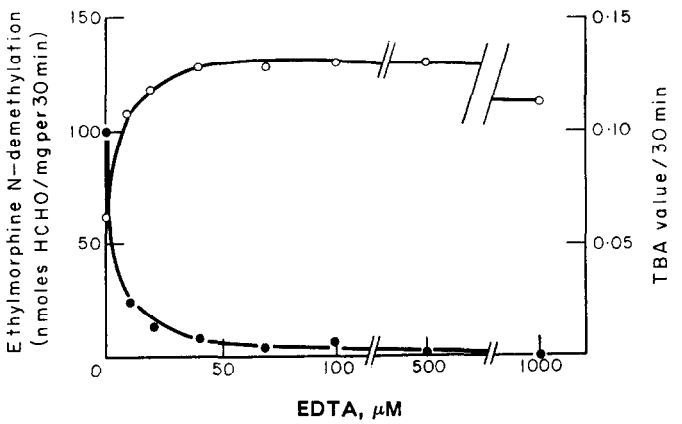

FIG. 3. Effects of EDTA on lipid peroxidation and ethylmorphine *N*-demethylation by liver microsomes of rats. Experimental conditions were as described for Fig. 2 except that ethylmorphine was used as a substrate. ○——○, Ethylmorphine *N*-demethylation; ●——●, lipid peroxidation.

between lipid peroxidation and rate of drug metabolism, ethylmorphine *N*-demethylation was measured in the presence of additional inhibitors of lipid peroxidation (Table 1). In the cases examined, ethylmorphine *N*-demethylation tended to be stimulated by addition of inhibitors of lipid peroxidation. The effects of EDTA on aminopyrine and ethylmorphine *N*-demethylations might reflect effects on the stability

TABLE 1. EFFECTS OF *O*-PHENANTHROLINE, *a,a'*-DIPYRIDYL, Co^{2+} AND Mn^{2+} ON LIPID PEROXIDATION AND ETHYLMORPHINE *N*-DEMETHYLATION*

Additions	Ethylmorphine *N*-demethylation (nmoles HCHO/mg/30 min)	TBA value
None	85·6 ± 8·0	0·078 ± 0·003
o-Phenanthroline (0·05 mM)	142·9 ± 2·9†	0·005 ± 0†
a,a'-Dipyridyl (0·1 mM)	121·4 ± 2·4†	0·006 ± 0†
$CoCl_2$ (0·5 mM)	145·2 ± 3·1†	0·003 ± 0·001†
$MnCl_2$ (0·1 mM)	104·7 ± 5·4	0·006 ± 0·001†

* Experimental conditions were as described for Fig. 3 except that various inhibitors of lipid peroxidation were used instead of EDTA and microsomal protein added was 2–3 mg. Values represent mean ±S.E. (n = 3).

† Differs significantly ($P < 0·05$) from corresponding control value.

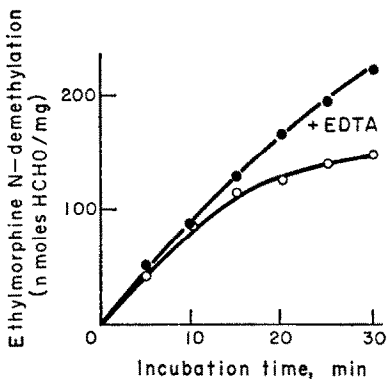

FIG. 4. Effect of EDTA on time course linearity of ethylmorphine *N*-demethylation by liver microsomes of rats. Rats weighing about 140 g were used. Microsomes (1·89 mg of protein) were incubated with ethylmorphine (1 mM), 0·8 ml of 0·2 M Na-K phosphate buffer, pH 7·4, and NADPH-generating system (prepared as described in Fig. 1), and with (●——●) or without (○——○) EDTA (0·1 mM) in a final volume of 2·5 ml.

of microsomal enzymes during the period of incubation. As shown in Fig. 4, the effect of EDTA on stability of ethylmorphine *N*-demethylase was examined by comparing the formation of formaldehyde against incubation time. EDTA effectively prolonged the linearity. In this case, it is possible that EDTA may prevent the impairment of the drug-metabolizing enzyme system by NADPH-dependent lipid peroxidation in rat liver microsomes. In order to test this possibility, the effect on codeine demethylase activity of preincubation of rat liver microsomes with an NADPH-generating system was studied. In this experiment, microsomes were preincubated for varying lengths of time with a NADPH-generating system at 37°, then incubation media, supplemented with additional NADPH-generating system, EDTA to block further lipid peroxidation and codeine, were incubated for an additional 15 min at 37°. The preincubation of microsomes produced a marked decrease in the demethylation of codeine and a concomitant increase in lipid peroxidation (Fig, 5).

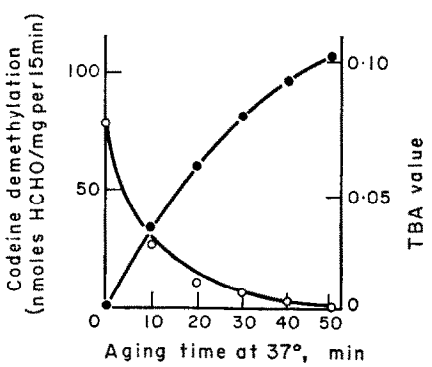

FIG. 5. Effect of aging of rat liver microsomes with NADPH-generating system on rate of codeine demethylation. Rats weighing about 110 g were used. One ml of microsomal suspension (2·65 mg of protein/ml) was aged at 37° aerobically with NADPH-generating system (prepared as described in Fig. 1) and 0·8 ml of 0·2 M Na-K phosphate buffer, pH 7·4, in a final volume of 2·0 ml. After aging, NADPH-generating system, codeine (5 mM) and EDTA (0·1 mM) were added and incubated at 37° for 15 min. ○——○, Codeine demethylation; ●——●, lipid peroxidation.

From the above findings, the authors conclude that stimulation of lipid peroxidation delays the rate of drug oxidation and, conversely, that inhibition of lipid peroxidation accelerates the rate of drug oxidation. This led us to examine further the kinetics of drug oxidation. When one investigates the kinetic constants of drug-metabolizing enzymes, one often uses varying concentrations of substrates near their K_m-values. Then if the drug substrates inhibit lipid peroxidation to a degree which varies with the concentrations of substrates employed, apparent activities of these enzymes should be less at low concentrations of substrate during the incubation period, since low substrate concentrations have little capacity to inhibit lipid peroxidation, while, on the other hand, apparent activities of these enzymes should be near their true values in the region of rather high concentrations of the substrates since high substrate concentrations appear to inhibit lipid peroxidation more strongly. Therefore, if this hypothesis on the effects of lipid peroxidation is correct, the kinetic constants of drug-metabolizing enzymes do not reflect the true properties of the enzymes, and the kinetic constants usually measured should be larger than those corrected for the effects of lipid peroxidation, since the lower the concentrations of the substrates employed, the more the inactivation of the enzymes depending upon lipid peroxidation occurs. To examine this hypothesis, the effects of EDTA on the rate of ethylmorphine *N*-demethylation and lipid peroxidation were tested (Fig. 6). Ethylmorphine inhibited lipid peroxidation with increasing concentration. Ten mM ethylmorphine inhibited lipid peroxidation by about 70 per cent. In the presence of complete inhibition of lipid peroxidation by EDTA, the rate of ethylmorphine *N*-demethylation was markedly increased. The apparent stimulation by EDTA was more pronounced if low concentrations of ethylmorphine were employed. This observation is in agreement with our

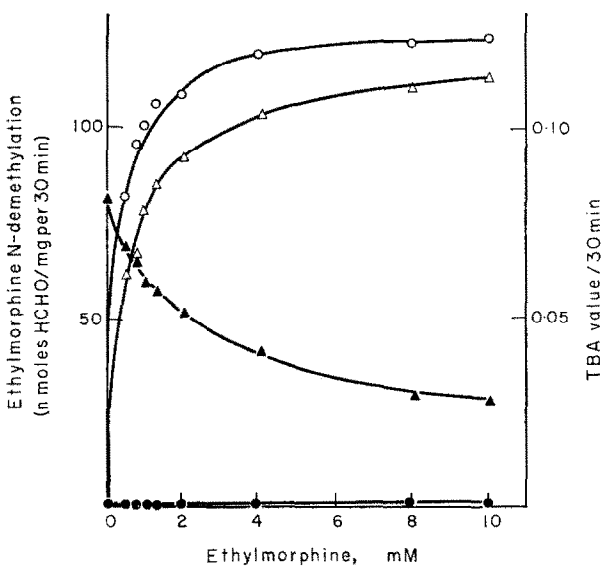

FIG. 6. Effects of EDTA on ethylmorphine *N*-demethylation and lipid peroxidation. Rats weighing about 140 g were used. The composition of the incubation mixture was as described for Fig. 1 except that EDTA (0·1 mM) was added instead of ferrous ion, various concentrations of ethylmorphine were employed and microsomal protein added was 5·50 mg. Ethylmorphine *N*-demethylation: with EDTA (○——○), without EDTA (△——△); lipid peroxidation; with EDTA (●——●), without EDTA (▲——▲).

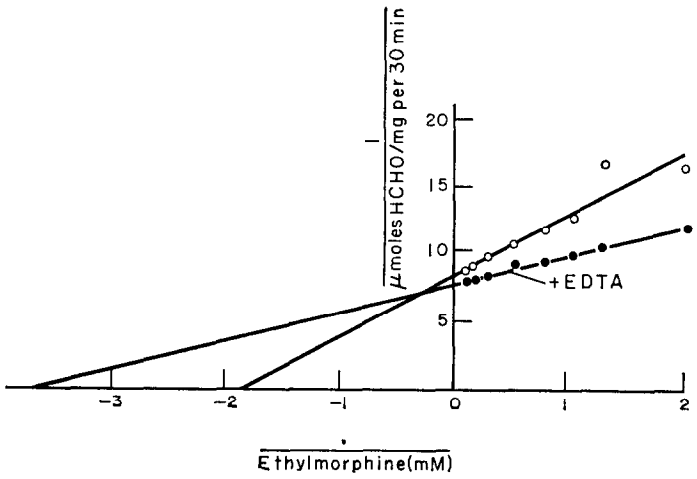

FIG. 7. Effect of EDTA on Lineweaver–Burk plots of ethylmorphine *N*-demethylation. The experimental conditions were the same as described in Fig. 6. Ethylmorphine *N*-demethylation: with EDTA (●——●), without EDTA (○——○).

hypothesis that low concentrations of ethylmorphine only partially inhibit lipid peroxidation. The Lineweaver–Burk plots of this reaction are shown in Fig. 7. As expected, the slope in the presence of EDTA was less than that without EDTA. Thus the K_m-value was changed to a significantly smaller value. The effects of EDTA on the K_m-values of several other substrates are shown in Table 2. In all cases, addition of EDTA to the incubation mixture produced changes in the Michaelis constants to smaller values. Pederson and Aust[31] and Aust and Stevens[32] have reported that there might be two enzymes acting as aminopyrine *N*-demethylases, for two K_m values are obtained when the activity of aminopyrine *N*-demethylation is measured as formaldehyde formation. Two K_m-values were also obtained in our experiment, but both values were changed to smaller ones. One may assume that addition of EDTA to the incubation mixture causes changes in the inhibition constants of drug-metabolizing enzymes by SKF 525-A, since EDTA produced apparent activation of drug-meta-

TABLE 2. EFFECTS OF EDTA ON K_m VALUES FOR ETHYLMORPHINE, CODEINE AND AMINOPYRINE DEMETHYLATION AND ANILINE HYDROXYLATION*

	N	K_m (mM)	
		−EDTA	+EDTA (0·1 mM)
Ethylmorphine *N*-demethylation	4	0·527 ± 0·038	0·291 ± 0·020†
Codeine demethylation	4	0·435 ± 0·041	0·268 ± 0·085†
Aminopyrine *N*-demethylation	5	1·020 ± 0·044	0·597 ± 0·044†
		2·757 ± 0·080	0·743 ± 0·018†
Aniline hydroxylation	3	0·058 ± 0·001	0·024 ± 0·001†

* All K_m values were obtained from Lineweaver–Burk plots using ethylmorphine (0·5 to 10 mM), codeine (0·5 to 10 mM), aminopyrine (0·5 to 10 mM) and aniline (0·01 to 50 mM) as substrates. Values represent mean ±S.E.

† Differs significantly ($P < 0·05$) from corresponding control value.

TABLE 3. EFFECTS OF EDTA ON INHIBITION CONSTANT OF SKF 525-A FOR DRUG-METABOLIZING ENZYMES*

Substrate	Inhibition constant (μM)		Type of inhibition
	−EDTA	+EDTA (0·1 mM)	
Ethylmorphine	7·7	3·9	Competitive
Aminopyrine (2·0–10·0, mM)	51·6	23·9	Competitive
Aminopyrine (0·75–2·0 mM)	26·0	12·9	Non-competitive
Aniline	1·057 (mM)	0·360 (mM)	Non-competitive

* Rats weighing about 110 g were used. In ethylmorphine *N*-demethylation, the inhibition constant for SKF 525-A was calculated from Dixon plots. The experiment was performed using 0·5 and 2·0 mM of ethylmorphine and 4·0 to 10 μM of SKF 525-A. In aminopyrine *N*-demethylation and aniline hydroxylation, the in hibition constants were calculated from Lineweaver–Burk plots. The experiments were performed using 0·75 to 10 mM of aminopyrine, 0·02 to 0·15 mM of aniline and 0·2 mM of SKF 525-A.

bolizing enzymes and SKF 525-A has an ability to block lipid peroxidation. We also examined this possibility using ethylmorphine, aminopyrine and aniline as substrates (Table 3). In all cases, EDTA produced changes in the inhibition constants of SKF 525-A to smaller values.

DISCUSSION

EDTA inhibited lipid peroxidation and apparently stimulated the rate of *N*-demethylation of ethylmorphine and aminopyrine. There have been reports in the literature showing that EDTA did not stimulate apparent activities of drug-metabolizing enzymes. Anders[16] reported that aniline hydroxylation was not increased by the addition of EDTA to inhibit lipid peroxidation. However, this result is compatible with the present experiments since 1 mM aniline, which was employed by Anders, inhibits lipid peroxidation completely and, therefore, a further effect of EDTA in inhibiting lipid peroxidation would not be anticipated. Also, Wills[14] reported that addition of EDTA (1 mM) to the incubation mixture did not stimulate aminopyrine *N*-demethylation; this may have been the result of the excess amount of EDTA employed, because as shown in Fig. 2 in this paper, aminopyrine *N*-demethylation activity is increased by the addition of low concentrations of EDTA, but the activity is gradually reduced if above 20 μM EDTA was added.

Wills[33] reported that phosphate stimulates lipid peroxidation. Thus, it may be possible to assume that the effects of EDTA on activities of drug-metabolizing enzymes were obtained depending upon phosphate or on the ferrous ion which is a contaminant in the phosphate. To examine the possibility, an experiment similar to that shown in Fig. 3 was performed, using 0·2 M Tris–HCl buffer (pH 7·4) instead of 0·2 M phosphate buffer (pH 7·4). The experiment gave a similar result to that shown in Fig. 3 (data not shown).

REFERENCES

1. H. E. MAY and P. B. MCCAY, *J. biol. Chem.* **243,** 2288 (1968).
2. W. G. JR. NIEHAUS and B. SAMUELSON, *Eur. J. Biochem.* **6,** 126 (1968).
3. Y. IMAI and R. SATO, *Biochim. biophys. Acta* **36,** 571 (1959).
4. Y. IMAI, and R. SATO, *Biochim. biophys. Acta* **42,** 164 (1960).
5. M. D. CHAPLIN and G. J. MANNERING, *Molec. Pharmac.* **6,** 631 (1970).

6. K. C. LEIBMAN and R. W. ESTABROOK, *Molec. Pharmac.* **7,** 26 (1971).
7. A. Y. H. LU and M. J. COON, *J. biol. Chem.* **243,** 1331 (1968).
8. A. Y. H. LU, K. W. JUNK and M. J. COON, *J. biol. Chem.* **244,** 3714 (1969).
9. M. J. COON and A. Y. H. LU, in *Microsomes and Drug Oxidations* (Ed. J. R. GILLETTE *et al.*) p. 151. Academic Press, New York (1969).
10. H. W. STROBEL, A. Y. H. LU, J. HEIDEMA and M. J. COON, *J. biol. Chem.* **245,** 4851 (1970).
11. A. Y. H. LU, H. W. STROBEL and M. J. COON, *Molec. Pharmac.* **6,** 213 (1970).
12. M. J. COON, A. P. AUTOR and H. W. STROBEL, *Chem. Biol. Interact.* **3,** 248 (1971).
13. S. ORRENIUS, G. DALLNER and L. ERNSTER, *Biochem. biophys. Res. Commun.* **14,** 329 (1964).
14. E. D. WILLS. *Biochem. J.* **113,** 333 (1969).
15. T. E. GRAM and J. R. FOUTS, *Arch. Biochem. Biophys.* **114,** 331 (1966).
16. M. W. ANDERS, *Biochem. Pharmac.* **18,** 2561 (1969).
17. S. E. LEWIS, C. F. WILKINSON and J. W. RAY, *Biochem. Pharmac.* **16,** 1195 (1967).
18. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
19. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **94,** 22 (1965).
20. R. KATO and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **150,** 279 (1965).
21. T. NASH, *Biochem. J.* **55,** 416 (1953).
22. E. D. WILLS, *Biochem. J.* **99,** 667 (1966).
23. P. HOCHSTEIN, K. NORDENBRAND and L. ERNSTER, *Biochem. biophys. Res. Commun.* **14,** 323 (1964).
24. F. MARKES and E. HECKER, *Hoppe-Seyler's Z. physiol. Chem.* **348,** 727 (1967).
25. E. D. WILLS and A. E. WILKINSON, *Biochem. J.* **99,** 657 (1966).
26. E. D. WILLS, *Biochem. J.* **113,** 325 (1969).
27. M. A. PETERS and J. R. FOUTS, *Biochem. Pharmac.* **19,** 533 (1970).
28. P. HOCHSTEIN and L. ERNSTER, *Biochem. biophys. Res. Commun* **12,** 388 (1963).
29. E. D. WILLS, *Biochem. J.* **123,** 983 (1971).
30. E. D. WILLS, *Biochem. Pharmac.* **21,** 239 (1972).
31. T. C. PEDERSON and S. D. AUST, *Biochem. Pharmac.* **19,** 2221 (1970).
32. S. D. AUST and J. B. STEVENS, *Biochem. Pharmac.* **20,** 1061 (1971).
33. E. D. WILLS, *Biochem. J.* **113,** 315 (1969).