REVERSAL OF THE INHIBITORY EFFECT OF LIPID PEROXIDES ON THE HEPATIC CYTOCHROME P-450 MONOOXYGENASE SYSTEM BY A SOLUBLE FACTOR FROM LIVER AND A COMMERCIAL ISOCITRIC DEHYDROGENASE PREPARATION FROM HOG HEART

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SUMMARY. A factor (SF) contained in the 105,000 x g supernatant fraction of rat liver homogenate, previously described as a requirement for the maximal activity of the cytochrome P-450-dependent monooxygenase system of hepatic microsomes, was found to function by reversing the inhibitory effect of TPNH-supported microsomal lipid peroxidation. Commercial hog heart isocitric dehydrogenase preparations contain a large amount of SF or a substance like SF.

In a communication published recently in this journal (1) we described a soluble factor (SF) contained in the 105,000 x g supernatant fraction of rat liver homogenate which was required for maximal activity of the cytochrome P-450-dependent monooxygenase system of hepatic microsomes. We suggested that SF might function "as an unknown component of the monooxidase system, as an activator of the system, or by removing an inhibitor of the system." The last of these three possibilities has proven to be the case. We have demonstrated that SF "stimulates" the monooxygenase system by reversing the inhibitory effect of TPNH-supported lipid peroxidation.

MATERIALS AND METHODS. Microsomes and the 105,000 x g supernatant fraction containing SF were prepared from livers of male Holtzman rats (180-250 g) as described previously (1). Monooxygenase activity was determined by measuring the amount of formaldehyde formed from ethylmorphine N-demethylation as described previously (2). Lipid peroxidase activity was estimated by measuring the amount of malondialdehyde formed throughout the incubation period (3). Highly purified (Type IV) hog heart isocitric dehydrogenase (ICDH, E.C.1.1.1.42) was purchased from Sigma; its activity was redetermined using an ICDH assay kit No. 175 (Sigma).

RESULTS. In the previous study (1) a 15 min incubation period was employed in the determination of ethylmorphine N-demethylase activity. A great deal of variation among experiments was observed in basal demethylase activity (microsomes minus SF) whereas activity was relatively uniform when SF was added. Consequently, the stimulatory activity of SF in different experiments varied from as little as 10% to as much as 100%. An increased incubation period was included among a number of other procedures designed to improve the SF assay. Increasing the incubation period from 15 min to 30 min greatly increased the SF effect and this was due entirely to prolongation of the linearity of the reaction (Fig. 1). This suggested that (a) contaminating SF contained in the microsomes was being destroyed during incubation, or (b) that some inhibitory substance was being generated during incubation; SF either destroyed this substance or prevented its formation. In either case, preincubation might be expected to improve the SF assay above that already achieved by extending

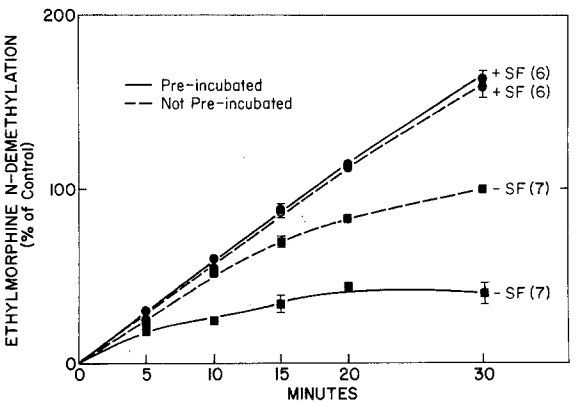

Figure 1.
Effect of soluble factor (SF) on the rate and linearity of the N-demethylation of ethylmorphine by preincubated and nonpreincubated hepatic microsomes from the rat. The preincubation mixture consisted of the following constituents in 4.5 ml of .044 M phosphate buffer (pH 7.4): TPN (.44 mM), G-6-P (4.4 mM), G-6-P dehydrogenase (2 units), Mg^{++} (2.22 mM), semicarbazide HCl (8.33 mM), and microsomes (2.0 mg of protein). Preincubation time was 0 or 10 min. The reaction was started by the addition of 0.5 ml of an ethylmorphine HCl solution (final concentration: 2 mM) at 0 time and continued for intervals between 5 and 30 min. Preincubation and incubation were conducted at 37° in air with shaking. In this and other figures, numbers in parentheses = number of experiments, vertical bars represent S.E., and SF = 105,000 x g supernatant fraction of rat liver homogenate, which supplied 1.2 mg of protein/ml of final incubation mixture. 100% of control ethylmorphine N-demethylation = 4.39 ± .49 nmoles of HCHO formed/mg of protein/min.

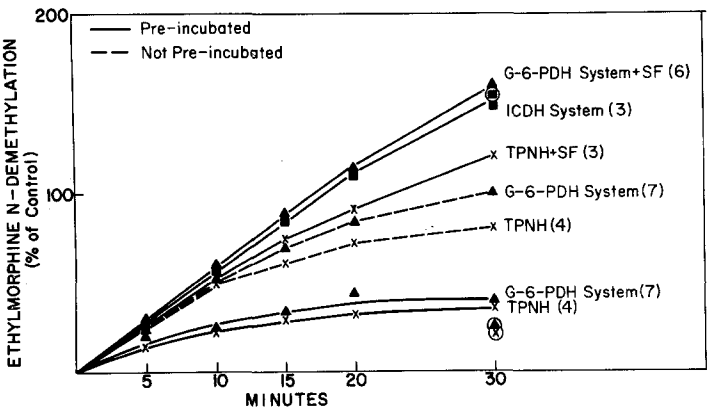

Figure 2.
Effects of preincubating hepatic microsomes with various sources of TPNH on the N-demethylation of ethylmorphine by preincubated and nonpreincubated hepatic microsomes from the rat. Preincubation and incubation conditions are the same as those given in Fig. 1 except that sources of TPNH were varied as indicated. The isocitric dehydrogenase (ICDH)-TPNH-generating system consisted of TPN (.37 mM), isocitric acid (3.7 mM) and ICDH (.33 units/ml of final incubation mixture); the G-6-P dehydrogenase-TPNH generating system was the same as that given in Fig. 1; when added without a generating system, the TPNH concentration was 4 mM. In several cases when preincubated microsomes were incubated with substrate for 30 min, respective sources of TPNH were added again at 0 time along with the substrate; values obtained from these experiments are circled. These studies were included to rule out the possibility that TPNH-generating systems may have degenerated during prolonged incubation. 100% of control ethylmorphine N-demethylation = 4.39 ± .49 nmoles of HCHO formed/mg of protein/min.

the incubation period to 30 min. When the incubation mixture was preincubated for 10 min in the absence of SF before starting the reaction with ethylmorphine, demethylase activity was only about half that observed with nonpreincubated microsomes; in the presence of SF, demethylase activity was unaltered by preincubation (Fig. 1).

If an inhibitory substance was being generated, it was important to know whether or not its generation required TPNH. In the experiment shown in Fig. 1, TPNH, generated by the glucose-6-phosphate dehydrogenase (G-6-PDH) system, was present in the medium during preincubation and incubation. When the TPNH-generating system was omitted during preincubation, demethylase activity was the same as that observed with nonpreincubated preparations. The depressant effect of preincubation on demethylation was not seen when DPNH was substituted for the TPNH-generating system. In what at the time might have been considered

an unnecessary experiment, TPNH per se or the isocitrate dehydrogenase (ICDH)-TPNH generating system was substituted for the G-6-PDH system. Preincubation with TPNH was as effective as preincubation with the G-6-PDH system in lowering basal demethylase activity, but less effective in supporting demethylation in the presence of SF, which was anticipated because of the known inhibitory effect of TPN formed during incubation (4). However, much to our surprise, the ICDH system behaved very much like SF (Fig. 2).

Because the lipid peroxidase system is known to require TPNH and because lipid peroxides are thought to inhibit the monooxygenase system (5-8), our observation that TPNH was required for preincubation to depress demethylase activity pointed to the possibility that lipid peroxides might be the offending agents. The lipid peroxidase system requires iron, and small amounts of iron are known to contaminate the incubation medium. EDTA inhibits the system by chelating iron (5). In Fig. 3, it may be seen that EDTA duplicates almost exactly the effect of SF (Fig. 1) on the linearity of the demethylase reaction and on the reversal of the depression of demethylation produced by preincubation.

The EDTA study raised the possibility that the ICDH system produced its SF-like effect because isocitric acid acted as a chelating agent. The experi-

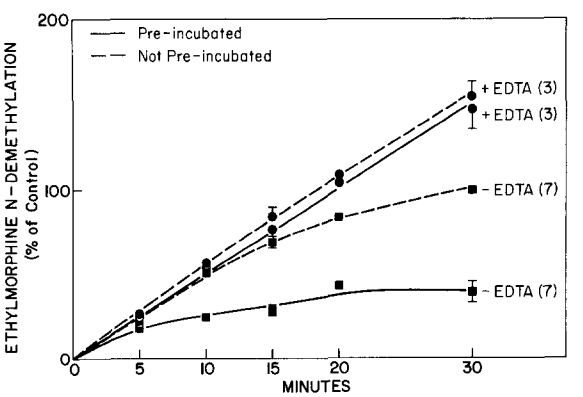

Figure 3.
Effect of EDTA on the rate and linearity of the N-demethylation of ethylmorphine by preincubated and nonpreincubated hepatic microsomes from the rat. Conditions were those given in Fig. 1. The concentration of EDTA was 0.05 mM. 100% of control ethylmorphine N-demethylation = 4.39 ± .49 nmoles of HCHO formed/mg of protein/min.

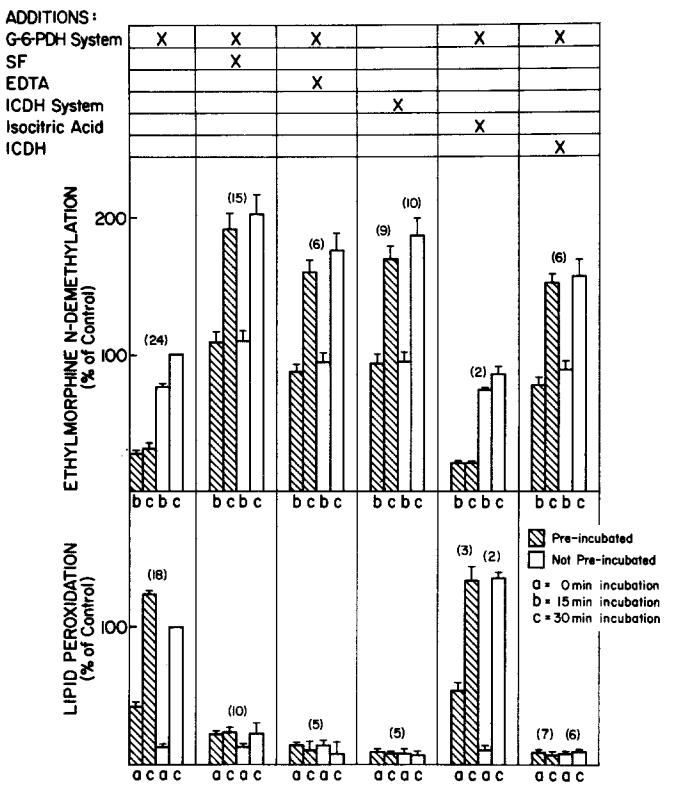

Figure 4.
Effects of SF, EDTA and a commercial preparation of hog heart isocitric dehydrogenase (ICDH) on ethylmorphine N-demethylase and lipid peroxidase activities of preincubated and nonpreincubated hepatic microsomes from the rat. Conditions were those given in Fig. 1. The ICDH system is described in Fig. 2. When used without each other the concentrations of isocitric acid and ICDH were the same as when they were used together. The concentration of EDTA was 0.05 mM. 100% of control ethylmorphine N-demethylation and lipid peroxidation = 3.85 ± .20 nmoles of HCHO formed/mg of protein/min and ΔOD_{535nm} .94 ± .08/mg of protein/30 min, respectively.

ment summarized in Fig. 4 tested this possibility and further established the role of peroxidation in the inhibition of demethylase activity. In summary, this experiment showed that monooxygenase and peroxidase activities are inversely related and that ICDH preparations almost abolish peroxidase activity and increase monooxygenase activity, but isocitric acid does neither.

The effects of varying concentrations of the ICDH preparation on demethylation and lipid peroxidation are illustrated in Fig. 5. An increase in demethylase activity coincident with a decrease in peroxidase activity is seen

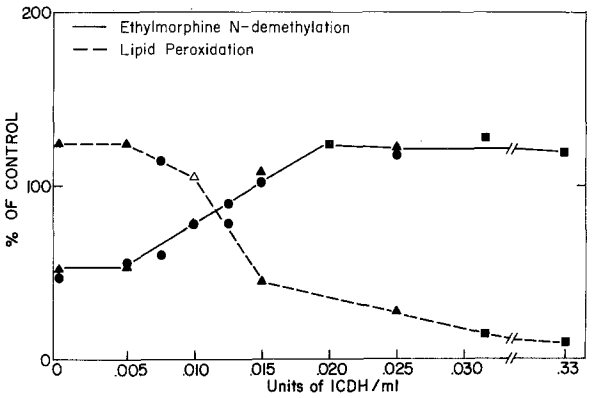

Figure 5.
Effects of varying concentrations of hog heart isocitric dehydrogenase (ICDH) preparation on the ethylmorphine N-demethylase and lipid peroxidase activities of preincubated hepatic microsomes from the rat. Conditions were the same as those given in Fig. 1 (including the addition of the G-6-P-TPNH generating system) except that the incubation time was 30 min in all cases. The commercial ICDH preparation contained 36 ICDH units and 10 mg of protein/ml; its ICDH activity was redetermined just prior to use as described in MATERIALS AND METHODS. 100% of control ethylmorphine N-demethylation and lipid peroxidation = 2.28 ± .32 nmoles of HCHO formed/mg of protein/min, and ΔOD_{535nm} 1.00 ± .09/mg protein/30 min, respectively. Symbols represent numbers of experiments: triangles, 3; circles, 2; and squares, 1.

with increasing ICDH activity. Maximum effects were observed with an amount of ICDH preparation containing 0.02 units of ICDH activity/ml of incubation medium. About 5 µg of protein is contained in the commercial hog heart ICDH preparation supplying 0.02 ICDH units.

DISCUSSION. These studies support the current view that the microsomal monooxygenase system is inhibited by lipid peroxides generated by TPNH-dependent peroxidases. They also explain the general manner in which SF "stimulates" the monooxygenase system, namely by preventing the inhibitory effect of lipid peroxides. This conclusion was made by Kamataki et al (8) from similar studies employing a soluble fraction from liver.

It has not been determined whether SF prevents the formation of lipid peroxides or whether it prevents formed lipid peroxides from exerting an inhibitory effect. In the first case, SF might prevent the formation of lipid peroxides by inhibiting the peroxidase directly or by preventing iron, an

essential component of the peroxidase system, from participating in the reaction. In the second case, SF might react preferentially with the lipid peroxides thereby preventing their reaction with components of the monooxygenase system. At first glance, the latter possibility seemed likely because ICDH is extremely sensitive to inactivation by linoleic acid hydroperoxide, putatively because the lipid peroxide reacts very readily with its sulfhydryl groups (9). Accordingly, lipid peroxides generated during incubation would not inhibit the monooxygenase system because they would react first with the sulfhydryl groups of isocitric dehydrogenase. Fractionation studies, the details of which we intend to publish soon, showed this not to be the case. The hog heart ICDH preparation was fractionated on a Sephadex G-75 column. The elution pattern, monitored at 280 nm, revealed a large and small peak. The material represented by the small peak contained all of the SF activity as determined by its ability to "stimulate" demethylase activity and to depress peroxidase activity; it possessed no ICDH activity. The large peak possessed all of the ICDH activity and no SF activity. Studies employing a calibrated Sephadex G-75 column suggest that the molecular weight of the SF contained in the commercial ICDH preparation is between 10,000 and 12,000.

Two components containing SF were obtained when the 105,000 x g supernatant fraction from liver homogenate was fractionated on a G-75 Sephadex column, one with a molecular weight of about 25,000 and the other with a molecular weight of about 10,000-12,000. The component with the smaller molecular weight contained SF activity, but no ICDH activity. The component with the larger molecular weight contained both ICDH and SF activities. However, when individual fractions of the eluate were assayed, little correlation was seen between ICDH activity and SF activity. This might suggest that the SF factor is normally complexed with ICDH and that it dissociates on standing or during fractionation on the column.

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REFERENCES.

1. Nelson, D. W., Lorusso, D. J., and Mannering, G. J., (1973), *Biochem. Biophys. Res. Comm.*, 53, 995-1001.
2. Correia, M. A., and Mannering, G. J., (1973), *Mol. Pharmacol.*, 9, 455-469.
3. Ernster, L., and Nordenbrand, K., (1967), *Methods in Enzymology*, (Estabrook, R. W., and Pullman, M. E., Eds.), Vol. X, pp. 574-580, Academic Press, New York.
4. Orrenius, S., (1965), *J. Cell Biol.*, 26, 713-723.
5. Wills, E. D., (1969), *Biochem. J.*, 113, 325-332.
6. Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Poyer, J. L., and McCay, P. B., (1973), *Arch. Biochem. Biophys.*, 158, 842-852.
7. Jacobson, M., Levin, W., Lu, A. Y. H., and Kuntzman, R., (1973), *Drug Metab. Disp.*, 1, 766-774.
8. Kamataki, T., Ozawa, N., Kitada, M., Kitagawa, H., and Sato, R., (1974), *Biochem. Pharmacol.*, 23, 2485-2490.
9. Green, R. C., Little, C., and O'Brien, P. J., (1971), *Arch. Biochem. Biophys.*, 142, 598-605.