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Effect of α-Tocopherol upon Lipid Peroxidation and Drug Metabolism in Hepatic Microsomes[1]

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In an *in vitro* system consisting of rat liver 9000*g* supernatant fraction and a TPNH-generating system, significant lipid peroxidation was observed during incubation at 37°C; negligible peroxidation occurred under similar conditions with rabbit liver. With rat liver, the addition of hexobarbital or codeine slightly stimulated peroxidation, but the addition of aminopyrine, zoxazolamine, aniline, or 3,4-benzpyrene to the system markedly reduced or abolished it. Homogenization of the liver in the presence of α-tocopherol abolished lipid peroxidation when incubation was subsequently carried out either in the presence or absence of hexobarbital or codeine. When incubated with rat liver supernatant fraction, hexobarbital and codeine were metabolized linearly with time for relatively short periods; early plateaus in activity suggested enzymic inactivation. Although lipid peroxidation was abolished by α-tocopherol, no effect was observed on the time course curves of either hexobarbital or codeine metabolism, suggesting that peroxidation is not responsible for the inactivation of rat liver microsomal drug-metabolizing enzymes.

A considerable body of evidence has accumulated indicating that under appropriate conditions *in vitro*, mammalian liver preparations (homogenates, microsomal plus soluble fractions, washed mitochondria, or washed microsomes) undergo lipid peroxidation and form a material (malonaldehyde) which produces a chromogen with thiobarbituric acid (TBA) (1–4, 15, 17). When various metallic ions such as Fe^{++} or Cu^{++} or ascorbic acid are added to liver preparations *in vitro*, marked stimulation of lipid peroxidation has been observed (4–6). Moreover, certain antioxidants (α-tocopherol, vitamin K_1) and metal chelators (α,α′dipyridyl, EDTA, 8-hydroxyquinoline) can abolish either spontaneous or stimulated lipid peroxidation. Recently (7), it has been reported that lipid peroxidation in rat liver microsomes leads to an early inactivation of enzymes which synthesize l-ascorbic acid and that the addition of antioxidants or metal chelators has a marked preserving or stabilizing effect on the enzymes. Further, in washed rat liver microsomes, the generation of TBA-reacting material, indicative of lipid peroxide formation, was markedly enhanced during TPNH oxidation (8). This suggested a causal relationship between lipid peroxidation and TPNH oxidation.

Most hepatic microsomal drug-metabolizing enzymes require TPNH and O_2 for maximal activity (9, 10). Previous work in this laboratory has shown that rabbit liver microsomes metabolize drugs linearly with time for relatively prolonged periods (45–90 minutes) while rat liver microsomes display early deviations from linear metabolism and early plateaus in activity which are independent of co-factor and substrate concentrations and are not the result of product inhibition (11). A preliminary observation, indicating more extensive lipid peroxidation with rat liver microsomes than those prepared from rabbit liver, suggested a possible relationship between these phenomena.

METHODS

Adult male Dutch rabbits and Holtzman rats were maintained on standard laboratory chow

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which contained supplementary quantities of tocopherols. Animals were killed by cervical dislocation and their livers were homogenized in 2 volumes of 1.15% KCl (pH 7.4). The 9000 *g* supernatant fraction containing microsomal and soluble enzymes was prepared by centrifuging the homogenate at 9000 *g* for 20 minutes at 0–4°C in an angle centrifuge. Incubations were carried out in a Dubnoff shaking apparatus at 37°C under an atmosphere of oxygen. Incubation media contained liver supernatant fraction, drug substrate, a TPNH-generating system (TPN, glucose 6-phosphate, nicotinamide, and Mg^{++}) and 0.1 *M* phosphate buffer (pH 7.35) to a final volume of 5.0 ml. Side-chain oxidation of hexobarbital and O-demethylation of codeine were determined as previously described (12, 13). Micro-Kjeldahl determinations of supernatant fraction nitrogen content were made according to Juchau *et al.* (14). Lipid peroxidation was assayed by the development of a TBA-chromogen according to Hunter *et al.* (4).

RESULTS

It was found with rat liver that the volume of tissue supernatant fraction present in a fixed-volume incubation mixture greatly influenced the TBA reaction (Fig. 1). It can be seen that, with amounts of supernatant fraction smaller than 0.1 ml or larger than 1.0 ml in a total volume of 5 ml, little lipid peroxidation was demonstrable. Similar findings have been reported by Ghoshal and Recknagel (15). Regardless of the amount of rabbit liver supernatant fraction present, however, little or no TBA reacting material could be found.

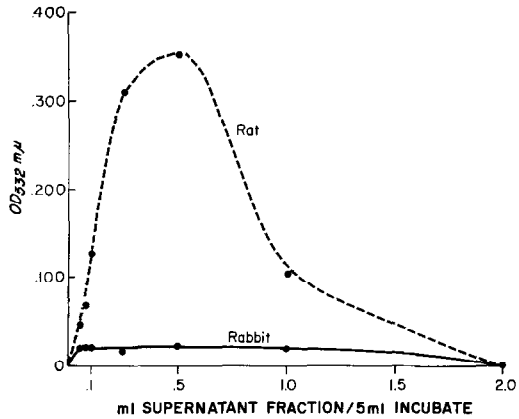

FIG. 1. Effect of varying the volume of liver supernatant fraction on lipid peroxidation. Supernatant fraction (9000 *g*) was incubated under O_2 at 37° for 30 minutes in the following system: TPN, 0.6 μmole; glucose 6-phosphate, 25 μmoles; nicotinamide, 100 μmoles; and $MgSO_4$, 25 μmoles. The mixture was brought to a final volume of 5 ml with 0.1*M* phosphate buffer (pH 7.35). Aliquots were assayed for lipid peroxidation by the thiobarbituric acid reaction (4).

In an effort to determine the role of various constituents of the incubation mixture in lipid peroxidation in rat liver, an experiment was carried out in which individual components were omitted. Table I summarizes the results. When 0.5 ml of rat liver supernatant fraction was incubated with a TPNH-generating system under O_2, significant lipid peroxidation occurred; addition of hexobarbital caused a slight stimulation. It is clear that the TPNH-generating system plays a central role in peroxidation since its omission markedly reduced the amount of TBA-reacting material produced. Similar observations have been made with rat liver microsomes (8, 16). It would further appear that TPN-TPNH-TPN cycling is important in peroxidation in rat liver since incubation of TPNH with supernatant

TABLE I

LIPID PEROXIDATION WITH RAT LIVER SUPERNATANT FRACTION[a]

Supernatant fraction	G-6-P Nicotinamide $MgSO_4$	TPN	Hexobarbital	(TPNH)	Lipid peroxidation, OD 532 mμ
+	+	+	+	−	.600
+	+	+	−	−	.475
+	+	−	+	−	.210
+	−	+	+	−	.015
+	−	−	−	−	.110
+	−	−	−	+	.060
+	−	−	+	−	.125
−	+	+	+	−	.00
+	+	+	+	−	.00 (Zero time)

[a] The incubation mixture consisted of the following: rat liver supernatant fraction, 0.5 ml; glucose 6-phosphate, 25 μmoles; nicotinamide, 100 μmoles; $MgSO_4$, 25 μmoles; TPN (or TPNH), 0.6 μmole; hexobarbital, 3 μmoles; and 0.1 *M* phosphate buffer (pH 7.35) to a final volume of 5 ml. Incubation was for 30 minutes under O_2 at 37°C. At the end of the incubation period, 2 ml of the incubation mixture was transferred to a tube containing 0.5 ml 40% TCA and 0.25 ml 5 *N* HCl and assayed for lipid peroxides (4)

fraction resulted in minimal peroxide formation.

The data in Fig. 1 suggested that the early deviation from linear drug metabolism previously observed with rat liver supernatant fraction (11) might be associated with microsomal lipid peroxidation which could lead to inactivation of microsomal drug metabolizing enzymes. Similarly, these data would be consistent with the observation that rabbit liver supernatant fractions metabolize drugs linearly with time for prolonged periods since, as shown in Fig. 1, rabbit preparations produced no detectable peroxides.

It is known that antioxidants markedly diminish lipid peroxide formation in liver preparations *in vitro* (4–6). Accordingly, pooled rat livers were minced and divided into two portions; one portion was homogenized with 2 volumes 1.15% KCl. The other portion was homogenized in the same medium to which α-tocopherol was added (50 mg α-tocopherol and 50 mg α-tocopheryl acetate per 10 ml KCl solution). Concurrent lipid peroxide assays and drug metabolism studies were run on 9000*g* supernatant fractions prepared from these homogenates. The data are summarized in Figs. 2 and 3 and indicate a clear divergence between lipid peroxidation and drug enzyme inactivation. Thus, although lipid peroxidation was abolished by α-tocopherol, the antioxidant had no effect on the microsomal enzymes which *O*-demethylate codeine or oxidize hexobarbital. It would appear, therefore, that lipid peroxidation and drug-enzyme inactivation in the rat are coincidentally but not causally related.

Orrenius *et al.* (16) observed that drugs undergoing oxidation demethylation, such as codeine and aminopyrine, markedly diminished lipid peroxidation in rat liver microsomes *in vitro*. Accordingly, we determined the effect on lipid peroxidation of several commonly used drug substrates. The results are summarized in Table II and show that hexobarbital and codeine had no effect or a slight stimulatory effect on peroxidation in liver 9000*g* supernatant fractions. Zoxazolamine markedly reduced peroxidation and aminopyrine, aniline, and

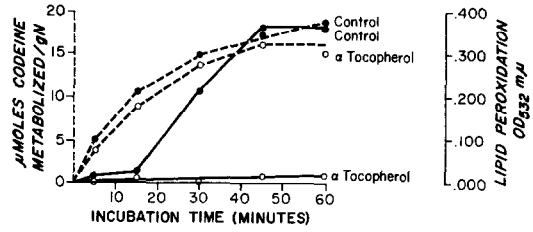

FIG. 2. Effect of α-tocopherol on lipid peroxidation and codeine *O*-demethylation by rat liver supernatant fraction. Incubation mixture was same as in Table I except codeine (10 μmoles) replaced hexobarbital. Tocopherol was added to the liver during homogenization as described in *Results*. All assays were done in duplicate as previously described (4, 13, 14). Codeine metabolism is expressed as μmoles metabolized/gm Kjeldahl N, lipid peroxidation as OD of the TBA chromogen. Solid lines, lipid peroxidation; broken lines, codeine metabolism.

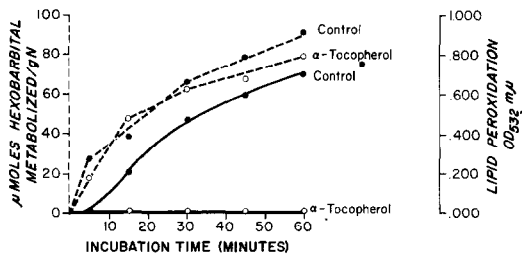

FIG. 3. Effect of α-tocopherol on lipid peroxidation and side-chain oxidation of hexobarbital by rat liver supernatant fraction. Incubation conditions are same as in Table I. Tocopherol was added to the liver during homogenization as described in *Results*. All assays were done in duplicate as previously described (4, 12, 14). Data are expressed as in Fig. 2. Solid lines, lipid peroxidation; broken lines, hexobarbital metabolism.

3,4-benzpyrene essentially abolished it. Similar results were obtained with washed microsomes.

It is of interest that the metabolisms of aminopyrine, zoxazolamine, aniline, and 3,4-benzpyrene by rat liver microsomes proceed linearly with time for relatively prolonged periods (11, 21); these substrates afforded considerable protection against lipid peroxidation (Table II). Conversely, hexobarbital and codeine, whose metabolisms by rat liver microsomes deviate early from linearity and reach early plateaus, provided no protection against peroxide formation.

TABLE II

EFFECT OF DRUGS ON LIPID PEROXIDATION IN RAT LIVER SUPERNATANT REACTION

System	Lipid peroxidation OD 532 mμ
Complete system[a] (no drug substrate)	.315
+ Hexobarbital, 3 μmoles	.365
+ Codeine, 10 μmoles	.340
+ Aminopyrine, 40 μmoles	.005
+ Zoxazolamine, 3 μmoles	.100
+ Aniline, 10 μmoles	.025
+ 3,4-Benzpyrene, 0.6 μmole	.020

[a] The complete system consisted of: rat liver supernatant fraction, 0.5 ml; glucose 6-phosphate, 25 μmoles; nicotinamide, 100 μmoles; $MgSO_4$, 25 μmoles; TPN, 0.6 μmole; and 0.1 *M* phosphate buffer (pH 7.35) to a final volume of 5 ml. Other conditions were the same as in Table I.

DISCUSSION

The present study was carried out to determine if the relatively early loss of drug-metabolizing enzyme activity observed in *in vitro* incubations of rat liver microsomes could be associated with the production of lipid peroxides. Previous studies had shown (11, 21) that rat liver microsomal preparations lost enzyme activity upon incubation much more rapidly than similar preparations from rabbit liver. The present study showed that more TBA reacting material was formed by rat liver microsomes than by rabbit liver microsomes.

However, our results also showed that inhibition of peroxide generation by α-tocopherol had no effect on the period of linearity of enzymic *O*-demethylation of codeine or side-chain oxidation of hexobarbital by rat liver preparations. Other workers have shown (7) that the microsomal enzymes which catalyze ascorbic acid biosynthesis are inactivated by lipid peroxides and that inhibition of peroxidation by added α-tocopherol stabilizes these enzymes and prolongs their activity *in vitro*. These results and ours on drug metabolism suggest that the microsomal drug-metabolizing enzymes may have a greater resistance to the denaturing effects of lipid peroxides than the enzymes involved in ascorbic acid synthesis. Similarly, other investigations have shown differential effects of lipid peroxidation on enzyme activity. Hochstein and Ernster (18) found that concomitant with peroxidation of rat liver microsomal lipids there was essentially complete inactivation of glucose 6-phosphatase and marked loss of DPNH cytochrome *c* reductase activity while TPNH cytochrome *c* reductase activity was unaffected. Moreover, in studies of the influence of lipid peroxides on the activity of 18 enzymes from diverse sources, Wills (19) found that some enzymes such as urease and papain were strongly inhibited while others such as catalase and D-amino acid oxidase were not affected. Sulfhydryl-containing enzymes seemed to be particularly labile to peroxide inactivation. Relationships between lipid peroxidation and inactivation of mitochondrial and microsomal enzymes have been nicely reviewed by Tappel (20) and probably involve both direct effects on enzymes as well as effects on the phospholipid membranes of subcellular organelles.

There is no readily apparent explanation for the marked difference between rabbit and rat livers with respect to peroxide formation under the incubation conditions employed here. Although both species received laboratory chow supplemented with vitamin E, it is possible that rats have higher basal requirements for the vitamin and were therefore relatively vitamin E-deficient. It may be that lipids in rabbit liver supernatant fraction do not generate peroxides to the extent seen with rat liver. There may be differences in endogenous levels of protective materials between the species. It is also possible that rabbit liver preparations rapidly metabolize or bind peroxides, their biological precursors, or their decomposition products which react with TBA.[2] Under the conditions employed here, however, it is clear that TBA-reacting material develops more readily with rat liver than with rabbit liver.

It is of interest (Table II) that the addi-

[2] This proposition does not appear tenable however, since additional experiments have shown that the addition of rabbit liver supernatant fraction to a system containing rat liver supernatant fraction and a TPNH-generating system did not depress lipid peroxidation.

tion of drugs to a mixture consisting of rat liver supernatant fraction and a TPNH-generating system may either increase or essentially abolish lipid peroxidation. For those substrates which strongly inhibit lipid peroxidation, the suggestion could be made that drug metabolism and lipid peroxidation share a common factor (a peroxide), and that when a drug substrate is present this factor is utilized for metabolism of the drug rather than for lipid peroxidation. Orrenius *et al.* (16) have made a similar proposal to account for the inhibition of lipid peroxidation by drugs which undergo oxidative demethylation such as aminopyrine and codeine.

Even though all known oxidative drug metabolisms catalyzed by microsomal enzymes can be written as hydroxylation reactions (9) which use an activated oxygen moiety (peroxide), it is apparent from our results that not all drug substrates have a higher affinity for such peroxides than do the microsomal lipids. Thus hexobarbital and codeine certainly do not depress lipid peroxidation by rat liver microsomes (Table II). It should be noted that we found aminopyrine strongly inhibited lipid peroxidation but codeine did not. This contrasts with the work of Orrenius *et al.* (16) which indicated that both aminopyrine and codeine inhibited lipid peroxidation by rat liver microsomes. We have no explanation for this discrepancy.

One is left with the conclusion that lipid peroxidation and drug metabolism may be related, but can also be separated. The relationship would seem to be closer for substrates like aminopyrine and aniline than for hexobarbital or codeine.

It seems obvious that the inactivation of drug-metabolizing enzymes in rat liver microsomes which occurs during incubation *in vitro* involves more than the deleterious effects of lipid peroxidation. Thus one can prevent lipid peroxidation by α-tocopherol without affecting the loss of hepatic microsomal drug metabolizing enzyme activity. Moreover, separate experiments showed that α-tocopherol had no effect on the time-course of the metabolism of aniline, a substrate which itself abolishes lipid peroxidation. Further studies on this mechanism of enzyme inactivation, particularly the differences between enzyme stability in rat and rabbit liver preparations, are in progress.

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