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INHIBITION OF HEPATIC MIXED FUNCTION OXIDASE ACTIVITY BY PROPYL GALLATE*

CHUNG S. YANG† and FREDERICK S. STRICKHART

Department of Biochemistry, New Jersey Medical School, Newark, N.J. 07103, U.S.A.

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Abstract—Propyl gallate was found to inhibit microsomal benzpyrene hydroxylase activity and demethylase activity with ethylmorphine, aminopyrine or benzphetamine as a substrate. The extent of inhibition with different substrates varied with the age and diet of the animals. The benzpyrene hydroxylase activity of the microsomes of the 3-methylcholanthrene-treated rats was shown to be less susceptible to propyl gallate inhibition. Propyl gallate does not inhibit the NADPH-dependent reduction of cytochrome P-450; therefore, the site of inhibition is not on NADPH-cytochrome c reductase as suggested previously. Propyl gallate interacts with cytochrome P-450 to produce a positive absorption peak around 420 nm, and it may also interfere with the binding of a type I substrate, benzphetamine. It inhibits ethylmorphine demethylation by a noncompetitive mechanism and aminopyrine demethylation by a mixed mechanism. The mode of propyl gallate inhibition and the implications of these observations are discussed.

THE BIOCHEMICAL actions of various antioxidants are of current interest due to their wide occurrence in food and their possible protective roles in liver damage,[1] aging[2] and carcinogenesis.[3,4] Propyl gallate is one of the most frequently used antioxidants in the food industry.[5] This compound has been found to attenuate several adverse effects caused by the administration of carbon tetrachloride in rats.[6,7] Torrielli and Slater[8] have reported that propyl gallate inhibited microsomal aminopyrine demethylase and NADPH-cytochrome c reductase activities, and suggested that the site of inhibition was on the flavoprotein, NADPH-cytochrome c reductase.

The liver microsomal mixed function oxidase system‡ is known to play key roles in the biotransformations of steroid hormones, drugs, carcinogens and other compounds.[9,10] In the present study, the interactions of propyl gallate with this important enzyme system are investigated. We found that propyl gallate inhibits benzpyrene hydroxylase activity and the oxidative demethylations of ethylmorphine, aminopyrine and benzphetamine. The extent of inhibition was found to be affected by age and diet. Further investigation revealed that the site of inhibition is not on the NADPH-cytochrome c reductase[8] but on cytochrome P-450. The mechanism of inhibition and the implications of these observations are discussed.

MATERIALS AND METHODS

Chemicals. *n*-Propyl gallate, 3,4-benzypyrene (benzo[a]pyrene), NADPH, NADP, isocitrate dehydrogenase (type IV), DL-isocitric acid, cytochrome c, bovine serum

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‡ This system is also known as the microsomal mono-oxygenase or oxidase system.

albumin and 3-methylcholanthrene were obtained from Sigma Chemical Co. Recrystallization of propyl gallate did not change its inhibitory properties. Phenobarbital (sodium salt) and ethylmorphine-HCl were gifts from Merck & Co. 3-Hydroxybenzpyrene, α-benzphetamine-HCl and aminopyrine were kindly supplied by Dr. H. V. Gelboin of NIH, the Upjohn Co. and Sterling–Winthrop Research Institute respectively.

Microsomes. Male Sprague–Dawley rats (70 g) were obtained from Marland Breeding Farms (West Milford, N.J.) and were maintained on laboratory chow before use. The animals, usually 3–5 in a group, were sacrificed by decapitation. The livers were excised and washed in cold homogenizing buffer before being homogenized in 6 vol. of 0·05 M Tris–HCl buffer, pH 7·4, containing 1·15% KCl. The microsomal fractions were isolated by centrifuging the postmitochondrial supernatant at 105,000 *g* for 90 min. The microsomal pellet was washed once and resuspended in 0·25 M sucrose. The microsomal suspension in small portions under nitrogen atmosphere was rapidly frozen in a dry ice–acetone bath and stored at −20°. The microsomes were usually used within a week of preparation. Freshly thawed microsomes were used for the experiments, and their mixed function oxidase activities were found not to be different from the freshly prepared microsomes. Prolonged storage, however, resulted in lower enzymic activities. Protein was determined by the method of Lowry *et al.*[11]

Assay of benzpyrene hydroxylase activity. Benzpyrene hydroxylase was assayed in duplicate by published methods[12,13] with slight modifications. Aqueous solutions of propyl gallate were freshly made for each experiment. A typical reaction mixture of 1 ml contained 0·1 M potassium phosphate buffer, pH 7·3, 3 mM $MgCl_2$, 0·1 mM EDTA, 0·4 mM NADPH and microsomes corresponding to 40–220 μg protein. After preincubation at 37° for 1 min, the reaction was initiated by the addition of 80 nmoles 3,4-benzpyrene (in 0·04 ml methanol) and the flask was shaken at 37° for 10 min. The reaction was stopped by the addition of 1·0 ml acetone and 3·0 ml hexane. The mixture was shaken for an additional 10 min to extract the phenolic metabolites of benzpyrene into the organic phase. A 2-ml aliquot of the organic phase was extracted with 4·0 ml of 1 N NaOH. The fluorescence of the hydroxylated products in the alkaline phase was measured in a Farrand spectrophotofluorometer with activation at 396 nm and fluorescence at 522 nm. These values were compared to a standard curve of authentic 3-hydroxybenzpyrene to calculate the amount of products formed. The entire assay was carried out in dim light. The differences between a duplicated set of assays were less than 10 per cent. Under these assay conditions, the hydroxylase activity was proportional to the incubation time and the amount of microsomes used in the assay.

Assays of oxidative demethylase activities. The oxidative demethylase activities were assayed by the method of Orrenius.[14] The incubation system contained 40 mM Tris–HCl, pH 7·2, 5 mM $MgCl_2$, 150 mM KCl, 5 mM ethylmorphine (or aminopyrine, or benzphetamine), 10 mM isocitrate, 0·4 unit isocitrate dehydrogenase and microsomes corresponding to 2–3·5 mg protein. After preincubation at 37° for 1 min, the reaction was initiated by the addition of NADP to give a concentration of 0·5 mM in a final volume of 2·0 ml. The mixture was incubated for 15 min at 37° and stopped by the addition of 0·25 ml of 25% $ZnSO_4$ solution and 0·25 ml saturated $Ba(OH)_2$ solution. The amount of formaldehyde formed was measured by the Nash

reaction.[15] Under these assay conditions, the formaldehyde formed was proportional to the time of incubation and the amount of microsomes present in the reaction mixture.

RESULTS

Inhibition of the mixed function oxidase activities by propyl gallate. The *N*-demethylation of various drugs and the hydroxylation of aryl hydrocarbons are two of the well known reactions catalyzed by the microsomal mixed function oxidase system. Propyl gallate was found to inhibit both types of reactions (Fig. 1). It was more sensitive toward the benzpyrene hydroxylase activity with an IC_{50}* of about 50 μM. Aminopyrine demethylase and benzphetamine demethylase were inhibited by propyl gallate to the same extent with an IC_{50} of about 140 μM, whereas the demethylation

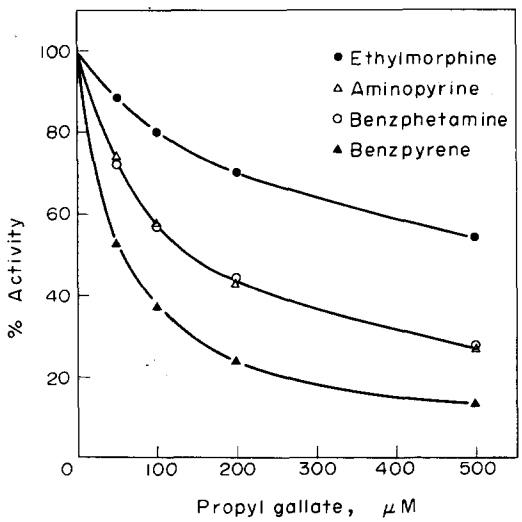

FIG. 1. Inhibition of the mixed function oxidase reactions by propyl gallate. Microsomes were made from rats with an approximate body weight of 220 g. For the demethylase assays with ethylmorphine, aminopyrine and benzphetamine, microsomes equivalent to 3·5 mg protein were used, and the formaldehyde (HCHO) produced by the uninhibited reactions was 4·8–5·6 nmoles/min/mg. For benzyprene hydroxylation reactions, 0·18 mg microsomal protein was used and the activity of the uninhibited reaction was 0·8 nmole product formed/min/mg.

of ethylmorphine was less susceptible to the inhibition with an IC_{50} of more than 500 μM. The fact that propyl gallate inhibited all the above mentioned reactions suggest that it is a true inhibitor of the mono-oxygenase system itself, and the inhibition is not due to the alteration of the substrates, products or the NADPH-generating system. When propyl gallate, at a dosage of 300 mg/kg body weight, was injected intraperitoneally into the rats at 24 hr before the experiment, no induction of the mixed function oxidase system was observed. The microsomes from propyl gallate-treated animals had lower demethylase activities, possibly due to the inhibitory action of the propyl gallate attached to these microsomes.

* An IC_{50} is the concentration of the inhibitor which exerts a 50 per cent inhibition.

During the course of this investigation, it was observed that propyl gallate was more inhibitory to some microsomal preparations and less inhibitory to others, especially with the ethylmorphine demethylase assay. This inconsistency was not due to the storage of the microsomes nor to the amount of microsomes used in the assays. With the ethylmorphine demethylase assay, it was consistently observed that the rats which had been fasted for 2 days were always inhibited by propyl gallate to a greater extent than the non-fasted controls. The average of five experiments showed that 500 μM of propyl gallate caused a 70 per cent inhibition of the ethylmorphine demethylase activity of the fasted rats compared to a 50 per cent inhibition for the non-fasted control. With the age groups studied, it appeared that the older the animal, the less sensitive it was toward propyl gallate inhibition. For example, 500 μM of propyl gallate usually caused a 40–45 per cent inhibition of the ethylmorphine demethylase of the 6- to 8-week-old rats and a 70–75 per cent inhibition for the 4- to 5-week-old rats. It has been suggested that, in liver microsomes, there is a mixture of different forms of cytochrome P-450, each with different specificities toward the various substrates that are metabolized by the mixed function oxidase system.[10] The presently observed variations may be a result of the change of cytochrome P-450 composition, due to age or diet. The different cytochrome P-450 species presumably are inhibited by propyl gallate to a different extent. This point is demonstrated in the following section by using different inducers of the microsomal mono-oxygenase system.

Inhibition pattern on the phenobarbital and 3-methylcholanthrene-induced system. Phenobarbital is known to induce cytochrome P-450, and the induced species are more active in catalyzing the demethylation reactions than the benzpyrene hydroxylation reaction.[10,13,16] On the other hand, 3-methylcholanthrene is known to induce a different species of cytochrome, usually known as cytochrome P-448 or P_1-450 which is more efficient in catalyzing the benzpyrene hydroxylation, but less active with the demethylation reactions.[10,13,16] As shown in Fig. 2, the microsomes of the rats that had been treated with phenobarbital had a slightly higher benzpyrene hyd-

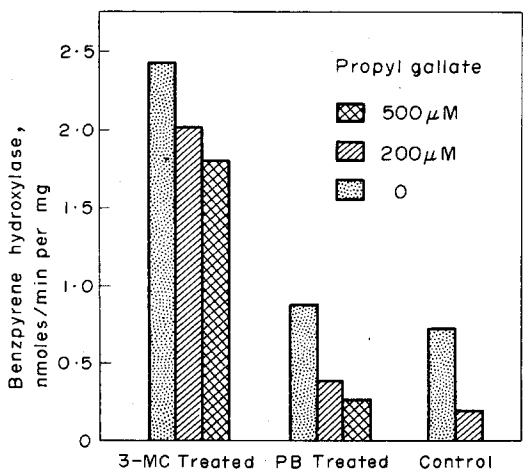

FIG. 2. Inhibition pattern of the induced oxidase system. Rats (*ca.* 150 g in body weight) were injected intraperitoneally, per 1 g body weight, with 100 μg phenobarbital (PB) in saline or 25 μg 3-methylcholanthrene (3-MC) in corn oil, 24 hr before they were sacrificed. Benzpyrene hydroxylase activity was assayed with microsomes corresponding to 0·193–0·224 mg protein.

roxylase activity and were slightly less susceptible to propyl gallate inhibition. The 3-methylcholanthrene-induced system had a much higher specific activity than the untreated control and was inhibited by propyl gallate to a much lesser extent, as shown by the fact that 200 μM of propyl gallate only caused a 17 per cent inhibition compared to a 75 per cent inhibition on the control group. This lack of inhibition was not due to the presence of more mono-oxygenase enzymes in the microsomes of the 3-methylcholanthrene-treated animals. As shown in Fig. 3, 500 μM of propyl gallate caused approximately a 25 per cent inhibition with all the microsome concentrations used, from 45 to 225 μg/ml, indicating that the extent of inhibition is independent of the enzyme concentration. With regard to the ethylmorphine demethylase activity, both the phenobarbital- and 3-methylcholanthrene-induced systems were slightly more sensitive to propyl gallate inhibition than the control. For example, 200 mM propyl gallate inhibited both the induced oxygenase systems by about 55 per cent and the control by 35 per cent.

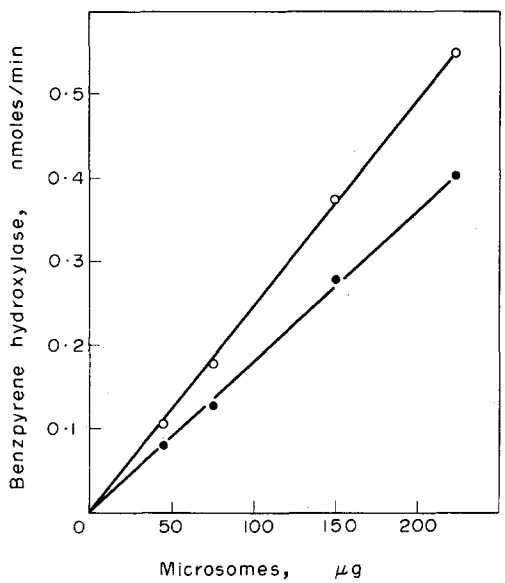

FIG. 3. Effects of the quantity of microsomes to the extent of inhibition of benzpyrene hydroxylase activity by propyl gallate. Microsomes of 3-MC-treated rats (same as Fig. 2) were used. Key ●—●, in the presence of 500 μM propyl gallate; and O—O, in the absence of propyl gallate.

Effects of propyl gallate on the NADPH-dependent reductases. In order to study the interactions between propyl gallate and the NADPH-cytochrome c assay system, some experiments of Torrielli and Slater[8] were repeated. As shown in Fig. 4, cytochrome c was readily reduced non-enzymically by propyl gallate. A separate study indicated that the rate of reduction was dependent on the concentrations of both cytochrome c and propyl gallate. Therefore, if propyl gallate was added to the reductase assay mixture, an enhanced rate of cytochrome c reduction would be seen due to the combined results of an enzymic and a non-enzymic reaction. When the non-enzymic rate was balanced out by adding the same concentrations of cytochrome c and propyl gallate into the reference cuvette, the enzyme-catalyzed cytochrome c reduction was

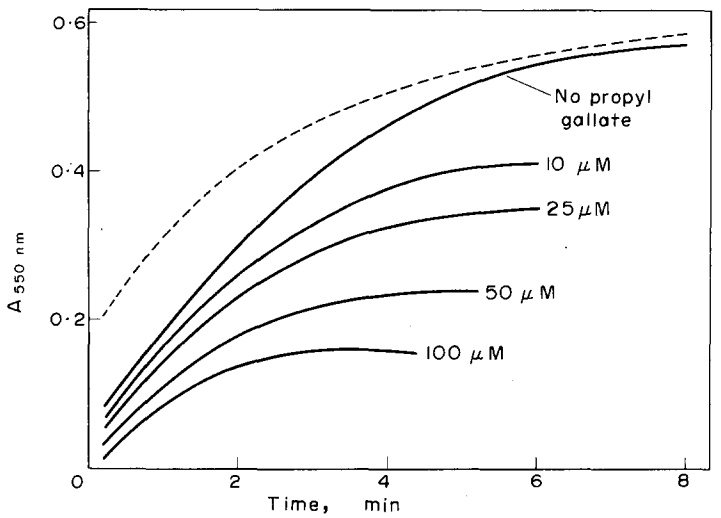

FIG. 4. Interaction of propyl gallate with the NADPH-cytochrome c assay system. For the solid lines, the assay system consisted of 0·1 M potassium phosphate buffer, pH 7·3, 3 mM $MgCl_2$, 0·1 mM EDTA, 1 mM KCN, 35 μM cytochrome c and microsomes (70 μg protein), in a final volume of 1·0 ml in both the sample and reference cuvettes. The reaction was initiated by the addition of propyl gallate to both the sample and reference cuvettes and 0·2 nmole NADPH to the sample cuvette, and both cuvettes were mixed rapidly. The absorbance change at 550 nm was recorded with a Cary 17 recording spectrophotometer.[17] The time between NADPH addition and the initial recording was about 15 sec. The broken line is a trace of the non-enzymic reduction of 35 μM cytochrome c by 100 μM propyl gallate.

found to level off rather quickly (Fig. 4). This is probably because some of the cytochrome c was reduced by propyl gallate and became unavailable to the reductase. However, even in the presence of 10 or 25 μM of propyl gallate, the initial rate of the NADPH-cytochrome c reductase reaction still remained uninhibited. At higher propyl gallate concentrations, the measurement of the initial rate became difficult due

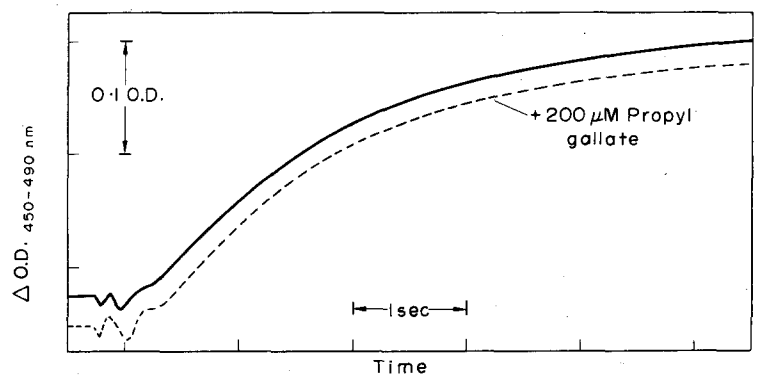

FIG. 5. Effect of propyl gallate on the NADPH-cytochrome P-450 reductase. Microsomes from fasted rats were used for the assay according to published method.[18,19] The reaction mixture contained 40 mM Tris–HCl buffer, pH 7·3, 10 mM $MgCl_2$, 5 mM ethylmorphine-HCl and 0·5 mM NADPH in a final volume of 2·5 ml. The cytochrome P-450 concentration was 3·3 nmoles/ml. The system was gassed for 12 min with CO (deoxygenated with anthraquinone-2-sulfonate and dithionite), and the reaction was initiated by mixing NADPH with the other components of the reaction mixture. The rate of cytochrome P-450 reduction was assayed by recording the $\Delta A_{450-490\ nm}$ with an Aminco–Chance double-beam spectrophotometer.

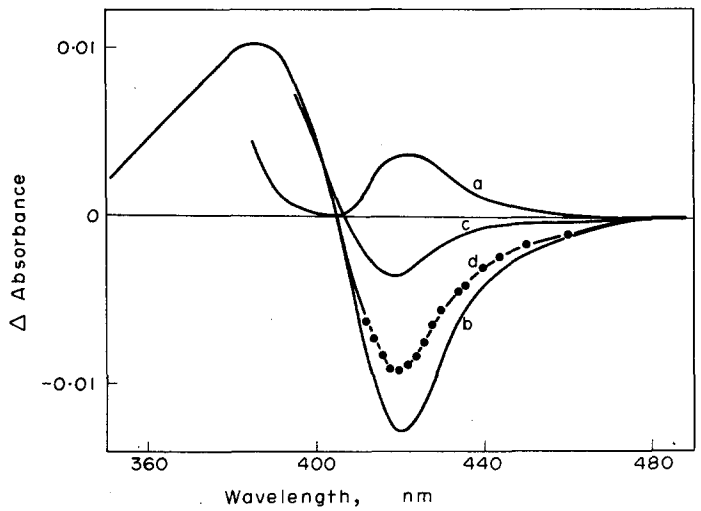

FIG. 6. Propyl gallate-induced spectral changes in microsomes. Liver microsomes were suspended to 1·6 mg protein/ml in 3 ml of 50 mM Tris–HCl buffer, pH 7·5, containing 10 mM $MgCl_2$ and 150 mM KCl; spectra were recorded with an Aminco–Chance double-beam spectrophotometer. Key: (a) 0·5 mM of propyl gallate in sample cuvette and 15 μl acetone in both the sample and reference cuvettes; (b) 0·05 mM of benzphetamine in sample cuvette; (c) 0·5 mM of propyl gallate was added to b; and (d) algebraic summation of spectra a and b.

to the non-linearity. In studying the mechanism of propyl gallate inhibition, the use of the cytochrome c reductase assay, therefore, can be misleading, especially if the initial rate is not used.

If NADPH-cytochrome c reductase were the site of propyl gallate inhibition, then the NADPH-dependent reduction of cytochrome P-450 should also be inhibited. Since propyl gallate does not reduce cytochrome P-450, its effect on the microsomal

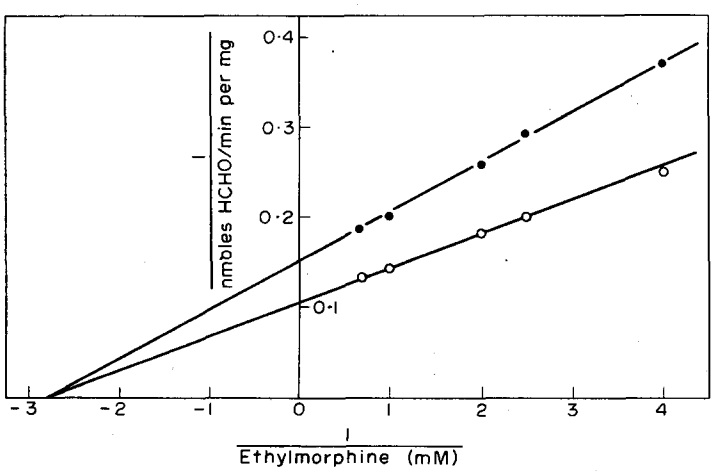

FIG. 7. Lineweaver–Burk plots of the propyl gallate inhibition of ethylmorphine demethylase. Assay conditions were similar to the standard assay, except 2·2 mg protein (microsomes), a 10-min incubation time and various concentrations of ethylmorphine were used. Key: O—O, in the absence, and ●—● in the presence of 100 μM of propyl gallate.

electron transfer can be accurately measured. As shown in Fig. 5, the microsomal NADPH-cytochrome P-450 reductase activity was not affected by propyl gallate. This result indicates that the inhibition site is not on the flavoprotein NADPH-cytochrome c reductase.

Site of propyl gallate inhibition. The interaction of propyl gallate with cytochrome P-450 is indicated by the appearance of an absorption peak around 420 nm in the difference spectrum (Fig. 6). The left part of the spectrum (below 400 nm) was masked by the absorbance of propyl gallate. While the nature of the interaction is not known, the peak at the 420 nm region is similar to the "reverse type I spectrum" described by Schenkman *et al.*[20] and Cinti *et al.*[21] Propyl gallate also reduced the magnitude of the benzphetamine-induced type I spectral change (as shown by the difference in magnitude of the negative peaks of curves c and d in Fig. 6), suggesting that propyl gallate may affect the binding of type I substrates to cytochrome P-450.

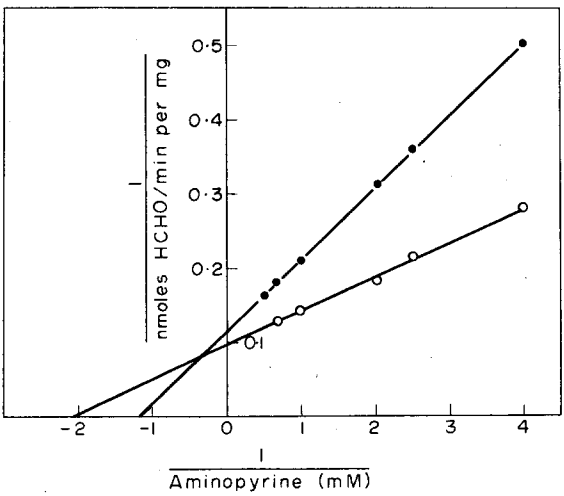

FIG. 8. Lineweaver–Burk plots of the propyl gallate inhibition of aminopyrine demethylase. Assay conditions were similar to those of Fig. 7, except 2·4 mg protein (microsomes) and aminopyrine were used. Key: O—O, in the absence, and ●—● in the presence of 50 μM of propyl gallate. Linear regression analysis with a computer gave the same results. The intercepts on the ordinate are 0·0906 ± 0·0044 (O—O) and 0·1136 ± 0·0029 (●—●). They are significantly different ($P < 0·01$).

The kinetic data in Fig. 7 show that propyl gallate decreases the V_{max} but does not alter the K_m of the ethylmorphine demethylase reaction, indicating a non-competitive inhibition mechanism. However, with aminopyrine as substrate, propyl gallate decreased the V_{max} and increased the K_m of the demethylation reaction (Fig. 8) in a manner similar to the mixed or dual mechanism described by Sasame and Gillette.[22]

DISCUSSION

The presently observed inhibitions of the microsomal mixed function oxidase activities by propyl gallate are consistent with the report of Torrielli and Slater[8] that propyl gallate inhibited the aminopyrine demethylation reaction and prolonged hexobarbital sleeping time in rats. The extent of inhibition depends on the concentration of the inhibitor and is independent of the amount of microsomes used in the assay.

The different extents of inhibition observed with different substrates may be interpreted by the hypothesis that these substrates do not have the exact same binding site on the enzyme or their metabolism involves rate-limiting steps that can be affected differently by propyl gallate. The results may also be interpreted with the view that microsomes consist of a mixture of different cytochrome P-450 species and each has different substrate specificities. The results in Fig. 2 demonstrate that the catalytic activities of different cytochrome P-450 species are inhibited to different extents by propyl gallate.

The data in Figs. 4 and 5 indicate that propyl gallate does not inhibit the electron transfer between NADPH and cytochrome P-450, and the inhibition site is not on NADPH-cytochrome c reductase as previously suggested.[8] The assignment of cytochrome P-450 as the site of inhibition is consistent with the present results. The difference spectra in Fig. 6 suggest that propyl gallate may interact with cytochrome P-450 and affect the binding of benzphetamine, a type I substrate. Although precautions are required in applying Michaelis–Menten kinetics in multi-enzyme systems, the data in Fig. 7 seem to indicate that propyl gallate does not compete with ethylmorphine for the substrate binding site. The results with aminopyrine as substrate (Fig. 8) suggest that propyl gallate may bind to the aminopyrine site to exert the competitive inhibition and also to a second site to inhibit the demethylation non-competitively. The mechanisms of inhibition of many compounds are known to vary with the substrate and possible explanations of this phenomenon have been advanced by Gillette *et al.*[23] It has been reported that free radicals such as superoxide may be involved in the mono-oxygenase reactions.[24,25] It remains to be determined whether the inhibitory action of propyl gallate is related to its free-radical scavenging ability. The detailed mechanism of the inhibition by propyl gallate, along with many other well known inhibitors of drug metabolism,[9,26] remains to be investigated.

Propyl gallate reacts readily with radical species and strongly inhibits the NADPH-dependent lipid peroxidation of microsomes[8] with an IC_{50} of about 10 μM* which is at least 5–10 times more sensitive than its inhibition of the mono-oxygenase system. The protective effects of propyl gallate on carbon tetrachloride poisoning are probably not due to the blockade of the conversion of CCl_4 to an active metabolite such as $\cdot CCl_3$; rather, it may impair the harmful free-radical reactions which can be initiated by $\cdot CCl_3$.

Propyl gallate, along with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are widely used food additives. Recently, BHA and BHT have also been found to be inhibitors of the microsomal mono-oxygenase system.* These are antioxidants approved for use in food, known not to accumulate in the body and appear to be practically nontoxic.[5] However, whether the dietary antioxidants would reach a level to affect the biotransformations of drugs, hormones and carcinogens remains to be determined. Age and dietary factors may play an important role in determining the extent of the inhibitions by these phenolic antioxidants. The present study has provided some basic information about the interactions of propyl gallate with the microsomal mixed function oxidase system. This type of knowledge may be useful in dealing with subjects such as food additives, drug metabolism and other biological problems in which free-radical mechanisms are involved.

* C. S. Yang, unpublished results.

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