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INHIBITION OF NADPH-CYTOCHROME *c* REDUCTASE BY PROPYL GALLATE

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Abstract—The free radical scavenger propyl gallate has been found to strongly inhibit the microsomal enzyme NADPH-cytochrome *c* reductase when added in concentrations greater than 10 μM. The inhibition is not competitive with NADPH or cytochrome *c*. Other free radical scavengers studied (Promethazine, vitamin E, cysteamine, or diphenyl-*p*-phenylene diamine) did not have any inhibitory action on the reductase. The action of propyl gallate is discussed in terms of its *in vivo* action in modifying drug activity.

A NUMBER of pathological disturbances of the liver including those produced by acute doses of carbon tetrachloride or ethanol are believed to involve free radical intermediates having only transient existence but possessing high chemical reactivity.[1] Carbon tetrachloride, for example, has to be metabolised by the NADPH-cytochrome P_{450} electron transport chain of the endoplasmic reticulum before it can manifest its necrogenic action on the liver;[2] the active metabolites are believed to be free radical in nature.[3] In addition, an acute dose of ethanol has been reported to increase the concentration of lipid peroxides in the endoplasmic reticulum of the liver;[4] lipid peroxidation is of course a free radical-motivated reaction. As a consequence of these and many other related observations there have been numerous attempts to modify the liver damage produced by such toxic substances by treatment with free radical scavenging agents. The agents most studied in this respect have been vitamin E, *N,N'*-diphenyl-*p*-phenylene diamine and promethazine.

More recently the powerful free radical scavenger propyl gallate, widely used as an antioxidant in the food industry, has been found to attenuate the high liver triglyceride levels in rat liver resulting from the administration of carbon tetrachloride.[5] During studies of the action of propyl gallate and other scavengers on microsomal enzymes *in vitro* it was observed that propyl gallate strongly inhibits the activity of NADPH cytochrome *c* reductase, the first component of the NADPH-cytochrome P_{450} chain. The reductase is a flavoprotein in which the flavine prosthetic group oscillates between the fully reduced structure and the half-reduced semi-quinone free radical form.[6] In this paper we give our results of an investigation on this inhibitory property of propyl gallate.

METHODS

Female albino rats, body weight approx. 120 g, were used in this investigation. Liver tissue was removed immediately after killing rats by cervical dislocation and

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the liver was homogenized in ice-cold 0·25 M sucrose. The liver homogenate was centrifuged twice at 11,700 $g_{av.}$ for 10 min and the supernatant suspension thereby obtained was centrifuged at 157,000 $g_{av.}$ for 30 min. The clear supernatant obtained from this centrifuging step was rejected and the microsomal pellet was rinsed briefly with ice-cold 0·15 M KCl and resuspended in ice-cold 0·15 M KCl, such that 1 ml of the suspension contained the microsomes equivalent to 1 g wet wt. of liver. Protein was estimated by the colorimetric method of Lowry *et al.*[7] using crystalline bovine plasma albumin as standard. The interaction of propyl gallate and other drugs with the microsomal fraction to give a difference spectrum was studied by the method of Schenkman *et al.*,[8] using a Unicam SP800 recording spectrophotometer. NADPH-cytochrome *c* reductase was assayed by a modification of the method of Ernster *et al.*[9] The spectrophotometer cuvettes contained the following components in a volume of 3·55 ml: Tris buffer, pH 7·4, 248 μmoles; cytochrome *c*, 0·9 mg; KCN, 2 μmoles; 0·5 ml of microsome suspension freshly diluted 1:30 with ice-cold 0·15 M KCl; the reaction was started by the addition of NADPH (0·1 μmole) to one of the cuvettes. The reaction was monitored at 550 nm using a Unicam SP800 recording spectrophotometer. NADPH-neotetrazolium reductase was assayed by a small modification of the method of Slater and Sawyer[10] using neotetrazolium chloride that had been purified by the method of Jones;[11] the neotetrazolium concentration was one-half of that used previously and the incubation time at 37° was 10 min. The demethylation of amino pyrine was measured by a modification of the method of Orrenius[12] in which the NADPH generating system was replaced by glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (5·6 i.u.), and nicotinamide was replaced by acetamide (105 mM). Lipid peroxidation in the presence of ADP and ferrous ions was measured in microsomal suspensions by the method described by Slater[3] using an oxygen electrode assembly.

RESULTS

Propyl gallate strongly inhibits the demethylation of amino pyrine (Table 1). The inhibition produced by propyl gallate was apparent at 30 μM in the presence of 5 mM

TABLE 1. EFFECT OF PROPYL GALLATE ON THE DEMETHYLATION OF AMINOPYRINE BY RAT LIVER MICROSOME SUSPENSIONS

Expt.	Microsomes (ml)	Propyl gallate (μM)	Formaldehyde produced (nmoles/min/ml microsomes)
A	0·05	—	28
		34	21
		68	14
	0·10	—	29
		34	23
		68	18
B	0·20	—	20
		500	1
		1000	0

For details of estimations see the Methods.

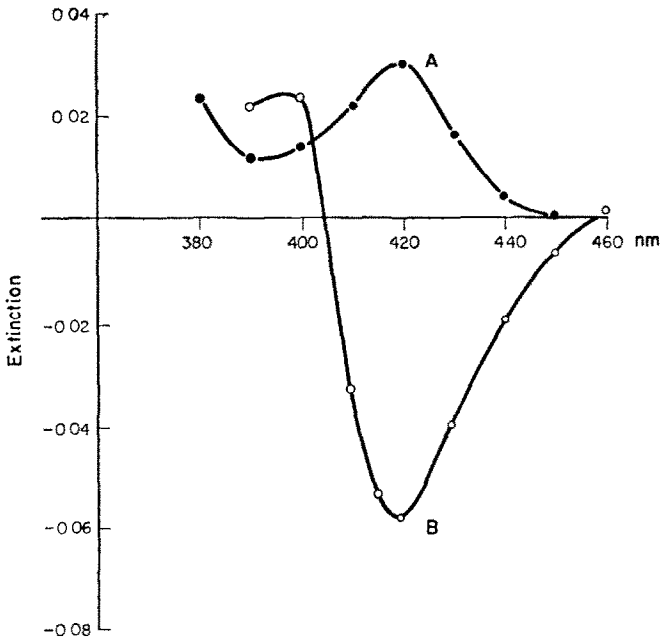

FIG. 1. Difference spectra obtained by mixing rat liver microsomes in phosphate buffer (approx. 2 mg microsomal protein/ml 0·1 M phosphate buffer, pH 7·4) with (a) propyl gallate and (b) β-diethylaminoethyl-3,3′-diphenyl propyl acetate (SKF 525A). For other details see Schenkman *et al.*[8] The final concentrations of the drugs used were (a) 6 mM; (b) 0·18 mM.

aminopyrine. This suggests that propyl gallate is not inhibiting aminopyrine metabolism by direct competition with the substrate for the cytochrome P_{450} site. However, a weak interaction of propyl gallate with cytochrome P_{450} is suggested by the demonstration of a difference spectrum of rather small intensity that was obtained by mixing propyl gallate with a microsomal suspension (Fig. 1). The maximum at 420 nm is similar to that given by Type II substances[8] and the rise in extinction below 400 nm is due to the absorption of propyl gallate itself. If propyl gallate inhibits aminopyrine metabolism by interacting with a more proximal component than cytochrome P_{450},

TABLE 2. EFFECT OF PROPYL GALLATE ON OXYGEN UPTAKE COUPLED TO LIPID PEROXIDATION IN RAT LIVER MICROSOMAL SUSPENSIONS IN THE PRESENCE OF ADP AND FERROUS IONS

Propyl gallate (μM)	Oxygen uptake (%)
0·0	100
2·0	79
3·0	73
6·1	33
61·0	5
610	1

The method used is that described by Slater.[3]

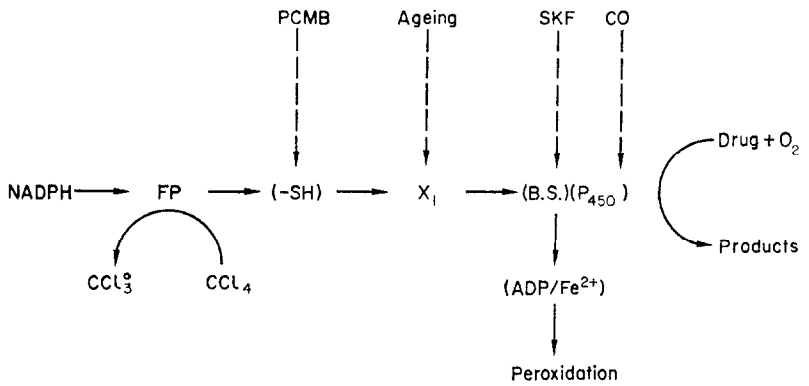

FIG. 2. Components of the microsomal NADPH-cytochrome P_{450} electron transport chain showing interaction sites of drugs undergoing metabolism at the P_{450} end, and the branch points to neotetrazolium reduction and to lipid peroxidation in the presence of ADP and ferrous ions. Abbreviations: FP, the NADPH-flavoprotein that exhibits NADPH cytochrome *c* reductase activity; X_1, rate limiting region postulated by Dallner *et al.*,[13] PCMB, *p*-chloromercuribenzoate; B.S., binding site(s) for drug interactions; CO, carbon monoxide.

it can be expected to inhibit NADPH-neotetrazolium reductase which couples with the electron transport chain in the vicinity of the rate limiting factor *X* (see Ref. 13 and Fig. 2). In fact propyl gallate (18 and 36 μM) inhibited neotetrazolium reductase by 16 and 31 per cent respectively during a 10 min incubation at 37°. Propyl gallate also strongly inhibited oxygen uptake coupled to lipid peroxidation in the presence of ADP and ferrous ions (see Table 2); approx. 5 μM propyl gallate inhibited this free

TABLE 3. EFFECT OF PROPYL GALLATE ON THE ACTIVITY OF NADPH CYTOCHROME *c* REDUCTASE IN RAT LIVER MICROSOMAL SUSPENSIONS

Expt.	Concentration of propyl gallate (μM)	NADPH-cytochrome *c* reductase activity (%)
1	Zero	100
	1·7	85
	3·3	70
2	Zero	100
	3·3	75
	6·6	59
	9·9	50
3	Zero	100
	10·0	51
	19·0	36
4	Zero	100
	7·8	76
	78·0	0
	780·0	0

For details of experimental procedure see the Methods. Four other experiments covering the same concentration range for propyl gallate were also performed and gave results consistent with those reported below.

radical mediated reaction by 50 per cent. The effect of propyl gallate on NADPH-cytochrome *c* reductase is shown in Table 3. It can be seen that propyl gallate causes a 50 per cent inhibition of the reductase at a final concentration of approx. 10 μM. The inhibition of the reductase by propyl gallate is not via a competition with NADPH; similar inhibitions of cytochrome *c* reductase were observed when the concentration of NADPH was varied between 14 and 56 μM. Variations in concentration of cytochrome *c* in the reaction mixture had little effect on the inhibitory action of propyl gallate showing that the inhibition was not due to a competition with cytochrome *c*; the concentration of cytochrome *c* was varied over the range 1·4–50·7 mg/100 ml incubation mixture.

A number of other free radical scavenging agents were studied for their inhibitory actions on NADPH cytochrome *c* reductase. Table 4 gives the results obtained. It can be seen that at concentrations of approx. 10 μM diphenyl-*p*-phenylene diamine,

TABLE 4. EFFECT OF VARIOUS FREE RADICAL SCAVENGERS ON NADPH-CYTOCHROME *c* REDUCTASE ACTIVITY IN SUSPENSIONS OF RAT LIVER MICROSOMES

Agent	Concentration (μM)	NADPH-cytochrome *c* reductase activity (%)
1. Promethazine	0	100
	6·5	110
	13·0	118
	19·5	130
	26·0	127
2. Cysteamine	0	100
	4·2	100
	8·5	115
	17·0	107
3. α-Tocopherol	0	100
	7·0	107
	14·0	103
	21·0	112
4. DPPD	0	100
	8·6	95
5. BHT	0	100
	3·5	92
	7·0	84
	14·0	85
6. Phenobarbitone	0	100
	6·6	101
	66	110

For other details see the Methods. Abbreviations: DPPD, *N'N*-diphenyl-*p*-phenylene diamine; BHT, 2,6-di-*t*-butyl-*p*-cresol; α-tocopherol, α-α-tocopherol polyethylene glycol 1000 succinate. Values are expressed as percentages of the control activities; in eleven preparations studied the control activity was 0·055 ± 0·003 μmoles cytochrome *c* reduced/min/mg microsomal protein (mean ± S.E.M.).

α-tocopherol polyethylene glycol succinate, cysteamine, and promethazine had no pronounced inhibitory effects; indeed, most of the agents studied stimulated the activity of the reductase. Butylated hydroxytoluene produced a small inhibition of NADPH-cytochrome *c* reductase when added a final concentration of 5 μM or greater. In contrast, promethazine, DPPD, and α-tocopherol polyethylene glycol 1000 succinates strongly inhibit two other microsomal reactions that involve free radical intermediates: the microsomal lipid peroxidation stimulated by ADP and ferrous ions[3] and the microsomal lipid peroxidation stimulated by low concentrations of carbon tetrachloride.[14]

DISCUSSION

Of the free radical scavengers tried, only propyl gallate decreased NADPH-cytochrome *c* reductase substantially at a concentration of approximately 10 μM. This suggests that the inhibition is not a general property of free radical scavengers. The inhibition does not appear to involve a competition between propyl gallate and either cytochrome *c* or NADPH. No alteration in the inhibitory effect of propyl gallate was observed when the agent was recrystallised from water. The mechanism of the interaction is not known.

The observation that propyl gallate decreases the activity of the NADPH cytochrome P_{450} chain by an interaction with the proximal flavoprotein is important. It can be expected that propyl gallate will thereby decrease the metabolism of all drugs that interact with the NADPH cytochrome P_{450} chain, wherever they interact with it. Most drugs, of course, interact at the P_{450} site and the metabolism of such drugs is decreased by concomitant dosing with SKF 525A. Other drugs may interact with proximal components of the chain as is the case with CCl_4, and here it may be expected that propyl gallate will be an effective inhibitor of the drug's metabolism whereas SKF 525A will be ineffective. The evidence obtained *in vivo* on this point in relation to CCl_4 is consistent with this conclusion.[5,15] Direct evidence that propyl gallate interferes with drug metabolism *in vivo* as well as *in vitro* is that it prolongs hexobarbital sleeping time in rats (M. V. Torrielli and G. Ugazio, to be published).

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