Biochem. J. (1977) **164**, 473–475
Printed in Great Britain

The Effects of Antioxidants on the Metabolism and Mutagenicity of Benzo[*a*]pyrene *in vitro*

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(*Received* 22 *February* 1977)

Antioxidants inhibit the rat liver microsomal mixed-function-oxidase-catalysed hydroxylation of benzo[*a*]pyrene. These antioxidants also decrease the formation of mutagenic products from benzo[*a*]pyrene as judged by the Ames bacterial-mutagenicity assay [B. N. Ames, J. McCann & E. Yamazaki (1975) *Mutat. Res.* **31**, 347–364]. It is suggested that antioxidants exert their protective effect against cancer by inhibiting the formation of carcinogenic metabolites.

Antioxidants are commonly added to human and animal food as preservatives for polyunsaturated lipids and other ingredients subject to spoilage by oxidation. Numerous studies indicate that these compounds produce a variety of physiological effects in animals, and their role as protective agents against the deleterious effects of various carcinogens is becoming increasingly apparent (Cumming & Walton, 1973; Grantham *et al.*, 1973; Ulland *et al.*, 1973; Speier & Wattenberg, 1974; Wattenberg, 1972, 1973, 1974, 1975). Particularly well examined are the effects of the phenolic antioxidants butylated hydroxytoluene and butylated hydroxyanisole, which are extensively used in food, and of ethoxyquin, which is widely used in commercial animal diets. Cumming & Walton (1973) found that feeding butylated hydroxytoluene to mice for 4 weeks gave significant protection against mortality caused by a variety of carcinogens. The addition of butylated hydroxytoluene and butylated hydroxyanisole to diets containing the carcinogens benzo[*a*]pyrene and dimethylbenzanthracene showed pronounced suppression of neoplasia of the forestomach in mice (Wattenberg, 1972, 1973). Similar protection has been observed with the antioxidants ethoxyquin and disulfiram (Wattenberg, 1973, 1974). Wattenberg (1975) has suggested that the decrease in cancer of the stomach in the U.S.A. might be due, at least in part, to the protective effects of the antioxidants used as food additives. In any case the actual mechanism by which the various antioxidants inhibit chemical carcinogenesis has not been determined. In mice fed with butylated hydroxyanisole under conditions that inhibit benzo[*a*]pyrene carcinogenesis, alterations in the metabolism of benzo[*a*]pyrene were found (Speier & Wattenberg, 1975). Thus incubation of benzo[*a*]pyrene and calf thymus DNA with liver microsomal fractions from butylated hydroxyanisole-fed mice showed about one-half the binding of benzo[*a*]pyrene metabolites to DNA as compared with the controls (Speier & Wattenberg, 1975). Yang *et al.* (1975) found that butylated hydroxyanisole and butylated hydroxytoluene bind to rat liver cytochrome *P*-450, produce a substrate complex with a 'type I' difference spectrum, and inhibit benzo[*a*]pyrene hydroxylation non-competitively. Cytochrome *P*-450, which exists in multiple forms, is the terminal oxidase and substrate-binding site of the mixed-function-oxidase system responsible for the oxidation of a variety of compounds including carcinogens (Conney, 1957). Other components of the system required for oxidative metabolism include NADPH, the flavoprotein NADPH–cytochrome *P*-450 reductase, phospholipid and molecular oxygen (Lu & Coon, 1968). Torrielli & Slater (1971) concluded that the site of inhibition by propyl gallate was the flavoprotein NADPH–cytochrome-*P*-450 reductase, as small amounts of the antioxidant propyl gallate inhibited NADPH–cytochrome *c* reductase activity as well as aminopyrine demethylation by rat liver microsomal fractions.

In the present study we examined the effect of various antioxidants on the flavoprotein NADPH–cytochrome *P*-450 reductase (assayed as NADPH–menadione reductase) and on benzo[*a*]pyrene hydroxylation. Since Ames and his co-workers (McCann *et al.*, 1975) have shown that there is a strong correlation between mutagenicity and carcinogenicity of chemicals, we also checked the effect of some of these antioxidants on the mutagenicity of benzo[*a*]-pyrene metabolites in *Salmonella typhimurium* strain TA 98. Our results indicate that none of the antioxidants studied (including propyl gallate) inhibits the flavoenzyme NADPH–cytochrome *P*-450 reductase, but they do inhibit benzo[*a*]pyrene hydroxylation as well as its conversion into mutagenic metabolites.

Materials and Methods

NADPH and benzo[*a*]pyrene were purchased from the Sigma Chemical Co. (St. Louis, MO,

U.S.A.). Ethoxyquin was a gift from the Monsanto Chemical Co., St. Louis, MO, U.S.A. All other chemicals and reagents were of the highest grade commercially available. *S. typhimurium* strain TA 98 was obtained from Dr. Bruce Ames (University of California, Berkeley, CA, U.S.A.). Microsomal fractions were obtained by differential centrifugation of homogenates from rat livers by established methods (Remmer *et al.*, 1967).

Benzo[a]pyrene hydroxylase

Hydroxybenzo[*a*]pyrene was measured fluorimetrically as described by Cantrell *et al.* (1976). The incubation mixture contained (in a final volume of 1.0ml): 0.25mg of microsomal protein, 100μmol of Tris/HCl (pH7.5), 1μmol of NADPH and the appropriate antioxidant when added (dissolved in water or acetone and added at the desired concentration in a volume of 10μl or less). The reaction was started by the addition of 70nmol of benzo[*a*]pyrene in 20μl of acetone and terminated 10min later by adding 0.2ml of formaldehyde (37%, v/v) followed by 1ml of 1M-NaOH. Fluorescence of 3-hydroxybenzo[*a*]pyrene was measured at 515nm with excitation at 467nm.

NADPH–cytochrome P-450 reductase

This was assayed by measuring the rate of oxidation of NADPH at 340nm in the presence of the artificial electron acceptor menadione. The reaction was carried out in 3ml spectrophotometer cells, and the reaction mixture contained (in 3ml): 0.5mg of microsomal protein, 0.6μmol of KCN, 300μmol of Tris/HCl (pH7.5), 0.3μmol of menadione in 10μl of acetone, and the appropriate antioxidant in the desired concentration. The reaction was started by the addition of 0.3μmol of NADPH to the sample cuvette.

Bacterial-mutagenicity assay

The method used for testing the mutagenicity of benzo[*a*]pyrene in *S. typhimurium* was described by Ames *et al.* (1975). Strain TA 98 requires histidine for growth and this requirement forms the basis of the assay. The bacteria were grown overnight in nutrient broth and diluted to 2×10^9 bacteria/ml with 5mM-potassium phosphate/150mM-NaCl, pH7.4. A portion (0.1ml) of this bacterial suspension was added to a mixture consisting of: microsomal protein, 0.4mg; potassium phosphate buffer (pH7.4), 100μmol; benzo[*a*]pyrene, 5μg dissolved in dimethyl sulphoxide; NADPH, 2.5μmol; antioxidants when added (dissolved in water or dimethyl sulphoxide); and Top agar (1.5ml). The final volume of the mixture was 2.5ml. The volume of dimethyl sulphoxide per plate was less than 100μl and this had no effect on strain TA 98, as was shown by Ames *et al.* (1975). After mixing, the contents were poured on to a Petri dish (100mm diam.) containing 20ml of a minimal glucose/agar medium. After 2 days the bacteria that reverted to histidine independence were counted.

Results and Discussion

The effect of several antioxidants on the NADPH-dependent menadione reduction and benzo[*a*]pyrene hydroxylation is shown in Table 1. All the antioxidants except glutathione inhibited benzo[*a*]pyrene hydroxylation, yet none of them had any effect on the flavoprotein. This indicates that the antioxidants do not inhibit benzo[*a*]pyrene hydroxylation by virtue of their effect on the flavoprotein but must do so by a more direct effect on cytochrome *P*-450. Torrielli & Slater (1971) found that propyl gallate inhibited the NADPH-dependent reduction of cytochrome *c* and the demethylation of aminopyrine in microsomal fractions, and on this basis concluded that the flavoprotein NADPH–cytochrome *P*-450 reductase was the site of inhibition. We have found that propyl gallate as well as pyrogallol, *NNN'N'*-

Table 1. *Effect of antioxidants on NADPH-dependent menadione reduction and benzo[a]pyrene hydroxylation in rat liver microsomal fractions*

Activities are expressed relative to 'None' (=100). The specific activity of NADPH–menadione reductase was 230nmol/min per mg of protein; that of benzo[*a*]pyrene hydroxylation was 1.08nmol/min per mg of protein.

Antioxidant added	Concn.	Activity: NADPH-dependent menadione reductase	Activity: NADPH-dependent benzo[a]pyrene 3-hydroxylation
None		100	100
NNN'N'-Tetramethyl-*p*-phenylenediamine dihydrochloride	20μM	101	50
Butylated hydroxy-toluene	125μM	100	72
Butylated hydroxy-anisole	25μM	105	54
	125μM	97	12
Nordihydroguariaretic acid	25μM	106	35
	125μM	97	0
Propyl gallate	25μM	102	82
	125μM	104	29
Ethoxyquin	25μM	98	35
Glutathione	1mM	102	100
Ascorbate	1mM	102	69
Pyrogallol	100μM	96	30

Table 2. *Effect of antioxidants on benzo[a]pyrene metabolism as assayed by Salmonella typhimurium (strain TA 98) mutagenicity in the presence of rat liver microsomal fraction and NADPH*

The assay was performed as described in the Materials and Methods section. The 'no microsomal fraction' control gave 20 revertants per plate, and this value has been subtracted from the values listed in the Table.

Antioxidant	Concn.	Revertants of strain TA 98 per plate
None		384
Butylated hydroxyanisole	100 μM	193
Ethoxyquin	25 μM	148
Pyrogallol	100 μM	156
Glutathione	1 mM	92

tetramethyl-*p*-phenylenediamine, nordihydroguaiaretic acid and ascorbate will reduce cytochrome *c* very rapidly in the absence of NADPH and microsomal fraction. Yang & Strickhart (1974) also reached similar conclusions with propyl gallate. Glutathione reduced cytochrome *c* slowly, whereas butylated hydroxytoluene and butylated hydroxyanisole were ineffective. This is in agreement with results of Torrielli & Slater (1971), which show that butylated hydroxytoluene and butylated hydroxyanisole do not inhibit microsomal NADPH–cytochrome *c* reductase. Thus, if a mixture of microsomal fraction, cytochrome *c* and the antioxidant were preincubated, some or all of the cytochrome *c* would be reduced. Subsequent addition of NADPH would show a partial reduction of cytochrome *c* or no reduction at all, depending on the amount of oxidized cytochrome *c* left in the cuvette at the time of addition of NADPH. When menadione is used as the electron acceptor in place of cytochrome *c*, its lower redox potential prevents it from being reduced chemically by the antioxidants. Under these conditions the antioxidants (at least at the concentrations used) have no effect on the flavoprotein.

Table 2 lists the effect of some of these antioxidants on the mutagenicity of benzo[*a*]pyrene metabolites in *S. typhimurium* strain TA 98. All of the compounds tested inhibited the mutagenicity of benzo[*a*]pyrene in strain TA 98 in the presence of microsomal fraction and NADPH. In the absence of microsomal fraction, these compounds had no effect on the growth of strain TA 98. In view of the high correlation between carcinogenicity and mutagenicity, the results in Table 2 suggest that the protective effect of these antioxidants *in vivo* may be due to their ability to inhibit the formation of carcinogenic metabolites from benzo[*a*]pyrene and other polycyclic hydrocarbons in man and animals. Glutathione was found to have no effect on benzo[*a*]pyrene hydroxylation, but it inhibited the mutagenicity of benzo[*a*]pyrene in strain TA 98. The reason for this is not clear, but it may be due to the fact that glutathione can react with toxic metabolites like epoxides to form relatively harmless glutathione conjugates, a process catalysed by a transferase (Nemoto *et al.*, 1975).

This work was supported by the National Cancer Institute of Canada.

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