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Effect of Phenolic Antioxidants on The Toxicity of Pentachlorophenol in Short-term Bacterial Bioassays

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Antioxidants have been extensively used for the prevention of lipid oxidation (TU 1980). Such widespread useage has lead to numerous toxicological studies to assess their potential hazards (CHIPAULT 1962; HALLIDAY et al. 1980; OHTA et al. 1980; SHELEF & CHIN 1980). However, little information is available concerning the interactions of antioxidants with toxic substances. In the present study, pentachlorophenol (PCP) was chosen as the test chemical to use in combination with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Pentachlorophenol is widely distributed in the environment (AHLBORG & THUNBERG 1980; GEGEFUGI et al. 1979) and has been used as a pesticide, wood preservative, and as a retardant for biodegradation of textiles. Also, it has recently been found in food products (HEIKES 1980; HEIKES & GRIFFITT 1980).

Most tests using microorganisms have been used to provide a predictive index of carcinogenicity (DE LA IGLESIA et al. 1980; OHTA et al. 1980; SALEH 1980). For instance, SHELEF & CHIN (1980) have reported an enhancement of the mutagenicity of aflatoxin B1 by low concentrations of both BHA and BHT using the Ames test. However, bacteria can also be very useful for obtaining initial toxicity information (TREVORS et al. 1981). The present study was undertaken to investigate the effects of non-toxic concentrations of BHA and BHT on the lethal toxicity of PCP using a bacterial species as the test organism.

MATERIALS and METHODS

Butylated hydroxytoluene (99%) and PCP (99+% high purity grade) were obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). Butylated hydroxyanisole (ACS grade) was acquired from BDH Chemicals Ltd. (Poole, England).

Pentachlorophenol, BHA and BHT were freshly prepared for each bioassay by separately dissolving each compound in 95% ethanol and filter-sterilizing the solution through a 0.20-μm average pore size Millipore membrane. The sterile solutions were added as 0.1-mL volumes to either resting cell suspen-

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sions or growing cells in shaken flasks. The ethanol solvent had no effect on bacterial growth or survival of the test organism.

Pseudomonas fluorescens (ATCC 11250) was used as the test organism throughout the present study. This organism has been previously used in toxicity tests with PCP (TREVORS et al. 1981). Cells were grown at 20°C with shaking at 120 rpm in 250-mL Erlenmeyer flasks containing 75 mL of nutrient broth (Difco, 4 g/L). Log phase cells were aseptically harvested by centrifugation at 8,000 x g , washed three times in sterile 150 mM phosphate buffer (pH 7), and resuspended in 17 mL of the same buffer to yield a cell density of 4×10^9 cells/mL. A set of controls received the solvent only and the test series were treated with either various concentrations of PCP combined with one of the antioxidants, or PCP alone. Another series was treated with either BHA or BHT at concentrations ranging from 10 to 50 µg/mL. Each cell suspension was incubated for 1 h at 20°C while shaking at 120 rpm. Then, 0.1 mL of the suspension was removed and serial decimal dilutions were prepared in the phosphate buffer. Viable cells were determined by the spread plate technique on nutrient agar plates. All plates were incubated at 20°C for 36 h at which time the number of viable cells were determined. Mortalities are expressed as the mean and standard error of the mean for triplicate trials and the Student's t test was used for statistical analyses of the expected and observed mortalities.

The effect of the antioxidants on the toxicity of PCP was also assessed using actively growing cells of P. fluorescens. A 250 mL Erlenmeyer flask containing 75 mL of nutrient broth was inoculated with 0.5 mL (equivalent to 6×10^8 cells/mL) of log phase cells. The controls and treatments were essentially the same as used in the toxicity tests on resting cells. The antioxidants and PCP were added as 0.1-mL quantities to the flasks which were then incubated at 20°C with shaking at 120 rpm. Growth was determined in triplicate flasks by measuring the optical density at 650 nm (OD_{650}). The EC_{50} (effective concentration that inhibited the amount of growth occurring after 12 h by 50%) was calculated using the probit analysis procedure in the SAS User's Guide (1979).

RESULTS and DISCUSSION

When the effects of non-toxic concentrations of BHA on the toxicity of PCP to the test organism were measured (Table 1), BHA at concentrations of 10 and 25 µg/mL in combination with PCP concentrations of 5 µg/mL or more was capable of significantly increasing the mortality above the expected value (i.e. the value obtained using PCP alone). When BHT was used at either 10 or 25 µg/mL in combination with PCP, the mortality of the test organism was again significantly

increased above the expected mortality, except when PCP was used at a concentration of 1 µg/mL (Table 2). When used alone, both BHA and BHT alone were non-toxic at concentrations as high as 50 µg/mL. Thus both BHA and BHT, which are related phenolic antioxidants, enhanced the toxicity of PCP.

When the effect of PCP on growing cells was determined at a concentration of 10 µg/mL, bacterial growth was not inhibited, with the cell biomass being the same as the control at the end of the 12-h incubation period (Table 3). However, when PCP was used at 75 µg/mL, there was a distinct delay in the onset of the log phase of growth as well as a marked decrease in both the rate of growth and the final cell yield. The effects of the antioxidants on growing cells were also examined and in the presence of PCP plus BHA the degree of inhibition was greater than for cells treated with only PCP (Table 3). Indeed, a PCP concentration of 75 µg/mL combined with either 10 or 25 µg/mL of BHA completely inhibited growth. Actively growing cultures of the test organism were less sensitive to BHT plus PCP than to BHA plus PCP. The EC_{50} values for PCP plus 10 µg/mL of BHA, PCP plus 25 µg/mL of BHA, PCP plus 10 µg/mL of BHT, and PCP plus 25 µg/mL of BHT were 38, 28, 54, and 43 µg/mL, respectively. Pentachlorophenol at 75 µg/mL with 10 µg/mL of BHA or BHT inhibited growth 100% and 88.2%, respectively, suggesting that even relatively low concentrations of either antioxidant were capable of increasing the toxic effect of PCP.

The enhancement of the toxicity of PCP by BHA and BHT is of considerable significance. It has been reported that these two antioxidants can increase the mutagenicity of aflatoxin B1 (SHELEF & CHIN 1980). On the other hand, a protective effect of BHA and BHT with a variety of carcinogens has been found (SULLIVAN et al. 1978). In addition, BHA can evoke a group of reactions which have the overall effect of increasing detoxification of foreign chemicals (WATTENBURG 1979). These particular antioxidants have also been used as food additives (JOHNSON 1971).

The actual mechanism by which the antioxidants enhanced PCP toxicity in the present study is not known. It is possible that they affected PCP uptake into the cells by changing membrane permeability or function. The toxic effects of PCP itself have been attributed to its ability to uncouple oxidative phosphorylation (AHLBORG & THUNBERG 1980). In addition to this uncoupling effect, PCP is an inhibitor of the terminal enzyme P-450 in liver microsomes. However, regardless of the mechanism of the toxic enhancement, the results obtained using the short-term bacterial bioassay system show a toxic interaction. The interaction of antioxidants with PCP in other test systems, such as animals, should probably be examined.

TABLE 1

Effect of BHA on the toxicity of PCP to *P. fluorescens*.

Treatment (µg/mL)			
PCP	BHA	Expected mortality (%)	Observed mortality (%)
0.0	0.0	0.0	0.0
0.0	10.0	-	0.0
0.0	25.0	-	0.0
0.0	50.0	-	0.0
1.0	0.0	-	0.0
5.0	0.0	-	0.0
10.0	0.0	-	0.0
25.0	0.0	-	14.4 ± 2.2
35.0	0.0	-	30.1 ± 1.5
50.0	0.0	-	73.9 ± 3.8
1.0	10.0	0.0	0.0
5.0	10.0	0.0	52.1 ± 2.2 *
10.0	10.0	0.0	90.2 ± 1.1 *
25.0	10.0	14.4 ± 2.2	91.6 ± 0.1 *
35.0	10.0	30.1 ± 1.5	91.7 ± 1.2 *
50.0	10.0	73.9 ± 3.8	95.9 ± 0.1 *
1.0	25.0	0.0	0.0
5.0	25.0	0.0	51.5 ± 1.5 *
10.0	25.0	0.0	99.9 ± 0.1 *
25.0	25.0	14.4 ± 2.2	98.9 ± 0.2 *
35.0	25.0	30.1 ± 1.5	98.8 ± 0.1 *
50.0	25.0	73.9 ± 3.8	98.9 ± 0.2 *

* Significant difference between expected and observed (p = 0.05)

TABLE 2

Effect of BHT on the toxicity of PCP to *P. fluorescens*.

Treatment (µg/mL)			
PCP	BHT	Expected mortality (%)	Observed mortality (%)
0.0	0.0	0.0	0.0
0.0	10.0	-	0.0
0.0	25.0	-	0.0
0.0	50.0	-	0.0
1.0	0.0	-	0.0
5.0	0.0	-	0.0
10.0	0.0	-	0.0
25.0	0.0	-	14.4 ± 2.2
35.0	0.0	-	30.1 ± 2.5
50.0	0.0	-	73.9 ± 3.8
1.0	10.0	0.0	0.0
5.0	10.0	0.0	70.1 ± 1.1 *
10.0	10.0	0.0	82.6 ± 1.4 *
25.0	10.0	14.4 ± 2.2	87.1 ± 2.7 *
35.0	10.0	30.1 ± 1.5	95.8 ± 0.1 *
50.0	10.0	73.9 ± 3.8	96.9 ± 0.1 *
1.0	25.0	0.0	0.0
5.0	25.0	0.0	46.7 ± 1.6 *
10.0	25.0	0.0	51.5 ± 2.6 *
25.0	25.0	14.4 ± 2.2	77.9 ± 0.2 *
35.0	25.0	30.1 ± 1.5	95.8 ± 0.1 *
50.0	25.0	73.9 ± 3.8	91.2 ± 0.1 *

* Significant difference between expected and observed ($p = 0.05$)

TABLE 3

Effects of PCP, and combinations of PCP, BHA and BHT on the growth of *P. fluorescens*.

Treatment (µg/mL)			OD_{650} *	
PCP	BHA	BHT	after 12 h	Percent inhibition
0.0	0.0	0.0	0.85 ± 0.01	control
10.0	0.0	0.0	0.85 ± 0.01	0.0
25.0	0.0	0.0	0.72 ± 0.03	15.3
35.0	0.0	0.0	0.70 ± 0.02	17.7
50.0	0.0	0.0	0.62 ± 0.04	27.1
75.0	0.0	0.0	0.30 ± 0.05	64.7
0.0	10.0	0.0	0.85 ± 0.02	0.0
0.0	25.0	0.0	0.86 ± 0.01	0.0
0.0	0.0	10.0	0.85 ± 0.07	0.0
0.0	0.0	25.0	0.85 ± 0.03	0.0
10.0	10.0	0.0	0.66 ± 0.05	22.4
25.0	10.0	0.0	0.59 ± 0.04	30.6
35.0	10.0	0.0	0.56 ± 0.02	34.1
50.0	10.0	0.0	0.32 ± 0.01	62.4
75.0	10.0	0.0	0.0	100.0
10.0	25.0	0.0	0.52 ± 0.06	38.8
25.0	25.0	0.0	0.42 ± 0.01	51.0
35.0	25.0	0.0	0.39 ± 0.01	54.1
50.0	25.0	0.0	0.17 ± 0.02	80.0
75.0	25.0	0.0	0.0	100.0
10.0	0.0	10.0	0.72 ± 0.02	15.3
25.0	0.0	10.0	0.72 ± 0.01	17.7
35.0	0.0	10.0	0.69 ± 0.03	18.8
50.0	0.0	10.0	0.62 ± 0.02	28.2
75.0	0.0	10.0	0.10 ± 0.02	88.2
10.0	0.0	25.0	0.72 ± 0.01	15.3
25.0	0.0	25.0	0.70 ± 0.01	17.7
35.0	0.0	25.0	0.68 ± 0.01	20.0
50.0	0.0	25.0	0.19 ± 0.04	77.7
75.0	0.0	25.0	0.09 ± 0.01	89.4

* mean ± S.E.M. (n=3)

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