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Registry No. 2-Chloro-4-(isopropylamino)-6-[(carboxypentyl)amino]-*s*-triazine, 98849-84-4; atrazine, 1912-24-9; cyanuric chloride, 108-80-5; isopropylamine, 75-31-0; aminocaproic acid, 1319-82-0.

Halogenated Hydrocarbon and Hydroperoxide Induced Lipid Peroxidation in Rat Tissue Slices

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Damage to biological systems exposed simultaneously to more that one toxicant may be amplified over damage produced by individual toxicants. Thiobarbituric acid reactive substances (TBARS) were measured in rat tissue slices to investigate lipid peroxidation induced by halogenated hydrocarbons (HHC), *tert*-butyl hydroperoxide (BHP), or a combination of the two types of toxicant. TBARS production by liver slices incubated with 1 mM HHC occurred in the following order: $BrCCl_3$ > CCl_4 = $C_2H_2Br_2$ > $C_2H_4Cl_2$; 1 mM $C_2H_2Br_4$, $C_2H_2Cl_4$, CH_2Br_2, CH_2Cl_2, or $C_2HBrClF_3$ or 0.05 mM BHP did not increase TBARS production. TBARS production by liver slices incubated with BHP was synergistic in combination with $BrCCl_3$, CCl_4, $C_2H_4Br_2$, $C_2H_4Cl_2$, $C_2H_2Br_4$, $C_2H_2Cl_4$, or $C_2HBrClF_3$, depending upon concentrations used. A synergistic effect of BHP and $BrCCl_3$ was shown in heart slices, but not in kidney, testes, lung, or spleen slices. Synergism between the HHC and BHP was dependent upon the HHC, oxidant concentrations, and the individual tissue. This dependency may reflect the mechanisms by which these compounds are metabolized in the individual tissues as well as other tissue variables that affect lipid peroxidation.

Many halogenated hydrocarbons (HHC) are found in drinking water (Ronsen, 1980) and in food products (Moon et al., 1986), and lipid hydroperoxides can develop in many food products (Chan, 1987). Various HHC and organic peroxides are powerful inducers of lipid peroxidation in tissues. Lipid peroxidation and its associated reactions are closely associated with tissue damage. Recent reviews (Reynolds and Moslen, 1980; Brattin et al., 1985) show the complexity of HHC stimulation of lipid peroxidation and tissue damage even when reactions are limited to those initiated by carbon tetrachloride (CCl_4) and related HHC. Among hydroperoxide oxidants, *tert*-butyl hydroperoxide (BHP) and cumene hydroperoxide have been studied the most (Sies, 1985). Investigations by Weiss

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Table I. Production of TBARS by Liver Slices Incubated with HHC, BHP, or HHC plus BHP

halogenated hydrocarbon	TBARS,[a] nmol MDA equiv/mL medium per 100 mg slices			synergism,[b] % change in TBARS	
	1 mM HHC	1 mM HHC + 0.05 mM BHP	1 mM HHC + 0.2 mM BHP	1 mM HHC + 0.05 mM BHP	1 mM HHC + 0.2 mM BHP
$BrCCl_3$	2.48 ± 0.54	3.81 ± 0.15	4.99 ± 0.52	52.4[c]	27.6[d]
CCl_4	1.16 ± 0.32	1.56 ± 0.28	3.78 ± 0.63	32.2[e]	45.9[d]
$C_2H_4Br_2$	1.15 ± 0.25	1.66 ± 0.33	5.02 ± 1.59	41.8[f]	94.6[d]
$C_2H_4Cl_2$	0.53 ± 0.15	0.73 ± 0.09	3.37 ± 0.39	32.7[d]	71.9[e]
$C_2H_2Br_4$	0.41 ± 0.08	0.45 ± 0.04	3.51 ± 0.24	4.7	90.8[e]
$C_2H_2Cl_4$	0.40 ± 0.05	0.46 ± 0.05	2.80 ± 0.35	9.5	53.0[e]
CH_2Br_2	0.35 ± 0.08	0.43 ± 0.03	2.02 ± 0.76	16.2	13.4
CH_2Cl_2	0.39 ± 0.08	0.49 ± 0.09	1.70 ± 0.23	19.5	-6.6
$C_2HBrClF_3$	0.41 ± 0.10	0.47 ± 0.07	2.41 ± 0.40	9.3	31.0[e]

[a] Control liver slices, 0.34 ± 0.08; liver slices + 0.05 mM BHP, 0.36 ± 0.05; and liver slices + 0.2 mM BHP, 1.77 ± 0.45. Reported values are means ± SD (n = 3–16). [b] The formula for calculation of synergism is given in Materials and Methods. [c] $P < 0.001$. [d] $P < 0.01$. [e] $P < 0.05$. [f] $P < 0.02$.

and Estabrook (1986) illustrate the information needed to gain a perspective of hydroperoxide-dependent lipid peroxidation mechanisms. Since exposure to toxicants in the environment and workplace may involve more than one toxicant, there is need for simple assays that show biological responses to a combination of toxicants such as halogenated hydrocarbons and hydroperoxides.

A series of studies (Gavino et al., 1984; Sano et al., 1986; Fraga et al., 1987–1989; Fraga and Tappel, 1988) on the effects of some toxicants as inducers of lipid peroxidation in tissue slices has been carried out in this laboratory. These studies have shown that, as biological samples, tissue slices offer some advantages over subcellular components and single cells. A number of HHC induce lipid peroxidation, and the amounts of thiobarbituric acid reactive substances (TBARS) produced by rat liver slices incubated with some of these HHC correlate inversely with the LD_{50} of these HHC for the rat (Fraga et al., 1987). BHP also stimulates lipid peroxidation in slices of a number of rat tissues. Bromotrichloromethane ($BrCCl_3$) induced lipid peroxidation in tissue slices was shown by production of pentane and ethane (Gavino et al., 1984) and TBARS (Gavino et al., 1984; Sano et al., 1986). The use of tissue slices to evaluate toxicants could be an important advance, since decreasing the number of experimental animals used in research is a very important current issue (Holden, 1986; Cartmill, 1986). When treated with either $BrCCl_3$ or BHP, tissue slices released TBARS into the medium (Sano et al., 1986). Studies of lipid peroxidation resulting from mixed toxicants for which the mechanisms of the individual toxicants are not well-known are necessarily empirical and not mechanistic. The present study continued investigations of lipid peroxidation induction in tissue slices, and the combined effects of HHC and BHP are reported.

MATERIALS AND METHODS

Animals and Preparation of Tissue Slices. Male Sprague–Dawley rats weighing 400–430 g were fed a standard rat diet. In our experience, the tissues of rats in the weight range of 100–450 g have responded similarly to lipid peroxidation inducers so far tested. The choice of the larger animals for these studies provided more tissue so that fewer animals were required. The rats were decapitated, and tissues were removed, rinsed, and immersed in ice-cold Krebs–Ringer phosphate buffer. Liver, kidney, heart, and spleen were cut into 0.5-mm slices with a Stadie-Riggs tissue slicer. Lung and testes were sliced with a razor blade. Two or three slices of tissue (80–90 mg) were placed into a 15-mL serum bottle with 5 mL of Krebs–Ringer phosphate buffer/10 mM glucose equilibrated with 100% oxygen.

Chemicals and Incubation Procedure. $BrCCl_3$ and CCl_4 were purchased from Eastman Kodak Co., Rochester, NY, and 1,2-dibromoethane ($C_2H_4Br_2$), 1,2-dichloroethane ($C_2H_4Cl_2$), 1,1,2,2-tetrabromoethane ($C_2H_2Br_4$), 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$), halothane ($C_2HBrClF_3$), dichloromethane (CH_2Cl_2), and dibromomethane (CH_2Br_2) were purchased from Aldrich Chemical Co., Milwaukee, WI. BHP was from Polysciences, Inc., Warrington, PA. These HHC were chosen as representative of good initiators of lipid peroxidation. Data for lipid peroxidation initiated by other HHC, including chloroform, are available in a previous publication (Fraga et al., 1987). The HHC and BHP were diluted with dimethyl sulfoxide and Krebs–Ringer phosphate buffer, respectively. Immediately prior to incubation of the slices, 3-μL portions of HHC and/or BHP solutions were added. Equivalent amounts of solvents added to controls did not increase production of TBARS. Incubations were done at 37 °C for 2 h in a shaker–water bath with a rotation rate of 120 cycles/min. Following incubation, the tissue slices wer removed, the medium was centrifuged at 12000g for 20 min, and the supernatant was immediately analyzed for TBARS.

Analysis of TBARS. TBARS were analyzed fluorometrically with some modifications to the method described by Yagi (1976). A 0.5-mL aliquot of centrifuged medium was mixed with 0.5 mL of 3% sodium dodecyl sulfate, followed by addition of 2 mL of 0.1 N HCl, 0.3 mL of 10% phosphotungstic acid, and 1 mL of 0.7% 2-thiobarbituric acid (Sigma Chemical Co., St. Louis, MO). The mixture was heated in boiling water for 45 min and then cooled in ice. TBARS were extracted into 5 mL of 1-butanol. The tubes were briefly centrifuged, and TBARS were measured spectrophotofluorometrically at 515-nm excitation and 555-nm emission. 1,1,3,3-Tetramethoxypropane, which converts quantitatively to malonaldehyde (MDA), was used as a standard, and TBARS were expressed as MDA equivalents per milliliter of medium per 100 mg of tissue.

Analysis of Data. Synergism is defined as the cooperative action of discrete components to produce a total effect that is greater than the sum of the individual effects. The synergistic effects of HHC and BHP on induction of lipid peroxidation were calculated from the mean values as percent increase in TBARS = [([HHC + BHP] - [control]/[BHP] - [control] + [HHC] - [control]) - 1] × 100%, which simplifies to [([HHC + BHP]/[BHP] - [control] + HHC]) - 1] × 100%. Within the brackets were the amounts of TBARS produced by tissue slices treated with the indicated compounds. The significance of differences between data for single inducers and the controls was determined by application of Student's t-test. Determination of the significance of synergism (percent change in TBARS) between groups was determined by two-way analysis of variance for the mean values.

RESULTS

Production of TBARS by Liver Slices. Table I shows that liver slices incubated with 1 mM $BrCCl_3$, CCl_4, $C_2H_4Br_2$, or $C_2H_4Cl_2$ produced more TBARS than did control slices ($p < 0.001$). The order of potency as peroxidation inducers was $BrCCl_3 > CCl_4 = C_2H_4Br_2 > C_2H_4Cl_2$. $C_2H_2Br_4$, $C_2H_2Cl_4$, CH_2Br_2, CH_2Cl_2, $C_2HBrClF_3$, each at 1 mM, and BHP at 0.05 mM were not peroxidation initiators under the test conditions used. There was a 5.2-fold increase in TBARS when 0.2 mM BHP was

Table II. Production of TBARS by Various Tissues Incubated with HHC plus BHP

halogenated hydrocarbon	tissue	TBARS,[a] nmol MDA equiv/mL medium per 100 mg slices				synergism,[b] % change in TBARS: 1 mM HHC + 0.2 mM BHP
		control	0.2 mM BHP	1 mM HHC	1 mM HHC + 0.2 mM BHP	
$BrCCl_3$	kidney	0.24 ± 0.06	0.98 ± 0.26	1.78 ± 0.30	2.40 ± 0.31	-4.8
	heart	0.22 ± 0.08	1.24 ± 0.08	0.49 ± 0.11	1.87 ± 0.32	23.8[c]
	testes	0.22 ± 0.04	0.75 ± 0.16	0.29 ± 0.02	0.89 ± 0.14	8.5
	lung	0.35 ± 0.08	0.58 ± 0.05	0.50 ± 0.07	0.81 ± 0.03	11.0
	spleen	0.45 ± 0.06	0.80 ± 0.25	0.96 ± 0.30	1.27 ± 0.20	-3.1
$C_2H_4Br_2$	kidney	0.20 ± 0.08	0.86 ± 0.18	0.68 ± 0.15	2.09 ± 0.26	56.0[d]
	heart	0.18 ± 0.04	1.09 ± 0.05	0.26 ± 0.05	1.42 ± 0.34	21.4
	testes	0.20 ± 0.06	0.87 ± 0.12	0.30 ± 0.06	1.19 ± 0.46	22.7
	lung	0.28 ± 0.08	0.44 ± 0.10	0.33 ± 0.10	0.60 ± 0.26	22.4
	spleen	0.32 ± 0.03	0.60 ± 0.18	0.68 ± 0.06	1.02 ± 0.32	6.2

[a] Values reported are means ± SD (n = 2–8). [b] The formula for calculation of synergism is given in Materials and Methods. [c] $P < 0.05$. [d] $P < 0.001$.

added to liver slices. Table I reports a synergistic effect of 0.05 mM BHP with either 1 mM $BrCCl_3$, CCl_4, $C_2H_4Br_2$, or $C_2H_4Cl_2$ on the production of TBARS. There was also a synergistic effect of 0.2 mM BHP with all of the 1 mM HHC tested except for CH_2Br_2 and CH_2Cl_2.

Production of TBARS by Other Tissues. The respective nanomoles of TBARS ± SD produced per milliliter per 100 mg of kidney slices incubated with 1 mM $BrCCl_3$, CCl_4, $C_2H_4Br_2$, $C_2H_4Cl_2$, $C_2H_2Br_4$, $C_2H_2Cl_4$, CH_2Br_2, CH_2Cl_2, and $C_2HBrClF_3$ were, with number of samples given in parentheses, 1.78 ± 0.30 (8), 0.84 ± 0.07 (5), 0.94 ± 0.25 (4), 0.41 ± 0.08 (4), 0.31 ± 0.08 (4), 0.28 ± 0.02 (4), 0.34 ± 0.02 (3), 0.32 ± 0.04 (3), and 0.39 ± 0.05 (4). Of these compounds, only $BrCCl_3$, CCl_4, $C_2H_4Br_2$, and $C_2H_4Cl_2$ significantly ($p < 0.01$) stimulated production of TBARS above control values of 0.2–0.24 (Table II). Table II shows the amount of TBARS produced by kidney, heart, testes, lung, and spleen incubated with combinations of 0.2 mM BHP with 1 mM $BrCCl_3$ or 1 mM $C_2H_4Br_2$. Significantly more TBARS were produced by all the tissues incubated with 0.2 mM BHP than by control tissues. Both $BrCCl_3$ and $C_2H_4Br_2$ induced production of TBARS by kidney and spleen slices. There was a synergistic effect of $BrCCl_3$ and BHP in heart slices but not in kidney, testes, lung, or spleen slices. There was also a synergistic effect of 1 mM $C_2H_4Br_2$ and 0.2 mM BHP on the productions of TBARS by kidney slices.

Dependence of TBARS Production on HHC Concentration. Liver slices were incubated with 0.2 mM BHP and 0.2–1.2 mM $BrCCl_3$, $C_2H_2Br_4$, or $C_2H_4Br_2$, the three HHC that exhibited the greatest synergism with BHP (Figure 1). The synergistic effect of $BrCCl_3$ with BHP was greatest with low concentrations of $BrCCl_3$. With $C_2H_2Br_4$ and $C_2H_4Br_2$, the synergistic effect increased with increasing concentrations of the HHC. While 0.2–1.2 mM $C_2H_2Br_4$ did not initiate a significant amount of lipid peroxidation, when combined with 0.2 mM BHP the effect on TBARS production was synergistic at 1 and 1.2 mM $C_2H_2Br_4$.

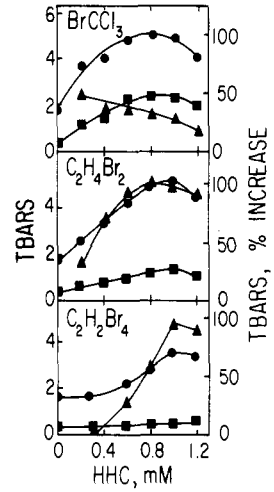

Figure 1. Effect of *tert*-butyl hydroperoxide (BHP) and various concentrations of halogenated hydrocarbons (HHC) on release of thiobarbituric acid reactive substances (TBARS) from liver slices. Liver slices were incubated for 2 h with $BrCCl_3$, $C_2H_4Br_2$, or $C_2H_2Br_4$. TBARS produced by (■) HHC alone or (●) HHC plus 0.2 mM BHP. (▲) Percentage increase in TBARS produced when HHC was added together with BHP. Points are the means of data from two to four experiments.

DISCUSSION

The use of whole animals for study of the complex processes of lipid peroxidation may make interpretation of results on specific organ responses difficult because of the interaction of the circulatory, respiratory, digestive, and other physiological systems. Tissue homogenates and subcellular fractions as models have provided useful information on damage to biological systems; however, the integrated functions of intact tissue are missing in these models. A tissue slice system is intermediate in complexity between strictly physiological and biochemical systems. Useful information relevant to damage to intact organs should be obtained in tissue slices undergoing oxidative or peroxidative challenge since the slices maintain much of their integrity for a matter of hours. A tissue slice model can also provide information on differences in susceptibility of a number of individual organs to oxidant damage. Because a number of slices can be prepared from a single organ, a reduction in the numbers of animals required is a potential asset for this model as compared with animal testing of toxicants where a whole animal is required per treatment.

TBARS are widely used as a measure of lipid peroxidation in biological materials. TBARS measured in slices of tissues in earlier studies (Gavino et al., 1984) were shown to correlate with other products of lipid peroxidation, namely ethane and pentane. Further, results obtained with use of this tissue slice assay system were shown to be in agreement with those obtained with use of microsomal and homogenate systems (Fraga et al., 1988).

The concentration of HHC used in these studies was chosen because the concentration of 1 mM $BrCCl_3$ produced the maximum amount of TBARS in the tissue slice assay system (Sano et al., 1986). A linear TBARS output was stimulated by $BrCCl_3$ over the concentration range of 10 μM to 1 mM. Concentrations of 0.05 and 0.2 mM BHP were chosen because these concentrations pro-

duced less than one-fourth of the maximum amount of TBARS for this initiator, and thus these concentrations were approximated as suitable for testing for a synergistic response in TBARS production. BHP stimulated approximately a linear TBARS output in the liver slice system for the BHP concentration range 0.05–2 mM (Sano et al., 1986).

Among the HHC tested as lipid peroxidation inducers in tissue slices, $BrCCl_3$ and CCl_4 were the most potent and dihalomethanes and tetrahaloethanes were ineffective or weak initiators. The relative potencies of the various HHC for production of TBARS by tissue slices were approximately the same as observed by measurement of MDA production in microsomes (Slater and Sawyer, 1971); pentane production by rats (Sagai and Tappel, 1979); TBARS, ethane, and pentane production by liver slices (Gavino et al., 1984); and TBARS production by tissue slices (Fraga et al., 1987, 1988). These comparisons show that TBARS produced by tissue slices reflect the potency of the HHC for induction of lipid peroxidation in various biological systems.

The dihaloethanes $C_2H_4Br_2$ and $C_2H_4Cl_2$ significantly stimulated the release of TBARS from liver slices. Dihaloethanes are mutagenic (Rannug et al., 1978) and carcinogenic (International Agency for Research on Cancer, 1979). These compounds also induce liver and kidney lesions after long-term inhalation by animals (Nitschke et al., 1981). The bioactivation of dihaloethanes may involve formation of GSH conjugates by GSH *S*-transferase (van Bladeren et al., 1979), aldehydes such as bromoacetoaldehyde (Hill et al., 1978), or radicals (Tomasi et al., 1985) mediated by the cytochrome P-450 enzyme system. The activated products may bind to nucleic acid or protein (van Bladeren et al., 1980; Sundheimer et al., 1982; White et al., 1984). Since TBARS (MDA and lipid peroxides) cause cleavage of double-stranded DNA (Inouye, 1984; Ueda et al., 1985) and have been reported to initiate and promote carcinogenesis (Ames, 1983; Shamberger et al., 1974), dihaloalkane-induced lipid peroxidation may play an important role in these reactions.

Some of the compounds used in mixtures, $BrCCl_3$ plus BHP and $C_2H_4Br_2$ plus BHP, are among the most potent known inducers of lipid peroxidation in tissues. The synergistic effect of BHP with dihalo- and tetrahaloethanes was greater with bromo-substituted compounds than with chloro-substituted compounds, which is consistent with the relative strength of the bond dissociation energies of carbon–halogen bonds (Br < Cl). BHP might accelerate dehalogenation with resultant formation of free radicals, or the reaction might be carried out by direct activation of BHP or mediation of the cytochrome P-450 system. Involvement of the cytochrome P-450 pathway may be supported by the finding that synergistic effects were greatest in tissues that have been reported to have high microsomal enzyme activities (Benedetto et al., 1981).

Although 1 mM tetrahaloethanes caused the production of only small amounts of TBARS by liver slices, when they were added with 0.2 mM BHP TBARS, production was synergistic. A similar synergistic effect was observed in rat liver slices incubated with tetrahaloethanes, where the release of total ethane and pentane was greater from vitamin E depleted liver slices than from control slices (Gavino et al., 1984).

The synergistic effects of $BrCCl_3$ with BHP were similar to those of some haloethanes with BHP. The activation of $BrCCl_3$ is easily accomplished in the absence of a peroxide, since the dissociation energy of the C–Br bond is small (Walling, 1957). The difference in synergistic effects of the various HHC with BHP may be attributed to the magnitude of the dissociation energies of the individual HHC (Burdino et al., 1973).

This study has shown that some halogenated hydrocarbons that are widely used as industrial chemicals, solvents, fumigants, and lead scavengers in gasoline have a synergistic effect with BHP on the production of TBARS from liver slices. Assay of TBARS in the medium in which liver slices are incubated with hepatotoxic chemicals is a useful means to evaluate the capacity of these chemicals to initiate lipid peroxidation. $BrCCl_3$-induced high-level lipid peroxidation in heart tissue was reported previously (Sagai and Tappel, 1979). Major variables among the tissues include polyunsaturated lipid content, activity of activator systems such as cytochrome P-450, inducer concentration and structure, antioxidant content, and yield of TBARS per peroxidation step. Some of these factors have been discussed by Dianzani and Ugazio (1978). Studies of mixed toxicants as inducers of lipid peroxidation need to be viewed as exploratory research. The results of this study showed the interesting and important phenomenon of synergism, which cannot yet be explained adequately. The mechanisms by which individual toxicants induce lipid peroxidation are not well-known; thus, studies of the effects of mixtures of toxicants are empirical rather than mechanistic. Mechanistic explanations will require further research. Although the mechanisms of synergism are not understood, it is significant that damage induced by certain toxicants in the environment may be synergistically enhanced in the presence of other toxicants. Since millions of tons of hundreds of types of chemicals are pumped into the atmosphere and dumped onto the land and into waterways from industrial and agricultural sources, concern over enhanced and combined effects of toxicants and potential toxicants on living systems is justified.

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Registry No. BHP, 75-91-2; $BrCCl_3$, 75-62-7; CCl_4, 56-23-5; $C_2H_4Br_2$, 106-93-4; $C_2H_4Cl_2$, 107-06-2; $C_2H_2Br_4$, 79-27-6; $C_2H_2Cl_4$, 79-34-5; CH_2Br_2, 74-95-3; CH_2Cl_2, 75-09-2; $C_2HBrClF_3$, 151-67-7.

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