Development of Enzyme Immunoassay for the Detection of Triazine Herbicides

Bohn Dunbar,[†] Bruce Riggle,*[,†,‡] and Gordon Niswender[§]

Northern Plains Area, Central Great Plains Research Station, USDA—ARS, P.O. Box 400, Akron, Colorado 80720, and Colorado State University, Ft. Collins, Colorado 80523

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. Antisera were obtained from rabbits immunized with a conjugate of caproic acid–atrazine [2-chloro-4-(isopropylamino)-6-[(carboxypentyl)amino]-*s*-triazine] and bovine serum albumin (BSA). The assay's detection limit for atrazine was 0.1 ng/mL. Propazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine] and azidoatrazine [2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] were 87 and 58% cross-reactive, respectively, while simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine] and ametryn [2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine] were 10 and 7% cross-reactive, respectively. Four triazine compounds, cyanazine [2-chloro-4-[(1-cyano-1-methylethyl)amino]-6-(ethylamino)-*s*-triazine], dichloroatrazine [2,4-dichloro-6-(isopropylamino)-*s*-triazine], hexazinone [3-cyclohexyl-6-(dimethylamino)-1-methyl-*s*-triazine-2,4-(1*H*,3*H*)-dione], and metribuzin [4-amino-6-*tert*-butyl-3-(methylthio)-*as*-triazin-5(4*H*)-one] were not cross-reactive. Of five atrazine metabolites, only deethylatrazine [2-chloro-4-amino-6-(isopropylamino)-*s*-triazine] was cross-reactive at 6%, while hydroxyatrazine [2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], deisopropylatrazine [2-chloro-4-amino-6-(ethylamino)-*s*-triazine], diaminoatrazine [2-chloro-4,6-diamino-*s*-triazine], and cyanuric acid [trihydroxytriazine] were not cross-reactive. Eight non-triazine pesticides were assayed, and none were cross-reactive.

Immunoassay procedures have been developed for a number of pesticides (Ercegovich et al., 1981; Fatori and Hunter, 1980; Gee et al., 1988; Newsome and Shields, 1981; Wie and Hammock, 1982; Wie et al., 1982; Newsome, 1985; Dreher and Podratzki, 1988; Wing et al., 1978). In addition, enzyme-linked immunosorbent assays (ELISA) have been developed for atrazine and other triazine herbicides (Bushway et al., 1988; Huber, 1985; Huber and Hock, 1985). Bushway et al. (1988) developed an assay for atrazine using a hapten/protein conjugate that was prepared by first derivatizing atrazine at the chloro position and then covalently attaching it to BSA. Huber (1985) developed an assay for atrazine and ametryn using a hapten/protein conjugate that was prepared by first converting ametryn to ametryn sulfoxide and then attaching it to hemocyanin. Ametryn is similar in structure to atrazine except for a methylthio group in place of the chloro group. Huber and Hock (1985) developed an assay for terbutryn using a hapten/protein conjugate that was prepared by first converting terbutryn to terbutryn sulfoxide and then attaching it to BSA. The triazine herbicide assays cited above showed cross-reactivity for a number of different triazine compounds.

Triazine herbicides such as atrazine, propazine, simazine, ametryn, and cyanazine have been used for a number of years and provide a relatively cost-effective and efficient means of controlling many different grass and broadleaf weeds. Because Federal and State regulations require pesticide residue analysis, there is a need to expand all available options for analysis. Currently, triazine residues are analyzed by either gas chromatography (Lee and Chau, 1983; Sirons et al., 1973) or high-performance liquid chromatography (Vermeulen et al., 1982; Vickrey et al., 1980). While these methods are accurate, they can also be time-consuming and expensive. The demand for more cost-effective methods that are also efficient, rapid, and dependable may be partially filled by the successful use of enzyme-linked immunoassays. The purpose of this study was to develop an ELISA for the identification and quantification of atrazine using a hapten with a carboxylic group that was used to form an amide linkage with protein.

MATERIALS AND METHODS

Reagents and Equipment. Triazine herbicides and metabolites were provided by Ciba-Giegy Corp. (Greensboro, NC). Dichloroatrazine was provided by Shell Chemical Co. (Modesto, CA). BSA, rabbit serum albumin (RSA), Tween-20, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) diammonium salt (ABTS), *N*,*N*-dimethylformamide (DMF), and Freund's complete adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO). Biotinylated goat anti-rabbit IgG and horseradish peroxidase–streptavidin conjugate were obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals were of reagent grade or better and were used as obtained. Buffers were prepared as follows. Carbonate/bicarbonate buffer solution: Na_2CO_3 (1.59 g), $NaHCO_3$ (2.93 g), and distilled water (1 L). Phosphate-buffered saline solution (PBS): NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g), KH_2PO_4 (0.2 g), $CaCl_2$ (0.1 g), $MgCl_2$ (0.1 g), and distilled water (1 L). Citrate/phosphate buffer: 27.8 mL of citrate buffer (citrate (960 mg) and distilled water (50 mL)), 22.2 mL of phosphate buffer (Na_2HPO_4 (2.68 g) and distilled water (50 mL)), and 50 mL of distilled water. All stocks and standard solutions were stored at 4 °C. Proton magnetic resonance (^{1}H NMR) spectral analysis was done with a Bruker ACE-300 operating at 300 MHz. Infrared spectral (IR) analysis was done with a Nicolet 5PC FTIR spectrometer. Mass spectral analysis was done with a VG micromass 16F mass spectrometer. ELISA plates (Immulon 2) were obtained from Dynatech (Torrance, CA). Absorbance readings for plates were done with a Model MR600 microplate reader from Dynatech.

Hapten Preparation. The hapten, 2-chloro-4-(isopropylamino)-6-[(carboxypentyl)amino]-*s*-triazine, was designed to produce antibodies against atrazine and is referred to as caproic acid–atrazine. The hapten was prepared as follows: Cya-

[†] USDA—ARS.

[‡] Current address: ICI Americas Inc., 1200 South 47th St., Box 4023, Richmond, CA 94804.

[§] Colorado State University.

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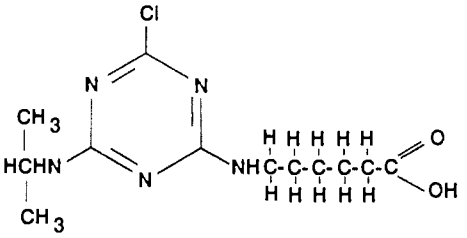


caproic acid atrazine

nuric chloride (5 g) was dissolved in toluene (100 mL), and then 60 mM NaOH (8 mL) was added. This was followed by the addition of 50 mM isopropylamine (3 mL). The mixture was stored for 1 h at approximately 20 °C, and the pH was kept between 11 and 12 with 60 mM NaOH. This was followed by the addition of 50 mM aminocaproic acid (6.56 g) dissolved in 5 M NaOH (10 mL). The pH was maintained between 11 and 12 with dilute NaOH, and the mixture was continually stirred. Toluene was removed by reduced pressure on a rotary evaporator, the residue was dissolved in H_2O (50 mL), and the pH was adjusted to 3 with dilute HCl. The resulting precipitated acidic material was filtered off and washed with H_2O to remove NaCl. The yield was 13.5 g. A portion of the material (2 g) was dissolved in ethyl acetate and eluted on a silica gel column with ethyl acetate–hexane (80:20). Eluted material was dried by a rotary evaporator. Residue was recrystallized from the solvent two times with chloroform and one time with acetone. The yield was 900 mg. A portion of the purified material was placed on thin-layer chromatographic silica gel plate and developed in ethyl acetate–hexane (80:20). A single spot with R_f 0.65 was found by UV absorption, which was followed by iodine vapor. The hapten structure was confirmed by ^1H NMR, IR, and MS analyses: ^1H NMR ($CDCl_3$, DMF, from TMS) δ 1.2 (d, 6 H, $(CH_3)_2$), 1.4 (m, 2 H, $CH_2CH_2CH_2COOH$), 1.6 (m, 4 H, $NHCH_2CH_2CH_2CH_2$), 2.3 (t, 2 H, CH_2COOH), 3.4 (t, 2 H, $NHCH_2$), 4.2 (m, 1 H, $CH(CH_3)_2$), 6.6 (d, 1 H, $NHCH(CH_3)_2$), 6.8 (t, 1 H, $NHCH_2$), 11.7 (br s, COOH); IR (KBr) 3250 (NH str), 2960, 2900, 2850 (CH aliphatic str), 1698 (C=O str), 1628, 1602, 1576 (C=N str) cm^{-1}; MS (70 eV) m/e (relative intensity) of 58 (100), 68 (37), 91 (30), 96 (72), 158 (50), 172 (35), 186 (27), 187 (33), 200 (86), 214 (35), 228 (26), 242 (78), 286 (26), 301 (15) (parent ion), 302 (4), 303 (4), 304 (1); mp 165–166 °C. Anal. Calcd for $C_{12}H_{20}N_5O_2Cl_5$: C, 47.76; H, 6.68; N, 23.21; Cl, 11.75. Found: C, 47.89; H, 6.89; N, 22.97; Cl, 11.89.

Conjugate Preparation. The hapten was conjugated to BSA and RSA by a mixed-anhydride method. A 150-mg portion of the 0.5 mM hapten was dissolved in dry DMF (7 mL) and 1.0 mM tributylamine (240 μL). To this mixture was added 0.5 mM isobutyl chloroformate (66 μL). The reaction was allowed to proceed at 5–10 °C in an ice bath for 30 min. This mixture was then added to a stirred cooled solution of BSA or RSA (1 g) in H_2O (3.6 mL), 1 N NaOH (1 mL), and DMF (20 mL). The resulting mixture was continually stirred for 30 min. The solution was adjusted to pH 8 with NaOH and brought to room temperature. The solution was dialyzed against running water for 72 h and then adjusted to pH 4 with dilute HCl. The solution was centrifuged at 500g, and the resulting precipitate was washed with cold acetone. The precipitate was suspended in water and redissolved by NaOH to pH 7.8. The material was dialyzed against running water for 8 h and then lyophilized. The yield was approximately 900 mg. The calculated ratio (w/w) of hapten to protein was approximately 7:1.

Antibody Preparation. Anti-atrazine antibody preparation was as follows. Three New Zealand rabbits were intradermally inoculated with the hapten/BSA conjugate (500 μg) and complete Freund's adjuvant, boosted at monthly intervals, and bled from the ears on a weekly basis after the second injection. Approximattely 30 mL/animal of whole blood was collected and centrifuged to separate blood cells from serum. The titer was determined from sera from two rabbits. Cross-reactivity was determined from serum from one rabbit.

Plate-Coating Procedure. A stock solution of hapten/RSA conjugate was prepared by first sonicating the hapten/RSA conjugate (900 mg) in DMF (1 mL) for 1 min and then adding carbonate/bicarbonate buffer solution (50 mL) (pH 9.5). The hapten/RSA conjugate solution was added at 200 μL/well to a 96-well polystyrene microtiter plate. The plate was covered with parafilm and stored at 4 °C for a minimum of 12 h. Plates were washed two times with PBS that contained Tween-20 (TW). Sites not occupied by hapten/RSA conjugate were blocked by 1% gelatin in PBS at 900 μL/well for 2 h after which plates were washed two times with PBS/TW.

Titer Determination. Rabbit antisera titers were determined as follows. Serial dilutions of sera in PBS were prepared at 100 μL/well and allowed to attach to the hapten/RSA conjugate for 2 h at 37 °C. Plates were then washed two times with PBS/TW. Biotinylated goat anti-rabbit IgG at a 1:1000 dilution in PBS/TW was added at 100 μL/well and allowed to attach to rabbit serum for 2 h at 37 °C. The plate was washed two times with PBS/TW. Streptavidin peroxidase at a 1:1000 dilution in PBS/TW was added at 100 μL/well and allowed to attach to the biotin portion of the biotinylated goat antibody for 2 h at 37 °C. The plate was washed two times with PBS/TW. Substrate consisting of ABTS (100 mg) in 0.03 M citrate/phosphate buffer (21 mL) (pH 4.4) and 30% H_2O_2 (2 μL) was added at 100 μL/well and kept at room temperature. No stopping agent was used. After a 10-min incubation period, the plate was measured at 410 nm (Amersham protocol) on a microplate spectrophotometer.

Immunoassay for Atrazine. The same procedure for determining serum titers was used, but with two modifications. A solution of atrazine was added at 100 μL/well. Diluted rabbit antiserum (dilution established from titer determination) was then immediately added at 100 μL/well. Both compound and antiserum were incubated together for 45 min before the plate was washed with PBS/TW. The remainder of the assay was the same as the titer determination. As more atrazine was added to the well, it was attached to antibody and then removed by washing. As a consequence, less antibody was available for attachment to the hapten/RSA conjugate. The amount of streptavidin-peroxidase present to react with substrate was inversely proportional to the amount of atrazine added to the well. Atrazine was assayed at dilutions of 0.1, 1, 10, 100, 1000, and 10 000 ng/mL. Other triazine compounds (Tables I and II) were assayed in the same manner. Stock solutions of triazine compounds were made with distilled water and 2% DMF. Non-triazine (Table III) compounds were assayed at a 10 000 ng/mL dilution.

Antibody Specificity. Antibody specificity was determined by measuring the cross-reactivity of compounds structurally similar to atrazine. The cross-reactivity of a compound was determined as [moles of atrazine at the assay midpoint]/[moles of structurally related compound at the assay midpoint] × 100 = % cross-reactivity. Cross-reactivity tests were done on rabbit 3 serum. Cross-reactivity of the hapten, caproic acid–atrazine, was not measured.

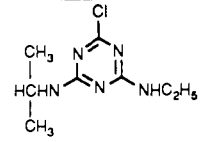
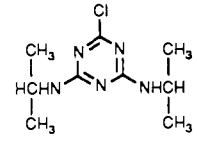
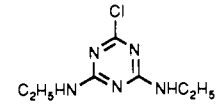
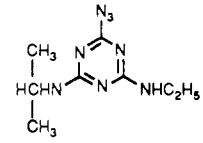
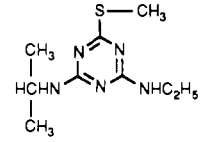
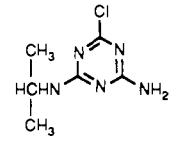
RESULTS AND DISCUSSION

Antiserum. Rabbits immunized with the caproic acid–atrazine/BSA conjugate produced antibodies against atrazine. Rabbit 3 antiserum, which had been collected 6 months after immunization, was used in the assay as described in this report.

Titer Results. Antisera binding capability to the hapten/RSA conjugate was demonstrated (Figure 1). Antisera dose–responses were similar for rabbits 1 and 3. The antisera titer, which is the dilution required for 50% of maximum absorbance, was 1:5000 for both rabbits. Preimmune sera showed no antibody response and was identical for both rabbits. Results showed that no antibodies against atrazine were present in either rabbit at the time of immunization.

RSA, Gel, and BSA Responses. RSA and gel were not cross-reactive (not shown in a table) to rabbit 3 antiserum. This demonstrated that antibodies did not attach to the RSA portion of the hapten/RSA conjugate and did not attach to the gel block. However, BSA was 100%

Table I. Triazine Compounds with Cross-Reactivities at 1000 ng/mL or Less

name	chemical name	formula	cross reactivity, %
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine		100
propazine	2-chloro-4,6-bis(isopropylamino)-*s*-triazine		87
simazine	2-chloro-4,6-bis(ethylamino)-*s*-triazine		10
azidoatrazine	2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine		58
ametryn	2-ethylamino-4-(isopropylamino)-6-(methylthio)-*s*-triazine		7
deethylatrazine	2-chloro-4-amino-6-(isopropylamino)-*s*-triazine		6

cross-reactive (not shown in a table). This high cross-reactivity was due to antibody attachment to BSA. This was an expected result since a hapten/BSA conjugate was used for immunization.

Assay and Cross-Reactivity Results. The assay's limit of detection for atrazine with rabbit 3 antiserum was 0.1 ng/mL. This was similar to some earlier findings. Huber (1985) detected atrazine at 1.1 ng/mL with rabbit antiserum developed with an ametryn sulfoxide hapten, while Bushway et al. (1988) detected atrazine at 0.1 ng/mL with rabbit antiserum developed using a 2-chloro position derivatized atrazine hapten.

Of all the other triazine compounds assayed, propazine was the most cross-reactive (Table I). However, propazine cross-reactivity results varied somewhat from those of earlier reported studies. Bushway et al. (1988) reported almost identical cross-reactivity for both atrazine and propazine, while Huber (1985) reported only half as much cross-reactivity for propazine as compared to atrazine.

Results for other triazine herbicides were as follows. Simazine was one-tenth as cross-reactive as atrazine, while ametryn was less than 10% cross-reactive. Both Huber (1985) and Bushway et al. (1988) reported cross-reactivity for both simazine and ametryn. However, cyanazine, hexazinone, metribuzin, and dichloroatrazine were not cross-reactive (Table II). Bushway et al. (1988) reported cross-reactivity with cyanazine and hexazinone but not with metribuzin.

Of the metabolites assayed (Tables I and II), only deethylatrazine was cross-reactive, while hydroxyatrazine, deisopropylatrazine, diaminoatrazine, and cyanuric acid were not cross-reactive (Table II). Bushway et al. (1988) reported cross-reactivity for both deethylatrazine and hydroxyatrazine.

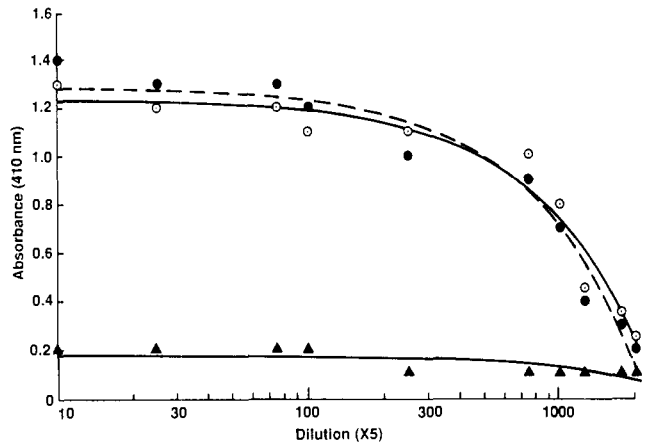


Figure 1. Dose–responses of rabbit serums to the hapten/RSA conjugate as measured by an enzyme-linked color change: (⊙) rabbit I; (●) rabbit 3; (▲) preimmune.

None of the assayed non-triazine compounds (Table III), which represented a wide spectrum of pesticide chemistry, were cross-reactive.

Comparison Analysis. Comparison analysis of compound structures showed that a combination of the chloro and isopropylamino groups resulted in the highest cross-reactivity values. Both atrazine and propazine had this combination. Substitution of the isopropyl group with an ethyl group, as demonstrated by simazine, resulted in a major loss of cross-reactivity. Removal of the ethyl group, as demonstrated by deethylatrazine, also resulted in a major loss of cross-reactivity. Removal of both the isopropyl and ethyl groups, as demonstrated by diaminoatrazine, resulted in a complete loss of cross-reactivity. Sub-

Table II. Triazine Compounds with No Cross-Reactivities at 10 000 ng/mL or Less

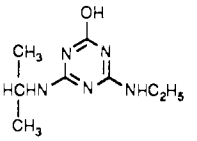
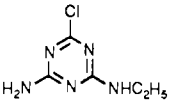
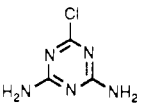
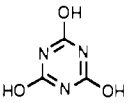
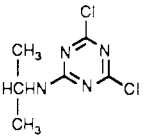
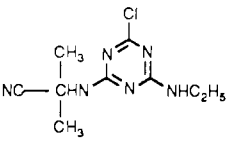
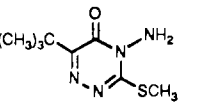
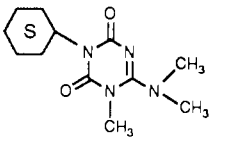
name	chemical name	formula
hydroxyatrazine	2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine	
deisopropylatrazine	2-chloro-4-amino-6-(ethylamino)-*s*-triazine	
diaminoatrazine	2-chloro-4,6-diamino-*s*-triazine	
cyanuric acid	trihydroxytriazine	
dichloroatrazine	2,4-dichloro-6-(isopropylamino)-*s*-triazine	
cyanazine	2-chloro-4-[(1-cyano-1-methylethyl)amino]-6-(ethylamino)-*s*-triazine	
metribuzin	4-amino-6-*tert*-butyl-3-(methylthio)-*as*-triazin-5(4*H*)-one	
hexazinone	3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione	

Table III. Non-Triazine Compounds with No Cross-Reactivities at Concentrations of 10 μg/mL or Less

name	chemical name
trifluralin	2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine
butylate	*S*-ethyl diisobutylthiocarbamate
glyphosate	*N*-(phosphonomethyl)glycine
pichloram	4-amino-3,5,6-trichloropicolinic acid
alachlor	2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide
cinmethylin	*exo*-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1]heptane
fluazifop	(±)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid
paraquat	1,1′-dimethyl-4,4′-bipyridinium ion

stitution of the chloro group with a hydroxy group, as demonstrated by hydroxyatrazine, resulted in a complete loss of cross-reactivity. This may have been the result of the acidic nature of the triazine hydroxy moiety. Substitution of the chloro group with a methylthio group, as demonstrated by ametryn, resulted in a major loss of cross-reactivity. However, substitution of the chloro group with an azido group, as demonstrated by azidoatrazine, resulted in only a moderate reduction of cross-reactivity. The high level of cross-reactivity for azidoatrazine suggests that the molecular configuration of the azido group was similar to that of the chloro group with respect to antibody recognition.

Azidoatrazine has been used as a ^{14}C-labeled marker in locating the chloroplast protein to which atrazine binds (Pfister et al., 1981; Steinback et al., 1981). Assay results suggest that antiserum in conjunction with azidoatrazine could be used to locate the atrazine site of action in chloroplasts.

Conclusions. The assay could not discriminate between atrazine and propazine nor could it discriminate between low concentrations of atrazine/propazine (0.1–1 ng/mL) and higher concentrations of simazine, ametryn, and deethylatrazine (100–1000 ng/mL). However, the assay's ability to detect low concentrations of atrazine/propazine could increase its potential usefulness as a rapid screening method for both herbicides. The requirement for higher concentrations of simazine, ametryn, and deethylatrazine makes the assay impractical for screening these three compounds. A significant finding was that hydroxyatrazine was not cross-reactive as was the case with the assay reported by Bushway et al. (1988).

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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

Mitsuaki Sano and Al L. Tappel*

Department of Food Science & Technology, University of California, Davis, California 95616

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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