Mechanisms producing lethal cell injury by bromobenzene were explored in primary cultures of rat hepatocytes. Cells from phenobarbital-pretreated males were killed by bromobenzene from 0.1 to 0.7 mM. Cell death was preceded by a decline in glutathione and was prevented by SKF 525A. Cell killing was also reduced by promethazine, 1α-tocopherol acetate, butylated hydroxytoluene, N, N'-diphenyl-p-phenylenediamine (DPPD), cysteine, N-acetylcysteine, α-mercaptopropionyl glycine, and β-mercaptoethanol. After 18–20 h, cell death was proportional to the extent of the covalent binding of [14C]bromobenzene to total cellular proteins in all cases. The time course, however, of the accumulation of covalently bound metabolites did not correlate with the time course of the cell death. Furthermore, the antioxidant DPPD prevented the cell death for 8–10 h without reducing the extent of covalent binding. Cell death during the first 6–10 h was accompanied by lipid peroxidation assessed by accumulation of malondialdehyde and by the appearance of conjugated dienes in cellular phospholipids. DPPD as well as the other protective agents reduced this lipid peroxidation in proportion to the reduction of the cell death. The data are consistent with two mechanisms of lethal cell injury in bromobenzene-intoxicated hepatocytes. The first is responsible for the death of cells during the first 10 h and is probably based on the peroxidative decomposition of phospholipids. The second kills the cells with a slower time course and is unrelated to lipid peroxidation. The extent of the cell killing by this second mechanism is proportional to the extent of covalent binding of bromobenzene metabolites to total proteins. However, the extent of covalent binding cannot be a direct measure of the mechanism producing lethal cell injury. The data define, therefore, conditions with which the peroxidation of lipids and the covalent binding of reactive metabolites may and, in turn, may not be related to the genesis of irreversible cell injury in bromobenzene hepatotoxicity.

The mechanisms whereby chemicals lethally injure cells remain a central concern of biochemical toxicology. This problem has been pursued largely by following the intracellular fate of particular chemicals. Such an approach has been applied to a variety of toxins acting on a number of cell systems. The results have been generally quite consistent and have produced two major conclusions. First, most toxic chemicals are not biologically active but must be converted to reactive metabolites usually by the target cell itself. Target cell specificity and the actual extent of cell injury is, in turn, dependent upon this metabolism. Second, the critical metabolites exert their biological action by covalently binding to cellular macromolecules.

Cellular injury mediated by the interaction of chemically reactive metabolites with macromolecules has been invoked to account for the liver cell death produced by a wide variety of xenobiotic chemicals and drugs (for a recent review see Ref. 1). The evidence to support such a role for covalent binding is largely circumstantial and based on the persistent correlation between the extent of binding and the severity of the accompanying liver cell necrosis. There is little direct evidence to substantiate the hypothesis that covalent binding to macromolecules can produce cell injury that results in necrosis. In particular, the molecular targets which interact with reactive metabolites and lead to cell death have not been identified. In addition, the functional consequences of such interactions between the chemical toxin and key cellular targets have rarely been considered.

We have begun to study the mechanism(s) mediating the bromobenzene killing of cultured hepatocytes in the attempt to define the cellular events linking the metabolism of chemical toxins with their biological consequences. Much of the evidence implicating the covalent binding of reactive metabolites to the subsequent cell death is based on bromobenzene-induced liver necrosis in intact rats (2–7). Previous studies have shown that isolated hepatocytes metabolize bromobenzene and that this metabolism can result in lethal cellular injury (8–11).

In the present study monolayer cultures of hepatocytes were utilized rather than short term suspensions of these cells. Using this system data are presented establishing that the killing of cultured hepatocytes reproduces the essential features of the toxicology of bromobenzene in the intact animal. A number of ways were then defined of protecting the hepatocytes from the cell killing by bromobenzene. The relationship between cell death and the covalent binding of bromobenzene was assessed with each protective agent. Conditions are described with which the killing of the hepatocytes could be dissociated from the covalent binding of metabolites of...
to the cells at a final concentration of viable cells, the cultures were rinsed once with prewarmed Hanks’ dead cells. The cultures were then incubated in complete lactic dehydrogenase (14) was assayed either by trypan blue exclusion (13) or by the release of aldehydes (Philadelphia). It was dissolved in culture medium and added to the cultures at the concentrations indicated in the text. SKF was added to the cultures at the times indicated in the text. SKF was the generous gift of the Smith Kline and French Laboratories, Inc. Male rats pretreated with phenobarbital were given sodium phenobarbital by intraperitoneal injections (80 mg/kg body weight/day) for 5 days. All animals were fasted overnight prior to use.

Isolated hepatocytes were prepared by collagenase perfusion according to Seglen (12). Yields of 2–4 × 10⁶ cells/liver with 85–90% viability (trypan blue) were routinely obtained. The hepatocytes were plated in sterile plastic 25-cm² flasks (Corning) at a density of 10,000 cells/cm² or in 75-cm² flasks (Corning) at a density of 50,000 cells/cm² in Williams’ E medium (Flow Laboratories, Inc.) containing 10% heat-inactivated (56 °C for 10 min) fetal calf serum (Grand Island Biological Co.), 10 IU/ml of penicillin, 10 μg/ml of streptomycin, and 0.02% of fetal bovine serum. Williams’ E plus serum, penicillin, streptomycin, and insulin is referred to below as complete Williams’. After incubation in an atmosphere of 5% CO₂-95% air for 2 h to allow the attachment of viable cells, the cultures were rinsed once with prewarmed Hank’s balanced salt solution (Flow Laboratories, Inc.) to remove unattached dead cells. The cultures were then incubated in complete William’s with the additions indicated in the text. Viability of the cultured cells was assayed either by trypan blue exclusion (13) or by the release of lactic dehydrogenase (14) as described previously.

Bromobenzene (Aldrich) was diluted in dimethyl sulfoxide and added to the cultures at the concentrations indicated in the text. SKF 525A was the generous gift of the Smith Kline and French Laboratories (Philadelphia). It was dissolved in culture medium and added to the cells at a final concentration of 1 × 10⁻⁵ M. 1-Cysteine (Sigma), N-acetyl-1-cysteine (Calbiochem-Behring), and α-mercapto- pionyl-1-glycine (Calbiochem-Behring) were dissolved in 0.9% NaCl and added to the cultures at a final concentration of 4, 2.5, and 4 μM, respectively. α-Tocopherol acetate (Sigma), butylated hydroxytoluene (Sigma), butylated hydroxyanisole (Sigma), and N,N’-diphenyl-p-phenylenediamine (Eastman) were dissolved in dimethyl sulfoxide and added to the cultures at a final concentration of 32, 0.9, and 1 μM, respectively. Promethazine (Eliks-Sinn, Inc.) was obtained as a 25 mg/ml solution, diluted with 0.9% saline, and added to the cultures at a final concentration of 10 μg/ml.

GSH was determined by the fluorometric method of Hissin and Hilf (15). The covalent binding of [¹⁴C]bromobenzene to protein was measured after incubating the cells for the times indicated in the text with a total of 1 × 10⁶ cpm of bromo-[¹⁴C]benzene (5.8 mCi/mmol, Amersham Corp.). Except for the concentration dependence study (Fig. 5), the final concentration of [¹⁴C]bromobenzene was always 0.5 μM. At the end of the incubation, the medium was aspirated and 5 ml of 10% trichloroacetic acid were added. The hepatocytes were scraped, the suspension was recovered by centrifugation, and the proteins were prepared according to Rao and Recknagel (16). The dry protein residue was weighed in tared scintillation vials, dissolved in 1 ml of NCS tissue solubilizer (Amersham Corp.) and counted in 15 ml of a toluene-based solution.

Malondialdehyde was measured fluorimetrically by adaptation of the method of Yagi (17). At the times indicated, 0.5 ml of the culture medium was removed for lactic dehydrogenase determination, and 500 μl of 0.5% trichloroacetic acid was added to achieve a final concentration of 5%. The cells and medium were recovered, homogenized with a Polytron (Brinkmann Instruments), and centrifuged for 30 min at 35,000 rpm in a 50ti rotor of a Beckman ultracentrifuge. To 2 ml of the clear supernatant were added 2 ml of 0.6% 2-thiobarbituric acid (Sigma). The mixture was stirred and then boiled for 10 min. The colored product was extracted by adding 1.6 ml of 1-butanol (Fisher), mixing, and centrifuging at maximum speed in a tabletop centrifuge for 15 min. To 1 ml of the butanol extract was added an additional 1 ml of butanol. The fluorescence of this extract was read in a Perkin-Elmer MPF 43A spectrofluorimeter at 515 nm excitation and 553 nm emission. Standards were prepared by diluting malondialdehyde bis(dimethylacetate (98%+, Aldrich) 1:1000 in methanol. The resulting solutions were mixed 1:1 with 0.2 M HCl and left overnight at room temperature. 1.65 ml was added to 8.35 ml of methanol. Appropriate aliquots of this solution containing 1.25 to 5 nmol of malondialdehyde were added to 4.5 ml of complete Williams’ medium and treated as the regular cultures. In all cases, the excitation and emission spectra of the experimental samples were identical with that of the standards.

RESULTS

Bromobenzene-induced Liver Cell Death—Freshly isolated hepatocytes were plated in plastic flasks and allowed to attach for 2 h prior to treatment with bromobenzene. Hepatocytes from male or female rats were insensitive to 0.5 mM bromobenzene over the course of a 20-h exposure. When male rats were pretreated for 3 days with phenobarbital (80 mg/kg body weight/day), their cultured hepatocytes were now sensitive to bromobenzene. Fig. 1 illustrates the time course of the cell killing by 3 different doses of bromobenzene. The viability of the cells was assessed by the exclusion of trypan blue (left panel) and, in the same flasks, by the release of lactic dehydrogenase into the culture medium (right panel). Trypan blue exclusion was shown previously to correlate with the plating efficiency of hepatocytes and with their ability to fluorescently.
following hydrolysis of fluorescein diacetate (13). Fig. 1 documents that release of lactic dehydrogenase similarly correlates with the ability to exclude trypan blue. The two assays gave virtually identical results. In contrast, the loss of intracellular K⁺ or the content of ATP can be dissociated from the ability to exclude trypan blue or the release of lactic dehydrogenase. ¹ The loss of K⁺ or the ATP content, therefore, do not necessarily reflect viability of cultured hepatocytes and were not used in the present study.

As shown in Fig. 1, the viability of control cells (closed circles) was greater than 90% of the initial value after 20 h. Bromobenzene reduced the viability of the cells in a time- and dose-dependent manner. While there was some variability among cell preparations, most of the cell killing generally occurred by 6-8 h with only a slight increase in the number of dead cells between 8 and 20 h. Fig. 2 summarizes the relationship between the extent of cell killing and the concentration of bromobenzene.

The following data suggest that sensitivity to bromobenzene is a consequence of its enhanced metabolism. Hepatocytes isolated from phenobarbital-treated males have an increased content of cytochrome P-450. Within 30 min, bromobenzene reduced the GSH content in sensitive hepatocytes to only 25% of the initial level (Fig. 3). There were no differences in the initial content of GSH in hepatocytes from untreated male rats, and bromobenzene had no effect on their GSH concentration. The cytochrome P-450-dependent mixed function oxidase of the liver endoplasmic reticulum is inhibited by the phenothiazine SKF 525A (21). Liver cell death in the intact phenobarbital-pretreated rat is also prevented by SKF 525A (22). Similarly, addition of 10 μM SKF 525A to the culture medium prevented the bromobenzene-induced killing of the cultured hepatocytes (Fig. 4).

The above data are consistent with previous investigations employing suspensions of isolated hepatocytes (8-11). Cultured hepatocytes reproduce the major features of the toxic effect of bromobenzene in the intact animal. Sensitivity to bromobenzene in culture is dependent upon prior induction of cytochrome P-450 by phenobarbital administration to the intact animal. Bromobenzene depletes induced hepatocytes of GSH prior to the appearance of cell death, and the cell killing can be prevented by an inhibitor of mixed function oxidase activity, SKF 525A.

The experimental strategy employed in this study was to alter the reaction of cultured hepatocytes to bromobenzene in a number of ways and to then assess the effect of such manipulations on the cell killing and the covalent binding of metabolites of [¹⁴C]bromobenzene. In addition to SKF 525A, two broad classes of chemicals, sulphydryl compounds and antioxidants, were found to be effective in reducing the extent of the killing of cultured hepatocytes by bromobenzene. The specific compounds and their actions are detailed below. We note, however, that including SKF 525A a number of different chemicals were thus available with which to perturb the reaction of cultured hepatocytes to bromobenzene. The effect of these agents on the covalent binding of [¹⁴C]bromobenzene to cellular macromolecules was examined next.

Cell Death and the Covalent Binding of [¹⁴C]Bromobenzene—Cultured hepatocytes were incubated with [¹⁴C]bromobenzene at concentrations from 0.1 to 0.7 mM. After 20 h,

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Fig. 2. Dose dependence of the killing of cultured hepatocytes by bromobenzene. Cultured hepatocytes from phenobarbital-induced male rats were treated with increasing concentrations of bromobenzene, and the viability of the cells was determined after 18 h by the content of lactic dehydrogenase in the medium. Results are the mean ± S.D. of the determinations on 3 separate flasks.

Fig. 3. GSH content of bromobenzene-treated hepatocytes. Hepatocytes from phenobarbital-induced male rats were treated with 0.5 mM bromobenzene (○). Control cells (●) received dimethyl sulfoxide alone. At the times indicated, the GSH content of the cells was measured as described under "Materials and Methods."

Fig. 4. Prevention by SKF 525A of the bromobenzene killing of cultured hepatocytes. Hepatocytes from phenobarbital-induced males were treated with 0.5 mM bromobenzene (A), 0.5 mM bromobenzene plus 10 μM SKF 525A (●) or with dimethyl sulfoxide alone (○). At the times indicated, the percentage of viable cells was determined by the lactic dehydrogenase content of the medium. Results are the mean ± S.D. of the determinations on 3 separate flasks.

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D. R. Petrovich and J. L. Farber, unpublished results.
the extent of cell killing was measured by the lactic dehydrogenase content in the medium, and the covalent binding of \([^{14}C]\)bromobenzene to total cellular proteins was determined. In this manner, cell killing and the extent of covalent binding were quantitated in the same cultures. The dose dependence of the cell killing was essentially as illustrated in Fig. 2. The dose dependence of the covalent binding to protein illustrated in Fig. 5 closely paralleled that of the cell killing. The amount of covalent binding of \([^{14}C]\)bromobenzene rose sharply with increasing concentration to about 0.3 mM. The rate of increase then declined with a plateau reached in the extent of binding between 0.5 and 0.7 mM bromobenzene.

Fig. 6 illustrates a representative time course of the covalent binding to protein with 0.5 mM \([^{14}C]\)bromobenzene. The numbers in parentheses are the percentage of dead cells at the respective times. The greater proportion of the total covalent binding occurred within 2 h of exposing the cells to bromobenzene. At this time, however, only about 10% of the cells were dead. This represented only about 20% of the total number of cells that died by the end of the experiment. Between the 2nd and the 18th hours, the covalent binding increased only very slightly. It was during this time, however, that 80% of the cell death occurred.

The insensitivity of uninduced hepatocytes from male or female rats was reflected in very little covalent binding (Table I). Each sulfhydryl compound reduced the extent of covalent binding in proportion to the degree of protection against the killing (Table I). Reduction in covalent binding with each of the compounds listed in Table I was observed as early as 30 min after exposure to \([^{14}C]\)bromobenzene. The percentage decrease in covalent binding was then constant between 30 min and 18 h. In Fig. 7 are plotted the data from Fig. 5 and Table I against the viability of the cells determined at 20 h by the release of lactic dehydrogenase into the culture medium. A calculated correlation coefficient of 0.90 emphasizes the close correlation between the two sets of measurements.

The antioxidants vitamin E and promethazine reduced the extent of both covalent binding and cell death in a manner similar to the sulfhydryl compounds (Table I). At all times between the addition of \([^{14}C]\)bromobenzene and the last time point at which binding and cell death were quantitated (18–10 h later), both measurements were reduced proportionately by vitamin E and promethazine. There was no dissociation between covalent binding and cell death with these two antioxidants as with the sulfhydryl compounds.

The antioxidants butylated hydroxyanisole, butylated hydroxytoluene, and DPPD differed. With these agents there was dissociation to a variable extent between the covalent binding of \([^{14}C]\)bromobenzene metabolites in bromobenzene-intoxicated hepatocytes and the death of the hepatocytes. DPPD was the most striking in its ability to dissociate such a dissociation, and we concentrate in what follows on it. The data in Table II compare with two different doses of bromobenzene (0.25 and 0.5 mM) the effect of 1 mM DPPD on the time course of the cell killing and covalent binding. For up to 10 h after treatment with bromobenzene, DPPD had a very significant protective effect on the extent of cell killing by both doses of bromobenzene. In contrast, DPPD had no
Malondialdehyde with that of the death of the cells during malondialdehyde in the cultures and by the appearance of covalent binding and cell death in the DPPD-protected cells. The data in Figs. 5 and 6 and Table I were plotted against the extent of cell killing determined by the lactic dehydrogenase content in the medium from the same flasks.

Fig. 7. Correlation between the covalent binding of bromobenzene metabolites and the extent of cell killing. The data in effect on the extent of covalent binding with the low dose of bromobenzene and actually increased the amount of such binding with the larger dose of bromobenzene. After 18 h, DPPD no longer prevented the cell killing with either dose of bromobenzene. These data suggest that the cell killing by bromobenzene for up to 10 h is not necessarily a consequence of the extent of covalent binding of reactive metabolites as assessed by the binding of [14C]bromobenzene to total cellular proteins.

Lipid Peroxidation in Bromobenzene-intoxicated Hepatocytes—Since DPPD is a potent antioxidant, its ability to prevent cell death between 0 and 10 h could result from an inhibition of lipid peroxidation. Such an action would then explain the lack of a correlation during these times between covalent binding and cell death in the DPPD-protected cells. The presence of lipid peroxidation in bromobenzene-intoxicated hepatocytes was demonstrated by the accumulation of malondialdehyde in the cultures and by the appearance of conjugated dienes in isolated cellular phospholipids.

Fig. 8 compares the time course of the accumulation of malondialdehyde with that of the death of the cells during the first 6 h after treatment of the hepatocytes with 0.5 mM bromobenzene. The data document a close temporal association between accumulation and cell death. This association was further documented by examination of the effect of the protective agents on the formation of malondialdehyde. Table III compares the cell death and the formation of malondialdehyde in cells treated with bromobenzene and four different protective agents including DPPD. Prevention of cell death was accompanied by prevention of malondialdehyde formation in each case. The presence of any of the sulfhydryl protective compounds in the tissue culture medium interfered with the malondialdehyde assay. The effect of these compounds on lipid peroxidation was examined by their influence on the formation of conjugated dienes in cellular phospholipids.

When lipids containing fatty acid dienes or polyenes are peroxidized, there is a shift in double bond position leading to conjugation. This involves initial abstraction of H- from a doubly allylic position followed by a double bond migration. Conjugated dienes result which show an intense absorption at 233 nm. The presence of conjugated dienes has been widely used for detection and estimation of lipid peroxidation in liver cell injury by hepatotoxic agents. Fig. 9 illustrates the absorp-

**TABLE II**

**Dissociation by DDPD of cell death from the covalent binding of [14C]bromobenzene metabolites**

| Time  | DPPD   | 0.25 mM Bromobenzene | 0.5 mM Bromobenzene |
|-------|--------|----------------------|---------------------|
|       | µM     | Cell death* | Covalent binding† | Cell death | Covalent binding |
| 1½    | ~      | 11.2      | 2.1                 | 28.4      | 2.7              |
| 4     | ~      | 3.6      | 3.2                 | 61.2      | 3.8              |
| 6     | ~      | 3.7      | 3.2                 | 5.2       | 5.9              |
| 10    | ~      | 4.1      | 3.2                 | 71.6      | 4.9              |
| 18    | ~      | 4.8      | 2.8                 | 73.1      | 4.4              |

* Results are the average of duplicate determinations on each of two separate cultures. Duplicates agreed in all cases within ±10%.

**TABLE III**

**Protection against cell death and malondialdehyde formation in bromobenzene-intoxicated hepatocytes**

| Treatment        | Cell death* | Malondialdehyde† |
|------------------|-------------|------------------|
|                  | % lactic dehydrogenase release | nmol/mg protein |
| None             | 0.8 ± 0.4 | 0.26 ± 0.03 |
| 0.5 mM Bromobenzene | 55.6 ± 1.1 | 1.41 ± 0.07 |
| Bromobenzene + SKF 525A | 1.3 ± 1.1 | 0.16 ± 0.04 |
| Bromobenzene + promethazine | 4.0 ± 2.8 | 0.16 ± 0.03 |
| Bromobenzene + vitamin E | 4.6 ± 0.5 | 0.23 ± 0.06 |
| Bromobenzene + DPPD | 3.0 ± 1.0 | 0.11 ± 0.03 |

* Results are the mean ± S.D. of separate determinations on each of 3 separate cultures.
Mechanisms of Cell Injury

Conjugated dienes could be detected in the phospholipids extracted from the cultured hepatocytes as early as 30 min following treatment with bromobenzene. This is prior to the onset of cell death and before significant formation of malondialdehyde could be detected as illustrated in Fig. 8. The formation of conjugated dienes was then used to assess the effect of sulfhydryl agents on the course of lipid peroxidation in the bromobenzene-intoxicated cells. The difference between the A_{233} of phospholipids from control and treated cells was used to quantitate the extent of formation of conjugated dienes. The data in Table IV document a proportional reduction in the extent of formation of conjugated dienes and in the cell death by cysteine, N-acetylcysteine, and α-mercaptopropionyl glycine.

This close correlation between the cell killing and the peroxidation of cellular phospholipids that was evident with the protective agents held over the entire course of an 18- to 20-h exposure to bromobenzene. The exception was again DPPD. The data in Table II indicate that DPPD was effective in preventing the cell death produced by bromobenzene up to 10 h after exposure to the toxin. By 18 h DPPD had no effect on the extent of the cell killing. DPPD did, however, prevent the formation of malondialdehyde and the appearance of conjugated dienes throughout this entire time. At times less than 10 h, therefore, DPPD could dissociate the cell killing from the extent of the covalent binding of [14C]bromobenzene. After 10 h, it could similarly dissociate the cell killing from the manifestations of lipid peroxidation.

This action of DPPD suggested the final series of experiments. It was possible that the peroxidation of cellular phospholipids in the bromobenzene-intoxicated cells was an effect of their death rather than presumably a cause. Such an interpretation was also suggested by the presence of ferric iron and ascorbic acid in the tissue culture medium in all of the above studies. This possibility was considered by killing the hepatocytes by mechanisms that very likely did not involve lipid peroxidation and then looking for evidence of peroxidation in the lipids of the dead cells. The results of such a study are illustrated in Fig. 10. The cultured hepatocytes were treated with the toxic calcium ionophore A23187. Within 15 min, over 40% of the cells were dead. By 30 min, almost 70% of the cells had died. Malondialdehyde could not be significantly detected in the cultures until at least 2 h after exposure to A23187. The amount of malondialdehyde was significantly less when virtually all the cells were killed by A23187 than the amount measured with bromobenzene when only 50% of the cells were dead (Table II). A difference in the relationship between the lipid peroxidation and the cell death with A23187 and bromobenzene was also evident in the effect of the protective agents. None of the agents effective against bromobenzene-induced liver cell death had any activity against the toxicity of A23187. The antioxidants promethazine, vitamin E, and DPPD did, however, prevent the lipid peroxidation that followed the death of the cells induced by A23187. The sulfhydryl agents did not reduce the lipid peroxidation following upon the A23187-induced cell death.

Similar results were obtained with a second agent that killed the cells by a mechanism that is also probably unrelated to lipid peroxidation. Melittin is the main cytolytic component of bee venom (22). Fig. 10 indicates that 0.05 μg/ml of melittin killed 50% of the hepatocytes within 1 h. At this time there was a little or no detectable malondialdehyde. Over the course, however, of the next 5 h, there was a significant production of malondialdehyde without any further increase in the number of dead cells. As with A23187, antioxidants added to the culture medium effectively prevented the formation of malondialdehyde without reducing the number of dead cells. There is clearly, therefore, lipid peroxidation in cultures containing dead hepatocytes. This lipid peroxidation, however, can be readily dissociated from the death of the cells produced by A23187 and melittin. Such lipid peroxidation is not necessarily

![Graph](http://www.jbc.org/Downloadedfrom)

**Fig. 9.** Conjugated dienes in the phospholipids prepared from bromobenzene-intoxicated hepatocytes. Hepatocytes from phenobarbital-induced male rats were treated with 0.5 mM bromobenzene (BrBz), bromobenzene plus 10 μg/ml of promethazine (Prom). Control cells (Ct) received dimethyl sulfoxide alone. After 3 h, total lipids were extracted from the cells, and phospholipids were suspended in cyclohexane at a concentration of 0.5 mg/ml. Illustrated are the absorption spectra of these lipids between 300 and 200 nm. The inset is the difference spectrum of the phospholipids from bromobenzene-treated and control cells.

![Table IV](http://www.jbc.org/Downloadedfrom)

**Table IV** Prevention against cell death and conjugated diene formation in bromobenzene-intoxicated hepatocytes

| Treatment* | Cell death | Conjugated dienes† |
|------------|------------|--------------------|
| Bromobenzene | 61 | 0.649 |
| Bromobenzene + cysteine | 14.7 | 0.216 |
| Bromobenzene + N-acetylcysteine | 13.6 | 0.162 |
| Bromobenzene + α-mercaptopropionyl | 10.2 | 0.067 |

*In each case the cultures were treated with bromobenzene alone or with bromobenzene plus the additions indicated for 6 h. Control cells received MeSO4.

†Results are the difference between the A_{233} of phospholipids isolated from the pooled cells in 2-3 separate 75-cm² flasks of control cultures and cultures treated as indicated.
Mechanisms of Cell Injury

related to the presence of ferric iron or ascorbic acid in the culture medium. The same extent of malondialdehyde formation was observed in medium containing neither iron nor ascorbic acid or ascorbic acid alone.

**DISCUSSION**

The objective of the studies presented above was to define the mechanisms whereby the metabolism of the model hepatotoxin bromobenzene leads to lethal liver cell injury. Previous studies of bromobenzene hepatotoxicity suggested that the cell death is related to the covalent binding of reactive metabolites to cellular macromolecules (2–7). The data in this report throw new light on the role of such covalent binding and suggest that the induction of lipid peroxidation may also participate in the pathogenesis of lethal cell injury.

Use of a system as employed here is dependent upon its reproducing the essential features of the action of bromobenzene in the intact animal. Without such a relationship, analysis of the cell culture model would not be relevant to previous studies. The initial results reported here establish that the formation of conjugated dienes in total phospholipids as early as 1/2 h after treatment with bromobenzene. Malondialdehyde accumulation was first measurable at 1 h. The amount of malondialdehyde in the cells and medium increased in parallel with the increasing number of dead cells. During this time, each of the agents that modified the reaction of the liver cells to bromobenzene reduced the manifestations of the peroxidation of lipids in proportion to the reduction in the number of dead cells.

These results, however, do not unequivocally establish a causal relationship between lipid peroxidation and the development of lethal cell injury. The major alternative explanation of the same data is that lipid peroxidation is the effect rather than the cause of the cell death. The killing of the cultured hepatocytes by A23187 and melittin was used to evaluate this alternative interpretation. With both these toxins lipid peroxidation developed but was delayed following the death of the hepatocytes. In addition, the cell death could be clearly dissociated from resulting lipid peroxidation. Antioxidants prevented lipid peroxidation without having any effect on the cell killing by either A23187 or melittin. Lipid peroxidation occurring with bromobenzene, therefore, differs in two ways from that which can be established as an effect of cell death. Bromobenzene-induced lipid peroxidation is detected simultaneously with or even possibly before the death of the cells, and it cannot be dissociated from the cell killing during the first 10 h of bromobenzene intoxication.

With the antioxidants promethazine and vitamin E and all the sulphydryl compounds, a reduction in lipid peroxidation is very likely the result of a reduction in bromobenzene metabolism. This is suggested by the parallel decrease in both lipid peroxidation and the covalent binding of [14C]bromobenzene. Reduction in covalent binding by the antioxidants is difficult to explain if their action is related to events subsequent to the formation of bromobenzene epoxides. More likely they are reacting with activated oxygen species that are responsible for the oxidation of bromobenzene and, most likely, also for the induction of lipid peroxidation. The sulphydryl compounds could be acting independently (as nucleophilic traps or by maintaining GSH levels) to prevent both covalent binding and lipid peroxidation. The close association, however, between these two effects suggests that the sulphydryls are also interacting with the mixed function oxidase in a manner that again inhibits bromobenzene metabolism.

With DPPD, metabolism was dissociated from lipid peroxidation and cell death. In this case, covalent binding was unaffected or actually increased at a time when lipid peroxidation and cell death were reduced. Two consequences of the metabolism of bromobenzene are suspected as responsible for the appearance of lipid peroxidation. The first is an acute oxidative stress that results from the generation of activated oxygen species (O2· or H2O2) accompanying the metabolism of many mixed function oxidase substrates. A branch point exists in the P-450 catalytic cycle at the introduction of the second electron (23). Active turnover by the P450 system involves production of hydrogen peroxide. As much as 55% of consumed oxygen appears as H2O2 in the presence of substrate (24). This will impose an oxidative stress on the cell that is dealt with by mechanisms dependent, at least in part, on glutathione (25, 26). The second factor, then, is the depletion of glutathione as a consequence of its reaction with the elec-
trophic products of bromobenzene metabolism. The depletion of GSH overpowers the cellular defenses against oxidative stresses. The result is peroxidation of cellular phospholipids.

While lipid peroxidation may play a role in the pathogenesis of lethal cell injury during the first 6–10 h after exposure to bromobenzene, there must be a second mechanism that is responsible for the death of the cells at later times. Again with DPPD the peroxidation of cellular lipids could be dissociated from the death of the cells at times between 8 and 20 h. The most obvious candidate for the responsible second mechanism is the covalent binding of reactive metabolites of bromobenzene to cellular macromolecules. The data presented above, however, place some restraints on the extent to which covalent binding can account for the cell death.

The present report confirms the conclusion of previous studies in intact animals (2–7) that the extent of liver necrosis is closely correlated with the extent of the covalent binding of [14C]bromobenzene to total cellular proteins. With cultured hepatocytes there was a parallelism between the extent of cell death and the extent of the covalent binding of [14C]bromobenzene when both were assessed at times later than 10 h after treatment with the toxin. The correlation between the extent of covalent binding and cell killing does not, however, necessarily establish a cause and effect relationship. The data simply imply that such a mechanism would be responsible for the death of the cultured hepatocytes and, in turn, in the necrosis of liver cells in the intact animal.

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