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FINAL REPORT

BENZENE MECHANISTIC RESEARCH PROJECT

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SUMMARY

The overall objective of the Benzene Mechanistic Research Project was to investigate the processes by which benzene induces solid tumors in various tissues of the rat. The strategy was to use these data to identify a biochemical or genotoxicity endpoint that would serve as a dose-response correlate for carcinogenicity. Determination of the dose-response for this endpoint over a range of doses at and well below those evaluated in cancer studies could permit, by correlation, determination of the shape of the dose-response curve for carcinogenicity over the same range, and would provide a measure of benzene dose at the site of action. The information gained would provide a sound scientific basis for estimation of risk from exposure to low doses of benzene, and thus, potentially influence benzene regulation.

The experimental design involved conducting research in three areas: metabolism and pharmacokinetics, DNA adduct formation, and genetic toxicology. DNA adduct formation was the primary candidate for a dose-response correlate, with other genotoxicity endpoints as backup possibilities. Ultimately, the information gained would be integrated into a comprehensive model of benzene solid-tumor carcinogenicity.

In the area of pharmacokinetics and metabolism, an integrated model of benzene metabolism was developed based on significant new information about disposition after oral administration. Early studies showed that at doses below about 0.15 mg/kg, essentially all administered benzene is converted to phenolic conjugates; the predominant species found in blood is phenyl sulfate.

Coupled with this observation was the finding that target tissues are relatively rich in sulfo-deconjugation enzymes (sulfatases), but poor in the enzymes responsible for detoxification (conjugation) activity. Conversely, most nontarget tissues showed the opposite profile. A further enzymatic difference in target and nontarget tissues, reported by other researchers and confirmed in our studies, was that target tissue possesses relatively high peroxidase activities.

Taken together, these findings support a model of carcinogenicity involving liver metabolism of benzene to phenol, conjugation to produce phenyl sulfate, and export of the conjugate to other tissues via the bloodstream. In target tissues, the phenyl sulfate can be deconjugated to phenol by the action of sulfatases, which, in turn, can be further metabolized to reactive intermediates by a combination of P-450-linked monooxygenases and peroxidases. It may be these reactive intermediates which are responsible for the genotoxicity, and ultimately, carcinogenicity of benzene.

The nature of the genotoxic events presumed to account for initiation of carcinogenicity in target tissues was the subject of the other two areas of research, DNA adduct analysis and genetic toxicology. The original program proposed to use DNA adducts as the dose-

response correlate for carcinogenicity. The selection of this endpoint was based on extensive data demonstrating a strong association between adduct-forming capability and carcinogenicity. However, exhaustive analysis using the extremely sensitive ^{32}P-postlabeling method failed to show any significant adduction of DNA in target tissues following oral administration of benzene for periods as long as one year.

Direct labeling studies with ^{3}H- or ^{14}C-benzene showed some adduction, but no selectivity between target and nontarget tissues. These results implied (a) that neither benzene nor its metabolites form DNA adducts in vivo, or (b) the adducts formed are not of a type amenable to detection by the postlabeling method (i.e. non-aromatic adducts, DNA-protein crosslinks, or labile adducts lost during the DNA isolation procedure). It was clear from these studies that DNA adduction would not serve as a dose-response correlate for solid-tumor carcinogenicity.

In a related project, we investigated the possibility that benzene exerts part of its biological effect by *noncovalent* binding to receptors or receptor-like sites in target tissues. Other researchers had reported interactions of this kind, and suggested that they might be involved in the promotion of carcinogenesis by means analogous to those observed for potent tumor promoters like tetradecanoylphorbol acetate (TPA). While our studies showed some competitive binding of benzene to blood cells or blood cell membranes, no pattern consistent with specific receptor interaction was observed. While these studies were not sufficient to rule out an involvement of receptor binding in benzene carcinogenicity, this research was discontinued in favor of more promising approaches.

The genetic toxicology studies were directed toward investigating benzene and its metabolites with respect to: (a) mutagenicity and/or clastogenicity in standard genetic toxicology assays; (b) capability to produce adducts in cell or tissue cultures *in vitro*; (c) ability to induce single- or double-strand breaks in cultured cells; and (d) potential to induce cytogenetic damage (chromosome aberrations, sister chromatid exchange or micronucleus formation) in target tissue cell populations exposed to benzene *in vivo*.

The project resulted in new findings in all four areas. The mutagenicity studies demonstrated, for the first time, unequivocal mutagenicity of benzene in the mouse lymphoma L5178Y TK+/- assay in the presence of rat S9 metabolic activation. Assay of the principal metabolites of benzene in the same test system showed each to be mutagenic, with some expressing mutagenicity only in the presence of metabolic activation, while others were mutagenic in both activated and nonactivated tests. The di- and tri-hydroxy metabolites were especially potent mutagens.

DNA adduct-forming capability was assessed in the mouse lymphoma cell line, as well as in explants of rat Zymbal gland tissue in culture. Benzene itself did not produce DNA adducts in either test system, but all of the metabolites produced adducts in the tissue culture system. The adducts from each metabolite were distinct, suggesting that the production of active electrophiles involved minimal changes in chemical structure, possibly

simple conversion of hydroxyl-compounds to semiquinone radicals.

DNA strand-break analysis showed that benzene and its metabolites were not active in inducing strand breaks in mouse lymphoma cells in the absence of metabolic activation (S9). However, benzene, when tested with S9, produced a linear increase in single-strand breaks over a range of doses associated with mutagenicity in the same cell line. The metabolites were not tested in assays with S9-activation.

While the information gained from the genotoxicity studies described above was, in many cases, novel, and contributed to the general model of benzene solid-tumor carcinogenicity, none of the endpoints was adaptable as a dose-response correlate for carcinogenicity *in vivo*. However, the fourth area of research, the investigation of cytogenetic damage in explant cultures following *in vivo* exposure to benzene, was ideally suited to that objective.

In these studies, a method was developed whereby Zymbal gland tissue from rats treated in acute or chronic dosing regimens could be explanted into culture. Within 24 hrs large numbers of cells migrate out of the explant and attach to the plastic substrate. In these cell populations are large numbers of mitotic cells, indicating a healthy, proliferative culture. The cells in these explant cultures are amenable to a variety of cytogenetic assays, including chromosome aberration studies, sister chromatid exchange analysis, and assessment of micronucleus induction.

The latter endpoint, which reflects either chromosome damage induced by direct interaction of a genotoxicant with cellular chromatin, or interference with the proper segregation of genetic material to daughter cells during mitosis, was selected as the endpoint with the highest potential for correlation with carcinogenicity. Studies conducted during the last six months of the project indicated that this endpoint could provide a dose-response correlate, making possible the determination of the shape of the dose-response curve for Zymbal gland carcinogenicity to doses as low as 5 mg/kg. Single doses, three consecutive doses, and chronic dosing for as long as six months all induced an excess of micronucleated cells in the explant cultures.

Detailed studies, with doses selected for maximum statistical significance of results, were underway at the time of conclusion of the project. It is expected that these experiments will establish a dose-response curve for micronucleus induction in Zymbal cells, which can then be used to predict the dose-response for solid tumor carcinogenesis in the rat, thus accomplishing the major specific aim of the project. In terms of the overall objective, developing a better understanding of the mechanisms involved in solid tumor carcinogenesis, the new data gained from this project can be integrated into a model consistent with this and most other studies of benzene carcinogenicity. This model is shown schematically in the accompanying figure.

BACKGROUND

1.0 Overall Objectives

The Benzene Mechanistic Research Project was initiated in April of 1987. The overall objective of the research was to provide scientific input to improve the estimation of the carcinogenic risk associated with low-dose exposure to benzene. In the absence of experimental data to the contrary, risk estimation models assume that risk is a linear function of dose at low doses and that no threshold exists. Direct measurement of benzene carcinogenicity in the dose range of interest is impractical because the number of experimental animals required for adequate statistical significance is prohibitive.

Our approach was directed toward developing sensitive, dose-responsive genetic and pharmacokinetic endpoints that correlate with carcinogenicity at doses used in published cancer studies[1,2]. Then, if one assumes that the correlation between a given endpoint and carcinogenicity holds over the entire dose range, the dose-response curve for carcinogenesis at low doses could be predicted from the correlation. Alternatively, the endpoint could be used for dosimetry to relate site-specific concentrations of metabolites to administered benzene doses.

2.0 Selection of8 a Dose-Response Correlate

The endpoint selected for initial investigation was DNA-adduct formation. There were two reasons for this choice: (1) mechanistic involvement of the endpoint -- DNA adduct formation has been strongly associated with carcinogenesis, and is generally held to be a critical step in the initiation process; and (2) sensitivity -- the ^{32}P-postlabeling method offers extremely sensitive detection and quantification of adducts (1 adduct in 10^9 nucleotides or 1 per cell). Other potential correlates included in the research proposal were cytogenetic damage (chromosomal aberrations or sister chromatid exchanges) and the generation and/or accumulation of specific metabolite(s) in target tissue(s) (quantification of *effective dose*).

3.0 Selection of a Target Tissue

Much of the benzene mechanistic research prior to this project was directed toward an understanding of benzene's ability to induce blood dyscrasias including leukopenia, lymphocytopenia, agranulocytosis, thrombocytopenia, aplastic anemia and myelogenous leukemia. The target tissue of interest in these studies was the bone marrow --primary site of hematopoiesis. However, as demonstrated by Maltoni et al[1] and the National Toxicology Program[2], benzene also induces tumors in a variety of *solid* tissues of the rat.

The principal site of solid tumor carcinogenesis in these studies was the Zymbal gland, a sebaceous tissue which in rodents produces and secretes lipid (oily wax) into the ear duct. As a model system, the Zymbal gland has several advantages over other target tissues, including the following:

(1) It is relatively homogeneous with respect to cell type (squamous epithelial and sebaceous cells). This reduces the complexity of conducting biochemical studies in heterogeneous tissues such as bone marrow;

(2) It is comprised of cells undergoing differentiation from stem- to secretory cell types, and this process may accelerate the expression of genetic damage. It also makes the tissue more amenable to *in vitro* cell or tissue culture;

(3) Its metabolic enzymes are capable of efficient oxidation of benzene and its proximal metabolites by either one- or two-electron mechanisms.

Its disadvantage is its small size -- on the order of 15 mg of tissue per adult rat.

4.0 Selection of an Animal Model

The original project proposal called for research to be conducted in two phases, the mechanistic work followed by a lifetime carcinogenicity study in C. Maltoni's laboratory. To maintain consistency with Maltoni's extensive historical database, the Sprague Dawley rat was chosen as the animal model for the mechanistic studies. For most work, female rats were used, rather than males, because they showed a higher incidence of Zymbal gland tumors.

5.0 Approach to the Mechanistic Research Project

The research program was divided into three subprojects: pharmacokinetics and metabolism, DNA adducts and related endpoints, and genetic toxicology/cell and tissue culture studies, each of which is summarized in the detailed report which follows. These subject areas were selected in keeping with the ultimate goal of the project, i.e. to identify a dose-response correlate for carcinogenicity at low doses. The formation of DNA-adducts in target tissues was considered a good candidate for such an endpoint. The pharmacokinetics and metabolism work was designed to provide information about the generation of adduct-forming species, their further metabolism or partitioning into target tissues, and their detoxification and elimination. The genetic toxicology endpoints, e.g. mutagenicity and clastogenicity, would be interpreted as sequelae of adduct formation

and steps along the pathway to tumor formation. Integrating information from each of the areas would, at the end of the project, permit us to design a testable model of benzene's mechanism of action in solid tumor formation.

6.0 References

1. Maltoni, C., Conti, B., Cotti, G., and Belpoggi, F. (1985) Experimental studies on benzene carcinogenicity at the Bologna Institute of Oncology: current results and ongoing research. *Am. J. Ind. Med.* **7**: 415-446.

2. Huff, J.E. (1986) Toxicology and carcinogenesis studies of benzene (CAS no. 71-43-2) in F344/N rats and B6C3F$_1$ mice (gavage studies), *Technical Report no. 289*. National Toxicology Program, Research Triangle Park, NC.

PHARMACOKINETICS AND METABOLISM

1.0. Benzene Pharmacokinetics

1.1 Tissue distribution and bioaccumulation studies.

Target tissue specificity for toxicity or tumorigenesis is often related to differences in absorption, distribution (i.e. accumulation) and elimination. It was thought that sequestration of benzene, which is very lipophilic, in Zymbal gland (a sebaceous tissue), nasal cavity tissue, mammary gland, etc. might account for their high susceptibility to benzene-induced tumorigenesis. In support of such a possibility, experimentation has shown that benzene metabolites have been observed to accumulate in bone marrow -- a major target for benzene hematotoxicity and leukemogenesis.[1-4]

However, we have shown that single or multiple dose administration of ^{14}C-benzene followed by monitoring of blood and tissue levels of radioisotope did not lead to the accumulation of benzene residues in the Zymbal gland. Apparently, this fatty sebaceous tissue does not serve as a "sink" for benzene; rather, our results indicate that benzene is absorbed, rapidly distributed to both target and non-target tissues, and then catabolized with no selective partitioning of radioactivity into target tissues (Table 1).

In the absence of an apparent role for bioaccumulation in target organ susceptibility, other factors such as differences in metabolism and elimination kinetics were investigated. In examining these differences, we were interested not only in determining metabolite profiles, but also in delineating the spectrum of enzymes involved in benzene metabolism in both target and nontarget tissues.

1.2 Evaluation of detoxification and first pass metabolism.

First-pass metabolism of benzene by the liver occurs extensively at low oral doses, and benzene is almost completely converted to water-soluble conjugated products. At higher doses, saturation of metabolism leads to the formation of greater amounts of unconjugated, free phenolic products and reduction of the rate of benzene oxidation.

1.3 Comparison of target and nontarget tissues.

It is not known whether tissue susceptibility to benzene carcinogenesis is the result of differences in the absorption, metabolism and/or elimination of benzene by target and non-target organs. Studying the pharmacokinetics (time-course) of these processes provides information on whether tissues differ in ability to take up and metabolize benzene or in ability to detoxify reactive metabolites. Differences in elimination half-lives and other kinetic endpoints are indicators of tissue specificity and saturation of metabolism and shed light on the importance of certain toxification or detoxification pathways.

Pharmacokinetic studies revealed biphasic elimination kinetics for radioactivity in both non-target tissues (Figures 1-3, Table 2). In the Zymbal gland, the half-life for the rapid phase was 2.4- 2.8 hr while the half-life of the slow phase was 18-21 hr (Table 2). The rapid phase of elimination was similar in all tissues (2.1 to 3.2 hrs) but kidney (4.2 hr). The half-lives of the slow phase of elimination, however, varied considerably from 11 to 29 hr.

Pharmacokinetic studies of benzene in blood revealed that the elimination half-lives of benzene at 15, 150 and 500 mg/kg doses were dose-dependent as evidenced by AVC (Figure 4).

The following conclusions can be drawn from the pharmacokinetic studies:

1. Pharmacokinetic analysis does not indicate that the kinetics of tissue uptake or distribution are significantly different between target and non-target tissues. Hence, selective absorption and distribution do not explain the greater susceptibility of Zymbal gland toward tumor formation.

2. However, the large differences in the radioactivity elimination half-lives (slow phase) among tissues is in accord with the possibility that differential metabolism or covalent binding might have occurred.

2.0 Benzene Metabolism

2.1 Metabolite profiling from in vivo *studies.*

It is generally accepted that the toxic effects of benzene result from the metabolism of benzene to electrophilic intermediates capable of interacting covalently with crucial macromolecules.[1-4,7-11] Tissue specific differences in metabolism might therefore account for the greater susceptibility of some tissues (e.g. Zymbal gland, oral and nasal cavity, mammary gland) to solid tumor formation.

We determined the *in vivo* metabolite profile in target and non-target tissues in order to identify possible genotoxic metabolite(s) and provide information on bioactivation and detoxification pathway(s). These studies showed that the metabolite profile in Zymbal gland is different from that found in other target and non-target tissues. Differences were apparent not only in the non-conjugated metabolite fraction but also in the water-soluble conjugated fraction (Table 3). Hydroquinone and phenol were the major free and unconjugated benzene metabolites found in target and non-target tissues, while phenylsulfate was the major water-soluble metabolite found in blood, bone marrow and oral cavity tissue (Figure 5). However, there was a striking absence of phenylsulfate in the Zymbal gland and nasal cavity tissue.

These findings suggest that oxidative metabolism and/or conjugation in Zymbal gland and nasal cavity tissue is fundamentally different from that of other tissues. These differences must reflect distinctive patterns of conjugative and/or deconjugative (i.e., hydrolytic) enzymes. We speculate that phenylsulfate in the blood (systemic circulation) is taken up by the Zymbal gland and other target tissues; there phenylsulfate is rapidly hydrolyzed by sulfatase enzymes to yield phenol, which, in turn, can be oxidized or glucuronidated. We further speculate that the demonstrated presence of sulfatases in the Zymbal gland provides a mechanism by which sulfate conjugates of phenolic benzene metabolites are further activated or inactivated. Follow-up studies (see below) were carried out to substantiate the proposed mechanistic scheme.

2.2 Metabolism in Zymbal gland tissue culture.

Zymbal gland tissue culture experiments with ^{14}C-benzene were conducted as part of an effort to determine the extent of benzene metabolism in this organ. In these studies, Zymbal gland cultures were treated with ^{14}C-benzene at a concentration of 750 μg/ml and incubated at 37°C for 6 and 24 hrs. Culture medium incubated with ^{14}C-benzene for similar lengths of time served as negative controls for these studies. Preliminary radiometric HPLC analyses of the media at the end of the incubation period (6 or 24 hrs) showed no significant benzene metabolites. The method was capable of distinguishing 2% of total radioactivity as metabolites in the presence of excess parent ^{14}C-benzene.

The absence of measurable amounts of metabolites in the culture medium does not preclude the possibility that reactive benzene metabolites are formed; it only implies that if they are formed, they are rapidly and covalently bound to Zymbal gland macromolecules (e.g. proteins and nucleic acids). This study illustrates the difficulties in obtaining definitive data on the metabolism of volatile compounds like benzene, particularly in tissue culture systems where the amount of actively metabolizing tissue is extremely limited. These difficulties led us to pursue alternative *in vitro* methods (e.g. microsomal fractions) for investigating benzene metabolism in target tissues.

2.3 In vitro metabolism studies with microsomal enzymes.

Although our *in vivo* studies identified benzene metabolites in the Zymbal gland and other target tissues, it was not clear whether they had been produced there or produced elsewhere (e.g. in the liver) and transported there by systemic circulation. In addition, it has not been demonstrated that Zymbal gland can oxidatively metabolize benzene to reactive intermediates. Postlabeling studies on Zymbal gland tissue from *in vitro* cultures provided circumstantial evidence that benzene and other xenobiotics are metabolized to reactive, adduct-forming intermediates. In addition, Pohl and Fouts[12] have reported that Zymbal gland homogenates metabolize benzo(a)pyrene and 7-ethoxycoumarin via cytochrome P450-dependent monooxygenases.

Early attempts to adopt the method of Pohl and Fouts to study the metabolism of

benzene met with limited success. Homogenates and microsomal fractions showed little metabolism of benzene, presumably because of the volatility of benzene and the very small amounts of tissue available for study. As with the tissue culture studies described above, these results pointed up the need for specialized methods for metabolic studies in micro systems. Subsequent refinements of our *in vitro* techniques have allowed us to characterize important enzyme systems involved in the metabolism of benzene and its metabolites in the Zymbal gland and other target tissues (e.g. nasal and oral cavity tissues, mammary gland, and bone marrow).

2.4 Sulfatases, phenyl sulfate and other sulfate conjugates.

In vivo metabolic profiling studies showed the distinct absence of phenylsulfate in Zymbal gland and nasal cavity tissue. We speculated that this qualitative difference might relate to the distribution and/or activity of conjugating (e.g., sulfotransferases) and deconjugating or hydrolytic (e.g., sulfatases) enzymes in the tissues. Therefore, extensive studies were carried out to identify and quantitate sulfohydrolysis and sulfoconjugation in target and non-target tissues.

Metabolism studies showed that Zymbal gland, mammary gland, and oral cavity all possess phenylsulfatase activity but lack detectable sulfoconjugation activity toward phenol (Table 4). Of the target tissues studied, Zymbal gland showed the highest sulfatase activity toward phenylsulfate (Table 4). Our results indicate that the Zymbal gland has the ability to hydrolyze phenylsulfate, catecholsulfate and hydroquinonesulfate (Table 5). Furthermore, all target tissues studied showed sulfatase activity but no sulfotransferase activity (except nasal cavity). Such tissue specific differences in metabolism may be important in target tissue susceptibility to benzene-induced tumor formation.

Although nasal cavity tissue showed phenol sulfoconjugating activity, other factors may explain its susceptibility, one of which may be the high capability of the nasal mucosa to convert benzene directly to phenol, hydroquinone and catechol. Hydroquinone production in nasal cavity tissue was found to be about 8 times higher than that in the liver. A second possibility is that sulfatase and sulfotransferase are highly compartmentalized in nasal cavity tissue. Hence, localization of the metabolite, phenylsulfate, in sulfatase-rich regions can lead to hydrolysis to phenol. Such compartmentalization of enzyme systems has been well-documented in the liver and extrahepatic tissues.[13]

The preponderance of sulfo-hydrolytic enzymes in the Zymbal gland probably accounts for the absence of appreciable phenylsulfate in this target tissue. Moreover, in an indirect way, it may also contribute to the greater susceptibility of Zymbal gland to the carcinogenic effects of benzene, inasmuch as it provides a plausible delivery mechanism by which protected sulfate conjugates produced in the liver can reach target tissues, be hydrolyzed, and then further oxidized to reactive intermediates.

2.5 Lipid peroxidation as a biochemical endpoint.

Some xenobiotics have been reported to exert their toxic effects through the peroxidation of membrane lipids and the subsequent cellular damage this induces.[14] Lipid peroxidation is often the result of attack by reactive intermediates that either are themselves free radicals or are able to induce the formation of reactive oxygen species. Alternatively, lipid peroxidation can be induced by interference with cellular defenses, such as reduced glutathione, which protect against the cell-mediated production of reactive oxygen.

In addition to the damage mediated by lipid peroxidation, free radicals can also exert toxic effects through DNA strand-breakage and/or direct covalent binding to DNA bases. Polyhydroxyaromatic compounds, such as hydroquinone and catechol, have been reported to generate free radicals or radical intermediates.[15-17] Since the Zymbal gland is a sebaceous tissue possessing peroxidative activity, one might suspect that this organ would be susceptible to benzene-induced lipid peroxidative events involving radical intermediates. Studies were, therefore, undertaken to determine if benzene or its metabolites are capable of inducing lipid peroxidation and whether lipid peroxidation can be used as a sensitive biochemical marker for benzene-induced toxicity.

Repeated oral or intraperitoneal exposure to benzene (1000 mg/kg daily) for seven days did not produce an increase in lipid peroxidation products in liver relative to levels in control animals (Table 6). Oral benzene exposure did, however, cause a statistically significant depletion of tissue GSH in Zymbal gland, nasal cavity and liver after one week exposure. In target tissues, Zymbal gland and nasal cavity tissue, benzene exposure decreased GSH levels by 29% and 43%, respectively, relative to levels found in the same tissues of control animals (Table 7). In liver, GSH levels were depleted by 44%, while in kidney GSH levels increased by 14% over those of controls. Significant depletion of tissue glutathione stores is indirect evidence that intermediate(s) are reacting with the nucleophilic glutathione molecule.

The inherent ability of benzene and five of its metabolites to induce lipid peroxidation was assessed using liver homogenate. Neither benzene nor the metabolites produced any measurable effect (Table 8). Positive control studies using Fe^{+2} and ADP carried out under similar conditions generated a five-fold increase in lipid peroxidation products compared to levels in untreated controls. Thus, although direct attempts to measure lipid peroxidation in the Zymbal gland and other target tissues were hindered by insufficient amounts of tissue, the absence of a significant effect in liver tissue either *in vivo* or *in vitro* strongly suggests that free radicals play at most a minor role in benzene-induced toxicity.

Our lipid peroxidation study also produced important ancillary findings: benzene exposure induced marked depletion of hepatic glutathione stores together with a 13-fold increase in urinary thioether levels (Figure 6). Taken together, these results imply that benzene is metabolized to potentially reactive intermediates in the liver, and that hepatic

glutathione stores play an important role in detoxification and in protecting tissue macromolecules against injury. But in target tissues like Zymbal gland and nasal cavity, reactive metabolites may have more serious consequences in that the concentrations of GSH and other protectants are presumably significantly lower in these tissues. Subsequent *in vitro* work with Zymbal gland homogenates (see below) demonstrated that 2hydroquinone significantly depletes GSH in this tissue.

2.6 Glutathione depletion and trapping of reactive intermediate.

Glutathione plays a crucial role in protecting cells against attack by reactive intermediates. The depletion of this cellular nucleophile leaves tissues vulnerable to toxic effects such as adduction, cross-linking, and DNA strand scission. Benzene and five of its metabolites were evaluated for their ability to alter liver GSH levels. In addition, studies were carried out to trap reactive intermediates using GSH.

Incubation experiments with liver homogenates showed that 1,2,4-benzenetriol, *t,t*-muconaldehyde, hydroquinone and catechol caused statistically significant decreases in GSH levels (Table 9). 1,2,4-benzenetriol showed the greatest ability to deplete GSH, followed by *t,t*-muconaldehyde, hydroquinone and catechol. The ability of hydroquinone to deplete hepatic GSH stores was mirrored by the formation of an equivalent amount of the corresponding glutathione conjugate, 2-(*S*-glutathionyl)hydroquinone (HQ-SG). These findings indicated that hydroquinone-mediated reduction in glutathione levels was directly related to formation of a reactive intermediate and its conjugation with glutathione to form HQ-SG, and is not related to oxidation of GSH to GSSG. The reactive intermediate, 1,4-benzoquinone, was monitored by trapping with glutathione and measuring the resulting 2-(*S*-glutathionyl)hydroquinone (HQ-SG) conjugate using HPLC with an electrochemical detector. At an applied voltage of 0.7 mv, the assay was very specific for the HQ-SG conjugate (Figure 7), as indicated by comparison to an authentic standard.

Incubations with phenol and benzene did not cause statistically significant depletion of GSH stores, but they did lead to the formation of detectable amounts of 2-(*S*-glutathionyl)-hydroquinone. Phenol produced 2.5 nmole/mg protein of HQ-SG while benzene yielded 0.3 nmole/mg protein of the same adduct.

The ability of various target tissues to metabolize hydroquinone to benzoquinone was measured using the HPLC-EC procedure described above. Zymbal gland, bone marrow and oral cavity produced the highest rate of formation (per mg of protein) of the reactive metabolite, 1,4-benzoquinone, as measured by the amount of HQ-SG adduct formed. In liver, formation of HQ-SG from hydroquinone occurred at one-third the rate observed in the Zymbal gland. Mammary gland and nasal cavity were also capable of metabolizing hydroquinone, but produced HQ-SG at a slower rate than Zymbal gland or oral cavity. Rates of formation of HQ-SG in nontarget tissues such as brain and kidney were slow.

Several groups[16,17] have reported that horseradish peroxidase (HRP) metabolizes *p*-hydroquinone and catechol to reactive quinonoid intermediates (1,4-benzoquinone and 1,2-benzoquinone, respectively) that can be trapped with thiols to give thioether conjugates. Experiments were carried out with HRP to substantiate the oxidation of *p*-hydroquinone to 1,4-benzoquinone; using GSH to trap the generated quinonoidal species, we found that HQ-SG was rapidly formed (21,000 mmoles HQ-SG/min/mg HRP protein) when HRP was incubated with hydroquinone and hydrogen peroxide. (Also see findings under lipid peroxidation for depletion of GSH in liver, Zymbal gland, and nasal cavity tissue *in vivo*.)

Results from our *in vivo* and *in vitro* studies indicate that tissue GSH stores are depletable by benzene and its metabolites. This depletion is due in part to the formation of glutathione adducts or conjugates in tissues and their subsequent excretion as thioether conjugates in the urine. The increased production of thioethers in the urine is indirect evidence that electrophilic intermediates were generated during benzene metabolism *in vivo*. The ability of free benzene metabolites like 1,2,4-benzenetriol, *t,t*-muconaldehyde, hydroquinone and catechol to deplete GSH stores *in vitro* is further evidence for the participation of reactive intermediates in the depletion process.

2.7 Oxidative metabolic capability of Zymbal gland and other target tissues.

The inherent ability of target tissues to oxidatively metabolize benzene may also be an important factor in explaining tissue susceptibility. The formation of free and unconjugated phenol, catechol or hydroquinone directly from the metabolism of benzene would demonstrate that the target tissues have the ability to generate phenolic species *in situ* and that first-step metabolism in the liver with transport to target tissues via the blood supply is not obligatory.

All target tissues investigated demonstrated the ability to metabolically transform benzene to phenol and hydroquinone, with nasal cavity tissue possessing the highest metabolic capability (Figure 8). Catechol formation was very low but detectable in all tissues. In general the formation of phenolic metabolites from benzene followed the order: phenol > hydroquinone > > catechol. The high metabolic activity in the nasal cavity is of particular interest. In fact, nasal cavity tissue homogenates metabolized benzene more rapidly (per mg of protein) than did liver homogenates. The ability of nasal tissue to metabolize xenobiotics is not surprising since it has been well documented that the nasal mucosa possesses considerable cytochrome P450 activity.[18,19]

Additionally, we have shown that hydroquinone arises from the further metabolism of initially formed phenol in target tissues like the Zymbal gland; this was demonstrated by using phenol as substrate. These findings indicate that all target tissues have the ability to form free phenol and hydroquinone *in situ* from benzene directly. Hence, several mechanisms account for the presence of free phenol or hydroquinone in target tissue: (1) they can be formed directly from metabolism of benzene; (2) they can be delivered to the

target tissue in the free form via transport from the liver; (3) they can be formed from the hydrolysis of sulfate or glucuronide conjugates of phenol or hydroquinone in target tissue.

2.8 Peroxidase activity in Zymbal gland and other tissues.

In many tissues, peroxidases are important mediators of oxidative metabolism.[20,21] For example, in the bone marrow, these enzymes have been shown to catalyze the oxidation of benzene to intermediates which covalently bind to tissue macromolecules.[7,9,16,17] Similarly, peroxidases have also been shown to oxidize hydroquinone to benzoquinone, which then reacts with GSH or other cellular nucleophiles such as DNA or protein. Yet there have been relatively few studies comparing peroxidase activities in target and non-target tissues for solid tumor formation.

Using guaiacol as a substrate, we measured tissue peroxidase activity by a standard spectrophotometric technique. Peroxidase activity was observed in all target tissues; the order of activity: ZG > oral cavity >> nasal cavity > mammary gland (Figure 9). Non-target tissues (e.g. liver, kidney, brain) demonstrated no peroxidase activity toward guaiacol or dianisidine. Bone marrow, as expected, showed significant peroxidase activity, presumably attributable to myeloperoxidases.

The presence of peroxidase activity in all target tissues (Zymbal gland, oral cavity, nasal cavity, and mammary gland), but not in non-target tissues (liver, kidney, and brain) is very significant. It emphasizes the importance of peroxidase as a common activating system in target tissues. The ability of peroxidase to metabolize phenol, hydroquinone, catechol, and 2,2′- and 4,4′-biphenols to reactive intermediates has been demonstrated by others.[16,17,22] Covalent binding of benzene metabolites to protein and the formation of glutathione adducts have also been demonstrated by others to occur in the presence of peroxidases. Finally, Zymbal gland peroxidase(s) have been proposed to be important in the metabolic activation of other Zymbal gland carcinogens such as aminostilbene derivatives.[23]

We have demonstrated in other experiments that hydroquinone-GSH adducts [2-(*S*-glutathionyl)hydroquinone] can be formed in Zymbal gland homogenates incubated with hydroquinone. The rate of formation of HQ-GSH adducts in target tissue appears to correlate with the relative amounts of peroxidase present (Figure 10). The finding of higher peroxidase activity in target as compared to nontarget tissues contributes to a possible biochemical and enzymological explanation for the increased sensitivity of target tissues to the toxic effects of benzene.

3.0 References

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Table 1. Concentration of radioactivity in various tissues 1 hr following oral administration of 0.15, 1.5 and 15 mg/kg ^{14}C-benzene.

	μg Benzene equivalents/g or mL, ppm[a]		
Tissue	0.15 mg/kg	1.5 mg/kg	15 mg/kg
Zymbal gland	0.034 ± 0.006	0.380 ± 0.059	3.2 ± 0.4
Nasal cavity	0.044 ± 0.008	0.547 ± 0.123	2.4 ± 0.8
Oral cavity	0.035 ± 0.001	0.359 ± 0.017	2.4 ± 0.2
Mammary gland	0.028 ± 0.008	0.373 ± 0.055	6.6 ± 1.4
Blood	0.086 ± 0.004	0.769 ± 0.073	6.3 ± 0.9
Bone marrow	0.058 ± 0.005	0.490 ± 0.077	10.1 ± 1.3
Liver	0.198 ± 0.006	2.043 ± 0.195	12.8 ± 1.4
Kidney	0.254 ± 0.005	1.926 ± 0.174	12.2 ± 1.2

[a]Values represent mean ± SEM for three animals.

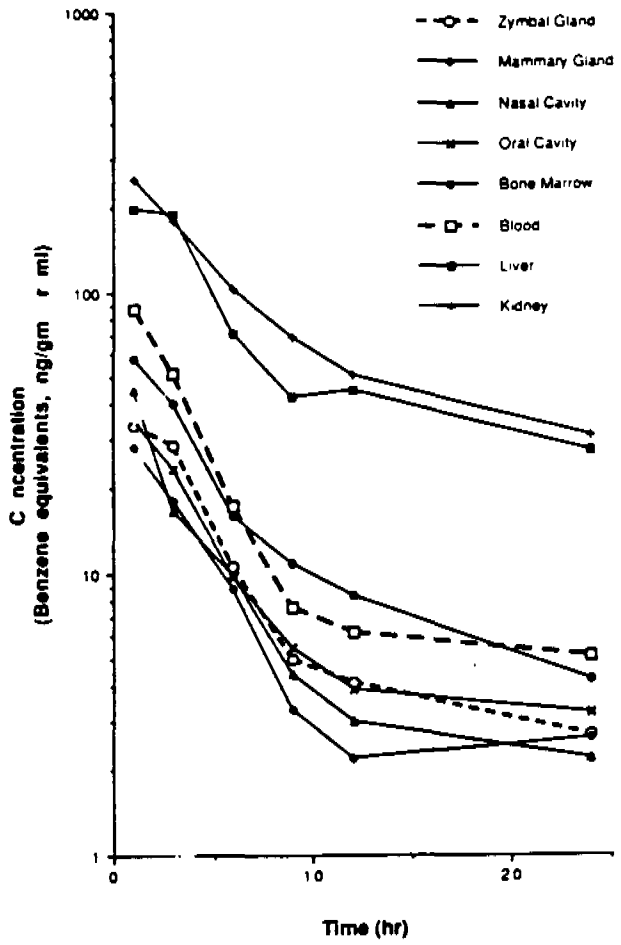

FIGURE 1. ^{14}C concentrations in the Zymbal gland, and various other target and nontarget organs in Sprague-Dawley rats following a single oral dose of benzene (0.15 mg/kg). Points represent the means of three animals.

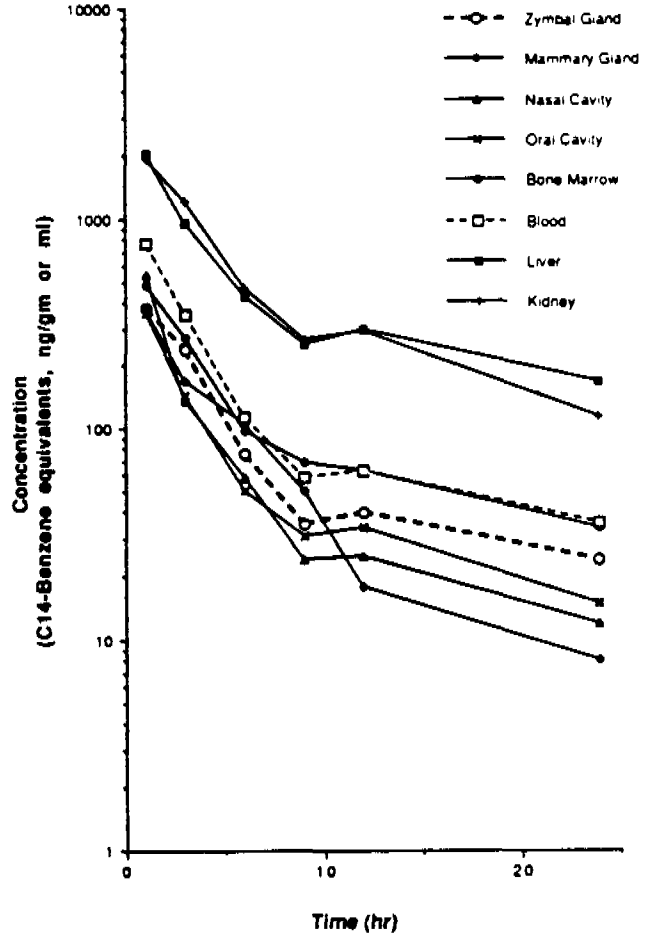

FIGURE 2. ^{14}C concentration in the Zymbal gland and various other target and nontarget organs in Sprague-Dawley rats following a single oral dose of benzene (1.5 mg/kg). Points represent the means of three animals.

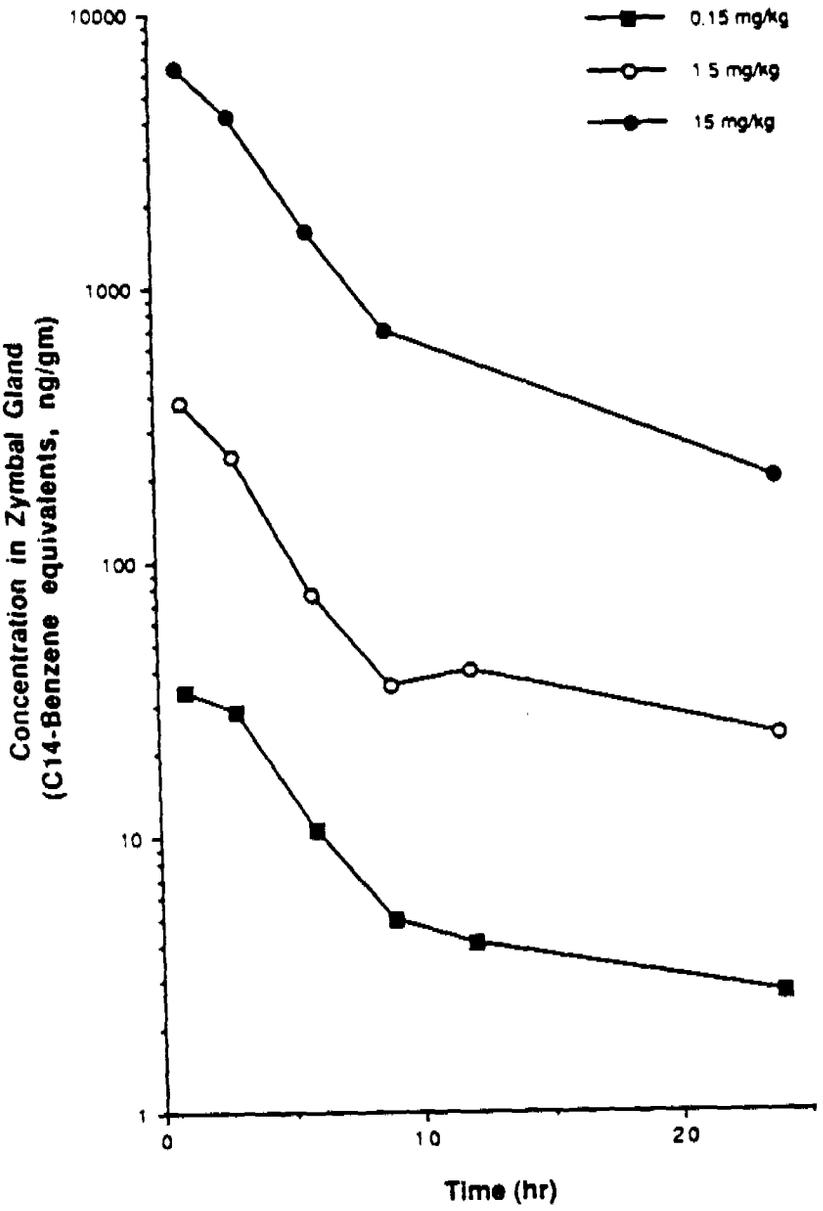

FIGURE 3. Comparison of ^{14}C levels in the Zymbal gland in rats given a single oral low dose of benzene of 0.15, 1.5 and 15 mg/kg. Points represent the means of three rats. For the 15 mg/kg dose group, tissues were not taken at the 12-hr time interval.

Table 2. Half-life of elimination of radioactivity from Zymbal gland and other tissues.[a]

Tissue	Dose, mg/kg	Rapid phase, $t_{1/2}$, hr	Slow phase, $t_{1/2}$, hr
Zymbal gland	0.15	2.8	18
Zymbal gland	1.5	2.4	21
Zymbal gland	15.0	2.5	8[b]
Blood	0.15	2.2	29
Blood	1.5	2.1	23
Blood	15.0	2.5	8[b]
Mammary gland	0.15	2.6	NC[c]
Nasal cavity	0.15	2.6	17
Oral cavity	0.15	2.9	23
Bone marrow	0.15	3.2	11
Liver	0.15	2.8	21
Kidney	0.15	4.2	14

[a]Half-life of elimination of radioactivity for the rapid and slow phases was estimated by visualizing the best line graphically on semilog plots and then best-fitting the data points for each phase using an exponential curve-fitting program on an HP 41CX calculator.

[b]Only two data points were available for determining half-life of the slow phase.

[c]Not calculable.

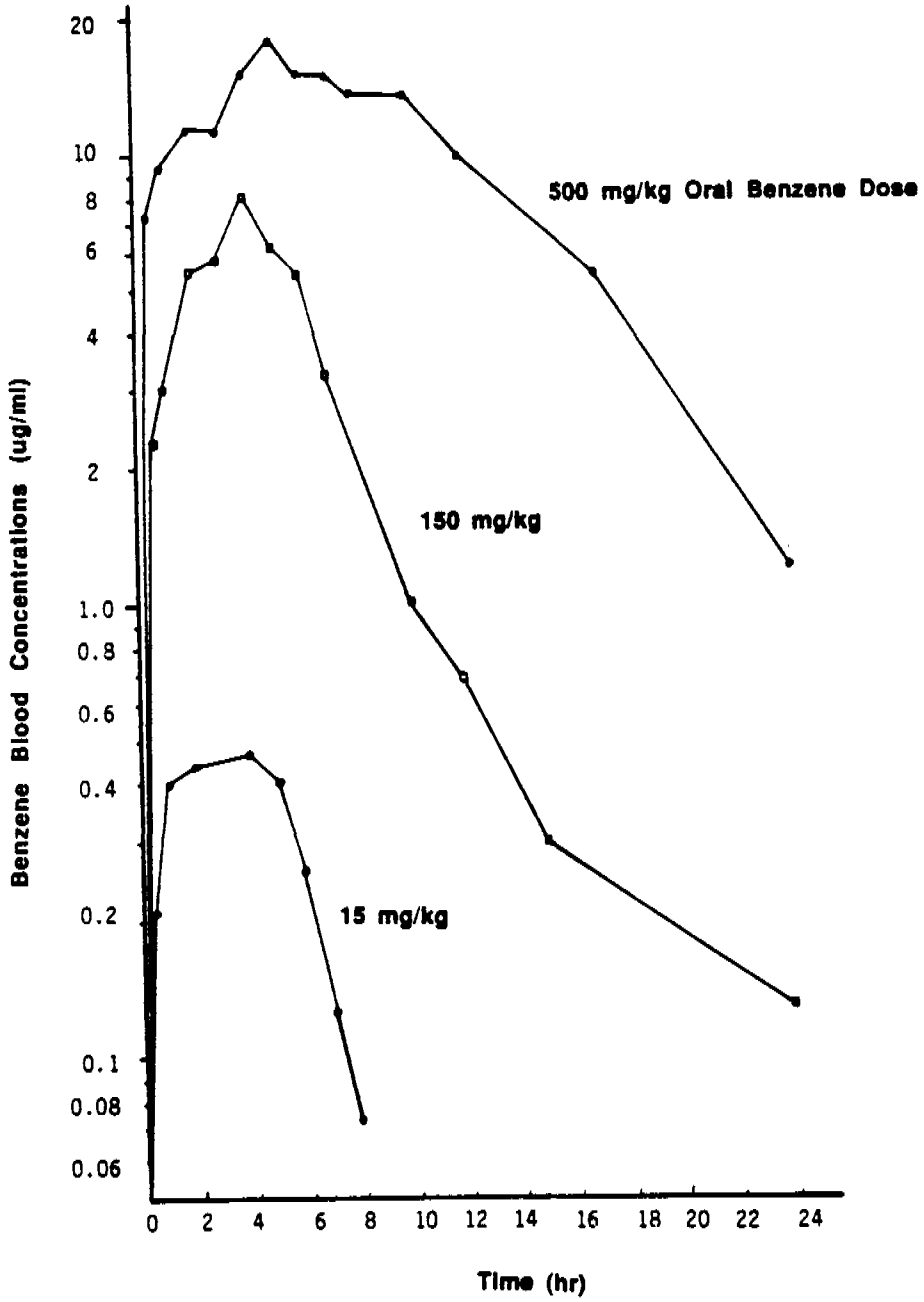

FIGURE 4

Table 3. Relative percentage of benzene metabolites in Zymbal gland and other tissues 1 hr after a 15 mg/kg ^{14}C-benzene oral dose.[a]

Metabolite	% Total radioactivity in ethyl acetate or aqueous fractions in tissue							
	Zymbal gland	Nasal cavity	Oral cavity	Bone marrow	Mammary gland	Blood	Liver	Kidney
Unconjugated metabolites in ethyl acetate fraction[b]								
Hydroquinone	30	11	53	–[d]	–[d]	64	89	65
Catechol	ND[c]	ND	ND	–	–	ND	ND	ND
Phenol	3	29	31	–	–	2	3	26
Unidentified	67 (1 major peak)	60 (1 major, 1 minor peak)	16	–	–	34	8 (2 peaks)	9 (2 peaks)
Water-soluble metabolites in aqueous fraction[b]								
Phenyl sulfate	ND	ND	62	66	–[d]	83	26	23
Phenyl glucuronide	35	18	3	ND	–	2	2	3
Muconic acid	ND	6	14	11	–	6	5	15
Hydroquinone glucuronide	ND	ND	4	2	–	2	8	6
Unidentified and other metabolites	65 (1 major, 2 minor peaks)	76 (major peak, 23.5 min, catechol glucuronide, 53%)	18 (4 minor peaks)	22 (4 minor peaks)	–	6 (2 minor peaks)	56 (major peak at 5 min, 27%)	53 (2 peaks at 3-5 min, 31%)

[a]See "Materials and Methods" for metabolite isolation procedure and HPLC conditions for separation of metabolites in the ethyl acetate fraction and in the aqueous fraction.

[b]HPLC retention times of unconjugated metabolites: 1,2,4-triol (3.0 min); hydroquinone (4.5 min); catechol (9.0 min); phenol (20 min). HPLC retention times (ion-pair) for water-soluble metabolites: hydroquinone glucuronide (14.0 min); triol glucuronide (major isomer, 16.0 min); muconic acid (17.5 min); phenyl glucuronide (21 min); catechol glucuronide (22.5 min); phenylsulfate (31 min).

[c]ND, not detected.

[d]Ethyl acetate extraction was carried out on bone marrow homogenate, but evaporation of solvent yielded little if any radioactive residue for HPLC analysis. Radioactivity lost most likely parent material. Similar findings occurred with mammary gland, radioactivity was not found in the evaporated ethyl acetate fraction or aqueous fraction.

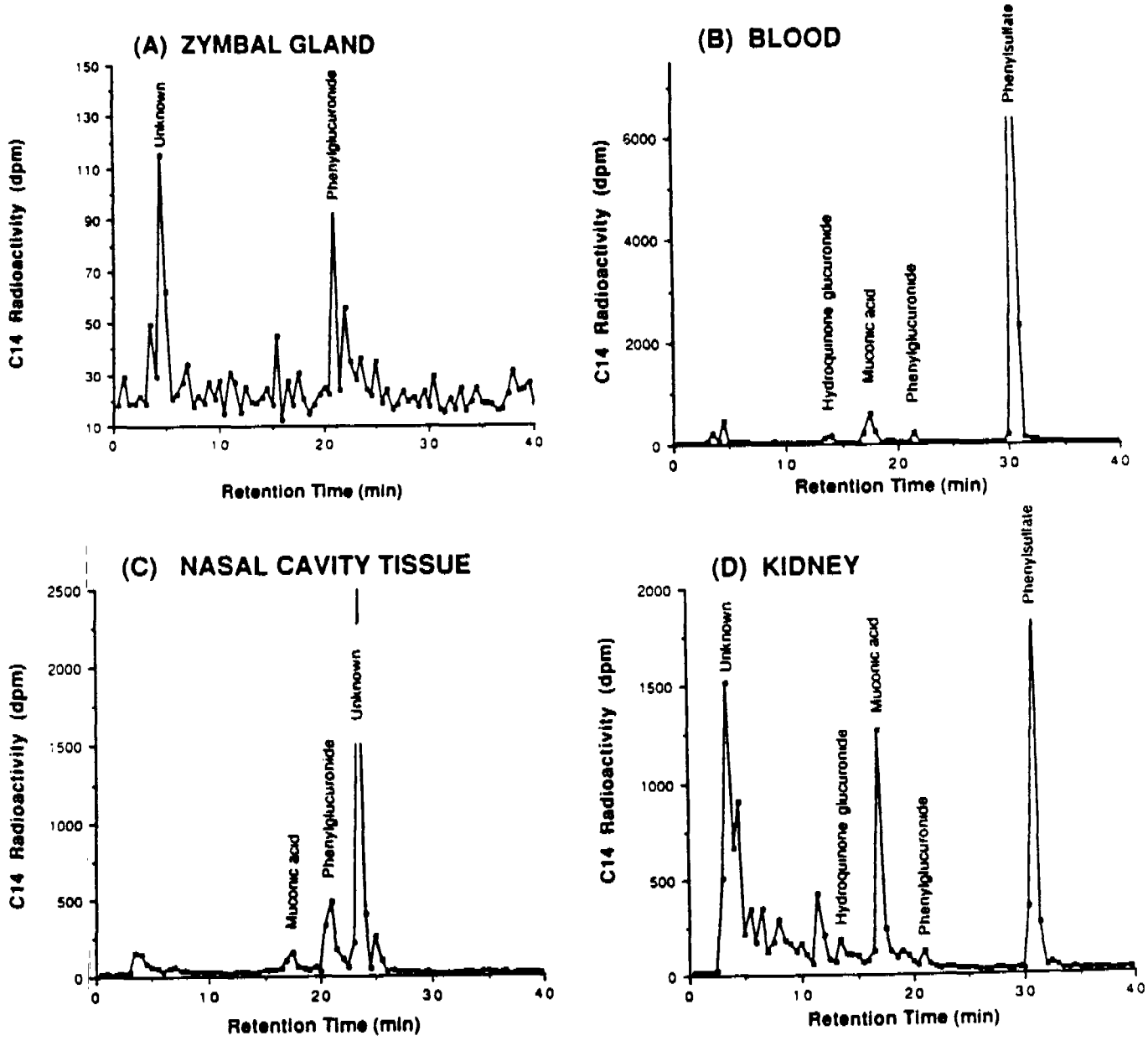

FIGURE 5 HPLC radioactivity profile of the water-soluble metabolite fraction isolated from various tissues and from blood 1 hr after female Sprague-Dawley rats were orally administered 15 mg/kg ^{14}C-benzene in olive oil: (*A*) Zymbal gland; (*B*) blood; (*C*) nasal cavity tissue; (*D*) kidney. Isolation procedures and ion-pair HPLC conditions are given in the materials and methods section. See Table 3 for relative percentages of each identified benzene metabolite and their HPLC retention times.

TABLE 4

Phenylsulfatase and Phenolsulfoconjugating Activity in Target and Nontarget Tissue Homogenates.

Tissue	Sulfatase	Sulfotransferase
	pmoles/min/mg protein[a,b]	pmoles/min/mg protein[c]
Zymbal Gland	145 ± 33	ND[d]
Nasal Cavity	13 ± 3	318 ± 23
Bone Marrow	35 ± 1	ND
Oral Cavity	65 ± 2	ND
Mammary Gland	17[e]	ND
Liver	150 ± 17	335 ± 14
Blood	ND	ND
Brain	ND	39 ± 2
Kidney	45 ± 16	9 ± 1

a. Sulfatase activity was determined by measuring the ability of the tissue to hydrolyze phenylsulfate to phenol. The phenol liberated was quantitated by HPLC-EC. Values shown represent mean ± standard deviation (n=3).

b. When 4-methylumbelliferylsulfate is used as substrate, the following sulfatase activity (all values in units of nmoles/min/mg protein) was found for the tissues: Zymbal gland (2.39 ± 0.19); nasal cavity (0.72 ± 0.24); bone marrow (0.67 ± 0.08); liver (2.76 ± 0.92); blood (ND); brain (0.45 ± 0.04); kidney (0.31 ± 0.04).

c. Sulfotransferase or sulfoconjugating activity was determined by measuring the ability of the tissue to conjugate ^{14}C-phenol with sulfate (PAPS); the ^{14}C-phenylsulfate formed was quantitated by HPLC-radiometric analysis. Values shown represent mean ± standard deviation (n=3).

d. ND = not detected. Limit of sensitivity for sulfatase assay was 2 pmole/min/mg protein and 4 pmole/min/mg protein for the sulfotransferase assay.

e. Average of two determinations.

TABLE 5

Tissue Sulfatase Activity Toward Sulfate Conjugates of Phenol, Catechol, Hydroquinone and 4-Methylumbelliferone (4-MU)

Tissue	Phenyl Sulfatase	Catechol Sulfatase	Hydroquinone Sulfatase	4-MU Sulfatase
		pmole/min/mg protein		
Zymbal Gland	145 ± 33	171 ± 15	7 ± 2	2390 ± 190
Nasal Cavity	13 ± 3	172 ± 17	6 ± 1	720 ± 240
Liver	150 ± 17	242 ± 18	7 ± 2	2760 ± 920

Values shown represent the mean ± standard deviation (n=3). See Methods section for experimental details to sulfatase assay for substrates.

TABLE 6

Lipid Peroxidation in Liver After Benzene Exposure[a]

Treatment	Thiobarbituric Acid Reactive Material ($OD_{520-535}$)[b]
Control	0.049 ± 0.006
Benzene	0.040 ± 0.010

a. Sprague-Dawley rats were treated intraperitoneally with 1000 mg/kg/day benzene for seven days; control animals received olive oil. Lipid peroxidation was measured by the method of Uchiyama and Mihara (1978) which follows the formation of thiobarbituric acid (TBA)-reactive products.

b Values shown are expressed in units of the optical density at 520-535 nm for TBA-reactive products ($OD_{520-535}$), and represent the mean ± standard deviation for four animals.

TABLE 7

Glutathione Levels in Target and Nontarget Tissues After Benzene Exposure[a]

Tissue	Treatment	mcg Glutathione[b]/g tissue or mg protein[c]
Liver	Benzene	1.41 ± 0.50*
	Olive Oil	2.53 ± 0.15
Kidney	Benzene	1.57 ± 0.06*
	Olive Oil	1.37 ± 0.07
Nasal	Benzene	2.27 ± 0.14[c]*
	Olive Oil	3.96 ± 0.41[c]
Zymbal	Benzene	9.18 ± 1.00[c]*
	Olive Oil	13.03 ± 1.70[c]

a. Sprague-Dawley rats were treated intraperitoneally with 1000 mg/kg/day benzene for seven days; control animals received olive oil.

b. Glutathione levels were determined by the spectrophotometric method of Ellman (1959). Values shown represent the mean ± standard deviation of GSH levels in four animals.

c. These values are expressed as mcg glutathione/mg protein. The protein content was determined using the Bradford method.

* Values differ significantly ($p<0.05$) from control as determined by paired Student's *t*-test.

TABLE 8

Lipid Peroxidation in Liver Homogenate After Incubation with Benzene and Its Metabolites[a]

Substrate	Thiobarbituric Acid Reactive Material [b]
Control	0.024 ± 0.002
Benzene	0.023 ± 0.006
Phenol	0.039 ± 0.007[c]
Catechol	0.026 ± .0007
Hydroquinone	0.025 ± 0.001
Muconaldehyde	0.024 ± 0.001
1,2,4-Benzenetriol	0.020 ± 0.002
$FeSO_4$/ADP[d]	0.112 ± 0.012

a. Lipid peroxidation was measured by the method of Uchiyama and Mihara (1978) which follows the formation of thiobarbituric acid (TBA)-reactive products.

b. Values shown are expressed in units of the optical density at 520-535 nm for TBA-reactive products ($OD_{520-535}$), and represent the mean ± standard deviation (n=3).

c. The elevated level of TBA-reactive material observed here for phenol was not reproduceable in subsequent experiments.

d. 200 mcM $FeSO_4$ /1 mMADP was used as a positive control for lipid peroxidation.

TABLE 9

Glutathione Levels and HQ-SG[a] Adduct Levels in Liver Homogenate After Incubation with Benzene and Its Metabolites

Substrate	Glutathione Concentration	HQ-SG Concentration
	nmoles GSH/mg protein[b]	nmoles HQ-SG/mg protein[b,c]
Control	82.4 ± 4.9	None Detected
Benzene	80.1 ± 1.9	0.3 ± 0.2
Phenol	76.9 ± 2.9	2.5 ± 0.2
Catechol	66.4 ± 1.3*	Not Determined[d]
Hydroquinone	45.9 ± 0.6*	41.6 ± 3.5
t,t -Muconaldehyde	18.2 ± 0.9*	Not Determined[d]
1,2,4-Benzenetriol	13.0 ± 0.9*	Not Determined[d]

a. HQ-SG = 2-(S-glutathionyl)hydroquinone. See Figure 4 for structure of HQ-SG.
b. Values shown represent the mean ± standard deviation (n=3).
c. HQ-SG [2-(S-glutathionyl)hydroquinone] is the glutathione adduct formed from the reaction of 1,4-benzoquinone and GSH (Lunte and Kissinger, 1983, 1984). HPLC-EC was used to detect and quantitate HQ-SG. The limit of detection of the HPLC-EC assay was 50 pmole/mg protein.
d. Not determined since substrate was not expected to form HQ-SG. Attempts to detect and quantitate glutathione adducts derived from incubation of benzenetriol, muconaldehyde and catechol were unsuccessful. Large amounts of polar HPLC-eluting and easily oxidizable components interfered with assay.
* Values differ significantly ($p<0.05$) from control as determined by a paired Student's *t*-test.

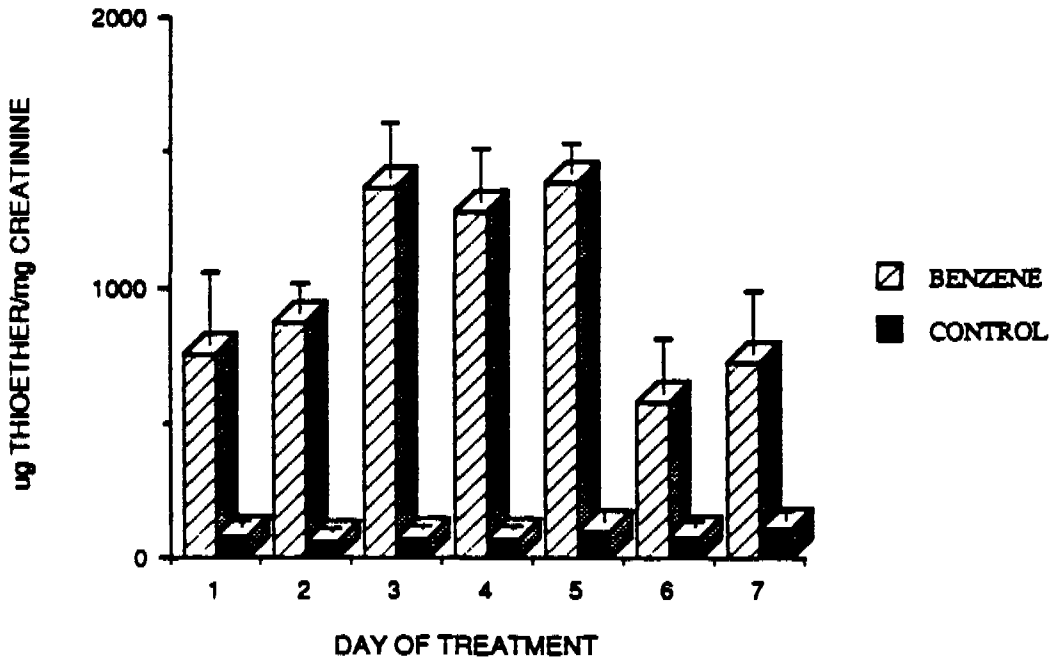

FIGURE 6. Thioether levels in urine from Sprague-Dawley rats treated intraperitoneally with 1000 mg/kg/day benzene for seven days; control animals received olive oil. Each bar represents the concentration of thioether formed normalized to the amount of creatinine in each urine sample. Values given are the mean ± standard deviation for four animals.

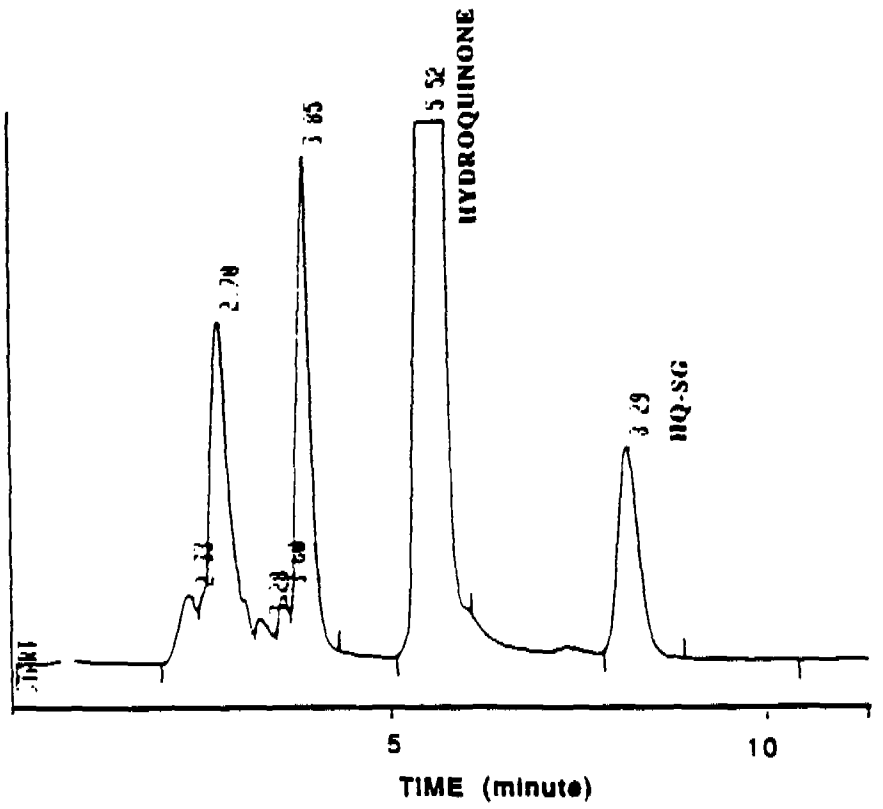

FIGURE 7. Chromatogram of reaction mixture in which hydroquinone and glutathione were incubated with tissue (10.000 x g) homogenate. Major peaks are labeled with both retention time (minute) and identity of eluting compound. HQ-SG = 2-(S-glutathionyl)-hydroquinone. Reaction and HPLC-EC were carried out as described under Methods for trapping of reactive metabolites *in vitro* with glutathione.

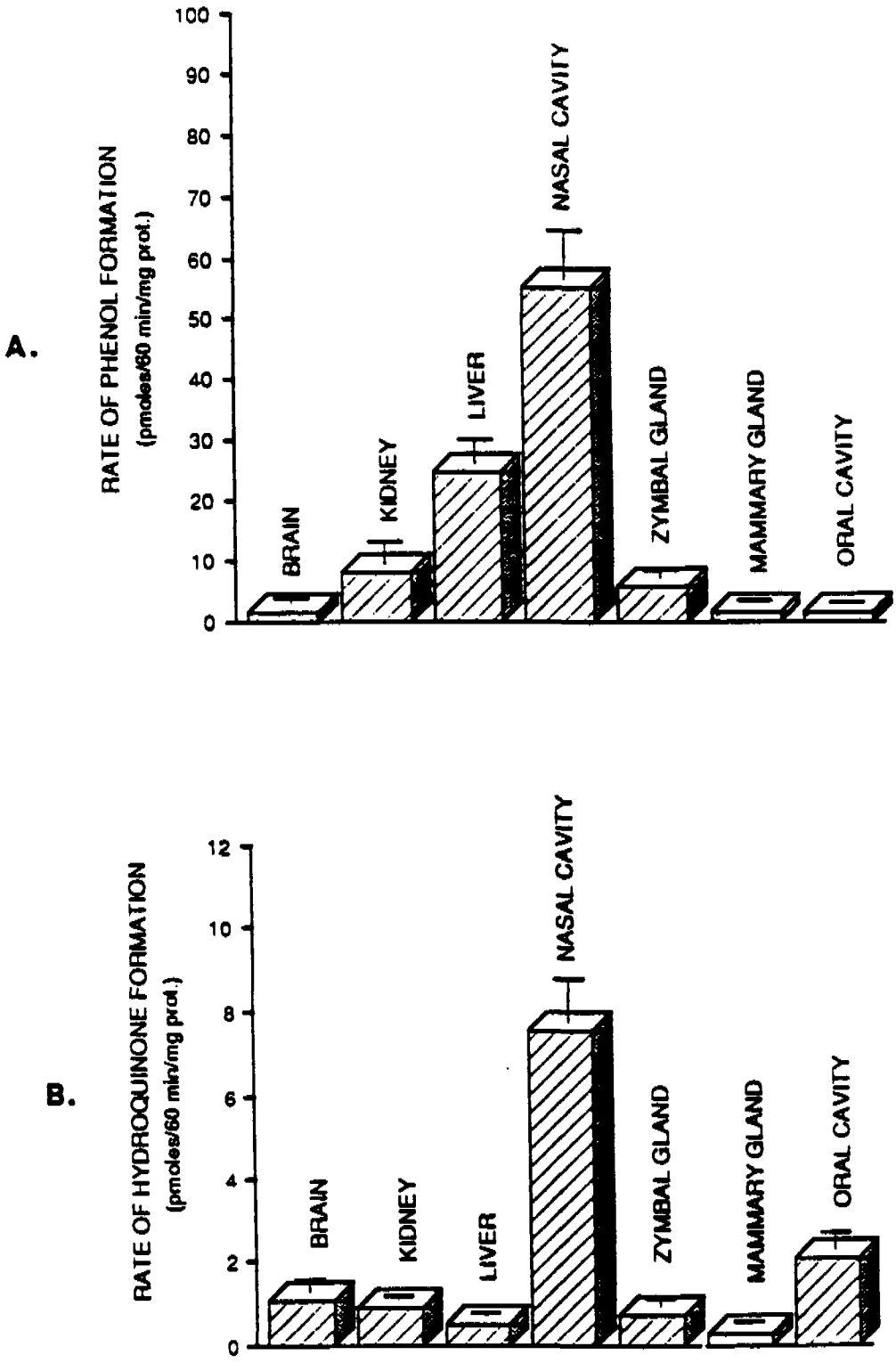

FIGURE 8. Production of the metabolites, phenol and hydroquinone, from ^{14}C-benzene incubated in target and nontarget tissue (10,000 x g) homogenates in the presence of NADPH. Each bar represents the rate of formation of either phenol (A) or hydroquinone (B). Values are the mean ± standard deviation (n=3). Boiled liver homogenate produced no detectable phenolic metabolites.

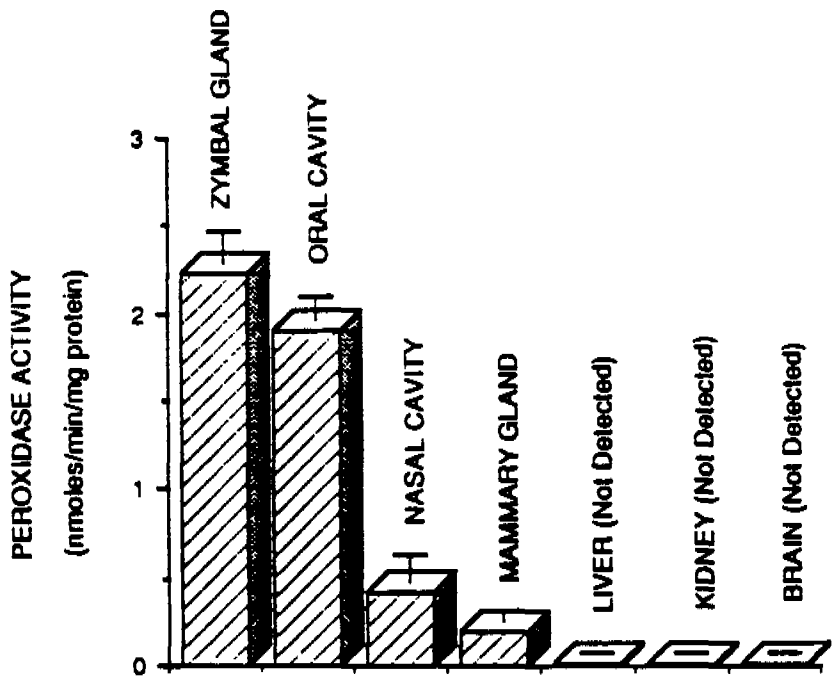

FIGURE 9. Peroxidase activity in target and nontarget tissues. Each bar represents specific peroxidase activity toward guaiacol as substrate. Values given are the mean ± standard deviation for three determinations. Liver, kidney and brain homogenates showed no detectable peroxidase activity; lower limit of detection for peroxidase assay was 0.1 nmole/min/mg protein. Bone marrow peroxidase activity was 295 ± 10 nmoles/min/mg protein with guaiacol as substrate.

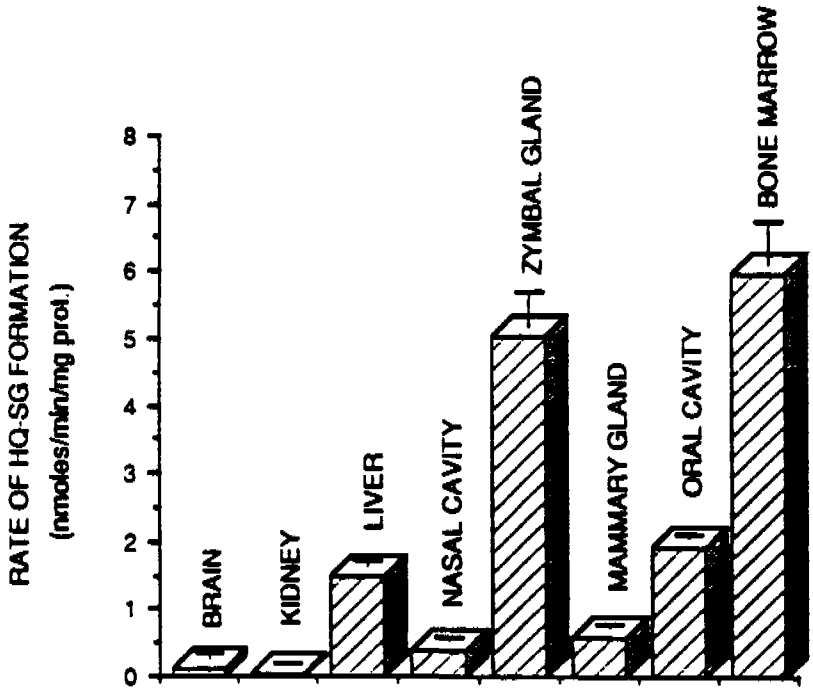

FIGURE 10. Trapping of reactive hydroquinone metabolite with glutathione in target and nontarget tissue (10,000 x g) homogenates. Each bar represents the rate of formation of the hydroquinone-glutathione conjugate 2-(S-glutathionyl)-hydroquinone (HQ-SG). Values are the mean ± standard deviation (n=3).

DNA ADDUCTS AND RELATED STUDIES

1.0 Postlabeling Studies

1.1 Postlabeling assay: Validation for benzene and metabolites.

The postlabeling assay is a highly sensitive technique for the detection of DNA adducts derived from structurally diverse aromatic carcinogens[1,2]. Detection limits are in the range of 1 adduct in 10^{9-10} DNA nucleotides, or about 1 adduct per cell.

For this project, the technique was validated and optimized using DNA modified *in vitro* by direct reaction with benzoquinone. The nuclease P1-enhanced postlabeling assay revealed the presence of one major adduct and several minor ones. The major adduct, representing about 82% of the total derivatives, appeared to be a guanine adduct, since it resembled chromatographically a major product formed by the reaction of benzoquinone with deoxyguanosine 3′-monophosphate, determined by HPLC and NMR analyses[3] to be (3′-OH)benzetheno-(N1,N2)deoxyguanosine. Alternative enhancement procedures such as extraction with butanol[4] and retention on reversed-phase TLC[5] gave much lower recovery of benzoquinone-DNA adducts as compared with nuclease P1-enhancement.

Dose-related DNA adducts were also detectable in Zymbal glands, a target organ for benzene carcinogenesis, when the tissue was treated in culture with benzene and its aromatic metabolites, phenol, hydroquinone, benzoquinone, catechol, and 1,2,4-benzenetriol (see section 1.2). Taken together, these results indicate that the nuclease P1 version of the ^{32}P-postlabeling assay is applicable for the sensitive measurement of adducts derived from benzene and its aromatic metabolites.

1.2 Benzene and metabolites produce DNA adducts in cultured Zymbal glands.

Tissue from rat Zymbal glands can be cultured for up to 48 hr with retention of metabolic capabilities, as demonstrated in validation studies using the known carcinogens, dimethylbenz(a)anthracene and 2-acetylaminofluorene. Patterns of adducts produced *in vitro* by these compounds were similar to those observed after *in vivo exposure*.

Similarly, various metabolites of benzene, i.e. phenol, hydroquinone, benzoquinone, catechol, and benzenetriol formed relatively high levels of DNA adducts (50-2000 adducts per 10^9 N) in Zymbal glands treated in culture, as measured by postlabeling. The adduct pattern was characteristic for each compound tested, suggesting the formation of compound-specific, DNA-binding electrophiles, e.g. phenol to phenoxy radical, hydroquinone to *p*-benzosemiquinone, catechol to *o*-benzosemiquinone, etc. Benzene produced low levels of adducts (i.e. about 0.5 adducts per 10^9 N) in culture.

Attempts to improve benzene adduct yields through variation of experimental conditions were unsuccessful. These modifications included: (1) incubation of tissue in a benzene-saturated atmosphere to maximize exposure; (2) incubation in a screw cap vial with zero-head space to prevent volatility losses; and (3) reducing benzene concentrations to 750, 375, and 188 μg/ml in order to reduce the possibility of toxic inhibition of activation enzymes. Zymbal glands were found to be metabolically inactive when incubated in a closed, zero-head space system, as evidenced by the absence of adducts from the positive control compound, dimethyl-benz(a)anthracene.

1.3 DNA adducts were not detected in mouse lymphoma cells treated with benzene or its metabolites.

Benzene, phenol, hydroquinone, catechol, and 1,2,4-benzenetriol are mutagenic in the mouse lymphoma assay with S9 metabolic activation. Highest concentrations of these compounds studied were, respectively, 8.8 μM, 5.7 μM, 1.1 mM, 2.5 mM, and 6.3 mM.

However, DNA adducts were not detected by the ^{32}P-postlabeling assay in mouse lymphoma cells treated with mutagenic doses of these compounds, although 1 out of 3 experiments showed dose-dependent formation of very low levels of a single adduct with benzene. These findings show that the mutagenicity of benzene and its metabolites is not a result of covalent adduction of DNA. Shorter incubation times, lower concentrations of test compound, or the difference in metabolic activation (rat liver S-9 vs. endogenous Zymbal enzymes) may account for the lack of adduct formation in mouse lymphoma cells as compared with Zymbal gland cultures.

1.4 DNA adducts were not detected in rats treated with benzene.

Following validation and optimization of the postlabeling assay with *in vitro* adducts (see sections 1.1 and 1.2), it was applied to the detection of DNA adducts *in vivo*. In an acute study, female Sprague-Dawley rats treated by oral gavage with a single dose of 500 mg/kg of benzene in 3 ml/kg olive oil did not show adducts by postlabeling either 6 or 24 hr after dosing. DNA samples from the kidney, liver, mammary gland, Zymbal gland, and bone marrow were assayed.

The absence of adducts after a single administration prompted us to examine DNA from animals treated with multiple doses. For this purpose, female Sprague-Dawley rats were treated by oral gavage with 200-500 mg/kg of benzene once a day, 5 days/week for 1 week, 5 weeks, and 10 weeks. At 24 hr after the last dosing, adducts were not detected in DNA samples of the liver, kidney, bone marrow, or mammary gland at any of the above time points. With Zymbal gland DNA, three week spots at levels totaling a maximum of 4 lesions per 10^9 DNA nucleotides were seen only after 10 weeks of treatment. These adducts did not chromatographically correspond to the major adducts detected with various aromatic metabolites in Zymbal gland tissue culture (see sections 1.1 and 1.2). Consequently this finding required confirmatory experiments.

A third *in vivo* experiment was, therefore, carried out. Female Sprague-Dawley rats were dosed by oral gavage with 200 mg/kg/day, 5 days/week for 10 weeks, 18 weeks, and 50 weeks. At 24 hr after the last dosing, DNA samples isolated from the liver, mammary gland, and Zymbal gland did not show adducts by nuclease P1-enhanced postlabeling for any of time points studied.

Taken together our studies indicate that benzene does not form *in vivo* DNA adducts which correspond to those derived from its aromatic metabolites, phenol, hydroquinone, benzoquinone, catechol, or benzenetriol. Although we detected low levels of unidentified adducts in some experiments, it is unlikely that these adducts can serve as sensitive markers for measuring low benzene exposures.

1.5 DNA adducts were not detected in rats dosed with phenol or phenol/hydroquinone.

The metabolites of some carcinogens have been shown to produce higher amounts of adducts than the parent compound itself. Thus, even though adducts were not detected after administration of benzene, the possibility remained that bypassing early steps in benzene metabolism by dosing with metabolites could lead to adduct formation. To test this proposal, we evaluated the adduct-forming capability of phenol and phenol/hydroquinone. Co-administration of phenol and hydroquinone has been shown to produce bone marrow suppression in mice similar to that produced by benzene[6]. Dosing of female Sprague-Dawley rats at 75 mg/kg phenol or phenol/hydroquinone for 4 days, followed by postlabeling analysis of adducts 24 hr later showed no adducts in the liver, Zymbal gland, or bone marrow. This result implies that phenol and hydroquinone are not likely to be mediating benzene-induced toxicity and carcinogenicity via covalent DNA adduction.

1.6 The postlabeling assay detects ring-opened benzene adducts, but these adducts are not present in vivo.

In addition to forming aromatic metabolites, benzene forms a polar ring-opened metabolite, muconaldehyde. Adducts formed from this compound are not detected under chromatographic conditions used for the analysis of adducts from aromatic metabolites. An alternative reversed-phase chromatography system was found suitable for the detection of muconaldehyde-DNA adducts, which were synthesized *in vitro* at relatively high levels of 4×10^5 lesions per 10^9 nucleotides. The detection limit of the assay for these adducts was 3 adducts per 10^{6-7} DNA nucleotides, about 3-4 orders of magnitude less sensitive than that for aromatic metabolite adducts. No muconaldehyde adducts were detected *in vivo* in the liver or Zymbal gland DNA of rats orally dosed with 200 mg/kg/day of benzene, 5 days/week for 18 weeks.

1.7 Benzene does not cause oxidative DNA damage.

Benzene metabolism produces hydroquinone and quinone. These compounds, through redox cycling, can produce oxygen radicals that, in turn, can initiate the formation of hydroxyl radicals. The hydroxyl radical is able to react with DNA to produce thymine glycols and 8-hydroxyguanine. We sought to measure thymine glycols as indicators of oxidative DNA damage by benzene. Thymine glycol adducts generated at relatively high levels by the reaction of osmium tetroxide with DNA were used for the development of sensitive postlabeling assays. Using this assay, we found no evidence of thymine glycol adducts in Zymbal gland DNA samples isolated from female Sprague-Dawley rats treated with 200 mg/kg benzene for 1, 5, 10, 18, and 50 weeks or in liver DNA after 18 weeks of treatment. These results suggest that benzene does not cause detectable levels of oxidative DNA damage. The detection limit was about 1 adduct in 10^7 DNA nucleotides.

2.0 Direct Labeling Studies

2.1 Radiolabeled benzene binds to DNA, RNA and proteins in rat tissues.

Failure to detect adducts by the postlabeling method can not be taken as an conclusive evidence that a compound does not form adducts, since some types of adducts are not amenable to enzymatic labeling and/or chromatographic recovery. Therefore, we have evaluated the DNA binding capability of benzene by the conventional technique, i.e. using radiolabeled benzene.

Pretreatment of female Sprague-Dawley rats i.p. with 500 mg/kg benzene for 5 days showed little or no induction in the binding of ^{3}H- or ^{14}C-benzene to rat liver DNA. Hydrolysis of this DNA to the nucleotide or base level followed by HPLC separation gave two peaks, which resolved from unmodified nucleotides or bases. Alkali treatment of the DNA prior to hydrolysis did not alter the HPLC profile, showing that the adducts are not alkali-labile *N*-7 guanine derivatives. When the two HPLC peak materials were isolated and postlabeled, no adducts were detectable, suggesting that direct labeled adducts are not amenable to detection by postlabeling.

To measure binding of benzene to various cellular macromolecules, rats pretreated by oral gavage with 25 mg/kg benzene for 6 weeks were given i.p. 0.31 mg/kg ^{3}H-benzene in 0.9% sodium chloride. At 24 hr after dosing, binding to various tissue proteins, (DNA + RNA), and DNA was evaluated (Tables 1 and 2). ((DNA+RNA) represents total nucleic acid preparation, while DNA represents the fraction obtained by treatment of (DNA+RNA) with ribonuclease.) Binding to (DNA+RNA) was 3 times higher than to proteins for the mammary gland, oral cavity, and Zymbal glands, but was only 1.3 times higher for liver. The (DNA+RNA) fraction also exhibited 8-25 times higher binding than the DNA fraction for the liver, mammary gland, and oral cavity, while the Zymbal gland DNA contained no radioactivity.

These results indicate that benzene metabolites primarily react with cytoplasmic electrophiles, and that RNA is a better binding substrate than proteins. Moreover, there was no correlation between a tissue's susceptibility to tumorigenesis and the extent of binding to DNA and proteins. For example, liver, a non-target organ, showed higher binding for these macromolecules than the target organs, mammary gland, oral cavity, and Zymbal gland. Lower binding values for DNA suggest that either benzene electrophiles are poorly transported across the nuclear membrane or that DNA in chromosomes is well protected from electrophilic attack.

Detection of protein binding in both target and non-target tissues allowed comparison of amino acid adduct profiles. Such comparisons could reveal electrophile differences among the tissues. Liver ^{3}H-protein hydrolysate showed two major and three minor amino acid adduct peaks (Figure 1). A similar pattern was obtained with liver ^{14}C-proteins. Zymbal gland protein hydrolysate exhibited one major peak, which was present at significant levels in the other target organs, oral cavity, and mammary gland, but was a minor component in the liver protein hydrolysate (Table 3). These results suggest that protein-binding electrophiles quantitatively varied among target and non-target tissues. Whether this difference relates to differential susceptibility of tissues to tumorigenesis remains to be elucidated.

3.0 Specific Binding Studies

3.1 Studies on benzene-specific binding sites on the cell membrane.

A chemical can induce or contribute to the induction of cancer either by causing an irreversible genetic change (initiation) or by causing an epigenetic change which involves induction of cell proliferation (promotion) or both. Compounds inducing cell proliferation often show binding to specific sites on the cell membrane; this binding triggers a cascade of events leading to a stimulation of biochemical pathways involved in cell division. We have, therefore, investigated the possibility that benzene's carcinogenicity is mediated by metabolite initiation and parent compound promotion.

To determine if benzene shows specific interaction with receptors in target tissue, we have performed competitive binding studies using labeled and unlabeled benzene. Preliminary results showed competitive benzene binding or compartmentalization in rat bone marrow and blood cells (Figures 2 and 3). However, several hundred-fold excess of cold benzene was required for competition, suggesting that the interaction is unlikely to be receptor mediated. A possible explanation for such low level competition is that unlabeled benzene is merely inhibiting the uptake or facilitating the release of radiolabeled benzene from a storage compartment.

We have also shown that toluene and xylene compete with benzene -- apparently somewhat more effectively than benzene itself. Rat bone marrow homogenate showed

3 times higher benzene binding than liver homogenate (Table 4), while no binding was observed with the Zymbal gland homogenate. Whatever the mechanism of benzene binding to tissue homogenate, our results show that this phenomenon does not correlate with a tissue's susceptibility to solid tumorigenesis.

4.0 Summary

DNA adducts were produced *in vitro* by benzene and its aromatic metabolites in cultured rat Zymbal gland, a target for benzene solid tumor carcinogenesis. These adducts were detected by the ^{32}P-postlabeling assay at a frequency as low as 1 adduct per 10^9-10^{10} DNA nucleotides. Corresponding adducts were not formed in this organ when rats were treated with multiple doses of benzene, phenol, or phenol plus hydroquinone, indicating that covalent DNA damage by aromatic metabolites is not likely to play a role in benzene-induced carcinogenesis. *In vivo* DNA damage by the ring opened product, muconaldehyde, or by benzene-induced oxygen radicals also was not detected. However, the postlabeling assay was 100-1000 times less sensitive for adducts derived from these compounds than those from aromatic metabolites.

Use of radiolabeled benzene showed that some covalent DNA adducts occur in target (Zymbal gland, oral cavity) and non-target tissues (liver) of rats, but the extent of DNA damage was not correlated with the tissue's susceptibility to tumorigenesis. These adducts may be labile and therefore not detected under the conditions used in the postlabeling assay. Studies aimed at determining the persistence and repair of these adducts would help determine their role, if any, in carcinogenesis.

The possible role of benzene as a promotor of carcinogenesis was investigated by attempting to identify specific membrane binding sites in target tissue. However, no receptors were detected in homogenates of Zymbal gland tissue using standard competitive binding protocols.

In conclusion, benzene neither induces DNA adducts nor interacts with specific binding sites in target tissue. Therefore, neither of these endpoints serves as a correlate for benzene solid tumor carcinogenicity.

5.0 References

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Table 1. Covalent Binding of ^{3}H-benzene to protein and nuclei acid fraction in target and non-target tissues of rats treated with 0.31 mg/kg

Tissue	pmole of ^{3}H-benzene equivalents bound per mg marcomolecular fraction		
	Protein	Nucleic acids	DNA
Liver	0.166	0.216	0.025
Mammary gland	0.108	0.365	0.0015
Oral cavity	0.070	0.239	0.013
Zymbal gland	0.061	0.172	ND

Protein and nucleic acid fractions were isolated by standard solvent extractions and precipitation with acetone or ethanol. The pellets were exhaustively washed until no counts detected in the washes. Protein was estimated using Bio-Rad protein assay kit; nucleic acid fraction was measured spectrophotometrically assuming 1 mg DNA equivalent to 20 absorbing units at 260 nm. A portion of macromolecular fraction was digested and measured for radioactivity. DNA samples were prepared from the nucleic acid fractions by proteinase K and ribonuclease treatments, solvent extractions, and alcohol precipitation.

Table 2. Distribution of radioactivity into protein and DNA of target and non-target tissues following 0.31 mg/kg ^{3}H-benzene[a]

Tissue	pmole of ^{3}H-benzene equivalents bound per mg macromolecule		No. of adducts per 10^9 DNA nucleotides
	Protein	DNA	
Bone Marrow	0.074 + 0.015	0.089 + 0.009	27.6
Liver	0.290 + 0.116	0.022 + 0.003	6.9
Mammary gland	0.159 + 0.093	0.003 + 0.002	1.0
Oral Cavity	0.051 + 0.018	0.022 + 0.010	6.7
Zymbal gland	0.061	NM[b]	NM

[a] See footnotes of Table 1 for macromolecular extractions and radioactive determinations. The values represent mean + standard deviation obtained for three rats. In the case of Zymbal glands, the tissue was pooled prior to macromolecular isolation.

[b] NM = Not measurable.

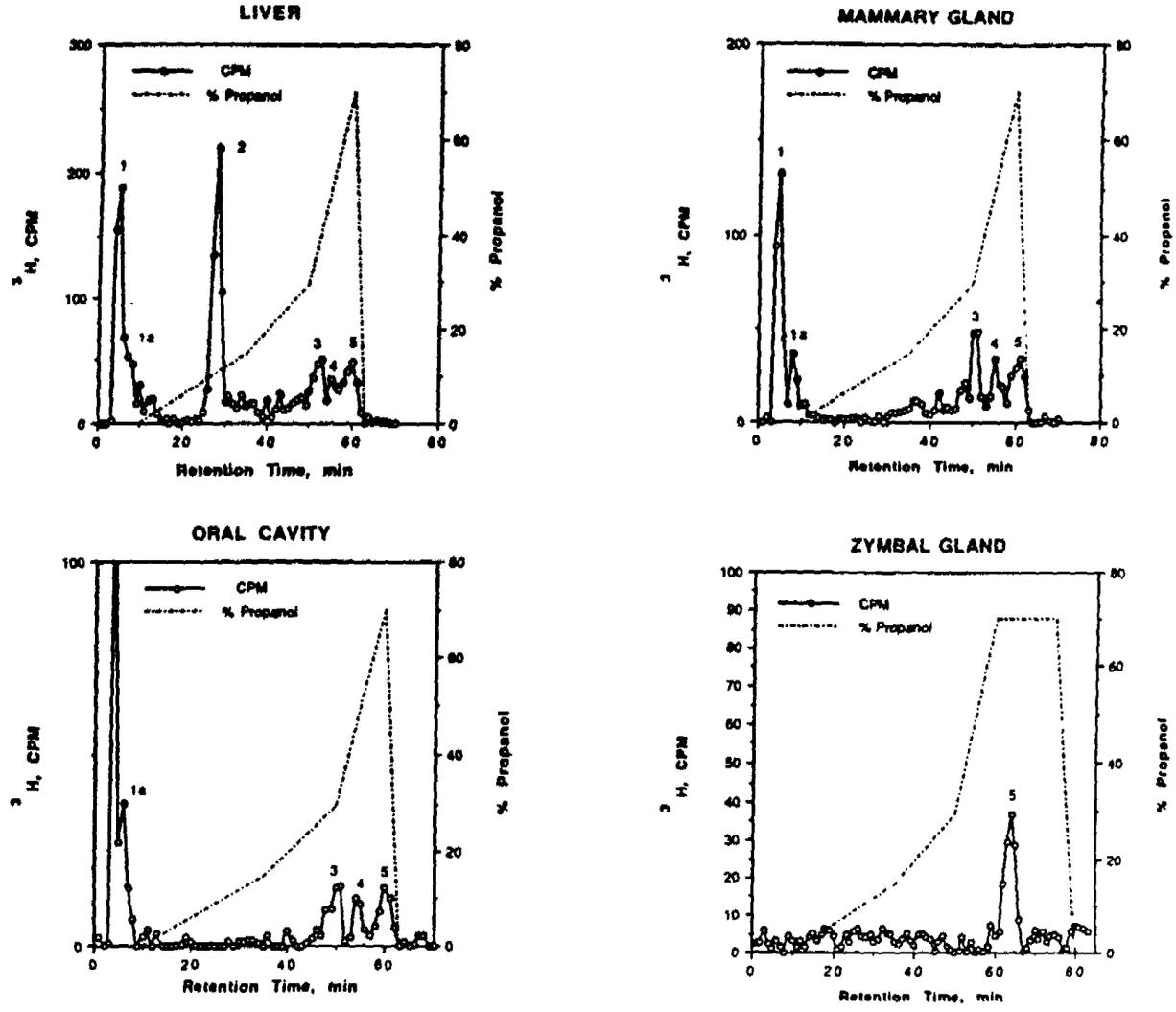


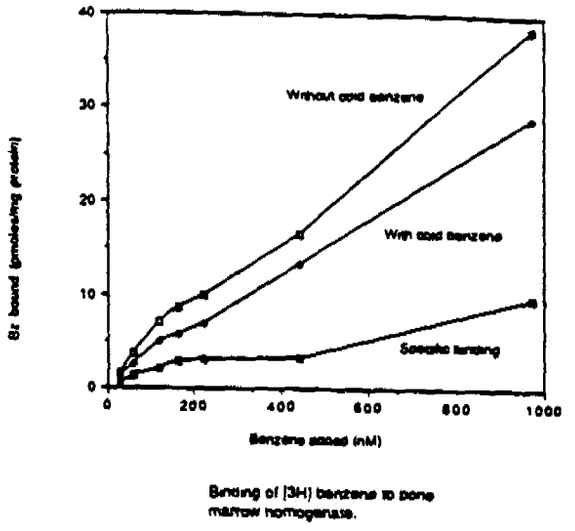
Fig. 1. HPLC profiles of protein hydrolysates from the indicated tissues. Female Sprague-Dawley rats pretreated with 25 mg/kg benzene were dosed with ^{3}H-benzene (0.31 mg/kg, Sp. Act. 4.1 Ci/mmol) and harvested the tissues at 24 hr later. Tissue proteins were isolated by a solvent extraction method and hydrolyzed with 6 N HCl at 100 °C for 18 hr. The hydrolysates were resolved by reversed-phase HPLC using starting buffer 0.017 M sodium citrate containing 0.3% sodium dodecyl sulfate and a gradient of n-propanol. Fractions (1 ml) were collected, and their radioactivity was determined by counting in Beckman^R liquid scintillation counter after the addition of 10 ml of liquid scintillator.

Table 3. Relative proportions of individual amino acid-benzene adducts in tissue protein hydrolysate[a]

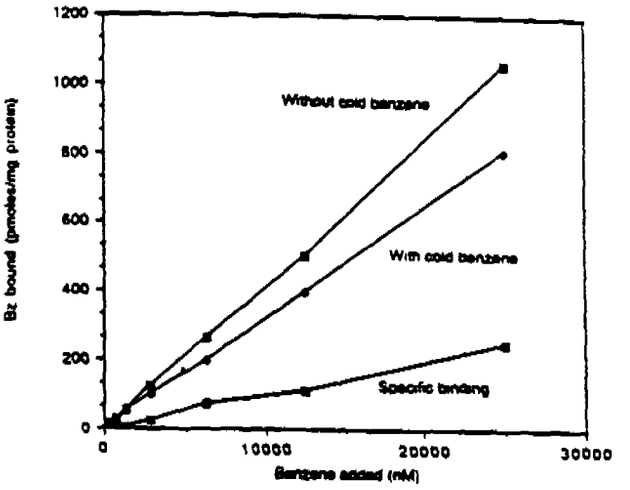
Peak no.	Relative percentage			
	Liver	Mammary gland	Oral cavity	Zymbal gland
2	51.3	ND[b]	ND	ND
3	19.1	35.7	39.9	ND
4	10.7	28.8	23.9	ND
5	18.9	35.5	36.2	100

[a] See Figure 1 for HPLC profiles of tissue protein hydrolysates. Radioactivity in individual peaks shown in Fig. 1 was used to calculate relative proportions of each peak.

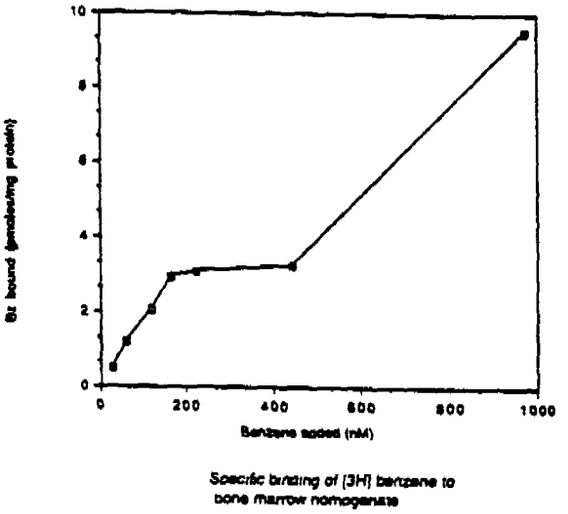
[b]Not detected.



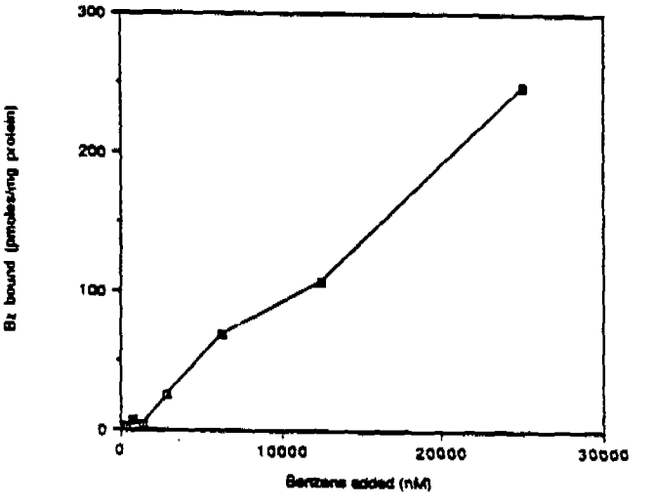
Binding of [3H] benzene to bone marrow homogenate.



Binding of [14C] benzene to bone marrow homogenate.



Specific binding of [3H] benzene to bone marrow homogenate



Specific binding of [14C] benzene to bone marrow homogenate.

Fig. 2. Binding of ^{3}H- and ^{14}C-benzene to rat bone marrow homogenate. Bone marrow homogenate (0.3 mg) prepared from female Sprague-Dawley rats was incubated at 2 °C for 10 min with different concentrations of ^{3}H- or ^{14}C-benzene with or without cold benzene (1300 nM) in 0.2 ml phosphate buffer, pH 7.4. After incubation, the reaction mixture was centrifuged at 18,000 g for 45 min at 2 °C. The radioactivity in the pellets was determined by counting in Beckman^{R} liquid scintillation counter after addition of 10 ml of liquid scintillator.

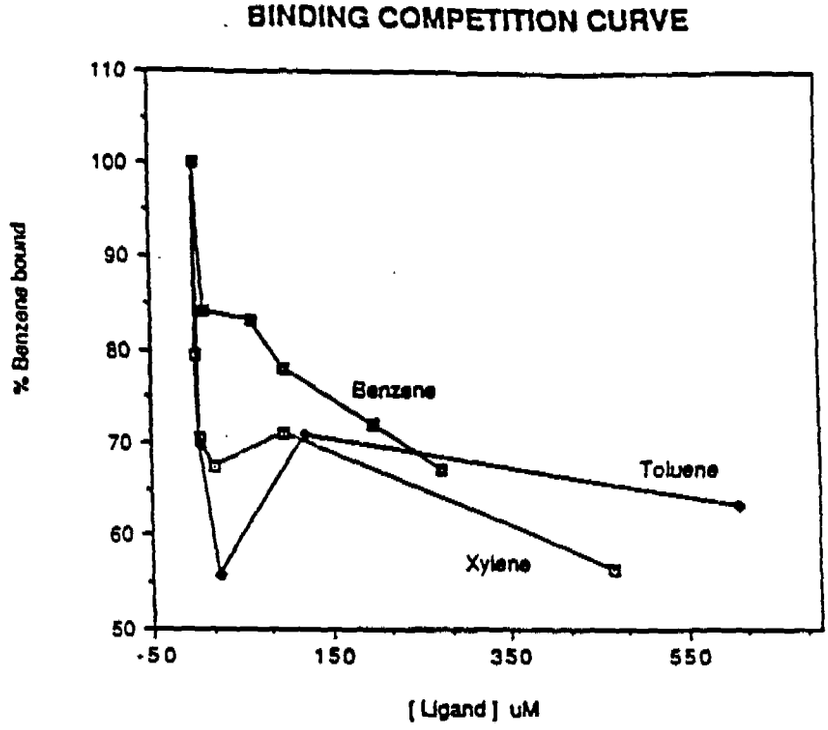


Fig. 3. Competition of binding of ^{3}H-benzene to rat bone marrow homogenate by benzene, toluene, and xylene. The binding of ^{3}H-benzene (about 100 nM) to bone marrow homogenate (0.3 mg) was measured in the absence or presence of the indicated concentrations of cold benzene, toluene, and xylene. The binding assay was performed as given in the legend of Figure 2.

Table 4. Comparison of benzene-specific binding activity in the homogenates of rat bone marrow and liver.

Tissue	^{3}H-Benzene added (nM)	^{3}H-Benzene bound (pmole/mg protein)
Bone marrow	118	2.06
	222	3.09
Liver	114	0.81
	227	1.17

The binding assay was performed as given in the legend of Figure 2. The binding of the indicated concentration of ^{3}H-benzene to tissue homogenate (0.3 mg) prepared from female Sprague-Dawley rats was measured in the presence or absence of cold benzene (1.3 mM). The decrease in binding values in the presence of cold benzene is considered to represent benzene bound.

GENETIC TOXICOLOGY/CELL AND TISSUE CULTURE STUDIES

1.0 Mutagenesis Testing

In most mutagenicity assays, benzene has been shown to be inactive[1], while in chromosome-level tests (i.e. cytogenetics assays), it has proven to be a very potent clastogen, inducing aberrations, sister chromatid exchanges, and micronuclei[2]. We reexamined benzene's genotoxicity in test systems designed to overcome certain procedural problems in most previous studies including (a) volatility losses, (b) potentially inadequate or inappropriate induction of S-9, and (c) test methods relatively insensitive to the detection of chromosomal damage as a mutagenic event.

1.1 Ames test results for benzene using Aroclor- and benzene-induced S-9.

Benzene was tested in the Ames *Salmonella* pre-incubation assay in strains TA98 and TA100, with and without metabolic activation provided by Aroclor 1254-induced or benzene-induced rat S-9. Although toxicity was evident in the assay, indicating interaction of benzene with the bacteria, no mutagenicity was observed. Since this result agreed with previously published Ames test data for benzene, and did not suggest any advantage to benzene induction of S-9, more detailed studies were not undertaken.

1.2 Mouse lymphoma L5178Y TK+/- mutagenesis assay of benzene.

In studies conducted in parallel to the Ames test described above (Section 1.1), benzene was also tested in the mouse lymphoma mutagenesis assay in the presence and absence of rat liver S-9 induced by Aroclor 1254 or benzene. Two test methodologies were employed: the standard procedure of Clive et al.[3], and an abbreviated method developed in the Mobil Toxicology Division, which allows microscopic detection of mutant clones at approximately the 2-32 cell stage. The latter method offers increased sensitivity by permitting enumeration of all mutant clones, instead of only clones which grow to sufficient size to be visible on a soft agar plate.

Tables 1 and 2 show the results of the testing with metabolic activation. Benzene was weakly mutagenic in the standard assay (Table 1), as evidenced by a dose-response and a doubling of mutant frequency relative to the untreated control. Table 2 compares the results of the abbreviated and standard assays. Again, clear evidence of mutagenicity was obtained, with a greater than three-fold increase in mutant frequency observed in the abbreviated assay. No evidence of mutagenicity was seen in the nonactivated test.

1.3 Postlabeling analysis of mouse lymphoma cells treated with benzene.

The observation of benzene mutagenicity in the mouse lymphoma assay prompted us to

examine concurrently treated cells for the presence of DNA adducts. The initial experiment, using the extremely sensitive ^{32}P-postlabeling method, showed the presence of a single dose-related adduct. This result suggested that benzene's mutagenicity might be mediated by adduct formation. However, several subsequent postlabeling analyses failed to confirm this finding, and we concluded that alternative mechanisms must be involved in benzene mutagenicity. The postlabeling studies are described in greater detail in the "DNA Adducts and Related Studies" section of this report.

1.4 Mouse lymphoma testing of benzene metabolites.

Dependence of mutagenicity on the presence of S-9 activation indicates that only metabolite(s), and not the parent compound itself, are mediating the genotoxic effects. That being the case, determination of which metabolites are mutagenic can lead to a better understanding of the parent compound's mechanism of action and the critical pathways leading to expression of its carcinogenicity.

The following benzene metabolites were tested for mutagenic activity in the mouse lymphoma assay: phenol, hydroquinone, catechol, 1,2,4-benzenetriol, benzoquinone, and *t,t*-mucondialdehyde. The results of that testing are shown in Table 3. The highest mutagenicity is shown by the di- and trihydroxy benzenes, which are active both in the presence and absence of metabolic activation. Significant activity in the absence of S-9 was also seen for benzoquinone and *t,t*-mucondialdehyde, but not for benzene itself or phenol.

These results suggest that expression of genotoxicity in this test system is dependent on the formation of multiply hydroxylated metabolites. If this interpretation is correct, then the significant mutagenic activity of these compounds in the absence of exogenous metabolic activation may reflect either their direct reaction with DNA, or, more likely, their further oxidation to highly reactive species. Data from the *in vitro* Zymbal gland tissue culture system (see "DNA Adducts and Related Studies") suggests that these species may be semiquinone radicals, such as are formed by chemical oxidation of di- and trihydroxy benzenes.

1.5 Postlabeling analysis of mouse lymphoma cells treated with benzene metabolites.

The potent mutagenicity of hydroxylated metabolites of benzene in the mouse lymphoma assay could be mediated either by the formation of metabolite-DNA adducts or by alternative DNA damaging mechanisms such as strand breaks, cross links, or base hydroxylation. Postlabeling analysis of mouse lymphoma cells treated with phenol, hydroquinone, catechol, benzoquinone, or 1,2,4-benzenetriol at mutagenic doses produced no evidence of significant DNA adduction, indicating that the further activation of these metabolites by rat liver S-9 does not lead to their direct covalent binding to DNA. These results shifted the emphasis of the *in vitro* genetic toxicology work toward the alternative endpoints cited above.

2.0 DNA Strand-Break Analyses

The absence of DNA adducts in mouse lymphoma cells treated with mutagenic doses of benzene and major metabolites prompted an investigation of alternative mechanisms for induction of DNA damage and mutation. The known clastogenicity of benzene and several of its hydroxylated metabolites pointed toward DNA strand scission as a promising endpoint. Pellack-Walker[4], in an investigation of benzene genotoxicity, used the assay of Rydberg[5] as a rapid screening test for strand-break induction. We adopted the same method for analysis of strand-breaks in the mouse lymphoma L5178Y system.

2.1 Strand-break analysis of benzene and metabolites: Nonactivated system.

Table 4 shows the results of strand break analysis for mouse lymphoma cells treated with mutagenic doses of benzene, phenol, hydroquinone, catechol, 1,2,4-benzenetriol, and benzoquinone. There was no evidence of significant strand-break induction by any of the compounds, indicating that, in the absence of S-9, no reactive intermediates are generated in the test system.

2.2 Strand-break analysis of benzene: Activated system.

The results of testing in the nonactivated system showed that neither benzene nor its hydroxylated metabolites were themselves capable of directly or indirectly interacting with DNA to induce strand scission. Therefore, we used an S-9 activated system to determine if enzymatic and/or other biochemical reactions could produce reactive DNA-damaging species either directly from benzene or via its known hydroxylated metabolites. The data in Table 5 and Figure 1 show a dose-related increase in DNA strand breaks over a range of doses previously shown to be mutagenic to mouse lymphoma cells. These results are consistent with several features of benzene's genotoxicity: (1) benzene is mutagenic in the mouse lymphoma mutagenicity assay with activation (Section 1.2); (2) benzene is a potent clastogen in mouse bone marrow cells; and (3) benzene does not produce DNA adducts at doses shown to be mutagenic to mouse lymphoma cells (Section 1.3).

3.0 In Vitro Cytogenetic Analyses

3.1 Introduction and rationale.

The known clastogenicity of benzene, as well as the ability of benzene, phenol, hydroquinone, catechol, 1,2,4-benzenetriol, and benzoquinone to induce sister chromatid exchanges[6] suggested that the mutagenicity of these compounds in the mouse lymphoma assay might be mediated by chromosome-level damage unrelated to adduct

formation. This conjecture was further strengthened by the observation of DNA strand-break induction by benzene in the presence of S-9. We therefore selected the most potent benzene-derived mutagen in the mouse lymphoma system, catechol, for investigation of clastogenicity *in vitro*.

3.2 Aberration induction in Chinese hamster ovary (CHO) cells by catechol.

The results of cytogenetic analysis of CHO cells treated with catechol in the presence and absence of Aroclor 1254-induced rat liver S-9 are presented in Table 6. The data show that catechol is a potent clastogen in both the activated and nonactivated systems. These results are particularly noteworthy inasmuch as similar doses of catechol failed to produce DNA adducts or DNA strand-breaks in mouse lymphoma cells. Taken together, these data and those from previous studies suggest that benzene exerts its genotoxic effects primarily through chromosome-level damage rather than via adduction, point-mutation, or even the simple induction of single-strand breaks.

4.0 Other Endpoints for Genotoxicity In Vitro

4.1 Protein adduction by benzene.

Mouse lymphoma cells were treated with ^{14}C-benzene, and nuclear and cytoplasmic fractions were isolated by differential centrifugation. Liquid scintillation counting of these fractions showed significant binding of radiolabel to the cytoplasmic fraction, but little incorporation of label into the cell nuclei. Repeated washing of the fraction failed to release label, suggesting that a benzene metabolite had bound covalently to macromolecules, probably cytoplasmic proteins. While these results cannot be considered definitive, they do suggest that certain benzene metabolites are capable of covalent adduction of cellular macromolecules, but that virtually all of the reactive species are consumed in reactions with cytoplasmic nucleophiles.

4.2 Protein adduction by catechol.

An experiment analogous to that described in Section 4.1 was done using ^{14}C-catechol. This potent mutagen in mouse lymphoma cells produced significant binding to cellular protein, which survived solubilization in sodium dodecyl sulfate (SDS) and mercaptoethanol and electrophoresis in SDS polyacrylamide gels. The profile of counts recovered from an electrophoretic separation of mouse lymphoma proteins is shown in Figure 2. Virtually all the proteins separated on the gel show significant levels of bound radioactivity. This result is consistent with the presence of a highly reactive metabolic intermediate that rapidly binds to nucleophiles near its point of formation. If correct, this inference supports the conclusions drawn in Section 4.1 above, and suggests a mechanism for induction of chromosome-level damage in the absence of detectable DNA adducts or strand breaks, i.e. protein-protein or DNA-protein crosslinking at critical sites

in chromatin. While such a mechanism is difficult to test, and was therefore beyond the scope of the original project, it does offer a consistent explanation for the genotoxicity and adduct findings in this study.

5.0 Zymbal Gland Tissue Culture Studies

5.1 Introduction.

In vivo studies of Zymbal gland metabolism and the processes leading to carcinogenesis are severely hampered by the small size of the gland as well as the inability to administer doses of test compound high enough to produce measurable biochemical effects. These problems can be partially overcome by excising the gland from the animal, maintaining its viability in tissue culture, and dosing with high concentrations of test compound. For this approach to be valid, metabolism in tissue culture must mimic that *in vivo* for a sufficient time period to produce the biochemical effects of interest. Early in the Mechanistic Research Project, we developed a tissue culture system for Zymbal gland that accomplished this objective.

5.2 In vitro *metabolism studies using Zymbal gland tissue culture.*

Culture medium from fragment cultures of Zymbal gland treated with ^{14}C-benzene was extracted with ethyl acetate, and the extract separated by HPLC. Radioactivity was monitored using a radiometric detector. No metabolite in excess of 2% of parent compound (benzene) concentration was detected after either 6 or 24 hr of culture. Either benzene itself is not efficiently metabolized by Zymbal tissue (as suggested by numerous subsequent studies), or the levels of metabolites formed in this experiment were too low to be detected by the analytical method employed. Details of this study are presented on page 5 of this report.

5.3 ^{32}P-postlabeling analysis of Zymbal gland DNA from in vitro *culture.*

Zymbal gland in tissue culture was treated with benzene and major metabolites of benzene, including phenol, hydroquinone, catechol, 1,2,4-benzenetriol, and benzoquinone. DNA was extracted from the tissue at various time points following administration, and ^{32}P-postlabeling analysis used to detect any DNA adducts formed. These experiments are described in detail on page 31 of this report.

In short, the studies showed that each of the metabolites produced a significant level of adduction; the adduct pattern for each was unique. Benzene itself produced little or no adduction. These results are consistent with a one-step activation of each metabolite via a free-radical mechanism to a DNA adducting species. Although the significance of this finding with respect to *in vivo* metabolism is difficult to assess, it does illustrate that benzene-derived DNA adducts are detectable by the postlabeling method -- a result of

some significance in light of the absence of postlabeling adducts in the *in vivo* studies.

6.0 Zymbal Gland Cell Culture Studies

6.1 Introduction.

The project proposal for the Benzene Mechanistic Research Program emphasized two potential dose-response correlates for benzene solid tumor carcinogenesis: DNA adducts and cytogenetic endpoints such as chromosome aberrations, sister chromatid exchange, or micronucleus induction. Our proposal to use DNA adduct formation as a correlate was based upon the strong association between this endpoint and the initiation of carcinogenesis; the selection of the alternate cytogenetic endpoint acknowledged the known potent clastogenicity of benzene in bone marrow cells. As it became evident that benzene induces few if any DNA adducts, methods were developed to permit analysis of chromosomal damage in Zymbal gland and other solid tissue targets.

6.2 Micronucleus assay in primary cultures from Zymbal gland explants.

The development of a method for producing *in vitro* cultures by outgrowth of cells from Zymbal gland tissue explants made possible the assessment of cytogenetic damage incurred *in vivo* following benzene administration. Early experiments using this system were directed toward the detection of chromosome aberrations or higher frequencies of sister chromatid exchange, but during evaluation of the slides made from the explant cultures, an apparent effect on micronucleus frequency was noted. Examination of a series of cultures derived from rats treated with varying concentrations of benzene revealed a clear-cut dose-response for micronucleus induction (data not shown).

Exploiting this finding, we designed additional experiments to delineate the dose-response curve for micronucleus induction; first, to determine if the curve paralleled that for cancer induction in Zymbal gland, and second, to extend the curve into the dose range of particular interest in the project, i.e. between 0 and 50 mg/kg. Several oral dosing regimens, emphasizing different regions of the dose response curve, were employed in treating both Sprague-Dawley female and Fisher 344 male rats. The data from these studies are shown in Tables 7-12.

The data show a consistent induction of micronuclei following a single dose, three consecutive daily doses, or chronic (26 weeks) dosing with benzene. Sprague Dawley female and Fisher male rats, both susceptible to Zymbal gland carcinogenesis, appear to be equally susceptible to cytogenetic damage. Based on the data acquired thus far (Figure 3), the dose-response curves for carcinogenesis and micronucleus induction appear to follow a similar pattern of decline with decreasing dose, and clearly, the micronucleus endpoint has sufficient sensitivity to establish a dose-response at or below the 10 mg/kg level.

Although both sets of data in Figure 3 are well-fit by a linear function of dose, there is still insufficient precision in the micronucleus data to establish the shape of the curves below 25 mg/kg. Experiments designed to accurately determine background frequency and low-end dose-response are described below.

6.3 Detailed examination of the dose-response for micronucleus induction.

The strong likelihood that cytogenetic damage is involved in the initiation of solid tumor carcinogenesis in rat Zymbal gland suggests that the dose-response curve for micronucleus induction will follow the same pattern as that for carcinogenicity. Thus, determination of the shape of this curve at low doses could reveal whether benzene induction of solid-tumors follows linear kinetics to zero dose, as is assumed in the absence of data to the contrary, or if it is sub- or supralinear. Determination of an "effective threshold" might also be possible. In order to accomplish these goals, both the background rate and the induction frequency of micronuclei must be precisely determined in the range between zero dose and the minimum observably carcinogenic dose.

7.0 References

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3. Clive, D., Johnson, K.O., Spector, J.F., Batson, A.G., and Brown, M.M.M. (1979) Validation and characterization of the L5178Y TK +/- mouse lymphoma mutagen assay system. *Mutat. Res.* **59**: 61-108.

4. Pellack-Walker, M. (1986) Mechanism associated with benzene-induced DNA damage. Doctoral Dissertation, Department of Pharmacology, Case Western Reserve University, University Microfilms International, Ann Arbor, MI.

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TABLE 1

Mutagenicity of benzene in the Mouse Lymphoma L5178Y TK+/- Mutagenesis Assay.

TEST MATERIAL	DOSE *ul/ml*	MUTANT FREQUENCY X 10^4	TOTAL GROWTH %
BENZENE	0.8	0.56[a]	38.6, 33.3[b]
	0.6	0.50	61.6, 76.9
	0.4	0.46	113, 149
DMBA	5.0 *ug/ml*	4.30	40.9, 58.7
UNTREATED CONTROL	----	0.24	100

[a]Mutant frequencies are means of two determinations.

[b]Total growths are values for individual cultures.

TABLE 2

Comparison of benzene mutagenicity in the standard and abbreviated Mouse Lymphoma Mutagenesis Assays using Aroclor 1254- and benzene-induced rat liver S-9.

TEST MATERIAL	DOSE *ul/ml*	S-9	MUTANT FREQUENCY X 10^4	
			STANDARD ASSAY	ABBREVIATED ASSAY
BENZENE	0.8	A[a]	0.49	2.75
BENZENE	0.8	B	0.15	0.91
DMBA	5.0 *ug/ml*	A	3.84	22.7
DMSO	10.0	B	0.18	0.78

[a]A is Aroclor 1254-induced, B is benzene-induced rat liver S-9.

TABLE 3

Relative mutagenic potencies of benzene and major metabolites in the Mouse Lymphoma L5178Y TK+/- Mutagenesis Assay.

COMPOUND	S-9	RELATIVE POTENCY
BENZENE	–	0
	+	2
PHENOL	–	0
	+	2
HYDROQUINONE	–	7
	+	3
CATECHOL	–	4
	+	7
BENZENETRIOL	–	3
	+	5
BENZOQUINONE	–	5
	+	2
MUCONALDEHYDE	–	3
	+	0

TABLE 4

Induction of DNA single-strand breaks by benzene and its metabolites in the absence of metabolic activation.

COMPOUND	CONCENTRATION	PERCENT SINGLE STRAND
BENZENE[a]	1.2 *ul/ml*	29.9
	1.0	29.4
	0.8	27.7
	0.6	28.1
	0.0	24.6
PHENOL	1.0 *ul/ml*	19.9
	0.5	11.3
	0.25	11.0
	0.1	10.2
HYDROQUINONE	10.0 *ug/ml*	15.2
	7.5	18.3
	5.0	21.7
	2.5	7.3
CATECHOL	100.0 *ug/ml*	6.2
	75.0	6.0
	50.0	6.2
	25.0	6.9
	0.0	6.5
BENZOQUINONE	1.25 *ug/ml*	7.2
	1.00	5.7
	0.75	6.3
	0.50	11.1
BENZENETRIOL	25.0 *ug/ml*	13.0
	15.0	20.2
	5.0	13.0
	1.0	18.0
	0.0	16.8

[a]Averaged data

TABLE 5

Induction of DNA single-strand breaks by benzene in the presence of metabolic activation.

CONCENTRATION (*ul/ml*)	PERCENT SINGLE STRAND
1.2	63.1
1.2	65.9
1.2	65.0
MEAN	**64.7**
1.0	63.9
1.0	61.1
1.0	56.5
MEAN	**60.5**
0.8	48.8
0.8	48.6
0.8	47.9
MEAN	**48.4**
0.6	42.7
0.6	44.1
0.6	39.2
MEAN	**42.0**
0.0	28.9
0.0	27.3
0.0	25.3
MEAN	**27.2**

FIGURE 1

DOSE RESPONSE CURVE FOR INDUCTION OF SINGLE-STRAND BREAKS BY BENZENE IN THE PRESENCE OF S9

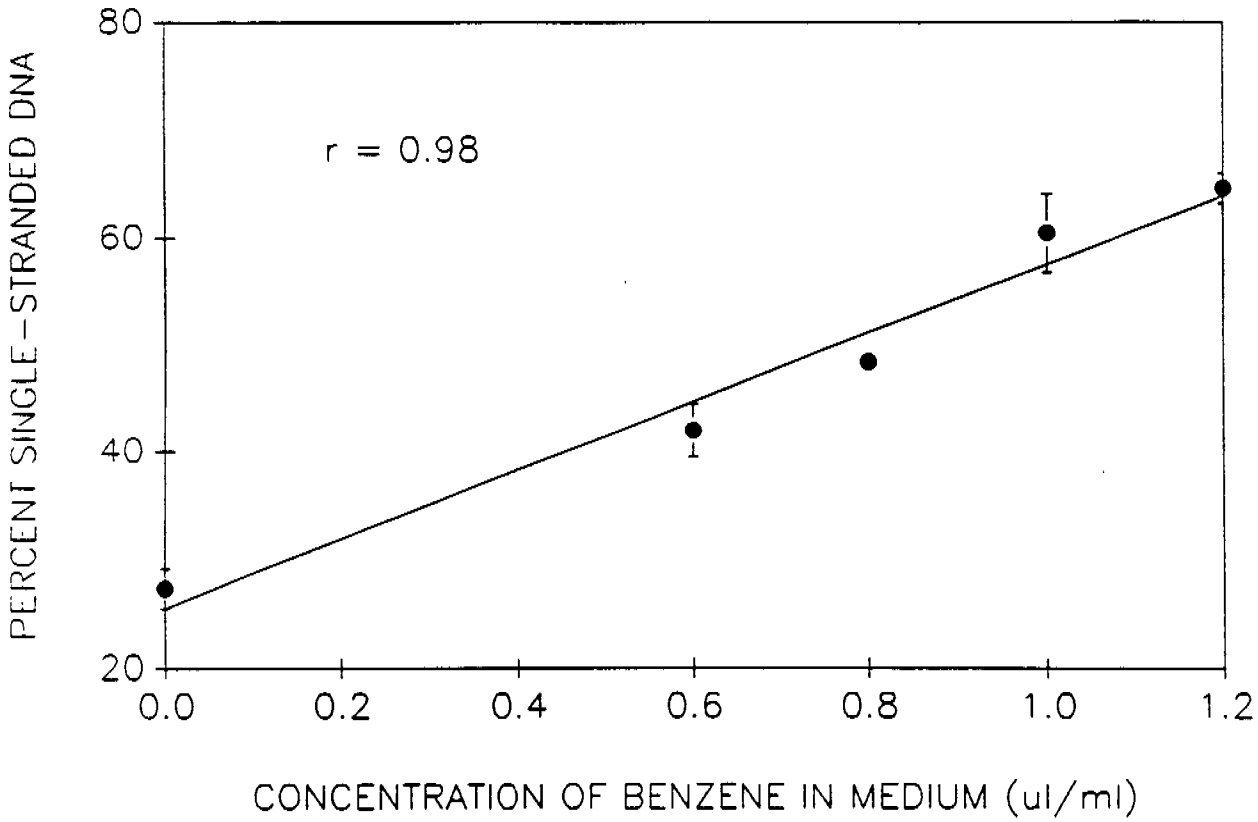


TABLE 6

Induction of chromosomal aberrations in Chinese hamster ovary (CHO) cells by catechol.

DOSE	EFFECTS
Without S-9	
100 *ug/ml*	21% of cells with aberrations 7% with chromatid breaks/deletions 7% with chromatid exchanges 3% with chromosome breaks 1% with dicentric chromosome 1% with ring chromosome 4% of cells had > 10 aberrations 5% of cells were pulverized
Negative Control	4% of cells with aberrations 3% with chromatid breaks/deletions 1% with chromosome breaks
With S-9	
100 *ug/ml*	17% of cells with aberrations 6% with chromatid breaks/deletions 2% with chromatid exchanges 2% with chromosome breaks 1% with dicentric chromosome 2% with double minutes 3% of cells with > 10 aberrations 5% of cells were pulverized
Negative Control	6% of cells with aberrations 2% with chromatid breaks/deletions 2% with chromosome breaks 2% with chromatid exchanges

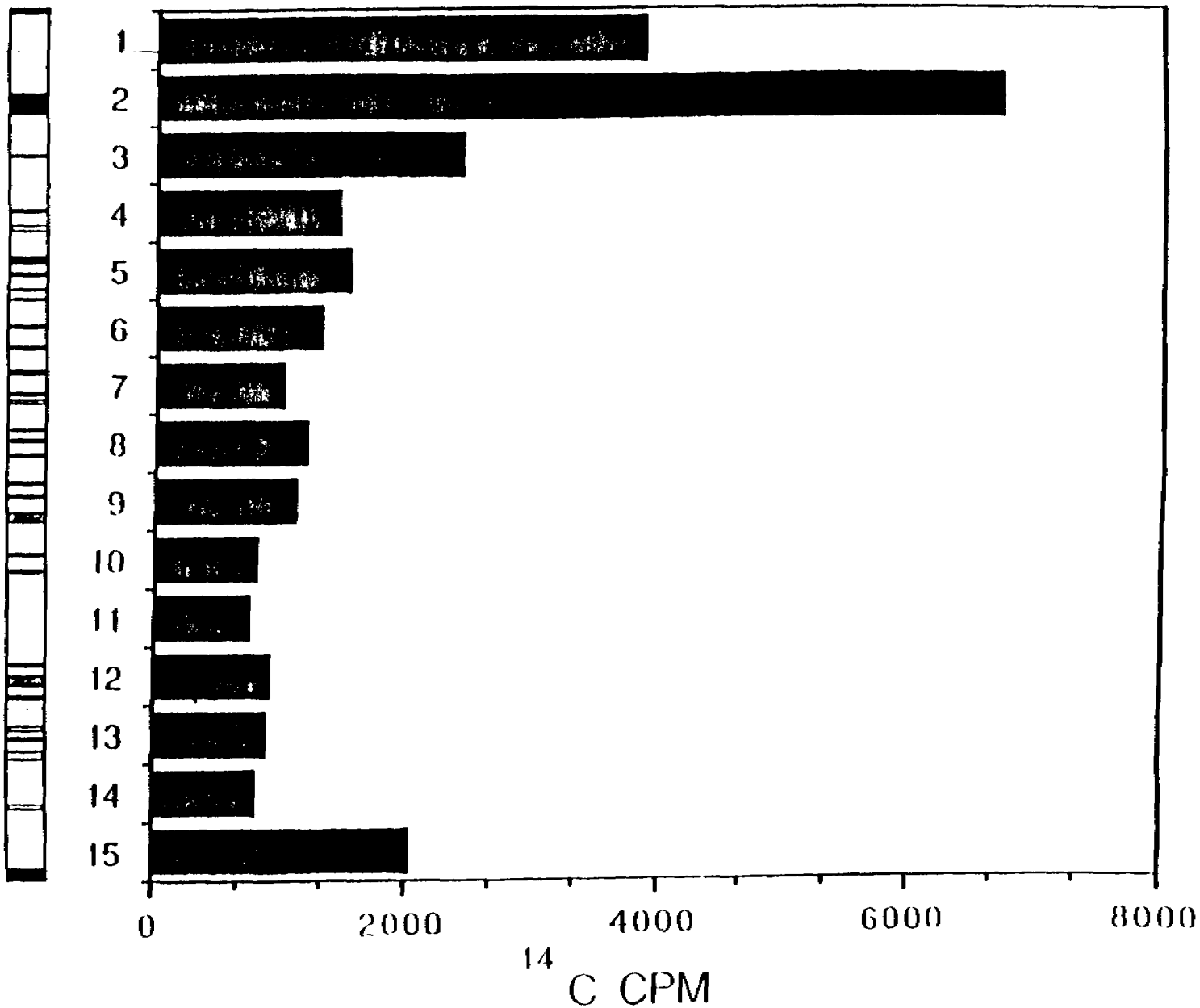


FIGURE 2 : Association of ^{14}C-catechol-derived Radioactivity with Mouse Lymphoma Cell Proteins Separated by SDS-polyacrylamide Gel Electrophoresis

TABLE 7

Induction of micronuclei in Zymbal gland cells after a single in vivo *dose of benzene.*

SPRAGUE-DAWLEY FEMALE RATS

DOSE *(mg/kg)*	TOTAL CELLS SCORED	MICRONUCLEATED CELLS	FREQUENCY PER THOUSAND
0	4079	42	10
1000	2852	102	36

TABLE 8

Induction of micronuclei in Zymbal gland cells after three consecutive daily doses of benzene.

SPRAGUE-DAWLEY FEMALE RATS

DOSE *(mg/kg/day)*	TOTAL CELLS SCORED	MICRONUCLEATED CELLS	FREQUENCY PER THOUSAND
0[a]	1845	10	5
0	1899	21	11
250	3182	57	18
250	2521	63	25

[a]Vehicle controls. Duplicates are individual animals from two separate experiments.

TABLE 9

Induction of micronuclei in Zymbal gland cells after six months dosing with benzene.

SPRAGUE-DAWLEY FEMALE RATS

DOSE *(mg/kg/day)*	TOTAL CELLS SCORED	MICRONUCLEATED CELLS	FREQUENCY PER THOUSAND
0	2053	9	4
1.5	2024	17	8
25	2035	40	20
250	2013	40	20

TABLE 10

Induction of micronuclei in Zymbal gland cells after three consecutive daily doses of benzene.

SPRAGUE-DAWLEY FEMALE RATS

DOSE (mg/kg/day)	FREQUENCY MICRONUCLEATED CELLS PER THOUSAND	
	FIRST SCORING[a]	SECOND SCORING
0.0	11	9
0.0[b]	12	9
12.5	14	16
12.5	14	13
25	24	15
25	18	19
50	9	12
50	21	21
100	34	28
100	25	18
200	60	41
200	56	36

[a]Second scoring was performed to test reproducibility of frequencies.
[b]Duplicates are individual animals.

TABLE 11

Induction of micronuclei in Zymbal gland cells after three consecutive daily doses of benzene.

FISHER 344 MALE RATS

DOSE (mg/kg/day)	FREQUENCY OF MICRONUCLEATED CELLS PER THOUSAND (Each entry is the frequency per 1000 cells)				
0.0	8	7	9	9	10
0.0	7	7	8	8	10
1.0	15	11	10		
1.0	13	11	11	13	10
5.0	19	16	14	15	
5.0	14	16	14	16	
10	19	18	17	15	
10	18	19	14	18	
20	21				
20	20				
30	24				
30	24				
40	34				
40	21	29			
50	32				
50	31				
100	40				
100	43				
200	45				
200	52				

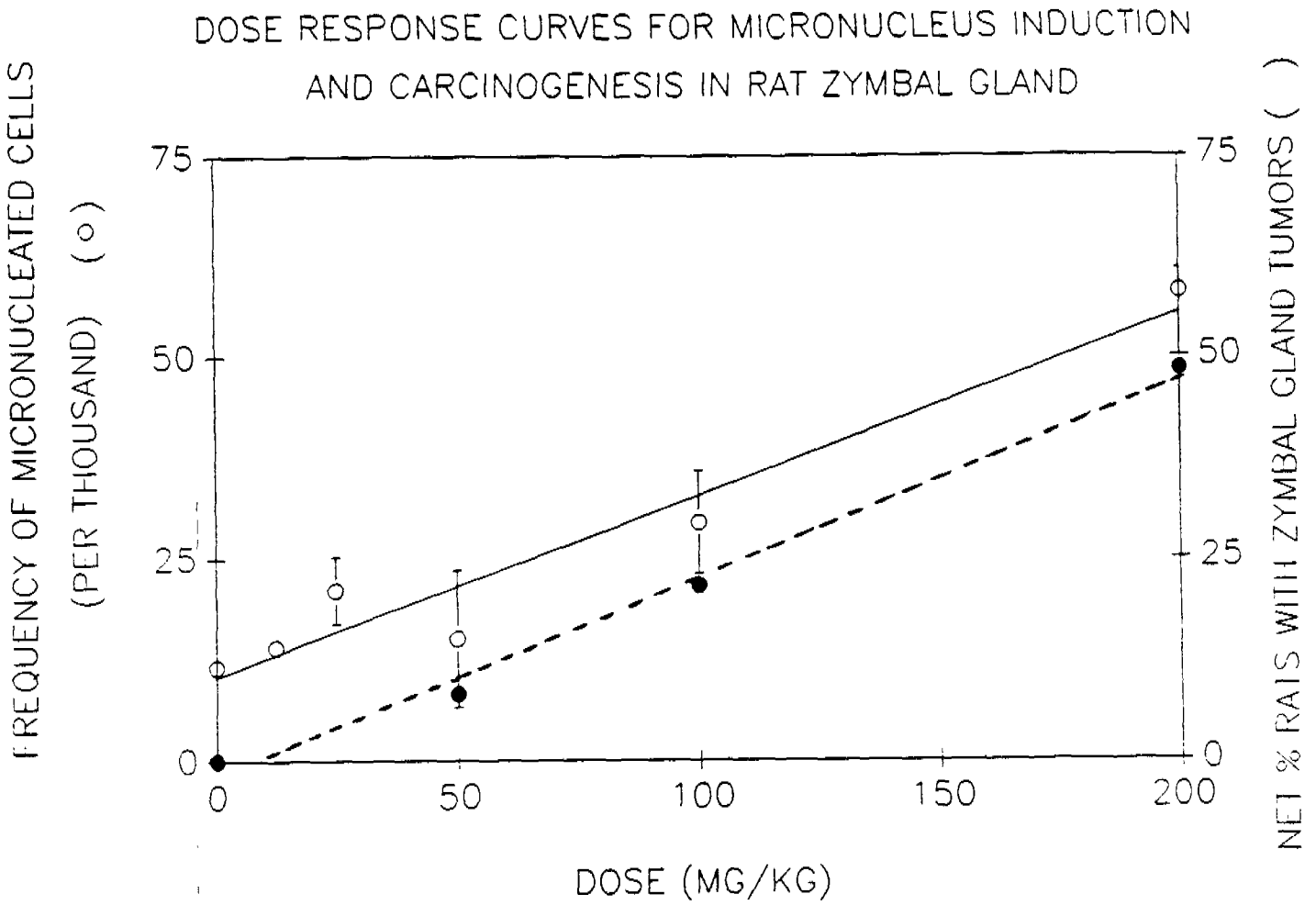
TABLE 12

Induction of micronuclei in Zymbal gland cells after three consecutive daily doses of benzene.

FISHER 344 MALE RATS

DOSE *(mg/kg/day)*	MICRONUCLEATED CELLS IN 1000 CELLS SCORED
NEGATIVE CONTROL	11
	2
	9
	4
	3
VEHICLE CONTROL	3
	4
	5
	2
	15
1.0	4
	5
	3
	8
	8
10	10
	17
	10
	13
	11
100	16
	18
	17
	20

FIGURE 3



PUBLICATIONS DERIVED FROM THE RESEARCH PROJECT

Published:

1. Low, L.K., Meeks, J.R., Norris, K.J., Mehlman, M.A., and Mackerer, C.R. (1989) Pharmacokinetics and metabolism of benzene in Zymbal gland and other key target tissues after oral administration in rats. *Environ. Health Persp.* **82**: 215-222.

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3. Reddy, M.V., Blackburn, G.R., Schreiner, C.A., Mehlman, M.A., and Mackerer, C.R. (1989) ^{32}P analysis of DNA adducts in tissues of benzene-treated rats. *Environ. Health Persp.* **82**: 253-258.

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In Press:

1. Reddy, M.V., Blackburn, G.R., Bleicher, W.T., Irwin, S.E. Mehlman, M.A., and Mackerer, C.R. (1990) ^{32}P-Postlabeling assay of DNA adducts formed *in vitro* and *in vivo* with benzene and metabolites: New assays to measure adducts as 5'-^{32}P-labeled dinucleotides and nucleoside monophosphates. To be published in Proceedings of the International Meeting "*Biomonitoring and Carcinogen Risk Assessment*" held on July 27-28, 1989 at Cambridge, U.K.

Submitted:

1. Reddy, M.V., Bleicher, W.T., and Blackburn, G.R. Nuclease S1-mediated enhancement of ^{32}P-postlabeling assay for aromatic carcinogen-DNA adducts. Submitted to *Carcinogenesis.*

2. Lambert, C.E., Low, L.K., Meeks, J.R., Naro, P.A., and Mackerer, C.R. Tissue specific metabolism of benzene in Zymbal gland and other solid tumor target tissues in rats. Submitted to *Carcinogenesis.*

In preparation:

1. Nucleic acid and protein adducts in tissues of benzene-treated rats as measured by radiolabeled benzene: Lack of correlation between DNA adduct level and tissue susceptibility to tumorigenesis.

2. A rat Zymbal gland tissue culture system that mimics *in vivo* activation of structurally diverse carcinogens to adduct-forming species, as detected by the ^{32}P-assay.

3. Evaluation of benzene-binding proteins (receptors) in various tissue homogenates.

4. Comparative evaluation by various enhancement procedures of ^{32}P-postlabeling of DNA adducts derived from benzoquinone, 2-acetylaminofluorene, and benzo[a]pyrene.

5. A survey of the genotoxicity of benzene and its major metabolites in mouse lymphoma L5178Y cells: Mutagenesis at the TK locus, DNA strand-break induction, cytogenetic effects and DNA-adduct formation as measured by ^{32}P-postlabeling analysis.

6. A method for primary culture of epithelial cells from rat Zymbal gland, a target for carcinogenesis by hydrocarbons and aromatic amines.

7. Benzene induces a dose-dependent increase in micronuclei in Zymbal gland cells of rats.

8. Induction of micronuclei in explant cultures of Zymbal gland cells by carcinogens from diverse chemical classes.

9. Comparison of the inducibility of micronuclei in cultured cells derived from Zymbal gland, preputial gland, mammary gland, and skin of rats and mice: Correlation with tissue susceptibility to carcinogenesis.

10. Effects of repeated benzene exposure on tissue lipid peroxidation and glutathione levels.

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2. Reddy, M.V., Blackburn, G.R., Schreiner, C.A., Mehlman, M.A., and Mackerer, C.R. (1988) ^{32}P-Analysis of DNA adducts in tissues of benzene-treated rats. *Ibid.*

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8. Reddy, M.V., Bleicher, W.T., Blackburn, G.R., and Mackerer, C.R. (1990) Nucleic acid and protein adducts in tissues of benzene-treated rats. *Ibid.*