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BIOTRANSFORMATION AND MUTAGENICITY OF BENZIDINE

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BIOTRANSFORMATION AND MUTAGENICITY

OF BENZIDINE

BY

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DISSERTATION

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ABSTRACT

BIOTRANSFORMATION AND MUTAGENICITY
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MELISSA LEA ROCHKIND

University of New Hampshire, December, 1983

This study was undertaken to investigate whether the priority pollutant benzidine could be biotransformed by bacteria from natural environments and to determine what effect such biotransformation would have on the mutagenicity of the compound. Bacteria were collected from sites of toxic waste dumping and from estuarine, lake, and ocean locations. Sediment and water samples as well as bacterial isolates were incubated in media containing 100 $\mu g\ ml^{-1}$ $^{14}C(U)$-benzidine (specific activity 6.76 mCi/mmol) supplemented with ammonia, phosphate and ferrous iron in aged seawater (salinity $26^{o}/oo$). Pure cultures were grown overnight in reduced-nutrient broth and washed prior to resuspending in the same volume of experimental media and incubating at ambient temperature ($22-28^{o}C$) in darkness with shaking. Detection of metabolites by thin layer chromatography and subsequent autoradiography revealed that benzidine is biotransformed by unacclimated bacteria from polluted sites and from estuaries. Benzidine could not serve as a sole source of carbon and energy for growth. At a low concentration (1 $\mu g\ ml^{-1}$) of benzidine

all the substrate was transformed to one major product; at 100 $\mu g\ ml^{-1}$ two transformation products appeared but most of the benzidine remained unchanged. Benzidine was also transformed abiotically to mutagenic compounds but not the same ones seen after bacterial biotransformation. Upon extended incubation, bacteria transformed the metabolites back to benzidine indicating that the biotransformations were ring substitutions but not ring cleavage. Benzidine was mutagenic after microsomal activation in the Ames test, the fluctuation test using Ames tester strain TA98, and the inductest-β-galactosidase assay. The transformation products were mutagenic without requiring prior microsomal activation. The inductest-β-galactosidase assay (Elespuru and Yarmolinsky, 1979) was modified extensively to increase its sensitivity for detecting benzidine as a mutagen.

INTRODUCTION

The increasing awareness of environmental pollution has led to many studies which investigated the capability of bacteria to degrade the chemical pollutants. In 1973 the Environmental Protection Agency published a list of priority pollutants and called for environmental fate and effects studies on them. Although benzidine appeared on that list, it has not been well studied. Benzidine is used in dye manufacturing and in clinical tests for hemoglobin, and in addition is a potential waste product of coal-conversion processes.

This study was undertaken to investigate whether benzidine was mineralized by environmental bacteria, and if not, whether the transformation products which might derive from the action of bacteria on benzidine were less mutagenic than the authentic benzidine. These results would contribute to an understanding of the fate of benzidine in the environment as well as giving information which might be extrapolated to the class of aromatic amines which includes other types of dyes. This study would also respond to the EPA's call for environmental fate and effects studies of the environmental priority pollutants.

Environmental sediment and water samples from areas around toxic waste dumps and from estuaries, lakes and the ocean were collected and placed into a mineral salts medium containing 100 mg per liter benzidine. These samples were incubated at ambient temperature for six days and then the samples were extracted with ethyl acetate and metabolites of benzidine separated on a thin layer chromatogram. Radioactive ^{14}C-benzidine spots were visualized on an autoradiograph and potential

mineralization followed by liquid scintillation counting.

Three mutagenesis assays were used to detect the mutagenicity of benzidine and its metabolites. The Ames test and the microtiter fluctuation test were used essentially without modification. The inductest-β-galactosidase assay was modified extensively to increase its sensitivity for a wide range of mutagens.

Results of these studies indicate that benzidine is cometabolized by environmental isolates from toxic waste sites and from the Great Bay estuary. Two major transformation products appeared and were mutagenic. Benzidine was not mutagenic without metabolic activation. Formation of these products was maximal after five days' incubation and then disappeared gradually, until at 34 days of incubation these products had essentially completely reverted to authentic benzidine. Sequential incubation of two strains of bacteria did not enhance transformation. The presence of light during the incubation had no effect.

During this study no bacteria able to grow on benzidine as the sole source of carbon were isolated. Benzidine was not found to be highly toxic for bacteria.

The conclusions of this study are that benzidine can be transformed reversibly by environmental bacteria to products which are mutagenic and act as direct mutagens. Under the conditions employed in this study benzidine was not mineralized to nontoxic products. The modifications to the inductest-β-galactosidase assay developed here increase its sensitivity as a mutagenesis assay for benzidine and establish the potential of this assay to be a method of choice for routine monitoring of mutagenesis.

I. LITERATURE REVIEW

The realization that chemicals, once deposited, can persist in the environment has led to an increased interest in determining the effects that microorganisms might have on those chemicals. The earliest compounds studied included humic substances, which remain after the natural decomposition of plants, animals, or microorganisms and can persist as long as 2,000 years, and peats, which can last 34,000 years (Alexander, 1965). Other compounds which persist in the environment include some hydrocarbons and detergents (Alexander, 1965). These studies led to establishment of the principle of molecular recalcitrance, that some compounds in environments degrade very slowly, if at all (Alexander, 1965). This principle offset the theory of microbial infallibility, which was a widely held view that there existed somewhere in the world bacteria capable of degrading any organic compound (Alexander, 1965).

A molecule may be recalcitrant for several reasons. The compound may be in a microenvironment which protects it from microbial attack, such as sorbed to clay or protected within a nonmetabolizable shield (Floodgate, 1979; Alexander, 1965). Temperature is of critical importance, governing enzymatic reaction kinetics, microbial growth rates, and the solubility of the compound (Colwell and Sayler, 1978). The chemical may only be recalcitrant in certain locations or at certain times of the year, not correlating with temperature (Rubin, Subba-Rao, and Alexander, 1982). The environment may be lacking some factor essential for bacterial growth, such as water, a terminal electron acceptor, or a nitrogen or phosphorus source. Oxygen is often required

for reasons other than its use as a terminal electron acceptor (Floodgate, 1979; Alexander, 1965). Alternatively, a factor may be present which is toxic or inhibitory to bacteria.

Bacteria may not possess the required enzymes, or they may not possess the transport mechanism needed for the chemical to enter the cell and be in proximity to the enzymes. Many chemicals possess substituents which render the molecule inaccessible to enzymes which might otherwise act readily on some portion of it. The enzymes may be inactivated by sorption to clays or colloids or inhibited by the presence of their substrates or products (Rubin, Subba-Rao, and Alexander, 1982; Perry, 1979; Alexander, 1965; Foster, 1962). While the sorption of pollutants to particulates in the water may be beneficial by bringing the chemical into close association with attached bacteria, such partitioning may concentrate the chemical to toxic levels which inhibit biodegradation (Colwell and Sayler, 1978).

With an awareness of the potential for molecular recalcitrance, attention turned to the herbicides and pesticides, chemicals which kill unwanted insects, animals, and foliage while ideally not harming any other component of the ecosystem. In practice, these chemicals often affect other portions of the environment as well. Such chemicals have been recovered in areas far removed in time and space from their origin, appearing in groundwater far below the soil surface, in rivers and ponds, and bioconcentrated through the food chain in animals and birds (Alexander, 1965).

Some decontamination of the environment may occur via nonbiological degradation of these pesticides or sorption and inactivation by clays and other colloids. However, biological activity is often the most

important means of removing these chemicals (Alexander, 1965). The ideal result of biological activity is mineralization to inorganic compounds. Bacteria grow on the substrate, deriving carbon and energy from it resulting in an increase in biomass. Usually, mineralization results in detoxication, unless the inorganic compound formed (such as nitrate) causes other problems in the environment (Alexander, 1981).

Some studies have demonstrated that chemicals added to environmental samples disappear in nonsterile samples but not in sterile samples, although no organism capable of using the chemicals as sources of carbon or energy were found (Alexander, 1981). These chemicals included DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (Rosenberg and Alexander, 1980b; Focht and Alexander, 1971). This phenomenon has been called cometabolism or cooxidation (Alexander, 1979; Perry, 1979; Horvath, 1972; Jensen, 1963; Foster, 1962; Leadbetter and Foster, 1959). Bacteria utilize another substrate as a source of carbon and energy while its enzymes incidentally are modifying the compound of interest. The cometabolite itself cannot be used as a source of energy (Jacobson, O'Mara, and Alexander, 1980). The appearance of products is related stoichiometrically to disappearance of the cometabolite. Transformation of the cometabolite is associated with increased oxygen consumption (Hulburt and Krawiec, 1977). The term cooxidation has been used to describe cometabolic processes which involve oxidative sequences (Jacobson, O'Mara, and Alexander, 1980; Alexander, 1979).

The use of these terms has been criticized for giving a new status to processes which are well-described in the cell (Hulburt and Krawiec, 1977). The stoichiometric appearance of products with a rise in oxygen

consumption is consistent with the definition of aerobic catabolism. The failure of the cell to replicate may be due to a lack of some essential nutrient, but other metabolic activities may continue and an increase in biomass may occur. Further, the "coincidental" use of an enzyme may not be wasteful and may indicate a specificity for that molecule (Hulburt and Krawiec, 1977). Direct evidence for cometabolism has come from the use of ^{14}C-labelled substrates. These studies have demonstrated a change in the chemistry of the cometabolized compound without incorporation of ^{14}C label into the cell nucleosides (Alexander, 1981). The process by which resting cells which do not increase in biomass transform a substrate is considered bioconversion (Perry, 1979). This effect might occur because an enzyme of low specificity produced by the bacteria is in close proximity to the chemical. Oligotrophs show evidence of ability to mineralize compounds without assimilation, indicating complete cometabolism in environments with low concentrations of substrate and other organics (Rubin, Subba-Rao, and Alexander, 1982).

Nonmineralizing transformations, either cometabolism or bioconversion, are marked by a slow disappearance of the compound without a rise in the bacterial population. The substrate is not mineralized; rather, it is transformed to other compounds via small structural changes in the molecule. These changes are often too small to result in detoxication because the structural feature associated with toxicity is not affected (Alexander, 1981). It has been postulated that the ability of a microorganism to cometabolize but not mineralize a substrate is related to enzyme specificity (Alexander, 1981). Many enzymes catalyze reactions involving different but chemically related substrates. If the product formed is not an appropriate substrate for another enzyme present, that

product accumulates and further biodegradation is blocked. There are several possible consequences of such partial degradation. Detoxication may result (Rosenberg and Alexander, 1980a). Alternatively, the original substrate may not be harmful, but the result of bacterial metabolism may be an activated species which is hazardous (Alexander, 1981). For example, phenoxy herbicides in soil are converted to phytotoxic intermediates rather than nontoxic chlorophenols (Gutenmann et al., 1964). A third consequence may be a change such that the original substrate with activity against one organism may be converted by microorganisms to a form which affects other organisms (Alexander, 1981). The original substrate may be metabolized easily by some microorganisms, but by chance be incorporated into another pathway which yields a product which is much more stable in the environment, as is the case with DDT (Metcalf et al., 1972).

The location of the substituent on a molecule is important. Ortho and meta dihydroxyphenols follow separate pathways into the tricarboxylic acid cycle (Dagley, 1971). The meta substituted methylbenzoic acid, m-toluic acid, is more readily oxidized than the ortho and para derivatives. Benzoic acid is more readily oxidized than its dihydroxy derivatives, which in turn are more active than the trihydroxybenzoic acids. Benzene is highly resistant to oxidation, but the presence of an amino group (aniline) allows measurable oxidation. However, the presence of a second amino group (p-phenylenediamine) increases resistance to oxidation (Tabak et al., 1964). The monochloro and nonchlorinated analogues of DDT are cometabolized, while DDT or p,p'-dichlorobenzophenone are not (Focht and Alexander, 1971). The enzymes involved in

ring hydroxylation and ring fission are substrate specific and are inducible (Colwell and Sayler, 1979).

The processes by which bacteria act on pesticides apply to other classes of chemicals. In 1973 the Environmental Protection Agency (EPA) published its first list of priority pollutants (Federal Register, 1973) and research into microbial action on these classes of chemicals increased. The list of compounds which had been introduced into the environment and subsequently were found to be mutagenic or carcinogenic expanded rapidly, so that by 1978, over 2,400 chemicals were listed by the National Institute for Occupational Safety and Health (NIOSH) as being suspect carcinogens (Federal Register, 1980).

Benzidine

Benzidine (4,4'-diaminobiphenyl) (bzd) appeared on the first EPA list of priority pollutants (Federal Register, 1973). It is a carcinogen widely used in making dyes for the paper, textile, and leather industries. These are direct dyes and therefore have also been used widely by craft workers and the general public (NIOSH, 1980). There are more than 200 bzd-based dyes, of which 30 are commercially important in the United States; 17 of which are manufactured in the United States (NIOSH, 1980). Bzd has also been used as a plasticizer, in hair dyes, and in testing for occult blood. Bzd is a potential waste product of coal-conversion plants as well (Zierath et al., 1980).

Bzd was established as a cause of bladder cancer because of the easily traceable rise in incidence of this disease concomitant with the establishment of a dyestuff industry in various countries. Bladder cancer was first reported in 1895 in Germany in dyestuff workers, and this relationship was repeated in Italy, Switzerland, Russia, Austria,

France, and the United Kingdom. Kimono painters in Japan who used their mouths to wet brushes developed oral and other cancers from bzd-based dyes. The relationship between bladder cancer and bzd-based dyes was noted in the United States in 1931, and a 1934 report estimated that 4.5% of dyestuff workers contracted bladder cancer (Haley, 1975).

Studies have shown that the time from exposure to first occurrence of cancer ranges form 8 to 41 years with a mean of 16 years (Zavon et al., 1973). The severity and duration of exposure may determine occurrence of bladder cancer but not the length of the induction period (Haley, 1975).

In factories with shoddy work practices bzd metabolites can be detected in the urine of workers, although where better standards of hygiene are practiced bzd or its metabolites are not detectable (Meal et al., 1981).

Chemistry of Benzidine and Its Dyes

Bzd ($C_{12}H_{12}N_2$) has a molecular weight of 184.23 and is relatively insoluble (one gram dissolves in 2,500 ml cold water). The octanol-water coefficient (K_{ow}) is 46.0, and the ionization constants for pKb_1 and pKb_2 are 4.3 and 3.3, respectively (Zierath et al., 1980; Windholz et al., 1976).

The chemical synthesis of bzd begins with nitrobenzene or azobenzene, which is reacted with zinc and aqueous sodium hydroxide to form hydrazobenzene. Hydrazobenzene undergoes acid rearrangement with hydrochloric acid to form bzd.2HCl or with sulfuric acid to form bzd-sulfate. Subsequent diazotization forms diazonium salts, which when complexed with an aryl amine or phenol and a sodium salt will form an azo dye with azo linkages in the para position. These are direct dyes which will

combine with cloth or other materials without requiring the use of a mordant (Haley, 1975; Conant, 1936). The diazo linkage can be broken by enzymatic, thermal, or irradiation processes to yield free bzd (NIOSH, 1980; Zollinger, 1961).

Sorption of Benzidine by Sediments

Bzd has a size and shape which allow it to obtain close contact with clay surfaces and to participate directly in charge transfer reactions at both crystal edges and planar surfaces (Theng, 1971). Sorption appears to be a two stage process, with bzd saturating the exterior surfaces of the particles in a monolayer. The sites are filled in order of increasing net negative charge on the clays which are neutralized by the bzd radicals. The formation of the complex is reversible, with unchanged bzd and clay obtained from extracting the complex (Tennakoon, Thomas, and Tricker, 1974; Hauser and Leggett, 1940).

The formation of a bzd-clay complex in aqueous solutions gives rise to color formation which is responsive to pH. At an acid pH (0.5-1.5), bzd exists as a divalent radical-cation and forms a yellow bzd-clay complex. Above pH 4.2 bzd is a monovalent radical-cation which results in a blue bzd-clay complex. Between these values a mixture of the two species exists. The "bzd-blue" reaction is an oxidation involving the transfer of an electron from bzd amino groups to the clay with ferric ions functioning as electron acceptor sites. The unpaired electron conjugates with the aromatic ring and achieves resonance with both rings resulting in both the stability and blue color of the complex. An additional proton is added to the nitrogen atom at low pH, hindering resonance of one ring and therefore giving the yellow complex. Under still more acid conditions, both amino groups form salts and the complete

inhibition of resonance results in a colorless state. The pH dependent color lightens and then disappears upon drying but is restored when wetted. The sequence of color change (blue to green to yellow to colorless) is the same as when the sample is acidified (Zierath et al., 1980; Tennakoon, Thomas, and Tricker, 1974; Theng, 1971; Hauser and Leggett, 1940).

Sorption of bzd to natural sediments and soils has been investigated (Hassett et al., 1980; Zierath et al., 1980). Fourteen samples of soil and sediments from various rivers produced isotherms which were fitted to the Freundlich equation $C_s = k_d C_w^{1/n}$ when 1/n was kept constant at 0.5. C_s equals the concentration of bzd in nmoles/gram of soil or sediment, C_w equals the equilibrium solution concentration in nmoles/ml, and k_d and 1/n are constants (Zierath et al., 1980). K_d values correlated strongly with pH. The pH of the system controls the amount of bzd in the ionized form and thus the sorption of bzd to sediments. Sorption increases as pH decreases for any particular sediment sample. Sorption of bzd is also correlated strongly with surface area. The addition of organic materials coats the sediment particles and lowers the sorption capacity of the sediment for ionized bzd (Hassett et al., 1980). For neutral organics in general, sediment sorption coefficients for various sediments are nearly constant when the partition coefficient is normalized to the organic carbon content of the sediment. However, for sediments with low organic carbon content clay surface structure rather than organic carbon content controls sorption (Brown and Flagg, 1981; Karickhoff, 1981; Hassett et al., 1980; Zierath et al., 1980; Karickhoff, Brown, and Scott, 1979). When the sorbing medium is considered to be the total soil plus organic matter rather than the mass of sediment

alone, strong correlation of sorption with sorbing medium is found for certain herbicides and insecticides, even at very low concentrations of organics (Lambert, 1968). Deviations were postulated to be due to experimental error, a false assumption that all the organic matter participates in sorption, or the soil itself showing anomalous characteristics.

In Vivo Metabolism of Benzidine

Morton et al. (1979, 1981) proposed that in the liver of rats, mice, and hamsters bzd is metabolized to N-acetylbzd, which forms N,N'-diacetylbzd and then N-hydroxy-N,N'-diacetylbzd. This product via an N,O-acyltransferase or sulfotransferase forms reactive derivatives which can bind to RNA, react with methionine, and record as positive in the Ames mutagenesis assay. N-hydroxy-N,N'-diacetylbzd may also be a carcinogen. In the rat the N,O-acyltransferase activates bzd to a glucuronide while in the guinea pig a deacetylase performs the same function (Bos et al., 1981). A sulfotransferase was isolated from rat livers only, and therefore would not be responsible for extrahepatic tumors in the rat or other species (King and Olive, 1975). Several bzd metabolites were recovered in the urine of dogs fed bzd, including 3-hydroxybzd, 4-amino-4-hydroxybiphenyl, mono- and diacetylbzd, and conjugated 3-hydroxybzd in addition to unchanged bzd (Sciarini, 1957). Monkeys fed bzd excreted bzd and monoacetylbzd (Haley, 1975). An analysis of the urine of humans exposed to bzd revealed 3.6-5.6% bzd, 1.6-5.4% monoacetylbzd, 5.1-10% diacetylbzd, and 78.5-89.7% 3-hydroxybzd, while other studies have recovered N-hydroxyacetylaminobzd as well (Haley, 1975) (Figure 1).

Figure 1. Chemical structure of benzidine and some of its metabolites.

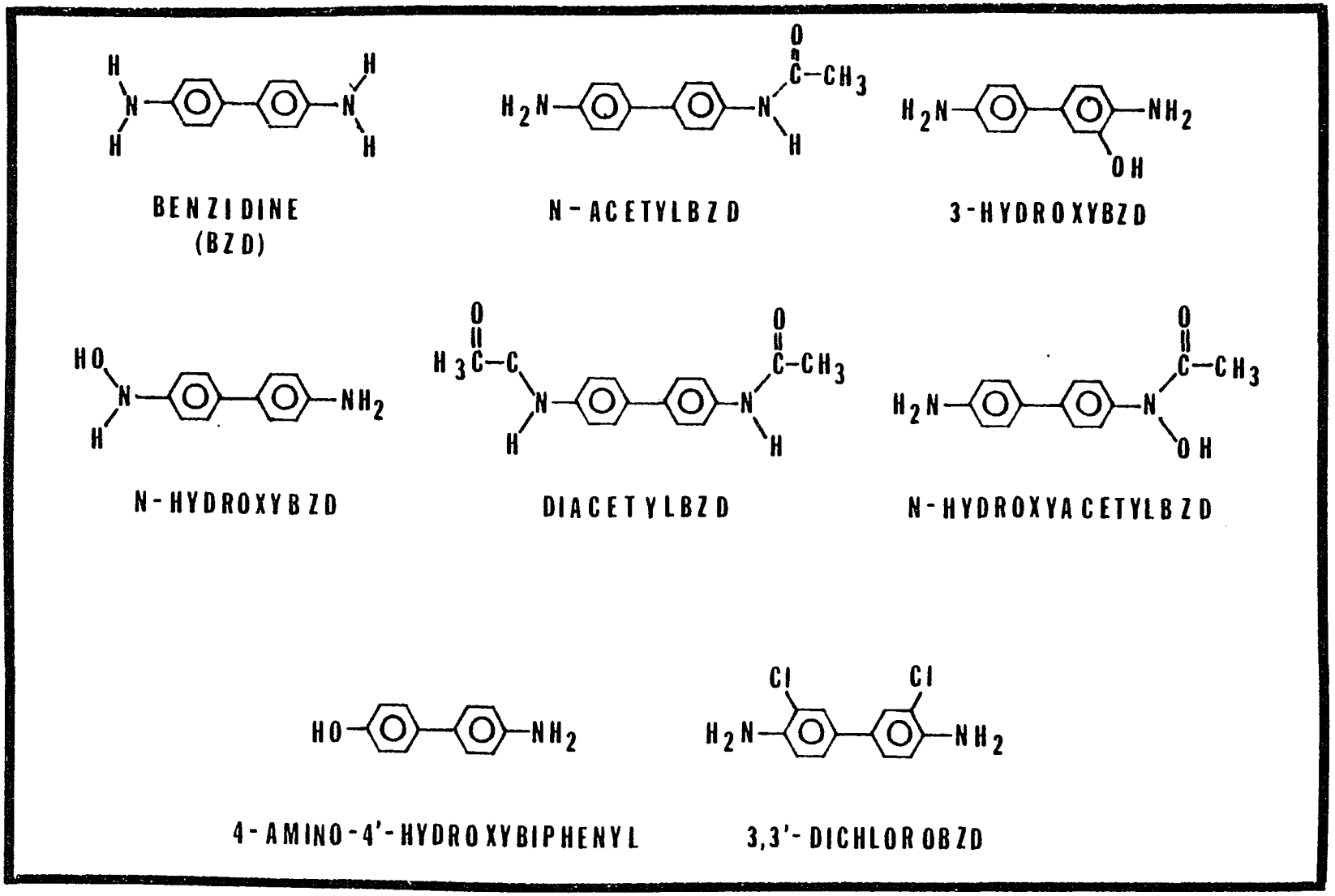
BENZIDINE
(BZD)
N-ACETYLBZD
3-HYDROXYBZD
N-HYDROXYBZD
DIACETYLBZD
N-HYDROXYACETYLBZD
4-AMINO-4'-HYDROXYBIPHENYL
3,3'-DICHLOROBZD

Figure 1.

In Vivo Metabolism of Benzidine-Based Dyes

The metabolism of azo compounds in mammals has been studied since 1911. Early workers established that several pathways existed for the metabolism of these compounds and that some of these pathways can also occur in humans (Walker, 1970). Azo reduction has also been shown to occur in the human placenta in the presence of NADPH (Miyadera, 1975).

Many animals possess azoreductases which will split bzd-based dyes at the azo linkage to yield free bzd and other products. This has been demonstrated in the hamster, rhesus monkey and dog (Lynn et al., 1980; Haley, 1975; Rinde and Troll, 1975). Dogs, rabbits and humans are the only animals known to develop bladder cancer after exposure to bzd-based dyes (Haley, 1975). Species differences in response to carcinogens have been noted for many other carcinogens. For example, N-2-fluorenyl-acetamide becomes a proximate carcinogen after undergoing N-hydroxylation in the rat, while the guinea pig lacks the N-hydroxylase and is resistant to this carcinogen (Haley, 1975).

Free bzd as well as monoacetylbzd, bzd conjugates, and low levels of 3,3'-dimethylbzd (o-toluidine) and 3,3'-dimethoxybzd (o-dianisidine) have been recovered in the urine of humans exposed to bzd-based dyes (Lowry et al., 1980; Lynn et al., 1980). The recovery of free bzd is far more than can be accounted for by the amount of bzd present as an impurity in the dyes, and is similar to the amount recovered after exposure to bzd itself (Lynn et al., 1980).

Bacterial Metabolism of Benzidine and Benzidine-Based Dyes

The anaerobic metabolism of bzd-based dyes to bzd in bacterial suspensions derived from rat and monkey intestinal contents and human feces, and from both whole cell and cell-free extracts of a human intestinal

anaerobe has been demonstrated (Cerniglia et al., 1982; Hartman, Fulk, and Andrews, 1978). Members of the mammalian gut flora reduce azo compounds (Walker, 1970; Roxon et al., 1967). Martin and Kennelly (1981) noted that rat liver microsomal azoreductases do not reduce bzd-based dyes well, and postulated that intestinal microorganisms perform this function in vivo. The importance of gut bacteria in reducing azo linkages could account for the failure of rats injected intraperitoneally to reduce some azo dyes (Miyadera, 1975).

Escherichia coli and soil bacteria have also been shown to perform azo reductions (Yoshida et al., 1981). Cloth dyes with Direct Black 38, a bzd-based dye, yielded bzd when incubated with raw river water for 72 hours. The cloth faded during this time (Yoshida et al., 1981; NIOSH, 1980). While bzd-based dyes have not been shown to penetrate skin, bzd readily does, and NIOSH (1980) warned that organisms on the skin such as E. coli may act on dyes in clothing to yield free bzd.

Bzd itself has not been well studied as a substrate for bacterial metabolism in the environment. Only one study has been published, in which bzd was incubated with river water but was not transformed after nine weeks' incubation (Yoshida et al., 1981). The same river water degraded a dye to yield free bzd.

Bzd has been subjected to aerobic wastewater treatment studies to determine the effect of bzd on respiration of activated sludge microflora and the degree to which bzd was transformed in the system. Bzd was resistant or inhibitory to unacclimated activated sludge microflora (Malaney et al., 1967). Extended acclimated sludge microflora completely oxidized continuous doses of 1 mg per liter bzd in two weeks but less completely oxidized bzd at dosages up to 30 mg per liter after six to

nine weeks (Tabak and Barth, 1978). Most municipal facilities have far shorter retention times for activated sludge. At concentrations up to 500 mg per liter, different activated sludge microflora showed various levels of bzd depletion. None of the metabolites of bzd which are suspected carcinogens were detected after sludge treatment (Baird, Carmona, and Jenkins, 1977).

Bzd was also studied in relation to its effect upon the nitrifying bacteria Nitrosomonas sp. and Nitrobacter sp. present in nitrification-type waste treatment processes (Hockenbury and Grady, 1977). While nitrate oxidation by Nitrobacter sp. was not affected, bzd did inhibit ammonia oxidation by Nitrosomonas sp. by 84% at 100 mg per liter bzd and 12% at 10 mg per liter bzd.

Carcinogenicity and Macromolecular Binding of Benzidine

In humans exposure to bzd can cause bladder tumors, papilloma, chronic cystitis, and hematuria, the latter often the first sign of exposure (Haley, 1975). Miller et al. (1956) found bzd to be a weak carcinogen, 2-methyl-N,N'-diaminobzd a moderate carcinogen, and 2,2'-dimethyl-N,N'-diacetylbzd to have no carcinogenic activity.

Bzd complexes to albumin in the serum of exposed workers (NIOSH, 1980). It is also taken up readily by the liver, kidney, and lung in rats (Martin and Ekers, 1980). The greatest binding is to RNA followed by DNA and then protein. At the time of maximal binding to DNA (at 24 h post-injection), one molecule of bzd was bound per 29,400 DNA bases. This amount dropped by approximately one-third in 72 h, but remained at that level for at least four weeks post-injection (Martin and Ekers, 1980). Binding appeared to occur via the nitrogen atom, only

slightly disturbing the aromatic protons. Any of these effects may be important in some instances of cancer. An alteration in RNA can alter the RNA-to-DNA information transfer which causes misinformation to be integrated into the host genome (Temin, 1974). Alterations of protein molecules, for example DNA polymerase, might give rise to altered and heritable genomes resulting from increased error rates (Miller and Miller, 1977).

Bzd is a proximate carcinogen which must be metabolized prior to interaction with nucleic acids and proteins (Weber, 1978). The ultimate carcinogenic form is an electrophilic reactant which has the potential to attack nucleophilic centers such as the nitrogen and oxygen atoms of nucleic acid bases or amino acid residues, the C-8 of guanine, and the sulfur atoms of methionine and cysteine (Miller and Miller, 1971; Miller, 1970).

Mutagenesis Assays

Mutagens and carcinogens may affect either germ cells, causing abnormal heritable genes, or somatic cells, giving rise to cancers (Nagao, Sugimura, and Matsushima, 1978). Both activities act on DNA, and for all chemicals extensively studied the occurrence of mutagenicity in bacteria and in higher organisms after exposure is well correlated (Ames, 1971). Humans may be able to detoxify some chemicals which cause mutations in bacteria or may be able to repair the mutation effectively, but the potential for DNA interaction still exists should the detoxification or repair systems fail. About 90% of carcinogens are mutagens and about 87% of noncarcinogens are nonmutagens (Ames, 1979; McCann and Ames, 1976; McCann et al., 1975).

Many chemicals are actually procarcinogens and as such would not interact with bacterial DNA. However, addition of mammalian mixed-function oxygenases and an NADPH-generating system results in the formation of metabolites which are the ultimate carcinogens (Ames et al., 1973; Garner, Miller, and Miller, 1972).

Studies confirming the covalent nature of the binding between a mutagen and DNA have shown that the addition of exogenous RNA or DNA to bacterial cultures decreases the sensitivity of the bacteria to metabolites of aflatoxin B_1 (Garner, Miller, and Miller, 1972). For all chemical carcinogens that have been well-studied, protein-, RNA-, and DNA-bound derivatives of the carcinogens have been found in the target tissues (Miller and Miller, 1977; Heidelberger, 1975). In most cases, the level of these macromolecule-bound derivatives corresponds to the carcinogenic response (Miller and Miller, 1977). Exceptions occur, due in part to the two stage process for tumor formation (initiation and promotion) and in part to the finding that not all of the binding to macromolecules by chemicals results in tumor formation (Miller and Miller, 1977).

Ultimate carcinogens as well as most mutagens are electrophilic reactants (Miller and Miller, 1977). Exceptions include base analog mutagens and simple (nonbinding) frameshift mutagens. The similarity in active forms of chemical carcinogens and mutagens has underscored the utilization of mutagenesis assays to prescreen potential carcinogens. While in normal cellular metabolism electrophiles join with nucleophiles at enzyme surfaces in a highly regulated manner, chemical carcinogens usually attack cellular nucleophiles nonenzymically and with little

discrimination (Miller and Miller, 1977). Thus, many such reactions are of little importance to the cell, and only a few may lead to the induction of tumors.

DNA lesions and repair systems are similar in human and bacterial cells, providing additional justification for using bacteria to detect damage to DNA (Devoret, 1979). Testing a chemical for mutagenicity using bacterial systems before conducting animal carcinogenicity tests has become widespread. A typical bacterial test exposes as many as 10^9 cells to the given test dosage (one plate or test tube) and results are read in 48 to 96 hours. Bacterial tests are cheaper, faster, more sensitive, and statistically more accurate than animal studies while yielding information that can often be extrapolated to higher organisms (Ames, 1979; Devoret, 1979). Routine use of these tests while developing a drug or chemical can warn of mutagenicity early enough to remove a contaminant or develop a nonmutagenic derivative (McCann and Ames, 1976).

Ames Test

The most widely used bacterial test for mutagenicity is the Salmonella/mammalian-microsome assay, commonly known as the Ames test (Devoret, 1979; Hollstein et al., 1979; Ames et al., 1973). The underlying principle is that a specific mutation can be reverted back to the wild type by the same processes that cause forward mutations. By using tester bacteria with a mutation in a known locus, these back mutations can be isolated and quantified. The limitation to this design is that the back mutation must occur at a specific site. However, this is balanced by the great number of cells tested and the increased sensitivity of the test.

The Ames test detects mutagens requiring metabolic activation by incorporating a mix of liver homogenate and NADPH-generating system, known as the S9 mix. The tester bacteria for the Ames test are several auxotrophic mutants of Salmonella typhimurium LT-2 (Ames, Lee, and Durston, 1973). Each has a specific type of mutation in the locus governing production of the amino acid histidine. Two strains (TA1535 and TA100) have a base pair substitution and three (TA1537, TA1538, and TA98) can detect frameshift mutations. Strains TA100 and TA98 are derivatives of TA1535 and TA1538, respectively, which have a plasmid (resistance transfer factor) which increases error-prone repair. This plasmid increases the sensitivity of the test for certain classes of compounds (Devoret, 1979).

All five strains contain two additional mutations which increase their sensitivity to mutagens. The first is the loss of the lipopoly-saccharide barrier which coats the surface of bacteria, rendering the cell wall more permeable to potential mutagens. The second mutation causes loss of the excision repair system, the cell's primary mechanism for repairing DNA damage. Thus, mutations that do occur are more likely to be expressed (Ames, Lee, and Durston, 1973).

The Ames test requires use of petri plates containing a defined salts agar medium. The chemical to be tested, the bacterial tester strain, the S9 mix if desired, and a trace amount of histidine are mixed and with melted agar as the carrier are poured over the basal agar medium. The trace amount of histidine permits five to six rounds of replication which allows the mutagen to be incorporated into the DNA. This allows enough growth to form a faint background lawn of bacteria on the plate. Cells which revert to histidine independence will form visible

colonies which are counted after two days' incubation. The number of spontaneous mutants is determined on control plates (lacking added mutagen) and subtracted from experimental plates.

A difficulty with this test is that histidine in the top agar permits spontaneous mutations for several rounds of replication, while the mutagen may diffuse to the lower agar or become inactivated. This might bias the test in favor of detecting spontaneous mutations over induced mutations, and may therefore fail to detect some weak mutagens (Green et al., 1976).

Bzd is a frameshift mutagen, reverting strains TA1538 and TA98 after microsomal activation with the S9 mix. Strain TA1538 is reverted at a rate of 1.4 revertants per nmole, causing bzd to be listed as a moderately mutagenic compound (Ashby, Styles, and Callander, 1980; Nagao, Sugimura, and Matsushima, 1978; McCann et al., 1975; Ames et al., 1973). By comparison, 4-aminobiphenyl provokes 31 revertants per nmole and 2-aminobiphenyl 0.51 revertants per nmole. Other well-known mutagens revert TA1538 at rates of 80 revertants per nmole for ethidium bromide, 121 revertants per nmole for benzo(a)pyrene, 1375 revertants per nmole for N-methyl-N'-nitro-N-nitrosoguanidine, and 7057 revertants per nmole for aflatoxin B_1. The range of mutagenic potency is over a millionfold, but is dependent on the parameters of the test, including choice of tester strain, efficiency of the S9 mix, and the properties of the chemical itself (McCann and Ames, 1976).

Other metabolites of some bzd-based dyes have also been found to be mutagenic. Direct Black 38 breaks down mainly to monoacetylbzd and both the dye and its metabolite are mutagenic after microsomal activation.

Pigment Yellow 12 metabolizes to several dichlorobzd derivatives, all of which are strong frameshift mutagens (Lazear et al., 1979).

Many carcinogens are frameshift mutagens (Ames et al., 1973). Such a carcinogen is often a flat aromatic molecule which intercalates in the DNA base-pair stack, stabilizing the shift in pairing sequences. During DNA replication or repair an additional base pair is added or deleted. Simple intercalation into the DNA helix is insufficient to induce a mutation (Garner and Nutman, 1977). A frameshift mutagen which also contains a side chain capable of covalently reacting with DNA would be a much more powerful carcinogen (Ames et al., 1973).

Modified Fluctuation Test

The fluctuation test of Luria and Delbruck (1943) has been modified to permit the detection of both direct-acting mutagens and mutagens requiring microsomal activation. It has been used with yeasts, fungi, and several strains of bacteria as the tester organisms (Hollstein et al., 1979; Green et al., 1977; Green, Muriel, and Bridges, 1976). Briefly, this method involves treating auxotrophic mutant cells (such as one of the Ames Salmonella strains) with a mutagen for a short period of time, and then diluting cells in 100 test tubes to very low concentration in a broth medium with a limiting concentration of required nutrient (histidine). After incubation the number of tubes with growth are counted. The advantage of this test is that both spontaneous and induced mutations had to occur during the preincubation step; thus the spontaneous mutations do not carry more weight. This method can detect some very weak mutagens (Green et al., 1977).

The test has been modified to a microtiter plate scale. The preincubation mix is dispensed in 0.2 ml aliquots into the 120 wells of

microtiter plates. After incubation the number of positive wells is counted. The technique is equally as sensitive as the macroscale (test tube) method (Gatehouse, 1978). The microtiter test has been combined with the Ames strains to result in a procedure approximately 2.4 times more sensitive that the Ames pour plate method (Levin, Blunt, and Levin, 1981).

Inductest

If a bacterial cell containing a prophage is damaged by a mutagen, the prophage will be induced to begin replicating, eventually to lyse the cell and form a plaque on a lawn of suitable host bacteria (Moreau, Bailone, and Devoret, 1976). The number of cells in which this occurs can be quantitated. An advantage of this method over a mutagenicity test is that the bacterial cell need not survive the mutagen treatment for prophage induction to be successful. Toxic mutagens therefore will still give a positive response (Devoret, 1979). The tester strains containing a prophage all have the envelope and excision repair mutations described for the Ames strains.

Bzd was tested and was negative in this test (Speck, Santella, and Rosenkranz, 1978). These results are somewhat in question, however, since another mutagen also reported to be negative (mitomycin C) was a positive mutagen in experiments reported in the paper originally describing the test (Moreau, Bailone, and Devoret, 1976).

A modification of the inductest was developed by constructing a fusion phage which upon induction synthesizes few phage proteins but large quantities of β-galactosidase (Elespuru and Yarmolinsky, 1979). The lacZ gene coding for this product is stripped of its normal promoter and fused to the operon controlled by the prophage lambda promoter. Upon

induction, β-galactosidase production increases, and this can be measured spectrophotometrically (Miller, 1972). This test removes the requirements for bacterial viability and capacity for phage infection and production (Elespuru and Yarmolinsky, 1979). Moreover, the test can be completed within one day. The test is more sensitive than a mutagenesis assay for such types of mutations as strand breaks or for toxic mutagens, but may be less sensitive for certain other mutagens (i.e., MNNG or ethylmethane sulfonate) (Elespuru and Yarmolinsky, 1979).

II. MATERIALS AND METHODS

Materials

Benzidine dihydrochloride was ACS reagent grade (Matheson Coleman & Bell) (referred to throughout this work as benzidine or bzd). Uniformly labelled ^{14}C-benzidine (not the dihydrochloride form) (New England Nuclear) was stored at $^{-}20^{\circ}$C. The purity of the radiolabelled compound was 98% (New England Nuclear) and the purity of the bulk bzd was not known.

The seawater which was used in all experiments unless otherwise noted and in media preparation was collected at New Castle Island, Portsmouth, NH. The seawater was aged to obtain a homogenous supply with minimal organic matter by filtering it through a glass fiber filter and storing at 4°C in darkness for a minimum of 30 days (ZoBell, 1946). Sediment was collected from Lake Sebago, ME, dried at 170°C to permit pulverization and sterilized by drying at 170°C for 4 h. This sediment was used for experiments using sediment as a sorptive surface for the bzd, but was not used as a source of bacteria.

Strains and Cultural Conditions

Eighteen samples of sediment or water were collected and tested for the ability of their microflora to degrade bzd. These included a sample provided by the EPA from a polluted site in Texas (sample Conroe 5B-1) and a matched sample from a similar but nonpolluted site (Conroe 7F-1). A sample of sediment from beneath a polluted water table in Long Island, NY was also provided by the EPA (sample Sunrise 231). Six samples of mixed water and sediment were collected at the

Keefe Toxic Waste Dump site, Raymond, NH, by the EPA. Five samples of sediment and two samples of water were collected from Great Bay, NH from June through September in two years at depths ranging from tidal to 15 meters. Seawater from Odiorne Point, Rye, NH and sediment from Lake Sebago, ME were collected in June. These samples were used as mixed microflora and in addition isolates were obtained and tested independently. Identifications of some of these strains are shown in the Results.

Ten bacterial strains were obtained from the UNH Microbiology Department culture collection. These included Arthrobacter crystallopoietes ATCC 15481; Alcaligenes marinus ATCC 25374 isolated in Great Bay (Cobet et al., 1970); Paracoccus denitrificans ATCC 51637; Proteus vulgaris ATCC 13315; Proteus vulgaris strain X-19; Pseudomonas alcaligenes ATCC 14909; Pseudomonas putida ATCC 795; P. putida biovar B strain Roche ATCC 29735, a marine form; Pseudomonas stutzeri ATCC 17588; and Pseudomonas sp. 130, a marine form isolated by J. Shewan.

The basal incubation medium (BIM) contained per liter, 100 mg bzd (0.389 mM, specific activity 6.76 mCi/mmol), 10^{-3}M $(NH)_2SO_4$, $5x10^{-4}$M Na_2HPO_4, and $3.6x10^{-6}$M $FeSO_4 \cdot 7H_2O$. All components were added aseptically from sterile stock solutions (with the exception of bzd) to aged seawater adjusted to a salinity of 25 ± 1^{o}/oo with distilled water. Bzd crystals were added directly. The pH after autoclaving was 7.8 ± 0.2. In some experiments 0.5% glucose or 2% sediment were added. BIM was used for all experiments; changes in concentration of bzd or type of seawater are noted in the experimental results as necessary.

The standard incubation conditions were used for all experiments unless otherwise noted. They included 6 days' incubation in darkness on a rotary shaker set at 150 rpm. Experiments were always conducted at ambient temperature which ranged from 22-28°C. Experimental flasks were 250 ml Erlenmeyer flasks containing 100 ml medium.

Isolation of Environmental Strains

Portions of environmental samples were placed in BIM lacking ^{14}C-bzd with bzd as the sole carbon source. After 6 days' incubation the cultures were streaked for isolation on plates of nutrient broth medium (NBM) (4 g per liter nutrient broth (Difco), 15 g per liter agar, aged seawater of 25°/oo salinity). Resulting colonies were transferred to NBM slants, stored at 4°C and transferred monthly.

Experimental Procedure

When sediment samples were collected, they were added to BIM at 2% concentration (w/v). Water samples were diluted by 25% with distilled water to prevent precipitation of salts and the medium salts and bzd were added directly. Incubation was for 6 days under standard incubation conditions (ambient temperature, darkness, rotary shaking).

Both before and after incubation 5 ml of the mixed sample were withdrawn for determination of pH and radioactivity and isolation of bacteria.

Bacteria in pure culture were prepared for testing in the following manner. Cultures were started from NBM slants and were grown overnight in 100 ml NBM lacking agar. After overnight incubation the culture was centrifuged and resuspended in 100 ml BIM for incubation under standard conditions. Five ml were withdrawn before and after incubation for determination of pH and radioactivity and isolation of bacteria.

Extraction of Organics from Media and Sediment

The remainder the experimental flasks was centrifuged at 10,000 rpm for 10 min. The supernatant fluid was shaken successively with three volumes of ethyl acetate (N.F., Baker) (20, 20, 10 ml) and the solution separated using a separatory funnel. Sediment was mixed three times successively with ethyl acetate (20, 20, 10 ml). Each time it was vortexed briefly and placed in a sonicating water bath (Bransonic) for 2 min. When there was no sediment in the experimental flasks, the bacteria were removed by centrifugation and were not extracted with ethyl acetate. In all cases, the three ethyl acetate fractions were combined and evaporated to dryness under a stream of flowing air. The residue was resuspended in 5 ml ethyl acetate, the suspension evaporated, and finally resuspended in 1 ml ethyl acetate before refrigeration in Hungate tubes with rubber septa.

Radioactivity Determination

Aliquots of samples (0.1 to 1 ml as necessary for sufficient radioactivity) were collected for liquid scintillation counting and were added to 3 ml water plus 10 ml Liquiscint fluor (National Diagnostics) in glass scintillation vials (VWR). The liquid scintillation spectrometer was a Beckman LS7000, programmed for internal correction for quenching (H number method) for a maximum counting time of 5 min.

Samples from the experimental flasks were collected before incubation, after incubation, after centrifugation, after extraction, before evaporation of the ethyl acetate washes, and before storing the concentrated ethyl acetate extract. Fractions of the bacterial pellet (0.5 ml of a resuspended solution) and the sediment (10 mg wet weight) were also counted.

Areas of interest on thin layer chromatograms were scraped directly into scintillation vials, water and fluor added as above and counted.

Thin Layer Chromatography

Aliquants of the ethyl acetate extracts sufficient to contain 20,000 cpm of radioactive material (30-300 µl) were spotted onto a silica gel 60 F_{254} thin layer chromatogram plate with plastic backing (Baker). Plates were developed by ascending chromatography to a height of 10 cm using a mixture of ethyl acetate and butanol (99:1) in an unequilibrated atmosphere in a glass chamber (Morton, King, and Baetcke, 1979). Resultant spots were viewed under a shortwave ultraviolet lamp.

Autoradiography

Developed thin layer chromatograms were placed in contact with x-ray film (Kodak XAR-5) for at least 2 weeks, and were developed in a Kodak RP X-Omat automatic processor.

Gas Chromatography-Mass Spectrometry

A sample of a degradation product separated on a thin layer chromatogram was eluted with methylene chloride (UV Spectrometry grade, Baker). This sample was subjected to gas chromatography on a Hewlett Packard Model 5992 gas chromatograph-mass spectrometer using an SE54, 25 m capillary column. The temperature was started at 100°C, increased to 265°C and held for 45 min.

Sorption of Benzidine to Sediment

Experiments were performed to determine the recovery of bzd from sediment as follows. From 100 to 1,000 mg sediment were mixed with 10 ml BIM in test tubes. This medium contained 100 µg ml^{-1} bzd. After

shaking for 24 h to obtain equilibrium (Zierath et al., 1980) the contents were centrifuged and extracted.

An experiment was performed to determine the amount of bzd sorbed to 20 mg ml^{-1} sediment at various concentrations of dissolved bzd. Various amounts of bzd were dissolved in seawater. An equal amount of ^{14}C-bzd was added to each solution (pH 7.8) and 10 ml added to 200 mg sediment. The tubes were shaken vigorously for 12 h and then allowed to sit motionless for 2 h, during which time all visible sediment settled. An aliquot of the supernatant fluid was counted for radioactivity and the amount of bzd sorbed determined by difference.

Abiotic Transformation of Benzidine

Sterile flasks containing 100 ml media and 2 g sediment were either covered with foil or extensively sparged with oxygen-free nitrogen and stoppered. The media contained 0.5% glucose and was adjusted to pH 7, although upon mixing with sediment the pH equilibrated to pH 7.8. All flasks were incubated under standard conditions. The ethyl acetate aliquots used in extracting the flasks were evaporated under either forced air or oxygen-free nitrogen, and the concentrated extract chromatographed on a thin layer plate.

Bacterial Growth on Benzidine

All strains were inoculated into test tubes of BIM containing either 1, 10, or 100 µg ml^{-1} bzd and incubated at 22°C for 4 to 6 weeks. Tubes that looked turbid were streaked onto plates containing 100 or 300 µg ml^{-1} bzd (the latter a saturated solution). Growth on plates was monitored for 4 weeks. Strains that showed any evidence of growth (appearance of a film or microcolonies) were tested further as follows.

Incorporation Plate Test. Overnight cultures of bacteria (0.1 ml) were spread on plates containing BIM made with defined salts seawater (Lyman and Fleming, 1940) and purified agar (15 g per liter, Difco). Control plates lacked bzd. Plates were incubated at 22°C in a humidified dessicator jar and checked for growth during 4 weeks before discarding a negative result.

Gradient Plate Test. Plates of media of the same composition as the control plates above were streaked with 0.1 ml of overnight cultures of bacteria. After the streaks dried, 10 mg bzd crystals were spotted in the center of each plate. Gradually (over several days) some of the bzd soaked into the agar.

Growth in Broth. Bacteria were grown overnight in NBM and the following day were washed once with sterile aged seawater as described previously. Portions were inoculated into 50 ml BIM in 250 ml sidearm flasks and incubated under standard conditions. Growth was monitored by following absorbance at 600 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb).

Sequential Incubation

The spent medium from incubation of one culture was centrifuged. The supernatant fluid was supplemented with 10^{-3}M $(NH)_2SO_4$, $5x10^{-4}$M Na_2HPO_4, and $3.6x10^{-6}$M $FeSO_4 \cdot 7H_2O$. This medium was divided into portions and used as the incubation medium for other strains of bacteria. These flasks were incubated and extracted in the same way as other experimental flasks.

Ames Test

The Ames plate incorporation test was conducted according to the procedure of Ames, McCann, and Yamasaki (1975) with the following modifications. The basal medium was Davis Minimal Salts Agar (Davis and Mingioli, 1950). The top agar contained 0.8% rather than 0.6% agar as the lower concentration did not solidify reliably. Since slightly larger glass plates were used rather than plastic plates, 3 ml of top agar were added per plate. This procedure meant that more histidine was available to the cells, but it did not interfere with the ability to detect revertant colonies over the lawn of nonrevertants. Plates were read after 72 hours' incubation at 37°C and each point represents the mean of three plates. The five tester strains were received from B. Ames and maintained in the UNH Microbiology Department. Before use they were checked for conservation of mutant characteristics (Ames, McCann, and Yamasaki, 1975). The liver microsomal fraction (S9 mix) for this test was prepared from rats of unknown strain obtained from a local pet store and were induced prior to sacrifice with a polychlorobiphenyl mixture (Aroclor 1254). Preparation of the S9 mix was conducted according to the method of Ames, McCann, and Yamasaki (1975).

Modified Fluctuation Test

The microtiter fluctuation test was performed according to the procedure of Levin, Blunt, and Levin (1981) except that microtiter plates contained 96 wells. Results were calculated using the Chi-square statistic and are reported as significant ($P < 0.05$) or highly significant ($P < 0.01$) mutagenicity.

Inductest

The fusion phage strain of Escherichia coli BR513 (ATCC 33312) was obtained and frozen as recommended (Elespuru and Yarmolinsky, 1979). The rat liver microsomal fraction was purchased (Litton Bionetics, Charleston, SC). The original procedure for the inductest (Elespuru and Yarmolinsky, 1979) gave unsatisfactory results (discussed in detail in Results), so several modifications were made. Chloramphenicol and ampicillin were not used. An overnight culture of the indicator strain was diluted 1:25 and incubated to an A_{600} of 0.3-0.6 (3 to 4 h). The Activation Mix A contained per ml, 3.14 µmol NADP, 7.44 µmol glucose-6-phosphate, 10 µmol $MgCl_2$, 76 µmol NaCl, 0.05 to 0.1 ml S9 Mix, 0.39 to 0.44 ml phosphate buffer (0.2 M, pH 7.4) (total volume S9 plus buffer equal to 0.49 ml), and 0.5 ml LBE medium (Elespuru and Yarmolinsky, 1979). Cells were incubated in the presence of the mutagen for up to 4 h. The β-galactosidase assay was performed according to the method of Miller (1972) including treatment of cells with chloroform and sodium lauryl sulfate. The units of β-galactosidase were calculated according to the formula of Miller (1972).

A complete protocol for the Inductest including these modifications is given in the Appendix.

III. RESULTS

Identification of Environmental Strains

Of the numerous bacterial strains isolated from environmental sediments and water samples, six were used intensively in these studies. An attempt was made to identify these six along with the laboratory contaminant strain SVR appearing on a plate during the course of these studies and a strain designated B1 which was isolated from Great Bay by others and grew on a plate containing hexadecane as the sole source of carbon (Table 1).

Bacterial Growth on Benzidine

All the strains of bacteria which were isolated from environmental samples incubated in BIM medium as well as the laboratory strains listed previously were tested for their ability to grow on bzd as the sole source of carbon and energy. Bacteria were inoculated into BIM supplemented with 1, 10, or 100 $\mu g\ ml^{-1}$ bzd and incubated under standard conditions for as long as 6 weeks. Some strains appeared to grow at one or more concentrations of bzd as noted by visual check of turbidity (Table 2). These were streaked directly onto plates containing 100 or 300 (saturated) $\mu g\ ml^{-1}$ bzd (Table 2). These strains were also inoculated into BIM in sidearm flasks. The optical density of the culture was followed for 2 weeks without noting any rise in turbidity (Table 3). The same flasks were seeded again with a fresh aliquot of bacteria and the optical density monitored. After an additional two weeks there was still

Table 1. Biochemical and cultural characteristics, source, and taxon of bacteria isolated during this study.

Strain	Source	Gram Reaction[a,b]	Shape[a,b]	O_2 Requirement[c]	Motile[a,b]	Oxidase[d]	O/F[e]	Colony Morphology[a]	Taxon[e]
B1	Great Bay[f]	-	rod	fa	-	-	F	pink, smooth, flat	Flavobacterium
CJ	Great Bay	-	rod	fa	+	+	F	translucent, flat, spreading	Vibrionaceae
K5	Keefe Waste Site	-	rod	fa	+	-	F	translucent, small, shiny	nd
K6	Keefe Waste Site	-	rod	fa	-	-	nd	translucent, small, shiny	nd
SF	Long Island, NY contaminated area	-	rod	a	-	+	-	creamy yellow, old colonies with sunken centers	nd
SG	Long Island, NY contaminated area	-	rod	a	+	+	nd	white, raised, sunken centers	Pseudomonaceae
SPK	Great Bay	-	rod	fa	+	+	F	translucent, flat, spreading	Vibrionaceae
SVR	laboratory	+	rod[g]	nd	-	+	nd	creamy, large, smooth	Bacillus

[a] determined from cultures on NBM nutrient agar incubated 18 h at 22°C.
[b] determined from cultures in NBM broth incubated 18 h at 25°C with shaking.
[c] Oxidation/Fermentation test, determined from Hugh & Leifson's O/F Medium (Difco) made with 25°/oo seawater supplemented with glucose or sucrose; anaerobic tubes overlaid with mineral oil. fa=facultative anaerobe, a=aerobe, F=fermentative, -=unable to utilize substrates, nd=not determined.
[d] tetramethyl-p-phenylene dihydrochloride method from colonies described in footnote (a).
[e] identifications verified in Buchanan and Gibbons (1974).
[f] hexadecane degrading organism isolated by E. Rose, UNH Microbiology Department.
[g] endospores observed.

Table 2. Bacterial growth at 22°C in BIM broth or agar plates supplemented with benzidine. All cultures were transferred directly from test tubes to plates.

Bacterial Culture	Benzidine ($\mu g\ ml^{-1}$)				
	Broth → transfer → Agar				
	1	10	100	100	300
K1	+[a]	+	+	-	-
K3	+	+	+	-	-
K4	+	+	-	-	-
K5	+	+	-	+	+
K6	+	+	-	+	+
15MY	-	-	+	-	-
CORE	+	-	-	-	-
B1	nd	nd	nd	+	+
CJ	-	-	-	+	-
SPK	-	-	-	+	-
SF	-	-	-	+	-
SG	-	-	-	+	+
SVR	-	-	-	+	+
P. denitrificans	nd	nd	nd	+	-
P. stutzeri	nd	nd	nd	+	-

[a] +=growth; -=no growth; nd=not determined.

Table 3. Growth of bacteria in BIM medium supplemented with 100 μg ml^{-1} benzidine at 25°C in darkness with shaking at 150 rpm in sidearm flasks.

Bacterial Culture	Days post inoculation[a]					
	0	2	12	16	22	32
K5	0.05[b]	0.03	0.03	0.11	0.09	0.06
K6	0.05	0.01	0.00	0.03	0.02	0.02
CJ	0.03	0.00	0.00	0.05	0.04	0.04
SF	0.05	0.04	0.04	0.08	0.05	0.04
SG	0.03	0.00	0.00	0.09	0.08	0.05
SVR	0.05	0.04	0.02	0.09	0.06	0.05

[a]Overnight cultures washed in sterile seawater were inoculated into sidearm flasks to the concentration represented by the OD_{600} at day 0 and incubated at 22-28°C. At day 16, a fresh overnight washed culture was added to raise the density of cells to the OD_{600} shown (visible turbidity).

[b]Optical density measured at 600 nm in a Bausch & Lomb Spectronic 20 spectrometer.

no evidence of growth. By the third direct subculture into BIM medium containing bzd as the sole source of carbon the ability to grow in this medium was lost by all strains.

Since these strains originally appeared to grow when bzd was the sole source of carbon, another experiment was performed to determine if these strains required any specifically added carbon to sustain growth. They were streaked for isolation onto plates of defined salts medium (Lyman and Fleming, 1940) solidified with purified agar with and without bzd (100 ug ml^{-1}). After 12 days' incubation all strains except SG and SVR showed comparable growth on each type of medium. Those two strains were reincubated an additional 14 days but no growth was seen on any plate. Thus, growth originally seen on bzd plates may not have been a consequence of the mineralization of bzd.

The two concentrations of bzd tested in those experiments were 100 and 300 $\mu g\ ml^{-1}$. To determine how other concentrations of bzd would affect the growth of these bacteria, gradient plates were constructed so that the concentration of bzd decreased from a saturated area in the center of the plate to minimal or zero concentration on the edges. Control plates had no bzd. Strains K1, K3, K4, K5 (all isolated from the Keefe Toxic Waste Site) and CJ (isolated from Great Bay sediment) failed to grow on either plate. These strains may have already lost the ability to grow in a low nutrient medium lacking added carbon. Three strains, 15MY (from Great Bay sediment), SF (from Sunrise 231), and SVR (the laboratory contaminant) grew on the plate lacking bzd but only grew on the outer 25% of the plate containing bzd (approximated by measuring the diameter of the plate). Strain SG (from Sunrise 231) grew on the plate lacking bzd but failed to grow on the plate spotted with bzd.

Thus, for these four strains high concentrations of bzd inhibited growth when no other source of carbon was present.

The presence of carbon might either mitigate or enhance the toxic effect of bzd on these bacteria. The degree of toxicity of bzd in the presence of nutrients was determined by repeating the spot test and plate incorporation test with nutrient agar plates (NBM medium) (Table 4). When 2, 5, or 8 mg bzd were spotted on a plate, zones of inhibition ranged from 0 to 5 mm from the edge of the bzd spot. Otherwise growth was continuous and abundant. In another experiment bzd was incorporated into the molten agar before pouring the plates. When 2 or 5 mg bzd was added to the plate, all strains grew over the entire plate. When 10 mg bzd was added to 20 ml molten agar per plate, not all bzd dissolved. Therefore these plates included some particulate bzd. Strains K5, K6, and CJ grew across the entire plate regardless of the particulate bzd. However, strains SF, SG, and SVR were inhibited in the areas of the particulate bzd only. Since the amount of bzd that remained particulate varied among plates, no direct comparison was made from strain to strain. However, these two experiments showed that bzd was not inhibitory to growth of these bacteria on nutrient media except when particulate bzd was present.

Extraction Procedure

The procedure developed for extracting bzd from seawater was successful in removing more than 90% of the radioactivity when the medium was sterile and contained no added glucose. When bacteria were incubated in the medium, however, the extraction efficiency was variable and ranged from 70 to 100% (Table 5). Less than 10% of the radioactivity was trapped in the pelleted bacteria and about 1% remained unextracted.

Table 4. Growth of bacteria on nutrient agar plates supplemented with benzidine as a center spot added after inoculation of the plates with 0.1 ml bacterial culture (spread plate), incubated at 27°C in darkness for two days.

Bacterial Culture	Benzidine Spotted (mg)		
	2	5	8
K5	<1[a]	4	4
K6	1	3	3
CJ	<1	0	3
SF	2	5	2
SG	3	4	5
SVR	2	3	3

[a]Zone of inhibition (mm) measured from edge of benzidine spot.

Table 5. Radioactivity in flasks before and after incubation, and recovery in extracted ethyl acetate, in bacterial pellet, and unextractable fraction.

			After Extraction							
Sample	(1) Before Incubation	(2) After Incubation	(3) Bacteria	%	(4) Extract	%	(5) Unextracted	%	(3)+(4)+(5)	%
Control	670[a]	720	--	-	670	-	8	1	680	94
K5	720	660	17	3	440	67	8	1	470	71
K6	710	650	38	6	550	84	8	1	590	91
CJ	720	680	42	6	630	93	9	1	680	101
SF	700	720	31	4	460	64	9	1	500	70
SG	720	680	31	5	550	82	9	1	590	88
SVR-1	720	770	27	4	570	74	9	1	600	79
SVR-2	680	610	55	9	420	68	8	1	480	79

[a]Kilo counts per min.

The loss of radioactivity occurred during the extraction procedure. This radioactivity may have been trapped in the film of bacterial cell material which remained on the sides of the flasks. Repeated washings of the flask with ethyl acetate did not remove the radioactivity.

A statistical evaluation was performed using the t test for comparison of sample means with paired observations (Steel and Torrie, 1960) on the data recorded for radioactivity of the samples before and after incubation from Table 5. The t statistic was 1.05 while $t_{.05,7df} = \pm 2.365$; therefore, the hypothesis that the sample means came from the same population was accepted.

A loss of extraction efficiency was also noted in all flasks containing glucose. For example, in sterile flasks, the extraction procedure removed 98% of the radioactivity when glucose was absent but only 72% when glucose was present. This factor was not related to the pH of the media (Table 6). These variations in extraction efficiency indicated that the affinity of bzd ^{14}C-congeners for the organics present in bacterial cultures and glucose-containing media was greater than their affinity for ethyl acetate.

The extraction procedure removed on average 30% of the radioactivity from sediments (Table 7). Most of this was removed in the supernatant fluid, and the ethyl acetate washes removed very little.

Sorption of Benzidine to Sediments

Bzd sorbed strongly to sediments, reducing extraction efficiency. In an experiment to establish the effect of various concentrations of sediment on the extraction efficiency of media containing 100 μg ml^{-1} bzd, concentrations of sediment greater than 20 mg ml^{-1} reduced extraction efficiency. At 100 mg ml^{-1} sediment, the extraction efficiency was 22%

Table 6. pH and percent unextractable radioactivity in flasks of BIM media supplemented with or lacking glucose, after incubation at room temperature for 6 days with shaking.

Sample	Glucose	pH Before Incubation	pH After Incubation	Percentage Unextracted Radioactivity
Control	+	7.10	7.88	28
	-	7.05	7.79	2
K5	+	6.00	2.97	21
	-	6.91	7.67	2
K6	+	6.98	4.70	29
	-	7.04	7.88	2
SVR	+	6.80	3.64	44
	-	6.86	8.00	2

Table 7. Percentage ^{14}C-benzidine extracted from sediment. Test tubes contained one gram sediment in 10 ml mineral salts medium (BIM) supplemented with 100 µg ml^{-1} benzidine and were shaken 24 h at 25°C.

	Trial				
	1	2	3	4	Average
Supernatant Fluid	18[a]	15	14	14	15
Wash 1	8	7	5	5	6
Wash 2	2	5	5	3	4
Wash 3	9	4	5	2	5
Total % Extracted	37	31	29	25	30

[a]Percentage of total ^{14}C-benzidine added to test tube.

of that at 20 mg ml^{-1} (Figure 2). Therefore, if sediment was used as a sorbing medium in experiments, it was used at a concentration of 20 mg ml^{-1}.

This concentration of sediment was used to construct a standard curve of bzd sorption at various concentrations of bzd. At the lowest concentration tested, 0.03 ng ml^{-1} bzd, 94% of the bzd was bound to the sediment (Figure 3). With increasing concentration total bzd the percentage bound bzd decreased to 23% at 100 µg ml^{-1} bzd.

The sorption of bzd to sediments was not affected by the amount of glucose present (Figure 4). Over a range of glucose concentration from 0-5%, the amount of bzd which remained in the supernatant fluid after equilibration was constant, for bzd concentrations ranging from 0.03 to 100 µg ml^{-1}.

Abiotic Transformation of Benzidine

Upon extraction with ethyl acetate and subsequent thin layer chromatography, all flasks including sterile controls gave rise to multiple spots. These spots could have resulted from the use of forced air for evaporating the ethyl acetate extracts causing abiotic transformation of bzd. This hypothesis was tested by incubating a series of flasks under either forced air or nitrogen and subsequently evaporating them under either air or nitrogen. Multiple spots appeared on the thin layer chromatograms under all combinations of gas treatments (Table 8). Contact of the media with air during incubation or during the extraction procedure did not cause the oxidation of bzd. Air was used for both the incubation gas and the evaporation gas in all experiments, and thin layer chromatograms of experimental flasks were compared to the sterile control flask which was incubated and evaporated at the same time using the same procedure.

Figure 2. Extraction of benzidine (as measured by counts per minute of ^{14}C-benzidine) from sediment at various concentrations in seawater supplemented with 100 µg ml^{-1} benzidine plus negligible quantity of ^{14}C-benzidine. Equilibration occurred during 24 hours shaking at 25°C. Circles represent the amount removed by the ethyl acetate extractions and triangles represent the amount remaining in the supernatant fluid.

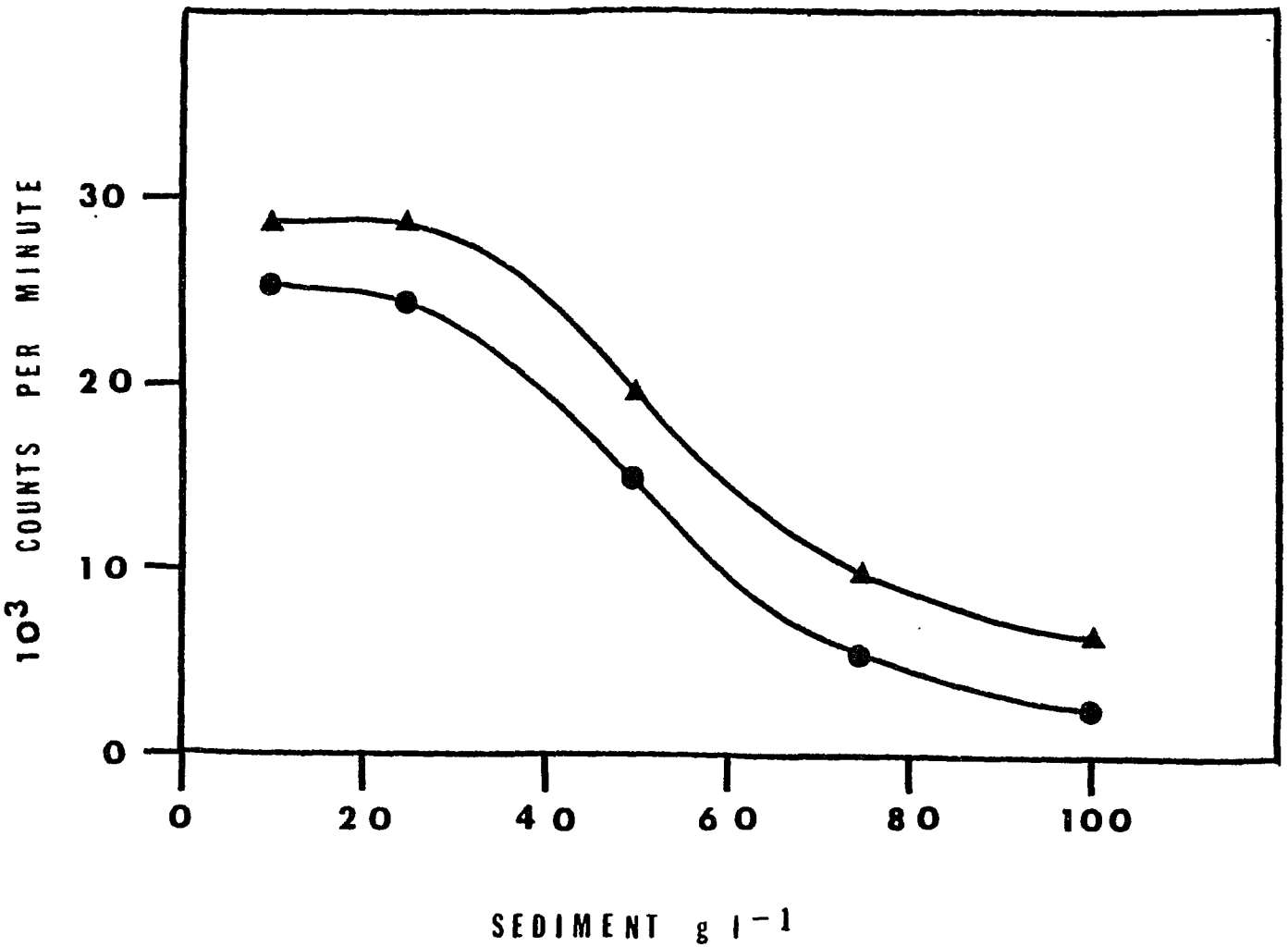

Figure 2.

Figure 3. Percentage of benzidine (as measured by counts per minute of ^{14}C-benzidine) bound to 20 mg ml^{-1} sediment at various concentrations of benzidine.

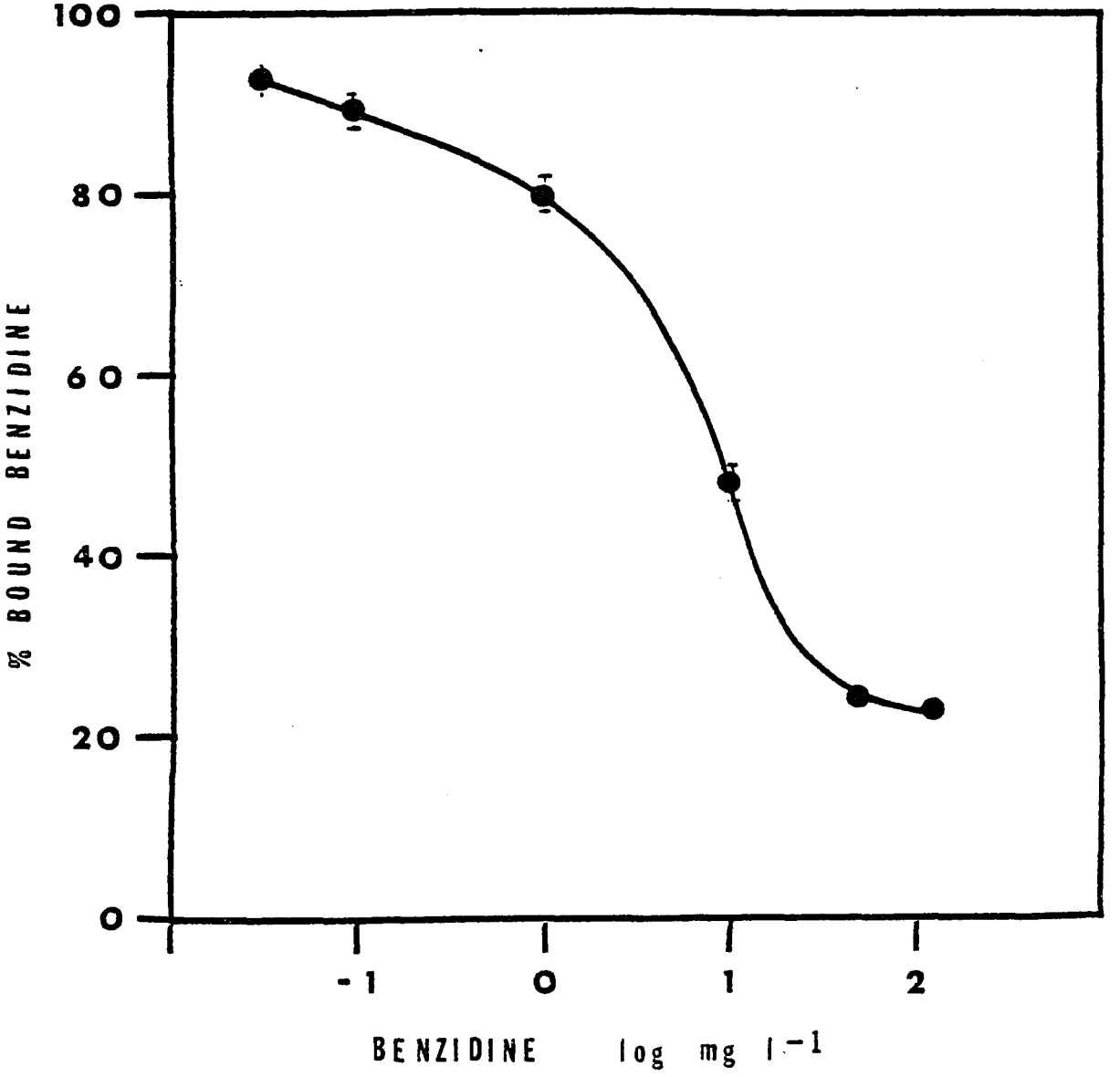

Figure 3.

Figure 4. Percentage of benzidine remaining in the supernatant fluid after equilibration with 20 mg ml^{-1} sediment in BIM containing various amounts of benzidine and glucose. Benzidine concentrations: (□) 0.03 μg ml^{-1}, (●) 0.10 μg ml^{-1}, (△) 1 μg ml^{-1}, (■) 10 μg ml^{-1}, (○) 50 μg ml^{-1}, (▼) 100 μg ml^{-1}.

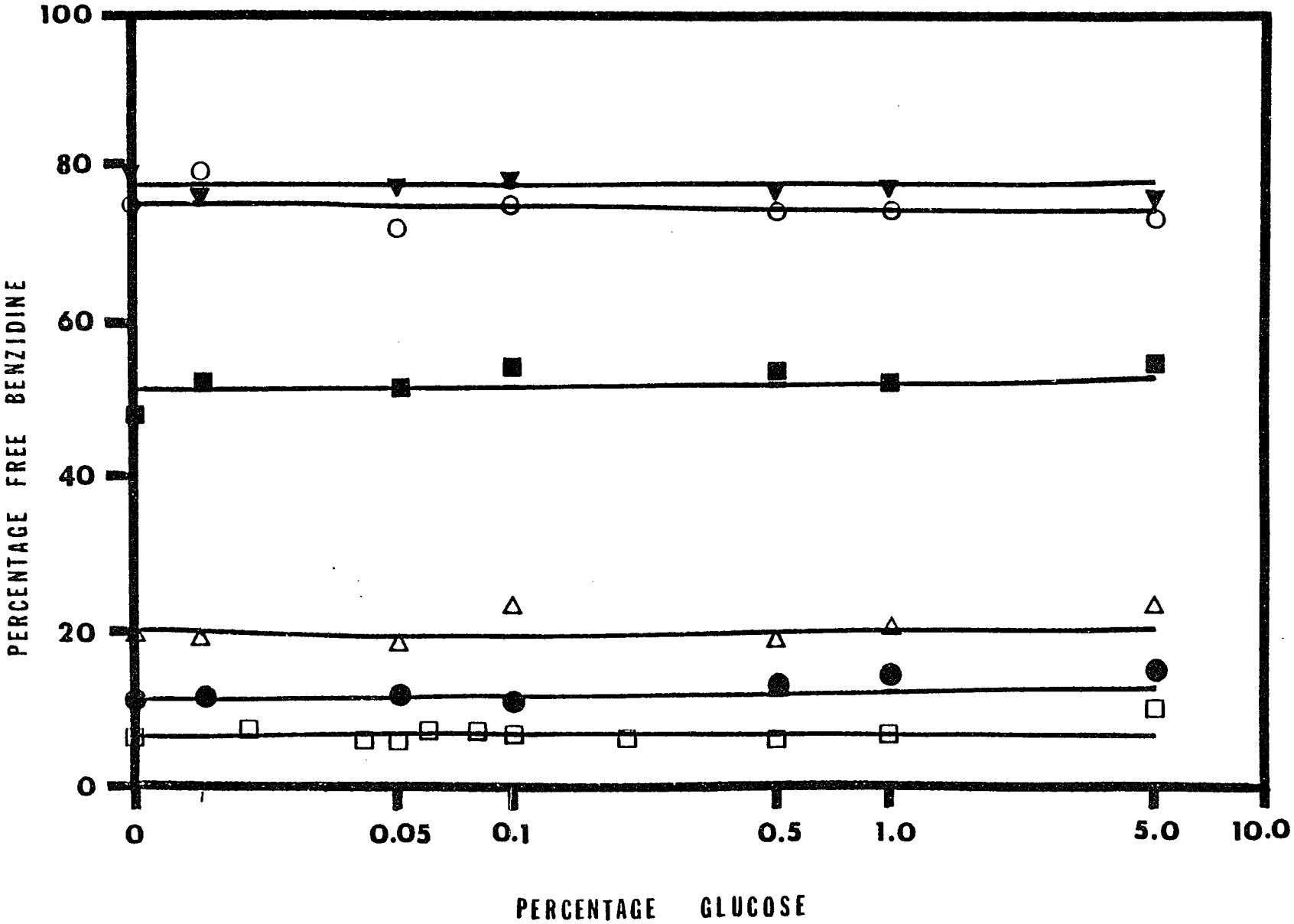
100
80
60
40
20
0
PERCENTAGE FREE BENZIDINE
0
0.05
0.1
0.5
1.0
5.0
10.0
PERCENTAGE GLUCOSE

Figure 4.

Table 8. Thin layer chromatographic analysis of sterile benzidine incubation media (BIM) incubated and/or extracted under nitrogen or forced air, after incubation for 6 days at 25°C with shaking.

Incubation Gas:	N_2	N_2	air	air
Evaporation Gas:	N_2	air	N_2	air
Spot A	0.94[a]	0.96	0.95	--
Spot B	0.85	0.84	--	0.86
Spot C[b]	0.72	0.73	0.72	0.75
Spot D	0.61	0.61	0.61	0.63
Spot E.	--	--	--	0.56

[a] R_f in ethyl acetate:1-butanol (99:1) solvent system; -- indicates no spot appeared.

[b] Benzidine Standard R_f = 0.75.

The combination of bzd and ethyl acetate did not cause multiple spots on thin layer chromatograms; the sole spot chromatographed in the range R_f=0.75-0.79. The R_f varied slightly due to the level of saturation of the solvent in the atmosphere and to the distance of the solvent front. In part this is a mathematical artifact. Authentic bzd was always chromatographed on every plate to provide an external standard.

Incubation in Fresh Water Medium

Since the bacteria isolated from the Keefe toxic waste site were not known to be acclimated to the salinity used in these experiments some of them were tested for their ability to degrade bzd in fresh water. None of the strains tested showed increased ability to degrade bzd in fresh water. This information was obtained by cutting out the spots from thin layer chromatograms and counting the radioactivity in each spot. As an example, for strain K2, more than 75% of the radioactivity remained in the spot cochromatographing with bzd, with about 10% remaining at the origin (Table 9). Although another spot with an R_f=0.66 showed measurable radioactivity, the fresh water flasks showed less of this product than the seawater flasks. Since all strains used in these experiments grew readily in a nutrient medium of the chosen salinity, strains were not tested routinely for transformation of bzd in fresh water.

Biotransformation of Benzidine

Biotransformation of bzd as detected by thin layer chromatography was tested at two concentrations of bzd. At a low concentration (1 $\mu g\ ml^{-1}$) strains B1, K5, P. denitrificans, and P. stutzeri failed to metabolize bzd. The amount of transformation of bzd was similar to

Table 9. Benzidine transformation in fresh water and seawater in sterile flasks and in the presence of bacterial strain K2, after incubation for 6 days at room temperature in darkness with shaking. Radiolabel analysis of thin layer chromatogram spots.

	Percentage of Recovered ^{14}C			
	Fresh water		Seawater	
R_f	Sterile	Strain K2	Sterile	Strain K2
0.75[a]	79	82	77	77
0.66	5	6	10	7
0.57	4	2	4	4
0.41	1	1	1	2
origin	11	9	8	10

[a]Benzidine Standard R_f = 0.78, ethyl acetate:1-butanol (99:1) solvent system.

that of the sterile control flask, in which abiotic transformation was noted. However, when strains SF, SG, and CJ were incubated in the medium most of the radioactivity on the thin layer chromatogram appeared not at a spot corresponding to bzd but at a unique spot with R_f=0.30 (Figure 5). This compound did not appear in the control flask. Most of the bzd appeared to be transformed by these three strains of bacteria. However, mineralization to CO_2 did not occur, since the amount of radioactivity in the flasks after incubation was within 10% of that before incubation. The t statistic calculated to compare sample means, paired observations was 5.05. Therefore the hypothesis that the means were from the same population was rejected ($t_{.05,8df}$=2.306) (Table 10). The data indicate that in all cases the radioactivity after incubation was lower than the radioactivity at the start of the experiment, which would explain the statistical result obtained. However, as previously stated bzd sorbs to organic compounds on the glass and in this experiment the radioactive bzd comprised a significant fraction of the total bzd. Thus, proportionately more ^{14}C-bzd would be sorbed than in experiments with more unlabeled bzd present. A reduction in recovered radioactivity of 10% would be explained by this factor. At this low concentration of bzd, not enough radioactive material could be extracted to perform mutagenesis assays. Therefore, transformation experiments were repeated at the higher concentration of 100 μg ml^{-1} bzd.

At this concentration, strain CJ again transformed bzd to a compound chromatographing at R_f=0.32 (Figure 6). Strains SVR and K6 transformed bzd to a compound chromatographing at R_f=0.49 and gave slight evidence of transforming bzd to a spot at R_f=0.32 (Figure 7). Strains K5 and P. denitrificans did not appreciably transform bzd. A spot

Figure 5. Autoradiograph of thin layer chromatogram of bacterial cultures incubated in BIM medium supplemented with 1 $\mu g\ ml^{-1}$ benzidine and glucose for six days at 25°C in darkness with shaking. Lanes: (1) SF, (2) P. stutzeri, (3) P. denitrificans, (C) Control, (5) SG, (6) K5, (7) CJ, (8) B1, (BZD) Benzidine standard.

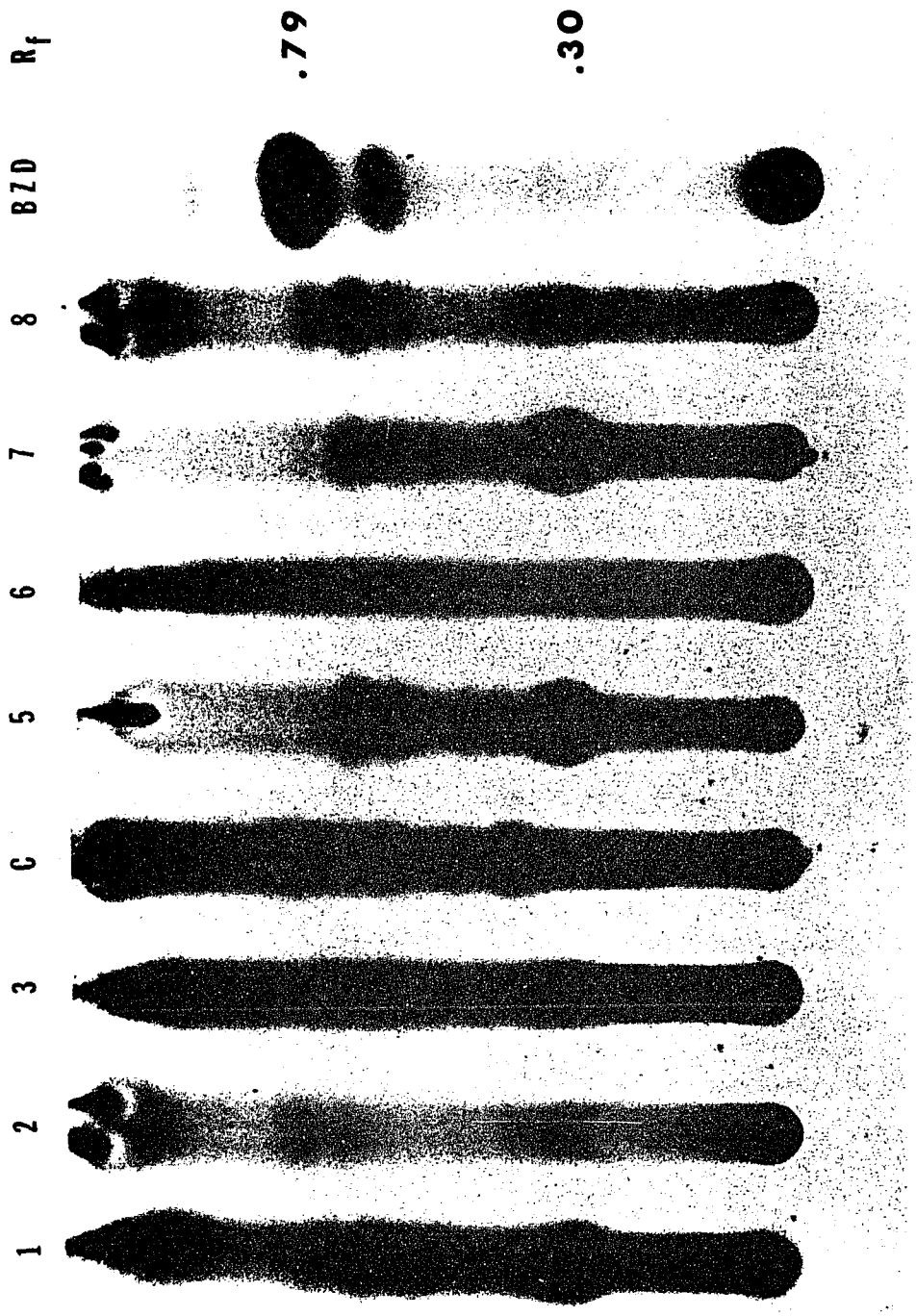

Figure 5.

Table 10. Radioactivity and pH of flasks before and after incubation of bacteria in BIM media supplemented with 1 μg ml^{-1} benzidine, incubated at 25°C 6 d in darkness with shaking.

	Before Incubation	After Incubation		
Strain	radioactivity[a]	radioactivity[a]	%	pH
Control 1	760	750	99	7.92
Control 2	780	750	96	7.85
B1	730	670	92	3.16
K5	750	740	99	2.90
CJ	730	670	92	3.81
SF	740	700	95	3.33
SG	760	700	92	3.37
P. stutzeri	750	710	95	2.91
P. denitrificans	740	670	90	3.57

[a]Kilo counts per min.

Figure 6. Autoradiograph of thin layer chromatogram of extracts from bacterial cultures incubated in BIM media supplemented with 100 $\mu g\ ml^{-1}$ benzidine for six days at 25°C in darkness with shaking. Lanes: (1) strain CJ in media with glucose, (2) CJ in media lacking glucose, (3) P. denitrificans with glucose, (4) P. denitrificans without glucose, (C+) Control with glucose, (C-) Control without glucose, (BZD) Benzidine standard, (8) Benzidine incubated with S9 mix for 30 minutes prior to spotting on thin layer chromatogram.

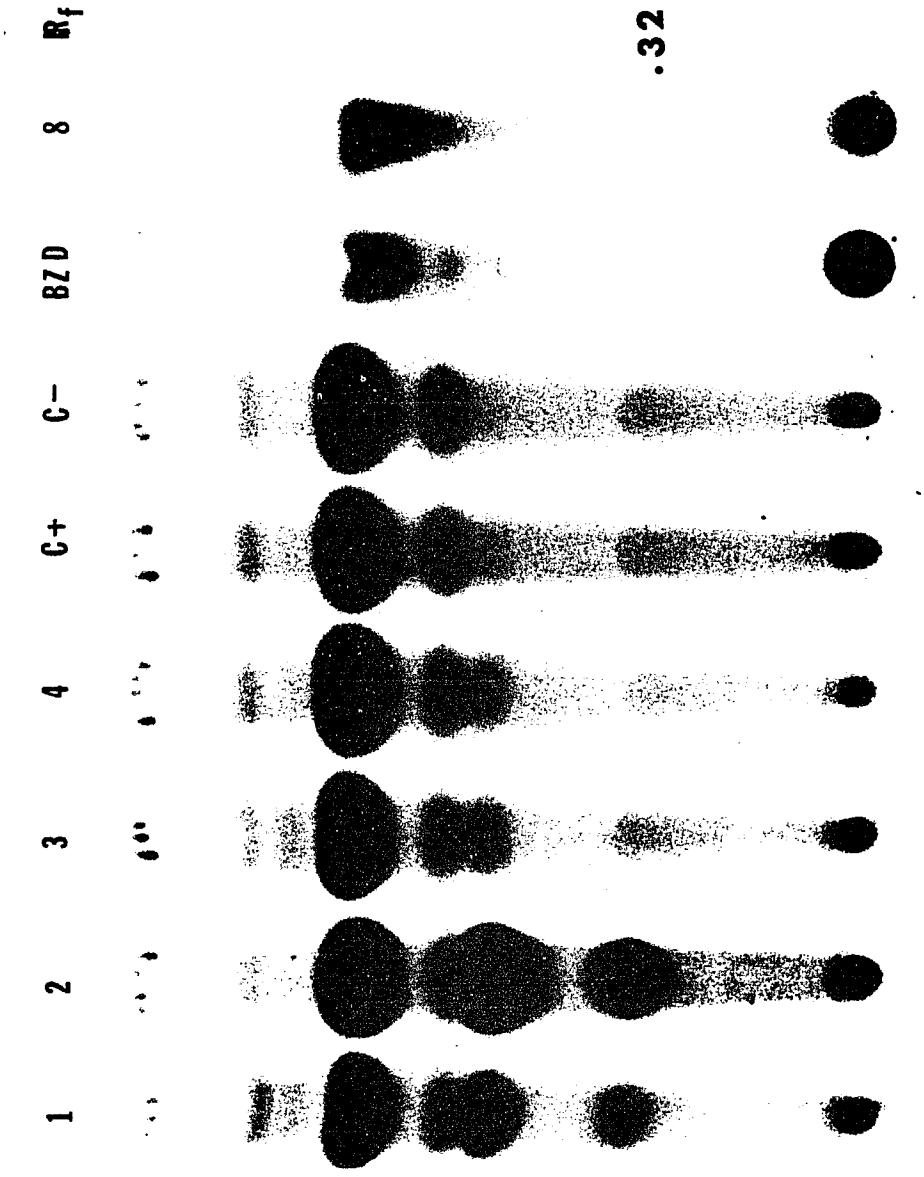

Figure 6.

Figure 7. Autoradiograph of thin layer chromatogram of extracts from bacterial cultures incubated in BIM media supplemented with 100 µg ml^{-1} benzidine in the presence or absence of 5 mg ml^{-1} glucose for six days at 25°C in darkness with shaking. Quantity of extracts applied to thin layer chromatogram not corrected for concentration of benzidine. Lanes: (C+) Control flask supplemented with glucose, (C-) Control flask without glucose, (3) K5 with glucose, (4) K5 without glucose, (5) K6 with glucose, (6) K6 without glucose, (7) SVR with glucose, (8) SVR without glucose, (BZD) Benzidine standard.

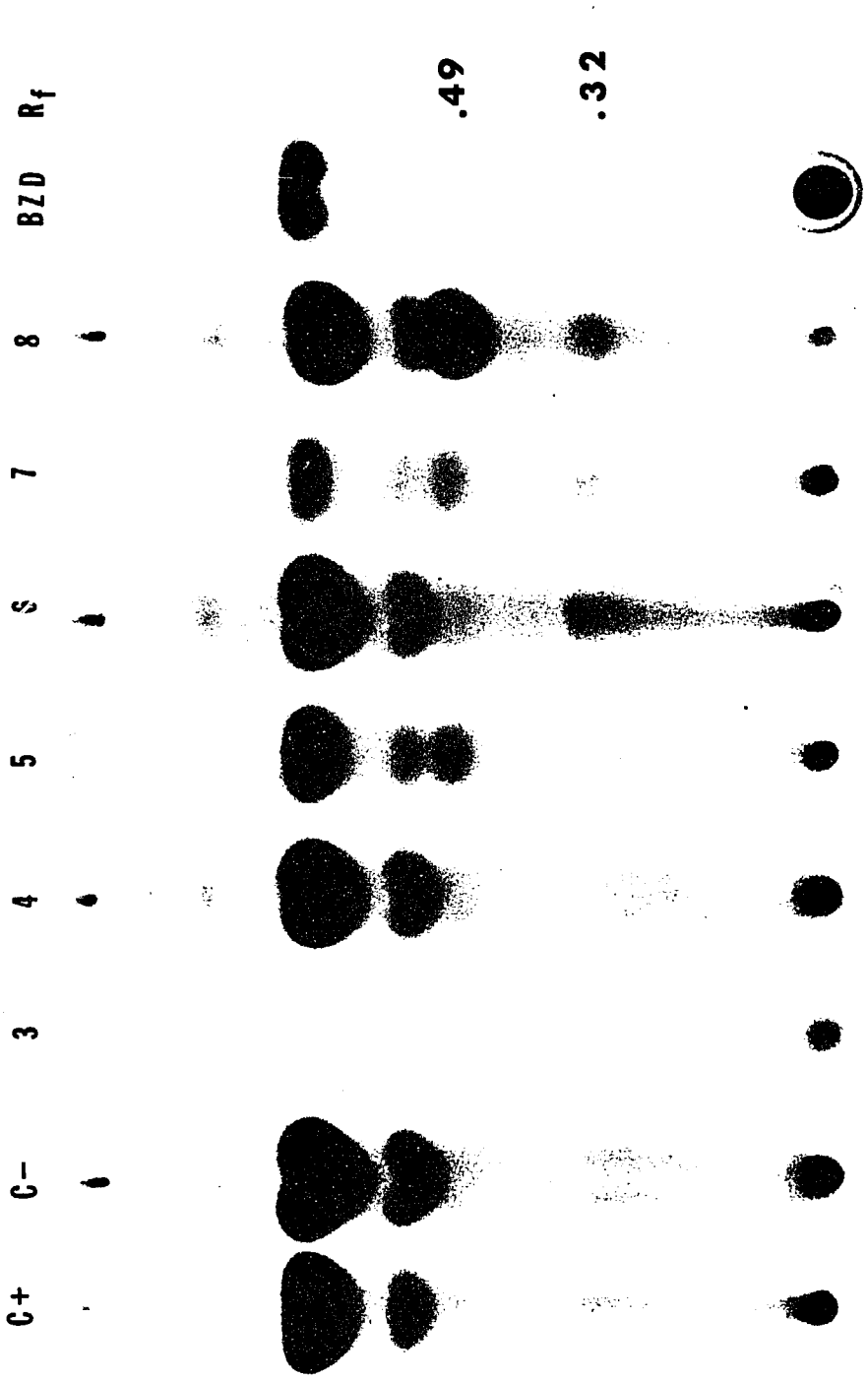

Figure 7.

cochromatographing with authentic bzd was isolated from all flasks. This spot was more intense on the autoradiograph than any other, indicating that although some transformation of bzd took place, most of the bzd was not acted on. This experiment gave the first evidence of loss of extraction efficiency when glucose was present, as indicated by the lowered level of radioactivity in lanes of extracts of glucose-containing flasks including the unseeded control, compared to equivalent incubations in flasks not supplemented with glucose. In later experiments the application of extract to the thin layer chromatography plate was normalized to the amount of radioactivity present.

Many of the spots appearing on the autoradiograph (Figure 6) were not apparent on the thin layer chromatogram (Figure 8), corroborating the necessity of using autoradiography to detect biotransformation. No spots appeared on the thin layer chromatogram that were not also present on the autoradiograph, indicating that the extraction procedure did not cause nonradioactive compounds in the bacteria or media to chromatograph.

The experiment was repeated under the same conditions except that defined salts seawater was used (Lyman and Fleming, 1940), and the ethyl acetate extracts applied to the thin layer chromatogram were normalized to result in the application of approximately 20,000 cpm. The resultant autoradiograph (Figure 9) shows that strain CJ gave a spot at R_f=0.32. Strains CJ, SF, SG, and SVR all showed a strong spot at R_f=0.51. Strains K5 and K6 did not show greater transformation of bzd than the control. In all cases at this higher concentration of bzd, the spot corresponding to bzd was the strongest on the chromatogram. During incubation strain SF developed a pink pigment in the supernatant

Figure 8. Thin layer chromatogram of extracts from bacterial cultures incubated in BIM media supplemented with 100 μg ml^{-1} benzidine for six days at 25°C in darkness with shaking. Lanes: (1) strain CJ in media with glucose, (2) CJ in media lacking glucose, (3) P. denitrificans with glucose, (4) P. denitrificans without glucose, (C+) Control with glucose, (C-) Control without glucose, (BZD) Benzidine standard, (8) Benzidine incubated with S9 mix for 30 minutes prior to spotting on thin layer chromatogram.

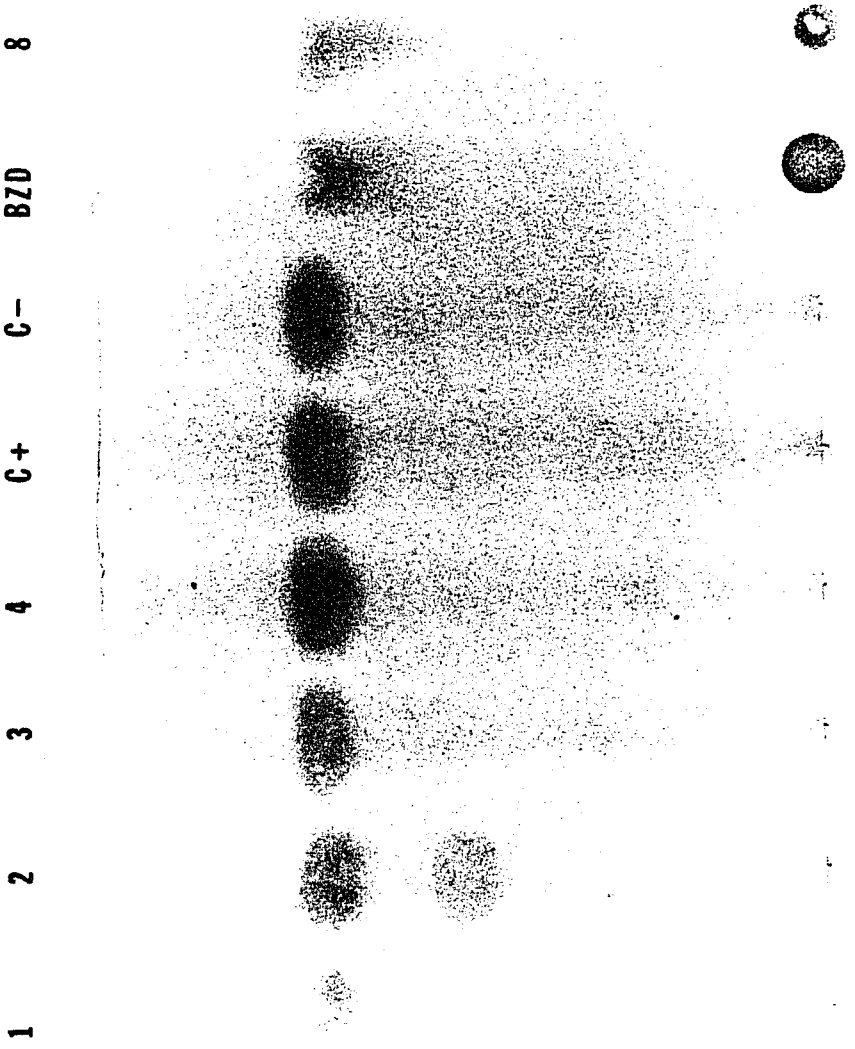

Figure 8.

Figure 9. Autoradiograph of thin layer chromatogram of extracts from bacterial cultures incubated in BIM media made with defined salts seawater supplemented with 100 $\mu g\ ml^{-1}$ benzidine for six days at 25°C in darkness with shaking. Quantity of extracts applied to thin layer chromatogram adjusted to result in application of approximately 20,000 cpm radioactivity. Lanes: (C) Control, (2) K5, (3) K6, (4) CJ, (5) SF, (6) SG, (7) SVR, (BZD) Benzidine standard.

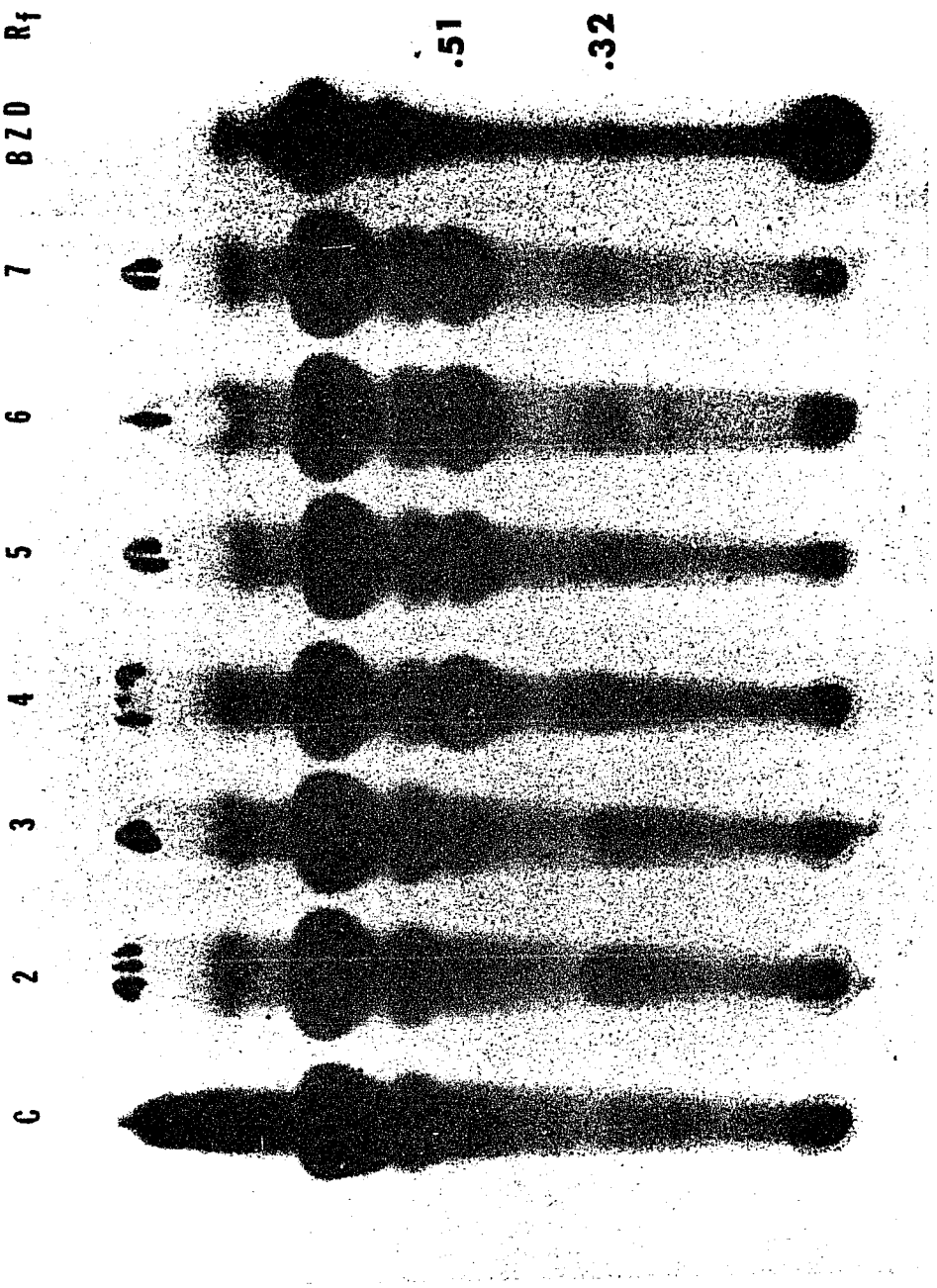

Figure 9.

fluid which was water soluble and was extracted in ethyl acetate. However, there was no indication of this product either on the thin layer chromatogram or on the autoradiograph. There was no evidence of mineralization of bzd by any strain, as the amount of radioactivity after incubation was within 4% of that before incubation (Table 11). The t statistic was 0.60 ($t_{.05,6df}$=2.447) indicating acceptance of the hypothesis that the sample means came from the same population. The bzd standard chromatographed in the last column shows a ghost spot which may have been due to carryover from a previous column via an improperly cleaned syringe.

Similar experiments were performed with other strains of bacteria using media containing 100 $\mu g\ ml^{-1}$ bzd. Only the strains that showed some transformation of bzd were reported here. The other strains tested showed no evidence of ability to transform bzd. None of the strains which did show evidence of ability to transform bzd appeared to mineralize bzd.

The spot at R_f=0.51 from a strain CJ culture was subjected to gas chromatography-mass spectrophotometry in an attempt to identify it. However, under the extraction procedure noted in Materials and Methods, the compound did not elute from the column. Further steps to identify it would include derivatization and use of a different column, and this was not within the scope of this project.

Speed of Transformation of Benzidine by Strain CJ

The transformations of bzd noted in the previous experiments occurred after 6 days' incubation. Another experiment was performed to determine when transformation was maximal. Strain CJ was incubated in

Table 11. Conservation of radioactivity in BIM medium supplemented with 100 µg ml^{-1} benzidine during incubation of six bacterial isolates for six days at 25°C in darkness with shaking.

Bacterial Culture	Total Kilocounts per Minute		Percentage Remaining
	Before	After	
Control	690	710	103
K5	620	640	103
K6	680	660	97
CJ	700	670	96
SF	610	600	98
SG	660	660	100
SVR	640	630	98

one liter of BIM and aliquots removed daily for six days and then at intervals for a total of 34 days (Table 12). Formation of the biotransformed product (chromatographing from R_f=0.54-0.57) peaked 5 days after incubation began but the percentage of total radioactivity chromatographing at this zone decreased from its peak at 5 days to the end of incubation, at which time the concentration of this product was lower than at the start of incubation. Mineralization did not occur, as total radioactivity after incubation was not lower than radioactivity at the beginning of the experiment. The change in percentage of radioactivity in each of the spots of the chromatogram indicate that by 34 days after incubation the compound had reverted to a compound chromatographing at the bzd locale. The second compound appeared to be a transient alteration of bzd, indicating a reversible addition to the bzd molecule but not ring cleavage.

Sequential Bacterial Incubation

Several strains of bacteria transformed bzd to other products. An experiment was performed to explore whether these bacteria acting sequentially could transform bzd further than any strain acting in pure culture could. Strain CJ was selected as the bacterium for the first stage of incubation and was incubated in one liter of BIM under standard incubation conditions. After 6 days' incubation, the CJ medium was centrifuged and additional ammonia, iron and phosphate added in the concentrations normally used. This solution became the incubation medium for strains SG, SF, K5, SVR, and K6 as well as another culture of strain CJ. In addition, an aliquot of the first stage medium was refrigerated to provide a control and another aliquot was allowed to continue incubation without interruption. After 6 days all flasks were extracted.

Table 12. Radioactivity before extraction and percent of total radioactivity applied in spots recovered from thin layer chromatogram of strain CJ incubated in BIM medium supplemented with 100 µg ml^{-1} benzidine, incubated with shaking at 25°C in darkness.

	Days After Inoculation									
	0	1	2	3	5	6	8	20	27	34
cpm ml^{-1}	6390	7000	8620	7620	7810	7390	8520	5960	8120	7910
R_f range										
0.88-0.93	5[b]	3	3	2	2	3	3	5	4	3
0.73-0.76[a]	77	81	77	74	75	76	83	82	87	89
0.64-0.65	7	5	5	6	4	4	2	3	3	3
0.54-0.57	5	6	9	11	14	12	11	7	3	3
0.34-0.35	3	2	2	2	3	2	1	1	1	1
origin	3	3	4	5	2	3	1	2	1	1

[a]Benzidine Standard R_f = 0.74, ethyl acetate:1-butanol (99:1) solvent system.
[b]Percentage of total radioactivity applied.

The autoradiograph resulting from extraction of these flasks and subsequent thin layer chromatography (Figure 10) was analyzed along with scintillation counting of the spots cut out from the thin layer chromatogram (Table 13). CJ(o) represents the amount of transformation achieved after the first stage of incubation and is the standard against which additional transformation was measured. CJ denotes the aliquot which was incubated without interruption throughout both stages of the experiment, and CJ(r) is the CJ culture freshly inoculated at the beginning of the second stage. Because the extracts were normalized so that approximately 20,000 cpm were applied to the chromatogram, different absolute quantities of extract were applied and this affected the migration of the spots. Therefore, equivalent spots have slightly different R_f values and are combined within an R_f range.

The results from both the autoradiograph and the radioactivity of the spots indicate that after the first stage CJ(o) transformed 11% of the bzd to a product chromatographing at R_f=0.52-0.54. When an aliquot was incubated an additional 6 days, more than half of this second compound disappeared. Strains SG, SF, K5, and CJ also promoted disappearance of this compound. Strain K6 neither caused this compound to disappear nor enhanced its appearance or the appearance of any additional spot. The total radioactivity in these flasks did not decrease during incubation. These strains acting sequentially after strain CJ did not transform bzd to any greater degree than did any strain acting in pure culture.

With strain SVR, however, 30% of the bzd was transformed to this compound. There also appears to be a second transformation product accumulating at R_f=0.27-0.34. A Gram stain of the flask contents after

Figure 10. Autoradiograph of thin layer chromatogram of bacterial isolates in the supernatant fluid from a prior incubation of strain CJ in BIM media supplemented with 100 μg ml^{-1} benzidine incubated six days, centrifuged and the supernate supplemented with ammonia, ferrous sulfate, and phosphate. CJ(o) represents the extract after the first stage of incubation, CJ(r) represents the first stage which continued incubation without interruption through the second stage, and the other six isolates represent the second stage incubation, which continued for six days at 25°C in darkness with shaking. Lanes: (1) SG, (2) SF, (3) K5, (4) SVR, (5) CJ, (6) CJ(r), (7) K6, (8) CJ(o), (BZD) Benzidine standard.

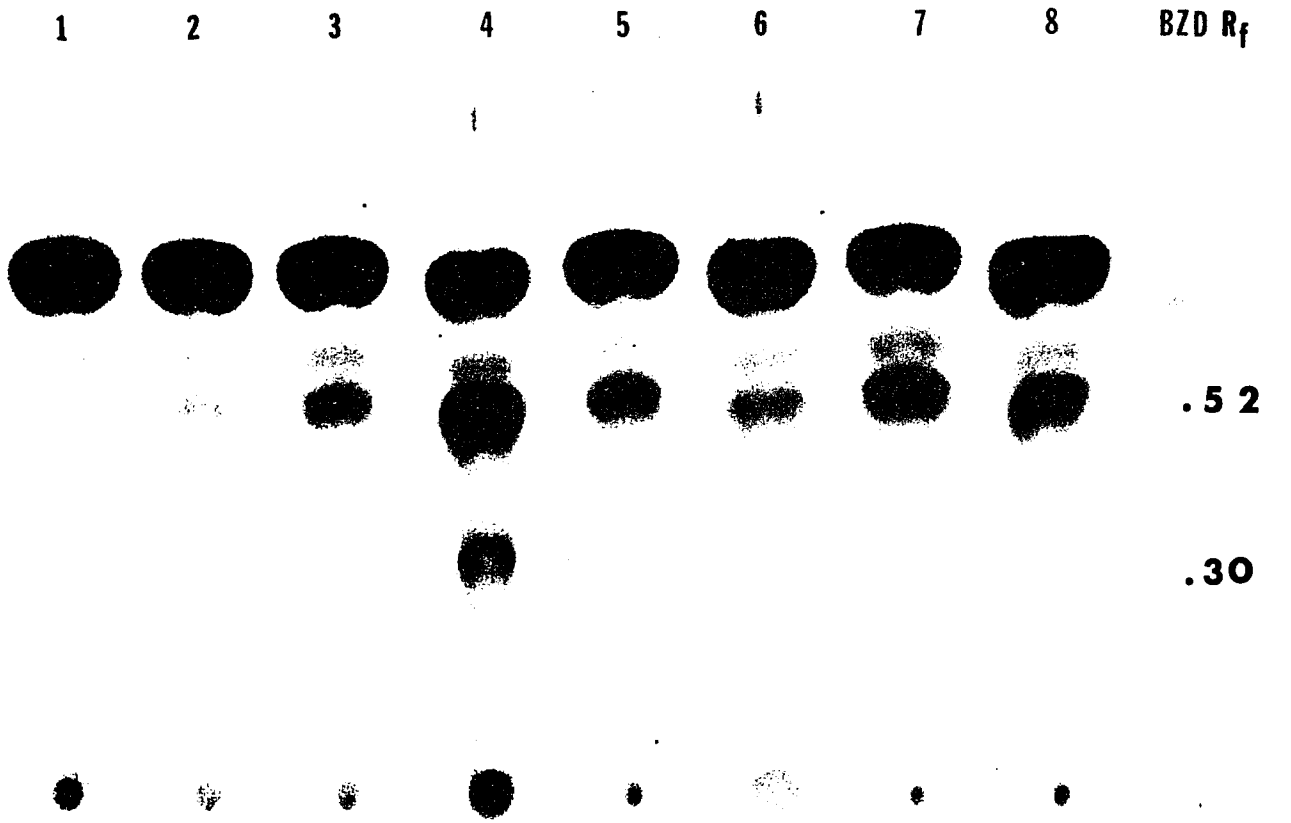

Figure 10.

Table 13. Incubation of bacterial isolates in the supernatant fluid from a prior incubation of strain CJ in BIM supplemented with 100 μg ml^{-1} benzidine, incubated 6 days, centrifuged and the supernatant fluid supplemented with ammonia, ferrous sulfate, and phosphate (see text for concentration). CJ(o) represents the extract after the first stage of incubation, CJ(r) represents the first stage which continued incubation without interruption through the second stage, and the other six isolates represent the second stage incubation, which continued for 6 days at 25°C in darkness with shaking. Spots from thin layer chromatographic analysis of ethyl acetate extracts were cut out and the radioactivity in the spots counted.

	Kilocounts per minute (percentage of total in parenthesis)							
R_f Range	CJ(o)	CJ(r)	SG	SF	K5	SVR	CJ	K6
0.82-0.87	0.37(2)[a]	0.56(3)	0.16(1)	0.20(1)	0.21(1)	0.37(2)	0.47(3)	0.37(2)
0.71-0.73[b]	16.0(79)	16.0(84)	18.0(88)	16.0(88)	16.0(84)	9.0(52)	14.0(81)	13.0(75)
0.52-0.54	2.0(11)	0.73(4)	0.28(1)	0.37(2)	0.87(5)	5.0(30)	1.0 (7)	2.0(12)
0.27-0.34	0.33(2)	0.20(1)	0.25(1)	0.22(1)	0.19(1)	0.97(5)	0.26(1)	0.23(1)
origin	0.25(1)	0.34(2)	0.36(2)	0.28(2)	0.27(1)	0.70(4)	0.22(1)	0.23(1)
other	1.0 (6)	1.0 (6)	1.0 (7)	1.0 (6)	2.0 (8)	1.0 (6)	1.0 (6)	1.0 (8)
Total	20	19	20	18	19	18	18	17

[a] Percentages reflect rounding errors.

[b] Benzidine Standard R_f = 0.72, ethyl acetate:1-butanol (99:1) solvent system.

incubation showed a mixed culture with a minority of cells being Gram positive Bacillus sp. characteristic of strain SVR and the majority of the cells being Gram negative rods characteristic of strain CJ. Although most of the cells of SVR died during the incubation since few cells proportionally were recovered, the mixed culture appeared to prevent the reversion of this compound back to bzd while also providing enough nutrients to permit strain CJ to grow and additionally metabolize bzd. Thus, more bzd was converted to this product, but transformation to another product or mineralization did not occur.

Effect of Light on Biodegradation

All experiments had been conducted in darkness to minimize the possible photolysis of bzd. To determine whether biotransformation would be affected by light, paired flasks were incubated either in darkness or exposed to room light. Results showed that there was no difference between light and dark flasks for any of the strains tested (K5, K6, CJ, SF, SG, SVR) or for the controls (data not shown). All showed the same degree of transformation that they had shown in previous experiments.

Portions of the ethyl acetate extracts were saved from all experiments and were used in mutagenesis assays to establish whether transformation of bzd affected its mutagenic potential.

Ames Test

Bzd was mutagenic in the Ames test against the frameshift mutants Salmonella typhimurium TA1538 and TA98 in the range from 70 to 200 μg bzd per plate in the presence of 20 μl ml^{-1} S9 mix (Figure 11). There was no mutagenic activity of bzd in the absence of this liver microsomal

Figure 11. Response of Ames tester strains TA1538 (●) and TA98 (■) to various concentrations of benzidine in the Ames test shown as number of revertants per plate, average of three plates each.

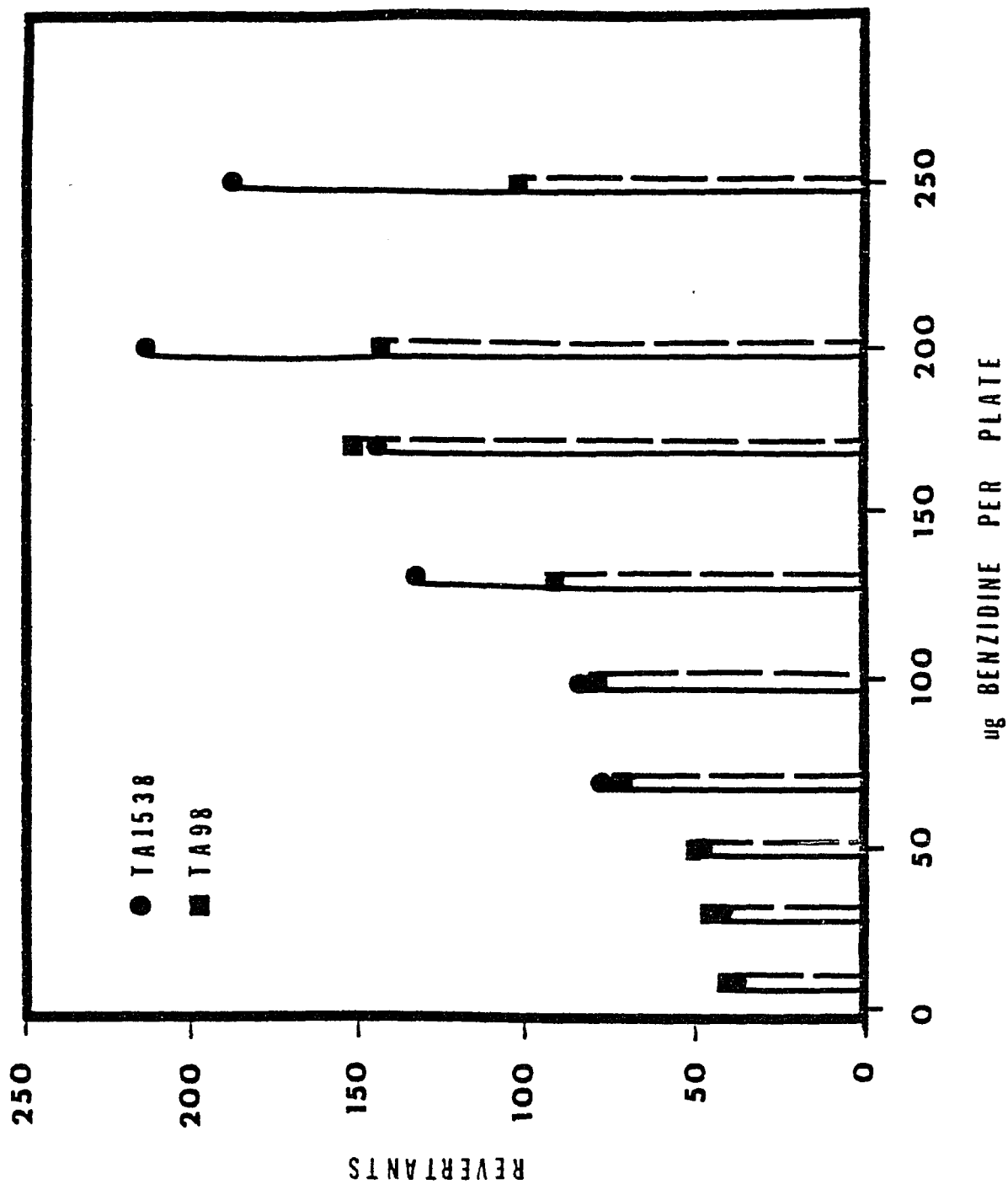
REVERTANTS
250
200
150
100
50
0
● TA1538
■ TA98
0
50
100
150
200
250
ug BENZIDINE PER PLATE

Figure 11.

activation mix. The mutagenic activity of extracts from sterile control flasks containing seawater based media and sediment, and from both seawater based and fresh water based sediment-containing flasks incubated with strain K2 were tested in this assay at approximately 100 μg "equivalent bzd radioactivity" per plate. All extracts showed significant mutagenesis for TA98 after microsomal activation. There was no activity in the absence of the S9 mix. Tester strain TA100 (not a frameshift mutant) did not respond to any extract or to bzd (150 μg per plate). These data supported the literature reports of the mutagenicity of bzd and substantiated the conservation of mutagenicity of bzd during the extraction procedure. However, other assays were explored to provide an alternative method of measuring mutagenicity.

Modified Fluctuation Test

Results with several standard carcinogens indicated that the modified fluctuation test is sensitive for at least some types of mutagens. Nitroquinoline-N-oxide resulted in highly significant mutagenesis over the entire range tested, 20 to 200 ng per assay. Daunomycin was not mutagenic over the range 200 ng to 10 μg per assay. Benzo(a)-pyrene required microsomal activation, and in the presence of 50 μg ml^{-1} S9 mix was mutagenic at 10.5 μg per pretreatment assay. The pretreatment included the S9 mix and was a twelvefold concentration of the amount added to the microtiter plates. Below 7.5 μg benzo(a)pyrene was not mutagenic, and at 15 μg was mutagenic but showed evidence of inhibiting the bacterial response.

Bzd was tested over the range 100 ng to 120 mg per assay. Below 100 μg bzd was not mutagenic. Between 100 and 500 μg bzd was mutagenic

(Figure 12), while at 500 µg bzd was mutagenic in the absence or presence of the S9 mix, indicating that a mutagenic impurity may have been present as a contaminant. At 100 mg bzd was inhibitory and the number of revertants decreased.

Extracts from sediment and supernatant portions of sterile flasks and flasks of P. denitrificans and K5 incubations were tested in this assay at two concentrations each. The sterile control supernatant fluid was mutagenic at both 0.22 and 2.2 µg bzd per assay. The sterile control sediment extract was mutagenic at both 0.05 and 0.5 µg bzd per assay. P. denitrificans sediment extract was mutagenic at 0.014 and 0.14 µg bzd per assay, although the supernatant fluid was mutagenic at 0.08 but not at 0.8 µg bzd per assay. The results which showed mutagenesis indicated that a compound was present which did not require metabolic activation. The three samples which were not mutagenic at the higher concentration contained some type of inhibitor of mutagenic expression, although the number of positive wells was always higher than the number of spontaneous mutants. Thus the samples did not appear to be toxic. When the samples were pretreated with the S9 mix, none was mutagenic although all showed at least some positive wells. However, since this step required twelve time the concentrations of materials and solvent as noted in the Materials and Methods, there is a possibility of interference with DNA replication or expression of the mutation. Therefore, the results of pretreating the extracts with S9 could not be interpreted.

Several extracts of bacterial treatments which appeared to have no effect on bzd were tested in this assay without microsomal activation. All the extracts tested (P. putida supernatant fluid, sterile control sediment and supernatant fluid, P. stutzeri sediment, and A. crystal-

Figure 12. Response of Ames tester strain TA98 in the modified fluctuation test to various concentrations of benzidine (▲) and benzo(a)pyrene (●), reported as number of turbid wells per 96-well microtiter plate. Lower dashed line marks significant deviation from control ($P < 0.05$) and higher dashed line marks highly significant deviation from control ($P < 0.01$).

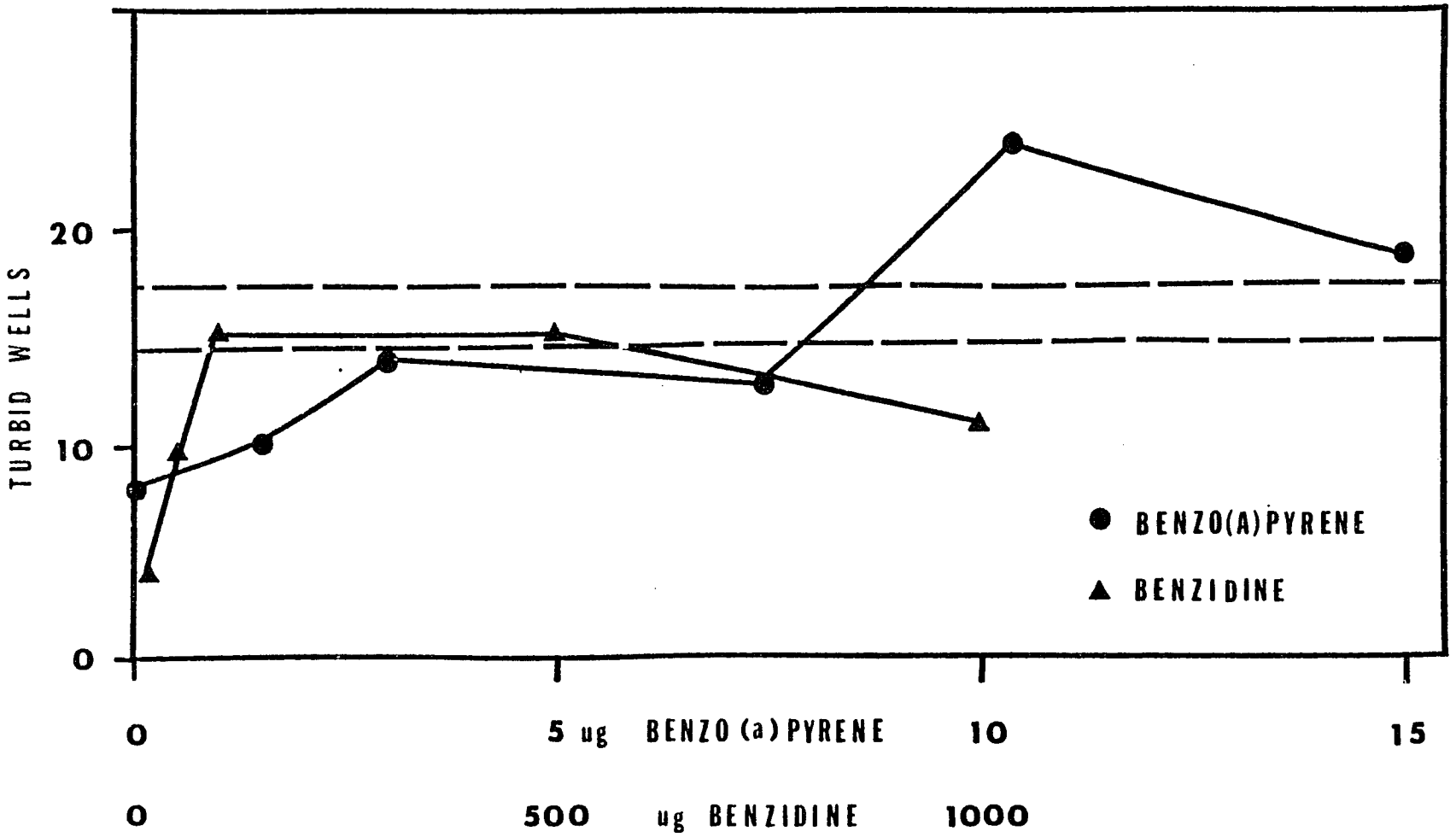
TURBID WELLS
20
10
0
BENZO(A)PYRENE
BENZIDINE
0
5 ug BENZO (a) PYRENE
10
15
0
500 ug BENZIDINE
1000

Figure 12.

lopoietes sediment) were negative in this test at both 2.5 and 5 mg per assay, indicating that there was no transformation of bzd to an active mutagen. In all cases the number of positive wells was very close to the spontaneous rate of mutation, indicating that there was no toxicity.

In all assays, even significant results calculated according to the Chi square statistic were close to the spontaneous rate of mutation. Consistently there was a difference of very few turbid wells between positive and negative results. Therefore, a more sensitive assay was explored.

Inductest

When the inductest-β-galactosidase procedure of Elespuru and Yarmolinsky (1979) was followed, bzd did not induce mutagenesis. The standard mutagens MNNG and aflatoxin B_1 did show activity. In an attempt to make this test more sensitive, several modifications were tried.

Although the procedure suggests leaving out treatment with chloroform and sodium lauryl sulfate prior to beginning the β-galactosidase assay, these steps were required to express the β-galactosidase production. Control tubes without treatment yielded 12 units β-galactosidase while equivalent tubes that were treated with chloroform and sodium lauryl sulfate yielded 132 units β-galactosidase. Therefore, these steps were included. The justification for omitting these steps was that the envelope mutation permits entry of the β-galactosidase complexing agent. Since it appeared that this was not the case, the strain was checked for sensitivity to large molecules by streaking on a plate of eosin-methylene blue agar (Difco). Only microcolonies formed after three days' incubation. Therefore the envelope mutation was apparent although it seemed to be selective.

Another modification to the procedure involved the time of incubation. When the tester strain was incubated with mitomycin C for four hours rather than two, more induction was observed at much lower concentrations (Figure 13). The production of β-galactosidase was linear from one to four hours (Figure 14).

The presence of ampicillin in the medium reduced the production of β-galactosidase. Without ampicillin in the medium mitomycin C induced much higher production of β-galactosidase and maximum sensitivity was at a much lower concentration than the corresponding experiment with ampicillin in the medium (Figure 15). A standard curve of mitomycin C-induced production of β-galactosidase was constructed (Figure 16). Ethidium bromide was tested over the range 0.1 to 5 µg per assay but did not stimulate β-galactosidase production, although there was no evidence of inhibition. Nalidixic acid was tested over the range 0.1 to 150 µg per assay and was mutagenic at 50 and 75 ug per assay. MNNG was tested over the same range, and was mutagenic at the lowest concentrations, 0.1 to 1.5 ug per assay. Above this level inhibition was evident, since the level of β-galactosidase was less than the control. None of these mutagens require microsomal activation.

Bzd and benzo(a)pyrene both required microsomal activation and after four hours' incubation gave low but measurable β-galactosidase production (Figure 17). The reported results (0 to 70 units) are net of the control (cells plus S9 mix plus dimethylsulfoxide, in which the mutagens were dissolved) which induced production of 232 β-galactosidase units. In this experiment 50 µl S9 was added per ml activation mix.

When 100 ul ml^{-1} S9 was added, bzd activated β-galactosidase production but the results were erratic. At other concentrations of S9

Figure 13. β-galactosidase production by E. coli K12 BR513 when incubated with mitomycin C for two hours (▲) and four hours (●), ampicillin present.

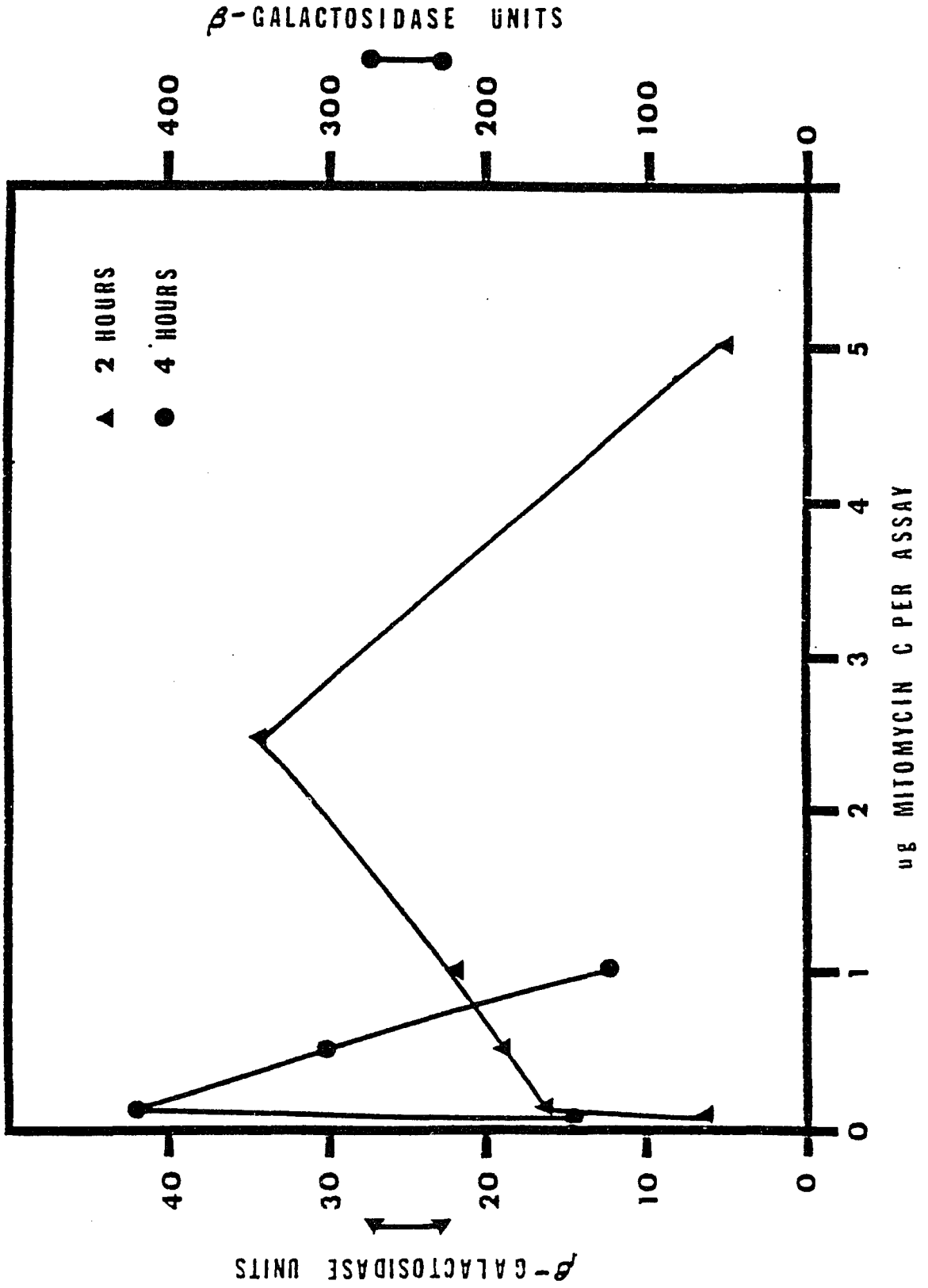
β-GALACTOSIDASE UNITS
400
300
200
100
0
2 HOURS
4 HOURS
0
1
2
3
4
5
ug MITOMYCIN C PER ASSAY
40
30
20
10
0
β-GALACTOSIDASE UNITS

Figure 13.

Figure 14. β-galactosidase production by E. coli K12 BR513 after induction with 0.5 µg mitomycin C per tube (●) and uninduced (▲), ampicillin present.

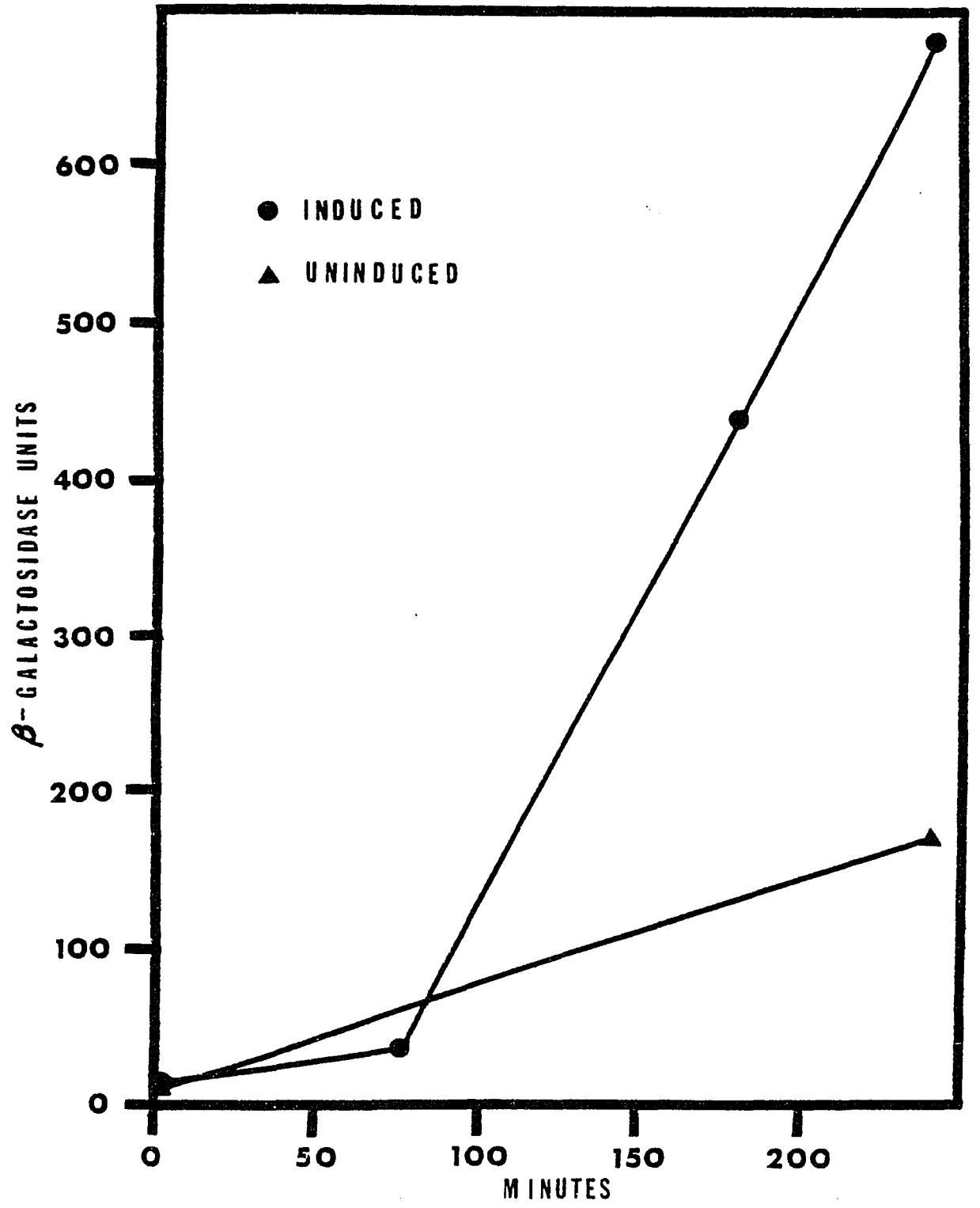
INDUCED
UNINDUCED
β-GALACTOSIDASE UNITS
600
500
400
300
200
100
0
0
50
100
150
200
MINUTES

Figure 14.

Figure 15. β-galactosidase production by E. coli K12 BR513 after four hours preincubation with 0.5 μg mitomycin C per tube in the presence (■) and absence (●) of ampicillin.

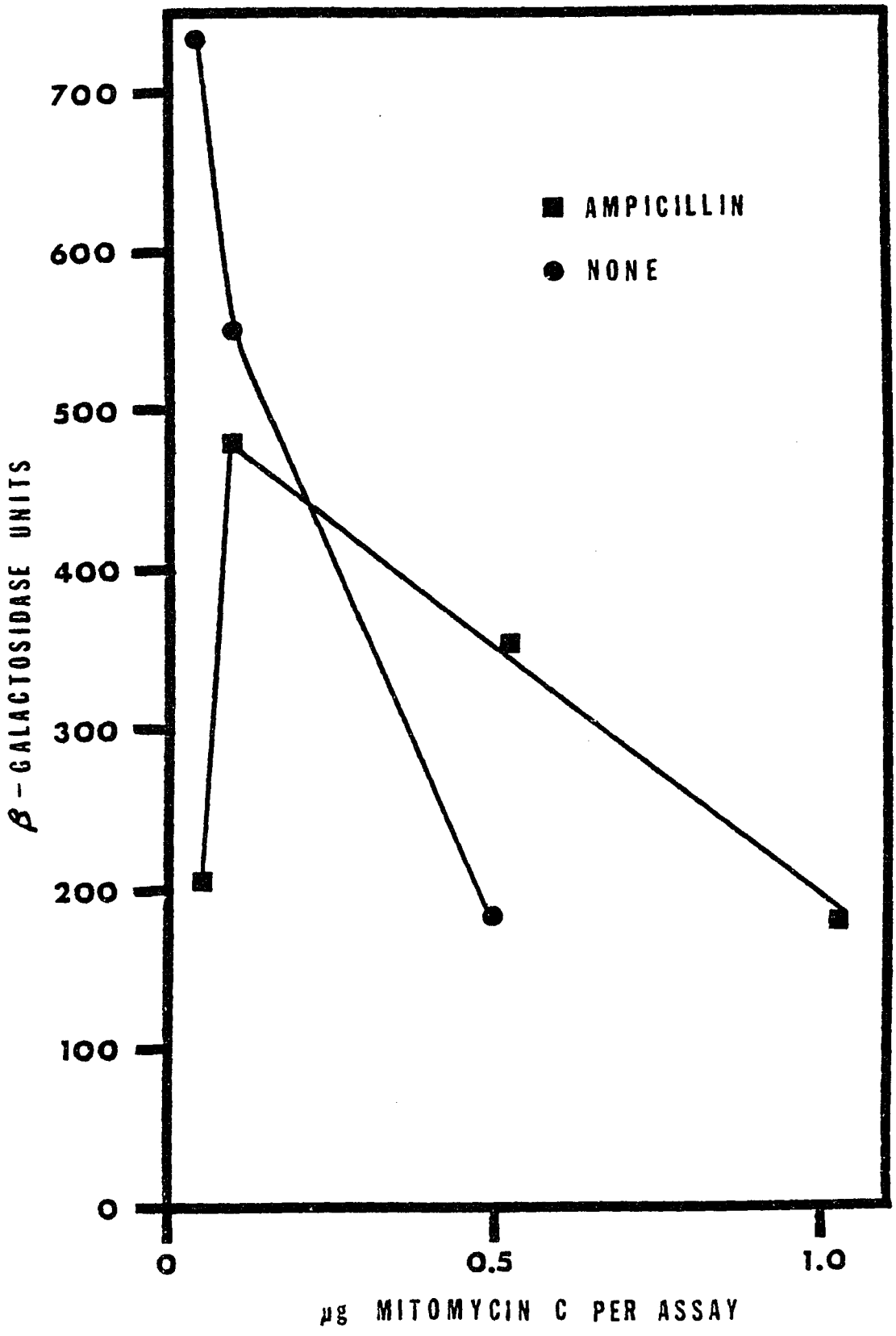
700
600
500
400
300
200
100
0
β - GALACTOSIDASE UNITS
AMPICILLIN
NONE
0
0.5
1.0
μg MITOMYCIN C PER ASSAY

Figure 15.

Figure 16. β-galactosidase production by E. coli K12 BR513 after preincubation with mitomycin C for four hours in the absence of ampicillin.

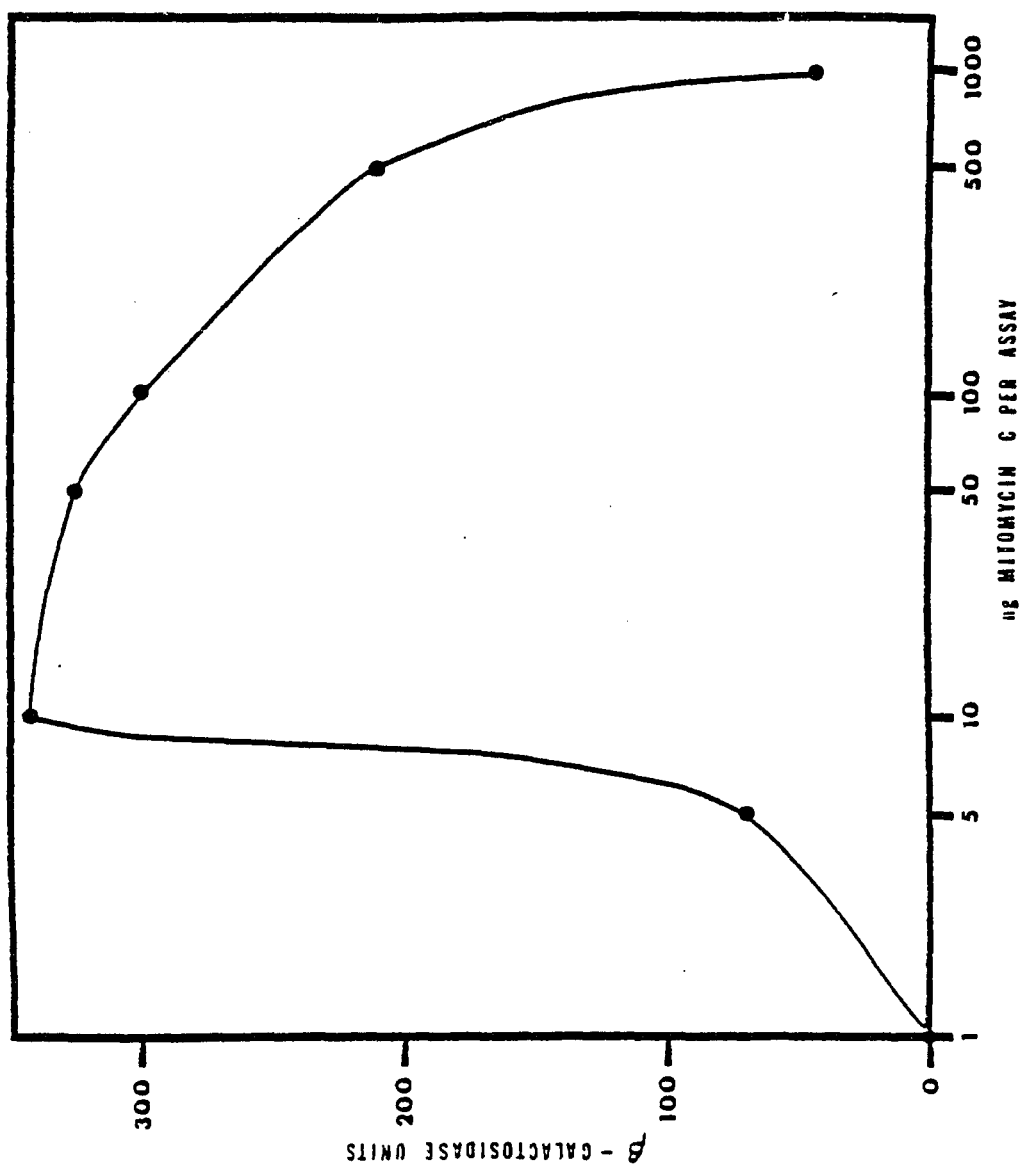
β - GALACTOSIDASE UNITS
300
200
100
0
1
5
10
50
100
500
1000
µg MITOMYCIN C PER ASSAY

Figure 16.

Figure 17. β-galactosidase production by E. coli K12 BR513 after induction by benzidine (●) and benzo(a)pyrene (■) for four hours without ampicillin, in the presence of S9 (50 µl per ml activation mix). Units are net of endogenous production (232 units).

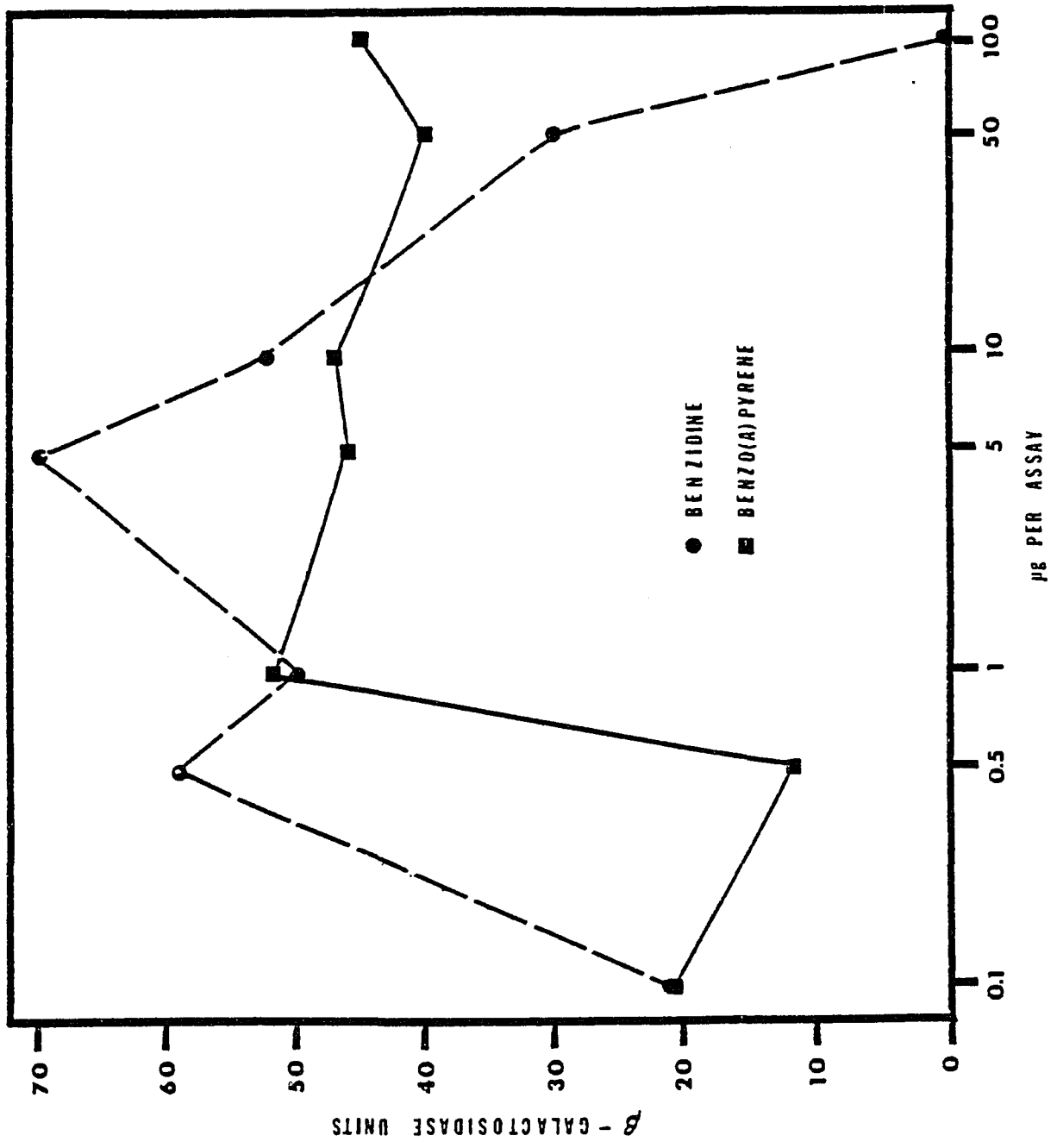
β - GALACTOSIDASE UNITS
70
60
50
40
30
20
10
0
0.1
0.5
1
5
10
50
100
μg PER ASSAY
BENZIDINE
BENZO(A)PYRENE

Figure 17.

from 25 to 100 $\mu l\ ml^{-1}$ bzd was slightly active or inactive. Benzo(a)-pyrene used as a standard mutagen requiring microsomal activation also gave erratic results over the range of S9 mix added. Aflatoxin B_1 which also requires microsomal activation consistently promoted β-galactosidase production.

While the results using the S9 mix were equivocal for bzd, the assay seemed to be sensitive for mutagens which did not require microsomal activation. Therefore, extracts from experimental flasks were tested at 5 and 50 μg "equivalent bzd radioactivity" in the presence and absence of 80 $\mu l\ ml^{-1}$ S9 mix. In the presence of S9 only the sterile control extract showed activity (37 units net) (Table 14). All other extracts failed to induce β-galactosidase production. In the absence of S9 mix all treatments showed some activity, but all showed less activity than the sterile control with the exception of strain K6 or the higher concentration of test sample. At the lower concentration strains SF and SG showed one-third less activity and strain CJ showed half the activity of the control. This is consistent with the reversion of the unidentified transformation product back to native bzd during incubation and indicated that the other compounds formed when bzd is incubated in sterile media or with bacteria are mutagenic without requiring metabolic activation.

In every experiment, production of β-galactosidase was much higher in samples containing the S9 mix (e.g., 232 units) than in parallel samples lacking any S9 mix (e.g., 4 units). This occurred as well in control flasks lacking any other source of mutagen. This assay depends upon induction of the lambda prophage promoter, not upon provision of histidine as for the standard Ames assay. Therefore, it appeared that a factor capable of inducing the prophage promoter was present in the S9 mix.

Table 14. β-galactosidase production by E. coli K12 BR 513 in response to treatment with extracts of bacterial cultures incubated with BIM medium.

Bacterial Culture	Equivalent Benzidine (ug)[a]			
	5		50	
	S9	-	S9	-
Control	0[b]	132	37	65
CJ	0	68	0	57
K5	0	122	0	34
K6	0	123	0	100
SVR	0	133	0	59
SF	0	92	0	84
SG	0	92	0	85

[a]Equivalent benzidine calculated as total radioactivity present in sample.

[b]Units β-galactosidase. Endogenous β-galactosidase production (4 units in absence of S9 mix, 253 units in presence of S9 mix) has been subtracted from all values.

IV. DISCUSSION

Benzidine appeared on the EPA's first priority pollutant list, which was part of the settlement of a lawsuit requiring the EPA to consider the promulgation of effluent standards for certain compounds (Patterson and Kodukala, 1981). Since that time many analyses of water, wastewater, and industrial effluents for these pollutants have been undertaken. Bzd's occurrence has not been widely reported. In a comprehensive list of the frequency of organic compounds in water, bzd was listed twice (Shackelford and Keith, 1976). Bzd is detectable (>25 ug per liter) in the effluent or sludge of some wastewater treatment plants (Feiler, 1980). Bzd seems to have been of minor interest to the EPA, however, as in recent acclimation/degradation studies on most of the organic priority pollutants bzd was one of 18 out of 112 which were not tested (Patterson and Kodukala, 1981). When bzd was treated by activated sludge or aerated lagoon wastewater treatment processes, bzd was incompletely degraded at levels as low as 11.9 µg per liter (Patterson and Kodukala, 1981). Areas that are in proximity to dye manufacturing plants harbor levels as high as 2,000 µg ml^{-1} of aromatic amines derived from dyes (Nelson and Hites, 1980). The production of bzd-based dyes in 1978 was 1.7×10^6 lb while an additional 1.6×10^6 lb were imported. Bzd is receiving renewed interest as well because of the potential for its presence in coal-conversion plants (Forney et al., 1974). Thus, although the EPA has given bzd little attention, the potential for bzd to be a pollutant still remains high.

The environmental samples collected for this study included some from the toxic waste dumps, although no information was available on the nature of the waste material. Other samples were collected from relatively pristine estuarine areas where extensive industrial waste dumping was not known to occur. The results showed that bzd was not highly toxic to these bacteria in the presence of nutrients (Table 4). Therefore, bzd would not be expected to be a threat to the bacterial ecosystem. These sites were chosen to provide a wide range of environments which might harbor bacteria capable of biotransforming bzd.

Since bzd was not mineralized extensively in this study, the bacterial strains were not subjected to extensive bacterial tests for identification. In most cases, identification could be made to the family taxon (Table 1). Environmental isolates are difficult to identify. In a study using numerical taxonomy techniques to identify 153 environmental isolates using 158 characteristics, 48% remained unidentifiable (Mallory and Sayler, 1983). This study included 13 reference cultures, and concluded that potential biodegradative populations are representative of the diverse populations found in uncontaminated fresh water environments.

None of the bacteria obtained could use bzd as a sole source of carbon (Table 3). No strain could be kept on media with bzd as the sole source of carbon for longer than three successive transfers (Table 2). However, some strains, particularly CJ isolated from Great Bay sediment, and SF, SG, and K6 isolated from toxic waste dumps, were able to transform bzd when incubated with glucose or other nutrients or when resting cell suspensions were incubated with bzd in the absence of an additional carbon source (Figures 5, 6, 7, 9, 10). These four

strains as well as the laboratory contaminant SVR, identified as a Bacillus sp., formed two products from bzd within six days (Table 15). However, upon prolonged incubation the four environmental isolates caused the reversion of the products back to bzd, indicating that the products were a transient change in the bzd molecule but not ring cleavage, which would have been an irreversible alteration to the molecule. Ring cleavage would have been followed by mineralization of at least part of the molecule with concomitant loss of total radioactivity. This was never observed.

There was a certain amount of abiotic transformation of bzd. The greatest number of transformation products was seen in the control flask which contained 1 $\mu g\ ml^{-1}$ bzd. However, although these spots are represented in the experimental flasks as well, strains SF, SG and CJ metabolized bzd to a unique compound (Figure 5). At a higher concentration of bzd, abiotic transformation of bzd was represented by only one additional product (Figures 6, 7, 9). Additional transformation of bzd by bacteria is clearly seen in the presence of unique spots on the chromatogram. After incubation for 30 days, the abiotic transformation product (Figure 14, R_f = 0.64-0.65) was also reverted to some degree back to authentic bzd by strain CJ.

The activity of SF, SG and CJ in causing the reversion of products to bzd was consistent with the results obtained in the β-galactosidase assay, in which these samples were less active than the control (Table 14). A greater proportion of bzd in the sample would be reflected in less mutagenic activity in the absence of S9 mix, since bzd itself is not mutagenic without microsomal activation. The control flask showed some mutagenic activity but did not contain any bacteria which would

Table 15. Effect on benzidine of bacterial cultures incubated for six days at 25°C in darkness with shaking, in BIM medium supplemented with 1 or 100 µg ml^{-1} benzidine.

Transformation to Product A[a]	Transformation to Product B[b]	Transformation From Product B to Benzidine[c]	No Effect
CJ	CJ	CJ	B1
SF	SF	SF	*P. denitrificans*
SG	SG	SG	*P. stutzeri*
SVR	SVR	K5	
K6	K6		

[a] R_f = 0.30, Figure 5; R_f = 0.32, Figures 6, 7 and 9.

[b] R_f = 0.49, Figure 6; R_f = 0.51, Figure 9.

[c] R_f = 0.52-0.54, Figure 10; Product B formed by action of strain CJ on 100 µg ml^{-1} benzidine.

mitigate this activity.

It appears, therefore, that environmental bacteria have the capacity both to activate bzd to a direct-acting mutagen and to convert such mutagens back to the proximate mutagen bzd. These activities might depend on the type of bacteria present, and might be an equilibrium process performed as a consequence of other enzymatic activity by the bacteria.

Aerobic degradation of molecules containing ring structures requires the presence of two hydroxyl groups on the ring located either ortho or para to each other (Gibson, 1980; Chapman, 1972). These substitutions on bzd are unlikely because of the electron-withdrawing nature of the amino groups and the stability conferred by resonance across both rings (Stock, 1968) (Figure 18).

At a low concentration of bzd (1 $\mu g\ ml^{-1}$), essentially all the available bzd appeared to be biotransformed, while at 100 $\mu g\ ml^{-1}$ most of the bzd remained unchanged. This is consistent with studies of the behavior of bzd in wastewater treatment plants which indicate that the acclimated sludge microflora could oxidize bzd completely at 1 $\mu g\ ml^{-1}$ but could not oxidize bzd completely at 30 $\mu g\ ml^{-1}$ (Tabak and Barth, 1978). The bacteria in this study were not acclimated to the presence of bzd, yet still showed the same degree of efficiency in oxidizing low concentrations of bzd. It appears, therefore, that the capacity to oxidize bzd is an inherent characteristic of bacterial metabolism but not a characteristic which either requires or is enhanced by acclimation.

Since bzd was not mineralized extensively, it was of interest to determine whether the abiotic and biotic transformation products were mutagenic. Bzd itself is a mutagen after metabolic activation, and the

Figure 18. Resonance structures of benzidine at pH > 4.2 (Boyd, 1973; (Stock, 1968).

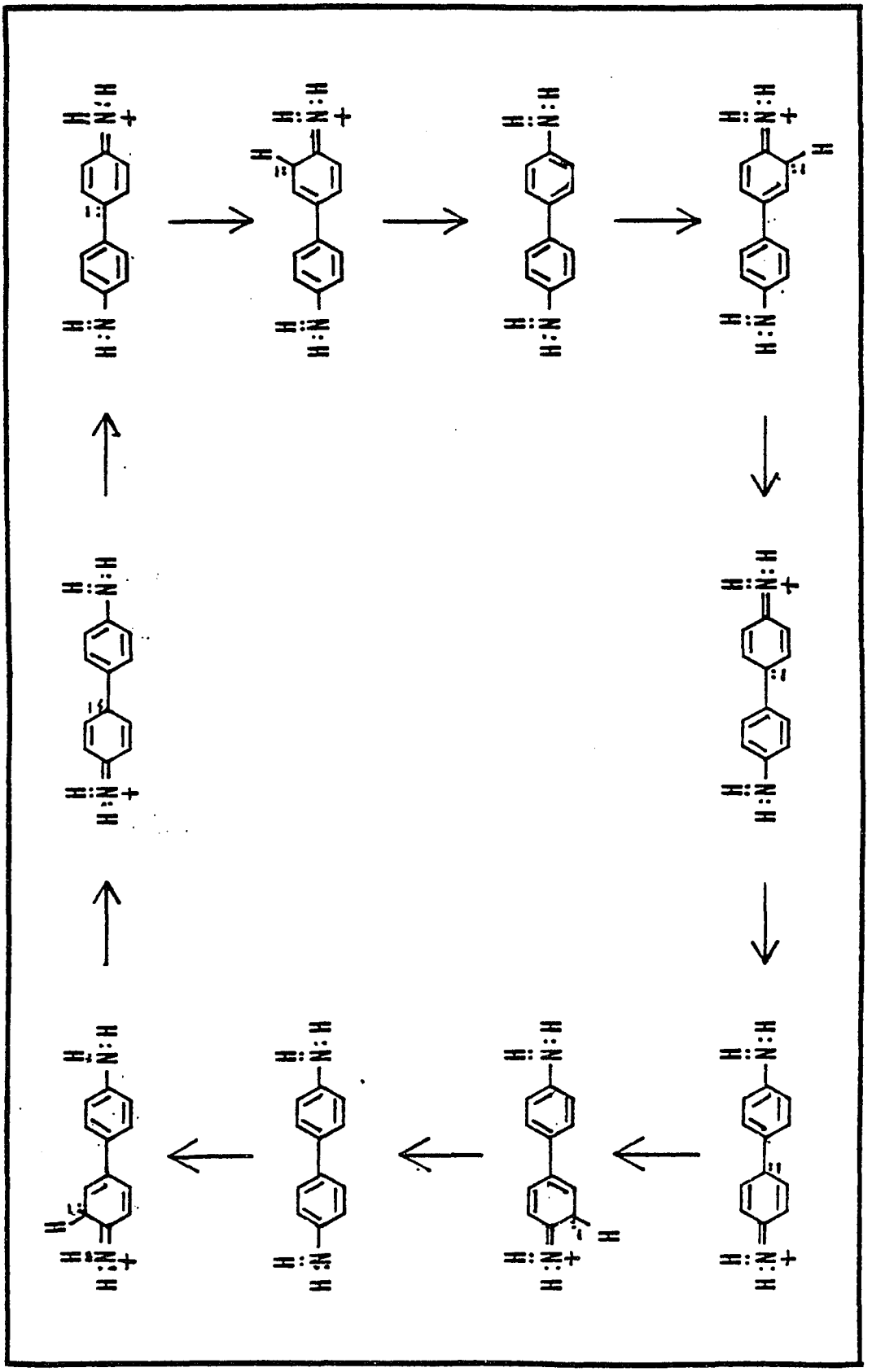

Figure 18.

effect of environmental transformation could be either to render bzd nonmutagenic, mutagenic without requiring prior metabolic activation, or no effect. Initially, the Ames test was utilized to obtain this information. A difficulty with performing the Ames test for mutagenicity was the relatively high quantities of materials required, including the mutagen. Bzd was mutagenic over the range 70-200 µg per plate (0.27-0.78 µmoles per plate) (Figure 11). The narrow window of activity in the mutagenesis assays which used the Ames strains has been explained by the presence of prophages in all the Ames S. typhimurium strains (Wheeler, Halula and Demeo, 1981). Prophage induction, which generally occurs at a higher concentration of mutagen than does mutagenesis, is accompanied by a decrease in mutagenicity as a result of cell lysis and death.

Recently the Ames assay was modified specifically to optimize the mutagenic activity of bzd (Prival and Mitchell, 1982). Modifications included changing the source and concentration of the liver microsomal S9 fraction, changing the cofactors, and including a preincubation step. With these modifications considerable mutagenic activity was observed at 0.03 µmoles per plate, an order of magnitude more sensitive than the results reported here. Their studies indicate that rat liver S9 rapidly detoxifies bzd or its metabolites. This may explain in part the variable results obtained from the fluctuation test and the inductest with regard to the mutagenicity of bzd. Both tests are more sensitive than the Ames test and detoxification effects may be more apparent.

The inductest requires that the bacterial "SOS" DNA repair system be activated, which in turn activates the lambda promoter causing β-galactosidase production to be initiated. The modified protocol for the

inductest developed here worked well in this assay for the direct acting mutagens such as mitomycin C (Figure 16) as well as for aflatoxin B_1 which required microsomal activation for expression of β-galactosidase activity. However, it did not yield reliable results for bzd and benzo-(a)pyrene. The mode of action of bzd upon DNA, that of causing mutations by intercalating and then covalent binding, may fail to induce the SOS response or may induce it only after a delay. Either action could interfere with the time-dependent production of β-galactosidase.

These results point out the selective nature of the responses of test bacteria in mutagenesis assays. The first bacterial mutagenesis assay was published ten years ago (Ames et al., 1973) and since then many other types of microbial mutagenesis assays have been developed (Devoret, 1979). New information indicates that these tests may need to be modified to optimize them for particular classes of compounds (Prival and Mitchell, 1982).

Of particular concern has been the nature and source of the S9 microsomal fraction. Comparisons of the hepatic microsomal fractions from hamsters, rats, mice, guinea pigs and humans have demonstrated differences in the capacity of each to activate specific promutagens to ultimate mutagens, and in the case of rats, strain specific differences have been detected (Prival and Mitchell, 1982; Yoshikawa et al., 1982; Raineri et al., 1981). The crude S9 mix contains azoreductases which deactivate mutagens, ring hydroxylation/conjugation enzymes which also deactivate mutagens, and oxidative enzymes for hydroxylamine formation which is an activation process (Ashby and Styles, 1978). The total effect of the S9 mix is competitive among these enzymes. The anti-tumor drug procarbazine (N-isopropyl-α-(2-methylhydrazino)-p-toluamide)

is metabolized by the S9 fraction to toxic products with a resultant decrease in the number of revertants of S. typhimurium TA98 in the Ames assay (Lackner and Schüpbach, 1981). Aflatoxin B_1 metabolism by the S9 mix results in a product bactericidal for all S. typhimurium strains, when aflatoxin B_1 is present at levels greater than 100 ng (Wheeler, Halula and Demeo, 1981). The net effect of the S9 mix on a particular compound can be altered by adding a competitive detoxification substrate for the deactivating enzymes, for example 4-dimethylaminoazobenzene to trap the ring hydroxylation/conjugation enzymes (Ashby and Styles, 1978). The promutagen 2-acetylaminofluorene is activated to the mutagen N-hydroxyacetylaminofluorene in the presence of S9 mix; however, this conversion is decreased in the presence of the azo dye amaranth (FD&C Red Dye No. 2) (McCalla et al., 1981). The activity of amaranth was found to be due to its capacity to reoxidize NADPH to $NADP^+$, thereby reducing the NADPH available for the necessary mutagen activation reactions. However, it is uncertain whether such modifications make the Ames test more representative of mammalian systems or whether the use of such competitive substrates lessens the overall utility of the assay (Ashby and Styles, 1978).

Such competitive activity could be a factor in the study reported here. Some of the metabolites of bzd could involve azo linkages or other features with the capacity to oxidize NADPH and consequently interfere with the activation of bzd and its analogues to mutagenic forms. This is supported by the finding that increasing the levels of the S9 mix increased the sensitivity of the inductest-β-galactosidase assay.

Increasing the concentration of S9 in the Ames assay has been found to increase the number of revertant colonies in the absence of

added mutagen (Kazmer, Katz and Weinstein, 1983). This has been shown not to be due to the amount of histidine in the S9 fraction (Kazmer, Katz and Weinstein, 1983) and has been postulated to be due to promutagens in the enrichment media used to grow the inocula (Vithayathil et al., 1978). However, the inductest uses a defined medium not expected to be contaminated with a significant concentration of promutagens.

The results reported here concerning the mutagenic potential of the S9 mix itself with regard to promoting β-galactosidase activity in the inductest, appear to be the first such report. These results, if consistent for other types of mutagenesis assays, may underline the need for better standardization of the S9 mix used in all such assays or perhaps the need for purifying the liver microsomal mix further prior to its use.

The revised protocol for the inductest-β-galactosidase assay reported here makes this assay more sensitive for a wide range of compounds. An additional modification in choice of liver microsomal S9 fraction may increase its utility for bzd as well. This assay can be completed within one day, is economical of materials and time, and the spectrophotometric results are obtained easily. With the increased sensitivity obtained here, it has the potential to become a method of choice for routine screening of compounds for mutagenicity.

APPENDIX

APPENDIX

Protocol for Inductest-β-Galactosidase Assay for Detection of Mutagens

(modified from Elespuru and Yarmolinsky, 1979)

1. Grow Escherichia coli K12 BR513 (ATCC 33312) overnight in LBE medium at 37°C with shaking.

2. Dilute culture 1:20 in LBE medium and continue growth until culture reaches OD_{600} of about 0.4 (Bausch & Lomb Spectronic 20) (about 3 hours).

3. Dilute culture 1:10 in either LBE medium or Activation Mix A warmed to 37°C just prior to use.

4. Add 0.5 ml of diluted suspension to tubes containing 10 ul of sample to be tested.

5. Cover with parafilm and incubate 4 hours at 37°C with shaking.

6. Add 0.2 ml suspension to 1.8 ml cold Z Buffer. Assay may be interrupted here and samples refrigerated overnight.

7. Warm samples to 28°C.

8. Add 4 drops chloroform and 2 drops 0.1% sodium lauryl sulfate. Vortex 10 seconds.

9. Add 0.4 ml oNPG solution and incubate at 28°C for 10 minutes to 3 hours, until visible yellow color develops. Time minutes of reaction.

10. Add 1.0 ml Na_2CO_3 (1 M) with mixing to stop reaction.

11. Read at both A_{420} and A_{550}. Calculate β-galactosidase units:

$$\text{Units} = 1000 \frac{(A_{420} - 1.75A_{550})}{t \times v \times OD_{600}} \quad \text{(Miller, 1972)}$$

t = minutes of color development
v = volume of cells used (= 0.1 for this procedure, based on doubling the reaction volumes specified in Miller (1972).
OD_{600} = culture optical density at time of mutagen addition.

12. Subtract appropriate control:
(a) Cells + carrier solvent + Activation Mix A
(b) Cells + carrier solvent

MEDIA

A. LBE Medium

	per liter
bactotryptone	10 g
yeast extract	5 g
NaCl	10 g
1 M tris(hydroxymethyl)aminomethane	5 ml

Autoclave, then add:

50X Medium E	4 ml
20% glucose solution	10 ml

B. 50X Medium E

In 670 ml distilled H_2O, dissolve successively:

$MgSO_4 \cdot 7H_2O$	10 g
Citric acid$\cdot H_2O$	100 g
K_2HPO_4 (anhyd.)	500 g
$NaNH_4HPO_4 \cdot 4H_2O$	175 g

Final volume is approximately one liter.

C. Z Buffer

$Na_2HPO_4 \cdot 7H_2O$	16.1 g
$NaH_2PO_4 \cdot H_2O$	5.5 g
KCl	0.75 g
$MgSO_4 \cdot 7H_2O$	0.246 g
β-mercaptoethanol	2.7 ml

Do not autoclave. Prepare freshly daily.

D. Medium A

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Na·citrate$\cdot 2H_2O$	0.5 g

E. oNPG Solution (o-nitrophenyl-β-D-galactopyranoside)

4 mg/ml in Medium A

Prepare just before use and keep on ice.

F. Activation Mix A

	per ml
NADP	2.4 mg
glucose-6-phosphate, disodium salt hydrate	2.9 mg
NaCl	4.4 mg
$MgCl_2$ (0.2 M)	0.1 ml
Na_2PO_4 (0.2 M, pH 7.4)	0.3 ml
S9 liver microsomal fraction	0.1 ml
LBE Medium	0.5 ml

Prepare just before use and keep on ice. Do not refreeze S9 fraction.

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