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CELL TRANSFORMATION AND MUTABILITY OF DIFFERENT GENETIC LOCI IN MAMMALIAN CELLS BY METABOLICALLY ACTIVATED CARCINOGENIC POLYCYCLIC HYDROCARBONS

Eliezer Huberman

Biology Division, Oak Ridge National Laboratory,

Oak Ridge, Tennessee 37830

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INTRODUCTION

Polycyclic hydrocarbons are widely distributed in the human environment. Some members of this class of chemicals can induce malignant tumors in a variety of experimental animals, thus suggesting a serious potential hazard for humans. Therefore carcinogenic polycyclic hydrocarbons have been used extensively as investigative research tools for understanding the mechanisms whereby chemicals induce malignancies. It has previously been shown that these hydrocarbons bind to DNA in cells derived from susceptible animals, and that there is a correlation between their carcinogenic potency and the extent of this binding (6, 28, 37). Such experiments suggest that, in the process of their interaction with cellular DNA, carcinogenic polycyclic hydrocarbons may induce mutations, some of which may involve genes that control malignancy (5, 25). Thus, mutagenesis studies in mammalian cells can be of value in determining the mode of action of such chemical carcinogens.

Many chemical carcinogens, including polycyclic hydrocarbons, have to be metabolized by the mixed-function oxidases and related enzymes (43, 48) in order to exert such biological properties as cytotoxicity (9, 14, 30), virus induction (22), transformation (15-17, 23, 39), and mutagenesis (1, 17, 20, 24, 26, 27). However, not all mammalian cells, including cell lines suitable for studies on mutagenesis, can metabolize these hydrocarbons (26, 30). We have, therefore, developed a cell-mediated mutagenesis assay in which the mutagenic effect of carcinogenic polycyclic hydrocarbons can be easily tested in mammalian cells without having to isolate the chemically reactive mutagenic metabolites (3, 26). In this assay, cells with the appropriate markers for mutagenesis are co-cultivated with lethally

irradiated cells that can metabolize polycyclic hydrocarbons. During co-cultivation, the reactive metabolites appear to be transmitted from the cells that metabolize the hydrocarbons and induce mutations in the cells with the appropriate markers.

Using this assay, we were able to demonstrate a relationship between the carcinogenic potency of polycyclic hydrocarbons and their ability to induce mutations in mammalian cells (27) and to identify the mutagenic metabolites of benzo(a)pyrene (BP), one of the most common of these compounds (27). In studying mutagenesis by the carcinogens, it is important to measure the degree of mutability of different genetic loci to determine whether carcinogens mutate genes that control malignant transformation as a result of a random hit. We have, therefore, tested the mutability by carcinogenic polycyclic hydrocarbons of three independent genetic markers by means of the cell-mediated assay (27). The genetic markers used affect the surface membrane, nucleic acid synthesis, and protein synthesis, respectively. The membrane mutation was detected by resistance to ouabain (2), the mutation for nucleic acid synthesis by resistance to 8-azaguanine (8), and the mutation for protein synthesis by temperature resistance in cells with a temperature-sensitive leucyl-tRNA synthetase (52). In addition, by comparing transformation and mutation frequencies induced in normal diploid golden hamster embryo cells by BP and one of its metabolites, we were able to estimate the genetic target size for cell transformation in vitro (24).

METHODS

Direct Mutagenesis Assay in Chinese Hamster V79 Cells

The direct mutagenesis assay was performed as previously described (20, 21, 29). The V79 cells (derived from a clone of V79-4 cells kindly supplied by E. H. Y. Chu, University of Michigan, Ann Arbor) were seeded at 200 cells per 50-mm Petri dish to determine cloning efficiencies, and at 2×10^4 and 10^5 cells per 50-mm Petri dish for selection of 8-azaguanine- and ouabain-resistant mutants, respectively. The hydrocarbons were added for 3 hr at 16–18 hr after cell seeding, and the medium with the added hydrocarbons was then replaced by fresh medium without the hydrocarbon. For selection of mutants, a final concentration of 0.2 mM 8-azaguanine or 1 mM ouabain (Sigma Chemical Co., St. Louis, Mo.) was added 2 days after treatment with the hydrocarbons. In the experiments with 8-azaguanine, the medium was replaced every 2 days with a medium containing fresh 0.2 mM 8-azaguanine, whereas ouabain was left in the original medium. Colonies were counted, after staining with Giemsa, at 6–8 days for cloning efficiency, 12–14 days for 8-azaguanine resistance, and 16–18 days for ouabain resistance.

Cell-Mediated Mutagenesis Assay

The cell-mediated assay was performed as described (18, 26, 27). Chinese hamster ovary ts cells (derived from a clone isolated from the ts-H1 cells kindly supplied by G. F. Whitmore, Ontario Cancer Institute, Toronto, Canada) and V79 cells were seeded at 3×10^5 cells in 4 ml of medium on a 5000-R-irradiated monolayer of 2×10^6 polycyclic hydrocarbon-metabolizing golden hamster embryo cells. The hydrocarbons were added 5 hr later in 1 ml of medium. In some experiments, 7,8-benzoflavone at a final concentration

of 3 μg/ml or aminophylline (Sigma Chemical Co., St. Louis, Mo.) at 0.1 mM were added 2 hr before treatment with the hydrocarbons. The treated V79 and ts cells were incubated for 2 and 3 days, respectively. The cultures were then dissociated with 0.25% trypsin solution and seeded on 50-mm Petri dishes: 200 cells per dish for cloning efficiency, 2×10^4 cells per dish for selection of 8-azaguanine-resistant mutants, and 10^5 cells per dish for selection of ouabain- or temperature-resistant mutants. Selection of the 8-azaguanine and ouabain mutants was performed as in the direct mutagenesis assay. For the selection of temperature-resistant mutants, the ts cells were shifted from 34° ± 0.5°C to 30° ± 0.5°C 3 days after cell seeding and were stained with Giemsa 2–3 weeks later.

The mutation frequency for resistance to 8-azaguanine, ouabain, or temperature was calculated per 10^6 survivors, based on the cloning efficiency and the number of cells seeded for mutant selection. The co-cultivation of irradiated normal golden hamster cells with V79 or ts cells did not change the spontaneous mutation frequency for these three genetic markers. The heritability of the drug- and temperature-resistant colonies was shown from experiments with 29 isolated ouabain-resistant colonies, 17 isolated 8-azaguanine-resistant colonies, and 10 temperature-resistant colonies, all of which remained resistant to the drug or temperature used for at least 1 month under nonselective conditions (20, 26, 27, 32).

Mutagenesis and Cell Transformation of Normal Cells

To test mutagenesis and cell transformation in normal cells, primary golden hamster embryo cells were seeded at 1.5×10^6 cells in 8 ml of medium per 100-mm Petri dish and were treated a day later with BP or its (±)-trans-7,8-diol metabolite. After 3 days' treatment with the hydrocarbons, the cultures were washed with fresh medium without the hydrocarbons,

and the cells were dissociated with trypsin solution. The cells were seeded at 5×10^2 to 5×10^3 cells on a 5000-R-irradiated feeder layer of 5×10^4 rat-embryo cells per 60-mm Petri dish to determine cloning efficiency and the number of transformed colonies, and at 10^5 cells per 50-mm Petri dish to determine the number of ouabain-resistant colonies. Cloning and transformation efficiencies were determined 10 days after cell seeding from Giemsa-stained Petri dishes as previously described (3, 25). Cells seeded for mutant selection were treated 2 days after seeding with a final concentration of 0.1 mM ouabain, and the number of mutants was counted 18 days later after staining with Giemsa. The heritability of ouabain resistance was shown from experiments with five isolated ouabain-resistant colonies, all of which remained resistant to the drug after 10–12 days' growth in the absence of ouabain (24).

The normal golden hamster embryo cells and V79 cells were grown in Eagle's medium with a fourfold concentration of amino acids and vitamins (H-21 Grand Island Biological Co., NY) and with 10% fetal calf serum; incubation was at 37° ± 0.5°C. The ts cells were grown in NCTC 135 medium (Grand Island Biological Co., NY) with 10% fetal serum and were incubated at 34° ± 0.5°C. All incubation was done in humidified incubators supplied with a constant flow of 10% CO_2 in air.

RESULTS

Cell-Mediated Mutagenesis by Carcinogenic Polycyclic Hydrocarbons

Considerable progress has been made in developing models for the study of chemical mutagenesis in mammalian cells (8, 34, 52). Various investigators (15, 20, 21, 23) obtained

positive correlations between mutagenicity and cell transformation with nitrosoguanidine, N-acetoxy-N-2-fluorenylacetamide, and some epoxides of polycyclic hydrocarbons by use of resistance to 8-azaguanine as a marker (8). However, no such correlations were obtained with the polycyclic hydrocarbons themselves since the cell lines used in chemical mutagenesis do not metabolize these carcinogens (26). To obtain better correlations, we developed a cell-mediated mutagenic assay with carcinogenic hydrocarbons in which Chinese hamster cells, which are susceptible to mutagenesis, are co-cultivated with lethally irradiated rodent cells that can metabolize these compounds (18, 26, 27). During co-cultivation, the reactive metabolites are apparently transferred from the metabolizing cell to the cells used for testing mutagenesis, where they induce mutations.

To determine the relationship between mutagenesis and carcinogenicity, a series of 11 polycyclic hydrocarbons with different degrees of carcinogenicity were tested in the cell-mediated mutagenesis assay for the induction of ouabain-resistant mutants (Table I). T-I After co-cultivation, four carcinogenic hydrocarbons, 7,12-dimethylbenz(a)anthracene, benzo(a)pyrene, 3-methylcholanthrene, and 7-methylbenz(a)anthracene, induced ouabain-resistant mutants whereas five noncarcinogenic hydrocarbons, benzo(e)pyrene, benz(a)anthracene, phenanthrene, pyrene, and chrysene, were not mutagenic (Table I). Dibenz(a,c)anthracene and dibenz(a,h)anthracene, which have been reported as noncarcinogenic in golden hamster (33, 45), showed a weak mutagenic effect. In the presence of aminophylline, which enhanced polycyclic hydrocarbon metabolism (26, 28, 31, 56), there was a two- to fourfold increase in mutagenicity with BP and 3-methylcholanthrene. Dibenz(a,c)anthracene, which showed a low degree of mutagenicity without aminophylline, showed a less than twofold

increase of mutagenicity with aminophylline. Dibenz(a, h)anthracene, which was similar to dibenz(a,c)anthracene without aminophylline, showed a tenfold increase in mutagenicity with aminophylline (Table II). These results indicate that there is a relationship between mutagenesis and the degree of carcinogenicity of polycyclic hydrocarbons after treatment with aminophylline. T-II

In addition to ouabain resistance, mutagenesis was determined with two other genetic markers: 8-azaguanine resistance and temperature resistance in cells with a temperature-sensitive leucyl-tRNA synthetase (Table III). The results indicate that the noncarcinogenic hydrocarbons pyrene and phenanthrene do not induce mutation, while 3-methylcholanthrene and BP enhanced the mutation frequency by 60–280 times over background for the three genetic markers tested. These results indicate that chemical carcinogens mutate different genetic loci in mammalian cells and may, therefore, induce mutations of other genetic loci, some of which may involve genes that control malignancy. T-III

Identification of the Mutagenic Metabolite of Benzo(a)Pyrene

In view of the relationship between chemical carcinogenesis and mutagenesis, it is important to identify the active metabolites that are mutagenic for mammalian cells. In these experiments, an attempt was made to identify the mutagenic metabolites of the carcinogenic hydrocarbon BP. In order to test for mutagenicity without further metabolism of the hydrocarbon, BP and 23 of its derivatives were tested for mutagenesis to ouabain resistance with the V79 Chinese hamster cells, which do not metabolize polycyclic hydrocarbons. The derivatives included the (±)-trans-4,5-, 7,8-, and 9,10-dihydrodiols; two stereo isomers of trans-7,8-dihydrodiols; the 1-, 3-, 6-, 7-, 8-, and 9-mono-hydroxy

derivatives; the 6-methyl and 6-methoxy derivatives; the K-region epoxide (4,5-oxide); and two isomeric diol-epoxides — the (±)-r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydro derivative (diol-epoxide I) and the (±)-r-7,t-8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydro derivative (diol-epoxide II, ref. 12), as well as 4 tetrols and 2 triols that are hydrolysis and reduction products of the two diol epoxides (57–61). The results indicated that BP and all the derivatives, with the exception of the epoxides, were either not mutagenic or gave maximally up to fourfold higher mutation frequencies than the untreated control. The K-region epoxide, as in other experiments (17), and the diol-epoxides I and II were the most active mutagenic derivatives, and their mutagenic response increased as a function of the hydrocarbon concentration (Fig. 1). At an equimolar concentration of 0.7 μM, the diol-epoxide I showed a mutation frequency that was about 2000-fold higher than that obtained with the K-region or diol-epoxide II (Fig. 1). F-1

In addition to the above experiments, BP and its derivatives, excluding the epoxides, were tested in the cell-mediated assay. The results indicate that in this assay the trans-7,8-dihydrodiols and BP were mutagenic (Fig. 2). The most active mutagenic hydrocarbon was (-)-trans-7,8-dihydrodiol, and activity decreased in the order (±)-trans-7,8-dihydrodiol, (+)-trans-7,8-dihydrodiol, and BP. All the other derivatives tested were either inactive or gave up to about threefold higher mutation frequencies than the untreated controls. At an equimolar concentration of 0.4 M, the (-)-trans-7,8-dihydrodiol was about two-, six-, and tenfold more than the (±)-trans-7,8-diol, (+)-trans diol, and BP respectively, and the cell-mediated mutagenesis by the trans-7,8-diols was inhibited by the mixed-function oxidase inhibitor 7,8-benzoflavone. These results, and other studies, indicate that the F-2

(-)-trans-7,8-dihydrodiol, a major metabolite of BP (47, 61), is further metabolized to a diol-epoxide (49, 59) and that this diol-epoxide [diol-epoxide I (24, 29, 46)] is the major mutagenic metabolite of BP.

In vitro cell transformation studies in golden hamster cells (which are able to metabolize polycyclic hydrocarbons) with some of the above metabolites indicated that, on a dose basis, the most active transforming metabolite of BP was diol-epoxide I, followed in decreasing order by (±)-trans-7,8-dihydrodiol and BP (39, 41). This suggests that the same metabolites are responsible for mutagenesis and transformation.

Relationship Between Mutagenesis and Transformation of Normal Cells by Benzo(a)Pyrene and Its Derivative 7,8-trans-dihydrodiol

The previous results, as well as other experiments (3, 16), indicate that there is a relationship between the degree of carcinogenicity of polycyclic hydrocarbons and their ability to induce cell transformation and somatic mutants in mammalian cells. It seems also that the same reactive metabolites of chemical carcinogens are responsible for both events. In view of this, cell transformation, which results from a one-hit event induced by the carcinogen (10, 25), may result from a somatic mutation at one of the same or different genes that control the phenotypic expression of the transformed state. In order to estimate the genetic target size for cell transformation, it is important to compare the frequency of cell transformation to the frequency of a mutation at one locus under similar conditions.

We have carried out such an experiment to determine mutation and transformation frequencies in normal golden hamster embryo cells treated with the common carcinogen BP and its derivative (±)-trans-7,8-dihydrodiol. The frequency of transformed colonies has

been compared with the frequency of mutation for ouabain resistance, a mutation associated with the surface membrane Na+/K+ ATPase (2). This mutation was chosen because of its low spontaneous frequency and the ease with which it can be detected in normal diploid cells.

The results have shown that BP and its 7,8-dihydrodiol derivative can induce both cell transformation and mutagenesis for ouabain resistance in normal diploid cells, and the ratio between both events is about 20:1 with both hydrocarbons (Table IV). Studies reported by Ts'o et al. (53) indicated similar results, although with 2–4 times higher ratios. (T-IV)

Since ouabain resistance is presumably due to mutation at one locus (2), it can be suggested that transformation, as measured by the appearance of colonies with a random pattern of cell growth, has a target equivalent to about 20 such genes. There may thus be this number of the same or different genes, the mutation of any one of which is the single hit required for transformation. The number of genes involved in transformation may be even smaller than about 20 if the genes for transformation are located at hot spots which have a higher mutation frequency.

CONCLUSION

Treatment of experimental animals with chemical carcinogens, including some polycyclic hydrocarbons, can result in the formation of malignant tumors. The process whereby some chemicals induce malignancy is as yet unknown. However, in a model system using mammalian cells in culture, it was possible to show that the chemical carcinogens induce malignant transformation rather than select for pre-existing tumor cells (3, 16, 25, 44).

In the process of the in vitro cell transformation, the normal cells, which have an oriented pattern of cell growth, a limited life-span in vitro, and are not tumorigenic, are converted into cells that have a hereditary random pattern of cell growth, the ability to grow continuously in culture, and the ability to form tumors in vivo (3, 11, 16).

This stable heritable phenotype of the transformed cells is similar to that of cells derived from spontaneous or experimentally induced tumors. Such stable heritable phenotype changes may arise from alteration in gene expression due to a somatic mutation after interaction of the carcinogen with cellular DNA (6, 43). In the present experiments we have shown that metabolically activated carcinogenic polycyclic hydrocarbons which have been shown to bind to cellular DNA (4, 6, 13, 28, 36, 49, 51, 54) induce somatic mutations at different genetic loci in mammalian cells (17, 18, 26, 27, 29, 46, 51, 53, 55) and that there is a relationship between the degree of mutant induction and the degree of carcinogenicity of the different hydrocarbons tested (18, 27, 42). Our results, and other studies, have also shown that the somatic mutations were induced by metabolites rather than the hydrocarbons themselves (18, 27, 29, 32, 40, 42, 55), and in the case of BP a 7,8-diol-9,10-oxide was identified as the major mutagenic metabolite (29, 46). Other experiments have indicated that this metabolite may also be responsible for cell transformation (39, 41), and presumably for tumor formation by BP as well (7, 35, 38, 50).

Based on these studies, it was possible to estimate the genetic target size for transformation by comparing in the same cells the frequency of cell transformation and mutation for ouabain resistance (which is presumably due to a mutation at one locus) induced by BP and one of its major metabolites (24). The results indicated that the target size for

transformation is 20 times larger than that determined for ouabain resistance. However, if the genes that control cell transformation are located at hot spots, this number may also be lower. These results suggest that cell transformation, as determined by a hereditary pattern of cell growth, is due to a mutation, and that this mutation can occur in one out of a small number of the same or different genes. However, cell transformation is presumably only an initial step in the process of chemical carcinogenesis. Thus, additional genetic and epigenetic events may take place in the process of tumor formation in the whole animal (19, 53).

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TABLE I

Induction of ouabain-resistant mutants in the cell-mediated assay by different carcinogenic hydrocarbons[a]

Hydrocarbon	Concentration of hydrocarbon (μg/ml)	Number of ouabain-resistant mutants per 10^6 survivors
Control	0	1
Benzo(e)pyrene	1	1
Phenanthrene	1	1
Pyrene	1	1
Benz(a)anthracene	1	2
Chrysene	1	2
Dibenz(a,c)anthracene	1	3
Dibenz(a,h)anthracene	1	4
7-Methylbenz(a)anthracene	1	24
3-Methylcholanthrene	1	108
Benzo(a)pyrene	1	121
7,12-Dimethylbenz(a)anthracene	0.1	66

[a]With the exception of benzo(e)pyrene, the data are based on results from Huberman and Sachs (27).

TABLE II

Induction of ouabain-resistant mutants in the cell-mediated assay by carcinogenic polycyclic hydrocarbons after treatment with (+) or without (-) aminophylline[a]

Hydrocarbon	Number of ouabain-resistant mutants per 10^6 survivors	
	(-)	(+)
Control	1	1
Pyrene	1	1
Phenanthrene	1	1
Dibenz(a,c)anthracene	3	5
Dibenz(a,h)anthracene	4	46
3-Methylcholanthrene	108	413
Benzo(a)pyrene	121	214

[a] Cells were treated with 1 μg/ml of the polycyclic hydrocarbons and 0.1 mM aminophylline. Data are based on results from Huberman and Sachs (27).

TABLE III

Mutability of different genetic loci in the cell-mediated assay by carcinogenic hydrocarbons[a]

Hydrocarbon	Number of mutants per 10^6 cells		
	Temperature-resistant	Ouabain-resistant	8-Azaguanine-resistant
Control	0.6	1	60
Pyrene	0.9	1	50
Phenanthrene	0.7	1	80
3-Methylcholanthrene	125	108	3660
Benzo(a)pyrene	170	121	4250

[a] Cells were treated with 1 μg/ml of the polycyclic hydrocarbons. Data are based on results from Huberman and Sachs (27).

TABLE IV

Transformation and mutagenesis by benzo(a)pyrene and its 7,8-dihydrodiol[a]

Hydrocarbon	Concentration ($\mu g/ml^{-1}$)	Cloning efficiency (%)	Number of transformed colonies per 10^6 CFC[b] (A)	Number of ouabain-resistant colonies per 10^6 CFC (B)	A/B
None	—	12.5	—	0.04	—
Benzo(a)pyrene	1	1.2	4300	213	20
7,8-Dihydrodiol	0.1	2.6	4700	193	24
	0.3	1.3	8200	451	18
	1.0	0.9	6300	419	15

[a] Data from Huberman et al. (24).

[b] CFC = colony-forming cells.

Figure Legends

Fig. 1. Induction of ouabain-resistant mutants in the non-metabolizing V79 cells by benzo(a)pyrene K-region epoxide and diol-epoxide derivatives. The figure is based on results from Huberman et al., (29).

Fig. 2. Induction of ouabain-resistant mutants in the cell-mediated assay by benzo(a)pyrene and the racemic and stereoisomers of trans-7,8-dihydrodiol. The figure is based on results from Huberman et al. (32).

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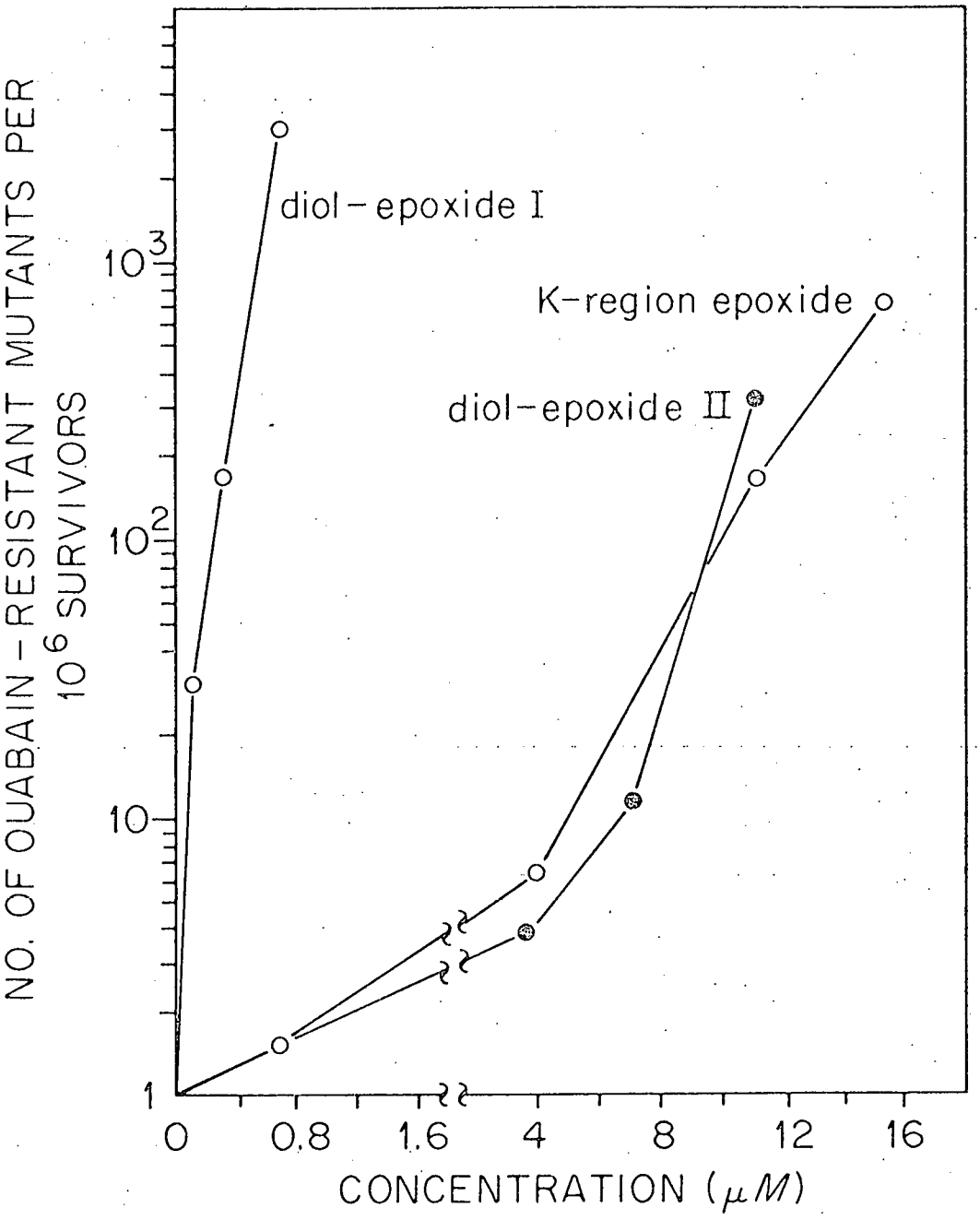
NO. OF OUABAIN-RESISTANT MUTANTS PER 10^6 SURVIVORS
10^3
10^2
10
1
diol-epoxide I
K-region epoxide
diol-epoxide II
0
0.8
1.6
4
8
12
16
CONCENTRATION (μM)

Fig. 1

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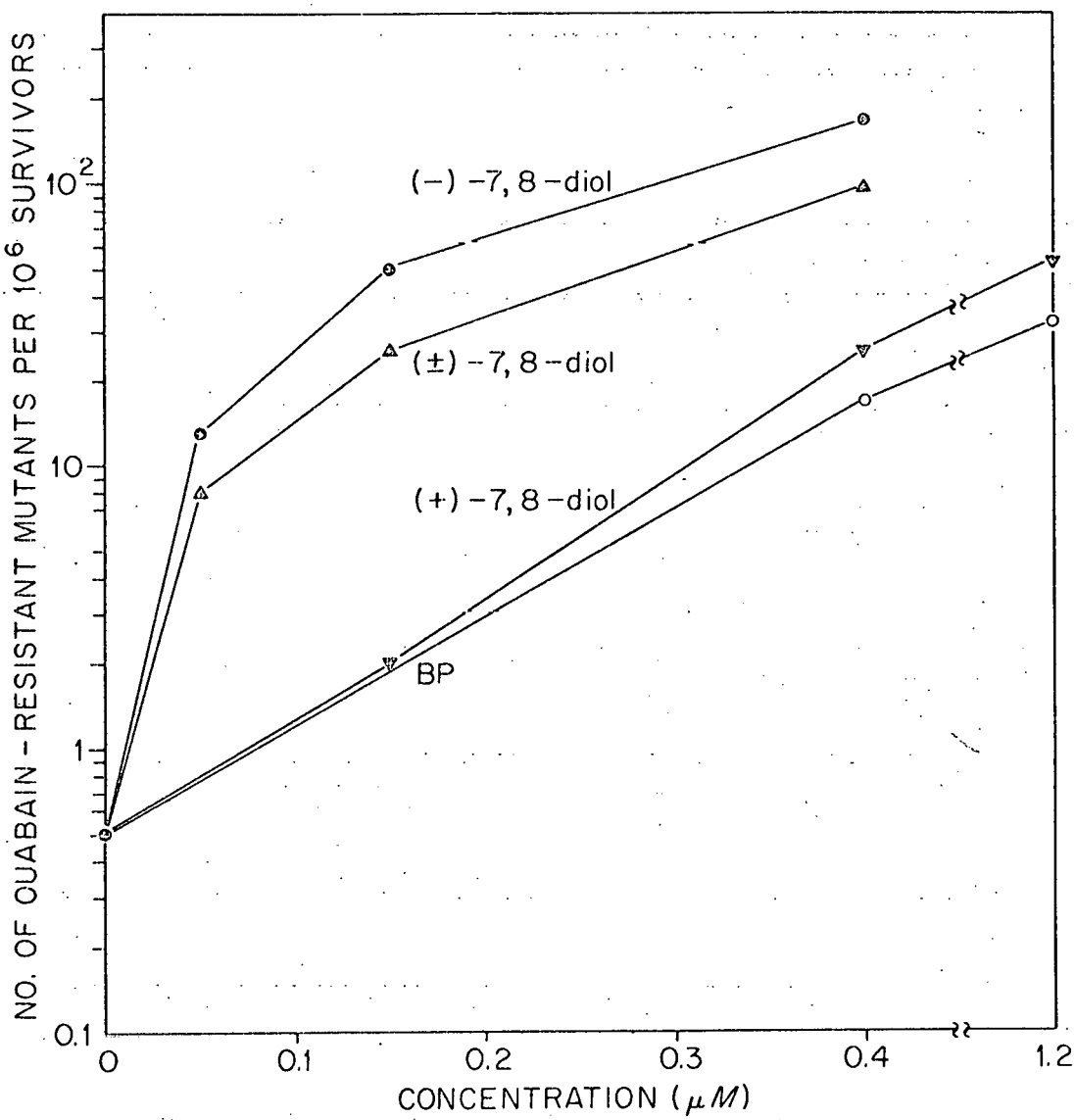
NO. OF OUABAIN-RESISTANT MUTANTS PER 10^6 SURVIVORS
10^2
10
1
0.1
(−) −7, 8 −diol
(±) −7, 8 −diol
(+) −7, 8 −diol
BP
0
0.1
0.2
0.3
0.4
1.2
CONCENTRATION (μM)

Fig. 2