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Genetics

Mutability of different genetic loci in mammalian cells by metabolically activated carcinogenic polycyclic hydrocarbons

(ouabain, temperature, and 8-azaguanine resistance/membrane mutants/aminophylline/Chinese hamster cells)

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ABSTRACT **The relationship between carcinogenesis and mutagenesis in mammalian cells has been determined with 10 polycyclic hydrocarbons with different degrees of carcinogenicity. Mutagenesis was determined in Chinese hamster cells with genetic markers that affect the surface membrane, nucleic-acid synthesis, and protein synthesis. The mutations were characterized by resistance to ouabain, 8-azaguanine, and temperature. Mutagenesis by the carcinogens required metabolic activation and this was provided by the presence of lethally irradiated metabolizing cells. The degree of carcinogenicity was related to the degree of mutagenicity for all three genetic markers. The most potent carcinogen, 7,12-dimethylbenz[*a*]anthracene, gave the highest mutagenicity and mutagenicity was obtained with 0.01 μg/ml. Treatment of the cells with aminophylline, which increases polycyclic hydrocarbon metabolism, increased mutagenesis by the carcinogens. It is suggested that such an experimental system with these and other mammalian cells should be useful as a sensitive assay for hazardous environmental chemicals.**

Chemical carcinogens can bind to the DNA of susceptible mammalian cells (1, 2) and may induce mutations, some of which may involve the genes that control malignancy (3–7). Studies on the mutagenesis of carcinogens can, therefore, be of value in elucidating the mechanism of carcinogenesis. In addition to studying mutagenesis in microorganisms (8), it is, of course, important to study mutagenesis of carcinogens in mammalian cells (9–11) from species susceptible to carcinogenesis. Since carcinogenicity may be associated with mutation of specific loci, it is also necessary to test the mutagenicity of carcinogens at different loci. The present experiments were undertaken to determine the spectrum of mutations that can be induced in mammalian cells by different carcinogens and the relationship of this mutagenicity to carcinogenicity. We have used in these studies three different genetic markers which affect the surface membrane, nucleic-acid synthesis, and protein synthesis. The membrane mutation was detected by resistance to ouabain (12), the mutation for nucleic-acid synthesis by resistance to 8-azaguanine (13), and the mutation for protein synthesis by temperature resistance in cells with a temperature-sensitive leucyl-tRNA synthetase (14).

Many chemical carcinogens, including polycyclic hydrocarbons, have to be enzymatically metabolized into reactive compounds that produce the biological effects (8, 10, 11, 15–19). Not all mammalian cells, including cell lines suitable for studies on mutagenesis, can metabolize chemical carcinogens (15, 16, 20). We have, therefore, developed a cell-mediated mutagenesis assay (11) in which the mutagenic effect of chemical carcinogens can be tested in mammalian cells without having to isolate the reactive metabolic intermediates. In this assay, cells with the appropriate markers for mutagenesis are cocultivated with lethally irradiated cells that contain the enzymes required for metabolic activation. During cocultivation, the reactive metabolites appear to be transmitted from the metabolizing cells and induce mutations in cells with the appropriate markers. We have now used this assay to study the mutagenicity of 10 polycyclic hydrocarbons with different degrees of carcinogenicity.

Abbreviations: BA, benz[*a*]anthracene; BP, benzo[*a*]pyrene; DB[*a,c*]A, dibenz[*a,c*]anthracene; DB[*a,h*]A, dibenz[*a,h*]anthracene; DMBA, 7,12-dimethylbenz[*a*]anthracene; MBA, 7-methylbenz[*a*]anthracene; MCA, 3-methylcholanthrene.

MATERIALS AND METHODS

Chemicals. Benz[*a*]anthracene, benzo[*a*]pyrene, chrysene, 7,12-dimethylbenz[*a*]anthracene, aminophylline, 8-azaguanine, and ouabain were obtained from Sigma Chemical Co., St. Louis, Mo.; pyrene, from Eastman Kodak Co., Rochester, N.Y. Phenanthrene was kindly supplied by A. Haddow of the Chester Beatty Research Institute, London; and 7-methylbenz[*a*]anthracene by M. S. Newman of Ohio State University, Columbus, Ohio. 8-Azaguanine was dissolved in dimethylsulfoxide, the polycyclic hydrocarbons in acetone, ouabain and aminophylline in growth medium. The final concentration of dimethylsulfoxide and acetone in growth medium was 0.5%. This solvent concentration did not affect cell growth and cloning efficiency or induce mutations.

Cell Cultures. Secondary cultures from whole golden hamster embryos, which served as metabolizing cells, and Chinese hamster V79 cells were cultured as described (11). The Chinese hamster ovary ts cells, derived from a clone isolated from the temperature-sensitive ts H1 cells (14) kindly supplied by G. F. Whitmore (Ontario Cancer Institute, Toronto, Canada) were grown in NCTC 135 medium (Grand Island Biological Co., N.Y.) with 10% fetal calf serum incubated at 34 ± 0.5°. Five milliliters of medium were used per 50 mm tissue culture petri dish (Falcon Co., Oxnard, Calif.) and the cultures were incubated in humidified incubators supplied with a constant flow of 10% CO_2 in air. There was no detectable mycoplasma contamination as tested on mycoplasma agar (21). Normal hamster cells were irradiated with 5000 R from a cobalt source (α-beam 150A Atomic Energy of Canada), seeded at 2×10^6 cells per 50 mm petri dish, and used for experiments 1 day after irradiation. These cells had about a 70% plating efficiency. 3×10^5 V79 or ts cells were seeded per petri dish on top of the irradiated normal cells. Since the V79 and ts cells were smaller than the irradiated cells, they could be distinguished for cell counting. Both types of cells grew to a similar density on the irradiated normal golden hamster cells as on the surface of a petri dish without the irradiated normal cells. The metabolism of benzo[*a*]pyrene to water-soluble products was measured after 2 days at 37° or 3 days at 34° as described (16, 20).

Mutations for Resistance to Ouabain or 8-Azaguanine.

To test for these mutations, 3×10^5 V79 cells were seeded in 3 ml of medium, the hydrocarbons were added 3–5 hr later in 1 ml of medium, and the cultures were incubated for 2 days. In some experiments a final concentration of 0.1 mM aminophylline was added 2 hr before treatment with the hydrocarbons. The cells were then dissociated with 0.25% trypsin solution (1:300, Grand Island Biological Co., N.Y.) and seeded to determine cloning efficiency and the frequency of resistant mutants. The colonies were counted after staining with Giemsa. Cloning efficiency was determined by counting the number of colonies in six to eight petri dishes per point 7–8 days after seeding 200 cells per petri dish with 5 ml of medium.

The frequency of ouabain-resistant mutants was determined from 32 to 48 petri dishes per point, by counting colonies 14–16 days after seeding 10^5 cells per petri dish. The frequency of 8-azaguanine resistant mutants was determined from 16 petri dishes after seeding 2×10^4 cells per petri dish. For selection of mutants, ouabain or 8-azaguanine at final concentrations of 1 mM or 0.2 mM, respectively, were added 2 days after seeding. In the cultures treated with 8-azaguanine, the medium was replaced every 2 days with medium containing fresh 0.2 mM 8-azaguanine.

Mutation for Temperature Resistance. To test for these mutations, 3×10^5 ts cells were seeded in 4 ml of medium, the hydrocarbons were added 5 hr later in 1 ml of medium, and the cultures were incubated for 3 days at 34° ± 0.5°. The cultures were then dissociated with 0.25% trypsin solution and seeded to determine cloning efficiency and the frequency of temperature-resistant mutants. The colonies were counted after staining with Giemsa. The cloning efficiency was determined by counting the number of colonies in six to eight petri dishes per point 10–12 days after seeding 200 cells per petri dish in 5 ml of NCTC 135 medium incubated at 34° ± 0.5°. The frequency of temperature-resistant mutants was determined from 24 to 32 dishes per point, by counting colonies 18–21 days after seeding 10^5 cells per petri dish. For selection of temperature-resistant mutants, the ts cells were shifted from 34° ± 0.5° to 39° ± 0.5° incubation 3 days after cell seeding.

The mutation frequency for resistance to ouabain, 8-azaguanine, or temperature was calculated per 10^5 or 10^6 survivors, based on the cloning efficiency and the number of cells seeded for mutant selection. The cocultivation of irradiated normal golden hamster cells with V79 or ts cells did not change spontaneous mutation frequency for these three markers. All the results were based on three to six experiments per point. The number of mutants after induction with the carcinogens varied up to about 25% in the different experiments. In all other cases, the number of mutants in different experiments varied up to 35%.

RESULTS

Benzo[*a*]pyrene Metabolism in the Cells Used in the Assay for Mutagenesis. Experiments on the metabolism of the carcinogenic hydrocarbon benzo[*a*]pyrene (BP) to water-soluble products have shown that both types of Chinese hamster cells, the V79 and ts cells, used for mutagenesis did not metabolize BP into water-soluble products. Irradiated normal golden hamster cells which were used for the metabolic activation of the polycyclic hydrocarbons metabolized BP into water-soluble products. Treatment of the irradiated cells, seeded at 2×10^6 cells per 50 mm petri dish, with 0.1–1 μg/ml of BP has shown that there was a proportional increase in water-soluble products in relation to the dose of carcinogen. At 1 μg of BP/ml, 0.6 and 0.9 μg/ml of water-soluble products were produced in cells incubated at 34° and 37°, respectively. Cocultivation of the irradiated normal golden hamster cells with the nonmetabolizing V79 or ts cells did not change the BP metabolism by the normal cells.

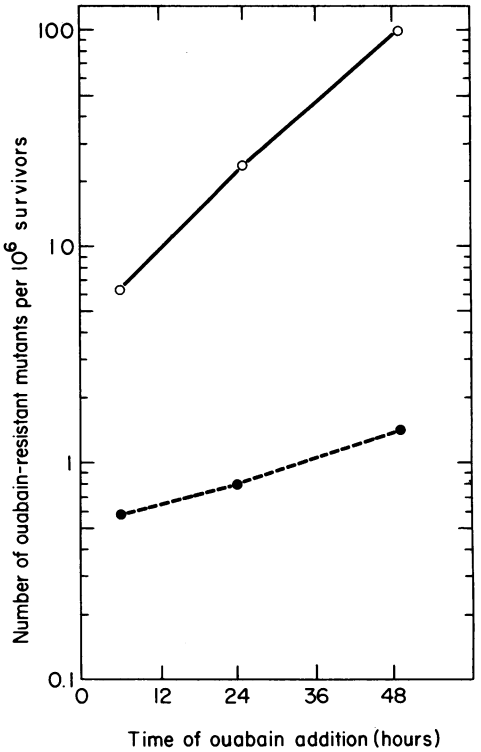

FIG. 1. Expression time for the induction of ouabain-resistant mutants by 1 μg/ml of benzo[*a*]pyrene (BP). ●, solvent-treated cells; ○, BP-treated cells. Two days after treatment with the solvent or BP there were 3.2×10^6 and 2.1×10^6 V79 cells per petri dish, respectively. The cloning efficiencies of the solvent- and BP-treated cells were 86% and 15%, respectively. Ouabain was added to the cells at different times after seeding.

Induction of Ouabain-Resistant Mutants by Benzo[*a*]pyrene. Treatment with the carcinogen hydrocarbon BP of V79 Chinese hamster cells cocultivated with metabolizing irradiated normal cells can induce mutations from ouabain susceptibility to ouabain resistance. Experiments on the time required for the optimal expression of these mutants have shown that after treatment with 1 μg/ml of BP the number of mutants per 10^6 survivors increased from 4.2 when ouabain was added 3 hr after seeding, up to 102 when ouabain was added 2 days after seeding (Fig. 1). At later times, the cultures were confluent and the cells started to degenerate even in the absence of ouabain. In all the following experiments we, therefore, used 48 hr after cell seeding as the optimal time for expression of drug resistance.

Nine ouabain-resistant mutants obtained after BP treatment, each from a different petri dish, were isolated and the progeny were tested for their resistance to different concentrations of ouabain. The cells were tested after they had been cultured in the absence of ouabain for about 2 months, i.e., about 100 cell generations. The results indicate (Fig. 2) that all nine mutants were still resistant to ouabain and that all showed a similar degree of resistance, as had also been found with mutants resistant to 8-azaguanine (11). The ouabain-resistant mutants cocultivated with the metabolizing cells were as susceptible to the cytotoxic effect of BP as the parental V79 wild-type cells.

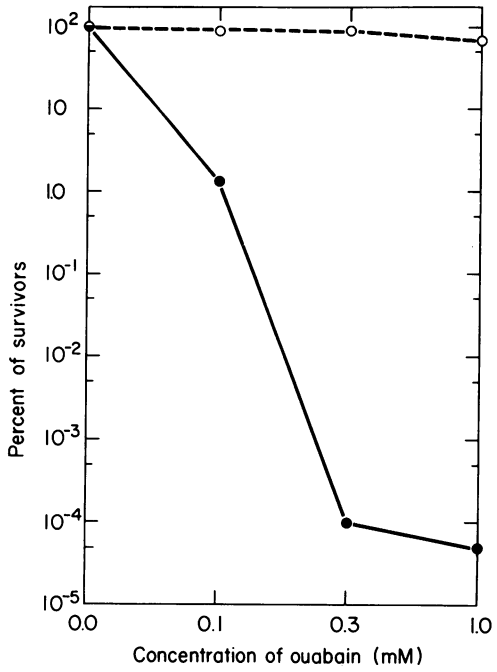

FIG. 2. Susceptibility to the cytotoxic effect of ouabain. ●, wild-type V79 cells; ○, nine isolated ouabain-resistant mutants. The untreated cells had about a 90% cloning efficiency. The percent survivors was calculated from the ratio between the cloning efficiencies of cells treated with ouabain and untreated cells.

Table 2. Induction of ouabain- and 8-azaguanine-resistant mutants by different chemical carcinogens

Treatment	Cloning efficiency (%)	Ouabain-resistant mutants per 10^6 survivors	8-Azaguanine-resistant mutants per 10^5 survivors
Solvent	92*	1	6
Pyrene	94*	1	5
Phenanthrene	79*	1	8
Chrysene	85*	2	9
BA	92*	2	9
DB[*a*,*c*]A	95*	3	22
DB[*a*,*h*]A	79*	4	17
MBA	61*	24	75
MCA	49‡	14	72
	41†	38	152
	38*	108	366
BP	47‡	18	54
	27†	45	128
	21*	121	425
DMBA	50§	22	41
	2‡	66	281

The cells were treated for 2 days with the polycyclic hydrocarbons with *1 μg/ml, †0.3 μg/ml, ‡0.1 μg/ml, or §0.01 μg/ml. After treatment there were with 0.01–0.1 μg/ml of DMBA, 0.3–1 μg/ml of BP, and 0.3–1 μg/ml of MCA, 1.0 to 2.5 × 10^6 V79 cells per petri dish. In all other cases, there were 2.7 to 3.6 × 10^6 V79 cells per petri dish.

Reconstruction experiments have shown that there was a complete survival of mutant cells cocultivated with V79 wild-type cells. Addition of 30 or 300 mutant cells to 3 × 10^5 wild-type cells before treatment with BP for 2 days yielded the frequency of mutants expected from the sum of the induced and added mutants (Table 1). These results indicate that the carcinogen induced the mutants and did not merely select for preexisting mutants in the population.

Induction of Ouabain- and 8-Azaguanine-Resistant Mu-

Table 1. Survival and induction of ouabain-resistant mutants in a reconstruction experiment

Treatment	Ouabain-resistant cells / wild-type cells	Cloning efficiency (%)	Ouabain-resistant mutants per 10^6 survivors
Solvent	$\frac{0}{3 \times 10^5}$	89	1
BP	$\frac{0}{3 \times 10^5}$	25	104
Solvent	$\frac{30}{3 \times 10^5}$	80	98
BP	$\frac{30}{3 \times 10^5}$	28	208
Solvent	$\frac{300}{3 \times 10^5}$	75	1029
BP	$\frac{300}{3 \times 10^5}$	23	1074
Solvent	$\frac{3 \times 10^5}{0}$	83	—
BP	$\frac{3 \times 10^5}{0}$	22	—

Ouabain-resistant cells were mixed with wild-type V79 cells before seeding on the irradiated normal cells. Two days after treatment with 1 μg/ml of BP, the cells were seeded for measuring cloning efficiency and the frequency of mutations. There were then 3.4 to 3.6 × 10^6 wild-type V79 and/or mutant cells after solvent treatment and 2.0 to 2.4 × 10^6 cells after BP treatment.

tants by Different Carcinogens. In order to determine the relationship between the carcinogenicity of a polycyclic hydrocarbon and its mutagenicity for ouabain and 8-azaguanine resistance, we have compared 4 carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA), BP, 3-methylcholanthrene (MCA), and 7-methylbenz[*a*]anthracene (MBA), and 6 hydrocarbons that have been reported to be noncarcinogenic for hamsters (22, 23), pyrene, phenanthrene, chrysene, benz[*a*]anthracene (BA), dibenz[*a,c*]anthracene (DB[*a,c*]A), and dibenz[*a,h*]anthracene (DB[*a,h*]A). None of these 10 compounds induced mutations for either of these two markers in the absence of metabolizing cells. After cocultivation with metabolizing cells, the four carcinogenic hydrocarbons induced mutations for both markers, whereas, four noncarcinogenic hydrocarbons, pyrene, phenanthrene, chrysene, and BA, were not mutagenic. DB[*a,c*]A and DB[*a,h*]A, which have been reported to be noncarcinogenic for hamsters (22, 23), showed a weak mutagenic effect with both markers. The frequency of mutations with the carcinogens was dose dependent (Table 2) and the degree of mutagenesis by BP was related to the amount of its metabolism. DMBA, the most potent of the carcinogens, showed the highest induction of mutation and a significant degree of mutagenicity was obtained with 0.01 μg/ml. Pyrene, phenanthrene, chrysene, and BA were not mutagenic or cytotoxic even at a concentration of 10 μg/ml. Two phenol derivatives of BP which are cytotoxic did not induce a significant number of mutations for either of these two markers. This indicates that the degree of mutagenicity is not necessarily related to the degree of cytotoxicity.

Induction of Temperature-Resistant Mutants. Three carcinogens, DMBA, BP, and MCA, and three noncarcinogens, pyrene, phenanthrene, and BA, were tested for mutagenicity for ability to grow at 39°, using ts Chinese hamster cells which cannot grow at 39°. As with the other markers, none of these six hydrocarbons changed the spontaneous mutation frequency for temperature resistance in the absence of metabolizing cells. On cocultivation with metabolizing cells, the three carcinogenic hydrocarbons were mutagenic and the three noncarcinogenic hydrocarbons were not mutagenic. The frequency of induced mutation by the carcinogens was dose dependent and the most potent carcinogen, DMBA, gave the highest mutation frequency (Table 3).

The properties of 10 temperature-resistant mutants induced by these carcinogens were tested after about 1 month, i.e., about 40 generations' growth at 34°. Seven of these mutants gave similar cloning efficiencies at 34°, 37°, and 39°. The other three mutants gave similar cloning efficiencies at 34° and 37°, but had a low cloning efficiency at 39°. The wild-type cells had a lower cloning efficiency at 37° than at 34° (Fig. 3). These results indicate that with this marker seven out of ten isolated mutants remained temperature resistant at 39°.

Increase in Mutagenicity by Carcinogens after Treatment with Aminophylline. Treatment with aminophylline, an inhibitor of cyclic nucleotide phosphodiesterase, can, in the appropriate cells, including normal golden hamster cells, result in an increase in the enzymatic metabolism of carcinogenic hydrocarbons (24, 25). We have, therefore, tested whether treatment of cells with aminophylline can increase the mutagenicity of carcinogens. The results (Table 4) indicate that there was a 2- to 4-fold increase in mutagenicity with MBA, MCA, and BP. DB[*a,c*]A, which showed a low degree of mutagenicity without aminophylline, showed a less than 2-fold increase with aminophylline. DB[*a,h*]A, which is not carcinogenic for hamsters but is carcinogenic for mice and rats (22, 23), was similar to DB[*a,c*]A without aminophylline, but showed with aminophylline a 10-fold increase in mutagenicity for ouabain resistance.

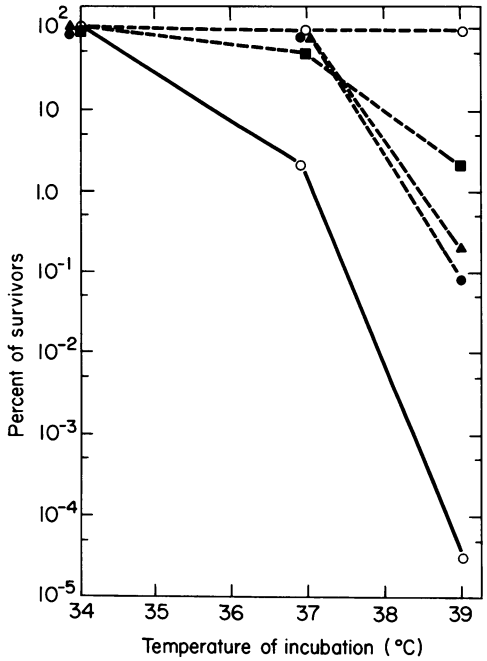

FIG. 3. Colony formation at different temperatures. O—O, temperature-sensitive cells; O- - - -O, seven isolated temperature-resistant mutants; ▲, ■, ●, three other temperature-resistant mutants. The percent of survivors was calculated from the ratio of cloning efficiency at 37° or 39° to the cloning efficiency at 34°. The cloning efficiency at 34° was about 100% for the ts cells and 70–90% for the temperature-resistant mutants.

DISCUSSION

Chemically nonreactive carcinogens, such as polycyclic hydrocarbons, have to be metabolized by cellular enzymes into

Table 3. Induction of temperature-resistant mutants by different chemical carcinogens

Treatment	Concentration (μg/ml)	Cloning efficiency (%)	Temperature-resistant mutants per 10^6 survivors
Solvent	0	93	0.6
Pyrene	1.0	88	0.9
Phenanthrene	1.0	88	0.7
BA	1.0	78	0.7
BP	0.1	67	21
	0.3	45	56
	1.0	9	170
MCA	0.1	78	20
	0.3	32	46
	1.0	10	125
DMBA	0.01	65	51
	0.1	3	494

Three days after treatment with the polycyclic hydrocarbons at 34°, the cells were seeded to determine the cloning efficiency of the ts cells and the frequency of temperature-resistant mutants. For selection of temperature-resistant mutants, the ts cells were shifted from 34° to 39°, 3 days after cell seeding. At the time when the cells were seeded for cloning efficiency and the frequency of mutations, there were after treatment with 0.01–0.1 μg/ml of DMBA, 0.3–1 μg/ml of BP or MCA, 0.5 to 1.9 × 10^6 ts cells per petri dish. In all other cases, there were 2.1 to 2.3 × 10^6 ts cells per petri dish.

Table 4. Induction of ouabain- and 8-azaguanine-resistant mutants by chemical carcinogens after treatment with aminophylline

Treatment	Cloning efficiency (%)	Ouabain-resistant mutants per 10^6 survivors	8-Azaguanine-resistant mutants per 10^5 survivors
Solvent	93	1	6
Pyrene	74	1	6
Phenanthrene	83	1	5
Chrysene	86	1	8
BA	81	3	12
DB[*a,c*]A	70	5	38
DB[*a,h*]A	43	46	87
MBA	22	NT*	189
MCA	4	413	960
BP	9	214	985

Cells were treated with 1 μg/ml of the polycyclic hydrocarbons and 0.1 mM aminophylline. Two days after treatment there were 1.7 to 2.3 × 10^6 V79 cells per petri dish with BP, MCA, and MBA. In all other cases, there were 2.6 to 3.0 × 10^6 V79 cells per petri dish.

* NT, not tested.

reactive metabolites that are responsible for biological effects, including mutagenicity (10, 11, 15–18). We have developed a cell-mediated mutagenesis assay (11), in which Chinese hamster cells, which are useful cells for mutagenesis but cannot metabolize the carcinogens, are cocultivated with lethally irradiated cells that can metabolically activate these compounds. The reactive metabolites produced by the metabolizing cells are apparently transferred to the cells used for testing mutagenesis and induce mutations. In this system, carcinogens can be tested without having to isolate the reactive metabolites.

In the present experiments we have shown with this system that carcinogenic polycyclic hydrocarbons can mutate Chinese hamster cells at three different genetic loci. These genetic loci affect the surface membrane (12), the control of nucleic-acid synthesis (13), and protein synthesis (14). In view of the significance of membrane changes in malignant cell transformation (6), the induction of mutations at a specific site on the membrane (12) appears to be of particular interest. With the polycyclic hydrocarbons tested, there was a relationship between carcinogenicity and mutagenicity for all three genetic markers. The most potent carcinogen, DMBA, gave the highest mutagenicity and a significant degree of mutagenicity was obtained with 0.01 μg/ml. It will be of interest to determine the spectrum of mutations of other genetic loci in mammalian cells with these and other carcinogens. It will also be important to determine the relationship between mutagenesis of specific loci and virus expression (3, 4).

No mutagenicity was obtained with any of the 10 compounds in the absence of cells with the appropriate metabolizing enzymes. The metabolism of polycyclic hydrocarbons can be increased by treating cells with aminophylline (24, 25). The present experiments have also shown that this treatment increases mutagenesis. Two of the compounds tested, DB[*a,c*]A and DB[*a,h*]A, have been reported to be noncarcinogenic for hamsters (22, 23) and both show only a weak mutagenic effect. However, after treatment of cells with aminophylline, there was a 10-fold increase in mutagenicity with DB[*a,h*]A but not with DB[*a,c*]A. Pyrene, chrysene, and phenanthrene, which are noncarcinogenic in all rodents tested, did not show any significant mutagenicity either with or without aminophylline. It can be concluded that cell-mediated mutagenesis with the present and other mammalian cells, including hepatocytes, and treatment with aminophylline, can provide a sensitive assay for hazardous chemicals.

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