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QUANTITATIVE MAMMALIAN CELL GENETIC TOXICOLOGY: STUDY OF THE CYTOTOXICITY AND MUTAGENICITY OF 70 INDIVIDUAL ENVIRONMENTAL AGENTS RELATED TO ENERGY TECHNOLOGIES AND 3 SUBFRACTIONS OF A CRUDE SYNTHETIC OIL IN THE CHO/HGPRT SYSTEM[1]

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INTRODUCTION

As science and technology advance, an extraordinary quantity of natural and synthetic chemicals is introduced continuously into our environment. Through the conventional animal tests, some of these environmental chemicals have been found to be either highly toxic, mutagenic, carcinogenic, or teratogenic. Epidemiological studies have shown that among these harmful chemicals, a few also exhibit such detrimental effects in the human population. Because of the high cost and long duration required for the animal experiments, such tests have been confined to only a very small fraction of these environmental agents. Thus, the biological effects of the great majority of these chemicals, including ingredients of our daily foods and drugs, remain either incompletely tested or unknown.

During the past few years, evidence has accumulated that a high percentage (80-90%) of human cancer is linked to exposure to industrial and environmental chemicals identifiable as carcinogens (23, 44). Since the expense of animal tests preclude their routine use to identify environmental carcinogens, many short-term assays have been developed as initial carcinogen screening tests. Studies of mutagenesis and DNA-repair in microorganisms, especially Salmonella typhimurium and Escherichia coli, have established that approximately 90% of chemical carcinogens cause mutation induction or DNA damage in these

bacteria (2, 3, 26, 27, 38, 39, 42, 45, 46). Such findings imply that the microbial tests are useful to identify not only potential mutagens but also carcinogens in the environment.

In view of the intrinsic limitation of the microbial assay to respond to certain classes of chemicals, such as the apparent failure of the Salmonella assay to demonstrate that carcinogenic halogenated hydrocarbons and metallic compounds are mutagenic (27), it appears that no single test system will give 100% correlation between mutagenicity and carcinogenicity. The use of a battery of tests rather than any single test in isolation has thus been proposed to reduce the probability of false negative (e.g., known carcinogens are not mutagenic) and false positives (e.g., known non-carcinogens are mutagenic) (4, 37).

It has been recognized that studies of mutagenesis in prokaryotes may not reveal some fundamental mechanisms of mutagenesis in mammals, because mammals differ from prokaryotes in their level of organization and repair of DNA, mechanisms of metablism of chemicals, and other related functions. Some bacterial mutagens such as caffeine and hydroxyamine do not appear to be mutagenic in mammalian cells, while agents such as nickel and beryllium compounds are mutagenic in mammalian cells but not in the Salmonella system (Couch, D. B., J. R. San Sebastian, and A. W. Hsie, unpublished, 27). In addition, it is well known that chromosomal abnormality is a major cause of inheritable

human diseases, and is often associated with the process of malignancy. The great majority of chemical carcinogens are known to induce chromosomal aberrations (1, 24), or sister chromatid exchange (1). Diethylstilbestrol, a synthetic hormone associated with human cancer in women, causes chromosomal aberrations in cultured mammalian cells (24), but not mutation-induction in Salmonella (27). Clearly, mammalian cell systems offer additional advantages for studying genetic toxicity at the chromosome and chromatid level, which is not available in bacterial assays.

Since the observation that treatment of mammalian somatic cells with conventional mutagens such as ethyl methanesulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) cause an increase in the number of cell variants that differ from parental cells in either nutritional requirement (5, 36) or drug sensitivity (5), there has been much interest in utilizing a quantitative mammalian cell mutation system for studying mechanisms underlying the process of mammalian mutation and additionally, for assessing the genetic hazard of environmental agents to the human population. Several mammalian cell mutation systems, especially those utilizing resistance to purine analogues such as 8-azaguanine (AG) and 6-thioguanine (TG) as a genetic marker (6), have been developed for such purposes. The selection for mutation induction to purine analogue resistance is based on the fact that the

wild-type cells containing hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity are capable of converting the analogue to toxic metabolites, leading to cell death; the presumptive mutants, by virtue of the loss of HGPRT activity, are incapable of catalyzing this detrimental metabolism and, hence, escape the lethal effect of the purine analogue (Table 1). T-1

The near-diploid Chinese hamster ovary (CHO) cell line has been chosen for our study because a mutation assay, referred to as CHO/HGPRT system, has been well defined (7-10, 16-22, 29-33). We have used CHO cells because these are perhaps the best characterized mammalian cells genetically (35, 40). They exhibit high cloning efficiency, achieving nearly 100% under normal growth conditions, and are capable of growing in a relatively well-defined medium on a glass or plastic substratum or in suspension with a population doubling time of 12-13 h. In addition, the cells have a stable, easily recognizable karyotype of 20 or 21 chromosomes (depending on the subclone) (11), and are suitable for studying mutagen- or carcinogen-induced chromosome and chromatid aberrations (1) and sister chromatid exchanges (1, 24) (Table 2). T-2

METHODS AND MATERIALS

Cell Culture

All studies to be described have employed a subclone of CHO-K_1 cells (25), designated as CHO-K_1-BH_4(16). It was isolated following selection in F12 medium containing aminopterin (10 μM) (16). Cells are routinely cultured in Ham's F12 medium (Pacific Biological Co.) containing 5% heat-inactivated (56°C, 30 min), extensively dialyzed fetal calf serum (Pacific Biological Co.) (medium F12FCM5) in plastic tissue culture dishes (Falcon or Corning Glass Works) under standard conditions of 5% CO_2 in air at 37°C in a 100% humidified incubator. These cells grow in aminopterin containing medium as well as in regular medium with 5 or 10% dialyzed fetal calf serum with a population doubling time of 12-13 h. Cells are removed with 0.05% trypsin for subculture and the number determined with a Coulter counter (model B, Coulter Electronics).

Treatment With Chemicals

We have standardized treatment procedures which are found to be suitable for various chemicals (16, 29). Briefly, CHO cells are plated at 5 x 10^5 cells/25 cm^2 bottle in medium F12FCM5. After a 16- to 24-h growth period (cell number = ∿ 1.0-1.5 x 10^6 cells/plate), the cells are then washed once with saline G, and sufficient serum-free F12 medium added to bring the final volume to 5 ml after the addition of various

amounts of microsome preparation (up to 1 ml) and 50 µl of chemical, usually dissolved in dimethylsulfoxide. Chemical and/or microsomes are omitted from some plates to provide controls. The microsomal preparation has been prepared from Aroclor 1254-induced male Sprague-Dawley rat livers in this laboratory according to the method of Ames et al. (3) and the microsome mix for biotransformation contains (per ml) 33 µmoles KCl, 8 µmoles $MgCl_2$, 4 µmoles NADP, 5 µmoles glucose-6-phosphate, 100 µmoles phosphate buffer (pH 7.4), and 0.2 ml microsome fraction. Cells are then incubated for 5 h, and washed three times with saline G before 5 ml of F12FCM5 are added. Following overnight incubation, cells are trypsinized and plated for cytotoxicity and specific gene mutagenesis to be described below. Treatment with physical agents have been described in detail elsewhere (17, 19, 29, 30).

Cytotoxicity

The effect of chemical on the cellular cloning efficiency is determined using the treated cells described above. For an expected cloning efficiency higher than 50%, 200 well-dispersed single cells are plated, and, for an expected survival lower than this, the number of cells plated are adjusted accordingly to yield 100-200 surviving colonies after standard incubation in medium F12FCM5 for 7 days. At the end of the incubation period, the plates are fixed with 3.7%

formalin, stained with a dilute crystal violet solution and the colonies enumerated. A cluster of more than 50 cells growing within a confined area is considered as a colony. Control cells, which do not receive treatment with mutagen, usually give 80% or higher plating efficiency under this condition. The solvent and microsome mix either singly or in combination does not affect the cellular cloning efficiency. The effect of carcinogen on the cloning efficiency is expressed as percent survival relative to the untreated controls.

Specific Gene Mutagenesis

The CHO/HGPRT system has been defined in terms of medium, TG concentration, optimal cell density for selection (and, hence, recovery of the presumptive mutants), and expression time for the mutant phenotype (16, 29). For the determination of mutation-induction, the treated cells are allowed to express the "mutant phenotype" in F12 medium for 7-9 days, at which time mutation induction reaches a maximum which is maintained thereafter (as long as 35 days examined) for several agents (EMS, MNNG, ICR-191, X-ray, and UV) irrespective of concentration or intensity of the mutagen (29-32). Routine subculture is performed at 2-day intervals during the expression period, and at the end of this time the cells are plated for selection in hypoxanthine-free F12FCM5 containing 1.7 μg/ml (10 μM) of TG at a density of 2.0×10^5 cells/

100-mm plastic dishes (Corning or Falcon), which permits 100% mutant recovery in reconstruction experiments (29). We find the use of dialyzed serum particularly important, presumably due to potential competition between hypoxanthine and TG for transport into the cells and for catalysis by HGPRT (29). After 7 to 8 days in the selective medium, the drug-resistant colonies develop; they are then fixed, stained, and counted. Such a protocol permits the maximum yield of TG resistant variants selected of which >98% have highly reduced HGPRT activity by various physical and chemical agents (7-10, 16-22, 29-33). Mutation frequency is calculated based on the number of drug-resistant colonies per survivor at the end of the expression period.

RESULTS

Characteristics of the CHO/HGPRT System: Evidence of the Genetic Basis of Mutation at a Specific Locus.

Conclusive, direct proof of the genetic origin of mutations in somatic cells should theoretically rely on demonstration that the affected hereditary alteration has resulted in a modified nucleotide sequence of the specific gene, causing modified coding properties which result in the production of altered protein with changes in the amino acid sequence. In the absence of such proof, one must rely on indirect criteria which are consistent with the concept that the observed phenotypic

variations are genetic in nature. Such criteria include stability of altered phenotype, mutagen-induced increase of occurrence of stable variants, biochemical and physiological identification of the variant phenotype, chromosomal localization of the affected gene, etc. (6, 35, 40, 43).

Over the past 4 years, we have used the assay protocol described (16, 29) and have found in approximately 400 experiments that the spontaneous mutation frequency lies in the range of 1-5 x 10^6 mutant/cell. Various physical and chemical agents are capable of inducing TG resistance. Among all chemical mutagens examined, mutation induction occurs as a linear function of the concentration (7-10, 16-22, 29-33). For example, mutation frequency increases approximately linearly with EMS concentration in this near-diploid cell line, conforming to the expectation that mutation induction occurs in the gene localized at the functionally monosomic X chromosome. However, in the tetraploid CHO cells, EMS does not induce an appreciable number of mutations, even at very high concentrations, as predicted theoretically (18).

We have been unable to detect any spontaneous reversion with 13 TG-resistant mutants, all of which contain low, yet detectable, HGPRT activity. Over 98% of the presumptive mutants isolated either from spontaneous mutation or as a result of mutation induction are sensitive to aminopterin, incorporate hypoxanthine at reduced rates, and have less than 5% HGPRT activity (29). Studies in progress have also shown that

mutants containing temperature-sensitive HGPRT activity can be selected, suggesting that mutation resides in the HGPRT structural gene (J. P. O'Neill and A. W. Hsie, unpublished observations).

The CHO/HGPRT system appears to fulfill the criteria for a specific gene locus mutational assay (Table 3) and should be valuable in studying mechanisms of mammalian cell mutagenesis and as a system to determine the mutagenicity of various physical and chemical agents. T-3

Mutagenicity of 70 Individual Energy Technology-Related Environmental Agents.

Polycyclic hydrocarbons (total of 27): One of the most ubiguitous environmental organic pollutants in our environment is polycyclic hydrocarbons many of which are carcinogenic. Coal- and synthetic fuel-related energy technologies and gasoline-driven autometives often generated high level of polycyclic hydrocarbons which are detectable in urban air and water. We have studied the mutagenicity of benzo(a)pyrene [B(a)P] and its 19 metabolites, including 11 phenols, 3 epoxides, 3 diols, and 2 diolepoxides. For comparison, benzo(e)pyrene [B(e)P] and pyrene were added to this study. Also included were benz(a)anthracene (BA) and 4 related compounds, 7,12-dimethyl BA, anthracene, and 2 phenolic derivatives of BA. The carcinogenic polycyclic hydrocarbons [B(a)P,

BA, and 7,12-dimethyl BA] require metabolic activation to be mutagenic. Weak carcinogen B(e)P is less mutagenic than B(a)P. The noncarcinogenic polycyclic hydrocarbons pyrene and anthracene are nonmutagenic even with metabolic activation. B(a)P-4,5-epoxide and B(a)P-7,8-diol,9-10-epoxide(Syn) are mutagenic. Since CHO cells cannot activate procarcinogens such as B(a)P, these cells appear to be most useful in screening for the mutagenicity of metabolites such as those of B(a)P (Hsie, A. W., and P. A. Brimer, unpublished). Because of the limited availability of B(a)P derivatives, some of the experiments remain to be pursued in detail.

Metallic compounds (total of 15). The carcinogenic and mutagenic potential of certain toxic metallic compounds has become an environmental concern especially with the increasing large scale coal mining and coal-firing power plants. We found that $MnCl_2.4H_2O$, $FeSO_4.7H_2O$ and $CoCl_2.6H_2O$ are mutagenic, $NiCl_2.6H_2O$, $BeSO_4.4H_2O$ and $CdCl_2$ are weakly mutagenic. Cis $Pt(NH_3)_2Cl_2$, an antitumor agent, is also mutagenic. Determination of metal mutagenicity is apparently complicated by the ionic composition of the medium. For example, we found that the mutagenicity and cytotoxicity of $MnCl_2$ were abolished by the excess of $MgCl_2$. The unusual environment required for demonstration of mutagenicity of $MnCl_2$ makes assessment of its biological hazard difficult. This too may account in part for varying results obtained in studying the mutagenicity of $AgNO_3$, $CaCl_2$, $Pb(CH_3COO)_2.3H_2O$, RbCl, H_2SeO_3, $TiCl_4$ and $ZnSO_4.7H_2O$ (10; Couch, D. B., J. R. San Sebastian, N. L. Forbes and A. W. Hsie, unpublished).

Nitrosamines and related compounds (total of 16): Nitrosamines are potent carcinogens for various animal species. They are of environmental concern because it is known that oxides of nitrogen produced at high temperature in the internal combustion engines and coal-fired power plants can react with atmospheric water to form nitrosamines. Nitrosamines can also be formed in human stomach by a reaction between a common meat preservative, sodium nitrite, and various secondary and tertiary amines, many of which are often used as counter or prescription drugs.

All nitrosamines require metabolic activation to be both cytotoxic and mutagenic. In addition to investigating the two common aliphatic nitrosamines, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), we have also studied the mutagenicity of 11 cyclic nitrosamines including 3 nitrosopiperidines, 3 nitrosopyrrolidines, 3 nitrosopiperazines, 2 nitrosomorpholines. Also included are the nitrosamine-related chemicals dimethylamine, formaldehyde and sodium nitrite. We have found that all 9 carcinogenic nitrosamines (DMN, DEN, 2-methyl-1-nitrosopiperidine, 3,4-dichloro-1-nitrosopiperidine, nitrosopyrolidine, 3,4-dichloro-nitrosopyrrolidine, 1,4-dinitrosopiperazine, 1,5-dinitrosohomopiperazine, nitrosomorpholine) are mutagenic and all 4 noncarcinogenic nitrosamines (2,5-dimethylnitrosopiperidine, 2,5-dimethylnitrosopyrrolidine, 1-nitro-sopiperazine and nitrosophenmetrazine) are not mutagenic. Formaldehyde

and sodium nitrite are not mutagenic and dimethylamine is mutagenic at high concentrations. (San Sebastian, J. R., D. B. Couch, and A. W. Hsie, unpublished). Variable carcinogenicity data on the latter 3 chemicals existed in the literature.

Quinoline compounds (total of 5). One class of potential environmental contaminants from fossil-fuel energy is heterocyclic compounds such as quinolines. Quinoline, a known carcinogen, is mutagenic with metabolic activation. Another carcinogen, 4-nitroquinoline-1-oxide, is highly mutagenic; its mutagenicity decreases when assayed in the presence of the activation system. The carcinogenicity of 8-hydroxy-, 8-amino-, 8-nitro-quinoline are not known, but they exhibit variably weak mutagenicity in preliminary experiments (San Sebastian, J. R. and A. W. Hsie, unpublished).

Physical agents (total of 7). The mutagenicity of both ionizing radiation such as X-ray and nonionizing physical agents such as UV light has been demonstrated. Fluorescent white-, black-, and blue-lights are slightly cytotoxic and mutagenic. Sunlamp light is highly cytotoxic and mutagenic, exhibiting the biological effects with 15 sec of exposure under conditions recommended by the manufacturer for human use. Cytotoxic and mutagenic effects are observed after 5 min of sunlight exposure; responses vary with hourly and daily variations in solar radiation. In view of man's constant exposure to various

light sources, demonstration of their genetic toxicity suggests that daily exposure to these light sources, especially sunlight, should be minimized (17, 19, 30). The demonstration that the CHO/HGPRT system is capable of quantifying the cytotoxic and mutagenic effect of sunlight recommends it as a model mammalian cell system for studies of the genetic toxicology of sunlight per se and of the interactive effects between sunlight and other physical and chemical agents, leading ultimately to a better understanding of the effects of sunlight on humans and the environment.

Mutagenicity of 39 Other Chemicals.

Direct-acting alkylating agents and related compounds (total of 11). Included are 10 alkylating agents: 2 alkylsulfates [dimethysulfate (DMS), diethylsulfate (DES)], 3 alkyl alkanesulfonates [methyl methanesulfonate (MMS), EMS, and isopropyl methanesulfonate (iPMS)], 2 nitrosamidines [MNNG and N-ethyl-N'-nitrosoguanidine (ENNG)], 3 nitrosamides [N-methyl-N-nitrosourea (MNU), N-ethyl-N-nitrosourea (ENU), and N-butyl-N-nitrosourea (BNU)] and a structural analogue of MNNG, N-methyl-N'-nitroguanidine (MNG). Among the alkylsulfates and alkanesulfonates, cytotoxicity was found to decrease with the size of the alkyl group: DMS>DES; MMS>EMS>iPMS. The mutagenicity based on mutants induced per unit mutagen concentration is DMS>DES; MMS>EMS>iPMS. However, when comparisons were made at 10% survival, mutagenic potency was: DES>DMS; EMS>iPMS>MMS. Among the nitroso-compounds, the order of the mutagenicity based on 10% survival is

MNNG>ENNG>MNU>ENU>BNU. This is the same order of potency as observed for mutation induction per unit mutagen concentration. MNG is not mutagenic (7-9; Couch, D. B., J. R. San Sebastian and A. W. Hsie, unpublished).

Heterocyclic nitrogen mustards - ICR compounds (total of 10): A series of heterocyclic nitrogen half-mustards, the ICR-compounds, has been developed at the Institute for Cancer Research as antitumor agents. Apparently, the biological activities of these compounds are associated with their ability to intercalate and covalently bind nucleic acid. Ten ICR-compounds (ICR-191, -170, -292, -372, -340, -191-OH, -170-OH, -292-OH, -372-OH, and -340-OH) have been studied. The 2-chloroethyl side chain of the first 5 compounds (e.g., ICR-191, etc.) has been replaced by a hydroxy group in the latter 5 (e.g., ICR-191-OH, etc.). The 10 compounds differ in the heterocyclic nucleus (methoxy acridine for ICR-191 and -170, benz(a)acridine for -292, and azaacridine for -372 and -340) and the alkylating side chain (the same secondary amine for ICR-191 and -372, and same tertiary amine for -170, -292 and -340). Those with 2-chloroethyl side chains are highly mutagenic, with the tertiary amines 3 to 5 times more mutagenic than the secondary amines. The 4 hydroxy derivatives are not mutagenic, but remain highly toxic, indicating that although the 2-chloroethyl group (nitrogen half-mustard) is needed for mutagenicity, its replacement with hydroxy group does not alter cytotoxicity. Cytotoxicity and mutagenicity of

ICR-compounds appear to be dissociable (32, 33; Fuscoe, J. C., J. P. O'Neill and A. W. Hsie, unpublished).

Aromatic amines (total of 5): Many aromatic amines are human carcinogens. We have shown that the carcinogens 2-acetylaminofluorene and its N-hydroxy- and N-acetoxyl-derivatives are mutagenic while fluorene, a noncarcinogenic analogue, is not mutagenic. 1-hydroxy-2-acetylaminofluorene appears to be mutagenic at a very high concentration in one preliminary experiment (Hsie, A. W., W. N. C. Sun and P. A. Brimer, unpublished).

Miscellaneous compounds (total of 13). Three commonly used organic solvents (acetone, dimethylsulfoxide, and ethanol) are noncarcinogenic and do not appear to be mutagenic. All four metabolic inhibitors (cytosine arabinoside, hydroxyurea, caffeine, and cycloheximide) are nonmutagenic in a preliminary study without coupling with the metabolic activation system. Hydrazine and hycanthone appear to be direct-acting mutagens. N^6, $O^{2'}$-dibutyryl adenosine 3':5'-phosphate, an analogue of adenosine 3':5'-phosphate, an important effector of growth and differentiation in many biological systems, is not mutagenic. The pesticides captan and folpet are mutagenic. The mutagenicity of an artificial sweetener, saccharin, appears to be variable; its determination is complicated by the requirement of high concentrations to yield any biological effect (J. P. O'Neill and A. W. Hsie, unpublished).

Correlation of Mutagenicity in the CHO/HGPRT Assay With Reported Carcinogenicity in Animal Tests.

Among a total of 109 chemical and physical agents studied, at different stages of completion, 56 have been reported to be either carcinogenic or noncarcinogenic in animal studies. Mutagenicity in the CHO/HGPRT assay of 54 of these agents correlated well with documented animal carcinogenicity. The concurrence (i.e. known carcinogens are mutagenic and noncarcinogens are nonmutagenic in CHO/HGPRT assays) of each class of agents is: direct-acting alkylating agents and relatives, 11/11 (100%); ICR compounds, 5/6 (83.33%); metallic compounds, 4/4 (100%); polycyclic hydrocarbons, 6/6 (100%); aromatic amines, 4/4 (100%); miscellaneous chemicals, 5/5 (100%); and physical agents, 3/3 (100%) (Table 4). The existence of a high correlation [54/56 (96.43%)] between mutagenicity and carcinogenicity speaks favorably for the utility of this assay in prescreening the carcinogenicity of chemical and physical agents. However, this result should be viewed with caution since, so far, only limited classes of chemicals have been tested and some of the preliminary results remained to be confirmed. T-4

A possible false negative was formaldehyde, which has been shown to be either carcinogenic or noncarcinogenic depending on the means of exposure to the test animal. Apparently, a false positive was ICR-191, a potent bacterial mutagen, which has been shown to be noncarcinogenic in a recent study.

A Study of EMS Exposure Dose: Differential Effects on Cellular Lethality and Mutagenesis.

Earlier, we have found that EMS-induced mutation frequency to TG resistance is a linear function over a large range of mutagen concentrations (0.013-0.8 mg/ml) including both the shoulder region (0-0.1 mg/ml) and the exponentially killing portion (0.1-0.8 mg/ml) when cells were treated for a fixed period of 16 h. To investigate whether EMS-induced mutagenesis can be quantified further, cells were treated with several concentrations of EMS for intervals of 2-24 h. Mutation induction increases linearly with EMS concentrations of 0.05-0.4 mg/ml for incubation times of up to 12-14 h. However, cell survival decreased exponentially with time over the entire 24 h period. This difference in the time course of cellular lethality vs mutagenicity might be due to the formation of toxic, nonmutagenic breakdown products in the medium with longer incubation times, or might reflect a difference in the mode of action of EMS in these two biological effects. Further studies using varying concentrations (0.05-3.2 mg/ml) of EMS for 2-12 hr showed that the manifestation of cellular lethality and mutagenesis occurs as a function of EMS exposure dose in that the biological effect is the same for different combinations of concentration multiplied by duration of treatment which yield the same product. From these studies the mutagenic potential of EMS can be described as 310×10^{-6} mutants (cell mg ml^{-1} h)$^{-1}$.

Thus, the CHO/HGPRT appears to be suitable for dosimetry studies which are essential for our understanding of the molecular mechanisms involved in mammalian mutagenesis (31).

Screening for the Mutagenicity of Fractionated Synthetic Fuel.

In addition to studying the mutagenicity of individual environmental agents such as polycyclic hydrocarbons, quinolines, nitrosamines, metallic compounds etc., we have found that the CHO/HGPRT assay can detect the cytotoxicity and mutagenicity of a crude organic mixture, in this case, the subfractions of crude synthetic oil (fractioned by M. R. Guerin of Analytical Chemistry Division, ORNL) supplied by Pittsburgh Energy Research Center. The acetone effluent (which contains tentatively identifiable heterocyclic nitrogen compounds) derived from the basic fraction is most mutagenic in the presence of metabolic activation system (Table 5) (Hsie, A. W., W. N. C. Sun and P. A. Brimer, unpublished). T-5
Earlier, it appeared that the extreme toxicity of the unfractionated crude fuel prevents meaningful mutagenicity studies in the CHO/HGPRT system (Hsie, A. W., and P. A. Brimer, unpublished). The chemistry (14), mutagenicity in microbial systems (12) and environmental testing (13) of the Synfuel are presented elsewhere in this Symposium.

Preliminary Development and Validation of the CHO Genetic Toxicity Assay for the Simultaneous Determination of Cytotoxicity, Mutagenicity, Chromosome Aberrations and Sister-Chromatid Exchanges.

We have so far shown that CHO cells are useful to study the cytotoxicity and mutagenicity of various individual physical and chemical agents as well as crude organic mixture. The CHO cells and other hamster cells in culture were also found to suitable for studying carcinogen-induced chromosome and chromatid aberrations (1, 15, 24, 28) and sister chromatid exchange (1, 34, 41). In our preliminary studies, we have found that these assays are useful in evaluating the cytogenetic effects of benzo(a)pyrene and dimethylnitrosamine using CHO cells coupled with the standard microsome preparation described earlier (San Sebastian, J. R. and A. W. Hsie, unpublished). Therefore, under proper experimental design, it appears feasible to determine simultaneously carcinogen-induced cytotoxicity, mutagenicity, chromosome aberration and the sister chromatid exchange in the same chemical-treated CHO cells.

The successful development and validation of the multiplex CHO cell genetic toxicity system will be extremely valuable from both the scientific and economical points of view in genetic toxicology, because this system will allow the simultaneous determination of 4 distinct biological effects: cytotoxicity or cloning efficiency measures the reproductive capacity of a single cell to develop into a colony;

single gene mutagenesis involves changes in the nucleotide sequence of DNA of a specific gene resulting in the acquisition of a novel or altered phenotype; chromosome aberrations involve microscopically identifiable changes in the number and/or structure of the chromosome; and sister chromatid exchange measures the extent of double-strand exchange in the DNA duplex after breaks and rejoining of subunits of chromatids each of which consists of one DNA duplex.

SUMMARY AND CONCLUSIONS

Conditions necessary for quantifying mutation-induction to 6-thioguanine resistance, which selects for >98% mutants deficient in the activity of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) in a near-diploid Chinese hamster ovary (CHO) cell line, referred to as CHO/HGPRT system, have been defined. Employing this mutation assay, we have determined the mutagenicity of diversified agents including 11 direct-acting alkylating agents, 16 nitrosamines, 10 heterocyclic nitrogen mustards, 15 metallic compounds, 5 quinolines, 5 aromatic amines, 27 polycyclic hydrocarbons, 13 miscellaneous chemicals, 7 ionizing and non-ionizing physical agents. The direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine is mutagenic while its non-carcinogenic analogue N-methyl-N'-nitro-N-nitroguanidine is not. Coupled with the rat liver S_9- activation system, procarcinogens such

as nitrosopyrrolidine, benzo(a)pyrene, and 2-acetylaminofluorene are mutagenic while their analogues 2,5-dimethylnitrosopyrrolidine, pyrene and fluorene are not. The mutagenicity of the 56 agents documented to be either carcinogenic or non-carcinogenic correlated well [54/56 (96.43%)] with the reported animal carcinogenicity. A possible false negative was formaldehyde and a false positive was ICR-191. Preliminary studies show that the acetone effluent (tentatively identifiable as heterocyclic nitrogen compounds) derived from the basic fraction of a synthetic crude oil is the most mutagenic fraction. The assay, thus, appears to be applicable for monitoring the genetic toxicity of crude organic mixtures in addition to diverse individual chemical and physical agents. The quantitative nature of the assay enables a study of EMS exposure dose: the mutagenic potential of EMS can be described as 310×10^{-6} mutants (cell mg ml^{-1} $h)^{-1}$. It is also feasible to expand the CHO/HGPRT system for quantifying cytotoxicity and mutagenicity to determination of chromosomal aberrations and sister chromatid exchanges in cells treated under identical conditions which allows a simultaneous study of these four distinctive biological effects.

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Table 1

CHO/HGPRT Mutation Assay[a]

(1) Enzyme system:

$$\begin{matrix} \text{H,} \\ \text{G} \\ \text{(or TG, AG)} \end{matrix} \xrightarrow{\text{HGPRT}} \begin{matrix} \text{IMP,} \\ \text{GMP} \\ \text{(or TG, AG)MP} \end{matrix}$$

(2) Mutation induction and selection for variants and revertants:

(a) wild type $\xrightarrow[\text{(induced by physical or chemical agents)}]{\text{mutation}}$ variant cell

genotype $HGPRT^+$ — $HGPRT^-$

phenotype TG^s, — TG^r,

aminopterin positive — aminopterin negative

(b) Variant selection is based on resistance to TG

(c) Selection of revertants is based on growth in presence of aminopterin.

(3) Characterization of TG^r variants:

(a) Direct enzyme assay for conversion of [^{3}H] hypoxanthine to [^{3}H]IMP.

(b) Cellular incorporation of [^{3}H]hypoxanthine into cellular macromolecules as revealed by either direct radioactivity measurements or autoradiographic determination.

(c) Sensitivity of clonal growth to aminopterin (10 μM) in medium F12FCM5 which contains hypoxanthine (30 μM), glycine (100 μM), and thymidine (3 μM).

[a] Table 2 of ref. 21.

TABLE 2

CHARACTERISTICS OF CHO CELLS

(1) Exhibit a stable karyotype over 20 years with a modal chromosome number of 20 which has a distinctly recognizable morphology.

(2) Have a colony-forming capacity of nearly 100% in a defined growth medium.

(3) Grow well in either monolayer or suspension with a relatively short population doubling time of 12-14 h.

(4) Are genetically and biochemically well characterized, with many genetic markers available, including auxotrophy, drug resistance, temperature sensitivity, etc.

(5) Respond well to various synchronization methods, including the mitotic detachment procedure which facilitates cell cycle study.

(6) Are useful in somatic cell hybridization experiments because they readily hybridize with different cell types, including human cells; when the CHO-human cell hybrid is formed there is subsequent rapid, preferential loss of human chromosome, which facilitates the assignment of marker genes to specific chromosomes or linkage groups in the human karyotype.

(7) Quantitatively respond to various physical and chemical mutagens and carcinogens with high sensitivity.

(8) Adapt to mutation induction either through coupling with a microsome activation system or through host (mouse) mediation.

(9) Are capable of monitoring induced mutation to multiple gene markers, chromosome aberration, and sister chromatid exchange in the same mutagen-treated cell culture.

TABLE 3

CHO/HGPRT Mutation Assay: Genetic Basis of Mutation at HGPRT Locus in TG-Resistance Selection[a]

(1) Spontaneous mutation frequency at 1-5 x 10^{-6} mutant/cell.

(2) Mutation induction by physical and chemical agents with linear dose-response relationship.

(3) Frequency of spontaneous reversion less than 10^{-7} reversion/cell.

(4) Failure to induce mutation in near-tetraploid cell lines.

(5) Altered HGPRT activity in mutants.

  (a) 1170/1189 (98.4%) mutant colonies are aminopterin negative.

  (b) 121/122 (99.2%) mutant colonies show reduced hypoxanthine incorporation by autoradiography studies.

  (c) 81/83 (97.6%) isolated mutant clones show reduced HGPRT enzyme activity.

[a] Table 3 of Ref. (29).

TABLE 4

CORRELATION OF MUTAGENICITY[a] IN THE CHO/HGPRT ASSAY WITH REPORTED CARCINOGENICITY[b] IN ANIMAL TESTS[c]

Agent[d]	Total number studied	Concurrence[e]	False negatives[f]	False positives[g]
Energy Technology related				
Polycyclic hydrocarbons	27	6/6 (100%)	0	0
Metallic compounds	15	4/4 (100%)	0	0
Nitrosamines and relatives	16	14/15 (93.33%)	1/15 (6.67%)	0
Quinolines	5	2/2 (100%)	0	0
Physical agents	7	3/3 (100%)	0	0
Subtotal	70	29/30 (96.67%)	1/30 (3.33%)	0
Other Chemicals				
Direct-acting alkylating agents and relatives	11	11/11 (100%)	0	0
ICR compounds	10	5/6 (83.33%)	0	1/6 (16.67%)
Aromatic amines	5	4/4 (100%)	0	0
Miscellaneous chemicals	13	5/5 (100%)	0	0
Subtotal	39	25/26 (96.15%)	0	1/26 (3.85%)
All agents	109	54/56 (96.43%)	1/56 (1.79%)	1/56 (1.79%)

Table 4 - continued

[a]Agents studied are found to be either mutagenic (regardless of "mutagenic potency") or nonmutagenic. The mutagenicity is assayed either directly or coupled with a metabolic activation system *in vitro* or *in vivo*. In the S_9-coupled assay the microsome used was prepared from Aroclor 1254-induced male Sprague-Dawley rat livers. The effects of either other inducers or conditions of the activation system have not been investigated extensively and are under study.

[b]Agents studied are denoted either as carcinogenic or noncarcinogenic or uncertain based primarily on published data from USPHS (44) and IARC (23) regardless of "carcinogenic potency." Carcinogenicity of many compounds is not yet available. The search for such data is admittedly neither exhaustive nor updated.

[c]In part from Table VIII of ref. 21.

[d]The data are compiled from all agents studied, excluding those whose carcinogenicity is either unknown or uncertain. Thus, only 56 out of 109 agents studied are compiled in this table.

[e]Known carcinogens are mutagenic and noncarcinogens are nonmutagenic in CHO/HGPRT assays, i.e., MNNG, ICR-292, Ni, benzo(a)pyrene, hycanthone, UV, etc.

[f]Known carcinogens are nonmutagenic in CHO/HGPRT assays, e.g. formaldehyde.

[g]Known noncarcinogens are mutagenic in CHO/HGPRT assays, e.g. ICR-191.

TABLE 5

CYTOTOXICITY AND MUTAGENICITY OF SUBFRACTIONS OF SYNFUEL A BASIC FRACTIONS[a]

Subfraction[b]	Concentration (μg/ml)	Relative cloning efficiency (%)		Observed mutation frequency (TG mutants/10^6 cells)	
		$-S_9$	$+S_9$	$-S_9$	$+S_9$
Benzene	0.25		92		4
	1		102		<1
	2.5		117		1
	10	109	91	<1	1
	25		90		16
	50		71		13
	100	<0.2	0.2	-	25
Isopropanol	0.25		95		1
	1		94		1
	2.5		103		6
	10	108	95	<1	4
	25		102		16
	50		82		7
	100	<0.2	58	-	2
Acetone	0.25		89		6
	1		101		5
	2.5		93		<1
	5		100		9
	10	58	96	13	22
	25	22	56	6	46
	50	<0.3	4	15	49
	100	<0.2	<0.2	-	135
Controls					
EMS	200			279	-
B(a)P	8			-	557
Solvent				4	9

[a] Unpublished data of Hsie, A. W. and P. A. Brimer.

[b] See ref. 14 for details about the chemical separation of Synfuel A.