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UTILIZATION OF A QUANTITATIVE MAMMALIAN CELL MUTATION SYSTEM, CHO/HGPRT, IN EXPERIMENTAL MUTAGENESIS AND GENETIC TOXICOLOGY*

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Introduction

It has been recognized recently 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 metabolism of chemicals, and other related functions. 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) causes an increase of cell variants that differ from the parental cells in either nutritional requirements [4,21,35] or drug sensitivity [4], there has been much interest in utilizing a quantitative 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 have been developed for such purposes [5].

Since the discovery that mammalian cells resistant to the purine analogue 8-azaguanine (AG) [or, more recently, 6-thioguanine (TG)] can be selected after treatment with chemical mutagens, a variety of mutagenesis studies have utilized resistance to purine analogues as a genetic marker [4,5].

The selection of 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. Because of the relative ease of

obtaining, scoring, and characterizing resistance to purine analogues and of studying the forward as well as the reverse mutation (resistance to aminopterin), this drug-resistance marker has been widely used in mammalian cell mutagenesis studies. The HGPRT gene is on the functionally monosomic X chromosome in diploid mammalian somatic cells. Only one X chromosome is functional genetically: male cells have one functional X chromosome because the Y chromosome does not contain genes known to be on the X chromosome, and female cells also have only one functional X chromosome because the other X chromosome has been inactivated during early embryogenesis according to the X-inactivation mechanism proposed originally by Russell [37] and Lyon (24]. Therefore, one would expect that mutation of the diploid cells at the HGPRT locus would be similar to that of the bacteria [34]. This attribute greatly facilitates studies of induction of mutation and the subsequent analyses of mutagenesis.

Despite the remarkable progress in somatic cell mutagenesis studies over the years [5], there remain numerous conflicting reports concerning conditions affecting mutagenesis, including even the most fundamental problem of the genetic basis of mutation induction to purine analogues. To clarify aspects of these problems, we have redefined conditions necessary for the selection of purine analogue resistance, utilizing Chinese hamster ovary (CHO) cells clone K_1-BH_4 and TG to measure mutation induction at the HGPRT locus; we refer to the assay as the CHO/HGPRT system or assay.

CHO cells were chosen for the study because they are perhaps the best characterized mammalian cells genetically and are suitable for studies in mutagenesis [22, 34]. In addition, 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 short population doubling time of 12–13 h. They have a stable, easily recognizable karyotype of 20 or 21 chromosomes (depending on the subclone) which appears to have undergone extensive chromosomal rearrangement [9]. This may have resulted in many hemizygous regions, since numerous phenotypically recessive mutations have been isolated [40] (Table I). (T-I)

Purine analogues AG and TG are known to differ in the mechanism by which they cause growth inhibition and cellular lethality to mammalian cells [36]. The choice of TG over AG as a selective agent has been prompted by reports that TG-resistant variants result from an alteration or loss of the enzyme HGPRT [3, 39], inplying that such mutations occur at the structural gene which confers HGPRT activity. In addition, we have found that AG at modest concentrations lacks the stringency in selecting variants affected at the HGPRT locus, and at higher levels exhibits general cytotoxicity leading to lethal effects to both the wild-type and the HGPRT-deficient variants [12].

Materials and Methods

Development of a stringent selective system

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 [13, 28]. Routinely, approximately 1×10^6 cells were exposed to a mutagen for a fixed

period of time, usually 16 h, which covers slightly over one population doubling time. After the mutagen was removed, the treated cells were allowed to express the "mutant phenotype" in F12 medium for 7-9 days, at which time mutation induction reached a maximum which was 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 (Fig. 1). Routine subculture was performed at 2-day intervals during the expression period, and at the end of this time the cells were plated for selection in hypoxanthine-free F12 medium 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 (Fig. 2). We find the use of dialyzed serum particularly important, presumably due to potential competition between hypoxanthine and TG for catalysis by HGPRT (Fig. 3) and for transport into the cells [1]. After 7 to 8 days in the selective medium, the drug-resistant colonies developed; they were then fixed, stained, and counted. Such a protocol permits the maximum yield of variants selected at the HGPRT locus by various physical and chemical agents [7, 13, 14, 16, 18, 28, 29, 31]. Mutation frequency was calculated based on the number of drug-resistant colonies per survivor at the end of the expression period. Weighted least-squares regression analysis was employed to determine the shape of the curves for mutation induction and cell survival [13].

F-1

F-2

F-3

Coupling of the microsome activation system to the CHO/HGPRT assay

Promutagens or procarcinogens are inactive by themselves but are converted to "active principles" through biotransformation. The CHO/HGPRT assay can be coupled with the so-called S_9 fraction derived from rat liver microsome activation preparation [29] as first described by Krahn and Heidelberger for V79 cells [23] for determining mutagenicity of procarcinogens such as benzo(a)pyrene, benz(a)anthracene, and dimethylnitrosamine. In our studies, the microsome fraction prepared from Arocolor 1254-induced male Spraque-Dawley rat livers was the only activation source employed. Numerous factors which might affect metabolic activation both qualitatively and quantitatively, such as differences in organ, sex, animal species, and inducer await further studies.

Host-mediated CHO/HGPRT assay

An alternative method of achieving metabolic activation is the use of host-mediated assay [10]. Comparison of mutagenic activity of a compound in the host-mediated assay with that in direct tests can indicate whether activation or detoxification has occurred. Nude mice, or their phenotypically normal littermates, and BALB/c mice have been used as hosts for CHO/HGPRT assays to determine the mutagenicity of dimethylnitrosamine, benzo(a)pyrene, and EMS [17, 18].

Results and Discussions

Evidence for the genetic origin of mutation induction in the CHO/HGPRT system

Employing the assay protocol described in Materials and Methods and outlined in Table II, we have bound that the spontaneous mutation frequency lies in the range

(T-II)

of $1-5 \times 10^{-6}$ mutant/cell, as found in approximately 400 experiments over the past 4 years. 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, 12-14, 16, 18, 28-31]. For example, mutation frequency increases approximately linearly with EMS concentration in a near-diploid CHO 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. The virtual absence of EMS-induced mutation to TG resistance in tetraploid cells is expected, as the theoretically predicted mutation frequency would be the spontaneous frequency (which is estimated to be 10^{-12} mutants/survivor) plus the square of the induced mutation frequency at the corresponding EMS concentration in the diploid cells (which is no greater than 5×10^{-6} mutants/survivor) [12]. 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 [12, 28]. Studies in progress have also shown that mutants containing temperature-sensitive HGPRT activity can be selected at a relatively high frequency, suggesting that mutation resides in the HGPRT structural gene.

The CHO/HGPRT system appears to fulfill the criteria for a specific gene locus mutational assay (Table III) and should be valuable in studying mechanisms

of mammalian cell mutagenesis and in determining the mutagenicity of various physical and chemical agents.

Dose-response relationship for EMS-mediated mutation induction and cell lethality

The first quantitative mutagenesis experiment performed was the dose-response of EMS. We found that EMS-induced mutation frequency to TG resistance is a linear function over a large range of mutagen concentrations when cells are treated for a fixed period of 16 h. The mutation-induction line can be adequately described by $\underline{f}(\underline{x}) = (8.73 + 3.45\ \underline{x}) \times 10^{-6}$ where $\underline{f}(\underline{x})$ is the fitted mutation frequency and $\underline{x}$ is the EMS concentration. These EMS concentrations cover a wide range of the survival curve, including the shoulder region (0–100 μg/ml of EMS), where no appreciable loss of cellular lethality occurs, and the straight portion (100–800 μg/ml of EMS), where cell killing increases nearly exponentially with increasing mutagen concentrations (Fig. 4) [13]. Apparently, no threshold effect is exhibited for mutation induction by EMS. It is worth noting that mutation frequency reaches approximately 350×10^{-6} at 100 μg/ml of EMS (from the spontaneous frequency of 9×10^{-6}) while no significant cell killing occurs. This experiment demonstrates that mutation induction could occur without significant loss of cell survival and that it is a more sensitive parameter of genetic toxicity than cell lethality as far as agents such as EMS are concerned.

F-4

Later experiments show that many physical and chemical agents exhibit an "EMS-type" curve of interrelationships of cell survival and mutation induction [7, 14, 16, 18, 29, 31]. However, there are agents, such as MNNG, which do not

exhibit an appreciable shoulder region in the survival curve, and mutation induction always occurs concomitantly with the loss of cell survival [7,28,30].

Apparent dosimetry of EMS-induced mutagenesis

To investigate whether EMS-induced mutagenesis can be quantified further, cells were treated with several concentrations of EMS for intervals of 2–24 h. A linear increase in mutation frequency was found with EMS concentrations of 50–400 μg/ml for incubation times of up to 12–14 hr. 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. However, the increases in both cell lethality and induced mutation with incubation times of up to 12 h allow a study of the apparent dosimetry of this chemical mutagen. For better defined dosimetry studies, cultures were incubated with varying concentrations of EMS (0.05–3.2 mg/ml) for 2–12 h. Here we define the apparent dose of EMS to be the product of the EMS concentration and the treatment time. The response to mutagen treatment (dose) should be the same for different combinations of concentration multiplied by duration of treatment which yield the same product. As shown in Fig. 5, the decrease in cell survival was exponential and the increase in mutation frequency was linear with dose. From these studies the mutagenic potential of EMS can be described as 310×10^{-6} mutants (cell mg $ml^{-1}h)^{-1}$.

(F-5)

These results demonstrate that the mutagenicity of EMS in mammalian cells follows a linear dosimetry relationship and that a quantitative study of chemical

mutagenesis in mammalian cells can be performed.

Structure–activity relationship of alkylating agents and ICR compounds

The quantitative nature of mutation induction by EMS [12, 13, 31] and ICR-191 [29] suggests the applicability of the CHO/HGPRT system for comparing the mutagenicity and cytotoxicity of various direct-acting alkylating agents and ICR compounds.

Comparative mutagenicity and cytotoxicity of alkylsulfate and alkanesulfonate derivatives. Dose-response relationships of cell killing and mutation induction of two alkylsulfates [dimethylsulfate (DMS) and diethylsulfate (DES)] and three alkyl alkanesulfonates [methyl methanesulfonate (MMS), EMS, and isopropyl methanesulfonate (iPMS)] have been studied, and certain features of these relationships are summarized here (Table IV) [7, 8]. Under the experimental conditions employed, (T-IV) cytotoxicity decreased with the size of the alkyl group: DMS>DES; MMS>EMS>iPMS. All agents produced linear dose response of mutation induction: based on mutants induced per unit mutagen concentration, DMS>DES; MMS>EMS>iPMS. However, when comparisons were made at 10% survival, mutagenic potency was: DES>DMS; EMS>iPMS>MMS.

Mutagenicity and cytotoxicity of congeners of two classes of nitroso compounds. Using the CHO/HGPRT system, we have compared the cytotoxicity and mutagenicity of two nitrosamidines [MNNG and N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)] and three nitrosamides [N-methyl-N-nitrosourea (MNU), N-ethyl-N nitrosourea (ENU), and N-butyl-N-nitrosourea (BNU)] differing in the nature of their alkylating group. Based on 10% survival data, the following order of cytotoxicity was observed: MNNG>ENNG>MNU>ENU>BNU. This is the same order of potency as observed for

mutation induction per unit concentration of mutagen (Table V) [6,7]. (T-V)

Mutagenicity of heterocyclic nitrogen mustards (ICR compounds). The six compounds chosen (ICR-191,-170,-292,-372,-191-OH, and -170-OH) contain structural similarities and differences which allow the roles of both the heterocyclic nucleus and the alkylating side chain in the mutagenic activity to be determined. The first four contain a single two-chloroethyl group (nitrogen half-mustard) on the side chain and are mutagenic, with the tertiary amine types (170 and 292) 3 to 5 times more mutagenic than the secondary amine types (191 and 372). The two remaining compounds (191-OH and 170-OH) are not mutagenic, indicating that the two-chloroethyl group is needed for mutation induction [29,30].

Mutagenicity of 79 physical and chemical agents: Alkylating chemicals, ICR compounds, metallic compounds, polycyclic hydrocarbons, nitrosamines, miscellaneous organic chemicals, X-ray, UV light, and other nonionizing agents

So far mutagenicity has been studied in 79 physical and chemical agents, 42 of which have documented carcinogenicity or noncarcinogenicity [19,41]. Included in this study were several classes of chemicals such as polycyclic hydrocarbons and nitrosamines which require coupling of metabolic activation systems to be mutagenic and cytotoxic. As a means of validating the CHO/HGPRT assay as a prescreen for carcinogens, several noncarcinogenic structural analogues of carcinogens were also included. Most of the mutagenicity studies were conducted at cell survivals ranging from 100 to 10%, and some to 1%. Several points can be made:

Alkylating agents and related compounds (total of eleven). Included in the mutagenicity study are two alkylsulfates, three alkanesulfonates, two nitrosamidines,

three nitrosamides, and N-methyl-N'-nitroguanidine. All ten alkylating agents (Tables IV and V) are carcinogens and are highly mutagenic. The fact that the carcinogen MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) is mutagenic and the structural analogue N-methyl-N'-nitroguanidine is neither carcinogenic nor mutagenic implies that nitrosation is required for both mutagenicity and carcinogenicity.

Heterocyclic nitrogen mustards — ICR compounds (total of six). Two ICR compounds (170 and 292) are carcinogens [32] and are highly mutagenic. Noncarcinogenic ICR-191 [32] is an active mutagen in the CHO/HGPRT system and was shown to be a powerful frameshift mutagen in the Salmonella mutation system [2,25,26].

Metallic compounds (total of eight). The mutagenicity of four carcinogenic metals (Ni, Be, Cd, and Co salts) can be demonstrated in this system. The CHO/HGPRT system appears to be useful for studying mutagenesis of metallic compounds whose mutagenicity was not detectable in the Salmonella system [2,25,26].

Polycyclic hydrocarbons (total of 27). We 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 and pyrene were added to this study. Also included were benz(a)anthracene (BA) and four related compounds (7,12-dimethyl BA, anthracene, and two phenolic derivatives of BA). The carcinogenic polycyclic hydrocarbons (B(a)P, BA, and 7,12-dimethyl BA) require metabolic activation to be mutagenic. The noncarcinogenic polycyclic hydrocarbons pyrene and anthracene are nonmutagenic even with metabolic activation. 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 the 4,5-epoxide of B(a)P [11].

Nitrosamines and related compounds (total of ten). All four carcinogenic nitrosamines (dimethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine, and 2-methyl nitropiperidine) also require metabolic activation to be mutagenic, while noncarcinogenic ones (2,5-dimethyl nitrosopyrrolidine and 2,5-dimethylpiperidine) are not mutagenic in this system. The requirement of nitrosation for both mutagenicity and carcinogenicity can be demonstrated from the observations that carcinogenic dimethylnitrosamine is mutagenic with activation, while dimethylamine is not mutagenic irrespective of activation. Formaldehyde, a metabolite of dimethylnitrosamine, is carcinogenic in some systems but not in others [27]; its mutagenicity is not evident in this system. Neither nitrate nor nitrite appears to be mutagenic [38].

Miscellaneous compounds (total of ten). Three commonly used organic solvents (acetone, dimethylsulfoxide, and ethanol) are noncarcinogens and do not appear to be mutagenic. All four metabolic inhibitors (cytosine arabinoside, hydroxyurea, caffeine, and cycloheximide) are nonmutagenic in a preliminary study without the addition of S_9. Hydrazine and hycanthone appear to be direct-acting mutagens because they are mutagenic without S_9. $N^6, O^{2'}$-dibutyryl adenosine 3':5'-phosphate, an analogue of adenosine 3':5'-phosphate, an inportant effector of growth and differentiation in many biological systems, is not mutagenic [15].

Physical agents (total of seven). The mutagenicity of both ionizing radiation such as X-ray [29] and nonionizing physical agents such as UV light [14] can be demonstrated. Fluorescent white, black, and blue lights were slightly lethal and mutagenic [18]. Sunlamp light was highly lethal and mutagenic, exhibiting the biological effects within 15 s of exposure under conditions recommended by the

manufacturer for human use [16]. Lethal and mutagenic effects were observed after 5 min of sunlight exposure (Fig. 6); responses varied with hourly and daily variations in solar radiation (Table VI) [16]. In view of man's constant exposure to and use of various light sources, demonstration of their genetic toxicity indicates that daily exposure to these light sources, especially sunlight should be minimized. The demonstration that the CHO/HGPRT system is capable of quantifying the lethal 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 man and the environment.

F-6

T-VI

Preliminary validation of the CHO/HGPRT assay in predicting chemical carcinogenicity

Mutagenicity in the CHO/HGPRT assay of 42 chemicals (whose carcinogenicity or noncarcinogenicity has been demonstrated [19,41]) correlated well [40/42 (95.24%)] with the reported carcinogenicity in the animal tests (Table VII). The existence of such a good correlation 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 only limited classes of chemicals have been tested so far.

T-VII

A possible false negative was shown for formaldehyde, which has been shown to be either carcinogenic or noncarcinogenic depending on the means of exposure to the test animals [27]. Apparently, a false positive was ICR-191, a well-known potent mutagen in Salmonella [2,25,26], which has been shown to be noncarcinogenic in a recent study [32].

Concluding remarks

We feel that we have now come close to optimizing conditions necessary for quantifying mutation induction to TG resistance in the CHO/HGPRT system, which selects for >98% mutants deficient in the activity of HGPRT specified by a gene on the X chromosome, in a near-diploid CHO cell line. In all chemical mutagens studied in the CHO/HGPRT system, mutation induction increases linearly as a function of the concentration without an apparent threshold effect. In contrast to the linear dose response of EMS-induced mutation in the diploid cells, EMS failed to induce mutations in near-tetraploid CHO cells, consistent with the notion that induced mutation occurs at the gene or chromosomal level. As an example of dosimetry studies, EMS produced 310×10^{-6} mutants (cell mg $ml^{-1}h)^{-1}$. The sensitive and quantitative nature of this assay has been utilized to study the structure—activity (mutagenicity) relationship of chemicals.

In addition to being capable of determining mutagenicity of direct-acting agents, the system can detect mutagenicity of procarcinogens by coupling with a standard microsome activation system or through a host (BALB/c mouse)-mediated assay. Thus far, mutagenicity has been determined in diversified ionizing and nonionizing physical agents such as X-ray, UV light, sunlight, etc., as well as in various chemicals, including alkylating agents, ICR compounds, polycyclic hydrocarbons, nitrosamines, metallic compounds, etc.; a total of 79 were examined, of which 43 have documented carcinogenicity or noncarcinogenicity. Mutagenicity as determined in the CHO/HGPRT assay appears to correlate well [40/42 (95.24%)] with the reported carcinogenicity in animals within these limited chemical groups.

To ensure that the determination of mutagenicity using the CHO/HGPRT system can be employed as a valid prescreen for environmental carcinogens, we have extended our studies to include various other major groups of experimental chemical carcinogens and their structurally related noncarcinogenic congeners [20,26,33]. We believe the results of this validation study should give a further assessment of the utility of the CHO/HGPRT system in both experimental mutagenesis and genetic toxicology.

References

1 Alford, B. L. and E. M. Barnes, Hypoxanthine transport by cultured Chinese hamster lung fibroblasts, J. Biol. Chem., 251 (1976) 4823–4827.

2 Ames, B. N., J. McCann and E. Yamasaki, Methods for detecting carcinogens and mutagens with Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31 (1975) 347–364.

3 Chasin, L. A., The effect of ploidy on chemical mutagenesis in cultured Chinese hamster cells, J. Cell. Physiol., 82 (1973) 299–308.

4 Chu, E. H. Y. and H. V. Malling, Chemical induction of specific locus mutations in Chinese hamster cells in vitro, Proc. Natl. Acad. Sci. U.S.A., 61 (1968) 1306–1312.

5 Chu, E. H. Y. and S. S. Powell, Selective systems in somatic cell genetics, Adv. Hum. Genet., 7 (1976) 189–258.

6 Couch, D. B. and A. W. Hsie, Mutagenicity and cytotoxicity of congeners of two classes of nitroso compounds in Chinese hamster ovary cells, submitted to Mutation Res.

7 Couch, D. B. and A. W. Hsie, Dose-response relationships of cytotoxicity and mutagenicity of monofunctional alkylating agents in Chinese hamster ovary cells, Mutation Res. 38 (1976) 399 (abstr).

8 Couch, D. B., N. L. Forbes and A. W. Hsie, Comparative mutagenicity of alkylsulfate and alkanesulfonate derivatives in Chinese hamster ovary cells, submitted to Mutation Res.

9 Deaven, L. and D. Peterson, The chromosome of CHO, an aneuploid Chinese hamster cell line: G-band, C-band and autoradiographic analysis, Chromosoma, 41 (1973) 129–144.

10 Gabridge, M. G. and M. S. Legator, A host-mediated assay for the detection of mutagenic compounds, Proc. Soc. Exp. Biol. Med., 130 (1969) 831–834.

11 Hsie, A. W. and P. A. Brimer, unpublished results.

12 Hsie, A. W., P. A. Brimer, R. Machanoff and M. H. Hsie, Further evidence for the genetic origin of mutations in mammalian somatic cells: The effects of ploidy level and selection stringency on dose-dependent chemical mutagenesis to purine analogue resistance in Chinese hamster ovary cells, Mutation Res. (in press, 1977).

13 Hsie, A. W., P. A. Brimer, T. J. Mitchell and D. G. Gosslee, The dose-response relationship for ethyl methanesulfonate-induced mutations at the hypoxanthine–guanine phosphoribosyl transferase locus in Chinese hamster ovary cells, Somat. Cell Genet., 1 (1975) 247–261.

14 Hsie, A. W., P. A. Brimer, T. J. Mitchell and D. G. Gosslee, The dose-response relationship for ultraviolet light-induced mutations at the hypoxanthine–guanine phosphoribosyl transferase locus in Chinese hamster ovary cells, Somat. Cell Genet., 1 (1975) 383-389.

15 Hsie, A. W., D. B. Couch and P. A. Brimer, unpublished results.

16 Hsie, A. W., A. P. Li and R. Machanoff, A fluence-response study of lethality and mutagenicity of white, black, and blue fluorescent lights, sunlamp, and sunlight irradiation in Chinese hamster ovary cells, Mutation Res. (in press, 1977).

17 Hsie, A. W., R. Machanoff, D. B. Couch and J. M. Holland, Mutagenicity of dimethylnitrosamine as studied by a quantitative host-mediated specific-locus mutation assay in Chinese hamster ovary cells, submitted to Mutation Res.

18 Hsie, A. W., R. Machanoff, D. B. Couch and J. M. Holland, A quantitative host-mediated mutational assay of the hypoxanthine–guanine phosphoribosyl transferase locus in Chinese hamster ovary cells, Mutation Res. (abstr) (in press, 1977).

19 IARC, IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man. Vol. 1–10, IARC, Lyons, 1972–76.

20 Ishidate, M., Jr. and S.Odashima, Chromosome tests with 134 compounds on Chinese hamster cells in vitro: A screening for chemical carcinogens, Mutation Res., 48 (1977) 337–354.

21 Kao, F. T. and T. T. Puck, Induction and isolation of nutritional mutants in Chinese hamster cells, Proc. Natl. Acad. Sci. U.S.A., 60 (1968) 1275–1281.

22 Kao, F. T. and T. T. Puck, Quantitation of mutagenesis by physical and chemical agents. J. Cell. Physiol., 74 (1969) 245–260.

23 Krahn, D. F. and C. Heidelberger, Liver homogenate-mediated mutagenesis in Chinese hamster V79 cells by polycyclic aromatic hydrocarbons and aflatoxins, Mutation Res., 46 (1977) 27–44.

24 Lyon, M. F., Sex chromatin and gene action in the mammalian X chromosome, Am. J. Hum. Genet., 14 (1962) 135-148.

25 McCann, J.and B. N. Ames, Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals: Discussions. Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 950–954.

26 McCann, J., E. Choi, E. Yamasaki and B. N. Ames, Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 5135–5139.

27 Nettesheim, P., unpublished results.

28 O'Neill, J. P., P. A. Brimer, R. Machanoff, G. P. Hirsch and A. W. Hsie, A quantitative assay of mutation induction at the hypoxanthine–guanine phosphoribosyl transferase locus in Chinese hamster ovary cells: Development and definition of the system, Mutation Res. (in press, 1977).

29 O'Neill, J. P., D. B. Couch, R. Machanoff, J. R. San Sebastian, P. A. Brimer and A. W. Hsie, A quantitative assay of mutation induction at the hypoxanthine–guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Utilization with a variety of mutagenic agents, Mutation Res. (in press, 1977).

30 O'Neill, J. P., J. N. Fuscoe and A. W. Hsie, Mutagenicity of heterocyclic nitrogen mustards (ICR compounds) in cultured mammalian cells, submitted to Cancer Res.

31 O'Neill, J. P. and A. W. Hsie, Chemical mutagenesis of mammalian cells can be quantified, Nature (in press, 1977).

32 Peck, R. M., T. K. Tan and E. B. Peck, Pulmonary carcinogenesis by derivatives of polynuclear aromatic alkylating agents, Cancer Res., 36 (1976) 2423–2427.

33 Pienta, R. J., J. A. Poiley and W. B. Lebherz, Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable in vitro bio-assay for identifying diverse carcinogens, Int. J. Cancer, 19 (1977) 642–655.

34 Puck, T. T., The Mammalian Cell as a Microorganism, Holden-Day Inc., San Francisco, 1972.

35 Puck, T. T. and F. T. Kao, Treatment of 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants, Proc. Natl. Acad. Sci. U.S.A., 58 (1967) 1227-1234.

36 Roy-Burman, P., Analogues of nucleic acid components. Recent Results Cancer Res., 25 (1970) 9–35.

37 Russell, L. B., Genetics of mammalian sex chromosomes, Science, 133 (1961) 1795–1803.

38 San Sebastian, J. R., D. B. Couch and A. W. Hsie, unpublished results.

39 Sharp, J. D., N. E. Capeechi and M. R. Capeechi, Altered enzymes in drug resistant variants of mammalian tissue culture cells, Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 3145–3149.

40 Siminovitch, L., On the nature of heritable variation in cultured somatic cells, Cell, 7 (1976) 1–11.

41 USPHS, Survey of Compounds Which Have Been Tested for Carcinogenic Activity. USPHS Publication No. 149 (1972–73).

TABLE I

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 hydridization 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 abberation, and sister chromatid exchange in the same mutagen-treated cell culture.

TABLE II

CHO/HGPRT MUTATION ASSAY

(1) Enzyme system:

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

(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]hypoxantine into cellular macromolecules as revealed by either direct radioactivity measurement 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).

TABLE III

CHO/HGPRT MUTATION ASSAY: GENETIC BASIS OF MUTATION AT HGPRT LOCUS IN TG-RESISTANCE SELECTION [a]

(1) Spontaneous mutation frequency at $1-5 \times 10^{-6}$ 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 sensitive.

  (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] Data from (6-8, 11-18, 28-31 and 38).

TABLE IV

CYTOTOXICITY AND MUTAGENICITY OF ALKYL SULFATES AND ALKANESULFONATES[a]

Compound	Concentration (μM) to produce 10% survival	Mutation frequency (mutants/10^6 survivors)	
		At 10% survival	Per μM mutagen
DMS	89	92	1.0
DES	2760	780	0.3
MMS	95	140	1.5
EMS	3700	1550	0.4
iPMS	4540	435	0.1

[a]Data from [7, 8, 13, 31].

TABLE V

CYTOTOXICITY AND MUTAGENICITY OF NITROSOGUANIDINES AND NITROSOUREAS[a]

Compound	Concentration (μM) to produce 10% survival	Mutation frequency (mutants/10^6 survivors)	
		At 10% survival	Per μM mutagen
MNNG	0.34	200	590
ENNG	6.1	522	86
MNU	86	243	3
ENU	1200	550	0.5
BNU	2540	200	0.08

[a]Data from [6,7,28].

TABLE VI

LETHALITY AND MUTAGENICITY OF SUNLIGHT AT DIFFERENT TIMES OF DAY[a]

Time	Ambient temperature (°C)	Solar radiation ($g\ cal/cm^2\ min$)	Loss of relative survival (%)	Observed mutation frequency (TG mutants/10^6 cells)
6:00 a.m. (control)	17.2	3.8	0	5
6:45 a.m.	17.2	10.6	34	5
9:00 a.m.	21.7	55.1	55	12
12:30 p.m.	28.9	75.0	65	147
4:30 p.m.	30.6	37.1	40	32
6:30 p.m.	26.1	9.8	25	10

[a]Data from [16]. All experiments were performed on July 28, 1975, with an exposure time of 10 min, except the untreated control.

TABLE VII

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

Agent[c]	Concurrence[d]	False negatives[e]	False positives[f]
Alkylating agents and relatives	11/11 (100%)	0	0
ICR compounds	2/3 (66.7%)	0	1/3 (33.3%)
Metallic compounds and relatives	4/4 (100%)	0	0
Polycyclic hydrocarbons	6/6 (100%)	0	0
Nitrosamines and relatives	9/10 (90%)	1/10 (10%)	0
Miscellaneous agents	5/5 (100%)	0	0
Physical agents	3/3 (100%)	0	0
All agents	40/42 (95.24%)	1/42 (2.38%)	1/42 (2.38%)

[a]Agents studied are found to be either mutagenic, regardless of "mutagenic potency," or nonmutagenic. Since only limited experiments were performed on a small sample of each derivative of B(a)P, these results should be viewed as preliminary in nature; more studies on these compounds are under way. The mutagenicity is assayed either directly or coupled with a metabolic activation system in vitro or in vivo. In the S9-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. In the host-mediated assay, uninduced congenitally athymic BALB/c ("nude") mice were used. Equally reproducible results were found using BALB/c mice recently [17]. In either case, the effects of inducers for metabolic activation are yet to be determined.

Table VII (continued)

[b] Agents studied are denoted either as carcinogenic or noncarcinogenic or uncertain based primarily on published data from USPHS [41] and IARC [19] regardless of "carcinogenic potency." Carcinogenicity of many compounds is not yet available. The search for such data is admittedly neither exhaustive nor updated.

[c] The data are compiled from all agents studied, excluding those whose carcinogenicity is either unknown or uncertain. Thus, only 42 out of 79 agents studied are compiled in this table.

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

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

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

Figure legends

Fig. 1. Expression time of HGPRT- (TG-resistant) mutant phenotype. CHO cells (clone K_1-BH_4) were plated at 5×10^5 cells/100-mm plate in Ham's F12 medium containing 5% dialyzed fetal calf serum and were incubated at 37° C in 5% CO_2 in air in a 100% humidified incubator. After 24 h (cell no. = $1-1.5 \times 10^6$ cells/plate) either (A) EMS or (B) ICR-191 was added (time = 0), and the cultures were incubated for 16 h. The cultures were washed 3 times with saline, the cells were removed by trypsinization, and approximately $0.5-1.0 \times 10^6$ cells were subcultured every 48 h. Mutant selection was performed at day 0 and at 2-day intervals thereafter. For selection of the mutant phenotype, cells were plated at 2×10^5 cells/100-mm plate (5 plates = 10^6 cells) in hypoxanthine-free F12 medium containing 5% dialyzed fetal calf serum and 10 μM TG. For cloning efficiency, 200–1000 cells were plated in triplicate in the same medium without TG. After 7 days of growth, colonies were fixed, stained, and counted. Mutation frequency was calculated as the number of mutant colonies/10^6 cells (corrected for the cloning efficiency at the time of selection). (A) Cells treated with EMS concentrations (μg/ml) at : 0 (△), 50 (▲), 100 (□), 200 (■), or 400 (○) were determined as described. (B) Cells treated with ICR-191 concentrations (μg/ml) at: 0 (△), 0.005 (▲), 0.1 (□), 0.25 (■), and 0.5 (○) were determined as described. The average spontaneous mutation frequency was $2-3.6 \times 10^{-6}$. (From [28,29,31]).

Fig. 2. Effect of $HGPRT^+$ cell number on recovery of $HGPRT^-$ cells in TG medium. Relative cloning efficiency of $HGPRT^-$ cells is plotted vs the number of $HGPRT^+$ (wild-type) cells. $HGPRT^-$ cells (200) were plated in the presence of varying numbers of wild-type cells in TG medium on 100-mm plates. After 7 days the colonies were fixed, stained, and counted. The $HGPRT^-$ cells are MNNG-A, [○, absolute cloning efficiency = 0.88; HGPRT activity (% of wild type) = 9.4] and EMS 50-1 [●, cloning efficiency = 0.94; HGPRTase activity (% of wild type) = 5.4]. The arrow marks a cell number of 2×10^5 cells/100-mm plate. (From [28]).

Fig. 3. Lineweaver-Burke graph of CHO cell HGPRT activity. The reaction contained hypoxanthine over a concentration range of 0.5–10 μM, in the absence (△, ▲) or presence (○, ●) of 10 μM TG with 5 μg (open symbols) or 10 μg (closed symbols) cell extract protein. Velocity (v) is expressed in nmol/min per mg protein. From the intercepts, the apparent K_m for hypoxanthine is 2.5 μM and the apparent K_i for TG is 2.6 μM. (From [28].)

Fig. 4. Colony-forming ability and mutation induction of CHO-K_1-BH_4 cells treated with various EMS concentrations for 16 h under the standard conditions described in the text. The survival curve (○) was constructed from the pooled data of two separate experiments by fitting the data to the "multitarget model":

$$R_i = 1 - (1 - e^{-Bx_i})^N,$$

were B and N are fitted coefficients. The line (△) was constructed using weighted least-square analysis from the pooled data of two separate experiments. The straight-line fit of mutation frequency, $f(x)$, in mutants/10^6 survivors as a function of EMS concentration, x, in μg/ml is $f(x) = (8.73 + 3.45x) \times 10^{-6}$. The 95% confidence limits for the slope are 3.02×10^{-6}, 3.88×10^{-6}. (From [13].)

Fig. 5. Apparent dosimetry for EMS cellular lethality (A) and induced mutation frequency (B). Cells were treated with various concentrations (mg/ml) of EMS for 2 (△), 4 (▲), 8 (□), or 12 h (▩). After treatment the cultures were washed, and single-cell survival was determined after a total time of 24 h. Cells were plated for selection on day 9 of expression, as in Fig. 1. Spontaneous mutation frequency = 2.3×10^{-6}. In (A) the linear fit of the survival curve was found over 1.6–6.4 mg (of EMS)/ml^{-1} h through fitting to the "multitarget model." The extrapolation number, N is 1.74, with 95% confidence limits of 1.18, 2.58; the slope, B, is -0.248, with 95% confidence limits of -0.303, -0.188. In (B) the straight-line fit for the

pooled data on mutation induction over the dose range 0.64–6.4 mg (of EMS)/ml per h is $\underline{f}(\underline{x}) = (5.58 + 310.77\underline{x}) \times 10^{-6}$. (From [31].)

Fig. 6. Colony-forming ability (A) and mutation induction (B) of CHO-K_1-BH_4 cells as a function of sunlight exposure time on July 17, 1975. For analysis of the survival curve, the linear fit was determined over the exposure-time range of 12–60 min through fitting to the "multitarget model." The extrapolation number, $\underline{N}$, is 1.94, with 95% confidence limits of 1.15 and 3.27; the slope, $\underline{B}$, is -3.56×10^{-2}, with 95% confidence limits of -4.33×10^{-2} and -2.86×10^{-2}. For analysis of the mutation-induction curve, the linear fit was determined for an exposure period of 0–60 min. The straight-line fit is $\underline{f}(\underline{x}) = (1.29 + 6.99\underline{x}) \times 10^{-6}$. The 95% confidence limits for the slope are 5.26×10^{-6}, 8.73×10^{-6}. Nearly identical curves for cell survival and mutation induction were found in experiments performed on July 18, 1975, with weather conditions (furnished by the U.S. Department of Commerce Weather Bureau at Oak Ridge) almost identical to those of July 17, 1975. (From [16].)

32884-C-1

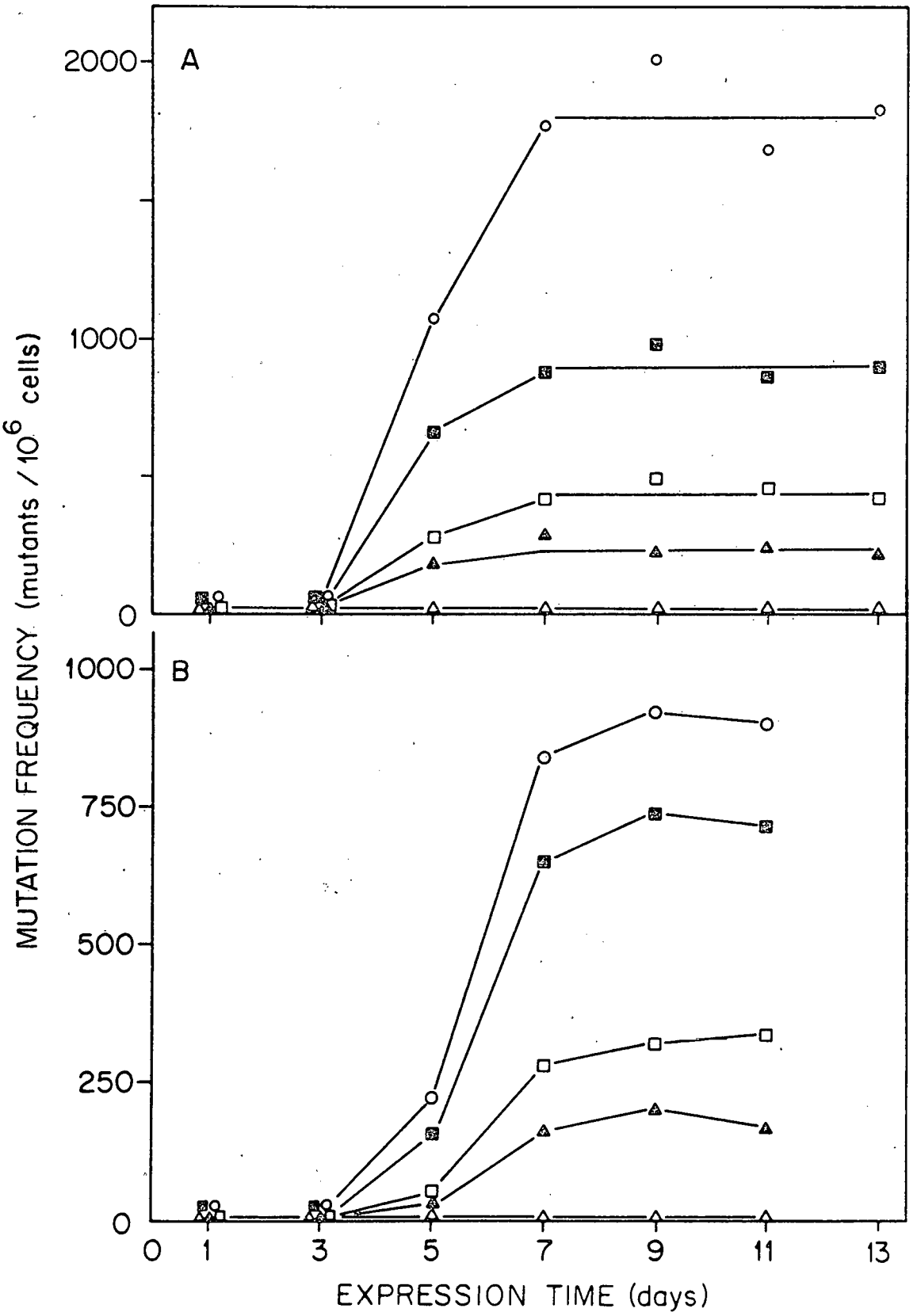
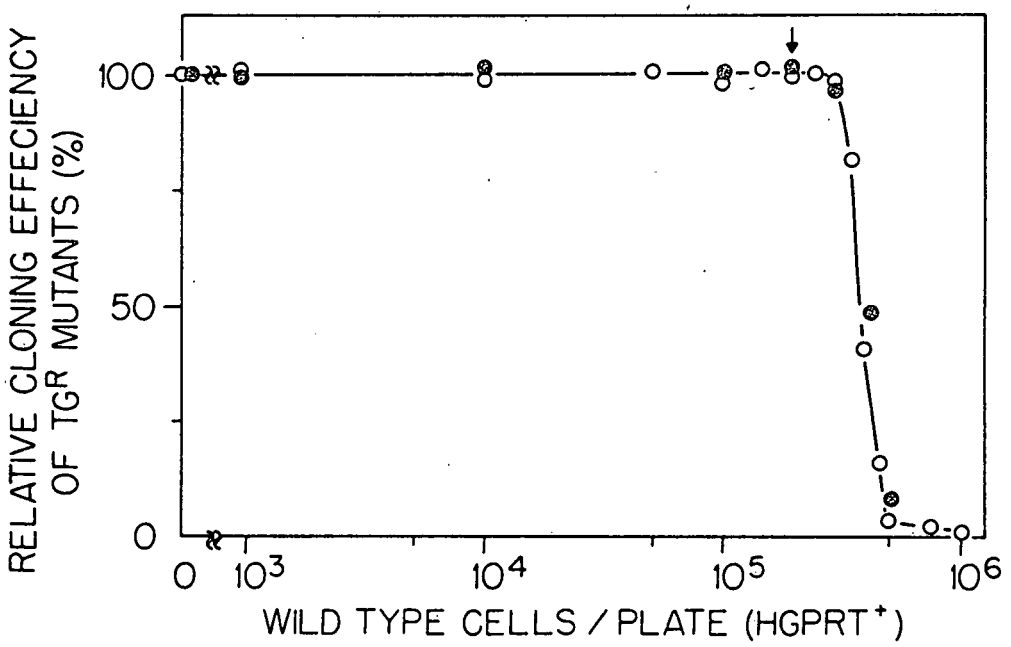


Fig 1

32668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OF TG^R MUTANTS (%)
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0
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10^4
10^5
10^6
WILD TYPE CELLS / PLATE (HGPRT^+)

Fig 2

32674

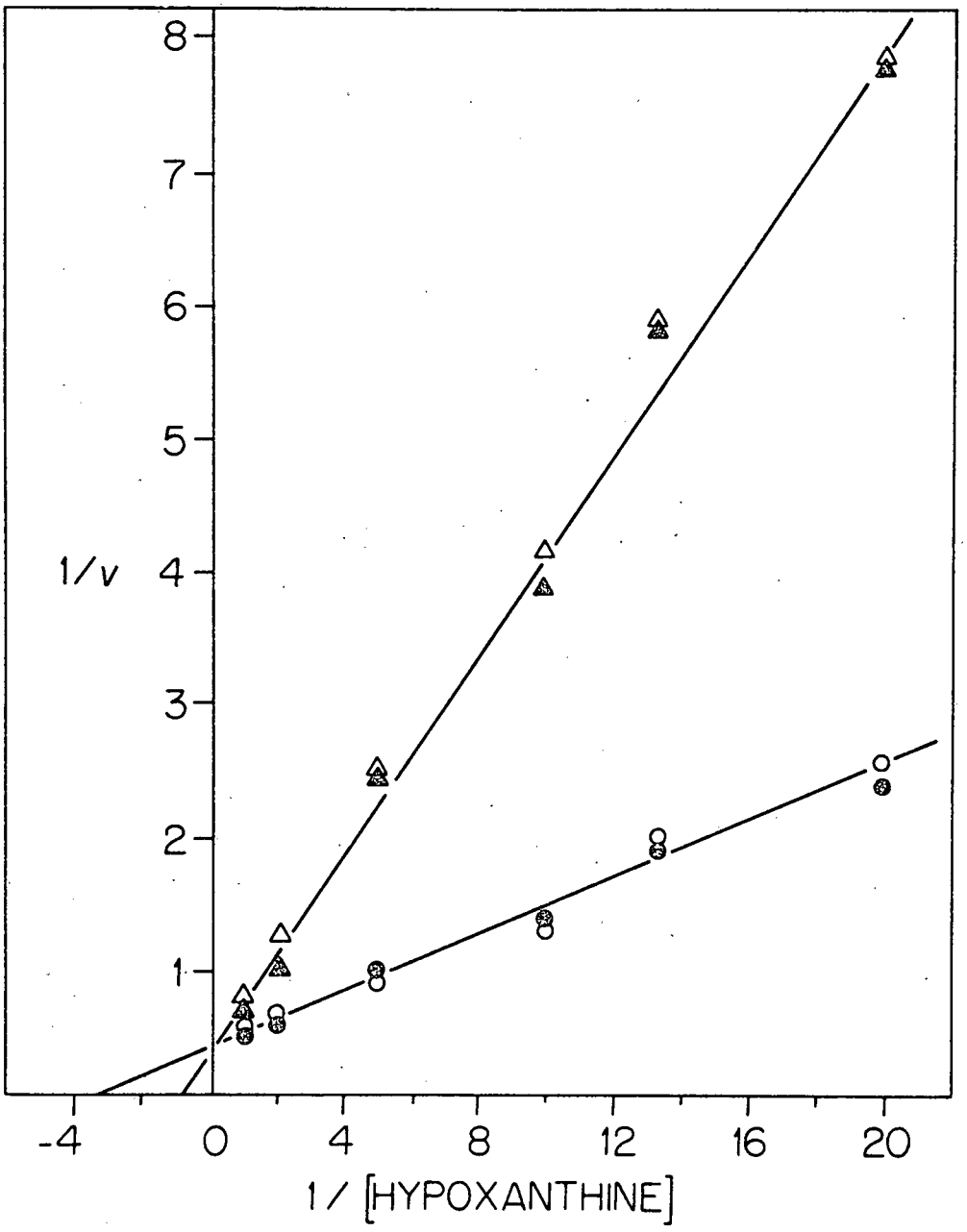
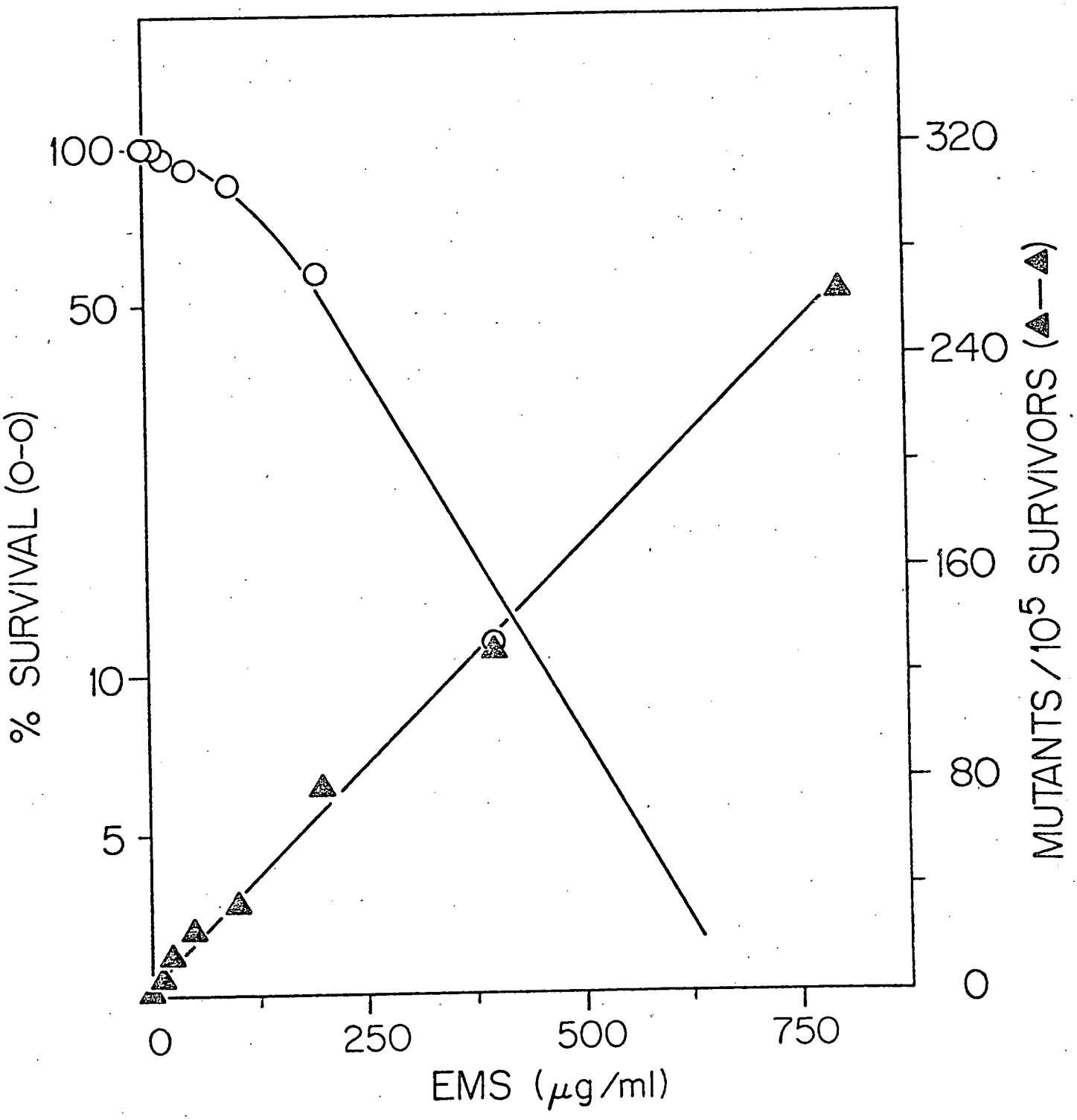


Fig. 3

31559-1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240
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80
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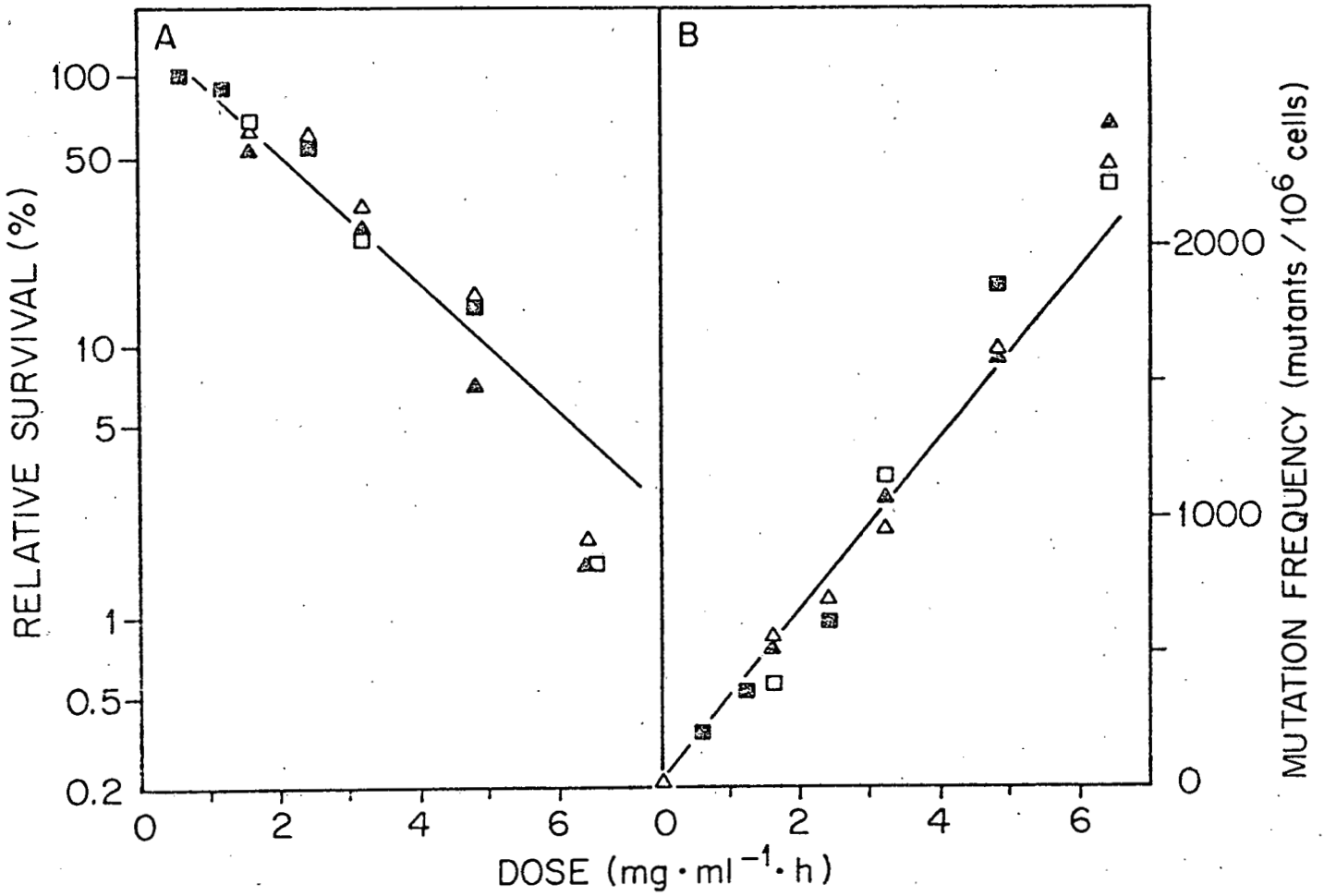
0
250
500
750
EMS (μg/ml)

Fig 4

32887-1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2
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6
0
2
4
6
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1000
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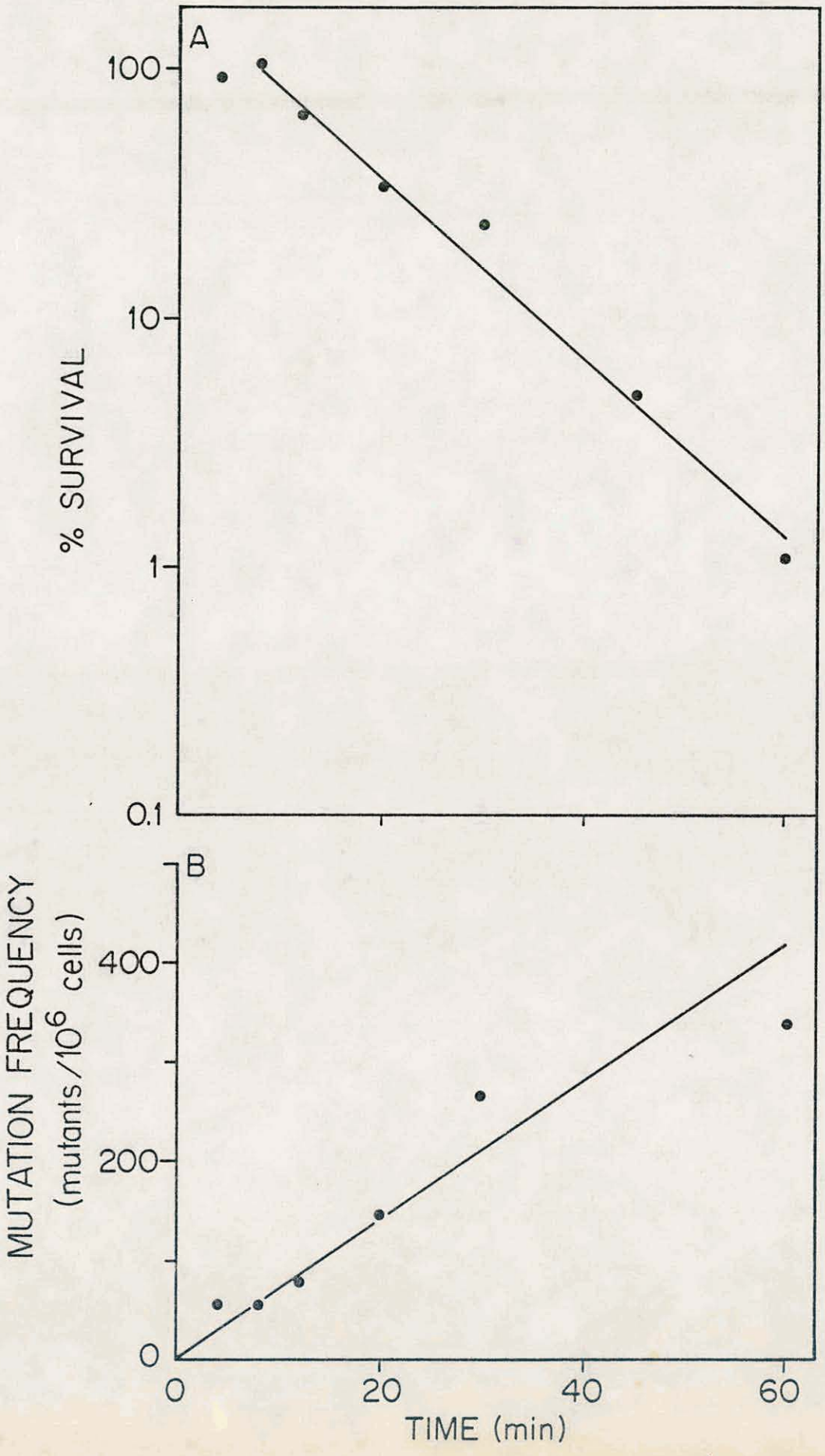
MUTATION FREQUENCY (mutants / 10^6 cells)
DOSE (mg · ml^{-1} · h)

Fig 5

32889-1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MUTATION FREQUENCY
(mutants/10^6 cells)
0
20
40
60
TIME (min)

Fig 6