

A mutagen-testing assay based on heterogeneity in diameter and integrated optical density of mammalian cell colonies

(Chinese hamster ovary cells/semisolid substrate/automated cell biology/toxicity testing)

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ABSTRACT We investigated the effects of the well-known mutagenic agents ethyl methanesulfonate (EtMes), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), and ICR-191 on colonies of the Chinese hamster ovary line CHO cultured on a semisolid substrate. These agents induced heterogeneity in diameter and integrated optical density of colonies as determined by computer-assisted photography and subsequent analysis of the images of the colonies. When CHO colonies were exposed to agents such as urethane that are not known to be mutagenic in mammalian systems or to activation-requiring mutagens such as cyclophosphamide, there was no noticeable effect on the distribution of colony diameter and volume. Similarly, nonmutagenic agents such as dimethyl sulfoxide (Me₂SO) also did not induce heterogeneity in colony diameter and integrated optical density. Our observations recommend the use of agar-grown mammalian cell colonies for predictive testing of chemical mutagens and carcinogens in a simple, *in vitro* mammalian cell assay. This assay system, unlike other mammalian cell culture assays, allows detection and measurement of the simultaneous effects of chemical mutagens on several genetic and non-genetic targets and, thus, may emulate more closely the potential hazards of these agents *in vivo*.

Over the last few years, our laboratory has been engaged in the study of three-dimensional structures developed *in vitro* by cells of the Chinese hamster ovary cell line CHO on semisolid substrates such as agar. We have found that this culture system is extremely sensitive to small changes in growth medium, such as addition of very low doses of growth hormones and drugs that do not produce observable effects when using conventional culture methods on solid substrates or in suspension (1, 2).

Simultaneously, for the last few years, there has been a considerable movement for adopting alternatives to whole animals in the field of toxicity testing. The *in vitro* techniques that have been developed in response to this movement for screening mutagens and carcinogens have been of considerable use in genotoxic screening. The Ames test (3), based on the mutagenicity of the test substance in *Salmonella*, is the most extensively used *in vitro* assay. Similarly, the detection of forward mutations at specific loci in cultured mammalian cells appears to be a promising tool for the evaluation of potentially hazardous chemicals responsible for genetic damage and, thus, causal in carcinogenesis (4, 5).

Mutagens and carcinogens are generally known to be quite reactive and are able to attack a large variety of molecules and organelles within the cell. However, the most widely used *in vitro* toxicity assays are focused upon a single target, DNA. Colony formation on agar is a complex developmental phenomenon that involves cell-cell interaction, cell-substratum interaction, surface tension, cell movement, and several other factors. Therefore, we expected that this sys-

tem would be considerably more vulnerable and, hence, sensitive to the effects of potentially hazardous chemicals compared with a system scored only for a few specific gene products.

The agar-grown CHO colony assay described in this report is insufficient by itself for testing toxic agents requiring metabolic activation because of the lack of activating enzymes (6). However, because of the continuing effort in this field toward the development of mammalian homologs to the Ames test, methods have been developed that make use of exogenously supplied activating enzymes in a test system lacking such enzymes but suitable by other criteria (6).

The data reported in this study on the sensitivity of agar-grown CHO colonies to mutagens and carcinogens suggest the usefulness of this system in predictive toxicity testing for potentially hazardous agents.

MATERIALS AND METHODS

Cell Culture. All of these studies were carried out with CHO cells designated CHOSCI. This is a stable clone in terms of colony morphology and size and has been described in detail by Konrad *et al.* (7). Cells were routinely maintained in monolayer culture in α minimal essential medium containing 10% fetal calf serum in a humidified incubator at 37°C in 7.5% CO₂/92.5% air. All cultures were routinely screened for mycoplasma contamination by the Hoechst DNA stain and were found to be free of mycoplasma.

Mutagen Treatment. A freshly thawed vial was used for each experiment. Logarithmically dividing cultures were trypsinized and seeded in replicate with 5×10^5 cells per 25-ml T flask (Corning). Twenty-four hours later, fresh medium supplemented with various concentrations of ethyl methanesulfonate (EtMes), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), ICR 191, cyclophosphamide, or urethane was added to the cultures and allowed to incubate for 18 hr. Cultures were then washed three times with serum-free medium and allowed to recover and stabilize for 7 days before being used for agar studies. In the studies involving the effects of dimethyl sulfoxide (Me₂SO) on CHO colonies on agar, the experimental details on drug exposure and subsequent analysis of colonies are as described (2).

Phenotypic Expression. Mutagen-treated cultures were expanded as monolayers during a 7-day recovery period. Single cells were then inoculated for clonal growth on a 0.5% Noble agar gel supplemented with 10% fetal calf serum in α minimal essential medium in 100-mm Petri dishes by means of "Cyclops," a computer-assisted device for inoculation and dispensation of chemicals, using streams of electrostatically controlled liquid droplets, and for time-lapse photography of growing colonies. The spacing of droplets carrying single cells was adjusted to yield ≈ 100 colonies per dish.

Abbreviations: EtMes, ethyl methanesulfonate; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; Me₂SO, dimethyl sulfoxide.

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Incubation of dishes was carried out at 37°C for 20 days in 7.5% CO₂/92.5% air at 100% relative humidity.

Computer-Assisted Photography and Data Collection. Colonies were photographed 20 days after the initial inoculation of single cells by using the Cyclops photography setup for dark-field photography. The colonies were photographed by scattered light with Kodak SO-115, a fine-grain, high-resolution film. The film was developed in a Jamieson automatic film processor with Kodak Versamat 641 developer to achieve a contrast index of about 2. Details of photometry, film densitometry, and the software requirements for analysis of colony size and mass are as described (8). In essence, in order to determine the colony diameter and integrated optical density, the image of each colony is scanned with a flying spot scanner, which acts as an optical microdensitometer. In conjunction with a PDP-KL10 computer, the data obtained with the scanner can provide quantitative detail for a large number of colonies, thus allowing a statistically meaningful interpretation of the results.

RESULTS AND DISCUSSION

Effects of EtMes, MNNG, or ICR 191 Treatment of CHO Cells on Colony Diameter and Integrated Optical Density. CHO cells were treated for 18 hr with the alkylating agent EtMes at 50–200 µg/ml or MNNG at 0.3–1 µg/ml or with the nitrogen mustard ICR-191 at 0.3–1.5 µg/ml 1 week before plating on a 0.5% Noble agar gel. All three compounds have been found to be potent mutagens and carcinogens by a variety of screening systems (6, 9–11). We investigated the effects of these agents in an *in vitro* mammalian cell assay, the agar-grown CHO colony assay, which has been shown to be extremely sensitive to growth effectors (1, 2). We demonstrated that a one-time treatment of CHO cells with these agents followed by a 7-day recovery period to return the cultures to the original growth rate and eventual plating on agar results in the expression of heterogeneity in colony diameter and integrated optical density in treated cultures that is absent in the controls. In other words, the majority of the colonies in the control population fall within a narrow size range, whereas mutagen-treated cells give rise to colonies that are often larger or smaller than those of the control population. Fig. 1 illustrates the mutagen dose-dependent increase in heterogeneity in CHO colony diameter. In other words, the relative homogeneity of the control population, which is evident as a peak in the medium-size range, can be seen in Fig. 1 *a*, *e*, and *i*. Lower concentrations of mutagens perturb the distribution to a much smaller extent than do the higher concentrations, demonstrating a specific, dose-dependent response. The colonies represented in these histograms were approximately circularly symmetric and increased in thickness monotonically from edge to center as described by Konrad *et al.* (8) and Couch *et al.* (12). Colonies with asymmetrical shapes due to contact with adjacent colonies, etc., were not included in the analysis because their "real profile" could not be measured. Attempts to measure heterogeneity in colony diameter can also be made on colonies propagated on a solid substratum such as plastic or glass. However, previous reports from this laboratory have shown that a semi-solid substratum such as agar is much more effective in demonstrating low dose sensitivity and variant response (1, 2).

A mutagen dose-dependent increase in heterogeneity was also observed with regard to another colony characteristic, namely the integrated optical density of the image of a colony on black and white film in the Cyclops dark-field photographic system. It is closely related to the total light scattered by a colony during photography and has previously been shown to be proportional to the total number of cells in the colony (8). The effects of various doses of MNNG on the integrated optical density of CHO colonies are shown in Fig. 2. A similar dose-dependent increase in the heterogeneity of

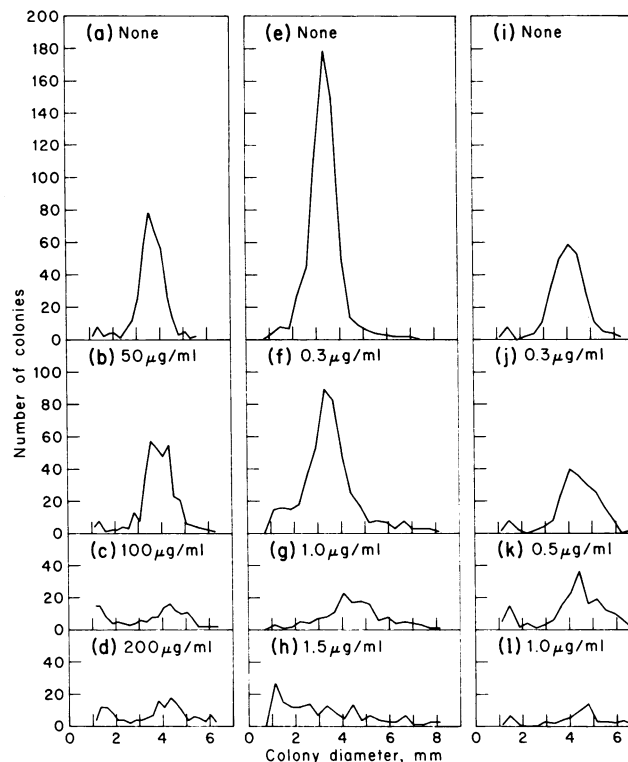


FIG. 1. Histogram representation of the effects of mutagen treatment on the diameter of 20-day-old CHO colonies growing on agar. CHO cells were treated with mutagens, allowed to recover and to propagate in the absence of mutagen for 7 days, and then plated on a minimal essential medium containing 0.5% Noble agar and 10% fetal calf serum. (*a–d*) Effects of EtMes. Curves represent data on 1143 colonies. (*e–h*) Effects of MNNG. Curves represent data on 2183 colonies. (*i–l*) Effects of ICR-191. Curves represent data on 884 colonies. All data represent those colonies that were acceptable for image analysis on the basis of circularity and nonoverlap parameters described by Konrad *et al.* (8).

the colony integrated optical density also was induced by EtMes and ICR-191.

It is interesting to note that the ratio of mean sizes of large and small colonies (about 5 mm for large colonies and 2.5 mm for small colonies) is much larger than the ratio of integrated optical densities of the two classes of colonies (about 193 units for large colonies and 185 units for small colonies). Because integrated optical density is roughly proportional to cell number, we conclude that cell number (or mean cell division time) is less sensitive to mutagens than is colony diameter. Colony diameter is influenced by many factors in addition to cell number, including cell size, cell–cell adhesion, cell–substratum adhesion, colony surface-tension effects, competition for utilization of nutrients, and cell motility. Since colony diameter is sensitive to all of these factors in addition to effective cell-division time, it is plausible that it should be more sensitive to mutagens than is cell number alone (as measured, for instance, by integrated optical density).

Mutagen treatment and subsequent occurrence of small colony variants in terms of diameter and integrated optical density could be interpreted as being due to mutagen-related cytotoxicity. The 7-day recovery period allowed after an 18-hr mutagen treatment apparently returned cultures to the original growth rate and, therefore, was sufficient to eliminate such cytotoxic effects. Thus, we interpret the appearance of small colonies to be the result of combined genetic and epigenetic effects. Furthermore, the increased frequency of larger colonies with increasing mutagen dosage also

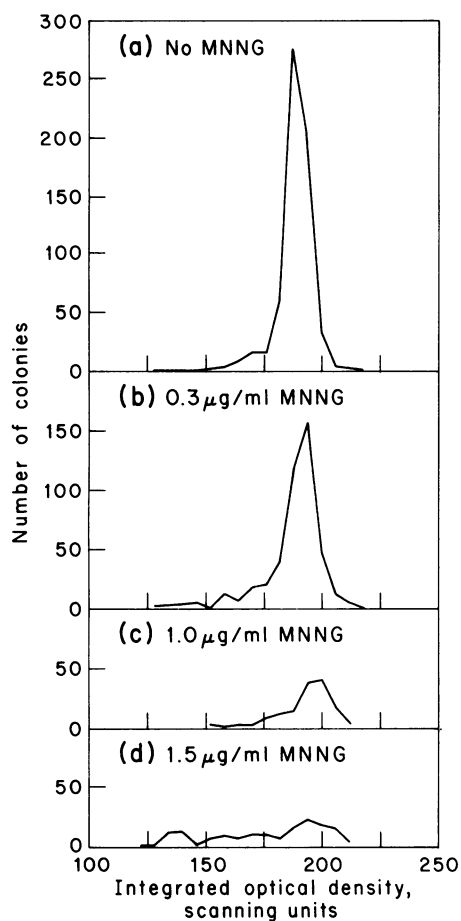


FIG. 2. Histogram representation of the effect of MNNG treatment on the integrated optical density of 20-day-old CHO colonies growing on agar. The distribution of colony diameter of this population is illustrated in Fig. 1 *e-h*. (a) Control, no MNNG. (b-d) Effect of increasing concentrations of MNNG.

supports this interpretation of the results.

In order to compare EtMes, MNNG, and ICR-191 in terms of the extent of induction of heterogeneity by each agent, we calculated the area under the major peak between the diameters of 2.5 and 5 mm in the control populations shown in Fig. 1 *a, e*, and *i*. This range of diameters represents a predominantly medium-sized population. The area of the histogram to the left of this region represents small colonies, whereas the area to the right represents large colonies. MNNG appears to be the most potent agent in inducing heterogeneity in colony diameter because our calculations in Table 1 demonstrate that, with this agent, the medium-sized population

Table 1. Distribution* of colony diameter in the presence of MNNG, ICR-191, and EtMes

Mutagen	Dose, $\mu\text{g/ml}$	% of colonies		
		Small (<2.5 mm)	Medium (2.5-5 mm)	Large (>5 mm)
MNNG	0	8.9	86.3	4.7
	0.3	17.6	72.7	9.5
	1.5	43.6	36.4	19.8
ICR-191	0	4.3	86.1	9.4
	0.3	8.4	68.0	23.5
	1.0	13.8	65.5	20.5
EtMes	0	3.8	94.4	1.7
	50	6.0	87.2	6.6
	200	35.0	52.0	12.8

*Data represent measurements on ≈ 4000 colonies.

drops from 86% of the total in the controls to 36% of the total at the highest dose of 1.5 $\mu\text{g/ml}$. With ICR-191, the medium-sized colonies decreased from 86% in the controls to 65% of the total at the highest dose of 1 $\mu\text{g/ml}$. EtMes appears to be relatively less effective because the medium-sized colony count dropped from 94% in the control to 52% of the total at 200 $\mu\text{g/ml}$, a concentration 200 times higher than the highest ICR-191 dose used in these experiments. Other investigators have determined the number of mutants generated at the hypoxanthine phosphoribosyltransferase locus by these three mutagens, and an analysis of their data demonstrates the same order of potency as we have found in our assay system, which depends on the effects of these agents on colony characteristics. In other words, MNNG generates the most mutants, EtMes, the least number of mutants, and ICR-191, an intermediate number of mutants at equimolar doses of all three mutagens under comparable conditions (13). However, a comparison of the sensitivities of the two methods to the presence of mutagens favors the agar-grown CHO colony assay. For example, the number of mutants generated at the hypoxanthine phosphoribosyltransferase locus by EtMes at 200 $\mu\text{g/ml}$ is 0.08% of clonable cells (14). At the same EtMes concentration, in our assay system, we find that 42% of clonable cells relative to control cells have deviated from the medium-sized colony norm, thus indicating an appreciable advantage in sensitivity (Table 1). Similar conclusions can be reached for MNNG and ICR-191. More sophisticated statistical criteria of change in the histograms describing colony characteristics would show even greater sensitivity than the crude measure used in Table 1.

Since these results reflect only phenotypic measurements, they could be a consequence of a large variety of DNA sequence changes, including point mutations, deletions, frame shifts, and rearrangements. They also could result from other mechanisms for moderately stable changes, sometimes called epigenetic events. All such mechanisms could contribute collectively to the high sensitivity of this assay method.

Heritability of Colony Size and Integrated Optical Density in Subclones of Mutagenized Cultures. In order to test the nature of the changes brought about by a mutagen, resulting in a certain colony diameter and integrated optical density phenotype, we plucked colonies from agar of either a small diameter (<1 mm) or a large diameter (≈ 5 mm). These colonies were dispersed into one pooled single-cell suspension derived from 10 small colonies and one derived from 10 large colonies and replated on agar. Cells from the large colonies gave rise to predominantly large colonies, and cells from small colonies generated mostly small colonies. Measurements on the colony integrated optical density and diameters of populations derived from the large and small colonies are presented as histograms in Fig. 3. The two phenotypes appear to be quite stable, though some heterogeneity is apparent. Heterogeneity is well-known in cell culture. Clones from a common parental line can differ in morphology, growth properties, karyotype, antigens, tumor-forming capacity *in vivo*, and in transplantability (15-18). Colony parameters such as morphology can be stably inherited in some CHO clones, whereas in others heterogeneity develops (7). Such heterogeneity is probably controlled by both genetic and epigenetic events. An example demonstrating the close association of epigenetic changes and mutation has been reported by Harris and Collier (19) in the development of thymidine kinase-deficient mutants of V79 Chinese hamster cells by long-term culture in the presence of BrdUrd. For any mode of inheritance of colony characteristics and despite the presence of inherent heterogeneity, the CHO agar colony assay is particularly useful for studying potential mutagens and carcinogens, mainly because these agents introduce a much higher level of heterogeneity that can be readily detected in this assay system.

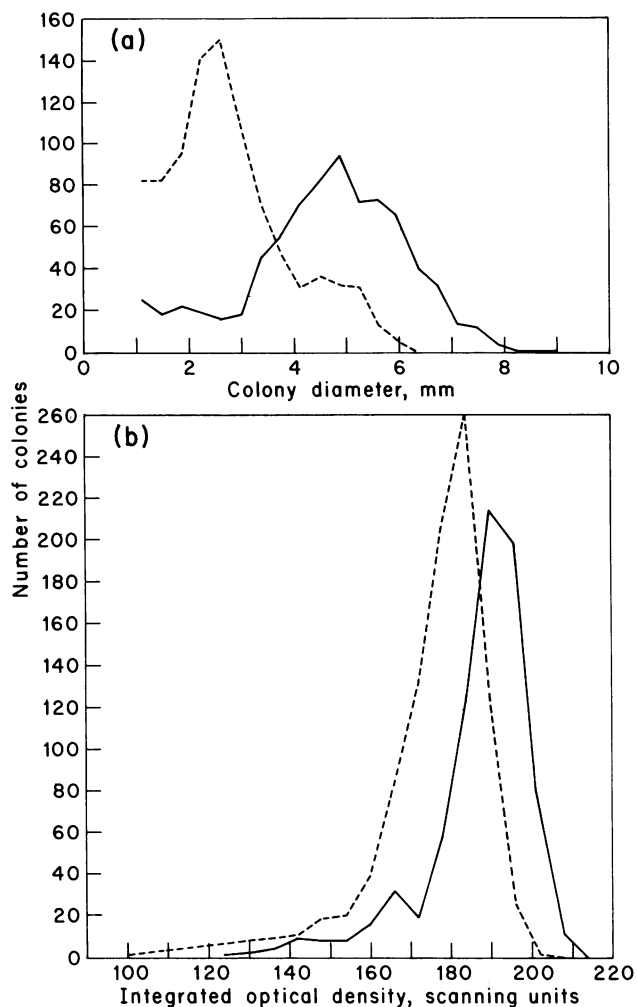


FIG. 3. Histogram representation of the distribution of colony diameter and integrated optical density of large and small subclones. Ten colonies of <1 mm or 10 colonies >5 mm were picked, pooled, and replated. (a) Colony diameter of 785 large colonies that resulted from replating the pool of 10 large colonies (—) and 928 small colonies that resulted from replating the pool of 10 small colonies (---). (b) Integrated optical density of colonies shown in a.

Effects of Cyclophosphamide and Urethane Treatment of CHO Cells on Colony Diameter and Integrated Optical Density. Logarithmically growing cultures of CHO cells were exposed to cyclophosphamide and urethane at various concentrations for 18 hr. Both of these agents have been tested frequently by others in a variety of short-term mutagenicity tests (11). Cyclophosphamide is an indirect carcinogen requiring metabolic conversion to the active mutagenic form. It has been found to be unequivocally positive in a large number of short-term tests (11). Our data on the effects of cyclophosphamide on CHO agar-grown colonies are shown in Fig. 4. At concentrations ranging from 0.1 to 10 mM cyclophosphamide, we have been unable to observe any deviation in colony diameter or integrated optical density from the untreated control population. This result was expected because CHO cells are known to lack the activation enzymes required for cyclophosphamide mutagenicity (6).

For a complete evaluation of the ability of predictive toxicity tests to discriminate between active and inactive compounds, it is required that an analysis of data be performed on noncarcinogens as well. The compound urethane has been designated as weak or negative in a total of 14 different toxicity tests. It has not yet been found to be positive in any known test (11). The effects of urethane on the agar-grown CHO colony diameter and integrated optical density are

shown in Fig. 4. As described above for cyclophosphamide, urethane at concentrations of 0.1 to 10 mM was ineffective in inducing noticeable heterogeneity. The data for untreated or cyclophosphamide- or urethane-treated cells are so similar that all the curves appear superimposed.

Effects of Me₂SO Treatment of CHO Cells on Colony Diameter and Integrated Optical Density. The effects of Me₂SO on agar-grown CHO colonies have been described in detail (2). It seems appropriate to present some of the data from the previous studies for the purpose of comparison with the effects of mutagens on specific colony parameters. Mutagens were applied as a one-time short exposure (18 hr), whereas Me₂SO was mixed in with the nutrient agar medium and cells were continuously kept in the presence of this compound. Increasing concentrations of Me₂SO did not induce appreciable heterogeneity in colony diameter or integrated optical density (Fig. 5), unlike the mutagens EtMe₃, MNNG, or ICR₁191. Instead, with 0.5% Me₂SO, there is a specific increase in colony diameter previously reported to be associated with specific biochemical changes (2). Increased diameter in these studies was not accompanied by increased integrated optical density, thus indicating that colonies were not larger in terms of the number of cells per colony but were flatter in the presence of Me₂SO. Larger doses of Me₂SO resulted in smaller colonies, but none of the Me₂SO effects were inherited as seen in subsequent experiments.

General Conclusions. Because all of the mechanisms by which chemical carcinogens induce cancer are not known, it

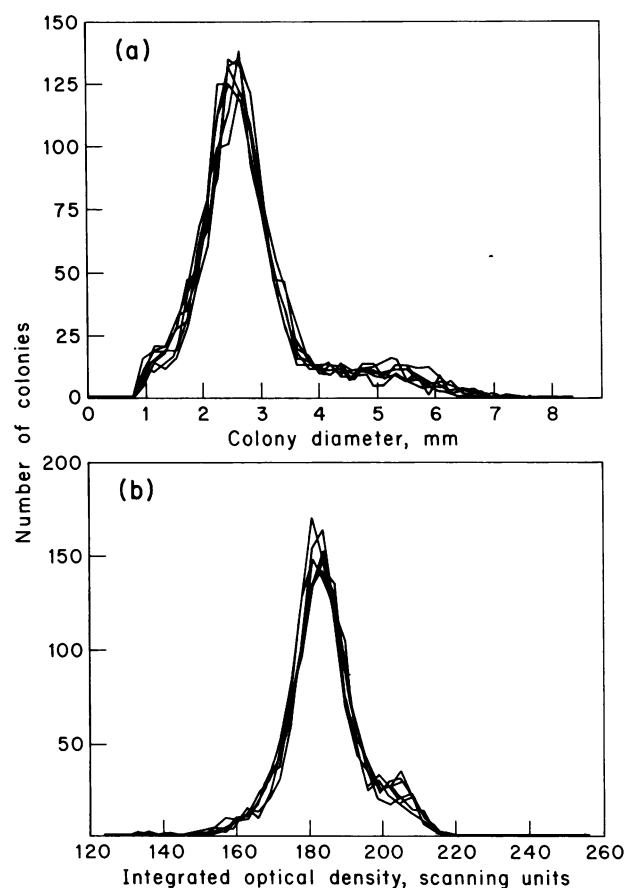


FIG. 4. Histogram representation of cyclophosphamide or urethane treatment on the diameter of 20-day-old CHO colonies on α minimal essential medium containing 0.5% Noble agar and 10% fetal calf serum (a). Curves represent 10,933 colonies. (b) Colony integrated optical density of the same population. Both compounds were used at concentrations of 0, 0.1, 1, or 10 mM. Note that for all doses of both compounds, experimental and control curves are identical.

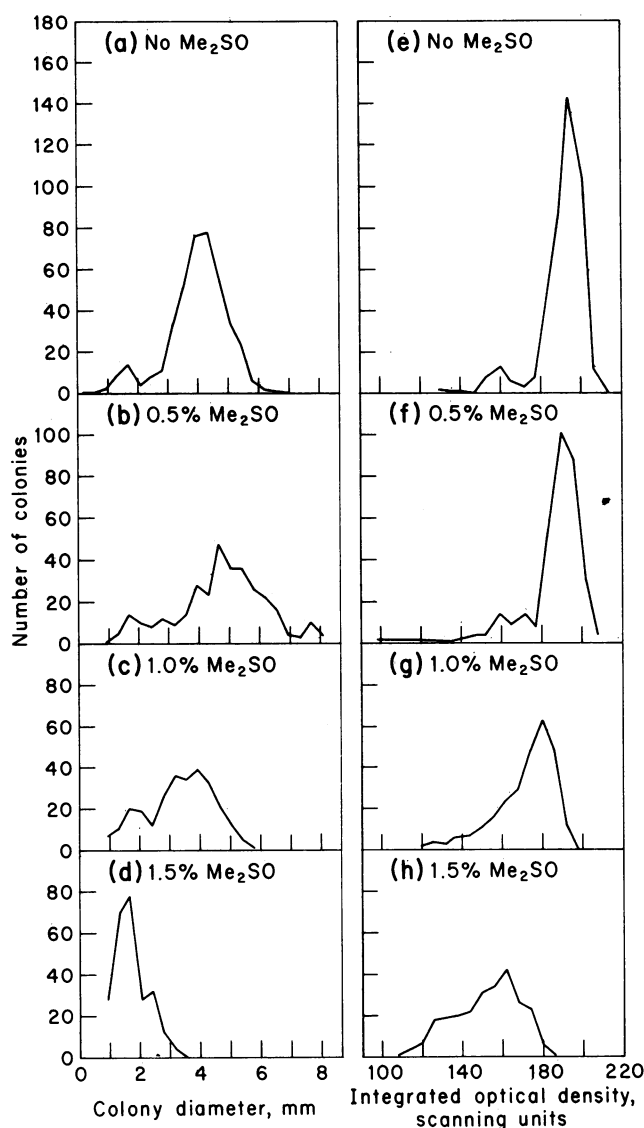


FIG. 5. Histogram representation of the effects of Me_2SO treatment on the diameter (a-d) and integrated optical density (e-h) of 20-day-old CHO colonies growing on agar. Me_2SO was mixed with the agar-containing growth medium at concentrations of 0, 0.5, 1, or 1.5% (vol/vol) and was present throughout the duration of the experiments. Curves represent data on 1276 colonies.

seems wisest to examine a number of different predictive tests that may depend on a variety of cellular targets, validating each test by exposure to a number of known carcinogens and noncarcinogens. The agar-grown CHO colony assay described here has responded well in sensitivity, specificity, and predictive value to various classes of chemicals, including direct-acting mutagens, indirect mutagens, nonmutagens, and known growth factors. The particular advantage of this assay is its ability to detect a wide range of both genetic and epigenetic effects resulting from exposure to chemical agents in this mammalian test system. It is not necessary to understand and specify in advance the precise targets that will be sensitive to a new and untested agent. Greater overall sensitivity and a broad range of applications result from non-specificity. Further work is necessary to determine the optimal expression time for measuring the colony diameter and integrated optical density and to find the lowest dose at

which a signal can be detected. Effects of indirect carcinogens in the presence of an activating system also must be explored. Other cell lines with different sensitivities should be added to the test. Computer-aided analysis of more elaborate aspects of colony morphology has been used in other applications of this technology (12) and would be expected to increase the sensitivity in this application because shape-dependent parameters other than diameter and integrated optical density may be especially sensitive to some transformation events. Finally, it should be noted that the equipment constructed for use in this work is unique, complex, and expensive. It can readily do large numbers of samples accurately and rapidly and can measure parameters subtler than colony diameter and integrated optical density. Simple diameter measurements can, of course, be done by hand without the use of a machine or with simple colony-scanning machines that are now available commercially.

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