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CELLULAR HEREDITY IN HAPLOID CULTURES OF SOMATIC CELLS

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Jerome J. Freed, Ph.D.

The Institute for Cancer Research
The Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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DISCLAIMER

CONTENTS

I. ABSTRACT

We report here studies in progress on cultured frog and fish cells, exploring the relation between the frequency of mutation after ultraviolet irradiation and the pathway through which DNA repair takes place. Our rationale is that the mutant frequency induced by a UV exposure is determined not only by the dose delivered but by the fidelity of the DNA repair process. Since frog cells express photoreversal enzyme, we can determine experimentally whether repair takes place by error-free photoreversal or by other, error-prone, mechanisms. An important question is whether an inducible, error-prone mutagenic form of repair is demonstrable.

During the past year we worked out methods necessary to determine UV survival and mutant frequency over a range of UV exposures. Using these methods, we have tested for alteration of the UV survival curve by previous conditioning exposures in frog cells and examined UV survival and photoreversal capacity in fish cells. The relation between UV survival and induction of ouabain resistance by an alkylating agent (MNNG) has been examined as a background for further studies with UV.

A procedure intended to accomplish DNA-mediated transfer of frog DNA photolyase enzyme to Chinese hamster cells is described.

II. INTRODUCTION

In our proposal last year we outlined an investigation of the relation of DNA repair to mutagenesis in cultures of haploid frog cells. Since repair of ultraviolet (UV) induced DNA damage in these cells proceeds mainly by photoreversalin the light and by postreplicational repair synthesis in the dark, a photoreversal test can be used to associate the origin of drug resistant phenotypes with pyrimidine dimers in DNA. Since photoreversal can be induced at various times following exposure to UV, it is possible to examine the time course of mutation fixation in these cells in much the same way as in bacteria (1). Our observations on repair functions in haploid cell line ICR 2A are summarized in an attached manuscript (Appendix A) and a discussion of the photoreversal test is given in a second manuscript (Appendix B). Using ICR 2A cells, we undertook to investigate how the pathway employed for DNA repair influences the probability of mutation. A salient problem was to determine whether exposure to conditioning doses of UV induced changes in the cells that would alter the extent or the fidelity of DNA repair following subsequent UV exposures. In other words, do frog cells express an inducible error-prone mutagenic form of DNA repair like the SOS system of bacteria (2)?

Induction of new forms of repair of UV damaged DNA might be expected to alter the survival curve, enhance the rate of repair of DNA molecules or increase the yield of mutants in split dose experiments. During the past year, we have tested the first of these propositions and developed methods that permit attack on the latter two. In our previous work (Appendix A) we exposed cells to UV while they were attached to the surface of UV transparent petri dishes. While this procedure gives good uniformity of UV exposure and facilitates dosimetry, it limits the number of cells that can be irradiated in biochemical or mutagenesis experiments. Furthermore, it introduces undesirable variability in estimates of survival and thus produces noisy survival curves. Therefore, we found it advisable to work out a method to irradiate the cells in suspension as described below. Using this method, we tested for the broadening of the shoulder of the UV survival curve following exposure to conditioning doses that would be expected if there were a quantitative increase in overall DNA repair capacity. The experiments summarized below indicated that such quantative changes must be small. Tests for qualitative changes, i.e., changes in the fidelity of repair, are to be continued in the coming year as outlined in the accompanying research proposal.

The capacity for photoreversal is expected in fish cells and we were interested in knowing whether fish cells could be employed in much the same way as our ICR 2A cells. Accordingly we carried out some studies using FHM, a vigorous, established, near-diploid cell line, that we anticipated might give us more precise estimates of survival and mutant frequency in consequence of its more rapid multiplication and higher plating efficiency. Survival curves were prepared and substantial capacity for photoreactivation was demonstrated, but the response of FHM cells to ouabain selection in mutagenesis assays was unsatisfactory. While ouabain at micromolar concentrations blocked colony formation by FHM cells, the blocked cells remained

attached and apparently viable so that mutant selection could not be carried out at high cell densities, a substantial disadvantage.

A specific aim proposed last year was to isolate UV-resistant cell strains. This work has been initiated in connection with other gene transfer experiments that were carried out during the past year to study the genetics of thymidine kinase in ICR 2A cells. Chinese hamster cell line CHO-K1 is widely used for mutagenesis experiments but like other mammalian cells it lacks photoreversal function. By exposure of CHO-K1 cells to high molecular weight frog DNA we sought to introduce a functional DNA photolyase gene. UV-resistant derivatives obtained by the cycling procedure described in this report are now being examined for photoreactivation capacity. If we can introduce the photoreversal function into CHO cells these should provide an exceptionally favorable material for a variety of mutagenesis experiments.

III. METHODOLOGY

The method of UV irradiation of attached cells used in our previous work has several disadvantages that led us to seek a satisfactory method of irradiating suspended cells. In our previous work, replicate monolayer cultures in UV-transparent petri dishes were exposed to various levels of UV fluence, then detached with trypsin, counted with a Coulter counter and plated out to determine survival at the various doses. To obtain well spread and therefore uniformly irradiated cells, no more than 5×10^5 cells plate could be employed. Among the problems experienced were variation in survival behavior from experiment to experiment; furthermore, the relatively small number of cells that could be irradiated at one time hindered mutagenesis assays. Since separate cultures were used for each exposure in construction of survival curves, errors in cell enumeration were directly expressed as variation in the estimate of surviving fraction. Many of these difficulties could be eliminated, we felt, if a single large suspension of cells could be irradiated and aliquots removed after increments of exposure. A further advantage of such a procedure was anticipated in mutagenesis experiments designed to study the dependence of mutant frequency on UV fluence: at high doses with consequent low surviving fractions, very large cell populations ($> 10^7$ cells) must be used for each data point.

We began by defining "steady state" conditions for propagating cell stocks to be used for irradiation. Using the Coulter counter to enumerate cells, T-150 flasks are inoculated with 5×10^6 cells in 25 ml of growth medium. After three days the cultures are fed by complete change (25 ml) of growth medium. After seven days, the cells are harvested with trypsin and used:

a. to seed additional "steady state" cultures,
b. to obtain 10^7 cells for UV irradiation and
c. to determine cellular characteristics used for quality control.

Table 1 illustrates the stabilization in several cellular characteristics that took place as a cell population was shifted from ordinary propagation to growth under these controlled conditions. Stabilization of cell volume and content of UV absorbing material (mainly ribosomal RNA) was of particular

interest since these would be expected directly to affect the pyrimidine dimer content resulting from exposure to a given UV fluence.

Using these cells, we developed the following standard irradiation procedure. The trypsinized cells are centrifuged and resuspended in PBSI, a calcium and magnesium free Dulbecco's phosphate buffered saline containing 0.1 mM EDTA. Our experiments normally employ 1 x 10^7 cells suspended in 10 ml of PBSI. The suspension is placed in a 6 cm Permanox UV-transparent plastic petri dish and a steel needle sealed in a glass capillary tube is floated on the surface. A magnet placed above the petri dish cover is rotated at 60 RPM in order to keep the cells uniformly suspended and agitated. Irradiation is from below, using 254 nm UV isolated from a germicidal mercury lamp by a Corning 7-54 glass filter. The average fluence to which a cell is exposed in such a system may be estimated from the Morowitz correction (3), knowing the incident fluence from measurements made with a photometer (Appendix A). Morowitz's correction compensates for the "sieve effect," i.e., the shielding of cells by the interposition of other nearly opaque cells closer to the radiation source. The magnitude of the Morowitz correction is a function of the measured transmission of the cell suspension as shown in Figure 1. Transmissions of cell suspensions (10 ml in a 6 cm dish) at various cell densities were determined in our irradiation apparatus as shown in Figure 2. Note that when samples of 10^7 cells are used, the value of the Morowitz correction is small, i.e., there is relatively little shielding of one cell by another.

Using this system we found that cell distribution remained uniform and aggregation was prevented for up to 30 minutes, a sufficient time to deliver substantial doses of 254 nm. The plating efficiency of unirradiated cells was not reduced during this time in the apparatus.

To determine survival curves, the cell suspension was sampled by withdrawing 0.10 or 0.20 ml portions (1-2 x 10^5 cells) prior to irradiation and then following increments of exposure. For example, the apparatus was adjusted to deliver an average fluence of 3 Jm^{-2} min^{-1} to the cells, the suspension was stirred, sampled and then sampled again following successive 1 minute exposures. The samples were delivered into 10 ml volumes of growth medium, mixed and then distributed to groups of petri plates to allow colony formation. The colonies were subsequently stained, counted and survival calculated as the ratio of mean colony number after any total fluence to mean colony number from the unirradiated sample. This procedure gives results as shown in Figure 3, which may be contrasted with the noisier data shown in Appendix A.

Finally, it should be noted from Figures 1 and 2 that the density of the irradiated cell suspension may be increased several-fold without producing undesirable elevation of the Morowitz correction. This is convenient when large cell populations are to be handled, as in mutagenesis experiments or in cycling to select UV resistant isolates.

IV. ULTRAVIOLET INACTIVATION AFTER CONDITIONING DOSES

One of the specific aims proposed last year was to test for enhancement of repair capacity by examining effects on the form of the survival curve of previous conditioning UV exposures intended to elicit the induction of new repair enzymes. If the shoulder of the survival curve is a reflection of total repair capacity, as appears to be the case from studies with yeast and from our own comparisons of haploid and diploid survival in frog cells, then enhancement of repair should tend to broaden the shoulder (increase D_q). If, on the other hand, conditioning exposures change only the fidelity of the repair process, the survival curve should remain unaltered.

Using the suspension irradiation method discussed above we have exposed ICR 2A cells to conditioning doses and after various times to a subsequent series of exposures in order to plot survival curves. The magnitude of the conditioning exposure was chosen to be approximately equal to D_q, 2 Jm^{-2} so that this dose of UV did not itself reduce the surviving fraction. Following the conditioning dose, the cells were returned to growth medium for times ranging from 1 to 26 hours to allow enzyme induction to take place. The cells were then resuspended and given the second series of irradiations.

A series of split-dose curves, with delays of 1 to 6 hours is shown in figure 3. There were no shifts in the curves larger than experimental error. With longer delays, shown in figure 4a, the curves show a smaller slope and are upwardly inflected, as if there were a subpopulation of highly resistant cells. The magnitude of this effect appears to be greatest between 10 and 20 hours following the conditioning dose, as shown in figure 4b, in which survival at two intermediate doses is plotted against delay time. Such effects on the survival curve are difficult to interpret as induction of additional repair capacity; they might reflect partial cell cycle synchrony imposed by the experimental manipulations, shielding of some part of the cell population, perhaps by cell surface materials, or blocked DNA synthesis resulting from the conditioning dose. We conclude that while split-dose experiments do alter the form of the survival curve, the changes are not of the form most readily associated with induction of additional repair capacity.

V. MUTATION TO OUABAIN RESISTANCE IN ICR 2A FROG CELLS

To make an adequate estimation of mutagenesis it is necessary to be able to determine the dependence of mutant yield on the extent of interaction with mutagen. Is the relationship a linear one or is there a threshold effect? In published mutagenesis experiments with various cultured cell lines, evidence has been presented for both forms of relationship. In order to determine the behavior of our ICR 2A cells we have carried out some studies with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in order to make comparisons with the effects of UV. Since MNNG is a powerful alkylating agent it readily leads to base substitution. The compound is highly reactive so that the half life of the chemical in culture medium is short, on the order of 30 minutes. Therefore,

freshly prepared solutions of MNNG at various concentrations yield a series of pulse-like exposures to mutagen. This work was carried out using ouabain resistance since this phenotype should be a good indicator of base substitution. Ouabain resistance arises through the appearance of a Na^+/K^+-dependent ATPase that no longer binds the inhibitor (4). The appearance of such an altered enzyme is likely to require changes at specific sites in the protein sequence and therefore would be expected to be produced most effectively by mutagenic agents like MNNG that lead to base substitutions in DNA. In haploid ICR 2A cells, a single step selection following MNNG exposure gives highly resistant mutants: the ID_{50} for ouabain is typically increased more than 1000-fold. This is consistent with alteration in a single structural gene for the ATPase, so that all ATPase molecules in the plasma membrane of the mutant cells are replaced by the resistant type.

The experimental material for these experiments was the ICR 2A strain TK4 which is doubly marked by lack of two forms of thymidine kinase (5). These cells form favorable colonies well attached to the growth surface and thus are convenient for scoring mutant frequency.

Experiments were carried out by treating replicate monolayer cultures with differing concentrations of MNNG left in contact with the cells for 6 hours. Immediately after mutagen treatment, cell samples were removed to determine the efficiency of plating and the remainder of the cells propagated for 10 days in standard growth medium to allow expression of mutation. The cells were then detached with trypsin, counted in a Coulter counter and known numbers of cells exposed to growth medium containing 1 mM ouabain and 1 mM BUdR. The latter drug was added to screen out reversions affecting either form of thymidine kinase. Colonies of resistant cells formed and the flasks were stained and scored. The appearance of experimental and control flasks is shown in Figure 5.

The data obtained are shown in Figure 6. Note that the survival curve is a simple exponential as first observed in CHO-K1 cells by Kao and Puck (6). They suggested that MNNG might inhibit repair functions and therefore eliminate any shoulder on the survival curve but there is no direct evidence in support of this explanation. The induction of ouabain-resistant clones was a linear function of mutagen dose up to 1.5 μg/ml MNNG, corresponding to a surviving fraction of 0.4. The maximum induced mutant frequency was nearly 500-fold greater than among untreated cells.

These results may be contrasted with the results obtained with 254 nm radiation when ICR 2A cells were exposed to a single acute dose and both DNA repair and subsequent cell multiplication took place in the dark. In such experiments, (e.g., see Appendix B), mutant frequency is 3- or 4-fold elevated at survival levels corresponding to those giving maximal mutant frequency with MNNG. These observations are consistent with repair of UV damage by relatively error free pathways in uninduced ICR 2A cells but with a higher error frequency repairing the damage due to an alkylating agent.

These studies indicate the suitability of mutation to ouabain resistance as a useful assay system for ICR 2A cells. It is clear that after treatment with an appropriate mutagen ICR 2A give rise to substantial frequencies of

easily selected mutants and that the spontaneous mutation frequency is low. We will employ this procedure to estimate the frequency of mutation induced by graded UV exposures following conditioning doses.

VI. FISH CELLS AS EXPERIMENTAL MATERIAL

Since our studies make use of photoreversal capacity as a tool for investigation of mutagenesis we could employ not only amphibian cells but other vertebrate cells that express the photoreversal function. Cells of marsupials, although capable of photoreversal, appear to be less effective in carrying this out (7). We therefore evaluated the use of fish cells, since these would be expected to be fully capable of photoreactivation (8) and to allow propagation at high temperatures, giving the advantage of faster multiplication. The cells studied were strain FHM, a permanent line established from the fathead minnow (9) which has been used extensively to propagate viruses of poikilothermic vertebrates. Since these cells may be grown at 32° and are extremely vigorous we felt it would be worthwhile to investigate whether they had properties suitable for our work.

To summarize these experiments briefly, a number of growth media and incubator temperatures were investigated. While mass cultures were readily propagated at 32°C, the efficiency of plating at this temperature was zero in all the media tested. At 26°C, on the other hand, efficiency of plating was greater than 20% and colonies were distinct and readily scorable. The population doubling time at 26° in L-15 medium was about 24 hours. These observations suggested that at the higher temperatures FHM cells were no longer capable of sufficiently rapid biosynthesis of some limiting nutrilite. A nutritional investigation to ascertain the limiting compounds has not been carried out.

Using 26°C and L-15 medium, we investigated the UV survival and photoreactivation properties of FHM cells, employing the suspension irradiation method. As shown in the left panel of figure 7, if FHM cells were maintained in the dark following 254 nm irradiation, they yielded a survival curve of conventional form, with both D_q and D_o near 2 Jm^{-2}. However, if UV was followed by 20 minutes' exposure to visible light at 850 $\mu W/cm^2$, survival was substantially increased as shown in the right panel of figure 7. If higher doses of 254 nm radiation are given, photoreactivation under these conditions gives only partial restoration of survival. As shown in figure 8, cells irradiated with increments of UV totalling 12 Jm^{-2} gave the survival curve shown in the left panel. After exposure to visible light at 850 $\mu W/cm^2$ for times up to 60 minutes survival reached a plateau at a level corresponding to survival after UV at 6 Jm^{-2}, giving a photoreactivable section of about one-half.

While these results were encouraging, investigation of oubain resistance in these cells revealed difficulties. Colony formation at low cell densities is prevented by low concentrations of ouabain as shown in Figure 9. The ID_{50} lies near 3×10^{-8} M. However when mass cultures were exposed to selective media containing ouabain at concentrations as high as 1×10^{-5} M, the majority of the cells remained attached and apparently viable; surviving colonies of resistant cells could not be detected above this background. If such cultures

were harvested after 11 days exposure to ouabain at 3×10^{-6} M, (i.e., 100 times the ID_{50} value for wild-type cells) the efficiency of plating as a function of ouabain concentration was indistinguishable from that of control cells. Our interpretation of this finding is that FHM cells must differ from frog cells in their response to ouabain: it is cytostatic rather than cytotoxic. ICR 2A cultures, on the other hand, respond to ouabain quite differently. After a few days the majority of the cells detach and leave a low background of attached cells against which surviving colonies can easily be detected. FHM cells lack this differential response. While it would be desirable to explore the effects on FHM cells of selective concentrations of ouabain greater than 1×10^{-5} M, we have suspended these experiments in favor of use of the ICR 2A frog cells on which we have more substantial information and which give cleaner assays of ouabain resistance.

VII. ULTRAVIOLET RESISTANT CELLS: GENE TRANSFER EXPERIMENTS

With the development of adequate methods for accomplishing DNA-mediated gene transfer (DNA transformation) in cultured cells (10, 11), it may become possible to construct novel cells with regard to DNA repair functions. In our own work, it would be valuable to introduce DNA photolyase activity into CHO-K1 cells, which are a well-understood experimental material for assay of mutagenesis. Since we were carrying out DNA-mediated gene transfers in frog and mouse cells as part of our studies of thymidine kinase genetics, it seemed worthwhile adding this feature to the experiments intended to isolate UV-resistant cell strains that we proposed last year. These DNA transformation studies were carried out with the assistance of a summer volunteer student, and were essentially an off-shoot of Dr. Hepfer's studies with frog cells.

CHO-K1 cells were used as recipients, and were treated in monolayer culture with calcium phosphate-precipitated Rana pipiens liver DNA of high molecular weight as described by Wigler *et al.* (10). This procedure, in our hands, gives a good yield of thymidine kinase transformants when TK-mouse cells are treated with high molecular weight mouse liver DNA. The recipient cells were propagated for 1 week to allow expression of any acquired functions. They were then subjected to three cycles of a stringent selection procedure designed to enrich for cells capable of photoreversal. Cell suspensions prepared as described above were irradiated with 254 nm using an exposure of 40 Jm^{-2}, giving a surviving fraction near 0.001 in control CHO-K1 cells. The irradiated suspensions were thereafter illuminated at 1100 $\mu W\ cm^{-2}$ for three hours, washed, resuspended in growth medium and incubated at 37°C until the flasks were repopulated. These progeny were again resuspended and the process repeated. Following the third cycle of this selection screen, the cells were plated out in Permanox petri plates and allowed to form colonies of 50 to 100 cells at about 100 - 200 colonies per plate. These plates were then washed, covered with PBS and exposed to 254 nm radiation (60 Jm^{-2}) and subsequently to visible light (3 hours at 1100 $\mu W\ cm^{-2}$). The PBS was removed, growth medium added and the plates incubated for an additional week. Under these conditions, wild-type CHO-K1 colonies are eliminated, i.e., the probability of even one member of the colony surviving is very low. If a substantial fraction of the colony continues to multiply it is likely to have been composed of UV resistant variants. Since visible light is employed, the selection favors transformants that have acquired photoreversal enzyme activity. A number of colonies that were of large size one week following the irradiation and photoreversal sequence have been isolated and are being propagated as cloned strains. Experiments are in progress to determine a) whether they yield altered UV survival curves and b) whether they exhibit photoreactivation.

Obviously, several kinds of UV-resistant variants might survive the selection procedure outlined above. DNA repair functions normally expressed in CHO-K1 might be augmented, functions like excision repair that are repressed in CHO-K1 might be derepressed, or frog photolyase might be acquired. These possibilities will be examined in the coming year.

VIII. LITERATURE CITED

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IX. PROJECT STAFF

The studies reported above were carried out at The Institute for Cancer Research, Fox Chase Cancer Center, in the laboratory of Dr. Jerome J. Freed. The work of the laboratory during the reporting period consisted entirely of studies in the genetics of cultured cells. The participation of individual members of the staff in specific projects is indicated above.

	Effort	Source of Salary*
Jerome J. Freed, Ph.D., Member	50%	CA-06927
Carol E. Hepfer, Ph.D., Post-doctoral Associate (from June 1978)	100%	CA-09035
Sharon Howard, B.S., Sr. Research Technician (until October 1977)	100%	CA-05959
Stephen K. Shuman, B.S., Research Technician I (July 1978 to August 1978)	100%	EY-76-S-02-3110
Todd M. Kelman, B.S., Research Technician I (from September 1978)	100%	EY-76-S-02-3110
A. Catherine Bluett, Technician II	50%	EY-76-S-02-3110
Suzanne Rivitz, Volunteer Student Assistant (July-August 1978)	100%	NA

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CA-05959 "Genetic Studies on Haploid Cells in Culture," Jerome J. Freed, Principal Investigator, National Cancer Institute.

X. PUBLICATIONS

The following have appeared since our last annual report:

Freed, Jerome J., editor, Mechanisms of Cellular Control: Symposium of the 28th Annual Meeting, The Tissue Culture Association. In Vitro 14:62ff, 1978.

Freed, Jerome J., Lorraine H. Toji and Arthur E. Greene On the "Lucke tumor" origin of cell line LT-1. J. Nat'l. Cancer Inst. 60:493-495, 1978.

Freed, Jerome J. Tables of culture media for cells of Amphibia and Reptilia, pp. 17-24 in M. Rechcigl, editor, CRC Handbook of Nutrition, vol IV G, CRC Press, Cleveland, 1977.

Viceps-Madore, D. and L. Mezger-Freed Studies on DNA repair in frog and human cells exposed to an acridine half-mustard (ICR 191) and to MNNG. Mutation Res. 49:407-419, 1978.

Viceps-Madore, D. Effects of an acridine half-mustard (ICR 191) on growth and ploidy of frog cells in culture. J. Cell. Physiol. 94:187-196, 1978.

In press:

Freed, Jerome J., Frank A. Angelosanto and Ronald H. Hoess Photoreversal as a tool for study of mutagenesis in haploid frog cell cultures. J. Cell Biol. (abstract).

In preparation:

Freed, Jerome J., Ronald H. Hoess, Frank A. Angelosanto and Holman C. Massey, Jr. Ultraviolet irradiation of haploid and diploid frog cells: survival and modes of DNA repair.

Freed, Jerome J., Holman C. Massey, Jr., Frank A. Angelosanto and Ronald H. Hoess Photoreversal test of the genetic origin of variants in cell culture.

Table 1

Characteristics of ICR 2A cells after shift to "steady state" conditions.

Subculture Generation[a]	Modal Volume[b]	Lowry Protein[c]	A_{260} per Cell[d]
62	0.68	0.90	0.81
63	0.81	0.70	1.13
64	0.87	0.53	1.04
65	1.07	1.08	1.13
66	1.16	0.95	1.12
67	1.01	0.97	0.96
68	1.01	1.00	1.01
69	1.04	-	-
70	1.00	1.00	1.00

[a] Cells were transferred weekly, beginning with subculture generation 62, as described in the text.

[b] Modal volume of trypsin-detached cells was estimated using a Coulter counter, model P-128 size distribution analyzer and X-Y recorder output. Steady-state value was 2520 μm^3.

[c] Total protein per cell was determined, using the Lowry procedure, on samples of cells enumerated with the Coulter counter. Steady state value was 440 pg/cell.

[d] Samples of cells enumerated with the Coulter counter were extracted for 20 min in 1 ml of 5% perchloric acid at 95°C, centrifuged, and the absorbance of the extract at 260 nm was determined with a Gilford spectrophotometer model 240. Steady state value was 1.15×10^{-6}/cell.

FIGURE LEGENDS

Figure 1. Working curve for application of the Marowitz correction (3). The relation between average irradiation of a particle and the energy incident on the suspension may be estimated provided that agitation is sufficient to maintain the suspension uniform. Abscissa is measured transmittance of cells relative to the same volume of diluent.

Figure 2. Experimental measurements of transmittance of cell suspensions in the irradiation apparatus. The number of cells shown on the abscissa was suspended in 10 ml of diluent in a standard 6 cm dish and the transmittance of the agitated suspension was measured at 254 nm. Data shown are for fish cells (FHM), haploid frog cells (TK4) and Chinese hamster cells (CHO).

Figure 3. Survival curves of ICR 2A cells, at various times after delivery of a conditioning dose of 2 Jm^{-2} at 254 nm. The curves are displaced arbitrarily along the abscissa to facilitate comparisons of shape.

Figure 4a. Survival curves of ICR 2A cells, at various times after delivery of a conditioning dose of 3 Jm^{-2} at 254 nm. At 8, 14 and 20 hours delay, the curves are inflected, suggesting a resistant function.

Figure 4b. Data from figure 4a replotted to show the relation between surviving fraction and time between expsoures at low doses.

Figure 5. Stained culture flasks after selection with ouabain. Control flask has one colony (marked by circle), flask treated with 1.5 µg/ml MNNG has numerous colonies.

Figure 6. Effect of treatment with nitrosoguanidine on the ICR 2A derivative TK4c11. Upper panel, survival of colony forming capacity; lower panel, yield of colonies resistant to 1 mM ouabain.

Figure 7. Survival curves of FHM cells after 254 nm radiation. Left, single acute doses, recovery in the dark. Right, exposed to visible light for 20 minutes following UV.

Figure 8. Time course of photoreactivation in FHM cells following exposure to 12 Jm^{-2} (left panel shows survival curve, right panel restoration of survival by visible light).

Figure 9. Plating efficiency of FHM cells as a function of ouabain concentration. The ID_{50} lies near 3×10^{-8} M.

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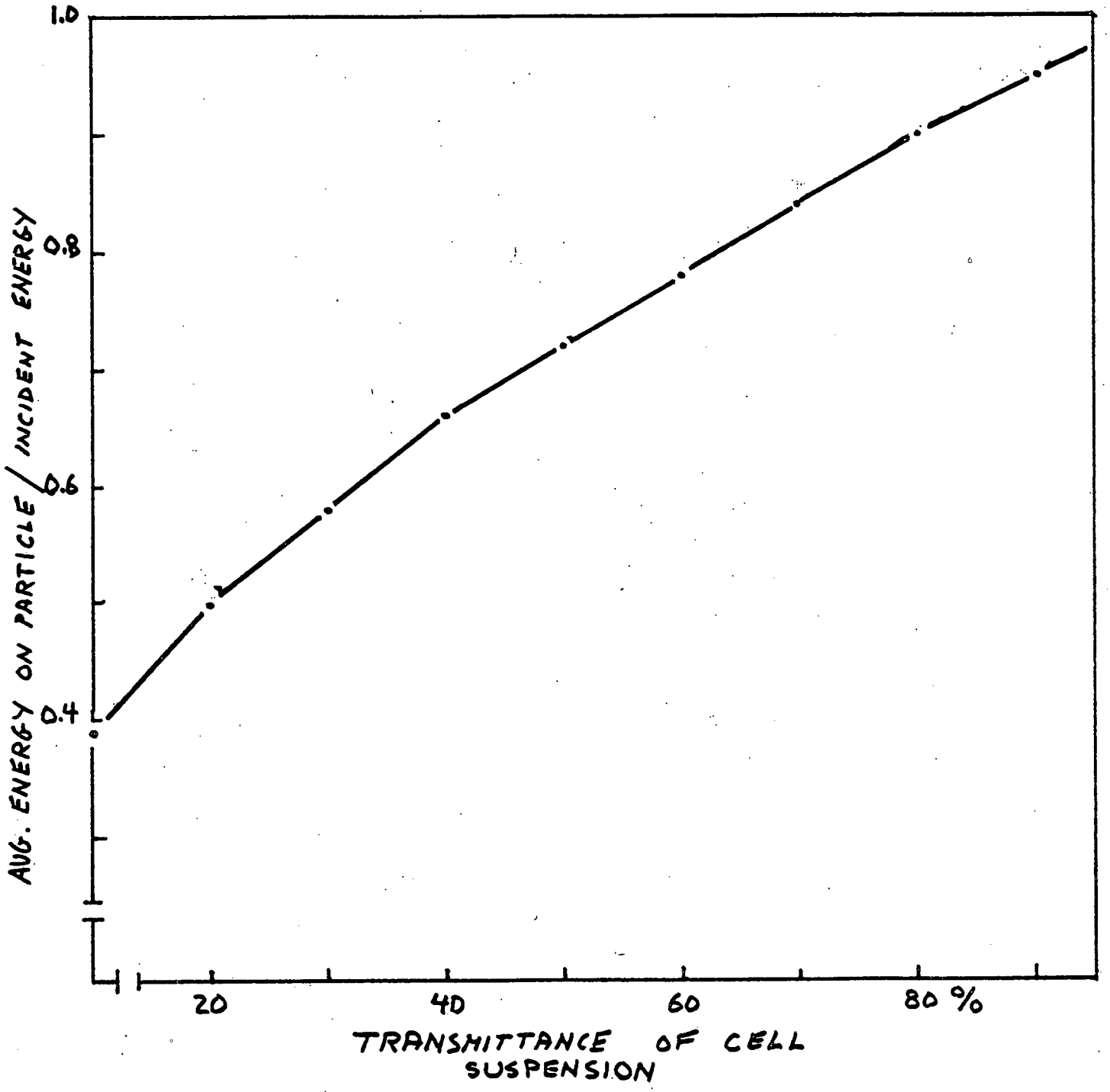
Figure 1


1.0
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AVG. ENERGY ON PARTICLE / INCIDENT ENERGY
20
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60
80 %
TRANSMITTANCE OF CELL SUSPENSION

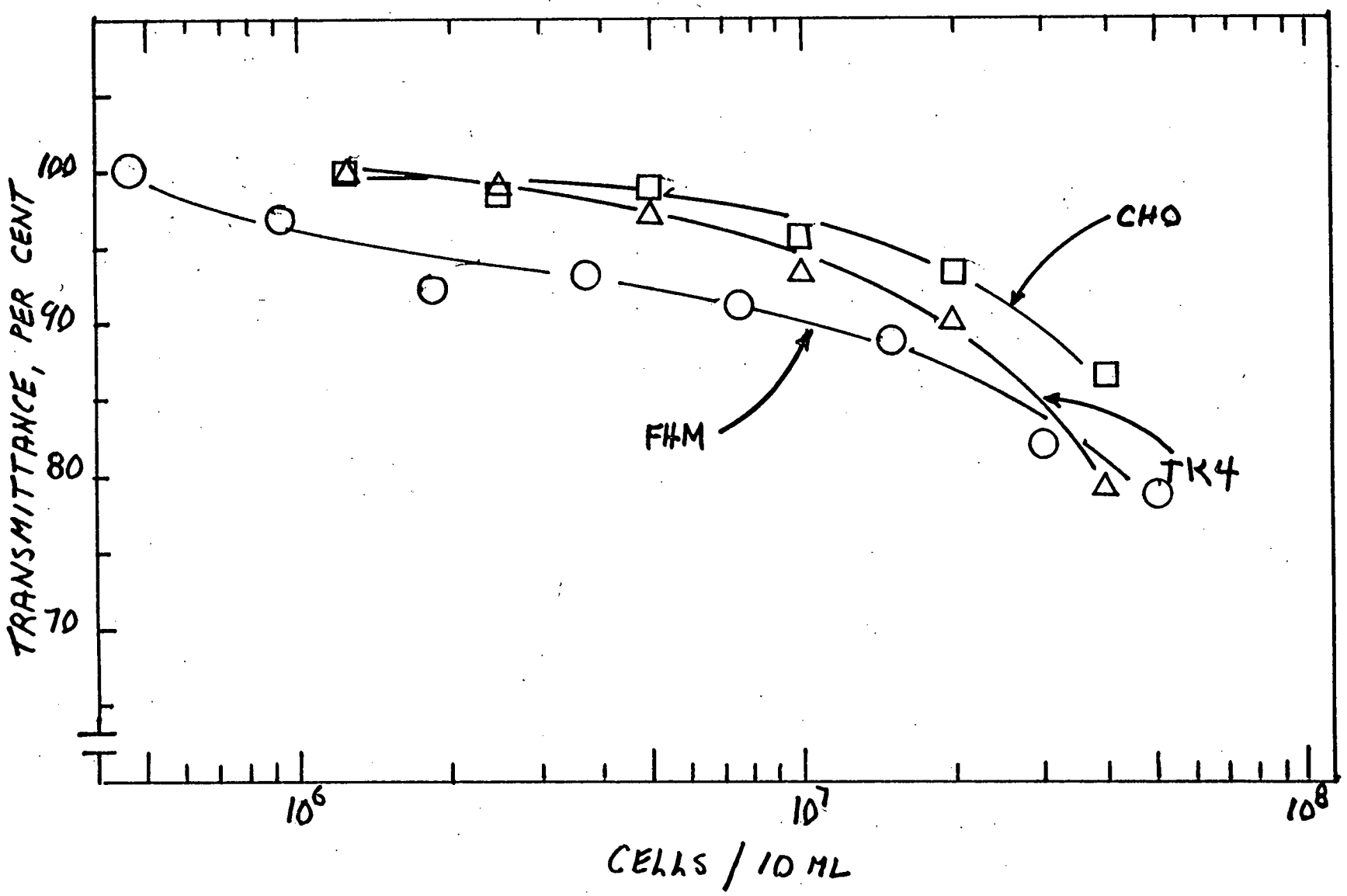

TRANSMITTANCE, PER CENT
100
90
80
70
10^6
10^7
10^8
CELLS / 10 ML
CHO
FHM
TK4

Figure 2

Figure 3

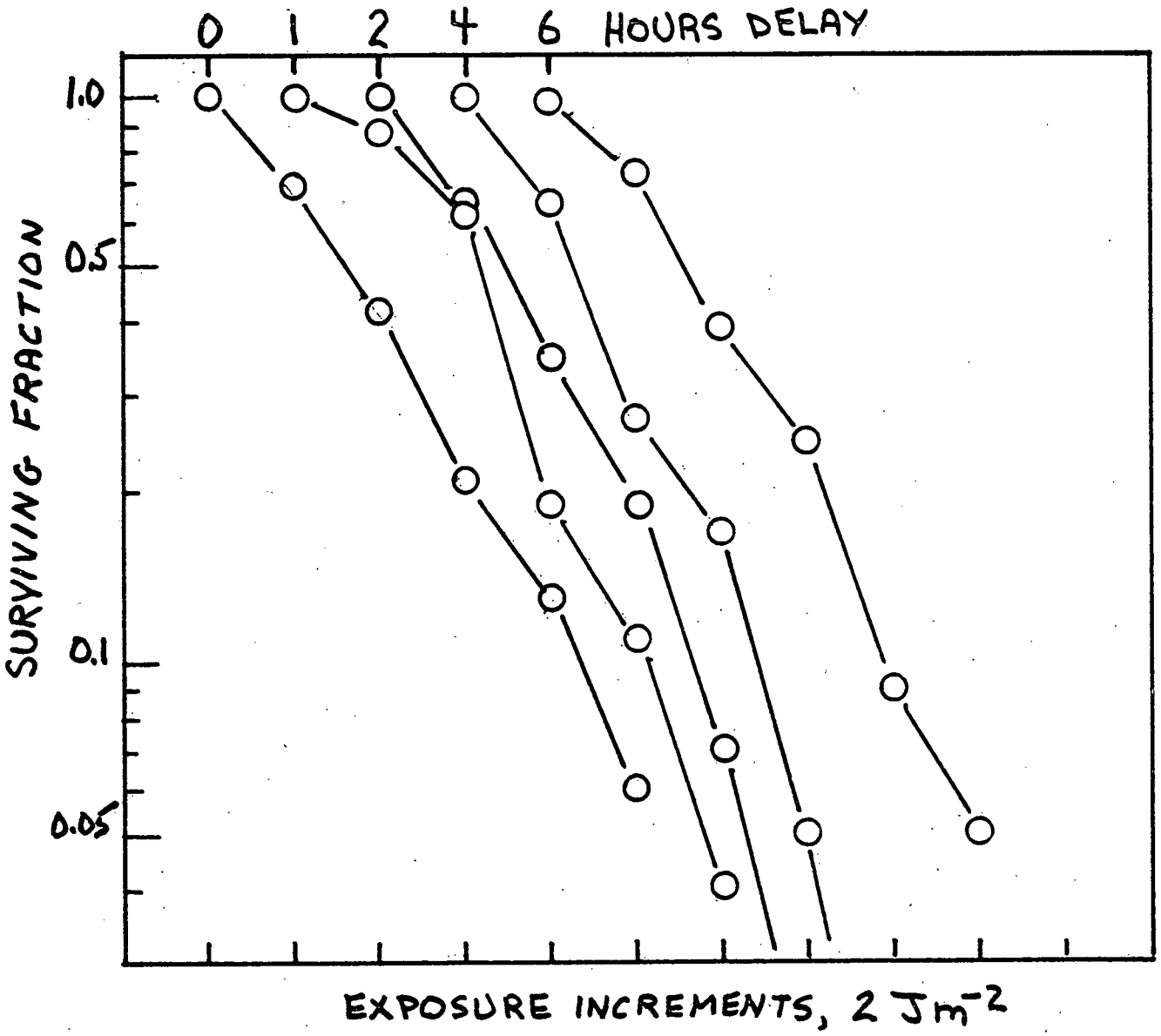
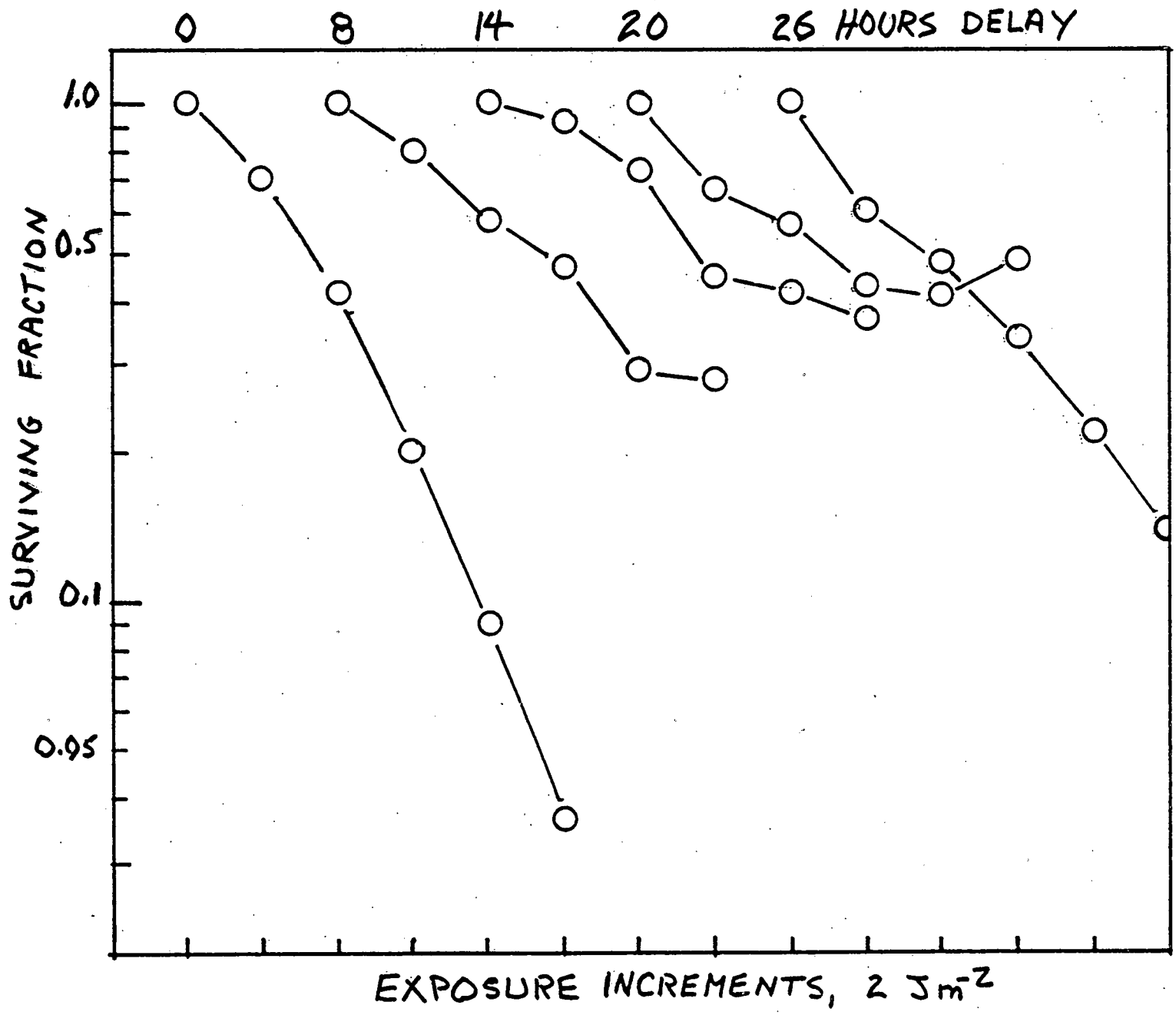
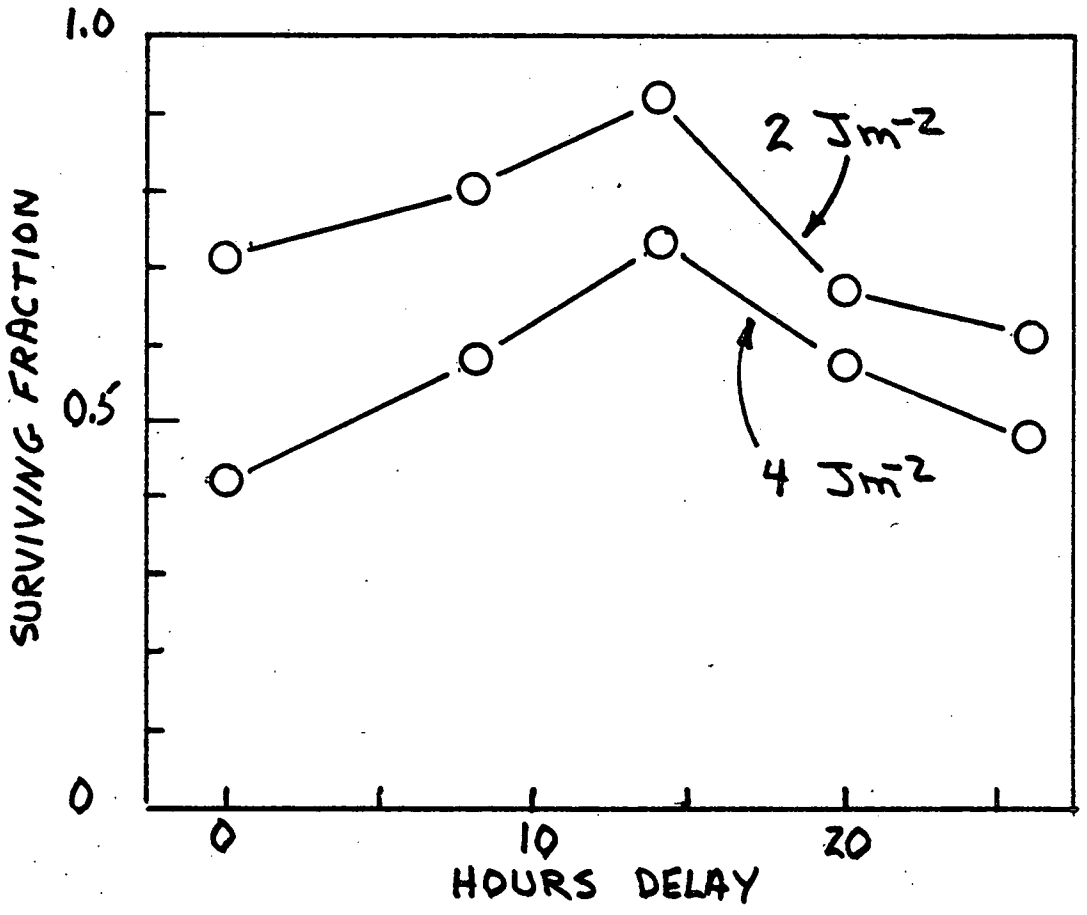

0 1 2 4 6 HOURS DELAY
1.0
0.5
0.1
0.05
SURVIVING FRACTION
EXPOSURE INCREMENTS, 2 Jm⁻²

Figure 4a


0
8
14
20
26 HOURS DELAY
1.0
0.5
0.1
0.05
SURVIVING FRACTION
EXPOSURE INCREMENTS, 2 Jm⁻²


1.0
0.5
0
SURVIVING FRACTION
2 Jm⁻²
4 Jm⁻²
0
10
20
HOURS DELAY

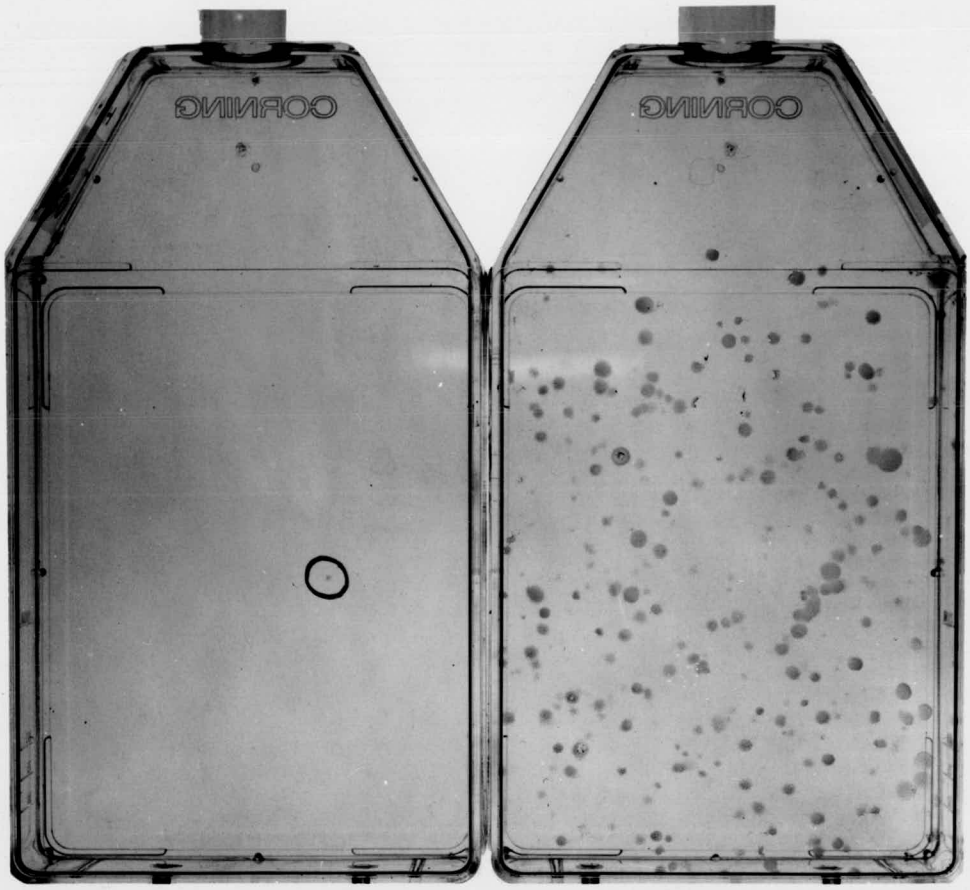

CORNING
CORNING

Figure 6

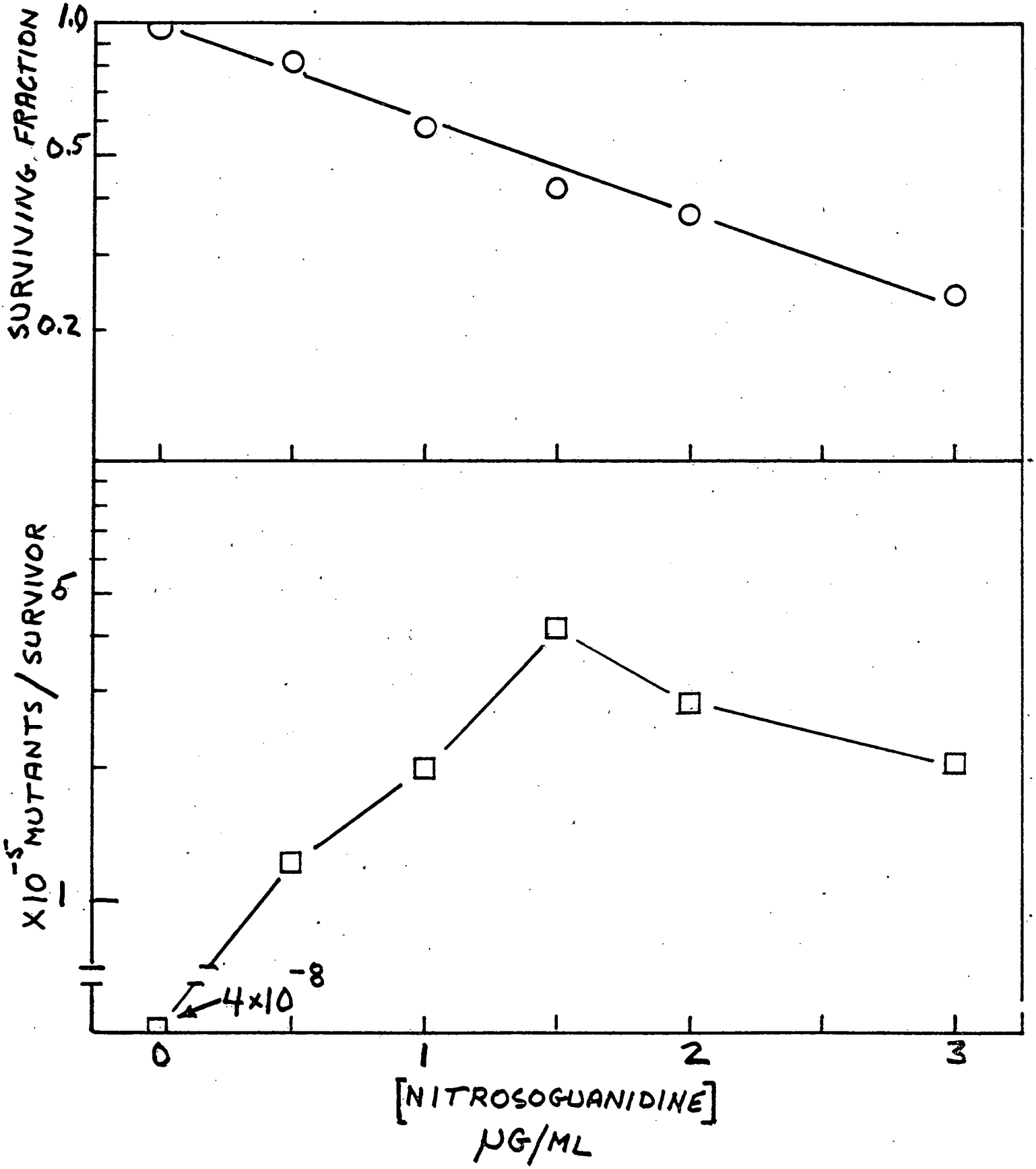

SURVIVING FRACTION
1.0
0.5
0.2
X10^-5 MUTANTS / SURVIVOR
5
1
4x10^-8
0
1
2
3
[NITROSOGUANIDINE]
µG/ML

Figure 7

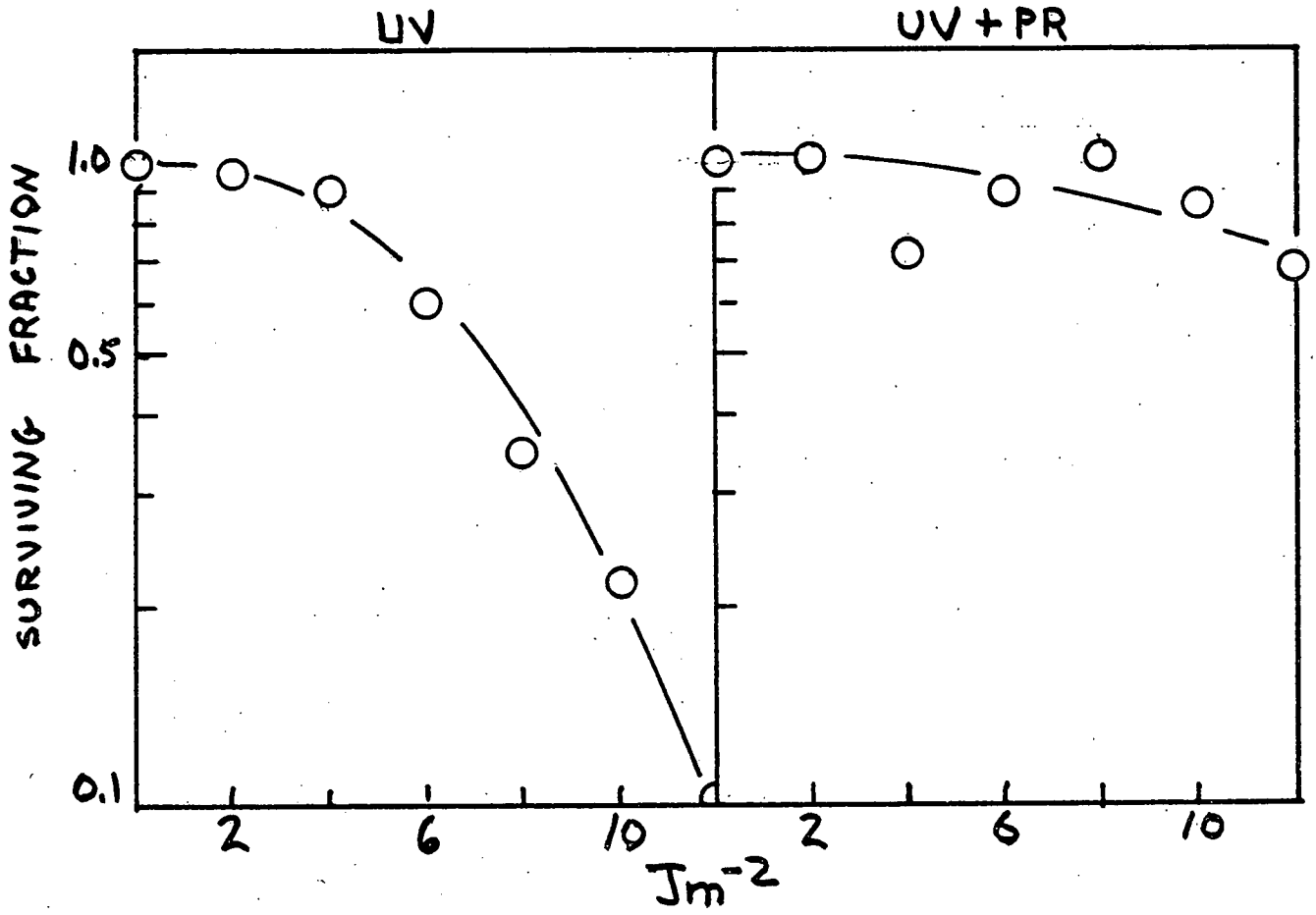


Figure 8

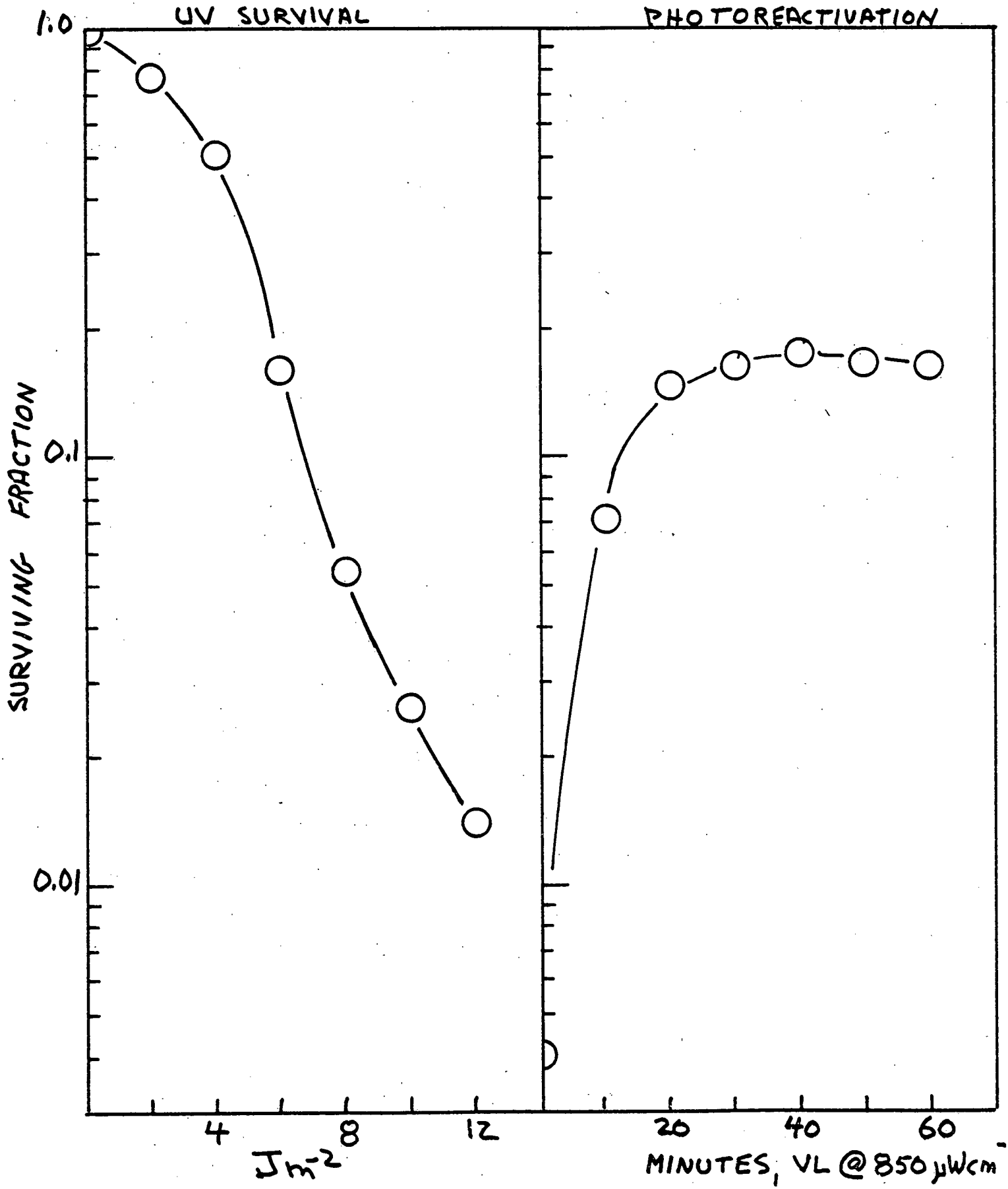
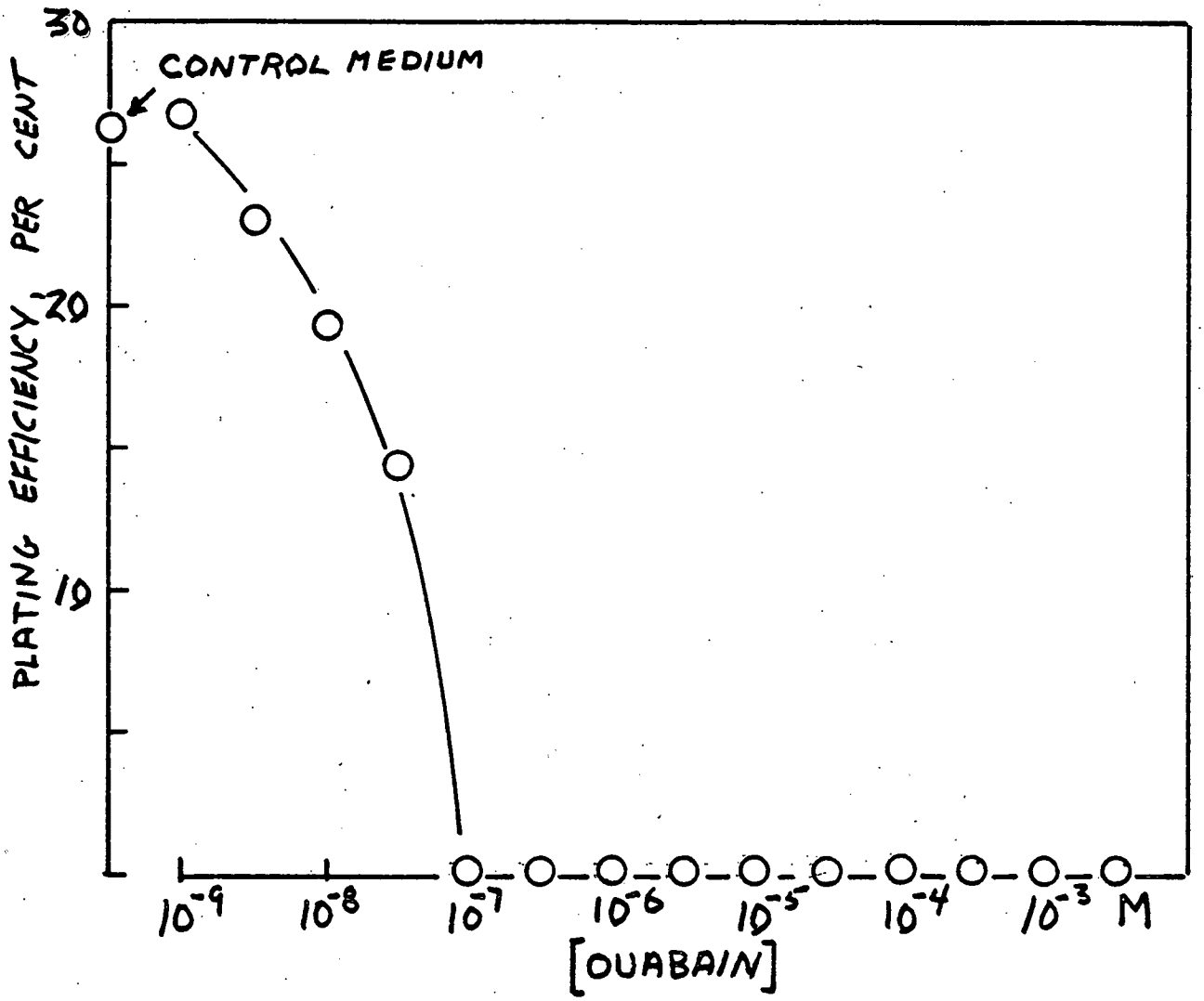

UV SURVIVAL
PHOTOREACTIVATION
1.0
0.1
0.01
SURVIVING FRACTION
4
8
12
Jm-2
20
40
60
MINUTES, VL @ 850 μWcm-2

Figure 9


30
20
10
CONTROL MEDIUM
PLATING EFFICIENCY, PER CENT
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
M
[OUABAIN]

ØØBREAKØØ

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COO-3110-31

CELLULAR HEREDITY IN HAPLOID CULTURES OF SOMATIC CELLS

Progress Report for Period
August, 1978 through September, 1979

Jerome J. Freed, Ph.D.

The Institute for Cancer Research
The Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

October, 1979

Prepared for

THE U.S. DEPARTMENT OF ENERGY

UNDER CONTRACT NO. DE-AC02-76-EVO-3110

- CONTENTS -

SUMMARY

During the past year we have continued our studies of the relation of ultraviolet mutagenesis to DNA repair in cultures of the haploid frog cell line ICR 2A. Our method of irradiation of cells in suspension was improved by construction of an improved detector with major sensitivity to the 254 nm Hg resonance line, to give better estimates of actual exposure of the cells. Using this method, dose-response and dose-fractionation studies on irradiation of ouabain resistance were carried out. The UV induction of this phenotype in the ICR 2A cell line was found to be less than that necessary for adequate analysis of dose-response curves. Cell fusion experiments using frog and mouse cells revealed an enhancement of mutagenesis in the mouse parent that will be explored in further work.

BACKGROUND: UV SURVIVAL AND DNA REPAIR IN ICR 2A

The properties of our haploid frog cell line with regard to single cell survival after UV and the modes of DNA repair that they express have been described (Freed et al., 1979). These cells readily photoreverse pyrimidine dimers, but excision does not appear to provide a quantitatively important pathway since few dimers are lost during incubation in the dark and "unscheduled DNA synthesis" is saturated at low doses. Survival curves for the haploid cells and their diploid derivatives were indistinguishable, save that exponentially growing diploids showed an increased Dq value (width of shoulder at low fluence). Details will be found in the attached reprint.

These repair properties make it possible to test whether UV induction of variant phenotypes is photoreversible and thus likely to reflect a direct effect on DNA. Using ouabain resistance and thioguanine resistance as the induced phenotypes, we sought to test whether photoreversal prior to mutation expression reduced the yield of resistant colonies. Experiments of this kind are described in the attached manuscript, which proposes that photoreversibility might be a general test for the genetic origin of UV-induced cell culture variants. A problem is that neither of these phenotypes is induced in sufficient numbers by UV (maximum increase in frequency less than 10-fold) to demonstrate the effect satisfactorily.

IRRADIATION AND DOSIMETRY

Improvements in our UV irradiation protocol now provide more satisfactory data. Figure 1, showing a typical ICR 2A survival curve by the new method, may be compared to figures 2 and 3 in the attached reprint. The improvements stem from a suspension irradiation procedure and better dosimetry.

Suspension irradiation is carried out by dispersing 1×10^7 cells in 10 ml of calcium- and magnesium-free phosphate buffered saline plus EDTA; the suspension is stirred in a UV-transparent petri dish during exposure from below. 254 nm radiation is obtained from a low pressure Hg discharge tube, air-cooled to reduce generation of wavelengths other than the 254 nm resonance line, and is passed through a Corning 7-54 filter to reduce contaminating visible light. Samples of 100 to 200 microliters are removed before exposure and at intervals during the irradiation. The samples are diluted in growth medium in petri dishes to give inocula of a size determined by the expected surviving fraction. After formation of colonies, the dishes are stained, colonies counted and surviving fraction calculated.

This suspension irradiation procedure demands that the average exposure of the cells be calculated from the incident fluence by correcting for the sieve effect, i.e., shielding of cells by cell intervening in the beam. Such a correction can be calculated from measurements of the apparent transmittance of the cell suspension at the wavelength of interest (Morowitz, 1950). When we attempted to make such measurements with our Tektronix J-16 digital photometer, we encountered a problem of detector response, leading to overestimation of the transmittance of cell suspensions. Although germicidal lamps are often treated as sources of nearly pure 254 nm radiation, there is significant emission of other longer wavelengths.

The problem comes from the response of the J-6504 silicon photodiode sensor, which falls rapidly with decreasing wavelength in the UV·B range. Thus, response is biased in favor of the longer Hg wavelengths and against the 254 nm major output. Since cells are strongly absorbing at 254 nm but reasonably transparent above 280 nm, we could not make valid estimates of the sieve effect.

To deal with this, we modified the photometer probe to respond only to absorbed wavelengths. A short-wave UV/visible transducer was attached to the probe (figure 2). Radiation passes a 7-54 visible-blocking filter to remove fluorescent light or stray visible and strikes a layer of willemite powder phosphor (Zn_2SiO_4:Mn) which produces visible light that is measured by the photodiode. With this detector, it can be calculated that 90% of the response to an Hg low pressure discharge will be to the 254 nm line, 10% to 313 nm. This was confirmed by experiments with a glass filter, which blocks 254 nm but passes 313 nm. Linearity of response, measured by an inverse square method, yielded a mean deviation of 1.4% (figure 2). Absolute calibration by chemical actinometry gave a sensitivity of 3.6 ergs/min/mm^2 per photometer scale unit. Since our work involves fluences near 50 ergs/min/mm^2, resolution is satisfactory both for fluence measurement and for determination of the Morowitz correction.

Typical transmittance measurements of cell suspensions made with this instrument are shown in figure 3. With samples of 1 x 10^7 cells per dish, 254 nm transmittances fall between 40% and 60%; average exposure of the cells is about 75% of incident fluence, convenient for our work.

OUABAIN RESISTANCE AND UV, DOSE FRACTIONATION

The purpose of these experiments was to determine the curve for induction of ouabain-resistant mutants as a function of increasing UV exposure to describe alterations in the curve for fractionated doses and to determine the effects of varying the time between doses. Our method was to irradiate samples of 10^7 cells by the suspension method described above, to return the cells to growth medium for the chosen delay period, then to carry out a second irradiation by the same procedure. The samples were then transferred to 150 cm^2 culture flasks in growth medium and propagated for 7 days to allow expression of the resistant phenotype. This expression time had been found to produce full yield of nitrosoguanidine-induced mutants in prior experiments. The cells were then subcultured to 75 cm^2 flasks in medium supplemented with 1 mM ouabain, incubated in the dark for 14 days and then fixed and stained to score surviving colonies.

In prior experiments, we had found that these conditions were satisfactory for detection of UV or nitrosoguanidine induced mutants. Recovered single-step mutants showed an extreme resistant phenotype, as would be anticipated for haploid cells expressing a single altered allele coding for Na^+/K^+-dependent ATPase, the target gene product in this selective system. Recovery of added mutants appeared to be independent of cell density, up to the limit set by the tendency of dense monolayer cultures to "peel" from the growth surface during selection in ouabain-containing medium, carrying away resistant cell colonies.

The results of these assays showed a restricted induction of ouabain resistant clones when UV was used as a mutagen. With single doses up to 10 Jm^{-2} (average surviving fraction 0.04), no more than 10 colonies per assay were

recovered. The limits of expectation at 5% level of significance were therefore so large that neither dependence on dose nor dependence on fractionation could be established. These results were consistent with our earlier studies, using single doses; however, we did not observe the increased mutant yield we had expected at higher doses of UV.

This low yield after UV treatment of ICR 2A contrasts with the results of treatment with the alkylating agent nitrosoguanidine. In such experiments, a maximal enhancement of mutant frequency (at least 100-fold) was obtained at a surviving fraction of about 0.4.

It is not clear whether the low yield reflects specific properties of the frog gene for Na^+/K^+-dependent ATPase, or a problem in detecting the mutant phenotype. Exposure of mammalian cells to comparable UV fluence (resulting in equivalent pyrimidine dimer formation) yields larger numbers of mutants in a dose-dependent fashion (Chan and Little, 1978; Chang et al., 1978a, 1978b). Since our recovery of added mutants in reconstruction experiments is adequate, we tend to favor the view that the phenomenon reflects insensitivity of the gene itself to UV mutagenesis. It should be noted that in order for ouabain resistance to be expressed, there must be a base substitution in one or more of the critical codons that specify amino acids defining the ouabain-binding site on the protein. Therefore, this kind of mutation might be very sensitive to sequence differences defining mutagen specificity at the molecular level.

GENE TRANSFER EXPERIMENTS

We have used cell fusion and DNA uptake methods to explore transfer of frog genes to mammalian cells. Since interspecific transfers among mammals are feasible, we wished to determine if genetic material from more distantly related vertebrates could be handled in the same way. DNA-mediated gene transfer (Bacchetti and Graham, 1977; Wigler et al., 1977, 1978) is carried out by exposing cells to DNA co-precipitated with $CaPO_4$ which is taken up by phagocytosis. A selective system is then applied to isolate rare clones that have replicated, transcribed and expressed a particular DNA sequence.

Our experiments sought to detect transfer of frog genes for thymidine kinase (TK) or photoreversal enzyme (PRE) to mammalian recipient cells. Pilot experiments were carried out to transform mouse LM/TK^- cells with the purified 3.4 kilobase restriction enzyme fragment that codes for herpes simplex viral TK (Wigler et al., 1977). We were able to obtain transformants by this technique and at lower frequency by using high molecular weight DNA from mouse liver (Wigler et al., 1978). However, similarly prepared DNA from wild-type ICR 2A frog cells failed to elicit transformed colonies from 2×10^8 LM/TK^- cells treated.

DNA from ICR 2A cells and from frog liver was also applied to Chinese hamster CHO-K1 cells in an attempt to transfer PRE activity. To select recipients expressing this enzyme, the cultures were exposed to successive cycles of UV radiation and photoreversing illumination and the survivors were tested for alterations in survival curve and for dose-modifying effect of exposure to visible light. No significant alterations were detected.

Thus, any transfer of these frog genes was too rare for us to observe. It is possible that there is a block to expression of introduced DNA sequences when a sufficiently great evolutionary distance separates donor and recipient.

In related experiments, a cell fusion method was employed to combine frog and mouse genomes. The fusion method, using polyethylene glycol (PEG) as the fusogen, in our hands yields viable hybrids in intraspecific frog cell crosses employing our TK^- OUA^R "universal hybridizer" test strain from ICR 2A. Using this procedure, the revertable TK^- mouse cell strain B82 was fused with wild-type KR 2A frog cells. The cultures were selected at 37°C (to eliminate unfused frog parents) in HAT medium containing 1 mM thymidine (Hi-HAT), to eliminate unfused B82 mouse cells and permit survival only of cells expressing TK_B, the frog thermostable cytosol deoxypyrimidine kinase (Freed and Hames, 1976). The frequency of Hi-HAT resistant colonies rose from about 1×10^{-8} to 2.5×10^{-7} when the B82 cells were treated with PEG in the presence of ICR 2A cells. The effect required both ICR 2A cells and exposure to PEG. Representative resistant clones were isolated, expanded and shown to express a thymidine kinase with the electrophoretic mobility of the mouse cytosol enzyme. No amphibian chromosomes were detected cytogenetically. Thus, the evidence suggests that these strains are (mouse) revertants.

Why is TK reversion in the mouse cells enhanced by fusion with frog cells? Two classes of explanation seem worth considering. First, fusion with frog cells, by introduction of frog chromosomes that are subsequently degraded, may have induced a mutagenic repair phenomenon resembling radiation-enhanced-reactivation. Alternatively, introduction of the functional frog gene product, TK_B, may have permitted DNA synthesis in the presence of an imbalanced deoxynucleotide pool, a known mutagenic influence. In future experiments to detect the first class of effects, we wish to use purified DNA to avoid the possibility of effects of the second class.

STATISTICAL EVALUATION METHODS

The purpose of this work was to facilitate hypothesis testing in the interpretation of single cell survival and mutagenic response data. A particular aim was to provide objective methods for ascertainment of the best-fit curve and the confidence limits of the coefficients that describe it. At present, the survival curve analysis methods are complete, and curve-fitting methods for mutagenic response data are under development.

Single cell survival data in the form of colony counts per plate are provided to the ICR Biomathematics Laboratory for entry into the computer procedure. Their program calculates the surviving fractions at each dose, determines the best fit to the standard survival equation and displays the curve with proper coordinates, data points and values of D_o and D_q together with their confidence limits. Hard copies are printed. To compare two sets of survival data, the separately estimated values of the curve coefficients are assigned in various combinations to test whether these give significantly better fit than if the same values were used for both populations. Significance is tested by the sampled permutation procedure described in the attached reprint (Freed et al., 1979).

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Chan, G. L. and J. B. Little 1978 Induction of ouabain-resistant mutations in $C_3H10T_{\frac{1}{2}}$ mouse cells by ultraviolet light. Proc. Nat. Acad. Sci. USA 75:3363-3366.

Chang, C. C., J. E. Trosko and T. Akera 1978a Characterization of ultraviolet-light induced ouabain-resistant mutations in Chinese hamster cells. Mutation Res. 51:85-98.

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Morowitz, H. J. 1950 Absorption effects in volume irradiation of microorganisms. Science 111:229-230.

Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. Cheng and R. Axel 1977 Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223-232.

Wigler, M., A. Pellicer, S. Silverstein and R. Axel 1978 Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. Cell 14:725-731.

STAFF

	Effort	Source of Salary*
Jerome J. Freed, Ph.D., Member	50%	CA-06927
Carol E. Hepfer, Ph.D., Post-doctoral Associate	100%	CA-09035
Todd M. Kelman, B.S., Research Technician I (until August 1979)	100%	DE-AC02-76 EVO 3110
Mary Ellen Croke, B.A., Research Technician I (from August 1979)	100%	DE-AC02-76 EVO 3110
A. Catherine Bluett, Technician II	50%	DE-AC02-76 EVO 3110
Stephen K. Shuman, B.S., Summer Assistant	(summer)	RR-05539
Suzanne Rivitz, Summer Assistant	(summer)	RR-05539

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PUBLICATIONS

The following have appeared since our last annual report:

1978 Freed, Jerome J., Frank A. Angelsanto and Ronald H. Hoess Photoreversal as a tool for study of mutagenesis in haploid frog cell cultures. J. Cell Biol. 79:390 (abstract).

1979 Freed, Jerome J., Ronald H. Hoess, Frank A. Angelsanto and Holman C. Massey, Jr. Survival and DNA repair in ultraviolet-irradiated haploid and diploid frog cells. Mutation Res. 62:325-339.

Reprint + abstract Removed

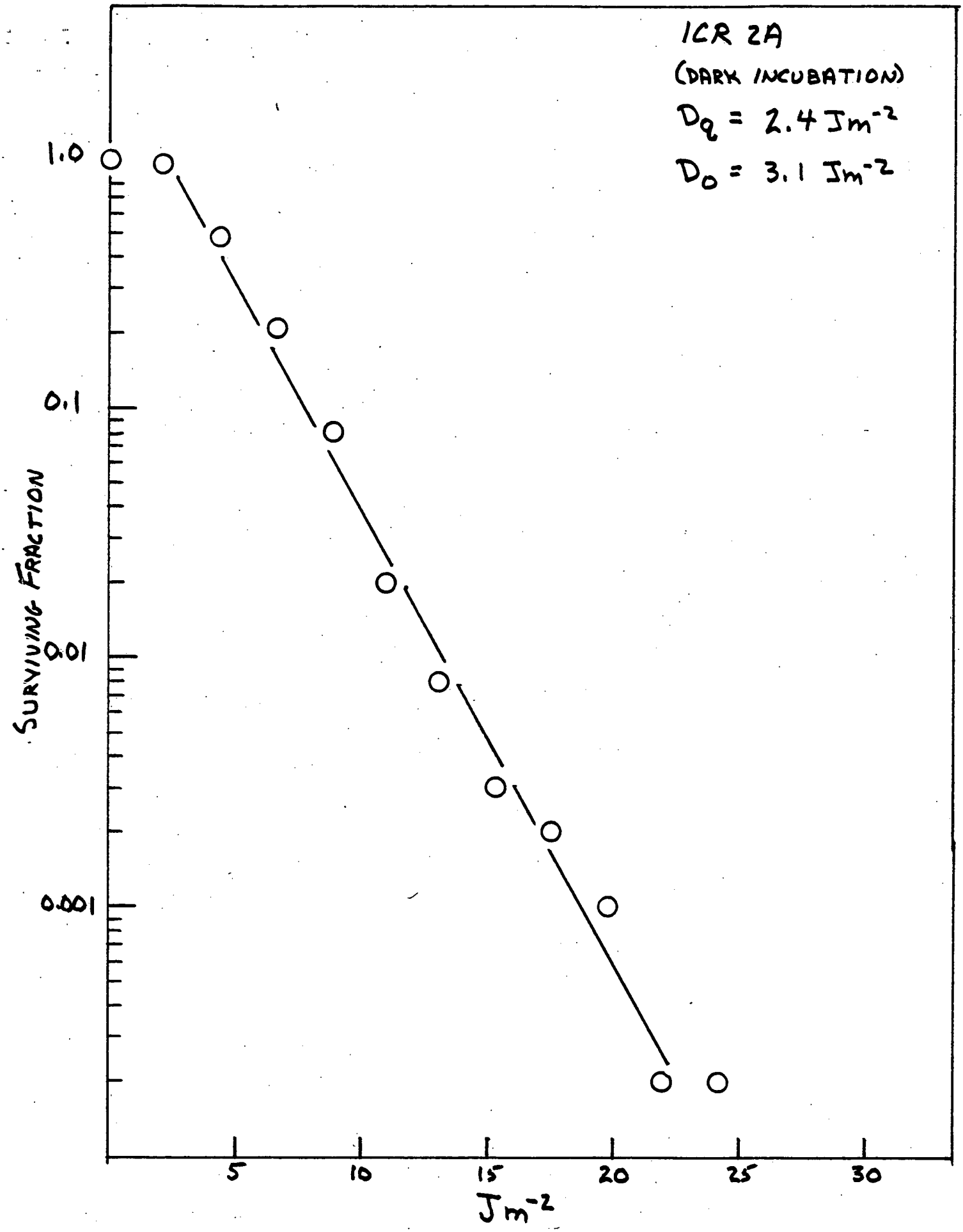

Figure 1. Haploid frog cell UV survival curve from a single experiment by the suspension method described in the text.

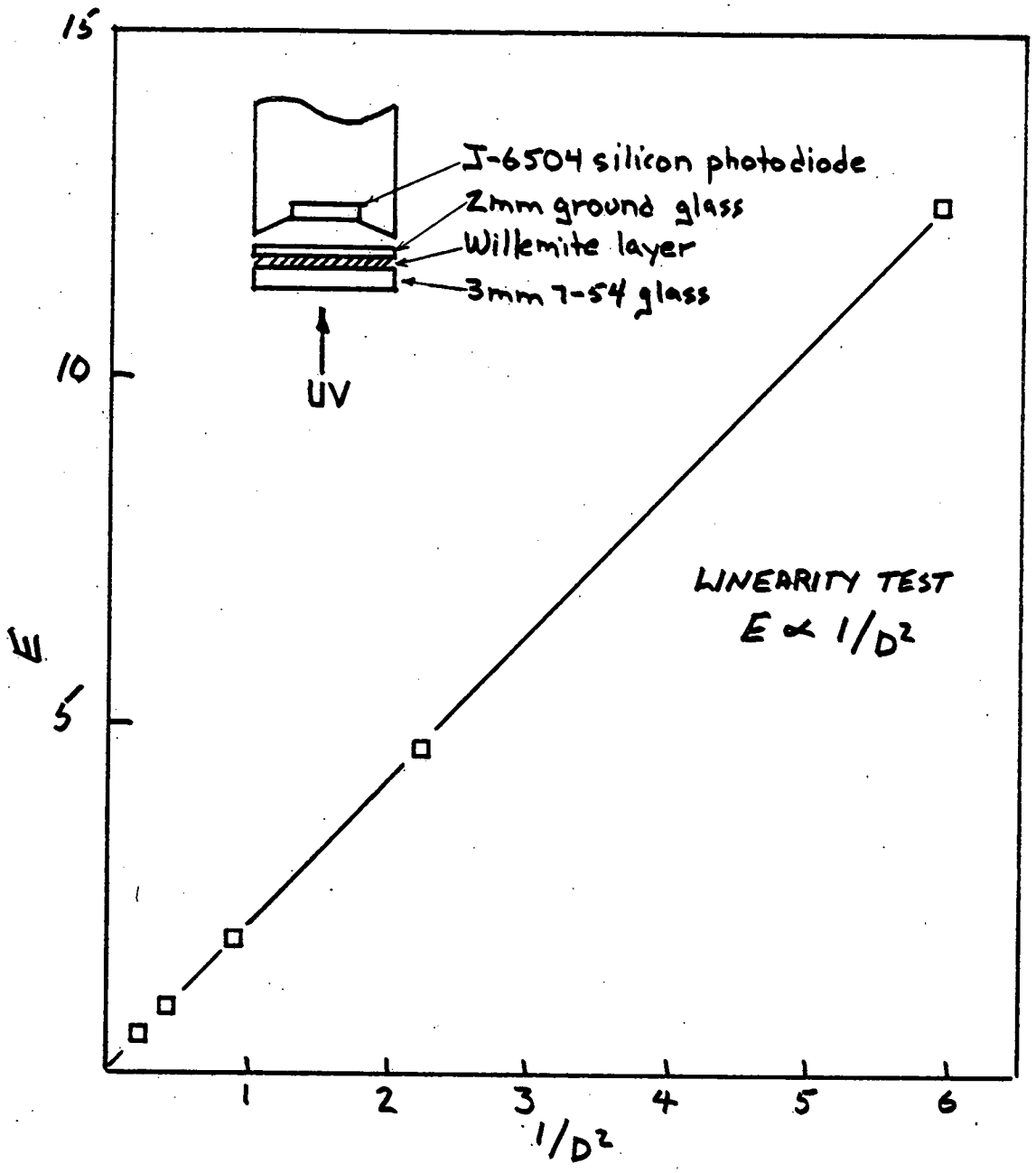

Figure 2. 254 nm-specific transducer attachment to a Tektronix radiometric probe and test of its linearity. D is the distance between the transducer and a 1 cm diameter source of 254 nm UV; E represents arbitrary units of photometer response.

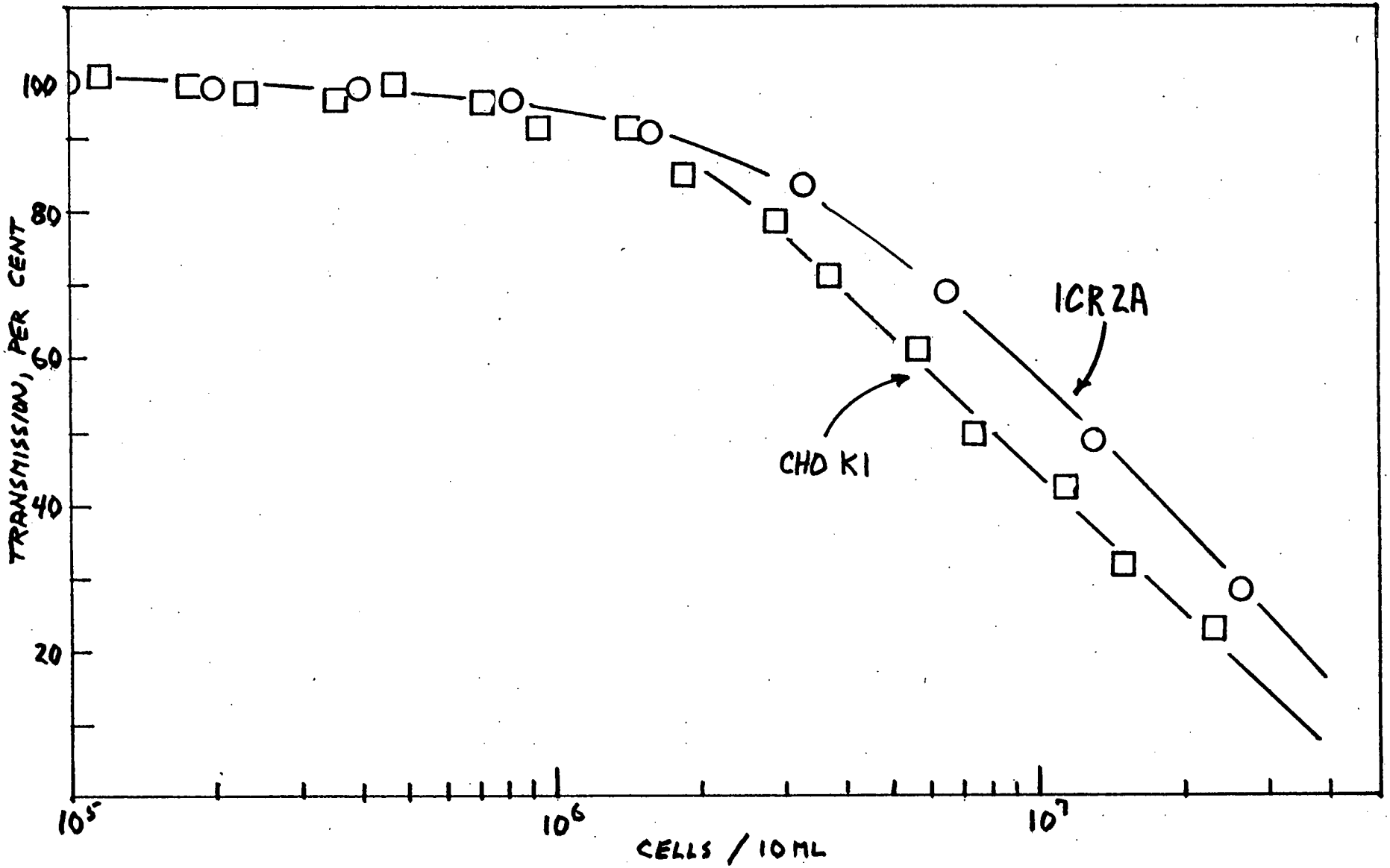

Figure 3. Transmission of cell suspensions, in the irradiation apparatus, as a function of cell density, as measured with the 254 nm-specific transducer.