A STUDY OF THE EFFECTS OF 60-HZ ELECTRIC FIELDS ON THE CLONING EFFICIENCIES OF CHINESE HAMSTER OVARY CELLS

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March 30, 1987

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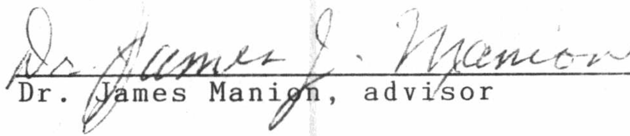

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ABSTRACT

Chinese hamster ovary (CHO) cells were exposed to 60-Hz electric fields at 18 V/m and 5 V/m, and the cloning efficiencies compared. Statistical studies failed to show a significant dose response for the field values tested. However, with regard to the 5 V/m field, a trend of 85% viability compared to sham-exposed cells was discovered.

In general, cloning efficiencies for sham-exposed controls were low. Various techniques were employed to increase the efficiencies of the controls. Suggestions for improving controls and the overall prodceure are discussed.

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INTRODUCTION

The use of electric power in today's industrial society is on the rise, and man-made electric and electromagnetic environments are present in ever-increasing levels and types. With this increased application of electric power, the probability of exposure of organisms, including man, has risen concommitantly. The frequencies of these fields range from 0-Hz to thousands of hertz. This paper is concerned with electric fields of very low frequency (60-Hz).

Many studies have been and are currently being done to detect effects on cell growth, metabolism, carcinogenesis, and mutation frequencies due to interaction with extremely low-frequency electric and/or magnetic fields. While it is now known that living organisms are indeed affected by electric fields, the mechanisms and interactions of such fields remain, for the most part, unknown.

Electric field studies employ various methods of detection of cellular effects. The means utilized in this study is a measurement of cell viability. Chinese hamster ovary cells (CHO) are cultured and exposed to 60-Hz electric fields of two different magnitudes. A known number of cells are plated and incubated. Viable cells will form visible colonies which can be counted and related to the original number dispensed. In this way, percent viability can be determined.

LITERATURE REVIEW

Nordstrom et al. (1980) performed epidemiological studies at the University of Umeå in Sweden which indicated an increased frequency of chromosomal aberrations in cultured lymphocytes of switchyard workers. There was also an increased frequency of abnormal pregnancies as evidenced by an increased number of children with congenital malformations. All workers in these studies had been exposed to 50-Hz electric fields which emanated from high-voltage equipment (cited in Mild et al. 1982).

Smith and Pilla (1982) showed that pulsed electromagnetic fields affected limb regeneration rates in salamanders. Basset et al. (1979) reported that electromagnetic fields of 20-40 microsecond pulses promoted bone fracture healing in dogs and humans (cited in Dihel, Smith-Sonneborn, and Middaugh 1985). Electric field strengths of 2-4 KV/cm have been observed to cause human erythrocyte hemolysis (cited in Mild et al. 1982). Sale and Hamilton (1967) and Hulsheger and Niemann (1980) reported that high-voltage pulsed fields have also had deleterious effects on bacteria and yeast cells (cited in Mild et al. 1982). An electric potential across the cell membrane is induced by the electric field. At a critical value of 1V, it is assumed the membrane experiences dielectric breakdown, causing changes in ion permea-

bility, and eventually lysis of the cells.

Systems for exposing cultured cells to extremely low frequency electric fields have been developed. Cell membrane damage in cultured human leucocytes has been observed with an electric field value of 2.6 KV/cm (Mild et al. 1982). Other biochemical responses encountered include: increased DNA synthesis (Rodan et al. 1978), altered Ca^{+2} transport across the plasma membrane (Basset 1982), and increases in transcription (Goodman et al. 1983). In addition, increased lysosomal activity (Norton 1982), and altered glycosaminoglycan content (Norton 1982) have also been observed.

Pulsed electromagnetic fields of 72-Hz may alter permability of cellular membranes to induce changes in ion transport. Dihel, Smith-Sonneborn, and Middaugh (1985) demonstrated increased rates of cell division in mutant strains of *Paramecium*. The mutants differed from the wild-type with respect to Ca^{+2} transport mechanisms.

These cellular responses have been observed in a wide array of cells and organisms. With this in mind, it has been suggested that pulsed electromagnetic fields might perturb cell membranes and cause significant alterations in ion transport and/or binding properties (Dihel et al. 1985).

METHODS AND MATERIALS

Exposure System

The system used to expose the cells operated at a frequency of 60-Hz. It consisted of five main parts (see fig. 1):

1) Isolation Transformer:
   The isolation transformer isolated the ground of the building power supply from the in vitro chambers. Its use was imperative for the safety of the system.
2) Control Variac:
   The output of this unit supplied the necessary voltage to energize the system. In combination with current-setting resistors, it determined the magnitude of the electric field.
3) System Unit:
   The System Unit determined exposure levels for the eight exposure chambers. The System Unit box consisted of eight pairs of connectors on top, between which resistors were connected that determined the magnitude of the electric field in the chambers. Due to the potentially high voltage of this unit, operation was only possible upon closure of a protective plastic shield.
   The System Unit utilized an electronic circuit that mea-

sured the current passing through the individual chambers. An external ac voltmeter was used to adjust individual chamber currents. The System Unit was calibrated such that with a switch position of ÷1 and x1, one volt (rms) corresponded to 1 mA of current passing through each chamber.

4) Exposure Trays:

Plexiglass trays equipped with four pairs of electrical connectors were capable of holding four exposure chambers each. Exposure trays were, in addition, equipped with electrical connectors so that they were connected to the System Unit via electric cables. The trays used in sham-exposed chambers had no such connectors, nor wiring, so that they recieved no current in any chamber.

5) Exposure Chambers (Electric Field (ELF) Tubes):

The exposure chambers consisted of two glass cylindrical tubes joined at sintered glass junctions (see fig. 2). Rubber stoppers with carbon electrodes were placed in each end. In addition, F-12 medium agar plugs were placed in each end. This prevented direct contact of the electrodes with the cell suspension to inhibit electrolysis products from interfering with cell metabolism. The top of each ELF tube had an access port. A rubber cap held an air exchange tube that was made from a bent Pasteur pipette. The tip was placed through the cap, and the open end plugged with sterile cotton.

Operation of the System Unit:

First, we determined the magnitude of the chamber current. The chamber current is directly proportional to the electric field in the chamber (see fig. 3). The fullness of the cell chamber and the conductivity of the F-12 culture medium determines this relationship. Our chambers were completely full, and we assumed the conductivity to be 1.9 S/m. Next, we chose the resistors to create the appropriate electric field. The desired voltage drop was approximately 50V across each resistor. This was calculated by multiplying the chamber current by the resistance value according to Ohm's law (V=IR).

We read the electric field value of each chamber by setting the channel switch to the appropriate corresponding position. In our exposures, we used resistors of 10 KΩ and 2 KΩ to approximate electric field values of 5 V/m and 18 V/m, respectively. The variac position was set between 50 and 54 volts in each case. The precision of the electric field values was not critical.

Exposure:

Chinese hamster ovary (CHO) cells were used in the exposures. They were grown in 75 ml. plastic tissue culture flasks. We used F-12 tissue culture medium containing 10% heat inactivated fetal bovine serum. The cells were incubated in 5% CO_2 at 37°C., until they were 85% confluent.

Trypsin was used to harvest the cells from the surface

of the flasks. Total cell counts were performed on a Coulter particle counter. We used 10% F-12 culture medium to dilute the cells to approximately 10^4/ml. Ten milliters of this suspension were pipetted under aseptic conditions to each exposure chamber via the access port. In this manner, twelve such tubes, containing 10^5 cells each, were prepared at one time. Four tubes were positioned in each of three exposure trays. The variac was set at approximately 50V, and the trays were connected to the System Unit. With a set of four 10 KΩ resistors in place, we attained an electric field of 5.3 V/m for one tray. The other set of four 2 KΩ resistors yielded field values of 18.4 V/m. In addition to the eight exposed tubes, we placed a set of four sham-exposed tubes in an unwired exposure tray. The trays were placed on separate shelves in an incubator. The cells were exposed to the field for a twenty-four hour period at 37° C. The CO_2 level was 7.5%

After exposure, the cells were dispensed into six-well tissue culture plates in the following manner (The entire procedure was done under a laminar flow hood):

a) The media was drawn off from each ELF tube with an aspirator, and cells were harvested from the glass bottoms with trypsin.

b) Once the cells were detached, enough media was added, via the access port, to dilute and inactivate the trypsin.

c) Total cell counts were performed separately for each ELF tube on a Coulter counter.

d) The diluted cell suspensions were aliquoted separately into plastic 50 ml. test tubes which also contained 35 ml. of F-12 medium with 5% serum. The final concentration was approximately 100 cells/ml.

e) Aliquots of 1.0 ml. were then dispensed into each of the six wells of the culture plates. In addition, each well contained 2.0 ml. of 5% F-12 medium. A set of five plates was prepared for each ELF tube.

f) The plates were incubated, completely undisturbed, for eight days at 37° C.

After incubation, the media was carefully aspirated from each well. The cells were fixed by addition of a 10% formalin solution for ten minutes. The formalin was then drawn off. Cell colonies were stained with crystal violet, rinsed with tap water, and air dried. The colonies in each well were counted on a Biotran colony counter.

Control Check:

It was necessary to check the cloning efficiencies of the control tubes. To do this, we cultured CHO cells in two plastic 75 ml. tissue culture flasks, which were used as shams. We then cultured cells in two ELF tubes. In addition, we determined the percent viability by comparing one untreated flask and one untreated ELF tube against a flask and ELF tube which had been irradiated. Each vessel was seeded with 10^5 CHO cells in 10% F-12 medium and incubated until confluent. The vessels were placed on ice. One flask and one glass tube were exposed to x-radiation, and the other pair were kept on ice. None of the cells were

exposed to an electric field.

After exposure, 100 cells were dispensed into each well along with 2.0 ml. of 5% F-12 medium. Five plates were prepared from the cells in each vessel. The plates were incubated for eight days at 37° C. After incubation, the cells were fixed, stained, and counted.

RESULTS

Sham-exposed cells consistently failed to show cloning efficiencies better than 70%. That is, out of the 100 cells dispensed, at most we observed a mean of only 70 colonies. In general, the counts were much lower (see fig. 4). Subsequent comparison of exposed cells were made to the controls. The following values are expressed in terms of "percent of sham".

In the case of the 18 V/m field, four exposures were performed. Two of the exposures showed a cloning efficiency greater than the sham-exposed cells, at 122% and 102%. Two of the exposures showed efficiencies less than the sham, at 60.3% and 70.0% (see fig. 5). This is a range of over 60%.

For the 5 V/m exposure, plating efficiencies were much more consistent. Five exposures were performed. In each case, the number of viable colonies arising following exposure was less than the number of sham-exposed cells. These values are given in figure 6. The median value was 84.1% of the sham, and the range was only 9.0%.

Due to the low number of colonies which grew in the shams, it was necessary to improve the cloning efficiencies in this group for comparative reasons. It was suspected that the agar used in the end plugs had cytotoxic effects. To test this, we cultured cells in plastic 25 ml. tissue

culture flasks under two conditions. Ten milliters of 1% ion agar were added to each of two flasks, and two flasks contained no agar. CHO cells were grown in each flask in 10% F-12 medium. These flasks were incubated together until they were 85% confluent. These cells were not exposed to any electric field. Cells were harvested with trypsin and brought up in 5% F-12 medium. Total cells counts were performed, and 100 cells were dispensed into each well as described in the previous section. Five plates were prepared from each flask. These plates were incubated for eight days. The cells were fixed, stained, and counted.

The cells grown in the presence of ion agar showed a 90% decreased plating efficiency compared to the non-agar exposed cells (see fig. 7).

Results of Control Check:

We observed an average of 60 colonies per well from the untreated plastic flask. Cloning of cells from the glass ELF tube was 62% as efficient, with an average of 37 colonies per well (see fig. 8).

In the irradiated plastic flask, we observed an average of 31 colonies per well. From the irradiated glass ELF tube, we observed the lowest cloning efficiencies of all four vessels. The average was 65% of the irradiated sham, or approximately 20 cells per well.

We also compared the results of the irradiated vessels with their untreated counterparts. This was to check the consistency of the percent of cells killed by the x-radiation

In the case of the plastic flasks, 49.3% were killed by the radiation. In the case of the glass ELF tubes, 46.8% of the cells were killed. This is evidence that the radiation killed the same percentage of cells in each vessel.

DISCUSSION AND CONCLUSIONS

Initial experimental runs showed low cloning efficiencies for sham-exposed cells. Several changes in cell processing techniques were made in hopes of improving cell health.

It was thought that the cells may have been overcrowded while in the exposure chamber. The chamber volume was doubled to 20 ml., but this seemed to have no significant effect in increasing cell counts.

A fresh and more active trypsin solution was used to detach cells from the vessels in order to decrease the length of time in which the cells were exposed to this protease. This significantly increased the number of viable CHO in our controls.

We also suspected that clumping of the CHO cells was giving erroneous readings on the Coulter counter. Thus, cells were kept on ice and gently pipetted up and down several times prior to diluting and dispensing to minimize clumping. This seemed to improve the cloning efficiencies in the controls, as well as in the exposed cells.

For exposed cells, no dose response was observed. In the case of the 18 V/m field, no consistent or repeatable results were observed. Two of the four exposures showed cloning efficiencies greater than sham exposed cells, and two of them showed cloning effiencies less than the shams.

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The most plausible explanation for these results is human error and/or variations in procedure, experimental conditions, or both.

On the other hand, while no consistent relationship can be shown between the two doses of electric fields, there is a notable trend with respect to the 5 V/m field. Within each experimental run, there was consistency in the percent viability of the exposed cells with their respective shams, with only a 9.0% difference between extreme values. Although these results cannot be considered significant due to the low numbers of colonies in the controls, it is interesting to note this tendency in spite of less than optimal control conditions.

One possible explanation may be that there is some unknown critical electric field value, which is neither so large that it exerts general toxic effects on the cells, nor so small such that is is incapable of inducing changes in cell function. If this is the case, then the critical value would be smaller than 18 V/m and probably closer to 5 V/m.

As mentioned earlier, control colony counts were too low to be of immediate value. It is necessary to increase them consistently to at least 80 or 90 colonies per well. Our results indicate the main cause of decreased cell viability to be the agar used in the end plugs. This substance may have been responsible for as much as 90% of the cell death observed. This is supported by the experimental results

of the effects of 1% ion agar on cell viability. The purpose of the end plugs is to prevent direct contact of the carbon electrodes with the growth medium, as electrolysis products may somehow interfere with cell membrane function. A semi-solid material, with conductivity similar to that of the growth medium, and without cytotoxic constituents must be found before any meaningful data can be obtained. We are presently searching for such a suitable material.

An alternative, but more costly, approach could be made in the design of the exposure chambers. It is apparent that cells attach and grow better on plastic substrates than on those of glass. If the chambers were composed of plastic, an increase in cell retrival may be possible. The disadvantages are that this would require redesign of the chamber, and the chambers would not be reusable. Both of these factors would greatly increase the cost of the experiment.

From this study, it cannot be concluded with certainty that the electric fields used induced cell death, but some of the results seem to indicate this as a possibility. When improvements in our control groups are made, additional studies may provide us with more significant findings.

Exposure System - Electric Fields

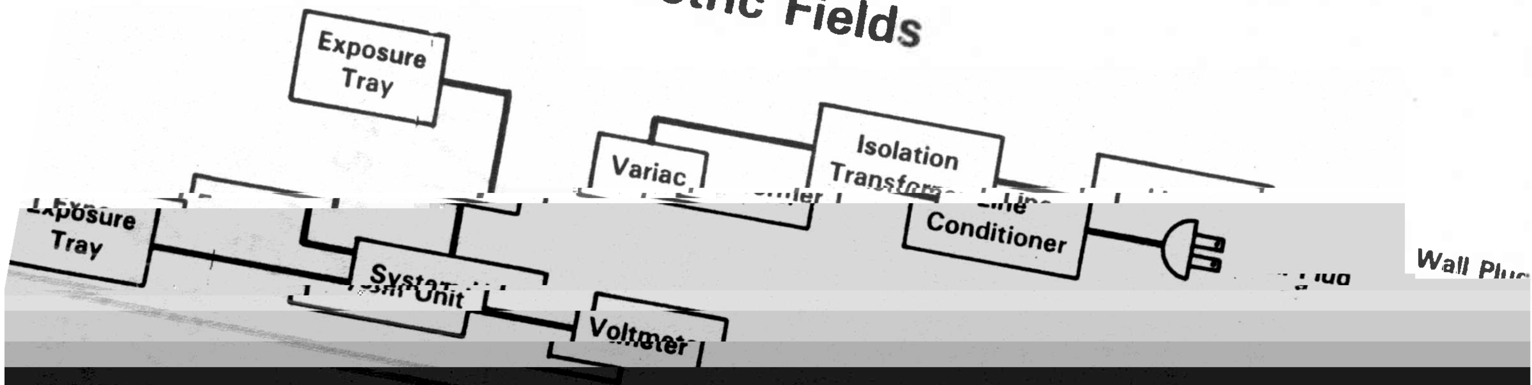

Figure 16

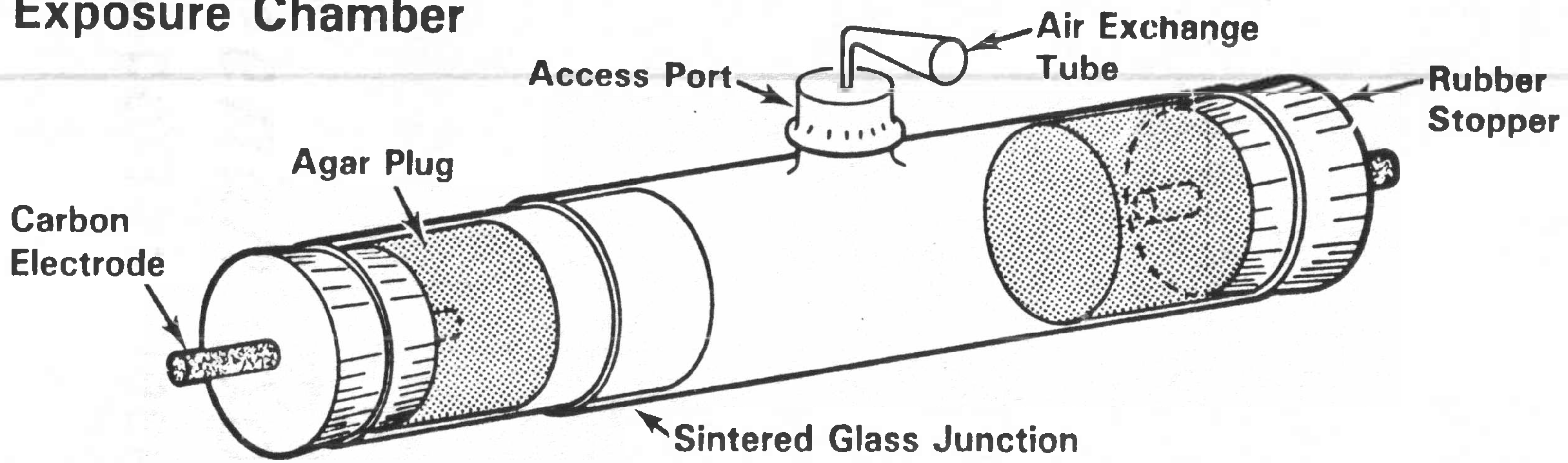

Figure 2

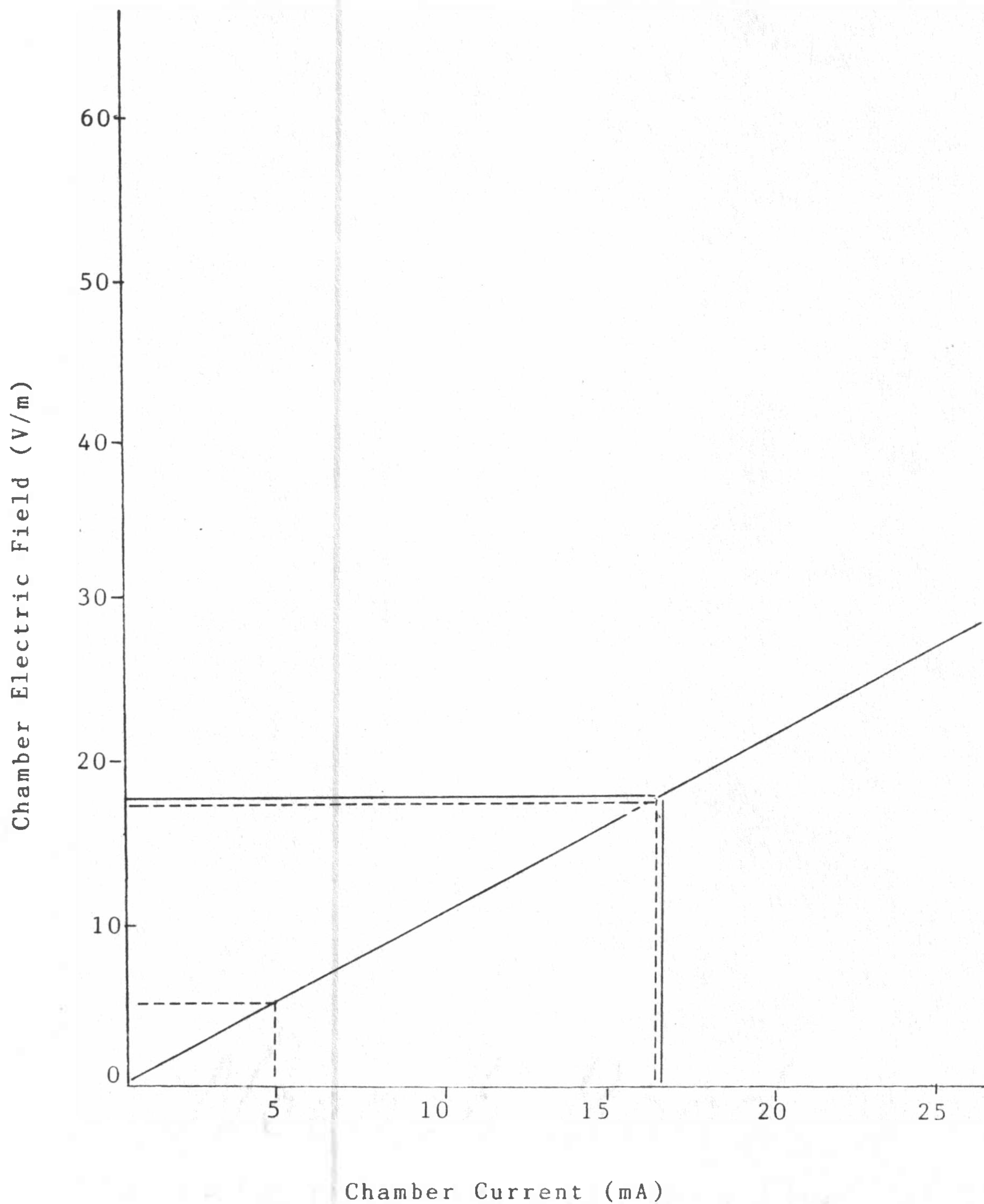

Figure 3--Estimation of electric field in glass ELF tube. Chamber electric fields, assuming the central volume between the chamber agar plugs is completely full. A medium of conductivity 1.9 S/m was assumed.

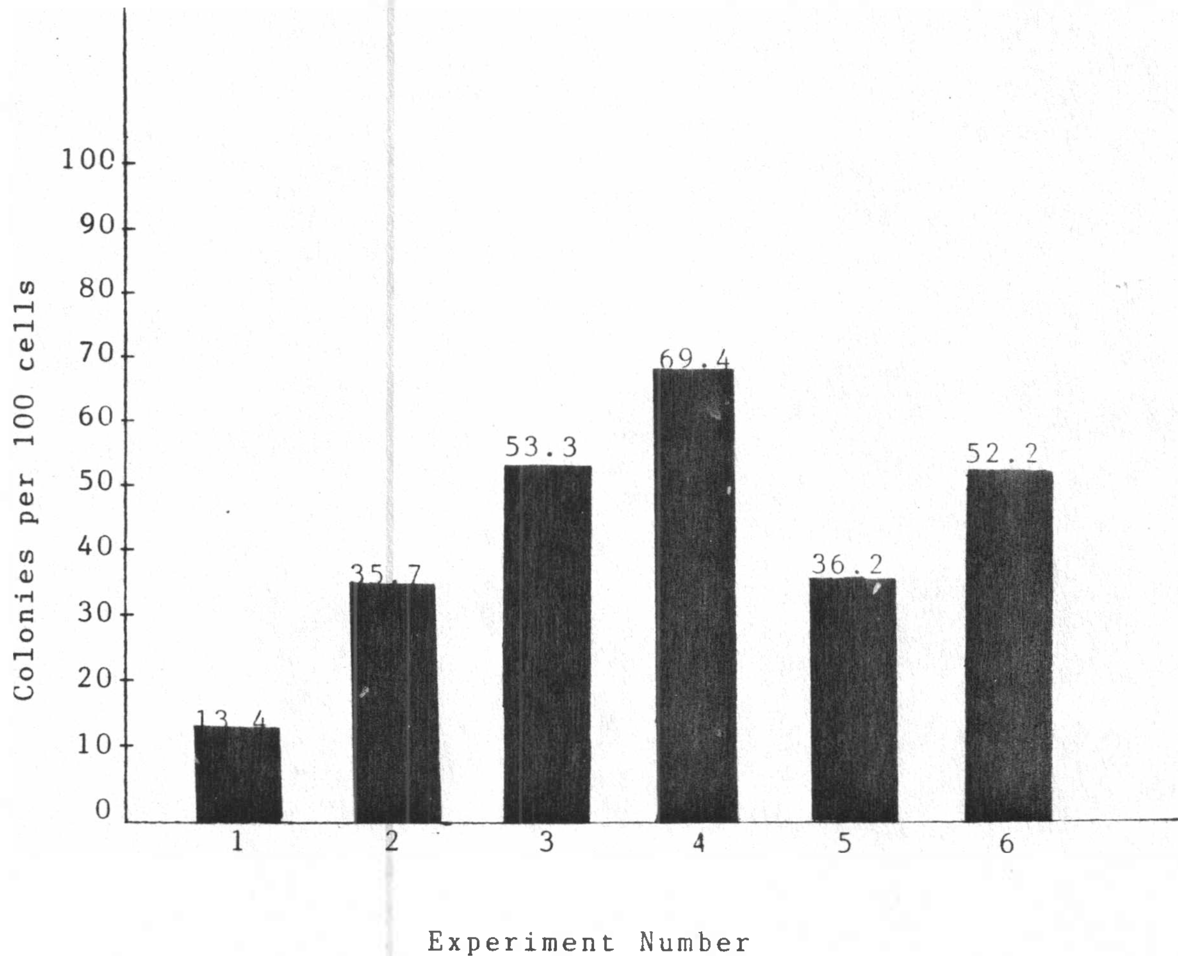

Figure 4--Summary of the cloning efficiencies of sham-exposed cells. None of the cells were exposed to an electric field. The numbers expressed are the mean number of cells which grew in each of 120 culture wells. Overall cell counts in the controls were low. For statistically significant results, 80 to 90 clones per well were needed.

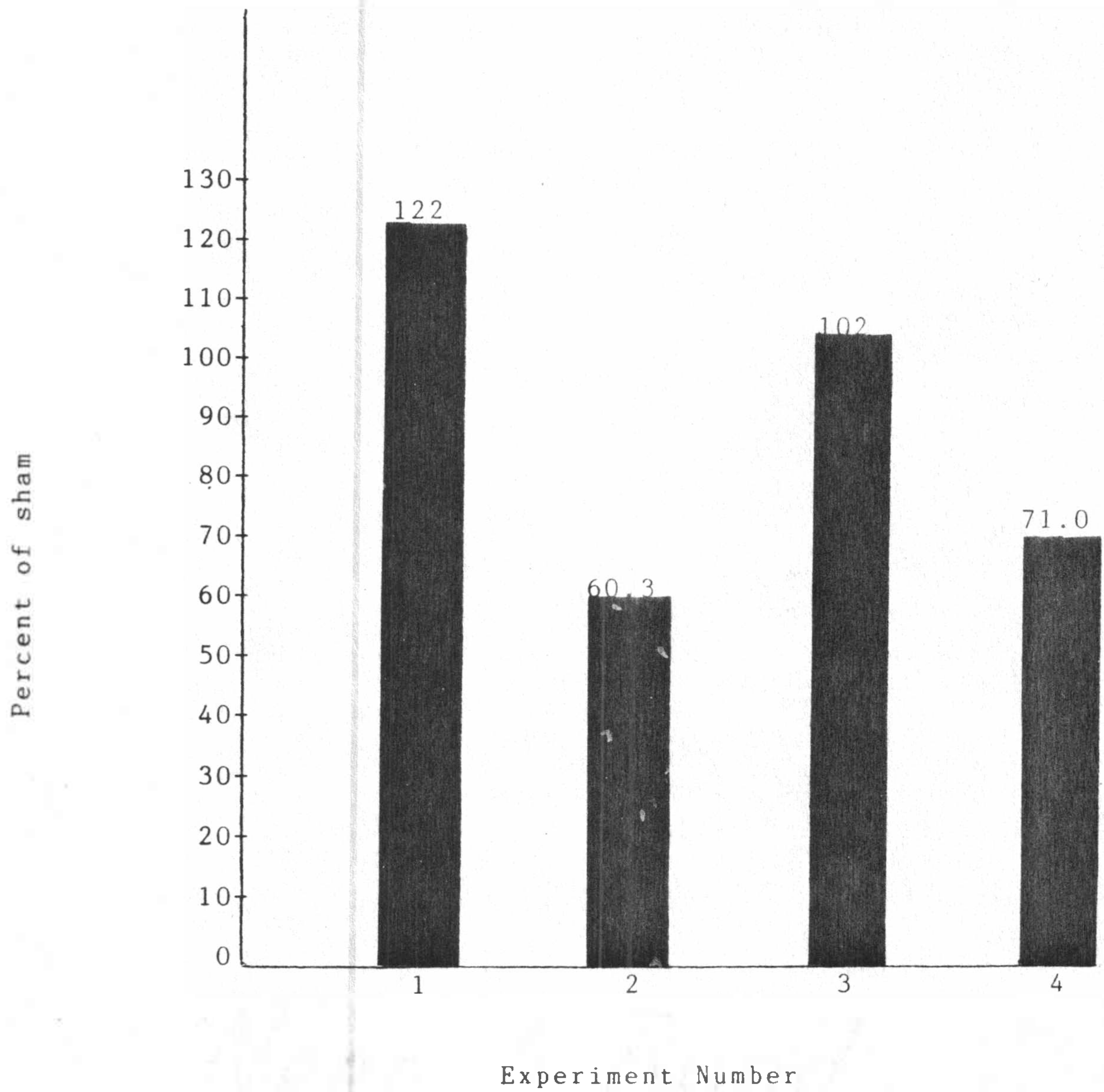

Figure 5--Summary of the cloning efficiencies of cells exposed to an 18 V/m electric field. Experiment numbers correspond to those of the controls. The mean number of cells per 120 wells was computed and expressed as a ratio of the sham mean.

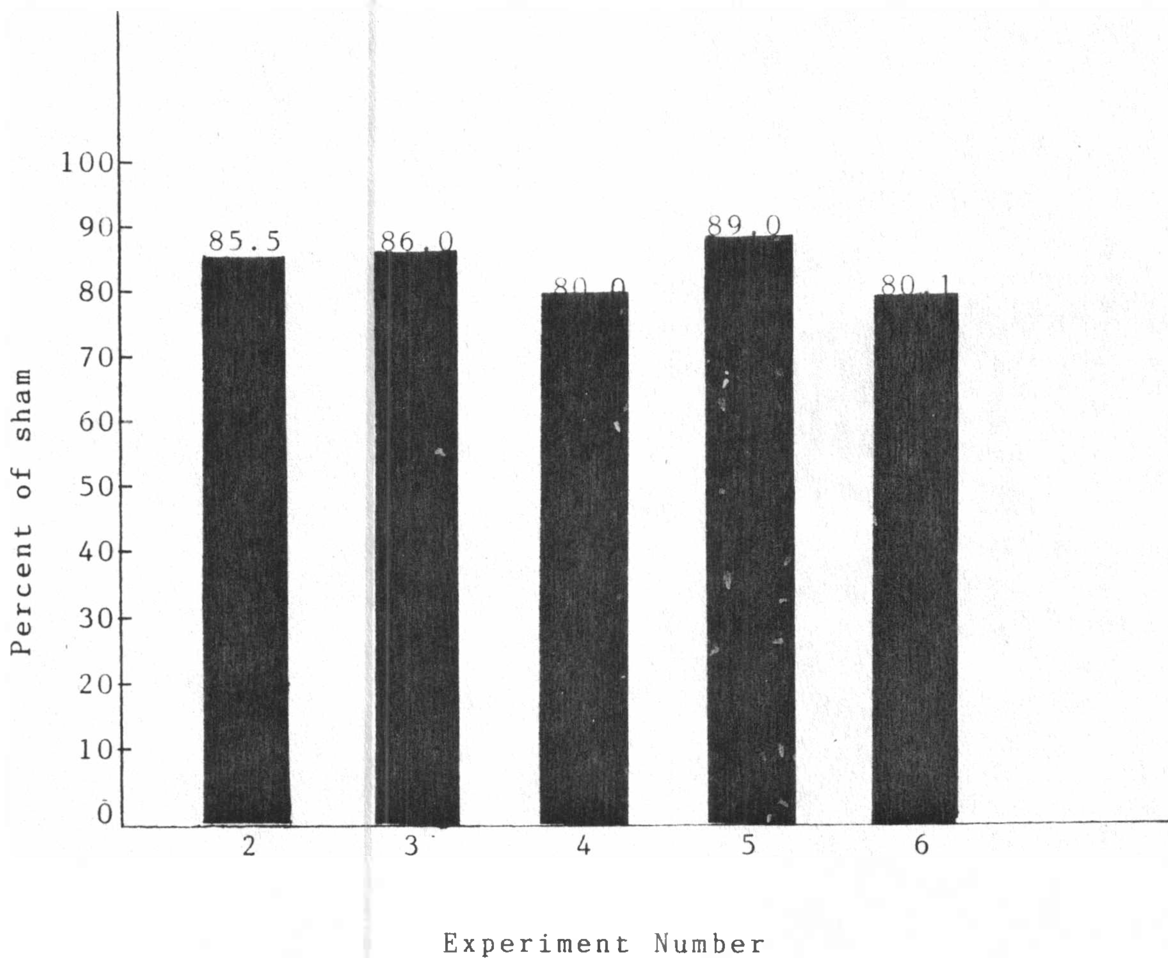

Figure 6--Summary of the cloning efficiencies of cells exposed to a 5 V/m electric field. Experiment numbers correspond to those of the controls. The mean number of cells per 120 wells was computed and expressed as a ratio of the sham mean.

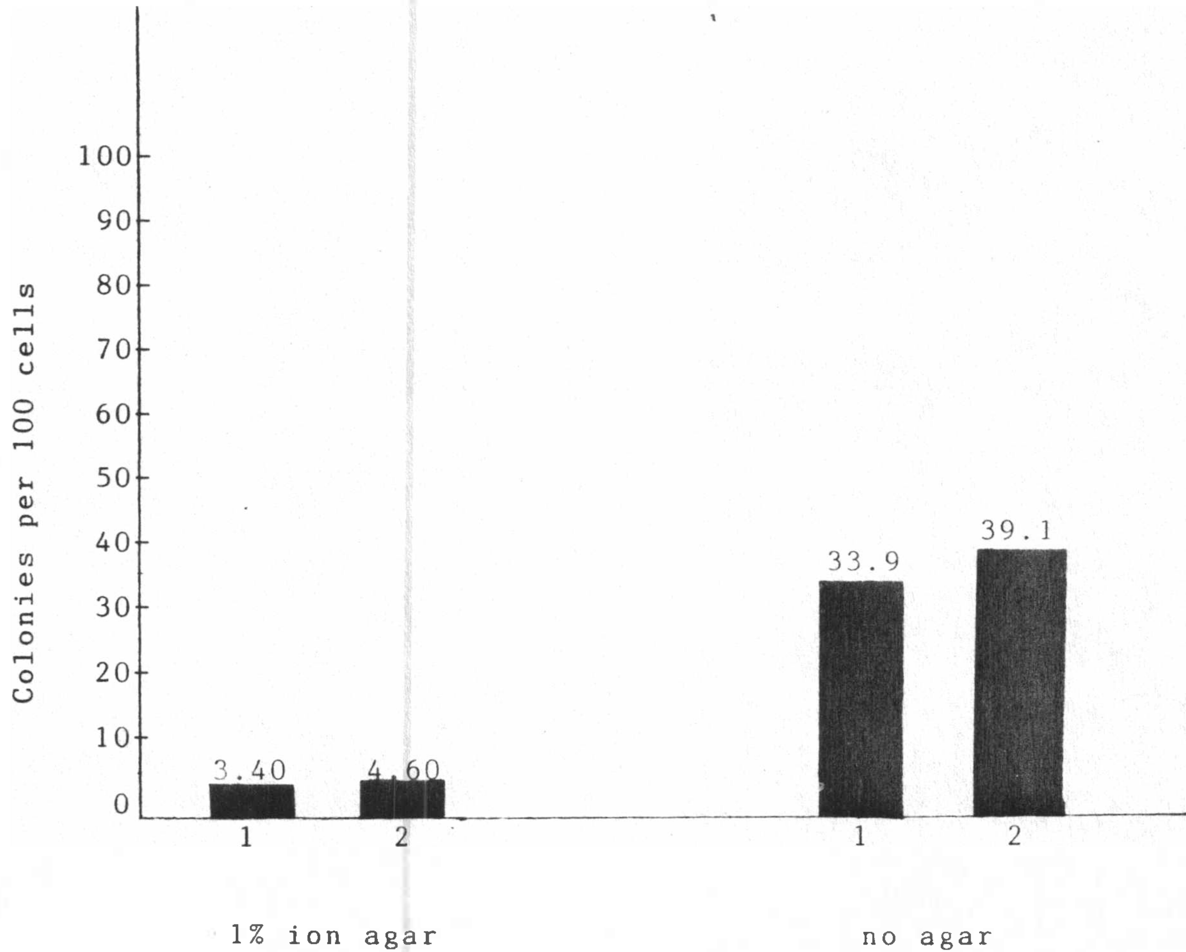

Figure 7--Effects of 1% ion agar on cell viability. Each number expressed is the mean number of cell colonies which grew in each of 18 tissue culture wells. The cells grown in the presence of 1% ion agar showed a 90% decreased plating efficiency compared to those grown only in the F-12 medium.

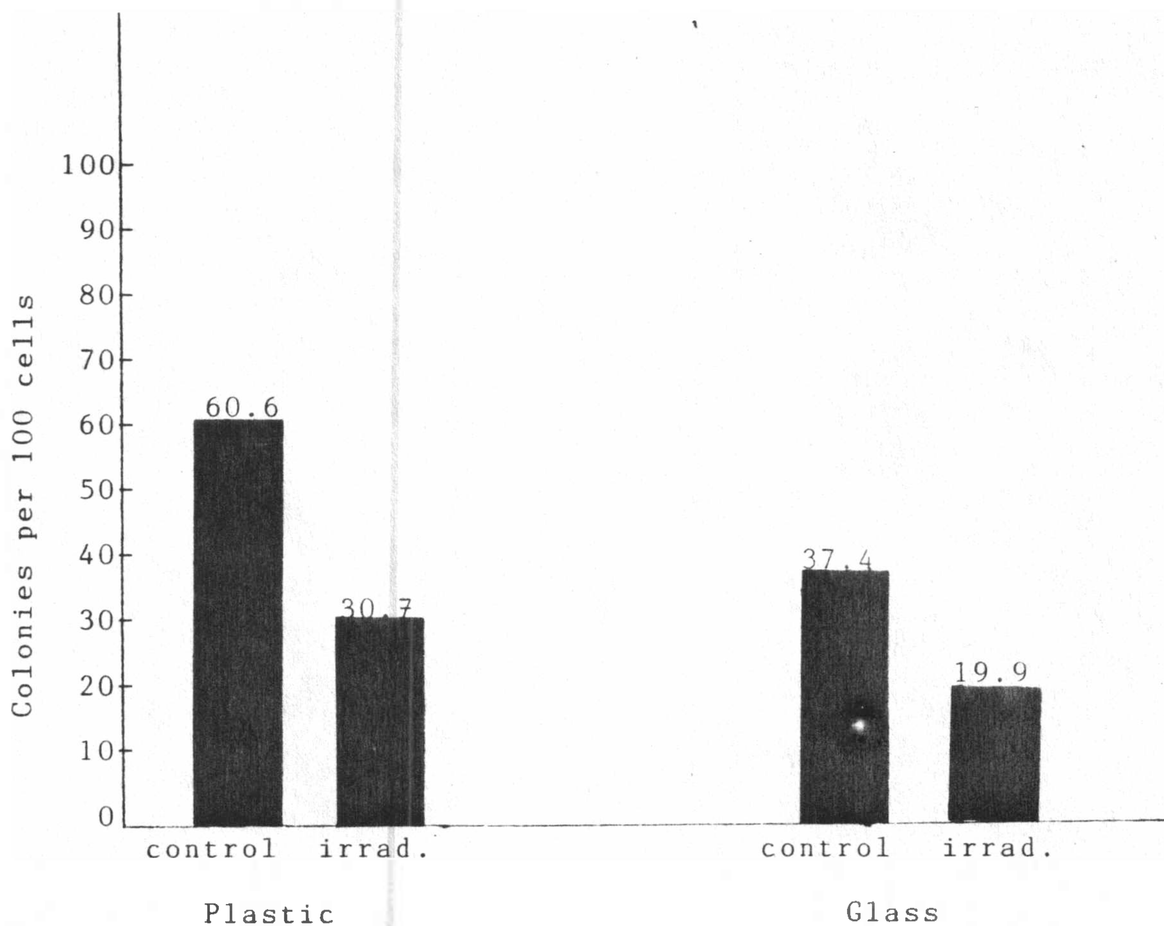

Figure 8--Summary of control check. Treatment with x-radiation in the plastic and glass vessels killed 49.3% and 46.8% of the cells, respectively. Cells grew better in the plastic vessels than in the glass tubes. The ratios of the means of the plastic to the glass were 1.62 and 1.54 for the non-radiated controls and the radiated vessels, respectively.

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