BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF DEFICIENT MAMMALIAN CELLS IN CULTURE

by Paul Todd, Donner Laboratory, University of California, Berkeley, California

Biophysical Society
8th Annual Meeting
Chicago, Illinois
February 26-28, 1964

HISTORY

In 1953 Zirkle and Tobias[1] issued a report on the role of LET and ploidy in what was considered the recessive lethal killing of yeast by ionizing radiation. Following this same line of research, Tobias and Stepka, writing in 1955[2], isolated yeast colonies from irradiated cultures and found them to bear their sublethal damage indefinitely or until spontaneous "mutations" repopulated the cultures with normal cells. This phenomenon was termed "mutation to increased radiosensitivity," and the cells were said to bear unpaired defects. This interpretation was later brought under question by Magni[3].

Ruddle[4], working at the University of California, X-irradiated cultures of diploid pig kidney cells and isolated stable cell lines with heritable alterations of their karyotypes. Sinclair[5] noted, as had Puck[6] and Elkind[7] before him, that surviving colonies in irradiated cultures are morphologically non-uniform. The mean colony size was found to decrease with increasing dose, and this can be seen in the histogram of Figure 1, which represents results obtained in the present work, using a long-term line of human kidney cells obtained from Dr. G. W. Barendsen's laboratory in Rijswijk, the Netherlands[8]. Sinclair found that isolated cells from small colonies among survivors to X-irradiation grew more slowly, were more sensitive to inhibition of their colony forming ability by X-rays, and consistently had much lower plating efficiencies. These data are corroborated in a general way by the analogous results obtained with

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T1 cells in Figure 2.

Sinclair[9] reported that there were no obvious relationships of these phenomena to the number of chromosomes in these cells which were said to bear "heritable radiation damage."

METABOLIC DEFICIENCIES

Human kidney cells designated clone T91M in the previous figure were serially propagated from a small colony developed from a single cell after 500 rads of X-irradiation and tested for their ability to utilize oxygen, on the basis that they might be remotely related to respiratory deficient yeasts (petites) which appear frequently in irradiated cultures, as noted by Raut and Simpson[10]. Figure 3 shows a typical oxygen consumption experiment using classical manometric techniques.

It can be said that the deficient cells do consume oxygen and that their respiratory pattern differs from that of the unirradiated parent line. The levelling of the curves is evidently due to substrate exhaustion. Equal concentrations of both cell types were used in these experiments, due to the influence of cell concentration on the rates of metabolic processes[11].

The deficient line T91M did not respond appreciably to growth in improved medium. Its doubling time in Eagle's MEM[12] with 10% fetal bovine serum was 28 hours, compared to 27.5 hours in a medium similar to Elkind and Sutton's HU-15[7]. T1 cells normally double every 22 to 24 hours.

RADIOBIOLOGICAL STUDIES

The interpretation of the survival curves in Figure 2 is not simple, and cannot be logically developed without recourse to other

experiments on T1 cells in our laboratory. Survival curves determined under various conditions can be described operationally as consisting, in part, of a product of an exponential inactivation curve and a dose-response curve for several identical recoverable sub-lethal sites, 911 of which must be inactivated to kill a single cell. This situation is described by equation (1).

$$S = e^{-D/D_1}\left[1 - (1 - e^{-D/D_2})^n\right] \qquad (1)$$

The values of D_1, D_2, and n are not immutable, but the T1 cell survival curve of Figure 2 agrees roughly with

$$D_1 = 500 \text{ rads}$$

$$D_2 = 240 \text{ rads}$$

$$n \approx 3.0,$$

after correction for the average number of cells per colony at the time of exposure to X-rays. The plotted curve in Figure 2 corresponds to equation (1) with these values.

The radiobiologically important question is the following: Does the inherited sublethal damage correspond to the normally recoverable radiosensitive sites? If so, the corresponding mathematical expression must apply. The applicability of the appropriate analytical description is a necessary, but not sufficient condition to identify the inherited damage with recoverable sites.

Equation (1) is obtained from the general form shown in equation (2).

$$S = e^{-D/D_1} \sum_{m=0}^{m=n-1} \frac{n!}{m!\,(n-m)!} (e^{-D/D_2})^{n-m} (1 - e^{-D/D_2})^m \qquad (2)$$

If one of the n sites is already inactivated, then the expression is

summed from m = 0 to m = n-2. The resulting expression for n = 3 is equation (3)

$$S = e^{-D/D_1}\left[e^{-2D/D_2}(3 - 2e^{-D/D_2})\right] \quad (3)$$

If one uses the same values for the mean lethal doses, namely

$$D_1 = 500 \text{ rad}$$

$$D_2 = 240 \text{ rad},$$

equation (3) is the curve which is plotted for the survival of T91M cells in Figure 2. One necessary condition appears to be satisfied for the identification of heritable damage with the recoverable sites.

On the other hand, if the damage which results in deficient cells is registered at recoverable sites, then the recovery process should be equally applicable for the production of deficient cells as for radiation lethality. In other words, fractionated doses should produce fewer deficient cells than a single, instantaneous dose of X-rays. That T1 cells recover from sublethal radiation events during the first post-radiation generation is shown in Figure 4. The ratio of curve C to curve B is about 2.6, which indicates at least that number of recoverable radiosensitive sites. However, if the histograms of Figure 1 are compared with histograms obtained from cultures exposed to two doses of X-rays separated by 25 hours (see Figure 5), it appears that fractionated doses are just as effective as single doses in the production of deficient colonies. Note, for example, that 1100 rads, when fractionated in this manner, is equivalent to about 900 rads for cell killing but is much more effective for the production of deficient colonies.

The production of deficient colonies (as evaluated by postradiation

colony size distribution) by carbon-ion irradiation was investigated. Figure 6 indicates that high LET radiation is very efficient for the process if the top row of histograms (X-rays) is compared with the lower row (C ions). Much lower doses of carbon ions were delivered. Inactivation experiments with carbon ions indicate that accumulated sublethal damage plays little or no role in cell killing by high LET radiations. Evidently, deficient cells are produced in these experiments independently of lesions registered in the recoverable sites.

Evidently the appearance of a deficient cell is not identical with the registration of a sublethal number of radiation lesions. The only consistent interpretation, then is to suppose that the damage to such cells is registered as a metabolic derangement which limits the ability of the cells to reverse accumulated sublethal events. The metabolic deficiencies are expressed, for example, in the reduced growth rate and oxygen consumption of the dificient cells.

CYTOGENETIC STUDIES

In consideration of Puck's view that the principal radiation damage is registered in the chromosomes[13], T91M cells were examined cytogenetically and found to contain, in general, fewer chromosomes than T1 cells, as indicated in Figure 7. Both cell lines are highly aneuploid, and the distribution of chromosome number in T91M cells could result from the fortuitous selection of such a clone from the parent line, irrespective of its history.

Ruddle was able to select from irradiated cultures clones with stable abnormal marker chromosomes. An experiment was designed to determine whether or not such cells were identical with deficient cells. Clearly the experiment could not be performed with aneuploid T1 cells,

so a line of diploid Chinese hamster fibroblasts was established from a bone-marrow aspirate from which a diploid clone had been selected and designated M3-1.

Small aberrant clones were selected from a culture which had been exposed to 500 rads of X-radiation and allowed to grow 2 1/2 weeks post radiation. Four such clones were selected for an experiment in which radiation survival, growth rate, and karyotype were analysed simultaneously. The growth and survival kinetics of these four clones are summarized in Figure 8. The chromosome number distributions of each of these lines is shown in Figure 9. In accord with similar observations by Sinclair, the numbers of chromosomes cannot be said to change. The idiograms of diploid cells from each clone are shown in Figure 10 in comparison with that of the parent M3-1 clone and a normal schematic idiogram[14]. Apparently the heritable deficiencies are not visibly registered in the chromosomes.

One deficient hamster cell clone was tested for the respiratory effect, and the finding was positive, as indicated in Figure 11. Evidently a correlation exists between human and hamster cells with regard to these deficiencies.

DISCUSSION

The frequency with which these deficiencies occur indicates, at first glance, that, where large fractionated doses are involved, such as in tumor therapy, the increased radiosensitivity of the deficient cells may over-ride the importance of repopulation and cellular recovery of the Elkind type (illustrated in Figure 4). In vivo experiments, however, suggest that this is not the case, as has been demonstrated in the classical studies of Blair and his co-workers. Either the phenomenon of

deficient cells is an artifact of *in vitro* experimentation, or their sublethal damage can be reversed in the chemically complete *in vivo* milieu.

The mechanism of inheritance of these persisting deficiencies remains a mystery, and we are currently preparing a series of biochemical studies to further investigate the genetic and environmental control of carbohydrate metabolism in mammalian cells.

ACKNOWLEDGMENTS

The advisory support of Dr. Cornelius A. Tobias is gratefully appreciated. This research was jointly supported by the USAEC and the NASA.

REFERENCES

(1) R. E. Zirkle & C. A. Tobias: Arch. B & B 47, 282 (1953)

"Effects of Ploidy & LET on Radiobiological Survival Curves"

(2) C. A. Tobias & B. Stepka: University of California Radiation Laboratory Report UCRL-1922, 40 (1955)

"Mutation to Increased Radiation Sensitivity in Yeast"

(3) G. E. Magni: Rad. Res. Suppl. 1, 347-356 (1959)

"Genetic Effects of Radiation on Yeast Cells and Genetic Control of Radiosensitivity"

(4) F. H. Ruddle: Cancer Res. 21, 885-894 (1961)

"Chromosome Variations in Cell Populations Derived from Pig Kidney"

(5) W. K. Sinclair: in Radiation Effects in Physics, Chemistry, and Biology Ed. . . Ebert & A. Howard 113 (North-Holland, 1963)

(6) T. T. Puck: Progr. in Biophys. 10, 237 (1960)

"In vitro Studies on the Radiation Biology of Mammalian Cells"

(7) M. M. Elkind & H. Sutton: Rad. Res. 13, 556 (1960)

"Radiation Response of Mammalian Cells Grown in Culture I. Repair of X-Ray Damage in Surviving Chinese Hamster Cells"

(8) G. W. Barendsen, T. L. J. Beusker, A. J. Vergroesen, & L. Budke: Rad. Res. 13, 841 (1960)

"Effects of Different Ionizing Radiations on Human Cells in Tissue Culture II. Biological Experiments"

(9) W. K. Sinclair: Rad. Res. 21, 581-611 (1964)

"X-Ray Induced Heritable Damage (Small-Colony Formation) in Cultured Mammalian Cells"

(10) C. Raut and W. L. Simpson: Arch. Biochem. and Biophys. 57, 218-228 (1955)

"The Effect of X-Rays and of Ultraviolet Light of Different Wavelengths on the Production of Cytochrome-Deficient Yeasts"

(11) H. T. Zwartouw & J. C. N. Westwood: Brit. J. Exp. Path. 39, 529 (1958)

"Factors Affecting Growth and Glycolysis in Tissue Culture"

(12) H. Eagle: Science 130, 432 (1959)

"Amino Acid Metabolism in Mammalian Cell Cutures"

(13) T. T. Puck: Am. Naturalist 94, 95 (1960a)

"The Action of Radiation on Mammalian Cells"

(14) G. Yerganian: in Methodology in Mammalian Genetics, Ed. W. J. Burdette, p. 469 (Holden-Day, San Francisco, 1963)

"Cytogenetic Analysis"

FIGURE CAPTIONS

Figure 1.

Colony size distributions of T1 cells exposed to various doses of 50 kVp X-rays and allowed to grow 12 days. The ordinate is number of cells per 0.1 mm. diameter interval. Uppermost size class is artificially more populous due to the inclusion of all colonies greater than 1.0 mm. in diameter. Ordinate of the control culture was multiplied by 1/2 to give it a scale comparable to irradiated cultures.

Figure 2.

Growth and X-ray survival curves of T91M deficient human kidney cells (solid circles) compared to those of normal T1 cells (open circles). P. E. = 137.5 $\pm$ 11.0% for T1 cells and 38.8 $\pm$ 11.9% for T91M cells.

Figure 3.

Oxygen consumption curves for parallel cultures of T1 cells and T91M cells in MEM spinner medium initially containing 1 g/liter glucose and no pyruvate. Initial slopes are nearly equal, but total substrate utilization appears to be less.

Figure 4.

Survival curves for T1 cells: A; 4 hours after plating (open circles); B: 25 hours after plating (solid circles); C: 25 hours after plating and 21 hours after an initial X-ray dose of 500 rads (squares). Doubling time was about 25.0 hours for unirradiated cells; P. E. = 86.0 $\pm$ 8.2%.

Figure 5.

The effect of dose fractionation on the production of deficient colonies of T1 cells by 50 kVp X-rays. Ordinate is the per cent of total colonies per 0.1 mm. diameter interval, and the two smallest size classes are lumped at high doses. Top row: single doses; bottom row: two doses separated by 25 hours.

Figure 6.

Colony size distribution of T1 cell cultures exposed to various doses of 50 kVp X-rays and C^{12} ions (LET = 2200 MeV-cm^2/g). Histograms are formed on the same basis as described in Figure 1. 12 days of growth.

Figure 7.

Comparison of chromosome number distributions for T1 cells and T91M cells.

Figure 8.

Growth and radiation survival curves for four sublines of Chinese hamster cells derived from single irradiated M3-1 cells. Dashed curves correspond to results expected with M3-1 cells. The radiation survival curve of line M147E did not appear to differ from the normal. Plating efficiencies were as follows:

M147F 53.7 $\pm$ 2.9%

M147M 48.6 $\pm$ 5.9%

M147J 52.7 $\pm$ 5.3%

M147E 21.1 $\pm$ 4.6%

Figure 9.

Chromosome number distributions of cultures of four sublines of Chinese hamster cells derived from single irradiated M3-1 cells. These are the same lines as were used to obtain the data of Figure 8. Number of chromosomes per cell was determined by counting the number of chromosomes in each observed cell twice[4].

Figure 10.

Idiograms of the four sublines of Chinese hamster cells described in the preceding two figures. Typical strict diploid cells were chosen for constructing idiograms. No gross abnormalities were observed in any of the four sublines. Normal and schematic [14] idiograms of M3-1 cells and *in vivo* Chinese hamster cells, respectively, are given for comparison.

Figure 11.

Oxygen consumption curves for parallel cultures of M3-1 and M147M cells in MEM spinner medium initially containing 1 g/liter glucose and no pyruvate. Initial slopes are similar, but total substrate utilization by M147M appears to be less.

FIGURE 1

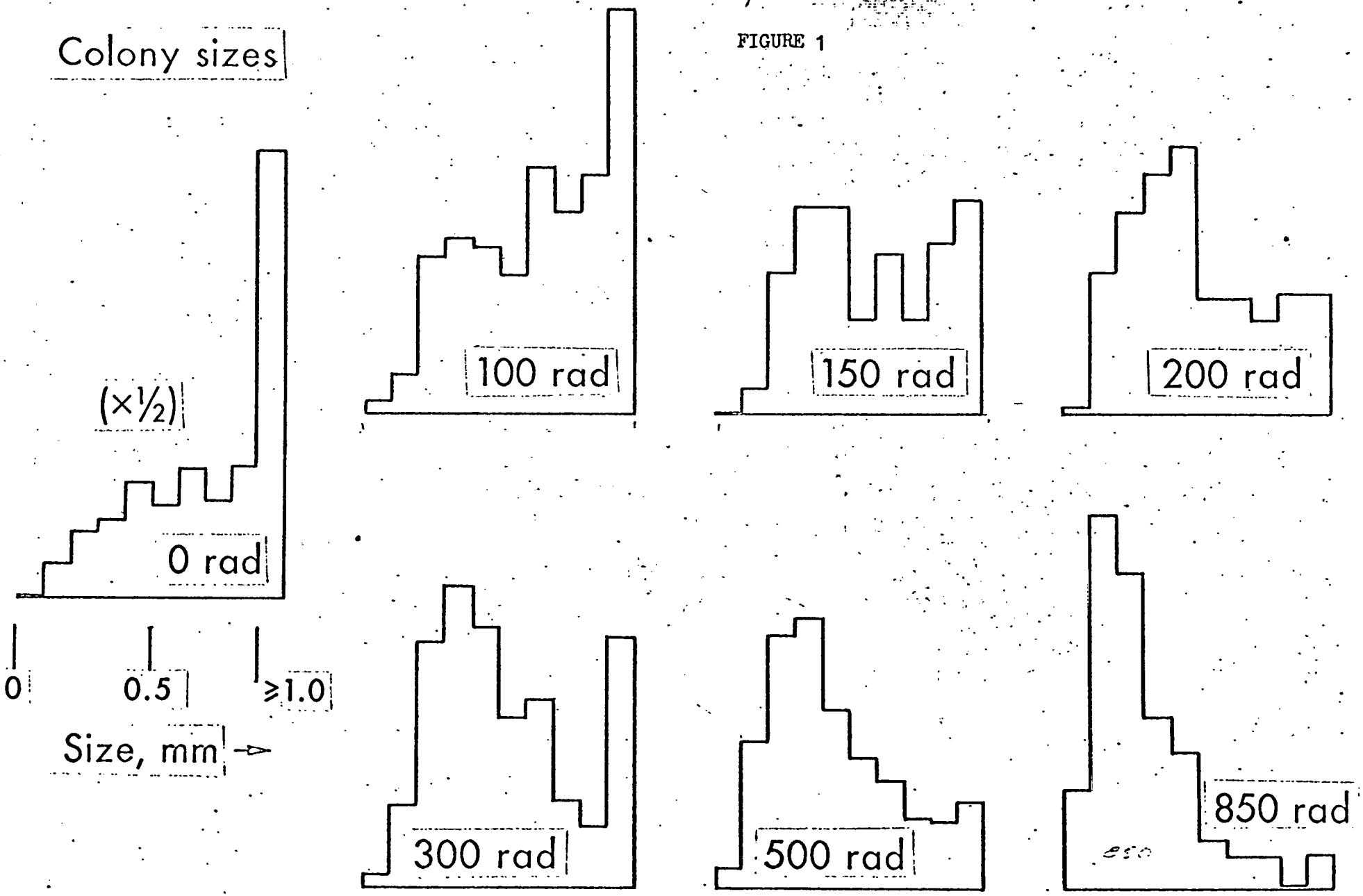

FIGURE 1

FIGURE 2

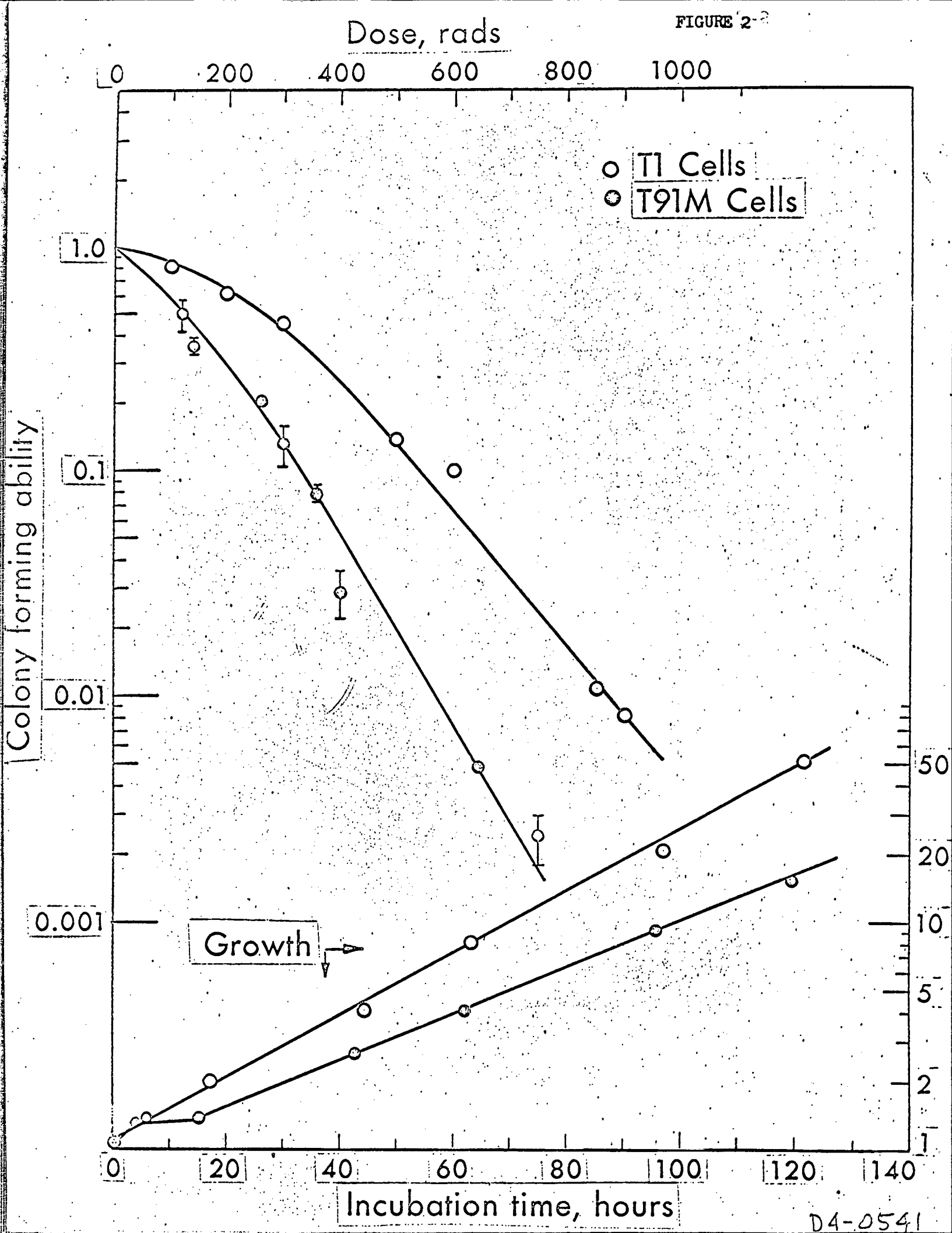

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FIGURE 7-3

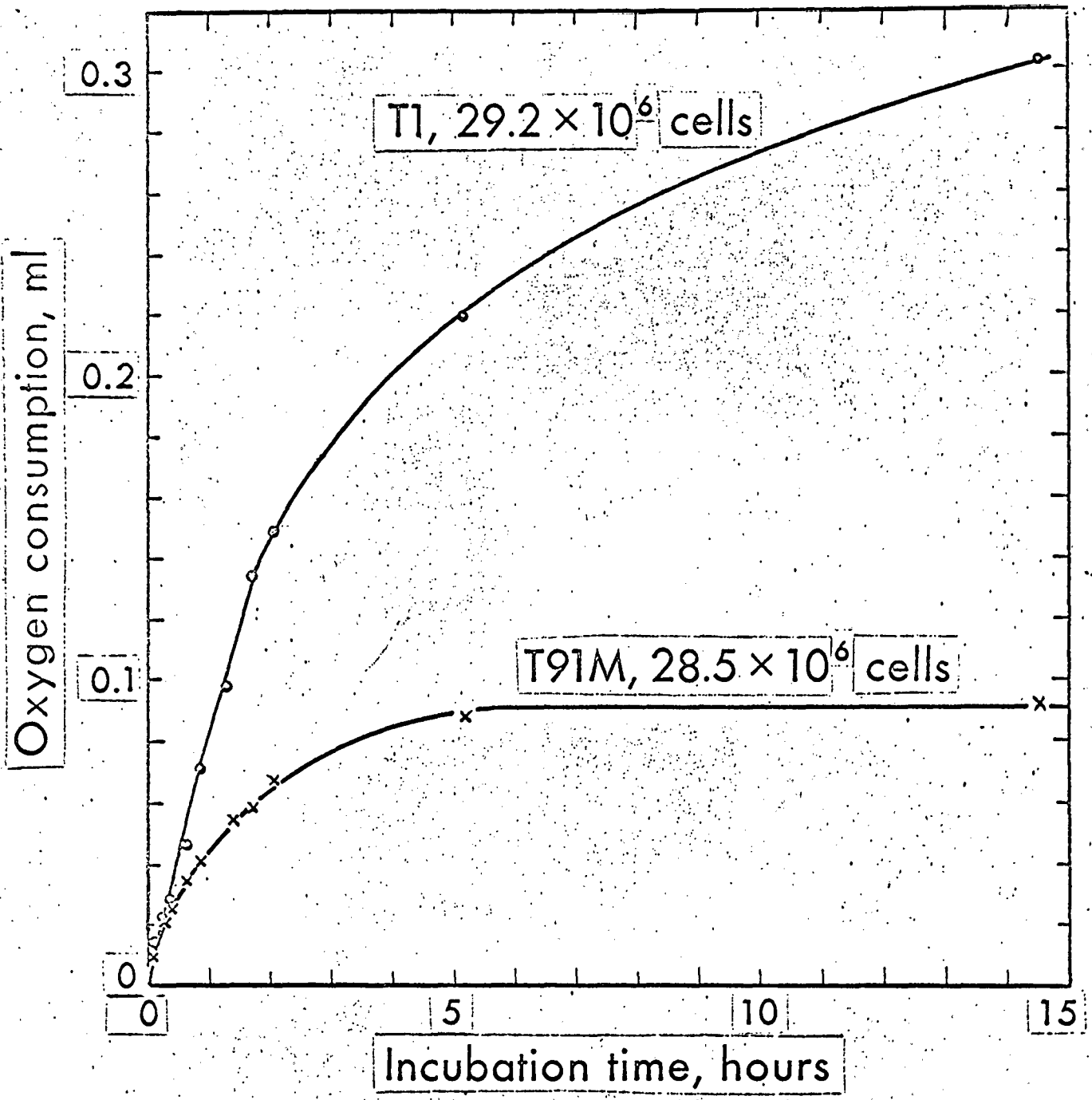
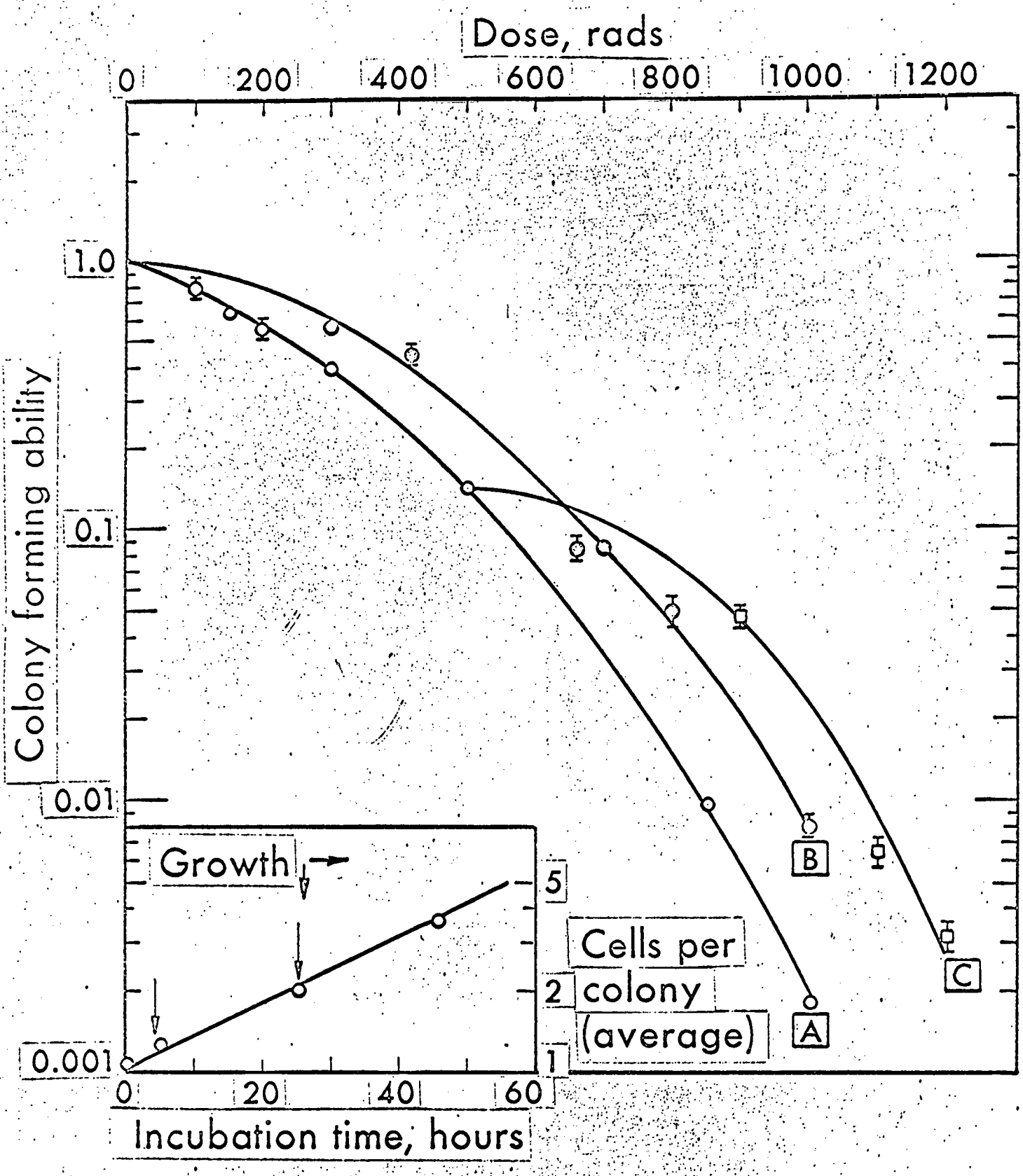

0.3
0.2
0.1
0
T1, 29.2 × 10⁶ cells
T91M, 28.5 × 10⁶ cells
Oxygen consumption, ml
0
5
10
15
Incubation time, hours

FIGURE 4



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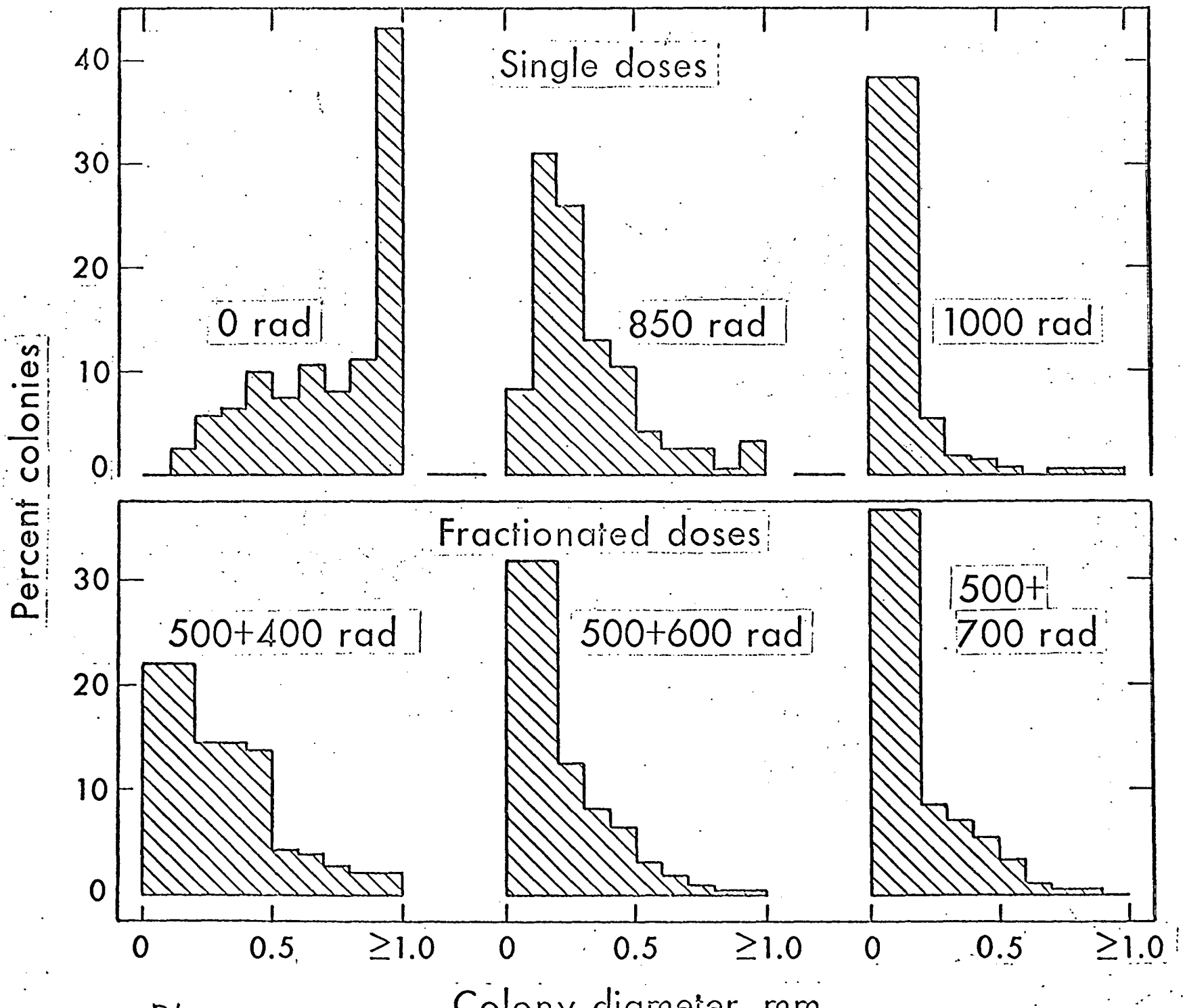

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FIGURE-5

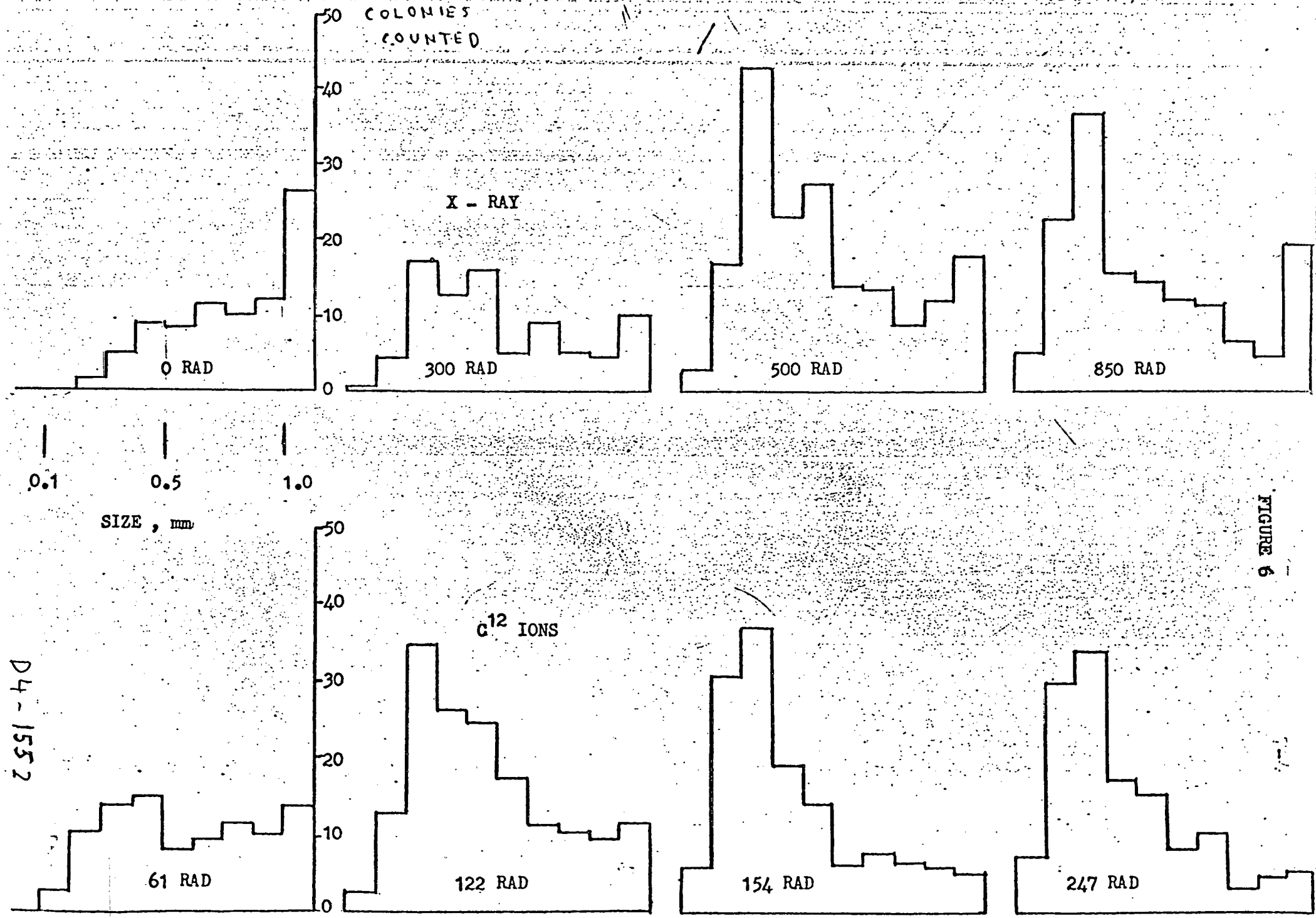

FIGURE 6

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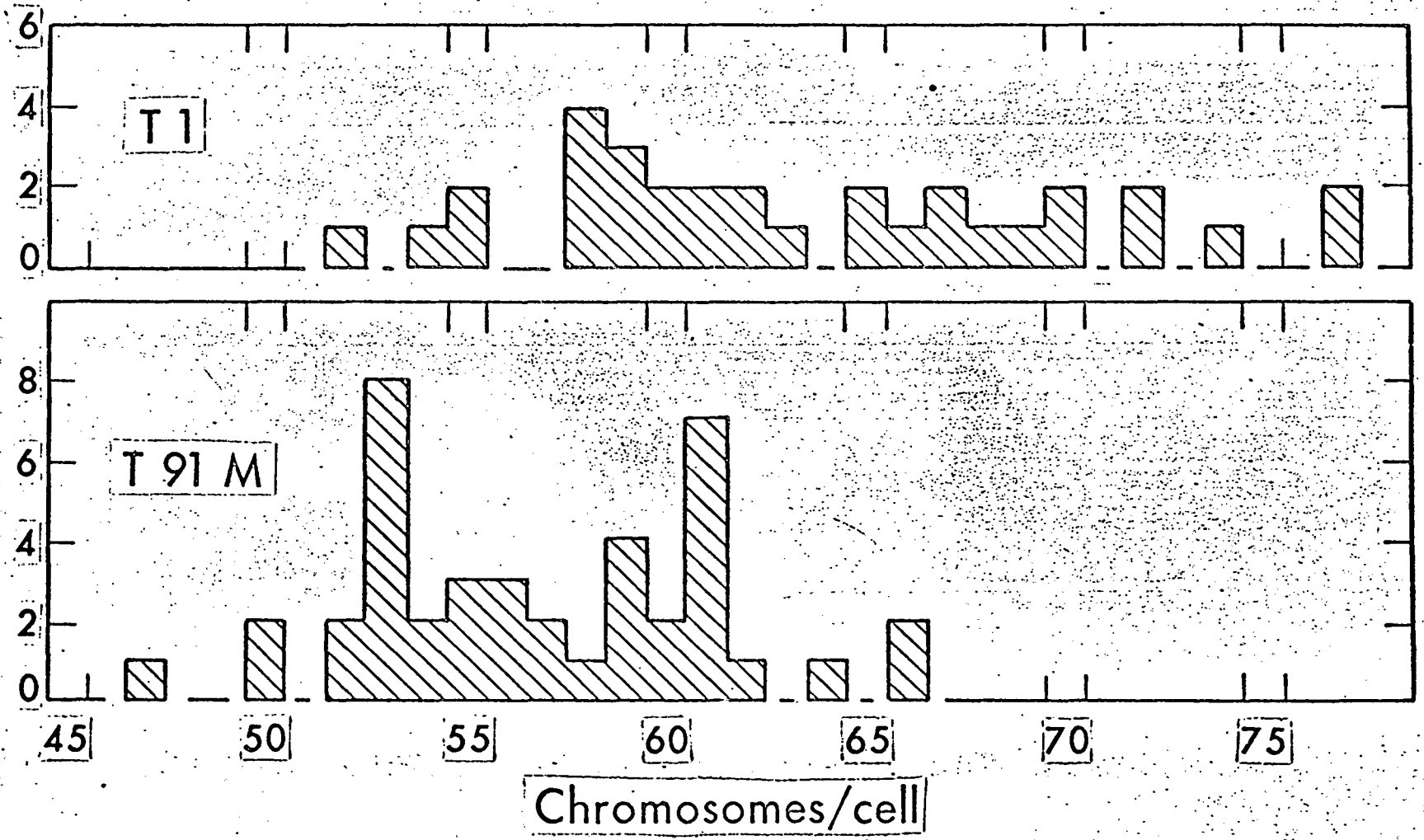

FIGURE 7-

D4- 1861

FIGURE-8

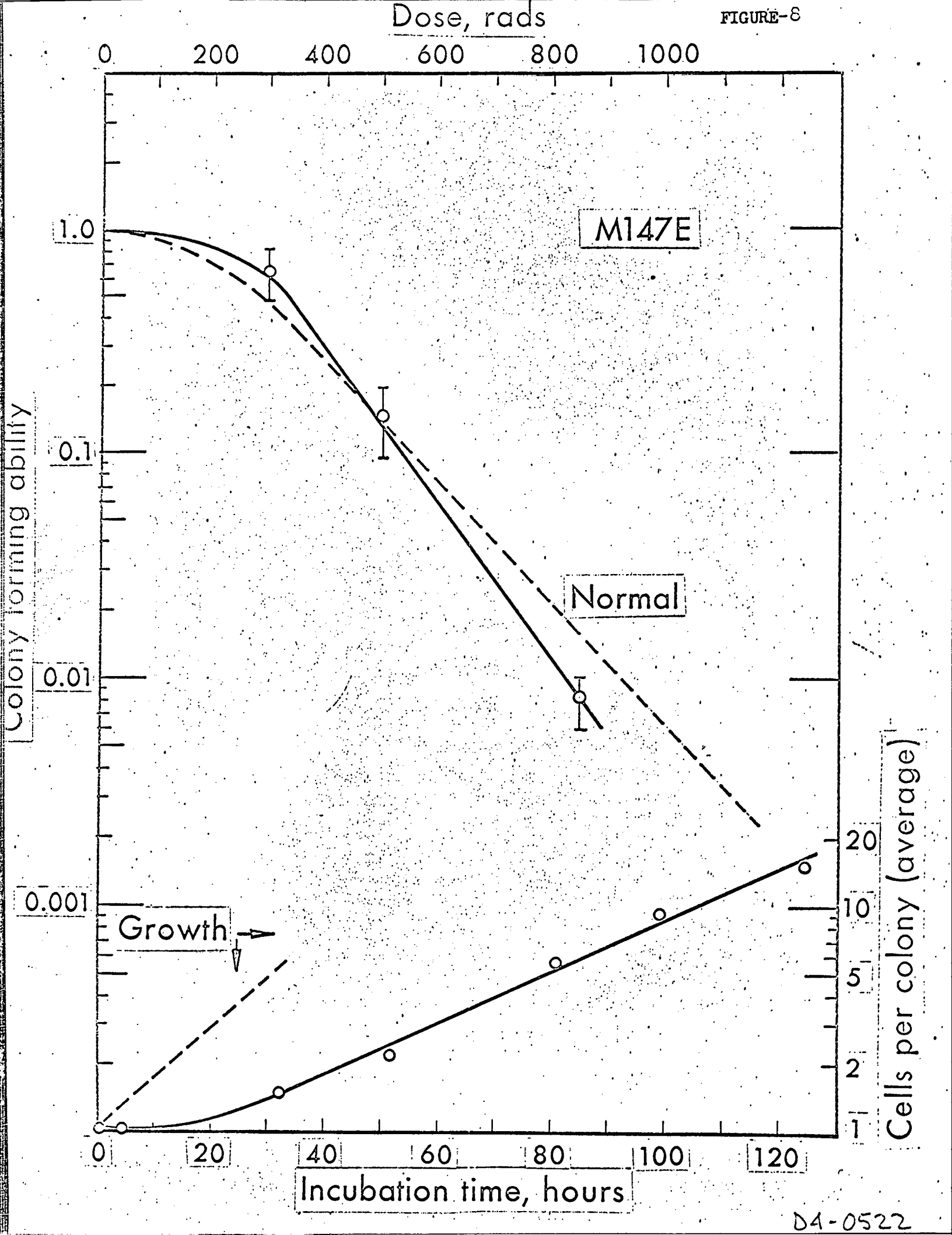

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FIGURE-8

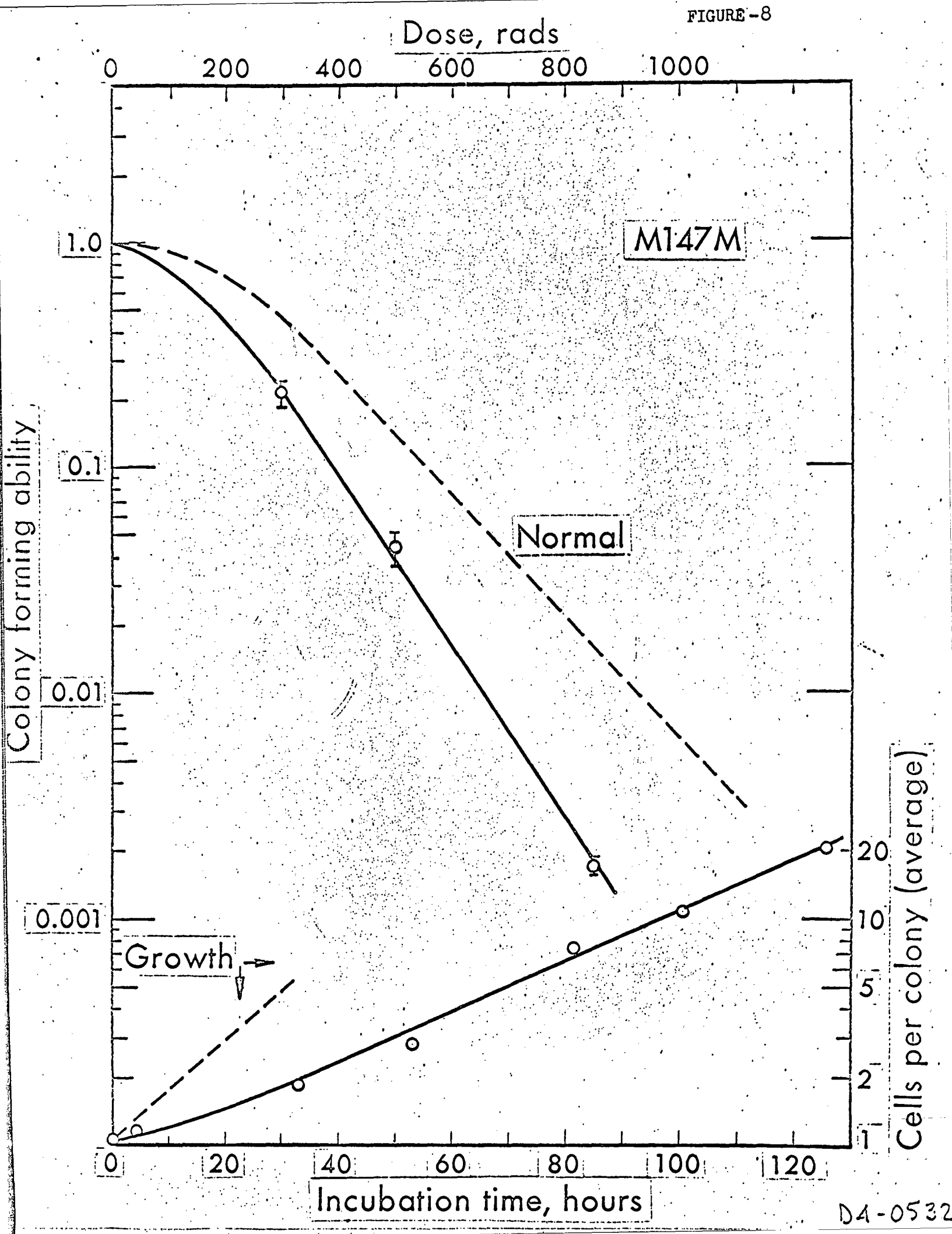

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FIGURE-8

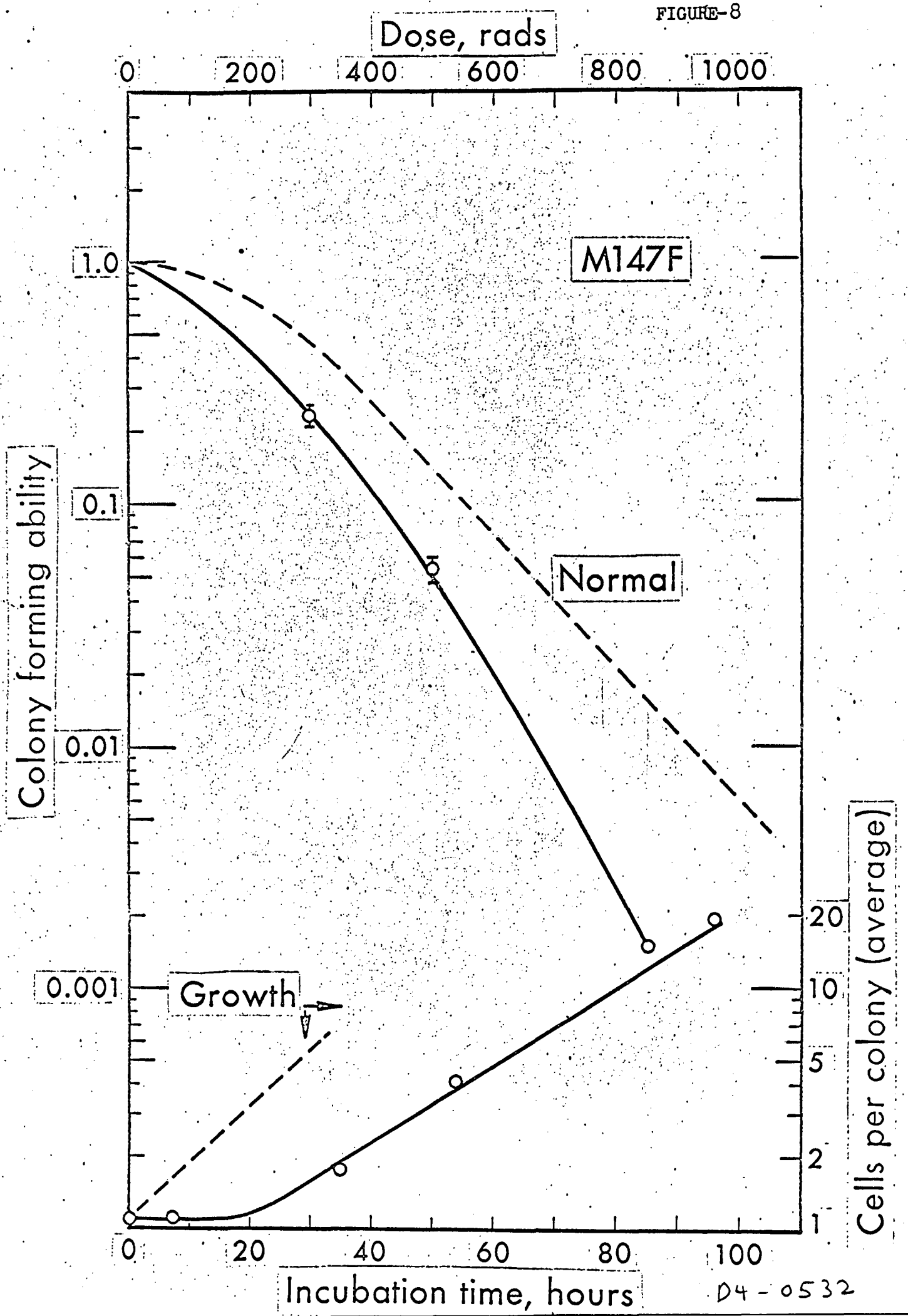
Dose, rads
0
200
400
600
800
1000
M147F
1.0
0.1
0.01
0.001
Colony forming ability
Normal
Growth
Cells per colony (average)
20
10
5
2
1
0
20
40
60
80
100
Incubation time, hours
D4-0532

FIGURE-8

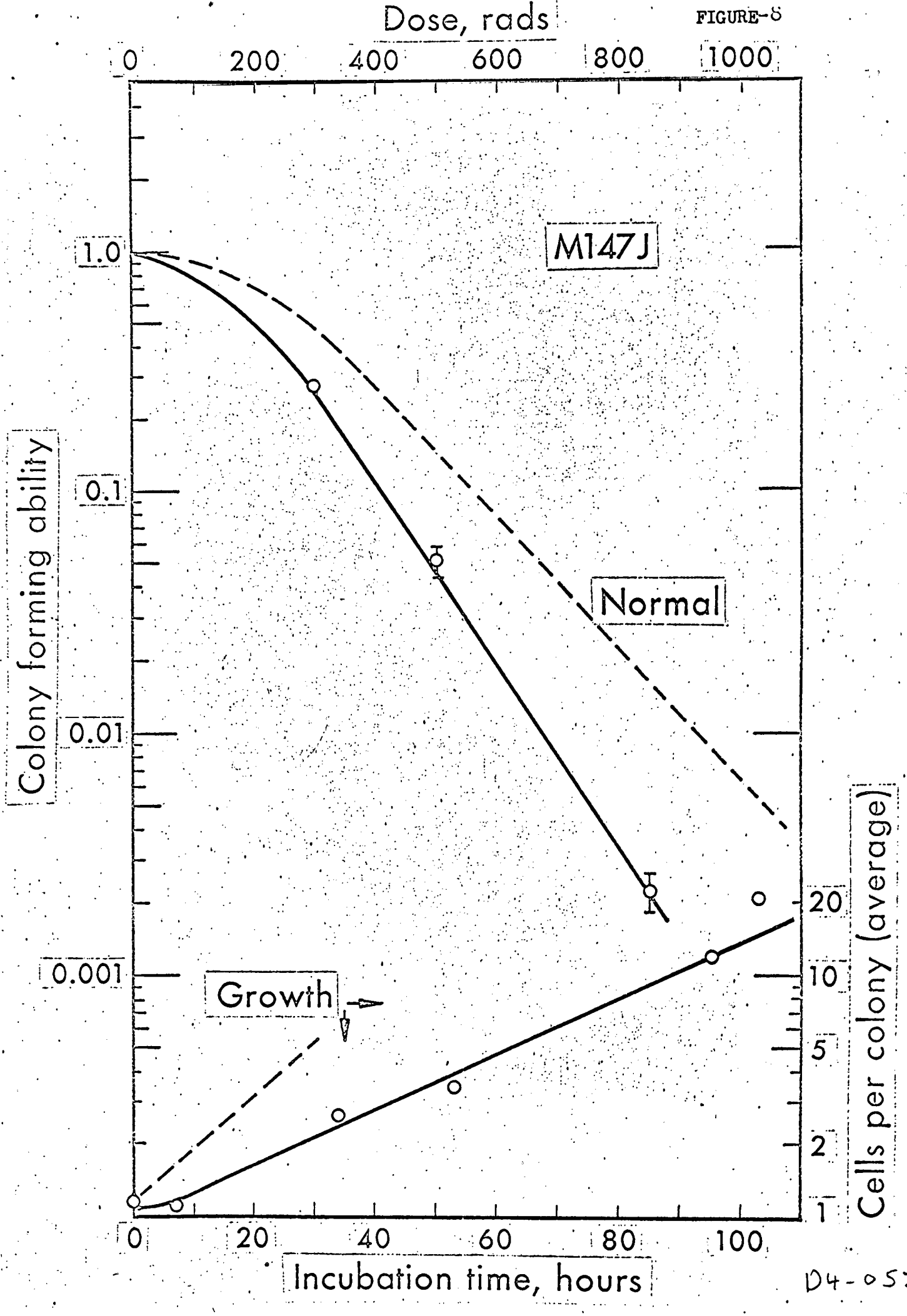

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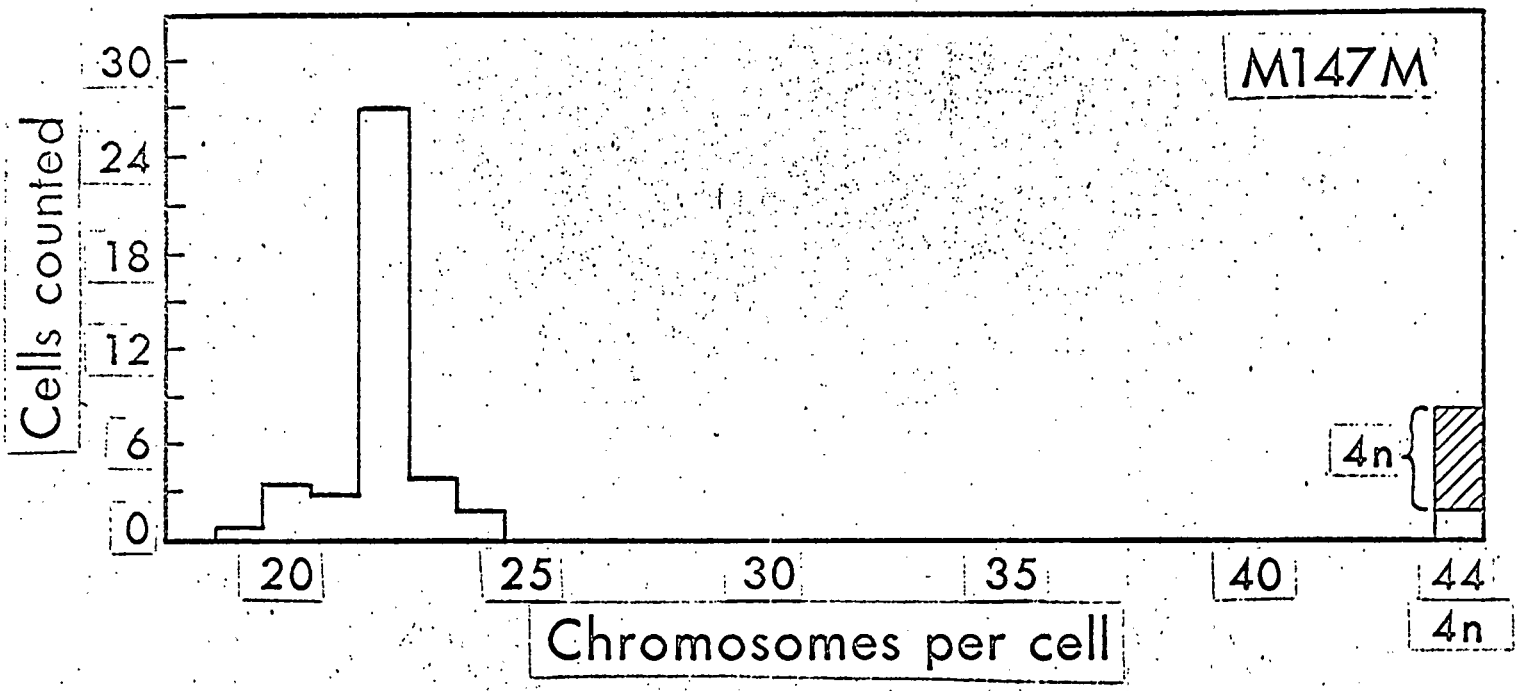
FIGURE-9



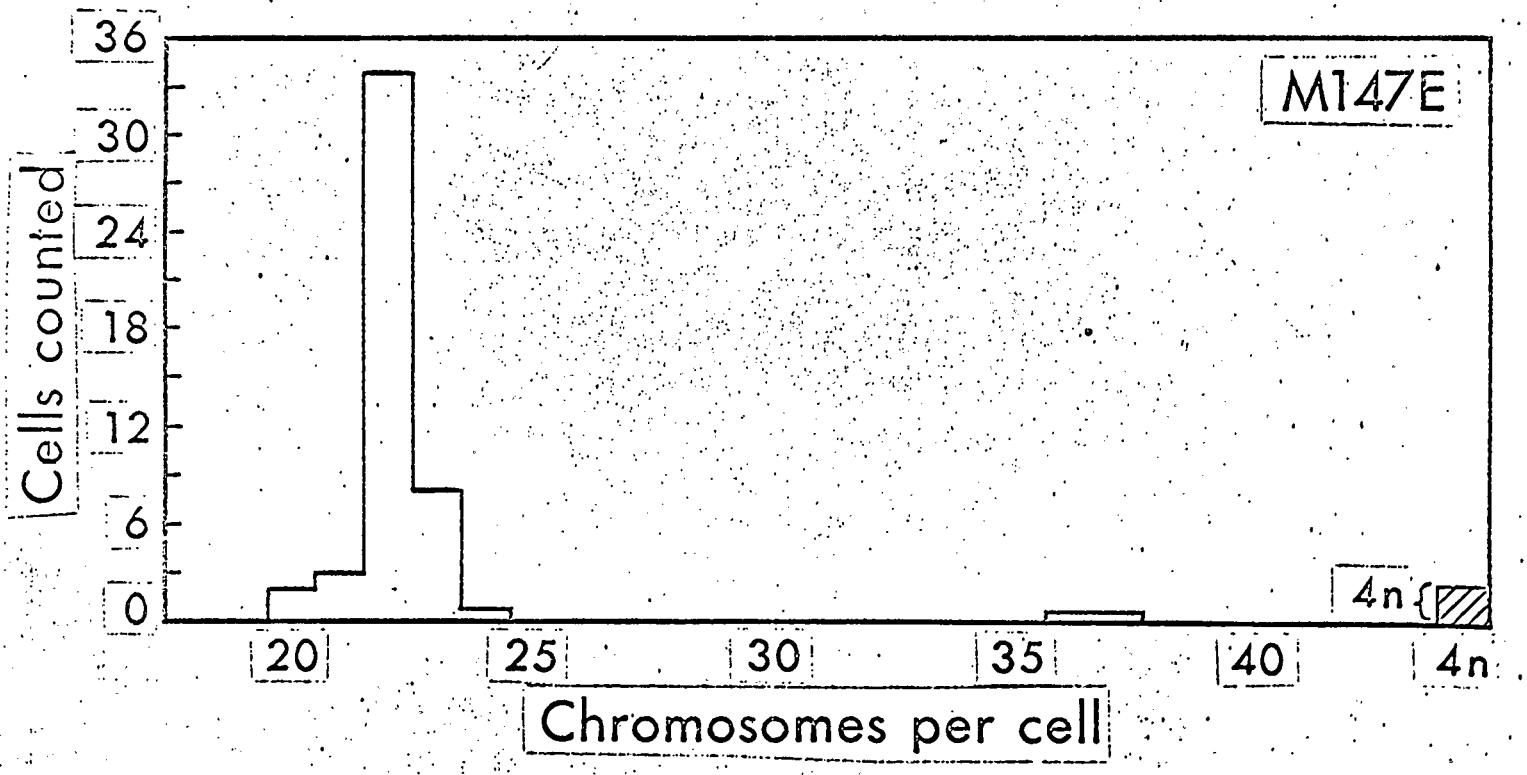


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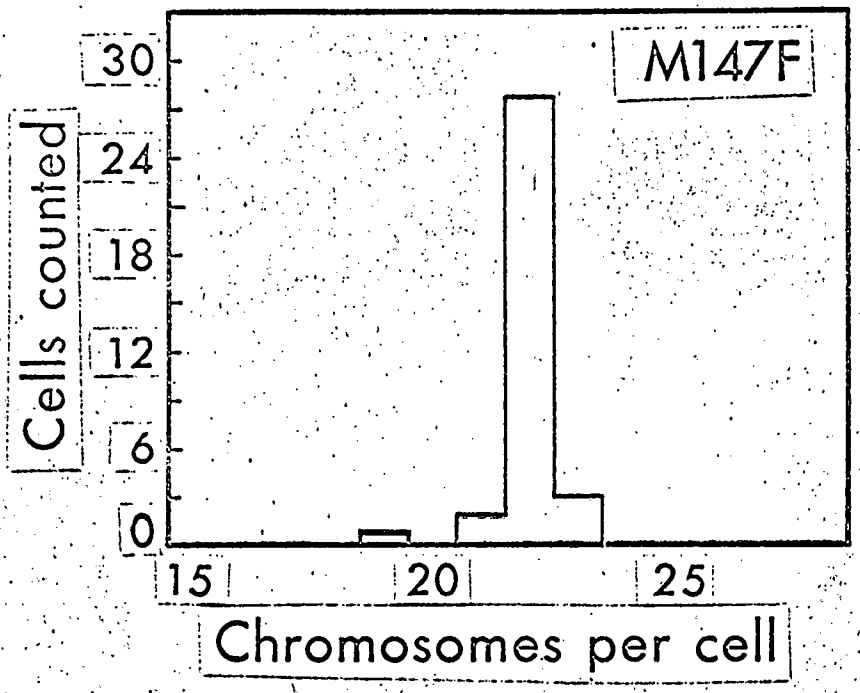
FIGURE-9



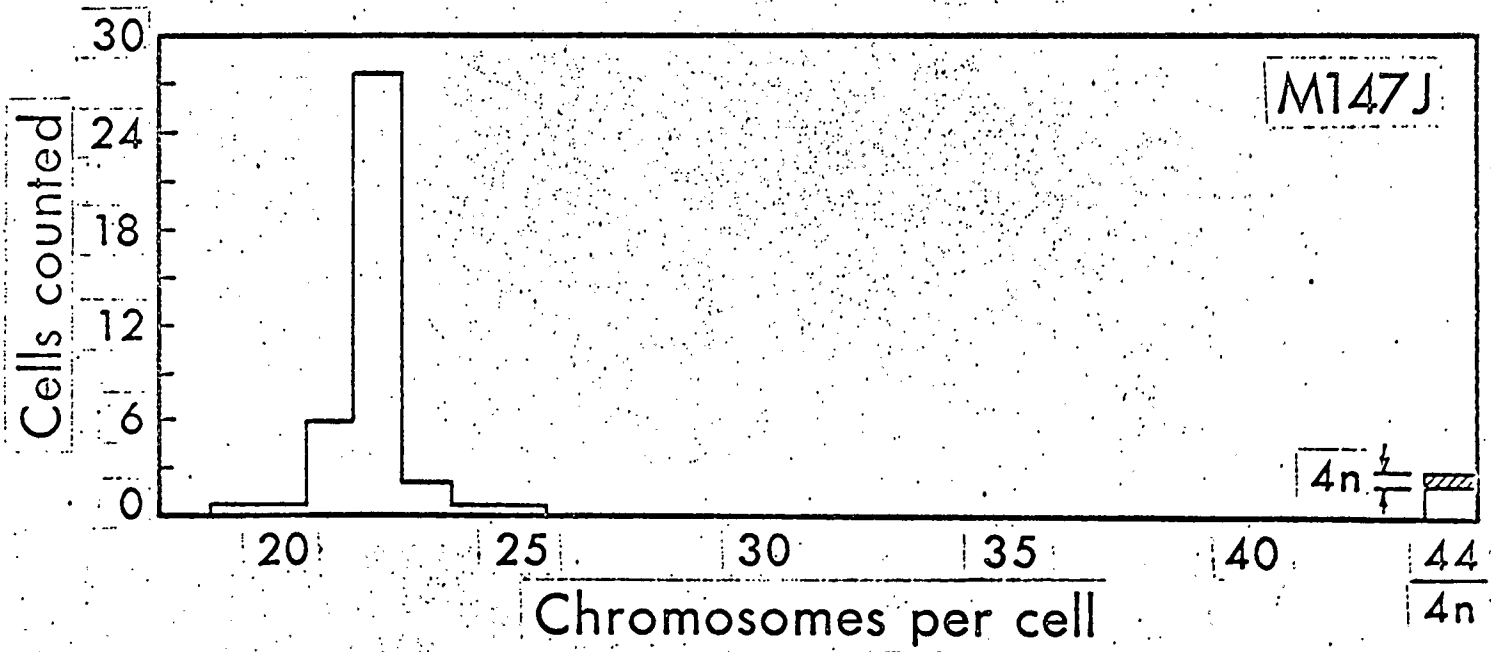


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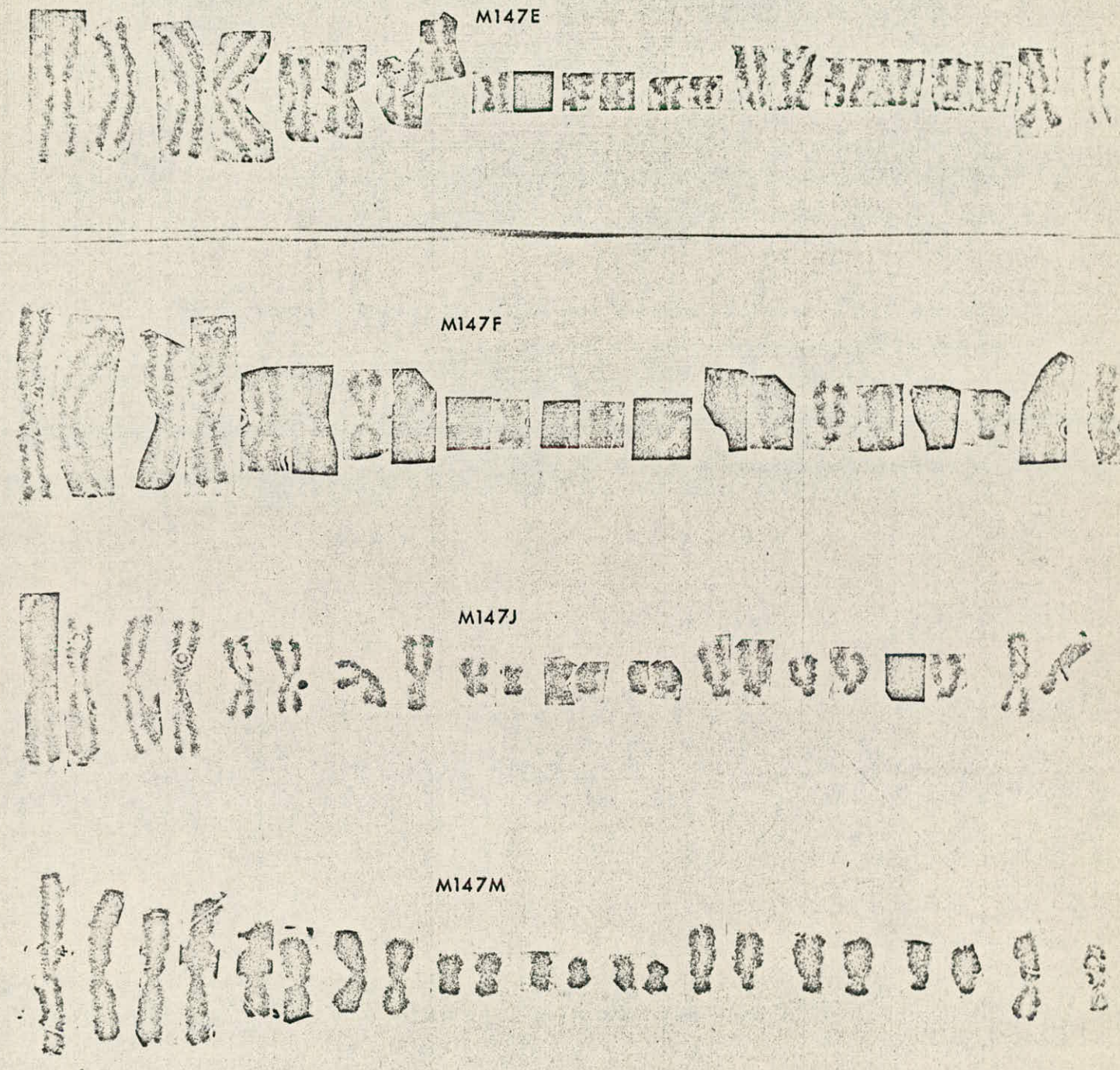
FIGURE -10



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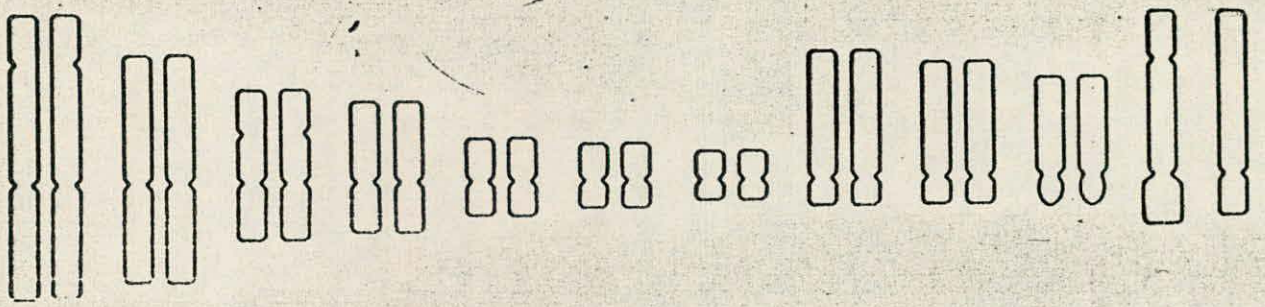


M3-1

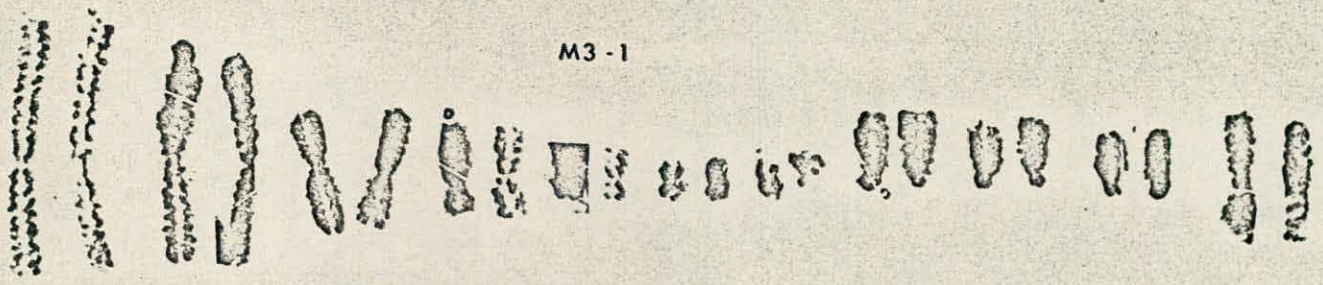
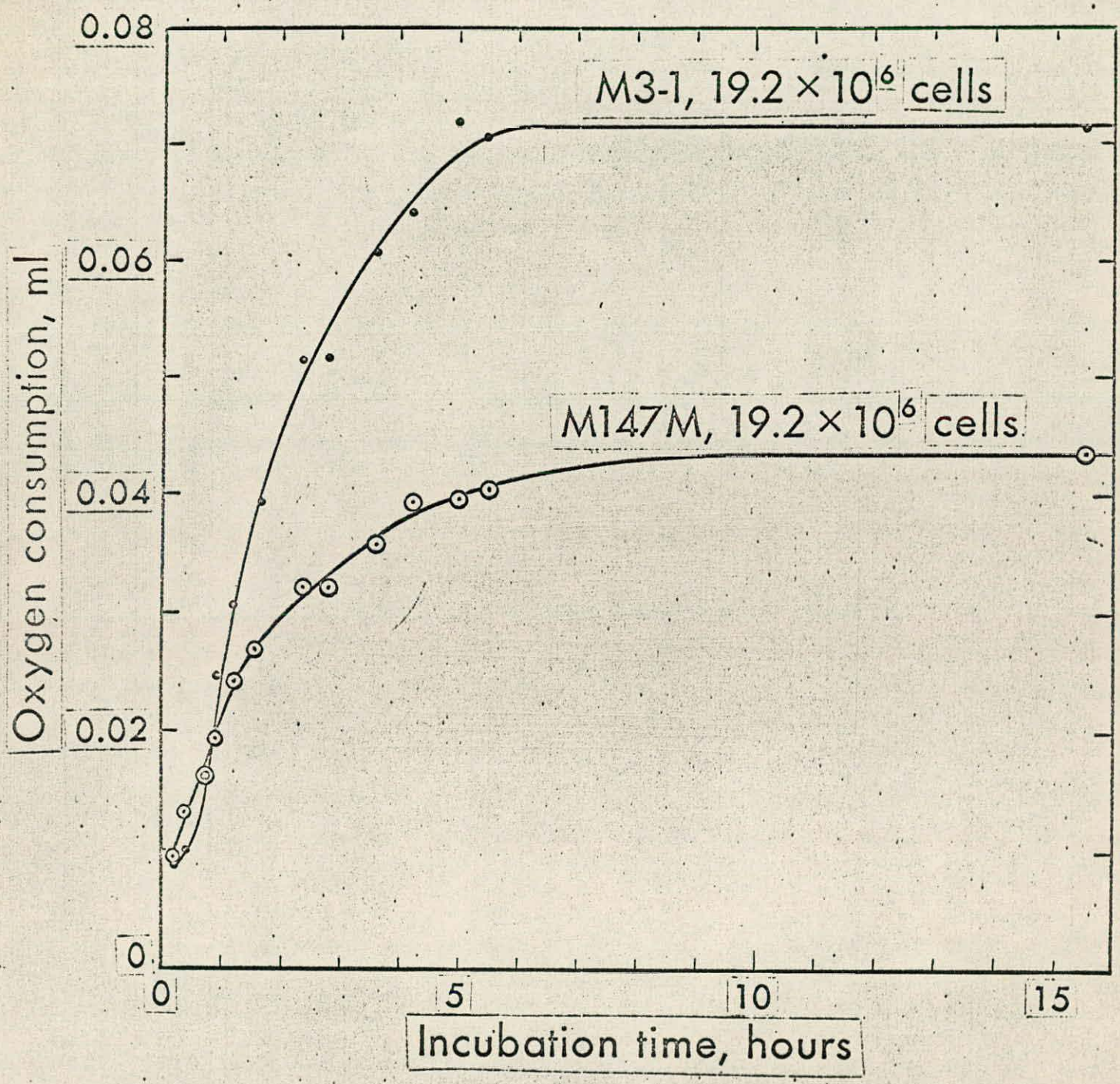


FIGURE 10

FIGURE-11



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