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Quantitative Plating of Gamma-Irradiated Cultures of a Blue-Green Alga

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ABSTRACT — A method for quantitative recovery of the blue-green alga, *Anacystis nidulans*, after treatment with gamma rays is described, and data for the survival of the alga after exposure to a gamma source is presented. No conclusions could be drawn by applying a "multi-target" model relating dosage to survival. Application of the data to a revised target-theoretical expression which included the effects of a dose-dependent repair mechanism indicates that such a mechanism is present in *A. nidulans*. The validity of these dose-response models is discussed.

Anacystis nidulans is a small, unicellular blue-green alga which grows rapidly in simple, defined, inorganic media. For the past few years I have been engaged in research aimed at demonstrating genetic recombination in this organism and thereby showing that some form of sexuality occurs in the Cyanophyta. Part of this research involved establishing a method for the quantitative recovery of viable cells on agar plates and for the determination of the number of survivors after given doses of gamma irradiation. These results are presented and two of the simpler hypotheses relating dosage to survival applied to them.

In order to investigate physiological or genetic characteristics of a micro-organism quantitatively, it is necessary to develop methods for counting individual cells. *A. nidulans* can be counted in a Coulter electronic counter and, with difficulty, in a haemocytometer. Such counts determine the total number of cells present.

To determine the effect of gamma irradiation, a method is needed for estimating the number of viable cells present in a population. The classical microbiological technique for such determinations is a plate count. A sample of the population that is to be assayed is diluted, and small portions are spread on to petri plates containing medium that has been supplemented with agar. The preparations are then incubated, and the number of colonies that form on the surface of the agar is counted. It is assumed that each colony represents the products of a single cell. Therefore the number of cells in the original population can be calculated from the colony count by multiplying by the appropriate dilution factors. To use this technique it is first necessary to verify experimentally the assumption that each colony on the agar plates represents a clone, i.e. derived from a single cell.

The ability of *A. nidulans* to form clones on agar plates was tested with a sample of cells from a vigorously growing, aerated culture. Cells in one part of the sample were counted in a Coulter Counter after appropriate dilution with Millipore filtered 0.95% NaCl solution. The rest of the sample was used for a viable count determination. A measured portion was diluted with sterile mod-

ified Kratz and Myers medium C (Van Baalen, 1965), from which manganese was omitted from the A5 microelements solution, and 0.1 ml samples placed in 10 plastic petri dishes on the same medium to which 1.5% agar had been added.

The suspensions were then spread over the surface of the agar by means of a sterile glass rod and incubated at 25°C under 10 Champion 40 watt fluorescent lamps producing 1.2×10^4 ergs/cm^2 per second incident light on the plates. After about 10 days colonies appeared on the surface of the agar, and these were counted.

The cell count determined with the Coulter Counter was 5.89×10^8 cells/ml. The viable count was 6.58×10^8 cells/ml. The number of viable cells apparently exceeded the total number of cells present. The most likely explanation for this discrepancy is the inherent variability in the plate-counting method. In another experiment, five separate viable count determinations were made, each based on colony counts of five plates. The average count was 6.16×10^7 cells/ml with a standard error of the mean of 0.72×10^7 (about 11.5% of the mean).

The similarity between viable and cell counts indicates that *A. nidulans* is capable of forming clonally derived colonies on a simple inorganic medium. This result conflicts with previous reports which have suggested that *A. nidulans* could not be plated quantitatively (Kumar, 1962; Pikálek, 1967) or precautions had to be taken to prevent the formation of a lethal peroxide (Marler and Van Baalen, 1965) or that the agar had to be autoclaved separately from the medium in order to prevent the formation of toxic products (Allen, 1968).

Action of Gamma Irradiation on *A. nidulans*

A. nidulans was grown at 28°C in 600 ml Bellco culture tubes 3.5 inches from three 40 watt Champion fluorescent lamps producing 1.6×10^4 ergs/cm^2 per second incident light. The cultures were vigorously aerated with water-saturated, sterile air. Samples of 2.0 ml were dispensed to small, sterile glass tubes plugged with cotton which were placed in a cylindrical configuration five inches from 10 Co^{60} pencils yielding 1.394 Kr/minute of gamma irradiation. At timed intervals the tubes were removed from the gamma source and the algal suspensions were diluted appropriately and viable count deter-

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minations made. Five plates were spread for each exposure. Three such experiments were performed. The results are given in Table 1.

Interpretation of survival data

Several hypotheses have been proposed relating dosage to survival. I have applied the data in Table 1 to two of them. The "multi-target" model (Zimmer, 1961; Hutchinson and Pollard, 1961) presupposes that there are m target molecules per cell each with a volume v, and that all m targets have to be hit in order to kill a cell. For $m = 1$, the fraction of cells killed should be independent of the number present so that $dN/NdD = -k$, where N represents the number of cells surviving, D is dose and k is a constant. Thus: $N/N_o = ^{-kD}$, where N/N_o represents the surviving fraction. The fraction that dies is $1 - N/N_o$ or $1 - e^{-kD}$. The constant, k, is in fact the target volume, v. The expression $1 - e^{-vD}$ may be regarded as the probability of the radiation hitting one target. The probability of hitting m targets in a single cell, simultaneously, is therefore $(1 - e^{-vD})^m$. The surviving fraction, S, may thus be represented as:

$$S = 1 - (1 - e^{-vD})^m$$

Expanding this expression by the binomial series yields:
$$S = 1 - (1^m - m(1^{m-1})e^{-vD} + m(m-1)1^{m2}e^{-2vD}/2 \ldots)$$
At high doses the terms after $m(1^{m-1})e^{-vD}$ (which is equal to me^{-vD}) ten to zero and may be discarded. Thus for high D:

$$S = 1 - 1 + me^{-vD}$$

And: $\ln S = \ln m - vD$

This means that if the logarithm of the surviving fraction is plotted against dose, the curve becomes linear at high dosage. In addition, extrapolation of this linear portion back to zero dose yields m, the number of target molecules per cell.

TABLE 1. Action of gamma irradiation on *Anacystis nidulans*.
Fraction surviving

Dose (Kr)	Experiment 1	Experiment 2	Experiment 3
0	1.0000	1.0000	1.0000
4	0.7008	1.0295	0.9414
8	0.5239	0.4612	1.7029
12	0.4343	0.2841	1.2300
16	0.5022	0.4539	0.9915
20	0.3500	—	0.7767
24	—	0.1845	0.4253
28	—	0.3472	0.5195
32	—	—	
			0.2315
			0.3166
			0.0169
			0.0231
50	—	—	

In Fig. 1 the combined data of the three experiments has been used to plot a dose-response curve based on the "multi-target" hypothesis. The constants v and m were determined from the relationship between dose and survival at high dosage. A linear regression line was drawn through the log fraction survival for dosage values equal and greater than 20 Kr. When this line is extrapolated to zero dose, an abscissa value of 6.55 is obtained. (This
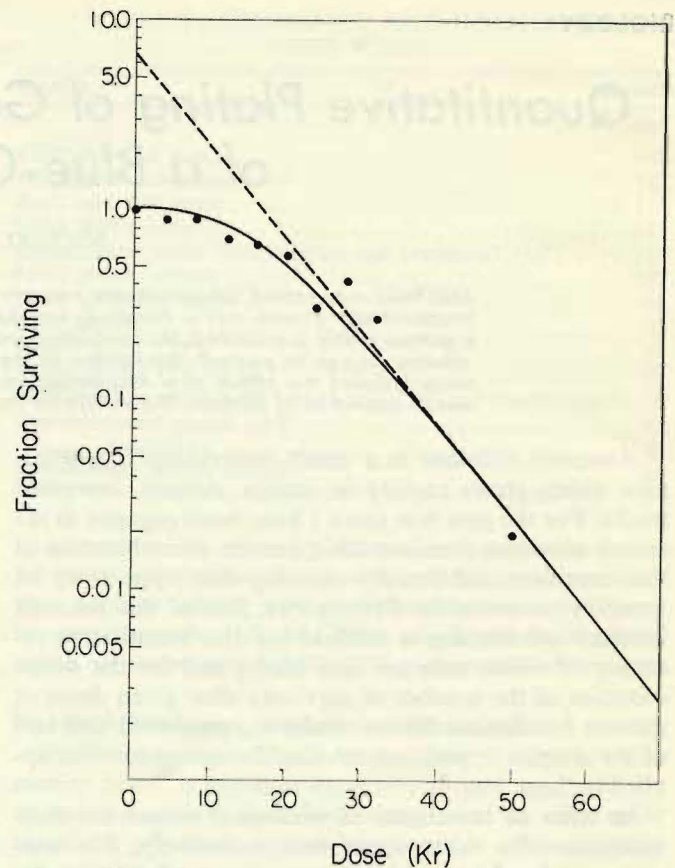

FIGURE 1. Survival of gamma-irradiated *A. nidulans*. Application of the data to the "multi-target" model. Broken line represents a regression line through the data points at high dose. The solid line represents the theoretical expression, $S = -(1-e^{vD})^m$, where $m = 6.55$ (the intercept of the regression line at zero dose) and $v = 0.12$ (the slope of the regression line).

is a minimum value, as at $D = 50$ the second and succeeding terms of the binomial expansion contribute significantly to the curvature of the graph.) This represents the number of target molecules per cell, m. The slope of the regression line gives the constant v and has a value of 0.12.

The second model applied to the data is that of Haynes (1966). This hypothesis is based on the assumption that damage to the target molecules causes the cells to die exponentially but that there is a repair mechanism within the cells which is responsible for the shoulder on the initial part of the dose-response curve. The relation between fraction surviving and dose is:

$$\ln S = -F(D) + G(D)$$

where $F(D)$ represents the way in which the number of targets damaged increases with dose and $G(D)$ represents the way in which they are repaired. As the number of target molecules struck is a function of the number present,

$$-F(D) = -kD$$

The repair mechanism, $G(D)$, is described by the simplest saturation function that: (1) Passes through the origin — at zero dose no defects are repaired as none have
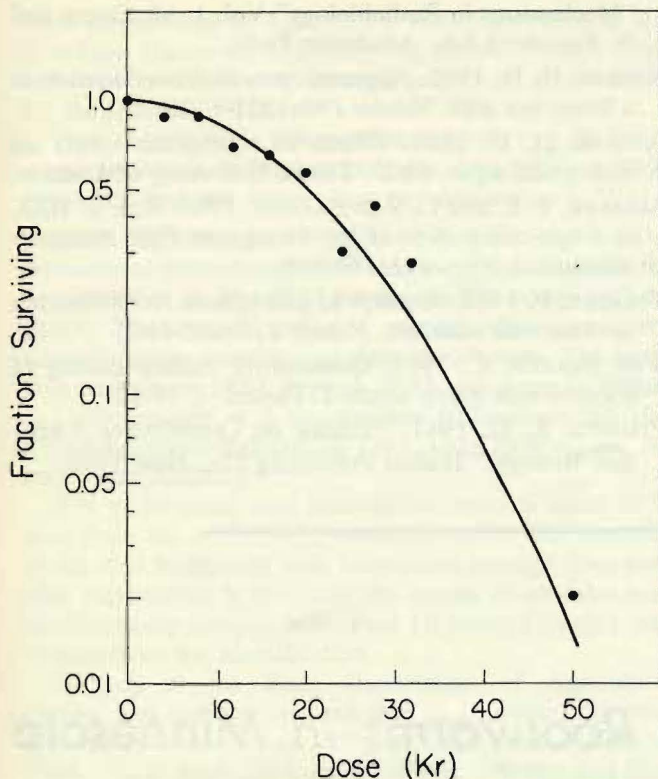
FIGURE 2. Survival of gamma-irradiated *A. nidulans*. Application of the data to Haynes' (1966) model, $\ln S = -kD + \alpha(1-e^{\beta D})$, where $\beta = 0.0005$, $k = 6.386$, and $\alpha = 12776$.

been made; (2) at low doses the number of defects repaired is proportional to dose; and (3) the capacity for repair saturates at high doses. The function suggested by Haynes is:

$$G(D) = \alpha(1 - e^{-\beta D})$$

Thus the expression for S becomes:

$$\ln S = -kD + \alpha(1 - e^{-\beta D})$$

The parameters k, α and β represent respectively the way in which the target molecules are inactivated, the maximum number of potentially lethal hits which can be repaired and the manner in which repair saturates with increasing dose.

The grouped data of all three experiments has been used in an attempt to estimate the parameters k, α, and β by means of a least squares fit. This statistical method showed that the value of β which best fits the data lies between 0.0001 and 0.005. Fig. 2 shows the combined experimental data fitted to the theoretical curve where $\alpha = 0.0005$, $k = 6.386$ and $\alpha = 12776$. The values used by Haynes were $\beta = 0.00277$, $k = 1.29$ and $\alpha = 466$. The theoretical curves generated by either of these sets of parameters give a reasonably good representation of both Haynes' and my data, despite the difference in magnitude of the values.

Estimate of Viable Cells

Although the plating technique is subject to a high degree of variability, it can be used as an estimate of the number of viable cells present in a population of *A. nid-*

ulans. Of greater significance is the fact that the progeny of a single cell within a large population of cells can be isolated.

Thus if a suspension of several million cells is plated on a selective medium, containing for example streptomycin, a single mutant cell resistant to the antibiotic can grow, form a colonoy and be subcultured. In this way many mutant strains can be isolated and used for a variety of genetic and physiological experiments.

In order to evaluate either of the models applied to the survival data, it is necessary to know the site of action, or target molecules, of the radiation. There is good evidence, at least for UV and X-rays, that DNA is the principle macromolecule to be affected (Haynes, 1966). If DNA is the target molecule, then the constant, m, of the "multi-target" hypothesis represents the average number of DNA molecules per cell. In *A. nidulans* m has a value of at least 6.55. Although the possibility cannot be absolutely excluded at present, it is difficult to accept this value as an estimate of the number of genomes in a cell of the alga.

Thus the results of my experiments call into question the validity of the "multi-target" model. Evidence from organisms of known ploidy casts further doubt on the efficacy of the model. Davies (1967) presented survival data for haploid and diploid gamma-irradiated *Chlamydomonas reinhardi*. The extrapolation values (m) for these strains did not conform to the simple relationship predicted by the "multi-target" hypothesis. There was, however, a longer initial shoulder in the dose-response curves of the diploid strains, indicating that the survival of the cells is at least partly a function of their DNA content.

Haynes' model which incorporates the effects of a repair mechanism is supported by some experimental evidence. Haynes (1966) compared the dose-response curve of *Escherichia coli* B/r, in which there is an initial shoulder, to *E. coli* B$_{s-1}$, a mutant strain lacking a DNA repair mechanism, which showed no initial shoulder but died exponentially. It appears therefore, that at least part of the shoulder in the dose-response curve is due to the repair of DNA within the cells. As *A. nidulans* shows such an initial shoulder, it can be inferred that such a mechanism exists in the alga.

The basic assumptions of both the models are supported by some experimental evidence. The "multi-target" model states basically that the shoulder of the dose-response curve is a function of the number of DNA molecules per cell. Haynes' model relates this shoulder to a repair mechanism. There is evidence for both suppositions—experiments with *Chlamydomonas* (Davies, 1967) and Haynes' experiments with *E. coli* described above. It may therefore be necessary to incorporate both of these assumptions in a single model before a satisfactory relationship between dosage and survival can be postulated.

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BIOLOGY

Northern and Western Corn Rootworms in Minnesota

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ABSTRACT — The occurrence and population changes of the northern corn rootworm, *Diabrotica longicornis* (Say), and the western corn rootworm, *D. virgifera* LeConte, were reviewed on the basis of information assembled at the Department of Agriculture of the State of Minnesota; and the Department of Entomology, Fisheries, and Wildlife and the Extension Entomologist's Office of the University of Minnesota.

The review shows no record of the northern species in Minnesota before 1899, but it appeared between 1899 and 1915. Populations were probably very low until 1932. Sporadic infestations were reported between 1932 and 1953. Progressive increase in infestation from 1954 parallelled an increase in continuous planting of corn. The western species reached Minnesota in 1961. By 1968 it was found in practically all counties where the northern species was found. There was no displacement of the northern species by the western in Minnesota, as happened in some other localities.

The northern corn rootworm, *Diabrotica longicornis* (Say), has been in Minnesota for many years, but the western corn rootworm, *D. virgifera* LeConte, is a relatively newcomer to the state. It is acknowledged that infestations of both species have been encouraged by continuous planting of corn. The present review attempts to establish, by retrospect, the occurrence of both species and the increase of infestation as related to continuous corn planting in Minnesota.

Information was gathered from several sources: published material; and files, correspondence, and interim reports at the Division of Plant Industry, Department of Agriculture, State of Minnesota; the Department of Entomology, Fisheries, and Wildlife; and the Extension Entomologist's Office, University of Minnesota. The events are presented chronologically in several periods.

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BEFORE 1899. The earliest mention of corn rootworm in Minnesota publications was by Dr. Otto Lugger, the state entomologist, in his report of 1899, as follows:

"In the western and central states a third species is found, the "corn root *Diabrotica*," named as from its long feelers. Its larva is a very serious pest. As this species winters in the egg state in corn fields, a simple rotation is all that is necessary to keep it in check. It is not found in Minnesota, at least no specimens have been seen or received, while *D. 12-punctata* and *D. vittata* are very common, and the latter seems to be rapidly on the increase."

From the above, it seems fairly certain that *D. longicornis* was not present in Minnesota at that time.

1899 TO 1931. No further information was available until 1916, when C. W. Howard reported "The western (*sic*) corn root-worm (*Diabrotica longicornis*) has been reported in Minnesota for the first time during the past summer, appearing in several widely separated localities in the southern quarter of the state."

Further search of the literature and the files of the