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EVIDENCE FOR RECOVERY FROM X-RAY DAMAGE IN CHLAMYDOMONAS

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August 1957

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ABSTRACT

The effect, on subsequent colony formation, of irradiation of cells of Chlamydomonas reinhardi with X rays at a high dose rate has been studied. A survival curve was obtained, the shape of which cannot be accounted for by a mechanism involving killing by a specific number of quanta. As in organisms whose survival curves are simpler, death rarely occurs until one to five cell generations have elapsed after irradiation. The LD_{50} is approximately 4.5 kiloroentgens.

When exposure to the X-ray beam is interrupted for about 15 minutes, survival of a given dose is increased, provided that the cells are at a suitable temperature during the treatment. At just above 0° C, little or no increase occurs even after an hour. Under favorable conditions, the second dose kills fewer cells than it would if given immediately after the first dose. When the number of cells killed by the first dose is accounted for, however, the fraction killed by the second dose is at least as great as would be expected if the first dose had not been given.

Two or three interruptions of the dose result in higher survival than a single interruption, but not high enough to suggest than an irradiated cell population can tolerate more irradiation than a previously unirradiated population. There is some evidence that illumination between irradiations enhances the increase in survival.

These observations can be reconciled with target theory by making assumptions which deprive the theory of much of its usefulness. It is suggested that the effect of interruption of the exposure is due to recovery, possibly associated with a metabolic process, rather than to a change caused by irradiation itself. It is further suggested that considerations of "hit number" may not be applicable to this organism.

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INTRODUCTION

The algal flagellates have been subjected to relatively few radiobiological studies. In an early experiment, Holweck and Lacassagne irradiated the colorless chlamydomonad Polytoma uvella with α particles.[1,2] Ralston studied the effects of X rays on Dunaliella salina.[3] Although both these organisms are presumably haploid,[4] both exhibit multiple-hit survival curves, as does the colorless euglenid Astasia longa.[5] Pandorina shows a cumulative lethal effect without recovery.[6] Nybom has reported nonexponential survival curves in the green algae, Chlamydomonas eugametos, C. moewusii, and C. reinhardi, after exposure to x-rays.[7] Because Nybom's irradiations were performed in conjunction with genetic experiments, the haploid condition of these cells seems reasonably certain.

If more than one quantum must be absorbed to inactivate a single target, the last absorption can be thought of as the lethal event, and the preceding ones as sensitizing events. Reversibility of the "sensitizing" process would be manifested by an increased yield of survivors of a given dose when the exposure is interrupted, and sufficient time permitted to elapse before it is resumed. Sax found such a dose-fractionation effect in the production of two-hit chromosome aberrations in Tradescantia.[8]

MATERIALS AND METHODS

Chlamydomonas reinhardi Dangeard, the organism used in this investigation, is an algal flagellate which has recently become a tool for the study of microbial genetics. The cultures employed were provided through the generosity of Drs. Russell A. Eversole and E. L. Tatum. They are derived from a strain which, since its isolation by Dr. G. M. Smith, has been subjected to a number of genetic experiments.[7,9-13] Tetrad analysis by the authors cited above shows the organism to be haploid and uninucleate. After mating, it undergoes crossing-over in the four-strand stage.

From the pure cultures that had been supplied, several single-colony isolates were made. Those of the "plus" mating type are designated by odd numbers, those of the "minus" by even numbers.

Except where otherwise indicated, growing motile cultures were used. These were diluted and spread on agar surfaces in 10-cm petri plates just before irradiation. Dr. M. B. Allen was extremely helpful in suggesting a liquid medium that produces rapid growth with a negligible number of cell aggregates. It consists of:

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*National Science Foundation Postdoctoral Fellow, 1956-57.

KNO_3	0.02 M,
$MgSO_4$	0.001 M,
$CaCl_2$	0.0005 M,
KH_2PO_4, K_2HPO_4	about 0.003 M, added after autoclaving, to pH 7 - 7.5,
trace elements:	1 part per 1000 of the mixture described by Eversole,[12] modified by the omission of $CaCl_2$.

Cultures were aerated in test tubes with a mixture of 4% CO_2 and 96% air. Growth on this medium is autotrophic sensu stricto, since there is practically no reduced nitrogen nor carbon present.

The addition of 1% agar and 0.015 M sodium acetate was found to make a suitable solid medium for irradiation and plate counts.

Unfiltered x-rays from a 50-kvp beryllium-window x-ray tube (Machlett OEG-60) operated at 50 kv and 25 ma, were used. Measurements with a beryllium-window ionization chamber had shown the dose rate at the distance at which samples were exposed (15.6 cm) to be 250 r/sec over the entire area occupied by the Petri dish. Plating prior to irradiation made possible the application of uniform doses of unfiltered x-rays, resulting in this high dose rate.

The plates were spread in a planned sequence, to insure detection of any change in viability prior to irradiation. After irradiation, all plates were incubated in the light for 5 to 7 days. Colony counts were then made, in order to determine the fraction surviving. The above procedures have been described in detail.[14]

RESULTS

I. Survival Curve: Uninterrupted Irradiation

Uninterrupted irradiation at 250 r/sec resulted in survival curves of the multihit type.

The radiosensitivity of the cells was constant throughout all the experiments (see Table I) except No. 30. Within the limits of experimental error, the survival curve can be represented by the function

$$S = e^{-kD^2}$$

or

$$\ln S = -kD^2,$$

where S is the fraction surviving, D is the dose, and k is a positive constant. By use of the least-squares method, with the restriction that S = 1 when D = 0,[14] k was evaluated. Data for doses greater than 10 kr were omitted, because they were based on rather small samples. The value obtained

Table I

Fraction of Chlamydomonas reinhardi surviving after continuous x-irradiation

Expt. Strain	4 CR1	6 CR1	7 CR1	14 minus	15 CR1	17 CR1	18 CR32	23 CR1	28 CR1	34 CR1	
Dose (kr)											e^{-kD^2} from fitted curve
1.25				0.910		0.938	0.870				0.949
2.50	0.833[a]	0.803	0.743	0.764	0.822	0.831	0.758	0.835	0.777		0.810
5.00	0.463[a]	0.435	0.440[a]	0.466	0.422	0.439	0.415			0.410	0.430
6.00									0.262[a]	0.285	0.297
6.25							0.202				0.268
7.00									0.225[a]	0.171	0.191
7.50		0.128	0.112	0.162	0.174						0.150
10.00		0.040									0.035
11.25			0.011[b]								0.014
15.00 (separate experiment)				0.0003[b]							0.0005

[a]Based on fewer than 125 survivors

[b]Based on fewer than 4 irradiated plates

Experiment 7 involved nitrogen-depleted cells, Experiment 17 involved slant-grown cells motilized in nitrogen-free medium, Experiment 18 involved cells from which the elements Ca and Mg had been withheld, and Experiment 23 involved cells that had been suspended for a few hours in a 0.002M $MnCl_2$. All others were performed on freshly grown cells from liquid cultures.

was $k = 0.03376\ kr^{-2}$, resulting in a survival curve that nearly matches the curve obtained by Nybom using suspensions of haploid cells.[7] The fitted curve is shown in Figure 1. From the equation, the LD_{50} is 4.53 kr. Some spurious exponential curves were obtained on cells dying because of ionic inadequacy of the diluent.

Although the equation that has been used to fit the survival curve is not an equation customarily used to represent a multiple-hit curve, examination of its characteristics shows that it represents some cumulative process. In addition to killing part of the population, any dose renders the remainder of the population more susceptible to killing by further irradiation than it was before.

In Table I, 26 out of 30 measurements agree within 15% with the survival quotients calculated from the curvilinear regression equation. Hence this procedure seems justified. No significant effect was observed due to 22 hours of nitrogen starvation, to withholding calcium and magnesium from the growth medium for 17 hours, to growth on mineral agar followed by 25 hours of exposure to nitrogen-free liquid medium to induce motility, nor to 4 hours of illumination in a medium to which 0.002 M $MnCl_2$ had been added. Nitrogen starvation was effected by incubation and aeration in the light in mineral medium from which KNO_3 was omitted. The enlarged size of the cells was evidence that they were actually nitrogen-deplected.[15]

Microscopic observation of young colonies indicated that, although division was probably delayed in all irradiated cells, death seldom occurred until the ill-fated cells had undergone one to five divisions. The progeny of the non-survivors, although few in number, were large in size.

II. Dose Fractionation

Division of the dose into portions separated by an interval of 10 to 55 minutes resulted in a significant increase in survival. The extent of this increase varied between plates and between experiments.

In estimating the amount of recovery occurring, it was assumed that only survivors of the first dose were benefited by the interval between doses. It had been found that when irradiation was continuous, a cell that had survived some irradiation was less likely to survive further irradiation than an unirradiated cell. A response to the second dose that was equal to that which would have occurred if the first dose had not been given was defined as 100% recovery. In other words, full recovery means no interaction between the doses. The regression equation was used to estimate the survival values corresponding to 0% and 100% recovery. The actual survival usually fell between these two values (see Table II).

When two doses are given such that the survival from each alone would be, respectively, S_1 and S_2, 100% recovery from the first dose corresponds to a net survival equal to S_1S_2. The unfractionated, or zero-recovery, survival is always less than this. When multiple doses were used, the product $S_1S_2S_3$ or $S_1S_2S_3S_4$ was computed to determine the survival corresponding to 100% recovery.

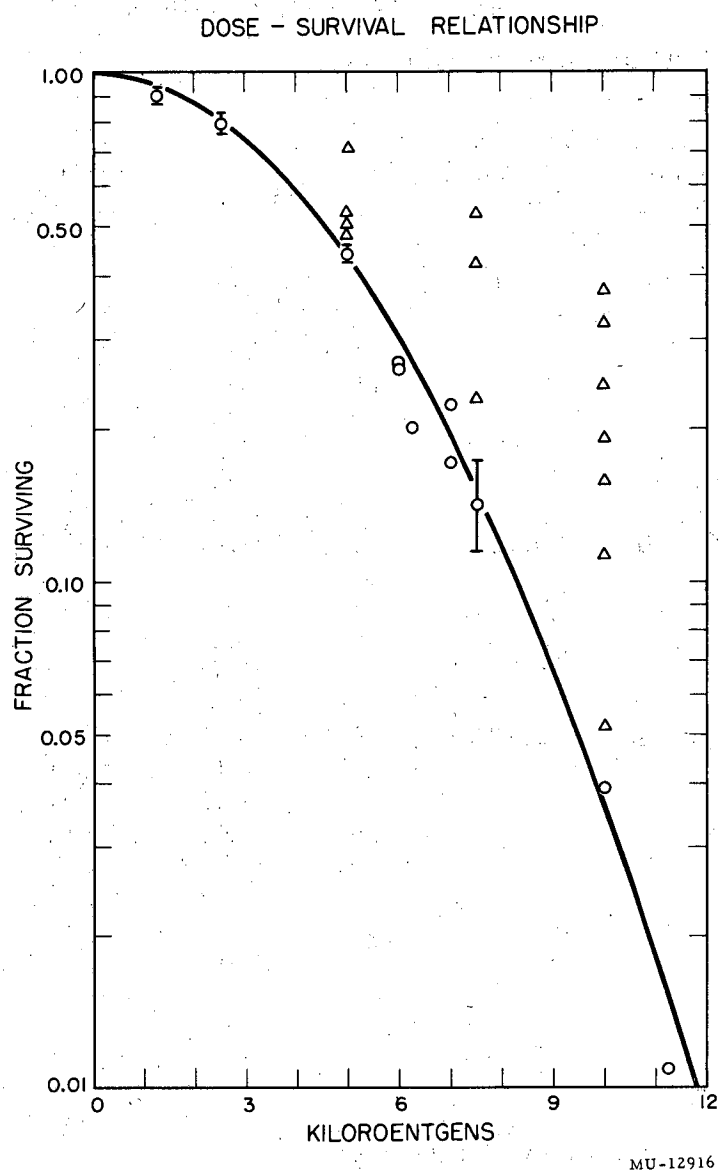

Fig. 1. Dose-survival relationship in Chlamydomonas reinhardi subjected to x-rays.

O Unfractionated Dose

△ Fractionated Dose

Vertical lines: Standard deviation of observations

Curve: Calculated survival curve for unfractionated doses

Table II

Dose-fractionation data

(For comparison with controls, see Table III)

Dose (kr)	Survival if no recovery	Survival if 100% recovery	Expt. No.	Strain	Conditions during interval	Interval (min)	Survival observed
2 x 2.5	0.430	0.656	24	CR1		45	0.509
			25	CR1		10, 20	0.537
			25	CR1		5	0.470
			26	CR1		20	0.712
			28	CR1		15	0.495
5.0 + 2.50	0.150	0.348	25	CR1		20	0.230
2 x 5.0	0.348	0.185	31	CR1	30°C	15-55	0.124(A)
			31	CR1	5°C	15-55	0.0571(A)
3 x 2.5	0.150	0.531	26	CR1		2 x 20	0.530
			28	CR1		2 x 15	0.422
4 x 2.5	0.0348	0.430	26	CR1		3 x 20	0.372
			28	CR1	light	3 x 15	0.322
			28	CR1	dark	3 x 15	0.246
			29	CR32	light	3 x 15	0.193(E)
			29	CR32	dark	3 x 15	0.158(E)
4 x 2.5 (after dark incubation)	0.15E		30	CR26	light	3 x 15	0.370
			30	CR26	dark	3 x 15	0.255

(A): adjusted for apparent dilution error

(E): obtained by extrapolation

Table III

Statistical analysis of data in Table II

Experiment number	Treatment compared	Mean count	Degrees of freedom	Student's t[a]	p[b]
24	Fract.	65.2			
	Unfract.	46.6	8	2.437	0.05
25	Fract.	29.0			
(5 kr)	Unfract.	21.6	13	2.563	0.05
25	Fract.	12.4			
(7.5 kr)	Unfract.	8.8	8	1.446	0.2
26	(All doses fractionated)				
28	Fract.	35.4			
(7.5 kr)	Unfract.	22.0[c]	13	2.843[c]	0.02[c]
30	Fract.	90.0			
(light)	Unfract.	53.75[c]	6	4.887[c]	0.01[c]
30	Fract.	62.0			
(dark)	Unfract.	53.75[c]	6	1.858[c]	0.2[c]
31	Fract.	142.35			
(30°C)	Unfract.	46.2	20	8.559	0.00
31	Fract.	65.56			
(5°C)	Unfract.	46.2	19	1.805	0.1
28	Light	27.0			
(10 kr)	Dark	20.6	8	1.308	0.3
29	Light	377.5			
	Dark	308.75	6	3.197	0.02
30	Light	90.0			
	Dark	62.0	6	3.870	0.01
31	30°C	142.35			
	5°C	65.56	31	9.460	0.00

[a] degrees of freedom = number of plates minus 2

[b] p = probability of as large a value of ± if treatment has no effect

[c] unfractionated plates received lower total dose

All experiments except Numbers 26 and 29 included enough unfractionated exposures to show whether the cells were behaving as predicted by the regression equation. In all except Number 30, which involved preirradiation treatment consisting of incubation in the dark, it was concluded that the cells were of normal radiosensitivity.

The amount of recovery was always positive but never clearly greater than 100%. Although division of the total into three or four exposures produced up to 10 times as many survivors as a single application of the same total dose, the percent recovery was comparable with that from two exposures. Survival of series of 1, 2, 3, and 4, exposures of 2.5 kr each, indicated that recovery continued even after three exposures.

Figure 2 shows that the effect increases rapidly for the first 15 or 20 minutes after exposure, when growing cells are used. The data on nitrogen-depleted cells, which show slower recovery, are regarded as preliminary. Percent recovery, in Figs. 2 and 3, is obtained by calculating the effective reduction of the first dose with respect to its sensitizing effect on those cells that survived it. From the regression equation, percent recovery is linearly related to the logarithm of the number surviving both doses.

III. Effect of Illumination on Recovery

The effect of illumination during the recovery interval upon the survival after successive exposures to 2.5 kr was tested. Although there was no effect after two and three exposures, a small but consistent enhancement of recovery by illumination was evident after four exposures. This effect did not depend on illumination prior to the first irradiation.

IV. Effect of Temperature on Recovery

The effect of temperature during the recovery interval was more marked. Two groups of plates were irradiated with two doses of 5 kr. One group was kept at 30°C until it received the second dose, while the other was kept at a temperature between 0° and 5° C. The fractionation intervals varied from 1/2 to 55 minutes. Pre- and postirradiation handling of the plates and the exposure sequence were randomized to make certain that the treatment of the two groups was the same except for temperature. Although, in the 30° series, a maximum increase of about threefold in the yield of viable cells was reached in about 15 minutes, the 5° series showed relatively little recovery even after 55 minutes. Forty-eight plates received fractionated doses in this experiment. Five plates, at room temperature, received a control dose (Table IV).

Another series at 10° and 24.5° C, using two 4-kr doses, gave a similar result, as shown in Fig. 3.

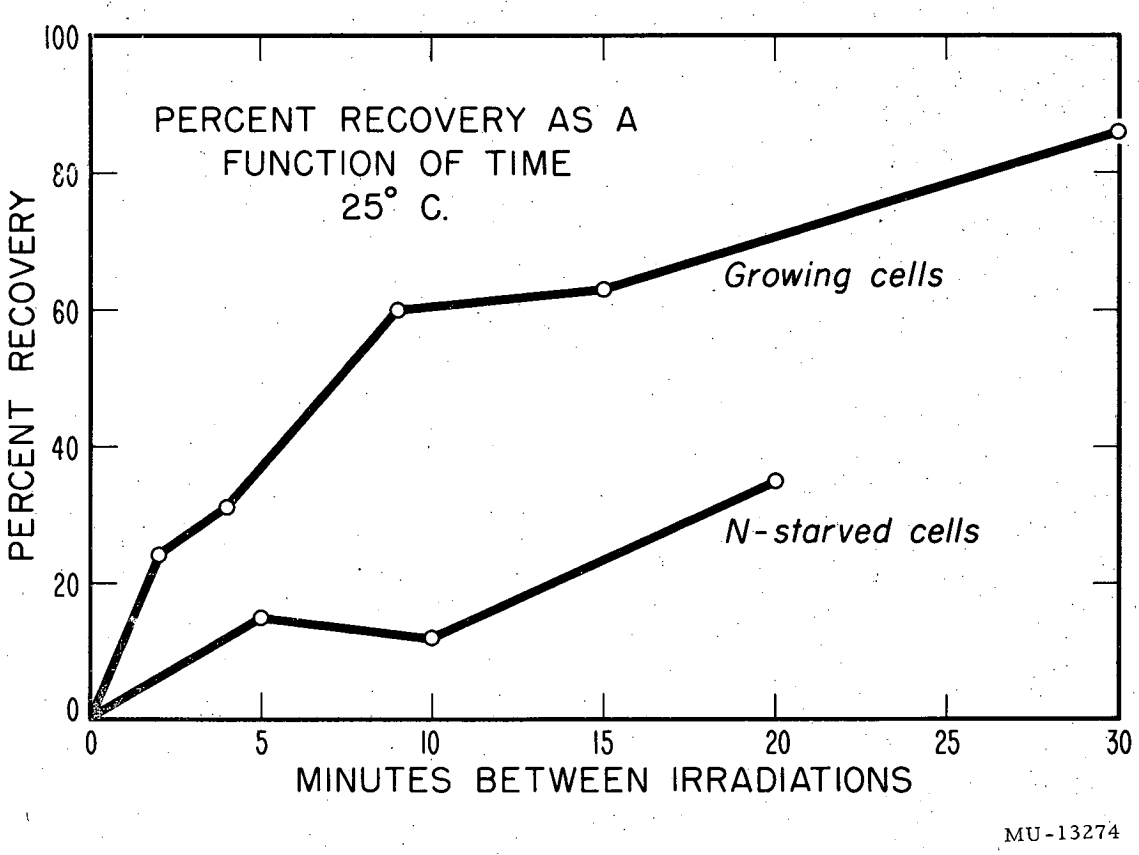

Fig. 2. Recovery of *Chlamydomonas reinhardi* from x-rays as a function of time. Dose: 4 kr + 4 kr at 25° C.

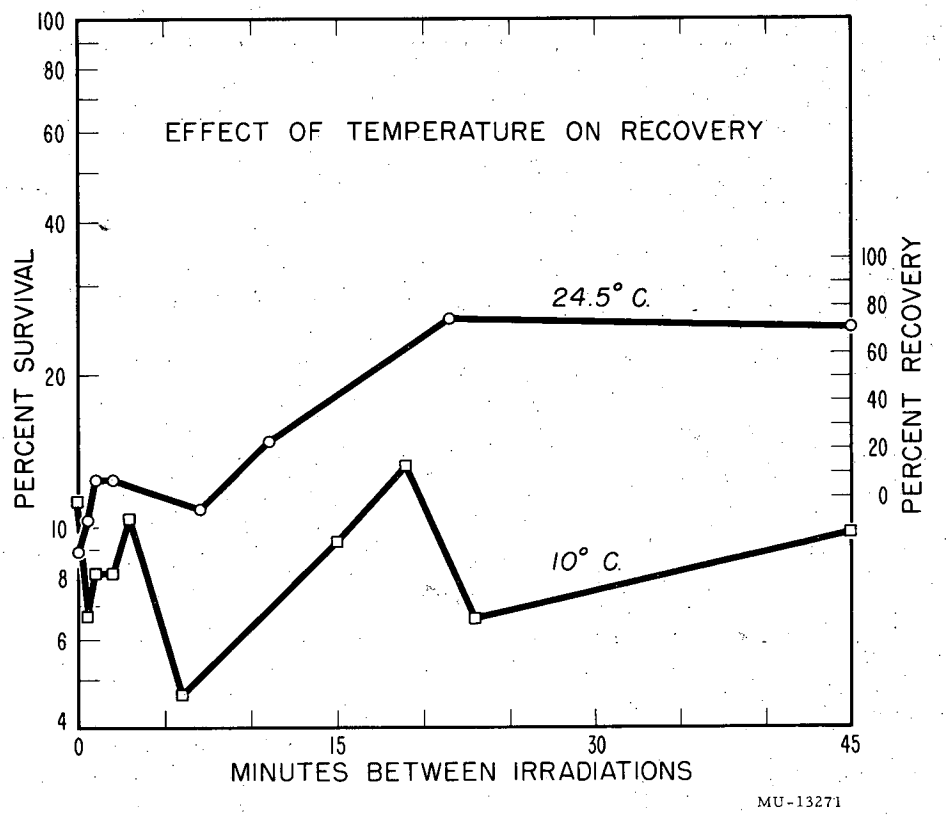

Fig. 3. Effect of temperature on recovery of *Chlamydonomas reinhardi* from x-rays. Dose: 4 kr + 4 kr.

Table IV

Experiment 31. Relation between recovery time, recovery temperature and plate count.

Time (min)	Count at 30°C	Unfractionated Count	Count at 5° C
0		61 49 29 53 39 Mean: 46.2	
1/2	62		42
1	82		-
2	51		63
4	75		-
5	-		47
6	80		-
7	-		64
9	100		46
12	112		47
14	89		43
15 to 55	142.35 mean of 17 plates, none less than 100 (See Tables II and III)		65.56 mean of 16 plates, only one greater than 100 (See Tables II and III)

DISCUSSION

An indication that cell division, rather than cell enlargement, is blocked in the progeny of irradiated cells is found in their increased size.[16]

It can be shown that a one-hit mechanism of cell death implies a negative slope of the survival curve at the point corresponding to zero dose, regardless of complications that may arise at higher doses because of inhomogeneity or sensitization of the population.[17] Since the empirical equation,

$$S = e^{-kD^2} ,$$

which fits the survival curve obtained, initially has a slope of zero, it suggests that multiple-hit mechanisms predominate in x-ray killing in *Chlamydomonas reinhardi.*

The equation

$$S = e^{-kD^2} ,$$

can be derived in several ways, depending upon the assumptions made regarding the nature of the primary radiobiological event. Although the applicability of this equation is not in itself evidence in favor of any specific mechanism, it is useful in assessing the extent of "sensitizing" and "lethal" damage.

For any survival equation, the probability that a cell that survives a dose D will fail to survive a dose D + dD is equal to $-(1/S)(dS/dD)\,dD$. The quantity $-(1/S)(dS/dD)$, therefore, measures the sensitization of that part of the population which survives the dose D. In this case the sensitization is equal to 2kD--i.e., is proportional to the dose already received. The sensitization diminishes considerably during the first 5 to 15 minutes after exposure, but never drops below its initial value.

A number of quanta of irradiation must be absorbed by a cell of this species before death can occur. At the instant when irradiation is terminated, some cells have undergone a modification that will prove fatal even if they are not irradiated again. Merely delaying the second irradiation will not reactivate these cells. Other cells have been rendered more liable to killing by the second dose, if it is given immediately, than they were originally. This increased susceptibility becomes less evident, under appropriate conditions, after time has elapsed. The time necessary is short compared with the generation time.

So far as their response to further irradiation is revealed by survival studies, the increased radiosensitivity of irradiated cells diminishes with time. Two explanations are possible. The effect of prior irradiation may be reversible, or the cell may undergo changes that reduce its sensitivity to further damage without repairing the damage already done. The first explanation would include all recovery mechanisms.

The term "recovery" implies not merely a spontaneous decay of the products of irradiation, but a process by which these products are removed by the cell, or by which the damage wrought by them is repaired. A process in which the metabolic equipment of the cell plays no role might more aptly be termed reversal.

In the reactivation of Escherichia coli by postirradiation treatment, several observations justify use of the term recovery. In addition to being peculiarly dependent on temperature, this reactivation occurs only in the presence of a rich organic nutrient medium. No single substance has been found that will suffice.[18] The nutritional requirements of the recovery process, as well as its optimum temperature, differ from those for growth. Some metabolic process, which is more crucial for irradiated cells than for normal cells, is probably responsible.

Another illustration of metabolic recovery is found in the effect of fractionated doses on plant chromosomes. In Vicia faba, Wolff and Luippold find that respiratory activity during the recovery interval is necessary for restitution.[19] The ultimate consequences of the radiation-induced lesion depend upon physiological events occurring after its induction.

The temperature dependence of the dose-fractionation effect in Chlamydomonas suggests the probable involvement of cellular metabolism. At temperatures just above freezing, the process is not merely delayed; it is virtually abolished. Since cultures can be stored for weeks at this temperature, without the fatal consequences that ensue in stale medium at higher temperatures, and without growing, it is certain that metabolism is inhibited at 0° C to 10°C. The enhancement of recovery by light may indicate that photosynthesis can provide energy for recovery.

In C. reinhardi, recovery occurs at temperatures close to the optimum for growth, and on minimal medium. In E. coli, where recovery occurs at suboptimal temperatures, an enriched medium is required. The relationship between growth temperature and recovery temperature parallels the relationship between the nutritional requirements for growth and for recovery.

If the increase in survival accompanying dose fractionation is due to neither reversal of, recovery from, nor restitution of radiation damage, the irradiated cells must become resistant to further damage, after a few minutes. This effect could have several consequences. If two equal doses are given, 100% recovery implies that the same fraction of viable cells is killed by the second dose as by the first dose If the second dose is less effective than the first dose, apparent recovery in excess of 100% could occur. The data do not show that the relative probability of surviving the second dose is ever greater than that of surviving the first.

Since irradiation apparently retards cell division in the survivors, any resistant state induced by preirradiation might also be manifested by lag-phase cells. Nitrogen-starved cells, which do not divide, might be expected to exhibit radioresistance. Such resistance was not observed. The culture treated with manganous chloride and the one deprived of calcium and magnesium, although apparently not growing, showed normal sensitivity.

The increased resistance of unilluminated cells had been explained by Nybom as a consequence of the protective effect of anoxia.[7] That the protective effect of dose fractionation is not due to the inhibition of photosynthetic production of oxygen, however, is shown by the enhancement of the effect by illumination. While this enhancement could be explained by

inadequate temperature control, its failure to appear until after the fourth exposure suggests the depletion of some photosynthetic product.

The existence of a postirradiation refractory state, although not impossible, is not suggested by the results of this experiment, since the only observed effects of irradiation, other than death, are a transient increase in sensitivity to further irradiation and the inhibition or one or more processes involved in cell duplication. It seems unlikely that the fractionation effect is due to inhibition of cellular activity, since it is enhanced by treatment that stimulates activity. It seems even less likely that the effect is due to radiation-induced stimulation of cellular activity, at doses which kill a substantial part of the population and which delay division of the survivors.

Nybom interprets the great difference in sensitivity to immediate and delayed (end-point) killing as evidence of a genetic mechanism.[7] Although the shape of the survival curve excludes any appreciable kill by one-hit mutations, killing by "two-hit" chromosomal aberrations is not excluded. The response of Chlamydomonas to dose fractionation resembles that of plant chromosomes. Wolff and Luippold have postulated a rejoining system which must function in order that chromosome breaks may restitute.[20] The rejoining system may be blocked by a variety of agents that inhibit either respiration or oxidative phosphorylation. Since the recovery of Chlamydomonas is blocked by suboptimal temperature, and reduced in the absence of a major energy source, light, it seems possible that the recovery system in this organism is identical with the rejoining system. Although the minuteness of the chromosomes prohibits a direct cytological test of this hypothesis, it is hoped that experiments now in progress will define the recovery system more precisely.

Metabolic recovery seems, at present, to be the most plausible explanation of the dose-fractionation effect. Whether this recovery consists of the repair of chromosome breaks, or whether it consists of reversal of some other incomplete radiation-induced process, is not yet clear.

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