

EFFECT OF SUCCESSIVE X-IRRADIATION ON *NOCARDIA CORALLINA*¹

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Since no satisfactory single criterion has been found for the determination of ploidy in microorganisms, it is necessary to correlate results from several experimental approaches in order to make interpretations in terms of ploidy. One such criterion that can be used is the application of the unpaired defect theory of Tobias (1952). According to this theory, sublethal irradiation of a diploid culture results in the formation of unpaired defects (recessive lethals?) which will sensitize the culture to subsequent irradiation. This theory is based on the concept that a significant cellular damage from irradiation is genetic, although it does not exclude the idea that irradiation can cause many other types of damage. Certainly the ploidy of a microorganism and some measure of its resistance to irradiation must necessarily be related, even though resistance can occur independently of ploidy changes as in the case of *Escherichia coli* strain B/r (Witkin, 1947).

Cytological studies on *Nocardia corallina* (Webb, *et al.*, 1954) suggested that the coccoidal stage of growth of this organism should be a stable diploid. This was evidenced by the fragmentation of hyphal growth which yielded binucleate bacillary cells, which in turn yielded uninucleate coccoidal cells. The nuclear events involved in the transition from a binucleate to a uninucleate cell were not observable, but a nuclear fusion was suggested. Since no uninucleated, haploid phase appears to exist in the life cycle of *N. corallina* (Webb and Clark, 1957) direct comparative radiobiological survivor studies as done in the polyploid series of yeasts (Latarjet and Ephrussi, 1949) were not possible. Therefore, the unpaired defect theory was used as a basis for a study of the coccoidal cells of this organism.

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MATERIALS AND METHODS

The organism used in these experiments was *N. corallina* strain 4273 of the American Type Culture Collection, which was maintained in stock on nutrient agar (Difco) containing 1 per cent fructose. Transplants for experiments were made on fructose agar and incubated for four days at 29 C, at which time the cells were in the coccoidal stage of growth. Growth on the four day slants was harvested in sterile saline and shaken vigorously on a vibratory shaker to break up clumps. The addition of glass beads to the suspension often aided in breaking clumps of cells. The shaken suspension was then centrifuged at slow speeds to remove clumps which were still present. The centrifugate was examined by phase microscope, and unless this final suspension contained at least 90 per cent single cells, it was not used for irradiation experiments. Cytological examination using the crystal violet nuclear stain (Chance, 1952) was done to assure that the coccoids were not germinating and thus producing a binucleate state.

The cell suspensions were standardized turbidimetrically and subjected to X-irradiation from a Picker portable field unit at a dose of 1,500 roentgens per minute as determined by a Victoreen 250 r dosimeter. Plate counts made on nutrient agar at time intervals during the irradiation gave the dose-survivor response of the culture. A mass culture was made from the colonies developing on the plates made from the irradiated aliquot which yielded 75 per cent survivors of the parent strain (18,000 r dose). The mass culture was transferred to fructose agar and incubated for 4 days. The resulting culture was subjected to the standardized procedure of suspension in saline, shaking, centrifuging, and microscopic examination, and then subjected to X-irradiation to give the second successive irradiation. Colonies developing on the plates from the aliquot irradiated with 18,000 r were carried through the identical procedure for the third successive irradiation. All subsequent successive irradiations followed an identical procedure.

It should be noted that each successive irradiation involved at least two life cycles of the organism; first, as it developed to yield colonies on the irradiation sample plate, and second, when the mass survival culture was transferred to fructose agar and incubated.

Micrococcus pyogenes var. *aureus* FDA 209 was grown on nutrient agar and handled in much the same manner as *N. corallina*. Cultures used for irradiation were 6-8 days old and were cytologically examined to insure the absence of the diploid state, cross septation, and active nuclear division. Mechanical shaking was normally adequate to break up clumps and centrifugation was not necessary.

Cytological studies were made on the successively irradiated cultures using the crystal violet nuclear stain of Chance (1952).

RESULTS

The X-ray survivor response of the parent or "normal" culture of *N. corallina* was a multi-event type (figure 1, curve 1). No calculations were made on hit multiplicity since too many factors may be involved in radiation damage and this organism is not homogenous in regard to radiation sensitivity of the individual cells (Frady and Clark, 1956). Repeated experiments under the experimental conditions used resulted

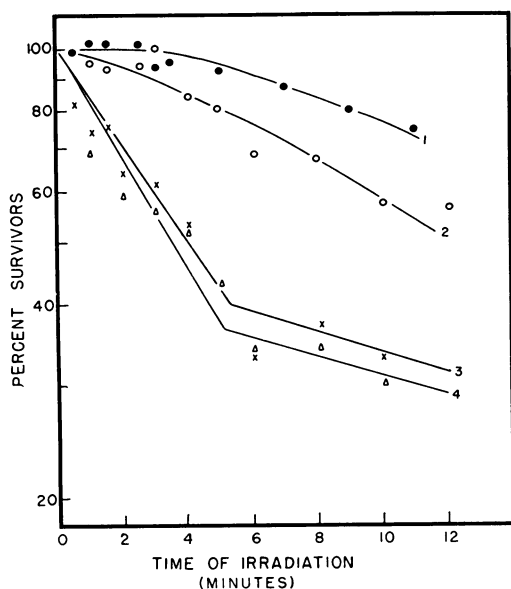


Figure 1. The effect of four successive X-irradiations on the dose survivor response of *Nocardia corallina* coccoids.

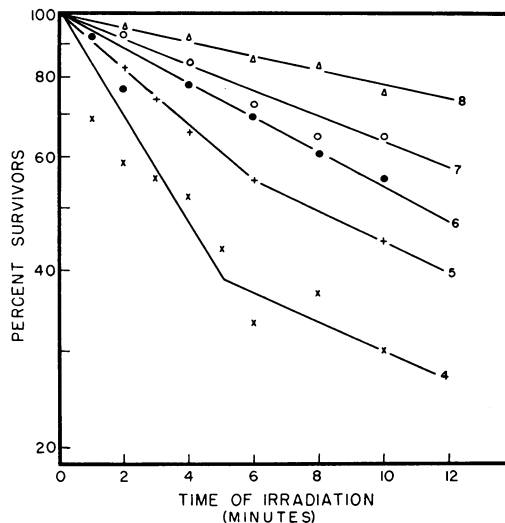


Figure 2. The effect of the fourth through eighth successive X-irradiation on the dose survivor response of *Nocardia corallina* coccoids.

in a similar survival response of the parent strain. The dose-survivor response of the survivors from the first 18,000 r dose was still of the multi-event type (figure 1, curve 2) but more sensitive to radiation than the parent strain. The dose-survivor response became approximately exponential, or a single event type, after the third irradiation and remained approximately exponential throughout all subsequent irradiations. Maximum sensitivity was obtained on the fourth successive irradiation (figure 1, curve 4) after which the successively irradiated cultures became more resistant (figure 2). The maximum resistance was obtained after the eighth successive irradiation (figure 2, curve 8). Additional successive irradiation resulted in a slight decrease in resistance through the eleventh irradiation, followed by variability in resistance on subsequent irradiations as shown in figure 3. In each case the dose-survivor response remained exponential.

The result of four successive irradiations of *Micrococcus aureus* is shown in figure 4. This culture was grown to yield haploid cells (Clark and Webb, 1955) and shows the stability of radiation resistance in a haploid cell.

Cytological studies of cells after each successive irradiation revealed no detectable nuclear change. The cells did decrease in size, the smallest size being about half that of the parent coccoid. The smallest cells were found after the eighth succes-

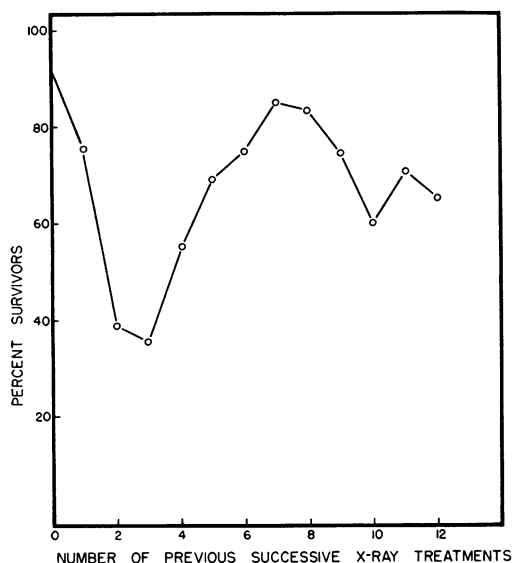


Figure 3. Survival of *Nocardia corallina* coccoids at a constant X-ray dose after previous X-radiations.

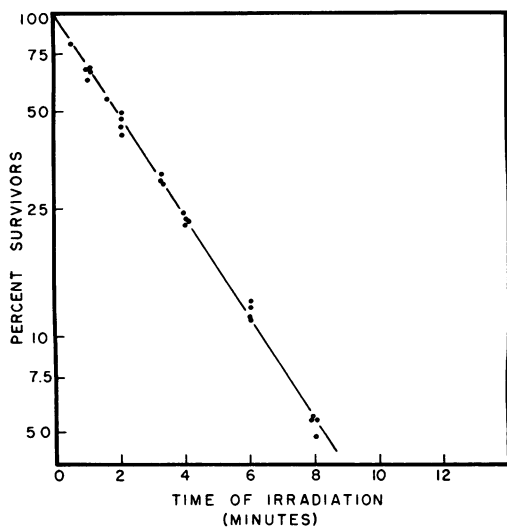


Figure 4. The effect of four successive X-irradiations on *Micrococcus aureus*.

sive irradiation, and the cells remained small during all subsequent irradiations.

DISCUSSION

The interpretation of the kinetics of radiation induced inactivation must be approached with caution. The dose-survival curve is a measure of the cumulative total of the various reactions which lead to inactivation of cell division as a

function of radiation dose. In actuality, the experimental approach used with microorganisms detects the inhibition of visible colony formation, and in some cases several cell divisions have been found to occur between the time of irradiation and the cessation of observable cell activity. The accumulation of evidence to date indicates that there is more than one mechanism involved in radiation damage; however, the significance of radiation induced recessive lethals in higher forms of life as well as much of the work on lower forms suggests that recessive lethal formation is one of the causes of radiation damage in microorganisms (see Zelle and Hollaender, 1955, for excellent review). Data such as presented by Atwood (1952) and Norman (1951) are best interpreted on the basis of lethal mutations.

The results reported by Tobias (1952) and in this paper are strongly suggestive of the recessive-lethal mutation concept, since the irradiation damaged survivors are more sensitive to subsequent radiation. The normal selective action of irradiation should result in either a more resistant culture or a culture of equal sensitivity if no inheritance of damage were involved. The fact that the cultures made from radiation survivors were more sensitive than the parent strain is indicative of the inheritance of some type of radiation induced damage. This could be due to chromosomal aberrations or gene mutation. Heterokaryon formation could not explain the results since the coccoidal cells were always uninucleate. However, it is probable that the radiation damaged haploid nuclei in the bacillary cells survived because of the heterokaryotic state existing in this phase of growth.

The low dose of irradiation used in the survivor experiments probably served to minimize some of the instantaneous radiation effects such as have been reported by Kimball (1955). The low dose also increased the probability of the isolation of survivors which had unpaired defects. Because of the low dose, the change toward maximum sensitivity occurred more slowly and made it possible to study the intermediate stages. As shown in figure 1, the first successive irradiation resulted in a population of survivors which displayed a multi-event response (curve 2) but was more sensitive than the parent culture. Since the dose-survival curve is an indication of population response rather than cellular response, these results are best explained on the basis of the heterogeneity of the culture. Many of the cells

had sustained unpaired defects, but many were undamaged. The over-all result of such mixture was the sensitive, multi-event response. The third and fourth successive irradiation culture gave virtually identical responses and represented the maximum sensitivity obtained in these experiments. The survivor response became a single-event type and remained approximately so throughout all subsequent irradiations. In these cultures the maximum level of unpaired defects had been obtained and any additional gene damage resulted in pairing of the recessive lethal alleles. This would yield an inactivation response that was exponential.

The survivor-response from the third and fourth successive irradiations displayed a "tail" indicating the presence of very resistant cells. Microscopic examination proved that this resistance could not be due entirely to clumps. It therefore appeared that a culture of *N. corallina* may contain a small percentage of cells which are significantly more resistant to irradiation than the normal cells. The output of our X-ray equipment was too limited to detect these resistant forms in a normal culture, and their occurrence was too low to be revealed by the selective action of the first two irradiations. During these irradiations the resistant cells apparently sustained unpaired defects since the survivor response was approximately exponential when they were detected. However, it is probable that the sigmoid-portion of the survival curve was obscured by the presence of the more sensitive, defective normal cells, and that the response did not become truly exponential until the seventh or eighth successive irradiation. Inherent experimental error precludes the exact determination of this point. Resistant cells were isolated from the nonirradiated parent culture and were found to yield a multiple-event survivor response.

After the eighth successive irradiation, the survivors again displayed an increasing sensitivity. This suggests that the cells composing the culture displaying maximum resistance were not saturated with respect to unpaired defects. Cultural response after the tenth successive irradiation was varied, probably because of cultural heterogeneity as well as inherent experimental error. Since two life cycles occurred between each successive irradiation, it would be expected that cultural heterogeneity would result from recom-

ination, which has been shown to exist in this organism (Webb, 1956).

In using the concept of recessive lethals to explain these results, it should be pointed out that tentative calculations (Tobias, 1952) indicate between 15 and 20 sensitive sites in *N. corallina*. The haploid response curve was estimated from the fourth successive X-irradiation on the assumption that a population carrying the maximum number of unpaired defects would approach the haploid sensitivity. These sites are undoubtedly only the most radiation sensitive ones which are associated primarily with the mechanisms of cell division. Mutations leading to biochemical deficiencies would not be expressed under the experimental conditions used. It was found that a culture appeared to be more sensitive to irradiation when the irradiated cells were plated in a minimal medium rather than in nutrient agar. This is at least in part due to the induced biochemical deficiencies.

The response of the haploid cells of *M. aureus* to successive irradiation (figure 4) was compatible with the unpaired defect theory. No resistant cells were present in the cultures tested as evidenced by the stability of the radiation response on successive irradiation.

An alternate explanation for the results reported in this paper, which cannot be completely discounted, is the haploidizing effect of radiation. Lederberg *et al.*, (1951) reported that X-rays induced segregation in the "het" strain of *E. coli* strain K-12. A similar action could account for the results reported here. However, the *E. coli* "het" is a non-disjunctional diploid which is normally unstable. There is no good evidence that irradiation will induce segregation in a normal diploid, and there is much evidence that irradiations can exert a polyploidizing effect on cells. Extensive cytological studies were performed on *N. corallina* immediately after irradiation and, although these studies will not be reported in detail since no interpretation is yet possible, no cytological evidence of reduction-division was found. In numerous studies of various isolates of *N. corallina* carried out in this laboratory, no evidence has been found that a culture consisting predominately of haploid coccoidal cells can exist, although the position of the few "three spot coccoids" observed by Webb and Clark (1957) is still unknown.

Extended work on cell variability has shown that the tentative interpretation of an exponen-

tial response in a coccoidal cell culture being due to a haploid state (Frady and Clark, 1956) was based on insufficient data. Coccoidal cells have been isolated that yield cultures which give a resistant, apparently exponential survivor response; however, cultural heterogeneity appears to be the cause of these results rather than haploidy. On the basis of these results, it now appears unlikely that induced segregation is involved in this case.

Regardless of whether the results are due to unpaired defects or induced segregation, or other cause, there is little evidence that X-irradiation induces radiation sensitivity in a population of haploid cells. The concept of ploidy in a cell involves both cytological and genetical aspects. Although in higher forms of life it is possible to demonstrate both cytological and genetical evidence of the state of ploidy, it is as yet quite speculative to accept cytological evidence alone in microorganisms. Likewise genetical evidence of a diploid state in microorganisms is usually very fragmentary, the best such evidence being presented by Lederberg, *et al.* (1951) for the non-disjunctional diploid strain of *E. coli* strain K-12. The accumulation of evidence now available indicates that the coccoidal cell of *N. corallina* is at least diploid.

SUMMARY

Cultures made from cells of *Nocardia corallina* which had survived an 18,000 r dose of X-ray were more sensitive to X-radiation than the parent cells. Maximum sensitivity was obtained after four successive irradiations of survivors, after which the radiation response became more resistant. This was due to the selection of a normally occurring resistant coccoidal cell which was initially present in a very small percentage of the total population. A culture of *Micrococcus aureus* treated in a similar manner showed no change in survival response on successive irradiation.

These results conform with the unpaired-defect theory of Tobias applied to a diploid cell. Although induced segregation of a diploid would cause similar results, experimental findings favor the theory of recessive lethal formation. Regardless of which theory is found to be more correct in interpreting the results, it appears probable that only a diploid or mixoploid population would yield the results obtained. The use of suc-

cessive irradiation as a supplemental tool in determining the ploidy of microorganisms should prove to be valuable.

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