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Induction of Provariants as a Function of Dose of Ultraviolet Radiation

With an appendix on the contribution of differential effects upon survival of provariant and nonvariant spores in the modifications of induced variant frequency resulting from post-irradiation treatments

BY S. D. WAINWRIGHT* AND ANN NEVILL

Biology Branch, Atomic Energy of Canada Ltd, Chalk River, Ontario

SUMMARY: With spores of *Streptomyces* sp. strain T12 irradiated at doses of ultraviolet light up to 1000 ergs/mm.² and then incubated in distilled water, both the proportion of survivors and the proportion of induced variants amongst the survivors rose progressively to maximal values as the period of incubation was increased. The maximal proportion of variants was attained before the maximal proportion of survivors. The rate of increase of survival and the final level of survival were enhanced in the presence of iodoacetate. The rate of increase of variant frequency was enhanced by iodoacetate, but the maximal proportion of variants was not raised. The changes of variant frequency resulting from post-irradiation treatments do not appear to be due to selectively greater increases in the survival of variant spores than of non-variant spores.

We have recently demonstrated (Wainwright & Nevill, 1955) that post-irradiation treatment of ultraviolet irradiated spores of *Streptomyces* sp. strain T 12 with iodoacetate or a peptone medium can modify both the extent of survival and the proportion of colonies showing radiation-induced heritable modifications of character. The effects of post-irradiation treatment with iodoacetate of spores irradiated at doses of radiation exceeding 400 ergs/mm.² were similar to the effects of a small decrease in the radiation dose. We have, therefore, attempted to determine whether independent modifications of the lethal and mutagenic effects of the radiation could be obtained with spores irradiated at these higher doses. A detailed study of the influence of the duration of the post-treatment has revealed modifications of the proportions of survivors without accompanying changes in variant frequency.

In addition, the nature of the relationship between the maximal proportion of variants obtained after suitable post-treatment and the dose of radiation received has been determined.

METHODS

Preparation of the spore suspensions of *Streptomyces* sp. strain T 12 and the experimental methods used are given in the accompanying paper (Wainwright & Nevill, 1955).

Cytological studies were made with spore preparations stained by the method of Robinow (1945).

* Atomic Energy of Canada Ltd Fellow.

RESULTS

Stimulation of the conversion of provariants* into variants

Examination of the data obtained in our earlier study of the effects of post-irradiation treatment with iodoacetate upon spores irradiated at a dose of 400 ergs/mm.² (Wainwright & Nevill, 1955) suggested that the higher the proportion of variant colonies obtained from irradiated control spores in-

Fig. 1. Enhancement of variant frequency by iodoacetate. Spores irradiated at a dose of 400 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated for 3 hr. at 37°. The variant frequency in the control suspension (distilled water) is plotted as ordinate. The increase in variant frequency due to treatment with iodoacetate, expressed as a percentage of the control value, is plotted as abscissa.

cubated in distilled water the smaller the effect of treatment with iodoacetate. The results of these and subsequent experiments are illustrated in Fig. 1. Similar inverse correlations have also been found (*a*) between the increase in proportion of variants induced by treatment with iodoacetate and the initial

* Our results indicate that ultraviolet radiation induces an unstable condition within a proportion of the irradiated spores. Under certain conditions these spores revert to stable non-variant spores and under other conditions these spores become stable variants. We therefore require a definition which will describe such modified spores.

Induced variant colonies can only arise from spores in which the radiation has induced the potential ability to express a heritable variation of character. However, the genotype of any given spore can only be determined after growth and development of a visible colony. The term 'provariant' is, therefore, used to describe those spores which are potentially able to give rise to a variant colony. This definition contains no implication concerning either the genotype or the viability of such spores immediately after cessation of the radiation.

proportion of variants observed with spores plated without post-treatment immediately after irradiation (zero-hour controls), and (b) between the increase in proportion of variants due to incubation in distilled water (3 hr. controls) and the initial proportion of variants (zero-hour controls).

The extent of the increases in variant frequencies resulting from treatment either in distilled water or with iodoacetate were not correlated with the corresponding increases in survival. Some of the most marked increases in survival were associated with small increases in the proportion of variants, and vice versa (see Wainwright & Nevill, 1955). Further, there was no correlation between the extent of the increase in survival due to treatment with iodoacetate and either the initial proportion of survivors from control unincubated spores or the final proportion of survivors from control spores incubated in distilled water. Similarly, the extent of the increase in proportion of survivors following incubation in distilled water could not be correlated with the proportion of survivors from unincubated control spores.

The effect of peptone and other N-sources in suppressing the increase in variant frequency (Wainwright & Nevill, 1955 and unpublished experiments) could not be correlated with the proportions of variants observed with control spores either before or after incubation in distilled water. No correlations were found between the effects of treatment with N-sources upon variant frequency and upon survival.

Influence of length of post-treatment

The effects of post-irradiation incubation for varying periods of time, with iodoacetate or in distilled water, upon the proportions of survivors and of induced variants among spores irradiated at 400 ergs/mm.² are illustrated in Fig. 2.

Three phases have been observed in the recovery of spores incubated in distilled water. The first, or lag, phase was characterized by a progressively rising rate of increase in viable count and was usually completed within 3–5 hr. (Fig. 2*a*). In the second phase the rate of increase in viable count was fairly constant and in the third phase the rate of recovery declined until a maximal level of survival was attained. There was some variation in the lengths of the various phases with different suspensions, and the lag period was frequently not observed (Fig. 2*b*). In the presence of iodoacetate no lag phase in the recovery process was ever observed, and the rate of increase in viable count was markedly raised (Fig. 2*a*). However, at the time of completion of the second phase of recovery in the control suspension the iodoacetate ceased to affect survival, and the subsequent rates of increase in viable count were approximately the same in the presence and absence of iodoacetate (Fig. 2*b*).

The proportion of spores yielding variant colonies observed after incubation in distilled water increased steadily at an approximately constant rate to reach an approximately constant maximal value after 24–48 hr. treatment. This maximal proportion of variants was attained before the maximum level of survival. Similar results were obtained with spores irradiated at doses

Fig. 2. Influence of post-irradiation treatment with and without iodoacetate. Spores irradiated at 400 ergs/mm^2 were diluted 4:5 with either distilled water or $2 \times 10^{-4} \text{ M-Na}$ iodoacetate (neutralized to pH 7.2) and incubated at 37° ; at intervals samples were plated. Broken lines: survival $\times 10^5$. Solid lines: % variants. Curves A and C: controls, distilled water. Curves B and D: $2 \times 10^{-4} \text{ M-iodoacetate}$. Figs. a and b represent two experiments of this kind.

exceeding 400 ergs/mm.² In the presence of iodoacetate the initial rate of increase in the proportion of variants was enhanced. However, stimulation of the increase in variant frequency stopped at a time coincident with the cessation of the stimulatory effects of iodoacetate upon survival, and thereafter the agent caused a progressive decline in variant frequency (Fig. 2b).

Experiments were performed in which portions of the irradiated spore suspensions were washed once with distilled water after a 3 hr. period of post-treatment, then resuspended in distilled water and re-incubated. The maximal proportion of variants obtained with spores treated with iodoacetate and subsequently washed was insignificantly different from that obtained with either washed or unwashed control spores incubated in distilled water (Table 1). As we were unable to prevent some loss of spores, it has not been possible to study in detail the effects of washing upon the course of subsequent increases in survival. However, it was noted that the rate of increase of survival of iodoacetate-treated spores was slightly increased after removal of the agent, whereas the rate of recovery of the spores incubated with distilled water was reduced to approximately one-half as a consequence of washing.

Table 1. *Influence of washing on maximal proportion of variants after post-treatment*

Spore suspensions irradiated at 400 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. After 3 hr. incubation portions of the suspensions were washed once with distilled water, resuspended in distilled water, and reincubated.

Maximal proportion of variants after post-treatment			
Control (distilled water)		2×10^{-4} M-iodoacetate	
Unwashed	Washed	Unwashed	Washed
60.8 $\pm$ 2.1	58.9 $\pm$ 2.6	56.8 $\pm$ 2.7	63.0 $\pm$ 4.5
65.6 $\pm$ 4.8	60.2 $\pm$ 2.5	50.7 $\pm$ 1.9	63.1 $\pm$ 4.9

No increase was observed in the viable count of unirradiated control suspensions over the period of these experiments. In fact, there was a slight progressive decrease which became significant after 72 hr. incubation. Further, there was no significant change in the proportion of spontaneous variants, which was of the order of 1–2 %. Hence it seems most improbable that either the increases in survival or in the proportions of variants can be attributed to germination of the spores, followed by subsequent fragmentation of the mycelium. Indeed, cytological examination of unirradiated control suspensions incubated for 72 hr. in either distilled water or 2×10^{-4} M-iodoacetate solution revealed that not more than 2 % of the spores had germinated, and for this small proportion of the spores the average number of nuclei present in the resulting mycelia was only 4.

The effects of post-irradiation incubation in distilled water or with iodoacetate upon spores irradiated at 200 ergs/mm.² are illustrated in Fig. 3. In distilled water there was a steady increase in both the viable count and the proportion of induced variants during the course of the 6 hr. period of incubation.

The rate of increase in viable count was raised for a brief period in the presence of iodoacetate. No significant effect of the agent upon the variant frequency was observed during the first 4 hr. of treatment, and thereafter it appeared to cause a reduction in the extent of the increase in the proportion of variants.

Fig. 3

Fig. 3. Influence of post-irradiation treatment with and without iodoacetate. Spores irradiated at 200 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. At intervals samples were plated. Broken lines: % survival. Solid lines: % variants. Curves A and C: controls, distilled water. Curves B and D: 2×10^{-4} M-iodoacetate.

Fig. 4

Fig. 4. Influence of post-irradiation treatment upon the dose/mutation curve. Irradiated spore suspensions were diluted 4:5 with distilled water and incubated at 37°. Curve A: suspensions plated immediately after irradiation and dilution. Curve B: suspensions plated after incubation for 24 hr. Curve C: suspensions plated after incubation for 48 hr. Curve D is the theoretical linear curve to be expected from the proportion of variants obtained at a dose of 100 ergs/mm.².

Influence of post-irradiation treatment upon the dose-survival and dose-mutation curves

It is usually assumed that radiation-induced mutations occur independently of each other, i.e. that the probability of a mutation taking place within any one cell is not affected by a prior mutation within that cell. In addition, it is usually assumed that the extent of the immediate response to irradiation of the cells (e.g. production of toxic chemical intermediates, induction of unstable states in the genes) is a linear function of radiation dose. If the lethal effects of the radiation not due to lethal mutation could be neglected, the shape of a curve showing induced mutant, or variant, frequency as a function of radiation dose could be predicted from these two assumptions. At very low radiation doses it would be expected that the curve would tend to be linear. However, as the dose increased so would the proportion of mutations which

would occur in cells in which a prior mutation had already occurred. Thus, the curve would actually show a less than linear increase in mutant frequency with increase of radiation dose.

The dose mutation (or variant) curves we have obtained experimentally deviate considerably from this theoretical curve. Usually the curve shows a peak (Fig. 4), the mutant frequency rising to a maximum and then decreasing again as the radiation dose is increased. Further, the initial increase in mutant frequency with increase of radiation dose is usually less than would be anticipated from a calculated theoretical curve based upon the frequency of mutants obtained at a low dose of radiation.

It is not our intention to discuss the various hypotheses which may be advanced to account for the shape of dose/mutation curves determined experimentally. Nor do we wish to illustrate the manner in which any one particular set of postulates can be used to account for the shape of curves obtained by experiment. Instead, we wish to note that one postulate which we have made is that the cell components responsive to the mutagenic activity of the radiation (and intermediary products) become 'saturated' at some intermediate dose of radiation.

Taking this postulate in conjunction with the assumptions previously noted, the theoretical dose/mutation curve would be expected to show a plateau. The inverse correlations noted between the extent of the effects of the post-treatments upon the induced variant frequency and the initial proportion of induced variants observed with unincubated controls suggested that the dose/mutation curve obtained after sufficient post-irradiation treatment to permit the attainment of the maximal variant frequency might correspond to this latter theoretical curve.

Table 2. *Influence of dose and post-treatment upon proportion of variants*

Samples of spore suspension irradiated at 400 ergs/mm.² were diluted 4:5 in either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. The maximal proportion of variants observed is compared with the initial proportion at 0 hr. treatment, and with the proportion of variants obtained after irradiation of the same stock suspension at 100 ergs/mm.² and plating after treatment in distilled water for 3 hr.

Expt. no.	Dose 100 ergs/mm. ² (A)	Maximal proportion of variants			Ratio B:A
		Dose 400 ergs/mm. ² , with post-treatment in			
		No treatment (0 hr. control)	Distilled water (B)	2 × 10 ⁻⁴ M- iodoacetate	
1	15.2 ± 1.1	33.5 ± 2.7	63.5 ± 3.6	60.4 ± 3.0	4.2
2	13.7 ± 0.9	42.6 ± 5.1	52.2 ± 2.4	47.8 ± 4.4	3.8
3	16.9 ± 3.6	36.8 ± 3.1	65.9 ± 3.8	—	3.9
4	15.3 ± 1.8	47.2 ± 4.3	68.9 ± 3.1	—	4.5
5	16.0 ± 1.6	42.1 ± 3.8	66.8 ± 4.6	—	4.2

Post-treatment of spores irradiated at 100 ergs/mm.² had previously been found to cause no significant effect upon the proportion of variants induced at this dose (Wainwright & Nevill, 1955). Thus, it was possible to compare the

maximal proportion of variants observed after post-treatment of spores irradiated at a dose of 400 ergs/mm.² with the proportion to be expected from the theoretical curve. The maximal proportion of variants after post-treatment either in distilled water or with iodoacetate was considerably higher than that expected from the theoretical model. Indeed, the maximal proportion found after incubation in distilled water roughly corresponded to that expected for

Fig. 5. Influence of post-irradiation treatment upon the dose/survival curve. Irradiated spore suspensions were diluted 4:5 with distilled water and incubated at 37°. Curve *A*: suspensions plated immediately after irradiation and dilution. Curve *B*: suspensions plated after incubation for 24 hr. Curve *C*: suspensions plated after incubation for 96 hr. The logarithm of the proportion of survivors is plotted against dose; the values are corrected for the slight progressive decrease in viable count of the unirradiated control suspension.

a linear dose/mutation curve (Table 2). A detailed study of the effects of post-irradiation incubation was therefore made, and the results of a typical experiment are illustrated in Figs. 4 and 5.

After incubation of irradiated spores in distilled water for 24 hr. the dose/variant-frequency curve (Fig. 4, curve *B*) was of the same form as the curve for spores plated immediately after irradiation (Fig. 4, curve *A*). There was a significant increase in the observed proportion of variants at all doses of radiation exceeding 100 ergs/mm.², but the dose corresponding to the peak of

the curve was unchanged. On incubation for another 24 hr. there was a further increase in the proportion of variants observed with spores irradiated at doses greater than 400 ergs/mm.², but a slight decrease in variant frequency for spores which had received smaller doses of radiation (Fig. 4, curve *C*). The proportion of induced variants observed for all doses of radiation decreased slightly, but insignificantly, upon further incubation.

The form of the dose/survival curve was unchanged by post-irradiation treatment (Fig. 5), although the extents of survival continued to increase throughout the whole period of the experiment (96 hr.). However, the value of the intercept of the linear portion of the curve increased during the first 24 hr. of treatment, and thereafter tended to decrease again to the value obtained with spores plated immediately after irradiation (Table 3, Expt. 1 corresponds to Figs. 4 and 5). There was considerable variation in the values of these intercepts for different experiments, but the same qualitative changes were observed in all experiments.

Table 3. *Influence of post-treatment on value of the ordinate intercept of dose/survival curves*

Irradiated samples of a spore suspension were diluted 4:5 with distilled water and incubated at 37°. Samples were plated at daily intervals and dose/log. survival curves were prepared from the results obtained. The values of the antilogarithm of the intercept *n* of the linear portions of the curves are recorded in the table.

Expt. no.	Value of intercept <i>n</i> on ordinate at hr.				
	0	24	48	72	96
1	16	50	35	30	30
2	50	100	100	100	50
3	270	350	300	250	250

DISCUSSION

An increase in survival not accompanied by any significant modification of the proportion of induced variants was observed during the course of post-irradiation treatments with iodoacetate or with distilled water of spores irradiated at all doses up to 1000 ergs/mm.² (Figs. 2, 4 and 5, and Wainwright & Nevill, 1955). A partial resolution of the lethal and mutagenic effects of ultraviolet radiation upon spores of *Streptomyces* sp. strain T 12 has, therefore, been demonstrated over the whole range of radiation doses up to 1000 ergs/mm.².

On the basis of results previously obtained (Wainwright & Nevill, 1955), sets of postulates were given which we believed to represent the minimum requirements for an explanation in terms of lethal and mutagenic 'poisons' of the observed effects of post-radiation treatments upon ultraviolet irradiated spores. The continued increase in survival without modification of the induced variant frequency observed with spores irradiated at doses of radiation exceeding 400 ergs/mm.² (Fig. 2) necessitates an increase in the minimal number of lethal and mutagenic poisons previously postulated. Results obtained in a survey of the effects of post-irradiation treatment with other agents of significance to cell metabolism upon spores of the *Streptomyces*

strain T 12 (in preparation) and upon cells of *Escherichia coli* (Dr Jacobs, personal communication) indicate that yet a further increase in this minimum number of poisons must be postulated.

The effects upon the induced variant frequency of post-irradiation treatment with a given agent have been found to depend upon the dose of radiation used (Fig. 4, and Wainwright & Nevill, 1955), and upon the length of the period of treatment (Figs. 2*b*, 4). Thus, any interpretation of our findings based solely, or partially, upon postulated different effects upon the viabilities of 'provariant' and non-variant spores must be very complex.

Nevertheless, in attempting to make any further analysis of the results obtained in this study, it is important to assess the possible contribution of different effects of the post-treatments upon the viabilities of 'provariant' and non-variant spores which could result in a selectively greater increase in the survival of provariant spores than of the non-variant spores.

For reasons given in detail in the Appendix, we believe that such differences in the effects of the post-treatments upon the viabilities of provariant and non-variant spores are of little, if any, importance in the observed phenomena.

Our results indicate that the increases in variant frequency observed as consequences of post-irradiation treatment cannot be attributed to the associated increases in survival. Hence, it appears that the radiation induces within a fraction of the spores an unstable provariant state which may either revert to the parental non-variant state or be converted to a stable variant condition. Further, the extent to which the provariant spores become stable variants is dependent upon the nature and duration of the post-treatment to which the irradiated spores are subjected. Similar conclusions have been made by Kaplan (1952, 1953) from a study of sectoring in colonies of *Serratia marcescens* (*Chromobacterium prodigiosum*).

Results obtained in a study of the effects of post-irradiation treatment upon the dose/variant-frequency curve differ markedly from results obtained by Hollaender & Emmons (1941) in a similar study with spores of *Trichophyton mentagrophytes* incubated in physiological saline. No explanation of this difference can be given at this time. The results obtained in our own study do not shed any light on the problem of the mechanism of action of the radiation in inducing the provariant state. However, the relationship observed between the maximal proportion of variants found with spores given a dose of 400 ergs/mm.² and the variant frequency for spores irradiated at 100 ergs/mm.² (Table 2) indicates a high degree of reproducibility in the relationship between induction of provariants and radiation dose.

Values of the intercepts at the ordinate of the linear portions of dose/survival curves have been considered to represent the average number of hits or critical events required to inactivate an irradiated cell. Attempts have been made to correlate this value with the average number per cell of some cell component: in particular, the average number of nuclei or sets of chromosomes (e.g. Atwood & Norman, 1949; Norman, 1951; Sarachek & Lucke, 1953). Variations in the values of such intercepts resulting from post-irradiation treatment of non-germinating, uninucleate spores (Fig. 5 and Table 3) clearly impose limits

upon the types of events which can be postulated as leading to cell inactivation, and indicate that killing occurs only as the result of a complex sequence of events.

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APPENDIX

The contribution of differential effects upon survival of provariant and non-variant spores in the modifications of induced-variant frequency resulting from post-irradiation treatments

As the period of post-treatment in distilled water of spores irradiated at 400 ergs/mm.² is increased, a stage is reached at which the extent of survival continues to rise but the proportion of variants remains unchanged (Fig. 2*b*). This indicates that if there are any differences in effects which result in a selection favouring provariant spores they progressively decrease during the course of the post-treatment. Our consideration must, therefore, be restricted entirely to effects which may be observed during the early period of treatment of populations of provariant and non-variant spores.

The extent of the increases in variant frequency observed after 3 hr. post-treatment, in distilled water or with iodoacetate (Fig. 1), of spores irradiated at 400 ergs/mm.² were inversely related to the variant frequency observed without post-treatment. The extent of these increases could not be correlated with either the initial level of survival (which varied in different experiments over a 50-fold range) or the extent of the increases in survival. Further, the total concentration of spores was approximately the same in all experiments. Thus, it would appear that the rate of increase in the survival of provariant spores relative to that of non-variant spores would be primarily determined

by the frequency of provariants actually present in the suspension, and would be largely independent of the absolute concentration of viable spores. Hence, as the variant frequency rose during the course of prolonged incubation, the selectively greater increase in the survival of provariant spores than non-variant spores would decrease and become negligible. The variant frequency would, therefore, be expected to rise to a maximal value presumably determined by the initial physiological condition of the spores before irradiation, the nature of the post-treatment applied and perhaps the radiation dose. The maximal variant frequency would not be directly determined by the frequency of variants observed with spores plated without post-treatment, but the time required for the attainment of that maximal value would be determined by the initial variant frequency.

Therefore, if the relative responses of radiation-induced provariant and non-variant spores to a constant selective environment were independent of the radiation dose, it would be expected that the effects of a given post-treatment upon the proportions of variants would (i) be greater upon spore suspensions irradiated at 100 ergs/mm.² than upon those irradiated at 400 ergs/mm.², and (ii) lead to the attainment of approximately the same maximal proportion of variants for all suspensions independently of radiation dose.

Neither prediction is in accord with the results obtained (Fig. 4 and Wainwright & Nevill, 1955). Further, postulates which could be used to explain the discrepancy between prediction and observation at low radiation doses are inadequate to explain the corresponding discrepancy at high radiation doses.

On the other hand, if the relative response of provariant and non-variant spores to a constant selective environment were a function of radiation dose, we would expect a progressive shift of the dose corresponding to the peak of the dose/mutation curve towards a higher dose during the course of the post-radiation treatment. The absolute extent of the shift cannot be predicted as the extent to which the survival of provariant spores would be selectively increased above that of non-variant spores is unknown.

The data presented in Fig. 5 show that the increase in variant frequency resulting from post-radiation incubation in distilled water of spores irradiated at doses exceeding 300 ergs/mm.² is very marked. With spores irradiated at lower doses the increase in variant frequency after similar treatment is smaller, or even negligible. However, there was no apparent change in the dose of radiation corresponding to the peak of the dose/mutation curve. Our data do not preclude the possibility of a small change of this type. Nevertheless, they do indicate that the extent of any such change is smaller than would be expected if the observed increases in variant frequency following incubation in distilled water were due solely, or even largely, to differences in the extents of the increase of survival of provariant and non-variant spores.

It appears, therefore, that differences in the effects of the post-treatments upon the viabilities of provariant and non-variant spores are of little, if any, importance in the phenomena which we have studied.

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