DOSE-RESPONSE RELATIONSHIPS IN RADIATION INDUCED MUTATION. SATURATION EFFECTS IN STREPTOMYCES

H. B. NEWCOMBE AND J. F. McGREGOR

Atomic Energy of Canada Limited, Chalk River, Ontario, Canada

Received January 20, 1954

VERY considerable effort has been devoted to showing that the frequencies of X-ray induced mutations, and in particular the recessive lethal mutations of Drosophila, rise linearly with increasing dose; and the theoretical implications of this relationship have been much discussed. Special interest therefore attaches to KELNER's (1948) demonstration of a clear-cut exception to the general rule in X-irradiated spores of Streptomyces, such that at high doses the capacity for mutational response appears to be saturated and further increases in exposure fail to cause the expected rise in mutant frequency.

This exceptional dose-response relationship is of additional interest because it resembles closely the corresponding nonlinear relationship for ultraviolet-induced mutation found in almost all organisms studied, and thus suggests similarities in the modes of action of X-rays and ultraviolet (and also that the commonly observed difference in dose-response relationship may perhaps be quantitative rather than qualitative).

For these reasons a further detailed study of the saturation effects in Streptomyces was carried out and is described in this paper.

MATERIALS

A pigmented laboratory strain (T12) of Streptomyces, having a deep orange leathery base with abundant pale orange aerial mycelium, was used throughout. Clearly scorable colony-morphology and color mutations occur with high frequencies in spores irradiated with X-rays or with ultraviolet. All of these mutations are stable in that they do not revert to normality except as an infrequent event in certain mutant lines. Most however are unstable in that a high proportion of the spores give rise to further variant forms (for a detailed description of the induced mutants see NEWCOMBE 1953).

The material lends itself to precise quantitative studies since: (1) the spores are uninucleate, (2) suspensions with little or no clumping are readily prepared by blending and filtration, and (3) large numbers of induced mutations can be scored simply by observing the colonies which develop from irradiated spores. (In our experiments these were plated on asparagine, peptone, $K_2HPO_4 \cdot 3\ H_2O$, 0.5 each; glucose 10, agar 15, water 1000.) Including preliminary experiments, over a half million colonies were examined, of which over 50,000 were mutant.

It is apparent that the bulk of the Streptomyces mutants constitute a special class, resembling in certain respects the " variegated " forms of higher

organisms. However, there is now a clear demonstration that a large group of " variegations " in Zea can arise through an initial chromosome breakage involving a particular heterochromatic region (McCLINTOCK 1951), and there is no reason to suppose that the present changes are other than chromosomal or genic.

THE EFFECT OF DOUBLING X-RAY OR GAMMA DOSE

The dose-mutation relationships were studied by comparing the effects of a single and a double exposure to X-rays at different dose levels (see table 1), the single exposures being 1, 2, 4, 8 and 16 minutes at 50 cm. from the target of a G.E. X-ray machine operating at 2000 K.V.P. and 1.5 ma. (approx. 500 r per minute). Ten ml of a spore suspension suitably diluted with liquid

TABLE 1

Effect of doubling the dose of X-rays on mutation and survival (combined results from ten replicate experiments for each pair of doses).

Dose (min.)	Mutations				Survival	
	Mutant colonies	Total colonies	Av. % induced mutants	Av. obs./exp.	Av. % survival	Av. obs./exp.
0	336	68,563	0.00		100.0	
1	1,917	58,817	2.75		86.3	
1	1,890	56,363	2.89		82.7	
1 + 1	2,902	49,300	5.43	0.98[1]	72.4	1.03[2]
0	30	5,052	0.0		100.0	
2	239	3,200	9.6		62.0	
2	241	2,789	10.4		53.5	
2 + 2	322	1,958	19.2	1.12[3]	34.2	1.11[4]
0	26	5,198	0.0		100.0	
4	1,400	10,618	13.2		50.7	
4	1,064	9,240	12.3		44.7	
4 + 4	1,256	5,795	23.6	1.00[5]	26.6	1.22[6]
0	60	15,291	0.0		100.0	
8	1,509	8,042	18.4		34.4	
8	1,539	7,126	21.5		31.2	
8 + 8	1,533	5,676	26.0	0.72[7]	11.8	1.18[8]
0	20	5,639	0.0		100.0	
16	1,694	6,322	26.5		8.3	
16	1,253	5,209	23.7		7.2	
16 + 16	1,329	8,968	14.4	0.30[9]	2.4	4.51[10]

Irradiated in nutrient medium at 50 cm from the target (dose rate = approximately 500 r/minute).

Observed/expected; values for individual replicate experiments:

[1]1.00, 0.99, 0.93, 0.96, 0.88, 0.94, 0.95, 1.02, 1.08, 1.01.
[2]1.00, 1.06, 0.98, 0.98, 1.25, 1.14, 0.97, 0.98, 1.05, 0.90.
[3]0.87, 2.18, 0.94, 1.88, 1.14, 0.89, 0.86, 0.71, 0.86, 0.82.
[4]0.94, 1.11, 1.55, 1.02, 1.16, 0.54, 0.69, 0.74, 2.06, 1.31.
[5]1.02, 1.24, 0.87, 1.26, 1.06, 0.92, 0.93, 0.99, 0.91, 0.77.
[6]1.13, 0.92, 1.38, 0.93, 1.12, 1.52, 1.83, 1.30, 1.34, 0.69.
[7]0.65, 0.73, 0.65, 0.86, 0.60, 0.65, 0.73, 0.77, 0.73, 0.86.
[8]1.14, 1.06, 1.53, 1.27, 1.12, 1.31, 0.72, 1.44, 0.70, 1.47.
[9]0.34, 0.25, 0.24, 0.27, 0.28, 0.33, 0.34, 0.36, 0.27, 0.31.
[10]2.86, 4.04, 2.62, 3.64, 6.15, 3.51, 5.64, 3.13, 3.89, 9.60.

medium (the one previously mentioned, minus the agar) were placed in each of four identical pillbox-shaped duraluminum boxes with watertight stainless steel lids. Two of the boxes were exposed once, one of them twice, and the fourth served as an unirradiated control (all four exposures being of the same duration). Ten replicate comparisons were made at each dose level, and to avoid bias the doubly exposed box received the first and third exposures, and the second and fourth, respectively in alternate experiments.

To ensure that all of the spores in a suspension were uniformly exposed, vigorous churning during the irradiation was achieved by incorporating a baffle in each container and rotating on a horizontal axis at a rate of 60 r.p.m. with the bottom of the box (3 mm thick duraluminum) facing the horizontal X-ray beam.

The dilution prior to the exposure was the same in all four boxes, and was arranged in the case of the comparisons at low doses so that plating 0.1 ml (per plate) without further dilution resulted in 20 to 100 colonies. In the case of comparisons at higher doses this could not be achieved for all four suspensions, a further dilution being necessary for the unirradiated suspension and sometimes also for those which had received the single dose.

In each replicate experiment the percent mutant colonies induced by the double exposure was compared with that expected on the basis of the two single exposures (assuming a linear dose-response relationship, allowance being made for the failure to discriminate between single and multiple induced genetic changes). The percent survival from the double exposure has also been compared with that expected from the two single exposures assuming an exponential relationship (linear production of lethal changes). The degree of accuracy which was achieved is indicated by the high level of consistency between replicates.

Special attention has been directed to the comparison at the lowest dose level (1, and 1 plus 1, minute). At this level mutation rises linearly and survival declines exponentially, the agreement between the observed and the expected effects of doubling the dose being extremely close. This linearity appears to hold up to approximately 10 or 20 percent mutations, and in this respect there is a close parallel with the corresponding relationship for recessive lethal mutations in Drosophila which also rise linearly with dose to approximately 15 percent (see OLIVER 1932; SPENCER and STERN 1948; and TIMOFÉEFF-RESSOVSKY, ZIMMER and DELBRÜCK 1935).

Under the conditions of these experiments, doubling the dose had progressively less effect at increasing doses, and at the highest level (16, and 16 plus 16, minutes) the percent mutant colonies was actually less in the case of the double exposure, by a factor of approximately two. In addition at this level the lethal effect of the double exposure, while not actually less than that of the single dose, was less than would be expected on the basis of an exponential survival. Thus it would seem that there is a tendency toward saturation at high doses, of the capacity of the spore suspension for further response both to the mutagenic and to the lethal effects of a further exposure.

It will be noted that the peak of the dose-mutation curve, occurring with

16 minutes (and 8 plus 8 minutes) exposure, is in the vicinity of 25 percent mutant colonies. In all, there are three groups of ten independent estimates of the mutational response at this dose and these show remarkable consistency, the averages for the three groups being 26.0, 26.5 and 23.7 percent, and the ranges within the groups being from 21.9 to 30.9, 23.1 to 28.6, and 16.6 to 27.8 percent respectively.

That this peak does not occur under all conditions, and that the percent mutant colonies may rise to higher values, is evident from two other series of experiments using X-rays, and gamma rays from a cobalt-60 source, respectively (see tables 2 and 3). The effect of doubling the dose was, as in the

TABLE 2

Effect of doubling the dose of X-rays on mutation (combined results from five replicate experiments for each pair of doses).

Dose (r)	Mutations			
	Mutant colonies	Total colonies	Av. % induced mutants	Av. obs./exp.
0	15	4,169	0.0	
4000	403	1,800	24.0	
4000	252	1,162	22.8	
4000 + 4000	262	980	27.2	.68[1]
0	5	1,585	0.0	
8000	736	2,631	27.1	
8000	661	2,366	28.4	
8000 + 8000	835	2,433	34.4	.72[2]
0	6	1,192	0.0	
16000	1,296	3,372	28.8	
16000	969	2,708	35.6	
16000 + 16000	1,002	2,122	47.0	.78[3]

Irradiated in normal saline at 16 cm from the target (dose rate = 2000 r/minute).
Observed/expected; values for individual replicate experiments:
[1].85, .67, .55, .61, .73.
[2].76, .63, .84, .74, .62.
[3].74, .71, .87, .79, .81.
Percent survival varied from 3 to 100 with 2 minutes exposure, 3 to 45 with 2 + 2 min., 12 to 50 with 4 min., 1 to 6 with 4 + 4 min., 1 to 7 with 8 min., .02 to .08 with 8 + 8 min.

previous high-dose experiments, less than linear, but the mutant frequency rose considerably above 25 percent, and in the X-ray experiments approached 50 percent without any evidence of a peak, or even a plateau, having been reached. No attempt was made to determine the factors responsible for the presence or absence of a peak (in the second of the two series of X-ray experiments undiluted spore suspensions in normal saline were irradiated at a higher intensity), but it is clear that both a "peaked" and a "flattened" type of response to increasing doses of an ionizing radiation can occur with this material. This represents another similarity with the non-linear ultraviolet dose-response relationships, which HOLLAENDER and EMMONS (1941)

TABLE 3

Effect of doubling the dose of gamma rays on mutation (combined results from three replicate experiments for each dose).

Dose (r)	Mutations			
	Mutant colonies	Total colonies	Av. % induced mutants	Av. obs./exp.
0	15	3951	0.0	....
25,000	996	4872	22.0	....
50,000	1442	5694	26.8	.70[1]
100,000	2540	6852	35.5	.77[2]

Irradiated in normal saline (dose rate = 5000 r/hour).
Observed/expected; values for individual replicate comparisons:
[1].78, .61, .71.
[2].72, .95, .53.
Percent survival varied from 1.0 to 8.7 at 25,000 r, .08 to .6 at 50,000 r, and from .006 to .6 at 100,000 r.

have shown to be readily convertible from the "peaked" to the "flattened" form by means of suitable post-treatments.

ULTRAVIOLET AND PHOTOREVERSAL EFFECTS

The ultraviolet dose-mutation curve for Streptomyces also reached a peak followed by a decline, and in a series of four preliminary experiments (2,323 mutants out of 14,791 colonies examined) the peak occurred at doses between 300 and 600 ergs per sq. mm. Choosing a single dose of 400 ergs per sq. mm the effect of doubling the exposure was to reduce the proportion of mutants to approximately one half (see table 4). It will be noted that these results are similar to those obtained in the comparison at the highest dose level in the first series of X-ray experiments (table 1) both with respect to the percentage of mutants from the single dose and to the extent of the reduction.

Post-treatment with visible light was also carried out, either of two possible effects being expected: (a) a reduction in the proportions of mutant

TABLE 4

Effect of doubling the dose of ultraviolet on mutation (combined results from seven replicate comparisons between the two doses).

Dose (ergs per mm^2)	Mutation			Av. ratio (800/400)
	Mutant colonies	Total colonies	Av. % mutants	
400	2,041	9,263	24.2	
800	378	3,992	9.6	.42[1]

Ultraviolet from a G.E. germicidal lamp.
Unirradiated controls averaged 0.6 percent mutant colonies.
Ratios of mutant frequencies (high dose/low dose) for the individual experiments:
[1].57, .65, .42, .64, .10, .34, .22.
Percent survival varied from .04 to 1.0 for the low, and from .0005 to .01 for the high dose.

TABLE 5

Effect of post-treatment with visible light on ultraviolet induced mutation (combined results from 11 experiments at 400 and 9 experiments at 800 ergs per sq. mm).

Dose (ergs per mm²)	Dark			Light			Av. ratio $\frac{L}{D}$
	Mutant colonies	Total colonies	Av. % mutants	Mutant colonies	Total colonies	Av. % mutants	
400	1,085	4,690	27.7	894	6,378	13.2	0.47[1]
800	337	2,902	12.9	757	3,316	24.8	2.09[2]

Ultraviolet as in previous table.

Visible light: ten minutes exposure at 37°C; filtered light from a G.E. AH-5 high pressure mercury lamp.

Unirradiated controls averaged 0.6 percent mutant colonies.

Percent survival varied from .01 to 1.0 and from .0001 to .01 at 400 and at 800 ergs per mm² respectively with no post-treatment.

Post-treatment with light increased the survival by factors of 20 to 3,000 and 20 to 10,000 at the two u.v. doses.

Ratios of mutant frequencies (light/dark) for the individual experiments:

[1] .38, .39, .24, .31, .27, .58, .56, .40, .47, .71, .86.

[2] 1.03, 1.53, 1.53, 2.05, 1.51, 2.52, 4.30, 2.64, 1.16.

colonies at all ultraviolet doses, with no change in the dose at which the peak frequency occurred, or (b) a reduction in effective dose, higher ultraviolet doses being required to reach the peak response, but with no change in the height of this peak. The experimental results (see table 5) show clearly the latter type of response, the light treatment being equivalent to halving the initial dose of ultraviolet. Thus at the low dose light reduced the mutant frequency, and at the high dose (where the mutant frequency was low) it raised it towards the peak value. This is in agreement with KELNER's (1949) "dose-reduction principle" (which appears to apply also where the u.v. dose-mutation curve is of the "flattened" or "plateau" type, NEWCOMBE and WHITEHEAD 1951).

It follows from this that the photoreversible intermediate must be produced linearly (or nearly so) with increasing ultraviolet doses. The subsequent non-

TABLE 6

Effect of post-treatment with visible light on X-ray induced mutation (combined results from five experiments).

Dose	Dark			Light			Av. ratio $\frac{L}{D}$
	Mutant colonies	Total colonies	Av. % mutants	Mutant colonies	Total colonies	Av. % mutants	
8000 r	634	1773	37.4	573	1730	37.1	.96[1]

X-irradiation as in table 2.

Visible light: as in previous table.

Unirradiated controls averaged 0.4 percent mutant colonies (5 out of 1691).

Percent survival varied from .3 to 10.2 for dark, and .3 to 6.2 for light. In all comparisons there was a slight decrease in survival with light (by factors of .85, .89, .79, .97, .67 respectively).

Ratios of mutant frequencies (light/dark) for the individual experiments:

[1] .78, .98, 1.23, .94, .89

TABLE 7

Effect of ultraviolet and gamma irradiation singly and combined (combined results from five replicate comparisons).

Treatment	Mutations			
	Mutant colonies	Total colonies	Av. % induced mutants	Av. obs./exp.
None	9	3,908	0.0	
U.V.	568	1,792	31.1	
γ	2,188	6,052	35.0	
U.V. + γ	1,015	2,306	43.6	0.81[1]

Ultraviolet dose 400 ergs per mm^2; gamma dose 25,000 r.
Percent survival was from .01 to .5 for U.V. alone, .1 to 30 for gamma alone, and from .0003 to .002 for the combined treatment.
Values from individual experiments:
[1].87, .94, .71, .78, .75.

linear mutational response could be due to some selection effect (e.g., differential killing of the more mutable cells, or of induced mutants or potential mutants), or to the saturation of some essentially intracellular response. The latter interpretation seems to have been largely neglected, but there is nothing to preclude the possibility of a nonlinear step in a chain of chemical (and/or structural) events leading to mutation.

As expected from previous studies, post-treatment with light had no corresponding effect on X-ray induced mutation (see table 6).

THE EFFECT OF COMBINED ULTRAVIOLET AND GAMMA IRRADIATION

As a similar saturation of the mutational response occurred both with ionizing radiation and with ultraviolet, it was of interest to know whether exposure to one of these agents reduced the capacity of a suspension to respond to the mutagenic effects of the other. In the experiments to test this, exposures to ultraviolet and to cobalt^{60} gammas were carried out singly and in combination (see table 7), and it was clear from the results that the ultraviolet did reduce the mutagenic effectiveness of the subsequent gamma irradiation.

TABLE 8

Effect of the sequence of combined ultraviolet and X-irradiation (combined results from five replicate experiments).

Treatment	Mutations			
	Mutant colonies	Total colonies	Av. % mutants	Av. ratio U.V. + X/X + U.V.
None	3	1508	0.2	
X + U.V.	1164	3233	36.2	
U.V. + X	809	2328	35.2	.98[1]

Ultraviolet dose 200 ergs per mm^2; X-ray dose 4 min. at 16 cm (= 8000 r).
Percent survival was from .1 to .6 for X + U.V. and from .1 to .2 for U.V. + X.
Values from individual experiments:
[1].83, .86, 1.10, 1.11, 1.04.

It can therefore be inferred that the saturation effects found with both the ionizing and the non-ionizing radiations, have a common mechanism. Apparently also it make no difference in which sequence the ultraviolet and the ionizing radiation are administered, survival and mutation being the same in either case (see table 8).

CONCLUSIONS

The frequency of induced colony morphology mutants in X-irradiated Streptomyces spores rises as a linear function of dose at low doses. At higher doses the response is less than linear, and under certain conditions a peak is reached and further increases in dose cause a decline in the proportion of mutants among the surviving spores. This dose-response relationship is similar to that for ultraviolet-induced mutation, both in Streptomyces and in practically all organisms studied, but seems to be unique for the ionizing radiations. It is emphasized however that the apparent linear X-ray-dose relationships observed in other materials do not rule out the possibility of a nonlinear response at doses too high for convenient study, and that in any case the nonlinear responses observed in ultraviolet- and X- or gamma-irradiated Streptomyces must have a common mechanism, since exposure to ultraviolet can partially saturate the capacity of a spore suspension to respond to the mutagenic effects of the ionizing radiations.

This does not mean that the initial effects of these two kinds of mutagenic agent are identical since that of ultraviolet can be partially reversed by light, the reversal in this material being equivalent to halving the original dose. The part of the ultraviolet effect which cannot be reversed is however indistinguishable by present methods from that of ionizing radiation, and all three mutagenic effects (ionization induced, reversible u.v. and stable u.v.) must be thought of as affecting in a similar manner the common mechanism by which the capacity of a spore suspension for mutational response becomes saturated.

Since with ultraviolet there is a long-lived (photoreversible) intermediate which is formed in direct proportion to the dose, it is an attractive speculation that the ionizing radiations may act through a similar long-lived (but photostable) intermediate, and that the nonlinear step in the induced mutation process may, with both agents, occur at some time after the irradiation.

It should be emphasized that there is as yet no means of determining whether the ultraviolet-induced intermediate arises through changes within the genes, or external to them (but perhaps localized in closely adjacent materials). And similarly, we cannot be certain whether the nonlinear step in the mutational response to both agents, is due to some *inter*cellular (selection) effect, or to the saturation of an essentially *intra*cellular capacity for response.

SUMMARY

In most organisms, X-ray induced mutations increase linearly with dose while the corresponding ultraviolet induced changes rise either to a plateau, or to a peak followed by a decline. Streptomyces spores are exceptional in

that the response to both agents is similar, and varies non-linearly with dose. A common cause is indicated, since combined treatment with ultraviolet and ionizing radiation has a less than additive effect. In the case of ultraviolet it has been shown that the long-lived (photoreversible) intermediate in the mutation process, must increase linearly with increasing dose; but it is not certain whether the final nonlinear response is due to some subsequent step in the intracellular processes leading to mutation, or to differential survival.

It is suggested that the apparently linear X-ray response curves found with other organisms might be nonlinear at doses too high for convenient study; and also that X-rays, like ultraviolet, might perhaps act through the production of a long-lived intermediate.

LITERATURE CITED

HOLLAENDER, A., and C. W. EMMONS, 1951 Wavelength dependence of mutation production in the ultraviolet with special emphasis on fungi. Cold Spring Harbor Symp. Quant. Biol. **9:** 179–186.

KELNER, A., 1948 Mutation in *Streptomyces flaveolus* induced by X-rays and untraviolet light. J. Bact. **56:** 457–465.

1949 Photoreactivation of ultraviolet-irradiated *Escherichia coli*, with special reference to the dose-reduction principle and to ultraviolet-induced mutation. J. Bact. **58:** 511–522.

MCCLINTOCK, B., 1951 Chromosome organization and genic expression. Cold Spring Harbor Symp. Quant. Biol. **16:** 13–47.

NEWCOMBE, H. B., 1953 Radiation induced instabilities in *Streptomyces*. J. Gen. Microbiol. **9:** 30–36.

NEWCOMBE, H. B., and H. A. WHITEHEAD, 1951 Photoreversal of ultraviolet-induced mutagenic and lethal effects in *Escherichia coli*. J. Bact. **61:** 243–251.

OLIVER, C. P., 1932 An analysis of the effect of varying the duration of X-ray treatment upon the frequency of mutations. Zeit. Ind. Abst. Ver. **61:** 447–488.

SPENCER, W. P., and C. STERN, 1948 Experiments to test the validity of the linear r-dose/mutation frequency relation in Drosophila at low-doses. Genetics **33:** 43–47.

TIMOFÉEFF-RESSOVSKY, N. W., K. G. ZIMMER, and M. DELBRÜCK, 1935 Über die Natur der Genmutation und der Genstruktur. Nachr. Ges. Wiss., Gottingen **1:** 189–245.