

DARK REACTIVATION IN ULTRAVIOLET IRRADIATED *ESCHERICHIA COLI*¹

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Reversal of the lethal effect of ultraviolet radiation on microorganisms by a variety of treatments has been demonstrated with two essentially different plating techniques. Cells may be plated immediately after irradiation, in which case reactivation occurs on the agar plate. By employing such a plating procedure, reactivation has been accomplished through the use of heat (Anderson, 1951), acetate (Ellison, *et al.*, 1955), and a recovery factor isolated from irradiated cells (Whitehead, 1955).

The second technique requires that the recovery treatment occur prior to plating. In this manner Kelner (1949a), with visible light, and Heinmets (1953) and Heinmets *et al.* (1954), with metabolic intermediates, were able to reactivate injured cells. Although Heinmets (1953) also was able to demonstrate limited reactivation in buffer alone, he attributed this recovery to "heat reactivation and possible limited multiplication." Recently Wainwright and Nevill (1955a, b, c, d) have been able to reactivate spore suspensions of *Streptomyces* by incubating the ultraviolet irradiated spores in distilled water alone or with various metabolic inhibitors prior to plating.

The data presented in this paper indicate that it is possible to reverse the effect of ultraviolet irradiation to an extent that equals photoreactivation when irradiated cells are incubated in only a saline solution, prior to plating.

MATERIALS AND METHODS

The organism principally employed in this study was *Escherichia coli* strain B obtained from the Johnson Foundation at the University

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of Pennsylvania. For comparative purposes, the Grant strain, *E. coli* C-30 was used, because of its increased resistance to radiation.

Stock cultures were maintained in litmus milk at -17°C . All growth media had the following composition: Sheffield N-Z-Case, 1.0 g; yeast extract, 1.0 g; K_2HPO_4 , 0.2 g; glucose, 0.1 g; and distilled water to 100 ml. The medium was adjusted to pH 7.0. Plate counts were made on the same medium to which 1.5 g of agar had been added. All growing cultures were incubated at 37°C .

Cells were prepared for experiments by transferring one loopful of a stock culture that had been incubated for 8 hours to 10 ml of the growth media. After 16 hours of incubation, the cells were harvested by centrifugation, washed twice with 10-ml portions of sterile physiological saline, and finally suspended in 10 ml of the same. A volume of this suspension was added to saline until an optical density (measured on an Evelyn photoelectric colorimeter) equivalent to 6 to 7×10^7 cells per ml was reached.

A 15-watt General Electric germicidal lamp adjusted to emit ultraviolet light at an intensity of $300 \text{ ergs} \times \text{min}^{-1} \times \text{mm}^{-2}$ was used as a source of radiation. Cell suspensions were irradiated in open petri dishes with agitation accomplished by an eccentric rotor working from a motor attached to the ring stand holding the open petri dish and adjusted to set up a series of standing waves in the suspension through its vibratory impulses.

For photoreactivation, 15 ml of irradiated cell suspensions were illuminated in 100-ml Kjeldahl flasks at 30°C . The light source was a 300-watt tungsten projection lamp in a projector with bellows fully contracted. The projector lens was adjusted to contact a 250-ml Florence flask filled with distilled water, for the purpose of focusing the beam of light and reducing the heat from the lamp. To obtain the highest light intensity,

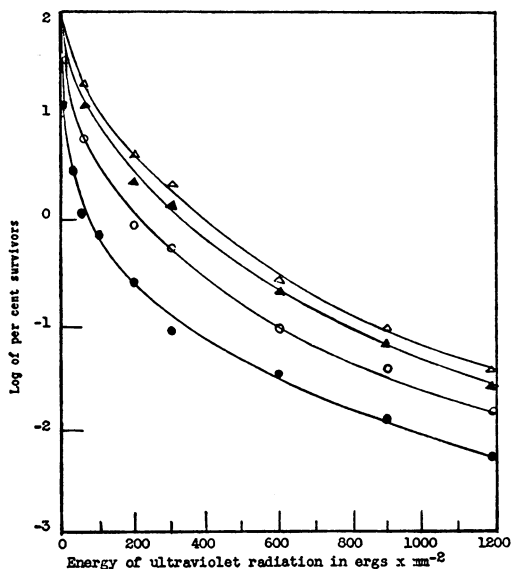


Figure 1. Reactivation of *Escherichia coli* strain B by holding in the dark 100 minutes prior to plating. ● Cells plated immediately after irradiation. ○ Cells held 100 minutes at 20 C before plating. ▲ Cells held 100 minutes at 30 C before plating. △ Cells held 100 minutes at 40 C before plating.

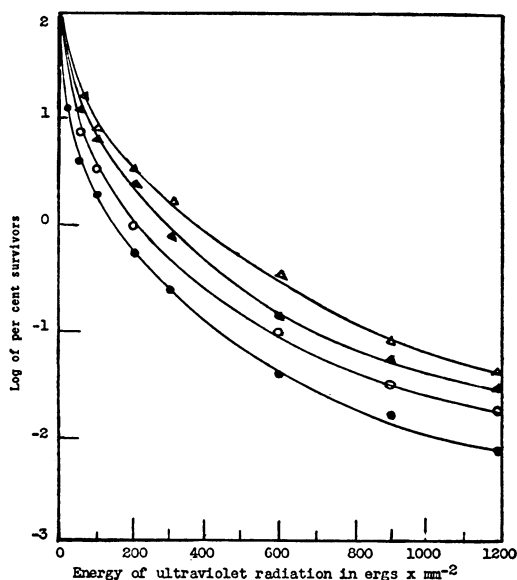


Figure 2. Reactivation of *Escherichia coli* strain B by holding in the dark 100 minutes prior to plating. ● Cells plated immediately after irradiation. ○ Cells held 100 minutes at 15 C before plating. ▲ Cells held 100 minutes at 25 C before plating. △ Cells held 100 minutes at 35 C before plating.

the cells were placed at the focal point of the beam in a glass-sided water bath.

Dark reactivation was carried out on 15-ml aliquots of irradiated cells in test tubes covered with aluminum foil and incubated in a water bath at the designated temperatures. The laboratory was illuminated by a yellow light during all irradiation and recovery procedures.

Suspensions were assayed in triplicate for viable cells by spreading 0.1 ml of suitable dilutions on the surface of previously prepared petri dishes containing the solid growth medium. Physiological saline was the diluent used at all times since distilled water killed as many as 50 per cent of the suspended *E. coli* strain B cells in an hour. The tips of all pipettes used in making dilutions and in plating were wiped with sterile filter paper. Colonies were counted after 24 hours incubation.

RESULTS

Saline suspensions of *E. coli* strain B irradiated with varying doses of ultraviolet light were held 100 minutes in the dark at various temperatures before plating and compared in terms of colony

counts to the same suspension plated immediately after irradiation. The data in figures 1 and 2 show that with this organism, an increase in the number of viable cells occurs when irradiated suspensions are held in the dark before plating. Recovery occurs at all levels of survival encompassed by the experiment, with temperature influencing the magnitude of recovery. More detailed information, obtained from figures 1 and 2, is shown in table 1 in terms of the dose

TABLE 1

Comparison of dose reduction ratios in *Escherichia coli* strain B held in the dark 100 minutes at various temperatures

Per cent survivors	Experiment 1			Experiment 2		
	20 C	30 C	40 C	15 C	25 C	35 C
63	1.6	2.6	3.3	1.1	1.8	1.9
50	1.6	2.6	3.2	1.1	1.7	2.0
20	2.0	3.3	4.2	1.2	1.7	2.1
10	2.0	3.0	4.5	1.2	1.8	2.3
4	2.0	3.4	4.4	1.5	2.6	3.3
1	2.7	4.0	5.0	1.5	2.0	2.8
0.25	2.1	2.8	3.2	1.3	1.7	2.1

reduction ratio as used by Kelner (1949b) to demonstrate the effect of visible light on irradiated cells of *E. coli* strain B/r. In this work, the dose reduction ratio is a comparison of the ultraviolet dose giving a specific survival when cells are plated immediately after irradiation, with the ultraviolet dose giving numerically the same survival when cells are held in the dark for a given period of time.

The data shown in experiment 1 of table 1 are not strictly comparable to those of experiment 2 of the same table, as they represent results obtained from two separate bacterial suspensions. It was noted during most of the experimental work reported here, that the amount of reactivation in a given period of time could not be duplicated from one experiment to the next, although the experimental conditions appeared to be identical. This observation has been made by other investigators (Heinmets *et al.*, 1954) concerned with reactivation processes. However, all general characteristics of dark reactivation are reproducible.

Evaluation of the data in table 1 discloses that the dose reduction ratios at various levels of survival and at any given temperature are not constant, but seem to be larger when the level of survivors is in the region of 1 to 4 per cent. Experiment 1 in this same table shows that reactivation increases with temperature, reaching a maximum value at 40 C, while in experiment 2 the maximum value is attained at 35 C. In both cases the highest temperature produced the most reactivation. The optimum temperature of reactivation will be shown in a later figure where the temperatures of 30, 35, and 40 C are incorporated in a single experiment.

The results of subjecting *E. coli* strain C-30 to varying doses of ultraviolet light followed by dark incubation at 20, 30 and 40 C are shown in figure 3 and table 2. It is apparent that recovery through holding does not occur to an appreciable extent in this organism; only a small amount of reactivation results at 20 C and a slight but definite killing effect takes place at 30 and 40 C.

To determine the effect of time of holding on reactivation, cells of *E. coli* strain B were irradiated with ultraviolet light at an energy level of 60 ergs \times mm⁻² and held for various periods at different temperatures. A graphic representation of the results is shown in figure 4. These two groups of curves with different points of origin

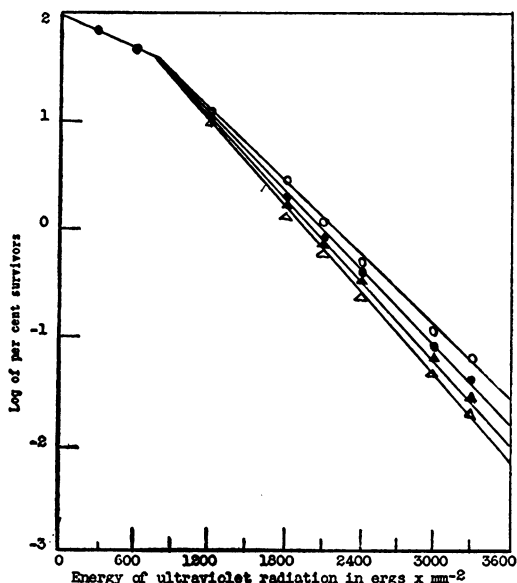


Figure 3. Reactivation of *Escherichia coli* strain C-30 by holding in the dark 100 minutes prior to plating. ● Cells plated immediately after irradiation. ○ Cells held 100 minutes at 20 C before plating. ▲ Cells held 100 minutes at 30 C before plating. △ Cells held 100 minutes at 40 C before plating.

TABLE 2

Comparison between dose reduction ratios of *Escherichia coli* strain B and strain C-30 held in the dark 100 minutes at various temperatures

Per Cent Survivors	<i>Escherichia coli</i> B			<i>Escherichia coli</i> C-30		
	20 C	30 C	40 C	20 C	30 C	40 C
10	2.0	3.0	4.5	1.02	0.96	0.94
8	2.0	3.0	4.6	1.01	0.95	0.94
4	2.0	3.4	4.4	1.05	0.97	0.95
1	2.7	4.0	5.0	1.05	0.95	0.93
0.25	2.1	2.8	3.2	1.05	0.95	0.93
0.06	1.6	2.2	2.4	1.06	0.95	0.93

are the results of separately irradiated suspensions which are not inactivated to the same degree even though exposed to an identical amount of ultraviolet light. Although the data between two experiments again are not strictly comparable, it was found that the sequence of temperature relationships remained constant from experiment to experiment, with incubation at 30 C always giving the most reactivation over long periods of holding and 35 C giving the most reactivation in

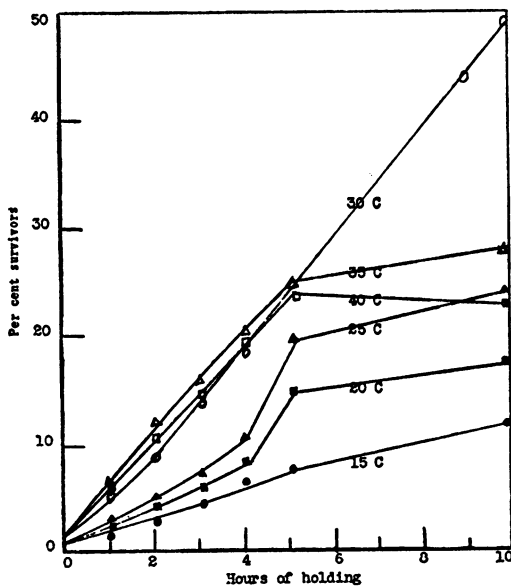


Figure 4. The influence of temperature and duration of dark incubation on recovery of *Escherichia coli* strain B.

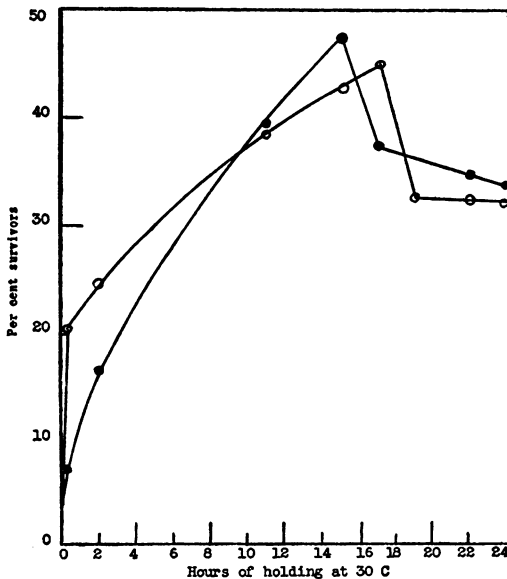


Figure 5. A comparison of dark recovery and mixed recovery treatment of *Escherichia coli* strain B. ○ Irradiated cell suspension exposed to white light for 10 minutes followed by dark incubation before plating. ● Irradiated cell suspension incubated entirely in the dark before plating.

100 minutes. The only differences found between experiments were the magnitude of reactivation and the rate at which it occurred.

It was of interest to determine if the effects of dark reactivation and photoreactivation were additive. The results of such an experiment are shown in figure 5 where irradiated cells exposed to 10 minutes of visible light are compared to irradiated cells kept entirely in the dark. The data show that no such additive effect occurs.

The irradiated cells used in the preceding experiment were also photoreactivated for 120 minutes at 30 C. The results obtained show that maximum reactivation occurs in 50 minutes with the number of survivors reaching 54 per cent. These data may be compared to the total dark reactivation curve in figure 5 where maximum reactivation is 48 per cent in 14 hours.

DISCUSSION

An increase in viable cells, comparable in magnitude to photoreactivation, takes place when irradiated cells of *E. coli* strain B are held for a period of time in the dark prior to plating. However, a much greater time period is required to produce the same degree of recovery in the dark than that required when light is used. Because photorecovery and dark recovery are not additive, it is possible to assume that only those injured cells that are reactivated by one process may be reactivated by the other. If this is the case, then dark reactivation may be thought of as a process which is accelerated by visible light. Alternately, if dose reduction ratios are taken to be symptomatic of basic relationships among the various types of reactivation, then dark reactivation may be considered to be inherently different (see table 1) from photoreactivation (Kelner, 1949b) where the effect of visible light is to reduce by a constant factor the effect of the ultraviolet dose, and to be different from Anderson's (1951) heat reactivation phenomenon, where dose reduction ratios decrease with decreasing survivors.

Heinmets (1953) and Heinmets and Kathan (1954) have stated that irradiation with ultraviolet light disrupts the normal metabolic pattern of the cell. A plausible mechanism of dark reactivation may entail a competition for a particular substance(s) by two possibly independent regulatory systems of the cell; namely, reproduction and repair. One of the functions of this sub-

stance(s) would be reparation of irradiation damage while an alternate function would entail a commitment to the reproductive process. Should this material be present in the cell in limiting quantities, then placing irradiated cells in a growth medium would block recovery through competition. That dark reactivation is essentially metabolic in essence, seems to be implied by those data illustrating the temperature dependence of the system. The fact that dark reactivation does not occur in *E. coli* strain C-30, may be due to an entirely different type of radiation injury which cannot be repaired by the cell under the experimental conditions employed.

Another possible interpretation of dark reactivation may be considered in terms of the findings of Whitehead (1955), who demonstrated that the plating of washed irradiated cells with the supernatant liquid from other irradiated cells resulted in more survivors than when the washed cells were plated by themselves. From these results he concluded that a recovery factor was released by injured cells. If such a factor is responsible for dark reactivation, it must be operative primarily during the incubation period prior to plating, since it is during this period that recovery occurs. Such recovery would be related to time of incubation in saline, as well as the ultraviolet dosage, and is therefore compatible with the data presented in this study.

The possibility that dark reactivation is a reflection of growth of viable cells remaining after irradiation rather than recovery of the inactivated cells cannot be overlooked. Garvie (1955) found that *E. coli* that had been washed and incubated in phosphate buffer for 24 hours showed an increase in viable cells, while in distilled water growth would occur only if a ratio existed of at least 10,000 dead cells to 1 viable cell.

That dark reactivation is not a result of growth can be demonstrated in several ways. Nonirradiated cells of *E. coli* strain B were incubated in saline at 37 C for periods up to 24 hours, with aliquots being plated at various intervals; no increase in viable cells was noted during this period of incubation. That dead cells in the saline suspension of irradiated cells did not promote growth may be inferred from the data (see figure 3) on the irradiation of *E. coli* strain C-30 which never recovered to any extent as a result of dark incubation. In all of the experiments that required

incubation periods of over 100 minutes, the ratio of dead cells to viable ones never exceeded 200 to 1, which according to the work of Garvie would not be expected to support growth.

SUMMARY

Ultraviolet irradiated cells of *Escherichia coli* strain B have been found to partially recover from the lethal effects of irradiation when incubated in the dark in physiological saline. Recovery occurred over a wide range of survival levels and was influenced by heat. The magnitude of reactivation closely approached that achieved by photoreactivation and was found to increase with temperature, the maximum amount of recovery being attained at 30 C. This phenomenon did not occur to any great extent in cells of *Escherichia coli* strain C-30.

REFERENCES

- ANDERSON, E. H. 1951 Heat reactivation of ultraviolet-inactivated bacteria. *J. Bacteriol.*, **61**, 389-394.
- ELLISON, S. A., ERLANGER, B. F., AND ALLEN, P. 1955 The chemical reversal of ultraviolet effects on bacteria. *J. Bacteriol.*, **69**, 536-540.
- GARVIE, E. I. 1955 The growth of *Escherichia coli* in buffer substrate and distilled water. *J. Bacteriol.*, **69**, 393-398.
- HEINMETS, F. 1953 Reactivation of ultraviolet inactivated *Escherichia coli* by pyruvate. *J. Bacteriol.*, **66**, 455-457.
- HEINMETS, F. AND KATHAN, R. H. 1954 Preliminary studies on the mechanism of biological action of ultraviolet irradiation and metabolic recovery phenomenon. *Arch. Biochem. and Biophys.*, **53**, 205-227.
- HEINMETS, F., LEHMAN, J. J., TAYLOR, W. W., AND KATHAN, R. H. 1954 The study of factors which influence metabolic reactivation of the ultraviolet inactivated *Escherichia coli*. *J. Bacteriol.*, **67**, 511-522.
- KELNER, A. 1949a Effect of visible light on the recovery of *Streptomyces griseus* from ultraviolet irradiation injury. *Proc. Natl. Acad. Sci. U. S.*, **35**, 73-79.
- KELNER, A. 1949b Photoreactivation of ultraviolet irradiated *Escherichia coli* with special reference to the dose reduction principle and to ultraviolet induced mutation. *J. Bacteriol.*, **58**, 511-522.
- WAINWRIGHT, S. D. AND NEVILL, A. 1955a Induction of provariants as a function of dose of ultraviolet radiation. *J. Gen. Microbiol.*, **12**, 13-24.

- WAINWRIGHT, S. D. AND NEVILL, A. 1955b Modifications of the biological effects of ultraviolet irradiation by postirradiation treatment with iodoacetate and peptone. *J. Gen. Microbiol.*, **12**, 1-12.
- WAINWRIGHT, S. D. AND NEVILL, A. 1955c Relationships between postradiation treatments with iodoacetate and light. *Can. J. Microbiol.*, **1**, 427-439.
- WAINWRIGHT, S. D. AND NEVILL, A. 1955d Some effects of postirradiation treatment with metabolic inhibitors and nutrients upon ultraviolet irradiated spores of *Streptomyces* T12. *Can. J. Microbiol.*, **1**, 416-426.
- WHITEHEAD, H. A. 1955 A factor released by irradiated cells that induces recovery of viability of ultraviolet irradiated bacteria. *Can. J. Microbiol.*, **1**, 266-270.