ELECTRON MICROSCOPY OF ULTRAVIOLET IRRADIATED BACTERIA AND THEIR INTERACTION WITH BACTERIOPHAGE

by

LOUIS W. LABAW, VERNON M. MOSLEY, AND RALPH W. G. WYCKOFF

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland (U.S.A.)

INTRODUCTION

It is generally accepted that viruses require living host cells for their growth and multiplication. Recently, however, it has been shown that bacteriophages can multiply in the presence of bacteria which have been killed in the sense of having been rendered incapable of the indefinitely continued multiplication needed to produce visible colonies. This "killing" of the bacteria has been brought about by treatment with chemicals such as mustard[1] or formaldehyde[2] or by exposure to ultraviolet irradiation[3]. It is of obvious importance to an understanding of both the nature of viruses and the action of these chemicals and radiations to know if the bacteria are immediately killed by these treatments or are injured so severely that they all succumb before producing visible colonies. If the first were true it would be necessary to conclude that bacteriophage could develop on dead bacteria; if the second is the case bacteriophage production may proceed on these damaged, but still viable, organisms.

Accumulating evidence suggests that this second alternative is the correct one. The recent demonstration[4] that microorganisms "killed" by ultraviolet irradiation can be "resuscitated" by strong illumination with visible light favours it. The electron microscopic examination of ultraviolet-treated bacteria described here points to the same conclusion.

EXPERIMENTAL

These experiments have been carried out using the B strain of *Escherichia coli* and the T2 strain of bacteriophage. The bacteria, from young and activity growing cultures, were obtained from a 1½ to 2-hour culture in tryptose phosphate broth. For irradiation such a culture was centrifuged and the sediment washed by suspension in physiological saline, recentrifugation and final resuspension in saline to give a bacterial concentration of around 10^9 per ml. This suspension was spread out in a very thin layer (approximately 2 mm) for exposure to the unfiltered radiation from an Hanovia Analytic type 500 watt, 115 volt, quartz-mercury ultraviolet lamp. Immediately after irradiation one part of the suspension was added to nine parts of fresh tryptose-phosphate broth with or without

References p. 334.

the addition of a broth suspension of bacteriophage to give an infection ratio of from 5 to 10 bacteriophage per bacterium. In some experiments, this broth suspension of the irradiated bacteria was incubated for various lengths of time, in others it was planted on tryptose agar plates for incubation. In all experiments the killing action of the ultraviolet was determined by planting suitable dilutions of both an unirradiated control and the irradiated sample on agar and incubating overnight for counts of developed colonies. Determinations of bacteriophage titre, where they were called for, were carried out by the plaque-count method of HERSHEY. Preparations for electron microscopy were made from both broth suspensions and incubated spreads on agar. After incubation of the broth suspensions enough formalin was added to make them one percent with respect to formaldehyde; this arrested growth and change. The suspensions were then centrifuged, washed with formalin-saline and formalin-water in the usual fashion, spread on formvar-covered grids and shadowed with palladium or gold-manganin. The plate cultures were "replicated" by being covered with collodion which was floated off and, after mounting on grids, shadowed for observation.

Since the killing of *E. coli* with ultraviolet proceeds in a semilogarithmically linear fashion, it is impractical to seek complete killing with this agent. In the present experiments, it was found that irradiation for fifteen seconds at a distance of one meter reduced about one-hundred-thousand fold the number of colonies developing after incubation. In other words, only about one out of 100 000 of the irradiated organisms could multiply to give a normal bacterial colony. When such an irradiated suspension was infected with T2 bacteriophage, it was, however, lysed in the usual way and yielded approximately the normal increase in the bacteriophage titre; this increase commonly ran between 10 and 100-fold depending on the titre of the infecting bacteriophage. Taking this exposure to radiation as a minimum, series of experiments were made using up to 300 times it in amount. Good yields of bacteriophage, as determined by plaque counts, were obtained from bacteria which had received from 5 to 10 times the minimum; after greater exposures the yield gradually was reduced until ultimately fewer particles were recovered than the number used for infection. The electron microscopic observations are compatible with these results.

A preliminary survey to determine the immediate and delayed effects of the radiation was made by electron microscopic examination of the irradiated organisms after periods of incubation up to four hours. This showed that even the prolonged irradiations have not produced immediate changes in the appearance of the bacteria. Under the conditions of cultivation and after suspension in saline, they have had the general appearance of Fig. 1. Both the unirradiated cultures and those organisms which have been irradiated for 15 seconds swell promptly and seem to be growing normally after incubation of from ½ to 2 hours (Fig. 2 and 3) in fresh broth. Since very few of these irradiated cells or their immediate and seemingly normal progeny can proceed to form visible colonies, it is clear that the lethal effects of the radiation must make themselves evident only after still longer incubation. After one minute irradiation and an hour's incubation, most cells still show signs of metabolic activity as evidenced by swelling and are reasonably normal (Fig. 4). With longer irradiation fewer normally growing cells are present after two hours incubation until after five minutes irradiation most bacteria appear coarsely granular and lysed.

In order to be able to trace the fate of the individual irradiated bacteria, a series of experiments was next made in which the ultraviolet-treated saline suspension was

References p. 334.

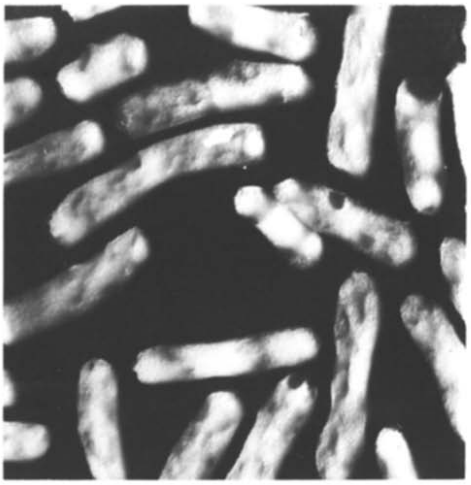

Fig. 1. Unirradiated colon bacilli from a 1½ hour broth culture after centrifugation and suspension in physiological saline. Magnification = 7000 ×

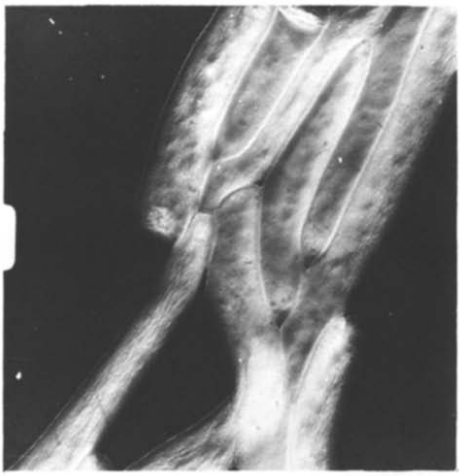

Fig. 2. Colon bacilli incubated for 2 hours in broth after 15 seconds irradiation in saline. There was widespread growth and some increase in mean cellular diameters. Magnification = 7500 ×

Fig. 3. Cells after 30 minutes incubation in broth following 15 seconds irradiation in saline. Magnification = 16000 ×

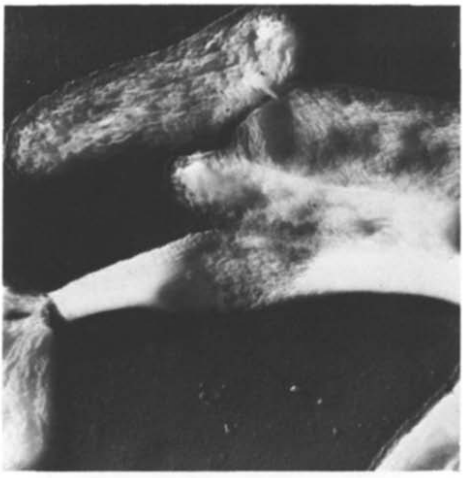

Fig. 4. Cells incubated 60 minutes in broth after one minute irradiation in saline. Most bacteria showed signs of metabolic activity. Magnification = 13000 ×

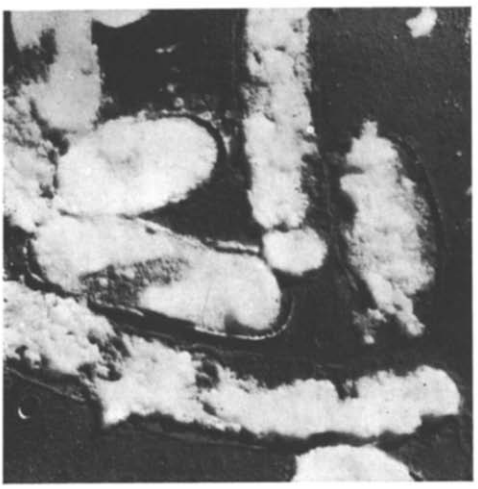

Fig. 5. Cells incubated four hours on agar after ten minutes irradiation in saline. Most cells appear ruptured and comparison with similar experiments involving shorter incubation shows no evidence of growth beyond the two hour stage. Magnification = 10000 ×

Fig. 6. Cells incubated one hour on agar after receiving twice the dose of radiation used for Fig. 5. Practically all cells appear ruptured. Magnification = 9500 ×

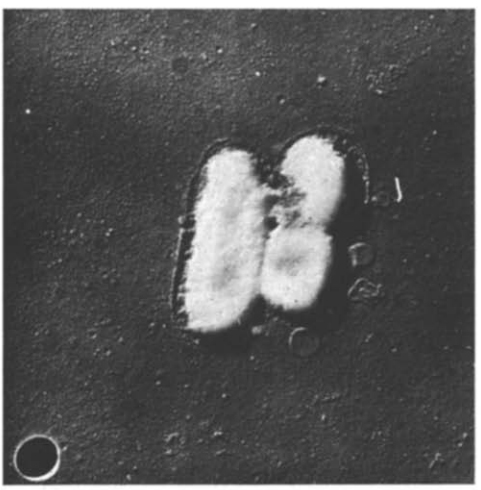

Fig. 7. Bacteria incubated one hour on agar after receiving eight times the dose of radiation used for Fig. 5. No cells showed evidence of metabolic activity. Magnification = 10000 ×

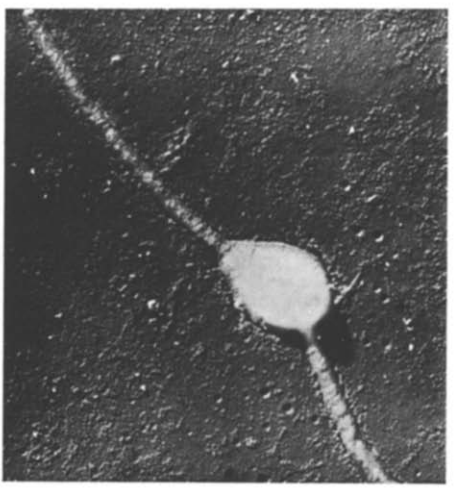

Fig. 8. Narrow tubes developing from an ultraviolet irradiated colon bacillus. Magnification = 10000 ×

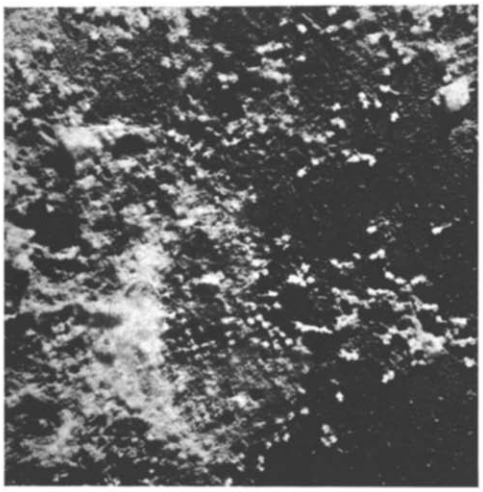

Fig. 9. Protoplasm containing many newly-formed bacteriophage particles resulting from the action for 60 minutes of T2 on colon bacilli irradiated for 15 seconds with ultraviolet. Practically no intact organisms could be found. Magnification = 12 000 ×

Fig. 10. Protoplasmic masses from the action for 40 minutes of T2 on *E. coli* irradiated for one minute. An excellent yield of phage and few intact bacteria. Magnification = 13 000 ×

Fig. 11. A typical field from *E. coli* which had been irradiated for two minutes before incubation with T2 for 40 minutes. Many lysed cells and new phage particles are present. Magnification = 12 500 ×

Fig. 12. *E. coli* irradiated for five minutes followed by 60 minutes incubation with T2. Some lysis and new phage. Magnification = 12 500 ×

seeded on agar and incubated for periods of time up to six hours. After one minute irradiation and two hours incubation innumerable micro-colonies consisting of a few normal bacteria were seen on the "pseudo-replicas" taken from the agar surfaces. The bacteria in these clusters did not seem more numerous after 4 or 6 hours incubation, and by then many of them had granulated and ruptured. Essentially the same result, involving some growth and multiplication during the first two hours of incubation with little thereafter, was found after irradiation for three minutes. There appears to have been some growth and occasionally a division after ten minutes irradiation, but these cells have been ruptured and fragments of protoplasm are strewn over the agar (Fig. 5) after even a short incubation. With twice this dose of radiation (administered over a shorter period of time by bringing the suspension to a distance of 50 cm from the source) practically all cells were ruptured after an hour incubation (Fig. 6). After still heavier doses of radiation the bacteria have failed to showeven this initialgrowth and subsequent lysis when incubated (Fig. 7).

An interesting form that has been seen several times in incubated cultures after moderate irradiation (of the order of three minutes) is shown in Fig. 8. These long thin tubes have never been seen detached from a bacterium or in a state of division.

In the next experiments, normal bacteria were seeded to agar surfaces before irradiation. In one test the seeded agar was irradiated immediately after inoculation, in the other after an hour's incubation to insure that the organisms were in a state of active growth. The results of both these experiments have agreed with the foregoing.

Several experiments have been made to observe the action of T2 bacteriophage on irradiated bacteria. After the 15 second treatment which reduced the number of developing colonies of bacteria to ca 10^{-5} of the bacteria present before irradiation, the phenomena of lysis and bacteriophage production were scarcely distinguishable from those seen with normal bacteria. In samples taken 10 minutes after mixing bacteriophage and bacteria, many cells were ruptured and there was some production of bacteriophage. In samples taken 20 minutes after mixture most cells were lysed, while after an hour's incubation there was complete lysis and a copious production of new bacteriophage (Fig. 9).

There is a similar complete lysis and extensive bacteriophage production with bacteria irradiated for one (Fig. 10) and two minutes, but the lytic process has been slower. Thus only a few of the cells irradiated for 2 minutes have been lysed after 20 minutes incubation, though lysis has been very extensive after 40 minutes (Fig. 11). It has been still slower with the bacteria irradiated for 3 minutes; then there has been practically no discernible lysis after 20 minutes. At the end of an hour's incubation, however, most cells have been lysed with the production of much new bacteriophage. Even with the cells that have received from 5 to 10 minutes exposure to the ultraviolet there has been some lysis and bacteriophage production (Fig. 12); but the protoplasm of most of these unswelled bacteria has been so granular as to prevent decision concerning the amount of new bacteriophage trapped within them.

In a last series of experiments a mixture of bacteriophage and irradiated bacteria was seeded to agar plates and incubated for from 60 to 90 minutes before the usual replication for electron microscopic observation. Complete lysis was observed in all cases, but the lysis of the more heavily irradiated cells has been like that obtained in the absence of bacteriophage; with them there has been no evidence that bacteriophage has been the cause of the lysis or has increased at their expense.

References p. 334.

DISCUSSION

The foregoing experiments indicate how ultraviolet light affects young cells of *E. coli*. They demonstrate that bacteria which have received many times the dose of radiation sufficient to prevent their indefinite multiplication are not immediately killed by this exposure. Instead such mortally damaged cells can continue to grow and undergo normal-seeming division even though they and all their daughter cells succumb after a few hours incubation. For the periods of irradiation used here, which did not allow more than one in about 100 000 of the irradiated organisms to proceed to normal colony formation, this period of bacterial survival appeared to be of the order of a couple of hours. Seemingly the metabolic activity of the damaged bacteria, as shown by cellular enlargement and division and as substantiated by phosphorus uptake studies, during this period of survival is diminished with increased exposure to radiation; there is little perceptible activity in bacteria which have received about 100 times the minimal dose of radiation used in these experiments.

The electron microscopic observations are compatible with the hypothesis that bacteriophage produced in cultures of *Escherichia coli* "killed" with ultraviolet light is developed through interaction with these mortally injured, but not yet "dead", cells. These damaged bacteria can be lysed in normal fashion by bacteriophage with the production of a large yield of new bacteriophage; but there is an absence of such bacteriophage from cells irradiated so heavily that they do not give evidence of further growth and multiplication.

SUMMARY

Electron microscopic observation shows that most bacteria in a suspension of *E. coli* receiving doses of ultraviolet light many times that sufficient to reduce plate counts of viable organisms 10^5 fold can grow and multiply for some time after irradiation. Evidence of this residual metabolism diminishes with increased dose of radiation. Such bacteria are lysed by bacteriophage with yields of new bacteriophage that also diminish with increased irradiation.

RÉSUMÉ

Des observations au microscope électronique nous ont montré que, dans une suspension de *E. coli* recevant des doses de lumière ultra-violette plusieurs fois supérieures à la dose qui suffit à diminuer 10^5 fois le nombre des organismes viables, la plupart des bactéries continuent à croître et à se multiplier pendant un certain temps après l'irradiation. Les effets visibles de ce métabolisme résiduel diminuent si l'irradiation augmente. De telles bactéries sont dissoutes par le bactériophage avec formation de quantitées nouvelles de bactériophage qui, elles aussi, diminuent si l'irradiation augmente.

ZUSAMMENFASSUNG

Beobachtungen mit dem Elektronenmikroskop haben gezeigt, dass in einer mit ultraviolettem Licht bestrahlten Suspension von *E. coli* (und zwar mehrmals die Dosis, welche die Anzahl der lebensfähigen Organismen 10^5 mal verringert) die meisten Bakterien noch einige Zeit leben und sich vermehren können. Bei verstärkter Bestrahlung verringern sich die Anzeichen dieser Rest-Metabolismus. Solche Bakterien werden durch Bakteriophagen aufgelöst, wobei neue Bakteriophagen entstehen, desto weniger je stärker die Bestrahlung.

REFERENCES

[1] R. M. HERRIOTT AND W. H. PRICE, *J. Gen. Physiol.*, 32 (1948) 63.
[2] L. W. LABAW, V. M. MOSLEY, AND R. W. G. WYCKOFF, *Science*, 110 (1949) 275.
[3] T. F. ANDERSON, *J. Bact.*, 56 (1948) 403.
[4] A. KELNER, *Proc. Nat. Acad. Sci.*, 35 (1949) 73.

Received February 14th, 1950