PHOTOREACTIVATION OF ULTRAVIOLET-INACTIVATED BACILLI

by

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Since KELNER's discovery of the phenomenon of photoreactivation (PHR) in 1949[1] many papers on this subject have been published[2-12]. The results of all these experiments suggest that PHR is a generally occurring phenomenon. However, no experiments have been published which show that the bacilli can be photoreactivated. JOHNSON[13] reported that *Bacillus cereus* did not show PHR after UV-inactivation. It may seem that with the bacilli PHR is not possible.

In order to make sure of this the author investigated three cultures of *Bacillus B. subtilis*, *B. cereus* and *B. mycoides*. The results showed that these bacilli were very sensitive to long-wavelength UV-radiation and short-wavelength visible light. When UV-irradiated bacteria suspensions were illuminated with a high dose-rate a small reactivation was observed after some minutes but on further illumination the number of surviving bacteria was reduced at a rather rapid rate. Sometimes no reactivation at all could be found. Clear results, however, were obtained by illuminating the UV-inactivated bacteria with a dose-rate of about 200 $\mu W/cm^2$. They show that the bacilli do not differ from other bacteria in so far as they can be reactivated by light after UV-inactivation. This was not the case with the spores of *B. subtilis*.

EXPERIMENTAL

1. *Cultures, materials*

The bacilli tested were *B. subtilis*, *B. cereus* and *B. mycoides*. They were grown in Difco nutrient broth, to which had been added 0.5% NaCl and 0.1% phosphate. The cultures were incubated at 30–37° C for 20 hours under continuous (magnetic) stirring. For experiments the cells were centrifuged down, resuspended in the same volume of phosphate buffer pH 7.0–7.1 (41 parts of KH_2PO_4, 9.078 g per l + 59 parts of $Na_2HPO_4 \cdot 2aq.$, 11.876 g per l + 100 parts of distilled water) and diluted 10 times with the same buffer.

After the radiation treatment 1.0 or 0.1 ml of a suitable dilution of the bacteria suspension was seeded in Difco nutrient agar at a temperature of about 45° C. To the agar had been added 0.5% NaCl, 0.1% phosphate and 1% glucose[14]. The agar plates were incubated at 30° C.

The spores of *B. subtilis* were obtained by washing them off from Difco nutrient agar cultures, which had been incubated at 30° C for three weeks. They were preserved at 4° C in distilled water. Less than 5% of vegetative forms were present.

2. *Inactivating ultraviolet irradiation*

The UV-radiation source was a low-pressure mercury discharge tube, Philips T.U.V. 15 W. Of the emitted radiation, over 80% had a wavelength of 2537 A. The incident radiation had a dose-rate of about 90 $\mu W/cm^2$.

10 ml of the bacteria suspension were pipetted into a sterile petri dish, which was placed in an ice-water bath in order to keep the temperature of the bacteria suspension low (about 5° C). The irradiation times varied from 2½ to 3 minutes.

References p. 211.

Between the various operations the bacteria suspensions were maintained at 0–1° C, unless otherwise stated.

The spore suspensions were irradiated in the same way.

3. *Recovering irradiation with long-wavelength UV and blue light (Illumination)*

The light source was a high-pressure mercury discharge tube fitted in a glass bulb, Philips HP 125. This tube emits no radiation below 3000 A, an appreciable quantity at 3130, 3655, 4047 and 4358 A and a little at 3342 A. Over the whole spectral region there is a small amount of radiation with a continuous spectrum.

About 4 ml of the bacteria suspension were pipetted into a quartz absorption cell. This cell was placed in a water bath behind a window at a distance of 25 cm from the lamp. The glass of this window absorbed radiation with wavelengths shorter than about 3000 A and transmitted 30% of the radiation at 3130 A. The temperature of the water bath was maintained between 1 and 3° C, except in some named experiments.

In nearly all experiments Schott light-filters were placed between the lamp and the absorption cell.

When monochromatic light was used, *i.e.* the 3 mercury lines 3655, 4047 and 4358 A, the dose-rate was measured. This was done by means of a selenium photo-cell which was calibrated against a thermopile.

The bacterial spores were illuminated in the same way.

RESULTS

1. *Lethal effect of radiations with wavelengths in the region of 3500 to 4500 A*

When no Schott light-filters were used, illumination of the bacteria had two effects:

(a) The UV-inactivated bacteria were (partly) reactivated.

(b) The living bacteria were inactivated at a rather rapid rate.

In order to make sure that the inactivation by the illumination was not due to small amounts of radiations of wavelengths shorter than 3200 A, experiments were repeated by introducing the Schott light-filter WG3, which absorbs these radiations. The same results were found when the UV-inactivated bacteria were illuminated with and without use of the WG3 filter. Fig. 1 shows the results of two of these experiments.

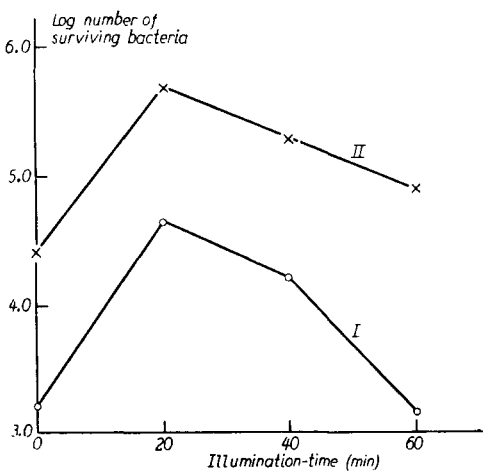

Fig. 1. Effect of illumination of UV-irradiated *B. subtilis*. Curve I. Initial log number of bacteria: 6.28, after UV-irradiation: 3.23. No light-filter used. Curve II. Initial log number of bacteria: 6.81, after UV-irradiation: 4.36. Schott light-filter WG3 used.

This inactivating effect was (greatly) reduced by using the Schott light-filter WG1 (4 mm), as shown in Table I. The latter filter cuts off the radiation at 3130 and 3655 A.

That the inactivating effect still occurs under these conditions is shown by experiments in which unirradiated *B. subtilis* and *B. cereus* were illuminated with monochromatic light.

The following light-filter-combinations were used: Schott filters WG3 + UG2 (both 2 mm); Schott filters BG2 + WG1 + Corning 4035 (all 4 mm); Schott filters BG12 + GG3 (both 2 mm); and in this way approximately monochromatic light at 3655, 4047 and 4358 A resp. was obtained.

Tables II and III show that inactivation occurred when the bacteria were illuminated with a dose-rate of 200 $\mu W/cm^2$ for each wavelength.

References p. 211.

TABLE I

EFFECT OF ILLUMINATION OF UV-IRRADIATED *B. subtilis* USING SCHOTT LIGHT-FILTER WG1 (4 mm)

Before UV	*Number of bacteria per ml in log units*			
	After 3 min UV	*After illumination for (min)*		
		20	40	60
6.81	4.36	5.74	6.04	6.20
6.78	4.04	5.88	5.95	6.04
6.15	2.70	4.15	4.18	4.08

TABLE II

INACTIVATION OF *B. subtilis* WITH LIGHT OF 3655, 4047 AND 4358 Å

Dose-rate 200 μW per cm^2.

Wavelength	*Number of bacteria per ml in log units*			
	Before illumination	*After illumination for (min)*		
		20	40	60
3655 Å	4.98	4.98	4.95	4.67
4047 Å	4.98	5.08	4.95	4.66
4358 Å	4.98	5.04	4.88	4.66

TABLE III

INACTIVATION OF *B. cereus* WITH LIGHT OF 3655, 4047 AND 4358 Å

Dose-rate 200 μW per cm^2.

Wavelength	*Number of bacteria per ml in log units*			
	Before illumination	*After illumination for (min)*		
		20	40	60
3655 Å	4.95	4.85	4.64	4.18
4047 Å	4.95	4.85	4.67	4.36
4358 Å	4.95	4.80	4.52	4.28

These and other experiments showed that the inactivation rates were about the same at the wavelengths used.

2. General experiments

Experiments, mainly performed with *B. subtilis* and *B. cereus*, gave the following results:

(a) The relative increase in survival due to PHR was greater with increasing UV-inactivation.

(b) The UV-inactivated bacteria, when kept at about 1° C, did not lose their ability for PHR for at least some hours.

(c) PHR was also achieved by illuminating the UV-inactivated bacteria at 20 and 30° C.

(d) Pre-illumination (Schott light-filters BG2 + WG1, both 4 mm) had no effect on subsequent UV-inactivation, not even if the temperature of the bacteria suspension was maintained between 0 and 3° C during all operations.

References p. 211.

(e) Light of wavelength greater than about 5200 A (Schott light-filter OG1 used) had no photoreactivating effect.

(f) The UV-inactivation temperature might be expected to have an influence on the PHR. This was investigated by irradiating the bacteria at 5, 20 and 40° C.

Illumination of these UV-inactivated bacteria suspensions gave the same PHR-rates, as shown in Table IV.

TABLE IV

INFLUENCE OF THE UV-IRRADIATION TEMPERATURE ON THE PHR OF *B. subtilis*

The bacteria were irradiated at 5, 20 and 40° C for 2½ min. PHR at 2° C. Schott light-filters used: WG2 + WG1 (both 4 mm).

UV-irradiation temperature	*Number of bacteria per ml in log units*			
	Before UV	*After UV*	*After illumination for (min)*	
			10	20
5	6.95	3.23	4.46	5.42
20	6.95	2.90	3.95	4.95
40	6.95	2.18	3.23	4.40

3. *The photoreactivating effectiveness of light of different wavelength*

KELNER[15] has determined the action spectrum for the PHR of UV-inactivated *Escherichia coli*. He found that PHR occurred in the region of 3650 to 4700 A, the most active wavelength lying near 3750 A.

The author investigated the effectiveness of monochromatic light with wavelengths of 3655, 4047 and 4358 A in photoreactivating UV-inactivated *B. subtilis* and *B. cereus*. Illumination was as described under RESULTS (1). Figs. 2 and 3 give the results of 2 experiments. They show that of the 3 wavelengths used, 4047 A is most effective while 4358 A is least effective. The most active wavelength probably lies near 4000 A, but of course the action spectrum must be determined to make sure of this.

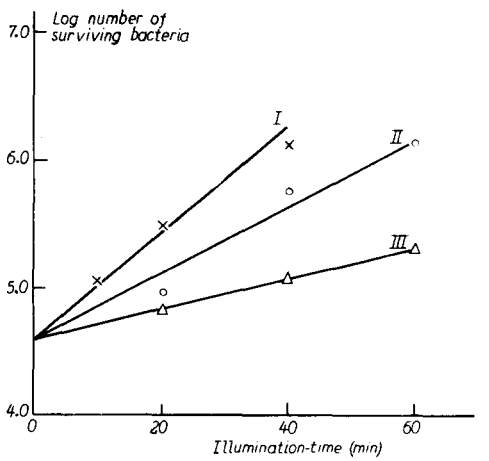

Fig. 2

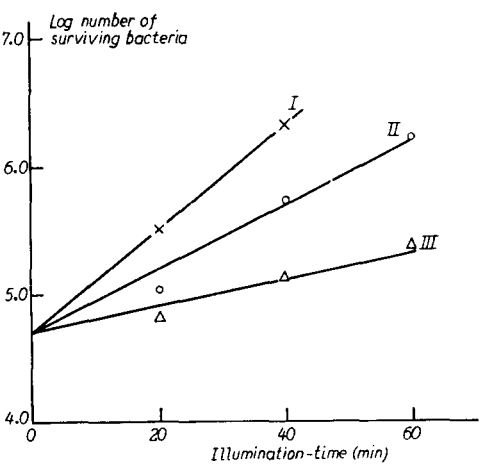

Fig. 3

Effectiveness of light of 3 different wavelengths on the PHR of *B. subtilis* (Fig. 2) and *B. cereus* (Fig. 3). Incident energy 200 μW/cm^2. Curve I: light with wavelength of 4047 A; Curve II: light with wavelength of 3655 A; Curve III: light with wavelength of 4358 A. Initial titers of bacteria in log units: 7.00.

References p. 211.

Inactivation by the illumination occurred simultaneously. This was investigated with unirradiated bacteria suspensions as mentioned above (see Tables II and III). Since this inactivation is of the same (small) magnitude for the 3 wavelengths the conclusions remain valid.

4. *Illumination of UV-irradiated spores*

Spores of *B. subtilis* showed no PHR after UV-inactivation. In some experiments the spores were heated to 40, 50 or 60° C prior to UV-irradiation and in other experiments after UV-inactivation, but still no PHR could be found.

DISCUSSION

The experiments, described in this paper, show that bacilli behave like other bacteria in so far as they can be photoreactivated after UV-inactivation.

The fact that PHR has not always been observed with bacilli might be explained by the sensitivity of bacilli to radiations with wavelengths in the region of 3500 to 4500 A. HOLLAENDER[16] reported that for *Escherichia coli* the 50 %-inactivation dose of light (mainly the 3 wavelengths also used here) is about $5 \cdot 10^8$ erg per cm^2. For *B. subtilis* this is about $7 \cdot 10^6$, that is, some 70 times less. *B. cereus* is still more sensitive. With greater dose-rates the inactivation might even be greater than the PHR, so that only a slight inactivation is found (unpublished data). It is very interesting that *B. subtilis* occurs in two forms of which the vegetative form shows PHR while the spore does not. We see here after UV-treatment a difference in response of these forms to long-wavelength UV-radiation and short-wavelength visible light. One may explain this on the basis of the typical structure differences between the spore and the bacterium, since PHR seems to be generally occurring. We know very little about cellular structures but nevertheless this fact may be of some help in elucidating the mechanism of photoreactivation.

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SUMMARY

The phenomenon of photoreactivation has been studied with three cultures of bacilli.

It has been shown that they could be reactivated by light with wavelengths of 3655, 4047 or 4358 A after ultraviolet inactivation. Since these radiations also have an inactivating effect, the photoreactivation may be small, even not noticeable, when the dose-rate of the incident light is too great.

Bacterial spores did not show photoreactivation.

RÉSUMÉ

Le phénomène de la photorestauration a été étudié pour trois cultures de bacilles.

Nous avons montré que, à l'état végétatif, des bactéries inactivées par l'ultraviolet de 2537 A peuvent être réactivées par une irradiation consécutive à longueur d'onde de 3655, 4047 ou 4358 A. Ces radiations ont également un effet bactéricide notable, ce qui complique et pourrait même dissimuler le phénomène de la photorestauration.

Pour les spores de *B. subtilis* aucun effet de photorestauration n'a été constaté.

References p. 211.

ZUSAMMENFASSUNG

Die Photoreaktivierung wurde an drei verschiedenen Bazillenkulturen untersucht.

Durch Ultravioletstrahlung von 2537 Å inaktivierte Bazillen werden im vegetativen Stadium durch Bestrahlung mit Licht von 3655, 4047 oder 4358 Å reaktiviert. Da diese Wellenlängen auch eine beträchtliche bakterizide Wirkung aufweisen, kann der Reaktivierungseffekt weniger deutlich oder sogar aufgehoben sein.

Sporen von *B. subtilis* zeigten keine Photoreaktivierung.

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