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The Mechanism of Ultraviolet Induction in Lysogenic *Staphylococcus aureus*

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SUMMARY

This investigation has been focused on the significance of residual growth in the latent period after ultraviolet irradiation of a lysogenic strain of *Staphylococcus aureus* (111). It was shown that the organisms divide once or twice before the development of free phage and the number of infective centres are increased by about 100% in 30 min. after irradiation.

When celbenin (sodium 6-(2,6 dimethoxy benzamido) penicillinate) was added to the culture after irradiation, the number of infective centres decreased progressively, and no free phage developed. A delay of 10, 20 or 30 min. in introduction of the drug allowed an increasing fraction of the infective centres to develop and to yield free phage.

These observations lead to the suggestion that ultraviolet irradiation inhibits the synthesis of new repressor of vegetative phage development, but has no influence on repressor molecules present beforehand. Residual growth of the irradiated bacteria serves to dilute the intracellular repressor concentration below an effective level.

INTRODUCTION

It is commonly accepted that the lysogenic state of a bacterial culture depends on the presence of a repressor of the vegetative development of the prophage. Prophage induction by ultraviolet irradiation (u.v.) should be a consequence of radiation damage to the repressor (Stent, 1963; Hayes, 1964; Braun, 1965). Alternatively, if the repressor is assumed to be unstable, induction could result from inhibition of its synthesis following irradiation and a consequent decrease in its intracellular concentration (Jacob & Monod, 1961). A third possibility seems not to have been advocated. The repressor might be resistant to u.v. effect, and stable, but diluted to an ineffective level by growth of the bacterial host. The repressor of the *lac*-operon of *Escherichia coli* has been shown to be stable in non-proliferating bacteria, but unstable during growth. This could be explained as dilution of the intracellular concentration leading to dissociation of the complex repressor molecule (Sadler & Novick, 1965). Experiments with heat induction of prophage in a mutant *E. coli* (λ) are likewise consistent with the hypothesis that induction may be due to dilution of the repressor consequent upon bacterial growth (Sussman & Jacob, 1962).

Observations on u.v. induction of a *Staphylococcus aureus* strain showed that the number of infective centres increased by more than 100% before free phage developed. This was not simply a consequence of slow intracellular phage development, but

proved to be a precondition for this development. These results seem to indicate that the repressor was not destroyed by the irradiation, nor was it unstable. The effect of irradiation is plausibly explained as an inhibition of synthesis of new repressor, while cell growth dilutes it below the effective intracellular concentration.

METHODS

Bacterial strains. The investigation was performed with *Staphylococcus aureus* III. This strain was susceptible only to phage ISRAEL, and it was lysogenic, as are all strains of this phage pattern (Sompolinsky, 1963). *S. aureus*, propagation strain for typing phage 80, was used as indicator for the phage released.

Media and drugs. Bacto Nutrient Broth; pH 7·2. Ten times concentrated Nutrient Broth (10 × NB) was also prepared. Bacto Brain heart infusion. This solidified with 2·0% Bacto agar was designated Brain heart infusion Agar. Tryptic Soy Agar was composed as follows: Bacto Tryptone, 17 g.; Phytone (Baltimore Biological Laboratories, Inc.), 3 g.; NaCl, 5 g.; Bacto agar, 20 g.; H_2O to 1 l.; pH 7·2. Nutrient soft agar: Bacto peptone, 5 g.; Bacto beef extract, 3 g.; $CaCl_2$, 0·15 g.; Bacto agar, 6 g.; H_2O to 1 l.; pH 7·2. Saline Buffer: 0·15 M-Na_2HPO_4, 61·1 ml.; 0·15 M-KH_2PO_4, 38·9 ml.; NaCl, 0·8%, 100 ml. Celbenin (Sodium 6-(2,6 dimethoxy benzamido)-penicillinate), was obtained from Bristol Laboratories, Syracuse, N.Y.

Phage induction. Bacteria were grown at 37° with aeration. When in the logarithmic growth phase they were washed twice and resuspended in ice-cold saline buffer. Density of the bacterial suspension corresponded to absorbency of 0·036–0·041 (filter 540 mμ). Eleven ml. of the suspension were irradiated with an ultraviolet lamp (Sterisol F. 1185/3 J, Original Hanau) at a distance such that the dose rate was 3·16 ergs $mm.^2/sec.^{-1}$. The culture was protected against visible light during the irradiation and the following 60 min.

One vol. of 10 × NB, prewarmed to 37°, was added to nine vol. of the irradiated bacterial suspension. Sodium citrate was added to a final concentration of 10 mM to prevent adsorption of the released phage. The culture was incubated at 37° with slight shaking. Samples of this culture were taken for analysis as indicated.

Infective centres were determined as the total number of plaque forming units, since the relative number of free phage particles was negligible during the first 60 min. after irradiation. Free phage was determined in the supernatant fluid after sedimentation of the bacteria by centrifugation at 2° (6200 *g*) for 20 min.

Enumeration of infective centres and free phage was performed by the agar layer method (Adams, 1959) using nutrient soft agar on a base layer of tryptic soy agar. Viable counts of bacteria were estimated by incorporating 0·2 ml. of appropriate dilutions in saline buffer into melted brain heart infusion agar (45°), plating and incubation for 24 hr (37°). At least three parallel determinations were performed. The bacterial suspension was shaken vigorously to break up possible clumps of bacteria, although no clumps were observed by phase-contrast microscopy of diluted suspensions.

The total number of bacteria were determined as indicated by Wright (see Gradwohl, 1956) by direct microscopy with a standard suspension of sheep erythrocytes for comparison. In each determination at least 500 bacteria were counted.

DNA was determined in washed bacteria after treatment with cold trichloracetic acid and extraction with 5% trichloracetic acid at 70° by the diphenylamine reaction (Burton, 1956).

RESULTS

Induction of Staphylococcus aureus III

The optimal dose of u.v. irradiation for induction was found to be 18 sec. (~ 57 ergs/mm.^{2}). With this dose, in a typical experiment, 23% of the bacteria formed infective centres and a further 48% were nonviable on plating. The remaining 29% retained colony-forming ability.

When the irradiated bacterial population was incubated in nutrient broth at 37°, turbidity increased and was more than doubled in 60 min. (Fig. 1). The total number of bacteria increased at almost the same rate as turbidity, whereas the rise in DNA was significantly slower during the first 30 min., probably indicating that during this period mainly cocci which had already doubled their genome before irradiation were dividing. While the number of colony-forming bacteria remained constant during the first hr after irradiation, the number of infective centres increased rapidly and was more than

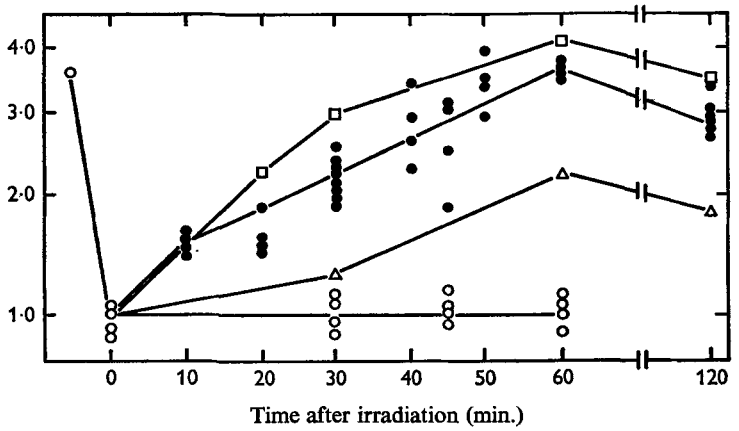

Fig. 1. Relative number of colony-forming staphylococci (○), relative turbidity (●), amount of DNA (△) and total number of staphylococci (□) in an irradiated culture of *Staphylococcus aureus* III. A logarithmic phase culture was washed, resuspended in saline buffer and irradiated with ultraviolet light (~ 57 ergs mm^2). The bacteria were incubated in nutrient broth at 37° and samples analysed as indicated (see Methods). The relative number of colony-forming cocci are from 4 independent experiments. Relative optical density from eight experiments are indicated; the curve indicates results from one typical experiment.

doubled in 30 min.; thereafter it remained more or less constant until free phage appeared (Fig. 2). The mean burst size was only 8 to 12 in this system. This was confirmed by the single cell method (Burnet, 1929). The burst size could be almost doubled by raising the concentration of NaCl in nutrient broth to 3·6%. Free phage accounted only for 10^{-4} of all infective centres at zero time, and was always less than 2% at the end of the first hr. At the end of 2 hr more than 90% of plaque forming particles were free phages. Though the detailed increase in infective centres and turbidity varied from one experiment to the other, in principle the course was always the same.

Since infective centres accounted for only a small fraction of the bacteria, the increase of infective centres alone cannot explain the increase in turbidity, DNA and total cell number during the first hr after irradiation. Furthermore, the number of

colony-forming cells was constant during this period. It was therefore assumed that the 'killed' cells underwent 1 to 2 cell divisions. These results indicate that the irradiation damage was selective for cocci in active replication. This fraction of the population was preferentially killed or induced, while other cells underwent dark repair.

Alternatively, one might try to explain the increase in the number of infective centres during incubation as only apparent. The fate of a fraction of the cocci, which would develop infective centres during growth in the broth, might be changed by early

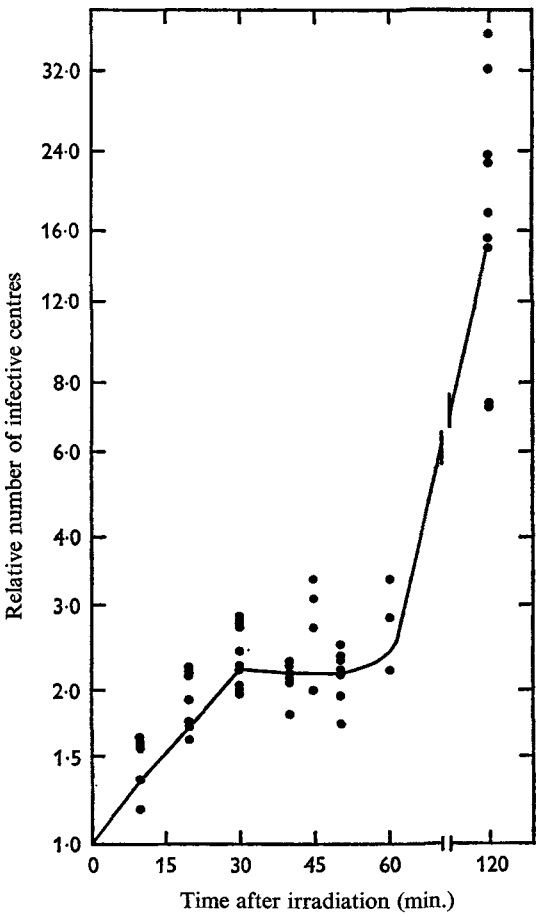

Fig. 2. Relative number of plaque forming units developing in a nutrient broth culture of *Staphylococcus aureus* 111 during 120 min. after ultraviolet irradiation (~57 ergs mm^{-2}). Results from ten independent experiments are plotted. The curve shows the results obtained in one of the experiments.

plating. The changed fraction would decrease when the period of cultivation in nutrient broth was increased. If these cocci are changed to colony-forming cocci this might explain the lack of increase in the number of these cells, provided the fraction of infective centres changed by plating at zero time equalled the obscured increase in the viable cells due to growth. The increase in turbidity, DNA and total number of bacteria

must then be explained by assuming that during the first hr in the liquid medium the 'dead' bacteria replicate together with the colony-forming units, and only the infective centres remain static in number.

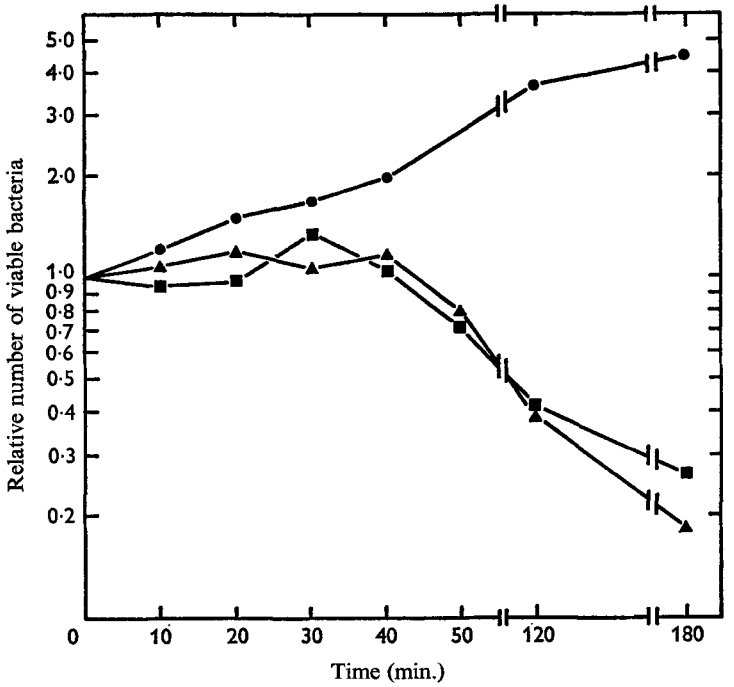

Fig. 3. The influence of celbenin on the number of colony-forming bacteria in a nutrient broth culture of *Staphylococcus aureus* 111. ●, Control; ■, celbenin 25 μg./ml.; ▲, celbenin 100 μg./ml.

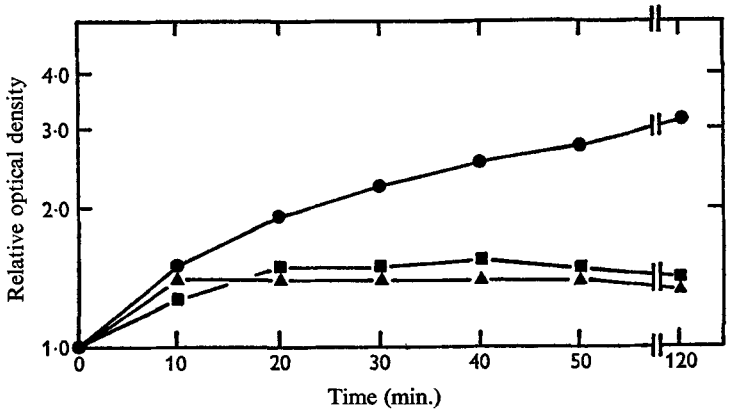

Fig. 4. The influence of celbenin on optical density of a culture of *Staphylococcus aureus* 111 in nutrient broth after irradiation with ultraviolet light (~57 ergs mm^{-2}). Conventions as Fig. 3.

Influence of celbenin on infective centres

Penicillin has no influence on the intracellular development of vegetative phage in *Staphylococcus aureus* (Price, 1948). Addition of penicillin to the induced culture might therefore be helpful in establishing whether the infective centres divide or not.

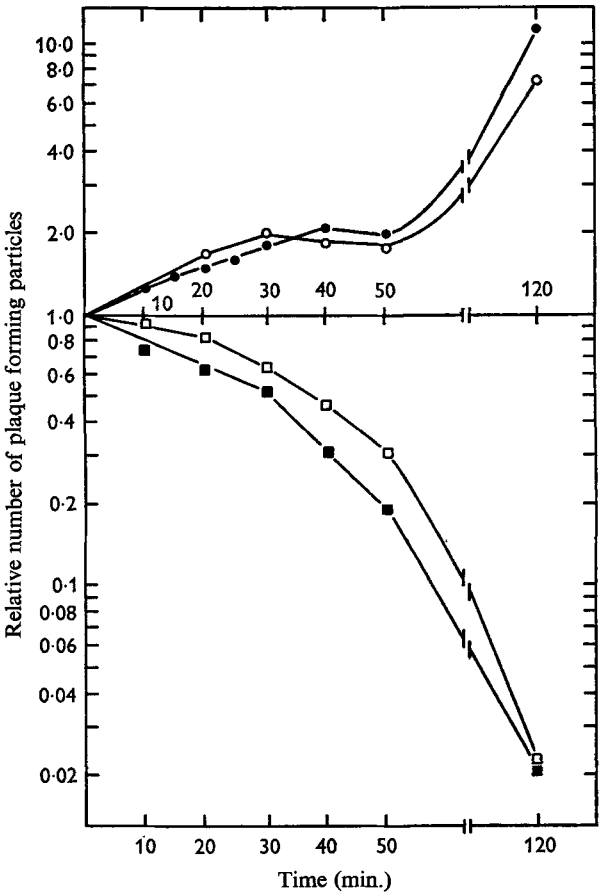

Fig. 5. The influence of celbenin (25 μg./ml. added at zero time) on the number of plaque forming units in a nutrient broth culture of *Staphylococcus aureus* III; the bacteria were irradiated with ultraviolet light (~57 ergs mm^{-2}) before incubation. ●, Control (Expt 1); ■, celbenin (Expt 1); ○, control (Expt 2); □, celbenin (Expt 2).

Since only growing cells are susceptible to penicillin, this drug would not be expected to kill infective centres if they are not dividing. Before considering the results, it should be recalled that penicillin killing of staphylococci generally occurs considerably later than the time at which the cell division would normally occur (Gardner, 1940; Murray, Francombe & Mayall, 1959). Since *S. aureus* III produces penicillinase, celbenin was used in these experiments. Fig. 3 shows the influence of 25 μg./ml. and 100 μg./ml. celbenin on a non-irradiated culture of *S. aureus* III. After 40 min. the number of viable cells was still 52 to 60% of the control, while it decreased to 11% after 2 hr, and 4 to 6% after 3 hr. The influence of celbenin on turbidity of an irradiated culture is

shown in Fig. 4. The influence of this drug on the development of infective centres was much more dramatic than might have been expected from Figs. 3 and 4. When the drug was added at zero time of the incubation in broth the number of plaque forming particles after 2 hr reached only 0·5 to 3·0% of the control, or 5 to 15% of the infective centres at zero time (Fig. 5). Furthermore, while in the control, after 120 min., the plaque forming units were mainly free phage, virtually no increase in the titre of free phage occurred in the celbenin-treated culture during 3 hr, so that it was possible to sediment by centrifugation (6200 *g* during 20 min.) almost all infective centres

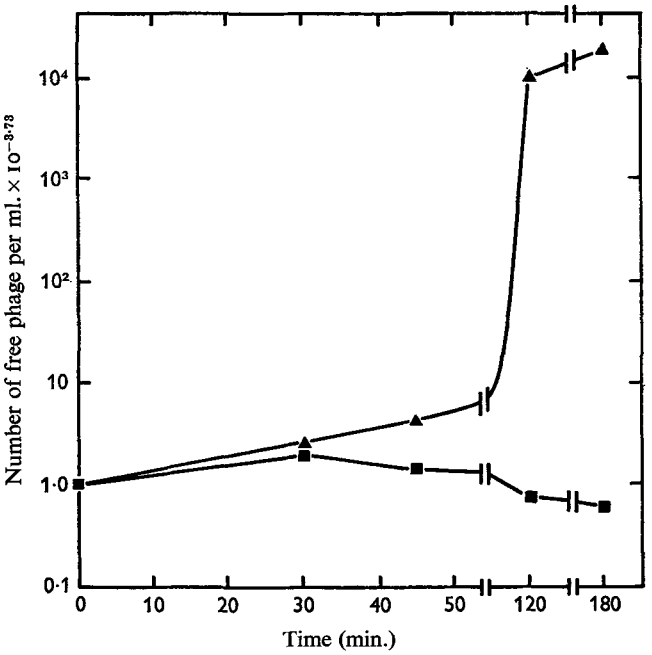

Fig. 6. The influence of celbenin (25 μg./ml.) on the development of free phage in a nutrient broth culture of *Staphylococcus aureus* 111 after ultraviolet irradiation (~ 57 ergs mm^2). At the indicated times, samples of the culture were centrifuged 20 min. at 6200 *g* (2°). The number of plaque forming units in the supernatant fluid was considered free phage. ■, celbenin; ▲, control.

(Fig. 6). The decrease in infective centres in the celbenin culture was progressive, being 15 to 32% of the number at zero time after 50 min., whereas this number had at least doubled in the control culture.

In another series of experiments, the drug was added at various times during 120 min. incubation in nutrient broth. The plaque forming particles were determined by plating after 120 min. One typical experiment is reported in Table 1. A delay of 10 min. in addition of the drug had a marked influence on the final number of plaque forming units. When the drug was added after 20 min. incubation, this number reached 47% of the control. This rapid abolition of the effect of the drug is to be expected, if phage development in the infective centres which have already divided is unaffected by the drug. One might have expected an even larger effect of a delay of 10 min. before the addition of the drug, since a relatively large fraction of the infective centres seem to divide during this period (Fig. 2). However, some of the cell divisions early after irradiation may not

produce mature cells for phage development, since the intracellular concentration of repressor might already have been increased in those cells, which were ready for cell division at the time of the u.v. irradiation. Additional growth of the host may be required to enable development of vegetative phage in these infective centres.

In every case, the high susceptibility of infective centres to celbenin shows that these cells divide. The increase in the number of infective centres and the lack of increase in the number of colony-forming units after irradiation must therefore be considered real.

Table 1. *The influence of celbenin on the number of plaque forming particles after ultraviolet irradiation*

Time of addition of celbenin (min.)	p.f.u.
0	0·14
10	1·66
20	8·94
30	11·43
40	14·29
No celbenin	18·17

A washed logarithmic phase culture suspended in saline buffer was irradiated with u.v. light (57 ergs mm^{-2}) and reincubated in nutrient broth at 37° for 120 min. Celbenin (25 μg./ml.) was added at the times indicated. P.f.u. expressed relative to 1·0 at zero time.

DISCUSSION

Residual growth of a u.v.-induced lysogenic bacterial culture was described many years ago. It has been reported that bacterial growth without cell division occurs in lysogenic *Escherichia coli* and *Pseudomonas pyocyanea* during the latent period before lysis (Jacob & Fuerst, 1958; Jacob & Wollman, 1953, 1959). On the other hand, Lwoff, Siminowitz & Kjeldgaard (1950), in a very careful investigation, showed that in a culture of lysogenic *Bacillus megaterium* which was induced by u.v., optical density increased 2 to 4 times before phage developed, and that each induced cell underwent two divisions in the latent period. These observations are in principle comparable to the behaviour of *Staphylococcus aureus* 111 described in this report. The period from the occurrence of intracellular mature phage until burst is similar after u.v.-irradiation and after infection from without (Jacob, 1954). The crucial question is whether this 'residual growth', either with or without cell division, is simply a consequence of the length of the latent period after induction, or whether the residual growth is necessary for phage development, and that the prolonged latent period is due to the time needed for this growth, before the prophage is able to begin its vegetative development.

We think that the celbenin experiment offers an answer to this question. When the drug was added to an induced culture, no phage developed in the infective centres. The influence of celbenin might partly be explained as lysis of dividing infective centres before infective phage was synthesized. But this cannot be the whole effect. Even in the few infective centres that were still found a long time after the drug was added, no vegetative development had occurred in the presence of the drug, since no increase in the number of free phage could be demonstrated after 2 and 3 hr (Fig. 6). These surviving infective centres had probably escaped the celbenin effect and had delayed

phage development for the same reason: they had not grown enough. The delay in growth may in itself be a consequence of the drug action (Gardner, 1940; Murray *et al.* 1959).

This effect cannot be explained as a direct inhibition of the prophage development by celbenin. Such an effect is not consistent with what is known about the action of penicillins. Furthermore, when celbenin was added a short time (10 to 30 min.) after incubation of the irradiated culture, a relatively large fraction of the infective centres were resistant and seemed to develop normally in the presence of the drug (Table 1). It may therefore be concluded that u.v. irradiation converted a fraction of the most actively growing cells in the culture into infective centres because growth is most probably a condition for vegetative phage development.

The notion that the repressor must be diluted by residual growth as a precondition for phage development could explain some facts which hitherto have been rather obscure. The latent period after u.v. irradiation is considerably longer than after infection from without (Lwoff, 1953; Bertani, 1958; Jacob & Wollman, 1953). It has been proposed to relate this prolonged latent period to photoreactivating activity (Lwoff, 1953; Jacob & Wollman, 1953) though the connexion is not quite clear. It is more plausibly explained as the period of residual growth necessary to dilute the repressor. Furthermore, according to this concept, inhibition of lysis after u.v. induction by decreasing the 'aptitude' (Lwoff, 1953) does not need any complex explanation. On this point it may be of interest to quote from Lwoff (1953): 'If bacteria are irradiated and then starved, neither growth nor lysis is observed. If the missing "growth substance" is added after 2 or 3 hr, residual growth takes place and lysis occurs.'

Recently, new significant evidence for the mechanism of u.v. induction of lambda prophage has appeared. U.v. induction of lambda lysogenic *Escherichia coli* seems to involve activation of synthesis of a substance which in turn inactivates the repressor (Hertman & Luria, 1967). Non-lysogenic segregates from abortive lysogenic cells lose their lambda immunity through growth by dilution out of the growth stable repressor. In these non-lysogenic immune cells, u.v. irradiation brings about a biochemical process which gradually abolishes the immunity to λ-phages on incubation. This process involves protein synthesis (Tomizawa & Ogawa, 1967*a*). However, another function of the lambda repressor, inhibition of the growth of r II mutants of phage T_4 (Benzer, 1955), is not damaged by the u.v. irradiation, and only lost when the repressor is diluted out by growth (Tomizawa & Ogawa, 1967*b*).

The present study has been focused on the importance of residual growth for phage development after u.v. irradiation. We have no data to indicate whether or not the process of induction in the strain of *Staphyloccus aureus* investigated by us is complex and involves the synthesis of a substance which advances de-repression of phage development. But if such a substance in itself was sufficient to abolish repression, our results would indicate that its synthesis was inhibited by celbenin. Celbenin should be expected specifically to inhibit the synthesis of cell mucopeptide, and this substance is not appealing as a candidate for repressor inactivation. On the other hand, it will not be too surprising if further studies will demonstrate dissimilarities in the ways in which induction is displayed in different lysogenic systems.

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