BIOCHEMICAL MODIFICATIONS IN LYSOGENIC *B. MEGATHERIUM* 899 (1) AFTER INDUCTION WITH ULTRAVIOLET LIGHT*

by

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INTRODUCTION

In lysogenic bacteria, bacteriophages are propagated through succeeding bacterial generations in a non-infectious form, the so-called "prophage"[1]. Development of infective phage particles may be induced in almost all the bacteria of lysogenic *B. megatherium* 899 (1) by irradiation with ultraviolet light[2]. After a definite latent period, mass lysis ensues, with liberation of bacteriophages into the medium, similar to the process observed during one-step growth of the T-phages on their *Escherichia coli* host. An important difference is found, however, when the optical density of mass cultures is observed during phage development. Bacterial growth, which is arrested immediately after infection of *E. coli* with T-phages[3] continues up to the moment of lysis, after induction of *B. megatherium*[2]. Such residual growth is also observed after the induction of other lysogenic strains, such as *E. coli* K-12[4], *Pseudomonas pyocyanea*[5], and *Staphylococcus aureus*[5]. This behaviour, however, cannot be considered to be characteristic of induction, as opposed to infection, since residual growth also occurs when sensitive bacteria are infected by phages originating from a lysogenic strain[2,4,5].

It seems justified, therefore, to distinguish between those phages which do, and those which do not, permit residual bacterial growth during their development. For convenience, phages which do not permit residual growth will be referred to as *virulent*, in contrast to the *temperate* phages carried by lysogenic strains.

Detailed investigations of the biochemical modifications occurring during the development of virulent phages have shown that synthesis of ribonucleic acid is arrested[6], that respiration continues but only at the rate existing at the time of infection[3,7] and that the synthesis of adaptive enzymes is inhibited[7]. The formation of virulent phages seems then to inhibit the synthesis of many bacterial components. Desoxyribonucleic acid synthesis is also blocked temporarily but recommences, and continues throughout the remainder of the latent period at a rate greater than that found just prior to infection[6].

To further compare the characteristics of the development of temperate and virulent phages, the biochemical changes occuring after induction of *B. megatherium* 899 (1) have been examined in the present work. It will be seen that the residual growth after

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induction of lysogenic strains is a manifestation of the fact that during the development of temperate phages there is much less interference with those bacterial components whose syntheses are blocked during growth of virulent phage.

The first part of this paper concerns the measurement of latent periods, and the effect of temperature on these periods, the effect of dose of U.V. light used for induction, and the significance of residual growth. The second part is devoted to the biochemical study. A preliminary report of this work has already been published[8].

MATERIALS AND METHODS

The lysogenic strain of *B. megatherium* 899(1), the sensitive indicator strains *mutilat* M and 899(dl), and the yeast-casein culture medium have previously been described[1,2,9]. In the experiments reported here, bacteria in their exponential growth phase were prepared by diluting a fully grown culture (in beef broth medium), 1:100 into yeast-casein medium, and allowing this culture to grow at 37° C with aeration. Under these conditions, strain 899(1) has a doubling time of 20–25 minutes at 37° C and 37–40 minutes at 27° C.

Optical densities were followed in a Meunier electrophotometer. They are expressed as graduations on the dial of the apparatus. These graduations were calibrated against the bacterial concentration by cell counts in a Bucher cell. With a cell of 1 cm light path, and a red filter, 100 graduations represent $3.4 \cdot 10^7$ cells/ml.

The ultraviolet irradiations were usually performed on cultures containing $1.2 \cdot 10^8$ bacteria/ml. These were then diluted into fresh medium in an Erlenmeyer flask, maintained at the appropriate temperature in a water bath and shaken for good aeration. Since the induction by U.V. light of lysogenic strains is reversed by the action of visible light[4], the flask was always kept in the dark.

For the induction, the culture was placed in Petri dishes, which were rocked continuously during the irradiation. The thickness of the irradiated layers never exceeded 2 mm. The ultraviolet light was furnished by a Hg vapour lamp at low pressure and high tension, which delivered an energy of 2000 ergs/mm^2/min^{-1}, at the surface of the liquid at a distance of 50 cm. Under these conditions at least 90% of the bacteria of a culture of *B. megatherium* 899(1) are induced by a dose of U.V. light of 250 ergs/mm^2.

The nucleic acids were extracted by the method developed by SCHNEIDER[10], ribonucleic acid being then determined by MEJBAUM's technique[11] and desoxyribonucleic acid by that of STUMPF[12].

THE KINETICS OF THE U.V. INDUCED *B. megatherium* LYSOGENIC SYSTEM

a. *Latent period*

The latent period of bacteriophage development is measured most accurately by titration of free bacteriophage in highly diluted cultures (one step-growth experiment[13]). Using this procedure, the appearance of free extra-cellular phage can be observed following the induction of *B. megatherium* 899(1). The results of such an experiment are shown in Fig. 1 where it is seen that a period of 43 minutes intervenes between the irradiation with U.V. light, and the first appearance of free phage in the medium. This period can be identified with the latent period intervening between infection of *E. coli* B and appearance of new phage.

Also presented on Fig. 1 is the curve of optical density of the mass culture after induction. After residual growth, the culture begins to clear at the same moment that the first phage particles can be detected in the medium. For convenience, the length of the latent period has usually been measured, in this study, by careful observation of the optical density of mass cultures.

b. *Intracellular phage development*

B. megatherium is lysed by lysozyme and this property may be utilised to follow the development of bacteriophage within the cells. The technique is analogous to that

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described by DOERMANN for *E. coli* infected with a T phage[14] except that lysozyme has the additional advantage of lysing the bacteria even when no intracellular phage is present[15]. If a one step growth curve is then carried out with induced *B. megatherium* by treating the cells with lysozyme at frequent intervals, results such as those shown in Fig. 1 are obtained, where it is seen that at 37° the first infective phage particles appear within the cells at 30 minutes after induction.

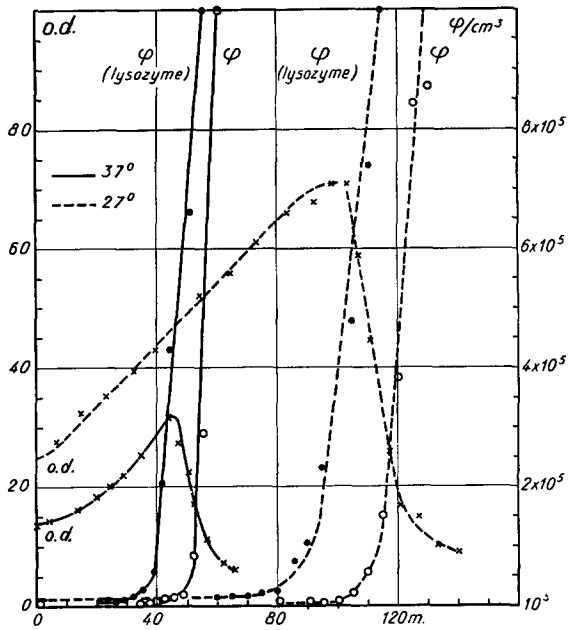

Fig. 1. Measurement of time elapsed after induction, for lysis and for the appearance of intra and extracellular phage at 27 and 37° C.

c. *Effect of temperature*

The extracellular and intracellular appearance of phages after induction has also been examined at the temperature of 27° C, and the results are shown in Fig. 1. The latent period has been lengthened to 102 minutes, and the first intracellular phage does not appear until 72 minutes after induction. The change of temperature from 37 to 27° C has therefore lengthened by the same factor, 2.4, the time required for appearance of both the first intracellular and extracellular phage.

d. *Effect of increased inducing doses of U.V. light*

The experiments described so far were effected with the minimal dose of U.V. light sufficient to induce phage development in at least 90% of the bacteria of a culture of *B. megatherium*, that is 250 ergs/mm². To examine the effect of higher doses, several aliquots of the same bacterial suspension were treated with different amounts of U.V. light and the optical density of the irradiated suspensions followed during subsequent incubation at 27° C. The results are represented in Fig. 2 from which it appears that, as the dose is increased, the residual growth rate is diminished and the latent period prolonged. Whereas the latent period is 98 minutes for a dose of 250 ergs/mm², it is increased to 280 minutes for a dose of 10,000 ergs/mm². A forty fold increase in dose has therefore tripled the length of the latent period. Similar results are found at 37° C where an increase of the minimal dose of 250 ergs/mm² to 3,000 ergs/mm² results in an increase of the latent period from 43 to 75 minutes.

These results are in accordance with those found by WEIGLE AND DELBRUCK with the K-12 strain of *E. coli*[4]. It appears probable that the increased dose affects the rate of most metabolic processes in the cell, and thus prolongs the period required for maturation of the bacteriophage particle. It is possible that, in addition, the prophages themselves are somewhat damaged by heavy doses of ultraviolet light and that this interferes as well with normal development. This latter effect would be analogous to the increased latent period exhibited by the survivors of U.V. irradiation of virulent phages[16].

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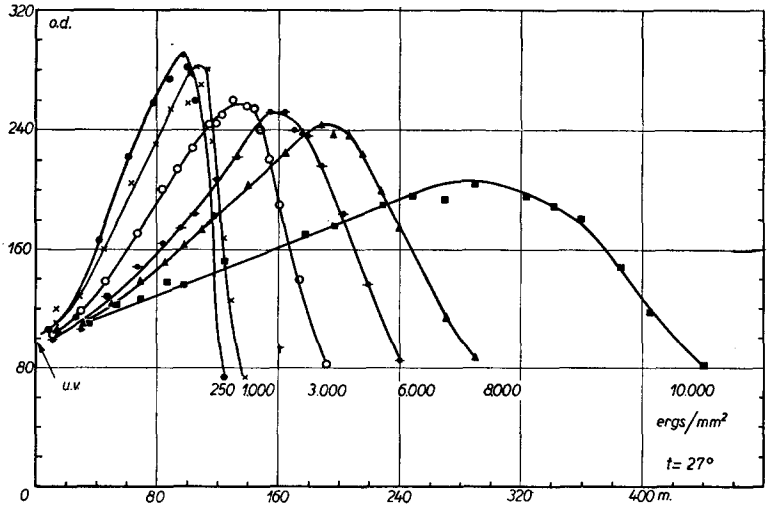

Fig. 2. Effect at 27° of increasing dose of ultraviolet light used for induction

e. *Residual growth*

It has been seen that one of the main differences between temperate and virulent phages is the residual bacterial growth exhibited by the former. This residual growth, up to the moment before lysis, is nevertheless less than that observed in a non-irradiated culture over the same period of time[2]. The decrease is in fact due to a continual change in growth rate during phage development of an irradiated culture, where the rate of increase of optical density continually diminishes (Fig. 1).

These modifications in the growth rate are probably manifestations of the bacteriophage reproduction which is taking place and not, as one might also suppose, of the lethal effects of the irradiation by U.V. light. This is supported by several different lines of evidence. If the non-lysogenic strains M or 899(d1) are irradiated with doses of U.V. light of up to 1,000 ergs/mm², very little diminution in the growth rate is observed. This dose is 4 times greater than the minimum required for induction. If, on the other hand, the sensitive strain M is infected by bacteriophage 1, a decrease in its growth rate similar to that of the induced lysogenic strain is observed[2]. Finally, it has recently been found that strain 899(1) may also be induced by certain chemical agents to form bacteriophage in almost the totality of the population[17]. For concentrations of inducing agent just sufficient to give total induction, the same characteristic residual growth curve is observed.

It will be shown later in this paper that the residual growth is not an artefact of optical density measurements, such as swelling, but corresponds to definite synthesis of bacterial matter. It should be mentioned that in strain 899(1) no bacterial division is observed during this residual growth[18].

BIOCHEMICAL MODIFICATIONS AFTER INDUCTION

Residual growth seems to be an expression of the possibility of bacterial synthesis during phage multiplication. In the following section, we will examine the evolution after induction of certain specific bacterial constituents.

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a. *Rate of respiration*

When *E. coli* is infected with T_2 or φ_2 bacteriophage, the rate of respiration during the latent period of virus multiplication remains equal to that prevailing at the moment of infection[3,7]. A similar experiment has been performed with 899 (1).

A culture containing $5 \cdot 10^7$ bacteria/ml was irradiated with a dose of 1000 ergs/mm^2. Immediately after irradiation, the suspension was divided into 11 Warburg flasks which were agitated at 28° C. 5 flasks served for the measurement of respiration rate, and 6 others were removed successively for optical density measurements.

The results of this experiment are shown in Fig. 3 where the average O_2 uptake over a period of 20 minutes, and the optical density are plotted against the time elapsed after induction. It is seen that the respiration rate continues to increase after induction and that this increase is parallel to the increase in optical density.

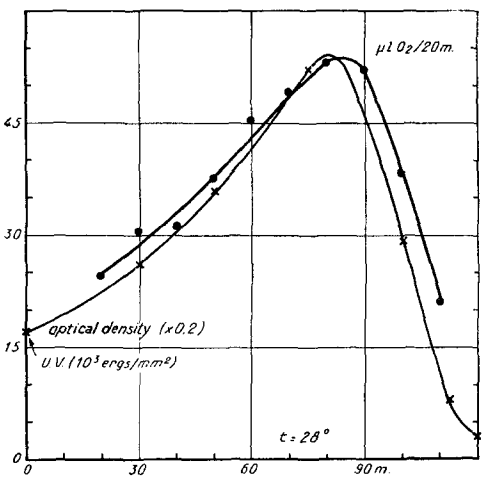

Fig. 3. Measurement of rate of respiration at 27° C after induction

Under the conditions of this experiment, the respiration rate may be considered as an over all measure of all those enzymes which affect the oxygen uptake. The results show, therefore, that the formation of bacteriophage in lysogenic bacteria does not block the synthesis of all or any of this system of enzymes, a result which is in sharp contrast to that found when *E. coli* B is infected with T_2 or φ_2 where no synthesis of respiratory enzymes seems to be possible after infection[3,7].

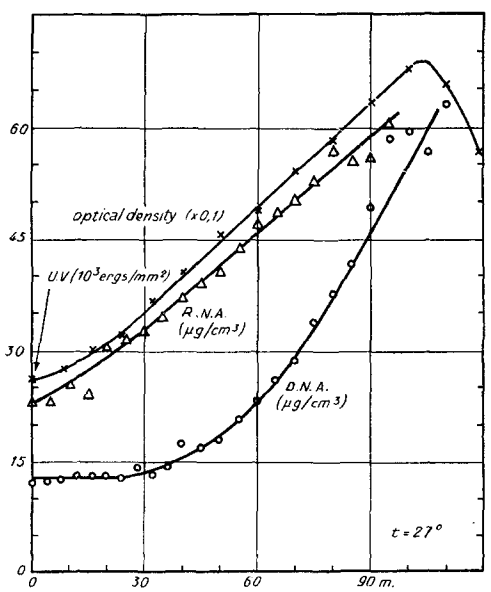

Fig. 4. The synthesis of ribonucleic acid and desoxyribonucleic acid at 27° C in *B. megatherium* after induction with U.V. light.

b. *The synthesis of ribonucleic acid (RNA)*

Ribonucleic acid has not been found in any phage examined heretofore, and it is understandable that, after infection with a virulent phage where no bacterial growth occurs, no increase in RNA is observed. The pattern of RNA development has been examined in an induced lysogenic strain.

A culture containing $5 \cdot 10^7$ bacteria/ml was irradiated with a U.V. dose of 1000 ergs/mm^2. This suspension was then diluted into fresh medium and shaken in a water bath at 27° C. At suitable intervals the optical density was read, and samples taken, immediately chilled to 0° C and centrifuged, washed with 2% trichloracetic acid (TCA) and then treated twice with hot 5% TCA. Under these conditions ribonucleic acid is extracted quantitatively[10]. The Mejbaum color reaction was carried out on these

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extracts, and the extinction of the color produced measured at 650 mμ in the Beckman spectrophotometer. The quantity of RNA was estimated by comparison with a calibration curve based on ribose as standard. All analyses were performed in duplicate.

Fig. 4 shows the results of this experiment. It is seen that as the optical density increases so does the quantity of ribonucleic acid, with the two curves actually being parallel. This again is in marked contrast to the results of T_2 infection of *E. coli* where *no* RNA is synthesized during phage development[6].

c. *The synthesis of desoxyribonucleic acid (DNA)*

One of the most striking biochemical consequences of phage development is the characteristic kinetics of desoxyribonucleic acid synthesis. It has been shown with virulent phages that, in a first stage, immediately following infection, synthesis is completely blocked, but is resumed later at a rate greater than that shown by uninfected normally dividing cells[6]. The latter result is coherent with the fact that complete phage consists in great part of DNA[3]. This is the situation for bacteria which do not grow after infection and which have their normal capacity for syntheses considerably impaired. In the following series of experiments we have examined the pattern of DNA synthesis in *B. megatherium* 899 (1).

The experimental technique is similar to that described in section b for the analysis of RNA except that larger samples were taken. The Stumpf color reaction was used and the extinction measured at 490 mμ. A sample of thymonucleic acid prepared in our laboratory was used as standard. The results have been represented graphically in Fig. 4 on normal, and in Fig. 5 on semilogarithmic coordinates.

It is seen that, as with infected *E. coli*, two definite stages may be observed. Even though, after induction, *B. megatherium* continues to grow, synthesis of DNA is completely stopped for 30 minutes. This blockage is followed by a second period of rapid DNA accumulation.

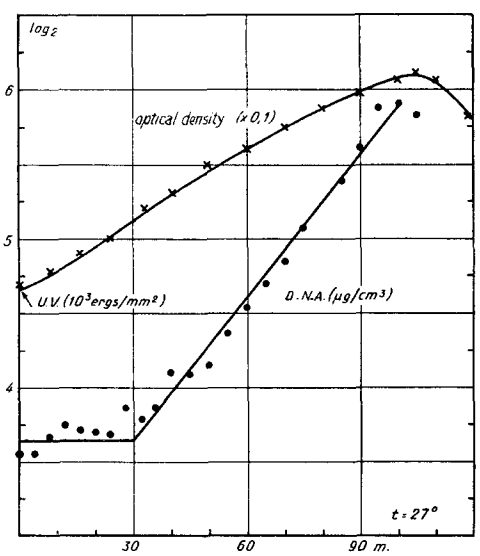

Fig. 5. The synthesis of desoxyribonucleic acid at 27° C in *B. megatherium* after induction with U.V. light. The ordinate values represented in Fig. 4 have been plotted here on a logarithmic scale.

If the sensitive strain M is irradiated with a similar U.V. dose, the blockage is not observed, that is, the synthesis of DNA is not affected. The accumulation curve of DNA is parallel to the optical density curve (Fig. 6). The cessation of DNA synthesis by 899 (1) after induction seems to be related, therefore, to phage development, and is not the consequence of the U.V. irradiation.

The blockage which lasts for about 30 minutes in the experiment described in Fig. 4 or 5 is only temporary, for synthesis recommences and at a very rapid rate. Discussion of the form of this curve is postponed until later. It should be noted that when synthesis has begun, the rate (doubling of quantity of DNA in 31 minutes at 27° C) is higher than the normal rate found when the non-induced bacteria are growing exponentially

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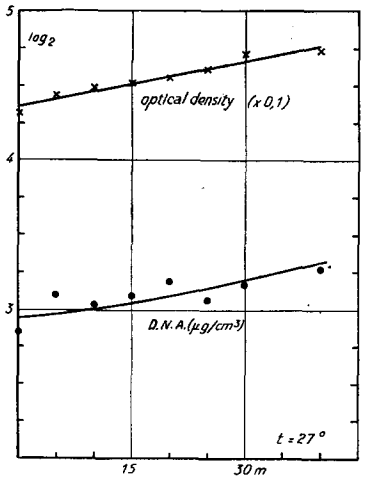

Fig. 6. The synthesis of desoxyribonucleic acid at 27° C in mutilat after irradiation with U.V. light.

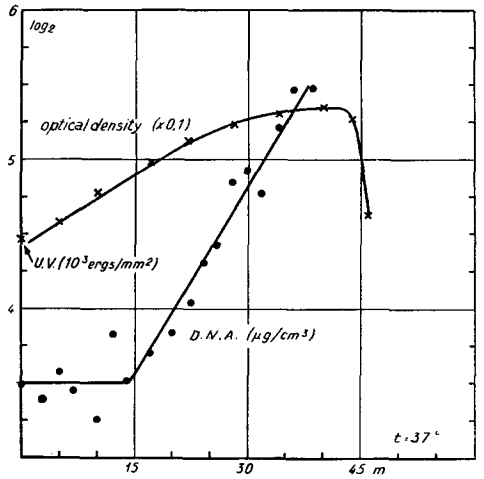

Fig. 7. The synthesis of desoxyribonucleic acid at 37° C in *B. megatherium* after induction with U.V. light.

(doubling in 37–40 minutes at 27° C). Thus, the two types of phages, virulent and temperate, which differ in so many respects in their effect on the biochemical activities of the host, do show very similar kinetics of DNA synthesis.

It has been seen that the latent period of phage development in lysogenic systems may be altered in at least two ways: by a change in dose of U.V., or by a change in temperature. Experiments were undertaken to determine if changes in these two factors would affect one or other of the two clearly demarcated periods of the DNA accumulation curve in a more marked fashion. Fig. 7 shows the DNA curve obtained at 37° C, and Fig. 8 a set of curves obtained at 27° C with an increased dose. In each case the accumulation curve has been plotted on semilogarithmic coordinates since the synthetic portion is then translated graphically as a straight line. It is seen that both the periods of arrested DNA synthesis and that of accelerated DNA synthesis are altered, and no simple relationship seems to exist between the lengths of time for each of these periods.

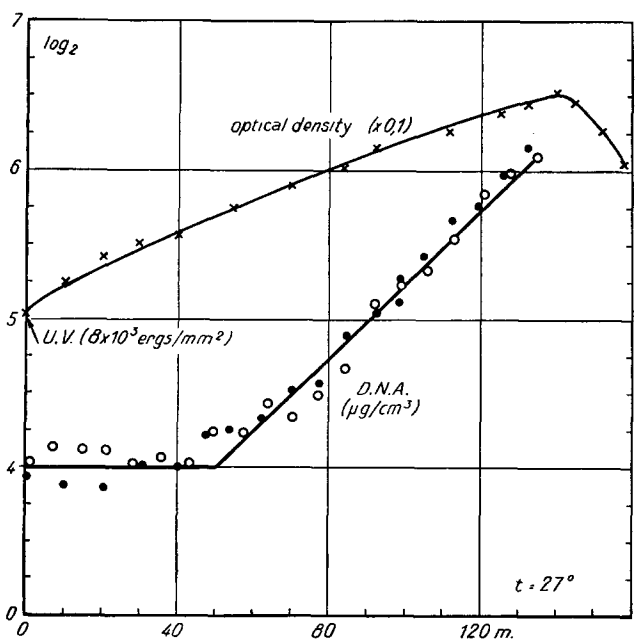

Fig. 8. The synthesis of desoxyribonucleic acid at 27° C in *B. megatherium* after induction with a heavy dose of U.V. light.

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DISCUSSION

One of the main interests in the comparative study of the biochemical aspects of bacteriophage production, is to determine on the one hand, what results are particular to any strain or to any system, and, on the other hand, what biochemical modifications are a general consequence of bacteriophage formation. The observations described in this investigation when compared with those found with the virulent phages, show that many of the results found heretofore are of the former variety. With *E. coli* B, on the one hand it had been shown that its growth, synthesis of respiratory enzymes and ribonucleic acid were severely inhibited after infection with T_2 or φ_2[3,6,7]. On the other hand, these synthetic processes are very slightly affected during the development of bacteriophage in induced *B. megatherium* 899(1). A similar situation is observed when the ability to synthesize adaptive enzymes is examined. *E. coli* B infected with φ_2 is incapable of induced enzymic biosynthesis[7], whereas two lysogenic bacterial strains *Pseudomonas pyocyanea*[19] and *E. coli* K-12[20] have been shown to be capable of this type of synthesis after U.V. induction. All these biochemical modifications seem, then, to be particular to the kind of system, bacteria-bacteriophage, studied.

However, this is not true for the kinetics of DNA synthesis. In all the systems studied up to now, the accumulation of DNA follows the same pattern, that is, with two regions, a first period in which no DNA increase is found, and a second stage where rapid synthesis is observed. The fact that *B. megatherium* is capable of other syntheses after induction, makes it all the more probable that the early blockage of DNA synthesis is a general phenomenon consequent to phage multiplication.

However, it should be emphasized that the measurement of DNA is a global one, and therefore does not give us any information about the fate of the bacterial DNA present in the bacterium at the moment of induction. In spite of the fact that no total increase of DNA is observed during the first part of the latent period, it is conceivable that bacteriophage DNA is being synthesized during this time. This conception implies that bacterial DNA is being continuously degraded and that the synthesis of phage DNA compensates this loss. The findings of KOZLOFF AND PUTNAM with *E. coli* infected with T_6r^+, that bacterial DNA is utilized for the formation of phage DNA[21] would seem to support this possibility.

It may be seen that in *B. megatherium* the quantity of DNA found just before lysis is 4 times that present initially. This would mean that of all the DNA finally present in the cell, 75% is synthesized *de novo*, and 25% comes from the bacterium. Of interest in this connection are the results found with *E. coli* B infected with T_6r^+ where it was shown that approximately 25% of bacteriophage phosphorus originated from the bacteria and the remainder was contributed by the medium[22]. The coincidence of these and our results may be fortuitous but they do indicate the possibility that during the phase of non-DNA-synthesis, bacterial DNA is being transformed into phage DNA.

The second part of the DNA accumulation curve is of interest in two respects. The fact that DNA is synthesized rapidly and at a rate greater than that of normally growing, non-induced cells, is similar to the situation found with infected *E. coli* B[6]. This seems to be a particular characteristic of phage synthesis. A study of the form of this curve is also revealing. COHEN showed that in infected *E. coli* DNA was formed at a constant rate during synthesis, and he concluded from this that this synthesis was therefore not autocatalytic[6,23]. In a more recent publication the same author, with

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ARBOGAST, has found that the linear portion of the curves of DNA synthesis is preceded by a phase in which the rate of accumulation increases progressively[24]. As we have shown in *B. megatherium* (Fig. 5, 7, 8), DNA is not formed at a constant rate and, in fact, seems to accumulate exponentially.

These results may be interpreted as follows: in bacteria where bacteriophage development is taking place the rate of synthesis of DNA is far superior to the rate existing during normal exponential growth. With infected *E. coli* B, all bacterial syntheses, including those of enzymes, seem to be inhibited. If, as is probable, the rate of synthesis of DNA is a function of the existing enzymes of the host, this rate should have a maximum limit. It is quite conceivable that during the first stages of DNA increase the existing enzymes can handle all the available substrate and that under these conditions one observes the true kinetic picture, that is an exponential curve. But once the rate of DNA formation becomes very rapid, the synthesis continues but only at a limited constant rate.

With *B. megatherium* where syntheses are still possible after induction, the rate of DNA accumulation can increase throughout almost the whole latent period, which one actually does observe.

LURIA's findings in studying spontaneous mutants of bacteriophages are also of interest in this connection[25]. He has shown that the distribution of these mutants is clonal, which suggests that during some phase of the phage growth cycle, we are dealing with a self reproducing population. In the case of *E. coli* B, if one assumes a large excess of DNA compared to the genetic units studied by LURIA, one might observe clonal appearance of mutants, and at the same time, linear synthesis of DNA. However in *B. megatherium*, the observed exponential increase in DNA may actually be the expression of a stage of self reproduction in bacteriophage multiplication.

It should be pointed out, however, that the type of DNA curve obtained may be affected by the heterogeneity of the population. In carrying out single burst experiments with *E. coli* K-12, WEIGLE AND DELBRUCK found a very large heterogeneity in the bacterial population with respect to the time required for the synthesis of mature phage[4]. More than 10 minutes separated the time of appearance of the first phage particles in a bacterium from the time where every bacteria contained at least one phage particle. Analogous results have been found with T4 phage by BENTZON, MAALOE AND RASCH[26].

It is possible that both these phenomena, that is, differences in times of development of bacteriophage in individual bacteria, and enzymic limitations, play some role in the DNA accumulation curves that have been found. However, these results emphasize the difficulty of interpreting the patterns of kinetic results in studying specific syntheses in bacterial populations. Until further techniques are elaborated, the form of a kinetic curve does not yield requisite information on the type of kinetics which is being followed. The type of curve one obtains is obviously a composite of processes within individual bacteria and renders interpretation difficult.

SUMMARY

1. The time elapsed between induction of *B. megatherium* 899(1) by irradiation with ultraviolet light, and formation of the first intra- and extra-cellular bacteriophages has been measured at 27 and 37° C. The latent period is prolonged by an increase in the inducing dose of ultraviolet light.

2. A study of the biochemical modifications occuring after induction of lysogenic *B. megatherium* has shown that, concomitant with the residual growth, respiration increases, and ribonucleic acid is synthesized. The synthesis of desoxyribonucleic acid is blocked during approximately the first third of the latent period. Rapid synthesis then commences and continues until the moment of lysis.

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3. The biochemical modifications during development of temperate phages in induced lysogenic bacteria have been compared with those found in bacteria infected with virulent phages, which block growth completely. Whereas growth, ribonucleic acid and enzyme synthesis does, or does not, occur, depending on the phage-host system studied, the pattern of desoxyribonucleic synthesis seems to be a common property of all phage-host systems examined heretofore.

RÉSUMÉ

1. Des mesures ont été faites à 27° et à 37° du temps écoulé entre l'irradiation de *B. megatherium* 899(1) par les rayons ultraviolets et l'apparition des bactériophages intra- et extra-bactériens. La durée de la période latente est prolongée lorsqu'on augmente la dose du rayonnement ultra-violet.

2. Après irradiation de *B. megatherium* lysogène, la respiration et la teneur en acide ribonucléique augmentent parallèlement à la courbe de croissance résiduelle. La formation d'acide désoxyribonucléique, par contre, est arrêtée pendant le premier tiers de la période latente. Une synthèse rapide reprend ensuite et se poursuit jusqu'au moment de la lyse.

3. Les modifications biochimiques qui se produisent pendant le développement d'un bactériophage tempéré chez les bactéries lysogènes induites, ont été comparées avec celles qui ont été décrites chez les bactéries infectées avec des bactériophages "virulents". Suivant le système considéré il y a, ou non, croissance bactérienne résiduelle, synthèse d'acide ribonucléique et d'enzymes, alors que la forme que revêt la courbe de synthèse de l'acide désoxyribonucléique est commune à tous les systèmes phages-bactéries étudiés jusqu'ici.

ZUSAMMENFASSUNG

1. Die Zeit, welche zwischen der Ultraviolett-Bestrahlung von *B. megatherium* 899(1) und dem Auftreten der ersten intra- und extrazellulären Bakteriophagen vergeht, wurde bei 27 und bei 37° gemessen. Die Dauer der latenten Periode nimmt zu, wenn die Bestrahlungs-Dosis erhöht wird.

2. Nach Bestrahlung von lysogenem *B. megatherium*, nimmt die Atmung und die Ribonukleinsäure-Synthese parallel mit dem Rest-Wachstum zu. Dagegen wird ungefähr während des ersten Drittels der latenten Periode keine Desoxyribonukleinsäure gebildet. Dann setzt eine rasche Synthese ein, welche bis zum Augenblick der Lyse fortdauert.

3. Die biochemischen Modifikationen, welche während der Entwicklung eines gemässigten Bakteriophagen in den induziert lysogenen Bakterien auftreten, wurden mit denjenigen verglichen, welche für mit virulenten Bakteriophagen infizierte Bakterien beschrieben worden sind. Letztere blockieren das Wachstum vollständig. Je nach dem untersuchten System, treten Bakterien-Wachstum, Ribonukleinsäure und Enzymsynthese wohl oder nicht auf; dagegen ist die Form der Kurve, welche die Desoxyribonukleinsäure-Synthese wiedergibt, für alle untersuchten Bakteriophag-Bakterien-Systeme dieselbe.

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