UNBALANCED GROWTH AND BACTERIAL DEATH IN THYMINE-DEFICIENT AND ULTRAVIOLET IRRADIATED *ESCHERICHIA COLI*[1]

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Cohen and Barner (1954) showed that a thymine-requiring strain of *Escherichia coli*, 15T⁻, progressively lost the capacity for multiplication when placed in a growth medium lacking thymine, and called such a phenomenon "thymineless death." They proposed the hypothesis that thymineless death was caused by unbalanced growth, i.e., a continued synthesis of bacterial protoplasm under conditions which inhibited the synthesis of deoxyribonucleic acid (DNA). Barner and Cohen (1956) further assumed that the unbalanced growth was also the cause of the secondary reduction of viable cell count of ultraviolet irradiated *E. coli* during the postirradiation incubation in a growth medium. In such bacteria, the synthesis of DNA is completely inhibited for a certain interval while the synthesis of ribonucleic acid (RNA) or protein proceeds without appreciable inhibition (Kelner, 1953; Kanazir and Errera, 1956; Harold and Ziporin, 1958; Okagaki, 1960). Therefore, the bacteria at this stage certainly show an unbalanced growth. However, Barner and Cohen (1956) did not analyze the chronological relationship of the secondary reduction of the viable cell count and the resumption of DNA synthesis in the irradiated bacteria.

In this paper we wish to present some evidence indicating that the unbalanced growth and the bacterial death, either in thymine-deficient *E. coli* 15T⁻ or in ultraviolet irradiated *E. coli* strains, are separable phenomena. A preliminary account of this work has already appeared (Okagaki, Kihara, and Sibatani, 1959).

EXPERIMENTAL METHODS

Bacterial strains. Escherichia coli, strains B, 15, 15T⁻ (Cohen) and 15T⁻ (Zamenhof) were used. The last named strain was probably the one called strain I by Zamenhof and Griboff (1954). All these strains were the same as employed by Okagaki (1959, 1960).

Growth and irradiation of bacteria. The growth media and procedures for growth experiments were much the same as those reported by Okagaki (1960). Usually a basal medium (tris (tris(hydroxymethyl)aminomethane)-glucose medium of Okagaki (1960)) and casamino acid medium (tris-glucose-casamino acid medium of Okagaki (1960)) were employed. Unless otherwise specified, bacteria were grown in 30 ml of casamino acid medium, 10 μg/ml thymine being added for the thymine-requiring strains, under constant aeration for 14 to 16 hr at 37 C and harvested by centrifuging. The bacteria were washed once with 0.1 M phosphate buffer or buffered saline (pH 7.0), resuspended in appropriate growth media to give a density of about 3×10^8 cells/ml, and subjected to "preincubation" for 3 to 6 hr until the late logarithmic phase was reached, when the turbidity was 80 to 90 per cent of the maximal attainable value and the viable count was 2 to 3×10^9 cells/ml. The turbidity was measured at 570 mμ by a Tokyo Photoelectric Laboratory model IV spectrophotometer. The strains 15T⁻ (Cohen) and 15T⁻ (Zamenhof) were "preincubated" with the addition of 2 μg/ml thymine, which was more than sufficient to support the bacterial growth under the conditions described. As mentioned above, however, a higher concentration of thymine was used in the first overnight subculture, in order to prevent possible thymineless death. In experiments in which the effects of casamino acid were examined, the preincubation was modified in various ways as will be described later. Then the centrifuged bacteria were washed as above and served as the material for ultraviolet irradiation or thymine-deficient incubation.

The ultraviolet irradiation was conducted exactly as described by Okagaki (1960). The irradiated or nonirradiated bacteria (3×10^9 cells/ml of 0.1 M phosphate buffer) were diluted

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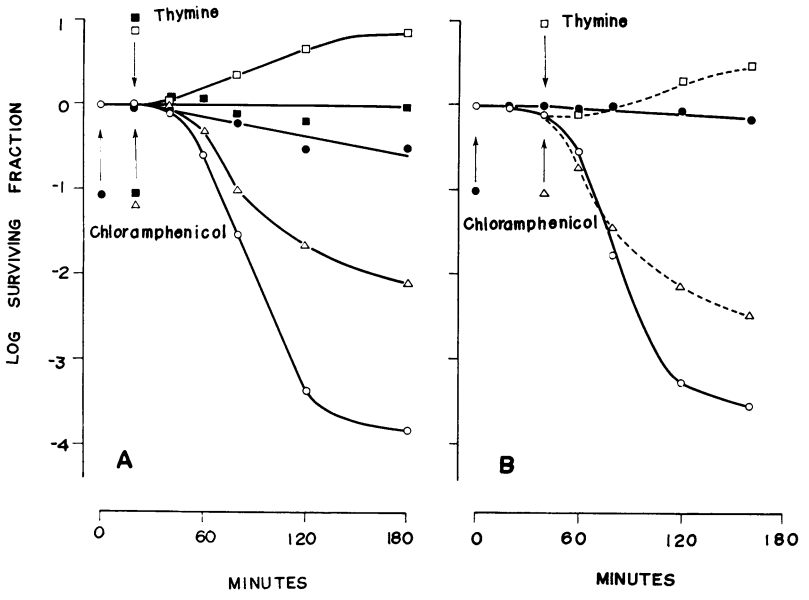

Figure 1. Effects of chloramphenicol or thymine on thymineless death of *Escherichia coli* 15T⁻ (Cohen) *A*. Bacteria preincubated in casamino acid medium with thymine (2 μg/ml) were shaken in the fresh casamino acid medium without thymine (○); 100 μg/ml chloramphenicol were added at 0 min (●): at 20 min were added: 2 μg/ml thymine (□), thymine + chloramphenicol (■) or chloramphenicol alone (△). *B*. In the same medium (○); chloramphenicol was added at 0 min (●); thymine (□) or chloramphenicol (△) was added at 40 min. Concentration of thymine and chloramphenicol was the same in all cases.

with 10 volumes of experimental medium and incubated at 37 C with constant aeration. Samples were withdrawn at intervals for measurements of viable cell count, optical density, or biochemical analysis. Necessary precautions were observed to avoid the photoreactivation in all the manipulations following the ultraviolet irradiation.

Analytical methods. These were exactly the same and the results presented generally in the same way as in a previous report (Okagaki, 1960). The turbidity and DNA content were expressed in per cent of the initial value (determined at the end of preincubation or immediately following irradiation), and plotted on the logarithmic scale in text figures.

RESULTS

Effects of chloramphenicol on thymineless death. With both thymine-requiring strains, 15T⁻ (Cohen) and 15T⁻ (Zamenhof), the typical "thymineless death" as observed by Cohen and Barner (1954) could be demonstrated. To block the protein synthesis, we used chloramphenicol at a concentration of 100 μg/ml. There was an immediate cessation of the increase in protein content of the culture when the antibiotic was added at this concentration. When chloramphenicol was added at the outset of thymine-deficient incubation of 15T⁻ (Cohen), the decrease in viable count or the "death" of bacteria[3] occurred extremely slowly (figure 1). This fact suggests that the imbalance between the DNA and protein syntheses might well be the cause of thymineless death, whereas the imbalance between DNA and RNA syntheses had no significant effect

[3] The objective of this paper is to present evidence against the theory of Cohen and Barner (1954) of the "lethal" effect of unbalanced growth. The only observed properties of the bacteria concerning their viability was the loss of their capacity to multiply on the nutrient agar. The mechanism of such an event and its relation to bacterial death are out of the scope of the present paper. For this reason, the words "death," "die," "lethal," etc. were used in this paper to refer simply to the events which result in the failure of the bacterium to form a visible colony on the nutrient agar.

upon the survival of the bacterium, for chloramphenicol at this concentration allowed the syntheses of nucleic acids to continue, although at a reduced rate.

If thymineless death were caused by the imbalance between DNA and protein syntheses, either the resumption of DNA synthesis or the complete inhibition of protein synthesis would equally be expected to prevent the thymine-deficient bacteria from dying. In experiments illustrated in figure 1, addition of thymine (2 μg/ml) 20 or 40 min after the start of a thymine-deficient incubation of 15T$^-$ (Cohen) immediately gave rise to an increase of viable cells. In contrast, addition of chloramphenicol at the 20th or 40th min of incubation without thymine failed to obviate the killing effect of thymine deficiency. The earlier addition of chloramphenicol was apparently more effective in preventing thymineless death. But even the addition of the antibiotic at the 40th min was effective in reducing the lethal effect of thymine deficiency during the later phase of thymine-deficient incubation. It should be noted here that practically all the bacteria had survived the unbalanced growth lasting 20 or 40 min, and yet the prevention of unbalanced growth at these stages by inhibiting protein synthesis failed to rescue the bacteria from dying in the thymine-deficient medium during a period of 100 min which followed. However, an addition of both thymine and chloramphenicol 20 min after the start of thymine-deficient incubation completely eliminated the killing effect.

Effects of amino acids on thymineless death. In experiments shown in figure 2, bacteria of the strain 15T$^-$ (Cohen) grown without contact with amino acids were preincubated for 40 min with or without casamino acid, and then transferred to a thymine-deficient basal medium. The bacteria exposed to casamino acid showed typical thymineless death, while those maintained free of casamino acid showed a reduced rate of thymineless death from 1 hr after the start of thymine-deficient incubation onward. The increase in turbidity was a little higher under the latter conditions, so that the reduction of the rate of thymineless death could not be correlated with the reduction of the growth rate. Simultaneous addition of chloramphenicol and casamino acid

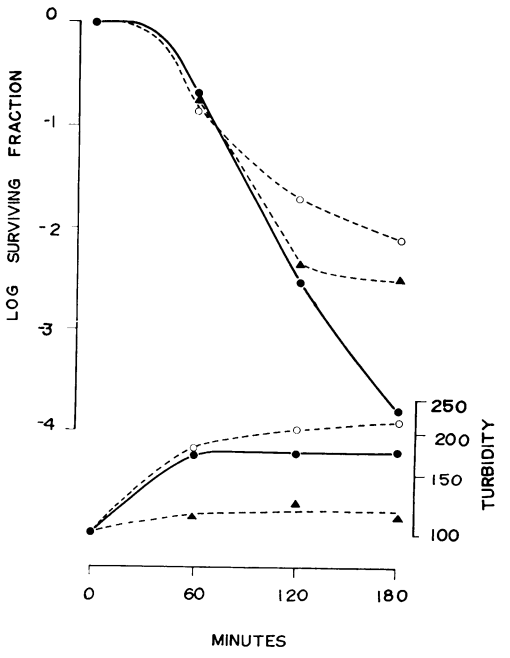

Figure 2. Effect of preincubation with casamino acid on thymineless death and on turbidity increase of *Escherichia coli* 15T$^-$ (Cohen) in the basal medium. The bacteria were grown overnight in the basal medium with 10 μg/ml thymine. The harvested bacteria were incubated (3×10^8 cells/ml) in the basal medium with 2 μg/ml thymine for 5 hr. The bacteria in the late logarithmic phase were harvested and shaken for additional 40 min in the basal medium containing either 2 μg/ml thymine (○), thymine + 0.1 per cent casamino acid (●), or thymine + casamino acid + 100 μg/ml chloramphenicol (▲). They were then centrifuged and placed in a thymine-deficient basal medium.

to the preincubation medium scarcely reduced the effect of amino acids.[4]

Figure 3 represents an experiment of reversed type. Bacteria of the same strain grown in casamino acid-thymine medium were preincubated for different intervals in a basal medium containing thymine. The effect of casamino acid on the rate of thymineless death was maintained in

[4] The reason that in this case the turbidity did not significantly increase during thymine-deficient incubation is not clear. In other experiments conducted under similar conditions, such growth inhibition was not observed. However, this single instance illustrated in figure 2 would suggest that typical thymineless death can occur without appreciable synthesis of bacterial protoplasm.

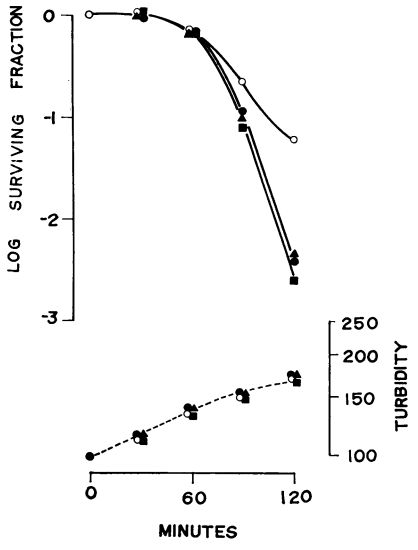

Figure 3. The loss of the effect of casamino acid during prolonged preincubation as detected by the rate of subsequent thymineless death in *Escherichia coli* 15T⁻ (Cohen). The bacteria in the late logarithmic phase grown in the casamino acid medium containing 2 μg/ml thymine were placed in the basal medium with 2 μg/ml thymine and incubated for 40 min (●), 2 hr (▲), or 4 hr (○). Then a portion of 4-hr culture was centrifuged, and the bacteria were further incubated in the basal medium containing both 2 μg per ml thymine and casamino acid for an additional 40 min (■). All these bacteria were washed and incubated in the basal medium.

a casamino acid-free medium for 2 hr, but it was largely lost by the 4th hr, so that the bacteria were killed in basal medium at a reduced rate. When such resistant bacteria were again incubated in casamino acid medium for 40 min, they showed the previous high sensitivity to the lethal action of thymine deficiency. It should be stressed here that the change in the death rate in these experiments was not accompanied by any significant difference in the growth rate.

Casamino acid was also effective in enhancing thymineless death even when added to the thymine-deficient medium instead of to the preincubation medium. This can be seen from figure 4. In this experiment, growth of bacteria in thymine-deficient casamino acid medium was somewhat enhanced, but growth in the medium lacking both thymine and casamino acid also proceeded without appreciable inhibition and eventually reached the same level as that in the medium containing casamino acid.

Thus, the contact with amino acids of the bacteria of the strain 15T⁻ (Cohen) either before or during thymine-deficient incubation seems to be a favorable condition for the full development of thymineless death. But such an enhancement of thymineless death, or its suppression in bacteria maintained in media free of casamino acid, cannot be correlated with alteration of the growth rate. However, it has so far not been possible to prevent the thymineless death completely by the modification of the nutritional

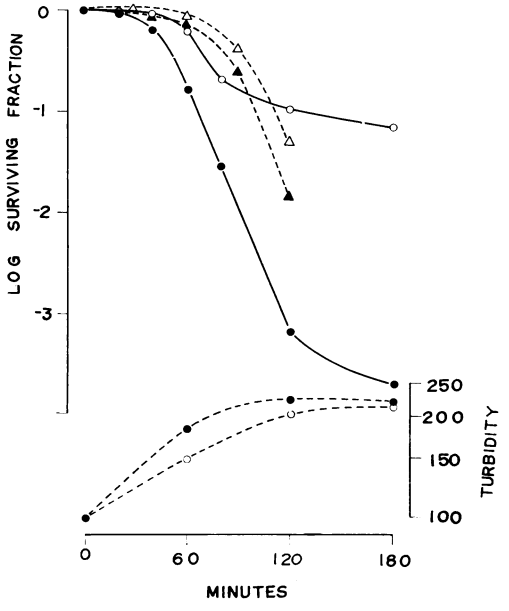

Figure 4. The effect of casamino acid added to the thymine-deficient medium on thymineless death and on the turbidity of *Escherichia coli* 15T⁻ (Cohen), as compared with the effect of preincubation with or without casamino acid on subsequent thymineless death in *Escherichia coli* 15T⁻ (Zamenhof). Bacteria of 15T⁻ (Cohen) were grown in the basal medium with 2 μg/ml thymine for 4.5 hr and then incubated in thymine-deficient basal (○) or casamino acid (●) medium. Bacteria of 15T⁻ (Zamenhof) were preincubated in two ways: in the basal medium with 2 μg/ml thymine for 5 hr (△); in the casamino acid medium with 2 μg/ml thymine for 3 hr (▲); and then they were transferred to the basal medium with 2 μg/ml thymine and incubated for only 40 min. They were centrifuged and incubated in the basal medium for the period indicated. Note the different experimental procedures for the two strains.

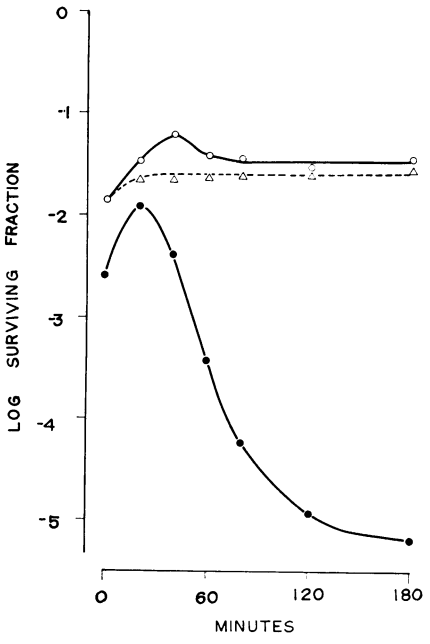

Figure 5. Effect of preincubation without casamino acid and of ultraviolet irradiation on subsequent thymineless death of *Escherichia coli* 15T⁻ (Cohen). The viable count before the irradiation is taken as unity. The bacteria were preincubated either in the casamino acid medium with 2 μg/ml thymine for 3 hr (●), or in the basal medium with thymine for 5 hr (○). They were then harvested, washed, ultraviolet irradiated for 100 sec (at 1 m from a 15 W Toshiba (Mazda) germicidal lamp), and finally placed in the basal medium. A portion of the bacteria preincubated without casamino acid was also placed in the basal medium containing 100 μg/ml chloramphenicol (△).

conditions alone, even after a prolonged incubation in the basal medium as shown in figure 2.

The thymineless death of strain 15T⁻ (Zamenhof) was found to be less sensitive to the nutritional conditions. As shown in figure 4, the trend of thymineless death of this strain was more or less comparable after preincubating without casamino acid for either 40 min or 5 hr, although the death rate was somewhat lower in the latter case.

Effects of ultraviolet irradiation. As can be seen from figure 5, the bacteria of 15T⁻ (Cohen) which had been preincubated with casamino acid and had survived ultraviolet irradiation showed a typical curve of thymineless death, whereas survivors among those preincubated for 5 hr without casamino acid and irradiated under similar conditions did not die in the basal medium without thymine during a period of 3 hr. Naturally the latter bacteria did not proliferate in the thymine-deficient medium in this experiment. A small peak of viable count at the beginning of thymineless growth was due to recovery from the lethal effect of ultraviolet irradiation (Cohen and Barner, 1954; Barner and Cohen, 1956; Okagaki, 1960). Such bacteria also failed to show chloramphenicol death of ultraviolet irradiated bacteria, as pointed out by Okagaki (1960) and illustrated in figure 5 for comparison. Incidentally, in the experiment shown in figure 5, the survival of casamino acid-pretreated bacteria following irradiation was 0.2 per cent, whereas that of untreated cells was 1.5 per cent. Since exactly the same trend was repeatedly noticed in a series of similar experiments, it appears that the radiosensitivity of the bacteria is slightly enhanced by preincubating them with amino acids. In a duplicate experiment giving similar results, it was ascertained that such a vast change as noted in figure 5 in the development of thymineless death of irradiated bacteria was not accompanied by any significant difference in turbidity increase. In the presence of thymine also, the preincubation with casamino acid did not affect the rate of increase in viable count of ultraviolet irradiated bacteria.

It can thus be concluded that, in bacteria of strain 15T⁻ (Cohen) maintained in media free of casamino acid before and during thymine deficiency, ultraviolet irradiation may completely abolish the lethal effect of thymine deficiency upon the bacteria surviving irradiation, and that the exposure of such bacteria to casamino acid during the preincubation effectively induced thymineless death. As in the unirradiated bacteria of this strain, the contact with casamino acid during thymine-deficient incubation was also effective in inducing thymineless death. This is illustrated in figure 6. Again, there was no significant change in the rate of turbidity increase whether the capacity for multiplication of bacteria was greatly decreased or completely maintained.[5] A similar experiment was conducted

[5] In a few experiments, ultraviolet irradiated 15T⁻ (Cohen) maintained in casamino acid-free media showed thymineless death to some extent (about 10 per cent survival) after 3 hr, but it could not be correlated with the difference in

with strain 15T⁻ (Zamenhof). In this strain, thymineless death after ultraviolet irradiation could not be prevented by maintaining the bacteria in a casamino acid-free medium (figure 6). However, survival following irradiation was unusually low in this particular experiment, so that any definite conclusion about the possible difference between the two strains must await further confirmation to be made at similar survival levels.

The effect of casamino acid on the sensitivity of strain 15T⁻ (Cohen) to the lethal action of thymine deficiency was most pronounced when it was added to the ultraviolet irradiated bacteria. This system seemed, therefore, to be convenient for tracing the active entities in casamino acid and for testing the effect of some other compounds. DL-Alanine, DL-valine, L-leucine, DL-serine, L-methionine, DL-asparagine, L-glutamic acid, L-lysine, L-arginine, L-phenylalanine, and L-tryptophan were tested under the same conditions as given in figure 6, and the viable count after 3-hr thymine-deficient incubation was determined; all the compounds, at a concentration of 1 mM, were effective in promoting thymineless death. Moreover, 1 mM cytosine or α-ketoglutaric acid also enhanced death, whereas the effects of some other amino acids and nucleic acid bases have so far not been conclusive. The individual compounds at the concentration tested were always less effective than 0.1 per cent casamino acid. However, the effect of casamino acid did not significantly vary between a concentration range of 0.1 to 10 mg/ml.

Period of unbalanced growth in ultraviolet irradiated E. coli. We followed the change in viability and the course of DNA synthesis in ultraviolet irradiated *E. coli* strain 15 shaken in the basal medium. As was expected, there was no marked inhibition of RNA and protein syntheses in these bacteria. Typical results are shown in figure 7. In this experiment, the bacteria in the late logarithmic phase were irradiated with two different doses of ultraviolet light, yielding 35[6] and 0.25 per cent survival. In agreement with the observations of Barner and Cohen (1956), there was a several-fold increase in viable count within 20 to 40 min which indicated restoration of the bacteria initially killed by the irradiation and did not represent cell multiplication. Upon further incubation in the same medium, the revived bacterial population again started to lose the capacity for multiplication. This is what we call in this paper "secondary death." It was this phase of death which Cohen and Barner (1954) and Barner and Cohen (1956) considered to be caused by unbalanced growth. The maximal point of viable count, between the phase of

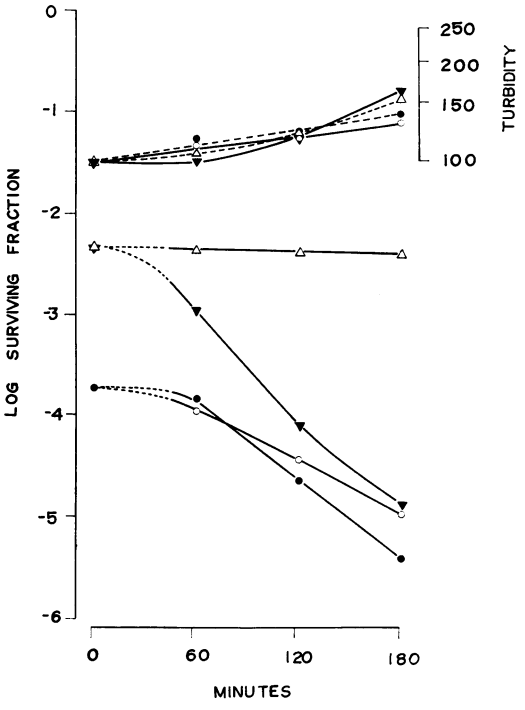

Figure 6. Effect of casamino acid added to the thymine-deficient medium on thymineless death in ultraviolet irradiated *Escherichia coli* 15T⁻ (Cohen) and 15T⁻ (Zamenhof). Preincubation in the basal medium containing 2 μg/ml thymine for 5 hr. Ultraviolet irradiation as in figure 5 for 100 sec. The bacteria of 15T⁻ (Cohen) were placed in the basal (△) or casamino acid (▼) medium; the bacteria of 15T⁻ (Zamenhof) were likewise placed in the basal (○) or casamino acid (●) medium

survival level following irradiation. The reason for such a fluctuation of results is not clear.

[6] Probably some restoration occurred in this case between the termination of irradiation and sampling (the necessary manipulation took about 5 min), since the subsequent growth curve extrapolated back to the point of about 10 per cent survival. In other experiments this usually coincided with the initial survival as Barner and Cohen (1956) had shown.

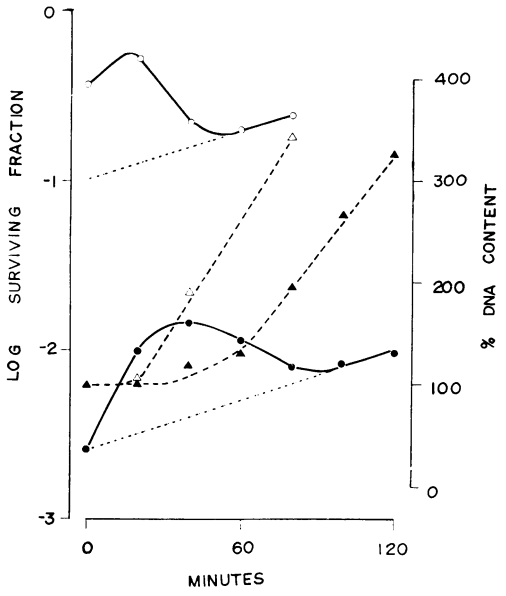

Figure 7. Change in viable cell count and synthesis of DNA in ultraviolet irradiated *Escherichia coli* 15. Bacteria were irradiated as in figure 5 for 30 (*open markings*) or 100 (*solid markings*) sec, and incubated in the basal medium. *Circles* for viable count and *triangles* for relative DNA content of the culture. The absolute amount of DNA-P and RNA-P per initial unirradiated bacterium as estimated from the viable count was 0.45×10^{-9} and 2.55×10^{-9} μg, respectively.

restoration and that of secondary death, occurred earlier and the rate of secondary death was higher with the smaller dose of ultraviolet light. It may also be seen from figure 7 that the inhibition of DNA synthesis lasted longer with the higher dose of ultraviolet light. This last point confirms the observations of Kanazir and Errera (1956).

There is no evidence indicating that secondary death begins earlier than the time when the viable cell count reaches its maximum but is masked by the restoration (Barner and Cohen, 1956). It is, therefore, not reasonable to assume that the onset of secondary death did not coincide with the peak of the survival curve. It should now be noted in figure 7 that DNA synthesis resumed at about the same time as the viable count began to decline. Consequently, there was an active synthesis of DNA while the restored bacteria were dying. This observation clearly speaks against the hypothesis that secondary death is caused by imbalance between the syntheses of nuclear and cytoplasmic material. This death could not be the consequence of unbalanced growth which had occurred prior to the resumption of DNA synthesis in the irradiated bacteria, because in the thymine-deficient culture of 15T$^-$ (Cohen) the addition of thymine and hence the resumption of DNA synthesis prevented, without delay, the bacteria from dying (figure 1), showing that unbalanced growth had no delayed effect on the viability of bacteria once the DNA synthesis was resumed. Essentially similar observations were made with *E. coli* strain B, although in this strain the occurrence of secondary death was far less clear-cut, as was already noted by Barner and Cohen (1956) and Okagaki (1960).

DISCUSSION

The effect of inhibition of protein synthesis upon the survival of thymine-deficient *E. coli* 15T$^-$ was studied by Barner and Cohen (1957) who used some amino acid-requiring mutants of this strain. The deficiency of the required amino acid led to the reduced rate of thymineless death but failed to abolish it completely. It is likely that in these cases the block of protein synthesis was not complete. The over-all results of Barner and Cohen (1957) were similar to those of our experiments in which protein synthesis was blocked by chloramphenicol some time after the start of incubation without thymine.[7] When chloramphenicol was added at the outset of incubation without thymine, thymineless death was completely prevented, as shown by Billen (1959) and by our own experiments.

From the results illustrated in figure 1, it may be assumed that the absence of thymine rather than the imbalance between protein and DNA syntheses caused the decrease in viable count of thymine-requiring *E. coli*. The thymine deficiency seems to require some protein synthesis in order to develop its bactericidal effect. Probably the earlier cessation of protein synthesis due to the addition of chloramphenicol at the 20th and 40th min of thymine-deficient incubation reduced the lethal effect of thymine deficiency in a much later phase of incubation. In view of these findings, one of the necessary conditions for thy-

[7] After completion of this manuscript, we were notified that essentially similar observations have just been reported by Galland and Suskind (Bacteriol. Proc., **1960,** 178) who seem to have reached similar conclusions regarding thymineless death.

mineless death may be formulated as a limited synthesis of protein in the absence of thymine followed by the persistence of thymine deficiency irrespective of the presence or absence of the protein synthesis.[8]

The effects of amino acids and of ultraviolet irradiation on thymineless death are more difficult to interpret. Concerning this point, there are certain similarities between thymineless death and "ultraviolet-chloramphenicol death" as described by Okagaki (1960), but they may be distinguished in several respects. The possible relationship between the two deaths remains completely obscure. It should only be mentioned here that strain 15T⁻ (Cohen), unlike 15T⁻ (Zamenhof), can be made resistant to either death by treating the bacteria with amino acids under appropriate conditions.

In any case, evidence presented in this paper will be sufficient for excluding the possibility that unbalanced growth *per se* is the cause of thymineless death, because either one may occur independently of the other. However, it is difficult to analyze further the necessary conditions for thymineless death from the results presented here. The mechanism of thymineless death may very well involve entities other than DNA. Indeed, the occurrence of several thymidine diphosphate sugar compounds in *E. coli* has recently been reported by Okazaki, Okazaki, and Kuriki (1960) and by Strominger and Scott (1959). Such compounds may participate in cell wall formation, and thymineless death may be the consequence of an imbalance related to the synthesis of cell wall.

According to the original concept of Barner and Cohen (1956), bacterial death by unbalanced growth after ultraviolet irradiation related only to secondary death, i.e., the secondary reduction of viability during postirradiation incubation, but not to the initial reduction of viable cells observed immediately after ultraviolet irradiation. They assumed, on the basis of the following observations, that secondary death was caused by unbalanced growth: the rate of secondary death was that of thymineless death; and secondary death was inhibited if metabolism and cell growth were inhibited. This latter observation was confirmed and extended by Okagaki (1959). However, unbalanced growth, as indicated by complete inhibition of DNA synthesis, ought to occur to practically the whole population of irradiated bacteria, whereas secondary death seems to attack only those cells which have recovered from the initial lethal effect of ultraviolet light. This occurs because the exponential increase in viable count of the irradiated bacteria which had survived initial and secondary death usually extrapolated back to the point of survival determined immediately after ultraviolet irradiation in both Barner and Cohen's (1956) and our own experiments. Furthermore, the rate of secondary death is lowered by prolongation of the period of ultraviolet-induced unbalanced growth. Also, secondary death cannot be prevented by the resumption of DNA synthesis, but rather the former begins concomitant with the latter. None of these features of secondary death supports the theory that it is caused by unbalanced growth.

Harold and Ziporin (1958) presented ample evidence for excluding the possibility of unbalanced growth as the basis of bactericidal action of mustards, the effect of which on DNA synthesis in *E. coli* closely parallels that of ultraviolet irradiation. Stuy (1958) also reported that vegetative cells of *Bacillus cereus* were killed by ultraviolet irradiation without undergoing unbalanced growth. Their conclusions are not at all surprising, because they were dealing with only initial death, or at least could not discriminate initial death from secondary death because of the nature of the agent used or the technique employed, while secondary death itself has now been proved to be independent of unbalanced

[8] If unbalanced growth is defined as the imbalance between the syntheses of DNA and protein, such a formulation certainly implies that limited initial unbalanced growth is a prerequisite for thymineless death. However, it is shown in this paper that such unbalanced growth alone can not cause bacterial death. Of course, it is difficult at the moment to decide whether limited protein synthesis without thymine rather than initial slight unbalanced growth (irrespective of thymine deficiency) is responsible for the later development of thymineless death. However, unbalanced growth as defined above and thymineless death can be separated partially or entirely under various experimental conditions, so that it would be much more valuable to formulate some of the necessary conditions for thymineless death as above and avoid the use of the rather ambiguous expression "unbalanced growth."

growth. Recently Alper and Gillies (1960) suggested that in *E. coli* B the initial lethal action of ultraviolet irradiation which can be restored by agents inhibiting certain metabolic processes and slowing down the growth is due to some lethal sort of unbalanced growth. That conditions affecting the metabolism or growth may enhance the restoration was also demonstrated by Barner and Cohen (1956) and Okagaki (1959, 1960). However, the initial lethal effect cannot be correlated with unbalanced growth without DNA synthesis, because the prevention of protein synthesis by chloramphenicol or in phosphate buffer-glucose medium at the time when DNA synthesis is resumed also effects a considerable recovery of *E. coli* B (Okagaki, 1959, 1960; Okagaki and Sibatani, 1960). Also, Doudney (1959) has recently suggested that restoration is related to partial prevention of unbalanced growth. But experiments of Gillies and Alper (1959), Alper and Gillies (1960), Okagaki (1960), and Okagaki and Sibatani (1960) do not support such a view.

Kanazir and Errera (1959) showed that a marked decrease in adenosine triphosphate (ATP) content takes place while the DNA synthesis is just being resumed in ultraviolet irradiated *E. coli* B. This may be related to the development of secondary death. Indeed, Okagaki (1959) found that secondary death was prevented to some extent by ATP added to the culture of *E. coli* strain 15 when the bacteria were just starting to die by secondary death. ATP added to the growth medium also acted to enhance restoration (Okagaki, 1959). However, restoration can proceed even without external energy supply (Barner and Cohen, 1956). The whole situation involving restoration and secondary death is thus complicated, and the elucidation of the underlying mechanism must await further investigation.

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SUMMARY

Bacteria of *Escherichia coli* strain 15T$^-$ (Cohen) were treated with chloramphenicol or casamino acid either before or during thymine-deficient incubation, or they were irradiated with ultraviolet light immediately before the start of incubation without thymine. By such treatments it was possible to dissociate thymineless death, i.e., the decrease in viable count of thymine-requiring bacteria grown in the absence of thymine, and unbalanced growth, i.e., the increase in protoplasmic mass of bacteria without the synthesis of DNA, which is realized in thymine-requiring bacteria by the deprivation of thymine. Thus, unbalanced growth could be impaired or completely abolished without greatly affecting thymineless death and *vice versa*.

In ultraviolet irradiated bacteria of *E. coli* strain 15, the decline of the viable count, or secondary death, which followed the initial restoration of irradiated bacteria on incubation in a liquid growth medium, set in at the time when ultraviolet-inhibited deoxyribonucleic acid synthesis was resumed, namely when unbalanced growth terminated.

These observations appear to invalidate the idea that unbalanced growth *per se* has any lethal effect. They would therefore indicate that neither thymineless death nor secondary death of ultraviolet irradiated *E. coli* is the direct consequence of unbalanced growth.

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