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Thymineless Death in *Escherichia coli*: Strain Specificity

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Thymineless death of various ultraviolet (UV)-sensitive strains of *Escherichia coli* B and K-12 was investigated. It was found that *E. coli* B, B_{s-12}, K-12 *rec-21*, and possibly K-12 Lon$^-$, all sensitive to UV, were also sensitive to thymine starvation. However, other UV-sensitive strains of *E. coli* were found to display the typical resistant-type kinetics of thymineless death. The correlation of these results with various other cellular processes suggested that the filament-forming ability of the bacteria might be involved in the mechanism of thymineless death. It was apparent from the present results that capacity for host-cell reactivation, recombination ability, thymine dimer excision, and probably induction of a defective prophage had little to do with determining sensitivity to thymine deprivation.

Recently, Cummings and Taylor (5) reported that *Escherichia coli* B, in addition to being sensitive to ultraviolet (UV) irradiation and to mitomycin C (MC) treatment, also displayed an unusual sensitivity to thymine deprivation. Upon being deprived of thymine, *E. coli* B immediately began losing its ability to form colonies and was termed *sensitive* to thymine starvation. In contrast, thymine-requiring derivatives of *E. coli* strains such as B/r, 15 (4, 13), and K-12, were *resistant*, in that all exhibited a lag of about 50 min prior to the onset of thymineless death. Some other UV-sensitive strains of *E. coli* were also examined, and while these strains were sensitive to UV and to MC, their response to thymine deprivation was essentially identical to that of the resistant strains. Similarly, Mennigmann (24) found that *E. coli* B, but not some other UV-sensitive strains, was much more sensitive to 5-fluorodeoxyuridine (FUDR) than was *E. coli* B/r. From these results, it was apparent that sensitivity to one or more lethal agents did not necessarily correlate with sensitivity to thymine deprivation. Of the sensitive strains investigated by Cummings and Taylor (5), one was deficient in its ability to undergo recombination (3), and another was unable to excise thymine dimers (15). It was concluded, therefore (5), that a single defect in deoxyribonucleic acid (DNA) repair (1, 31, 32; B. H. Rosenberg and D. E. Packer, Abstr. Biophys. Soc., p. 39, 1967) could not satisfactorily account for both sensitivity to thymineless death and sensitivity to either UV or MC.

An important result of these earlier studies was that two different types of response to thymine deprivation were known: sensitive and resistant. Aside from the influence of this finding in understanding the phenomenon of thymineless death, it is necessary to determine whether *E. coli* B is unique in its response to thymine deprivation. Hill and Feiner (14) presented in detail the properties of some UV-sensitive mutants of *E. coli* B, and Howard-Flanders (1, 15, 16; P. Howard-Flanders and A. J. Clark, *personal communication*) has described many different UV-sensitive mutants of *E. coli* K-12, including a recombination-deficient mutant basically different from those mutants previously observed (2, 3). A number of these bacterial strains have been characterized with regard to their response to thymine starvation, and this communication will present evidence that *E. coli* B is one of many strains sensitive to thymine starvation.

MATERIALS AND METHODS

Bacterial strains. Thymine-requiring strains of *E. coli* were obtained by use of the aminopterin selection technique (27, 36). In addition to the recombination deficient (Rec$^-$) strain studied previously (JC1569 *rec-1* and its parent JC1557, Rec$^+$; 3, 5), a second Rec$^-$ strain, AB2470 *rec-21*, was obtained from P. Howard-Flanders. This Rec$^-$ strain differs physiologically from JC1569 in that it does not degrade its DNA during growth (2; P. Howard-Flanders and A. J. Clark, *personal communication*). P. Howard-Flanders also supplied five other UV-sensitive strains of *E. coli* K-12: AB1899 and 2426, each Lon$^-$ (16); AB1884, *uvrC;* AB1885, *uvrB;* and AB1886, *uvrA*. Each of the last three UV-sensitive strains is unable to repair UV-irradiated T1 bacteriophage and fails to excise thymine dimers (15). The wild-type Rec$^+$

strain (AB1157) from which these strains were derived was also obtained from P. Howard-Flanders. The UV-sensitive mutants of *E. coli* B (14), B_{s-1}, B_{s-2}, B_{s-3}, B_{s-4}, B_{s-8}, B_{s-11}, and B_{s-12}, as well as the parent strain *E. coli* B, were obtained from R. F. Hill and R. B. Setlow. These bacterial strains were grown aerobically in a glucose-phosphate salts minimal medium at 37 C with required supplements. Changes of medium were achieved by 800-fold dilution into glucose-phosphate salts containing no thymine. For uniform results, thymineless death characteristics of all the bacterial strains examined were obtained in the presence of 1% Casamino Acids (Difco). In some cases, the lag in thymineless death (especially for the Lon^- strains) was often prolonged in the absence of Casamino Acids. This was probably because growth conditions were better in the presence of Casamino Acids or because intracellular pools were more efficiently depleted, as was suggested by Freifelder (10), who used nucleoribosides, in particular uridine, to decrease the lag time. In our hands, the addition of nucleoribosides did not facilitate thymineless death as well as did Casamino Acids. Samples were taken at intervals and assayed for viable cells on tryptone (Difco)-agar plates supplemented with 30 μg of thymine per ml.

UV irradiation was carried out as previously described (5).

RESULTS

Strain specificity. Hill and Feiner compared the properties of some UV-sensitive mutants of *E. coli* B and found that of 12 such mutants there were 9 different phenotypes (14). Some were more UV-sensitive than the parent *E. coli* B, and most had lost their predilection toward filament formation after UV irradiation. Figure 1 illustrates the thymineless death characteristics of seven of these UV-sensitive mutants of *E. coli* B. As can be seen, *E. coli* B and B_{s-12} have essentially the same sensitivity to thymine deprivation in that the loss of colony-forming ability commences almost immediately. The results obtained here on the Hill strain B were the same as those obtained previously (5) on an *E. coli* B obtained from S. Luria. Except for *E. coli* B_{s-4}, all of the UV-sensitive mutants of strain B responded to thymine deprivation only after a lag of 38 to 55 min. This variability in the lag time may reflect a graduated resistance to thymine starvation or may result from differences in the rates of depletion of thymine from intracellular pools. The possibility that these strains were "leaky" in their requirement for thymine was excluded on the basis that only stringent colonies were isolated and that "leaky" mutants would most likely lead to an increase in the ultimate surviving fraction rather than a prolonged lag time. It should be emphasised that all these B_s strains were directly isolated from *E. coli* after

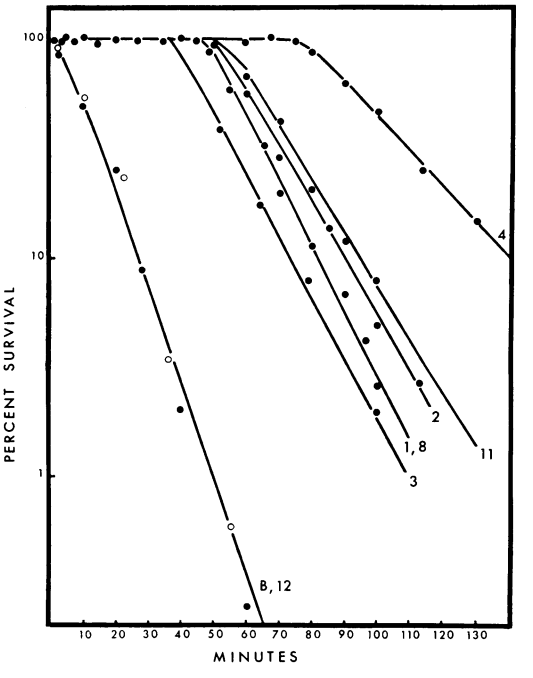

FIG. 1. *Thymineless death of Escherichia coli* B_s. *The open circles refer to data obtained with the parent B from which all the* B_s *strains (closed circles) were derived. As can be noted, only E. coli B and* B_{s-12} *responded to thymine deprivation in a sensitive manner. Strains* B_{s-1}, B_{s-2}, B_{s-3}, B_{s-8}, B_{s-11} *responded in essentially the same resistant manner as did B/r (5). Strain* B_{s-4} *was consistently the least sensitive strain with respect both to the initial lag and to the ultimate rate of thymineless death.*

UV irradiation and all were more UV-sensitive than the parent strain B (14). Of these mutants, only B_{s-3} and B_{s-12} retained the propensity of strain B to form filaments after UV irradiation, only B_{s-11} had the same sensitivity as its parent B to crystal violet, and strains B_{s-1}, B_{s-3}, B_{s-8}, and B_{s-12} no longer had the ability to repair UV-treated T1 bacteriophage (14). To minimize the possibility that particular thymine mutants were examined here, several single-colony isolates from separate aminopterin selections were studied; all had the same UV sensitivity as the original thymine-nonrequiring bacterium, and all displayed the same response to thymine deprivation. It should be noted that, although all the results reported here were obtained with the so-called high-thymine (20 μg/ml) requiring strains (27), the same results were also observed with low-thymine (2 μg/ml) requiring strains of *E. coli* B, B_{s-1} and B_{s-11}.

In a similar manner, various UV-sensitive strains of *E. coli* K-12 were compared with wild-

type K-12. It was found (Fig. 2) that *uvrA*, *B*, or *C* mutants had the same thymineless death characteristics as did the UV-resistant wild-type parent strain. All these strains lost their colony-forming ability as a result of thymine deprivation only after a lag of about 55 min. This behavior was essentially the same as that obtained previously with *E. coli* K-12 *rec-1* (5). In contrast, *E. coli* K-12 *rec-21* proved to be sensitive to thymine deprivation; this was the only UV-sensitive K-12 strain examined which responded to thymine starvation in a manner similar to *E. coli* B and B_{s-12}. Two Lon^- strains were studied [each isolated by UV irradiation from AB1157 (16)], and both showed intermediate sensitivities to thymine starvation by responding after a lag of about 20 min. In the absence of Casamino Acids, this lag was extended to about 32 min. It may well be that these Lon^- strains are actually more sensitive to thymine deprivation than it would appear. A distinguishing characteristic of Lon^- strains is

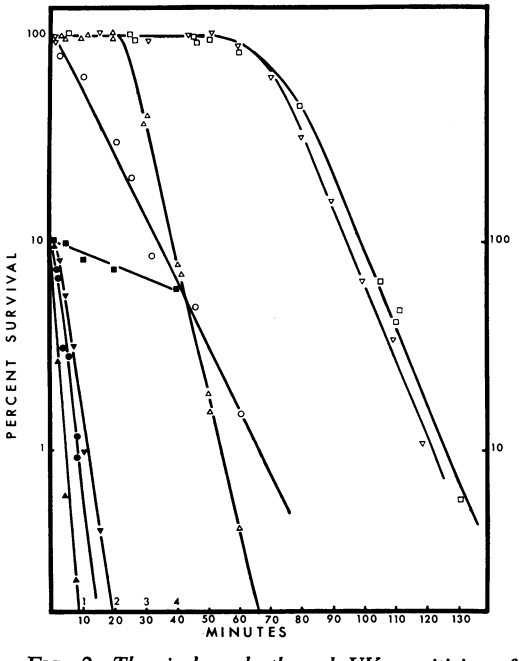

FIG. 2. *Thymineless death and UV sensitivity of Escherichia coli K-12. Closed symbols refer to UV (abscissa 0-4 min; right side ordinate) and open symbols refer to thymineless death kinetics (abscissa 0-130 min; left side ordinate);* (▽, ▼) *uvrA, B, or C;* (□, ■) *wild-type;* (○, ●) *rec-21; and* (△, ▲) *Lon⁻. The plot representing uvrA, B, or C is an average, and no significant differences were observed between these UV-sensitive strains. Of all the K-12 strains, only rec-21 and possibly the Lon⁻ strains (the average of two strains) were sensitive to thymine starvation.*

their glossy, mucoid-like appearance on minimal medium-agar plates; that is, these strains tend to form filaments during growth in minimal medium (16). The function of the Casamino Acids in reducing the lag time may be to break up these filaments. That other events may be occurring during the 20-min lag may be indicated by the fact that, once death commences in Lon^- strains, it does so at a rate faster than that noted in any of the other strains examined. It is difficult to state the ultimate rate of death with much assurance, however, since this factor depends strongly on the generation time of the organism. Nevertheless, in the cases observed, the generation times of the *E. coli* B Lon^- and *rec-21* strains were about 40, 55, and 70 min, respectively, and the ultimate exponential death half-times observed were 6 to 7 min, 4 to 5 min, and 11 to 12 min for *E. coli* B or B_{s-12}, Lon^-, and *rec-21* strains, respectively.

Filament formation. The filament-forming ability of the bacterial strains must be evaluated in considering a possible mechanism to account for the phenomenon of thymineless death. *E. coli* B, B_{s-3}, B_{s-12}, and K-12 Lon^- are all known to form extensive filaments after UV irradiation (14, 16). Of these, *E. coli* B and B_{s-12}, and possibly K-12 Lon^-, are sensitive to thymineless death. The ability to form filaments can be determined either by direct observation in a light microscope (14) or by the rescue effect of pantoyl lactone (37), a precursor of pantothenic acid. In Fig. 3, the effect of pantoyl lactone on *E. coli* B can be observed. It can be noted that pantoyl lactone had a marked restorative effect on *E. coli* B exposed either to thymine starvation or to UV irradiation. Presumably, pantoyl lactone inhibits the formation of filaments (37) and allows the bacteria to develop into visible colonies. For purposes of monitoring the various bacterial strains for their response to pantoyl lactone, only the effect on UV survival was measured. In this regard, *E. coli* K-12 Lon^- is already known to be rescued by pantoyl lactone (18), and the results on the other organisms of interest are presented in Fig. 4. As can be seen, *E. coli* B_{s-3} and B_{s-12} are readily rescued by pantoyl lactone. On the other hand, pantoyl lactone has little or no reactivating action on *E. coli* K-12 *rec-21*. As shown by Kneser (18), another type of Rec^- similar to *rec-1* was also not reactivated by pantoyl lactone. In our hands, pantoyl lactone actually inhibited growth of the *rec-21*, but not of *rec-1*, *uvrB*, or any of the other strains examined. After 72 hr of incubation in the presence of 0.6% pantoyl lactone, *rec-21* failed to produce visible colonies.

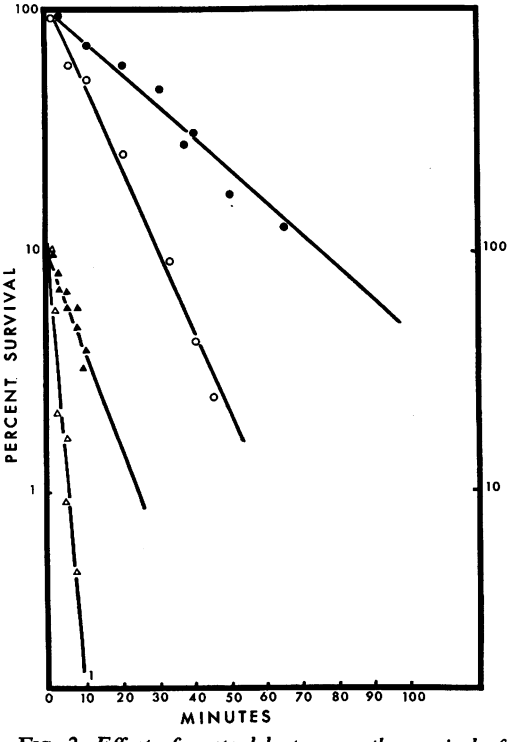

FIG. 3. *Effect of pantoyl lactone on the survival of Escherichia coli B (Luria). Open symbols, plated without pantoyl lactone; closed symbols, plated with 0.3% pantoyl lactone. (○, ●; 0-100 min abscissa, left side ordinate) refers to thymineless death kinetics and (△, ▲; 0-1 min abscissa, right side ordinate) refers to UV inactivation. In each case, pantoyl lactone restored the colony-forming ability to about the same extent.*

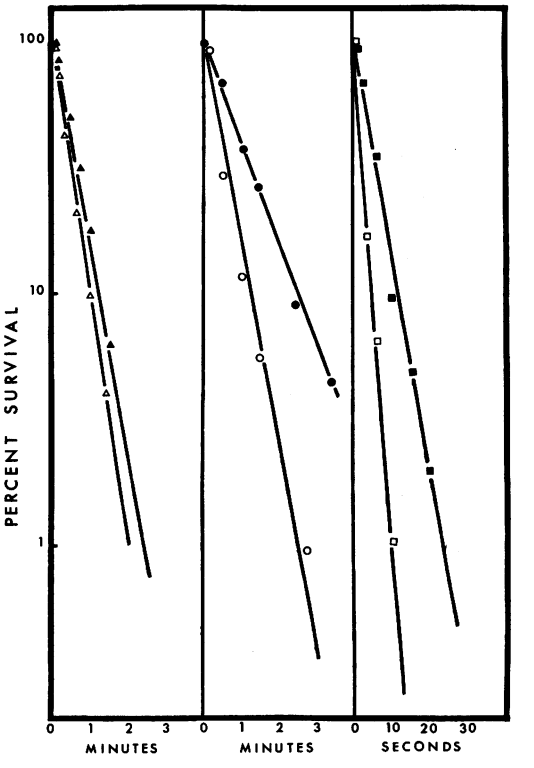

FIG. 4. *Effect of pantoyl lactone on the UV survival of (△, ▲) Escherichia coli K-12 rec-21; (○, ●) E. coli B_{s-3}; and (□, ■) E. coli B_{s-12}. Open symbols, plated without pantoyl lactone; closed symbols, plated with 0.3% pantoyl lactone. E. coli B_{s-3} and B_{s-12} responded in essentially the same manner to pantoyl lactone but in the case of the rec-21 strain, little, if any, restorative effect was observed. Increasing the pantoyl lactone concentration enhanced the reactivation effect on E. coli B, B_{s-3}, and B_{s-12}, but little or no effect was observed on E. coli rec-1, uvrB, or B_{s-1}.*

For this reason, 0.3% pantoyl lactone was used in these experiments. Of the strains examined, *E. coli* B, B_{s-3}, and B_{s-12} were reactivated by pantoyl lactone, and no particular effect was noted in strains *E. coli* B_{s-1}, K-12 *rec-1*, K-12 *rec-21*, and K-12 *uvrB*. As shown by Hill and Feiner (14), *E. coli* B, B_{s-3}, and B_{s-12} had essentially the same filament-forming ability. This ability to form extensive filaments after UV irradiation is readily observable in the light microscope (14). Figure 5 shows representative photographs of the three strains found to be sensitive to thymineless death. As was indicated by the results with pantoyl lactone, *E. coli* B and B_{s-12} displayed many long filaments 3 hr after sufficient UV irradiation, whereas *E. coli rec-21* showed a few short filaments and mostly single cells. With the same technique, *E. coli* B_{s-3} gave essentially the same results as B and B_{s-12}, whereas *E. coli* B/r and *E. coli* K-12 AB1157, the parent of *rec-21*, had occasional short filaments and mostly single cells. It would appear, therefore, that, although *E. coli* B, B_{s-12}, and Lon$^-$ all form extensive filaments and are all sensitive to thymine starvation, there are strains which are sensitive to thymine starvation (*rec-21*) but do not form extensive filaments and there are strains which do form extensive filaments (B_{s-3}) but are not sensitive to thymine starvation.

Induction. Since thymine starvation is known to induce prophage (17, 22) and colicin formation (9, 23), it has been suggested (24, 35) that thymineless death could be caused by the induction of a defective prophage. In support of this hypothesis, Frampton and Brinkley (8) studied four strains of thymine-requiring *E. coli* 15, strains known to produce colicins, and found that, after exposure to UV, extracts from lysed cells contained some complete, but mostly

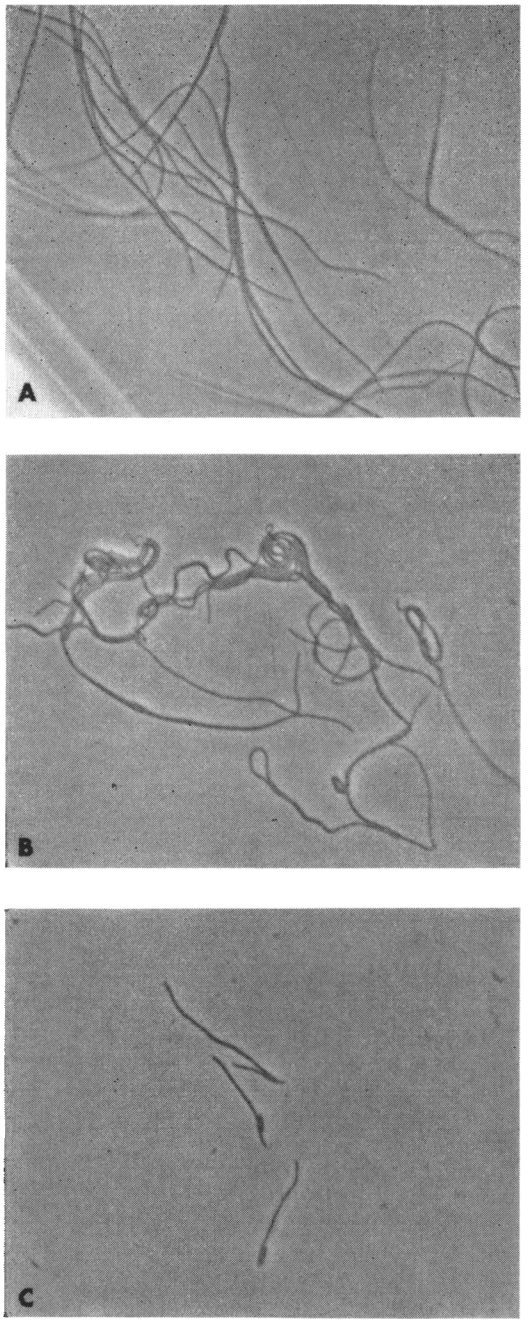

FIG. 5. *Light micrographs of the three strains found to be sensitive to thymine starvation: (A) Escherichia coli B; (B) E. coli* B_{s-12}*; (C) E. coli K-12 rec-21. Following the procedure of Hill and Feiner (14), the bacteria were UV-inactivated to 1% survival, and then allowed to grow for 3 hr on tryptone-thymine-agar plates. Pictures were taken with a Leitz phase-contrast microscope on Polaroid film at a magnification of about 700:1.*

incomplete, phage particles of morphology different from any phage previously seen associated with *E. coli*. None of these particles was found to be infective when *E. coli* B, C, or 15 was used as indicator strain. We therefore examined concentrated extracts from 10^{10} to 2×10^{10} cells of *E. coli* B and B/r, both wild-type and thymine-requiring strains, using either MC (28) or thymine starvation to induce a hypothetical defective prophage. We were not able to find any convincing evidence with electron microscopy for the presence of defective phages or phage parts in the lysates. Some substructures somewhat similar to T-even phage parts were observed rarely in only a few preparations, but no recognizable phage particles were seen.

It was readily shown that induction of a normal phage had little or no effect on the kinetics of thymineless death (Fig. 6). *E. coli* K-12 *rec-1*, *rec-21*, *uvrB*, and two wild-type thymine-requiring strains lysogenized with λ bacteriophage all responded to thymine deprivation in the same manner as did the nonlysogenic parents. In addition, no significant differences were noted in the kinetics of induction of λ bacteriophage by

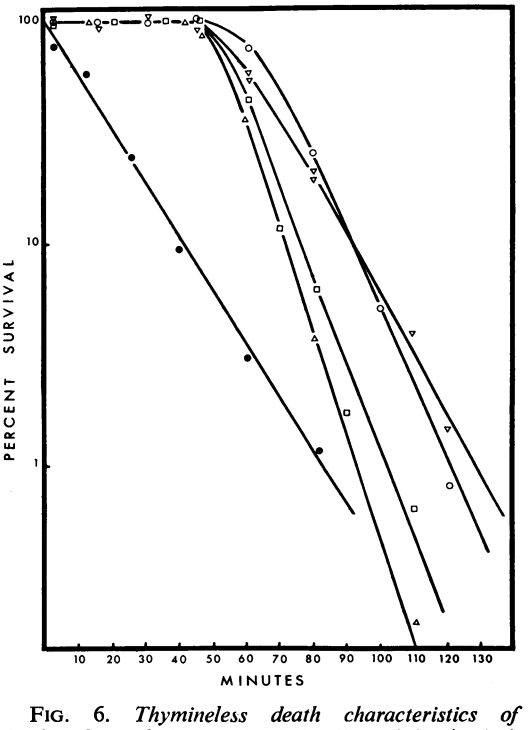

FIG. 6. *Thymineless death characteristics of Escherichia coli K-12* (λ). (○) *AB1157 Rec*$^+$; (△) *JC1557 Rec*$^+$; (▽) *JC1569 rec-1*; (□) *uvrB; and* (●) *AB2470 rec-21. Note that little or no change were observed compared with the kinetics contained in Figure 2.*

MC, thymine deprivation, or heat (19) in *E. coli* K-12 AB1157, *rec-21*, or *uvrB*. These studies on lysogenic strains also revealed another physiological difference between *rec-1* and *rec-21* in that induction of λ in *rec-21* was reduced only 5- to 10-fold, whereas in *rec-1* induction was reduced at least 1,000-fold (P. Howard-Flanders and A. J. Clark, *personal communication*).

It has also been suggested that ribonucleic acid (RNA) synthesis (6, 12, 13), and in particular messenger RNA synthesis (31), is required in order that the bacteria suffer thymineless damage. It is difficult to test this hypothesis (31) directly, but it is possible to induce protein synthesis and observe the effect on thymineless death. In the case of *E. coli* B and B/r, no changes were observed in the kinetics of thymineless death which occurred during the induction of either β-galactosidase (29) or D-serine deaminase (30), or both proteins simultaneously, even though the specific activity of each enzyme induced increased 4- to 14-fold. In agreement with Luzzati (20), who showed a reduction in messenger RNA synthesis during thymineless death, measurable enzyme induction ceased when the bacteria had undergone thymineless death to the extent of 5 to 10% survival.

DISCUSSION

From a variety of *E. coli* strains, three and possibly four were found to be sensitive to thymine deprivation: *E. coli* B, B_{s-12}, K-12 *rec-21*, and possibly K-12 Lon^-. Although each of these strains was sensitive to UV or MC, the range and type of sensitivity varied greatly. *E. coli* B, B_{s-12}, and K-12 Lon^- all form filaments extensively, and hence are rescued by pantoyl lactone; *E. coli* B_{s-12} is unable to repair UV-treated T1 bacteriophage, and K-12 *rec-21* is unable to undergo genetic recombination. On the basis of these results and the results obtained with other bacteria, host-cell reactivation, recombination ability, and thymine dimer excision do not appear to be particularly relevant in determining sensitivity to thymine deprivation. The most common feature seems to be filament-forming ability. However, one strain sensitive to thymine starvation (*rec-21*) did not form filaments and was not rescued by pantoyl lactone, whereas another strain (B_{s-3}) was not sensitive to thymine starvation but did form filaments and was rescued by pantoyl lactone. It seems likely that there are many mechanisms involved in repairing lesions created in DNA, by whatever means, and that each type is genetically determined and independent of the other types of repair. In this regard, three levels of repair of thymine dimers in vivo have been described by Setlow et al. (34) using *E. coli* B_{s-1}, B_{s-3}, and B/r, all of which we now know to have essentially the same resistance to thymine starvation. It may also be that the mechanism(s) of repairing lesions caused by thymine starvation does not involve directly the repair of DNA but rather that the DNA lesion observed (31) after thymine deprivation may be a secondary effect occurring after a lethal event elsewhere. Recently, in fact, Rosenberg and Packer (Abstr. Biophys. Soc., p. 39, 1967) reported that abnormal methylation occurs in the absence of thymine. The existence of two distinct modes of thymineless death may enable us to determine genetically a site (or sites) controlling sensitivity to thymine deprivation, and thus specify its relationship to other metabolic events.

A major difficulty in obtaining evidence concerning the mechanism of thymineless death is that thymine deprivation may interfere with many cellular processes. Along with UV and MC, thymine starvation is known to induce prophages (17, 22), and hence prophage induction must be considered as a possible cause of thymineless death. In a variety of UV-resistant or UV-sensitive strains, we were not able to demonstrate any effect of a normal prophage, such as λ, on the kinetics of thymineless death, but this did not exclude the presence of a defective prophage (24, 35). There is no doubt that some thymine-requiring bacteria have been shown (8) to yield unique defective phage parts. Although we were not able to demonstrate that such phage parts were induced in *E. coli* B or B/r, one could argue that here the prophage was so defective that even substructural pieces were not formed. However, it is not apparent how a defective prophage could be the primary cause for two different types of thymineless death in organisms derived from a common parent. In addition, there is little evidence that *E. coli* B or B/r produce colicins and, in fact, it has been shown that *E. coli* B/r differs from *E. coli* 15 (7, 8) in its ability to synthesize RNA after exposure to UV or X rays. The strains of *E. coli* 15 in which defective phage parts were induced showed an increased synthesis of RNA, whereas *E. coli* B/r showed an inhibited synthesis of RNA. Similarly, although Mennigmann and Szybalski (25) detected single strand breaks in DNA isolated from *B. subtilis*, a strain known to harbor a prophage, while it was undergoing thymineless death, several laboratories (11, 13, 21, 26) including our own were unable to detect any such physical changes in DNA isolated from *E. coli* B, B/r, or 15. Recently, D. W. Smith and P. C. Hanawalt (Abstr. Biophys. Soc., p. 78, 1967) made the observation that thymineless

death occurs in pleuropneumonia-like organisms, and suggested that it is quite unlikely that this smallest living organism could harbor any type of prophage. Hence, an important feature of thymineless death is its ubiquitous nature, and any proposed mechanism for thymineless deaths should be applicable to a great many organisms which differ in many respects.

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