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R Factor Elimination During Thymine Starvation: Effects of Inhibition of Protein Synthesis and Readdition of Thymine

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R factor 1818 is shown to be eliminated from a thymineless strain of *Escherichia coli* J6-2 (R-1818) during thymine starvation. Readdition of thymine to the starved cultures produces a partial recovery in viable count but does not affect the proportion of R^- cells. The R factor is not cured from exponential- or stationary-phase cultures which are starved of required amino acids as well as thymine, nor from cells which are deprived of thymine in the presence of chloramphenicol. However, in both of these cases, the extent of thymineless death is reduced. It is suggested that protein synthesis is a requirement for R-1818 elimination, and the possible nature of this protein is discussed.

Suboptimal growth of thymine-requiring auxotrophs in low concentrations of thymine produces elimination of F prime and colicinogenic factors from *Escherichia coli* (2). In a previous report (22), we have shown that when thymine-requiring strains of *E. coli* and *Klebsiella aerogenes*, which harbor the R factor 1818, are undergoing thymineless death, the occurrence of R^- clones may be over 100 times greater than in control, unstarved cultures. Both suboptimal growth in low concentrations of thymine and thymine starvation eliminate episomal elements which are refractory to the classic acridine curing treatments (13, 28).

The reason why thymine-requiring cells lose viability when deprived of thymine is still unknown, but the necessity of concurrent protein synthesis has been implicated by the fact that chloramphenicol (Cm), which inhibits protein synthesis (9, 30), reduces the extent of thymineless death. In the presence of Cm, either the death rate is decreased (10) or the proportion of cells that survive inactivation is increased (4). Prophage induction has been implicated as contributory to thymineless death (14). In the absence of prophage induction, Donachie and Hobbs (7) produced a complete reversal of thymineless death by the readdition of thymine. However, Cummings and Kusy (4), also working with nonlysogenic strains, found that the addition of thymine did not produce immediate recovery from thymine starvation but that when chloramphenicol and thymine were added recovery did occur.

In order to initiate an investigation into the basic mechanism responsible for R-1818 elimination during thymine starvation, we have examined the effects of the inhibition of protein synthesis upon thymine-starved R^+ bacteria. Similar results were obtained when protein synthesis was inhibited either by Cm or by depriving the cultures of essential amino acids. This communication also reports how the restoration of thymine to thymine-starved R^+ cultures affects R factor elimination.

MATERIALS AND METHODS

Bacterial strains. *E. coli* J6-2 *pro*$^-$ *his*$^-$*trp*$^-$*lac*$^-$ F^- (3), a derivative of *E. coli* K-12, and a thymineless mutant (= J6-2 *thy*$^-$) were used. Strain J6-2 *thy*$^-$ was isolated by trimethoprim selection (26) and requires approximately 30 μg of thymine per ml for optimal growth. R factor 1818 (= R-46 of Meynell and Datta, reference 18), which conveys resistance to ampicillin (Am), streptomycin (Sm), sulfonamides (Su), and tetracycline (Tc), was transferred into both strains from *E. coli* K-12 *met*$^-$ (R-1818), by the method of Smith (25).

Media. Davis and Mingioli (DM; 6) liquid and solid minimal media were prepared as described by Smith (24). Oxoid nutrient broth no. 2 and MacConkey agar were supplied by Oxoid Ltd., Southwark Bridge Road, London, S.E.1. Growth supplements were used as described previously (24, 25).

Antibiotics. Chloramphenicol (Park, Davis and

Co. Ltd.), streptomycin sulfate (Glaxo Ltd.), and tetracycline hydrochloride (Cyanamid Ltd.) were generous gifts from the respective manufacturers.

Thymine starvation and estimation of R factor content. Exponential-, or overnight stationary-phase cultures of strain J6-2 *thy*$^-$ (R-1818) were grown at 37 C in DM medium containing thymine at 60 μg/ml, as well as required amino acids. The bacteria were washed in DM salts solution (without glucose, thymine, or amino acids) and resuspended in prewarmed DM medium, containing the necessary supplements, to give a viable count of approximately 5 $\times$ 10^6/ml. Samples were removed at intervals, diluted in nutrient broth, and plated on MacConkey agar plus 60 μg of thymine per ml. After overnight incubation, clones were replica plated onto fully supplemented DM medium and onto fully supplemented medium with Tc or Sm added at 10 μg/ml. R$^-$ clones were scored as those which did not grow in the presence of either Tc or Sm but which grew on plates containing no antibiotic.

RESULTS

Strain J6-2 *thy*$^-$ (R-1818) requires a high level of thymine for optimal growth and is relatively resistant (17) to thymineless death. In thymineless conditions its viability decreased after a lag period of approximately 45 min, with a half-life of about 20 min. Readdition of thymine to starved cultures permitted regrowth with an increase in turbidity which continued for at least 5 hr without any detectable lysis of the culture. Hence, strain J6-2 *thy*$^-$ (R-1818) does not seem to harbor a prophage, since if it were lysogenic, lysis should take place under these conditions (14).

Effect of amino acid starvation. It has been proposed that only the fraction (approximately 97%) of cells from an exponential culture which are synthesizing deoxyribonucleic acid (DNA) at the time thymine is removed are susceptible to thymineless death under conditions of protein synthesis inhibition (12). Hanawalt (11) has suggested that messenger ribonucleic acid (RNA) synthesis is a requirement for this sector of thymineless death, in that the template DNA could be left in a labile state, or the messenger RNA produced during thymine starvation might code for some lethal protein which would be synthesized on the readdition of the necessary supplements.

In the experiments performed here (Fig. 1 and Table 1), it was found that the viable count of exponential cultures of strain J6-2 *thy*$^-$ (R-1818) in medium lacking both thymine and required amino acids was still 5.8% of its initial value after 4 hr, whereas in cultures containing no thymine but supplemented with amino acids the count fell to 0.017% after the same period. This observation is in good agreement with the suggestion that only the fraction of cells which are actively synthesizing DNA when thymine is removed are susceptible to thymineless death under conditions of protein synthesis inhibition (11, 12). In support of this view are the findings (Fig. 2, Table 2) that when stationary-phase cultures, in which only a small proportion of cells are actively engaged in DNA synthesis, were placed in media lacking thymine and amino acids the viable count fell to only 59% of its initial value after 4 hr.

After 3 or 4 hr of thymineless death in the presence of required amino acids, the viable counts of exponential-phase cultures of strain J6-2 *thy*$^-$ (R-1818) (Fig. 1) were 100 times less than those of stationary-phase cultures (Fig. 2) under the same conditions. However, the proportion of R$^-$ cells was approximately the same in each case (compare Table 1 with Table 2). There would appear to be no correlation, therefore, between the level of thymineless death (as determined by the proportion of surviving cells) and the extent of R-1818 elimination. This makes it very unlikely that the mechanism of R-1818 curing by thymine starvation is due to selection of the small proportion of spontaneous R$^-$ segregants (ca. 0.1%) which are present in unstarved cultures of strain J6-2 *thy*$^-$ (R-1818). Indeed, we have shown previously (22) that the number of R$^-$ cells in a culture of *K. aerogenes* strain 418 *thy*$^-$ (R-1818) actually increased during thymine starvation.

After 5 hr of thymine starvation of stationary-phase strain J6-2 *thy*$^-$ (R-1818) in the absence of required amino acids, the proportion of R$^-$ cells present in the culture was scarcely more than in control, unstarved cultures (Table 2). Similarly, no R factor elimination occurred when log-phase cultures were starved for thymine under conditions of amino acid deprivation, although the viable count of these cultures fell to 5% of its initial value after 4 hr (Table 1). The viable count of exponential cultures, starved of thymine but supplemented with amino acids, fell to 5% after 2 hr, but, in this case, the proportion of R$^-$ cells increased about 100-fold (Table 1). Thus, although viability can decline by 95% during thymine starvation in the absence of protein synthesis, it would appear that a specific protein must be produced if R-1818 is to be eliminated during thymineless death.

Control cultures which were starved of amino acids but supplemented with thymine

showed no change in viability or R factor content during up to 5 hr of treatment. Fully supplemented cultures grew exponentially with no increase in the proportion of R^- cells (Fig. 1 and 2; Tables 1 and 2).

Effect of thymine starvation in the presence of chloramphenicol. Incubation of strain J6-2 *thy*$^-$ (R-1818) in thymineless media containing required amino acids and various concentrations of Cm decreased the death rate and increased the proportion of surviving cells (Fig. 3). Increasing the concentration of Cm from 10 to 100 μg/ml slightly reduced this protective effect. Even in the presence of Cm, the surviving fraction of cells decreased to 1% or less of the initial viable count, but, at all con-

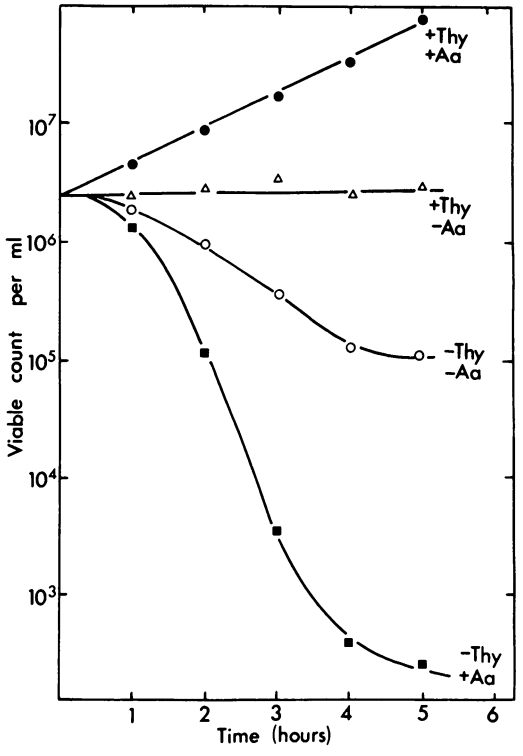

FIG. 1. *Effect of thymine starvation in the presence or absence of required amino acids on the viability of exponential cultures of Escherichia coli strain J6-2 thy*$^-$ *(R-1818). Thy = thymine; Aa = amino acids. Presence and absence of supplements denoted by + and −, respectively.*

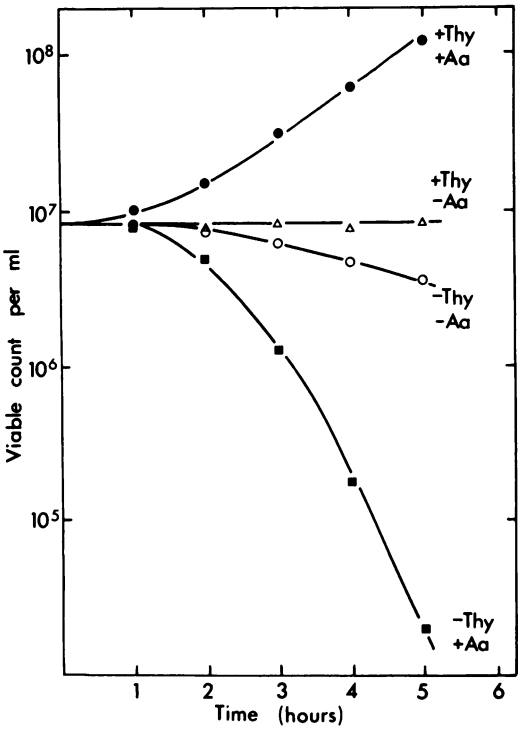

FIG. 2. *Effect of thymine starvation in the presence or absence of required amino acids on the viability of stationary-phase cultures of Escherichia coli strain J6-2 thy*$^-$ *(R-1818). Symbols as in Fig. 1.*

TABLE 1. *R factor elimination from thymine-starved exponential cultures of Escherichia coli strain J6-2 thy*$^-$ *(R-1818)*[a]

Time of thymine starvation (min)	+Thy +Aa		−Thy −Aa		+Thy −Aa		−Thy +Aa	
	% Initial count	% R^- cells	% Initial count	% R^- cells	% Initial count	% R^- cells	% Initial count	% R^- cells
120	354	<0.39	40	0.10	100	<0.21	5.0	9.8
180	667	<0.38	16	0.30	100	<0.19	0.15	12.8
240	1,350	<0.65	5.8	0.63	100	<0.19	0.017	7.8

[a] Cultures were grown to late log phase (2×10^8/ml), washed, and resuspended in DM medium with (+) or without (−) thymine (Thy) or amino acids (Aa) to give a viable count of approximately 5×10^6/ml, and incubation was continued. Samples were removed at intervals and plated on MacConkey agar plus thymine (60 μg/ml). Clones which arose after overnight incubation, were replica plated onto fully supplemented DM medium + Sm or Tc at 10 μg/ml and onto fully supplemented DM medium without either antibiotic. R^- clones were scored as described in Materials and Methods.

TABLE 2. *R factor elimination from thymine-starved stationary-phase cultures of Escherichia coli strain J6-2 thy⁻ (R-1818)*[a]

Time of thymine starvation (min)	+Thy +Aa		-Thy -Aa		+Thy -Aa		-Thy +Aa	
	% Initial count	% R⁻ cells	% Initial count	% R⁻ cells	% Initial count	% R⁻ cells	% Initial count	% R⁻ cells
120	186	<0.18	91	<0.29	100	<0.26	64	2.9
180	395	<0.62	75	<0.34	100	<0.24	16	19.7
240	753	<0.36	59	<0.66	100	<0.28	2.2	16.5
300	1,494	<0.31	46	<0.90	100	<0.22	0.26	5.7

[a] Overnight cultures were treated as described in Table 1, abbreviations as in Table 1.

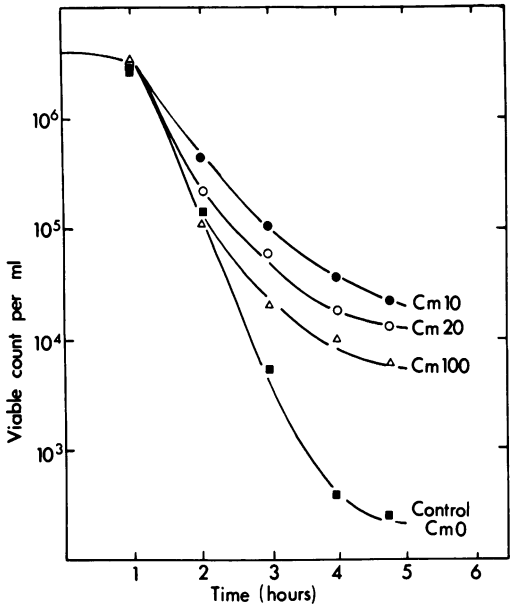

FIG. 3. *Effect of chloramphenicol on thymineless death of exponential cultures of Escherichia coli strain J6-2 thy⁻ (R-1818). Figures give concentrations of chloramphenicol in micrograms per milliliter.*

TABLE 3. *Effect of chloramphenicol (Cm) on R factor elimination from thymine-starved exponential cultures of Escherichia coli strain J6-2 thy⁻ (R-1818)*[a]

Time of thymine starvation in presence of Cm (min)	R factor elimination (%)			
	0[b]	10[b]	20[b]	100[b]
0	<0.55			
120	7.5	<0.33	<0.30	<0.40
180	14.4	<0.61	<0.46	<0.74
285	10.2	0.24	<0.30	<0.31

[a] Late log-phase cultures (2×10^8/ml) were washed and resuspended in fully supplemented DM medium with or without Cm at various concentrations. Samples were taken at various time intervals and plated, and the resulting clones were tested for R factor content as described in Table 1.

[b] Cm concentration in micrograms per milliliter.

centrations of Cm tested, no significant elimination of R-1818 was observed (Table 3). Thus, Cm treatment during thymine starvation produced the same net result as that seen during amino acid deprivation. Exponential cultures lost viability, although at a reduced rate, to between a 1% and 0.5% survival level, but no significant R factor elimination was produced.

Effect of readdition of thymine to thymine-starved cultures. Donachie and Hobbs (7) showed that, in the absence of prophage induction, cultures that had undergone thymineless death exhibited a rapid and complete recovery of viable count when thymine was added. However, Cummings and Kusy (4) could only produce similar results if Cm, as well as thymine, was added to the cultures. We therefore determined the conditions necessary for recovery of viability in exponential cultures of strain J6-2 *thy⁻* (R-1818) which had been subjected to thymine starvation.

It can be seen from Fig. 4 that the addition of thymine plus Cm (15 μg/ml) after 2 hr of thymine starvation prevented further death but produced no recovery in viability. Cm added alone increased the bactericidal effect of thymine starvation. On the other hand, the addition of thymine alone caused a rapid increase in viable count to occur, which proceeded at a doubling time of about 15 min. The mean generation time of this strain grown under these conditions is about 50 min. This indicated that the initial increase in viable count produced by the readdition of thymine was not due to growth and division of the surviving fraction of viable cells, but that some repair of the lesion(s) induced by thymine starvation was taking place. Subsequent to

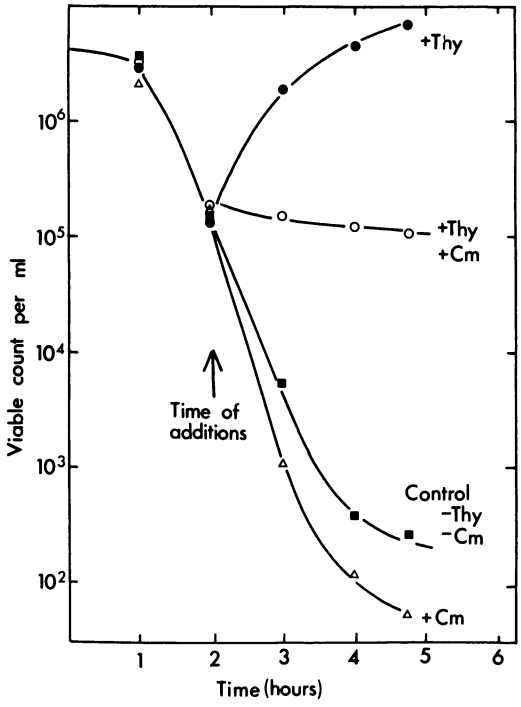

FIG. 4. *Recovery of Escherichia coli strain J6-2 thy⁻ (R-1818) from thymineless death. Exponential cultures were deprived of thymine for 2 hr. Thymine (60 μg/ml) was then added to one (●); thymine (60 μg/ml) + chloramphenicol (15 μg/ml) to another (○); chloramphenicol (15 μg/ml) to a third (△); and the fourth was left as control (■).*

this initial recovery phase, growth was observed which proceeded with a doubling time of about 50 min. Our results are therefore in agreement with those of Donachie and Hobbs (7) rather than with those of Cummings and Kusy (4).

Another experiment was done to test whether the readdition of thymine also permitted the recovery of stationary-phase cells, and to test whether the timing of thymine addition was significant. The R factor content of these cells was followed during their recovery from thymine starvation. The results (Fig. 5) show that the time at which thymine was readded had little affect on the manner in which viability returned after thymineless death, providing that the addition was made when a significant fraction of the cells ($\geq$ 90%) had undergone thymineless death. With such cultures, thymine-initiated recovery produced about a tenfold increase in viable count which was then followed by growth and division at the normal rate. Recovery of viability was not accompanied by a decrease in the proportion of R⁻ cells in the culture (Table 4). Greater than 10% of clones from cells which had undergone thymine starvation for 2 hr or more were found to be R⁻, compared with less than 0.23% from control, unstarved cultures. Where significant R factor elimination had occurred, the proportion of R⁻ cells did not change substantially during periods of up to 4 hr of regrowth in thymine (Table 4). This indicates that R-1818 was not distributed to R⁻ daughter cells by mating on the restoration of thymine and suggests that repair of plasmid DNA did not occur even though some chromosomal repair had occurred, as evidenced by the recovery in viability on the reintroduction of thymine.

DISCUSSION

The initiation of a round of DNA replication in *E. coli* requires the synthesis of several proteins, one of which is inhibited by 25 μg of Cm per ml and another by 150 μg of Cm per ml (15). It is likely that the synthesis of these proteins would also be blocked by amino acid deprivation. If only those cells that are actively synthesizing DNA are susceptible to

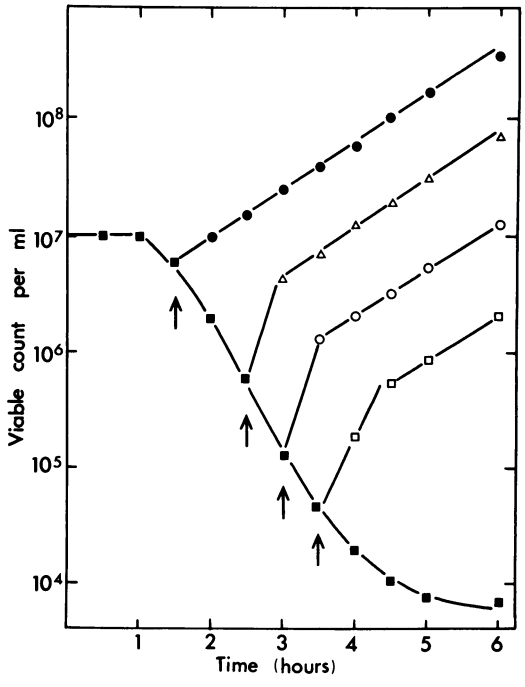

FIG. 5. *Effect of readdition of thymine to starved cultures of Escherichia coli strain J6-2 thy⁻ (R-1818). Stationary-phase cultures were deprived of thymine for varying times, after which thymine at 60 μg/ml was added to the medium. ●, 90 min; △, 150 min; ○, 180 min; □, 210 min.*

TABLE 4. *R factor content of thymine-starved Escherichia coli strain J6-2 thy⁻ (R-1818) to which thymine has been readded*[a]

Time of thymine readdition (min)	Time of incubation after readdition of thymine (min)	R⁻ cells (%)
0 (control)		<0.23
120	0	11.4
	30	8.6
	240	8.9
180	0	20.1
	30	17.6
	180	19.5
210	0	18.1
	30	15.9
	90	17.3
	150	22.3

[a] Washed overnight cultures of strain J6-2 *thy⁻* (R-1818) were deprived of thymine in otherwise fully supplemented DM medium for the times indicated. Thymine was then added at 60 μg/ml, and clones from cells sampled after various periods of incubation in the presence of thymine were tested for R factor content by replica plating as described in Table 1.

thymineless death (11), a correlation would be expected between the level of survivors in thymineless cultures deprived of amino acids and in amino acid-supplemented cultures starved of thymine in the presence of Cm. In agreement with this, we have found that the survivor level in the presence of Cm concentrations which would block the synthesis of at least one of the initiator proteins (Fig. 3) is approximately that produced by concurrent thymine and amino acid starvation (Fig. 1). However, Cummings and Kusy (5) have shown that survival from thymineless death in *E. coli* B does not necessarily correlate with a finished round of DNA replication. They conclude that some other event, possibly the inhibition of protein synthesis, is responsible for the "immune" state. However, their effect may be strain-specific, because they show that *E. coli* B recovers from thymineless death provided that protein synthesis is inhibited when thymine is added, which contrasts with the results of Donachie and Hobbs (7) and the results presented here (Fig. 4 and 5) with *E. coli* 15 and *E. coli* K-12, respectively.

Chloramphenicol, in the concentrations used, inhibits protein synthesis without affecting RNA synthesis (21), and, although amino acid deficiency in a stringent strain represses ribosomal and transfer RNA synthesis, it has little effect on messenger RNA synthesis (20). Hanawalt (11) has suggested that the lethal effect of thymine starvation in the absence of required amino acids could be due to a protein, coded for by this RNA, which would be synthesized on readdition of thymine. However, if postinhibitory translation were responsible for the lesions which cause R-1818 elimination, such curing should have been demonstrated in cultures deprived of thymine under conditions of protein synthesis inhibition, since the lethal protein could still be synthesized when amino acids were returned to the culture or Cm was removed. We have shown (Tables 1, 2, and 3) that amino acid starvation or Cm abolishes R-1818 curing from thymine-deprived cultures, and it is therefore probable that the mechanism of elimination is dependent upon protein synthesis during the actual period when thymine is absent from the medium.

Thus, although it would seem that two sectors of thymineless death exist, one of which may possibly be ascribed to lethal messenger RNA synthesis and another which has a requirement for protein synthesis, the elimination of R factor 1818 during thymine starvation appears to be due solely to the latter cause, i.e., protein must be synthesized.

Single-strand breaks, or nicks, occur in episomal (8) and chromosomal (27) DNA during thymine starvation, which Friefelder (8) has suggested are caused by production or activation of an endonuclease, or inhibition of a ligase. Weissbach and Schuster (29) have shown that, during thymine starvation of thymine-requiring host cells, an endonuclease is produced by the bacteriophage lambda that causes the disappearance of its closed circular form. The nicked circles were not reconstituted when thymine was returned to the medium, and further experiments showed that nicking was prevented by Cm, i.e., endonuclease production was inhibited (29). The similarities between their results and those presented here suggest at least three possible models for the mechanism of R-1818 elimination: (i) thymine starvation induces the synthesis of an endonuclease which inactivates the R factor genome relative to the chromosome; (ii) an exonuclease is induced which preferentially degrades R factor DNA from a nicked site; or (iii) R-1818 is less susceptible to ligase repair activity than the host chromosome.

It should be remembered that the process of elimination is taking place against a background of thymineless death in the host popu-

lation. This could be due to chromosome damage, produced either by nucleases or as a consequence of DNA transcription in the absence of ligase action.

ACKNOWLEDGMENT

We are grateful to Lorraine Pascoe for her excellent technical assistance.

ADDENDUM IN PROOF

D. Freifelder and E. Levine (Biochem. Biophys. Res. Commun. 46:1782–1787, 1972) have recently shown that extracts of thymine-starved *E. coli* contained an endonuclease not present in unstarved cells, whereas the level of DNA ligase activity was unaffected by thymine starvation.

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