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*NONCONSERVATIVE DNA REPLICATION IN BACTERIA AFTER THYMINE STARVATION**

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The phenomenon of thymineless death, first described over ten years ago by Barner and Cohen,[1] remains poorly understood, although much information about it has been accumulated. The loss in viability during thymine deprivation was initially ascribed to unbalanced growth,[1] as cytoplasmic syntheses continue in the absence of chromosomal replication. This concept was subsequently refined when it was shown that specifically RNA synthesis, but not protein synthesis, is a prerequisite for thymineless death.[2, 3] Cells that have completed a round of DNA replication but have not initiated the next round are immune to the pathological effects of thymine deprivation;[4] thus, a direct involvement with the chromosomal replication cycle is apparent.

In addition to lethality, many other events have been correlated with thymine deficiency. Thymine deprivation is mutagenic[5–8] and recombinogenic.[9–11] It induces the production of colicins,[12] effects the induction (or diversion) of prophage,[13, 14] and causes the premature initiation of the DNA-replication cycle.[15] Many of these consequences of thymine deprivation parallel those of ultraviolet (UV) irradiation; and, in fact, a synergism has been observed between thymineless

death and both UV inactivation[16, 17] and death due to decay of P^{32} incorporated into the DNA of bacteria.[18]

It has recently been demonstrated in our laboratory that a nonconservative mode of DNA replication occurs in bacteria after UV irradiation,[19] and that this constitutes the postulated repair synthesis that follows the excision of photoproducts from the DNA.[20, 21] It was therefore of interest to examine the qualitative nature of DNA replication that immediately follows thymine starvation. The results of our studies implicate the repair replication mechanism in the phenomenon of thymineless death.

Materials and Methods.—The thymine-requiring *Escherichia coli* strain TAU-bar[3] was cultured aerobically in a glucose-salts synthetic medium at 37°C with required supplements as previously described.[3, 4] Changes of medium were accomplished by rapid filtration.[4]

Bacterial DNA was uniformly prelabeled with C^{14}-thymine (Calbiochem) at 2 μg/ml and 25 mc/mM or lower specific activity. Density labeling involved the substitution of 5-bromouracil (BU) at 2 μg/ml or H^3-BU (Nuclear-Chicago) at 0.5 μg/ml and 2 c/mM in the growth medium in place of thymine. Isotope incorporation and growth were stopped abruptly by dilution of the culture with an equal volume of ice-cold "NET"[22] buffer containing 0.01 *M* KCN. The cells were harvested by filtration, resuspended in 2 ml NET, and lysed with lysozyme (Worthington, N.J.).[22] Following rapid freezing and thawing, the resulting lysate was brought to 2.5 ml in NET and extracted with an equal volume of 9:1 chloroform-octanol. The aqueous phase was subsequently decanted for density gradient analysis. Reproducibly high yields of DNA, 95 per cent or better, were obtained by this method, but it should be pointed out that the method selectively discriminates against DNA near the normal growing point[22] in the chromosome.

Preparative density-gradient equilibrium sedimentation and radioisotope assays were performed as previously described.[19] For density analysis of denatured DNA, 0.6 ml 0.4 *M* K_2HPO_4-K_3PO_4 buffer at pH 12.45 was added to the sample.[23]

Results.—Density labeling of DNA during its replication *in vivo* can be used to distinguish between the semiconservative mechanism and other possible mechanisms for the duplication of the molecule.[24] Thus, in Figure 1*a* the incorporated tritium activity following H^3-BU labeling of a normal culture of bacteria in balanced growth yields the expected density distribution for semiconservative replication of chromosomal segments.[24] If a period of thymine deprivation is interposed between normal growth and the introduction of the H^3-BU label, a different density distribution is obtained, as evident in Figure 1*b* and *c:* a substantial amount of H^3 is found in the density region between normal thymine-containing DNA and BU hybrid, semiconservatively replicated DNA. The relative amount of newly synthesized material in this intermediate density region is positively correlated with the duration of thymine starvation. This pattern of BU incorporation is qualitatively similar to that previously reported for ultraviolet-irradiated bacteria.[19]

The unusual mode of DNA replication observed here is not related to the premature initiation of chromosomal replication reported by Pritchard and Lark,[15] since we perform the thymine starvation in the absence of required amino acids, a condition that does not lead to reinitiation of chromosomal replication. We have experimental verification of this point.

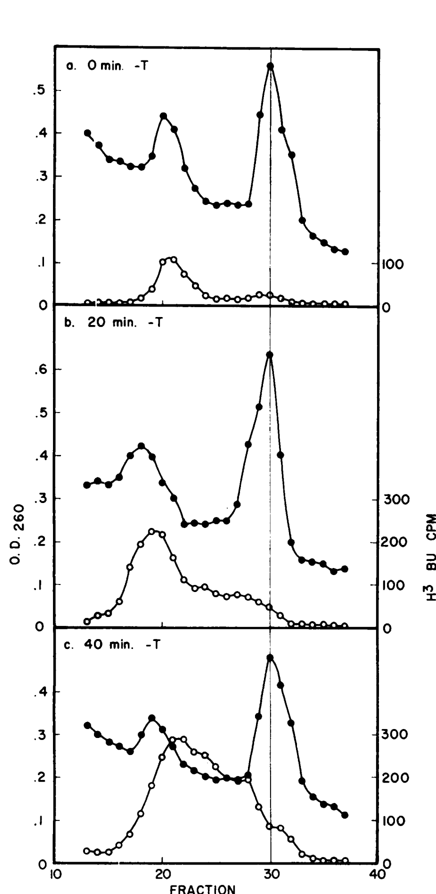

FIG. 1.—Density distributions of DNA fragments isolated from bacteria grown in H^3-BU following thymine deprivation. A 25-min period of growth in medium containing unlabeled BU preceded the period of thymine deprivation and growth in H^3-BU so that transition point fragments would not be isotopically labeled. (*a*) Control: no thymine deprivation before a 10-min period of growth in H^3-BU; (*b*) 20-min thymine deprivation before 10-min growth in H^3-BU; (*c*) 40-min thymine deprivation before 10-min growth in H^3-BU. ●——●, Optical density at 260 mμ; ○——○, tritium activity, from growth in H^3-BU.

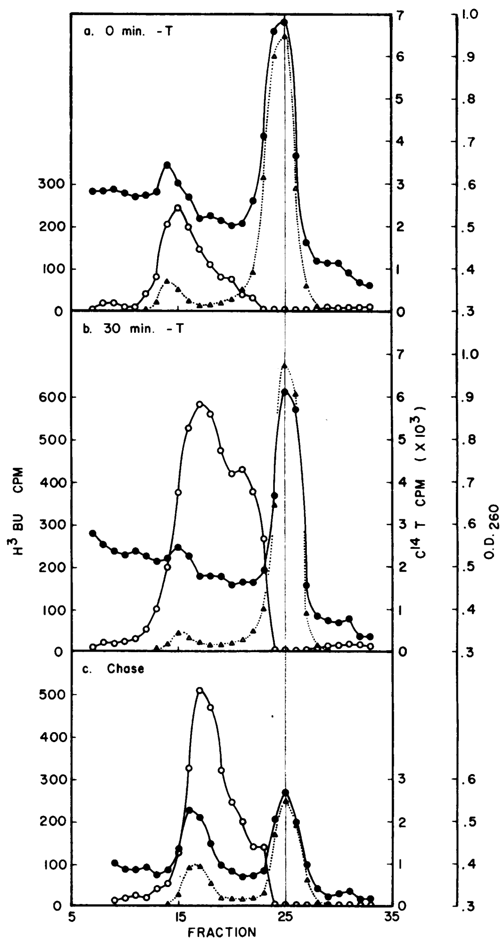

FIG. 2.—Effect of further growth in unlabeled BU on density distribution of H^3-BU incorporated following thymine deprivation. The DNA was uniformly prelabeled with C^{14}-thymine at 5 mc/mM (see *Methods*); a 15-min period of growth in medium containing unlabeled BU preceded the period of thymine deprivation and H^3-BU incorporation. (*a*) Control, same as Fig. 1*a*; (*b*) 30-min thymine deprivation before 10-min growth in H^3-BU; (*c*) as in (*b*), except that 60-min growth in medium containing unlabeled BU followed the incorporation of H^3-BU. ●——●, OD_{260}; ○——○, tritium activity; △. . . △, C^{14} activity.

Previous studies with this bacterial system have resolved three distinct classes of DNA fragments that may appear between normal and hybrid densities in a BU labeling experiment. The fragments containing transition points[25] are eliminated from analysis in our experiments by growth in a medium containing unlabeled BU prior to the onset of thymine starvation or growth with H^3-BU. Fragments containing the replication point are eliminated by the chloroform-octanol step in the DNA preparation.[22] The third class of intermediate density DNA has been observed as the result of random nonconservative replication after ultraviolet[19] or nitrogen

mustard[26] treatment of bacteria. The following experiments were performed to determine whether some DNA replication after thymine starvation is also nonconservative.

It is characteristic of repair-replicated DNA that it is not shifted from the intermediate density region into the hybrid band upon continued incubation of the bacteria with density label. It is clear from Figure 2 that this is also true for intermediate density DNA obtained after thymine starvation. (This experiment was performed in the absence of amino acids so that no portion of the chromosome would replicate twice during the incubation.) The pattern of DNA synthesis following thymine starvation could not involve the incomplete filling in of a daughter strand along the parental template, since, were this the case, a continued incubation with BU should have eventually shifted the density to hybrid as remaining gaps were filled.

The effect of further fragmentation by sonication is demonstrated in Figure 3. It is clear that sonication of the intermediate density DNA does not resolve it into fragments of different densities but merely results in broadening of the band width, as expected upon fragmentation of homogeneous material. This result is

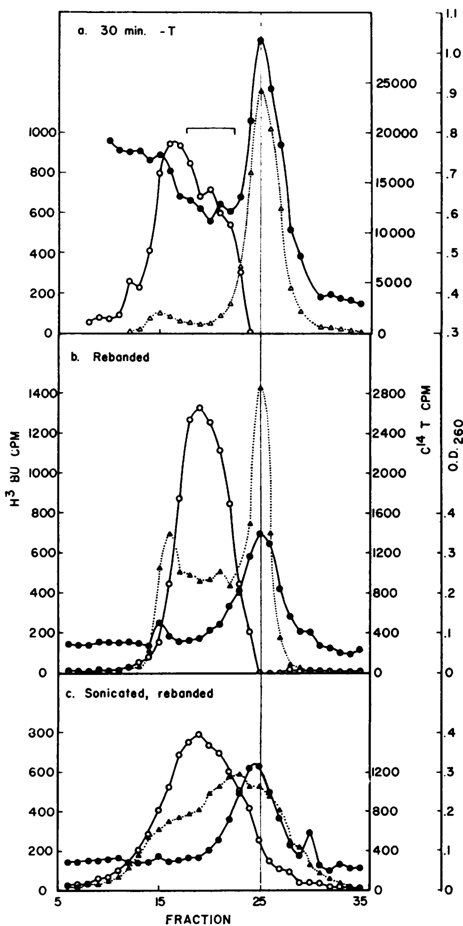

FIG. 3.—Rebanding of intermediate density fractions. The DNA was uniformly prelabeled with C^{14}-thymine at 12.5 mc/mM. A 15-min period of growth in medium containing unlabeled BU preceded the period of thymine deprivation and H^3-BU incorporation. (*a*) 30-min thymine deprivation before 10-min growth in H^3-BU; (*b*) rebanding in a second CsCl gradient of fractions indicated by bracket in (*a*); unlabeled TAU-bar DNA was added as an optical density marker; (*c*) as in (*b*), except that material was sonicated for 1 min under conditions previously described.[19] ●——●, OD_{260}; O——O, tritium activity; Δ...Δ, C^{14} activity.

in marked contrast to that obtained when DNA molecules containing transition points or replication points are subjected to fragmentation,[22] but consistent with the idea that the density label has been incorporated into a number of relatively short segments in the DNA.

In order to determine whether both strands of DNA contained density label, material was isolated from the gradients illustrated in Figure 4 and then rebanded in alkaline cesium chloride gradients to denature the DNA. An important point that is evident in Figure 4 is that only a very small fraction of the parental DNA (C^{14} prelabel) is involved in the intermediate density DNA. It is apparent in Figure 5 that the H^3-BU-containing DNA from the intermediate density region

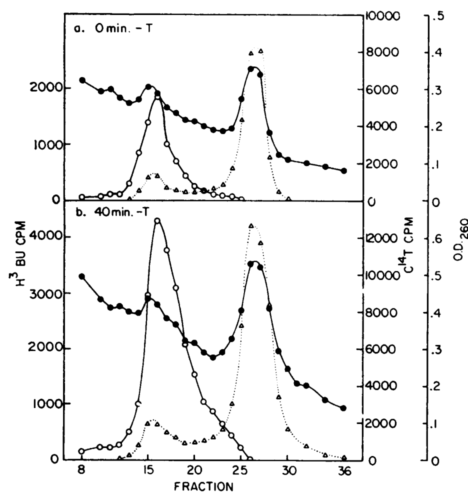

FIG. 4.—Preparation of intermediate density material for alkaline density gradient rebanding. The DNA was uniformly prelabeled with C^{14}-thymine. A 20-min period of growth in medium containing unlabeled BU preceded thymine deprivation and incorporation of H^3-BU. (*a*) Control: no thymine deprivation before 12-min growth in H^3-BU; (*b*) 40-min thymine deprivation before 12-min growth in H^3-BU. ●——●, OD_{260}. O——O, tritium activity; △. . . △, C^{14} activity.

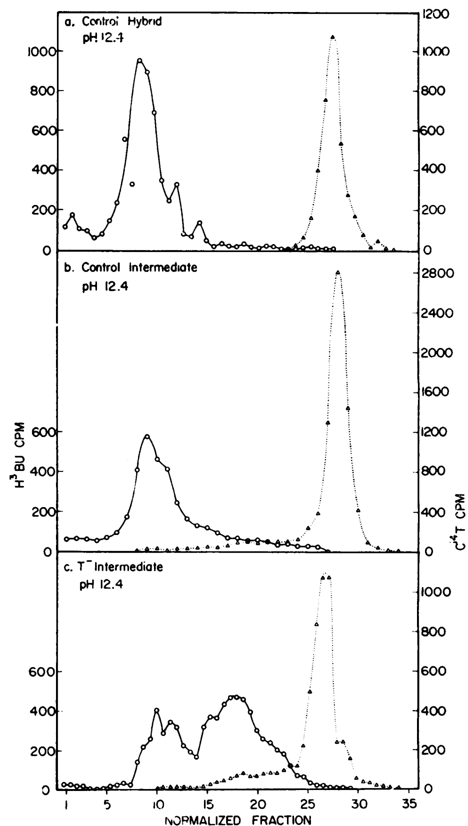

FIG. 5.—Rebanding at pH 12.4 of fractions from Fig. 4. (*a*) Fraction 15 from Fig. 4*a* rebanded at pH 12.4; (*b*) Fractions 20 and 21 from Fig. 4*a*, pooled and rebanded at pH 12.4. (*c*) Fractions 20 and 21 from Fig. 4*b*, pooled and rebanded at pH 12.4. O——O, Tritium activity; △. . . △, C^{14} activity.

resolves into two bands upon denaturation: single strands containing complete replacement of thymine sites by BU, and a material that bands between the positions of single-strand DNA containing thymine and single-strand DNA containing BU. The C^{14} prelabel material bands predominantly at the position of strands containing thymine, but there is a significant tail on the denser side. Some of the

C^{14} in this tail can certainly be accounted for by transition-point fragments (containing C^{14}-thymine and unlabeled BU at the point of transfer from thymine medium to BU medium), and our selection of the intermediate density region for rebanding enriches for such fragments. However, the C^{14} in the tail from material isolated from the thymineless preparation represents about twice as much material as that from the control, relative to total C^{14}-thymine counts recovered. Thus, roughly half of the intermediate density C^{14} label can be accounted for on the basis of transition points, and the remaining half may be associated with the H^3 material which bands at the same density. Our interpretation of this result is that the isolated single strands in this region contain parental C^{14} thymine, and also many short regions of H^3-BU label, incorporated nonconservatively.

Discussion.—We have presented evidence that a small amount of bacterial DNA is replicated nonconservatively following thymine starvation. What does this observation add to our understanding of the molecular mechanism of thymineless death and related phenomena?

Nonconservative DNA synthesis has been observed to occur as a step in the repair of specific lesions introduced into the DNA by ultraviolet light and other deleterious agents.[19, 26] The demonstration of nonconservative DNA synthesis following thymine starvation extends the observed parallels between UV irradiation and thymine starvation, suggesting that both treatments result in damage to the DNA and necessitate repair replication to restore the DNA to pretreatment condition. Yet there are important differences between the pattern of nonconservative DNA synthesis observed after UV irradiation and those reported here. In the case of low-dose UV irradiation, the bulk of isotopically labeled BU is incorporated without any detectable shift of density; that is, the amount of BU substituted for thymine to effect repair replication of the UV lesions is insufficient to effect a noticeable shift in the density of the DNA fragment banded in the CsCl density gradient.[19] After thymine starvation, however, the unusual fragments have densities clearly intermediate between normal and hybrid. Thus the degree of substitution of BU for thymine in the fragments isolated following thymine starvation is greater than that after low doses of UV. The patterns of rebanding of intermediate density material from the thymineless growth experiment are also similar to those reported for repair.[19, 26] It is important to emphasize again, however, that the amount of this unusual DNA is a small fraction of the total DNA.

Previously reported observations of repair replication were in response to clearly detectable chemical modifications of the DNA, e.g., thymine dimers formed by UV irradiation, or an alkylation product formed upon treatment with nitrogen mustard.[19, 26] What is the nature of the lesion induced by thymine starvation that requires repair replication to cure? We consider two alternatives: (1) thymine deprivation in some way directly induces lesions in the DNA, which then are recognized by the repair replication machinery; and (2) lesions are suffered by the DNA as part of the normal cell metabolism, but in the absence of thymine repair replication cannot be completed. Of these alternatives we feel that the second is the more likely. It is difficult to conceive of direct effect on the DNA of withholding thymine, except possibly at the growing-point region itself; it also seems unlikely that all the manifestations of thymineless growth can be due to damage of the growing-point region itself.

An apparent requirement for thymineless damage to be sustained by the bacteria is RNA synthesis in the absence of concomitant DNA synthesis.[2] More specifically, it has been suggested that the synthesis of messenger RNA is required.[3] There is evidence that only one strand of the DNA is read in the synthesis of messenger RNA, and current thinking on the mechanism of transcription suggests that the DNA double helix must unwind to allow RNA transcription.[27-30] It is difficult to see how unwinding to allow transcription could occur simultaneously at many points along the *E. coli* chromosome without a certain number of single-strand breaks. This proposition, that transcription introduces single-strand breaks, has the important implication that the repair enzymes may have functions that are not restricted to the repair of specific chemical damage to the DNA, but more generally are involved with the normal processes of replication and transcription. Evidence that single-strand breaks can be repaired following their production by methylmethane sulfonate has been reported.[31, 32] Also there is some evidence that a low level of random nonconservative DNA replication does occur in *E. coli*[33] and *Bacillus subtilis*[34] not undergoing normal DNA replication.

It has been proposed that genetic recombination in bacteria proceeds by breakage and reunion and that at least the reunion step utilizes the repair replication machinery.[35-37] It is in this connection significant that recombination-deficient mutants of *E. coli* are also UV-sensitive.[37] The recombination-deficient mutants are able to excise thymine dimers, and presumably are blocked at a later step in the repair process. We predict that a thymineless mutant of a recombination-deficient strain would be strikingly more sensitive to thymine deprivation than a recombination-sufficient strain.

Our model provides a plausible explanation for the mutagenicity and possibly the recombinogenicity and prophage induction effect of thymine deprivation. The base pair error resulting in a transition mutation[8] may occur either during attempted repair in the absence of exogenous thymine, or during repair after plating in the presence of thymine. The apparent role of breakage in genetic recombination, and the parallel between recombination and incorporation of prophages as suggested by the Campbell model,[38] suggests that the induction of single-strand breaks in the DNA would increase the frequency of these phenomena.

We propose, then, that upon removal of thymine, transcription continues, concomitantly inducing single-strand breaks in the DNA. Experimental evidence for single-strand breaks under thymineless conditions in *B. subtilis* has been presented.[39] The repair replication machinery recognizes these breaks and begins to excise nucleotides to produce a gap, as part of the normal repair replication process. But in the absence of thymine the repair DNA polymerase is unable to fill the gap so formed; meanwhile the excision enzyme or enzymes continue to enlarge the gap. Upon readdition of thymine (or BU) to the culture, normal replication resumes, and in addition the repair DNA polymerase proceeds to fill the gaps. A possible cause of lethality may be the arrival of the normal growing point at an unfilled gap. This model, we think, accounts for the observed parallels between thymine deprivation and UV irradiation, the requirement for RNA synthesis for the pathogenic effects of thymine deprivation, and the observed pattern of DNA synthesis after thymine deprivation.

Summary.—An unusual mode of DNA replication occurs in a small fraction of

the DNA in *E. coli* TAU-bar following thymine deprivation. The physical nature of this newly replicated DNA has been studied and it appears similar in most respects to the nonconservative repair replication of lesions induced by ultraviolet irradiation in bacterial DNA. A model is discussed in which the routine transcription of messenger RNA on the DNA results in single-strand breaks which must be repaired. The model accounts for many of the observed pathological effects of thymine deprivation in bacteria.

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