JOURNAL OF BACTERIOLOGY, Mar. 1979, p. 1243-1252
0021-9193/79/03-1243/10$02.00/0

Vol. 137, No. 3

Enzymatic Degradation of Uracil-Containing Deoxyribonucleic Acid

V. Survival of *Escherichia coli* and Coliphages Treated with Sodium Bisulfite

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Received for publication 10 October 1978

A number of mutants of *Escherichia coli* defective in the *ung* gene (structural gene for uracil-deoxyribonucleic acid [ura-DNA] glycosylase) are shown to be abnormally sensitive to treatment with sodium bisulfite when compared with congenic *ung*$^+$ strains. These results provide further evidence that sodium bisulfite causes the deamination of cytosine to uracil in DNA and that ura-DNA glycosylase is required for the repair of U-G mispairs. The effect of the chemical is apparently selective with respect to base damage; coliphages containing cytosine in their DNA are inactivated by treatment with sodium bisulfite, whereas those containing hydroxymethylcytosine are not. ura-DNA glycosylase and the major apurinic-apyrimidinic endonuclease of *E. coli* may function in the same repair pathway, since the extent of inactivation of a congenic set of strains which are *ung xth* (structural gene for the major apurinic-apyrimidinic endonuclease of *E. coli*) or *ung xth*$^+$ is the same.

A number of studies in recent years have supported the existence of a base excision repair mode for the removal of uracil from the DNA of living cells. Experimental evidence for this repair mode is based partly on the isolation of an enzyme termed uracil-DNA glycosylase (ura-DNA glycosylase) from a number of procaryote and eucaryote sources (2, 7, 9, 11, 17, 20, 23). In vitro the enzyme catalyzes the release of uracil as the free base from either single- or double-stranded DNA or deoxyribopolymers (2, 23). Studies with *Escherichia coli* have shown that uracil normally incorporated into DNA during DNA synthesis is excised in wild-type cells but not in mutants defective in ura-DNA glycosylase (*ung*). Such mutants consequently accumulate significant amounts of uracil in their DNA (16, 29, 39-42). Similar results have been obtained with *Bacillus subtilis* (28, 38) and with polyoma DNA (1).

Uracil in DNA can also be generated by the deamination of cytosine. This process occurs spontaneously in a temperature-dependent reaction with measurable rate constants (24). Cytosine deamination is greatly facilitated by treatment with certain chemicals that promote this reaction (14, 31, 32, 34, 37, 43). We have previously reported that *ung* mutants of *E. coli* are abnormally sensitive to nitrous acid compared with *ung*$^+$ controls (3). However, this compound has pleiotropic effects on DNA. In addition to the deamination of cytosine, it promotes the deamination of adenine and guanine and is also reported to cause cross-links (6). To study the molecular mechanisms of uracil excision repair, including the biochemical steps that follow removal of the base, we have sought an agent that is more selective in its effect on DNA.

Studies on the interaction of sodium bisulfite with bases, nucleosides, nucleotides, and DNA suggest that this chemical may fulfill the selectivity we seek. Sodium bisulfite has been reported to cause adduct formation with both thymine and cytosine and to promote DNA-protein cross-linking and free radical formation (14). However, in a recent comprehensive review, Hayatsu (14) has indicated that bisulfite-induced deamination of cytosine in DNA is highly selective under defined experimental conditions. Under these conditions no deamination of adenine or guanine moieties is detected. Although bisulfite-thymine adducts are formed, these are transient and readily reversible (15). In the present studies we show that treatment of *E. coli* with sodium bisulfite under defined experimental conditions causes inactivation of the organism and that all *ung* strains tested are more sensitive to this inactivation than congenic *ung*$^+$ strains. Strains defective in the major apurinic-apyrimidinic (AP) endonuclease of *E. coli*

(*xth*) are also abnormally sensitive to treatment with sodium bisulfite; however, strains bearing the double mutation *ung xth* are no more sensitive than either congenic single mutant. (The term AP endonucleases was adopted for general use at the ICN-UCLA Winter Conference on DNA Repair Mechanisms [see reference 25] to designate a general class of endonucleases that catalyze hydrolysis of phosphodiester bonds in DNA containing either apurinic or apyrimidinic sites.) On the other hand, mutants defective in both DNA polymerase I activity and ura-DNA glycosylase activity are more sensitive than congenic *ung* mutants. In addition, we show that T-odd phages are sensitive to sodium bisulfite, whereas T-even bacteriophages (which contain hydroxymethylcytosine [HMC] in their DNA instead of cytosine) are insensitive to inactivation by sodium bisulfite under our experimental conditions.

MATERIALS AND METHODS

Bacterial and phage stocks. Table 1 lists the bacterial strains used and their origins. All strains were maintained on 1.5% agar plates with 1% tryptone (Difco), 0.5% yeast extract (Difco), and 1% sodium chloride. Bacteriophages T2, T3, T4, T5, and T7 are maintained in our laboratory. Phage T4 2095 was generously provided by John S. Wiberg and contains the following mutations: *amN*55×5 (gene 42); *amE*51 (gene 56); *nd*28 (gene *denA*); H23 (*denB*); *alc* (gene *alc*). This mutant was grown in *E. coli* strain K803 to generate phages containing HMC in their DNA. When grown on strain B834 this phage has most, if not all, of its HMC replaced by cytosine (J. S. Wiberg, personal communication). To prepare phage with cytosine-containing DNA, strain T4 2095 was plaque purified on *E. coli* K803 and then grown in *E. coli* B834 with four serial passages. For all phage production cells were grown in double-strength nutrient broth and infected at a multiplicity of approximately 1.0. Cultures were incubated with shaking at 37°C for 3 h, after which chloroform was added to complete cell lysis.

Enzymes. Purified *E. coli* ura-DNA glycosylase was a gift from Bruce Duncan.

Measurement of bacterial sensitivity to sodium bisulfite. For experiments with stationary-phase cells, bacteria were grown overnight in double-strength nutrient broth (Difco) at 37°C. Cells were harvested by centrifuging 10.0 ml of the culture at 6,000 × *g* for 10 min, and the pellets were resuspended in 10.0 ml of 0.1 M sodium phosphate buffer, pH 5.3. For experiments with logarithmically growing cells, bacteria from overnight cultures were diluted 1:10 in double-strength nutrient broth and grown to a density of 2×10^8 cells per ml. Cultures (50 ml) were centrifuged as described above and resuspended in 10.0 ml of 0.1 M sodium phosphate buffer, pH 5.3. In all experiments in which pairs of strains were compared in logarithmic phase, growth rates were monitored and found to be essentially identical.

Sodium bisulfite solution was prepared immediately

TABLE 1. *Bacterial strains used*

Strain		Genotype	Source
W3110		*thyA deo-?*	Bruce Duncan (4)
PA3306		*thi-1 argH1 nadB4 purI66 lacY1 malA1 xyl-7 ara-13 mtl-2 gal-16 rspL9 tonA2 supE44*	Bruce Duncan (4)
BD10		W3110, *ung-1*	Bruce Duncan (4)
BD13		W3110, *ung-2*	Bruce Duncan (4)
BD1117	congenic[a]	*thi-1 argH1 lacY1 malA1 mtl-2 xyl-7 ara-13 gal-6 rpsL9 tonA2* λ *supE44*	Bruce Duncan
BD1118		BD1117, *ung-1*	Bruce Duncan
BD1119		BD1117, *ung-1*	Bruce Duncan
BD1180	congenic[a]	*thi-1 pyrE lac*(MS286) φ80dII *lac*(BK1) Str^r	Bruce Duncan
BD1179		BD1180, *ung-1*	Bruce Duncan
BD1154	congenic[a]	*thi-1 argH1 lacY1 malA1 xyl-7 rha-6 ara-13 gal-7 rpsL9 tonA2 supE44* $T2^r$ *rel-1?*	Bruce Duncan
BD1153		BD1154, *ung-1*	Bruce Duncan
BKT406		BD1154, *polA1*[b]	Bik-Kwoon Tye
BKT411		BD1153, *polA1*[b]	Bik-Kwoon Tye
BW273	congenic[a]	*Hfr rel-1 ton-10* at 81 min	Bernard Weiss
BW271		BW273, *ung-1*	Bernard Weiss
BW275		BW273, *ung-1* Δ(*pncA-xth*)	Bernard Weiss
BW277		BW273, Δ(*pnc-xth*)	Bernard Weiss

[a] The term congenic refers to a set of strains derived by phage P_1 transduction from a single parent. In one case strain BKT406 was derived from parent strain BD1154, whereas strain BKT411 was derived from parent strain BD1153. However, BD1154 and 1153 are also related by P_1 transduction.

[b] The *polA* mutation is derived from strain BT4113, which at 30°C has approximately 20% of the wild-type DNA polymerase activity and about 80% of the wild-type 5′ → 3′ exonuclease activity (30).

before use as follows. A solution of 1.0 M sodium bisulfite and 0.05% sodium thioglycolate was prepared in 0.2 M sodium phosphate buffer (pH 6.0), to give a final pH of 5.3. The solution was sterilized through a prewashed 0.45-μm Millex filter (Millipore Corp.). Sterile sodium bisulfite (18.0 ml) was transferred to sterile, screw-capped, 25-ml Erlenmeyer flasks. Bacterial suspension (2.0 ml at 10^9 cells per ml) was added to each, and the flasks were gassed for approximately 30 s with nitrogen and then sealed tightly. The flasks were incubated in a shaking water bath at 37°C. At designated time intervals 1.0 ml of each sample (containing 10^8 cells) was removed and added to 9.0 ml of sterile 0.1 M Tris-hydrochloride buffer, pH 8.5. A second dilution (1:10) was immediately made in the same buffer, and samples were placed on ice for a minimum of 10 min before making serial dilutions in 0.1 M Tris-hydrochloride buffer at pH 8.5 and plating on yeast extract-tryptone plates. After removal of samples for each time point, the flasks were regassed with nitrogen before continuing the incubation. The pH of each incubation with sodium bisulfite was measured before and after the experiment and showed very little, if any, change. Control incubations without the addition of sodium bisulfite were performed in 0.9 M NaCl plus 0.2 M sodium phosphate buffer, pH 5.3.

Measurement of phage sensitivity to sodium bisulfite. Phage were diluted into a solution containing 0.1 M NaCl, 1.0 mM $MgCl_2$, and 0.1 mM $CaCl_2$ to give a final concentration of 10^8 plaque-forming units/ml in all cases except the experiments with strain T4 2095, in which case the starting concentration of both the mutant and its wild-type T4 partner were 10^6 plaque-forming units/ml. Treatment with sodium bisulfite was carried out as described for the bacteria. In most cases phage were titrated on *E. coli* BD1117 (*ung*$^+$). Strain 2095 and its wild-type T4 partner were titrated on *E. coli* K803. Control incubations were performed with phage in 0.9 M NaCl in 0.2 M sodium phosphate buffer, pH 5.3.

Assay of ura-DNA glycosylase. All *ung* strains were tested for ura-DNA glycosylase activity and found to contain less than 1% wild-type levels. Cells were grown to stationary phase in double-strength nutrient broth and harvested by centrifugation at 6,000 × *g* for 10 min. Cells were resuspended in 10 mM Tris-hydrochloride buffer, pH 7.5, and sonically treated in a Sonifier cell disruptor, model 350 (Branson Sonic Power). The extract was clarified by centrifugation at 27,000 × *g* for 90 min at 4°C and made 5.0 M with respect to NaCl. Nucleic acid was removed from the supernatant by phase partitioning in polyethylene glycol 6000 and dextran 500 (10). The polyethylene glycol phase was extensively dialyzed against 10 mM Tris-hydrochloride buffer (pH 7.5) containing 6% polyethylene glycol. This fraction was assayed for ura-DNA glycosylase activity according to the procedure of Duncan et al. (5). Incubation mixtures (0.15 ml) contained [^{3}H]polydeoxyuridylic acid (75 pmol as nucleotide), 10.0 mM Tris-hydrochloride buffer, pH 7.5, 25 mM NaCl, 1 mM EDTA, 0.1% bovine serum albumin, and 2 to 20 μg of protein. Incubations were for 20 min at 37°C. ura-DNA glycosylase activity was measured by the release of acid-soluble radioactivity as described previously (5).

Detection of uracil in the DNA of bacteria and bacteriophage. DNA from *E. coli* strain 15 T and bacteriophages T2 and T5 were labeled with [^{3}H]-thymine and purified as previously described (10). The specific activity of the *E. coli* DNA was 3.5×10^3 cpm/μg, and those of T2 and T5 DNA were 2.54×10^4 and 5.66×10^4 cpm/μg, respectively. *E. coli* DNA (5.0 ml at 440 μg/ml), T2 DNA (1 ml at 18 μg/ml), and T5 DNA (1 ml at 12 μg/ml) in 10 mM Tris-hydrochloride buffer (pH 7.6) plus 1 mM EDTA were alkali denatured by titrating with 1 N NaOH to a final pH of 12.3. The DNAs were held at this pH for 10 min and then neutralized with 1 N HCl and dialyzed against 2 liters of 10 mM Tris-hydrochloride buffer, pH 7.6. One milliliter of each DNA sample was mixed with an equal volume of 0.1 M sodium phosphate buffer, pH 5.3. Each solution was placed in a small vial and made 0.9 M with respect to sodium bisulfite and 0.05% with respect to sodium thioglycolate. The vials were gassed with nitrogen, sealed, and incubated in a shaking water bath at 37°C for 2 h, after which the pH of the DNA solutions was adjusted to 8.4 with 1 M Tris base and each solution was kept on ice for 30 min. The DNA was then dialyzed against 10 mM Tris-hydrochloride buffer, pH 8.1.

Sodium bisulfite-treated DNA was incubated with purified *E. coli* ura-DNA glycosylase. Incubation mixtures (50 μl) contained *E. coli* DNA (1.9 μg), T2 DNA (0.19 μg), or T5 DNA (0.28 μg), 1 mM EDTA and 10 mM Tris-hydrochloride buffer (pH 7.6) and 34 U of ura-DNA glycosylase; control incubations contained no enzyme. Incubations were at 37°C for 60 min, after which 50 μl of each sample was layered onto density gradients containing 5 to 20% sucrose in 0.1 N NaOH with 0.4 M NaCl and 0.1 mM EDTA. The gradients were centrifuged in an SW 50.1 rotor (Beckman Instruments). (See legend 6 for details.) Fractions were collected, and the radioactivity was measured by liquid scintillation spectroscopy.

RESULTS

Incubation of *E. coli* with 0.9 M sodium bisulfite under the conditions described in Materials and Methods results in inactivation of cells (Fig. 1). This killing is not a result of the acid pH or of the relatively high ionic strength of the bisulfite as indicated by the results obtained in control incubations at pH 5.3 and 0.9 M NaCl (Fig. 1). In any single experiment in which the inactivation of congenic *ung*$^+$ cells was compared, the extent of killing was identical within experimental error. However, despite the establishment of a standard experimental protocol, variation in the absolute amount of cell killing for any given strain was observed in different experiments. This variation probably arises from the marked lability of sodium bisulfite. In addition, we have observed that the sensitivity of different wild-type strains of *E. coli* to sodium bisulfite varies, and for this reason we have mainly considered comparisons between congenic strains.

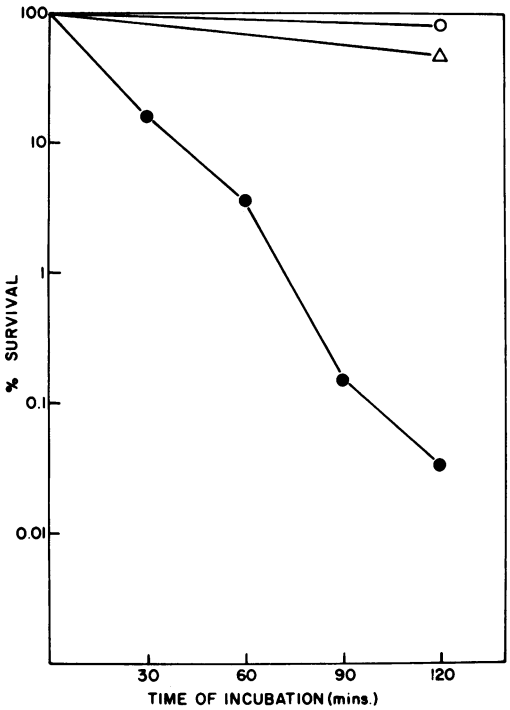

FIG. 1. *Effect of 0.9 M sodium bisulfite at pH 5.3 and 0.9 M sodium chloride at pH 5.3 on the survival of stationary-phase E. coli. Symbols:* (●) *strain BD1180 (ung⁺) treated with sodium bisulfite;* (○) *strain BD1180 (ung⁺) treated with sodium chloride;* (△) *strain BD1179 (ung) treated with sodium chloride. The survival of these strains when tested in logarithmic growth in the presence of 0.9 M sodium chloride at pH 5.3 was essentially identical to the results shown in stationary phase. Further experimental details are provided in the text.*

We found that all strains tested were significantly more sensitive to sodium bisulfite when treated in the logarithmic phase of growth than when treated in the stationary phase of growth (Fig. 2). As indicated under Materials and Methods, to minimize this factor as an experimental variable, all strains tested in any single experiment were grown to the identical cell density, and their growth kinetics were routinely monitored.

The differential sensitivity of *ung*⁺ and *ung* strains to sodium bisulfite was tested in an extensive series of experiments with cells in either the stationary (14 experiments) or the logarithmic (14 experiments) phase of growth. Four different sets of congenic strains (Table 1) were examined as well as two original *ung* isolates (Table 1). The latter were compared directly with the wild-type parent from which they were derived by mutagenesis (4) (Table 1). Of the total experimental trials, in only two experiments (one in logarithmic phase and one in stationary phase) with the congenic pair BD1117/BD1118 was there an equivocal result. In all other cases the *ung* strain was reproducibly more sensitive to sodium bisulfite than the *ung*⁺ partner. Results of typical experiments with the congenic pair BD1117/BD1119 tested in logarithmic and stationary phases are shown in Fig. 2. Qualitatively similar results were obtained with the other congenic strains tested (data not shown). Our general impression based on multiple experiments is that the difference in cell killing between *ung* and *ung*⁺ strains was greater in the stationary than in the logarithmic growth phase. However, in most instances the shape of the survival curves was not sufficiently linear to accurately measure quantitative differences in the slope of the curves.

The sensitivity of strain BW275 (*xth ung*) was no greater than that of either of the congenic strains BW277 (*xth ung*⁺) and BW271 (*xth*⁺ *ung*) (Fig. 3). Strain BKT406 (*polA ung*⁺) was more sensitive to sodium bisulfite than the congenic strain BD1153 (*polA*⁺ *ung*) (Fig. 4a). However in independent experiments, strain BKT411 (*polA ung*) showed the same sensitivity as the congenic strain BKT406 (Fig. 4b).

We also tested the sensitivity of a number of bacteriophages to sodium bisulfite treatment. In all cases the T-odd series of bacteriophages (which contain cytosine in their DNA) were slightly sensitive to the chemical, whereas the T-even phages (which contain HMC in their DNA) were insensitive (Fig. 5a). In addition, when phage T4 2095 was grown under conditions promoting the retention of cytosine in its DNA, selective inactivation was observed compared to wild-type phage T4 (Fig. 5b).

When [^{3}H]thymine-labeled *E. coli* DNA was denatured in alkali and then incubated with sodium bisulfite under the conditions used for bacterial inactivation, sites sensitive to purified *E. coli* ura-DNA glycosylase were detected by alkaline lability (data not shown). Such sites were also detected in bisulfite-treated denatured T5 DNA, but not in T2 DNA similarly treated (Fig. 6). Based on weight average molecular weight differences, we calculated the presence of approximately one endonuclease-sensitive site per 6.4×10^6 daltons of T5 DNA.

DISCUSSION

We previously suggested the use of the term "base excision repair" to make the distinction between excision repair involving the removal of free bases from DNA and that involving removal of nucleotides (nucleotide excision repair) (5, 8). A number of authors (3, 18, 40; T. Lindahl, Prog. Nucleic Acid Res. Mol. Biol., in press) have

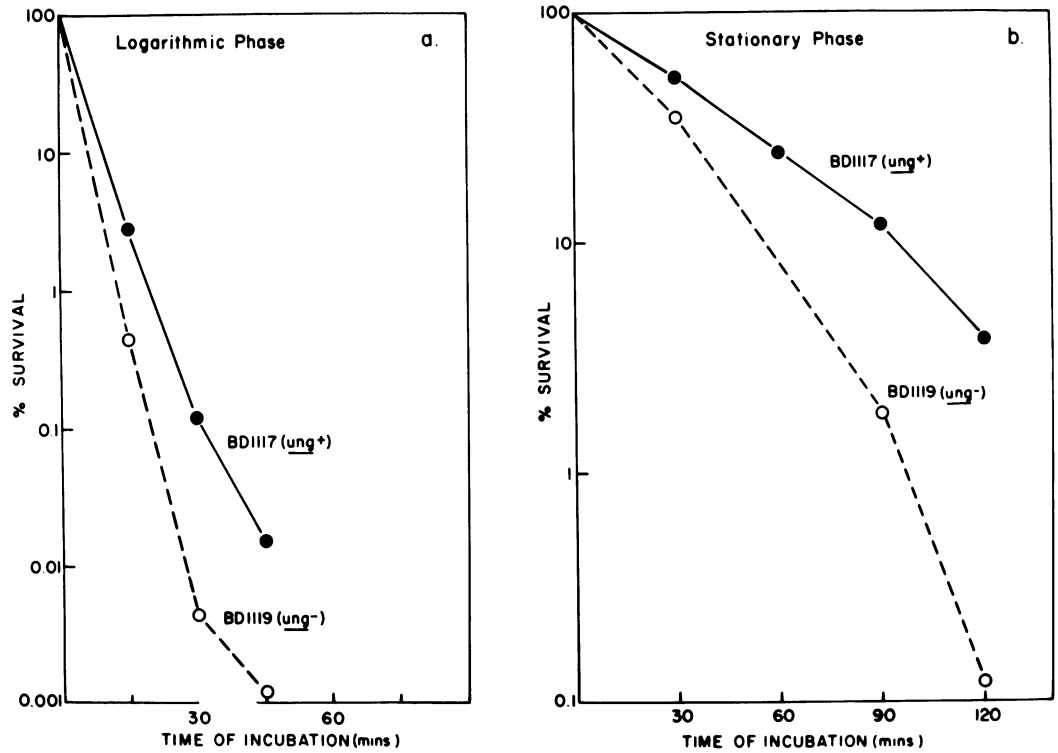

FIG. 2. *Comparative sensitivity of strains BD1117 (ung⁺) and BD1119 (ung) to treatment with sodium bisulfite in logarithmic phase (a) or stationary phase (b). See text for further experimental details.*

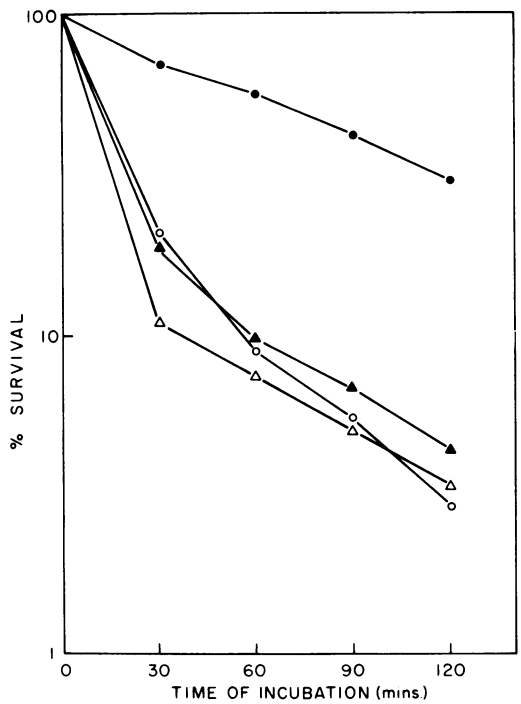

FIG. 3. *Comparative sensitivity of strains BW273 (ung⁺ xth⁺) (●), BW271 (ung xth⁺) (△), BW275 (ung xth) (▲), and BW277 (ung⁺ xth) (○) to sodium bisulfite. The cells were grown to stationary phase. See text for further experimental details.*

proposed models for base excision repair, all of which include the initial enzyme-catalyzed removal of the free base followed by endonucleolytic incision at the sites of base loss, excision of the deoxyribose residue (possibly with other nucleotides), DNA resynthesis, and rejoining. Experimental support for such a general mechanism of repair comes from both enzymological and biological studies. Concerning the former, there are two classes of enzymes that are uniquely suited for a direct role in such a model, DNA glycosylases and AP endonucleases. The isolation and properties of a number of examples of both classes of enzymes have recently been reviewed by Lindahl (22; Lindahl, in press), Friedberg et al. (7), and Linn (25).

Biological evidence for a base excision mode in *E. coli* during the repair of DNA containing U:A base pairs has been provided (29, 39–42, 44). Uracil is incorporated into DNA, occasionally replacing thymine during DNA replication. The extent of uracil incorporation is apparently a reflection of the intracellular dUTP pool size, since in dUTPase-defective strains (*dut*) this

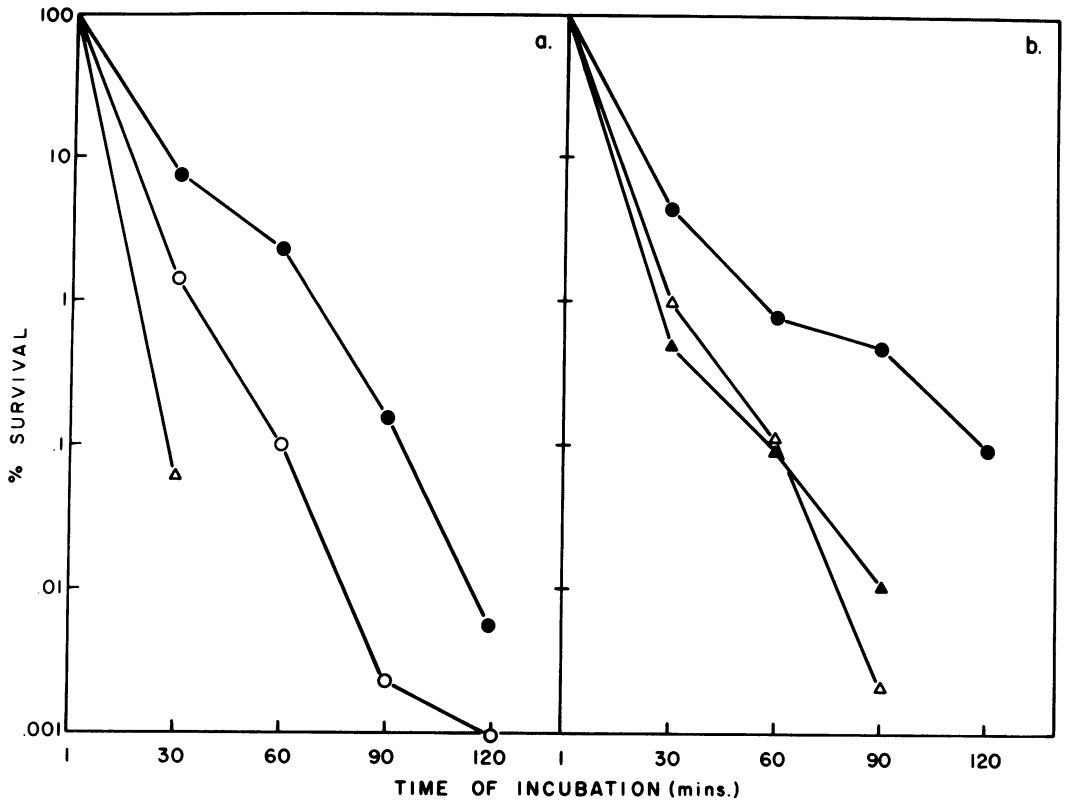

FIG. 4. *Comparative sensitivity of strains BD1154 (ung⁺ polA⁺), BD1153 (ung polA⁺), BKT406 (ung⁺ polA), and BKT411 (ung polA) to sodium bisulfite. (a) ●, BD1154; ○, BD1153; △, BKT406. (b) ●, BD1154; △, BKT406; ▲, BKT411. Only a single data point (at 30 min of incubation) is shown for strain BKT406 in (a) since the 60- and 90-min time points showed 0.001% survival. The experiments were performed on cells in stationary phase.*

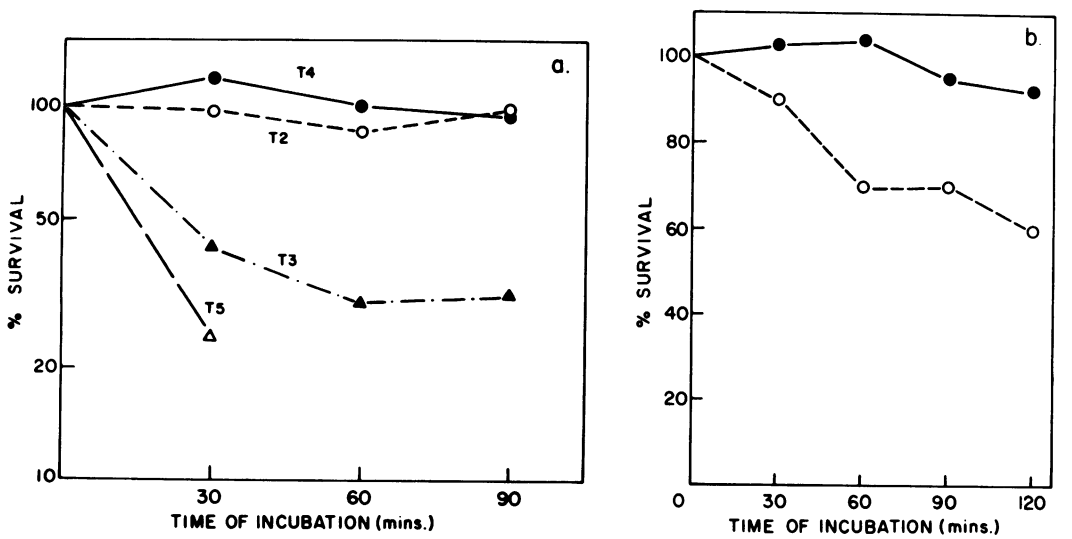

FIG. 5. *Comparative sensitivity of (a) T-odd and T-even phages and (b) phage T4 (wild type) (●) and T42095 (○) to treatment with sodium bisulfite. See text for further experimental details.*

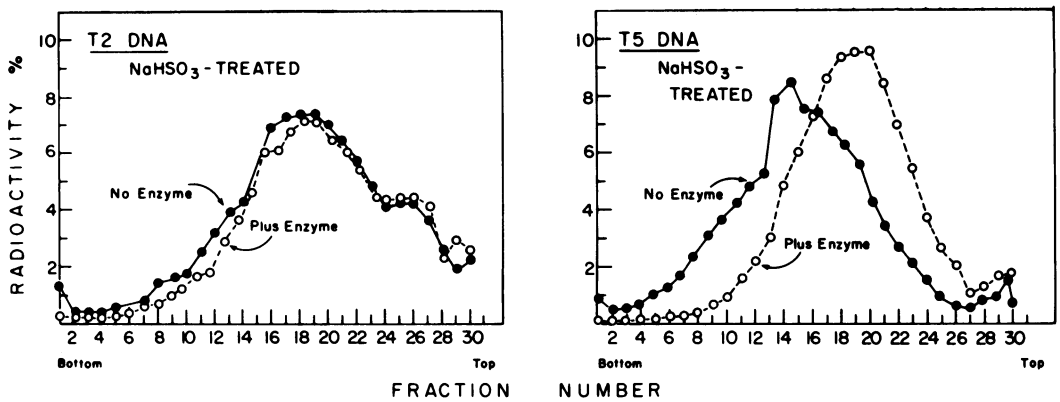

FIG. 6. *Sedimentation profiles of phage T2 DNA (left) and phage T5 DNA (right) treated with sodium bisulfite. Preparation of DNA, treatment with sodium bisulfite, and incubation with or without ura-DNA glycosylase are described in the text. The open symbols indicate incubation in the presence of ura-DNA glycosylase and the closed symbols indicate incubation in its absence. Phage DNA treated with sodium bisulfite was centrifuged for 3 h at 35,000 rpm.*

phenomenon is exaggerated. Under these conditions the presence of normal levels of ura-DNA glycosylase and AP endonuclease(s) results in strand breaks in newly replicated DNA, generating so-called short Okazaki fragments. These fragments are not observed in *ung* strains, however. In addition, the conversion of short DNA fragments to higher-molecular-weight DNA fails to occur in strains of *E. coli* defective in DNA polymerase I activity or DNA ligase. Thus, these studies support a model of base excision repair in *E. coli* involving at least ura-DNA glycosylase, AP endonuclease(s), DNA polymerase I, and DNA ligase.

We have investigated the molecular mechanisms of the repair of uracil present as a mispaired base in DNA (and hence potentially mutagenic) arising by cytosine deamination. The hydrolytic deamination of cytosine and cytidine has been investigated by Shapiro and Klein (35), and that of cytosine monophosphate has been investigated by Lindahl (19). More recently, Lindahl and Nyberg (24) investigated the heat-induced deamination of cytosine in single- and double-stranded *E. coli* DNA and in various deoxyribopolymers. At physiological ionic strength and pH the reaction for denatured *E. coli* DNA has been measured at a rate of $k = 2.2 \times 10^{-7}$ s^{-1} at 95°C. The expected rate at 37°C has been calculated at $k = 2 \times 10^{-10}$ s^{-1}. Cytosine deamination is effectively enhanced by a number of reagents such as nitrous acid (6, 31, 32) and sodium bisulfite (14, 15, 34, 36). We previously reported that mutants of *E. coli* defective in ura-DNA glycosylase, DNA polymerase I (either the polymerizing or the 5′ → 3′ exonuclease moiety of this enzyme), or exonuclease III (which is the major AP endonuclease activity in *E. coli*) are abnormally sensitive to nitrous acid treatment (3). More recently, Making and Munakata (27) reported that a mutant of *B. subtilis* defective in ura-DNA glycosylase activity (*urg*) was not abnormally sensitive to treatment with sodium nitrite. However, in our hands and in those of others (4), the preparation and use of nitrous acid for bacterial inactivation studies requires great care due to the extreme lability of the compound, and it is not clear that the procedure used by Makino and Munakata is comparable to that reported by Da Roza *et al.* (3). In our opinion, the stringent substrate specificity of ura-DNA glycosylase suggests that the increased sensitivity of *ung* mutants to nitrous acid results from the deamination of cytosine in vivo. However, in view of the pleiotropic effects of nitrous acid on DNA, including alteration of purines, depurination, and DNA cross-linking, the relationship of the *polA* and *xth* genes to the excision repair of uracil is less clear in that experimental system.

In the present studies we have investigated the repair of uracil arising from the interaction of cytosine in DNA with sodium bisulfite. This agent appears to be much more selective in its action than nitrous acid (14). Under the conditions used in our experiments purines are not deaminated, and although thymine-bisulfite adducts can be formed, their yield is very poor and they are readily reversible. Hayatsu et al. (15) found that treatment of thymine with 1 M sodium bisulfite at room temperature and pH 6 to 7 followed by dilution in phosphate buffer at pH 7 resulted in quantitative recovery of the starting material. Even heating at 60°C with 3 M sodium bisulfite at pH 6 did not result in thymine modification (15). The deamination of cytosine is

believed to occur in a three-step process involving (i) the acid-dependent formation of a cytosine–bisulfite adduct, (ii) its deamination to a uracil–bisulfite adduct, and (iii) the alkali-dependent loss of the bisulfite ion to give uracil. Sodium bisulfite is very readily oxidized, and free radicals so generated can be reactive toward nucleic acids. However, as pointed out by Hayatsu (14), the use of free radical scavengers or anaerobic conditions reduces this problem. Moreover, most of the free radical reactions are difficult to accomplish at high concentrations of bisulfite.

The present studies provide direct evidence that the treatment of DNA with sodium bisulfite under defined experimental conditions results in the formation of uracil. Thus, when either *E. coli* or phage T5 DNA are incubated with purified ura-DNA glycosylase from *E. coli* and then sedimented in alkaline sucrose gradients, alkaline-labile sites are detected only in the bisulfite-treated DNA. Since this enzyme probe does not recognize hydroxymethyluracil in DNA (9), our failure to observe enzyme-sensitive sites in the phage T2 bisulfite-treated DNA does not indicate the absence of this base. However, after treatment of intact phages with sodium bisulfite, the T-odd series were always more sensitive to killing than the T-even series. In addition, a mutant of T4 containing cytosine in its DNA rather than HMC was also more sensitive. These results suggest that deamination of HMC and/or of the glucosylated form of this base occurs at a significantly lower frequency than that of cytosine, if at all. According to Hayatsu (14), bisulfite reactions with 5-HMC derivatives have not been reported. Since the substitution in the 5-position of cytosine and its glucosylation are the only known features that distinguish the chemistry of DNA in the T-even and T-odd bacteriophages, the relative insensitivity of the former to sodium bisulfite supports the stringent specificity of this reagent for cytosine in DNA. An alternative explanation is that hydroxymethyluracil is indeed formed in the DNA of T-even phages treated with sodium bisulfite, but this base is efficiently repaired by an excision repair pathway involving a phage-coded hydroxymethyluracil-DNA glycosylase. However, we have failed to detect such an activity in extracts of T4-infected *E. coli* using labeled SPO1 DNA (which naturally contains hydroxymethyluracil) as a substrate (R. Simmons and E. C. Friedberg, unpublished data).

In our hands all *ung* strains of *E. coli* tested are reproducibly more sensitive to sodium bisulfite treatment than their congenic *ung*$^+$ parents. Thus, ura-DNA glycosylase is clearly involved in the repair of uracil arising from the deamination of cytosine in vivo. The observation that *E. coli* strains are more sensitive to sodium bisulfite in logarithmic than in stationary phase is consistent with the known affinity of the reagent for single-stranded DNA, since one might expect that in actively growing logarithmic-phase cultures, the concentration of single-stranded regions associated with DNA replication would be greater than in stationary-phase cells. However, such a direct correlation has not been experimentally demonstrated in our studies and other explanations are equally tenable.

Since the repair of uracil incorporated into DNA during semiconservative DNA synthesis has an apparent requirement for AP endonuclease(s), DNA polymerase I, and DNA ligase (39, 40, 42, 44), it is of interest to ask whether the repair of uracil arising by cytosine deamination (and hence mispaired with guanine) occurs by the same enzymatic pathway. Our observation that *xth* mutants defective in the major AP endonuclease of *E. coli* do not demonstrate an additive sensitivity to sodium bisulfite when coupled with an *ung* mutation suggests that these two genes function in a common pathway of repair. These results also suggest that other endonucleases reported to attack sites of base loss in DNA in vitro are not biologically relevant to this form of DNA repair. This is not too surprising since these enzymes have been reported to be present in small amounts in cell-free systems (12, 13, 26). Finally, the observation that *xth* mutants are as sensitive to sodium bisulfite as *ung* mutants argues against alternative repair mechanisms in *E. coli* such as the direct reinsertion of cytosine (or thymine in the case of U:A base pairs) after ura-DNA glycosylase-catalyzed base excision. These arguments are supported by the studies of Weiss et al. (44), who have observed that mutants defective in both dUTPase activity (*dut*) and the major AP endonuclease (*xth*) are inviable in the presence of the *ung*$^+$ genotype. In addition, studies by Friedberg and Lindahl (unpublished data) have failed to detect any activity in extracts of *E. coli* that inserts cytosine into *E. coli* DNA containing depyrimidinated sites. In these studies cytosine in DNA was deaminated by alkali treatment according to the procedure of Ullman and McCarthy (43) and the DNA was reannealed. The uracil present was removed by preincubation with purified ura-DNA glycosylase, and the DNA was incubated with extracts in the presence of [^{14}C]cytosine or -deoxycytidine.

The results obtained with the *polA ung* double mutant might suggest more than a single repair pathway operating after sodium bisulfite treatment of *E. coli*. In our view the most likely interpretation of the increased sensitivity of this

mutant is that DNA strand breaks which cannot be repaired by *polA* mutants are produced by free radicals generated during the incubation, despite the anaerobic conditions of the experiments. This notion is supported by our observations that when DNA is treated with sodium bisulfite in vitro, a significant reduction in the molecular weight of the DNA is observed relative to untreated DNA (unpublished data).

On the basis of these results we postulate that treatment of *E. coli* with sodium bisulfite causes two distinct effects on DNA. One effect is the deamination of cytosine to uracil, the repair of which requires ura-DNA glycosylase, AP endonuclease, and probably also DNA polymerase I and DNA ligase. The other effect is to cause strand breaks, the repair of which requires at least DNA polymerase I and presumably DNA ligase activity.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service research grant CA-12428 from the National Cancer Institute and NP 174B from the American Cancer Society, as well as contract EY-76-S-03-0326 PA 32 with the U.S. Department of Energy. R.S. was supported by an Anonymous Postdoctoral Fellowship in Medicine Fund and the Robert D. Plageman Memorial Fund. E.C.F. is the recipient of Research Career Development Award 5K04 CA 71005 from the National Cancer Institute.

We thank Thomas Bonura, Richard Cone, and Richard Reynolds for helpful discussions and criticisms.

LITERATURE CITED

1. **Brynolf, K., R. Eliasion, and P. Reichard.** 1978. Formation of Okazaki fragments in polyoma DNA synthesis caused by misincorporation of uracil. Cell **13**:573–580.
2. **Cone, R., J. Duncan, L. Hamilton, and E. C. Friedberg.** 1977. Partial purification and characterization of a uracil DNA N-glycosidase from *Bacillus subtilis*. Biochemistry **16**:3194–3201.
3. **Da Roza, R., E. C. Friedberg, B. K. Duncan, and H. R. Warner.** 1977. Repair of nitrous acid damage to DNA in *Escherichia coli*. Biochemistry **16**:4934–4939.
4. **Duncan, B. K., P. A. Rockstroh, and H. R. Warner.** 1978. *Escherichia coli* K-12 mutants deficient in uracil-DNA glycosylase. J. Bacteriol. **134**:1039–1045.
5. **Duncan, J., L. Hamilton, and E. C. Friedberg.** 1976. Enzymatic degradation of uracil-containing DNA. II. Evidence for *N*-glycosidase and nuclease activities in unfractionated extracts of *Bacillus subtilis*. J. Virol. **19**: 338–345.
6. **Freese, E.** 1971. Molecular mechanisms of mutations, p. 1–56. *In* A. Hollaender (ed.), Chemical mutagens. Principles and methods for their detection, vol. 1. Plenum Press, New York.
7. **Friedberg, E. C., T. Bonura, R. Cone, R. Simmons, and C. Anderson.** 1978. Base excision repair of DNA, p. 163–173. *In* P. C. Hanawalt, E. C. Friedberg, and F. C. Fox (ed.), DNA repair mechanisms. Academic Press Inc., New York.
8. **Friedberg, E. C., K. H. Cook, J. Duncan, and K. Mortelmans.** 1977. DNA repair enzymes in mammalian cells. Photochem. Photobiol. Rev. **2**:263–322.
9. **Friedberg, E. C., A. K. Ganesan, and K. Minton.** 1975. *N*-glycosidase activity in extracts of *Bacillus subtilis* and its inhibition after infection with bacteriophage PBS2. J. Virol. **16**:315–321.
10. **Friedberg, E. C., and J. J. King.** 1971. Dark repair of ultraviolet-irradiated deoxyribonucleic acid of bacteriophage T4. Purification and characterization of a dimer-specific phage-induced endonuclease. J. Bacteriol. **106**: 500–507.
11. **Friedberg, E. C., J. M. Rudé, K. H. Cook, U. K. Ehmann, K. Mortelmans, J. E. Cleaver, and H. Slor.** 1977. Excision repair in mammalian cells and the current status of xeroderma pigmentosum, p. 21–36. *In* W. W. Nichols and D. G. Murphy (ed.), DNA repair processes. Symposia Specialists, Miami, Fla.
12. **Gates, F. T., and S. Linn.** 1977. Endonuclease V of *Escherichia coli*. J. Biol. Chem. **252**:1647–1653.
13. **Gates, F. T., and S. Linn.** 1977. Endonuclease from *Escherichia coli* active upon duplex DNA damaged by ultraviolet light, osmium tetroxide, acid or X-rays. J. Biol. Chem. **252**:2802–2807.
14. **Hayatsu, H.** 1976. Bisulfite modification of nucleic acids and their constituents. Prog. Nucleic Acid Res. Mol. Biol. **16**:75–124.
15. **Hayatsu, H., Y. Wataya, Kai, and S. Lida.** 1970. Reactions of sodium bisulfite with uracil, cytosine and their derivatives. Biochemistry **9**:2858–2865.
16. **Hochhauser, S. J., and B. Weiss.** 1978. *Escherichia coli* mutants deficient in deoxyuridine triphosphatase. J. Bacteriol. **134**:157–166.
17. **Kuhnlein, U., B. Lee, and S. Linn.** 1978. Human uracil DNA N-glycosidase: studies in normal and repair defective cultured fibroblasts. Nucleic Acids Res. **5**:117–125.
18. **Laval, J.** 1977. Two enzymes are required for strand incision in repair of alkylated DNA. Nature (London) **269**:829–832.
19. **Lindahl, T.** 1967. Irreversible heat inactivation of transfer ribonucleic acids. J. Biol. Chem. **242**:1970–1973.
20. **Lindahl, T.** 1974. An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proc. Natl. Acad. Sci. U.S.A. **71**:3649–3653.
21. **Lindahl, T.** 1976. New class of enzymes acting on DNA. Nature (London) **259**:64–66.
22. **Lindahl, T., P. Karran, and S. Riazuddin.** 1978. DNA glycosylases of *Escherichia coli*, p. 179–182. *In* P. C. Hanawalt, E. C. Friedberg, and F. C. Fox (ed.), DNA repair mechanisms. Academic Press Inc., New York.
23. **Lindahl, T., S. Ljungquist, W. Siegert, B. Nyberg, and B. Sperens.** 1977. Properties of uracil-DNA glycosidase from *Escherichia coli*. J. Biol. Chem. **252**: 3286–3294.
24. **Lindahl, T., and B. Nyberg.** 1974. Heat-induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry **13**:3405–3410.
25. **Linn, S.** 1978. Workshop summary: enzymology of base excision repair, p. 175–178. *In* P. C. Hanawalt, E. C. Friedberg, and F. C. Fox (ed.), DNA repair mechanisms. Academic Press Inc., New York.
26. **Ljungquist, S.** 1977. A new endonuclease from *Escherichia coli* acting at apurinic sites in DNA. J. Biol. Chem. **252**:2808–2814.
27. **Makino, F., and N. Munakata.** 1977. Isolation and characterization of a *Bacillus subtilis* mutant with a defective *N*-glycosidase activity for uracil-containing deoxyribonucleic acid. J. Bacteriol. **131**:438–445.
28. **Makino, F., and N. Munakata.** 1978. Deoxyuridine residues in DNA of thymine-requiring *Bacillus subtilis* strains with defective *N*-glycosidase activity for uracil-containing DNA. J. Bacteriol. **134**:24–29.
29. **Olivera, B. M.** 1978. DNA intermediates at the *Escherichia coli* replication fork: effect of dUTP. Proc. Natl. Acad. Sci. U.S.A. **75**:238–242.
30. **Olivera, B. M., and F. Bonhoeffer.** 1974. Replication of *Escherichia coli* requires DNA polymerase I. Nature (London) **250**:513–514.
31. **Schuster, H.** 1960. Die Reaktionsweise der Desoxyribonucleinsäure mit salpetriger Säure. Z. Naturforsch. Teil

B **15**:298–304.

32. **Schuster, H.** 1960. The reaction of nitrous acid with deoxyribonucleic acid. Biochem. Biophys. Res. Commun. **2**:320–323.
33. **Sekiguchi, M., H. Hayakawa, F. Makino, K. Tanaka, and Y. Okado.** 1976. A human enzyme that liberates uracil from DNA. Biochem. Biophys. Res. Commun. **73**:293–299.
34. **Shapiro, R., B. Braverman, J. B. Louis, and R. E. Servis.** 1973. Nucleic acid reactivity and conformation. II. Reaction of cytosine and uracil with sodium bisulfite. J. Biol. Chem. **248**:4060–4064.
35. **Shapiro, R., and R. S. Klein.** 1966. The deamination of cytidine and cytosine by acidic buffer solutions. Mutagenic implications. Biochemistry **5**:2358–2362.
36. **Shapiro, R., R. E. Servis, and M. Welcher.** 1970. Reactions of uracil and cytosine derivatives with sodium bisulfite. A specific deamination method. J. Am. Chem. Soc. **92**:422–424.
37. **Shapiro, R., and H. Yamaguchi.** 1972. Nucleic acid reactivity and confirmation. 1. Deamination of cytosine by nitrous acid. Biochim. Biophys. Acta **281**:501–506.
38. **Tamanoi, F., and T. Okazaki.** 1978. Uracil incorporation into nascent DNA of thymine-requiring mutant of *Bacillus subtilis 168*. Proc. Natl. Acad. Sci. U.S.A. **75**: 2195–2199.
39. **Tye, B.-K., J. Chien, I. R. Lehman, B. K. Duncan, and H. R. Warner.** 1978. Uracil incorporation: a source of pulse-labeled DNA fragments in the replication of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. U.S.A. **75**:233–237.
40. **Tye, B.-K., and I. R. Lehman.** 1977. Excision repair of uracil incorporated in DNA as a result of a defect in dUTPase. J. Mol. Biol. **117**:293–306.
41. **Tye, B.-K., P.-O. Nyman, and I. R. Lehman.** 1978. Excision repair of uracil during replication of ϕX 174 DNA *in vitro*. Biochem. Biophys. Res. Commun. **82**: 434–441.
42. **Tye, B.-K., P. Nyman, I. R. Lehman, S. Hochhauser, and B. Weiss.** 1977. Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:154–157.
43. **Ullman, J. S., and B. J. McCarthy.** 1973. Alkali deamination of cytosine residues in DNA. Biochim. Biophys. Acta **294**:396–404.
44. **Weiss, B., S. G. Rogers, and A. F. Taylor.** 1978. The endonuclease activity of exonuclease III and the repair of uracil-containing DNA in *Escherichia coli*, p. 191–194. *In* P. C. Hanawalt, E. C. Friedberg, and F. C. Fox (ed.), DNA repair mechanisms. Academic Press Inc., New York.