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# Recovery from *N*-Hydroxyurethan-Induced Death

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Bacteria (*Escherichia coli*) can recover from the lethal action of *N*-hydroxyurethan when they are incubated in drug-free liquid medium. This recovery, which is dependent upon energy metabolism, does not occur on solid medium. Recovery is accompanied by repair of the cellular deoxyribonucleic acid (DNA). A bacterial mutant deficient in DNA polymerase was extremely sensitive to the lethal action of hydroxyurethan. Data are presented which support the concept that DNA replication and DNA repair are mediated by different enzymes and that only the former process is inhibited by hydroxyurea.

In an accompanying paper (12), it is reported that *N*-hydroxyurethan [HUN; HON(—H)—C(=O)—$OC_2H_5$] kills growing bacteria (*Escherichia coli*), and that cultures treated concomitantly with HUN and inhibitors of macromolecular biosynthesis were protected from this lethal action. It is shown that HUN-induced death is due primarily to a drug-induced alteration of the cellular deoxyribonucleic acid (DNA). In this report we describe experiments in which the consequences of removal of HUN from treated cultures were investigated.

## MATERIALS AND METHODS

**Materials.** *N*-hydroxyurethan was obtained either from New England Nuclear Corp., or from Aldrich Chemical Co., and hydroxyurea was from E. R. Squibb & Sons. Chloramphenicol was purchased from Parke, Davis & Co., sodium azide from Fisher Scientific Co., puromycin from Nutritional Biochemical Corp., and sodium arsenate from Mallinckrodt Chemical Works. $^3$H-uridine, $^3$H-deoxycytidine, and $^3$H-cytidine were obtained from Schwarz BioResearch, Inc., and $^3$H-thymidine was from International Chemical and Nuclear Corp.

**Methods.** Most of the procedures employed in these studies were described in the accompanying paper (12). HUN was removed from treated cultures by harvesting the bacteria, and the cultures were washed twice with physiological saline and resuspended in the original volume of fresh medium.

Colorimetric analyses for cellular DNA, ribonucleic acid (RNA), and proteins were done by a previously described procedure (18).

**Bacterial strains.** Unless indicated otherwise, all experiments described herein were performed with *E. coli* C600 growing in medium HA (17). *E. coli* K-12 $u^-$ $met^-$, a double auxotroph requiring uracil and methionine, was obtained from Rivka Rudner, Hunter College of the City of New York. For determination of the viability of these strains, portions (0.1 ml) of the cultures were plated either on Columbia base agar (Baltimore Biological Laboratories) or HA-containing agar (1.5% Difco agar in medium HA) plates.

*E. coli* p3478, a strain deficient in DNA polymerase (3), and the parent strain *E. coli* W3110 $thy^-$ were generously provided by John Cairns, Cold Spring Harbor Laboratory. These strains were grown in medium HA supplemented with thymine (5 μg/ml), and colony-forming units were enumerated by plating on an agar medium of the same composition.

## RESULTS

**Recovery of cells.** In the accompanying paper (12), it was shown that treatment of *E. coli* C600 with 0.05 M HUN for 3 hr resulted in extensive killing of the bacteria. The results of an experiment in which HUN was removed from a culture of *E. coli* C600 after 3.5 hr of treatment are shown in Fig. 1. The data indicate that after removal of the drug the bacteria recovered from its seemingly lethal effect. Recovery is defined here as an increase in the number of viable bacteria which is greater than that resulting from the division of those cells that were viable at the time of removal of HUN.

A control experiment (*not shown*) in which a growing culture of *E. coli* C600 was diluted to a density of about $10^4$ cells/ml (the approximate number of viable cells remaining after 3.5 hr of treatment with 0.05 M HUN) was also performed. The growth rate of this diluted culture was consistent with that obtained normally for this organism in medium HA, thus indicating

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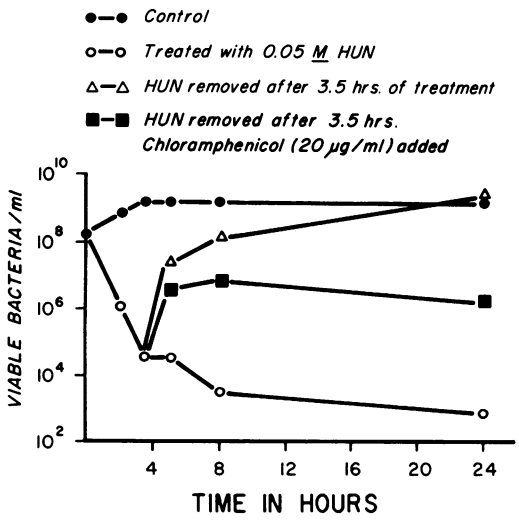


FIG. 1. *Recovery of Escherichia coli from the lethal action of N-hydroxyurethan (HUN). An exponentially growing culture of E. coli C600 was divided into portions, one of which received HUN (final concentration 0.05 M). After 3.5 hr of exposure to the drug, the control and a portion of the treated culture were chilled, and the remainder of the treated culture was washed free of HUN and resuspended in fresh medium HA. The cultures were incubated at 37 C and the number of viable bacteria was determined at intervals.*

that dilution of cells to $10^4$ per ml did not result in an increase in the growth rate.

Because the manner of removing the drug—dilution and immediate plating of the cells, as opposed to washing followed by aeration in fresh medium—influenced the number of bacteria that survived HUN treatment, it was necessary to determine whether the lethal effect was a function of the composition of the plating medium used, since Columbia base agar is more nutritious than the liquid medium (HA) in which exposure to the drug took place. To test this possibility, bacteria were exposed to HUN, and portions of the culture were removed and plated on both Columbia base and HA-agar plates. It was found (data not shown) that HUN-induced death was independent of the composition of the plating medium used.

To determine which metabolic processes were required for recovery, inhibitors of macromolecular biosyntheses were added when treated cells were resuspended in HUN-free medium. It was thus found that neither chloramphenicol (Fig. 1) nor puromycin (Table 1), two inhibitors of protein synthesis (4, 13), impaired the recovery of bacteria from HUN-induced death. Nor was DNA production required for the reversal of the effects of HUN, as the presence of hydroxyurea, an inhibitor of DNA synthesis (20), did not interfere with the initial rate of recovery (Table 1).

Uracil deprivation of a strain requiring this pyrimidine for growth revealed (Table 2) that RNA synthesis was also not required for the recovery process.

Having established that inhibitors of DNA, RNA, and protein synthesis did not prevent recovery from HUN-induced death, the possibility was considered that this was an energy-requiring process. Sodium azide, an inhibitor of oxidative phosphorylation (2, 10), was added, therefore, to washed cultures. In the presence of this inhibitor, the rate of repair was retarded and its extent was reduced (Table 1). Although $NaN_3$ is a potent inhibitor of oxidative phosphorylation, it does not inhibit the production of adenosine triphosphate (ATP) completely in *E. coli*, because this organism can ferment glucose, thus insuring a supply of ATP that is independent of oxidative phosphorylation. Because of the incomplete inhibition of ATP production by $NaN_3$, we used sodium

TABLE 1. *Effects of inhibitors on recovery from N-hydroxyurethan (HUN)-induced death*[a]

| Time after removal of HUN (hr) | Inhibitor tested[b] | Viable cells/ml | |
|---|---|---|---|
| | | Control | Inhibitor added |
| 0 | PM, 90 μg/ml | $7.8 \times 10^4$ | $7.8 \times 10^4$ |
| 1 | | $4.9 \times 10^6$ | $2.9 \times 10^6$ |
| 2.5 | | $1.1 \times 10^7$ | $5.9 \times 10^6$ |
| 4 | | $3.6 \times 10^7$ | $6.5 \times 10^6$ |
| 20.5 | | $3.7 \times 10^9$ | $2.0 \times 10^7$ |
| 0 | HU, 0.10 M | $5.2 \times 10^4$ | $5.2 \times 10^4$ |
| 4.5 | | $2.9 \times 10^7$ | $1.6 \times 10^7$ |
| 0 | $NaN_3$, 0.01 M | $5.4 \times 10^4$ | $5.4 \times 10^4$ |
| 1.5 | | $6.9 \times 10^6$ | $2.9 \times 10^5$ |
| 4 | | $8.0 \times 10^7$ | $3.4 \times 10^6$ |
| 0 | Arsenate, 0.10 M | $5.5 \times 10^4$ | $5.5 \times 10^4$ |
| 1 | | $2.4 \times 10^6$ | $1.2 \times 10^5$ |
| 2.5 | | $5.7 \times 10^6$ | $1.3 \times 10^5$ |
| 4 | | $2.5 \times 10^7$ | $1.7 \times 10^5$ |

[a] Exponentially growing cultures of *E. coli* were exposed to 0.05 M HUN for 3.5 hr, whereupon bacteria were harvested, washed free of HUN, suspended in fresh drug-free medium, and divided into two portions, one of which was supplemented with an inhibitor. The cultures were shaken at 37 C, and the number of viable cells was determined at intervals. Each set of experiments included the following controls: (i) an untreated culture, (ii) a culture not exposed to HUN but supplemented with an inhibitor (to test the effect of the inhibitor alone), and (iii) a culture exposed to HUN for the duration of the experiment.

[b] Abbreviations: HU, hydroxyurea; HUN, hydroxyurethan; $NaN_3$, sodium azide; PM, puromycin.

TABLE 2. *Effect of uracil starvation on the ability of a uracil-requiring strain to recover from N-hydroxyurethan (HUN)-induced death*[a]

| Time after removal of HUN (hr) | Viable cells/ml | |
|---|---|---|
| | Uracil added | Uracil absent |
| 0 | $2.9 \times 10^5$ | $2.9 \times 10^5$ |
| 1.5 | $8.9 \times 10^7$ | $3.2 \times 10^7$ |
| 20 | $1.3 \times 10^9$ | $6.3 \times 10^7$ |

[a] Bacteria (*E. coli* K-12 U⁻) in medium HA (17) containing uracil (25 μg/ml) were brought to the exponential growth phase, whereupon they were exposed to 0.05 M HUN for 3 hr. The bacteria were then washed free of HUN and resuspended in fresh medium. Half of the culture was allowed to recover in the presence of uracil (25 μg/ml), and the other half was incubated in the absence of this pyrimidine.

TABLE 3. *Effect of temperature on the recovery of N-hydroxyurethan (HUN)-treated bacteria*[a]

| Expt | Time after removal of HUN (hr) | Temp (C) | Viable cells/ml | |
|---|---|---|---|---|
| | | | Cells previously exposed to HUN | Control cultures not preexposed to HUN |
| I | 0 | | $3.2 \times 10^4$ | $1.6 \times 10^8$ |
| | 1.5 | 2 | $4.3 \times 10^4$ | $1.6 \times 10^8$ |
| | 3.5 | 2 | $5.0 \times 10^4$ | $1.6 \times 10^8$ |
| | 1.5 | 37 | $5.5 \times 10^6$ | $1.5 \times 10^9$ |
| | 3.5 | 37 | $5.6 \times 10^7$ | $2.1 \times 10^9$ |
| II | 0 | | $4.3 \times 10^4$ | $1.9 \times 10^8$ |
| | 1.5 | 23 | $7.1 \times 10^6$ | |
| | 3.5 | 23 | $1.5 \times 10^7$ | |
| | 1.5 | 37 | $1.4 \times 10^7$ | $1.5 \times 10^9$ |
| | 3.5 | 37 | $8.5 \times 10^7$ | $1.8 \times 10^9$ |
| III | 0 | | $3.1 \times 10^4$ | $1.3 \times 10^8$ |
| | 1.5 | 42 | $1.3 \times 10^7$ | |
| | 3.5 | 42 | $7.3 \times 10^7$ | $7.3 \times 10^7$ |
| | 1.5 | 37 | $9.5 \times 10^6$ | $1.8 \times 10^9$ |
| | 3.5 | 37 | $4.5 \times 10^7$ | $2.2 \times 10^9$ |

[a] Bacteria in the exponential growth phase were exposed to 0.05 M HUN for 3.5 hr, and were then washed free of HUN and incubated with aeration at the temperature indicated. At intervals, samples were withdrawn for the determination of the number of viable cells. Cultures previously unexposed to HUN served as controls to determine the effect of temperature shifts on the growth of normal *E. coli*.

arsenate, which blocks oxidative as well as glycolytic processes (24). It could thus be shown (Table 1) that this inhibitor blocked repair almost completely, and it thus appears that recovery is favored when ATP production is maximal.

The apparent need for ATP production should be reflected in a temperature dependence of the repair process. It was demonstrated (Table 3) that recovery was blocked at 2 C and retarded at 23 C.

To determine whether the repair process could be differentiated further from normal cellular replication, the effect of exposure to 42 C on the recovery process was investigated. It was thus shown (Table 3) that, although the rate of multiplication of normal cells decreased when shifted from 37 to 42 C, this shift had no effect on recovery, thereby indicating that repair is not subject to the normal constraints imposed upon bacterial division.

As length of exposure to HUN was increased, bacteria progressively lost the ability to recover from drug-induced death (Table 4). Recovery occurred after as much as 5 hr of exposure to HUN, and the process occurred at a slower rate after 6 hr of treatment. After 9 hr of treatment, there was no recovery during a 1-hr incubation in drug-free medium, and the process was completely inoperative after 13 hr of exposure to HUN.

**Metabolism of recovering bacteria.** Addition of $^3$H-thymidine to recovering and control bacteria (Fig. 2) revealed that, whereas incorporation of precursors into the DNA of control cells was exponential, there was a lag of approximately 60 min before appreciable incorporation into the recovering cells was observed. Similar results were obtained when incorporation of $^3$H-deoxycytidine into DNA was determined (*unpublished data*). Similarly, by using $^3$H-cytidine as a precursor of both RNA and DNA synthesis, it was shown (Fig. 3) that this precursor was incorporated into DNA of a control culture until the supply of cytidine was exhausted ($t$ = 60 min), and incorporation into DNA of recovering cells was greatly retarded. RNA synthesis proceeded linearly in both cultures, although it was slower in the recovering one. In confirmation of these results, it was shown (*unpublished data*) that the incorporation of uridine-*5-$^3$H* into RNA of recovering cells proceeded without delay, although at a slower rate than in the control culture.

It is noteworthy that, although hydroxyurea was an effective inhibitor of normal DNA synthesis (Fig. 4), it did not block effectively the incorporation of $^3$H-thymidine by cells recovering from the effects of HUN (Fig. 4).

Direct chemical analysis of control and recovering cultures revealed (Table 5) that, after 1.25 hr of incubation in drug-free medium, no increase in DNA content was observed, and protein and RNA contents increased by 8 and 18%, respectively. The macromolecular constituents of a control culture increased approximately threefold during the same period.

TABLE 4. *Effect of the length of exposure of bacteria to N-hydroxyurethan (HUN) on their ability to recover*[a]

| Expt | Time exposed to HUN (hr) | Time after removal from HUN (hr) | Viable cells/ml |
|---|---|---|---|
| I | 0 | 0 | $1.1 \times 10^8$ |
| | 3.5 | 0 | $4.5 \times 10^4$ |
| | | 0.5 | $2.6 \times 10^5$ |
| | | 1 | $1.7 \times 10^6$ |
| | | 2 | $3.8 \times 10^6$ |
| | | 4.5 | $1.3 \times 10^8$ |
| | 4 | 0 | $2.4 \times 10^4$ |
| | | 0.5 | $2.8 \times 10^5$ |
| | | 1.5 | $1.2 \times 10^6$ |
| | | 4 | $5.3 \times 10^7$ |
| | 4.5 | 0 | $1.8 \times 10^4$ |
| | | 1 | $3.4 \times 10^5$ |
| | | 3.5 | $2.3 \times 10^7$ |
| II | 0 | 0 | $1.6 \times 10^8$ |
| | 5 | 0 | $1.4 \times 10^4$ |
| | | 1 | $8.0 \times 10^5$ |
| | | 4 | $3.3 \times 10^7$ |
| | 6 | 0 | $1.5 \times 10^4$ |
| | | 3 | $9.2 \times 10^5$ |
| | 9 | 0 | $2.3 \times 10^3$ |
| | | 1 | $3.5 \times 10^3$ |
| III | 0 | 0 | $4.7 \times 10^7$ |
| | 2 | 0 | $4.8 \times 10^5$ |
| | 13 | 0 | $6.3 \times 10^3$ |
| | | 3 | $1.0 \times 10^4$ |

[a] Bacteria were brought to the exponential growth phase and exposed to 0.05 M HUN for the interval indicated. Cells were then washed free of HUN and resuspended in fresh medium. The number of viable cells was determined at intervals.

**Properties of the DNA of recovering bacteria.** DNA which had been prelabeled with $^3$H-thymidine was isolated from bacteria which had been treated with HUN, and also from bacteria which had been incubated in drug-free medium after treatment. Upon ultracentrifugal analysis of these samples on sucrose gradients, we observed a shift of the DNA from treated cells to a position higher in the gradient than the control DNA (Fig. 5), indicating a decrease in the molecular weight of DNA from HUN-treated cells. The DNA isolated from bacteria which recovered from HUN-induced death (i.e., 1 hr of incubation in drug-free medium) returned to a position in the gradient coincident with that of DNA from untreated cells (Fig. 5).

If DNA is the target of HUN damage, a point should be reached beyond which the DNA is irreparably damaged. This situation would be analogous to that described in Table 4, in which it was shown that recovery no longer occurred after extended exposure to HUN. This happened when

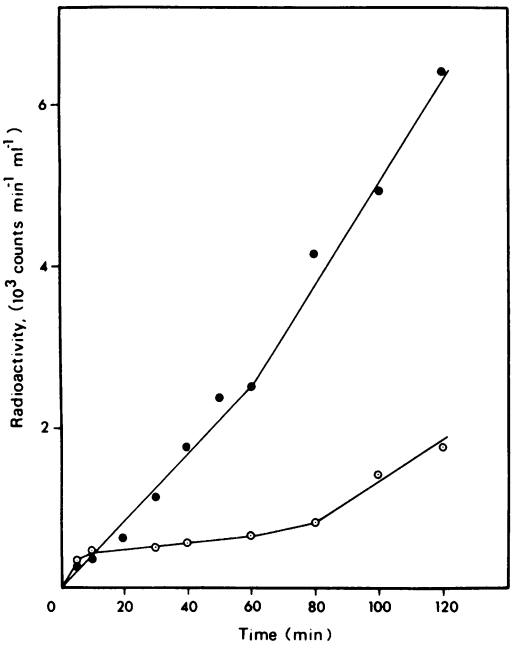


FIG. 2. *Incorporation of $^3$H-thymidine into normal cells and into cells after recovery from N-hydroxyurethan (HUN). Normal bacteria as well as those recovering from HUN (3.5 hr exposure) were resuspended in medium HA containing uridine ($1.3 \times 10^{-4}$ M) and $^3$H-thymidine ($1.3 \times 10^{-8}$ M, 11.3 Ci/mmole). At intervals, portions from each culture were withdrawn for determination of radioactivity incorporated into acid-insoluble form. ●, Control; ⊙, cells recovering from HUN.*

cells were exposed to HUN for 10.5 hr and subsequently aerated for 2 hr in drug-free medium (Fig. 6). The DNA from these bacteria actually decreased in size during incubation in drug-free medium (i.e., there was latent breakdown).

**Effect on a DNA polymerase-deficient mutant.** A bacterial strain deficient in DNA polymerase was extremely sensitive to the lethal action of HUN. This was in contrast to the behavior of the parent strain which was quite resistant to the action of this drug (Table 6). Co-treatment with HUN and chloramphenicol resulted in a protective effect on both strains (Table 6). Removal of HUN from the mutant strain permitted some recovery to occur (Table 7).

## DISCUSSION

The present study revealed that removal of HUN from cultures showing great reduction in the numbers of colony-forming bacteria as a result of exposure to the drug resulted in "revival" of treated bacteria. The observed increase in viability initially exceeded the growth rate of the bacterial strains used. The ability of bacteria to

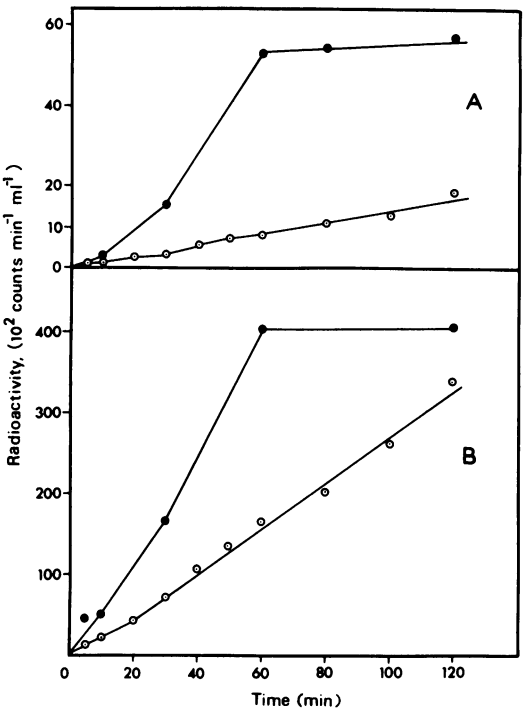


FIG. 3. *Incorporation of $^3$H-cytidine into normal and recovering cells. The procedure used was identical to the one described in the legend of Fig. 2 except that $^3$H-cytidine ($4.5 \times 10^{-6}$ M, 1.15 Ci/mmole) was used. ●, Control; ⊙, cells recovering fron N-hydroxyurethan treatment. A and B, Incorporation of $^3$H-cytidine into DNA and RNA, respectively.*

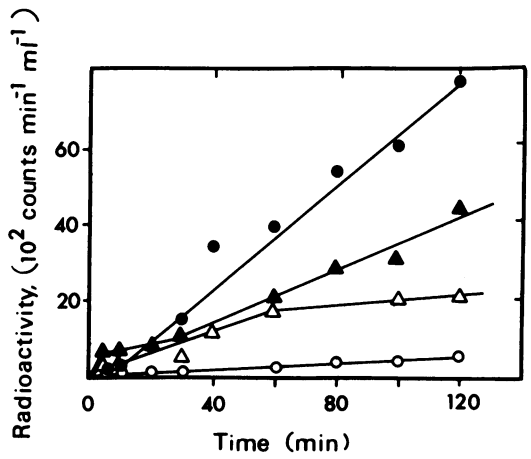


FIG. 4. *Effect of hydroxyurea on DNA synthesis by normal and recovering cells. The procedure used was essentially identical to the one described in the legend of Fig. 2. The final concentration of hydroxyurea was 0.2 M. ●, Control culture; ○, control culture + hydroxyurea; ▲, recovering culture; △, recovering culture + hydroxyurea.*

TABLE 5. *Colorimetric analysis of macromolecules before and after recovery*[a]

| Time (hr) | Conditions | DNA[b] | RNA[b] | Protein[b] |
|---|---|---|---|---|
| 0 | Control | 0.046 | 0.501 | 1.56 |
| | HUN[c] Treated and washed | 0.042 | 0.884 | 3.17 |
| 1.25 | Control | 0.172 | 1.62 | 5.05 |
| | Recovering from HUN | 0.042 | 1.07 | 3.42 |

[a] A culture of *E. coli* C 600 in the exponential phase of growth was divided into two portions; one portion was chilled; the other portion received HUN (final concentration, 0.05 M) and was aerated at 37 C for 3.5 hr. After this treatment, the HUN-containing culture was divided into two portions; one was chilled and washed in physiological saline, and the other was washed and resuspended in fresh medium. Both the control culture which had been chilled at the beginning of the experiment and the washed culture which had been resuspended in fresh medium were aerated at 37 C for 1.25 hr, after which they were chilled. All samples were precipitated with perchloric acid (final concentration 3%), and the determinations of the content of macromolecules were made.

[b] Values are expressed as mg/30 ml of culture.

[c] HUN, *N*-hydroxyurethan.

recover from the lethal effects of various treatments, including exposure to ultraviolet (22) and X rays (11) and alkylating agents (8, 16), has been studied extensively. In studies with all of these agents, DNA was shown to be the critical target, and damage to cellular DNA the basis of the lethal event. The data of Fig. 5 indicate that DNA of bacteria treated for 3 hr with 0.05 M HUN was smaller than that of normal cells; moreover, after removal of HUN the molecular weight of the DNA from "revitalized" cells was again identical with that of normal bacteria. These results can be interpreted as indicating that damage to DNA is the primary lethal event in HUN-induced death, since the repair of aberrant DNA is reflected, biologically, in a return of viability to the cells. This conclusion is supported by the findings that a bacterial strain deficient in DNA polymerase and therefore unable to repair damaged DNA (3) was much more sensitive to the lethal action of HUN (Table 6). Presumably the chemical basis of the damage is a reaction between HUN and DNA cytosine (14, and *unpublished data*). However, in vitro studies revealed (*unpublished data*, and K. P. Mullinix, Ph.D. thesis, Columbia Univ., 1969) that, in addition to a reaction with cytosine, HUN also induced scissions in the phosphodiester backbone of the DNA. The role of this reaction in HUN-induced death is unresolved.

Recovery from HUN occurred in the presence of inhibitors of DNA, RNA, and protein synthesis. It was reported previously that recovery from ultraviolet irradiation proceeded in the absence of uracil in a bacterial strain requiring this pyrimidine for growth, and excision of thymine dimers from the DNA of ultraviolet-irradiated

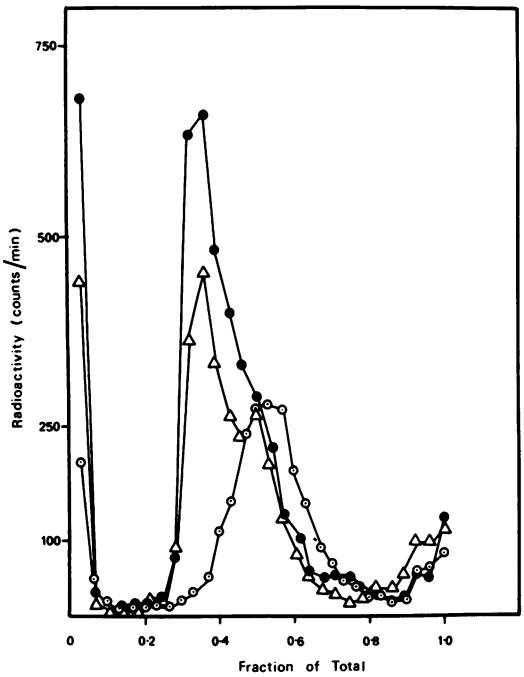


FIG. 5. *Zonal centrifugation of DNA from normal, N-hydroxyurethan (HUN)-treated, and recovered Escherichia coli. The DNA was layered on top of a gradient [5 to 20% sucrose in 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7]. The samples were spun at 30,000 rev/min for 3 hr in the SW50 rotor in a Spinco, model L-2 ultracentrifuge. The viability of the cultures from which DNA was isolated was as follows: control (t = 0), 7.7 × 10⁷; HUN-treated (3 hr), 5.5 × 10⁵; and recovered (1 hr), 3.6 × 10⁶ cells/ml. ●, Control; ⊙, HUN-treated; △, recovered.*

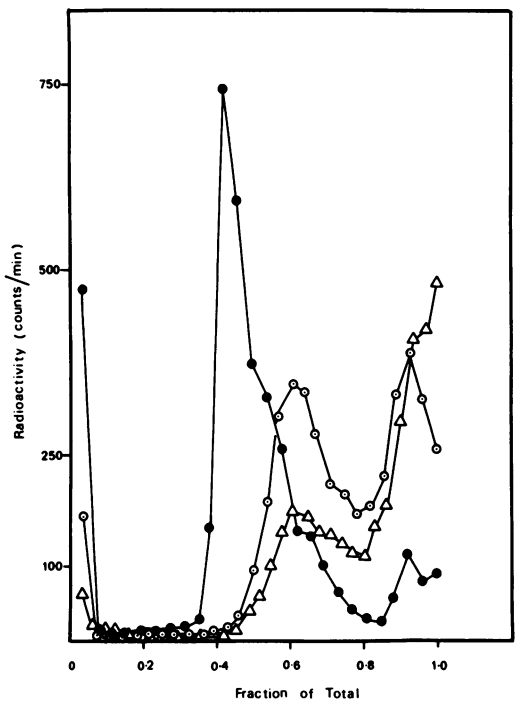


FIG. 6. *Zonal centrifugation of DNA from normal and recovered Escherichia coli. The experimental procedure was identical to the one described in the legend of Fig. 5. The viability of the cultures was: Control, 8.1 × 10⁷; HUN-treated (10.5 hr), 3.0 × 10⁴; recovered (2 hr), 7.4 × 10⁴. ●, Control; ⊙, HUN-treated; △, recovered.*

bacteria was not inhibited by chloramphenicol (9, 25). It was shown that HUN-induced death is an energy-requiring process, since it occurred neither in the presence of inhibitor of ATP production (Table 1) nor in the cold (Table 3). This dependence on the production of high-energy compounds might provide a clue to the basis of the recovery process. Since the composition of the plating medium did not influence survival and since essentially the only difference between liquid and solid media is the extent of aeration, it might be that cells vigorously aerated in suspension exhibit an enhanced rate of ATP production and that an unusually high level of ATP is necessary to repair the damaged DNA before some critical stage of the division cycle is reached. In this connection, it was shown that glucose was required for the repair of DNA after treatment of bacteria with ultraviolet light and methyl methanesulfonate (21, 23).

Results of experiments in which treated cells were aerated at 42 C are analogous to those obtained when ultraviolet-irradiated cells were incubated at elevated temperatures. It has been suggested (5) that repair of damaged DNA at 42 C indicates the possibility that repair of DNA and replication of the DNA proceed by means of two separate enzyme systems, since cell division but not repair is inhibited at nonphysiological temperatures. The isolation and characterization by deLucia and Cairns (3) of a bacterial mutant deficient in DNA polymerase, but perfectly capable of cellular replication, supports this hypothesis and suggests that DNA polymerase is the enzyme responsible for DNA repair (see also reference 6). The finding that DNA polymerase both in vivo and in vitro was resistant to inhibition by hydroxyurea (19; S. J. Jacobs, Ph.D. thesis, 1968), whereas cellular DNA synthesis was very sensitive to blockage by hydroxyurea (18–20), also suggests that two different enzymes are involved. The finding that the repair of HUN-treated cells took place in the presence of hydroxyurea (Table 1) and that $^3$H-thymidine was incorporated in recovering cells in the presence of hydroxyurea (Fig. 4) confirms the suggestion that DNA repair

TABLE 6. *Effect of N-hydroxyurethan (HUN) on a bacterial strain deficient in DNA polymerase*[a]

| Expt | Time (hr) | Additions | Viable bacteria/ml | |
|---|---|---|---|---|
| | | | Parent *E. coli* W3110 | Mutant *E. coli* p3478 |
| I | 0 | None | $1.6 \times 10^8$ | $1.2 \times 10^8$ |
| | 1 | 0.05 M HUN | $1.9 \times 10^8$ | $3.2 \times 10^6$ |
| | 2 | 0.05 M HUN | $1.3 \times 10^8$ | $3.1 \times 10^5$ |
| | 4 | 0.05 M HUN | $4.0 \times 10^7$ | $8.2 \times 10^3$ |
| | 4 | None | $2.2 \times 10^9$ | $5.3 \times 10^9$ |
| II | 0 | None | $1.5 \times 10^8$ | $5.5 \times 10^7$ |
| | 1 | 0.05 M HUN | | $1.5 \times 10^6$ |
| | 1 | CM, 20 μg/ml | | $3.2 \times 10^7$ |
| | 1 | HUN + CM | | $4.4 \times 10^7$ |
| | 2 | 0.05 M HUN | | $3.1 \times 10^4$ |
| | | HUN + CM | | $2.0 \times 10^7$ |
| | 4 | None | $2.8 \times 10^9$ | $2.4 \times 10^9$ |
| | | 0.05 M HUN | $1.2 \times 10^7$ | $1.1 \times 10^3$ |
| | | CM, 20 μg/ml | $2.0 \times 10^8$ | $2.4 \times 10^7$ |
| | | HUN + CM | $1.9 \times 10^8$ | $5.4 \times 10^6$ |

[a] Bacteria in medium HA (17) containing thymine (5 μg/ml) were brought to the exponential growth phase and then were distributed into flasks containing premeasured amounts of HUN (final concentration 0.05 M) or chloramphenicol (CM, 20 μg/ml), or both. The number of viable bacteria was determined by plating serial dilutions (0.1 ml) of cultures on HA agar containing thymine.

TABLE 7. *Recovery of a DNA polymerase-deficient strain from HUN-induced death*[a]

| Time after removal of HUN (hr) | Viable bacteria/ml | |
|---|---|---|
| | Parent *E. coli* W3110 | Mutant *E. coli* p3478 |
| 0 | $3.0 \times 10^5$ | $9.0 \times 10^3$ |
| 0.5 | $3.6 \times 10^6$ | $3.5 \times 10^5$ |
| 1.0 | $1.8 \times 10^7$ | $1.3 \times 10^6$ |
| 1.5 | $2.0 \times 10^7$ | $2.3 \times 10^6$ |

[a] The parent and mutant strains were exposed to 0.05 M HUN for 4 and 1 hr, respectively, whereupon the bacteria were washed free of HUN and resuspended in fresh medium.

and DNA duplication are separate processes and only the former is sensitive to hydroxyurea.

Although bacteria are able to repair damaged regions of cellular DNA, it is clear that this capacity is limited, and after extended treatment there are so many damaged regions in the DNA that repair cannot take place. This was shown for HUN-treated cells (Table 4 and Fig. 6), since that point at which there is no longer recovery of viability of treated cells can be correlated with the point after which the DNA is irreparably damaged. The ability of *E. coli* to recover from ultraviolet irradiation has also recently been correlated with the extent to which cellular DNA has suffered radiation damage (1).

Incorporation studies during the "recovery" phase revealed a lag in the synthesis of DNA, whereas RNA synthesis proceeded without delay, although at a slower rate than in normal cells. Colorimetric analyses (Table 5) indicated that during recovery there was no net synthesis of DNA, and only a small amount of net synthesis of RNA and protein. These results suggest that the small amount of (hydroxyurea-resistant) incorporation of $^3$H-thymidine into the DNA of recovering cells represents "repair synthesis" of damaged regions of the cellular DNA. It is proposed that the lag in incorporation of precursor into DNA during recovery is a measure of the time necessary for repair synthesis to occur, and that repair synthesis is necessary for a successful round of replication to take place. Since there is little net synthesis of RNA during this period, the incorporation of precursor into this macromolecule probably reflects the production of messenger RNA, which is rapidly turned over in *E. coli* (9). Similar patterns of incorporation of precursors into macromolecules have been observed by Swenson and Setlow in *E. coli* after ultraviolet irradiation (25). These investigators showed that RNA synthesis proceeded normally after irradiation, whereas there was a lag before DNA synthesis was restored.

In the accompanying paper (12), it was shown that treatment of *E. coli* with HUN in the presence of chloramphenicol resulted in protection of the bacteria from the lethal effects of HUN. Similarly, it was shown that *E. coli* C600/HUN, the mutant, with an increased resistance to HUN, was not killed when exposed to 0.05 M HUN. It is apparent, however, that in both cases there was degradation of cellular DNA upon exposure to HUN, although at a slower rate than in the case of sensitive cells treated with HUN alone. In view of the results presented above, which suggest repair of DNA during recovery, an explanation of the phenomenon of degradation of DNA accompanying protection from the lethal effects of HUN can be presented. The solubilization of DNA of cells treated with HUN is most probably a result of the occurrence of two separate events: (i) a reaction between HUN and DNA (14, and *unpublished data*) which causes chemical alterations in the DNA which eventually become so numerous that the DNA is degraded to acid-soluble fragments, and (ii) repair of HUN-induced damage to DNA which involves the removal of damaged portions which are then released into the acid-soluble pool.

The rate of HUN-induced damage to DNA is retarded in cells treated simultaneously with

chloramphenicol and HUN. Such a retardation was also seen in *E. coli* C600/HUN exposed to HUN. The role of chloramphenicol in cellular devitalization and DNA degradation is puzzling. It may, however, reflect the possibility that HUN reacts more readily with cytosine when DNA is single-stranded (*unpublished data*), and chloramphenicol, by preventing new cycles of DNA synthesis from being initiated (see reference 15), also blocks strand separation, thereby decreasing the number of HUN-sensitive sites (i.e., non-hydrogen-bonded cytosine residues).

The data presented herein indicate that repair enzymes are probably functioning in the presence of HUN and that initially a state of equilibrium between damage and repair exists, thereby accounting for the (bacteriostatic) delay before killing starts (Table 6). In bacteria with a reduced capacity to repair damaged DNA (i.e., deficient in DNA polymerase), no such delay was observed (Table 6).

To further elucidate the basis of the action of HUN, a study of the enzyme systems involved in the repair process is currently underway in this laboratory.

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