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Effects of *N*-Hydroxyurethan on Viability and Metabolism of *Escherichia coli*

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Exposure of growing cells to low levels of *N*-hydroxyurethan (approximately 0.05 M) resulted in loss of colony-forming ability. This was accompanied by degradation of cellular deoxyribonucleic acid. When the concentration of *N*-hydroxyurethan was increased to 0.2 M, bacteriostasis was the primary manifestation. The lethal action of the drug was prevented by cotreatment with chloramphenicol or azauracil or by uracil-deprivation of a bacterial strain requiring this pyrimidine for growth.

N-hydroxyurethan [HUN; HON(—H)—C(=O)—OC_2H_5] is a carcinogen and an inducer of chromosomal aberrations in plant and mammalian cells (3, 5, 6). In addition, HUN also possesses antitumor and antiviral properties (1, 13, 18).

An investigation of HUN was undertaken because of its relationships to hydroxyurea [HU; HON(—H)—C(=O)—NH_2], a selective and reversible inhibitor of deoxyribonucleic acid (DNA) synthesis (17, 31, 40, 45, 46) which exhibits promising antiviral and antineoplastic properties (7, 20, 21, 22, 23, 25, 30, 42, 44). The present report deals with the effects of HUN on bacterial growth and metabolism.

MATERIALS AND METHODS

Materials. *N*-hydroxyurethan was obtained either from New England Nuclear Corp., or from Aldrich Chemical Co. Hydroxyurea was from E. R. Squibb & Sons. Chloramphenicol was purchased from Parke, Davis & Co., Columbia-Base agar from Baltimore Biological Laboratories, and sodium azide from Fisher Scientific Co. ^{3}H-lysine and ^{3}H-uridine were from Schwarz BioResearch Inc., ^{14}C-thymidine from New England Nuclear Corp., and ^{3}H-thymidine from International Chemical and Nuclear. Scintillators were purchased either from Packard Instrument Corp. or from New England Nuclear Corp.

Bacterial strains and media. The properties of *Escherichia coli* C600 were described previously, as was the composition of the liquid growth medium HA (37). *E. coli* K-12 u^- met^- is a derivative of *E. coli* K-12 requiring uracil and methionine for growth. This strain was a gift from Rivka Rudner, Hunter College of The City University of New York. Viable bacteria were enumerated by plating serial dilutions (0.1 ml) of cultures on Columbia base agar.

Isolation of a mutant with an increased resistance to HUN. An exponentially growing culture of *E. coli* C600 was exposed to 0.05 M HUN for 24 hr and streaked on Columbia base agar plates containing 0.05 M HUN. After incubation, single colonies were picked and subjected to three additional cycles of treatment with 0.05 M HUN for 24 hr and streaking on HUN-containing plates, after which the resistance of the survivors to 0.05 M HUN was tested in liquid medium. Bacteria with an increased resistance to HUN were designated *E. coli* C600/HUN.

Metabolic techniques. Techniques for determining the incorporation of specific precursors of DNA, ribonucleic acid (RNA), and protein into acid-insoluble products have been described previously (38, 39, 41). Whenever radioactive thymidine was used, the medium was supplemented with uridine (366 μg/ml) to inhibit and repress thymidine phosphorylase (9).

Prelabeling of bacterial DNA was accomplished by inoculating bacteria into warm medium HA to which uridine (366 μg/ml) and radioactive thymidine were added. The bacteria were grown for several generations, harvested by centrifugation, and resuspended in fresh medium. The cells were then aerated at 37 C for 30 min to exhaust the pool of radioactive precursor. At this point, experiments were initiated.

Protoplasts were prepared by a modification of the procedure of Fraser and Mahler (15). Bacterial DNA was isolated from intact cells or from protoplasts by the technique described by Marmur (26) or by the procedure described by Bode and Kaiser (4) which was modified to include several extractions of the material at the interphase to increase the amount of DNA recovered. In some experiments, to minimize loss of bacterial DNA, protoplasts were allowed to lyse directly on gradients of sucrose. The procedure used to extract DNA did not influence the results obtained in this study.

Radioactive bacterial DNA was analyzed on gradients of sucrose (5 to 20%) in the SW50 rotor of a Spinco model L-2 ultracentrifuge. In some experiments,

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protoplasts were lysed directly on alkaline sucrose gradients, as described by McGrath and Williams (27). After centrifugation, drops were collected as previously described (14).

The bacterial DNA was also subjected to analysis on ECTEOLA-cellulose (2). Fractions eluted from the column were acidified with trichloroacetic acid, and the specimens were processed for determination of radioactivity rendered acid-insoluble.

Previously described techniques were used to band DNA specimens in gradients of cesium chloride (28, 43)

RESULTS

Effect of HUN on the growth of E. coli. Exposure of growing bacteria to 0.05 M HUN resulted in bacterial death and an inhibition of the increase in turbidity of the culture (Fig. 1). The latter is an indication of interference with protein synthesis (8). The effect of increasing concentrations of HUN on the growth of *E. coli* is illustrated in Table 1; it is noteworthy that exposure of bacteria to 0.2 M HUN resulted in a decreased bactericidal effect. As a matter of fact, whereas a 4-hr exposure of bacteria to 0.05 M HUN resulted in cellular death, a similar contact with 0.2 M HUN was bacteriostatic. The decreased bactericidal effect of elevated levels of HUN was accompanied by an increased inhibition of protein synthesis as determined by turbidity measurements (Table 1).

Bacteria selected for their increased resistance to HUN were also more resistant to the detrimental effects of HU (Table 2), thereby indicating a possible similarity in the modes of action of the two drugs.

Effects of metabolic inhibitors on the lethal action of HUN. Inhibitors of RNA or of protein synthesis (i.e., azauracil and chloramphenicol) afforded a great deal of protection to HUN-treated bacteria (Table 3). On the other hand, sodium azide, an inhibitor of oxidative metabolism, although it protected cells during the first 4 hr of treatment, did not do so when exposure to HUN was prolonged for 24 hr.

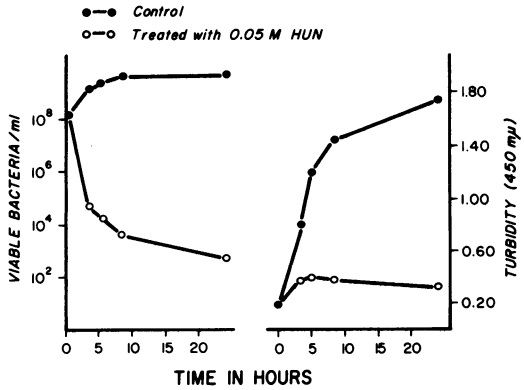

FIG. 1. *Effect of N-hydroxyurethan (HUN) on the growth of Escherichia coli C600.*

TABLE 1. *Effect of N-hydroxyurethan (HUN) concentration on the growth of Escherichia coli*[a]

Additions	Time (hr)	Viable bacteria/ml	Turbidity (450 nm)
None	0	1.2×10^8	0.22
	4	1.4×10^9	0.74
	24	3.8×10^9	1.5
0.05 M HUN	4	5.8×10^4	0.39
	24	8.7×10^3	0.34
0.20 M HUN	4	1.3×10^8	0.27
	24	3.5×10^6	0.25

[a] Bacteria in medium HA (37) were brought to the exponential growth phase, at which time they were distributed into flasks containing premeasured amounts of HUN. At intervals, the number of viable cells and the turbidity (at 450 nm on a Bausch & Lomb Spectronic 20) were determined.

TABLE 2. *Effect of N-hydroxyurethan (HUN) and HU on Escherichia coli C600 and E. coli C600/HUN*[a]

Time (hr)	Additions	Per cent survivors	
		E. coli C600	*E. coli* C600/HUN
0	None	100	100
2.5	None	390	320
	0.05 M HUN	0.48	42.0
	0.2 M HU	14.0	15.0
7.5	None	2,600	4,500
	0.05 M HUN	0.044	16.0
	0.2 M HU	0.12	7.5
24	None	9,000	9,400
	0.05 M HUN	0.0003	1.4
	0.2 M HU	0.007	2.6

[a] Medium HA was inoculated with either *E. coli* C600 or *E. coli* C600/HUN. When the cultures reached the early exponential phase of growth, they were each divided into three portions. One portion of each culture served as control; a second received HUN (final concentration 0.05 M); the third received HU (final concentration, 0.20 M). All cultures were aerated at 37 C, and the number of viable cells was determined at intervals. The number of cells at t = 0 was 6.1×10^7 for *E. coli* C600 and 3.1×10^7 for *E. coli* C600/HUN.

The requirement of RNA synthesis for the expression of the lethal action of HUN was confirmed by an experiment in which it was shown that uracil-requiring bacteria were protected from HUN-induced death when deprived of this pyrimidine (Table 4).

Metabolic effects of HUN. Studies on the rate of incorporation of specific radioactive precursors into macromolecular constituents revealed that RNA synthesis was inhibited without delay (Fig. 2) when cells were exposed to bactericidal levels

TABLE 3. *Effect of metabolic inhibitors on the bactericidal action of N-hydroxyurethan (HUN)*[a]

Time (hr)	Conditions[b]	Viable cells/ml
0	None	1.2×10^8
4	None	1.4×10^9
	AzaU (300 μg/ml)	1.6×10^8
	CM (20 μg/ml)	2.2×10^8
	NaN_3 (0.01 M)	1.5×10^8
	0.05 M HUN	5.8×10^4
	0.05 M HUN + AzaU	3.6×10^6
	0.05 M HUN + CM	6.7×10^7
	0.05 M HUN + NaN_3	4.3×10^7
	0.20 M HUN	1.3×10^8
	0.20 M HUN + AzaU	9.5×10^7
	0.20 M HUN + CM	1.5×10^8
	0.20 M HUN + NaN_3	9.5×10^7
24	None	3.8×10^9
	AzaU	9.3×10^8
	CM	7.2×10^7
	NaN_3	2.2×10^6
	0.05 M HUN	8.7×10^3
	0.05 M HUN + AzaU	7.0×10^4
	0.05 M HUN + CM	2.6×10^7
	0.05 M HUN + NaN_3	7.0×10^3
	0.20 M HUN	3.5×10^6
	0.20 M HUN + AzaU	1.6×10^7
	0.20 M HUN + CM	2.3×10^7
	0.20 M HUN + NaN_3	5.3×10^4

[a] An exponentially growing culture of *E. coli* C600 was divided into 12 portions, each of which was supplemented as indicated. The cultures were aerated at 37 C and the numbers of viable cells were determined at intervals.

[b] Abbreviations: AzaU, 6-azauracil; CM, chloramphenicol; NaN_3, sodium azide.

(0.05 M) of HUN. This was followed 4 min later by a decreased rate of protein production (Fig. 3). DNA synthesis, on the other hand, was quite insensitive to inhibition by HUN (Fig. 4); it was only after 50 min of exposure to HUN that the rate of DNA production was curtailed sharply (*unpublished data*). Similar results were obtained with *E. coli* C600/HUN, the bacterial strain with an increased resistance to the lethal effects of HUN (Fig. 2–4), except that protein synthesis in this strain was inhibited somewhat more rapidly than in the sensitive parent strain (Fig. 3).

The rapid effect of HUN on RNA synthesis was also observed when pulse-labeling experiments were performed. Thus, immediately upon addition of the drug, the amount of rapidly labeled RNA decreased by 37%, whereas 9 min later it was only 12.7% that of the control (Table 5).

The addition of HUN to growing bacteria did not result in gross structural alterations of the ribosomes which existed at the time the drug was added, nor were the ribosomes made in the presence of HUN abnormal (Fig. 5). This can be deduced from the superimposition of the absorbance (mainly preexisting ribosomes) and radioactivity (newly synthesized ribosomes) curves. Similarly, no differences were observed when ribosomes were analyzed in buffer containing 0.01 M Mg^{2+} (*unpublished data*). Finally, no change was observed in preexisting ribosomes by these same criteria when they were labeled with ^{3}H-uridine before exposure to HUN (*unpublished data*).

TABLE 4. *Effect of HUN on a uracil-requiring strain of Escherichia coli*[a]

Time (hr)	Additions	Viable Bacteria/ml	
		Uracil present	Uracil absent
0	None	7.6×10^7	1.2×10^8
1.5	None	1.1×10^9	1.4×10^8
	0.05 M HUN	2.1×10^5	1.0×10^8
3	None	1.2×10^9	1.5×10^8
	0.05 M HUN	1.7×10^5	5.5×10^7
23	None	1.5×10^9	1.6×10^8
	0.05 M HUN	1.4×10^4	3.5×10^6

[a] *E. coli* K-12, u$^-$met$^-$ was inoculated into medium HA containing uracil (25 μg/ml). Upon initiation of the exponential phase of growth, the culture was chilled and a portion was withdrawn, centrifuged, and suspended in medium HA (no uracil). The uracil-lacking culture was aerated at 37 C for 30 min to deplete the intracellular pool of uracil. At this point, both the depleted and the chilled cultures were divided, and portions of each received *N*-hydroxyurethran (HUN; final concentration 0.05 M). All cultures were aerated at 37 C and numbers of viable cells were determined at intervals.

Effect of HUN on cellular DNA. The HUN-induced killing of *E. coli* was accompanied by degradation of the cellular DNA to acid-soluble products (Table 6). An agent (chloramphenicol) capable of reducing the lethal action of HUN also decreased the drug-induced degradation of bacterial DNA (Table 6). Moreover, the DNA of *E. coli* C600/HUN was also depolymerized when these bacteria were exposed to HUN (Table 6). Because solubilization is a very insensitive assay for damage to DNA, since extensively degraded fragments can still be precipitated by trichloroacetic acid, additional criteria were used to detect structural changes of the cellular DNA.

Cesium chloride density gradient centrifugation of DNA from bacteria exposed to 0.05 or 0.2 M HUN for 24 hr revealed (Fig. 6) that, whereas the nucleic acid from cells treated with 0.2 M HUN appeared identical to that from untreated

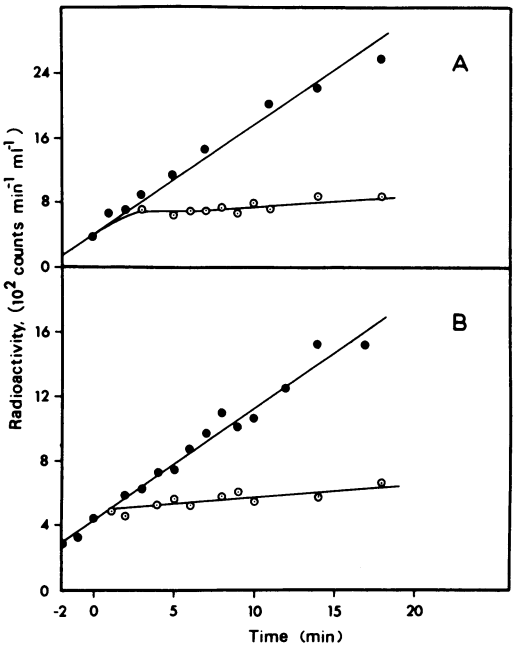

FIG. 2. *Effect of N-hydroxyurethan (HUN) on RNA synthesis. Bacteria (Escherichia coli C600) in medium HA were brought to the exponential growth phase, at which time ^{3}H-uridine (2×10^{-4} M, 2 Ci/mmole) was added. Two minutes later, half of the culture was withdrawn and added to a prewarmed flask containing HUN (final concentration 0.05 M). Samples were withdrawn at intervals for the determination of radioactivity incorporated into acid-insoluble form. A, E. coli C600; B, E. coli C600/HUN, the HUN-resistant strain. ●, Control; ⊙, HUN-treated.*

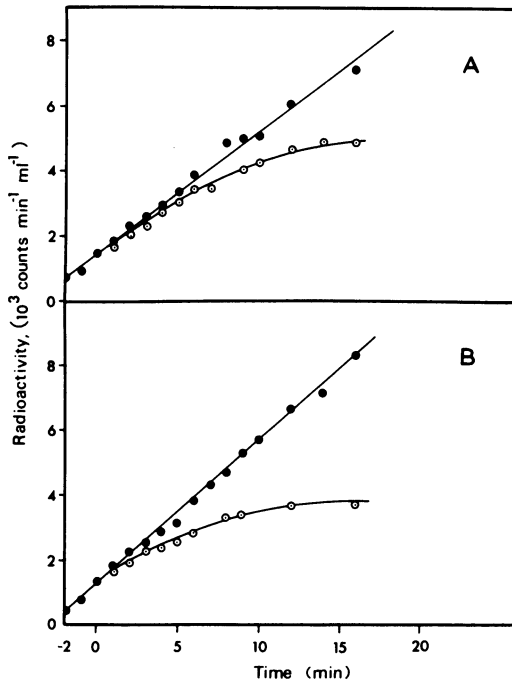

FIG. 3. *Effect of N-hydroxyurethan on protein synthesis. The procedure used was identical to the one described in Fig. 2 except that ^{3}H-lysine (5.4×10^{-5} M, 0.15 Ci/mmole) was used. A, Escherichia coli C600; B, E. coli C600/HUN. ●, Control; ⊙, HUN-treated.*

bacteria, the polydeoxynucleotidic material isolated from cells exposed to 0.05 M HUN failed to band, thus indicating extensive breakdown of cellular DNA by that concentration of HUN which also caused maximal killing.

The chromatographic fractionation of DNA on ECTEOLA-cellulose is based upon size, shape, composition, and charge of these macromolecules (2, 32, 33). Low molecular polynucleotides are eluted with dilute salt solutions, whereas larger molecules require increases in ionic strength and *p*H for elution. To minimize (see reference 38) artifacts due to isolation procedures (i.e., shear degradation), the DNA of cells to be treated with HUN was prelabeled with ^{3}H-thymidine and that of a control culture was prelabeled with ^{14}C-thymidine. After HUN treatment (24 hr), the two cultures were combined and DNA was isolated. The isolation procedure involved dialysis to remove phenol and, inherent in the procedure, is a preliminary fractionation by which the most degraded portions of DNA (i.e., those from HUN-treated cells) are removed selectively.

Analysis of such a doubly labeled preparation on ECTEOLA-cellulose columns revealed (Table 7) that DNA derived from HUN-treated cells (^{3}H-DNA) was recovered earlier (i.e., lower molecular weight) than the DNA of the control culture (^{14}C-DNA). The validity of the procedure was established by demonstrating that fractionation of doubly labeled DNA from untreated cells did not result in a preferential elution of ^{3}H-DNA.

Prolonged exposure to 0.05 M HUN is not required for extensive killing (Fig. 1). As a matter of fact, the lethal effect is at a maximum at the end of 4 hr of treatment; on the other hand, degradation of cellular DNA to acid-soluble products required at least 3 hr of exposure (Table 6, and *unpublished data*). These considerations suggested that cellular DNA be examined not at the end of 24 hr of treatment but approximately 4 hr after addition of HUN.

Analysis of such a specimen on a gradient of sucrose revealed (Fig. 7) that degradation of the DNA could be detected after 4 hr of incubation in the presence of HUN. Continued exposure to the drug resulted in a further progressive decrease in molecular weight of the DNA of treated cells (K. P. Mullinix, Ph.D. thesis, Columbia Univ.,

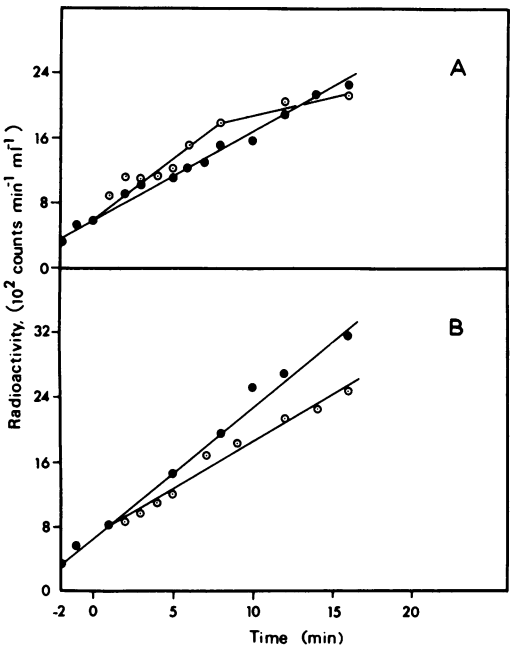

FIG. 4. *Effect of N-hydroxyurethan on DNA synthesis. The procedure used was identical to the one described in Fig. 2. The radioactive precursor was ^{3}H-thymidine (6.9×10^{-8} M, 11.3 Ci/mmole), "cold" uridine (1.4×10^{-4} M) was also added to inhibit and repress thymidine phosphorylase (9). A, Escherichia coli C600; B, E. coli C600/HUN. ●, Control; ⊙, HUN-treated.*

TABLE 5. *Effect of N-hydroxyurethan (HUN) on the synthesis of pulse-labeled RNA*[a]

Conditions	^{3}H-uridine incorporated[b]
No additions	33,984
^{3}H-uridine + HUN added simultaneously	24,138
HUN added at $t = -9$ min; ^{3}H-uridine added at $t = 0$	3,671

[a] Bacteria (*E. coli* C600) in medium HA at 30 C were brought to the exponential growth phase, at which time 1-ml portions of the culture were distributed into tubes containing premeasured amounts of ^{3}H-uridine (5.3×10^{-10} M; 2.1 μCi/ml) and HUN (final concentration 0.05 M). After 0.5 min of incubation, the cultures were supplemented with trichloroacetic acid (final concentration 5%) and processed for determination of radioactivity incorporated. Those cultures which were pretreated with HUN were handled as above, except that ^{3}H-uridine was added 9 min after the drug. Results are the averages of five separate determinations.

[b] ^{3}H-uridine incorporated during a 30-sec pulse. Results expressed as counts per minute per milliliter.

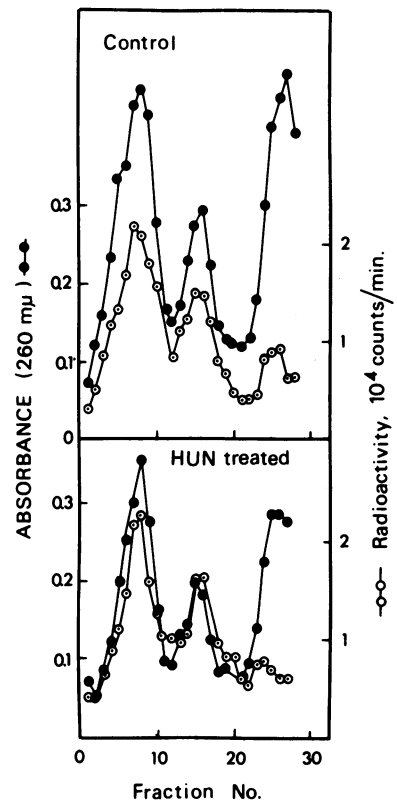

FIG. 5. *Properties of ribosomes isolated from N-hydroxyurethan (HUN)-treated cells. Bacteria in the exponential growth phase (2×10^8/ml) were divided into two equal portions (500 ml), one of which received HUN (0.05 M); 2 min later the cultures each received ^{3}H-uridine (2×10^{-4} M; 0.5 μCi/ml). After incubation for 0.5 hr, the cells were processed for extraction of ribosomes (34). Ribosomes were analyzed in linear gradients of sucrose (5 to 20%) in 0.01 M tris(hydroxymethyl)aminomethane, pH 7.0, containing 10^{-4} M Mg^{2+}. Centrifugation was carried out in the SW50 rotor of a Spinco model L-2 ultracentrifuge at 35,000 rev/min for 3 hr.*

1969). It was also found that the procedure used to isolate the DNA had no effect on the sedimentation profiles of the DNA (*unpublished data*, and K. P. Mullinix, Ph.D. thesis, Columbia Univ., 1969).

DISCUSSION

The effects of HUN on bacteria vary with concentration (Table 1). Surprisingly enough, low concentrations of the drug are bactericidal for *E. coli* C600 whereas higher levels (0.20 M) are bacteriostatic. There is a direct relationship, however, between HUN concentration and the extent to which protein synthesis is inhibited (Table 1).

TABLE 6. *Degradation of prelabeled cellular DNA by hydroxyurethan (HUN)*[a]

Expt	Bacteria	Additions	Time (hr)	Viable cells/ml	Radioactivity retained	
					Counts/min	Per cent of control
I	C600	None	0	8.2×10^7	5,154	100
			2	3.1×10^8	5,670	109
			3.5	1.1×10^9	5,801	113
			6.5	1.5×10^9	5,877	114
			22	4.0×10^9	5,077	98.5
			24		5,299	101
		HUN	2	2.0×10^6	4,876	94.6
			3.5	1.7×10^5	3,990	77.4
			6.5	2.2×10^4	2,684	52.1
			22	6.7×10^2	1,686	32.7
			24		1,665	32.3
II	C600	None	0	9.1×10^7	804	100
			23	6.3×10^9	760	94.6
		HUN	23	1.6×10^3	420	52.3
		HUN + CM	23	4.5×10^7	560	69.7
		CM	23	7.0×10^6	716	89.1
III	C600	None	0	1.0×10^8	4,398	100
			21		4,522	103
			26	3.6×10^9		
		HUN	21		2,447	55.3
			26	2.0×10^1		
	C600/HUN	None	0	9.1×10^7	4,151	100
			21		4,091	98.6
			26	3.1×10^9		
		HUN	21		2,273	54.8
			26	2.0×10^5		

[a] Bacterial DNA was prelabeled by growing cells in the presence of ^{14}C-thymidine (10^{-10} M; 0.2 μCi/ml). At the beginning of the experiment, the cultures were distributed into flasks containing: HUN (final concentration 0.05 M), chloramphenicol (CM; final concentration 20 μg/ml), or a mixture of HUN and CM. At intervals, samples were withdrawn for determination of viability, turbidity, and the amount of acid-precipitable material.

An examination of the viability and turbidities of treated cultures indicates that a condition resembling unbalanced growth (12) exists in cells exposed to 0.05 M HUN, i.e., protein synthesis accompanies cellular death. It was attempted, therefore, to determine whether bacteria could be protected from HUN-induced killing if they were exposed simultaneously to inhibitors of macromolecular biosynthesis. It was found (Tables 3 and 4) that RNA and protein synthesis were required for HUN-induced killing, because when these were blocked, as by the addition of chloramphenicol (16) or azauracil (19) or by uracil starvation of a bacterial strain requiring this pyrimidine for growth, the bacteria were protected. The inhibition of oxidative energy metabolism by sodium azide (11, 24) exerted a protective effect (Table 3) for a limited time only, presumably because in the presence of this inhibitor the cells were able to obtain some energy through glycolysis (10).

Since inhibitors of macromolecular biosynthesis protected bacteria from the lethal action of low concentrations of HUN, it can be inferred that higher levels of the drug (0.20 M) blocked these synthetic processes completely. This would then explain the survival of bacteria exposed to higher levels of HUN. This hypothesis was supported by the observation that, upon exposure of bacteria to 0.2 M HUN, the turbidity of the cultures did not increase. Thus it appears that macromolecular synthesis is required for the expression of the lethal damage induced by HUN.

HUN-induced killing is influenced by the conditions of aeration; in the absence of adequate aeration, the bactericidal effect of 0.05 M HUN is lessened. This situation prevails for many bactericidal agents and has been described previously in relation to the bactericidal effects of HU (35). It is possible that vigorous aeration is necessary for oxidation of HUN to an intermediate which is responsible for the lethal event. Evidence that

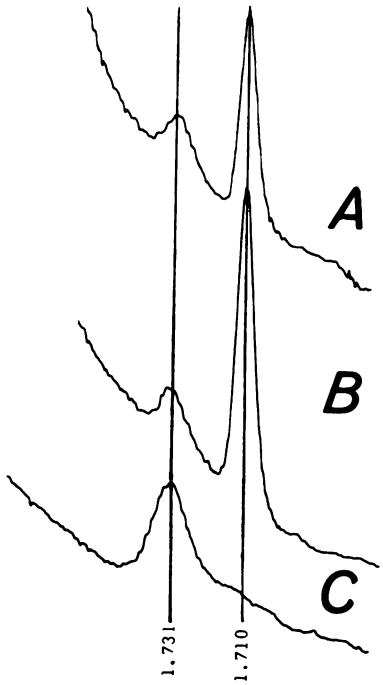

FIG. 6. *Effect of N-hydroxyurethan on the banding properties of bacterial DNA in gradients of cesium chloride. Bacteria in medium HA were brought to the exponential growth phase, whereupon portions of the culture were exposed to 0.05 and 0.2 M HUN for 24 hr and 37 C, at which time DNA was isolated by the procedure of Marmur (26). Portions of the DNA, together with a reference sample (DNA from Micrococcus lysodeikticus, 1.731 g/cm³), were placed in a CsCl solution (density 1.70 g/cm³). The samples were centrifuged at 44,740 rev/min for 24 hr, and photographs were taken. The buoyant densities were calculated by comparison with the position of the reference DNA. In the tracings, the band at the left represents the position of the marker DNA. A, Control; B and C, bacteria exposed to 0.2 and 0.05 M HUN, respectively.*

such an oxidation of HUN is involved in the in vitro degradation of DNA has been obtained (K. P. Mullinix, Ph.D. thesis, Columbia Univ., 1969). It is also probable that optimal killing occurs when the cells are incubated under conditions permitting maximal growth. Such conditions would afford the best opportunity for the expression of HUN-induced damage to a cellular constituent.

In an attempt to relate the action of HUN to that of HU, a bacterial mutant (*E. coli* C600/HUN) resistant to HUN was isolated. This strain was killed at a slower rate and to a lesser extent both by HUN and HU than was the parent strain (Table 2). This result was unexpected, because it had been reported previously

TABLE 7. *Fractionation of Escherichia coli DNA on ECTEOLA-cellulose*[a]

Eluant	DNA from untreated cells		DNA from HUN-treated cells	
	^{14}C[b]	Per cent of total	^{3}H[b]	Per cent of total
0.01 M Phosphate (*p*H 7) ..	0		29	0.01
0.25 M NaCl	0		17,525	6.4
0.50 M NaCl	386	1.7	102,050	37.1
2 M NaCl/0.1 M NH_3	8,812	37.7	84,446	30.7
2 M NaCl/1.0 M NH_3	14,023	60.0	69,397	25.3
0.5 M NaOH	146	0.6	1,316	0.5

[a] Bacterial cultures were brought to the exponential growth phase in a medium (HA) that was supplemented either with ^{3}H-thymidine (7.4×10^{-6} M; 0.5 μCi/ml) or ^{14}C-thymidine (1×10^{-10} M; 0.2 μCi/ml) and "cold" uridine (366 μg/ml), at which time the cells were harvested, washed, and suspended in prewarmed medium free of radiothymidine. At the end of 40 min of aeration at 37 C, the culture labeled with ^{3}H-thymidine received *N*-hydroxyurethan (HUN; final concentration 0.05 M) and the ^{14}C-labeled culture served as control. The cultures were aerated for 24 hr at 37 C, at which time they were combined and DNA was isolated (4) and analyzed on ECTEOLA-cellulose (2).

[b] Counts per minute.

(40) that a bacterial strain resistant to HU (*E. coli* C600/HU) was not cross-resistant to HUN.

The lethal action of HUN on *E. coli* C600 was accompanied by a slight increase in turbidity, but such was not the case for *E. coli* C600/HUN (K. P. Mullinix, Ph.D. thesis, Columbia Univ., 1969). In fact, whereas addition of HUN (0.05 M) to *E. coli* C600 resulted in a delayed inhibition of protein synthesis, there was no such delay when HUN was added to *E. coli* C600/HUN (Fig. 3). Since expression of the lethal action of HUN on *E. coli* requires active protein synthesis, it may be postulated that *E. coli* C600/HUN derives its increased resistance to HUN from the fact that its protein synthesis is also blocked without delay by HUN.

The data presented herein indicate that HUN causes profound alterations in the DNA of treated cells. It is apparent that, after long exposure to 0.05 M HUN, the cellular DNA is extensively degraded, as evidenced by the decrease in the amount of acid-precipitable material, the inability of DNA from treated cells to band in gradients of cesium chloride, the altered chromatographic profile of DNA from treated cells when subjected to analysis on ECTEOLA-cellulose, and the lowered sedimentation coefficient of DNA derived from bacteria exposed to HUN for short periods.

It is concluded from these observations that degradation of cellular DNA is the event primarily responsible for the lethal properties of HUN. If DNA degradation is the basis of HUN-induced killing, it can be predicted that agents which reduce the lethal effects of HUN should also interfere with the depolymerization of prela-

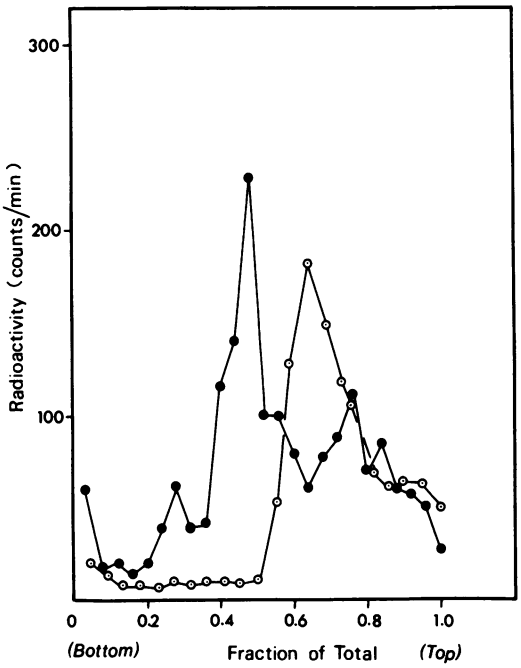

FIG. 7. *Analysis on gradients of sucrose of DNA from normal and HUN-treated cells. Cultures were labeled with ^{3}H- and ^{14}C-thymidine. The culture labeled with ^{14}C-thymidine served as control; the one containing ^{3}H-thymidine was exposed to 0.05 M HUN for 4 hr. (During exposure to HUN the viability of the culture decreased from 1.2×10^{8} to 2.6×10^{5} cells/ml.) Protoplasts were prepared (15) and layered onto a linear (5 to 20%) sucrose gradient (pH 12), and 0.1 ml of 0.5 N NaOH was added. After 15 min of standing at room temperature to insure complete lysing of the protoplasts, the materials were spun in the SW50 rotor of a Spinco model L-2 centrifuge for 90 min at 30,000 rev/min. Fractions were then collected and processed for determination of radioactivity.* ●, *^{14}C-DNA (control);* ⊙, *^{3}H-DNA (HUN-treated).*

beled DNA to acid-soluble products, and thus it was found that the rate of this solubilization was greatly reduced in the presence of chloramphenicol, an antagonist of the lethal action of HUN. However, after 23 hr of exposure to a mixture of the two drugs, the amount of acid-precipitable DNA was reduced by 30% even though the number of viable bacteria was not so affected. Although the initial rate of DNA degradation in *E. coli* C600/HUN was retarded, at the end of 24 hr of treatment the extent of this depolymerization was identical to that of the sensitive strain, but cellular death was decreased considerably in the resistant organism. This suggests that degradation of DNA of HUN-treated cells is the result of two separate occurrences: a reaction between HUN and DNA which results in alteration of the DNA and which in time becomes so extensive that degradation to acid-soluble fragments ensues. The rate of attack on the DNA is retarded in cells treated simultaneously with HUN and chloramphenicol. The solubilization which occurs under these conditions after extended exposure to HUN is a reflection of the ability of the bacteria to repair HUN-induced damage to DNA; this is discussed in detail in the accompanying paper (29).

Although the primary effect of HUN is on the structure of the cellular DNA, metabolically this is reflected in a rapid inhibition of RNA (Fig. 2) and more specifically messenger RNA (Table 5). This finding probably reflects the more rapid synthesis of this molecular species—as soon as the DNA template is damaged transcription ceases.

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