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Metabolic Processes Involved in Repair of *Escherichia coli* Cells Damaged by Exposure to Acid Mine Water†

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***Escherichia coli* was stressed by exposure to filter-sterilized acid mine water. Synthetic processes required for repair of sublethally injured survivors were studied by the addition of specific metabolic inhibitors to a resuscitation broth. Inhibitors of protein, RNA, DNA, lipid, and peptidoglycan synthesis as well as uncouplers and inhibitors of electron transport and ATPase activity were used. Acid mine water injury was severe, causing damage to the outer and cytoplasmic membranes. Repair of sublethally injured cells required protein, RNA, and lipid synthesis as well as a proton motive force.**

Formation of acid mine water (AMW) from abandoned coal mines and refuse piles is one of the most persistent industrial pollution problems in the United States, contributing to the degradation of over 10,000 miles (ca. 16,100 km) of rivers and streams (29). AMW is characterized by low pH and elevated concentrations of metal ions. AMW pollution affects waters used for human and animal consumption, recreation, and wastewater disposal.

Aquatic environments affected by AMW are often polluted by domestic or agricultural wastes or both. Assessment of water quality with bacterial indicator organisms requires that the actual number of bacteria present be detected accurately. Inability to detect stressed or injured indicator organisms can compromise the evaluation of the public health safety of a water supply. Previous studies in our laboratory have demonstrated that *Escherichia coli* was injured following exposure to AMW (15, 19, 42) and that the adverse effect of AMW on fecal-indicator detection can be reduced by resuscitation techniques (10, 16). Recently, we reported that AMW caused extensive structural damage to *E. coli* (43) and that an extended incubation period was required for repair of injury (42).

Improvements in detection methodologies for fecal indicators in AMW- contaminated waters depend on a better understanding of the mechanisms of injury. Although *E. coli* is injured by AMW exposure, the damage is reversible if the stressed cells are incubated under appropriate conditions of repair (42). We report here metabolic processes involved in the repair of AMW-damaged *E. coli*. Repair was monitored in the presence of specific metabolic inhibitors. Physiological processes required for repair can be identified by adding a specific inhibitor to the resuscitation broth to block a targeted metabolic process (4, 38, 39). Damaged cells are then unable to resuscitate or do so at a reduced rate if the blocked metabolic process is needed for repair. In the current study, resuscitation of AMW-damaged cells was significantly affected by inhibitors of the proton motive force and of synthesis of RNA, protein, and lipids. These observations extend our previous findings on AMW-stressed *E. coli* by identifying key physiological processes required for repair of injury.

MATERIALS AND METHODS

AMW. AMW was collected from an underground stream draining an area of active strip mining in West Virginia. Specific conductance, pH, acidity, and temperature were measured at the time of collection (2). Mean values for 66 samples were as follows: pH 3.02, standard deviation (SD) = 0.07; acidity, 1,755 mg of $CaCO_3$ per liter, SD = 262; specific conductance, 3,079 μS/cm, SD = 167; and temperature, 16.1°C, SD = 0.5. Concentrations of metals commonly associated with AMW were measured by atomic absorbance (2). Mean values (± SD, in milligrams per liter) for 11 samples were Al, 133.2 ± 7.1; Cd, not detected; Cu, 0.11 ± 0.03; Fe, 231.1 ± 13.5; Mn, 5.6 ± 1.1; Pb, 0.002 ± 0.001; Zn, 2.7 ± 0.7.

Test organism. *E. coli* B/5 (from P. Snustad, University of Minnesota) was maintained on Trypticase soy agar supplemented with 0.3% yeast extract (TSYA) and stored at 4°C. Prior to use, 100 ml of Trypticase soy broth fortified with 0.3% yeast extract (TSYB) was inoculated and incubated for 6 h at 35°C. Two loopfuls of culture were subsequently transferred into 100 ml of TSYB and incubated for 12 h at 35°C on a rotary shaker (125 cycles per min). Cells were pelleted by centrifugation for 10 min at 3,020 × *g* and washed twice in a volume of 0.1% peptone buffer (2) equal to that of the harvested culture. The final suspension contained approximately 1.2×10^9 CFU/ml.

AMW exposure and repair of injury. Washed cells in 2-ml samples were injured by exposure to 100 ml of filter-sterilized AMW by the method of Wortman and Bissonnette (42). This procedure caused injury and death indices of 98.3% (SD = 3.1%, *n* = 81) and 98.3% (SD = 2.6%, *n* = 81), respectively. After exposure, 2 ml of the inoculated AMW was withdrawn and placed in 98 ml of TSYB repair medium (with or without a specific metabolic inhibitor) and incubated at 35°C with constant agitation (125 cycles per min). The TSYB repair medium was identical in composition to the broth used to grow the organisms prior to AMW stress. Previous studies indicated that repair of AMW-damaged cells proceeded optimally under these conditions (42). At predetermined times, samples were removed from the repair broth, diluted, and surface plated in triplicate onto selective (TSYA plus 0.05% sodium deoxycholate [TSYDA]) and nonselective (TSYA) media. Following incubation at 35°C

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TABLE 1. MICs[a] and static concentrations[b] for *E. coli* B/5 exposed to various metabolic inhibitors

Inhibitor	Mode of action (reference)	MIC (μg/ml)	Static concn (μg/ml)	Sensitivity factor[c]
Nalidixic acid	Inhibition of DNA replication (14, 44)	1.9	1.5	1.2
Chloramphenicol	Inhibition of translation (34)	1.9	0.8	2.3
Rifampin	Inhibition of transcription (37, 41)	31.2	1.5	21.0
DNP	Uncoupling of respiration (21)	62.5	31.2	2.0
CCCP	Dissipation of ΔpH portion of proton motive force (21)	3.9	0.7	5.5
Cerulenin	Inhibition of lipid synthesis (11, 17, 33)	62.5	12.0	5.2
D-Cycloserine	Inhibition of peptidoglycan synthesis (31)	125.0	6.0	20.8
DCCD	Inhibition of ATPase (21, 22)	16.5	0.3	55.0
Potassium cyanide	Inhibition of electron transport (18)	31.2	8.0	3.9

[a] Determined by the macrodilution broth method (26).
[b] Greatest concentration of inhibitor not causing a significant reduction in the number of viable, AMW-injured cells during an initial 3-h incubation in TSYB.
[c] MIC/static concentration ratio.

for 24 ± 2 h, colonies were counted on a Quebec dark-field colony counter. Controls for these experiments consisted of injured bacteria in inhibitor-free repair broth and nonstressed 12-h-culture washed cells under identical repair conditions (TSYB with or without inhibitor).

Inhibitors. Nine inhibitors were used in the study. Chloramphenicol, rifampin, and nalidixic acid were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cerulenin, and D-cycloserine were obtained from Sigma Chemical Co., St. Louis, Mo. Potassium cyanide and 2,4-dinitrophenol (DNP) were obtained from Fisher Scientific Co., Fair Lawn, N.J. *N, N'*-Dicyclohexylcarbodiimide (DCCD) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. The reported modes of action of these inhibitors are given in Table 1.

The MIC for each compound was determined by an adaptation of the macrodilution broth method (26) in which TSYB was substituted for Mueller-Hinton broth. Results were determined at 6, 12, and 24 h by using a 12-h nonstressed culture. The 6-h MIC was used as a starting point to determine concentrations for examining the repair of AMW-injured cells.

AMW-exposed cells were placed into flasks containing twofold dilutions of the MICs for each inhibitor, and viability was monitored on the nonselective medium. The greatest concentration of each inhibitor which did not cause a significant decrease in cell number during a 3-h incubation at 37°C was considered static but not lethal; this concentration was used to examine the roles of various metabolic processes in repair. Several of the inhibitors did not show a static concentration when twofold dilutions were used; thus, subsequent determinations were made in smaller increments.

Assumptions, calculations, and statistical analyses. All viable cells (injured and noninjured) are capable of producing colonies on nonselective TSYA, whereas only repaired or uninjured cells can grow on the selective medium, TSYDA. Increased counts observed on TSYDA without a concomitant increase on TSYA are due to the repair of damaged organisms. Simultaneous increases in TSYDA and TSYA counts are due to cell division (39).

The percentage of dead cells was calculated as [(nonselective count before AMW exposure − nonselective count after AMW exposure)/(nonselective count before AMW exposure)] × 100. The percentage of injured cells was calculated as [(nonselective count after AMW exposure − selective count after AMW exposure)/(nonselective count after AMW exposure)] × 100.

Data were analyzed to determine the time needed by *E. coli* to repair injury in the absence and presence of the various inhibitors. Repair of AMW-stressed cells in the absence of the respective inhibitors was repeated in each of the separate experiments. This was necessary since there was variation in the level of injury from experiment to experiment, reflected by the amount of time needed to complete repair. This variation was not unexpected since we could not control the daily fluctuation of the chemical-physical composition of the AMW. Detailed repair curves are given for only three of the nine inhibitors, which were representative of compounds that had no effect on, enhanced, or inhibited resuscitation. Repair times for all the inhibitors were determined with the General Linear Models System of the Statistical Analysis System (SAS Institute Inc., Cary, N.C.); it was used to fit quadratic equations to the data points. Two equations were generated for each data set, one from counts made on TSYA and a second from counts made on TSYDA. Confidence limits of 95% were then calculated for each curve. The time needed for repair was determined as that point (to the nearest 0.1 h) at which the counts were not significantly different (overlapping of 95% confidence intervals).

Uptake of radiolabeled substrates. Uniformly labeled [^{14}C]uracil (62 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. L-[^{3}H]tryptophan (1.0 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Uracil (50 μCi) and tryptophan (200 μCi) were added to 98 ml of TSYB, which was then inoculated with AMW-injured bacteria as described above. Resuscitation was followed with triplicate plate counts made on TSYA and TSYDA. Simultaneously with the plate counts, a 5-ml sample of culture was removed and filtered through a 0.22-μm-pore-size membrane filter (GSWP 025 00; Millipore Corp., Bedford, Mass.) and washed three times with 5 ml of cold 5% trichloroacetic acid. The filter was dried, placed in 10 ml of scintillation cocktail, and counted in a liquid scintillation counter (46OCD; Packard Instrument Co., Inc., Rockville, Md.).

RESULTS AND DISCUSSION

Injury induced by AMW is reversible if the damaged cells are incubated under suitable conditions of repair (42). The need for a given physiological process during repair can be determined by supplementing the resuscitation broth with inhibitors to block metabolic processes (4, 38, 39). If the blocked metabolic process is needed for repair, the damaged cells are unable to resuscitate or do so at a reduced rate. The use of inhibitors in this manner is justified because repair processes are distinct from population growth, since repair can occur under conditions which prevent cell multiplication (25).

MIC and static concentration determinations. Initial studies compared the effects of MICs determined for healthy cells with those determined for AMW-damaged *E. coli*. These concentrations were unsatisfactory for repair studies because they often were lethal to injured cells; thus, the determination of static concentrations for AMW-exposed cells was necessary. Damaged cells were more sensitive than undamaged cells to all inhibitors tested. Differences in the sensitivity factor (ratio of MIC to static concentration) suggested a differential susceptibility of healthy and AMW-injured cells to these inhibitors (Table 1). In particular, the sensitivity factors were most pronounced for DCCD, rifampin, and D-cycloserine. Increased sensitivity of gram-negative bacteria to antibiotics and other metabolic inhibitors is indicative of damage to the outer membrane (39). Thus, the increased sensitivity of injured cells to the inhibitors suggested that the outer membrane sustained damage during AMW exposure. These findings support previous ultrastructure studies by Wortman et al. (43) which indicated that *E. coli* exposed to AMW experienced a considerable change in morphology, accompanied by leakage of cytoplasm and apparent damage to portions of the bacterial envelope. Sensitivity factors (Table 1) also indicated that the outer membranes of uninjured organisms excluded nalidixic acid, cerulenin, chloramphenicol, and KCN and more effectively than they excluded DCCD, D-cycloserine, and rifampin.

The concentrations of inhibitors used in this study often resulted in a reduction in the number of uninjured cells produced during a 6-h incubation. This decrease was equivalent only to a reduction of two cell divisions over the incubation period. Concern has been expressed about the action of subminimum inhibitory concentrations of antibiotics on susceptible organisms (30), yet studies have not clearly demonstrated different modes of action for these compounds at low concentrations (1, 30). Such effects do not appear relevant in our study because the concentration of inhibitor added to the repair broth was the MIC for damaged cells rather than that for healthy cells.

Compounds without effect on repair. Nalidixic acid, a potent inhibitor of DNA replication (14), was the only compound tested which had no effect on resuscitation (Fig. 1 and Table 2). Resuscitation was complete within 4 h regardless of the presence or absence or nalidixic acid. This inhibitor binds to DNA gyrase, interfering with the three-dimensional changes necessary for replication (44). Lack of replication during repair was expected since DNA synthesis should occur only after resuscitation is complete.

Compounds that inhibited repair. Chloramphenicol and rifampin were used to evaluate the need for protein and RNA synthesis during repair. Chloramphenicol, which binds to the 50S particle of procaryotic ribosomes (34), prevented repair, since none of the cells repaired themselves within the 3.5-h period required for resuscitation in the medium lacking the inhibitor (Fig. 2 and Table 2). Since the concentration of chloramphenicol added to the repair broth was not lethal, as indicated by constant nonselective counts during the incubation period and multiplication of the control culture, these data indicate that all exposed cells required translation for repair of AMW injury.

Rifampin binds specifically to the β subunit of bacterial RNA polymerase, thus interfering with DNA-dependent RNA synthesis (37, 41). RNA synthesis was required by a large portion of the resuscitating population (Table 2), perhaps reflecting the need for mRNA synthesis as well as tRNA and rRNA production. A static concentration for this inhibitor was difficult to determine because the difference between a concentration with no effect and one with a major effect was sharp. More than 90% of the AMW-exposed bacteria died in the presence of rifampin. These results suggest that RNA synthesis was needed for repair and that without it, the more severely damaged cells perished. Of the cells that survived, 91% required RNA synthesis.

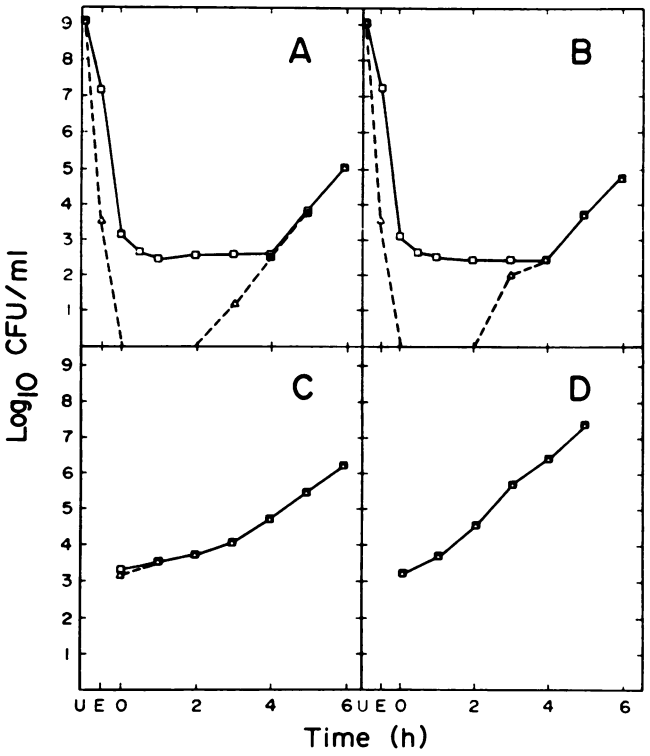

FIG. 1. Effect of nalidixic acid on the ability of *E. coli* B/5 to repair damage sustained during AMW exposure. A washed 12-h culture of *E. coli* B/5 was exposed for 1 min to a 3:1 (AMW-H_2O) dilution of sterile AMW, placed in TSYB containing 1.5 μg of nalidixic acid per ml, and incubated at 35°C. Uninjured cells were diluted in 0.1% peptone buffer, placed in TSYB containing 1.5 μg of nalidixic acid per ml, and incubated at 35°C. (A) Injured cells in TSYB plus nalidixic acid. (B) Injured cells in TSYB. (C) Uninjured cells in TSYB plus nalidixic acid. (D) Uninjured cells in TSYB. ——, Counts on TSYA; – – –, counts on TSYDA; U, cell counts before exposure to AMW; E, cell counts after 1 min of exposure to AMW; 0, cell counts from TSYB (repair broth) immediately after inoculation with AMW-exposed bacteria.

The proton ionophores DNP and CCCP are soluble in membrane lipids and function by reducing the ΔpH component of the electrochemical gradient (21). This proton shuttling activity can lead to a collapse or reversal of the $\Delta\psi$ if positively charged ions are not removed from the cell interior. Bacteria can use the electrochemical gradient for oxidative phosphorylation, active transport, osmotic regulation, and motility (12). Data from inhibitor studies utilizing CCCP and DNP indicate that any or all of these processes might be required for repair by a significant portion of the AMW-damaged cells (Table 2).

Cerulenin, a lipid synthesis inhibitor, blocks the condensation of acyl- and malonyl-thioesters (11, 17, 33), and addition of this inhibitor interfered with the repair of 91% of the AMW-injured bacteria (Table 2). Since virtually all lipids in gram-negative bacteria are associated with envelope

TABLE 2. Effect of inhibitors on repair of AMW-damaged *E. coli*

Inhibitor[a] and concn (μg/ml)	Time required for repair (h)		Effect on repair	% Effect[b]
	Inhibitor present	Inhibitor absent		
Nalidixic acid (8.0)	4.0	4.0	Neutral	0
Chloramphenicol (0.8)		3.5	Inhibition	100
Rifampin (1.5)	5.3	4.4	Inhibition	91
DNP (31.2)	4.9	3.1	Inhibition	93
CCCP (0.7)	4.0	3.5	Inhibition	46
Cerulenin (12)	4.2	2.8	Inhibition	91
D-Cycloserine (6)	2.7	3.1	Enhancement	79
DCCD (0.3)	2.0	2.2	Enhancement	59
KCN (1.5)	4.0	4.2	Enhancement	33

[a] Static concentration in parentheses.

[b] When the tested compound had an inhibitory effect on repair, this value reflects the percentage of the injured population which had not completed repair in the presence of the inhibitor when the injured control culture completed resuscitation. When the tested compound enhanced repair, this value reflects the percentage of the injured control population which was not repaired when the culture containing the inhibitor completed repair.

structures (27), the need for lipid synthesis indicated a significant amount of damage to the outer or cytoplasmic membranes or both. These findings agree with recently reported morphological studies of AMW-damaged bacteria which showed extensive membrane alterations (42).

Compounds that enhanced repair. Compounds that interfered with electron transport, ATPase function, and peptidoglycan synthesis improved the ability of the damaged bacteria to complete resuscitation, suggesting that elimination of nonessential synthetic activities might facilitate repair by permitting more efficient use of cellular resources. D-Cycloserine blocks peptidoglycan synthesis by interfering with the incorporation of D-alanine into peptide chains (31). The possibility that cell wall synthesis is nonessential to AMW-injured bacteria was suggested by a shortened repair period when this process was inhibited (Fig. 3 and Table 2).

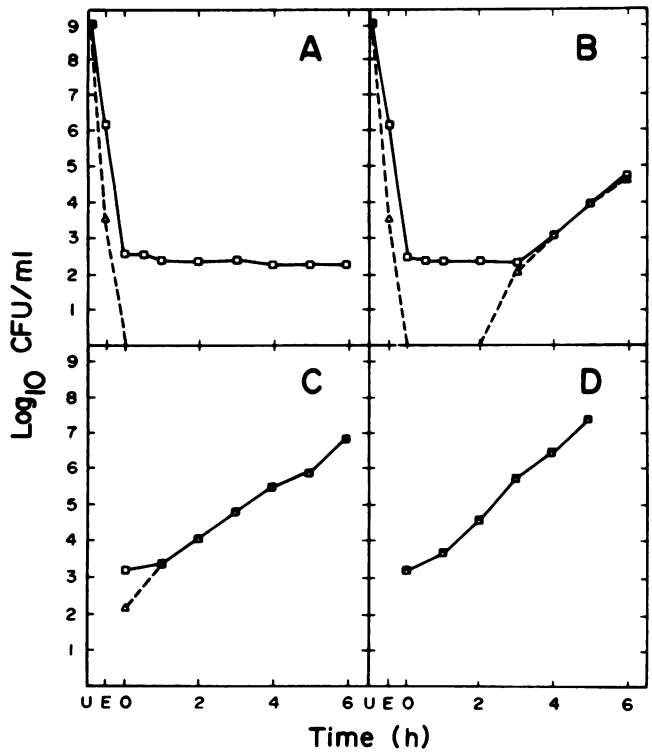

FIG. 2. Effect of chloramphenicol on the ability of *E. coli* B/5 to repair damage sustained during AMW exposure. A washed 12-h culture of *E. coli* B/5 was exposed for 1 min to a 3:1 (AMW-H_2O) dilution of sterile AMW, placed in TSYB containing 0.8 μg of chloramphenicol per ml, and incubated at 35°C. Uninjured cells were diluted in 0.1% peptone buffer, placed in TSYB containing 0.8 μg of chloramphenicol per ml, and incubated at 35°C. (A) Injured cells in TSYB plus chloramphenicol. (B) Injured cells in TSYB. (C) Uninjured cells in TSYB plus chloramphenicol. (D) Uninjured cells in TSYB. For explanation of symbols, see legend to Fig. 1.

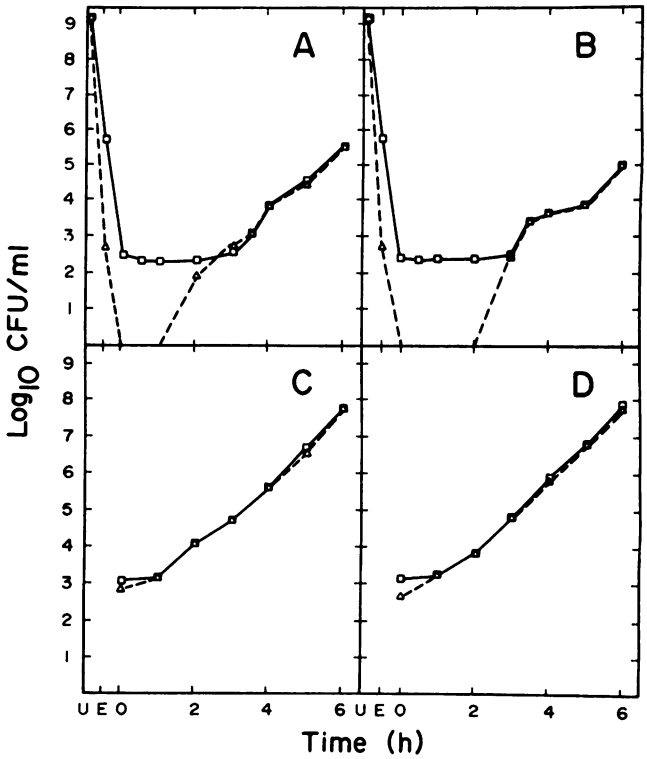

FIG. 3. Effect of D-cycloserine on the ability of *E. coli* B/5 to repair damage sustained during AMW exposure. A washed 12-h culture of *E. coli* B/5 was exposed for 1 min to a 3:1 (AMW-H_2O) dilution of sterile AMW, placed in TSYB containing 6.0 μg of D-cycloserine per ml, and incubated at 35°C. Uninjured cells were diluted in 0.1% peptone buffer, placed in TSYB containing 6.0 μg of D-cycloserine per ml, and incubated at 35°C. (A) Injured cells in TSYB plus D-cycloserine. (B) Injured cells in TSYB. (C) Uninjured cells in TSYB plus D-cycloserine. (D) Uninjured cells in TSYB. For explanation of symbols, see legend to Fig. 1.

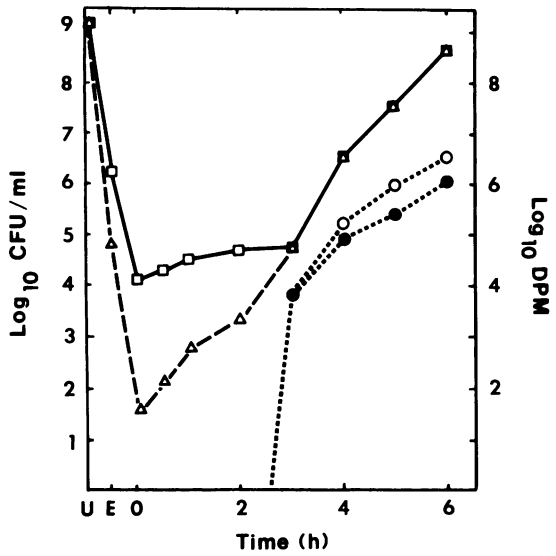

FIG. 4. Uptake of radiolabeled precursors during repair of AMW damage by *E. coli* B/5. A washed 12-h culture of *E. coli* B/5 was exposed for 1 min to a 3:1 (AMW-H_2O) dilution of sterile AMW, placed in TSYB containing 200 μCi of [^{3}H]tryptophan and 50 μCi of [^{14}C]uracil, and incubated at 35°C. [^{3}H]tryptophan (○) and [^{14}C] uracil (●) uptake are shown. For explanation of remaining symbols, see legend to Fig. 1.

Similarly, Calcott and MacLeod (6) observed that cell wall damage was not detrimental to the survival of freeze-damaged *E. coli*.

DCCD, which inhibits the function of membrane-bound ATPase, prevents oxidative phosphorylation as well as ATP hydrolysis by restricting the movement of protons through the membrane-associated F_0 subunit (20–23). The ability of DCCD to improve resuscitation of injured bacteria (Table 2) could indicate that the F_1 subunit was damaged or missing, effectively uncoupling the injured bacteria. The addition of DCCD would then prevent dissipation of the proton motive force by blocking the proton channel of the F_0 subunit. A similar phenomenon was reported for *E. coli* NR70, which was uncoupled due to a lack of the F_1 portion of the ATPase (40). Our results also suggest that the cytoplasmic membrane was damaged and initially could not maintain a charge separation. It appears that DCCD might have enhanced repair by preventing ATPase-mediated extrusion of protons, which would leak back into the cell through damaged membranes without having performed useful work.

Electron transport uses reducing power from specific substrates to move the protons from the interior of the cell to the exterior, thereby creating a ΔpH. Cyanide inhibits electron transport by interfering with the terminal oxidase of the respiratory chain (18). Inhibition of electron transport shortened the repair period of AMW-injured bacteria (Table 2). Preventing the oxidation of certain compounds by blocking electron transport might allow the energy stored in these substrates to be used for other cellular activities. The enhancement of repair by cyanide suggests that the cells were uncoupled by AMW damage to the cytoplasmic membrane and supports our findings with DCCD.

Uptake of radiolabeled substrates. Acid-stressed cells also were examined for the ability to take up radiolabeled tryptophan and uracil (Fig. 4). Tryptophan enters *E. coli* by a highly specific, inducible transport system which requires the presence of a proton motive force (20, 28). Labeled tryptophan did not enter the AMW-exposed cells until repair was complete (3.0 h), indicating that the electrochemical gradient was insufficient to activate transport or that proteins involved in transport had been damaged by AMW. These findings provide further evidence of membrane involvement during AMW injury. The pattern of uracil uptake was similar to that of tryptophan and did not occur until repair was complete (Fig. 4). Loss of transport function following injury has been observed in cells damaged by heating (24, 32), freezing (8), and contact with chlorine (9). Failure to transport substrates could result in starvation conditions for AMW-exposed cells. Cells would be forced to obtain carbon and energy needed for repair from endogenous reserves. Organisms rich in endogenous reserves are better able to survive stress (5). However, endogenous metabolism does not require the presence of specialized storage materials, because protein and RNA can be degraded in the absence of specific reserves (13, 35).

Conclusions. Processes needed for repair by AMW-injured *E. coli* were similar to those needed for repair of organic acid injury, which required RNA and protein synthesis as well as an energized membrane (4). Thermally injured *Pseudomonas fluorescens* also required RNA and protein synthesis for repair (32). Freeze-damaged *E. coli* showed cell wall and membrane damage (39). The damage suffered by AMW-exposed cells in our study was greater than that of *E. coli* injured by exposure to acetic acid (36), in which repair occurred rapidly, requiring only protein synthesis.

Cells which experienced injury as extensive as that described in this study would need significant amounts of synthetic activities to replace damaged membrane lipids and proteins. Even with repaired membranes, the cell is probably still not completely resuscitated. The effect of DNP and CCCP on repair indicated that an electrochemical gradient was essential for complete resuscitation. If cells were uncoupled by AMW damage and remained uncoupled until repair was complete, then ionophores would not facilitate resuscitation because cells would have no gradient to dissipate until repair was finished. But if the membrane were replaced first, the cell could hold a gradient which could then be used for other repair activities, such as active transport. This suggests that repair is occurring in a stepwise fashion. Several authors (3, 24) have concluded that all forms of injury have membrane involvement, and Calcott and MacLeod (7) found that viability was related to the degree of membrane damage. The damage to both the outer and cytoplasmic membranes resulting from AMW exposure substantiates these conclusions.

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