APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1979, p. 633-641
0099-2240/79/03-0633/09$02.00/0

Vol. 37, No. 3

Chlorine Injury and the Enumeration of Waterborne Coliform Bacteria

ANNE K. CAMPER AND GORDON A. McFETERS*

Department of Microbiology, Montana State University, Bozeman, Montana 59717

Received for publication 26 December 1978

Injury induced in *Escherichia coli* cells by chlorination was studied from a physiological standpoint. Predictable and reproducible injury was found to occur rapidly in 0.5 mg of chlorine per liter and was reversible under nonselective conditions. There was an extended lag period in the growth of chlorinated cells not seen in control suspensions followed by the resumption of logarithmic growth at a rate equaling that of control cells. The aldolase activity of cells chlorinated in vivo was equivalent to that obtained for control cells. Oxygen uptake experiments showed that chlorinated cells underwent a decrease in respiration that was not immediately repaired in the presence of reducing agents. This effect was more pronounced in rich media containing reducing agents. Uptake of metabolites was inhibited by chlorine injury as shown with experiments using ^{14}C-labeled glucose and algal protein hydrolysate.

When waterborne organisms of fecal origin are confronted with hostile chemical environments, they experience stress, injury, and finally death. Reports in the literature indicate that injury of a reversible nature occurs in cells that have been exposed to water (4), freezing in foods (25), and sanitizers (31). Other potential sources of injury may also be present in aquatic environments. The subject of chlorine-induced injury has been controversial, with some investigators stating that enumerated cells were not injured (7, 14), whereas others suggested that certain cells were capable of repairing the injury incurred (23, 22). It has also been proposed that the addition of certain metabolites aided in the reversal of injury (12) and that reducing agents would inhibit or reverse the oxidation caused by chlorine (16, 27).

Studies have shown that the membrane filtration procedure for enumerating coliform bacteria with the selective M-FC medium does not enumerate chlorine-injured cells (5, 10, 19, 21, 26, 32). The multiple-tube fermentation (most-probable-number) technique has provided higher estimates of bacterial numbers due to its initial nonselective medium (11), but it is time consuming and cumbersome. Improvements in the membrane filtration technique have been made for detecting chlorine-injured populations, but with little consideration of the cellular mechanism of chlorine damage (8, 30; R. A. Green, R. H. Border, and P. V. Scarpino, Abstr. Annu. Meet. Am. Soc. Microbiol. 1964, p. 34) or based on previously accepted modes of chlorine action (35). Many questions have been left unanswered with regard to the specific site(s) of chlorine injury in coliform bacteria.

The earliest research on the physiology of bacterial chlorine injury was reported in 1946 by Green and Stumpf (13). They suggested that chlorine acted as an inhibitor of glucose oxidation in cells. This work along with that of Knox et al. (18) attempted to demonstrate that chlorine specifically oxidized sulfhydryl groups of certain enzymes important in carbohydrate metabolism. They proposed that aldolase was the main site of action due to its essential nature in metabolism. An indirect substantiation of the aldolase hypothesis was published in 1953 (16). Skidal'skaya (34) and Venkobacher et al. (37) performed experiments investigating the effect of chlorine on bacterial dehydrogenases. Electron microscopic examinations (6) of cells treated with chlorine showed no morphological alteration. Other more recently considered modes of chlorine action have been: unbalanced metabolism after the destruction of key enzymes (39), disruption of protein synthesis (3), oxidative decarboxylation of amino acids to nitriles and aldehydes (29), reactions with nucleic acids, purines, and pyrimidines (13), the formation of chloro derivatives of cytosine (28), the creation of chromosomal aberrations (15), the induction of deoxyribonucleic acid lesions with the accompanying loss of deoxyribonucleic acid transforming ability (33), and inhibition of oxygen uptake and oxidative phosphorylation coupled with leakage of some macromolecules (38).

An understanding of chlorine injury physiology in indicator bacteria is important for optimal

enumeration of bacteria from chlorinated and receiving waters. The purpose of this research was to determine whether chlorine does produce injury in a bacterial population and whether that injury is reversible. Studies were also undertaken to determine the physiological site of chlorine injury.

MATERIALS AND METHODS

Organism. The organism used was a fecal *Escherichia coli* isolated from the East Gallatin River and maintained on stock culture medium (Difco, Detroit, Mich.). It produced a ++−− IMViC (indole, methyl red, Voges Proskauer, citrate) reaction, formed colonies with a green metalic sheen on Levin EMB agar (Difco), and produced gas in EC medium (Difco).

Media and reagents. The water used was processed through a Milli-Q water system (Millipore Corp., Bedford, Mass.) after single distillation. Peptone-phosphate buffer used as a diluent was prepared from a stock solution (1) with the addition of 0.1% peptone (Difco). Tris(hydroxymethyl)aminomethane (Tris) buffer (Sigma Chemical Co., St. Louis, Mo.) was used for the oxygraph and aldolase assays. A 0.05 M solution of pH 7.2 (35°C) was autoclaved and refrigerated until used. Chlorine demand-free water and sodium thiosulfate used to neutralize residual chlorine in samples were prepared according to "Standard Methods" (1). Solutions of sodium thioglycolate and glutathione (Sigma Chemical Co.) were prepared according to Stuart et al. (35) and refrigerated.

TSY broth was prepared by supplementing Trypticase soy broth (Difco) with 0.3% yeast extract (Difco) and sufficient glucose to make the final concentration 0.5% (wt/vol). M-FC broth (Difco) was prepared according to "Standard Methods" (1) not more than 4 h before use. Solid M-FC medium was made by the addition of 1.5% agar (wt/vol). The overlay medium was prepared as prescribed by Stuart et al. (35). The overlay medium was used as the index of total viable cells in the plate count procedure. The same medium without agar was used in the recovery experiments. Mineral salts medium (36) was filter sterilized through a 0.22-μm filter (Millipore Corp.) and refrigerated.

Cell enumeration and cell density procedures. The membrane filter technique as described in "Standard Methods" (1) for the enumeration of fecal coliforms was followed, using type HC filters and disposable plastic plates (Millipore Corp.). For each medium, duplicate plates of two or more dilutions per sample were used. The overlay plates were incubated at 35°C in a circulating air incubator, and the M-FC plates were held in a 44.5°C circulating water bath or block-type incubator (Millipore Corp.). The 24-h M-FC plate incubation began within 10 min of filtering. Spectrophotometric measurements of cell densities were determined by using the Varian Techtron model 635 spectrophotometer at 420 nm.

Chlorination procedure. Commercial chlorine bleach (Clorox) was purchased at least monthly and refrigerated. A new stock solution was made daily immediately before use. For most treatments, a 0.5-mg/liter final concentration was desired; therefore, a 500-mg/liter solution was prepared by volumetrically diluting 1 ml of the 5.25% sodium hypochlorite bleach with 99 ml of chlorine demand-free water. One milliliter of this solution was then transferred to 1 liter of a mixed bacterial suspension in the chlorination vessel. The *E. coli* culture was grown for 12 h at 35°C in TSY broth. The cells were centrifuged at 3,020 × *g* for 10 min and suspended in peptone-phosphate buffer. This was repeated twice, with the final suspension in sterile chlorine demand-free water of a volume equal to that of the growth medium (approximately 8×10^9 organisms per ml). A portion of washed cells was diluted 1:10 for a control, and the cells were kept on ice. A volume of 900 ml of sterile chlorine demand-free water was added to a sterile, acid-washed, foil-covered, 2-liter-capacity DeLong flask containing a magnetic stir bar. The water was stirred constantly at low speed. One hundred milliliters of the washed cells was added and mixed, and 1 ml was removed and added to an iced dilution blank for plate count. One milliliter of the chlorine stock solution was added, and timing commenced. At timed intervals, 1-ml samples were removed and added to dilution blanks, and 1 ml of sodium thiosulfate was added. All samples were iced until filtered. These experiments were performed at 22 to 25°C and pH 6.5.

Recovery experiments. Organisms that had been chlorinated for 8 min were used in this procedure, and enumeration was by the membrane filter technique. Four-milliliter samples of control and chlorinated cells were placed in separate flasks containing 36 ml of overlay broth. The zero-time samples were removed, and the flasks were incubated at 35°C. Samples were removed hourly, filtered, and incubated on M-FC and overlay media.

Oxygraph and aldolase assays. Control and chlorinated cells were centrifuged at 3,020 × *g* for 10 min at 1 to 3°C and suspended in one-fourth the original volume of 0.05 M Tris buffer. These concentrated cells were placed in glass-stoppered flasks and iced. Oxygraph experiments were performed immediately after concentration. The cell suspensions were refrigerated overnight and disrupted with sonic treatment before the aldolase assay. Sonic treatment for 15 min with a Bronwill Biosonic sonic oscillator set at 90% of maximal intensity was used and led to 98% disruption of the cells. The sonic treatment was done on ice in 3-min bursts, with cooling between treatments. This solution was diluted 1:10 with Tris buffer for the aldolase and protein determinations. The protein determination of Lowry et al. (20) was used, and a standard curve using bovine serum albumin was performed in conjunction with each set of protein determinations.

The aldolase assay used was a modification of the hydrazine assay as described by Jagannathan et al. (17) and performed according to the Worthington manual (Worthington Biochemicals Corp., Freehold, N.J.). Reaction progress was recorded with a Varian spectrophotometer at 240 nm. Each reaction was monitored for 3.5 min while temperatures of 35 or 45°C were maintained around the reaction cuvette by means of a Heto ultrathermostat model 623 EUL circulating heater. The hydrazine sulfate solution was allowed to reach room temperature before use, but the fructose 1,6-diphosphate and sonically disrupted cells were

iced. Triplicate readings were taken for each sample. Statistical analyses of the data obtained were performed on each group. The t value was determined by using pooled variance; the two-tailed P values were obtained by use of a t probability program run on a Xerox Sigma 7 computer.

Oxygraph assays were performed with a Gilson model KM oxygraph equipped with a YSI model 4004 Clark-type oxygen electrode. Constant chamber temperature was maintained at 35°C. One milliliter of iced cells was added to the chamber and allowed to equilibrate for 1 min, and 1 ml of substrate was then added and equilibrated for 30 s. Oxygen uptake was monitored for 3 min. The rates were determined from the slopes of the lines obtained, and results are expressed as the percentage of the slope of the chlorinated cells as opposed to that of the control cells.

Adenosine 5'-triphosphate (ATP) determinations. Cells were prepared as for the oxygraph experiments and extracted by a modification (L. E. Hersmann and K. L. Temple, Soil Sci., in press) of the method of Bancroft et al. (2). Readings were made in a DuPont Luminescence Biometer.

Labeled glucose and algal protein hydrolysate experiments. D-[U-^{14}C]glucose with a specific activity of approximately 5.0 mCi/mmol (New England Nuclear Corp., Boston, Mass.) and algal [U-^{14}C]protein hydrolysate with a specific activity of 1.0 mCi/mmol (International Chemical and Nuclear Co., Irvine, Calif.) were received in 0.05-mCi amounts. This was solubilized in distilled water, and portions containing 1/10 of the labeled material were used per 100 ml of organisms.

A standard liquid scintillation counting procedure was used with all the experiments. Air-dried filters containing bacteria were exposed to 105°C for 20 min, rolled, placed in Poly-Q scintillation vials (Beckman Instruments Co., Irvine, Calif.), and made transparent with 4 ml of toluene, and 9 ml of Aquasol liquid scintillation cocktail (New England Nuclear Corp.) was added. The vials were shaken and placed in a Beckman liquid scintillation counter and counted to 5% error. The counter was programed to perform labeled carbon counts and external standards on each vial.

Three types of labeling experiments were performed. One of these involved long-term labeling before chlorination. Two flasks of TSY broth (100 ml) were inoculated and incubated for 10 h at 35°C. One milliliter of labeled material was added to each flask, and incubation was resumed for an additional hour. One flask was prepared and chlorinated as described, and the second flask was prepared as for the unchlorinated control organisms. One hundred milliliters of the cells was centrifuged at 3,020 × g for 10 min and suspended in 100 ml of TSY at 35°C. Zero-time samples (10 ml) were removed, filtered through a type HC membrane filter (Millipore Corp.), and rinsed, and the filters were air dried. At the same sampling times, 1-ml samples were taken and the cell counts were determined as described above. Turbidities were also determined. Samples were removed at timed intervals for determination of radioactivity and cell numbers.

A second type of prechlorination labeling experiment involved short-term exposure; preparation was identical to that for the long-term procedure, except that labeling was limited to 10 min.

The third form of labeling experiments used exposure to the radioactive material after chlorination. The organisms were grown in TSY broth, and half were chlorinated as described. Four hundred milliliters of control cells diluted 1:10 and chlorinated cells were spun at 3,020 × g for 10 min and suspended in 100 ml of TSY broth at 35°C. One milliliter of labeled glucose or algal protein hydrolysate was added to each flask and allowed to incubate at 35°C for 10 min. The cells were then washed twice with chilled buffer and suspended in TSY broth at 35°C, and the zero-time samples were removed. The flasks were placed in a 35°C water bath, timing was commenced, and 10-ml samples were removed at timed intervals for radioactivity and absorbance analyses.

RESULTS

Laboratory chlorination. Initial experiments determined that a concentration of 0.5 mg of chlorine per liter was necessary to give reproducible and predictable chlorine injury in *E. coli*. With a starting bacterial concentration averaging 8.0 × 10^8 cells per ml, the results presented in Fig. 1 were observed. In most cases an approximate 90% injury rate, as determined by

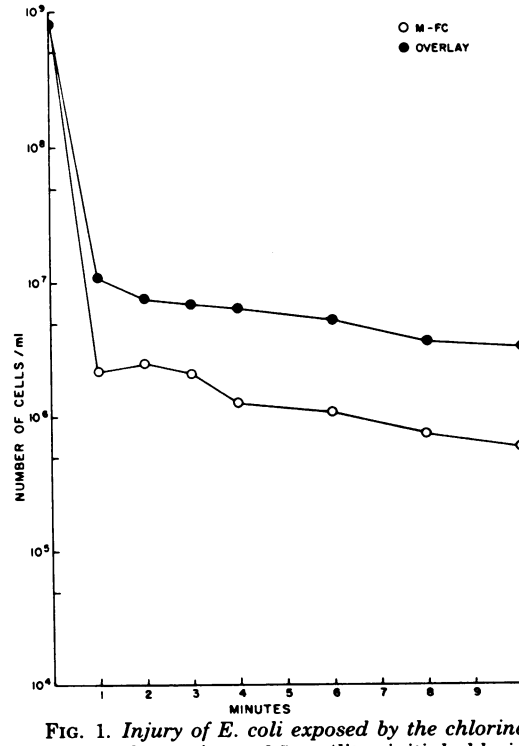

FIG. 1. *Injury of E. coli exposed by the chlorination procedure using a 0.5-mg/liter initial chlorine concentration at room temperature. Selective M-FC medium at 44.5°C and nonselective overlay at 35°C were used for enumeration.*

the difference between plate counts with selective and nonselective media, was obtained in 8 min of chlorine exposure. A large decrease in cell numbers on both media was observed at the 1-min sampling time, with a subsequent gradual decline in numbers of cells. In all instances when absorbance and plate count data were determined for the same sample, the plate counts decreased with time while the absorbance remained stable. If a richer medium such as Eagle was used, a higher count was obtained that was often twice the counts obtained with overlay medium.

Recovery of chlorinated organisms. Experiments were performed with chlorine-treated organisms to determine whether the injury was reversible (Fig. 2). The control organisms exhibited a short lag period and then entered into logarithmic growth with the overlay (nonselective) and M-FC (selective) media counts virtually superimposed. The chlorinated organisms demonstrated a different response. The chlorine-caused injury was not immediately reversed by incubation in the overlay broth, as shown by the difference in cell counts on the two media at zero time. After 2 h the counts obtained with overlay plates indicated active growth, which preceded the resumption of growth on the selective medium. After 4 h of incubation, the growth curves with the two media were superimposed.

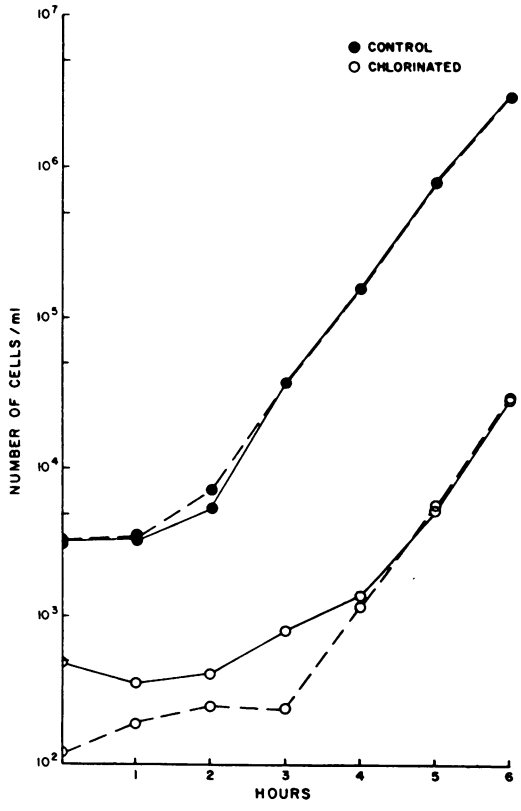

FIG. 2. *Repair in overlay broth of E. coli cells that were exposed to chlorine (0.5-mg/liter initial concentration) for 8 min. Control and chlorinated cells were enumerated over a 6-h growth period, using overlay (——) and M-FC (— —) media and the membrane filter technique.*

Aldolase. The aldolase activity of cell-free extracts was determined after the chlorination of intact cells in order to evaluate the hypothesis that impairment of this enzyme is the basis of chlorine injury and death. Table 1 compares the results of control cells with those that received 8 min of chlorine exposure. The variance between members of the set varied from 0.001 to 0.031. Of the eight experiments listed, six had P values of 0.01 or more, indicating no significant difference between the control and chlorinated aldolase activities. A positive percentage value indicates an increase in the aldolase activity of the chlorinated cells over the control cells. A similar trend was seen at 45°C; three of four P values indicated no significant difference between control and chlorinated aldolase activity. A comparison was made to see what effect chlorination would have on the expected Q_{10} relationship of the enzyme. With four experiments using control and chlorinated cells at the two temperatures, the increase in 45°C activity ranged from 1.34 to 2.54 times that of the 35°C activity. The mean Q_{10} value for the control cells was 1.75, compared with 1.51 for the chlorinated cells. The effect of chlorination on aldolase activity in crude cell extracts was also determined. In this set of tests, the cells were sonically disrupted, chlorine was added to the concentration of 0.5 mg/liter, and the activity of the enzyme was determined. An increase averaging 123% of the nonchlorinated extract was observed with the chlorinated solution in five trials.

Oxygraph determinations. Cellular respiration experiments were performed to determine whether certain media could evoke a rapid recovery from injury in the chlorine-treated bacteria. Duplicate samples were analyzed and averaged to give the final oxygen uptake rates for each experiment. A summary of the results from four experiments with five media is given in Fig. 3. These findings illustrate the effect of increasing media complexity on oxygen uptake. The initial minute of chlorination produced the greatest change. The two media that were poorest nutritionally (mineral salts and mineral salts + 2% glucose) produced the highest residual respiration after chlorination, and the plot exhibited a slight upward trend with prolonged exposure to chlorine, whereas the more complex media were less favorable for respiration.

TABLE 1. *Comparison of aldolase activity at 35°C obtained from control and 8-min chlorinated cells, with statistical information and the percentage increase of chlorinated values over control values*

Date	Control		Chlorinated				
	$\bar{X}^a$	s^2	$\bar{X}^a$	s^2	*t* statistic	*P*	%
8/26/76	1.206	0	1.300	0	—	0.000	7.8
9/14/76	1.450	0.001	1.503	0.031	0.5131	0.635	3.7
9/22/76	1.283	0.019	1.727	0.026	3.580	0.023	34.6
9/29/76	0.665	0.001	0.942	0.005	6.197	0.003	41.7
10/7/76	0.649	0.002	0.772	0.003	3.005	0.039	19.0
10/13/76	1.138	0.026	1.287	0.002	1.542	0.198	13.1
10/27/76	1.127	0.003	1.061	0.013	0.904	0.417	−6.2
11/9/76	0.778	0.008	0.856	0.001	1.423	0.228	10.0

[a] Numbers were obtained by averaging three specific enzyme activity values, which were standardized by comparison to total protein determinations.

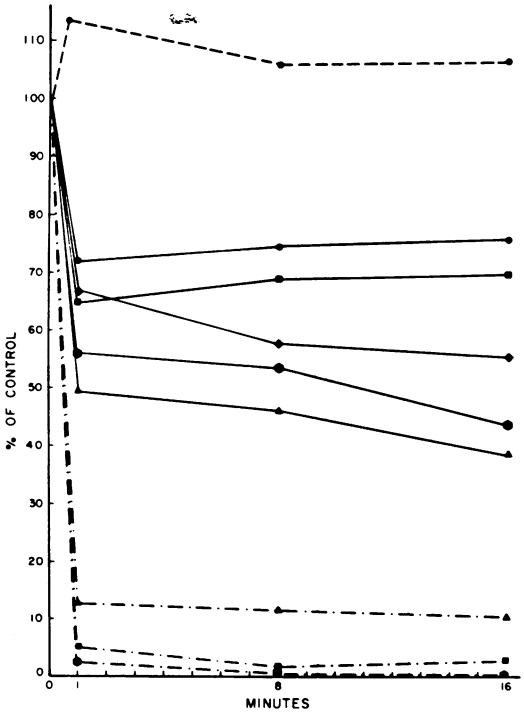

FIG. 3. *Effect of chlorine exposure for 0, 1, 8, and 16 min on oxygen uptake (——), aldolase activity (●— —●), ATP values (▲—·—▲), overlay plate count values (■—·—■), and M-FC plate count values (●—·—●) of E. coli. The cells in the oxygraph assays were inoculated into mineral salts medium (●——●), minerals salts + 2% glucose (■——■), mineral salts + 2% glucose + 0.025% glutathione and 0.025% sodium thioglycolate (◆——◆), mineral salts + 2% glucose + 0.025% glutathione + 0.025% sodium thioglycolate + 0.01% yeast extract (▲——▲), and TSY (●——●). Initial chlorine concentration was 0.5 mg/liter.*

Experiments were performed in which the chlorine was added directly to the cells in the oxygraph reaction vessel after the control rate of respiration had been established. These organisms displayed a reduction in oxygen uptake immediately upon introduction of the chlorine, with the most pronounced decrease in the mineral salts medium that declined by 41.2% of the control value. Mineral salts + 2% glucose, mineral salts + 2% glucose + reducing agents, and TSY exhibited decreases to 24.3, 24.1, and 18.9% of control, respectively.

ATP determinations. The ATP content of cells used for the oxygraph studies was determined to examine the possibility that chlorine acted as a respiratory uncoupler. The ATP results and plate counts obtained on overlay and M-FC are also given in Fig. 3. Again, there was a drastic decline in the first minute of chlorination followed by a gradual reduction. The plate counts with M-FC medium were very low (2.3 to 0.2% of control), the overlay values were slightly higher (5.2 to 3.0% of control), and the ATP values declined least of all (12.8 to 10.3% of control).

Radioactive labeling experiments. The possibility that chlorinated organisms had abnormal uptake of organic substrates was investigated by use of radioactive ^{14}C-labeled glucose and amino acids (algal protein hydrolysate).

The initial experiments involved the post-chlorination exposure of cells to labeled glucose to determine whether injured cells were capable of uptake (Table 2). Due to the similarity of the external standards, the counts per minute were not converted to disintegrations per minute. It can be seen that the control cells were much more capable of initial uptake of the labeled glucose. During the course of the experiment, the amount of label decreased in the control cells only slightly, with the first obvious difference occurring after 25 min of incubation. There was little change in the near background levels of the chlorinated cells.

To determine whether cells became leaky or their ability to metabolize labeled substrate was affected by chlorination, the organisms were labeled with [^{14}C]glucose before chlorine exposure.

A long-term labeling period of 1 h was performed to observe the effect on stable cellular constituents (Table 3), and short-term, 10-min labeling experiments were done to examine the fate of more transient cellular components. The level of radioactivity in the control bacteria decreased very little, with similar results in the chlorinated cells after a 2-h period of incubation in a rich nonselective medium. Short-term labeling experiments showed some similarity between control and chlorinated cells, with decreases of 44 and 33%, respectively, after 2 h of incubation. As in the long-term experiments, the initial label was slightly higher in the control organisms.

The possibility that these phenomena were limited to carbohydrate uptake was tested by the use of ^{14}C-labeled algal protein hydrolysate. Table 4 gives representative data obtained after the postchlorination uptake of labeled substrate. These results were very similar to those found with labeled glucose in that the control organisms were capable of greater uptake of amino acids than the chlorinated cells and the count decreased upon incubation, with a drop occurring after 25 min. The chlorinated cells were not capable of taking up the labeled substrate.

TABLE 2. *Comparison of radioactivity retention in control and 8-min chlorine-treated cells in TSY at 35°C*[a]

Min	Control		Chlorinated	
	External standard	cpm	External standard	cpm
0	6.21	200.93	6.21	13.95
5	6.14	197.34	6.11	13.95
10	6.22	190.47	6.17	13.95
15	6.10	196.71	6.12	14.12
20	6.22	197.36	6.10	14.89
25	6.08	163.39	5.99	14.38
30	6.30	146.59	6.02	14.37
Background	6.21	13.83		

[a] Cells were labeled with [^{14}C]glucose for 10 min after chlorination or no treatment (control).

TABLE 3. *Comparison of radioactivity retention in control and 8-min chlorine-treated cells in TSY at 35°C*[a]

Min	Control		Chlorinated	
	External standard	cpm	External standard	cpm
0	6.21	2,826.31	6.11	2,164.86
15	6.23	2,774.13	6.16	2,166.46
30	6.36	2,681.66	6.24	2,437.87
45	6.31	2,722.03	6.38	2,219.17
60	6.27	2,634.42	6.42	2,405.97
80	6.35	2,612.90	6.25	2,302.75
100	6.33	2,636.06	6.29	2,248.61
120	6.40	2,693.33	6.25	2,067.94
Background	6.36	18.13		

[a] Cells were labeled with [^{14}C]glucose for 1 h (long term).

TABLE 4. *Comparison of radioactivity retention in control and 8-min chlorine-treated cells in TSY at 35°C*[a]

Min	Control		Chlorinated	
	External standard	cpm	External standard	cpm
0	6.01	936.25	6.03	15.95
5	5.98	949.85	6.01	16.82
10	6.01	955.95	6.09	15.77
15	6.02	917.37	5.94	15.34
20	6.06	915.67	5.92	15.20
25	5.96	878.14	5.93	15.86
30	6.05	793.58	5.84	16.41
Background	6.02	15.20		

[a] Cells were labeled with ^{14}C-labeled algal protein hydrolysate for 10 min after chlorination or no treatment (control).

DISCUSSION

Recovery experiments were performed to determine whether chlorine injury was reversible under suitable conditions. The results obtained in this study clearly indicate that *E. coli* surviving chlorination were capable of repairing the resultant injury (Fig. 2). The eventual convergence of the M-FC and overlay counts after chlorination indicates that those cells capable of repair could do so after an extended lag period of 3 to 4 h. Further evidence of repair is shown by identical rates of growth of chlorinated and control cells after 2 h of nonselective incubation.

In earlier investigations (9, 16, 18, 34, 37), the primary implication that in vivo chlorine injury is due to sulfhydryl group oxidation was drawn from experiments using in vitro chlorination followed by decreases in enzyme activities that were also measured in vitro. The aldolase assays in this study showed that there was no decline in enzymatic activity after in vivo chlorination (Table 1). In addition, the experiments wherein hypochlorite was added directly to the crude cell-free extract yielded results similar to those from the in vivo studies. The exact meaning of this is unclear, but it is possible that the chlorine oxidized more highly reactive sites first in the crude extract and that the amount of chlorine in these experiments was insufficient to cause a measurable decrease in aldolase activity. Experiments were performed at 35 and 45°C to determine whether a temperature-sensitive lesion might be the cause of the lower numbers of chlorinated cells on M-FC medium incubated at 45°C. The results did not support this hypothesis, but rather showed a near-normal Q_{10} rela-

tionship in both chlorinated and control preparations.

The aldolase assays were followed by respiration experiments to examine the effects of chlorination on cellular metabolism. The general trend observed was that the oxygen uptake of cells decreased after chlorination, whereas the aldolase activity increased slightly (Fig. 3). Endogenous respiration with mineral salts medium showed less decrease than in more complex media. The addition of reducing agents or more complex media did not appear to be of any advantage to the cells during their short incubation, which would suggest that a simple reduction of the oxidized sites to their original state is not the primary mode of recovery. It therefore seems that the addition of metabolites or an extended incubation allows cells to actively repair their injury through synthetic processes.

The slight increase in aldolase activity suggested that chlorine may have acted as an uncoupler. However, there was a decrease in oxygen uptake, and the ATP values obtained (Fig. 3) would be within the range expected if a rich medium such as Eagle were used to enumerate the cells. In addition, the cells were capable of recovery, and this would not be anticipated in an uncoupler effect. When chlorine was added to the oxygraph vessel, there was an abrupt decline in the rate of oxygen uptake in the cells, which is also not indicative of uncoupling.

The possibility that chlorine acted on or near the cell surface was tested indirectly. Gram-negative cell walls and membrane configurations afford many potential reactive sites for chlorine. The experiments using ^{14}C-labeled glucose encompassed two related concepts: the ability of chlorinated cells to take up exogenous substrates, and the turnover of the incorporated carbon-containing compounds. If cell envelope damage were involved in chlorine injury, the uptake of ^{14}C-labeled glucose would likely be impaired. The first group of experiments involving the use of [^{14}C]glucose after chlorination showed a striking difference between uptake in chlorinated and control cells (Table 2). This suggested that chlorine-treated cells had an impaired carbohydrate transport system and were incapable of moving glucose across the cell membrane. During the incubation time used, some of the control cells divided, with a concurrent decrease in labeled carbon counts. The chlorinated cells exhibited a near-background count that could indicate an inability to take up the substrate. To test the possibility of radiolabel leaking from the cell, cells were labeled with [^{14}C]glucose before chlorination. With both the long- and short-term labeling, the decreases in the radioactivity of the control and chlorinated cells were very similar. This would suggest that at least a part of the carbon metabolism in the chlorinated cells continued at about the same rate as before and that accelerated leakage of the labeled compounds had not occurred. Stable materials (labeled with the long-term exposure) turned over at about the same rate after chlorination as before (Table 3). This trend was also observed in the short-term labeling of more transient cellular constituents, which appeared to be in conflict with the evidence that the cells were alive and capable of recovery. It was anticipated that the turnover of intracellular material would be accelerated in order that repair processes could occur during the chlorine-imposed discontinuation of carbohydrate transport processes. It is probable, however, that storage products that were used in this way were not appreciably labeled in our experiments.

Further investigation determined that the effect of chlorine on transport was not limited to a carbohydrate system. Labeled algal protein hydrolysate was used since amino acids are transferred via an active transport system. A repeat of the postchlorination uptake experiment with algal protein hydrolysate yielded essentially the same result as found with glucose; i.e., chlorinated cells were not able to take up label (Table 4).

The experimental results described in this report point to a new hypothesis regarding the injurious action of low concentrations of chlorine on *E. coli*. Membrane functions, especially those involved in the transport of extracellular nutrients, are affected. The cell envelope, due to its proximity to the environmental stress, appears to be a plausible site of chemical interaction. It seems unlikely that chlorine would actively seek out intracellular sulfhydryl groups and not react with the envelope. It should also be pointed out that 90% of the populations examined were injured and remained viable by using endogenous energy reserves until recovery took place. However, the remaining 10% that were not injured needed no repair to successfully grow on selective media.

In the past it has been recognized that many of the viable indicator bacteria present in chlorinated effluents are not enumerated with membrane filtration techniques (5, 10, 19, 21, 26, 32). Improvements in membrane filtration techniques and media have been reported to give results that are comparable with the MPN data when detecting chlorine-injured bacterial populations, but these methods were developed without consideration of the mechanism of chlorine action on the cell (8, 30; Green et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1964, p. 34) or were based on the previously accepted mode of

action of chlorine (35). Such procedures are successful with respect to the enumeration of a large segment of the chlorine-injured population. However, further investigations into the effects of chlorine on indicator bacteria might allow the development of even better bacterial enumeration methods to determine the public health safety of chlorinated waters and effluents. Disinfection technologies may also benefit from more precise physiological information about bacterial injury and death.

ACKNOWLEDGMENTS

We thank John Schillinger for his consultation during the laboratory work and assistance in the review of the paper. Susan Turbak and Bill Dockins are also thanked for their help in the preparation of the manuscript.

The Millipore Corporation is gratefully acknowledged for their provision of necessary equipment and materials.

This project was supported by funds from the U.S. Department of the Interior authorized under the Water Resources Research Act of 1964, Public Law 88-379, and administered through the Montana University Joint Water Resources Research Center (grants OWRR A-092 Mont. and OWRR A-099 Mont).

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