



Physiological studies of chlorine injury in *Escherichia coli*
by Anne Kosteczko Camper

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in Microbiology
Montana State University
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Abstract:

Injury induced in *E. coli* cells by chlorination was studied from a physiological standpoint. The chlorination procedure used consisted of exposure of cells directly to 0.5 mg/l chlorine in pH 6.5 water for timed intervals up to sixteen minutes at 22 - 25 C. Predictable and reproducible injury was found to occur.

The injury inflicted on the *E. coli* cells by the chlorinated environment was reversible under certain nutrient conditions such as in overlay broth. There was an extended lag period in chlorinated cell growth not seen in control cells followed by a resumption of logarithmic growth at a rate equaling that of control rate. The nontoxic and nonselective environment provides an opportunity for injured cells to repair themselves.

The aldolase activity of cells chlorinated *in vivo* was equal to or slightly higher than those values obtained with control cells. This implies that aldolase is not the primary site of chlorine action as previously suggested with *in vitro* experiments.

Oxygen uptake experiments showed that chlorinated cells undergo a decrease in respiration. This decrease is more pronounced in rich media containing reducing agents. The cells are not immediately repaired in the presence of the reducing agents, suggesting that synthesis of new material may be of greater importance than reversal of chlorine oxidation.

Uptake of metabolites was inhibited by chlorine injury as shown with experiments using labeled glucose and algal protein hydrolysate.

Labeling before chlorination demonstrated that turnover of intracellular material continues at about the same rate after chlorination as before. Amino acids are apparently turned over very rapidly in both control and chlorine treated cells.

The results of experiments given indicate that reversible injury occurs in *E. coli* cells exposed to commonly used concentrations of chlorine. Further, our findings suggest that the previously accepted mechanism of chlorine damage (aldolase inactivation through sulfhydryl oxidation) is invalid and that impairment of the normal transport physiology of the cell envelope should be considered.

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June 17, 1977

PHYSIOLOGICAL STUDIES OF CHLORINE INJURY
IN ESCHERICHIA COLI

by

ANNE KOSTECZKO CAMPER

A thesis submitted in partial fulfillment
of the requirements for the degree


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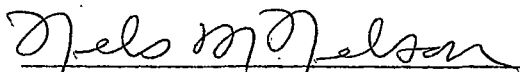
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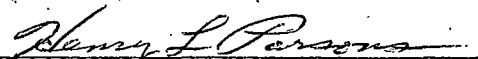
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TABLE OF CONTENTS

	<u>Page</u>
VITA	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	viii
ABSTRACT	ix
Chapter	
1. INTRODUCTION.	1
Statement of Purpose	3
2. MATERIALS AND METHODS	4
Test Organism	4
Media	4
Preparation of Glassware and Utensils	7
Cell Enumeration and Cell Density Procedures	8
Field Chlorination	9
Laboratory Chlorination with Membrane Diffusion Chambers	11
Laboratory Chlorination	11
Recovery Experiments	13
Preparation of the Cells for the Oxygraph and Aldolase Assays	15
Aldolase Assay and Protein Determination	15

	<u>Page</u>
Chapter	
Oxygraph Assay	16
ATP Determinations	17
Labeled Glucose and Algal Protein Hydrolysate Experiments	17
3. RESULTS	21
Field Chlorination	21
Laboratory Chlorination with Membrane Diffusion Chambers	27
Laboratory Chlorination	27
Comparison of Absorbance and Cell Counts Obtained by Plate Count	28
Recovery of Chlorinated Organisms	32
Aldolase	35
Oxygraph Determinations	40
ATP Determinations	42
Radioactive Labeling Experiments	43
4. DISCUSSION	52
5. SUMMARY	68
LITERATURE CITED	72

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Initial chlorine levels and plate count data obtained on M-FC (selective) and overlay (nonselective) media at timed exposures to chlorinated effluent on four dates using membrane diffusion chambers	22
2. Chlorine levels as determined initially and at timed intervals during experiments in chlorinated effluent on eleven selected dates . . .	26
3. Plate count data on M-FC and overlay media obtained with an initial chlorine level of 0.5 mg/l using the laboratory chlorination procedure	29
4. A comparison between plate count data, % injury, and absorbance at 420 nm obtained with exposure to chlorine on four dates	31
5. A comparison of aldolase activity at 35 and 45 C obtained from control and eight minute chlorinated cells with statistical information and the percent increase of chlorinated values over control values	36
6. A comparison of control and chlorinated aldolase activities at 35 and 45 C showing statistical and Q_{10} information	39
7. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ^{14}C glucose for ten minutes following chlorination or no treatment (control)	46
8. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ^{14}C glucose for one hour (long term) and ten minutes (pulse) before chlorination	48

<u>Table</u>	<u>Page</u>
9. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ¹⁴ C algal protein hydrolysate for ten minutes following chlorination or no treatment (control)	50
10. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ¹⁴ C algal protein hydrolysate for ten minutes before chlorination	51

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Progression of injury and die-off of <u>E. coli</u> suspended in chlorinated effluent in a membrane diffusion chamber using M-FC and overlay media . . .	23
2. Variation in die-off obtained on four dates when <u>E. coli</u> was suspended in chlorinated effluent . . .	24
3. Injury of <u>E. coli</u> exposed by the laboratory chlorination procedure using a 0.5 mg/1 initial chlorine concentration at room temperature	30
4. Repair in overlay broth of <u>E. coli</u> cells that were exposed to chlorinated effluent for 2½ hours	33
5. Effect of chlorine exposure for 0, 1, 8, and 16 minutes on oxygen uptake and aldolase activity of <u>E. coli</u>	41
6. Effect of chlorine exposure for 0, 1, 8, and 16 minutes on the concentration of ATP (fg/ml, 0.1 ml extract), plate counts obtained with overlay medium, and plate counts using M-FC medium	44

ABSTRACT

Injury induced in E. coli cells by chlorination was studied from a physiological standpoint. The chlorination procedure used consisted of exposure of cells directly to 0.5 mg/l chlorine in pH 6.5 water for timed intervals up to sixteen minutes at 22 - 25 C. Predictable and reproducible injury was found to occur.

The injury inflicted on the E. coli cells by the chlorinated environment was reversible under certain nutrient conditions such as in overlay broth. There was an extended lag period in chlorinated cell growth not seen in control cells followed by a resumption of logarithmic growth at a rate equaling that of control rate. The non-toxic and nonselective environment provides an opportunity for injured cells to repair themselves.

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Oxygen uptake experiments showed that chlorinated cells undergo a decrease in respiration. This decrease is more pronounced in rich media containing reducing agents. The cells are not immediately repaired in the presence of the reducing agents, suggesting that synthesis of new material may be of greater importance than reversal of chlorine oxidation.

Uptake of metabolites was inhibited by chlorine injury as shown with experiments using ¹⁴C labeled glucose and algal protein hydrolysate. Labeling before chlorination demonstrated that turnover of intracellular material continues at about the same rate after chlorination as before. Amino acids are apparently turned over very rapidly in both control and chlorine treated cells.

The results of experiments given indicate that reversible injury occurs in E. coli cells exposed to commonly used concentrations of chlorine. Further, our findings suggest that the previously accepted mechanism of chlorine damage (aldolase inactivation through sulfhydryl oxidation) is invalid and that impairment of the normal transport physiology of the cell envelope should be considered.

Chapter 1

INTRODUCTION

Indicator organisms in receiving waters and chlorinated effluents as isolated using various media have long been used to define the efficiency of wastewater treatment plants. The accepted thought is that chlorine as used in treating wastewater effluent will cause the rapid die-off of the bacterial population. This line of thought has created a pattern for the interpretation of results and the devising of media used in these tests by restricting the view to the alternatives of the live, healthy organism or the dead one. The concept of reversible injury has been applied in other situations of environmental stress, but has been largely ignored in the explanation of results obtained in the presence of chlorine. Knowledge of the physiological mode of action of chlorine on the cell and information on the reversibility of chlorine injury could be used to define the response of the organisms to various aquatic and nutritive environments and help formulate media for isolation and detection.

The appearance of increased numbers of organisms at some distance below the discharge of chlorinated effluent has been attributed to several factors, among which are regrowth of fecal coliforms (42), regrowth of nonfecal organisms (11), regrowth of nonfecal organisms plus an unidentified point source (28,43), and the upset of the predator-

bacteria balance resulting in bacterial multiplication (25). None of these instances considers the possibility that recovery or repair of injury induced by chlorination is responsible for the reappearance of the organisms.

The same mode of thinking appeared during the early research leading to the selection of various media used to enumerate organisms, particularly fecal coliforms, in chlorinated effluent. Since that time the concept of reversible injury resulting from chlorination and other environmental stresses has been established (6,35,40). It has been shown that the membrane filtration procedure, such as with M-FC medium, does not enumerate chlorine injured cells that are not dead (7,27,33). The multiple-tube fermentation technique (MPN) gives higher counts on the same sample because of its initial nonselective medium, but it is cumbersome and time consuming. Improvements in the media used with the membrane filtration technique to give results of equal or higher value than the MPN results when detecting chlorine injured populations of coliforms have been accomplished (39), but without consideration as to the mechanism of chlorine on the cell. However, a new method has been developed (46) which takes into account the proposed action of chlorine as described in earlier research. The basis for these improvements seemed legitimate, but many questions were left unanswered with regard to chlorine action on the basis of previous

research.

Statement of Purpose

The understanding of the physiology of chlorine injury in indicator bacteria is of utmost importance in the interpretation of bacterial counts obtained with various media from chlorinated effluent and receiving waters. The purpose of this research is to determine if chlorine does produce injury in addition to death in a bacterial population, and whether or not that injury is reversible under conducive conditions. Specific objectives are to determine the extent of this injury, the ability of the organisms to recover, and the general physiological site of action of chlorine in producing this injury.

Chapter 2

MATERIALS AND METHODS

Test organism

The organism that was used in all of the experiments was a water isolate taken from the East Gallatin River. It produced a ++-- IMViC reaction, formed metallic green sheened colonies on Levine EMB agar (Difco, Detroit, MI), and produced gas at 44.5 C in EC medium (Difco). The organism was maintained on stock culture agar (Difco) and transferred every four weeks. At intervals of about two months an EMB plate and IMViC series were inoculated and a Gram stain done to test for cultural and genetic purity.

Media

Water utilized

The water used for the media, buffers, and chlorine demand free water was either double distilled water that had been stored in glass or reagent grade water that had been processed by a Milli-Q water system (Millipore Corp., Bedford, MA) following single distillation.

Peptone-phosphate buffer

Peptone-phosphate buffer was utilized as a diluent throughout the course of these studies. The buffer was prepared by the use of phosphate stock solution (2) with the addition of 0.1% peptone (Difco) as suggested

by Sladek, et al. (45). This buffer was also used as a wash for the membrane filter technique. Dilution blanks were stored under refrigeration until used, at which time they were put on ice.

Tris buffer

The buffer used for the oxygraph and aldolase assays was prepared in accordance with the directions given in the product flier (Sigma Chemical Company, St. Louis, MO). The buffer chosen was a 0.05 M solution of pH 7.2 at 35 C. This buffer was autoclaved and refrigerated until used.

Chlorine demand free water

Chlorine demand free water was prepared according to Standard Methods for the Examination of Water and Wastewater (2). This water was used in the laboratory chlorination experiments and as a final wash for the organisms chlorinated by that procedure.

Sodium thiosulfate solution

The sodium thiosulfate utilized to neutralize the chlorine in samples was prepared in water to give a 10% solution and filter sterilized with a 0.45 um filter (Millipore). The sterile solution was kept under refrigeration.

Reducing agent stock solutions

Reducing agents were employed in the formulation of the overlay

medium and in the production of some of the oxygraph media. Solutions of 0.25% sodium thioglycollate (Sigma Chemical Co.) and 0.25% glutathione (Sigma Chemical Co.) were prepared with water and refrigerated until used.

TSY broth

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%. One hundred ml portions were dispensed into flasks, autoclaved, and used to grow the organisms utilized in the experiments.

M-FC

M-FC broth (Difco) was prepared according to Standard Methods (2). The broth was prepared no more than four hours prior to use. Solid M-FC was prepared by the addition of 1.5% agar (Difco). This was dispensed in seventeen ml quantities into sterile glass petri dishes.

Overlay medium

The overlay medium was prepared according to the method described by Stuart, et. al. (46). It consisted of the overlay portion of their IM-MF medium. Five ml quantities were dispensed after autoclaving into the same type of plates used for the M-FC broth and allowed to solidify. The overlay medium was used as the index of the total viable population in the plate count procedure. The same medium without agar

was used in the recovery experiments. The overlay plates were prepared the night before use and refrigerated or the day of use.

Dulbecco's modified Eagle medium

Eagle's medium (International Scientific Ind., Inc., Cary, IL) was prepared with one-half the amount of water directed by the manufacturer, filter sterilized through a 0.22 um filter (Millipore), and then mixed with an equal volume of autoclaved water plus 1.5% agar (Difco). The medium was dispensed in five ml volumes into sterile plastic petri dishes.

Mineral salts medium

Mineral salts medium was prepared (47), filter sterilized using a 0.22 um filter (Millipore), and refrigerated.

Desoxycholate lactose agar

Desoxycholate agar (DLA) (Difco) was prepared as directed by the manufacturer and utilized in recovery experiments as a selective medium.

Preparation of glassware and utensils

All glassware was machine washed, rinsed with distilled water, and air dried. Items that were acid washed were soaked in 10% HCl for a minimum of thirty minutes, rinsed six times with tap water followed by three rinses with single distilled water and one with double

distilled water or Milli-Q reagent grade water (Millipore), and dried. Glassware sterilized by autoclaving was covered with aluminum foil and processed for fifteen minutes at fifteen pounds pressure. Pipettes and glass petri dishes were placed in metal cans or boxes and oven sterilized at 350 F for three hours.

Cell enumeration and cell density procedures

Membrane filter technique

The membrane filter technique for the enumeration of fecal coliforms as stated in Standard Methods (2) was followed. Millipore type HC filters were used throughout this procedure. The media used (M-FC, overlay, Eagle's) were dispensed in 48 x 8.5 mm tight fitting plastic plates (Millipore). Duplicate plates of two or more dilutions per sample were performed with each medium. Plates incubated at 35 C (overlay and Eagle's media) were held in a circulating air incubator while a block incubator (Millipore) and circulating water bath at 44.5 C were used for the M-FC plates. M-FC plate incubation began within ten minutes of filtering and proceeded for twenty-four hours. Colony counts were made with a binocular microscope at fifteen x magnifications.

Pour plate procedure

This procedure was performed as suggested for the standard plate count in Standard Methods (2). Overlay and DLA pour plates were incubated at 35 C. M-FC pour plates were incubated for twenty-four hours

in a 45 C circulating air incubator. Colony counts were determined with a New Brunswick colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Surface plate procedure

Depending upon the experiment, overlay, M-FC and/or DLA plates were poured and allowed to solidify. One or 0.1 ml samples were pipetted onto the surface of the agar, spread with a sterile bent glass rod, overlaid with five ml of the same medium, and incubated as described above.

Spectrophotometric determination

Cell population was determined using the Varian Techtron spectrophotometer model 635 at 420 nm.

Field chlorination

Organism preparation

The EC+ E. coli culture was grown for twelve hours at 35 C in TSY broth. The cells were centrifuged at 3020 x g for ten minutes and resuspended in peptone-phosphate buffer. This procedure was repeated twice with the final suspension in sterile water. Organism concentrations of approximately 5×10^5 and 6×10^9 per ml were used. The suspensions were iced and transported to the field site.

Membrane diffusion chambers

Membrane diffusion chambers developed by McFeters and Stuart (32) were utilized for the field chlorination studies. Tear resistant micro-web filters (Millipore, WHWP 304 F1) were cut in circles and used. These membranes were sterilized by U.V. light for fifteen minutes per side. The chamber and dust caps were sterilized prior to assembly by autoclaving at fifteen pounds pressure for ten minutes. Chambers were assembled in the laboratory prior to transport.

Experimental location and sampling procedure

The chambers were loaded with a twenty ml sample using a sterile syringe and suspended in an eddy in the discharge ditch approximately five feet from the source of the effluent at the Bozeman Wastewater Treatment Plant. Immediately upon immersion a one ml sample was withdrawn with a sterile one ml syringe and labeled as the zero time sample. Timing began at that point and samples were withdrawn at intervals previously determined for each experiment. Before sample removal, the syringe was pumped ten times to ensure mixing of the chamber contents.

Samples taken from the chambers were deposited in a ninety-nine ml peptone-phosphate dilution blank containing one ml of a 10% sodium thiosulfate solution and shaken. If several samples were taken during the experiment, they were carried to the laboratory trailer at the

treatment plant where plating was performed. M-FC plates were incubated immediately after filtration in the portable black incubator. All plates were transported back to the laboratory at the termination of the experiment for incubation and counting.

Chlorine determinations

At the time the chamber was suspended in the effluent a sample of effluent was collected and taken to the treatment plant laboratory where chlorine concentrations were determined by the iodometric procedure described in Standard Methods (2). Samples were also taken during the experiment and at the termination in some instances.

Laboratory chlorination with membrane diffusion chambers

The procedure for the laboratory membrane diffusion chamber technique was a modification of the field chlorination studies in that the chambers were suspended in a five gallon plastic bucket. The bucket was filled with double distilled water and chlorine bleach (Chlorox) added to give a final concentration of 1.5 and 5.0 mg/l.

Laboratory chlorination

Preparation of the bleach solution

Commercial chlorine bleach (Chlorox) was purchased at least monthly during the course of the experiments and stored at refrigerator temperature as it has been demonstrated that the sodium hypochlorite

will dissipate with time. Chlorox was used as it has a 5.25% sodium hypochlorite concentration initially. A new stock solution was made each day immediately before it was to be used. For most of the chlorinations a 0.5 mg/l final concentration was desired, therefore, a 500 mg/l stock solution was prepared by diluting one ml of the bleach with ninety-nine ml of chlorine demand free water in a volumetric flask. One ml of this stock solution was then transferred to one liter of bacterial suspension in the chlorination vessel.

Preparation of the experimental chlorination vessel

A volume of 900 ml of sterile chlorine demand free water was added to a sterile, acid washed, foil covered two liter capacity DeLong flask containing a magnetic stir bar. The water was stirred constantly at a low speed. A styrofoam pad and wooden block were inserted between the flask and stirrer to reduce heat transfer from the stirrer to the water. One hundred ml of the washed E. coli culture were added to the flask and mixed thoroughly before the addition of the chlorine.

Sampling procedure

After the organisms were added to the flask and allowed to mix, the control organisms were removed and the one ml sample was transferred to a ninety-nine ml dilution blank containing iced peptone-phosphate buffer. One drop of a 10% sodium thiosulfate solution was added and the blank shaken well. One ml of the chlorine stock solution was then

added and timing commenced. At timed intervals one ml samples were removed and treated the same way as the control sample. All samples were iced until they could be filtered. Cell counts were done by the membrane filter technique and absorbances determined with these samples. When large volumes of sample were required, the aliquot was poured into a sterile flask containing 10% sodium thiosulfate solution, agitated, and iced. For a 200 ml volume, 0.5 ml of the sodium thiosulfate solution was used, and for a 400 ml sample 1.0 ml was used.

Preparation of the culture for chlorination

Cell preparation was the same as that for the field membrane diffusion chamber experiments except that final suspension was in a volume of chlorine demand free water equal to that of the growth medium (100 ml) to give a final concentration of approximately 7×10^9 organisms per ml. If a cell count of approximately 7×10^5 was used, the appropriate dilution was made with chlorine demand free water. When a large amount of control organisms were required two flasks of bacteria were grown and prepared as above. One 100 ml portion was added to the chlorination vessel and a 1:10 dilution made of the remaining organisms to give a desired quantity of control suspension and then iced.

Recovery experiments

Experiments were performed to determine if the chlorine injured organisms were capable of recovering if exposed to a suitable environ-

ment. Recovery experiments involved the use of organisms that had been exposed to chlorinated effluent for 2½ hours at the treatment plant or those that had been chlorinated by the laboratory procedure for eight minutes.

In the first group of experiments, treatment plant chlorinated organisms were transferred to the laboratory as previously described. The control organisms were retained in the refrigerator during the chlorination time. Four ml of the control and chlorinated bacteria were placed in separate flasks containing 36 ml of overlay broth. The zero time samples were removed and the flasks incubated at 35 C. Samples were removed hourly for six hours and plated by the surface plate method on DLA and overlay media. Incubation was at 35 C.

For the second group of experiments the procedure was changed and M-FC surface plates replaced the DLA plates.

A similar experiment was done where M-FC and overlay pour plates were used and incubated at 35 C as before. Another experiment was performed in which the M-FC plates were incubated at 45 C.

The final group of experiments utilizing field chlorinated organisms involved the performance of the membrane filter technique. M-FC broth plates were incubated at 44.5 C and overlay plates at 35 C.

The studies for which the organisms were chlorinated in the laboratory involved the use of the membrane filter technique as the cell count determination procedure.

Preparation of the cells for the oxygraph and aldolase assays

The 1:10 dilutions of the control and experimental cells removed from the chlorination vessel were centrifuged at 3020 x g for ten minutes at 1 - 3 C in sterile tubes. The supernatant was discarded to rid the cells of thiosulfate or cellular debris. This method insured that only intact cells were used for the oxygraph and aldolase assays. The pellet was then suspended in a volume of 0.05 M tris buffer one-fourth that of the original volume dispensed into the tubes. In most cases a volume of 200 ml was resuspended in a volume of 50 ml. The concentrated cells were then placed in fifty ml glass stoppered, acid washed flasks and immediately iced. Oxygraph experiments were done immediately after the organisms were concentrated. The concentrated cell suspension was then refrigerated over night to be disrupted by sonication the next morning for the aldolase assay.

Aldolase assay and protein determination

Sonication of the cells

It was determined through experimentation that the sonication time needed for 98% disruption of the cells used was fifteen minutes with the Bronwill Biosonic sonicator set at 90% of maximal intensity. This amount of sonication was accomplished in bursts of three minutes each with cooling periods allowed between sonications. A three minute burst resulted in a temperature rise of approximately 16 C if a volume of

forty ml was sonicated. To prevent enzyme denaturation the cell suspension was allowed to cool in an ice bath until it had reached 1 - 3 C before it was again treated by sonication. This sonicate was diluted 1:10 with tris buffer for the aldolase and protein determinations.

Protein determination

The protein determination of Lowry, et al. (30) was done with the crude extract. A standard curve utilizing bovine serum albumin was performed in conjunction with each protein determination. Absorbance readings were taken at 625 nm in the Varian spectrophotometer.

Aldolase assay

The aldolase assay used was a modification of the hydrazine assay as described by Jagannathan (24). Reaction progress was followed in the Varian spectrophotometer at 240 nm. The automatic chart was set at 10 mV and a speed of $3\frac{1}{2}$ cm/min. The reaction was monitored for $3\frac{1}{2}$ minutes. 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 used was allowed to reach room temperature prior to use in an attempt to minimize bubble formation on the cuvette walls. The fructose 1,6-diphosphate solution and sonicated cells were kept on ice. Triplicate readings were taken for each sample.

Oxygraph assay

Oxygraph assays were performed with a Gilson Oxygraph model KM equipped with a YSI model 4004 Clark oxygen probe. Constant temperature was maintained by a Heto ultrathermostat model 623 at 35 C. The substrates used were kept at room temperature. One ml of the concentrated cells which had been kept on ice was pipetted into the reaction chamber and allowed to equilibrate for one minute and one ml of substrate was then added and allowed to equilibrate for thirty seconds. The chart was then started and the course of oxygen uptake followed for three minutes. This was duplicated for each organism with each medium.

Media used in the oxygraph experiments included mineral salts medium, mineral salts medium plus 2% glucose, mineral salts medium plus 2% glucose plus 0.025% glutathione and 0.025% sodium thioglycollate, mineral salts medium plus 2% glucose and 0.025% glutathione and sodium thioglycollate plus 0.01% yeast extract, and TSY.

ATP determinations

Cells used for the oxygraph experiments were extracted by a modification (48) of the Bancroft, et al. procedure (3). ATP determinations were made in a DuPont Luminescence Biometer.

Labeled glucose and algal progein hydrolysate experiments

Preparation of the radioactive material

D-glucose [^{14}C (U)] with a specific activity of approximately 5.0 mCi/mmole (New England Nuclear, Boston, MA) was ordered in 0.05 mCi amounts. The glucose was dissolved in 10.2 ml double distilled water and two ml volumes were dispensed into vials. The vials were stoppered and frozen. Just prior to use one vial was thawed, two one ml quantities removed with one ml syringes and the syringes refrigerated if there was a slight delay before dispensing. A one ml quantity was used for each flask of organisms used in the experiments.

Algal protein hydrolysate [^{14}C (UL)] with a specific activity of 1.0 mCi/mmole (International Chemical and Nuclear Co., Irvine, CA) was ordered in 0.05 mCi amounts. Preparation and utilization was identical to the afore mentioned procedure.

Liquid scintillation procedure

Air dried filters were placed in a 105 C oven for twenty minutes to ensure complete dryness. The filters were rolled and placed in poly-Q scintillation vials (Beckman Instruments Co., Irvine, CA), made transparent with four ml of toluene, and nine ml of Aquasol liquid scintillation cocktail was added (New England Nuclear). The vials were shaken, wiped clean of fingerprints, and placed in a Beckman L.S.C. 100 counter set at 5% error, one hundred minutes termination time, and single automatic cycle. The counter was programmed to perform labeled carbon counts and external standards on each vial.

Pre-chlorination experimental design, long term labeling

Two flasks, each containing 100 ml TSY broth, were inoculated with the experimental organism and incubated for ten hours at 35C. One ml of labeled glucose was added to each flask and incubation was continued for one hour. One flask of organisms was prepared and chlorinated as previously described in the laboratory chlorination section. The other flask of organisms was prepared as described for the control organisms. One hundred ml of the chlorinated cells and 1:10 diluted control cells were centrifuged at 3020 x g for ten minutes and resuspended in one hundred ml of TSY at 35 C. Ten ml zero time samples were removed, filtered through a type HC Millipore filter, rinsed with chilled buffer, and allowed to air dry. These were used to determine radioactivity in the scintillation counter. At the same time, one ml samples were taken from the flasks and added to chilled ninety-nine ml peptone-phosphate buffer blanks. Appropriate dilutions were made and the membrane filter technique employed to determine cell numbers on M-FC and overlay media. Turbidities at 420 nm were also determined. The flasks were incubated at 35 C in a water bath during the experiment. Samples were taken at fifteen minute intervals during the first hour and at twenty minute intervals during the second hour.

Pre-chlorination experimental design, pulse labeling

The cell preparation was identical to the above with the exception that the exposure time to the labeled glucose was ten minutes instead of one hour. Ten ml samples were removed for radioactive analysis and absorbance readings at 0, 15, 30, 45, 60, 80, 100, and 120 minutes. Plate counts were performed with samples taken at 0, 30, 60, and 80 minutes by the membrane filter technique. The pre-chlorination algal protein hydrolysate experiments were identical to this.

Post-chlorination experimental design

In this group of experiments the organisms were grown in TSY as before, half were chlorinated by the laboratory method for eight minutes, the control cells diluted 1:10, 400 ml of control and chlorinated cells spun down at 3020 x g for ten minutes, resuspended in one hundred ml 35 C TSY broth plus one ml prepared labeled glucose or algal protein hydrolysate, and allowed to incubate at 35 C for ten minutes. The supernatant was discarded and the cells washed twice with chilled peptone-phosphate buffer. After the final centrifugation they were resuspended in 35 C TSY broth, zero time samples removed as previously described, placed in a 35 C water bath, and timing commenced. Ten ml samples were removed at five minute intervals for radioactive and absorbance analysis.

Chapter 3

RESULTS

Field Chlorination

The objectives of the project necessitated the development of a dependable and reproducible method by which organisms could be chlorinated. The first chlorination and injury experiments were performed in the chlorinated effluent at the Bozeman Wastewater Treatment Plant. Bacterial suspensions were removed from membrane diffusion chambers which had been immersed in chlorinated effluent and plated on selective and nonselective media to determine the extent of bacterial injury. The data in Figure 1 represent the desired result. The results showed the population exhibiting a difference in counts on selective (M-FCO) and nonselective (overlay) media beginning after one hour exposure to the chlorinated effluent, increasing to a maximal difference at 2½ hours, and then going into a death stage in which the counts on the two media were not different and dropped rapidly. A log or more separation in counts on selective and nonselective media at a predictable time was required for the performance of later experiments. Thirteen experiments with an initial cell population averaging 4.6×10^5 per ml and fourteen of an initial population of 5.6×10^9 per ml were performed. Figure 2 presents the data obtained on four dates. The numerical data for both the M-FCO and overlay counts for these experiments are given in Table 1. The initial counts (Figure 2) were comparable, but no

Table 1. Initial chlorine levels and plate count data obtained on M-FC (selective) and overlay (nonselective) media at timed exposure to chlorinated effluent on four dates using membrane diffusion chambers.

Date	Initial Chlorine mg/l	Medium	Plate count							
			0 hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr
8-19-75	.65	M-FC	3.4x10 ⁴	3.0x10 ⁴	3.1x10 ⁴	1.3x10 ⁴	4.5x10 ³	7.3x10 ³	1.0x10 ⁰	
		Overlay	4.1x10 ⁴	4.8x10 ⁴	5.2x10 ⁴	4.5x10 ⁴	5.9x10 ⁴	2.4x10 ³	3.9x10 ²	5.0x10 ¹
9-9-75	.97	M-FC	5.8x10 ⁴	4.4x10 ⁴	3.8x10 ⁴	2.2x10 ⁴	2.0x10 ⁴	1.6x10 ⁴	2.0x10 ⁴	3.2x10 ³
		Overlay	5.6x10 ⁴	4.5x10 ⁴	4.5x10 ⁴	4.5x10 ⁴	5.0x10 ⁴	2.1x10 ⁴	2.4x10 ⁴	4.3x10 ³
9-18-75	.70	M-FC	4.6x10 ⁴	1.1x10 ⁴	4.9x10 ⁴	3.1x10 ⁴	2.5x10 ³	2.4x10 ³	1.0x10 ²	1.0x10 ⁰
		Overlay	5.2x10 ⁴	2.4x10 ⁴	2.3x10 ⁴	2.0x10 ⁴	1.8x10 ⁴	1.2x10 ⁴	1.0x10 ²	1.0x10 ⁰
9-23-75	.75	M-FC	4.8x10 ⁴	2.0x10 ⁴	1.1x10 ⁴	4.3x10 ³	4.3x10 ³	1.8x10 ³	1.0x10 ⁰	
		Overlay	5.2x10 ⁴	3.1x10 ⁴	2.4x10 ⁴	2.2x10 ⁴	1.9x10 ⁴	7.3x10 ³	1.0x10 ⁰	

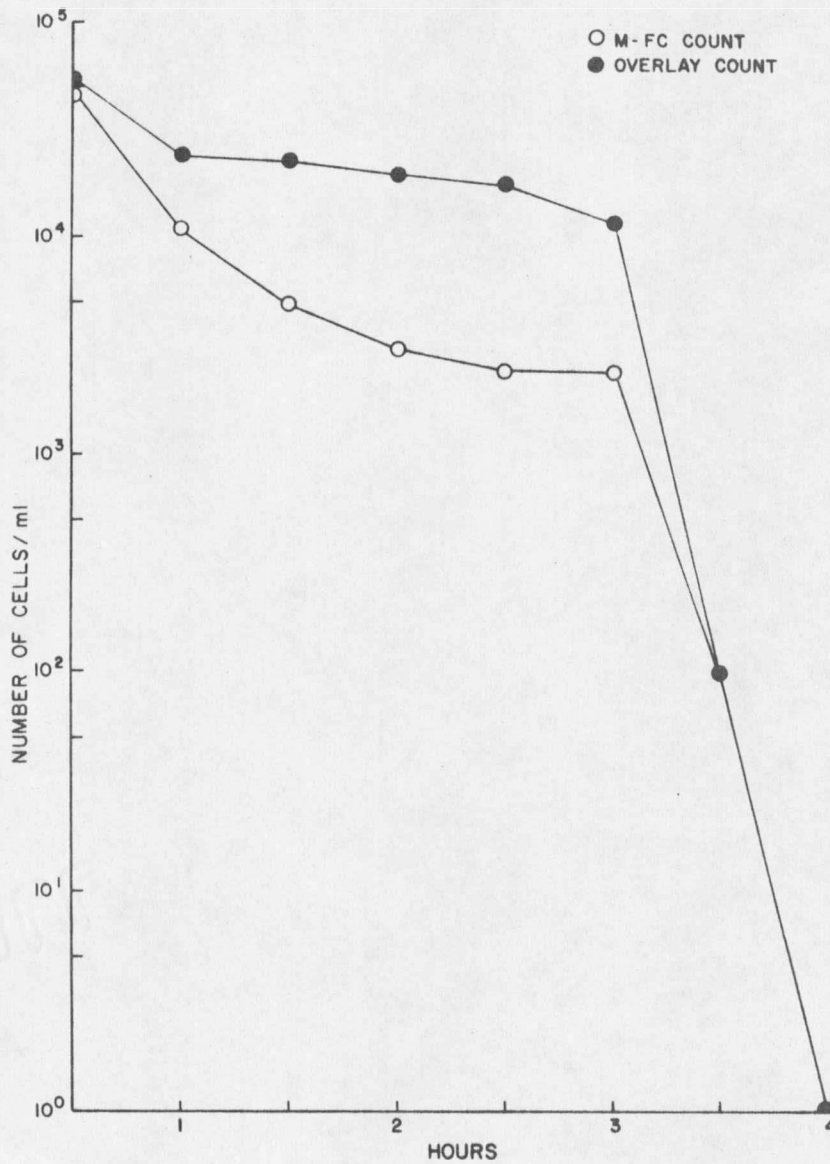


Figure 1. Progression of injury and die-off of *E. coli* suspended in chlorinated effluent in a membrane diffusion chamber using M-FC and overlay media.

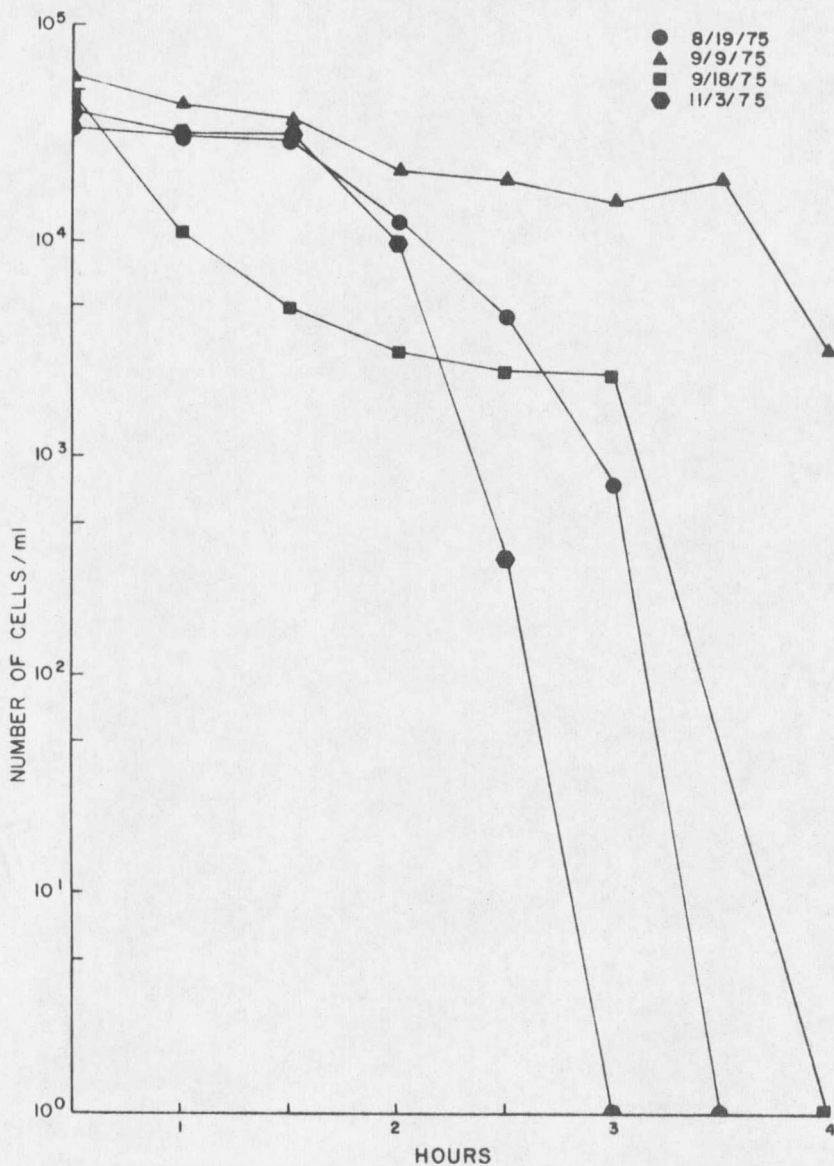


Figure 2. Variation in die-off obtained on four dates when *E. coli* was suspended in chlorinated effluent. Membrane diffusion chambers were used and cells were enumerated using M-FC medium.

predictions could be made once chlorination had commenced. Complete die-off occurred in three examples but at varying intervals with a decrease in viability of approximately four logs; in the fourth example the cell count decreased by only one log in four hours. Interestingly, the three experiments that showed complete die-off had similar initial chlorine levels of 0.65 - 0.75 mg/l while the experiment in which there was no die-off had a higher initial concentration of 0.97 mg/l. The shapes of the curves with comparable chlorine concentrations were not alike. The results obtained using the higher number of organisms were similar but not as dramatic.

Chlorine samples were taken at intervals during the course of some experiments and the values are given in Table 2. The chlorine concentrations in most instances were not constant. Some values were considerably less (a decrease from 0.97 to 0.29 mg/l) while others increased (0.15 to 0.30 mg/l) as the experiments progressed. In a few instances the levels did remain fairly stable. This table also shows that the initial chlorine levels varied from day to day and the range for these experiments was 1.80 to 0.15 mg/l. In nearly all cases the experiments were begun at the same predictable time of day as flow through the plant peaked. The chlorine flow into the chlorine contact chamber was not controlled by flow volume but rather by an arbitrary setting as indicated by a chlorine test of the effluent.

Interim and initial chlorine levels were not stable and time of

Table 2. Chlorine levels as determined initially and at timed intervals during experiments in chlorinated effluent on eleven selected dates.

<u>Date</u>	<u>Chlorine level, mg/l</u>			
	<u>Initial</u>	<u>1 hour</u>	<u>2½ hour</u>	<u>4 hour</u>
7-8-75	1.8			
7-14-75	.54			
7-29-75	.35			
8-6-75	1.05			
9-9-75	.97		.29	
9-11-75	1.03			.75
9-18-75	.70	.85	.70	.85
9-23-75				.45
10-28-75	.55		.70	.25
11-3-75	.30		.30	.45
12-9-75	.15			.30

exposure, based on initial chlorine concentration to give the desired level of injury, could not be predicted with much accuracy. Some recovery experiments were conducted with organisms chlorinated in the field. For these instances it was decided that injury, although not a predictable amount, would occur in most cases after approximately 2½ hours of contact time and the chance of encountering complete die-off was minimal. This method was acceptable for the recovery experiments but not for the physiological experiments that were to follow.

Laboratory chlorination with membrane diffusion chambers

In order to control the initial chlorine concentration, the field chlorination procedure was modified. The membrane diffusion chamber was suspended in a bucket containing a solution of known chlorine concentration. The progress of injury was monitored in the two bacterial suspensions as described above with samples removed every half hour. At the end of four hours, less than a half log drop in cell counts was seen and the M-FC curve was nearly superimposed on the overlay curve. Similar results were obtained with 1.5 and 5.0 mg/l initial chlorine concentrations.

Laboratory chlorination

The failure of the membrane diffusion chamber experiments to give the desired results prompted the design of a chlorination procedure in which the organisms would be directly exposed to the chlorine. The

chlorine concentration needed to give predictable injury was first determined. After several experiments with chlorine levels varying from 2.0 to 0.25 mg/l, a concentration of 0.5 mg/l was selected. Table 3 lists several groups of results obtained with an initial chlorine level of 0.5 mg/l. A graph of typical results obtained by this procedure is shown in Figure 3. In most cases a near 90% injury rate was obtained in eight minutes exposure with a decrease of about one log in viable population exhibited by the overlay plate counts. No final die-off was observed in the time span in which the experiments were run. As seen in the figure, an initial substantial decrease in cell number on both media was observed at the one minute sampling time and following this point the plot of cell number continued downward with a gradual slope. Initial experiments were carried out for up to twenty minutes, but it was observed that substantial and relatively predictable injury was present at the eight minute sampling time. Counts obtained on Eagle's medium, when used, were higher by approximately one log than the overlay plate counts.

Comparison of absorbance and cell counts obtained by plate count

Table 4 presents the comparison between methods that were used to determine viable cell number. The percent injury numbers were included to provide an indication of the progression of injury with time as calculated from the M-FC and overlay plate count data. The groups listed are representative samples of over forty experiments with varying

Table 3. Plate count data on M-FC and overlay media obtained with an initial chlorine level of 0.5 mg/l using the laboratory chlorination procedure.

Date	Plate count (organisms/ml)					
	0 min M-FC	0 min Overlay	1 min M-FC	1 min Overlay	8 min M-FC	8 min Overlay
5-6-76	8.6×10^8	8.6×10^8	3.5×10^6	2.9×10^7	5.0×10^5	1.3×10^7
5-11-76	8.7×10^8	8.8×10^8	2.6×10^6	3.5×10^7	9.4×10^5	5.3×10^6
5-13-76	8.2×10^8	8.1×10^8	2.2×10^6	1.1×10^7	7.6×10^5	3.6×10^5
5-27-76	8.4×10^8	8.4×10^8	3.1×10^7	6.7×10^7	9.7×10^6	3.0×10^7
6-3-76	8.3×10^8	8.3×10^8	--	2.6×10^7	2.6×10^5	8.7×10^5
6-15-76	8.4×10^8	8.5×10^8	2.6×10^7	9.2×10^7	8.3×10^6	1.8×10^7
8-17-76	8.3×10^8	8.4×10^8	5.0×10^8	6.1×10^8	2.4×10^8	2.8×10^8
8-25-76	7.9×10^8	8.2×10^8	5.9×10^8	7.3×10^8	2.4×10^8	3.0×10^8
9-14-76	8.0×10^8	8.3×10^8	3.1×10^7	1.1×10^8	5.0×10^6	2.1×10^7
9-21-76	7.2×10^8	7.3×10^8	1.1×10^6	2.0×10^6	1.7×10^4	2.9×10^5
9-28-76	8.2×10^8	8.0×10^8	5.5×10^5	3.5×10^6	3.7×10^5	7.9×10^5
10-5-76	7.5×10^8	7.5×10^8	4.2×10^6	8.2×10^6	1.0×10^6	1.4×10^6
10-12-76	7.0×10^8	7.1×10^8	5.3×10^7	6.6×10^7	4.8×10^6	9.4×10^6
10-26-76	8.2×10^8	8.4×10^8	8.9×10^5	3.1×10^6	5.4×10^5	9.6×10^5
11-9-76	7.0×10^8	7.1×10^8	5.5×10^7	1.2×10^8	1.6×10^7	3.4×10^7
11-30-76	7.7×10^8	8.2×10^8	4.9×10^6	1.1×10^7	3.7×10^6	7.0×10^6
3-23-77	7.8×10^8	8.4×10^8	5.1×10^7	9.1×10^7	1.0×10^7	3.2×10^7
3-25-77	8.6×10^8	8.4×10^8	9.2×10^6	3.0×10^7	4.3×10^6	1.4×10^7
3-29-77	6.7×10^8	7.7×10^8	6.2×10^7	9.3×10^7	1.0×10^7	3.7×10^7

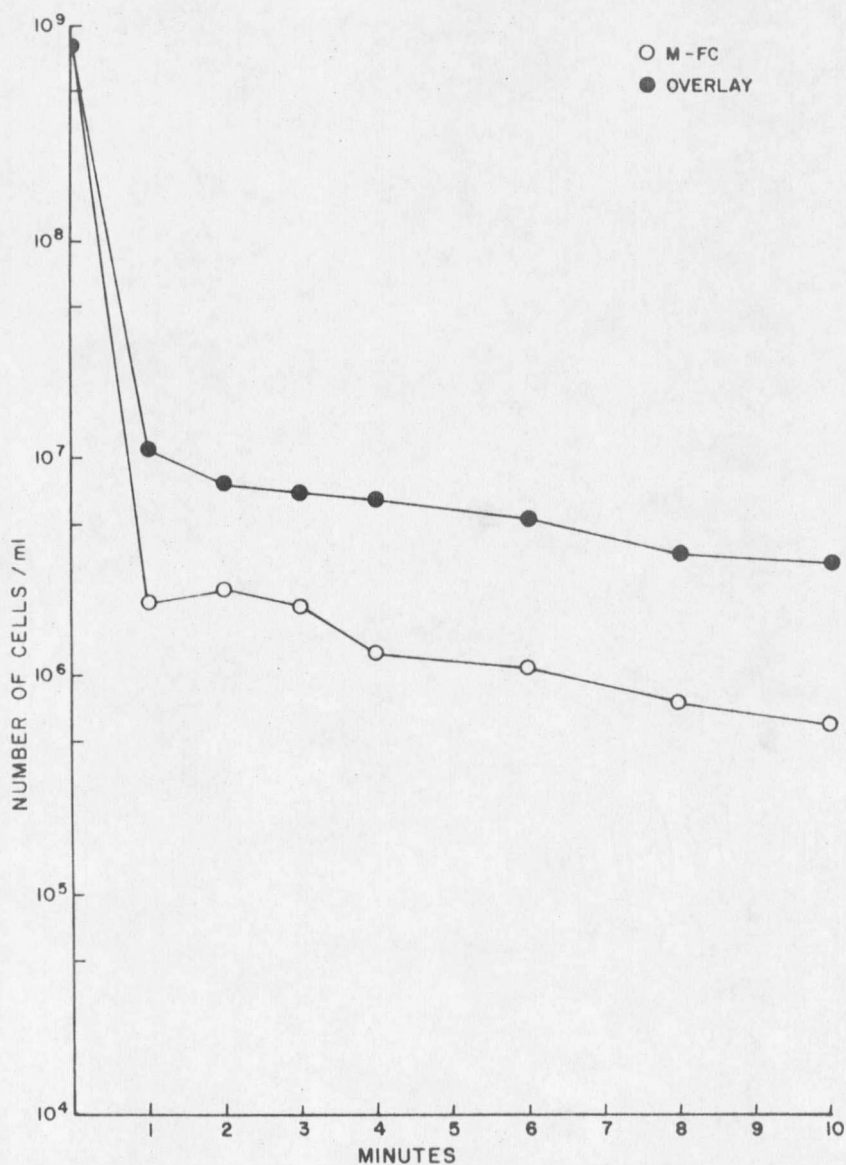


Figure 3. Injury of *E. coli* exposed by the laboratory chlorination procedure using a 0.5 mg/l initial chlorine concentration at room temperature. Selective M-FC incubated at 44.5 C and nonselective overlay at 35 C were used for enumeration.

Table 4. A comparison between plate count data, % injury, and absorbance at 420 nm obtained with exposure to chlorine on four dates.

<u>Date</u>	<u>Minutes</u>	<u>Plate count (#/ml)</u>		<u>% injury</u>	<u>Absorbance 420 nm</u>
		<u>M-FC</u>	<u>overlay</u>		
10-27-76	0	3.3×10^9	3.4×10^9	2.9	1.4
	1	3.6×10^6	1.2×10^7	70.0	1.5
	8	1.4×10^6	3.8×10^7	96.3	1.5
	16	5.6×10^5	2.9×10^6	80.7	1.5
11-11-76	0	2.8×10^9	2.8×10^9	0.0	1.8
	1	2.2×10^8	4.8×10^8	54.2	1.7
	8	6.4×10^7	1.4×10^8	54.3	1.7
	16	1.3×10^7	3.1×10^8	95.8	1.7
11-30-76	0	3.1×10^9	3.3×10^9	6.1	1.5
	1	2.0×10^7	4.4×10^7	54.5	1.3
	8	1.5×10^7	2.8×10^7	46.4	1.3
	16	4.8×10^6	1.6×10^7	70.0	1.3
1-14-77	0	3.3×10^9	3.3×10^9	0.0	1.6
	1	2.1×10^7	6.4×10^7	67.2	1.5
	8	9.2×10^6	3.4×10^7	72.9	1.6
	16	6.4×10^6	1.7×10^7	62.4	1.5

chlorinated times. It can be seen that the plate count values obtained with both selective and nonselective media decrease with chlorination time. There was a more pronounced decrease in cell numbers when M-FC was used although the overlay counts also decreased. The difference was represented as percent injury. During this time the absorbance readings decreased relatively little. In all instances where absorbance and plate counts were determined for same sample this relationship was seen; the plate count data were variable and the absorbance fairly stable.

Recovery of chlorinated organisms.

Experiments were performed with chlorine treated organisms to determine if injury was reversible. Both field and laboratory chlorination procedures were used to obtain the injured organisms. The general procedure consisted of treating the bacterial suspension by chlorination, resuspension in overlay broth, and cell number determination by plate count utilizing selective and nonselective media. A representative graph of the experimental results obtained is shown as Figure 4. The control organisms exhibited a short lag period and then entered into logarithmic growth. The overlay and selective media plots were very close and virtually superimposed. The chlorinated organisms, however, underwent a different response. There was an initial injury not immediately reversed by incubation in overlay broth as shown by the difference in cell counts at zero time on the

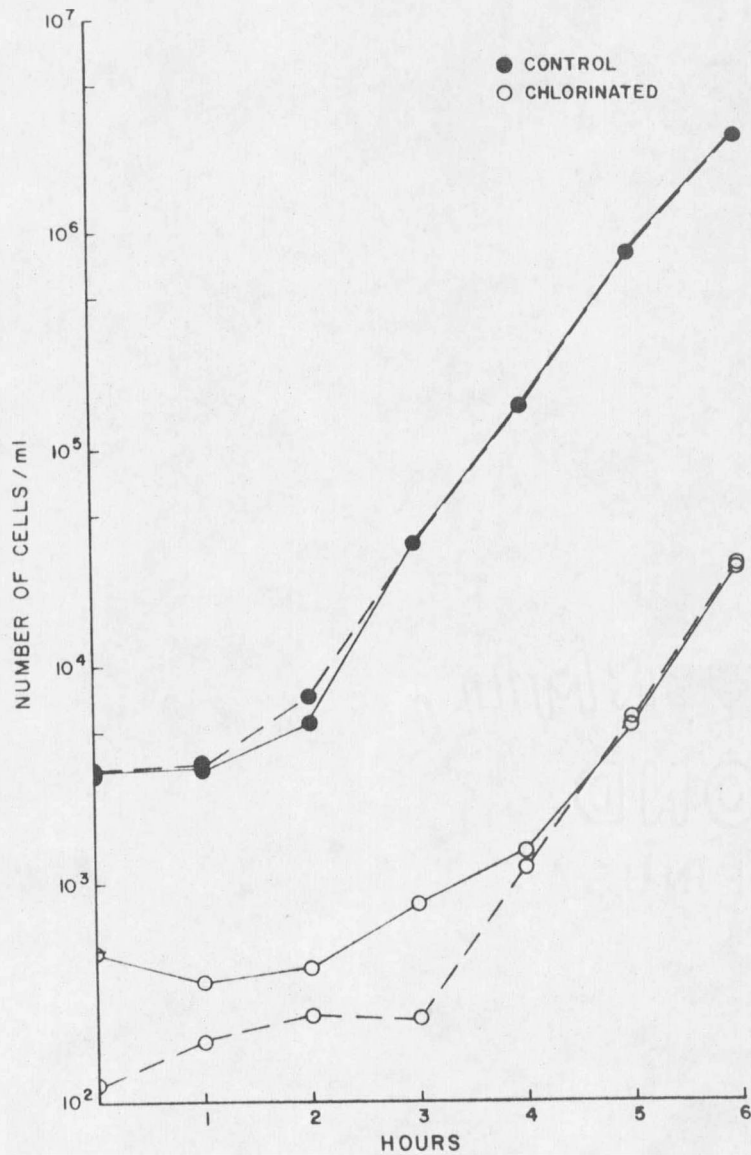


Figure 4. Repair in overlay broth of *E. coli* cells that were exposed to chlorinated effluent for $2\frac{1}{2}$ hours. Control cells and chlorinated cells were enumerated over a 6 hour growth period using overlay (—) and M-FC (— —) media and the membrane filter technique.

two media. The organisms first seemed to be able to regain growth capabilities on the selective medium after some recovery in the overlay broth as exhibited by the slight upward trend of the M-FC curve. The overlay plate counts remained stable but showed initiation of active growth before the plate counts done with the selective medium. This can be seen at the two hour point on the graph. After four hours the growth curves of the selective and nonselective media were superimposed.

The majority of the recovery experiments were performed with organisms chlorinated in the field, and no method was available to predict injury. Not all of the experiments yielded results identical to those shown in Figure 4, however, they are generally representative although recovery did not occur at the same time in each experiment. In experiments that were done using laboratory chlorinated organisms the same trend was present and the organisms did exhibit a more consistent initial injury.

Through the evolution of the recovery experimental design, different selective media and methods of plating were used. Incubation temperature of the selective medium was also changed. Initially, DLA and overlay plates were inoculated by the surface plate method and incubated at 35 C. M-FC and overlay pour plates incubated at 35 C as well as M-FC pour plates incubated at 45 C were also used. The membrane filter technique utilizing M-FC broth plates incubated at 44.5 C and overlay agar plates incubated at 35 C was also performed. Media

and temperature changes did not have much effect on the general response of the chlorinated organisms in the overlay broth. The control organisms were also not influenced by the changes. This was seen by comparing the graphs drawn for each experiment.

Aldolase

The aldolase activity in chlorinated and nonchlorinated cell preparations was determined in order to evaluate the hypothesis that impairment of this enzyme is the basis of chlorine injury and death. Sonicated control and chlorinated cells were analyzed spectrophotometrically for aldolase activity at constant temperatures of 35 and 45 C. Table 5 is a comparison of the results obtained with control cells and cells that had been chlorinated for eight minutes. The t value for each group was determined using pooled variance and the two tailed p values were obtained by the use of a t probability program run with a Xerox Sigma 7 computer. The first point to be considered is the variance between the members of each set. This ranged from 0.001 to 0.202. Of the twelve experiments listed, six had a p value of 0.01 or less, indicating a significant difference between the means of the chlorinated and control aldolase activities. The statistics were performed to substantiate the percentages given in the table which describe the difference between the two means. A positive value is indicative of an increase in the aldolase activity of the chlorinated cells over the control cells. In all but two instances the aldolase

Table 5. A comparison of aldolase activity at 35 and 45 C obtained from control and eight minute chlorinated cells with statistical information and the percent increase of chlorinated values over control values.

35C Date	Control			Chlorinated					
	Specific enzyme activity	\bar{x}	S^2	Specific enzyme activity	\bar{x}	S^2	t stat	P	%
8-26-76	1.206 1.206 1.206	1.206	0	1.300 1.300 1.300	1.300	0	-	.000	7.8
9-14-76	1.400 1.473 1.477	1.450	.001	1.516 1.711 1.283	1.503	.031	.5131	.635	3.7
9-22-76	1.146 1.229 1.473	1.283	.019	1.800 1.959 1.575	1.727	.026	3.580	.023	34.6
9-29-76	.694 .617 .685	.665	.001	.864 .925 1.036	.942	.005	6.197	.003	41.7
10-7-76	.583 .666 .699	.649	.002	.076 .845 .710	.772	.003	3.005	.039	19.0
10-13-76	.915 1.298 1.200	1.138	.026	1.255 1.346 1.260	1.287	.002	1.542	.198	13.1
10-27-76	1.100 1.072 1.209	1.127	.003	.921 1.198 1.065	1.061	.013	.904	.417	-6.2
11-9-76	.895 .680 .758	.778	.008	.891 .823 .855	.856	.001	1.423	.228	10.0

Table 5. Continued

45C Date	Control			Chlorinated					
	Specific enzyme activity	\bar{x}	S^2	Specific enzyme activity	\bar{x}	S^2	t stat	P	%
8-26-76	2.710 3.020 3.450	3.060	.092	2.270 2.270 2.270	2.270	0	4.514	.011	-34.8
9-14-76	2.099 1.959 1.866	1.975	.009	2.099 2.099 1.866	2.021	.012	.548	.613	7.0
9-22-76	1.719 2.047 1.842	1.869	.018	3.000 2.000 2.102	2.367	.202	1.838	.140	26.6
9-29-76	1.029 1.089 1.029	1.049	.001	1.512 1.512 1.414	1.479	.002	13.61	.000	41.0

activities of the chlorinated cells were higher than those of the control cells in the same experiment. One of these instances had a p value suggestive of no significant difference between the means. The range of percent increase when p is equal to or less than 0.01 for the other values was 7.8 - 41.7%. This trend was present at both 35 and 45 C. The overall indication is that when comparison is made between the control and chlorinated aldolase activities, the activity of the chlorinated cell aldolase is either higher than or equal to that of the control cell activity.

A comparison in the opposite direction between control cells at 35 and 45 C and chlorinated cells at the two temperatures is given in Table 6. This was done to see what effect chlorination would have on the expected Q_{10} relationship of that enzyme. All but one of the sets of results showed a significant difference between mean aldolase activity at 35 and 45 C. The increase in the 45 C activity is shown to range from 2.54 to 1.34 times that of the 35 C activity. The mean value of the control cells is 1.75 as compared to 1.51 for the chlorinated cell aldolase activity increase, giving a percent difference of only 13.7% between the means.

The effect of chlorine on control crude cell extract aldolase activity was also determined. In this instance the chlorine was added and activity determined. The average increase in activity was 123.7% over the cell extract not receiving chlorine when a 0.5 mg/l concentration

Table 6. A comparison of control and chlorinated aldolase activities at 35 and 45 C showing statistical and Q_{10} information.

Control Date	Specific enzyme activity	35C \bar{x}	S ²	Specific enzyme activity	45C \bar{x}	S ²	t stat	p value	Q_{10}
8-26-76	1.206 1.206 1.206	1.206	0	2.710 3.020 3.450	3.060	.092	10.590	.000	2.54
9-14-76	1.400 1.473 1.477	1.450	.001	2.099 1.959 1.866	1.975	.009	9.115	.001	1.36
9-22-76	1.146 1.229 1.473	1.283	.019	1.719 2.047 1.842	1.869	.018	5.280	.006	1.46
9-29-76	.694 .617 .685	.665	.001	1.029 1.089 1.029	1.049	.001	14.880	.000	1.58
								mean	1.74
Chlorinated									
8-26-76	1.300 1.300 1.300	1.300	0	2.270 2.270 2.270	2.270	0	-	.000	1.75
9-14-76	1.516 1.711 1.283	1.503	.031	2.099 2.099 1.866	2.021	.012	4.320	.010	1.34
9-22-76	1.800 1.959 1.575	1.727	.026	3.000 2.000 2.102	2.367	.202	2.319	.081	1.37
9-29-76	.864 .925 1.036	.942	.005	1.512 1.512 1.414	1.479	.002	11.120	.000	1.57
								mean	1.51

of chlorine was used. If 5.0 mg/l chlorine was introduced, there was still an increase of 107.2% over the control.

Oxygraph determinations

Cellular respiration experiments were performed with the oxygraph to determine if certain media could evoke a rapid recovery in the chlorine treated bacteria. The organisms and media were mixed for a very short time (30 seconds) and then the pattern of oxygen uptake was followed for a period of three minutes. During that time period the line produced by the oxygraph was virtually straight with a detectable slope. In a few instances the tracing had an initial steep slope and a less pronounced slope farther on. In these cases the slope was taken where the first straight line of consequence could be drawn. Duplicate samples were analyzed and averaged to give the final oxygen uptake reading. A summary of four experiments with five media is given in Figure 5. The percentages presented were obtained by determining the percent of control for each value for each date and then averaging the percentages to incorporate all of the experiments. The bottom section of the figure illustrates the effect of increasing media complexity on the oxygen uptake of organisms chlorinated for different times. As with the laboratory chlorination plate counts, the initial minute of chlorination produced the greatest change with slight spreading of the lines thereafter. The two media which were the poorest nutritionally (mineral salts and mineral salts plus 2% glucose)

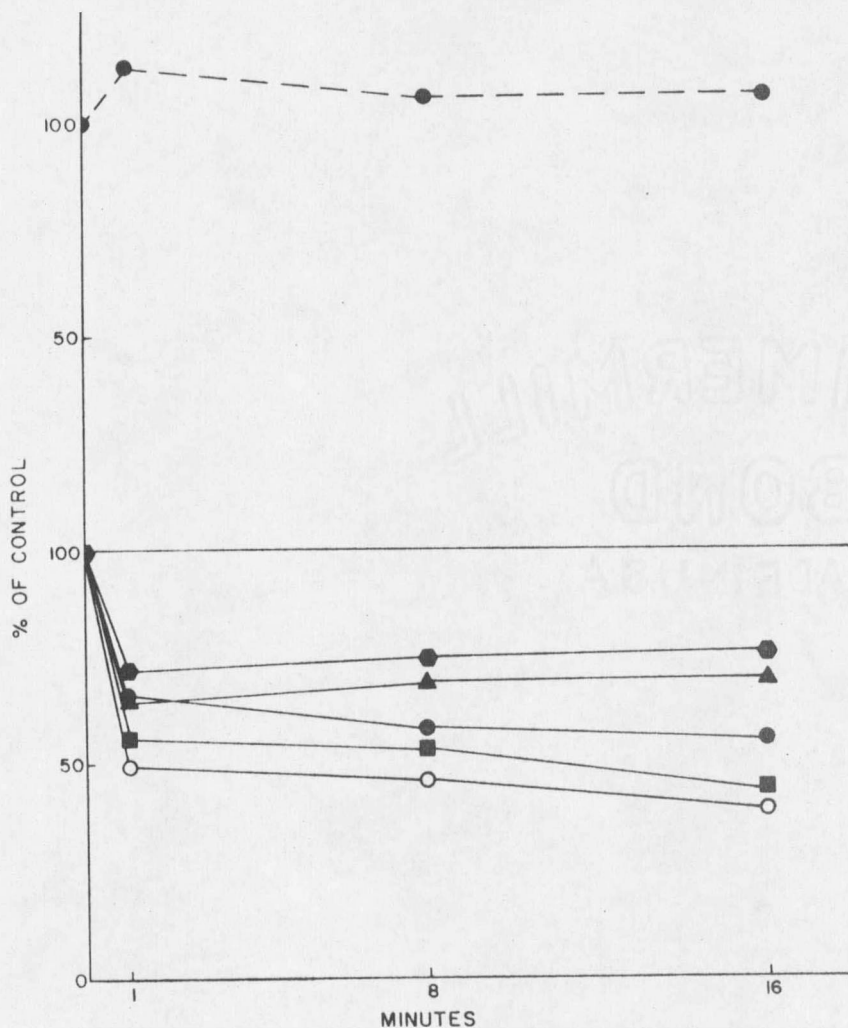


Figure 5. Effect of chlorine exposure for 0, 1, 8, and 16 minutes on the oxygen uptake (—) and aldolase activity (— —) of *E. coli*. The cells in the oxygraph assays were inoculated into mineral salts medium (●), mineral salts + 2% glucose (▲), mineral salts + 2% glucose + 0.025% glutathione + 0.025% thioglycollate (●), mineral salts + 2% glucose + 0.025% glutathione + 0.025% thioglycollate + 0.01% yeast extract (○), and TSY (■). The initial chlorine concentration was 0.5 mg/l.

exhibited a slight upward trend as chlorination progressed while the other three sloped slightly downward at the same relative angle. At the same time that the five oxygraph plots decrease, the aldolase activity increased. This is shown in the upper portion of the graph. Since the aldolase and oxygraph data were obtained from the same organisms, the composite graph reflects and simultaneous effect of chlorine on both functions. The aldolase plot exhibits an initial increase followed by a small decrease with prolonged exposure of the cells to chlorine.

Experiments were performed in which the chlorine was added directly to metabolizing control organisms in the oxygraph reaction vessel. The pattern of oxygen uptake had been followed for 90 seconds prior to the addition of sufficient chlorine to make the final concentration 0.5 mg/l, and monitored for an additional 90 seconds. This was performed with four of the media given on Figure 5. The organisms displayed a decrease in oxygen uptake immediately upon the introduction of the chlorine. The most pronounced decrease was in the mineral salts medium which displayed a 41.2% decline in uptake when compared to the control rate. Mineral salts plus 2% glucose, mineral salts plus 2% glucose plus reducing agents, and TSY exhibited percent decreases of 24.3, 24.1, and 18.9% respectively.

ATP determinations

The ATP content of concentrated cells used for the oxygraph studies and sonicated for the aldolase assays was determined in order to examine the possibility that chlorine acted as a respiratory uncoupler. It was also performed to identify a method to be used as a viable cell index. The graph comparing ATP values with plate count data is shown as Figure 6. The percent of control values were determined by dividing the values for each of four experiments by their control numbers and then taking the mean of the percentages. The figure represents the plot of the average percentages. All these plots showed a drastic decline in the first minute of chlorination and a gradual decline after that time. The M-FC values (percentage of control) were very low (2.32 - 0.21%) with the overlay determinations higher (5.19 - 3.04%) and the ATP values declined the least of all (12.80 - 10.32%). Examination of the raw data showed that when the M-FC counts decreased by three logs and the overlay counts by two logs, the ATP values declined by one log from the initial value to that obtained after sixteen minutes exposure to chlorine.

Radioactive labeling experiments

The possibility that chlorinated organisms had abnormal permeability and membrane functions was investigated by use of radioactive (^{14}C) labeled glucose and algal protein hydrolysate. Two types of experiments were performed with each substrate. In one instance the

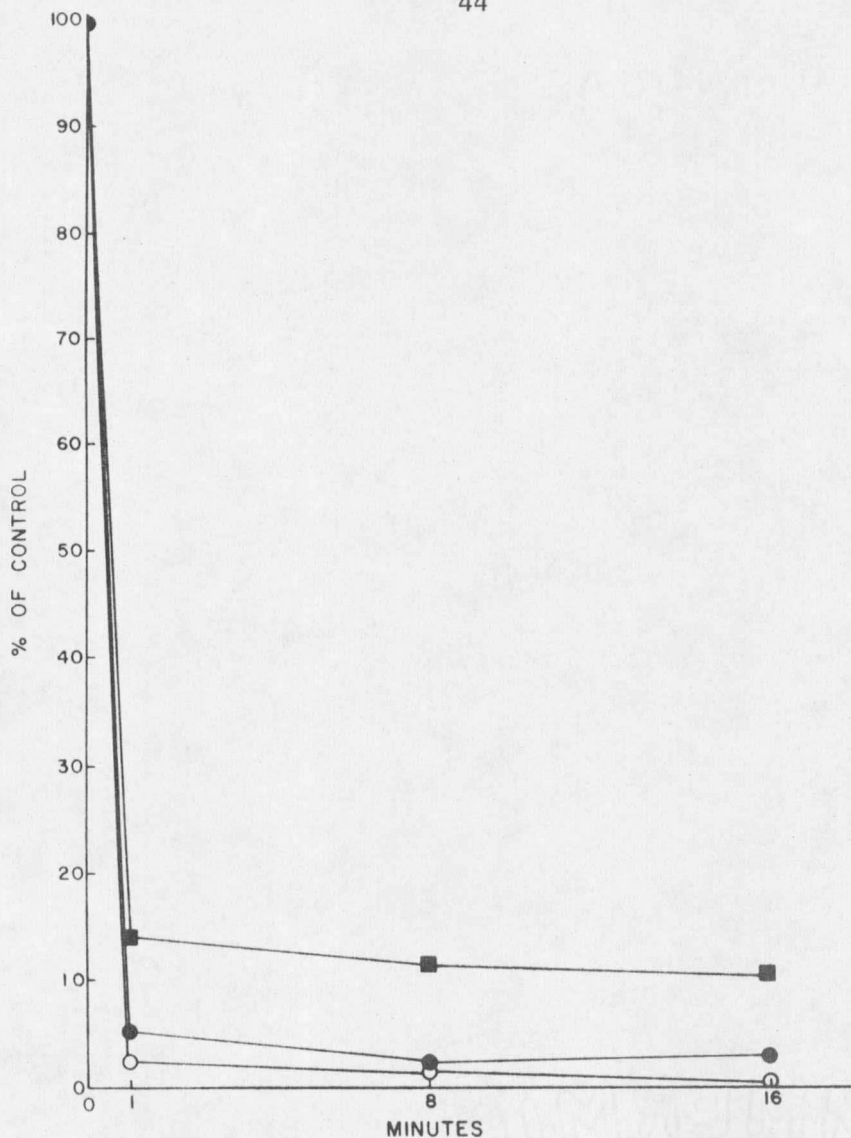


Figure 6. Effect of chlorine exposure for 0, 1, 8, and 16 minutes on concentration of ATP (fg/ml, 0.1 ml extract) (■), plate counts obtained with overlay medium (●), and plate counts using M-FC medium (○). The initial chlorine concentration was 0.5 mg/l.

bacteria were chlorinated, exposed to labeled substrate in TSY broth for a specified time, washed, resuspended in TSY, and sampled to detect the radioactivity. The second group of experiments involved the labeling of organisms in TSY prior to chlorination which was followed by washing, suspension in TSY, and sampling to obtain the counts.

The first set of experiments involved the use of labeled glucose after the cells had been chlorinated. This experiment was designed to determine if these injured organisms could take up glucose, and if so, the rate it disappeared from the cells during favorable growth conditions. Representative results of the six experiments are given in Table 7. Population determinations were done by absorbance and plate count each time that a sample was removed for analysis of radioactivity. The counts per minute given in the table were standardized per absorbance unit. Background counts were not subtracted from the readings. 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 organisms were much more capable of initial uptake of the labeled glucose. This value is 200.93 counts per minute as opposed to the near background level of 13.95 counts per minute exhibited by the chlorinated organisms. During the course of the experiment the amount of label per absorbance unit decreased in the control cells with the first obvious difference occurring after one generation time at 25 minutes incubation. The label within the chlor-

Table 7. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35° C. The cells were labeled with ^{14}C glucose for ten minutes following chlorination or no treatment (control).

<u>Minutes</u>	<u>Control</u>		<u>Chlorinated</u>	
	<u>External standard</u>	<u>Counts per min.*</u>	<u>External standard</u>	<u>Counts per min.</u>
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		

* All absorbances were identical, allowing for direct comparison of CPM for control and chlorinated cells.

inated cells remained very stable as did the absorbance during this time period.

In order to determine if the cells became leaky or their ability to dissimilate the label through metabolism was effected during chlorination, the organisms were labeled with ^{14}C glucose prior to chlorination. A long term labeling period of one hour was used to determine the effect of chlorine injury on stable cell constituents such as cell wall while a short term pulse label of ten minutes to examine the fate of transient components was also performed. Representative data are given in Table 8 for both instances. The calculations, as mentioned before, were used to obtain the radioactive data that are listed. The long term labeling results will be examined first. Organisms not receiving the chlorine had an initial count of 2826.31 which decreased relatively little to 2693.33 in two hours. The radioactivity of the chlorinated cell suspension began at a slightly lower level and decreased at approximately the same rate as the control organisms during the experiment (5% reduction vs. 2% reduction). The pulse labeling experiment showed the same sort of count decline, but the control cells showed a decrease of 44% and the chlorinated cells decreased by 33%. Once again, the control cells initially had a slightly higher count, and the rate of decline was somewhat similar between the columns.

The possibility that these phenomena were limited to carbohydrate uptake and assimilation was tested by the use of ^{14}C labeled algal

Table 8. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ¹⁴C glucose for one hour (long term) and ten minutes (pulse) before chlorination.

Minutes	Long term				Pulse			
	Control		Chlorinated		Control		Chlorinated	
	External standard	Counts per min.*	External standard	Counts per min.	External standard	Counts per min.	External standard	Counts per min.
0	6.21	2826.31	6.11	2164.86	6.01	144.27	6.07	114.34
15	6.23	2774.13	6.16	2166.46	6.15	141.96	6.09	123.55
30	6.36	2681.66	6.24	2437.87	6.02	147.05	6.09	104.03
45	6.31	2722.03	6.38	2219.17	5.98	131.90	5.94	101.52
60	6.27	2634.42	6.42	2405.97	6.12	132.66	6.05	102.75
80	6.35	2612.90	6.25	2302.75	6.06	132.89	5.93	104.77
100	6.33	2636.06	6.29	2248.61	5.93	104.37	5.90	88.44
120	6.40	2693.33	6.25	2067.94	5.54	81.02	5.96	76.15
Background	6.36	18.13			6.00	18.53		

* All absorbances were identical, allowing for direct comparison of CPM for control and chlorinated cells.

protein hydrolysate. The procedures used were the same as those employed for the labeled glucose experiments. The first group of experiments employed post-chlorination labeling. Table 9 is a sample of representative data from two experiments. The results obtained were very similar to those obtained with the labeled glucose; i.e., the control organisms were capable of an uptake of the labeled material at a higher level than the chlorinated cells and the count decreased upon incubation with a significant drop in counts occurring at about 25 minutes. The chlorinated cells did not take up the label, which is indicated by near background counts. When the control counts of algal protein hydrolysate uptake and those obtained with labeled glucose are compared, it can be seen that the cells took up more of the labeled algal protein hydrolysate during the same time period.

When the organisms were exposed to ^{14}C algal protein hydrolysate prior to chlorination, they were not as capable of its uptake as glucose during the same incubation time. Results obtained with cells chlorinated after labeling and those for the control cells are shown in Table 10. This was the only group of experiments in which the chlorinated cells had a higher initial and final count per minute value than the control cells, although that difference was always relatively small. There appeared to be some slight decrease in counts with time. In nearly all instances, the counts were not very far above background levels.

Table 9. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ^{14}C algal protein hydrolysate for ten minutes following chlorination or no treatment (control).

<u>Minutes</u>	Control		Chlorinated	
	<u>External standard</u>	<u>Counts per min.*</u>	<u>External standard</u>	<u>Counts per min.</u>
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		

* All absorbances were identical, allowing for direct comparison of CPM for control and chlorinated cells.

Table 10. Comparison of radioactivity retention in control and eight minute chlorine treated cells in TSY at 35 C. The cells were labeled with ^{14}C algal protein hydrolysate for ten minutes before chlorination.

<u>Minutes</u>	Control		Chlorinated	
	<u>External standard</u>	<u>Counts per min.*</u>	<u>External standard</u>	<u>Counts per min.</u>
0	6.30	15.44	5.94	21.44
15	5.99	16.96	5.79	21.14
30	5.97	18.38	5.96	21.50
45	6.25	16.81	6.21	25.85
60	6.34	18.03	6.22	22.14
80	6.14	18.39	6.16	22.49
100	6.04	15.98	6.13	19.34
120	6.14	13.81	6.21	18.56
Background	6.03	13.56		

* All absorbances were identical, allowing for direct comparison of CPM for control and chlorinated cells.

Chapter 4

DISCUSSION

When organisms of fecal origin are confronted with a hostile environment such as foreign aquatic surroundings or a chemical agent like chlorine, they will undergo injury and possibly subsequent death in response to this insult. Reports in the literature indicate that injury occurs in cells which have been exposed to an aquatic environment (6), to freezing in foods (35), and when treated with sanitizers (40). Additional causes of injury may be present in the environment. This injury is defined as the ability of the bacteria to grow in a nonselective medium and the inability of the same cells to grow in a selective medium.

Earlier it was thought that chlorine produced only death, and the concept of injury and the possible recovery of the chlorinated organisms from that damage was not considered. There arose some discussion at a later time concerning the possibility that some cells were actually only injured in a reversible manner and therefore capable of growth following repair under proper conditions. This initiated the controversy over the presence of injury in a chlorinated bacterial population and what the physiological and biochemical nature of that injury was. Some investigators said that some cells were not killed and later multiplied to produce a detectable count (14,20), while others suggested that the cells were capable of repairing whatever injury was

incurred and hence, able to reproduce (31,34). More importantly, it was discovered that the addition of certain metabolites would result in a reversal of the injury (17). The research presented in this thesis was done with a population of cells that were about 90% injured as determined with selective and nonselective media.

The earliest research on the physiology of chlorine injury was performed in 1946 by Green and Stumpf (15). They suggested that chlorine acted as a trace substance to inhibit glucose oxidation in cells, and therefore resulted in the death of the cell. This research and the work of Knox (26) attempted to demonstrate that chlorine specifically oxidized sulfhydryl groups of certain enzymes. Aldolase, an important enzyme in carbohydrate metabolism, was selected as the main site of chlorine action due to its nature as a sulfhydryl group containing enzyme and its essential role in glucose oxidation. The results of this research have been accepted since that time with one indirect substantiation published in 1953 (23). Other more recently suggested modes of action have been: 1. death is due to unbalanced metabolism after destruction of part of the enzyme system and recovery is suggested to occur when the cell replaces the damaged enzymes (50) 2. protein synthesis is inhibited (4) 3. chlorine reacts with alpha amino acids and causes oxidative decarboxylation to nitriles and aldehydes (58) 4. chromosomal aberrations (22) 5. reaction of chlorine with purines and pyrimidines (19) 6. chlorine reacts with

cytosine to produce chloro derivatives (37) and 7. there is an induction of DNA lesions and the accompanying loss of DNA transforming ability (41).

The importance of understanding the action of chlorine on indicator organisms such as E. coli became apparent with the discovery that the commonly used membrane filtration techniques do not enumerate chlorine injured organisms (7,28,33). This led to the controversy over whether or not the membrane filtration technique should be abandoned in favor of the more expensive and cumbersome multiple tube fermentation technique (MPN). This suggestion was based on the observation that the MPN technique utilizes an initial nonselective step which probably allows for recovery prior to exposure to a harsh selective medium. On the other hand, the membrane filtration technique employs a selective medium and temperature initially. The probability that the nonselective medium of the MPN technique (lactose broth at 35 C) allows for recovery is substantiated by the findings of Harris (16). He suggested that a nonselective medium and temperature of 37 C or lower depending upon the strain of organism will result in the increased recovery of organisms. The convenience of the membrane filtration technique encouraged investigators to explore the possibility that a medium could be devised to permit recovery, growth, and enumeration of chlorine injured cells with this procedure. A new method was proposed (39), but without knowledge of the physiology of chlorine

injury. A more recent design for assaying fecal coliforms in chlorinated waters utilizing the accepted hypothesis of chlorine injury as proposed in 1946 met with some success (46). This medium took into account the reversible nature of chlorine injury and utilized reducing agents as suggested by Ingols, et al. (23) and Mudge et al. (36) to inhibit or reverse the oxidizing nature of chlorine. Intermediary metabolites such as acetate and glycerol were also added as temporary carbon sources and a temperature shift procedure was employed of initial incubation at room temperature followed by incubation at 35 C and later at 45 C. Later research indicated that the temperature shift alone would result in the recovery of organisms similar to that obtained with the added metabolites plus the shift (29). These findings prompted our further investigation concerning the phenomenon of chlorine injury and the possible physiological action of this commonly used disinfectant.

The initial step taken in the present study was the selection of a chlorination procedure. In order to most closely approximate the actual in situ conditions, the membrane diffusion chambers were used in the chlorinated effluent of the Bozeman Wastewater Treatment Plant. The chlorine compounds present in effluent are diverse, and a combined chlorination effect from these agents was desired. The chlorine levels at the plant were highly variable and this created difficulty in establishing the extent of injury. There was some success with this method, but it proved to be time consuming and unreliable. In order

to expedite the chlorination procedure, the membrane diffusion chambers were used with an indoor controlled chlorination system. It was interesting to note that injury did not occur in the one hour exposure time even when a substantial amount of chlorine was introduced.

Likewise, injury often did not appear until the second hour of exposure when chambers were used at the treatment plant. This seemed to be an unrealistically low and delayed response since exposure during chlorination for twenty minutes in the chlorine contact chamber at the plant produced substantial bacterial killing and injury. It is proposed that the ability of chlorine to pass the membrane and enter the chamber was somehow impaired or delayed for a period of time. When injury did occur, such as at the treatment plant, it could be attributed to chlorine rather than aquatic injury due to the relatively short exposure time. Bissonnette (5) found that a log decrease in cell numbers on M-FC medium from control levels required an exposure of approximately one day in aquatic injury. The one log drop in counts at the treatment plant occurred in about $1\frac{1}{2}$ hours.

A different method of chlorination was then attempted utilizing a direct chlorine exposure method. It was thought that this procedure would result in rapid injury. Time of exposure and chlorine levels were determined in order that reproducible experiments could be performed. The number of organisms necessary for the physiological tests was determined and exposure time and chlorine levels were adjusted

to this number. This was considered an essential aspect as several investigators have shown that the action of chlorine is related to both the concentration of chlorine and the number of bacteria present (1,26). This was first ascertained mathematically by Chick (10), although his formula does not hold true for all pH and temperature ranges (9). Fair et al. (12) analyzed the data of Butterfield et al. and concluded that there must be three or four "hits" on an organism before death results with hypochlorous acid; this accounts for the deviation from Chick's first order law. The importance of temperature has been suggested (12). pH is also important as the maximum amount of HOCl available is present at approximately pH 6 (1,12), but this does not appear to be extremely critical when the contact time is ten minutes or less (13). In accordance with these findings, our experiments were performed with a temperature of 22 - 25 C, pH of about 6.5, and a relatively constant cell and chlorine concentration. Sodium hypochlorite was the source of the chlorine used since it is inexpensive, readily available, and it has been shown that aqueous hypochlorous acid is the most effective bacteriocidal form of chlorine. Other investigators have used chlorine dioxide (4,21) and chloramines (13,41), but these methods require specialized equipment in the case of chlorine dioxide or were not sufficiently effective, as with chloramines.

The laboratory chlorination procedure that was used produced the desired near log difference in counts on overlay and M-FC medium and a

predictable decrease in the number of cells enumerated by these media. Experiments discussed here required that some means of identifying viable cells be established. In addition, the rates of oxygen consumption as measured in the oxygraph studies needed to be correlated with a population of living cells. The use of plate counts as a viability index seemed inappropriate, especially when it was found that Eagle's medium gave higher counts than overlay medium. Cell numbers as determined by plate count procedures are a measure of only those cells capable of division. The measurement of cellular ATP levels was tried, but it also decreased less than the plate count numbers. Absorbance at 420 nm was selected since it remained relatively stable with exposure to chlorine. It was assumed that the time between chlorination and the performance of the other assays was sufficient to allow the dead cells present to undergo autolysis. The interference of cellular debris resulting from lysis of cells was eliminated by centrifuging the cells and discarding the supernatant.

Recovery experiments were performed to determine if the chlorine injury was reversible in suitable conditions. There has been much discussion on this subject and while it has been shown that repair will occur in aquatic injury (6), conflicting reports are given on repair from chlorine injury (14,17,20). The results obtained in these experiments clearly indicate that E. coli cells injured or surviving but not killed by chlorination were capable of repairing their injury

(Figure 4). Incubation of chlorine injured cells in a nutritionally complete medium followed by plating on selective and nonselective media support this conclusion. These organisms appear to repair their injury in overlay medium. Recovery was seen in the overlay broth but does not continue as readily when the cells are transferred to M-FC medium. The eventual convergence of the M-FC and overlay counts after incubation in overlay broth indicates that those cells capable of repair can do so after an extended lag period in overlay broth characterizing the repair process. Further evidence that repair occurs is given by comparison of the response of nonchlorinated and chlorinated cells on both media after about two hours incubation. The slopes of the plots from both media and both cellular conditions are identical, with the chlorinated slope being about one log lower than the control rate. This difference represents the portion of the population which was killed during chlorination. These experiments showed that the conditions used in the studies were suitable for repair. Although these nutrient conditions and temperatures are different than those in a receiving water environment, there is a possibility that repair can occur in the natural waters. Unpublished research performed by the author prior to this study implicates the probable involvement of repair in the observation of increased numbers of fecal coliforms at a point downstream from treatment plant effluent entrance.

These experiments that established the conditions of repair were

followed by physiological studies to investigate the site of chlorine injury. The work of Green (15) and Knox (26) suggests the involvement of sulfhydryl enzymes, particularly aldolase. They classified chlorine as a sulfhydryl enzyme inhibitor capable of oxidizing many of the cellular sulfhydryl containing enzymes. Their procedure involved the in vitro exposure of a partially purified cell free extract of E. coli to chlorine and then assaying the extract for aldolase activity. Other nonbacterial sulfhydryl-rich enzymes were tested in the same way. Ingols (23) supported this involvement of sulfhydryl groups in an indirect way by demonstrating that chlorine reacted with cysteine. Skidal'skaya (44) supposedly studied bacterial dehydrogenases, but the methods are not described clearly enough and it is difficult to determine if the system was in vivo or in vitro. In 1973, Venkobachar et al. (49) performed experiments in vitro with crude extracts and also in vivo. The in vitro experiments examined specific enzymes and the in vivo studies concerned only total dehydrogenase activity. They saw a correlation between the effect of chlorine on bacterial survival and dehydrogenase activity with intact cells, and a decrease in the activity of sulfhydryl group containing enzymes with exposure to chlorine in the crude extracts. But once again, the primary implication that chlorine injury is due to sulfhydryl group oxidation was drawn from in vitro chlorination procedures. It is important to note that most of these studies correlated an in vitro decrease in dehydro-

genase activity with a decline in cellular viability at the same chlorine concentration.

The aldolase experiments performed in this work involved in vivo chlorination followed by sonication and then the assay for aldolase activity. This experimental design satisfied the objection stated above to the correlation of in vitro experiments to in vivo phenomena. It seemed illogical that chlorine would pass by numerous exterior reactive binding sites and specifically act upon sulfhydryl groups in the cytoplasm in whole cells. In addition, the amount of chlorine that would be required to oxidize all reactive sites encountered while penetrating the cell envelope before entering the cytoplasm and then inactivating a sufficient number of enzymes to cause death appeared unrealistic. In this situation it would be more plausible to assume that some critical site exterior to the cytoplasm was at least partially responsible for the cellular injury. More evidence to support this idea will be presented later.

The aldolase assays showed that there was no decrease in activity of the enzyme after in vivo chlorination and, as stated previously, there was a slight increase. The reason for this increase was not ascertained. These results are in direct conflict with the previous in vitro analyses done by others and therefore not in accord with the commonly accepted concept of chlorine action. Therefore, there would appear to be another lesion responsible for the manifestation

of chlorine injury in E. coli. In addition, the experiments wherein hypochlorite was added directly to the crude extract yielded results similar to those from the in vivo studies; i.e., the activity did not decrease after chlorination. The exact meaning of this finding is unclear at this time, but it is possible that the chlorine oxidized more reactive sites first and the amount of chlorine was insufficient to cause a decrease in aldolase activity. An attempt was made to purchase purified bacterial aldolase for in vitro experiments but it was not available commercially so no further experiments were done on this aspect. It is important that muscle aldolase was used in the in vitro studies of most investigators. This is different than the bacterial enzyme, which may account for some of their observations. Aldolase activity was determined at 35 and 45 C to see if the elevated M-FC incubation temperature could be responsible for causing a decreased aldolase activity. If so, the idea of a temperature sensitive lesion could be used to explain the lower number of cells obtained with M-FC. The results (Table 6) did not support this notion but rather showed that there was a near normal Q_{10} relationship in both chlorinated and control cells.

All of the results obtained with aldolase indicate that it is not the critical site of injury and death due to chlorination as previously thought. It is unfortunate that this hypothesis has persisted for so long without the application of definitive experiments to test the

validity of in vitro results used to define in vivo effects.

The aldolase assays were followed by respiration experiments utilizing the oxygraph. Various media were used to test their effect on the oxygen uptake of control and chlorine treated cells. The general trend observed was that the oxygen uptake of cells decreased after chlorination while the aldolase activity increased slightly (see Figure 5). Endogenous respiration showed the least decrease. The addition of reducing agents or the presence of a more complex medium did not appear to be of any advantage to the cells in this short time incubation as it does during a long term incubation for over one hour. In fact, the more complex media seem to encourage a greater loss of oxygen uptake than did the less complex media. These data support the proposed concept that reducing agents do not reduce sites oxidized by chlorine. If this rationale applies to the long term incubation as well, it would then seem that the addition of metabolites or perhaps just an extended period of time in nonselective media would allow the cells to repair the injury instead of reversing it.

The increased aldolase activity suggests that chlorine may have acted as an uncoupler. However, the decrease in oxygen utilization along with a decline in ATP levels did not substantiate this idea. In addition, the cells were capable of recovering after an extended lag period which would not be predicted if chlorine acted as an uncoupler. When chlorine was added to the cells incubated in the

oxygraph flask there was a rapid decline in the rate of oxygen uptake. One would expect a rapid increase in oxygen uptake if the classic uncoupling reaction had occurred. The elimination of chlorine acting as an uncoupler was also of importance in supporting the decision made to use absorbance as the index of viability. If the oxygraph rates were standardized on the basis of cell counts obtained by plating, the chlorinated cells would have had a rate that was up to two logs higher than the control cells.

The approach then taken was an examination of the possibility that chlorine was acting on or near the cell surface. Gram negative cell wall and membrane configurations afford many potential reaction sites such as the associated proteins and perhaps even the reduced lipids. As already described, chlorine has been shown to react with alpha amino acids and dipeptides (38) and although oxidation of lipids by chlorine has not been investigated there is no obvious reason why it could not be possible. Electron microscopy work (8) on cells treated with chlorine showed no alteration of cellular configuration suggesting that the site of action might not be the peptidoglycan layer. No darkening or intracellular granules were observed after treatment, which is common with other treatments. This may indicate that the mechanism is not related to the oxidation or disruption of the cytoplasm.

The experimental design next devised encompassed two related

concepts; the ability of chlorinated cells to take up labeled material and the turnover of the labeled carbon that was incorporated within the cell. If membrane damage occurred during chlorination the uptake of labeled material should have been impaired, therefore, the first group of experiments involved labeling the cells with ^{14}C glucose following chlorination. A striking difference between uptake in the control and chlorinated cells was observed (Table 7). This would suggest that the chlorine treated cells had an impaired transport system and were incapable of moving glucose across the cell envelope. During the incubation time used, some of the control cells had time to replicate and a decrease in label resulted. The chlorinated cells probably did not recover in this time, but this could not be detected by the label due to the initial low level. The background counts may have been the result of the inability of the cell to retain label even if it had been transported if chlorine caused leakiness. To test this, cells were labeled with ^{14}C glucose prior to chlorination. With both long term and pulse labeling, the decrease in the levels of cellular label as control and chlorinated cells were incubated were very similar. This indicated that the carbon metabolism in the cells after chlorination continued at about the same rate as before. Transient (those materials labeled with the pulse exposure) and more stable materials (those labeled with the long term exposure) turned over at about the same rate after chlorination as before (Table 8).

This finding appeared to conflict with the evidence that the injured cells were alive and were able to recover. It was expected that the intracellular turnover of materials would be accelerated in order that repair processes could occur and the cell could then survive during the absence of transport. On the other hand, the cells could have survived on stored glycogen or used materials that were not labeled. The slightly lower initial counts with the chlorinated cells may indicate that there was some leakage and loss of labeled material, but this was of such small value that it could be accounted for in a difference in cell numbers which was not detected by the absorbance readings.

Further investigation was required to see if the chlorine injury of transport was limited to a phosphotransferase system of glucose or if it was more general. Labeled algal protein hydrolysate was utilized because amino acids were transferred via the active transport process. A repeat of the post-chlorination 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 the label (Table 9). For some undetermined reason, the cells labeled before chlorination did not display a significant amount of label (Table 10).

The experimental results using labeled compounds point to a new hypothesis on the action of chlorine i.e., that envelope functions, especially transport, are effected. Due to the proximity of

the envelope to the outer environment, this appears to be a reasonable site of chemical interaction, injury, and even death. This proposed mechanism has not been investigated due to the relative infancy of knowledge concerning membrane structure and function. It can be proposed that the cells remain alive after chlorine injury by turning over intracellular constituents until damage can be repaired and a sufficient level of transport is resumed. This hypothesis is not without its flaws, but it provides a much more plausible mode and describes the data obtained better than the site of action previously suggested. It is hoped that further investigation into the action of chlorine on membranes will be pursued as the understanding of membrane biochemistry and physiology is expanded.

Chapter 5

SUMMARY

The physiological site and recovery of chlorine injury were studied in E. coli following chlorination by several methods. Injury produced by exposure in membrane diffusion chambers at the Bozeman Wastewater Treatment Plant was found to be too variable and this system was replaced with a direct laboratory chlorination procedure. This method utilized chlorine bleach to give a concentration of 0.5 mg/l. The temperature (22 - 25 C), organism concentration (8×10^9 /ml), and pH (6.5) were kept relatively constant. A reproducible and relatively stable amount of chlorine injury was produced in a determined length of exposure, usually eight minutes.

E. coli cells were found to be able to repair their injury after about two hours in a rich overlay broth. Repair was characterized by the convergence of plots of plate count numbers from M-FC and overlay media and the subsequent resumption of logarithmic growth. Demonstration of repairable injury was an important aspect in the study which may explain the occurrence of increased numbers of fecal coliforms downstream from wastewater effluent discharge into the receiving water.

The commonly accepted site of chlorine damage, i.e. aldolase activity, was tested to determine if this enzyme was involved in the phenomenon of chlorine injury as observed in the experiments performed. In vivo chlorination of cells was followed by in vitro analysis of

enzyme activity. Aldolase activity did not decrease, but rather illustrated a slight upward trend after chlorination. Temperature elevation to 45 C from 35 C did not produce a temperature lesion that could be associated with the injury as a Q_{10} relationship was observed.

Oxygen uptake in chlorinated cells was lower than that of control cells. This was determined using an oxygraph and several media. The more complex media including reducing agents produced the greatest decrease in oxygen uptake after chlorine injury and the reversal of injury did not occur in the three minute incubation period. This indicated that repair probably is not due to simple reversal of chlorine oxidation.

Experiments with ^{14}C labeled glucose and algal protein hydrolysate were performed with the label administered both before and after chlorination. Pre-chlorination labeling illustrated that leakiness was probably not of major importance and that turnover occurred in chlorinated and control cells at about the same rate. Labeling after chlorination suggested that injury seriously impairs transport functions as chlorinated cells maintained only background levels of radioactivity.

The results obtained implicate the impairment of envelope physiology as a major site of chlorine injury and the absence of a

chlorine effect on aldolase. Further research on membrane function should, in time, allow the resolution of the exact site of injury.

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