MONTANA STATE UNIVERSITY

Copper injury of Escherichia coli
by Matthew Joseph Domek

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
© Copyright by Matthew Joseph Domek (1984)

Abstract:
Low levels of copper in chlorine-free distribution water caused injury of coliform populations. Monitoring of 44 drinking water samples indicated 64% of the coliform population was injured. Physical and chemical properties were measured including the concentrations of three heavy metals (copper, cadmium, and lead). Copper concentrations ranged from 0.007 to 0.54 mg/liter. Statistical analysis of all factors was used to develop a model to predict coliform injury. The model predicted almost 90% injury using a copper concentration near the mean observed value (0.158 mg/liter) in distribution waters. Laboratory studies using copper concentrations of 0.025 and 0.050 mg/liter in a carbonate buffer under controlled conditions of temperature and pH showed over 90% injury in 6 and 2 days, respectively.

Escherichia coli injured in drinking water or by copper showed decreased oxygen utilization. Oxygraph measurements of cells injured in drinking water showed 73 to 83% decrease in oxygen utilization when cells were injured 70 to 99.5%. Copper-injured cells had less than 25% the rate of oxygen utilization of the control cells. Respirometry experiments measured rates over a longer period of time and showed similar trends.

13Carbon-Nuclear Magnetic Resonance Spectroscopy (13C-NMR) and gas chromatography were used to identify differences in metabolism between healthy and injured populations of E. coli. The rate of glucose utilization by injured cells under anaerobic conditions was 64% of the healthy cells. The rates of accumulation of lactate and ethanol were 88 and 50% of the control, respectively. 13C-NMR studies of oxygenated cultures revealed differences in the accumulation of acetate and lactate. A large pool of glutamine accumulated in healthy but not injured cells. Additional studies revealed injured cells had a decreased ability to reduce 2-(p-iodopheny1)-5-phenyl tetrazolium chloride (INT) with a variety of carbohydrate substrates. When NADH was used as a substrate, injured cells reduced greater quantities of INT than the healthy cells. Copper-injured E. coli appeared to have impaired respiration as indicated by decreased INT reduction and other respirometric indices. A comparison of metabolic end products suggested that injured cells also had considerable differences in carbon flow compared with healthy cells indicating that the injured cells were being repaired.

COPPER INJURY OF <u>ESCHERICHIA</u> <u>COLI</u>

by

Matthew Joseph Domek

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

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

September 1984

ii

APPROVAL

of a thesis submitted by

Matthew Joseph Domek

This thesis has been read by each member of the thesis
committee and has been found to be satisfactory regarding
content, English usage, format, citations, bibliographic
style, and consistency, and is ready for submission to the
College of Graduate Studies.

8-31-84
Date

Chairperson,
Graduate Committee

Approved for the Major Department

9-5-84
Date

Head, Major Department

Approved for the College of Graduate Studies

9-20-84
Date

Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature_____

Date_____

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

ABSTRACT

Low levels of copper in chlorine-free distribution water caused injury of coliform populations. Monitoring of 44 drinking water samples indicated 64% of the coliform population was injured. Physical and chemical properties were measured including the concentrations of three heavy metals (copper, cadmium, and lead). Copper concentrations ranged from 0.007 to 0.54 mg/liter. Statistical analysis of all factors was used to develop a model to predict coliform injury. The model predicted almost 90% injury using a copper concentration near the mean observed value (0.158 mg/liter) in distribution waters. Laboratory studies using copper concentrations of 0.025 and 0.050 mg/liter in a carbonate buffer under controlled conditions of temperature and pH showed over 90% injury in 6 and 2 days, respectively.

Escherichia coli injured in drinking water or by copper showed decreased oxygen utilization. Oxygraph measurements of cells injured in drinking water showed 73 to 83% decrease in oxygen utilization when cells were injured 70 to 99.5%. Copper-injured cells had less than 25% the rate of oxygen utilization of the control cells. Respirometry experiments measured rates over a longer period of time and showed similar trends.

[13]Carbon-Nuclear Magnetic Resonance Spectroscopy (^{13}C-NMR) and gas chromatography were used to identify differences in metabolism between healthy and injured populations of E. coli. The rate of glucose utilization by injured cells under anaerobic conditions was 64% of the healthy cells. The rates of accumulation of lactate and ethanol were 88 and 50% of the control, respectively. ^{13}C-NMR studies of oxygenated cultures revealed differences in the accumulation of acetate and lactate. A large pool of glutamine accumulated in healthy but not injured cells. Additional studies revealed injured cells had a decreased ability to reduce 2-(p-iodophenyl)-5-phenyl tetrazolium chloride (INT) with a variety of carbohydrate substrates. When NADH was used as a substrate, injured cells reduced greater quantities of INT than the healthy cells. Copper-injured E. coli appeared to have impaired respiration as indicated by decreased INT reduction and other respirometric indices. A comparison of metabolic end products suggested that injured cells also had considerable differences in carbon flow compared with healthy cells indicating that the injured cells were being repaired.

INTRODUCTION

Isolation of Coliform Bacteria from Water

The evaluation of potable water supplies for total coliform bacteria is important in determining the sanitary qualitity of drinking water. Elevated levels of coliform bacteria indicate a contaminated source, inadequate treatment, or post-treatment deficiencies. The occurrence of injured coliform bacteria presents a significant problem in accurately estimating the number of indicator organisms in drinking water. Previous reports have documented that indicator bacteria become injured in drinking water (3) and surface water (8) and such injured bacteria are not enumerated when currently accepted media are used (37).

In general terms bacterial injury is defined as the inability of bacteria to grow under specified conditions that are satisfactory for uninjured cells (12). This may be assessed in different ways such as: measuring differences in the ability of bacteria to form colonies on minimal media versus complex media or loss of capability to grow in the presence of a selective agent (12). Sodium deoxycholate was used as a selective agent in this study since it is used in many different types of media used in

aquatic bacteriology and is known to prevent the growth of injured coliform bacteria (61).

Coliform bacteria may become injured by exposure to a variety of stressful factors in aquatic environments including: chlorine (11,13,40), heat (15,24,32), freezing (41,57), acid mine drainage (22), sunlight (25), and heavy metals (42, Domek, M.J., M.W. LeChevallier, and G.A. McFeters, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q6, p. 261). Injury of coliform bacteria by chlorine has been well-described (11,13,40) and is likely the major cause of damage to indicator organisms in drinking water. Metals can be another important cause of bacterial injury because of their common occurrence in drinking water (49,59).

Part A of this study was done to determine the role of metals in injury of Escherichia coli under conditions simulating municipal drinking water distribution systems. To accomplish this it was necessary to show that injured coliform bacteria could be found in drinking waters where chlorine was not present. In this part of the study, physical and chemical characteristics of water were assessed to determine the role of various factors in injury caused by distribution water. Laboratory experiments were done to test whether metals could cause injury at concentrations predicted from the physicochemical analyses of the water in which injury was observed. Strains of E. coli isolated from water were used in the experiments as

representatives of the coliform bacteria. The findings
that were reported in part A supported the conclusions that
metals, in particular copper, contributed to injury and
caused significantly reduced isolations of coliform
bacteria.

Metabolism of Injured Bacteria

Differences in bacterial growth on selective and non-
selective media reveal little specific information about
the nature of the bacterial injury caused by various
stresses. However, a considerable amount of literature is
available on cellular damage in injured bacteria caused by
treatments related to food processing (12). Freeze-injured
E. coli cells release amino acids, small molecular weight
RNA, and peptides (48,58). E. coli and Salmonella
typhimurium cells that are heat-injured release lipopoly-
saccharides (24,31). Heat injury to S. typhimurium causes
RNA degradation (38,56) and alters the transport of glucose
(55). Tomlins et al. reported that ribosomal regeneration
(68), and protein (67) and lipid (69) biosynthesis are
required for the recovery of heat-injured S. typhimurium.
Despite the information available regarding bacterial
injury related to food processing little is known about
water-related injury. Zaske et al. (77) showed that the
cell envelope of E. coli is damaged by exposure to water
that is high in conductivity. During the recovery period

aquatically injured E. coli exhibits an extended lag phase (76).

The major objective of part B was to develop an understanding of the physiological processes involved when coliform bacteria were injured in aquatic environments containing metals. Physiological alterations of E. coli injured by exposure to chlorine have been reported (13,75). There is however a paucity of physiological information describing coliform bacteria that have been injured in the aquatic environment where chlorine is either not present or negligible.

Toxic Effects of Metals on Microorganisms

There are reports about toxic effects of metals on microorganisms (for a review see Babich and Stotzky, 5). Singleton (62) observed a decrease in the diversity in a naturally occurring population of bacteria from fresh water when either mercury or copper was added to the system. A copper concentration of 0.05 mg/liter slows the growth rate of the alga, Selenastrum capricornutum, and 0.09 mg/liter of copper inhibits growth completely (6). Structural changes are observed when the alga, Ankistrodesmus braunii, and the blue green bacterium, Anabaena 7120, are grown in the presence of copper (45). Tyler reported that copper limits the rate of mineralization of nitrogen in acid forest soils (71) and decreases respiration rates of soil

microorganisms (41). A study by Albright et al. (1)
showed that a copper concentration of 0.01 mg/liter doubles
the rate of glucose turnover in a population of heterotrophic
bacteria, and that copper and zinc toxicity is additive
(2). Higher concentrations of copper are needed to cause
toxic effects when the cell density or amount of organic
matter in the system is increased. Lamb and Tollefson (36)
showed that copper at a concentration of 5.0 mg/liter
reduces by 90% the conversion of glucose to carbon dioxide
in activated sludge from a sewage plant. An exposure of
microorganisms in activated sludge to a concentration of
5.0 mg/liter of copper for one day causes nearly a 90%
reduction in oxygen utilization (30). Malaney et al. (43)
observed similar reductions in oxygen utilization when
bacteria are exposed to copper concentrations in the range
of 5.0 to 20.0 mg/liter.

Approach to Studying Injured Bacteria

A variety of techniques was used in part B to assess
physiological differences between the injured and non-
injured populations. Respirometry experiments were done on
cells injured by exposure to drinking water to investigate
possible differences in energy conserving physiology
between injured and non-injured E. coli (Dockins, W.S.,
M.J. Domek, and G.A. McFeters, Abstr. Annu. Meet. Am. Soc.
Microbiol. 1980, p. 179, N93). These preliminary

experiments revealed that E. coli injured in potable water
had impaired respiratory activity. Subsequent experiments
used E. coli cells injured by copper under carefully
controlled conditions. Injured E. coli were relatively
fragile compared to non-injured cells and did not withstand
many ordinary laboratory manipulations such as
centrifugation. Therefore techniques involving minimal
manipulation were employed whenever possible.

An active bacterial electron transport system (ETS)
reduces 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetra-
zolium chloride (INT) to INT- formazan (53) which was
measured spectrophotometrically (48). Injured E. coli
cells were tested for their ability to reduce this dye.

Metabolic differences between injured and healthy
organisms were obtained by using nuclear magnetic resonance
spectroscopy (NMR) to follow the fate of ^{13}C-enriched
glucose. This technique allowed monitoring of in vivo
metabolism under aerobic and anaerobic conditions (47).
Well-resolved spectra were obtained with 20 minute accumu-
lations of data. This method proved to be a powerful tool
in providing information about metabolic rates and pathways
without the need for analysis by extraction of the metabo-
lites or other destructive procedures. Also, gas chromato-
graphy was used to determine production of carbon dioxide
(CO_2) and hydrogen (H_2) gases during recovery from injury.

In addition to the basic physiological contribution of
this study, the information gained was used as part of a
larger concurrent study conducted in the laboratory to
develop a new medium (37) for the isolation and enumeration
of injured coliform bateria. Ultimately it is hoped that a
better understanding of aquatic injury is attained through
this work and other projects (9,13,45,68,69).

Statement of Objectives

Initial observations revealed that an E. coli
suspension in a membrane diffusion chamber became injured
in drinking water having less than a 0.1 mg/liter
concentration of chlorine. This prompted our efforts to
determine what type of physicochemical factors, other than
chlorine, might cause injury in a drinking water
distribution system. Additionally I sought to show that
naturally occurring coliform bacteria could be isolated
from distribution water thus establishing that the survival
of injured coliform bacteria was not an in vitro artifact.

After determining that copper injured E. coli in
drinking water, I was interested in the physiology of the
injured organism. We attempted several approaches in part
B. Respirometry experiments measured oxygen utilization.
[13]Carbon-NMR and gas chromatography were used to study the
fate of glucose. These methods were used to investigate
alterations in metabolism during the recovery of E. coli

injured by copper. INT was used to analyze electron
transport activity. The physiological data were also used
to delineate the metabolic lesion(s) caused by copper.

Materials and Methods

Organisms

The E. coli strains used in these studies were isolated by membrane filtration from the East Gallatin River near Bozeman, Montana. The isolates were identified and maintained as previously described (77).

Preparation and Laboratory Injury of Bacteria

Cultures were grown and prepared as before (45). Injury was assessed by differential counts of colony forming units (CFU) on the non-selective TLY (Trypticase Soy Broth without dextrose, Difco, 27.5 g; Lactose, 10.0 g; Yeast Extract, Difco, 3.0 g in 1.0 liter Milli Q water, Milli Q Reagent Grade Water Systems, Millipore) agar (15 g/liter, W/V), and selective TLY-D agar (TLY with 0.1 % [W/V] sodium deoxycholate) (45). The percent of injury was calculated as follows:

$$\frac{(CFU \text{ on } TLY) - (CFU \text{ on } TLY\text{-}D)}{CFU \text{ on } TLY} \times 100 = \% \text{ Injury}$$

Injured populations were obtained for some experiments by placing washed cells into membrane diffusion chambers (46) immersed in water drawn from a lightly used portion of the Bozeman, MT, drinking water distribution system (45).

All glassware needed for preparation of cells injured by copper was washed with nitric acid. Copper-injured populations were obtained by placing washed cells (approximately 1.0×10^5 CFU/ml) in $CaCO_3$ buffer and adding to the buffer various amounts of copper from a stock ($CuSO_4$) solution. $CaCO_3$ buffer was prepared by adding 0.168 g $CaCO_3$ to 1 liter Milli Q water, undissolved. After autoclaving and cooling to room temperature, $CaCO_3$ was dissolved by lowering the pH by adding 1N sulfuric acid (4.0 ml/liter) and then raising it by adding 1N sodium hydroxide to pH 7.0. The buffer solution was added to 125 ml glass-stoppered bottles. The bottles were sealed with silicone stopcock grease to maintain the pH after adding the copper solution and bacterial suspension.

Since physiological experiments were run over a relatively short time interval, high cell concentrations were used. All copper-injured cells used for physiology experiments were prepared using a copper concentration of 1.875 mg/liter and a cell concentration of approximately 1×10^9 CFU.

Physicochemical Analysis of Water

Water samples collected in sterile bottles were placed on ice, transported to the laboratory within 1 hour, and analyzed within 5 hours of collection. A complete description of sampling locations and coliform enumeration methods

has been previously published (37). Chemical and physical
analyses were done using published methods (28).

Copper Analysis

Water samples were acidified by adding 0.1% nitric acid
(v/v) and sodium nitrate was added as an electrolyte to
final concentration of 0.1 M. An average of 3 replicates
was used to determine copper concentrations by the
differential pulse polarographic method using a model 374
Polarographic Analyzer (Princeton Applied Research,
Priceton, New Jersey) (62,63).

Cadmium and Lead Analysis

The concentrations of these metals were analyzed in a
Woodriff Furnace Atomic Adsorption Spectrometer (33). The
average of 4 peak area measurements from replicate samples
were integrated to calculate each concentration.

Statistical Analysis

Statistical analyses including Pearson correlation
coefficients and multiple linear regressions were done on a
Honeywell CP-6 computer equipped with a Statistical Program
for the Social Sciences (SPSS). The regression model was
developed according to published techniques (50) with the
help of statistical consultant, Georgia Ziemba. Injury was
calculated for statistical analysis as the difference

between verified coliform counts on m-T7 agar and verified
coliform counts on m-Endo agar (21).

Oxygen Uptake Analyses

Oxygen uptake measurements of E. coli injured in
drinking water were done using a Gilson model KM Oxygraph
equipped with a model Y.S.I. 4004 Clark-type electrode.
The chamber of the instrument was maintained at $30^{\circ}C$. A
cell suspension (1.8 ml of 5.0 X 10^8 CFU/ml) in drinking
water was added to the chamber and allowed to equilibrate
for 2 minutes followed by the addition of 0.2 ml of
phosphate-buffered (pH 7.0) substrate (glucose or lactose;
1%, w/v). The oxygen uptake rates were determined graphi-
cally from the slope of the lines as compared with controls
without cells (13) as explained in Appendix A.

Oxygen uptake measurements of copper-injured E. coli
were done similarly using a Gilson model 5/6H Oxygraph
equipped with a model Y.S.I. 5331 Clark-type electrode.
Substrate solutions were phosphate-buffered (pH 7.0) 10 mM
(final concentration) glucose or lactose.

Manometric Methods

Additional oxygen uptake experiments were done using a
Gilson Differential Respirometer model IGP-14. The center
well of each flask contained a wick with 0.5 ml of 40% KOH
solution (w/v). The sidearm contained 1.0 ml of TY broth

(the same as TLY broth without the lactose) and 0.5 ml of 100 mM glucose solution. Flasks contained 1.0 ml suspension of approximately 5.0×10^8 CFU/ml E. coli, 2.0 ml Milli Q water, and 0.5 ml of mineral salts (MS) solution (pH 7.0) containing: K_2HPO_4, 7.0 g; $K_1H_2PO_4$, 3.0 g; Na citrate.2 H_2O, 0.1 g; $MgSO_4$. 7 H_2O, 0.1 g; $(NH_4)_2SO_4$, 1.0 g in 500 ml Milli Q water (53). After 20 minutes of temperature equilibration the contents of the sidearms were tipped into each flask. Replicate flasks (3 or 4) were shaken (100 rpm) at 30^oC and oxygen utilization was measured at intervals of 10 minutes (19).

NMR Methods

A 1.8 ml suspension of E. coli cells (adjusted to approximately 5×10^8 CFU/ml) was added to a 10 mm NMR tube containing 7.2 mg of [1-^{13}C] glucose (MSD isotopes) (20 mM final concentration) or 4.7 mg of [2^{13}C] succinate (Stohler Isotope Chemicals) (20 mM final concentration). Volumes of 0.1 ml MS solution and 0.1 ml TY4 broth (same as TLY broth but 4 times the concentration) were added to 1.8 ml of a cell suspension as buffer and additional nutrients. Oxygen (or nitrogen for anaerobic conditions) was bubbled through two coaxial tubes. The inner tube near the bottom of the NMR tube bubbled at a rate of 5 ml of gas per minute (Figure 1). The outer tube was placed just beneath the surface of the 2.0 ml sample and bubbled at rate of 20 ml

20 ml/min
(outer tube)

5 ml/min
(inner tube)

coaxial tubes

10 mm
NMR tube

2.0 ml sample

Figure 1.   Apparatus for bubbling gas into a 10 mm NMR
            tube containing a suspension of E. coli cells.

of gas per minute (73). The ^{13}C-NMR spectra of 62.83 MHz were obtained in Fourier transform mode on a Bruker WM-250 spectrometer (72). Chemical shifts of ^{13}C-containing compounds were compared relative to tetramethylsilane used as a zero ppm standard. The alpha C-1 resonance of glucose at 92.4 ppm was used as an internal reference. A repetition time of 1.01 seconds and 45 degree pulse width were used. Data (1200 scans) were collected every 20 minutes (Appendix B).

Gaseous End Products of Metabolism

A suspension of E. coli cells (3.6 ml, 5 X 10^8 CFU/ml) was added to 25 ml Erlynmeyer flasks that were fitted with No. 1.0 butyl rubber stoppers and gassed with nitrogen to purge the oxygen for anaerobic experiments. MS solution (0.2 ml) and TY4 broth (0.2 ml) were added. These flasks were incubated on a rotary shaker (100 rpm) at 35°C. Experiments were stopped at various time points by adding 1.0 ml of Zap solution (51) which consisted of 50% formaldehyde and 50% phosphoric acid (v/v).

Hydrogen was measured by removing part of the head space with a gas tight syringe (Becton, Dickinson) fitted with a Minvert valve (Supelco) and injecting the sample into a Carle model 8500 gas chromatograph (GC) having a thermal conductivity detector. The GC was equipped with a stainless steel column (2.3 meters by 3.18

mm O.D.) packed with Poroak N 80-100 mesh (Supelco). The
flow rate of helium was 21 ml of gas per minute. Area unit
responses were converted to umole concentrations with a
Spectra Physics model 4100 integrating computer
standardized against known concentrations of hydrogen (66).
Afterward carbon dioxide was analyzed similarly using
helium as a carrier gas at a flow rate of 21 ml per minute.

Respiration Measurements with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride

A 0.2 ml volume of cell suspension (approximately 5 X
10^8 CFU/ml) was added to 1.3 ml MS solution, 0.5 ml 0.2%
(w/v) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl
tetrazolium chloride (INT) solution, and 1.0 ml of
substrate solution (4,51). All substrates were 0.28 M and
dissolved in Triton X-100 solution. The Triton X-100
solution was made by adding 1.8 ml of a 10% solution (w/v)
of Triton X-100 to 98.2 ml of Milli Q water. NADH solution
was made by adding 10 mg NADH to 10 ml of Triton X-100
solution. The mixtures were incubated in triplicate at $35^{\circ}C$
on a rotary shaker (100 rpm) for 45 minutes and killed with
1.0 ml of Zap solution. The amount of INT-formazan
produced was estimated spectrophotometrically at 490 nm
(53) against a standard curve made with INT-formazan
dissolved in 95% ethanol and diluted in MS.

The glucose preincubation experiments were done by adding 0.2 ml cell suspension, 1.3 ml MS solution, 0.25 ml 80 mM glucose solution, and 0.25 ml of TY broth and the mixture was incubated for 45 minutes on a rotary shaker at 100 rpm. Then, 0.5 ml of INT solution and 0.5 ml of double strength Triton X-100 solution were added and the mixture was incubated for an additional 45 minutes. The cells were killed and analyzed as above.

Results

Part A - Injury in Relation to Water Chemistry

Preliminary experiments exposing E. coli in membrane
diffusion chambers (46) to distribution water with
negligible chlorine concentrations demonstrated greater
than 90% injury over a 5 day period (Figure 2). This level
increased to 99% with 7 days of exposure. At the same time
bacterial viability (CFU on the non-selective TLY medium)
within the test suspension remained high (>90%) over the
entire 7 day period. These inital observations prompted
further studies to determine the cause of bacterial injury
seen in distribution water where levels of chlorine were
either low (<0.1 mg/liter) or non-existent.

A survey was done using 44 drinking water samples
collected from two drinking water systems where chlorine
residuals were low in order to look for relationships
between injury and various physicochemical and
microbiological properties. The data for these properties
were the result of a collaboration among several
individuals within the laboratory. The data obtained were
presented in Table 1 with my contribution being the deter-
mination of the copper, lead, and cadmium concentrations.
Of particular interest were the low free and total chlorine

Figure 2. Differential plate count of E. coli cells exposed to drinking water containing less than 0.1 mg/liter of chlorine. Cells were plated on media that were nonselective, TLY (■), and selective,TLY-D (●).

Table 1. Physicochemical and microbiological properties of drinking water samples taken from several localities in southwestern Montana.

Microbiological, Physical or Chemical parameter	# of samples	unit of measure	Mean	Minimum	Maximum
Standard plate count bacteria	41	CFU's/ml	110.8	1.0	1270.0
Temperature	44	°C	10.5	4.0	26.0
Turbidity	44	nephlometer turbidity units	0.20	0.01	4.5
Conductivity	44	μ mho	403.1	32.0	650.0
pH	44	pH units	7.7	7.2	8.4
Free chlorine	44	mg/L	0.15	0.00	1.50
Total chlorine	44	mg/L	0.18	0.00	1.60
Phosphate	44	mg/L	.257	0.00	2.20
Nitrite	44	mg/L	.002	0.00	0.10
Nitrate	44	mg/L	1.21	0.05	1.76
Alkalinity[a]	43	mg/L	208.9	171.0	231.0
Hardness[a]	43	mg/L	222.7	88.0	278.0
Total organic carbon	37	mg/l	18.4	0.0	66.8
Copper	36	mg/L	0.117	0.007	0.540
Lead	40	mg/L	0.008	0.001	0.088
Cadmium	40	mg/L	0.001	0.000	0.011

[a] Measured as Ca CO_3 in mg/L.

residuals (mean 0.15 and 0.18 mg/liter respectively), and the high copper levels (median 0.117 mg/liter). Data from this collaborative survey were analyzed for correlations between properties measured and injury observed in waterborne bacteria within those samples. Significant (p <0.05) correlations are found between bacterial injury in drinking water and temperature, pH, copper, total organic carbon (TOC) as well as the sum of all metals tested (Table 2). None of the other properties listed in Table 1 show a significant simple correlation with injury in drinking water.

Injury is probably a result of complex interactions between the bacteria and the various physicochemical properties of drinking water. Simple correlations give some although limited insight into these problems. A statistical model was developed, using multiple linear regression to make a more comprehensive approach to determine the cause of bacterial injury in drinking water. All of the properties listed in Table 1 were used in building the model. Injury, however, was found to be influenced primarily by copper, temperature, pH, and alkalinity (Table 3). Among these four factors, copper is the most significant variable while the other properties probably influenced the availability and toxicity of copper ions in water. The coefficient of multiple determination, R_2, expressed as a percent, indicates that 50.8% of the

Table 2. Significant[a] correlation coefficients for injury in drinking water.

Parameter	Injury[b]		
	Correlation coefficient (r)	No. of samples	Significance (p)
Temperature	0.257	44	.044
pH	0.479	44	<.001
Copper	0.312	36	.032
Total organic carbon	0.301	37	.035
Metals[c]	0.537	44	<.001

[a] Only values significant at the 95 percent level (p $\leq$ 0.05) are presented.

[b] Injury is calculated as the difference between verified coliform counts on m-T7 agar minus the verified coliform count on m-Endo agar.

[c] The metals parameter represents the sum of copper, lead and cadmium values observed.

Table 3. Statistical model derived to predict coliform injury.

Model	Coefficient of Multiple Determination (R^2)	Number of Observations (N)	Total Squared Error (C)	F Values
Injury[a] = -261.7	0.508	30	5.7	-
+150.0 (Copper)				4.82
+ 2.04 (Temperature)				3.48
+ 21.69 (pH)				2.02
+ 0.314 (Alkalinity)				1.19

a. Injury is calculated as the difference between verified coliform counts on m-T7 minus the verified coliform count on m-Endo agar.

variation in injury was explained by the variables in the model. The total squared error for injury is 5.7. Other factors were also shown by statistical analysis to affect coliform injury. These factors included TOC, species of coliform, as well as free and total chlorine, but these properties either rarely occurred or were infrequently measured. Therefore, they could not be developed into an adequate model. Other factors that could not be measured, such as duration of aquatic exposure and the physiological state of the organism (7,14) as it entered the aquatic environment were also thought to be important.

The survey results were verified by laboratory experiments testing the capacity of copper, lead, and cadmium to cause measurable injury in a strain of E. coli isolated from water. Based on preliminary chemical analysis of water, metal levels for our first experiments were: copper, 0.25 mg/liter; cadmium, 0.017 mg/liter; and lead, 0.07 mg/liter. Initial experiments showed coliform injury occurred at these metal levels in reagent grade water, a phosphate-buffered system (pH 7.0), and in a carbonate-buffered system (pH 7.0). The carbonate-buffered system was preferred since this buffer is the dominant buffering system of most distribution waters (54) and metals were able to react without interference from phosphate or magnesium. The potential of these metals to cause injury was tested individually, and it was determined that copper

alone caused as much injury as a mixture of all the metals examined.

The progressive injury of E. coli caused by copper (0.05 mg/liter and 0.025 mg/liter) in a carbonate buffer is shown in Figure 3. Greater than 90% injury occurred in this system within 2 days (4°C) at 0.05 mg/liter copper. After 5 days exposure to 0.05 mg/liter copper, cells demonstrated greater than 99.5% injury. Injured cells started to die with longer contact times at this copper concentration resulting in the slight apparent decrease in injury seen on day 7. At lower copper concentrations (0.025 mg/liter), injury progressed more slowly taking 6 days to reach the 90% level (Figure 3).

This experiment was repeated with 6 other strains of E. coli isolated from water. Table 4 shows the typical pattern of progressive injury and loss of viability upon exposure to copper concentrations of 0.025 mg/liter and 0.050 mg/liter with one of the seven isolates. In comparisons among isolates, 6 out of 7 isolates showed less than 90% loss in viability in 5 days upon exposure to 0.025 mg/liter of copper, whereas 4 out of 7 isolates had greater than 90% loss in viability at 0.050 mg of copper per liter. At 0.025 mg/liter of copper 6 out of 7 isolates reached 95% injury in 3 days. Increasing the concentration to 0.050 mg/liter increased the degree and rate of injury observed. Only one isolate had greater than 63% injury in 5 days in
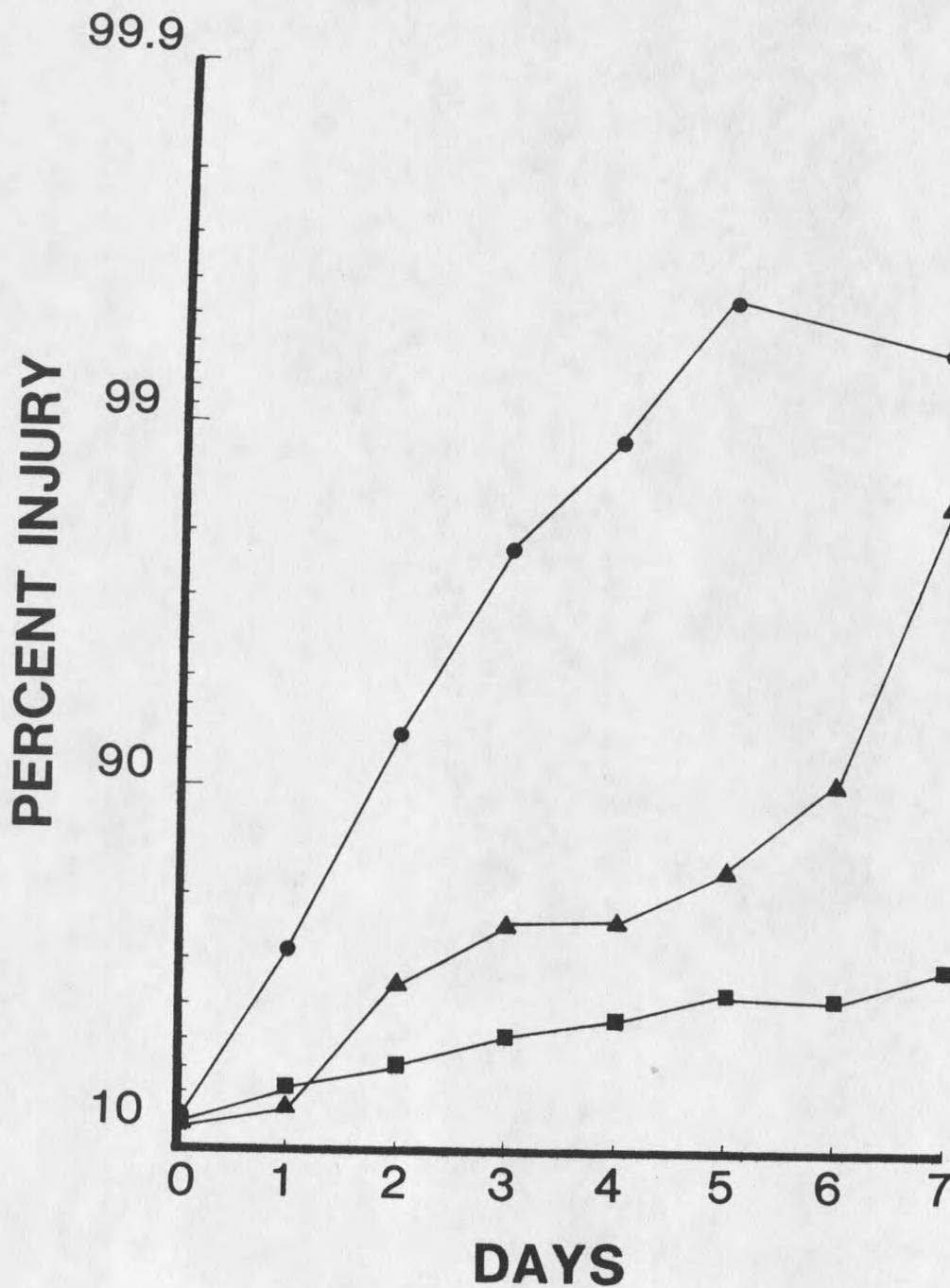
Figure 3.   Injury of E. coli cells exposed to copper in
             carbonate buffer. E. coli (1.1 x 10⁵
             CFU/ml) was exposed to 0.050 mg of Cu/liter
             (●), 0.025 mg of Cu/liter (▲), and carbonate
             buffer (pH 7.0) (■).

Table 4.   Effect of copper on viability and injury of _E. coli_, isolate No. 10.

| | Control[a] | | 0.025 mg/liter of copper[b] | | 0.050 mg/liter of copper | |
| | Percent | | Percent | | Percent | |
Days	Loss in viability[c]	Injury[d]	Loss in viability	Injury	Loss in viability	Injury
0	0	2.07	0	9.20	0	13.78
1	0.56	n.d.	12.27	90.48	27.04	n.d.
2	-10.56	49.66	23.93	93.44	77.55	99.89
3	8.47	47.33	41.72	95.70	n.d.	n.d.
4	3.95	n.d.	54.42	98.15	96.22	99.78
5	6.78	57.09	54.79	98.89	98.67	99.64

a. _E. coli_ cells (1 x 10^5 CFU/ml) suspended in a carbonate buffer.

b. _E. coli_ cells (1 x 10^5 CFU/ml) suspended in a carbonate buffer with a copper concentration of 0.025 mg/liter.

c. Loss in viability calculated as: $\frac{\text{day 0 - day X}}{\text{day 0}}$ x 100 = %.

d. Injury calculated as: $\frac{\text{TLY CFU - TLY-D CFU}}{\text{TLY CFU}}$ x 100 = %.

the control populations without copper and none had greater
than 28% loss in viability.

Raising the temperature above 4°C accelerated the rate
of copper injury. This conclusion is based on a limited
number of experiments. The time necessary to cause 90%
injury at 0.05 mg/liter copper was only 1 day at 22°C. In
additon, higher metal concentrations caused injury at a
faster rate (90% in less than 1 day at 4°C), but large
decreases in cell viability were also observed.

Part B - Physiology Studies

E. coli cells placed in membrane diffusion chambers and
exposed to distribution water from a quiescent portion
(i.e., no chlorine detected) of the Bozeman drinking water
distribution network were examined to determine the
physiological implications of aquatic injury. Initial
experiments revealed a trend of decreased oxygen use with
increased injury. Oxygen utilization (during metabolism of
lactose) measured on a Gilson Oxygraph over a short time
period (approximately 5 minutes) decreased 73 to 83% when
cells were injured 70 to 99.5%, respectively (Figure 4).
When cells were grown on glucose, oxygen utilization
decreased 47% for 70% injured cells (Figure 4). Similar
results were obtained with long term (2 hours) respirometry
experiments (Figure 5). Bacteria incubated in an enriched
medium containing either glucose or lactose required

Figure 4.   Short term uptake of oxygen by E. coli cells
            injured in drinking water. Substrates used
            were 1.0% glucose (bars) and 1.0% lactose
            (stippled).

Figure 5.   Oxygen uptake of injured E. coli measured
            during recovery in Tryptic Soy Broth. E. coli
            was injured in drinking water. The medium was
            supplemented with 0.5% glucose or 0.5% lactose.
            The numbers following the data curves indicate
            percent injury.

approximately 30 minutes (14% injured cells), 55 minutes

(55% injured cells), or 100 minutes (95% injured cells) to

utilize 10 ul oxygen per 10^8 viable cells. This oxygen

utilization level was never achieved by cells that were

99.5% injured regardless if they were grown on glucose or

lactose (Figure 5). Similar results were found when a

mineral salts medium containing either glucose or lactose

was used (Figure 6).

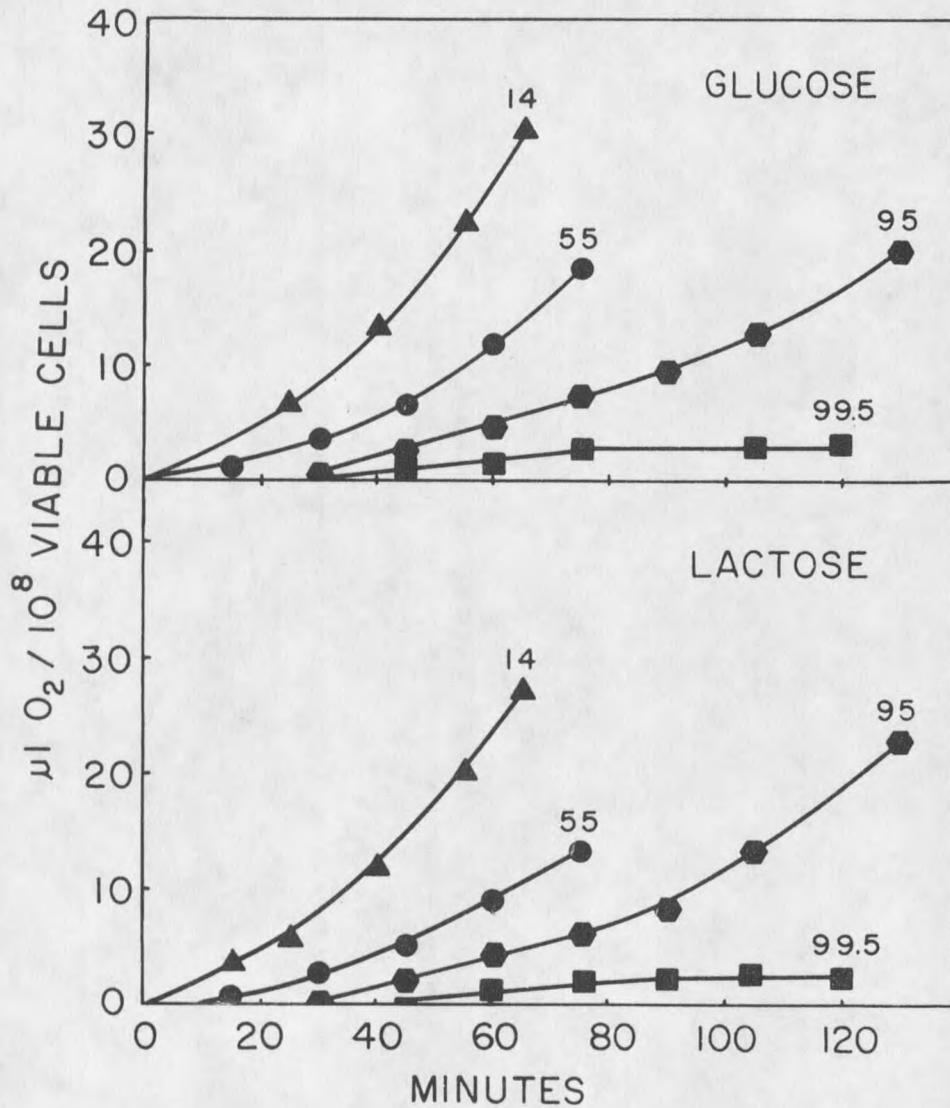Isolation of injured cells grown under aerobic and

anaerobic conditions also lended support to the above

experiments. Table 5 shows that injured E. coli cells were

isolated in approximately equal numbers under both aerobic

and anaerobic conditions. These results indicated that the

presence of oxygen was not required by injured cells during

recovery.

The oxygen uptake experiments were repeated using E.

coli injured by copper in a carbonate buffer thus isolating

the effect of copper injury from the variables that cannot

be controlled in drinking water. In these and subsequent

experiments the major comparisons were made between cells

exposed to copper in a carbonate buffer and cells starved

for the same amount of time (3 to 5 days) in the carbonate

buffer alone. Oxygen uptake measured by using a Gilson

Oxygraph showed that copper-injured cells had less than 25%

the rate of oxygen utilization of the control cells in

carbonate buffer using either glucose or lactose as a

Figure 6.   Oxygen uptake of injured E. coli measured
             during recovery in mineral salts medium.
             E. coli was injured in drinking water.
             Substrates were 0.5% glucose or 0.5%
             lactose. The numbers following the data
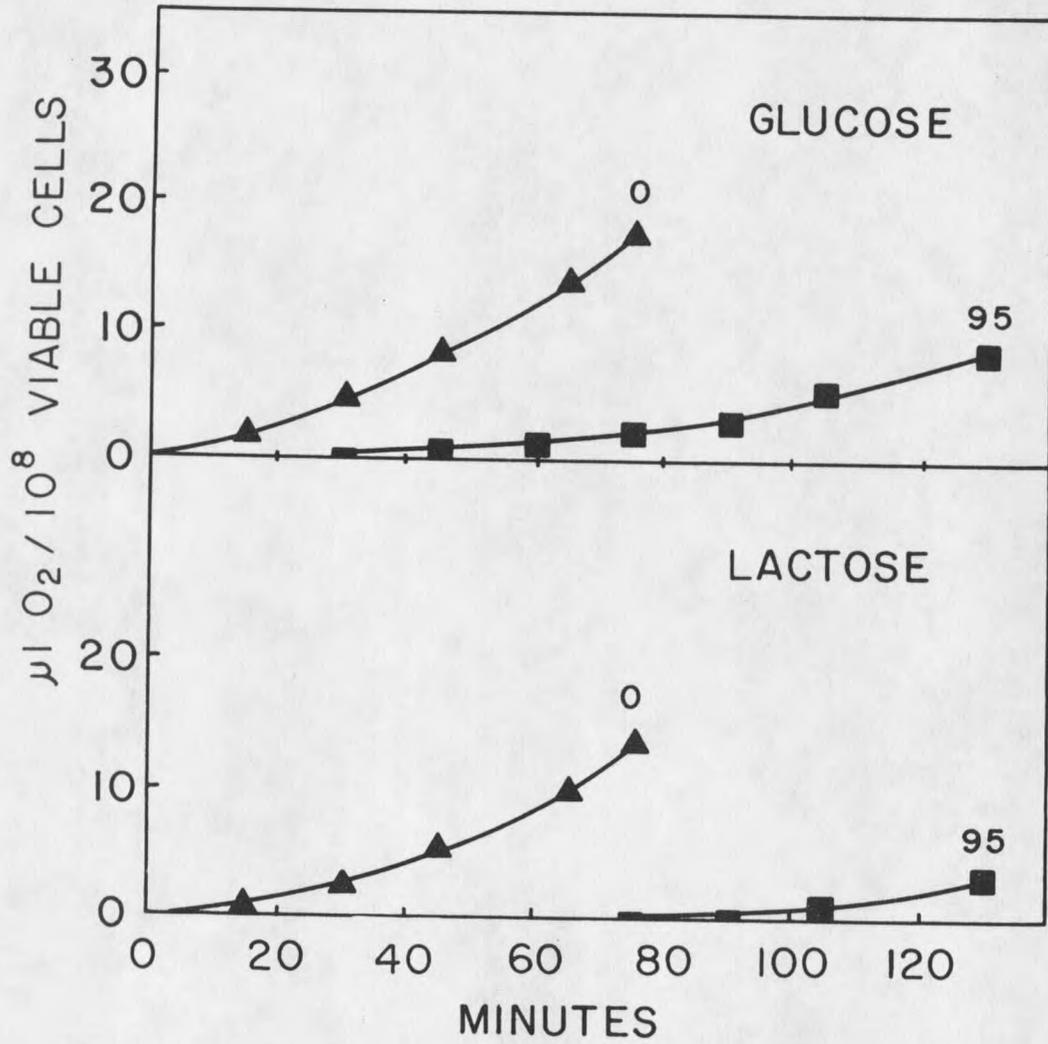   curves indicate percent injury.

Table 5. Aerobic and anaerobic plate count data with injured <u>E. coli</u>.

	Aerobic			Anaerobic		
No. Days Stress	TLY^a Count	$TLYD^b$ Count	$\%^c$ Injury	TLY^a Count	$TLYD^b$ Count	$\%^c$ Injury
0	1.13×10^9	9.97×10^8	11.8			
2	9.33×10^8	3.96×10^8	57.5			
4	8.17×10^8	1.81×10^8	77.9	7.30×10^8	2.30×10^8	68.2
6	2.10×10^8	7.43×10^6	96.5	1.80×10^8	7.70×10^6	96.17
0	1.21×10^9	1.02×10^9	16.4	1.08×10^9	1.01×10^9	7.3
2	8.80×10^8	6.70×10^8	23.9	8.46×10^8	5.73×10^8	32.3
4	5.10×10^8	1.40×10^8	72.6	6.80×10^8	1.83×10^8	73.0
6	1.87×10^8	1.51×10^7	92.0	2.20×10^8	1.17×10^7	94.6
7	9.50×10^7	3.16×10^5	99.7	1.97×10^8	5.00×10^5	99.8

a. TLY is Tryptic soy broth w/o dextrose + 0.3% yeast extract + 1.0% lactose + 1.5% agar

b. TLYD is TLY + 0.15% desoxycholate

c. % injury = $\dfrac{TLY\ count\ -\ TLYD\ count}{TLY\ count} \times 100$

34

substrate (Figure 7). E. coli cells that were depleted of their energy stores by incubation in a mineral salts medium at 35°C and identical controls not depleted were also tested. Both of these populations showed 33 to 44% greater oxygen uptake than the cell suspensions exposed to carbonate buffer indicating some damage had occurred to the cells in a carbonate buffer (Figure 7). Endogenous oxygen uptake (i.e., no exogenous carbon energy substrate provided for metabolism) was measured for each population. The injured population apparently had substantially less oxygen uptake than the other 3 populations, although it was difficult to be confident about these results since the signal to noise ratio was low.

Long term respirometry experiments measured oxygen uptake during the recovery period (several hours). In these experiments an enriched medium provided nutrients needed in recovery. The results of these experiments (Figure 8) showed that the cells injured by copper had 46.8% of the rate of oxygen uptake of the control population. Interestingly, this pattern was similar to Figure 5 where the 14% injured population (injured in drinking water) was similar to the control population and the 95% injured population (also Figure 5) was similar to cells injured by copper.

Figure 7.   Short term oxygen uptake by _E. coli_ injured by exposure to copper. Uptake measurements were made at $30^{o}C$ using phosphate-buffered substrates of 10 mM glucose (striped bars) and 10 mM lactose (open bars). The stippled portion of the bars represents the endogenous rate. _E. coli_ populations are labelled as follows: (A) washed uninjured cells, (B) cells after 3 hours depletion in a mineral salts medium without a carbon energy source at $35^{o}C$, (C) cells (control) after standing for 5 days in a carbonate buffer at $4^{o}C$, and (D) cells injured by copper after 5 days in a carbonate buffer at $4^{o}C$.

38

Figure 8. Manometric measurement of oxygen utiliza-
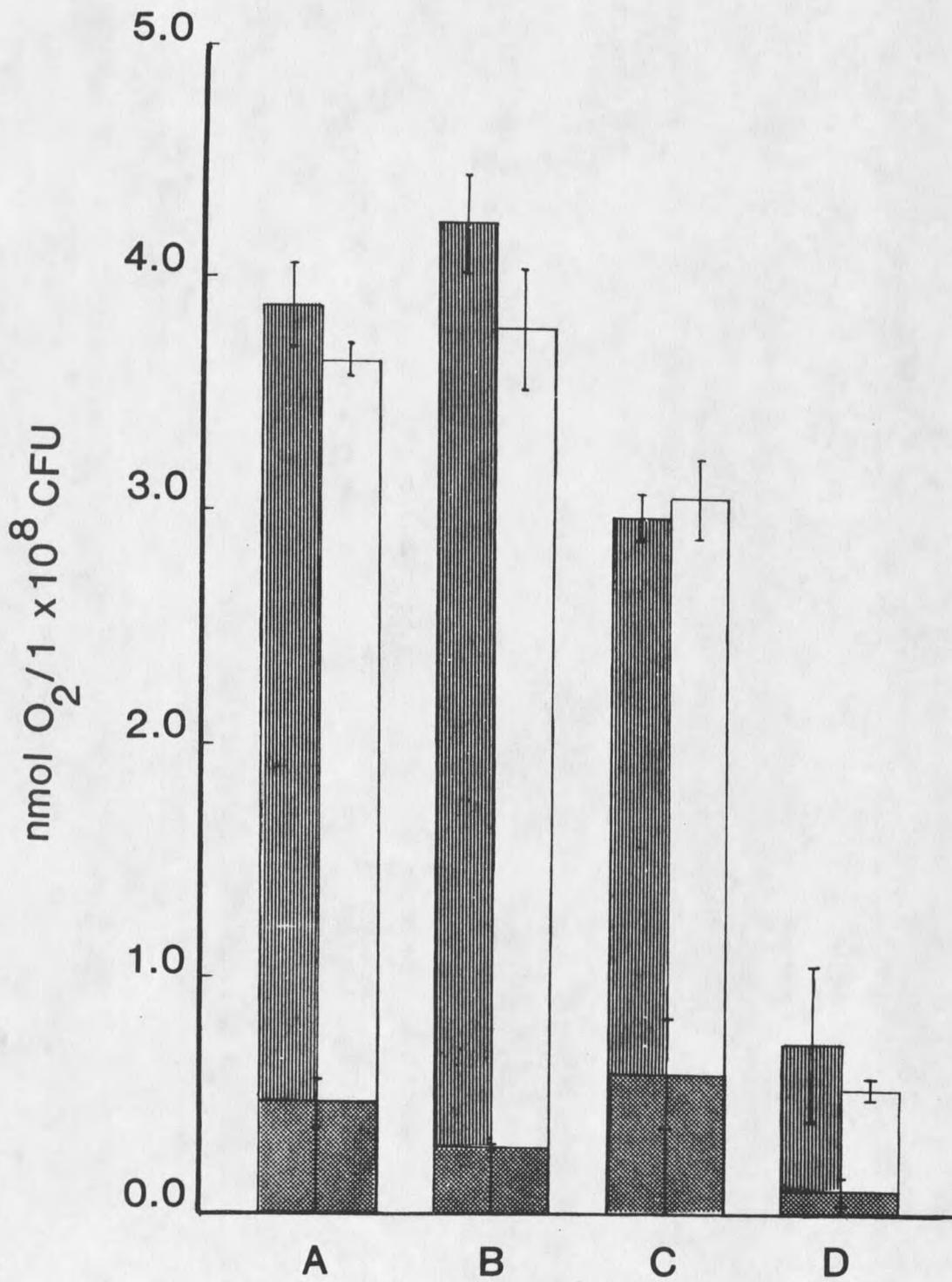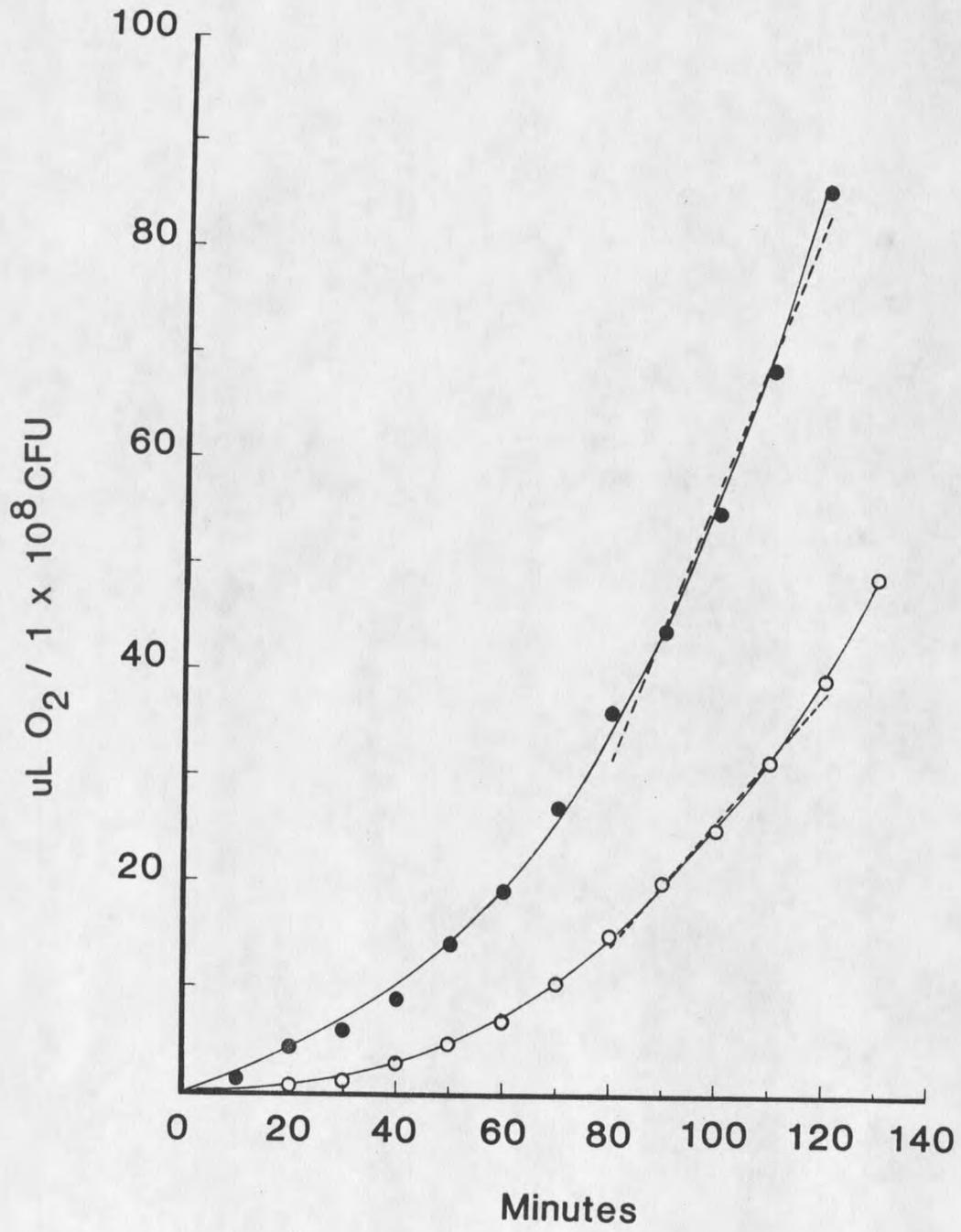tion by copper-injured E. coli. Control
cells (●) and injured cells (o) were
incubated at 30°C in a medium consisting
of phosphate-buffered TSB and Yeast
Extract. Only 3 data points had a S.D.
greater than 2.65. The dotted lines are
the linear regressions of the data from 80
to 120 minutes. Oxygen was used by con-
trol cells at 1.28 ul/min and by injured
cells at 0.60 ul/min.

Metabolism Studies of E. coli Injured by Copper

Since E. coli injured by copper displayed impaired aerobic respiration other evidence was sought to determine what type of metabolism E. coli used during repair. NMR was used to study the metabolism of glucose and revealed major differences of end product accumulations. The experiments were run under both aerobic and anaerobic conditions. The fate of glucose, labelled in the C-1 position, was followed. Since E. coli is a mixed acid fermenter, 3 products of the Embden-Meyerhoff-Parnass (EMP) pathway of glycolysis were measured, namely acetate, ethanol, and lactate. These spectra were accumulated anaerobically at 20 minute intervals (only 4 time points are shown) for control cells (Figure 9) and the injured cells (Figure 10). Integrals of the peak areas increased as concentrations of metabolic end products increased but different carbon resonances could not be quantitatively compared because of the Nuclear Overhauser Effect and lack of total relaxation. The spectral peaks observed were the alpha and beta peaks of the glucose C-1 position at 92.4 and 96.2 ppm, respectively. The C-2 through C-6 resonances of glucose were the natural abundance peaks of ^{13}carbon located between 60 and 80 ppm. The natural abundance of ^{13}carbon is approximately 1.1% and because there was a large quantity of glucose the C-2 through C-6 resonances

41

Figure 9.   NMR spectra of ^{13}C-labelled products of
            anaerobic glucose metabolism made by <u>E.
            coli</u> control cells. Acetate (A), lactate
            (L), ethanol (E), and succinate (S) were
            measured at 20 minute intervals but only 4
            time points are shown. The two large
            peaks at 92.4 and 96.2 ppm are the alpha
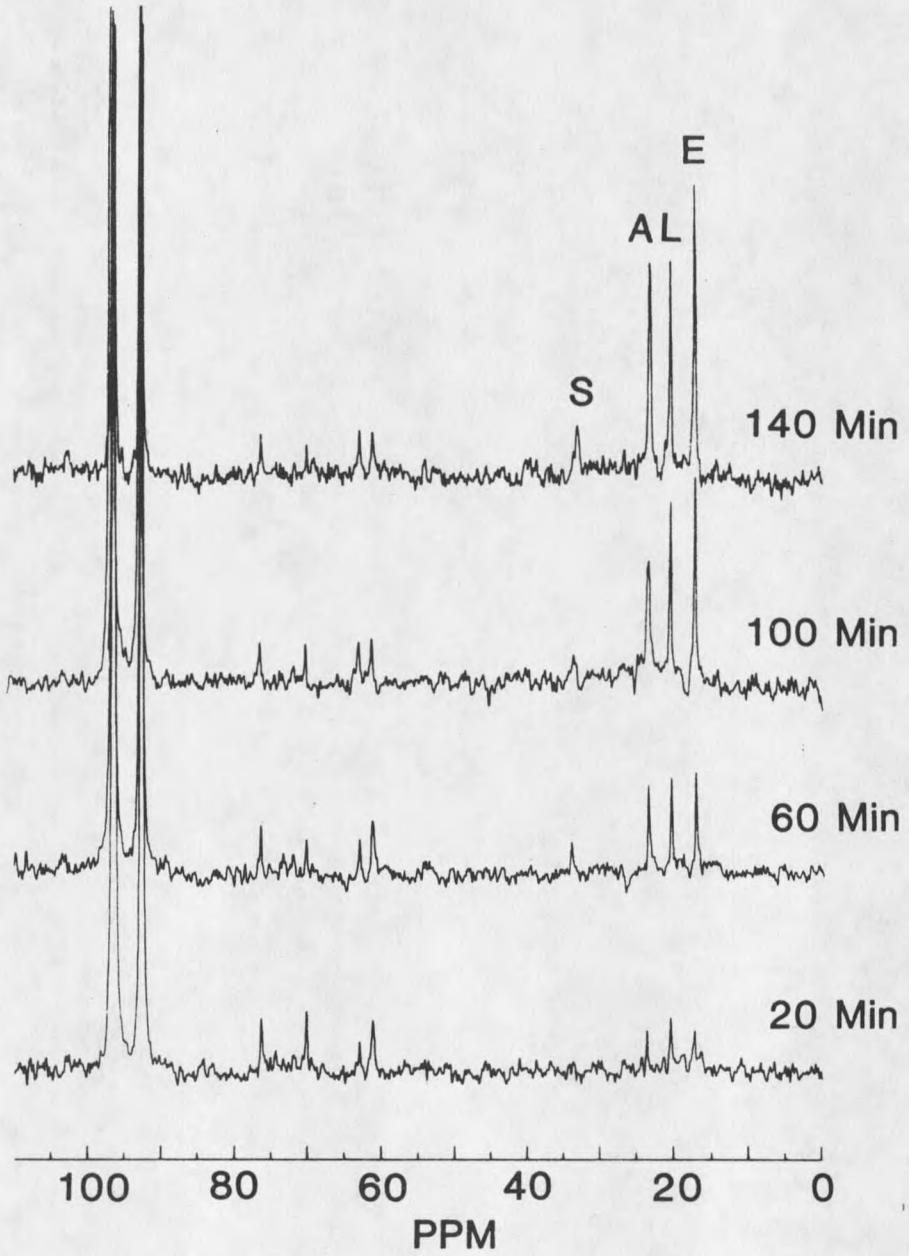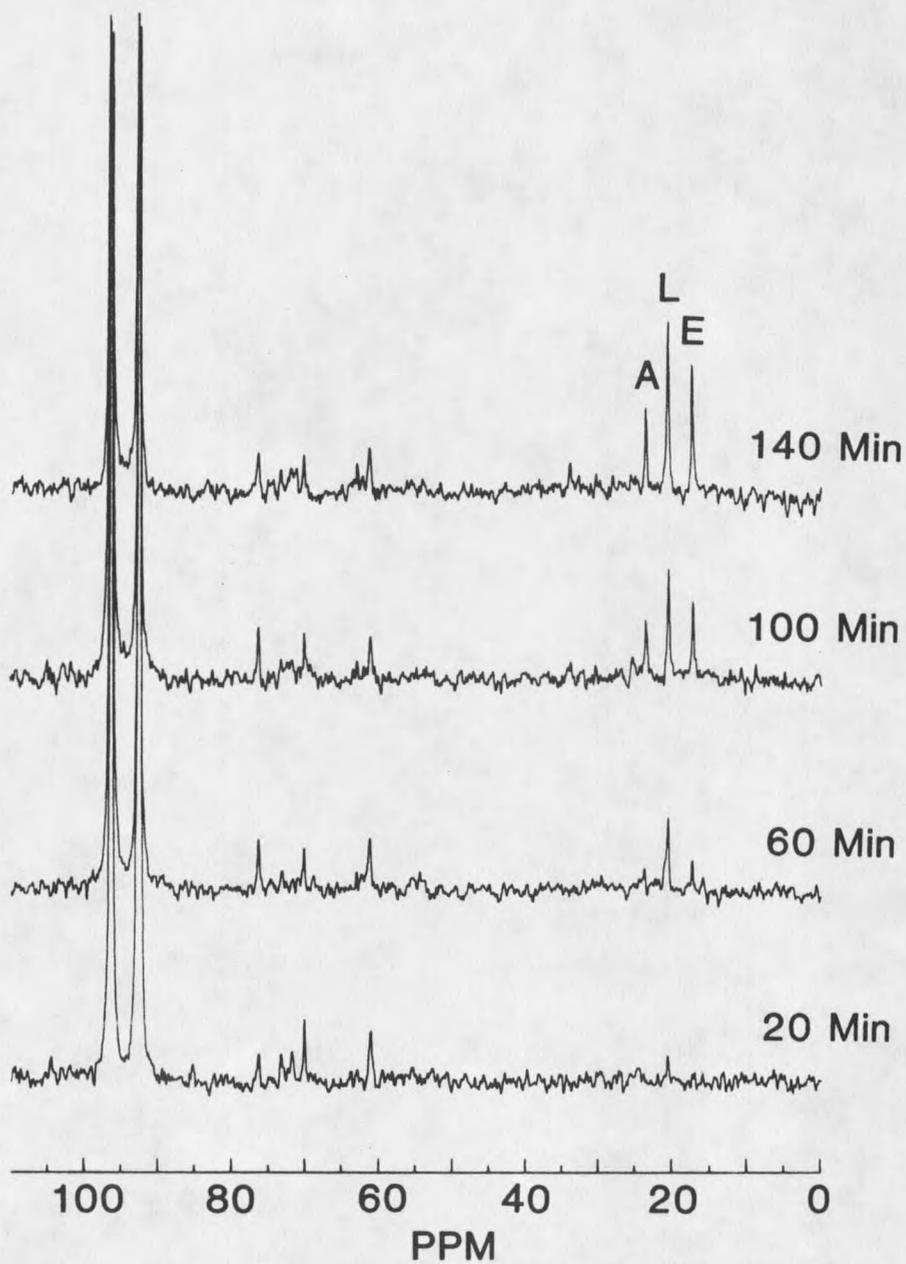   and beta peaks of glucose.

Figure 10. NMR Spectra of ^{13}C-labelled products of anaerobic glucose metabolism made by copper-injured _E. coli_. Peaks are labelled as in Figure 9.

were approximately 1.1% the size of the enriched alpha and beta C-1 peaks of glucose.

In addition to the EMP pathway end products, succinate is known to accumulate in mixed acid fermentations. Succinate was visible at a chemical shift of approximately 33ppm but not clearly distinguishable in the injured cells. In Figure 11 the integrals of the peaks for lactate and ethanol were graphed. The rate of accumulation of lactate in the injured population was 88% of the control cells and ethanol was produced in the injured population at only 50% the rate of the control population. Figure 12 shows that injured cells produced very little acetate and their rate of utilization of glucose was slower (64% of the control).

Formate, carbon dioxide gas (CO_2), and hydrogen gas (H_2) are also end products of mixed acid fermentations but could not be measured with the NMR procedure employed. Under experimental conditions formate would not accumulate but CO_2 and H_2 would be significant end products (20). Gas chromatography was used to measure H_2 and CO_2 accumulations. The injured population produced H_2 at 88.9% the rate of the control population and CO_2 at 88.4% the rate of the control (Figure 13).

Since oxygraph and respirometry studies had revealed decreased oxygen uptake by injured cells, it was of interest to investigate in more detail aerobic metabolism of injured cells. The working hypothesis was that injured

46

Figure 11. Accumulaton of lactate and ethanol by
copper-injured $\underline{E.}$ $\underline{coli}$ under anaerobic
conditions. Lactate (circles) and ethanol
(squares) were measured by NMR in arbi-
trary units (AU) at 4 time intervals.
Control (closed) and injured (open) cells
were incubated using 20 mM ^{13}C-labelled
glucose at 35°C and bubbled with nitrogen.
Linear regressions of all data had r
values of >0.97.

48

Figure 12. Accumulation of acetate by copper-injured
E. coli metabolizing ^{13}C-labelled glucose
under anaerobic conditions. Acetate
(squares) production and glucose (circles)
utilization were measured by NMR in arbi-
trary units (AU) at 4 time intervals.
Control (closed) and injured (open) cells
were incubated as in Figure 10. Linear
regressions of all data had r values of
>0.99 except acetate from injured cells
which had only 2 data points.

Figure 13. Production of CO_2 and H_2 by copper-injured
E. coli under anaerobic conditions. H_2
(circles) and CO_2 (squares) were measured
periodically as control (closed) and
injured (open) cells were incubated using
glucose as a carbon source on a rotary
shaker at $35^{\circ}C$.

cells may use fermentation pathways to compensate for loss of aerobic respiration.
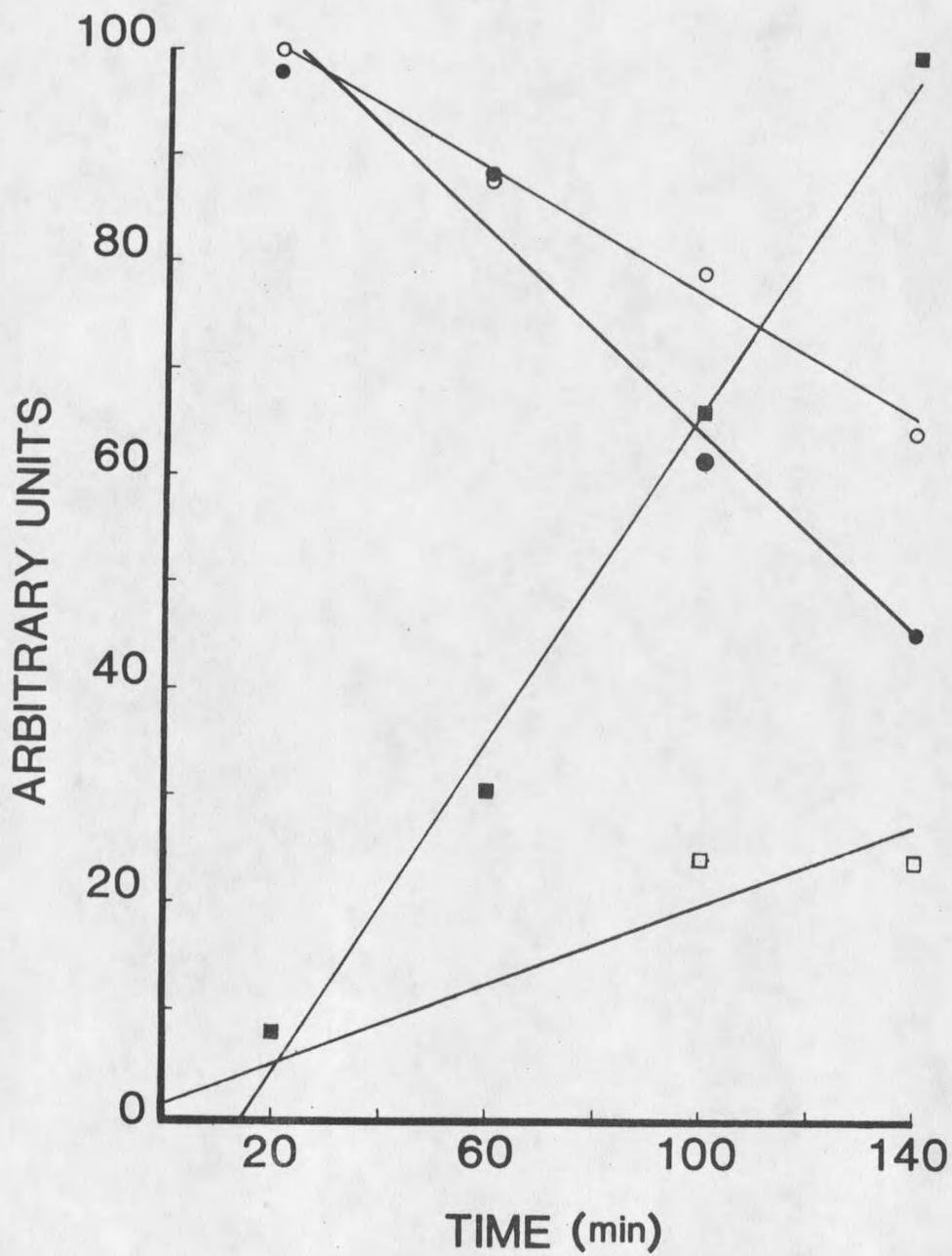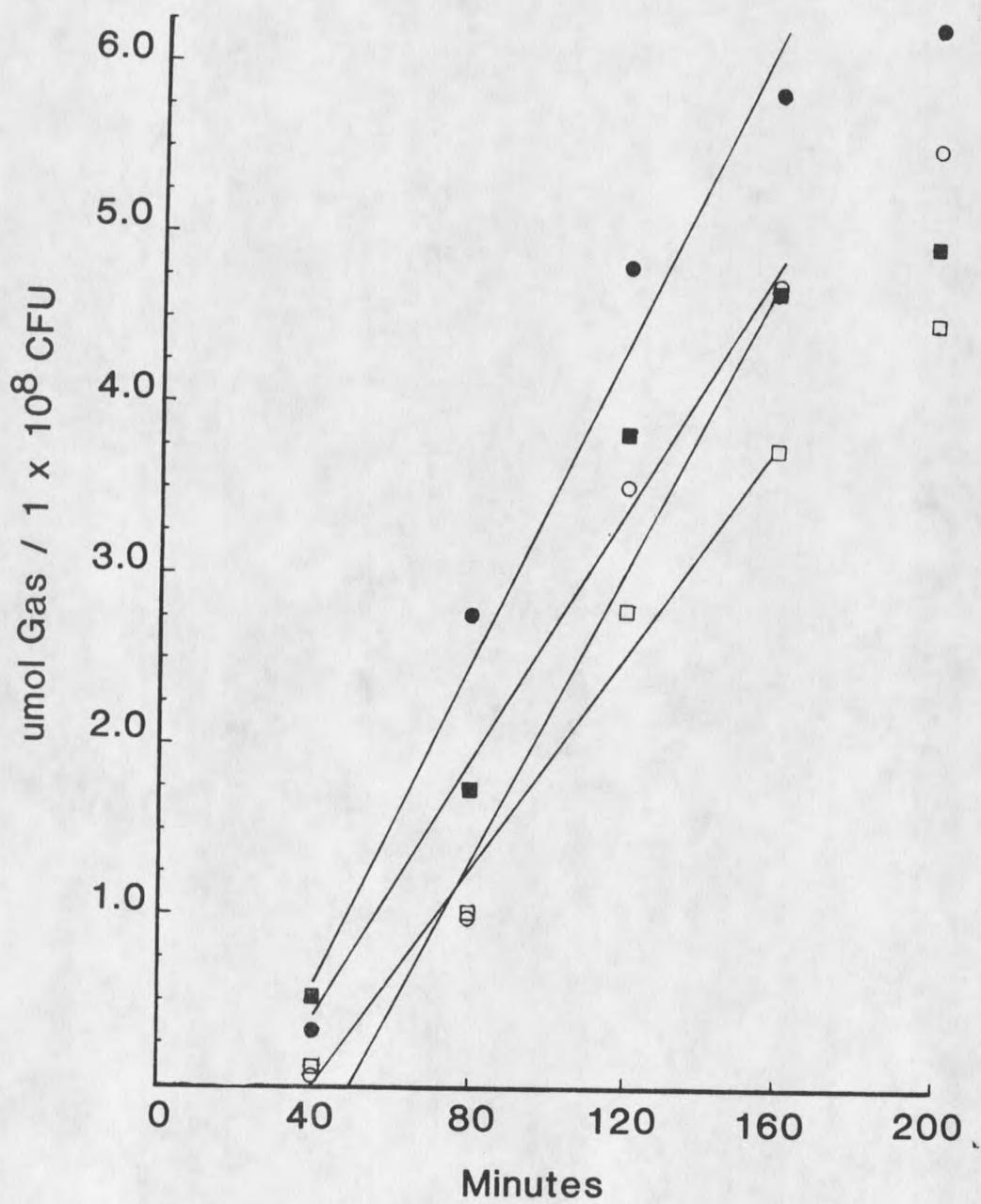
The NMR spectra of control and copper-injured cell suspensions under aerobic growth conditions showed major differences. Despite strongly aerobic conditions, E. coli did not completely metabolize carbon skeletons to CO_2 but produced some acid end products. Lactate and acetate were the two major peaks in both control (bottom, Figure 14) and injured cells (top, Figure 14). The control cells produced slightly more lactate and two large peaks representing acetate; these resonances corresponded to intracellular and an extracellular pools. The reason for the different peaks was that the number 2-carbon of acetate shifted its resonance downfield at the lower pH of the extracellular environment. The internal pH of the cell is approximately 7.6 and the medium had a pH of approximately 4.0 by the end of 2 hours. Figure 15 shows the spectra of the control cells (bottom) and supernatant fluid (top) of the control culture. Additionally, the resonances of acetate were measured in buffers at pH 7.6 and 4.0. The acetate in pH 7.6 buffer was put in a coaxial tube inside the NMR tube containing acetate in a pH 4.0 buffer. Again, separate peaks were distinguishable (data not shown).

The NMR spectra during aerobic metabolism produced one unexpected result, the accumulation of the amino acid glutamine. A large peak representing the number 4-carbon

53

Figure 14. NMR spectra of ^{13}C-labelled products of
glucose metabolism made under aerobic
conditions by copper-injured E. coli.
Each spectrum was accumulated for 140
minutes. The alpha and beta peaks of the
C-1 labelled glucose appear at 92.4 and
96.2 ppm. The natural abundance peaks of
glucose appeared between 60 and 80 ppm.
Other peaks are labelled as: G, gluta-
mine; Ai, acetate (intracellular); Ax,
acetate (external to the cell); and L,
lactate.

Figure 15. NMR spectra of excreted and intracellular
products of aerobic metabolism of glucose
by E. coli control cells. The E. coli was
grown with ^{13}C-labelled glucose under
aerobic conditions for 2 hours. The cells
were separated from the growth medium by
centrifugation. The supernatant fluid,
which was external to the cells, and the
cells, resuspended in carbonate buffer (pH
7.6), were scanned separately. Peaks are
labelled as in Figure 14.

of glutamine was present in the control cells and a clearly
evident but smaller peak in the injured cells (Figure 14).
This accumulation was external to the cell as seen in
Figure 15.

CO_2 was measured by a gas chromatographic method as in
the anaerobic experiment . Production of CO_2 under aerobic
conditions by control cells was 2.4 times the rate of the
injured cells (Figure 16). By contrast the difference was
far greater than the corresponding anaerobic experiment
(the control had only 1.2 times the rate of the injured
population as shown in Figure 13).

An experiment showing the utilization of glucose under
aerobic conditions was done over a longer period of time (4
hours) to completely use the added (20 mM) glucose (Figure
17). This experiment showed that the injured population
used glucose at 87% the rate of the control cells, and the
control population consumed 64% of the glucose after 2
hours.

There was a great difference in succinate utilization
between the two populations (Figure 18). The rate of
utilization seen in control cells increased steadily and
was linear near the end of the 3 hours. The injured cells
showed a more gradual increase or "lag" in the rate of
utilization. Rates were determined along portions of each
curve. The data points from 60 to 100 minutes in the
control population and from 140 to 180 minutes in the

58

Figure 16. Production of CO_2 by copper-injured <u>E. coli</u> under aerobic conditions. Control (o) and injured (o) cells were incubated with glucose on a rotary shaker at $35^{\circ}C$ and periodically fixed and analyzed for CO_2. Linear regressions of the data from 80 to 200 minutes compared rates. Control and injured cells had rates of 1.36 (r >0.99) and 0.56 (r >0.99) umol/min, respectively.

Figure 17.   Complete utilization of ^{13}C-labelled
glucose by copper-injured E. coli as
determined by NMR. Aerobic utilization of
^{13}C-labelled glucose (20 mM) by control
(●) and injured (○) cells was measured at
20 minute intervals as arbitrary units
(AU) (graph shows only 120 minutes). Con-
trol and injured cells had rates of -0.59
(r <-0.99) and -0.51 (r <-0.97) AU/min,
respectively.

Figure 18.   Succinate utilization by copper-injured $\underline{E.}$
$\underline{coli}$ as determined by NMR. ^{13}C-labelled
succinate (20mM) utilization by control
(●) and injured (o) cells measured as in
Figure 16.   Dotted lines represent linear
regressions of data.   Injured cells (140
to 180 min) compared to control cells (60
to 100 min) had the same rate of 0.50
AU/min.

injured population showed identical rates of utilization.

The rates of utilization for both populations were 0.502 AU

per minute and are shown as dotted lines in Figure 18.

The metabolic differences between control cells and

cells injured by copper were summarized in Tables 6 and 7.

The metabolic data of the cells injured by copper were

expressed as a percent of the control.

INT Reduction

E. coli populations were measured for their ability to

reduce INT to INT-formazan since that activity is

considered an indication of respiratory activity (4). INT

reduction was measured after incubation with a variety of

substrates (Table 8). In all experiments Triton X-100 was

used to increase solubility of the enzyme membrane complex

(34). Tricarboxcyclic acid (TCA) cycle intermediates,

succinate and malate, and TCA precursor, pyruvate, caused

greater reduction of INT by control cells than by the

injured cells.

In an another experiment, both populations were

preincubated with glucose to stimulate the TCA cycle and

subsequent flow of electrons down the electron transport

chain. The control population produced 2.44 times more

INT-formazan than the injured population and also produced

a greater concentration of INT-formazan than when the other

3 carbohydrates were used. However, when NADH was used as

Table 6. Anaerobic metabolism during recovery of <u>E. coli</u> injured by copper.

Time interval	Accumulation of End Product	Utilization of Substrate	
	CO_2	Glucose	Succinate
60 min	54[a]	91.5	92.3
120 min	53.5	79.0	59.4
180 min	46.3	98.1	n.d.[b]
Rate of accumulation or utilization	41.7[c]	87	21 44
Time interval (min)	(80-200)	(0-120)	(0-120) (120-180)

a. The quantity of end products accumulated (or substrate utilized by <u>E. coli</u> injured by copper expressed as a percent of the control.

b. no data

c. The difference in rate of accumulation of end product (or utilization of substrate) by <u>E. coli</u> injured by copper expressed as a percent of the control.

65

Table 7. Aerobic metabolism during recovery of E. coli injured by copper.

Time interval	Accumulation of End Product	Utilization of Substrate	
	CO_2	Glucose	Succinate
60 min	54[a]	91.5	92.3
120 min	53.5	79.0	59.4
180 min	46.3	98.1	n.d.[b]
Rate of accumulation or utilization	41.7[c]	87	21 44
Time interval (min)	(80-200)	(0-120)	(0-120) (120-180)

a. The quantity of end products accumulated (or substrate utilized by E. coli injured by copper expressed as a percent of the control.

b. no data

c. The difference in rate of accumulation of end product (or utilization of substrate) by E. coli injured by copper expressed as a percent of the control.

Table 8.   Reduction of INT by _E. coli_ injured by copper.

	Pyruvate[a]	Malate	Succinate	Glucose[c]	NADH
Control _E. coli_ population	14.1[b]	20.1	29.3	122.8	62.3
Injured _E. coli_ population	9.0	0.0	15.4	50.4	367.0

a.   All substrates 0.07 m (final concn) with 0.2% INT and 0.18% w/v Triton x-100.

b.   ug INT-formazan/ 1×10^8 cfu produced after 45 minutes incubation at 35°C.

c.   Cells were incubated for one hour with glucose as a substrate, then INT was added and incubated as above.

a substrate, the copper-injured cells reduced INT 5.89 times more than the control cells (Table 8). Interestingly, the amount of INT reduction in injured populations with NADH as a substrate surpassed any other combination of cell populations and substrates.

The addition of electron transport blocks such as sodium azide (0.2 nM, final concentrations) did not change the outcome of any of the INT experiments (data not shown). Pretreatment of the 2 E. coli populations with a mixture of toluene and acetone to increase cell membrane permeability also did not significantly change the amount of INT-formazan produced in either of the 2 populations (data not shown).

DISCUSSION

Part A - The Role of Metals in Causing
Injury of E. coli

Results of this study showed that E. coli (and, likely,
other coliform bacteria) was injured by copper in drinking
water. The levels of metals found in drinking water
samples in the Bozeman area were (table 1) not unusually
high and were similar to those reported in other localities
(23,35,49). In a study of selected trace metals in
household tap-water samples in Dallas, Texas, the copper
concentrations average 0.037 mg/liter (minimum, 0.004
mg/liter; maximum. 0.164 mg/liter) (49). A report of
metals in finished water supplies of the 100 largest cities
in the United States finds copper levels ranging from
<0.006 to 0.250 mg/liter (23). Another report of 380
finished drinking waters in the United States finds copper
present in 65.2% of the samples and average 0.043 mg/liter
(35).

Data from Figure 3 indicated that coliforms in many of
the nations potable water supplies would become injured
within 2 to 3 days because of exposure to metals naturally
found in drinking water. Sources of metals in distribution
waters include copper and brass material in pumps, pipes,
valves, and other appurtenances (49). Copper may also be

introduced as copper sulfate, used to control algal growth
in reservoirs (16), and as trace contaminants added with
flocculation material (49). In addition, copper may enter
surface water from geological sources. This probably
relates to previous investigations (8,46) suggesting that
differences in water chemistry in mountain streams in the
Bozeman area affect the level of injury and survival of
coliform bacteria in membrane diffusion chambers.
Therefore, this process may be especially important in some
areas (i.e., Montana) where copper is naturally abundant.

Statistical modeling of 30 drinking water samples in
the Bozeman area indicated the complex interaction of
ambient chemical and physical factors and injured E. coli
(Table 3). Temperature, pH, and alkalinity were implicated
as possible causative factors of injury and were properties
that affect the availability of copper (54). The statisti-
cal model was also used to predict the impact of copper
concentrations on coliform injury in drinking water. By
holding the properties of temperature, pH, and alkalinity
at the mean observed values in drinking water (Table 1),
the role of copper alone was evaluated as a causative
factor for injury. At the lowest copper concentration
observed in drinking water (0.007 mg/liter), the model
predicts no injury. Near the mean copper concentration
observed (0.158 mg/liter), the statistical model predicts a
10-fold (1 log) increase in injury. At the highest copper

level measured (0.54 mg/liter), the model predicts almost a 73-fold underestimation (on m-Endo medium) of actual coliform concentrations due to injury (Table 3).

Data presented in this report have several important implications in the areas of sample collection and coliform methodology relative to water analysis. Standard Methods for the Examination of Water and Wastewater (3) and the EPA Handbook for Evaluating Water Bacteriological Laboratories (26) both specify the adddition of ethylenediaminetetra acetic acid (EDTA) as a chelating agent to sample bottles used to collect water, wastewater, or industrial effluents thought to contain "high" concentrations of heavy metals. Similar instructions for collecting drinking water samples are given in those manuals (listed above) as well as the Handbook for Sampling and Sample Preservation of Water and Wastewater (74) and Microbiological Methods for Monitoring the Environment (10). These instructions specify the addition of EDTA (372 mg/liter) to bottles that are used to collect samples containing >0.01 mg/liter concentrations of heavy metals (10,74). Since the results of various studies (23,35,49), including this report, indicate that most drinking water contains >0.01 ug/liter concentration of copper alone, I recommended that EDTA be added routinely to all bottles used to collect water samples, especially if these samples will be stored or transported. I found that EDTA at a concentration of 25 ug/liter was sufficient to

prevent injury caused by copper (0.05 mg/liter) over a 5
day period (data not shown).

Part B - The Physiology of
Copper-Injured E. coli

Physiological aspects of this type of chlorine indepen-
dent aquatic injury were investigated to determine the
resulting metabolic lesion(s). E. coli injured in distri-
bution water showed a decreased use of oxygen. Additional-
ly, copper-injured E. coli showed this same defect. Of
particular interest was the oxygraph study (Figure 7) which
compared metabolically depleted and non-depleted E. coli
cells to control and copper-injured E. coli populations.
The control showed relatively little decrease in oxygen
utilization compared to both the depleted and non-depleted
cells indicating that copper, not the hypotonic environ-
ment, was the cause of significantly decreased oxygen
utilization.

Since coliform bacteria injured by copper recover after
a lag period (8), it was my intention to study the
metabolism during the recovery of injured cells. The data
suggested that the damage was caused by exposure to copper
but where the damage occurred was difficult to delineate.
If aerobic respiration were the only impairment one might
expect that under anaerobic conditions injured cells would
not show any metabolic differences. This was not the case.
In general the injured cells showed a slower rate of

anaerobic metabolism. I was unable to determine the precise location of the damage in the EMP pathway but the most likely possibility is the glucose uptake mechanism which consists of the phosphotransferase system (18) in the cell membrane. Copper attack on this complex may only partially decrease its efficiency. Therefore decreased uptake would result in the proportional decrease in end product accumulation.

The above model best fits the data because there was roughly a 10 to 15% decrease in the rates of end product accumulations. There were 2 exceptions to this decrease in rates (Figure 11, 12, and 13). The exceptions were acetate and ethanol production which were drastically reduced in the injured cells. These exceptions can be explained. If there were damage to the pyruvic decarboxylase, then pyruvate would not be decarboxylated and acetate, ethanol, CO_2, and H_2 would not accumulate. Since some of the products of the decarboxylation do accumulate (i.e., CO_2 and H_2) then the lack of accumulation of ethanol and acetate must occur for other reasons. I suggest that the carbon skeletons from acetyl CoA are used in the repair process. Therefore the 2 carbon skeletons from acetate and ethanol (which have acetyl CoA as a precursor) do not accumulate. The preceeding effects can be followed in Figure 19 which shows the carbon flow from glucose.
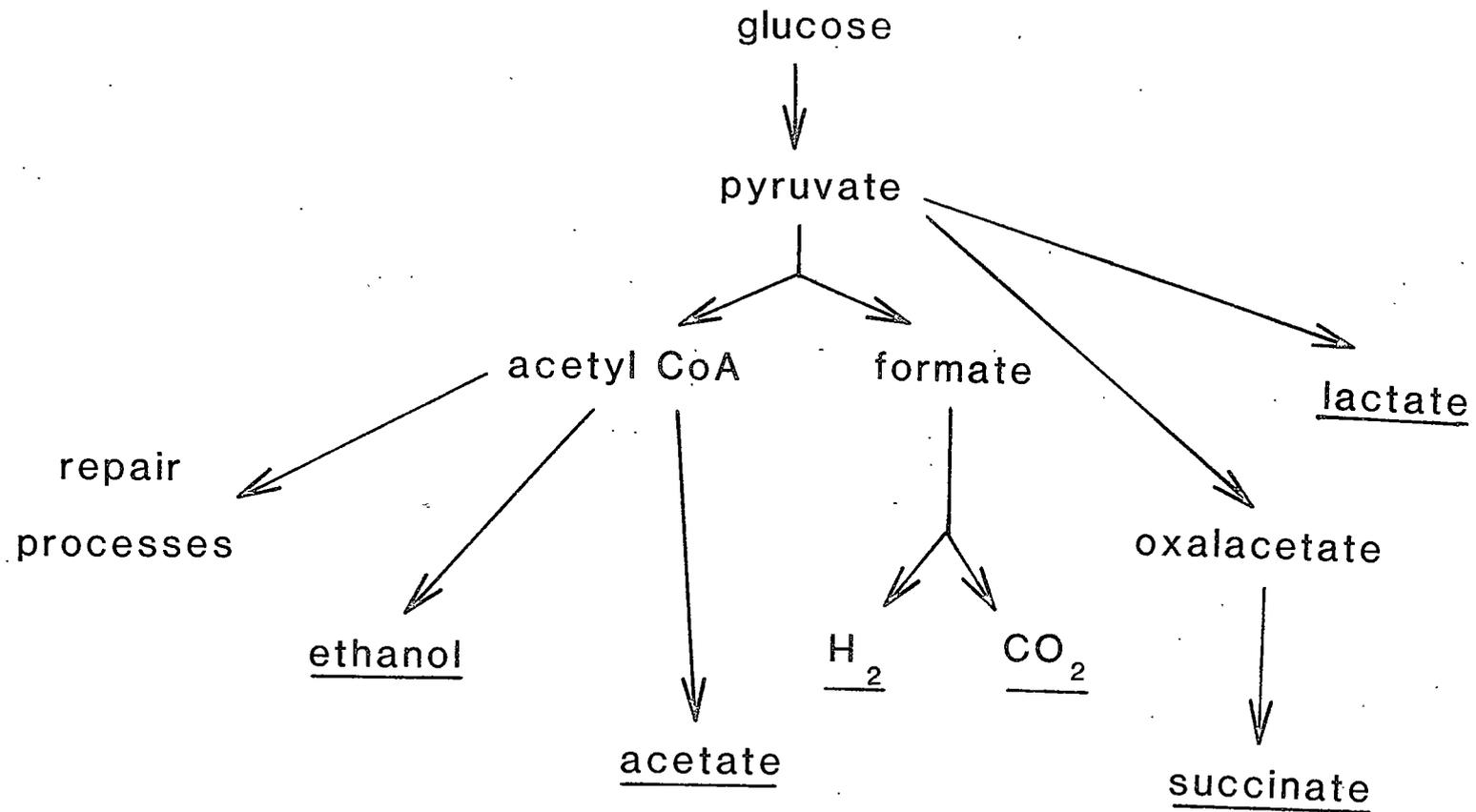
Figure 19.   The major end products of EMP pathway.   Underlined products
accumulated under anaerobic conditions (20) during the NMR
experiment.

The metabolic experiments done under aerobic conditions revealed similar trends in glucose utilization supporting a hypothesis that the glucose uptake mechanism has been damaged in injured cells. A greater difference in substrate utilization was seen when succinate was used as an energy source (Figure. 18). The utilization curves for succinate were similar to growth curves of control and injured cells. Injured cells typically showed a lag period of several hours before their growth rate parallels the growth rate of control cells. The slow utilization of succinate was probably related to damage to the TCA cycle. The damage to the TCA cycle is dicussed later.

The accumulation of glutamine in the control cell suspension showed that E. coli detoxified ammonia present in the medium. This process can be viewed as a storage mechanism since glutamine is an important source of nitrogen in anabolic cellular reactions (27,39). Copper-injured cells appeared to be using glutamine since it did not asccumulate. Heat-injured cells synthesize greater quantites of RNA and protein (67,68) during the repair process than the non-injured control cells. Glutamine most certainly plays a role (as a nitrogen source) in this process.

Since oxygen utilization was significantly depressed in cells injured by copper I suggest that the respiratory chain was damaged by copper. The respiratory chain is the

most likely target because it is membrane associated and therefore readily available for attack by copper. The cytochromes contain sulfhydral groups which are susceptable to binding by copper (47). This would disrupt electron transport. Indirect evidence supports the conclusion that the cytochromes were damaged by copper. INT is believed to be reduced at the level of coenzyme Q (51) (Figure 20). Apparently there was a copper-induced lesion at this site. It is possible that INT could be more easily reduced in injured cells since itis not competing for electrons with an intact electron transport chain. This is supported by the results which showed injured cells reduced greater amounts of INT than the controls when NADH was used as a substrate. If injured cells reduced greater quantites of INT simply because of increased permeability to NADH then treating control cells with an acetone-toluene mixture would cause increased reduction of INT. This was not the case. Greater reductions of INT by injured cells when NADH was used as as energy source was not an effect caused by greater permeability of the cell membrane. Therefore at least one lesion existed in the ETS of copper-injured _E. coli_ which prevented the flow of electrons to oxygen and allowed for a greater reduction of INT.

According to the above hypothesis, injured cells should reduce INT at a rate at least similar to control cells if TCA cycle intermediates are used as electron donors. This
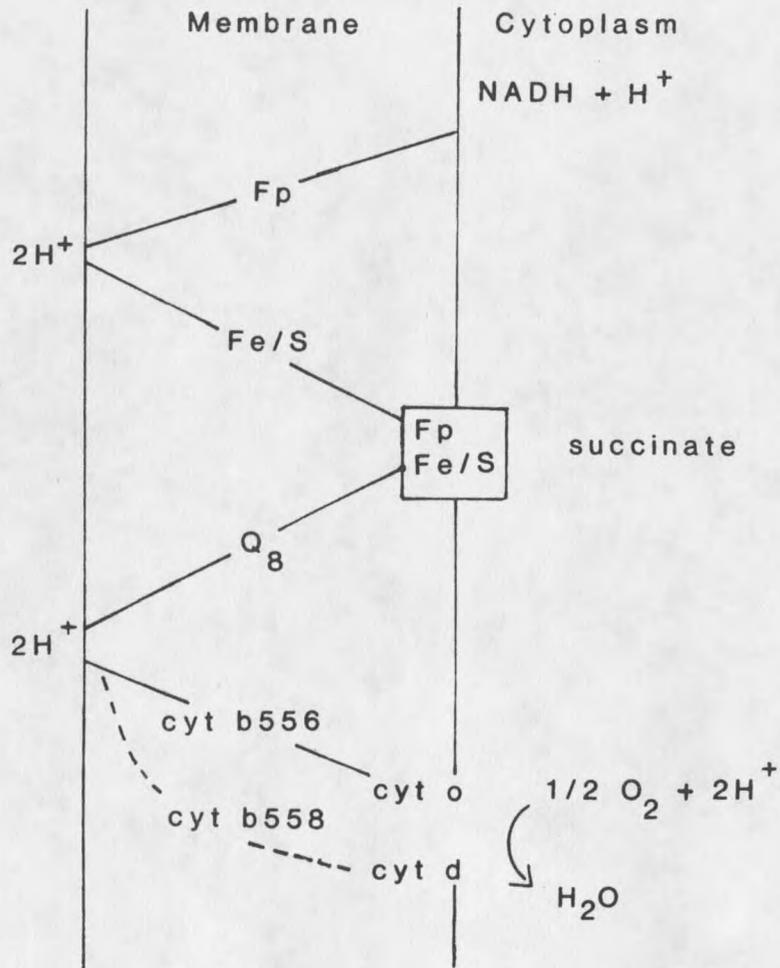
Figure 20. Electron transport system of E. coli. Abbreviations; Fp, Flavoprotein; Fe/S, iron-sulfur protein; Q, ubiquinone; cyt, cytochrome from a review by Haddock and Jones (29).

was not the case and apparently copper must affect the generation of NADH at the TCA cycle level. Using glucose or any of the TCA intermediates as substrates resulted in smaller amounts of INT reduction in the copper-injured cells. Since cells injured by copper did not reduce any INT when malate was used as a substrate it is possible that malate dehydrogenase was inactivated by copper. Additionally Daubner reported that E. coli exposed to water with a high mineral content has decreased ability to reduce INT when glucose is used as a substrate (17).

INT is used successfully as a means of determining numbers of respiring organisms (4). It is also used to determine potential respiration of the maximal capacity for oxygen utilization. INT is reduced to INT-formazan by one pair of electrons from the ETS (34) in the same way oxygen is reduced to water, thus it is used as an equivalent measurement of oxygen utilization. This method usually employs NADH as a substrate and is used instead of respirometry. Cells injured by copper showed a contradictory response of higher potential respiration than the control yet used less oxygen (as measured by respirometry). When using the INT method to measure respiration (i.e., quantity of INT reduced) the results must be interpreted with caution since INT reduction may not be equivalent to respirometry.

Summary

Microbiological, physical, and chemical properties of
drinking water including the concentrations of 3 heavy
metals cadmium, copper, and lead were measured and corre-
lated to injury. These studies revealed that the levels of
copper present in chlorine-free distribution water corre-
lated to injury of coliform populations. Statistical
analyses of all factors were used to develop a model to
predict coliform injury. The model predicted almost 90%
injury using a copper concentration near the mean observed
value (0.158 mg/liter) listed on Table 1. Laboratory
studies exposing E. coli to copper in concentrations and
under conditions similar to distribution waters supported
the field data by showing conclusively that copper caused
injury of E. coli.

E. coli injured by drinking water or by copper showed
decreased oxygen utilization. Oxygraph measurements of
cells injured in drinking water showed 73 to 83% decrease
in oxygen utilization when cells were injured 70 to 99.5%.
Copper-injured cells had less than 25% the rate of oxygen
utilization of the control cells. Respirometry experiments
measured rates over a longer period of time and showed
similar trends.

[13]Carbon-NMR and gas chromatography were used to
identify differences in metabolism between healthy and

injured populations of E. coli. The rate of glucose
utilization by injured cells under anaerobic conditions was
64% of the healthy cells. The rates of accumulation of
fermentation end products were decreased in the injured
cells. Lactate and ethanol were 88 and 50% of the control,
respectively. [13]Carbon-NMR studies of oxygenated cultures
revealed differences in the accumulation of acetate and
lactate. A large pool of glutamine accumulated in healthy
but not injured cells. A comparison of metabolic end
products suggested that injured cells had considerable
differences in carbon flow compared with healthy cells.
The reduced accumulations of ethanol and acetate by injured
cells suggest that acetyl CoA was used as a precursor in
the repair of injured cells.

Additional studies revealed injured cells had a
decreased ability to reduce INT during metabolism of a
variety of carbohydrate substrates. However, when NADH was
used as a substrate injured cells reduced greater quanti-
ties of INT than the healthy cells. Copper-injured E. coli
appeared to have impaired respiration as indicated by
decreased INT reduction and other respirometric indices.

LITERATURE CITED

LITERATURE CITED

1. Albright, L.J., J.W. Wentwort, and E.M. Wilson. 1972. Technique for measuring metallic salt effects upon the indigenous heterotrophic microflora of a natural water. Water Res. 6:1589-1596.

2. Albright, L.J. and E.M. Wilson. 1974. Sub-lethal effects of several metallic salts-organic compounds combinations upon the heterotroophic microflora of a natural water. Water Res. 8:101-105.

3. American Public Health Association. 1980. Standard methods for the examination of water and waste water. 15th ed. American Public Health Association, Washington, D.C.

4. Amy, P.S., C. Pauling, and R.Y. Morita. 1983. Starvation-survival process of a marine vibrio. 45:1041-1048.

5. Babich, H. and G. Stotzky. 1980. Environmental factors that influence the toxicity of heavy metal and gaseous pollutants to microorganisms.CRC Crit. Rev. Microbiol. 8:99-145.

6. Bartlett, L. ,W. Rabe and W.H. Funk. 1974. Effects of copper, zinc, a cadium on Selenastrum capricornutum. Water Research 9:179-185.

7. Berg, J.D., A. Matin, and P.V. Roberts. 1982. Effect of antecedent growth conditions on sensitivity of Escherichia coli to chlorine dioxide. Appl. Environ. Microbiol. 44:814-819.

8. Bissonnette, G.K., J.J. Jezeski, G.A McFeters, and D.G. Stuart. 1975. influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. 29:186-194.

9. Bissonnette, G.K., J.J. Jezeski, G.A. McFeters, and D.G. Stuart. 1977. Evaluation of recovery methods to detect coliforms in water. Appl. Environ. Microbiol. 33:590-595.

10. Bordner, R. and J. Winter (eds). 1978. Microbiological methods for monitoring the environment. U.S. Environmental Protection Agency, Cincinnati, OH.

11. Braswell, J.R.and A.w. Hoadley. 1974. Recovery of Escherichia coli from chlorinated secondary sewage. Appl. Microbiol. 28:328-329.

12. Busta, F.F. 1976. Practical implications of injured microorganisms in food. J. Milk Food Technol. 39:138-145.

13. Camper, A.K. and G.A. McFeters. 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. 37:633-641.

14. Carson, L.A., M.S. Favero, W.W. Bond, and N.J. Petersen. 1972. Factors affecting comparative resistance of naturally occurring and subcultured Pseudomonas aeruginosa to disinfectants. Appl. Environ. Microbiol. 23:863-869.

15. Clark, C.W. and Z.J. Ordal. 1969. Thermal injury and recovery of Salmonella typhimurium and its effect on enumeration procedures. Appl. Microbiol. 18:332-336.

16. Committee Report. 1983. Deterioration of water quality in large distribution reservoirs (open reservoirs). JAWWA 75:313-318.

17. Daubner, I. 1975. Changes in the properties of E. coli under the influence of water environments. Verh. Internat. Verein. Limnol. 19:2650-2657.

18. Dills, S.S., A. Apperson, M.R. Schimdt, and M.H Saier, Jr. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. 49:385-414.

19. Dockins, W.S. and G.A. McFeters. 1978. Fecal coliform elevated-temperature test: a physical basis. Appl. Environ. Microbiol. 36:341-348.

20. Doelle, H.W. 1969. Bacterial metabolism. Academic press. New York and London.

21. Domek, M.J., M.W. LeChevallier, and G.A. McFeters. 1984. Evidence for the role of heavy metals in the injury process of coliforms in drinking water. Appl. Environ. Microbiol. 48:289-293.

22. Double, M.L. and G.K. Bissonnette. 1980. Enumeration of coliforms from streams containing acid mine water. J. Water Poll. Contr. 52:1947-1952.

23. Durfor, C.N. and E.Becker. 1964. Public water supplies of the 100 largest cities in the United States, 1962. U.S. Geological Survey Water Supply Paper 1812. U.S. Government Printing Office, Washington D.C.

24. Egan, A.F. 1978. Enumeration of stressed cells of Escherichia coli. Can. J. Microbiol. 25:116-118.

25. Fujiioka, R.S., H.H. Hashimoto, E.B. Siwak, and R.H.F. Young. 1981. Effect of sunlight on survival of indicator bacteria in seawater. Appl. Environ. Microbiol. 41:690-696.

26. Geldriech, E.E. 1975. Handbook for evaluating water bacteriological laboratories. 2nd ed. U.S. Environmental Protection Agency, Cincinnati, OH.

27. Gottschalk, G. 1979. Bacterial metabolism, Springer-Verlag. New York.

28. Hach Chemical Company. 1978. Wastewater analysis handbook. Hach Chemical Company. Loveland, CO

29. Haddock, B.A. and C.W. Jones. 1977. Bacterial respiration. Bacteriol. Rev. 41:47-99.

30. Heukeklekian, H. and I. Gellman. 1955. Studies of biochemical oxidation by direct methods. IV. Effect of toxic metals ions on oxidation. Sewage and Industrial Wastes. 27:70-84.

31. Hitchner, B.J., F.H. Grau, and A.F. Egan. 1975. Significance of cell envelope in thermal death of Escherichia coli. Proc. Austal. Biochem. Soc. 8:73.

32. Iandolo, J.J. and Z.J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol. 91:134-142.

33. Jenke, D.R. and R. Woodriff. 1982. Applications of the Woodriff constant temperature graphite furnace atomizer to atomic spectroscopy. American Laboratory 14:14-26.

34. Jones, J.G. and B.M. Simon. 1979. The measurement of electron transport system activity in freshwater benthic and planktonic samples. J. Appl. Bacteriol. 46:305-315.

35. Kopp, J.F. 1969. The occurrence of trace metals in water. pp. 59-73. In D.D. Hemphill, ed. Proceedings of the Third Annual Conference of Trace Substances in Environmental Health. Univ. of Missouri, Columbia, MO.

36. Lamb, A. and E.L. Tollefson. 1973. Toxic effects of cupric, chromate and chromic ions on biological oxidation. Water Res. 7:599-613.

37. LeChevallier, M.W., S.C. Cameron, and G.A. McFeters. 1982. New medium for the improved recovery of coliform bacteria from drinking water. Appl. Microbiol. 45:484-492.

38. Lee, A.C. and J.M. Goepfert. 1975. Influence of selected solutes on thermallly induced death and injury of Salmonella typhimurium. J. Milk Food Technol. 38:195-200.

39. Lehninger, A. 1982. Biochemistry. Worth Publishers, Inc. New York.

40. Lin, S. 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollut. Control Fed. 45:498-506.

41. Mackey, B.M., C.M. Derrick, and J.A. Thomas. 1980. The recovery of sublethally injured Escherichia coli from frozen meat. J. Appl. Bacteriol. 48:315-324.

42. MacLoed, R.A., S.C. Kuo and R. Gelinas. 1967. Metabolic injury to bacteria. II. Metabolic injury induced by distilled H_2O or Cu^{++} in plating diluent. J. Bacteriol. 93:961- 969.

43. Malaney, G.W., W.D. Sheets, and R.Quillin. 1959. Toxic effects of metallic ions on sewage microorganisms. Sewage and Industrial Wastes. 31:1309- 1315.

44. Massalski, A., V.M. Laube, and D.J. Kushner. 1981. Effects of cadmium and copper on the ultrastructure of Ankistrodesmus braunii and Anabaena 7120. Microbiol. Ecol. 7:1183-193.

45. McFeters, G.A., S.C. Cameron, and M.W. LeChevallier. 1982. Influence of diluents, media and membrane filters on detection of injured waterborne coliform bacteria. Appl. Environ. Microbiol. 43:97-103.

46. McFeters, G.A. and D.G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24:805-811.

47. Moran, J.W., T.L. Smith, and L.D. Witter. 1976. Effect of potential water pollutants and enzyme inhibitors on an automated rapid test for _Escherichia coli_. Appl. Environ. Microbiol. 32:645-646.

48. Moss, C.W. and M.L. Speck. 1966. Release of biologically active peptides from _Escherichia coli_ at subzero temperatures. J. Bacteriol. 91:1105-1111.

49. The National Research Council. 1977. Drinking water and health. vol. 1. National Academy of Sciences, Washington, D.C.

50. Neter, J. and W. Wasserman. 1974. Applied linear statistical models. Richard D. Irwin, Inc, Homewood, IL.

51. Owens, T.G. and F.D. King. 1975. The measurement of respiratory electron-transport-system activity in marine zooplankton. Marine Biol. 30:27-36.

52. Packard, T.T. and M.L. Healy. 1968. Electrochemical standardization of the dehydrogenase assay used in the estimation of respiration rates. J. Mar. Res. 26:66-47.

53. Packard, T.T., M.L. Healy, and F.A. Richards. 1971. Vertical distribution of the activity of the respiratory electron transport system in marine plankton. Limnol. Oceanogr. 16:60-70.

54. Pagenkopf, G.K. 1978. Introduction to water chemistry. Marcell and Dekker. Inc. New York.

55. Pierson, M.D. and Z.J. Ordal. 1971. The transport of methyl alpha-D-glucopyranoside by thermally stressed _Salmonella typhimurium_. Biochem. Biophys. Res. Comm. 43:378-383.

56. Pierson, M.D., R.I. Tomlins, and Z.J. Ordal. 1971. Biosynthesis during recovery of heat-injured _Salmonella typhimurium_. J. Bacteriol. 105:1234-1236.

57. Ray, B. and M.L. Speck. 1973. Enumeration of _Escherichia coli_ in frozen samples after recovery from injury. Appl. Microbiol. 25:499-503.

58. Ray, B. and M.L. Speck. 1973. Freeze-injury in bacteria. C.R.C. Critical Rev., Clinical Laboratory Sciences. pp. 161-213.

59. Ridgeway, H.F. and B.H. Olson. 1982. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Appl. Environ. Microbiol. 44:972-987.

60. Sheusner, D.L., F.F. Busta, and M.L. Speck. 1971. Injury of bacteria by sanitizers. Appl. Microbiol. 21:41-45.

61. Sheusner, D.L., F.F. Busta, and M.L. Speck. 1971. Inhibition of injured Escherichia coli by several selective agents. Appl. Microbiol. 21:46-49.

62. Singleton, F.L. and R.K. Gutherie. 1977. Aquatic bacterial populations and heavy metals. I. Composition of aquatic bacteria in the presence of copper and mercury salts. Water Res. 11:639-642.

63. Skoog, D.A. and D.M. West. 1974. Analytical chemistry an introduction. Holt, Rinehart and Winston, Inc. New York.

64. Skoog, D.A. and D.M. West. 1980. Principles of Instrumental analysis. Saunders College. Phildelphia.

65. Tabor, P.S. and R.A. Nihof. 1982. Improved method for determination of respiring individual microorganisms in natural waters. Appl. Environ. Microbiol. 43:1249-1255.

66. Tayne, T.A. 1983. The fate of fermentation products and glycollate in hot spring microbial mats with emphasis on the role played by Chloroflexus aurantiacus. M.S. Thesis. Montana State Univ., Bozeman, MT

67. Tomlins, R.I. and Z.J. Ordal. 1971. Requirements of Salmonella typhimurium for recovery from thermal injury. J. Bacteriol. 105:512-518.

68. Tomlins, R.I. and Z.J. Ordal. 1971.Precursor ribosomal ribonucleic acid and ribosome accumulation in vivo during the recovery of Salmonella typhimurium from thermal injury. J. Bacteriol. 107:134-142.

69. Tomlins, R.I., G.L. Vaaler and Z.J. Ordal. 1972. Lipid biosynthesis during recovery of Salmonella typhimurium from thermal injury. Can. J. Microbiol. 18:1015-1021.

70. Tyler, G. 1974. Heavy metal pollution and soil enzymatic activity. Plant Soil. 41:303-311.

71. Tyler, G. 1975. Heavy metal pollution and mineralization of nitrogen in forest soils. Nature (London) 255:701-702.

72. Ugurbil, K., T.R. Brown, J.A. Den Hollander, P. Glynn, and R.G. Shulman. 1978. High-resolution ^{13}C nuclear magnetic resonance studies of glucose metabolism in Escherichia coli. Proc. Natl. Acad. Sci. USA. 75:3742-3746.

73. Ugurbil, K., P. Glynn, and R.G. Shulman. 1982. Phosphorus-31 nuclear magnetic resonance studies of bioenergetics in wild-type and adenosinetriphosphatase (1-) Escherichia coli cells. Biochemistry. 21:1068-1075.

74. United States Environmental Protection Agency. 1982. Handbook for sampling and sample preservation of water and wastewater. U.S.E.P.A., Cincinnati, OH EPA-60014-82-029.

75. Venkobachar, C., L. Iyengar, and A.V.S.P. Rao. 1976. Mechanism of disinfection: effect of chlorine on cell membrane functions. Water Res. 11:727-729.

76. Zaske, S.K., W.S. Dockins, J.E. Schillinger, and G.A. McFeters. 1980. New Methods to assess bacterial injury in water. Appl. Environ. Microbiol. 39:656-658.

77. Zaske, S.K., W.S. Dockins, and G.A. McFeters. 1980. Cell envelope damage in Escherichia coli caused by short-term stress in water. Appl. Environ. Microbiol. 40:386-390.

APPENDICES

APPENDIX A

Based on Henry's law and the Bunsen adsorption coeffecient the following equation was used to estimate the amount of oxygen dissolved in water (54) under condition of the experiment.

$$\frac{(a)(b)(c/d)(f)}{(e)} = g/liter\ (O_2 \cdot aq)$$

$$\frac{(0.02608)(0.21)(680/760)(31.9988)}{(22.414)} = g/liter\ (O_2 \cdot aq)$$

$$6.9958 \times 10^{-3} = (O_2 \cdot aq)$$

(a) or 0.02608   = Bunsen adsorption coeffient for oxygen at $30^\circ C$ and 760 mm Hg

(b) or 0.21   = percent of oxygen in atmosphere

(c) or 680   = approximate amount of mm Hg at 5000 feet elevation

(d) or 760   = mm Hg at 1.0 atmosphere

(e) or 22.414   = gas constant

(f) or 31.9988   = molecular weight of oxygen

g/liter (oxygen·aq) = grams of oxygen dissolved in 1 liter

Oxygen was measured as follows:

$$(rate_v - rate_x)\ (1 \times 10^8/CFU)(ug/ml)$$

$$= ug\ O_2\ ml^{-1}min^{-1}(1 \times 10^8)CFU^{-1}$$

$rate_v$ = rate determined with viable cell suspension measured as a slope on a oxygraph chart.

$rate_x$ = rate determined with a dead cell suspension as a measurement instrument drift.

APPENDIX B

Listed below are the additional parameters for the 13Carbon-NMR experiments described in the materials and methods section.

spectrometer frequency - 62.830 Hz

sweep width - 10,000 Hz

total data - 8K

relaxation delay - 0.6 sec

aquisition time - 0.41

repetion time - 0.01 sec

broad band proton decoupler power - 2 watt

pulse angle - 67 degrees

line broadening - 1.0 Hz

number of scans - 1200