To the Graduate Council:

I am submitting herewith a thesis written by David A. Armstrong entitled " Resistance to Copper Toxicity in a Spontaneously Generated Mutant of Salmonella typhimurium." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Kenneth J. Monty, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

RESISTANCE TO COPPER TOXICITY IN A SPONTANEOUSLY GENERATED MUTANT OF *SALMONELLA TYPHIMURIUM*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

David A. Armstrong

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I would also like to thank the other members of the Biochemistry Department for their constant support throughout this project.

For their advice and instruction in the electron microscopy work I am indebted to Dr. John Dunlap and Mr. Richard Williams.

ABSTRACT

The objective of this investigation was to define an experimental system using analytical electron microscopy as a complementary tool to traditional biochemical methods. Specifically, the system studied was the phenomenon of resistance to copper toxicity in a spontaneously generated mutant of Salmonella typhimurium strain LT2.

It was observed that this mutant, henceforth called CF, showed normal growth rates in minimal medium containing 0.01 M copper citrate, whereas the wildtype cells showed signs of metal toxicity at 10^{-5} M copper citrate levels. It was also noted that these mutants had the innate ability to actively accumulate the copper, which led to the possibility that these cells could be examined using energy dispersive X-ray spectroscopy, to determine if and where this accumulation of copper may be localized.

The accumulation of copper was highest at a period approximately 10 hours into resting phase, which will be refered to as PRP(copper peak of resting phase). Upon cell fractionization, a significant amount of copper was seen in both the cell extract and the cell membrane fraction. At PRP, copper levels from cell extracts of CF grown in copper showed up to a 60-fold increase over cell extracts from CF mutants grown in minimal medium that had not been supplemented with copper. It was found that CF cells harvested at PRP contain at least two copper binding factors, one of high molecular weight and

one of low molecular weight. The low molecular weight copper binder is the result of an inducible response to growth in copper and has an apparent molecular weight smaller than that of cytochrome c.

Also when comparing accumulation of copper in exponential or log phase growth of the wildtype vs. CF : the wildtype strain accumulates relatively high copper levels until mid-log phase and then growth ceases ; CF shows low levels of copper accumulation throughout log phase. This may indicate a possible copper exclusion phenomenon in the CF mutant which aids its adjustment to high copper levels in the growth medium.

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CHAPTER 1

INTRODUCTION

The accumulation of metals in eukaryotic systems has been observed as a common occurance, ranging from aluminum deposition in Alzheimer's disease (1) to calcium deposition in vascular tissues (2). Other occurances of metal deposition, specifically copper deposition include: Wilson's disease, an autosomal recessive inherited disease with copper accumulation in human liver and brain tissue (3); toxicity due to copper accumulation in the gill tissue of the clam species Protothaca staminea (4); the sequestering of copper in an evolutionarily lower scale eukaryote, Saccharomyces cerevisiae (5), and copper accumulation in a cultured cell system, a Morris rat hepatoma cell line (6). Now it is not surprising that eukaryotes have evolved systems for somehow sequestering copper, as this atom has a two-way effect on eukarotic cells: on one hand the aerobic reactions with copper and ascorbate produce superoxide anion, which subsequently forms toxic oxygen species thought to be lethal to cells (7,8); and on the other hand, copper is an essential cofactor in a variety of metabolic reactions (e.g. cytochrome c oxidase (9) and lysyl oxidase activities (10)). And it is interesting to note at this point that,in situations in which copper exhibits toxic effects, organisms have learned to cope through the inducible synthesis of a

low molecular weight metallothionein-like copper binding protein in numerous cases (4,5,11,12,13,14).

The number of reported occurances of copper accumulation in prokaryotic systems is quite low as compared to their eukaryotic counterparts. Among the copper containing proteins in prokaryotes are the azurins or the blue copper proteins, which have molecular weights in the 14,000 dalton range. They have been found in species of Pseudomonas and Bordatella, but not in E. coli, Salmonella or Bacilli. These small blue copper proteins are generally thought to function as electron transferases(15). The plastocyanins, a second group of copper-containing proteins, found in all photosynthetic organisms including blue-green algae, have a molecular weight of approximately 11,000 daltons and are thought to function also in redox roles(15). Bacteriocupreins, the Cu-Zn superoxide dismutases which catalyze the decomposition of superoxide radical and thus protect aerobes from the toxicity of reduced oxygen species(16,17,18,19,20) have been purified and characterized from two bacterial species, Photobacterium leiognathi(21), a luminescent marine symbiont, and Caulobacter crescentus(22), a pond bacterium.

In another case, Higham describes the synthesis of metallothionein-related proteins by a metal-resistant strain of Pseudomonas putida(23). These three proteins, isolated from cell extracts of P. putida, complex cadmium, zinc and copper. It is interesting to note that the three low molecular weight metal-binding proteins are synthesized at different times in the typical

bacterial growth curve: CdBP 1 (Mol. Wt. 6700) produced throughout exponential growth; CdBP 2 (Mol.Wt. 6900) during late exponential growth; and CdBP 3 (Mol.Wt. 3600) during stationary or resting phase. Also examined in Higham's study was the cysteine content in these proteins, comparing its correlation to the cysteine content of eukaryotic metallothioneins, and drawing on its implications in metal homeostasis and detoxification.

There have been several reports concerning Escherichia coli, which falls into the same taxonomic subdivision of enteric bacteria as does Salmonella, that these cells can be induced to form metal binding proteins when grown in a metal containing culture medium, or that they already contain a protein which has metal binding capacity. Mitra(24) describes at least one class of inducible proteins, apparent molecular weights of 55,000 to 65,000 daltons, that appear when E. coli are exposed to 3uM cadmium. Harmon(24) reports at least three heat stable proteins from E. coli crude extracts, molecular weights 33 Kd, 47 Kd, and 60 Kd, which bind calcium in buffers containing micromolar levels of calcium. Harmon suggests the possibility of an internal calcium regulatory role or calcium-dependent regulation of other processes by these proteins.

A number of authors have taken a more encompassing view of the metal accumulation phenomenon and made an attempt to examine the distribution within the prokaryotic cell. Pettersson et.al.(26) have examined cryosections of the cyanobacterium Anabaena cylindrica, using energy-dispersive X-ray microanalysis, to try and correlate

morphological data to their previous study(27), which indicated aluminum uptake by the cells. And more recently MaCaskie et al.(28) used X-ray microanalysis to identify a cadmium accumulation at the cell surface in a strain of Citrobacter.

Experimental Design

The question of tolerance and growth in high concentrations of copper by Salmonella typhimurium will be addressed on several levels. The first basic premise to be examined asks whether CF survives by :

a. excluding copper - so that its toxic effects are not seen within the cell;

b. accumulating copper - in some form that makes it more tolerable to cell growth.

If exclusion, can we determine the specific mechanism involved? If accumulation, in what form is the copper accumulated and can a localization of copper be identified? The first basic question will be addressed by monitoring copper levels of growing CF mutant cells using atomic absorption spectroscopy.

CHAPTER 2

MATERIALS and METHODS

Bacteria

All bacteria used in this study were subcultured from Salmonella typhimurium strain LT2. The mutant of interest, CF, was the result of a spontaneous mutation observed in LT2 wildtype cells. This mutant strain was generated in 10^{-4} M copper supplemented medium. These cells were susequently carried forward into medium in which the metal concentrations were increased up to 0.01 M copper citrate. The development of metal resistant cell lines was done by Dr. Kenneth J. Monty.

Media

Difco nutrient broth, nutrient agar, and bacto-agar were used for isolation and characterization of the bacterial cells. Growth media described by Vogel and Bonner(29) supplemented with 0.2% glucose was used in growth experiments. A typical 200 milliliter volume of copper supplemented growth medium was assembled as follows:

156 mls of glass distilled water
4.0 mls of concentrated E medium(29)
20 mls of 0.1 M Sodium Citrate/0.1 M Copper Chloride solution
20 mls of 2.0% glucose

Glucose and Copper Chloride supplied by Baker Chemical Company and Sodium Citrate supplied by Mallinckrodt Chemical Works. The 0.01 M

copper citrate chelating system used, was assembled to allow a continuous free copper concentration of 10^{-6} M in the growth medium.

Materials

Sodium Phosphate Monobasic, Sodium Phosphate Dibasic, Sodium Hydroxide, and Sodium Chloride were obtained from Mallinckrodt Chemical Works. Sodium Tartrate and Cupric Sulfate were purchased from Baker Chemical Company. Acetyl Cholinesterase from electric eel, bovine Immunoglobulin G, bovine Serum Albumin, cytochrome c from horse heart, Dextran, FAD and Percoll were obtained from Sigma Chemical Co.. Pharmacia Fine Chemicals Inc. supplied the Sephadex G-50 and G-200. And the Spectrapor membrane tubing was purchased from Spectrum Medical Industries Inc.. Centricon microconcentrators were supplied by Amicon and the Dye Reagent concentrate for protein assays was obtained from Bio-Rad.

Cell Growth Rates

Cell growth was monitored by measuring absorbance of 5 ml cultures in 15mm x 125mm culture tubes at 420 nanometers in a Bausch and Lomb Spectronic 20. The cultures were aerated by rotation at 60 rpm on an angled rotator wheel.

Protein Assays

The first protein determinations were done using the micro-biuret method from Itzhaki and Gill(30). Adopted later was the

Bradford dye-binding assay(31). Absorbance readings were obtained with a Beckman model DUR Recording Quartz Spectrophotometer.

Atomic Absorption Spectroscopy

An air-acetylene flame was used for copper determinations by flame atomization on an Instrumentation Laboratory aa/ae Spectrophotometer 551. Other operating conditions included: hollow cathode tube as a light source; lamp current of 5 milliamps; bandpass of 1 nanometer; absorbance monitoring at a wavelength of 324.7 nanometers.

Sonication and Separating Cell Fractions

Cells harvested at the appropriate time were sonicated for 60 seconds total with a Branson Instruments Inc., model LS 75 Probe Tip Sonifier. Three milliliters of cells suspended in 0.05 M sodium phosphate buffer (pH 7.0), were sonicated for 30 seconds, cooled in an ice-water bath, and sonicated an additional 30 seconds. All volumes of whole cell sonicate were recombined. That volume was spun down to separate a soluble fraction and a pellet as outlined by Borum(32) and Figure 1.* The contents of the soluble fraction will also be refered to as the cell extract. The fraction refered to as the pellet contained all fractionated membranes and any whole cells which survived sonication.

* All tables and figures may be found in the appendix.

Equilibrium Dialysis

Dialysis experiments were performed using specially constructed dialysis sample holders. Rigid-plastic tubes(25mm x 9mm), with open ends, were fit at both ends with a spectrapor membrane. The membrane, cut from dialysis tubing, was secured by overlaying the cylindrical plastic tubes with a slightly larger diameter ring of elastic rubber tubing. These 0.8 ml volume tubes were placed on a petri dish cover that had several 1 mm diameter holes bored into it. Under the petri dish cover was a stirring bar which allowed adequate stirring of the sodium phosphate dialysis buffer while maintaining the integrity of the dialysis tubing.

Percoll Density Gradient Centrifugation

Separation of the Blue cells from the White cells(refer to page 12) was accomplished using density gradient centrifugation. Percoll having an osmolarity of 300 mOs/kg H_2O was made by adding nine parts Percoll plus one part 0.9% sodium chloride. Twenty milliliters of cell suspension was carefully layered atop 20 mls of Percoll in plastic centrifuge tubes(30mm x 100mm), and spun in a Sorvall RC2-B centrifuge at 3000xg for 15 minutes at 4° C. Cells then can be separated from Percoll medium by washing with physiological saline(five volumes to one volume of cell suspension), several times.

Centricon 10 Microconcentrator Filtration

A volume of CF crude cell extract containing approximately two milligrams of protein as measured by the Bradford dye-binding assay, was applied to a Sephadex G-50 column(15cm x38.5cm), pre-equilibrated with 1×10^{-5} M copper chloride. The copper elution profile was monitored using atomic absorption spectroscopy. Eluted fractions showing the highest copper levels were recombined. A total of three milliliters of this eluate was placed in a Centricon 10 microconcentrator and spun for 15 minutes at 3500 r.p.m. in a Damon IEC HN-S centrifuge. A filtrate volume of 1.6 millilters was applied to the Sephadex G-50 column and fractions were collected, again monitoring the copper elution profile.

Electron Microscopy

All electron microscope work was done on either a Hitachi H-600 or a Hitachi H-800 Electron Microscope. Both are equipped with an EG&G Ortec Si(Li) X-ray Detector. And both function in the conventional transmission electron microscopy (CTEM) mode or for X-ray analysis, in the scanning transmission electron microscopy(STEM) mode. Generally the accelerating voltage used was 100 Kv. All energy dispersive spectroscopy was done on conventionally prepared thin sections (which will be described below) cut on either a Sorvall Porter-Blum MT1 Ultramicrotome or a Reichert OmU3 Ultramicrotome. Both glass and diamond knives were used in the

sectioning procedures. Copper grids(400 mesh) and aluminum grids(100 mesh) were supplied by Ted Pella Inc.. The copper grids were used exclusively for imaging while the aluminum grids were used in the X-ray detection.

The fixation procedure for Salmonella typhimurium modified from that of Hayat(33), was as follows:

1. Cells pelleted and fixed in 3% Glutaraldehyde in 0.1 M Phosphate Buffer pH 7 for 1 hour;
2. Cells repelleted; Glutaraldehyde decanted off, cells washed in 0.05 M Phosphate Buffer x 2;
3. Sample post-fixed in 2% Osmium Tetroxide for 1 hour;
4. Washed in Buffer;
5. Sample carried through a graded ethanol series as a dehydration step/ final dehydration in propylene oxide
6. Fixed cells allowed to equilibrate in a propylene oxide/ Epon (1:1) mixture for 12 hours;
7. Sample transfered directly to Epon 812 embedding media which was polmerized at 60^{o}C for 48 hours;

When individual samples were to be post-stained the procedure involved a 30 minute staining with aqueous saturated uranyl acetate followed by a 30 second staining with aqueous lead citrate pH 12.5 (33).

CHAPTER 3

RESULTS

Growth Rates : LT2 vs. CF

Growth of wildtype cells(strain LT2) ceases after three hours in 0.01 M copper citrate medium. By contrast, LT2 cells in minimal medium without copper, continue to to grow logarithmically. Growth rates of the mutant appear no different in the presence or absence of 0.01 M copper citrate,(Figure 2). Population limit, which corresponds to an optical density reading of 1.30 at 420 nanometers and a cell population of greater than 10^9 cells per milliliter, was achieved between 6-9 hours after the initial subculture transfer for LT2 and CF grown in the absence of copper. Population limit of CF grown in copper-supplemented medium was also reached after 6-9 hours of total growth; whereas LT2 cells grown in copper-supplemented growth medium showed their highest population density after three hours of growth, corresponding to an optical density reading of 0.15 and a cell population of approximately 10^8 cells per milliliter.

Copper Accumulation

The accumulation of copper was measured in both LT2 and CF by monitoring the micrograms of copper per milligram of total protein in each sample,(Figure 3). LT2 cells accumulated relatively high copper levels through several hours of exponential phase growth; this high

copper accumulation is followed by cessation of growth and presumably cell death(Figure 2A). On the other hand, CF cells appear to accumulate very little copper until late exponential phase, in fact the first three hours of growth show less than 0.05 micrograms of copper per milligram of protein and only after 4 hours of growth can significant levels of copper be noted(Figure 3B).

Furthermore, CF cells continue to accumulate copper from the medium through 30 hours of resting phase, with the peak copper accumulation seen at approximately 10 hours into resting phase,(Figure 3 C). The copper accumulation range seen in these experiments is illustrated in Table 1. Protein concentrations, copper concentrations, copper per milligram of protein and the percent of copper associated with separated cell fractions were determined in these experiments and the results are shown in Table 2.

An interesting phenomenon occurred approximately 15% of the time when cultures of 200 milliliters or greater volume were grown. What was seen was an apparent heterogenous copper accumulation among the mutant cells. After cells had been washed, there were clearly two layers of cells distinctly different in color. A blue layer of cells, apparently of higher density, pelleted first and then the more characteristically-seen white layer of CF cells pelleted atop the blue layer. The two differently colored cells, which can be separated by an <u>in situ</u> generated Percoll gradient, are shown in Figure 4.

Subsequent copper and protein analyses were done on the Blue layer and it was found that the amount of copper associated with the Blue cells was approximately 50 x greater than the copper concentrations in the White layer(Table 3).

Energy Dispersive X-ray Microanalysis

X-ray data indicating the presence of copper, confirming data from atomic absorption spectroscopy, was collected from CF Blue cells. The X-ray spectra are presented in Figure 5. No copper could be detected in the White cells using the same approach.

Column Chromatography

Gel filtration using a Sephadex G-200column(1.5cm x 42cm) was performed on CF cell extract. The first series of experiments, in which the column had been pre-equilibrated with 0.05 M sodium phosphate buffer(pH 7.0), showed only low levels of copper present in the eluted fractions(Figure 6A). The column was then re-equilibrated with sodium phosphate buffer containing 1×10^{-5} M copper chloride in an attempt to minimize copper diffusion away from any binder present. Results are shown in Figure 6B and 7A and B. Figure 6B illustrates the elution profile of CF cell extract taken from cells harvested 10 hours into resting phase grown in minimal media without added copper. There is copper binding seen in the high molecular weight fractions, but nothing significant above background in the low molecular weight fractions. Figure 7 illustrates the elution profiles from CF cell

extract taken from cells grown in the presence of minimal medium supplemented with 0.01 M copper citrate. Figure 7A is from cells harvested at mid-log phase growth amd Figure 7B is from cells harvested 10 hours into resting phase. As is the case with cells grown in minimal medium without added copper, in Figure 7 copper levels above background are noted in the high molecular weight or void volume region of the elution profile. But unlike cells grown in the absence of copper citrate we appear to have induced a low molecular weight binder, visible in elution profiles of Figure 7.

In order to obtain more information about this low molecular weight binder, we switched to a Sephadex G-50 column(15cm x 38.5cm) and again pre-equilibrated with 1×10^{-5} M copper chloride containing sodium phosphate buffer. The characteristic elution profiles are shown in Figure 8. The low molecular weight copper binder is centered at fraction 24, eluting just prior to Flavine-Adenine Dinucleotide. From the elution volumes(V_e) of known molecular weight standards a selectivity curve was constructed(34). Based on the linear relationship between K_{av} and log molecular weight of compounds, the apparent molecular weight of our copper binder is 1862 daltons(Figure 9).

Centricon 10 Filtration

When CF cell extract from cells that had been grown in copper citrate supplemented medium, was chromatographed on a Sephadex G-50 column fractions 24-26 showed copper levels above background. These fractions were combined and filtered through a Centricon 10 microconcentrator. The filtrate was then rechromatographed on the G-50 column monitoring the copper elution profile(Figure 10). Fractions 21-26 of filtrate eluent showed copper levels above background suggesting that the binder had passed through the Centricon 10 filter.

Equilibrium Dialysis

Dialysis of CF PRP crude cell extract was first done with dialysis membrane that had a molecular weight cut-off of 6000-8000 daltons. After 10 hours of dialysis against 0.05 M sodium phosphate buffer (pH 7.0), approximately 30% of the original copper was lost. Subsequent 10 hour dialysis of the same sample against the buffer containing 10^{-5} M copper chloride showed the ability of the sample to reacquire some of the lost copper. A second equilibrium dialysis was done from the same harvest of CF PRP cells. The dialysis membrane in this experiment had a molecular weight cut-off of 3500 daltons. After a 10 hour dialysis the sample lost only 19% of its original copper concentration. And after subsequent dialysis against a copper chloride containing buffer the sample showed 90% of its initial copper(Table 4).

CHAPTER 4

DISCUSSION

There are several points which have been established at this stage in examining the phenomenon of resistance to copper toxicity in our prokaryotic experimental system. First, we have established a mutant Salmonella typhimurium cell line that exhibits normal growth rates in minimal medium containing 0.01 M copper citrate. Secondly, these cells actively accumulate copper during resting phase of a typical bacterial growth curve. Thirdly, following whole cell sonication, the accumulated copper has been detected in both cell extract and fractionated membranes.

Previous works in which metal accumulation in prokaryotes has been noted(23,25) have focused their attention on crude cell extracts. This lab has also chosen to concentrate its efforts on cell extract derived from cells as illustrated in Figure 1.

Several of the characteristics of the elution profiles generated from cell extracts using column chromatography are notable at this point. First, the most notable copper peaks above background occur at the void volume and at a point in the elution corresponding to a molecular weight of approximately 1900 daltons(Figure 7). There also appears to be a small amount of copper noticable above background between these two prominent peaks. Another consistent feature of the

elution profiles is a large copper depletion or trough shaped region following the elution volume of the Sephadex columns.

The copper peak at low molecular weight elution we have attributed to a copper binding factor and at this point suggest the possibility that it plays some role in CF cells' resistance to copper toxicity. The copper peak associated with the high molecular weight fractions may also be attributed to some type of copper binding factor, but to this point that issue has not been addressed. Another possibility for the high and medium molecular weight fractions may be non-specific copper binding with proteins in the form of electrostatic interactions. The appearance of a trough in the elution profile provides evidence for a ligand binding situation as described by Hummel and Dreyer (35).

One experiment supporting the presence of a low molecular weight copper binder is the Centricon 10 filtration experiment. The presence of copper levels above background centered at fraction 23 indicates that our copper binder passed through the Centricon filter; thus has a molecular weight under 10,000 daltons.

Another experiment that involved an equilibrium dialysis of CF cell extract(outlined in previous chapters and Table 4), also supports the conclusion of a low molecular weight binding factor. When extract was dialyzed against sodium phosphate buffer alone with dialysis tubing that had a molecular weight cut-off of 6000-8000 daltons, the sample lost a portion of its copper. When that sample was re-dialyzed against the buffer containing 10^{-5} M copper chloride,

the cell extract had the tendency to regain some of the lost copper. However, when dialysis tubing of molecular weight cut-off 3500 daltons was used, the percent of copper lost after 10 hours was decreased and the reacquisition of copper markedly increased. The implication of these data is that with the higher molecular weight cut-off tubing, the binder was diffusing out of the dialysis cylinder and being lost with changing of the buffer.

With the smaller diameter tubing pores, there is a higher probability for the binder to remain within the dialysis cylinder and therefore a higher tendency to reacquire any free copper available on subsequent dialysis.

At this point we have no evidence to conclude to which class of macromolecules this copper binder may belong. Whether it is protein, nucleic acid or something else remains to be determined.

The initial focus of this study was to examine morphological data obtainable using electron microscopy and see how well it matched with the biochemical data. Ideally, we can use X-ray/elemental mapping to identify any localization of the copper and energy dispersive X-ray spectroscopy to quantitate the amount of copper present. Several problems which could not be fully anticipated, reduced our quantitative aspirations to qualitative.

The first complication arose in preparing the sample for X-ray analysis. The prefered method of sample preparation is by cryoprocedures(33), in which a sample is quick frozen at liquid nitrogen temperatures and then sectioned at low temperatures in a

cryoultramicrotome. The freezing of the sample is of utmost importance. It is done to prevent ice crystal formation and to minimize artifacts as a result of ice crystals. Unfortunately, this method of sample preparation was unavailable and samples were prepared by conventional fixation and embedding procedures as described in the methods chapter. It is well documented that many elements are leeched from samples during fixation, dehydration, and embedding(33). Thus the interpretation of the X-ray data from cells prepared this way is difficult.

CF white mutant cells(Figure 4), embedded in plastic, elicited no detectable copper X-ray signal. Whether leeching of copper during sample preparation was responsible or that the initial copper concentration in the cells was too low to be detectable by our system, remains an unanswered question at this point.

On the other hand, CF Blue cells did provide us with a small amount of information from the X-ray analysis. From thin sections of the blue cells we were able to detect a copper X-ray signal(Figure 5). Unfortunately the X-ray data as presented in Figure 5 is only consistent when the region analyzed held the morphological integrity normally associated with plastic - embedded cells(Figure 11). Some regions of the sections clearly showed broken cells and cell fragments. The X-ray information collected in these areas could not distinguish a comparmentalized copper concentration; implying that copper had left the cell and was dispersed within the plastic embedding resin.

Although the X-ray data presents limited success, it does agree with, and qualitatively confirm the atomic absorption spectroscopy information outlined in Table 3. The atomic absorption data says that blue cells contain about a 50 x higher copper concentration than the more characteristically seen white cells. And the X-ray data says, at that concentration of copper, enough remains after fixing and embedding to elicit a detectable copper signal.

The possibility still exists that when the prefered method of sample preparation becomes available (i.e. cryoprocedures), we will be able to more closely align the morphological data with the biochemical data.

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APPENDIX

LIST OF FIGURES

FIGURE

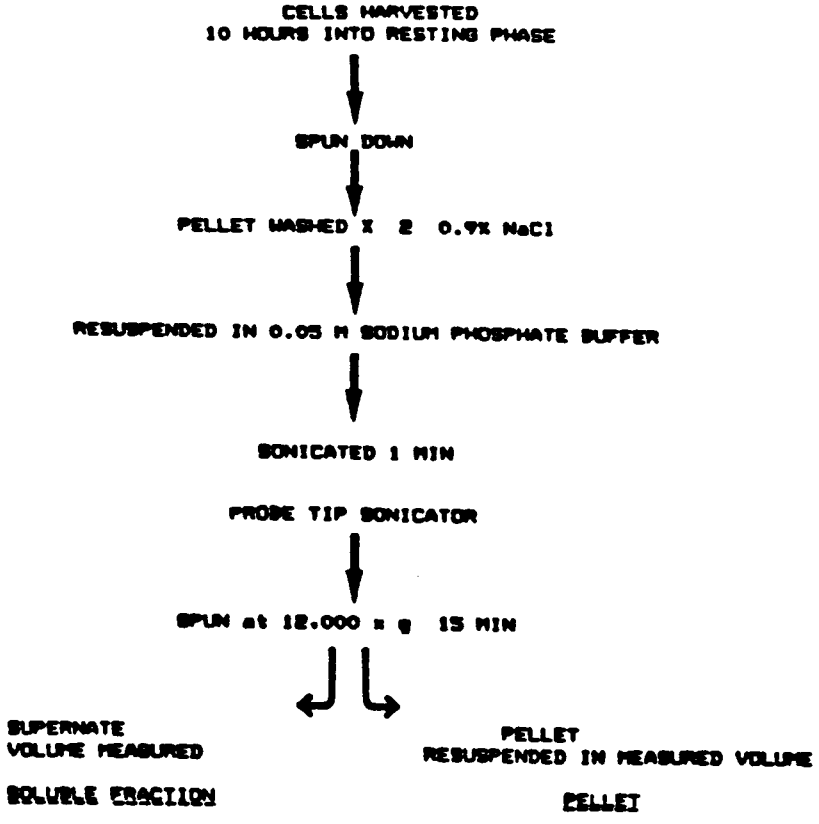


Figure 1. Protocol for Separating Cell Fractions

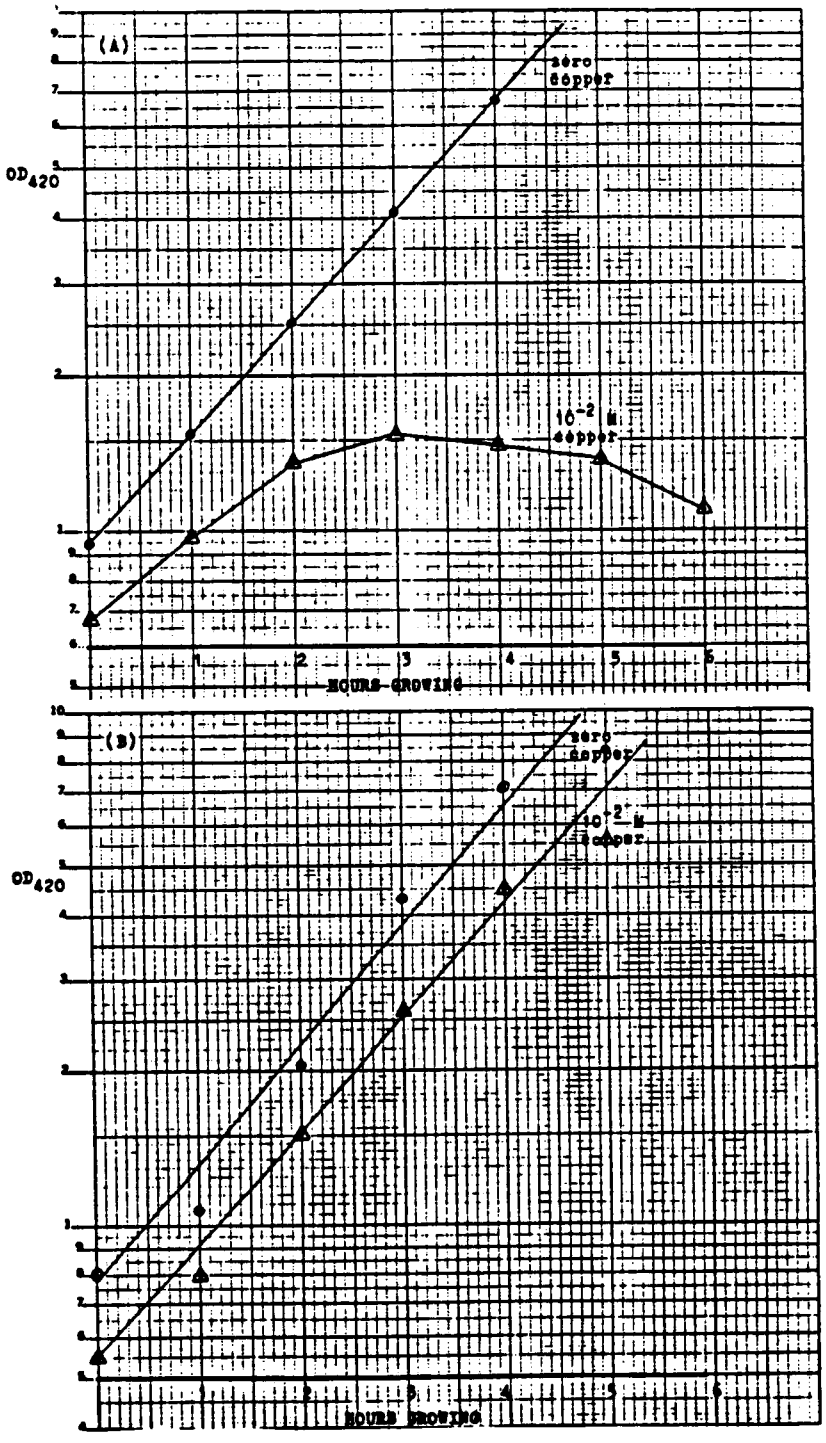


Figure 2. Log Phase Growth Rates of Wildtype and CF Cells

(A) : LT2 (Δ) with and (o) without copper
(B) : CF (Δ) with and (o) without copper as indicated

Figure 3. Monitoring of Copper Levels Throughout Cell Growth.

Measurements were done on washed whole cells prepared from individual 5.0 ml culture tubes containing minimal medium with or without copper as indicated. The copper levels presented are micrograms of copper per milligram of total cellular protein and are refered to as the Cu/protein ratioes. In this series of experiments the micro-biuret protein determination method was used for protein assay.
Absence of open or closed bars in 3A and B indicates a copper concentration of less than 0.05 micrograms per milligram of protein.

A : Cu/protein ratios associated with LT2 cells in log phase growth
B : Cu/protein ratios of CF mutant cells in log phase growth
C : Cu/protein ratios of CF cells monitored 30 hours into resting phase.

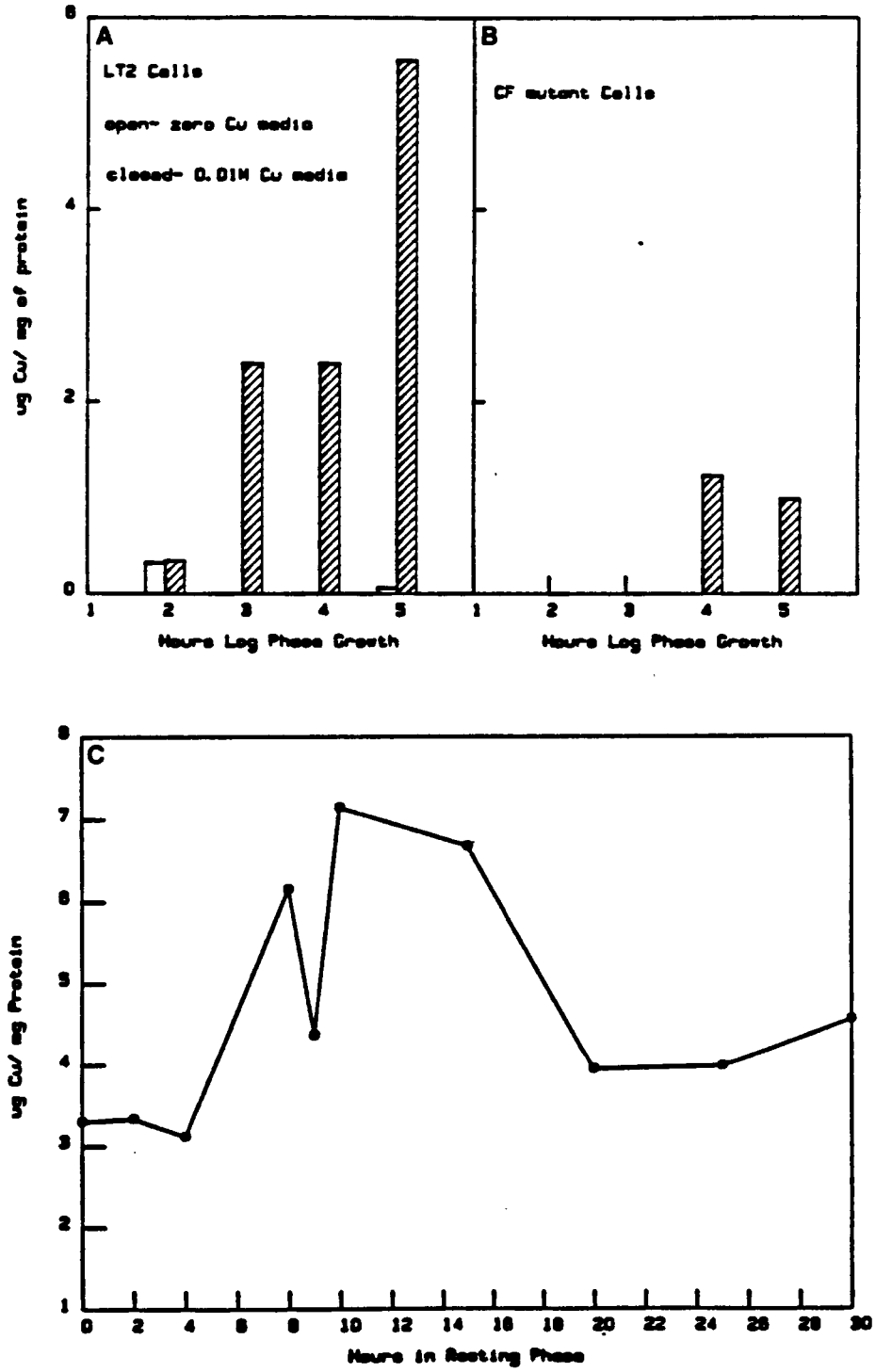

A
LT2 Cells
open- zero Cu media
closed- 0.01M Cu media
B
CF mutant Cells
ug Cu/ mg of protein
Hours Log Phase Growth
Hours Log Phase Growth
C
ug Cu/ mg Protein
Hours in Resting Phase

Figure 3.

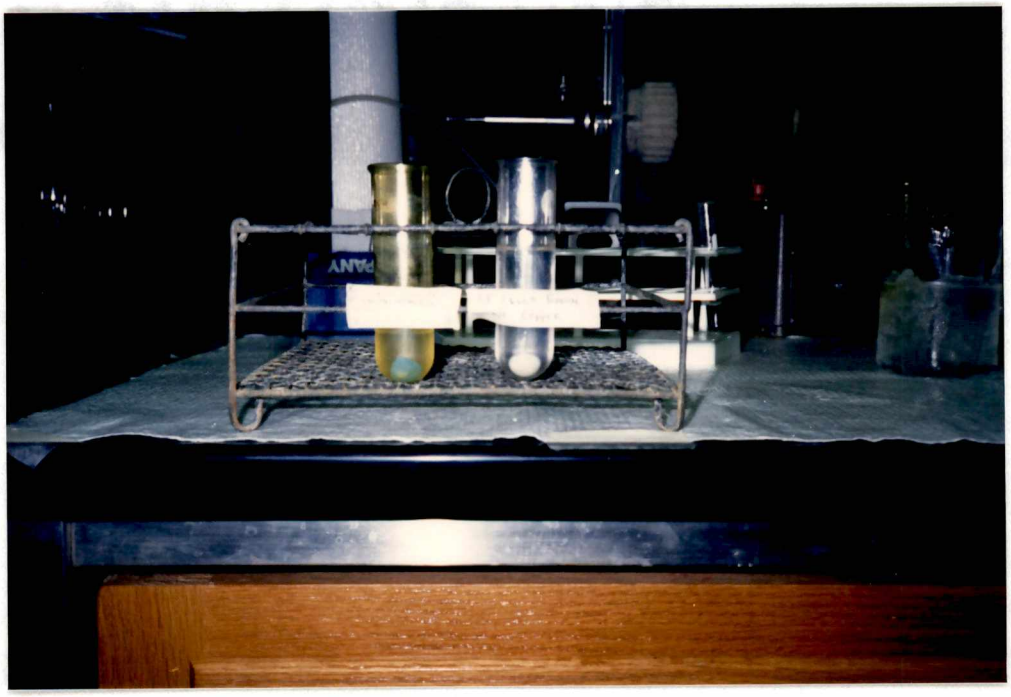


Figure 4. Mutant Cell Comparison.

Photograph comparing color of an apparent heterogeneous response in CF mutant cells grown in the presence of 0.01 M copper citrate.

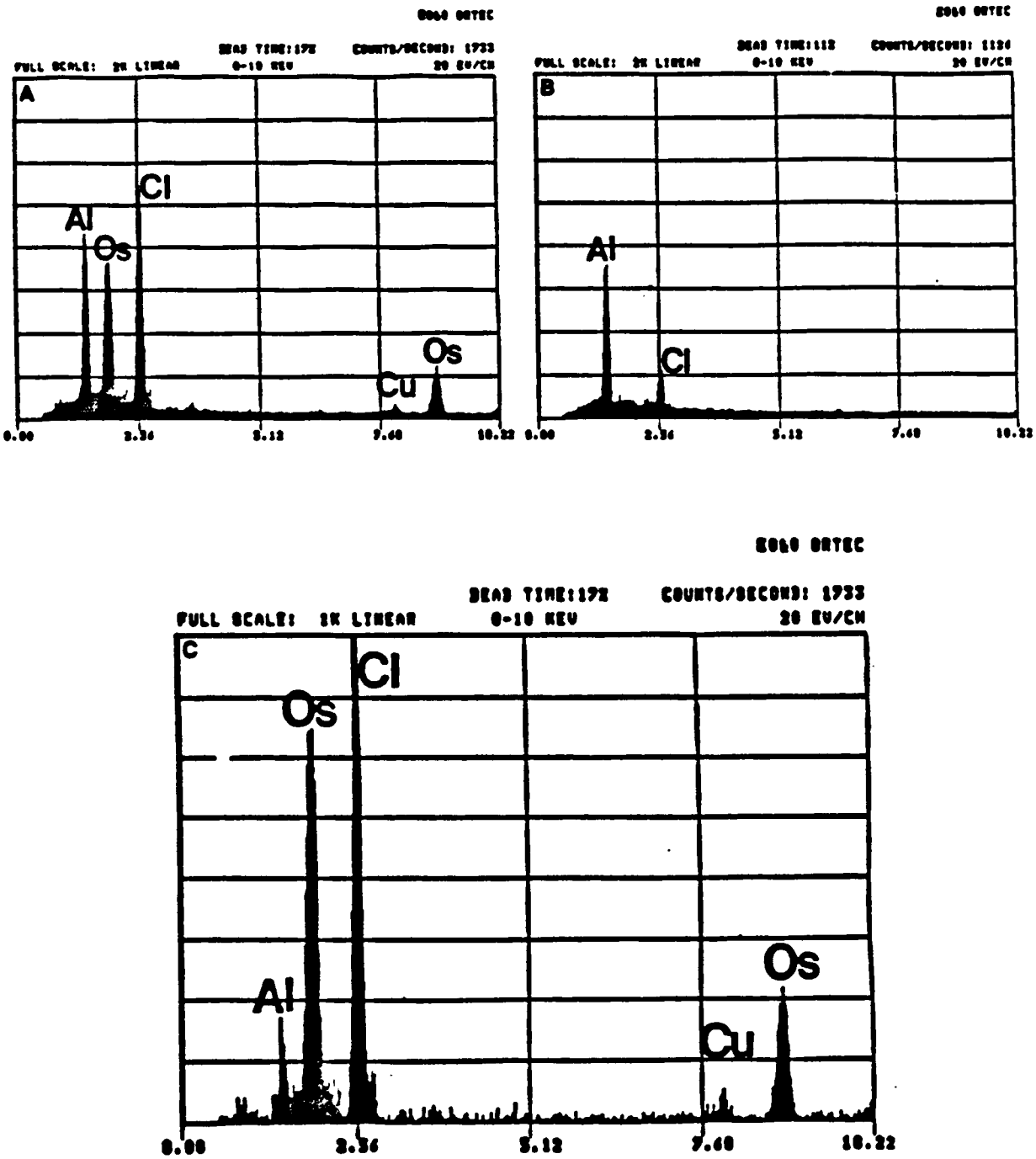
Left: CF Blue cells*
Right: CF White cells

* after rough separation by centrifugation

Figure 5. X-ray Microanalysis of CF Blue cells.

Samples were irradiated with the electron beam focused into a fine probe less than 2000 angstroms in diameter. X-rays were collected for 100 seconds. The samples had been post-fixed with Osmium tetroxide (Os); the grids used were Aluminium (Al); and Chlorine (Cl) is a component of the embedding resin. Characteristic copper peak seen at 8.049 keV.

A : Probe centered inside cell
B : Probe on resin alone (background)
C : X-ray spectrum of sample after background subtraction


EG&G ORTEC
DEAD TIME:17%
COUNTS/SECOND: 1933
FULL SCALE: 1K LINEAR
0-10 KEV
20 EV/CH
A
Cl
Al
Os
Os
Cu
0.00
2.56
5.12
7.68
10.22
EG&G ORTEC
DEAD TIME:11%
COUNTS/SECOND: 1134
FULL SCALE: 1K LINEAR
0-10 KEV
20 EV/CH
B
Al
Cl
0.00
2.56
5.12
7.68
10.22

EG&G ORTEC
DEAD TIME:17%
COUNTS/SECOND: 1933
FULL SCALE: 1K LINEAR
0-10 KEV
20 EV/CH
C
Cl
Os
Al
Os
Cu
0.00
2.56
5.12
7.68
10.22

Figure 5.

Figure 6. Sephadex G-200 Elution Profile of Cell Extract.

Following elution, each fraction was measured for relative protein content by Abs 280 nm (circles-solid lines) and micrograms of copper (plus signs-dotted lines). Cell extract was obtained from cells harvested 10 hours into resting phase.

A : Elution profile of cell extract passed through a column pre-equilibrated with 0.05 M sodium phosphate buffer(pH 7.0).

B : Elution profile of cell extract passed through column pre-equilibrated with sodium phosphate buffer which contained 1 x 10^{-5} M copper chloride. This allowed reading of a background level of copper and anything above background was noted.

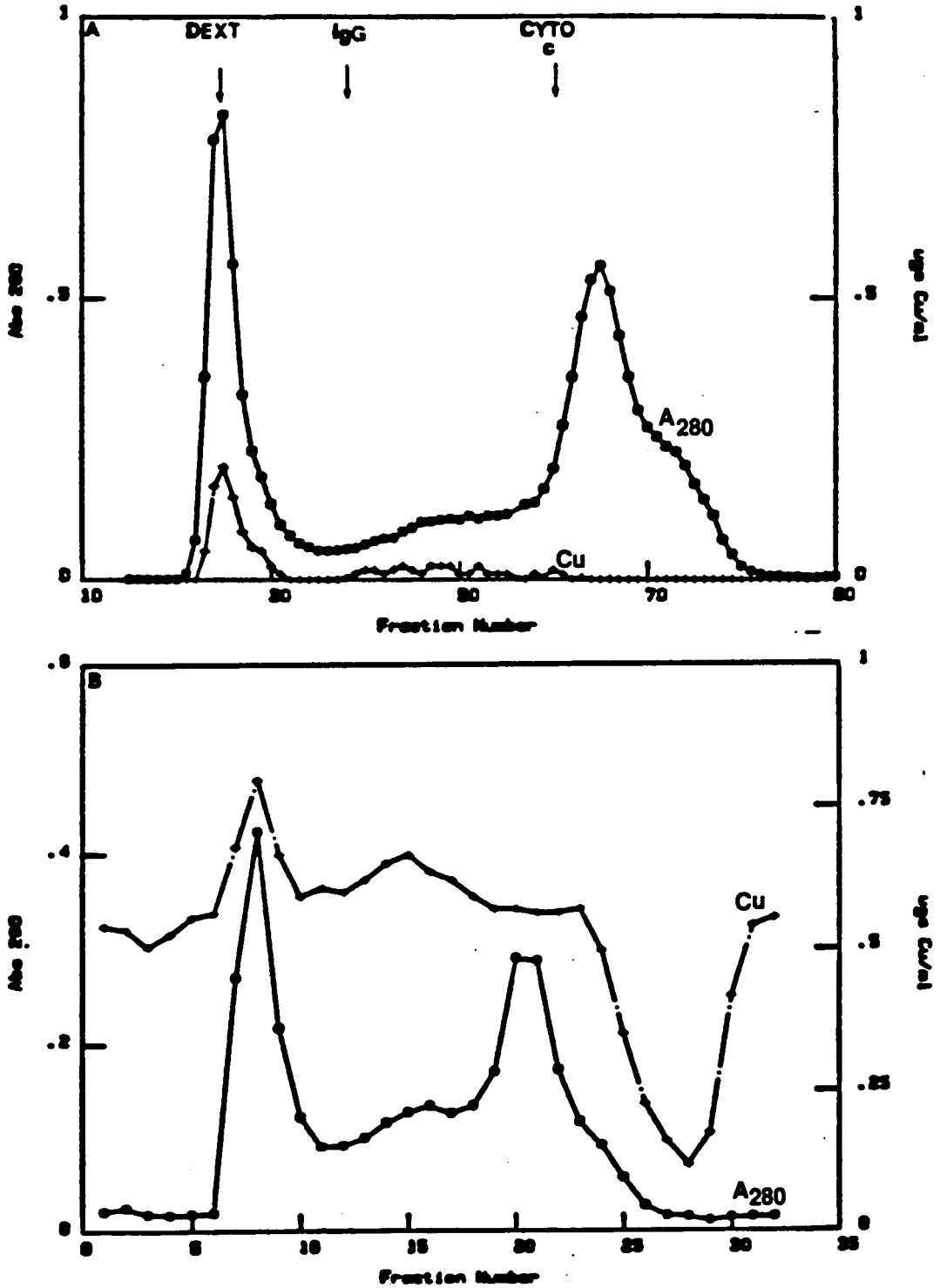

A
DEXT
IgG
CYTO c
A280
Cu
Fraction Number
B
Cu
A280
Fraction Number

Figure 6.

Figure 7. Sephadex G-200 Elution Profiles - Cells Grown in 0.01 M Copper Citrate.

The columns were pre-equilibrated with 1 x 10^{-5} M copper containing sodium phosphate buffer.

A : CF cell extract from cells harvested at mid-Log phase growth in copper supplemented minimal medium.

B : CF cell extract from cells harvested at 10 hours into resting phase. These cells were grown in minimal medium with added copper.

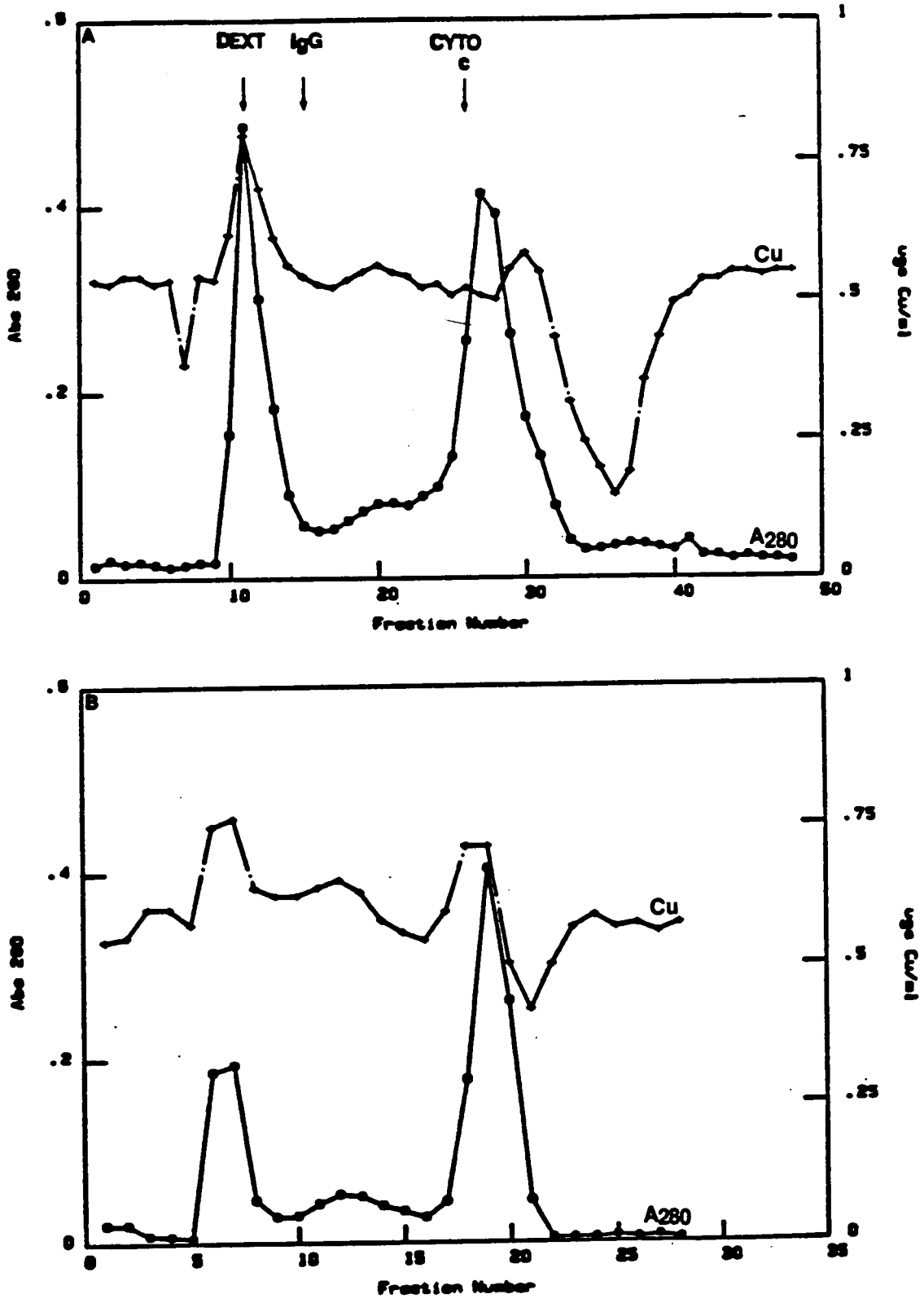

A
DEXT
IgG
CYTO
c
Cu
A280
Abs 280
ug Cu/ml
Fraction Number
B
Cu
A280
Abs 280
ug Cu/ml
Fraction Number

Figure 7.

Figure 8. Sephadex G-50 Elution Profile of CF Cell Extract.

Cell extract obtained from cells grown in copper supplemented medium, harvested 10 hours into resting phase. G-50 column was pre-equilibrated with 1 x 10^{-5} M copper containing sodium phosphate buffer.
Molecular weight estimate was done from the center of the copper peak, which was at Fraction 24.
Mol. Wt. Markers- Blue Dextran, cytochrome c (eluted with the sample), and FAD.

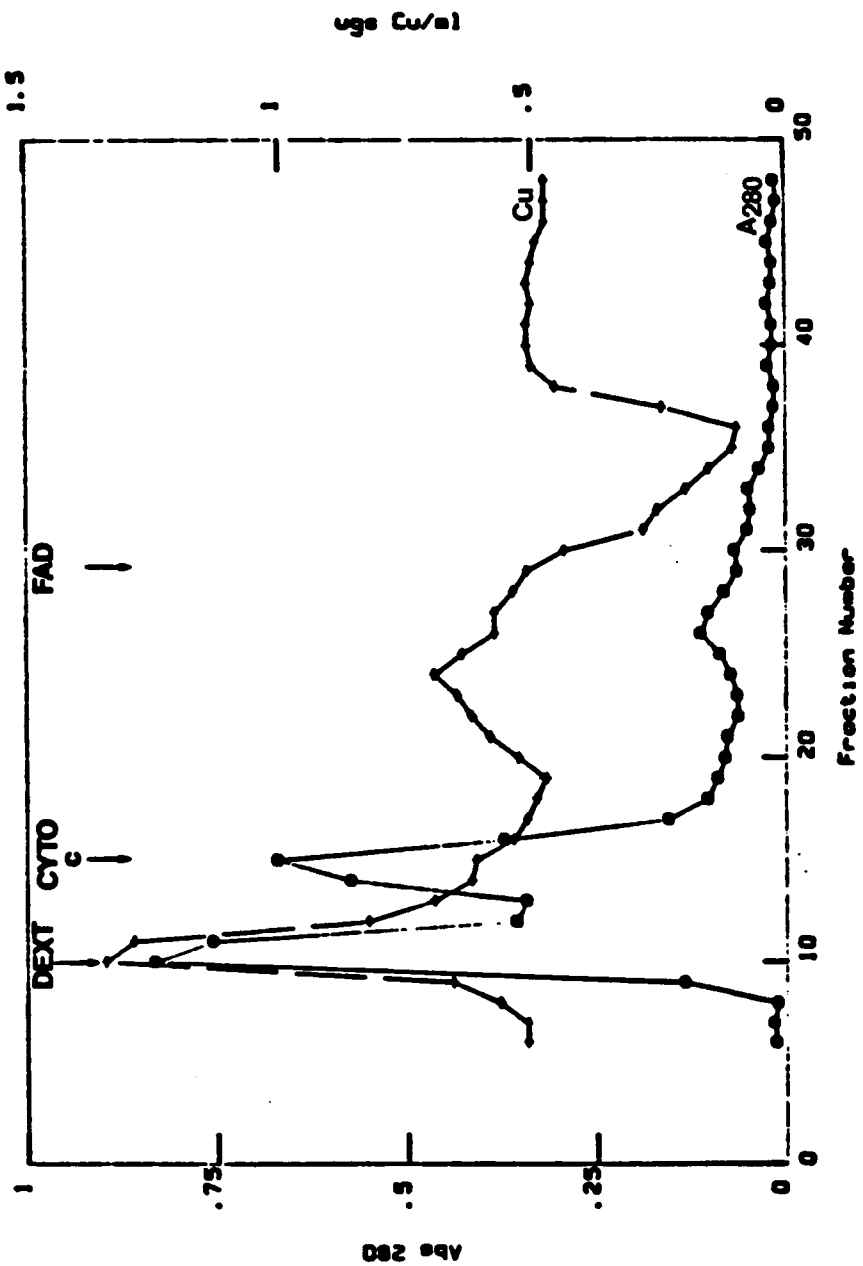

µgs Cu/ml
1.5
1
.5
0
50
40
30
20
10
0
Cu
A280
FAD
DEXT CYTO c
Fraction Number
Abs 280
1
.75
.5
.25
0

Figure 8.

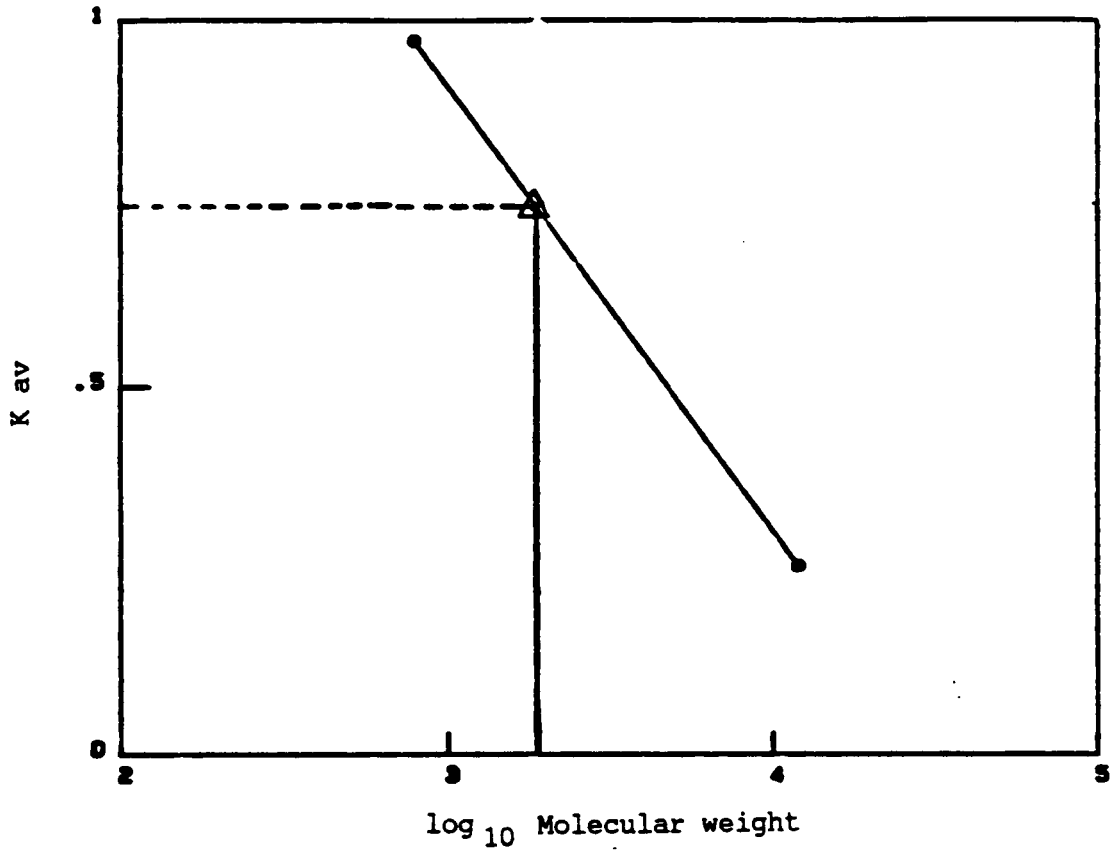


Figure 9 Selectivity Curve.

Illustrates the linear relationship between K av(the fractional volume of the stationary phase gel which is availible for diffusion of a given solute species) and the log of the molecular weight of that solute. FAD has a Mol. Wt. 785.5 dalts. and K av of 0.971; cytochrome c has a Mol. Wt. 12,000 dalts. and K av of 0.255.

Copper binder has a K av of 0.741.
Molecular Weight estimated in the 1800 - 1900 dalton range.

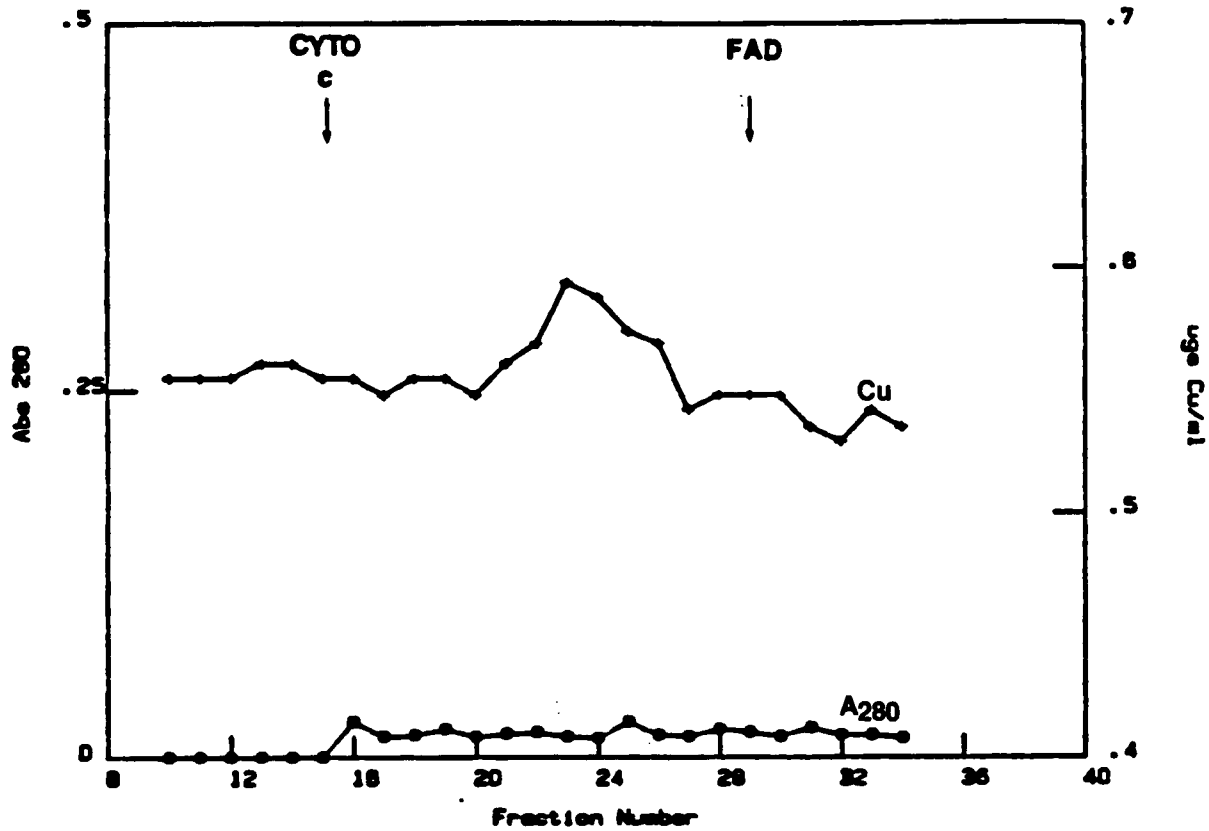


Figure 10. Sephadex G-50 Profile of Centricon 10 Filtrate.

There is almost no measurable absorbance at 280 nm. from the filtrate, but there is a noticable copper peak above background centered at fraction 23.

* The Centricon 10 microconcentrator filter has a exclusion limit of 10,000 daltons, thereby not allowing anything 10 Kd or larger to pass through as filtrate.

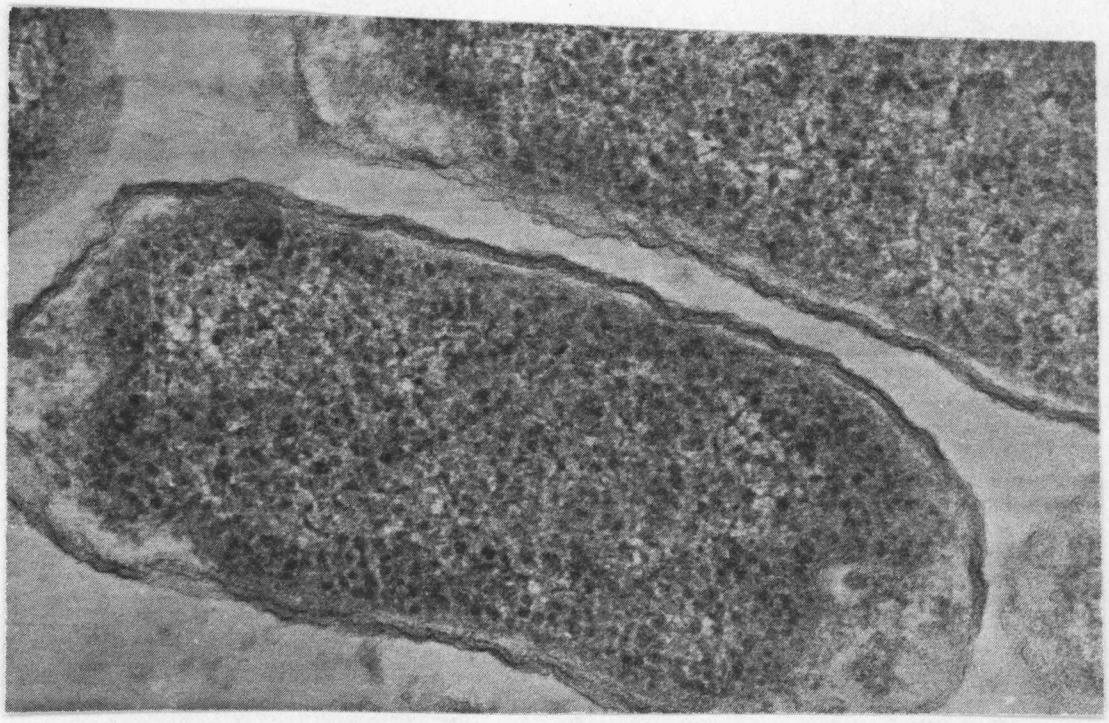


Figure 11. Typical Salmonella typhimurium cell, strain LT2.

Conventionally fixed, embedded, and sectioned.
(Magnification 50,000 x).

Table 1
Comparison of Copper Levels in CF Mutant
Cells Grown in the Absence of Copper vs.
Cells Grown in the Presence of Copper

	Cu Range (ugs Cu/mg Total Protein)
Cells Grown in Absence of Cu	0.00 - 0.941
Cells Grown in 10^{-2} M Cu*	1.00 - 9.168

All cells were harvested 10 hours into resting phase.

* In each individual experiment, the copper levels of CF cells grown in copper supplemented minimal medium was at least five times the copper levels of CF cells grown in minimal medium without copper.

Table 2

Cu and Protein Comparisons in Whole Cells and Cell Fractions of CF Mutants

	mg prot*/ml OCS	ug Cu/ml OCS	Cu/prot.	% Cu	% prot.
Whole Cells(Cu -)	0.660 (+/- 0.008)	0.078 (+/- 0.05)	0.118	--	--
Whole Cells(Cu +)	0.660 (+/- 0.001)	0.660 (+/- 0.05)	1.00	--	--
Cell Sonic.(Cu +)	0.580 (+/- 0.001)	0.775 (+/- 0.05)	1.33	--	--
Pellet (Cu +)	0.015 (+/- 0.0001)	0.149 (+/- 0.05)	0.257	18.3%	2.9%
Soluble (Cu +) Fraction	0.510 (+/- 0.0001)	0.664 (+/- 0.05)	1.145	81.7%	97.1%

(Cu -) - cells which have been grown in minimal medium without added copper

(Cu +) - cells grown in copper supplemented minimal medium

Cu/prot. - measured as micrograms of copper per milligram of total protein

ml OCS - refers to milliliter of original cell suspension

* Bradford method used for protein assay

Table 3

Copper and Protein Comparisons in Whole Sonicate of Cells
White CF vs. Blue CF

	mgs prot./ ml cell susp.	ug Cu/ ml cell susp.	ug Cu/ mg total prot.
White CF Sonicate	1.39	2.938	2.114
Blue CF Sonicate	0.27	27.492	101.82

Table 4

Soluble Fraction Dialysis

	No Dialysis	6 Hrs.	10 Hrs.	10 Hrs.+ Cu
Soluble[1] Fraction	2.52 (+/- 0.00)	2.195 (+/- 0.0006)	1.746 (+/- 0.007)	1.824 (+/- 0.0001)
Soluble[2] Fraction	2.32 (+/- 0.003)	2.02 (+/- 0.001)	1.874 (+/- 0.002)	2.073 (+/- 0.002)

1 - Dialysis tubing molecular weight cut-off 6000-8000 daltons

2 - Dialysis tubing mol. wt. cut-off 3500 daltons

All measurements are micrograms of copper per milliliter of original cell suspension.

VITA

David Alan Armstrong was born in suburban Boston, Massachusettes, August 31, 1962. He attended Boston College High School and completed high school in Exeter, New Hampshire following a move in 1978. He subsequently entered the University of New Hampshire, Durham, N.H. in September 1980. And graduated from that university with a Bachelor of Science degree in Biochemistry, May 1984. He then joined the Department of Biochemistry at the University of Tennessee, Knoxville in September 1984. There, under the direction of Dr. Kenneth J. Monty he was awarded a Master of Science degree in December 1987. Future plans include the pursuit of a Doctor of Philosophy degree concentrating in the field of Analytical Electron Microscopy.