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THE EFFECT OF COPPER ON PSEUDOMONAS CUPRODURANS, SP NOV

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THE EFFECT OF COPPER ON *PSEUDOMONAS CUPRODURANS*, SP. NOV.

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

THE EFFECT OF COPPER ON *Pseudomonas cuprodurans*, sp. nov.

by

Laurence R. McCarthy

A study of a copper-tolerant marine bacterium, *Pseudomonas cuprodurans*, sp. nov., was undertaken to determine its copper tolerance, factors affecting copper tolerance, and the physiological effect of copper stress on the bacterium.

P. cuprodurans grew optimally at 60-100% salinity. This salinity requirement reflected a need for sodium, magnesium, and calcium, but not for strontium, or potassium. Deletions of calcium or magnesium from the medium caused the formation of swollen cells and filaments.

The maximum copper concentration tolerated by *P. cuprodurans* in basal medium consisting of yeast extract, 1 g; peptone, 1 g; synthetic seawater, 750 ml; distilled water 250 ml (pH 7.0) was $2.25\text{-}2.50 \times 10^{-3}$ M. The copper tolerance of some non-marine bacteria in basal medium prepared with distilled water were: *Escherichia coli B*, 1×10^{-4} M; *Bacillus subtilus*, 5×10^{-5} M; *Staphylococcus aureus*, 1×10^{-4} M; and *P. aeruginosa*, 1×10^{-3} M.

The concentration of copper tolerated by *P. cuprodurans* decreased as the amount of organic matter in the medium was reduced. In copper supplemented basal medium, copper was present in both a labile (free) or non-labile (bound) form as determined by anodic stripping voltammetry. Labile copper concentration increased more rapidly than did non-labile

copper as the copper in the basal medium was increased to 1×10^{-3} M. Filter-sterilized basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper contained more labile copper than did autoclaved medium with the same copper concentrations. This indicated the exposure of more copper binding sites due to autoclaving.

P. cuprodurans in basal medium broth supplemented with 1×10^{-3} M copper had a 7-hr lag phase compared to a 1.5-hr lag phase in basal medium. Previous growth of P. cuprodurans in basal medium supplemented with 1×10^{-3} M copper reduced the lag phase of P. cuprodurans in basal medium supplemented with 1×10^{-3} M copper to 3-4 hr. Return of copper-stressed cells to basal medium and subsequent transfer to basal medium supplemented with 1×10^{-3} M copper produced, again, a 7-hr lag phase. During lag phase, P. cuprodurans populations remained stable under copper stress. Thus, adaptation rather than mutation played a significant role in the copper tolerance of P. cuprodurans.

P. cuprodurans in basal medium attained optimum growth at 33 C. P. cuprodurans grown in copper supplemented basal medium exhibited optimal growth at 26 C. Growth temperatures above 26 C caused copper-supplemented medium to become toxic to P. cuprodurans.

Increased copper supplementation to the basal medium caused no significant alteration of DNA, RNA, and protein synthesis in P. cuprodurans. The relationship of protein, dry weight and cell numbers was constant in basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper.

No significant morphological or ultrastructural alteration of P. cuprodurans occurred with growth in copper-supplemented basal medium.

The respiration of P. cuprodurans grown in basal medium decreased progressively in basal medium supplemented with the increased concentrations of copper. Growth of P. cuprodurans in copper-supplemented basal medium caused decreased respiration when studied in basal medium. Respiration was decreased only by copper concentrations exceeding that in which the organism was grown.

Triphenyl tetrazolium chloride overlays of P. cuprodurans colonies exhibited delayed reduction of triphenyl tetrazolium to triphenyl formazan as copper concentrations increased. Copper concentrations of 5×10^{-4} and 1×10^{-3} M caused respiratory deficient cells which upon transfer to basal medium reverted to respiratory sufficiency. Similar respiratory deficiencies were not obtained by growth of P. cuprodurans under maximum stress of nickel, zinc, cobalt and cadmium. Maximum copper stress did not cause respiratory deficiencies in E. coli B, S. aureus, or B. subtilis.

Growth of P. cuprodurans in copper-supplemented medium delayed reduction of triphenyl tetrazolium chloride, methylene blue, and dichloroindophenol in Thunberg tubes. This respiratory inhibition indicated that alteration in the electron transport system at the flavoprotein-quinone site occurred with growth of P. cuprodurans under copper stress.

Difference spectra performed on whole cell extracts of P. cuprodurans cultivated in basal medium unsupplemented and supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper revealed three cytochrome com-

ponents: $\underline{b}_1$, $\underline{c}$, and $\underline{o}$. Growth in increased copper stress decreased the relative quantity of cytochrome $\underline{c}$ and altered the relative quantity of cytochrome $\underline{b}_1$.

Uptake of ^{64}Cu occurred during logarithmic and stationary phases of growth. P. cuprodurans cells in basal medium unsupplemented and supplemented with copper contained progressively decreasing amounts of copper as late logarithmic phase was approached. Copper uptake in late logarithmic and stationary phases reached a constant level of copper.

Uptake of ^{64}Cu by stationary phase cells in basal medium supplemented with 1×10^{-4} M copper exhibited a rapid ionic binding of copper followed by an active diphasic uptake of the cation. The presence of basal medium was essential for the diphasic uptake. Chloramphenicol (100 ug/ml) eliminated the diphasic process indicating the requirement of protein synthesis for diphasic ^{64}Cu uptake.

Cells grown in copper supplemented basal medium had 95% of the total assimilated copper interior to the cell envelope. Biochemical extractions of P. cuprodurans cells grown in basal medium unsupplemented and supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper showed decreased amounts of copper associated with intermediary metabolytes and other small molecular weight compounds as copper concentrations were increased. Lipid extracts contained 8-10% of the total assimilated copper in all treatments. Seventy-five to eighty-five percent of the total copper was localized in the nucleic acid and protein fractions of all cells.

Copper tolerance by P. cuprodurans is dependent on the medium in

which the bacterium is grown and the ability of the bacterium to adapt to copper stress. The bacterium accumulates copper, concentrating 95% in its cytoplasm, predominately localized in the nucleic acid and protein fraction. Gross synthesis of DNA, RNA, and protein is unaffected by copper stress. Alteration of the electron transport system occurs with copper stress at the flavoprotein-quinone and cytochrome sites with decreased respiration occurring.

I. Introduction

Copper in low concentrations, serves as an essential growth factor for several life forms. In high concentrations, however, copper demonstrates extreme toxicity to biological systems. The toxic effect of elevated concentrations of the divalent cation resides in its ability to form stable complexes with ligand groups of biologically essential biochemical constituents of life, such as the sulfhydryl groups of enzymes, rendering them biologically inactive. Because of this reactivity, copper has been employed as an algicidal, fungicidal, and bactericidal agent. Studies of the effects of copper stress on the physiology of microorganisms have revealed alterations in energy metabolism, morphology, and respiration.

Roche (1966) isolated a marine bacterium, Pseudomonas X, which tolerated elevated concentrations of copper sulfate ($2.25\text{-}2.50 \times 10^{-3}$ M). P. X. was capable of concentrating copper from the growth medium on, or interior to the cell membrane.

Studies were undertaken in this dissertation to identify P. X. as a species, determine factors influencing copper tolerance, characterize the copper resistant capability, and to determine some of the biological effects of copper stress.

II. LITERATURE REVIEW

A. Copper In the Sea.

The major ionic constituents of seawater are weak in chemical and biological reactivity, and are termed conservative elements (Goldberg, 1965). Minor constituents of seawater, or non-conservative elements, possess high chemical and biological reactivity. Copper is a non-conservative element.

In the sea, copper possesses a neutral or positive charge (Goldberg, 1957). Because the sea is strongly oxidative, cuprous copper entering the sea from run-off is transformed rapidly to cupric copper (Harvey, 1963). The major inorganic species of copper in seawater is cupric sulfate (Goldberg, 1965).

The "Irving-Williams" series lists divalent metals in order of complex stability (Goldberg, 1965). Copper which is present above 1 ug/liter in the sea is the most reactive divalent metal ion of cations above this concentration. The concentration of copper in seawater varies, but 3 ug/liter is the best estimated value (Goldberg, 1965). Depth, location (proximity to land), and season of the year are factors affecting the variability of copper concentration, creating a range of less than 1 and up to 30 ug/liter (Galstoff, 1943; Chow and Thompson, 1952; Bougis, 1962; Atkins, 1953; Alexander and Corcoran, 1967; Meng-Cherego and Piccotti, 1959).

The relative reactivity of ions in seawater is expressed as residence time: the average time an element resides in the sea before being removed by precipitation of some other process. Copper has a residence

time of 5×10^{-4} years (Goldberg, 1965) which is a short to moderate period in relation to other constituents of seawater. Krauskopf (1956) found copper undersaturated when compared to determined saturation levels for the ion in seawater. Low copper concentrations (undersaturation) in seawater may be expected from the high reactivity of the ion (relative low residence time).

Copper in the sea is associated with organic matter (Sillen, 1961). Slowey and Hood (1966) found that copper in seawater is non-dialyzable, indicating interaction with organic molecules. As much as 50% of the total copper present in seawater samples could be extracted into chloroform (Slowey, Jeffrey and Hood, 1967). Corcoran and Alexander (1964) found that persulfuric acid treatment of seawater yielded higher measurement of copper due to release of copper from organic complexes than duplicate untreated samples. Williams (1969) found that 5-28% of copper in the sea to be in an organic associated form, and suggested that the association of copper with organic matter was not necessarily in the form of a chelate, but in a complexed form.

Copper may be removed from seawater by attachment to heavy organic aggregates (Krauskopf, 1956) or by chemical precipitation such as by hydrogen sulfide (Suckow and Schwartz, 1963). Due to its high biological reactivity, copper is concentrated by many marine organisms. Some determined values of copper concentration are: 1400 times in marine sponges (Bowen and Sutton, 1951); 100-600 times in seaweeds (Black and Mitchell,

1952); 400-90,000 times in plankton (Nicholls, Curl and Bowen, 1960); 100-200 times in pelagic fish (Goldberg, 1962). Copper was concentrated by marine scallops, oysters, and mussels (Brooks and Rumsby, 1965). Concentration of copper by pelagic fish and shellfish resulted from intake of plankton and copper-associated aggregates during the feeding process (Goldberg, 1962; Brooks and Rumsby, 1965). Some concentration of copper by marine organisms is nutritionally essential because of copper's role in enzymes (hemocyanin, respiratory pigment of crustaceans and mollusks; plastocyanin, a copper enzyme involved in photosynthesis in chloroplasts).

Thus, the concentration of copper in the sea is determined by the interaction of several factors: precipitation, levels of organic matter, biological demand, chemical reactivity, season of the year and others.

B. Nutritional Benefit of Copper.

1. Copper In Enzyme Systems.

Copper is one of several metal ions demonstrated as an essential nutrient for life forms. The nutritional benefit of copper is in the form of a cofactor in several enzyme systems (Porter, 1946; Dixon and Webb, 1964; Mahler, 1960). Mahler (1960) states that a metal may function in one of two enzymes types: metalloenzymes, and metal-activated enzymes. The following table abstracted from Mahler (1960) reveals some of the major differences in metal interactions in the two systems.

Parameter	Metalloenzyme	Metal-activated Enzyme
Attachment to protein	Firm, essentially irreversible	Loose, readily reversible
Dissociation constant	Virtually zero	Measurable
Ratio of metal to protein	Constant	Variable
Effect of metal content on enzyme activity	Direct proportionality	Modified Michaelis-Menten kinetics
Activity of enzyme in absence of metal	Nil	Frequently show some low activity
Specificity of metal	Very high	Similar ions substitute

Of known copper-enzymes, tyrosinase may be classified as a metal-activated enzyme as it shows some activity in the presence of iron and nickel (Williams, 1953). A second example of a metal-activated enzyme is malate dehydrogenase (decarboxylating) which is activated by manganese, cobalt, zinc, nickel, or copper (Dixon and Webb, 1964). The majority of copper-enzymes are metalloenzymes, with hemocyanin being a prime example (Williams, 1953).

Two different metal ions activate the same enzyme in two separate activities. An example is carboxypeptidase B (Dixon and Webb, 1964). In the presence of cobalt the enzyme functions as a peptidase, whereas in the presence of cadmium the enzyme functions as an esterase. Thus, metal ions play a role in regulation in such relationships.

Metal ions in metalloenzymes and metal-activated enzymes may function in several ways: 1. The metal may be the active site of the enzyme; 2. The metal ion may form a part of the enzyme's active site; 3. The

metal ion may attach at a non-active site causing alteration of the proteins structure which is necessary for function; 4. The metal ion may aid in the alignment of the substrate with the enzyme; 5. The metal ion may interact with the substrate altering the substrate conformationally in a manner necessary for enzyme action (Mahler, 1960).

Another class of metal associated enzymes have a flavin-metal complex as their prosthetic group. Copper functions in this manner in the cases of bacterial nitrate reductase (Dixon and Webb, 1964), butyryl coenzyme a dehydrogenase (Mahler, 1954), Pseudomonas stutzeri nitrite and nitric oxide reductases (Mahler, 1960), and Neurospora crassa hyponitrite reductase (Mahler, 1960).

To date, more than twenty copper proteins have been isolated and characterized. Of these proteins, the majority are enzymes associated with oxidations (oxidases). Such enzymes play an integral role in biological oxidations, and appear in a broad spectrum of organisms (Frieden, Osaki, and Kobayashi, 1965). Examples of such copper-enzymes are ascorbic acid oxidase, ceruloplasmin, laccase, uricase, diamine oxidase, monoamine oxidase, cytochrome oxidase (eucaryotic), hemocyanin, and tyrosinase. Other copper enzymes are: Beta-mercaptopyruvate transulfurase, and plastocyanin.

Among the copper-proteins characterized, some have been postulated to act as storage sites for the essential copper metal. Examples are: cerebrocuprein, erythrocuprein, hemocuprein, hematocuprein, milk copper protein, and neonatal hepatic mitochondrocuprein (Frieden, Osaki, and

Kobayashi, 1965; Porter, 1966).

Copper enzymes contain 1 (i.e. tyrosinase and hemocyanin), 4 (i.e. laccase) and 8 (i.e. ceruloplasmin and ascorbic acid oxidase) copper atoms per enzyme molecule. In copper-enzymes containing 4 or 8 copper atoms, electron spin resonance studies have revealed that 1/2 to 3/4 of the copper atoms are in the cuprous state, with the residual atoms in the cupric state (Malmstrom, 1965). Cuprous/cupric ions in these enzymes function by a mechanism not presently understood. With the exception of tyrosinase, the copper enzymes characterized are classified as metallo-enzymes, with copper proposed as the active site in a few.

2. Fungi.

Starkey (1955) has suggested that copper is required as a micro-nutrient by all microorganisms. Perlman (1949), Bortels (1927) and Foster (1939) have discussed the requirements for copper in fungi. In various species of fungi, copper is required for growth, spore formation, and catalase production. Because of the specific metal requirements of fungi, Nicholas (1952) suggested the use of Aspergillus niger for a microbiological assay of trace elements such as copper.

In eucaryotic, electron transfer the cytochrome oxidase component contains copper and is dependent on copper for its synthesis (Underwood, 1959). Copper residing between the two cytochrome a components probably functions as a transfer intermediate (Warton and Cusanovich, 1969; Beinert, 1966). There is no clear evidence for copper appearing in the cytochrome oxidase system of bacteria (Lucille Smith, personal communication).

3. Bacteria.

To date, only two copper-proteins (metalloenzymes) have been isolated from bacteria. P. aeruginosa contains the blue protein which is associated with the cytochrome oxidase system of the bacterium (Yamanaka, 1966; Tang and Coleman, 1968). In Bordetella pertussis a copper-enzyme, azurin, has been isolated (Frieden, Osaki, and Kobayashi, 1965). Azurin functions to oxidize intracellular reducing agents (i.e. cysteine). The reduced enzyme is re-oxidized by oxygen (Frieden, Osaki, and Kobayashi, 1965).

Copper-associated enzymes (copper-flavin prosthetic group) have been isolated from bacteria. No copper metal-activated enzymes have been isolated from bacteria, although such enzymes are thought to exist (Dixon and Webb, 1964).

Because of bacterial requirements for the transitional metals (i.e. copper), Weinberg (1957) suggested that antimicrobial activity of certain antibiotics (i.e. tetracyclines and streptomycin) may be partially explained by their ability to chelate essential metals, depriving the cell of their nutritional benefit.

4. Host-Parasite Relationships.

Copper plays an important role in host-parasite relationships. The presence of copper aids plants in resisting microbial infections (Weinberg, 1966). With most acute infections in man, there is a rise in serum copper levels (Cartwright, 1950; Raskovalov, 1963). This hypercupremia may suppress antibody formation in man. Drugs such as salicylates

may be therapeutic because of their ability to remove copper from the serum, returning it to tissue cells (from which the ion originated), allowing the immune response to function normally (Weinberg, 1966).

C. Copper Toxicity.

1. Biochemical Basis.

Because of its high biological reactivity, copper is quite toxic in high concentrations. Copper is one of the most toxic metal ions, with toxicity exhibited at levels below 1 mg/liter (Bowen, 1966).

The toxic action of copper results from its ability to form complexes with essential biochemical components of the living cell. Gurd and Wilcox (1956) have reviewed cationic interactions with proteins, peptides, and amino acids. Divalent heavy metal ions, according to Gurd and Wilcox, bind to amines and simple amino acids in the following decreasing order of affinity: Hg, Cu, Ni, Pb, Zn, Co, Cd, Mn, Mg, Ca, and Ba. Binding of divalent metal cations to carboxyl groups occurs in the following decreasing order of affinity: Cu, Ni, Zn, Co, Mn, Mg, Ca, and Ba (Gurd and Wilcox, 1956).

Other ligands appearing in the living cell are: phosphate sulfhydryl, and imidazole (Passow, Rothstein, and Clarkson, 1961). Copper reacts with all these ligands. Copper is one of the most reactive metal ions with sulfhydryl groups as present in cysteine-containing proteins and peptides (Passow, Rothstein, and Clarkson, 1961). Studies of copper (II) interactions with proteins such as bovine serum albumin revealed copper-binding to histidine residues (Bradshaw, Shearer and Gurd, 1968).

Such binding to histidine residues involves the imidazole group of the amino acid (Sarkar and Wigfield, 1967).

Due to this ability of copper to bind with protein residues several enzymes have been found which are inhibited by the cation: i.e. RNA polymerase ex rat liver nuclei (Novello and Stripe, 1969), bovine pancreatic ribonuclease (Saundry and Stein, 1967), alpha-oxoglutarate dehydrogenase (Webb, 1964), plant riboflavin kinase (Dixon and Webb, 1964).

Copper has been demonstrated to strongly interact with the DNA molecule (Schreiber and Daune, 1969; Bach and Miller, 1967; Miller and Bach, 1968; Sigel, 1968; Shapiro, Stannard, and Felsenfield, 1969; Zubay and Doty, 1958). Copper is the most firmly bound metal capable of joining with the DNA molecule (Eichorn et al, 1967). The effect of copper as observed in melting curve studies on DNA is to stabilize the DNA molecule raising the T_m and renaturing the molecule in subsequent cooling (Eichorn et al, 1967). Copper binds to guanine and cytosine residues as well as to the phosphate groups of the DNA molecule (Fritzsche and Zimmer, 1968).

Copper is bound by pteridines and riboflavin (Albert, 1951). Of the metals studied only Fe(II) and Cu(II) showed significant binding to riboflavin and pteroylglutamic acid, with the ferrous ion having the greatest avidity for the available ligands (Albert, 1951).

2. Effect of Copper on Fungi.

Because of its high reactivity copper has long been employed as a lethal agent for several types of microorganisms. The use of copper

sulfate as a fungicide has a long history and was originally used for the control of wheat bunt as early as 1761 (Woolman and Humphrey, 1924). The fungicidal activity of the cation is attributed to the affinity of copper for the fungal spore surface (McCallan and Miller, 1958; Miller and McCallan, 1957; Sussman and Lowry, 1955). Fungal spores attract the same quantity of copper to their surface with variable external concentrations of the cation (McCallan and Miller, 1958). Such uptake by fungal spores may be spared by the presence of other inorganic cations i.e. magnesium, calcium, and potassium (Marsh, 1945; Lin, 1940; Somers, 1963). As much as 36% of the copper taken up by fungal spores penetrates the spore membrane (Somers, 1963). Copper (2×10^{-4} M) greatly reduces the respiration rate of spores of Sclerotinia fructola (Marsh, 1945).

The greatest effect of copper on the fungal spore is in its prevention of germination. Because copper-treated spores germinate (100% germination) after removal of copper by dilute acid treatment, copper is considered to be a fungistatic agent rather than a fungicidal agent (McCallan and Miller, 1958). The effect of high concentration of copper associated with the spore surface is not fully manifested until the spores are germinated (Lowry, Sussman, and Von Boventer, 1957).

Although copper treatment of fungal disease is due to the ion's effect on the fungal spore, vegetative mycelia of certain fungi have been observed to undergo physiological changes with increased copper. Healy, Cheng and McElroy (1955) noted that additions of copper to the growth medium caused physiological alterations of Neurospora crassa: increase

in total protein nitrogen, lowering the rate of dye oxidase activity (thought to be indicative of cytochrome alteration), slight lowering in the levels of peroxidase, and a significant decrease in levels of peroxidase, and a significant decrease in levels of succinic dehydrogenase. Nicholas and Commisiong (1957) studying the effects of copper on N. crassa noted copper induced the following changes: increase in levels of cytochrome oxidase, decrease in levels of cytochrome c reductase, inhibition of acid phosphatase, and a decrease in nitrate reductase activity. In the cases of nitrate reductase and acid phosphatase, the effect of copper was found due to ion antagonism with molybdenum.

Considerable work has been performed on the action of copper upon yeast cells. Copper caused decreases in populations of Torulopsis homii subsequent to the attainment of stationary phase (Steenbergen, Steenbergen, and Weinberg, 1969). This loss in population is considered to be due to inhibition of secondary metabolism (inhibition of sulfhydryl containing enzymes), which resulted in a metabolic toxicity (Steenbergen, Steenbergen, and Weinberg, 1969). Passow and Rothstein (1960) have found that copper is able to rapidly penetrate the cell membrane of yeast causing relatively no alteration of the membrane permeability. The presence of 1.5×10^{-4} M copper caused alteration in the amino acid pool of Saccharomyces ellipsoideus with the loss of glycine and serine from the amino acid pool (Murayama, 1957).

Copper induced respiratory deficiencies in yeasts (using triphenyl tetrazolium chloride as an indicator of sufficiency/deficiency)

(Yanagashima, 1960; Lindegren, Nagai, and Nagai, 1958). Minagawa (1958) observed copper (1×10^{-3} M) to induce reduction in oxygen uptake of Saccharomyces ellipsoideus. Murayama (1961a) has shown that copper (1×10^{-3} M) affects both aerobic respiration and fermentation in S. ellipsoideus, with fermentative processes more affected by the cation. Murayama (1961b) investigating the affect of copper on S. ellipsoideus noted that copper caused interuption of the tricarboxylic acid cycle at succinate and fumarate levels. Copper greatly inhibited succinic dehydrogenase (Murayama, 1961c).

3. Effect of Copper on Algae.

Copper is a well established algicidal agent (Bowen, 1966). Rounsefell and Evans (1958) controlled an algal bloom of Gymnodinium breve (Florida red tide dinoflagellate) by maintaining a copper sulfate concentration of 180 ug/liter in neritic waters for a period of two weeks. A concentration of 30 ug/liter copper exhibited no effect on the phytoplankton of a saltwater lagoon (Marvin, Lansford, and Wheeler, 1961).

Greenfield (1942) has reported that Chlorella vulgaris was inhibited at a level of 6.4 ug/liter copper (approximately 1×10^{-7} M). Erickson, Lackie, and Maloney (in press) reported the following levels of copper toxicity for six species of estuarine algae: Amphidinium carteri, 50 ug/liter; Olisthodiscus luteus, 100 ug/liter; Cyclotella nana and Skeletonema costatum, 150 ug/liter; Isochrysis galbana, 200 ug/liter; and Dunaliella tertiolecta, greater than 450 ug/liter. These

values range between 7.88×10^{-7} M (50 ug/liter) to 7.1×10^{-6} M (450 ug/liter) copper.

Kanazawa and Kanazawa (1969) observed that division of Chlorella ellipsoideus was inhibited by 78 ug/liter copper (approximately 1.23×10^{-6} M) when the alga was cultivated in the absence of light. Short term exposure of Chlorella vulgaris to high concentrations of copper (2.0×10^{-4} to 2.6×10^{-2} M) inhibited oxygen uptake in unshaken cultures (Hassal, 1963). Cells studies in shaken cultures were observed to undergo no alteration in their oxygen uptake under the same copper stress. McBrien and Hassal (1965) noted that copper caused the release of potassium ions from Chlorella vulgaris in aerobic conditions. Under anaerobic conditions, copper exhibited significant inhibitory effects on respiration, photosynthesis, and growth of Chlorella vulgaris (McBrien and Hassal, 1967).

Habermann (1969) found copper in chloroplasts to act as a specific poison for the flavin-sensitized photooxidation of diketogulonic acid, the Mehler reaction, and whole cell photosynthesis. The action of copper can be reversed by the addition of increased levels of manganese to the culture medium.

4. Effect of Copper on Bacteria.

Bacteria concentrate inorganic metals from the media in which they are cultivated (Curran, Brunstetter, and Meyers, 1943; Knaysi, 1961). In sporeforming bacteria, the spore contains higher levels of minerals (i.e. copper) than does the vegetative cell (Curran, Brunstetter, and Meyers,

1943; Grelet, 1952). Kolodziej and Slepecky (1962) have shown copper to be essential for spore formation in a wide variety of spore-forming bacteria.

Copper stimulated pigment production in P. fluorescens var putida (Chakrabarty and Roy, 1964). Nitrate reductase in P. aeruginosa is stimulated in its production by small additions of copper sulfate to anaerobically incubated cells (Yamanaka, Kijimoto, and Okunuki, 1963).

Blundell and Wild (1969) observed copper to cause partial inhibition of protein and RNA synthesis when added to log phase cultures of E. coli M.R.E. 600. Jerebzoff (1967) has found copper to inhibit the division of Leptosphaeria michotii. This divisional inhibition was due to copper antagonism of iron (Jerebzoff, 1967). The addition of 5×10^{-6} M copper to the growth medium of B. megaterium prevented the development of phage synthesis, without noticeably affecting the host (Huybers, 1953). Hofsten (1962) has found that 2×10^{-6} M copper in minimal media was inhibitory to E. coli, with the organism being more affected when grown under anaerobic conditions. The toxic effect of copper was removed by the addition of metal-complexing agents or heat-killed bacteria (Hofsten, 1962).

Toxic effects of copper in water samples can be removed by the addition of thiosulfate (Hoather, 1957). Jones (1964) in studying the growth of E. coli B in glucose-supplemented seawater found that the toxic effect of seawater was decreased by autoclaving or treatment with chelating agents. Autoclaving seawater caused the formation of precipitates con-

taining heavy metal ions which effectively reduced their concentration in seawater (Jones, 1967a). The addition of 4 or 40 ug/liter copper to filter-sterilized, glucose-supplemented seawater lead to the progressive extension of the lag phase of E. coli B, with a concentration of 40 ug/liter proving inhibitory (Jones, 1967b). Toxicity of added copper was greatly reduced by the addition of 1×10^{-4} M cysteine, a known chelator of copper (Jones, 1967b).

Waksman, Johnstone, and Carey (1943) studying the effect of copper on marine bacteria noted that the addition of 2×10^{-4} M copper had relatively no effect on the total population in peptone-glucose supplemented seawater. The addition of 2×10^{-3} M copper lead to a 7-day lag in the development of a substantial population in the seawater samples. Roughly 20% of bacteria collected from submerged surface films were found capable of tolerating 2×10^{-3} M copper. Copper additions of 8×10^{-6}, 8×10^{-5}, 8×10^{-4}, and 2×10^{-3} M were found to cause a progressive decrease in the total oxygen consumption of the sampled marine populations (Waksman, Johnstone and Carey, 1943). Starr and Jones (1957) found that 16% of the total 175 isolates tested were able to tolerate a level of 8.2×10^{-4} M copper. Roche (1966) performing other ecological studies on copper tolerance found an average of 15.5% of marine bacteria tested capable of tolerating 1×10^{-3} M copper.

D. Copper Tolerance.

An intriguing aspect of the effect of copper on microorganisms is the study of copper-tolerant cells. To date there are three demonstrated

mechanisms for copper tolerance: acidity, hydrogen sulfide evolution, and genetic alteration.

1. Acidity.

Starkey and Waksman (1943) characterized two fungi (Acontium velatum and a fungus belonging to the Dematiaceae) capable of growth in synthetic medium at pH 0.7 and below. These organisms were capable of growth in media saturated with copper (approximately 25% copper sulfate; 1.16 M copper). As the pH of the medium increased towards neutrality the resistance to copper by both organisms rapidly decreased (R.L. Starkey, personal communication).

Booth and Murcer (1963) studying the copper tolerance in species of sulfur-oxidizing bacteria determined the following levels of copper tolerance: Thiobacillus thiooxidans, 20 g/liter (0.314 M); Thiobacillus concretovorus, 10 g/liter (0.157 M); and Ferrobacillus ferrooxidans, 10 g/liter (0.157 M). Each of these organisms grow well at pH levels below 3.0.

Ehrlich (1963) characterized the flora of microorganisms from acid (pH 2.5) mine waters having a copper concentration of 800 mg/liter (1.26×10^{-2} M). Major members of the flora were yeasts, protozoa and bacteria (members of the Thiobacillus-Ferrobacillus group).

Tolerance of such organisms to high concentrations of copper is believed due to the influence of pH on the surface molecules (proteins, etc.) of the organisms rendering the exterior the cell electropositive, repelling cations such as copper (Roche, 1966). Another factor is the

increase in hydrogen cations which may effectively compete for binding sites on the cell surfaces eliminating cations such as copper. Bradley and Parker (1968) studied the binding of aluminum (III) to Staphylococcus aureus 893 between pH 2.0 to 6.0. Increases in acidity detracted from the binding of the cation to the cell. The following values resulted from their experimentation:

pH	Bound aluminum, concentration x 10^{-6} M/g of dry weight
6.0	1180
4.0-4.4	240
3.9	Not detectable
2.0	Not detectable

2. Hydrogen Sulfide Evolution.

Booth and Murcer (1963) determined levels of copper tolerance for the following sulfate-reducing bacteria: Desulfovibrio desulfuricans, 50 mg copper/liter (7.8 x 10^{-4} M); D. desulfuricans (aestuarii), 20 mg copper/liter (3.12 x 10^{-4} M); D. orientis, 30 mg copper/liter (4.68 x 10^{-4} M); Clostridium nigrificans, 30 mg copper/liter (4.68 x 10^{-4} M).

Naiki (1961) found that copper tolerance by variants of S. ellipsoideus capable of growth in 1 x 10^{-3} M copper involves hydrogen sulfide production. Deprivation of a sulfur source in S. ellipsoideus greatly reduced the yeast's copper tolerance (Ashida and Nakamura, 1959). Kikuchi (1965) noted that hyper-hydrogen sulfide production by variants of S. cerevesiae enable them to tolerate as much as 2 x 10^{-3} M copper. Ashida

Higashi, and Kikuchi (1963) observed the presence of copper sulfide deposits about the periphery of copper resistant (tolerant) strains of S. ellipsoideus.

Ehrlich and Fox (1967) found two strains of Rhodotorula and one of Trichosporon capable of precipitating copper with hydrogen sulfide. Under anaerobic conditions, one strain of Rhodotorula was able to produce a five-fold increase in hydrogen sulfide, increasing the amount of copper precipitated. Ehrlich and Fox (1967) propose the use of such yeasts for reclamation of copper in mine waters.

3. Genetic Alteration.

Weed and Longfellow (1954) derived a small-colony mutant of E. coli (UV induced) which was able to tolerate a copper concentration of 5×10^{-5} M copper. This mutant could not grow in lactose, possessed a higher DNA : RNA ratio (twice that of the parent strain), and achieved lower maximal cell populations than did the parent strain. The mutant is also found to have a greater resistance to UV light.

Weed (1963) derived spontaneous mutants of B. subtilis capable of resisting concentrations of 4×10^{-4} M copper. These mutants were tryptophan auxotrophs (parent a tryptophan auxotroph) which were unable to be transformed to tryptophan independence (parent strain transformable) by the wild type organism. Large differences were noted in the base composition of the mutant's DNA.

Base	Parent strain	Mutant
Thymine	29.3%	17.9%
Adenine	28.8%	17.5%
Cytosine	21.5%	32.4%
Guanine	20.4%	32.4%

Lambina (1961) found that the resistance of a copper-sensitive strain of S. aureus was increased by transformation with the DNA of a resistant strain. Plasmids of S. aureus contain markers for resistance to metals such as lead and cadmium which may be true of copper (Richmond, 1968).

Resistant strains of S. cerevesiae to copper are formed by spontaneous mutations (Antoine, 1965). Ashida (1956) found that resistant strains of S. ellipsoideus were due to stable spontaneous mutations.

4. Possession of a Capsule.

A fourth postulated mechanism for copper tolerance (resistance) is the production or presence of a capsule or slime layer (Roche, 1966). Slime might serve to bind copper, preventing entry into the cell.

III. MATERIALS AND METHODS

A listing of chemicals used in this dissertation is shown in Table 1. The grade and manufacturer of these compounds is presented.

A. Organisms.

A culture of a bacterium identified as Pseudomonas sp. was isolated from Woods Hole seawater by Roche (1966). The organism was selected for study because of its ability to tolerate ($2.25\text{-}2.50 \times 10^{-3}$ M copper sulfate) and concentrate copper. This isolate was called Pseudomonas X by Roche (1966). When obtained, a streak culture was prepared and determined to be pure.

Cultures of P. aeruginosa, Escherichia coli, B, Staphylococcus aureus, and Bacillus subtilis obtained from the stock culture collection of the Department of Microbiology, University of New Hampshire, Durham, New Hampshire were also employed. All cultures were tested and determined to be pure.

B. Media.

The basal medium used in the majority of experiments consisted of: 1 g, yeast extract; 1 g, peptone; 750 ml synthetic seawater (Lyman and Fleming, 1940) and 250 ml distilled water (final salinity of 26 ± 1 ppt). After preparation the pH of the medium was adjusted with 0.1, 1.0, or 5.0 N sodium hydroxide (appropriately used to minimize volume changes) to 7.0. When agar was employed, 1.5% agar was added subsequent to the adjustment of the pH.

When the medium was supplemented with copper or other cations,

these additions were made prior to the adjustment of the pH. Erlenmeyer flasks containing the prepared media were covered with either aluminum foil or gauze-cotton (non-absorbant) plugs.

The basal medium was sterilized routinely by autoclaving at 121 C for 15 min.

C. Inoculum.

Unless otherwise stated the inoculum was prepared as follows: basal medium broth was inoculated with a loopfull of the marine pseudomonad (from an 18-hr basal medium slant culture) and grown to an optical density of 0.30 at 420 mu. One ml of this was then added to every 99 ml of the test medium (1.0×10^6 cells/ml final concentration).

D. Cell Density Measurements.

Optical density measurements (O.D.) were performed using one-half inch diameter cuvettes in the Bausch and Lomb Spectronic 20 colorimeter at 420 mμ. Zero transmittance was the uninoculated medium.

E. Glassware.

Borosilicate glassware used was first mechanically washed in a Heinicke glassware washer employing a distilled water rinse of 1.5 min. After air drying, the glassware was placed in a concentrated sulfuric-nitric acid bath (50/50, v/v) for a minimum of 6 hr. Upon removal from the acid bath, the glassware was rinsed 6 times with single distilled water, and 6 times with double glass distilled water. The glassware was air dried before use.

F. Aeration and Incubation of Cultures.

All broth cultures were shaken at a speed of 140 rpm on one of the following New Brunswick Scientific gyrorotary shakers: Model G-33, Metabolyte G-77 water bath shaker, or the Psychrotherm, Model G-26.

Cultures of P. X were incubated at 20 C unless otherwise stated. Cultures of P. aeruginosa, E. coli B, S. aureus, and B. subtilis were incubated at 37 C.

G. Maintenance and Preservation of Cultures.

The organisms were cultivated routinely on basal medium slants for 36 hr. Following growth, the slants were maintained at 4 C for 10-14 days before transfer to a fresh slant.

Prolonged preservation of P. X was accomplished by adding dimethyl sulfoxide (final concentration 14%) to midlog cells, and subsequently freezing 1.0 ml aliquots in small sterile screw-capped test tubes at -20 C (Green, 1966).

H. Enumeration.

Plate counts were performed using the spread plate technique (Buck and Cleverdon, 1960). Dilution blanks were prepared with 75% synthetic seawater adjusted to pH 7.0.

The Petroff-Hauser counting chamber was also employed in enumerating the marine pseudomonad. Previous to counting, the cells were fixed in 1.5% formaldehyde (final concentration) to stop motility and cell division. Such fixation was successful with no decrease in numbers during the process due to lysis. Cell counts remained stable for a period of at

least 5 days after fixation. Cells were examined in the counting chamber through the phase contrast alignment of the Zeiss WL Research Microscope (Carl Zeiss, Zena, Germany) at 1,000 X magnification.

I. Taxonomical Identification of the Isolate.

1. Cultural Characteristics.

Cultural characteristics of P. X were determined by the bacterium's growth on basal medium and Extract Agar (prepared with 75% synthetic seawater) plates and slants using the criteria appearing in the Manual of Microbiological Methods (1957) and Skerman (1967).

2. Stains.

Gram stain: Gram stains were performed through the use of the Hucker modification (American Public Health Association, 1960).

Spore stain: The spore stain was performed through the use of malachite green (Pelczar, 1965).

Flagellum stain: The flagellum stain was performed by the Leifson flagellum stain according to Skerman (1967).

Capsule stain: The capsule stain was performed by employing the Hiss capsule stain (Pelczar, 1965).

Shadow casting for electron microscopic examination of flagella: Mid-log cells grown in the basal medium were fixed in 2% glutaraldehyde for 30 min, washed three times in distilled water and placed on formvar coated copper grids. The grids were shadow cast with tungstic oxide at an angle of 30 degrees and examined

with the Akashi Tronoscope I electron microscope.

3. Differential Media.

Gelatin liquefaction: 10% gelatin was supplemented to the basal medium. Ten ml aliquots of the medium were dispensed into screw-capped test tubes and sterilized by autoclaving at 121 C for 15 min. After hardening at 20 C, the tubes were inoculated with a stab inoculum and reincubated at 20 C. The tubes were checked daily for evidence of liquifaction.

Indole production and nitrate reduction: Both these tests were performed using Tryptic Nitrate Broth prepared with 75% synthetic seawater, and autoclaved at 121 C for 15 min. Inoculated tubes of this medium were incubated both aerobically and anaerobically at 20 C. After growth, indole formation was tested by using Kovac's reagent in a spot test. Nitrate reduction was measured using the Griess-Ilosvay reagent.

Starch hydrolysis: Plates of Starch Agar were prepared with 75% synthetic seawater, and inoculated by streaking with P. X. After growth, starch hydrolysis was tested by flooding the plates with iodine solution (Pelczar, 1965).

Oxidase activity: Oxidase activity was tested using tetramethyl-paraphenylene diamine hydrochloride according to Skerman (1967).

Catalase activity: The presence of catalase was determined by adding 5 drops of 3% hydrogen peroxide to a fresh basal medium slant culture of P. X (Pelczar, 1965).

Litmus milk: Because of the difficulty in preparing Litmus Milk Media in 75% synthetic seawater, the components of the medium were prepared separately. Azolitmin (0.05 g) was dissolved in 30 ml of 100% synthetic seawater, followed by the addition of 2 drops of 1.0 N sodium hydroxide. Powdered Skim Milk (10.0 g) was dissolved in 70 ml of distilled water. These solutions were sterilized by autoclaving at 110 C for 10 min. After sterilization, the two solutions were mixed aseptically and placed in 10 ml volumes into sterile screw-capped test tubes. The tubes were inoculated with a drop of an 18-hr basal medium broth culture of P. X. The tubes were checked daily for growth and changes in the medium.

Citrate, acetate, succinate, and lactate utilization: Solutions (1.0%) of citrate, succinate, acetate, and lactate (sodium salts) were prepared in 75% synthetic seawater supplemented with 0.1% ammonium nitrate and 20 ug/ml arginine. Agar (1.5%) was added and the medium autoclaved at 110 C for 10 min. Following sterilization, agar plates were prepared and inoculated by streaking with P. X. The plates were incubated at 20 C and checked daily for the presence of growth.

Acetylmethylcarbinol formation: MR-VP medium was prepared in 75% synthetic seawater and inoculated with a drop of an 18-hr basal medium broth culture of P. X. Following growth, acetylmethylcarbinol formation was assayed by the method of Barrit (1936).

Carbohydrate utilization: Utilization of carbohydrates was examined by the use of Phenol Red Broth Base or a synthetic base. When Phenol Red Broth base was utilized, 0.5% carbohydrates were added to the base. The prepared medium was placed in 3.0 ml volumes into serological tubes with inverted Durham tubes and autoclaved at 110 C for 10 min. Following sterilization the tubes were inoculated with one drop of an 18-hr basal medium broth culture of P. X and checked daily for changes in the medium.

When employing the synthetic base for carbohydrate utilization, individual sugars were prepared separately from the medium base. Individual carbohydrates were prepared as 5% solutions in 75% synthetic seawater. These solutions were adjusted to a pH of 7.4 with sodium hydroxide and autoclaved at 110 C for 10 min. The basal portion of the medium consisted of a 75% synthetic seawater solution of 0.125% ammonium nitrate, 0.0025% phenol red, 0.0025% dibasic potassium phosphate, and 0.0025% arginine. This portion of the medium was adjusted to pH 7.4 with sodium hydroxide and dispensed in 2.4 ml volumes into serological tubes with inverted Durham tubes. The tubes were autoclaved at 110 C for 10 min. The complete carbohydrate tubes were prepared by adding 0.6 ml of the carbohydrate solution to the 2.4 ml of base. The completed medium contained 1.0% of the test carbohydrate. The prepared fermentation tubes were inoculated with a drop of an 18-hr basal medium broth culture of P. X. Uninoculated

controls were maintained simultaneously along with an inoculated control of the broth base (no carbohydrate additive).

Hydrogen sulfide production: Lead Acetate Agar, Triple Sugar Iron Agar and Kliegler's Iron Agar were prepared with 75% synthetic seawater. Slants of the basal medium with suspended lead acetate impregnated strips were also prepared. The prepared slants were inoculated with a combination stab-streak inoculum of P. X. and incubated at 20 C. The tubes were examined daily for growth and evidence of hydrogen sulfide production.

Motility: Motility was checked by hanging drop preparations and wet mounts using the Zeiss WL Research Microscope.

J. Polarographic Measurements.

Samples (10 ml) of prepared media were analyzed polarographically using a Sargent Model XXI polarograph (E.H. Sargent & Co., Chicago, Ill.). Before measurement, the sample was gassed with oxygen-free nitrogen for 10 min to prevent oxygen interference. A scan of -1.5 to 0.0 V was employed for the detection and measurement of copper.

K. Anodic Stripping Voltammetry Determinations.

Determinations employing this method were performed in the laboratory of Wayne Matson of Enviromental Science Associates, Cambridge, Massachusetts. Measurements were performed employing a MASA 2014 anodic stripping analyzer (Enviromental Science Associates, Cambridge, Mass.). Prior to analysis samples were purged with oxygen-free nitrogen gas for a period of 5 min. A plating potential of -0.800 V vs S.C.E. (standard calomel electrode) was

applied to all samples for a 10 min period. In measurement a sweep of 66 mv/sec was employed from the plating potential to 100 V vs S.C.E. Measurements were compared with standards prepared in distilled water and tested in the same manner.

L. Sampling Procedures Employed in Experimentation Involving ^{64}Cu.

Cell suspensions grown in the presence of ^{64}Cu for the study of copper uptake and localization were processed according to the following procedure.

1. 1.2 ml Samples.

When 1.2 ml samples were withdrawn from a test flask, 0.4 ml of 6% formaldehyde was added to stop the uptake of copper, cell division and motility of P. X. From these formalized suspensions, a 0.5 ml aliquot was removed for a cell count employing the Petroff-Hauser counting chamber. The remaining 1.1 ml of sample was placed in a 13 x 109 mm polycarbonate tube and centrifuged at 39,900 x g for 5 min. The supernatant was decanted and the cells washed in 1.1 ml of 75% synthetic seawater at the same centrifugation speed. The supernatant of this wash was removed and the pellet resuspended in 1.1 ml of 75% synthetic seawater. A 0.4 ml sample of this suspension was placed on a 25 mm Millipore HA (porosity 0.45 u) filter (Millipore Corp., Bedford, Mass.) in a microanalysis filter holder (Millipore Corp., Bedford, Mass.). When filtration was complete, 1.0 ml of 75% synthetic seawater was used to wash the cells on the filter.

Upon completion of the process, the filters were removed from the filter holders and dried under a heat lamp. Subsequent to drying, the

filters were placed in borosilicate scintillation vials (New England Nuclear Corp., Boston, Mass.) containing 10 ml of liquifluor solution prepared with toluene.

2. 0.9 ml Samples.

When 0.9 ml samples were employed, 0.3 ml of 6% formaldehyde was used as a fixative. When using this sample volume no portion was withdrawn for cell counts. The washing volume employed with this type of sample was 1.2 ml of 75% synthetic seawater.

3. Cell Extracts and Media.

When cell extracts were counted, 0.1 ml samples of the extracts were spotted on pieces of Whatman #2 filter paper and dried under a heat lamp. The dried filter papers were then placed in scintillation vials containing 10 ml of liquifluor solution.

In determining the precise amount of radioactivity available in the medium, 0.02 or 0.04 ml aliquots were removed prior to inoculation with the organism and treated in the same manner as the cell extracts.

4. Quantitation of ^{64}Cu.

All samples were counted with a Nuclear Chicago Model 720 scintillation counter (Nuclear Chicago, Chicago, Ill.).

Because ^{64}Cu possesses a half-life of 12.75 hr, it was necessary to compensate for changes in the quantity of isotope during the counting process. The first vial counted in a counting run was placed at the end of the same run and recounted. The elapsed time between this sample's first and second counting was noted. In knowing the difference in count

(degradation) and the elapsed time occurring between the sample's first and second counting, a degradation curve was constructed so that all vials could be standardized with respect to the first vial counted.

M. Electron Microscopy of Cells Grown in Copper-Supplemented Basal Medium.

A culture of P. X was sent, along with our dehydrated medium and copper sulfate, to the laboratory of George Hageage at the National Institute of Dental Health, National Institutes of Health, Bethesda, Maryland. In his laboratory, the medium was prepared and P. X grown as previously described. Cells grown in the basal medium and the basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper sulfate is harvested at an optical density of 0.85 at 420 mu. After harvesting by centrifugation, the cells were washed three times with 75% synthetic seawater.

After washing the fixation and staining process was begun by suspending the washed cell pellets in 75% synthetic seawater containing 6.25% glutaraldehyde for 12 hr at 4 C. The fixed cells then were harvested by centrifugation and enrobed in 2.0% molten agar (50 C). After the solidification of the agar, small blocks were cut and placed in 1.0% OsO_4 for 2 hr at ambient temperature. The blocks were collected by centrifugation and placed in 0.5% uranyl acetate for 1-2 hr at ambient temperature. The blocks were again harvested and subsequently dehydrated in an acetone series of 15, 30, 70, 90, and 100%. The blocks were placed in Vestopal W and polymerized at 60 C for 24 hr.

Thin sections were cut from the prepared blocks using an LKB ultramicrotome. Sections were collected and post-stained with saturated, aqueous

uranyl acetate for a 30 min period. Subsequent to post-staining, the sections were examined in a Siemens Elmiskop I electron microscope.

N. Determination of DNA : RNA : Protein Ratios.

Duplicate 100 ml portions of the following media were prepared and placed in 250 ml Erlenmeyer flasks: basal medium, and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper sulfate. The media were autoclaved at 121 C for 15 min, and inoculated with P. X. Upon attaining an optical density of 0.85 at 420 mu, the cells were harvested by centrifugation at 39,900 x g for 5 min at 4 C. The cells were washed three times in 50 ml volumes of 75% synthetic seawater. After washing, the cells were resuspended in 10 ml of 75% synthetic seawater and subjected to the following modification of the Schneider (1945) extraction technique.

Prior to extraction, duplicate 0.5 ml volumes of the suspension were removed and placed in pre-tared aluminum weighing pans for dry weight determinations. The pans were maintained at 110 C until a constant weight was obtained. A 0.5 ml aliquot was removed for Kjeldahl nitrogen determinations. A fourth 0.5 ml aliquot was removed and mixed with 0.5 ml of 3.0% formalin for a direct cell count. Following the withdrawal of these aliquots, the suspension was centrifuged at 39,900 x g, the supernatant decanted, and the extraction procedure begun.

The pellet was resuspended in 4.0 ml of 0.2 N perchloric acid and incubated for 15 min at 4 C. The suspension was centrifuged at 39,900 x g for 5 min and the supernatant saved. The residue was then suspended in 4.0 ml of an ethanol-ether solution (50/50, v/v). This new suspension was

maintained at 50 C for 30 min, and after incubation, centrifuged at 39,900 x g for 5 min at 4 C. The supernatant was saved and the pellet resuspended in 8.0 ml of 2.0 N perchloric acid. This suspension was incubated at 95 C for 10 min and then centrifuged at 39,900 x g for 5 min at 4 C, saving the supernatant for analysis. The residual pellet was saved for protein determinations, suspending the pellet in 4.0 ml of 1.0 N sodium hydroxide when the determinations were performed.

All 4.0 ml extracts were brought up to a final volume of 5.0 for analysis. The 8.0 ml extract was brought up to a final volume of 10 ml.

DNA was measured by the technique of Burton (1956) using DNA ex salmon sperm for a standard. RNA was determined by the method of Cerriotti (1955) using 2', 3'-adenosine monophosphate as a standard. Protein was measured by the method of Lowry et al (1951) using bovine serum albumin fraction V as a standard.

O. The Effect of Copper on Respiration.

One hundred ml portions of basal medium and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, 1×10^{-3} and 2×10^{-3} M copper sulfate were prepared in 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. The media were inoculated on separate days.

Upon attaining stationary phase, the cells were harvested by centrifugation at 39,900 x g for 5 min at 4 C. The cell pellets were washed in three 50 ml washes of 75% synthetic seawater. The final pellet was resuspended in 100 ml of 75% synthetic seawater. Duplicate 1.0 ml samples were removed and placed in pre-tared aluminum weighing pans for dry weight

determinations. The pans were maintained at 110 C until a constant dry weight was obtained from each. A 0.9 ml aliquot was removed and mixed with 0.3 ml of 6.0% formalin, and later the cells counted by use of the Petroff-Hauser counting chamber. A fourth aliquot was withdrawn for the determination of total protein as measured by Lowry et al (1951).

Respiration was measured by the use of the GR-20 Gilson differential respirometer (Gilson Medical Electronics, Milwaukee, Wisc.) using a reciprocal shaking speed of 140 rpm and an incubation temperature of 20 C. Flasks for the respirometer were prepared according to Table 2.

P. Triphenyl Tetrazolium Chloride (TTC) Overlay Technique for Determining Respiratory Sufficiency or Deficiency.

This procedure was based on that of Lindegren, Nagai, and Nagai (1958). In this method, the bacteria were cultivated on agar plates until good growth was apparent. After cultivation, the plates were overlayered with a 1.5% agar or a 10% gelatin solution containing 0.1% triphenyl tetrazolium chloride (TTC). The appearance of a red color (due to the reduction of TTC to a red formazan) associated with the bacterial colonies within one hr at 20 C was considered indicative of respiratory sufficiency. Reduction of TTC to the formazan in excess of one hr was considered as evidence of a respiratory deficiency.

The agar and gelatin solutions were prepared in 75% synthetic seawater, autoclaved at 121 C for 15 min and subsequently dispensed in 14 ml amounts into sterile test tubes. Triphenyl tetrazolium chloride was prepared separately as a concentrate (0.128 g/ml) and sterilized by filtration.

The "complete" overlay was prepared by adding 2.0 ml of the TTC concentrate (warmed to a compatible temperature) to either 14 ml of 50 C molten agar or 37 C molten gelatin.

Q. Qualitative and Quantitative Analysis of Cytochromes.

Five hundred ml volumes of media were prepared in one liter Erlenmeyer flasks. After preparation the flasks were inoculated with the standard inoculum and incubated at 20 C on a gyrorotary shaker at 140 rpm.

When cell densities attained an optical density of 0.85 at 420 mu, the cells were harvested by centrifugation at 19,900 x g for 10 min at 4 C. The cells were washed three times with 250 ml 75% synthetic seawater. After the final wash, the cell pellets were resuspended in 5.0 ml volumes of 75% synthetic seawater.

The final cell suspensions were disrupted by sonication employing a 100 watt M.S.E. sonicator (Measurement Scientific Electronics, London) with a sonic amplitude of 4 μ peak to peak. Six 1.0 min treatments were employed with a 1.0 min cooling time between treatments. Upon completion of the sonication process, wet mounts of the suspensions were prepared and examined under the phase microscope at 1,000 X magnification. The disruption process was considered complete if three or less intact cells per phase field were observed. If the disruptive process was found to be incomplete, then two additional sonication treatments were employed.

Following disruption the suspensions were centrifuged at 3,500 x g for 30 min to remove the residual intact cells. Following this centrifugation, the supernatant was removed and saved for spectrophotometric anal-

ysis of the cytochromes.

1. Difference Spectra.

Qualitative and quantitative measurements of the cytochromes were performed on the whole cell extracts employing the difference spectra technique of Chance (1954). In this procedure, the test cuvette was reduced by the addition of a few crystals of sodium dithionite.

2. Carbon Monoxide Spectra.

Upon completion of the difference spectra, the reference cuvette was reduced with sodium dithionite. Carbon monoxide was obtained by reacting formic acid and concentrated sulfuric acid in the presence of heat. Carbon monoxide generated in this manner was bubbled at 30 sec intervals into the test cuvette until no changes in the spectral analysis could be observed.

All spectral analyses were performed using a Cary model 15 spectrophotometer employing the one-tenth scale. A scan of 650 to 400 mμ was employed for all spectral analyses.

R. Thunberg Studies.

One hundred ml portions of basal media (unsupplemented and supplemented with copper) were prepared in 250 Erlenmeyer flasks. Following autoclaving at 121 C for 15 min, the media was inoculated with the standard inoculum and incubated at 20 C on a gyrorotary shaker. Upon attaining an optical density of 0.85 at 420 mμ, the cells were harvested by centrifugation at 39,900 x g for 5 min. The cell pellets were washed three times in 50 ml volumes of 75% synthetic seawater. The final pellet was resuspended in 25 ml

of synthetic seawater for use in the experiment.

Triphenyl tetrazolium chloride, methylene blue, and dichloroindophenol were employed as O/R dyes to measure rates of electron transport in P. X. All dyes were prepared as concentrates (0.0021 g/ml) in distilled water.

Thunberg tubes were prepared according to Table 3. The final concentration of all dyes employed was 0.0005%. After preparation, the tubes were equilibrated at 20 C for 20 min. The tubes were evacuated by use of a vacuum pump for 5 min, sealed, and the experiment started. All tubes were incubated at 20 C and measured at 1.0 min intervals at the appropriate wavelength using the Bausch and Lomb Spectronic 20 colorimeter. A common blank was prepared of a formalized suspension of the organism, and employed in all readings.

Reduction of TTC was measured at a wavelength of 590 mμ. Methylene blue and dichloroindophenol reduction were measured at 600 mμ.

S. Quantitation of Copper Uptake by Anodic Stripping Voltammetry.

Five hundred ml of basal medium and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper sulfate were prepared in one liter Erlenmeyer flasks and autoclaved at 121 C for 15 min. The media were then inoculated with the standard inoculum and incubated at 20 C at 140 rpm until the attainment of an optical density of 0.85 at 420 mμ. The cells of each flask were harvested at 19,900 x g for 10 min and washed in three 250 ml volumes of 75% synthetic seawater. The final pellet was resuspended in 10 ml of 75% synthetic seawater, and lypholized

in serum bottles. The resulting lypholized cells were mixed to a homogeneous powder with a mortar and pestel. Duplicate 1 mg samples of each powder were removed for the determination of total protein (Lowry et al, 1951). The remaining powder was weighed and placed into test tubes.

Each cell powder was digested in 70% perchloric acid (G.S. Smith) in the presence of heat until a clear solution was obtained. The resulting digest was brought up to 25 ml with double glass distilled water and 10 ml samples analyzed by anodic stripping voltammetry measurement (Methods section K).

IV. RESULTS

A. Species Identification of Pseudomonas X.

Roche (1966) isolated the bacterium from Woods Hole seawater identifying it as a member of the genus Pseudomonas (P. X.) Insufficient taxonomical study was performed to confer species identification or determine whether it was a new species. More work was undertaken here to establish the bacterium as a member of the genus Pseudomonas and identify it as a species.

1. Morphology.

The organism was a gram-negative rod (0.5-0.75 μ x 1.0-1.2 μ). On basal medium slant and in broth culture the bacterium was observed as singles, pairs, and short chains. In broth culture chain formation (8-15 cells) was prevalent in the logarithmic phase of growth. In stationary phase, cells previously attached in chains decreased to singles, pairs and short chains.

The bacterium was actively motile in hanging drop preparations and wet mounts. The flagellation of the organism was of interest because of its importance in the taxonomy of the genus Pseudomonas (Skerman, 1967). Leifson flagella stains and shadow cast preparations observed with the electron microscope (Fig 1) demonstrated a single polar flagellum associated with the organism as observed by Roche (1966).

Observations with spore and capsule stains showed the organism was non-sporulating and non-encapsulated.

2. Cultural Characteristics.

Basal Medium slant: Growth abundant, filiform, glistening, opaque, and cream in color.

Basal Medium plate: Colonies entire, convex, opaque, and cream in color. Colonies circular, 3-4 mm in diameter after 36 hr growth.

Extract Agar slant: Same as Basal Medium slant.

Extract Agar plate: Same as Basal Medium plate.

3. Differential Media.

Gelatin hydrolysis: Positive, crateriform after 30 hr.

Starch hydrolysis: Positive. Growth on starch plates noted after 72 hr. After 120-hr incubation, clear evidence of starch hydrolysis.

Indole formation: Negative. Good growth in the medium.

Nitrate reduction: Negative. Good growth in the medium when grown aerobically. No growth in medium when grown anaerobically.

Hydrogen sulfide production: Negative. Good growth on all media tested.

Litmus milk: No growth after 21-day incubation period.

Acetyl-methyl-carbinol: Negative.

Utilization of citrate: Small colonies (1-2 mm) after 48-hr incubation period. Subsequent transfers to second and third plates yielded the same growth.

Utilization of acetate: Same as citrate.

Utilization of succinate: Same as citrate.

Utilization of lactate: Negative. No growth after 21-day incubation period.

Carbohydrate utilization: In Phenol Red Broth Base after 2-day's incubation, growth with a pellicle was noted in the presence of arabinose, dextrin, dextrose, galactose, glycerol, lactose, mannitol, sucrose, and xylose. Acid reactions with no gas were noted in only two of these carbohydrate tubes: sucrose and dextrose. All other reactions where growth occurred were alkaline with no gas.

In the synthetic base the following reactions were observed: acid but no gas from cellobiose, dextrin, dextrose, galactose, glycerol, glycogen, lactose, maltose, mannitol and sucrose. All positive carbohydrate tubes were positive within 1-day's incubation with the exception of glycerol which showed a positive reaction on the 17th day of incubation.

The following carbohydrates prepared in the synthetic base were not utilized after 30 days of incubation: arabinose, cellulose, raffinose, ribose, sorbitol, and xylose.

An inoculated control of the synthetic base with no carbohydrate supplement showed no growth throughout the 30-day incubation at 20 C. Uninoculated controls of all the carbohydrate tubes showed no change throughout the 30-day incubation period.

Catalase: Negative.

Oxidase: Positive.

4. Temperature Requirement.

Basal medium broth cultures of P. X incubated at various growth temperatures revealed P. X capable of growth from 4 C (lowest temperature tested) through 33, but not 36 C. The optimum temperature of P. X was between 33-36 C (see section H of Results section). Maximum cell densities were attained in cultures incubated between 20-33 C.

5. Speciation

P. X was an obligate aerobe with definite requirements for inorganic ions which were satisfied by 75% synthetic seawater. Growth did not develop in media prepared solely with distilled water.

The differences noted in carbohydrate utilization using Phenol Red Broth Base and the synthetic base may be explained by noting that Phenol Red Broth Base contains a substantial amount of peptone (trypticase peptone, 10 g/liter). During peptone degradation ammonia is produced which masks acid production resulting from carbohydrate utilization (Stanier, Palleroni, and Doudoroff, 1966).

The characteristics of P. X determined in this dissertation correspond with descriptions for the genus Pseudomonas presented in Bergey's Manual of Determinative Bacteriology (7th edition, 1957) and Skerman's Guide to the Identification of the Genera of Bacteria (1967) establishing the bacterium as a member of the genus. No citings of a Pseudomonas species complying with the characteristics of this marine isolate were found in Bergey's 6th or 7th editions or other relevant literature (Payne, Eagon and Williams, 1961; Humm, 1946; Zobell and Upham, 1944; Colwell and

Sparks, 1967; O'Neil, Drisko and Hochman, 1961; Burkholder, Pfister and Leitz, 1966; Hayward, 1964; Hansen, Weeks and Colwell, 1960).

Shewan, Hobbs, and Hodgkiss (1960) developed a schema for the identification of certain gram-negative bacteria. Special attention was given to the genus Pseudomonas. Four major groupings of the genus Pseudomonas were established on the basis of carbohydrate utilization, pigment production, flagellation, and other criteria. P. X agreed best with the description of Group II of the schema. This group has P. fragi as its prototype.

More recent advances in the taxonomy of the genus Pseudomonas have been offered by Stanier, Palleroni, and Doudoroff (1966); and Colwell and Liston (1961 and 1961a). These authors have subjected bacteria to a number of substrates to determine utilization. Colwell and Liston (1961 and 1961a) have incorporated Adansonian taxonomy in classifying species of this genus. This work has not yet solidified into a standard schema for the identification of species within the genus Pseudomonas and has not been employed in this dissertation.

Because of the bacterium's lack of similarity to other members of the genus Pseudomonas, is described as a new species, P. cuprodurans, in accordance with the International Code of Nomenclature of Bacteria (1966). The species name complies with Recommendation 6a (1) of the code as it describes a property of the species: the ability to withstand copper ions (see section D of Results section).

In the taxonomic key for the genus Pseudomonas presented in Bergey's

7th edition (1957) this new species is placed with P. membranoformis.

P. cuprodurans differs from P. membranoformis in several characteristics:

Characteristic	P. membranoformis	P. cuprodurans
Flagellation	Lophotrichous	Monotrichous
Size of organism	0.9-1.2 μ x 3.5-4.8 μ	0.5-0.75 μ x 1.0-1.2 μ
Lactose oxidation	Negative	Positive
Starch hydrolysis	Negative	Positive
Possession of a capsule	Encapsulated	Non-encapsulated
Optimum temperature	20-25 C	30-33 C
Cultural characteristics	Colonies have membranous consistency	Colonies have non-membranous consistency

P. cuprodurans and P. membranoformis are placed in the same segment of the taxonomic key for the genus Pseudomonas (Bergey's 7th edition, 1957) because of the following characteristics: seawater requirement, gelatin liquefaction, possession of polar flagella, lack of growth in litmus milk, inability to produce indole, inability to produce hydrogen sulfide, and the ability to oxidize glucose.

A culture of P. cuprodurans will be sent to R.R. Colwell of Georgetown University for further study to substantiate this new species.

B. Salinity Requirement.

ZoBell and Upham (1944) defined a marine bacterium as one which required seawater upon initial isolation. P. cuprodurans required seawater

during its initial isolation (Roche, 1966). This seawater requirement has remained associated with P. cuprodurans; there is no growth in media prepared with distilled water. A salinity experiment was conducted to determine the effect of inorganic concentrations of seawater required by P. cuprodurans.

Basal medium was prepared in 100% synthetic seawater and distilled water. Seawater concentrations were varied by the method of continuous variation at 10% intervals; i.e. 80% seawater could be prepared by mixing 80 ml of the 100% synthetic seawater medium with 20 ml of the distilled water medium. One hundred ml aliquots at each salinity were placed in 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. Each flask was inoculated with 0.1 ml of an 18-hr broth culture of P. cuprodurans and shaken at 20 C. Growth of P. cuprodurans was determined by optical density measurements at 420 mμ using a Bausch and Lomb Spectronic 20 colorimeter (Table 4).

Optimal growth response of P. cuprodurans occurred at salinity levels of 60-100% (Table 4). A progressive delay and reduction in the optical density of P. cuprodurans cultures was observed when salinity levels were decreased below 60% until the growth limiting salinity of 20% was reached. Inoculated flasks containing medium prepared with 10 and 0% seawater were maintained at 20 C for 168 hr with no evidence of growth during this incubation.

The optimal range for salinity of 60-100% seawater demonstrated a definite seawater requirement indicating P. cuprodurans was a marine

bacterium by the definition of ZoBell and Upham (1944).

C. Major Ion Requirements of P. cuprodurans.

To furthur study the specific salinity requirements of P. cuprodurans a study of the bacterium's major ion requirements was undertaken. The individual constituents of 75% synthetic seawater appearing in Table 5 were prepared as 10 X solutions so that the addition of 10 ml of each constituent to a 250 ml Erlenmeyer flask would yield 100 ml of 75% synthetic seawater. Full or partial deletions of any salt could be made by the omission of 10 or less ml of a solution. When deletions were made, glycerol equal in molarity to the moles of the anion or cation deleted was added (glycerol not utilized by P. cuprodurans for 17 days; Results section A-3) to maintain the same osmotic pressure of the medium (MacLoed and Onofrey, 1956). Solutions (100 ml) containing the ionic deletions shown in Table 6 were prepared in 250 ml Erlenmeyer flasks with the organic constituents of the basal medium added to each. The prepared media were adjusted to pH 7.0 with 1.0 N NH_4OH, autoclaved at 121 C for 15 min, and inoculated with 0.1 ml of an 18-hr basal medium broth culture of P. cuprodurans. The inoculated flasks were incubated at 20 C at a shaking speed of 140 rpm with growth followed by optical density measurements at 420 mμ. Throughout their growth the cells were checked for alteration in morphology by microscopic observations under oil immersion made with the Zeiss WL Research Microscope.

1. Sodium Requirement.

Results appearing in Table 7 indicate a definite sodium requirement of P. cuprodurans as evidenced by the absence of growth with the deletion

of the sodium ion (Flask #1). Gradual increases in sodium levels from 3.492 mM to 138.28 mM sodium correspondingly heightened the growth response of P. cuprodurans with optimal growth attained at a sodium level of 138.28 mM (Table 7: Flask 4, 5, 6, and 7).

2. Magnesium Requirement.

When P. cuprodurans was cultivated in magnesium depleted medium optical density measurements could not be performed because of the formation of visible aggregates which were unevenly distributed in solution (Table 7: Flask #9). Cells comprising such aggregates were polymorphic with filamentous and round forms present (Fig 2). The addition of 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M $CuSO_4$ was observed to have no effect on the formation of such aberrant cells. The addition of 3.92 mM magnesium was sufficient to satisfy the bacterium's need for the ion (Table 7: Flask #10).

3. Calcium Requirement.

The deletion of calcium from the 75% synthetic seawater medium caused a reduction in the growth of P. cuprodurans (Table 7: Flask #13). Cells in this calcium-deleted medium were examined with the phase microscope and were large swollen cells with a diameter of approximately four times the length of the normal organism or 4.5 μ (Fig 3). The formation of these aberrant cells began in the lag phase of growth and swelling increased during subsequent growth. The presence of 1.48 mM calcium in the basal medium yielded a maximum growth response, but aberrant morphology in cells was not completely eliminated (less than 1.0% aberrant). The presence of 2.96 mM calcium in the basal medium yielded maximum growth with

no aberrant cells present, and hence was considered optimal for P. cuprodurans.

4. Other Ion Requirements.

No requirements for sulfate (Table 7: Flask #8), strontium (Flask #17), borate (Flask #18), or potassium (Flask #16) were observed in this experimentation. Requirements for these ions, especially potassium, may be satisfied by ionic contaminants present in the basal medium organic constituents: yeast extract, 10.5% ash (0.0126 g/100 ml of basal medium); peptone, 3.0% ash (0.003 g/100 of basal medium) (Fisher Scientific Co., 1966).

5. Defined Inorganic Constituents.

When the basal medium was prepared with 0.3098 M sodium, 39.2 mM magnesium and 7.4 mM calcium growth of P. cuprodurans was noted to equal that of the organism grown in basal medium (prepared with 75% synthetic seawater, Table 7: Flask #20). This clearly indicated the bacterium's requirements for only the three cations.

D. Copper Tolerance of P. cuprodurans.

Roche (1966) placed the maximum level of copper tolerance for P. cuprodurans (P. X.) at 2.25-2.50 x 10^{-3} M copper in 2216E medium (Oppenheimer and ZoBell, 1952). This medium contained 5.0 g/liter peptone, 1.0 g/liter yeast extract, and a small addition of ferric ion as ferric ammonium citrate (0.01 g/liter) (Roche, 1966). Since the basal medium contained 1.0 g/liter peptone, 1.0 g yeast extract, and no ferric ion additive, the maximum level of copper tolerance for P. cuprodurans was redetermined in the basal medium.

1. Broth Studies.

The basal medium (100 ml) supplemented with varied molarities of copper sulfate (1×10^{-5} to 3×10^{-3} M) was prepared in 250 ml Erlenmeyer flasks. All media were adjusted to pH 7.0 with sodium hydroxide and autoclaved at 121 C for 15 min. Each flask was inoculated with the standard inoculum (Methods section C) and incubated at 20 C on a gyrorotary shaker at 140 rpm. Optical density measurements at 420 mμ and pH measurements were performed during the growth of P. cuprodurans (Table 8).

No significant difference in the optical density of P. cuprodurans in the basal medium and the basal medium with copper supplementation up to 1×10^{-4} M was observed (Table 8). Supplementation of the basal medium with copper levels of 5×10^{-4} M and higher had the general effect of prolonging the lag phase of growth and decreasing the maximum optical density (Table 8).

The maximum level of copper tolerance for P. cuprodurans was $2.25\text{-}2.50 \times 10^{-3}$ M copper which was identical to the value determined by Roche (1966) (Table 8). Precipitation occurred in basal medium when supplemented with the toxic level of copper (2.50×10^{-3} M) after adjusting the pH to 7.0 prior to autoclaving. Precipitation increased with additional copper supplementation above the 2.50×10^{-3} M copper level.

Cells cultivated in high concentrations of copper (5×10^{-4} to 2.25×10^{-3} M) maintained normal morphology throughout their growth.

Measurements of pH with growth of P. cuprodurans demonstrated a decrease in the pH of the medium (approximately 0.2-0.3 pH units) during early growth (Table 9). After continued growth, the pH of the medium became

more alkaline, attaining a pH of 8.0-8.2 in the stationary phase of growth. Prolonged incubation of stationary phase cells led to a maximal pH value of 8.4 to 8.5 in the flasks (Table 9: 144 hr).

2. Copper Tolerance Studies With Agar Plates.

To determine if agar plates could be used for the accurate determination of copper tolerance, the media used in the previous section (Results section D-1) were prepared with 1.5% agar and agar plates prepared. The plates were inoculated by streaking a loopful of an 18-hr basal medium broth culture of P. cuprodurans upon them. The plates then were incubated for a 10-day period at 20 C, and checked for the presence of growth.

Results appearing in Table 10 show that the same level of copper tolerance could be derived for P. cuprodurans by this method. Colonies observed on plates of the basal medium containing 1-2.25 x 10^{-3} M copper developed a greenish tinge apparently indicating the concentration of copper by the colonies. Colonies developing on basal medium agar plates with 2.00-2.25 x 10^{-3} M copper were reduced in size, having a diameter of 1-3 mm after the 10-day incubation period compared to a 3-4 mm diameter of cells grown on basal medium agar.

3. Copper Tolerance Studies on Non-marine Bacteria.

Because of their reliability in determining copper tolerance, basal medium agar plates prepared with distilled water and 75% synthetic seawater were employed to determine the copper tolerance levels of the following four bacteria: E. coli B, P. aeruginosa, B. subtilis, and S. aureus. A 10-day incubation period at 37 C was employed for these studies.

B. subtilis was the least tolerant to the four bacteria tested being able to tolerate a copper level of 5×10^{-5} M (Table 11). This level of copper tolerance for B. subtilis was constant when the bacterium was cultivated on basal medium prepared with distilled water or 75% synthetic seawater. S. aureus tolerated a level of 1×10^{-4} M copper on both types of the prepared media (75% synthetic seawater or distilled water) (Table 12). E. coli B tolerated 1×10^{-4} M copper on media prepared with distilled water, and 4×10^{-4} M copper on media prepared with 75% synthetic seawater (Table 13). P. aeruginosa tolerated more copper than did the other three organisms, tolerating 1×10^{-3} M copper in both the distilled water and 75% synthetic seawater media (Table 14).

E. The Effect of Organic Matter on Copper Tolerance.

To determine the role of organic nutrients in copper tolerance of P. cuprodurans the following experiment was undertaken. Basal medium broth was prepared and diluted with 75% synthetic seawater to yield the following combined levels of peptone and yeast extract (50/50, w/w): 2.000, 1.000, 0.800, 0.600, 0.400, 0.200, 0.100, and 0.050 g/liter. Solutions of 100 ml of these levels of organic nutrients were prepared in triplicate. One flask from each level of organic supplement was untreated with copper, the second treated with 5×10^{-4} M copper, and the third with 1×10^{-3} M copper. All media were prepared in 250 ml Erlenmeyer flasks, adjusted to pH 7.0 with sodium hydroxide, and autoclaved at 121 C for 15 min.

An inoculum was prepared by aseptically harvesting 30 ml of a basal medium broth culture (O.D. at 420 mμ of 0.30) of P. cuprodurans by centri-

fugation at 19,900 x g for 5 min at 4 C. The pellet was washed aseptically three times with 15 ml of sterile 75% synthetic seawater at 19,900 x g for 5 min at 4 C. The final pellet was resuspended in 30 ml of sterile 75% synthetic seawater and 1.0 ml of this suspension used to inoculate each of the prepared flasks. Growth of P. cuprodurans was followed by optical density measurements at 420 mμ using sterile controls for zeroing the colorimeter.

Growth (as O.D.) of P. cuprodurans occurred at all levels of organic nutrients employed (unsupplemented with copper) with a linear relationship of growth to total organic matter occurring at the 0.200,0.100, 0.050 g/liter of total organic matter (Table 15). Copper at 1×10^{-3} M was tolerated at a total organic nutrient level of 0.800 g/liter, and toxic at a total organic nutrient level of 0.600 g/liter (Table 15). Copper at 5×10^{-4} M copper was tolerated in media containing 0.400 g/liter total organic nutrients, and toxic at 0.200 g/liter total organic matter (Table 15).

The presence of precipitation in the medium was noted when the levels of 1×10^{-3} and 5×10^{-4} M copper became toxic with decreased levels of total organic nutrients (0.600 and 0.200 g/liter respectively). In all instances when copper was added the lag phase of growth was extended and the maximum O.D. at 420 mu decreased, with these effects amplified as the copper supplements became more toxic with dilution of organic matter (Table 15).

Using the 2.25×10^{-3} M copper tolerance level determined for the basal medium (2.00 g/liter total organic nutrients) in Results section C-1 and C-2 and the values obtained here, a graph was prepared plotting the copper

tolerance level with the total level of organic nutrients (Fig 4). The three points determined all fall on a straight line, suggesting a possible linear relationship between level of organic nutrients and level of copper tolerance. Furthur study is needed to determine if this linear relationship is maintained at higher and lower levels of organic nutrients.

These results demonstrated that the levels of organic matter employed clearly affected the levels of copper tolerance for P. cuprodurans.

F. Copper Tolerance: An Adaptation or Selection Phenomenon?

It was previously observed that P. cuprodurans growing in high concentrations of copper (i.e. 1×10^{-3} M) had a long extended lag period before growth (increase in O.D.) (c.a. 8-10 hr in basal medium, Table 9 and Table 15). To determine if this extended lag period was due to adaptation or selection (i.e. mutation) processes the following experimentation was performed.

Duplicate 99 ml volumes of basal medium broth and basal medium broth supplemented with 1×10^{-3} M copper were prepared in 250 ml Erlenmeyer flasks, adjusted to a pH of 7.0 with sodium hydroxide, and autoclaved at 121 C for 15 min. Inocula were prepared by growing P. cuprodurans to an O.D. at 420 mμ of 0.30 in basal medium broth unsupplemented and supplemented with 1×10^{-3} M copper. One flask of each medium prepared was inoculated with 1.0 ml of each inoculum, and incubated at 20 C. To determine viable cell numbers, spread plates were performed on basal medium agar plates throughout the growth of P. cuprodurans.

1. Inoculum Grown in Basal Medium.

When this inoculum was employed to inoculate 99 ml portions of basal medium broth, P. cuprodurans had a 1-hr lag period before logarithmic growth (Fig 5). With this same inoculum, P. cuprodurans in the basal medium broth with 1×10^{-3} M copper was observed to have an 8-hr lag period before logarithmic growth (Fig 5). No die-off of P. cuprodurans in the basal medium broth with 1×10^{-3} M copper occurred indicating an adaptation of the bacterium to the copper stress (Fig 5). Die-off in the lag phase of growth in basal medium broth with 1×10^{-3} M copper would have been present if a selection process were involved in copper tolerance.

2. Inoculum Grown in Basal Medium Broth With 1×10^{-3} M Copper.

When this inoculum was employed to inoculate 99 ml of basal medium broth unsupplemented and supplemented with 1×10^{-3} M copper, P. cuprodurans grown in the basal medium broth was observed to have a 2-hr lag period before logarithmic growth (Fig 6). With this same inoculum, P. cuprodurans grown in the basal medium broth with 1×10^{-3} M copper, was observed to have a 3 to 4-hr lag period before logarithmic growth (Fig 6). The extension of the lag period in basal medium broth and decrease in the lag period in basal medium broth with 1×10^{-3} M copper shown in Fig 6 appeared as further evidence of adaptation to copper stress.

3. Stability of Copper Adaptation.

A 0.1 ml aliquot of the stationary phase basal medium broth culture of P. cuprodurans obtained in (Results section F-2, basal medium supplemented with 1×10^{-3} M copper) was used to seed another 100 ml of basal medium

broth. This basal medium broth culture was cultivated at 20 C to an O.D. at 420 mμ of 0.30. One ml aliquots of this culture were used to inoculate 99 ml portions of basal medium broth unsupplemented and supplemented with 1×10^{-3} M copper which were incubated at 20 C. Spread plates were performed on basal medium agar plates to follow viable cell numbers.

Results identical to those in Fig 5 were obtained in this experimentation (Fig 7). Results here show a reversion of copper-adapted P. cuprodurans cells to the control situation shown in Fig 5. If selection (mutation) played a role in copper tolerance this characteristic (copper tolerance) expressed in growth responses shown in Fig 6 should have remained stable and appeared in Fig 7.

G. Measurement of Copper Chelation in Basal Medium Broth.

Results section E established that the level of copper tolerance of P. cuprodurans was dependent on the level of organic nutrients in the growth medium (Table 15). An understanding of the state of copper in basal medium broth assists in understanding the phenomenon of copper tolerance.

1. Polarographic Measurement.

Polarography had been previously used for measurement of copper chelation in defined microbiological media (Avakyan and Rabotonova, 1966). An attempt was made here to measure the amount of copper chelation in the basal medium employing polarography.

Ten ml aliquots of basal medium broth and basal medium broth supplemented with 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M copper were subjected to polarographic measurement. The inorganic and organic components

of the basal medium represented too complex an electrolyte base for accurate measurement as they masked a defined copper wave. Because of this difficulty, polarographic measurements were abandoned.

2. Anodic Stripping Voltammetry.

Anodic stripping voltammetry (ASV) unlike polarography measures copper independent of the electrolyte base in which the copper resides. In ASV measurement of materials such as microbiological media two major forms of copper are identified. Labile copper is that ion which is free or loosely bound to organic molecules, and easily plated onto the graphite-mercury electrode for analysis. Non-labile copper refers to that portion of copper which is firmly bound (chelated) to organic material, and only plated onto the electrode after acid treatment which releases the ion from the chelate.

Synthetic seawater (75%) and basal medium broth supplemented and unsupplemented with 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M copper were prepared, and adjusted to a pH of 7.0 with sodium hydroxide. Portions of each solution were sterilized by filtration through Millipore HA filters (mean porosity of 0.45 u) or by autoclaving at 121 C for 15 min. Samples were diluted either 1:10 (2 ml in 18 ml) or 1:20 (1 ml in 19 ml) with distilled water and measured for the quantity of labile and non-labile copper.

a. Autoclaved Solutions.

Results for autoclaved media appearing in Table 16 demonstrated differences in the amounts of labile and non-labile copper with increased supplementation of copper to the basal medium. Both fractions of copper (labile and non-labile) were observed to increase with increases in copper

supplementation (Table 16). A more rapid increase in copper concentration occurred in the labile fraction (Table 16).

These results showed that copper was both in a free and bound state in basal medium broth supplemented with copper. These results were not indicative of equilibration of copper in the basal medium between the free and bound state. If equilibration were present in this system a constant percent of the total supplement for both labile and non-labile copper should have been apparent at all levels of supplementation (i.e. 50% labile and 50% non-labile).

b. Filter-Sterilized Solutions.

Results for filter sterilized solution appearing in Table 17 show differences in the levels of labile and non-labile copper with increased copper supplementation to the basal medium (1×10^{-4} to 2×10^{-3} M copper). Filter-sterilized basal medium supplemented with 1×10^{-4} to 1×10^{-3} M copper had consistently higher levels of labile copper and lower levels of non-labile copper than did autoclaved media containing the same copper supplement. This appeared to indicate that filter-sterilized basal medium possessed fewer copper binding sites than did autoclaved basal medium. This information implied that the autoclaving process serves to expose more binding sites for copper. An anomalous result was noted in filter-sterilized basal medium supplemented with 2×10^{-3} M copper. In this medium the amount of labile copper was found to diminish on a percent basis from the level of 1×10^{-3} M copper (Table 17). In this same medium the level of non-labile copper was observed to increase, opposed to the decreasing tendency observed with lower levels of copper supplementation (1×10^{-4} to 1×10^{-3} M) (Table 17).

H. Effect of Temperature on the Growth and Copper Tolerance of P. cuprodurans.

Experiments were designed to determine the optimal growth temperature of P. cuprodurans in basal medium unsupplemented and supplemented with copper. Two liters of the following media were prepared in 4-liter Erlenmeyer flasks, and adjusted to pH 7.0 with sodium hydroxide: basal medium, and basal medium with 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M copper sulfate respectively. Portions (100 ml) were placed into 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min.

Duplicate flasks of each medium were inoculated with the standard inoculum (Methods section C) and incubated at one of the following temperatures on a gyrorotary shaker at 140 rpm: 20, 24, 26, 28, 30, 33, and 36 C. Growth of P. cuprodurans was followed by optical density measurements at 420 mμ performed regularly at 4, 6, 8, 10, 13, 24, 28, 30, 34, 49, 53, and 77 hr. Optical density measurements between these times were performed to establish the growth rate of P. cuprodurans in the various media. The growth rate was defined as the change in optical density at 420 mu per hr in the logarithmic phase of growth.

Results appearing in Tables 18 through 23 showed that P. cuprodurans cultivated in the basal medium with no copper supplement attained maximal growth at 33 C. With basal medium, elevation in growth temperature from 20 to 33 C had the general effect of decreasing the lag period of P. cuprodurans and increasing the growth rate (Tables 18 through 23). No growth of P. cuprodurans occurred at 36 C in any of the media used in these experiments.

The characteristic decrease in lag phase and increase in growth rate was noted for P. cuprodurans in basal medium supplemented with copper (1×10^{-4} to 2×10^{-3}) from 20 to 26 C (Tables 18-20). Above 26 C growth of P. cuprodurans in copper supplemented basal medium was progressively delayed (Tables 21 to 23). Decreases in the maximum optical density attained by P. cuprodurans in basal medium supplemented with 1×10^{-4} to 2×10^{-3} M copper were observed at growth temperatures of 30 and 33 C (Tables 22 and 23).

In basal medium supplemented with 2×10^{-3} M copper, P. cuprodurans was observed to undergo a large amount of lysis upon attaining stationary phase at 33 C (Table 23). Lysis products were observed as visible aggregates in the medium. When cells in basal medium with 2×10^{-3} M copper were examined with the phase microscope at stationary phase, the cells were elliptically swollen and severely plasmolyzed, similar to those shown in Fig 3. Such aberrancies were not observed in earlier growth of the organism, and were considered as products of autolytic processes.

Since all growth temperatures could not be tested simultaneously in this experimentation a true direct comparison of growth in the tested media at the various temperatures connot be clearly established from these results. It may be inferred, however, that P. cuprodurans has its optimal growth temperature between 33-36 C in basal medium broth. The growth rates obtained for P. cuprodurans in the media tested were determined and plotted in Fig 8. Cells grown in copper supplemented media shared a common 26 C temperature optimum (Fig 8). Elevation in temperature beyond 26 C caused

decreases in the growth rate of P. cuprodurans in copper-supplemented media. Since these studies were performed in identical media using a standardized inoculum (all prepared simultaneously as a batch and subsequently divided into 100 ml portions), these observations are comparable although not performed simultaneously.

I. The Effect of Various Washing Solutions on P. cuprodurans.

1. Comparison of Washing Solutions.

Before engaging in physiological studies on P. cuprodurans, a washing solution was established for the duration of this dissertation. The following were employed experimentally as washing solutions: distilled water, 0.82 M glycerol, 75% synthetic seawater, 0.0392 M magnesium chloride, 0.0074 M calcium chloride, and a solution of both 0.0392 M magnesium chloride and 0.0074 M calcium chloride. Roche (1966) judged distilled water as an effective washing solution because of its ability to yield low levels of copper concentration by P. cuprodurans (P. X). Glycerol (0.82 M) was equivalent in osmotic pressure to 75% synthetic seawater (MacLoed and Onofrey, 1956). Synthetic seawater (75%) was employed as the ionic solution in which P. cuprodurans was grown. Individual solutions of magnesium, calcium, and magnesium and calcium together were employed because of their ability to prevent lysis of marine bacteria (Korngold and Kushner, 1968). Levels of calcium and magnesium employed were those found in synthetic seawater (Table 5).

To determine the effect of washing solutions upon P. cuprodurans, the solutions were prepared and adjusted to pH 7.0 with 1.0 and 0.1 N sodium

hydroxide. Logarithmic phase cells of P. cuprodurans were harvested at an optical density of 0.85 at 420 mμ. Such cells were in optimal growth and physiologically active for furthur study. Six 200 ml aliquots of a basal medium broth of P. cuprodurans in logarithmic growth culture were harvested at 19,900 x g for 10 min at 4 C and subsequently washed with three 15 ml volumes of one of the washing solutions. All washing supernatants were saved for furthur study.

The effectiveness of a washing solution was judged by three criteria: 1. Integrity of the centrifuged pellet after washing - a firm tight pellet was considered normal, whereas a loose pellet was considered abnormal and indicative of cell lysis. 2. Cell morphology - the presence vs the absence of normal morphology (i.e rounding of the cell). 3. Presence or absence of leakage material in ultraviolet absorption spectrum of a washing supernatant; with successive washes peak height of components of the spectrum should decrease in a normal washing process. Increases in the peak height of components of the spectrum with successive washes should then indicate leakage or lysis of cells.

Table 24 contains a summation of pellet and morphological observations obtained in this experimentation. All washes with the exception of 0.82 M glycerol and distilled water yielded favorable results by these criteria.

For comparison of the washing supernatant, the Hitachi-Perkin-Elmer-Coleman 124 recording spectrophotometer was employed. To prevent spectral changes due to increase in cuvette temperature, samples of the washing solutions were pre-incubated at 30 C for 30 min and subsequently analyzed

in the spectrophotometer equipped with a 30 C heating jacket. All cuvettes were acid cleaned in warm nitric acid and rinsed with distilled water and ethanol before use. For direct comparison of information each washing solution was standardized against the 0.82 M glycerol solution. Washing solutions containing magnesium chloride, magnesium chloride-calcium chloride, and 75% synthetic seawater exhibited a peak at 203 mμ as shown in Fig 9. All other washing solutions showed no peaks in the 320-200 mμ range examined and were compatible with the blank.

Aliquots (3.0 ml) of the washing supernatants were run against the 0.82 M glycerol blank. All such samples demonstrated two broad peaks occurring at 250 mμ and 203 mμ as shown in Fig 10. The heights of the two peaks were measured and corrected for the existing descrepancies existing between the washing solutions (Fig 11). Glycerol (0.82 M) and distilled water exhibited increases in the peak heights (203 and 250 mu peaks) with successive washes (Fig 11). This characteristic pattern was established as indicative of cell lysis, thus agreeing with the other two criteria employed.

The washing supernatants obtained from the use of the other four washing solutions exhibited a decrease in the height of both peaks (203 and 250 mμ) with successive washes. This is characteristic of a washing processes.

By the established criteria distilled water and 0.82 M glycerol were determined to be poor washing solutions as they led to leakage and lysis of P. cuprodurans. Magnesium chloride (0.0392 M), calcium chloride (0.0074 M),

magnesium chloride (0.0392 M) combined with calcium chloride (0.0074 M), and 75% synthetic seawater were determined acceptable washing solutions.

The 203 mμ peak appeared to be associated with the presence of magnesium in the washing supernatant, as it appeared only in washing solutions containing magnesium (Fig 9). The 250 mμ peak was indicative of basal medium constituents and their subsequent washing from the cell. No clear 260 mμ peak (nucleic acids) was observed in the instances of distilled water and 0.82 M glycerol washes where lysis was apparent. With cell lysis, a 260 mμ peak is evident due to the release of nucleic acids from the cell. It is felt that such a peak may have existed in the glycerol and distilled water washes, but was masked by the broad 250 mμ peak.

2. Magnesium Chloride as a Washing Solution.

Because of the preceding success using magnesium chloride for washing P. cuprodurans (Results section I-1), furthur study using magnesium chloride for washing P. cuprodurans was undertaken.

A basal medium broth culture (600 ml) of P. cuprodurans was grown to an optical density of 0.85 at 420 mμ. Aliquots (100 ml) of this broth culture were harvested by centrifugation at 19,900 x g for 10 min at 4 C. The supernatant was decanted and the pellet washed with one of the following magnesium solutions adjusted to pH 7.0: 49.2, 39.3, 29.5, 19.6, 9.8, and 4.9 mM magnesium chloride. Wash volumes of 7.5 ml were employed with the pellet checked for firmness and the cells for abnormal morphology with each wash. Ultraviolet spectra were performed on each of the washes using the corresponding solution as a blank.

In all solutions the pellet maintained its normal consistency, but rounded, plasmolyzed forms of P. cuprodurans were observed with the second washing with 4.9 and 9.8 mM magnesium chloride (Table 25).

Spectral analyses performed on the washing supernatants yielded the same 203 and 250 mμ peaks previously described in Results section I-1 (Fig 10). Quantitation of the peak heights appear in Fig 12. Close to a linear relationship occurred between the peak heights of the 203 mμ peak of the initial wash with varied concentrations of magnesium chloride (Fig 12).

Because of the alteration in cell morphology encountered at magnesium levels of 4.9 and 9.8 mM (Table 25), 19.6 mM magnesium chloride was selected for use as a washing solution.

3. Respirometry Studies Performed With the 4 g/liter Magnesium Chloride Washing Solution.

In initially studying respiration of P. cuprodurans, the 19.6 mM magnesium chloride washing solution was employed for the preparation of cells. With such studies, P. cuprodurans cells possessed an extremely low rate of respiration. To determine if this low rate of respiration was due to magnesium washing the following experiment was performed.

One hundred ml of the basal medium and the basal medium with 1×10^{-3} M copper were prepared, adjusted to pH 7.0 with sodium hydroxide, and autoclaved at 121 C for 15 min. The flasks were subsequently inoculated with the standard inoculum (Methods section C) and cultivated at 20 C to an O.D. of 0.85 at 420 mμ. Each 100 ml of broth culture was split into two 50 ml volumes and harvested by centrifugation at 39,900 x g for 5 min at 4 C.

Subsequent to harvesting one pellet derived from each medium was washed with 19.6 mM magnesium chloride, and the second pellet washed with 75% synthetic seawater. The final suspensions were prepared by resuspending each pellet in 75% synthetic seawater, and using this suspension for respirometry studies according to Table 2.

Results shown in Fig 13 show magnesium washed cells possessed greatly reduced rate of oxygen uptake when compared to cells washed with 75% synthetic seawater. Because of these results magnesium chloride washing was abandoned, and cells were washed with 75% synthetic seawater in preparing them for physiological study.

J. Determination of DNA : RNA : Protein Ratios.

Previous studies (Results section F) indicated that copper tolerance by P. cuprodurans involved an adaptive process. With such adaptation alteration of major biochemical constituents such as DNA, RNA and protein may have occurred. To determine if copper stress induced such biochemical alterations in P. cuprodurans, the ratios of DNA : RNA : protein were determined for the bacterium cultivated in basal medium broth supplemented with varied concentrations of copper (1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M). P. cuprodurans was grown to an optical density of 0.85 at 420 mμ in the prepared media and washed, extracted and analyzed according to Methods section N.

Results appearing in Table 26 indicated that no alteration of the DNA : RNA : protein ratio occurred with increased copper supplementation of the basal medium (1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M). Nitrogen present

in DNA : RNA : protein represented 95-97% of the cell nitrogen in the four situations tested. Ratios determined here complied with ranges for gram-negative bacteria (Luria, 1960).

No major alteration in the ratio of DNA : RNA : protein was associated with the adaptation of P. cuprodurans to copper stress.

K. Relationship of Cell Numbers to Protein and Dry Weight.

To determine the interrelationships of Lowry protein, dry weight, and cell numbers for P. cuprodurans grown in basal medium supplemented with varied concentrations of copper, the following experiment was performed. Several 99 ml portions of basal medium and basal medium supplemented with 1×10^{-4}, 5×10^{-4} and 1×10^{-3} M copper were prepared in 250 ml Erlenmeyer flasks, and autoclaved at 121 C for 15 min. Over a period of several days the flasks were inoculated with the standard inoculum and incubated at 20 C at 140 rpm until attaining an optical density of 0.85 at 420 mμ. The cells were harvested by centrifugation at 39,900 x g, washed three times with 75% synthetic seawater and a final suspension prepared.

Samples of the suspension were used for the determination of Lowry protein, dry weight and a Petroff-Hauser cell count. Samples were tested in triplicate to obtain accurate values. The relationship of protein and dry weight to cell numbers was identical for cells grown in the four different media employed (Fig 14). When tested throughout this dissertation these relationships were observed to remain constant.

L. Electron Microscopy of P. cuprodurans.

To determine if alteration in the ultrastructure of P. cuprodurans

occurred with increased copper stress, the bacterium was grown in basal medium supplemented with copper to an optical density of 0.85 at 420 mu and processed according to Methods section K for examination with the electron microscope.

No significant differences in ultrastructure were noted in cells grown in the basal medium (Fig 15) and basal medium supplemented with 1×10^{-4} to 1×10^{-3} M copper (Fig 16). Cells possessed a typical gram-negative multilayered cell wall with the dense-light-dense layers of the cytoplasmic membrane apparent. These electron micrographs of P. cuprodurans corresponded with those of Costerton et al (1967) and Wiebe (1968) for other marine pseudomonads.

M. The Effect of Copper on Oxygen Uptake by P. cuprodurans.

To determine if copper stress affected the respiratory activity, P. cuprodurans was cultivated in each of the following media until the attainment of stationary phase: basal medium, and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M copper. The cells were harvested and prepared for study according to Methods section O.

1. P. cuprodurans Cultivated in the Basal Medium.

Respiration of P. cuprodurans progressively decreased with increased concentration of copper (Fig 17). The respiration of P. cuprodurans was affected by all concentrations of copper added to the basal medium in the respiration flasks. No linear relationship between the concentration of copper supplement to the basal medium and corresponding decreases in respiration was noted.

2. P. cuprodurans Cultivated in Basal Medium Supplemented With 1 x 10^{-4} M Copper.

Respiration of P. cuprodurans cultivated in basal medium with 1 x 10^{-4} M copper was only altered in respiration flasks containing basal medium supplemented with 5 x 10^{-4} and 1 x 10^{-3} M copper (Fig 18). Because no difference was noted in the respiration of the organism in respiration flasks containing basal medium and basal medium with 1 x 10^{-4} M copper, adaptation of the bacterium to the 1 x 10^{-4} M copper supplement was apparent (Fig 18). This adaptation held for the three hr duration of this experiment as no difference between respiration in basal medium and basal medium supplemented with 1 x 10^{-4} M copper occurred (Fig 18).

3. P. cuprodurans Cultivated in Basal Medium Supplemented with 5 x 10^{-4} M Copper.

Respiration of P. cuprodurans cultivated in basal medium with 5 x 10^{-4} M copper was decreased only in respiration flasks containing basal medium with 1 x 10^{-3} M copper (Fig 19). Because no difference was observed in the respiration of P. cuprodurans in respiration flasks containing basal medium and basal medium supplemented with 1 x 10^{-4} and 5 x 10^{-4} M copper, adaptation of the bacterium to the 5 x 10^{-4} M copper supplement was apparent (Fig 19). This adaptation of the bacterium, as in the instance with cells cultivated in the basal medium supplemented with 1 x 10^{-4} M copper (Results section M-2), remained stable for the 3 hr duration of this experiment.

4. P. cuprodurans Cultivated in Basal Medium Supplemented with 1 x 10^{-3} M Copper.

Respiration of P. cuprodurans cultivated in basal medium supplemented

with 1 x 10^{-3} M copper was unaltered in respiration when tested in respiration flasks containing basal medium and basal medium supplemented with 1 x 10^{-4}, 5 x 10^{-4} and 1 x 10^{-3} M copper (Fig 20). This indicated adaptation of P. cuprodurans to the 1 x 10^{-3} M copper supplement. Such adaptation remained throughout the 3 hr period of examination as the respiration present in the four media tested exhibited the same rate of respiration.

5. P. cuprodurans Cultivated in Basal Medium Supplemented with 2 x 10^{-3} M Copper.

Respiration of P. cuprodurans cultivated in basal medium supplemented with 2 x 10^{-3} M copper was unaltered in respiration when tested in the respiration flasks containing basal medium, and basal medium supplemented with 1 x 10^{-4}, 5 x 10^{-4}, and 1 x 10^{-3} M copper (Fig 21). This indicated adaptation of P. cuprodurans to the 1 x 10^{-3} M copper supplement. Such adaptation remained throughout the 3 hr period of examination as the respiration present in the four media tested exhibited the same rate of respiration. The exact copper concentration of 2 x 10^{-3} M could not be tested in this experiment as large amounts of precipitation were noted when the medium was prepared as a 3X concentrate.

6. Respiratory Alteration of P. cuprodurans by Copper: A Die-off Phenomenon or a Respiratory Inhibition Phenomenon?

Decreased respiration occurring in respiration flasks containing levels of copper supplementation above which P. cuprodurans was cultivated were observed in Fig 17, 18, and 19. It was not known at the time of experimentation whether such decreased respiration was attributable to

decreased viable cell numbers or to an inhibitory effect of the copper ion on respiration. To determine the cause of the respiratory decreases, the following experiment was performed. P. cuprodurans was cultivated until the attainment of stationary phase in the following four media: basal medium, and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, 1×10^{-3} and 2×10^{-3} M copper. The cells were prepared for study as indicated in Methods section O. Identical mixtures to those in the respiration flasks of Results sections M-1, M-2, M-3, M-4, and M-5 were created in large sterile test tubes. Such test tubes were attached to the Gilson respirometer and incubated in accordance to the previous studies in Results section M. Duplicate 0.1 ml samples were removed from the test tubes at times corresponding to 0 and 180 min of incubation and plate counts performed on basal medium agar plates.

No decrease in viable cell numbers occurred during the period of respiratory study (Table 27). These results indicated that oxygen utilization decreases observed in Results section M could be attributed to respiratory inhibition by the copper ion.

7. Conclusions.

An adaptive phenomenon to copper similar to that observed in Results section F was apparent in these respiration studies (sections 1 through 5). Adaptation to copper was observed when P. cuprodurans was grown in basal medium supplemented with copper concentrations of 1×10^{-4} to 2×10^{-3} M, as cells maintained a constant oxygen utilization in basal medium supplemented to a concentration of copper in which cells were grown (Fig 16-21).

Decreased oxygen utilization occurred only in respiratory flasks containing basal medium supplemented with copper concentrations exceeding those in which P. cuprodurans was cultivated.

P. cuprodurans cultivated in basal medium supplemented with various concentrations of copper possessed progressively reduced oxygen uptake after 180 min in respiration flasks containing basal medium (Table 28). Thus, copper adapted cells were inhibited markedly in their respiration as a function of copper concentration.

Endogenous respiration at copper concentrations studied could not be performed because of lack of copper solubility in seawater without organic nutrients. Endogenous respiration of P. cuprodurans was also decreased when grown in increased concentrations of copper supplementation to the basal medium (Fig 17 through 21).

Oxygen utilization by P. cuprodurans was influenced strongly by both the copper concentration in the basal medium for growth and the copper concentration present in the respiratory vessel.

N. Respiratory Deficiency Induced by Copper.

Triphenyltetrazolium chloride (TTC) has been used to detect respiratory deficiencies in a variety of microorganisms such as yeast (Lindegren, Nagai, and Nagai, 1958). To determine if growth in copper-supplemented basal medium induced a respiratory deficiency in P. cuprodurans, the following experiment was performed. Agar plates of basal medium and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper were prepared and inoculated by streaking with freshly grown P. cuprodurans

cells. The inoculated plates were incubated at 20 C until well-developed colonies were apparent. The plates were overlayed with either a 1.5% agar or a 10% gelatin solution containing 0.1% TTC.

After overlaying, the plates were incubated at 20 C and examined every 5 min for the first hr of incubation, and at hourly intervals thereafter for the presence of red-reduced triphenylformazan. Respiratory sufficient colonies were defined as those capable of reducing the TTC within one hr of incubation (Lindegren, Nagai, and Nagai, 1958). Reduction of the dye after one hr was indicative of a respiratory deficiency (Lindegren, Nagai, and Nagai, 1958).

Growth of P. cuprodurans on basal medium agar plates supplemented with 5×10^{-4} and 1×10^{-3} M copper induced a respiratory deficiency after 6 and 12 hr, respectively (Table 29). P. cuprodurans colonies on basal medium agar plates reduced the TTC dye after 5 min incubation, whereas a nine-fold increase in TTC reduction to 45 min occurred on basal medium agar plates supplemented with 1×10^{-4} M copper. Although P. cuprodurans colonies on basal medium agar plates supplemented with 1×10^{-4} M copper had TTC reduction times extended beyond P. cuprodurans colonies on basal medium agar, such colonies were respiratory sufficient since TTC reduction occurred within one hr.

Agar and gelatin overlays were employed to determine if the 50 C temperature of the molten agar overlay was injurious to P. cuprodurans colonies. Identical TTC reduction times were noted for gelatin and agar overlays (Table 29).

1. Possible TTC-Copper Interactions.

To determine if the prolonged TTC reduction times with increased copper supplementation to the basal medium were due to TTC-copper interactions as indicated in Table 29, both 1.5% agar and 10% gelatin TTC overlays were supplemented with 1×10^{-3} M copper and used to overlay P. cuprodurans colonies on basal medium agar plates. A 5 min reduction time was recorded with both TTC overlays indicating that TTC-copper interactions were not responsible for respiratory deficiencies observed for P. cuprodurans colonies on basal medium agar plates supplemented with 5×10^{-4} and 1×10^{-3} M copper.

2. Reversibility of Respiratory Deficiencies Induced by Copper.

To determine if the respiratory deficiencies in Table 29 were reversible by removal of the copper stress, P. cuprodurans colonies on basal medium agar plates supplemented with 5×10^{-4} and 1×10^{-3} M copper were picked and streaked on basal medium agar plates. After development of the colonies, the plates were overlayed with 10% gelatin containing 0.1% TTC. The overlayed colonies reduced the TTC dye after 5 min of incubation indicating that the respiratory deficiency observed under copper stress was reversed upon copper removal.

3. Induction of Respiratory Deficiencies by Other Divalent Cations.

To determine if other divalent transitional elements induced respiratory deficiencies in P. cuprodurans the following experiment was undertaken. First, tolerance studies of P. cuprodurans to nickel, cobalt, zinc, and cadmium were performed using the agar plate technique (Results

section D-2). Results in Tables 30, 31, 32, and 33 demonstrated P. cuprodurans tolerated 1 x 10^{-3} M nickel, 3 x 10^{-4} M cobalt, 4 x 10^{-4} M zinc, and 5 x 10^{-5} M cadmium in basal medium. Supplementation of the basal medium with zinc and cadmium reduced the colony size of P. cuprodurans (Table 32 and 33).

P. cuprodurans was cultivated on basal medium agar plates supplemented with 1 x 10^{-4}, 5 x 10^{-4} and 1 x 10^{-3} M copper; 1 x 10^{-3} M nickel; 3 x 10^{-4} M cobalt; 4 x 10^{-4} M zinc; and 5 x 10^{-5} M cadmium. With the appearance of colonies on these plates, the plates were overlayed with 10% gelatin containing 0.1% TTC and incubated at 20 C. Maximum concentrations of nickel, cobalt, zinc, and cadmium tolerated by P. cuprodurans did not induce respiratory deficiencies (Table 34). Thus, induction of respiratory deficiencies in P. cuprodurans was a specific effect of copper rather than a general effect of divalent cations.

O. Thunberg Studies.

Data obtained in Results sections M and N demonstrated that increased copper supplementation to the basal medium caused decreased oxygen uptake and respiratory deficiencies in P. cuprodurans. To furthur investigate these respiratory alterations of the bacterium, the following experimentation was taken.

The use of Thunberg tubes with a variety of oxidation-reduction (O/R) dyes have been used to study electron transport in microorganisms (Tam and Wilson, 1941; Umbreit, Burris, and Stauffer, 1957). To study the electron transport system of P. cuprodurans, methylene blue (MB),

dichloroindophenol (DCIP) and sodium ferricyanide were used. Methylene blue and dichloroindophenol have been used as electron acceptors at the flavoprotein-quinone level of electron transport because of their comparable O/R potentials (Lardy, 1949). Ferricyanide accepts electrons at the cytochrome c level (Smith, 1968). TTC was also employed to determine where the dye accepted electrons. With the use of these oxidation-reduction dyes, it was possible to determine if the respiratory deficiency found in Results section N was reflective of changes in the electron transport system at or before the flavoprotein-quinone level, or at the cytochrome level.

Cells were cultivated and prepared for study according to Methods section R . All readings were performed using a common blank containing a formalized suspension of P. cuprodurans. Readings were standardized as changes in optical density occurring with a cell concentration of 5×10^9 cells/ml.

Difficulty was encountered in using sodium ferricyanide as an electron acceptor, for the electron transport system of P. cuprodurans was unable to transfer electrons to the ferricyanide. Attempts made with phenazine methylsulfate to couple electron transport with the ferricyanide were not successful. This difficulty was probably due to ionic interference from the seawater.

Results obtained from the TTC, DCIP, and MB dyes appear in Fig 22, 23, and 24, separately. With all dyes no reduction occurred during the 30 min incubation period when P. cuprodurans cells cultivated in basal

medium supplemented with 5×10^{-4} and 1×10^{-3} M copper were employed. Cells cultivated in basal medium with 1×10^{-4} M copper were noted to exhibit a slower rate of reduction of the three dyes when compared to cells cultivated under no copper stress. The slope obtained from cells cultivated in basal medium with 1×10^{-4} M copper were decreased by a factor of nine when compared to the non-copper stressed cells. This same nine-fold decrease was observed in the TTC overlays performed in Results section N (Table 29).

Thus, an alteration in electron transport occurred at or before the flavoprotein-quinone level of electron transport in P. cuprodurans cultivated in basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper.

P. Qualitative and Quantitative Measurement of the Cytochrome Components of P. cuprodurans.

Because of changes in electron transport and respiration caused by the addition of copper to the basal medium established in Results sections M through O, investigation of the cytochromes of P. cuprodurans was undertaken. P. cuprodurans was cultivated in basal medium supplemented with varied concentrations of copper sulfate to an optical density of 0.85 at 420 mu. The resulting cells were prepared for cytochrome study according to Methods section Q.

The resulting whole cell extracts were standardized according to protein determined by the method of Lowry et al (1951) and difference spectra performed (Methods section Q).

I. Qualitative Observations.

The difference spectrum obtained for whole cell extracts of P. cuprodurans cultivated in the basal medium demonstrated the presence of cytochrome alpha peaks at 559 and 551 mμ and beta peaks at 528 and 521 mμ (Fig 25). The gamma peaks of the two cytochromes were fused into one peak occurring at 426 mμ (Fig 25). The alpha and beta peaks of 558 and 528 mμ corresponded to listed values for cytochrome b_1 (Gel'man, Lukoyanova, and Ostovskii, 1967). The observed alpha peak of 551 mμ and beta peak of 521 mu corresponded to listed values for cytochrome c (Gel'man, Lukoyanova, and Ostovskii, 1967). Cytochromes b_1 and c have listed gamma absorption maxima at 426 and 416 mu respectively (Gel'man, Lukoyanova, and Ostovskii, 1967).

No peaks were observed between 650 mu and 590 mμ indicating the lack of presence of the Pseudomonas blue protein and any of the a cytochromes, which show absorption maxima in this region (Fig 25).

The difference spectrum obtained from whole cell extracts of P. cuprodurans cultivated in basal medium with 1×10^{-4} M copper exhibited alteration of the spectrum obtained from whole cell extracts of non-copper stressed cells demonstrated in Fig 25. Changes in the spectrum were observed to occur in four parts of the spectrum: A. alpha peaks, B. beta peaks, C. Depth of the 450-460 mμ trough, and D. Height of the gamma peak at 426 mμ (measured from minimum at 450-460 mμ to maximum at 426 mμ).

Growth of P. cuprodurans in 1×10^{-4} M copper caused fusing of the two cytochrome alpha peaks making neither clearly distinguishable (Fig 26). The 450-460 mμ trough maintained approximately the same depth as noted with

non-copper stressed whole cell extracts (Fig 26). The 426 mu gamma peak decreased slightly in height when compared to that of Fig 25, possibly indicating decreased cytochrome content.

The difference spectrum obtained from cells grown in 5×10^{-4} M copper showed furthur deviation from the control spectrum (Fig 25). The alpha and beta peaks were furthur distorted by growth in the 5×10^{-4} M copper stress (Fig 27). The 450-460 mμ trough decreased in depth, and the gamma peak decreased in height compared to the control spectrum (Fig 27).

Whole cell extracts of P. cuprodurans cultivated in 1×10^{-3} M copper demonstrated furthur alteration of the difference spectrum (Fig 28). The alpha and beta peaks of the cytochromes were reduced significantly and distorted, and the 450-460 mμ trough virtually eliminated (Fig 28). The height of the gamma peak at 426 mμ decreased and revealed the two gamma peaks of the cytochromes at 426 and 416 mμ (Fig 28).

Difficulty was encountered in performing carbon monoxide spectra on the cell extracts since the rate of gas evolution from the generator was difficult to regulate (Methods section Q). Vigorous bubbling of the extracts occurred with resulting aeration taking place. To make the cuvette contents again compatible for spectrophotometric study sodium dithionite was added to the test cuvette until compatibility with the reference was attained. When difference spectra were performed on the carbon monoxide treated whole cell extracts two peaks were observed to occur within the 600-520 mu spectrum at 570 and 540 mμ (Fig 29). The presence of a peak at 570 mu corresponded to a peak shift of cytochromes c and b_1 noted by White

(1962). The peak at 540 mμ corresponded to values cited for cytochrome o (White 1962; Gel'man, Lukoyanova, and Ostrovskii, 1967).

No quantitative evaluations were made concerning the possible alteration of the carbon monoxide spectra in copper-stressed whole cell extracts because of the inherent difficulty of reproducibility in the procedure.

2. Quantitative Measurement of Cytochromes b_1 and c.

Since cytochromes b_1 and c alpha peaks overlap in difference spectra, relative quantitation of the two cytochromes on the basis of absorption is difficult. Using the method of Sinclair (1970), contributions in absorption of one cytochrome to the other were corrected for and the true absorption of the cytochrome b_1 and c alpha peaks were determined.

Results in Table 35 demonstrated that increased copper supplementation to the basal medium caused corresponding decreased levels of cytochrome c. Additions of 1×10^{-4} and 5×10^{-4} M copper to the basal medium elevated levels of cytochrome b_1 above those obtained for non-copper stressed cells, whereas the addition of 1×10^{-3} M copper to the basal medium decreased the levels of cytochrome b_1 in the organism (Table 35). The ratio of cytochrome c to b_1 was lowered with additions of copper supplements to the medium (Table 35).

Thus, the cytochrome components of P. cuprodurans were cytochromes b_1, c, and o. Levels of cytochromes b_1 and c varied with increased copper supplementation to the growth medium; cytochrome b_1 increased relative to

cytochrome c. Cells cultivated in basal medium supplemented with 1×10^{-3} M copper contained significantly decreased relative amounts of cytochromes b_1 and c when compared to non-copper stressed cells.

Q. Induction of Respiratory Deficiencies by Copper in Other Bacteria.

To determine if high copper stress would induce respiratory deficiencies in other bacteria, E. coli B, B. subtilis, and S. aureus were streaked on basal medium agar plates containing the maximum copper supplement tolerated by each organism determined in Table 11 through 14. With the appearance of colonies after incubation at 37 C, the plates were overlayered with 1.5% molten agar (50 C) containing 0.1% TTC to check for the presence of respiratory deficiencies induced by growth in copper-stressed media. Growth in copper-supplemented medium did not induce a respiratory deficiency as indicated by TTC reduction times, in any of the bacteria tested (Table 36).

R. Copper Uptake During Growth of P. cuprodurans.

To determine if copper was taken up throughout the growth of P. cuprodurans in copper-supplemented basal medium, the following experiment was performed using the isotope ^{64}Cu (as $^{64}CuSO_4$) for the quantitation of copper uptake. Ninety-nine ml volumes of basal medium and basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper sulfate were prepared in 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. The media were seeded with equal amounts of the radionuclide, inoculated with the standard inoculum (Methods section C), and shaken at 140 rpm at 20 C. With the appearance of turbidity in the flasks, 1.2 ml samples were

withdrawn at regular intervals until the attainment of stationary phase. Such samples were assayed according to Methods L-1.

Copper was taken up throughout the phases of growth (Fig 30-33). Uptake of ^{64}Cu was essentially linear with time at all concentrations of copper (Fig 30 through 33). For comparison the results obtained were calculated as cpm/1 x 10^8 cells for all stages of growth (Table 37). Cells in early and mid-logarithmic growth possessed more copper than did cells in later phase of growth. Cells approaching late logarithmic and stationary phases of growth progressively decreased in their copper content until a constant amount of copper per cell was established (Table 37). Decreases in copper uptake by the cells could not be explained entirely by the dilution of copper per cell by cell division. Consequently, cells in these later stages of growth possessed a slower rate of ^{64}Cu uptake than did early and mid-logarithmic cells (Table 37). The ^{64}Cu level at which cells in late logarithmic and stationary phases stabilized their uptake decreased as copper supplementation to the basal medium was increased (Table 37). Copper was taken up throughout the growth phases of P. cuprodurans until a constant level of ^{64}Cu uptake was attained at a low level per cell.

S. Uptake of Copper by Stationary Phase Cells of P. cuprodurans.

1. Uptake of Copper by P. cuprodurans: An Active Transport System?

To determine if copper uptake by P. cuprodurans was due to an active transport system, 2,4-dinitrophenol (DNP) and sodium cyanide were employed as inhibitors. Such inhibitors are established as agents which

either uncouple oxidative phosphorylation (DNP) or inhibit electron transport (sodium cyanide) (Mahler and Cordes, 1966). Transport of copper in the presence of these inhibitors would be indicative of the absence of an active transport system. The lack of uptake of copper in the presence of the inhibitors and uptake in their absence would indicate active transport.

One hundred ml of basal medium broth was prepared in a 250 Erlenmeyer flasks, and autoclaved at 121 C for 15 min. The medium was then inoculated with the standard inoculum (Methods section C) and incubated at 20 C and shaken at 140 rpm. Upon attaining the stationary phase of growth, the cells were harvested aseptically by centrifugation at 39,900 x g for 5 min at 4 C. After removal of the supernatant, the cells were washed aseptically with three 50 ml volumes of sterile 75% synthetic seawater. After washing, the cells were resuspended in 100 ml of fresh sterile basal medium broth and used for study.

Three sterile 250 ml Erlenmeyer flasks were prepared for use according to Table 38. The cell suspensions were equilibrated at 20 C at 140 rpm for 30 min. After equilibration the inhibitors (DNP and sodium cyanide) were added and the flasks reincubated at 20 C for 15 min. The flasks were seeded with a 1×10^{-3} M copper solution containing ^{64}Cu. Samples were withdrawn from each flask at 0, 2, 4, 6, 8, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min of incubation and assayed according to Methods section L-2.

Cells of P. cuprodurans in all treatments underwent a rapid binding of ^{64}Cu during the initial 4 to 8 min of incubation (Fig 34). No furthur

significant uptake of ^{64}Cu occurred after 10 min in flasks containing the DNP and cyanide inhibitors (Fig 34). Untreated cells demonstrated a diphasic ^{64}Cu uptake subsequent to the rapid copper binding (Fig 34). Since the diphasic slopes of ^{64}Cu uptake were identical, the same uptake system was probably functioning in each phase of transport (Fig 34).

The uptake of copper by P. cuprodurans occurred in two stages: first, a rapid binding of copper; and second, a diphasic uptake which required energy. The rapid ^{64}Cu uptake was indicative of ionic binding to the cell because of the speed at which it occurred, and its presence in cells treated with transport inhibitors. The diphasic uptake in the absence of DNP and cyanide was indicative of a regulatory system, possibly a repressible enzyme.

2. Endogenous Uptake and the Effect of Chloramphenicol on Diphasic Active Transport.

If an active transport system with a repressible enzyme were present, the addition of 100 µg/ml of chloramphenicol (toxic to cells, growth inhibitory) would either stop the formation of a protein repressor, or interrupt systems relying on protein synthesis for regulation. Endogenous ^{64}Cu uptake was studied to determine if the basal medium was essential for the diphasic uptake.

One hundred and twenty ml of basal medium broth was prepared in a 250 ml Erlenmeyer flask and autoclaved at 121 C for 15 min. The medium was inoculated with the standard inoculum (Methods section C) and incubated at 20 C at 140 rpm. Following the attainment of stationary phase, the cells

were harvested aseptically in two 60 ml volumes at 39,900 x g for 5 min at 4 C. Each pellet was subsequently washed in three 30 ml volumes of sterile 75% synthetic seawater. After washing, one pellet was resuspended in fresh sterile basal medium broth, and the other pellet in sterile 75% synthetic seawater.

Three flasks were prepared according to Table 39. The cell suspensions were equilibrated at 20 C at 140 rpm for 30 min. Following this, chloramphenicol was added and the cells reincubated at 20 C for 15 min. Three ml of a 1 x 10^{-3} M copper solution containing ^{64}Cu was added to all flasks and the flasks incubated at 20 C at 140 rpm. Samples of 0.9 ml were removed from all flasks at 0, 2, 4, 6, 8, 10, 15, 30, 45, 60, 75, 90, 105, 120 min and assayed according to Methods section L-2.

Chloramphenicol-treated cells did not demonstrate the diphasic ^{64}Cu uptake, but rather showed an increased rate of ^{64}Cu uptake (Fig 35). The rate of ^{64}Cu uptake in chloramphenicol-treated cells did not parallel the slope of the diphasic uptake of the control cells in basal medium (Fig 35). The endogenous cells demonstrated only ionic binding of ^{64}Cu with no diphasic uptake (Fig 35).

Diphasic active transport of copper by P. cuprodurans necessitated the presence of the basal medium, as no transport after ionic binding was observed with endogenous cells. Diphasic active transport required the presence of protein synthesis in P. cuprodurans.

T. Physical Localization of Copper in P. cuprodurans.

Roche (1966), after spheroplasting P. cuprodurans cells, found the

majority of copper taken up to remain with the spheroplasts suggesting that transported copper was interior to the cell membrane. To determine the quantity of copper interior to the cell envelope (cell wall and cell membrane) the following experiment was performed.

Five hundred ml of basal medium broth supplemented with 1×10^{-3} M Cu was prepared in a one liter Erlenmeyer flask and autoclaved at 121 C for 15 min. The medium was seeded with isotope (^{64}Cu), inoculated with the standard inoculum, and incubated at 20 C at 140 rpm. Upon attaining an optical density of 0.85 at 420 mμ, the cells were harvested by centrifugation at 19,900 x g for 10 min at 4 C. The pellet was washed in three 250 ml volumes of 75% synthetic seawater, and finally resuspended in 5 ml of 75% synthetic seawater. Duplicate 0.2 ml samples of this suspension were placed on membrane filters (Millipore HA filters) and assayed for total cpm according to Methods section L.

The remaining suspension was sonicated according to Methods section Q, and the disrupted suspension centrifuged at 3500 x g for 30 min at 4 C to remove residual whole cells. The supernatant was saved for furthur analysis, and the pellet resuspended in 2.0 ml of 75% synthetic seawater. Duplicate 0.1 ml samples were withdrawn from this suspension and assayed for total counts according to Methods section L-3.

The 3,500 x g supernatant was centrifuged at 39,900 x g for one hr at 4 C to collect the envelope fragments in the suspension. The supernatant obtained was assayed for ^{64}Cu content by spotting duplicate 0.1 ml samples on Whatman #2 filter paper (Methods section L-3). The pellet obtained was

resuspended in 2.0 ml of 75% synthetic seawater and assayed for ^{64}Cu content according to Methods section L-3.

A recovery of 98.8% of the isotope present in the initial cell suspension was accomplished (Table 40). Only 4.67% of the isotope present in the 3,500 x g supernatant was associated with the 39,900 x g pellet (wall fraction) with the residual 95.37% located in the 39,900 x g supernatant (non-wall fraction) (Table 40). Thus, 95.38% of copper taken up by P. cuprodurans in basal medium supplemented with 1 x 10^{-3} M copper was localized interior to the cell envelope.

U. Localization of Copper by Biochemical Extraction.

To obtain furthur information regarding the location of copper in P. cuprodurans cells, biochemical extractions were employed. Application of this method had been employed previously to localize ^{191}Pt in E. coli B cells (Renshaw and Thomson, 1967). Such extractions would reveal the class of compounds to which copper was bound in P. cuprodurans.

Duplicate 99 ml volumes of basal medium and basal medium supplemented with 1 x 10^{-4}, 5 x 10^{-4}, and 1 x 10^{-3} M copper were prepared in 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. The flasks were seeded with ^{64}Cu and inoculated with the standard inoculum (Methods section C). All flasks were incubated at 20 C at 140 rpm until attainment of an optical density of 0.85 at 420 mμ. The cells were harvested washed and extracted as in Results section J. Samples (0.1 ml) of each extract were spotted on Whatman #2 filter paper according to Methods section L-3 and the isotope quantitated. The ratios of DNA : RNA : protein were determined to check

the validity of the extracts.

The ethanol-ether extract from all cells contained approximately the same level of isotope (Table 41). Quantities of isotope in the 0.2 N perchloric acid extract decreased with increased copper supplementation to a level of 5×10^{-4} M copper (Table 41). Large amounts of isotope were in the 2.0 N perchloric acid extract with a small amount in the 1.0 N sodium hydroxide extract (Table 41).

These results demonstrated that copper was distributed throughout the major biochemical constituents of the cells. The lipid extract (ethanol-ether) contained a fairly constant percentage of the copper transported into the cell indicating that the membrane of P. cuprodurans may equilibrate with the external copper concentration. The decreased levels of the isotope present in the 0.2 N perchloric acid with increased copper supplement to the growth medium may suggest a migration of copper in the cell with the ion binding to larger molecular weight intracellular ligands (Table 41). The finding of the majority of the copper present in the 2.0 N extract was thought to be due to the extreme strength of the acid (2.0 N perchloric acid) employed which released the cation from protein ligands. This would explain the finding of such a small amount of the isotope present in the sodium hydroxide extract. Thus, the level of isotope present in the 2.0 N perchloric acid and 1.0 N sodium hydroxide extracts was distributed between the DNA, RNA, and protein moieties.

Thus, 15-20% of the copper taken up by P. cuprodurans in the media tested is associated with lipid and small molecular weight components of

the cell (Table 41). The residual 80-85% of copper was present in the nucleic acid and protein components of the cell.

V. Quantitation of Copper Uptake by Anodic Stripping Voltammetry.

To measure the amount of copper taken up by P. cuprodurans, cells were prepared and quantitated for copper uptake according to Methods section S.

Copper concentration by P. cuprodurans increased as copper supplementation of the basal medium increased (Table 42). When calculated furthur to the amount of copper per 10^8 cells using the cell number relationship to protein (Fig 14), significantly lower amounts of copper uptake was found when these results (Table 42) were compared to those determined by isotope dilution (Results section Q: Fig 30 through 33).

The results obtained here agree with those determinations performed by Roche (1966). The discrepancy existing between isotope dilution determinations and these measurements will be considered in the discussion section.

V. DISCUSSION

A. Biological Effects of Copper.

1. DNA : RNA : Protein Ratios.

Copper stress caused decreased RNA and protein synthesis in bacteria (Blundell and Wild, 1969), and increased protein synthesis in fungi (Healy, 1955). Copper-resistant mutants of *E. coli* possessed increased amounts of nucleic acids (Weed and Longfellow, 1954), whereas copper-resistant mutants of *B. subtilis* demonstrated partial loss of their genome (Weed, 1963).

P. cuprodurans cultivated in basal medium with increased copper-stress exhibited no alteration of the cellular DNA : RNA : protein ratio (Table 26). Such measurements were quantitative only, and did not reflect qualitative changes in RNA or the protein species synthesized by *P. cuprodurans* under copper stress. The DNA : RNA : protein ratio indicated a lack of copper damage to essential nucleic acid polymerases unlike the copper-sensitive enzymes in eucaryotic systems (Novello and Stripe, 1969). Inhibition of such enzymes by copper would have caused an apparent alteration in the DNA : RNA : protein ratio.

2. Electron Transport and Respiratory Alteration.

a. Oxygen Utilization.

Oxygen utilization by *P. cuprodurans* decreased in copper-supplemented medium (Results section M-1: Fig 17). Copper stress has also been observed to decrease respiration in other microorganisms: algae (Kanazawa and Kanazawa, 1969; McBrien and Hassal, 1967; Hassal, 1963; Hassal, 1962),

yeast (Minagawa, 1958; Murayama, 1961a), copper-resistant bacteria (Weed and Longfellow, 1954) and two soil pseudomonads A-50 and C-1 (Sadler and Trudinger, 1967).

Growth in copper-supplemented basal medium reduced the immediate effect of copper on respiration, and decreased the oxygen uptake of P. cuprodurans in unsupplemented basal medium broth (Results section M-2 through M-5: Table 28). Respiration was only decreased in copper concentrations exceeding that in which the bacterium was cultivated (Results section M-2 through M-5). This minimizing of copper's respiratory effect by growth in copper-stressed medium indicated cellular adaptation to copper stress. Respiration studies of copper stress on P. A-50 revealed a similar respiratory adaptation (Sadler and Trudinger, 1967).

b. Triphenyl Tetrazolium Chloride Overlays.

P. cuprodurans colonies on copper-supplemented basal medium agar plates exhibited progressively delayed TTC reduction with increased copper (Table 29). Respiratory deficiencies induced by 5×10^{-4} and 1×10^{-3} M copper were reversed by growth of the respiratory-deficient bacteria on basal medium agar plates (Results section N-2). Maximum copper stress applied to B. subtilis, E. coli B and S. aureus did not cause delayed TTC reduction by the bacteria, this indicated that the formation of respiratory deficiencies was not a general effect of copper (Table 36). Maximum tolerated concentrations of divalent metals other than copper did not illicit delayed reduction of TTC by P. cuprodurans (Table 34). Thus, there was some degree of specificity of copper for P. cuprodurans for the

formation of respiratory deficiencies.

Hassal (1963) also observed that respiratory decreases induced by copper stress on Chlorella vulgaris were not induced by other heavy metal ions. Copper has induced respiratory deficiencies in yeast (Lindegren, Nagai, and Nagai, 1958; Yanagashima, 1957). Copper stress exhibited no effect on the respiration of E. coli (Jones, 1964)

c. Thunberg Studies.

Growth of P. cuprodurans in copper-supplemented medium caused delayed reduction of DCIP, MB, and TTC in Thunberg tubes (Results section O: Fig 22-24). Methylene blue (MB) and dichloroindophenol (DCIP) are established electron acceptors at the flavoprotein-quinone level of electron transport (Lardy, 1949; Dolin, 1961). Cells grown in basal medium supplemented with 1×10^{-4} M copper exhibited a nine-fold decrease in the reduction rate of MB, TTC, and DCIP when compared to the reduction rate of non-copper stressed cells. The extended reduction time of MB and DCIP by copper-stressed P. cuprodurans cells (Results section O) indicated alteration in the electron transport system at or before the flavoprotein-quinone level.

d. Cytochrome Studies.

Difference spectra performed on copper-stressed whole cell extracts demonstrated three cytochrome components in P. cuprodurans: cytochromes b_1, c, and o (Results section P-1). Alteration of cytochromes b_1 and c occurred in copper-stressed cells (Fig 26-28: Table 35). The predominant cytochrome change was a decreased cytochrome c content with increased concentrations of

copper in the growth medium (Table 35). Similar decreases in c-type cytochromes have been observed with N. crassa (Nicholas and Commisiong, 1957), S. ellipsoideus (Minagawa, 1958), and Pseudomonas A-50 (Sadler and Trudinger, 1967) when cultivated under similar copper stresses.

Unfortunately, whole cell extracts of P. cuprodurans did not demonstrate respiration, making the determination of DPNH and substrate-reducible cytochromes impossible. The availability of such respiring extracts would have permitted the determination of DPNH and substrate-reducible cytochromes, and the performance of time-course, in vitro studies of copper alteration of the electron transport system.

Difference spectra of whole cell extracts demonstrated the absence of peaks absorbing in the 590 to 650 mμ range, eliminating cytochrome a and the Pseudomonas blue protein. The Pseudomonas blue protein is a copper protein isolated from P. aeruginosa (Horio et al, 1958) which participates in the electron transport system (Dolin, 1961). Stimulation of cytochrome a synthesis by copper has been found in anaerobically incubated P. aeraginosa cells (Yamanaka, Kijimoto, and Okunuki, 1963).

The difference spectra performed indicated a progressive decrease in the depth of the 450-460 mμ trough in copper-stressed whole cell extracts (Fig 26-28) which is indicative of decreased flavoprotein content (Lehninger, 1970; White, 1962). These observations were consistent with Thunberg studies with methylene blue and dichloroindophenol (Results section 0).

e. Site of Electron Acceptance by Triphenyl Tetrazolium Chloride.

Triphenyl Tetrazolium Chloride in certain mammaliam systems

demonstrated electron acceptance at cytochromes a_1/a_3 (Slater, Sawyer and Strauli, 1963; Nachlas, Margulies and Seligman, 1960). Studies with rat liver mitochondria, however, have indicated that TTC accepts electrons at the flavoprotein-quinone level of electron transport (Sato and Sato, 1965). Decreased reduction of TTC (Results section N) did not agree with respiration decreases observed for cells treated with the same copper stress (Results section M). Agreement should have occurred if TTC accepted electrons at the oxygen terminal site of electron transport (cytochrome a_1/a_3 level). Thunberg studies (Results section O) of 1×10^{-4} M copper-stressed cells exhibited a nine-fold decrease in reduction rate of TTC, DCIP, and MB when compared to reduction rates obtained for non-copper stressed cells. Dichloroindophenol and methylene blue are established electron acceptors at the flavoprotein-quinone level (Dolin, 1961). The similarities in decreased reduction by 1×10^{-4} M copper-stressed cells of TTC, MB, and DCIP, coupled with the lack of agreement between TTC reduction and respiration data suggested that TTC accepts at the flavoprotein-quinone level of electron transport. Supportive evidence was found in the genus Streptococcus which possess no cytochromes, but reduce TTC (Slanetz and Bartley, 1957).

f. Copper Stress and the Electron Transport System of P. cuprodurans.

The electron transport system of P. cuprodurans was altered with increased copper concentrations at the flavoprotein-quinone and cytochrome sites. Nicholls and Malviya (1968) found zinc altered the electron transport system of Keilin-Hartree particles (sub-mitochondrial particles of

heart muscle) at two sites " . . . an initial one between cytochrome b and c_1 and a subsequent one at the flavoprotein level in the respiratory chain." Copper interactions with the electron transport system in P. cuprodurans closely parallel these findings of Nicholls and Malviya (1968).

No stoichiometric relation between oxygen utilization, TTC overlays, Thunberg studies, and cytochrome studies was obtained. It is possible that decreased reduction rates of MB, DCIP, and TTC induced by copper-stress reflected the disappearance or blockage of one electron transport pathway and a shift to a parallel or alternate pathway. Such an alternate pathway would be unable to transfer electrons to the TTC, DCIP, and MB acceptors employed. This hypothesis would explain the extended delays in DCIP, MB, and TTC reduction encountered (Results section N and O).

Possible permeability changes in the cell membrane to the oxidation-reduction compounds used may also explain the extension in reduction times encountered with increased copper stress. However, similar extension in dye reductions were not noted for non-marine bacteria under maximum copper stress (Results section Q), and were not encountered when P. cuprodurans was cultivated under maximum tolerated concentrations of other divalent transitional metals which should have also affected membrance permeability (Table 34).

The ability of copper stress to decrease both respiration and the quantity of cytochrome c was observed in P. cuprodurans. These same effects of copper stress have occurred in S. ellipsoideus (Minagawa, 1958; Murayama, 1961a) and in Pseudomonas A-50 (Sadler and Trudinger, 1967). The common

response by these microorganisms to copper stress suggested a common site of attack by the copper ion.

Mechanisms for copper alteration of the electron transport system are numerous. Copper has an extremely high avidity for sulfhydryl groups (Passow, Rothstein, and Clarkson, 1961) which are essential for electron transport (Barron et al, 1948; Haugaard et al, 1969).

Potential alteration of DPNH-producing pathways (i.e tricarboxylic acid cycle) may contribute to the copper-induced alteration of electron transport and oxygen uptake in P. cuprodurans. Isocitric dehydrogenase and succinic dehydrogenase were inhibited by copper stress in in vivo studies with S. ellipsoideus (Murayama, 1961d). Copper inhibited succinic dehydrogenase in N. crassa (Healy, 1955). Oxoglutarate reductase was partially inhibited by copper (Webb, 1964). Plant riboflavin kinase is inhibited by copper (Dixon and Webb, 1964).

Ion antagonism by copper in metalloflavoproteins may explain the decreased flavoprotein content observed in difference spectra (Results section P-1). Copper's affinity for flavins and flavoproteins have been reviewed by Albert (1951) and Ehrenberg and Hemmerich (1968). Thus, displacement of iron, or molybdenum co-factors of flavoproteins by copper may render a flavoprotein non-functional (Rajagopalan and Handler, 1968).

The observed electron transport alterations may indicate a secondary effect of increased copper-stress rather than a primary effect. Passow, Rothstein, and Clarkson (1961) have stated ". . . the inactivation of one sensitive site by a metal usually induces a whole sequence of secondary

changes which may affect the physiological state of the whole cell."

3. Morphological Alteration by Copper.

No morphological alteration of P. cuprodurans cells occurred when they were subjected to maximum copper stress (Results sections D-1 and L). Copper has induced divisional cessation accompanied by morphological aberrancies in several microorganisms: Chlorella ellipsoidea (Kanazawa and Kanazawa, 1969), B. stearothermophilus (Bubela, in press), A. marinus (Cobet, 1968), Leptosphaeria michotii (Jerebzoff, 1967) and P. C-1 (Sadler and Trudinger, 1967).

Ultrastructural studies performed by Roche (1966) demonstrated electron-dense granular deposits interior to the cell membrane of P. cuprodurans. Such granular deposits were hypothesized to be concentrated copper within the cell (Roche, 1966). These granular deposits were not observed in our ultrastructural studies (Fig 15 and 16). It is doubtful that the granular deposits observed by Roche represented copper deposits, as copper is electron transparent (Pihl, 1968). The electron dense areas are considered as osmium tetroxide deposits formed in the fixation and staining processes.

No significant alteration of the ultrastructure of P. cuprodurans occurred when exposed to a copper stress of 1×10^{-3} M, which may reflect the copper tolerance of P. cuprodurans.

4. The effect of Temperature on Growth and Copper Tolerance by P. cuprodurans.

Growth temperatures from 35 to 42 C caused an increased copper

toxicity in E. coli (Burke and McVeigh, 1967). Increasing incubation temperature for A. marinus from 35 to 40 C enhanced the toxic effect of nickel (Cobet, 1968). P. cuprodurans in basal medium demonstrated optimal growth at 33 C (Results section H). However, P. cuprodurans in copper-supplemented basal medium demonstrated optimal growth at 26 C for all concentrations of copper tested (1×10^{-4} to 2×10^{-3} M) (Fig 8). While incubation temperatures beyond 26 C enhanced the inhibitory effect of copper, some growth in copper-supplemented medium occurred at 33 C but not at 36 C.

The common 26 C temperature optimum demonstrated in the copper-supplemented medium tested may be due to a variety of factors. Some of the factors include: 1. the susceptibility of an enzyme which aids in the copper-tolerance to temperatures above 26 C optimum. 2. the increased uptake of copper by the cell grown at temperatures above 26 C. 3. the detrimental alteration of the plasma membrane under copper-stress conditions above the temperature of 26 C. 4. a physiological shift to a metabolic pathway inhibited by copper at temperatures above 26° C.

The significance of this common temperature optimum for copper-stressed cells is not understood and should be studied by future investigators.

B. Uptake of Copper by P. cuprodurans.

1. Isotope Measurements

a. Transport of Copper During Growth of P. cuprodurans.

Copper uptake was measured by ^{64}Cu, in all media employed

(Results section R: Fig 30-33). P. cuprodurans cells in earlier stages of logarithmic growth absorbed more copper than did cells in late logarithmic or stationary phases of growth (Fig 30-33; Table 37). Cell division alone could not account for the decreased absorbed copper observed in late logarithmic and stationary phase cells. This decreased copper uptake remained constant (Table 37) and may have represented a regulatory transport system for copper, such as a specific permease.

Sadler and Trudinger (1967) studied the effect of copper on Pseudomonas C-1 and observed that the organism on initial contact with copper stress, lost motility and was altered its morphology. The inhibitory effect of copper held for one hr, after which growth of the bacterium resumed. Upon introduction of copper stress to a broth culture of Pseudomonas C-1, copper uptake by the cell followed and indicated that

> "Initially copper was rapidly absorbed by the bacteria and amounts per cell were 2-3 times those found in growing cells at the same external concentration. Following initial binding, there was a progressive fall in the amount of copper bound per cell until, at the onset of growth, the value characteristic of that for bacteria growing at the particular external copper concentration, was reached." (Sadler and Trudinger, 1967)

The pattern of copper uptake by Pseudomonas C-1 and those of P. cuprodurans were quite similar except a decrease in copper associated with the cell occurred in P. cuprodurans during growth, whereas a reduction of cell copper in Pseudomonas C-1 occurred during a period of no growth with the development of a swollen morphology. The mechanism of copper uptake and regulation by these two bacteria may not be the same, but it is of interest that both accumulate a constant amount of copper.

b. Uptake of Copper by Stationary-phase Cells.

In all situations tested, P. cuprodurans cells demonstrated a rapid ionic binding of copper (Results section S). Rapid ionic binding of copper has been observed in fungal spores which is characteristic of trace metals (Marsh, 1945; McCallan and Miller, 1958). No initial binding of magnesium was observed to occur in E. coli (Silver, 1969).

Following ionic binding of copper to P. cuprodurans, a diphasic uptake of copper occurred which required energy (Fig 34 and 35). The presence of basal medium was necessary for the diphasic uptake of copper, since diphasic uptake did not occur with cells incubated endogenously (Fig 35). The basal medium may serve as either an energy source or a carrier for the transportation of copper into the cell.

The diphasic uptake of copper was eliminated after treatment of the cells with chloramphenicol (100 ug/ml) and replaced by a higher rate of copper uptake by the cell. This demonstrated a requirement for protein synthesis in the diphasic uptake process (Fig 35). The diphasic uptake of copper may be due to a repressible permease system for either copper or a basal medium carrier to which copper was bound (i.e. an amino acid). The effect of chloramphenicol could then be explained as due to the inhibition of synthesis of a protein repressor necessary for the diphasic uptake of copper.

c. Physical Localization of Copper.

The majority (95.38%) of copper assimilated by P. cuprodurans in basal medium supplemented with 1×10^{-3} M copper was located interior to

the cell envelope (Table 40). This finding is in agreement with chemical analysis performed by Roche (1966).

d. Chemical Localization of Copper.

Chemical localization of copper in cells of P. cuprodurans revealed changes in the amount of copper associated with intermediary metabolytes and small molecular weight components with increased copper stress (12% for non-copper stressed cells to 6.2% for 5×10^{-4} and 1×10^{-3} M copper stressed cells) (Table 41). The decreased copper content of the intermediary metabolyte fraction was accompanied by increased copper in the nucleic acid and protein fractions. Thus, increased copper stress altered the distribution of assimilated copper. The lipid extract of P. cuprodurans contained a stable percentage of copper (mean of 9.5%) when the bacterium was cultivated in copper stressed and non-copper stressed medium (Table 41). The relative stability of assimilated copper in the lipid extract suggests an equilibration between the external copper concentration and the amount of copper bound to the plasma membrane. Substantial amounts of assimilated copper (75%-80%) were extracted in the nucleic acid fraction of P. cuprodurans cultivated in the four media (Table 41). The high percentage of total copper associated with the nucleic acid fraction is due to the extreme strength of the acid employed (2.0 N perchloric acid) releasing copper from protein ligands. Thus, values obtained for the nucleic acid and protein extracts should be combined for discussion. The combined protein and nucleic acid extraction value increased from 78% to 83% of the accumulated copper as copper stress was increased to 1×10^{-3} M

(Table 41). The presence of intracellular copper associated with these macromolecular constituents was quite high. It may well be that a majority of the copper presently described as residing in the nucleic acid-protein fraction was in association with the protein fraction of the cell. Intracellular proteins responsible for the storage of copper have been described in mammalian systems (Porter, 1966; Vogel and Kemper, 1966). The ability of P. cuprodurans to synthesize proteins of this nature would enable the cell to tolerate high concentrations of intracellular copper.

2. Anodic Stripping Voltammetry Measurements.

Copper determinations by anodic stripping voltammetry (Table 42) revealed a significant departure from values obtained by isotope dilution methods (Table 37). Reasonable agreement between the two methods occurred with non-copper stressed cells: 0.274% copper uptake/10^8 cells as measured by anodic stripping voltammetry (Table 42); and 0.22% copper uptake/10^8 cells as measured by isotope dilution (Table 37). Copper stressed cells, however, did not demonstrate such agreement since uptake values/10^8 cells decreased more significantly with increased copper supplementation when measured by anodic stripping voltammetry than when measured by isotope dilution: P. cuprodurans grown in 1×10^{-3} M copper demonstrated a 0.0089% uptake/10^8 cells when measured by anodic stripping voltammetry (Table 42), and a 0.197 to 2.38% uptake/10^8 cells when measured by isotope dilution (Table 37).

Copper uptake in basal medium and basal medium supplemented with

1×10^{-4} M copper, measured by anodic stripping voltammetry, were in agreement with those determined by Roche (1966).

3. Comparison of Isotope and Anodic Stripping Voltammetry Measurement.

Descrepancies in copper uptake determined by anodic stripping voltammetry may have been due to the manner in which the copper was added to the basal medium (Results sections R and V). In the isotope procedure ^{64}Cu was added after autoclaving; while in the anodic stripping voltammetry measurement, copper was added before autoclaving. To determine if the manner in which copper was added to the basal medium was responsible for the observed descrepancy, basal medium was prepared, autoclaved, and then supplemented with 1×10^{-4} M copper. The distribution of labile and non-labile copper was determined by anodic stripping voltammetry measurement (Methods section S). The distribution of non-labile and labile copper in basal medium in which 1×10^{-4} M copper was added after autoclaving was identical to that of media in which the 1×10^{-4} M copper was added previous to autoclaving (Table 16). This indicated that the distribution of the cation was unaffected by the method of copper addition.

A study of data collected by other investigators regarding trace metal uptake: 1. uptake of cobalt by Proteus vulgaris (Neyland, Dunkel, and Schade, 1952); and 2. nickel uptake by A. marinus (Cobet, 1968) revealed parallel slopes of cation uptake with concentration as determined here with ^{64}Cu (Fig 36).

The descrepancy between ^{64}Cu and anodic stripping voltammetry uptake values requires furthur work to determine which best describes

copper accumulation by P. cuprodurans.

C. Copper Tolerance.

The basal medium played an integral role in the copper tolerance of P. cuprodurans and other bacteria. Reduction of the organic matter in the medium caused a reduction of copper tolerance by P. cuprodurans (Table 15: Fig 4). Levels of copper tolerance for copper-resistant mutants of B. subtilis (1×10^{-5} M) (Weed, 1963) and E. coli (5×10^{-5} M) (Weed and Longfellow, 1954) in defined media were exceeded by non-mutated basal medium grown cells of the same genera and species (Tables 11 and 13). Strain differences are a possible explanation for the increased copper tolerances noted, but chelation of copper by the basal medium plays an important role in increased tolerance levels for these bacteria. Chelating agents effectively reduced metal toxicity for E. coli (Jones, 1964; Jones, 1967b).

Adaptation of P. cuprodurans to copper stress was encountered in this research. Growth of non-copper stressed cells in basal medium supplemented with 1×10^{-3} M copper caused a 7-hr lag phase characterized by the absence of decreased viable cell numbers before logarithmic growth (Fig 5). Growth of 1×10^{-3} M copper-stressed cells in basal medium supplemented with 1×10^{-3} M copper was characterized by a 3-4 hr lag phase before growth (Fig 6). Return of 1×10^{-3} M copper-stressed cells to basal medium and subsequent transfer to basal medium supplemented with 1×10^{-3} M copper caused cells to again demonstrate the same 7-hr lag phase (Fig 7).

Growth of P. cuprodurans on basal medium agar supplemented with

either 5×10^{-4} or 1×10^{-3} M copper caused cells to become respiratory deficient (Results section N: Table 29). Transfer of a respiratory deficient colony to basal medium agar caused P. cuprodurans to revert to a respiratory sufficiency (Results section N-2)

Genetic alteration may be eliminated as a participant in the copper tolerance of P. cuprodurans. Copper induced TTC respiratory deficiencies (Results section N) and the more rapid growth response to copper stress (Results section F) were not stable characteristics as they were removed by growth in basal medium. If genetic alteration occurred such characteristics would have remained stable with growth in the absence of copper stress. Although not tested, it is highly probable that observed copper-induced effects such as cytochrome alteration, DCIP and MB delayed reductions, and respiration can be reverted to the unstressed state by transfer of the bacterium to non-copper stressed media.

Adaptation of this nature has been reported by Sadler and Trudinger (1967) who state ". . . bacteria grown in the presence of sublethal concentrations of copper exhibit an increased copper resistance compared with cells of the original inoculum. However, after one passage through a copper-free medium, this enhanced resistance is lost." Sadler and Trudinger (1967) have also found that such adaptation to copper is ion specific: "Resistance to copper was not acquired by bacteria after exposure to other metals such as cobalt, nickel, iron, manganese or zinc nor did acquisition of copper resistance diminish sensitivity to these other metals." It is not presently known if the adaptation of P. cuprodurans to copper ion is as

specific as found by Sadler and Trudinger (1967) for P. A-50. The adaptation to copper is concentration-dependent since respiration is only affected by concentrations of copper exceeding that in which the bacterium was grown. The adaptation phenomenon probably reflects physiological alterations due to copper stress.

Copper toxicity in P. cuprodurans may reside in the interaction of copper with the basal medium constituents. Results obtained from anodic stripping voltammetry analyses of autoclaved (Table 16) and filter-sterilized media (Table 17) were plotted as log of labile and non-labile copper concentration vs the log of the total copper supplement (Fig 37 and 38). Copper concentrations of 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M yielded a linear plot of labile and non-labile copper for both filter-sterilized and autoclaved media (Fig 37 and 38). Concentrations of labile copper in the filter-sterilized media (Fig 37) exceeded those of corresponding autoclaved basal medium supplemented with 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M copper (Fig 38). The decreased labile copper values for autoclaved media (Fig 38) are possibly due to the exposure of more ligand groups by the autoclaving process.

In filter-sterilized basal medium supplemented with 2×10^{-3} M copper, significant deviation of relationship of labile to non-labile copper was found (Fig 37). Slight deviation of the labile to non-labile relationship for autoclaved basal medium supplemented with 2×10^{-3} M copper also occurred (Fig 38). It was noted in tolerance studies performed with P. cuprodurans (Results sections D-1 and E) that copper toxicity always was

accompanied by a visible precipitate in the basal medium. Precipitation occurred when the pH of the medium was adjusted to neutrality. Precipitated copper in anodic stripping voltammetry measurements was included in non-labile copper values. The decrease in the rate of labile copper formed in filter-sterilized and autoclaved basal medium supplemented with 2×10^{-3} M copper may reflect the passage of the saturation point of the medium with labile copper and thus the precipitation of a small degree as increased non-labile copper (Fig 37 and 38). Thus, P. cuprodurans may survive in saturated copper medium and toxicity may result from the precipitation of an essential growth factor. Copper supplementation to synthetic media at neutral pH precipitated essential phosphates and prohibited the growth of Torula utilis (Avakyan and Rabotonova, 1966). Thus, the deprivation of an essential growth factor by over-saturating quantities of copper is a logical explanation for the toxicity values (Results sections D-1 and E). If this hypothesis is true, the true copper tolerance concentration for P. cuprodurans may be even higher than reported.

Alternate explanations for the copper toxicity exist:

1. Growth of P. cuprodurans in increased copper stress greatly affects respiration and electron transport (Discussion section A-2). At toxic concentrations of copper, components of the electron transport system may be decreased to such an extent that P. cuprodurans expires due to anoxia.

2. Essential protein enzymes may be inactivated by the binding of copper to sulfhydryl groups (Thimann, 1963) or to other ligands such as imidazole groups (Passow, Rothstein, and Clarkson, 1961).

3. Copper, being highly reactive, may combine with nutrients of the basal medium (i.e. and essential amino acid) effectively removing such nutrients by chelation and causing a nutrient deprivation in the medium.

4. Copper is able to combine firmly with the DNA molecule at guanine and cytosine residues and phosphate groups. Structural alteration of DNA may occur (i.e. crosslinking) rendering a part of the molecule non-functional (Eichorn and Clark, 1965).

5. Copper causes decreased intracellular magnesium levels of some pseudomonads to as low as 30% of the normal cell (Sadler and Trudinger, 1967). Magnesium depletion or starvation of the cell may occur and effect the stability of ribosomes (McCarthy, 1962).

6. Copper because of its high reactivity may cause damage to a cell by antagonism of essential ion cofactors (Abelson and Aldous, 1950).

7. In high concentrations of copper, there may be sufficient alteration of membrane permeability to disrupt transport processes (Fuhrman and Rothstein, 1968).

D. Speciation of P. cuprodurans.

The ability of P. cuprodurans to withstand high copper concentration is characteristic of the bacterium. Comparing the 2.25-2.50 x 10^{-3} M copper tolerance level for P. cuprodurans to ecological studies of copper tolerance performed by Waksman, Johnstone, and Carey (1943), Starr and Jones (1957) and Roche (1966) on marine bacteria, P. cuprodurans resists copper in the top 15% of the marine bacteria tested by these investigators. P. cuprodurans was initially isolated for its ability to tolerate copper

(Roche, 1966). This dissertation confirms that the epithet selected, describes the species.

P. cuprodurans is a marine bacterium since it corresponds to the definitions of marine bacteria set forth by ZoBell and Upham (1944) and MacLoed (1965) due to its requirements for seawater and sodium, respectively (Results sections B and C-1). P. cuprodurans demonstrated requirements for calcium, magnesium, and sodium, but not potassium or strontium for growth (Results section C). Deletions of calcium or magnesium from the growth medium caused development of aberrant morphology of P. cuprodurans (Fig 2 and 3). Magnesium deletions have illicited similar aberrant morphology in other bacteria (Webb, 1951; Shankar and Bard, 1952). No citings of morphological aberrancies due to calcium deletion have been found in the literature. The requirements for magnesium and calcium correspond with results of Eagon (1969) who found these two cations essential for the integrity of the cell wall of P. aeruginosa.

VI. BIBLIOGRAPHY

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Table 1. List of chemicals, bacteriological media, and reagents employed. The table is divided into four major categories: inorganic compounds, organic compounds, bacteriological media, and prepared reagents. The following abbreviations are employed: A.R.=analyzed reagent, Cert.=certified, Rgt.=reagent grade.

INORGANIC COMPOUNDS

Compound	Formula	Grade	Manufacturer
ammonium hydroxide	NH_4OH	Rgt.	Fisher Scientific Co., Medford, Massachusetts
ammonium nitrate	NH_4NO_3	A.R.	Baker Chemical Co., Phillipsburg, New Jersey
boric acid	H_3BO_3	Cert.	Fisher Scientific Co., Medford, Massachusetts
cadmium chloride	$CdCl_2$	Cert.	Fisher Scientific Co., Medford, Massachusetts
calcium chloride	$CaCl_2$	Cert.	Fisher Scientific Co., Medford, Massachusetts
cobalt chloride	$CoCl_2$	Cert.	Fisher Scientific Co., Medford, Massachusetts
copper sulfate	$CuSO_4 \cdot 5\ H_2O$	Cert.	Fisher Scientific Co., Medford, Massachusetts
copper sulfate isotope	$^{64}CuSO_4$	----	Cambridge Nuclear Corp., Cambridge, Massachusetts
hydrochloric acid	HCl	Rgt.	Fisher Scientific Co., Medford, Massachusetts
hydrogen peroxide	H_2O_2	Cert.	Fisher Scientific Co., Medford, Massachusetts
magnesium chloride	$MgCl_2 \cdot 6\ H_2O$	Cert.	Fisher Scientific Co., Medford, Massachusetts
nickel chloride	$NiCl_2 \cdot 6\ H_2O$	Cert.	Fisher Scientific Co., Medford, Massachusetts
nitric acid	HNO_3	Rgt.	Fisher Scientific Co., Medford, Massachusetts

(Table 1, cont'd)

INORGANIC COMPOUNDS

Compound	Formula	Grade	Manufacturer
perchloric acid	$HClO_4$	Rgt.	Allied Chemical Co., Morristown, New Jersey
potassium bromide	KBr	Cert.	Fisher Scientific Co., Medford, Massachusetts
potassium chloride	KCl	Cert.	Fisher Scientific Co., Medford, Massachusetts
potassium hydroxide	KOH	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium bicarbonate	Na_2CO_3	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium chloride	NaCl	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium cyanide	NaCN	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium dithionite	$Na_2S_2O_4$	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium ferricyanide	$NaFe(CN)_6$	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium fluoride	NaF	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium hydroxide	NaOH	Cert.	Fisher Scientific Co., Medford, Massachusetts
sodium sulfate	Na_2SO_4	Cert.	Fisher Scientific Co., Medford, Massachusetts
strontium chloride	$SrCl_2 \cdot 6\ H_2O$	Cert.	Fisher Scientific Co., Medford, Massachusetts
sulfuric acid	H_2SO_4	Rgt.	Fisher Scientific Co., Medford, Massachusetts
zinc chloride	$ZnCl_2$	Cert.	Fisher Scientific Co., Medford, Massachusetts

(Table 1, cont'd)

ORGANIC COMPOUNDS

Compound	Manufacturer
acetaldehyde	Eastman Organic Chemicals, Rochester, New York
adenosine-2'&3'-monophosphoric acid	Sigma Chemical Co., St. Louis, Missouri
arabinose	Fisher Scientific Co., Medford, Massachusetts
arginine	Eastman Organic Chemicals, Rochester, New York
azolitmin	Matheson, Coleman & Bell, Cincinnati, Ohio
bovine serum albumin fraction V	Fisher Scientific Co., Medford, Massachusetts
cellobiose	Matheson, Coleman & Bell, Cincinnati, Ohio
chloramphenical	Sigma Chemical Co., St. Louis, Missouri
dextrin	Pfanstehl Chemical Co., Waukegan, Ill.
dextrose	Fisher Scientific Co., Medford, Massachusetts
2,6-dichloroindophenol (DCIP) (2,6-dichlorobenzoneindophenol)	Fisher Scientific Co., Medford, Massachusetts
2,4-dinitrophenol (DNP)	Nutritional Biochemical Corp., Cleveland, Ohio
diphenylamine, certified	Fisher Scientific Co., Medford, Massachusetts

(Table 1, cont'd)

ORGANIC COMPOUNDS

Compound	Manufacturer
deoxyribonucleic acid (DNA) ex salmon sperm, A grade	Calbiochem, Los Angeles, California
dulcitol	Eastman Organic Chemicals, Rochester, New York
ether, anesthesia grade	Fisher Scientific Co., Medford, Massachusetts
formaldehyde, certified	Fisher Scientific Co., Medford, Massachusetts
formic acid, certified	Fisher Scientific Co., Medford, Massachusetts
galactose	Pfanstehl Chemical Co., Waukegan, Ill.
glycerol, certified	Fisher Scientific Co., Medford, Massachusetts
glycogen	Fisher Scientific Co., Medford, Massachusetts
inositol	Difco Laboratories, Inc., Detroit, Michigan
lactose	Difco Laboratories, Inc., Detroit, Michigan
lead acetate, A.R.	Mallincrodt Chemical Works, New York, New York
maltose	Fisher Scientific Co., Medford, Massachusetts
mannitol	Fisher Scientific Co., Medford, Mass.
methylene blue	Baltimore Biological Laboratory, Baltimore, Md.

(Table 1, cont'd)

ORGANIC COMPOUNDS

Compound	Manufacturer
orcinol	Fisher Scientific Co., Medford, Massachusetts
phenazine methosulfate	Mann Research Laboratory, New York, New York
phenol red	Baltimore Biological Laboratory, Baltimore, Md.
raffinose	Difco Laboratories, Inc., Detroit, Michigan
riboflavin	Nutritional Biochemical Corp., Cleveland, Ohio
sodium acetate, certified	Fisher Scientific Co., Medford, Massachusetts
sodium citrate, certified	Fisher Scientific Co., Medford, Massachusetts
sodium lactate, certified	Fisher Scientific Co., Medford, Massachusetts
sodium succinate, certified	Fisher Scientific Co., Medford, Massachusetts
sorbitol	Baltimore Biological Laboratory, Baltimore, Md.
sucrose	Fisher Scientific Co., Medford, Massachusetts
tetramethylparaphenylene diamine hydrochloride	Eastman Organic Chemicals, Rochester, New York
toluene, A.R.	Mallincrodt Chemical Works, New York, New York
triphenyl tetrazolium chloride	Eastman Organic Chemicals, Rochester, New York
xylose	Difco Laboratories, Inc., Detroit, Michigan

(Table 1, cont'd)

ORGANIC COMPOUNDS

Compound	Manufacturer
vitamin K	Nutritional Biochemical Corp., Cleveland, Ohio

BACTERIOLOGICAL MEDIA

Media	Manufacturer
Extract Agar	Baltimore Biological Laboratory, Baltimore, Md.
Gelatin, bacteriological	Fisher Scientific Co., Medford, Massachusetts
Kligler Iron Agar	Difco Laboratories, Inc., Detroit, Michigan
Lead Acetate Agar	Difco Laboratories, Inc., Detroit, Michigan
MR-VP Broth	Baltimore Biological Laboratory, Baltimore, Md.
Peptone (Lot # 762280)	Fisher Scientific Co., Medford, Massachusetts
Phenol Red Broth Base	Baltimore Biological Laboratory, Baltimore, Md.
Skim milk	Difco Laboratories, Inc., Detroti, Michigan
Starch Agar	Difco Laboratories, Inc., Detroit, Michigan
Triple Sugar Iron Agar	Baltimore Biological Laboratory, Baltimore, Md.
Tryptic Nitrate Broth	Difco Laboratories, Inc. Detroit, Michigan
Yeast Extract (Lot # 773224)	Fisher Scientific Co., Medford, Massachusetts

(Table 1, con't)

PREPARED REAGENTS

Compound	Manufacturer
Liquifluor	New England Nuclear, Corp. Boston, Massachusetts
Nessler's Reagent	Fisher Scientific Co., Medford, Massachusetts
Phenol Reagent, 2 N	Fisher Scientific Co., Medford, Massachusetts

Table 2. Contents of flasks for oxygen utilization. (Reference for Fig 13, 17, 18, 19, 20, 21)

Flask	KOH, ml	75% synthetic seawater, ml	Cell suspension, ml	3X Conc. of corresponding medium, ml
Endogenous	0.2	2.0	1.0	---
Basal medium	0.2	1.0	1.0	1.0
Basal medium with 1×10^{-4} M Cu	0.2	1.0	1.0	1.0
Basal medium with 5×10^{-4} M Cu	0.2	1.0	1.0	1.0
Basal medium with 1×10^{-3} M Cu	0.2	1.0	1.0	1.0

Note: Reference flask contained 15 ml of 75% synthetic seawater.

Table 3. Contents of Thunberg tubes for studies in electron transport. (Reference for Fig 22, 23, 24).

Dye tested	Cell suspension, ml	*TTC, ml	*MB, ml	*DCIP, ml	3X Conc. of Basal medium, ml
Triphenyltetrazolium chloride (TTC)	4.0	0.5	---	---	2.0
Dichloroindophenol (DCIP)	4.0	---	---	0.5	2.0
Methylene blue (MB)	4.0	---	0.5	---	2.0

* All dyes added to the sidearm of the Thunberg tubes, and mixed with cells at start of the experiment.

Table 4. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ 20 C in basal medium prepared with varied salinity.

Salinity	Incubation Time in hr																	
	7	9	10	12	13	14	15	16	17	18	20	22	24	36	48	54	60	72
100%	11	27	38	57	67	76	87	96	100	105	110	115	120	115	110	105	100	98
90%	12	29	40	60	70	80	91	99	100	105	110	115	120	115	110	105	99	93
80%	11	28	39	58	68	78	89	97	100	105	110	115	120	115	110	105	100	96
70%	12	29	39	57	69	79	89	98	100	105	110	115	120	115	110	105	100	95
60%	11	28	38	58	69	78	88	97	100	105	110	115	120	115	110	105	100	97
50%	3	12	27	42	62	62	72	83	92	100	105	110	115	115	110	105	100	98
40%	1	3	8	19	26	36	47	57	57	76	93	99	100	110	110	105	97	92
30%	0	0	4	11	17	26	35	45	55	64	74	85	93	105	105	100	92	89
20%	0	0	0	0	0	1	6	12	18	25	37	43	51	100	100	98	91	87
10%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5. List of components (g/liter) in synthetic seawater (Lyman and Fleming, 1940) and 75% synthetic seawater.

Component	Synthetic Seawater	75% Synthetic Seawater
NaCl	23.476 (0.4013 M)*	17.607 (0.3098 M)*
$MgCl_2$	** 4.981 (0.0523 M)	** 3.736 (0.0392 M)
Na_2SO_4	3.917 (0.0276 M)	2.938 (0.0207 M)
$CaCl_2$	1.102 (0.0099 M)	0.827 (0.0074 M)
KCL	0.664 (0.0089 M)	0.498 (0.0067 M)
$NaHCO_3$	0.192 (0.0023 M)	0.144 (0.0017 M)
KBr	0.096 (0.0008 M)	0.072 (0.0006 M)
H_3BO_3	0.026 (0.0044 M)	0.019 (0.0032 M)
$SrCl_2$	** 0.024 (0.00015 M)	** 0.018 (0.00011 M)
NaF	0.003 (0.00007 M)	0.002 (0.00005 M)
	Total = 34.482	Total = 25.861

* Molarity of component in synthetic seawater and 75% synthetic seawater.

** Component added in hydrated form:

$MgCl_2 \cdot 6\ H_2O$: 10.634 g/liter in synthetic seawater and 7.975 g/liter in 75% synthetic seawater.

$SrCl_2 \cdot 6\ H_2O$: 0.041 g/liter in synthetic seawater and 0.031 g/liter in 75% synthetic seawater.

Table 6. List of ionic deletions in 75% synthetic seawater prepared for the study of the major ion requirements of P. cuprodurans.

Flask #	Ionic deletion	Moles of cation remaining in 75% synthetic seawater *
1	All Na salts	0.00000 M Na
2	NaCl	0.04315 M Na
3	NaF, $NaHCO_3$, Na_2SO_4	0.30980 M Na
4	NaF, $NaHCO_3$, 0.27892 M NaCl, 0.01868 M Na_2SO_4	0.03492 M Na
5	NaF, $NaHCO_3$, 0.24894 M NaCl, 0.01661 M Na_2SO_4	0.06904 M Na
6	NaF, $NaHCO_3$, 0.21796 M NaCl, 0.01454 M Na_2SO_4	0.10426 M Na
7	NaF, $NaHCO_3$, 0.18698 M NaCl, 0.01297 M Na_2SO_4	0.13828 M Na
8	Na_2SO_4	0.31155 M Na
9	$MgCl_2$	0.00000 M Mg
10	0.03528 M $MgCl_2$	0.00392 M Mg
11	0.03136 M $MgCl_2$	0.00784 M Mg
12	0.02352 M $MgCl_2$	0.01568 M Mg
13	$CaCl_2$	0.00000 M Ca
14	0.00592 M $CaCl_2$	0.00148 M Ca
15	0.00444 M $CaCl_2$	0.00296 M Ca
16	All K salts	0.00000 M K
17	$SrCl_2$	0.00000 M Sr
18	H_3BO_3	0.00000 M H_3BO_3
19	No salts deleted	-------
20	All K salts, $SrCl_2$, H_3BO_3	0.0392 M Mg, 0.0074 M Ca, 0.3529 M Na

* For molarities of the individual salts contained in 75% synthetic seawater see Table 5.

Table 7. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 20 C in basal medium prepared with solutions containing deletions in the components of 75% synthetic seawater.

Flask ** #	Incubation Time in hr																	
	7	9	10	12	13	14	15	16	17	18	20	22	24	36	48	54	60	72
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	3	5	8	12	26	35	44	52	68	88	98	105	105	105	100	96
3	8	23	35	54	65	74	84	95	100	105	110	115	120	115	110	105	100	94
4	2	3	3	3	4	8	12	18	24	32	46	60	74	100	100	97	94	90
5	2	8	13	35	47	58	69	79	88	97	100	105	110	120	115	110	110	100
6	7	17	23	43	54	64	75	84	94	100	105	110	115	120	115	110	105	98
7	9	22	33	54	63	73	84	93	99	105	110	115	120	115	110	105	100	93
8	10	24	34	55	64	74	85	95	100	105	110	115	120	115	110	105	100	95
9	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	9	23	35	54	65	75	84	94	100	105	110	115	120	115	110	105	100	94
11	8	22	33	53	63	74	85	95	100	105	110	115	120	115	110	105	100	96
12	9	21	34	54	64	75	84	94	100	105	110	115	120	115	110	105	100	97
13	4	10	18	27	32	45	52	64	68	70	72	76	79	80	90	90	90	88
14	8	22	36	53	62	73	84	95	100	105	110	115	120	115	110	105	100	95
15	7	22	34	53	64	74	83	94	100	105	110	115	120	115	110	105	100	96

(Continued)

* Not determined = ND

** For deletions of components of 75% synthetic seawater present in flasks see Table 6.

(Table 7 continued)

Flask #	Incubation Time in hr																	
	7	9	10	12	13	14	15	16	17	18	20	22	24	36	48	54	60	72
16	8	22	33	52	62	71	82	92	100	103	110	115	120	115	110	105	100	97
17	9	21	34	53	63	73	84	95	100	105	110	115	120	115	110	105	100	95
18	9	22	35	54	63	74	84	96	100	105	110	115	120	115	110	100	100	93
19	9	23	36	54	64	74	85	96	100	105	110	115	120	115	110	105	100	94
20	8	23	35	53	63	74	84	95	100	105	110	115	120	115	110	105	100	96

Table 8. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 20 C in basal medium containing varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	6	8	10	12	18	24	36	48	54	72	96	120	144
Basal Medium	12	26	63	88	100	120	120	120	115	110	100	92	87	82
Basal Medium with 1.00×10^{-5} M Cu	11	25	62	87	100	120	120	120	115	110	100	94	89	83
Basal Medium with 5.00×10^{-5} M Cu	12	26	63	84	100	120	120	120	115	110	100	94	88	82
Basal Medium with 1.00×10^{-4} M Cu	12	26	64	85	100	120	120	120	115	110	100	96	90	85
Basal Medium with 5.00×10^{-4} M Cu	3	17	30	45	62	100	110	110	97	94	88	80	76	69
Basal Medium with 1.00×10^{-3} M Cu	0	1	5	8	11	35	64	105	105	105	99	96	90	83
Basal Medium with 1.25×10^{-3} M Cu	0	0	1	2	6	18	40	96	100	100	100	95	89	83
Basal Medium with 1.50×10^{-3} M Cu	0	0	0	0	1	3	11	54	92	96	96	95	92	88

(Continued)

(Table 8 continued)

Medium	Incubation Time in hr													
	4	6	8	10	12	18	24	36	48	54	72	96	120	144
Basal Medium with 1.75×10^{-3} M Cu	0	0	0	0	0	2	7	37	61	74	93	93	86	81
Basal Medium with 2.00×10^{-3} M Cu	0	0	0	0	0	0	1	13	41	57	86	90	90	87
Basal Medium with 2.25×10^{-3} M Cu	0	0	0	0	0	0	0	5	8	13	21	32	44	45
Basal Medium with 2.50×10^{-3} M Cu	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Basal Medium with 2.75×10^{-3} M Cu	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Basal Medium with 3.00×10^{-3} M Cu	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 9. pH changes during growth of P. cuprodurans at 20 C in basal medium with varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr									
	0	6	12	18	24	48	72	96	120	144
Basal Medium	7.0	6.7	7.1	7.8	8.0	8.0	8.3	8.5	8.5	8.5
Basal Medium with 1.00×10^{-5} M Cu	7.0	6.7	7.1	7.8	8.0	8.0	8.3	8.5	8.5	8.5
Basal Medium with 5.00×10^{-5} M Cu	7.0	6.7	7.1	7.8	8.0	8.0	8.3	8.5	8.5	8.5
Basal Medium with 1.00×10^{-4} M Cu	7.0	6.7	7.1	7.7	8.0	8.0	8.3	8.5	8.5	8.5
Basal Medium with 5.00×10^{-4} M Cu	7.0	6.8	7.0	7.4	7.6	7.9	8.3	8.5	8.5	8.5
Basal Medium with 1.00×10^{-3} M Cu	7.0	7.0	6.8	7.3	7.5	7.9	8.2	8.4	8.5	8.5
Basal Medium with 1.25×10^{-3} M Cu	7.0	7.0	7.0	6.8	7.3	7.8	8.1	8.3	8.4	8.5
Basal Medium with 1.50×10^{-3} M Cu	7.0	7.0	7.0	7.0	6.8	7.2	7.8	8.2	8.3	8.3

(Continued)

(Table 9 continued)

Medium	Incubation Time in hr									
	0	6	12	18	24	48	72	96	120	144
Basal Medium with 1.75×10^{-3} M Cu	7.0	7.0	7.0	7.0	6.9	6.9	7.3	7.6	7.9	8.2
Basal Medium with 2.00×10^{-3} M Cu	7.0	7.0	7.0	7.0	7.0	6.9	6.8	7.2	7.7	8.0
Basal Medium with 2.25×10^{-3} M Cu	7.0	7.0	7.0	7.0	7.0	7.0	6.9	7.1	7.5	7.7
Basal Medium with 2.50×10^{-3} M Cu	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Basal Medium with 2.75×10^{-3} M Cu	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Basal Medium with 3.00×10^{-3} M Cu	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0

Table 10. Growth of P. cuprodurans at 20 C on agar plates of the basal medium with varied concentrations of $CuSO_4$.

Medium	Growth/No Growth*
Basal medium	Growth
Basal medium with 1.00×10^{-5} M Cu	Growth
Basal medium with 5.00×10^{-5} M Cu	Growth
Basal medium with 1.00×10^{-4} M Cu	Growth
Basal medium with 5.00×10^{-4} M Cu	Growth
Basal medium with 1.00×10^{-3} M Cu	Growth
Basal medium with 1.25×10^{-3} M Cu	Growth
Basal medium with 1.50×10^{-3} M Cu	Growth
Basal medium with 1.75×10^{-3} M Cu	Growth
Basal medium with 2.00×10^{-3} M Cu	Growth
Basal medium with 2.25×10^{-3} M Cu	Growth
Basal medium with 2.50×10^{-3} M Cu	No Growth
Basal medium with 2.75×10^{-3} M Cu	No Growth
Basal medium with 3.00×10^{-3} M Cu	No Growth

* Growth or No Growth determined after 10-day incubation period at 20 C.

Table 11. Growth of B. subtilis after 10 day's incubation at 37 C on agar plates of the basal medium prepared with distilled water and 75% synthetic seawater and varied concentrations of $CuSO_4$.

Medium	Distilled Water	75% Synthetic Seawater
Basal Medium	Growth	Growth
Basal Medium with 1×10^{-5} M Cu	Growth	Growth
Basal Medium with 2×10^{-5} M Cu	Growth	Growth
Basal Medium with 3×10^{-5} M Cu	Growth	Growth
Basal Medium with 4×10^{-5} M Cu	Growth	Growth
Basal Medium with 5×10^{-5} M Cu	Growth	Growth
Basal Medium with 6×10^{-5} M Cu	No Growth	No Growth
Basal Medium with 1×10^{-4} M Cu	No Growth	No Growth

Table 12. Growth of S. aureus after 10 day's incubation at 37 C on agar plates of the basal medium prepared with distilled water and 75% synthetic seawater and varied concentrations of $CuSO_4$.

Medium	Distilled Water	75% Synthetic Seawater
Basal Medium	Growth	Growth
Basal Medium with 1×10^{-5} M Cu	Growth	Growth
Basal Medium with 5×10^{-5} M Cu	Growth	Growth
Basal Medium with 6×10^{-5} M Cu	Growth	Growth
Basal Medium with 7×10^{-5} M Cu	Growth	Growth
Basal Medium with 8×10^{-5} M Cu	Growth	Growth
Basal Medium with 9×10^{-5} M Cu	Growth	Growth
Basal Medium with 1×10^{-4} M Cu	Growth	Growth
Basal Medium with 2×10^{-4} M Cu	No Growth	No Growth
Basal Medium with 5×10^{-4} M Cu	No Growth	No Growth

Table 13. Growth of *E. coli B* after 10 day's incubation at 37 C on agar plates of the basal medium prepared with distilled water and 75% synthetic seawater and varied concentrations of $CuSO_4$.

Medium	Distilled Water	75% Synthetic Seawater
Basal Medium	Growth	Growth
Basal Medium with 1×10^{-5} M Cu	Growth	Growth
Basal Medium with 5×10^{-5} M Cu	Growth	Growth
Basal Medium with 8×10^{-5} M Cu	Growth	Growth
Basal Medium with 9×10^{-5} M Cu	Growth	Growth
Basal Medium with 1×10^{-4} M Cu	Growth	Growth
Basal Medium with 2×10^{-4} M Cu	No Growth	Growth
Basal Medium with 3×10^{-4} M Cu	No Growth	Growth
Basal Medium with 4×10^{-4} M Cu	No Growth	Growth
Basal Medium with 5×10^{-4} M Cu	No Growth	No Growth
Basal Medium with 1×10^{-3} M Cu	No Growth	No Growth

Table 14. Growth of P. aeruginosa after 10 day's incubation at 37 C on agar plates of the basal medium prepared with distilled water and 75% synthetic seawater and varied concentrations of $CuSO_4$.

Medium	Distilled Water	75% Synthetic Seawater
Basal Medium	Growth	Growth
Basal Medium with 1×10^{-5} M Cu	Growth	Growth
Basal Medium with 5×10^{-5} M Cu	Growth	Growth
Basal Medium with 1×10^{-4} M Cu	Growth	Growth
Basal Medium with 5×10^{-4} M Cu	Growth	Growth
Basal Medium with 6×10^{-4} M Cu	Growth	Growth
Basal Medium with 7×10^{-4} M Cu	Growth	Growth
Basal Medium with 8×10^{-4} M Cu	Growth	Growth
Basal Medium with 9×10^{-4} M Cu	Growth	Growth
Basal Medium with 1×10^{-3} M Cu	Growth	Growth
Basal Medium with 2×10^{-3} M Cu	No Growth	No Growth

Table 15. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 20 C in varied levels of the basal medium supplemented with 5×10^{-4} and 1×10^{-3} M $CuSO_4$.

Peptone, g/liter	Yeast Extract, g/liter	Level of $CuSO_4$	Incubation Time in hr														
			4	6	8	10	12	14	16	18	20	22	24	48	72	96	120	144
1.000	1.000	----------	11	25	58	79	97	105	110	115	120	120	120	115	93	85	76	68
0.500	0.500	----------	9	26	54	77	84	84	84	84	84	84	84	84	78	70	64	58
0.400	0.400	----------	9	26	54	71	71	71	71	71	71	71	71	71	67	60	49	35
0.300	0.300	----------	10	25	46	61	61	61	61	61	61	61	61	61	58	52	41	39
0.200	0.200	----------	9	23	37	44	44	44	44	44	44	44	44	44	42	37	32	24
0.100	0.100	----------	8	16	25	25	25	25	25	25	25	25	25	24	23	21	12	11
0.050	0.050	----------	6	7	11	11	12	12	12	12	12	12	12	12	11	11	8	7
0.025	0.025	----------	2	3	6	6	6	6	6	6	6	6	6	6	6	6	3	3
1.000	1.000	5×10^{-4} M	5	15	36	50	64	79	89	98	100	105	110	95	80	75	65	64
0.500	0.500	5×10^{-4} M	3	7	15	28	46	62	72	74	75	76	76	75	60	54	50	49
0.400	0.400	5×10^{-4} M	0	0	0	0	0	0	5	21	37	45	48	52	50	48	45	45
0.300	0.300	5×10^{-4} M	0	0	0	0	0	0	1	2	2	2	2	35	38	36	36	34

(continued)

(Table 15 continued)

Peptone, gg	Yeast Extract, g	Level of $CuSO_4$	Incubation Time in hr														
			4	6	8	10	12	14	16	18	20	22	24	48	72	96	120	144
0.200	0.200	5×10^{-4} M	0	0	0	0	0	0	0	0	0	0	0	1	3	6	10	8
0.100	0.100	5×10^{-4} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.050	0.050	5×10^{-4} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.025	0.025	5×10^{-4} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.000	1.000	1×10^{-3} M	0	0	9	22	38	52	66	81	93	99	100	105	100	90	88	86
0.500	0.500	1×10^{-3} M	0	0	0	0	0	2	3	4	4	4	4	46	52	54	52	50
0.400	0.400	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	6	8	10	10
0.300	0.300	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.200	0.200	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.100	0.100	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.050	0.050	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.025	0.025	1×10^{-3} M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 16. Distribution of labile and non-labile copper present in autoclaved 75% synthetic seawater and basal medium broth unsupplemented and supplemented with varied concentrations of $CuSO_4$.

Solution	Total Molarity of copper present	% Labile Cu	Molarity of Labile Cu	% Non-labile Cu	Molarity of Non-labile Cu
75% Synthetic Seawater	$8\text{-}9 \times 10^{-5}$	100.0	$8\text{-}9 \times 10^{-5}$	00.0	0.00
Basal Medium	1×10^{-5}	0.0	0	100.0	1×10^{-5}
Basal Medium with 1×10^{-4} M Cu	1×10^{-4}	10.7	1.07×10^{-5}	89.3	8.93×10^{-5}
Basal Medium with 5×10^{-4} M Cu	5×10^{-4}	27.9	1.39×10^{-4}	72.1	3.61×10^{-4}
Basal Medium with 1×10^{-3} M Cu	1×10^{-3}	40.3	4.03×10^{-4}	59.7	5.97×10^{-4}
Basal Medium with 2×10^{-3} M Cu	2×10^{-3}	46.8	9.36×10^{-4}	53.2	10.64×10^{-4}

Table 17. Distribution of labile and non-labile copper present in filter-sterilized 75% synthetic seawater and basal medium broth unsupplemented and supplemented with varied concentrations of $CuSO_4$.

Solution	Total Molarity of copper present	% Labile Cu	Molarity of Labile Cu	% Non-labile Cu	Molarity of Non-labile Cu
75% Synthetic Seawater	$8\text{-}9 \times 10^{-5}$	100.0	$8\text{-}9 \times 10^{-5}$	0.0	0
Basal Medium	1×10^{-5}	0.0	0	100.0	1.0×10^{-6}
Basal Medium with 1×10^{-4} M Cu	1×10^{-4}	12.5	1.25×10^{-5}	87.5	8.75×10^{-5}
Basal Medium with 5×10^{-4} M Cu	5×10^{-4}	30.9	1.55×10^{-4}	69.1	3.46×10^{-4}
Basal Medium with 1×10^{-3} M Cu	1×10^{-3}	45.2	4.52×10^{-4}	54.8	5.48×10^{-4}
Basal Medium with 2×10^{-3} M Cu	2×10^{-3}	39.1	7.82×10^{-4}	60.9	12.18×10^{-4}

Table 18. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 20 C in basal medium containing varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr														
	4	6	8	8.5	9	10	12	13	14	15	16	18	23	24	28
Basal Medium	10	32	56	62	69	82	--	105	--	--	--	--	--	120	120
Basal Medium with 1×10^{-4} M Cu	8	30	52	58	64	76	--	100	--	--	--	--	--	120	120
Basal Medium with 5×10^{-4} M Cu	2	14	21	--	--	39	56	64	72	81	--	--	--	110	110
Basal Medium with 1×10^{-3} M Cu	0	5	8	--	--	10	--	15	--	30	35	45	70	75	92
Basal Medium with 2×10^{-3} M Cu	0	0	0	--	--	0	--	0	--	0	--	--	--	0	0

(Continued)

(Table 18 continued)

Medium	Incubation Time in hr							
	30	34	49	50	52	53	55	77
Basal Medium	120	120	110	--	--	105	--	96
Basal Medium with 1 x 10^{-4} M Cu	120	120	110	--	--	105	--	90
Basal Medium with 5 x 10^{-4} M Cu	110	110	110	--	--	105	--	92
Basal Medium with 1 x 10^{-3} M Cu	96	100	105	--	--	105	--	95
Basal Medium with 2 x 10^{-3} M Cu	0	3	33	35	39	41	45	68

Table 19. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 24 C in basal medium containing varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	6	7	8	9	10	12	13	14	15	16	18	23	24
Basal Medium	16	46	58	71	83	96	--	110	115	--	--	--	--	120
Basal Medium with 1 x 10^{-4} M Cu	16	44	57	69	81	94	--	110	115	--	--	--	--	120
Basal Medium with 5 x 10^{-4} M Cu	11	25	--	40	49	57	74	82	91	--	--	--	--	110
Basal Medium with 1 x 10^{-3} M Cu	0	7	--	9	--	12	--	18	--	34	39	51	81	87
Basal Medium with 2 x 10^{-3} M Cu	0	0	--	0	--	0	--	0	--	--	--	--	--	--

(Continued)

(Table 19 continued)

Medium	Incubation Time in hr								
	28	30	34	49	50	52	53	55	77
Basal Medium	120	120	120	110	--	--	105	--	82
Basal Medium with 1×10^{-4} M Cu	120	120	120	110	--	--	105	--	84
Basal Medium with 5×10^{-4} M Cu	110	110	110	105	--	--	100	--	86
Basal Medium with 1×10^{-3} M Cu	93	100	105	105	--	--	95	--	88
Basal Medium with 2×10^{-3} M Cu	--	2	9	34	36	39	41	43	72

Table 20. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 26 C in basal medium with varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	6	7	8	9	10	12	13	15	16	18	24	28	30
Basal Medium	25	51	67	83	--	100	--	110	--	--	--	120	115	110
Basal Medium with 1 x 10^{-4} M Cu	23	50	67	84	--	100	--	110	--	--	--	120	115	110
Basal Medium with 5 x 10^{-4} M Cu	12	30	--	52	63	74	96	100	--	--	--	110	110	110
Basal Medium with 1 x 10^{-3} M Cu	8	13	--	18	--	24	--	33	46	53	66	92	98	100
Basal Medium with 2 x 10^{-3} M Cu	0	0	--	0	--	0	--	0	--	--	--	6	8	10

(Continued)

(Table 20 continued)

Medium	Incubation Time in hr						
	34	49	50	52	53	55	77
Basal Medium	100	93	--	--	84	--	71
Basal Medium with 1×10^{-4} M Cu	100	98	--	--	86	--	73
Basal Medium with 5×10^{-4} M Cu	105	100	--	--	93	--	88
Basal Medium with 1×10^{-3} M Cu	105	95	--	--	91	--	85
Basal Medium with 2×10^{-3} M Cu	14	50	53	59	62	67	72

Table 21. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 28 C in basal medium with varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	5	6	7	8	9	10	12	13	14	15	16	18	24
Basal Medium	34	52	71	89	100	--	110	--	120	--	--	--	--	120
Basal Medium with 1×10^{-4} M Cu	28	41	57	73	88	--	105	--	120	--	--	--	--	120
Basal Medium with 5×10^{-4} M Cu	12	--	21	--	33	40	48	64	75	83	--	98	--	110
Basal Medium with 1×10^{-3} M Cu	6	--	14	--	18	--	21	--	28	--	35	40	50	79
Basal Medium with 2×10^{-3} M Cu	0	--	0	--	0	--	0	--	0	--	--	--	--	7

(Continued)

(Table 21 continued)

Medium	Incubation Time in hr								
	28	30	34	49	50	52	53	55	77
Basal Medium	105	100	96	92	--	--	86	--	78
Basal Medium with 1×10^{-4} M Cu	110	105	100	95	--	--	89	--	79
Basal Medium with 5×10^{-4} M Cu	110	105	100	93	--	--	90	--	82
Basal Medium with 1×10^{-3} M Cu	88	93	100	105	--	--	100	--	89
Basal Medium with 2×10^{-3} M Cu	9	13	17	45	47	51	53	57	70

Table 22. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 30 C in basal medium with varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	5	6	7	8	9	10	12	13	14	15	16	18	23
Basal Medium	42	61	81	--	105	--	115	--	120	--	--	--	--	--
Basal Medium with 1 x 10 M Cu	26	38	52	66	80	--	100	--	115	--	--	115	--	--
Basal Medium with 5 x 10 M Cu	8	--	14	--	22	26	33	45	51	57	--	--	80	--
Basal Medium with 1 x 10 M Cu	2	--	8	--	12	--	16	--	23	--	30	33	40	60
Basal Medium with 2 x 10 M Cu	0	--	0	--	0	--	0	--	0	--	0	0	0	--

(Continued)

(Table 22 continued)

Medium	Incubation Time in hr									
	24	28	30	34	49	50	52	53	55	77
Basal Medium	115	110	105	99	89	--	--	86	--	73
Basal Medium with 1×10^{-4} M Cu	115	110	110	105	98	--	--	95	--	83
Basal Medium with 5×10^{-4} M Cu	100	96	91	87	85	--	--	81	--	77
Basal Medium with 1×10^{-3} M Cu	64	80	85	90	95	--	--	92	--	88
Basal Medium with 2×10^{-3} M Cu	3	6	9	14	38	40	44	46	50	64

Table 23. Growth of P. cuprodurans as O.D. (x 100) at 420 mμ at 33 C in basal medium with varied concentrations of $CuSO_4$.

Medium	Incubation Time in hr													
	4	5	6	7	8	10	12	13	14	15	16	18	23	24
Basal Medium	44	64	84	--	100	115	--	120	--	--	--	--	--	115
Basal Medium with 1×10^{-4} M Cu	23	32	43	54	65	87	--	110	--	--	--	--	--	110
Basal Medium with 5×10^{-4} M Cu	4	--	8	--	12	14	18	21	24	--	--	35	--	53
Basal Medium with 1×10^{-3} M Cu	0	--	0	--	3	6	--	12	--	16	--	--	--	38
Basal Medium with 2×10^{-3} M Cu	0	--	0	--	0	0	--	0	--	--	--	--	--	0

(Continued)

(Table 23 continued)

Medium	Incubation Time in hr									
	26	28	30	34	49	50	52	53	55	77
Basal Medium	--	105	100	96	86	--	--	78	--	70
Basal Medium with 1×10^{-4} M Cu	--	110	105	99	94	--	--	84	--	75
Basal Medium with 5×10^{-4} M Cu	59	65	68	64	58	--	--	54	--	52
Basal Medium with 1×10^{-3} M Cu	42	46	49	53	60	--	--	60	--	56
Basal Medium with 2×10^{-3} M Cu	0	1	1	1	32	34	38	40	42	29

Table 24. Observations of cell morphology and pellet consistency of P. cuprodurans cells washed with varied solutions.

Washing Solution	Wash # Morphology			Integrity of Pellet		
	1	2	3	1	2	3
Distilled Water	Normal	Abnormal*	Abnormal*	Normal	Mucoid	Mucoid with lysis products in washing supernatant
0.82 M glycerol	Normal	Abnormal*	Abnormal*	Normal	Mucoid	Mucoid with lysis products in washing supernatant
0.0397 M $MgCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
0.0074 M $CaCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
0.0392 M MgCl and 0.0074 M $CaCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
75% synthetic seawater	Normal	Normal	Normal	Normal	Normal	Normal

* Greater than 50% of cells lysed, lysis products evident.

Table 25. Observations of cell morphology and pellet consistency of P. cuprodurans cells washed with varied magnesium solutions.

Washing Solution g/liter	Wash # Morphology			Integrity of Pellet		
	1	2	3	1	2	3
10 g $MgCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
8 g $MgCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
6 g $MgCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
4 g $MgCl_2$	Normal	Normal	Normal	Normal	Normal	Normal
2 g $MgCl_2$	Normal	Abnormal*	Abnormal*	Normal	Normal	Normal
1 g $MgCl_2$	Normal	Abnormal*	Abnormal*	Normal	Normal	Normal

* Rounded morphology in approximately 1% of cells. Cell plasmolyzed with membrane drawn back from the cell wall.

Table 26. Ratios of DNA : RNA : Protein determined for P. cuprodurans cultivated at 20 C in basal medium containing varied concentrations of $CuSO_4$.

Medium	Total DNA per Extract, ug	Total RNA per Extract, ug	Total Protein per Extract, ug
Basal Medium	1,325 (1.00)*	2,475 (1.87)*	12,000 (9.05)*
Basal Medium	1,250 (1.00)	2,350 (1.88)	11,310 (9.05)
Basal Medium with 1×10^{-4} M Cu	1,155 (1.00)	2,185 (1.89)	10,480 (9.07)
Basal Medium with 1×10^{-4} M Cu	1,155 (1.00)	2,140 (1.85)	10,480 (9.07)
Basal Medium with 5×10^{-4} M Cu	1,000 (1.00)	1,860 (1.86)	9,040 (9.04)
Basal Medium with 5×10^{-4} M Cu	1,000 (1.00)	1,860 (1.86)	9,080 (9.08)
Basal Medium with 1×10^{-3} M Cu	1,125 (1.00)	2,030 (1.84)	10,200 (9.07)
Basal Medium with 1×10^{-3} M Cu	1,000 (1.00	1,850 (1.85)	9,060 (9.06)

* Ratios of constituents in parenthesis using DNA value as 1.00

Table 27. Plate counts (x 10^8) of P. cuprodurans on basal medium agar plates performed at 0 and 180 min of respiration studies.

Medium Cells Grown in for Study	Respiration Flask	Incubation Time in min 0	180
Basal Medium	Endogenous	6.00	5.90
Basal Medium	Basal Medium	6.05	6.00
Basal Medium	Basal Medium with 1 x 10^{-4} M Cu	6.10	6.10
Basal Medium	Basal Medium with 5 x 10^{-4} M Cu	5.90	6.00
Basal Medium	Basal Medium with 1 x 10^{-3} M Cu	5.90	6.05
Basal Medium with 1 x 10^{-4} M Cu	Endogenous	5.90	5.85
Basal Medium with 1 x 10^{-4} M Cu	Basal Medium	5.80	5.75
Basal Medium with 1 x 10^{-4} M Cu	Basal Medium with 1 x 10^{-4} M Cu	5.7	5.65

(Continued)

(Table 27 continued)

Medium Cells Grown in for Study	Respiration Flask	Incubation Time in min 0	180
Basal Medium with 1 x 10^{-4} M Cu	Basal Medium with 5 x 10^{-4} M Cu	5.65	5.70
Basal Medium with 1 x 10^{-4} M Cu	Basal Medium with 1 x 10^{-3} M Cu	5.90	5.85
Basal Medium with 5 x 10^{-4} M Cu	Endogenous	4.00	4.00
Basal Medium with 5 x 10^{-4} M Cu	Basal Medium	4.00	4.00
Basal Medium with 5 x 10^{-4} M Cu	Basal Medium with 1 x 10^{-4} M Cu	3.90	3.95
Basal Medium with 5 x 10^{-4} M Cu	Basal Medium with 5 x 10^{-4} M Cu	4.00	3.95
Basal Medium with 5 x 10^{-4} M Cu	Basal Medium with 1 x 10^{-3} M Cu	4.00	4.10

(Continued)

(Table 27 continued)

Medium Cells Grown in for Study	Respiration Flask	Incubation Time in min 0	180
Basal Medium with 1 x 10^{-3} M Cu	Endogenous	3.10	3.00
Basal Medium with 1 x 10^{-3} M Cu	Basal Medium	3.10	3.10
Basal Medium with 1 x 10^{-3} M Cu	Basal Medium with 1 x 10^{-4} M Cu	3.00	3.10
Basal Medium with 1 x 10^{-3} M Cu	Basal Medium with 5 x 10^{-4} M Cu	3.00	3.10
Basal Medium with 1 x 10^{-3} M Cu	Basal Medium with 1 x 10^{-3} M Cu	3.05	3.00
Basal Medium with 2 x 10^{-3} M Cu	Endogenous	2.50	2.50
Basal Medium with 2 x 10^{-3} M Cu	Basal Medium	2.55	2.50
Basal Medium with 2 x 10^{-3} M Cu	Basal Medium with 1 x 10^{-4} M Cu	2.50	2.55

(Continued)

(Table 27 continued)

Medium Cells Grown in for Study	Respiration Flask	Incubation Time in min 0	180
Basal Medium with 2×10^{-3} M Cu	Basal Medium with 5×10^{-4} M Cu	2.60	2.60
Basal Medium with 2×10^{-3} M Cu	Basal Medium with 1×10^{-3} M Cu	2.55	2.50

Table 28. Oxygen uptake after 180 min in basal medium broth by P. cuprodurans cultivated in basal medium broth supplemented with varied concentrations of $CuSO_4$.

Cultivation Medium	180 min Oxygen Uptake in ul/mg of dry wt.	% of non-copper stressed respiration
Basal Medium	624	100.0
Basal Medium with 1×10^{-4} M Cu	574	92.0
Basal Medium with 5×10^{-4} M Cu	345	55.5
Basal Medium with 1×10^{-3} M Cu	210	33.7
Basal Medium with 2×10^{-3} M Cu	138	22.5

Table 29. Reduction time of triphenyl tetrazolium chloride at 20 C by P. cuprodurans cultivated on basal medium agar plates supplemented with varied concentrations of $CuSO_4$.

Medium	Time for Reduction of TTC to Triphenyl formazan, min	
	1.5% Agar Overlay	10% Gelatin Overlay
Basal Medium	5	5
Basal Medium with 1×10^{-4} M Cu	45	45
Basal Medium with 5×10^{-4} M Cu	360	360
Basal Medium with 1×10^{-3} M Cu	720	720

Table 30. Growth of P. cuprodurans after 10 day's incubation at 20 C on basal medium agar plates with varied concentrations of $NiCl_2$.

Medium	Growth/No Growth	Colony diameter in mm
Basal Medium	Growth	3-4
Basal Medium with 1×10^{-5} M Ni	Growth	3-4
Basal Medium with 5×10^{-5} M Ni	Growth	3-4
Basal Medium with 1×10^{-4} M Ni	Growth	3-4
Basal Medium with 5×10^{-4} M Ni	Growth	3-4
Basal Medium with 6×10^{-4} M Ni	Growth	3-4
Basal Medium with 7×10^{-4} M Ni	Growth	3-4
Basal Medium with 8×10^{-4} M Ni	Growth	3-4
Basal Medium with 9×10^{-4} M Ni	Growth	3-4
Basal Medium with 1×10^{-3} M Ni	Growth	3-4
Basal Medium with 2×10^{-3} M Ni	No Growth	---

Table 31. Growth of P. cuprodurans after 10 day's incubation at 20 C on basal medium agar plates with varied concentrations of $CoCl_2$.

Medium	Growth/No Growth	Colony Diameter in mm
Basal Medium	Growth	3-4
Basal Medium with 1×10^{-5} M Co	Growth	3-4
Basal Medium with 5×10^{-5} M Co	Growth	3-4
Basal Medium with 1×10^{-4} M Co	Growth	3-4
Basal Medium with 2×10^{-4} M Co	Growth	3-4
Basal Medium with 3×10^{-4} M Co	Growth	3-4
Basal Medium with 4×10^{-4} M Co	No Growth	---

Table 32. Growth of P. cuprodurans after 10 day's incubation at 20 C on basal medium agar plates with varied concentrations of $ZnCl_2$.

Medium	Growth/No Growth	Colony Diameter in mm
Basal Medium	Growth	3-4
Basal Medium with 1×10^{-5} M Zn	Growth	2-3
Basal Medium with 5×10^{-5} M Zn	Growth	2-3
Basal Medium with 1×10^{-4} M Zn	Growth	2-3
Basal Medium with 2×10^{-4} M Zn	Growth	2-3
Basal Medium with 3×10^{-4} M Zn	Growth	2-3
Basal Medium with 4×10^{-4} M Zn	Growth	2-3
Basal Medium with 5×10^{-4} M Zn	No Growth	---

Table 33. Growth of P. cuprodurans after 10 day's incubation at 20 C on basal medium agar plates with varied concentrations of $CdCl_2$.

Medium	Growth/No Growth	Colony Diameter in mm
Basal Medium	Growth	3-4 mm
Basal Medium with 1 x 10^{-5} M Cd	Growth	1-2
Basal Medium with 2 x 10^{-5} M Cd	Growth	1-2
Basal Medium with 3 x 10^{-5} M Cd	Growth	1-2
Basal Medium with 4 x 10^{-5} M Cd	Growth	1-2
Basal Medium with 5 x 10^{-5} M Cd	Growth	1-2
Basal Medium with 6 x 10^{-5} M Cd	No Growth	---

Table 34. Reduction of triphenyl tetrazolium chloride at 20 C by P. cuprodurans cultivated in basal medium agar plates supplemented with varied concentrations of $CuSO_4$, $NiCl_2$, $CoCl_2$, $ZnCl_2$, and $CdCl_2$.

Medium	Time for Reduction of TTC to Triphenyl formanzan in min
Basal Medium	5 min
Basal Medium with 1×10^{-4} M Cu	45 min
Basal Medium with 5×10^{-4} M Cu	360 min
Basal Medium with 1×10^{-3} M Cu	720 min
Basal Medium with 1×10^{-3} M Ni	5 min
Basal Medium with 3×10^{-4} M Co	5 min
Basal Medium with 4×10^{-4} M Zn	5 min
Basal Medium with 5×10^{-5} M Cd	5 min

Table 35. Quantitation of relative absorbance of cytochromes b_1 and c alpha peaks from difference spectra performed on whole cell extracts of P. cuprodurans grown in basal medium supplemented with varied concentrations of copper.

Cultivation Medium	Relative Absorbance of Cytochrome c alpha peak	Relative Absorbance of Cytochrome b_1 alpha peak	Ratio of Cytochrome c to Cytochrome b_1
Basal Medium	26.49 (100.0%)*	24.62 (100.0%)*	1.076
Basal Medium with 1×10^{-4} M Cu	17.70 (68.8%)	28.13 (114.3%)	0.629
Basal Medium with 5×10^{-4} M Cu	13.01 (49.1%)	26.90 (109.3%)	0.487
Basal Medium with 1×10^{-3} M Cu	6.10 (23.0%)	19.38 (78.7%)	0.314

* Percent of value obtained with whole cell extracts of P. cuprodurans cultivated in the basal medium.

Table 36. Reduction time of triphenyl tetrazolium chloride (TTC) at 37 C by S. aureus, B. subtilis and E. coli B cultivated on basal medium agar plates supplemented with the maximum concentration of copper tolerated by each organism.

Medium	Organism	Time for Reduction of TTC to Triphenyl formazan, min
Basal Medium	E. coli B	5-10
Basal Medium	S. aureus	5-10
Basal Medium	B. subtilis	5-10
Basal Medium with 5×10^{-5} M Cu	B. subtilis	5-10
Basal Medium with 1×10^{-4} M Cu	E. coli B	5-10
Basal Medium with 1×10^{-4} M Cu	S. aureus	5-10

Table 37. Comparative uptake of ^{64}Cu by P. cuprodurans during growth at 20 C in basal medium with varied concentrations of $CuSO_4$.

Time, hr	cpm/ml of cell suspension	Cell Count x 10^8/ml	cpm/l x 10^8 cells/ml	Percent Uptake per 1 x 10^8 cells*
Cells Grown in Basal Medium				
7.0	6,750	2.70	2,500	0.849
8.0	8,920	5.20	1,719	0.584
8.5	10,705	10.60	1,010	0.343
9.0	11,530	17.00	678	0.230
9.5	13,365	21.20	630	0.214
10.5	18,900	29.10	650	0.221
11.0	19,710	30.10	655	0.222
12.0	21,410	32.10	668	0.227
Cells Grown in Basal Medium with 1 x 10^{-4} M Cu				
7.0	3,470	2.05	1,693	0.575
8.0	4,690	3.50	1,340	0.455
8.5	5,110	4.70	1,098	0.373
9.0	6,250	7.75	804	0.273
9.5	7,940	12.10	656	0.223
10.0	8,220	13.10	628	0.213
11.0	10,750	17.20	625	0.212
12.0	13,080	21.10	620	0.211
13.0	14,490	23.00	630	0.214

(Continued)

* Total cpm/ml of medium = 294,400

(Table 37 continued)

Time, hr	cpm/ml of cell suspension	Cell Count x 10^8/ml	cpm/1 x 10^8 cells/ml	Percent Uptake per 1x10^8 cells*
Cells Grown in Basal Medium with 5 x 10^{-4} M Cu				
8.0	2,245	1.73	1,298	0.441
9.0	3,435	3.38	1,017	0.349
9.5	5,710	5.20	1,098	0.373
10.5	6,040	10.05	575	0.195
11.0	7,605	14.30	536	0.182
11.5	9,150	15.40	594	0.202
12.0	9,380	17.60	533	0.181
13.0	11,300	21,10	535	0.182
14.0	12,840	23.60	544	0.185
Cells Grown in Basal Medium with 1 x 10^{-3} M Cu				
10.5	1,975	1.17	1,688	0.573
11.0	2,370	1.99	1,191	0.404
12.0	3,750	3.28	1,145	0.389
13.0	5,300	7.56	700	0.238
14.0	5,985	10.32	580	0.197
15.5	10,240	20.00	512	0.174
17.5	12,620	24.50	515	0.175

* Total cpm/ml of medium = 294,400 cpm

Table 38. Contents of flasks used for active transport studies (Results section 18a). Reference for Fig 32.

Contents	Flask		
	Uptake of ^{64}Cu in basal medium with 1 x 10^{-4} M Cu	Uptake of ^{64}Cu in basal medium with 1 x 10^{-4} M Cu and 1 x 10^{-3} M DNA	Uptake of ^{64}Cu in basal medium with 1 x 10^{-4} M Cu and 1 x 10^{-2} M NaCN
Basal Medium cell suspension, ml*	24.0	24.0	24.0
1 x 10^{-2} M 2,4-dinitrophenol,** ml	----	3.0	----
1 x 10^{-2} M NaCn, ml**	----	----	3.0
M Distilled Water, ml**	3.0	----	----
1 x 10^{-3} M $CuSO_4$ containing ^{64}Cu ***	3.0	3.0	3.0

* Equilibrated for 30 min at 20 C at 140 rpm

** Added after 30 min of equilibration, the flasks reincubated for 15 min at 20 C at 140 rpm after addition

*** Added at 0 min of experiment.

Table 39. Contents of flasks used for endogenous copper uptake of 1×10^{-4} M Cu and the effect of chloramphenicol on active transport by P. cuprodurans at 20 C in basal medium with 1×10^{-4} M $CuSO_4$.

Contents	Endogenous uptake of ^{64}Cu in 1×10^{-4} M Cu	Uptake of ^{64}Cu in basal medium with 1×10^{-4} M Cu	Uptake of ^{64}Cu in basal medium with 1×10^{-4} M Cu and Chloramphenicol (100 µg/ml)
75% Synthetic Seawater Cell Suspension, ml*	24.0	----	----
Chloramphenicol (1,000 ug/ml), ml**	----	----	3.0
Distilled Water, ml**	3.0	3.0	----
1×10^{-3} M $CuSO_4$ containing ^{64}Cu***	3.0	3.0	3.0

* Equilibrated for 30 min at 20 C at 140 rpm.

** Added after 30 min equilibration, the flasks reincubated for 15 min at 20 C at 140 rpm after addition.

*** Added at 0 min of experiment.

Table 40. Physical localization of ^{64}Cu in P. cuprodurans cultivated at 20 C in basal medium with 1 x 10^{-3} M $CuSO_4$.

1. Total cpm of whole cell suspension			740,978
a. Total cpm of 3,500 x g sediment	13,100		
b. Total cpm of 39,900 x g sediment (envelope fraction)	33,665	(4.62%)	
c. Total com of 39,900 x g supernatant (non-envelope fraction)	695,600	(95.38%)	
d. Total cpm of 3,500 x g supernatant (b + c)	719,665		
2. Total cpm accounted for (a + d)			732,765
3. Total experimental recovery of ^{64}Cu (2/1 x 100)			98.89%

* Percent of 3,500 x g supernatant (d)

Table 41. Localization of ^{64}Cu by biochemical extraction in P. cuprodurans cultivated at 20 C in basal medium supplemented with varied concentrations of $CuSO_4$. Results expressed as percent of total cpm.

Medium	Biochemical extract			
	0.2 N $HClO_4$ (small mol. wt compounds, intermediary metabolytes)*	Ethanol/Ether (Lipid)*	2.0 N HClO (RNA and DNA)*	1.0 N NaOH (protein)*
Basal Medium	12.01%	9.71%	75.31%	2.97%
Basal Medium	12.22%	10.05%	74.84%	2.89%
Basal Medium with 1×10^{-4} M Cu	9.30%	8.79%	77.93%	3.99%
Basal Medium with 1×10^{-4} M Cu	9.17%	8.93%	77.82%	4.08%
Basal Medium with 5×10^{-4} M Cu	6.20%	9.75%	80.39%	3.66%
Basal Medium with 5×10^{-4} M Cu	6.29%	9.66%	80.55%	3.49%
Basal Medium with 1×10^{-3} M Cu	6.23%	9.55%	79.77%	4.47%
Basal Medium with 1×10^{-3} M Cu	6.42%	9.55%	79.47%	4.56%

* Contents of extracts according to Munro and Fleck (1966)

Table 42. Uptake of copper by P. cuprodurans at 20 C in basal medium supplemented with varied concentrations of copper as measured by anodic stripping voltammetry.

Medium	ng Cu/mg protein	ng Cu/10^8 cells	% uptake per 10^8 cell
Basal Medium	59.37	1.74	0.274
Basal Medium$_{4}$ with 1 x 10^{-4} M Cu	106.33	3.22	0.0515
Basal Medium$_{4}$ with 5 x 10^{-4} M Cu	138.00	4.18	0.00131
Basal Medium$_{3}$ with 1 x 10^{-3} M Cu	187.83	5.70	0.0089%

Fig 1. Shadow cast preparation of P. cuprodurans demonstrating flagella (Magnification 25,000 X).

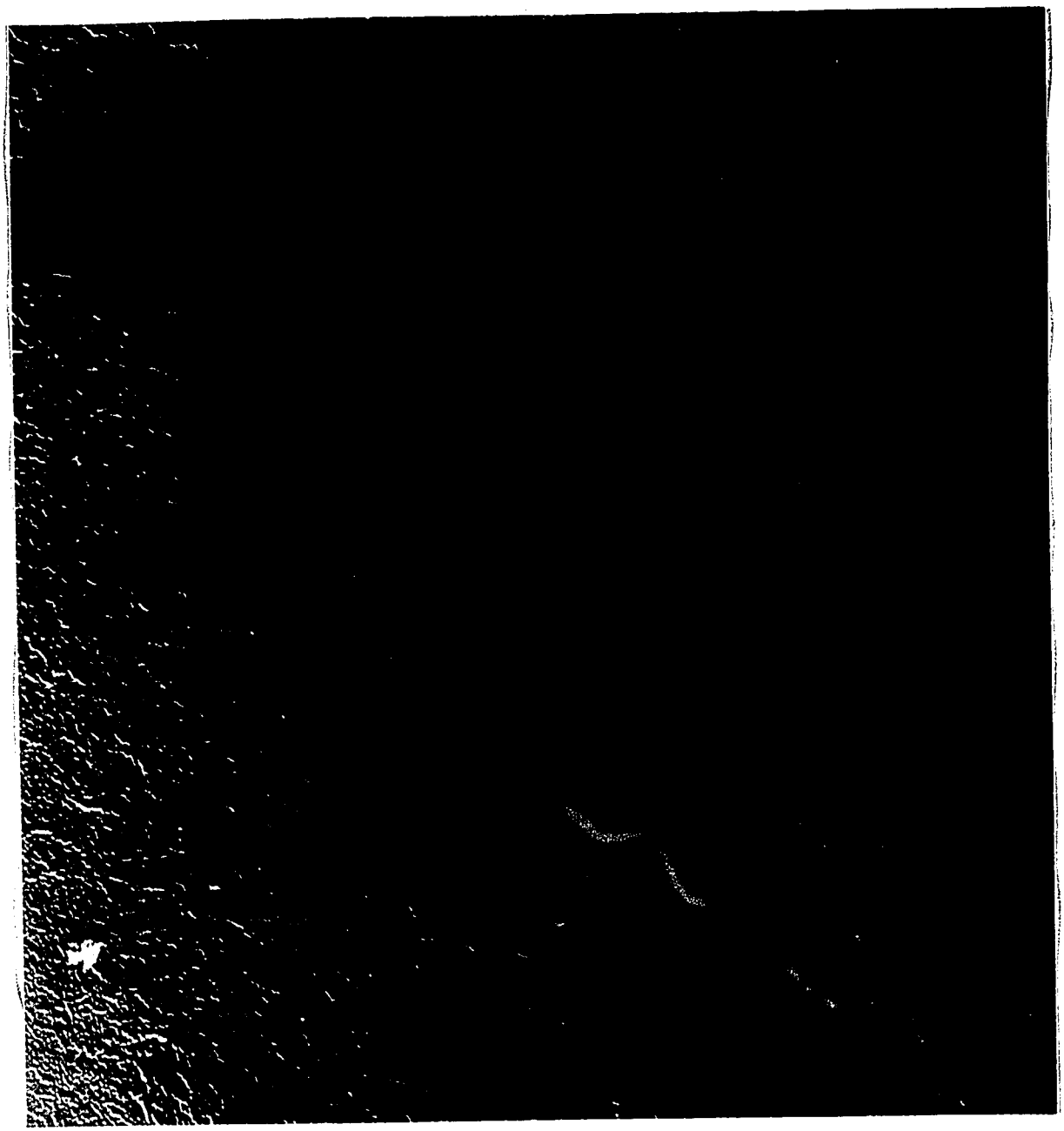


Fig 2. Aberrant morphology of P. cuprodurans grown in the absence of magnesium (Magnification 3,000 X).

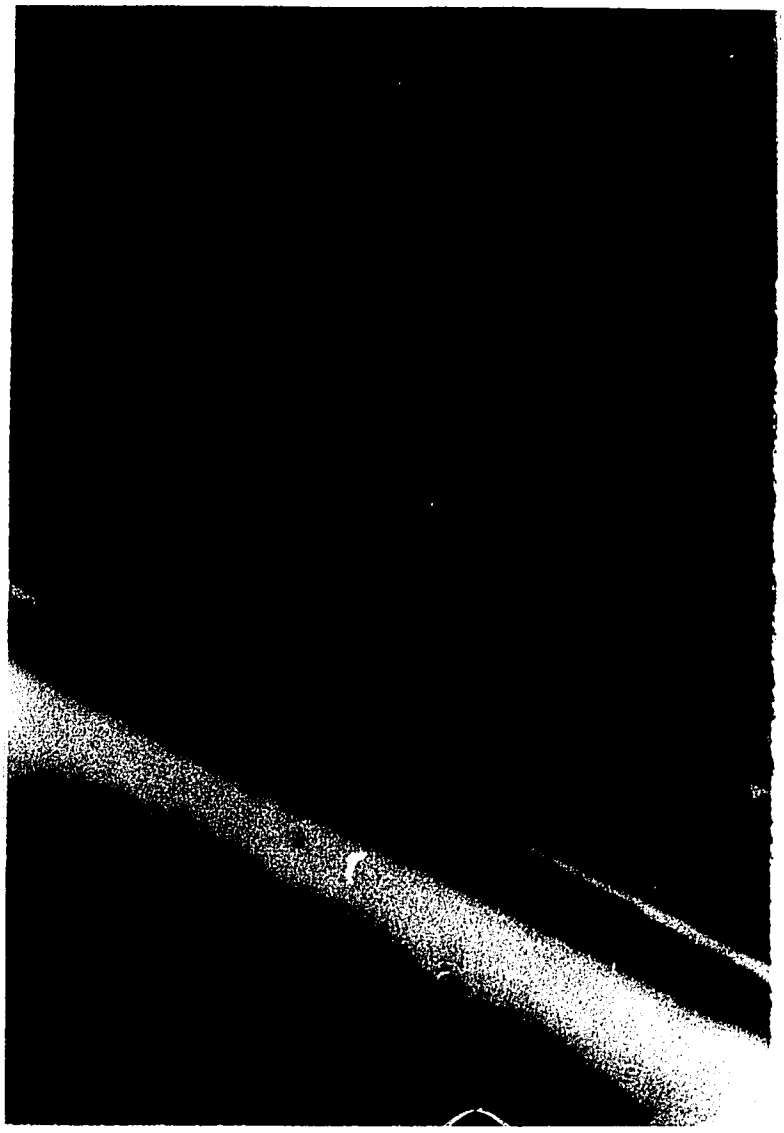


Fig 3. Aberrant morphology of P. cuprodurans grown in the absence of calcium (Magnification 3,000 X).

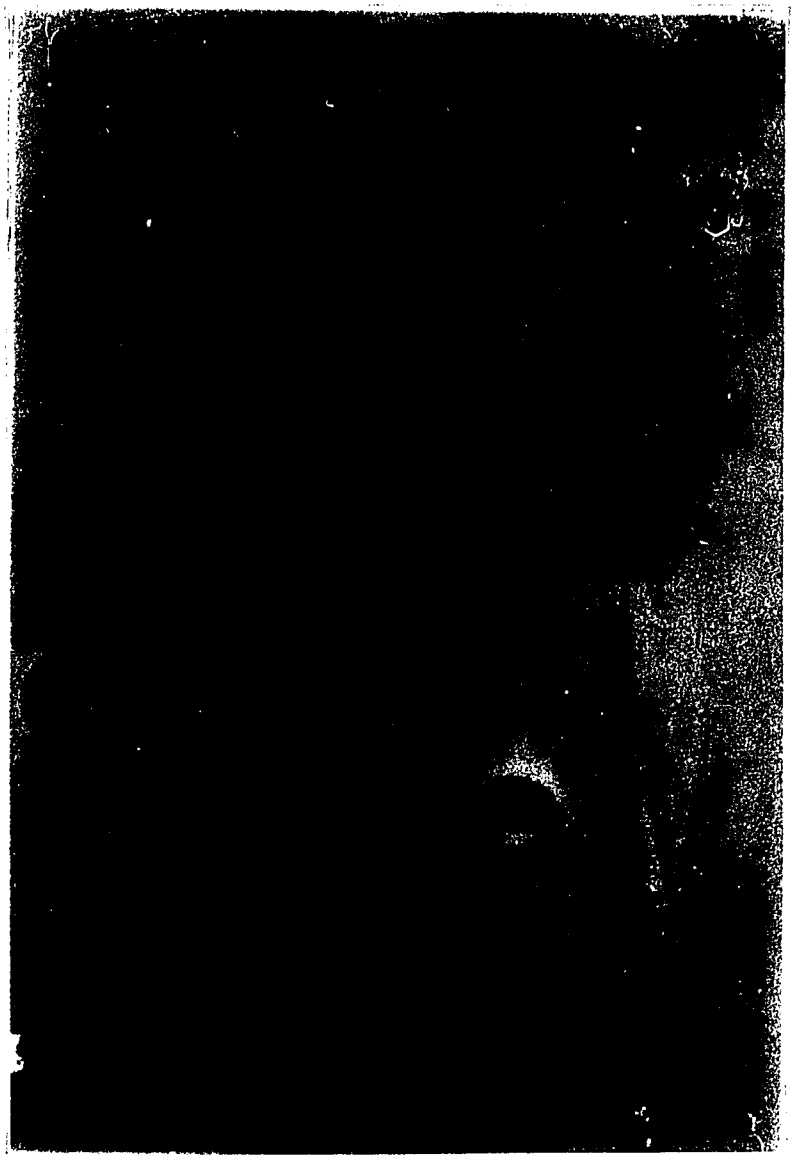


Fig 4. Log of copper tolerance of P. cuprodurans vs. log of total organic nutrients (mg).

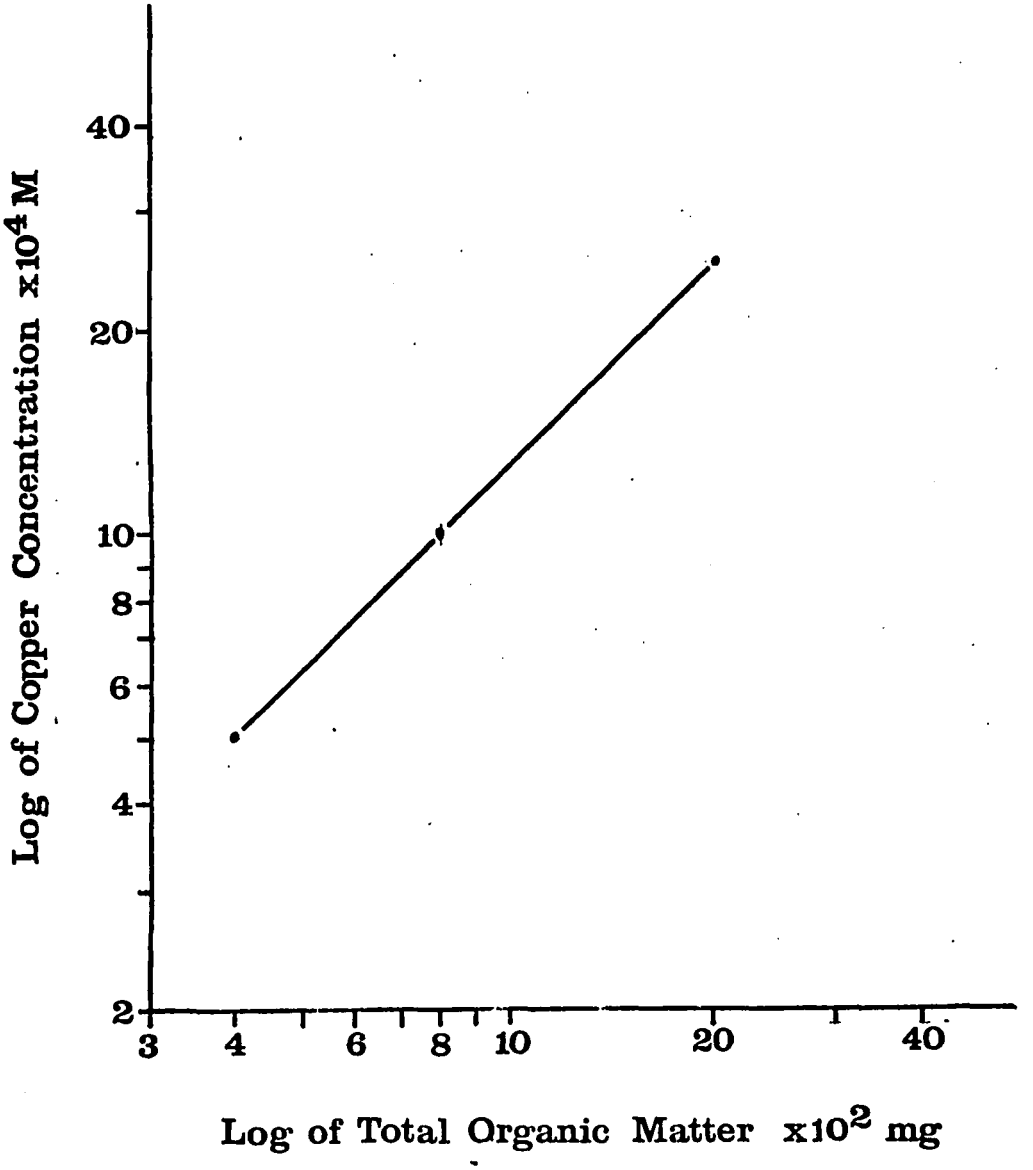

Log of Copper Concentration x10⁴ M
40
20
10
8
6
4
2
3
4
6
8
10
20
40
Log of Total Organic Matter x10² mg

Fig 5. Growth of P. cuprodurans at 20 C in basal medium broth unsupplemented and supplemented with 1×10^{-3} M $CuSO_4$ as determined by plate counts. Inoculum: standard inoculum in basal medium.

A - Cells grown in basal medium

B - Cells grown in basal medium with 1×10^{-3} M $CuSO_4$.

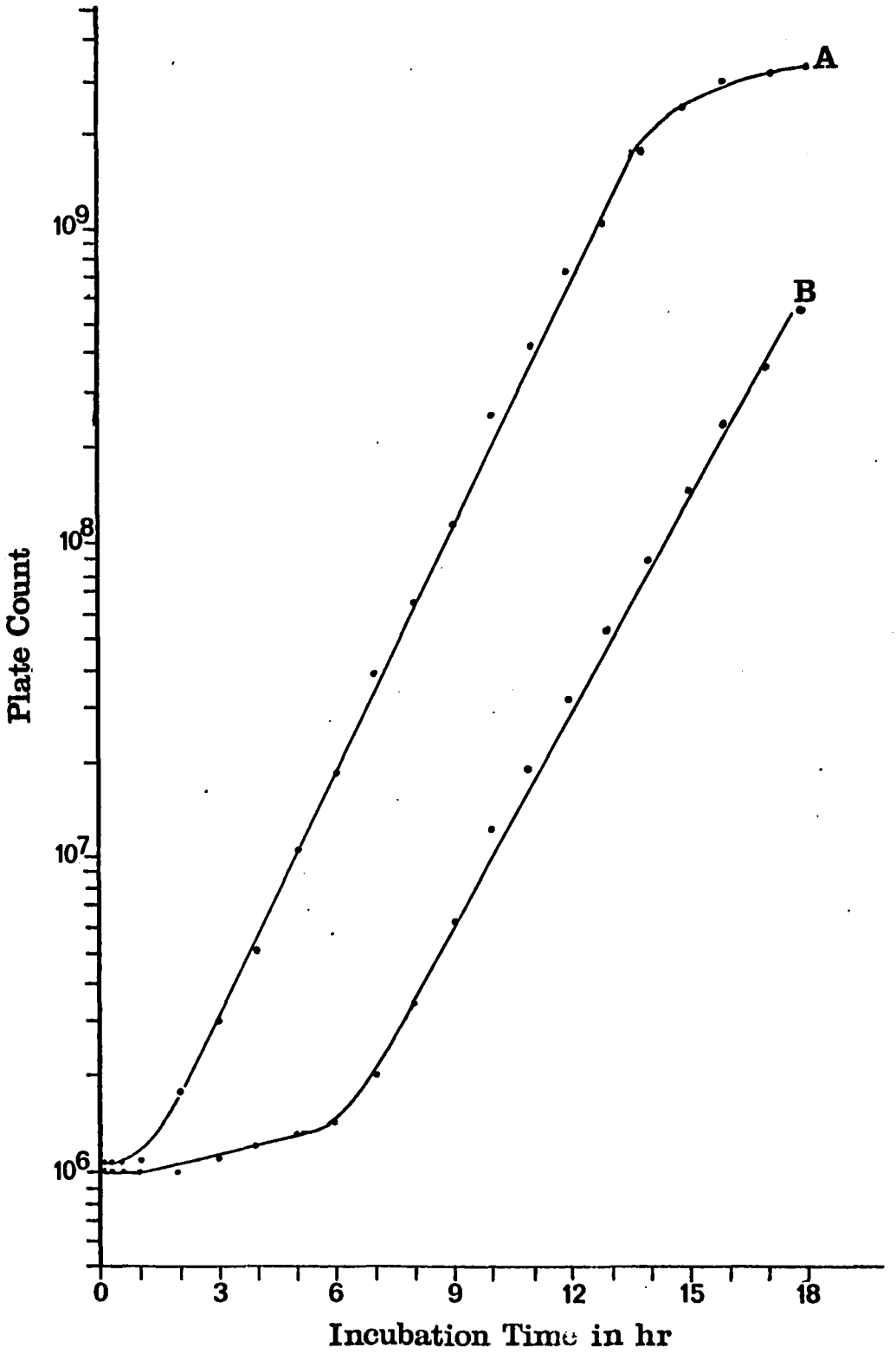

A
B
10^9
10^8
10^7
10^6
Plate Count
0
3
6
9
12
15
18
Incubation Time in hr

Fig 6. Growth of P. cuprodurans at 20 C in basal medium unsupplemented and supplemented with 1×10^{-3} M $CuSO_4$ as determined by plate counts. Inoculum: the standard inoculum was prepared in basal medium broth supplemented with 1×10^{-3} M $CuSO_4$.

A - Cells grown in basal medium

B - Cells grown in basal medium with 1×10^{-3} M $CuSO_4$

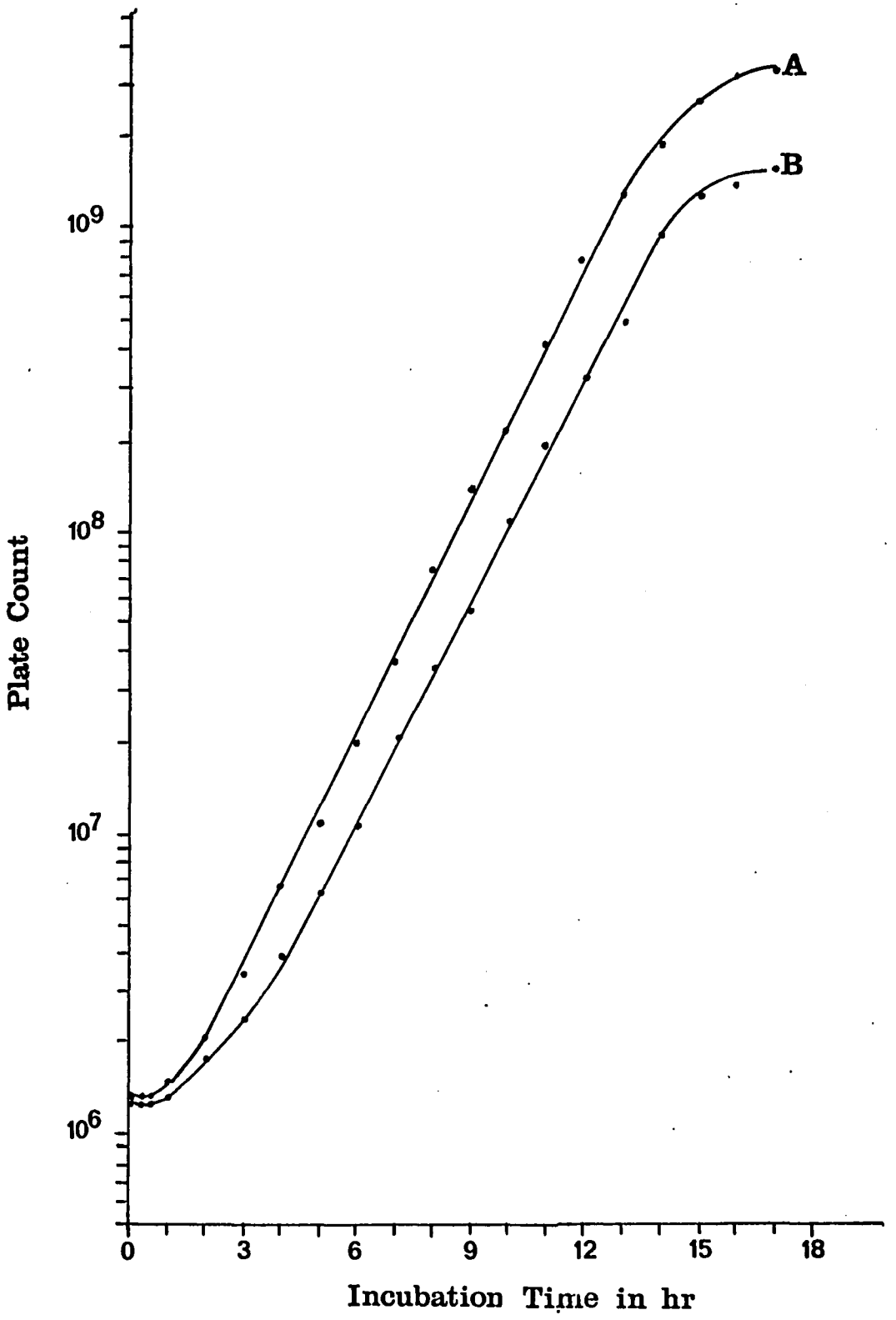

A
B
10^9
10^8
10^7
10^6
Plate Count
0
3
6
9
12
15
18
Incubation Time in hr

Fig 7. Growth of P. cuprodurans at 20 C in basal medium unsupplemented and supplemented with 1 x 10^{-3} M $CuSO_4$ as determined by plate counts. Inoculum: cells previously grown in the presence of 1 x 10^{-3} M $CuSO_4$ were used to inoculate a basal medium flask from which the standard inoculum was obtained.

A - Cells grown in basal medium

B - Cells grown in basal medium with 1 x 10^{-3} M $CuSO_4$.

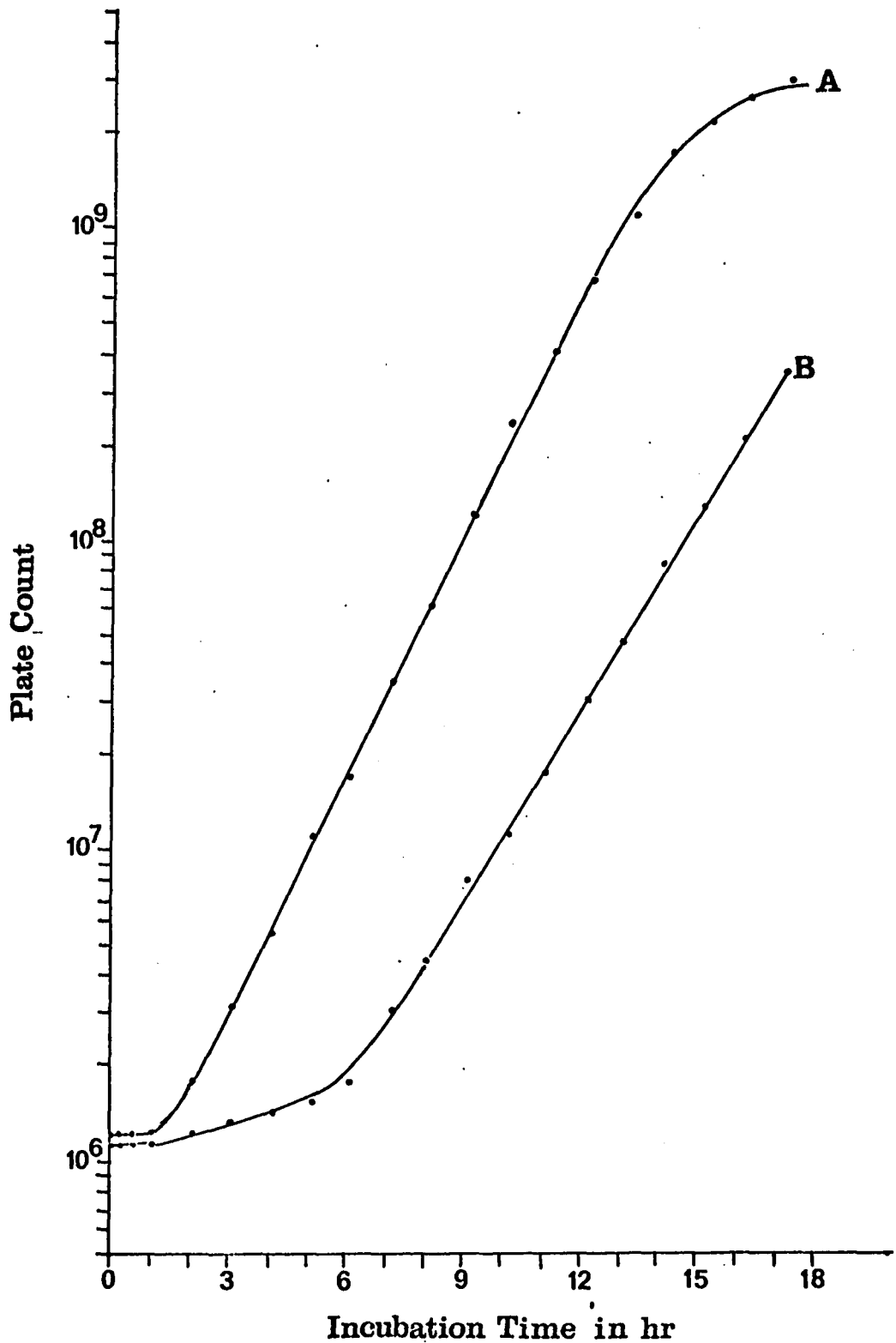

A
B
10^9
10^8
10^7
10^6
Plate Count
0
3
6
9
12
15
18
Incubation Time in hr

Fig 8. Comparative growth rates of P. cuprodurans in basal medium with varied concentrations of copper and varied temperature.

A - P. cuprodurans grown in the basal medium

B - P. cuprodurans grown in the basal medium with 1×10^{-4} M Cu

C - P. cuprodurans grown in the basal medium with 5×10^{-4} M Cu

D - P. cuprodurans grown in the basal medium with 1×10^{-3} M Cu

E - P. cuprodurans grown in the basal medium with 2×10^{-3} M Cu

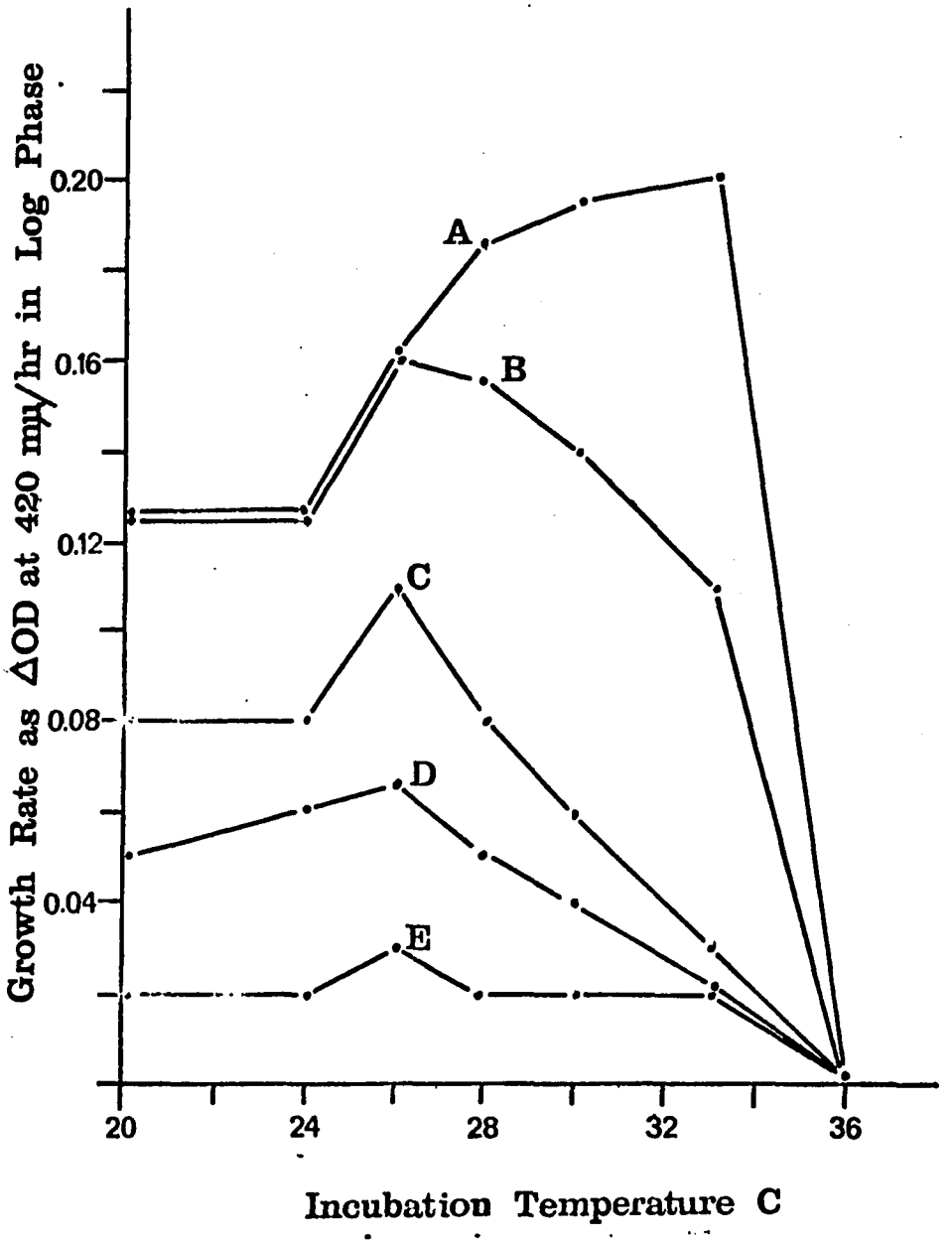

Growth Rate as ΔOD at 420 mμ/hr in Log Phase
0.20
0.16
0.12
0.08
0.04
A
B
C
D
E
20
24
28
32
36
Incubation Temperature C

Fig 9. Ultraviolet absorption spectrum of 0.0392 M magnesium chloride washing solution.

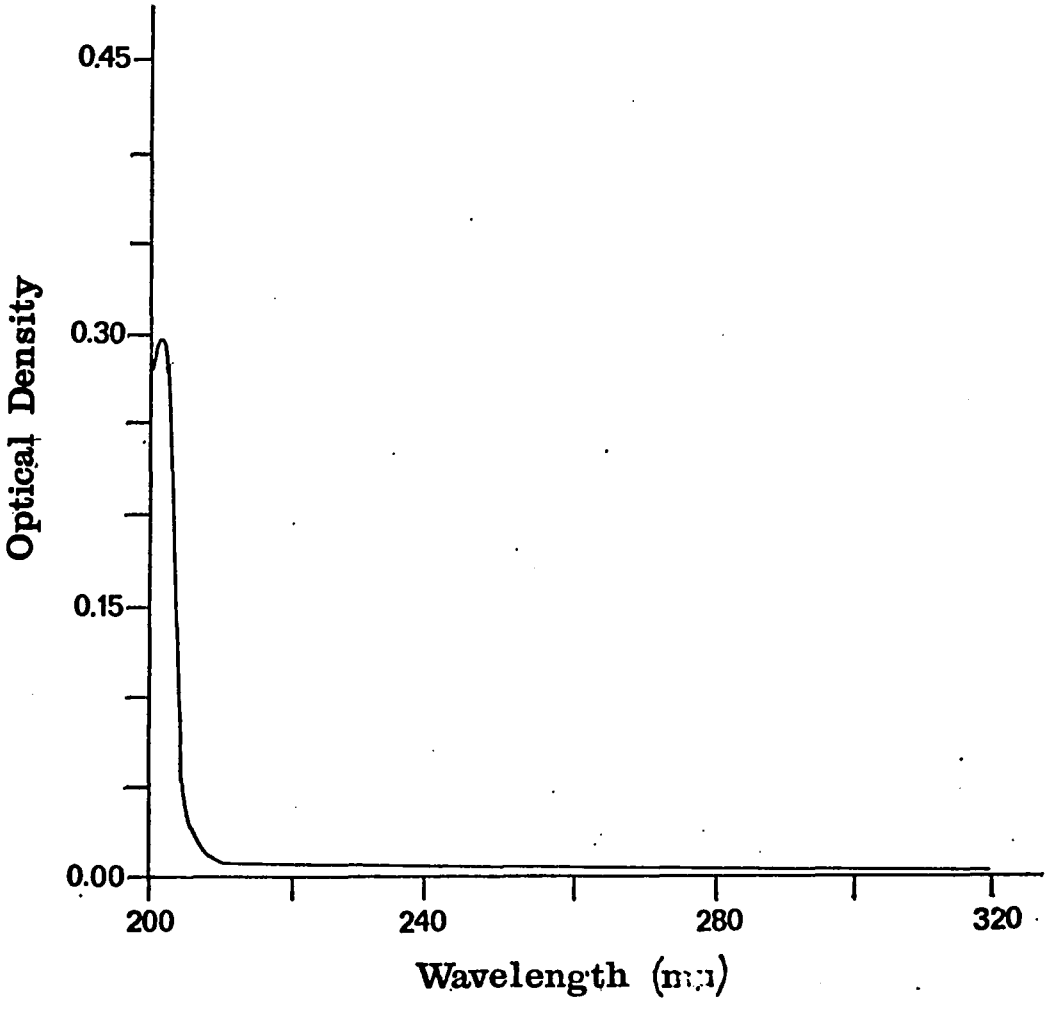

0.45
0.30
0.15
0.00
Optical Density
200
240
280
320
Wavelength (nm)

Fig 10. Ultraviolet absorption spectrum of second 0.0392 M magnesium chloride washing supernatant.

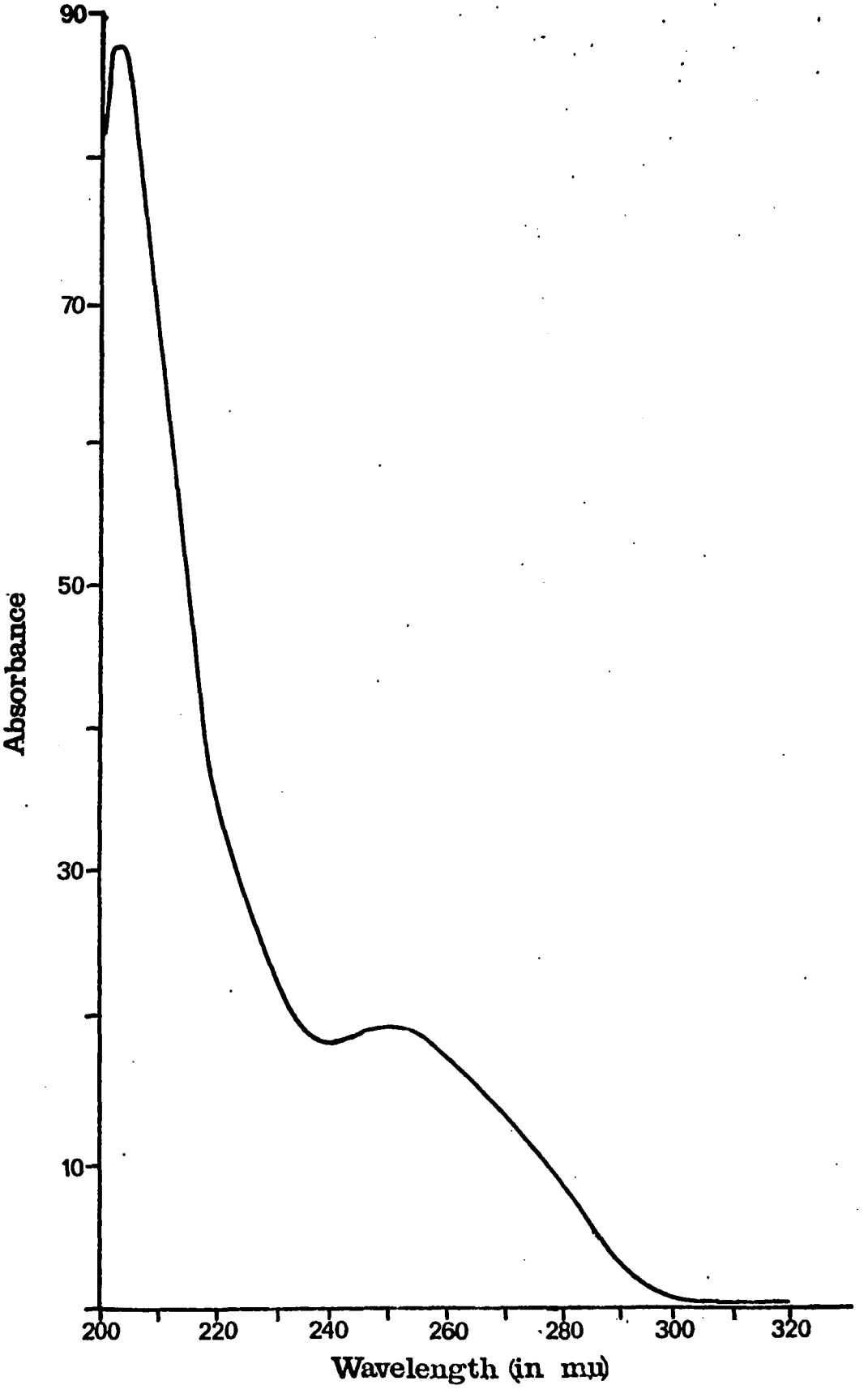

90
70
50
30
10
Absorbance
200
220
240
260
280
300
320
Wavelength (in mμ)

Fig 11. Comparative peak heights of the 203 and 250 mμ peaks obtained in the ultraviolet absorption spectra of various washing supernatants vs. 0.82 M glycerol.

A - 0.82 M glycerol

B - Distilled water

C - 0.0392 M magnesium chloride

D - 0.0074 M calcium chloride

E - 0.0392 M magnesium chloride and 0.0074 calcium chloride

F - 75% synthetic seawater

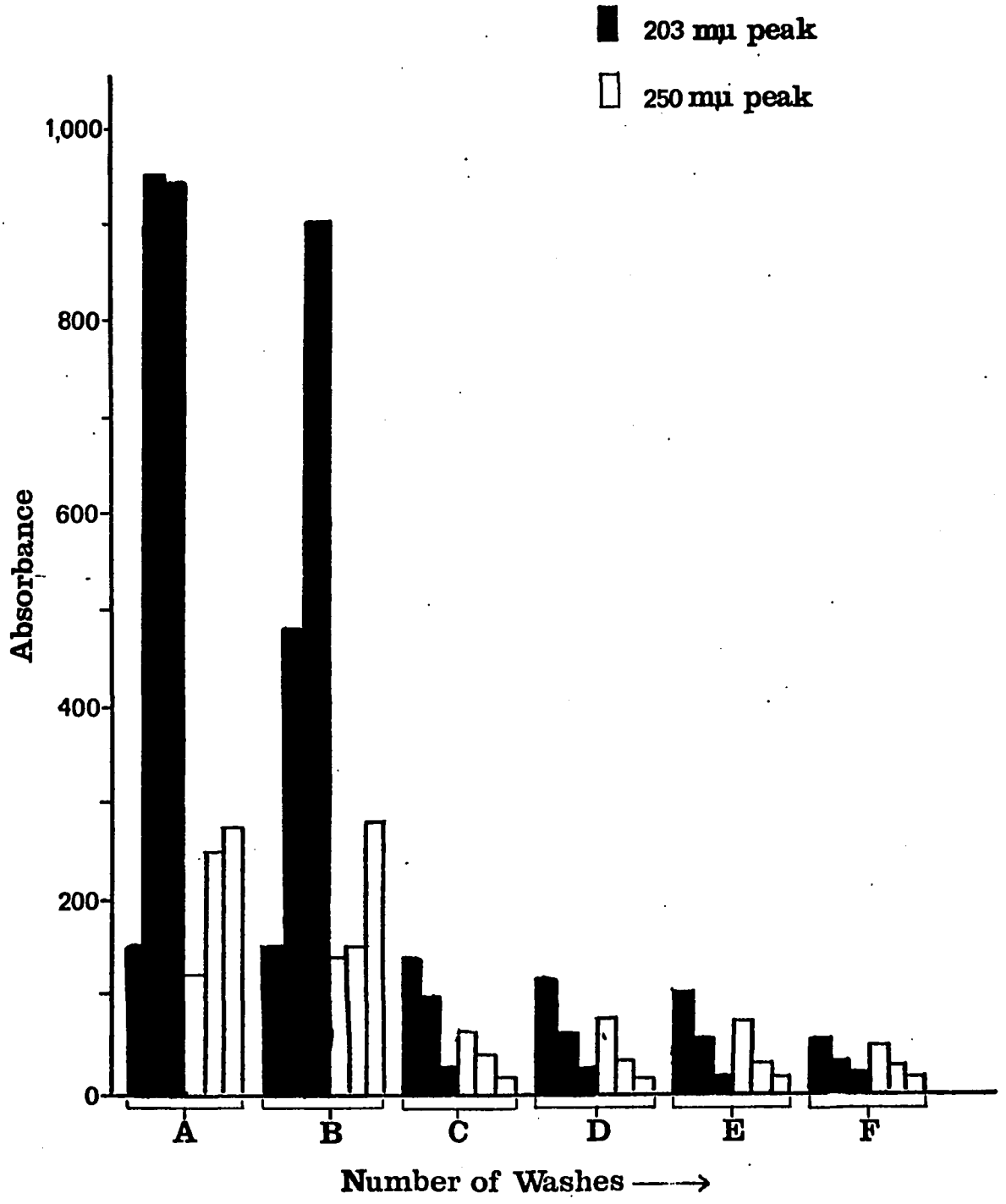

203 mμ peak
250 mμ peak
1,000
800
600
400
200
0
Absorbance
A
B
C
D
E
F
Number of Washes ⟶

Fig 12. Comparative peak heights of the 203 and 250 mμ peaks obtained in the ultraviolet absorption spectra of magnesium washing solutions vs 0.82 M glycerol.

A - 49.2 mM magnesium chloride

B - 39.3 mM magnesium chloride

C - 29.5 mM magnesium chloride

D - 19.6 mM magnesium chloride

E - 9.8 mM magnesium chloride

F - 4.9 mM magnesium chloride

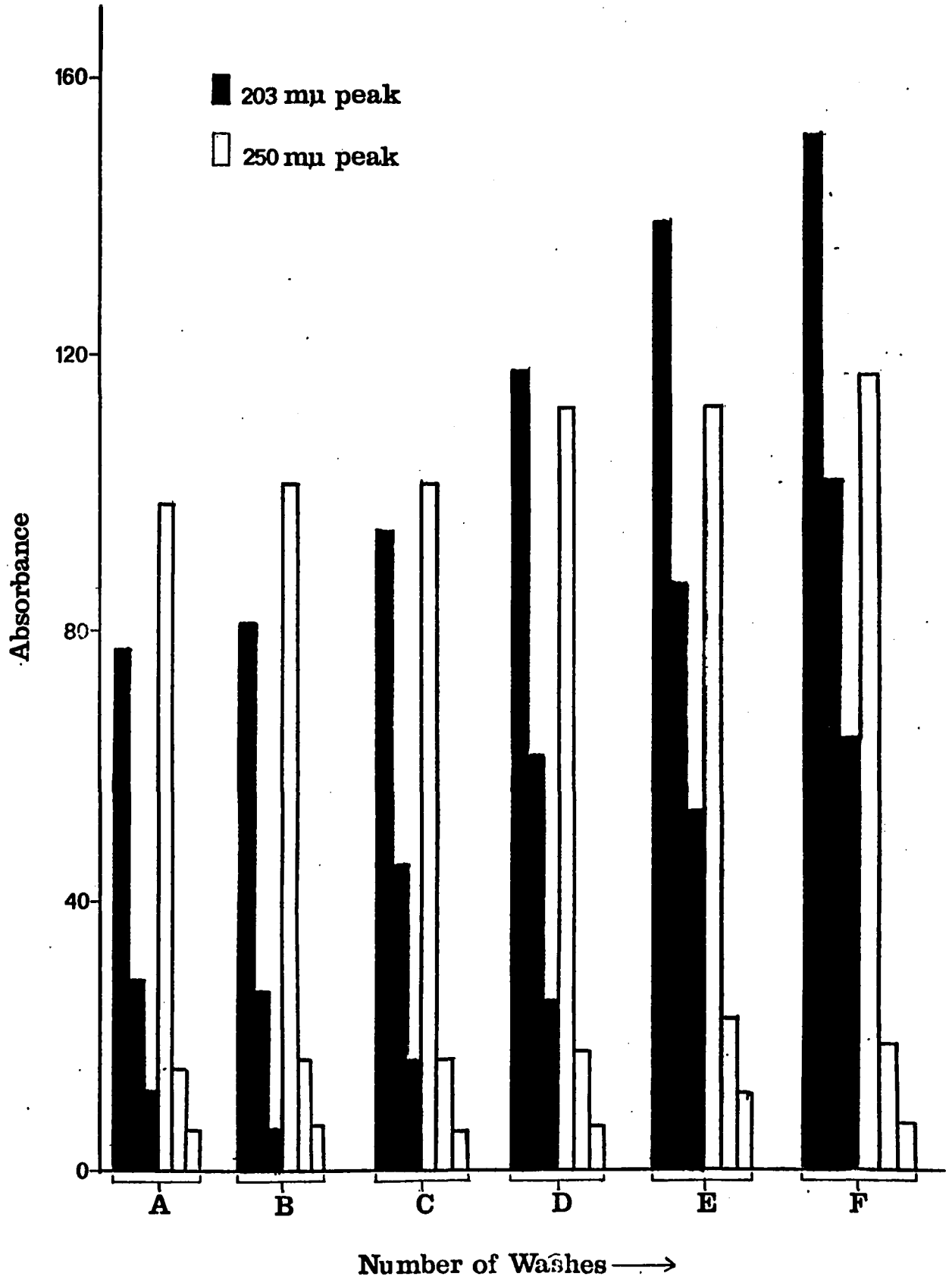

160
120
80
40
0
203 mμ peak
250 mμ peak
Absorbance
A
B
C
D
E
F
Number of Washes ⟶

Fig 13. Comparative respiration of P. cuprodurans cells grown in the basal medium unsupplemented and supplemented with 1×10^{-3} M copper and washed with either 75% synthetic seawater or a magnesium chloride solution (4 g/liter).

A - P. cuprodurans cells cultivated in basal medium broth and washed with 75% synthetic seawater.

B - P. cuprodurans cells cultivated in basal medium broth supplemented with 1×10^{-3} M Cu and washed with 75% synthetic seawater.

C - P. cuprodurans cells cultivated in basal medium broth and washed with magnesium chloride solution (4 g/liter).

D - P. cuprodurans cells cultivated in basal medium broth supplemented with 1×10^{-3} M Cu and washed with magnesium chloride solution (4 g/liter).

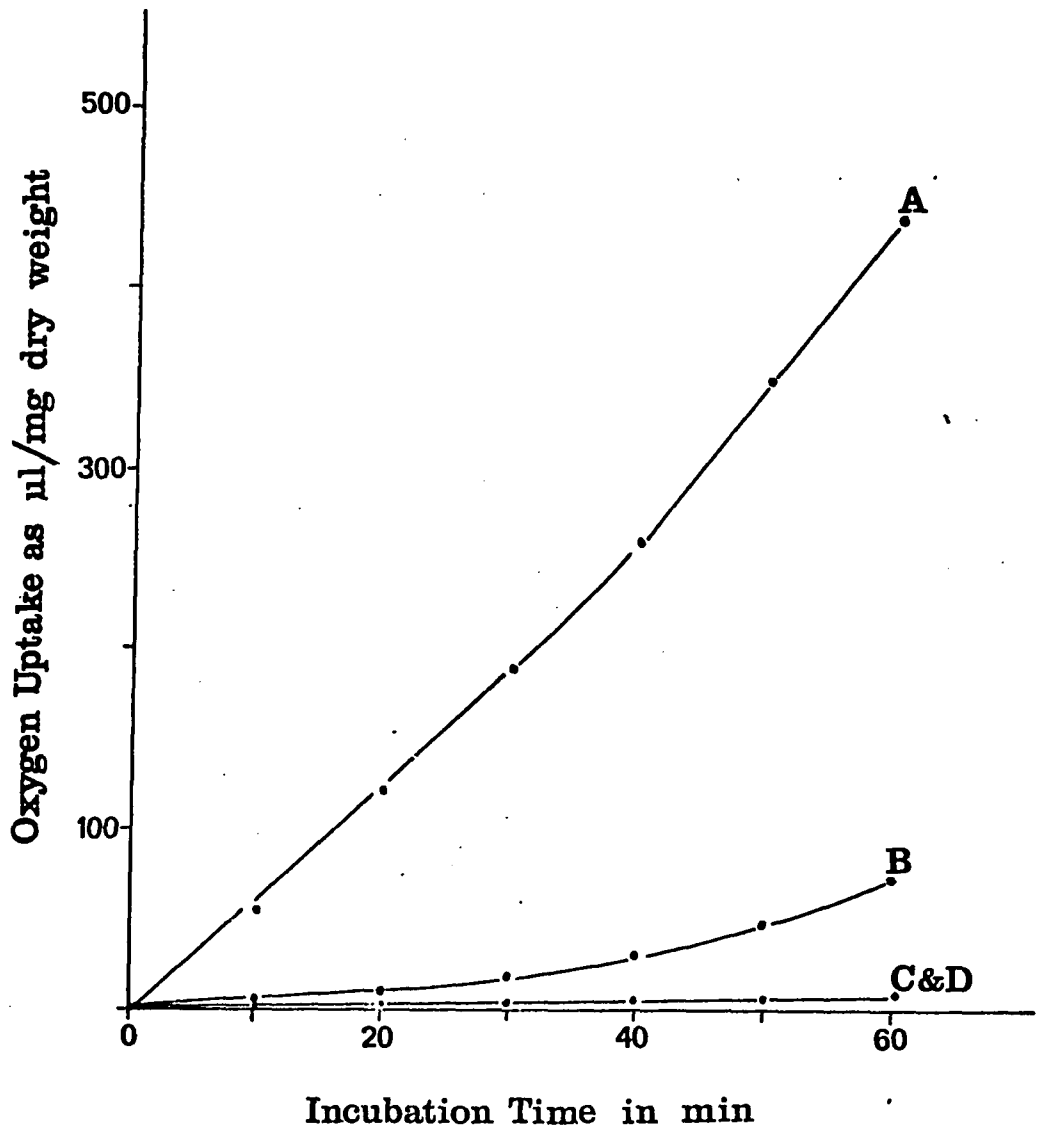

Oxygen Uptake as µl/mg dry weight
500
300
100
A
B
C&D
0
20
40
60
Incubation Time in min

Fig 14. Interrelationships of cell numbers, Lowry protein, dry weight of P. cuprodurans cells grown in basal medium supplemented with varied concentrations of $CuSO_4$.

- O Cells grown in basal medium
- ● Cells grown in basal medium with 1×10^{-4} M copper
- Δ Cells grown in basal medium with 5×10^{-4} M copper
- ▲ Cells grown in basal medium with 1×10^{-3} M copper

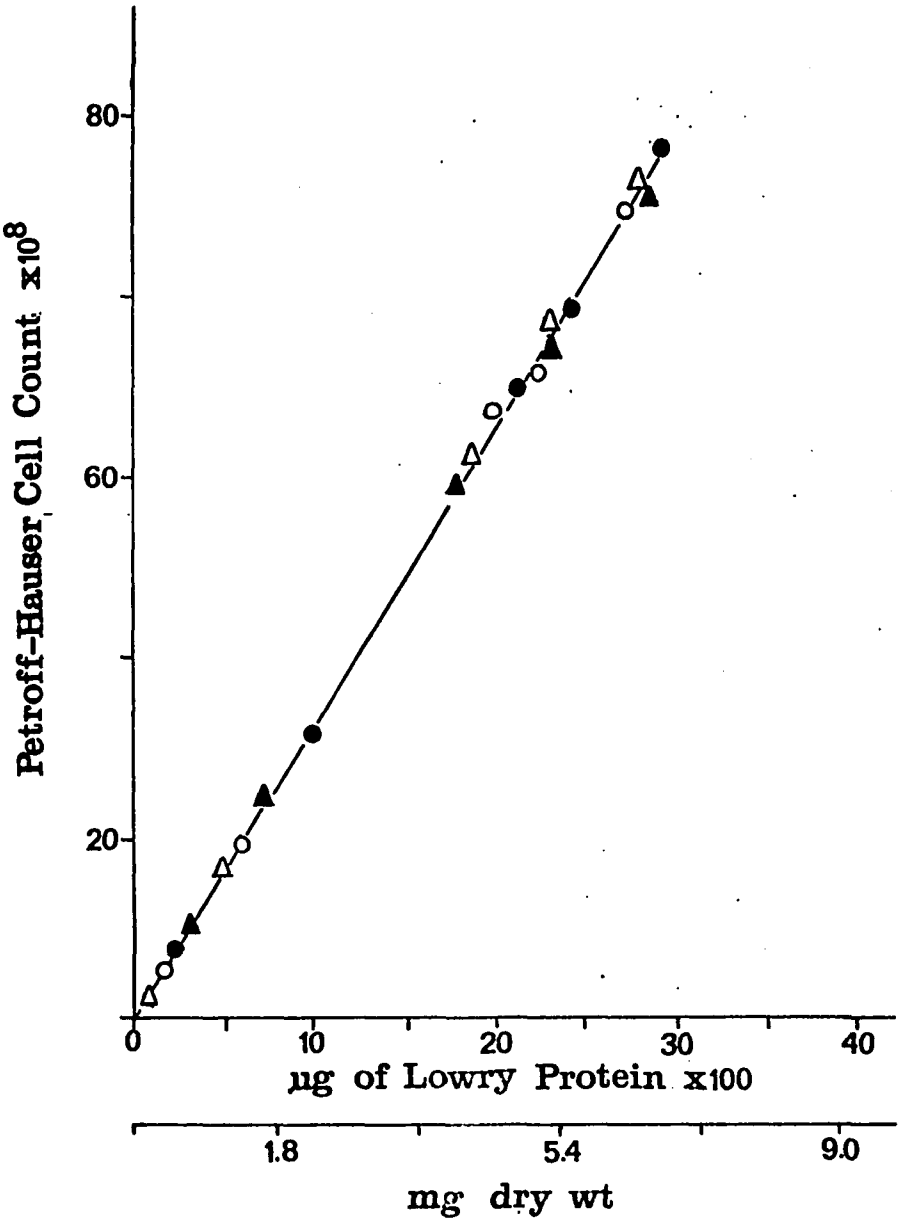

Petroff-Hauser Cell Count x10^8
80
60
20
0
10
20
30
40
µg of Lowry Protein x100
1.8
5.4
9.0
mg dry wt

Fig 15. Ultrastructure of P. cuprodurans cultivated at 20 C in the basal medium (Magnification 57,150 X).

R - Ribosomes

CM - Cytoplasmic Membrane

CW - Cell Wall

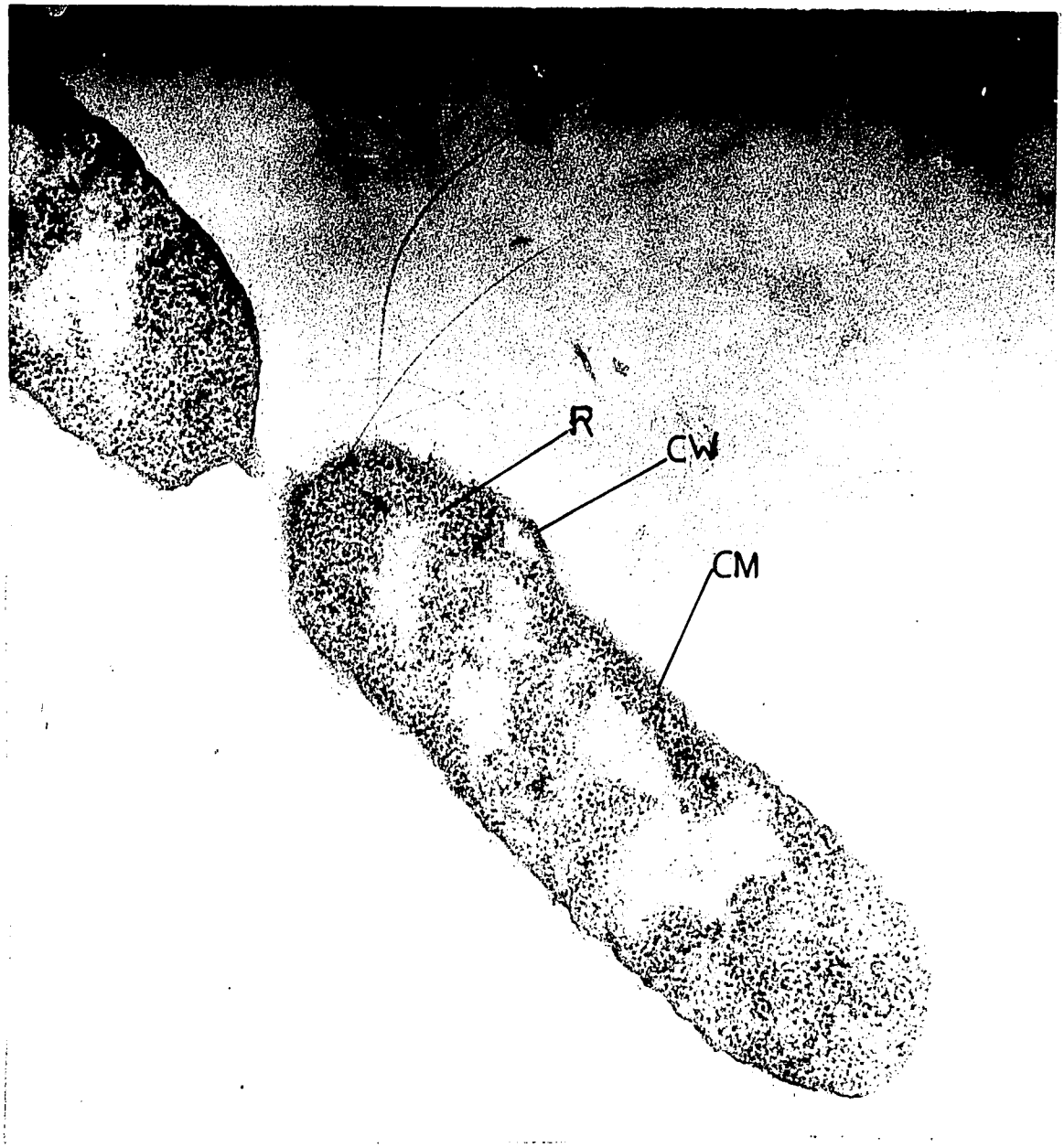

R
CW
CM

Fig 16. Ultrastructure of P. cuprodurans cultivated at 20 C in basal medium supplemented with 1 x 10^{-3} M Cu (Magnification 57,150 X).

R - Ribosomes

CM - Cytoplasmic Membrane

CW - Cell Wall

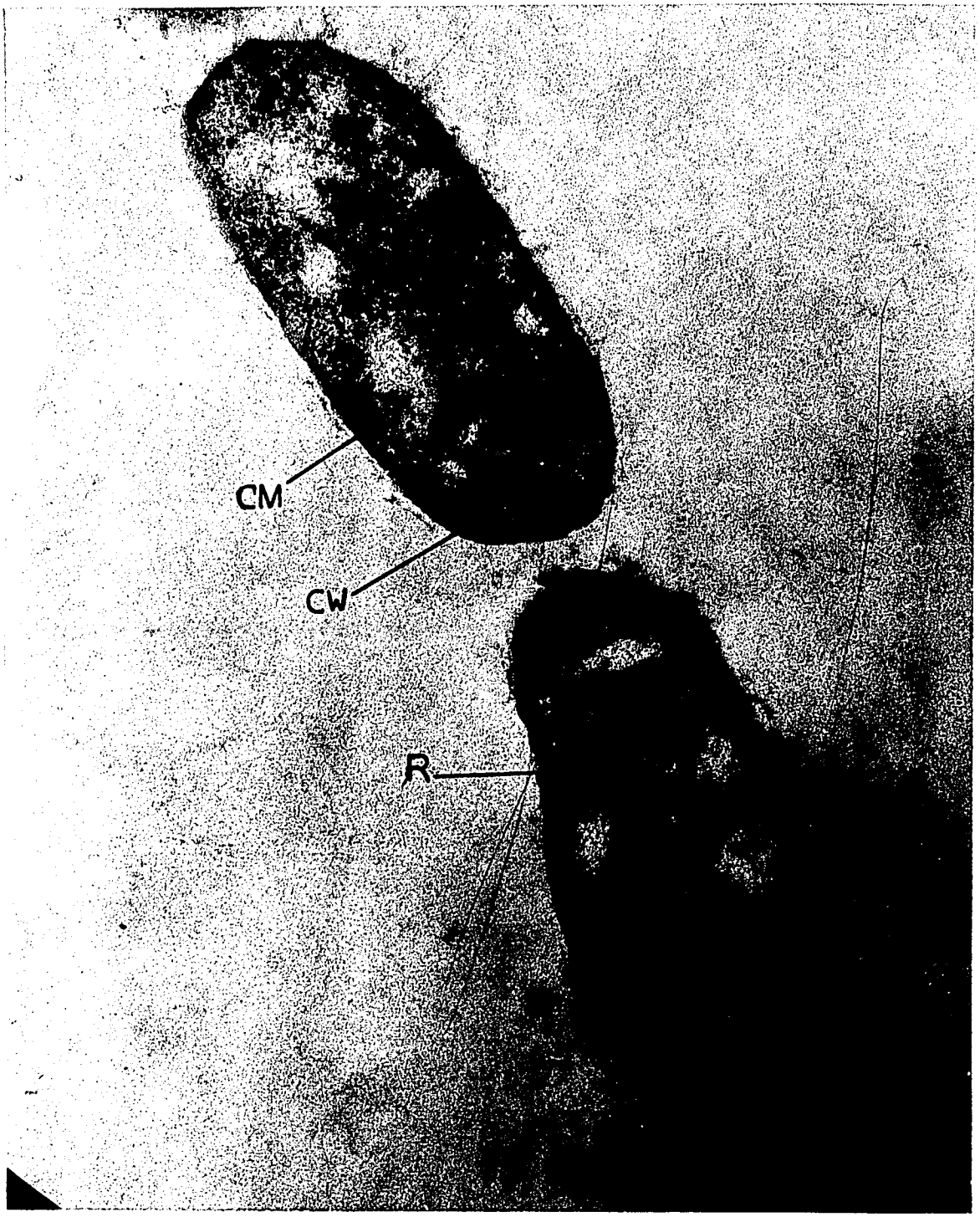

CM
CW
R

Fig 17. Oxygen uptake at 20 C of P. cuprodurans cultivated in basal medium broth and studied in basal medium broth with varied concentrations of $CuSO_4$.

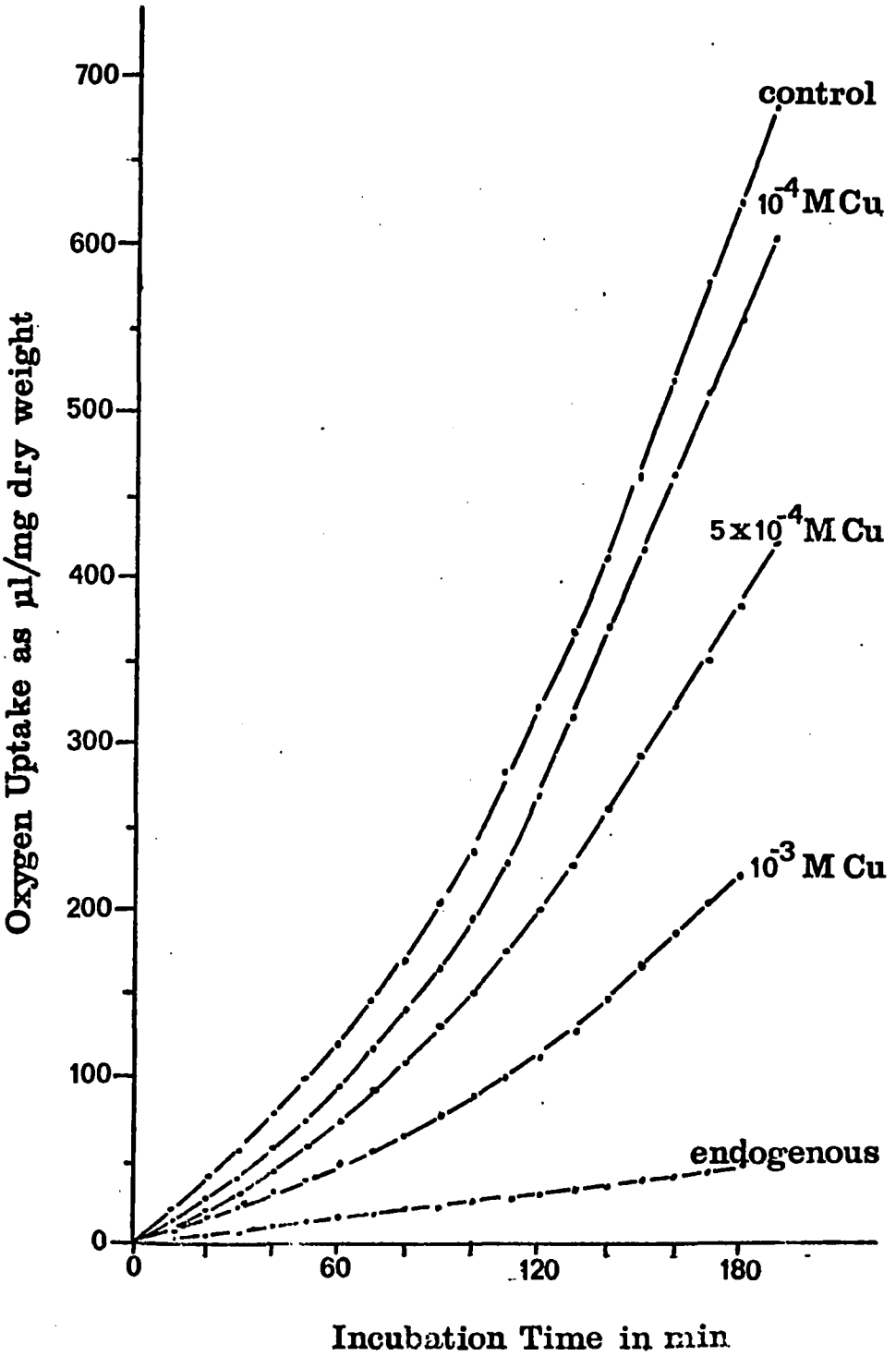

700
600
500
400
300
200
100
0
0
60
120
180
control
10-4 M Cu
5 x 10-4 M Cu
10-3 M Cu
endogenous
Oxygen Uptake as µl/mg dry weight
Incubation Time in min

Fig 18. Oxygen uptake at 20$_4$C of P. cuprodurans cultivated in basal medium with 1 x 10^{-4} M $CuSO_4$ and studied in basal medium with varied concentrations of $CuSO_4$.

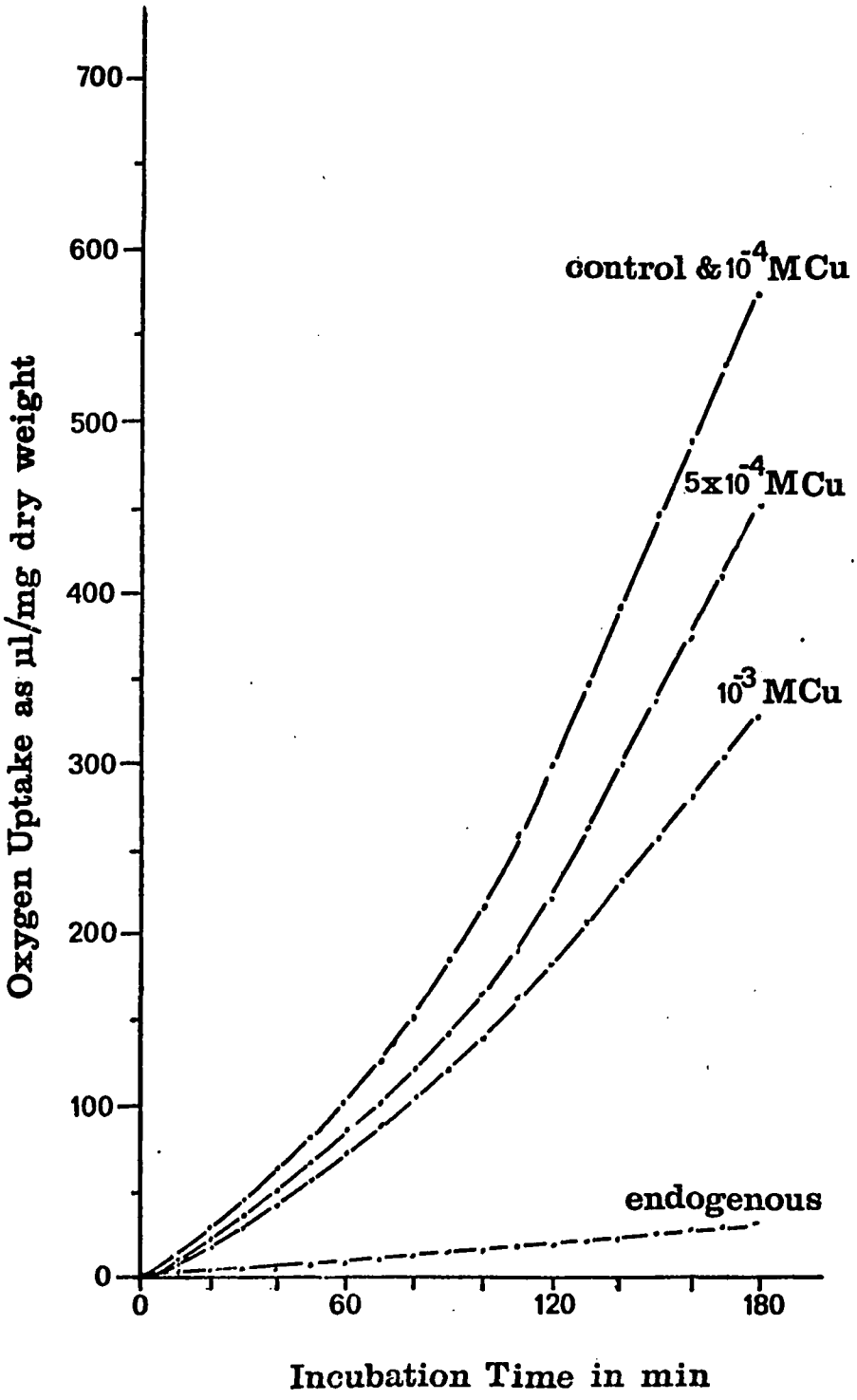

700
600
500
400
300
200
100
0
0
60
120
180
control & 10^{-4} M Cu
5×10^{-4} M Cu
10^{-3} M Cu
endogenous
Oxygen Uptake as µl/mg dry weight
Incubation Time in min

Fig 19. Oxygen uptake at 20$_4$C of P. cuprodurans cultivated in basal medium with 5 x 10^{-4} M $CuSO_4$ and studied in basal medium with varied concentrations of $CuSO_4$.

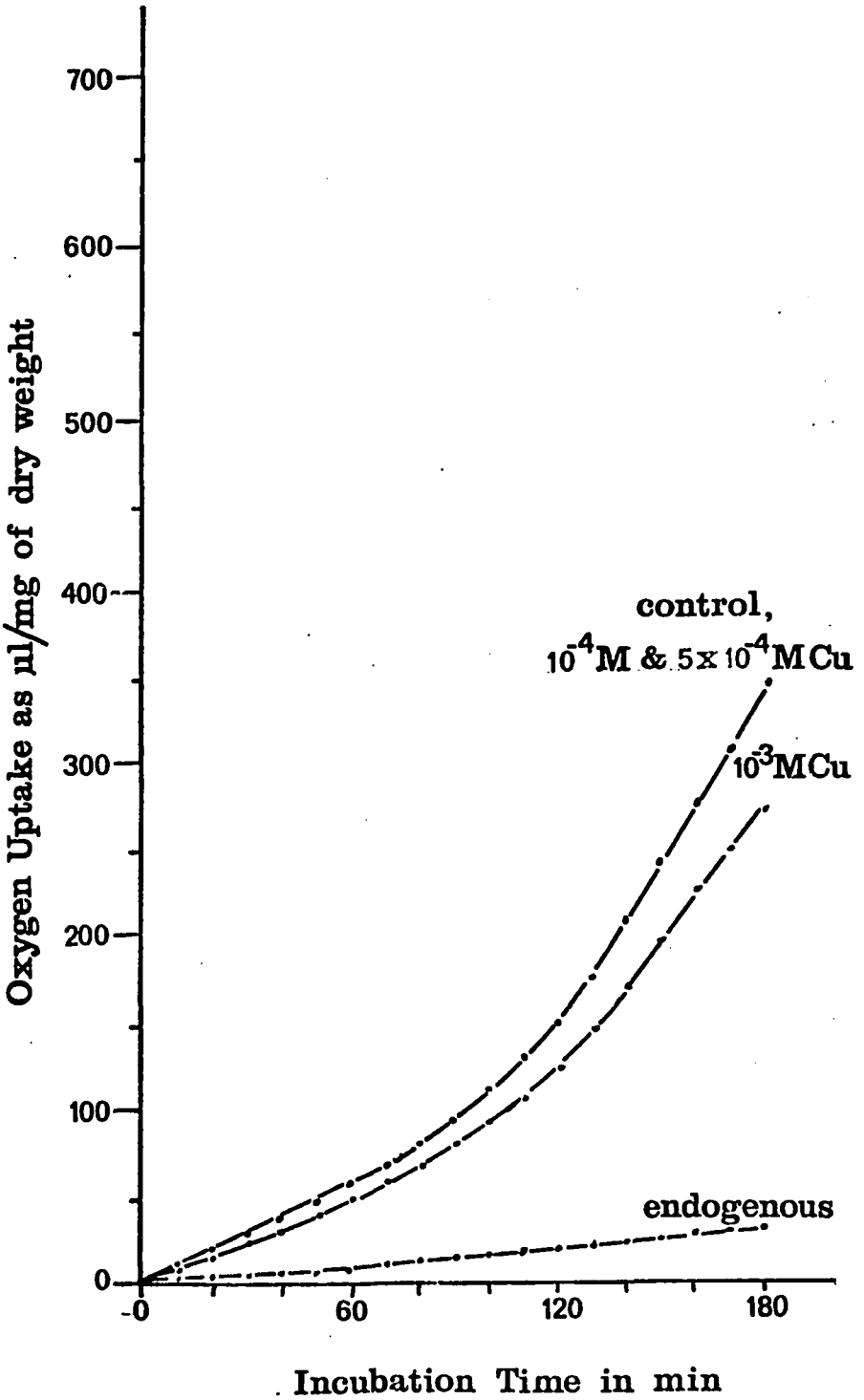

700
600
500
400
300
200
100
0
-0
60
120
180
Oxygen Uptake as µl/mg of dry weight
control,
10-4M & 5x10-4MCu
10-3MCu
endogenous
Incubation Time in min

Fig 20. Oxygen uptake at 20°C of *P. cuprodurans* cultivated in basal medium with 1×10^{-3} M $CuSO_4$ and studied in basal medium with varied concentrations of $CuSO_4$.

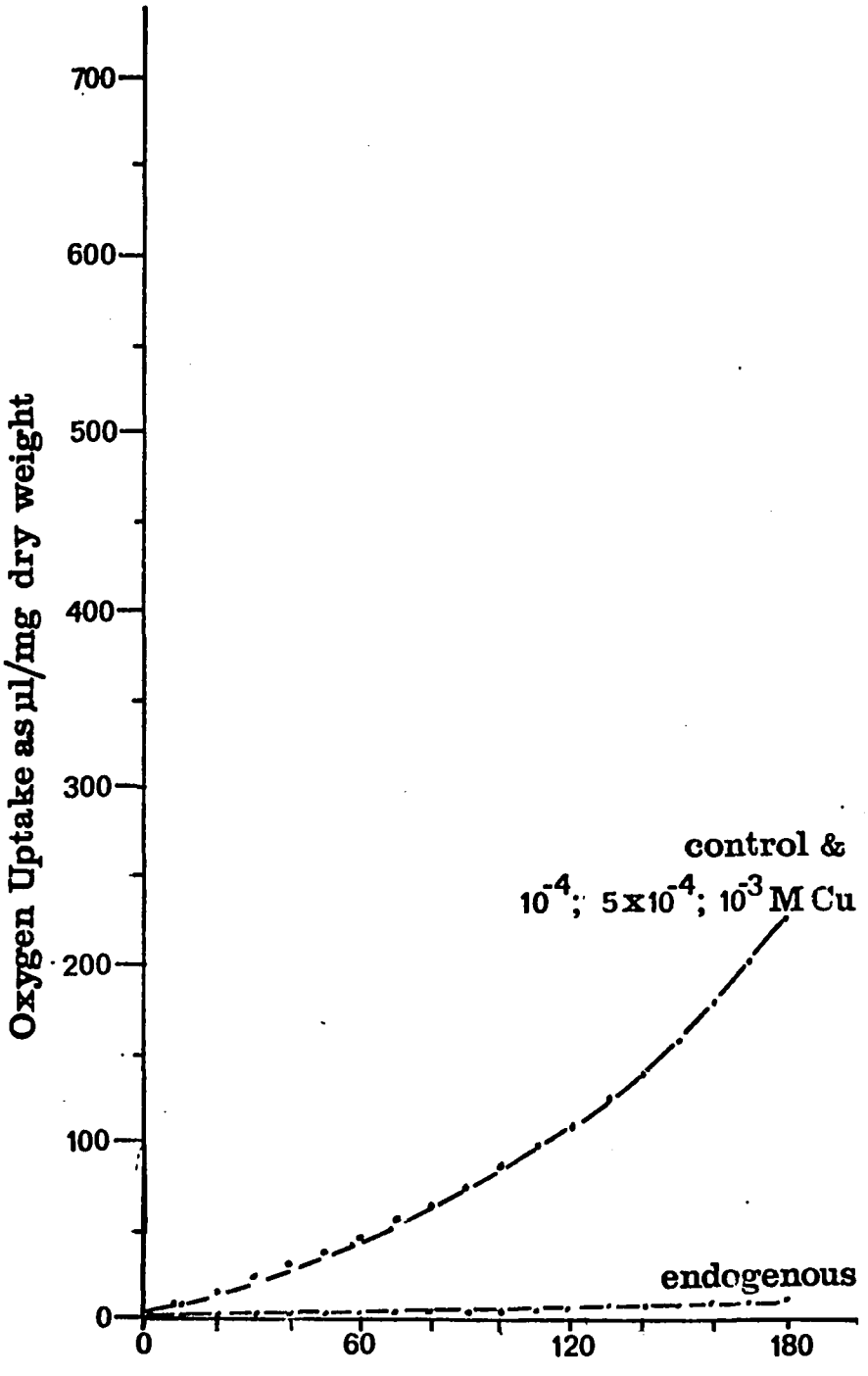

700
600
500
400
300
200
100
0
Oxygen Uptake as µl/mg dry weight
control &
10-4; 5x10-4; 10-3 M Cu
endogenous
0
60
120
180
Incubation Time in min

Fig 21. Oxygen uptake at 20°C of *P. cuprodurans* cultivated in basal medium with 2×10^{-3} M $CuSO_4$ and studied in basal medium with varied concentrations of $CuSO_4$.

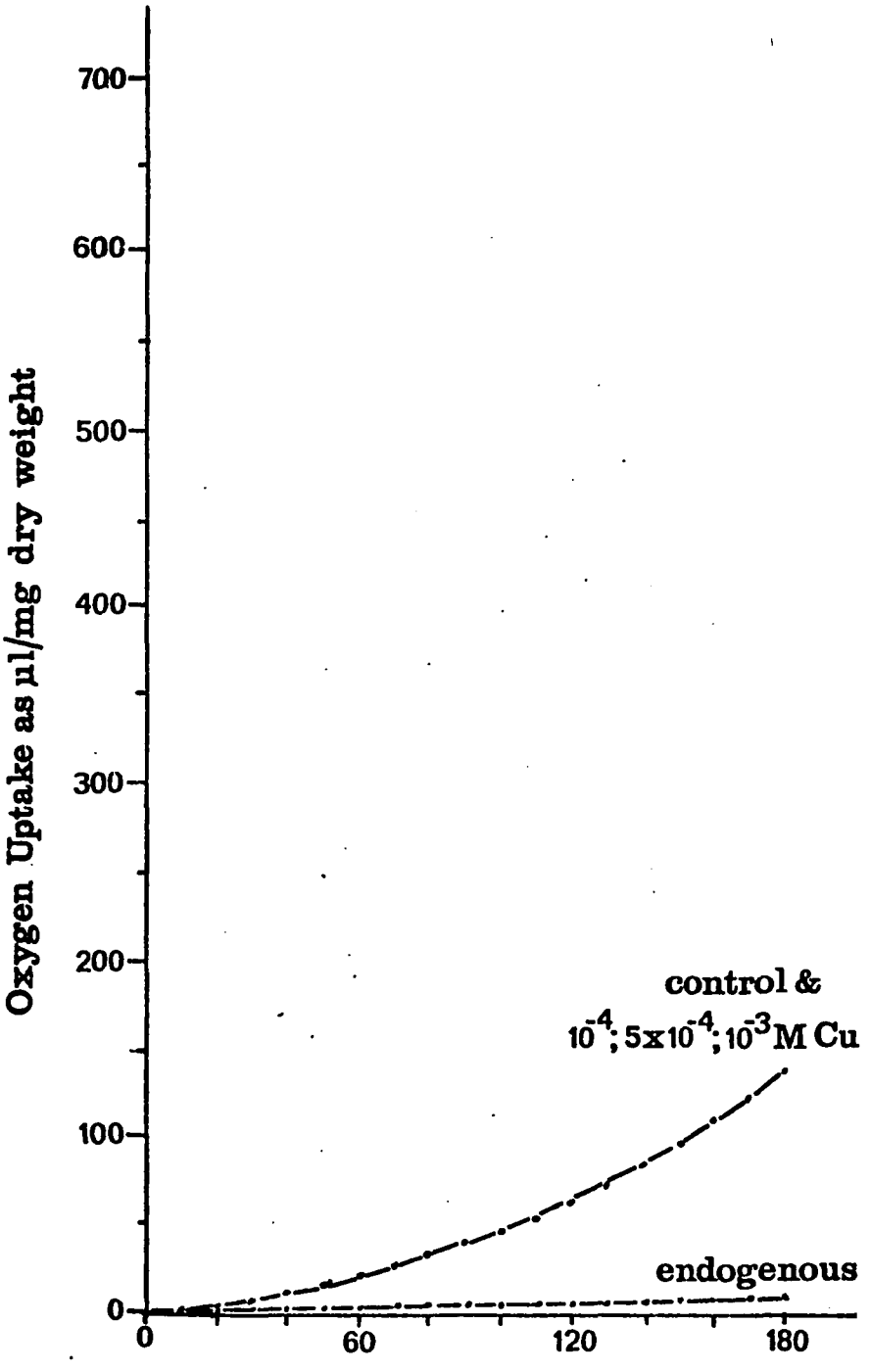

700
600
500
400
300
200
100
0
Oxygen Uptake as µl/mg dry weight
control &
10⁻⁴; 5x10⁻⁴; 10⁻³M Cu
endogenous
0
60
120
180
Incubation Time in min

Fig 22. Reduction of methylene blue at 20 C by P. cuprodurans cells cultivated in basal medium with varied concentrations of $CuSO_4$.

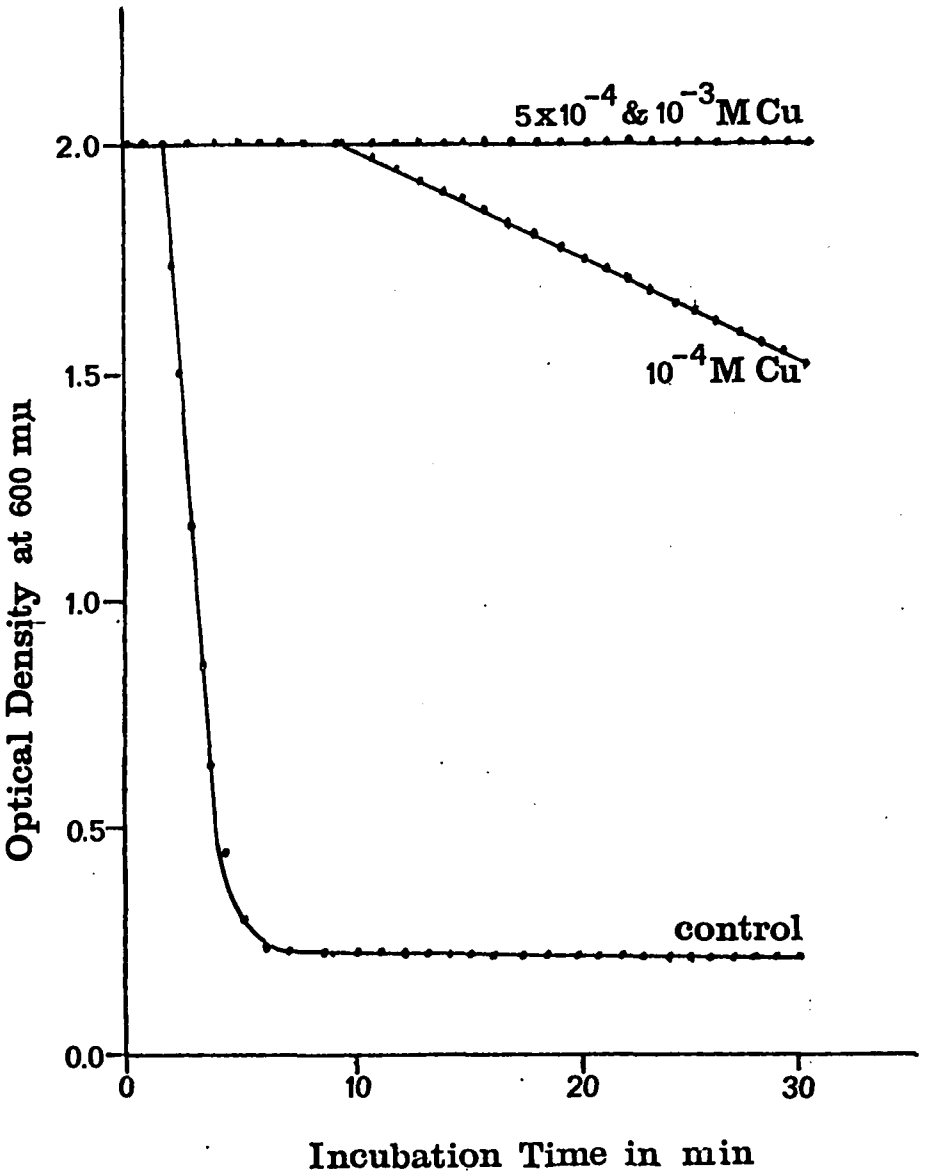

5×10^{-4} & 10^{-3} M Cu
10^{-4} M Cu
control
Optical Density at 600 mμ
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Incubation Time in min

Fig 23. Reduction of dichloroindophenol at 20 C by P. cuprodurans cells cultivated in basal medium with varied concentrations of $CuSO_4$.

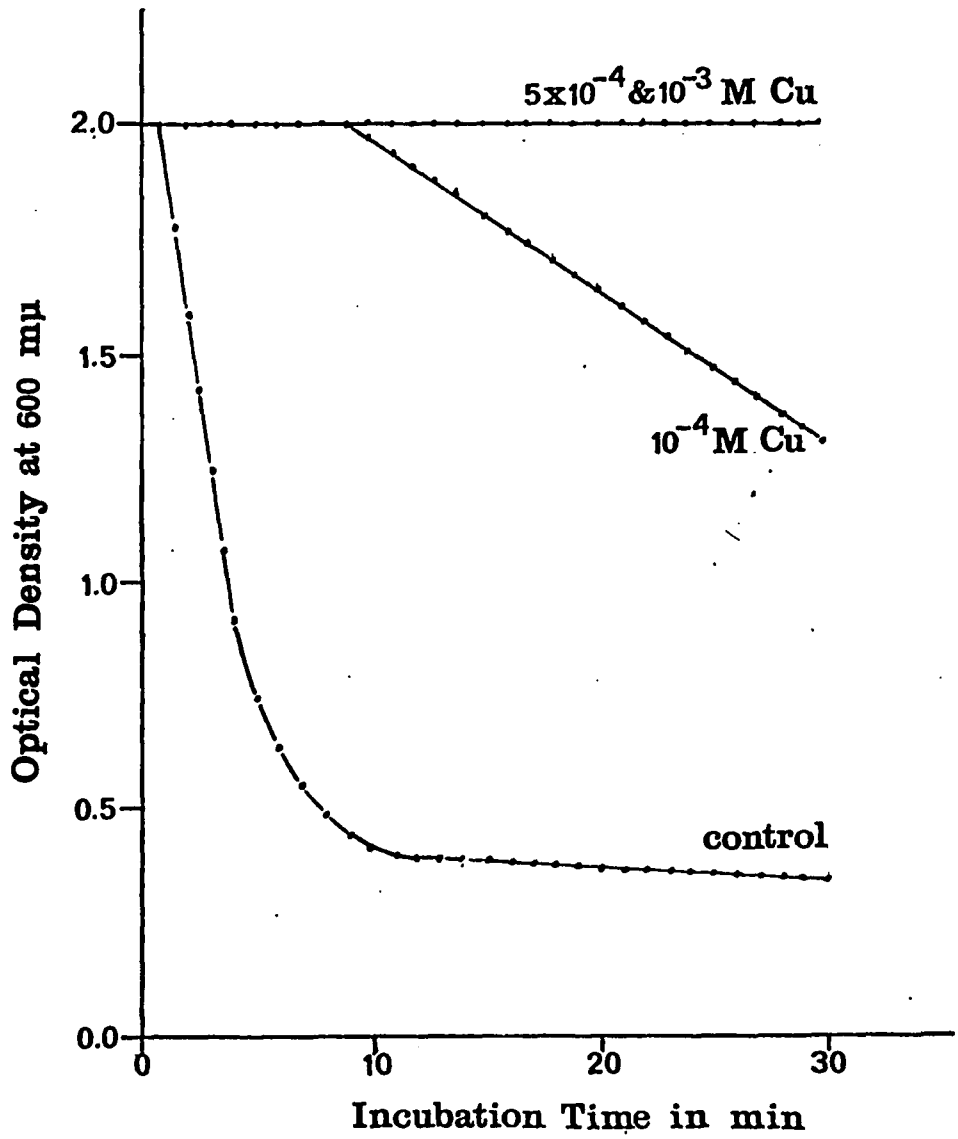

5x10^{-4}&10^{-3} M Cu
10^{-4}M Cu
control
Optical Density at 600 mμ
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Incubation Time in min

Fig 24. Reduction of triphenyltetrazolium chloride at 20 C by P. cuprodurans cells cultivated in basal medium with varied concentrations of $CuSO_4$.

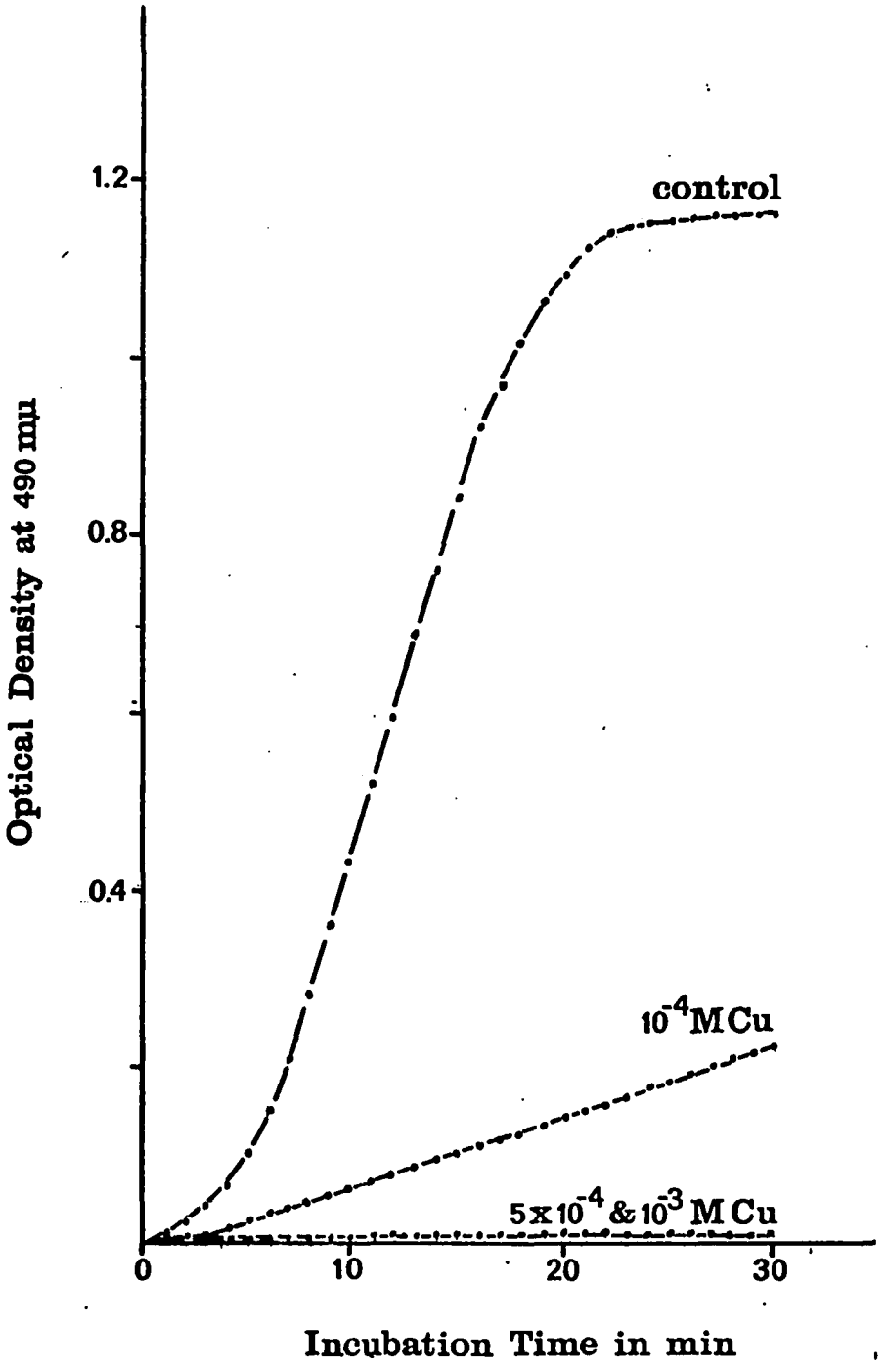

1.2
0.8
0.4
Optical Density at 490 mμ
control
10^{-4}M Cu
5×10^{-4} & 10^{-3} M Cu
0
10
20
30
Incubation Time in min

Fig 25. Difference spectrum (650-400 mμ) of whole cell extracts of P. cuprodurans cells cultivated in basal medium.

A - Cytochrome alpha peaks

B - Cytochrome beta peaks

C - 450-460 mu trough

D - Cytochrome gamma peak

Optical Density Increments cm-1
-0.06
0.00
0.06
0.12
400
480
560
640
Wavelength (mμ)
A
B
C
D

Fig 26. Difference spectrum (650-400 mμ) of whole cell extracts of P. cuprodurans cells cultivated in basal medium with 1 x 10^{-4} M $CuSO_4$.

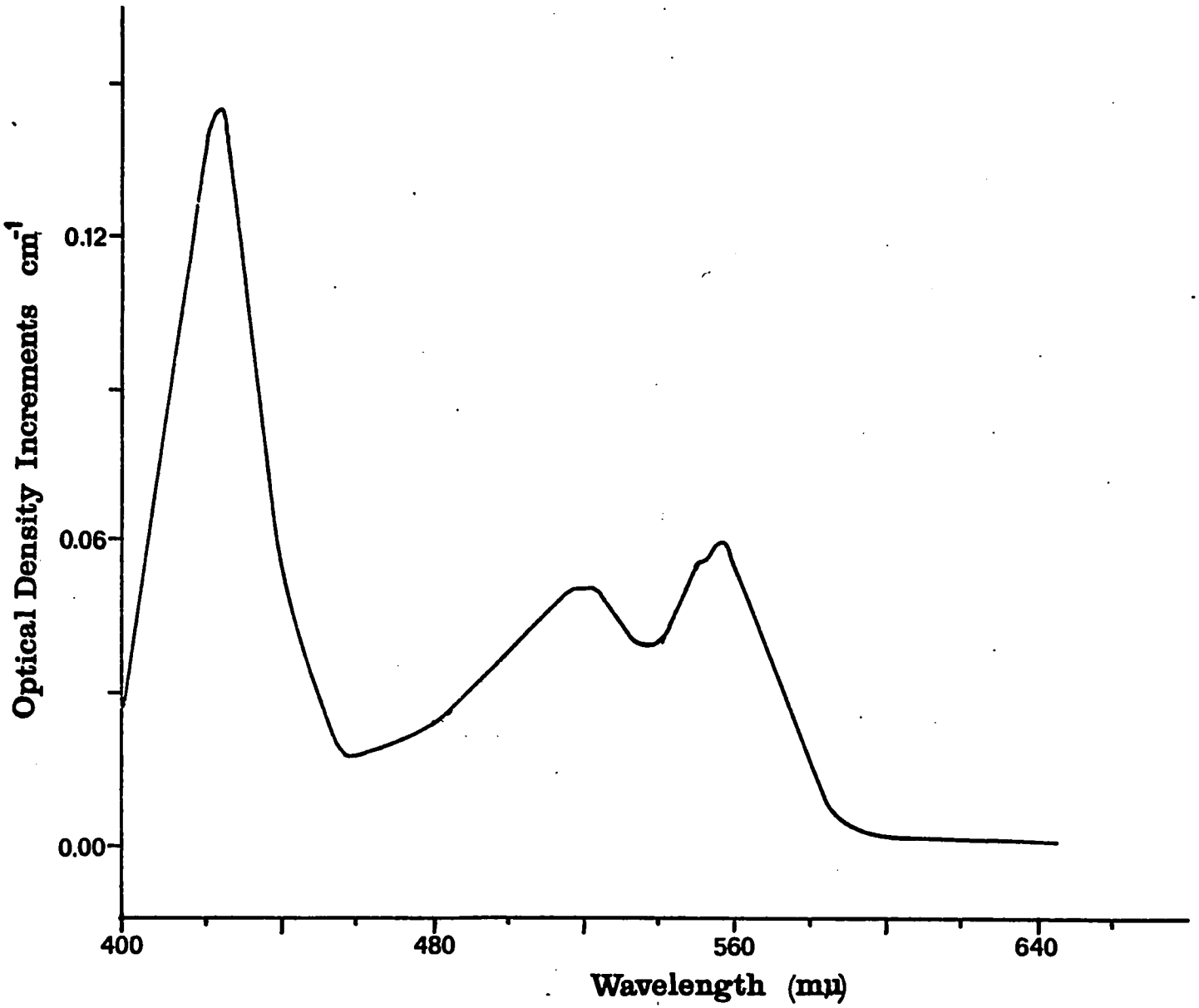

Optical Density Increments cm-1
0.12
0.06
0.00
400
480
560
640
Wavelength (mμ)

Fig 27. Difference spectrum (650-400 mμ) of whole cell extracts of P. cuprodurans cultivated in basal medium with 5×10^{-4} M $CuSO_4$.

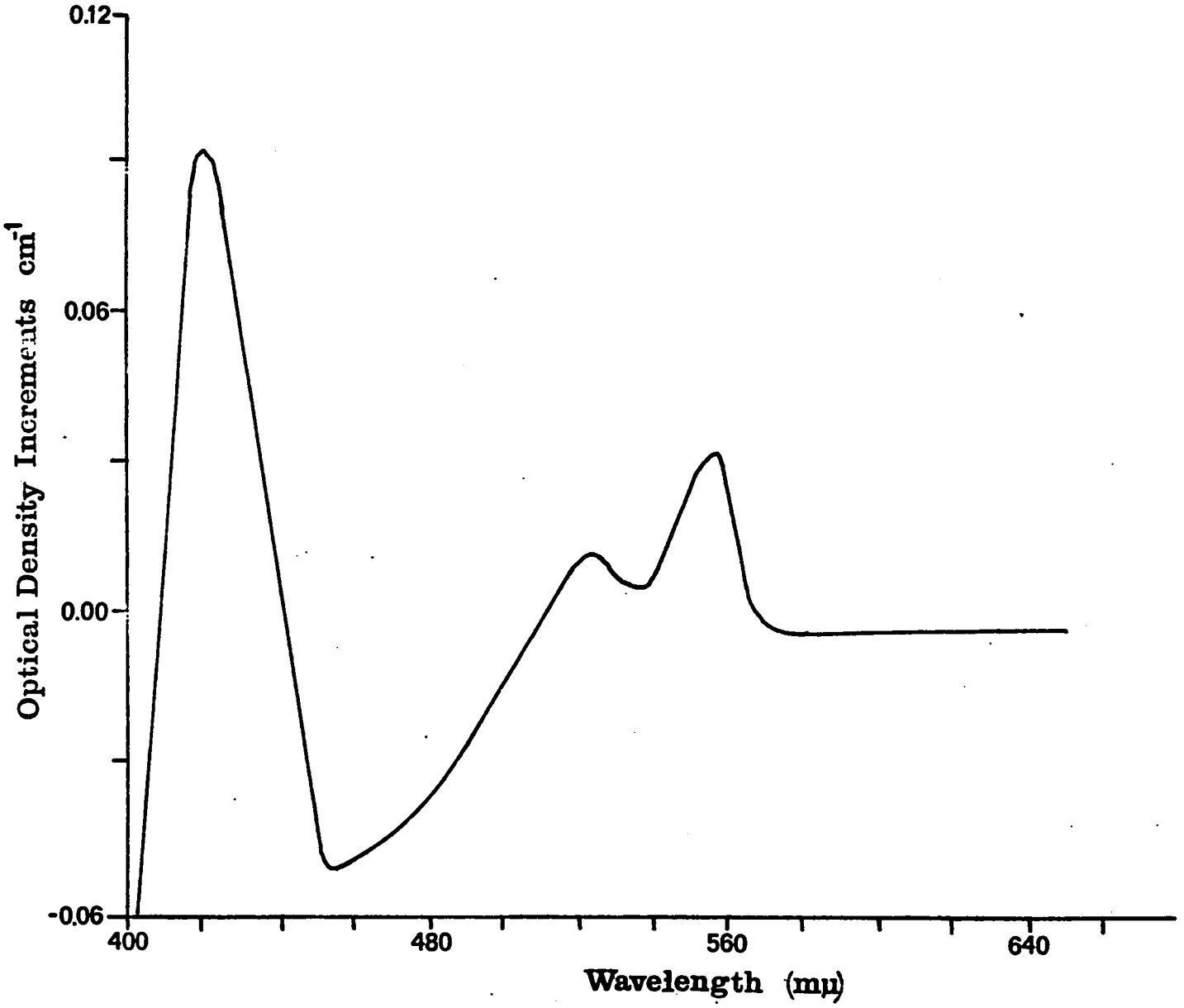

0.12
0.06
0.00
-0.06
Optical Density Increments cm-1
400
480
560
640
Wavelength (mμ)

Fig 28. Difference spectrum (650-400 mμ) of whole cell extracts of P. cuprodurans cultivated in basal medium with 1×10^{-3} M $CuSO_4$.

Optical Density Increments cm-1
0.00
0.06
0.12
400
480
560
640
Wavelength (mμ)

Fig 29. Carbon monoxide spectra (600-520 mμ) of whole cell extracts of P. cuprodurans cultivated in basal medium with varied concentrations of $CuSO_4$.

A - Cells grown in basal medium

B - Cells grown in basal medium with 1×10^{-4} M $CuSO_4$.

C - Cells grown in basal medium with 5×10^{-4} M $CuSO_4$.

D - Cells grown in basal medium with 1×10^{-3} M $CuSO_4$.

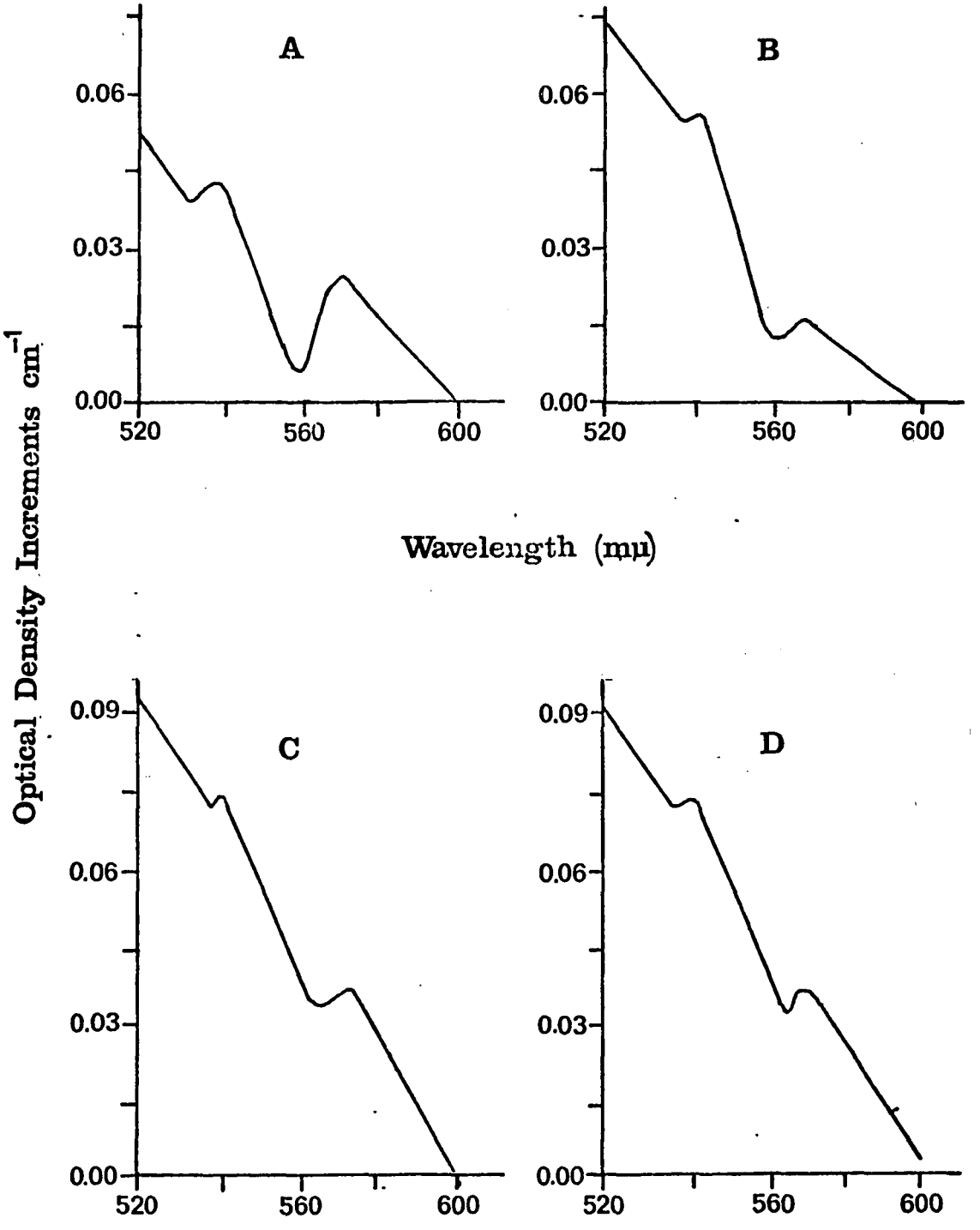

A
B
C
D
0.06
0.03
0.00
0.09
520
560
600
Optical Density Increments cm⁻¹
Wavelength (mμ)

Fig 30. Growth as Petroff-Hauser counts and ^{64}Cu uptake by P. cuprodurans at 20 C in basal medium.

O—O Cell numbers

●---● ^{64}Cu uptake

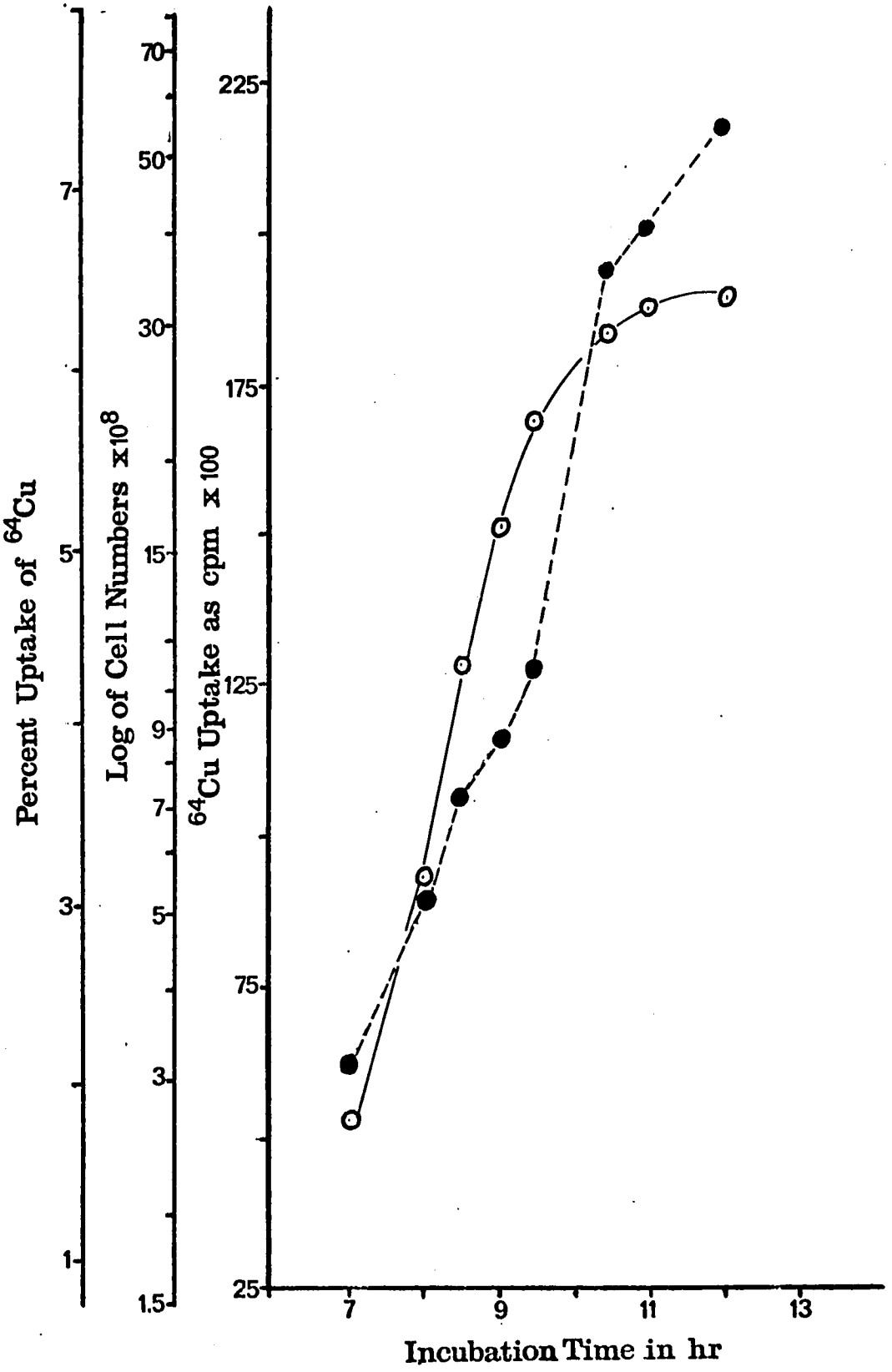

Percent Uptake of ^{64}Cu
Log of Cell Numbers x 10^8
^{64}Cu Uptake as cpm x 100
Incubation Time in hr
70
50
30
15
9
7
5
3
1.5
225
175
125
75
25
7
9
11
13

Fig 31. Growth as Petroff-Hauser counts and ^{64}Cu uptake by P. cuprodurans at 20° C in basal medium supplemented with 1×10^{-4} M $CuSO_4$.

O——O Cell numbers

●---● ^{64}Cu uptake

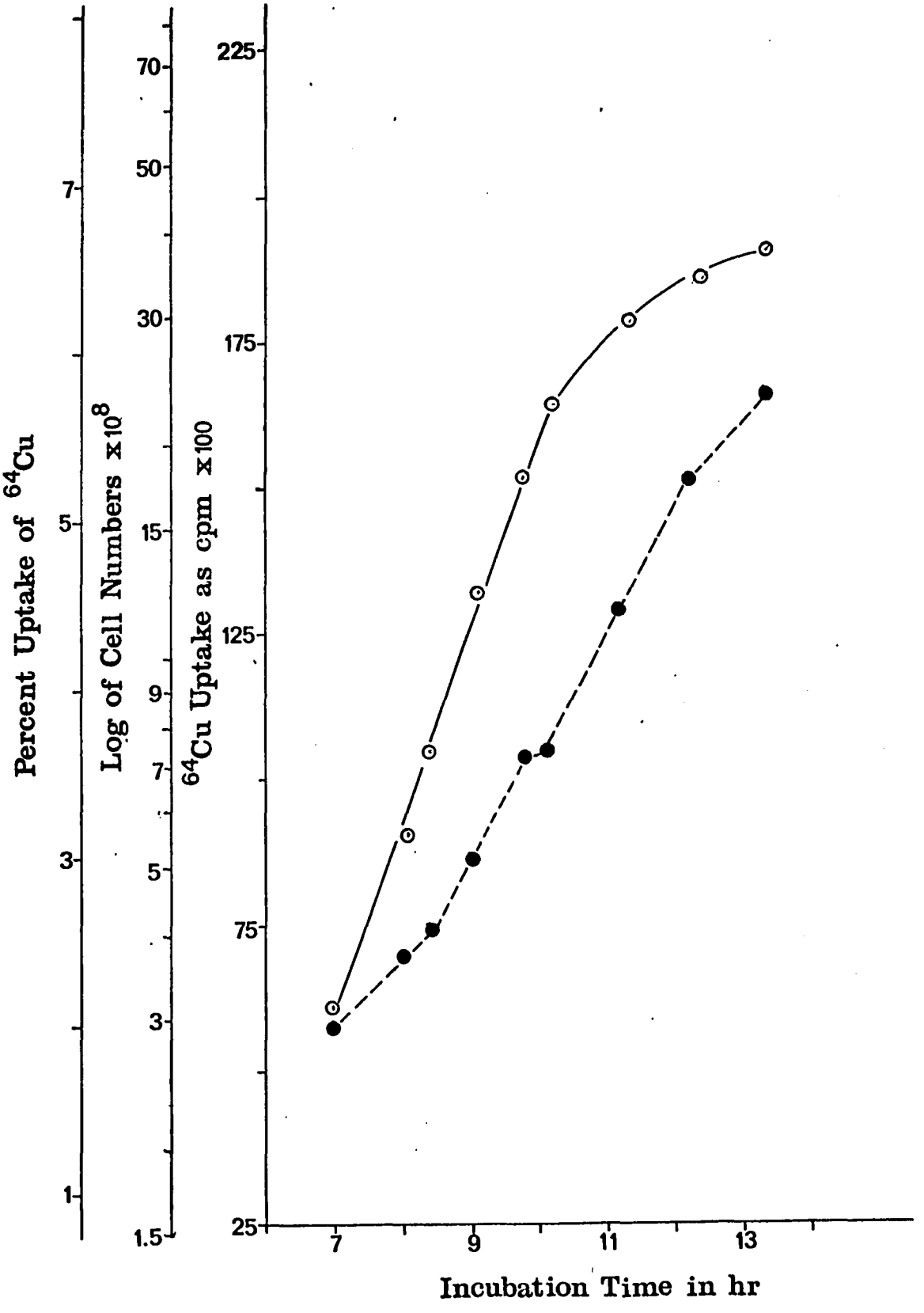

Percent Uptake of ^{64}Cu
Log of Cell Numbers x 10^8
^{64}Cu Uptake as cpm x100
Incubation Time in hr
7
5
3
1
70
50
30
15
9
7
5
3
1.5
225
175
125
75
25
7
9
11
13

Fig 32. Growth as Petroff-Hauser counts and ^{64}Cu uptake by *P. cuprodurans* at 20 C in basal medium supplemented with 5×10^{-4} M $CuSO_4$.

o——o Cell numbers

●---● ^{64}Cu uptake

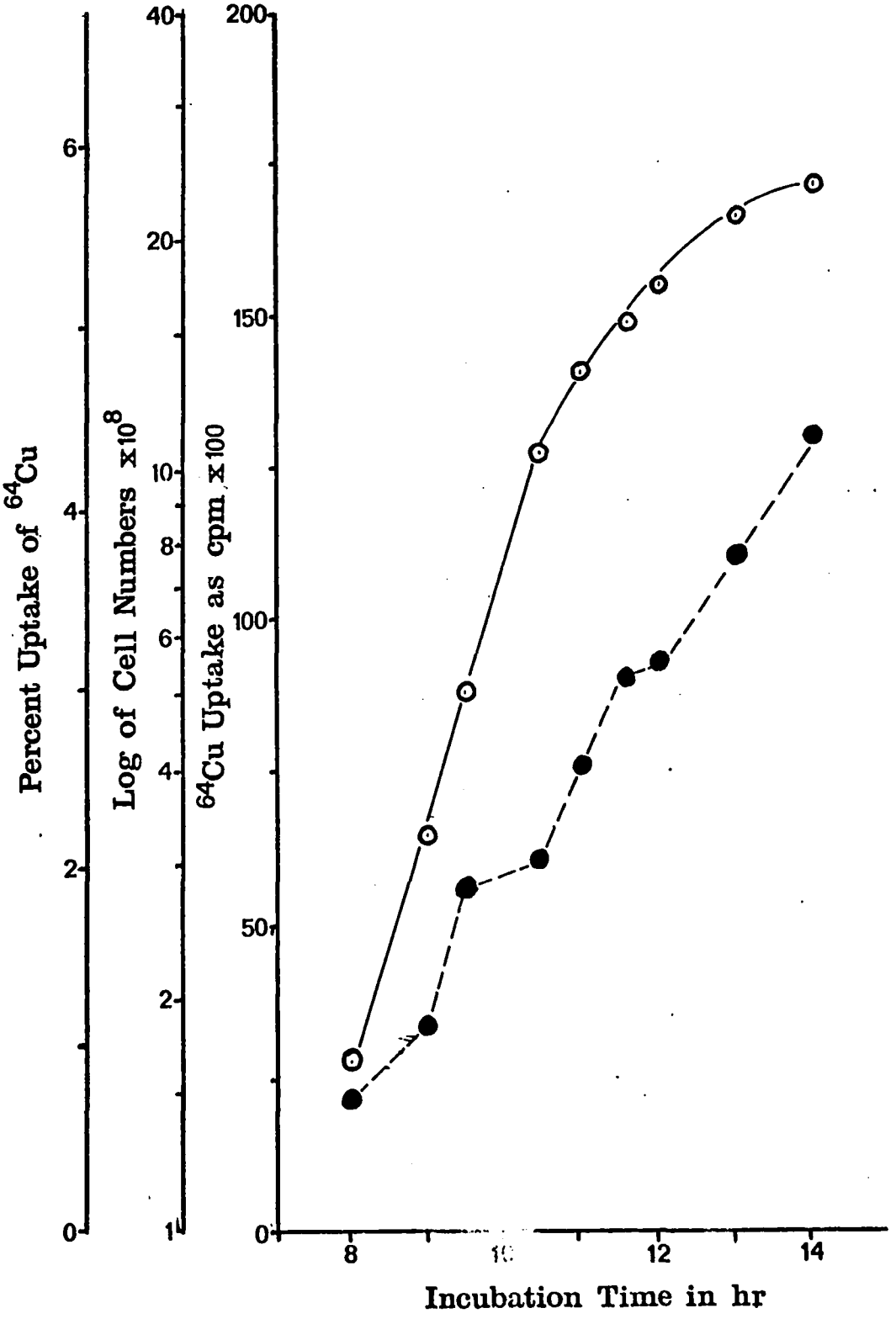

Percent Uptake of ^{64}Cu
Log of Cell Numbers x10^8
^{64}Cu Uptake as cpm x100
Incubation Time in hr
0
2
4
6
1
2
4
6
8
10
20
40
0
50
100
150
200
8
12
14

Fig 33. Growth as Petroff-Hauser counts and ^{64}Cu uptake by P. cuprodurans at 20 C in basal medium supplemented with 1×10^{-3} M $CuSO_4$.

o—o Cell numbers

●---● ^{64}Cu uptake

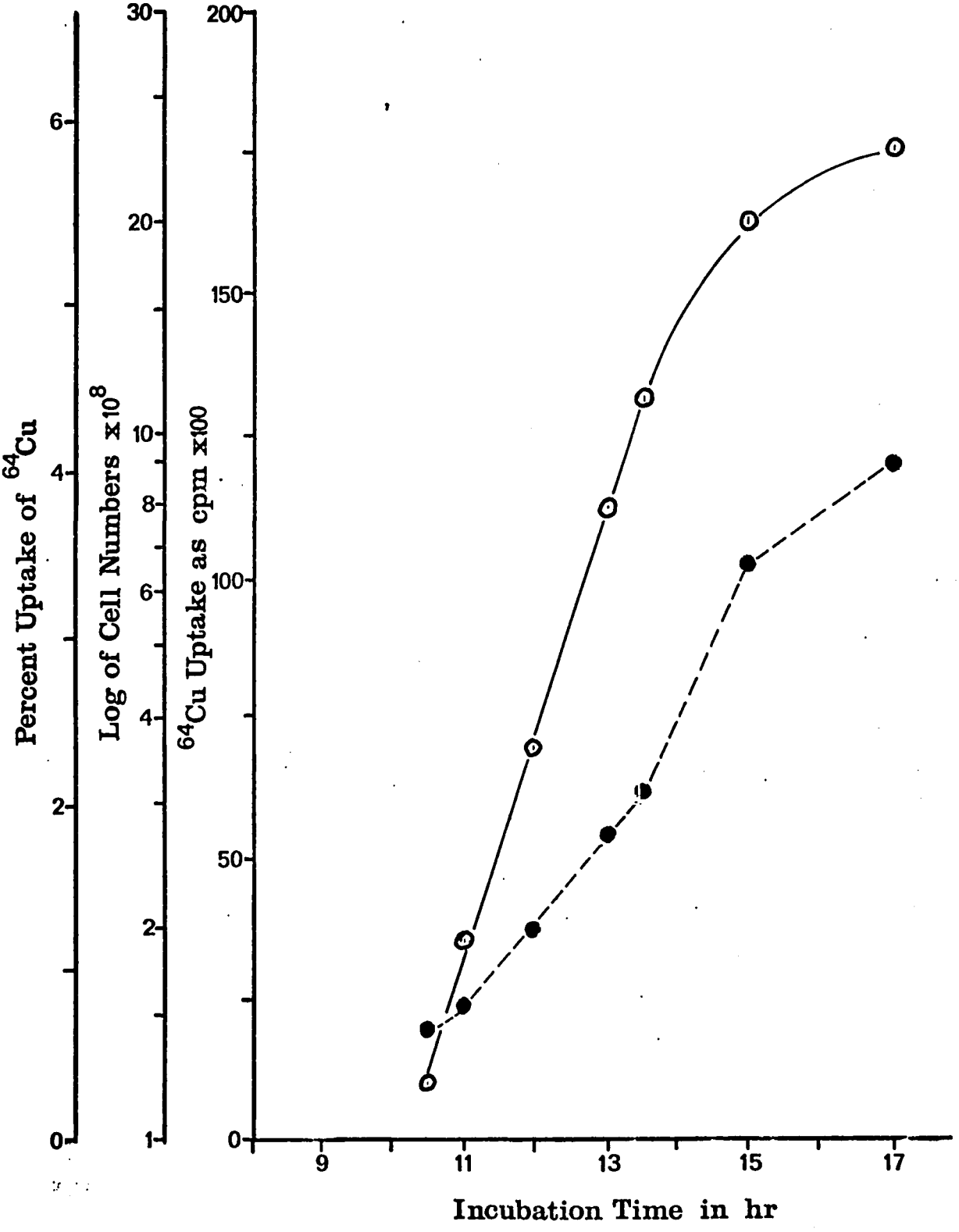

Percent Uptake of ^{64}Cu
Log of Cell Numbers x10^8
^{64}Cu Uptake as cpm x100
Incubation Time in hr
0
2
4
6
1
2
4
6
8
10
20
30
0
50
100
150
200
9
11
13
15
17

Fig 34. Effect of 2,4-dintrophenol and sodium cyanide on copper transport by P. cuprodurans at 20 C in basal medium supplemented with 1 x 10^{-4} M $CuSO_4$.

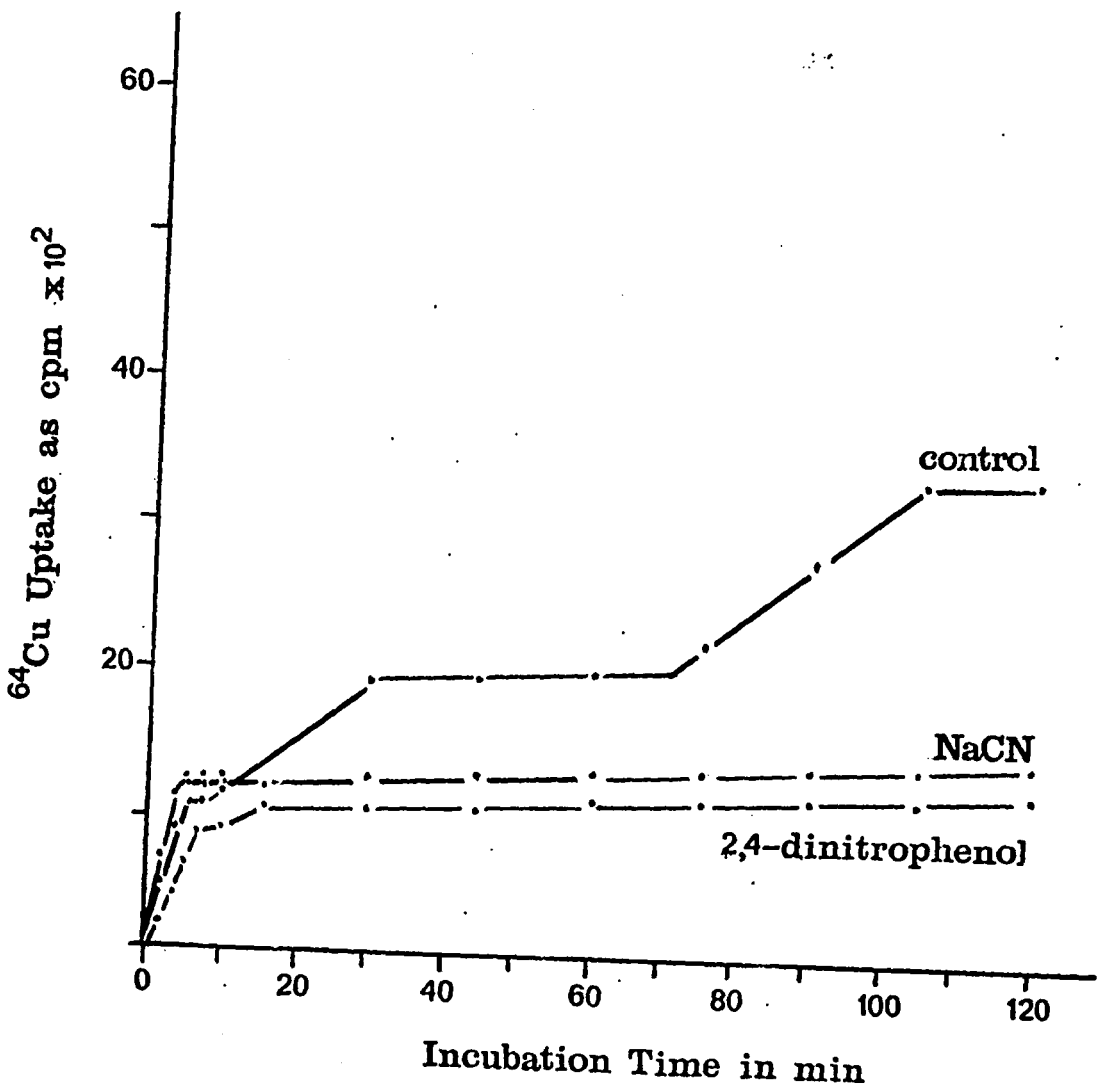

60
40
20
^{64}Cu Uptake as cpm x 10^2
control
NaCN
2,4-dinitrophenol
0
20
40
60
80
100
120
Incubation Time in min

Fig 35. Endogenous copper uptake and the effect of chloramphenicol on active transport of copper by P. cuprodurans at 20 C in basal medium supplemented with 1×10^{-4} M $CuSO_4$.

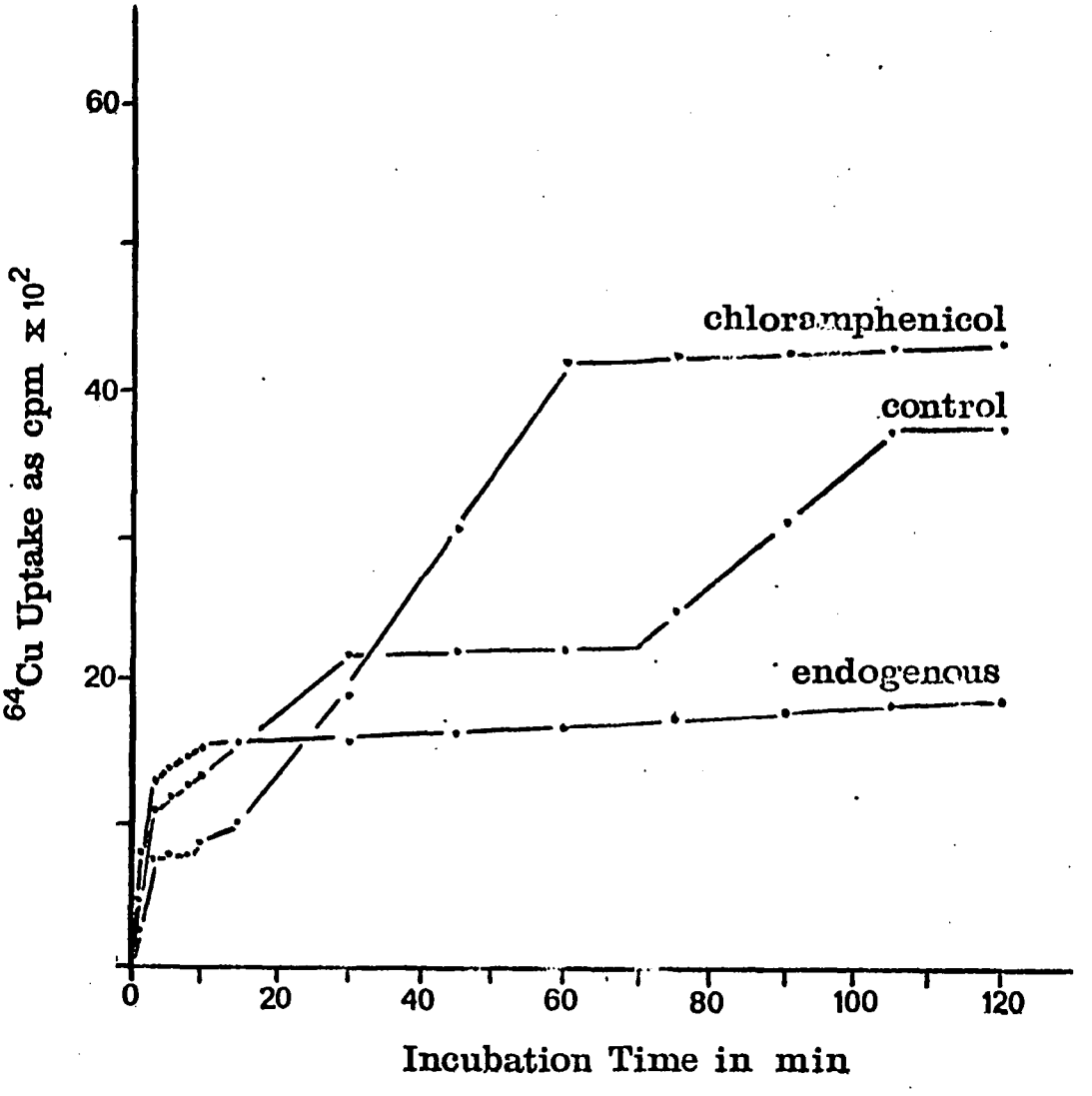

60
40
20
^{64}Cu Uptake as cpm x 10^2
chloramphenicol
control
endogenous
0
20
40
60
80
100
120
Incubation Time in min

Fig 36. Log of cation uptake vs log of extracellular cation concentration for P. cuprodurans, A. marinus, and P. vulgaris.

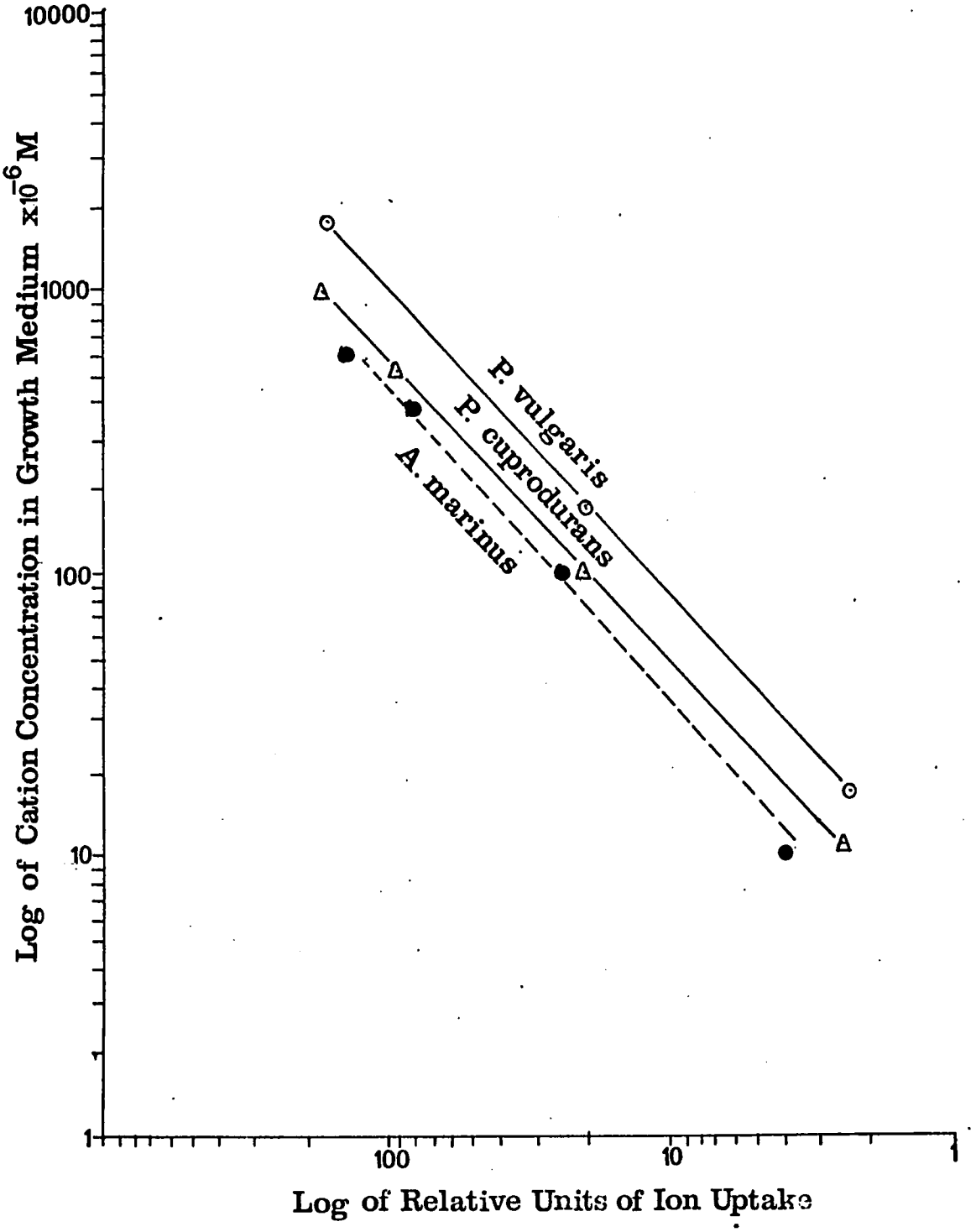

10000
1000
100
10
1
Log of Cation Concentration in Growth Medium x10⁻⁶M
P. vulgaris
P. cuproduran
A. marinus
100
10
1
Log of Relative Units of Ion Uptake

Fig 37. Log of labile and non-labile copper vs log of total copper supplement determined by anodic stripping voltammetry for filter-sterilized basal medium supplemented with copper.

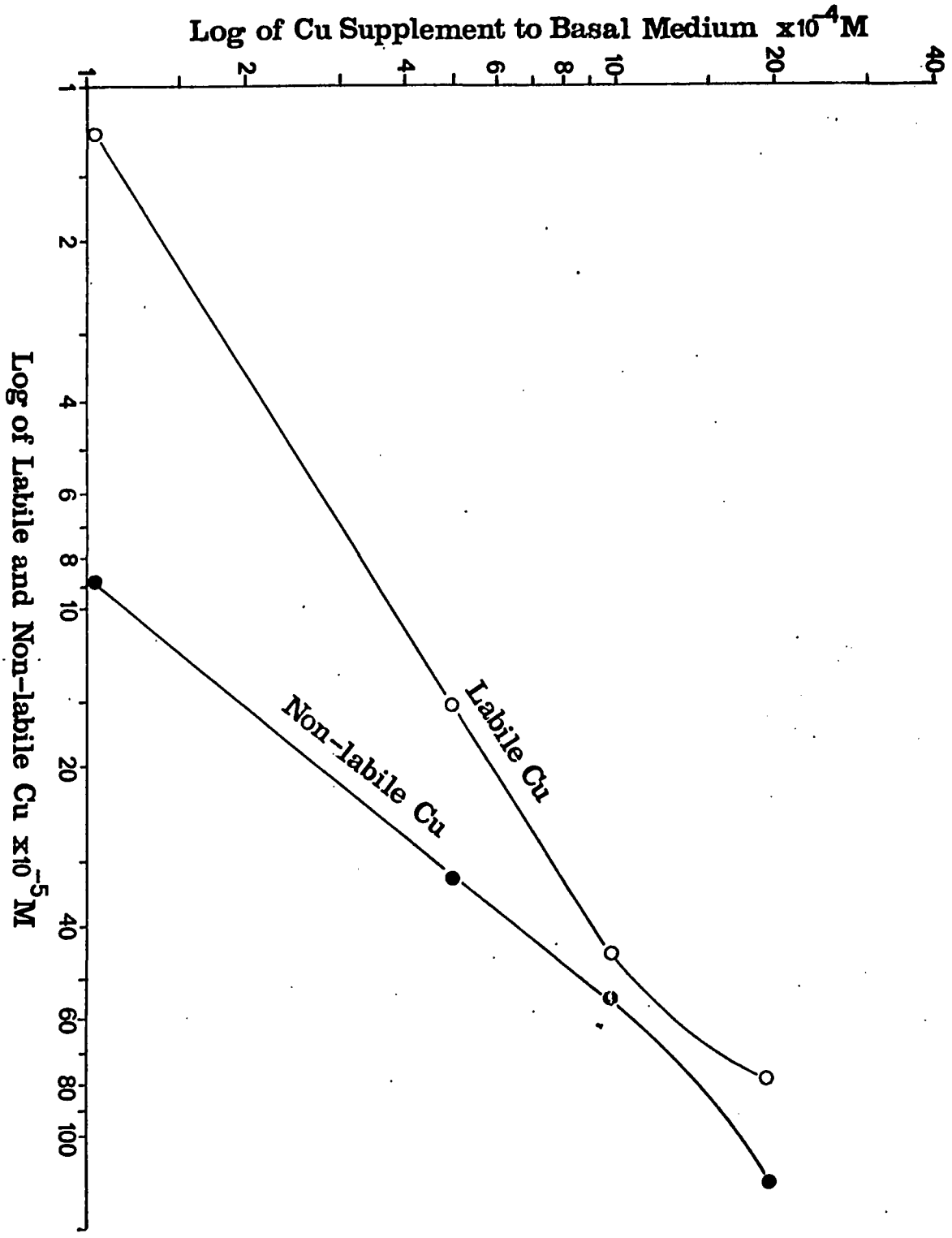
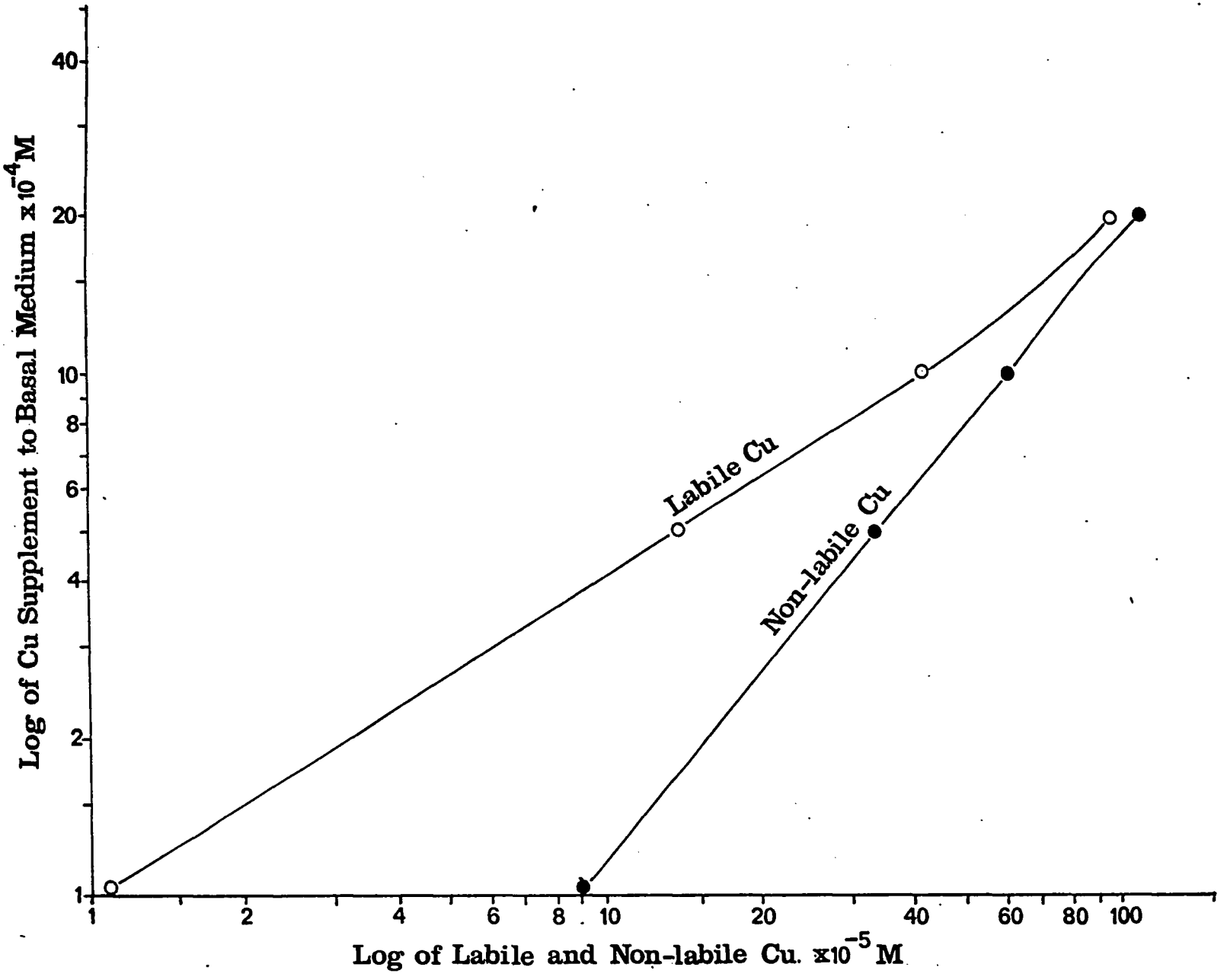

Log of Cu Supplement to Basal Medium x10⁻⁴M
1
2
4
6
8
10
20
40
Log of Labile and Non-labile Cu x10⁻⁵M
1
2
4
6
8
10
20
40
60
80
100
Labile Cu
Non-labile Cu

Fig 38. Log of labile and non-labile copper vs log of total copper supplement determined by anodic stripping voltammetry for autoclaved basal medium supplemented with copper.


Log of Cu Supplement to Basal Medium x10^{-4}M
40
20
10
8
6
4
2
1
Labile Cu
Non-labile Cu
1
2
4
6
8
10
20
40
60
80
100
Log of Labile and Non-labile Cu. x10^{-5}M