HEAVY METAL ION INTERACTION AND TRANSPORT WITH SYNTHETIC COMPLEXING AGENTS AND DETERGENT PHOSPHATE SUBSTITUTES IN AQUATIC SYSTEMS

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ABSTRACT

The chemical aspects of the copper micronutrient requirement for algae have been investigated. A reproducible copper requirement for Chlorella vulgaris and Oocystis marssonii was demonstrated. Optimal growth was observed above 40 micrograms/l for Oocystis and 30/l for Chlorella. A study of the effects of EDTA on the toxicity of copper to Chlorella showed that copper in chelated form was not toxic to these algae at concentrations up to 46 mg/l copper. When only sufficient chelating agent was present to keep the iron (III) in solution however, the toxic effects of copper were evident at 7.00 mg/l of copper.

A second aspect of the project involved the development of a simple, direct multiple standard addition method for the potentiometric analysis of copper in water with a solid-state copper ion-selective electrode. The technique is more sensitive than conventional atomic absorption analysis, though not so rapid. Measurements are made in a complexing antioxidant buffer medium containing acetate (to complex copper), fluoride (to complex iron), and formaldehyde (to provide a reducing medium).

Keywords - Copper*, algal culture*, chelating agents*, electroanalysis, potentiometry, eutrophication

3. Introduction and Objectives.

This research has had several objectives. One aspect of the research is a continuation of research involving the application of ion-selective electrodes to water analysis, which the principal investigator has been conducting since 1968. Specifically, on this project the copper electrode was used for very low level copper analysis required for copper deficiency studies with algae. A special standard addition technique was developed for use with the copper ion-selective electrode enabling low level copper analysis in natural waters and algal growth media.

A second aspect of the research involved a study of the copper micronutrient requirement for algae and copper toxicity to algae. Chemical aspects were emphasized.

A third aspect of the research had to do with the influence of chelating agents on metal ion availability to algae. Particular emphasis was put upon the influence of strong chelating agents on the availability of copper to algae both below and above the optimum levels of copper required for maximum algal growth. This kind of study is needed because of the use, and proposed use, of strong chelating agents as detergent phosphate substitutes. If these substitutes are approved for wide scale use, it must be assumed that until more adequate sewage treatment facilities are constructed throughout the nation, the phosphate substitutes will become important pollutants in many natural waters.

4. Statement of Problem.

See pages which follow.

5. Method of Investigation.

See pages which follow.

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ABSTRACT

A reproducible copper requirement for Chlorella vulgaris and Oocystis marssonii has been demonstrated by employing a precipitate-free medium deficient in EDTA. Optimal growth was observed above 40 µg/l Cu for Oocystis and 30 µg/l for Chlorella. The EDTA medium employed simplifies the interpretation of the effects of trace elements on algal growth. By varying the chelate concentration in the medium at a growth limiting concentration of copper, a dependence of the two organisms on the free cupric ion concentration was also demonstrated. An expression that allows the computation of $[Cu^{2+}]$ from a consideration of the medium macrocomponents is described. A study of the effects of EDTA on the toxicity of copper demonstrated that chelated copper showed no toxic effects at any environmentally realistic concentration (up to 46 mg/l Cu). At reduced chelate concentrations, however, toxic effects were evident. A simple, direct multiple standard addition method for the determination of trace levels of copper in natural waters using a solid-state cupric ion electrode is described. Sample pretreatment is not necessary. Measurements as low as 1 µg/l Cu are conveniently made in a 0.0500 M complexing, antioxidant acetate buffer which eliminates sample electrode interferences. A least-squares computer program for accurate electrode calibration is also described.

SECTION I

THE RELATIONSHIP BETWEEN THE CHEMISTRY OF COPPER AND ALGAL GROWTH

CHAPTER I

INTRODUCTION

The significance of trace-level substances in the environment and in living organisms is a topic which is now receiving increased attention. Much of the interest in trace substances has been generated by a growing, genuine concern over the chemical, biological, and physiological significance of "excesses" of these materials in the environment. The term "trace" is, however, a relative one. It implies a relationship to the concentrations of the macrocomponents in a given chemical system. Once the identity of the major components in a system are known, it is often possible to predict a deficiency or toxicity for the substances present at trace levels. The information may then be used to establish criteria to suggest corrective action, if necessary.

THE PROBLEM OF DETERMINING THE SIGNIFICANCE OF TRACE METALS IN NATURAL WATERS

One of the characteristics of the role played by physiologically active trace substances is their toxicity when their concentrations exceed a certain value. The role of trace substances such as trace elements in the metabolism of organisms is still not well understood. Information about the biological availability of trace elements is virtually nonexistent. Biological and chemical data from

personnel concerned with environmental quality are, however, slowly contributing to our knowledge in this area.

Water analysis commonly reveals a number of elements to be present at trace levels. A recent study has been made of trace metals in waters of the United States [1]. The minimum, maximum, and mean observed levels of dissolved trace elements as determined in this study are given in Table I along with the number of positive occurrences and the frequencies of detection for each element. Over 1500 samples were analyzed during a five-year period between 1962 and 1967. All analyses were performed using direct reading emission spectrographic procedures with preconcentration by evaporation, precipitation, or ion exchange. The persistence of the common trace elements in widely varying water types is certain evidence of their importance.

In 1962 the Public Health Service Drinking Water Standards were revised in recognition of man's changing environment and its effect on water supplies [2]. A summary of the standards for soluble trace elements is given in Table II. The standards were based upon the best and latest information available at the time of their establishment. In many instances absolute standards could not be established for several reasons.

In determining water quality requirements it is essential to recognize that there are tolerable, favorable, and

TABLE I

SUMMARY OF TRACE ELEMENTS IN WATERS OF THE UNITED STATES [1]

Elements	No. of Positive Occurrences	Frequency Of Detection, %	Observed Positive Values(µg/l) Min.	Max.	Mean
Zinc	1207	76.5	2	1183	64
Boron	1546	98.0	1	5000	101
Phosphorus	747	47.4	2	5040	120
Iron	1192	75.6	1	4600	52
Molybdenum	516	32.7	2	1500	68
Manganese	810	51.4	0.3	3230	58
Aluminum+	456	31.2	1	2760	74
Copper	1173	74.4	1	280	15
Barium	1568	99.4	2	340	43
Strontium	1571	99.6	3	5000	217

[1] 1,577 Samples (Oct. 1, 1962 - Sept. 30, 1967).

+1,464 Aluminum Analyses

TABLE II

USPHS DRINKING WATER STANDARDS FOR SOLUBLE TRACE ELEMENTS COMMONLY FOUND IN WATER

Metal	Max. Permissible Level (mg/l)
Arsenic	0.05
Barium	1.0
Cadmium	0.01
Chromium(VI)	0.05
Copper	1.0
Iron	0.3
Lead	0.05
Manganese	0.05
Silver	0.05
Zinc	5.0

essential levels of dissolved minerals as well as acute and chronic levels. The fact that species in different developmental stages may differ widely in their sensitivity to various materials must be taken into consideration. Differences in sensitivity are a function of age, sex, health, and history. The young and the unborn seem particularly sensitive. Substances in suspension as well as in solution may affect aquatic organisms directly or indirectly. The problem is further complicated by the fact the "safe level" depends upon other water quality characteristics. After substances enter the environment they may be diluted or concentrated by physical forces and may undergo chemical changes that affect their biological availability. If they are consumed by organisms they may be changed into materials that are more dangerous than the initial substances consumed. One example is methylmercury which is produced by anaerobic bacteria from inorganic mercury. At one time inorganic mercury was thought to settle safely into bottom sediments when discharged into water. However, it is now known that anaerobic bacteria methylate mercury to a soluble, and highly toxic form. Methylmercury is passed through the food chain by algae and fish, eventually reaching man. Interactions among organisms, plants, and water therefore preclude the establishment of a single set of universal criteria for water quality. The problem is further complicated

by a lack of basic information concerning the availability of nutrient and toxic substances to aquatic animal and plant life.

Water quality standards are usually based upon guidelines for human health standards. The routine surveillance of trace elements in water is concerned with those elements found primarily in solution largely because suspended matter is removed before the water is used for human consumption. Although this approach is basically sound, neither does it measure the total trace metal load in an aquatic system, nor does it suggest the suitability of the water source for purposes other than human consumption. Soluble trace elements certainly contribute to the overall water quality at their time of measurement, but it is the reservoir of suspended trace elements that determines how long the soluble trace elements may persist as conditions change. Very little is known about the distribution of trace elements associated with suspended matter. Water quality criteria are selected on the basis of the trace elements in solution. The presence of trace elements in the sediment load is ignored. The question of the availability of the various forms of the soluble trace element fraction as nutrient and toxic substances is far from answered. The effect of strong chelating agents on nutrient availability is also largely unknown.

The appearance of strong chelating agents in industrial wastes and the possible use of a strong chelating agent nitrilotriacetic acid (NTA) as a phosphate substitute in detergents has created considerable interest in synthetic chelating agents and their effect on water quality. Because of its concern with the effects of NTA in the aquatic environment, the Federal Government has banned the use of NTA until completion of further testing. If NTA had proven safe, an estimated 600 million pounds would have been used annually in detergents by 1973 [3]. Other chelating agents (EDTA, Deselex) are now finding limited use as detergent builders.

Essentially nothing is known about the relationship between strong chelates and trace element availability in aquatic ecosystems. The introduction of large quantities of strong chelate may seriously alter the distribution of the naturally occurring trace elements. They may solubilize metals normally found associated with suspended matter and enhance assimilation by aquatic organisms. The biodegradability and treatability of strong chelates in conventional waste treatment processes is at this time questionable and the accumulation of such substances in surface waters is a definite possibility.

THE DETERMINATION OF TRACE ELEMENTS IN NATURAL WATERS

Natural water is a generally more dilute solution than

most solution chemists are accustomed to working with. Although the general principles of solution chemistry are applicable to natural waters, factors other than the chemistry of a body of water contribute to its chemical composition. Natural water chemistry is the result of an enormously complex series of chemical, physical, and biological interactions. It therefore follows that some understanding of these processes is needed before one can act intelligently toward performing water analyses. A complete analysis is a function of all the physical, biological, and chemical factors that have contributed to the sample taken for analysis.

Sampling is a vital part of determining water composition and is perhaps the major source of error in the whole process of water analysis. Sampling problems are not well enough recognized and some emphasis upon them seems desirable. Natural waters are commonly poorly mixed. Thermal stratification and the associated changes in water composition are frequently observed effects. Trace elements are often influenced by the presence of oxidizing or reducing conditions, with the reduced species usually increasing in concentration with depth below the surface where bacterial action reduces the level of dissolved oxygen. Trace elements utilized by life forms in water are often considerably affected. Single samples can only be assumed to represent the

spot within the body of water from which they came at the time that they were taken.

The extent to which a small sample may be reliably considered representative of a large volume of material depends very much upon sampling technique. A sample composed of several small samples taken from systematically distributed points in a system is undoubtedly more representative of the whole system than a sample collected from a single point. Clearly the more portions taken, the more nearly the sample represents the original. Yet while one of the goals of water analysis may be to provide information about the composition of a whole body of water, the water within a certain region may be of greater interest. Information about the variation in composition from place to place or the variation in composition with time may be required. Choosing and designing a sampling system that will accomplish one or all of these goals requires considerable thought. In short, the purpose underlying an analytical study determines the sampling procedures that will be required.

The examination of water for trace elements on site is another important consideration in water analysis. Certain properties of water, especially pH, are intimately related to the environment of the body of water with which they are associated. They are likely to be altered by

storage. Under these circumstances a meaningful value can be obtained only in the field. Changes in parameters such as pH bring about other changes within the sample taken for analysis. For most waters, gas dependent or related equilibria are particularly sensitive to pH changes caused by sample agitation. The precipitation of metal carbonates and hydroxides from CO_2 containing samples is a common problem in sampling natural waters for trace elements. Quite often the very insoluble metal carbonates and hydroxides act as scavengers for other trace elements, coprecipitating trace elements even when their solubilities are not exceeded. Determinations which can be made by potentiometric methods have been mentioned in literature as especially adaptable to field work [4]. On-site sensors for continuous monitoring are also possible.

Suspended materials in natural waters may occur in a wide range of particle sizes and in light of the earlier discussion on the availibility of suspended vs. soluble trace metals to biological systems it is frequently necessary to distinguish between soluble and particulate species in water. As the wide size range of particulate matter may suggest, an exact definition of dissolved vs. particulate is not practical. The distinction is made using an operational rather than an exact definition of the two states. Where total and dissolved concentrations are to

be determined, the dissolved concentration is taken to be the amount present after filtration through a membrane filter with a nominal pore size of 0.45 microns. This procedure does not give an exact separation of particles of larger and smaller diameter than the nominal pore size due to entrapment of smaller colloidal species as well as the passage of larger species because of heterogeneity of pore size in the filter. Obviously filtration should be carried out as soon as possible after sample collection to minimize changes from one state to another.

Recent advances in analytical instrumentation have continued to decrease the minimum concentrations detectable in a given medium. Consequently, concentration levels formerly designated "undetectable" now fall well within the detection limits for modern instrumental techniques. Interest in the subject of trace elements has become intimately related to the advances in instrumental methods of analysis.

An adequate analytical procedure is the first requirement for the study of the effects of trace substances in any system. The trace elements commonly found in natural waters are found at concentrations less than 100 µg/l. Yet the determination of constituents occurring to the extent of 100 µg/l or less is as a rule difficult by the usual methods of trace analysis without invoking some method of

sample pretreatment. Pretreatment is necessary to eliminate interfering substances as well as to bring the element studied into the proper concentration range for the technique chosen.

The most general methods of trace elemental analysis that have appeared in the literature are distinguished by their sensitivity and accuracy for a wide variety of samples. The following techniques are commonly used for trace metal determinations.

(1) UV - Visible Spectrophotometry
(2) Emission Spectrography
(3) Polarography and Voltammetry
(4) Anodic Stripping Voltammetry
(5) Neutron Activation Analysis
(6) Atomic Absorption Analysis

No individual technique is universally applicable to all samples types and concentration ranges, but each is applicable to a number of elements. The choice of any one method, of course, depends upon the nature of the sample matrix as well as the sensitivity and accuracy required.

Probably the most widely accepted technique for the determination of metals is atomic absorption spectrophotometry. The technique is quite selective, but again except for a few metal ions such as Cd and Zn, conventional flame atomic absorption cannot be successfully employed below

the 100 µg/l level. Sample preconcentration must be employed at precisely the level where contamination from reagents and even laboratory water systems may have most serious consequences. Reagent contamination is difficult to detect when a sample preconcentration step is necessary. Low levels of contamination must be inferred from a series of standards and blanks run through the same preconcentration procedure as the samples taken for analysis. Sample preconcentration at low levels may also modify the physical and chemical characteristics of the species under consideration.

Since trace metals in the aquatic environment are frequently in the low µg/l range (10^{-7} to 10^{-9} M), one would hope to find a suitable analytical procedure that could be employed successfully for direct determinations on-site. Yet strangely enough, none of the commonly used trace analysis techniques is readily adaptable to on-site measurements. The spectrophotometric techniques can be used on site, but they lack the necessary sensitivity for measurements to be made directly. Although neutron activation offers the high sensitivity required, its use is restricted by the requirement for available reactor facilities. Anodic stripping voltammetry is also quite sensitive, but deposition times are quite dependent upon the sample matrix. Methods for trace metal analysis in natural waters capable

of performing *in situ* analysis on-site simply do not exist.

THE CONTROL OF ALGAL POPULATIONS

A large increase in population and the constant reuse of water for industrial and domestic use in this country have caused a number of problems with nuisance organisms in surface waters. Present methods of waste disposal have become inadequate in some instances and are intensifying the problem. As population and industrial demands increase, ground water supplies have not been able to keep pace. More and more cities and industrialized areas are turning to lakes, streams, and reservoirs for potable water supplies. While ground waters are relatively free of nuisance organisms, surface waters may contain organisms that complicate the development of suitable surface water supplies. Many organisms become problems in surface water supplies when they become overabundant, but among the most prevalent of the nuisance organisms are the algae. Algae are normally desirable inhabitants encountered in every water supply exposed to sunlight. Excessive growths of algae, however, commonly cause taste and odor problems, filter clogging, and unsightly surface mats. Some forms of the blue-green algae are capable of producing substances that are highly toxic to other forms of aquatic life, higher animals, and even man himself. An excessive growth of algae followed by

death and decay of the biomass consumes oxygen in water resulting in the condition called "eutrophication".

The numbers and kinds of algae found in surface waters depend upon environmental conditions. Industrial and domestic sewage wastes have increased the productivity of these waters by supplying nutrient materials for the growth of algae and other microorganisms. With the continued discharge of wastes and the associated increase in the productivity of surface waters, large "blooms" of algae have become common-place.

A number of materials have been investigated as methods of control or elimination of growths of algae, but copper sulfate is probably the most widely used algal control agent in the United States. Although it has been used for this purpose since the turn of the century, the details of its action in various types of water are still not fully known. The popularity of $CuSO_4$ is undoubtedly due to its low cost and high effectiveness. It is toxic to many algae at concentrations of 1 mg/l Cu or less while it is ordinarily nonlethal to fish at these strengths. In highly alkaline waters, however, it precipitates rapidly as $CuCO_3$ and in such instances is only effective for a short period of time following application. Recent work has also shown that organic matter present in bottom sediments as well as the nature of clay materials present in colloidal suspen-

sion determine the amount of copper removed from solution by adsorption [5].

Algae are not all equally susceptible to copper and this factor is often neglected in determining the concentration applied. The dosage is usually determined by the alkalinity of the water supply treated. If the bicarbonate alkalinity is less than 50 mg/l, a rate of ca. 0.9 lb/acre/ft is applied. Above 50 mg/l the dosage is 5 lbs/acre. In waters with high alkalinity, penetration depth is relatively unimportant since the rapid precipitation of the applied copper makes it ineffective below the surface. The application techniques for $CuSO_4$ are varied, but usually involves dragging a bag of $CuSO_4 \cdot 5H_2O$ behind a boat. A zig-zag pattern is followed from one side of the body of water to the other until it is felt suitable application has been made.

The obviously crude application techniques and the general lack of information concerning the fate of copper in surface waters indicate that analysis of the water in each individual application must be made to establish whether optimum control rates have been reached and to prevent further application when already dangerous levels of copper are present. Copper sulfate has been observed to sink immediately to the bottom when applied in crystalline form where it is rapidly adsorbed by bottom muds [6]. After combining with organic substances in these muds over many

years of use, copper concentrations may be so high in bottom sediments that sudden changes in water quality parameters or rapid mixing may dissolve toxic quantities of copper from bottom sediments. Contact of the sediment with pollutant chelating agents could solubilize dangerous levels of copper. The need for continued monitoring of copper levels in such cases is also obvious.

STATEMENT OF THE PURPOSE OF THE STUDY

In the context of the previously presented arguments, it became the purpose of this study (1) to investigate the relationship between algal growth and trace levels of cupric ion at nutrient and toxic concentrations for two representative organisms of the green algae, (2) to carry out these investigations in a well-defined laboratory medium where the concentration of soluble copper species could be definitely correlated to algal growth, (3) to study the influence of strong chelates on the availability of soluble copper to algal cells, (4) to investigate techniques of removing trace levels of contaminant copper from reagents used in the preparation of biological media, (5) to demonstrate the suitability of the cupric ion-selective electrode to the direct, on-site measurement of copper levels in natural waters, and (6) to investigate the utility of the cupric ion electrode as an aid in the preparation of metal-free reagents.

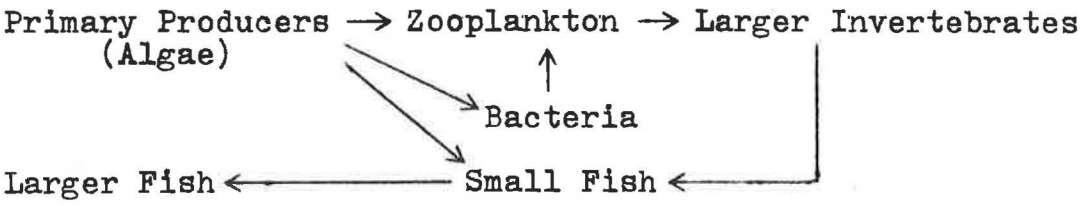
CHAPTER II

THE ROLE OF ALGAE IN SURFACE WATERS

Because there exists a variety of aquatic environments that often differ markedly in chemical and physical properties, it is not surprising that the many organisms found within them also differ widely. Among these organisms is a group known as the algae which often are the predominant microorganisms in surface waters. The algae occupy a unique position among the organisms of the aquatic world because they are able to utilize light energy in the process of reducing CO_2 to the oxidation state of cellular carbon. As a result, the algae are an important link in the food chain and are often called the primary producers in aquatic systems. Because the algae are the ultimate source of both cellular carbon and chemical energy for other organisms in aquatic systems, the biological activity of an aquatic ecosystem is very much dependent upon the rate of primary production. The biological activity within the ecosystem is in turn affected by the physical environment.

The energy found as organic matter in the primary producers reaches the later stages of the food chain in several ways. Some of the organic matter excreted in soluble form or from decaying algal cells serves as nutrient material for the growth of heterotrophic bacteria. The algae as well as the bacteria may also be consumed directly and in

this respect are a major source of food for the zooplankton and young fish. A simplified food chain for a surface aquatic zone can be represented as:



The principle importance of algae is, therefore, their ability to give rise to large quantities of organic matter in aquatic systems.

In the process of producing cellular carbon with the energy derived from photosynthetic processes, the algae acquire reducing power by using water as an electron donor. Oxygen is also produced from water as a by-product of the photosynthetic light reactions during daylight hours. As respiration is carried out by the nonphotosynthetic organisms, carbon dioxide is released and oxygen is consumed from the environment. For this reason, the levels of oxygen and carbon dioxide in aquatic environments depend to a large degree upon the relative rates of photosynthesis and respiration being carried on collectively by the algae, bacteria, and other organisms in the immediate area.

The algae make possible important chemical changes through the release of oxygen and the consumption of carbon dioxide during daylight hours. Oxygen is made available for respiration carried on by all types of organisms from

fish to the smallest bacteria. The algae constitute the primary source for the continuous daytime renewal of essential oxygen in lakes and reservoirs. Oxygen release by algae and oxygen uptake by aeration are the two primary sources of oxygen in flowing surface waters. As algae remove carbon dioxide from their surroundings they cause an alteration of the pH in surface waters. The pH in such a system will increase during daylight hours as a result of the following reaction:

$$HCO_3^- + H_2O \xrightarrow{h\nu} CH_2O + O_2 + OH^- \qquad (I\text{-}1)$$

At night when photosynthetic activity is at a minimum, the reaction is reversed and the pH decreases.

Obviously, lakes and rivers normally contain many genera of planktonic and benthic algae. Limited numbers of algae are not troublesome, but rather a necessary link in the aquatic food chain. Algae frequently do become a problem in surface waters because of their capacity for rather prolific growth under certain conditions. Algal abundance varies with the degree of enrichment with algal nutrients, the presence of toxic substances, temperature, turbidity, and other parameters. Under conditions where the proper nutrients are available, cell counts as high as 171,000 per ml have been recorded [7]. Such large quantities of algal material are the usual cause of difficulties (eutrophication) in surface waters. The simple solution to

the problems caused by excessive growths of algae would seem to be to have the proper kind of algae present in the appropriate amounts.

The abundance and types of algal flora in natural waters are a function of levels and balances in available nutrients. Yet all of the nutrients essential for optimal algal growth are not known. Quantitative data are nearly nonexistent. Some elements known to be important are nitrogen, magnesium, calcium, iron, silicon (for diatoms), sulfate, oxygen, and carbon. In many systems, the abundance of nitrogen and phosphorus determines algal production if other conditions are favorable. The algae also require trace levels of vitamins and a host of minor trace elements. Not only are the various nutrients important, but their relative abundances can be of even greater importance. Limited studies indicate that algae have phosphorus requirements differing several-fold, usually somewhere between 0.01 and 0.05 mg/l P. At these levels algal blooms may be expected when all other required nutrients are available.

The nitrogen-phosphorus ratio is also of importance. The ratio varies with water type, season, temperature, and geological formation and may range from 1:1 or 2:1 to 100:1. In natural waters the ratio is usually near 10:1 under "normal" conditions. Another important factor in plant growth is the availability of carbon dioxide and HCO_3^-

in a particular environment [8].

Under conditions where nitrogen-phosphorus ratios are optimal, the trace elements required as algal nutrients may become growth limiting. This may be particularly true in waters of high alkalinity where insoluble metal hydroxides and carbonates are the major metal containing species. Precipitated trace elements are generally not available as algal nutrients. Copper, zinc, and iron availability are affected.

CHAPTER III

REVIEW OF THE LITERATURE CONCERNING THE RELATIONSHIP BETWEEN TRACE METALS, STRONG CHELATES AND ALGAL GROWTH

The trace element requirement of the algae has become an important subject. The recognition that natural chelating agents (generally fulvic acids, often called the "yellow" or "humic" acids) as well as synthetic chelating agents from domestic and industrial wastes are present in surface waters has done much to clarify the relationship between nutrient and toxic levels of the trace elements and algal growth. The participation of many trace elements in metabolic functions has been demonstrated, but unfortunately, often in a manner where a quantitative estimate for a particular element cannot be made. It is sometimes difficult to decide whether the effects observed are due to changes in trace element concentrations or to other changes within the culturing system. An attempt to integrate information from widely divergent fields of study is made here, but only a brief summary of the more important publications on this subject can be given.

TRACE ELEMENT DEFICIENCY STUDIES

Research in trace element nutrition is frequently confused by the fact that the lack of any one of several trace metals may produce very similar symptoms, usually retarded

growth. Initial attempts at studying trace element deficiencies in algae were made in simple media sometimes without adequate control of experimental variables. Early work was further hampered by a lack of materials of suitable purity for use in the preparation of nutrient media. Contamination from culture containers and reagents has undoubtedly been the most serious problem in both laboratory and field methods of investigation of trace metal limiting systems. Recent improvements in reagents and purification procedures as well as analytical methods for checking contamination should greatly aid these studies in future years.

Of all the trace metals, copper is probably one of the most extensively studied yet little is known about the algal micronutrient requirement for it or the availability of the various forms of copper found in natural waters to algal cells. The literature concerning the algal micronutrient requirement for copper is limited to a single study in one paper [9]. The effects of the other required trace elements have been studied to a similar extent.

MOLYBDENUM

Molybdenum is among the trace elements essential for plant growth and has been demonstrated to be involved in nitrogen fixation and nitrate reduction. Anabena has been shown to require molybdenum when nitrate or nitrogen gas is

used as a nitrogen source, but not when ammonia is used [10]. The necessity of molybdenum for all groups of algae has yet to be demonstrated. In one of two of the most meaningful papers concerning trace metal requirements for the algae, Walker demonstrated that Chlorella pyrenoidosa required molybdenum when using nitrate, but not when ammonium ion or urea was used as a nitrogen source [9]. Molybdenum is a well-known cofactor of nitrate reductase where molybdenum appears to undergo cyclic valence changes between Mo(V) and Mo(VI) [11]. Goldman, suspecting that the very low concentrations of molybdenum found in natural waters might be of biological importance, found that the phytoplankton community in Castle Lake in Northern California responded to the addition of 50 µg/l Mo as sodium molybdate [12]. The distribution of molybdenum in the lake was followed colorimetrically. Molybdenum was found to remain in solution for over a year, through two periods of lake turnover.

VANADIUM

Vanadium has been shown by Arnon and Wessels to increase the dry weight of Scenedesmus obliquus but the omission of the element from the culture medium caused only a slight decrease in chlorophyll content per cell [13]. Vanadium could not be replaced by molybdenum or a variety of other elements. The response of laboratory cultures was

greatest at 20 µg/l V. The vanadium requirement appeared to be a thousand times greater than that for molybdenum. Goldman, in attempting to substitute vanadium for the molybdenum deficiency in Castle Lake, was not able to achieve any change in the phytoplankton community as a result of vanadium addition. Warburg found that vanadium stimulated CO_2 uptake but only at low light intensities [14]. Only V(V) proved effective as a source of the element. The necessity for vanadium in media prepared for algal growth seems to merit further investigation.

BORON

Boron has been reported necessary for the growth of Nostoc muscorum with the minimum amount of boron required for maximum growth about 9×10^{-6} M [15,16]. Contradictory conclusions have been drawn about the necessity of boron for Chlorella. Early work carried out in quartz containers failed to demonstrate any boron deficiency, but McIlrath and Skok found that boron-free cultures of Chlorella vulgaris increased in cell number when boron was added. The optimum concentration was 0.5 mg/l B [17]. Bowen and co-workers in a later study found that boron did not stimulate growth significantly at any level between 10 µg/l and 100 mg/l [18]. Fifty mg/l B was tolerated without affecting the growth rate. Wetzel, however, found that in Borax Lake, California where

the natural level of boron ranged from 440 to 850 mg/l, some increase in carbon assimilation could be obtained with boron additions of 50 and 100 mg/l [19]. Goldman later pointed out that the stimulation may have resulted from trace impurities or pH changes induced when large amounts of the boron-containing reagent were added [20]. Carefully controlled studies under conditions where boron containing glassware are avoided will be needed to establish the necessity of this element.

MANGANESE

Manganese has been demonstrated to be necessary for the growth of Chlorella by several workers [9,21,22]. Walker found 10^{-7} M to be sufficient for autotrophic growth of Chlorella in an EDTA containing medium [9], but in later work found that the Mn requirement under conditions of photoheterotrophic growth in the presence of EDTA was greatly enhanced. In the absence of EDTA, 2.5 µg Mn were required per gram of dried heterotrophically grown cells. It should be pointed out that the formation of glucose (or urea) metal ion complexes could have also enhanced the Mn requirement in absence of EDTA, so that the quantitative Mn requirement given above may apply only to the medium investigated. Later work concluded that around 10^{-7} M was required for autotrophic growth, although heterotrophic cultures showed deficiency symptoms

only when the concentration of Mn fell below 10^{-10} M [23]. Anacystis nidulans was apparently shown to give normal growth in the absence of added Mn [24].

ZINC

Zinc at concentrations of 10 µg/l to 100 µg/l has been shown to be required by several algae in an EDTA medium. Walker found that in a glucose-nitrate or urea-salts medium void of EDTA, zinc concentrations below 100 µg/l caused a marked decrease in growth for Chlorella pyrenoidosa [21]. A higher rate of growth was obtained in the urea-containing medium, but the point at which less than optimal growth was observed remained essentially the same. Photoheterotrophic growth in the presence of EDTA greatly increased the zinc requirement. Price and Vallee were able to show a zinc requirement for Euglena gracilis grown heterotrophically in a medium containing high concentrations of ammonium glutamate, sucrose, and malic acid [25]. Cell yields were measured by a turbidimetric procedure and trace metal salts were supplied as ultrapure salts. The macrocomponents of the medium were purified by extraction or ion exchange. The rate of growth of the organism was independent of the concentration of zinc in the culture medium from 10^{-6} to 3×10^{-5} M Zn. The growth of the organism between 0 and 15 µg/l Zn was a linear function of the concentration of the element.

COBALT

Cobalt has been shown to be required for optimal growth of a number of algae and is generally replaceable by vitamin B_{12}. The element is a known constituent of the vitamin. Holm-Hansen and co-workers have shown that cobalt is necessary for optimal growth of several blue-green algae [26]. Benoit observed that only 2 to 13 per cent of the cobalt present in pond water was tied up as the vitamin [27]. Goldman has found that eight out of ten lakes in New Zealand were deficient in cobalt and addition of the element increased photosynthetic carbon fixation [28]. In Castle Lake, where a high natural concentration of cobalt exists, the addition of 5 µg/l Co(II) proved inhibiting to the phytoplankton community.

IRON

The concentration of iron required for optimal growth of the algae is open to some question. Walker found that Chlorella pyrenoidosa grown photoheterotrophically in a glucose containing medium required approximately 1 mg/l Fe regardless of whether the iron was supplied as $Fe(CN)_6^{-3}$ or as the Fe(II)-EDTA complex [21]. Iron supplied as $FeSO_4$ was found to be less efficient as an iron source in the absence of EDTA with availability decreasing at higher pH values. The pH dependence is probably due to the formation of

insoluble Fe(III) hydroxide. In the presence of EDTA, $Fe(CN)_6^{-3}$ and $FeSO_4$ were found to be equally effective as iron sources. Other workers have reported that drastically different concentrations were required [29,30] for the same organism.

COPPER

The requirement of the algae for copper has been reported only once in the literature, although it is known to be involved in photosynthetic processes. Walker reported in 1953 that copper was essential for the growth of Chlorella pyrenoidosa grown photoheterotrophically and that copper concentrations less than 30 µg/l gave less than optimal growth of the organism [9]. Attempts to demonstrate a copper deficiency with autotrophically grown cells failed, presumably due to copper in either the reagents used to prepare the medium or in the CO_2-AIR mixture used to aerate the cultures. It should be noted, however, that a similar failure to show copper deficiency in Euglena gracilis occurred with cells grown heterotrophically [25]. Both studies were conducted in Pyrex containers. It is this writer's experience that even carefully cleaned Pyrex containers cannot be used for copper deficiency studies in the low µg/l range (see Chapter VII). Copper is particularly difficult to remove from distilled water. In one study, Nicholas found 400 µg/l Cu in water

removed from a tin-lined copper still after triple redistillation in Pyrex [31].

COPPER TOXICITY STUDIES

Because of the widespread interest in the use of copper as an algicide in natural waters, the toxicity of copper to the algae has been the subject of a number of studies. However, the availability and toxicity of the various forms of copper remains obscure.

The growth of Chlorella pyrenoidosa has been studied in a laboratory medium containing varying levels of copper [32]. A medium containing 250 mg/l EDTA and 3 mg/l citric acid was modified to exclude the two chelates since no influence of copper could be demonstrated at copper concentrations found in nature. Walker, however, had shown earlier that copper was a required nutrient below approximately 30 µg/l Cu [9]. Neither the source of the reagents used to prepare the culture medium nor a reagent cleanup procedure were mentioned. The medium without the chelating agents contained $Fe(OH)_3$ which was held in suspension by a continuous air flow through the cultures. The $Fe(OH)_3$ bound copper so tightly, that about 50 µg/l Cu was necessary to depress growth to the same degree in high iron containing media as 1 µg/l Cu in a growth medium containing 6 µg/l Fe. Additions of 1 and 5 µg/l Cu to the non-chelating medium at pH 8 were found to extend the log

phase to 24 and 48 hours, respectively, but the same rate of growth as in the absence of added copper was sustained in the log phase. The authors concluded that "it was possible for the culture to counteract the influence of copper after some time". The extention of the log phase is, however, normal when the concentration of a medium component is changed, and in light of previous studies, and data to be presented in this study, it is unlikely that copper at these concentrations is toxic to Chlorella. A similar experiment with the diatom Nitzschia palea showed that copper at a concentration of 12.5 µg/l and an initial cell concentration of 10^7 organisms/l inhibited growth for four days, but copper additions of 3.75 µg/l and 6.25 µg/l were essentially without effect.

Hassal, in studying the effects of copper on the respiration of Chlorella vulgaris, found that high concentrations of copper caused no respiratory inhibition for several hours in a pH 6.0, 10^{-3} M phosphate buffer [33]. If the cultures were shaken continuously in respirometer flasks, 0.1 M copper sulfate was not inhibitory for the short 7-20 hour periods of exposure. When shaking was stopped, however, concentrations of 2.0×10^{-4} M Cu decreased oxygen uptake by approximately a factor of four over a period of four hours. The decreased respiration was not closely related to the amount of copper applied or to the amount present in the cells.

The search for new and more efficient algicides has

been the subject of a number of papers. Palmer and Maloney tested the effects of 76 potential algicides on algal cultures grown in laboratory media [34]. Six representative cultures of algae were selected for their ability to produce rapid, uniform growth under laboratory conditions. During 21 day period of observation, the cell density in each flask was compared to that in control flasks. Copper sulfate proved to prevent growth or to greatly reduce growth in all six of the algae tested at a concentration of 2 mg/l $CuSO_4$(anhyd.). The medium used to test the effects of these materials contained 3 mg/l citric acid in addition to 3 mg/l ferric citrate at a relatively high pH. It is interesting to note that $Na_2CuEDTA$ applied at the rate of 2 mg/l caused no change in the rate of growth in these experiments.

It was concluded in a recent study of the effects of 74 potential algicidal materials on mat producing blue-green algae that copper is still the most suitable algicide for controlling or suppressing the growth of these alage [35].

Hassal has reported that copper is highly toxic under anaerobic conditions, but seldom reduces respiration for many hours at much higher concentrations in aereated cultures [36]. This difference in toxicity cannot be explained in terms of increased uptake. Comparison of copper uptake by dead and living cells show that dead cells absorb copper rapidly, but the total absorbed is the same as when copper kills the cells

by prolonged anaerobic contact. Two-thirds of the copper absorbed by living cells is retained after death, resisting washing with K_2SO_4 or distilled H_2O. In a later study, Hassal noted that Chlorella vulgaris released K^+ upon uptake of copper [37].

The sensitivity of algae towards copper prompted the systematic investigation of the metal and nonmetallic ion tolerance of Chlorella vulgaris [38]. It was found that of thirty metals, toxicity had a definite tendency to increase with increasing atomic number. Cobalt, nickel, and copper completely inhibited growth at very low concentrations ranging from 4.2×10^{-6} to 2×10^{-5} M. In view of their relatively low atomic numbers, the toxicity was regarded as a specific algotoxicity.

A survey of the toxicity of zinc, copper, and lead to Chlorophyta taken from flowing waters and studied laboratory conditions was made by Whitten [39]. Twenty populations each of Stigeoclonium tenue and Cladophora glomerata were investigated to see if any variation in metal resistance could be found in natural populations. All populations gave similar results for copper and lead with a slight variation in resistance to zinc.

Fitzgerald and Faust presented data from laboratory studies that five sources of copper appeared to be equally toxic to algae [40]. Microcystis aeruginosa and Chlorella

pyrenoidosa were used as test organisms. The toxicity of solutions of $CuSO_4$ and of a mixture of 1 part $CuSO_4$ and 2 parts citric acid to Chlorella were compared along with three commercial algicides a chelate-free medium (Allen's medium). Concentrations of 0.5 mg/l Cu or greater were toxic to Chlorella after seven days contact time whether they were supplied as copper sulfate or as the citric acid complex. In a second medium (Gorham's medium) maintained at pH 7, analyses of filtered and unfiltered samples at the start of a similar test and after 12 days treatment revealed that only 8 per cent of the copper from the $CuSO_4$ was soluble whereas 78 per cent of the copper from the $CuSO_4$-citric acid solution was in a soluble form. Unfortunately, the total copper concentration at which the analyses were carried out was not specified. The two sources appeared to be equally toxic to an unidentified Chlorophyte, indicating that either copper is toxic to algae regardless of whether it is in a soluble or insoluble form as the authors concluded or that the formation of insoluble copper species is necessary for a toxic effect and that copper added over and above the medium solubility has little effect.

The toxicity of copper sulfate to Chlorella was compared in Allen's medium (no chelate), Allen's medium with iron supplied as Fe(III) chelated with EDTA, and Gorham's medium containing Fe(III) citrate and 1 mg/l (3.4×10^{-6} M) EDTA. The results of these tests are given in Table III. One mg/l

TABLE III

COMPARISON OF TOXICITY OF Cu(II) IN ARTIFICIAL MEDIA TO CHLORELLA WITH VARYING METHODS OF SUPPLY OF Fe(III) AND WITH VARYING LEVELS OF CHELATE*

Medium	[Fe(III)]	[Chelate]	[Cu(II)] Required for Toxicity
Allen's	3 mg/l $FeCl_3$ (1.8×10^{-5} M)	———	0.25 mg/l Cu (4×10^{-6} M)
Modified Allen's	3 mg/l $FeCl_3$ (1.8×10^{5} M)	7.4 mg/l $Na_2EDTA \cdot 2H_2O$ (2.0×10^{-5})	2.0 mg/l Cu (3.2×10^{-5} M)
Gorham's	6 mg/l Fe(III) Citrate$\cdot 5H_2O$ (1.8×10^{-5} M)	6 mg/l Fe(III) Citrate$\cdot 5H_2O$ (1.8×10^{-5}) +6 mg/l Citric Acid (3.1×10^{-5}) +1 mg/l H_4EDTA (3.4×10^{-6})	0.5 mg/l Cu (8×10^{-6} M)

*Data Calculated from that Given in Reference [40]

$CuSO_4 \cdot 5H_2O$ is toxic to Chlorella in Allen's medium after 5 days, whereas 2 mg/l are required in Gorham's medium, and 8 mg/l are required in Allen's medium with EDTA chelated iron.

The toxicity of $CuSO_4 \cdot 5H_2O$ to Microcystis aeruginosa with four sources of iron are given in Table IV. Apparently an excess of a very strong chelate over iron in the medium decreases the toxicity of copper. The authors also presented data that 4 and 8 mg/l $CuSO_4 \cdot 5H_2O$ (1 and 2 mg/l Cu) did not kill Chlorella pyrenoidosa but caused virtually complete inhibition of growth after 14 days exposure. Subcultures of the original treated cultures grew at copper concentrations of 6.1 mg/l Cu.

Toth and Reimer investigated the behavior of copper in ponds when applied as $CuSO_4 \cdot 5H_2O$ [41]. Adsorption studies with humic acid and the clay minerals Kaolinite, Illite, and Montmorillonite showed that the amount of copper removed from solution after application will largely be determined by the suspended and bottom material. One gram additions of each of the minerals and 0.25 grams of humic acid were added to separate 100 ml volumes of copper sulfate solution in the pH range 3 to 5. Complete adsorption of copper occurred at 2.0 mg/l added $CuSO_4 \cdot 5H_2O$ (0.5 mg/l Cu) in all systems. This copper concentration represents 2 to 4 times the normal application rate for algal control. High rates of removal should be expected with sediments high in clays. The copper

TABLE IV

COMPARISON OF TOXICITY OF Cu(II) TO MICROCYSTIS AERUGINOSA IN GORHAM'S MEDIUM WITH VARYING METHODS OF SUPPLYING Fe(III) AND WITH VARYING LEVELS OF CHELATE*

[Fe(III)]	[Chelate]	[Cu(II] Required for Toxicity
3 mg/l $FeCl_3$ (1.8×10^{-5} M)	6 mg/l Citric Acid (3.1×10^{-5})	0.013 mg/l Cu (2×10^{-7} M)
6 mg/l Fe(III) Citrate·$5H_2O$ (1.8×10^{-5} M)	6 mg/l Citric Acid (3.1×10^{-5}) 3 mg/l Fe(III) Citrate (1.8×10^{-5})	0.013 mg/l Cu (2×10^{-7} M)
6 mg/l Fe(III) Citrate·$5H_2O$ (1.8×10^{-5} M)	6 mg/l Citric Acid (3.1×10^{-5}) 3 mg/l Fe(III) Citrate (1.8×10^{-5}) 1 mg/l H_4EDTA (3.4×10^{-6})	No Evidence of Toxicity at 0.076 mg/l Cu (1.2×10^{-6} M)
3 mg/l $FeCl_3$ (1.8×10^{-5} M)	6 mg/l Citric Acid (3.1×10^{-5}) 7.4 mg/l $Na_2EDTA \cdot 2H_2O$ (2.0×10^{-5})	No Evidence of Toxicity at 0.076 mg/l Cu (1.2×10^{-6} M)

*Data Calculated from that Given in Reference [40]

was strongly sorbed and 2 washings with 1 N HCl failed to completely release the fixed copper.

In an earlier paper concerning the precise control of algae in ponds, Toth and Reimer concluded that prior analysis of the water in individual ponds would be necessary to establish optimum control rates for copper [42]. Granulated copper sulfate was applied to two ponds at total concentrations of 0.250 mg/l Cu and to one at a total concentration of 0.187 mg/l Cu. Soluble copper concentrations in the waters never approached more than 50 per cent of that calculated from the amount of copper applied and essentially no stratification with depth was evident after 24 hours even though the copper sulfate crystals were observed to sink to the bottom upon application. Much of the copper apparently was sorbed by bottom muds.

CHAPTER IV

TECHNIQUES AND TEST CONDITIONS CHOSEN FOR THE STUDY OF ALGAL GROWTH

There are essentially two techniques for the study of the effects of nutrient and toxic substances on microorganisms in general use:

(1) a static test in which the test solution is not changed during the period of exposure to the organism.

(2) a flow-through test in which the test medium and test materials are continually renewed during the period of exposure.

Measurements made under flow-through test conditions are generally thought to be more reliable because under static conditions a significant fraction of the test material that is present initially may be consumed by the organism under study. The complicated work of building an elaborate supply system and metering devices, however, limits the utility of the flow-through system which may or may not represent conditions in actual aquatic environments where in many cases the concentration of certain nutrients or toxicants will not be constant, but will vary with time. The increased likelihood of trace contamination and the cost of maintaining such a system in studying the effects of trace metals makes the use of the flow-through test for the study of these substances nearly prohibitive.

Conditions that simulate the flow-through test can,

however, be approximated by the static test through the control of experimental variables and the time at which growth measurements are made. During the exponential growth phase, the rate of growth reaches a constant value. Nearly all the cells formed are viable, and consequently, cell mass and cell number increase at the same rate. Generally, the rate of microorganism growth is proportional to the concentration of its least available nutrient when that nutrient is growth limiting. Consequently, when exponential growth occurs, the concentration of the growth limiting nutrient remains essentially constant. The major disadvantage of the static test can be overcome, therefore, by measuring the microorganism growth in the late log phase where nutrient limited exponential growth is occurring, but where a sufficient density of organisms is not present to significantly reduce the initial quantity of the test material introduced into the medium or to produce metabolite waste products that would limit growth.

Another advantage of the flow-through system is that water soluble gas concentrations (CO_2, O_2, _etc._) and pH may be maintained at constant values because of the continued renewal of these materials as new medium is introduced. These parameters may also be controlled quite well under static conditions, especially when autotrophic organisms are studied, and the carbon source can be supplied as an air-

carbon dioxide mixture. The CO_2:AIR ratio can be used to control the pH of the medium quite well, especially when large population densities are not allowed. The general tendency for the pH to rise as algal growth proceeds in static systems is likewise lessened when results are interpreted early in the growth of the population.

Most of the studies concerned with the effects of trace metal concentrations on algal growth have measured the concentrations of materials that either increase or decrease physiological processes rather than the concentrations that cause a decreased rate of growth in the test population. Many of the measurements of cell growth are based upon chlorophyll production, a parameter which in itself may be strongly related to the concentration of the material under study. Under conditions where measurements can be made in a medium of low turbidity, and during or near the exponential growth phase, a simple turbidity measurement is both an accurate and rapid means of cell enumeration. A calibration curve of the absorbance of the cell suspension vs. dry cell weight per unit volume of medium and/or total cell count per unit volume of medium can be prepared. The rapidity, reproducibility, and precision with which turbidity measurements can be made is an added feature of this technique. Since growth measurement by turbidimetric techniques is nearly independent of the physiology of the cell, it is ideal for

the measurement of the growth rate when exponential growth is occurring and nearly all cells are viable. If turbidimetric measurements are made well into the statiomary phase, many of the cells in the medium will be nonviable, but still capable of scattering light. Total cell counts made under conditions of exponential growth are, however, a close approximation of the viable cell count.

Although the results of toxicity studies usually are reported as 96 hour median tolerance limits, ie., the concentration that kills 50 per cent of the test organisms within the specified time span, the death point of algal cells is difficult to determine except by subculturing. The measurement of the death point is relatively unimportant if a 50 per cent reduction in the rate of growth is used as a measure of the toxicity of the substance being tested. A growth rate assessment is also easier to evaluate and generally more reliable than subculturing to determine viable cell count. The point at which a 50 per cent reduction in the growth rate occurs is a useful quantity in evaluating growth inhibition when copper is used to control algal populations in natural waters.

CHAPTER V

A PRECIPITATE-FREE ALGAL GROWTH MEDIUM AND AN EXPRESSION FOR THE COMPUTATION OF TRACE ELEMENT SPECIES THEREIN

It seems that many earlier studies concerning the trace element nutrition of the algae were complicated by the lack of a suitable growth medium that would enable investigators to compute, within reasonable accuracy, the concentrations of the various trace element species found in solution. Most laboratory media are simply poorly defined chemically in terms of their trace element content. Prepared media that are not precipitate-free are probably the most difficult to handle mathematically. The introduction of the solid phase raises the question of whether certain equilibria are ever established. The "availability" of the metal hydroxides, carbonates, and phosphates to algal cells is currently open to question. Adsorption and coprecipitation phenomena which can occur in such systems leads to additional complications that defy simple chemical definition. To the author's knowledge, no attempt has been made to prepare a complete algal growth medium that was both suitable for growth and at the same time chemically well defined. Combining the information derived from observations made in simple, defined systems will allow a more meaningful interpretation of the complex interactions that occur in environmental systems.

An EDTA medium was chosen for use in this study to

facilitate the calculation of equilibrium concentrations of Cu^{2+}. The trace element composition of the medium is similar to that used by Walker [9]. The presence of the strong complexing agent prevented the formation of insoluble zinc and iron species which are probably not available for algal uptake. The addition of EDTA to the culture medium also allows the maintenance of comparatively large reservoirs of trace elements that can support rapid growth. Extended periods of rapid growth can be effected without requiring replacement of the medium components. The fraction of the total concentration of any one trace element that is depleted by cellular uptake in a properly designed medium will, therefore, be insignificant for short periods of growth. This means that the initial concentration of a trace component in the medium may be used to calculate the species present after algal growth has occurred.

The composition of the medium used in this study is given in Tables V and VI. The medium contains only enough chelating agent (EDTA) to keep the trace elements Cu, Fe, Zn, Mo, Ca, and Mn in solution. The medium contains a total chelatable trace metal ion concentration of 1.00×10^{-3} M and has a pH of 7.0 in equilibrium with air. In such a system, all the trace metal species more strongly complexed than Mg^{2+} by EDTA have their free concentrations determined by $[Mg^{2+}]/[Mg\text{-}EDTA^{2-}]$. If the pH is within an acceptable

TABLE V

CONTRIBUTION FROM MACRONUTRIENT, pH ADJUSTING, AND VITAMIN SOLUTIONS TO MEDIUM USED FOR ALGAL GROWTH AT A CONSTANT CONCENTRATION OF EDTA

From Macronutrient Solution:

Salt	Molarity (x 10^3)	mg/l Ion	Weighing Form	Wt. Salt (mg/l)
KNO_3	10.00	391 NO_3^-	KNO_3	1000
$MgSO_4$	5.32	129 Mg^{2+}	$MgSO_4 \cdot 7H_2O$	1311
KH_2PO_4	1.50	285 PO_4^{3-}	KH_2PO_4	204
K_2HPO_4	1.50		K_2HPO_4	261

From KOH Solution:

Salt	Molarity (x 10^3)	mg/l Ion	Weighing Form	Wt. Salt (mg/l)
KOH	2.6	———	KOH	———

From Vitamin Solution:

Vitamin	mg/l
Thiamine·HCl	200
Biotin	1.0
B_{12}	1.0

TABLE VI

CONTRIBUTION FROM TRACE ELEMENT SOLUTION TO MEDIUM USED FOR ALGAL GROWTH AT A CONSTANT CONCENTRATION OF EDTA

Component	Molarity ($\times 10^3$)	mg/l Ion	Weighing Form	Wt. Salt (mg/l)
$Na_2EDTA \cdot 2H_2O$	1.50	558	$Na_2EDTA \cdot 2H_2O$	558
Fe^{3+}	0.179	10.0	$Fe_2(SO_4)_3$	35.8
Ca^{2+}	0.399	16.0	$CaCO_3$	40.0
Zn^{2+}	0.331	21.6	$ZnSO_4 \cdot 7H_2O$	95.2
Mn^{2+}	0.091	5.0	$MnSO_4 \cdot H_2O$	15.0
Mo^{6+}	0.0042	0.40	$Na_2MoO_4 \cdot 2H_2O$	1.0
B^{3+}	0.092	1.00	H_3BO_3	5.70

Total Chelatable Trace Metals = 1.00×10^{-3} M

range (to be discussed later), the equilibrium $[Cu^{2+}]$ can be expressed in terms of the formal concentration of EDTA, C_Y, and Cu(II), C_{Cu}, expressed in moles/liter. The formation constant expression for the CuY^{2-} complex can be written in the following manner if it is assumed that essentially all the copper in solution is present as CuY^{2-}:

$$[Cu^{2+}] = \frac{C_{Cu}}{[Y^{4-}]\ K_{f,Cu}} \qquad \text{(I-2)}$$

where $K_{f,Cu} = 6.3 \times 10^{18}$, the formation constant of the Cu(II)-EDTA complex [43].

The formal concentration of Mg(II), C_{Mg}, is such that only a fraction of this ion is complexed. Using the same reasoning as above, the $[MgY^{2-}]$ and $[Mg^{2+}]$ are given by the following:

$$[MgY^{2-}] = C_Y - C_{TM} \qquad \text{(I-3)}$$

$$[Mg^{2+}] = C_{Mg} + C_{TM} - C_Y \qquad \text{(I-4)}$$

where C_{TM} is the total chelatable trace metal concentration.

Substituting these values into the formation constant expression for the Mg-EDTA complex, the concentration of free chelate can be expressed in the following manner:

$$[Y^{4-}] = \frac{C_Y - C_{TM}}{(C_{Mg} + C_{TM} - C_Y)\ K_{f,Mg}} \qquad \text{(I-5)}$$

where $K_{f,Mg} = 1.0 \times 10^{9}$, the formation constant of the Mg(II)-EDTA complex [43]. By inserting equation I-5 into equation I-2

and rearranging, the final expression for free cupric ion becomes:

$$[Cu^{2+}] = \frac{K_{f,Mg}}{K_{f,Cu}} \times \frac{C_{Mg} + C_{TM} - C_Y}{C_Y - C_{TM}} \times C_{Cu} \qquad (I\text{-}6)$$

$$= (\text{Term 1}) \times (\text{Term 2}) \times (\text{Term 3})$$

The effect of adding complexing agent under these conditions is to lower the concentration of free Cu^{2+}. A similar expression could be derived to describe the distribution of the other chelated trace metal species in the medium and their dependence on the concentration of chelate present. If the formal concentration of EDTA is varied from 1.1×10^{-3} M to 5.1×10^{-3} M, with $C_{Mg} = 5.32 \times 10^{-3}$ M and $C_{TM} = 1.00 \times 10^{-3}$ M, Term 2 ranges from 52.2 to 0.30 which corresponds to a 177-fold change in $[Cu^{2+}]$. The $[Cu^{2+}]$ is quite low (5×10^{-15} M even at a formal concentration of 1.10×10^{-3} M EDTA). Equation I-6 is accurate to within 1 per cent in the pH range from 6 to roughly 12. The lower pH limit is due to the dissociation of the MgY^{2-} complex and the upper limit to the precipitation of $Cu(OH)_2$.

CHAPTER VI

REAGENT PURIFICATION METHODS

A copper analysis of the reagent-grade materials to be used in the preparation of the growth medium for this study showed that at the concentrations necessary for algal growth, a significant amount of Cu^{2+} (5.3 µg/l) entered the growth medium as a reagent contaminant (Table VII). The analyses for contaminant copper were performed on concentrated solutions of each reagent against freshly prepared standards using a Perkin-Elmer Model 403 Atomic Absorption Spectrophotometer equipped with a three slot burner head. Reagent-grade materials typically contain 10^{-3} to 10^{-4} per cent copper. For a single reagent, a large variation in copper content was noted in freshly opened bottles from several manufacturers. Therefore, the reagents chosen for the preparation of the growth medium were carefully selected for their low copper content.

Since it was desired to study total Cu(II) concentrations as low as 1 µg/l, prior purification of the reagents used for the medium preparation was necessary. For purification purposes, the medium components were separated into a trace element solution and a macronutrient solution. A controlled potential electrolysis procedure was used on a 0.200 M solution of Na_2EDTA since it was found that this component was the major source of copper in the trace element solution.

TABLE VII

CONTRIBUTION OF EACH MEDIUM COMPONENT TO Cu^{2+} CONTAMINATION IN THE FINAL GROWTH MEDIUM AS DETERMINED BY ATOMIC ABSORPTION ANALYSIS

Medium Component	Grams Cu Per Gram of Reagent-Grade Material (x 10^6)	Cu^{2+} Contamination Due to Medium Component in Growth Medium (µg/l)
Trace Element Solution (EDTA)	——	1.2
Macronutrient Solution	——	3.6
K_2HPO_4	3.6	0.7
KNO_3	1.8	1.7
$MgSO_4 \cdot 7H_2O$	0.93	0.6
KH_2PO_4	2.5	0.6
KOH	3.0	0.5

Typically, the level of copper in the 0.200 M Na_2EDTA solution after electrolysis was 1 μg/l. The potential controlling apparatus was a simple electronic potentiostat, the design and principles of which are described elsewhere [44,45]. A circuit diagram is given in Figure 1. Approximately 24 hours were allowed for complete Cu^{2+} removal. Removal of dissolved oxygen was accomplished by bubbling tank nitrogen through the cell solution for 30 minutes prior to and during the electrolysis period. The nitrogen was first passed through a solution of Na_2SO_3 and then through a column of deionized water containing a mixed bed ion-exchange resin. The anode compartment was filled with 0.200 M Na_2EDTA.

A three electrode system was used to control the cathode potential at -0.500 volts *vs.* an Orion Model 90-02 double junction reference electrode (Orion Research, Inc., Cambridge, Mass.). The potential of this electrode is +5 mV *vs.* SCE when the Orion electrode inner chamber is filled with Orion 90-00-02 filling solution, and the outer chamber is filled with 10 per cent KNO_3. The use of a double junction reference electrode in this manner reduces the contamination of the cell solution due to flow of the filling solution from the reference electrode. The potential drift of the reference electrode was found to be *ca.* ±0.3 mV and non-cumulative over the 24 hour period during which drift measurements were made. The advantages of the double junction reference

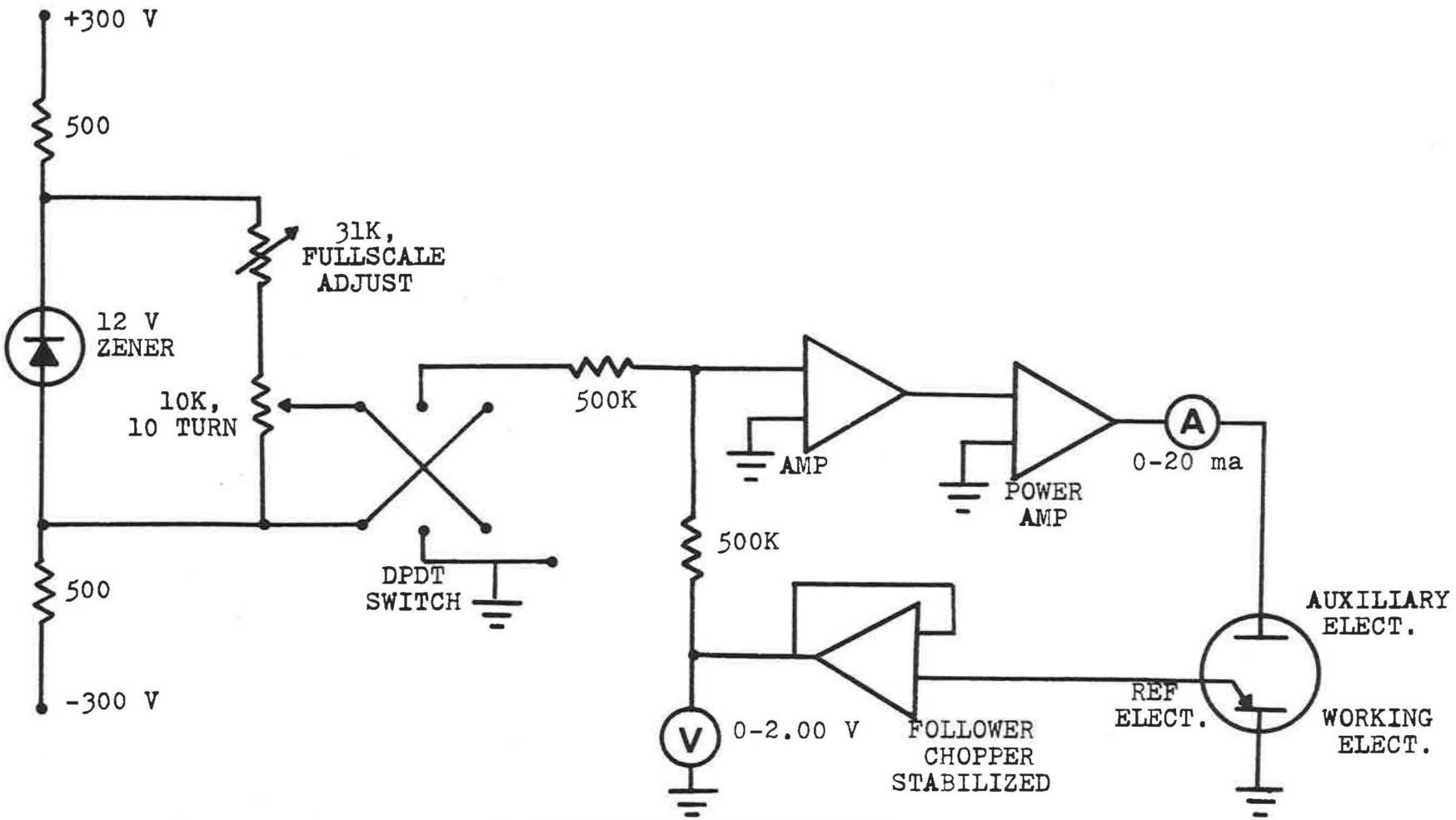


FIGURE 1. CIRCUIT DIAGRAM FOR POTENTIOSTAT USED TO REMOVE COPPER FROM Na_2EDTA USED IN TRACE ELEMENT SOLUTION.

electrode over the conventional glass frit, agar plug reference electrode under these conditions make it very attractive for use in other electrochemical techniques. An Orion Model 94-29A solid-state cupric ion activity electrode was used to follow the decrease in total copper during the electrolysis by measuring the potential of this electrode vs. the Orion reference electrode. A 25 ml mercury pool cathode was employed, and the mercury solution interface was continually renewed by means of a magnetically driven Teflon stirring bar. Approximately 300 ml of EDTA solution were treated each time. A typical curve showing the removal of copper is given in Figure 2.

It was found that a solvent extraction procedure was both a convenient and rapid means of removing contaminant copper from the macronutrients portion of the growth medium. The selection of an extraction system to be used in conjunction with biological media, however, involves the consideration of several factors in addition to those normally considered in selecting a good extraction system. The two most important of these are (1) the solubility of the extracting solvent and (2) the solubility of the extracting reagent in the aqueous solution to be treated. In light of these two factors, a number of the more commonly used extraction systems are not suitable for use in biological systems. For example, the very efficient extraction of trace metals into ketone

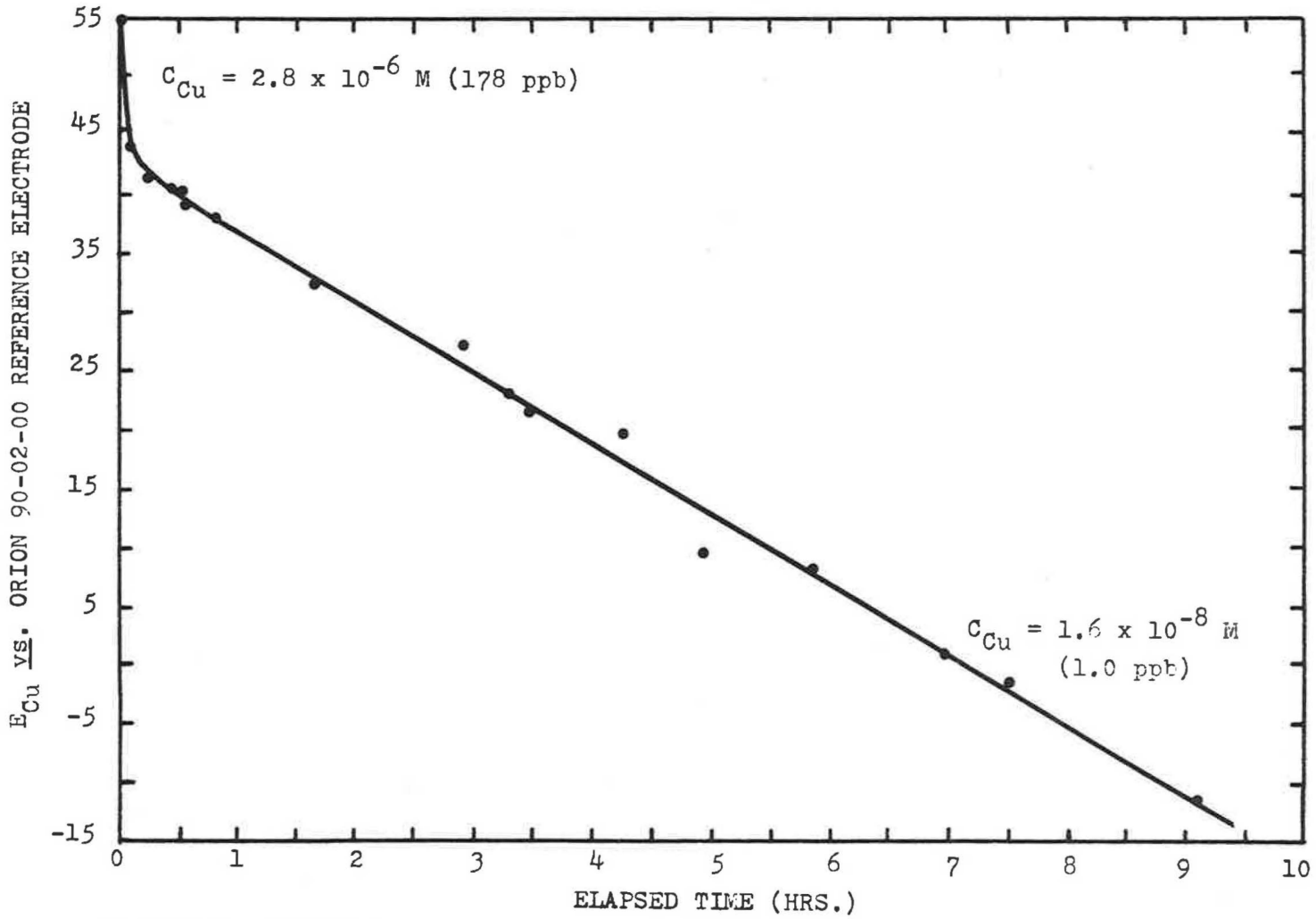


FIGURE 2. REMOVAL OF Cu(II) FROM 300 ml OF 0.200 M Na_2EDTA AT E_{App} = -0.500 V vs. A DOUBLE JUNCTION REFERENCE ELECTRODE USING A 25.0 ml Hg CATHODE. INITIAL CURRENT = 4.4 mA.

solvents with ammonium pyrrolidinecarbodithioate that is commonly used in atomic absorption analyses cannot be used since both the reagent and solvents are moderately soluble in water.

An extraction system that meets the solubility criteria is 0.01 per cent dithizone in CCl_4. In addition, it was found that the cupric ion electrode was useful in monitoring copper levels during dithizone extractions. Not only did the cupric ion electrode offer greater sensitivity than most other methods, but it also allowed continuous monitoring during the copper removal process. A plot of electrode potential *vs.* time elapsed during the extraction of Cu^{2+} from a tenfold concentrate of the macronutrients component of the algal growth medium is given in Figure 3. Approximately 400 ml of aqueous solution were treated with 25 ml of 0.01 per cent dithizone in CCl_4 in a four neck, 500 ml boiling flask with an over head glass stirrer to continually renew the aqueous-organic interface. A Teflon stopcock assembly was installed in the bottom of the flask to provide for the removal of the organic phase. Typically, two hours were allowed for complete extraction. The pH of the tenfold concentrate of the macronutrients solution is 7.21, and at this pH, dithizone is slightly soluble in the aqueous phase. In order to ensure that only a very small amount of the dithizone remained in the macronutrients solution, the rea-

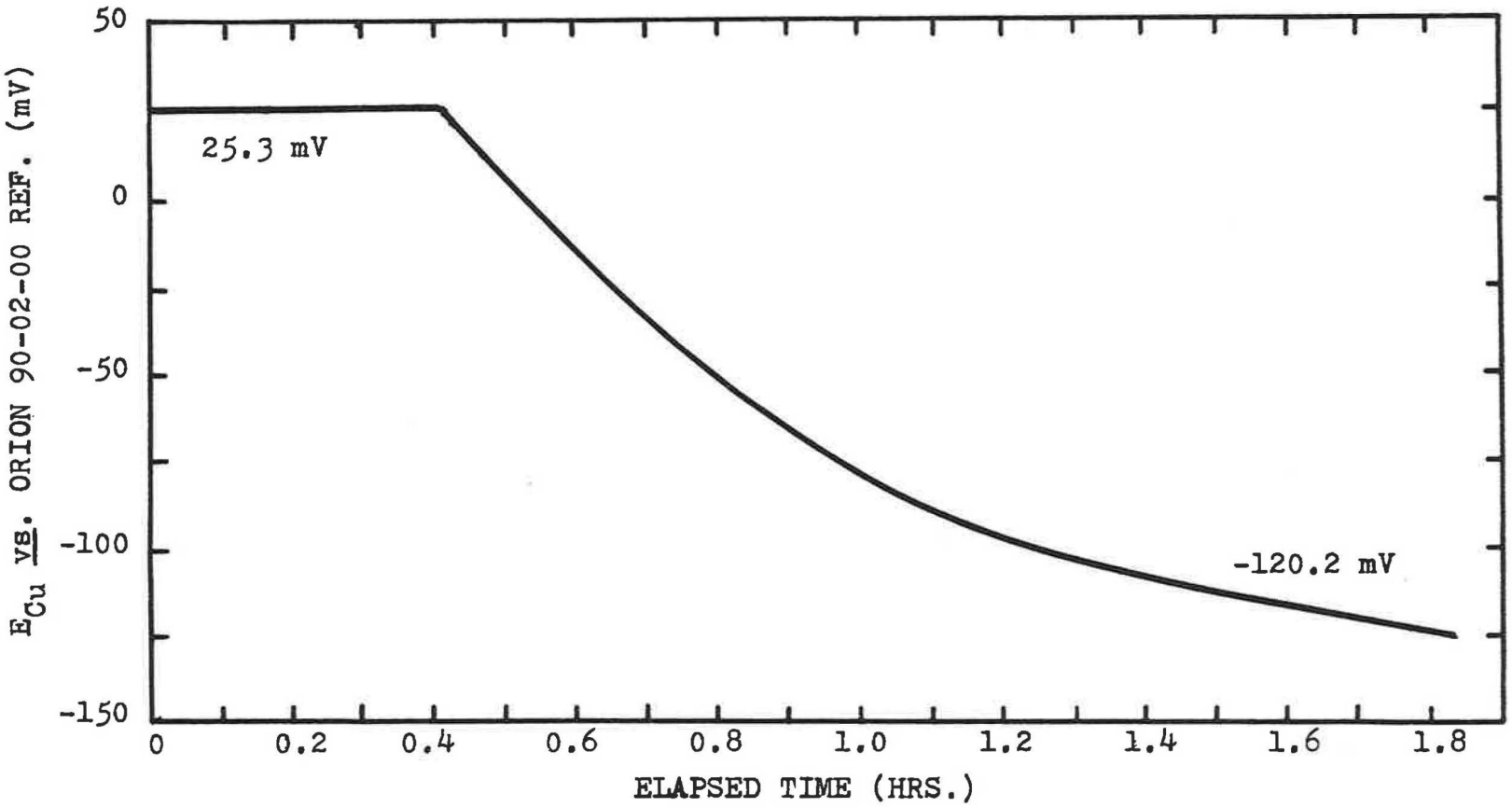


FIGURE 3. PLOT OF POTENTIAL FOR THE SOLID-STATE CUPRIC ION SELECTIVE ELECTRODE vs. TIME ELAPSED DURING EXTRACTION OF Cu^{2+} FROM 400 ml OF A TEN-FOLD CONCENTRATE OF A MACRONUTRIENTS SOLUTION INITIALLY 36.0 µg/l IN Cu(II) USING 25.0 ml OF 0.01% DITHIZONE IN CCl_4. AQUEOUS pH = 7.21.

gent was re-extracted from the aqueous phase with 25 ml of redistilled CCl_4 after the completion of each extraction.

CHAPTER VII

EXPERIMENTAL

The purpose of this investigation was to demonstrate the effect of cupric ion on the growth of two typical planktonic algae. Unialgal cultures of the two algae, Oocystis marssonii and Chlorella vulgaris, were obtained from the Starr collection of algae at Indiana University, Bloomington. The culture numbers are LB287 and LB398, respectively.

PREPARATION OF THE GROWTH MEDIUM

Two liter batches of a tenfold concentrate of the trace element solution were prepared from the appropriate reagent-grade materials and electrochemically-treated 0.200 M Na_2EDTA. The individual components were added to the solution in the order they are given in Table VI. After the addition of $Fe(SO_4)_3$ and $CaCO_3$, the suspended material was allowed to dissolve before additional material was added. Dissolution sometimes required as long as two hours at room temperature, but could be speeded up by warming.

The macronutrients solution was also prepared at a concentration ten times that in the final growth medium. The solution was prepared by adding the reagents with rapid stirring in the order they are given in Table V to approximately 300 ml of deionized, distilled water contained in a 500 ml volumetric flask. The resulting solution was then diluted to

approximately 200 ml of 1.00×10^{-3} M KNO_3. Ten ml of the resulting suspension were added to the culture medium just before dilution to final volume. Typical inocula were such that the absorbance of the final medium was the same as an uninoculated blank.

EVALUATION OF ALGAL GROWTH

The absorbance of the cell suspension was employed as a measure of cell yield. Both Oocystis and Chlorella are unicellular, nonfilamentous green algae which form homogeneous suspensions, thus permitting a turbidimetric determination of growth. The absorbance of an algal suspension is a function of both light scattering and absorption due to the cell pigments. For this reason, the absorbances of the cell suspension were measured at 560 nm where a minimum in the chlorophyll absorption spectrum exists for both organisms studied. It was felt that this approach would minimize the influence of the cell pigments on the measuring technique as the level of copper was varied. It was assumed that cell size remained essentially constant, and checks of the average cell size at widely varying copper levels proved to be nearly identical. The relationship between the dry weight of algal cells and absorbance was determined by diluting 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ml aliquots of the appropriate cell suspension to volume with fresh medium in a 10 ml volumetric

the mark. Stirring is necessary to prevent the precipitation of $Mg_2(PO_4)_3$. This solution was prepared fresh for each run and treated as described in the previous section.

A 6.50×10^{-2} M KOH solution for pH adjustment was prepared from previously standardized ca. 0.8 M ultrapure KOH (Ventron Corp., Beverly, Mass.). The 0.8 M solution was prepared by dissolving a ca. 10 gram stick of the solid 75 per cent ultrapure KOH in 200 ml of deionized, distilled water. A vitamin solution was prepared at a concentration 500 times that required in the final medium. A stock 3000 mg/l Cu solution was prepared from anhydrous $CuSO_4$ in 0.01 N H_2SO_4. The required standard copper solutions were prepared from the stock $CuSO_4$ solution by dilution. The appropriate volumes of the KOH, vitamin, trace element, and copper solutions were then used to prepare the final growth medium. All reagents were stored in new polyethylene bottles that were cleaned initially with HNO_3-H_2SO_4 cleaning solution and treated prior to filling with 0.05 M Na_2EDTA in 3 M NH_3 for 3-4 days.

SOURCE OF INOCULA

Inoculum cells were maintained in copper deficient media. Approximately 15 ml of the appropriate exponentially growing cell suspenion were centrifuged and washed with a 15 ml portion of 1.00×10^{-3} M Na_2EDTA. After two additional washings in 1.00×10^{-3} M KNO_3, the washed cells were suspended in

flask. The absorbances of the suspensions were measured at 560 nm using a Spectronic 20 colorimeter. The dry cell weight was determined by vacuum filtering 25 ml aliquots of cell suspension through 47 mm, 0.45 μ GA-6 Metricel filters (Millipore Corp., Bedford, Mass.). Two superimposed filters, matched in weight to within 0.1 mg, were assembled in a Pyrex filter holder. Twenty-five ml aliquots of sample were passed through both filters while carefully avoiding contact of the cell suspension with the walls of the filter holder. Both filters were, therefore, subjected to the same fluid flow, but the cell suspension was retained on the upper filter. After drying both filters, the weight of the lower filter was subtracted from that of the test filter to determine the weight of the collected cells. Triplicate analyses were made for both dry weight determinations. The linear relationship between absorbance and dry cell weight for each organism is shown in Figures 4 and 5. A similar plot of absorbance vs. number of cells/ml was also prepared for Chlorella. The plot was linear with a zero intercept. An absorbance of 0.400 represents 8.80×10^6 cells/ml.

CULTURE CONDITIONS

Cultures were grown for 4 days in exactly 250 ml of media contained in new 500 ml polyethylene wash bottles treated as described previously. Attempts to demonstrate copper deficien-

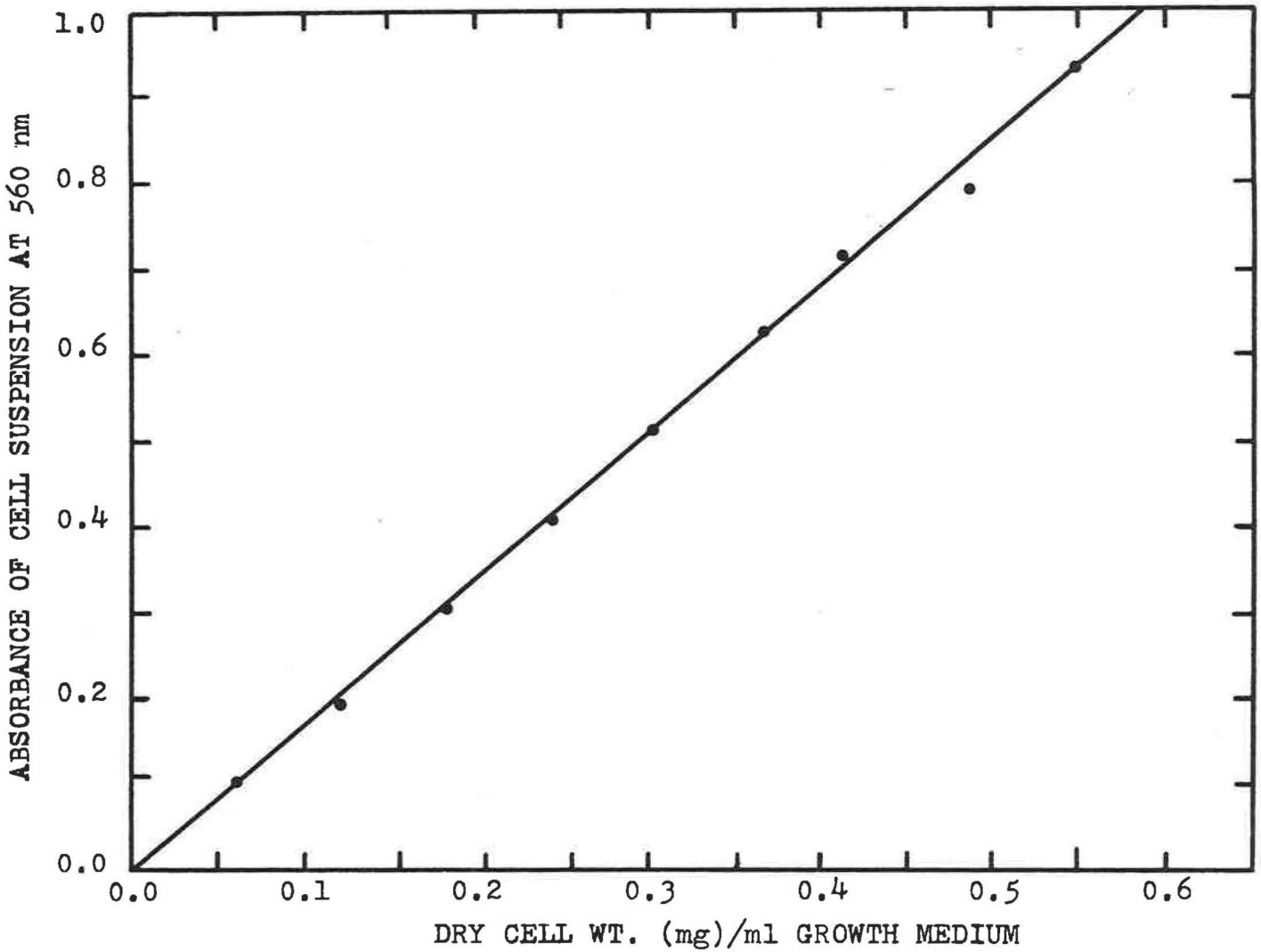


FIGURE 4. PLOT OF ABSORBANCE OF CELL SUSPENSION FOR OOCYSTIS MARSSONII vs. WEIGHT OF DRY CELLS/ml GROWTH MEDIUM.

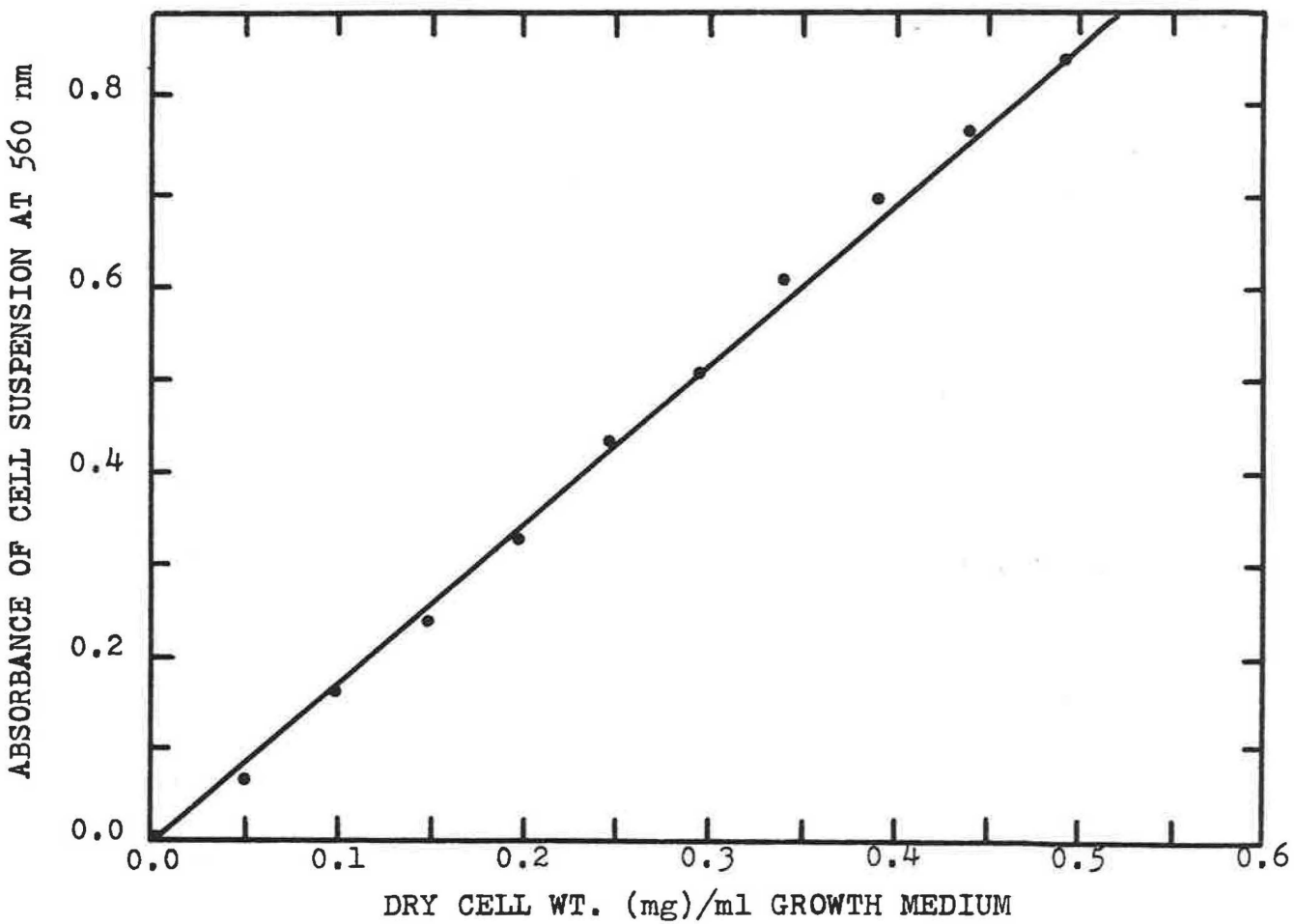


FIGURE 5. PLOT OF ABSORBANCE OF CELL SUSPENSION OF CHLORELLA VULGARIS vs. WEIGHT OF DRY CELLS/ml GROWTH MEDIUM.

cies in new Pyrex flasks treated in the same manner as polyethylene ware usually gave erratic results, presumably due to copper contamination from the glass. All operations were carried out at room temperature (25-27° C). The growing cultures were agitated continuously by means of a slow-speed shaker plate. Four 40 watt Gro-Lux fluorescent lamps provided overhead illumination through a plastic light diffusing panel attached to the lamp fixture. Light intensity measurements showed, however, that only a fraction of the area under the lamps received the same intensity of illumination. Due to the high nutrient to cell ratio in the medium, cultures containing optimal quantities of copper for growth show a strong dependence on light intensity. Consequently, cell culturing was confined to the area receiving the same intensity of illumination.

A CO_2-AIR mixture bubbled through the cultures via a Pyrex manifold served as both a carbon source and a means of pH control. The gas mixture was hydrated and washed by bubbling through a gas mixing chamber filled with deionized, distilled water. Two additional washings were accomplished by bubbling the gas mixture through water containing a mixed bed ion exchange resin. Gas delivery to each chamber was accomplished through medium porosity gas filter candles. Gas flow rates were measured by calibrated flow meters that could be switched in or out of the delivery system by means of

three-way Teflon stopcocks. The air supplied was enriched to nearly 4 per cent CO_2 by supplying 100 per cent CO_2 to the mixing chamber at a rate of 20 ml/min and air at the rate of 500 ml/min. After equilibration with the gas mixture, the initial medium pH is approximately 6.1 and at the end of 96 hours of optimal growth is 6.3. The pH of the medium in equilibrium with air is 7.0.

CHAPTER VIII

RESULTS AND DISCUSSION

A reproducible copper requirement for both Chlorella vulgaris and Oocystis marssonii was demonstrated by employing the previously described medium at an EDTA concentration of 1.50×10^{-3} M. Figures 6 and 7 are cell yield curves obtained by adding varying amounts of copper to the copper deficient medium while keeping the concentration of the complexing agent constant. Maximum growth is observed above 40 µg/l Cu for Oocystis and 30 µg/l Cu for Chlorella. The minimum level of total copper for maximum growth of Chlorella is virtually identical to that obtained by Walker for Chlorella pyrenoidosa in a similar medium, but under conditions of photoheterotrophic rather than photoautotrophic growth [9]. The curves were taken from earlier work completed at a total Mg(II) concentration of 2.60×10^{-3} M rather than 5.32×10^{-3} M. From these two studies and the earlier work by Walker it may be concluded that there is probably a reproducible micronutrient requirement for copper for the green algae. It is also likely that the variation in copper requirement within this grouping is small. Additional evidence for a copper requirement by Chlorella is given in Figure 8 where the growth of the organism is measured as a function of time. An early portion of the log phase is shown at copper concentrations of 20 and 60 µg/l. At both concentrations, the log absorbance vs. time curve is

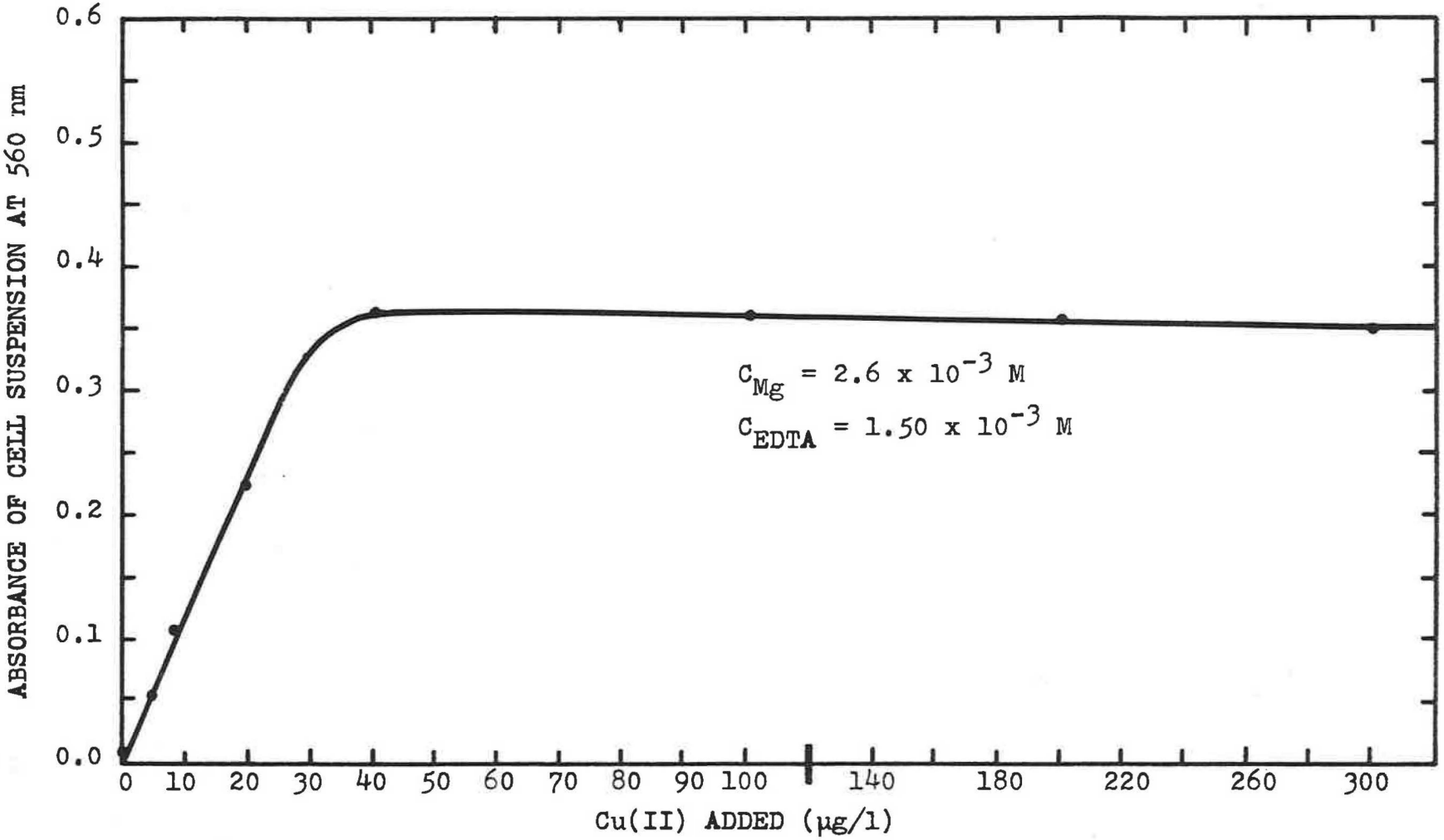


FIGURE 6. CELL YIELD CURVE FOR THE COPPER DEFICIENCY STUDY OF OOCYSTIS MARSSONII AT A CONSTANT CONCENTRATION OF EDTA AND Mg(II).

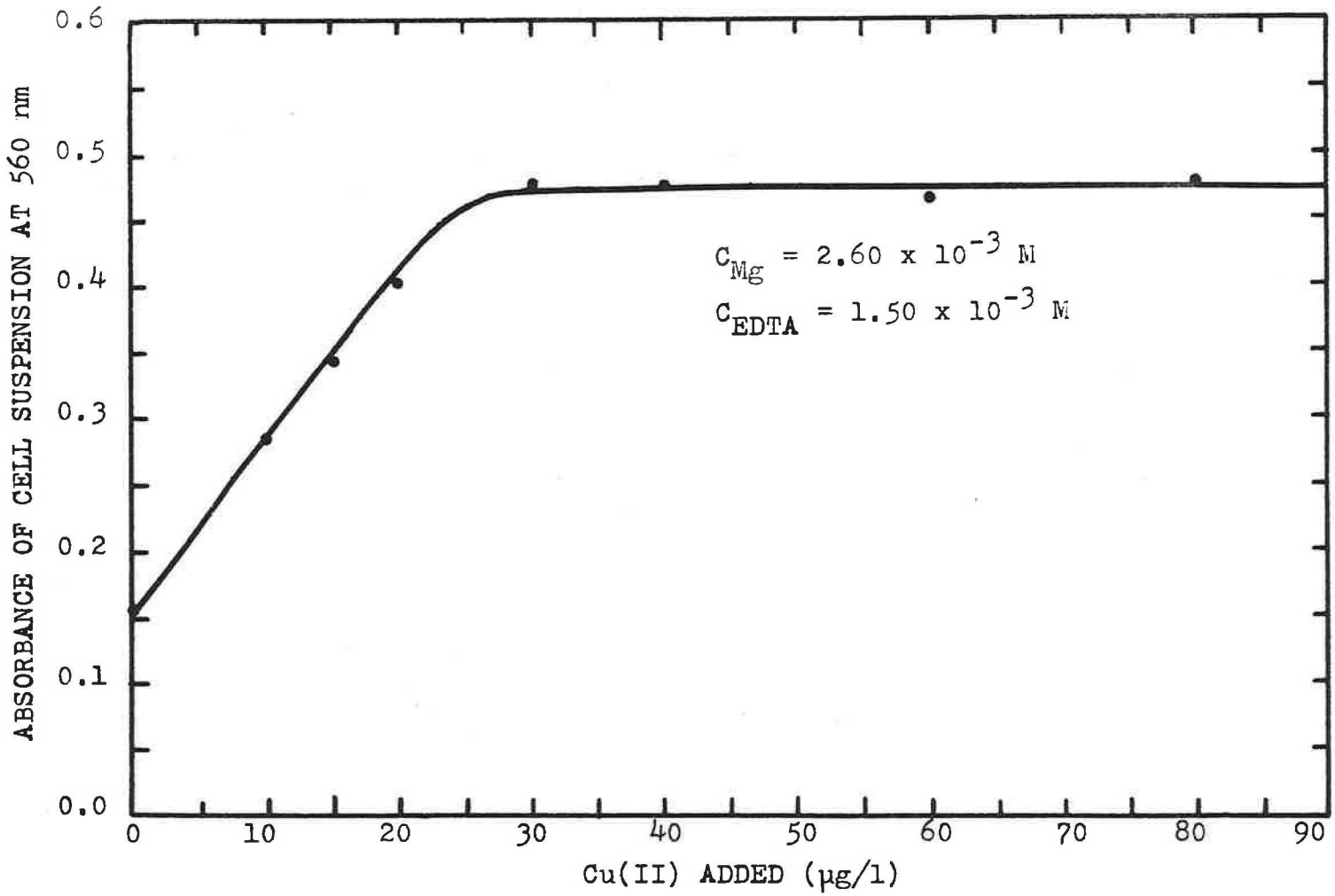


FIGURE 7. CELL YIELD CURVE FOR THE COPPER DEFICIENCY STUDY OF CHLORELLA VULGARIS AT A CONSTANT CONCENTRATION OF EDTA AND Mg(II).

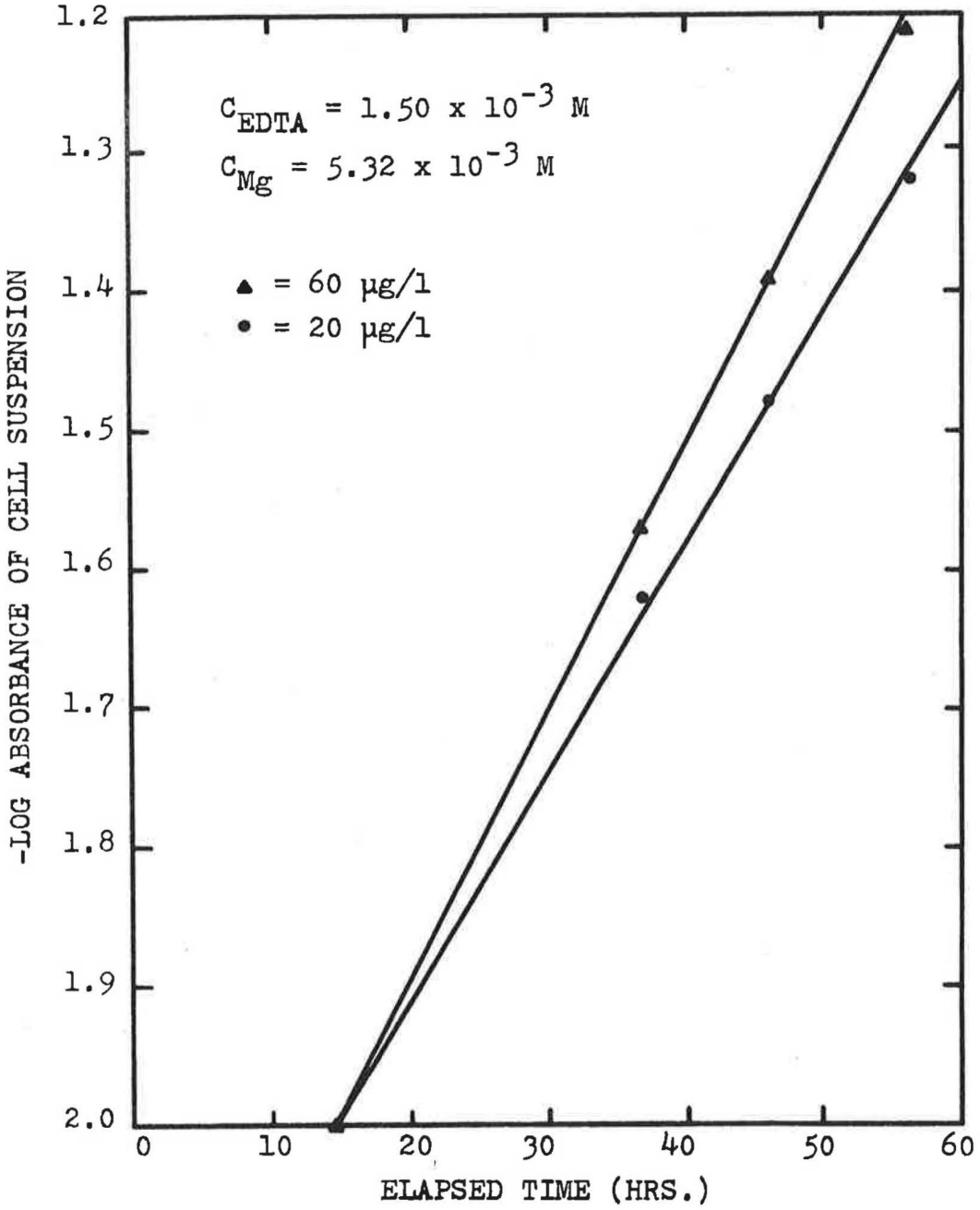


FIGURE 8. ABSORBANCE OF CELL SUSPENSION DURING THE EARLY PORTION OF THE LOG PHASE FOR CHLORELLA VULGARIS AT LIMITING AND OPTIMAL Cu LEVELS.

linear but the slope of the curve at 20 μg/l Cu is less than that at the optimal level of 60 μg/l. Growth relationships of this type are typical of those obtained for microorganisms in media containing a constant, but growth limiting concentration of nutrient. The data presented in Figure 8 were taken at a slightly lower light intensity than normal with a second experimental set up, and hence the maximum rate of growth presented is lower than that normally achieved.

Experiments of this nature are quite useful for discerning whether a particular trace metal is required for growth, but they do not generally establish a quantitative basis for a required trace element. Although the particular type of medium employed in this study in light of the mathematical development developed in Chapter V of this section gives the experimenter a high degree of confidence that the observations made are a result of a deficiency of the trace element studied, there are several copper species present in solution and any one or all could be "available" for use by algal cells. Increasing the total copper level in the medium increases both the level of $CuEDTA^{2-}$ and Cu^{2+}.

Additional evidence about the availability of the copper species in solution may be obtained by varying the chelate concentration in the medium. Since a level of 40 μg/l total Cu(II) is the minimum required by Oocystis for optimal growth at a total EDTA concentration of 1.50×10^{-3} M, one might

expect from a consideration of Figure 6 and equation I-6, that an increase in the total concentration of EDTA at a constant total concentration of 40 μg/l Cu or less would cause a corresponding decrease in the rate of algal growth. While the level of total copper and hence $CuEDTA^{2-}$ remains essentially constant under such conditions, the level of Cu^{2+} in the medium decreases considerably. A growth dependence of this nature would indicate the $CuEDTA^{2-}$ is generally unavailable for use by the organisms being studied and allow one to estimate the Cu^{2+} concentration required to support optimal growth.

In focusing attention on the copper species in solution it must be pointed out that as the EDTA concentration is increased, the free concentrations of all the chelated trace elements in the medium decrease, as does the free Cu^{2+} concentration. If the other chelated trace elements are present in sufficient quantity, however, the decrease in concentration of these species will not be sufficient to bring about a deficiency of the other trace elements in the medium. It is important to note that the concentration of total copper chosen is nearly growth limiting before the EDTA level is increased. When all the chelatable trace elements are present in sufficient quantity, increasing the level of chelate at a higher (say greater than 200 μg/l) copper level should have no effect on the growth of the organism. Furthermore, increasing the

total copper concentration at high concentrations of chelate and a growth limiting Cu^{2+} concentration should also increase the rate of growth if only Cu^{2+} is growth limiting.

The relationship between the concentration of Cu^{2+} and the level of EDTA in the medium is shown in Figure 9 where Term 2 from equation I-6 is plotted *vs.* the total concentration of EDTA at two total concentrations of Mg(II). In both cases there is a general decrease in free Cu^{2+} with increasing EDTA. In varying the chelate concentration from 1.50 to 2.50×10^{-3} M EDTA, the free Cu^{2+} concentration decreases from 4 to 6 fold depending upon the level of Mg(II) chosen. The values of Term 2 for two concentrations of Mg(II) at several total concentrations of EDTA are given in Table VIII.

Apparently, there is a dependence on the free Cu^{2+} level for the two algae studied as is shown in Figures 10 and 11 where the absorbance of the algal suspensions are plotted *vs.* the $[Cu^{2+}]$ calculated from equation I-6. The total concentration of EDTA was varied from 1.50 to 2.50×10^{-3} M in the *Chlorella* study and from 1.50 to 3.10×10^{-3} M in the *Oocystis* study at a total copper concentration of 30 µg/l. A free Cu^{2+} concentration above 1.2×10^{-16} M for *Chlorella* and 1.6×10^{-16} M for *Oocystis* produces optimal growth. The effect was demonstrated to be a function of copper by increasing the free copper level at high concentrations of chelate. Cell suspensions prepared by employing high total concentrations of

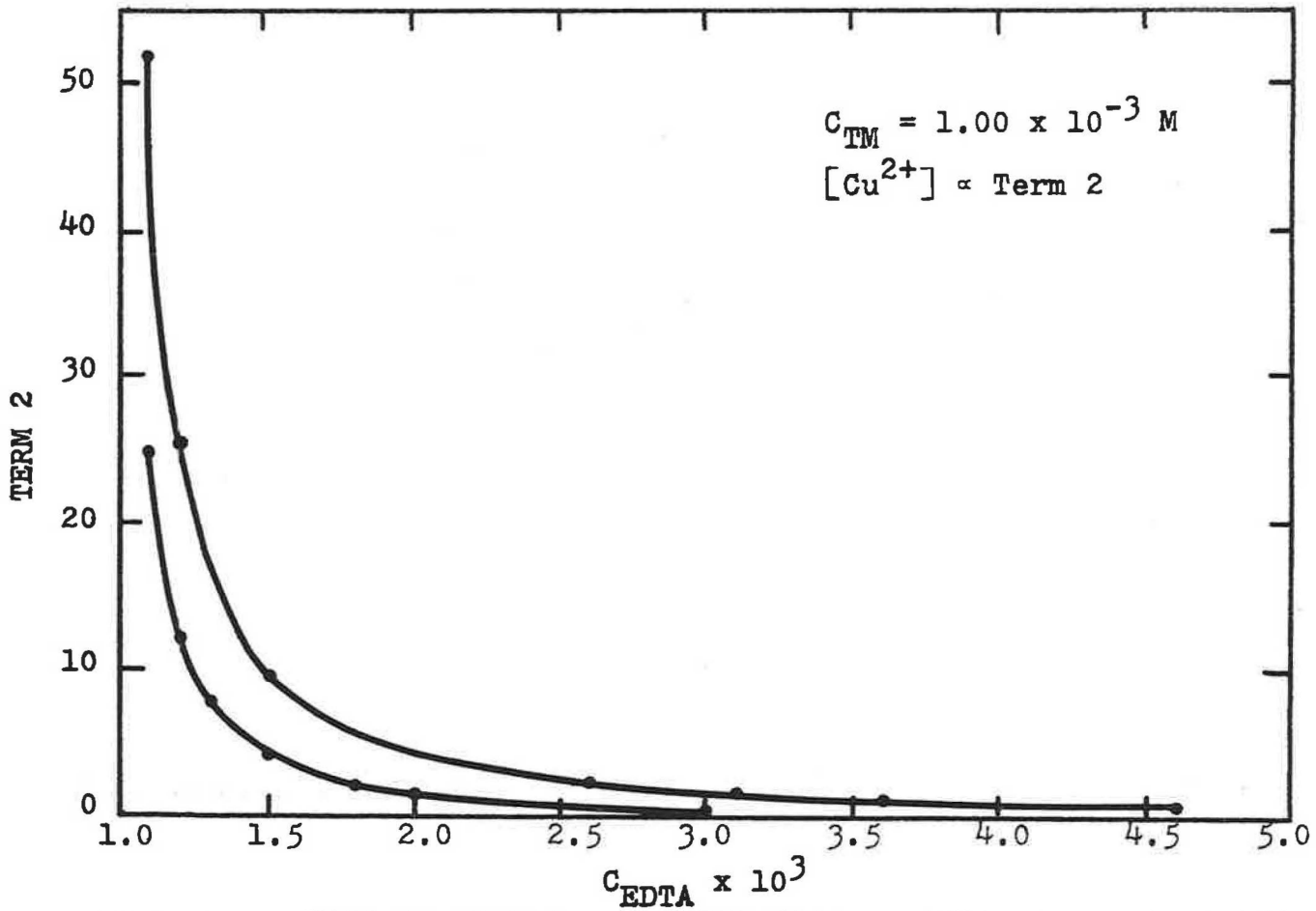


FIGURE 9. PLOT OF TERM 2 vs. TOTAL CONCENTRATION OF EDTA AT TWO LEVELS OF Mg(II).

TABLE VIII

VALUES OF TERM 2 IN EXPRESSION FOR THE FREE CONCENTRATION OF COPPER IN ALGAL MEDIUM AT VARYING LEVELS OF EDTA AND TWO FORMAL CONCENTRATIONS OF Mg(II)*

$C_{EDTA} \times 10^3$ (Moles/l)	Term 2 at $C_{Mg} = 5.32 \times 10^{-3}$ M	Term 2 at $C_{Mg} = 2.60 \times 10^{-3}$ M
1.10	52.2	25.0
1.20	25.5	12.0
1.30	16.7	7.67
1.40	12.3	5.50
1.50	9.64	4.20
1.60	7.87	3.33
1.70	6.60	2.71
1.80	5.65	2.25
1.90	4.91	1.89
2.00	4.32	1.60
2.10	3.84	1.36
2.20	3.43	1.17
2.30	3.09	1.00
2.40	2.80	0.86
2.50	2.55	0.73
3.00	1.66	0.30
3.50	1.13	0.04
4.00	0.77	——
4.50	0.52	——
5.00	0.33	——

*$C_{TM} = 1.00 \times 10^{-3}$ M

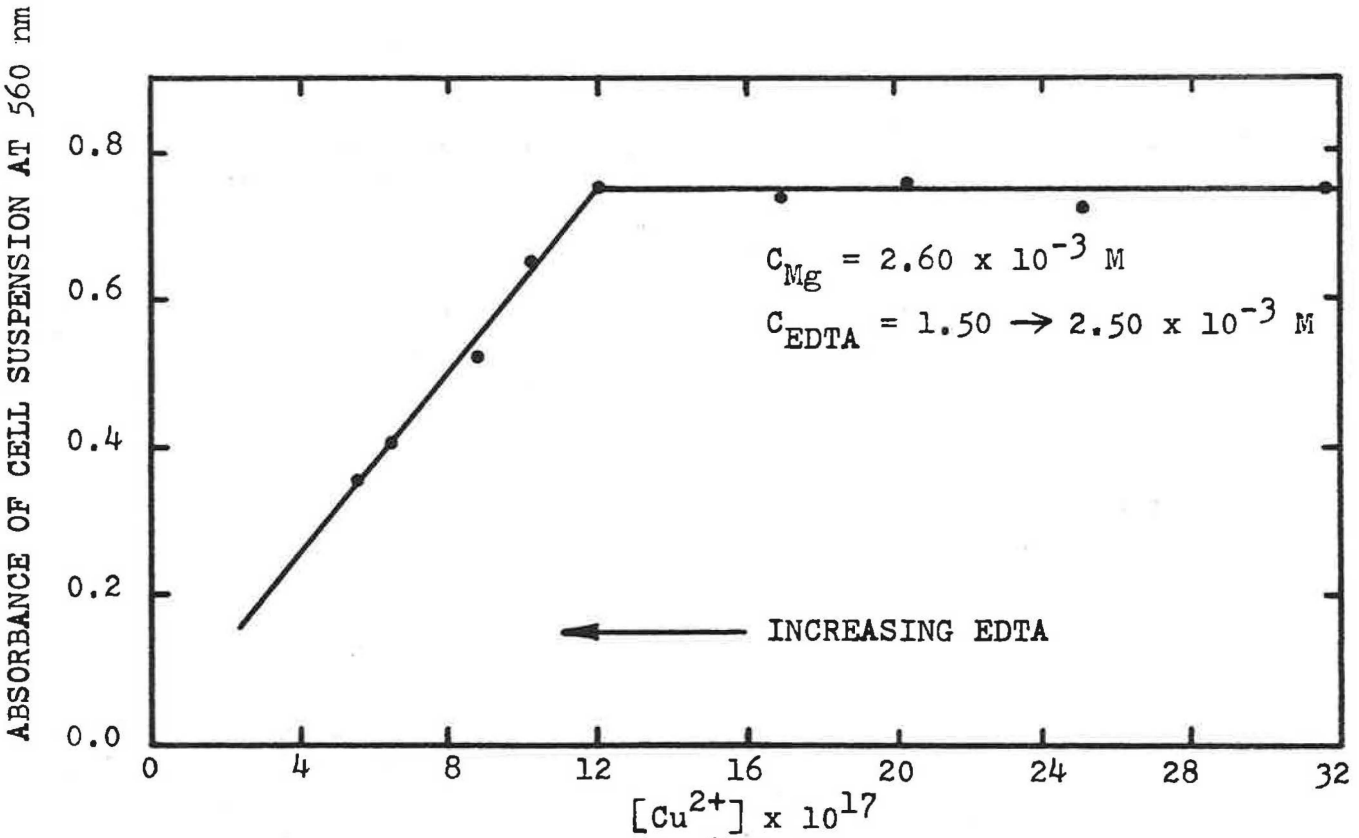


FIGURE 10. CELL YIELD vs. $[Cu^{2+}]$ CALCULATED FOR INCREASING EDTA LEVELS FOR CHLORELLA VULGARIS AT A CONSTANT TOTAL COPPER LEVEL OF 30 µg/l.

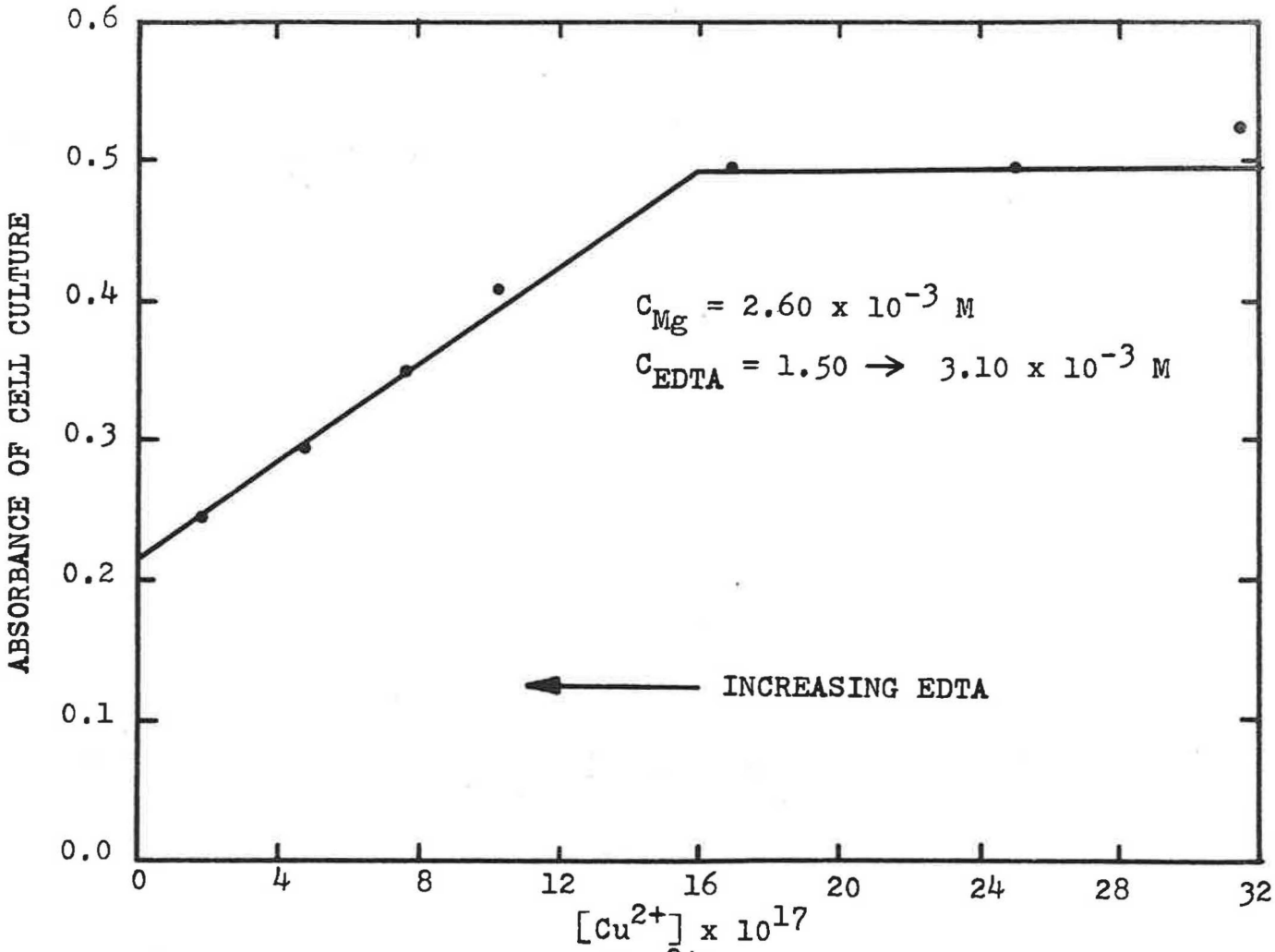


FIGURE 11. CELL YIELD vs. $[Cu^{2+}]$ CALCULATED FOR INCREASING EDTA LEVELS FOR OOCYSTIS MARSSONII AT A CONSTANT TOTAL COPPER LEVEL OF 30 µg/l.

both Cu(II) and EDTA but with free cupric ion concentrations corresponding to those in Figures 10 and 11 showed increased growth.

Furthermore, an increase in the concentration of chelate from 1.50 to 2.50 x 10^{-3} M at a much higher level of total copper causes no decrease in the rate of growth as is shown in Figure 12.

The role of natural and synthetic chelating agents found in aquatic systems is apparently quite important. In light of the very low copper levels required for optimal growth, it is possible that much of the difference between the so called "available concentrations" of the trace elements and their total concentrations may be explained by the presence of chelating agents. The data presented also suggest that the stimulatory effect of chelates on algal growth when added to natural waters is due to the creation of a reservoir of soluble trace metal species which allows the rapid replacement of the fraction of the element depleted by biological uptake. In addition, studies designed to illustrate the required levels of trace elements to algae must necessarily include the addition of a strong chelate so that the very low concentrations required can be studied in terms of much higher total levels of the medium components.

A study of the effects of EDTA on the toxicity of copper to Chlorella led to the collection of some rather surprising

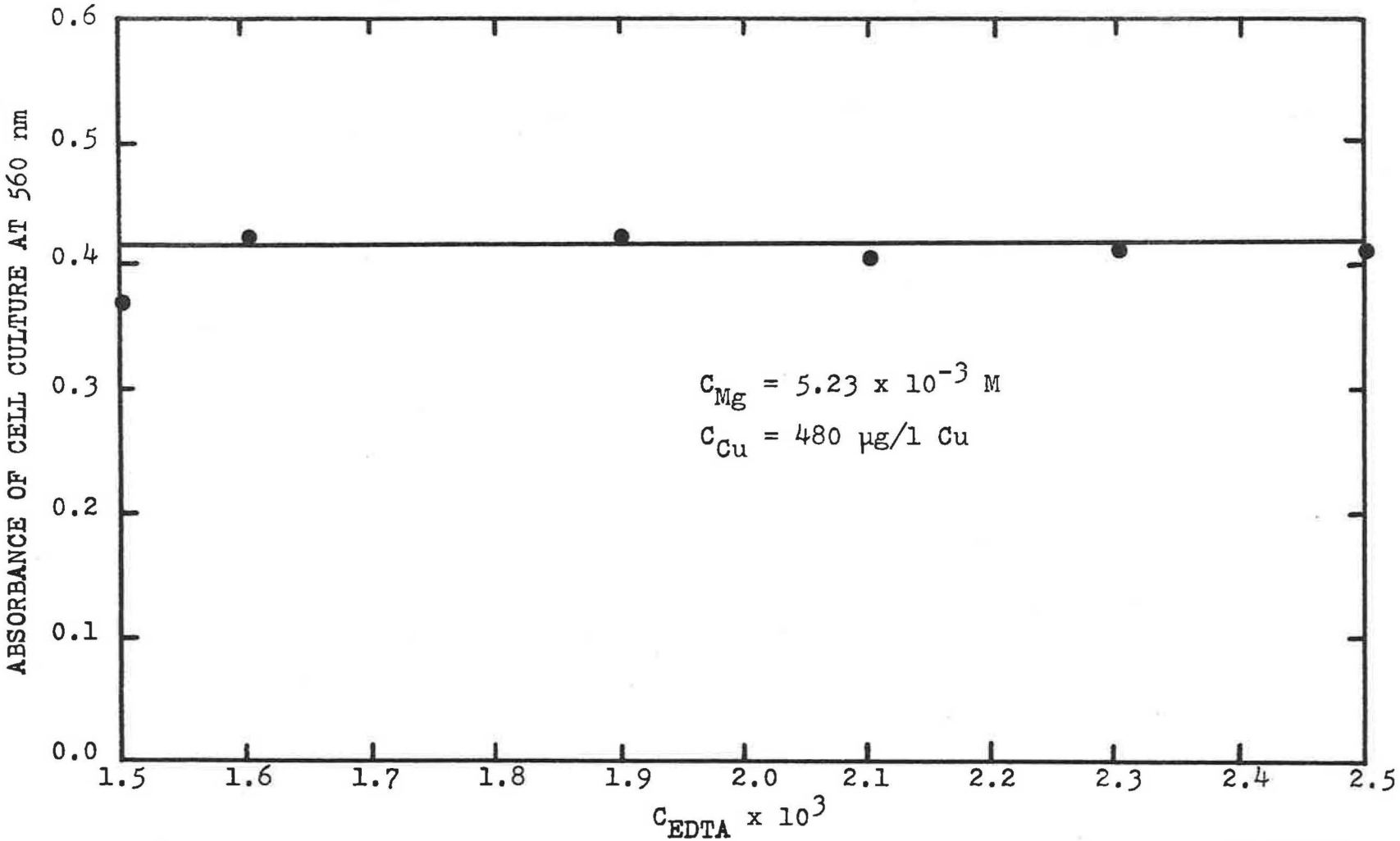


FIGURE 12. CELL YIELD FOR CHLORELLA VULGARIS AT A CONSTANT INTERMEDIATE TOTAL COPPER LEVEL OF 480 µg/l AND INCREASING EDTA.

data. Initially it was felt that a study similar to that conducted at deficient levels of copper might lead to some insight into the effects of the free copper fraction on the toxicity of copper. As the level of copper was increased, however, it was found that in the presence of EDTA sufficient to chelate any added copper, the rate of growth of Chlorella was unaffected by the concentration of total copper from 30 to 12,000 µg/l (1.89×10^{-4} M) Cu as is shown in Figures 13 and 14. At 12,000 µg/l Cu the total chelatable trace element concentration is 1.19×10^{-3} M while the EDTA level is 1.50×10^{-3} M. Figure 15 is a plot of the rate of growth of Chlorella in the log phase showing again that the rate of growth at 240 and 12,000 µg/l Cu was indeed identical. An additional run where copper concentrations up to 46,000 µg/l Cu were employed showed no toxic effects. In all cases, the medium was precipitate-free. From the above studies it is apparent that in the presence of EDTA, concentrations of copper that are normally growth inhibiting to algae have no effect on the rate of growth of Chlorella.

Since chelated copper was non-toxic at any environmentally realistic concentration, an additional study was undertaken in which the level of EDTA in the medium was reduced to exactly the total concentration of Fe(III). A 1.79×10^{-2} M (1000 mg/l Fe) stock Fe(III)EDTA solution was prepared by equilibrating 3.33 g of $Na_2EDTA \cdot 2H_2O$, 1.85 g $Fe_2(SO_4)_3$, and

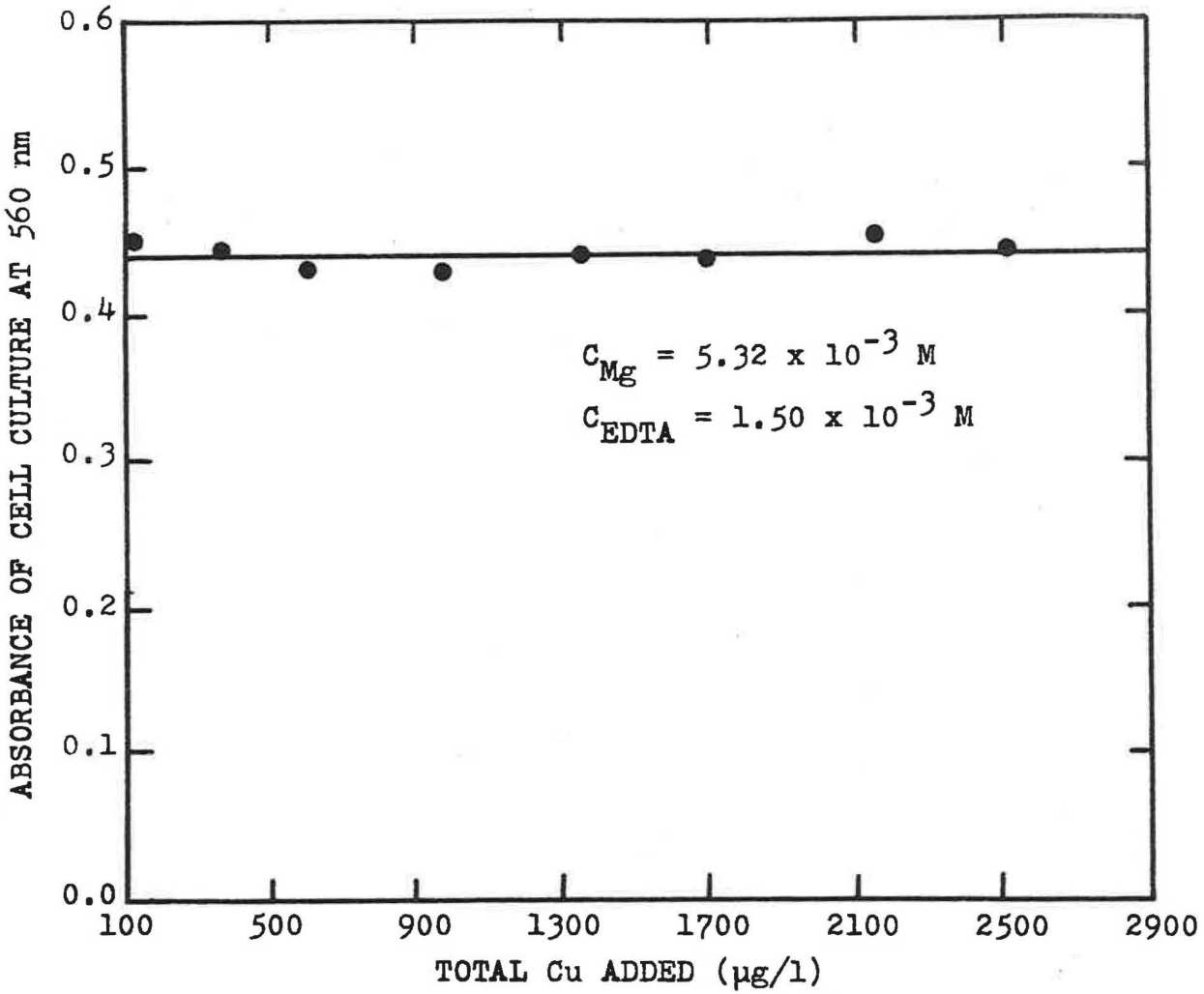


FIGURE 13. PLOT OF CELL YIELD vs. TOTAL COPPER ADDED AT INTERMEDIATE COPPER LEVELS FOR CHLORELLA VULGARIS AT A CONSTANT LEVEL OF EDTA AND Mg(II).

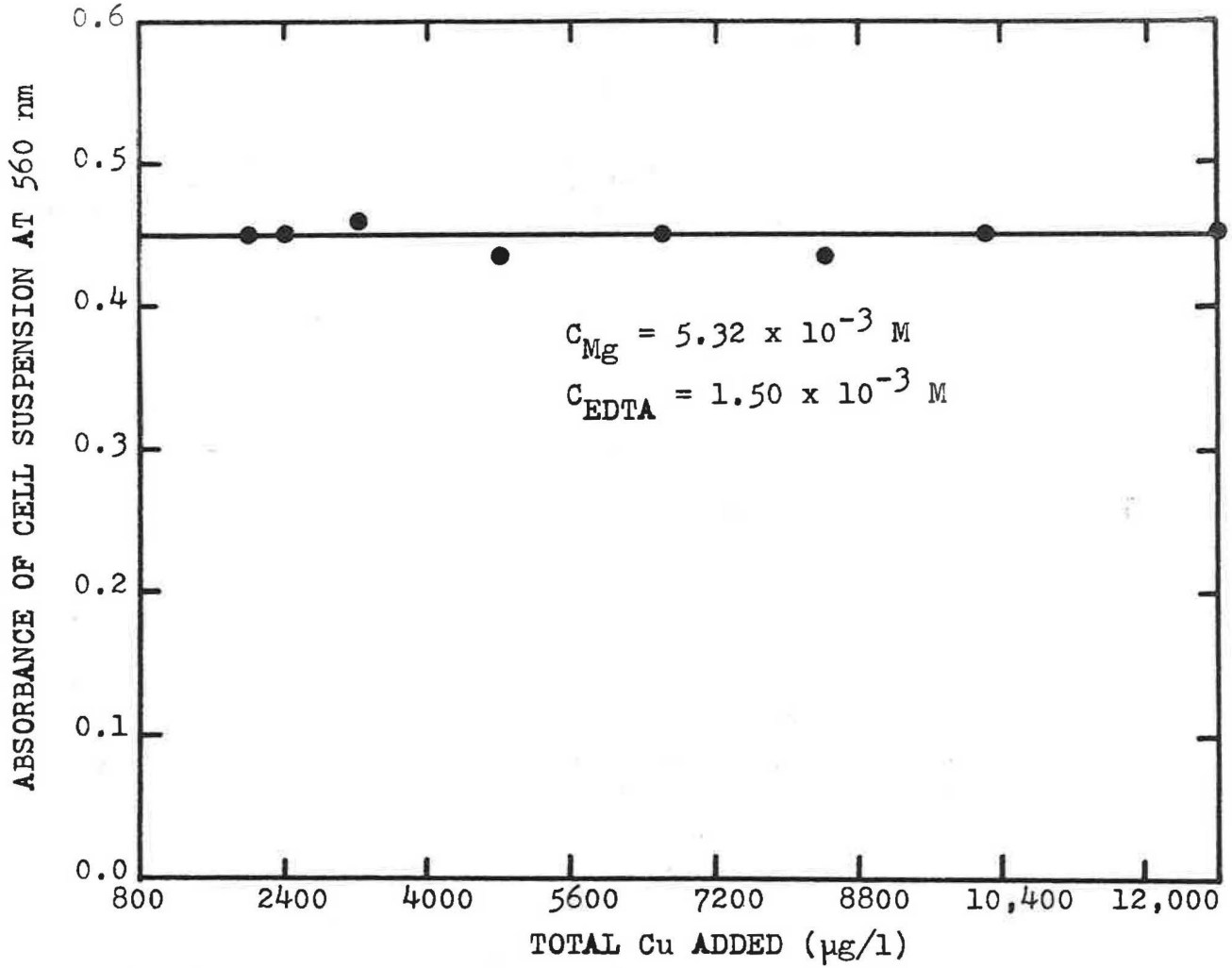


FIGURE 14. PLOT OF CELL YIELD vs. TOTAL COPPER ADDED AT HIGH COPPER LEVELS FOR CHLORELLA VULGARIS AT A CONSTANT LEVEL OF Mg(II) AND EDTA.

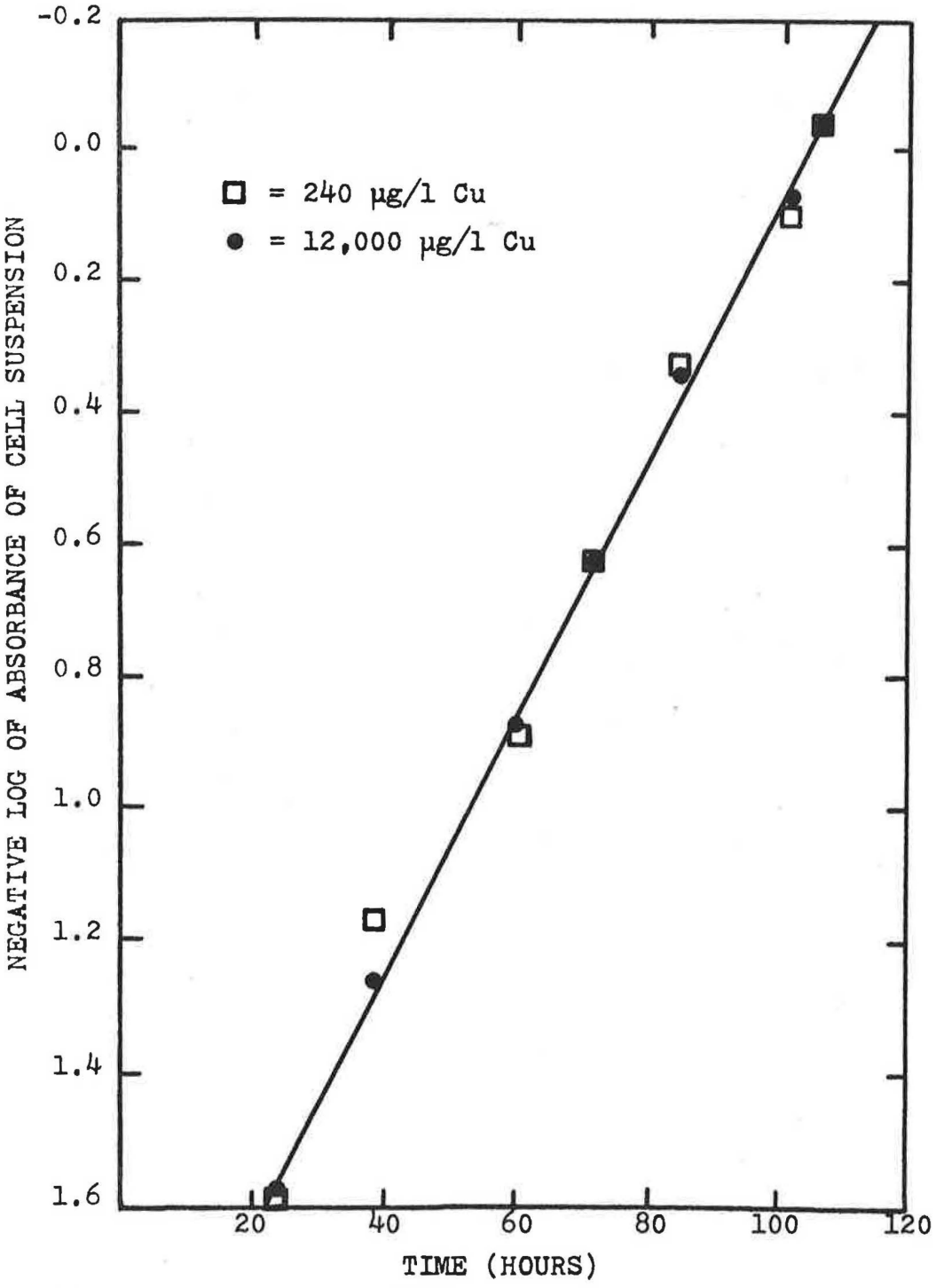


FIGURE 15. PLOT OF ALGAL GROWTH IN LOG PHASE AT TWO CONCENTRATIONS OF Cu(II) FOR CHLORELLA VULGARIS.

8.8 ml of 2.00 N KOH in ca. 450 ml of deionized, distilled water. Warming is necessary. A final pH readjustment of pH 6 was made with 2.00 N KOH and the mixture was allowed to re-equilibrate overnight. After dilution to the mark in a 500 ml volumetric flask, the suspension was filtered through a 0.45 μ Millipore filter and stored in a polyethylene bottle. The trace element solution was prepared as before except Fe(III)EDTA was substituted for the $Fe_2(SO_4)_3$ and $Na_2EDTA \cdot 2H_2O$ normally added. The growth medium was prepared as before except that the pH adjusting solution was omitted. The medium prepared at the reduced EDTA level is slightly turbid (ca. 88 per cent transmittance at 560 nm) and has a pH of 6.4 in equilibrium with air. Carbon dioxide supplied at the rate of 4 ml/min at an air flow rate of 500 ml/min produced an initial medium pH of 6.1, the same as that employed at higher EDTA levels in earlier work. Figures 16 and 17 show that total copper concentrations from 60 to 7000 μg/l exhibited little or no toxic effect in this medium, but at approximately 7000 μg/l the abrupt onset of copper toxicity was observed. A determination of the solubility of copper in this medium was made by atomic absorption analysis. After 24 hours of equilibration at pH 6.1, a 50 ml portion of medium containing 12.0 mg/l total copper was filtered through a 0.45 μ Millipore filter and quickly acidified with 0.1 ml of concentrated HNO_3. Subsequent analysis by atomic absorption revealed the

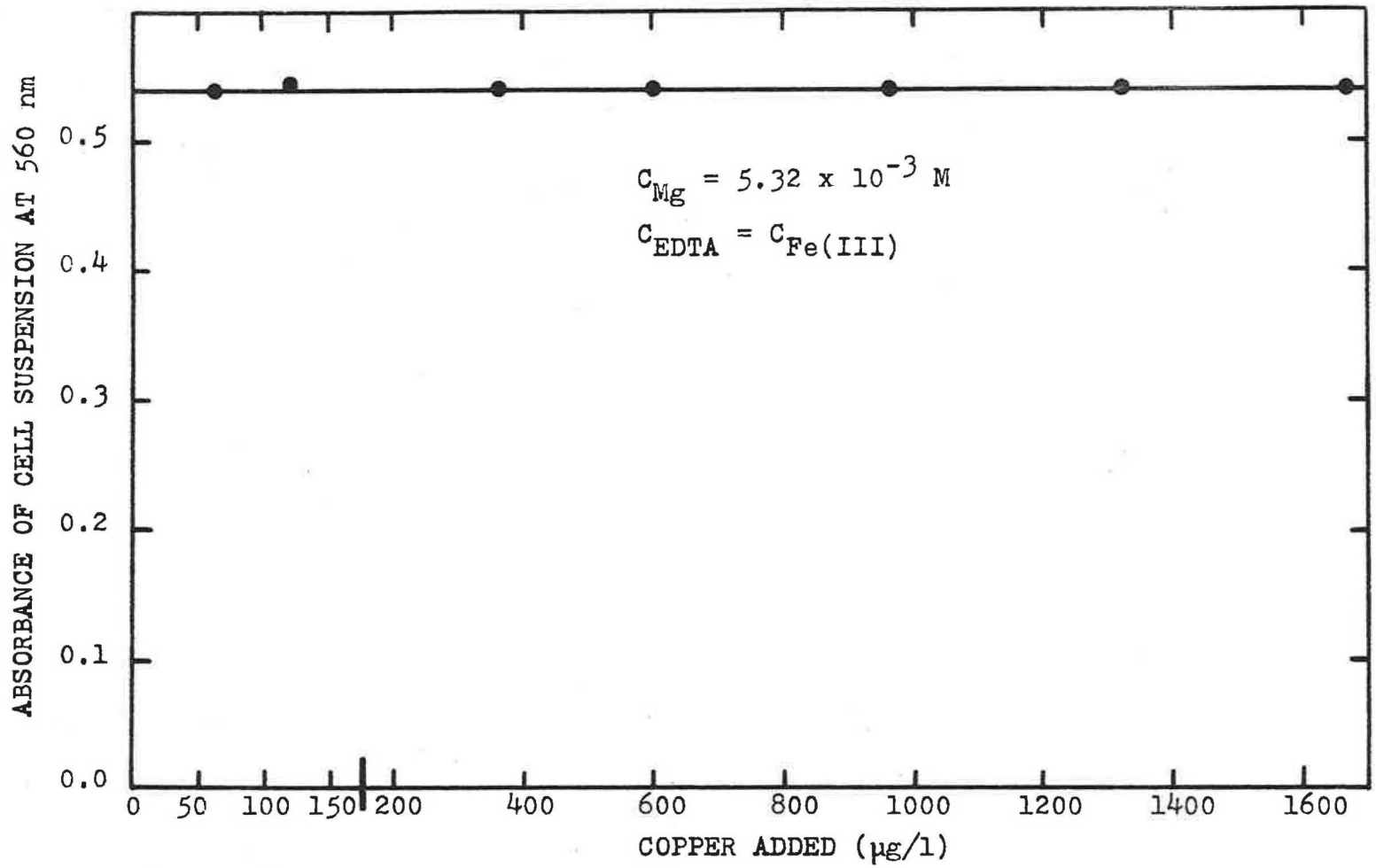


FIGURE 16. CELL YIELD vs. TOTAL COPPER ADDED AT INTERMEDIATE COPPER LEVELS FOR CHLORELLA VULGARIS AT A CONSTANT LEVEL OF EDTA AND Mg(II) IN A MEDIUM CONTAINING ONLY ENOUGH EDTA TO CHELATE Fe(III).

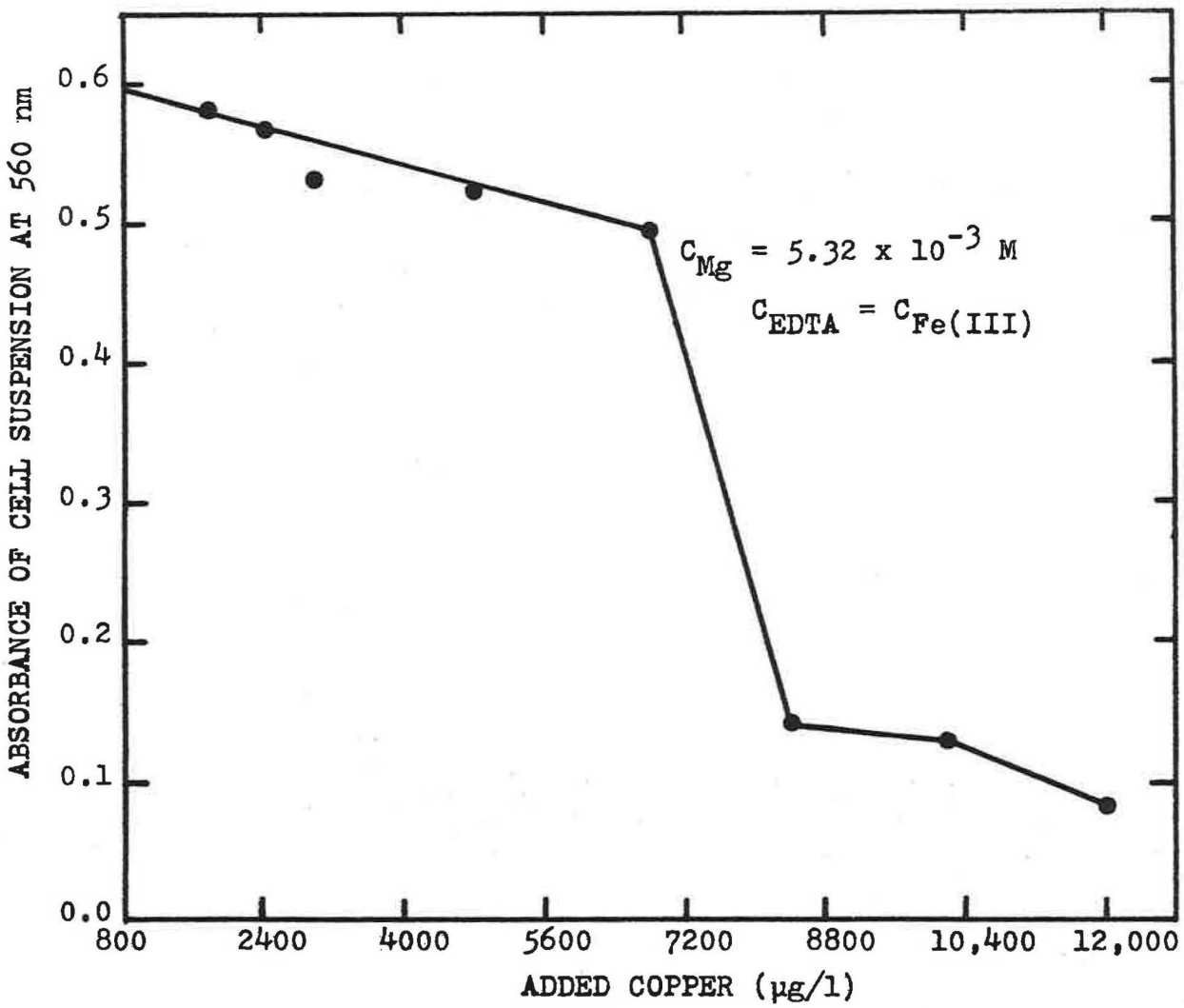


FIGURE 17. PLOT OF ABSORBANCE OF CELL SUSPENSION OF CHLORELLA VULGARIS IN GROWTH MEDIUM CONTAINING ONLY Fe(III)EDTA.

solubility of copper at pH 6.1 was approximately 7100 µg/l, almost exactly the concentration at which the onset of copper toxicity occurred. Although it is far from firmly established, the correlation between the toxicity of copper to Chlorella and the formation of insoluble copper species would seem to indicate a possible relationship between the two phenomenon. A further study of this aspect is to be made in the near futrue. It is also apparent that careful attention must be paid to the techniques and growth media employed when studies of trace element deficiencies and toxicities are to be directly compared.

CHAPTER IX

CONCLUSION AND SUMMARY - PART I

A reproducible copper requirement for Chlorella vulgaris and Oocystis marssonii has been demonstrated by employing a medium deficient in EDTA. Optimal growth was observed above 40 µg/l Cu for Oocystis and 30 µg/l Cu for Chlorella. Below these levels, the rate of exponential growth was demonstrated to be copper limited. By varying the chelate concentration in the growth medium at a growth limiting concentration of copper, a dependence of the growth of the two organisms on the free copper concentration was also demonstrated. Free Cu^{2+} concentrations above 1.2×10^{-16} M for Chlorella and 1.6×10^{-16} M for Oocystis produced optimal growth. The effect was demonstrated to be a function of free copper by increasing the free copper level at high concentrations of chelate.

A study of the effects of EDTA on the toxicity of copper to Chlorella showed that copper in chelated form was non-toxic to Chlorella at any concentration investigated (up to 46 mg/l Cu). At a reduced chelate concentration equal to exactly the concentration of Fe(III) in the medium, however, the toxic effects of copper were evident at 7.00 mg/l Cu. The solubility of copper under the conditions the experiment was run correlated well with the onset of toxicity, suggesting a relationship between the two phenomena.

The EDTA-deficient medium employed in this study enables simplifying the interpretation of the effects of trace elements on microorganism growth since the study of trace elements is often complicated by the medium employed. Complete culture media prepared in the absence of complexing agents necessarily contain insoluble forms of at least some of the required trace elements. In precipitate-free media containing chelating agents, the details of interrelated metal-chelate equilibria can be computed and understood. This lends strong credence to the belief that the physical symptoms developed as a particular trace element is eliminated from the medium are indeed a result of the deficient element and not due to other changes occurring within the system. The assumption has to be made that the presence of the chelating agent in the medium does not enhance the requirement for the trace elements, but in light of the very small concentrations required, this is unlikely. Precipitate-free media are also advantageous for the study of non-ionic toxic substances since the adsorption of these materials on insoluble matter may very well affect their availability.

The investigation of each trace element required for algal growth is an individual problem in itself due to the varying levels of most of the trace elements in the materials normally used for medium preparation. The demonstration that some of the more esoteric trace elements are required by the

algae is difficult since the addition of trace quantities of these materials as contaminants is difficult to avoid. Perhaps the only way to show such requirements is to employ media prepared from all ultrapure materials. To the authors's knowledge such a study has never been undertaken, although ultrapure trace element sources have been employed in several studies.

SECTION II

A DIRECT METHOD FOR THE DETERMINATION OF COPPER IN NATURAL WATERS USING THE CUPRIC ION-SELECTIVE ELECTRODE AND A MULTIPLE STANDARD ADDITION TECHNIQUE

CHAPTER I

ION-SELECTIVE ELECTRODES - INTRODUCTION

Although ion-selective electrodes are useful for a number of somewhat specialized applications, they have not come into general use in routine analytical work. This is largely due to the fact that these devices are not specific sensors and are usually subject to a number of interferences. Among the most selective of the group are the fluoride ion, cupric ion, and ammonia electrodes. Because of interference problems, the application of ion-selective electrodes to real samples where the exact nature of the sample matrix is not known has met with only limited success. Early work in this field was further complicated by the lack of a suitable reference electrode that was usable in a wide variety of samples. Over-zealous claims concerning the capability of these devices have discouraged extensive study of their applications in medicine, biology, environmental studies, and related fields.

The solid-state cupric ion electrode is among the most selective of the ion-selective electrodes and can be used to measure cupric ion levels of less than 1 µg/l under the proper conditions. Only silver ion, mercuric ion, ferric ion, and high levels of cadmium are serious interferences. In Section I it was demonstrated that at very low levels (20 to 40 µg/l Cu) copper is a required algal nutrient and

that at moderately higher levels it is toxic to the same organisms. As a result, copper is often applied to surface waters as copper sulfate to control algal populations. Soluble copper concentrations of 2000 µg/l or less are usually the maximum safe levels for fish, so the range over which the level of soluble copper may be used as an algicide in aquatic systems is quite narrow. Since a variable fraction of the copper applied to an aquatic system remains in solution, a routine analytical procedure adaptable to the on-site measurement of soluble copper would be of interest to persons concerned with water quality. The availability of the solid-state cupric ion-selective electrode makes the feasibility of such a technique an attractive possibility. This section describes a technique for the determination of copper in natural waters as well as a wide variety of electrolyte materials. Measurements of total copper at concentrations of less than 1 µg/l are easily made in a complexing antioxidant buffer (CAOB). Sample preconcentration or pretreatment is not necessary.

THE CUPRIC ION-SELECTIVE ELECTRODE

The sensing element of the cupric ion electrode is a mixture of cupric and silver sulfides. The level of free cupric ion in solution is determined by its effect on a low level of sulfide released by the electrode sensing element which in turn affects the free silver activity at the elec-

trode surface. Any metal ion which forms a more insoluble sulfide than cupric ion acts as a possible electrode interference by changing the free sulfide activity in equilibrium with the electrode sensing element. Therefore, ionic materials such as Hg(II) and Ag(I) must either be absent from the samples to be measured or present at very low levels. Ferric ion levels greater than one-tenth the expected cupric ion level also interfere.

In the absence of interfering substances, the cupric ion electrode responds to cupric ion activity. The electrode develops a potential proportional to the logarithm of the activity of free cupric ion in solution. At 25° C, it exhibits typical Nernstian response; approximately 29.6 mV for each ten-fold change in cupric ion activity. The electrode response is described by a modified form of the Nernst equation:

$$E = E_c + \frac{2.303\ RT}{2F} \log a_{Cu}2+ \qquad \text{(II-1)}$$

where: E = the observed electrode potential.

E_c = a "constant" term which is due to internal filling solutions, liquid junction potentials and the reference electrode used.

$\frac{2.303\ RT}{2F}$ = the Nernst factor (29.6 mV at 25° C).

$a_{Cu}2+$ = the cupric ion activity in the sample.

The free cupric ion concentration and the cupric ion activity are related by a parameter called the single ion

activity coefficient (f). The activity coefficient is a variable quantity and depends upon the total ionic strength of the sample (μ) in a manner predicted by the extended form of the Debye-Hückel equation:

$$\log f_i = \frac{-A\, Z_i^2\, \mu^{\frac{1}{2}}}{1 + \mathring{a} B\, \mu^{\frac{1}{2}}} \qquad \text{(II-2)}$$

where

$$\mu = \frac{1}{2} \Sigma\, C_i\, Z_i^2 \qquad \text{(II-3)}$$

In equation II-2, Z_i is the charge on the ion i, A and B are constants, and $\mathring{a}$ is the "ion-size parameter" which is the same order of magnitude as the ionic diameter of the ion in question. Values of $\mathring{a}$ may be found tabulated in the literature [47]. In the expression for the ionic strength, C_i and Z_i are the molarity and charge of each ionic species in solution. A summation is made for all ionic species present. The Debye-Hückel equation actually gives an activity coefficient on the mole fraction scale, but for dilute solutions the difference between the two scales is negligible. Equation II-2 may be used to accurately predict the relationship between activity and concentration up to ionic strengths of ca. 0.1 molar. At concentrations above 0.1 M, the single ion activity coefficient depends on the ionic composition of the solution and cannot be reliably predicted. Since f is a function of ionic strength, electrode measurements made at constant ionic strength may be correlated directly with stand-

ards of known concentration.

CHAPTER II

THE THEORY OF STANDARD ADDITION FOR MEASUREMENTS MADE WITH ION-SELECTIVE ELECTRODES

There are three basic measuring techniques commonly used in conjunction with ion-selective electrodes:

(1) Direct Potentiometry

(2) Potentiometric Titrimetry

(3) Standard Addition

Probably the most commonly used measuring technique of the three is direct potentiometry. In this technique a calibration curve (a plot of E vs. log concentration) is prepared using a medium as similar as possible to the sample medium. In order to overcome effects caused by variations in total ionic strength, the ionic strength of both sample and standard solutions are adjusted by adding a high level of noninterfering electrolyte. In this method, sample concentrations are determined by relating measured electrode potentials to a previously prepared calibration curve.

Cation measurements by direct potentiometry are especially difficult to make in samples where the exact nature of the sample matrix is unknown. For cation measurements made in samples containing complexing anions, electrode potentials are related to a calibration curve prepared in a medium in which non-complexing anions are substituted for the complexing anions found in the sample. Cation measurements

made at or near neutral pH values with cations that form very insoluble hydroxides pose a special problem unless a complexing agent is employed to keep the measured ions in solution. When stable metal-hydroxide complexes are formed, a suitable buffer must be employed to decrease the electrode pH dependence. This is especially important in samples containing carbon dioxide where stirring may cause large changes in pH.

Direct potentiometric measurements with ion-selective electrodes are further complicated by long term potential drift which is a problem inherent in these devices. While the electrode sensitivity to the sensed ion remains constant, with time there is a great tendency for changes to occur in the "constant" term in the Nernst-like equation describing the response of these devices. As a consequence, day-to-day changes in the measured potentials for identical standard solutions as high as $\pm$ 10 mV and typically $\pm$ 5 mV are common for divalent electrodes even when precise temperature control is employed. Changes in liquid-junction potentials with sample manipulation further decrease precision.

Ion-selective electrodes are not inherently precise measuring devices. Because they respond to concentration changes in a logarithmic manner, a small uncertainty in potential measurement is magnified into a comparatively large uncertainty in concentration. A potential measure-

ment made with an uncertainty of only 2 mV causes a relative concentration uncertainty of 8 per cent for a monovalent ion and 16 per cent for a divalent ion. For this reason frequent electrode recalibration is always necessary when direct potentiometry is employed with ion-selective electrodes.

Since ion-selective electrodes are not perfectly selective, they may also respond to other substances in solution as well as to the ion of interest. Direct potentiometric methods give no indication that interfering ions which are contributing to the measured electrode potential may be present. Measurements made under these conditions obviously lead to incorrectly determined concentrations.

In contrast to direct potentiometric methods, potentiometric titrations offer high accuracy and precision. Because the change in electrode potential with volume of standard solution added rather than the absolute value of the electrode potential is of interest in this technique, any uncertainty in the measured electrode potential is minimized. As a result, the precision and accuracy of a potentiometric titration often approaches that of the volumetric equipment employed. The endpoint of the titration reaction is signaled by a potential "break". Since the sharpness of the potential break is determined by the degree of reaction completeness, potentiometric titrations are not generally employed to determine trace quantities and only rarely are they

applied to solutions more dilute than 0.001 M.

An additional restriction that severly limits the application of titration prcedures to real samples is the requirement that the titrant react only with the material to be titrated. It is often the presence or absence of side reactions that determines the suitability of a titration procedure for a given analytical purpose. Titrimetric methods with ion-selective electrodes are relatively time consuming, although this disadvantage sometimes is overcome by using automated techniques.

The problems associated with potentiometric measurements made with ion-selective electrodes can be minimized by employing the technique of standard addition. Standard addition is a quite convenient method for the total concentration of individual substances even in very complex systems. The technique allows trace level determinations to be made in systems where many materials exist at high concentrations and in most instances can be used in the presence of complexing agents.

The technique of standard addition involves observing the change in electrode potential developed in a known volume of sample (V_o) upon the addition of a small volume of standard solution (V_s) containing a known total concentration of the ion being measured (C_s). The original sample concentration is then calculated from the observed change in

electrode potential. No calibration curve is required and only a knowledge of the electrode sensitivity to the material of interest is necessary. Since the technique requires only the addition of a standard solution, it is applicable to all concentration ranges over which the electrode employed is responsive. When standard addition is used for trace analysis, sample contamination is minimized because the electrodes need not be moved from solution to solution.

The successful standard addition step is normally completed in a manner so that:

(1) The addition of standard solution causes an insignificant change in the original sample volume.

(2) The addition of standard solution causes an insignificant change in ionic strength.

(3) The fraction of electroactive ion that is complexed remains unchanged as the addition of standard is made.

(4) Electrode interferences are not present in amounts that will affect electrode response.

(5) The electrode employed responds in a Nernstian manner in the concentration region where measurements are being made.

Restriction three is the most serious limitation to the standard addition technique. In samples containing complexing agents, only the free metal ion fraction is sensed by an ion-selective electrode. The fraction of metal ion M that is uncomplexed in the presence of the complexing species X, Y, ... is given by:

$$\phi = [1 + B_{1,x}C_x + B_{2,x}C^2{}_x + B_{3,x}C^3{}_x + \ldots + B_{1,y}C_y + B_{2,y}C^2{}_y + B_{3,y}C^3{}_y + \ldots]^{-1} \quad \text{(II-4)}$$

where $C_x + C_y$ are the concentrations of free ligands X and Y. The B terms are the over-all formation constants for the series of complexes MX_1, MX_2, MX_3, ... and MY_1, MY_2, MY_3, ... Since ϕ can only be kept constant by keeping the free concentration of ligands essentially unchanged, the concentration of ligands present must be in large excess of M so that the amount of ligand consumed by the addition of a standard solution of M will be small in comparison to the total free ligand concentration in the sample. This difficulty can be overcome experimentally by purposely adding a large excess of a strong complexing agent to the samples to be analyzed assuming that the activity of the sought-for ion is not lowered below the limit of electrode response.

The first step in employing the standard addition technique with a cation-selective electrode is to measure the initial electrode potential in a sample before the addition of standard. The electrode response to the initial free concentration ([M]) of the ion being measured is:

$$E_o = E_c + S \log [M] \quad \text{(II-5)}$$

where E_o = the measured initial electrode potential.

E_c = the portion of the total potential due to references and internal solutions.

S = an experimentally determined electrode sensi-

tivity to the ion M; or the Nernst factor, 2.303 RT/ZF where R and F are constants, T is the temperature in degrees Kelvin, and Z is the charge on the ion M.

The above expression may be related to the total initial concentration of M through equation II-4. Because the free concentration of M can always be expressed as some fraction of the total soluble concentration of M, equation II-5 can be rewritten as:

$$E_o = E_c + S \log \phi_o C_o \qquad \text{(II-6)}$$

where C_o is the initial total (free + complexed) concentration of M.

The next step is to add a small known volume of standard M solution. The final total concentration of M is then the sum of the initial concentration and the change in concentration induced through the addition of standard, ie.,

$$C_f = \Delta C + C_o \qquad \text{(II-7)}$$

The final electrode potential is:

$$E_f = E_c + S \log \phi_f C_f \qquad \text{(II-8)}$$

On taking the difference between the initial and final potentials, the E_c term subtracts out, and

$$E = E_f - E_o = S \log \left[\frac{\phi_f C_f}{\phi_o C_o}\right] \qquad \text{(II-9)}$$

If the fraction of the total concentration of M which is free is not changed by the addition of standard, then $\phi_f = \phi_o$ and equation II-9 becomes:

$$\Delta E = S \log \left[\frac{C_f}{C_o}\right] \qquad \text{(II-10)}$$

Substituting the value of C_f from equation II-7 and dividing equation II-10 by S yields:

$$\frac{\Delta E}{S} = \log \left[\frac{\Delta C}{C_o} + 1\right] \qquad \text{(II-11)}$$

Letting Z = antilog $\frac{\Delta E}{S}$ and taking antilogarithms, equation II-11 becomes:

$$Z - 1 = \frac{\Delta C}{C_o} \qquad \text{(II-12)}$$

The change in concentration induced by the addition of standard is related to the concentration of standard by:

$$\Delta C = \frac{V_s C_s}{V_o + V_s} \qquad \text{(II-13)}$$

where C_s is the concentration of the standard solution, V_s is the volume of standard added, and V_o is the initial volume of sample.

Equation II-13 may be simplified if the volume of standard added is kept small in comparison to the initial volume of sample:

$$\Delta C = \frac{V_s C_s}{V_o} \qquad \text{(II-14)}$$

This requirement is easily met in applying standard addition to trace analysis where microliter additions of standard are conveniently made. Substituting the above expression into equation II-12 yields a function suitable for

the calculation of the original concentration (C_o) in the sample:

$$Z - 1 = \frac{C_s}{C_o V_o} \cdot V_s \qquad \text{(II-15)}$$

The above expression may be solved directly for C_o from the observed ΔE induced through a single addition of standard. Under such conditions, however, a standard addition analysis yields no more information about electrode interferences than does direct potentiometry. An alternate procedure is to perform a series of additions and prepare a plot of Z - 1 vs. V_s. Some information about electrode interferences (see Chapter III, this section) as well as a substantial improvement in precision may be obtained by performing a series of additions. A plot of Z - 1 vs. V_s will, therefore, be linear with a zero intercept if the analysis is carried out successfully. The slope of the resulting curve is then used to calculate the original sample concentration. The value of Z is calculated from the over-all change in potential at each point plotted, ie., $\Delta E_i = E_i - E_o$ not $E_i - E_{i-1}$. The precision to which C_o can be determined depends directly upon how well the values of C, V_s, V_o, ΔE, and S are known. The concentration and volume of standard as well as the initial volume of sample are easily determined. The greatest difficulty lies in determining accurate values of ΔE and S. As a consequence, ΔE values are usually made as large as possible,

but not so large as to affect the sample matrix. A reasonable compromise is to induce a potential change corresponding to an approximate 50 to 100 per cent change in the original sample concentration. The relationship between the change in electrode potential and per cent change in total concentration at 25° C is given in Table IX.

TABLE IX

THE RELATIONSHIP BETWEEN THE CHANGE IN ELECTRODE POTENTIAL AND PER CENT CHANGE IN TOTAL CONCENTRATION INDUCED AS A RESULT OF STANDARD ADDITION AT 25° C*

$\Delta E_{Divalent(mV)}$	$\Delta E_{Monovalent(mV)}$	% Change In Concentration
0.5	1.0	4.0
1.0	2.0	8.1
1.5	3.0	12.4
2.0	4.0	16.6
2.5	5.0	21.5
3.0	6.0	26.3
3.5	7.0	31.3
4.0	8.0	36.5
4.5	9.0	42.0
5.0	10.0	47.6
7.5	15.0	79.3
10.0	20.0	117.8
25.0	50.0	600.0

*Calculated from: % Change in $C = \frac{\Delta C}{C} \cdot 100 = (Z - 1) \cdot 100$, assuming theoretical slope of 59.16 mV for a monovalent electrode and 29.58 mV for a divalent electrode at 25° C

CHAPTER III

THE DETECTION OF ELECTRODE INTERFERENCES BY MEANS OF THE MULTIPLE ADDITION TECHNIQUE

In the presence of interfering substances, the response of the liquid ion exchanger (NO_3^-, ClO_4^-, *etc.*) and glass electrodes (H^+, Na^+, K^+, Ca^{2+}, *etc.*) is described by a modified form of equation II-1 [48]:

$$E = E_c + S \log (a + K_i a_i^{\frac{Z}{Z_i}}) \qquad \text{(II-16)}$$

where a is the activity of the primary ion the electrode is designed to measure, a_i is the activity of the interfering ion, K_i is the "selectivity constant" for the interfering ion, Z_i is the charge on the interfering ion, and Z is the charge on the primary electroactive ion. The other terms have been described previously. For two ions of the same charge, if K_i were exactly 1, the electrode would respond as well to the interfering ion as it does to the primary ion. Under these conditions if $a = a_i$, half of the electrode response is determined by each ion. However, it is found experimentally that the selectivity constant is not a true constant and varies to a certain extent if the concentration of the primary ion is varied widely [49]. In spite of this, selectivity constants are extremely useful in deciding whether a given glass or liquid ion exchange membrane electrode can be used for a particular application. For small

changes in the concentration of primary ion, K_i is approximately constant and equation II-16 describes the response of these devices quite well.

From a practical analytical standpoint it is not as important to know the nature of electrode interferences as it is to be able to detect them during routine analyses. Assuming the response of a liquid exchanger or glass electrode in a sample containing an interfering ion i can be related with fair accuracy to equation II-16, the presence of the interfering ion cannot usually be detected when multiple standard addition is employed. Consider a cell containing an electroactive ion and a single interfering ion where multiple standard addition is to be employed at constant ionic strength. The initial cell potential is described by:

$$E_o = E_c + S \log (C_o + K_i C_i) \qquad \text{(II-17)}$$

If a volume of standard V_s is added which is negligible in comparison to the sample volume V_o, C_i remains essential constant and the final potential is described by a similar expression:

$$E_f = E_c + S \log (C_f + K_i C_i) \qquad \text{(II-18)}$$

Subtracting equation II-17 from equation II-18, dividing by S, and substituting equation II-7 for C_f yields:

$$\frac{\Delta E}{S} = \log \left[\frac{\Delta C + C_o + K_i C_i}{C_o + K_i C_i} \right] \qquad \text{(II-19)}$$

Taking antilogs and rearranging gives the following expression:

$$Z = \frac{\Delta C}{C_o + K_i C_i} + \frac{C_o + K_i C_i}{C_o + K_i C_i} = \frac{\Delta C}{C_o + K_i C_i} + 1 \quad \text{(II-20)}$$

or

$$Z - 1 = \frac{\Delta C}{C_o + K_i C_i} = \frac{C_s}{V_o(C_o + K_i C_i)} \cdot V_s \quad \text{(II-21)}$$

Interferences of this type will, therefore, yield multiple addition plots which are linear and have a zero y-intercept. Except for a high apparent concentration of anlyte, such a plot will appear normal in all respects. Under these conditions, electrode interferences cannot be detected by multiple addition techniques in samples of varying composition. In some cases K_i may vary with C_f to such an extent that multiple addition plots are non-linear. When non-linear plots are observed, interferences should be suspected.

The multiple addition technique should be a valuable tool in evaluating the effects of interfering substances in model systems where the concentration of interfering ion is known. A single slope measurement yields the value of K_i which may be determined under conditions very similar to those to be expected in actual samples.

With solid-state electrodes, interferences are quite different and usually involved surface reactions that convert one of the components of the sensing element to a second

substance. As a result, the sensitivity of the electrode to the ion being measured changes. An overriding characteristic of interferences at solid-state electrodes is its abrupt onset when the ratio of interfering ion to the activity of the primary ion exceeds a critical value that is sometimes predictable from solubility considerations. Below the predicted value, no interference occurs, while above this value, the electrode response to the primary ion may be impaired. This behavior is to be contrasted with the behavior of liquid and glass membrane electrodes that show a gradual increase in the level of interference with increasing concentration of interfering ion. Because interferences at solid-state electrodes occur abruptly at discrete levels, a multiple addition plot will be non-linear in the presence of these substances. Therefore, electrode interferences can be detected easily when multiple standard addition is employed.

The response of the mixed cupric-silver sulfide membrane electrode to cupric ion is dependent upon the presence of CuS at the electrode surface and the establishment of an equilibrium with Cu^{2+} in the sample solution. The sample Cu^{2+} level controls the activity of Ag^{+} which is sensed at the electrode surface through the intermediate S^{2-} species released by the electrode [50]:

$$Cu^{2+} + Ag_2S \longrightarrow CuS + 2Ag^{+} \qquad \text{(II-22)}$$

In order for an interfering ion to displace copper from the above equilibrium, the ion activity ratio in solution must exceed the value given by the solubility products of the copper and the interfering metal sulfides. The calculation of electrode interference levels based upon solubility considerations may be too conservative in some cases, especially in solutions containing very low concentrations of interfering ion. An interfering ion reaching the CuS - Ag_2S membrane surface will react to convert a small amount of CuS to the corresponding more insoluble sulfide. Until the entire electrode sensing surface is converted, the electrode will continue to respond to the sample cupric ion activity.

Strong complexing agents and oxidizing agents may also cause measurement problems with the cupric ion electrode. Complexing agents interfere by causing the electrode to give erroneously high values of analyte. Oxidizing agents may cause electrode instability by oxidizing the low level of sulfide in equilibrium with the electrode sensing element.

CHAPTER IV

CHOICE OF BUFFER

The complexing antioxidant buffer (CAOB) chosen for use in this study was designed to effectively reduce electrode interferences from ferric ion and oxidizing agents and at the same time permit rapid and reliable low level copper measurements to be made. The formation of insoluble cupric hydroxides, carbonates, and phosphates greatly limits the pH range over which reliable cupric ion measurements can be made successfully. Low level measurements are usually restricted to systems yielding pH values of approximately 7 or less.

The choice of a buffering medium for cupric ion measurements also involves a number of considerations other than the desired pH. They include the following:

(1) Chemical compatibility with the electrode measuring system.

(2) Buffering capacity against the addition of both acid and base.

(3) A moderate complexing ability to decomplex Cu^{2+} from low levels of complexing materials found in natural water samples. (Low levels of complexing agents may cause non-linear multiple addition plots if the fraction of ion complexed changes upon the addition of standard.)

(4) The ability to prevent the precipitation of $Fe(OH)_3 \cdot X\ H_2O$ from ground water samples. (The precipitation of this material coats the copper electrode sensing element and causes the loss of Cu^{2+} from water samples through coprecipitation of Cu^{2+}.)

(5) The availability of buffer materials of ultrapure quality to prevent low level Cu^{2+} contamination from the buffer components themselves.

A buffer material that would seem to fulfill all of the above requirements is disodium ethylenediaminetetraacetic acid (Na_2EDTA). Unfortunately, at very low levels of Cu^{2+}, the cupric ion electrode does not function properly in this medium. The effect of EDTA is to increase the lower limit of detection of the electrode and high values for the sample copper level are reported. A multiple addition plot for the determination of copper in a 1.00×10^{-3} M Na_2EDTA solution is given in Figure 18. An atomic absorption analysis of a similar, but more concentrated EDTA solution reveals that the more dilute solution contains less that 1 µg/l total copper while the multiple addition plot indicates that over 2 mg/l total copper is present. A similar, although less marked effect, is observed in acidic solutions. Multiple addition plots for background copper in 0.100 N and 0.0100 N ultrapure acetic acid solutions is given in Figure 19. The level of copper in the 0.100 N solution is less than 1 µg/l.

A phosphate buffer system was also investigated. The response of the cupric ion electrode at pH 7.0 in phosphate buffer was satisfactory for the measurement of low copper levels in synthetic samples containing copper only. The precipitation of metal phosphates from water samples, however, was an annoying problem. Further investigation of this buffer

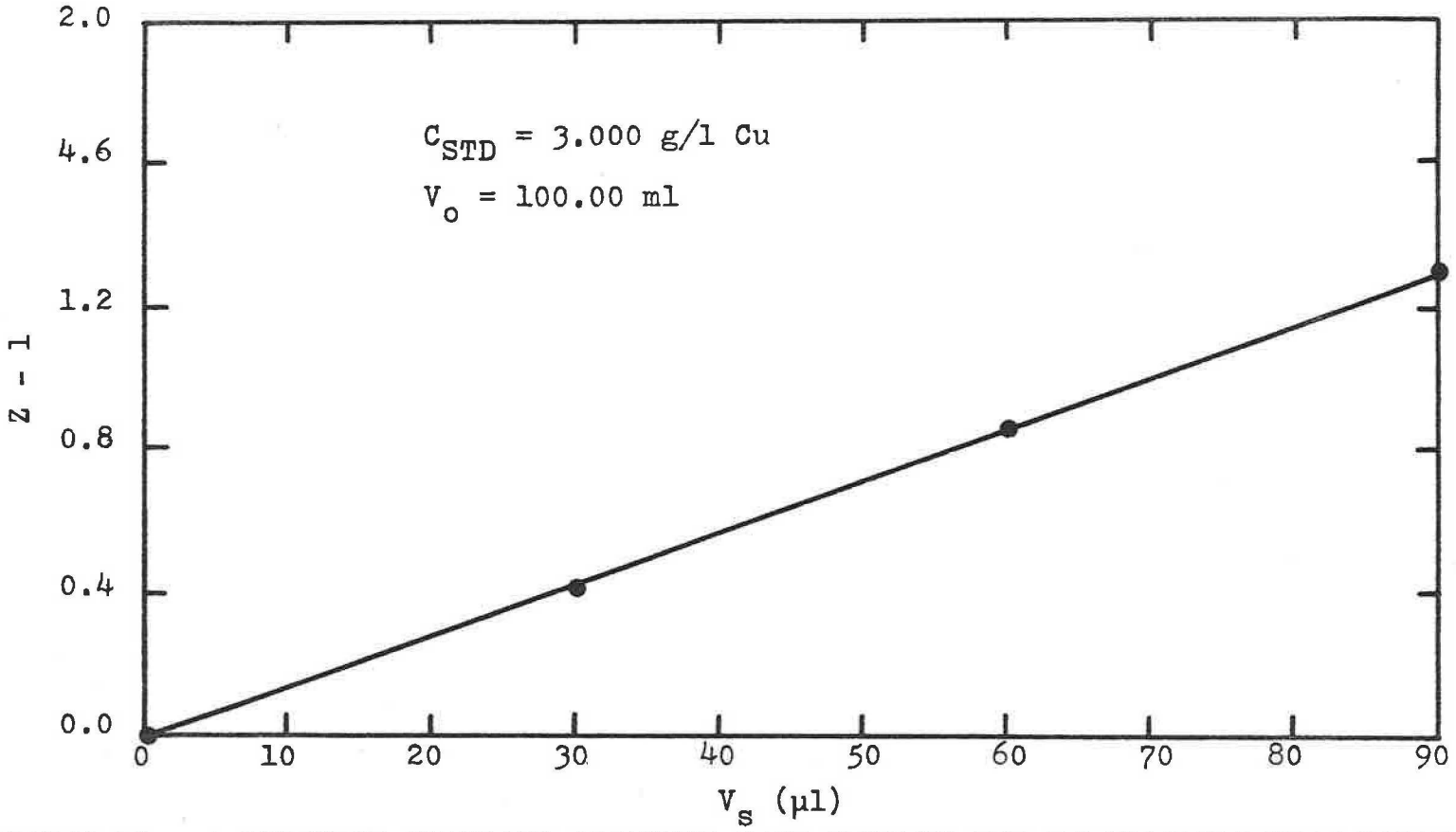


FIGURE 18. A MULTIPLE STANDARD ADDITION PLOT SHOWING THE ERRONEOUSLY HIGH VALUE FOR COPPER AS DETERMINED USING THE CUPRIC ION ELECTRODE IN 1.00×10^{-3} M REAGENT GRADE Na_2EDTA. $C_o < 1$ µg/l Cu BY ATOMIC ABSORPTION ANALYSIS.

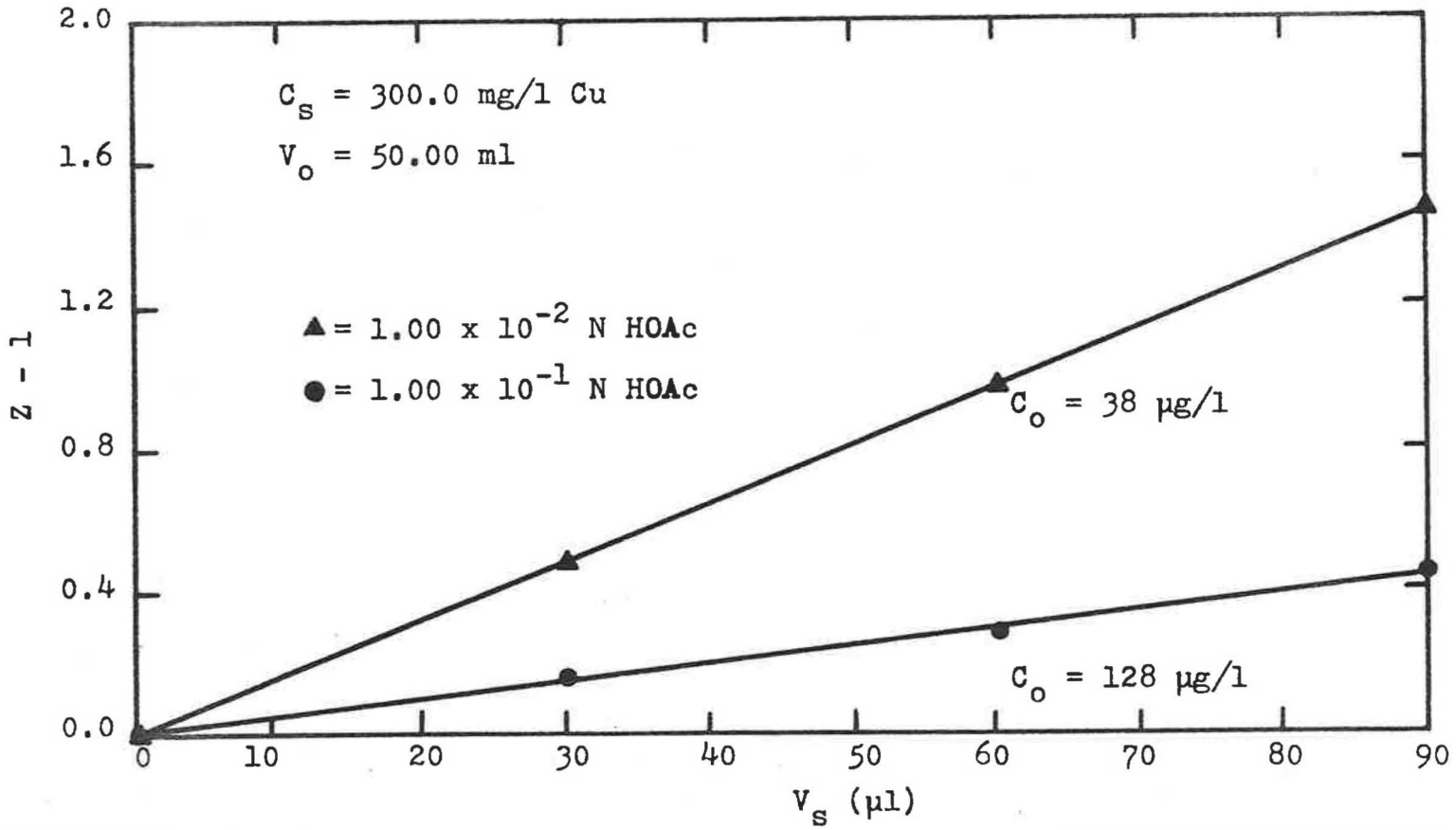


FIGURE 19. A MULTIPLE STANDARD ADDITION PLOT SHOWING THE ERRONEOUSLY HIGH VALUE FOR ANALYZED COPPER AS DETERMINED USING THE CUPRIC ION ELECTRODE IN ULTRAPURE ACETIC ACID. LOT ANALYSIS INDICATES < 1 µg/l Cu IN 0.100 N HOAc SAMPLE.

system was discontinued.

The complexing antioxidant buffer solution finally chosen for use in this study is a pH 4.8 ultrapure acetate buffer containing sodium fluoride and formaldehyde. Total copper measurements were easily made in this system down to levels as low as 1 μg/l Cu. A stock CAOB solution, 0.100 F in total acetate, 2.0×10^{-2} F in NaF, and 2.0×10^{-3} F in formaldehyde was used for the analysis of all water samples. Samples were analyzed by mixing equal amounts of the CAOB solution and sample. Acetate ion present in the CAOB solution serves to decomplex copper from the weak complexing materials normally found in natural water samples. The formation constant for 1:1 copper acetate is $10^{6.3}$ [51]. Sodium fluoride was added to the buffer mixture to prevent the precipitation of $Fe(OH)_3 \cdot X\ H_2O$ from ground water samples and to decrease Fe^{3+} electrode interference through complexation. Ferric ion interference is a two-fold problem. If the ferric ion level is comparable to the concentration of cupric ion in the sample, conversion of the electrode sensing element surface to FeS_2 is possible. A more serious problem occurs because Fe^{3+} is also a moderately strong oxidizing agent. Ferric ion and other oxidizing agents interfere with electrode measurements made in water samples presumably through the oxidation of intermediate sulfide at the electrode surface. The rapid oxidation of S^{2-} by Fe^{3+} even in

dilute solution is a well known reaction [52]. Severe electrode instability was noted when low level Cu^{2+} measurements were attempted in the presence of oxidizing agents. It was found that formaldehyde at concentrations comparable to that used in the CAOB solution was capable of stabilizing the cupric ion electrode when measurements were made in the presence of oxidizing agents. Hydroxylamine hydrochloride and ascorbic acid were also investigated for use as an antioxidants but both materials were capable of rapidly reducing even low levels of Cu^{2+}. It was found that 1×10^{-3} M formaldehyde solutions show no tendency to reduce Cu^{2+} under these conditions.

The use of ascorbic acid as an antioxidant in low level sulfide measurements is well established [53]. Antioxidants have not been used, however, in connection with low level Cu^{2+} measurements made at high levels of Fe(III) and other oxidizing agents.

CHAPTER V

A LEAST-SQUARES ELECTRODE CALIBRATION TECHNIQUE

It was noted in Chapter II of this section, that the parameter most difficult to determine in the employment of the standard addition technique with electrode measurements is S, the slope of a plot of E *vs.* log ion concentration. While many ion-selective electrodes are perfectly Nerstian in response, some of these devices exhibit response slopes as much as 2 or 3 millivolts less than the theoretical value for S. Much of the apparent deviation from theoretical response observed with these devices, however, is attributable to liquid junction potentials developed at the reference electrode employed [54]. Nevertheless, once the response of an electrode pair is determined, it remains constant so that the frequent redetermination of S is not necessary. The electrode response to the ion of interest must be evaluated to the highest degree of precision possible (*ie.* $\pm$ 0.1 mV) since standard addition techniques are strongly dependent upon the value of this parameter (see equation II-11).

Once the data points are positioned on an electrode calibration plot, there are three choices one can make as to how to find the "best" line through those points. As a first attempt, one can simply follow the usual technique of positioning an eye-estimated best line through the plotted points. Positioning the best line by this technique is very much

dependent upon the observer and is subject to personal bias. A second approach is to draw a line with the theoretical Nernst slope through the data points. There is, however, no experimental justification for such a procedure since a 1 or 2 mV adjustment in the slope of a line drawn through typical data can be made without making the line look any more or less correct. Furthermore, many electrode systems do not give theoretical Nernstian response due to a number of factors such as liquid junction potentials. A third method, and most likely the best, is to determine the slope of the best line mathematically by the method of least-squares. Using this technique removes the tendency for personal bias and allows a statistical error treatment of the experimental data to be made. It is possible to report slopes to a precision of $\pm$ 0.1 mV with justification.

To accomplish this task, a FORTRAN IV least-squares computer plotting program was written for the IBM 360 Model 65 computer system. The program calculates the activity or concentration of the solution resulting from the addition of concentrated electrolyte to a known initial volume of solution at a selected temperature. Data to be entered into the program are normally taken by adding a series of volumes of successively more concentrated standard solutions to a single known volume of sample. The volumes of each standard added are selected to give approximately equal spacing of

the resulting data points on a logarithmic plot. A 10 ml digital piston driven buret (DIGIPET) was found convenient for making additions to an initial 100 ml volume of solution. In cases where the maintenance of a constant ionic strength background is necessary, material used to adjust the ionic strength can be added to the titrant solutions. The preparation of calibration curves in this manner avoids the necessity of moving the electrodes from solution to solution and lessens the chance of contamination when low level measurements are made.

Ion-selective electrodes have a lower concentration limit below which their response becomes non-Nernstian. Because of this factor, a provision for the automatic selection of the first data point representing the lowest concentration considered to be part of the linear calibration curve is included as part of the program for the data reduction process. Only those data points that are considered part of the linear calibration curve are considered for least-squares analysis. The selection of the lower limit is based upon the computation of the average slope between each data point and the standard deviation of the computed slopes from the average. Points yielding between point slopes more than one standard deviation from the average are rejected. The entire computational process is repeated until no further points are rejected during an iteration. Automatic selection

of the first data point can be overridden by specifying the subscript on the first data pair to be taken for least-squares analysis. The values for the slope and intercept of the computed least-squares equation are printed giving the constant term in equation II-5 and the slope of the line through the data points, respectively. All computations are carried out in double precision arithmetic.

In order to plot the data and least-squares line accurately, a CalComp off-line pen plotting subroutine is included. Plots may also be made on the line printer. The choice of either type of plot or complete plot suppression may be selected by changing a single input parameter.

Program usage is limited to 50 data points at a time and the program recycles for multiple runs. Reading of input data is stopped by reading a 999 trailer card. The entire program is written from the standpoint of flexibility and simplicity for the user. The choice of either the concentration or activity mode and the implementation or deletion of many computational steps can be readily and easily accomplished. When the concentration mode is used, the computed concentrations are printed in both moles/l and mg/l of the ion sensed by the electrode.

When identical determinations are made, the input data for a given run may be simplified by using the information entered in the previous data set. In this case, all data

cards may be omitted except for the first two data cards and the cards for the new titrant volumes and the corresponding electrode potentials.

The least-squares subroutines give an accuracy of approximately 13 decimal digits when well-behaved synthetic data are entered. The least-squares matrix equations are solved by Gaussian elimination. Roundoff error is decreased by partial pivoting. The solution to the matrix equations is further improved by the Gauss-Seidel iterative technique if necessary [55]. In addition, the average error of estimate and standard error of estimate are computed. The standard error of estimate (SE_y) is the root mean-square of the Y deviations about the computed curve and, in this case, is an estimate of the precision to which S had been determined:

$$SE_y^2 = \frac{(Y_i - Y_{i,est})^2}{N} \qquad \text{(II-24)}$$

where N = the number of data pairs. The correlation coefficient (r), the degree of relationship between the x and y variables is also computed:

$$r^2 = \frac{\text{Explained variation in Y}}{\text{Total variation in Y}} \qquad \text{(II-25)}$$

Correlation coefficients in excess of 0.999 are typical with calibration curves prepared for the cupric ion electrode.

The cupric ion electrode was evaluated using the program in the region from 18 µg/l to 23 mg/l Cu at constant ionic strength vs. an Orion #90-02-00 double junction refer-

ence electrode with 10 per cent KNO_3 in the outer chamber. Typical results for a single run are 29.6 mV with a standard error of estimate of $\pm$ 0.1 mV. The average value of S for 4 runs is 29.6 mV with an average deviation of $\pm$ 0.3 mV.

A complete program listing and sample output follow. The variable names used in the program, their meanings, and their positions on the required data cards are given at the beginning of the listing. Additional information regarding the operation of the program is given at the beginning of each subroutine.

```
C      PROGRAM PCTPLT
C
C      ***********************************************************************
C
C      PURPOSE:
C      PROGRAM TO EVALUATE ELECTRODE RESPONSE VS. -LOG ACTIVITY OR
C      CONCENTRATION IN AN AQUEOUS SOLUTION AT ANY TEMPERATURE
C      AND TO PLOT THESE FUNCTIONS USING THE LEAST SQUARES METHOD
C      AND THE CALCOMP PLOTTER.  THE SLOPE AND THE INTERCEPT OF THE
C      LEAST SQUARES LINE ARE PRINTED.
C      SELECTION OF THE FIRST DATA PAIR TO BE CONSIDERED
C      PART OF THE LINEAR PORTION OF THE COMPUTED LEAST-SQUARES
C      CURVE IS DONE AUTOMATICALLY.
C      AUTOMATIC SELECTION OF THE FIRST DATA PAIR CAN, HOWEVER,
C      BE OVERRIDDEN BY SPECIFYING THE SUBSCRIPT ON THE FIRST DATA
C      PAIR TO BE TAKEN FOR LEAST-SQUARES ANALYSIS THROUGH THE
C      USE OF THE VARIABLE IFIRST.
C
C      ***********************************************************************
C
C      THE CONCENTRATION MODE IS USED WHEN PLOTS ARE MADE AT
C      CONSTANT IONIC STRENGTH OR WITH AN 'OUTER' REFERENCE
C      ELECTRODE THAT COMPENSATES FOR THE CHANGE IN ACTIVITY
C      COEFFICIENT.
C      WHEN THE ACTIVITY MODE IS USED, ACTIVITY COEFFICIENTS
C      ARE CALCULATED FROM THE EDHLL.
C      THE MAXIMUM NUMBER OF TITRANT SOLUTIONS IS FIVE, AND THE
C      MAXIMUM NUMBER OF DATA POINTS IS 50.
C      FACTOR = 0.75OO WHICH GIVES A PLOT SUITABLE FOR AN
C      8.5 X 11 SHEET OF PAPER.
C
C      ***********************************************************************
C
C      DESCRIPTION OF VARIABLES AND POSITION ON DATA CARDS
C      FOR EACH DATA SET
C
C      FIRST DATA CARD: (VALUE FOR SAME PUNCHED W/O TICK MARKS)
C         ADATE=DATE THE ANALYSIS WAS RUN (FORM=XX/YY/ZZ).
C                   (BEGINS IN COL 1, 8 COLS MAX)
C         SALT=THE NAME OF THE SALT USED FOR THE POTENTIAL PLOT.
C                   (BEGINS IN COL 10, 20 COLS MAX)
C         RFRNC=THE NAME OF THE REFERENCE ELECTRODE USED.
C                   (BEGINS IN COL 30, 20 COLS MAX)
C         SAME:IF SAME='YES',ALL DATA CARDS FOR THE RUN SPECIFIED
C              MAY BE OMITTED EXCEPT FOR THE FIRST DATA CARD
C              AND THE VALUES FOR VOLADD AND XMVOLT.
C              IF NEW ASSOCIATED DATA ARE TO BE READ IN, A
C              BLANK FIELD MAY BE INSERTED FOR SAME.
C              NOTE THAT SAME CANNOT BE DESIGNATED 'YES' FOR
C              THE FIRST DATA SET.
C                   (BEGINS IN COL 50)
C      SECOND DATA CARD:  (ALL DATA PUNCHED W/O DECIMAL POINT)
C         NTRNTS=THE NUMBER OF TITRANT SOLUTIONS USED TO MAKE THE PLOT.
C                   (ENDS IN COL 10, MAXIMUM VALUE=5)
C         NOACT:IF NOACT=1, E VS. -LOG CONCENTRATION IS COMPUTED
C               AND PLOTTED.  OTHERWISE E VS. -LOG ACTIVITY IS
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C               PLOTTED.
C                    (ENDS IN COL 2C)
C         IR:IF IR=1, AN ERROR ANALYSIS IS MADE ON THE LEAST-SQUARES
C                 RESIDUALS.
C                    (ENDS IN COL 3C)
C         NOLSQ:IF NOLSQ=1, NO LEAST SQUARES ANALYSIS IS
C               PERFORMED AND ONLY THE DATA VALUES ARE PLOTTED.
C                    (ENDS IN COL 4C)
C               IN THIS MODE, PLOTTING ON THE PRINTER WILL BE DELETED.
C         IPLOT:IF IPLOT=0 NO PLOTTING IS DONE
C                          1 DATA IS PLOTTED ON THE LINE PRINTER
C                          2 A CALCOMP PLOT IS MADE OF THE DATA
C                          3 PLOTS ARE MADE ON BOTH THE LINE
C                            PRINTER AND THE CALCOMP PLOTTER.
C                    (ENDS IN COL 5C)
C         IFIRST=0 IF AUTOMATIC SELECTION OF THE FIRST DATA PAIR LYING
C                  IN THE LINEAR PORTION OF THE COMPUTED LEAST-SQUARES
C                  CURVE IS DESIRED.
C                          OTHERWISE:
C                = THE SUBSCRIPT ON THE FIRST DATA PAIR TO BE USED FOR
C                  LEAST-SQUARES COMPUTATION.
C                    (ENDS IN COL 6C)
C      THIRD DATA CARD:  (DATA PUNCHED WITH DECIMAL PT.)
C         AP=THE ION-SIZE PARAMETER IN THE EDHLL TIMES 10**8.
C                    (COLS 1-10)
C         VOLIN=THE INITIAL VOLUME OF THE SOLUTION TO WHICH
C               THE TITRANT SOLUTIONS ARE ADDED.
C                    (COLS 11-20)
C         DFACTR=A VOLUME CORRECTION FACTOR WHICH ALLOWS THE
C                TITRATION TO BE PERFORMED AT ONE TEMP. T AND THE
C                TITRANT TO BE ADDED AT ROOM TEMP.
C                DFACTR=DH2O(AMBIENT DEG C)/DH2O(T DEG C)
C                    (COLS 21-30)
C         ATERM=THE A CONSTANT IN THE EDHLL ON THE MOLARITY
C               SCALE AT THE TEMPERATURE THE DATA ARE TAKEN.
C                    (COLS 31-40)
C         BTERM=THE B CONSTANT IN THE EDHLL ON THE MOLARITY SCALE
C               TIMES 10**-08 AT THE TEMP. THE DATA ARE TAKEN.
C                    (COLS 41-50)
C         FWTION=THE FORMULA WEIGHT OF THE ION TO WHICH
C                THE ELECTRODE IS RESPONSIVE.
C                    (COLS 51-60)
C      FOURTH DATA CARD:  (ALL DATA PUNCHED W/O DECIMAL PT.)
C         NOPTS(1)=THE NUMBER OF DATA POINTS TAKEN USING THE
C                  FIRST TITRANT SOLUTION.
C                   (ENDS IN COL 10)
C                   .
C                   .
C                   .
C         NOPTS(NTRNTS)=THE NUMBER OF DATA POINTS TAKEN USING THE
C                       LAST TITRANT SOLUTION.
C                    (ENDS IN COL NTRNTS X 10)
C      FIFTH DATA CARD (OMIT IF NOACT=1) (PUNCHED W/C DECIMAL PT.)
C         IPOS=THE CHARGE ON THE POSITIVE ION OF THE SALT USED.
C                    (ENDS IN COL 10)
C         INEG=THE CHARGE ON THE NEGATIVE ION OF THE SALT USED.
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C                    (ENDS IN COL 2C)
C          NPIONS=THE NUMBER OF MOLES OF POSITIVE ICNS PRCDUCED/
C                 FORMULA WT. SALT.
C                    (ENDS IN COL 3C).
C          NNIONS=THE NUMBER OF MOLES OF NEGATIVE ICN PRCDUCED/
C                 FORMULA WT. SALT.
C                    (ENDS IN COL 4C)
C          ZTERM=NORMALLY, THE Z TERM IN THE EQUIL SQUARED,IE,
C                THE CHARGE ON THE ELECTROACTIVE ION SQUARED.
C                IF HOWEVER, A CATION AND AN ANION RESPONSIVE
C                ELECTRODE ARE USED SIMULTANEOUSLY THIS TERM
C                BECOMES |Z(+)*Z(-)| WHICH IS NUMERICALLY=
C                |IPOS*INEG| WHICH ARE DESCRIBED BELOW.
C                    (ENDS IN COL 5C)
C       SIXTH DATA CARD: (OMIT IF NOACT=1) (PUNCHED WITH DECIMAL PT.)
C          DH2O=THE DENSITY OF WATER AT THE TEMP.THE DATA WERE TAKEN.
C                    (COLS 1-1C)
C          DSOLN=THE DENSITY OF THE SOLUTION WHEN CONC.=C.1M.
C                    (COLS 11-20)
C          WSOLUT=THE FORMULA WT. OF THE SOLUTE ADDED.
C                    (COLS 21-3C)
C       SEVENTH TO SEVENTH+NTRNTS DATA CARD: (ONE VALUE/CARD)
C          CONC(I)=THE CONCENTRATION OF THE I'TH TITRANT SOLUTION USED.
C                    (BEGINS IN COL 1, IN THE FORM X.XXXE-YY)
C       SUCCEEDING DATA CARDS: (ONE VALUE/CARD PUNCHED WITH DECIMAL PT.)
C          VOLADD(I)=THE BURET READING IN MILLILITERS AFTER THE ADDITION
C                    OF TITRANT CORRESPONDING TO XMVOLT(I).
C                    (COLS 1-10)
C       DATA CARDS FOLLOWING VOLADD(I) CARDS: (PUNCHED WITH DECIMAL PT.)
C          XMVOLT(I)=THE POTENTIAL READINGS IN MILLIVOLTS COR-
C                    RESPONDING TO THE ABOVE VALUES OF VOLADD(I).
C                    (COLS 1-1C)
C
C       READING IS STOPPED BY READING A 999 TRAILER CARD,SO THE LAST
C       CARD IN THE DATA DECK MUST HAVE 999 PUNCHED IN COLS 1-3.
C
C       ***********************************************************************
C
        IMPLICIT REAL*8 (A-H,O-Z)
        DIMENSION C(1C),X(5C),Y(5C),NOPTS(5),YEST(5C)
        DIMENSION STRNTH(5C),SLOPE(5C),PPM(5C)
        DIMENSION XMVOLT(5C),VOLADD(5C),ACTVTY(5C),ACONC(5C),CONC(5)
        DIMENSION XARRAY(52),YARRAY(52),YY(52),ACTCOF(5C),XLOGCF(5C)
        REAL*4 XARRAY,YARRAY,YY,CS(1C),FACTER/C.75CC/
        COMMON ADATE,SALT,REFNC,NOLSQ,NOACT
        EQUIVALENCE (ACTVTY,ACONC),(Y,XMVOLT)
        INTEGER YES/'YES '/,SAME,SALT(5),ADATE(2),REFNC(5),ZTERM
C
C       ***********************************************************************
C
        DATA NINES/'999 '/,KEY/C/
C
C       ***********************************************************************
C
   41 FORMAT('1RUN',I3,5X,2A4//)
   42 FORMAT(' THE FOLLOWING DATA WERE TAKEN USING ',5A4/
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     1'OREFERENCE ELECTRODE= ',5A4//)
  43 FORMAT(' INITIAL VOLUME=',F6.2,'ML',5X,'NUMBER OF DATA POINTS= ',
     1I2//)
  44 FORMAT('0',10X,'TITRANT ',I1,5X,'CONCENTRATION=',1PD10.3/
     110X,'BURET VOLUMES',3X,'ELECTRODE POTENTIAL(MV)')
  45 FORMAT(12X,F7.3,12X,F7.1)
  99 FORMAT(5I10/3F10.0)
 100 FORMAT(6I10)
 101 FORMAT(6F10.0)
 102 FORMAT(F10.0)
 103 FORMAT(D9.3)
 105 FORMAT(2A4,1X,5A4,5A4,A3)
 200 FORMAT(10X,'CONCENTRATION',5X,'ACTIVITY COEFF.',5X,'ACTIVITY',7X,'
     1POTENTIAL (MV.)')
 201 FORMAT(6X,1PD17.8,5X,0PF10.5,5X,1PD17.8,5X,0PF10.5)
 202 FORMAT(7X,'CONCENTRATION(PPM)',3X,'CONCENTRATION(MOLES/L)',
     14X,'POTENTIAL(MV.)')
 203 FORMAT(6X,1PD17.8,6X,D17.8,10X,0PF10.5)
 902 FORMAT('0EVALUATING',I2,' TERMS IN POLYNOMIAL LEAST SQUARES EQUATI
     1ON')
 904 FORMAT(1X,'THE MEAN SQUARE DEVIATION FROM THE COMPUTED CURVE IS',
     1F10.5,/1X,'THE STANDARD DEVIATION IS',F10.5,//)
1493 FORMAT  (' THE FOLLOWING VALUES WERE USED TO DETERMINE THE POINTS
     1CHOSEN FOR THE LEAST SQUARES LINE'//13X,'SLOPE',10X,'LOG ACTIVITY'
     1,3X,'POTENTIAL (MV.)'/10X,'BETWEEN PTS.')
1494 FORMAT  (' THE FOLLOWING VALUES WERE USED TO DETERMINE THE POINTS
     1CHOSEN FOR THE LEAST SQUARES LINE'//13X,'SLOPE',13X,'LOG CONC.',
     13X,'POTENTIAL (MV.)'/10X,'BETWEEN PTS.')
      KK=0
    8 READ(5,105) ADATE,SALT,REFNO,SAME
      IF(ADATE(1).EQ.NINES) GO TO 611
      KK=KK+1
      WRITE(6,41) KK,ADATE
      WRITE(6,42) SALT,REFNO
      IF(SAME.EQ.YES) GO TO 9
      READ(5,100)NTRNTS,NOACT,IR,NOLSQ,IPLOT,IFIRST
      READ(5,101)AP,VOLIN,DFACTR,ATERM,BTERM,FWTION
      READ(5,100)(NOPTS(J),J=1,NTRNTS)
      IF(NOACT.EQ.1) GO TO 14
      READ(5,99) IPOS,INEG,NPIONS,NNIONS,ZTERM,CH2C,DSOLN,WSOLUT
      NIONS=NPIONS+NNIONS
      XIONS=NIONS
      XNIONS=NNIONS
      XPIONS=NPIONS
      ENEG=INEG
      EPOS=IPOS
   14 NADD=0
      DO 334 J=1,NTRNTS
      NADD=NADD+NOPTS(J)
  334 CONTINUE
      READ(5,103)(CONC(KI),KI=1,NTRNTS)
    9 WRITE(6,43) VOLIN,NADD
      READ(5,102)(VOLADD(IK),IK=1,NADD)
      READ(5,102)(XMVOLT(JK),JK=1,NADD)
      NN=1
      NUMBER=0
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      DO 346 I=1,NTRNTS
      NUMBER=NUMBER+NCPTS(I)
      WRITE(6,44) I,CONC(I)
      DO 345 K=NN,NUMBER
      WRITE(6,45) VOLADD(K),XMVOLT(K)
  345 CONTINUE
      NN=NUMBER+1
  346 CONTINUE
      WRITE(6,41) KK,ADATE
      IF(NOACT.EQ.1) GO TO 80
      WRITE(6,200)
      GO TO 82
   80 WRITE(6,202)
   82 MT=0
      IMMOLS=0
      VOLUME=VOLIN
      DO 2 L=1,NTRNTS
      NN=MT+1
      MT=NCPTS(L)+MT
      DO 1 I=NN,MT
      IF(I.EQ.NN) GO TO 10
      VOLUME=VOLUME+VOLADD(I)-VOLADD(I-1)
      IMMOLS=IMMOLS+(VOLADD(I)-VOLADD(I-1))*CONC(L)
      ACONC(I)=IMMOLS/(VOLUME*DFACTR)
      IF(NOACT.EQ.1) GO TO 81
      GO TO 15
   10 VOLUME=VOLUME+VOLADD(I)
      IMMOLS=IMMOLS+VOLADD(I)*CONC(L)
      ACONC(I)=IMMOLS/(VOLUME*DFACTR)
      IF(NOACT.EQ.1) GO TO 81
   15 STRNTH(I)=5.0D-01*(EPOS**2*ACONC(I)*XPIONS+ENEG**2*ACONC(I)*
     1XNIONS)
      XLGACF(I)=(-ATERM*DELCAT(ZTERM)*DSQRT(STRNTH(I)))/(1.0D0+BTERM*AP
     1*DSQRT(STRNTH(I)))
      ACTCOF(I)=DEXP(2.3025855093*XLGACF(I))
C     THE NEXT STATEMENTS CORRECT THE ACTIVITY COEFFICIENT ON THE MOLE
C     FRACTION SCALE TO THE MOLARITY SCALE USING THE APPROXIMATION
C     THAT THE DENSITY OF THE SOLUTION AT ANY CONCENTRATION ABOVE
C     0.01 MOLAR IS EQUAL TO THE DENSITY OF THE SOLUTION AT 0.1 MOLAR
      IF(STRNTH(I).LE.0.01) GO TO 16
      ACTCOF(I)=ACTCOF(I)*(DH2O/(DSOLN+1.0D-03*ACONC(I)*(XIONS*1.8020D1-
     1WSOLT)))
   16 ACTVTY(I)=ACTCOF(I)*ACONC(I)
      WRITE(6,201)ACONC(I),ACTCOF(I),ACTVTY(I),XMVOLT(I)
      GO TO 1
   81 PPM(I)=ACONC(I)*FWTION*1.0D3
      WRITE(6,203) PPM(I),ACONC(I),XMVOLT(I)
    1 CONTINUE
    2 CONTINUE
C     BEGIN LEAST SQUARES PROGRAM HERE FOR THE EQN. Y=C(1)+C(2)X WHERE
C     X=+LOG(ACTIVITY),C(2)=SLOPE OF THE LEAST SQUARES CURVE, AND
C     C(1)=CONSTANT.
      IF(NOLSQ.EQ.1) GO TO 888
      WRITE(6,41) KK,ADATE
      IF(NOACT.EQ.1) GO TO 5
      WRITE(6,1493)
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      GO TO 6
    5 WRITE(6,1494)
    6 CONTINUE
      DO 12 I=1,NACD
      X(I)=DLOG1C(ACTVTY(I))
   12 CONTINUE
C     DETERMINE FIRST POINTS TO BE TAKEN FOR LEAST-SQUARES ANALYSIS.
      NUMBER=IFIRST
      IF(IFIRST.NE.0) GO TO 13
      CALL SRCH(X,Y,NACD,NUMBER,SLOPE)
   13 M=2
      WRITE(6,41) KK,ADATE
      WRITE(6,902) M
      CALL SUBLSQ(X,Y,M,NUMBER,NACD,1,C,COEFF,STDEST,AVEEST,STDDEV,YEST)
  888 CONTINUE
C     PLOTTING ROUTINES BEGIN HERE
      IF(IPLOT.EQ.0) GO TO 8
      DO 600 I=1,NACD
      XARRAY(I)=X(I)
  600 YARRAY(I)=Y(I)
      IF(NOLSQ.EQ.1) GO TO 605
      DO 601 I=1,NACD
  601 YY(I)=C(1)+C(2)*XARRAY(I)
  605 CALL CALREG(XARRAY,YARRAY,YY,NACD,NUMBER,KEY,FACTER)
      IF(NOLSQ.EQ.1) GO TO 8
      IF(IPLOT.EQ.2) GO TO 8
      DO 75 I=1,M
      CS(I)=C(I)
   75 CONTINUE
      CALL REGPLT(XARRAY,YARRAY,NACD,NUMBER,CS,M,0)
      GO TO 8
  611 IF(KEY.EQ.1) CALL CALENC
      WRITE(6,3333)
 3333 FORMAT('1')
      STOP
      END
```

```
      SUBROUTINE CALREG (X,Y,YEST,NUMBER,BEGIN,KEY,FACTER)
C
C     ******************************************************************
C
C     PURPOSE:
C        SUBROUTINE TO PLOT A SCATTER DIAGRAM AND A REGRESSION LINE FOR
C        UP TO 50 X,Y DATA PAIRS.
C
C     ******************************************************************
C
C     DESCRIPTION OF VARIABLES:
C
C        X,Y,YEST,NUMBER,BEGIN - SEE SUBROUTINE PRTREG.
C        KEY = A VARIABLE THAT SIGNALS WHEN THE PROPER CALCOMP
C              INITIALIZATION HAS BEEN ACCOMPLISHED.
C        FACTER = A PARAMETER THAT ALLOWS ADJUSTMENT OF THE SIZE OF THE
C                 CALCOMP PLOT MADE.
C
C     ******************************************************************
C
C     THIS SUBROUTINE HAS BEEN MODIFIED FOR USE IN PROGRAM QGRADE AND
C     THE VARIABLES ADATE,TITLE1,TITLE2,NOLSQ, AND NOACT ARE A RESULT
C     OF THAT MODIFICATION.
C     THIS SUBROUTINE USES SUBROUTINE CALCOM WHICH PERFORMS THE
C     NECESSARY CALCOMP INITIALIZATION.  IT IS PECULIAR TO THE UNIV.
C     OF MO. COMPUTING CENTER.
C     TO USE THIS SUBROUTINE AT ANOTHER INSTALLATION THE STANDARD
C     CALCOMP INITIALIZATION SUBROUTINES SHOULD BE SUBSTITUTED.
C
C     THIS SUBROUTINE ALSO REQUIRES CALCOMP FINALIZATION THROUGH THE
C     USE OF SUBROUTINE CALEND, ALSO PECULIAR TO THE UNIV. OF MO.
C     COMPUTING CENTER.
C
C     ******************************************************************
C
      SUBROUTINE CALREG(X,Y,YEST,NUMBER,BEGIN,KEY,FACTER)
      DIMENSION X(52),Y(52),YEST(52)
      INTEGER BEGIN,TITLE1(5),TITLE2(5),ADATE(2)
      COMMON ADATE,TITLE1,TITLE2,NOLSQ,NOACT
C     IF SUBROUTINE HAS NOT BEEN CALLED BEFORE(KEY~=1), DO
C     CALCOMP INITIALIZATION.
      IF(KEY.EQ.1) GO TO 608
      CALL CALCOM (16H MIKE SMITH 012 )
      CALL FACTOR(FACTER)
      CALL PLOT (0.0,1.13333,-3)
      KEY=1
      GO TO 609
  608 CALL PLOT(0.0,1.8,-3)
  609 CALL SCALE(X,8.0,NUMBER,1)
      CALL SCALE(Y,11.0,NUMBER,1)
      IF(NOACT.EQ.1) GO TO 603
      CALL AXIS(0.0,0.0,13H-LOG ACTIVITY,-13,8.0,0.0,X(NUMBER+1),
     *          X(NUMBER+2))
      GO TO 604
  603 CALL AXIS(0.0,0.0,18H-LOG CONCENTRATION,-18,8.0,0.0,X(NUMBER+1),
     *          X(NUMBER+2))
```

```
  604 CALL AXIS(0.0,0.0,23HPOTENTIAL IN MILLIVOLTS,23,11.0,90.0,
     *          Y(NUMBER+1),Y(NUMBER+2))
      IF(NOLSQ.EQ.1) GO TO 605
      YEST(NUMBER+1)=Y(NUMBER+1)
      YEST(NUMBER+2)=Y(NUMBER+2)
C     NUMBER=THE NUMBER OF DATA POINTS, BEGIN=THE SUBSCRIPT ON THE
C     FIRST LEAST SQUARES DATA PAIR
      LEFT=NUMBER-BEGIN+1
      CALL LINE(X(BEGIN),YEST(BEGIN),LEFT,1,0,0)
  605 CALL LINE(X,Y,NUMBER,1,-1,0)
      CALL SYMBOL(1.5,10.75,0.21,ADATE,0.0,8)
      CALL SYMBOL(1.5,10.25,0.21,21HPLOT OF POTENTIAL VS.,0.0,21)
      IF(NOACT.EQ.1) GO TO 606
      CALL SYMBOL(1.5,9.75,0.21,16H-LOG ACTIVITY OF,0.0,16)
      CALL SYMBOL(1.5,9.25,0.21,TITLE1,0.0,20)
      CALL SYMBOL(1.5,8.75,0.21,11HREFERENCE: ,0.0,11)
      CALL SYMBOL(999.,999.,0.21,TITLE2,0.0,20)
      GO TO 607
  606 CALL SYMBOL(1.5,9.75,0.21,21H-LOG CONCENTRATION OF,0.0,21)
      CALL SYMBOL(1.5,9.25,0.21,TITLE1,0.0,20)
      CALL SYMBOL(1.5,8.75,0.21,11HREFERENCE: ,0.0,11)
      CALL SYMBOL(999.,999.,0.21,TITLE2,0.0,20)
  607 CALL PLOT(15.0,-20.0,-3)
      RETURN
      END
```

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G LEVEL  20                    MAIN                DATE = 72211          13/56/37

      C     SUBROUTINE SRCH (X,Y,NACC,BEGIN,SLOPE)
      C
      C     ***************************************************************
      C
      C     PURPOSE:
      C        SUBROUTINE TO CHOOSE THE FIRST DATA POINTS TO BE USED
      C        FOR LEAST-SQUARES ANALYSIS.
      C
      C     ***************************************************************
      C
            SUBROUTINE SRCH (X,Y,NACC,BEGIN,SLOPE)
            IMPLICIT REAL*8 (A-H,O-Z)
            DIMENSION SLOPE(50),X(50),Y(50)
            INTEGER BEGIN
      C     DETERMINE SLOPES BETWEEN PTS AND PRINT OUT
            BEGIN=1
            SUM=0.0D0
            SUMSQ=0.0D0
            WRITE(6,1492) X(1),Y(1)
       1492 FORMAT(30X,F10.5,5X,F10.5)
            DO 1 I=2,NACC
            IM1=I-1
            SLOPE(IM1)=(Y(I)-Y(IM1))/(X(I)-X(IM1))
            SUM=SUM+SLOPE(IM1)
            SUMSQ=SUMSQ+SLOPE(IM1)*SLOPE(IM1)
      C     WRITE OUT SLOPES AND DATA POINTS
            WRITE(6,1495) SLOPE(IM1),X(I),Y(I)
       1495 FORMAT(10X,F10.5/30X,F10.5,5X,F10.5)
          1 CONTINUE
            WRITE(6,4951)
       4951 FORMAT('1ITERATION FOR THE FIRST DATA POINT TO BE TAKEN FOR LEAST-
           *SQUARES ANALYSIS'//' AVERAGE SLOPE',5X,'STANDARD DEVIATION',5X,
           *'# PTS REJECTED',2X,'# PTS KEPT')
      C     FIND THE AVERAGE SLOPE
            RACC=DFLOAT(NACC-1)
          5 IF(BEGIN.EQ.NACC) RETURN
            AVE=SUM/RACC
      C     FIND STD DEV OF SLOPES
            STDDEV=DSQRT(DABS((SUMSQ-(SUM*SUM)/RACC)/(RACC-1.0)))
            KEY=0
            J=BEGIN+1
            DO 2 I=J,NACC
            IM1=I-1
            STDTST=1.0*STDDEV
            IF(DABS(SLOPE(IM1)-AVE).LE.STDTST) GO TO 3
            SUM=SUM-SLOPE(IM1)
            SUMSQ=SUMSQ-SLOPE(IM1)*SLOPE(IM1)
            BEGIN=BEGIN+1
            KEY=KEY+1
            GO TO 2
          3 IF(KEY.EQ.0) GO TO 4
            I=NACC-BEGIN+1
            RACC=DFLOAT(I-1)
            K=BEGIN-1
            WRITE(6,9514) AVE,STDDEV,K,I
       9514 FORMAT(3X,F10.5,9X,F10.5,15X,I2,13X,I2)
```

```
      GO TO 5
    2 CONTINUE
      GO TO 5
    4 IF(BEGIN.EQ.1) WRITE(6,5149)
 5149 FORMAT('CALL DATA POINTS INCLUDED IN LEAST-SQUARES COMPUTATION')
      RETURN
      END
```

```
C      SUBROUTINE SLBLSQ(X,Y,M,BEGIN,NUMBER,IR,C,COEFF,STDEST,AVEEST,     SLSQ  10
C                        STDDEV,YEST)                                     SLSQ  20
C                                                                         SLSQ  30
C      ***********************************************************************SLSQ  40
C                                                                         SLSQ  50
C      PURPOSE:                                                           SLSQ  60
C                                                                         SLSQ  70
C      UTILIZATION OF THE LEAST SQUARES METHOD FOR THE                    SLSQ  80
C      CURVE FITTING OF DATA PAIRS (X(I),Y(I)) FOR EQUATIONS              SLSQ  90
C      OF THE FORM Y = A(1)+A(2)X+A(3)X**2+A(4)X**3+...+A(M)X**(M-1)      SLSQ 100
C      FOR M=2 TO 10.                                                     SLSQ 110
C      ***********************************************************************SLSQ 120
C                                                                         SLSQ 130
C      DESCRIPTION OF VARIABLES:                                          SLSQ 140
C                                                                         SLSQ 150
C      X(I)=THE INDEPENDENT VARIABLE                                      SLSQ 170
C      Y(I)=THE DEPENDENT VARIABLE                                        SLSQ 180
C      M=THE DESIRED NUMBER OF TERMS FOR THE LEAST SQUARES                SLSQ 190
C        EQUATION(M=2 TO 10.)                                             SLSQ 200
C      BEGIN=THE SUBSCRIPT ON THE FIRST DATA PAIR TO BE TAKEN FOR         SLSQ 210
C            LEAST SQUARES ANALYSIS                                       SLSQ 220
C      NUMBER=THE # OF DATA PAIRS                                         SLSQ 160
C      IR=IF IR=1, AN ERROR ANALYSIS IS DONE ON THE RESIDUALS             SLSQ 230
C      C=THE ARRAY OF CONSTANTS WHICH APPEAR IN THE COMPUTED EQN.         SLSQ 240
C        (RETURNED)                                                       SLSQ 250
C      COEFF=THE COEFFICIENT OF CORRELATION  (RETURNED)                   SLSQ 260
C      STDEST=THE STANDARD ERROR OF ESTIMATE FOR THE                      SLSQ 270
C             COMPUTED EQUATION.  (RETURNED)                              SLSQ 280
C            =SQRT((YEST(I)-Y(I))/(NUMBER-BEGIN+1))  (RETURNED)           SLSQ 290
C      STDDEV=THE STANDARD DEVIATION OF THE Y-VALUES FROM                 SLSQ 300
C             THEIR MEAN.  (RETURNED)                                     SLSQ 310
C      AVEEST=THE AVERAGE ERROR OF ESTIMATE  (RETURNED)                   SLSQ 320
C                                                                         SLSQ 330
C      ADDITIONAL SUBROUTINES REQUIRED: CVER,GAUSS,CORR                   SLSQ 340
C                                                                         SLSQ 350
C      ***********************************************************************SLSQ 360
C                                                                         SLSQ 370
       SUBROUTINE SLBLSQ(X,Y,M,BEGIN,NUMBER,IR,C,COEFF,STDEST,AVEEST,     SLSQ 380
      *                  STDDEV,YEST)                                     SLSQ 390
       REAL*8 X(50),Y(50),A(10,10),B(10),C(10),D(10,10),P(10),YEST(50)    SLSQ 400
       REAL*8 COEFF,STDEST,AVEEST,STDDEV,APASS(10,10)                     SLSQ 410
       INTEGER BEGIN                                                      SLSQ 420
C      BEGIN SUMMATION FOR PRODUCTS TO BE INSERTED INTO THE NORMAL EQNS.  SLSQ 430
       IF(NUMBER.EQ.BEGIN) GO TO 934                                      SLSQ 440
       N=M-1                                                              SLSQ 470
       NX2=N+N                                                            SLSQ 480
       DO 10 J=1,NX2                                                      SLSQ 490
       P(J)=0.0                                                           SLSQ 500
       DO 10 I=BEGIN,NUMBER                                               SLSQ 510
    10 P(J)=P(J)+X(I)**J                                                  SLSQ 520
C      SET UP COEFFICIENT MATRIX                                          SLSQ 530
       DO 30 I=1,M                                                        SLSQ 540
       DO 30 J=1,M                                                        SLSQ 550
       K=I+J-2                                                            SLSQ 560
       IF (K) 29,29,28                                                    SLSQ 570
    29 A(I,J)=P(K)                                                        SLSQ 580
```

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      GO TO 30                                                          SLSQ 590
   29 A(1,1)=NUMBER-BEGIN+1                                             SLSQ 600
   30 CONTINUE                                                          SLSQ 610
C     FORM THE RIGHT-HAND SIDE MATRIX                                   SLSQ 620
      B(1)=0.0                                                          SLSQ 630
      DO 15 I=BEGIN,NUMBER                                              SLSQ 640
   15 B(1)=B(1)+Y(I)                                                    SLSQ 650
      DO 22 I=2,M                                                       SLSQ 660
      B(I)=0.0                                                          SLSQ 670
      DO 22 J=BEGIN,NUMBER                                              SLSQ 680
   22 B(I)=B(I)+Y(J)*X(J)**(I-1)                                        SLSQ 690
C     SAVE MATRIX A BY SETTING EQUAL TO APASS                           SLSQ 700
      DO 23 I=1,M                                                       SLSQ 710
      DO 23 K=1,M                                                       SLSQ 720
   23 APASS(I,K)=A(I,K)                                                 SLSQ 730
C     SOLVE THE MATRIX EQUATION BY INVERTING MATRIX A.                  SLSQ 740
      I=1                                                               SLSQ 750
   18 CALL OVER(APASS,D,M,K,I)                                          SLSQ 760
C     MATRIX D IS INVERSE OF MATRIX A                                   SLSQ 770
      IF(K.EQ.1) RETURN                                                 SLSQ 780
      DO 55 I=1,M                                                       SLSQ 790
      C(I)=0.0                                                          SLSQ 800
      DO 54 J=1,M                                                       SLSQ 810
   54 C(I)=C(I)+D(I,J)*B(J)                                             SLSQ 820
   55 CONTINUE                                                          SLSQ 830
C     WRITE OUT THE COEFFICIENTS OF THE EQUATION                        SLSQ 840
  800 WRITE(6,902)                                                      SLSQ 850
  902 FORMAT(//' THE FOLLOWING VALUES ARE THE COEF. OF THE COMPUTED EQUASLSQ 860
     1TION'/' THEY ARE PRINTED IN ASCENDING ORDER'/                     SLSQ 870
     2'OBY DIRECT SOLUTION')                                            SLSQ 880
      DO 900 I=1,M                                                      SLSQ 890
  900 WRITE(6,901) I,C(I)                                               SLSQ 900
  901 FORMAT(1X,'C(',I2,')= ',1PD15.8)                                  SLSQ 910
C     IMPROVE INITIAL ESTIMATE OF SOLUTION BY GAUSS-SEIDEL              SLSQ 920
C     ITERATIVE METHOD                                                  SLSQ 930
      CALL GAUSS(A,B,C,M,10)                                            SLSQ 940
C     IF IR=1, COMPUTE THE MEAN SQUARE ERROR                            SLSQ 950
      IF(IR.NE.1) GO TO 888                                             SLSQ 960
      CALL CORR(M,NUMBER,BEGIN,X,Y,C,YEST,COEFF,STDEST,STDDEV,AVEEST)   SLSQ 970
      WRITE(6,904) COEFF,STDEST,AVEEST,STDDEV                           SLSQ 980
  904 FORMAT('0THE CORRELATION COEFFICIENT= ',F8.5/                     SLSQ 990
     *'0THE STANDARD ERROR OF ESTIMATE= ',1PD13.5/                      SLSQ1000
     *'0THE AVERAGE ERROR OF ESTIMATE= ',D13.5/                         SLSQ1010
     *'0THE STANDARD DEVIATION OF THE AVERAGE ESTIMATE= ',D13.5)        SLSQ1020
      GO TO 888                                                         SLSQ1030
  934 WRITE (6,935)                                                     SLSQ1040
  935 FORMAT('ONLY ONE POINT TAKEN FOR LEAST SQUARES ANALYSIS'/         SLSQ1050
     1' NO LEAST SQUARES ANALYSIS WILL BE PERFORMED')                   SLSQ1060
  888 RETURN                                                            SLSQ1070
      END                                                               SLSQ1080
```

```
C      SUBROUTINE OVER(A,D,M,NCINV,IPRINT)                                    OVER  10
C                                                                             OVER  20
C      ***********************************************************************OVER  30
C                                                                             OVER  40
C      PURPOSE:                                                               OVER  50
C         SUBROUTINE TO COMPUTE THE INVERSE OF AN M*M SQUARE MATRIX BY        OVER  60
C         GAUSSIAN ELIMINATION.  ROUNDOFF ERROR IS DECREASED BY PARTIAL       OVER  70
C         PIVOTING.                                                           OVER  80
C                                                                             OVER  90
C      DESCRIPTION OF PARAMETERS:                                             OVER 100
C         A =     THE M*M MATRIX TO BE INVERTED.                              OVER 110
C         D =     THE COMPUTED M*M INVERSE OF MATRIX A (RETURNED)             OVER 120
C         M =     THE NUMBER OF ROWS & COLUMNS IN MATRICES A & D.             OVER 130
C         NCINV- A VALUE OF 1 IS RETURNED FOR NCINV IF INVERSION CANNOT       OVER 140
C                BE COMPLETED.  (A SINGULAR MATRIX IS ENCOUNTERED)            OVER 150
C                (SINGULAR MATRIX = ONE WITH A ZERO OR NEAR-ZERO              OVER 160
C                DIAGONAL ELEMENT)                                            OVER 170
C         IPRINT-IF IPRINT EQUALS 1, THE INVERTED MATRIX IS PRINTED IN        OVER 180
C                ROW MAJOR ORDER.                                             OVER 190
C                                                                             OVER 200
C      ***********************************************************************OVER 210
C                                                                             OVER 220
       SUBROUTINE OVER(A,D,M,NCINV,IPRINT)                                    OVER 230
C      MATRIX INVERSION BY ELIMINATION WITH PARTIAL PIVOTING                  OVER 240
C      ORIGINAL MATRIX=A, INVERSE MATRIX=D                                    OVER 250
       REAL*8 A(10,10),D(10,10)                                               OVER 260
       DOUBLE PRECISION EPS,ATMP,DTMP,AMAX,DIV,AMULT                          OVER 270
       EPS=1.0D-12                                                            OVER 280
       NCINV=0                                                                OVER 290
C      CONSTRUCT IDENTITY MATRIX D(I,J)=I                                     OVER 300
       DO 6 I=1,M                                                             OVER 310
       DO 5 J=1,M                                                             OVER 320
       IF(I-J) 4,3,4                                                          OVER 330
     3 D(I,J)=1.0                                                             OVER 340
       GO TO 5                                                                OVER 350
     4 D(I,J)=0.0                                                             OVER 360
     5 CONTINUE                                                               OVER 370
     6 CONTINUE                                                               OVER 380
C      LOCATE MAXIMUM MAGNITUDE A(I,K) ON OR BELOW MAIN DIAGONAL              OVER 390
       DO 45 K=1,M                                                            OVER 400
       IF (K-M) 12,30,30                                                      OVER 410
    12 IMAX=K                                                                 OVER 420
       AMAX=DABS(A(K,K))                                                      OVER 430
       KP1=K+1                                                                OVER 440
       DO 20 I=KP1,M                                                          OVER 450
       IF(AMAX-DABS(A(I,K))) 15,20,20                                         OVER 460
    15 IMAX=I                                                                 OVER 470
       AMAX=DABS(A(I,K))                                                      OVER 480
    20 CONTINUE                                                               OVER 490
C      INTERCHANGE ROWS IMAX AND K IF IMAX .NE. TO K                          OVER 500
       IF(IMAX-K) 25,30,25                                                    OVER 510
    25 DO 29 J=1,M                                                           OVER 520
       ATMP=A(IMAX,J)                                                         OVER 530
       A(IMAX,J)=A(K,J)                                                       OVER 540
       A(K,J)=ATMP                                                            OVER 550
       DTMP=D(IMAX,J)                                                         OVER 560
```

```
      D(IMAX,J)=D(K,J)                                                  OVER 570
   29 C(K,J)=DTMP                                                       OVER 580
   30 CONTINUE                                                          OVER 590
C     TEST FOR SINGULAR MATRIX                                          OVER 600
      IF (DABS(A(K,K))-EPS) 93,93,35                                    OVER 610
   35 CONTINUE                                                          OVER 620
C     DIVIDE PIVOT ROW BY ITS MAIN DIAGONAL ELEMENT                     OVER 630
      DIV=A(K,K)                                                        OVER 640
      DO 38 J=1,N                                                       OVER 650
      A(K,J)=A(K,J)/DIV                                                 OVER 660
   38 D(K,J)=D(K,J)/DIV                                                 OVER 670
C     REPLACE EACH ROW BY LINEAR COMBINATION WITH PIVOT ROW             OVER 680
      DO 43 I=1,N                                                       OVER 690
      AMULT=A(I,K)                                                      OVER 700
      IF (I-K) 39,43,39                                                 OVER 710
   39 DO 42 J=1,N                                                       OVER 720
      A(I,J)=A(I,J)-AMULT*A(K,J)                                        OVER 730
   42 C(I,J)=C(I,J)-AMULT*D(K,J)                                        OVER 740
   43 CONTINUE                                                          OVER 750
   45 CONTINUE                                                          OVER 760
      IF(IPRINT.NE.1) GO TO 99                                          OVER 770
      WRITE(6,120)                                                      OVER 780
      WRITE(6,110) (( C(I,J),I=1,N),J=1,N)                              OVER 790
   99 RETURN                                                            OVER 800
   93 WRITE(6,113) K                                                    OVER 810
      NOINV=1                                                           OVER 820
      RETURN                                                            OVER 830
  110 FORMAT(2X,1PD15.8)                                                OVER 840
  113 FORMAT(' SINGULAR MATRIX FOR K =',I2,' INVERSION NOT COMPLETED'/) OVER 850
  120 FORMAT(' ELEMENTS OF INVERSE IN ROW-MAJOR ORDER'//)               OVER 860
      END                                                               OVER 870
```

```
C      SUBROUTINE GAUSS (A,B,XK,N,ITMAX)                                GAUS  10
C                                                                       GAUS  20
C      ******************************************************************GAUS  30
C                                                                       GAUS  40
C      PURPOSE:                                                         GAUS  50
C         SUBROUTINE TO SOLVE THE N*N MATRIX EQUATION AX=B BY THE GAUSS-GAUS  60
C         SEIDEL ITERATIVE METHOD.  COLUMN MATRIX B IS INPUT INTO COLUMN GAUS  70
C         N+1 OF THE N*N+1 AUGMENTED MATRIX A.  AN INITIAL ESTIMATE OF  GAUS  80
C         THE SOLUTION MATRIX X IS SUPPLIED EITHER AS A ZERO MATRIX, AN GAUS  90
C         ARBITRARY ESTIMATE, OR BETTER AS THE APPROXIMATE SOLUTION     GAUS 100
C         OBTAINED BY A DIRECT METHOD (SUCH AS THE GAUSS-JORDAN METHOD). GAUS 110
C         THE MAXIMUM NUMBER OF ITERATIONS USED TO IMPROVE THE SOLUTION GAUS 120
C         MAY BE SPECIFIED.  NORMALLY ONE OR TWO ITERATIONS WILL SUFFICE.GAUS 130
C         WHEN THE NUMBER OF LEADING ZEROS IN THE ERROR COMPONENT EPS   GAUS 140
C         APPROACHES THE NUMBER OF DECIMAL PLACES CARRIED IN THE        GAUS 150
C         COMPUTATIONS, THEN THE IMPROVEMENT BY FURTHUR ITERATION IS    GAUS 160
C         NEGLIGIBLE.  AT THIS POINT THE SYSTEM IS CONSIDERED TO HAVE   GAUS 170
C         CONVERGED ON THE 'EXACT' SOLUTION.                            GAUS 180
C                                                                       GAUS 190
C      ******************************************************************GAUS 200
C                                                                       GAUS 210
C      DESCRIPTION OF PARAMETERS                                        GAUS 220
C         A = N*N +1 AUGMENTED MATRIX WHERE COLUMN B IS READ INTO       GAUS 230
C             COLUMN N SO THAT A(I,N+1) = B(I).                         GAUS 240
C         B = THE COLUMN (RIGHT-HAND SIDE MATRIX) OF LENGTH N.          GAUS 250
C         XK = THE SOLUTION MATRIX, AND THE INITIAL ESTIMATE OF THE     GAUS 260
C              TRUE SOLUTION.  IF XK(1) IS SET EQUAL TO 9.99D02 THEN THE GAUS 270
C              INITIAL ESTIMATE OF XK(I) USED IS ZERO.                  GAUS 280
C         N = THE NUMBER OF EQUATIONS.  THE NUMBER OF ELEMENTS IN MATRIX GAUS 290
C             A = THE DIMENSIONS OF MATRIX A.  (MAX VALUE = 10)         GAUS 300
C         ITMAX = THE NUMBER OF ITERATIONS ALLOWED TO OBTAIN THE TRUE   GAUS 310
C                 SOLUTION TO MATRIX X.                                 GAUS 320
C         EPS = CONVERGENCE CRITERIA, NORMALLY ABOUT 10**-12.           GAUS 330
C                                                                       GAUS 340
C      ******************************************************************GAUS 350
C                                                                       GAUS 360
C      REFERENCE:                                                       GAUS 370
C         MCCALLA,T.R., 'INTRODUCTION TO NUMERICAL METHODS & FORTRAN    GAUS 380
C         PROGRAMMING', PP. 176-185, JOHN WILEY & SONS, INC., NEW YORK. GAUS 390
C                                                                       GAUS 400
C      ******************************************************************GAUS 410
C                                                                       GAUS 420
       SUBROUTINE GAUSS(A,B,XK,N,ITMAX)                                 GAUS 430
       IMPLICIT REAL*8 (A-H,O-Z)                                        GAUS 440
       DIMENSION A(10,11),XK(10),XKP1(10),B(10)                         GAUS 450
       DATA EPS/1.0D-8/                                                 GAUS 460
       NP1=N+1                                                          GAUS 470
C      SET MATRIX B=N+1TH COL OF MATRIX A                               GAUS 480
       DO 5 I=1,N                                                       GAUS 490
     5 A(I,NP1)=B(I)                                                    GAUS 500
C      IF XK(1) IS NOT 999. SET XK(I)= TO ZERO                          GAUS 510
       IF(XK(1).NE.9.99D02) GO TO 1                                     GAUS 520
       DO 20 I=1,N                                                      GAUS 530
    20 XK(I)=0.0D0                                                      GAUS 540
     1 K=1                                                              GAUS 550
C      DIVIDE ITH EQUATION BY DIAGONAL TERM A(I,I)                      GAUS 560
```

```
      DO 15 I=1,N                                                    GAUS 570
      DIV=A(I,I)                                                     GAUS 580
      DO 10 J=1,NP1                                                  GAUS 590
   10 A(I,J)=A(I,J)/DIV                                              GAUS 600
   15 CONTINUE                                                       GAUS 610
C     CALCULATE (K+1)ST ITERATES XKP1(I) OF VARIABLE XK(I)           GAUS 620
   21 DO 30 I=1,N                                                    GAUS 630
      XKP1(I)=A(I,NP1)                                               GAUS 640
      DO 25 J=1,N                                                    GAUS 650
      IF(J-I) 22,25,24                                               GAUS 660
   22 XKP1(I)=XKP1(I)-A(I,J)*XKP1(J)                                 GAUS 670
      GO TO 25                                                       GAUS 680
   24 XKP1(I)=XKP1(I)-A(I,J)*XK(J)                                   GAUS 690
   25 CONTINUE                                                       GAUS 700
   30 CONTINUE                                                       GAUS 710
C     WRITE OUT SOLUTION X(I)                                        GAUS 720
      WRITE(6,120) K                                                 GAUS 730
      WRITE(6,110) (I,XKP1(I),I=1,N)                                 GAUS 740
C     TEST CONVERGENCE OF ITERATION                                  GAUS 750
      DO 40 I=1,N                                                    GAUS 760
      IF(DABS(XKP1(I)-XK(I))-EPS) 40,40,50                           GAUS 770
   40 CONTINUE                                                       GAUS 780
      GO TO 99                                                       GAUS 790
C     IF CONVERGENCE,RETURN.  OTHERWISE,                             GAUS 800
C     REPLACE KTH ITERATES BY K+1 ITERATES                           GAUS 810
   50 IF(K-ITMAX) 51,55,55                                           GAUS 820
   51 K=K+1                                                          GAUS 830
      DO 52 I=1,N                                                    GAUS 840
   52 XK(I)=XKP1(I)                                                  GAUS 850
      GO TO 21                                                       GAUS 860
   55 WRITE(6,115) ITMAX                                             GAUS 870
   99 RETURN                                                         GAUS 880
  110 FORMAT(1X,'C(',I2,')= ',1PD15.8)                               GAUS 890
  115 FORMAT('FAILURE TO CONVERGE AFTER ',I2,' ITERATIONS')          GAUS 900
  120 FORMAT('0ITERATION ',I2)                                       GAUS 910
      END                                                            GAUS 920
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G LEVEL  20                    MAIN                DATE = 72211          13/56/37

C      SUBROUTINE CORR(M,NUMBER,BEGIN,X,Y,C,YEST,COEFF,STDEST,STDDEV,     CORR  10
C                      AVEEST)                                            CORR  20
C                                                                         CORR  30
C      ***********************************************************************CORR  40
C                                                                         CORR  50
C      PURPOSE:                                                           CORR  60
C         SUBROUTINE TO COMPUTE THE COEFFICIENT OF CORRELATION, STANDARD  CORR  70
C         ERROR OF ESTIMATE, AND AVERAGE ERROR OF ESTIMATE FOR            CORR  80
C         APPROXIMATING POLYNOMIAL EXPRESSIONS OF THE FORM:               CORR  90
C                                                                         CORR 100
C         YEST = C(1) + C(2)X + C(3)X**2 + ... + C(M)X**(M-1).           CORR 110
C                                                                         CORR 120
C      ***********************************************************************CORR 130
C                                                                         CORR 140
C      DESCRIPTION OF PARAMETERS:                                         CORR 150
C         M =     THE NUMBER OF TERMS IN THE POLYNOMIAL USED TO ESTIMATE YCORR 160
C         NUMBER=THE NUMBER OF X,Y DATA PAIRS                             CORR 170
C         BEGIN =THE SUBSCRIPT ON THE FIRST X,Y DATA PAIR USED IN THE     CORR 180
C                COMPUTATION OF YEST.                                     CORR 190
C         X,Y =   THE X,Y DATA PAIRS FOR WHICH YEST HAS BEEN COMPUTED.    CORR 200
C         C =     THE VECTOR OF M COEFFICIENTS COMPUTED TO ESTIMATE Y.    CORR 210
C         YEST =  THE POLYNOMIAL ESTIMATE FOR Y COMPUTED FROM X & C.      CORR 220
C         COEFF= THE COEFF OF CORRELATION OF Y ON X (RETURNED)            CORR 230
C         STDEST=THE STANDARD ERROR OF ESTIMATE. (RETURNED)               CORR 240
C         STDDEV=THE STANDARD DEVIATION OF THE Y VALUES FROM THE          CORR 250
C                AVERAGE Y. (RETURNED)                                    CORR 260
C         AVEEST=THE AVERAGE ERROR OF ESTIMATE. (RETURNED)                CORR 270
C                                                                         CORR 280
C      ***********************************************************************CORR 290
C                                                                         CORR 300
       SUBROUTINE CORR(M,NUMBER,BEGIN,X,Y,C,YEST,COEFF,STDEST,STDDEV,     CORR 310
      *                AVEEST)                                            CORR 320
       IMPLICIT REAL*8 (A-H,O-Z)                                         CORR 330
       DIMENSION X(50),Y(50),YEST(50),C(10)                              CORR 340
       INTEGER BEGIN                                                     CORR 350
C      COMPUTE THE SUM OF SQUARES OF RESIDUALS(YEST-Y(I)) AND YEST(I)     CORR 360
       SQRSD=0.0                                                         CORR 370
       RESID=0.0                                                         CORR 380
       N=M-1                                                             CORR 390
       DO 932 I=BEGIN,NUMBER                                             CORR 400
       YEST(I)=C(M)                                                      CORR 410
       DO 903 J=1,N                                                      CORR 420
   903 YEST(I)=YEST(I)*X(I) + C(M-J)                                     CORR 430
       DIFF=DABS(YEST(I)-Y(I))                                           CORR 440
       RESID=RESID + DIFF                                                CORR 450
   932 SQRSD=SQRSD + DIFF*DIFF                                           CORR 460
C      COMPUTE THE SQUARE OF THE STD ERROR OF ESTIMATE(STDEST)            CORR 470
       RNUMBR=NUMBER-BEGIN+1                                             CORR 480
       STDEST=SQRSD/RNUMBR                                               CORR 490
       YSUM=0.0                                                          CORR 500
       YSQR=0.0                                                          CORR 510
C      COMPUTE THE SQUARE OF STD DEV OF AVE Y BY CODING METHOD            CORR 520
       DO 1032 I=BEGIN,NUMBER                                            CORR 530
       YSUM=YSUM + Y(I)                                                  CORR 540
  1032 YSQR=YSQR + Y(I)*Y(I)                                             CORR 550
       STDDEV=(YSQR-YSUM*YSUM/RNUMBR)/RNUMBR                             CORR 560
```

```
G LEVEL  20                    CORR                DATE = 72211          13/56/37

      C     COMPUTE THE COEFF OF CORRELATION(COEFF)                                  CORR 570
            COEFF=1.C-STDEST/STDDEV                                                  CORR 580
            COEFF=DSQRT(COEFF)                                                       CORR 590
      C     COMPUTE THE STD ERROR OF ESTIMATE, STDDEV, AND AVE ERR OF ESTIMATECORR 6CC
            STDDEV=DSQRT(STDDEV)                                                     CORR 610
            STDEST=DSQRT(STDEST)                                                     CORR 620
            AVEEST=RESID/RNUMBR                                                      CORR 630
            RETURN                                                                   CORR 640
            END                                                                      CORR 650
```

```
G LEVEL  2C                  MAIN              DATE = 72211          13/56/37

C                                                                               RPLT  1C
C     ********************************************************************RPLT  2C
C                                                                               RPLT  3C
C     SUBROUTINE REGPLT(X,Y,NPTS,BEGIN,C,N,IPRINT)                              RPLT  4C
C                                                                               RPLT  5C
C     ********************************************************************RPLT  6C
C     DESCRIPTION OF PARAMETERS:                                                RPLT  7C
C                                                                               RPLT  8C
C     X,Y = THE DATA SET FOR WHICH THE REGRESSION LINE IS TO BE PLOTTED RPLT  9C
C     NPTS = THE NUMBER OF POINTS PER ARRAY                                     RPLT 10C
C     BEGIN = THE SUBSCRIPT ON THE FIRST X,Y DATA PAIR                          RPLT 11C
C             USED FOR LEAST-SQUARES ANALYSIS                                   RPLT 12C
C     C = THE COEFFICIENTS IN THE LEAST-SQUARES POLYNOMIAL.                     RPLT 121
C     N = THE NUMBER OF TERMS IN THE LEAST-SQUARES POLYNOMIAL.                  RPLT 122
C     IXLNTH = THE NUMBER OF CHARACTERS IN THE TITLE FOR THE X AXIS             RPLT 13C
C     XTITL = THE TITLE FOR THE X AXIS                                          RPLT 14C
C     IYLNTH = THE NUMBER OF CHARACTERS IN THE TITLE FOR THE Y AXIS             RPLT 15C
C     YTITL = THE TITLE FOR THE Y AXIS                                          RPLT 16C
C     SYMBOL = SYMBOLS FOR PLOTTING CURVES                                      RPLT 17C
C              SYMBOL(1) IS FOR REGRESSION LINE                                 RPLT 18C
C              SYMBOL(2) IS FOR DATA POINTS                                     RPLT 19C
C     IPRINT - IF IPRINT EQUALS 1 THEN THE X,Y DATA SET IS PRINTED              RPLT 20C
C              BEFORE PLOTTING IS PERFORMED.                                    RPLT 21C
C                                                                               RPLT 22C
C     THE X ARRAY MUST BE IN ASCENDING ORDER BEFORE PLOTTING IS DONE.           RPLT 23C
C     IF THE RANGE OF VALUES TO BE PLOTTED IS SUCH THAT AN F6.2 FORMAT          RPLT 24C
C     IS NOT SUITABLE FOR THE Y AXIS, CHANGE THE EXPRESSION IN PARENS           RPLT 25C
C     FROM STATEMENT 301 TO (1X,1PE9.2).                                        RPLT 26C
C     IF THE X ARRAY CHANGES SIGN, THE STEP TO INCREASE THE SIZE OF             RPLT 27C
C     XFIRST SHOULD BE OMITTED.                                                 RPLT 28C
C                                                                               RPLT 29C
C     ********************************************************************RPLT 30C
C                                                                               RPLT 31C
      SUBROUTINE REGPLT(X,Y,NPTS,BEGIN,C,N,IPRINT)                              RPLT 32C
      DIMENSION X(52),Y(52),C(10),YPR(11)                                       RPLT 33C
      INTEGER BEGIN                                                             RPLT 34C
      INTEGER*2 SYMBL1,SYMBL2,CLT(102),BLANK/'  '/,ASTER/'* '/                  RPLT 35C
      LOGICAL*1 XTITLE(48),YTITLE(48),JUNK(100)/100*' '/                        RPLT 36C
      COMPLEX*16 XTITL(3),YTITL(3)                                              RPLT 37C
      EQUIVALENCE (XTITL,XTITLE),(YTITL,YTITLE)                                 RPLT 38C
C                                                                               RPLT 39C
C     ********************************************************************RPLT 40C
C                                                                               RPLT 41C
      DATA SYMBL1/'* '/,SYMBL2/'X '/                                            RPLT 42C
      DATA IXLNTH,XTITL/29,'LOG CONCENTRATIC','N OR ACTIVITY'/                  RPLT 43C
      DATA IYLNTH,YTITL/23,'ELECTRODE POTENT','IAL(MV)'/                        RPLT 44C
C                                                                               RPLT 45C
C     ********************************************************************RPLT 46C
C                                                                               RPLT 47C
C     FIND THE LARGEST AND SMALLEST VALUE OF Y IN THE Y ARRAY                   RPLT 48C
      YMAX=Y(1)                                                                 RPLT 49C
      YMIN=Y(1)                                                                 RPLT 50C
      DO 11 I=1,NPTS                                                            RPLT 51C
      IF(YMAX-Y(I)) 12,10,13                                                    RPLT 52C
   12 YMAX=Y(I)                                                                 RPLT 53C
      GO TO 10                                                                  RPLT 54C
```

```
     13 IF(YMIN-Y(I)) 10,10,14                                          RPLT 550
     14 YMIN=Y(I)                                                       RPLT 560
     10 CONTINUE                                                        RPLT 570
     11 CONTINUE                                                        RPLT 580
C       SCALE THE X ARRAY                                               RPLT 590
        XSCALE=(X(NPTS)-X(1))/49.0                                      RPLT 600
C       SCALE THE Y ARAAY                                               RPLT 610
        DELTAY=YMAX-YMIN                                                RPLT 620
        KK=0                                                            RPLT 630
        IF(DELTAY-1.0) 600,600,602                                      RPLT 640
    600 KK=KK+1                                                         RPLT 650
        B=10.**(KK-1)                                                   RPLT 660
        YFAC=DELTAY*B                                                   RPLT 670
        IF(YFAC.LT.1.0) GO TO 600                                       RPLT 680
        GO TO 601                                                       RPLT 690
    602 KK=KK+1                                                         RPLT 700
        B=0.1**(KK-1)                                                   RPLT 710
        YFAC=DELTAY*B                                                   RPLT 720
        IF(YFAC.GT.10.0) GO TO 602                                      RPLT 730
    601 CONTINUE                                                        RPLT 740
        IF(YFAC.GT.8.) GO TO 650                                        RPLT 750
        IF(YFAC.GT.5.) GO TO 651                                        RPLT 760
        IF(YFAC.GT.2.) GO TO 652                                        RPLT 770
        IF(YFAC.GT.1.) GO TO 654                                        RPLT 780
        DELTAY=1.0                                                      RPLT 790
        GO TO 653                                                       RPLT 800
    650 DELTAY=10.0                                                     RPLT 810
        GO TO 653                                                       RPLT 820
    651 DELTAY=8.0                                                      RPLT 830
        GO TO 653                                                       RPLT 840
    652 DELTAY=5.0                                                      RPLT 850
        GO TO 653                                                       RPLT 860
    654 DELTAY=2.0                                                      RPLT 870
    653 CONTINUE                                                        RPLT 880
        IF (IPRINT.NE.1) GOTO 655                                       RPLT 890
        WRITE(6,201)                                                    RPLT 900
    201 FORMAT(' ',17X,'XARRAY',15X,'YARRAY'/)                          RPLT 910
        WRITE(6,203)(X(I),Y(I),I=1,NPTS)                                RPLT 920
    203 FORMAT(5X,1PE20.8,5X,E20.8)                                     RPLT 930
    655 YSCALE=DELTAY/(B*10.0**2)                                       RPLT 940
C       CENTER AND PRINT TITLE FOR Y AXIS                               RPLT 950
        NSKIP=(100-IYLNTH)/2                                            RPLT 960
        WRITE(6,100) (JUNK(I),I=1,NSKIP),(YTITLE(I),I=1,IYLNTH)         RPLT 970
    100 FORMAT('1',16X,100A1//)                                         RPLT 980
C       PRINT SCALE FOR Y AXIS                                          RPLT 990
        YPR(1)=YMIN                                                     RPLT1000
        DO 90 K=1,10                                                    RPLT1010
     90 YPR(K+1)=YPR(K)+YSCALE*10.0                                     RPLT1020
        WRITE(6,301) YPR                                                RPLT1030
    301 FORMAT('0',8X,11(3X,F7.2))                                      RPLT1040
        WRITE(6,302)                                                    RPLT1050
    302 FORMAT(18X,'.         .         .         .         .         .  RPLT1060
       1         .         .         .         .         .')            RPLT1070
        WRITE(6,307)                                                    RPLT1080
    307 FORMAT(17X,'*************************************************************RPLT1090
       1*************************************************')            RPLT1100
```

```
      C     FIND THE X VARIABLES AND CENTER THE TITLE FOR THE X AXIS          RPLT111C
            NSKIP=(5C-IXLNTH)/2                                               RPLT112C
            LAST=NSKIP+IXLNTH                                                 RPLT1130
      C     THE FOLLOWING STEP INSURES THAT XPR WILL EXCEED X(L)              RPLT114C
      C     BY(0.C1% OF X(1))                                                 RPLT115C
            SIGN=-1.C                                                         RPLT1160
            IF(X(1).GT.0.0) SIGN=-SIGN                                        RPLT1170
            XFIRST=X(1)+1.COE-04*X(1)*SIGN                                    RPLT118C
      C     J=THE # OF COLS PRINTED, L=SUBSCRIPT ON DATA PAIR UNDER           RPLT1190
      C     CONSIDERATION FOR PRINTING                                        RPLT12CC
            J=1                                                               RPLT121C
            L=1                                                               RPLT1220
            K=0                                                               RPLT1230
            OUT(102)=ASTER                                                    RPLT124C
            NM1=N-1                                                           RPLT1250
          145 UP=J-1                                                          RPLT1260
            XPR=XFIRST+UP*XSCALE                                              RPLT127C
            XCALC=X(1)+UP*XSCALE                                              RPLT1275
            DO 155 I=1,1C1                                                    RPLT1280
          155 OUT(I)=BLANK                                                    RPLT1290
            IF(L.LT.BEGIN) GO TO 165                                          RPLT1300
      C     CALCULATE THE POINT ON REGRESSION LINE                            RPLT1310
            YEST=C(N)                                                         RPLT1320
            DO 16C I=1,NM1                                                    RPLT1330
          160 YEST=YEST*XCALC+C(N-I)                                          RPLT1340
            JT=(YEST-YMIN)/YSCALE+1.C                                         RPLT135C
            OUT(JT)=SYMBL1                                                    RPLT136C
          165 IF(X(L)-XPR) 15C,15C,17C                                        RPLT1370
          150 JT=(Y(L)-YMIN)/YSCALE+1.C                                       RPLT138C
            OUT(JT)=SYMBL2                                                    RPLT139C
            L=L+1                                                             RPLT1400
      C     PRINT THE LINE                                                    RPLT1410
          17C IF(J-1.LT.NSKIP) GO TO 175                                      RPLT142C
            IF(J.GT.LAST) GOTO 175                                            RPLT1430
            K=K+1                                                             RPLT1440
            WRITE(6,1CC0) XTITLE(K),XCALC,OUT                                 RPLT1450
         1CC0 FORMAT(4X,A1,1X,1PE1C.3,1X,'*',1C2A1)                           RPLT1460
            GOTO 176                                                          RPLT1470
          175 WRITE(6,1C01) XCALC,OUT                                         RPLT148C
         1C01 FORMAT(5X,1X,1PE1C.3,1X,'*',1C2A1)                              RPLT1490
          176 J=J+1                                                           RPLT1500
            IF(J-5C) 145,145,186                                              RPLT151C
          186 WRITE(6,3C7)                                                    RPLT152C
            WRITE(6,999)                                                      RPLT1530
          999 FORMAT('1')                                                     RPLT154C
            RETURN                                                            RPLT155C
            END                                                               RPLT1560
```

RUN 1 11/21/70

THE FOLLOWING DATA WERE TAKEN USING COPPER SULFATE

REFERENCE ELECTRODE= ORION

INITIAL VOLUME=101.0ML NUMBER OF DATA POINTS= 16

TITRANT 1 BURET VOLUMES	CONCENTRATION= 4.721D-05 ELECTRODE POTENTIAL(MV)
0.600	-1.4
1.100	5.1
2.000	12.2
3.200	17.4
5.000	23.1
8.300	29.8

TITRANT 2 BURET VOLUMES	CONCENTRATION= 4.721D-04 ELECTRODE POTENTIAL(MV)
0.600	36.4
1.300	41.5
2.600	47.9
4.600	54.0
8.000	60.9

TITRANT 3 BURET VOLUMES	CONCENTRATION= 4.721D-03 ELECTRODE POTENTIAL(MV)
0.500	66.0
1.400	72.6
2.700	78.1
5.000	84.6
8.700	90.1

RUN 1 11/21/70

CONCENTRATION(PPM)	CONCENTRATION(MOLES/L)	POTENTIAL(MV.)
1.77149020D-02	2.78799213D-07	-1.40000
3.23182737D-02	5.08628795D-07	5.10000
5.82470563D-02	9.16699029D-07	12.20000
9.21220238D-02	1.44982726D-06	17.40000
1.41496387D-01	2.22698679D-06	23.10000
2.27792353D-01	3.58502287D-06	29.80000
3.90318877D-01	6.14288444D-06	36.40000
5.77704416D-01	9.09198011D-06	41.50000
9.19486261D-01	1.44709830D-05	47.90000
1.43007007D 00	2.25066111D-05	54.00000
2.25810380D 00	3.55383035D-05	60.90000
3.52174657D 00	5.54256621D-05	66.00000
5.76947643D 00	9.08006992D-05	72.60000
8.95667412D 00	1.40961192D-04	78.10000
1.44295771D 01	2.27094383D-04	84.60000
2.28145630D 01	3.59058278D-04	90.10000

RUN 1 11/21/70

THE FOLLOWING VALUES WERE USED TO DETERMINE THE POINTS CHOSEN FOR THE LEAST SQUARES LINE

SLOPE BETWEEN PTS.	LOG CONC.	POTENTIAL (MV.)
	-6.55471	-1.4CCCC
24.89378		
	-6.29360	5.1CCCC
27.75326		
	-6.03777	12.2CCCC
26.11891		
	-5.83868	17.4CCCC
30.58237		
	-5.65230	23.1CCCC
32.39943		
	-5.44551	29.8CCCC
28.21955		
	-5.21163	36.4CCCC
29.94959		
	-5.04134	41.5CCCC
31.70835		
	-4.83950	47.9CCCC
31.80196		
	-4.64769	54.0CCCC
34.78058		
	-4.44930	60.9CCCC
26.42293		
	-4.25629	66.0CCCC
30.78670		
	-4.04191	72.6CCCC
28.79425		
	-3.85090	78.1CCCC
31.38477		
	-3.64379	84.6CCCC
27.64395		
	-3.44484	90.1CCCC

ITERATION FOR THE FIRST DATA POINT TO BE TAKEN FOR LEAST-SQUARES ANALYSIS

AVERAGE SLOPE	STANDARD DEVIATION	# PTS REJECTED	# PTS KEPT
29.54936	2.72244	1	15

RUN 1 11/21/70

EVALUATING 2 TERMS IN POLYNOMIAL LEAST SQUARES EQUATION
ELEMENTS OF INVERSE IN ROW-MAJOR ORDER

2.15023107D 00
4.29925588D-01
4.29925588D-01
8.87114458D-02

THE FOLLOWING VALUES ARE THE COEF. OF THE COMPUTED EQUATION
THEY ARE PRINTED IN ASCENDING ORDER

BY DIRECT SOLUTION
C(1)= 1.94707906D 02
C(2)= 3.02760410D 01

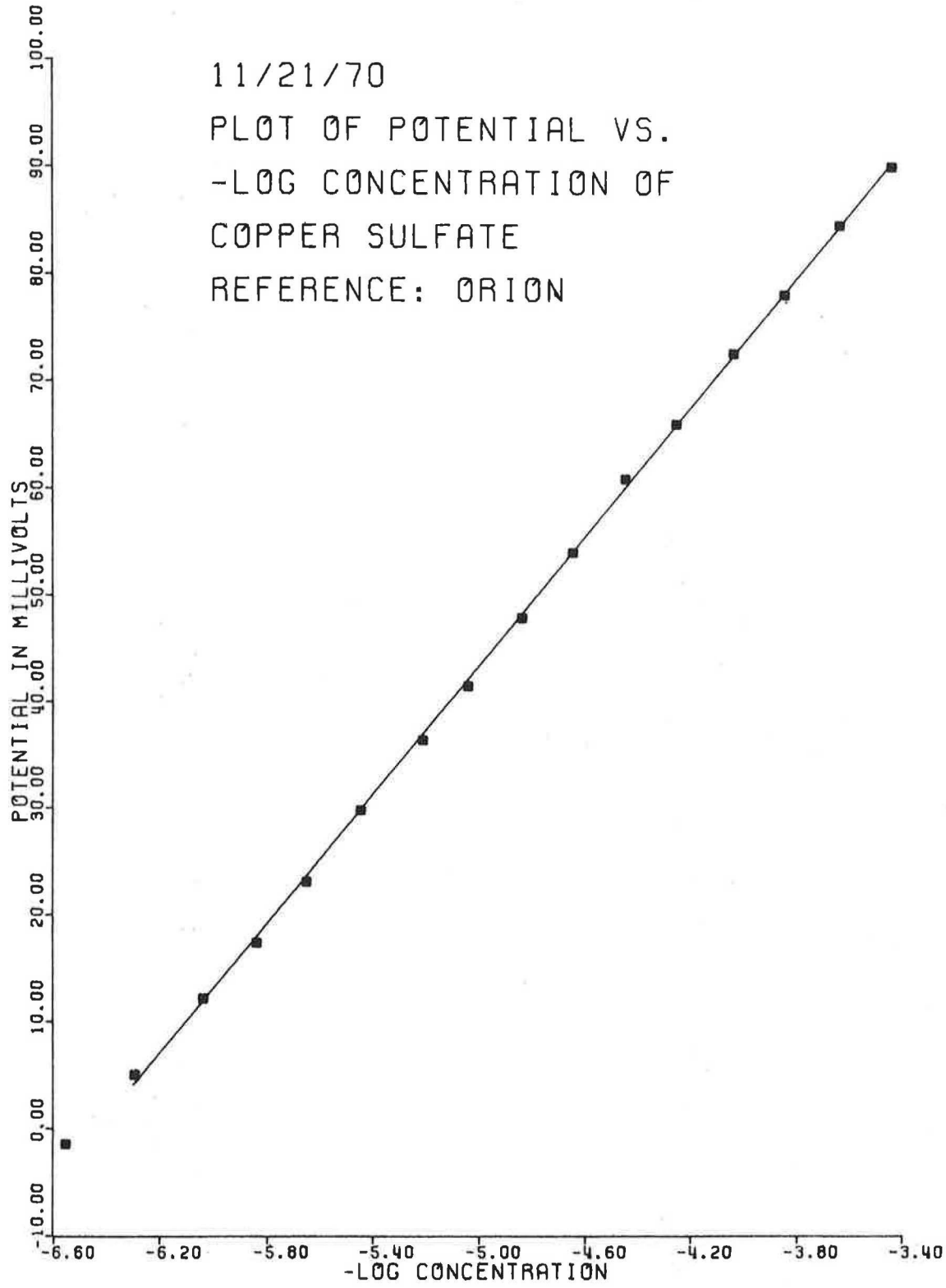
ITERATION 1
C(1)= 1.94707906D 02
C(2)= 3.02760410D 01

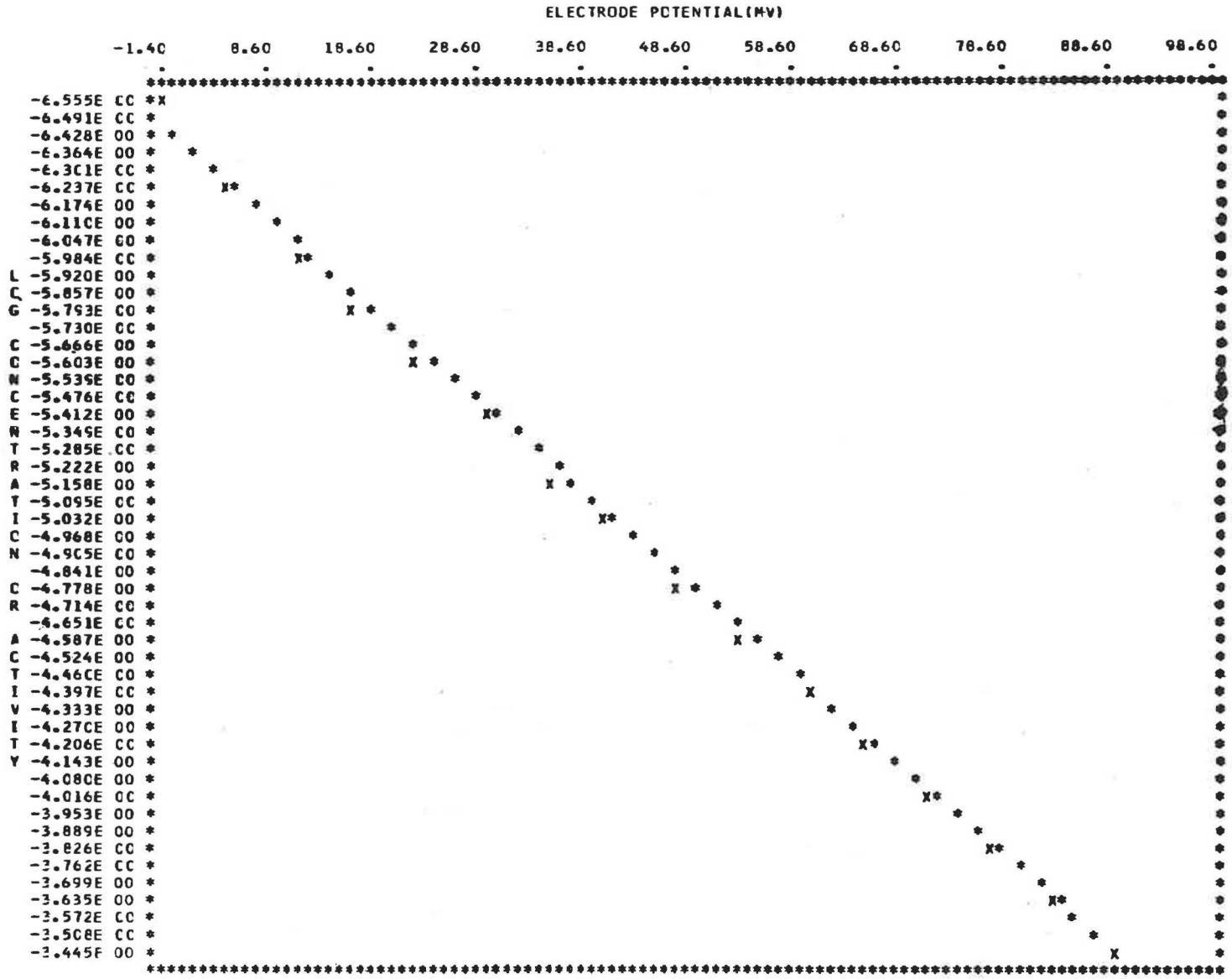
THE CORRELATION COEFFICIENT= 0.99984

THE STANDARD ERROR OF ESTIMATE= 4.62774D-01

THE AVERAGE ERROR OF ESTIMATE= 3.68935D-01

THE STANDARD DEVIATION OF THE AVERAGE ESTIMATE= 2.62501D 01


11/21/70
PLOT OF POTENTIAL VS.
-LOG CONCENTRATION OF
COPPER SULFATE
REFERENCE: ORION
POTENTIAL IN MILLIVOLTS
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
-6.60
-6.20
-5.80
-5.40
-5.00
-4.60
-4.20
-3.80
-3.40
-LOG CONCENTRATION


ELECTRODE POTENTIAL(MV)
-1.40
8.60
18.60
28.60
38.60
48.60
58.60
68.60
78.60
88.60
98.60
LOG CURRENT

CHAPTER VI

EXPERIMENTAL

After choosing a suitable buffer system, the feasibility of using the cupric ion-selective electrode for direct total soluble copper measurements in natural waters was investigated. The multiple standard addition technique described previously was used for all measurements. Direct potentiomety was abandoned at this point largely because of the difficulty with which interfering substances are detected by the technique.

Trace metal analysis at the low ppb level places extreme demands upon reagent quality. It was found that ordinary reagent grade materials are not suitable buffer materials for low level copper measurements since they invariably contain 10^{-3} to 10^{-4} per cent of the common trace elements. Consequently, ultrapure or specially treated materials are required for all measurements. Extreme care must also be taken to avoid sample contamination due to the adsorption of copper on container walls. Chromic acid cleaning solutions and metal stirrers must be avoided. All of the necessary reagents described below were stored in new, carefully cleaned polyethylene bottles.

APPARATUS

All measurements were made in a jacketted Pyrex cell

thermostatted at 25.00 ± 0.05° C by means of water circulated through the cell jacket from a constant temperature bath. A diagram of the cell is given in Figure 20. The cell was fitted with a Teflon cover to prevent the introduction of foreign materials during analysis and to aid in temperature control. A cell with the dimensions indicated was found convenient for sample volumes from 50 to 100 ml. The drain and stopcock assembly at the bottom of the cell was included to allow thorough, one-way flushing of the cell between samples. A small amount of methylene blue chloride was added to the circulating water to eliminate the slight response of the cupric electrode to ambient light levels. Uniform stirring was accomplished by means of a spiral shaped glass stirrer driven by a HI-TORQUE lab stirrer operating at approximately 260 rpm. A motor driven overhead stirrer of this type results in significantly more stable electrode potentials than does the typical magnetically driven stirring bar. An Orion 94-29A solid-state Cupric Ion Electrode and an Orion 90-02 double junction reference electrode were used for all measurements. Ten per cent KNO_3 was used in the outer chamber of the reference electrode. This electrode combination is a remarkably stable system when used with the stirring apparatus described. Once electrode equilibration was achieved, the measured electrode potential for the system was stable to within ± 0.1 mV often for periods of hours at trace Cu^{2+} levels.

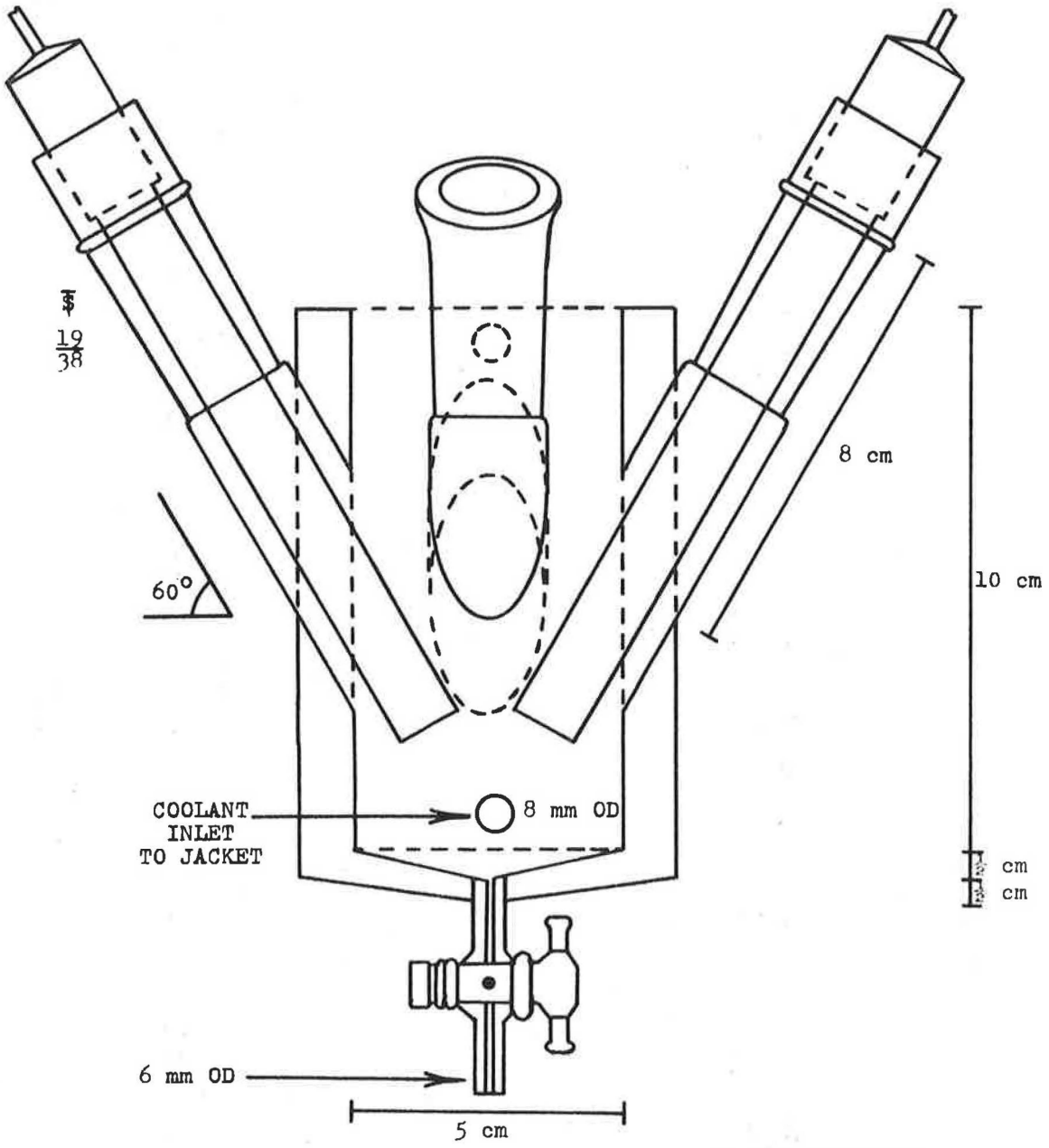


FIGURE 20. JACKETED PYREX CELL USED FOR CUPRIC ION MEASUREMENTS

Electrode potentials were measured with an Orion Model 801 digital pH meter. A Texas Instruments Servoriter II recorder was connected to the recorder output terminals of the Orion meter and adjusted so that a meter reading of 200 mV gave a full scale defection. A chart speed of 20 cm/hr was employed. The use of a recorder was found necessary in order to discern the slow potential drift that occurs as the cupric ion electrode equilibrates in samples containing very low levels of copper.

Additions of standard copper solution were made with a Hamilton 50 µl gas-tight syringe equipped with a Chaney adapter and a pipet delivery needle. A syringe delivery device, however, is not recommended because of the necessity of frequent recalibration. A piston driven microburet or Eppendorf pipet should prove superior.

CLEANING OF GLASSWARE

The glassware used in this study was cleaned with a 3:1 (v:v) mixture of concentrated H_2SO_4 and HNO_3 acids. The acid mixture was found to be a satisfactory substitute for chromic acid cleaning solution. The rinsing of the cell, electrodes, and glassware between runs was accomplished with a solution of 1.0×10^{-3} M Na_2EDTA which was followed by a thorough rinsing with deionized distilled water.

STANDARD Cu(II) SOLUTION

A stock 3000 mg/l Cu^{2+} solution was prepared by carefully dissolving 7.5359 g of anhydrous $CuSO_4$ in ca. 500 ml deionized distilled water acidified with 0.10 ml of concentrated H_2SO_4 and diluting to volume in a 1 liter volumetric flask. Standard Cu^{2+} solutions were prepared daily by making appropriate dilutions of the above solution.

COMPLEXING ANTIOXIDANT BUFFER (CAOB)

A 0.10 M complexing antioxidant buffer of pH 5.0 was prepared by mixing 100.0 ml of 1.00 N Aristar acetic acid (Gallard-Schlesinger, Inc.), 63.5 ml of 1.00 N ultrapure KOH (Alfa Inorganics), 0.84 g of ultrapure NaF (Alfa Inorganics), and 2.0 ml of 1.0 M formaldehyde solution (Matheson Coleman and Bell). The resulting mixture was diluted to volume in a 1 liter volumetric flask. Buffer solutions prepared as described are typically 1 μg/l in copper.

PROCEDURE FOR WATER ANALYSIS

Natural water samples were analyzed by adding 50.00 ml of sample to 50.00 ml of CAOB. The initial electrode potential and the potentials after each of 3 additions of standard Cu^{2+} solution were recorded. A plot of $Z - 1$ vs. V_s (see Chapter II, this section) was prepared and the concentration of copper in the diluted sample was determined from the slope of the resulting linear plot. The original sample concentra-

tion is twice the value determined because of dilution. The concentration and volume of the standard Cu^{2+} solution were chosen such that the initial ΔE for the first addition of standard corresponded to an approximate doubling of the initial copper concentration. At the 30 µg/l Cu level ca. 20 minutes are required for the initial electrode equilibration. Equilibration times between additions at this level are typically 10 minutes. Initial electrode equilibration times varied from less than one minute at 1 mg/l Cu to as long as one hour around 1 µg/l Cu. A recorder trace for the multiple addition analysis of 9.0 and 900 µg/l Cu samples is given in Figure 21.

Water samples taken for analysis were collected with a 100 ml plastic syringe and expelled into a clean polyethylene bottle through a plastic 25 mm Swinnex filter unit containing a 0.22 µ Millipore filter. A 75 ml aliquot of the filtrate was then transferred to a 180 ml polyethylene bottle containing exactly 75 ml of CAOB for storage. A 100 ml aliquot of the resulting solution was then used for analysis. Samples that are to be stored for extended periods of time may be filtered, acidified with 0.10 ml of concentrated HNO_3/75 ml of sample, and stored in polyethylene bottles.

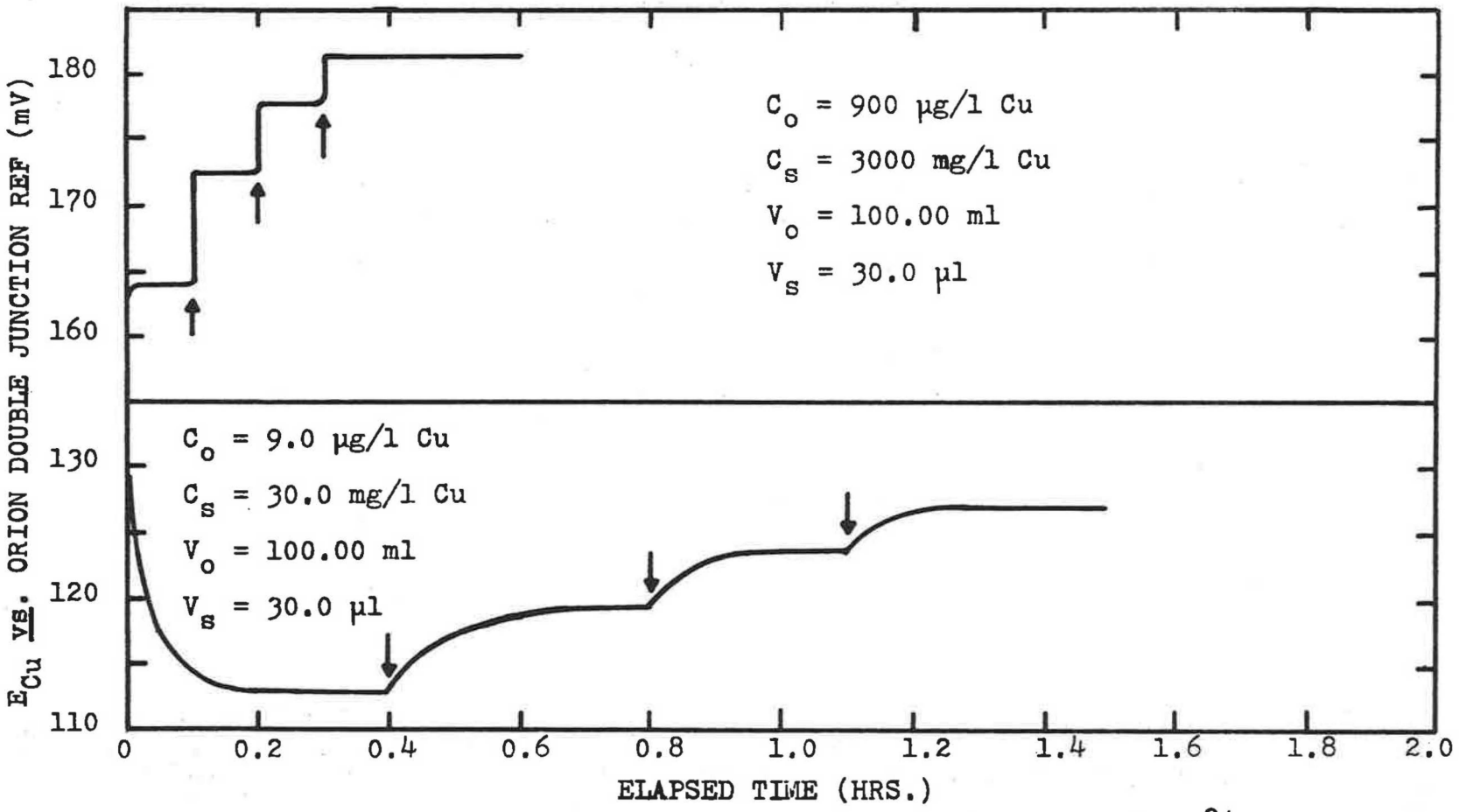


FIGURE 21. TIME RESPONSE FOR THE ANALYSIS OF 9.0 AND 900 µg/l Cu^{2+} IN CAOB BY MULTIPLE STANDARD ADDITION. ADDITION OF STANDARD MARKED BY ARROWS.

CHAPTER VII

RESULTS AND DISCUSSION

In performing trace analysis using standard addition techniques and the cupric ion electrode, it is desirable to know (1) an accurate value of S, the electrode slope sensitivity to copper for use in calculating Z, (2) the level of copper contamination in the reagents used for analysis, and (3) the nature of the electrode response to Cu^{2+} changes down to the level of contaminant copper in the reagents employed. All these parameters may be determined by means of a technique that will be termed "addition-calibration". After measuring the initial electrode potential in the buffer medium to be tested, a single standard addition step is performed approximately doubling the original copper concentration. A calibration curve of E vs. log concentration is then prepared through a series of additions of standard Cu^{2+} solution 100 times more concentrated than that used for the single addition step. Then, using the value of S determined from the calibration curve, the apparent concentration of copper corresponding to the initial electrode potential is computed from equation II-15. If the addition point lies on an extension of the calibration curve, the electrode responds in a Nernstian manner down to the copper contamination level calculated. The method is unique in that it gives the background copper level and validates the electrode response slope in the medium where

measurements are to be made. The value of S is determined near the detection limit rather than at a higher level of analyte where extrapolation into the region of interest is necessary. All operations must be carried out at constant ionic strength. An improved value for S may be determined if the entire data set is fed into the electrode calibration program described in Chapter V of this section.

An addition-calibration plot is given in Figure 22 for the CAOB solution. The background level of 1.2 µg/l Cu is typical for CAOB solutions prepared from ultrapure materials. The copper electrode response in the CAOB solution is Nernstian to the background copper level. The value of S determined by least-squares analysis is 29.7 mV with a standard error of estimate of 0.1 mV. The copper contamination level in the CAOB solution as determined by multiple standard addition is given in Figure 23. The results for triplicate analyses at this level were 1.15, 1.32, and 1.21 µg/l Cu.

In order to ensure that copper could be successfully determined at varying levels and in order to estimate the precision with which trace level copper measurements could be made in the CAOB solution, triplicate analyses of 0.050 M CAOB solution spiked with copper were run. The values of copper found were corrected for 1.6 µg/l Cu contamination in the CAOB as determined by triplicate multiple addition analyses. Copper samples spiked at the levels of 9.0, 27.0,

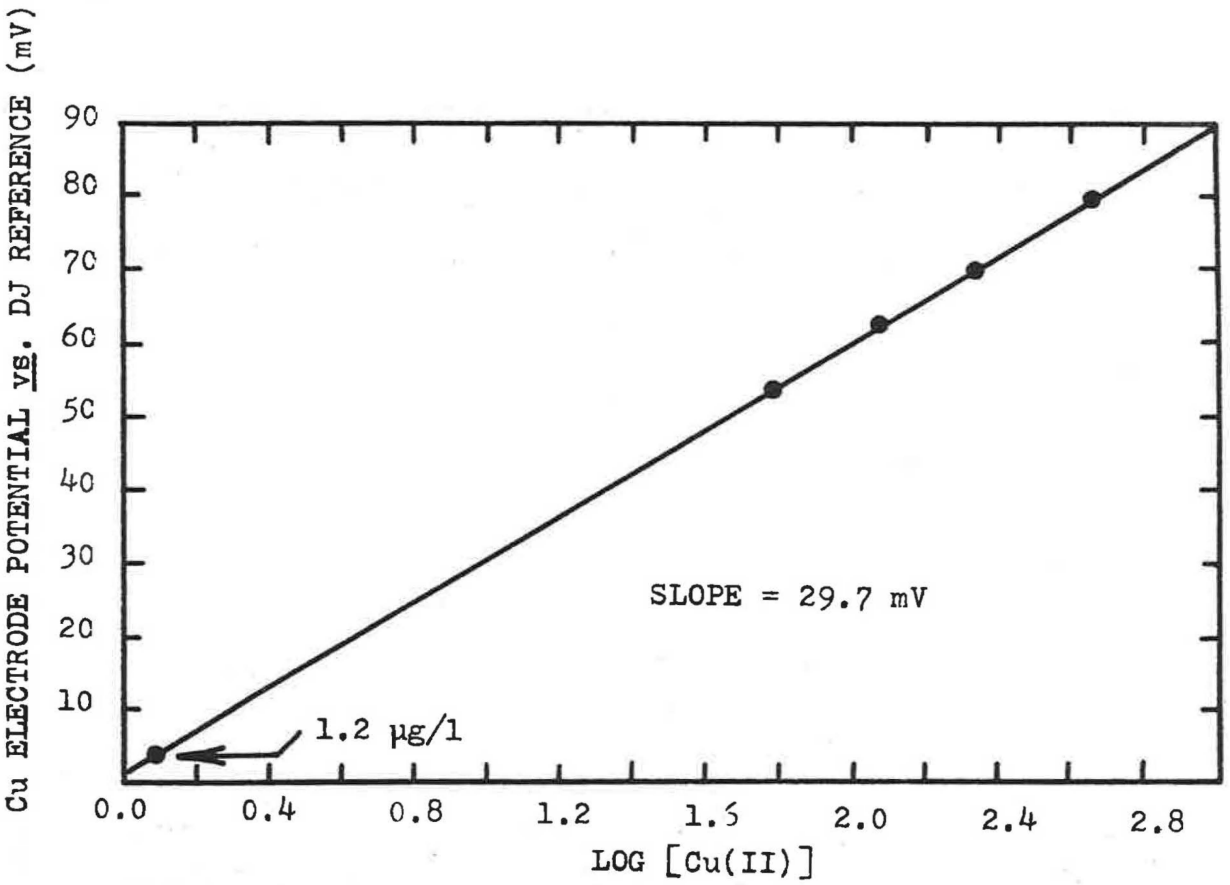


FIGURE 22. THE DETERMINATION OF THE BACKGROUND LEVEL OF Cu IN CAOB SOLUTION AT pH 5.0.

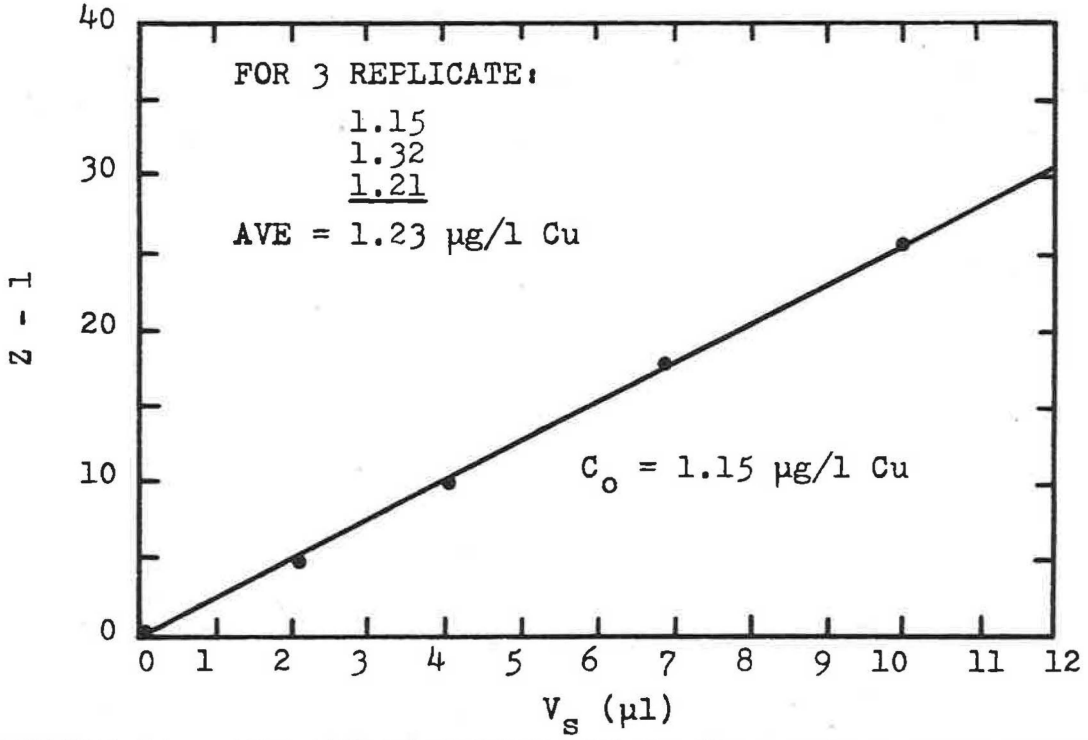


FIGURE 23. THE DETERMINATION OF Cu IN 0.050 M CAOB SOLUTION BY MULTIPLE STANDARD ADDITION AT pH 5.0. V_o = 100.00 ml.

90.0, and 900 µg/l were prepared by adding 30.0 µl of 30.00, 90.00, 300.0, and 3000. mg/l standard copper solutions to 100.00 ml volumes of CAOB solution. The results of this study are given in Table X. The average per cent deviation over the entire range investigated was 0.9. The precision estimates given here represent values determined under optimal conditions where precise temperature control was employed.

A considerable increase in precision is observed when multiple rather than single standard addition is employed in low level copper analyses. This effect is shown in Table XI. Identical samples of spiked 0.050 M CAOB solution were analyzed for copper by both single and multiple standard addition in the range from 9.0 to 90.0 µg/l Cu. The multiple addition analysis was carried out in each case with three additions of standard. The known amounts of copper were added to 100.00 ml of CAOB solution as 30.0 µl volumes of the appropriate standard copper solutions as described previously. The improvement in precision in the range investigated when multiple addition is employed is over 100 per cent.

The results from a typical copper determination made on a natural water sample are given in Figure 24. Multiple standard addition was employed, and the linearity of the multiple addition plot illustrates the effectiveness of the buffer system in decomplexing copper from ligands normally found in water samples. The water sample is University tap water. The

TABLE X

RESULTS FOR TRIPLICATE ANALYSIS OF Cu SPIKED 0.050 M CAOB SOLUTION BY MULTIPLE ADDITION AT VARYING LEVELS OF Cu(II)*

Sample Number	(µg/l) Cu Spike	Total (µg/l) Cu Found			Ave. (µg/l) Cu Found	% Deviation
		1	2	3		
1	9.0	8.0	10.2	8.5	8.9	1.1
2	27.0	26.2	28.0	25.9	26.7	1.1
3	90.0	94.2	92.6	86.2	91.0	1.1
4	900.0	901.	901.	901.	901.	0.1

*Corrected for 1.6 µg/l Cu Contamination in CAOB

TABLE XI

COMPARISON OF PRECISION FOR SINGLE VS. MULTIPLE STANDARD ADDITION IN Cu SPIKED 0.050 M CAOB SOLUTION AT LOW Cu LEVELS*

Sample Number	µg/l Cu Added	Single Addition		Multiple Addition	
		µg/l Cu Found	% Deviation	µg/l Cu Found	% Deviation
1	9.0	10.8	20.0	9.8	8.9
2	18.0	18.9	5.0	18.0	0.0
3	27.0	25.5	5.6	26.7	1.1
4	36.0	36.4	1.1	38.6	7.8
5	90.0	79.6	11.6	88.2	2.0
		Ave. % Dev. = 8.7		Ave. % Dev. = 4.0	

*Corrected for 1.2 µg/l Cu Contamination in CAOB

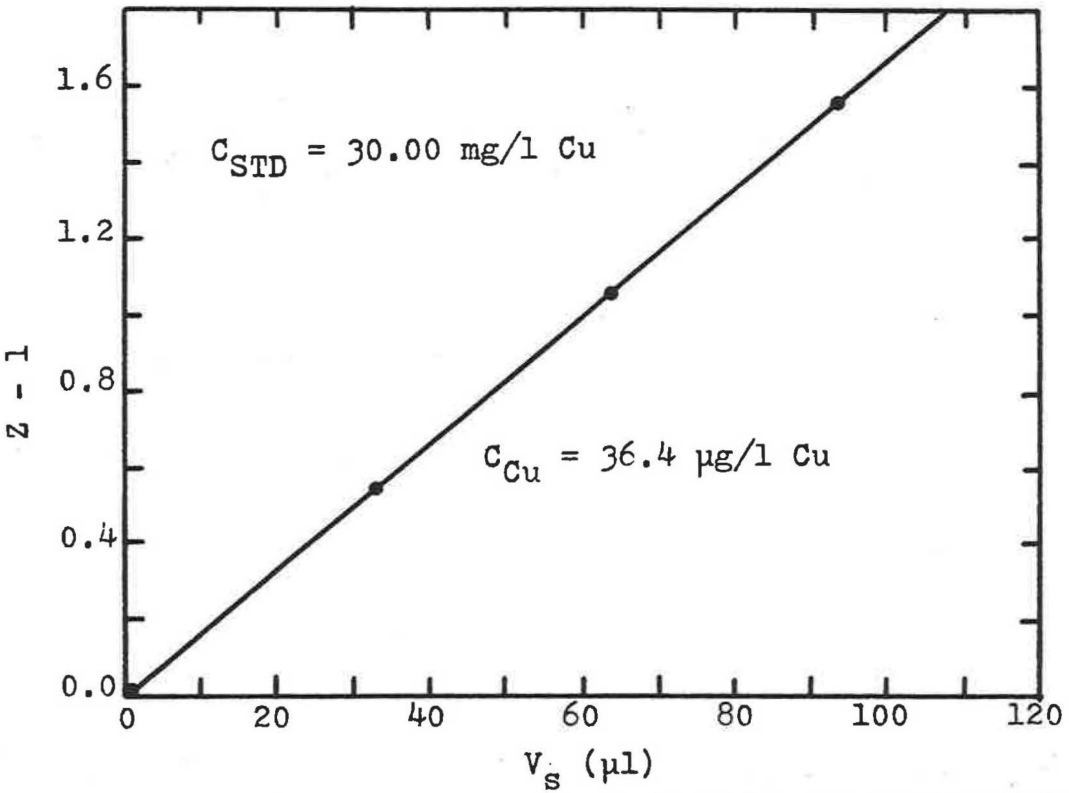


FIGURE 24. THE DETERMINATION OF Cu IN UNIVERSITY TAP H_2O BY MULTIPLE STANDARD ADDITION. V_o = 50.00 ml BUFFER + 50.00 ml SAMPLE.

composition of the sample matrix is given in Table XII. Water samples from a variety of sources were analyzed using the multiple addition technique and no difficulties were encountered. There is, however, the possibility of precipitating CaF_2 from hard waters extremely high in Ca^{2+} when the F^- containing CAOB solution and sample are mixed. In cases where samples of this nature are encountered, precipitation can be prevented by reducing the formal concentration of F^- in the CAOB solution.

The loss of copper from untreated water samples was especially evident for ground water samples. Up to 90 per cent losses of copper from untreated water samples stored overnight in polyethylene bottles was observed. A multiple standard addition plot for a sample of University tap water taken at the same time as the sample analyzed in Figure 24 is shown in Figure 25. The level of copper in the sample was reduced from 36.4 µg/l to 3.3 µg/l after storage of the untreated sample for 17 hours.

The recovery of known amounts of copper from natural water samples and one tap water sample (sample # 4) was also investigated. The water samples analyzed contained from 3.3 to 46.8 µg/l Cu. After the copper level in the original filtered water sample was determined, an identical 50.00 ml sample was filtered and mixed with 50.00 ml of CAOB solution. Thirty microliters of a 60.00 mg/l Cu solution were then

TABLE XII

COMPOSITION OF UNIVERSITY TAP WATER*

Component	Concentration (mg/l)
Ca^{2+}	59.2
Mg^{2+}	27.2
Na^{+}	46.5
K^{+}	5.6
SO_4^{2-}	16.3
HCO_3^{-}	357.4
NO_3^{-}	0.3
CO_3^{2-}	0.0
Cl^{-}	32.9
F^{-}	1.2
Fe	0.02
Al	<0.1
SiO_2	8.0
Mn	0
Zn	1

pH = 7.2

*Source: Department of Public Health and Welfare of Missouri, Division of Health, Environmental Services Laboratory

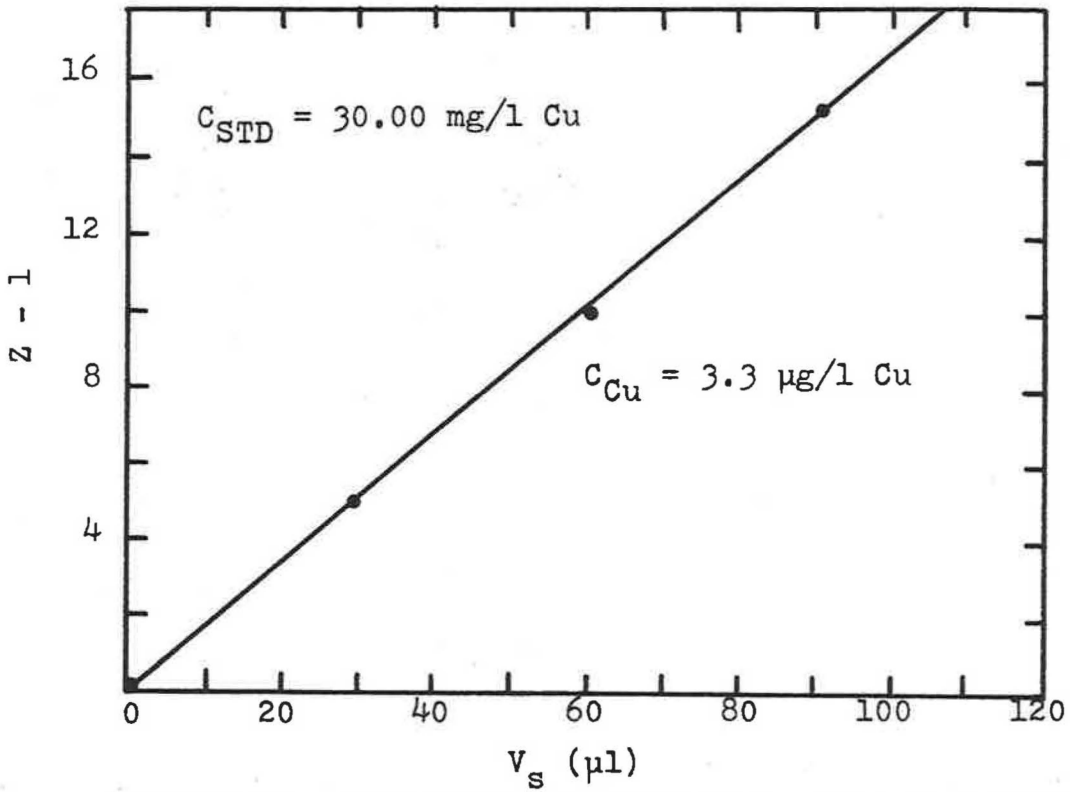


FIGURE 25. THE DETERMINATION OF Cu IN A H_2O SAMPLE BY MULTIPLE STANDARD ADDITION AFTER STORAGE FOR 17 HRS W/O TREATMENT. V_o = 50.00 ml H_2O + 50.00 ml SAMPLE.

added to the sample-buffer mixture and the sample was analyzed for copper by multiple standard addition. The spike added corresponds to 9.0 μg/l additional copper in the original sample. The recovery data for six samples are given in Table XIII. The average per cent recovery is 102.9 with a standard deviation of 7.5 per cent.

INTERFERENCE STUDY

The influence of foreign ions was studied by adding a known quantity of the respective ion to 100 ml aliquots of 0.050 M CAOB solution spiked with 90.0 μg/l Cu. The resulting mixture was analyzed for copper by multiple standard addition. A total concentration ratio of foreign cation to copper of approximately 100 was established by adding a 9.0 mg/l spike of foreign ion to the copper containing CAOB solution that was pre-equilibrated with the electrode measuring system at 25.00° C. Higher ratios of cationic interferences were not investigated because of the possible interference from contaminant copper in the reagent grade metal ion salts used to prepare the foreign ion standard solutions. Ferric ion salts are especially high in copper and often contain as much as 10^{-2} per cent contaminant copper. Foreign ion solutions were prepared in 0.01 N sulfuric or nitric acid. The acidified Fe(II) solution was stabilized by preparation in the presence of iron wire and was used as

TABLE XIII

RECOVERY STUDY FOR KNOWN AMOUNTS OF Cu IN CAOB SOLUTION ADDED TO FILTERED WATER SAMPLES

Sample #	µg/l Cu Found In Sample	µg/l Cu Added	Theoretical µg/l Cu After Addition of Spike	µg/l Cu Found In Spike	% Recovery Of Spike
1	16.3	9.0	25.3	10.2	113.3
2	32.6	9.0	41.6	8.8	97.8
3	46.8	9.0	55.8	9.9	110.0
4	36.4	9.0	45.4	9.2	102.2
5	3.3	9.0	12.3	8.4	93.3
6	16.3	9.0	25.3	9.1	101.1
				Average % Recovery:	102.9
				Standard Deviation:	7.5

rapidly as possible.

Chloride and bromide ion interferences were investigated at concentrations of 0.010 M by adding 1.00 ml of the appropriate 1.00 M standard solution to exactly 100 ml of CAOB spiked with 90.0 µg/l Cu. The apparent copper concentration determined by multiple addition was corrected for the 1 per cent volume change.

The results of the interference study are shown in Table XIV. Only Fe(III) and Cd(II) seem to affect the electrode in the CAOB solution at concentrations approximately 100 times the level of copper. The multiple addition plots for the Cd(II) and Fe(III) studies were, however, linear and the apparent copper concentration determined in each case agreed with the amount added within experimental error. The +0.9 mV shift in electrode potential observed upon the addition of 9.0 mg/l Fe(III) to the CAOB solution corresponds to a possible change in the total copper concentration of 6.6 µg/l Cu. An analysis of the 3000 mg/l Fe(III) stock solution by atomic absorption showed the solution to contain 1.1 mg/l Cu which corresponds to an additional 3.3 µg/l Cu contributed to the CAOB solution from the stock Fe(III) solution. The average value for triplicate determinations of copper in this solution was 97.1 µg/l Cu. Subtracting the 3.3 µg/l Cu contamination from the Fe(III) solution leaves a deviation of 4.2 per cent from the level of copper known to be present, which is within experimental error.

TABLE XIV

EFFECT OF FOREIGN IONS

The following materials had no observable effect at the concentrations indicated on the equilibrated copper electrode system in CAOB spiked with 90.0 µg/l Cu. A normal multiple addition plot was obtained in all ceses.

Added As	Possible Interference	Concentration moles/l	mg/l
NaCl	Cl^-	1.0×10^{-2}	355
NaBr	Br^-	1.0×10^{-2}	799
$FeSO_4 \cdot 7H_2O$	Fe^{2+}	1.6×10^{-4}	9.0
$NiCl_2 \cdot 6H_2O$	Ni^{2+}	1.5×10^{-4}	9.0
$Co(NO_3)_2 \cdot 6H_2O$	Co^{2+}	1.5×10^{-4}	9.0
$ZnSO_4 \cdot 7H_2O$	Zn^{2+}	1.4×10^{-4}	9.0
$Pb(NO_3)_2$	Pb^{2+}	4.3×10^{-5}	9.0

The following materials caused the listed changes in electrode potential under the conditions given above. A normal addition plot was obtained in both cases.

Added As	Possible Interference	E(mV)	Concentration moles/l	mg/l
$CdCl_2$	Cd^{2+}	-0.4	8.0×10^{-5}	9.0
$Fe(NO_3)_2 \cdot 9H_2O$	Fe^{3+}	+0.9*	1.6×10^{-4}	9.0

*See Text

CHAPTER VIII

CONCLUSION AND SUMMARY - PART II

A simple, direct multiple standard addition method has been developed for the determination of copper in natural waters at trace levels using an Orion solid-state cupric ion electrode. While the technique is not as rapid as the commonly used flame atomic absorption technique, it does possess superior sensitivity. The adaptability of potentiometric devices to on-site measurements should make the technique quite useful to those concerned with water quality and the control of algal populations in natural waters.

Sample pretreatment (ie., digestion, separation, etc.) is not necessary. Measurements are made in a 0.05 M complexing antioxidant acetate buffer (CAOB) containing sodium fluoride and formaldehyde. The buffer solution contains NaF to complex Fe(III) which decreases electrode interference from Fe^{3+} and to prevent the precipitation of $Fe(OH)_3 \cdot X\ H_2O$ from ground water samples. The formaldehyde in the buffer solution provides a reducing medium to prevent electrode interference from oxidizing agents. Acetate ion in the buffer system serves to decomplex copper from mild complexing agents found in natural water samples. The large excess of acetate over copper in the sample-buffer mixture allows the fraction of copper that is complexed in the medium to remain constant as the addition of copper standard is made.

Triplicate analyses of 0.050 M CAOB solution spiked with copper at the levels of 9.0, 27.0, 90.0, and 900 μg/l were carried out to estimate the precision with which trace copper measurements could be made using multiple standard addition. The average per cent deviation in the range investigated was 0.9 when precise temperature control was employed. A considerable improvement in precision was observed when multiple rather than single standard addition was employed.

The recovery of known amounts of copper from previously analyzed water samples was also investigated. The average per cent recovery for 9.0 μg/l additions of copper to the sample in the CAOB solution was 102.9 with a standard deviation of 7.5 per cent.

An interference study was made for several foreign ions. It was found that Fe(II), Ni(II), Co(II), Zn(II), and Pb(II) at concentrations approximately 100 times the level of copper and 0.01 M Cl^- and Br^- had no effect on the electrode system in the CAOB solution. Only Fe(III) and Cd(II) seemed to affect the electrode in the CAOB solution at concentrations 100 times the level of copper. The multiple addition plots prepared in the presence of either ion, however, were linear and the apparent copper concentration determined in each case agreed with the amount added within experimental error.

An "addition-calibration" method which combines a standard addition step with a calibration curve for the determination of S, the electrode slope sensitivity, was developed. The technique also yields information about the linearity of the E vs. log concentration plot near the background contamination level in the medium in which sample measurements are to be made.

A computer program was written in order to determine the least-squares line through experimental data for plots of E vs. log concentration or activity. A provision was made for accurately plotting the experimental data and the computed least-squares line through the data points using a CalComp plotter. The program was used to evaluate the response of the cupric ion electrode in the CAOB solution used for copper determinations in natural waters. The average slope sensitivity of the cupric ion electrode vs. a double junction reference electrode the CAOB solution was determined to be the theoretical 29.6 mV at 25.00° C with an average deviation of ± 0.3 mV.

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VITA

Michael James Smith was born on February 18, 1945 in E. St. Louis, Illinois, the son of Mr. and Mrs. James F. Smith. He lived in Troy, Illinois during his early years where he attended public schools in the Triad School District. He graduated from Troy Public School in June, 1959 and from Triad High School in June, 1963. In the fall of 1963, he began his college career at Southern Illinois University. He received the Bachelor of Arts degree from the same institution in June, 1967. The author entered graduate school in the fall of 1967 at the University of Missouri, Columbia. He received the Master of Arts degree in June, 1969. After serving in the United States Army as a Combat Engineer, he returned to graduate school at the University of Missouri and expects to receive the Doctor of Philosophy degree in December, 1972. The author will join the faculty at Wright State University in Dayton, Ohio in September, 1972.

9. Training Accomplished.

One Ph.D. candidate, two Master's candidates, and one undergraduate student received financial support from the grant. In addition, during July and August of 1971, nine students working on an NSF Student Originated Studies project worked on various aspects of the research for a brief time.

On a particularly encouraging note, Dr. Michael Smith, who completed his Ph.D. thesis on the project, had no difficulty finding an excellent job and had a number of other inquiries regarding possible employment opportunities. It is the feeling of the principal investigator that there is a growing demand for Ph.D. chemists who through their research and course work have tha capability of doing applied research in the area of aquatic chemistry. Dr. Smith's success in finding employment would indicate that this is the case.

10. References.

(See body of the report.)

11. Appendices.

(None)

8. Publications, Reports, Papers, Talks Presented.

No publications have yet come from this work, although several are in press or to be submitted. The following papers on this research were presented orally:

Paper Title	Meeting
"Studies of the Effect of Cupric Ion Activity on Algal Growth. The Use of the Cupric Ion-Selective Electrode in Natural Aquatic Systems."	162nd National American Chemical Society Meeting, Washington, D. C., September, 1971.
"The Removal of Trace Metal Contaminants from Biological Systems."	7th Midwest Regional American Chemical Society Meeting, St. Louis, Mo., Oct., 1971.
"Influence of Chelating Agents on Heavy Metal Ion Availability in Natural Waters."	163rd National American Chemical Society Meeting, Boston, Mass., April, 1972. (To be published as part of a symposium volume.)
"Ion-Selective Electrodes in Environmental Research."	141st Meeting of the Electrochemical Society, Houston, Texas, May, 1972. (This was an invited paper to a symposium on Electrochemical Contributions to Environmental Protection. It will be published as part of a symposium volume.)
"Use of the Copper Ion-Selective Electrode in the Investigation of the Copper Requirement of Algae."	Northwest Regional American Chemical Society Meeting, Corvallis, Oregon, June, 1972.
"The Solid-State Copper Ion-Selective Electrode for Low Level Copper Analysis."	First Rocky Mountain Regional American Chemical Society Meeting, Fort Collins, Col., July, 1972. (Part of a symposium on Advances in Electrochemical Science.)