Spectrophotometric determination of iron, copper and zinc in a single aliquot of serum using 2-amino-5-bromo-pyridylazo resorcinol.

Narace Dyal Seudeal
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SPECTROPHOTOMETRIC DETERMINATION OF IRON, COPPER AND ZINC IN A SINGLE ALIQUOT OF SERUM USING 2-AMINO-5-BROMO-PYRIDYLazo RESORCINOL

BY
Narace Dyal Seudeal

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
1985
ABSTRACT

SPECTROPHOTOMETRIC DETERMINATION OF IRON, COPPER AND ZINC
IN A SINGLE ALIQUOT OF SERUM USING 2-AMINO-5-
BROMO-PYRIDYLazo RESORCINOL

by

Narace Dyal Seúdeal

A modified procedure was developed to synthesize the
reagent 2-amino-5-bromo-pyridylazo resorcinol, Br-PAR, with
the advantages of higher purity and less time required for
its synthesis. Br-PAR was found to be more sensitive than
PAR and most of the other reagents used to determine the
major trace elements iron, copper and zinc in aqueous and
biological samples.

The analytical studies dealt with the determination of
iron, copper and zinc individually as well as sequentially
using the synthesized Br-PAR as the common ligand for all
three metals. The molar absorptivities for iron, copper
and zinc as determined were 66,500, 72,200, and 91,100-
cm²mol⁻¹, respectively, and Beer's law was obeyed up to
300μg/dL for all three metals. Each metal was assayed in
turn from a mixture of all three by first determining the
absorbance due to the reaction of the three metals, then
sequentially determining the absorbance drop due to the
presence of copper by selective masking of it with cyanide, and then the absorbance drop due to zinc by selectively masking with EDTA. The residual absorbance was due to iron.

This sequential method was then applied to serum samples and normal ranges were derived that agreed with those found in the literature. Recovery of each metal was quantitative and ranged from 94-104%. Precision studies showed coefficients of variation of less than 2% for both within-run and between-run studies.
DEDICATION

To my loving wife, Donna; my parents, Olive and Eddie; my brother, Mohan and my sisters, Doreen, Vigai and Brehase with deepest love and profound gratitude.
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I would sincerely like to thank my advisor, Dr. R. J. Thibert, for his financial and moral support throughout the entire period of this study. Moreover, I must thank him for his supervision and direction during my entire M.Sc. program.

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% per cent
kg kilogram
g gram
mg milligram
µg microgram
M molar
µM micromolar
L liter
dL deciliter
mL milliliter
µL microliter
h hour
d day
min minute
TIBC total iron binding capacity
S.D. standard deviation
TPTZ 2,4,6-tripyridyl-s-triazine
PDT 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine
PDTS PDT-sulfonate
TCA trichloroacetic acid
β-CI-PAN β-chloro-pyridylazo napthol
PAR pyridylazo resorcinol
EDTA ethylenediaminetetraacetic acid
m.p. melting point

xii
°C  degree Celsius
v/v  volume by volume
cm  centimeter
mm  millimeter
N  normality
nm  nanometer
λ  wavelength
Br-PAR  2-amino-5-bromo-pyridylazo resorcinol
AA  ascorbic acid
r  correlation coefficient
A  absorbance
n  number of samples
S.E.M.  standard error of the mean
C.V.  coefficient of variation
σ_n-1  standard deviation at n-1 degrees of freedom
CHAPTER I

INTRODUCTION

A. PHYSIOLOGY AND METABOLISM OF IRON, COPPER AND ZINC

1. Iron

The amount of iron in an individual is approximately 4 to 5 g of which 70 to 75% has an active and vital physiological role and the remaining 25 to 30% is present in various storage forms which can be readily mobilized if needed as shown in Fig. 1 (1). Hemoglobin contains about two-thirds of the iron in the adult human and 5% can be found in myoglobin. The heme containing enzymes of the mitochondria contain 0.1% of the total iron despite their importance in the transformation of energy. Flavin iron enzymes, such as succinic dehydrogenase contain a smaller fraction. Catalase in red blood cells contain about 0.1% of the total iron, however, there is no estimate on flavoproteins containing non-heme iron in multiple sulfur linkages. A small amount of iron circulates in plasma combined with transferrin. The remainder is stored mainly in the liver, spleen, bone marrow and muscle as ferritin and hemosiderin.

Iron is removed from senescent red blood cells by the
FIGURE 1
PATHWAYS OF IRON METABOLISM

Legend

FIGURE 1

Ingested food iron
(Fe$^{+2}$ and Fe$^{+3}$)
20 mg/day

Fe$^{+3}$-ferritin in intestinal
mucosal cell

Excretion of
unabsorbed Fe
in stool

Excretion of Fe
through urine and
skin (less than .1%)

Functional iron (Fe$^{+2}$ or Fe$^{+3}$)
e.g., enzymes 1%

Fe$^{+3}$-ferritin or
hemosiderin

Myoglobin (Fe$^{+2}$)
3-5%

Fe$^{+3}$-transferrin and
Fe$^{+3}$ in plasma

R.E. system

Hematopoietic organs

Hemoglobin (65-70%)

Loss of iron due to bleeding
breakdown of hemoglobin in reticuloendothelial cells in the
liver, spleen and bone marrow. Most of this iron is then
released to circulating transferrin and delivered to
erthrocyte precursors in the bone marrow, where it is
reutilized for hemoglobin synthesis. Thus, iron is conserved
and normally there is a relatively small requirement for
absorption of dietary iron to maintain body iron balance.
This "closed-circuit" is illustrated in Fig. 2 (2). Daily
requirements for iron are approximately 1 mg in the adult male
and 1½ mg in women of childbearing age.

Three plasma proteins, i.e., transferrin, haptoglobin
and hemopexin, transport iron in the circulatory system.
Transferrin is the major iron-binding protein, while
haptoglobin binds hemoglobin. Hemopexin binds heme and
transport it to the liver. At any given time, about 3-4 mg
of iron is present in the circulation bound to plasma
transferrin, a β-globulin having a molecular weight of
77,000 (3). The major function of this protein is to
transport inorganic iron from the alimentary tract and
ericuloendothelial system to the erythroid marrow.

Iron absorption from the gut has been extensively
studied but a totally acceptable mechanism for its absorption
has not been found. McLaren et al. (4) describe the
absorption of iron into phases. In the intraluminal phase,
they contend that iron may be divided into heme and non-heme
FIGURE 2

SUMMARY OF UNIQUE "CLOSED-CIRCUIT" METABOLISM OF IRON IN THE BODY

Legend

iron pools. Heme is found primarily in such proteins as hemoglobin and myoglobin, while non-heme iron is present in eggs, vegetables and other foodstuffs in the form of ferrous and ferric salts. Some inorganic iron (non-hemé) is also complexed by starch, protein, fiber and phosphate-rich substances such as phytate or vitallin (5). Because of the affinity of phosphorus for iron, foods rich in phosphorus decrease iron absorption. The iron from complexes with starch, protein and some phytate can be released by digestion but that complexed with fiber is not released and thus, passes out into the feces.

The intestinal epithelial cells are capable of taking up iron from the gut lumen in either the heme or non-heme form. This uptake occurs most efficiently in the duodenum (6). The exact mechanism of absorption is not well understood. The processes of iron absorption and release are summarized in Fig. 3 (7), which shows that on demand iron is released from the mucosal cells into the blood where it circulates mainly as Fe\(^{2+}\)-transferrin, which in turn is in equilibrium with an extremely small amount of free Fe\(^{3+}\).

Iron is stored primarily in hepatocytes and reticulendothelial cells in the form of ferritin and hemosiderin. Ferritin is a predominantly intracellular protein consisting of 24 subunits of 19,000 to 21,000 daltons each (8). Hemosiderin appears to be an iron-dense material
FIGURE 3

INTRALUMINAL FACTORS AND MUCOSAL PROCESSES IMPORTANT IN IRON ABSORPTION

Legend

consisting of multiple aggregates of ferritin molecules in which the iron cores have coalesced, probably as a consequence of protein loss (9).

The iron content of the body is regulated by control of absorption of iron from the gut (10). The excretion of iron in the feces amounts to 5–25 mg or more each day, but all except 0.2–0.5 mg is iron that has remained unabsorbed from food. About 1 mg is secreted into the bile per day but nearly all is reabsorbed and recycled. Excretion in urine is about 0.2–0.3 mg daily. The consensus on skin excretion is 0.5 mg (11) with greater losses in sweat at higher temperatures. Thus, total losses range between 0.6–1.0 mg daily under ordinary circumstances. These are balanced by absorption of an equal amount.

2. Copper

A 70-kg human body has about 80–120 mg copper. The daily requirement for humans is 2.5 mg. Large amounts of copper can be found in shellfish, oysters, organ meats, legumes, dried vegetables and cocoa. In the human, the highest concentrations of copper in decreasing order can be found in the liver, brain, heart, kidney and other tissues. About 10% of all copper in the human body is present in the liver and about one-half the total is found in bone and muscle because of their large masses. Copper in the blood occurs in erythrocytes, bound to albumin and complexed with
ceruloplasmin and amino acids. The mean value for erythrocytes in 980 μg/L (15.5 μM) and the major portion of copper occurs in superoxide dismutase. Plasma has about 1.09 mg/L of which 93% is bound to ceruloplasmin (12). The remainder is copper bound to albumin.

The two fractions of serum copper appear to maintain separate pathways in the hepatocytes, where ceruloplasmin copper becomes more firmly attached to cytochrome oxidase than that bound by albumin (13). Their transport in the cell is mediated by carrier proteins that differ in size (14) of which plasma albumin appears to be the first carrier to which absorbed or injected copper is bound. Copper complexed with albumin rapidly disappears from the blood with a concomitant increase in hepatic copper content as copper bound to ceruloplasmin.

Copper is absorbed from the stomach by at least two mechanisms (15). One is an energy-dependent process, is facilitated by amino acids, and probably represents the absorption of copper complexes of amino acids. A larger portion of the copper is absorbed by the second mechanism and is bound to two protein fractions in the intestinal mucosa. One of these proteins is the copper enzyme, superoxide dismutase. The second protein is rich in sulfhydryl groups and has the characteristics of metallothionein.

In the liver, copper is largely sequestered with acid
phosphatase rich pericanalicular lysosomes. The bile is the major pathway for copper excretion (16) where it is closely associated with taurochenodeoxycholate (17). Very little copper is excreted in the urine, about 5–24 μg/24 h.

Copper can be found in many enzymes and some are presented here. Apart from binding copper, ceruloplasmin aids in the mobilization of iron from ferritin in the liver and other iron-storage sites. To form stable complexes with transferrin, it must be converted to the ferric form. Conversion is accomplished by ceruloplasmin (18). Cytochrome oxidase contains one atom of copper for each atom of iron and molecule of heme. A severe deficiency of copper is accompanied by a decrease in capacity for oxidative phosphorylations in liver, muscle and nervous tissue. This effect is attributed to the impaired synthesis of this enzyme (19). Copper is a component of the monoamine oxidase system present in elastic and connective tissues that catalyses oxidative deamination of lysine in peptide chains to amino adipic semialdehyde (20), which forms the cross-linkages of vascular tissues (21). Superoxide dismutases are copper containing proteins isolated from erythrocytes (erythrocuprein). The function of the dismutase is the conversion of superoxide free radical anions into oxygen and hydrogen peroxide as follows:

\[ 2H^+ + O_2^- + O_2^- \rightarrow O_2 + H_2O_2 \]

Additional enzyme systems include tyrosinase, ascorbic acid oxidase, uricase, aminolevulinate dehydratase and galactose
oxidase.

3. Zinc

A 70-kg human body has about 1.4 to 2.3 g of zinc. The adult ingests about 10-15 mg daily and absorbs about 5 mg primarily from the small intestine. The erythrocytes contain about 75-85%, primarily in the zinc metalloenzyme, carbonic anhydrase. Plasma contains about 12-22% and leucocytes about 3% of the total zinc in whole blood of humans (22). The normal zinc level is about 100 μg/dL. Much of the zinc is present in the bone and skin. The visceral organs contain about 15-55 μg/g of fresh tissue and prostate and tapetum of the eye are noteworthy for having exceptionally high concentrations. The zinc levels vary from 300 ± 20 μg/dL in earlier years to 95 ± 12 μg/dL in later years. The concentrations of zinc in serum are higher by 5 to 15 μg/dL than those in plasma, mainly because zinc is released from platelets during clot formation (23).

Paris and Vallee (24) indicate that 30-40% of the zinc in serum is firmly bound to $\alpha_2$-macroglobulin and the remaining zinc is loosely bound to albumin. According to Giroux and Henkin (25), 1 μg/dL of human serum zinc is complexed with amino acids cysteine and histidine on the basis of in vitro addition of these acids to albumin.

Normally, only a small percentage of ingested dietary zinc is absorbed. Absorption is difficult to precisely ascertain, because excretion of zinc is nearly all via the gut.
Data on site(s) of absorption in man and on mechanism(s) of absorption (whether by active, passive, or facultative transport) are meager. Evans (26) proposed a mechanism whereby the pancreas secretes a compound into the duodenum that binds zinc which is then transported into the epithelial cell where the metal is transferred to binding sites on the basolateral plasma membrane. Albumin interacts with the plasma membrane and removes zinc from the receptor sites.

Several factors influence the absorption and retention of zinc and thus its availability from the diet. Phytate (myo-inositol hexamonophosphate), which is present in cereal grains, markedly impairs the absorption of zinc as was first shown by O'Dell and Savage (27). High fiber intake prevents the absorption of zinc as the zinc fiber complex is not degraded by the digestive secretions and is thereby lost in the feces still bound to the fiber (5).

Various studies by Vallee (22) have shown that zinc is a constituent of a number of metalloenzymes. Some of these enzymes are shown in Table I. If related enzymes from different species are included, then over 80 zinc metalloenzymes are now on record. The metal is present in several dehydrogenases, aldolases, peptidases and phosphatases.

The primary excretory pathway for zinc is the gastrointestinal tract. The mechanism of excretion is unknown. Small amounts of zinc are lost in the urine, 300–700 μg/24 h. About 1 mg/L may be lost in sweat and the amount of zinc in the stool
TABLE I

ZINC METALLOENZYMES

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Yeast, human liver</td>
</tr>
<tr>
<td>D-Lactate cytochrome oxidase</td>
<td>Yeast</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>Beef, pig muscle</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>Yeast</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>E. coli</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>E. coli, sea urchin</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>Avian myeloblastosis virus</td>
</tr>
<tr>
<td>Mercaptopyruvate sulfur transferase</td>
<td>E. coli</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>E. coli</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>Pig kidney, lens</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>Beef, human pancreas</td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>Beef, pig pancreas</td>
</tr>
<tr>
<td>Carboxypeptidase C</td>
<td>Pseudomonas stutzeri</td>
</tr>
<tr>
<td>Dipeptidase</td>
<td>Pig kidney</td>
</tr>
<tr>
<td>Neutral protease</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>Alkaline protease</td>
<td>Escherichia feundi</td>
</tr>
<tr>
<td>AMP aminohydrolase</td>
<td>Rabbit muscle</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Yeast, aspergillus niger</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Erythrocytes</td>
</tr>
<tr>
<td>δ-Aminolevulinic acid dehydratase</td>
<td>Beef liver</td>
</tr>
<tr>
<td>Phosphomannose isomerase</td>
<td>Yeast</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>Yeast</td>
</tr>
</tbody>
</table>

Taken from Vallee (22).
seems to be related to the amount in the diet (28).

B. CLINICAL SIGNIFICANCE OF IRON, COPPER AND ZINC

1. Iron

The clinical significance of serum iron content is depicted in such disease states as severe anemias, iron poisoning and iron overload (29). Anemias due to a deficiency of iron are of three basic types:

(a) anemias caused by excessive loss of body iron as in menstrual bleeding, lesions of intestines, malignancies, infections and parasites;

(b) anemias due to malabsorption of iron. The poor availability of iron from cereal diets is a major cause of iron deficiency in less developed countries. Iron intakes may be high, exceeding by several fold the estimated requirements yet fail to yield the 4 to 10 mg per day needed by adolescents and adults in the available form;

(c) anemias associated with nutritional deficiencies such as in infancy as a consequence of undue dependence on milk which contains little iron.

As a result of iron deficiency, markedly lowered cytochrome oxidase activities in cells obtained by biopsy of buccal mucosa have been observed (30). Disturbances of bone marrow function and the decreased production of erythrocytes along with abnormal size cells are also consequences of iron
deficiency (31).

High serum or plasma iron can be seen in cases of decreased iron utilization, decreased formation of blood as in lead poisoning or pyridoxine deficiency and also in situations in which increased release of iron from body stores occurs, e.g., release of ferritin in necrotic hepatitis (32). Increases in serum or plasma iron may be seen in states characterised by increased red cell destruction such as in hemo-lytic anemia and decreased survival time of red blood cells as in chromium poisoning. Elevated levels can also be seen in conditions in which the process of iron storage is defective as in pernicious anemia and in conditions in which there is an increased rate of absorption.

In contrast to anemias, iron poisoning and iron over-load are less prevalent but still dangerous. The oral intake of excessive amounts of iron, such as ferric sulphate, can be toxic and result in death. Increased body iron stores are associated with greater accumulations of these iron containing proteins, and progressive iron overloading favours the conversion of ferritin to hemosiderin. The body is capable of storing relatively large quantities of iron in ferritin or hemosiderin apparently without undue effects, but when the iron load is above normal or when the storage capacity has been exceeded; tissue damage may ensue. The syndrome of hemo-chromatosis is thought to be attributable to the toxic effects of excess iron in parenchymal cells of the liver and other involved organs. This accumulation of iron and the development
of organ damage generally occur over a period of many years.

Alterations in the level of serum iron and total iron binding capacity are summarized in Table II.

2. Copper

Three distinct syndromes of copper deficiency have been described in infants:
(a) a moderate to severe anemia in infants whose diets were based on cow's milk (33);
(b) characterized by chronic diarrhea and
(c) a sex-linked recessive disorder with retardation of growth, defective keratinization and pigmentation of the hair ("kinky hair"), hypothermia, degenerative changes in the skeleton and progressive mental deterioration (34).

Decreased serum copper concentrations may be due to malabsorption from celiac disease. (35), and sprue (tropical and non-tropical) (36). Prolonged administration of penicillamine also decreases copper. Several instances of copper-dependent anemias have been described in children (37). Enzootic neonatal ataxia which results in uncoordination of movement and gross ataxia are symptoms found in copper deficient neonates of many species (38). Various studies correlating symptoms and pathological findings with depleted activities of the copper enzyme, cytochrome oxidase, indicate that decreased activity of this enzyme results in the clinical and pathological findings typical of enzootic neonatal ataxia (39). Defects in
### TABLE II

**SERUM IRON AND TIBC VALUES IN VARIOUS DISEASES**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Serum Iron</th>
<th>TIBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency anemia</td>
<td>↓ ↓</td>
<td>↑</td>
</tr>
<tr>
<td>(dietary, malabsorption, chronic hemorrhage, late pregnancy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia of chronic infections</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Anemia of neoplastic disease</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Hemolytic anemia</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Hemosiderosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Chronic Liver disease</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Obstructive jaundice</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Polycythemia</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Nephrosis</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Hodgkin's disease (terminal)</td>
<td>↑ ↑</td>
<td></td>
</tr>
</tbody>
</table>

*^a^Taken from: Tietz, N.W. (1976) in Fundamentals of Clinical Chemistry (Tietz, N.W., ed.), p. 923, W.B. Saunders Co., Toronto*
connective tissue formation have also been observed (40).

Increased copper accumulation in the organism, either chronic as in Wilson's disease or acute as in copper poisoning, can cause brain damage (41). Ceruloplasmin concentration in serum increase in pregnancy reaching values at parturition that are about two times those found in non-pregnant women. Women on contraceptive medications containing estrogens also show marked increase in copper (42).

Increased levels have been observed in several illnesses. According to Sass-Korstak (43) these include acute and chronic infections, disseminated lupus erythematosis, leukemias, malignant neoplasia, granulomatous diseases and peripheral vascular diseases. However, not all patients show increases. Increases have also been observed in major surgery and myocardial infarction which probably is due to stress (43). Both acute and chronic liver diseases also show increases.

The most significant clinical application of copper determinations is in the diagnosis of hepatolenticular degeneration, Wilson's disease. This disease is associated with a decrease in the synthesis of ceruloplasmin which results in a low serum level of this enzyme (<20 μg/100 mL). The amount of free and albumin copper, however, is greater than normal, and this is attributed to a greater-and uncontrolled rate of absorption of copper. Despite this fact, the total serum copper concentration is generally decreased (because of low ceru-
lopasmin values). The amount of copper deposited in tissues (e.g., liver and brain) is greatly increased and there is also an increased urinary excretion of copper, possibly due to the increase in free serum copper. Thus, the determination of serum ceruloplasmin, of the total serum copper and of urinary copper are of great help in the diagnosis of this disease (43).

The interrelationship between copper and iron metabolism indicates that some copper deficiencies are associated with iron deficiencies (44). Also, others have shown that altered copper and iron levels are associated with certain carcinomas (45). Copper depleted animals loose the ability to utilize stored iron because of the failure of the ferrous to ferric conversion (46). As a result, abnormally enlarged iron stores may accompany an iron deficiency type anemia.

3. Zinc

The sign of zinc deficiency in childhood is retarded growth from infancy to adolescence. In the adolescent, there is the failure to undergo the rapid sexual maturation and growth spurt characteristic of this stage of development (47). Administration of available zinc to affected dwarfs resulted in dramatic stimulation of growth and sexual development (48, 49). Under dietary conditions prevailing in Iranian villages, the response was slow but unmistakable (50). The village diets do not lack zinc but deficiency develops because of
interference with uptake of zinc from the intestine by dietary fiber and to a lesser extent, phytate, both being present in the Iranian diet in unusually large amounts.

The clinical significance of zinc was shown by Moynahan and Barnes who used zinc sulfate to treat a girl with acrodermatitis enteropathica with dramatic therapeutic results (51). The disorder is a recessively inherited disease due to zinc deficiency (52). There is a defect in zinc absorption that may be due either to an absence of a low molecular weight zinc binding ligand in the pancreas (53) or to a defect in prostaglandins, which are known facilitators of zinc absorption (54). The clinical features include a bullous pustular dermatitis about the mouth, genital areas and limbs. Diarrhea with and without malabsorption may occur and irritability, restlessness and anorexia are present. Growth and sexual development are stunted or very markedly delayed.

Acute changes of plasma zinc concentrations fall sharply as a result of acute stress such as that produced by myocardial infarction (55) or the injection of pyrogens (56). Chronic changes include pregnancy in which there is a progressive decrease of zinc concentration in plasma (57) due to demands of fetus. Low plasma zinc is found in liver cirrhosis of alcoholic origin. Some two-thirds of such patients have plasma zinc concentrations that are below the normal mean by more than 3 SD (58). Zinc deficiency can also be caused by prolonged administration of penicillamine for the treatment of
Wilson's disease. Low plasma zinc concentrations are found in sickle cell disease (59). Administration of zinc may have a striking effect on wound healing (60) only when a zinc deficiency exists (61). After major operative trauma, such as that with gastric surgery and cholecystectomy, there is a significant decrease in plasma zinc levels, the decrease becoming maximal about six hours after surgery and returns to normal within two to three days. Women on oral contraceptives have lower plasma zinc levels (62). Low levels of zinc have also been observed in Crohn's disease (63). Patients with carcinoma of digestive organs show low zinc but high plasma copper levels (64). Plasma zinc in bronchogenic carcinoma is also low (65). Patients with cirrhosis have decreased plasma zinc levels and increased urinary excretion of zinc (66).

Evaluation of the microenvironment, especially with respect to trace metals, may prove to be a crucial factor in the etiology, diagnosis, and treatment of cancer. Zinc deficiency is associated with profound impairment of cellular and humoral immune functions, which show improvement upon zinc supplementation. Therefore, it is not surprising that in serum, decreased zinc levels are associated with poor prognosis in patients with malignancies. Changes in serum zinc levels, serum copper levels and Cu/Zn ratio have been reported to be useful indication of disease activity, prognosis and response to treatment in hematopoietic malignancies, gastrointestinal malignancies, sarcomas and epidermoid cancers of head and neck (67).
Bodgen et al. (68) have reported that plasma and whole blood copper concentrations are increased but plasma and whole blood zinc are decreased in tuberculosis. Thus, the Cu/Zn ratios are markedly higher in tuberculosis patients. Plasma Cu/Zn ratios have been used for monitoring the response of leukemia patients to chemotherapy (69). It is believed also to be of value for monitoring osteosarcoma patients. Bodgen et al. (70) found high plasma Cu/Zn ratios in cerebral infarction patients.

The exact relationship between seminal plasma zinc and fertility is not clear. Zinc is secreted into the seminal plasma by the prostate gland (71). It has been demonstrated that zinc levels are excellent indicators of prostatic secretory function. Zinc has been implicated in the antibacterial activity of prostatic fluid and has been shown to be decreased in prostatitis (72). In addition, decreased zinc levels have been correlated with decreased fertility potential (73). Treatment of infertile patients with oral zinc sulfate results in improved sperm motility and three out of twenty pregnancies have been confirmed.

The prostate gland contains zinc in higher quantities than any other soft tissue in the human body (71). The exact mechanism by which zinc affects sperm function is unclear. Millar et al. demonstrated that zinc is needed for the maintenance of intact germinal epithelium and for spermatogenesis in the
rat (74). Lindholmer and Elgasson have shown that epididymal as well as mature spermatozoa contain zinc and magnesium in high concentrations (75).

Zinc, testosterone and dihydrotestosterone concentrations have been measured in normal prostatic tissue and in specimens obtained from untreated patients with benign prostatic hyperplasia and carcinoma of the prostate. This suggests that discrimination analysis combining the hormonal data into a single variable is a reliable test for distinguishing between benign prostatic hyperplasia and carcinoma of the prostate in patients. Since these patterns, particularly those associated with neoplasia, precede the clinical manifestations, they may be used as an index for predicting the onset of carcinoma in the prostate gland and be of value in monitoring the progress of this disease (76).

C. COLORIMETRIC REAGENTS FOR IRON, COPPER AND ZINC

Many methods have been proposed to determine iron, copper and zinc in serum and other biological samples. This section presents a brief overview of some of the more common reagents which have been used as chromogens in the colorimetric determination of these metals.

The majority of reagents used to determine iron contain the functional group \(-N=C-\) and \(-N=C-N\), known as the ferroin and terroin groups, respectively. The earliest of these ferroin group reagents are 2,2'-bipyridine and 1,10-phenanthroline (77), however, their sensitivities and specificities
are very poor. Later Case (78) synthesized several derivatives of 1,10-phenanthroline and noted that one of them showed much better sensitivity for iron, viz. bathophenanthroline. The molar absorptivity for this reagent is 22,400 at 534 nm compared to 11,100 for 1,10-phenanthroline. Still working on ferroin group compounds, others (79) synthesized various substituted triazines and found that the most useful of these was 2,4,6-tripryidyl-s-triazine (TPTZ) with a molar absorptivity of 24,100. Moreover, TPTZ is water soluble. Later syntheses of substituted triazines yielded 3-(4-phenyl-2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PPDT) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT) (80). The sulfonated form of the latter (PDTS) is known as ferrozine and seems to be the most useful of these triazines and is widely utilized in clinical laboratories today. However, it still presents the problems of poor sensitivity (for pediatric samples) and specificity. Other important ferroin-type compounds used for iron determination in the past include tripyridine (81), terosine (82), terosole (83) and terosite (84).

Other reagents not belonging to ferroin group include acetylacetone (85), nitroso-R-salt (86), 4-hydroxy-phenyl-3-carboxylic acid (87), and 3-hydroxyquinoline (88). Thiocyanate (89) was one of the oldest methods used to determine iron. Others include dimethylglyoxime (90), pyrocatechol (91), and pyramidone (92). Apart from these reagents being very insensitive and non-specific, the methodologies in which
they are used are very long, tedious and most of them involve the usage of organic solvents which is gradually being phased out in the clinical laboratories. Table III gives a summary of some of the more important iron reagents with their molar absorptivities.

The ferroin family of reagents has also been successfully used to determine Cu⁺¹ (93). The reagent 2,2'-biquinoline (molar absorptivity, 6,220 or cuproine synthesized by Breckenridge et al. (94) only gave a colored complex with copper and the well-known reaction of iron with the ferroin group was prevented due to steric hindrance. This specificity for copper opened the way for a number of derivatives of cuproine to be synthesized in an attempt to find a reagent that is just as specific for copper but more sensitive since the molar absorptivity of cuproine is only 6,220 (95). Thus, Smith and McCurdy introduced a new reagent for copper, 2,9-dimethyl-1,10-phenanthroline or neocuproine, with the same selectivity for copper but still not sensitive enough (molar absorptivity, 7,950) (96). However, further work by Smith and Wilkins (97) resulted in the reagent, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline or bathocuproine, which was just as selective for copper but with a higher sensitivity (molar absorptivity, 14,600). Bathocuproine is still used very widely in many clinical laboratories today.

Many other reagents not belonging to the ferroin-class of reagents have been used to determine copper. Some of the ear-
TABLE III

MOLAR ABSORPTIVITIES OF Fe^{2+} WITH VARIOUS LIGANDS

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Wavelength (nm)</th>
<th>Molar absorptivity$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridyl benzodiazepine-2-one-type</td>
<td>580</td>
<td>17,400</td>
</tr>
<tr>
<td>Bathophenanthroline</td>
<td>534</td>
<td>22,150</td>
</tr>
<tr>
<td>Bathophenanthroline disulfonate</td>
<td>534</td>
<td>22,140</td>
</tr>
<tr>
<td>Tripyridyl-s-triazine</td>
<td>593</td>
<td>22,600</td>
</tr>
<tr>
<td>2,6-Di-[pyridyl-(2)-4-p-methoxy-phenyl] pyridine</td>
<td>570</td>
<td>26,900</td>
</tr>
<tr>
<td>DMPP sulfonic acid salt</td>
<td>570</td>
<td>26,900</td>
</tr>
<tr>
<td>Ferrozine</td>
<td>562</td>
<td>27,900</td>
</tr>
<tr>
<td>Terrosine</td>
<td>569</td>
<td>22,000</td>
</tr>
<tr>
<td>Terosole (4-methyl)</td>
<td>575</td>
<td>26,300</td>
</tr>
<tr>
<td>Terosole (4-ethyl)</td>
<td>570</td>
<td>27,100</td>
</tr>
<tr>
<td>Terosite (4-phenyl)</td>
<td>583</td>
<td>30,200</td>
</tr>
<tr>
<td>Tripyridyl-2,2',2''-tripyridine (terpyridine)</td>
<td>552</td>
<td>12,500</td>
</tr>
<tr>
<td>Chromazurol-3</td>
<td>630</td>
<td>168,000</td>
</tr>
<tr>
<td>BPTFS$^{b}$</td>
<td>565</td>
<td>32,000</td>
</tr>
</tbody>
</table>

$^{a}$ Molar absorptivities are expressed in cm$^2$ mol$^{-1}$.


$^{b}$ 2,4-Bis(5,6-diphenyl-1,2,4-triazin-3-yl)pyridinetetrasulfonic acid.
liest were ethyl xanthate (98), diethylthiocarbamate (99) and diethyldithiocarbamate (molar absorptivity, 8,000) (99). Apart from being non-specific and insensitive, the complexes /cherlates of the reagents with Cu^2+ are quite unstable in light (100). Dibenzylidithiocarbamate is more specific and more sensitive but the complex must be extracted into CCl₄ (101). A very well-known reagent, prepared by Emil Fisher, was dithizone (102). The molar absorptivity for the copper-dithizionate complex was 35,000 (93). However, dithizone combines with many other metals forming different colors. The dithizone reaction can be made more selective for a given metal by adjusting the pH or by using chelating agents. These, however, increase the complexity and tediousness of the method. Potassium cyanide or potassium thiocyanate form a blue color with copper in the presence of a tincture of guaiacum resin (103). Other reagents that have been used include dimethylglyoxime (104), benzaminosemicarbazide (105), urobilin (106) and β-napthol (107). Cuprizone, bis-cyclohexanone-dioxalyl-dihydrazone, was first used by Nilsson (108). A molar absorptivity of 16,000 was reported. The determination of copper with a commercially available disulfiram derivative, bis(1-piperidyl-carbonyl) disulfide with a molar absorptivity of 37,500 has been used by Carter (109). 1,5-Diphenyl carbodylsamine was first used by Caseneuve as a sensitive reagent for copper (110) with a molar absorptivity of 58,000. However, this reagent is difficult to prepare, water insoluble and not
very stable in an ethanolic solution. The spectrophotometric
determination of copper with N,N,N',N'-tetraethylthiuram
disulfide (TETD or antabuse or disulfiram) has been used by
Matsuba and Takahashi (111) to determine copper with a repor-
ted molar absorptivity of 29,900, however, they encountered
problems with a TCA/HCl filtrate. Watkins and Zak used glacial
acetic acid to overcome this problem (112).

Not as many colorimetric reagents have been used to deter-
mine zinc in serum as compared to those available for iron and
copper. Some of the earlier reagents used for different media
include xylenol orange (113), 8-hydroxyquinoline (114), Eri-
ochrome blue black R (115), 4-chlororesorcinol (116) and nitro-
quinaldine acid (117). Zincon (2'-hydroxy-5'-sulfoformazyl-
benzene) has been used quite readily to determine serum zinc
(118). Of lesser importance are dithizone (119) and di-β-
napthylthiocarbazone (120). Pyridylazo napthol (PAN) (121,
122); β-Cl-PAN (123) and pyridylazo resorcinol (PAR) have
been used for zinc determination in various media (124).
Only PAN seems to have any usefulness in serum zinc determina-
tion with a molar absorptivity of 58,000. A demasking techni-
que for measuring zinc in the presence of other metals is
given below (125).

Metals + Masking Agent → Masked Solution
(cyanide)

Masked Solution + Color Reagent → Blank

Blank + Differential Demasking → Zinc Ions
(chloral hydrate)

Zinc Ions + Zincon → Color Reaction
D. DETERMINATION OF ONE METAL IN THE PRESENCE OF THE OTHER

Numerous chromogenic reagents have been recommended for the determination of copper, iron or zinc individually; however, relatively few of these are practical for the simultaneous determination of any two or all three. Some systems are presented here as examples of simultaneous determinations.

1,10-Phenanthroline, 2,2'-bipyridine and related compounds have been used by various methods for the simultaneous determination of iron and copper in serum (126-128). Iron and copper were also determined simultaneously with 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT) (129). Watkins et al. (130) determined copper, iron and zinc from a single small sample. In one procedure, the determination of copper and iron were made on one aliquot of filtrate by two sequential reactions (bathocuproine and bathophenanthroline) followed by the determination of zinc on a second aliquot by differential demasking of the zinc cyanide complex with chloral hydrate and measuring the zinc by zincon.

Parker and Griffin (131) used 2,2'-bipyridine and sodium diethyldithiocarbamate and an extraction step to determine iron and copper. Wilkins and Smith introduced a method in which the copper is extracted as the cuprous-1,10-phenanthroline derivative in n-octyl alcohol and the ferrous 1,10-phenanthroline complex remains in the aqueous layer (132). Zak and Ressler
used 1,10-phenanthroline and neocuproine for the analysis of iron and copper by the use of simultaneous mathematical equations and also by extraction techniques. Another method used bathophenanthroline and bathocuproine where one of the metals was determined in aqueous solution with a sulfonated reagent and the other was extracted into an organic solvent containing the other unsulfonated reagent (134). Bathophenanthroline sulfonate and bathocuproine sulfonate have been used for the determination of copper and iron by first forming the copper-bathocuproine sulfonate complex followed by the addition of bathophenanthroline sulfonate to form the iron-bathophenanthroline sulfonate complex (135).

A more detailed look is presented here on the masking and demasking techniques used to determine these metals using cyanide and EDTA with the chromogen.

PAR forms chelates with a variety of metals. This low selectivity is a hindrance to its application in many systems, often requiring separation as a prior step before color development and measurement. Recent work by Totsuyanagi et al. has shown that among some twenty common metals only iron and palladium persist as PAR chelates under reducing conditions in the presence of excess EDTA (136). None of the other metals normally found in serum persist as PAR chelates under these conditions.
Me + PAR $\rightarrow$ Me-PAR
Me-PAR + EDTA $\rightarrow$ Me-EDTA + PAR
(Me= Cu; Zn; Co; Ni)
Fe(II)-PAR + EDTA $\rightarrow$ Fe(II)-PAR + EDTA
or Pd(II)-PAR

Feldkamp et al. studied the effect of EDTA on Fe(II) and Zn(II) chelates with PAR at room temperature. When mixtures of two different concentrations of Zn(II)-PAR chelates with fixed amount of Fe(II)-PAR chelate were treated with EDTA, both came down to a common absorbance level representing the Fe(II)-PAR chelate alone. Thus, it appears that the binding constant of the Fe(II)-PAR chelate is sufficiently strong to resist the complexing action of EDTA (137). Nickel and cobalt are sensitive reactors with PAR and could resist EDTA action if they were present in their higher oxidation states, but this is not a problem in a reducing medium. Only palladium, not a normal constituent of serum, can react with PAR and resist the action of EDTA.

The determination of zinc as the zinconate in a serum centrifugate containing interfering copper and iron was made possible by the use of a system in which all three metals were first bound as their cyanide complexes (125). Then zinc, the easiest of the three complexes to destroy, was differentially demasked by the reaction with chloral hydrate. The zinconate formed was measured immediately before the other metals were also released from their complexes to show
interferences. Due to the instability of the ferric and
cupric cyanide complexes in the presence of chloral hydrate,
the idea of measuring the zinconate quickly becomes diffi-
cult. However, the formation of ferrocyanide and cupro-
cyanide complexes seem to be much more stable in the pre-
sence of chloral hydrate.

\[
\text{[Zn(CN)₄]⁺} + 4 \text{CCl}_3\text{CH(OH)} \rightarrow \text{chloral hydrate}
\]
\[
\text{Zn}^{+2} + 4 \text{CCl}_3\text{CH(OH)}\text{CN} \rightarrow \text{cyanochlorohydrin}
\]
\[
\text{Zn}^{+2} + \text{Zincon} \rightarrow \text{Zinconate}
\]

E. THE STUDY

As mentioned before, many reagents have been proposed to
determine iron, copper and zinc in biological fluids, however,
the majority of these present some form of serious disadvan-
tage to limit their clinical usefulness. Such disadvantages
include: non-specificity; insensitivity; difficulty in syn-
thesis; poor water solubility; instability of the color of
metal chelates/complexes; and tediousness of the methods
themselves. The reagent PAR has been used to determine all
three metals in aqueous media in the past. Two molar
absorptivities of 56,000 and 50,000 have been reported by
Yotsuyanagi et al. (136) and Paschal et al. (138) for the Fe^{+2}
chelate in serum. Serum zinc has also been determined using
PAR(124). Busev and Ivanov (139) prepared the brominated derivative (Br-PAR) and it was observed to be quite sensitive for indium and cobalt. However, many other metals react to give sensitive reactions with this reagent which was only prepared in 97% purity (based on only nitrogen analysis). The synthesis took two days with a complex workup including the bubbling of CO₂ through the reaction mixture.

The goals of this research are: (i) to prepare a purer sample of Br-PAR by a simpler method thus making it readily available; (ii) to determine iron, copper and zinc using this reagent since it is more sensitive than PAR and most other reagents used to determine these metals; (iii) to develop a methodology to determine each metal in the presence of the other and the application of the methods developed to serum.
CHAPTER II

EXPERIMENTAL

A. SYNTHESIS OF 2-AMINO-5-BROMO-PYRIDYLAZO RESORCINOL

1. Synthesis of Diazotate
   (i). Reagents

   Methanol, diethyl ether, ethanol, petroleum ether (m.p. 80°C), acetone, isobutanol, sodium nitrite, sodium chloride; sodium bicarbonate, sodium metal and sulphuric acid were purchased from Fisher Scientific Co., Don Mills, Toronto, Ontario, M3A 1A9. Ethanol was dried by the Lund-Bjerrum method (140). Diethyl ether was dried with thinly pressed ribbons of cleaned sodium metal. The latter, cut into small pieces and stored in mineral oil, was cleaned by sequential rinses in petroleum ether until the latter appeared colorless.

   2-Amino-5-bromopyridine and resorcinol were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI, 53233.

   Isobutyl nitrite was prepared by a method similar to that of Noyes (141) for n-butyl nitrite: A solution was prepared with 3.80 g sodium nitrite and 15 mL water in a flask surrounded by an ice-salt mixture and the solution was stirred until the temperature fell to 0°C. A mixture of
1.0 mL water, 1.36 mL concentrated sulfuric acid (specific gravity 1.84) and 4.57 mL isobutyl alcohol was cooled to 0°C and then introduced slowly (via a pipet) beneath the surface of the nitrite solution with stirring and the temperature maintained at ± 1°C. After addition was completed, the mixture was allowed to stand for 0.5 h and the lower aqueous layer was removed and the isobutyl nitrite layer washed twice with 1.0-mL portions of a solution containing 0.02 g sodium bicarbonate and 0.25 g sodium chloride in 1.0 mL water. The isobutyl nitrite was separated and dried over 0.2 g anhydrous sodium sulfate.

(ii). Equipment

Powerstat and Mantle: A powerstat (120V) available from the Superior Electric Co., Bristol, Connecticut, and a 100-mL mantle available from Glas-col Apparatus Co., Terre Haute, Indiana, were used to generate heat for the refluxing. Both were distributed by Fisher Scientific Co. (Canada).

Equipment for Br-PAR Analysis: Sodium analysis was done by flame emission spectrometry on a IL-250 Atomic Absorption Emission Spectrometer. The 1H-NMR spectrum of Br-PAR was obtained from an EM-360 NMR Spectrometer available from Varian Instrument Division, 611 Hansen Way, Palo Alto, CA. The infrared spectrum was obtained from a Perkin-Elmer 180 Recorder Console from Perkin-Elmer, Norwalk, Connecticut.
(iii). Procedure

A 3-necked, 100-mL round-bottomed flask containing a magnetic stirring bar was surrounded by a mantle which was connected to a powerstat (120V). The center neck of the flask was connected to a reflux condenser which was then attached to a nitrogen bubbler. Another neck was connected to a nitrogen supply and the third neck was closed by a stopper and was only used for the addition of chemicals into the flask.

The entire apparatus was flushed with nitrogen gas at approximately 30 bubbles per min via the bubbler. During the addition of nitrogen, the stopper was removed and 20 mL dried ethanol was poured into the flask. This was immediately followed by the slow addition of 0.60 g of cleaned sodium pieces. Care must be taken not to add the sodium pieces too rapidly otherwise a vigorous to a violent reaction may occur. After flushing the apparatus out with nitrogen, 4.04 g of 2-amino-5-bromopyridine were added to the dissolved sodium in ethanol. The stopper was then inserted into the flask and the nitrogen flow rate adjusted immediately to about 30 bubbles per min. This must be done immediately otherwise the sudden increased pressure in the flask may cause the stopper to be violently displaced or the flask may explode.

The mixture was stirred continuously (magnetic stirrer) and refluxed gently for 30–40 min. Then the stopper was removed, while a flow rate of nitrogen in excess of 30 bubbles
per min was maintained throughout the apparatus (prevents air from entering the flask). Freshly prepared isobutyl nitrite (3.0 mL) was poured into the flask and stoppered immediately.

The flow rate was re-adjusted to about 30 bubbles per min and the contents were gently refluxed for 3.5 to 4 h under continuous stirring.

After reflux was completed, the flask was disconnected, cooled in air for about 3 min and then immersed in ice-cold water for 30-45 min. The brown solid was isolated by filtration through a sintered glass crucible and then washed 4 times with 7 mL diethyl ether (dried) per washing. The isolated sodium diazotate was dried in air and weighed.

2. Coupling of Diazotate to Resorcinol

A resorcinol solution was prepared by mixing 90 mL ethanol, 30 mL methanol, 6 mL water and 5.0 g resorcinol in a 200-mL round-bottomed flask. To this 1.7 g diazotate were added and stirred for 10 h in the dark at a temperature of 40-45°C. Darkness is maintained by covering the entire flask with aluminum foil and a temperature of 40-45°C is obtained by partially immersing the flask into water being continuously flowed from the hot tap.

After the coupling reaction was completed, the volume of the solution was reduced by two-thirds via a rotary evaporator. Dried diethyl ether was added until no more precipitate formed and the brown solid was filtered, washed 4 times with 7 mL diethyl ether per washing, dried in air and weighed.
3. Characterisation of Br-PAR

The compound synthesized was characterized by UV, $^1H$-NMR, thin-layer chromatography, as well as sodium and elemental analyses. The latter (C, H, N, and Br) were done at Guelph Chemical Laboratories, Silvercreek Rd., Guelph, Ont., N1H 1E7.

B. ANALYTICAL STUDIES

1. Equipment

**Balance:** A Mettler PC 1200N balance supplied by Fisher Scientific Co. (Canada), was used for measuring values above 1 g and a Mettler Type H15 balance from the same company was used for weights less than 1 g.

**Glassware:** Test tubes (13 x 100 mm), volumetric flasks and pipets, Pyrex beakers and other glass material used in this study were obtained from Fisher Scientific Co. (Canada) and were cleaned in the following way:

The glassware was soaked in dilute HCl (25% v/v) for a day and then rinsed with metal-free water (see Materials, page 41). In studies which directly involved the determination of the trace metals, the glassware (pipets and test tubes) were rinsed in addition with a 0.030% of PAR solution and then rinsed with metal-free water.

**Micropipettors:** An Oxford pipettor, (200 μL) available from Canadian Laboratory Supplies, Ltd., Toronto, Ontario, M5Z 2H4 and Gilson Pipetman Models P-200D and P-1000D with disposable pipet tips C20 and C200 purchased from Mandel
Scientific Co., Ltd., Ville St. Pierre, P.Q., H8R 1A3, were used in this study. The pipet tips were cleaned in the same way the test tubes were cleaned.

**pH Meter:** A Pražens- pH meter B510, Metrohm Herisau distributed by Fisher Scientific Co. (Canada), equipped with a glass electrode from Graphic Controls, Buffalo, NY, 14240, was used for pH measurements.

**Spectrophotometer:** A Shimadzu UV-Visible Recording Spectrophotometer UV-240 connected to a Shimadzu Graphic Printer PR-1, distributed by Teckscience Ltd., Toronto, Ontario, M8H 5T4, was used for the spectrophotometric studies.

**Vortex:** A Vortex-Genie obtained through Scientific Industries, Inc., Bohemia, NY, 11719, was used to mix solutions in test tubes.

**Cuvets:** Two cuvets of 1.00-cm pathlength each and available from Beckman Instruments, Inc., Scientific Division, Toronto, Ontario, M8Z 5T2, were used for the spectrophotometric studies.

2. Materials

**Water:** Deionized distilled water was purified by a Syn- bron/Barnstead Ultrapure cartridge with a Bantam Demineralizer and supplied by Barnstead Still and Sterilizer Co., Boston 31, MA, 02132 and distributed by Fisher Scientific Co. (Canada), was used throughout this study.

**Chemicals:** L-Ascorbic acid, trichloroacetic acid,
sodium cyanide, disodium ethylenediaminetetraacetate (Na$_2$EDTA) and sodium hydroxide were obtained from Fisher Scientific Co. (Canada). The following atomic absorption standards were purchased from Aldrich Chemical Co.; Fe in 2% HNO$_3$ (1000 µg/mL), Zn in 2% HCl (1000 µg/mL) and Cu in 2% HNO$_3$ (1001 µg/mL). Boric acid was obtained from BDH Chemicals, Toronto, Ontario, M9K 1H9.

3. Reagents

**Borate Buffer:** A solution was made by adding 7.75 g boric acid to 50 mL H$_2$O and 20 mL 4N NaOH (16.0 g/100 mL) in a 250-mL volumetric flask and swirled until the boric acid was dissolved. This was then diluted to 250 mL with metal-free H$_2$O. The pH should be 9.6-9.8.

**Trichloroacetic Acid Stock Solution:** This was prepared by dissolving 100 g trichloroacetic acid with metal-free water to a final volume of 100 mL. This was refrigerated and used as required.

**Working Trichloroacetic Acid Solution:** A 30-mL aliquot of the stock TCA was diluted to 100 mL with metal-free water in a volumetric flask.

**Sodium Cyanide Stock Solution:** A solution was prepared by weighing 0.150 g sodium cyanide and dissolving it in metal-free water to a final volume of 100 mL.

**Working Sodium Cyanide Solution:** A 2.0-mL aliquot of stock sodium cyanide solution was diluted to 6 mL with metal-
free water.

**Disodium Ethylenediaminetetraacetate Solution:** Disodium ethylenediaminetetraacetate (6.0 g) was dissolved with metal-free water to final volume of 100 mL.

**Stock Copper, Iron and Zinc Solutions:** These were prepared individually by taking 5.0 mL of the respective metal solutions (atomic absorption standards) and diluting them to 100 mL with metal-free water in a volumetric flask. This procedure yielded concentrations of 50 μg/mL.

**Standard Iron, Copper and Zinc Solutions:** The solutions were prepared according to Table IV. The mixed metal standards (1:1:1) were prepared according to Table V.

### 4. Methods

(a). **Individual Determination of Metals**

(i). **Determination of Zinc:** A 0.30-mL sample was pipetted into a test tube and 0.30 mL of working TCA solution and 0.30 mL ascorbic acid solution were added to the sample. The mixture was mixed thoroughly and 0.80 mL of it was added to a cuvet containing 0.50 mL Br-PAR solution and 0.127 mL 4N NaOH, then 1.5 mL of buffer were added and mixing was done using a pipet. A blank solution was prepared in the same way but the iron solution was replaced by H₂O. The test solution was read against the blank at 510 nm.

(ii). **Determination of Copper:** The exact procedure as described for iron was followed except that a copper metal solution was used.
### TABLE IV

**PREPARATION OF METAL STANDARDS**

<table>
<thead>
<tr>
<th>Volume of stock metal solution (mL)</th>
<th>µg/dL</th>
<th>µM Fe</th>
<th>µM Cu</th>
<th>µM Zn</th>
</tr>
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<tbody>
<tr>
<td>1.00</td>
<td>50</td>
<td>7.6</td>
<td>7.9</td>
<td>9.0</td>
</tr>
<tr>
<td>1.50</td>
<td>75</td>
<td>11.5</td>
<td>11.8</td>
<td>13.4</td>
</tr>
<tr>
<td>2.00</td>
<td>100</td>
<td>15.3</td>
<td>15.7</td>
<td>17.9</td>
</tr>
<tr>
<td>2.50</td>
<td>125</td>
<td>19.1</td>
<td>19.7</td>
<td>22.4</td>
</tr>
<tr>
<td>3.00</td>
<td>150</td>
<td>22.9</td>
<td>23.6</td>
<td>26.9</td>
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<td>4.00</td>
<td>200</td>
<td>30.6</td>
<td>31.5</td>
<td>35.8</td>
</tr>
<tr>
<td>5.00</td>
<td>250</td>
<td>38.2</td>
<td>39.3</td>
<td>44.8</td>
</tr>
<tr>
<td>6.00</td>
<td>300</td>
<td>45.9</td>
<td>47.2</td>
<td>53.7</td>
</tr>
</tbody>
</table>

*Each volume of stock metal solution of iron or copper or zinc was diluted to 100 mL with metal-free water.*

*Stock metal concentration: 50 µg/mL*
### TABLE V

PREPARATION OF MIXED METAL STANDARDS

<table>
<thead>
<tr>
<th>Volume of stock metal solutions (mL)</th>
<th>Final concentration of metal (μg/dL)</th>
<th>(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>Cu</td>
<td>Zn</td>
</tr>
<tr>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
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<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
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<tr>
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<td>3.00</td>
</tr>
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<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>6.00</td>
<td>6.00</td>
<td>6.00</td>
</tr>
</tbody>
</table>

---

*a* The volumes of stock metal solutions for iron, copper, and zinc were mixed in a 100 mL volumetric flask and then diluted to the mark with H₂O.

*b* Each stock metal concentration: 50 μg/dL.
(iii). Determination of Iron: The exact procedure for zinc was followed except that an iron metal solution was used.

(b). Sequential Determination of Iron, Copper and Zinc

A 0.30-mL sample of the metals solution (1:1:1) was pipetted into a test tube and 0.30 mL of working TCA solution and 0.30 mL ascorbic acid solution were added to the sample. The tube was covered with a piece of parafilm and then spun in a centrifuge for 10 min. An aliquot of 0.80 mL of the mixture was pipetted into a clean cuvet containing 0.127 mL of 4N NaOH and 0.50 mL Br-PAR solution. Finally, 1.5 mL borate buffer were added and the solution was mixed by vigorous pipetting. A blank was prepared in exactly the same way except that the metals solution was replaced by 0.30 mL metal-free water. The absorbance of the test solution was read at 510 nm against the blank and this reading was taken as the total absorbance due to Fe$^{+2}$, Cu$^{+1}$ and Zn$^{+2}$. Then 80 $\mu$L of the working sodium cyanide solution were added to the test solution and the blank and both mixed thoroughly with separate pipet tips. This reading was taken after 5 min and represented the absorbance that is only due to Fe$^{+2}$ and Zn$^{+2}$. Finally, 400 $\mu$L of Na$_2$EDTA solution were added to both cuvets and again mixed with separate pipet tips and read at 510 nm after 5-10 min. This absorbance is due only to iron.
C. CLINICAL INVESTIGATION

1. Equipment

These were similar to the ones described in ANALYTICAL STUDIES (CHAPTER II B.1, p. 40).

2. Materials

The same chemicals were used for the determination of all three metals as described in ANALYTICAL STUDIES (CHAPTER II B.2, p. 41).

3. Reagents

The same reagents were used as described in ANALYTICAL STUDIES (CHAPTER II B.3, p. 42).

4. Methods

The methods for serum iron, copper and zinc determination were the same as those described in ANALYTICAL STUDIES (CHAPTER II B.4, p. 43), except that serum samples were substituted for metal standards.

Sera were pooled, centrifuged and stored in the freezer (-4°C) for several days and were taken out and thawed when needed. Within-run and between-run precision studies were carried out for each metal determined. Specific amounts of each metal were added to pooled serum samples for recovery studies.
CHAPTER III

RESULTS AND DISCUSSION

A. SYNTHESIS OF 2-AMINO-5-BROMO-PYRIDYLAZO RESORCINOL

1. Synthesis of Diazotate

The sodium diazotate of 2-amino-5-bromopyridine

was synthesized by Chichibabin's method (142) with

isobutyl nitrite instead of isopentyl nitrite (143) (see

Fig. 4). The diazotate was obtained in about 60% yield

as a light brown, powdery solid. The successful preparation

of this diazotate depended mainly on the purity of the starting materials and the order of addition. Dried ethanol and

freshly prepared isobutyl nitrite must be used as traces of

water or other impurities may destroy the highly reactive

2-amino-5-bromo-pyridyl anion produced as a consequence of

the strong base generated by sodium in ethanol. The alkoxide

is generated by pure sodium metal instead of sodium

amide. The latter is not very stable and the reproducibility

of the yield of the diazotate varies considerably when it is used. Because of the reactivity of the pyridyl anion, nitrogen is flushed through the apparatus at all times. After one-half hour of refluxing (i.e., sufficient time to convert all the amino-bromo-pyridine to the anionic form), the addition of isobutyl nitrite causes the diazotate to be formed which
FIGURE 4
SYNTHEtIC PATHWAY FOR Br-PAR

Legend

An alkoxide solution is prepared and 2-amino-5-bromo pyridine is refluxed in it for 5 min after which isobutyl nitrite is added to the mixture while maintained under nitrogen. The sodium diazotate is then reacted with excess resorcinol for 10 h at 42-45°C. The product isolated is the monosodium monohydrate salt of Br-PAR.
A. Preparation of Diazotate

\[
\text{Br} \quad \text{NH}_2 
\rightarrow \text{Na/EtOH} \quad \Delta; 30\text{min} \quad \rightarrow \text{Br} 
\]

Isobutyl nitrite
\[\Delta; 30\text{min}\]

B. Coupling of Diazotate to Resorcinol

\[
\text{Br} \quad \text{N=N-ONa} \quad \rightarrow \quad \text{OH} \quad \text{OH} \quad \rightarrow \text{Br} 
\]

42-45°C
10h
is quite stable in air. The diazotate formed is more stable than typical diazonium salts.

2. Coupling of Diazotate to Resorcinol

The coupling procedure used to synthesize 2-amino-5-bromo-pyridylazo resorcinol (monosodium monohydrate salt) was much simpler than that of Busev and Ivanov (139). The previous method involved the bubbling of CO₂ through the mixture for 24 h which is time consuming, more expensive and tedious. In preparations of PAR involving the bubbling of CO₂, sodium carbonate is formed as an impurity, and is very difficult to remove. Thus, in the proposed procedure, CO₂ was eliminated and the temperature was raised to 42-45°C. This prevented the formation of sodium carbonate and the coupling step was effected in less than half the time. Moreover, instead of the neutral Br-PAR being isolated, the monosodium monohydrate salt was obtained which conferred an additional advantage of water solubility to the molecule. The solvent system for the coupling was also modified and the one that seemed to work best was ethanol:water:methanol as compared to only ethanol for previous coupling procedures. Also, an excess of resorcinol was dissolved in the solvent system and then the sodium diazotate was added to the solution (see Fig. 4).

In order to isolate the Br-PAR salt, diethyl ether was used. Other solvents such as petroleum ether (b.p. 80°C),
benzene, acetone or chloroform produced oily precipitates which were very difficult to solidify. Diethyl ether provides the added advantage of removing the unreacted resorcinol as the latter is very soluble in it and the Br-PAR salt is not. In all future studies, Br-PAR salt is referred to as Br-PAR.

3. Characterization of Br-PAR.

The spectroscopic results compare very favorably with the literature values. The absorption spectra as a variation of pH are shown in Fig. 5. The maxima in acidic solutions is 420 nm and in alkaline solutions is 500 nm. The isosbestic point is 452 nm. Values reported for acidic and alkaline media for Br-PAR are 420 nm and 500 nm, respectively, with an isosbestic point of 455 nm (139). The $^1$H-NMR (in deuterated methanol) and IR (in KBr) spectra were recorded but these showed considerable overlapping and were not very useful. However, the IR spectrum showed a strong peak at 3450 cm$^{-1}$ and a weaker one at 1615 cm$^{-1}$ suggesting \(-\text{OH}\) and \(-\text{N-N}\) vibrational frequencies, respectively. The compound was also analyzed by thin-layer chromatography with a solvent system of isopropanol:methyl ethyl ketone:ammonia solution (4:3:3) (145). An $R_f$ of 0.88 was obtained for Br-PAR as compared to 0.80 for PAR itself (145). Finally, the compound was analyzed by a reference laboratory for the elements carbon, hydrogen, nitrogen and bromine. The results for these analyses along with sodium analysis are as follows:
FIGURE 5

EFFECT OF pH ON AN AQUEOUS SOLUTION OF Br-PAR

Legend

Concentration of Br-PAR in cuvet: $4.57 \times 10^{-3}$ M

pH of various aqueous solutions of Br-PAR:

- A. 4.0
- B. 6.0
- C. 7.0
- D. 8.5
- E. 9.0
- F. 9.5
- G. 10.0
- H. 11.0
- I. 12.0
- J. 13.0

Solutions A–D were made up with phosphate buffers and solutions E–J were made up with borate buffers.
Anal. Calcd. for C₁₁H₂₀₂₃N₃BrNa:

C, 39.55; H, 2.70; N, 12.50; Br, 23.95; Na, 5.88.

Found: C, 39.65; H, 2.68; N, 11.99; Br, 26.10; Na, 6.65.

Busev and Ivanov (139) prepared this compound in 97% purity
based on only nitrogen analysis determined by the reduction
of the azo-linkage with chromous sulfate. The per cent nitro-
gen in nitrogen containing heterocyclics and the presence of
bromine in nitrogen containing compounds is usually underes-
timated by the Kjeldahl's method (146).

5. ANALYTICAL STUDIES

1. Chelates of Br-PAR with Iron, Copper and Zinc

(a). Stability of an Aqueous Solution of Br-PAR

The absorbances of an aqueous solution of Br-PAR were
measured over a period of several days to check the stabi-
ility of the reagent. The results are shown in Table VI.
The reagent is still stable at 12 days or more. Earlier
studies with PAR (147) indicated that an alkaline, aqueous
stock solution of PAR deteriorates over a period of 6-10
days and this was overcome by using an ethanolic solution
of PAR. This was not necessary in this study.

(b). Effect of pH on Metal Chelate Formation

The effect of pH upon the formation of the ligand-
metal chelates was studied by adjusting the pH of the reac-
tion mixture. The absorption curves for the iron chelate
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Absorbance&lt;sup&gt;b,c&lt;/sup&gt; (510 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.337</td>
</tr>
<tr>
<td>2</td>
<td>0.334</td>
</tr>
<tr>
<td>3</td>
<td>0.337</td>
</tr>
<tr>
<td>4</td>
<td>0.330</td>
</tr>
<tr>
<td>5</td>
<td>0.331</td>
</tr>
<tr>
<td>6</td>
<td>0.338</td>
</tr>
<tr>
<td>7</td>
<td>0.333</td>
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<tr>
<td>11</td>
<td>0.335</td>
</tr>
<tr>
<td>12</td>
<td>0.336</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of Br-PAR in cuvet: $1.45 \times 10^{-4}$ M

<sup>b</sup> Absorbances are the averages of triplicate experiments.

<sup>c</sup> Absorbances were read against water.
with Br-PAR as a function of pH are shown in Fig. 6. The maximum wavelength occurs at 510 nm and the maximum absorbance occurs at a pH of 9.6. The absorption curves as a function of pH for the copper chelates are shown in Fig. 7. These show a maximum wavelength at 520 nm with a maximum absorbance at a pH of 9.0. The absorption curves as a function of pH for the zinc chelates are shown in Fig. 8. The maximum wavelength occurs at 500 nm and the maximum absorbance occurs over a wide range of pH from 9.0 to 12.0. The wavelength of maximum absorption for each chelate did not differ by very much, thus, the absorption maximum of 510 nm for all three metal chelates was selected for future studies as this wavelength did not result in any significant change in absorbances of the chelates formed. The absorbances as a function of pH at 510 nm for each metal chelate are shown in Table VII and these are plotted in Fig. 9. The curves for all three metals reach a maximum at a pH between 9.0 and 12.0. The zinc chelate has a broad pH range of 9.0–12.0, the copper chelate has a pH range of 9.0–10.0. Considering all the information available from these studies a pH of 9.6 and a wavelength of maximum absorption of 510 nm were selected as the optimum pH and wavelength to determine each metal in the absence or in the presence of the other two.

(c). Effect of Order of Addition

The effect of the order of addition on the formation of metal-ligand chelates was checked in order to determine the
FIGURE 6

EFFECT OF pH ON THE ABSORBANCES OF IRON CHELATE WITH Br-PAR

Legend

Concentration of Br-PAR in cuvet: $1.80 \times 10^{-5}$ M.
Concentration of Fe$^{+2}$ in cuvet: $1.79 \times 10^{-4}$ M.

pH of various solutions of iron chelate with Br-PAR:

- A. 4.0
- B. 6.0
- C. 7.0
- D. 9.0
- E. 9.6
- F. 10.0
- G. 11.0
- H. 12.0

Spectra were recorded against water.
Solutions A–C were made up with phosphate buffers and solutions D–H were made up with borate buffers.

The wavelength of maximum absorption occurs at 510 nm and the maximum absorbance is noted at a pH of about 9.6.
FIGURE 7
EFFECT OF pH ON THE ABSORBANCES OF COPPER CHELATE WITH Br-PAR

Legend

Concentration of Br-PAR in cuvet: \( 4.69 \times 10^{-5} \) M.
Concentration of \( Cu^{+1} \) in cuvet: \( 4.71 \times 10^{-4} \) M.

pH of various solutions of copper chelate with Br-PAR:

- A. 4.0
- B. 6.0
- C. 7.0
- D. 9.0
- E. 9.5
- F. 10.0
- G. 11.0
- H. 12.0

Spectra were recorded against water.
Solutions A-C were made up with phosphate buffers and
solutions D-H were made up with borate buffers.

The wavelength of maximum absorption occurs at 520 nm
and the maximum absorbance is noted at a pH of about 9.0.
EFFECT OF pH ON THE ABSORBANCES OF ZINC CHELATE WITH Br-PAR

Legend

Concentration of Br-PAR in cuvet: \(4.49 \times 10^{-5}\) M.
Concentration of Zn\(^{+2}\) in cuvet: \(4.59 \times 10^{-4}\) M.

pH of various solutions of zinc chelate with Br-PAR:

<table>
<thead>
<tr>
<th>A</th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>6.0</td>
</tr>
<tr>
<td>C</td>
<td>7.0</td>
</tr>
<tr>
<td>D</td>
<td>9.0</td>
</tr>
<tr>
<td>E</td>
<td>9.6</td>
</tr>
<tr>
<td>F</td>
<td>10.0</td>
</tr>
<tr>
<td>G</td>
<td>11.0</td>
</tr>
<tr>
<td>H</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Spectra were recorded against water.

Solutions A-C were made up with phosphate buffers and solutions D-H were made up with borate buffers.

The wavelength of maximum absorption occurs at 500 nm and the maximum absorbance is observed over a wide range of pH of 9.0 to 12.0.
TABLE VII
EFFECT OF pH ON THE ABSORBANCES OF IRON, COPPER AND ZINC CHELATES FORMED WITH Br-PAR AT 510 nm

<table>
<thead>
<tr>
<th>pH</th>
<th>Fe^{2+}</th>
<th>Cu^{1+}</th>
<th>Zn^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>0.168</td>
<td>0.370</td>
<td>0.144</td>
</tr>
<tr>
<td>6.0</td>
<td>0.370</td>
<td>0.632</td>
<td>0.522</td>
</tr>
<tr>
<td>7.0</td>
<td>0.398</td>
<td>0.740</td>
<td>0.585</td>
</tr>
<tr>
<td>9.0</td>
<td>0.525</td>
<td>0.846</td>
<td>0.880</td>
</tr>
<tr>
<td>9.6</td>
<td>0.543</td>
<td>0.836</td>
<td>0.884</td>
</tr>
<tr>
<td>10.0</td>
<td>0.521</td>
<td>0.821</td>
<td>0.876</td>
</tr>
<tr>
<td>11.0</td>
<td>0.508</td>
<td>0.800</td>
<td>0.879</td>
</tr>
<tr>
<td>12.0</td>
<td>0.504</td>
<td>*0.736</td>
<td>0.876</td>
</tr>
</tbody>
</table>

*aAbsorbances were obtained from Figs. 6-8.*
FIGURE 9

EFFECT OF pH ON THE ABSORBANCES OF IRON, COPPER AND ZINC CHELATES FORMED WITH Br-PAR

Legend

○——○ Iron chelate
Concentration of Br-PAR in cuvet: 1.80 x 10^{-5} M.
Concentration of Fe^{2+} in cuvet: 1.79 x 10^{-4} M.

x——x Zinc chelate
Concentration of Br-PAR in cuvet: 4.49 x 10^{-5} M.
Concentration of Zn^{2+} in cuvet: 4.59 x 10^{-4} M.

●——● Copper chelate
Concentration of Br-PAR in cuvet: 4.49 x 10^{-5} M.
Concentration of Cu^{+1} in cuvet: 4.71 x 10^{-4} M.

The absorbances were read at 510 nm. Maximum absorbances for iron, copper and zinc occur at a pH of 9.6, 9.0-10.0 and 9.0-12.0, respectively.
FIGURE 9

[Graph showing absorbance vs pH]

- Absorbance scale from 0.0 to 1.0
- pH scale from 4.0 to 12.0

Lines representing different absorbance values at various pH levels.
best order of addition of the reagents to form the chelates of iron, copper and zinc. These are shown in Table VIII. The order of metal added to TCA followed by the addition of ascorbic acid, sodium hydroxide, Br-PAR and finally buffer produced maximum absorbances for all three metals and, thus, was used for all subsequent studies. The addition of NaOH to the sample, TCA, ascorbic mixture neutralized it sufficiently such that when the Br-PAR is added followed by the buffer, the buffering capacity is still maintained at the required pH.

(d). The Effect of the Concentration of Br-PAR on the Formation of the Chelates; Formulas of such Chelates

The amount of ligand required in the reactions of Br-PAR with Cu⁺¹, Zn⁺² and Fe⁺², respectively, in order to achieve full color formation for that amount is shown in Table IX. Different volumes of stock Br-PAR and a standard of 100 μg/dL metal solution were used to determine these metals as outlined in ANALYTICAL STUDIES (CHAPTER II B.4, p. 43). The absorbances were monitored for about 20 min at 510 nm. Clearly, all three metals reached maximum absorbances with volumes ranging from 0.25-0.50 mL Br-PAR. The volume of Br-PAR used for all subsequent studies was 0.50 mL.

The chelates are formed instantly with maximum color development. Due to the time required for mixing, absorbances could only be taken after 0.5 min. Once formed, the color intensity virtually remained the same for 20 min or more (see Table X). Thus, these metals can be determined immediately upon the addition of reagents.
### TABLE VIII

**EFFECT OF THE ORDER OF ADDITION OF REAGENTS ON THE FORMATION OF METAL CHELATES**

<table>
<thead>
<tr>
<th>Order of addition</th>
<th>Absorbances (510 nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zn&lt;sup&gt;+2&lt;/sup&gt; c</td>
<td>Cu&lt;sup&gt;+1&lt;/sup&gt; c</td>
<td>Fe&lt;sup&gt;+2&lt;/sup&gt; c</td>
</tr>
<tr>
<td>M → TCA → AA → NaOH → Br-PAR</td>
<td></td>
<td>0.216</td>
<td>0.167</td>
<td>0.160</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M → TCA → AA → Buffer</td>
<td></td>
<td>0.122</td>
<td>0.135</td>
<td>0.141</td>
</tr>
<tr>
<td>Br-PAR → NaOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer → NaOH → M → TCA</td>
<td></td>
<td>0.077</td>
<td>0.107</td>
<td>0.121</td>
</tr>
<tr>
<td>AA → Br-PAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOH → Br-PAR → M → TCA</td>
<td></td>
<td>0.211</td>
<td>0.170</td>
<td>0.158</td>
</tr>
<tr>
<td>AA → Buffer&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: M = Metal (Zn, Cu, or Fe); AA = Ascorbic acid; TCA = Trichloroacetic acid.

<sup>b</sup>Absorbances are averages of duplicate experiments.

<sup>c</sup>Concentration of metal in cuvet: Zn<sup>+2</sup> = 2.32 x 10<sup>-6</sup> M; Cu<sup>+1</sup> = 2.38 x 10<sup>-6</sup> M; Fe<sup>+2</sup> = 2.71 x 10<sup>-6</sup> M.

<sup>d</sup>Concentration of Br-PAR in cuvet: 1.52 x 10<sup>-4</sup> M.

<sup>→</sup> "followed by the addition of "
TABLE IX
EFFECT OF Br-PAR CONCENTRATION ON METAL CHELATE FORMATION

<table>
<thead>
<tr>
<th>Volume of Br-PAR(^a) (mL)</th>
<th>Absorbances (510 nm)(^b)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn(^{+2}) c</td>
<td>Cu(^{+1}) c</td>
<td>Fe(^{+2}) c</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.102</td>
<td>0.080</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.108</td>
<td>0.084</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>0.112</td>
<td>0.091</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.120</td>
<td>0.099</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.126</td>
<td>0.100</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.129</td>
<td>0.105</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>0.35</td>
<td>0.128</td>
<td>0.104</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.127</td>
<td>0.109</td>
<td>0.107</td>
<td></td>
</tr>
<tr>
<td>0.45</td>
<td>0.125</td>
<td>0.106</td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.126</td>
<td>0.103</td>
<td>0.102</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Concentration of Br-PAR: 8.98 \times 10^{-4} M.

\(^b\)Absorbances are the averages of duplicate experiments.

\(^c\)Concentration of each metal standard used: 100 \mu g/dL.
TABLE X

STABILITY OF METAL CHELATES WITH Br-PAR as a FUNCTION OF TIME

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Absorbances (510 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn$^{+2}$ d</td>
</tr>
<tr>
<td>0.50</td>
<td>0.125</td>
</tr>
<tr>
<td>1.00</td>
<td>0.126</td>
</tr>
<tr>
<td>2.00</td>
<td>0.127</td>
</tr>
<tr>
<td>3.00</td>
<td>0.128</td>
</tr>
<tr>
<td>4.00</td>
<td>0.128</td>
</tr>
<tr>
<td>8.00</td>
<td>0.127</td>
</tr>
<tr>
<td>10.00</td>
<td>0.128</td>
</tr>
<tr>
<td>14.00</td>
<td>0.128</td>
</tr>
<tr>
<td>16.00</td>
<td>0.129</td>
</tr>
<tr>
<td>18.00</td>
<td>0.128</td>
</tr>
<tr>
<td>20.00</td>
<td>0.128</td>
</tr>
</tbody>
</table>

a Concentration of Br-PAR in cuvet: $1.534 \times 10^{-4}$ M.

b Time: Time at which absorbances were read after mixing of reagents.

c Absorbances were the averages of duplicate experiments.

d Concentration of metal in cuvet: Zn$^{+2} = 1.39 \times 10^{-6}$ M; Cu$^{+1} = 1.43 \times 10^{-6}$ M; Fe$^{+2} = 1.63 \times 10^{-6}$ M.
The stoichiometric composition of the metal chelates was determined by the mole ratio method (see Fig. 10). A 2:1 ratio of Br-PAR to metal ion was observed for all three metals, see APPENDIX A.

(e). Individual Determination of Metals

(i). Determination of Zinc: The procedure was followed as outlined in ANALYTICAL STUDIES (CHAPTER II B. 4, p. 43). The absorption spectra for various zinc concentrations with Br-PAR are shown in Fig. 11. The spectra show a single maximum wavelength and at 510 nm, the average molar absorptivity is 9.11 x 10^4 cm^2 mol^-1 with δ_n=1 = 1696, Table XI. The regression line is shown in Fig. 12 and corresponds to the equation y = 0.1256x + 0.0029 with a correlation coefficient of 0.9992. Beer's law is followed up to a concentration of 300 μg/dL. The molar absorptivity is increased by 11% over PAR which has a molar absorptivity of 8.11 x 10^4 cm^2 mol^-1, 36% over PAN which has a molar absorptivity of 5.8 x 10^4 cm^2 mol^-1.

(ii). Determination of Copper: The procedure was followed as outlined in ANALYTICAL STUDIES (CHAPTER II B.4, p. 43). The absorption spectra for the various copper concentrations with Br-PAR are shown in Fig. 11. The spectra show a single wavelength of maximum absorption and at 510 nm, the molar absorptivity is 7.22 x 10^-4 cm^2 mol^-1 with δ_n=1 = 1047 (see Table XI). An increase of 23% over Cu-PAR chelate is observed with a molar absorptivity of 5.89 x 10^4 cm^2 mol^-1 (144), 80% over bathocuproine (a recent derivative of cuproine).
FIGURE 10
FORMATION OF THE METAL CHELATES

Legend

For Iron
Stock Br-PAR concentration: $7.16 \times 10^{-5}\text{M}$
Stock metal ion concentration: $7.16 \times 10^{-5}\text{M}$
Final concentration of iron in cuvet: $8.59 \times 10^{-6}\text{M}$

For Copper
Stock Br-PAR concentration: $3.15 \times 10^{-5}\text{M}$
Stock metal ion concentration: $3.15 \times 10^{-5}\text{M}$
Final concentration of copper in cuvet: $3.78 \times 10^{-6}\text{M}$

For Zinc
Stock Br-PAR concentration: $3.06 \times 10^{-5}\text{M}$
Stock metal ion concentration: $3.06 \times 10^{-5}\text{M}$
Final concentration of zinc in cuvet: $5.16 \times 10^{-6}\text{M}$

The volume of stock metal solution used in each case was constant while the volume of the ligand (Br-PAR) was increased to give ratios of moles ligand to moles metal ion of 0 to 6.

Iron plot
Copper plot
Zinc plot
FIGURE 10

[Graph showing the relationship between absorbance and the ratio of moles Br-PAR to moles metal, with absorbance values ranging from 0.00 to 0.90 and ratio values ranging from 0 to 6.]
FIGURE 11
ABSORPTION SPECTRA OF ZINC, IRON AND COPPER CHELATES WITH Br-PAR

Legend

A. Zinc spectra
B. Copper spectra
C. Iron spectra

Concentrations of metal standards (µg/dL):
   a. 50 e. 150
   b. 75 f. 200
   c. 100 g. 250
   d. 125 h. 300

Zinc calibration curve: \( y = 0.1256x + 0.0029 \)  
\( r = 0.9992 \); molar absorptivity = 91,100 cm\(^2\)mol\(^{-1}\).

Copper calibration curve: \( y = 0.1024x + 0.0014 \)  
\( r = 0.9997 \); molar absorptivity = 72,200 cm\(^2\)mol\(^{-1}\).

Iron calibration curve: \( y = 0.1007x + 0.0106 \)  
\( r = 0.9996 \); molar absorptivity = 66,500 cm\(^2\)mol\(^{-1}\).
TABLE XI
MOLAR ABSORPTIVITIES FOR IRON, COPPER AND ZINC CHELATES WITH BR-PAR

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Absorbances (510 nm)</th>
<th>Molar absorptivities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 (7.6)</td>
<td>0.062</td>
<td>0.051</td>
</tr>
<tr>
<td>75 (11.5)</td>
<td>0.094</td>
<td>0.080</td>
</tr>
<tr>
<td>100 (15.3)</td>
<td>0.128</td>
<td>0.104</td>
</tr>
<tr>
<td>125 (19.1)</td>
<td>0.161</td>
<td>0.129</td>
</tr>
<tr>
<td>150 (22.9)</td>
<td>0.194</td>
<td>0.154</td>
</tr>
<tr>
<td>200 (30.6)</td>
<td>0.245</td>
<td>0.204</td>
</tr>
<tr>
<td>(31.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE XI CONTINUED

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>250</td>
<td>250</td>
<td>0.317</td>
<td>0.258</td>
<td>0.260</td>
<td>9.10</td>
</tr>
<tr>
<td>(38.2)</td>
<td>(39.3)</td>
<td>(44.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>300</td>
<td>300</td>
<td>0.384</td>
<td>0.310</td>
<td>0.316</td>
<td>9.18</td>
</tr>
<tr>
<td>(45.9)</td>
<td>(47.2)</td>
<td>(53.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average molar absorptivities

9.11 7.22 6.65

\[ a \text{Absorbances are averages of triplicate experiments.} \]

\[ b \text{Molar absorptivities are expressed in cm}^2\text{mol}^{-1} \times 10^{-4}. \]
FIGURE 12

CALIBRATION CURVE FOR ZINC CHELATE WITH Br-PAR

Legend

100 μg/dL = 15.3 μM
200 μg/dL = 30.6 μM
300 μg/dL = 45.9 μM

Concentration of other standards in μM are shown in Table XI.

Regression line: y = 0.1256x + 0.0029 (r = 0.9992).
Average molar absorptivity: 9.11 x 10^4 cm^2 mol^-1,
(ε_n-1 = 1696 cm^2 mol^-1).

Absorbances are averages of triplicate experiments.
with a molar absorptivity of $1.46 \times 10^4$ cm$^2$mol$^{-1}$, 89% over
diethylidithiocarbamate which has a molar absorptivity of
$8.00 \times 10^3$ cm$^2$mol$^{-1}$ and 59% over antabuse which has a molar
absorptivity of $2.99 \times 10^4$ cm$^2$mol$^{-1}$.

The regression line is shown in Fig. 13 and is rep-
resented by the equation $y = 0.1024x + 0.0014$ with a correlation
coefficient of 0.9997. Beer's law is observed up to a concen-
tration of 300 g/dL.

(iii) Determination of Iron: The procedure was fol-
lowed as outlined in ANALYTICAL STUDIES (CHAPTER II B.4, p. 46).
The absorption curves for various concentrations of iron are
shown in Fig. 11. The iron spectra show two maximum wave-
lengths at 510 nm and 715 nm. These double absorption maxima
were also observed by Feldkamp et al. (137) for the iron
chelate with PAR but was not reported by other workers (136,
138). The molar absorptivities for iron with Br-PAR are
$6.65 \times 10^4$ cm$^2$mol$^{-1}$ and $2.81 \times 10^4$ cm$^2$mol$^{-1}$ at 510 nm and
715 nm, respectively. The former represents a 16% increase
over Fe$^{2+}$-PAR with a molar absorptivity of $5.60 \times 10^4$ cm$^2$mol$^{-1}$
and 25% increase over $5.00 \times 10^4$ cm$^2$mol$^{-1}$ again for Fe$^{2+}$-PAR
chelate. 100% increase is seen over most of the other reagents
(see Table III). Even the second peak at 715 nm is quite sen-
sitive and its molar absorptivity of $2.81 \times 10^4$ cm$^2$mol$^{-1}$ com-
pares quite favorably with those of other commonly used re-
agents (see Table III). As seen in the absorption spectra
FIGURE 13
CALIBRATION CURVE FOR COPPER CHELATE WITH Br-PAR

Legend

$100 \ \mu g/dL = 15.7 \ \mu M$
$200 \ \mu g/dL = 31.5 \ \mu M$
$300 \ \mu g/dL = 47.2 \ \mu M$

Concentration of other standards in $\mu M$ are shown in Table XI.

Regression line: $y = 0.1024x + 0.0014 \ (r = 0.9997)$

Average molar absorptivity: $7.22 \times 10^4 \ \text{cm}^2\text{mol}^{-1}$
($\varepsilon_{\text{M}} = 1047 \ \text{cm}^2\text{mol}^{-1}$).

Absorbances are averages of triplicate experiments.
(see Fig. 14), none of the other metal chelates absorb at this wavelength, therefore, iron can be selectively determined in the presence of copper and zinc at this wavelength with no masking/demasking procedures necessary.

The regression line is shown in Fig.15 and is represented by the equation $y = 0.1007x + 0.0106$ ($r = 0.9996$). Beer's law is followed at a concentration of 300 $\mu g/dL$.

The absorption spectrum (total) of a 1:1:1 mixture ($\mu g/dL$) of zinc, iron and copper was recorded along with the individual spectrum of each metal to check the additive effect of the metals at 510 nm. For the concentrations of each metal shown in Fig. 14A, a total absorbance for the 1:1:1 mixture ($\mu g/dL$) of 0.849 was obtained as compared to 0.849 for the addition of individual absorbances of iron, copper and zinc determined individually under the same conditions. At another concentration of each metal, 0.963 was obtained for a total absorbance of the 1:1:1 mixture ($\mu g/dL$) compared to 0.961 for the total of the individual absorbances, Fig. 14B. Thus, the absorbances of the individual chelates are additive at 510 nm. No color from either chelate enhances or decreases the intensity of the color from the other chelates at this wavelength.
FIGURE 14

ABSORPTION SPECTRA OF INDIVIDUAL AND MIXED 1:1:1 CHELATES OF IRON, COPPER AND ZINC WITH BR-PAR.

Legend

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance (510 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{+2}$ = 4.26 x 10$^{-6}$M</td>
<td>0.259</td>
</tr>
<tr>
<td>Cu$^{+1}$ = 3.75 x 10$^{-6}$M</td>
<td>0.261</td>
</tr>
<tr>
<td>Zn$^{+2}$ = 3.64 x 10$^{-6}$M</td>
<td>0.329</td>
</tr>
<tr>
<td>Total</td>
<td>0.849</td>
</tr>
</tbody>
</table>

Fe$^{+2}$ + Cu$^{+1}$ + Zn$^{+2}$ in above concentrations 0.849

A.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance (510 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{+2}$ = 4.87 x 10$^{-6}$M</td>
<td>0.300</td>
</tr>
<tr>
<td>Cu$^{+1}$ = 4.28 x 10$^{-6}$M</td>
<td>0.303</td>
</tr>
<tr>
<td>Zn$^{+2}$ = 4.16 x 10$^{-6}$M</td>
<td>0.360</td>
</tr>
<tr>
<td>Total</td>
<td>0.963</td>
</tr>
</tbody>
</table>

Fe$^{+2}$ + Cu$^{+1}$ + Zn$^{+2}$ in above concentrations 0.961

B.

a. Copper spectra
b. Iron spectra
c. Zinc spectra
d. Metals mixed together spectra
FIGURE 15
CALIBRATION CURVE FOR IRON CHELATE WITH Br-PAR

Legend

100 \( \mu \text{g/dL} \) = 17.9 \( \mu \text{M} \)
200 \( \mu \text{g/dL} \) = 35.8 \( \mu \text{M} \)
300 \( \mu \text{g/dL} \) = 53.7 \( \mu \text{M} \)

Concentration of other standards in \( \mu \text{M} \) are shown in Table XI.
Regression line: \( y = 0.1007x + 0.0106 \) (\( r = 0.9996 \))
Average molar absorptivity: \( 6.65 \times 10^4 \ cm^2\text{mol}^{-1} \), \( (\epsilon_{n-1} = 3431 \ cm^2\text{mol}^{-1}) \).
Absorbances are averages of triplicate experiments.
2. Sequential Determination of Iron, Copper and Zinc

(a). **Order of Addition of Reagents on the Formation of Mixed Chelates**

The order of addition of reagents on the formation of metal chelates was reported in Table VIII. For a 1:1:1 mixture of the metals, the orders of addition of the reagents that seemed to work best were virtually the same:

i. mix the sample with TCA and ascorbic acid and then add an aliquot of this mixture to a solution of Br-PAR and sodium hydroxide in the cuvet and finally add the buffer;

ii. mix the sample, TCA, ascorbic acid and sodium hydroxide and then add an aliquot of this mixture to the Br-PAR solution and finally add the buffer.

The first order of addition was followed for all subsequent studies.

(b). **Optimum Cyanide and EDTA Concentrations**

It is a well known fact that iron, copper and zinc ions bind cyanide and EDTA (148, 149). Thus, to determine these metals in the presence of one another, a study of the addition of cyanide and EDTA separately to these metal chelates with Br-PAR is necessary. Different amounts of cyanide were added to the individual chelates of iron, copper and zinc with Br-PAR and the absorbances were measured after 5 min. The results are shown in Table XIII and in Fig. 16.

The addition of varying amounts of cyanide to the copper
TABLE XII
EFFECT OF VARYING AMOUNTS OF CYANIDE ON THE ABSORBANCES OF THE INDIVIDUAL CHELATES OF IRON, COPPER AND ZINC WITH Br-PAR

<table>
<thead>
<tr>
<th>Amount of cyanide&lt;sup&gt;a&lt;/sup&gt; (μg)</th>
<th>Absorbances (510 nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Cu&lt;sup&gt;1+&lt;/sup&gt;</td>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.222</td>
<td>0.207</td>
<td>0.191</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.217</td>
<td>0.016</td>
<td>0.190</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.210</td>
<td>0.014</td>
<td>0.191</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.200</td>
<td>-0.009</td>
<td>0.188</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.205</td>
<td>0.010</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.205</td>
<td>0.009</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.199</td>
<td>0.012</td>
<td>0.187</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration of working sodium cyanide solution: 50 mg/dL. Corresponding aliquots were taken to contain the amounts shown.

<sup>b</sup>The absorbances are averages of duplicate experiments. These were done using the procedure on p. 46.

<sup>c</sup>Concentration of each metal standard: 200 μg/dL.
FIGURE 16

EFFECT OF VARYING AMOUNTS OF CYANIDE ON THE INDIVIDUAL CHELATES OF IRON, COPPER AND ZINC WITH BR-PAR

Legend

- - - - - Effect on zinc chelate
\[\times\times\times\] Effect on copper chelate
- - - - - Effect on iron chelate
chelate resulted in the immediate displacement of Br-PAR and the binding of cyanide, even at the lowest concentrations shown, to the cuprous ion with a subsequent decrease in absorbance to approximately zero. The addition of cyanide to the iron chelate, however, had no effect at even the highest concentration shown and thus, the iron chelate remained intact with little change in the absorbance observed. The zinc chelate seemed to have a slight effect on the displacement of Br-PAR by the cyanide as the concentration of the cyanide was increased. From the data presented in Fig. 16, a value of 50 μg sodium cyanide was used for all future binding studies. It can be seen from the plot for the case of the copper chelate that the cyanide has a greater affinity for copper than Br-PAR does. Also, it can be seen in Fig. 16 that cyanide has a lesser affinity for zinc and iron than Br-PAR does.

The same experiments were repeated but with EDTA instead of cyanide. Different amounts of EDTA were added to individual chelates of iron, copper and zinc with Br-PAR. At all values used (i.e., 3.7 to 9.3 mg), Br-PAR was replaced by EDTA in the zinc and copper chelates with a concomitant decrease in absorbances to about zero. However, EDTA did not displace the Br-PAR from the iron chelate since the absorbance was virtually unchanged upon the addition of EDTA. Thus, for future sequential analyses, a value of 7.44 mg of EDTA was used.
(c). **Addition of Optimum Concentrations of Cyanide and EDTA to Various Amounts of Metal Chelates.**

In part (b) of this section, the optimum amounts for the binding of cyanide and EDTA to metal chelates for future sequential studies were determined. It would be important to find out if these optimum concentrations are still applicable at different concentrations of the respective metal chelates. Thus, the optimum concentration of cyanide was added to three metal chelate concentrations of each metal. The time-scan curves were recorded and are shown in Fig. 17. Clearly, all three concentrations for each metal showed the same binding characteristics. As mentioned in section (b), the same amount of cyanide has the same effect on all three copper chelate concentrations. However, at this cyanide concentration, there is no real effect on the absorbances of the various concentrations of the iron and zinc chelates with Br-PAR.

In separate experiments, the optimum EDTA concentration was added to three metal chelate concentrations of each metal. The time-scan curves were recorded and are shown in Fig. 18. Again, the observations for all three concentrations of each metal agree with those in section (b).
FIGURE 17

EFFECT OF OPTIMUM CYANIDE CONCENTRATION ON VARIOUS
CONCENTRATIONS OF IRON, COPPER AND ZINC CHELATES
WITH Br-PAR

Legend

Time-scan surves:
a. 100 μg/dL of metal standard
b. 150 μg/dL of metal standard
c. 200 μg/dL of metal standard

A. Zinc chelate scans.
B. Copper chelate scans.
C. Iron chelate scans.
FIGURE 18

EFFECT OF OPTIMUM EDTA CONCENTRATION ON VARIOUS CONCENTRATIONS OF IRON, COPPER AND ZINC CHELATES WITH Br-PAR

Legend

a. 100 µg/dL metal standard
b. 150 µg/dL metal standard
c. 200 µg/dL metal standard

A. Zinc chelate scans.
B. Copper chelate scans.
C. Iron chelate scans.
(d). **Sequential Addition of Cyanide and EDTA to the Metal Chelates of Iron, Copper and Zinc with Er-PAR**

In parts (b) and (c) in this section, the optimum concentrations of cyanide and EDTA were found and their effects on different concentrations of the metal chelates were assessed, respectively. It would be interesting to find out if there is any effect on the sequential addition of cyanide and EDTA to the various concentrations of metal chelates, i.e., whether the simultaneous presence of cyanide and EDTA in the testing solutions contribute to any positive or negative interferences. At all three concentrations of iron used, linearity prior to and after the addition of cyanide was observed (see Fig. 19). Subsequent addition of EDTA did not change the absorbances by very much. The addition of cyanide to the copper chelates reduced the absorbances to approximately zero and these were not depressed further upon the subsequent addition of EDTA, i.e., they remained at or about zero (see Fig. 20). For the three concentrations of zinc chelates shown, little effect was observed upon the addition of cyanide and linearity was maintained prior to and after its addition (see Fig. 21). The subsequent addition of EDTA reduced all the absorbances to approximately zero. From these studies, it can safely be assumed that the sequential addition of cyanide and EDTA to the metal chelates did not produce any positive or negative interferences when both were present together.
FIGURE 19

EFFECT OF SEQUENTIAL ADDITION OF CYANIDE AND EDTA TO IRON CHELATE WITH Br-PAR

Legend

a. Concentration of iron standard: 100 μg/dL
b. Concentration of iron standard: 200 μg/dL
c. Concentration of iron standard: 300 μg/dL
FIGURE 20
EFFECT OF SEQUENTIAL ADDITION OF CYANIDE AND EDTA TO COPPER CHELATE WITH BR-PAR

Legend

a. Concentration of copper standard: 100 \( \mu \text{g/dL} \)

b. Concentration of copper standard: 200 \( \mu \text{g/dL} \)

c. Concentration of copper standard: 300 \( \mu \text{g/dL} \)
FIGURE 21
EFFECT OF SEQUENTIAL ADDITION OF CYANIDE AND EDTA TO
ZINC CHELATE WITH BR-PAR

Legend

a. Concentration of zinc standard: 100 μg/dL
b. Concentration of zinc standard: 200 μg/dL
c. Concentration of zinc standard: 300 μg/dL
(e). Determination of Iron, Copper and Zinc

As a result of studies described in (a), (b), (c) and (d) of this section, the following scheme is proposed to determine iron, copper and zinc in the presence of each other:

1. SAMPLE: \( \text{Fe}^{2+} \cdot \text{Cu}^{2+} \cdot \text{Zn}^{2+} \) + Br-PAR → TOTAL ABSORBANCE: \( \text{Fe}^{2+} \cdot \text{Cu}^{+} \cdot \text{Zn}^{2+} \)

2. \( \text{A} \) + CN\(^-\) → ABSORBANCE DUE TO: \( \text{Fe}^{2+} \cdot \text{Zn}^{2+} \)

3. \( \text{B} \) + EDTA → ABSORBANCE DUE TO: \( \text{Fe}^{2+} \)

4. \( \text{A} \) - \( \text{B} \) → ABSORBANCE DUE TO: \( \text{Cu}^{+} \)

5. \( \text{A} - [\text{C} + \text{D}] \) → ABSORBANCE DUE TO: \( \text{Zn}^{2+} \)
The sequential determination of all three metals in the presence of each other 1:1:1 mixture (μg/dL) at various concentrations was carried out as outlined in ANALYTICAL STUDIES (CHAPTER II B. 4, p. 46). Time-scan curves for the sequential determination of these metals are shown in Fig. 22. The absorption spectra for the 1:1:1 mixture of the metals present are shown in Fig. 23 and these are represented by the regression line in Fig. 24 which corresponds to the equation \( y = 0.3027x + 0.0031 \) \((r=0.9997)\). The curve is linear up to 300 μg/dL of metals used. The molar absorptivities obtained by this procedure for all three metals are shown in Table XIII. These molar absorptivities are comparable to those obtained for the determination of the individual metals (in the absence of the other two (see Table XI). Based on the latter molar absorptivities and the absorbances derived from the sequential determination of the metals (see Table XIII), the recoveries obtained for these metals by the sequential procedure for zinc, copper and iron were calculated. These results are shown in Table XIV. Recoveries ranging from 95 to 107% were obtained for all three metals.

The individual calibration curves for zinc, iron and copper determined by the sequential method of a 1:1:1 mixture are shown in Figs. 25-27, respectively. All three curves follow Beer's law up to 300 μg/dL for the respect-
FIGURE 22
SEQUENTIAL DETERMINATION OF IRON, COPPER AND ZINC
USING Er-PAR

Legend

Concentration of a 1:1:1 mixture of each metal:

a. 100 µg/dL  
   e. 200 µg/dL  

b. 125 µg/dL  
   f. 250 µg/dL  

c. 150 µg/dL  
   g. 300 µg/dL  

d. 175 µg/dL

Experiments were done in triplicates.

Regression line for zinc: \(y = 0.1084x + 0.0022\)  
\((r = 0.9981)\) molar absorptivity 91,700 cm\(^2\)mol\(^{-1}\).

Regression line for copper: \(y = 0.1057x + 0.0074\)  
\((r = 0.9980)\) molar absorptivity = 72,400 cm\(^2\)mol\(^{-1}\).

Regression line for iron: \(y = 0.0898x + 0.0066\)  
\((r = 0.9986)\) molar absorptivity = 66,800 cm\(^2\)mol\(^{-1}\).
FIGURE 23

ABSORPTION SPECTRA OF METAL CHELATE MIXTURES

Legend

Concentration of a 1:1:1 mixture of each metal:

a. 100 µg/dL
b. 125 µg/dL
c. 150 µg/dL
d. 175 µg/dL
e. 200 µg/dL
f. 250 µg/dL
g. 300 µg/dL
FIGURE 24
CALIBRATION CURVE FOR A 1:1:1 MIXTURE OF IRON, COPPER AND ZINC

Legend

<table>
<thead>
<tr>
<th></th>
<th>Fe+² (μM)</th>
<th>Cu⁺¹ (μM)</th>
<th>Zn⁺² (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/dL</td>
<td>17.9</td>
<td>15.7</td>
<td>15.3</td>
</tr>
<tr>
<td>200 μg/dL</td>
<td>35.8</td>
<td>31.5</td>
<td>30.6</td>
</tr>
<tr>
<td>300 μg/dL</td>
<td>53.7</td>
<td>47.2</td>
<td>45.9</td>
</tr>
</tbody>
</table>

Regression line: y = 0.3027x + 0.0031 (r = 0.9997)

Experiments were done in triplicate.
<table>
<thead>
<tr>
<th>Concentration of each metal $^b$ (1:1:1) in a single aliquot ($\mu g/dL$) $^c$</th>
<th>A = total $=Zn+Cu+Fe$</th>
<th>B = add $CN^{-}$ $=Zn+Fe$</th>
<th>C = A - B $=Cu$</th>
<th>D = add EDTA $=Fe$</th>
<th>E = A - [C + D] $=Zn$</th>
<th>Absorbance (510 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.304</td>
<td>0.203</td>
<td>0.101</td>
<td>0.095</td>
<td>0.108</td>
<td>(7.24)</td>
</tr>
<tr>
<td>125</td>
<td>0.380</td>
<td>0.261</td>
<td>0.119</td>
<td>0.114</td>
<td>0.147</td>
<td>(6.82)</td>
</tr>
<tr>
<td>150</td>
<td>0.458</td>
<td>0.307</td>
<td>0.151</td>
<td>0.144</td>
<td>0.163</td>
<td>(7.21)</td>
</tr>
<tr>
<td>175</td>
<td>0.530</td>
<td>0.354</td>
<td>0.176</td>
<td>0.166</td>
<td>0.188</td>
<td>(7.21)</td>
</tr>
<tr>
<td>200</td>
<td>0.613</td>
<td>0.406</td>
<td>0.207</td>
<td>0.191</td>
<td>0.215</td>
<td>(7.42)</td>
</tr>
<tr>
<td>250</td>
<td>0.768</td>
<td>0.500</td>
<td>0.264</td>
<td>0.231</td>
<td>0.273</td>
<td>(7.57)</td>
</tr>
</tbody>
</table>
TABLE XIII CONTINUED

<table>
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<tr>
<th></th>
<th>0.904</th>
<th>0.592</th>
<th>0.304</th>
<th>0.273</th>
<th>0.330</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7.26)</td>
<td>(6.49)</td>
<td>(9.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average molar absorptivity</td>
<td>7.24</td>
<td>6.68</td>
<td>9.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Concentration of each metal in cuvet (μM)</th>
<th>Recoveries</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn(^{+2})</td>
<td>Cu(^{+1})</td>
</tr>
<tr>
<td>1.20</td>
<td>1.19</td>
<td>1.40</td>
</tr>
<tr>
<td>1.50</td>
<td>1.61</td>
<td>1.65</td>
</tr>
<tr>
<td>1.80</td>
<td>1.79</td>
<td>2.09</td>
</tr>
<tr>
<td>2.09</td>
<td>2.06</td>
<td>2.44</td>
</tr>
<tr>
<td>2.39</td>
<td>2.36</td>
<td>2.87</td>
</tr>
<tr>
<td>2.99</td>
<td>3.00</td>
<td>3.66</td>
</tr>
<tr>
<td>3.59</td>
<td>3.62</td>
<td>4.21</td>
</tr>
</tbody>
</table>

\(^{a}\)Recoveries were calculated from the following molar absorptivities:

Zn = 9.11 x 10\(^{4}\) cm\(^{2}\) mol\(^{-1}\); Cu = 7.22 x 10\(^{4}\) cm\(^{2}\) mol\(^{-1}\); Fe = 6.65 x 10\(^{4}\) cm\(^{2}\) mol\(^{-1}\).
FIGURE 25

CALIBRATION CURVE FOR ZINC DETERMINED BY THE SEQUENTIAL METHOD USING Br-PAR

Legend

100 $\mu g/dL = 15.3 \mu M$

200 $\mu g/dL = 30.6 \mu M$

300 $\mu g/dL = 45.9 \mu M$

Regression line: $y = 0.1084x + 0.0022$ ($r = 0.9981$).

Molar absorptivity: $9.17 \times 10^4 + 2989 \text{ cm}^2 \text{ mol}^{-1}$.

Experiments were done in triplicate.
FIGURE 26

CALIBRATION CURVE FOR IRON DETERMINED BY THE SEQUENTIAL METHOD USING Br-PAR

Legend

\[ 100 \mu g/dL = 17.9 \mu M \]
\[ 200 \mu g/dL = 35.8 \mu M \]
\[ 300 \mu g/dL = 53.7 \mu M \]

Regression line: \( y = 0.0898x + 0.0066 \) (\( r = 0.9986 \)).

Molar absorptivity: \( 6.68 \times 10^4 \pm 1506 \text{ cm}^2 \text{ mol}^{-1} \).

Experiments were done in triplicate.
FIGURE 26

Absorbance vs. Concentration (μg/dL)

Absorbance

Concentration (μg/dL)
FIGURE 27

CALIBRATION CURVE FOR COPPER DETERMINED BY THE SEQUENTIAL METHOD USING Br-PAR

Legend

100 µg/dL = 15.7 µM
200 µg/dL = 31.5 µM
300 µg/dL = 47.2 µM

Regression line: \( y = 0.1057x + 0.0074 \) (\( r = 0.9980 \)).

Molar absorptivity: \( 7.24 \times 10^4 + 2262 \) cm\(^2\) mol\(^{-1}\).

Experiments were done in triplicate.
tive metal.

In certain pathological conditions a 1:1:1 ratio of the metals is usually not observed (149) and since it has been reported that the presence of increasing amounts of a particular metal may interfere with the determination of the other (150) a study was carried out to check the effect of increasing amounts of one metal on the determination of the others by the sequential method.

The absorbances obtained for various ratios of metals are plotted in Figs. 28-30. From these curves, it is quite clear that there is little effect of any one metal on the determination of the other.

C. CLINICAL STUDIES

1. Normal Ranges

A study was carried out on the sequential determination of iron, copper and zinc in serum for an adult population. No extensive statistical analyses are reported here since a small number of samples (n=10) were analyzed. For those specimens analyzed (in duplicate), the zinc concentrations found varied from 66-179 μg/dL (11.9-32.2 μM) with a mean of 105 μg/dL (18.9 μM). The iron concentrations found varied from 45-185 μg/dL (6.8-28.2 μM) with a mean of 77 μg/dL (11.8 μM). The copper concentrations found varied from 64-180 μg/dL (10.0-28.4 μM) with a mean of 75 μg/dL (11.8 μM).
Figure 28

Effect of Iron Concentration on the Sequential Determination of Copper and Zinc Using Br-PAR

Legend

- - - Represents the iron curve
- - - Represents the zinc curve
- - - Represents copper curve

Beer's law is observed for the varied concentrations of iron.

Regression line for the iron curve:

\[ y = 0.1195x + 0.0243 \quad (r = 0.9991) \]

Standards were made to contain the ratios indicated in \( \mu g/dL \) and then each standard was carried through the sequential procedure (p.46) to determine each metal, e.g., a 1:1:2 standard would be equivalent to 150:150:300 \( \mu g/dL \).
FIGURE 29

EFFECT OF COPPER CONCENTRATION ON THE SEQUENTIAL DETERMINATION OF IRON AND ZINC USING Br-PAR

Legend

- Represents the iron curve
- Represents the zinc curve
- Represents the copper curve

Beer's law is observed for the varied concentrations of copper.

Regression line for the copper curve: $y = 0.1470x + 0.0053$ ($r = 0.9998$).

Standards were made to contain the ratios indicated in $\mu g/dL$ and then each standard was carried through the sequential procedure (p. 46) to determine each metal, e.g., a 1:1:2 standard would be equivalent to 150:150:300 ($\mu g/dL$).
FIGURE 30
EFFECT OF ZINC CONCENTRATION ON THE SEQUENTIAL DETERMINATION OF COPPER AND IRON USING Br-PAR

Legend

- Represents the iron curve
○ Represents the zinc curve
x Represents the copper curve

Beer's law is observed for the varied concentrations of zinc.
Regression line for the zinc curve: $y = 0.1550x + 0.0023$ ($r = 0.9992$).

Standards were made to contain the ratios indicated in μg/dL and then each standard was carried through the sequential procedure (p.46) to determine each metal, e.g., a 1:1:2 standard would be equivalent to 150:150:300 (μg/dL).
The normal values reported for serum zinc, iron and copper are 60-130 μg/dL (10.8-23.4 μM), 57-194 μg/dL (8.66-29.49 μM) and 70-140 μg/dL (11.06-22.12 μM), respectively.

2. Recovery Studies

Recovery studies were done for the sequential determination of iron, copper and zinc in serum. Sera were pooled and the metal concentrations determined. Then known amounts of iron, copper and zinc were added to an aliquot of pooled sera and the resultant concentration of each metal was determined by the sequential method. The results are shown in Table XV. The recovery for iron varied from 95-103% with an average of 99.6%. The recovery for copper varied from 96-104% with an average of 99.6%. The recovery for zinc varied from 97-101% with an average of 98.6%.

3. Precision Studies

Several sera were pooled and analyzed for the evaluation of within-run and between-run reproducibility of the sequential procedure. For the within-run study, the pooled sera was analyzed 10 times within the same analytical batch for three different runs over three days. For the between-run, the pooled sera was assayed in duplicate over six analytical batches. Table XVI summarizes the results obtained for all three metals. The within-run coefficient of variations for iron, copper and zinc were 1.50%, 0.90% and 1.68%, respectively. The between-run coefficient of
<table>
<thead>
<tr>
<th>Serum base value (µg/dL)</th>
<th>Each metal added (µg/dL)</th>
<th>Concentration expected (µg/dL)</th>
<th>Concentration found (µg/dL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe  Cu  Zn</td>
<td></td>
<td>Fe    Cu    Zn</td>
<td>Fe    Cu    Zn</td>
<td></td>
</tr>
<tr>
<td>91  79  170</td>
<td>63</td>
<td>154   142   233</td>
<td>146   133   228</td>
<td>95  94     98</td>
</tr>
<tr>
<td>118</td>
<td>208</td>
<td>208   197   288</td>
<td>210   199   282</td>
<td>101  101   98</td>
</tr>
<tr>
<td>167</td>
<td>258</td>
<td>258   246   337</td>
<td>263   241   317</td>
<td>102  98    94</td>
</tr>
<tr>
<td>210</td>
<td>301</td>
<td>301   289   380</td>
<td>292   286   388</td>
<td>97  99     102</td>
</tr>
<tr>
<td>250</td>
<td>341</td>
<td>341   329   420</td>
<td>351   342   412</td>
<td>103  104   99</td>
</tr>
</tbody>
</table>
TABLE XVI
PRECISION STUDIES FOR THE SEQUENTIAL DETERMINATION OF IRON, COPPER AND ZINC

<table>
<thead>
<tr>
<th>Metals</th>
<th>n</th>
<th>Mean (μg/dl)</th>
<th>S.D. (μg/dl)</th>
<th>S.E.M. (μg/dL)</th>
<th>C.V. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>3</td>
<td>90.7</td>
<td>1.36</td>
<td>0.785</td>
<td>1.50</td>
</tr>
<tr>
<td>Copper</td>
<td>3</td>
<td>70.6</td>
<td>0.63</td>
<td>0.367</td>
<td>0.90</td>
</tr>
<tr>
<td>Zinc</td>
<td>3</td>
<td>97.6</td>
<td>1.64</td>
<td>0.947</td>
<td>1.68</td>
</tr>
<tr>
<td>Between-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>6</td>
<td>87.9</td>
<td>1.63</td>
<td>0.665</td>
<td>1.86</td>
</tr>
<tr>
<td>Copper</td>
<td>6</td>
<td>71.6</td>
<td>0.58</td>
<td>0.240</td>
<td>0.82</td>
</tr>
<tr>
<td>Zinc</td>
<td>6</td>
<td>98.0</td>
<td>1.76</td>
<td>0.719</td>
<td>1.80</td>
</tr>
</tbody>
</table>

See APPENDIX B for definitions of the statistical terms used in this Table.
variations for iron, copper and zinc were 1.86%, 0.82% and 1.80%, respectively.

The effect of varied concentrations of magnesium and calcium on the determination of iron is shown in APPENDIX C.
CHAPTER IV
SUMMARY AND CONCLUSIONS

A. SYNTHESIS OF 2-AMINO-5-BROMO-PYRIDYLazo RESORCINOL

The synthesis of Br-PAR was carried out in two steps. The diazotate was first formed by the reaction of 2-amino-5-bromo-pyridine and freshly prepared isobutyl nitrite in a basic alcoholic medium. The isolated sodium diazotate was then coupled to an excess of resorcinol in a ethanol:water:methanol solvent system. The use of such a solvent system along with the temperature of the reaction (42-45°C) and an excess of resorcinol were some of the conditions that allowed for a more rapid synthesis of this compound.

The final product isolated from the coupling step was analyzed by spectroscopy and for per cent elemental composition. All results indicate a compound which correspond to the monosodium monohydrate derivative of Br-PAR. Thus, the reagent Br-PAR was synthesized in less than half the time required for the original synthesis and in higher purity (139).

B. ANALYTICAL STUDIES

An aqueous solution of the reagent Br-PAR showed very little increase or decrease in absorbance after 12 days. The variation of pH on aqueous solutions of Br-PAR showed two maximum wavelengths of absorption corresponding to acidic and alkaline media and an isosbestic point of 452 nm. These correlate...
quite well with those found in the literature (139). The variation of pH on the formation of metal chelate with Br-PAR and iron, copper and zinc gave a common optimum pH of 9.6. The order of addition of reagents and the effect of the concentration of Br-PAR on the stability of the metal chelates were also investigated.

Using the established optimum conditions, methods were developed to determine each metal with Br-PAR in the absence of the others. The molar absorptivities as determined in this way were 66,500, 72,200 and 91,700 cm² mol⁻¹, respectively, and Beer's law was obeyed up to 300 µg/dL. The effects of the addition of cyanide and EDTA individually to metal chelates were also studied. The addition of cyanide to iron and zinc chelates with Br-PAR did not result in any displacement of the Br-PAR, but when cyanide was added to the copper chelate with Br-PAR, displacement of the Br-PAR was observed. EDTA displaced the Br-PAR from copper and zinc chelates but showed no effect on the iron chelate. Based on these observations, a sequential method was developed to determine these three metals in the presence of one another. The molar absorptivities for iron, copper and zinc determined sequentially were 66,800, 72,400 and 91,700 cm² mol⁻¹, respectively, and Beer's law was obeyed up to 300 µg/dL.

C. CLINICAL STUDIES

A sample population (n=10) was studied and normal ranges found for iron, copper and zinc were 45 to 185 µg/dL,
64 to 180 \mu g/dL and 66 to 179 \mu g/dL, respectively.

Known amounts of each metal were added to pooled sera and the resultant concentration for each metal was determined by the sequential procedure. Recovery of the metals ranged from 94 to 104%. Precision studies for within-run and between-run analyses show coefficient of variations less than 2%.
APPENDIX A

CALCULATION OF FORMATION CONSTANTS OF METAL CHELATES

The formation constants can be calculated from the instability constants:

\[ K_f = \frac{1}{K_{\text{inst}}} \]

\[ K_f \text{ is the formation constant and } K_{\text{inst}} \text{ is the instability constant} \]

The equation used to calculate the instability constants for iron, copper and zinc chelates with Br-PAR is the following:

\[ K_{\text{inst}} = \frac{L}{1-a} \alpha^3 C^2 \]

for a 1:2 chelate (see reference below for the derivation of this equation).

\[ C = \text{concentration of chelate in mol L}^{-1} \text{ and } \alpha = \text{degree of dissociation} \]

\[ \alpha = \frac{E_m - E_s}{E_m} \]

\[ E_m \text{ is the maximum absorbance of a given amount of metal in the presence of excess Br-PAR.} \]

\[ E_s \text{ is the value obtained when the same amount of metal is mixed with a stoichiometric amount of Br-PAR.} \]

For a solution that was \(8.59 \times 10^{-6}\) M iron, \(E_m\) and \(E_s\) were 0.530 and 0.480, respectively (see Fig. 10). Thus, \(K_{\text{inst}} = 9.4 \times 10^{-13}\) and \(K_f = 1.06 \times 10^{12}\).

For a solution that was \(3.78 \times 10^{-6}\) M copper, \(E_m\) and \(E_s\) were 0.564 and 0.508, respectively (see Fig. 10). Thus, \(K_{\text{inst}} = 6.35 \times 10^{-14}\) and \(K_f = 1.58 \times 10^{13}\).

For a solution that was \(5.16 \times 10^{-6}\) M zinc, \(E_m\) and \(E_s\) were 0.704 and 0.648, respectively (see Fig. 10). Thus, \(K_{\text{inst}} = 5.78 \times 10^{-14}\) and \(K_f = 1.73 \times 10^{13}\).

APPENDIX B

STATISTICS

C.V. Coefficient of variation is the standard deviation expressed as a percentage of the mean. It expresses relative precision over a wide range of values.

S.D. Standard deviation is the square root of the variance. It is inversely proportional to precision, thus, when S.D. increases, precision decreases.

V Variance is the sum of the squares of the deviations from the average divided by the degrees of freedom.

S.E.M. Standard error of mean normalizes the standard deviation.

FORMULAS

\[
\begin{align*}
\text{C.V.} & = \frac{100 \times \text{S.D.}}{\bar{x}} \\
\text{S.D.} & = \sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}} \\
\text{S.D.} & = \sqrt{V} \\
V & = \frac{\sum (x-\bar{x})^2}{(n-1)} \\
\text{S.E.M.} & = S_{\bar{x}} = \frac{S}{\sqrt{n}} \\
r & = \frac{n\Sigma xy - \Sigma x \Sigma y}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}} \\
y & = a + bx \\
b & = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\Sigma (x-\bar{x})^2} \\
a & = \bar{y} - b\bar{x}
\end{align*}
\]

$\bar{x}$ = mean

$n-1$ = degrees of freedom

$x$ = individual value

$n$ = runs

$S$ = S.D.

$r$ = correlation coefficient

$V$ = variance
APPENDIX C

EFFECT OF MAGNESIUM AND CALCIUM ON THE DETERMINATION OF IRON

Legend

A. Effect of magnesium concentration on iron determination
   h. Curve for a 200 µg/dL iron standard
   i. Curve for a 200 µg/dL iron standard containing 2 mg/dL magnesium
   j. Curve for a 200 µg/dL iron standard containing 4 mg/dL magnesium
   k. Curve for a 200 µg/dL iron standard containing 6 mg/dL magnesium

B. Effect of calcium concentration on iron determination
   a. Curve for a 200 µg/dL iron standard
   d. Curve for a 200 µg/dL iron standard containing 5 mg/dL calcium
   c. Curve for a 200 µg/dL iron standard containing 10 mg/dL calcium
   e. Curve for a 200 µg/dL iron standard containing 15 mg/dL calcium

All of the above curves were obtained by following the procedure as outlined in ANALYTICAL STUDIES (CHAPTER II 3.4, p.46).
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