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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

COMPLEXATION STUDIES OF 5,5'-DINITRO BAPTA AND
MAG-QUIN-1, WITH THE DIVALENT CATIONS OF CADMIUM,
LEAD, ZINC, COPPER, NICKEL, AND CALCIUM

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

LAURA ISABELLE HALEY

Norman, Oklahoma
1999

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A Dissertation APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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ABSTRACT

The protonation constants of 1,2-bis[2-[bis[carboxymethyl]amino]-5-nitrophenoxy]ethane - (5,5'-dinitro-BAPTA, DNB) and 6-Methoxy-2-methyl-8-aminoquinoline-*N,N*-diacetic acid - (Mag-quin-1, MQ1) and their complex formation constants with the divalent cations of lead, cadmium, copper, zinc, nickel, and calcium, have been determined in aqueous solution at 25.0 °C. Values of these constants were determined using potentiometric and spectrophotometric titration techniques.

For DNB the log values for the first three stepwise, mixed-mode protonation constants were: $\log K_{H1} = 5.36 \pm 0.01$, $\log K_{H2} = 4.51 \pm 0.01$, and $K_{H3} = 3.30 \pm 0.01$. Titrations of DNB with Cd^{2+} , Pb^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , and Ca^{2+} at fixed values of pH produced changes in the UV-vis spectra which were analyzed to give conditional complex formation constants, K'_{MDNB} . Data from potentiometric pH titrations of solutions containing DNB and metal salt were analyzed to determine complex formation constants as well. Log values for the 1:1 metal-DNB complex formation constants with these ions are: $\text{Cd}^{2+} = 8.68$; $\text{Pb}^{2+} = 7.35$; $\text{Cu}^{2+} = 7.24$; $\text{Zn}^{2+} = 6.54$; $\text{Co}^{2+} = 4.60$; $\text{Ni}^{2+} = 3.53$; and $\text{Ca}^{2+} = 2.75$. Evidence for the formation of a 2:1 copper to DNB complex was apparent in titration data from both methods. All DNB experiments were performed at $I = 0.1 \text{ M}$ (KNO_3). The stability constants with these metals indicate a retention of selectivity pattern with a lowered complexation strength in comparison to the parent compound BAPTA(5,5' = H) of about 10^4 .

Log values for the first three stepwise, mixed-mode protonation constants of MQ1 are: $\log K_{H1} = 7.57 \pm 0.01$, $\log K_{H2} = 3.73 \pm 0.01$, and $K_{H3} = 2.41 \pm 0.02$ at $I = 0.05$ (TEAP). Log values for the 1:1 metal to MQ1 complex formation constants of MQ1 with the indicated metal ions at $I = 0.05$ (TEAP) are: $\text{Cd} = 7.98$; $\text{Pb} = 9.38$; $\text{Cu} = 13.8$; $\text{Zn} = 9.61$; $\text{Ni} = 11.5$; $\text{Ca} = 3.46$. The formation of a 1:2 copper to MQ1 complex was indicated. These values indicate a retention of affinity for smaller metal ions with decreased affinity for the larger ions Ca^{2+} , Cd^{2+} , and Pb^{2+} in comparison to quin-2, the parent compound.

Chapter I

Introduction

Metal ions have long been known to play significant roles in biological function. Although their importance is apparent, the mechanisms by which they function have been difficult to determine. A major hindrance to understanding the means by which metal ions affect biological function is the lack of means to determine the concentrations of these ions, especially in the presence of other ions, often present at higher concentration. The main challenge has been to devise satisfactory means for measuring and controlling metal ion concentrations, particularly inside cells. Indicators, substances which exhibit changes in absorbance or fluorescence when they bind metal ions, and metal ion buffers have been the most popular tools for this purpose. Early indicators had several shortcomings such as weak affinity, poor selectivity, formation of complexes with multiple stoichiometries¹, and difficult or disruptive methods for introduction into cells². Major advances have been made in the development of selective indicators for measurement of calcium and a wealth of information on its roles in cellular function has accumulated^{3,4,5}. The roles of trace metal ions in physiological systems are becoming more apparent and indicators selective for these ions at their low concentrations are needed to help determine the mechanisms by which they exert both healthy and harmful effects.

Although the effects of deficiency or excess of many trace metals are known, the mechanisms through which they achieve these effects are not understood. Even

with the more abundant metal ions the mechanisms through which they act have only recently been explored. The importance of calcium and other metals in catalysis, transport and the stabilization of protein structure has long been appreciated but details of chemical mechanisms by which these processes take place are more recently discovered⁶. The chemical basis of many biological activities is still being explored and many new processes and important functions of metal ions have become apparent. Selective indicators for the measurement of metal ion concentrations are needed to help answer many questions such as: How much of these ions are present in the environment? How do they pass through membranes? How are they are carried through the body? How and where do they ultimately manifest their biological functions? Can they be removed from a reactive environment?

The growth in understanding of the importance of calcium to cellular function emphasized the need for improved tools to study the role of calcium in physiological function and escalated the search for effective means of controlling and measuring metal concentrations. These compounds allow us to investigate the effects of metal ions on vital processes such as the transmission of nerve impulses^{37,8,9}, muscle contraction^{410,11}, auditory transduction¹², immune response^{13,14}, the activation of enzymes^{5,15} and cell division¹⁶. The largest problem faced in developing these tools has been competitive binding by cations other than the one being studied, especially cations such as Mg^{2+} which may be present in much greater concentration than that of the ion of interest. The most popular tools have been buffers and proteins which would bind calcium and maintain a consistent concentration of free Ca^{2+} , and indicators which bind calcium with resultant changes detectable by spectroscopic methods.

Metal ion buffers are used to maintain concentrations of metal ions in biological media at essentially constant levels. As metal ions are removed from or added to the system, by biochemical processes or other means, the concentration of free metal ions is maintained by the equilibrium reactions of ions chelated by the buffering substance. These buffers are useful in studies concerning metal ion transport¹⁷, hormone regulation^{18,19}, enzyme inhibition, activation, and reaction rates²⁰, the toxicity of metal ions such as lead and cadmium^{21,22}, and in controlling the catalytic action of metal ions in chemical and industrial processes. The level at which free metal ion concentrations are maintained is dependent on the conditional stability constant of the metal-buffer complex at the pH of the system. Therefore it is useful to have buffers with a range of stabilities in order to match the requirements of a particular system²³. Stability constants are affected by the pH of the system due to competitive binding of protons. If the buffer ligand has protonation constants ($\log K_H$) above the pH being studied, the apparent stability constants can vary significantly with small changes in pH. Other considerations in physiological studies are solubility of complexes and metabolism of the ligand²⁴.

Metal indicators are tools that allow determination of metal ion concentrations, static or transient, by spectroscopic methods. In the search for, or development of, adequate indicators many factors must be considered. Desired properties for indicators to be used for measuring metal ion concentrations in biological systems are:

- 1) Linear response to metal complexation which is achieved when there is a high affinity of the indicator ligand for the metal ion of interest.
- 2) Sensitivity in measurement which is accomplished through high quantum efficiency and/or molar absorptivity at the wavelength of maximum absorbance or emission.

3) High selectivity for the metal ion of interest compared to more abundant ions such as Ca^{2+} and Mg^{2+} .

4) Insensitivity to pH to avoid significant changes in formation constants with relatively small changes in pH. Protonation constants ($\log K_H$) for the indicator should be below the pH of experiments.

5) Rapid kinetics of complexation and dissociation reactions. The unprotonated form of the indicator usually reacts most rapidly because binding sites are not blocked by protons.

6) Absorbance at long wavelengths to avoid spectral interferences from endogenous species and to avoid damage to cells that can be caused by intense UV light.

Requirements for effective metal ion buffers are similar to those for indicators except that spectral characteristics are often unnecessary or undesirable.

Metal ion indicators and buffers to be used to measure and control intracellular concentrations have additional requirements in that they must be non-toxic to cells and have a convenient and effective means of being introduced into cells.

EGTA (ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid) was the first buffer to have found significant use in biological research where high selectivity for Ca^{2+} over Mg^{2+} was required.¹ The selectivity for calcium over magnesium resulted from the spacing (cavity size) and number of donor atoms (6 oxygen, 2 nitrogen) of EGTA with respect to the larger size and coordination number of calcium as opposed to magnesium. Magnesium has a small ionic radius (0.65 Å), making it a good polarizer with a strong preference for a coordination number of 6 and an octahedral geometry²⁵. Calcium has a larger ionic radius (0.99 Å) and exhibits variable coordination numbers from 6-9 with no strong directionality in bonding²⁶.

The small size and geometric preference of Mg^{2+} restrict the binding of EGTA whereas the calcium ion can bind to all the donor atoms with no steric or geometric restriction. Unfortunately EGTA is not selective for calcium over protons and both of the amine nitrogens are protonated in the physiological pH range. Because protons block the binding sites, the rate at which EGTA forms complexes with calcium was slow thus limiting its use for buffering rapid calcium transients. The $\log K_{\text{H}}$ values of EGTA ($\log K_{\text{H2}} = 8.90$ and $\log K_{\text{H1}} = 9.52^{27}$) also cause the complex formation equilibria to be strongly pH dependent in the physiological pH range.

The problem of lowering proton affinity while maintaining the selectivity for calcium over magnesium was effectively dealt with by Roger Tsien in the invention of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, BAPTA¹. In BAPTA the cavity size of EGTA is maintained and benzene rings replace the methylene groups connecting the ether oxygens to the nitrogens. Figure I.1 compares the structures of EGTA and BAPTA tetraanions.

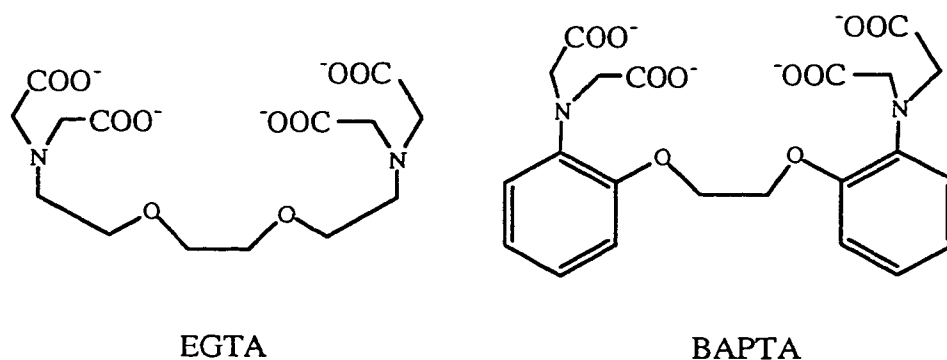


Figure I.1 Structures of EGTA and BAPTA

With the incorporation of the benzene rings the nitrogen atoms are part of an aniline type structure and the lone pair electrons associated with them are delocalized through extended conjugation of the ring through the nitrogen atoms. Thus, in the unbound state the tetraanion of BAPTA has absorbance characteristics similar to an unprotonated dialkylaniline. In the complexed state the nitrogen lone pairs form bonds with the metal ions disrupting the extended conjugation. This results in significant changes in the absorbance spectrum as the wavelength maximum shifts to a position similar to that found for benzene. The conjugation of the aniline groups makes the nitrogens more acidic, lowering the pH at which they are protonated. Therefore, in BAPTA all log K_H values are below 7.0 and binding rates of calcium are faster and less sensitive to small changes in pH during experiments. Tsien also surmised that indicator properties of the BAPTA molecule could be adjusted by “tinkering with the molecular design”. In the years following the introduction of BAPTA a large number of derivatives for different types of applications have been developed^{28,29,30,31,,23}. Although BAPTA itself has several limitations as an indicator and is mainly used as a calcium buffer, it has been the prototype molecule for most indicators used to study calcium in physiological media today.

The affinity of BAPTA for calcium has been manipulated by the addition of electron-withdrawing and electron-donating groups in an attempt to create a family of calcium buffers effective over the entire physiological range of calcium concentrations²³. In these simple derivatives of BAPTA, addition of electron withdrawing groups lowers the affinity for calcium. Replacing the hydrogens para to the nitrogen atoms with bromine reduced the affinity for calcium by a factor of ten²³. Addition of electron donating groups to the aromatic ring increases the affinity for calcium. Replacing the hydrogens in the para position with methyl groups resulted in

a three-fold increase in calcium affinity²³. As expected, the addition of substituents to the aromatic ring has an effect on the protonation constants for the aniline nitrogens similar to its effect on calcium binding¹. Figure I.2 shows the structures of 5,5'-dibromo- and 5,5'-dimethyl BAPTA along with several other BAPTA derivatives.

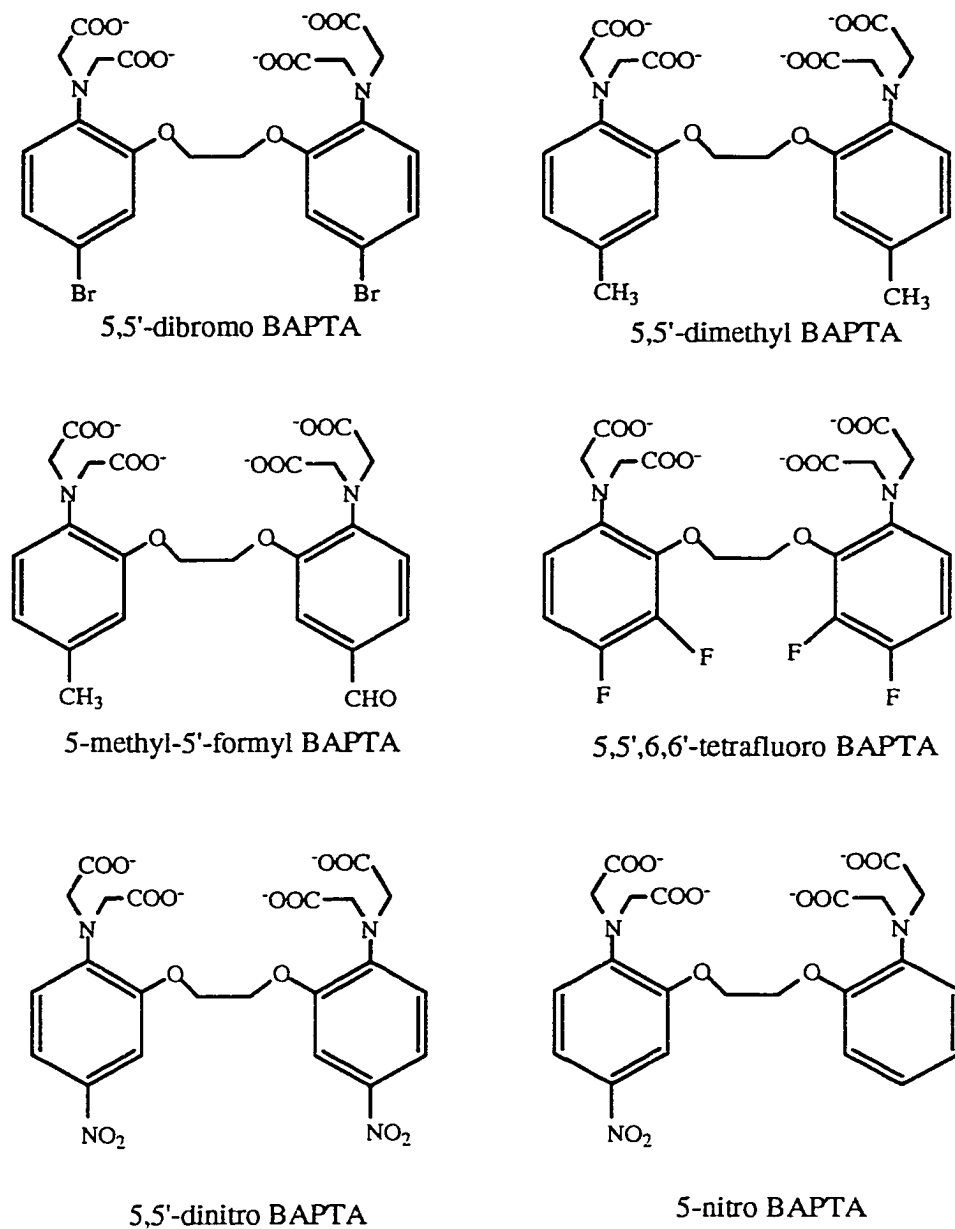


Figure I.2 Structures for some simple BAPTA derivatives.

Though BAPTA exhibits fluorescence behavior, with an emission peak at 363 nm, quantum efficiency is low and BAPTA is classified by commercial suppliers as a nonfluorescent indicator. Derivatives of BAPTA with longer excitation wavelengths and higher quantum efficiency were created by adding chromophores with extended conjugation. Several of these derivatives are shown in Figure I.3. The first of these was called quin-2 because of the substitution of a quinoline group for one of the benzene rings with the further addition of a methoxy group meta to the 8-amino group. The absorbance, as well as the fluorescence, is shifted to longer wavelength with the addition of the larger chromophore and different spectra are observed for the calcium and magnesium complexes. The difference in the spectra between the calcium and magnesium complexes of quin-2 is explained as a result of magnesium binding only the quinoline half of the molecule and leaving the absorbance due to the aniline moiety unaffected, whereas calcium binds to both ends of the molecule. Other popular BAPTA derivatives excited by UV light are indo-1 and fura-2²⁸. Along with the many advantages provided by these compounds were a few disadvantages due to the necessity for excitation in the UV region². Later derivatives incorporated rhodamine and fluorescein fluorophores to allow excitation at longer wavelength, and are named rhod-2 and fluo-3, respectively. Additional derivatives have been designed to provide increased fluorescence intensity at lower indicator concentration and to allow other specialized applications. Dextran conjugate derivatives have been developed to minimize translocation, compartmentalization, leakage, and binding to proteins³².

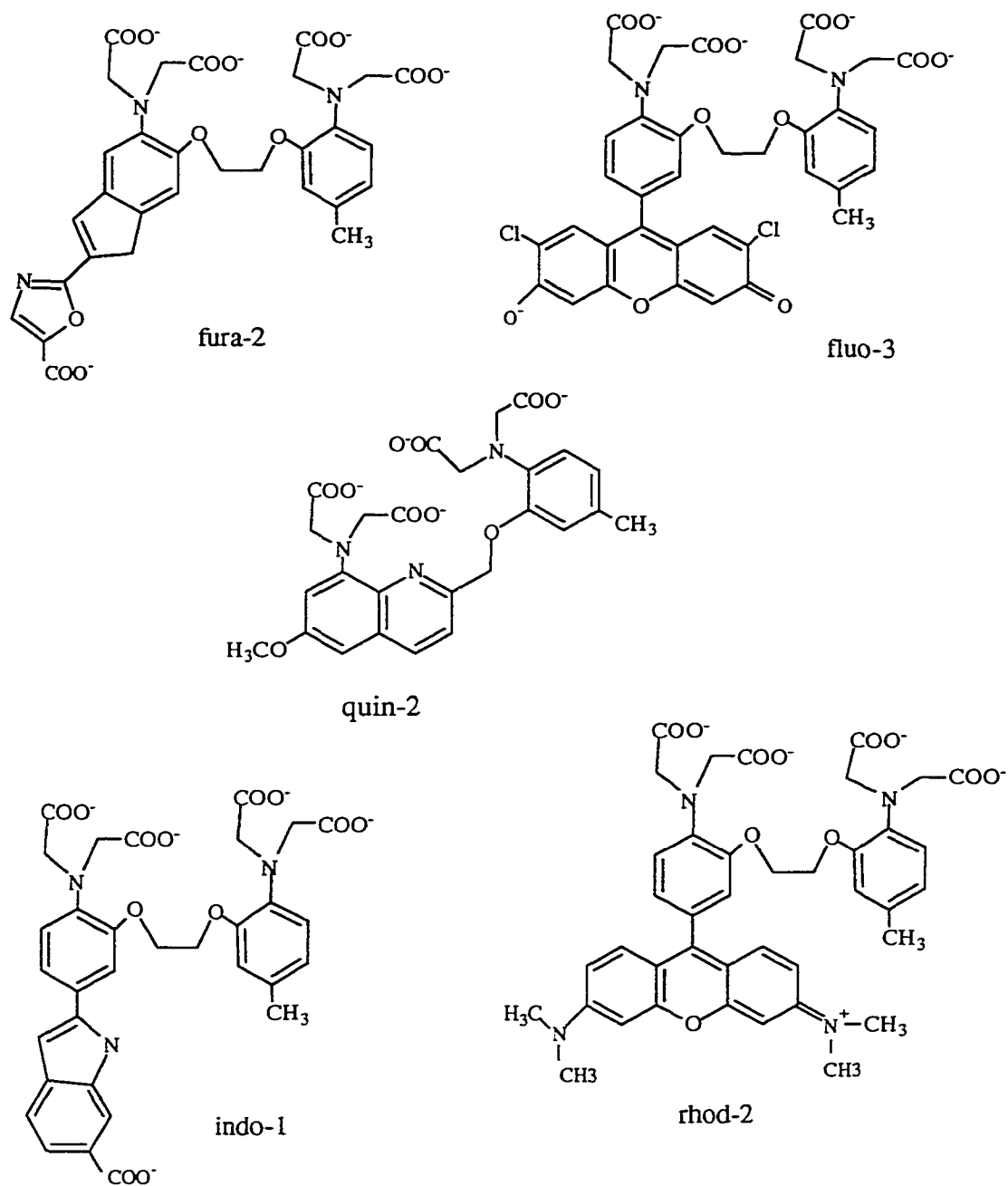


Figure I.3 Structures of fluorescent Ca^{2+} indicators related to BAPTA.

Many BAPTA derivatives have been synthesized that use the position (δ) of ^{19}F NMR signals and integration of peak areas as indicators of Ca^{2+} concentration^{33,34}. These include simple fluorinated derivatives of BAPTA and quin-2, and some with further modifications such as alkyl substitution of the aminodiacetic groups³⁵, or methylation of the benzene rings³⁶ to increase sensitivity and selectivity.

Magnesium is a metal cation of considerable physiological interest. Due to its small radius the magnesium ion has binding properties very different from calcium. The small ionic radius of Mg^{2+} makes it more vulnerable to steric hindrance effects especially since it is fairly rigid in its adherence to octahedral symmetry. There are very few effective probes for the study of Mg^{2+} in biological systems. Evidence that magnesium binds only the quinoline half of the quin-2 molecule prompted development of indicators designed to be selective for magnesium over calcium. Fluorescent magnesium indicators have been developed, based on existing fluorescent Ca^{2+} indicators such as fura-2, indo-1, and quin-2. It was thought that removing the half of the molecule not used in binding magnesium would lower the affinity for calcium and leave the molecule more selective for magnesium. These indicators are named according to the calcium indicator from which they were derived, i.e., mag-fura-2, mag-indo-1, mag-quin-1, and mag-quin-2. Binding of Mg^{2+} produces spectral changes similar to those observed for Mg^{2+} binding by the parent calcium indicators. These indicators are still selective for calcium over magnesium, but to a smaller degree. However, because the concentration of magnesium ($\sim\text{mM}$) is much higher than the concentration of calcium ($< \mu\text{M}$) it is still possible to measure magnesium concentration with little interference due to calcium binding. These indicators tend to form 1:2 complexes, metal to ligand, which can lead to complications in analysis. The

characteristics of metal binding by mag-quin-1 were explored and the results are reported and discussed in this paper. The structure of mag-quin-1 is shown in Figure I.5.

In addition to Ca^{2+} , Mg^{2+} , K^+ , and Na^{2+} there are several transition metal ions essential to human health. Low levels of these ions are necessary for proper physiological functioning and serious problems can result from deficiency or excess in these levels. These ions are present in physiological media at concentrations much lower than those for calcium and magnesium. Zinc is the second most abundant of the essential transition metals yet the concentration of free Zn^{2+} in most cells is less than 1 nM³². Copper, nickel and cobalt have even lower concentrations. Zinc is known to play a central role in the immune system³⁷ and functions in the regulation of cell and tissue growth as well as in apoptosis³⁸. Zinc is essential to reproductive processes³⁹, gene expression⁴⁰, and neurotransmission^{41,42}. Copper is the third most abundant essential transition metal and is important in the prevention of anemia⁴³ and some enzymatic functions²⁶. Menkes' kinky hair disease is a fatal, genetic copper deficiency⁴⁴. Wilson's disease is also a genetic disorder of copper metabolism and results in toxic accumulation of copper in the liver.⁴⁵ Nickel and cobalt have limited known function in higher lifeforms. Well recognized function is limited to vitamin B₁₂ for cobalt. Nickel is required in the enzyme urease and may function as a redox center in other biological sites. Deficiencies of nickel and cobalt can result in growth depression, anemia, and impaired reproduction.⁴⁶ Excess cobalt can result in heart failure or hyperthyroidism while an overabundance of nickel can cause lung cancer⁴⁷.

Lead, cadmium, and mercury are non-essential elements and are lethal toxins in our bodies. While our bodies may contain two grams of zinc, only 10 mg of cadmium can produce severe effects. Cadmium has toxic effects in cardiac tissue⁴⁸,

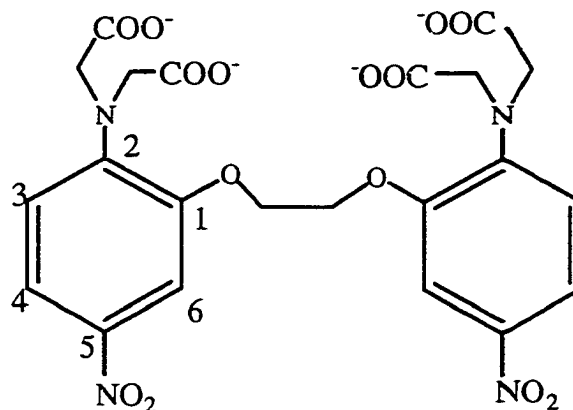
impairs liver and kidney function⁴⁹, and is a well known carcinogen.⁴⁷ Cd^{2+} can bind to Zn^{2+} sites in enzymes, and can be transported through calcium channels in cells⁵⁰. Cadmium can substitute for calcium in bone causing demineralization, shrinkage, and brittleness of bone⁵¹. Lead can produce anemia, by inhibiting enzymes involved in hemoglobin production, and stimulate lipid oxidation⁵². Bound to organic ligands, lead can cross the blood brain barrier resulting in damage to the central nervous system⁵³. Lead has also been shown to mimic calcium in cellular processes such as activation of protein kinase C⁵⁴. The tendency of divalent cations of Cd, Pb, Cu, Zn, Co, and Ni to form stable complexes, especially when binding to nitrogen and sulfur atoms, allows these metals to be very competitive in binding biological sites. The toxicity of metal ions such as lead and cadmium often results from their replacement or blocking of essential biological cations in their endogenous binding sites.

The strong complexes formed by these cations may also have a beneficial application in the development of indicators for studies of their function in cells. Indicators will need to have affinity for the ion of interest far greater than the affinity for calcium and magnesium in order to accomplish quantitative measurement of the lower concentrations of heavy metal ions in biological media. Indicators with binding constants for heavy metals several orders of magnitude greater than their binding constants for calcium will be needed to overcome the proportional differences in concentration to allow quantitative determination of heavy metal ions in the presence of the more abundant calcium ions.

Principles utilized in the design of Ca^{2+} indicators can be useful in development of indicators for other metal ions. The sizes and coordination numbers of different ions are important in ligand design, as demonstrated by the behavior of Ca^{2+} and Mg^{2+} with EGTA. Cd^{2+} and Ca^{2+} have virtually the same ionic radii²⁴ and charge density.

Cadmium has few requirements for binding configuration due to the completely filled d orbitals. Therefore, a binding cavity size that shows selectivity for Ca^{2+} should be suitable for Cd^{2+} .

The objective of this work is to determine the properties of indicator compounds with low calcium affinity and ascertain the viability of their use as indicators for other metal ions of biological, medical, or environmental significance. The indicators examined are derivatives of BAPTA and quin-2. The BAPTA derivative chosen for study is 5,5'-dinitro-BAPTA (DNB), which has electron withdrawing nitro groups substituted on the phenyl ring in the positions para to the aniline nitrogen atoms as shown in Figure I.4. This indicator was reported to have a calcium complexation constant 4 orders of magnitude lower than that of BAPTA²³. If the same selectivity pattern is retained, DNB should have complexation constants for Cd^{2+} , Pb^{2+} , and Zn^{2+} of approximately 10^7 to 10^9 and may prove suitable for determinations of these metal ions. The protonation constants of DNB, the metal-ligand stoichiometry and stability constants for its complexes with cadmium, lead, copper, zinc, nickel, cobalt, and calcium, and the spectral characteristics of its complexes are investigated and discussed in this work.



5, 5'-dinitro BAPTA (DNB)

Figure I.4 Structure of DNB

Quin-2 and BAPTA type buffers tend to form binuclear complexes with Mg^{2+} and some first row transition metal cations, particularly Ni^{2+} and Cu^{2+} . Therefore, indicators are needed that are suitable for smaller cations with coordination numbers equal to or less than six. The second indicator examined, called mag-quin-2, is related in structure to quin-2 and was originally designed as a probe for magnesium ion. The structure of mag-quin-1 (MQ1) is shown in Figure I.5. In mag-quin-1 the ether linkage and phenyliminodiacetic acid group constituting half of the quin-2 molecule are not present (see Figure I.3). The protonation constants of MQ1 and the stoichiometry, UV-vis absorbance spectral characteristics, and stability constants of its complexes with cadmium, lead, copper, zinc, nickel, and calcium are investigated in light of the need for indicators for metals with smaller ionic radius.

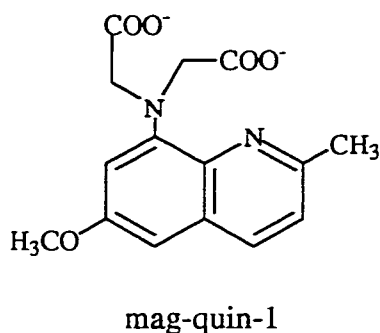


Figure I.5 Structure of MQ1

To ascertain the usefulness of DNB and MQ1 as buffers and indicators for trace metal concentration determinations we have investigated their complexation characteristics with protons and several trace metals. Protonation constants for both of these compounds were determined by UV-vis spectrophotometric and potentiometric pH titration. Conditional stability constants of metal-ligand complexes were determined by spectrophotometric titration of the ligands with metal salts at constant pH. Stability constants were also determined by potentiometric titration of metal ligand solutions.

Indicators that will selectively bind heavy metals at concentrations in the nanomolar range in the presence of micromolar calcium are necessary for effective study of trace metals in biological systems. Such indicators would also be of significant value for determination of metal contamination in environmental studies and industrial processes. The intelligent use of effective indicators for trace metals can lead to major advances in our understanding of the chemical mechanisms through which they function.

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Chapter II

EXPERIMENTAL

I Reagents

Distilled Deionized Water - D.D. H_2O . Aqueous solutions were prepared using deionized water which was further purified by distillation in a Corning Model MP-3A Mega-Pure distillation system.

Perchloric Acid - $HClO_4$. Solutions of $HClO_4$ were made from filtered 70% redistilled acid (GFS Chemicals) and were standardized potentiometrically with standardized base.

Hydrochloric Acid - HCl . Solutions of HCl were made from filtered reagent grade (Fisher Scientific) concentrated acid and were standardized by titration with standardized base potentiometrically or using phenolphthalein as indicator.

Sodium Hydroxide - $NaOH$. Stock solutions of $NaOH$ were made by diluting either 50% w/w concentrate (Mallinckrodt or Fisher Scientific) or ampoules of Dilut-it (J.T. Baker) with freshly boiled, D.D. H_2O and storing under nitrogen. Titrants were made from the stock solutions or by direct dilution from the 50% solution. Solutions were standardized potentiometrically using oven dried potassium hydrogen phthalate (KHP) (primary standard, Mallinckrodt or primary standard, Fisher Scientific). These solutions were titrated with either HCl or $HClO_4$ on a daily basis and the results were used to determine the extent of CO_2 contamination by the method of Gran¹.

Tetraethylammonium Hydroxide - $(\text{CH}_3\text{CH}_2)_4\text{NOH}$, Et_4NOH . Stock solutions of $(\text{CH}_3\text{CH}_2)_4\text{NOH}$ were made from filtered, 20% w/v concentrated base (Acros chemical) by diluting with boiled D.D. H_2O and were stored under nitrogen. Stock solutions were standardized by KHP using phenolphthalein as indicator. Titrants made from these solutions were titrated with HClO_4 to check for CO_2 absorption by the method of Gran¹.

Non-complexing Tertiary Amines All buffers used were of the type described by Yu et al.² as non-complexing tertiary amines to avoid interference due to metal binding. The buffers 2-[*N*-morpholino]ethanesulfonic acid, (MES, $\text{pK}_a = 6.19$), 3-[*N*-morpholino] propanesulfonic acid (MOPS, $\text{pK}_a = 7.09$), piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES, $\text{pK}_{a1} = 2.81$, $\text{pK}_{a2} = 6.77$), and piperazine-*N,N'*-bis[4-butanesulfonic acid] (PIPBS, $\text{pK}_{a1} = 4.39$, $\text{pK}_{a2} = 8.74$) were purchased from Sigma Chemical Company. *N,N'*-diethylpiperazine (DEPP, $\text{pK}_{a1} = 4.67$, $\text{pK}_{a2} = 8.83$) was not commercially available and was synthesized in this laboratory.

Tetraethylammonium Perchlorate - $(\text{CH}_3\text{CH}_2)_4\text{NClO}_4$, TEAP. TEAP was prepared by titrating 20% tetraethylammonium hydroxide with 70% redistilled perchloric acid to one drop past the equivalence point. The equivalence point is marked by a sharp decrease in pH, at which addition of one drop of base will result in a sharp increase in pH while one drop of acid will reverse this and result in an acidic pH. The solution was chilled to crystallize the product, which was then filtered and redissolved. This process was repeated 4 times for to insure purity. The purified compound was then dried overnight in a vacuum oven at 70 °C.

Potassium Nitrate - KNO_3 . KNO_3 (analytical reagent grade, Mallinkrodt) was oven dried at 90 °C for at least two hours prior to use.

Sodium Nitrate - NaNO_3 . NaNO_3 (analytical reagent grade, J. T. Baker) was also dried two hours in an oven at 90 °C before being used.

Lead Perchlorate - $\text{Pb}(\text{ClO}_4)_2$. $\text{Pb}(\text{ClO}_4)_2$ was prepared by reacting PbO (99.9995%, Alfa Aesar) with a slight excess of 70% redistilled HClO_4 . The resulting solution was standardized by titration with EDTA using methylthymol blue as indicator and hexamethylene tetramine as buffer.

Zinc Perchlorate - $\text{Zn}(\text{ClO}_4)_2$. $\text{Zn}(\text{ClO}_4)_2$ was prepared from ultrapure ZnO (99.99%, Alfa Aesar) reacted with 70% redistilled HClO_4 . The product was then crystallized and washed with diethyl ether. Stock solutions were standardized with EDTA using Eriochrome Black T (EBT) as indicator with pH adjusted to 10.0 using ammonia/ammonium chloride buffer.

Cadmium Perchlorate - $\text{Cd}(\text{ClO}_4)_2$. $\text{Cd}(\text{ClO}_4)_2$ was prepared by reacting CdCO_3 (99.999%, Alfa Aesar) with 70% redistilled HClO_4 . The product was then crystallized and washed with diethyl ether. Stock solutions were standardized with EDTA using EBT as indicator at pH 10.0.

Nickel Perchlorate - $\text{Ni}(\text{ClO}_4)_2$. $\text{Ni}(\text{ClO}_4)_2$ was prepared previously in this laboratory by Thomas³, by reacting NiCO_3 with HClO_4 and recrystallizing from hot water. Stock solutions were standardized by titration with EDTA, at pH 10.0, using murexide as indicator.

Metal Chlorides Calcium chloride - CaCl_2 (99.999%, puratronic); cobalt chloride - CoCl_2 (99.998%, puratronic); and copper chloride, CuCl_2 (99.999%, puratronic) were used as obtained from Aldrich. Stock solutions of these metal salts were standardized by titration with EDTA using EBT, Xylenol Orange, and FSB, respectively, as indicators.

Calcium Nitrate - $\text{Ca}(\text{NO}_3)_2 \cdot \text{Ca}(\text{NO}_3)_2$ (99.997%, Aldrich) was used as purchased. Stock solutions were standardized by titration with EDTA using EBT as indicator.

Details regarding the methods used for standardization of metal ion solutions are from Vogel⁴.

6-Methoxy-2-methyl-8-aminoquinoline-*N,N*-diacetic acid - (Mag-quin-1, MQ1). MQ1 was used as purchased from Molecular Probes. MQ1 solutions were standardized by spectrophotometric titration at 262 nm with standard $\text{Pb}(\text{ClO}_4)_2$.

1,2-bis[2-[bis(carboxymethyl)amino]-5-nitrophenoxy]ethane - (5,5'-dinitro-BAPTA, DNB). DNB was commercially available and was used as purchased from Molecular Probes. Stock solutions were made by weighing 5,5'-dinitro-BAPTA into a flask, adding a weighed amount of inert electrolyte (TEAP or KNO_3) to establish an ionic strength of 0.1 M, and diluting with D.D. H_2O . Known volumes of standardized NaOH were added to facilitate dissolution. The stock solution was then standardized by spectrophotometric titration at 430 nm with $\text{Cd}(\text{ClO}_4)_2$ or $\text{Pb}(\text{ClO}_4)_2$.

II Synthesis of *N,N'*-Diethylpiperazine

N,N'-Diethylpiperazine (DEPP) was synthesized from piperazine and ethyl bromide in a solution of K_2CO_3 in D.D. H_2O by a method described by Yu et al.² The reaction took place in the biphasic mixture, during 40 hrs of vigorous stirring, after which the two phases were separated and the aqueous layer was extracted twice with diethyl ether. The two organic layers were combined, washed twice with a saturated NaCl solution, and dried over MgSO_4 . After filtering, the solution was concentrated by evaporation and purified by distillation at 168 °C. NMR and mass spectral data indicate the desired product was obtained and showed no contaminant peaks.

III pH Measurements

Measurements of pH were accomplished using a Fisher pH meter, model 825MP or with a Corning pH meter, model 125, and an Orion Ross semi-micro pH electrode, model 8103. The internal filling solution for the electrode was 3M NaCl. A three-point calibration of the pH meter/electrode system was performed daily using standard commercial buffers (Gram-Pac, Fisher) of pH values 4.01, 6.86, 9.18. pH 9.18 buffer solutions were made with freshly boiled water and stored under N₂ gas to minimize CO₂ contamination.

IV Ligand Standardization

The concentration of MQ1 and DNB in stock solutions was determined by spectrophotometric titration. A sample of the stock solution was diluted 1 to 10 and adjusted to pH 7.0 with filtered Et₄NOH. An aliquot of 2.50 mL of the dilute solution was titrated with 4.0 uL aliquots of standard solution of Pb(ClO₄)₂ or Cd(ClO₄)₂ until the spectra no longer changed with subsequent additions of metal. Data for the absorbance at 430 nm (λ max) as a function of the total metal concentration were divided into two subsets as shown in Figure II.1. Data from each subset were fit to a linear model using least squares. The resulting linear equations were combined to find the value of $[\text{Cd}^{2+}]_{\text{tot}}$, where the line segments intersect. This intersection corresponds to the x -axis value for the equivalence point for the titration assuming that a 1:1 metal ligand complex is formed and that the metal reacts quantitatively (> 99% of the total metal added complexes with the ligand at each point). Therefore, the total cadmium

concentration at the intersection is equal to the total ligand concentration in the dilute solution. The ligand concentration in the dilute solution can be multiplied by the dilution factor to obtain the ligand concentration of the stock solution.

Autopipets (Gilson and Eppendorf) were used to deliver aliquots of metal solutions. These pipets were calibrated by comparison of the mass of delivered D.D. H₂O with the indicated volume.

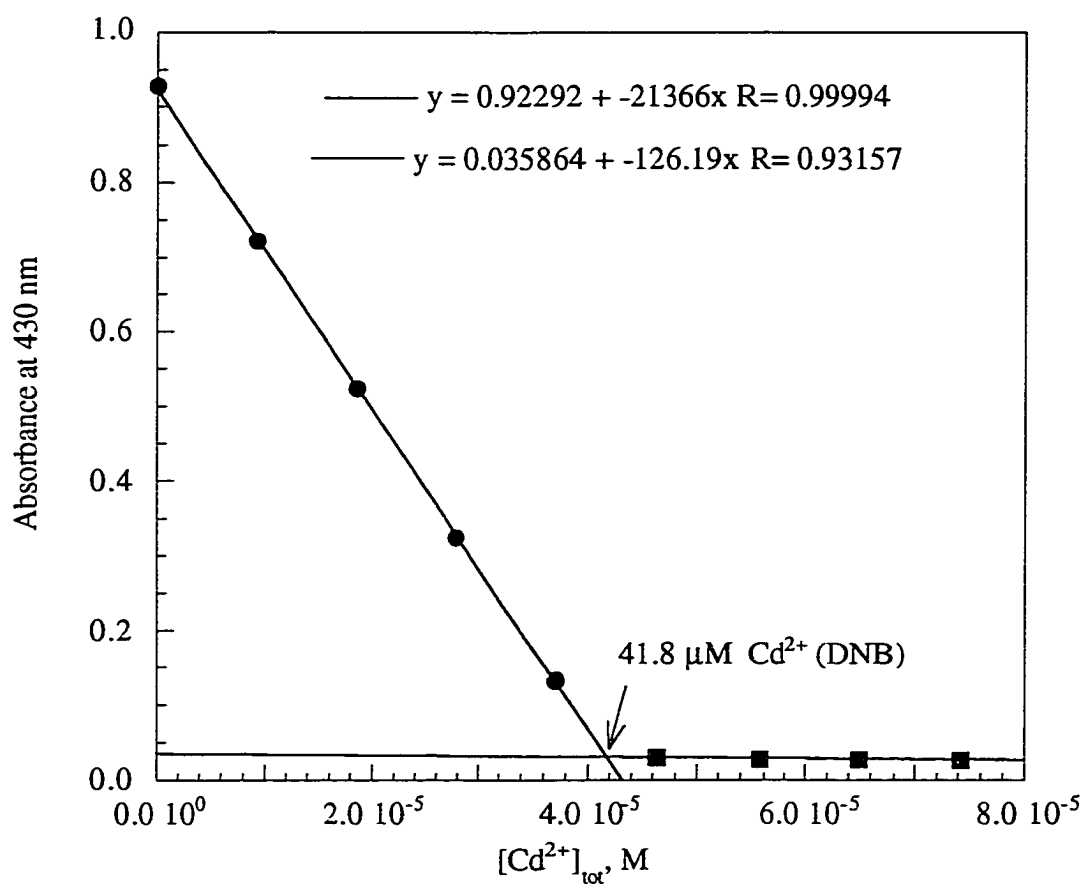


Figure II.1 Plot of absorbance at 430 nm versus concentration of total added cadmium for the standardization of DNB at pH 7.0. The calculated intersection of the two linear segments occurs at $x = 4.18 \times 10^{-5} M$.

V Determination of Percent CO₂ in Titrant

The extent of CO₂ contamination in basic titrants was determined using the pH(observed) and volume of titrant added (mL) data from an automated titration of the base solution with strong acid solution (HCl or HClO₄). The automated titration system consisted of a Metrohm model 655 digital buret and a Fisher 825MP pH meter, with an Orion Ross 8103 semimicro pH electrode, and was controlled by a Zenith 158-42 computer⁵. The resulting data were analyzed by the method of Gran^{1,6}. In this method the quantity ϕ is plotted against the volume of acid added (V_{acid}). The value of ϕ is determined by the equation,

$$\phi = (V_{\text{base}} + V_{\text{acid}}) \times 10^{\pm \text{pH}} \quad (2.1)$$

In this expression V_{base} refers to the volume of base being titrated and pH refers to the recorded readings from the pH meter. The values of ϕ_1 obtained for the acidic region ($10^{+\text{pH}}$ is used in these calculations) and ϕ_2 obtained for the basic region ($10^{-\text{pH}}$ is used) yield separate straight lines that intersect the x axis. The difference in volume between the intersections is related to the amount of carbonate present in the base solution. An example of a Gran plot is shown in Figure II.2. The lines cross the x axis at 9.28 mL and 9.53 mL. The percentage of CO₂ of the total base present is calculated using eq 2.2.

$$\% \text{ CO}_2 \text{ in base} = \frac{\text{intercept } \phi_2 - \text{intercept } \phi_1}{\text{intercept } \phi_2} \times 100\% \quad (2.2)$$

In this example the calculated CO_2 is 2.6%. When dealing with very dilute bases contamination by CO_2 is virtually instantaneous upon contact with air and solutions are kept under N_2 at all times. Titrant base solutions were discarded when calculated CO_2 was greater than 6.0%.

The delivery apparatus of the automated titration system was calibrated by comparison of the mass of D.D. H_2O delivered versus the recorded volume. The pH electrode/meter was calibrated daily as described in section III.

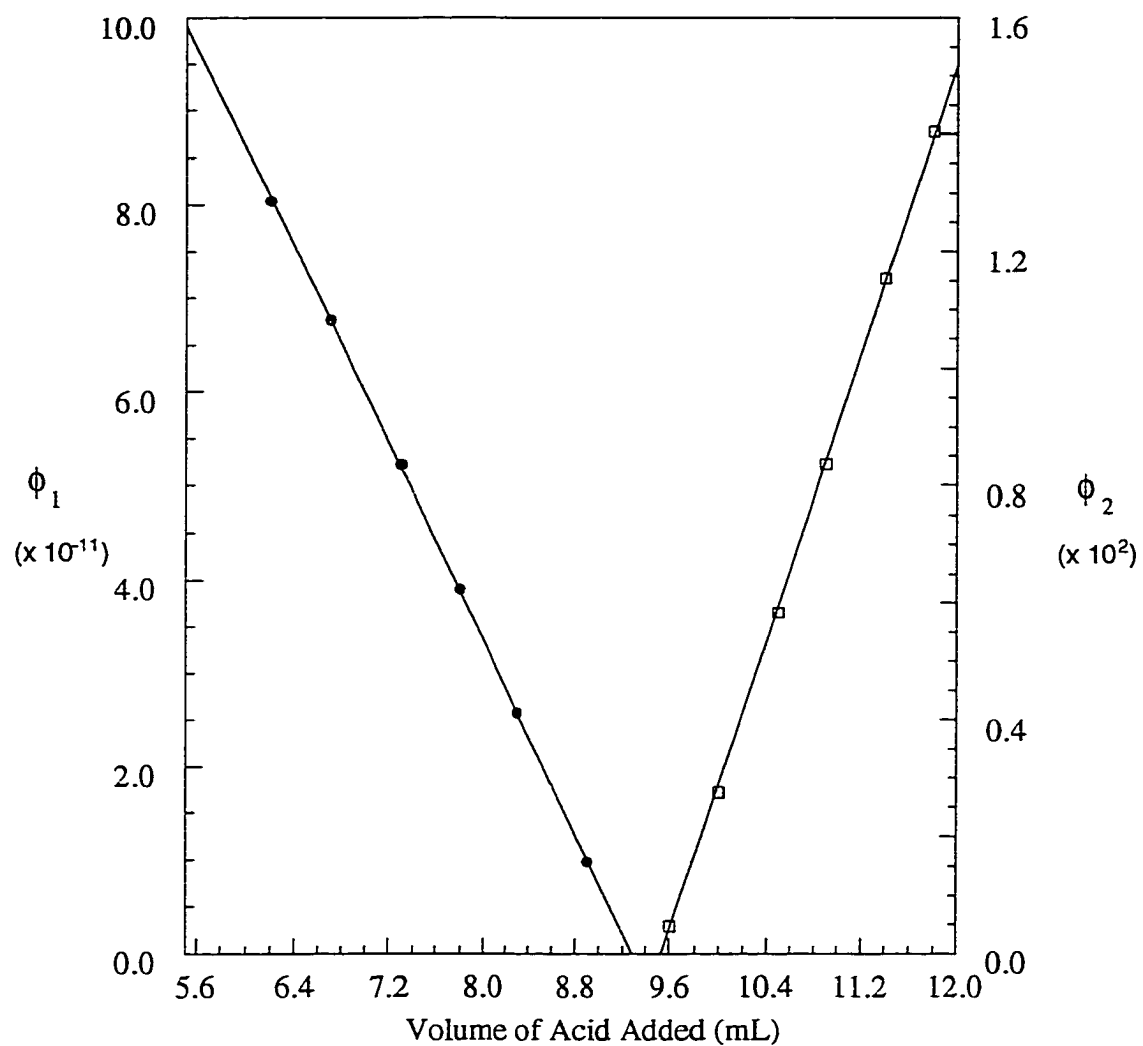


Figure II.2 Gran plot from a titration of NaOH with HCl. The difference in the x axis intercepts is used to determine carbonate contamination in base solutions.

VI Determination of Protonation Constants (K_H) of Ligands

Protonation constants for MQ1 and DNB were determined by both spectrophotometric and potentiometric methods.

A *Spectrophotometric Studies*

Ligand solutions were buffered with MES, PIPES, DEPP, MOPS and PIPBS at concentrations of 5 mM each. Ionic strength was maintained at 0.05 M for MQ1 solutions and 0.1 for DNB solutions by addition of Et_4NClO_4 or KNO_3 , respectively. EDTA at a concentration of 0.5 mM was employed to chelate any metal impurities in the solutions. Solutions containing all components of the sample solutions except the ligand were used as a reference for each data set collected. pH was adjusted to the desired value using filtered solutions of Et_4NOH and HClO_4 at various dilutions.

Spectrophotometric data, obtained as a function of pH in the range of 2.0-10.0, were used to calculate protonation constants. Spectra were acquired at a constant temperature of 25.0 °C which was maintained using external temperature baths attached to both the titration vessel and the cuvette holder. The solutions were prepared with ligand concentrations in the 40 to 65 μM range to give absorbance (maxima) ≤ 1.6 . The pH was determined using an Orion Ross 8103 semimicro pH electrode connected to the Fisher Scientific Accumet pH meter. After each adjustment an aliquot of the solution (ca. 2.5 mL) was transferred to a 1.0 cm pathlength cuvette. The cuvette was placed in the thermostated cell holder and allowed to equilibrate thermally for 2-3 minutes. Then the spectrum was scanned from 190 nm to 800 nm with a Hewlett Packard 8452A diode array spectrophotometer using a 1.0 second integration time.

B *Potentiometric Studies*

The potentiometric data for MQ1 was obtained by titrating 5.0 mL of an unbuffered aqueous solution containing 0.860 mM MQ1 with 5.21 mM Et₄NOH. Measured volumes of standardized HClO₄ were added to adjust the pH to 2.5 ± 0.2 and partially neutralize the solution. TEAP was added to all MQ1 solutions and titrants to maintain ionic strength at 0.05 M.

Potentiometric data for DNB were obtained by titration of DNB solutions with volumes ranging from 5.00 to 25.00 mL and with ligand concentrations ranging from 0.191 mM to 0.376 mM. DNB solutions were prepared with KNO₃ to maintain ionic strength at 0.1 M. Titrant solutions used for DNB potentiometric titrations ranged in concentration from 5.0-15.0 mM NaOH contained sufficient KNO₃ to maintain ionic strength at 0.1 M.

Potentiometric titrations for both ligands were conducted at 25.0 °C with continuous evacuation of CO₂ by N₂. All titrants were standardized by KHP and checked for CO₂ contamination daily, as described previously. Titrations and data acquisition were accomplished using the autotitrator system described in section V.

VII Measurement of Stability Constants

Stability constants for the metal ligand complexes formed by both MQ1 and DNB were determined spectrophotometrically and potentiometrically.

A *Spectrophotometric studies*

Spectrophotometric titrations to determine stability constants were conducted at 25.0 °C and constant pH using a HP 8452A diode array spectrophotometer. The

spectrum of a reference solution was recorded and subtracted from each spectrum in the titration. Each titration was performed by addition of 3.0 or 4.0 mL aliquots of standardized metal solutions directly to 2.50 mL of ligand solution in the cuvette. After each addition of metal solution the cuvette was inverted several times to ensure adequate mixing and the cuvette was returned to the thermostated cell holder. For most titrations 2 minutes were allowed for the reaction to be complete and the solution to regain the prescribed temperature. In titrations with nickel this time was extended to 3 minutes. The absorbance of the solution was then scanned from 190 - 800 nm. In addition to the spectrum for each aliquot, baseline corrected absorbance at selected individual wavelengths was recorded for use in the graphing program. All solutions contained 5.0 mM buffer. The buffer used in each solution was dependent on the pKa of the buffer and the desired pH. PIPES was used in solutions titrated in the 3.0 -3.6 and 6.8 - 7.2 pH ranges. PIPBS was used as the buffer at pH 4.0. From pH 4.5 -5.1 and 9.0 DEPP was used to buffer the solution. MES was used to buffer solutions at pH 6.0 - 6.5. The solution pH was adjusted, using filtered HClO_4 or Et_4NOH , to the desired value before each titration and checked after the titration was complete using an Orion Ross 8103 semimicro electrode and a Corning model 125 pH meter. Temperature was maintained by use of an external waterbath.

Cadmium, nickel, and zinc standard metal stock solutions were made from the crystallized perchlorate salts. Lead perchlorate solution was prepared by reaction of ultrapure PbO with 70% redistilled HClO_4 , without crystallization. Calcium nitrate was used for the standard calcium solution. The copper and cobalt standard solutions were prepared from commercially obtained ultrapure chloride salts. All metal salts used were of $\geq 99.999\%$ purity on the basis of metals to avoid interference from metal contaminants.

MQ1 solutions contained 62.0 μM for all titrations except those with cadmium and copper where the MQ1 concentration was 45.5 μM . The ionic strength of all MQ1 solutions was maintained at 0.05 M using TEAP. Titrations with calcium solution were analyzed at wavelengths unaffected by nitrate absorbance.

DNB solutions ranged from 29.0 mM to 54.7 mM in DNB and ionic strength was maintained at 0.1 M using TEAP or KNO_3 . These solutions were titrated with 3.0 or 4.0 μL aliquots of dilute standardized metal solutions. For titrations using calcium nitrate solution, the nitrate absorbance was subtracted from each spectrum. This was accomplished by adding equal volumes of calcium nitrate to the sample and reference solutions at each point in the titration.

B Potentiometric Studies

Complexation constants for MQ1 and DNB were obtained by analysis of data from the potentiometric titration of unbuffered solutions containing a mixture of the ligand and the metal ion of interest. The ratio of metal ion to ligand concentration was approximately 1:1 in most cases. Standardized stock solutions of ultrapure metal perchlorates, chlorides and nitrates were diluted to obtain the desired metal concentrations. Potentiometric data was collected using the automated titrator system with temperature controlled at 25.0 $^{\circ}\text{C}$ and continual flow of N_2 to evacuate CO_2 . CO_2 and moisture traps were installed on the N_2 gas line to prevent CO_2 contamination from this source. All titrant bases were standardized with KHP and checked for CO_2 contamination.

For MQ1 titrations 5.0 mL samples contained 0.860 mM MQ1, excess acid, metal ion, and sufficient TEAP to maintain ionic strength at 0.05. The sample was titrated with standardized Et₄NOH of approximately 5.0 mM concentration. Titrant solutions had ionic strength maintained at 0.05 with TEAP. Data sets were collected in this manner for the complexation of MQ1 with lead, zinc, nickel, cadmium, calcium and copper.

For DNB, sample solutions contained DNB, in concentrations ranging from 0.1910 mM to 0.3765 mM, metal ion, and sufficient KNO₃ to maintain ionic strength at 0.1 M. The volume of sample used ranged from 5.0-25.0 mL. Titrants were 5 - 15 mM in NaOH with sufficient KNO₃ to maintain ionic strength at 0.1 M. Titrant solutions were made daily by direct dilution of the 50% w/w NaOH concentrate. Data sets were collected for the complexation of DNB with cadmium, lead, copper, zinc, cobalt and nickel.

The autotitrator system provided a data file consisting of the observed pH and volume of base added at each point in the titration. The potentiometric titration data were used by the program BEST⁷ to obtain the stability constants of the metal-ligand complexes. This program also requires accurate values of the initial volume, millimoles of ligand present, millimoles of metal present, the molarity of the titrant base, and millimoles of excess acid added, along with the protonation constants for the ligand.

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Chapter III

RESULTS

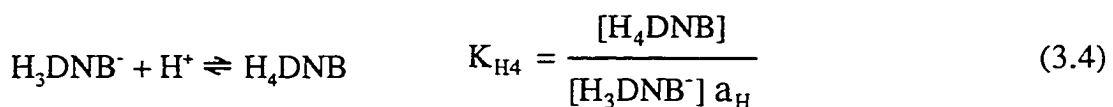
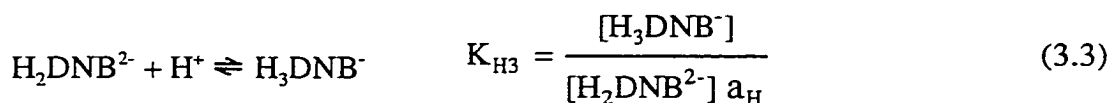
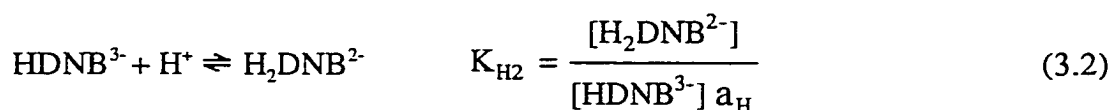
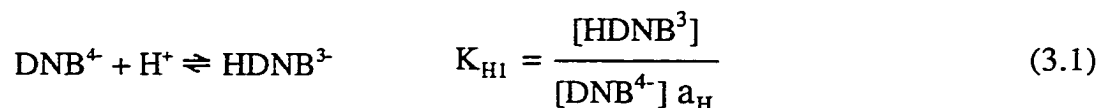
I 5,5'-dinitro-BAPTA (DNB)

A Protonation Constants of DNB

The structure of DNB (Figure I.4) reveals six possible protonation sites on this ligand. Stepwise protonation constants were determined using potentiometric (pH) and spectrophotometric techniques. The fourth, fifth and sixth protonation steps occur at pH values too low to be measured accurately by the potentiometric method. They can be determined spectrophotometrically only if there is a measurable change in absorbance. In the fully deprotonated state the molecule is symmetrical and the sites available for protonation on either side are chemically equal. The neutral form of DNB is obtained after the fourth protonation step. The first three stepwise mixed-mode protonation constants were determined potentiometrically and spectrophotometrically. The fourth stepwise protonation constant was not determined for DNB but is shown as eq 3.4 in the following equations which describe four of the six steps in the stepwise protonation of DNB.

Equations 3.1 to 3.4 list the protonation reaction steps and the stepwise, mixed-mode protonation constant (K_{Ht}) is shown to the right of the reaction. Mixed-mode constants reflect only quantities directly obtainable from the data such as pH, a_{H^+} , and

the ratio of protonated (or complexed) ligand to unprotonated (or uncomplexed) ligand. The mixed-mode protonation constants (eqs 3.1 - 3.4) are related to the thermodynamic constants by the ratio of the activity coefficients for the protonated and unprotonated species. Units for equilibrium constants are M^{-1} but are omitted throughout the text.



The value a_{H} is calculated from the measured pH values according to the equation

$$a_{\text{H}} = 10^{-\text{pH}} \quad (3.5)$$

1 *UV-vis Spectrophotometric Method*

Figure III.1 shows changes in the UV-vis spectra of DNB as the pH is varied. The unprotonated ligand (pH 7.93) has a peak of maximum absorbance, λ_{max} , at 431 nm. As the pH decreases λ_{max} shifts to shorter wavelength. An isosbestic point is observed at 412 nm in spectra from the pH range 4.5 to 7.0. The presence of an isosbestic point is evidence that the two components involved are the only absorbing components present. The isosbestic point is shown in greater detail in Figure III.2. As the pH is decreased further the isosbestic point is lost and λ_{max} decreases in intensity and shifts to shorter wavelength.

Figure III.3 is a semi-log plot of the measured absorbance (A_i) versus a_{H} at a single wavelength. The absorbance changes occur over five orders of hydrogen ion activity indicating more than one protonation process. In the case of DNB these absorbance changes overlap; therefore, distinct regions for each protonation are not observed and a definite number of individual protonation steps is not readily seen. Plots of absorbance versus pH at several different wavelengths were fit to equations derived to calculate two, three, and four protonation constants. The results obtained when fitting only two constants gave visibly poor fits, while those obtained for models with three and four constants fit the observed data more closely. For the plots in which four constants were fit, the fits were good but the errors associated with the refined K values were much larger than those calculated for the third order polynomial used to fit the model with three protonation constants. In some cases the calculated error was larger than the value of the calculated parameter, or negative K values were obtained which have no physical meaning. For these reasons the model based on three protonation

steps was used and the expression used to calculate the fit is given in eq 3.6. This expression defines the observed absorbance, A_i , at any given wavelength as

$$A_i = \frac{A_3 K_{H1} K_{H2} K_{H3} a_H^3 + A_2 K_{H1} K_{H2} a_H^2 + A_1 K_{H1} a_H + A_0}{K_{H1} K_{H2} K_{H3} a_H^3 + K_{H1} K_{H2} a_H^2 + K_{H1} a_H + 1} \quad (3.6)$$

where K_{H1} , K_{H2} , and K_{H3} are the protonation constants defined in eqs 3.1-3.3. A_0 , A_1 , A_2 and A_3 are the limiting absorbance values for the unprotonated, monoprotated, diprotated and triprotonated forms of the ligand, respectively. Estimates for the parameters K_{H1} , K_{H2} , K_{H3} , A_0 , A_1 , A_2 , and A_3 were refined by fitting the A_i and a_H data to eq 3.6. This was accomplished by a curve fitting program, in the commercial program called Kaleidagraph. The curve fitting program uses a nonlinear least-squares approach to minimize the sum of squares of the difference between the experimental values of a_H and values calculated from the initial estimates of the variable parameters. Inset tables show parameters with standard error. Throughout the text, the parameters and errors in the tables contain a fixed number of digits which may overestimate the precision. The program is based on the Levenberg-Marquardt algorithm¹.

The refined values for these parameters calculated from A_i data at 460 nm are displayed in the inset table of Figure III.3. However, as may be inferred from Figure III.1, data at different wavelengths may give different K_H values because one protonation reaction may be marked by a substantially greater change in absorbance than the other reaction steps and, therefore, give reliable information for only the single protonation. This is illustrated by the absorbance behavior observed at 412 nm. Because an isosbestic point occurs at this wavelength, in the pH range of the first two protonation steps, only the third protonation step is observed based on a change in

absorbance. Therefore, only that protonation constant can be calculated. The individual wavelengths at which the most precise fits resulted were 460 nm and 430 nm, when all three protonation constants are being considered. A plot of A_i versus a_H at 412 nm showed a large change in absorbance for the third protonation and was fit to a similar but simpler equation to solve for one protonation constant. Table III.1 lists the values for K_{H1} , K_{H2} , K_{H3} , the absolute error associated with each, and the log values which were obtained at these wavelengths for three separate titrations.

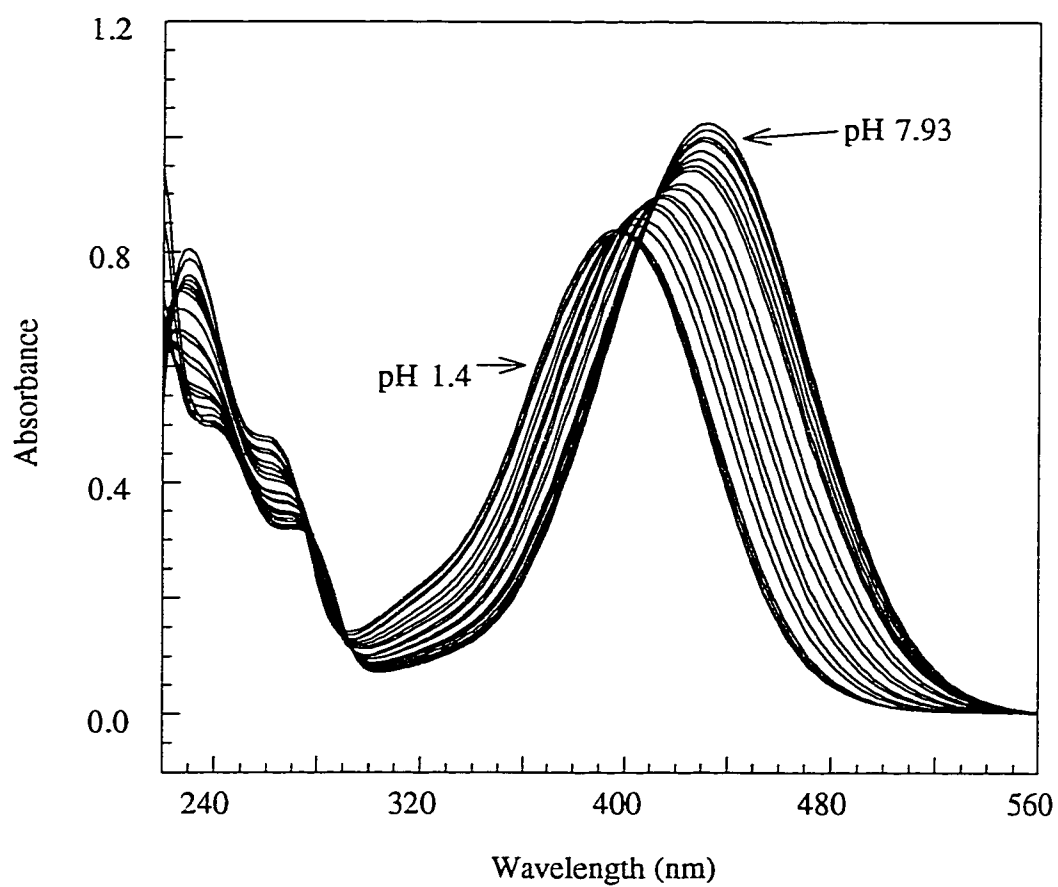


Figure III.1 Spectra from a UV-vis pH titration of DNB at 25.0 °C. The solution contained 40.0 μM DNB, buffered with PIPES, MES, MOPS and DEPP at 5 mM each, 0.5 mM $\text{Na}_2\text{H}_2\text{EDTA}$, and TEAP to maintain ionic strength at 0.1 M.

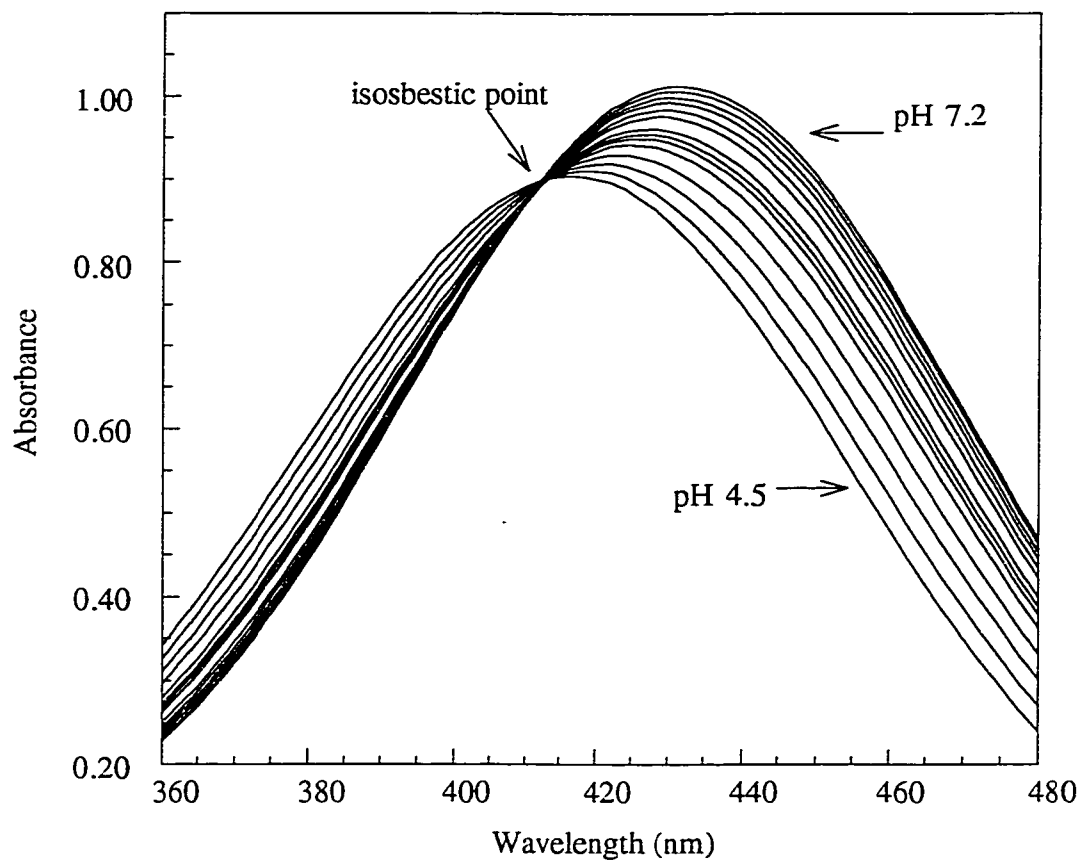


Figure III.2 Expanded view of Figure III.1 containing only the spectra recorded at pH between 4.5 and 7.0 in the region near the isosbestic point at 412 nm.

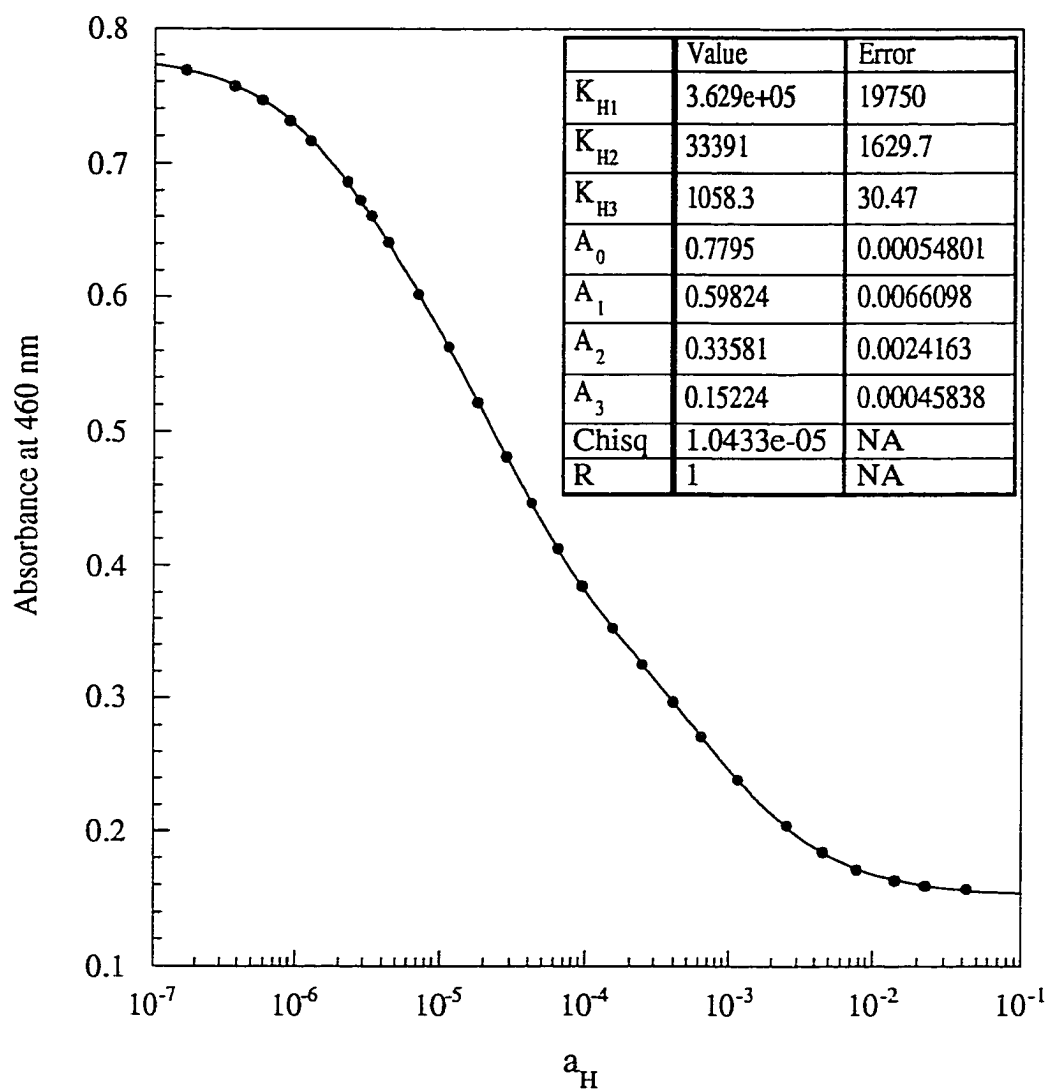


Figure III.3 Plot of absorbance at 460 nm as a function of a_H , taken from spectra in Figure III.1. The data was fit to eq 3.6. The solid line was calculated using the values of the parameters listed in the inset table.

Table III.1 Calculated values for the mixed-mode protonation constants of DNB from spectrophotometric titrations^{a,b}

Exp. No.	λ (nm)	K_{H1} ($\times 10^{-5}$)	K_{H2} ($\times 10^{-4}$)	K_{H3} ($\times 10^{-3}$)	$\log K_{H1}$	$\log K_{H2}$	$\log K_{H3}$
1	460	3.63 ± 0.20	3.39 ± 0.16	1.06 ± 0.03	5.56 ± 0.02	4.52 ± 0.02	3.02 ± 0.01
1	430	4.27 ± 0.32	3.07 ± 0.21	1.00 ± 0.02	5.63 ± 0.03	4.49 ± 0.03	3.00 ± 0.01
1	412			1.08 ± 0.02			3.03 ± 0.01
2	460	2.24 ± 0.45	1.87 ± 0.52	0.68 ± 0.16	5.35 ± 0.09	4.27 ± 0.12	2.83 ± 0.10
3	460	2.25 ± 0.38	1.88 ± 0.41	0.68 ± 0.13	5.35 ± 0.07	4.27 ± 0.09	2.84 ± 0.08
3	430	2.20 ± 0.40	1.23 ± 0.38	0.65 ± 0.10	5.34 ± 0.08	4.09 ± 0.13	2.81 ± 0.07

^a $25.0 \pm 0.1^\circ\text{C}$, ionic strength = 0.1 M with TEAP. ^b Uncertainty expressed as standard error.

2 Potentiometric Method

The automated titration system employed in the potentiometric studies provides data in terms of pH observed and volume of titrant added. These data were then analyzed using the program PKAS created by Motekaitis and Martell.¹ The program PKAS calculates protonation constants from pH titration data by minimizing the difference in the squares of the weighted pH residuals. The equations used to calculate, this quantity, called sigma, are

$$\text{sigma} = \left(\frac{U}{N} \right)^{1/2} \quad (3.7)$$

where

$$U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2 \quad (3.8a)$$

$$w = 1/(p[H]_{i+1} - p[H]_{i-1})^2 \quad (3.8b)$$

and

$$N = \sum w \quad (3.8c)$$

Values of $p[H]_{\text{calc}}$ are calculated for each titration point using estimates of K_{Hi} and mass balance equations for total ligand and total hydrogen ion (acid added) concentrations. The latter are determined using known values of the initial mmoles ligand, mmoles added acid (or base) present in the initial volume, and formality of the titrant base added. Due to limited solubility of the neutral form of the ligand (H_4DNB) in aqueous solution, one to two equivalents of NaOH were added to the ligand solution before the titration was started. Therefore, only two, or in one case three, of the protonation constants could be obtained from analysis of the titration data. Figure III.4 shows a representative plot of the experimental titration curve overlaid with the curve calculated by the PKAS program. Data from two of the titrations were also analyzed using the program BEST also created by Motekaitis and Martell.¹ The program BEST uses a similar algorithm to refine the data but gives values in terms of overall constants, β , as opposed to the stepwise constants given by PKAS. Overall constants are values for the total reaction and are related to the

stepwise constants in a cumulative manner, such that $\beta_n = K_1 K_2 \dots K_n$. Further explanation of β values is given in section I.C.2 of this chapter. The values for $\log K_{\text{H}}$ obtained from the PKAS program, and the $\log \beta$ values from the BEST program for the different titrations are shown in Table III.2.

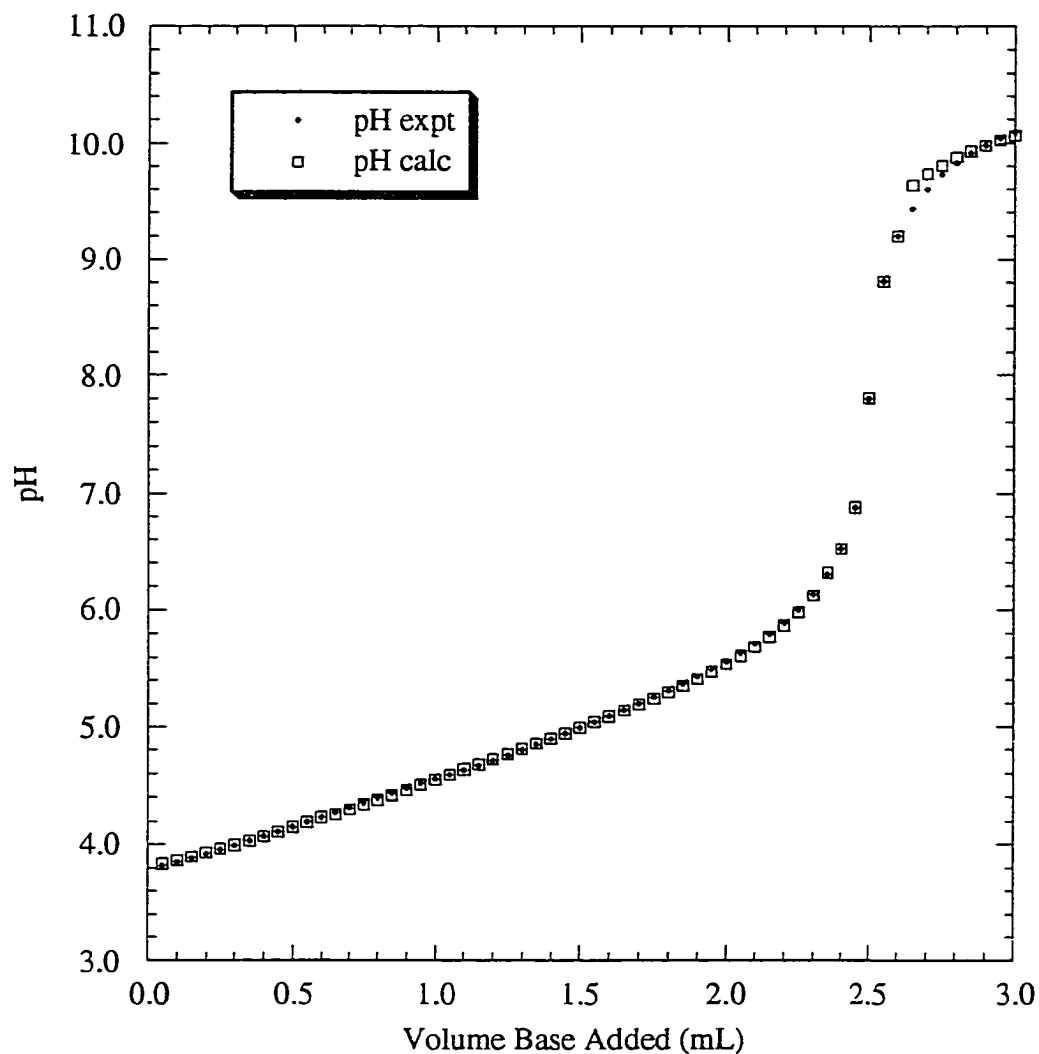


Figure III.4 Experimental titration curve obtained from the titration of 25.0 mL of 0.376 mM DNB with 9.85 mM NaOH at 25.0 °C and 0.1 M ionic strength (KNO_3). The experimental curve is overlaid with the theoretical curve calculated using the known values of ligand and base concentrations and the resolved protonation constants.

Table III.2 Log K values of DNB calculated using PKAS and BEST^a

run	method	$\log K_{H1}^b$	$\log K_{H2}^b$	$\log \beta_{H2}^c$	$\log K_{H3}^b$	$\log \beta_{H3}^d$	sigma ^e
1	PKAS	5.36 ± 0.05	4.53 ± 0.01		3.45 ± 0.01		0.035
1	BEST	5.40	(4.45) ^c	9.95	(3.45) ^c	13.40	0.017
2	PKAS	5.25 ± 0.02	4.46 ± 0.005				0.015
2	BEST	5.24	(4.60) ^c	9.84			0.020
3	PKAS	5.40 ± 0.01	4.48 ± 0.003		3.46 ± 0.001		0.005
4	PKAS	5.18 ± 0.03	4.58 ± 0.02		3.30 ± 0.01		0.017

^a 25.0 °C, ionic strength = 0.1 M (TEAP). ^b Uncertainty expressed as standard deviation in pH for individual segments of titration curve. $\text{sigma} = (U/N)^{1/2}$; as defined in eq 3.7 and 3.8. ^c $\log \beta = \sum \log K_{Hi} \cdot (i=1-n)$. ^d $\log K_{Hi} = \log \beta_{Hi} - \log \beta_{Hi-1}$. ^e Uncertainty in standard deviation of pH for entire titration.

B Stoichiometry and Spectral Properties of DNB Complexes at pH 7.0

To determine spectral characteristics and stoichiometry of DNB with various metals, UV-vis spectrophotometric titrations of DNB with cadmium, lead, copper, zinc, cobalt, nickel, and calcium were done at pH 7.0. This pH is in the physiological range and, as shown below, is sufficient to cause almost complete deprotonation of DNB. Taking $\log K_{H1} = 5.38$ as the average from the potentiometric and spectrophotometric titrations, the ratio of $[\text{HDNB}^3]$ to $[\text{DNB}^4]$ at a given pH may be calculated by rearranging eq 3.1.

$$\frac{[\text{HDNB}^{3-}]}{[\text{DNB}^{4-}]} = K_{\text{H1}} a_{\text{H}} = (2.4 \times 10^5)(1.0 \times 10^{-7}) = 0.024 \quad (3.9)$$

Thus, at pH 7.0, 97.6% of the total DNB concentration is in the DNB^{4-} form. Similar calculations show that the percentage of ligand in the $\text{H}_2\text{DNB}^{2-}$ and H_3DNB^- forms is much less than 0.01% at pH 7.0. This implies that interference due to side reactions of the ligand with protons will be minimal resulting in a low sensitivity to minor pH changes in measurements made at this pH.

For the spectrophotometric titrations at pH 7.0 the DNB solution was 0.041 mM with ionic strength maintained at 0.1 M with KNO_3 . The temperature was controlled at 25.0 °C. The cadmium, lead, copper, zinc and nickel solutions were prepared from the perchlorate salts. Cobalt solutions were prepared from the chloride salt. For calcium solutions the nitrate salt was used and the absorbance of the nitrate was subtracted out by titrating the reference solution and recording that absorbance as the new reference at each measurement. The resulting spectra represent absorbance as a function of metal concentration. From the spectra it was possible to determine wavelengths of maximum absorbance (λ_{max}), molar absorptivity at λ_{max} (ϵ_{max}) and isosbestic points, observed during complex formation, for most of the complexes. ϵ_{max} was calculated according to the Beer-Lambert Law as shown in eq 3.10. In this equation b refers to the pathlength of the cell, which was 1 cm in all instances, and c was the molar concentration of the complex, which was assumed to be the same as the initial concentration of the ligand.

$$\epsilon_{\lambda} = \frac{A_{\lambda}}{bc} \quad (3.10)$$

The characteristics mentioned above are listed in Table III.3.

The concentration of metal required to complex all of the DNB present is an indication of the ratio in which DNB and the metal combine. In odd numbered Figures III.5 through III.17 the spectra obtained are shown. In even numbered Figures III.6 through III.18 plots of the fraction of the total absorbance change versus the ratio of metal to ligand are shown. These plots are constructed from spectral data and concentration data in the following manner. The absorbance value, at a particular wavelength, at which further addition of metal causes no further change is subtracted from the absorbance of DNB, at that same wavelength, before any metal has been added. In the case of calcium, nickel and cobalt the total absorbance change if all the ligand were bound by the metal ions was estimated by curve fitting the absorbance data as a function of metal added and extrapolating to the point that the curve shows saturation behavior (the absorbance value no longer changes). This value represents the total change in absorbance at that wavelength for complex formation. The absorbance change measured for each of the increasing concentrations of added metal are divided by the total change in absorbance to give the fraction of the total change associated with each concentration and are plotted on the y axis. The total concentration of metal at each addition of an aliquot is divided by the total concentration of ligand in the titration solution. This is the ratio of total metal to total DNB and is plotted on the x axis. In each plot a dotted line is drawn at the 1:1 ratio of metal to ligand and a sloped line is drawn from the origin to the point representing total absorbance change (1.0 on the y axis) and 1:1 concentration ratio (1.0 on the x axis). This line traces the linear change

that would occur if the ligand were being bound with the metal in an analytical manner. In other words, if all the metal at each aliquot was bound by the ligand the absorbance change resulting would follow this line exactly. If the 1:1 complexation reaction is the only reaction taking place no further change in absorbance will be seen with the addition of excess metal ion. The horizontal dotted line represents the lack of change in absorbance observed under the described conditions. If the metal ions are bound by the ligand quantitatively the stoichiometry of the reaction can be inferred from the data.

As an example, Figure III.6 shows the behavior of cadmium as it reacts with DNB. The fraction of the total absorbance change associated with each aliquot is as would be expected for a quantitatively bound ion and traces the sloped line to the equimolar total absorbance change mark exactly. Further addition of cadmium causes no further change in absorbance and follows the horizontal dotted line for the rest of the titration. Therefore, it is reasonable to assume that DNB binds cadmium in a 1:1 ratio. Similar behavior was observed in the cases of lead, copper and zinc. Zinc showed a slight deviation from quantitative binding, being only about 95% bound at the stoichiometric ratio, but followed the pattern closely enough to indicate formation of a 1:1 complex. The case of copper is interesting in its complexity. The fraction of absorbance plot indicates 1:1 binding and no further reaction, but closer inspection of the spectra contradicts this scenario. Immediately following the addition of one equivalent of copper ion the isosbestic point at 367 nm is no longer maintained indicating a further reaction of the CuDNB complex but resulting in no noticeable change in absorbance at the wavelength used in the fraction of absorbance plot. Figures IV.4 a) and b) highlight the changes at this isosbestic point. These figures are in the discussion section along with further discussion of this anomaly.

The fraction of absorbance change plots for cobalt, nickel and calcium show substantial deviation from the quantitative formation of a 1:1 metal DNB complex. In

the case of cobalt it seems that approximately 75% of the cobalt is bound at the 1:1 stoichiometric mark. Nickel is indicated to be less than 15% bound at this mark and calcium less than 5%. This is an indication of a lack of affinity for these cations by DNB. The behavior shown does not indicate other stoichiometries for these complexes or rule out the formation of the one to one complex exclusively.

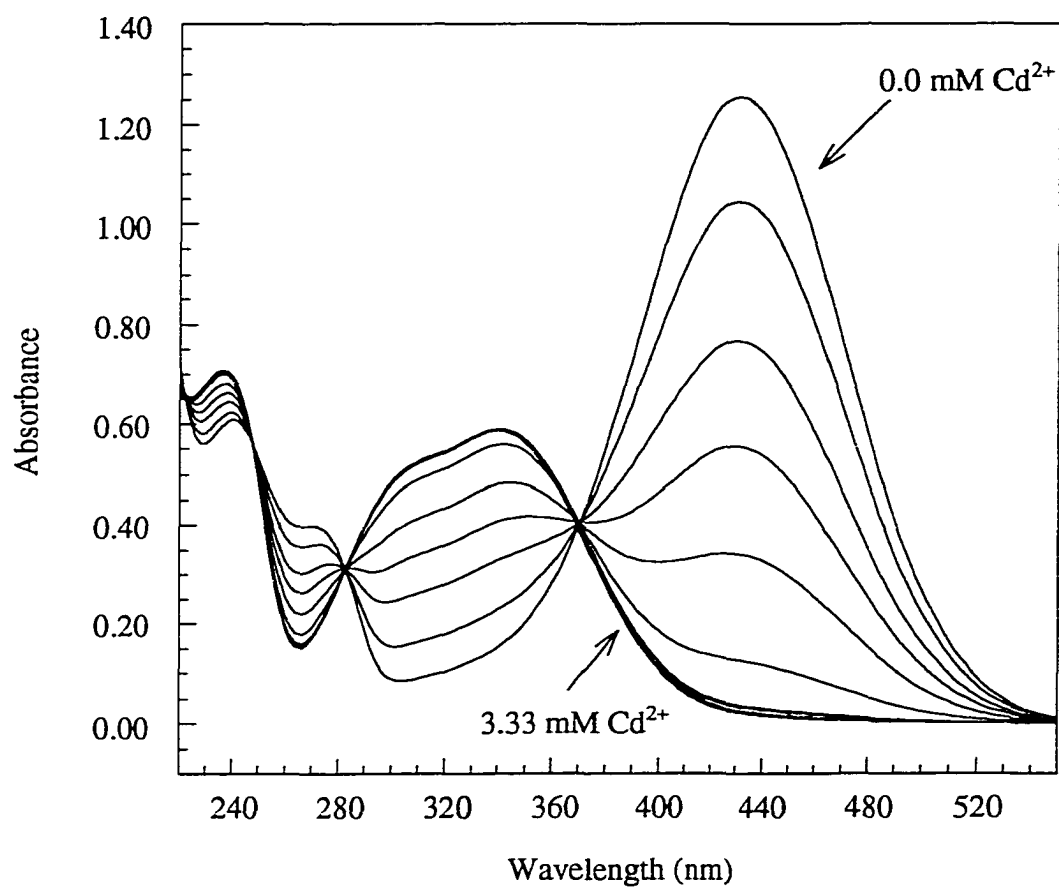


Figure III.5 Spectra from the titration of DNB with cadmium at 25.0 °C. 4.0 μL aliquots of 4.18 mM Cd^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB, buffered to pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

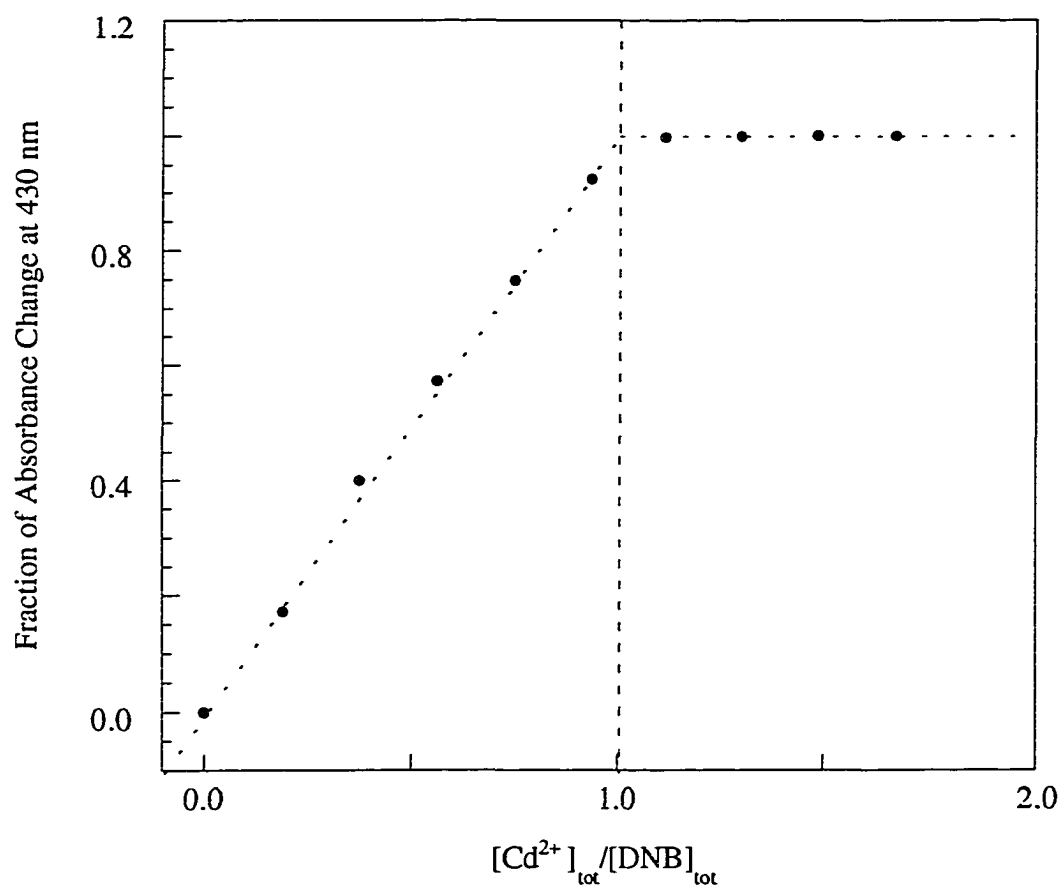


Figure III.6 Plot of the fraction of the absorbance change resulting from each aliquot of cadmium added versus the ratio of total cadmium to total DNB.

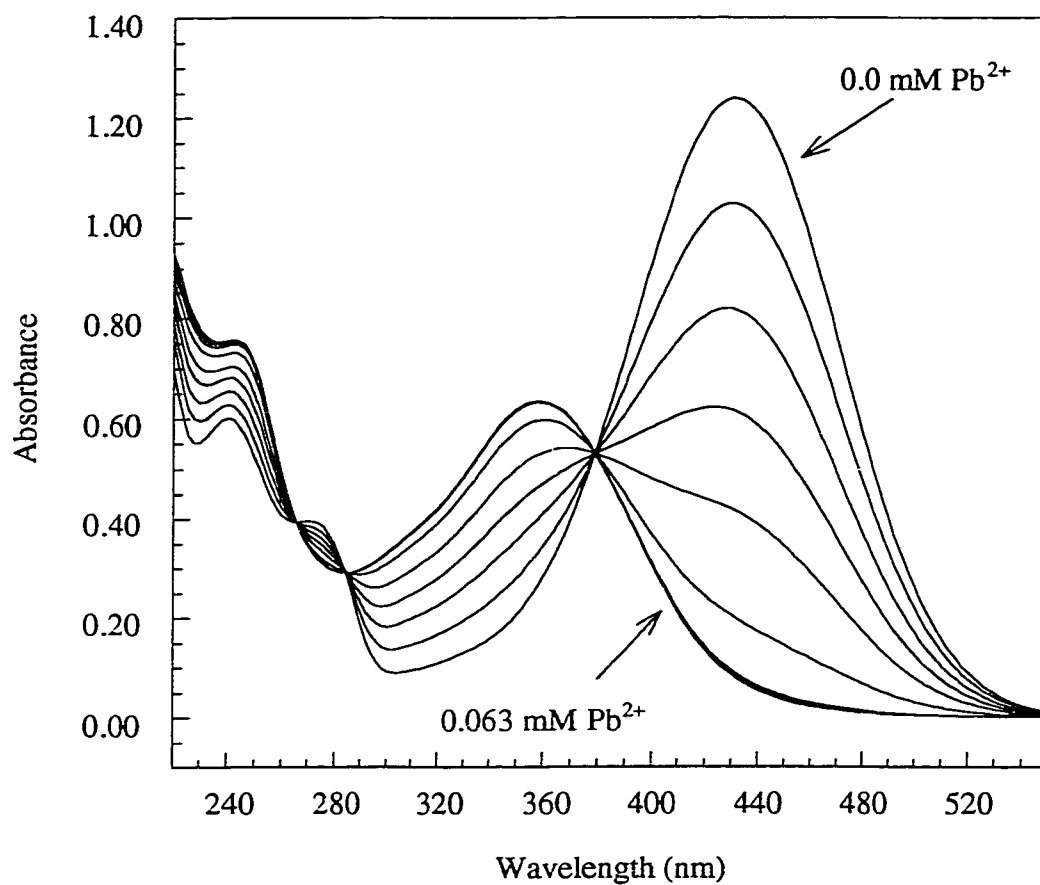


Figure III.7 Spectra from the titration of DNB with lead at 25.0 °C. 4.0 μL aliquots of 4.96 mM Pb^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB, buffered at pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

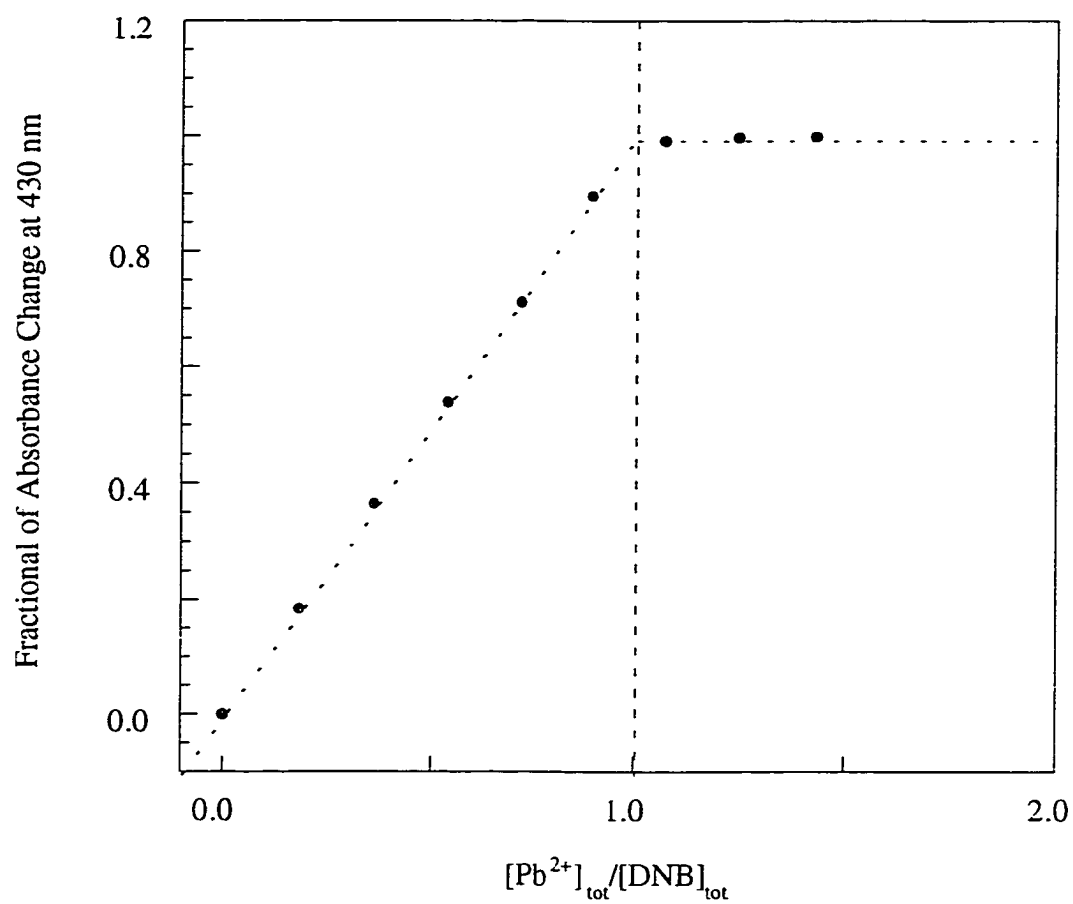


Figure III.8 Plot of the fraction of the absorbance change resulting from each aliquot of lead added versus the ratio of total lead to total DNB.

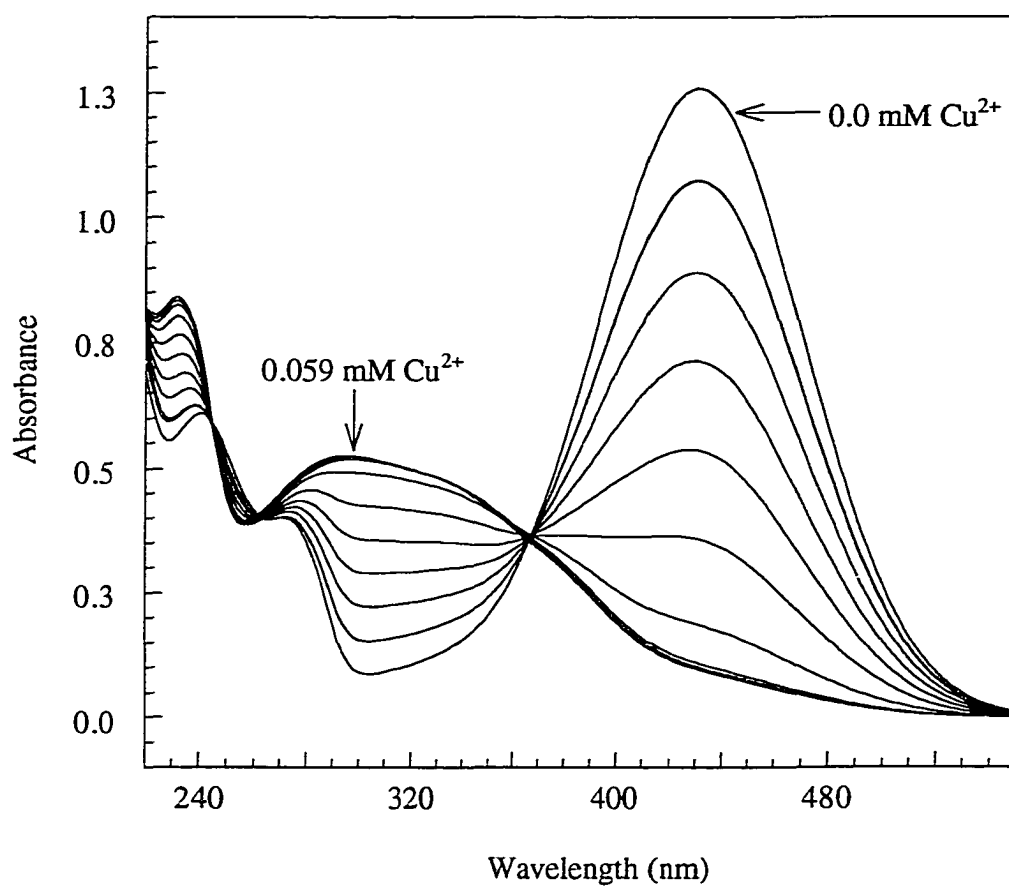


Figure III.9 Spectra from the titration DNB with copper at 25.0 °C. 4.0 μL aliquots of 3.75 mM Cu^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB, buffered at pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

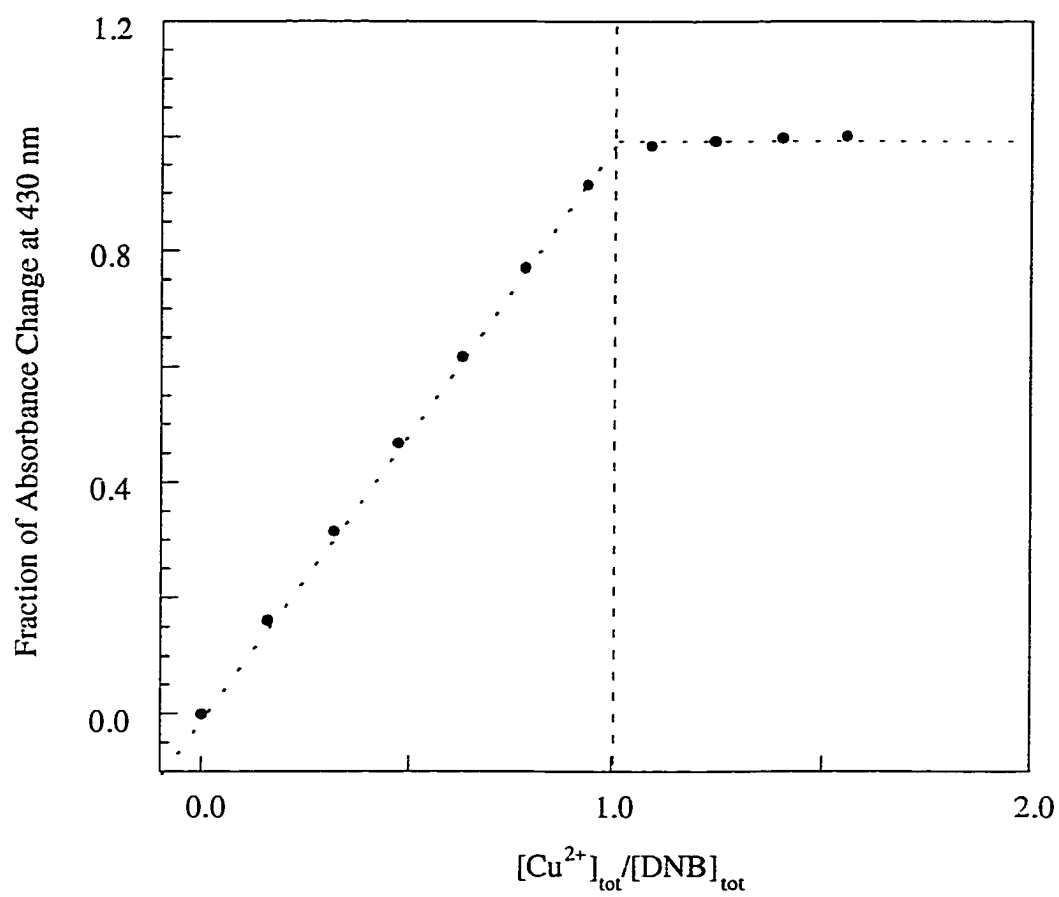


Figure III.10 Plot of the fraction of the absorbance change resulting from each aliquot of copper added versus the ratio of total copper to total DNB.

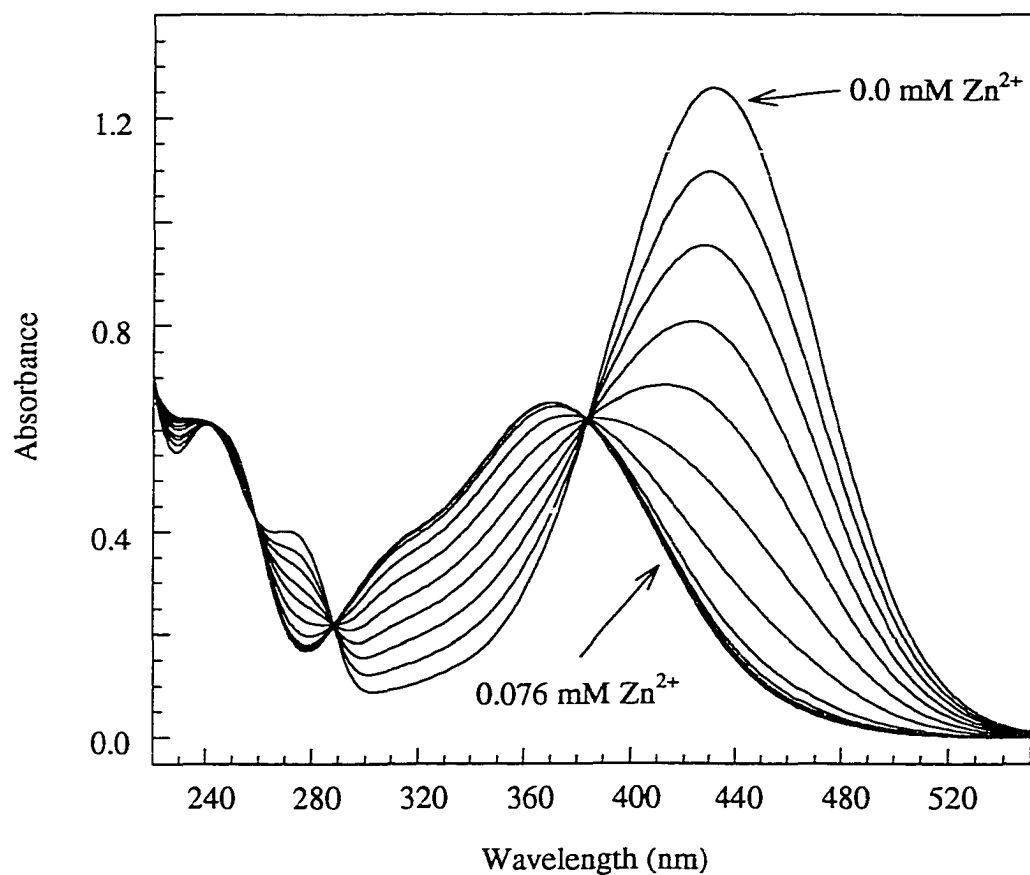


Figure III.11 Spectra from the titration of DNB with zinc at 25.0 °C. 4.0 μL aliquots of 3.75 mM Zn^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB, buffered at pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

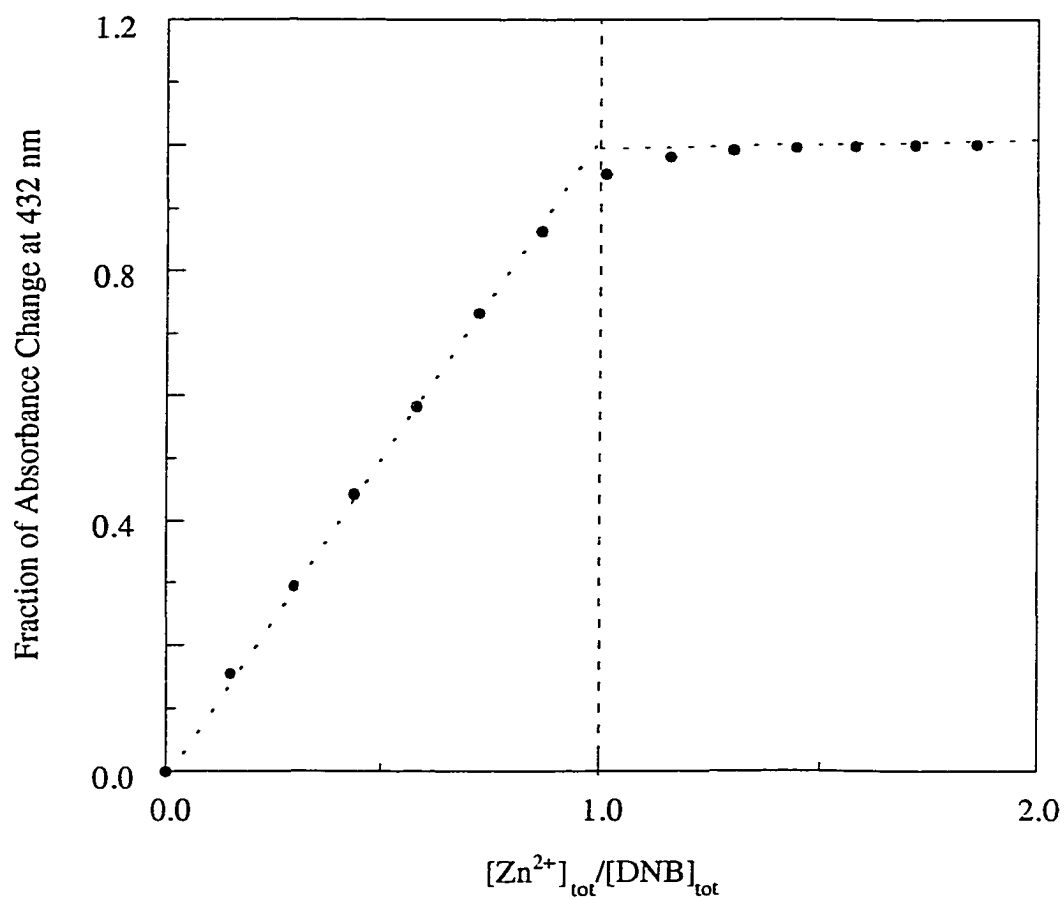


Figure III.12 Plot of the fraction of the absorbance change resulting from each aliquot of zinc added versus the ratio of total zinc to total DNB.

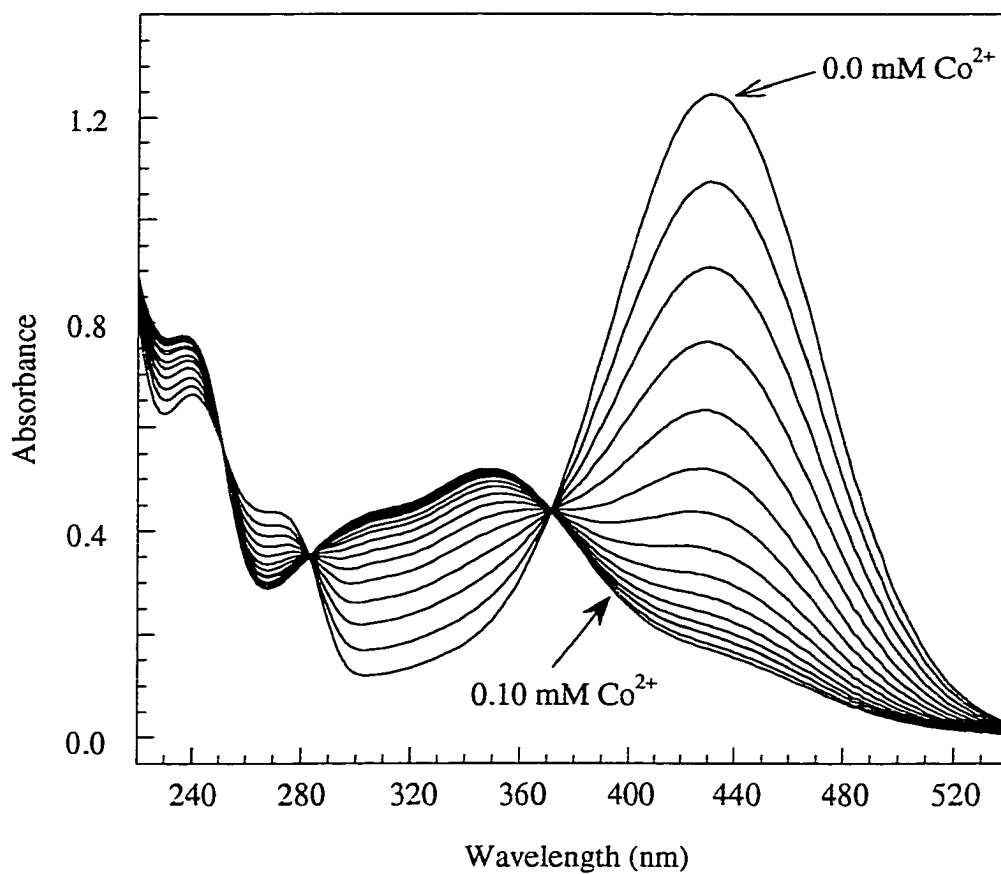


Figure III.13 Spectra from the titration of DNB with cobalt at 25.0 °C. 4.0 mL aliquots of 4.00 μM Co^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB buffered at pH 7.0 with 5.0 mM PIPES at an ionic strength of 0.10 M (KNO_3).

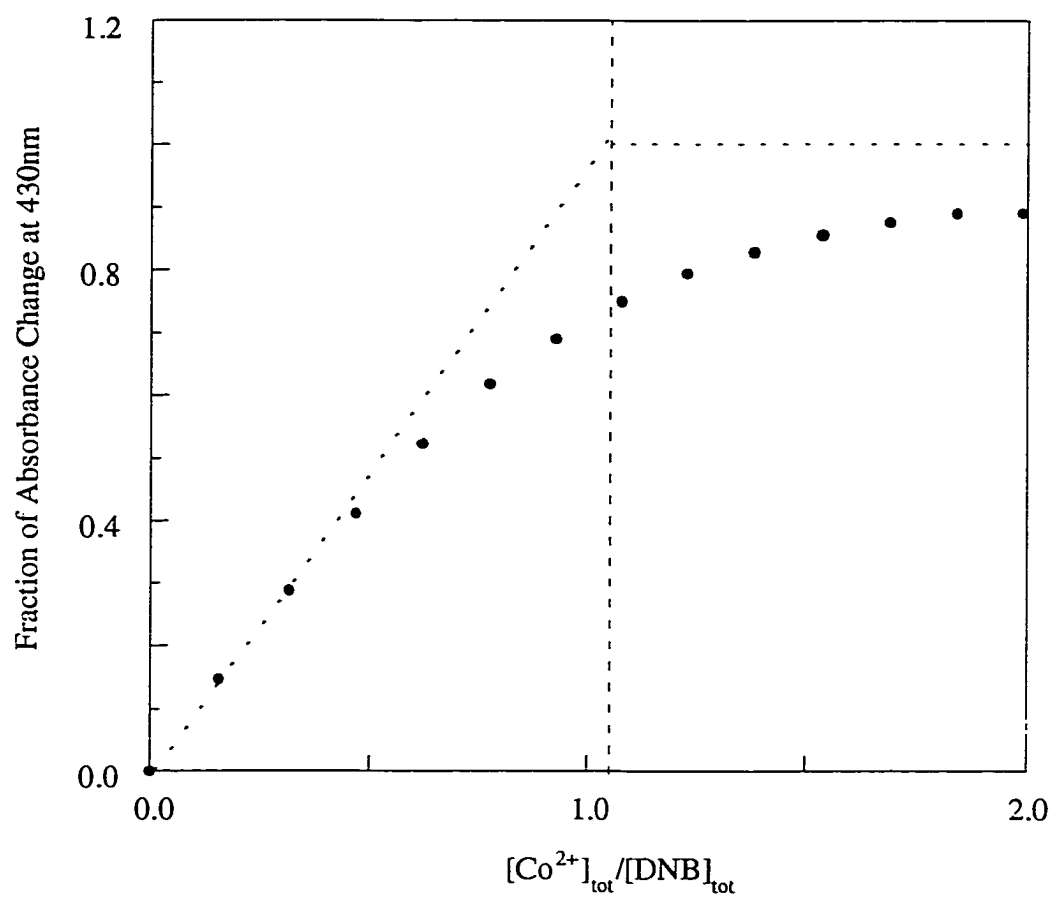


Figure III.14 Plot of the fraction of the absorbance change resulting from each aliquot of cobalt added versus the ratio of total cobalt to total DNB.

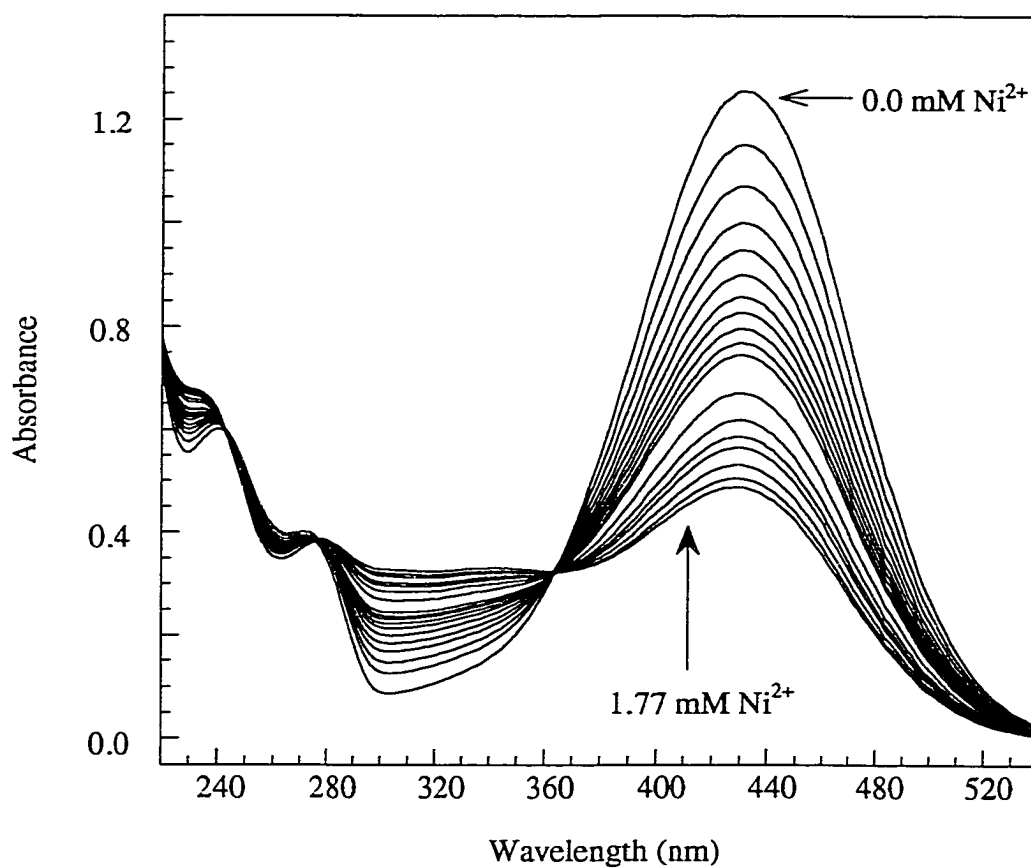


Figure III.15 Spectra from the titration of DNB with nickel at 25.0 °C. 4.0 μL aliquots of 20.0 mM, 93.05 mM, and 186.1 mM Ni^{2+} were used to titrate 2.5 mL of a solution of 0.041 μM DNB, buffered at pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

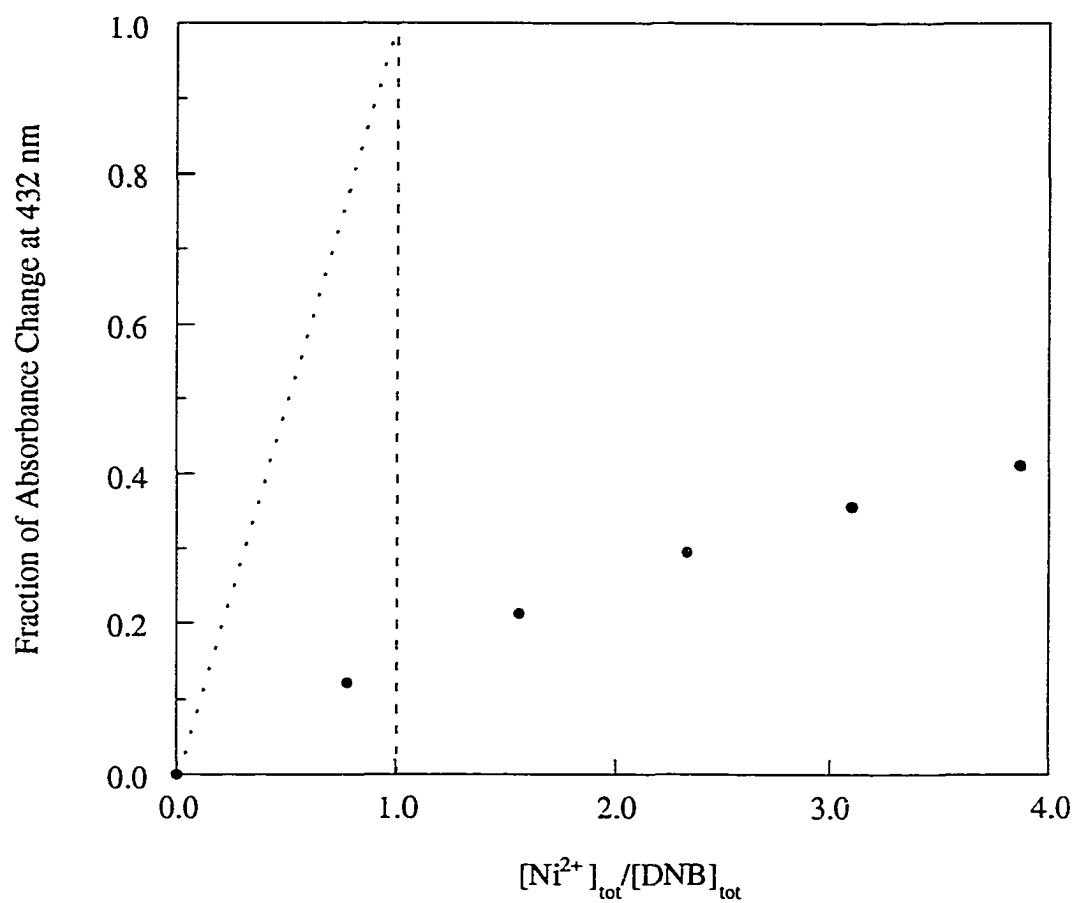


Figure III.16 Plot of the fraction of the absorbance change resulting from each aliquot of nickel added versus the ratio of total nickel to total DNB.

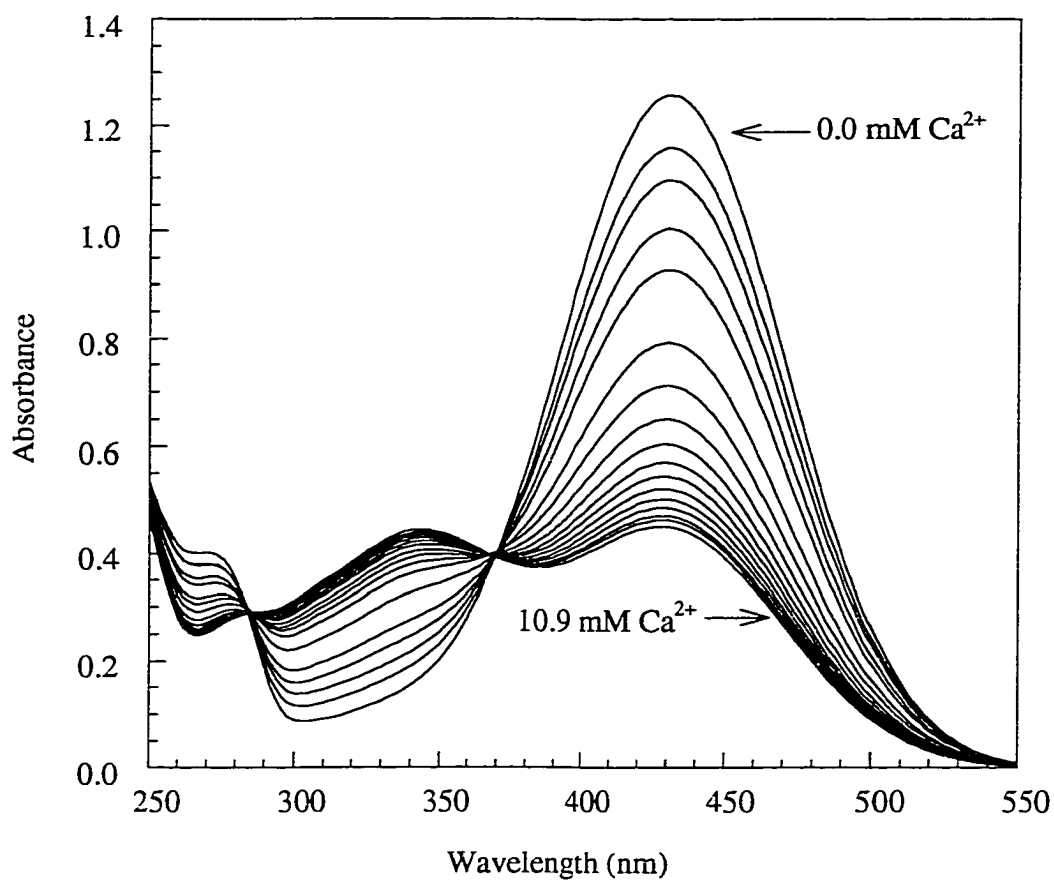


Figure III.17 Spectra from the titration of DNB with calcium at 25.0 °C. 4.0 μL aliquots of 7.38 mM, 29.52 mM, 0.1095 M and 2.651 M Ca^{2+} were used to titrate 2.5 mL of a solution containing 0.041 μM DNB, buffered at pH 7.0 with 5.0 mM PIPES, at an ionic strength of 0.10 M (KNO_3).

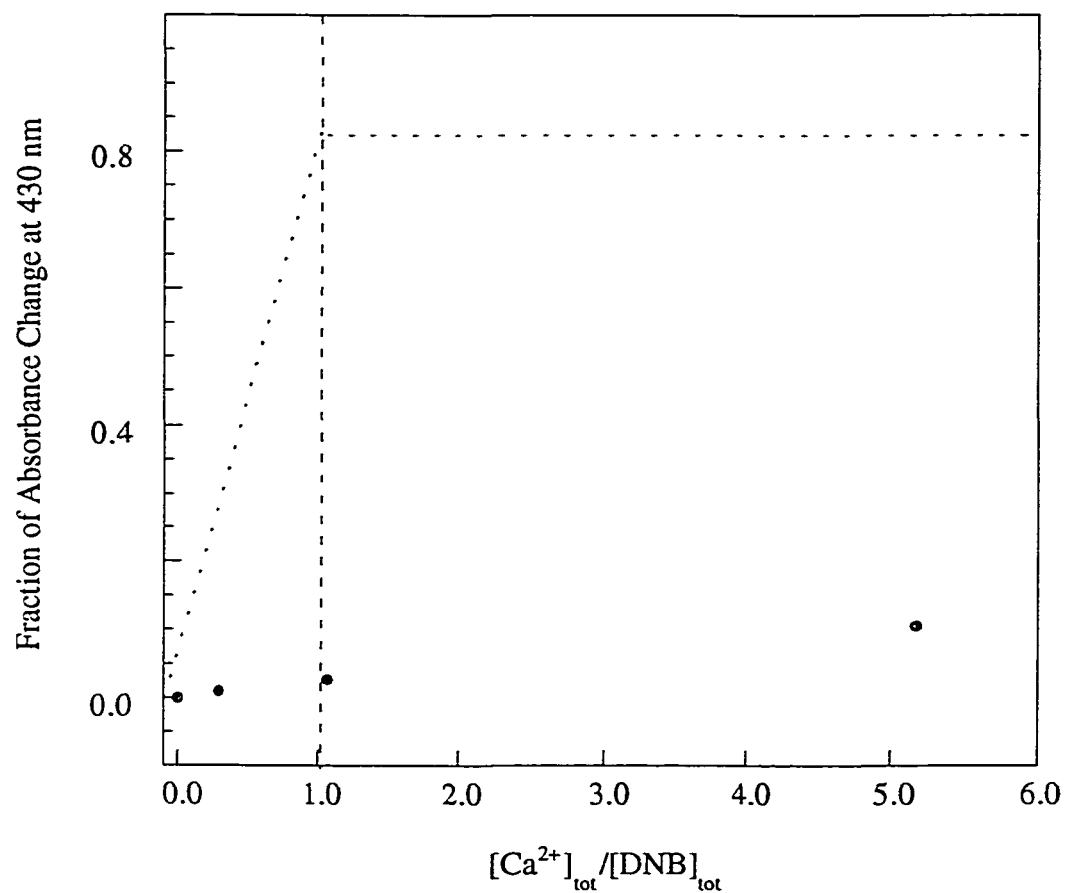


Figure III.18 Plot of the fraction of the absorbance change resulting from initial aliquots of calcium added versus the ratio of total calcium to total DNB.

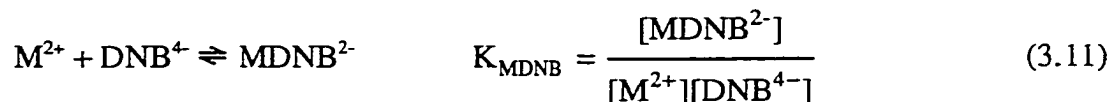
Table III.3 Metal to ligand stoichiometry and spectral properties for DNB complexes^a

Complex	Stoichiometry (M:L)	$\lambda_{\max}$ (nm)	$\epsilon_{\lambda_{\max}}$	Isosbestic points (nm)
DNB ⁴⁻		430	30,600	246, 282, 370
CdDNB	1:1	340	14,400	267, 285, 368
PbDNB	1:1	358	15,500	245, 262, 378
CuDNB	1:1, (2:1) ^b	294	12,900	240, 258, 368
ZnDNB	1:1	370	15,900	240, 258, 290, 386
CoDNB		348	12,700	252, 282, 370
NiDNB				242, 276, 362
CaDNB		344	11,951	285, 368

^a 25.0 °C, ionic strength 0.1 M (TEAP), pH 7.0. ^b The formation of a 2:1 complex is suggested by the loss of the isosbestic point at high [Cu²⁺].

C Complex Formation Constants of DNB

The formation of one to one complexes of DNB with various divalent metals is depicted by the following reaction equation and equilibrium expression



The stoichiometry in eq 3.11 is indicated from the UV-vis experiments at pH 7.0 (Figures III.5 - III.18). Side reactions of DNB and the metal-DNB complexes with protons as well as alternative stoichiometries for the metal-DNB complexes, may complicate the analysis. Therefore, data from both UV-vis spectrophotometric titrations and potentiometric titrations are needed to indicate directions to take in further analysis of the data.

1 UV-vis spectrophotometric determination of complexation constants

Odd numbered figures, from Figure III.19 through III.27, and Figures III.30 and III.33 are spectra resulting from the titration of DNB with standard solutions of metal salts. Specific conditions of individual experiments are indicated in the captions below each figure. Even numbered Figures III.20 through III.28, and Figures III.29, III.31, III.32, III.33 and III.35 are the corresponding plots of absorbance at a particular wavelength versus free metal concentration. The A_λ versus $[M^{2+}]$ data from these plots are fit to eq 3.13 with the parameters and calculated values displayed in the inset tables. The equilibrium expression defined in eq 3.11 is based on the total initial concentration

of the ligand being in the DNB^{4-} form and the total equilibrium concentration of the complexed ligand being in the MDNB^{2-} form. Due to side reactions of these species with protons this may or may not be an acceptable assumption at a given pH. Equation 3.9 showed that, at pH 7.0, 97.6% of the total ligand was present as DNB^{4-} . As the pH decreases the percentage of ligand in a protonated form increases. Therefore, the fraction of the free ligand concentration present as DNB^{4-} is dependent upon pH. Similar protonation side reactions of the metal-ligand complex may result in interference as well. The pH dependence for each species is signified by a prime sign. Thus, the conditional (pH dependent) stability constant is denoted as K''_{MDNB} and is defined by the relationship,

$$K''_{\text{MDNB}} = \frac{[\text{MDNB}]'}{[\text{M}][\text{DNB}]} \quad (3.12)$$

where $[\text{MDNB}]' = [\text{MDNB}^{2-}] + \Sigma[\text{MH}_i\text{DNB}^{i-2}]$, $[\text{DNB}]' = [\text{DNB}^{4-}] + [\text{HDNB}^{3-}] + [\text{H}_2\text{DNB}^{2-}] + [\text{H}_3\text{DNB}] + [\text{H}_4\text{DNB}]$, and $[\text{M}]'$ is the free metal ion concentration. For each titration the absorbance values, A_i , are related to $[\text{M}]'$ by the equation,

$$A_i = \frac{A_1 K''_{\text{MDNB}} [\text{M}]' + A_0}{K''_{\text{MDNB}} [\text{M}]' + 1} \quad (3.13)$$

where A_1 and A_0 are the limiting absorbance values for the metal-ligand complex and the uncomplexed ligand, respectively. Initial estimates for calculated parameters, A_1 , A_0 , and K''_{MDNB} must be included. Estimates of A_1 and A_0 are obtained from the spectra.

Initial estimates of K''_{MDNB} are obtained from fractional absorbance versus metal:ligand ratio plots. The estimate of K''_{MDNB} is most easily calculated at the point where total metal (C_M) and total ligand (C_L) concentrations are equal. The fraction of the total absorbance change which has occurred at this point is linearly related to the fraction of the total ligand that has been complexed so that, if f equals the fraction of the total absorbance change then $[\text{ML}]' = f C_L$. The concentrations of uncomplexed reactants $[\text{M}]'$ and $[\text{L}]'$ are then determined by subtracting $[\text{ML}]'$ from C_L (or C_M). The estimated concentrations can then be used in eq 3.12 to calculate an estimate for K''_{ML} . The measured absorbance, A_i , is plotted versus the total metal concentration, C_M , and the data is fit to equation 3.13 to obtain a new value for K''_{MDNB} . The calculated K''_{MDNB} value is then used to calculate the concentration of the metal-ligand complex at each step in the titration, using the following equation

$$[\text{MDNB}]' = \frac{K''_{\text{ML}}(C_{\text{DNB}} + C_M) + 1 - \sqrt{(K''_{\text{ML}}(C_{\text{DNB}} + C_M) + 1)^2 - 4K''_{\text{ML}}^2 C_{\text{DNB}} C_M}}{2K''_{\text{ML}}} \quad (3.14)$$

where C_{DNB} is the total concentration of DNB. The new calculated values of $[\text{MDNB}]'$ are then subtracted from the total metal concentration to get a new set of free metal concentrations and the curve fitting process is repeated using the estimated values of K''_{MDNB} , A_0 , and A_1 from the previous refinement cycle. These calculations continue until the resulting parameters (K''_{ML} , A_0 , A_1) are constant within 2% between iterations.

Table III.5 shows the data from the iterative process with lead at pH 3.5 to illustrate how the free metal concentrations change through the successive iterations and Figure III.20 shows the resulting curve fits from the initial $[\text{Pb}]'$ values, and the $[\text{Pb}]'$ values for the 2nd, 7th and 8th iteration cycles.

Figures III.21 to III.35 show titration spectra and plots of absorbance vs $[\text{M}^{2+}]$ for each of the other metal ions. In the latter, the values of the parameters listed in the inset tables and the corresponding calculated curve are from the final cycle for the refinement process described above.

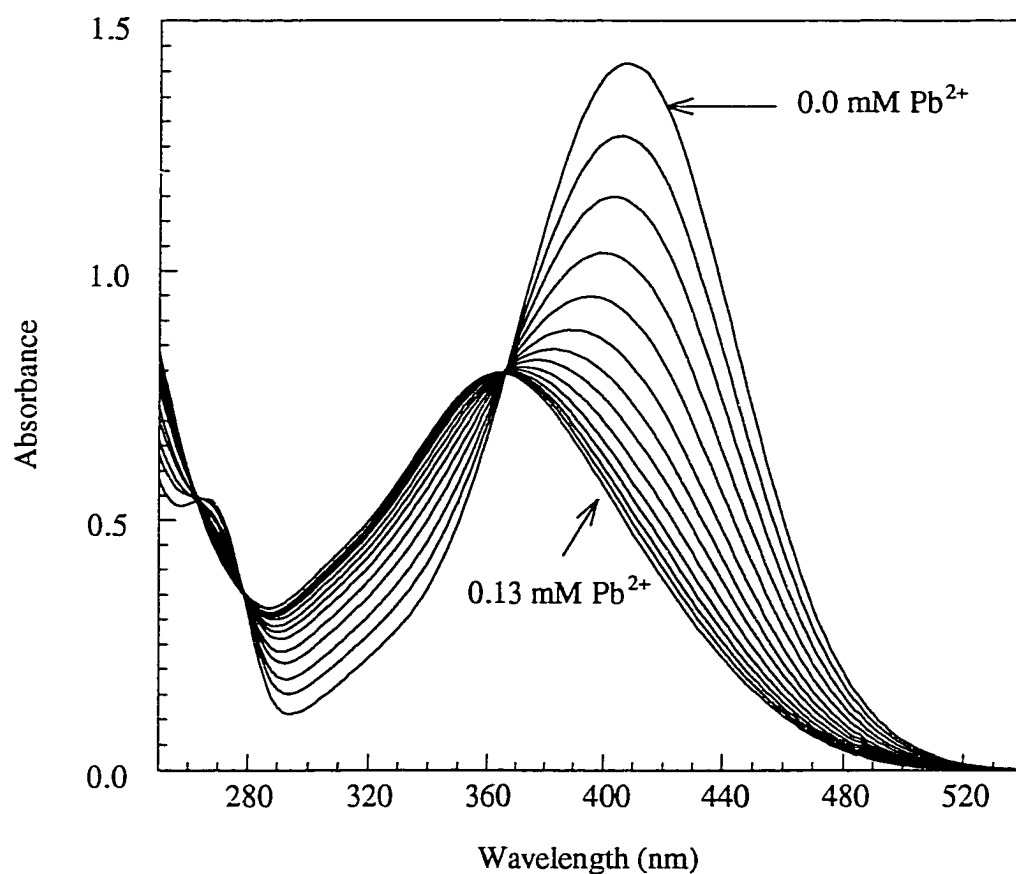


Figure III.19 Spectra from the titration of DNB with lead at pH 3.5. 4.0 μL aliquots of 6.375 mM Pb^{2+} were used to titrate 2.5 mL of 54.6 μM DNB solution which was buffered at pH 3.5 with 5.0 mM PIPES. The titration was done at 25.0 $^{\circ}\text{C}$ and at 0.10 M ionic strength (KNO_3).

Table III.4 Data from the successive approximation of free lead ion in the titration of 54.6 μM DNB at pH 3.5^{a,b,c}

$[\text{Pb}^{2+}]_{\text{total}}$	Abs (430nm)	Pb2	Pb3	Pb4	Pb5	Pb6	Pb7	Pb8	Pb9
0.00	1.1717	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10.20	1.0326	6.84	5.79	5.36	5.17	5.09	5.05	5.03	5.02
20.30	0.9103	14.00	12.10	11.30	11.00	10.80	10.80	10.70	10.70
30.50	0.7927	21.50	18.90	17.80	17.40	17.20	17.10	17.00	17.00
40.50	0.6925	29.30	26.10	24.90	24.30	24.10	24.00	23.90	23.90
50.60	0.6037	37.20	33.70	32.30	31.70	31.40	31.30	31.30	31.20
60.60	0.5362	45.40	41.60	40.10	39.50	39.20	39.10	39.00	39.00
70.60	0.4825	53.80	49.80	48.20	47.60	47.30	47.10	47.10	47.10
80.60	0.4327	62.30	58.20	56.60	55.90	55.60	55.50	55.40	55.40
90.50	0.3916	70.90	66.70	65.10	64.50	64.20	64.10	64.00	64.00
100.00	0.3622	79.60	75.40	73.80	73.20	72.90	72.80	72.70	72.70
110.00	0.3367	88.40	84.20	82.70	82.00	81.80	81.60	81.60	81.50
120.00	0.3171	97.20	93.10	91.60	91.00	90.70	90.60	90.50	90.50
130.00	0.2980	106.00	102.00	101.00	100.00	99.70	99.60	99.60	99.50

^a 25.0 °C, ionic strength 0.1M (TEAP). ^b All concentrations listed in μM units. ^c Heading numbers (e.g. Pb2) denote the number of iterations completed.

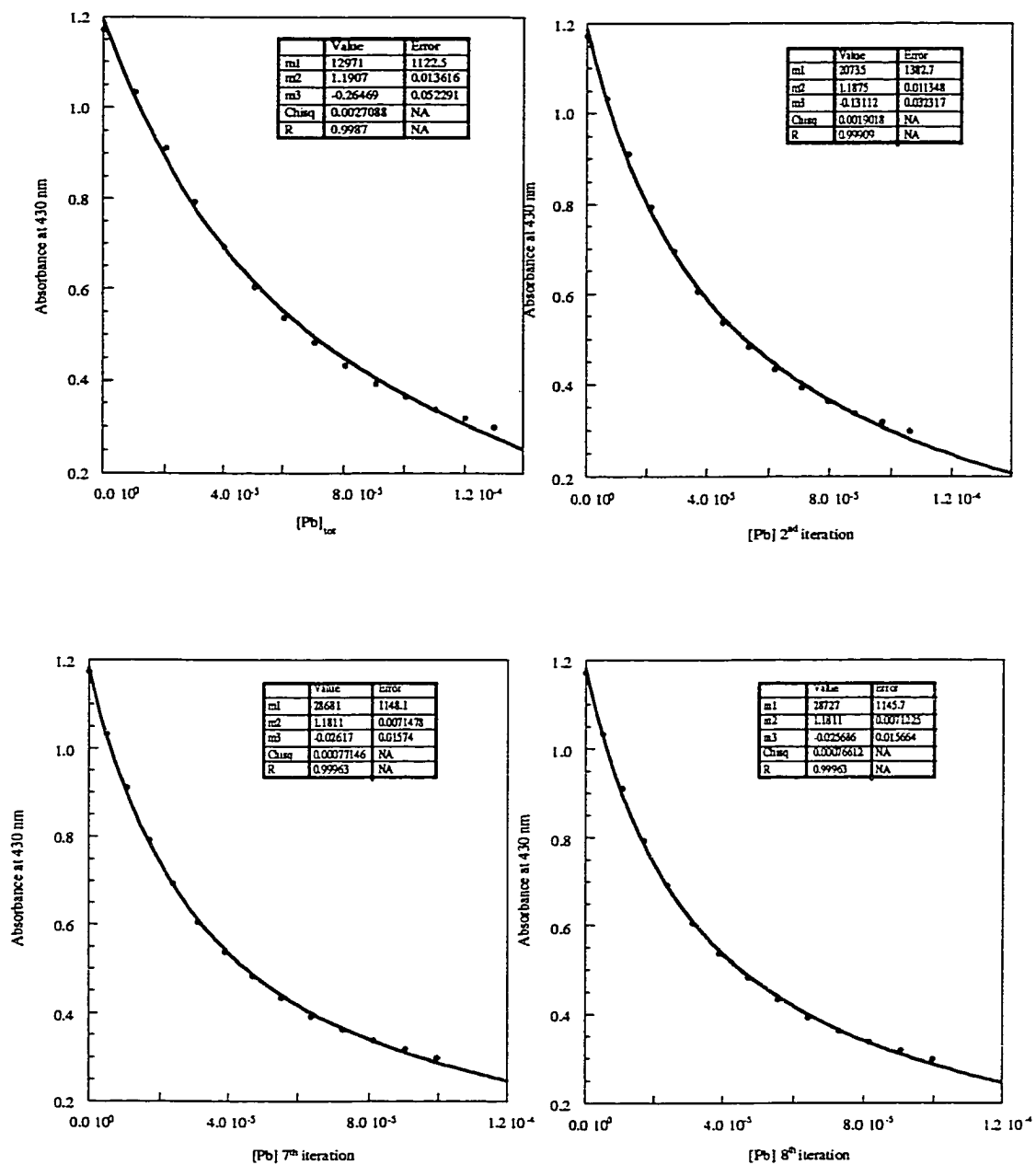


Figure III.20 Plots of absorbance, at 430 nm, versus free lead ion, fit to eq 3.13 from data at various stages in the successive approximation of K_{PbDNB}^n .

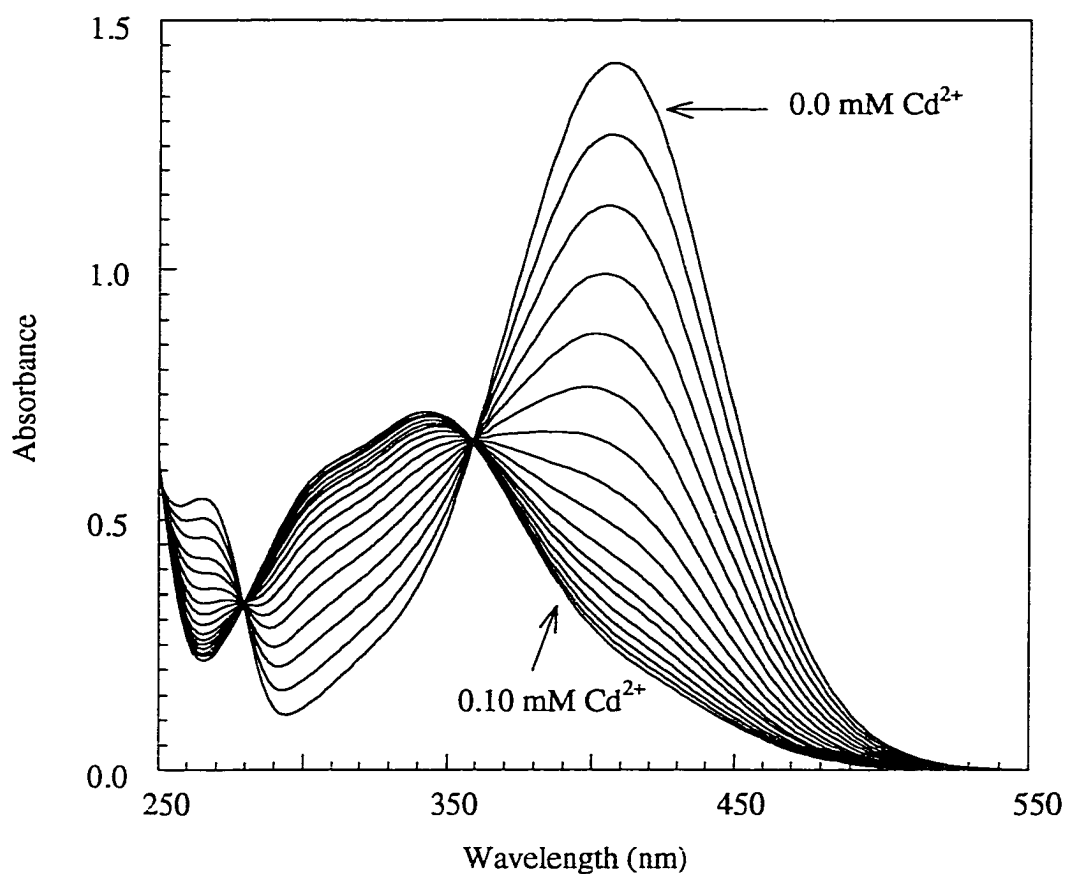


Figure III.21 Spectra from the titration of DNB with cadmium at pH 3.5. 4.0 μL aliquots of 4.156 mM Cd^{2+} were used to titrate 2.50 mL of 54.6 μM DNB solution which was buffered at pH 3.5 with 5.0 mM PIPES. The titration was done at 25.0 $^{\circ}\text{C}$ at an ionic strength of 0.10 M (KNO_3).

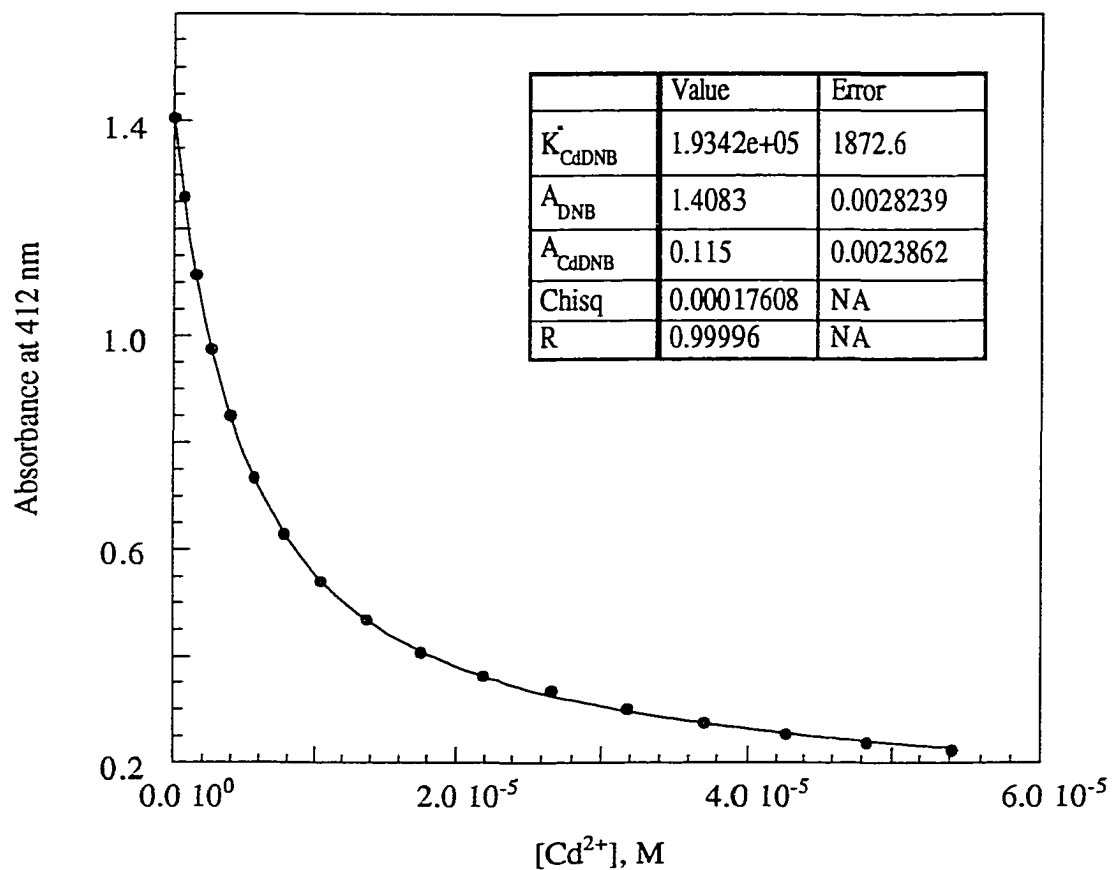


Figure III.22 Absorbance at 412 nm as a function of $[\text{Cd}^{2+}]$ at pH 3.5. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

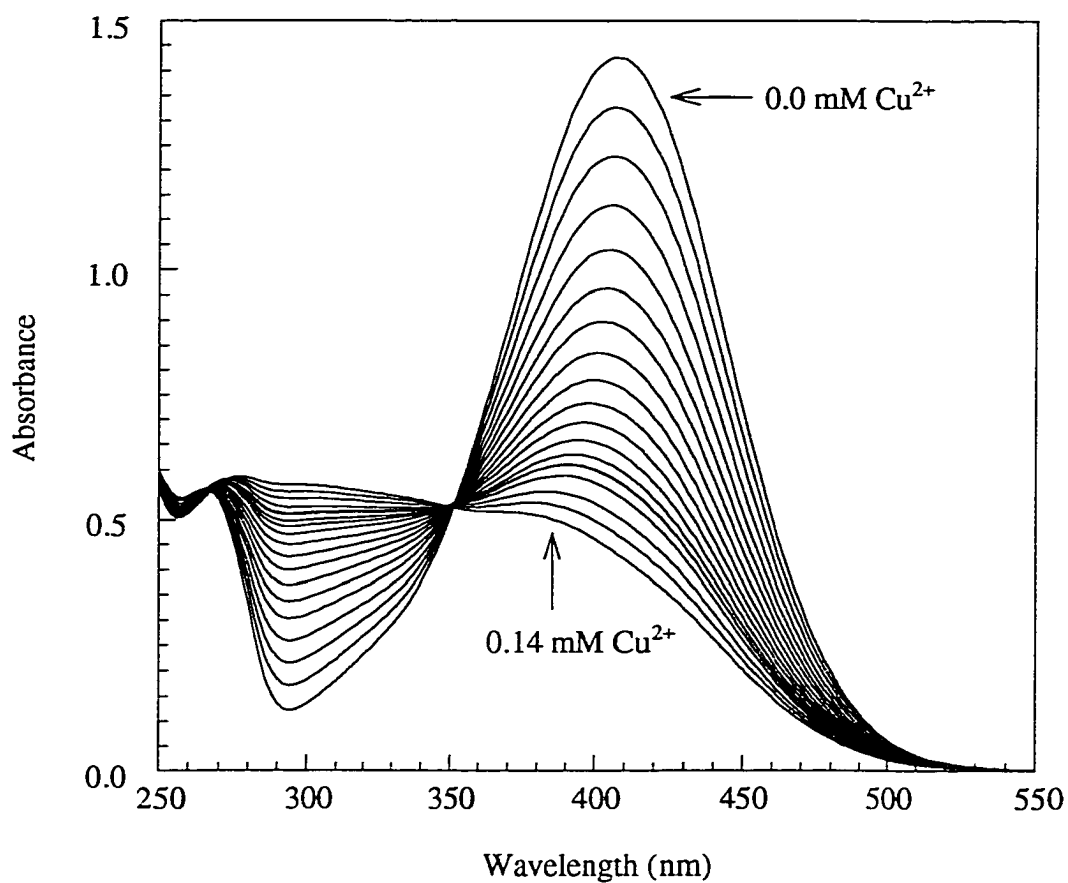


Figure III.23 Spectra from the titration of DNB with copper at pH 3.5. 2.50 mL of 54.6 μM DNB solution, buffered at pH 3.5 with 5.0 mM PIPES, was titrated with 4.0 μL aliquots of 4.096 mM Cu^{2+} . The titration was done at 25.0 $^{\circ}\text{C}$ at an ionic strength of 0.10 M (KNO_3).

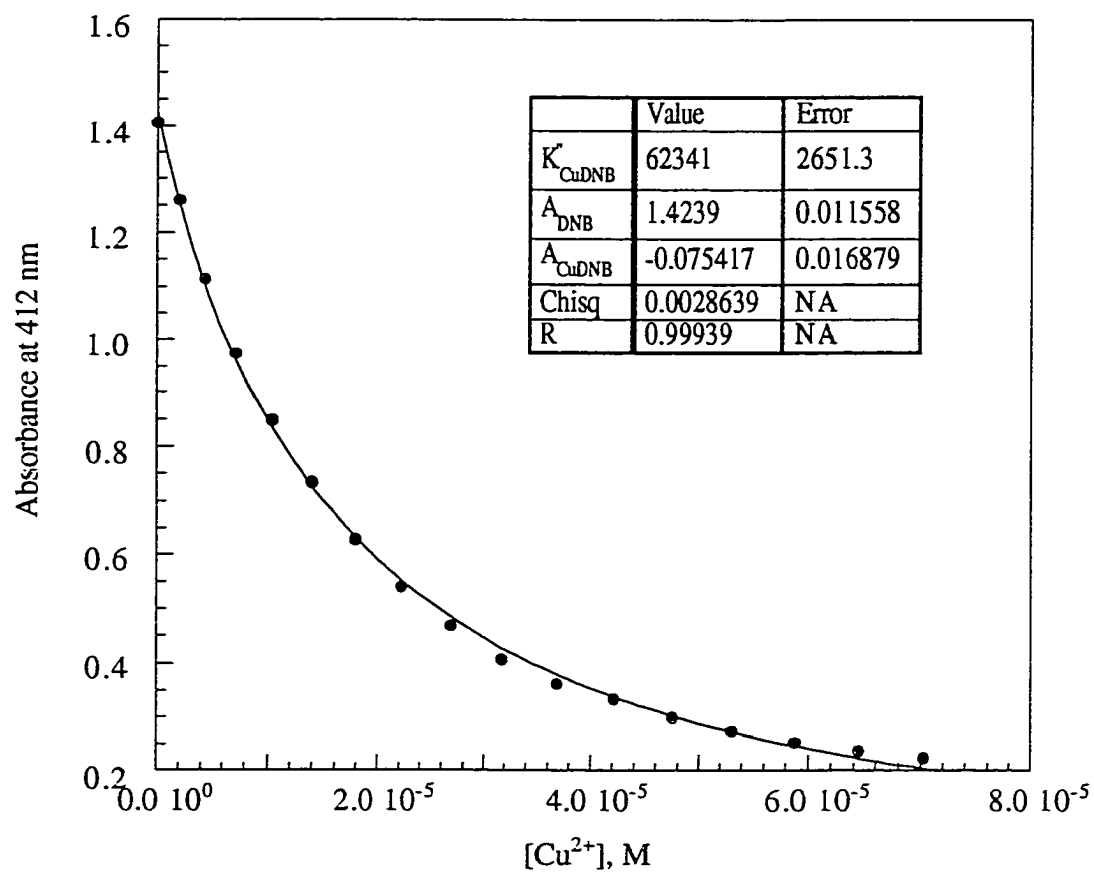


Figure III.24 Absorbance at 412 nm as a function of $[\text{Cu}^{2+}]$ at pH 3.5. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

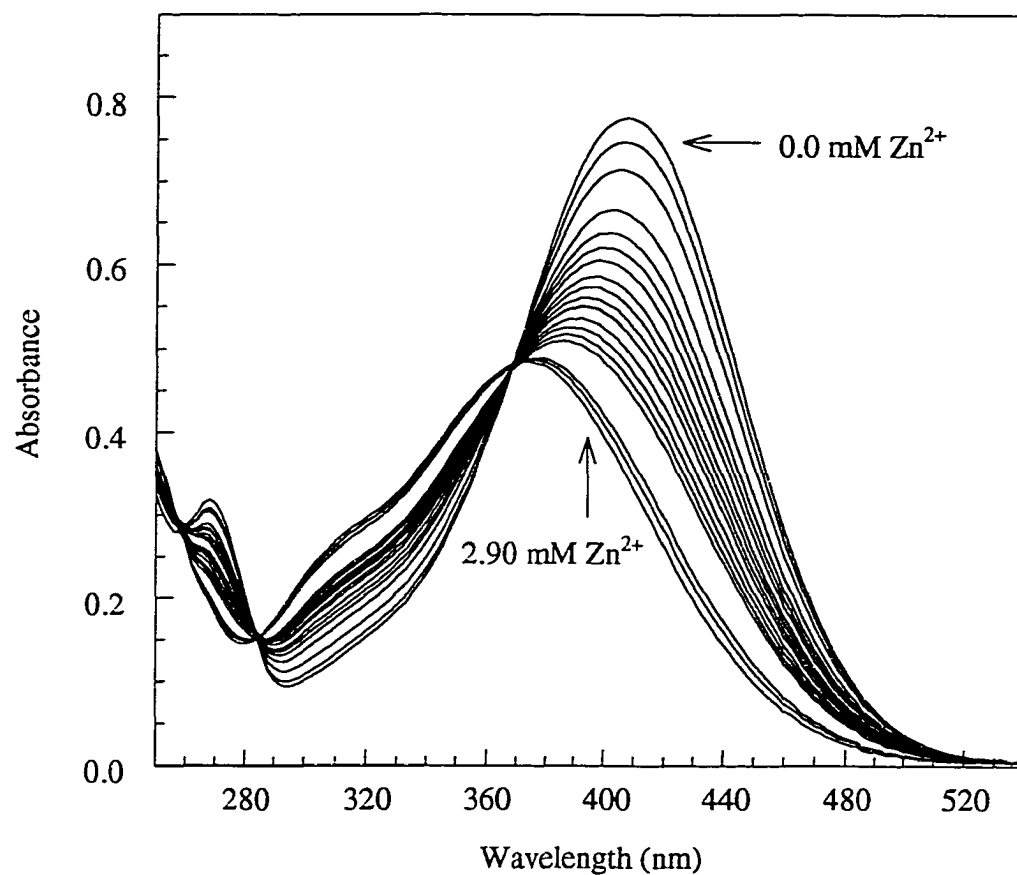


Figure III.25 Spectra from the titration of DNB with zinc at pH 3.5. 4.0 μL aliquots of 20.0 mM and 0.284 M Zn^{2+} were used to titrate 2.50 mL of 35.0 μM DNB solution, buffered at pH 3.5 with 5.0 mM PIPES. The titration was done at 25.0 $^{\circ}\text{C}$ at an ionic strength of 0.10 M (KNO_3).

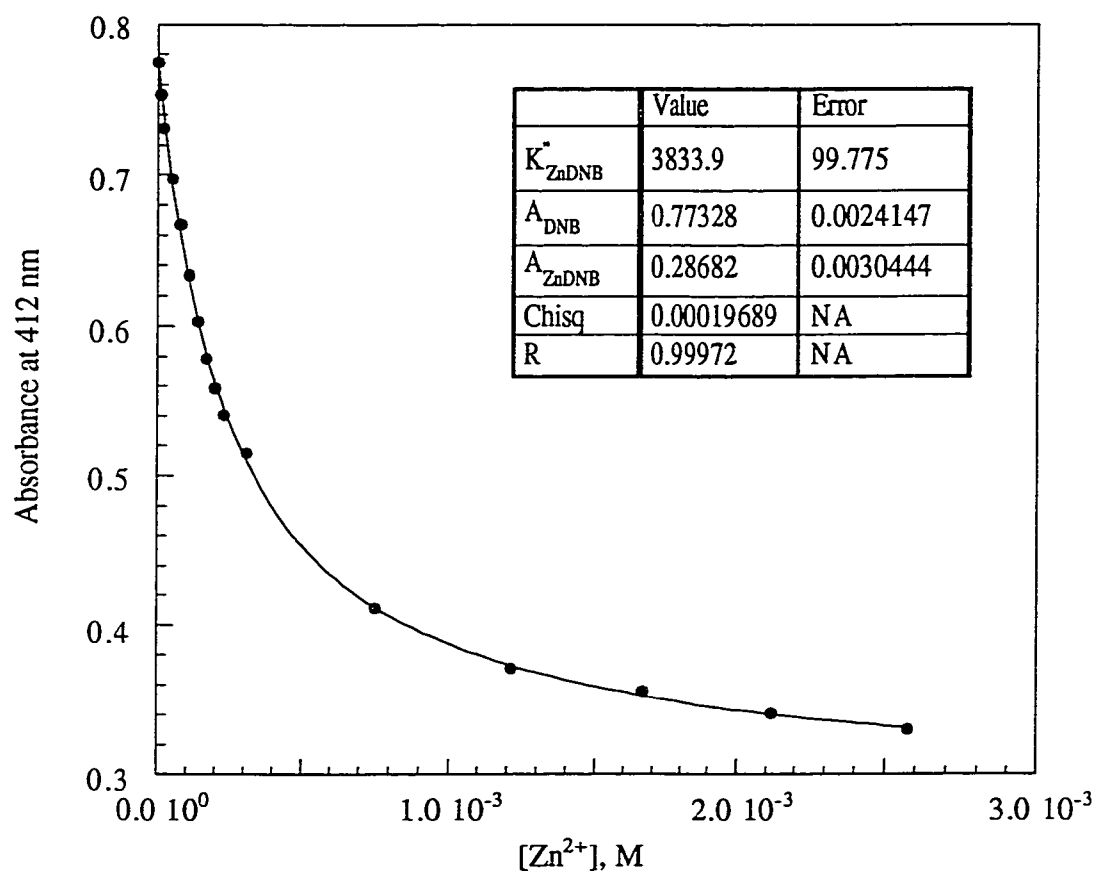


Figure III.26 Absorbance at 412 nm as a function of $[Zn^{2+}]$ at pH 3.5. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

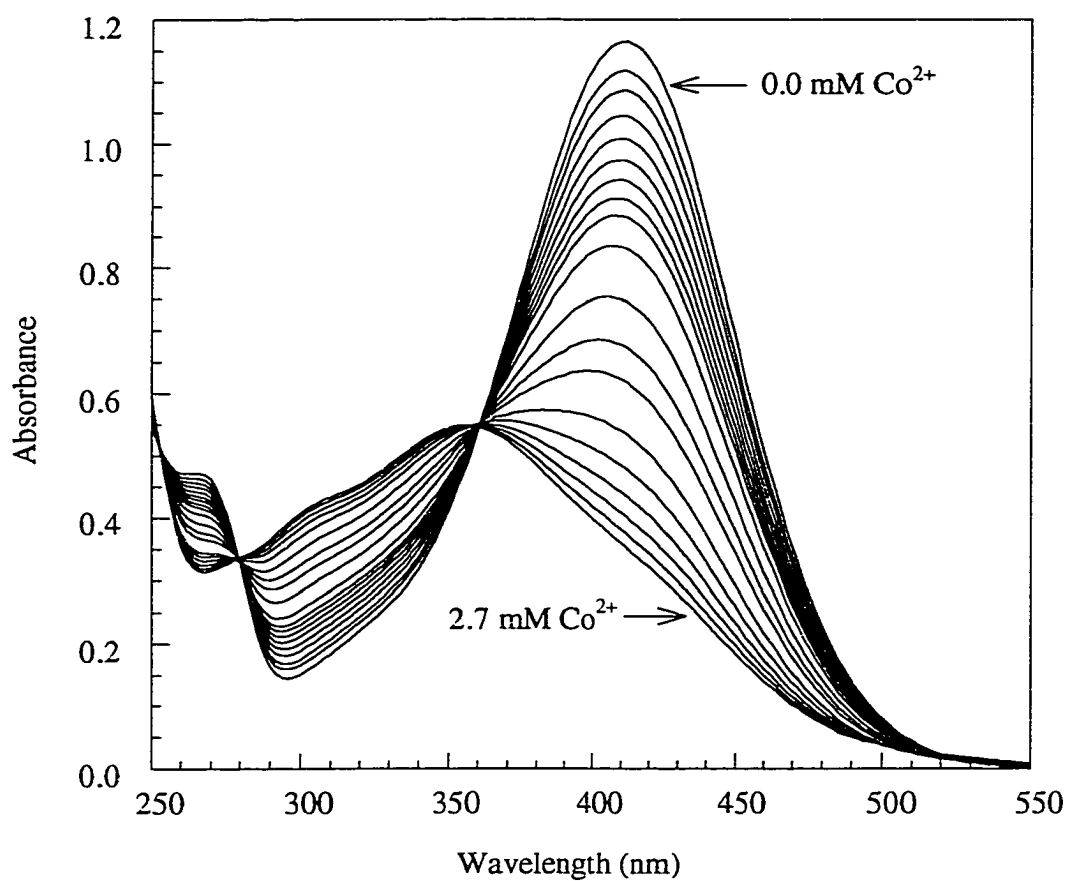


Figure III.27 Spectra from the titration of DNB with cobalt at pH 4.0. 4.0 μL aliquots of 3.96 mM, 19.8 mM and 99.0 mM Co^{2+} were used to titrate 2.5 mL of 34.0 μM DNB solution, buffered at pH 4.0 with 5.0 mM PIPBS. The temperature was controlled at 25.0 $^{\circ}\text{C}$ and ionic strength was maintained at 0.10 M (KNO_3).

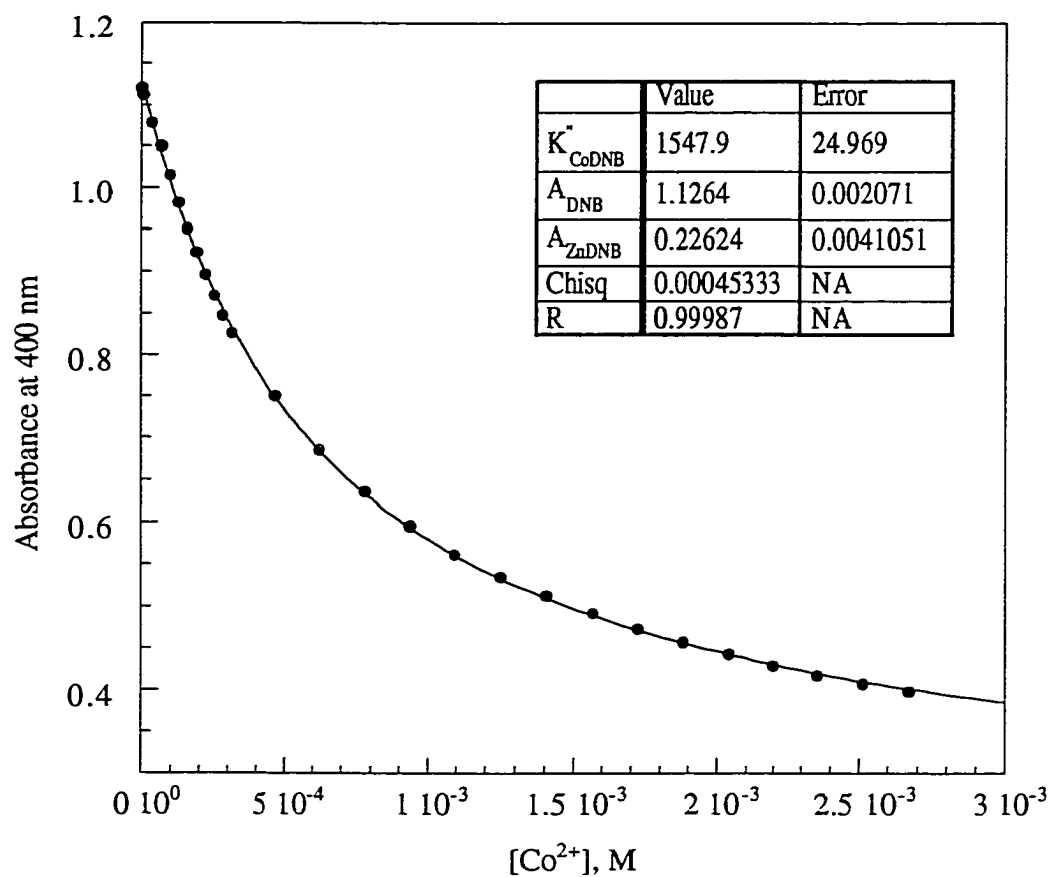


Figure III.28 Absorbance at 412 nm as a function of $[\text{Co}^{2+}]$ at pH 4.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

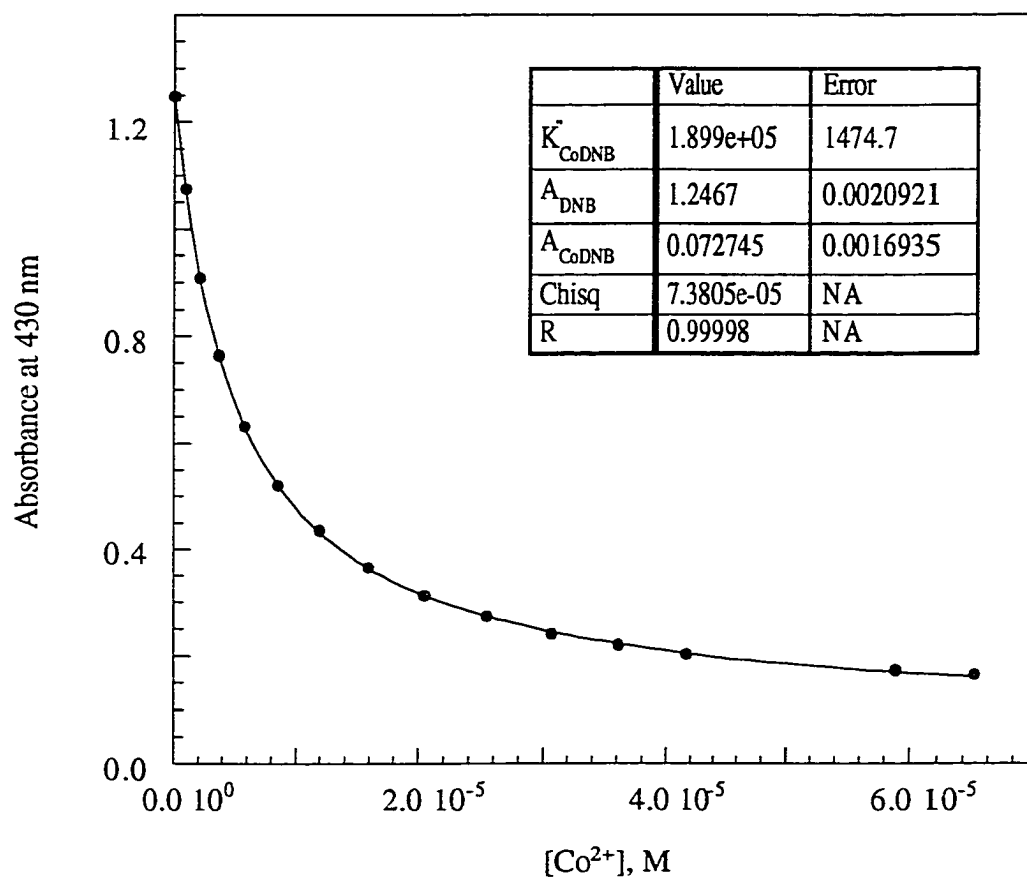


Figure III.29 Absorbance at 412 nm as a function of $[\text{Co}^{2+}]$ at pH 7.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

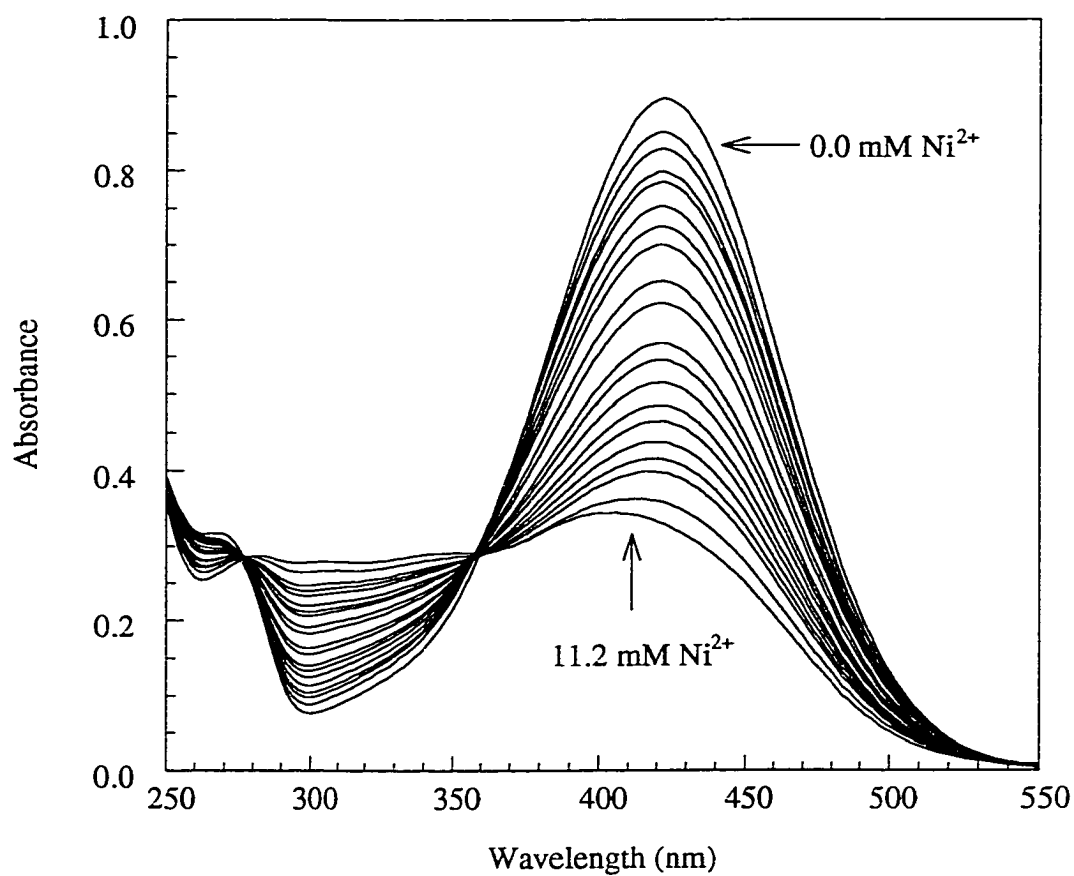


Figure III.30 Spectra from the titration of DNB with nickel at pH 5.0. 4.0 μL aliquots of 4.36 mM, 93.1 mM, 0.1861 M, and 0.931 M Ni^{2+} were used to titrate 2.5 mL of 35.0 μM DNB solution which was buffered at pH 5.0 with 5.0 mM DEPP. The titration was done at 25.0 $^{\circ}\text{C}$ at an ionic strength of 0.1 M (KNO_3).

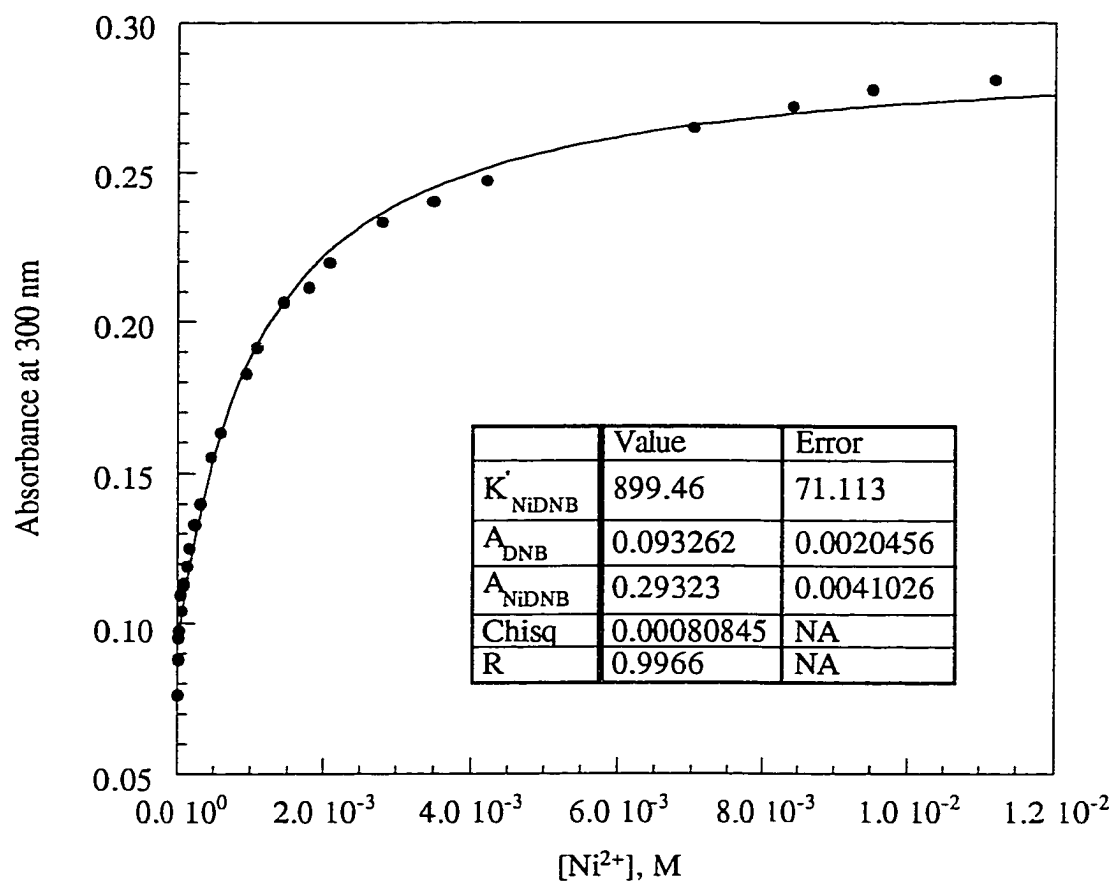


Figure III.31 Absorbance at 300 nm as a function of $[Ni^{2+}]$ at pH 5.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

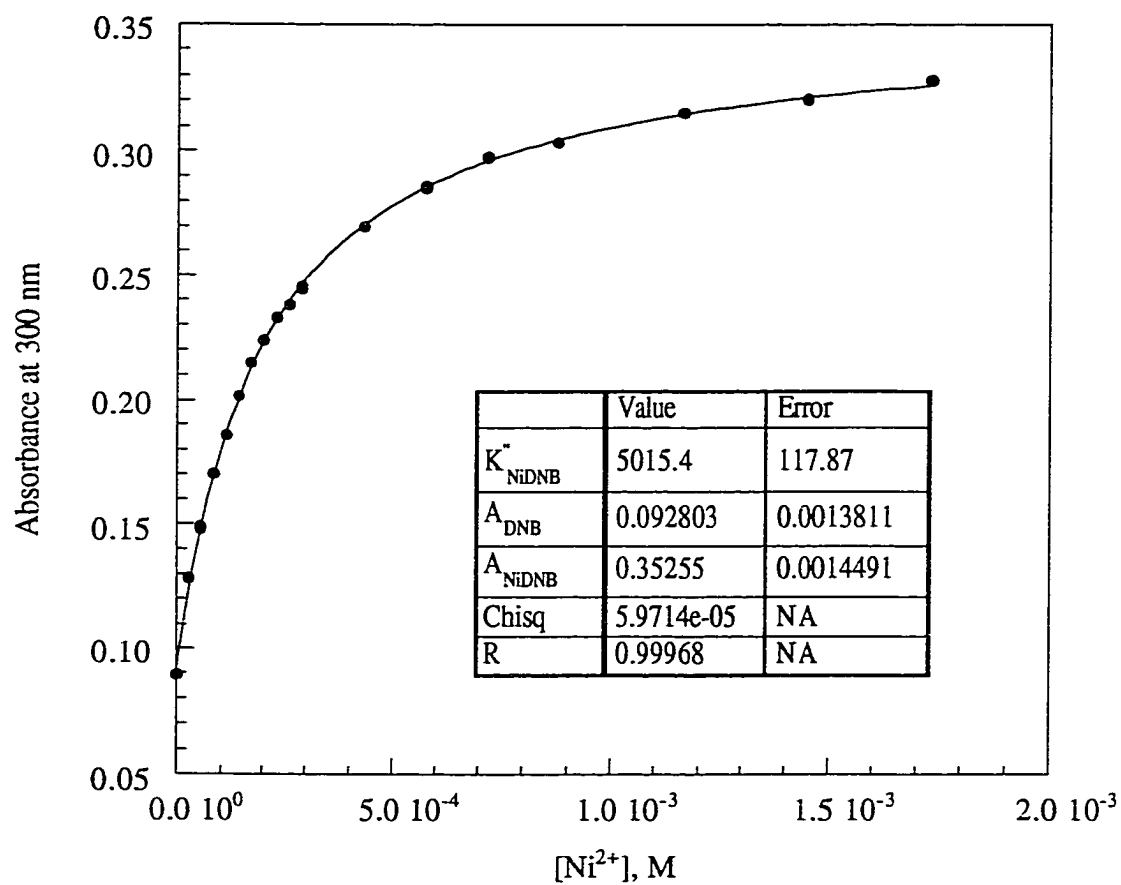


Figure III.32 Absorbance at 300 nm as a function of $[Ni^{2+}]$ at pH 7.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

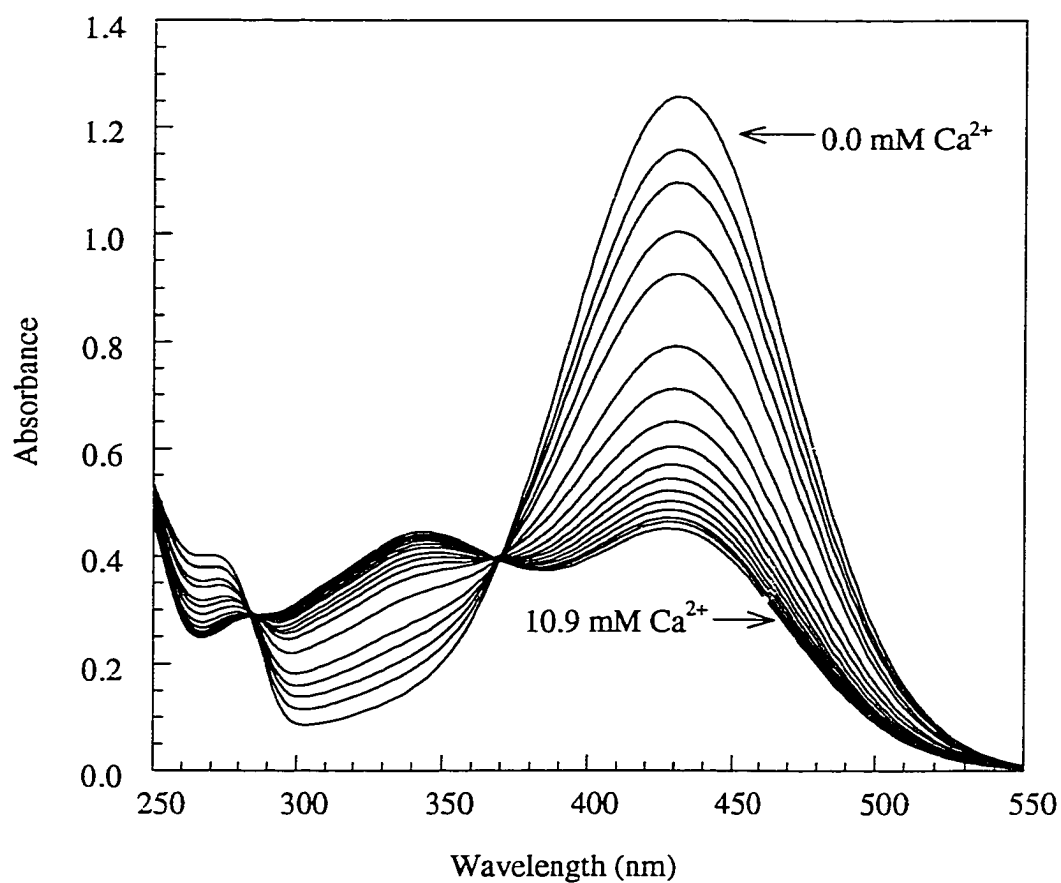


Figure III.33 Spectra from the titration of DNB with calcium at pH 7.0. 4.0 μL aliquots of 7.38 mM, 0.1095 M, 2.651 M Ca^{2+} were used to titrate 2.5 mL of 0.41 μM DNB solution which was buffered at pH 7.0 with 5.0 mM PIPES. The titration was done at 25.0 $^{\circ}\text{C}$ at 0.1 M ionic strength (KNO_3).

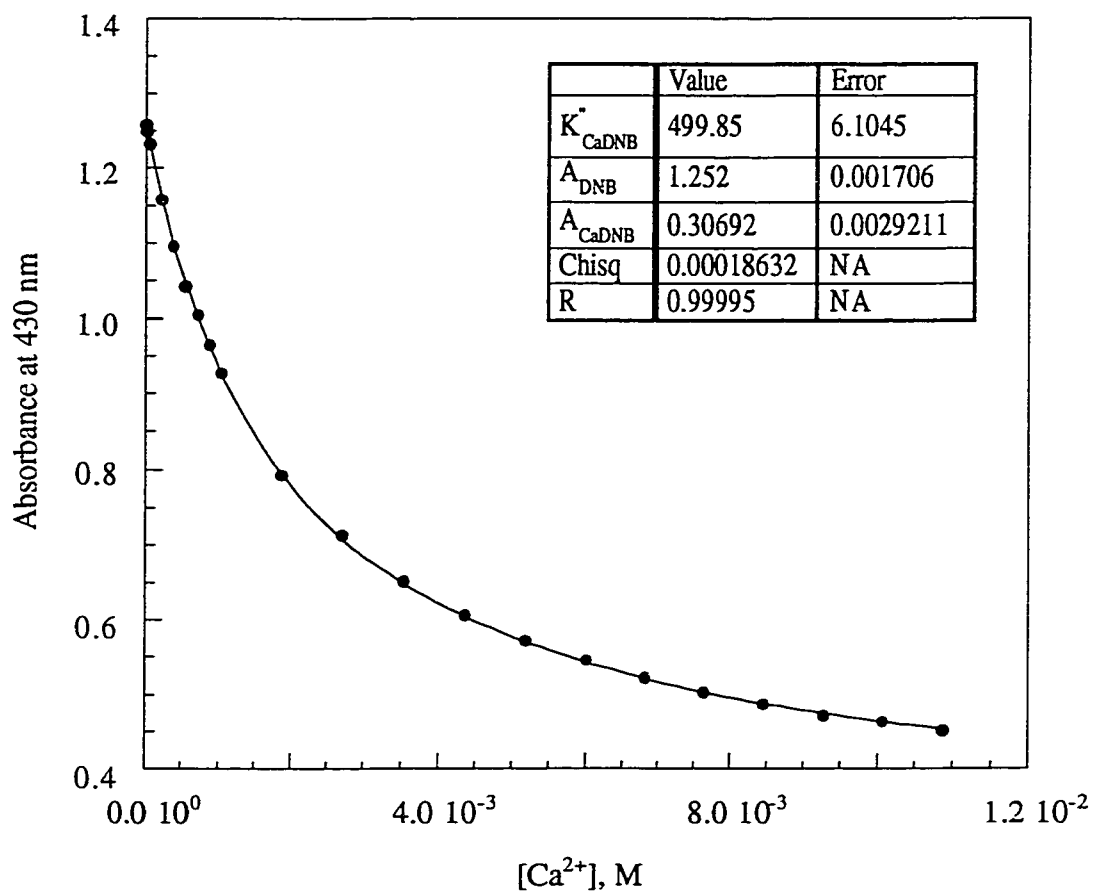


Figure III.34 Absorbance at 430 nm as a function of $[\text{Ca}^{2+}]$ at pH 7.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

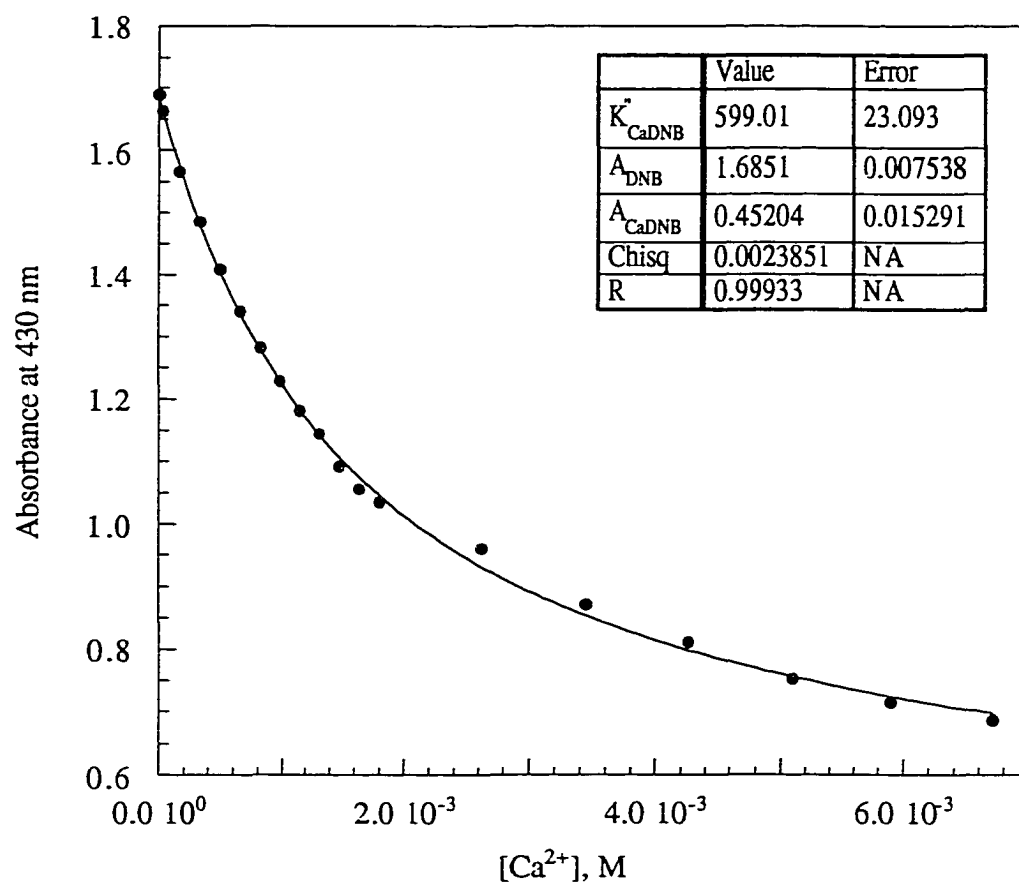


Figure III.35 Absorbance at 430 nm as a function of $[\text{Ca}^{2+}]$ at pH 9.0. The solid line was calculated from eq 3.13 using the derived parameters listed in the inset table.

The pH dependence of K''_{MDNB} due to side reactions of DNB with protons can be accounted for by the relationship,

$$K''_{\text{MDNB}} = \frac{[\text{MDNB}]'}{[\text{M}^{2+}][\text{DNB}]'} = \frac{[\text{MDNB}]' \alpha_{\text{DNB}}}{[\text{M}^{2+}][\text{DNB}^{4-}]} = K'_{\text{MDNB}} \alpha_{\text{DNB}} \quad (3.15)$$

where

$$\alpha_{\text{DNB}} = \frac{[\text{DNB}^{4-}]}{[\text{DNB}]'} = (1 + K_{\text{H1}} a_{\text{H}} + K_{\text{H1}} K_{\text{H2}} a_{\text{H}}^2 + K_{\text{H1}} K_{\text{H2}} K_{\text{H3}} a_{\text{H}}^3) \quad (3.16)$$

Table III.5 lists the values for K''_{MDNB} , α_{DNB} , and K'_{MDNB} for each metal.

Table III.5 Conditional stability constants, α_{DNB} factor, corrected stability constants, and log stability constants for DNB with divalent metal ions^a

Metal	pH	K_{ML}''	error ^b	α_{DNB}	K_{ML}'	log K_{ML}'
Cd	3.5	2.11×10^5	2.36×10^3	6.40×10^{-4}	3.29×10^8	8.52
Zn	3.5	3.83×10^3	9.98×10^1	6.40×10^{-4}	5.99×10^6	6.78
Pb	3.5	2.87×10^4	1.15×10^3	6.40×10^{-4}	4.49×10^7	7.65
Cu	3.5	6.23×10^4	2.65×10^3	6.40×10^{-4}	9.74×10^7	7.99
Ni	5.0	8.99×10^2	7.11×10^1	2.43×10^{-1}	3.70×10^3	3.57
Ni	7.0	5.02×10^3	1.18×10^2	9.77×10^{-1}	5.13×10^3	3.71
Co	4.0	1.55×10^3	2.50×10^1	8.00×10^{-3}	1.93×10^5	5.29
Co	7.0	2.02×10^5	1.90×10^3	9.77×10^{-1}	2.07×10^5	5.32
Ca	7.0	5.08×10^2	6.56	9.77×10^{-1}	5.19×10^2	2.72
Ca	9.0	5.98×10^2	2.31×10^1	1.00	5.99×10^2	2.78

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Uncertainty in terms of standard error.

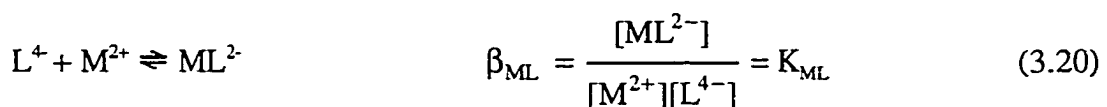
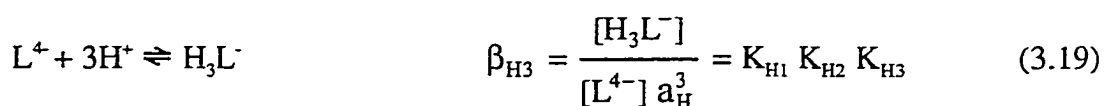
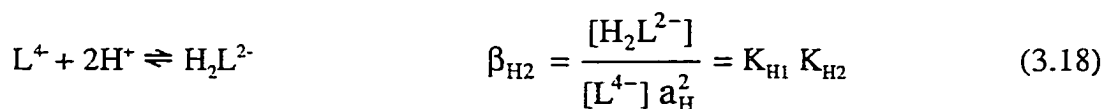
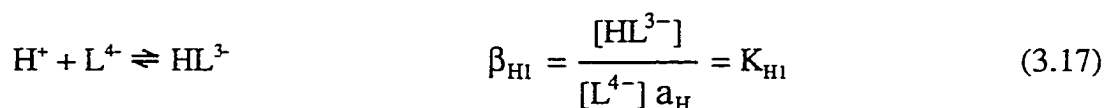
2 Potentiometric Determination of Stability Constants

The complex formation constants of DNB were determined by analysis of data obtained from potentiometric titrations. Each data set consisted of the values of the observed pH and volume of titrant added at each point in the titration. The titrations were accomplished by the automated titration system that measured the variable $-\log[H^+]$ as a function of the volume of titrant added. The titrated samples consisted of 10.00 - 25.00 mL of solution containing 0.192 to 0.377 mM DNB, an approximately equimolar concentration of metal, and sufficient KNO_3 to maintain ionic strength at 0.1 M. The titrant was prepared by diluting 50% w/w NaOH with freshly boiled D.D. H_2O to give an NaOH concentration of approximately 5 - 10 mM. The titrant was then standardized with KHP and tested for CO_2 contamination as described in chapter II.V. All titrations were conducted at 25.0 °C.

The program BEST² was used to calculate the stability constants of the DNB complexes for cadmium, lead, copper, zinc, cobalt, and nickel. In addition to the raw titration data, this program requires the known concentrations of the sample solution components, the initial volume of the sample solution and the titrant concentration. The program calculates stability constants based on mass balance constraints for each component of the system, summed over all species present, at each point in the titration.

Each set of data was input into the BEST program along with a user defined model based on the protonation and metal complex formation reactions of the ligand. The input for the model consists of the stoichiometric coefficients of the components in each equilibrium to be considered along with the known value, or an estimate, of the overall equilibrium constant for that reaction. An initial, simple model for DNB is illustrated by the reactions and equilibrium expressions given by eqs 3.17 to 3.20. This model consists of equilibria for the first three protonation reactions (eqs 3.17 - 3.19) and the

formation of a 1:1 metal-ligand complex (eq. 3.20). The known values for the formality of the metal and DNB components of the titration solution and the titrant, NaOH, are entered into the file for that particular titration along with the model for the DNB-metal system involved. All constants included in the model are expressed in terms of overall formation constants, $\log \beta$ values. Overall constants are cumulative in terms of stepwise constants. This is illustrated below in the expressions for β_{H1} and $\log \beta_{H1}$ of the mono-, di-, and triprotonated forms of DNB, as well as the metal-ligand complex. The stepwise expressions of K_{H1} for protonation of DNB are given in eqs 3.1 - 3.3. In eqs 3.17 - 3.20 and 3.24 - 3.26 the term L^{4-} refers to the tetraanion of DNB.

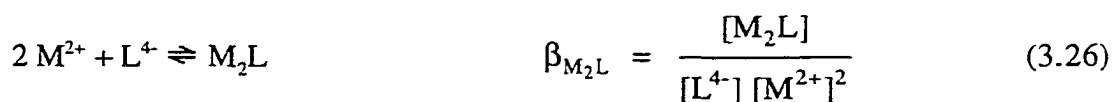
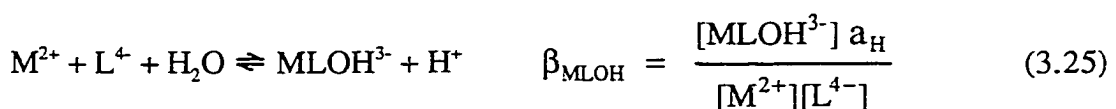
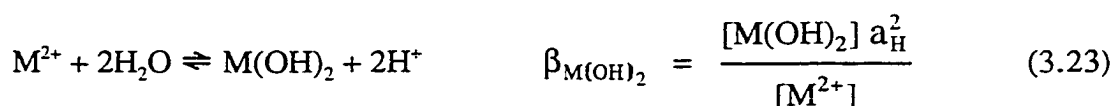
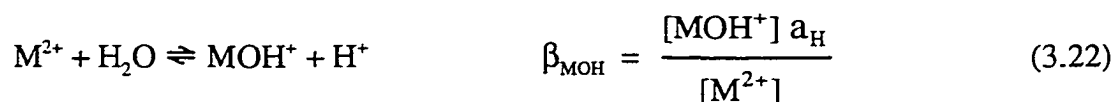


The estimated stability constants for the metal-ligand complexes are refined by the program to minimize the quantity sigma, which is described in eqs 3.7 and 3.8, while known equilibrium constants are held constant. In models used in this study the $\log \beta_{H1}$ and $\log \beta_{H2}$ values determined earlier were held constant during the calculations. $\log \beta_{H3}$ was not considered to have been determined with a sufficient degree of accuracy and the program was allowed to refine this value within limits. If refinement of the metal-ligand stability constant is not sufficient to allow a sigma of less than 0.01 with the experimental data, other complexes are added to the model. Values for complexes included in the model were considered to be only initial guesses and were allowed to refine over a broad range. The initial model is revised by addition of other possible complexes, such as metal hydroxides, other metal-ligand complexes, protonated complexes, and hydroxy adducts of the complex. Each equilibrium to be considered in the model is input with the stoichiometric coefficients of its components along with the known value, or an estimate, of the overall equilibrium constant for that reaction. Stability constants for metal hydroxides, obtained from the literature³, were corrected to the appropriate ionic strength using the Davies equation, as shown below,

$$\log \gamma_i = -1/2z_i^2 \left[\frac{\sqrt{I}}{1 + \sqrt{I}} - 0.3 I \right] \quad (3.21)$$

where γ_i is the activity coefficient of an ion of charge z , at an ionic strength of I . These corrected values were held constant in calculations by the program. Additional complexes with DNB were allowed to refine in the same manner as the 1:1 metal-ligand

complex. After each additional complex is added to the model all values not initially held constant were again refined by the program until no further minimization of sigma could be obtained. Overall reactions leading to formation of complexes included in the final models are shown below with definitions for their corresponding β values. Reactions for the formation of hydroxy species are expressed in terms of acid hydrolysis reactions.



In most cases the data collected past pH 7.0 were excluded due to a systematic deviation from the theoretical curve that may be attributed to CO₂ contamination, a slight contamination of the ligand solution or precipitation effects due to insolubility of uncharged metal hydroxides or complexes. The data used to determine the complexation constants is in the portion of the curve included and omitting data past pH 7.0 had no significant effect on the calculated constants but in some cases decreased the value of sigma appreciably.

A protonated complex species (MHL⁻) was required to fit some data sets and was included in each model with the exception of the copper DNB model. In the case of the model for copper with DNB, a binuclear species was included and the protonated complex was excluded after determining that no reasonable values could be obtained for protonated complexes in this system. Most of the data sets required the inclusion of a hydroxy-complex (MLOH³⁻) to minimize the fit as well. The species included in the final models for each DNB-metal system are listed in Tables III.6 - III.11 along with the known or derived values of log β for each species. Table III.12 lists the average log β values for the 1:1 complex species of DNB with each of the metals studied.

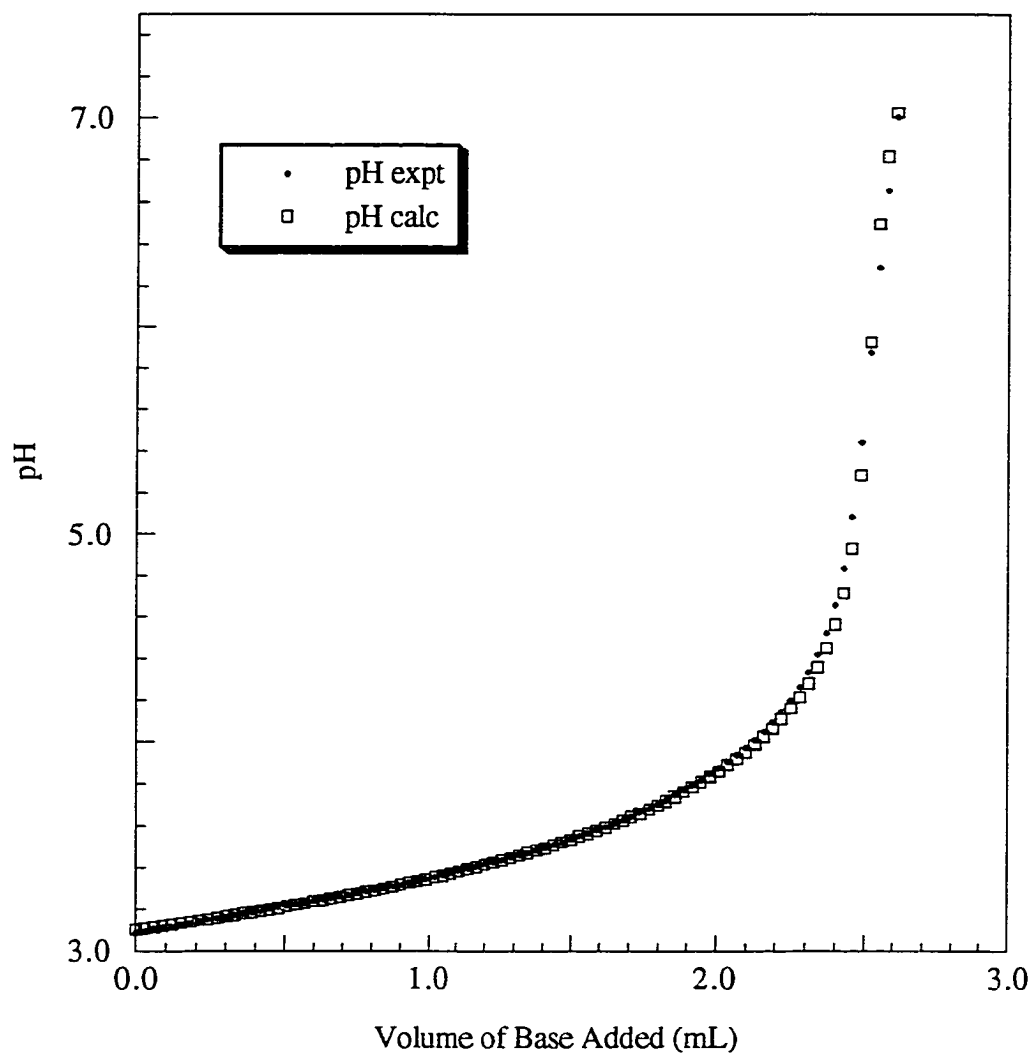


Figure III.36 Potentiometric titration curve for 20.0 mL of a solution containing 0.192 mM DNB, 0.191 mM $\text{Cd}(\text{ClO}_4)_2$ at an ionic strength of 0.1 M (KNO_3) at 25.0 °C. The titrant is 6.934 mM NaOH with ionic strength maintained at 0.1 M with KNO_3 . The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.6, Exp. 1.

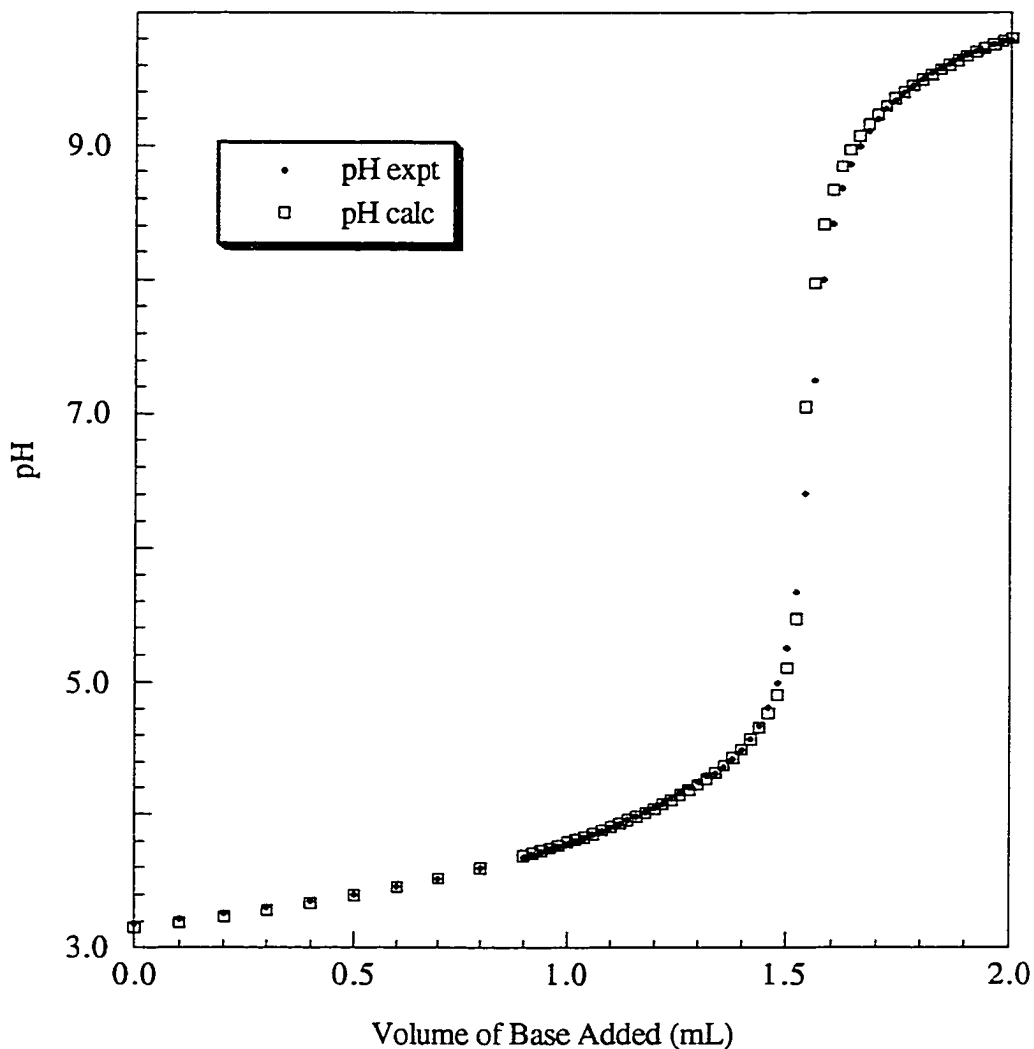


Figure III.37 Potentiometric titration curve for 10.0 mL of a solution containing 0.377 mM DNB, 0.372 mM $\text{Pb}(\text{ClO}_4)_2$ at an ionic strength of 0.1 M (KNO_3) at 25.0 °C. The titrant is 7.94 mM NaOH with ionic strength maintained at 0.1 M (KNO_3). The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.7, Exp. 1.

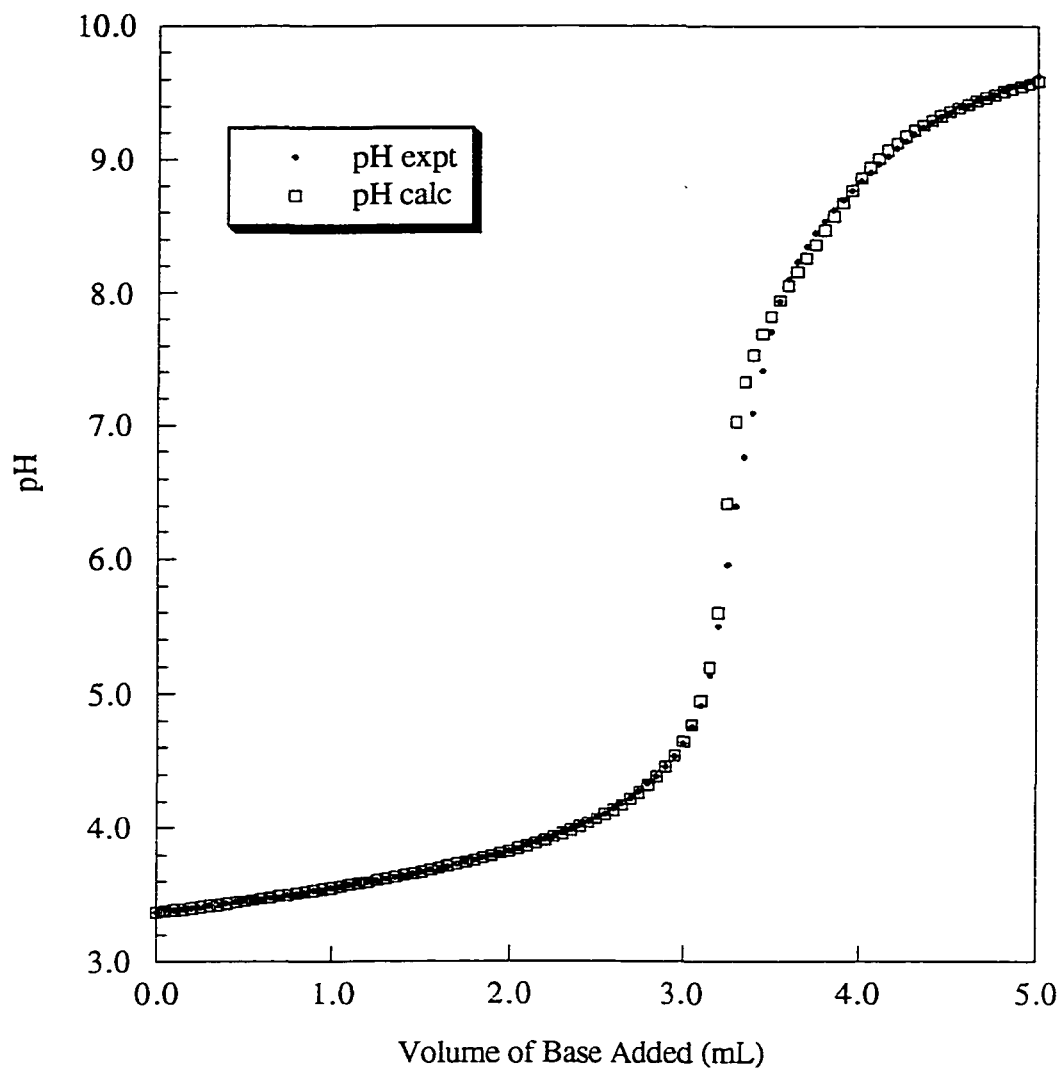


Figure III.38 Potentiometric titration curve for 25.0 mL of a solution containing 0.219 mM DNB, 0.200 mM CuCl_2 at an ionic strength of 0.1 M (KNO_3). The titrant is 6.67 mM NaOH with ionic strength maintained at 0.1 M with KNO_3 . The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.8, Exp. 1.

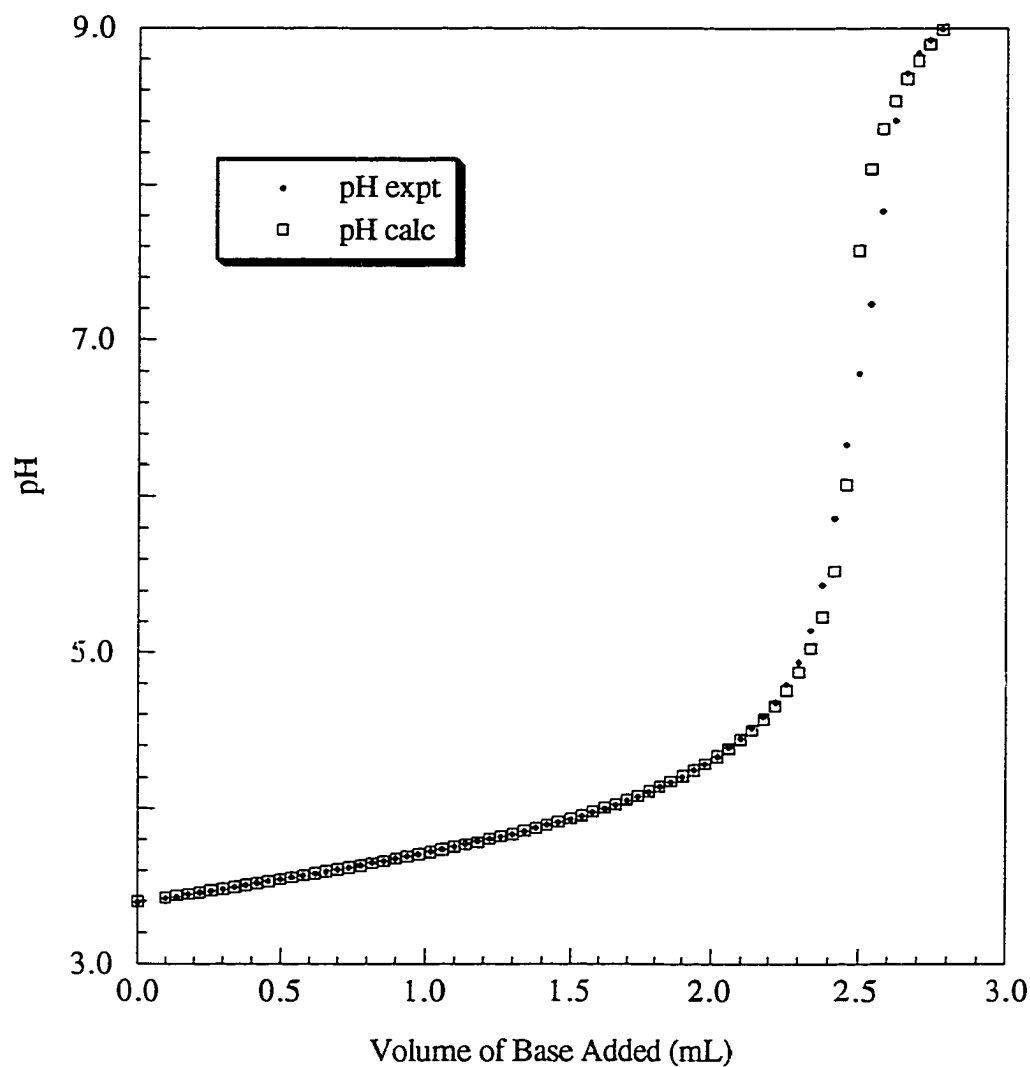


Figure III.39 Potentiometric titration curve for 20.0 mL of a solution containing 0.192 mM DNB, 0.191 mM $\text{Zn}(\text{ClO}_4)_2$ at an ionic strength of 0.1 M (KNO_3). The titrant is 7.56 mM NaOH with ionic strength maintained at 0.1 M with KNO_3 . The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.9, Exp. 1.

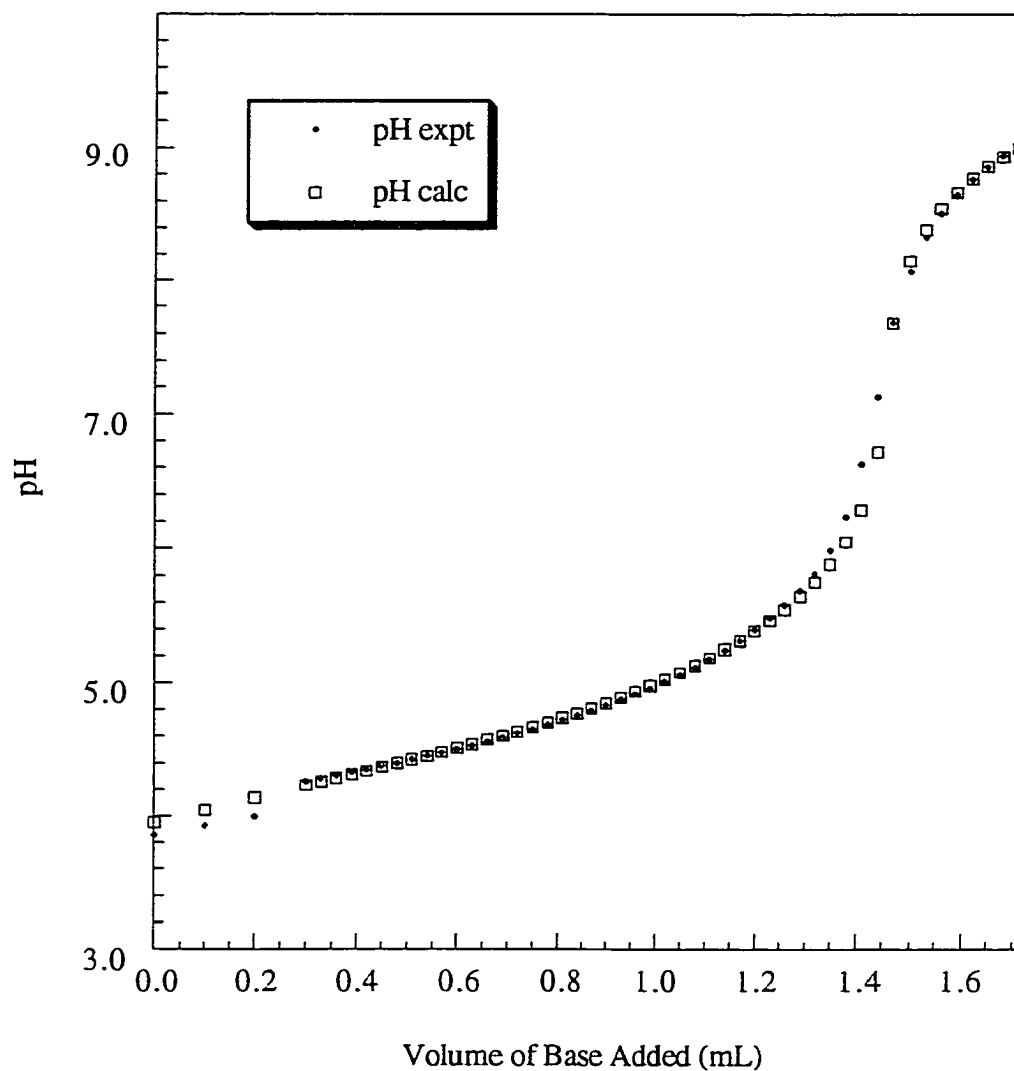


Figure III.40 Potentiometric titration curve for 10.0 mL of a solution containing 0.377 mM DNB, 0.376 mM CoCl_2 at an ionic strength of 0.1 M (KNO_3). The titrant is 8.16 mM NaOH with ionic strength maintained at 0.1 M with KNO_3 . The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.10, Exp. 1.

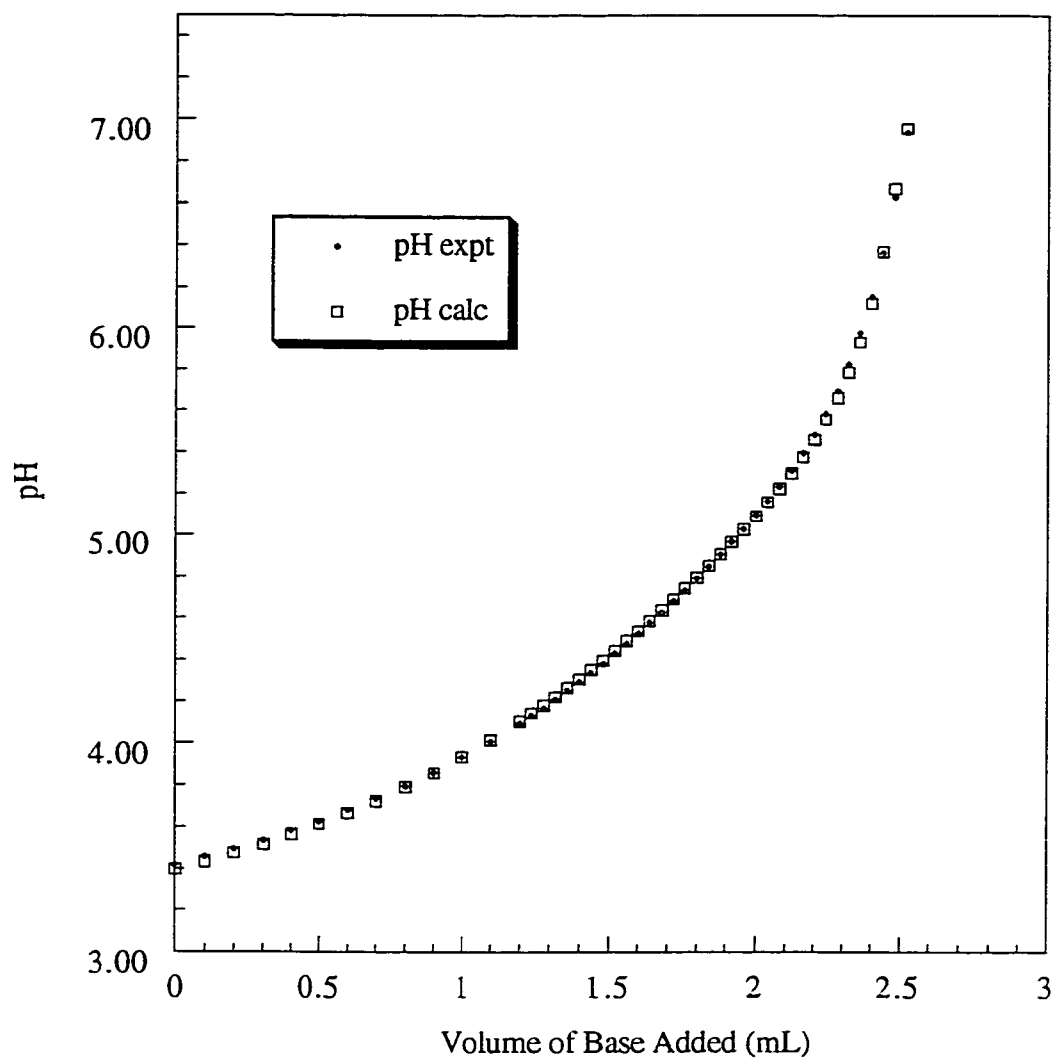


Figure III.41 Potentiometric titration curve for 20.0 mL of a solution containing 0.192 mM DNB, 0.191 mM $\text{Ni}(\text{ClO}_4)_2$ at an ionic strength of 0.1 M (KNO_3). The titrant is 7.56 mM NaOH with ionic strength maintained at 0.1 M with KNO_3 . The calculated pH values are obtained from the program BEST using refined values for the model containing the species listed in Table III.11, Exp. 1.

Table III.6 Log β_x values from potentiometric titrations of DNB and cadmium^a

Exp. No.	<u>Identity of complex species x</u>								sigma ^e
	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>CdOH^d</u>	<u>Cd(OH)₂^d</u>	<u>CdDNB^c</u>	<u>CdHDNB^c</u>	<u>CdDNBOH^c</u>	
1	5.40	9.88	12.62	-10.3	-20.4	9.15	11.49	1.34	0.0058
2	5.40	9.88	13.19	-10.3	-20.4	8.48	12.08	0.362	0.0160
3	5.40	9.88	13.24	-10.3	-20.4	8.70	11.89	1.76	0.0084

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = $(U/N)^{1/2}$, as defined in eqs 3.7 - 3.8.

Table III.7 Log β_x values from potentiometric titrations of DNB and lead^a

Exp. No.	<u>Identity of complex species x</u>								sigma ^e
	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>PbOH^d</u>	<u>Pb(OH)₂^d</u>	<u>PbDNB^c</u>	<u>PbHDNB^c</u>	<u>PbDNBOH^c</u>	
1	5.40	9.88	13.44	-7.8	-17.18	7.15	10.29	0.0884	0.0078
2	5.40	9.88	13.19	-7.8	-17.18	7.38	11.09	-0.6165	0.0120

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = $(U/N)^{1/2}$, as defined in eqs 3.7 - 3.8.

Table III.8 Log β_x values from potentiometric titrations of DNB and copper^a

Exp.	<u>Identity of complex species x</u>								
No.	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>CuOH^d</u>	<u>Cu(OH)₂^d</u>	<u>CuDNB^c</u>	<u>Cu₂DNB^c</u>	<u>CuDNBOH^c</u>	sigma ^e
1	5.40	9.88	13.27	-7.68	-16.18	7.16	10.61	-1.06	0.0057
2	5.40	9.88	13.19	-7.68	-16.18	7.31	10.31	-0.8279	0.0079

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = (U/N)^{1/2}, as defined in eqs 3.7 - 3.8.

Table III.9 Log β_x values from potentiometric titrations of DNB and zinc^a

Exp.	<u>Identity of complex species x</u>								
No.	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>ZnOH^d</u>	<u>Zn(OH)₂^d</u>	<u>ZnDNB^c</u>	<u>ZnHDNB^c</u>	<u>ZnDNBOH^c</u>	sigma ^e
1	5.40	9.88	13.23	-9.08	-17.89	6.40	9.38	-2.64	0.007
2	5.40	9.88	13.32	-9.08	-17.89	6.57	8.31	-2.36	0.011

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = (U/N)^{1/2}, as defined in eqs 3.7 - 3.8.

Table III.10 Log β_x values from potentiometric titrations of DNB and cobalt^a

Exp.	<u>Identity of complex species x</u>							
No.	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>CoOH^d</u>	<u>Co(OH)₂^d</u>	<u>CoDNB^c</u>	<u>CoHDNB^c</u>	sigma ^e
1	5.40	9.88	13.89	-9.78	-18.86	4.33	8.88	0.0220
2	5.40	9.88	13.89	-9.78	-18.86	3.71	8.31	0.0525
3	5.40	9.88	13.98	-9.78	-18.86	4.12	4.85	0.0194

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = (U/N)^{1/2}, as defined in eqs 3.7 - 3.8.

Table III.11 Log β_x values from potentiometric titrations of DNB and nickel^a

Exp.	<u>Identity of complex species x</u>								
No.	<u>HDNB^b</u>	<u>H₂DNB^b</u>	<u>H₃DNB^c</u>	<u>NiOH^d</u>	<u>Ni(OH)₂^d</u>	<u>NiDNB^c</u>	<u>NiHDNB^c</u>	<u>NiDNBOH^c</u>	sigma ^e
1	5.40	9.88	13.44	-9.98	-20.09	3.58	8.53	-3.62	0.0057
2	5.40	9.88	13.48	-9.98	-20.09	3.41	7.70	-4.42	0.0187

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section III.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell², adjusted for ionic strength. ^e sigma = (U/N)^{1/2}, as defined in eqs 3.7 - 3.8.

Table III.12 Summary of complexation constant values obtained from potentiometric data^a

Metal	Exp. No.	log K _{MDNB}	sigma ^b
Cd	1	9.15	0.0058
Cd	2	8.48	0.0160
Cd	3	8.70	0.0084
Pb	1	7.15	0.0078
Pb	2	7.38	0.0120
Cu	1	7.16	0.0057
Cu	2	7.31	0.0079
Zn	1	6.40	0.0071
Zn	2	6.57	0.0105
Co	1	4.33	0.0220
Co	2	3.71	0.0525
Co	3	4.12	0.0194
Ni	1	3.58	0.0057
Ni	2	3.41	0.0187

^a 25.0 °C, ionic strength 0.1 M (KNO₃). ^b sigma = (U/N)^{1/2} as defined in eqs 3.7 and 3.8.

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2. Motekaitis, R. J.; Martell, A. E., *Determination and Use of Stability Constants*, 2nd ed., VCH Publishers, Inc., New York, NY, 1992.
3. Smith, R.M.; Martell, A.E.; Motekaitis, R.J. *NIST Critically Selected Stability Constants of Metal Complexes Database*; Version 5, U.S. Department of Commerce, Gaithersburg, MD, 1998.

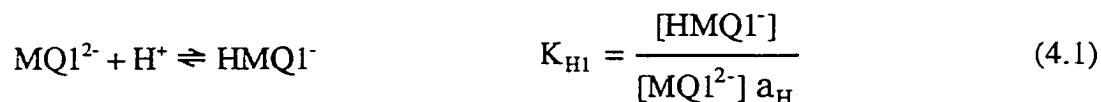
Chapter IV

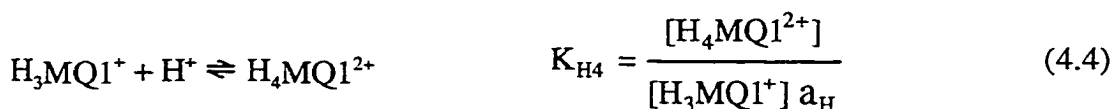
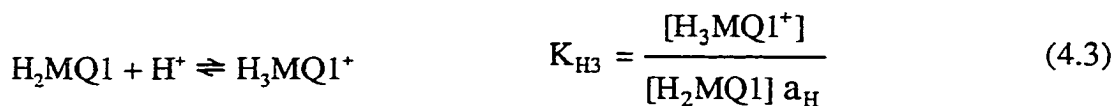
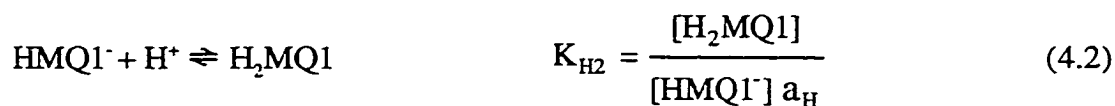
RESULTS

I Mag-quin-1

A Protonation Constants of MQ1

Four protonation sites can be seen in the structure of MQ1 shown in Figure I.5. Two of the four sites are carboxylate groups attached to an aniline nitrogen, which is a third site. The remaining protonation site is the quinoline nitrogen. The neutral form of the ligand is achieved after the second protonation step. UV-vis spectrophotometric pH titrations were used to determine macroscopic, mixed-mode, protonation constants for three of these four protonations. The fourth protonation occurs under very acidic conditions and was not determined in this study. Two of the four constants were also determined potentiometrically. The stepwise protonation reactions of MQ1 and associated equilibrium expressions are shown below in eqs 4.1 - 4.4.





1 UV-vis spectrophotometric Method

Spectrophotometric titrations were carried out using sample solutions containing 62.0 μM MQ1, MES, MOPS, DEPP, PIPES and PIPBS at 5.0 mM each, 0.2 mM $\text{Na}_2\text{H}_2\text{EDTA}$, and sufficient TEAP to maintain the ionic strength at 0.05. Temperature was controlled at 25.0 °C by external water baths attached to both the titration vessel and the cell holder. The total pH range included was from 9.5 - 1.3. Titrations to determine protonation steps at high and low pH were also done separately.

Figure IV.1 shows representative spectra obtained for a spectrophotometric titration covering the full pH range. Several isosbestic points are apparent but the spectra show that several protonation steps occur in the pH range employed. The isosbestic points are not readily apparent when spectra from the whole pH range are plotted together. The spectra are displayed in two pH ranges to show the behavior more

clearly. Figure IV.2 shows spectra for the pH range 9.4 - 5.1, while Figure IV.3 shows representative spectra for the pH range 5.1 through 1.3. In Figure IV.2 the spectra are plotted from 310 nm to 460 nm to provide a better view of isosbestic points at 344 and 377 nm. The presence of an isosbestic point indicates that only two absorbing species are present in this pH range. This is consistent with a single protonation step. This protonation step would be presumed to be the first step, K_{H1} , and the two absorbing species $MQ1^{2-}$ and $HMQ1^-$. In Figure IV.3 the same wavelength range was used to show that the isosbestic points seen from pH 9.4 - 5.1 are not seen in the lower pH region. Closer inspection of the spectra indicate isosbestic points at 382 nm, in the pH range 4.5 - 3.0, and at 372 nm for $pH \leq 2.5$. These isosbestic points indicate further protonation steps consistent with the formation of the H_2MQ1 and H_3MQ1^+ forms of the ligand respectively.

Figure IV.4 shows a plot of absorbance at a single wavelength (268 nm) versus a_H for an entire titration. The solid line is the result of a curve fit based on eq 4.5 (which defines A_i in terms of three protonation constants).

$$A_i = \frac{A_3 K_{H1} K_{H2} K_{H3} a_H^3 + A_2 K_{H1} K_{H2} a_H^2 + A_1 K_{H1} a_H + A_0}{K_{H1} K_{H2} K_{H3} a_H^3 + K_{H1} K_{H2} a_H^2 + K_{H1} a_H + 1} \quad (4.5)$$

In this expression K_{H1} , K_{H2} , and K_{H3} are defined in eqs 4.1 - 4.3, A_0 is the absorbance of the unprotonated ligand, A_1 is the absorbance of the monoprotonated ligand, A_2 is the absorbance of the diprotonated ligand, A_3 is the absorbance of the triprotonated ligand, and a_H is the hydrogen ion activity. The value a_H is calculated from the measured pH values according to the equation:

$$a_H = 10^{-\text{pH}} \quad (4.6)$$

Because the first protonation occurs at a much higher pH than the other protonation steps, there is little overlap in the absorbance changes associated with the subsequent protonation steps. Therefore it is possible to fit the data for K_{H1} separately as well as part of the total change. Figure IV.5 is a plot of absorbance at 400 nm versus a_H for the part of the titration related to the first protonation and is fit with the much simpler equation

$$A_i = \frac{A_1 K_{H1} a_H + A_0}{K_{H1} a_H + 1} \quad (4.7)$$

(where A_0 , A_1 , K_{H1} and a_H have the definitions previously assigned for eq 4.5).

Figure IV.6 is a plot of absorbance at 254 nm versus pH for a titration in the pH range where the second and third protonation steps occur. These data are fit with an equation including only the second and third protonation steps.

$$A_i = \frac{A_3 K_{H2} K_{H3} a_H^2 + A_2 K_{H2} a_H + A_1}{K_{H3} K_{H2} a_H^2 + K_{H2} a_H + 1} \quad (4.8)$$

The protonation constants and their log values from different data sets and various wavelengths are listed in Table IV.1.

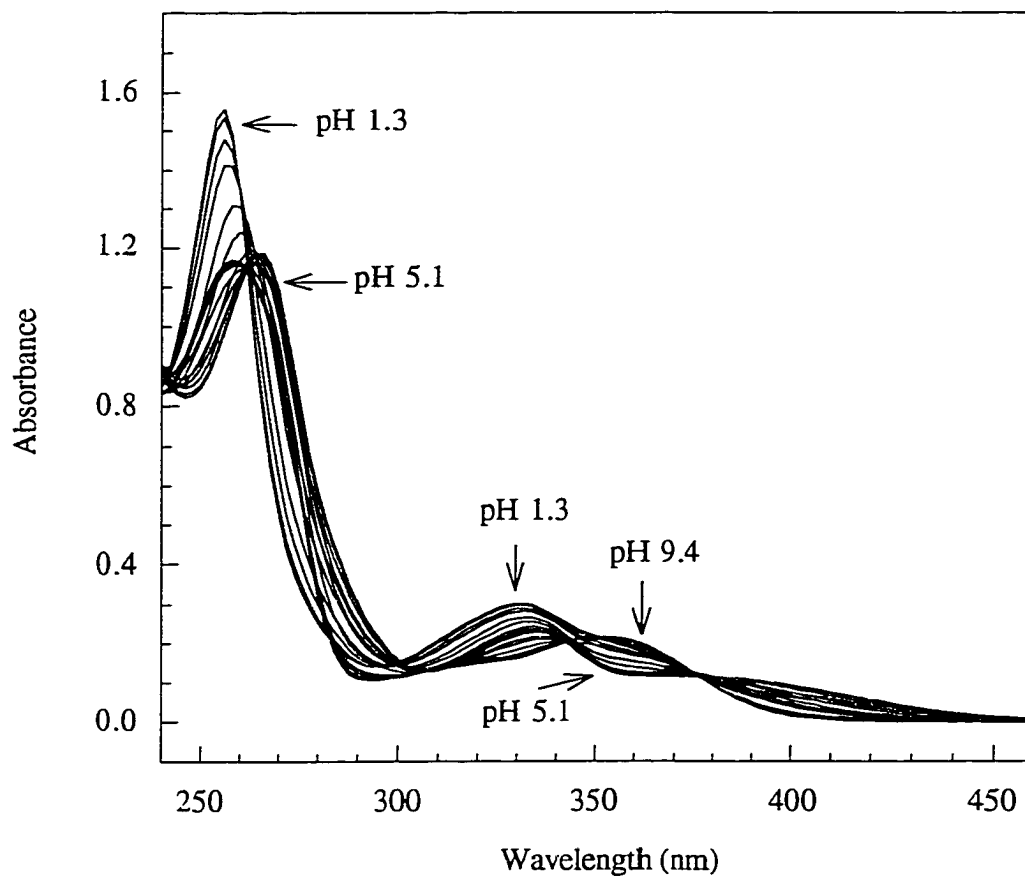


Figure IV.1 Spectra from the spectrophotometric titration of MQ1 at 25.0 °C. 2.50 mL of a solution containing 62.0 μM MQ1, 5.0 mM each of MES, MOPS, DEPP, PIPES and PIPBS, 0.2 mM EDTA and ionic strength 0.05 (TEAP) was titrated from pH 9.4 to pH 1.3 by addition of small aliquots of dilute HClO_4 .

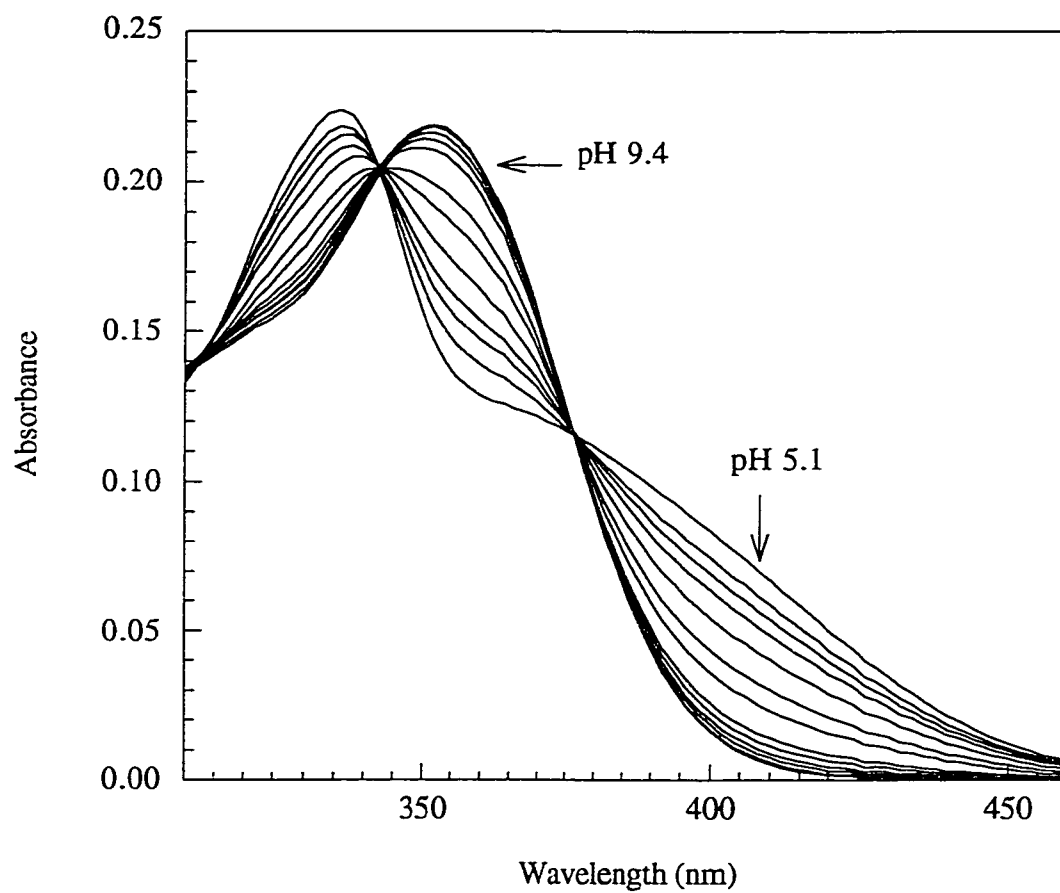


Figure IV.2 Spectra from the titration of 62.0 μM MQ1 expanded to show the isosbestic points present from pH 9.4 through pH 5.5.

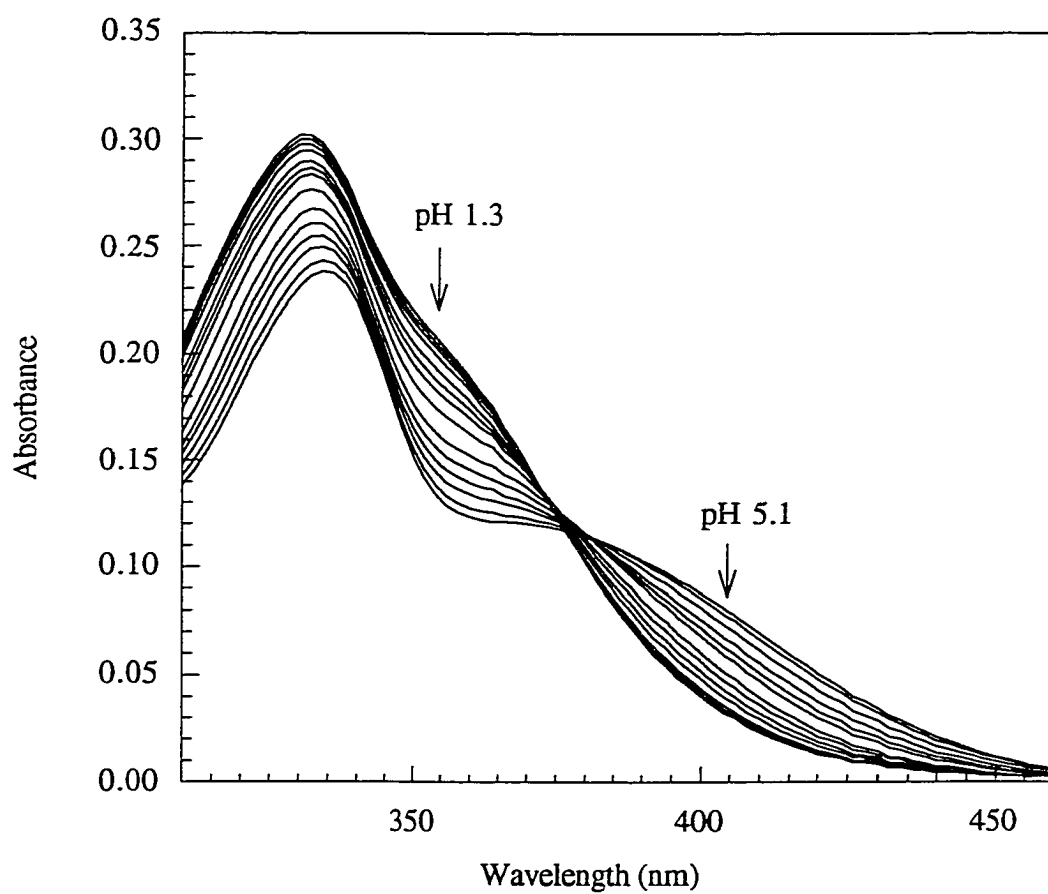


Figure IV.3 Spectra from the titration of 62.0 μM MQ1, in the pH range 5.5 - 1.3, showing the loss of isosbestic points at 344 and 377 nm. New isosbestic points appear at 382 nm (pH 4.5 - 3.0) and 372 nm (pH $\leq$ 2.5).

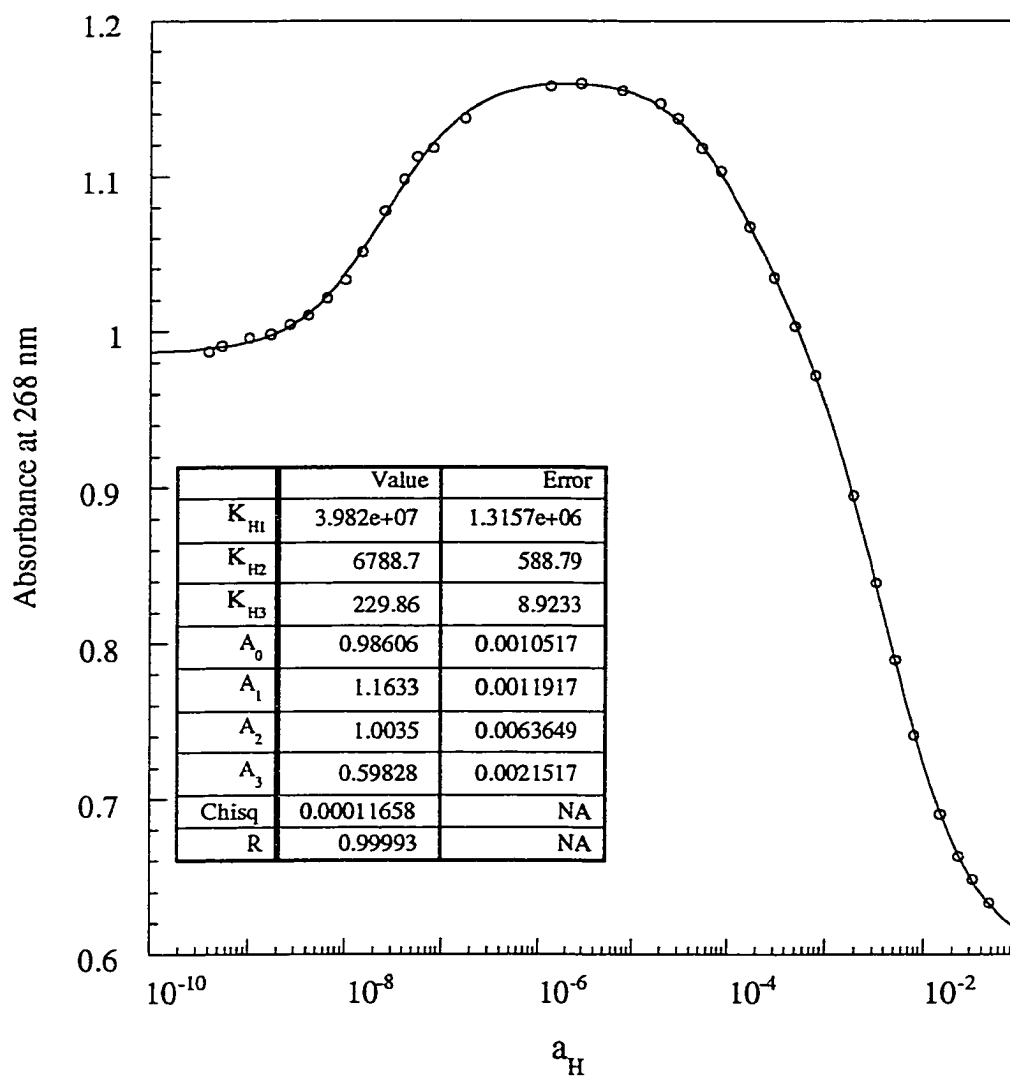


Figure IV.4 Plot of absorbance at 268 nm versus a_H for the titration of MQ1 shown in Figure IV.1. The data were fit to eq 4.5 and the solid line was calculated using the parameters listed in the inset table.

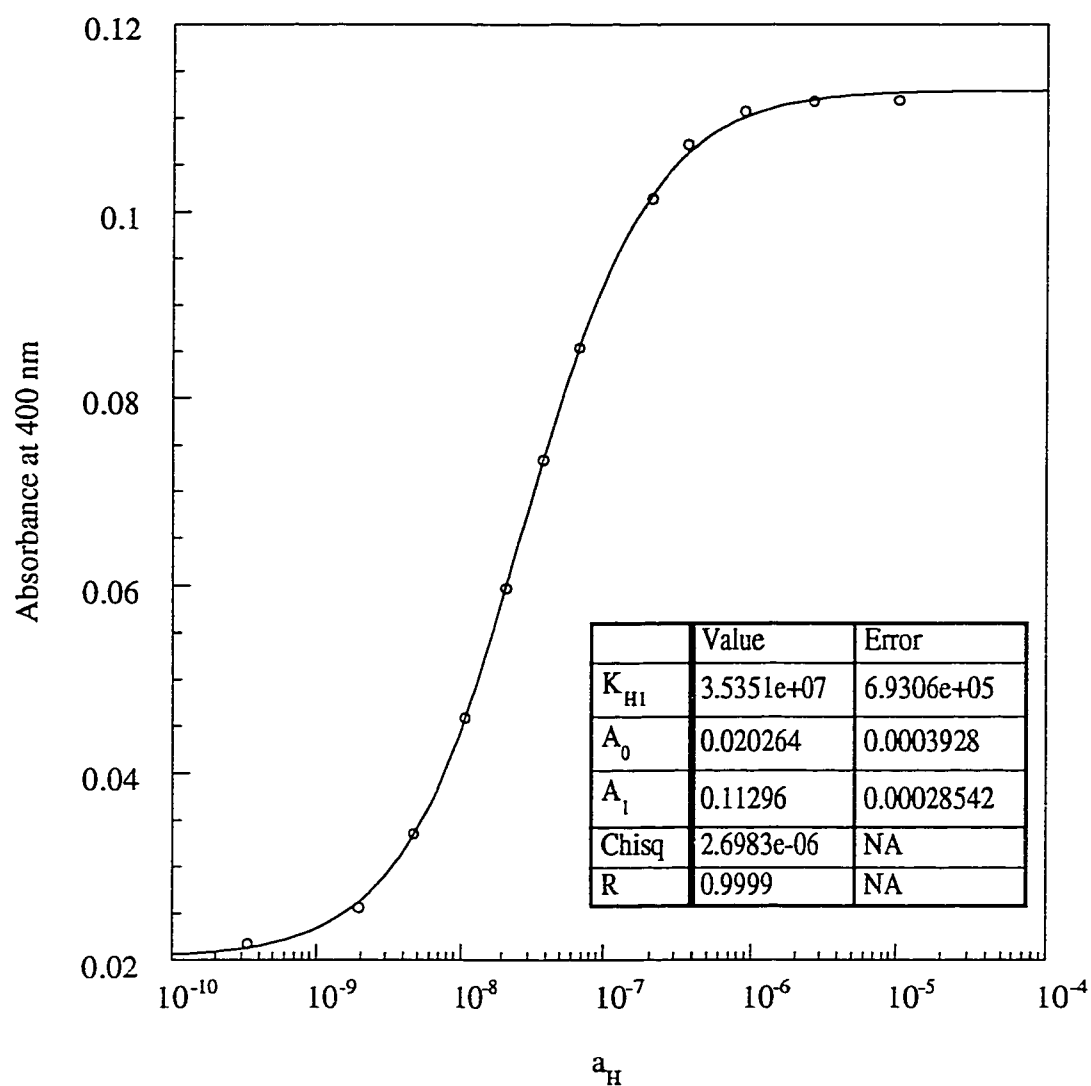


Figure IV.5 Plot of absorbance at 400 nm versus a_H for a titration of MQ1 at high pH. The data were fit to eq 4.7 and the solid line was calculated using the parameters listed in the inset table.

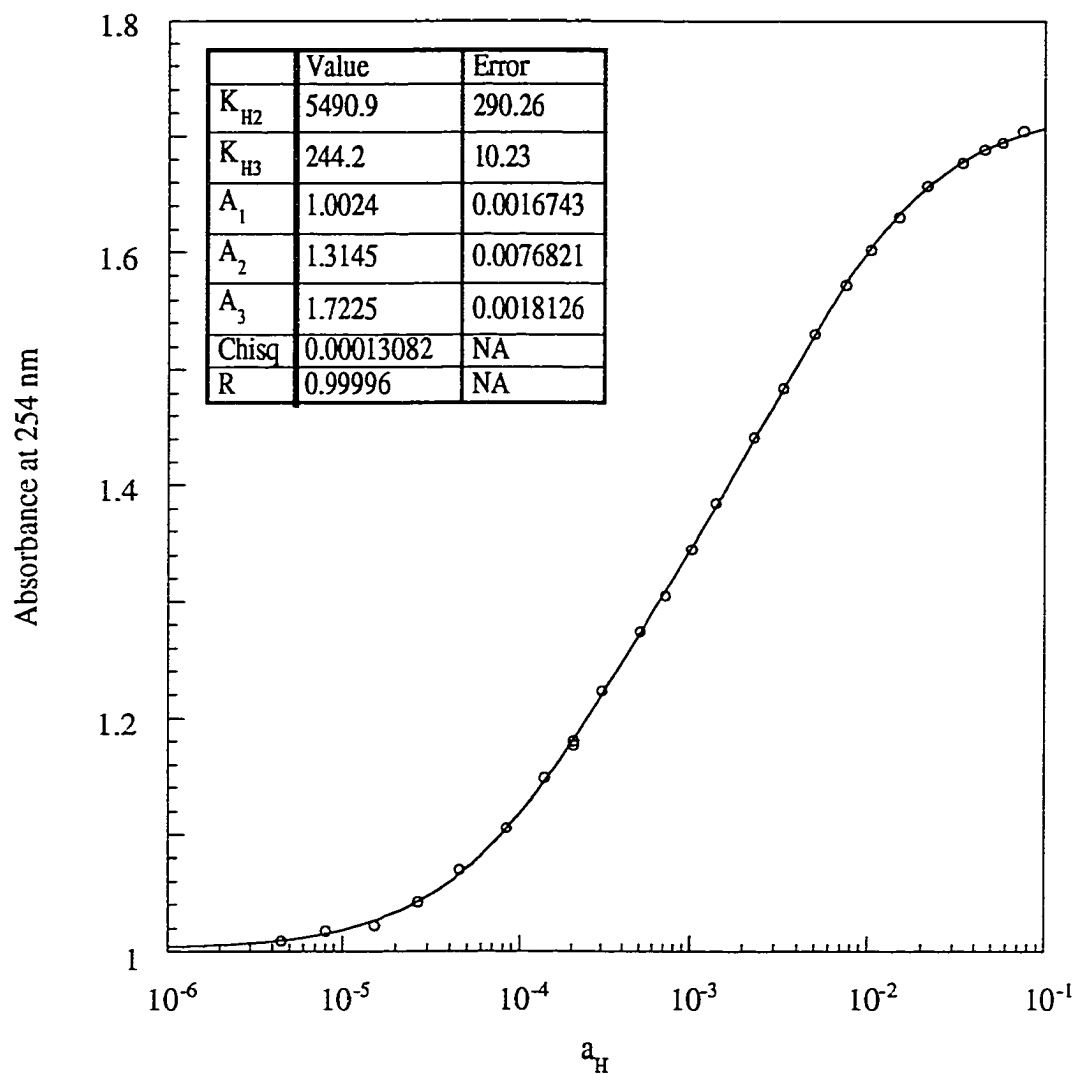


Figure IV.6 Plot of Absorbance at 254 nm versus a_H from the titration of MQ1 at lower pH. The data were fit to eq 4.8 and the solid line was calculated using the parameters listed in the inset table.

Table IV.1 Mixed-mode protonation constants with log values for MQ1 calculated at various wavelengths^{a,b}

Data Set	λ (nm)	K_{H1}^c ($\times 10^{-7}$)	K_{H2}^c ($\times 10^{-3}$)	K_{H3}^c	log K_{H1}	log K_{H2}	log K_{H3}
1MQ1	268	3.98 ± 0.13	6.79 ± 0.59	230 ± 8.9	7.60	3.83	2.36
	400	3.67 ± 0.05	6.05 ± 0.59	256 ± 23	7.56	3.78	2.41
	254	3.44 ± 0.12	5.08 ± 0.43	289 ± 20	7.54	3.71	2.46
	352	3.50 ± 0.07	4.50 ± 0.68	227 ± 28	7.54	3.65	2.36
	286	3.59 ± 0.06	4.13 ± 0.45	219 ± 38	7.56	3.62	2.34
2MQ1	286		4.83 ± 0.50	335 ± 53		3.68	2.52
	268		4.83 ± 0.30	262 ± 7.9		3.68	2.42
	254		5.49 ± 0.29	244 ± 10		3.73	2.39
3MQ1	400	3.68 ± 0.07			7.55		
	254	3.45 ± 0.16			7.54		
	268	3.94 ± 0.23			7.60		
	286	3.73 ± 0.11			7.57		
	352	3.35 ± 0.08			7.52		

^a25.0 °C, ionic strength 0.05 M (TEAP). ^b Data sets are from titrations in differing pH ranges. ^c Uncertainty expressed as standard error.

1 Potentiometric Method

In potentiometric titrations to determine protonation constants for MQ1, 5.0 mL samples of solution contained 0.930 mM MQ1, known amounts of HClO_4 to partially neutralize the ligand, and sufficient TEAP to maintain the ionic strength at 0.05 M. The solutions were titrated at 25.0 °C with 5.21 mM or 4.96 mM Et_4NOH containing sufficient TEAP to maintain ionic strength at 0.05 M. The automated titration system collected data as pH observed and volume of Et_4NOH added. These data were used by the PKAS¹ program to calculate the first two protonation constants. The potentiometric methods employed in these titrations were not suitable to determine the protonations occurring at very acidic pH and reliable values were not calculated by PKAS for the third and fourth protonation steps.

Figure IV.7 shows the curve obtained from a potentiometric titration of MQ1, overlaid with the curve calculated by PKAS based on the derived values of K_{H1} , the mmoles of MQ1 present, the volume of the initial solution, the volume and formality of standardized base added and mmoles of excess acid included in the initial solution. The values of $\log K_{\text{H1}}$ and $\log K_{\text{H2}}$ calculated by PKAS, the standard deviation in log units determined using eq 3.7 and 3.8, and the overall sigma value (standard deviation in % $[\text{H}^+]$) are given in Table IV.2.

Table IV.2 Mixed-mode protonation constants for MQ1 from potentiometric measurements^a

Data Set	$\log K_{\text{H1}}$	sigma K_{H1} ^b	$\log K_{\text{H2}}$	sigma K_{H2} ^b	σ % ^c
1	7.55	0.04	3.85	0.05	5.72
2	7.62	0.04	3.67	0.02	8.26

^a 25.0 °C, ionic strength 0.05 M (TEAP). ^b sigma = $(U/N)^{1/2}$; where $U = \sum w(p[\text{H}]_{\text{obs}} - p[\text{H}]_{\text{calc}})^2$ and $N = \sum w$. ^c Uncertainty in terms of standard deviation in % $[\text{H}^+]$ for the entire titration.

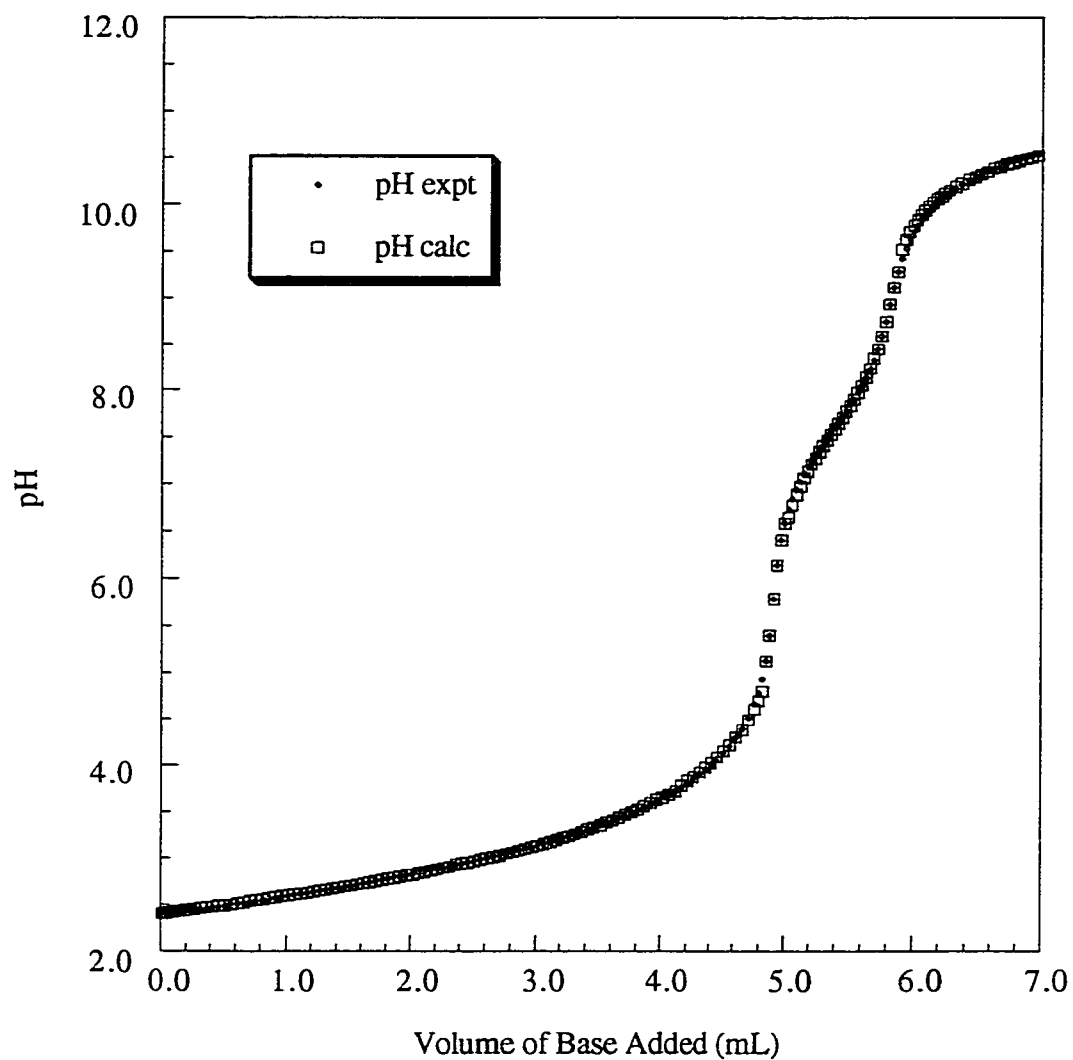


Figure IV.7 The potentiometric titration curve of 0.930 mM MQ1 with 5.21 mM Et_4NOH , ionic strength 0.05 M (TEAP), at 25.0 °C. The experimental curve is overlaid with the curve calculated by PKAS.

A Stoichiometry and Spectral Characterization of MQ1

UV-vis spectrophotometric titration was used to investigate the spectral properties and complexation stoichiometry of MQ1 with copper, nickel, cadmium, lead, zinc and calcium. The ligand solutions contained 49.0 - 62.0 mM MQ1, 5.0 mM MES or PIPES buffer, and sufficient TEAP to maintain ionic strength at 0.05 M. These experiments were conducted at pH 6.2 - 7.2, to approach physiological pH, with temperature maintained at 25.0 °C. Even numbered Figures IV.8 - IV.18 are spectra from these titrations. Odd numbered Figures IV.9 - IV.19 are plots in which the fraction of the absorbance change at a specific wavelength is plotted against the ratio of total metal to total ligand. In the fraction of absorbance plots the vertical dashed line highlights the 1:1 stoichiometric ratio and the slanted dashed line traces the linear response that would be obtained if the metal is quantitatively complexed by the ligand with 1:1 stoichiometry. The horizontal dashed line denotes the lack of absorbance change that would occur when all the ligand has been complexed and no other complex formation was affecting the absorbance. The behavior of MQ1 when titrated with lead illustrates this behavior. Figure IV.11 shows that for each addition of an aliquot of lead the line indicating quantitative binding is followed almost exactly, indicating $\geq 99\%$ binding of the lead with MQ1. Past the vertical line designating equal concentrations of lead and MQ1, no further absorbance change is observed. The behavior shown by lead indicates essentially complete binding of one molecule of MQ1 to each lead ion. Analogous plots for cadmium, copper, zinc and nickel also indicated quantitative binding by MQ1 with a 1:1 stoichiometry. No plot is shown for the titration of MQ1 with calcium due to the lack of an absorbance change in any stoichiometrically relevant range. This behavior does not rule out one to one binding of calcium by MQ1 but does imply that MQ1 has little affinity for calcium.

From the spectra it was possible to determine wavelengths of maximum absorbance (λ_{max}), molar absorptivity at λ_{max} (ϵ_{max}), and isosbestic points observed during complex formation, for most of the complexes. ϵ_{max} was calculated using the Beer-Lambert Law, eq 4.9, and the recorded absorbance at λ_{max} .

$$\epsilon_{\lambda} = \frac{A_{\lambda}}{bc} \quad (4.9)$$

The pathlength (b) was 1.0 cm in all cases. The molar concentration of the metal-ligand complex (c) was assumed to be the same as the initial concentration of MQ1 for metals that were quantitatively bound.

In the case of calcium the nitrate salt was used and many spectral characteristics were unobtainable due to the absorbance maximum of the nitrate in the region of the spectrum where λ_{max} for the CaMQ1 complex occurs. In the case of copper isosbestic points are blurred and the experimental lines deviate somewhat from the theoretical lines. This may be caused by a tendency of Cu^{2+} to form complexes containing two ligand molecules when an excess of ligand is present and will be discussed further in chapter IV.

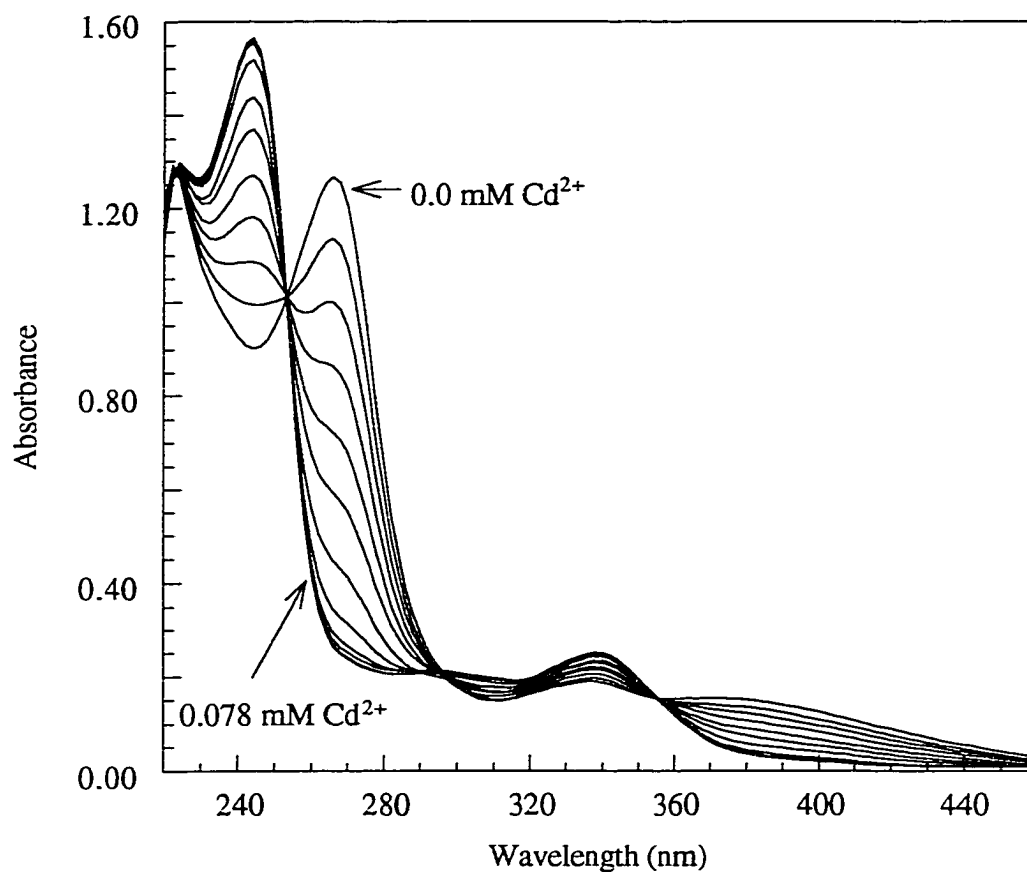


Figure IV.8 Spectra from the titration of MQ1 with cadmium at 25.0 °C. 4.0 μL aliquots of 4.16 mM Cd^{2+} were used to titrate 2.50 mL of 49.0 μM MQ1 solution, buffered at pH 6.8 with 5.0 mM PIPES, at an ionic strength of 0.05 M (TEAP).

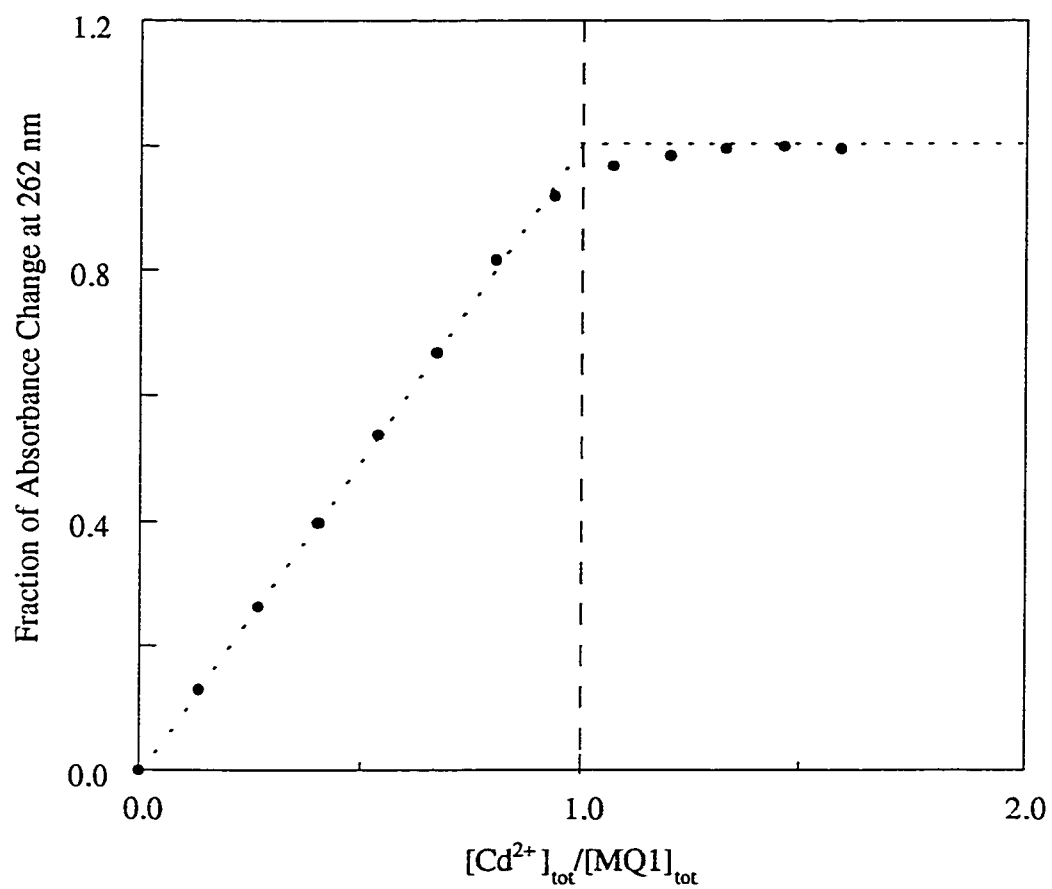


Figure IV.9 Fraction of the absorbance change versus the ratio of total cadmium to total MQ1 from the titration of MQ1 at pH 6.8.

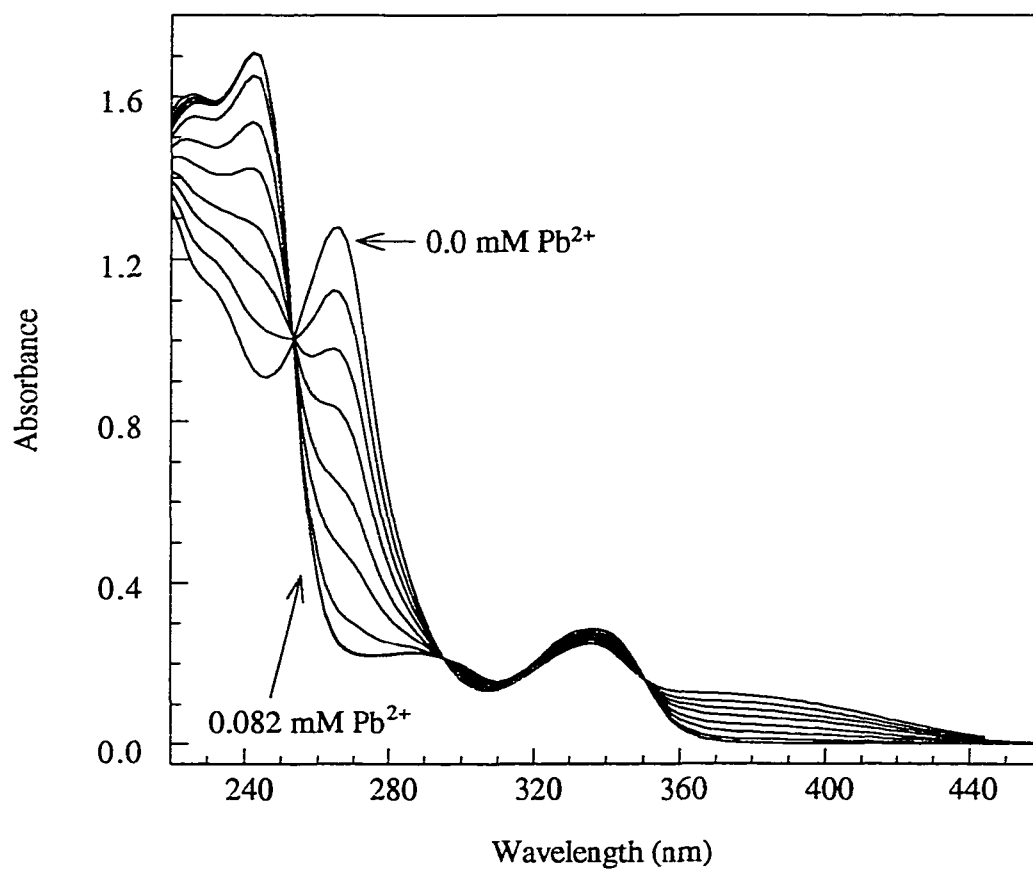


Figure IV.10 Spectra from the titration of MQ1 with lead at 25.0 °C. 4.0 μL aliquots of 6.38 mM $\text{Pb}(\text{ClO}_4)_2$ 2.50 mL of 62.0 μM MQ1 solution, buffered at pH 6.4 with 5.0 mM MES, at an ionic strength of 0.05 M (TEAP).

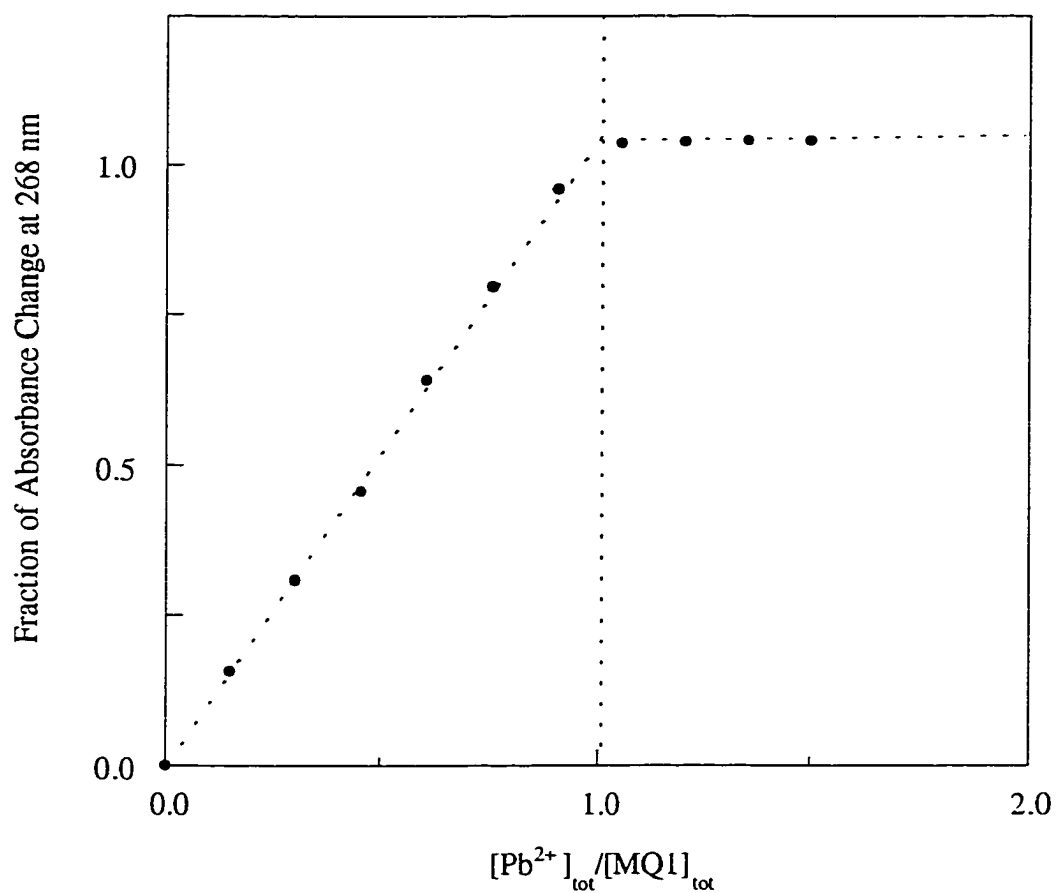


Figure IV.11 Fraction of the absorbance change as a function of the ratio of total lead to total MQ1 at pH 6.4.

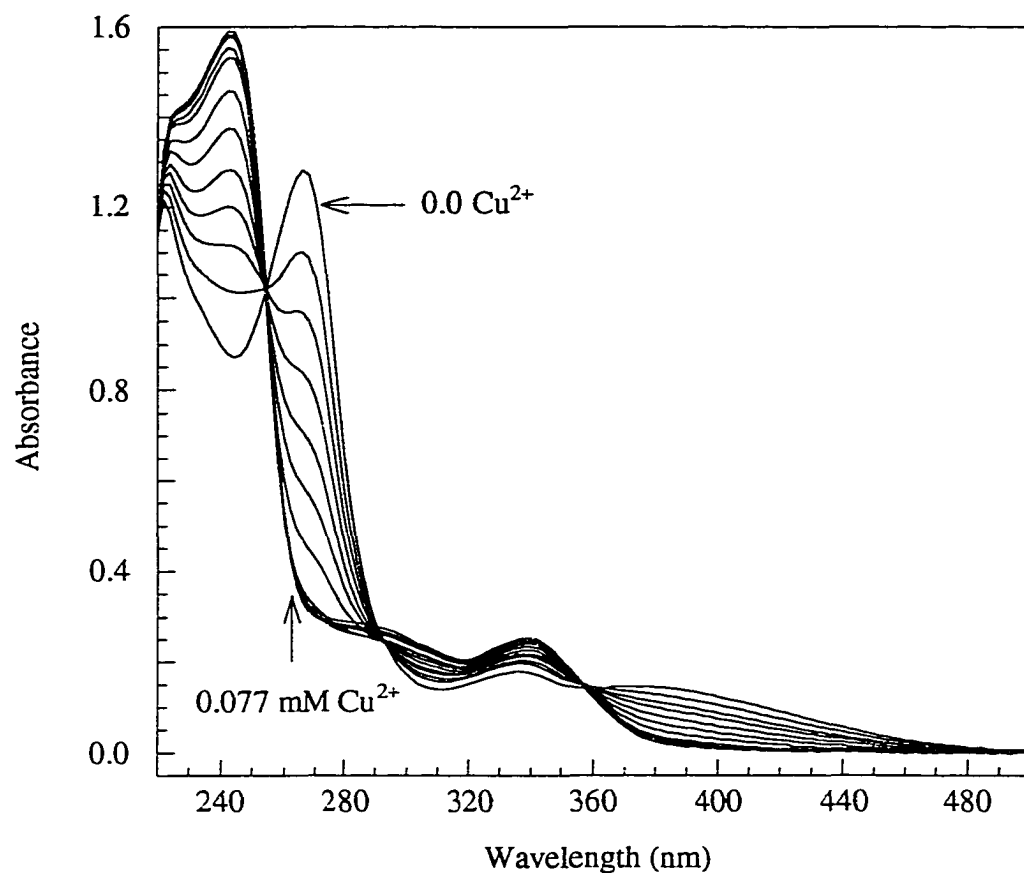


Figure IV.12 Spectra from the titration of MQ1 with copper at 25.0 °C. 4.0 μL aliquots of 4.10 mM Cu^{2+} were used to titrate 2.50 mL of 49.0 μM MQ1 solution, buffered at pH 6.5 with 5.0 mM PIPES, at an ionic strength of 0.05 M (TEAP).

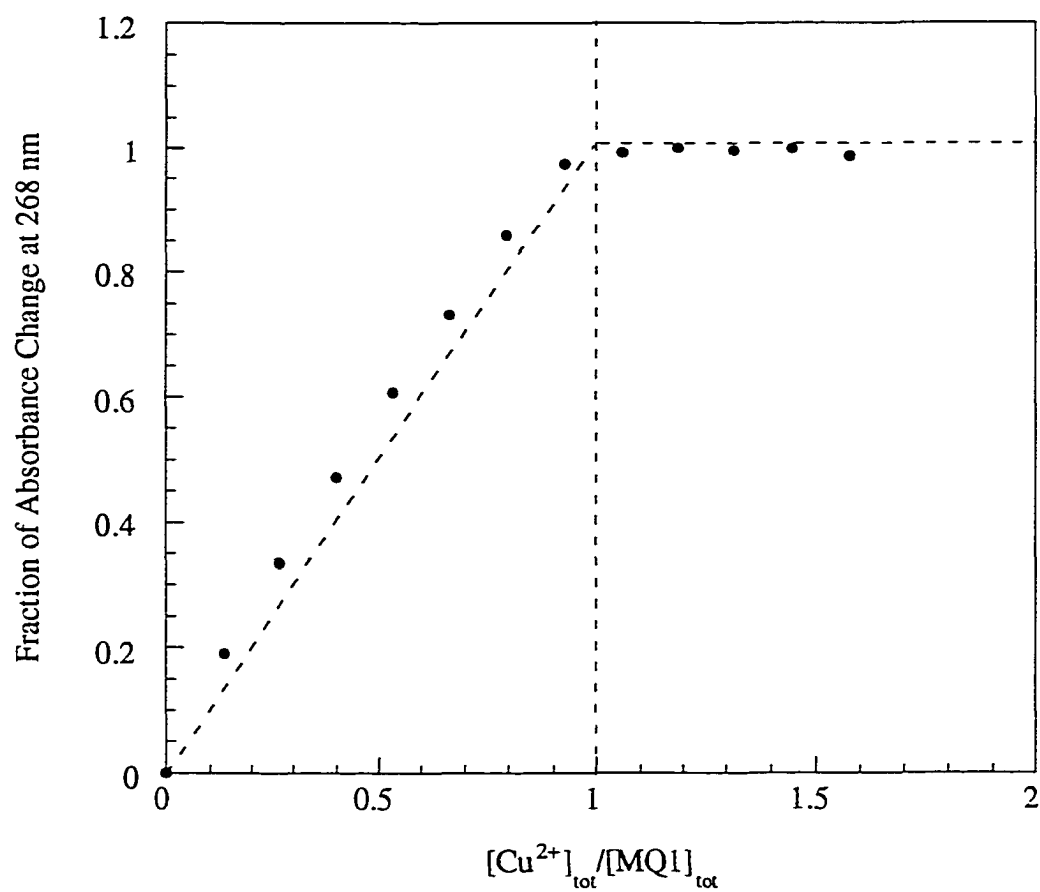


Figure IV.13 Fraction of the absorbance change versus the ratio of total copper to total MQ1.

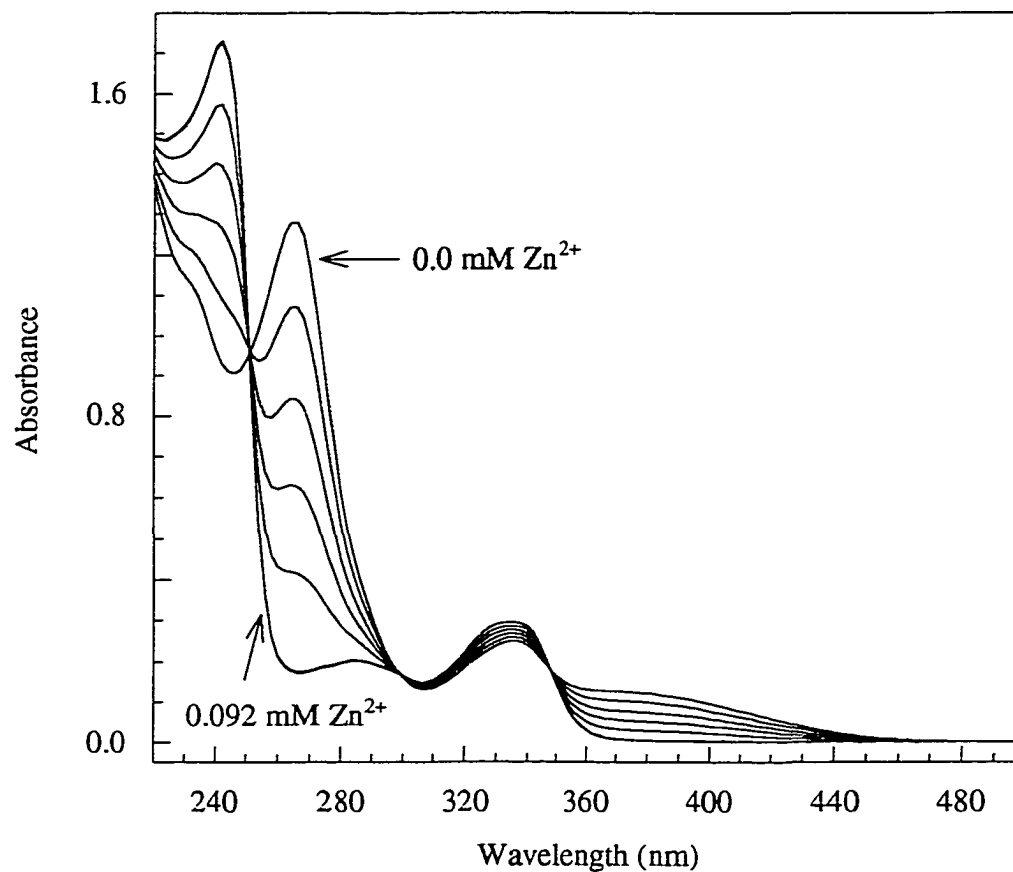


Figure IV.14 Spectra from the titration of MQ1 with zinc at 25.0 °C. 4.0 μL aliquots of 7.30 mM $\text{Zn}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution, buffered at pH 6.4 with 5.0 mM MES, at an ionic strength of 0.05 M (TEAP).

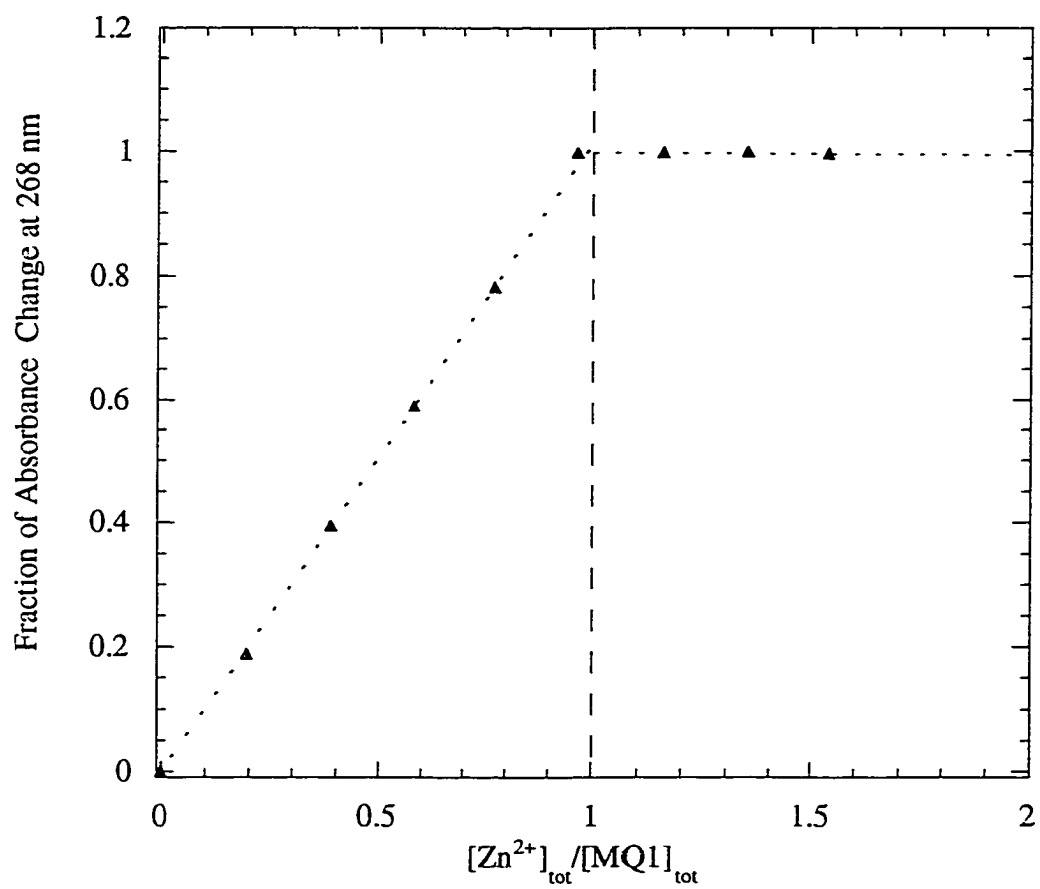


Figure IV.15 Fraction of the absorbance change as a function of the ratio of total zinc concentration to total MQ1 concentration.

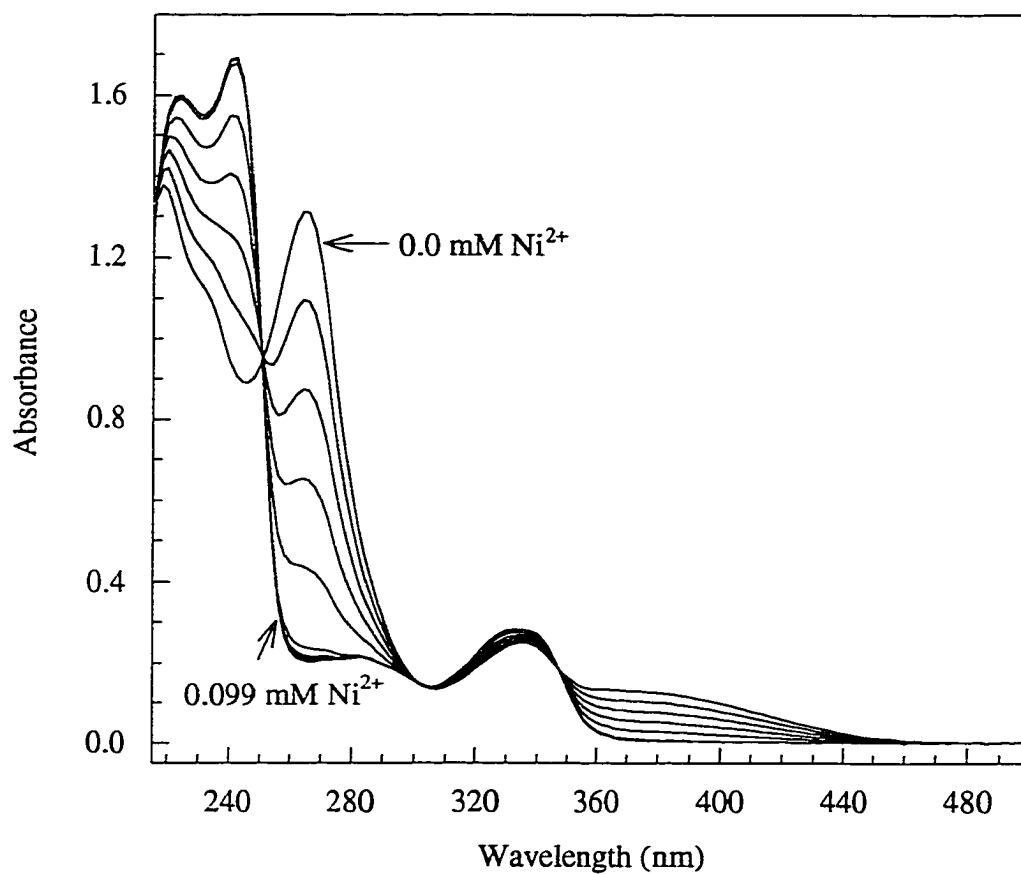


Figure IV.16 Spectra from the titration of MQ1 with nickel at 25.0 °C. 3.0 μL aliquots of 9.31 mM $\text{Ni}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 56.0 μM MQ1 solution, buffered at pH 6.2 with 5.0 mM MES, at an ionic strength of 0.05 (TEAP).

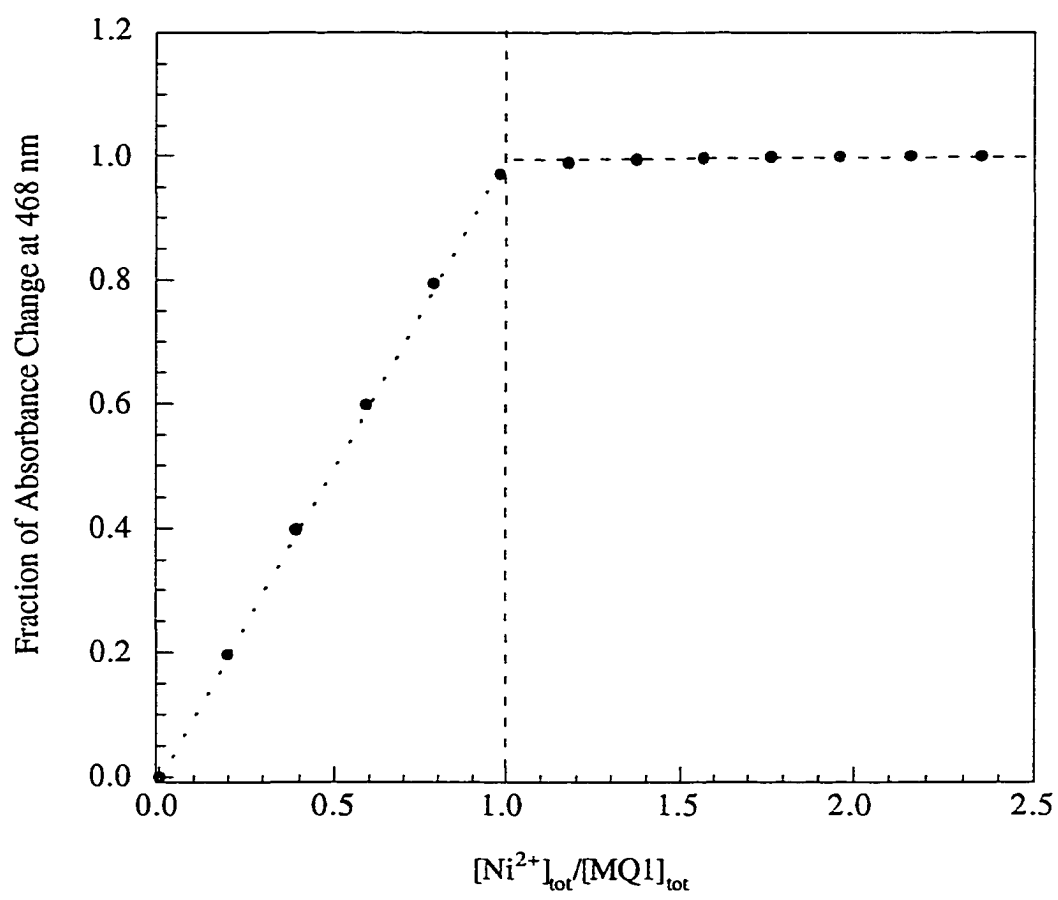


Figure IV.17 Fraction of the absorbance change versus the ratio of total nickel to total MQ1 at pH 6.2.

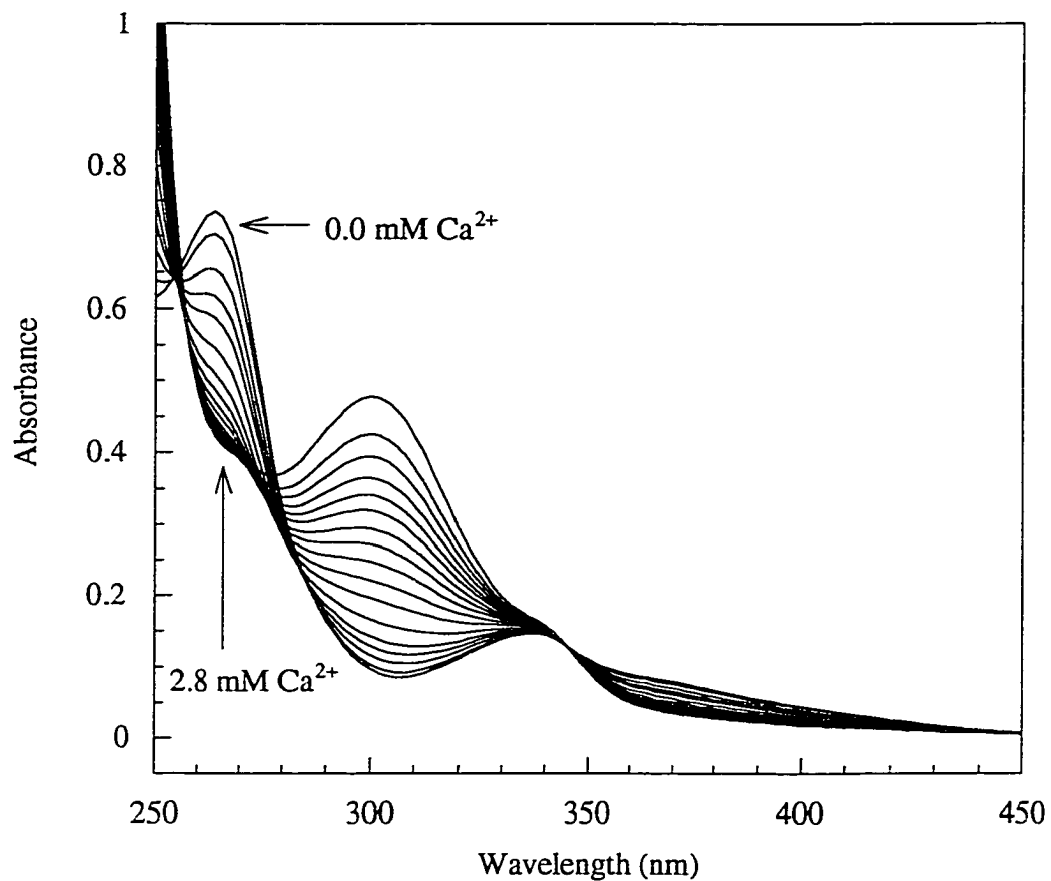


Figure IV.18 Spectra from the titration of MQ1 with calcium at 25.0 °C. 4.0 μL aliquots of 0.265 M and 0.530 M $\text{Ca}(\text{NO}_3)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution, buffered at pH 7.15 with 5 mM PIPES, at an ionic strength of 0.05 (TEAP).

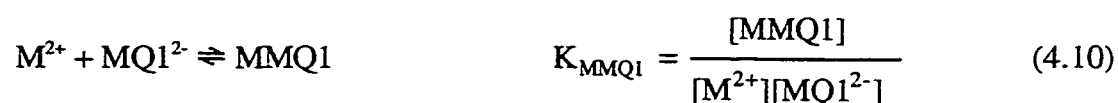
Table IV.3 Spectral characteristics and stoichiometry for MQ1 complexes^a

Complex	pH	Stoichiometry	λ_{max} (nm)	$\epsilon_{\lambda_{\text{max}}}$	Isosbestic points (nm)
MQ1 ²⁻	9.4		258	18,710	
			352	3,520	
HMQ1 ⁻	5.6		266	19,130	312, 344, 377
			337	3,610	
CdMQ1	6.8	1:1	244	31,600	254, 295, 352
PbMQ1	6.4	1:1	242	27,600	254, 295, 352
Cu MQ1	6.5	1:1	242	32,300	252, 292?, 355
Zn MQ1	6.4	1:1	240	27,900	252, 298, 348
NiMQ1	6.2	1:1	240	30,200	248, 300, 346
CaDNB	7.2				342

a) 25.0 °C, ionic strength 0.05 M (TEAP).

A Complex Formation Constants of MQ1

Formation of 1:1 complexes of MQ1 with divalent metal ions is described by the reaction equation



Spectrophotometric titrations of MQ1 with metal ions indicate that this is the stoichiometry of the complex formed by MQ1 with most of the various metals investigated in the pH range 6.2 - 7.2. Although this stoichiometry is indicated, further investigation employing potentiometric titration or other pHs may reveal more complex interactions or interference due to side reactions. Therefore, interpretation of data for one method may warrant further refinements of the results obtained from other methods. The following sections give results from UV-vis spectrophotometric titrations and potentiometric titrations..

1 UV-vis spectrophotometric determination of complex constants

Data obtained from spectrophotometric titration of MQ1 solutions with metal ions were used to determine complexation constants. Each titration used 2.5 mL of solution containing 49.0 or 62.0 μ M MQ1, 5.0 mM PIPES, and sufficient TEAP to maintain the ionic strength at 0.05 M. The solutions were buffered at pH 3.0, except for the solution titrated with calcium which was buffered at pH 7.2. The titration temperature was controlled at 25.0 °C using a water-jacketed cell holder. The titration was performed by

addition of small (3.0 - 4.0 μL) aliquots of standardized metal solution directly to the cell. The cell was inverted several times, to ensure adequate mixing, then placed back in the cell holder. Two minutes were allowed for the mixture to reach thermal and chemical equilibrium before the spectrum was scanned from 190 - 200 nm using the diode array spectrophotometer with an integration time of 1 second. Even numbered Figures IV.19. through IV.29 show the UV-vis spectra from titrations of MQ1 with cadmium, lead, copper, zinc, nickel and calcium. Specific information about concentrations of MQ1 and the metal titrants for each titration are given in the figure captions.

Data from these sets of spectra were used in the successive approximation process described below to approach the actual metal ion concentrations, $[\text{M}^{2+}]$, and fit the calculated values to obtain conditional, mixed-mode stability constants. The constants are called conditional constants due to pH-dependent side reactions of the ligand which may cause the percentage of the ligand concentration present in a specific state of protonation to vary significantly with pH. The dependence of these constants on the protonation state of the ligand is signified by a prime sign. A further interference is possible due to side reactions of the metal-MQ1 complex, and is signified by an additional prime sign. Therefore, the conditional stability constant is denoted as K''_{MMQ1} . K''_{MMQ1} is based on the reaction in eq 4.10 and is defined by the relationship:

$$K''_{\text{MMQ1}} = \frac{[\text{MMQ1}']}{[\text{M}'][\text{MQ1}']} \quad (4.11)$$

In this expression $[\text{MMQ1}]' = [\text{MMQ1}] + \Sigma[\text{MH}_i\text{MQ1}]$, $[\text{MQ1}]' = [\text{MQ1}^{2-}] + [\text{HMQ1}^-] + [\text{H}_2\text{MQ1}] + [\text{H}_3\text{MQ1}^+] + [\text{H}_4\text{MQ1}]$, and $[\text{M}]'$ is the concentration of uncomplexed metal ion. The absorbance values for each titration are related to $[\text{M}]'$ by the equation,

$$A_i = \frac{A_1 K_{\text{MMQ1}}'' [\text{M}]' + A_0}{K_{\text{MMQ1}}'' [\text{M}]' + 1} \quad (4.12)$$

where A_1 and A_0 are the limiting absorbance values of the metal-ligand complex and the free ligand, respectively.

Initial estimates for the calculated parameters A_1 and A_0 are taken from the spectra, and an estimate of K_{MMQ1}'' is obtained from fraction of absorbance change versus metal:ligand ratio plots. Using these initial estimates, the A_i , $[\text{M}]'$ data are fit to eq 4.12 using the total metal concentration as an initial estimate of free metal ion, $[\text{M}]'$. The curve-fitting program produces refined values for the adjustable parameters. The value of K_{MMQ1}'' obtained from this fit is then used to calculate the concentration of the metal-ligand complex using the following equation,

$$[\text{MMQ1}]_i = \frac{K_{\text{MMQ1}}'' (C_{\text{MQ1}} + C_{\text{M}}) + 1 - \sqrt{(K_{\text{MMQ1}}'' (C_{\text{MQ1}} + C_{\text{M}} + 1)^2 - 4K_{\text{MMQ1}}''^2 C_{\text{MQ1}} C_{\text{M}}}}{2K_{\text{MMQ1}}''} \quad (4.13)$$

where C_{MQI} and C_{M} represent the total ligand and total metal concentrations, respectively, and $[\text{MMQI}]_i'$ is the metal-ligand complex concentration calculated for the i^{th} iteration titration point. New values are obtained for the of metal-ligand complex for each step in the titration. These new values are then subtracted from the total metal concentrations to get a new set of free metal concentrations, $[\text{M}]'$. The adjusted values of $[\text{M}]'$ are then fit to eq 4.12 using the calculated values from the previous fit as the estimates of the parameters. The second calculated value of K_{MMQI}^* is used in eq 4.13 to recalculate values for $[\text{M}]'$. This process is repeated until the calculated parameters converge to values that do not vary by more than 2.0% from the previous iteration.

Even numbered Figures IV.20 - IV.30 are plots of the measured absorbance after each addition of metal solution versus the final calculated metal ion concentrations. The curve-fitting procedure is applied and the values for the estimated variables in eq 4.12 are listed in the inset tables. The value K_{MMQI}'' in each inset table is the result from the curve-fitting process using the final free metal approximation. The K_{MMQI}'' values displayed in the inset tables represent the refined values of the stability constants.

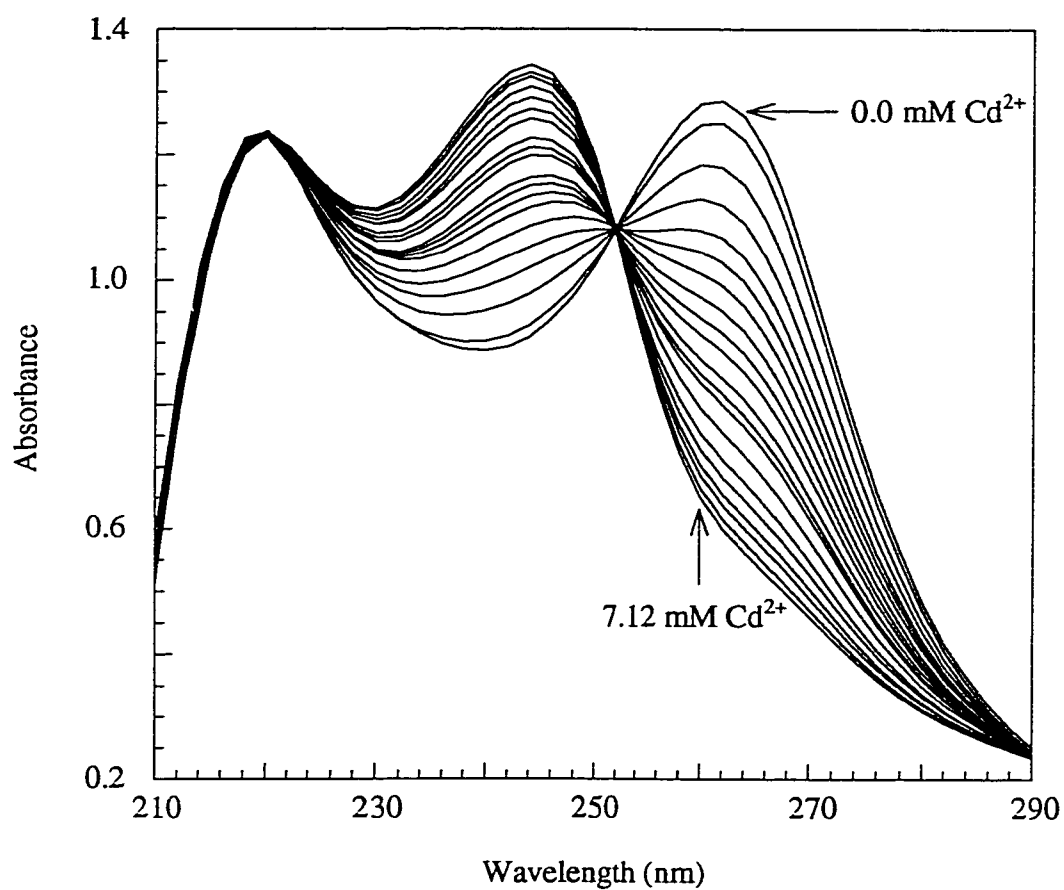


Figure IV.19 Spectra from the titration of MQ1 with cadmium. 4.0 μL aliquots of 4.156 mM $\text{Ni}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 49.0 μM MQ1 solution buffered at pH 3.0 with 5.0 mM PIPES at 25.0 $^\circ\text{C}$ and ionic strength 0.05 M (TEAP).

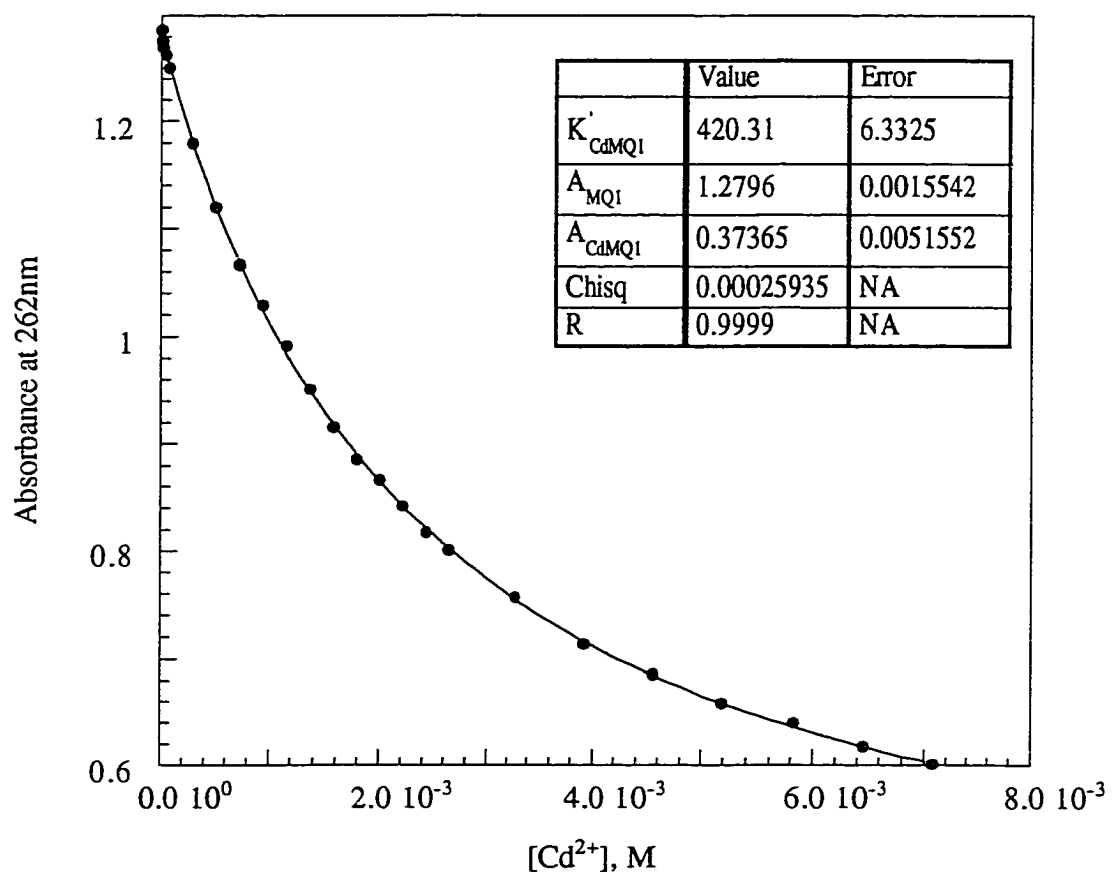


Figure IV.20 Absorbance at 262 nm as a function of the final approximation of cadmium ion concentration at pH 3.0. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

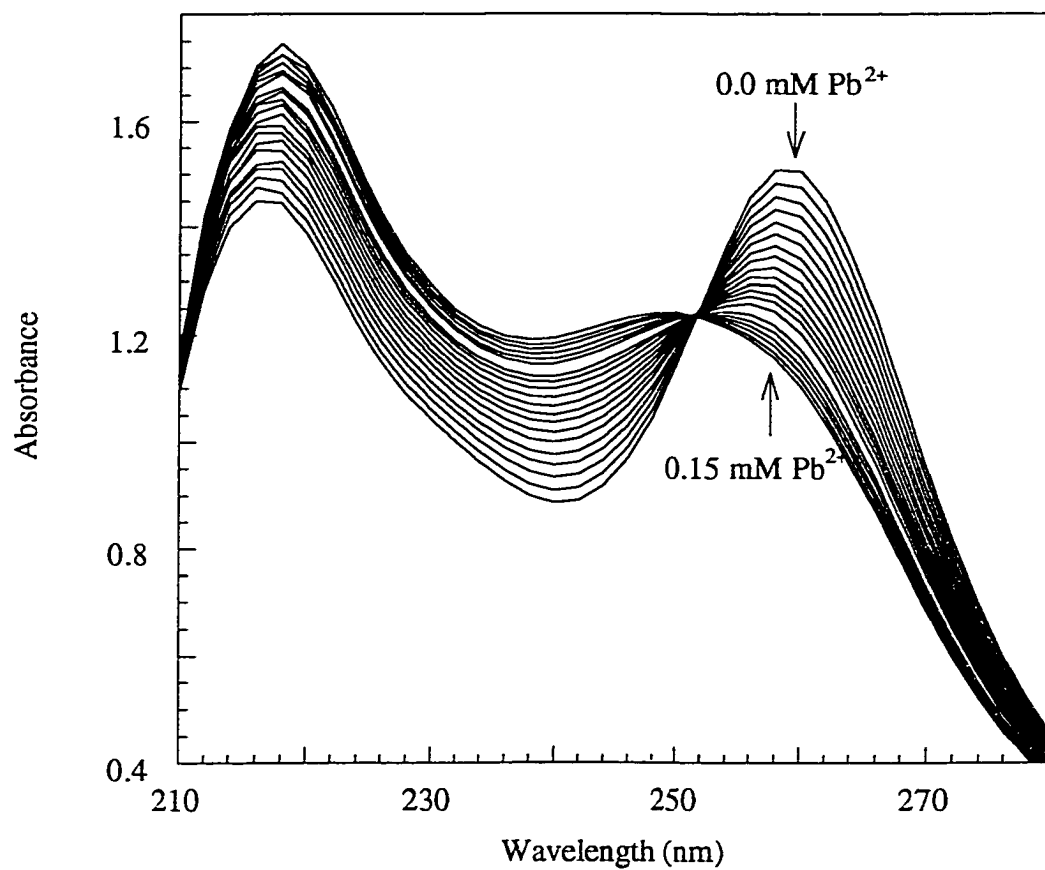


Figure IV.21 Spectra from the titration of MQ1 with lead at 25.0 °C. 3.0 μL aliquots of 6.4 mM $\text{Pb}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution, buffered at pH 3.0 with 5.0 mM PIPES, at an ionic strength of 0.05 M (TEAP).

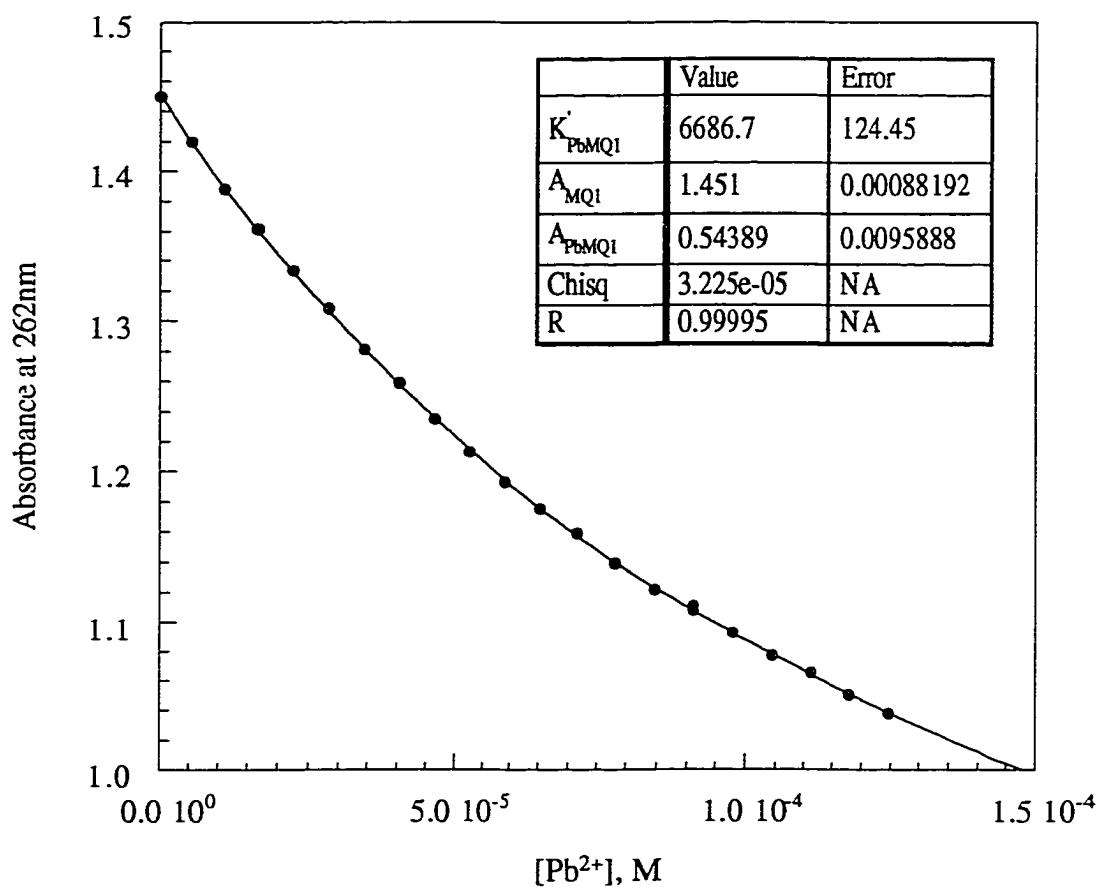


Figure IV.22 Absorbance at 262 nm and pH 3.0 as a function of the final approximation of free lead ion. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

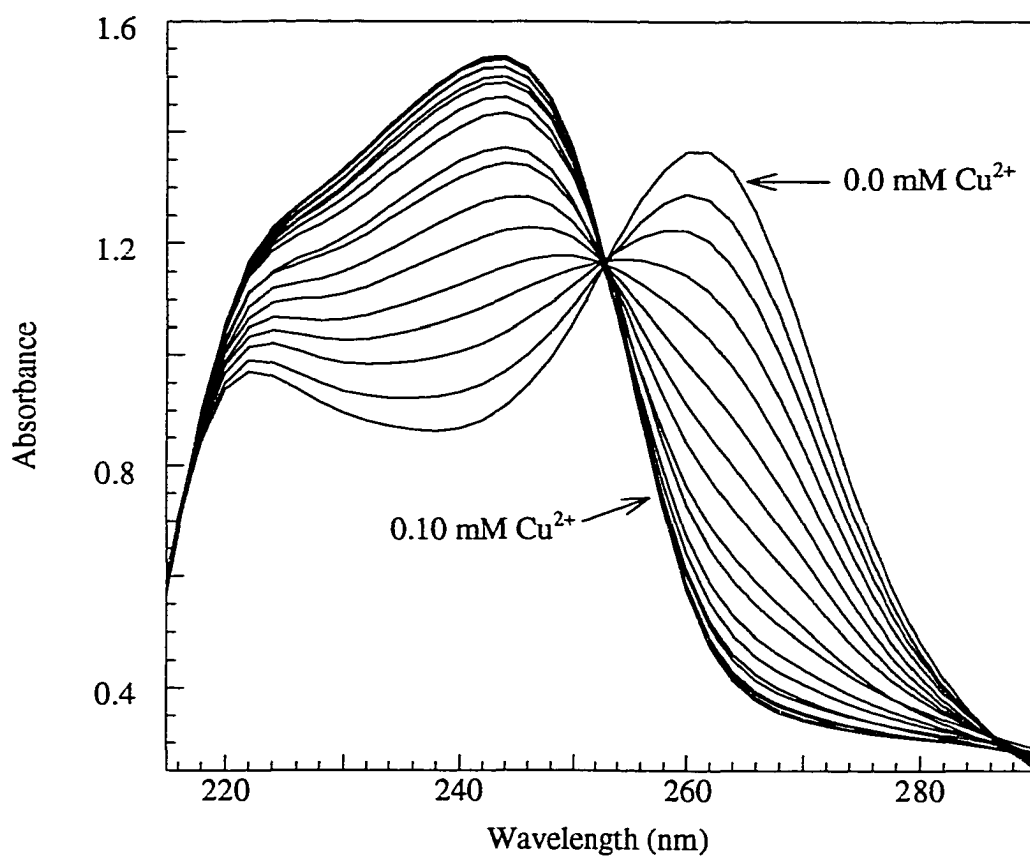


Figure IV.23 Spectra from the titration of MQ1 with copper at 25.0 °C. 3.0 μL aliquots of 4.096 mM CuCl_2 were used to titrate 2.50 mL of 49.0 μM MQ1 solution buffered at pH 3.0 with 5.0 mM PIPES at an ionic strength of 0.05 M (TEAP).

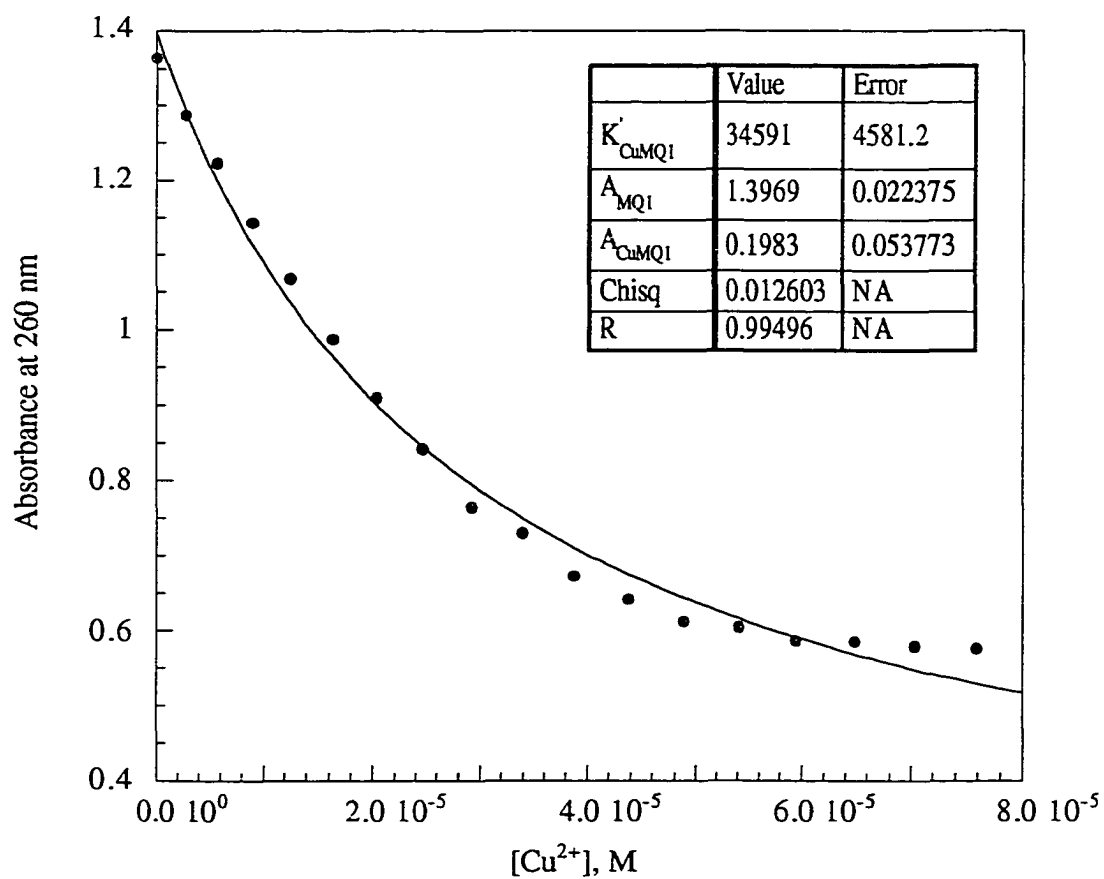


Figure IV.24 Absorbance at 260 nm at pH 2.8 as a function of the final approximation of free copper ion concentration. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

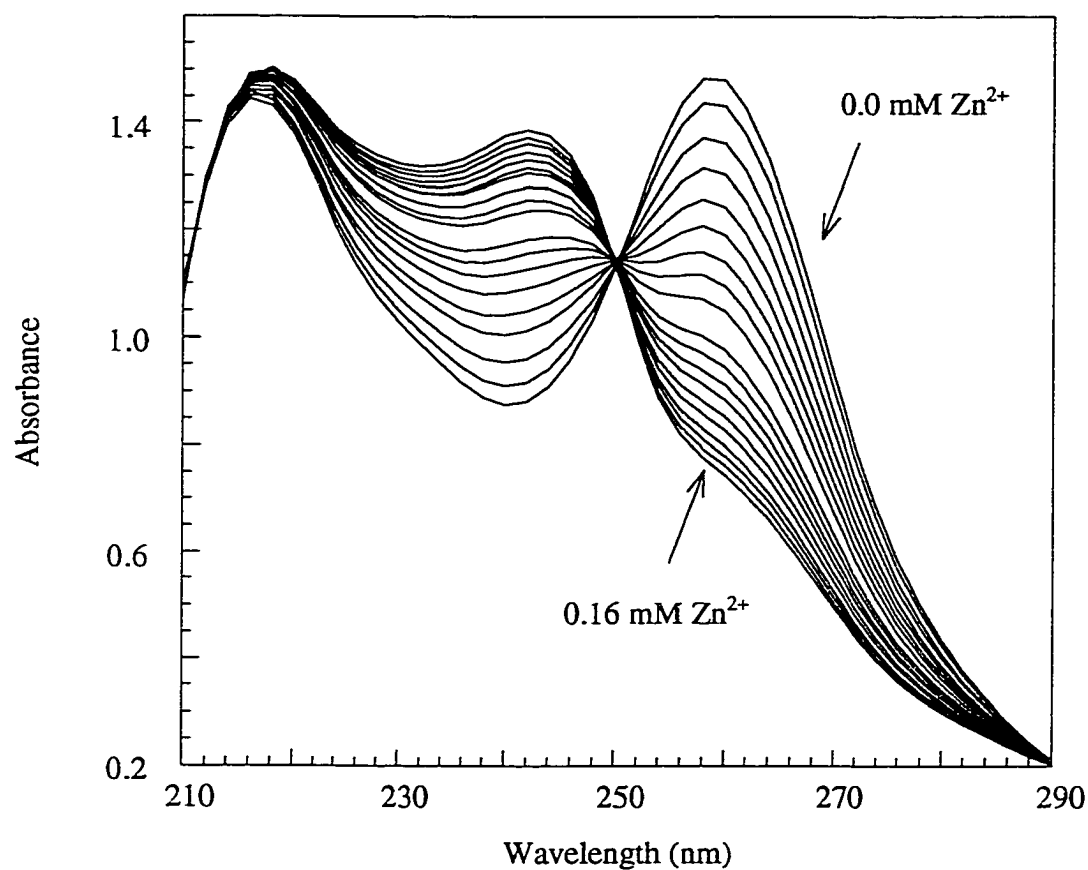


Figure IV.25 Spectra from the titration of MQ1 with zinc at 25.0 °C. 3.0 μL aliquots of 7.5 mM $\text{Zn}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution buffered at pH 3.0 with 5.0 mM PIPES at an ionic strength of 0.05 M (TEAP).

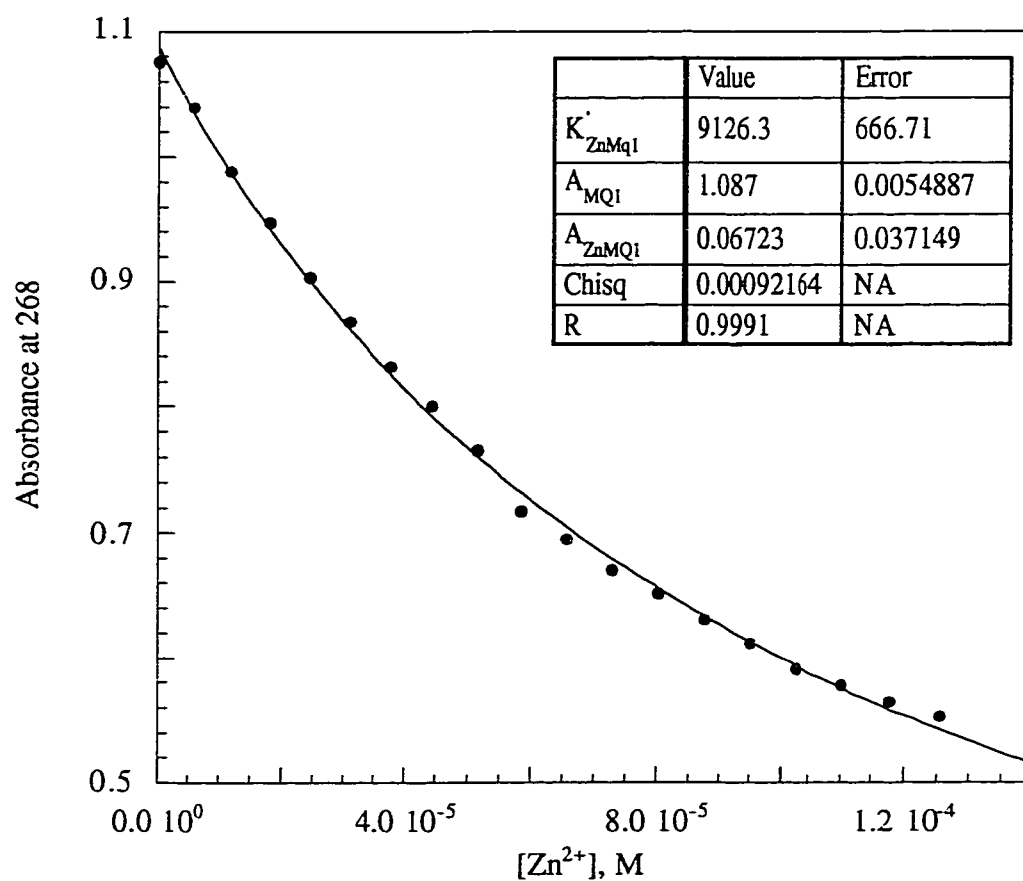


Figure IV.26 Absorbance at 268 nm at pH 3.0 as a function of the final approximation of free zinc ion concentration. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

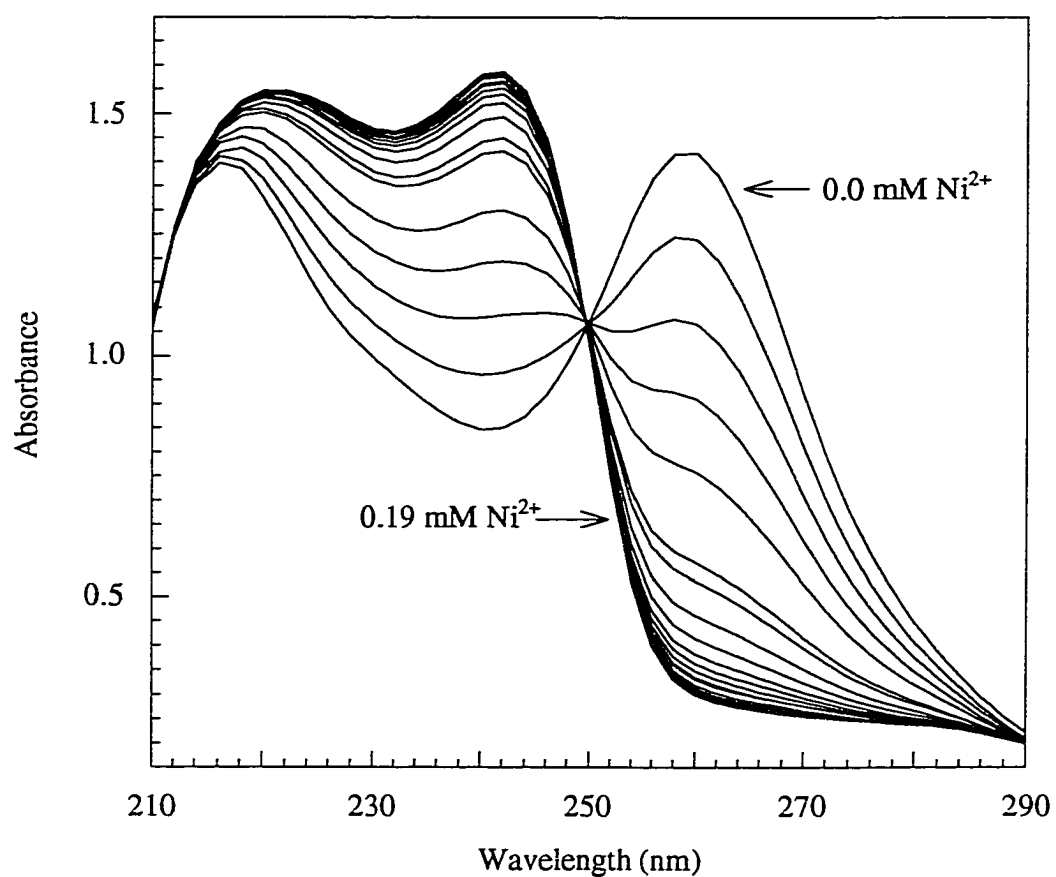


Figure IV.27 Spectra from the titration of MQ1 with nickel at 25.0 °C. 3.0 μL aliquots of 9.31 mM $\text{Ni}(\text{ClO}_4)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution buffered at pH 3.0 with 5.0 mM PIPES at an ionic strength of 0.05 M (TEAP).

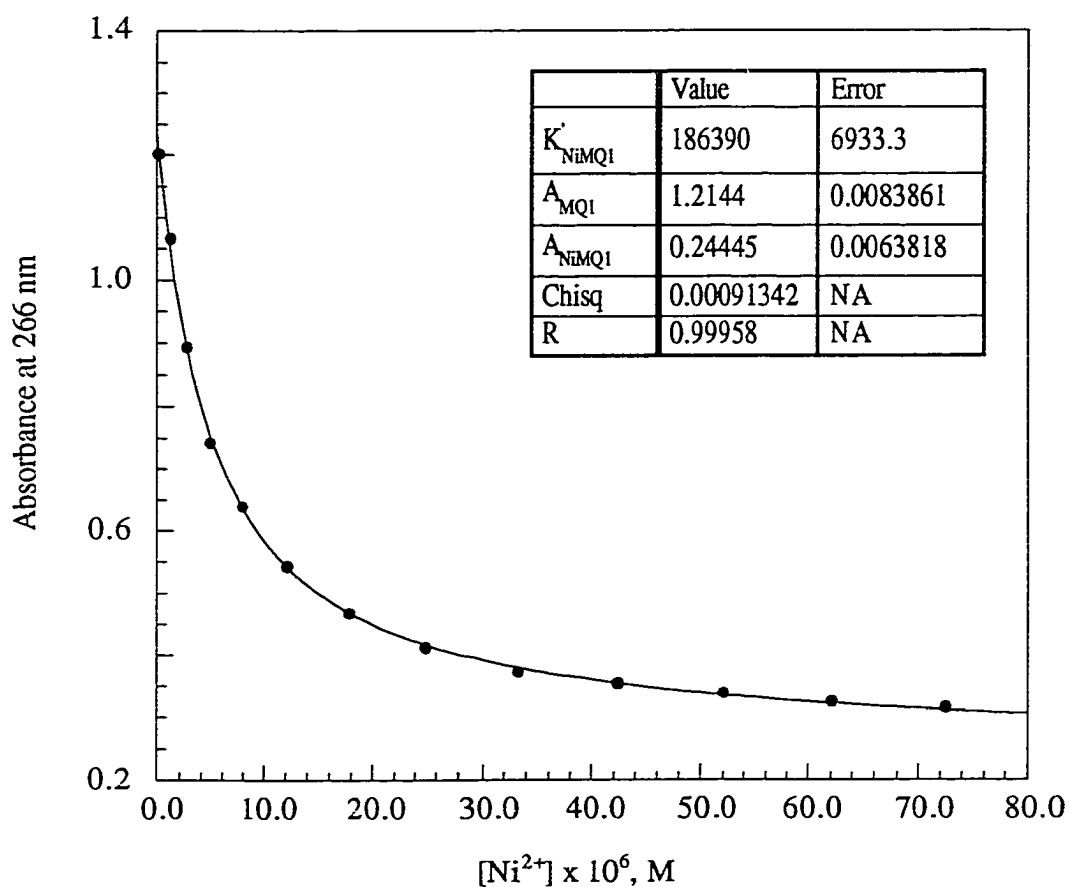


Figure IV.28 Absorbance at 266 nm at pH 3.0 as a function of the final approximation of free nickel ion concentration. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

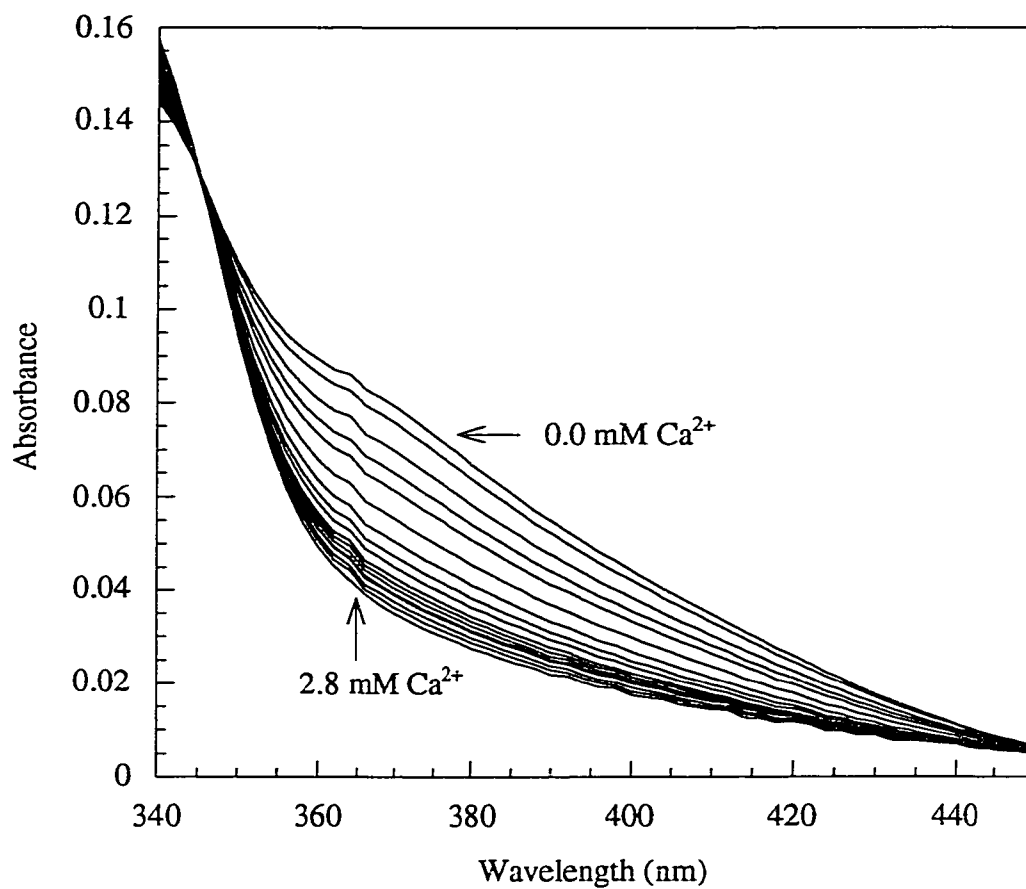


Figure IV.29 Spectra from the titration of MQ1 with calcium at 25.0 °C. 4.0 μL aliquots of 264.7 mM, and 529.5 mM $\text{Ca}(\text{NO}_3)_2$ were used to titrate 2.50 mL of 62.0 μM MQ1 solution buffered at pH 7.2 with 5.0 mM PIPES at an ionic strength of 0.05 M (TEAP).

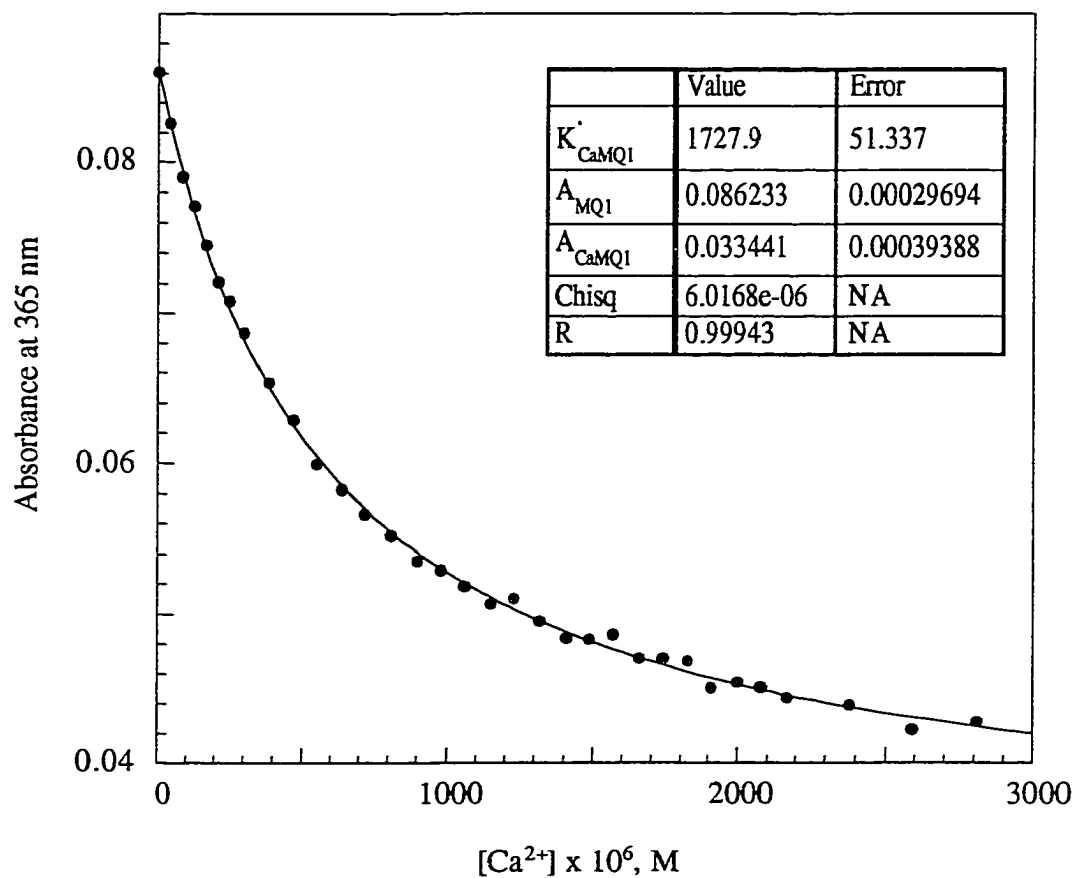


Figure IV.30 Absorbance at 365 nm at pH 7.2 as a function of the final approximation of free calcium ion concentration. The solid line was calculated from eq 4.12 using the derived parameters listed in the inset table.

The pH dependence of K''_{MMQ1} , due to side reactions of MQ1 with protons, can be accounted for by the relationship:

$$K''_{MMQ1} = \frac{[MMQ1]'}{[M^{+2}][MQ1]'} = \frac{[MMQ1]'\alpha_{MQ1}}{[M^{+2}][MQ1^{2-}]} = K'_{MMQ1}\alpha_{MQ1} \quad (4.14)$$

where

$$\alpha_{MQ1} = \frac{[MQ1^{2-}]}{[MQ1]'} = (1 + K_{H1}a_H + K_{H1}K_{H2}a_H^2 + K_{H1}K_{H2}K_{H3}a_H^3)^{-1} \quad (4.15)$$

Table IV.4 lists the values for K''_{MMQ1} , α_{MQ1} , and K'_{MMQ1} for each metal.

Table IV.4 Conditional stability constants, α_{MQ1} factor, partially corrected stability constants, and $\log K'_{\text{ML}}$ for MQ1 with divalent metal ions^a

Metal	pH	K'_{ML}	error ^b	α_{MQ1}	K'_{ML}	$\log K'_{\text{ML}}$
Cd	3.0	420	6.3	2.95×10^{-6}	1.42×10^8	8.15
Pb	3.0	6.69×10^3	1.2×10^2	2.95×10^{-6}	2.27×10^9	9.36
Cu	2.8	3.46×10^4	4.6×10^3	1.11×10^{-6}	3.11×10^{10}	10.5
Zn	3.0	9.13×10^3	6.7×10^2	2.95×10^{-6}	3.09×10^9	9.49
Ni	3.0	1.86×10^5	6.9×10^3	2.95×10^{-6}	6.32×10^{10}	10.8
Ca	7.2	1.73×10^3	51	0.303	5.70×10^3	3.76

^a25.0 °C, ionic strength 0.05 M (TEAP). ^bUncertainty expressed as standard error.

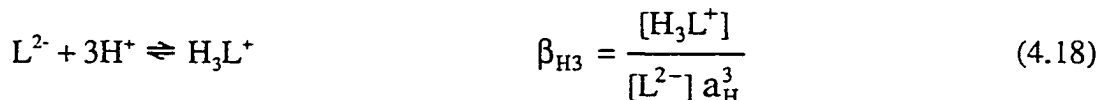
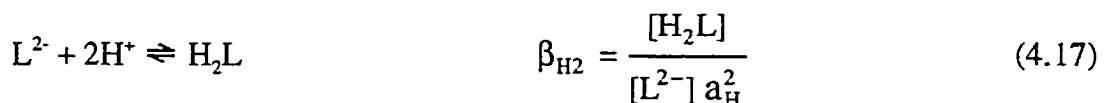
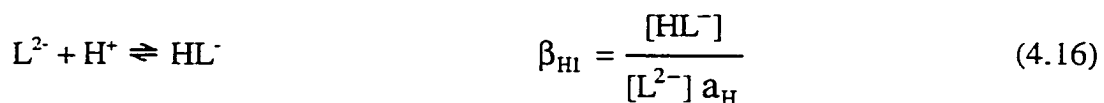
1 Potentiometric Method

The complex stability constants of MQ1 were determined by analysis of data sets, consisting of observed pH and volume of Et₄NOH added, obtained in potentiometric titrations. Titrated samples were 5.20 -5.29 mL of solution containing 5.0 mL of 0.860 mM MQ1 solution with sufficient TEAP to maintain ionic strength at 0.05 M, 0.036 mL of 0.1086 M HClO₄, and 0.19 - 0.29 mL of standardized metal solution. The titrations were accomplished by the automated titration system which measured the variable -log[H⁺] as a function of the volume of titrant added. The titrant was Et₄NOH diluted from a stock solution with freshly boiled D.D. H₂O to give a concentration of approximately 5 mM. The titrant was then standardized with KHP and tested for CO₂ contamination as described in Chapter II.V.

Computer calculations of stability constants of the MQ1 complexes for cadmium, lead, copper, zinc, nickel, and calcium for each data set were accomplished using the program BEST². The measured variable, pH observed, is compared to the pH calculated from a model and the sum of the weighted squares of differences between the measured and calculated pH values is minimized in the calculation of the stability constant. The overall fit, sigma, defined by eqs 3.7 and 3.8, is minimized as described previously in section I.A.2 of this chapter. The program calculates stability constants based on mass balance constraints for each component of the system, summed over all species present, at each point in the titration.

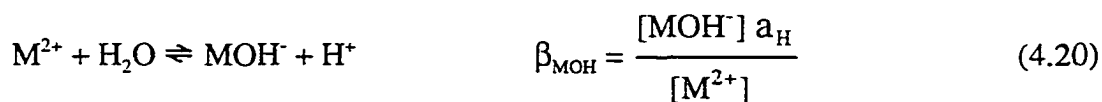
Each set of data was input into the program BEST with a model describing a simple system based on the protonation constants for MQ1 determined in this study, and an estimate of the stability constant for the 1:1 complex of MQ1 with the specific metal involved. The known values for the formality of the metal, MQ1 and excess acid components of the titration solution and the titrant, Et₄NOH are entered into the file for

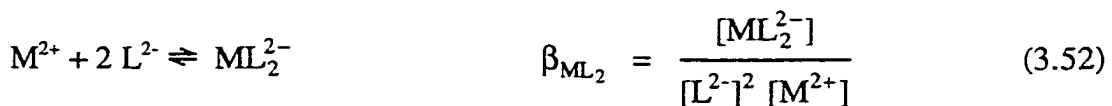
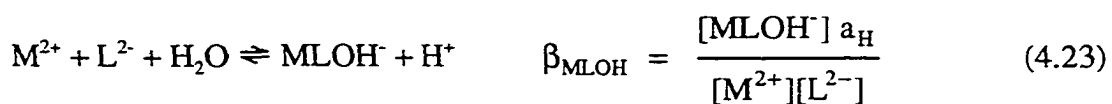
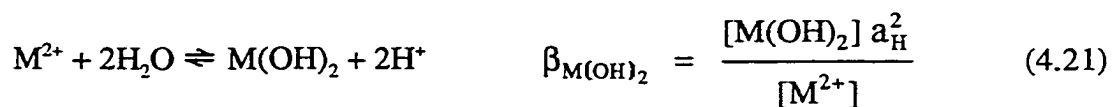
that particular titration along with the model for the MQ1/metal system involved. All constants included in the model are expressed in terms of overall complex formation ($\log \beta$) values. Overall constants are cumulative in terms of stepwise constants. This is illustrated below in the expressions for β_{H} and $\log \beta_{\text{H}}$ of the mono-, di-, and triprotonated forms of MQ1 as well as the metal-ligand complex. The stepwise expressions of K_{H} for the protonation of MQ1 are given in eqs 4.1 - 4.4. In eqs 4.16 - 4.19 the term L^{2-} refers to the dianion of MQ1.



The estimated stability constant for the metal-ligand complex is refined by the program while known values are held constant. In models used in this study the $\log \beta_{\text{H1}}$ and $\log \beta_{\text{H2}}$ values determined earlier were considered known and held constant in the

calculations. $\log \beta_{H_3}$ was not considered to have been determined with a sufficient degree of accuracy and the program was allowed to refine this value within limits. If refinement of the metal ligand stability constant is not sufficient to allow an acceptable fit ($\sigma < 0.01$) with the experimental data other complexes are added to the model and the program allowed to refine the original complex constant as well as estimates input for any added complex. Some of the complexes considered in the analysis of MQ1 were protonated metal-ligand complexes, metal ligand hydroxides, metal hydroxides and various stoichiometric combinations of metal and ligand (especially 2:1 Ligand to metal complexes). When addition of a species made a significant improvement in the fit of the model it was kept as part of the model. If no significant contribution to goodness of fit was observed the species was removed. Stability constants for metal hydroxides obtained from literature³, were corrected to the appropriate ionic strength using the Davies equation (eq 3.21). These $\beta_{M(OH)_i}$ values were held constant during the refinement process. Many different complexes and combinations of complexes were tried in the models for each MQ1 metal system. The final model represents the complexes present at $> 5\%$ for that system at some point in the pH range studied. Overall reactions leading to formation of complexes included in the final models are shown below with definitions for their corresponding β values. Reactions for the formation of hydroxy species are expressed as acid hydrolysis reactions.





The goodness of fit between the experimental data and the values of pH calculated based on the model can be visualized by overlaying the plot of the experimental titration curve with a curve for the values calculated by BEST. This type of plot is shown for one data set from each of the six metal-ligand systems in Figures IV.31 - IV.36. Tables IV.5 - IV.10 show the model used in each metal ligand system. The values given for $\log \beta_x$ are averages from the data in section I-A of this chapter, published values corrected for ionic strength, or derived by the program BEST, as noted in each table.

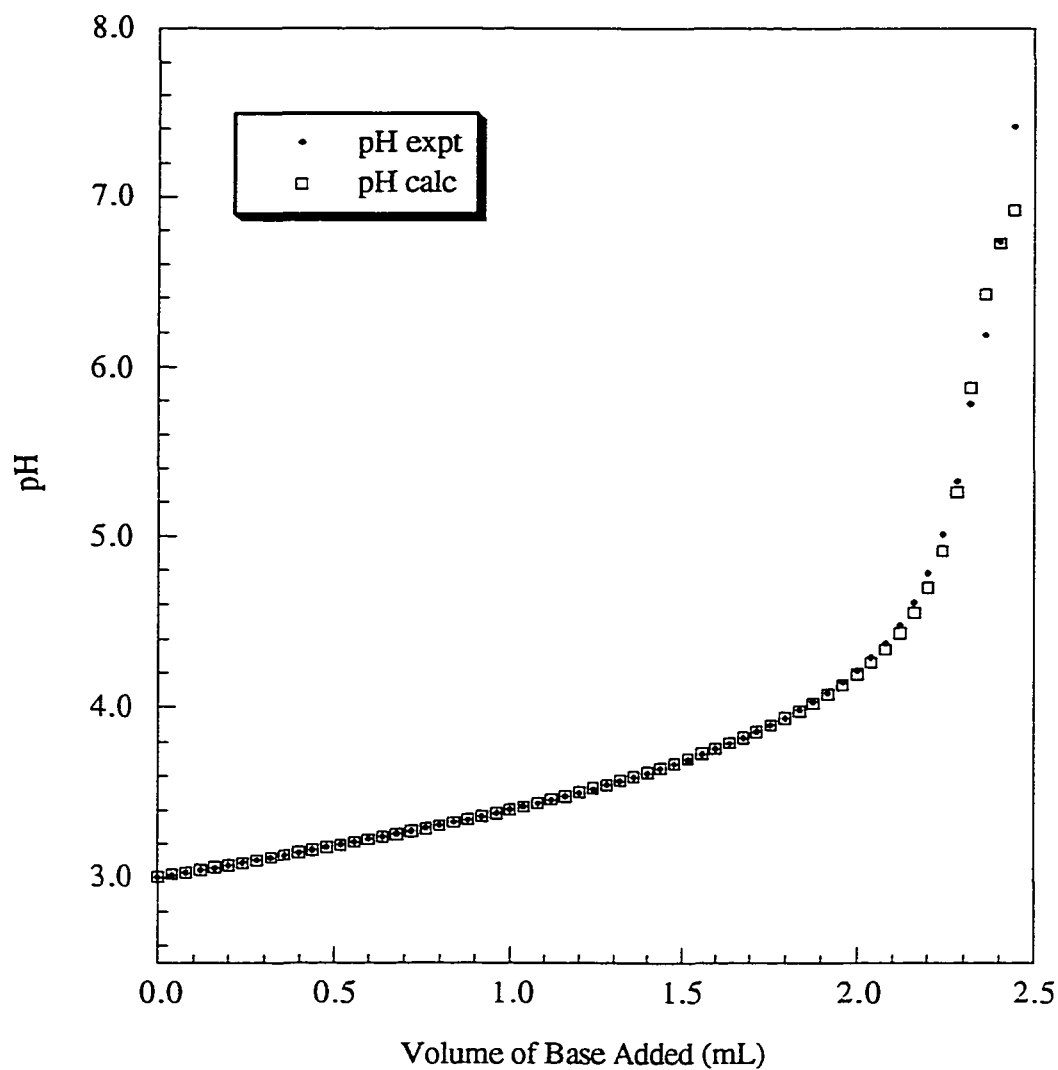


Figure IV.31 Plot of pH vs volume of base for the potentiometric titration of 5.28 mL of a solution containing 0.815 mM $\text{Cd}(\text{ClO}_4)_2$ and 0.814 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at 25.0 °C using 4.96 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.5, exp. 1.

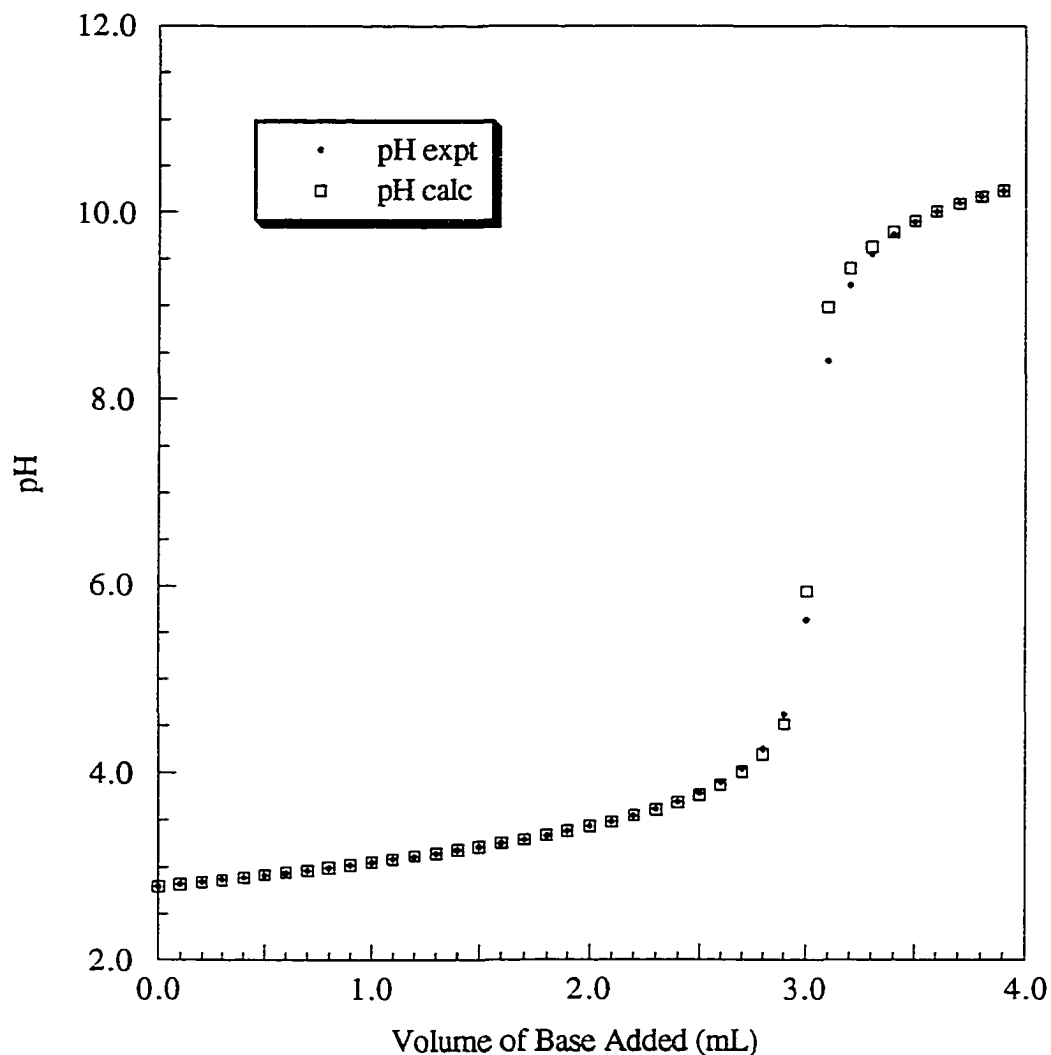


Figure IV.32 Plot of pH vs volume of base for the potentiometric titration of 5.25 mL of a solution containing 0.819 mM $\text{Pb}(\text{ClO}_4)_2$ and 0.819 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at at 25.0 °C using 4.96 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.6, exp. 1.

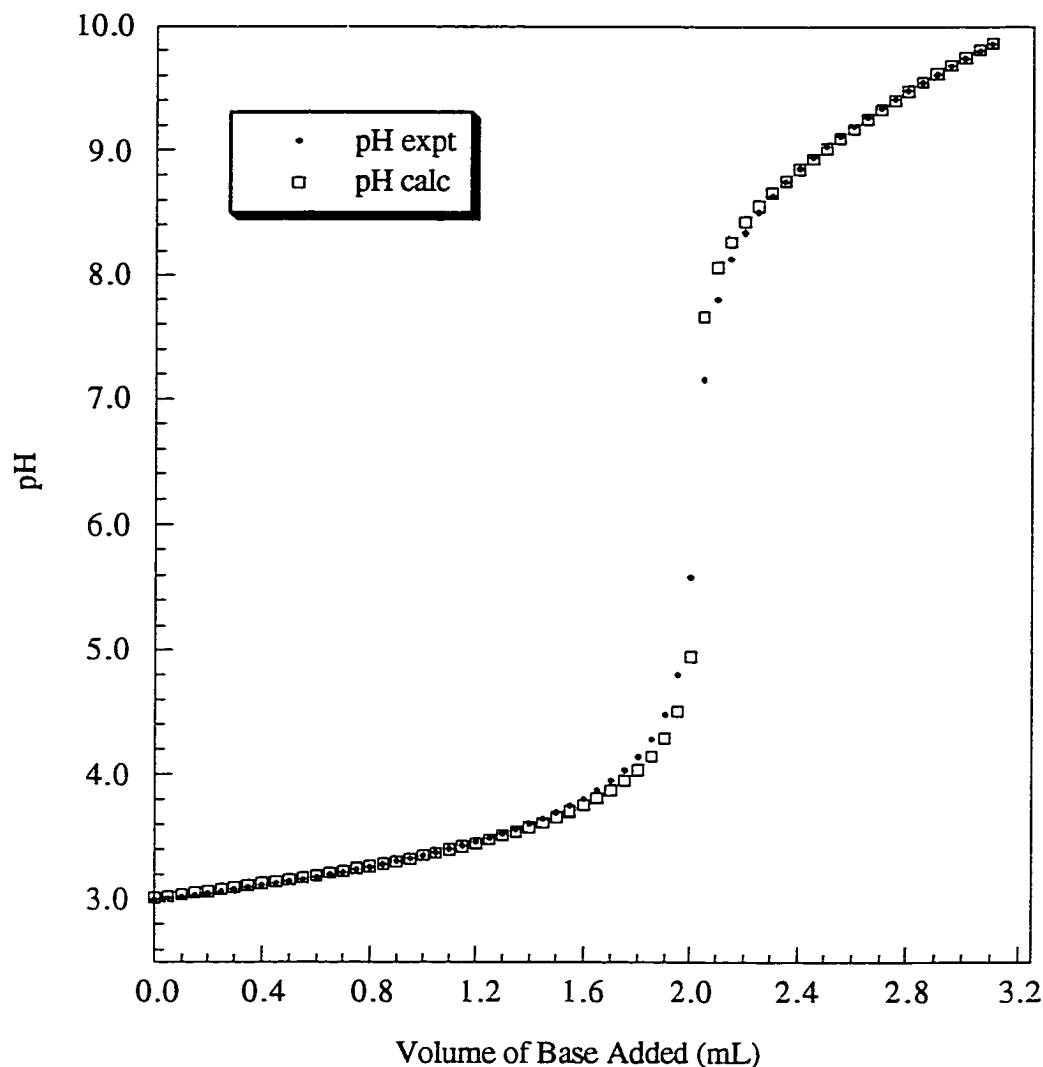


Figure IV.33 Plot of pH vs volume of base for the potentiometric titration of 5.20 mL of a solution containing 0.804 mM CuCl_2 and 0.827 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at 25.0 °C using 4.58 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.7, exp. 1.

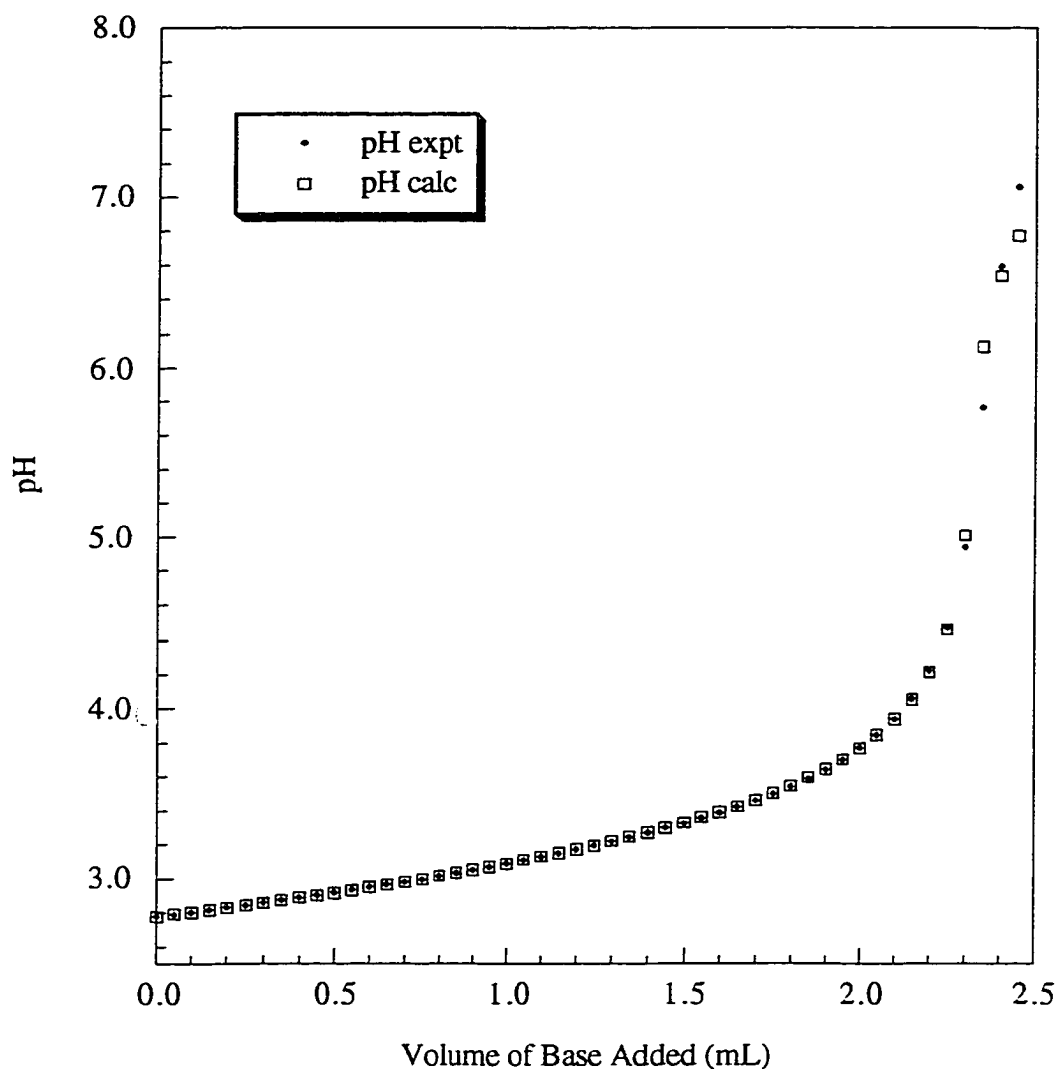


Figure IV.34 Plot of pH vs volume of base for the potentiometric titration of 5.25 mL of a solution containing 0.811 mM $\text{Zn}(\text{ClO}_4)_2$ and 0.819 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at 25.0 °C using 5.20 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.8, exp. 1.

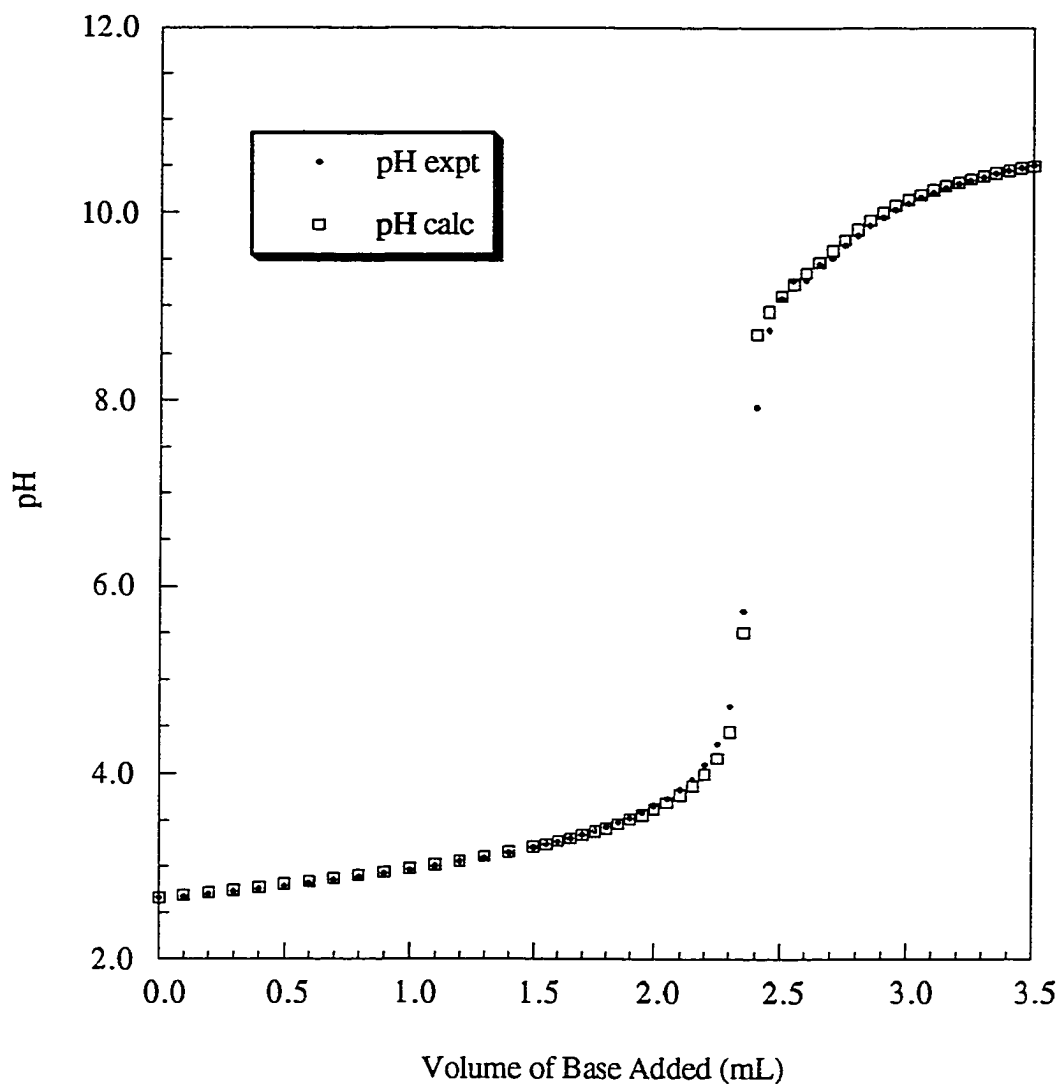


Figure IV.35 Plot of pH vs volume of base for the potentiometric titration of 5.29 mL of a solution containing 0.945 mM $\text{Ni}(\text{ClO}_4)_2$ and 0.813 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at 25.0 °C using 4.96 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.9, exp. 1.

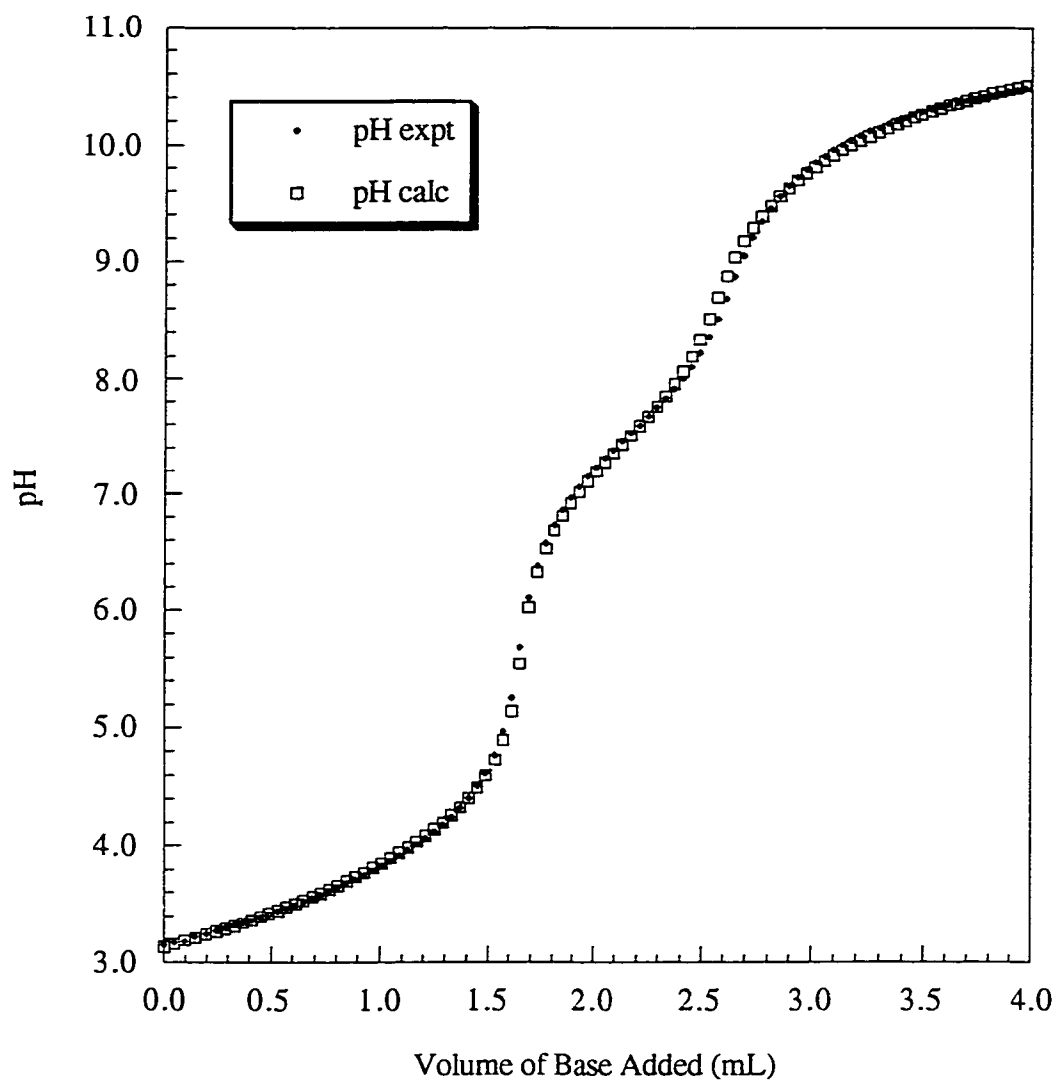


Figure IV.36 Plot of pH vs volume of base for the potentiometric titration of 5.25 mL of a solution containing 0.688 mM $\text{Ca}(\text{NO}_3)_2$ and 0.819 mM MQ1 at an ionic strength of 0.05 M (TEAP). The titration was carried out at 25.0 °C using 4.96 mM Et_4NOH . The calculated pH values are obtained from the program BEST using refined values of the equilibrium constants for the species in the model defined Table IV.10, exp. 1.

Table IV.5 Log β_x values from potentiometric titrations of MQ1 and cadmium^a

Exp. No.	Identity of complex species x						sigma ^d
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₃MQ1^b</u>	<u>CdMQ1^c</u>	<u>CdHMQ1^c</u>	<u>CdMQ1OH^c</u>	
1	7.56	11.40	13.67	8.26	11.35	0.566	0.005
2	7.56	11.40	13.67	7.88	10.68	-4.82	0.007

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.6 Log β_x values from potentiometric titrations of MQ1 and lead^a

Exp. No.	Identity of complex species x						sigma ^d
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₃MQ1^c</u>	<u>PbMQ1^c</u>	<u>PbHMQ1^c</u>	<u>PbMQ1OH^c</u>	
1	7.56	11.40	13.67	9.09	11.30	-0.672	0.006
2	7.56	11.40	13.67	9.97	12.86	2.40	0.005

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.7 Log β_x values from potentiometric titrations of MQ1 and copper^a

Exp. No.	Identity of complex species x					sigma ^d
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₂MQ1^c</u>	<u>CuMQ1^c</u>	<u>CuMQ1₂^c</u>	
1	7.56	11.40	13.68	13.96	24.05	0.018
2	7.56	11.40	13.67	13.87	24.03	0.027
3	7.56	11.40	13.68	13.94	24.13	0.044

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.8 Log β_x values from potentiometric titrations of MQ1 and zinc^a

Exp. No.	Identity of complex species x						sigma ^d
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₂MQ1^b</u>	<u>ZnMQ1^c</u>	<u>ZnHMQ1^c</u>	<u>ZnMQ1OH^c</u>	
1	7.56	11.40	13.67	9.87	11.82	2.38	0.003
2	7.56	11.40	13.67	9.74	12.32	2.13	0.005

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.9 Log β_x values from potentiometric titrations of MQ1 and nickel^a

Exp. No.	Identity of complex species x							sigma ^e
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₃MQ1^c</u>	<u>NiOH^d</u>	<u>Ni(OH)₂^d</u>	<u>NiMQ1^c</u>	<u>NiMQ1OH^c</u>	
1	7.56	11.40	13.92	-10.02	-17.88	12.12	-0.1671	0.015

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d Values fixed during refinement; from Smith and Martell² adjusted for ionic strength. ^e sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.10 Log β_x values from potentiometric titrations of MQ1 and calcium^a

Exp. No.	Identity of complex species x					sigma
	<u>HMQ1^b</u>	<u>H₂MQ1^b</u>	<u>H₃MQ1^c</u>	<u>CaMQ1^c</u>	<u>CaMQ1OH^c</u>	
1	7.56	11.40	13.92	3.15	-6.19	0.026

^a 25.0 °C, ionic strength 0.1 M (TEAP). ^b Values fixed during refinement; from section IV.A. ^c Values refined by program BEST. ^d sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{\text{obs}} - p[H]_{\text{calc}})^2$ and $N = \sum w$.

Table IV.11 Summary of complexation constant values for 1:1 complexes of MQ1 obtained from potentiometric data^a

Metal	Exp. No.	log K_{MMQ1}	sigma ^b
Cd	1	8.26	0.005
Cd	2	7.88	0.007
Pb	1	9.09	0.006
Pb	2	9.97	0.004
Cu	1	13.68	0.017
Cu	2	13.87	0.027
Cu	3	13.94	0.044
Zn	1	9.87	0.003
Zn	2	9.74	0.005
Ni	1	12.12	0.015
Ca	1	3.15	0.026

^a 25.0 °C, ionic strength 0.1 M (KNO₃). ^b sigma = $(U/N)^{1/2}$; where $U = \sum w(p[H]_{obs} - p[H]_{calc})^2$ and $N = \sum w$

References

1. Motekaitis, R. J.; Martell, A. E., *Can. J. Chem.*, **1982**, 60, 2403 -2409.
2. Motekaitis, R. J.; Martell, A. E., *Determination and Use of Stability Constants*, 2nd ed., VCH Publishers, Inc., New York, NY, 1992.
3. Smith, R.M.; Martell, A.E.; Motekaitis, R.J. *NIST Critically Selected Stability Constants of Metal Complexes Database*; Version 5, U.S. Department of Commerce, Gaithersburg, MD, 1998.

Chapter V

DISCUSSION

Knowledge of the importance of metal ions in biological processes brought a demand for tools to aid in the study of the physiological functions of metal ions. Initially, intracellular calcium was the main focus of research, but as knowledge of cellular processes increased more metal ions were recognized as vital to physiological processes. The demand for nondestructive methods for measuring and controlling intracellular concentrations of metals increased with the knowledge of the importance of the roles these ions play. The most widely used techniques for measuring intracellular metal ion concentration have employed substances which undergo a change in absorbance or fluorescence upon binding to specific metal ions. The requirements for these indicators are the same as those for metal indicators for other types of research with a few added constraints. Desirable characteristics for metal chelating indicators are selectivity, speed of interaction, large spectral changes at easily measured wavelengths, and quantitative, linear response to metal ion concentration. Added requirements for studies involving cells are a lack of toxicity, insensitivity to protons in the 6.8 - 7.8 pH range, and a convenient method for loading the indicator into cells. These indicators must be selective for the ion they are used to study while being relatively insensitive to other ions, such as protons, Mg^{2+} and Ca^{2+} , that may be present in comparatively significant concentration. The need for rapid metal ion complexation calls for indicators whose binding sites are not blocked by protons in the pH range of the measurements. Indicators need also to exhibit quantitative binding at one to one, metal-ligand,

stoichiometry of interaction to allow linear calibration and avoid complicated mathematical treatments.

I 5,5'-Dinitro BAPTA

A PROTONATION CONSTANTS OF DNB

The demand for indicators that bind quickly and selectively while remaining insensitive to protons in the pH range of physiological studies requires that all ligand protonation constants be sufficiently low, below 10^7 , to ensure essentially complete deprotonation at pH 7.0. Therefore, the values obtained for the protonation constants of DNB are very important to any discussion of the utility of this ligand. In addition, the protonation constants of the ligand are critical to the analysis of absorbance and potentiometric data in determining spectral characteristics, stoichiometry, and complexation constants.

I Mean Values

Protonation constants of DNB were determined using UV-vis spectrophotometric and potentiometric methods with ionic strength maintained at 0.10 M with KNO_3 and 25.0 °C in both methods to facilitate comparisons between constants obtained for this ligand and published values obtained for the parent compound, BAPTA, at the same ionic strength and temperature. Only the first three protonation constants were calculated using the spectrophotometric data. Three protonation constants were also calculated from some of the potentiometric data using the PKAS¹ program. Values obtained for the first and second protonation constants agreed well between the two methods. Values for the third protonation constant showed considerably more variation, and those obtained by spectrophotometric titration with

relative error greater than 5% were not included in calculations of the overall mean value for the two methods. Table V.1 lists the average values of the protonation constants of DNB that were obtained using each method and the average values of the two methods.

Table V.1 Values for protonation constants of DNB from Potentiometric (P) titrations^a and average values from each Spectrophotometric (S) titrations^b along with composite values for both methods^{c,d}

Titration	$\log K_{H1}$	$\log K_{H2}$	$\log K_{H3}$
S1	5.58 ± 0.02	4.51 ± 0.02	3.02 ± 0.01
S2	5.35 ± 0.09		
S3	5.35 ± 0.05		
P1	5.38 ± 0.05	4.53 ± 0.01	3.45 ± 0.01
P2	5.24 ± 0.02	4.46 ± 0.01	3.46 ± 0.01
P3	5.40 ± 0.01	4.48 ± 0.03	
P4	5.18 ± 0.03	4.58 ± 0.02	3.30 ± 0.01
composite	5.35 ± 0.13	4.51 ± 0.05	3.31 ± 0.21

^a Error expressed in terms of sigma as defined in eqs 3.7 - 3.8. ^b Error expressed in terms of standard deviation. ^c 25.0 °C, I = 0.1 M (KNO₃). ^d Values with error greater than 5% not included.

2 General Trends

The protonation constants for DNB were compared to values obtained for the parent compound, BAPTA, reported by Tsien² and Yuchi et al.³. These investigators reported significantly different values for the first protonation constant but similar values for the second, as seen in Table V.2. DNB shows a lowering of the first two protonation constants as compared to either set of values for BAPTA. The values for the third protonation constant of DNB and BAPTA are similar.

The structure of DNB given in Figure I.4 shows six protonation sites; four carboxylate groups, and two aniline nitrogens. In the unprotonated form the molecule is symmetrical, and the two aniline groups are chemically equal just as they are in the parent compound BAPTA. The values of constants for the first three protonation steps are closer for DNB than for the parent compound. This results in a greater overlap in protonation states for DNB than BAPTA but also ensures almost total deprotonation of this ligand in the physiological pH range. At pH 6.8 at least 96% of the ligand exists in the unprotonated form. This is illustrated in Figure V.1 which shows a species distribution plot for DNB, based on the overall mean values for the first three protonation constants listed in Table V.1. The lack of multiple protonation states in the physiological pH range gives greater independence from small changes in pH which may occur during measurements of intracellular metal ion concentrations. For comparison, a species distribution plot using the first four protonation constants of BAPTA is shown in Figure V.2. Values for the first and second protonation constants are those published by Tsien² and the third and fourth values are those determined by Yuchi et al.³ The higher value of K_{H1} for BAPTA results in a more complex distribution

of indicator species in the physiological pH range; thus increasing the sensitivity of the spectral measurements to small changes in pH.

Table V.2 Comparison of protonation constants for DNB^{a,b} and BAPTA^c

Ligand	Method	log K _{H1}	log K _{H2}	log K _{H3}	log K _{H4}
DNB	spectral	5.43 ± 0.13	4.51 ± 0.02	3.02 ± 0.01	ND
DNB	potentio- metric	5.30 ± 0.18	4.51 ± 0.05	3.40 ± 0.09	ND
DNB	overall	5.35 ± 0.13	4.51 ± 0.05	3.31 ± 0.21	ND
BAPTA ^b	potentio- metric	5.95 ± 0.02	5.44 ± 0.02	3.48 ± 0.04	2.67 ± 0.04
BAPTA ^c	potentio- metric	6.36 ± 0.1	5.47 ± 0.1	ND	ND
EGTA ^d		9.40	8.79	2.70	1.90

^a 25.0 °C, ionic strength 0.1 M (KNO₃). ^b Error expressed as unweighted mean ± 1 standard deviation. ^c 25.0 °C, ionic strength 0.1 M (KNO₃), ref. 3. ^e 22 ± 2 °C, ionic strength 0.1 M (KNO₃), ref. 2. ^d 25.0 °C, ionic strength 0.1 M (KNO₃), ref. 4.

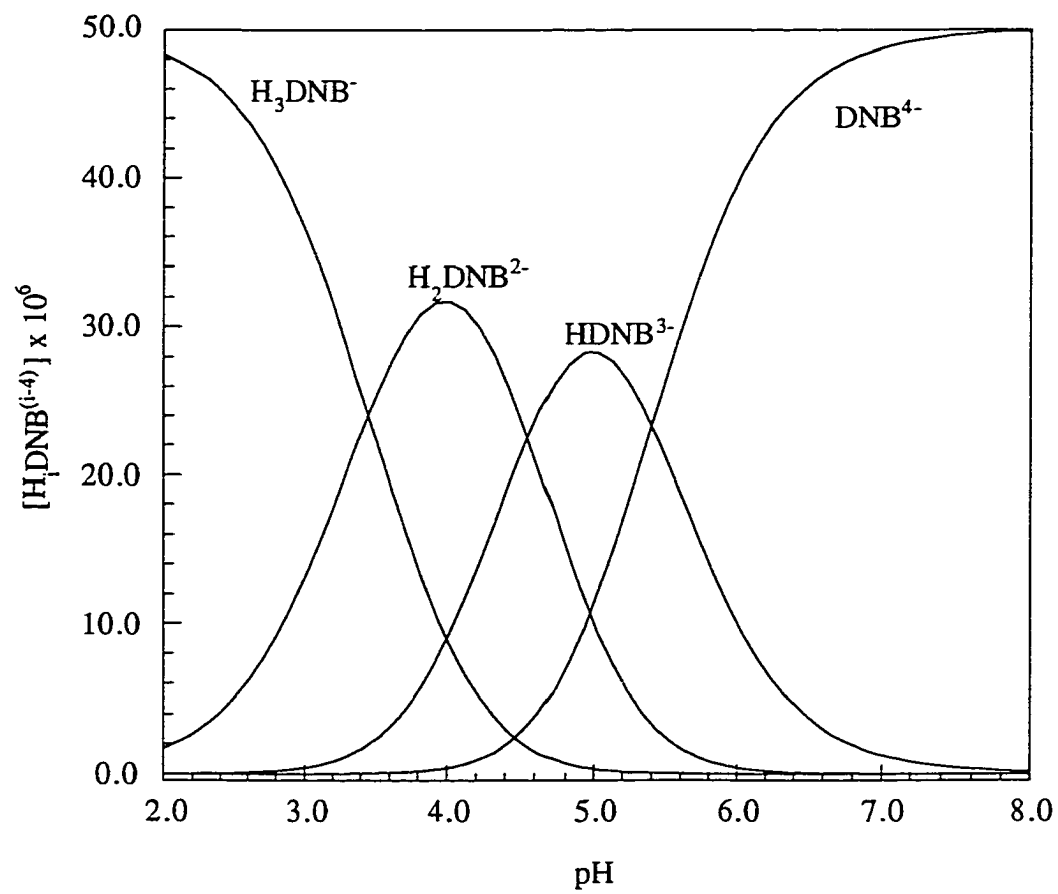


Figure V.1 Species distribution plot for various protonation states of DNB.

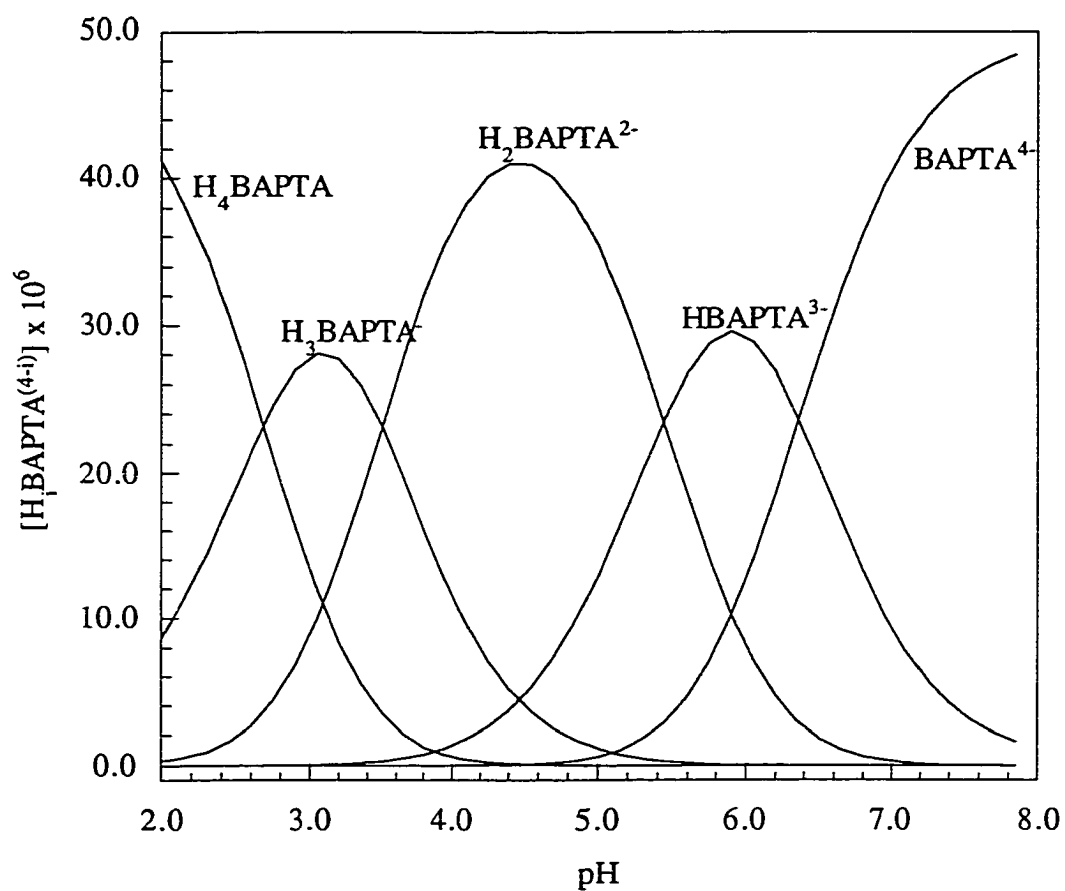


Figure V.2 Species distribution of protonation states of BAPTA as a function of pH using K_{H1} and K_{H2} from Tsien² and K_{H3} and K_{H4} from Yuchi et al³.

3 Specific Characterization of Protonation Constants

The addition of the nitro substituents lowers the values for $\log K_{H1}$ and $\log K_{H2}$ by approximately 1 log unit in all cases except in comparison with the value of $\log K_{H1}$ determined by Yuchi et al.³ Table V.3 shows the difference in values determined for BAPTA and DNB for each set of BAPTA values along with the difference in $\log K_{H1}$ and $\log K_{H2}$ for each individual set of determinations. The extent of effect of the nitro substituent is similar except when the first protonation constant determined by Yuchi et al. is considered. In comparison to the values for BAPTA reported by Tsien², the first two protonation constants of DNB were equally effected by the addition of the nitro substituents. However, compared to the results of Yuchi et al. the first protonation step was affected less than the second protonation step.

Table V.3 Comparison of values of protonation constants

	$\Delta \log K_{H1}$	$\log K_{H1}$	$\Delta \log K_{H2}$	$\log K_{H2}$	$\Delta \log K_{H2}$
BAPTA (Tsien)		6.36	<-----> 0.89	5.47	
	0.99				0.98
DNB		5.37	<-----> 0.96	4.49	
	0.57				0.95
BAPTA (Yuchi)		5.95	<-----> 0.51	5.44	

For a ligand with two equivalent, but independent protonation sites the minimum difference between K_{H1} and K_{H2} should be a factor of four, or 0.6 log units⁵. The factor of four comes from consideration of the microscopic protonation constants of the identical sites in relation to the macroscopic protonation constants. This minimum difference in macroscopic protonation constants is observed in EGTA ($\Delta \log K = 0.61$, Table V.2). The values obtained by Yuchi et al. differed by less than the minimum theoretical value. The K_{H1} and K_{H2} values reported by that group differ only by a factor of 3.2. Given the discrepancy in the values reported for K_{H1} this raises some doubt about the lower K_{H1} value although it was reported to be consistent in measurements with six different electrolytes. The values reported by Tsien were about 0.9 log units apart, or a factor of 7.8. This difference is similar to that between the corresponding values determined for DNB. For DNB the difference between values of K_{H1} and K_{H2} was 0.78 log units, for potentiometric determinations, and 1.11 log units, for spectrophotometric determinations. The average difference for both methods was 0.96 log units, or a factor of 9.1. The larger differences observed for protonation constant values of DNB and BAPTA values reported by Tsien indicate that the first protonation has some effect on the second protonation site. Although the sites are remote and equivalent they are not completely independent of each other. The difference could be seen as that between a site with a charge of -4 becoming -3 as opposed to two completely independent sites with charges of -2. A study of ionic strength effects on the calcium affinity of 5,5'-dibromo BAPTA, in which best results were obtained by treating the ligand as a sphere with a charge of -4 in the center⁶ supports the theory of interaction between the carboxylates. In 5,5'-dibromo BAPTA a similar difference in protonation constants is observed. For the dibromo compound $\log K_{H1}$ and $\log K_{H2}$ were determined to be 5.47 and 4.57, respectively⁷. This is a difference of 0.90 log units and also indicates interaction between the two binding sites. It is also noteworthy

that while the drop in protonation constant values for the dibromo derivative compared to those for BAPTA are similar to those observed for DNB the drop in metal complexation constants are far greater for DNB than the dibromo compound.

When looking at simpler analogous compounds it is seen that a nitro substituent can have a relatively large effect on basicity. Aniline has a protonation constant of 4.59 while 4-nitroaniline has a protonation constant of only 0.98.⁸ The dramatic drop in basicity is due to delocalization of the nitrogen lone pair electrons through an extended resonance framework involving the phenyl group. Though both molecules exhibit resonance through the aniline nitrogen, the nitro substituent contributes further delocalization of π electrons and further extension of the planar σ frame through the nitrogen of the nitro group as well. This is illustrated in Figure V.3.

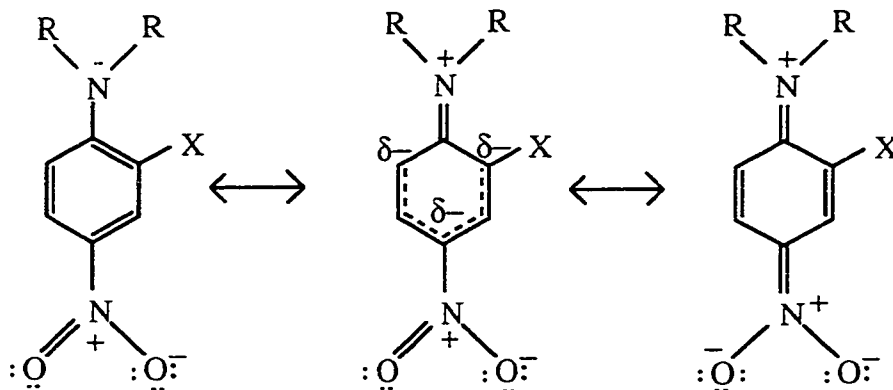


Figure V.3 Resonance structures illustrating the extension of conjugation through the aniline and nitro group nitrogen atoms.

The change in the value for K_{H} from BAPTA to DNB is relatively small compared to the difference between aniline and 4-nitroaniline even though similar resonance structures can be drawn for the two unsubstituted and the two substituted compounds.

The large shift to longer wavelength for the absorbance maximum seen when looking at DNB^+ (λ_{max} 432 nm) relative to BAPTA (λ_{max} 287 nm) seems to indicate a

substantial degree of resonance stabilization in the nitro substituted derivative. The spectral changes observed with a decrease in pH, to the point that the molecule is mostly diprotonated (ca. pH 4), do not occur to the same extent as when a metal ion is complexed indicating that the resonance stabilization is only partially destroyed. There are two identical aniline type groups to consider in DNB and binding of protons by the molecule does not affect the spectra as much as the binding of a metal ion in which both sides of the molecule are involved. If an aniline nitrogen were indeed the first protonation site, and the absorbance contributed by each half of the molecule were equal, binding of a proton should shift the absorbance peak to half the extent as metal binding. This is not the case with DNB; after the second protonation should be complete the absorbance maximum seen at 430 nm has only been diminished by about 20% of the total change that is seen with complexation of metals. Protonation of the aniline nitrogen would destroy the extended resonance. It is possible that the molecule assumes a conformation in which the four carboxylate groups are brought together. Polycarboxylates can have relatively high values for the first protonation constant. Tricarballic acid, a tricarboxylic acid has a $\log K_{H1}$ value of 5.82 at 25.0 °C and 0.1 M ionic strength⁴. Butane-1,2,3,4-tetracarboxylic acid has a $\log K_{H1}$ value of 6.51 under the same conditions⁴. If the first protonation sites were carboxylates, protonation would have little effect on the formation of resonance structures.

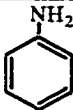
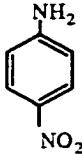
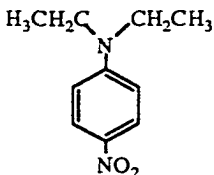
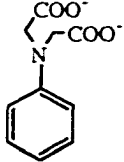
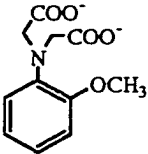
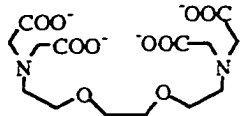
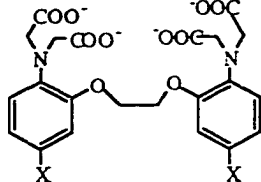
Although steric effects do not usually play a large part in acid base reactions they commonly affect resonance stabilization and have significant effects on resonance promoted acidity. In order for resonance stabilization of the electron pair on the amine nitrogen to occur, the group involved must become coplanar with the conjugated system (phenyl ring). In the case of aniline and 4-nitroaniline there are no hindrances to the amine and nitro groups becoming coplanar. In contrast, BAPTA and DNB have a large

bulky substituent at the position ortho to the amine, as well as the acetate groups attached to the amine. The acetate groups may rotate away from the ortho group and make it more difficult for the aniline nitrogen to become planar with the ring⁹. This would cause the the same effect in BAPTA as in DNB but could account for some of the difference in the effect of addition of a para nitro group observed between 4 nitroaniline and BAPTA. The effect of an ortho substituent on *N*-phenyliminodiacetic acid is shown by comparison to *N*-(2-methoxyphenyl)-iminodiacetic acid (half BAPTA). In the latter the resonance structures become less important due to steric hindrance by the methoxy substituent. As a result, the lone pair on the amine nitrogen is more localized and the basicity is increased. Thus, half BAPTA has a higher protonation constant ($\log K_H = 5.58$)⁴ than *N*-phenyliminodiacetic acid ($\log K_H = 5.04$).¹⁰ The effect on acidity of substitution at the aniline nitrogen of a para-nitroaniline compound can be seen by comparison of 4-nitroaniline and *N,N*-diethyl-4-nitroaniline. When the hydrogens of the aniline nitrogen are replaced with ethyl groups the basicity of the molecule increases. The protonation constant increases from $\log K_H = 0.97$ (4-nitroaniline) to $\log K_H = 1.75$ (*N,N*-diethyl-4-nitroaniline)¹¹. Values for the first protonation constants of some aniline type compounds are listed in Table V.4.

The relatively minor effect of the para nitro substituents on the first two protonation constants of DNB is an anomaly that is not easily explained. The steric effects of the substituents near the amine group could inhibit resonance to some extent but individual differences for each added group do not seem to equal the difference between the large drop in the first protonation constant for 4-nitroaniline relative to aniline and the comparatively small change in acidity seen between the more sterically hindered BAPTA and DNB molecules. The spectral characteristics of both DNB and BAPTA are indicative of substantial resonance contributions of extended conjugation of the benzene rings and suggest that steric hindrances do not overcome the ability of the

aniline nitrogen to become coplanar with the benzene ring to a very large degree. Compared to BAPTA, the drop in both protonation constants ($\Delta K_{H1} = 0.76$, $\Delta K_{H2} = 0.90$) and calcium complexation constant ($\Delta K_{Ca} = 1.17$) of the dibromo derivative⁷ are similar, but with DNB the drop in protonation constants is much less than that for complexation constants. The experimental evidence suggests that the addition of the nitro substituents has delocalized the lone pair electrons and increased the acidity of the aniline nitrogen to the extent that the carboxylate groups serve as the first protonation site(s). Because aniline nitrogens are not protonated first, the spectral features associated with the extended resonance are not disrupted until the third protonation step takes place.

Table V.4 Protonation constants for aniline and aniline derivatives

Compound	log K_{H1}	Structure
Aniline ^a	4.62	
4-Nitroaniline ^a	0.99	
<i>N,N</i> -Diethyl-4-nitroaniline ^b	1.75	
<i>N</i> -Phenyliminodiacetic acid ^a	5.02	
<i>N</i> -(2-Methoxyphenyl)-iminodiacetic acid ^a	5.58	
EGTA ^a	9.32	
BAPTA ^c	6.36	$x = H$ 
5,5'-dinitro BAPTA ^d	5.38	$x = NO_2$
5,5'-dibromo BAPTA ^c	5.6	$x = Br$

^a25.0 °C, I = 0.1 M, ref. 4. ^b25.0 °C, ref. 11. ^c22 ± 2 °C, I = 0.1 M, ref. 2. ^d25.0 °C, I = 0.1 M ^e22 ± 2 °C, I = 0.1 M, ref. 7.

B *Characterization of the Spectra of Metal-DNB Complexes*

Large spectral changes, a quantitative linear response, and one to one metal-ligand stoichiometry are desirable characteristics for ligands used as indicators. Large absorbance changes allow for greater sensitivity in quantitative analysis. Linearity of response and a single stoichiometry for binding interactions allow simple mathematical treatment and the use of linear calibration graphs for evaluation of unknown concentrations. To determine if the complexation reactions of DNB possessed these characteristics, experiments were conducted to evaluate the behavior of DNB with the divalent cations of lead, cadmium, copper, zinc, cobalt, nickel, and calcium. The shift in wavelength of maximum absorbance, molar absorptivity, and the binding ratio of DNB with Cd^{2+} , Pb^{2+} and Zn^{2+} were determined. The results are listed in Chapter III, Table III.3.

Large spectral changes and linear response were observed for the binding of DNB with cadmium, lead, copper and zinc. Figure V.4 shows the spectrum for 41 μM DNB at pH 7.0 along with the spectra for the cadmium, lead, zinc and copper complexes, at the same concentration and at the same pH. As can be seen, the changes are significant and varied for each complex.

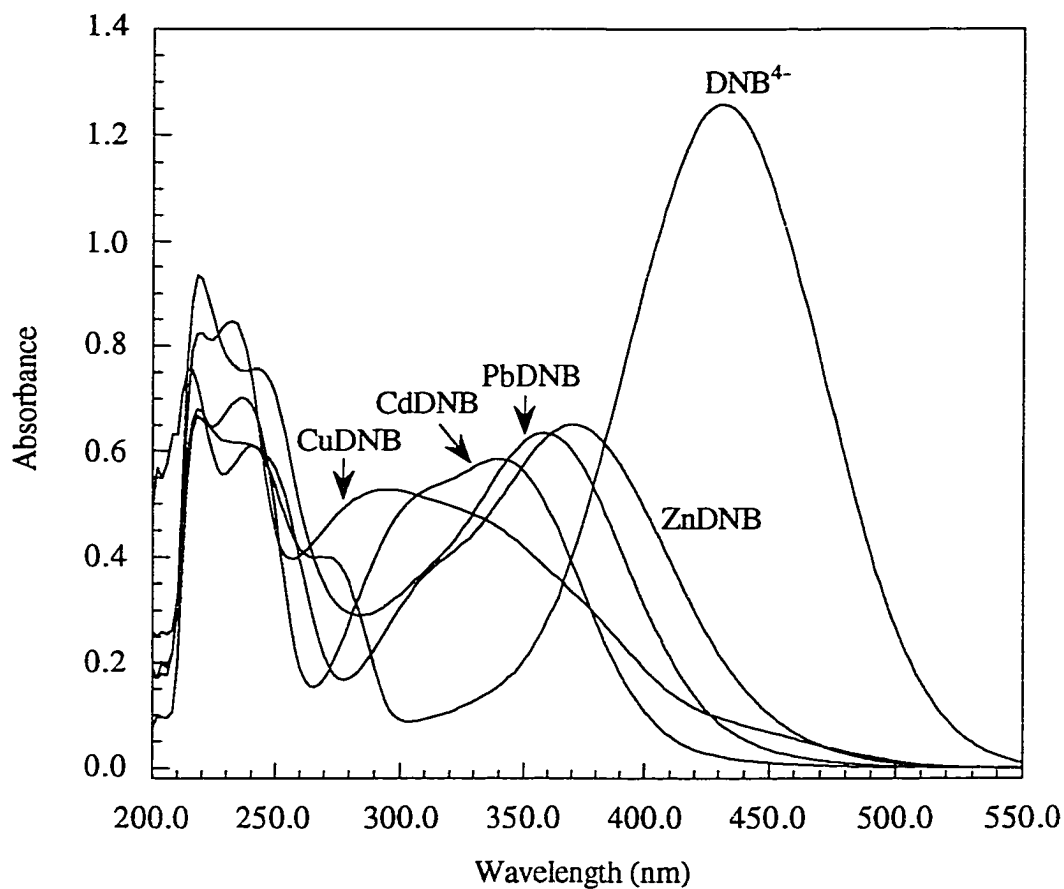


Figure V.4 Spectrum of 41 μM DNB and spectra of CdDNB, PbDNB, ZnDNB and CuDNB all with ionic strength controlled at 0.1 M (KNO_3). All solutions contained 41 μM DNB at pH 7.0 and at 25.0 $^\circ\text{C}$. The spectrum for CuDNB was obtained at with 42 μM added copper. Spectra for complexes of DNB with Cd^{2+} , Pb^{2+} and Zn^{2+} contain excess metal ion.

Co^{2+} shows linear response at low metal to ligand concentration ratios but, as seen in Figure III.14, absorbance changes decrease as this ratio approaches one. Nickel complexation is only about fifteen percent by the 1:1 stoichiometric ratio for 41 μM DNB as seen in Figure III.16. Calcium complexation with DNB by the same concentration ratio is less than one percent at similar ligand concentration. This does not imply that the stoichiometry of complexes formed between DNB and these ions is other than one to one but that these ions are not strongly bound by DNB. The lack of quantitative binding of Co^{2+} , Ni^{2+} and Ca^{2+} by DNB would preclude its utility as a desirable indicator for these ions but could prove to be a valuable characteristic when trying to determine more strongly bound ions when these ions, especially Ca^{2+} or Ni^{2+} , are present.

Examination of the spectra for the titrations of DNB with each of the metals mentioned, except copper, suggests that only a single complex species (presumably 1:1) is formed. In each case at least two well-defined isosbestic points are present. Copper shows three clear isosbestic points (240, 258 and 368 nm) at metal-ligand ratios less than one, but the isosbestic points at 258 and 368 nm disappear as the metal concentration exceeds the ligand concentration. The isosbestic point at 368 nm is shown in Figures V.5 a. for $[\text{Cu}]_{\text{tot}}$ up to the 1:1 stoichiometric ratio and Figure V.5 b. for the whole titration. This behavior indicates formation of a new complex, presumably with a two to one copper to DNB ratio. The formation of the binuclear species is supported by the potentiometric titrations as well. The addition of this species to the model for the copper-DNB system was necessary to achieve a satisfactory fit in calculations with BEST. The formation of binuclear copper complexes has been reported for both BAPTA³ and its parent compound EGTA^{12,13,14}, thus it is not surprising to find evidence for similar behavior with DNB as well.

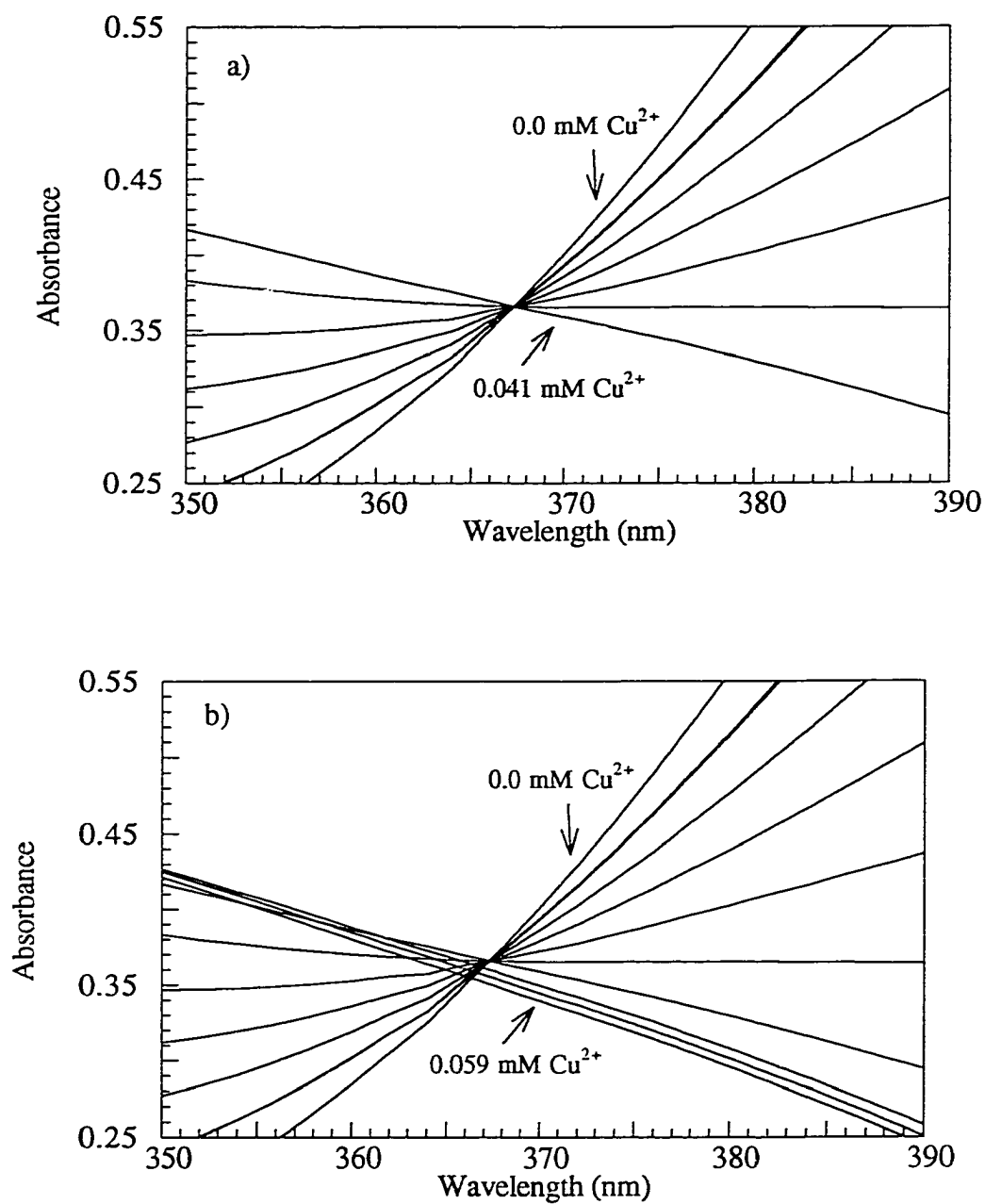


Figure V.5 a) A clear isosbestic point is shown in the titration of $0.041 \mu\text{M}$ DNB with Cu^{2+} at pH 7.0. b) In the same titration the isosbestic point is lost as excess Cu^{2+} is added.

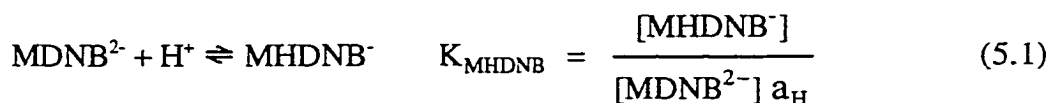
Binuclear species were also reported for BAPTA complexes with Ni^{2+} and Mg^{2+} ^{15,3}. The binding of DNB with Mg^{2+} is very weak and is not a problem at any stoichiometry. In the case of nickel, no evidence was observed for binuclear species formation with DNB. Given the low affinity of DNB for Ni^{2+} , binuclear species formation would only be observed at large concentrations (>0.5 mM) of excess nickel. Spectrophotometric titrations of ~ 40 micromolar DNB with Cd^{2+} and with Pb^{2+} , with final total metal concentrations as large as 3 - 5 mM, showed no spectral changes past the 1:1 ratio that might be associated with formation of a binuclear metal-DNB complex. The complex stoichiometry suggested for the interaction of DNB with copper may complicate determinations especially in view of the small spectral changes observed for 2:1 complex formation compared to spectral changes for 1:1.

The binding properties of DNB with lead, cadmium, and zinc show that it could prove useful as an indicator for these metals in biological or environmental work. Because indicator concentrations must be higher than concentrations of the ion they are measuring, formation of the copper binuclear species may not inhibit its quantitation with DNB. Species distribution calculations showed that in solutions containing 50 μM Cu^{2+} and 50 μM DNB less than 3% of DNB is complexed by two copper atoms. Solutions of DNB exhibit a bright yellow color (almost orange at high pH). As the complexation reaction with metal ions proceeds the absorbance maximum shifts to a shorter wavelength no longer in the visible range and the color fades. The large spectral changes that occur when DNB binds metals and the sensitivity allowed by its large molar absorptivity suggest potential for use as a tool for spectroscopic measurements of metal concentration or contamination at micromolar levels.

C. Complexation Constants of DNB with Divalent Metal Ions

I Mean Values

Data from the potentiometric titrations were input into the program BEST and the model was refined to determine the stability constants for the metal complexes formed. In most cases a protonated 1:1 complex, MHDNB, was required to fit the titration curve and, for consistency, was included in all models except for copper-DNB. The reaction and mixed-mode equilibrium expression for the protonation reaction of the metal-DNB complex are shown below.



In the final model for the copper-DNB system, the binuclear species was included and the protonated complex was omitted. The calculated stability constants for the protonated metal-DNB complexes obtained from potentiometric titration varied over a wide range for each complex. Based on results for BAPTA³ the expected value of K_{MHDNB} for each metal ligand complex was in the range of 2.7 - 4.25 log units (because this was the range for the same complexes with BAPTA). The average values obtained from calculations using BEST fell within this range for all metal-ligand complexes except the nickel-DNB complex. The average value for this complex was 4.62 log units which is slightly higher than the value of 4.24 obtained for the nickel-BAPTA protonated complex. The complex formation constant of calcium with DNB was not determined potentiometrically due to the low affinity of this ion for DNB.

Determinations of the complex formation constants of DNB with cadmium, lead, copper, zinc, cobalt, nickel, and calcium were done using UV-vis spectrophotometric to complement the studies using potentiometric methods. The results of the spectrophotometric titrations were analyzed to obtain the conditional stability constants and then corrected for side reactions of the ligand with protons using the α_{DNB} (eq 3.16) values for the pH at which the titration was carried out. The formation of protonated metal-DNB complexes required a second correction factor, α_{MDNB} , (based on average K_{MHDNB} values) for analysis of the spectrophotometric data.

The data used to calculate the average K_{MHDNB} values for DNB are from the potentiometric data analyzed using BEST and shown in Tables III.6 - III.11. This information was obtained in the form of overall formation constants. The values of K_{MHDNB} are extracted by using the equation for the overall formation constant for the protonated complex.

$$\beta_{\text{MHDNB}} = K_{\text{MDNB}} K_{\text{MHDNB}} \quad (5.2)$$

or

$$\log \beta_{\text{MHDNB}} = \log K_{\text{MDNB}} + \log K_{\text{MHDNB}} \quad (5.3)$$

The average values of K_{MHDNB} were used to calculate the correction factor, α_{MDNB} , using Eq 5.4.

$$\alpha_{\text{MDNB}} = \frac{[\text{MHDNB}^-]}{[\text{MDNB}^{2-}] + [\text{MHDNB}^-]} = \frac{1}{1 + K_{\text{MHDNB}} a_{\text{H}}} \quad (5.4)$$

The values for the correction factors, α_{MDNB} and α_{DNB} , at the titration pH used, are combined with the conditional stability constant, K''_{MDNB} , to give the intrinsic stability constant, K_{MDNB} , according to the equation

$$K_{\text{MDNB}} = \frac{K''_{\text{MDNB}} \alpha_{\text{MDNB}}}{\alpha_{\text{DNB}}} \quad (5.5)$$

The values obtained from spectrophotometric titrations for the conditional stability constants and the values of the intrinsic stability constants after correction for protonation reactions of both the ligand and the metal-ligand complex are listed in Table V.5.

Table V.6 lists the average values of stability constants for metal complexes with DNB, from each method and the overall mean values for both methods,. The second potentiometric value for cobalt was not included due to the poor fit (large sigma). The values from spectrophotometric titrations with copper were excluded from the overall average for lack of a correction factor for the formation of the binuclear species.

Table V.5 Results from correction of spectrophotometric complexation constants for side reactions of DNB and metal-DNB complexes with protons^a

Metal	pH	K''_{ML}	α_{DNB}	α_{MDNB}	K_{ML}	$\log K_{ML}$
Cd	3.5	2.11×10^5	6.40×10^{-4}	0.74	2.45×10^8	8.39
Pb	3.5	2.87×10^4	6.40×10^{-4}	0.72	3.21×10^7	7.51
Cu	3.5	6.23×10^4	6.40×10^{-4}	ND	9.73×10^7	7.99
Zn	3.5	3.83×10^3	6.40×10^{-4}	0.77	4.60×10^6	6.66
Co	4.0	1.55×10^3	8.00×10^{-3}	0.21	1.62×10^5	4.61
Co	7.0	2.02×10^5	9.77×10^{-1}	1.00	2.07×10^5	5.32
Ni	5.0	8.99×10^2	2.43×10^{-1}	0.71	2.61×10^3	3.42
Ni	7.0	5.02×10^3	9.77×10^{-1}	1.00	5.12×10^3	3.71
Ca	7.0	5.08×10^2	9.77×10^{-1}	ND	5.20×10^2	2.72
Ca	9.0	5.98×10^2	1.00	ND	5.98×10^2	2.78

^a 25.0 °C, ionic strength 0.1M (KNO₃).

Table V.6 Average values of stability constants for DNB from spectrophotometric and potentiometric methods^{a,b}

DNB			
Metal	log K _{ML} (spectral)	log K _{ML} (pot)	log K _{ML} (composite) ^c
Cd ^d	8.39 ± 0.01 (1)	8.78 ± 0.01 (3)	8.7 ± 0.3 (4)
Pb ^d	7.51 ± 0.02 (1)	7.26 ± 0.01 (2)	7.4 ± 0.2 (3)
Cu ^d	(7.99 ± 0.02 (1))	7.24 ± 0.08 (2)	7.2 ₄ ± 0.08 (2) ^e
Zn ^d	6.66 ± 0.01 (1)	6.48 ± 0.01 (2)	6.54 ± 0.13 (3)
Co	4.96 ± 0.35 (2)	4.22 ± 0.02 (2) ^f	4.6 ₀ ± 0.5 ₂ (4) ^f
Ni	3.56 ± 0.16 (2)	3.50 ± 0.01 (2)	3.53 ± 0.14 (4)
Ca	2.75 ± 0.03 (2)		2.75 ± 0.03 (2)

^a 25.0 °C, 0.1 M ionic strength (KNO₃). ^b (n) = number of titrations averaged. ^c Error expressed as standard deviation for the mean of all measurements from both methods unless otherwise specified. ^d Error expressed as standard error from nonlinear least squares. All other errors expressed as standard deviation of unweighted means. Potentiometric titrations only. ^e Exp.2 of potentiometric measurements not included.

2 Comparison with BAPTA

The average values of K_{ML} for each one to one metal-DNB complex are listed in Table V.7 with the corresponding values with BAPTA³. In Figure V.6 the average values obtained for logarithmic stability constants of each metal-DNB complex are plotted vs published values of constants for the same complexes with BAPTA. This plot illustrates the linear correlation between the logarithmic stability constants of DNB complexes and those of BAPTA complexes. The ordering of the metals according to binding strength is the same for both ligands, $Cd > Cu \approx Pb > Zn > Co > Ni > Ca$. The equation for the line shown is $y = -4.07 + 1.02x$. The slope of 1.0 and y-intercept of -4 indicate retention of selectivity with an overall drop in strength of complexation equivalent by 4 log units. Complexation selectivity is maintained but affinity is lowered for all metals to much the same degree

The large spectral changes observed for metal binding with DNB are consistent with destruction of the extended conjugation involving the aniline nitrogen atoms as the lone pair electrons of those atoms localize to coordinate the metal ion. The large changes seen in spectra from metal titrations of DNB are as expected, but occur to a smaller extent for the first two protonation steps.

Table V.7 Stability Constants of DNB and BAPTA Complexes with Divalent Metal Ions^a

Metal	DNB		BAPTA ^b	
	$\log K_{ML}^c$	$\log K_{MHL}^d$ ($\log K_{M_2L}^e$)	$\log K_{ML}$	$\log K_{MHL}$ ($\log K_{M_2L}$)
Cd^{2+}	8.78	3.04	12.2	2.70
Pb^{2+}	7.26	3.14	11.3	3.52
Cu^{2+}	7.23	(3.22)	11.7	3.65 (5.0)
Zn^{2+}	6.48	2.98	9.33	3.32
Co^{2+}	4.60	4.55	8.67	3.25
Ni^{2+}	3.49	4.62	7.76	4.24
Ca^{2+}	2.75		6.78	3.3

^a 25.0 °C, ionic strength 0.1M (KNO₃). ^b Published values from ref. 3. ^c For the reaction, $M + L \rightleftharpoons ML$. ^d For the reaction, $ML + H \rightleftharpoons MHL$. ^e For the reaction, $ML + M \rightleftharpoons M_2L$.

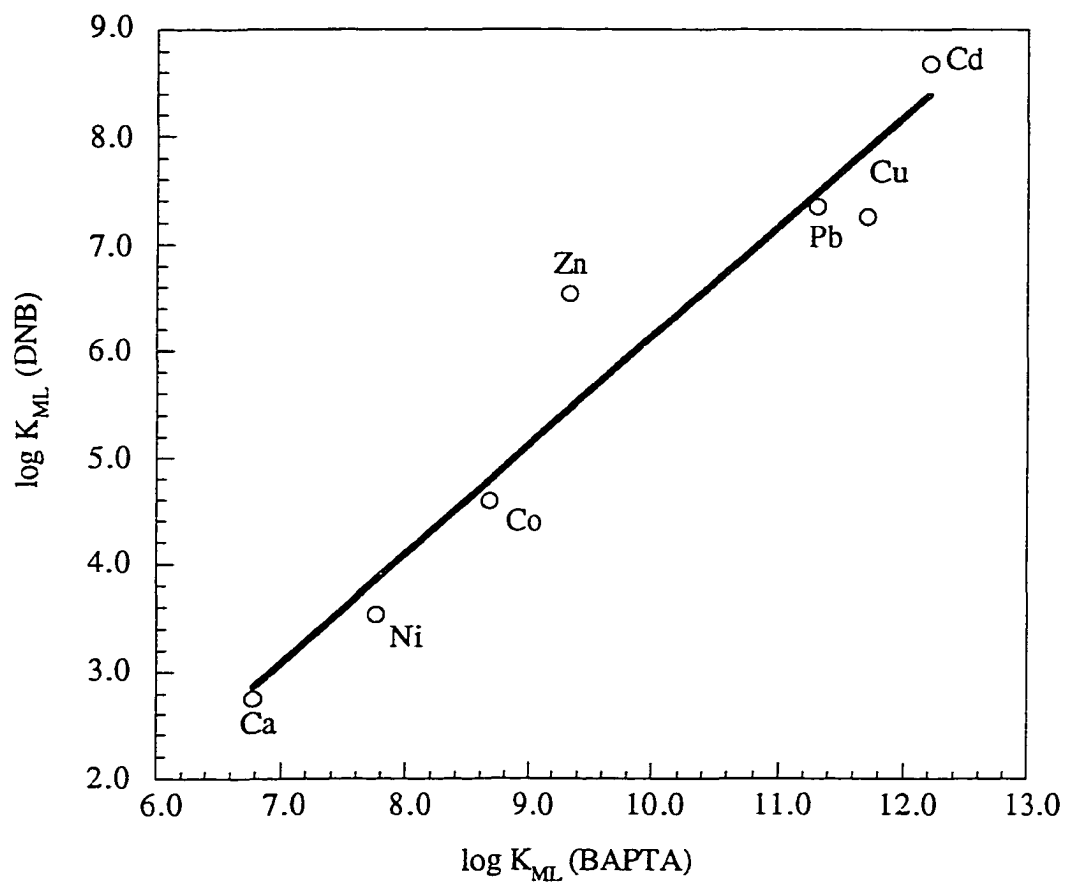


Figure V.6 Plot of the logarithmic stability constants of DNB complexes vs those of BAPTA complexes. The straight line results from a linear fit of the log values.

II Mag-Quin-1

MQ1 (mag-quin-1) was named after the calcium chelator quin-2 because of their structural and spectral similarities (structures are shown in Chapter I Figures I.4 and I.5). Quin-2 is from the first generation of calcium indicators designed by Tsein^{2,16}. Quin-2 was the first of the BAPTA derivatives designed to measure Ca^{2+} concentrations by fluorescence. Quin-2 and other BAPTA derivatives have been designed based on calcium selectivity of the spacing of donor atoms in EGTA. This spacing produces a binding cavity which fits well around a calcium ion but fits the smaller Mg^{2+} ion poorly. Evidence suggests that Mg^{2+} ions bind with only one end of one of these indicators. Effective indicators for measuring Mg^{2+} concentrations are much needed and hard to find. The selectivity for calcium based on the size of the binding cavity along with the evidence that Mg^{2+} binds only half this cavity, led to the idea of a Mg^{2+} indicator consisting of only half the calcium indicator structure yet retaining the ease of loading and some spectral properties. MQ1 consists of the more fluorescent half of quin-2. The affinity for Ca^{2+} was greatly diminished in these molecules though still greater than that for Mg^{2+} . The diminished affinity for Ca^{2+} also makes this type of indicator a candidate for measuring other metal ions in calcium containing media.

A Protonation Constants

The conditional stability constant of an indicator for any metal ion is dependent upon its side reactions with protons. Therefore, the protonation constants of an indicator are necessary to determine the effective metal binding constant at a given pH. The use of indicators having protonation constants ($\log K_H$) above the experimental pH requires careful consideration of the sensitivity of measurements to pH. Indicators

sensitive to pH changes in the range of the experiment call for more stringent pH control with non-interfering buffers. In some physiological experiments pH buffering other than by endogenous substances is not possible or desirable, thus, pH sensitive metal ion indicators would be less reliable.

1 Mean Values

The first three protonation constants for MQ1 were determined by UV-vis spectrophotometric titration at 25.0 °C with ionic strength maintained at 0.05 M with TEAP. Potentiometric methods were used to determine the first two protonation constants at the same temperature and ionic strength. The average values obtained in each titration, for each method and from results of both methods are listed in Table V.8. The average values for both methods were used to calculate the species distribution plot shown in Figure V.7, using the program Comics¹⁷. At pH 6.0, greater than 95% of the ligand is present in the monoprotinated form. At pH 6.5 this percentage drops to 90%, and at pH 7.0, 32% of the ligand is present as MQ1²⁻. This is illustrated in Figure V.8 by the steep slope of the two species around pH 7.0 and indicates a sensitivity of the absorbance to slight changes in pH in this range. The species distribution plot indicates areas relatively insensitive to pH to be between 5.0 and 6.2 or above 9.0. These pH values are outside the range of most physiological studies; therefore, pH buffers would be needed to stabilize measurements.

Table V.8 Average values for the protonation constants of MQ1 from spectrophotometric (S^a) and potentiometric (P^b) titrations^{c,d}

	$K_{H1} (x 10^{-7})$	$\log K_{H1}$	$K_{H2} (x 10^{-3})$	$\log K_{H2}$	K_{H3}	$\log K_{H3}$
S1	3.64 ± 0.21	7.56 ± 0.03	5.31 ± 1.10	3.72 ± 0.09	244 ± 29	2.39 ± 0.05
S2			5.05 ± 0.38	3.70 ± 0.03	280 ± 48	2.45 ± 0.07
S3	3.63 ± 0.23	7.55 ± 0.03				
Average Spectral ^e	<u>3.63 ± 0.04</u>	<u>7.56 ± 0.03</u>	<u>5.21 ± 0.87</u>	<u>3.72 ± 0.07</u>	<u>258 ± 39</u>	<u>2.41 ± 0.07</u>
P1		7.55 ± 0.04		3.85 ± 0.05		
P2		7.62 ± 0.04		3.67 ± 0.02		
Average Potentio ^f		<u>7.58 ± 0.03</u>		<u>3.76 ± 0.09</u>		
Composite ^g		<u><u>7.57 ± 0.03</u></u>		<u><u>3.74 ± 0.08</u></u>		<u><u>2.41 ± 0.07</u></u>

^a Average values of calculations at different wavelengths for each titration. ^b Values from individual measurements. ^c 25.0 °C, 0.05 M ionic strength (TEAP). ^d Error expressed in terms of standard deviation. ^e Average value of all calculations for all titrations. ^f Average of values from titrations. ^g Average of all titrations from both methods.

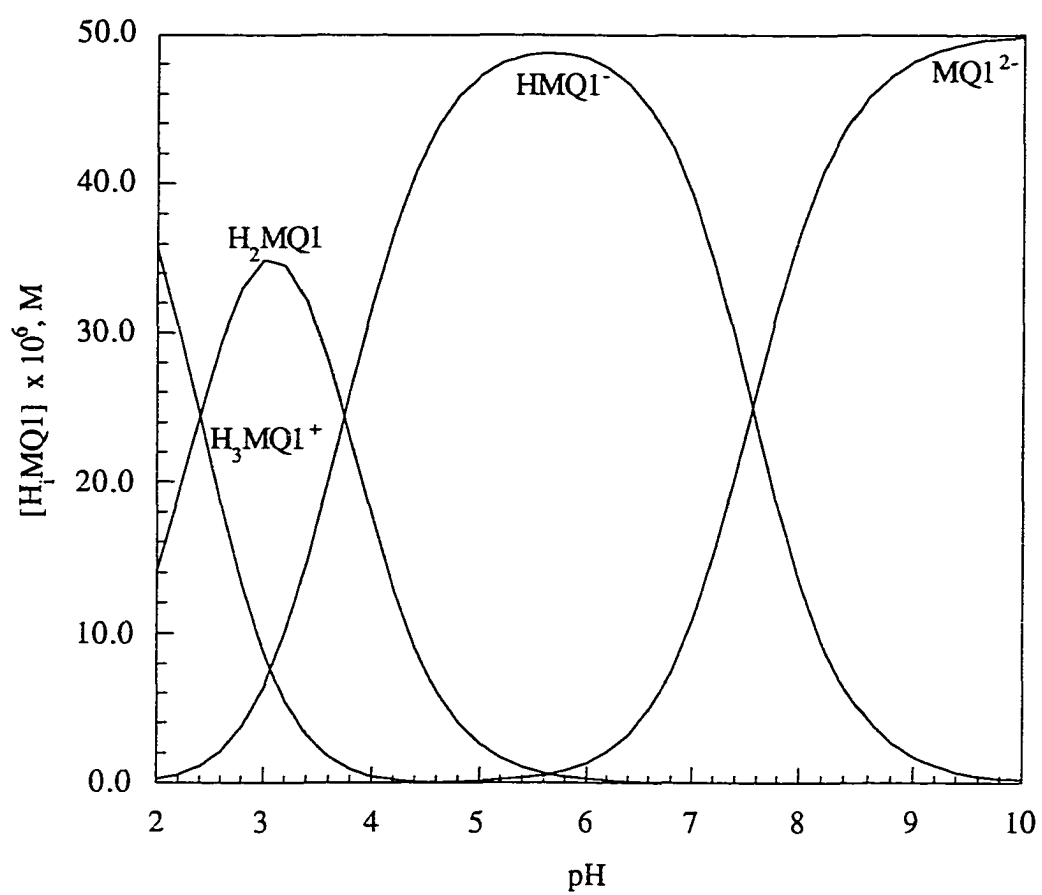


Figure V.7 Species distribution plot for the first three protonation reactions of MQ1 from pH 2.0 - 10.0.

2 Comparison to quin-2

In comparison to quin-2 the first protonation constant is significantly higher causing the undesirable pH sensitivity. The second protonation site for quin-2 is for the missing aniline nitrogen so is not comparable to MQ1. The second and third protonation constants in MQ1 are essentially the same as the third and fourth protonation constants recorded for quin-2. The average values obtained for MQ1 are listed in Table V.9 along with the values of protonation constants for quin-2.³

The cause of the increase in the first protonation constant for MQ1 compared to quin-2 is unclear. Both compounds have a methyl(ene) group at the 2 position. Quin-2 has an ether oxygen in place of the hydrogen in MQ1. The inductive effects of the electron withdrawing, methyl ether linkage would have to be fairly large to account for such an increase in acidity. Electron donating effects of a methyl group are usually small and probably would not cause a great increase in basicity. A similar increase in protonation constant is seen for a derivative of quin-2, quene-1, in which the ether linkage is replaced by a double bonded carbon. Quene-1¹⁸ was determined to have a protonation constant of 7.3 at 37.0 °C, and ionic strength of 0.15 M.

Table V.9 Comparison of log K_{H} values for MQ1^a and quin-2^b

	log K_{H1}	log K_{H2Q2}	log $K_{\text{H2MQ1}}/K_{\text{H3Q2}}$	log $K_{\text{H3MQ1}}/K_{\text{H4Q2}}$	log K_{H5Q2}
MQ1	7.57		3.73	2.41	
quin-2	6.25	5.30	3.59	2.67	2.0

^a 25.0 °C, ionic strength 0.05 M (TEAP). ^b 25.0 °C, ionic strength 0.1 M (KNO₃) Yuchi et al.⁵

B Characterization of MQ1-metal complexation spectra

The spectral characteristics of MQ1 were investigated to determine the response upon metals ion complexation. UV-vis spectral changes for the binding of cadmium, lead, copper, zinc, nickel, and calcium by MQ1 were analyzed to determine shifts in wavelength of maximum absorbance, molar absorptivity, and in some cases the binding ratio. These results are displayed in Chapter IV, Table IV.3.

A substantial spectral response was observed for most metals investigated with increases in absorbance around 240 nm and 340 nm and decreasing absorbance in the wavelength region of the unbound MQ1 peak maxima at 268 nm and 370 nm. Calcium spectra are complicated by the absorbance of nitrate at wavelengths below 350 nm, but show a small, measurable decrease in absorbance from 350 - 420 nm. For all metals investigated clean isosbestic points were observed throughout the titrations. All metals except calcium showed a quantitative linear response with greater than 99% complexation when the metal to ligand ratio was 1:1. Spectra for 49.0 μM MQ1 are shown in Figures V.8 and V.9 overlaid with spectra of metal-MQ1 complexes of equal concentration to illustrate the spectral changes which occur when MQ1 forms a complex with each metal ion.

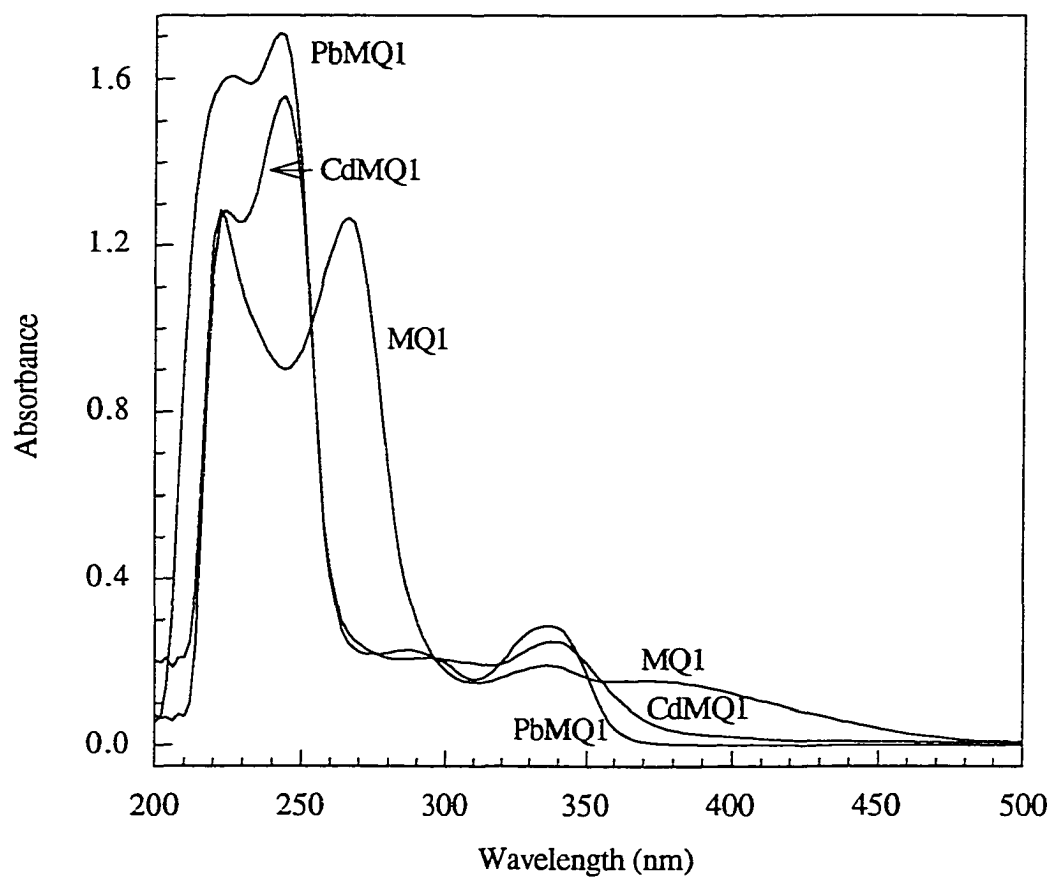


Figure V.8 Spectral changes between unbound MQ1 and CdMQ1 and PbMQ1 complexes at pH 6.5. All solutions contain 49 μM MQ1 and excess metal at 25.0 $^{\circ}\text{C}$ and ionic strength 0.05 M (TEAP).

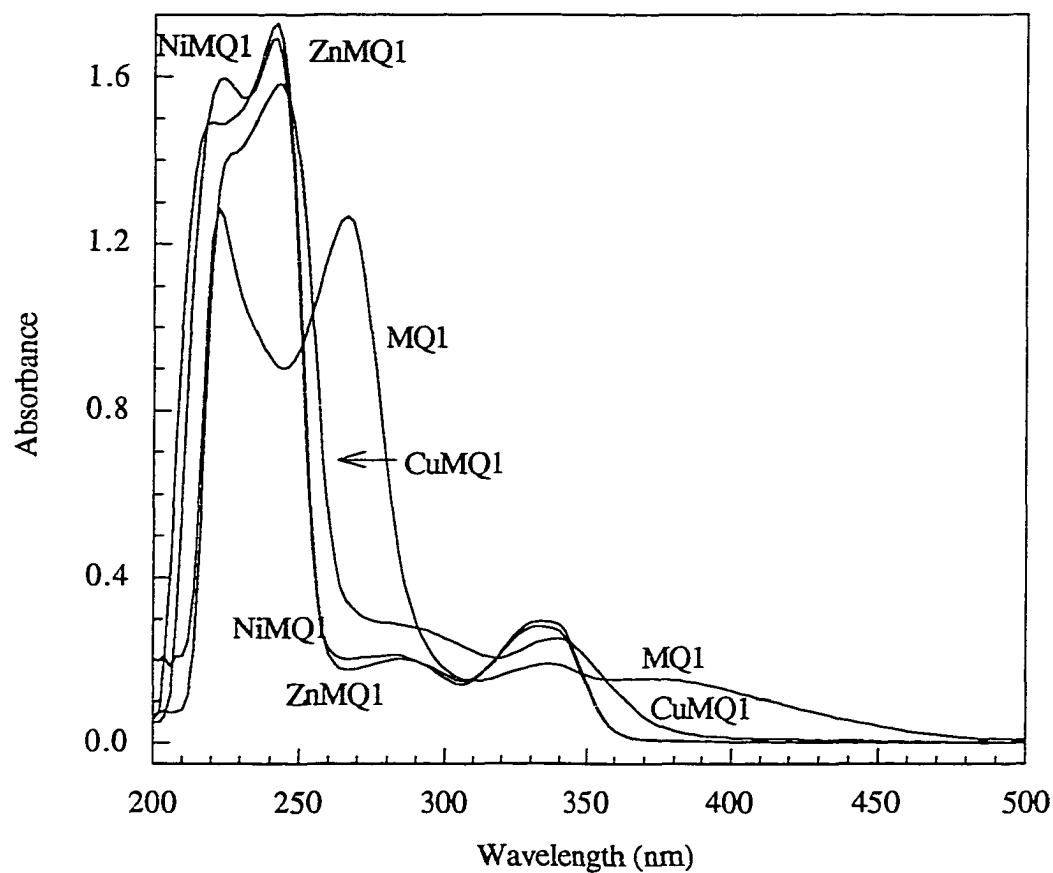


Figure V.9 Spectral changes between unbound MQ1 and ZnMQ1, CuMQ1 and NiMQ1 complexes at pH 6.4. All solutions contain 49 mM MQ1 and excess metal at 25.0 °C and ionic strength 0.05 M (TEAP).

C Formation Constants of MQ1 with Divalent Metal Ions

Spectrophotometric and potentiometric titration methods were used to determine complex formation constants of MQ1 with cadmium, lead, copper, zinc, nickel, and calcium. The conditional complex formation constants obtained for the pH of the titrations were corrected for protonation side reactions of MQ1 as described in Chapter IV.I.C. Data from the analysis of potentiometric data indicated the formation of protonated complexes for cadmium-MQ1, lead-MQ1, and zinc-MQ1 as well. The formation constants determined using the spectrophotometric method were corrected for these side reactions, at the pH of the titration. The K_{MHMQ1} values used in eq 5.6 were determined in potentiometric experiments.

$$\alpha_{MMQ1} = \frac{1}{1 + K_{MHMQ1}a_H} \quad (5.6)$$

The values obtained for formation constants of MQ1-metal complexes determined from UV-vis spectrophotometric titrations before and after correction for protonation side reactions are listed in Table V.10.

Table V.10 Formation constants from correction of spectrophotometric conditional formation constants for protonation reactions of MQ1 and metal-MQ1 complexes^a

Metal	pH	K''_{ML}	error ^b	α_{MQ1}	K'_{ML} ^c	α_{MMQ1}	K_{ML}	log K_{ML}
Cd	3.0	420	6.3	2.95×10^{-6}	1.42×10^8	0.53	7.53×10^7	7.88
Pb	3.0	6.69×10^3	1.2×10^2	2.95×10^{-6}	2.27×10^9	0.74	1.68×10^9	9.23
Cu	2.8	3.46×10^4	4.6×10^3	1.11×10^{-6}	3.11×10^{10}			10.5
Zn	3.0	9.13×10^3	6.7×10^2	2.95×10^{-6}	3.09×10^9	0.84	2.60×10^9	9.49
Ni	3.0	1.86×10^5	6.9×10^3	2.95×10^{-6}	6.32×10^{10}			10.8
Ca	7.2	1.73×10^3	51	0.303	5.70×10^3			3.76

^a 25.0 °C, ionic strength 0.05 M (TEAP). ^b Uncertainty expressed as standard error.

^c $K'_{ML} = K''_{ML} / \alpha_{MQ1}$.

1 Mean Values

Quin-2 was reported to form complexes with magnesium², copper³ and nickel¹⁵ having 2:1 metal-ligand stoichiometry. Analysis of potentiometric data for a solution containing approximately equimolar MQ1 and copper indicated the formation of a two to one, MQ1 to copper, species as well as the one to one complex. Using the successive approximation process, attempts to fit the spectrophotometric data for copper to a model with 1:1 stoichiometry did not give a satisfactory convergence after as many as 30 iterations calculated. Visual inspection of the curve-fit of the final free copper concentrations, Figure IV.24, shows a poor fit. Because of the poor fit and inability to correct the calculation of the formation constant of the one to one complex for the

presence of the two to one complex, the spectrophotometric titration value was not included in the calculation of the composite stability constant.

A large discrepancy can be seen between the spectral and potentiometric values for nickel as well. No evidence was found for formation of a $\text{Ni}(\text{MQ1})_2$ complex from the potentiometric data. For spectrophotometric titrations the successive approximation of free nickel concentrations converged to values that changed less than 2% between iterations and the calculated curve fit the final concentration data well. Although the apparent stability constant determined at pH 3.0 is at the upper limits of the range suitable for the spectrophotometric titration method, values from both spectrophotometric and potentiometric determinations were kept and included in the calculation of the composite formation constant of this complex.

Calculations of formation constants for MQ1 with calcium varied by 0.61 log units between the two methods. Significant changes in dissociation constants resulting from changes in ionic strength have been reported for similar Ca^{2+} chelators⁶. Given the concentration of calcium required to reach the degree of complexation necessary for curve-fitting calculations of the stability constant of the CaMQ1 complex for the spectrophotometric method, ionic strength differences could account for part of the difference between the two methods. Calcium causes only a small perturbation of the potentiometric titration curve of MQ1 which could lead to less accuracy in calculations. For these reasons, values determined by both methods were included in the mean value of the of the formation constant for this complex.

The values determined by both methods are listed in Table V.11 along with the composite values. Values determined for cadmium, lead and zinc complexes with MQ1 agreed well between the two methods and showed no evidence for the formation of complexes with additional stoichiometries.

Table V.11 Average values for formation constants of MQ1 with divalent metal ions from spectrophotometric and potentiometric methods^{a,b}

Metal	log K _{ML} (spectral)	log K _{ML} (potentiometric)	log K _{ML} (composite)
Cd	7.88 ± 0.01 (1)	8.07 ± 0.19 (2)	8.0 ± 0.2
Pb	9.23 ± 0.01 (1)	9.53 ± 0.44 (2)	9.4 ± 0.5
Cu	10.49 ± 0.06 (1)	13.83 ± 0.13 (3)	13.8 ± 0.1 ^c
Zn	9.41 ± 0.03 (1)	9.80 ± 0.07 (2)	9.7 ± 0.2
Ni	10.80 ± 0.02 (1)	12.12 ± 0.02 (1)	11.5 ± 0.7
Ca	3.76 ± 0.01 (1)	3.15 ± 0.03 (1)	3.5 ± 0.3

^a 25.0 °C, ionic strength 0.05 M (TEAP). ^b (n) = number of titrations averaged.
^cSpectral value not included.

2 Comparison with quin-2

The log K_{ML} values for complexes of Mg^{2+} , Ni^{2+} , Ca^{2+} , Cd^{2+} , and Pb^{2+} with both MQ1 and quin-2²³ are listed in Table V.11 and are plotted vs the ionic radii of the metal ions¹⁹ in Figure V.10. Values for the complexes of quin-2 with Zn^{2+} and Cu^{2+} were not available due to precipitation problems³. From this plot the significant effect on the stability of MQ1 complexes with larger ions can be seen. The ligand, quin-2, has eight donor atoms; two nitrogens and two carboxylate oxygens on the quinoline moiety and a nitrogen, ether oxygen and two carboxylate oxygens on the half BAPTA portion. The small size of Ni^{2+} and Mg^{2+} ions along with a strong preference for octahedral stereochemistry of ligands (coordination number of six) keeps these ions from being able to bind through all the donor atoms available in quin-2. Smaller ions were thought to bind to only the half of quin-2². The quinoline nitrogen is a more attractive donor atom for Mg^{2+} , Ni^{2+} , Zn^{2+} than the ether oxygen and thus makes the quinoline portion of the quin-2 molecule the more logical binding site. This idea seems to be corroborated, at least in the case of Ni^{2+} and Mg^{2+} , by the minor effect on binding strength caused by the loss of the non-binding half of the molecule. Larger metal ions, such as Ca^{2+} , Cd^{2+} , and Pb^{2+} , with no rigid geometrical requirements imposed by partially filled d orbitals can complex with greater numbers of donor atoms. These metal ions can complex through all 8 of the donor atoms in quin-2. The loss of the aniline nitrogen, ether oxygen and 2 carboxylate oxygens from the missing half of the quin-2 molecule excludes the energy derived from binding at these donor atoms and thereby lowers the stability constant for the metals that were able to use these sites.

Table V.12 Formation constants of MQ1 and quin-2 complexes with divalent metal ions^a

Metal ion	MQ1 log $K_{ML}^{b,c}$	quin-2 log $K_{ML}^{b,d}$
Cd ²⁺	7.98	12.26
Pb ²⁺	9.38	12.24
Ni ²⁺	11.5	11.62
Ca ²⁺	3.46	7.28
Mg ²⁺	2.89 ^d (2.17) ^e	2.7 ^f

^a 25.0 °C. ^b For the reaction, $ML + H \rightleftharpoons MHL$. ^c ionic strength 0.05 M (TEAP). ^d ionic strength 0.1 M (KNO₃), ref. 3. ^e Ref. 20. ^f Ref. 19. ^g Ref. 2.

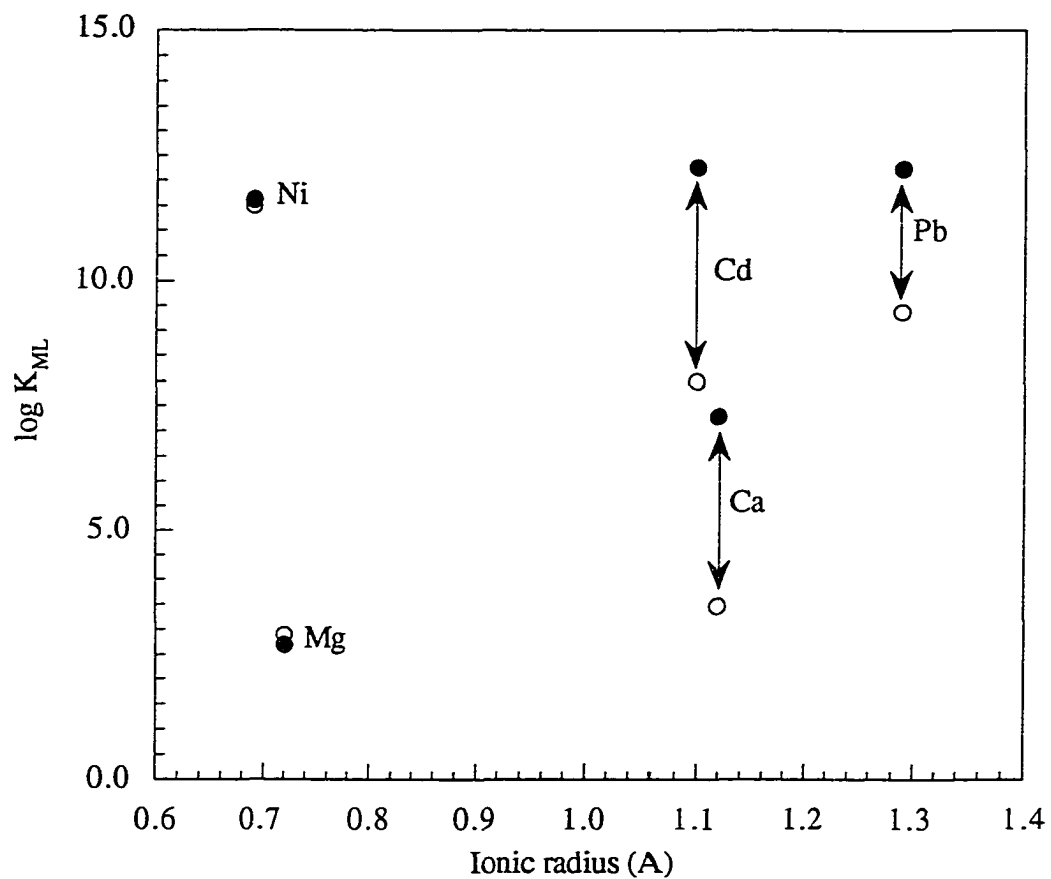


Figure V.10 Plot of K_{ML} for quin-2 (○), and MQ1 (●) vs ionic radius. Effective radii with coordination number 6 for Mg^{2+} and Ni^{2+} , coordination number 8 for Cd^{2+} , Ca^{2+} , Pb^{2+} , ref. 22.

III Conclusions

Indicators useful for measuring metal ions are vital to understanding the role each of these ions play in health and disease. They are also necessary to evaluate levels of contaminants in industrial and environmental media. The main problem faced in developing indicators is interference from more abundant ions. Many tools have been developed to study Ca^{2+} in the presence of more abundant ions such as Mg^{2+} and protons but as yet, few are available for the study of trace metals in the presence of calcium.

In physiological media calcium is present at about 0.1 to 1.0 micromolar levels while trace metals are at or lower than nanomolar in concentration. In general, the difference in stability constant values (K_{ML}) between the target ion (lower concentration) and the interfering ion (higher concentration), should exceed the relative concentration difference by at least a factor of 10. For 1 nM M^{2+} in the presence of 10 μM Ca^{2+} the stability constant for M^{2+} needs to be greater than that for Ca^{2+} by a factor of at least 10^5 .

In the case of DNB, the calcium complex has a formation constant of about 2.75 log units, therefore a stability constant greater than 7.75 is required. The cadmium-DNB complex has a stability constant almost 6 orders of magnitude larger than that of calcium-DNB. The stability constant for the complex of DNB with lead is greater than that for calcium by about $10^{4.5}$. This is the order of selectivity demonstrated by BAPTA but in DNB the affinity for calcium has been lowered to the extent that calcium binding would be negligible at normal concentration levels while complexation to cadmium would be complete. Because of this and the longer wavelength absorbance of DNB compared to BAPTA, DNB would be the better indicator for Cd^{2+} in physiological studies.

The formation constant of the nickel-DNB complex is also much lower than that for the cadmium-DNB complex. It should be possible to determine Cd^{2+} in the presence of greater concentrations of Ni^{2+} . This could be a useful indicator for some industrial applications, specifically in waste from factories where nickel-cadmium batteries are manufactured.

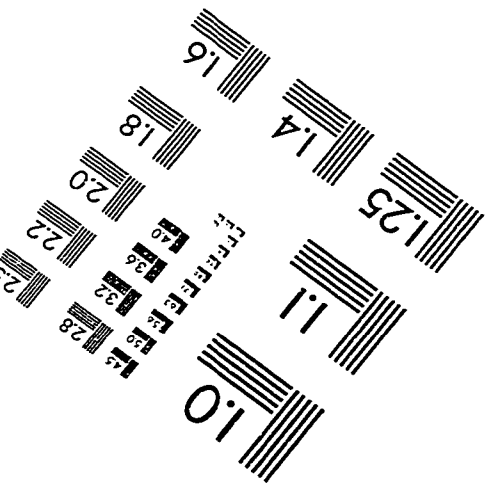
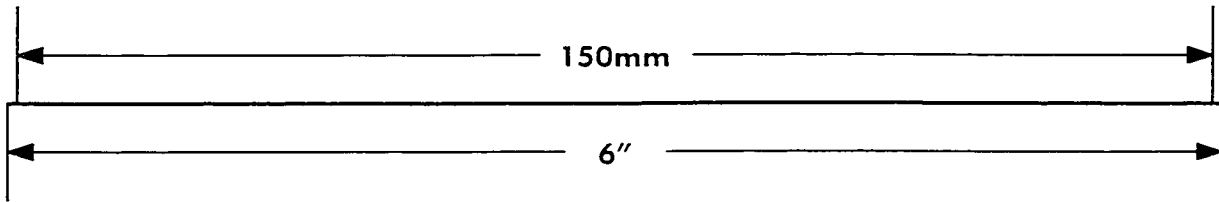
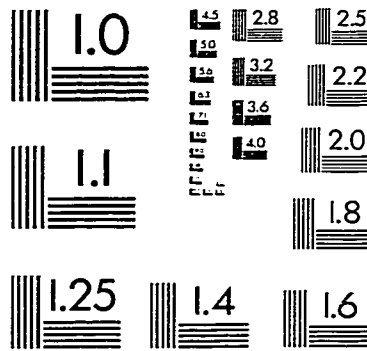
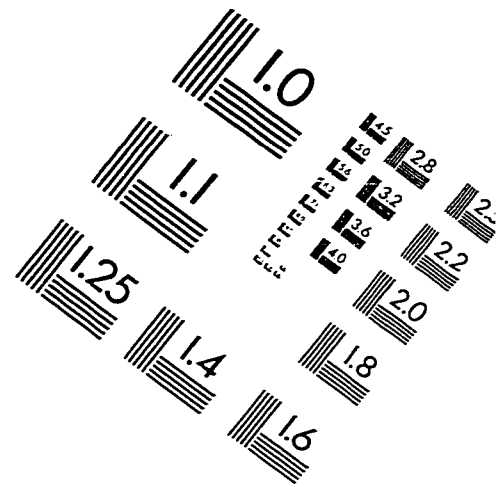
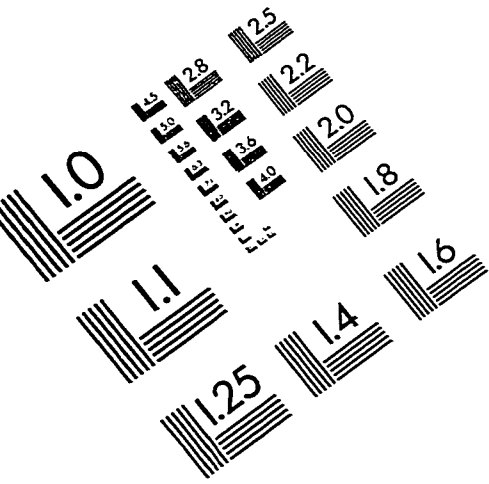
MQ1 has very different properties than DNB, complexing more strongly to some of the smaller metal ions. Ni^{2+} and Zn^{2+} are complexed strongly compared to Ca^{2+} , and only complexes with 1:1 metal-ligand stoichiometry were observed at the concentrations investigated. Of the trace metals examined, all but Cd^{2+} complex MQ1 with conditional formation constants, at pH 7, at least 5 orders of magnitude greater than for Ca^{2+} . Cu^{2+} complexes with two ligands bound to one copper ion are indicated and may complicate the use of MQ1 as an indicator for this metal ion. Although pH sensitivity would still need to be considered, MQ1 should easily indicate zinc, nickel and lead ions without interference from intracellular Ca^{2+} . The molar absorptivity of MQ1 is higher at shorter wavelengths (220 - 280nm) where interference from endogenous cellular compounds can be a problem. Although MQ1 absorbance may be less sensitive at longer wavelengths than DNB, it has the additional advantage of fluorescence.

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IMAGE EVALUATION TEST TARGET (QA-3)



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