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Compounds with two dissimilar chelating centers

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Recommended Citation

COMPOUNDS WITH TWO DISSIMILAR CHELATING CENTERS

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Analytical Chemistry

Approved:

In Charge of Major Work

Head of Major Department

Dean of \Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1966

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INTRODUCTION

The determination of calcium and magnesium in biological liquids has become more and more important in recent years. Many body disorders are now diagnosed by chemical examination of the changes in the calcium and magnesium levels in body liquids. Because the sample for analysis must be withdrawn from the patient, it is necessary that the sample be as small as possible and this in turn necessitates that the methods of analysis be appropriate to small amounts of material. Fluorometric methods of analysis are ideal for such analytical problems owing to the inherently great sensitivity of fluorescence measurements. Neither calcium nor magnesium is inherently fluorescent and for the fluorometric determination of these elements reagents are needed which undergo changes in fluorescence on union with the ions of the elements.

The reagent Calcein was introduced by Ellingboe and Diehl (8) in 1956 for the fluorometric determination of calcium. Calcein (I) is derived from fluorescein by the introduction of two methyleneiminodiacetic acid groups (R)

$$R = -CH_2-N^+ \begin{matrix} CH_2-CO_2^- \\ H \\ CH_2-CO_2H \end{matrix}$$
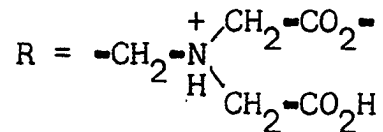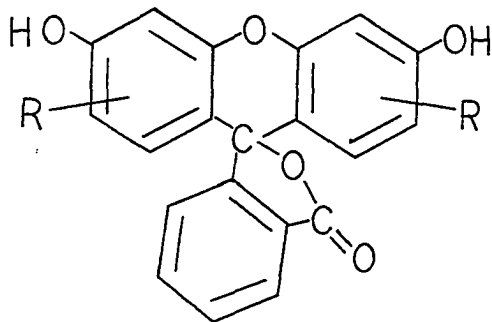
I

The calcium derivative of Calcein is intensely fluorescent and Calcein was initially proposed as an indicator for the ethylenediaminetetraacetate (EDTA, $H_2Y^=$) titration of calcium in the presence of magnesium, the main reaction in this titration being

$$Ca^{++} + H_2Y^= = CaY^= + 2H^+$$
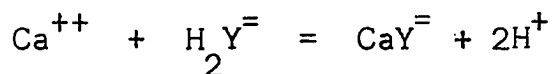
(Solution buffered at pH 12.5+)

At the end-point the calcium is extracted from the calcium-Calcein compound by the EDTA, that is, by the EDTA no longer required for the main reaction, and the fluorescence disappears. Calcein has been widely used, the current (1966) bibliography on it numbering about eighty papers; a review of the literature to 1964 is found in the monograph of Diehl (7). Attempts to use Calcein as originally prepared for the direct fluorometric determination of calcium were disappointing although a paper describing such a method was actually published (14); this picture is changing as a result of work on the preparation of metal-free Calcein currently (1966) under way at Iowa State University. The structure of Calcein is still in doubt although some work has been published on the subject (Wallach and Steck (26), Bozhevol'nov and Kreingol'd (3).

A different type of chelating compound, the hydroxyazo compounds, was also studied by Diehl and Ellingboe and in 1956 (9, 12) they established the minimum structural require-

ments for an azo compound to react with calcium and magne-
sium. Two hydroxy groups, o- and o'-, respectively, to the
azo group, were found necessary and sufficient:
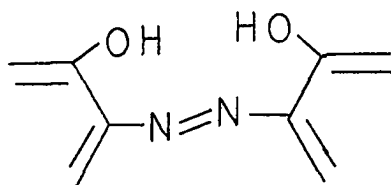
II

The combining ratio of metal and azo compound was invariably
one to one. For the o,o'-dihydroxyazo compounds studied,
the formation constant toward magnesium was greater than that
toward calcium, the ratio of the two being in the range of
50 to 1 to 1000 to 1. Of some thirty azo compounds studied,
one compound proved remarkable, o,o'-dihydroxyazobenzene
(H_2Dhab, III), the simplest member of the group:
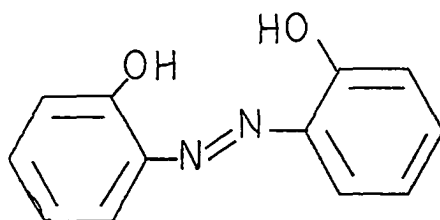
III

Diehl and Ellingboe (9) found that this compound did not re-
act with calcium but did with magnesium, the formation con-
stant toward magnesium being $K_{MgDhab} = 7.1 \times 10^4$. In the
effort to exploit o,o'-dihydroxyazobenzene as an analytical
reagent, H. Diehl et al. (10) found that the magnesium

derivative was fluorescent. An excellent method was then developed for the fluorometric determination of micro amounts of magnesium in the presence of calcium (10).

In Calcein, the methyleneiminodiacetic acid group (IV R of structure I)

$$-CH_2-\overset{+}{\underset{H}{N}}\overset{\displaystyle CH_2-CO_2H}{\underset{\displaystyle CH_2-CO_2^-}{<}}$$
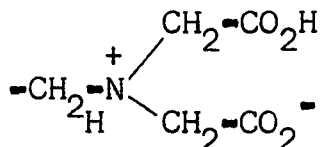
IV

was introduced into the molecule of fluorescein. The methyleneiminodiacetic acid group, by virtue of the two carboxyl groups and the amino nitrogen atom has chelating properties, that is, will unite with metal atoms to form ring compounds, and its presence in the fluorescein molecule give the latter the property of reacting with metal ions. The methyleneiminodiacetic acid group is half of the molecule of ethylenediaminetetraacetic acid (EDTA, V).

$$\underset{\displaystyle ^-O_2C-CH_2}{\overset{\displaystyle HO_2-CH_2}{>}}\overset{+}{\underset{H}{N}}-CH_2-CH_2-\overset{+}{\underset{H}{N}}\overset{\displaystyle CH_2-CO_2H}{\underset{\displaystyle CH_2-CO_2^-}{<}}$$
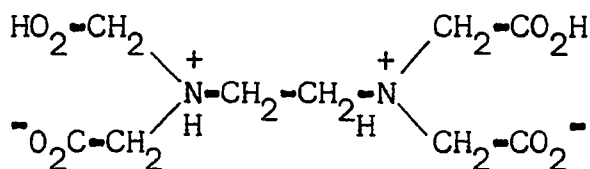
V

Whereas ethylenediaminetetraacetic acid acts as a sexadentate (literally, six-toothed) chelating molecule, becoming attached to the metal atom by six bonds (through the four oxygen atoms of the carboxyl groups and the two nitrogen atoms, the meth-

yleneiminodiacetic acid group acts as a tridentate (three-
toothed) chelating group. The fluorescence of fluorescein
is dependent on the hydrogen ion concentration of the solu-
tion in which it is dissolved, that is, fluorescein acts as
a fluorescent acid-base (pH) indicator. In Calcein there
is thus superimposed on the acid-base-fluorescence indicator
function the chelating function. The two functions inter-
act for the fluorescence of Calcein is dependent on the
union with a metal ion as well as on pH. Hence the action
and use of the compound, Calcein, as a metallofluorochromic
indicator.

In o,o'-dihydroxyazobenzene, the chelating action is
centered in the o,o'-dihydroxyazo grouping (II). o,o'-
Dihydroxyazobenzene itself is highly colored, the color in
solution being dependent on the hydrogen ion concentration
of the solution, that is, it functions as an acid-base in-
dicator although a poor one. It is non-fluorescent but on
combination with magnesium undergoes a change in color and
becomes fluorescent.

In the compounds described in this thesis the methylene-
iminodiacetic acid group has been introduced into the o,o'-
dihydroxyazobenzene molecule. The new compounds differ from
Calcein and from o,o'-dihydroxyazobenzene in having two
different chelating functions in the same molecule, the
o,o'-dihydroxyazo group and the iminodiacetic acid group.

These chelating functions are, of course, superimposed on the acid-base indicator property. The new compounds, thus were expected and proved to form metal derivative and to be responsive in color and fluorescent to both hydrogen ion and metal ion concentration. It was of interest to learn which chelating group, or if both, unites with the metal ions. It is of interest, too, to learn if the metal derivatives are highly colored or fluorescent and of use in chemical analysis.

PREPARATION OF 1-[3'(N,N-BIS(CARBOXYMETHYL)AMINOMETHYL)-2'
-HYDROXY-1'-BENZENEAZO]-2-HYDROXYBENZENE AND
1-[3'(N,N-BIS(CARBOXYMETHYL)AMINOMETHYL)-2'-HYDROXY-1'
-BENZENEAZO]-3-(N,N-BIS(CARBOXYMETHYL)AMINOMETHYL)
-2-HYDROXYBENZENE

Experimental Work

Apparatus

Measurements of pH were made with a Corning Model 10
pH meter equipped with a Beckman No. 40495 high alkalinity
glass electrode and a Beckman asbestos fiber type saturated
Calomel electrode.

Nuclear Magnetic Resonance spectra were made with a
Varian Associates A-60 Nuclear Magnetic Resonance Spectro-
meter.

Mass spectra were obtained on an Atlas MAT Bremer
Massenspectrometer CH4.

Reagents

o-Nitrophenol, melting point: 45°, was obtained from
Eastman Kodak Chemical Division, Kingsport, Tennessee, and
was not further purified.

Trifluoroacetic acid, spectral quality, was obtained
from Eastman Kodak Chemical Division, Kingsport, Tennessee.

Disodium iminodiacetate dihydrate was obtained from
Geigy Chemical Corporation, Ardsley, New York and was not

further purified.

Sephadex G-10 was obtained from Pharmacia Inc., Newark, New Jersey.

o,o'-Dihydroxyazobenzene, melting point: 174°, was prepared according to the procedure outlined by R. Olsen (17). It is also available in satisfactory quality from the G. Fredrich Smith Chemical Company, Columbus, Ohio.

The buffers used to standardize the pH meter were Beckman 3506 - pH 4 buffer solution and Beckman 3505 pH 10 buffer solution.

All reagent chemicals used were of reagent grade quality.

All the water used was distilled and deionized by passage through Amberlite MB-1 ion-exchange resin.

Mannich condensation of o,o'-dihydroxyazobenzene, formaldehyde, and disodium iminodiacetate dihydrate

Fifty grams of o,o'-dihydroxyazobenzene was dissolved in 300 ml. of dimethylsulfoxide. This solution was poured into a 1l., three-necked round bottom flask fitted with a condenser, a stirrer, and a thermometer. To this stirred solution was slowly added 57 g. of disodium iminodiacetate dihydrate. The temperature was raised to 72° and maintained at that temperature. A solution containing 27.1 ml. of 37 percent formaldehyde in methanol and 75 ml. of dimethylsulfoxide was slowly added through the condenser. The mixture was held at 72° for eight hours with constant stirring.

The resultant mixture was added to 1.5 l. of water; the precipitate which formed was filtered off. This solid residue was amorphous and dark brown with admixed greenish-yellow particles. It contained unreacted starting material and tar. The filtrate was acidified with hydrochloric acid bringing the pH to 2. The red-brown precipitate which formed was filtered off. This precipitate was dissolved in sodium hydroxide, filtered, and reprecipitated with hydrochloric acid. The precipitate was filtered, triturated with water and filtered again. The precipitate was triturated with ethanol, filtered, and dried in a vacuum oven at 60°. The amount of impure product obtained was 30 g.

Separation and purification of 1-[3'(N,N-bis(carboxymethyl) aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene and 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'- benzenazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2- hydroxybenzene

The solid material obtained from the Mannich condensation described above was subjected to descending paper chromatography to estimate the number of components present. A few milligrams of product was dissolved in sodium hydroxide and spotted on filter paper. The paper was eluted with a solvent containing: 10 percent phenol, 60 percent ethanol, and 30 percent water. The color of the bands themselves was used to estimate the number of compounds present. At least four different bands

were observed. After the chromatogram was sprayed with an aqueous solution containing magnesium ions, two of the bands were found to fluoresce.

A column of Sephadex G-10, 80 cm. long and 1.6 cm. in diameter was prepared. The column was equilibrated with 0.001 M sodium hydroxide as this was used as the eluent. To start a run on the chromatography column, 0.4 g. of the impure product from the Mannich condensation reaction was dissolved in the minimum amount of 1.0 M sodium hydroxide. The pH of this solution was adjusted to 10.5. The eluent was allowed to drain to the top of the Sephadex and the solution to be chromatographed was introduced at the top of the column. After the sample had been absorbed onto the top one inch of the column, elution was begun with the 0.001 M sodium hydroxide. The flow rate was adjusted to 10.5 ml./cm.^{2}/hr. by means of a pinch clamp on the outlet of the column.

As the elution proceeded, four distinct bands became apparent. Each band was collected in a separate beaker and the total amount of eluent was recorded. The material in each beaker was precipitated with hydrochloric acid, filtered, washed, and dried in a vacuum oven at 60°. The precipitate from each fraction was weighed. The weight of each precipitate was converted to weight percent of the weight of the total material recovered. This weight percent was plotted as an area against volume of eluent to construct

the elution curve shown in Figure 1.

The first fraction recovered seemed to be a mixture of high molecular weight compounds. This material did not melt, but turned black and charred above 200°. No further work was done with this material. The second fraction recovered was a yellow compound that melted sharply, melting point: $184\text{-}185^{\circ}$. The third fraction consisted of a red compound, melting point: $225\text{-}226^{\circ}$. The red compound decomposed with effervescence at the melting point. The fourth fraction was brown in color and very hygroscopic. No melting point could be obtained below 300°.

Identification of 1-[3'(N,N-bis(carboxymethyl)aminomethyl) -2'-hydroxy-1'-benzenazo]-2-hydroxybenzene

The yellow compound, isolated from the second fraction from the Sephadex column, was examined further. This material was not soluble in benzene or ethyl alcohol. The material could be dissolved in sodium hydroxide and reprecipitated with hydrochloric acid. This procedure gave an amorphous precipitate with a melting point of $184\text{-}185^{\circ}$.

Elemental analysis (Galbraith Laboratories, Knoxville, Tennessee): Found C 56.86; H 4.76; N 10.80; calculated for $C_{17}H_{17}O_6N_3$ (one methyleneiminodiacetic acid group), molecular weight 359.3: C 56.82; H 4.77; N 11.69.

A determination of the equivalent weight of this material was made by performing a potentiometric titration with sodium

Figure 1. Elution curve for the separation of products
from the Mannich condensation reaction.

    I   High molecular weight material (brown)

    II   Mono-methyleneiminodiacetic acid substituted
           on o,o'-dihydroxyazobenzene (yellow compound)

    III   Di-methyleneiminodiacetic acid substituted on
           o,o'-dihydroxyazobenzene (red compound)

    IV   Unknown (brown compound)
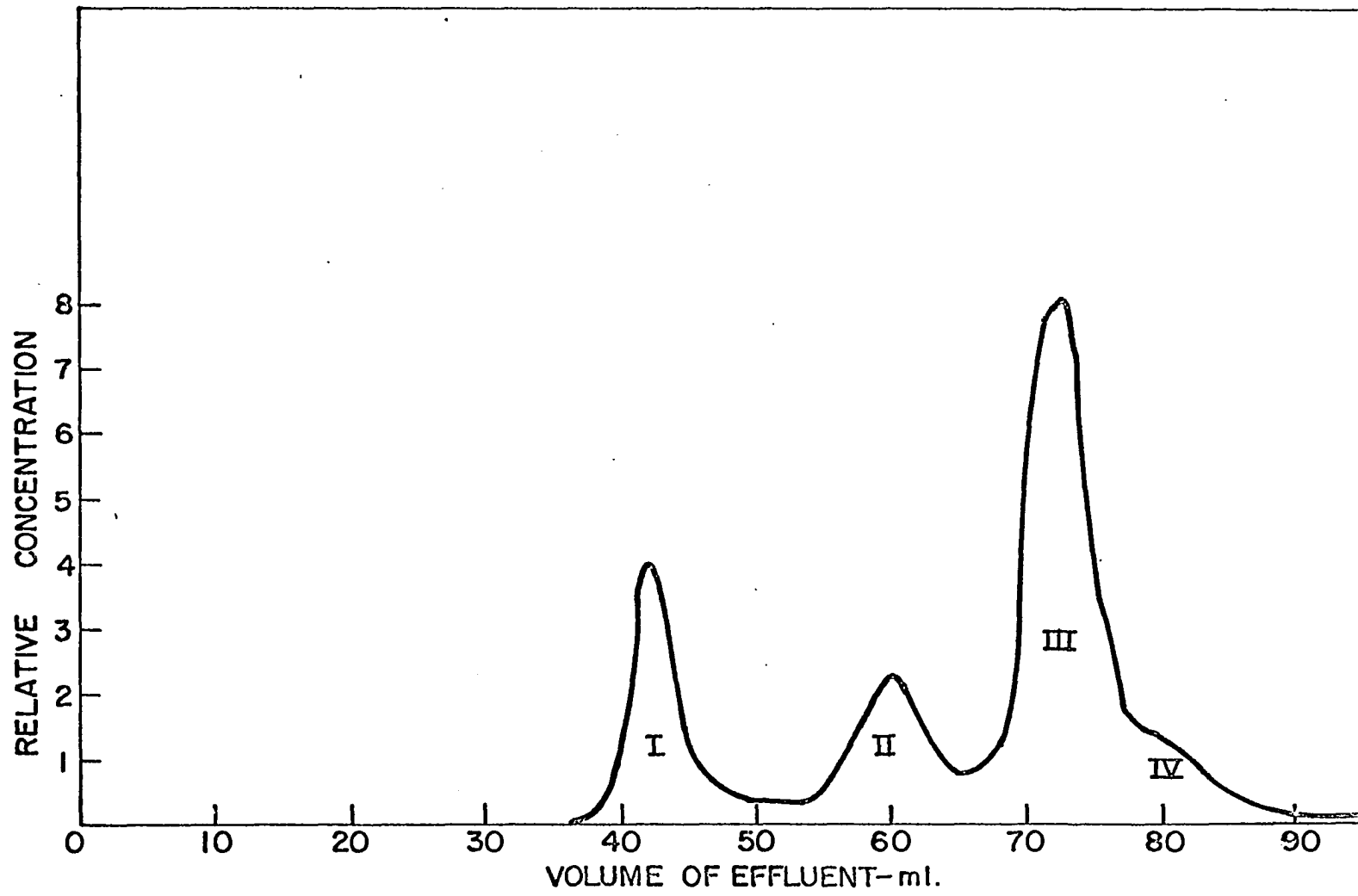
hydroxide. For this, 0.1693 g. of the yellow compound was added to 30 ml. of water that was 0.1 M in potassium chloride. A suspension was formed from which the solid dissolved during the titration, the last solid dissolving just before the first end point was reached. The suspension was titrated with 0.0647 N sodium hydroxide. The titration curve is shown in Figure 2. Equivalent weight found (first end point): 379.6; calculated for one replaceable hydrogen atom 359.3.

To determine the amount of metal ion impurities in the yellow compound, 0.2237 g. of the yellow material was weighed into a platinum crucible. The crucible was heated with a bunsen burner until all the carbonaceous material burned away. The amount of residue was found to be 0.5 mg.

A thermogravimetric analysis was made to determine the amound of water present in the sample. To accomplish this, 0.1003 g. of the yellow compound was weighed into a small platinum crucible and placed on a thermobalance. The weight loss versus temperature graph obtained showed a loss of 3.0 mg. up to the point where the compound melted and decomposed.

Nuclear magnetic resonance spectra were obtained using the Varian A-60 on a solution of the compound in deuterium oxide saturated with potassium carbonate, in dimethyl-sulfoxide at 90°, and in trifluoroacetic acid. The spectrum in trifluoroacetic acid was the most satisfactorily resolved. The spectrum obtained on the compound dissolved in tri-

Figure 2. Potentiometric titration of 1-[3'(N,N-bis
(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-2-hydroxybenzene (H_4Idhab)
with sodium hydroxide
   Weight of compound: 0.1693 g.
   Concentration of sodium hydroxide: 0.0647 N.

a The number of moles of base added per mole of
   acid

a (MOLES OF BASE ADDED PER MOLE OF ACID)

pH

SODIUM HYDROXIDE — ml.

16
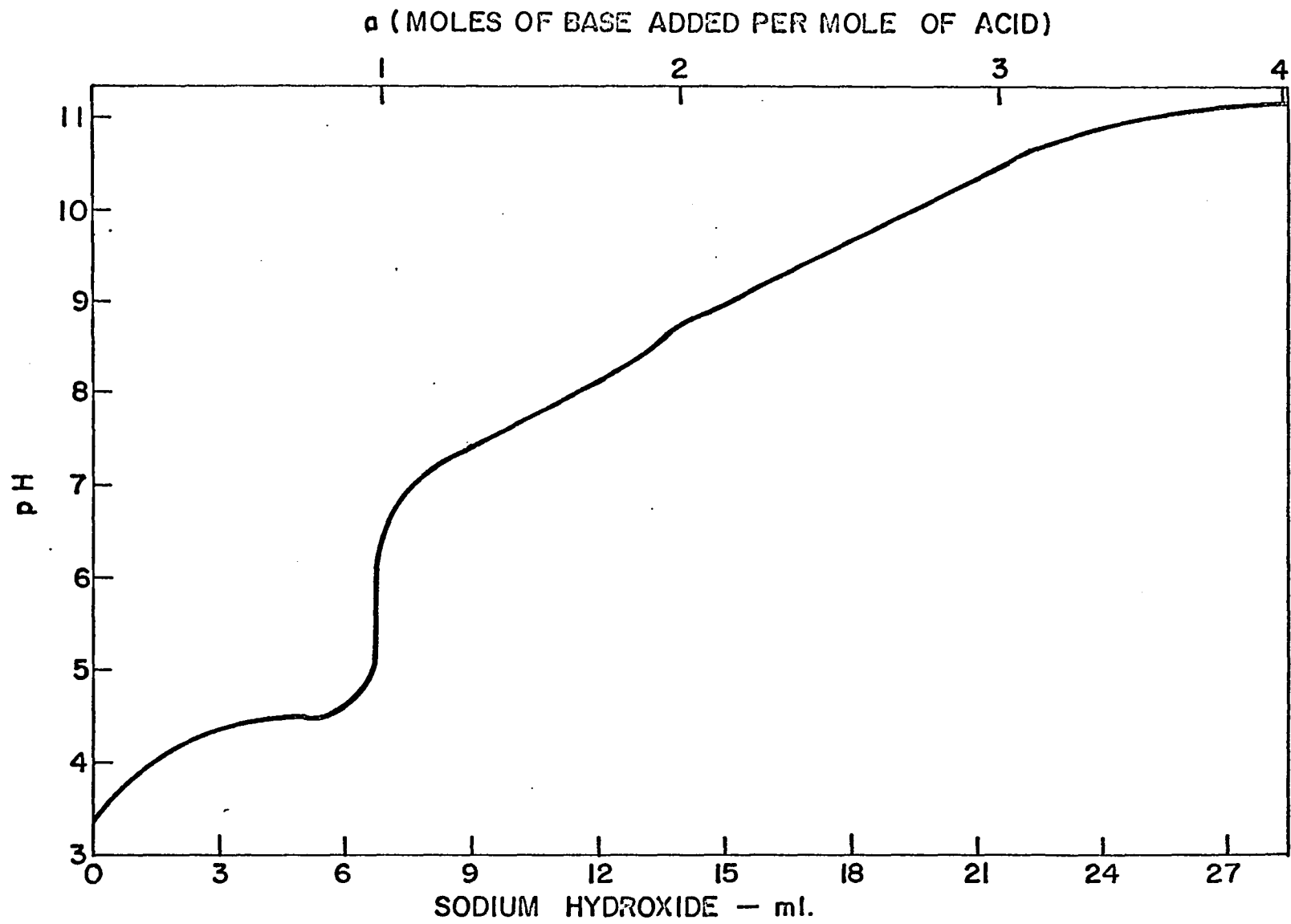
fluoroacetic acid is shown in Figure 3. An integration of the peaks which was obtained at the same time is also shown in Figure 3. Peaks were found at 4.55 p.p.m., 4.90 p.p.m., and in the region from 7.25 p.p.m. to 8.47 p.p.m.

A sample of this compound was submitted for mass spectrographic analysis on the Atlas MAT Bremen Massenspektrometer CH4. Major mass peaks for the ion fragments were found at 327 atomic mass units (a.m.u.), 271 a.m.u., 242 a.m.u., 228 a.m.u., 210 a.m.u. and many more at lower atomic mass units.

Identification of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene

The red compound isolated from the third fraction from the Sephadex column was examined further. This material was not soluble in alcohol or benzene. The material was soluble in sodium hydroxide and could be reprecipitated by adding hydrochloric acid. The precipitate that formed was amorphous and melted over the range 225-226°.

Elemental analysis: Found (Huffman Laboratories, Inc., Wheatridge, Colorado) C 52.24; H 4.86; N 10.91; calculated for $C_{22}H_{24}O_{10}N_4$ (two methyleneiminodiacetic acid groups), molecular weight 504.4: C 52.38; H 4.76; N 11.11.

A determination of the amount of metal impurity in this compound was performed by weighing 0.6473 g. of the red compound into a weighed platinum crucible. The sample was

Figure 3. Nuclear magnetic resonance spectrum of 1-[3'
(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-
1'-benzeneazo]-2-hydroxybenzene.

A Hydrogen atom resonance due to the hydrogen
   atoms on the methylene groups between the
   nitrogen atom and the carboxyl groups on the
   side chain

B Hydrogen atom resonance due to the hydrogen
   atoms on the methylene group between the
   phenyl ring and the nitrogen atom

C Hydrogen atom resonance due to the hydrogen
   atoms on the aromatic ring

D Integration of areas under the peaks
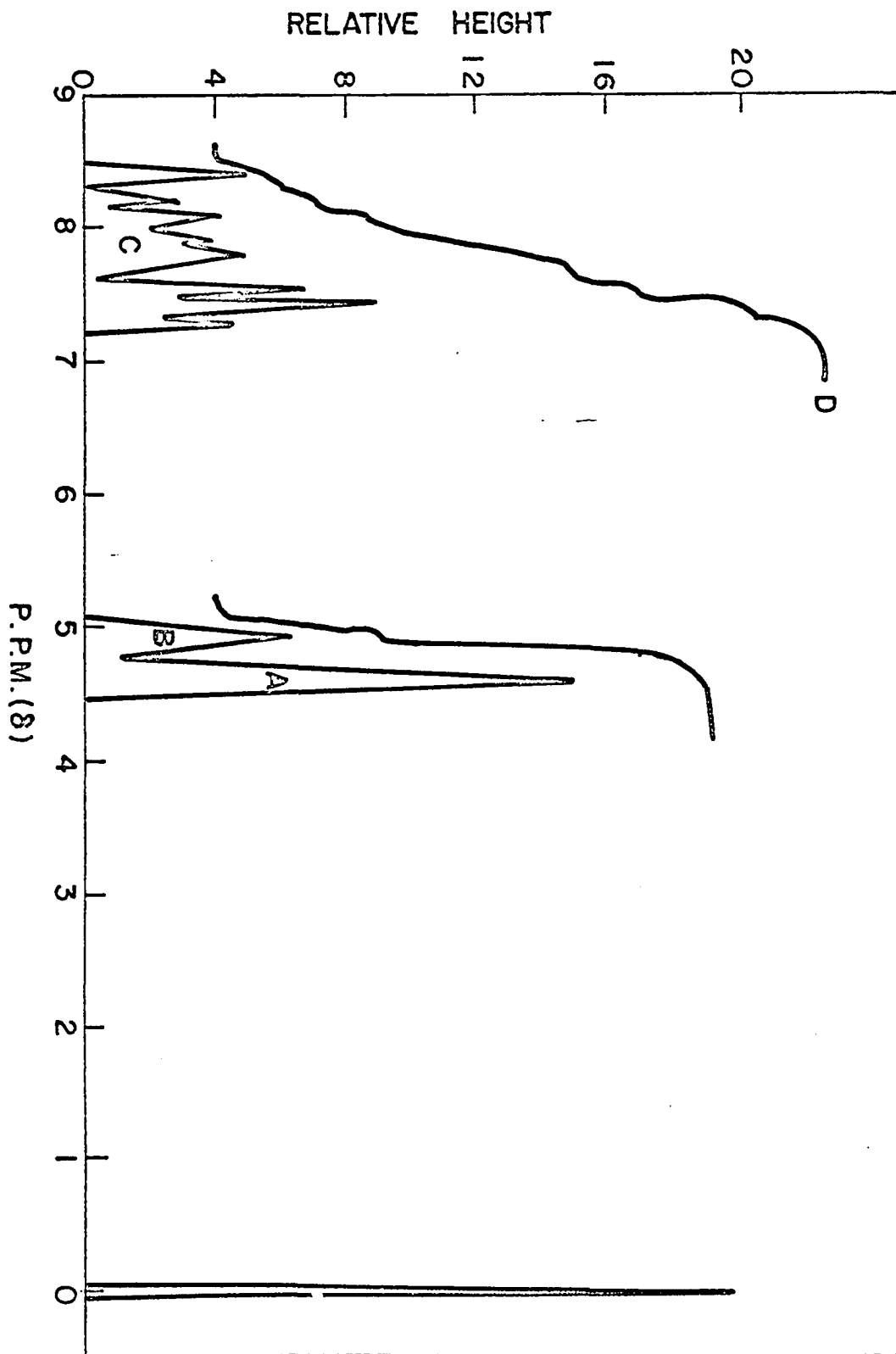
burned slowly over a Bunsen burner. The residual material was made up of metallic oxides. The residue amounted to 0.6 mg.

The equivalent weight of the red compound was determined by potentiometric titration with alkali. To 0.1605 g. of the red compound was added 30 ml. of 0.1 M potassium chloride solution. A suspension was formed from which the solid dissolved slowly on titration; the last of the solid passing into solution just before the first end point was reached. The mixture was titrated with 0.0933 N sodium hydroxide. This titration curve is shown in Figure 4. Equivalent weight found (first end point): 268.0; calculated for one replaceable hydrogen atom: 504.4, for two replaceable hydrogen atoms: 504.4/2 = 252.2.

A second potentiometric titration was performed: 0.1732 g. (3.44 x 10^{-4} moles) of the red compound in the presence of 3.44 x 10^{-4} moles of magnesium iodate was titrated with 0.09326 N sodium hydroxide. The titration curve is shown in Figure 5. Equivalent weight found (first end point): 133.5; calculated for three replaceable hydrogen atoms, 504.4/3 = 168.2, for four replaceable hydrogen atoms, 504.4/4 = 126.1.

The nuclear magnetic resonance spectrum of the red compound dissolved in trifluoroacetic acid was obtained on the Varian A-60 Nuclear Magnetic Resonance Spectrometer.

Figure 4. Potentiometric titration of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab) with sodium hydroxide
        Weight of compound taken: 0.1605 g.
        Concentration of sodium hydroxide: 0.0933 N.

a   The number of moles of base added per mole of acid

Figure 5. Potentiometric titration of 1-[3'(N,N-bis (carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl) aminomethyl)-2-hydroxybenzene (H_6Iidhab) in the presence of magnesium iodate with sodium hydroxide

Weight of compound taken: 0.1732 g. (3.44 x 10^{-4} moles)
Moles of magnesium iodate tetrahydrate added: 3.44 x 10^{-4}
Concentration of sodium hydroxide: 0.0933 N.

a   The number of moles of base added per mole of acid

a (MOLES OF BASE ADDED PER MOLE OF ACID)

SODIUM HYDROXIDE - ml
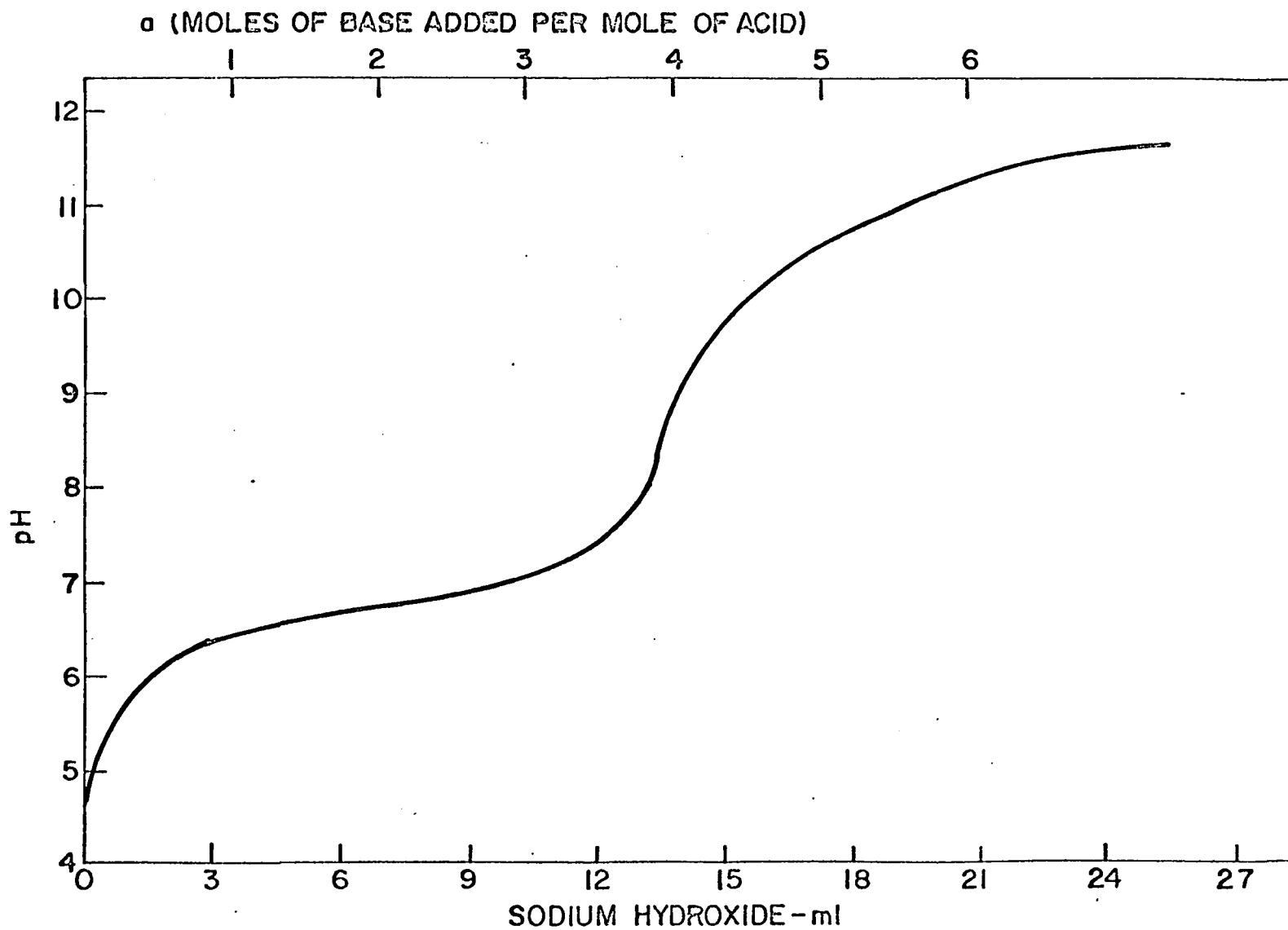
This spectra and the integration are shown in Figure 6. Peaks were found at 4.55 p.p.m., 4.98 p.p.m., and in the range from 7.10 p.p.m. to 8.18 p.p.m.

A sample of the red compound was submitted for mass spectrographic analysis in the Atlas MAT Bremen Massen-specktrometer CH4. The spectrum indicated major ion fragment peaks at 450 a.m.u., 451 a.m.u., 365 a.m.u., 342 a.m.u., 329 a.m.u., 320 a.m.u., 302 a.m.u., 451 a.m.u., 284 a.m.u., 271 a.m.u., 255 a.m.u., 244 a.m.u., 228 a.m.u., 210 a.m.u. and many more fragments of lower atomic mass units.

An attempt was made to split the azo linkage in the red compound by reducing the azo linkage with titanous chloride in sulfuric acid. Also sodium sulfide was used as a reducing agent. It was not possible to recover the substituted aminophenols. Oxidation of the compound with hydrogen peroxide in sulfuric acid was performed but the substituted nitrophenols could not be recovered.

Results and Discussion

Mannich condensation of o,o'-dihydroxyazobenzene, formaldehyde, and disodium iminodiacetate dihydrate

The Mannich condensation reaction has been run in many different solvent systems depending upon the relative solubilities of the reactants and products. In this study, the reaction was attempted in aqueous solution, in glacial acetic

Figure 6. Nuclear magnetic resonance spectrum of 1-[3'
(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-
1'-benzeneazo-3-(N,N-bis(carboxymethyl)amino-
methyl)-2-hydroxybenzene.

A Hydrogen atom resonance due to the hydrogen
   atoms on the methylene groups between the
   nitrogen atom and the carboxyl groups on the
   side chain

B Hydrogen atom resonance due to the hydrogen
   atoms on the methylene group between the phenyl
   ring and the nitrogen atom

C Hydrogen atom resonance due to the hydrogen
   atoms on the aromatic ring

D Integration of areas under the peaks
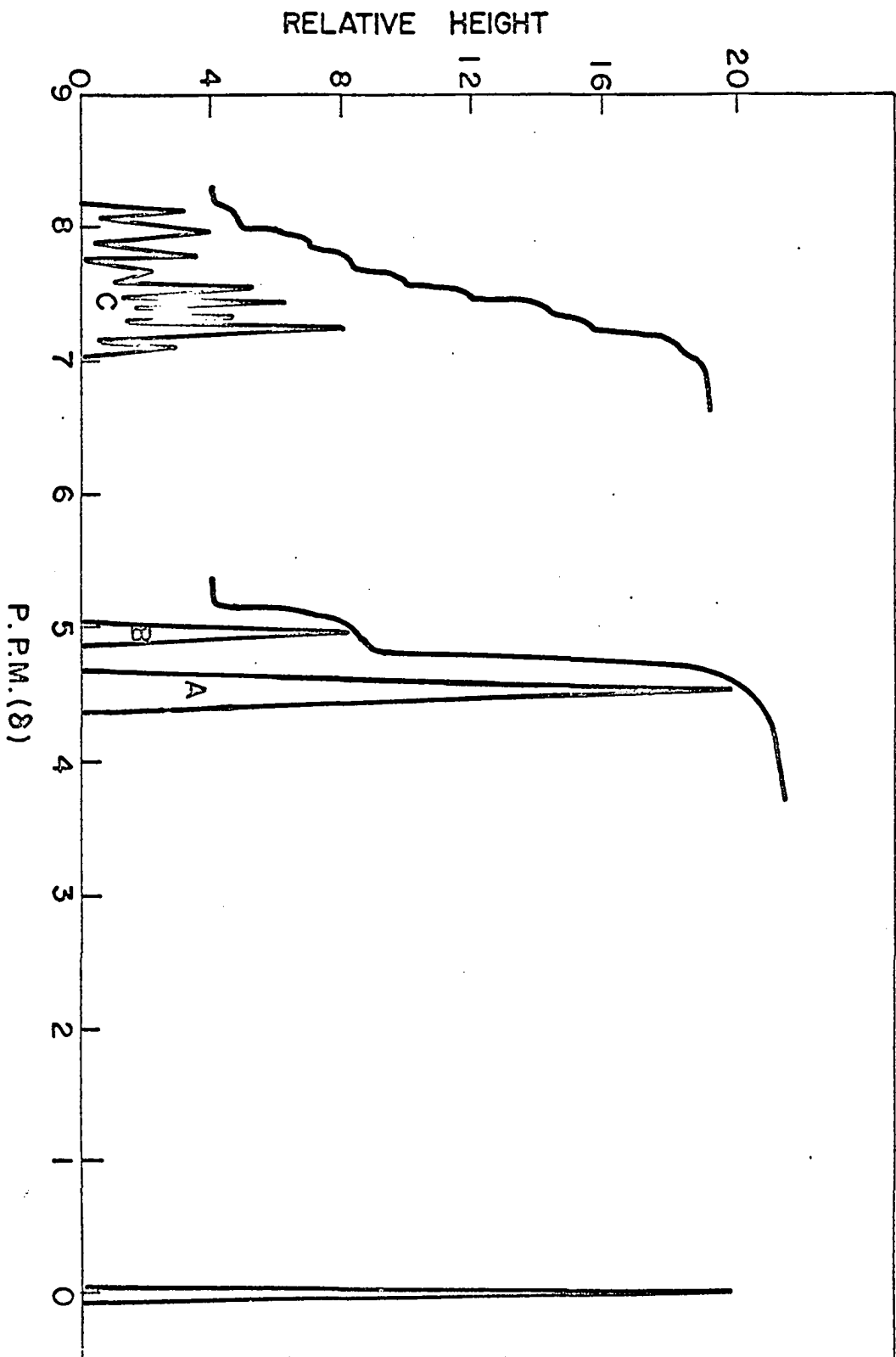
acid, and in dimethylsulfoxide. In the case of the aqueous
solution, the yields were low even after prolonged reaction
time. Also, the o,o'-dihydroxyazobenzene was not very sol-
uble in this system. In the case of glacial acetic acid,
the yields were low; but in addition, it proved difficult
to remove the acetic acid from the final product for the
odor of acetic acid could be detected above the dry product.
Dimethylsulfoxide was found to be the solvent of choice.
All of the reactants were soluble in dimethylsulfoxide,
although the disodium iminodiacetate dihydrate dissolved
with difficulty. The unreacted o,o'-dihydroxyazobenzene
could then be recovered by diluting the reaction mixture
with a large amount of water and filtering off the resulting
precipitate. The condensation products were then precipitated
from the filtrate by the addition of acid, and the product
washed free of dimethylsulfoxide.

Separation and purification of 1-[3'(N,N-bis(carboxymethyl)-
aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene and
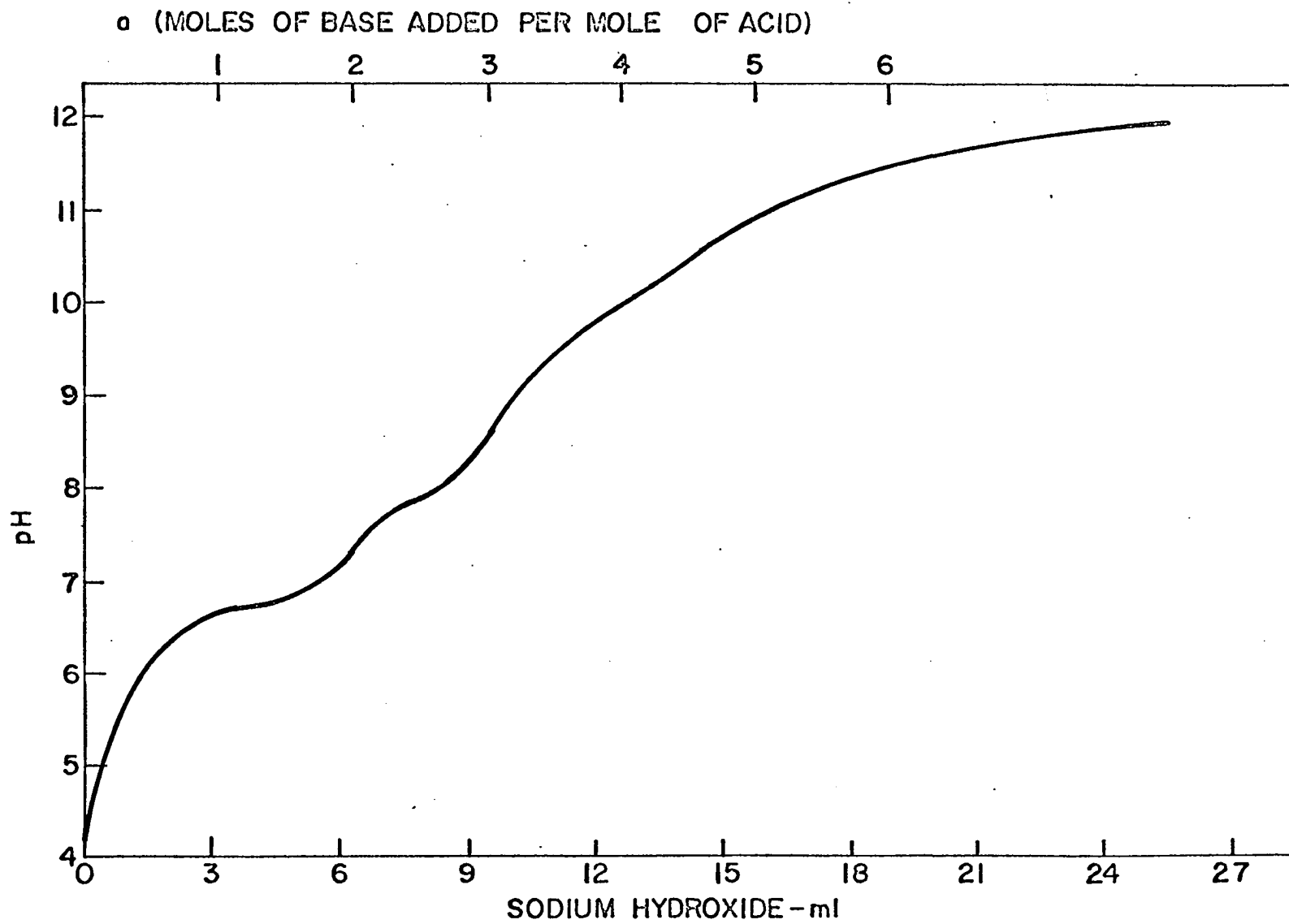1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-3-(N,N,-bis(carboxymethyl)aminomethyl)-2-
hydroxybenzene

Various methods were employed in attempts to isolate and
purify the products from the Mannich condensation of
o,o'-dihydroxyazobenzene, formaldehyde, and disodium
iminodiacetate dihydrate. Crystallization procedures

failed, because the material was not appreciably soluble in any of the solvents tried other than basic solvents. From basic solvents, the material precipitated only on the addition of acid with no apparent separation taking place.

Separation was achieved by a combination of gel filtration and chromatography on Sephadex G-10. Sephadex G-10 is a high molecular weight dextran polymer that excludes from the interstices of the polymer molecules larger than about molecular weight 700. Sephadex G-10 thus has been used as the column material for gel filtration experiments for the quantitative separation of molecules of high molecular weight from materials of low molecular weight; the high molecular weight material moving off the column first. Sephadex G-10 also acts as an adsorbent on which materials can be separated by chromatography. The factors involved in such separations are molecular size, molecular shape, and the presence of functional groups which can interact with the hydroxy groups of the dextran polymer. In this work, it was found that the material obtained from the Mannich condensation reaction separated according to molecular weight with the smallest molecule eluting first.

It proved possible to separate the products from the Mannich condensation reaction into four fractions by chromatography on Sephadex G-10. The first fraction contained high molecular weight materials that did not melt below 300°,

but slowly darkened and charred. The second fraction contained a yellow compound melting sharply at 184-185°. This material was not appreciably soluble in alcohol or benzene and thus differed from o,o'-dihydroxyazobenzene monohydrate, melting point: 174°, which is very soluble in ethyl alcohol and benzene. The third fraction consisted of a red compound, melting point: 225-226°. The red compound was also insoluble in ethyl alcohol and benzene. The fourth fraction was collected in a large volume of eluent and could not be obtained in sufficient quantity to purify. It was brown in color and showed no definite melting point, charring slowly above 200°.

It was thus possible to separate and purify two different compounds from the material produced by the Mannich condensation reaction and subsequent chromatography on Sephadex G-10.

Identification of 1-[3'(N,N-bis(carboxymethyl)aminoethyl -2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene

The yellow compound, precipitated from the second fraction of the Sephadex G-10 chromatography, was examined further. Results of analysis for carbon, hydrogen, and nitrogen indicated that one methyleneiminodiacetic acid group had been substituted for a hydrogen atom on the molecule of o,o'-dihydroxyazobenzene. This material is subsequently designated by the abbreviation H$_4$Idhab.

The equivalent weight found by the potentiometric titration (379.6, page 14) was greater than the calculated value (359.3) by twenty atomic weight units. Only a small part of this discrepancy can be ascribed to an impurity of metal ions, because the amount of residue found after burning a sample of H_4Idhab amounted to only 0.2 percent. The high equivalent weight is not explained by the presence of water of hydration inasmuch as the elemental analysis is correct for the anhydrous compound and no loss in weight was observed on heating a sample of material up to its melting point in the thermogravimetric balance. This problem was left unresolved.

The nuclear magnetic resonance spectra of the yellow compound dissolved in trifluoroacetic acid is shown in Figure 3. The assignment of peaks is as follows: the peak at 4.55 p.p.m. was assigned to the hydrogen atoms of the methylene groups between the nitrogen atom and the carboxyl groups of the side chain; the peak at 4.90 p.p.m. was assigned to the hydrogen atoms on the methylene group between the phenyl ring and the nitrogen atom; the peaks in the region 7.25 p.p.m. to 8.47 p.p.m. was assigned to the aromatic hydrogen atoms. No particular assignment was made for the individual aromatic hydrogen atoms. The phenol hydrogen atoms were not found in the spectra, presumably, because the phenol hydrogen atoms are rapidly exchanging with the hydrogen atoms of the

trifluoroacetic acid.

The integration of the areas under the peaks in the nuclear magnetic resonance spectra gave a ratio of seven for the aromatic hydrogen atoms to two for the methylene hydrogen atoms between the phenyl ring and the nitrogen atom to four for the methylene hydrogen atoms between the nitrogen atom and the carboxyl group. This is the ratio to be expected from the integration of the nuclear magnetic resonance spectra of 1-[3-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzenazo]-2-hydroxybenzene.

The mass spectrographic analysis of the yellow compound showed no parent ion peak. High molecular weight carboxylic acids typically do not show a parent ion peak, because the compounds decarboxylate in the electron beam used for ionization. A rational structural assignment was made to describe the mass peaks of the molecular ion fragments that were observed. For example the mass peak at 271 a.m.u. can be explained by a structure in which the compound has lost two molecules of carbon dioxide ($359 - 2 \times 44 = 271$). In itself, this analysis was of no use in elucidating the structure of this compound; but in relationship to the mass spectrum of the red compound this analysis gives some insight into both structures.

This data and data from the red compound (given later) show that in the yellow compound the methyleneiminodiacetic

acid group is probably substituted in the 3 position on the aromatic ring adjacent to the hydroxyl group. Therefore, the compound is assigned structure VI, 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzenazo]-2-hydroxybenzene:
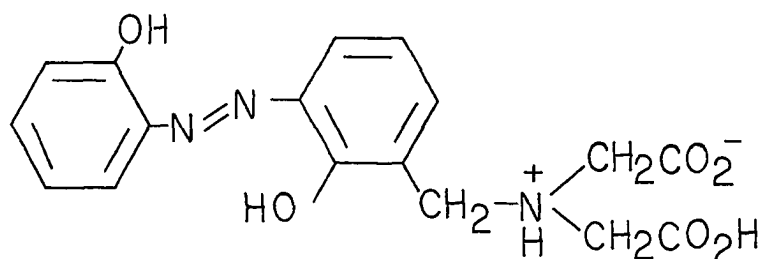
VI

Identification of 1-[3'(N,N-bis(carboxymethyl)aminomethyl) -2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)amino-methyl)-2-hydroxybenzene

The method of identifying the red compound isolated from the third fraction of the Sephadex G-10 chromatography paralleled closely the method used to identify the yellow compound. Results of analysis for carbon, hydrogen, and nitrogen indicated that two methyleneiminodiacetic acid groups had been substituted for hydrogen atoms on the molecule of o,o'-dihydroxyazobenzene. This material is subsequently designated by the abbreviation H_6Iidhab.

The equivalent weight of H_6Iidhab as determined by

potentiometric titration with alkali proved high, 268.0

in contrast to the theoretical value of 252.2 (504.5/2,

two replaceable hydrogen atoms). The equivalent as de-

termined by potentiometric titration in the presence of an

equal molar quantity of magnesium ion with alkali also

proved high, 133.5, calculated on the basis of four re-

placeable hydrogen atoms the theoretical would be 504.4/4

= 126.1. The presence of two molecules of water of crystal-

lization in the compound which might explain the high values

is ruled out by the elemental analysis and by the thermo-

gravimetric analysis which showed very little loss in weight

at temperatures up to the melting and decomposition point.

The presence of metal ions in the compound was ruled out by

the fact that less than one tenth of one percent ash was

found on burning the sample.

The nuclear magnetic resonance spectrum of the red

compound dissolved in trifluoroacetic is shown in Figure 6.

The assignment of peaks is as follows: the peak at 4.55

p.p.m. was assigned to the hydrogen atoms of the methylene

groups between the nitrogen atoms and the carboxyl groups

of the side chain; the peak at 4.98 p.p.m. was assigned to

the hydrogen atoms on the methylene groups between the phenyl

ring and the nitrogen atoms; the peaks in the region from

7.10 p.p.m. to 8.18 p.p.m. were assigned to the aromatic

hydrogen atoms. Thus, the nuclear magnetic resonance spectrum

of the red compound is very similar to the nuclear magnetic
resonance spectrum of the yellow compound. The difference
between the two spectra lies in the arrangement of peaks in
the aromatic region. The singlet peak farthest down field
in the spectrum of the yellow compound is missing in the
spectrum of the red compound, and the whole aromatic pattern
in the spectrum of the red compound appears more symmetrical.
This indicated that in the red compound the two methylene-
iminodiacetic acid groups are being affected by the same
magnetic environment. There is also an indication of a one,
two, three hydrogen atom pattern, indicating that the three
hydrogen atoms are situated on adjacent carbon atoms. This
is a further indication that the methyleneiminodiacetic acid
is adjacent to the phenolic group.

The mass spectrum of the red compound did not show a
parent ion peak. On comparison of the mass spectrum of the
red compound with that of the yellow compound, no molecular
ion fragments were found above the background peaks. Mass
peaks were found which were ascribed to molecular fragment
ions that could be produced from a doubly substituted moiety.
These mass peaks were found at 450 a.m.u., 451 a.m.u., and
365 a.m.u. It was found that in the mass spectrum of the
red compound the intensity of mass peak at 150 a.m.u. in-
creased relative to the intensity of the mass peak at 109
a.m.u. as compared to the same mass peaks in the mass

spectrum of the yellow compound. The mass peak at 150 a.m.u. is ascribed to a molecular ion fragment that is substituted with a methyleneiminodiacetic acid group, and the mass peak at 109 a.m.u. is ascribed to a molecular ion fragment that is not substituted with a methyleneiminodiacetic acid group. This indicated that the two methyleneiminodiacetic acid groups are substituted on different aromatic rings.

It was not possible to isolate the substituted amino-phenols produced on the reduction of the red compound with titanous chloride or sodium sulfide. In both cases the compound lost its color indicating a splitting of the azo linkage, but no material could be isolated other than tar. The substituted aminophenols were probably oxidized by air during the attempted isolation. Oxidation of the azo linkage by hydrogen peroxide in sulfuric acid was also unsuccessful owing to polymer formation.

It is possible to get some insight into the substitution pattern of the methyleneiminodiacetic acid on the o,o'-dihydroxyazobenzene by examining molecular models. Starting with the assumption that the phenyl groups are trans to each other and that the phenol protons are hydrogen bonded to the pi-electron cloud of the azo double bond, (this is indicated by the shift to very low field of the phenol protons in the nuclear magnetic resonance spectrum of o-o'-dihydroxyazobenzene in dimethylsulfoxide) it is possible to see that substitution

at the 6 and 6' positions are very hindered. The other

positions: 3,3', 4,4' and 5 and 5' are all equally avail-

able for substitution. L. Hunter and R. S. Barnes (13)

showed that chlorination with dichloroamine T of o,o'-

dihydroxyazobenzene gave substitution in the 3,3' and 5,5'

positions. Thus, the phenol was the component influencing

substitution. The active chlorinating species in di-

chloroamine T is Cl^+ and the active species in the Mannich

condensation reaction is ^+CHO so that the reaction mechanism

is probably similar. This argument eliminates the 4 and 4'

sites from consideration. The only reason for eliminating

the 5 and 5' sites from consideration is that the nuclear

magnetic resonance spectrum of the red compound shows a

hint of a one, two, three pattern in the aromatic region

of the spectrum. This pattern is not possible if the sub-

stitution is present at the 5 and 5' positions. The red

compound is therefore assigned structure VII, 1-[3'N,N-bis

(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-

(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene

$(H_6 Iidhab)$:

VII
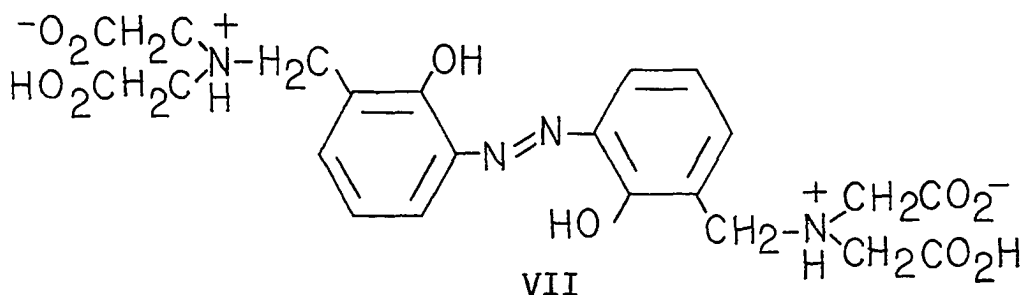
PROPERTIES OF 1-[3'N,N-BIS(CARBOXYMETHYL)

AMINOMETHYL)-2'-HYDROXY-1'-BENZENEAZO]

-2-HYDROXYBENZENE AND 1-[3'(N,N-BIS(CARBOXYMETHYL)

AMINOMETHYL)-2'-HYDROXY-1'-BENZENEAZO]

-3-(N,N-BIS(CARBOXYMETHYL)AMINOMETHYL)-2-HYDROXYBENZENE

Experimental Work

Apparatus

Fluorescence spectra were obtained on an Aminco-
Kiers spectrophotofluorometer. This instrument makes it
possible to scan both the exciting and the fluorescent
light. The instrument was equipped with a Osram xenon lamp
as the light source and a 1P28 photomultiplier as the de-
tector. The slit arrangement was as follows: excitation
beam 3,2 and 3 mm. in order; fluorescent beam 3,2,3 and 5
mm. This arrangement was a compromise between the arrange-
ment permitting greatest sensitivity and the arrangement
showing greatest resolution. A 1 cm., square, quartz cell
was used. Spectra were recorded on a Mosey Model 2-S X-Y
recorder.

Absorption spectra were obtained on the Cary Model 14
spectrophotometer.

All spectrophotometric data used in obtaining combining
ratios and formation constants were obtained using a Beckman
DU spectrophotometer.

All other instruments used were discussed in part II.

Reagents

The buffers used in the determination of acid dissociation constants spectrophotometrically were prepared according to R. Bates (2) and were of constant ionic strength. The pH 10 buffer was prepared from reagent grade ammonium hydroxide and ammonium chloride as described by H. Diehl and G. F. Smith (11).

Stock solutions of o,o'-dihydroxyazobenzene (5.30 x 10^{-4} M), 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (3.30 x 10^{-4} M), and 1-[3'(N,N-bis(carboxylmethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)-aminomethyl)-2-hydroxybenzene (3.97 x 10^{-4} M) were prepared within a few hours of use. Small quantities of potassium hydroxide were used to facilitate dissolution. Solutions on which the spectra were run were prepared by mixing 5 ml. of the desired stock solution of dye, 4 ml. of 1 x 10^{-3} M metal ion (if desired), 10 ml. of buffer solution, and dilution to 50 ml. with 0.1 M potassium chloride. The pH of each of the solutions was checked and found to be within 0.05 pH units of the value reported.

All of the other reagents were described in part II.

Determination of the acid dissociation constants of o,o'-dihydroxyazobenzene

The absorption spectra were obtained for o,o'-dihydroxy-

azobenzene (H_2Dhab) at pH 7.99, pH 10.15, and pH 14.0.

The solutions were prepared by mixing 5 ml. of the 5.30

x 10^{-4} M H_2Dhab stock solution, 10 ml. of the appropriate

buffer and dilution to 50 ml. with 0.1 M potassium chloride.

The resultant solution was placed in a 1 cm. cell and the

absorption spectrum recorded automatically on a Cary Model

14 spectrophotometer. The absorption spectra are shown in

Figure 7.

The acid dissociation constants for H_2Dhab were de-

termined spectrophotometrically by preparing forty-five

different solutions each at a different pH and measuring

the absorbance of the resulting solution. Five milliliters

of the 5.30 x 10^{-4} M H_2Dhab stock solution was added with

10 ml. of the appropriate buffer to a 50 ml. volumetric

flask and diluted to volume with 0.1 M potassium chloride.

The absorbance of each solution was determined on a Beckman

DU spectrophotometer at 390 mμ. and 480 mμ. The pH of

each solution was checked with a pH meter calibrated with

buffer solutions of known pH. The value of absorbance

measured were plotted versus pH for each wavelength sep-

arately. The graph is shown in Figure 8. The acid dis-

sociation constants determined from the graph were: pK_1 =

8.9, pK_2 = 11.1.

Figure 7. Absorption spectrum of 5.57 x 10^{-5} M
o,o'-dihydroxyazobenzene

    A   pH 7.99

    B   pH 10.15

    C   pH 14.0
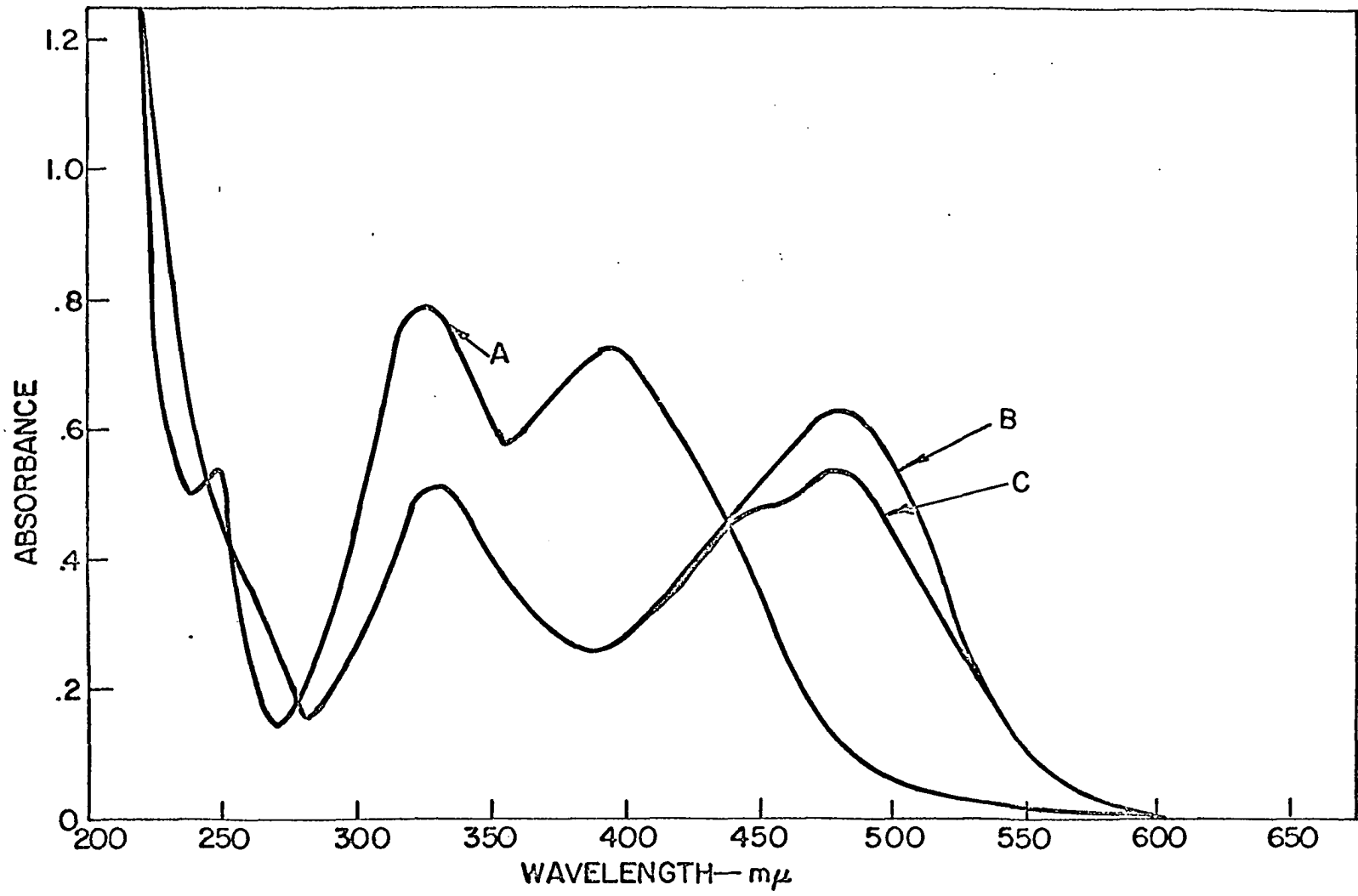
Figure 8. Absorbance versus pH of 5.30 x 10^{-5} M
o,o'-dihydroxyazobenzene

A Wavelength: 480 mμ
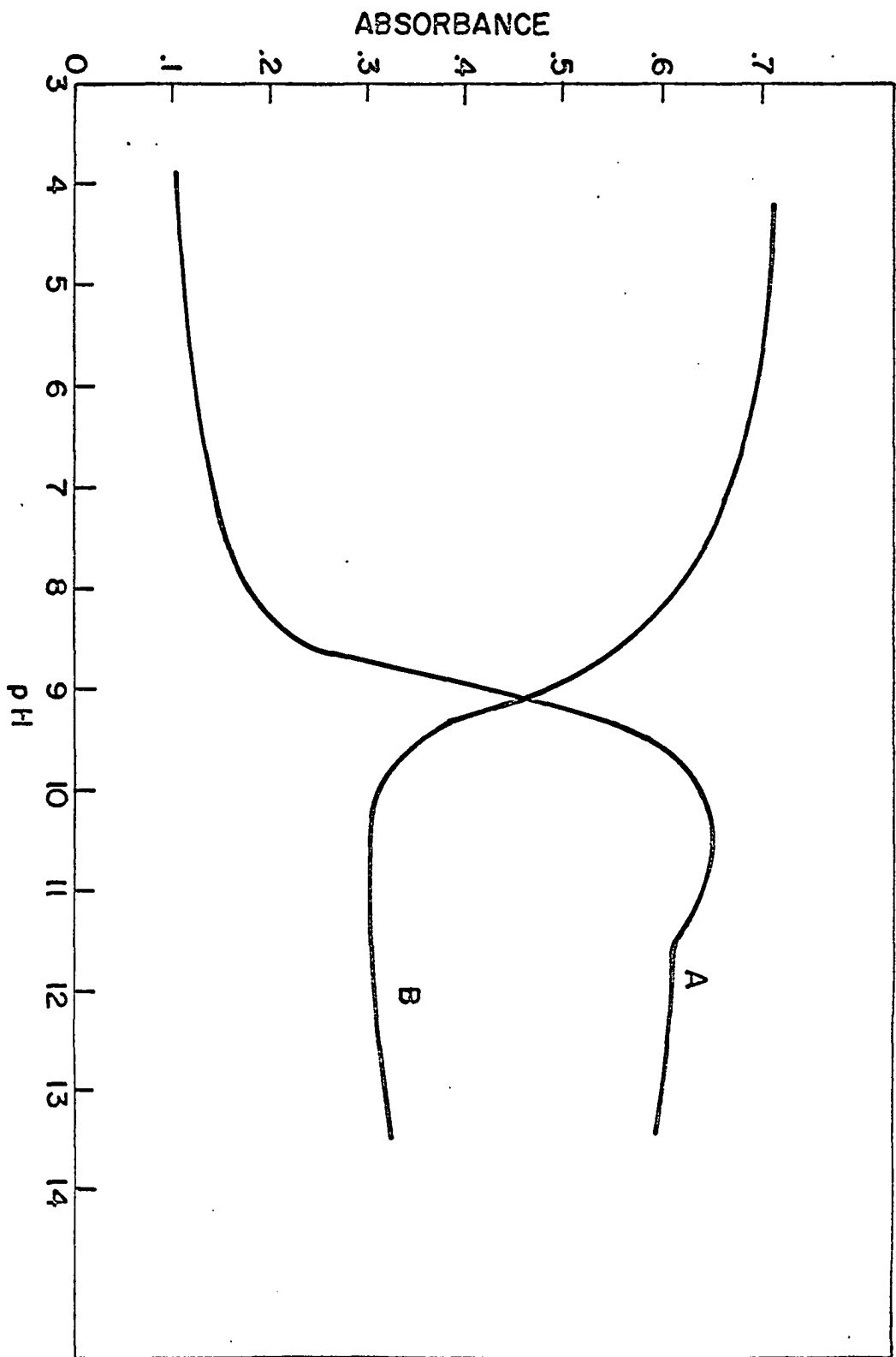
B Wavelength: 390 mμ

ABSORBANCE

pH

A

B

Determination of the acid dissociation constants of
1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-2-hydroxybenzene

The absorption spectra of 1-[3'(N,N-bis(carboxymethyl)
aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene
(H_4Idhab) was obtained at pH 2.2, pH 9.7, and pH 14.0.
Five milliliters of 5.0 x 10^{-4} M H_4Idhab stock solution was
added with 10 ml. of the appropriate buffer to a 50 ml.
volumetric flask. The flask was diluted to volume with
o.1 M potassium chloride solution. The absorption spectra
obtained on these solutions are shown in Figure 9.

One acid dissociation constant of H_4Idhab could be
determined spectrophotometrically. This was done by adding
5 ml. of the 3.30 x 10^{-4} M H_4Idhab stock solution to 10 ml.
of the appropriate buffer to a 50 ml. volumetric flask and
dilution to volume with 0.1 M potassium chloride. The ab-
sorbance of each of the thirty-five different solutions was
read on a Beckman DU spectrophotometer at 390 mμ. and 480 mμ.
This absorbance was plotted versus pH for each wavelength.
The resultant graph is shown in Figure 10. The value of the
pK_a determined was 7.8.

A potentiometric titration of 0.1693 g. of H_4Idhab was
performed using 0.0647 M sodium hydroxide. The graph of this
titration curve is shown in Figure 2. The data from this graph
was used to calculate the dissociation constants of H_4Idhab.

Figure 9. Absorption spectra of 5×10^{-5} M 1-[3'(N,N-bis (carboxymethyl)aminomethyl)-2'-hydroxy-1'- benzeneazo]-2-hydroxybenzene (H_4Idhab)

A   pH 2.2

B   pH 9.7

C   pH 14.0
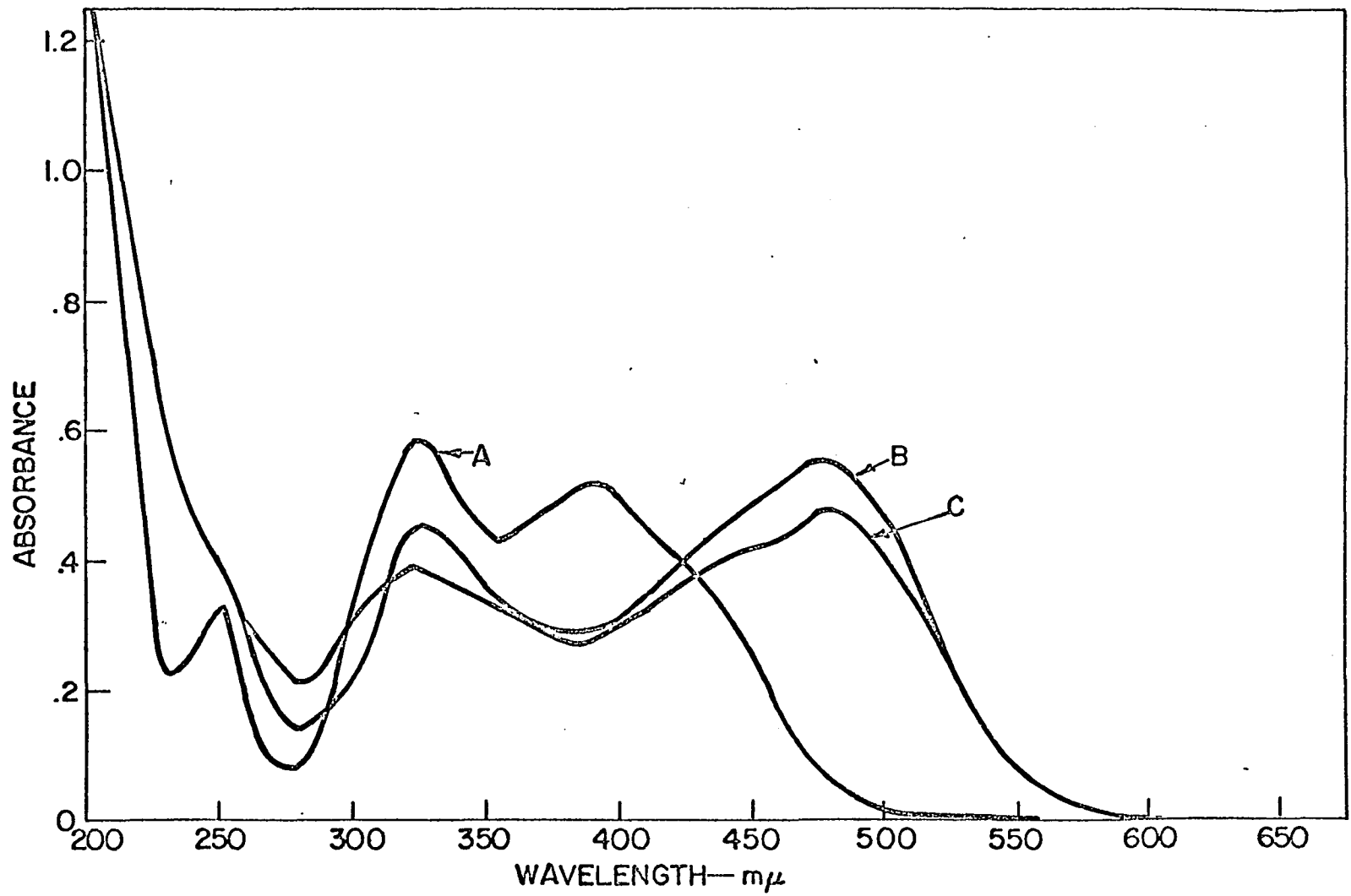
Figure 10. Absorbance versus pH of 3.30×10^{-5} M 1-[3'
(N,N-bis(carboxymethyl)aminomethyl)-2'-
hydroxy-1'-benzeneazo]-2-hydroxybenzene
(H_4Idhab).

A Wavelength: 480 mμ.
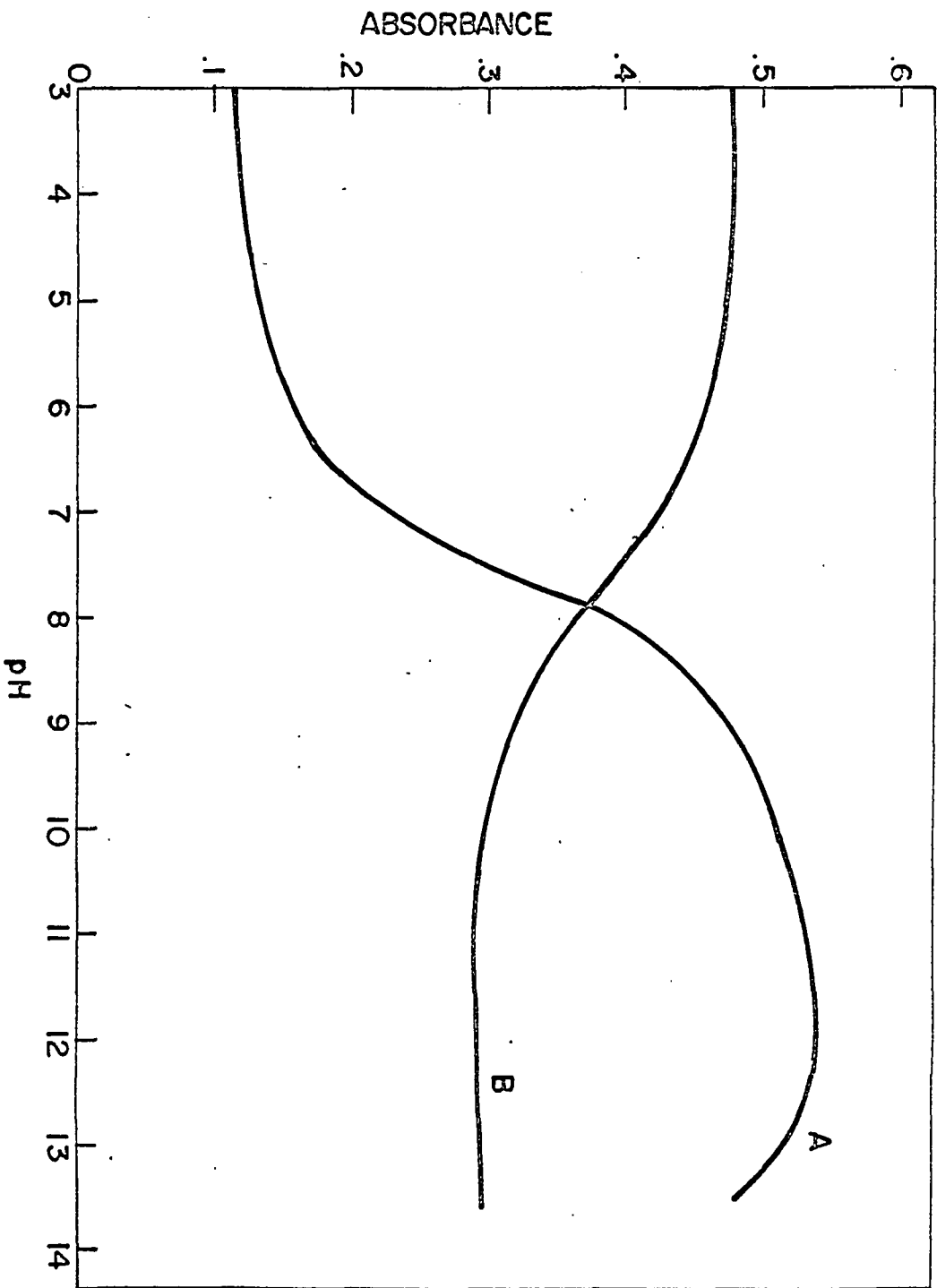
B Wavelength: 390 mμ.

Determination of the acid dissociation constants of 1-[3' (N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo] -3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene

The absorption spectra of 1-[3'(N,N-bis(carboxymethyl) aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxy-methyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab) was obtained at pH 2.2, pH 9.7, and pH 14.0. Five milliliters of 5.0 x 10^{-4} M H_6Iidhab stock solution was added to 10 ml. of the appropriate buffer in a 50-ml. volumetric flask. The solution was diluted to the mark with 0.1 M potassium chloride solution. The absorption spectra obtained on these solutions are shown in Figure 11.

One acid dissociation constant of H_6Iidhab could be obtained spectrophotometrically. This was obtained by the addition of 5 ml. of 3.97 x 10^{-5} M H_6Iidhab with 10 ml. of the appropriate buffer to a 50-ml. volumetric flask. The solution was diluted to the mark with 0.1 M potassium chloride. The absorbance of these thirty-five different solutions was measured on a Beckman DU spectrophotometer and plotted versus the pH of the particular solution. The graph is shown in Figure 12. The value of the acid dissociation constant found from this graph (pK_{a3}) was 7.7.

A potentiometric titration of 0.1605 g. of H_6Idhab was performed using 0.0933 M sodium hydroxide. The solution was kept at ionic strength 0.1 with potassium chloride. The

Figure 11. Absorption spectra of 5×10^{-5} M 1-[3'(N,N-bis
(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-3-[N,N-bis(carboxymethyl)amino-
methyl]-2-hydroxybenzene (H_6Iidhab)

A   pH 2.2

B   pH 9.7

C   pH 14.0
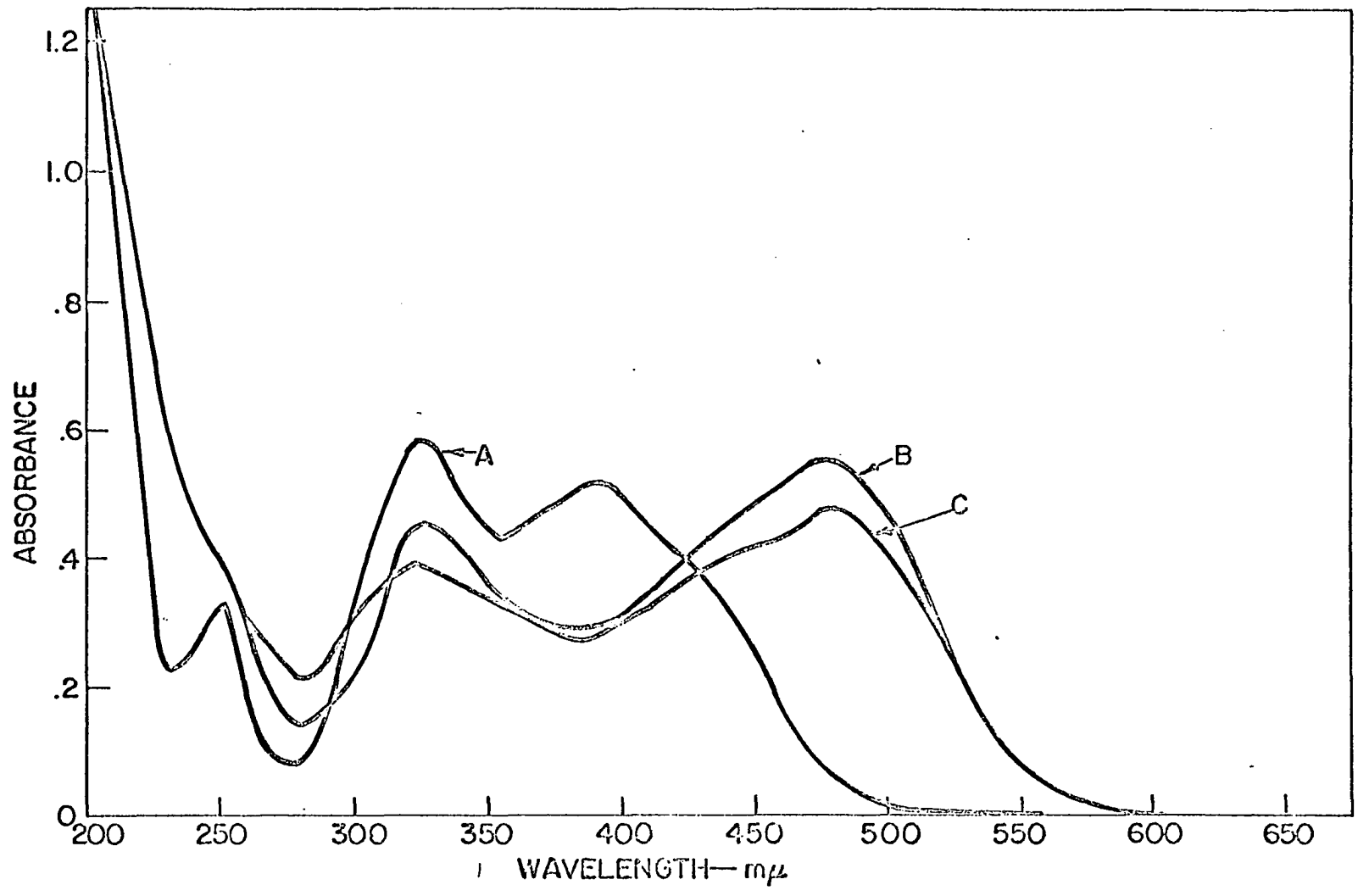
Figure 12. Absorbance versus pH of 3.97×10^{-5} M 1-[3'
(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-
1'-benzeneazo]-3-(N,N-bis(carboxymethyl)amino-
methyl)-2-hydroxybenzene (H_6Iidhab).

A Wavelength: 480 mµ

B Wavelength: 390 mµ
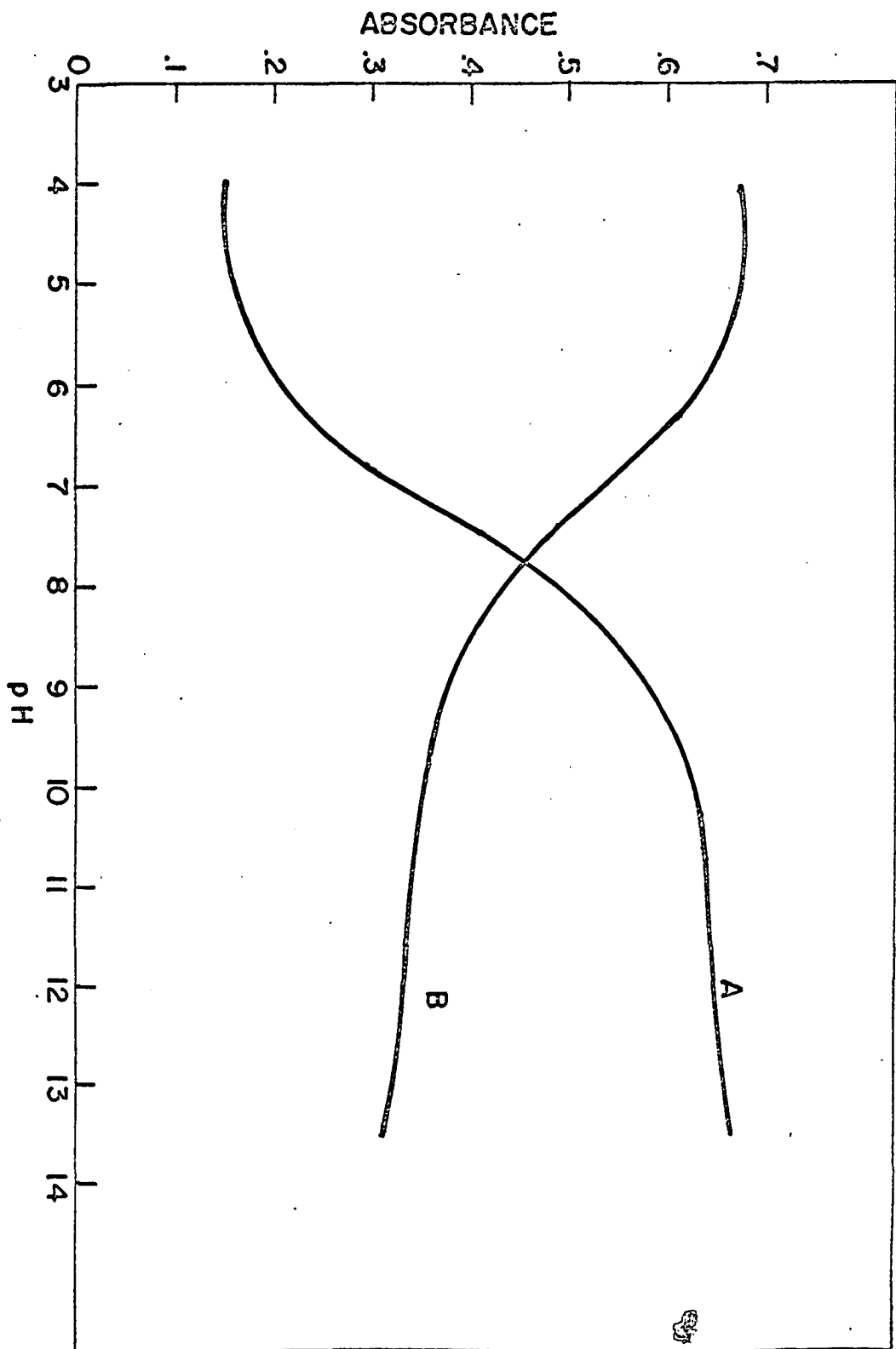
graph of the titration curve is shown in Figure 4. This data was used to calculate the acid dissociation constants.

The solubility of H_6Iidhab at various pH values before the first end point was determined in order to calculate the solubility product constant. The solubility was determined by adjusting the pH of a solution containing excess H_6Iidhab to a desired value and stirring for an hour. The solution was filtered. Five milliliters of the filtrate was adjusted to pH 10.0 with buffer and diluted to 100 ml. in a volumetric flask. The amount of H_6Iidhab in each solution was determined spectrophotometrically on a Beckman DU spectrophotometer at 480 mμ. The results are shown in Table 1. This data made it possible to calculate pK_{a1} and pK_{a2}.

Table 1. The "apparent solubility" of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzenene as a function of pH.

pH	"Apparent Solubility" of H_6Iidhab
4.39	9.35×10^{-5}
4.90	2.02×10^{-4}
5.80	1.19×10^{-3}
5.90	1.29×10^{-3}
6.25	2.04×10^{-3}

Determination of the formation constants of the calcium and magnesium compounds of 1-[3'(N,N-bis(carboxymethyl)amino-methyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene

To determine the formation constants of the magnesium compounds of H_4Idhab, two different potentiometric titrations were performed. The first was the titration of a mixture of 0.1063 g. (2.97 x 10^{-4} moles) of H_4Idhab and 1.1850 g. (2.96 x 10^{-3} moles) of magnesium iodate tetrahydrate with 0.0933 M sodium hydroxide. This titration curve is shown in Figure 13. The second was a titration of a mixture of 0.0841 g. (2.34 x 10^{-4} moles) of H_4Idhab and 0.0998 g. (2.34 x 10^{-4} moles) of magnesium iodate tetrahydrate with 0.0925 M sodium hydroxide. This titration curve is shown in Figure 14.

To determine the formation constant of the calcium compound of H_4Idhab, again two titrations were necessary. In the first titration, a mixture of 0.1032 g. (2.88 x 10^{-4} moles) of H_4Idhab and 0.4120 g. (2.88 x 10^{-3} moles) of calcium chloride dihydrate was titrated with 0.0933 M sodium hydroxide. This titration curve is shown in Figure 15. In the second titration, a mixture of 0.0998 g. (2.78 x 10^{-4} moles) of H_4Idhab and 0.0408 g. (2.78 x 10^{-4} moles) of calcium chloride dihydrate was titrated with 0.0925 M sodium hydroxide. This titration curve is shown in Figure 16.

Figure 13. Potentiometric titration of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) in the presence of magnesium iodate with sodium hydroxide

Weight of compound taken: 0.1063 g. (2.98 x 10^{-4} moles)

Weight of magnesium iodate tetrahydrate added: 1.1850 g. (2.98 x 10^{-3} moles)

Concentration of sodium hydroxide: 0.0933 N.

a The number of moles of base added per mole of acid
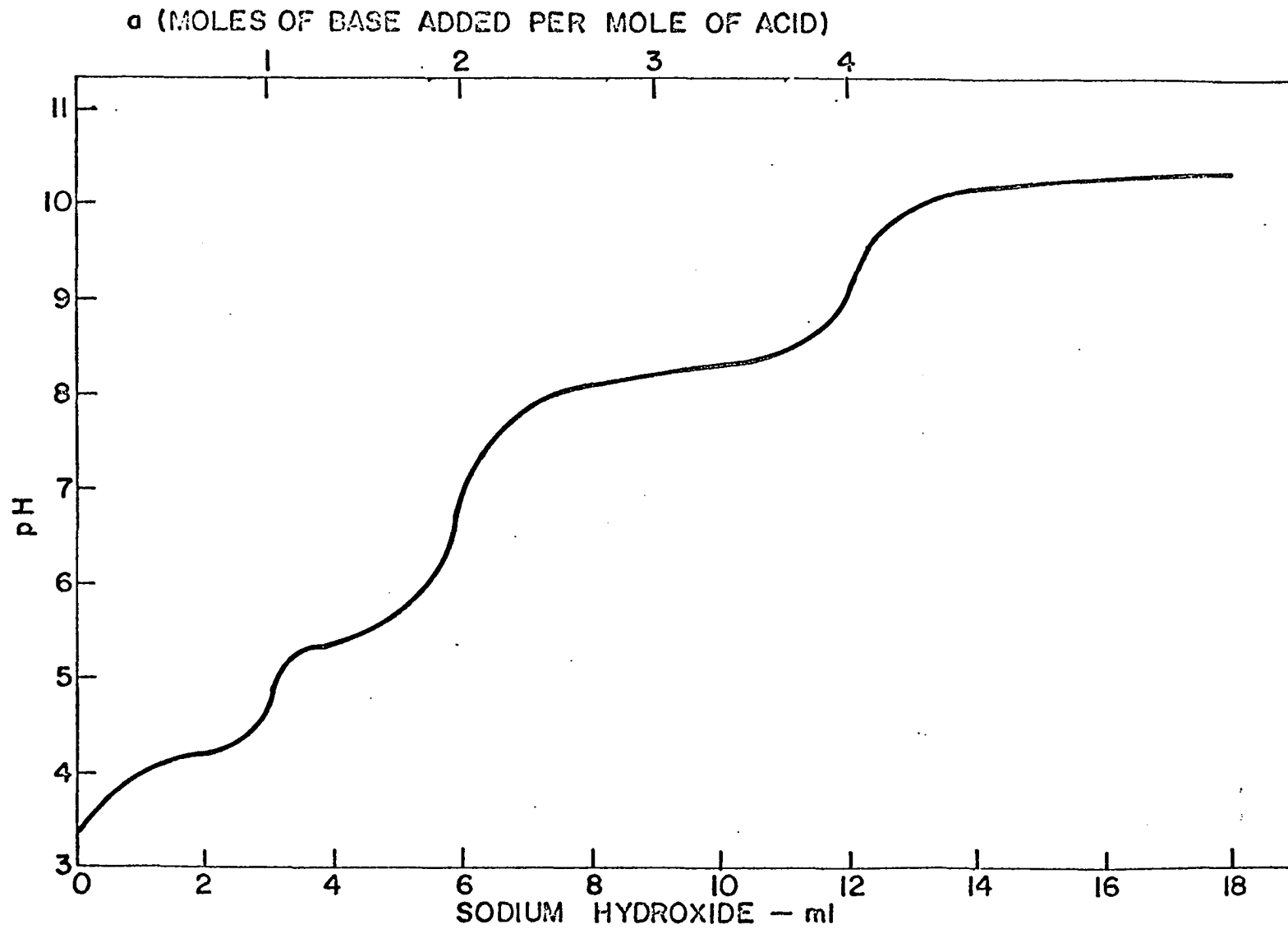
Figure 14. Poteniometric titration of 1-[3'(N,N-bis(carboxy-
methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-
hydroxybenzene (H_4Idhab) in the presence of mag-
nesium iodate with sodium hydroxide
Weight of compound taken: 0.0841 g. (2.34
x 10^{-4} moles)
Weight of magnesium iodate tetrahydrate
added: 0.0998 g. (2.34 x 10^{-4} moles)
Concentration of sodium hydroxide: 0.0925 N.

a Moles of base added per mole of acid
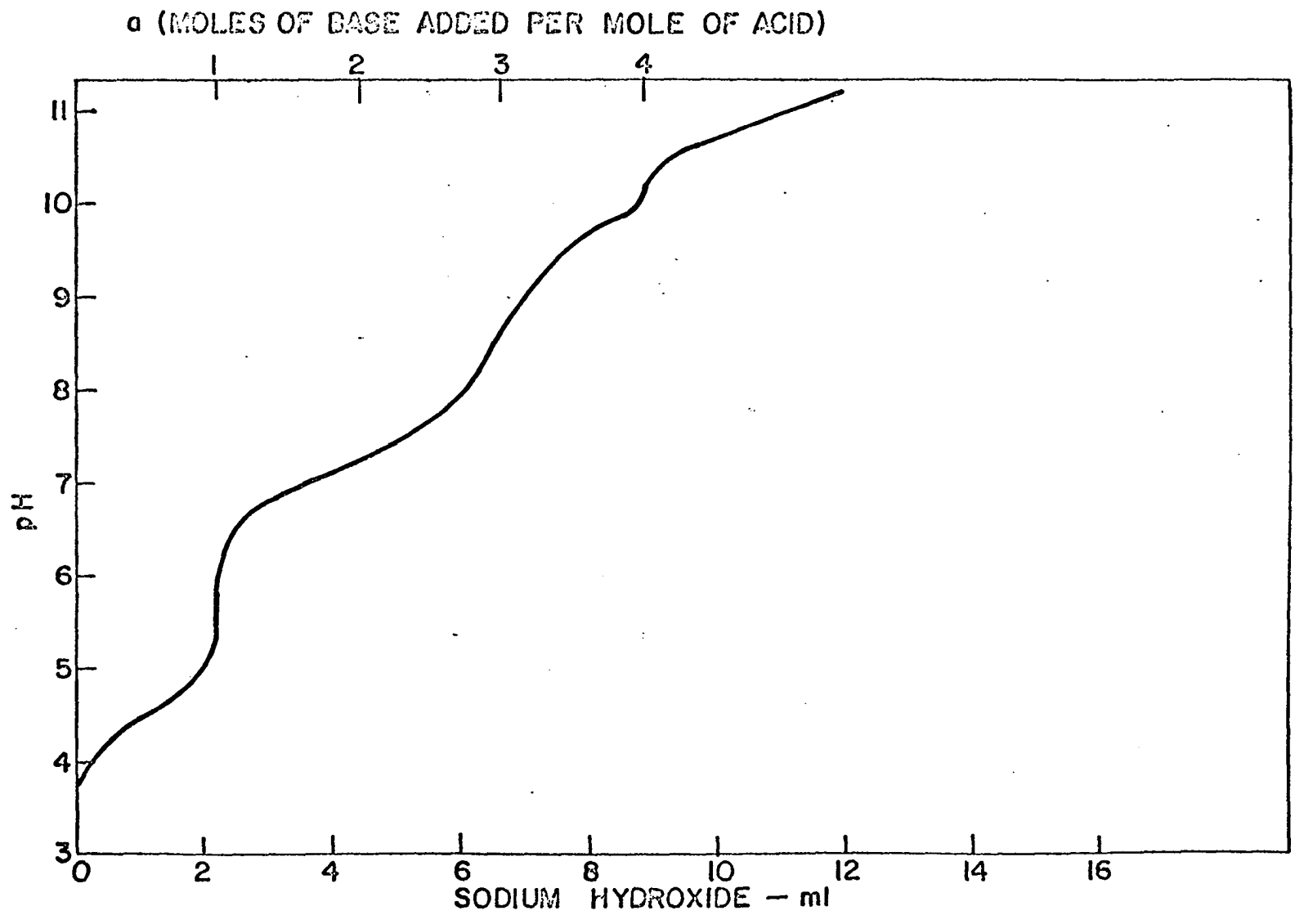
Figure 15.   Potentiometric titration of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) in the presence of calcium chloride with sodium hydroxide
        Weight of compound taken: 0.1032 g. (2.88 x 10^{-4} moles)
        Weight of calcium chloride dihydrate added: 0.4120 g. (2.88 x 10^{-3} moles)
        Concentration of sodium hydroxide: 0.0933 N.

a   Moles of base added per mole of acid

a (MOLES OF BASE ADDED PER MOLE OF ACID)

pH

SODIUM HYDROXIDE — ml
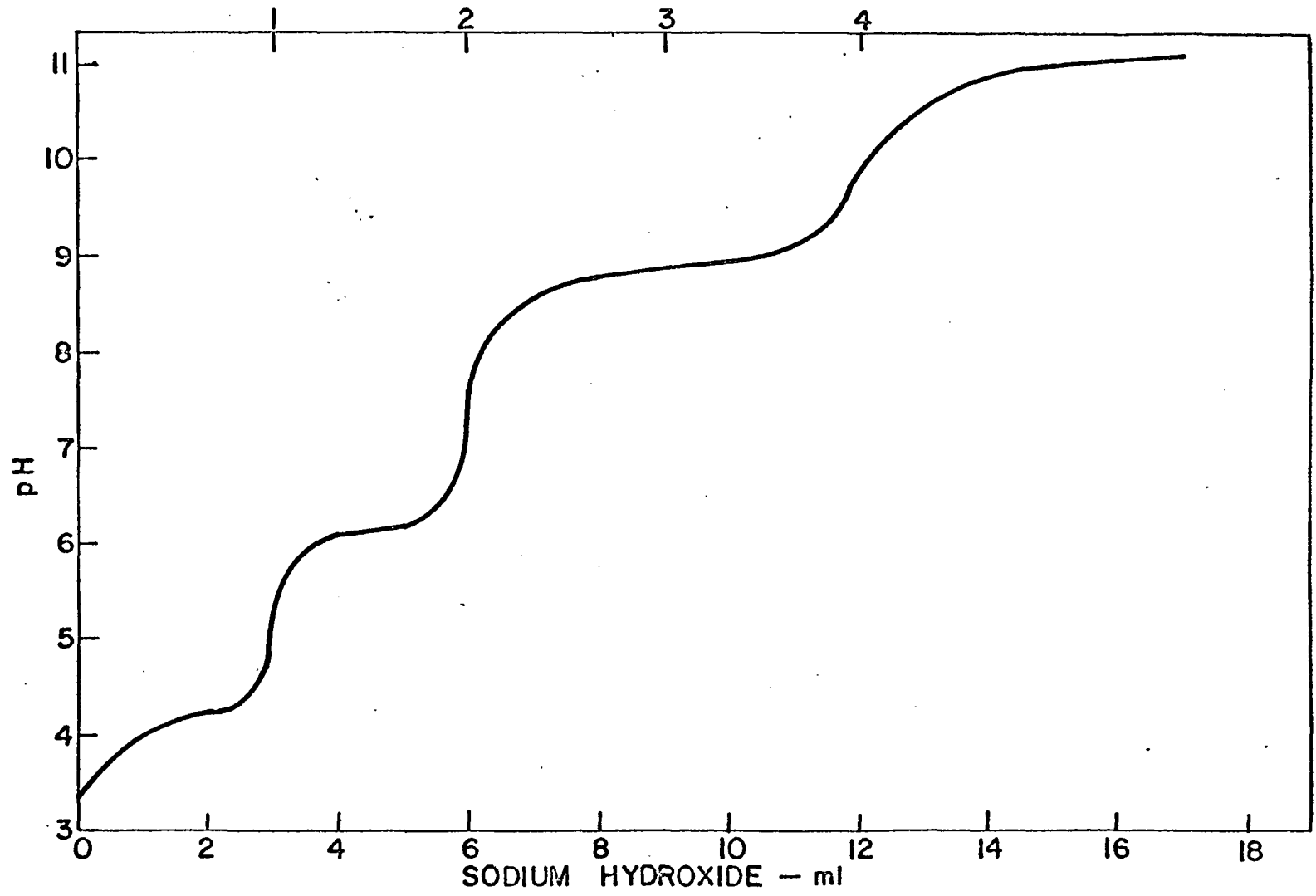
Figure 16. Potentiometric titration of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) in the presence of cal-cium chloride with sodium hydroxide

     Weight of compound taken: 0.0998 g. (2.78 x 10^{-4} moles)
     Weight of calcium chloride dihydrate added: 0.0408 g. (2.78 x 10^{-4} moles)
     Concentration of sodium hydroxide: 0.0925 N.
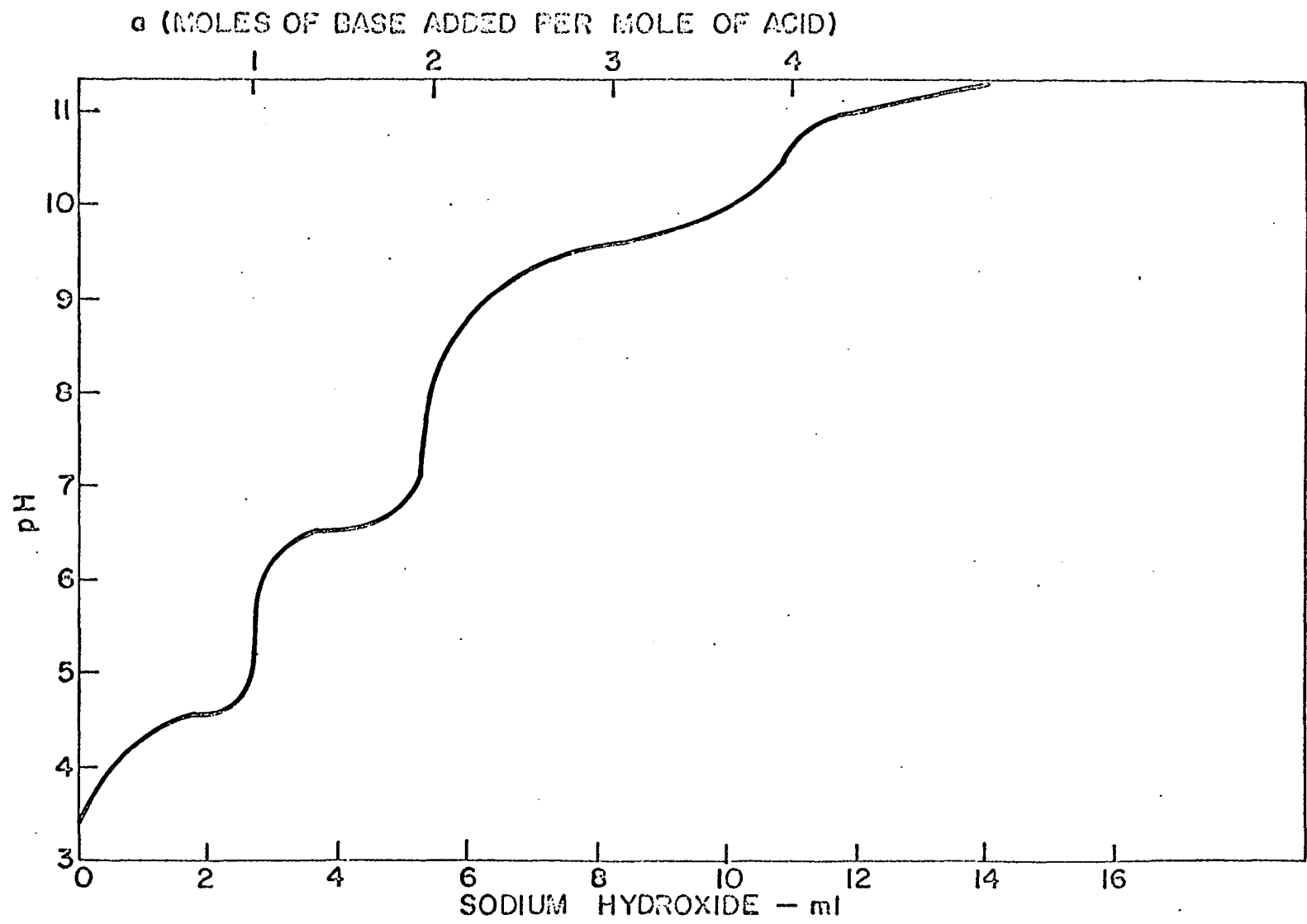
a Moles of base added per mole of acid

Determination of the formation constants of the calcium and

magnesium compounds of 1-[3'(N,N-bis(carboxymethyl)amino-

methyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)

aminomethyl)-2-hydroxybenzene

In order to determine the formation constants of the

calcium compounds of H_6Iidhab, two potentiometric titrations

were performed. In the first titration, a mixture of 0.1620

g. (3.18 x 10^{-4} moles) of H_6Iidhab and 4.720 g. (3.20 x 10^{-2}

moles) of calcium chloride dihydrate was titrated with 0.0933

M sodium hydroxide. This titration curve is shown in Figure

17. In the second titration, a mixutre of 0.2459 g (4.87 x

10^{-4} moles) of H_6Iidhab and 0.0839 g. (4.87 x 10^{-4} moles) of

calcium sulfate dihydrate was titrated with 0.0933 M sodium

hydroxide. This titration curve is shown in Figure 18.

To determine the formation constants of the magnesium

compounds of H_6Iidhab, again two potentiometric titrations

were performed. In the first titration, a mixture of 0.1852

g. (3.64 x 10^{-4} moles) of H_6Iidhab and 1.240 g. (3.63 x 10^{-3}

moles of magnesium iodate tetrahydrate was titrated with

0.0933 M sodium hydroxide. This titration curve is shown in

Figure 19. In the second titration, a mixture of 0.1732 g.

(3.44 x 10^{-4} moles) of H_6Iidhab and 0.1216 g. (3.44 x 10^{-4}

moles) of magnesium iodate tetrahydrate with 0.0933 M sodium

hydroxide. This titration curve is shown in Figure 5.

In order to further show the combining ratio of magnesium

Figure 17. Potentiometric titration of 1-[3'(N,N-bis(carboxy-
            methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-
            (N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene
            (H$_6$Iidhab) in the presence of calcium chloride with
            sodium hydroxide
                    Weight of compound taken: 0.1620 g. (3.18
                    x 10^{-4} moles)
                    Weight of calcium chloride dihydrate added:
                    4.7167 g. (3.21 x 10^{-2} moles)
                    Concentration of sodium hydroxide: 0.0933 N.

         a Moles of base added per mole of acid

Figure 18. Potentiometric titration of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab) in the presence of calcium sulfate with sodium hydroxide

      Weight of compound taken: 0.2459 g. (4.87 x 10^{-4} moles)

      Weight of calcium sulfate dihydrate added: 0.0839 g. (4.87 x 10^{-4} moles)

      Concentration of sodium hydroxide: 0.0933 N.

a Moles of base added per mole of acid

69

Figure 19.   Potentiometric titration of 1-[3'(N,N-bis(carboxy-
             methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-
             (N,N-bis(carboxymethyl)aminomethyl)-2-hydroxy-
             benzene (H_6Iidhab) in the presence of magnesium
             iodate with sodium hydroxide
                  Weight of compound taken: 0.1852 g. (3.63
                  x 10^{-4} moles)
                  Weight of magnesium iodate tetrahydrate added:
                  1.5467 g. (3.63 x 10^{-3} moles)
                  Concentration of sodium hydroxide: 0.0933 N.

         a   Moles of base added per mole of acid
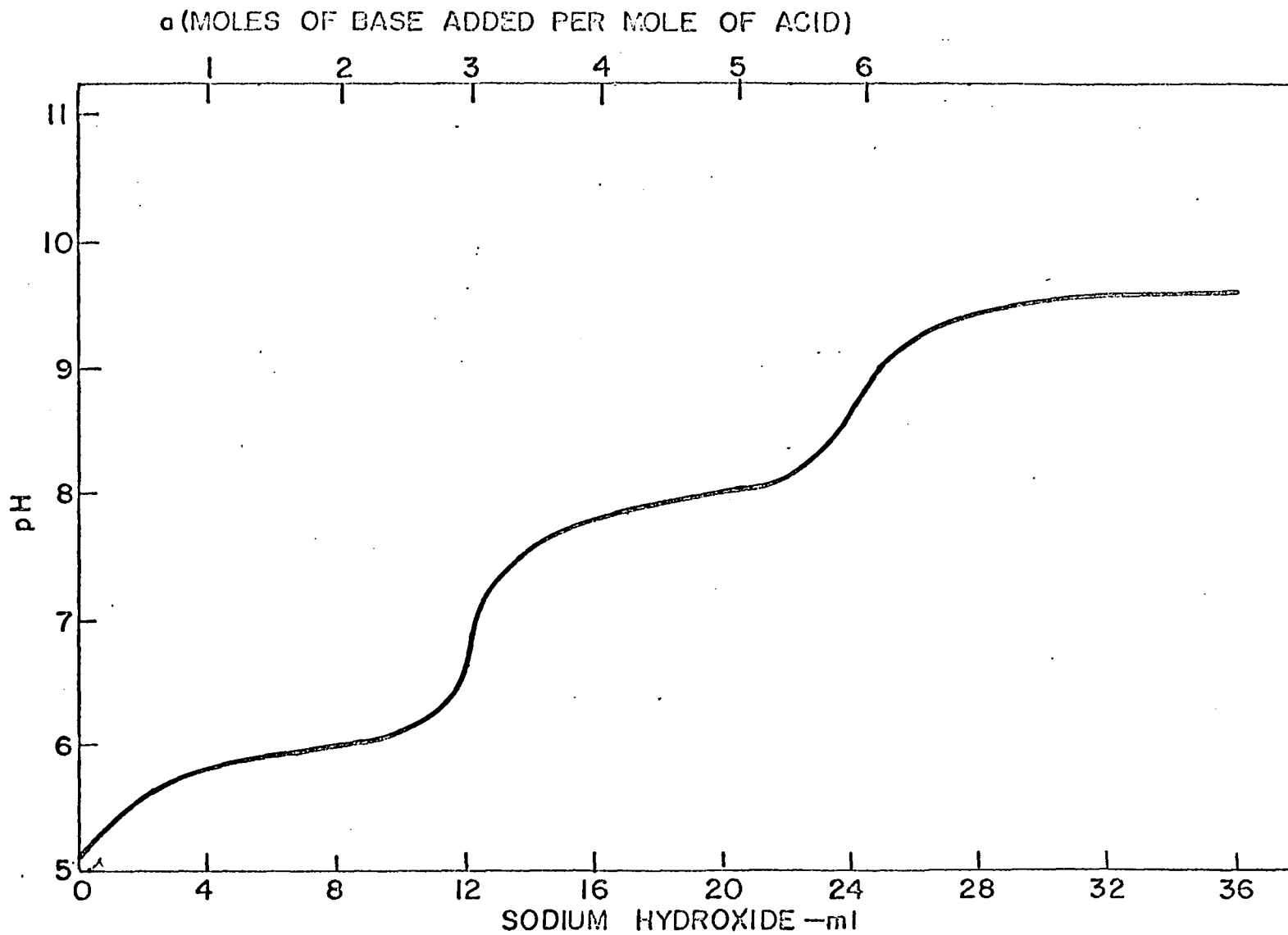
with H_6Iidhab, a spectrophotometric titration of H_6Iidhab

with magnesium iodate tetrahydrate at pH 10.0 was performed

in the following manner: 5 ml. of 3.18 x 10^{-4} M H_6Iidhab,

10 ml. of pH 10.0 buffer, and varying amounts of 1 x 10^{-4} M

magnesium iodate were added to a 50-ml. volumetric flask;

and the solution was diluted to the mark with 0.1 M potassium

chloride. The difference in absorbance of the solution con-

taining magnesium and the solution containing no magnesium

used as a blank at 480 mμ. was determined. This value was

plotted versus the concentration of magnesium. This graph

is shown in Figure 20. The same solutions were examined in

a spectrophotofluorometer with an excitation wavelength of

480 mμ. and an emission wavelength of 579 mμ. The values of

log 100/F where F is relative fluorescence was plotted versus

magnesium concentration. This graph is also shown in Figure

20.

Fluorescence study of the magnesium compound of 1-[3'(N,N-bis
(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-
hydroxybenzene

The fluorescence spectrum of H_4Idhab was obtained on a

solution containing 5 ml. of 3.44 x 10^{-4} M H_4Idhab, 2 ml. of

2 x 10^{-3} M magnesium iodate tetrahydrate, and 10 ml. of pH

10.0 buffer in 50 ml. of solution. The excitation and emis-

sion spectra are shown in Figure 21.

The dependency of the fluorescence and absorption of

Figure 20. Change in absorbance and fluorescence versus mag-
nesium ion concentration of 1-[3'(N,N-bis(carboxy-
methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-
(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxy-
benzene (H_6Iidhab) at pH 10.

A ΔA (change in absorbance) versus magnesium ion
concentration

B Log 100/F versus magnesium ion concentration

n Moles of magnesium added per mole of acid

74
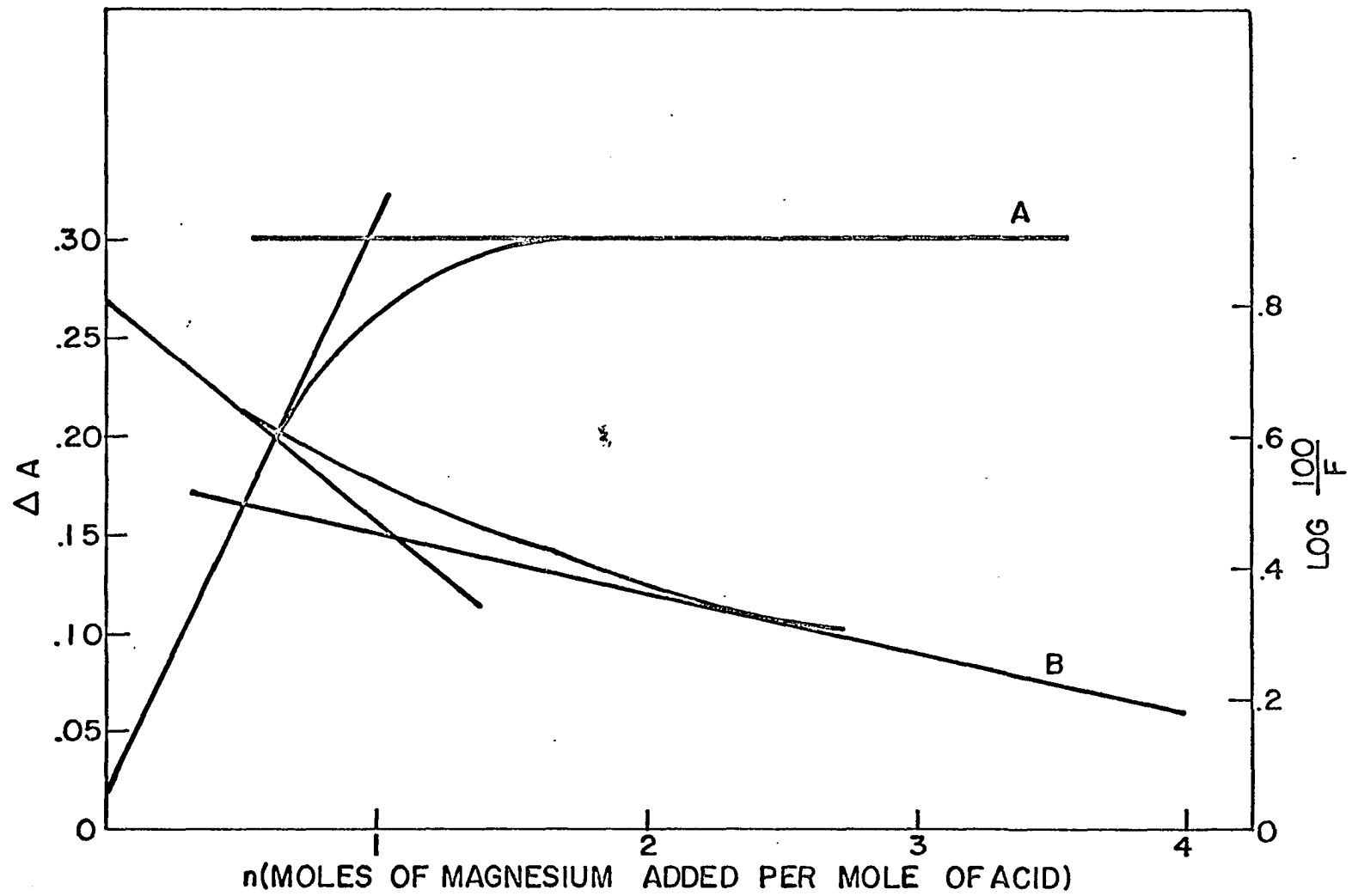
Figure 21. Fluorescence excitation and emission spectra of a solution 3.44×10^{-5} M in 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) and 8.0×10^{-5} M in magnesium iodate at pH 10.

A Excitation Spectra

B Emission Spectra
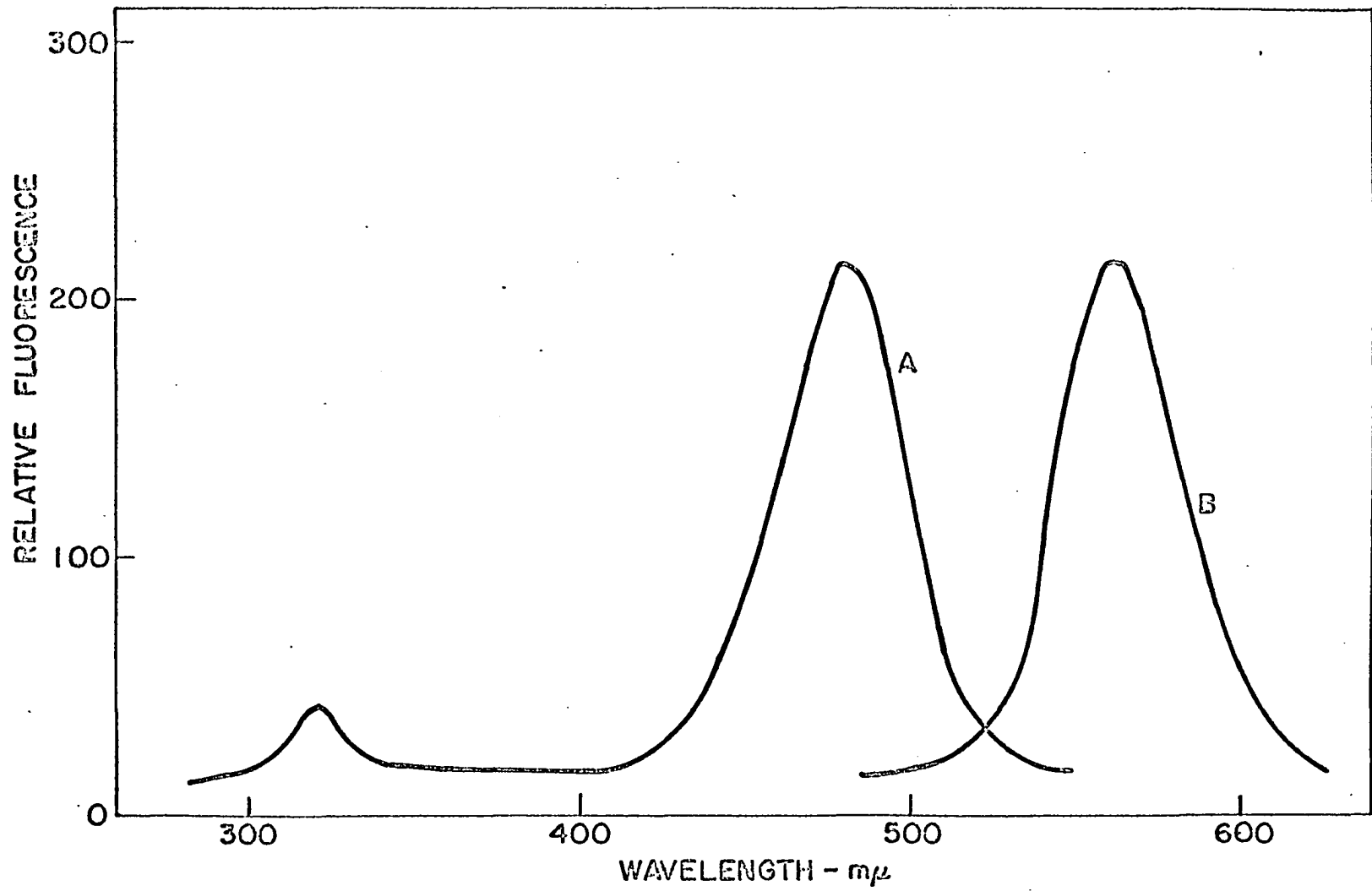
pH was examined by adding 5 ml. of 3.44 x 10^{-4} M H_4Idhab, 2 ml. of 2 x 10^{-3} M magnesium iodate, and 10 ml. of the appropriate buffer to a 50-ml. volumetric flask. The solution was diluted to the mark with 0.1 M potassium chloride solution. The relative fluorescence of each of the thirty-five different solutions was measured with the excitation monochromator set at 480 mμ. and the emission monochromator set at 565 mμ. The values of the relative fluorescence were converted to log 100/F and plotted against the pH of the particular solution. The graph of this data is shown in Figure 22.

The same solutions described above were used to determine the effect of pH on absorbance. Each solution was measured in the Beckman DU spectrophotometer at 480 mμ. The absorbance was plotted versus pH, and the graph is shown in Figure 22.

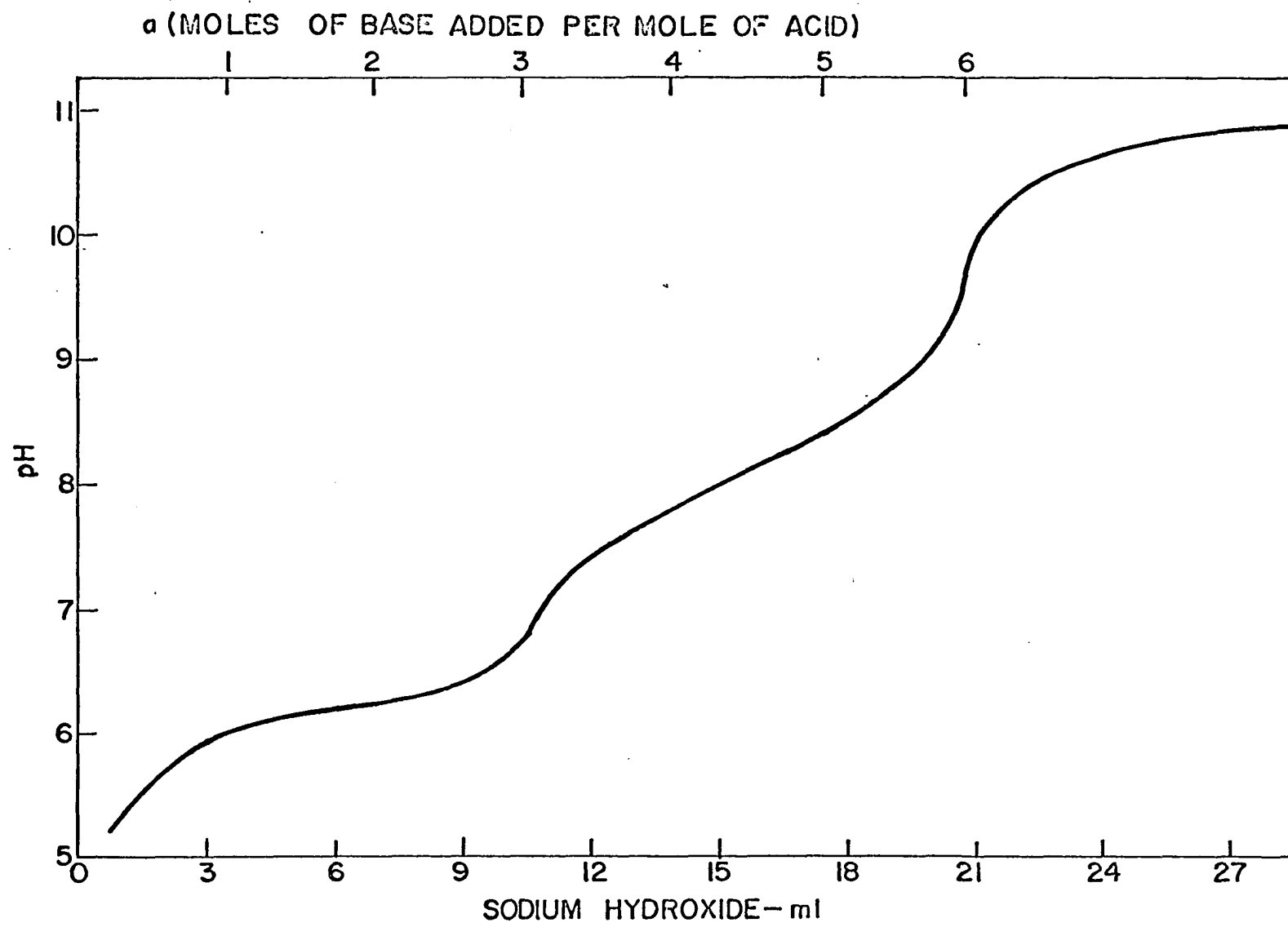Fluorescence study of the magnesium compound of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene

The fluorescence spectrum of H_6Iidhab was obtained on a solution that contained 5 ml. of 3.18 x 10^{-4} M H_6Iidhab, 2 ml. of 2 x 10^{-3} M magnesium iodate tetrahydrate and 10 ml. of pH 10.0 buffer in 50 ml. of solution. The excitation and emission spectra are shown in Figure 23.

The dependence of fluorescence and absorbance on pH

Figure 22. Absorbance and fluorescence versus pH of a
            solution 3.44 x 10^{-5} M in 1-[3'(N,N-bis(carboxy-
            methyl)aminomethyl-2'-hydroxy-1'-benzeneazo]-2-
            hydroxybenzene (H_4Idhab) and 8.0 x 10^{-5} M in
            magnesium iodate. Excitation wavelength: 480
            mμ. Emission wavelength: 565 mμ.

            A   Absorbance versus pH

            B   Relative fluorescence versus pH
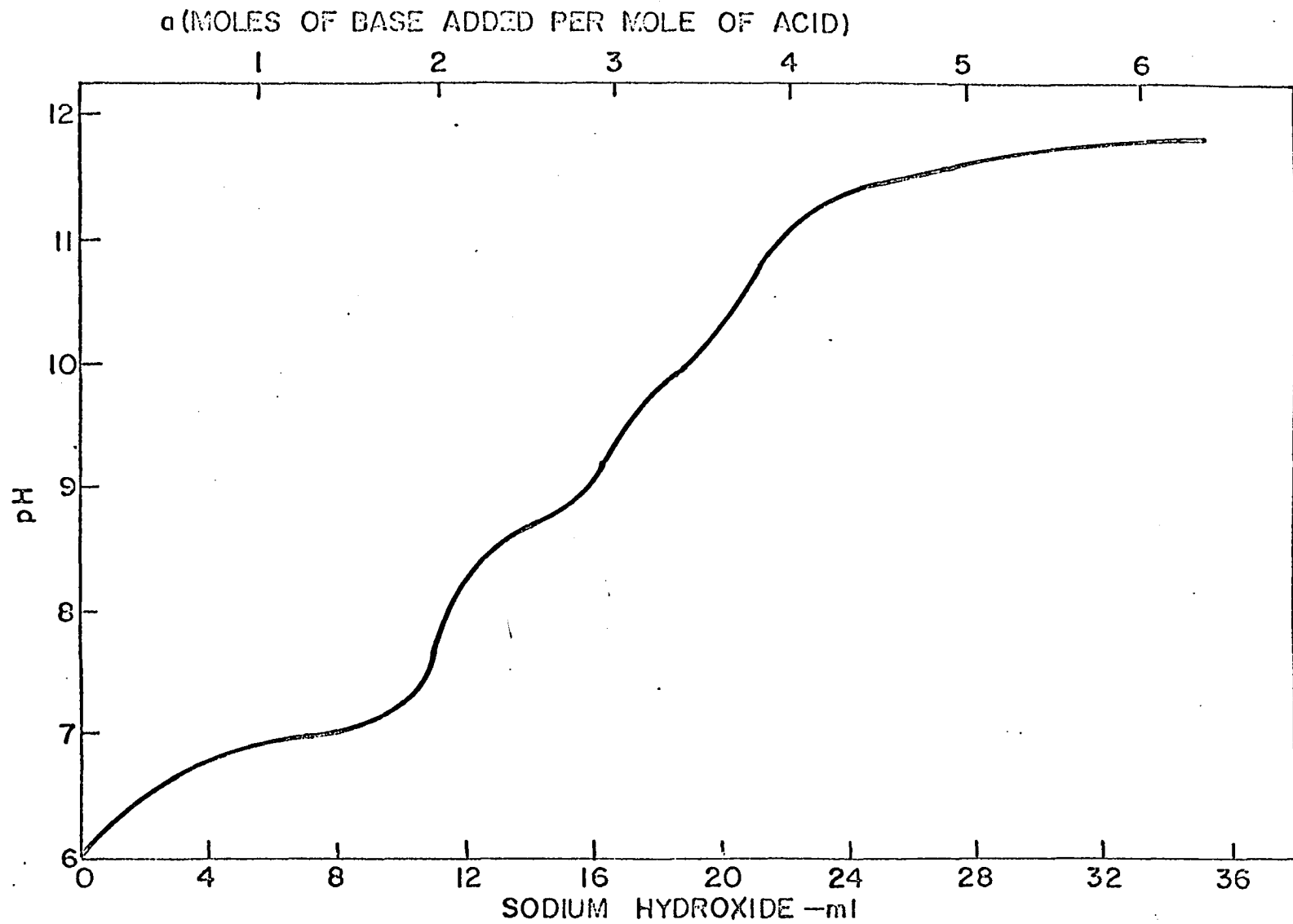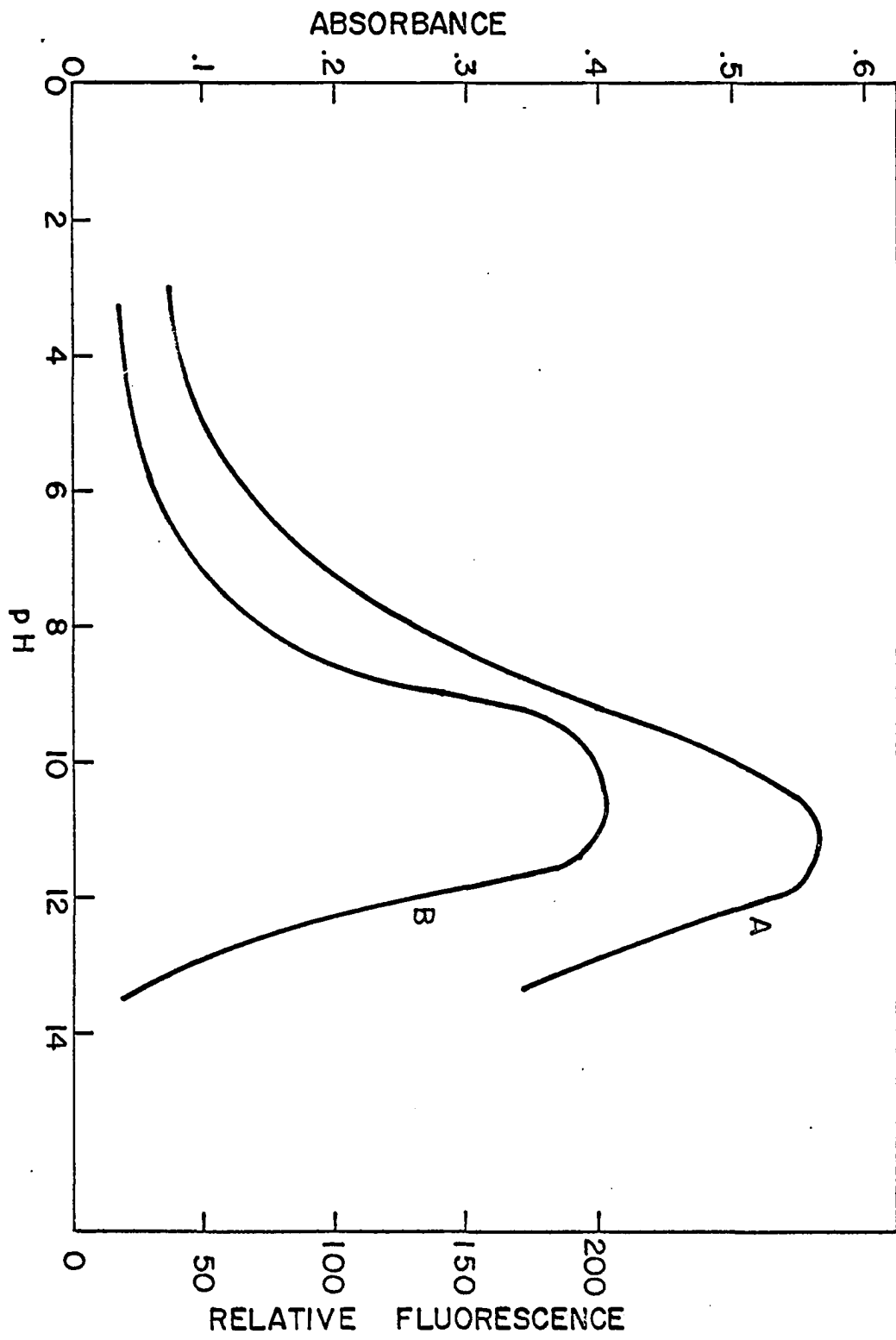
Figure 23. Fluorescence excitation and emissibn spectra of a solution 3.18 x 10^{-5} M in 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxy-benzene (H_6Iidhab) and 8.0 x 10^{-5} M in magnesium iodate at pH 10.

A   Excitation spectra

B   Emission spectra

RELATIVE FLUORESCENCE

WAVELENGTH - mμ

81
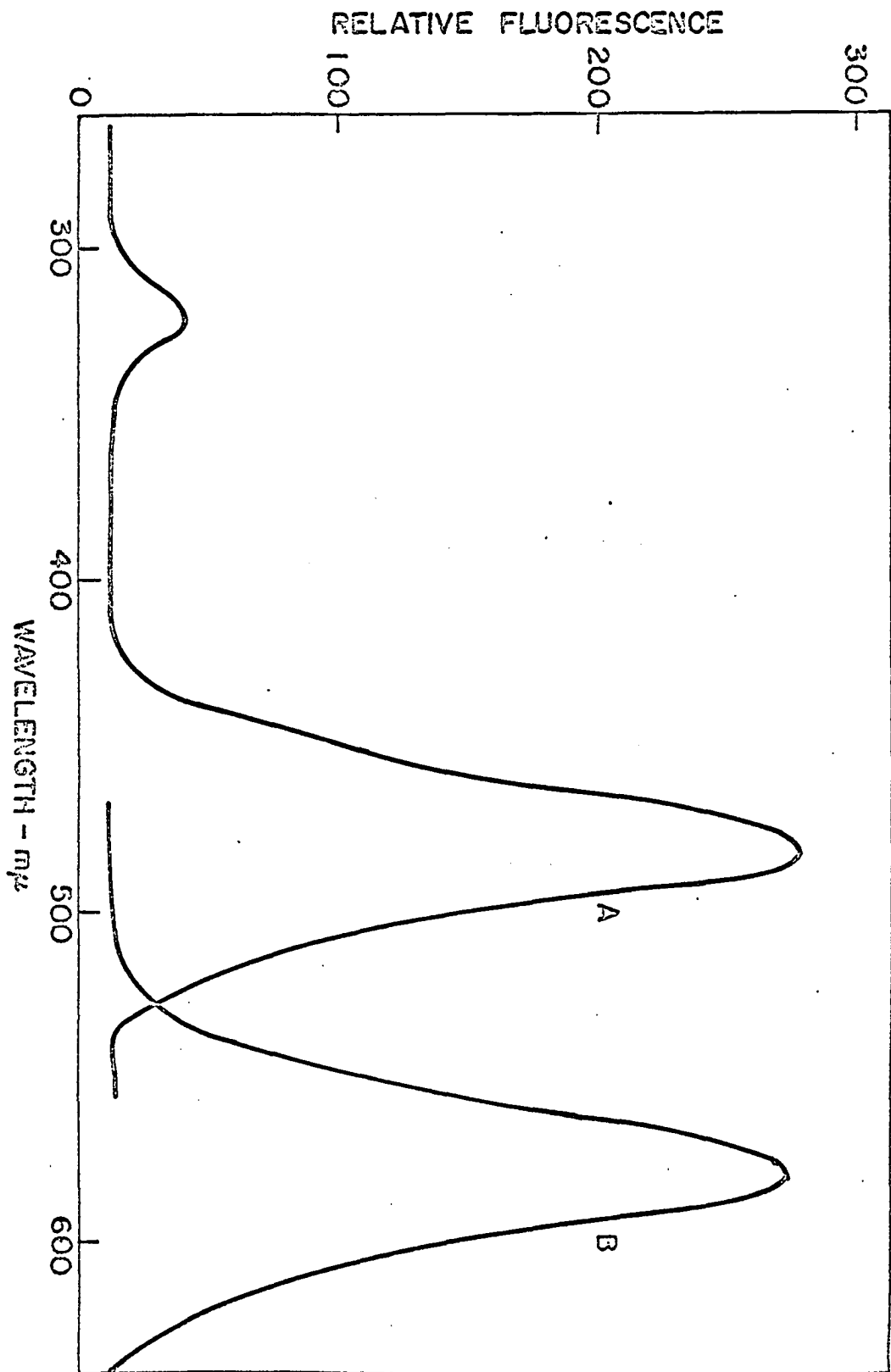
of the compound was determined by adding 5 ml. of 3.18 x

10^{-4} M H_6Iidhab, 2 ml. of 2 x 10^{-3} M magnesium iodate and

10 ml. of an appropriate buffer to a 50-ml. volumetric

flask. The solution was diluted to the mark with 0.1 M

potassium chloride. The relative fluorescnece of each of

thirty-five different solutions was measured on the Aminco-

Kiers spectrophotofluorometer. The excitation monochromator

was set at 480 mμ. and the emission monochromator was set at

565 mμ. The relative fluorescence values were converted to

log 100/F and plotted versus the pH of each solution. The

resultant graph is shown in Figure 24. The same solutions

were examined with a Beckman DU spectrophotometer at 480 mμ.

The absorbance of each solution was plotted versus pH.

This graph is also shown in Figure 24.

Results and Discussion

Determination of the acid dissociation constants of
o,o'-dihydroxyazobenzene

The determination of the acid dissociation constants

of o,o'-dihydroxyazobenzene (H_2Dhab) was first reported by

J. Ellingboe (12). He found pK_{a1} = 7.7 and pK_{a2} = 11.4 from

a plot of absorbance versus pH. J. R. Kirby, R. M. Milburn,

and J. H. Saylor (15) reported only one value, pK_{a1} = 9.3,

for H_2Dhab, stating that the second acid dissociation con-

stant could not be obtained spectrophotometrically. The

Figure 24. Absorbance and fluorescence versus pH of a
solution 3.18 x 10^{-5} M in 1-[3'(N,N-bis(carboxy-
methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-
(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxy-
benzene and 8.0 x 10^{-5} M in magnesium iodate.
Excitation wavelength: 480 mμ. Emission wave-
length: 579 mμ.

A   Absorbance versus pH

B   Relative fluorescence versus pH
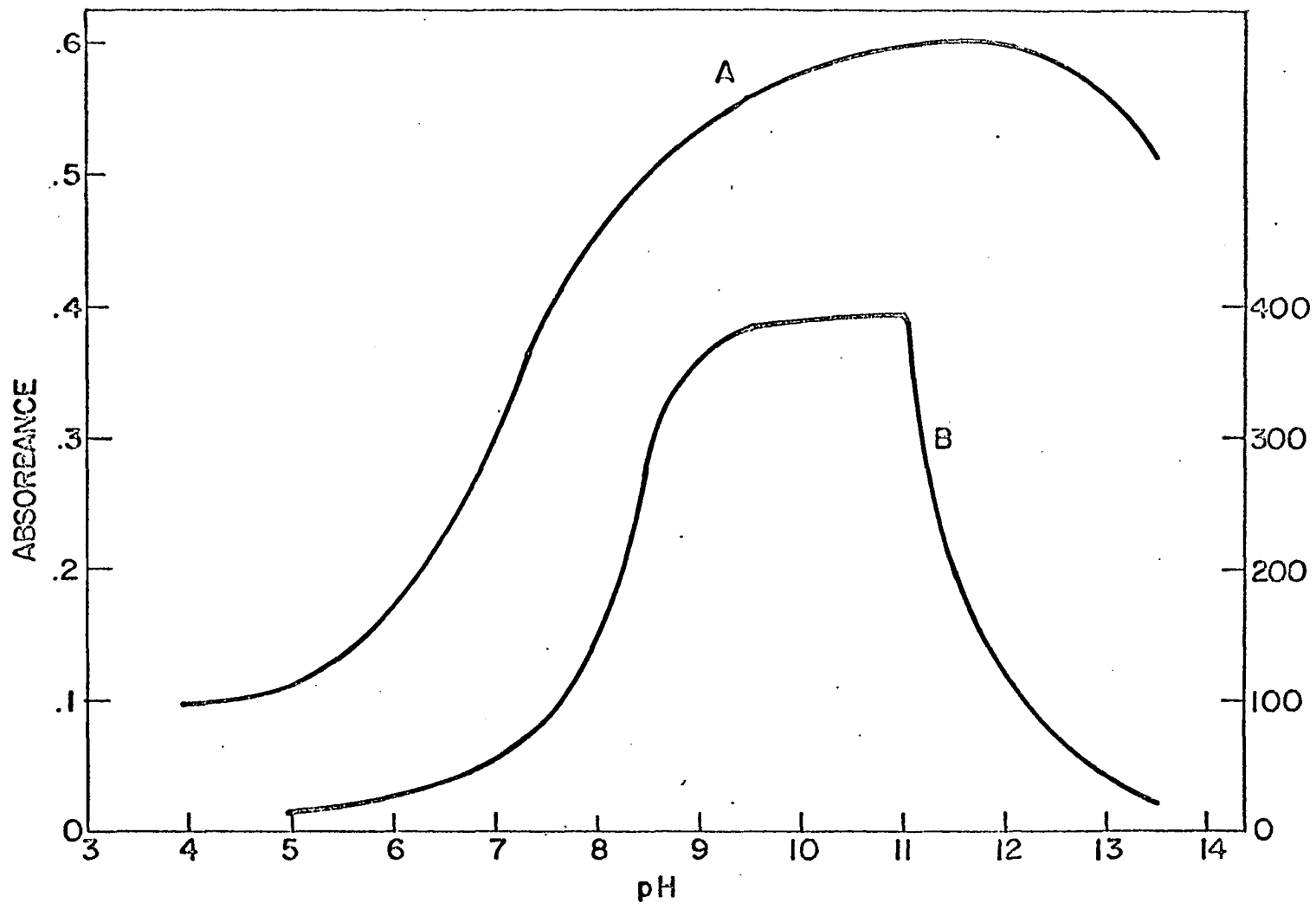
discrepancy between these two reported acid dissociation constants lies in the fact that the J. R. Kirby, Milburn and Saylor measurements were made on solutions 35 percent in ethyl alcohol, and the pH values reported were those obtained using a pH meter standardized with aqueous buffer; the values obtained are thus only apparent pH values. In the present work, the acid dissociation constants of H_2Dhab were again measured so that direct comparison could be made of these constants with those of the new compounds prepared, H_4Idhab and $H_6Iidhab$.

The absorption spectra shown in Figure 7 indicate that the absorption of H_2Dhab changes most rapidly with pH at 390 mμ. and 480 mμ. Measurements were made at both of these wavelengths to determine the acid dissociation constants. The graphs shown in Figure 8 show two inflections in the curve of absorbance at 480 mμ. and only one inflection for the curve of absorbance at 390 mμ. The inflection point of the curve is the point at which the pH equals the acid dissociation constant. The acid dissociation constants obtained from the graph are, therefore, $pK_{a1} = 8.9$ and $pK_{a2} = 11.1$.

The discrepancy between the values described here and the ones reported by J. Ellingboe (12) probably lie in the fact that the Ellingboe measurements were made on much more dilute solutions, the highest absorbance reading being only 0.070 and thus subject to considerable error.

The best values for the acid dissociation constants of H_2Dhab are probably those reported here: $pK_{a1} = 8.9$, $pK_{a2} = 11.1$.

Determination of the acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene

The determination of the acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) was made using potentiometric and spectrophotometric data. The compound was not completely dissolved at the point half-way to the first end-point. Therefore, a value for the first acid dissociation constant could not be obtained directly from this point as is frequently done, and it was necessary to calculate the constant from data at points between the half-way point and the end-point, points at which the material was completely in solution.

Because of the relatively large size of the first break the second acid dissociation was not considered in calculating the first, that is, the material was considered as monobasic acid:

$$H_4Idhab = H^+ + H_3Idhab^- \qquad (1)$$

The first ionization constant was then calculated using the equation defining the constant:

$$K_{a1} = \frac{(H^+)(H_3Idhab^-)}{(H_4Idhab)} \qquad (2)$$

and data from the titration curve shown in Figure 2. The average pK_{a1} found from calculations at three different points was 3.8.

The second acid dissociation constant was obtained directly from the graph in Figure 2 assuming that pH = pK_{a2} at (a) = 1.5 where (a) is the number of moles of base added per mole of acid in the titration. The small second break in the titration curve indicates that the third replaceable hydrogen atom is also being titrated to a small extent so that the pK_{a2} determined in this manner is not highly ac- curate. As a rough approximation pK_{a2} = 7.6. A better value was obtained by using the spectrophotometric method.

The absorption spectra shown in Figure 9 indicated that at least one of the acid dissociation constants of H_4Idhab could be determined spectrophotometrically. The maximum changes in absorbance with pH occurred at 390 mμ. and 480 mμ. These are the same wavelengths that were found for H_2Dhab, so it was assumed that this change was associated with the neutralization of one of the phenolic hydrogen atoms. The plot of absorbance versus pH shown in Figure 10 indicates only one good inflection point for both the wavelengths monitored. There is a hint of a second in- flection point above pH 13. The inflection point comes at pH 7.8, thus, the value of pK_{a2} = 7.8.

The titration curve of H_4Idhab (Figure 2) shows no

distinct breaks for the third and fourth replaceable hydrogen atoms. Values for the third and fourth dissociation constants were determined from the potentiometric data using the method devised by H. Ackermann and G. Schwarzenbach (1). The argument is as follows: The apparent degree of neutralization (a) is taken from the graph, and the true degree of neutralization (g) is determined from the equation defining (g) shown in equation (3).

$$g = a + \frac{(H^+) - (OH^-)}{C_s} \qquad (3)$$

In this equation, C_s is the total concentration of H_4Idhab present in all forms.

(g) can also be expressed in terms of the species present in solution.

$$g = \frac{(H_3Idhab^-) + 2(H_2Idhab^{-2}) + 3(HIdhab^{-3}) + 4(Idhab^{-4})}{(H_4Idhab) + (H_3Idhab^-) + (H_2Idhab^{-2}) + (HIdhab^{-3}) + (Idhab^{-4})}$$

$$(4)$$

If the value of (a) is chosen at sufficiently high pH, the amount of H_4Idhab, and H_3Idhab^- present is negligible and can be disregarded. Equation 4 simplifies to

$$g = \frac{2(H_2Idhab^{-2}) + 3(HIdhab^{-3}) + 4(Idhab^{-4})}{(H_2Idhab^{-2}) + (HIdhab^{-3}) + (Idhab^{-4})} \qquad (5)$$

Introducing the equation defining the acid dissociation constants

$$K_{a1} = \frac{(H^+)(H_3Idhab^-)}{(H_4Idhab)}, \qquad K_{a2} = \frac{(H^+)(H_2Idhab^{-2})}{(H_3Idhab^-)},$$

$$K_{a3} = \frac{(H^+)(HIdhab^{-3})}{(H_2Idhab^{-2})}, \quad \text{and} \quad K_{a4} = \frac{(H^+)(Idhab^{-4})}{(HIdhab^{-3})} \qquad (6)$$

gives

$$g = \frac{\dfrac{2K_{a1}K_{a2}}{(H^+)^2} + \dfrac{3K_{a1}K_{a2}K_{a3}}{(H^+)^3} + \dfrac{4K_{a1}K_{a2}K_{a3}K_{a4}}{(H^+)^4}}{\dfrac{K_{a1}K_{a2}}{(H^+)^2} + \dfrac{K_{a1}K_{a2}K_{a3}}{(H^+)^3} + \dfrac{K_{a1}K_{a2}K_{a3}K_{a4}}{(H^+)^4}} \qquad (7)$$

Introducing new unknowns $X = K_{a3}$ and $Y = K_{a3}K_{a4}$; and re-arranging terms gives

$$X \cdot \frac{K_{a1}K_{a2}(g-3)}{(H^+)^3} + Y \cdot \frac{K_{a1}K_{a2}(g-4)}{(H^+)^4} + \frac{K_{a1}K_{a2}(g-2)}{(H^+)^2} = 0 \qquad (8)$$

The values of X and Y were determined in the following manner: Two values of (a) were chosen above a = 2 and the corresponding value of (g) calculated from equation 3. These values of (g) were separately inserted into equation 8 with the corresponding value of (H^+) and the values of K_{a1} and K_{a2} determined above. This gave two equations with two unknowns. These equations were solved with the help of a determinant. The results are shown in Table 2.

In summary then, the values obtained for the acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)amino-methyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene are:

$pK_{a1} = 3.8$, $pK_{a2} = 7.8$, $pK_{a3} = 9.7$, and $pK_{a4} = 11.2$.

Table 2. Summary of data used in calculating the third and fourth acid
dissociation constants of 1-[3'(N,N-bis(carboxymethyl)amino-
methyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab).

a	(H^+)	g	X	Y	pK_{a3}	pK_{a4}
2.50	2.50×10^{-10}	2.49				
			2.24×10^{-10}	1.43×10^{-21}	9.65	11.19
3.25	1.48×10^{-11}	3.24				
2.75	1.00×10^{-10}	2.74				
			2.21×10^{-10}	1.30×10^{-21}	9.66	11.23
3.10	2.50×10^{-11}	3.09				

Determination of the acid dissociation constants of 1-[3' (N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo] -3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene

The determination of acid dissociation constants for 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3'-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab) was made by using a combination of methods. The potentiometric titration of H_6Iidhab shown in Figure 4 indicates two distinct breaks, one at a = 2 and one at a = 3, where (a) is the number of moles of base added per mole of acid. The compound did not completely dissolve in this titration until immediately before the first end point at a = 2. Immediately before the first end point the pH changed very rapidly with very small increments of base added. This made it difficult to calculate accurately the acid dissociation constants from data at such points. Instead, the method suggested by D. Davidson (6) was used to calculate the first two acid dissociation constants and the solubility product constant of H_6Iidhab. This method required the use of the data in Table 1 on the solubility of H_6Iidhab as a function of pH.

Using the following equations, the acid dissociation constants K_{a1}, K_{a2}, and the solubility product constant were calculated for H_6Iidhab. The symbol "S" represents the apparent solubility, K_{b2}, and K_{b1} represent the dissociation

constants for the conjugate bases of K_{a1} and K_{a2}. K_{b2} and K_{b1} are defined by the following equations:

$$K_{b2} = \frac{(H_6 Iidhab)(OH^-)}{(H_5 Iidhab^-)(H_2 O)} \quad \text{and} \quad K_{b1} = \frac{(H_5 Iidhab^-)(OH^-)}{(H_4 Iidhab^{-2})(H_2 O)} \quad (9)$$

The relation between K_{b2} and K_{a1} is the following:

$$K_{a1} \times K_{b2} = K_w \quad (10)$$

where K_w is the dissociation constant for water equal to 1×10^{-14}.

Adding equations 11, 12, and 13, and combining the dissociation constants shown at the right of the equation gives an equation for the dissolution of $H_6 Iidhab$ equation 14.

$$H_6 Iidhab_{(solid)} = H_6 Iidhab_{(dissolved)} \quad K_s \quad (11)$$

$$H_6 Iidhab + OH^- = H_5 Iidhab^- + H_2 O \quad 1/K_{b2} \quad (12)$$

$$H_5 Iidhab + OH^- = H_4 Iidhab^{-2} + H_2 O \quad 1/K_{b1} \quad (13)$$

$$H_6 Iidhab_{(solid)} + 2OH^- = H_4 Iidhab^{-2} + 2H_2 O \quad K_s/K_{b2}K_{b1} \quad (14)$$
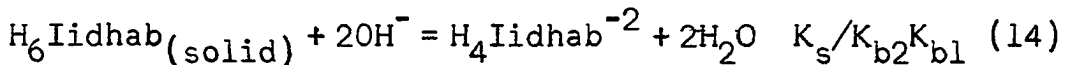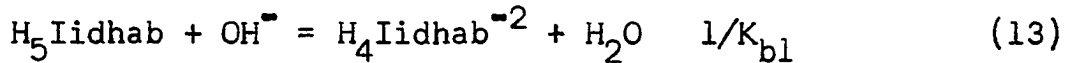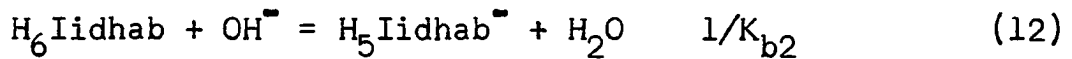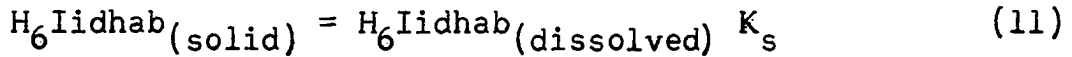
The activity of a solid is unity, and the concentration of water does not change appreciably so that the equilibrium constant for equation 14 can be written

$$\frac{(H_4 Iidhab^{-2})}{(OH^-)^2} = \frac{K_s}{K_{b2}K_{b1}} \quad (15)$$

Solving for $H_4Iidhab^{-2}$, multiplying both top and bottom of the right hand equation by $(H^+)^2$ and introducing the relationship between K_a, and K_b (equation 10) gives

$$(H_4Iidhab^{-2}) = \frac{K_s(OH^-)^2(H^+)^2}{K_{b2}K_{b1}(H^+)^2} = \frac{K_sK_{a1}K_{a2}}{(H^+)^2} \tag{16}$$

The apparent solubility "S" can be expressed in terms of the species present in solution

$$S = (H_6Iidhab)_{(dissolved)} + (H_5Iidhab) + (H_4Iidhab) \tag{17}$$

Introducing the constants defining the concentrations above

$$S = K_s + \frac{K_sK_{a1}}{(H^+)} + \frac{K_sK_{a1}K_{a2}}{(H^+)^2} \tag{18}$$

Let $X = K_s$, $Y = K_sK_{a1}$, and $Z = K_sK_{a1}K_{a2}$.

$$X + \frac{Y}{(H^+)} + \frac{Z}{(H^+)^2} = S \tag{19}$$

Choosing three different values for "S" from Table 1 and the appropriate pH values gave three equations in three unknowns. These equations were solved with the help of a three by three determinant. The data used to make these calculations are shown in Table 3.

The average values obtained are $K_s = 6.0 \times 10^{-6}$, $pK_{a1} = 4.2$, and $pK_{a2} = 5.8$.

The third dissociation constant pK_{a3} of $H_6Iidhab$ was determined spectrophotometrically. The absorption spectra

Table 3. Summary of the data used to calculate the solubility product constant and the first two acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab).

S	pH	Y	Z	K_s	pK_{a1}	pK_{a2}
9.35×10^{-5}	4.49					
2.02×10^{-4}	4.90	1.71×10^{-10}	1.83×10^{-16}	3.24×10^{-6}	4.28	5.74
1.29×10^{-3}	5.90					
1.19×10^{-3}	5.80					
2.04×10^{-3}	6.75	6.62×10^{-10}	1.20×10^{-15}	8.50×10^{-6}	4.11	5.82
9.35×10^{-5}	4.49					

shown in Figure 11 indicates that the maximum change in

absorbance with pH occurs at 390 mμ. and 480 mμ. This change

in the absorption spectra is the same as that observed in

o,o'-dihydroxyazobenzene. The change is, therefore, at-

tributed to the neutralization of the first phenolic hydrogen

atom. The graph of absorbance at 390 mμ. and 480 mμ. versus

pH is shown in Figure 12. This graph shows only one inflection

point for each curve. The inflection point occurs at pH 7.7,

indicating, therefore, that pK_{a3} = 7.7.

The last three acid dissociation constants of H_6Iidhab

were calculated from the potentiometric titration curve shown

in Figure 4 using the method of H. Ackermann and G. Schwarzen-

bach (1). The argument is as follows: The true degree of

neutralization (g) is determined from the apparent degree

of neutralization (a) by the defining equation

$$g \equiv a + \frac{(H^+) - (OH^-)}{C_s} \qquad (20)$$

where C_s is the amount of H_6Iidhab present in all forms.

(g) can also be expressed in terms of the species in solution

$$g = (H_5Iidhab) + 2(H_4Iidhab^{-2}) + 3(H_3Iidhab^{-3}) + 4(H_2Iidhab^{-4})$$

$$+ 5(HIidhab^{-5}) + 6(Iidhab^{-6}) / (H_6Iidhab) + (H_5Iidhab^-) +$$

$$(H_4Iidhab^{-2}) + (H_3Iidhab^{-3}) + (H_2Iidhab^{-4}) + (HIidhab^{-5}) +$$

$$(Iidhab^{-6}) \qquad (21)$$

By choosing values of (a) at sufficiently high pH the amounts

of H_6Iidhab, H_5Iidhab$^-$, and H_4Iidhab^{-2} can be disregarded. This simplified equation 21 to

$$g = \frac{3(H_3\text{Iidhab}^{-3}) + 4(H_2\text{Iidhab}^{-4}) + 5(\text{HIidhab}^{-5}) + 6(\text{Iidhab}^{-6})}{(H_3\text{Iidhab}^{-3}) + (H_2\text{Iidhab}^{-4}) + (\text{HIidhab}^{-5}) + (\text{Iidhab}^{-6})}$$

$$(22)$$

Introducing the defining equation for the acid dissociation constants

$$K_{a1} = \frac{(H^+)(H_5\text{Iidhab}^-)}{(H_6\text{Iidhab})} \quad, \quad K_{a2} = \frac{(H^+)(H_4\text{Iidhab}^{-2})}{(H_5\text{Iidhab}^-)} \quad,$$

$$K_{a3} = \frac{(H^+)(H_3\text{Iidhab}^{-3})}{(H_4\text{Iidhab}^{-2})} \quad, \quad K_{a4} = \frac{(H^+)(H_2\text{Iidhab}^{-4})}{(H_3\text{Iidhab}^{-3})} \quad,$$

$$K_{a5} = \frac{(H^+)(\text{HIidhab}^{-5})}{(H_2\text{Iidhab}^{-4})} \quad, \quad K_{a6} = \frac{(H^+)(\text{Iidhab}^{-6})}{(\text{HIidhab}^{-5})} \quad (23)$$

$$g = \frac{3K_{a1}K_{a2}K_{a3}}{(H^+)^3} + \frac{4K_{a1}K_{a2}K_{a3}K_{a4}}{(H^+)^4} + \frac{5K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}}{(H^+)^5}$$

$$\frac{6K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}K_{a6}}{(H^+)^6} \Bigg/ \frac{K_{a1}K_{a2}K_{a3}}{(H^+)^3} + \frac{K_{a1}K_{a2}K_{a3}K_{a4}}{(H^+)^4} +$$

$$\frac{K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}}{(H^+)^5} + \frac{K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}K_{a6}}{(H^+)^6} \qquad (24)$$

Introducing new unknowns $X = K_{a4}$, $Y = K_{a4}K_{a5}$ and $Z = K_{a4}K_{a5}K_{a6}$ and rearranging gives

$$X \cdot \frac{K_{a1}K_{a2}K_{a3}(g-4)}{(H^+)^4} + Y \cdot \frac{K_{a1}K_{a2}K_{a3}(g-5)}{(H^+)^5} + Z \cdot \frac{K_{a1}K_{a2}K_{a3}(g-6)}{(H^+)^6} +$$

$$\frac{K_{a1}K_{a2}K_{a3}(g-3)}{(H^+)^3} = 0 \qquad (25)$$

Choosing three different values of (a) and the corresponding values of pH, the three values of (g) were calculated. Introducing these values of (g) and (H^+) into equation 25 gave three equations and three unknowns. These equations were solved with the help of a determinant. A summary of the data used is shown in Table 4.

The average values obtained for the fourth, fifth, and sixth dissociation constants are $pK_{a4} = 9.6$, $pK_{a5} = 10.6$, and $pK_{a6} = 11.5$.

A summary of the acid dissociation constants of H_2Dhab, H_4Idhab, and $H_6Iidhab$ is shown in Table 5.

The acid dissociation constants of H_2Dhab, H_4Idhab, and $H_6Iidhab$ were compared in order to assign the replaceable hydrogen atoms to particular acid functions. The only acid functions in H_2Dhab are the phenolic groups, the very weakly basic character of the azo group precluding it from the present discussion. The values of pK_{a1} and pK_{a2}, 8.9 and 11.2, respectively, fall in the usual range for phenols and differ from each other as expected when hydrogen bonding is possible. The value of the pK of phenol (C_6H_5OH) is 9.98 and values for substituted phenols vary from 7.1 to 10.3

Table 4. Summary of the data used to calculate the fourth, fifth and sixth acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl-2-hydroxy-benzene (H_6Iidhab).

a	pH	g	X	Y	Z	pK_{a4}	pK_{a5}	pK_{a6}
3.50	9.55	3.49						
4.50	10.55	4.44	2.30×10^{-10}	5.53×10^{-21}	2.06×10^{-32}	9.64	10.62	11.69
5.25	11.14	4.99						
3.75	9.82	3.74						
4.75	10.70	4.68	2.32×10^{-10}	5.37×10^{-21}	1.14×10^{-32}	9.64	10.64	11.42
5.50	11.40	5.20						

Table 5. Summary of acid dissociation constants of
o,o'-dihydroxyazobenzene (H_2Dhab), 1-[3'(N,N-
bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-2-hydroxybenzene (H_4Idhab), and
1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-
hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)
aminomethyl)-2-hydroxybenzene (H_6Iidhab).

H_2Dhab		H_4Idhab		H_6Iidhab	
pK_1 = 8.9	P1	pK_1 = 3.8	C	pK_1 = 4.2	C1
pK_2 = 11.1	P2	pK_2 = 7.8	P1	pK_2 = 5.8	C2
		pK_3 = 9.7	P2	pK_3 = 7.7	P1
		pK_4 = 11.2	A	pK_4 = 9.6	P2
				pK_5 = 10.6	A
				pK_6 = 11.5	A2

P1 Replaceable hydrogen atom of first phenolic group

P2 Second phenolic group

C   Carboxyl group

C2 Second carboxyl group

A   Ammonium ion

A2 Second ammonium ion

depending on the nature and position of the substituent. The acidity of the phenolic group is greatly reduced by hydrogen bonding; thus, for the isomeric dyes Orange I and Orange II

VIII

Orange I

$pK_{a2} = 8.2$

IX
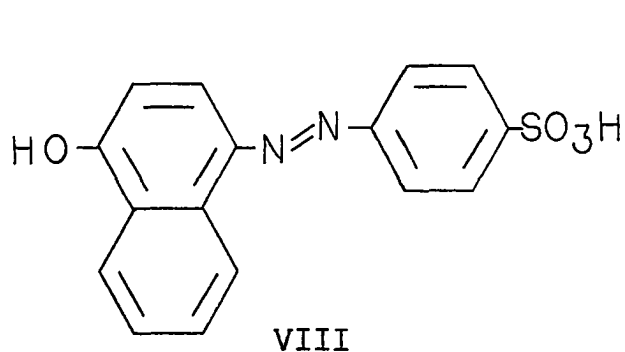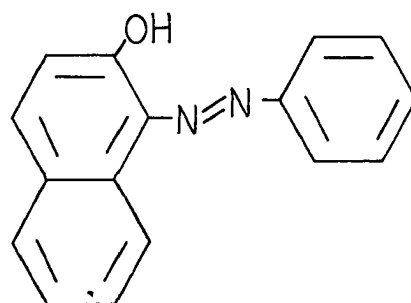
Orange II

$pK_{a2} = 11.4$

the constants differ (25), (16) by more than 10^3. In o,o'-dihydroxyazobenzene it appears then that one phenolic group is normal, the other hydrogen bonded

X
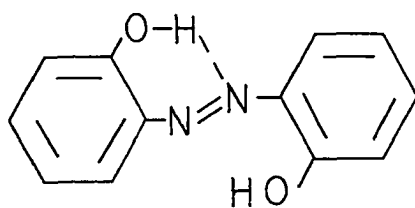
and that the conceivable hydrogen bonding of both groups

XI
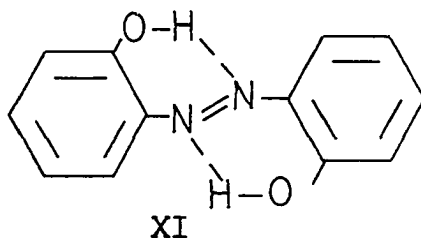
does not occur.

The tautomeric character of p-hydroxyazo compounds has now been well established, that is, that such compounds exist as mixtures of the true hydroxyazo (structure XII) and the hydrazone forms (structure XIII), thus

XII   XIII
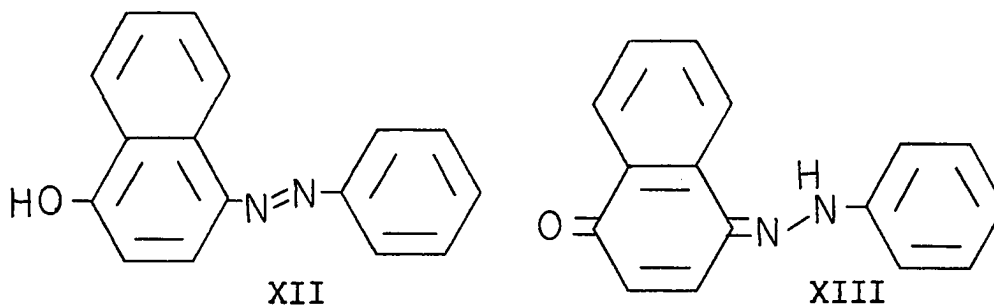
A fine review of the literature on this subject appears in Zollinger (27). The tautomeric character of o-hydroxyazo compounds was much harder to prove owing to the hydrogen bonding which occurs and it was only in 1952 that Burawoy (4, 5) succeeded in establishing the existence of the two forms

XIV   XV

XVI   XVII
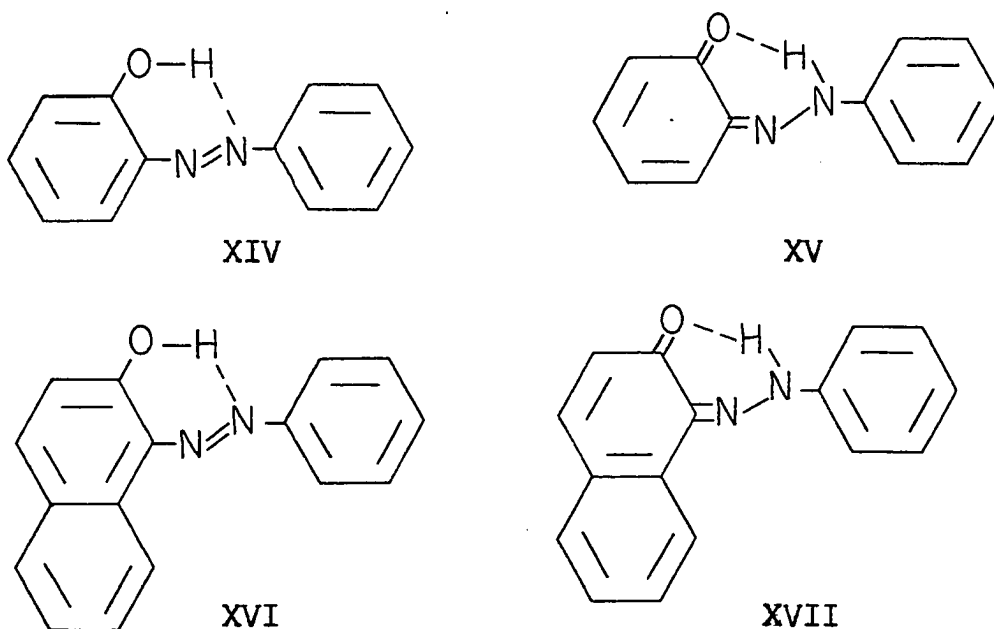
Apparently the 2-phenylazophenols are predominantly in the hydroxyazo form (XIV) and the 1-phenylazo-2-naphthols predominantly in the hydrazone form (XVII). The evidence was adduced from absorption spectra in different solvents and comparison with the O- and N- methyl ethers. This subject is certainly pertinent to o,o'-dihydroxyazobenzene but because of the complexity of the argument no attempt has been made in this thesis to extend the treatment to o,o'-dihydroxyazobenzene or to the new compounds described, and the hydroxyazo compounds are formulated throughout the thesis in the hydroxyazo structure.

Turning to 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab), the four replaceable hydrogen atoms come respectively from the carboxyl group, a phenolic group, the second phenolic group, and the ammonium ion. Of the two carboxyl groups present, one is already dissociated owing to the transfer of the hydrogen atom to the imino group (zwitter ion formation); the second, free carboxyl group is a stronger acid than expected owing to the positive charge on the neighboring ammonium ion. The same phenomenon occurs in iminodiacetic acid

$$H_2\overset{+}{N}\Big\langle\begin{array}{l}CH_2-CO_2^-\\CH_2-CO_2H\end{array}$$
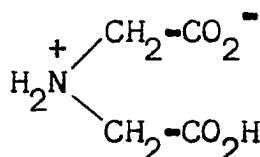
XVIII

pK$_1$ = 2.54   pK$_2$ = 9.12

(Carboxyl)   (Ammonium)

The second and third replaceable hydrogen atoms of H_4Idhab (pK_2 = 7.8 and pK_3 = 9.7) are derived from the phenolic groups, the neutralization being accompanied by a change in the ultraviolet absorption. The first of these is a considerably stronger acid than a normal phenol, presumably because of the positive charge on the ammonium group
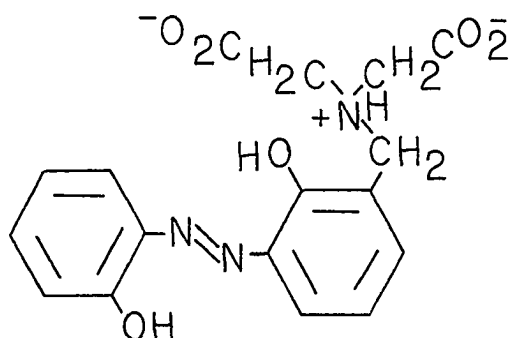
XIX

The argument is that adopted by Schwarzenbach, Anderegg and Sallmann (20, 21) for the analogous compound 2[N,N-bis(carboxymethyl)aminomethyl]phenol (abbreviated H_3Phim and shown in structure XX in the H_2Phim-form).

XX

pK_1 = 2.2 pK_2 = 8.17 pK_3 = 11.79

(Carboxyl)   (Phenol)   (Ammonium)
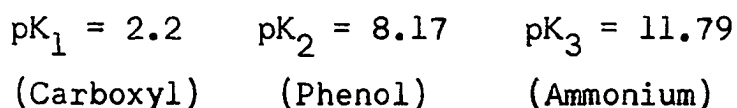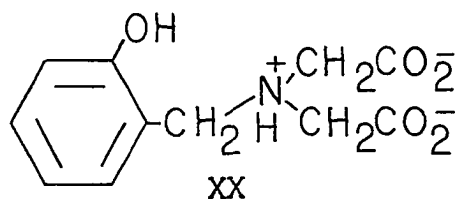
The acid dissociation constant of the second phenolic group of H_4Idhab is the same as that of phenol and it is uncertain

whether this hydrogen atom is hydrogen bonded or not.

The hydrogen atom on the ammonium group is titrated at still higher pH (corresponding to the conversion of ammonium chloride to ammonium hydroxide by sodium hydroxide). The acid dissociation constant (pK_4 = 11.2) is somewhat greater than the corresponding one for phenolmethyleneiminodiacetic acid (pK_3 = 11.79) but both are considerably weaker than that of iminodiacetic acid (pK_2 = 9.12). Schwarzenbach (20) explains this by assuming hydrogen bond formation of the ammonium hydrogen atom with the neighboring phenolate ion

XXI
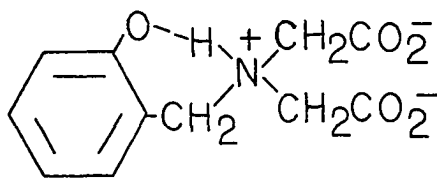
that is, the more tightly bound hydrogen ion would have less tendence to dissociate.

The reasoning is much the same for 1-[3'(N,N-bis(carboxymethyl)aminomethyl-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis (carboxymethyl)aminomethyl)-2-hydroxybenzene (H_6Iidhab), the situation being more complicated because of the second methyleneiminodiacetic acid group. A zwitter ion is formed with each methyleneiminodiacetic acid group and the free carboxyl group is present on each, pK_1 = 4.2, pK_2 = 5.8. The two phenolic groups have almost exactly the same acidity (pK_3 = 7.7, pK_4 = 9.6) as those of H_4Idhab. The acid

dissociation constants of the two ammonium ions differ by one order of magnitude (pK_5 = 10.6 and pK_6 = 11.5) and straddle that of H_4Idhab (pK_4 = 11.2). Analogous compounds bearing two methyleneiminodiacetic acid groups are: ethylenediaminetetraacetic acid (structure V, page 4) for which the negative logarithims of the acid dissociation constants are

Ethylenediaminetetraacetic Acid

Carboxyl	Ammonium
pK_1 = 1.99	pK_3 = 6.16
pK_2 = 2.67	pK_4 = 10.26

and phenol-2,6-bi(methyleneiminodiacetic acid) (20)
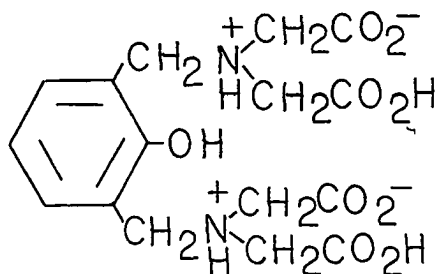
XXII

Carboxyl	Phenol	Ammonium
pK_1 = 2.0	pK_3 = 6.65	pK_4 = 9.74
pK_2 = 2.9		pK_5 = 11.4

The behavior of the three compounds as acids runs parallel with the increasing complexity showing up as expected.

Thus, the characteristics of H_4Idhab and H_6Iidhab as acids appear understandable in terms of the nature and number

of the dissociable groups present. Quantitatively, the most distinctive difference between H_4Idhab and $H_6Iidhab$ and the simple, analogous compounds bearing the same number of methyleneiminodiacetic acid groups appears in the appreciably smaller dissociation (larger pK) of the free, titratable carboxyl groups. Thus, for the free carboxyl groups

Iminodiacetic acid (structure XVIII)	$pK_1 = 2.54$
Methyliminodiacetic acid	$pK_1 = 2.12$
Phenol-2-methyleneiminodiacetic acid (structure XX)	$pK_1 = 2.2$
H_4Idhab (structure VI)	$pK_1 = 3.8$

and

Ethylenediaminetetraacetic acid (structure V)	$pK_1 = 1.99$
	$pK_2 = 2.67$
Phenol-2,6-bis(methyleneiminodiacetic acid) (structure XXII)	$pK_1 = 2.0$
	$pK_2 = 2.9$
$H_6Iidhab$ (structure VII)	$pK_1 = 4.2$
	$pK_2 = 5.8$

It is evident from the overall nature of the acid dissociation constants that the methyleneiminodiacetic acid groups of H_4Idhab and $H_6Iidhab$ are present as zwitter ions and that the positive charge on the ammonium group, which accounts for the strongly acid character of the free carboxyl group

in the analogous compounds, has been reduced. Inasmuch as
the acid group of phenol-2-methyleneiminodiacetic acid (XX)
is not similarly weakened, the azo group must be involved
someway. One explanation is that the oxygen atom of the
phenol group is bonded both to the azo group and to the
ammonium nitrogen atom thus drawing off part of the positive
charge of the ammonium ion.
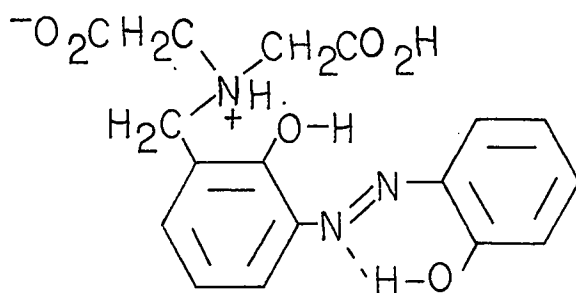
XXIII

The effect is even greater on the second free carboxyl group
of H_6Iidhab (pK_1 = 4.2, pK_2 = 5.8) for the second free
carboxyl group is weaker than a normal carboxyl group (acetic
acid, pk = 4.7), as if the entire charge on the ammonium ion
were withdrawn.
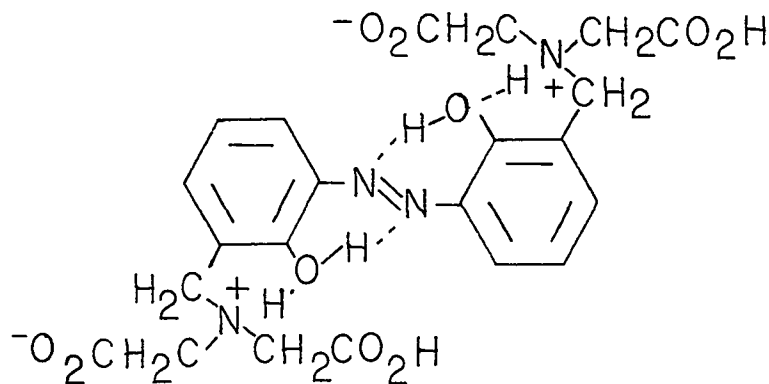
XXIV

As written, structure XXIV is highly symmetrical and no difference is shown in the hydrogen bonding. Such difference may exist and account for the difference in pK_1 and pK_2 or the explanation may be the more simple, dynamic one that as neutralization of the first carboxyl progresses, the concommitant increase in the negative charge changes the charge distribution throughout the molecule in such a way as to make the other free carboxyl groups a weaker acid.

<u>Determination</u> <u>of</u> <u>the</u> <u>formation</u> <u>constants</u> <u>of</u> <u>the</u> <u>calcium</u> <u>and</u> <u>magnesium</u> <u>compounds</u> <u>of</u> <u>1-[3'(N,N-bis(carboxymethyl)amino-</u> <u>methyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene</u>

The formation constants for calcium and magnesium uniting with H_4Idhab to form MIdhab^{-2} and M_2Idhab were determined by the method devised by G. Schwarzenbach, G. Anderegg, and R. Sallman (20). Use of this method requires two sets of data for each metal. One set of data is obtained from the potentiometric titration of H_4Idhab in the presence of excess metal ion with sodium hydroxide. Such titration curves for calcium and for magnesium are shown in Figures 13 and 15. The second set of data is the potentiometric titration of H_4Idhab in the presence of an equimolar concentration of metal ion with sodium hydroxide. These titration curves are shown in Figures 14 and 16.

The argument is as follows: The graph of the titration curve with excess metal ion has three distinct breaks. The

first break is assigned to the first acid dissociation con-
stant of H_4Idhab and does not seem to be affected by compound
formation. This is also shown by the observation that the
absorbance or fluorescence, as shown in Figure 22, does not
increase until a pH of 5 is reached. The other two breaks
in the titration curves result from compound formation. The
second and third buffer regions are quite flat, and apparent
acid dissociation constants K'_{H_4Idhab} and K''_{H_4Idhab} were
calculated for each of these regions. The second buffer
region extends over the range of neutralization of one re-
placeable hydrogen atom, thus K'_{H_4Idhab} can be determined
from equations 26 and 27.

$$H_3A \longrightarrow H_2A + H^+ \tag{26}$$

$$K'_{H_4Idhab} = \frac{(H^+)(H_2A^{-2})}{(H_3A^-)} \tag{27}$$

The results of these determinations of K'_{H_4Idhab} are shown in
Table 5.

The third buffer region extends over thr range of neu-
tralization of two replaceable hydrogen atoms and the over-
all apparent acid dissociation constant K''_{H_4Idhab} was cal-
culated using equation 28 and 29.

$$H_2A^{-2} = 2H^+ + A^{-4} \tag{28}$$

$$K''_{H_4Idhab} = \frac{(H^+)^2(A^{-4})}{(H_2A^{-2})} \tag{29}$$

The results of these determinations of K''_{H_4Idhab} are present are shown in Table 6.

Table 6. The apparent dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) in the presence of excess calcium and magnesium.

Metal	a	(H^+)	K'_{H_4Idhab}	K''_{H_4Idhab}
Ca	1.5	9.35×10^{-7}	9.35×10^{-7}	
Mg	1.5	4.68×10^{-6}	4.68×10^{-6}	
Ca	3.5	1.0×10^{-9}		3.0×10^{-18}
Mg	3.5	3.16×10^{-9}		3.0×10^{-17}

The reactions which take place on neutralization in the presence of excess metal ion are

$$H_4Idhab = H_3Idhab^- = H_2Idhab^{-2} = HIdhab^{-3} = Idhab^{-4}$$
$$+ \qquad + \qquad MIdhab$$
$$MH_2Idhab \quad MHIdhab^- \quad M_2Idhab \qquad (30)$$
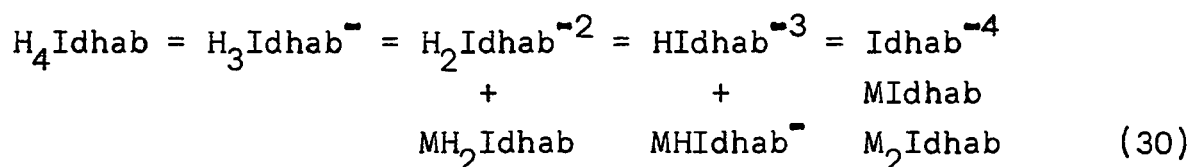
Therefore, K'_{H_4Idhab} can be expressed in terms of the species present in solution in the pH range indicated

$$K'_{H_4Idhab} = \frac{(H^+)[(MH_2Idhab) + (H_2Idhab^{-2})]}{(H_3Idhab^-)} \qquad (31)$$

Defining

$$K^M_{MH_2Idhab} = \frac{(MH_2Idhab)}{(M^{+2})(H_3Idhab^-)} \qquad (32)$$

and

$$K_{a_2} = \frac{(H^+)(H_2Idhab^{-2})}{(H_3Idhab^-)} \tag{33}$$

equation 31 can be expressed as

$$K'_{H_4Idhab} = K_{a_2}(1 + (M^{+2})K^M_{MH_2Idhab}) \tag{34}$$

Similarly K''_{H_4Idhab} can be expressed as

$$K''_{H_4Idhab} = \frac{(H^+)^2[(Idhab^{-4}) + (MIdhab^{-2}) + M_2Idhab]}{(MH_2Idhab) + (H_2Idhab^{-2})} \tag{35}$$

Substituting

$$K^M_{MIdhab^{-2}} = \frac{(MIdhab^{-2})}{(M^{+2})(Idhab^{-4})} \tag{36}$$

$$K^M_{M_2Idhab} = \frac{(M_2Idhab)}{(M^{+2})(MIdhab^{-2})} \tag{37}$$

and

$$K_{a_3}K_{a_4} = \frac{(H^+)^2(Idhab^{-4})}{(H_2Idhab^{-2})} \tag{38}$$

into equation 35 gives

$$K''_{H_4Idhab} = \frac{Ka_3Ka_4\,[1 + (M^{+2})K^M_{MIdhab^{-2}} + (M^{+2})^2(K^M_{MIdhab^{-2}})(K^M_{M_2Idhab})]}{1 + (M^{+2})K^M_{MH_2Idhab}} \tag{39}$$

The value of $K^M_{MH_2Idhab}$ can be calculated using equation 34, the values of K'_{H_4Idhab} given in Table 6 and the acid

dissociation constants Ka_2 given in Table 5. The values are shown in Table 7. Equation 39 contains two unknown quantities K^M_{MIdhab}-2 and $K^M_{M_2Idhab}$, therefore a value for K^M_{MIdhab} must be obtained by an independent method.

Table 7. The formation constants of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) and calcium and magnesium as calculated using K'_{H_4Idhab}.

	Calcium	Magnesium
$K^M_{MH_2Idhab}$	$1.1 \times 10^{+3}$	$5.6 \times 10^{+3}$

To obtain a value for $K^M_{MIidhab}$-2, the data from the titration of an equimolar mixture of H_4Idhab and metal ion with sodium hydroxide must be used. These titration curves are shown in Figures 14 and 16, magnesium or calcium, respectively, being the metal ion. These two curves are markedly different. The shape of the titration curve when an equimolar amount of calcium is present is quite similar to the shape of the curve when an excess of calcium is present. The titration curves with magnesium present are not so similar. For this reason, the dissociation constants for the two metals will be discussed separately.

In the case of the titration curve of equimolar calcium and H_4Idhab, (Figure 16), the species present at a = 2 are

only CaH_2Idhab and H_2Idhab^{-2}. Comparing the titration curve of H_4Idhab (Figure 2) and the curve of Figure 16, it is seen that the pH in the titration of Figure 16 is considerably lower at every point between a = 2 and a = 4 than the pH in the titration of Figure 2. Thus, it can be assumed that the amount of uncombined H_2Idhab^{-2} is negligible and that the observed pH is due almost entirely to the dissociation of CaH_2Idhab.

The two replaceable hydrogen atoms in CaH_2Idhab are lost almost simultaneously so that the two acid dissociation constants must be calculated using an equation similar to equation 8. The values calculated for $K^H_{CaH_2Idhab}$ and $K^H_{CaHIdhab^-}$ are shown in Table 8.

Table 8. The acid dissociation constants of the calcium and magnesium compounds of 1-[3'(N,N-bis(carboxymethyl) aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxy-benzene (MH_2Idhab).

Metal	a	pH	g	X	Y	$K^H_{MH_2Idhab}$	$K^H_{MHIdhab^-}$
Ca	2.5	9.18	2.5	7.6×10^{-9}	1.3×10^{-19}	7.6×10^{-9}	1.69×10^{-11}
	3.5	9.71	3.5				
Mg	2.75	7.82	2.75			4.5×10^{-8}	
Mg	3.5	9.41	3.5				3.92×10^{-10}

As shown by the titration of equimolar magnesium and H_4Idhab (Figure 14), the acid MgH_2Idhab is a much stronger acid than MgHIdhab⁻ or CaH_2Idhab. Assuming that essentially all of the H_4Idhab is present as MgH_2Idhab at a = 2, the acid dissociation constant $K^H_{MgH_2Idhab}$ can be determined from Figure 14 at a = 2.5 and $K^H_{MgHIdhab}$ can be determined at a = 3.5. This is possible because MgH_2Idhab and MgHIdhab⁻ are stronger acids than H_2Idhab⁻² and HIdhab⁻³ and the formation constant is sufficiently high that all of the H_4Idhab can be assumed to be present in the combined form. The resultant acid dissociation constants for the magnesium compounds are shown in Table 8.

Using the acid dissociation constants of H_4Idhab (Table 5), the acid dissociation constants for MH_2Idhab, and MHIdhab⁻, and the data from the titration curves shown in Figures 14 and 16, the values of $K^M_{MH_2Idhab}$ and K^M_{MIdhab}⁻² were calculated. The argument for calculating $K^M_{MH_2Idhab}$ is as follows: The total concentration

$$C = (H_4Idhab) + (H_3Idhab^-) + (H_2Idhab^{-2}) + (HIdhab^{-3})$$
$$+ (Idhab^{-4}) + (MH_2Idhab) + (MHIdhab^-) + (MIdhab^{-2})$$

$$(40)$$

The terms containing only hydrogen can be written in terms of H_2Idhab⁻² and the terms containing metal can be written in terms of MH_2Idhab. Introducing the values of (α) and (γ) defined by

$$\alpha = \frac{(H^+)^2}{Ka_1 Ka_2} + \frac{(H^+)}{Ka_2} + 1 + \frac{Ka_3}{(H^+)} + \frac{Ka_3 Ka_4}{(H^+)^2} \tag{41}$$

$$\gamma = 1 + \frac{K^H_{MH_2 Idhab}}{(H^+)} + \frac{K^H_{MH_2 Idhab} K^H_{MHIdhab}}{(H^+)^2} \tag{42}$$

into equation 40 gives

$$C = \alpha(H_2 Idhab^{-2}) + \gamma(MH_2 Idhab) \tag{43}$$

The amount of sodium hydroxide consumed at any point in the titration can be calculated from the expression

$$a \cdot c = (H_3 Idhab^-) + 2(H_2 Idhab^{-2}) + 3(HIdhab^{-3}) + 4(Idhab^{-4})$$
$$+ 2(MH_2 Idhab) + 3(MHIdhab^-) + 4(MIdhab^{-2}) + (OH^-)$$
$$- (H^+) \tag{44}$$

The terms again can be separated into ones containing metal and ones containing no metal. The values are expressed in terms of $H_2 Idhab^{-2}$ and $MH_2 Idhab$. Introducing the values of (β) and (δ) defined by

$$\beta = \frac{(H^+)}{Ka_2} + 2 + \frac{3Ka_3}{(H^+)} + \frac{4Ka_3 Ka_4}{(H^+)^2} \tag{45}$$

$$\delta = 2 + \frac{3(K^H_{MH_2 Idhab})}{(H^+)} + \frac{4(K^H_{MH_2 Idhab})(K^H_{MHIdhab}^-)}{(H^+)^2} \tag{46}$$

into equation 44 gives

$$a \cdot c = (H_2 Idhab^{-2}) + (MH_2 Idhab) + (OH^-) \_ (H^+) \tag{47}$$

The total metal concentration (c) can be expressed as

$$c = (M^{+2}) + (MH_2Idhab) + (MHIdhab^-) + (MIdhab^{-2}) \qquad (48)$$

Introducing the value of (γ) defined above gives

$$c = (M^{+2}) + (MH_2Idhab) \qquad (49)$$

Equation 43, 47 and 49 define three equations with three unknowns. Each unknown can be expressed in terms of the constants. Thus,

$$(H_2Idhab^{-2}) = \frac{c - \gamma \left[\dfrac{\alpha a c - \beta c + \alpha(H^+) - \alpha(OH^-)}{\alpha\delta - \beta\gamma}\right]}{\alpha} \qquad (50)$$

$$(M\,H_2Idhab) = \frac{\alpha a c - \beta c + \alpha(H^+) - \alpha(OH^-)}{\alpha\delta - \beta\gamma} \qquad (51)$$

$$(M^{+2}) = c - \gamma \left[\frac{\alpha a c - \beta c + \alpha(H^+) - \alpha(OH^-)}{\alpha\delta - \beta\gamma}\right] \qquad (52)$$

Substituting the values of (M^{+2}), (MH_2Idhab), and (H_2Idhab^{-2}) obtained using equations 50, 51 and 52 into equation 32 the values of $K^M_{MH_2Idhab}$ shown in Table 9 were calculated. These values were compared with those given in Table 6.

Table 9. Formation constants of the calcium and magnesium compounds of 1-[3'(N,N-bis(carboxymethyl)amino-methyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab).

Metal	$K^M_{MH_2Idhab}$	$K^M_{MIdhab^{-2}}$	$K^M_{M_2Idhab}$
Ca	$3.25 \times 10^{+3}$	$8.9 \times 10^{+3}$	$1.8 \times 10^{+3}$
Mg	$4.5 \times 10^{+3}$	$2.2 \times 10^{+5}$	$9.6 \times 10^{+2}$

The value of $K^M_{MIdhab}{}^{-2}$ can be obtained in a manner similar to the way in which $K^M_{MH_2Idhab}$ was obtained. Equation 40 can be written in terms of $Idhab^{-4}$ and $MgIdhab^{-2}$. Introducing new values of (α') and (γ') so that

$$\alpha' = 1 + \frac{(H^+)}{Ka_4} + \frac{(H^+)^2}{Ka_4Ka_3} + \frac{(H^+)^3}{Ka_4Ka_3Ka_2} + \frac{(H^+)^4}{Ka_4Ka_3Ka_2Ka_1} \tag{53}$$

$$\gamma' = 1 + \frac{(H^+)}{(K^H_{MHIdhab}{}^-)} + \frac{(H^+)^2}{(K^H_{MHIdhab}{}^-)(K^H_{MH_2Idhab})} \tag{54}$$

then

$$c = \alpha'(Idhab^{-4}) + \gamma'(MIdhab^{-2}) \tag{55}$$

Equation 44 can also be expressed in terms of $Idhab^{-4}$ and $MIdhab^{-2}$. Introducing new values of (β') and (δ') so that

$$\beta' = 4 + \frac{3(H^+)}{Ka_4} + \frac{2(H^+)^2}{Ka_4Ka_3} + \frac{(H^+)^3}{Ka_4Ka_3Ka_2} \tag{56}$$

$$\delta' = 4 + \frac{3(H^+)}{(K^H_{MHIdhab}{}^-)} + \frac{2(H^+)^2}{(K^H_{MHIdhab}{}^-)(K^H_{MH_2Idhab})} \tag{57}$$

then

$$a \cdot c = \beta'(Idhab^{-4}) + \delta'(MIdhab^{-2}) + (OH^-) - (H^+) \tag{58}$$

Equation 48 can also be expressed in terms of $MIdhab^{-2}$ so that

$$c = (M^{+2}) + \gamma'(MIdhab^{-2}) \tag{59}$$

Equations 55, 58 and 59 have the same form as equations

43, 47 and 49, but with new unknowns and new constants.

Thus $Idhab^{-4}$ was substituted for H_2Idhab^{-2}, $MIdhab^{-2}$ for

MH_2Idhab, α' for α, and β' for β, γ' for γ and δ' for δ in

equations 50, 51, and 52. The resultant values were sub-

stituted into equation 36 to give values for $K^M_{MIdhab^{-2}}$.

These values are shown in Table 9.

Using the values of $K^M_{MH_2Idhab}$, K^M_{MIdhab} and K''_{H_4Idhab}

equation 39 was used to calculate values of $K^M_{M_2Idhab}$. These

results are shown in Table 9.

Determination of the formation constants of the calcium and magnesium compounds of 1-[3'(N,N-bis(carboxymethyl)amino-methyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl) aminomethyl)-2-hydroxybenzene

The first and second formation constants for one and

two metal ions uniting with $H_6Iidhab$ to form $MIidhab^{-4}$ and

$M_2Iidhab^{-2}$ were determined by the method devised by G.

Schwarzenbach, G. Anderegg, and R. Sallmam[20]. This

method requires two sets of data for each metal. The first

is the titration of the chelating agent in the presence of

a large excess of metal ion with sodium hydroxide. These

titrations are shown in Figures 17 and 19. The second set

of data is the titration of the chelating agent in the

presence of an equimolar concentration of metal ion with

sodium hydroxide. These titrations are shown in Figures

5 and 18.

The argument is as follows: The plot of the titration curve with excess metal ion shows two distinct regions of compound formation. It appears that a compound $MH_3Iidhab^-$ and a compound $M_2Iidhab^{-2}$ are formed. The graph of change of absorbance versus pH (Figure 24) shows that no compound formation occurs before pH 6.0, therefore, the first two acid dissociation constants can be disregarded in calculating the formation constant for $MH_3Iidhab^-$. The first break in the titration curves shown in Figures 17 and 19 is fairly large so that the last apparent acid dissociation constant can be found from the graphs. The apparent acid dissociation constant $K'_{H_6Iidhab}$ in each case was determined at a = 2.5. At that point the apparent acid dissociation constant equals the hydrogen ion concentration. This cannot be done with the desired accuracy on the small graph presented here but was done on a larger graph. The value of the apparent acid dissociation constants when calcium or magnesium is present are shown in Table 10.

The second part of the titration curves in Figures 17 and 19 appear as though a tribasic acid were being titrated. By choosing values in the region from a = 3.5 to a = 5.5 and finding the corresponding hydrogen ion concentration, the values of the second apparent acid dissociation constant can be calculated using equations 38 and 39.

Table 10. The apparent acid dissociation constants of 1-[3'(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxybenzene (H_6Iidhab) in the presence of excess calcium and magnesium.

Metal	a	(H^+)	$K'_{H_6Iidhab}$	$K''_{H_6Iidhab}$
Ca	2.50	3.16×10^{-7}	3.16×10^{-7}	
Mg	2.50	7.76×10^{-7}	7.76×10^{-7}	
Ca	5.5	2.76×10^{-7}		1.1×10^{-26}
Ca	5.0	3.98×10^{-9}		1.3×10^{-26}
				ave 1.2×10^{-26}
Mg	5.0	7.58×10^{-9}		8.7×10^{-25}
Mg	5.25	6.17×10^{-9}		7.0×10^{-25}
				ave 7.9×10^{-25}

$$H_3A^{-3} = 3H^+ + A^{-6} \qquad\qquad (60)$$

$$K''_{H_6Iidhab} = \frac{(H^+)^3 (A^{-6})}{(H_3A^{-3})} \qquad\qquad (61)$$

The values calculated for the second apparent acid disociation constant when calcium or magnesium is present are shown in Table 10.

In reality, metal ions are present during the titration and the following reactions take place on neutralization with

alkali.

$$H_6 Iidhab = H_5 Iidhab^- = H_4 Iidhab^{-2} = \frac{H_3 Iidhab^{-3}}{+} = \frac{H_2 Iidhab^{-4}}{+}$$
$$MH_3 Iidhab^- \qquad MH_2 Iidhab^{-2}$$

$$= \frac{HIidhab^{-5}}{+} = \frac{Iidhab^{-6}}{+}$$
$$MHIidhab^{-3} \qquad MIidhab^{-4}$$
$$+$$
$$M_2 Iidhab^{-2}$$

$$(62)$$

Therefore, $K'_{H_6 Iidhab}$ can be expressed in terms of the species present in solution.

$$K'_{H_6 Iidhab} = \frac{(H^+) \, [(MH_3 Iidhab^-) + (H_3 Iidhab^{-3})]}{(H_4 Iidhab^{-2})} \qquad (63)$$

Remembering that

$$K^M_{MH_3 Iidhab^-} = \frac{(MH_3 Iidhab^-)}{(M^{+2})(H_3 Iidhab^{-3})} \qquad (64)$$

and

$$K_{a3} = \frac{(H^+)(H_3 Iidhab^{-3})}{(H_4 Iidhab^{-2})} \qquad (65)$$

then equation 63 can be expressed as

$$K'_{H_6 Iidhab} = K_{a3}(1 + (M^{+2})K^M_{MH_3 Iidhab^-}) \qquad (66)$$

Similarly $K''_{H_6 Iidhab}$ can be expressed as

$$K''_{H_6Iidhab} = \frac{(H^+)^3[(MIidhab^{-4}) + (M_2Iidhab^{-2}) + (Iidhab^{-6})]}{(MH_3Iidhab^-) + (H_3Iidhab^{-3})} \qquad (67)$$

Substituting

$$K^M_{MIidhab^{-4}} = \frac{(MIidhab^{-4})}{(M^{+2})(Iidhab^{-6})} \qquad (68)$$

$$K^M_{M_2Iidhab^{-2}} = \frac{(M_2Iidhab^{-2})}{(M^{+2})(MIidhab^{-4})} \qquad (69)$$

and

$$K_{a4}K_{a5}K_{a6} = \frac{(H^+)^3(Iidhab^{-6})}{(H_3Iidhab^{-3})} \qquad (70)$$

into equation 70 gives

$$K''_{H_6Iidhab} = \frac{K_{a4}K_{a5}K_{a6}[1 + (M^{+2})K^M_{MIidhab^{-4}} + (M^{+2})^2}{}$$

$$\frac{(K^M_{MIidhab^{-4}})K^M_{M_2Iidhab^{-2}}]}{} \Big/ 1 + (M^{+2})$$

$$K^M_{MH_3Iidhab^-} \qquad (71)$$

The values of $K^M_{MH_3Iidhab^-}$ can be calculated using

equation 66 and the values of K' from Table 10 and the acid

dissociation constant K_{a4} given in Table 5. These values

are shown in Table 11. Equation 71 contains two unknown

quantities $K^M_{MIidhab^{-4}}$ and $K^M_{M_2Iidhab^{-2}}$, therefore, a value

for $K^M_{MIidhab^{-4}}$ must be obtained by an independent method.

To obtain a value for $K^M_{MIidhab^{-4}}$, the data from the

Table 11. The formation constant of 1-[3'(N,N-bis(carboxy-methyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxy-benzene (H_6Iidhab) with calcium and magnesium calculated using K'.

	Calcium	-	Magnesium
$K^M_{MH_3Iidhab^-}$	2.7×10		$6.3 \times 10^{+2}$

titration of an equimolar mixture of H_6Iidhab and metal ion with sodium hydroxide must be used. In this titration, there is no formation of a bimetallic compound and the values of the acid dissociation constants $K^H_{MH_3Iidhab^{-3}}$, $K^H_{MH_2Iidhab^{-4}}$, and $K^H_{MHIidhab^{-5}}$ can be found from the graphs shown in Figure 5 and 18. The necessity of evaluating these acid dissociation constants will be seen later. The value of $K^H_{MH_3Iidhab^{-3}}$ where the metal is calcium can be taken directly from the graph shown in Figure 18 where a = 3.5. At this point the hydrogen ion concentration is equal to the acid dissociation constant.

The value of $K^H_{MH_3Iidhab^{-3}}$ when the metal is magnesium had to be obtained from the data contained in the graph shown in Figure 5. In this case the titration curve is essentially flat in the region from a = 1 to a = 3.75. It is the acid dissociation before the first break that must

be obtained. In this case the acid dissociation constant

was calculated at a = 3.75 assuming that because of the large

break in the curve the species $MH_2Iidhab^{-2}$ would be the only

species present other than the non-neutralized species

$MH_3Iidhab^-$. The values for the first acid dissociation

constants of $K^H_{MH_3Iidhab^-}$ are shown in Table 12 when calcium

or magnesium is the metal present.

Table 12. The first acid dissociation constant of the cal-
cium and magnesium compound of 1-[3'(N,N-bis
(carboxymethyl)aminomethyl)-2'-hydroxy-1'-
benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)
-2-hydroxybenzene $(MH_3Iidhab^-)$.

Metal	a	(H^+)	$K^H_{MH_3Iidhab^-}$
Ca	3.5	7.6×10^{-11}	7.6×10^{-11}
Mg	3.75	2.82×10^{-8}	8.5×10^{-8}

The values of $K^H_{MH_2Iidhab^{-4}}$ and $K^H_{MHIidhab^{-5}}$ were ob-

by solving simultaneous equations similar to the ones de-

scribed previously (equation 8). The values for $K^H_{MH_2Iidhab^{-4}}$,

and $K^H_{MHIidhab^{-5}}$ are shown in Table 13 for calcium or mag-

nesium present.

Using the acid dissociation constants of $H_6Iidhab$

(Table 5), the acid dissociation constants for $MH_3Iidhab^-$

(Tables 12 and 13), and the data from the titration curves

Table 13. Data used to calculate the last two acid dissociation constants of the calcium and magnesium compound of 1-[3'(N,N-bis(carboxymethyl) aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl) aminomethyl-2-hydroxybenzene ($MH_3Iidhab^-$).

Metal	a	pH	g	X	Y	$K^H_{MHIidhab^{-2}}$	$K^H_{MHIidhab^{-3}}$
Ca	4.5	11.59	0.4	1.4×10^{-12}	1.9×10^{-24}	1.4×10^{-2}	1.4×10^{-12}
	5.5	11.70	1.33				
Mg	4.5	9.85	1.49	1.2×10^{-10}	9.7×10^{-22}	1.2×10^{-10}	8.4×10^{-12}
	5.5	10.92	2.33				

(Figures 5 and 18) the values of $K^M_{MH_3Iidhab^-}$ and $K^M_{MIidhab^{-4}}$ were calculated. The argument for calculating $K^M_{MH_3Iidhab^-}$ is as follows: The total concentration of $H_6Iidhab$ in all forms (C) can be expressed as

$$C = (H_6Iidhab) + (H_5Iidhab^-) + H_4Iidhab^{-2}) + H_3Iidhab^{-3})$$

$$+ (H_2Iidhab^{-4}) + (HIidhab^{-5}) + (Iidhab^{-6}) + (MH_3Iidhab^-)$$

$$+ (MH_2Iidhab^{-2}) + (MHIidhab^{-3}) + (MIidhab^{-4}) \qquad (72)$$

The terms containing only hydrogen can be written in terms of $H_3Iidhab^{-3}$ and the terms containing metal can be written in terms of $MH_3Iidhab^-$. Introducing the values of (α_1) and (γ_1) defined by

$$\alpha_1 = \frac{(H^+)^3}{K_{a1}K_{a2}K_{a3}} + \frac{(H^+)^2}{K_{a2}K_{a3}} + \frac{(H^+)}{K_{a3}} + 1 + \frac{K_{a4}}{(H^+)} + \frac{K_{a4}K_{a5}}{(H^+)^2}$$

$$+ \frac{K_{a4}K_{a5}K_{a6}}{(H^+)^3} \qquad (73)$$

$$\gamma_1 = 1 + \frac{K^H_{MH_3Iidhab^{-3}}}{(H^+)} + \frac{K^H_{MH_3Iidhab^-}K^H_{MH_2Iidhab^{-2}}}{(H^+)^2}$$

$$+ \frac{K^H_{MH_3Iidhab}\ K^H_{MH_2Iidhab}\ K^H_{MHIidhab^{-3}}}{(H^+)^3} \qquad (74)$$

into equation 72 gives

$$C = \alpha_1(H_3Iidhab^{-3}) + \gamma_1(MH_3Iidhab^-) \qquad (75)$$

The amount of sodium hydroxide consumed at any point in the

titration can be calculated from the expression

$$a.c = (H_5 Iidhab^-) + 2(H_4 Iidhab^{-2}) + 3(H_3 Iidhab^{-3})$$

$$+ 4(H_2 Iidhab^{-4}) + 5(HIidhab^{-5}) + 6(Iidhab^{-6})$$

$$+ 3(MH_3 Iidhab^-) + 4(MH_2 Iidhab^{-2}) + 5(MHIidhab^{-3})$$

$$+ 6(MIidhab^{-4}) + (OH^-) - (H^+) \tag{76}$$

The terms again can be separated into ones that contain no metal and ones that do contain metal. The values are then expressed in terms of $H_3 Iidhab^{-3}$ and $MH_3 Iidhab^-$. Introducing the values of (β_1) and (δ_1) defined by

$$\beta_1 = \frac{(H^+)^2}{K_{a2}K_{a3}} + \frac{2(H^+)}{K_{a3}} + 3 + \frac{4K_{a4}}{(H^+)} + \frac{5K_{a4}K_{a5}}{(H^+)^2} + \frac{6K_{a4}K_{a5}K_{a6}}{(H^+)^3} \tag{77}$$

$$\delta_1 = 3 + \frac{4(K^H_{MH_3 Iidhab^-})}{(H^+)} + \frac{5(K^H_{MH_3 Iidhab^-})(K^H_{MH_2 Iidhab^{-2}})}{(H^+)^2}$$

$$+ \frac{6(K^H_{MH_3 Iidhab^-})(K^H_{MH_2 Iidhab^{-2}})(K^H_{MHIidhab^{-3}})}{(H^+)^3} \tag{78}$$

into equation 76 gives

$$a.c = \beta_1(H_3 Iidhab^{-3}) + \delta_1(MH_3 Iidhab^-) + (OH^-) - (H^+) \tag{79}$$

The total metal concentration (c) can be expressed as

$$c = (M^{+2}) + (MH_3 Iidhab^-) + (MH_2 Iidhab^{-2}) + (MHIidhab^{-3})$$

$$+ (MIidhab^{-4}) \tag{80}$$

Introducing the value of (γ_1) defined above gives

$$c = (M^{+2}) + \gamma_1(MH_3Iidhab^-) \qquad (81)$$

Equations 75, 79, and 81 define three equations with three unknowns. These three equations are of the same form as equations 43, 47, and 49, but with different constants and different unknowns. Thus, the unknowns $H_3Iidhab^{-3}$, $MH_3Iidhab^-$ and M^{+2} can be substituted for H_2Idhab^{-2}, MH_2Idhab and M^{+2}; by also substituting the constants α_1 for α, β_1 for β, γ_1 for γ, and δ_1 for δ in equations 50, 51, and 52.

Introducing the values of (M^{+2}), $(MH_3Iidhab^-)$ and $(H_3Iidhab^{-3})$ calculated using equation 50, 51, and 52 into equation 64, the values of $K^M_{MH_3Iidhab^-}$ shown in Table 14 were calculated. These values were compared with the values shown in Table 11.

Table 14. Formation constants for the calcium and magnesium compounds of 1-[3'(N,N-bis(carboxymethyl)amino-methyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis (carboxymethyl)aminomethyl)-2-hydroxybenzene ($H_6Iidhab$).

	$K^M_{MH_3Iidhab^-}$	$K^M_{MIidhab^{-4}}$	$K^M_{M_2Iidhab^{-2}}$
Ca	$2.61 \times 10^{+1}$	$2.88 \times 10^{+3}$	$1.83 \times 10^{+2}$
Mg	$1.13 \times 10^{+3}$	$4.06 \times 10^{+6}$	$1.99 \times 10^{+5}$

The value of $K^M_{MgIidhab}-4$ can be obtained in a similar manner. Equation 72 can be written in terms of $Iidhab^{-6}$ and $MgIidhab^{-4}$. Introducing new values of (α'_1) and (γ'_1)

$$\alpha'_1 = 1 + \frac{(H^+)}{K_{a6}} + \frac{(H^+)^2}{K_{a6}K_{a5}} + \frac{(H^+)^3}{K_{a6}K_{a5}K_{a4}} + \frac{(H^+)^4}{K_{a6}K_{a5}K_{a4}K_{a3}}$$

$$+ \frac{(H^+)^5}{K_{a6}K_{a5}K_{a4}K_{a3}K_{a2}} + \frac{(H^+)^6}{K_{a6}K_{a5}K_{a4}K_{a3}K_{a2}K_{a1}} \tag{82}$$

$$\gamma'_1 = 1 + \frac{(H^+)}{K^H_{MHIidhab}-3} + \frac{(H^+)^2}{K^H_{MHIidhab}-3K^H_{MH_2Iidhab}-2}$$

$$+ \frac{(H^+)^3}{K^H_{MHIidhab}-3K^H_{MH_2Iidhab}-2K^H_{MH_3Iidhab}-} \tag{83}$$

into equation 72 gives

$$c = \alpha'_1(Iidhab^{-6}) + \gamma'_1(MIidhab^{-4}) \tag{84}$$

Equation 76 can be expressed in terms of $Iidhab^{-6}$ and $MIidhab^{-4}$. Introducing new values of (β'_1) and (δ'_1)

$$\beta'_1 = \frac{(H^+)^5}{K_{a2}K_{a3}K_{a4}K_{a5}K_{a6}} + \frac{2(H^+)^4}{K_{a3}K_{a4}K_{a5}K_{a6}} + \frac{3(H^+)^3}{K_{a4}K_{a5}K_{a6}} + \frac{4(H^+)^2}{K_{a5}K_{a6}}$$

$$+ \frac{5(H^+)}{K_{a6}} + 6 \tag{85}$$

$$\delta'_1 = 6 + \frac{5(H^+)}{K^H_{MHIidhab}-3} + \frac{4(H^+)^2}{K^H_{MHIidhab}-3K^H_{MH_2Iidhab}-2}$$

$$+ \frac{3(H^+)^3}{K^H_{MHIidhab}-3K^H_{MH_2Iidhab}-2K^H_{MH_3Iidhab}-} \tag{86}$$

and inserting these values into equation 76 gives

$$a.c = \beta_1^{'}(\text{Iidhab}^{-6}) + \delta_1^{'}(\text{MIidhab}^{-4}) + (\text{OH}^{-}) - (\text{H}^{+}) \quad (87)$$

Equation 80 can also be expressed in terms of MIidhab^{-4} so that

$$c = (\text{M}^{+2}) + \gamma_1^{'}(\text{MIidhab}^{-4}) \quad (88)$$

Equation 84, 87, and 88 have the same form as equations 43, 47, and 49, but with new unknowns and new constants. Thus, Iidhab^{-6} can be substituted for $\text{H}_2\text{Idhab}^{-2}$, MIidhab^{-4} for MH_2Idhab, $\alpha_1^{'}$ for α, $\beta_1^{'}$ for β, $\gamma_1^{'}$ for γ, and $\delta_1^{'}$ for δ. The resultant values were inserted into equation 68 to give a value for $K^{M}_{\text{MIidhab}^{-4}}$. These values are shown in Table 14.

Using the values of $K^{M}_{\text{MH}_3\text{Iidhab}^{-}}$, $K^{M}_{\text{MIidhab}^{-4}}$, and $K^{"}_{\text{H}_6\text{Iidhab}}$, equation 71 was used to calculate values of $K^{M}_{\text{M}_2\text{Iidhab}^{-2}}$. The results are shown in Table 14.

A summary of the formation constants with magnesium and calcium of H_2Dhab, H_4Idhab, and H_6Iidhab is given in Table 15.

For comparison the formation constants of calcium and magnesium toward various analogous compounds have been assembled in Table 16.

It is obvious at once that the specificity of o,o'-dihydroxyazobenzene for magnesium has been destroyed by the introduction of the methyleneiminodiacetic acid groups into the molecule although preference for magnesium has been retained. Thus, H_4Idhab, the compound bearing one

Table 15.   Summary of the formation constants of the calcium and magnesium
            derivatives of o,o'-dihydroxyazobenzene (H_2Dhab), 1-[3'(N,N-bis
            (carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxy-
            benzene (H_4Idhab) and 1-[3'(N,N-bis(carboxymethyl)aminomethyl)
            -2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl
            -2-hydroxybenzene ($H_6Iidhab$).

Metal	$logK^M_{MDhab}$	$logK^M_{MIdhab}-2$	$logK^M_{M_2Idhabj}$	$logK^M_{MIidhab}-4$	$logK^M_{M_2Iidhab}-2$
Ca	----[a]	3.95	3.26	3.46	2.26
Mg	4.85[b]	5.33	2.98	6.61	5.30

[a]Reported by J. Ellingboe (12) that no compound formed.

[b]Reported by J. Ellingboe (12).

Table 16. Formation constants of the calcium and magnesium derivatives of various related compounds.

	log K	
	Calcium	Magnesium
Iminodiacetic acid[a] (structure XVII, page 102) (literature reference 24)	2.59	2.94
Methyliminodiacetic acid (22)	2.75	3.44

Phenol-2-methyleneiminodiacetic acid (H_3Phim; structure XX, page 103) (20)

	$\log K^M_{MHPhim}$	$\log K^M_{MPhim}$
Ca	3.00	6.74
Mg	----	7.28

Ethylenediaminetetraacetic acid (structure V, page 4) (18, 23)

	$\log K_{MHL}$	$\log K_{ML}$
Ca	3.51	10.70
Mg	2.28	8.69

Phenol-5-methyl-2,6-bis(methyleneiminodiacetic acid) (H_5Phdiim; structure XXII, page 105) (20)

	$\log K^M_{MH_2Phdiim}$	$\log K^M_{MHPhdiim}$	$\log K^M_{MPhdiim}$	$\log K^M_{M_2Phdiim}$
Ca	2.8	6.67	8.4	4.0
Mg	----	6.8	8.0	5.4

[a]Values given here, superseding those of reference (16), are those of Schwarzenbach and Senn; see Stability Constants. The London Chemical Society. London. 1957, Part I, page 23.

methyleneiminodiacetic acid group unites with both calcium
and magnesium, and indeed forms two compounds with each metal,
in the ratios of one to one (metal to H_4Idhab) and to two to
one. The formation constant of the one to one magnesium
compound is greater than that for calcium (log K = 5.33 and
3.95, respectively, (Table 15)). For each metal, the dif-
ference between the first and second formation constants is
not great indicating that the second metal atom enters the
molecule with about the same ease, this difference being
much less for calcium than magnesium.

It is of interest to speculate as to where the first
metal atom enters the molecule. It is evident from the se-
quence of titration curves: H_4Idhab alone with sodium
hydroxide (Figure 2), H_4Idhab plus an equimolar amount of
magnesium with sodium hydroxide (Figure 14), and H_4Idhab
plus a ten-fold excess of magnesium with sodium hydroxide
(Figure 13), that the first and second phenol groups and to
a lesser extent the ammonium ion are implicated when the
magnesium is present in an equimolar amount (compare Figures
2 and 14), and that the ammonium ion is completely involved
when an excess of magnesium is present (note the extensive
change at a = 4 on passing from Figure 14 to Figure 13). It
thus appears that the first atom of magnesium enters the
o,o'-dihydroxyazo group and the second the iminodiacetic
acid group, the picture being somewhat confused by overlap

of the two reactions, the formation constants differing only by a factor of 220 or so. The formation constants of o,o'-dihydroxyazo compounds toward magnesium are always greater, by 10^2 to 10^5, than those toward calcium (Diehl and Ellingboe (9)) and the respective constants of the one to one compounds of H_4Idhab are in line with this.

In the compound bearing two methyleneiminodiacetic acid groups H_6Iidhab, the preference for magnesium over calcium is even more pronounced and extends to the two to one compounds. A steric effect reflecting the larger atomic radius of the calcium ion is probably entering here. The azo molecule undoubtedly lies in the transconfiguration, but even so with the constituents lying 1,2,3, on each ring, space is at a premium.

The titration curves show no evidence of a three to one compound being formed between the metal and H_6Iidhab. The formation constants for the two to one compound are smaller than for the one to one compound so that if the three to one compound formed, its formation constant would be smaller still. The size of the compound with two metal atoms present would also discourage the formation of the three to one compound. For these reasons the three to one compound was disregarded in this discussion.

Fluorescence study of the magnesium compound of 1-[3'(N,N-bis
(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-
hydroxybenzene (H$_4$Idhab)

The fluorescent properties of H$_4$Idhab were examined and
it was found that over the pH range from 6 to 13 H$_4$Idhab
alone did not fluoresce nor did the calcium derivative. The
only compound found to fluoresce was the magnesium derivative.

The fluorescence spectra of the magnesium compound of
H$_4$Idhab, shown in Figure 21, indicates that the wavelength of
maximum excitation occurs at 480 mμ. and the wavelength of
maximum emission occurs at 565 mμ.

The change in fluorescence of the magnesium compound of
H$_4$Idhab with pH follows closely the change in absorption with
pH as shown in Figure 22. The initial increase is caused by
compound formation and the very rapid decrease at pH above
11.5 is caused by the formation of non-dissociated magnesium
hydroxide.

Fluorescence study of the magnesium compound of 1-[3'(N,N-bis
(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-
bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (H$_6$Iidhab)

The examination of the fluorescent properties of the
calcium, magnesium, barium and strontium compounds of H$_6$Iidhab
indicated that the free acid, the calcium, barium and strontium
compounds did not fluoresce in the pH range 6 to 13. The

magnesium compound was the only compound tested that fluoresced.

The fluorescence spectra of the magnesium compound of H_6Iidhab shown in Figure 23, indicate the wavelength maximum for excitation at 480 mμ. and the wavelength maximum for emission at 579 mμ. Thus, the emission curve shows a shift to longer wavelength with increased substitution.

The change in fluorescence of the magnesium compound of H_6Iidhab with pH follows the change in absorption with pH closely as shown in Figure 24. The fluorescence reaches a broad plateau from pH 9.3 to 11.0 where the fluorescence is essentially constant. This mades possible the quantitive determination of magnesium using solutions buffered in this region.

A summary of the fluorescence data for H_2Dhab, H_4Idhab and H_6Iidhab with magnesium present is shown in Table 17. This shows that with increasing substitution on the o,o'- dihydroxyazobenzene moiety the emission wavelength maximum shifts to longer wavelength and the intensity of the fluorescence becomes much less.

Table 17. Summary of fluorescence data of o,o'-dihydroxyazo-
benzene (H$_2$Dhab), 1-[3'(N,N-bis(carboxymethyl)
aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxy-
benzene (H$_4$Idhab) and 1-[3'(N,N-bis(carboxymethyl)
aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-
bis(carboxymethyl)aminomethyl)-2-hydroxybenzene
(H$_6$Iidhab) with magnesium present.

Compound	Excitation	Emission	Relative Fluorescence
H$_2$Dhab	480 mμ.	557 mμ.	0.220
H$_4$Idhab	480 mμ.	565 mμ.	0.095
H$_6$Iidhab	480 mμ.	579 mμ.	0.035

SUMMARY

Two new o,o'-dihydroxyazo compounds have been synthesized bearing one and two methyleneiminodiacetic acid groups, respectively. The methyleneiminodiacetic acid groups were introduced into the molecule of o,o'-dihydroxyazobenzene by the Mannich condensation with formaldehyde and iminodiacetic acid. The crude product which formed was separated by chromatography on Sephadex G-10 into four fractions, two of which yielded pure products, a yellow monomethylene-iminodiacetic acid compound and a red di(methyleneiminodiacetic acid) compound.

On the basis of elemental analysis, equivalent weight determination, nuclear magnetic resonance data, and mass spectrographic data structures have been assigned to the compounds, to the yellow compound (for convenience designated H_4Idhab, H_2Dhab being the parent o,o'-dihydroxyazobenzene) the structure 1-[3'(N,N-bis(carobxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (structural formula VI, page 33), and to the red compound designated H_6Iidhab) the structure 1-[3'N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-(N,N-bis(carboxymethyl)aminomethyl)-2-hydroxybenzene (structural formula VII, page 37).

The acid dissociation constants of both the yellow and the red compounds have been determined and the various acid functions assigned to specific groups in the molecule.

H_4Idhab proved to have four replaceable hydrogen atoms,
H_6Iidhab six. The characteristics of the compounds as acids
indicated that the iminodiacetic acid groups are present as
zwitter ions. The negative logarithms of the dissociation
constants and the respective groups are

H_2Dhab	H_4Idhab	H_6Iidhab
pK_1 = 8.9 P1	pK_1 = 3.8 C	pK_1 = 4.2 C1
pK_2 = 11.1 P2	pK_2 = 7.8 P1	pK_2 = 5.8 C2
	pK_3 = 9.7 P2	pK_3 = 7.7 P1
	pK_4 = 11.2 A	pK_4 = 9.6 P2
		pK_5 = 10.6 A
		pK_6 = 11.5 A2

P representing a phenolic group, C a carboxyl group, and A
an ammonium ion. The values of these various acid dissociation
constants are of the same magnitude as those of similar groups
in analogous, compounds bearing methyleneiminodiacetic acid
groups, except that the carboxyl groups are considerably
weaker.

The formation constants of the calcium and magnesium
derivatives of both new compounds have been determined. Cal-
cium and magnesium form both one to one and two to one com-
pounds with H_4Idhab, the formation constants of the species
formed being

$$\log K^{Ca}_{CaH_2Idhab} = 3.5 \quad \log K^{Ca}_{CaIdhab^{-2}} = 4.0 \quad \log K^{Ca}_{Ca_2Idhab} = 3.2$$

$$\log K^{Mg}_{MgH_2Idhab} = 3.7 \quad \log K^{Mg}_{MgIdhab}{}^{-2} = 5.3 \quad \log K^{Mg}_{Mg_2Idhab} = 3.0$$

Calcium and magnesium form both one to one and two to one compounds with $H_6Iidhab$, the formation constants of the ions formed being

$$\log K^{Ca}_{CaH_3Iidhab}{}^- = 1.4 \quad \log K^{Ca}_{CaIidhab}{}^{-4} = 3.5 \quad \log K^{Ca}_{Ca_2Iidhab}{}^{-2} = 2.3$$

$$\log K^{Mg}_{MgH_3Iidhab}{}^- = 3.1 \quad \log K^{Mg}_{MgIidhab}{}^{-4} = 6.6 \quad \log K^{Mg}_{Mg_2Iidhab}{}^{-2} = 5.3$$

The specificity of o,o'-dihydroxyazobenzene for magnesium is lost in these substituted compounds although a preference for magnesium is retained. It is likely that the first magnesium atom to enter the molecule of both H_4Idhab and $H_6Iidhab$ does so by attachment to the o,o'-dihydroxyazo group. That the formation constants of both calcium derivatives is smaller than for the corresponding magnesium derivatives of $H_6Iidhab$ is probably a steric effect reflecting the larger atomic radius of calcium and space restrictions resulting from the proximity of the seven functional groups.

A fluorescence study of both H_4Idhab and $H_6Iidhab$ showed that only magnesium forms fluorescent compounds with the new compounds. The fluorescence spectra of the magnesium compounds compared with that of the magnesium compound of H_2Dhab showed that the wavelength of maximum fluorescence increases with increasing substitution and that the intensity of the fluorescence decreases with increasing substitution.

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ACKNOWLEDGMENTS