1981

A study of complexometric reagents for serum total calcium determinations.

John Gordon. Parks
University of Windsor

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A STUDY OF COMPLEXOMETRIC REAGENTS FOR SERUM TOTAL CALCIUM DETERMINATIONS

BY

JOHN GORDON PARKS

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

Windsor, Ontario, Canada

1981

ABSTRACT

A STUDY OF COMPLEXOMETRIC REAGENTS FOR SERUM TOTAL CALCIUM DETERMINATIONS

by

JOHN GORDON PARKS

o-Cresolphthalein, fluorescein, phenolphthalein, thymolphthalein, and phenolsulfonephthalein complexone were examined for their suitability in determining serum total calcium spectrophotometrically. o-Cresolphthalein complexone was the only complexone that did not show a wavelength maxima shift. The effects of temperature, pH, and expected interferences were studied with the commonly employed o-cresolphthalein complexone reagents. The stability of the reagents, the stability of the metal-complex and relative sensitivities for the various buffer and solvent systems were compared. Reagents with 2-amino-2-methyl-1-propanol demonstrated remarkable stability.

Fluorescein complexone was also examined and optimized in the fluorescent mode. Protein interference was a problem.

A spectrophotometric method using o-cresolphthalein complexone is recommended.

ACKNOWLEDGEMENTS

Without the generous help and guidance of my co-advisors, Dr. R. J. Thibert and Dr. Bennie Zak, this work would not have been started, let alone accomplished. The many people who are responsible for the facilities made available to me at The University of Windsor and the Schiffman Library of Wayne State University deserve special mention. In addition, and of the greatest significance was the help and friendliness of all those people around me: Dr. K. E. Taylor, Dr. N. F. Taylor, Dr. P. B. Taylor, Dr. G. C. Moses, all my fellow chemistry graduate students; Dr. T. A. Hyde, Miss E. Chape and all the laboratory organization at Hotel Dieu of St. Joseph Hospital; M. R. Goodwin and E. Olivero of the Salvation Army Grace Hospital; the AACC Michigan section; and our chemistry department secretaries. All of the above have made me feel welcome.

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LIST OF ABBREVIATIONS

λ	wavelength
A	absorbance
AMP	2-amino-2-methyl-1-propanol
BMDP	Biomedical Computer Programs
CAPS	3-(cyclohexylamino) propanesulfonic acid
CPC	o-cresolphthalein complexone
CaICPC	one calcium ion chelated to one complexone
CaIICPC	two calcium ions chelated to one complexone
DEA	diethylamine
DMSO	dimethylsulfoxide
EG	ethylene glycol
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
n	number of samples
NCCLS	National Committee for Clinical Laboratory Standards
nm	nanometers
S.D.	standard deviation

CHAPTER I

INTRODUCTION

A. PATHOLOGICAL SIGNIFICANCE

Unlike most substances in serum, total calcium in a healthy adult is regulated over the very narrow reference range from 2.25 mmol L^{-1} to 2.65 mmol L^{-1} (9.0 mg/dL to 10.6 mg/dL)*; and therefore, total calcium requires a more precise method of analysis than is usual in the clinical laboratory. A value over 2.75 mmol L^{-1} might indicate primary hyperparathyroidism. Hypercalcemic coma is associated with levels over 3.375 mmol L^{-1}. Hypocalcemic tetany is observed with values under 1.75 mmol L^{-1} (1). Barnett suggested that the medical coefficient of variation for calcium is 2.27% indicating that if a patient's serum total calcium result increased as little as 0.06 mmol L^{-1}, the physician may start corrective treatment. At the present level of the art, a good total calcium procedure has a coefficient of variation of about 2.8% which presents the real possibility that a physician may treat the patient

*1 mg/dL Ca = 0.25 mmol L^{-1} Ca

for a variation due to the methodology used to determine the total calcium level (2).

Serum calcium is widely determined as total calcium by means of an automated o-cresolphthalein complexone spectrophotometric procedure although numerous attempts have been made to find a superior method. Several literature reviews of complexometric methods for calcium have been published (3-6).

The original o-cresolphthalein complexone reagent developed for the continuous-flow AutoAnalyzer® system (7) has been modified by the addition of 8-hydroxyquinoline to mask magnesium (8, 9) and cyanide to complex copper, iron, and zinc as well as to stabilize the base (9). Problems arising from the dialysis procedure (10, 11) have led to a widespread acceptance of direct total calcium methods, thus eliminating the need for the dialysis step (12-14), and effecting a procedure more suitable to automated, discrete and centrifugal methodologies (15).

The acceptance of the automated methodologies has occurred even in the face of the National Committee for Clinical Laboratory Standards (NCCLS) preferring atomic absorption as the reference method for total calcium in serum (16).

Since only ionized calcium, representing about 45% of the total calcium, is biologically active (17), many attempts have been made to measure this fraction directly rather than calculate it from the serum total calcium and protein. Most promising is the recent introduction of the ion-selective electrode for calcium (3-7, 18-23) although it is not without problems (24-27). Dialyzable calcium using ultrafiltrates of serum as an estimate of ionized calcium is of interest but has not met with widespread acceptance (17, 28, 29).

B. THE STUDY

Since the direct method proposed by Baginski et al* (13) and the modifications proposed by Clark et al* (14) are the most widely used methods which determine serum total calcium in the laboratory today, my attention was directed towards the optimization of their parameters.

Of academic interest is the suggestion as described in Figure 1 that the o-cresolphthalein complexone which absorbs at 575 nm chelates two calcium ions to produce CaIICPC which also happens to absorb at 575 nm. Color is removed as well as

*For clarity these reagents are referred to as the Baginski and Clark reagents in this thesis.

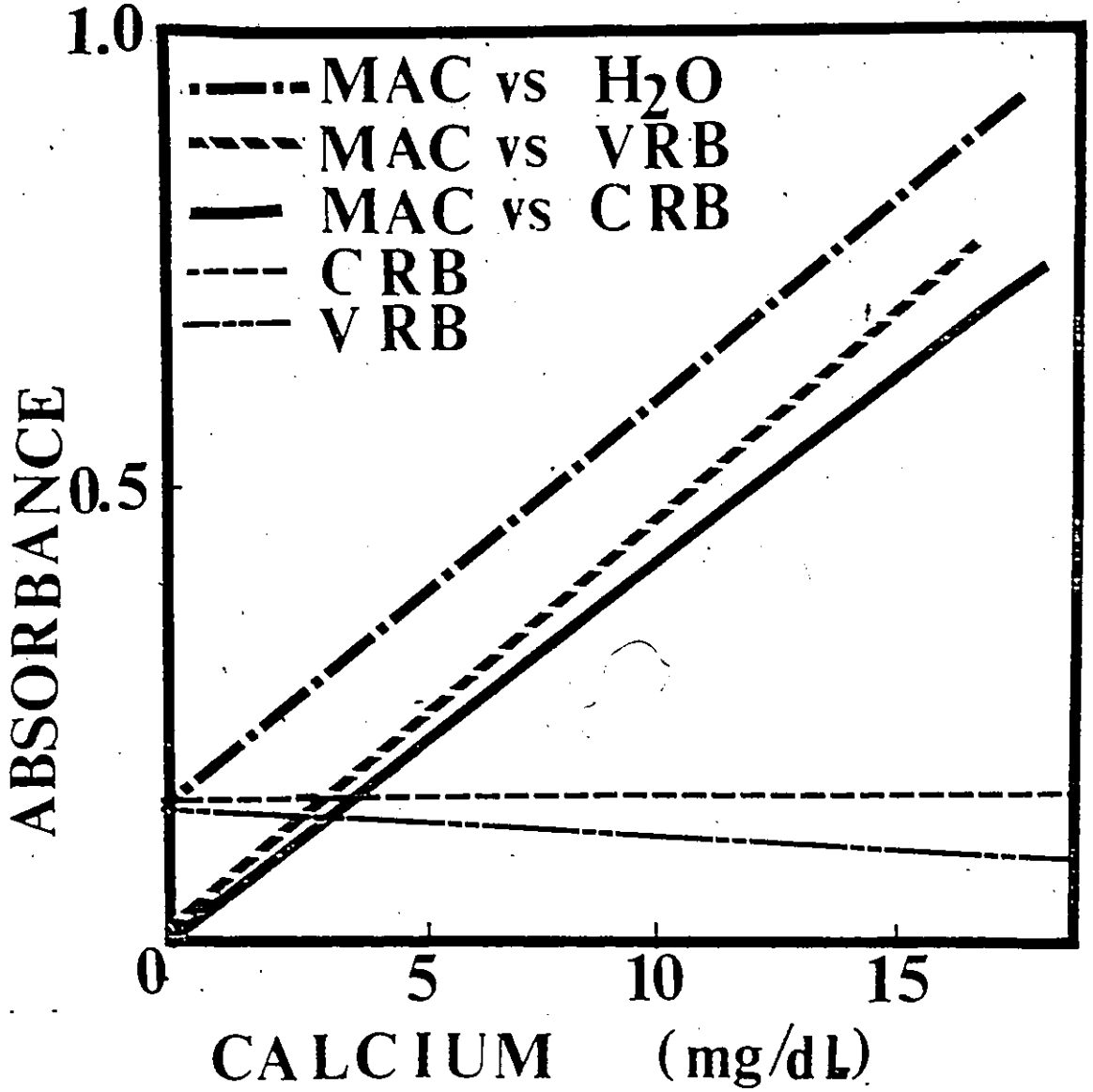
FIGURE 1

CLARK'S BLANKING HYPOTHESIS

Legend

The measured absorbance curve (MAC) against a water (H_2O) blank and the MAC against the continuous o-cresolphthalein complexone reagent blank (CRB) are parallel as expected. But, if for every CaIICPC absorbing at 575 nm one CPC weakly absorbing at 575 nm is removed, the true blank would be a decreasing variable reagent blank (VRB) and the true absorbance line from CaIICPC would be represented by the MAC vs. VRB line (14).

FIGURE 1


1.0
0.5
0
ABSORBANCE
MAC vs H_2O
MAC vs VRB
MAC vs CRB
CRB
VRB
5
10
15
CALCIUM (mg/dL)

added at 575 nm. The normal blanking procedure is therefore not adequate to reflect the total color produced, but a decreasing variable blank is necessary which would produce a true absorbance line representing the CaIICPC versus calcium concentration. Because the error is stoichiometric and both CPC and CaIICPC absorb at 575 nm, linearity is maintained. Since the procedure depends on comparing unknown samples to reference samples analyzed in the same manner, this blanking problem is only of academic interest and would never lead to erroneous results.

For this reason, different complexones were examined to see if they have any advantage over o-cresolphthalein complexone. The primary study compared the most commonly employed spectrophotometric reagents with respect to reagent stability, the effect of temperature, and interferences. The final portion of the investigation dealt with an optimization of a fluorescent procedure in an attempt to find a more sensitive method for serum total calcium.

CHAPTER II

SPECTROPHOTOMETRIC DETERMINATIONS

A. INTRODUCTION

Several compounds similar to o-cresolphthalein complexone (CPC) as shown in Figure 2 were examined in order to further enlighten the blanking hypothesis and determine if they would be more suitable for the determination of calcium. Schwarzenbach (30) predicted bathochromic shifts in the absorbing wavelength maxima with the addition of alkyl groups to the rings; therefore, it was anticipated that these complexones would absorb at a different wavelength after chelating calcium ions than they would when they were free of calcium.

The four complexones examined form zwitterions in solution. For each iminodiacetate group, one of the carboxyl group hydrogens is more closely associated with the nearby nitrogen. As the pH increases the other carboxyl group hydrogen and the 4,4'-hydroxyl groups dissociate. With the phenolphthalein compounds the lactone ring formed by the ionic oxygen of the COO^- attaching to the central

FIGURE 2

COMPLEXONES STUDIED

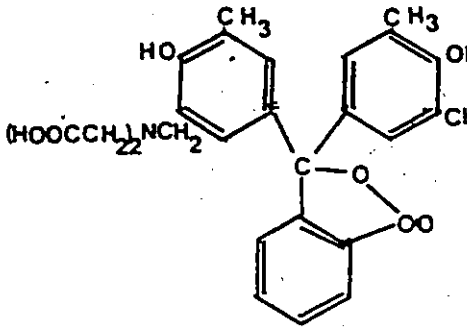
Legend

The four complexones which were studied are shown as the sodium salts. They are all similar to o-cresolphthalein complexone.

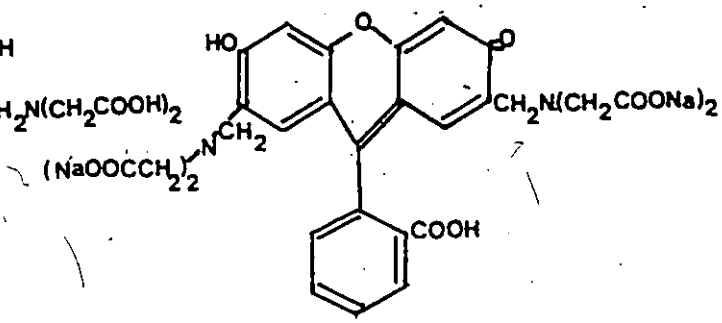
FIGURE 2

COMPLEXONES STUDIED

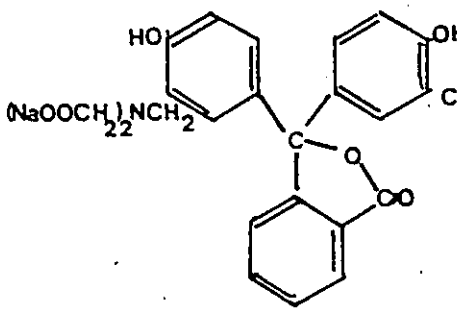
CRESOLPHTHALEIN



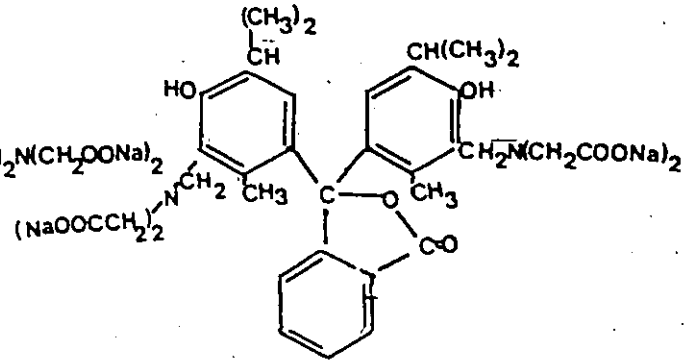
FLUORESCEIN



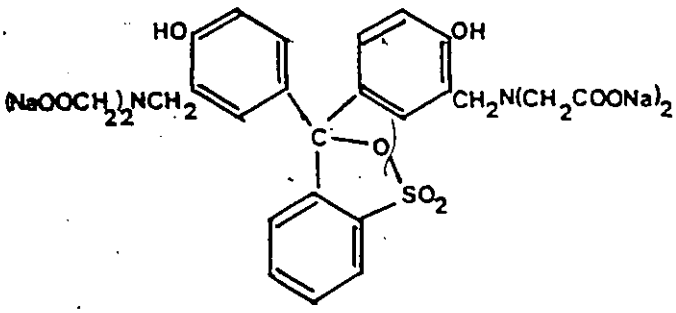
PHENOLPHTHALEIN



THYMOLPHTHALEIN



PHENOLSULFONEPHTHALEIN



carbon breaks when the auxochromic oxygens are deprotonated in the basic solution. The compound then absorbs light. Due to the 2-2'-ether bridge of the fluorescein complexone, and the sulfonate ring of the phenolsulfonephthalein complexone, similar effects are exhibited by these compounds. For all the complexones, the greatest molar absorptivity occurs at the pH when the chelation of a calcium ion displaces the hydrogen bridging the auxochromic oxygen and the nitrogen of the iminodiacetate group.

It is also to be noted that the ionic form of the complex reacts with the ionic form of calcium which is in equilibrium with the non-ionic calcium in solution. The stability constant is large enough to remove calcium from protein in serum. The color formed by the metal-complex is indicative of the total amount of calcium present.

B. EXPERIMENTAL

1. Materials

Glassware: Pyrex beakers and borosilicate test tubes were used after being washed by a Meile dishwasher model G19 with an automatic acid rinse. An

investigation showed little difference in absorbance using new Pyrex, polystyrene, or borosilicate tubes. Hand-washed and acid-rinsed tubes demonstrated less precision in these absorbance studies when compared to dishwasher washed and rinsed borosilicate and Pyrex tubes. The NCCLS method (16) discussed an elaborate procedure for minimizing the ubiquitous calcium contamination. Many shorter procedures are available (8, 31, 32).

Water: Deionized distilled water (0.2 MΩ) using a Barnstead column from Barnstead, Boston, Mass., 02132, was used throughout the studies. A sample of deionized distilled water (7 MΩ) from Hotel Dieu of St. Joseph Hospital's laboratory was tested but proved to have a comparable amount of calcium contamination as the water prepared as above.

Calcium standards: For the 1.25 mmol L^{-1} to the 3.75 mmol L^{-1} range, standards were purchased from Sigma Chemical Co., St. Louis, MO, 63178. For all other work, the standards were prepared from a stock calcium solution (1 g L^{-1}) obtained by dissolving 250 mg of primary standard grade anhydrous $CaCO_3$ (99.96% pure) obtained from Ventron Co., Alfa Products, Montreal, which had been oven-dried for ten hours, in a

100-mL volumetric flask with 0.1 mL of concentrated hydrochloric acid (obtained from Fisher Scientific Co., Toronto, Ont., M3A 1A9) and diluting it to volume (13, 14) and then varifying the prepared 1.25 mmol L^{-1} with the Sigma 1.25 mmol L^{-1} standard. The standards were sorted in polyethylene.

Interference standards: Bovine serum albumin from Sigma Chemical Co., MO, 63172, was run through a column containing "Amberlite" resin IR-120 (H), analytical grade, from BDH Chemicals, Toronto, Ont., M8Z 1K5, in order to first remove the calcium residue. Magnesium was obtained by dissolving 1.0 g of pure magnesium turnings from Fisher Scientific Co., Toronto, Ont., M3A 1A9 in 100 mL of water and 8 mL of hydrochloric acid and then diluting to 1 L (7). Also, salicylate from Fisher Scientific Co. which can complex with iron (30) and was observed to have a wavelength maximum at 520 nm and absorb to some extent at 575 nm was used to see if there were any other reactions that it may enter that are a problem. The new Kodak Ektachem 400 calcium determination has a problem with salicylate interference (33). All interference standards were prepared as described in Table I.

TABLE I

INTERFERENCE STANDARDS

Tube	Calcium mmol L^{-1}	Magnesium mmol L^{-1}	Bovine albumin g L^{-1}	Salicylate mg L^{-1}
1	2.5	1.125	20	0
2	2.5	1.125	60	0
3	2.5	1.125	42	0
4	2.5	2.5	42	0
5	1.25	1.125	42	0
6	3.75	1.125	42	0
7	2.5	1.125	42	500

Baginski Reagents: These were prepared as described in the paper by Baginski et al. (13). CPC (40 mg) was dissolved in 1.0 mL of HCl in a 50-mL beaker and then washed into a 1-L volumetric flask containing 1 dL of dimethylsulfoxide (DMSO) and 2.5 g of 8-hydroxyquinoline (Fisher Scientific Co., Toronto, Ont., M3A 1A9, ACS grade). Water was added to volume and the solution mixed. The diethylamine (DEA) buffer was prepared by adding 40.0 mL of DEA from J. T. Baker Chemical Co., Phillipsburg, N.J. 08865, which was distilled to remove the yellow color which developed with age, to a 1-L volumetric flask containing 0.5 g of potassium cyanide (from Fisher Scientific Co., ACS grade). The solution was diluted to volume and stored in polyethylene bottles.

Similar molar equivalent reagents to the Baginski-CPC reagents were prepared by adding 44 mg of thymolphthalein complexone, 39.2 mg of fluorescein complexone, 40.4 mg of phenolphthalein complexone, and 38.5 mg of phenolsulfonaphthalein complexone, all obtainable from Eastman Kodak, Rochester, N.Y., 14850. The DEA buffer was similarly made up to 4.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 80.0, 100.0, and 140.0 mL L^{-1} concentrations for a pH range.

Clark Reagents: These four reagents were made similar to those described in the paper by Clark et al. (14). To a 500-mL volumetric flask, 15 mg of CPC were dissolved in about 5 mL of water, 37.5 mL of either ethylene glycol (EG) from Fisher Scientific Co., Toronto, Ont., M3A 1A9 or DMSO were then added with 500 mg of 8-hydroxyquinoline and dissolved. Then 12.5 mL of 2-amino-2-methyl-1-propanol (AMP) from Sigma, St. Louis, Mo, 63172, or the molar equivalent of 10.25 mL of DEA was added and the solution was diluted to the mark with water and mixed.

A new organic buffer, 3-(cyclohexylamino)propanesulfonic acid (CAPS) from Hoechst Can. Ltd., Montreal, P.Q., H4R 1R6 was also tried with a reagent composed of 7.5 mL of DMSO, 3.18 mg CAPS, 3 mg CPC, and 100 mg of 8-hydroxyquinoline diluted to 100-mL and adjusted to pH 10.2 with sodium hydroxide (34).

EGTA solution: A 1% saturated solution was prepared from ethyleneglycol N,N^1-tetraacetic acid obtained from Sigma, St. Louis, MO, 63172.

pH buffer standards: 0.1 M NaOH with either 0.05 M sodium bicarbonate or 0.05 M disodium hydrogen phosphate both from Fisher Scientific Co. were used to prepare standards for the pH meter

immediately before use (35). Short-range Accutint pH test papers, from Canlab, Toronto, M8Z 2H4, were used to verify the accuracy of the standards and the stability of the sodium hydroxide solution.

2. Apparatus

Micropipettor: Oxford pipettor, available through Canadian Laboratory Supplies Ltd., Toronto, Ontario, M8Z 2H4.

Spectrophotometer: Beckman Acta MVI, Beckman Instruments Inc., Analytical Instrument Sales and Service Division, Toronto, Ontario, M8Z 5T1.

pH meter: A Corning Digital 111 pH meter distributed by Canadian Laboratory Supplies Ltd., Toronto, Ontario M8Z 2H4 and an Accumet model 120 from Fisher Scientific Co. were both used with an electrode obtained from Graphic Controls, Buffalo, N.Y., 14210.

Balance: A Sauter balance supplied by August Sauter of America, New York, N.Y. 085280, was used for weights below one gram and a Mettler PC 4400 Delta Range distributed by Fisher Scientific Co. was used for values above one gram.

Calculator: A Commodor 91-90R hand calculator distributed by Consumer's Distributing, Windsor,

Ontario, and the University of Windsor's WYLBUR terminal to access the central terminals' Biomedical Computer Programs (BMDP), were used.

3. General Methods

Although serum total calcium is determined almost solely on automated systems, a manual adaptation of the automated methodologies was used in this study.

Into a 13x100 mm test tube, 60 µL of calcium standard, water, or EGTA solution were pipetted. Either 3.0 mL of the Baginski-complexone reagent and then 3.0 mL of the Baginski-DEA buffer, or 6.0 mL of the Clark reagent were added with volumetric pipets. The test tube was covered with parafilm and the mixture was then mixed by inverting several times and read against a water, a normal blank, or a blank with EGTA at the appropriate wavelength maximum for each complexone.

C. RESULTS AND DISCUSSION

1. Preliminary Investigations

A check of the micropipetor's precision and accuracy produced a mean of 60.7 µL of water (S.D. =

0.09 µL, n = 30) with a single tip whereas a mean of 60.1 uL of water (S.D. = 0.20 µL, n = 30) was obtained with a new tip for each sample. This corresponds well with the manufacturer's suggested tolerance of 0.5 µL at 60 µL.

Variation of the slitwidth of the spectrophotometer with CaIICPC (13) indicated that a narrow slitwidth may produce slightly greater molar absorptivity but much less precision of absorbance readings at low concentrations. The accepted practice of using the programmed slit adjust after setting a bandwidth of 2.5 nm at 500 nm with the dynode was chosen.

2. Other Complexones

Baginski-type reagents of all four CPC-like complexones over a range of DEA buffer concentrations from 4 to 140 mL L^{-1} pointed to 40 mL L^{-1} DEA buffer as the most effective for calcium sensitivity.

It was noted that thymolphthalein complexone was highly hydroscopic and as such easily dissolved in water, but its large granular crystals made it difficult to weigh. The spectra of thymolphthalein complexone with various calcium concentrations are presented in Figure 3. Linearity appeared to be

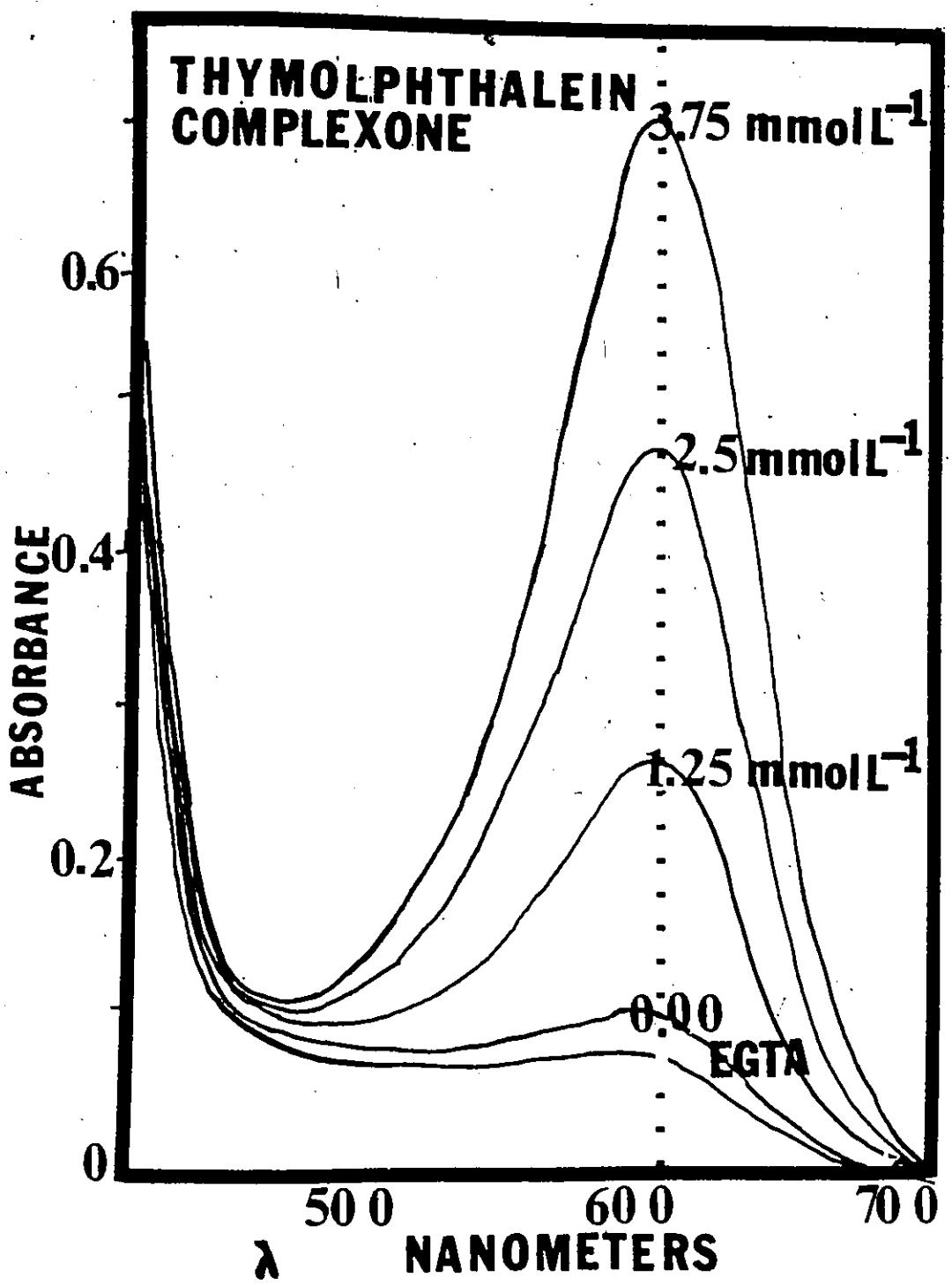
FIGURE 3

SPECTRA OF THE THYMOLPHTHALEIN COMPLEXONE REAGENT SHOWING THE EFFECTS OF CALCIUM

Legend

Thymolphthalein complexone reagent (see Materials) was reacted with 0.00, 1.25, 2.50, 3.75 mmol L^{-1} of calcium, respectively, and the spectra were read against water. The spectrum of EGTA, which complexes with calcium, plus the reagent is also presented to demonstrate the quantity of calcium contamination.

FIGURE 3


THYMOLPHTHALEIN COMPLEXONE
3.75 mmol L⁻¹
2.5 mmol L⁻¹
1.25 mmol L⁻¹
0.00
EGTA
ABSORBANCE
0.6
0.4
0.2
0
500
600
700
λ
NANOMETERS

excellent. A hypsochromic shift in the wavelength maximum (610 nm) of the spectra was noted with increased calcium concentrations. The molar absorptivity of the Ca-thymolphthalein was about one-half that of CaIICPC whose spectra for various calcium concentrations are presented in Figure 4. Note that the absorbance scale is different between Figures 3 and 4. Excellent linearity was noted with the CPC reagent. The increase in absorbance at 400 nm was due to the presence of 8-hydroxyquinoline in all the reagents. It was also noted that the absence of 8-hydroxyquinoline in the thymolphthalein-complexone reagent would increase the absorbance due to calcium by about 30% suggesting that the Ca-thymolphthalein stability constant is close to that of the Ca-8-hydroxyquinolate constant.

The spectra of phenolsulfonephthalein complexone with various calcium concentrations are shown in Figure 5. A problem occurred due to the formation of a fine precipitate with time even with as little as 60 μL of 3.75 mmol L^{-1} of calcium present. The extremely high blank proved unacceptable for the determination of calcium. A hypsochromic shift in the wavelength maxima (570 nm) was also noted.

FIGURE 4

SPECTRA OF o-CRESOLPHTHALEIN COMPLEXONE BAGINSKI REAGENT SHOWING ITS REACTION WITH CALCIUM

Legend

Shown are typical absorbance maxima at 575 nm for the physiological range of serum calcium. The CPC reagent was reacted with 0.00, 1.25, 2.50, and 3.75 mmol L^{-1} of calcium, respectively, and the spectra were read against water. The spectrum of the EGTA plus CPC was presented.

FIGURE 4

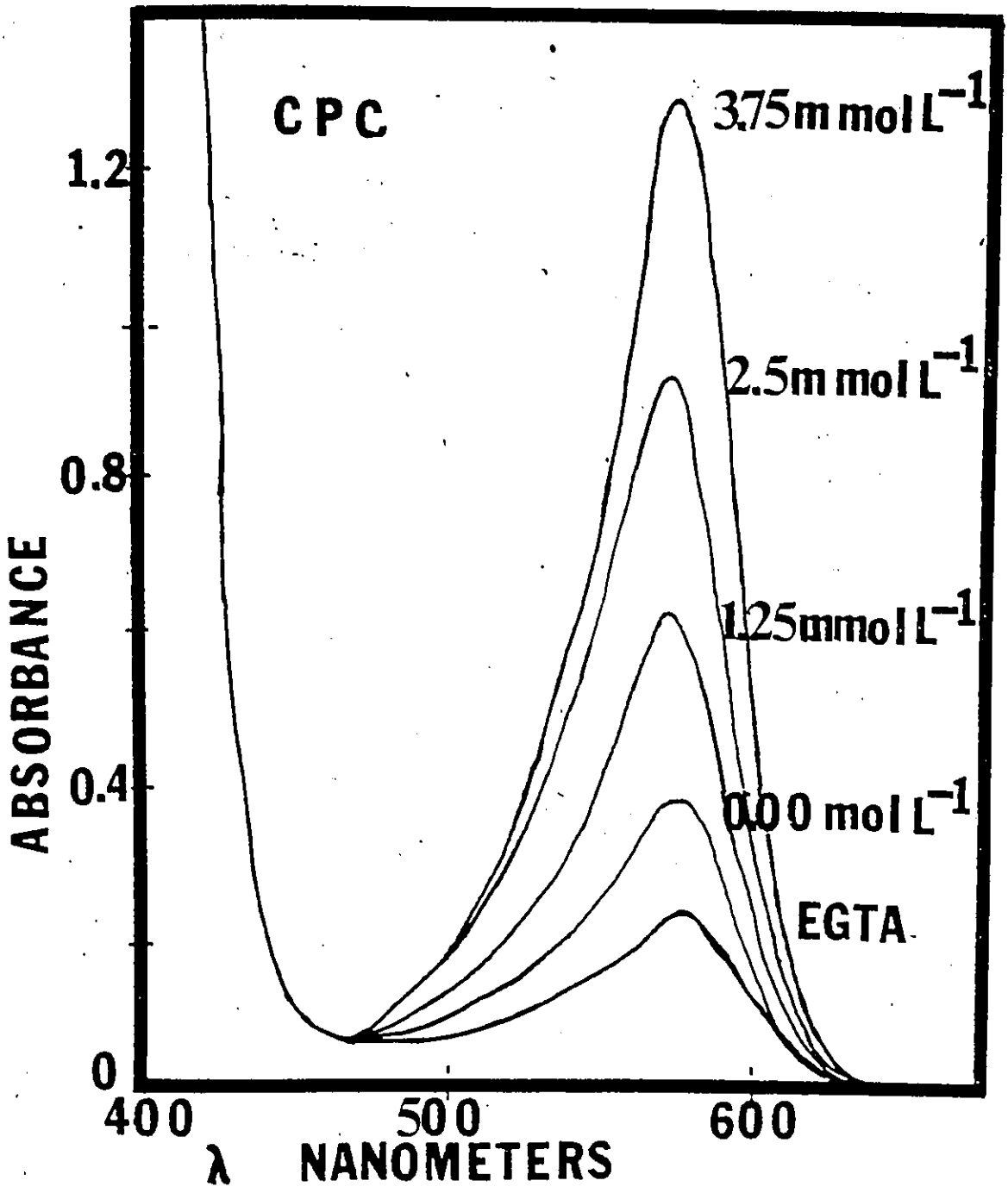

CPC
3.75m mol L⁻¹
2.5m mol L⁻¹
1.25mmol L⁻¹
0.00 mol L⁻¹
EGTA
ABSORBANCE
1.2
0.8
0.4
0
400
500
600
λ NANOMETERS

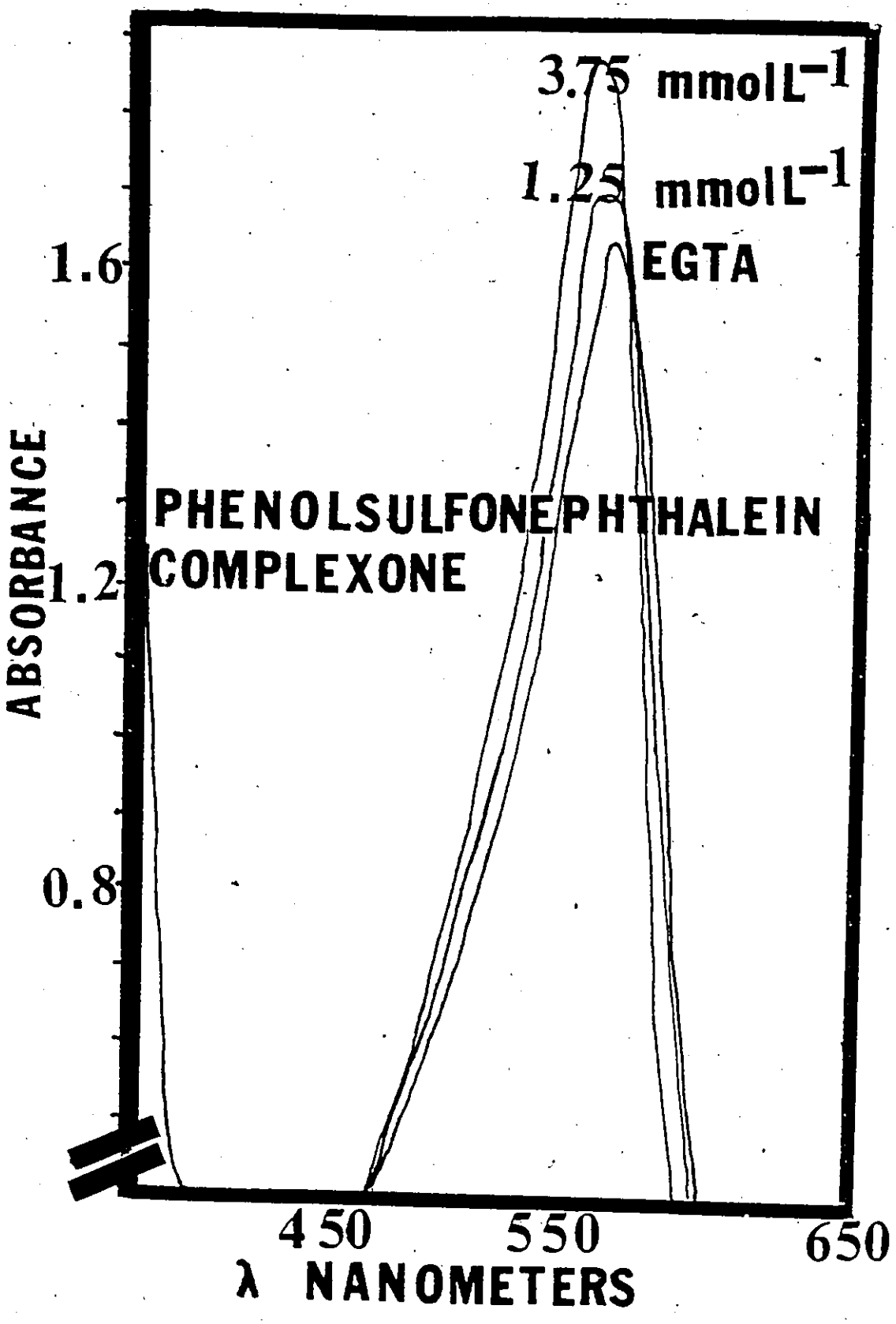
FIGURE 5

SPECTRA OF PHENOLSULFONEPHTHALEIN COMPLEXONE SHOWING ITS REACTION WITH CALCIUM

Legend

The phenolsulfonaphthalein reagent was used with 0.00, 1.25, 2.50, and 3.75 mmol L^{-1} of calcium, respectively. The spectra for 3.75 mmol L^{-1} of calcium, 2.5 with 1.25 mmol L^{-1} of calcium which were concurrent and the EGTA sample which would indicate the absorbance of a blank without any calcium contamination, are presented. All spectra were read against a water blank.

FIGURE 5


3.75 mmolL⁻¹
1.25 mmolL⁻¹
EGTA
PHENOLSULFONEPHTHALEIN
COMPLEXONE
ABSORBANCE
1.6
1.2
0.8
450
550
650
λ NANOMETERS

Ca-phenolphthalein studies were performed as above resulting in only marginal increases in absorbance with increasing calcium concentrations.

The spectra of fluorescein complexone resulting from various concentrations of calcium are presented in Figure 6. The spectra also showed a hypsochromic shift in the wavelength maximum (505 nm). Although extreme green fluoresence was noted with the presence of calcium, little difference in absorbance was observed.

An attempt was made to use the Simplex method (36-40) to optimize the components of the thymolphthalein complexone reagent noting the success Olansky had with the CPC reagent (37). The base, solvent, 8-hydroxyquinoline, and thymolphthalein complexone were varied noting a maximum absorbance for calcium, and minimum absorbance for magnesium and albumin, from the standards described in Table I. This proved to be more time-consuming than was anticipated due to problems in weighing 0.003 mg of thymolphthalein accurately and the lack of stability of the reagents; and therefore, Simplex was abandoned in favor of the greater understanding of the system that was obtained by varying one component at a time.

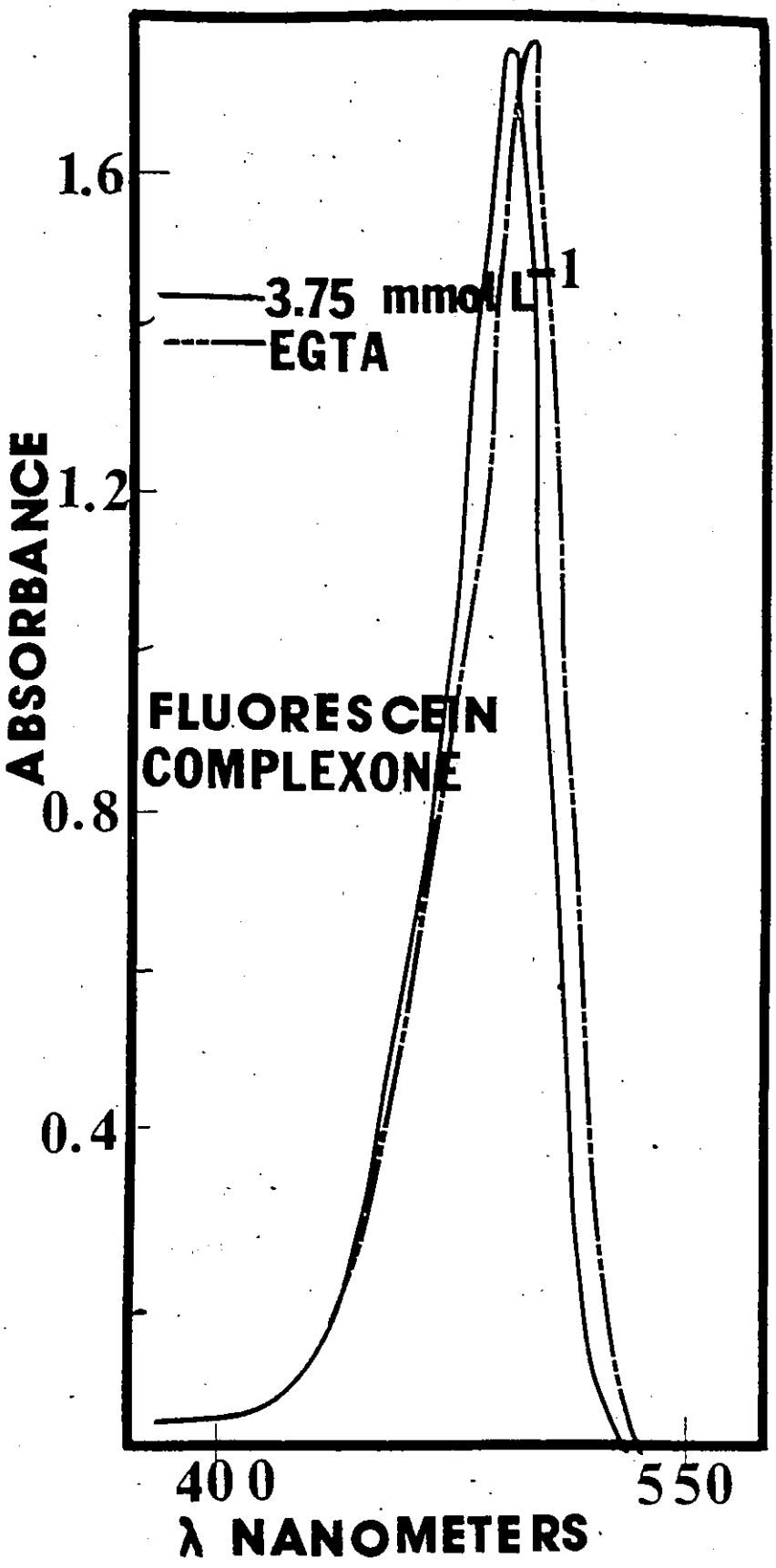
FIGURE 6

SPECTRA OF FLUORESCEIN COMPLEXONE SHOWING ITS REACTION WITH CALCIUM

Legend

The fluorescein complexone reagent was used with various calcium standards, and the resultant spectra were read against a water blank. The 3.75 mmol L^{-1} of calcium spectrum and the EGTA spectrum representing the magnitude of the blank without any calcium contamination are presented.

FIGURE 6


1.6
1.2
0.8
0.4
ABSORBANCE
3.75 mmol L⁻¹
EGTA
FLUORESCEIN
COMPLEXONE
400
550
λ NANOMETERS

It was also noted that thymolphthalein complexone had been used in the past (41-45) and that molar absorptivity in this study was similar to that previously described (44, 45).

Figure 7 demonstrates the calibration curve obtained with a Clark-type reagent, showing excellent linearity and a bathochromic shift with increased calcium. There was no significant interference from magnesium, albumin, or salicylate as indicated from the standards described in Table I. It is apparent that the slope of the calibration curve or molar absorptivity was about half that of CPC. Therefore, due to its relatively lower sensitivity, thymolphthalein cannot replace CPC as the preferred complexone.

The Yoe-Jones' plot, Figure 8, indicated that one calcium ion combined with one thymolphthalein and not two calcium ions as is the case with CPC (Figure 9). Seven times the concentration of thymolphthalein did not improve the molar absorptivity, and therefore, presumably this did not bring the law of mass action into play.

A measurable shift in the wavelength maximum occurred when calcium was added (Figure 10), adding to the evidence that the blanking hypothesis as des-

FIGURE 7

CALIBRATION CURVES FOR THYMOLPHTHALEIN COMPLEXONE REAGENT

Legend

Thymolphthalein complexone reagent was reacted with 0.00, 1.25, 1.875, 2.50, 3.125, 3.75, 5.00, and 6.25 mmol L^{-1} of calcium standards, respectively. Each tube was read with a water blank (solid line), a normal reagent blank, and a reagent blank containing EGTA. The difference between the normal reagent blank and the reagent blank containing EGTA is indicative of the background calcium contamination present.

FIGURE 7

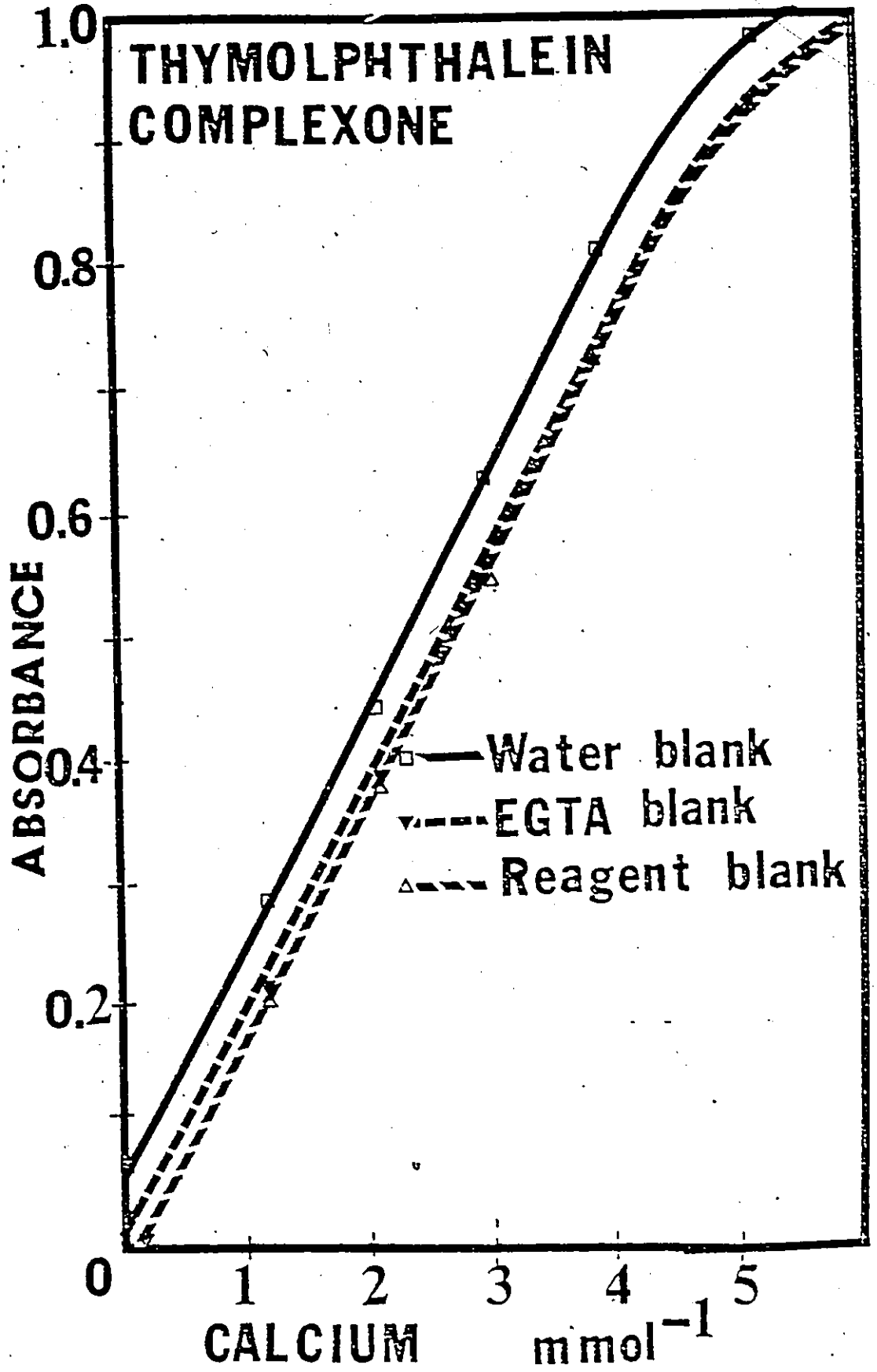

THYMOLPHTHALEIN
COMPLEXONE
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
CALCIUM mmol^{-1}
Water blank
EGTA blank
Reagent blank

FIGURE 8

YOE-JONES' PLOT WITH THYMOLPHTHALEIN COMPLEXONE

Legend

The absorbances of the ratios of [Ca] over [thymolphthalein complexone] of 0.3, 0.45, 0.6, 0.75, 0.9, 3.0, 6.0, 12.0, and 18.0 were obtained and plotted.

FIGURE 8

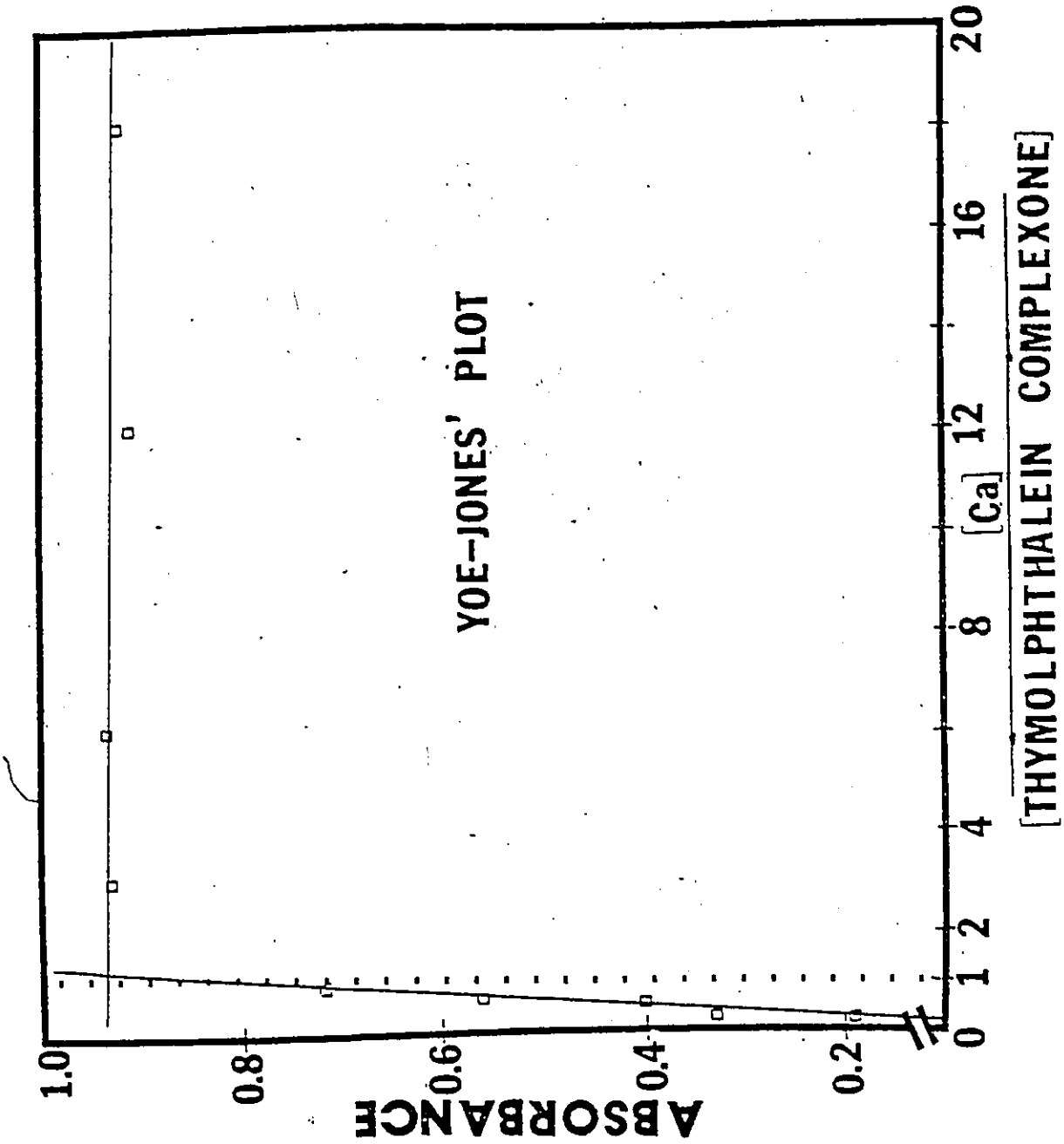

YOE-JONES' PLOT
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
1
2
4
8
12
16
20
[Ca]
[THYMOLPHTHALEIN COMPLEXONE]

FIGURE 9

YOE-JONES' PLOT WITH THE
BAGINSKI REAGENT

Legend

Two calcium atoms combine with each o-cresolphthalein complexone molecule and since it was linear between an absorbance of 0.1 and 1.2 there appeared to be a direct reaction and no intermediate CaICPC formed.

FIGURE 9

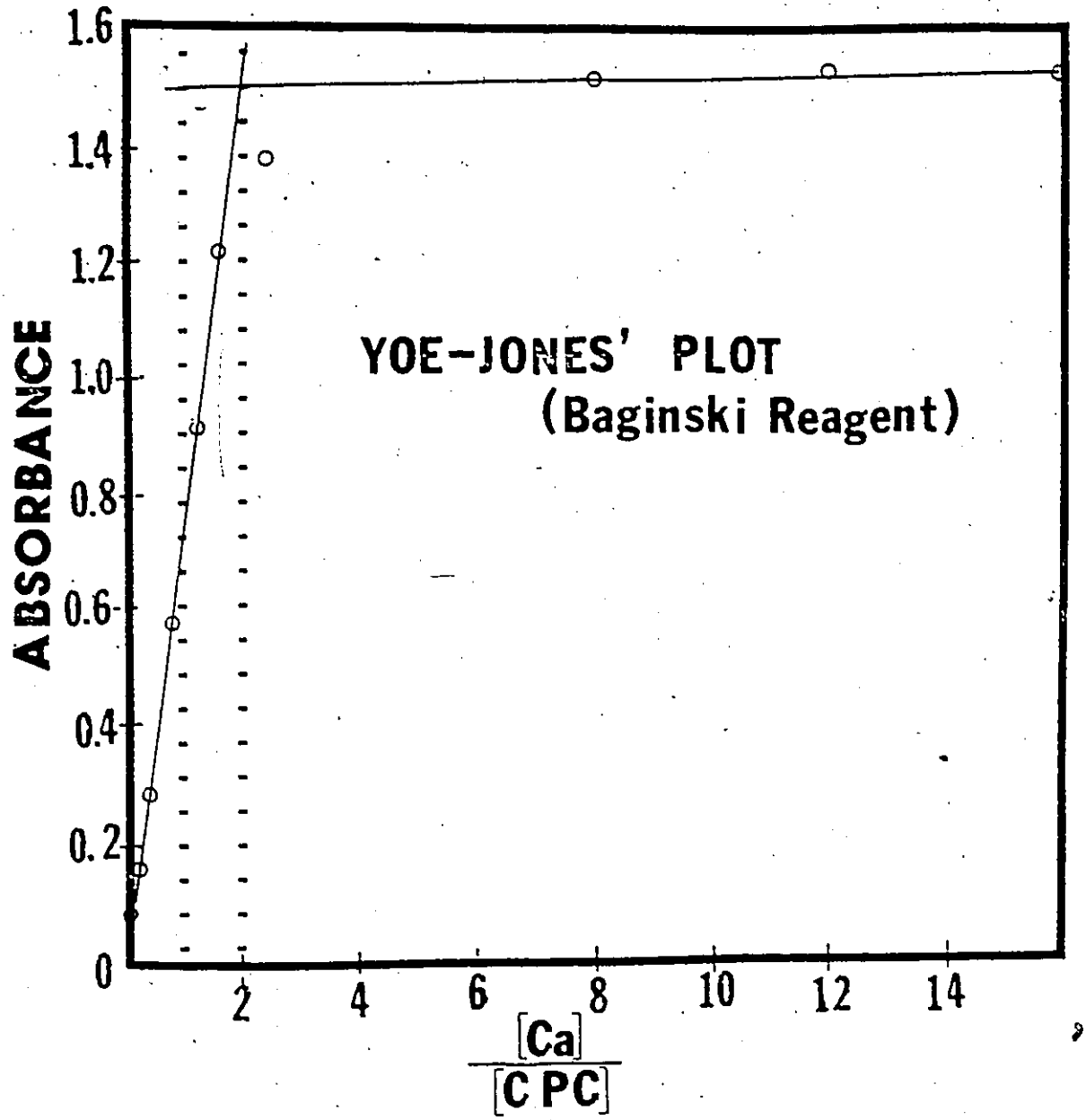

YOE-JONES' PLOT
(Baginski Reagent)
ABSORBANCE
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
[Ca]
[CPC]

FIGURE 10

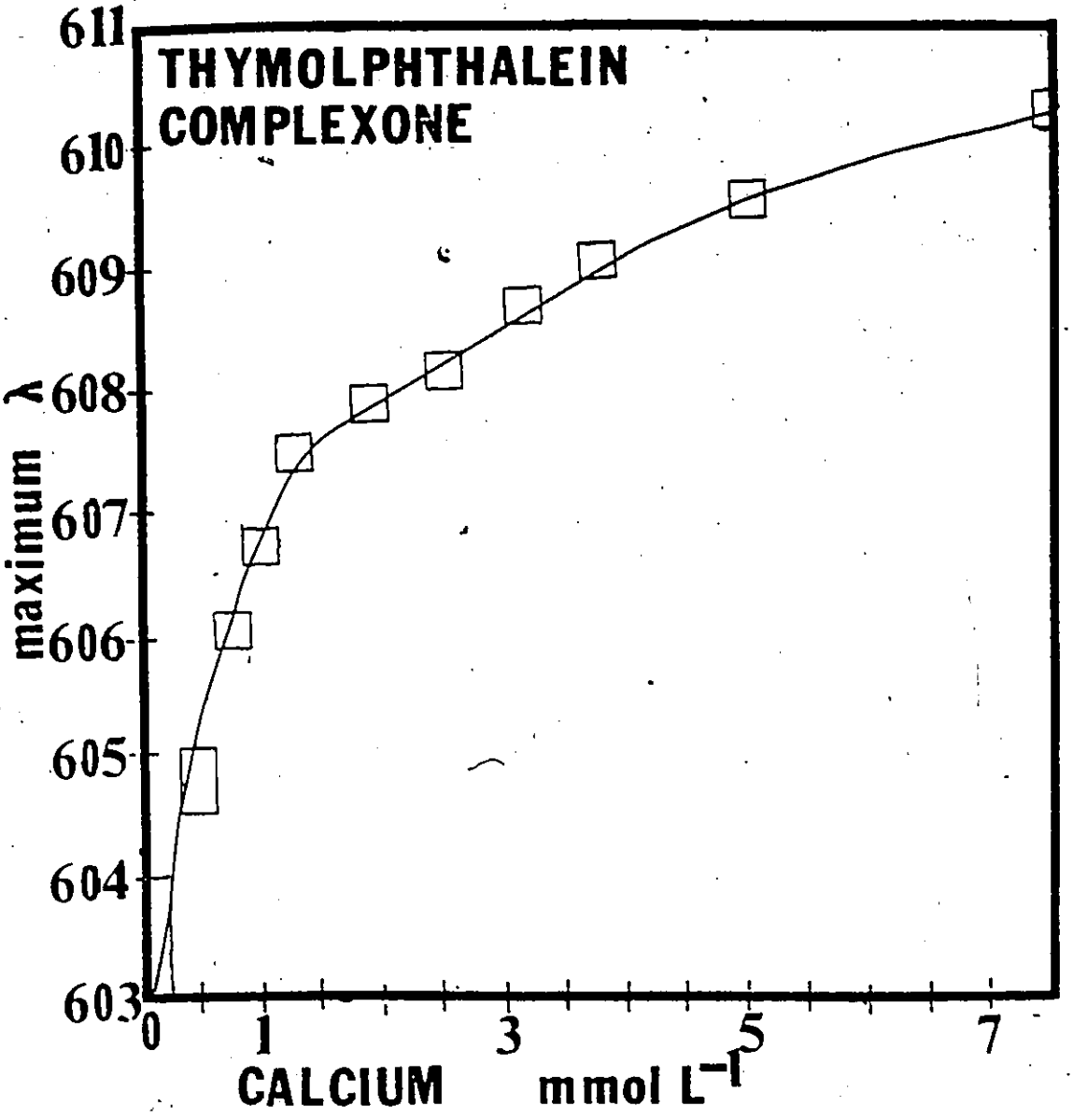
THYMOLPHTHALEIN COMPLEXONE AND

WAVELENGTH MAXIMA

Legend

Solutions with thymolphthalein complexone reagent and calcium standards of 0.00, 1.25, 1.85, 2.50, 3.125, and 3.75 mmol L^{-1}, respectively, were used to determine their wavelength maxima by adjusting the wavelength selection until the maximum absorbance was observed.

FIGURE 10


THYMOLPHTHALEIN
COMPLEXONE
611
610
609
608
607
606
605
604
603
maximum λ
0
1
3
5
7
CALCIUM mmol L⁻¹

cribed by Clark does in fact exist for this complexone as well.

3. Stability

The stability problem for the Baginski and four different Clark reagents containing CPC is presented in Figures 11 through 15.

For the Baginski reagent (Figure 11) the sensitivity (slope) was remarkably stable with the age of the reagent and then slowly decreased over a period of a week. The decrease in sensitivity was very pronounced with the CaIICPC.

The Clark reagents containing DEA (Figures 12 and 14) would result in an absolute error if the standards were read and then several hours later the patients' samples were read and all were reacted at the same time. As the reagent aged, a loss of both sensitivity and linearity resulted. If standards were determined using a new reagent, and then hours later the patients' samples were determined with the same reagent, a relative error would result. With the EG-DEA reagent the errors could be so large that a calcium concentration of 2.5 mmol L^{-1} would read 3.0 mmol L^{-1} if either the reacted compound or the reagent was two hours old.

FIGURE 11

DMSO-DEA-BAGINSKI REAGENT STABILITY

Legend

The effect of a 1.75-hour delay between the time of the reaction and the time that the test tubes are read is obtained by comparing the 0-time curve with the 1.75-h Ca-CPC curve. The 0-time curve was obtained by reacting freshly prepared reagent with 0.00, 2.50, 3.75, 5.00, 7.50, and 25.0 mmol L^{-1} of calcium, respectively. The 1.75-hour Ca-CPC curve was obtained by rereading the same tubes 1.75 hours later.

The effect of age on the reagent is seen by comparing the 0-time curve with the 2-h reagent curve. The 2-h reagent curve was obtained by reacting similar calcium standards with reagent that was two hours old.

During the study readings were taken every fifteen minutes but the data presented conveys the correct concept. The Ca-CPC faded at a constant rate. The age of the reagent resulted in a slight improvement initially and then in a slight deterioration. All readings were taken with a water blank and the reactions were carried out at 25°C.

FIGURE 11

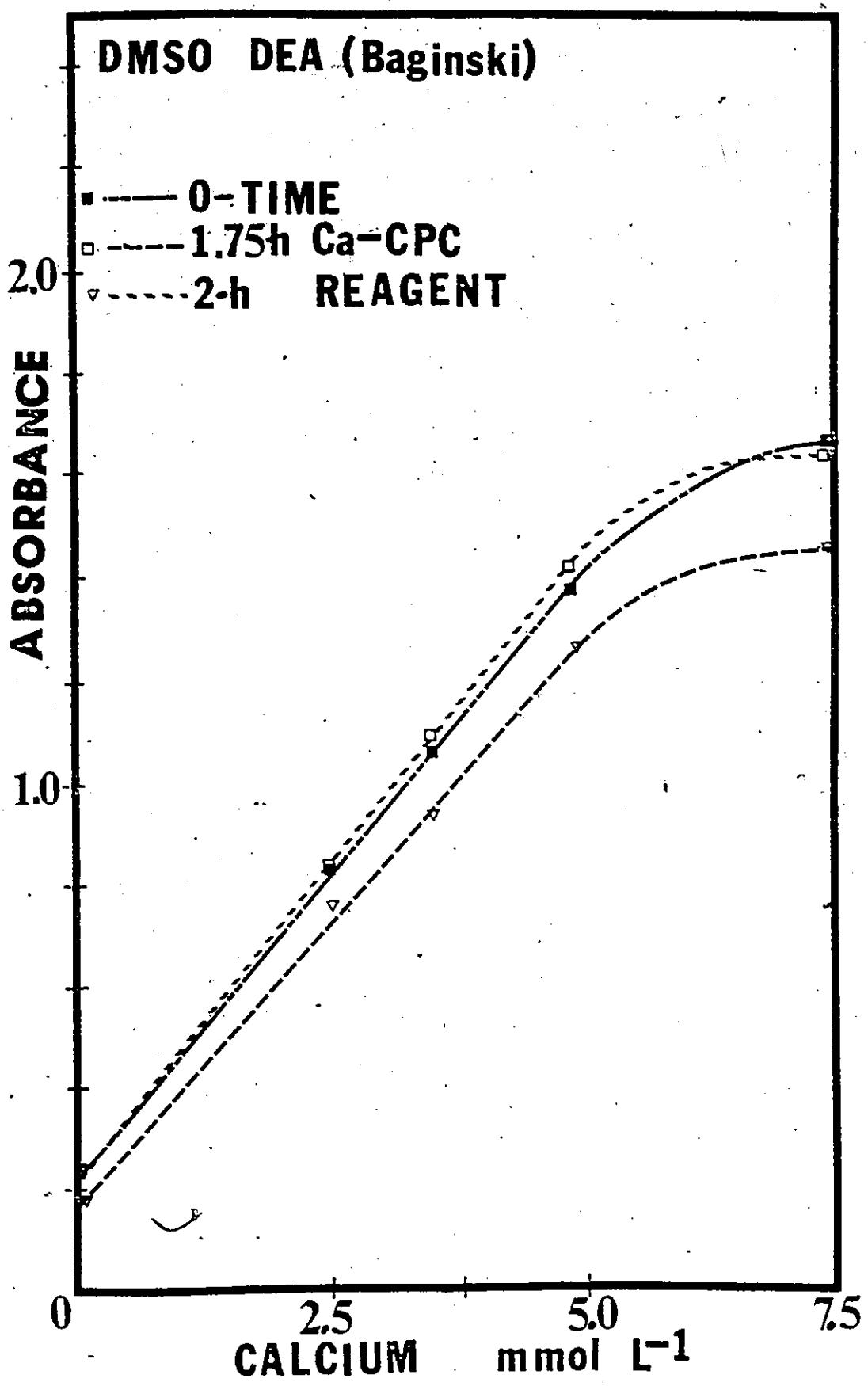

DMSO DEA (Baginski)
0-TIME
1.75h Ca-CPC
2-h REAGENT
ABSORBANCE
2.0
1.0
0
2.5
5.0
7.5
CALCIUM mmol L-1

FIGURE 12

DMSO-DEA-CLARK REAGENT STABILITY

Legend

The effect of a delay in the reading of the reaction is obtained by comparing the 0-time curve with the 2.5-hour Ca-CPC curve. The reagent was reacted with 0.00, 2.50, 3.75, 5.00, 7.50, and 25.0 mmol L^{-1} of calcium standards, respectively, and read against a water blank at 25°C. After 2.5 hours the same tubes were reread in a similar manner.

The effect of the age of the reagent is obtained by comparing 0-time with 1.25 and 2.75-hour reagent curves. After 1.25 hours and 2.75 hours similar calcium standards were reacted with the reagent and read against a water blank and the reactions were conducted at 25°C.

FIGURE 12

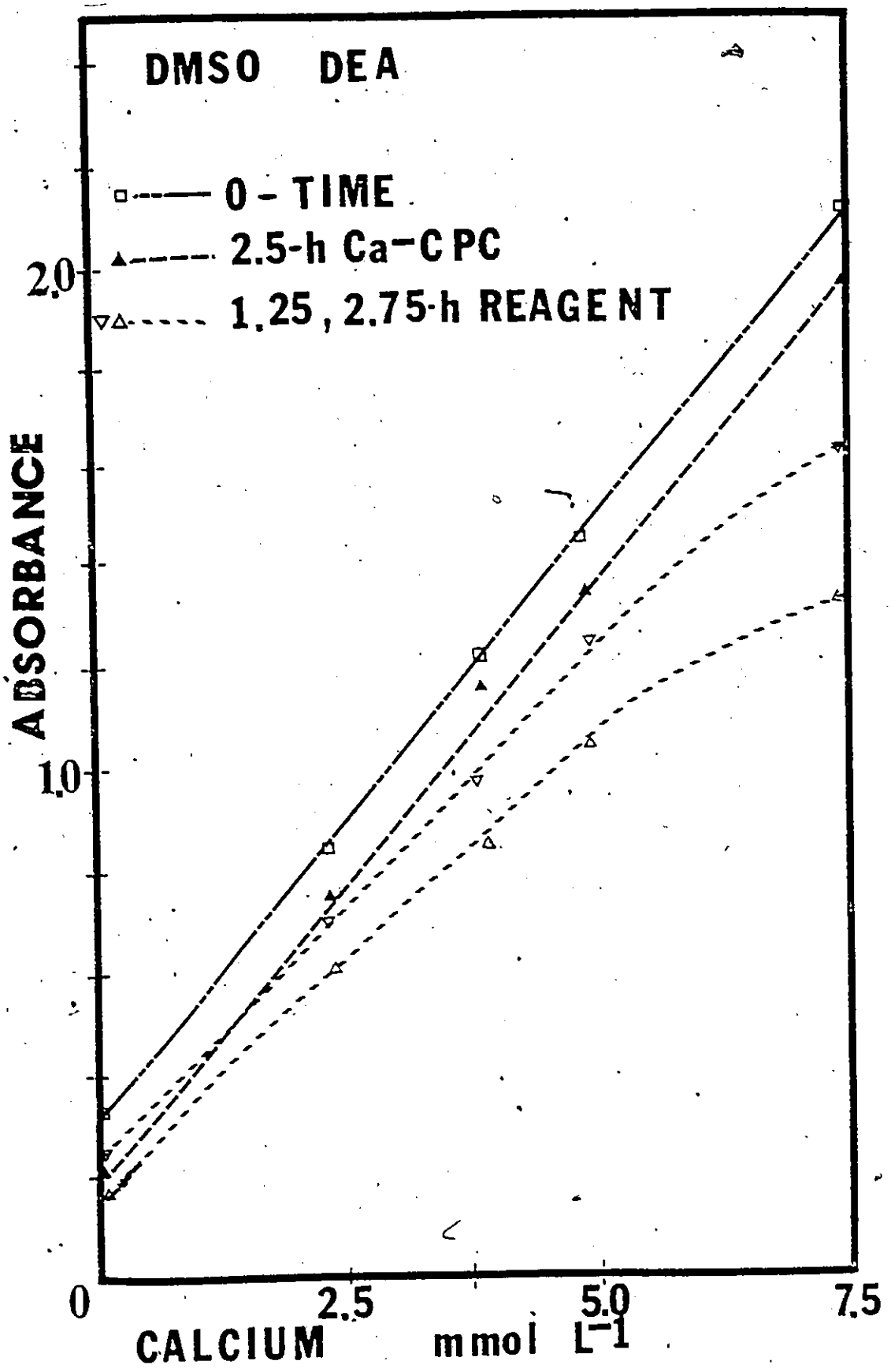

DMSO DEA
0 - TIME
2.5-h Ca-CPC
1.25, 2.75-h REAGENT
ABSORBANCE
2.0
1.0
0
2.5
5.0
7.5
CALCIUM mmol L⁻¹

FIGURE 13

DMSO-AMP-CLARK REAGENT STABILITY

Legend

Similar readings of a new reagent (0-time), a new reagent 2.5 hours after it was reacted with various calcium levels, and reagent which was 2.25 hours old are presented. All absorbances are read against water and the reaction was carried out at 25°C.

The data presented is representative of least squares regression lines obtained from readings made every fifteen minutes.

FIGURE 13

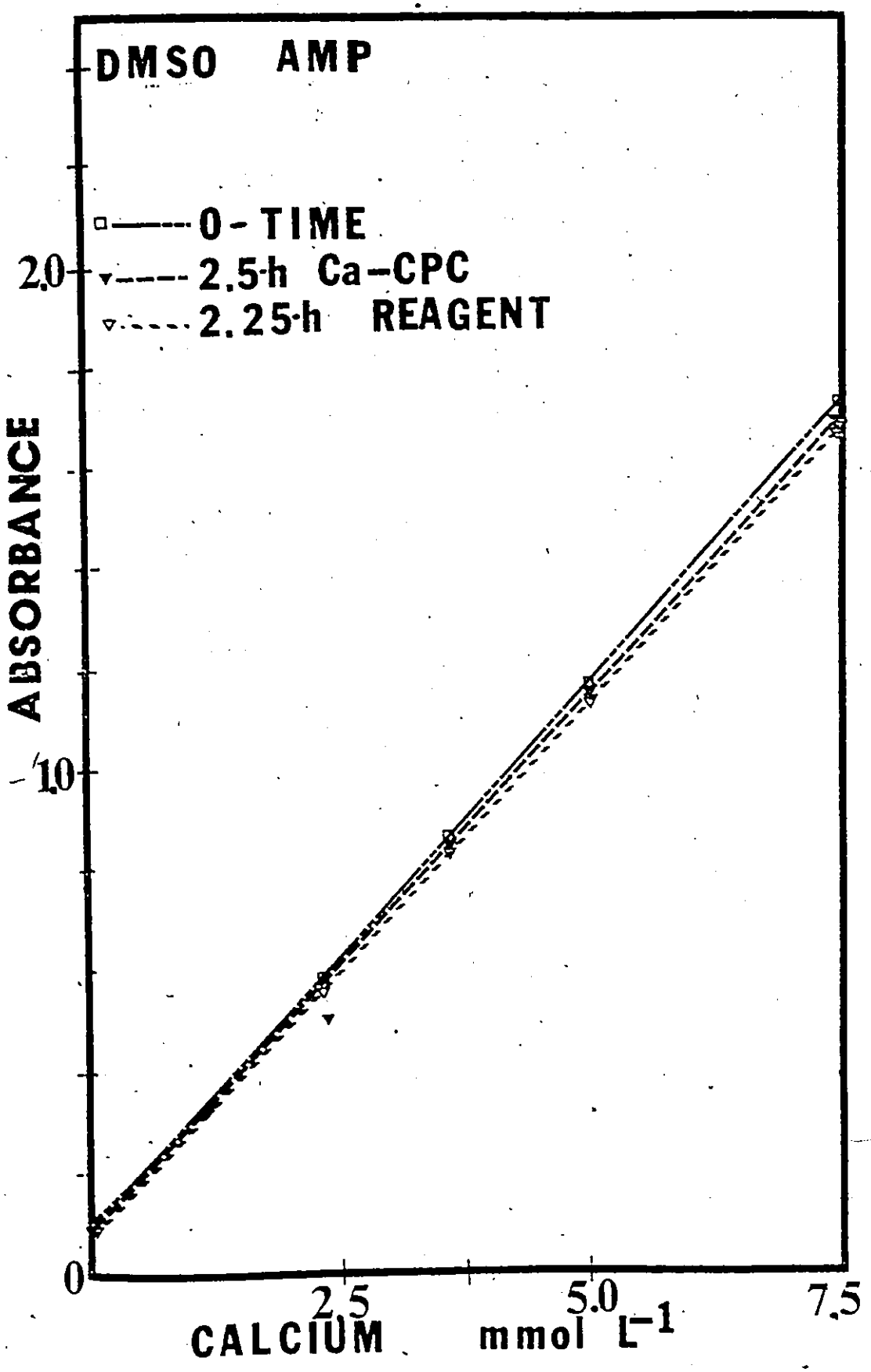

DMSO AMP
0-TIME
2.5-h Ca-CPC
2.25-h REAGENT
ABSORBANCE
2.0
1.0
0
2.5
5.0
7.5
CALCIUM mmol L-1

FIGURE 14

EG-DEA-CLARK REAGENT STABILITY

Legend

Absorbances for solutions of 0.00, 2.50, 3.75, 5.00, 7.50, and 25.0 mmol L^{-1} of calcium were read against a water blank with fresh reagent (0-time), 0.75-hour old reagent, 1.75-hour old reagent, and fresh reagent which was not read until 2 hours after it was reacted with the calcium. All reagents were at 25°C.

The data presented is representative of least-squares regression lines obtained from readings made every fifteen minutes.

FIGURE 14

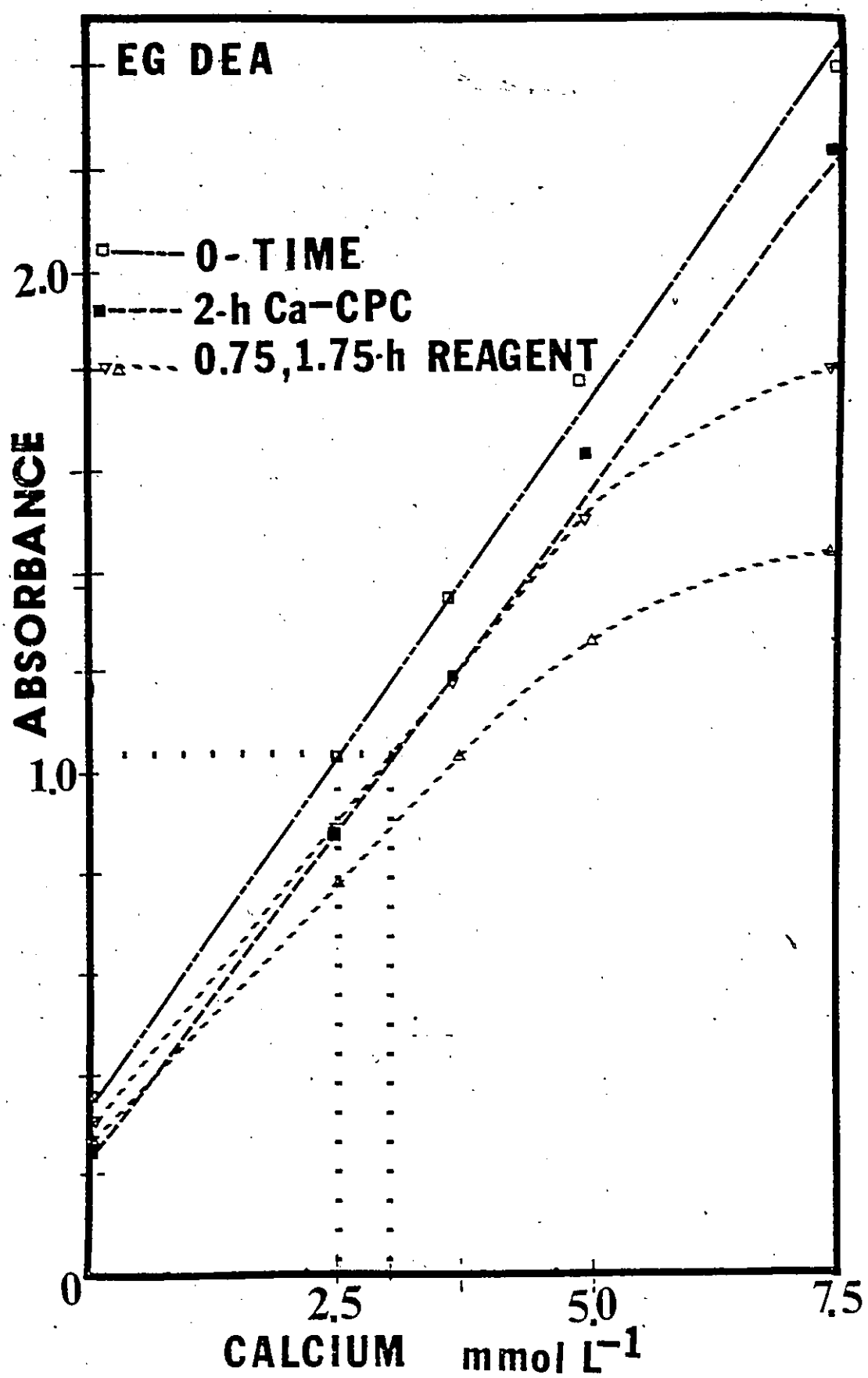

EG DEA
0-TIME
2-h Ca-CPC
0.75,1.75-h REAGENT
ABSORBANCE
2.0
1.0
0
2.5
5.0
7.5
CALCIUM mmol L-1

FIGURE 15

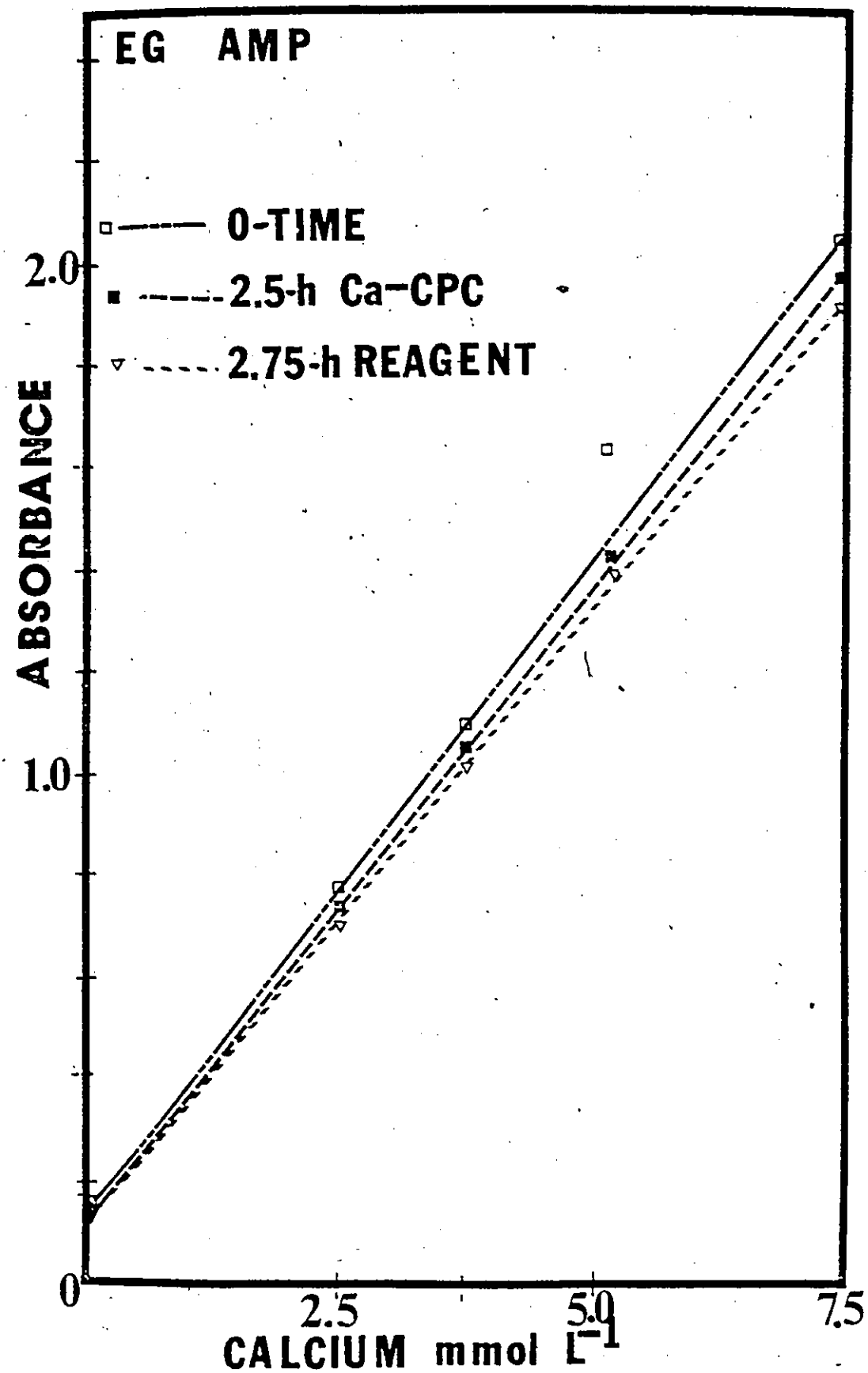
EG-AMP-CLARK REAGENT STABILITY

Legend

Absorbances for solutions of 0.0, 2.5, 3.75, 5.0, 7.5, and 25.0 mmol L^{-1} of calcium were read against a water blank with fresh reagent (0-time), 2.75-hour old reagent, and fresh reagent which had reacted with the calcium levels 2.5 hours previously. All readings were taken at 25°C.

The data presented is representative of readings made every fifteen minutes. The lines were obtained by least squares regression.

FIGURE 15


EG AMP
0-TIME
2.5-h Ca-CPC
2.75-h REAGENT
ABSORBANCE
2.0
1.0
0
2.5
5.0
7.5
CALCIUM mmol L-1

The Clark reagents containing AMP (Figures 13 and 15) demonstrated remarkable stability with respect to both the reagent and the Ca-complex. The Baginski reagent (Figure 11) as well as the Clark reagents containing AMP showed excellent reagent stability over a period of a week.

As a result of the reagent stability problem as well as the Ca-complex stability problem which was of more concern in the manual method than the automated method, all further readings were performed within a few minutes after their reaction and only newly-made reagents were used.

4. Temperature

Although serum total calcium by CPC is normally performed at ambient temperature, the effect of change in temperature is rather dramatic. Only in Gindler's paper on thymolphthalein complexone was it mentioned that a temperature increase decreases absorbance (44, 45). Generally, temperature is ignored (3, 9, 13-15).

Figure 16 demonstrates the dramatic effect temperature has on a complexing reaction, the buffer systems, or the dielectric constant of the reagents. With the DMSO-AMP reagent, an error of 0.25 mmol L^{-1}

FIGURE 16

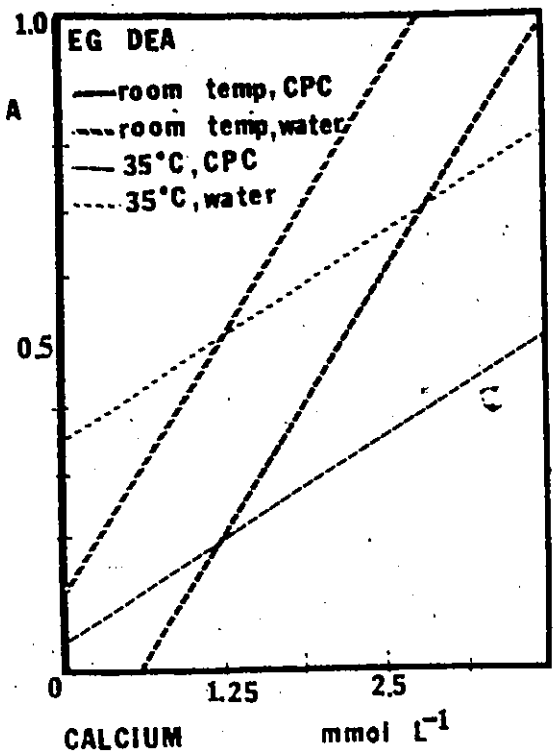
THE EFFECT OF TEMPERATURE ON THE CLARK REAGENTS

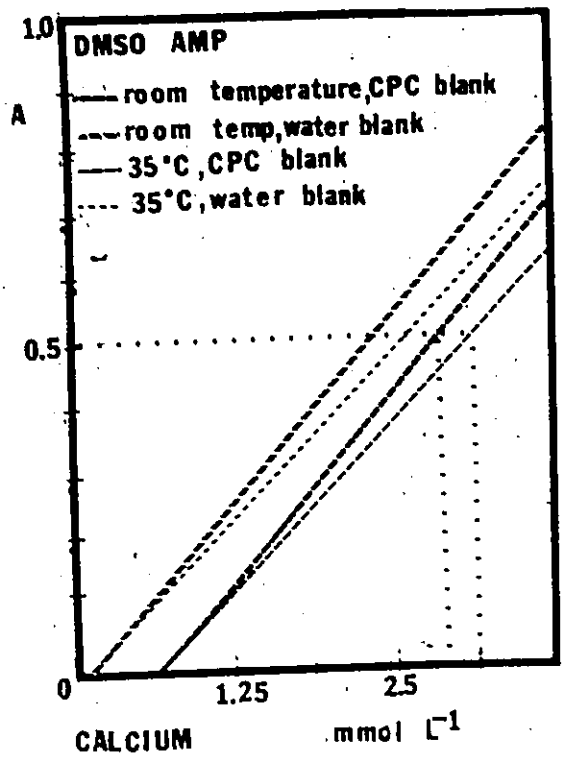
Legend

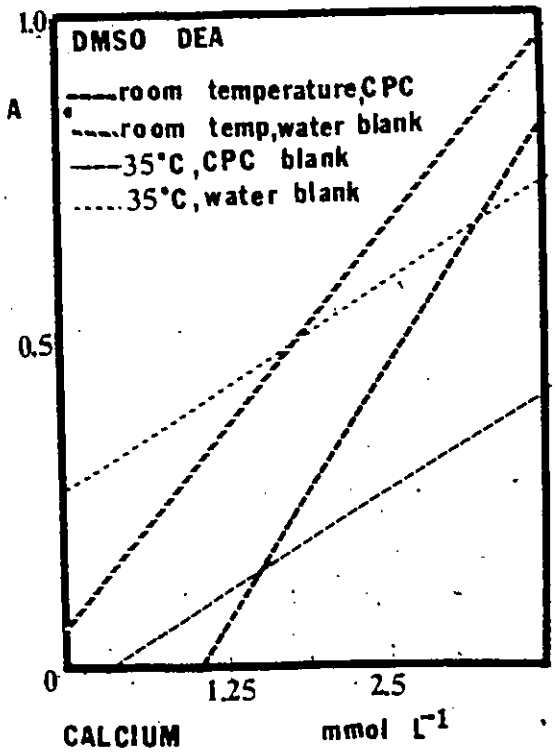
To demonstrate the decrease in sensitivity (slope) with the increase in temperature, the same twenty-four test tubes were read at room temperature and then three hours later at 35°C. Four intermediate temperatures were read but the dramatic change occurred near 35°C. Calcium levels of 0.00, 1.25, 1.875, 2.5, 3.125, and 3.75 mmol L^{-1} were determined and read against a water blank as well as a normal reagent blank.

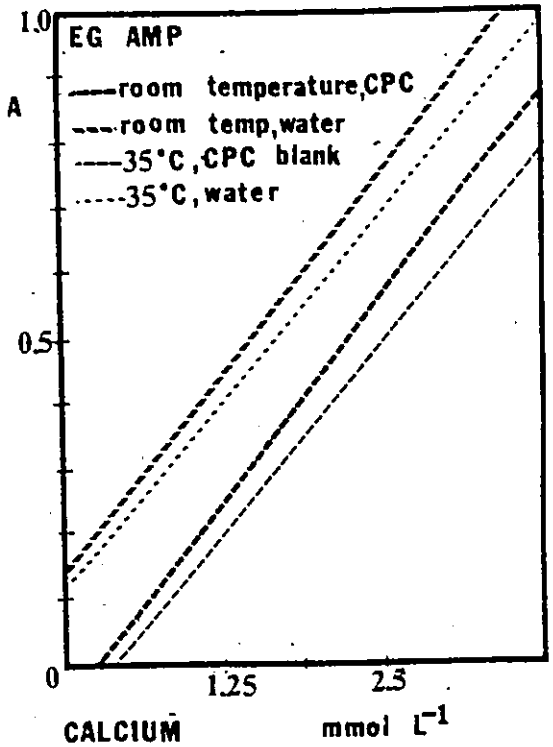
The lines were obtained by least squares regression analysis.

FIGURE 16


EG DEA
room temp, CPC
room temp, water
35°C, CPC
35°C, water
A
1.0
0.5
0
1.25
2.5
CALCIUM
mmol L⁻¹


DMSO AMP
room temperature, CPC blank
room temp, water blank
35°C, CPC blank
35°C, water blank
A
1.0
0.5
0
1.25
2.5
CALCIUM
mmol L⁻¹


DMSO DEA
room temperature, CPC
room temp, water blank
35°C, CPC blank
35°C, water blank
A
1.0
0.5
0
1.25
2.5
CALCIUM
mmol L⁻¹


EG AMP
room temperature, CPC
room temp, water
35°C, CPC blank
35°C, water
A
1.0
0.5
0
1.25
2.5
CALCIUM
mmol L⁻¹

of calcium could result from the rise of the temperature to 35°C inside an automated machine. Due to the three hours required to raise the temperature some decrease in sensitivity was expected with the DEA reagents.

pH is a constant dependent on temperature (46). The negative log of the ionization constant for water at 24°C is 14.000; and therefore, neutral pH at 24°C is 7, but at 35°C, the constant is 13.680; and therefore, neutrality is 6.84 at 35°C (35). The Nernst equation predicts that as the temperature increases the pH measured by a glass-electrode will fall. At the high pH ranges that are being employed, there is a lowering of the pH due to the sodium ion concentration (46).

Every electrode has its own characteristics and the silver/silver chloride intervals-gelled, saturated KCl electrode employed, was recommended for use over the full pH range 0-14 at 0°C to +100°C. The pH of the deionized distilled water changed from a pH 5.87 at 10°C to 6.65 at room temperature and then stabilized at 6.76 at 40°C. Deionized distilled water with a trace of HCl decreased linearly from a pH of 4.93 at 10°C to 4.59 at 40°C. A 0.01 M solution of sodium hydroxide

measured pH 11.7 with narrow-range pH paper, and after calibrating the electrode with standard buffer solutions, the sodium hydroxide solution measured pH 10.41 to 10.50 from 10°C to 40°C, demonstrating the sodium error but exceptional stability with temperature. The buffer used to set pH 11 (50 mL of 0.05 M disodium hydrogen phosphate and 4 mL of 0.1 M sodium hydroxide), and confirmed with short-range pH paper, varied from 10.97 at 10°C up to 11.02 at 40°C. Since only small quantities of sodium were present in both the standardizing buffers and the solutions to be measured, and mindful that the electrode showed little response due to temperature at the desired pH, an attempt was made to show the effect of temperature on the base, and reagents (Figure 17).

With increasing temperature there is an increase in the ionization of the base which is counter-balanced by a decrease in the dielectric constant of the medium thereby decreasing the tendency towards charge separation and decreasing the orientation of solvent molecules around the solute ion. The apparent pH was expected to rise with temperature to a maximum and then, due to the

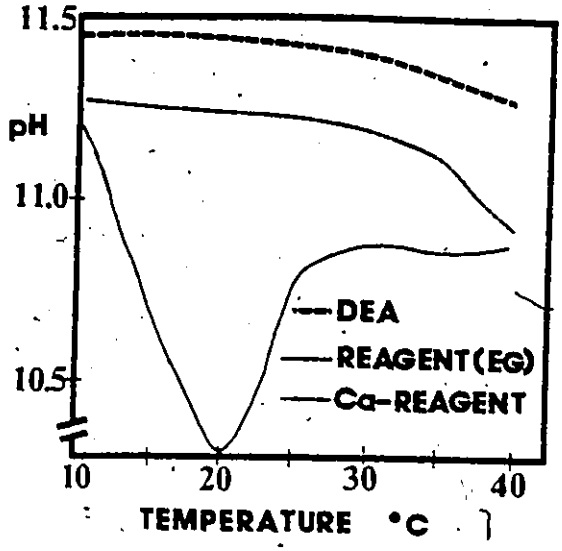
FIGURE 17

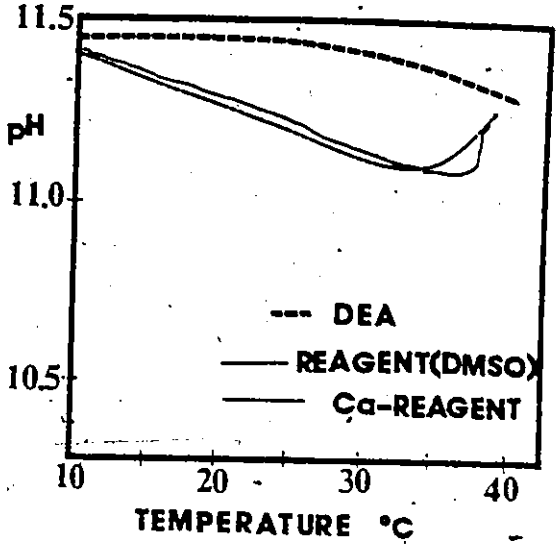
THE EFFECT OF TEMPERATURE ON THE APPARENT pH WITH CLARK REAGENTS

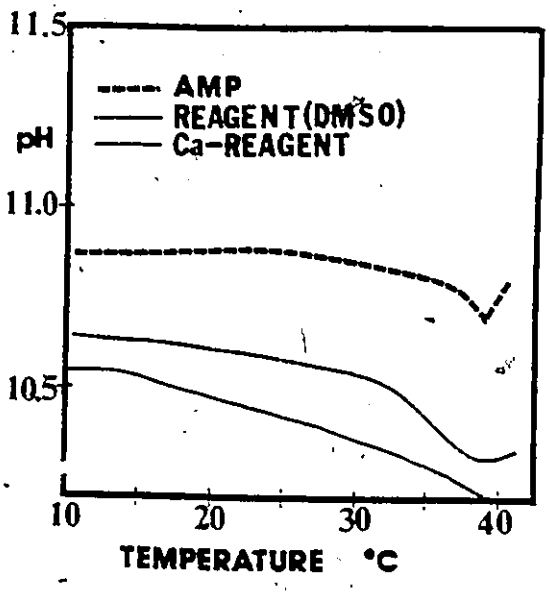
Legend

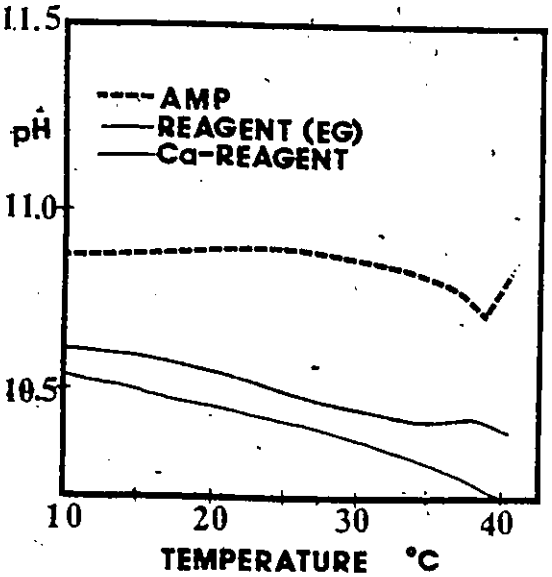
A 7.5-mL sample of the base (either DEA or AMP) diluted to 100 mL with water, the Clark reagent, or the Clark reagent with 3.75 mmol L^{-1} of calcium were heated to over 40°C and then the apparent pH was recorded as the temperature was lowered in a cold water bath.

FIGURE 17


11.5
pH
11.0
10.5
DEA
REAGENT(EG)
Ca-REAGENT
10
20
30
40
TEMPERATURE °C


11.5
pH
11.0
10.5
DEA
REAGENT(DMSO)
Ca-REAGENT
10
20
30
40
TEMPERATURE °C


11.5
AMP
REAGENT(DMSO)
pH
Ca-REAGENT
11.0
10.5
10
20
30
40
TEMPERATURE °C


11.5
AMP
REAGENT (EG)
pH
Ca-REAGENT
11.0
10.5
10
20
30
40
TEMPERATURE °C

effect of a lowering of the dielectric constant, decrease (47).

The addition of the organic solvent lowered the dielectric constant of the media, and therefore, the amount of dissociation of the buffer. The general lowering of the apparent pH with increasing temperature was therefore anticipated.

The presence of the complexing reagent, an acid, added an additional factor in the equilibrium. Also, the calcium itself caused a change. An exception occurred when calcium was added to a DMSO-DEA reagent lacking CPC, there was no change in the pH. The lowering of the apparent pH with the addition of calcium may be related to the release of the hydrogen associated with the nitrogen of the complexone.

It was also noted that the AMP-Clark reagents produced a lower apparent pH than the DEA-Clark reagents.

No explanation is readily available for the drop in the apparent pH at 20°C in the DEA-EG-Clark reagent.

The erratic change in apparent pH near 37°C would certainly affect calcium results. Since all

complexones are essentially modified pH indicators, they are very sensitive to changes in the apparent pH as illustrated by graphs presented in two studies (3, 18).

Alkaline phosphatase reagent with an AMP buffer (48) without an organic solvent in the mixture, proved to have a remarkably stable pH from 20° to 40°C.

Table II presents the slopes of the absorbances versus the calcium concentrations of 0.00, 1.25, 1.875, 2.50, 3.125, and 3.75 mmol L^{-1} with the Clark reagents varying temperature (n = 24). Slopes of the absorbances versus the calcium concentration for various Clark reagents were compared as a measure of molar absorptivities (L cm^{-1} mol^{-1}). Each reagent contained 30 mg L^{-1} of CPC. Using the definition of sensitivity as response per unit analyte, these slopes reflect the different reagents' sensitivities to calcium at different temperatures. Generally, the sensitivity to calcium decreased with an increase in temperature, except for the EG-DEA. DMSO-DEA at 20°C produced the greatest sensitivity.

TABLE II

SLOPES OF ABSORBANCES AGAINST CALCIUM CONCENTRATION WITH CLARK REAGENTS

Temperature ^{o}C	DMSO-DEA L $mmol^{-1}$	DMSO-AMP L $mmol^{-1}$	EG-DEA L $mmol^{-1}$	EG-AMP L $mmol^{-1}$
20	0.31	0.21*	0.17	0.28
25	0.28	0.19	0.19	0.28
30	0.27	0.19	0.22	0.27
35	0.26	0.19	0.25	0.27
40	0.26	0.17	0.24	0.24

*22^{o}C

Clark's blanking hypothesis obviates any spectrophotometric method of obtaining the stability constant, and hence the molar absorptivities. The ratio method requires two solutions with equal absorbances but different concentrations of both the calcium and the CPC. The logic is as follows:

$$\text{The stability constant} = \frac{[\text{CaIICPC}]}{[\text{Ca}]^2\,[\text{CPC}]}$$

From Beer's law: $[\text{CaIICPC}]_1 = kA$ solution 1

and $[\text{CaIICPC}]_2 = kA$ solution 2

If the absorbances of the above solutions were the same, then the concentration of CaIICPC was the same. Since the initial concentrations of the calcium and the CPC were known in both the first solution and the second, the problem resolves itself to two equations with two unknowns, similar to:

$$\text{Stability constant} = \frac{[\text{CaIICPC}]}{([\text{Ca}]_2 - [\text{CaIICPC}])^2\,([\text{CPC}]_2 - [\text{CaIICPC}])}$$

But since both CaIICPC and CPC absorb at 575 nm and conventional blanking overblanks, Beer's law would result in an equation with one constant for CaIICPC and another CPC.

Methods for determining stability constants with polarography, ion displacement, or from solubility measurements (49) were not considered feasible for this study.

5. A New Buffer System

A CAPS and DMSO Clark reagent was prepared requiring the addition of NaOH as described in Figure 18 and it was checked for its suitability as a buffer in a CPC reagent (Figure 19). Although its slope and stability were comparable to other systems studied, due to the fact that it was necessary to add sodium hydroxide to adjust the pH of the CAPS buffer, unlike the DEA and AMP buffer, the system was not studied any further.

6. Comparison of the Different Reagents

The first attempt to produce a standard curve with the Baginski reagent is shown in Figure 20A. The non-linearity and the fact that the CPC blank calibration curve does not go through the origin proved to be the result of storage of the calcium standards in glass for a week. Figure 20B demonstrates the calibration from Baginski reagent with standards stored in polyethylene. The CPC-

FIGURE 18

THE APPARENT pH WITH CAPS AND SODIUM HYDROXIDE

Legend

CAPS (2.62×10^{-2}M) with a pK_a of 10.4 in water (33) required 15 to 18 mL of 1 M NaOH to obtain an apparent pH of 10.2 in a solvent mixture of 75 mL of DMSO diluted to 1 L with water.

FIGURE 18

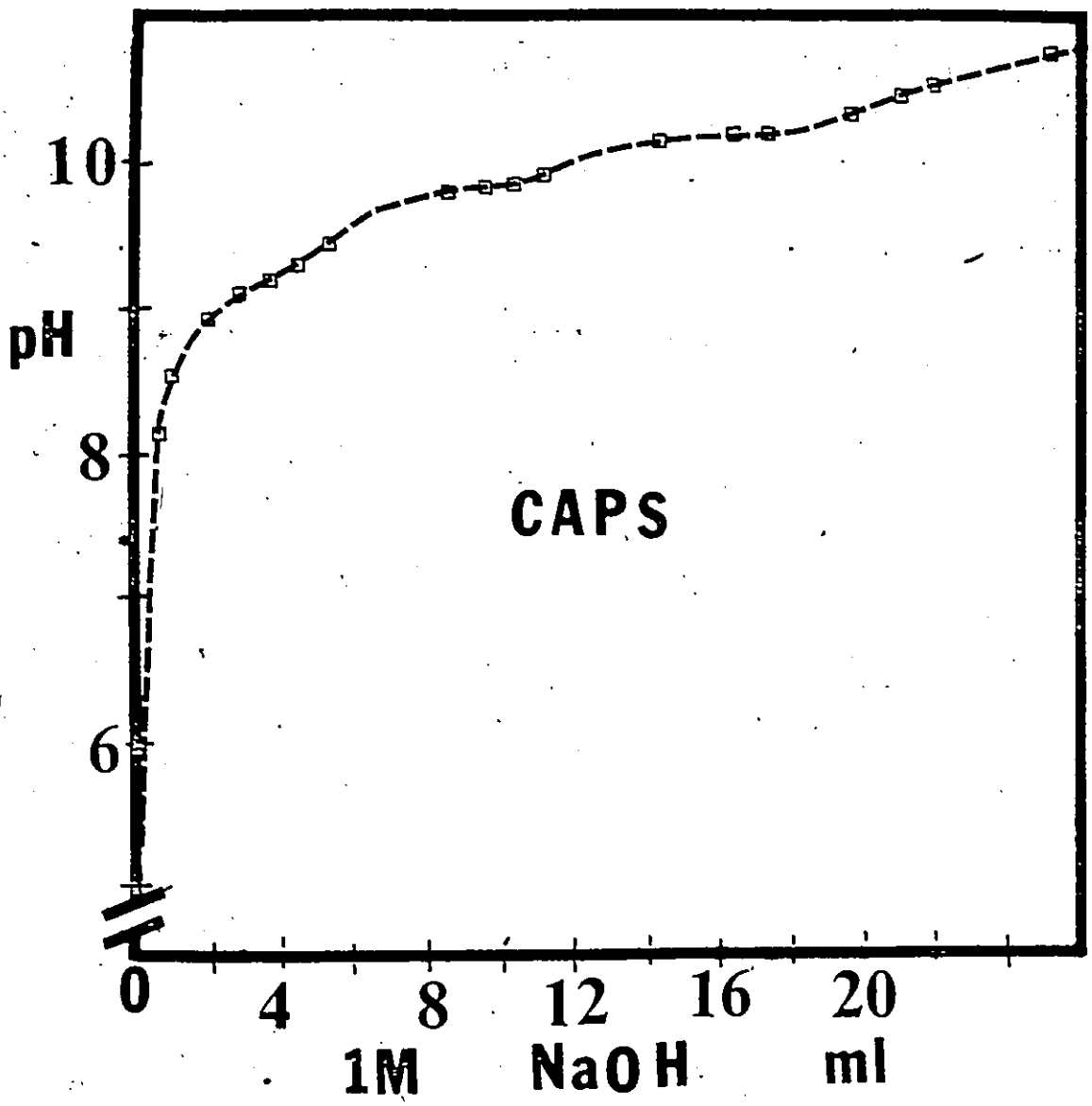


FIGURE 19

CALIBRATION CURVES FOR DMSO-CAPS REAGENT WITH VARIOUS LEVELS OF CALCIUM

Legend

Calibration curves (n = 32) for a reagent containing 5.798 g CAPS, 75 mL of DMSO, and 30 mg of CPC diluted to 1 L with water were obtained with calcium standards containing 2.50, 3.75, 5.00, 7.50, 10.00, 12.50, 18.75, and 25.00 mmol L^{-1}, respectively.

FIGURE 19

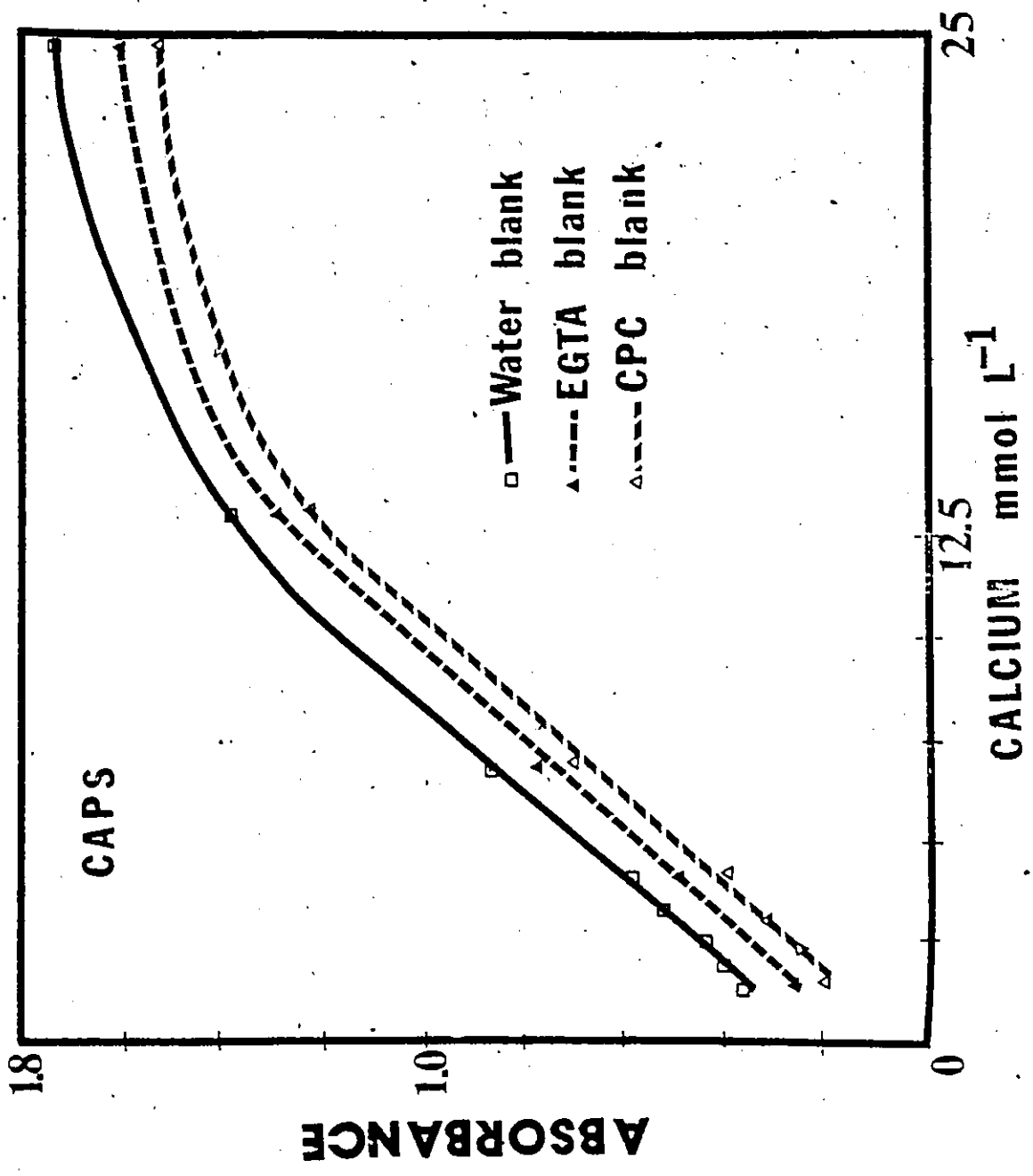

CAPS
Water blank
EGTA blank
CPC blank
ABSORBANCE
1.8
1.0
0
12.5
25
CALCIUM mmol L⁻¹

FIGURE 20

CALIBRATION CURVES FOR THE DMSO-DEA-BAGINSKI REAGENT

Legend

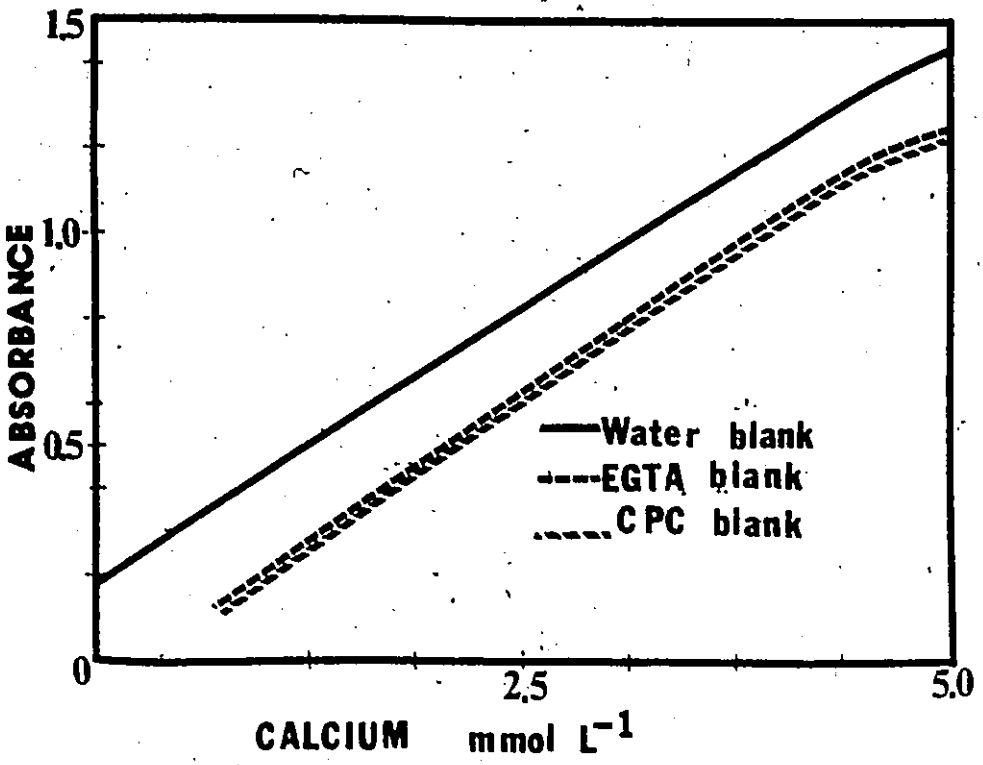
A: The Baginski reagent (13) was used at 25°C with calcium standards of 0.00, 1.25, 1.875, 2.50, 3.125, 3.75, 5.00, and 6.25 mmol L^{-1}, respectively. The standards were stored in glass.

B: The Baginski reagent (13) was also used with 0.00, 1.25, 1.875, 2.50, 3.125, 5.00, and 6.25 mmol L^{-1} of calcium standards, respectively. The standards were stored in polyethylene. The difference between the EGTA blank and the normal reagent blank is representative of the degree of calcium contamination.

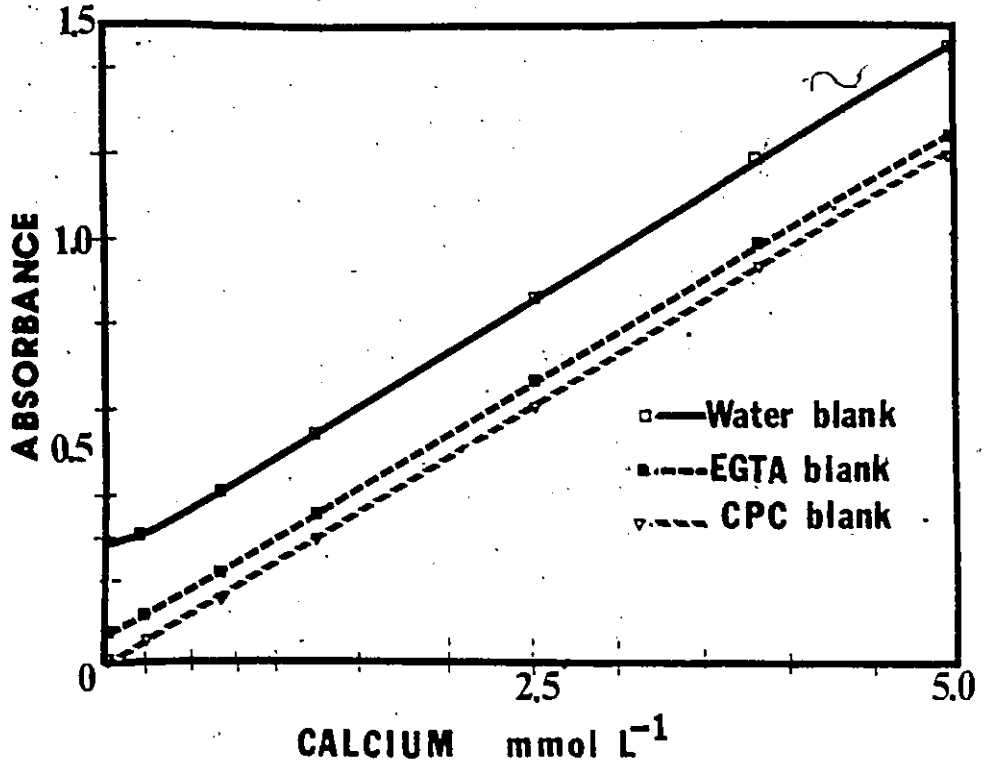
Lines were obtained by least squares regression.

FIGURE 20

A.


1.5
1.0
0.5
0
ABSORBANCE
Water blank
EGTA blank
CPC blank
2.5
5.0
CALCIUM mmol L^{-1}

B.


1.5
1.0
0.5
0
ABSORBANCE
Water blank
EGTA blank
CPC blank
2.5
5.0
CALCIUM mmol L^{-1}

blanked calibration curve passes through the origin and increased linearity was achieved throughout. Calcium adheres to glass. Similarly, all the Clark-reagent calibration curves are presented in Figure 21. EG-AMP reagent resulted in a slope second only to that of the Baginski reagent.

The data presented in Tables III to V compares the results of sets of 30 tubes from 0.00 to 3.75 mmol L^{-1} of calcium at 25°C. Regression lines were obtained from the least squares method commonly employed which assumes precise measurement of the independent x variable (calcium ion concentration). Since the variance of the error over the variance of the x value(Sex/Sx) did not exceed 0.2, correction for the lack of precision in x was unnecessary (42).

The comparison of the standard deviations of the different reagents from Table III indicates that the DMSO-DEA-Clark reagent has unusual precision. An unanticipated observation was that the Baginski reagent requiring an additional pipetting step was slightly more precise than most of the Clark reagents. A standard deviation of 0.07 mmol L^{-1} or 0.28 mg/dL of calcium is considered the state of the art for an

FIGURE 21

CALIBRATION CURVES FOR THE CLARK REAGENTS

Legend

The four different Clark reagents were used to obtain the calibration curves at 25°C. Standard solutions of calcium of 0.00, 1.25, 1.85, 2.50, 3.125, and 3.75 mmol L^{-1}, respectively, were used in this study (n = 24). The regression lines were obtained by least-squares (APPENDIX A).

FIGURE 21

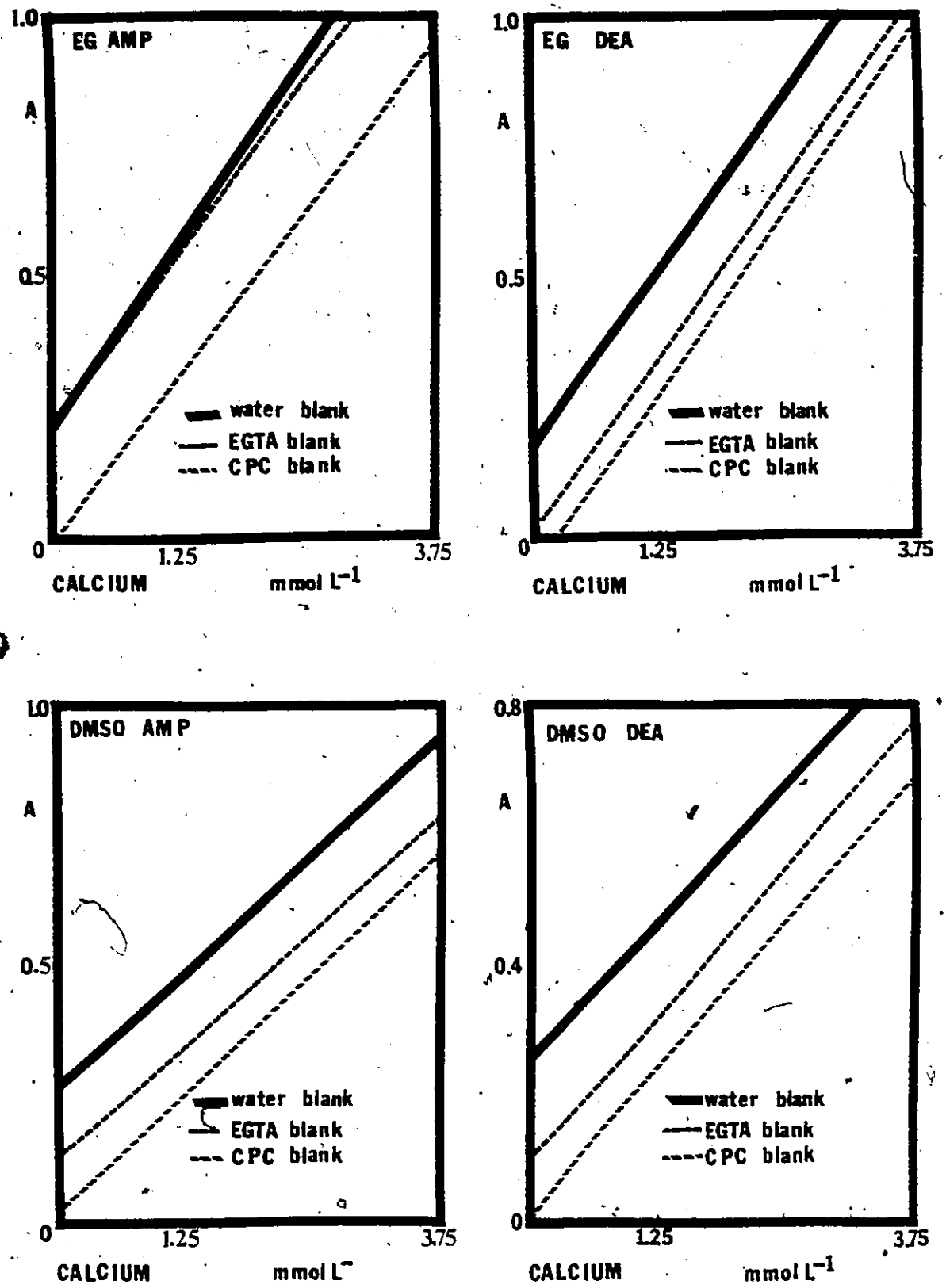

EG AMP
1.0
A
0.5
0
1.25
3.75
CALCIUM
mmol L⁻¹
water blank
EGTA blank
CPC blank
EG DEA
1.0
A
0.5
0
1.25
3.75
CALCIUM
mmol L⁻¹
water blank
EGTA blank
CPC blank
DMSO AMP
1.0
A
0.5
0
1.25
3.75
CALCIUM
mmol L⁻
water blank
EGTA blank
CPC blank
DMSO DEA
0.8
A
0.4
0
1.25
3.75
CALCIUM
mmol L⁻¹
water blank
EGTA blank
CPC blank

TABLE III

STANDARD DEVIATIONS COMPARING

DIFFERENT REAGENTS

Method	S.D.* mmol L^{-1}	S.D.* mg/dL
DMSO-DEA-Baginski	0.09	0.36
DMSO-DEA-Clark	0.05	0.19
DMSO-AMP-Clark	0.12	0.48
EG-DEA-Clark	0.08	0.35
EG-AMP-Clark	0.12	0.47

*Standard deviation of the calcium holding the absorbance fixed -- commonly known as the standard error of the estimate. A regression line was obtained with 0.00, 1.25, 1.825, 2.50, 3.125, and 3.75 mmol L^{-1} of calcium standards, respectively, and analyzed as shown in APPENDIX A. n = 30.

TABLE IV

ERROR IN THE ESTIMATES OF THE

REGRESSION COEFFICIENTS

Method	Regression coefficient L $mmol^{-1}$	Error L $mmol^{-1}$
DMSO-DEA-Baginski	0.27	5.6×10^{-3}
DMSO-DEA-Clark	0.19	1.4×10^{-3}
DMSO-AMP-Clark	0.18	3.9×10^{-2}
EG-DEA-Clark	0.25	5.2×10^{-3}
EG-AMP-Clark	0.27	6.9×10^{-3}

n = 30 for each study
See footnote to Table III for details of experimental method.

TABLE V

F-TESTS COMPARING THE DIFFERENT REAGENTS

Method	Goodness of fit*	Linearity**
DMSO-DEA-Baginski	2299.0	2.5
DMSO-DEA-Clark	0.3	16.5
DMSO-AMP-Clark	20.4	3.3
EG-DEA-Clark	2304.8	17.2
EG-AMP-Clark	1502.0	0.4

n = 30
See footnote to Table III for details of experimental method.
See APPENDIX B for a sample calculation.

*Also referred to as the significance of the regression $F[_{28}^{1}\ 0.5] = 4.2$

**$F[_{25}^{1}\ 0.5] = 4.25$

automated system (1), and therefore, only the DMSO-DEA-Clark reagent was acceptable. If these manual methods were to be automated an increase in precision would be expected.

Sensitivity as indicated by the slopes of the calibration curves or molar absorptivity proved that the EG-Clark reagents were notably superior to the DMSO-Clark reagents and that the DMSO-DEA-Baginski reagent was as good as the best Clark Reagent (Table IV).

Table V lists the results of the two different F-tests. The commonly employed F-test is referred to as the test for the goodness of fit which is often referred to as the significance of the regression. It was obtained by dividing the regression mean square by the error mean square (50-58). If the F-value is greater than 4.2 at a 0.05 significance level, the null hypothesis is rejected and a relationship exists between the independent and dependent variables. Woolf (54) presents a method of testing linearity of the regression of data with replicate measurements of the y values. If 'the total sum of squares' is equal to 'the deviation from linearity sum of squares' plus 'the regression sum of squares'

plus 'the within-groups sum of squares', 'the deviation from linearity sum of squares' can be obtained by subtraction. This F-test for linearity is determined by dividing the deviation from linearity mean square by the within-groups mean square. Any value smaller than 4.25 at a 0.05 significance level would indicate that a linear relationship exists. The DEA-Clark reagent lacked linearity possibly due to the lack of stability of the reagent as previously noted (Figures 12 and 14), since it did take about two hours to complete a set of readings.

7. Interferences

Using the standards in Table I, no evidence of interference due to magnesium, albumin or salicylate was noted. At a magnesium level of 14 mmol $\cdot L^{-1}$ with an EG-AMP-Clark reagent and 55 mmol L^{-1} with the Baginski reagent, a yellow precipitate was visible.

8. Comparison to the Reference Method

Since atomic absorption spectroscopy is the accepted reference method for serum total calcium

(16), calcium levels from patients' sera obtained from the clinical biochemistry laboratory at the Salvation Army Grace Hospital, Windsor, were statistically analyzed in order to compare the automated discrete analyzer methodology of the KDA (American Monitor Corp.) with atomic absorption spectrophotometry (Varian 1200 obtained from Varian Assoc. of Can., Ltd., Georgetown, Ont., L7G 2J4) at 422.7 nm on patients' sera.

The KDA employing a DMSO-AMP-Clark-like reagent compared to the atomic absorption (AA) with a correlation coefficient of 0.9932 (n = 57). Using the BMDP program of least squares (APPENDIX A), the equation comparing methods was:

KDA = 1.0397 AA - 0.13 (if x was atomic absorption)

or

AA = 0.949 KDA + 0.621 (if x was the KDA method).

The above equations are not equivalent even if they are rearranged.

As indicated by Cornbleet (50), the least squares method assumes the accurate measurement of x, and therefore, is not adequate for comparing methods if Sex/Sx was less than 0.2. The Deming correction which takes into account the error in the measurement of x

gave a slope of 1.050 rather than 1.0397. The Mandel estimate of the Deming slope was 1.0397. Since the estimate of the error of the coefficient of regression was 0.015, the corrections were of little significance, but only demonstrate the use of the calculations.

It is of interest that the Student t-test comparing the means of the two methods and the F ratio comparing variances both at 0.01 probability indicated that the data represented two different populations. The correlation coefficient was 0.9932.

Table VI compares the standard deviations of several commonly used methods.

D. CONCLUSIONS

The o-cresolphthalein complexone spectrophotometric determination of serum total calcium is superior to the other complexones examined, and due to the ease of its adaptation to automated methods, it may be superior to atomic absorption in a clinical setting.

Of the commonly used o-cresolphthalein complexone reagents in manual methods, the Baginski and EG-AMP-Clark reagent proved superior.

TABLE VI

STATISTICAL COMPARISON OF COMMON INSTRUMENTATION AND METHODOLOGIES*

Instrument and methodology	n**	Mean mg/dL	S.D. mg/dL	Coefficient of variation
Abbott (CPC-DMSO-DEA)	6	9.45	0.26	2.86
ACA (Dupont) (CPC-glycine buffer)	35	9.20	0.16	1.53
Gemeni (CPC-DMSO-AMP)	5	9.64	0.40	4.02
Hycel (CPC - DEA)	3	9.80	0.25	2.86
KDA (CPC-DMSO-AMP-Clark)	6	9.38	0.17	1.82
SMA II (Technicon) (CPC-0.25% 8-hydroxyquinoline and 0.3 N HCl.)	20	9.22	0.19	1.95
Atomic absorption	1	9.33	0.12	1.28

*Reference (59)

**n = the number of different laboratories which presented data, where each of their values are the means of usually in excess of thirty different determinations.

The DMSO-DEA Clark reagent proved to have a relatively lower molar absorptivity, a greater stability problem, a poor 'goodness of fit' for linear regression, and a non-linear regression. The DMSO-AMP-Clark reagent demonstrated a relatively low molar absorptivity. The EGA-DEA-Clark reagent lacked linearity.

Of the two preferred reagents, the Baginski reagent unexpectedly demonstrated more precision than the EG-AMP-Clark reagent even though the Baginski reagent required an extra pipetting step, thereby requiring an additional pump in an automated system. The EG-AMP-Clark reagent showed remarkable linearity relative to all other methods. The Baginski reagent contained 7.5 mg of 8-hydroxyquinoline per test whereas the Clark reagent contained only 3 mg of 8-hydroxyquinoline which was more difficult to dissolve in EG than DMSO (13, 14). 8-Hydroxyquinoline complexes with magnesium, and even though 3 mg was sufficient for the anticipated range, the extra 4.5 mg of 8-hydroxyquinoline in the Baginski reagent adds a measure of insurance against magnesium interference. The cyanide present in the Baginski reagent, but absent in the Clark reagent,

masks copper, iron, and zinc. Since these metals exhibit little or no interference, it is of little significance (14). The only advantage of EG-AMP-Clark reagent has over the Baginski reagent is that it eliminates the need for one pipetting step (pump tube). Each laboratory should conduct a comparison of the gains achieved by the EG-AMP-Clark reagent over the Baginski reagent on their particular automated system. If the Baginski reagent provided comparable precision and accuracy, the Baginski reagent should be preferred since it exhibits a higher degree of freedom from magnesium interference.

With respect to the thymolphthalein complexone, the shift in wavelength maxima due to the formation of the calcium complex serves to support the hypothesis of Clark (14). Since both the complexone and the metal-complexone of o-cresolphthalein complexone absorb at the same wavelength, linearity was maintained. o-Cresolphthalein complexone is therefore the ideal complexone for the determination of calcium.

CHAPTER III

FLUOROMETRIC DETERMINATIONS

A. INTRODUCTION

Many fluorescent complexometric methods for determining serum total calcium with fluorescein complexone (formerly known as calcein) have been published. The methods require the maintenance of a high pH for the development of the fluorescence. This has been accomplished with sodium hydroxide (60-66), potassium hydroxide (67, 68), or with glycine (69, 70). The Hill method (63, 66) which uses sodium hydroxide was tested but a problem with precision and stability as mentioned by Toffaletti (29) directed this study to the base systems of the Baginski reagent (13) or the Clark reagents (14) which proved to be more stable.

B. EXPERIMENTAL

1. Materials

Fluorescein complexone was obtained from Eastman Kodak, Rochester, N.Y., 14850, although the product

of G. Frederick Smith Chemical Co., Columbus, Ohio was reported to be superior (66). It has been suggested that fluorescein complexone is a mixture of three compounds (63).

All other materials were those used for the spectrophotometric determinations described in CHAPTER II, p. 10.

2. Apparatus

A Perkin-Elmer model 204 Fluorescence Spectrophotometer with a Perkin-Elmer model 150 Xenon Power Supply and model 56 Recorder was used and obtained from Perkin-Elmer Corp., Downsview, Ont., M3N 1Y4. Generally, the sensitivity control and the selector were set at 2 and 1/10, respectively. The 0% Adjustment knob was left on maximum due to the persistent high background fluorescence.

Other apparatus and glassware was as described for the spectrophotometric determinations described in CHAPTER II, p. 16.

3. General Method

The fluorescein complexone reagent was prepared for the fluorescent studies using the buffer system

described by Baginski for the spectrophotometric method with CPC (13). The instrument was allowed to warm up for an hour and then the wavelength settings were checked at an excitation wavelength of 365 nm and an emission wavelength of 448 nm with quinine sulfate (0.04 $\mu g\ mL^{-1}$ in 0.1 N H_2SO_4) (71). The silica gel crystals in the spectrofluorometer were routinely checked for dryness.

Into a test tube, 60 μL of a aqueous standard and then 3 mL of reagent containing 39.2 mg of fluorescein complexone followed by 3 mL of 40 $mL\ L^{-1}$ DEA were pipetted. The contents were mixed by inversion after covering the test tube with parafilm and immediately read in the spectrofluorometer.

The concentration of the DEA was varied, as well as the concentration of the fluorescein complexone in an effort to optimize the analytical parameters.

As a result of the above, a modified method containing a 20-μL sample of calcium and a 17.5 mg fluorescein-complexone-Baginski-like reagent was used. This optimized system was studied with respect to linearity with calcium concentration, the nature of the complex, and interferences.

C. RESULTS AND DISCUSSION

1. Preliminary

The Hill method lacked precision (S.D. = 0.5). Drift was noted with the spectrofluorometer on very humid days even when newly dried silica gel crystals were used. Calibration curves were not reproducible.

There was no convenient method of controlling the cuvet temperature. Hill did point out that a 1°C change in temperature would change the fluorescence by 2% (63). All readings were done at ambient temperature.

2. Optimum pH Studies

As a result of a compromise between a high blank and the quantity of fluorescence with calcium, a buffer system of 40 mL of DEA per liter resulting in an apparent pH of 11.3 was chosen as the optimum. The data are shown in Figure 22.

3. The Optimal Fluorescein-Complexone Concentration

The emission spectra of the Ca-complex of fluorescein complexone (39.2 mg L^{-1}) demonstrated

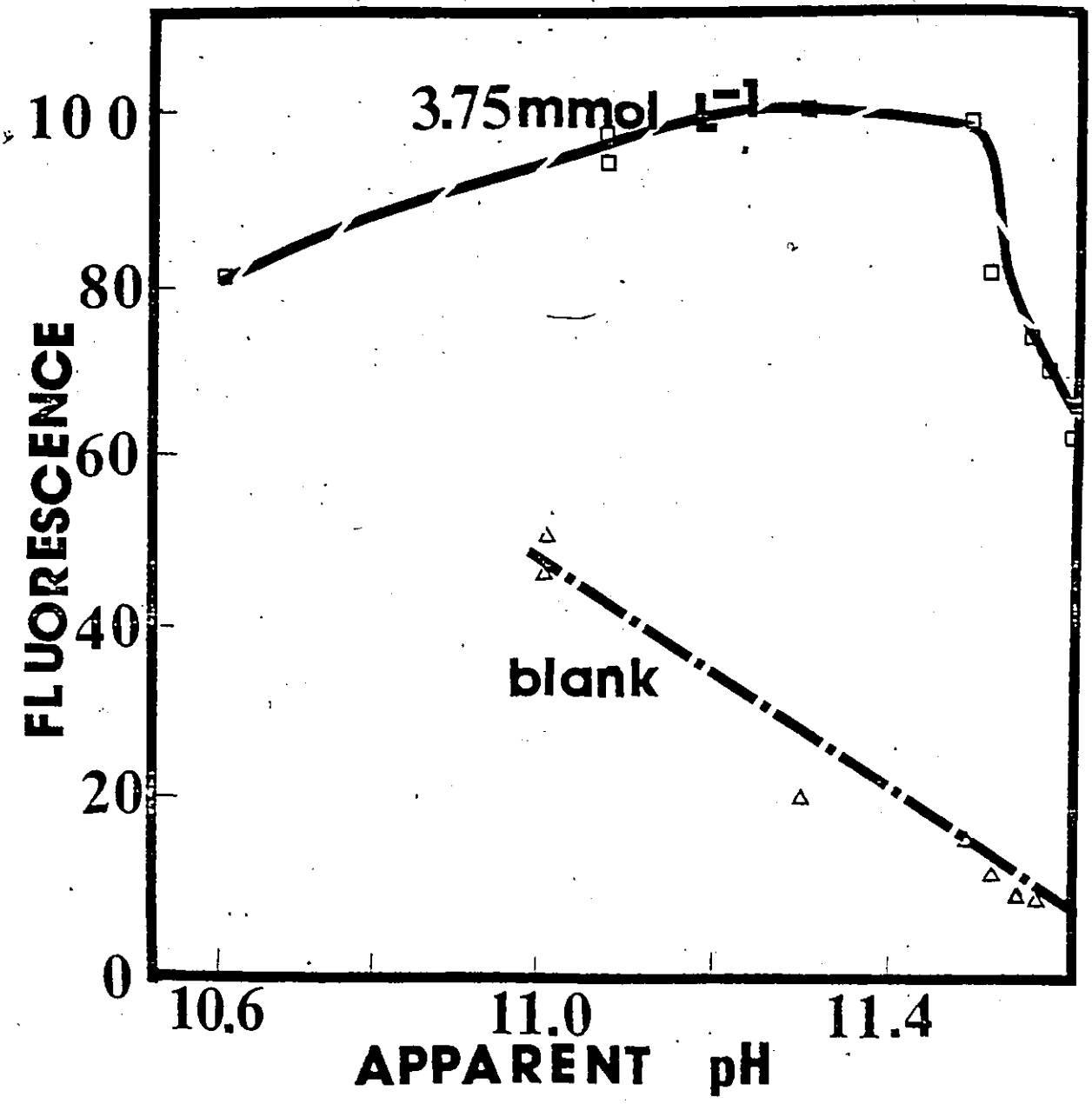
FIGURE 22

THE EFFECT OF APPARENT pH ON THE FLUORESCENCE OF FLUORESCEIN COMPLEXONE WITH 3.75 MMOL L^{-1} OF CALCIUM

Legend

The concentrations of the DEA in the alkaline buffer were varied through 4.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 80.0, 100.0, to 140.0 mL L^{-1}, respectively. The fluorescence of blanks and 3.75 mmol L^{-1} of calcium standards were measured using these buffers. The apparent pH of each blank and standard was measured. The settings for the sensitivity and selector of the spectrofluorometer were at 3 and 1/10, respectively. Excitation wavelength of 466 nm and emission wavelength of 522 nm were used.

FIGURE 22


3.75mmol L⁻¹
blank
FLUORESCENCE
100
80
60
40
20
0
10.6
11.0
11.4
APPARENT pH

a slight hypsochromic shift in the wavelength maximum (Figure 23) with increased concentration of calcium. The corresponding excitation spectra showed a maximum at 466 nm which increased out of proportion to the maximum at 489 nm with increasing calcium concentration (Figure 24).

These excitation spectra were difficult to repeat. A new peak at 521 nm (Figure 25) was present if old yellowed DEA was used. Figure 26 shows similar experimental data using freshly distilled DEA. The maximum wavelengths for the emission and excitation spectra varied with the concentration of the fluorescein complexone (Figures 25-27). Figure 26 demonstrated a hypsochromic shift with the excitation wavelength maxima and a corresponding bathochromic shift with the emission maxima with increasing fluorescein-complexone concentration. Linearity was in evidence in only a narrow portion of the fluorescein-complexone-concentration range studied as shown in Figure 27. Linearity was present at 39 mg L^{-1} of fluorescein complexone. The organic solvent DMSO with a dielectric constant lower than that of water enhanced the wavelength maximum of 466 nm as noted for 5 mmol L^{-1} of calcium in Figure 28.

FIGURE 23

EMISSION SPECTRA OF FLUORESCEIN COMPLEXONE SHOWING THE EFFECT OF VARIOUS CALCIUM CONCENTRATIONS

Legend

A two-solution Baginski-like reagent was used. The first solution contained 39.2 mg of fluorescein complexone, 1 dL of DMSO, and 2.5 g of 8-hydroxyquinoline diluted to one liter with distilled water. The second solution contained 40 mL of DEA with 0.5 g of potassium cyanide diluted to 1L with water. A sample (60 µl) was mixed with 3 mL of each solution. Samples contained 1.25, 2.50, 3.75, and 5.0 mmol L^{-1} of calcium standards, respectively. Excitation wavelength of 466 nm was used.

FIGURE 23

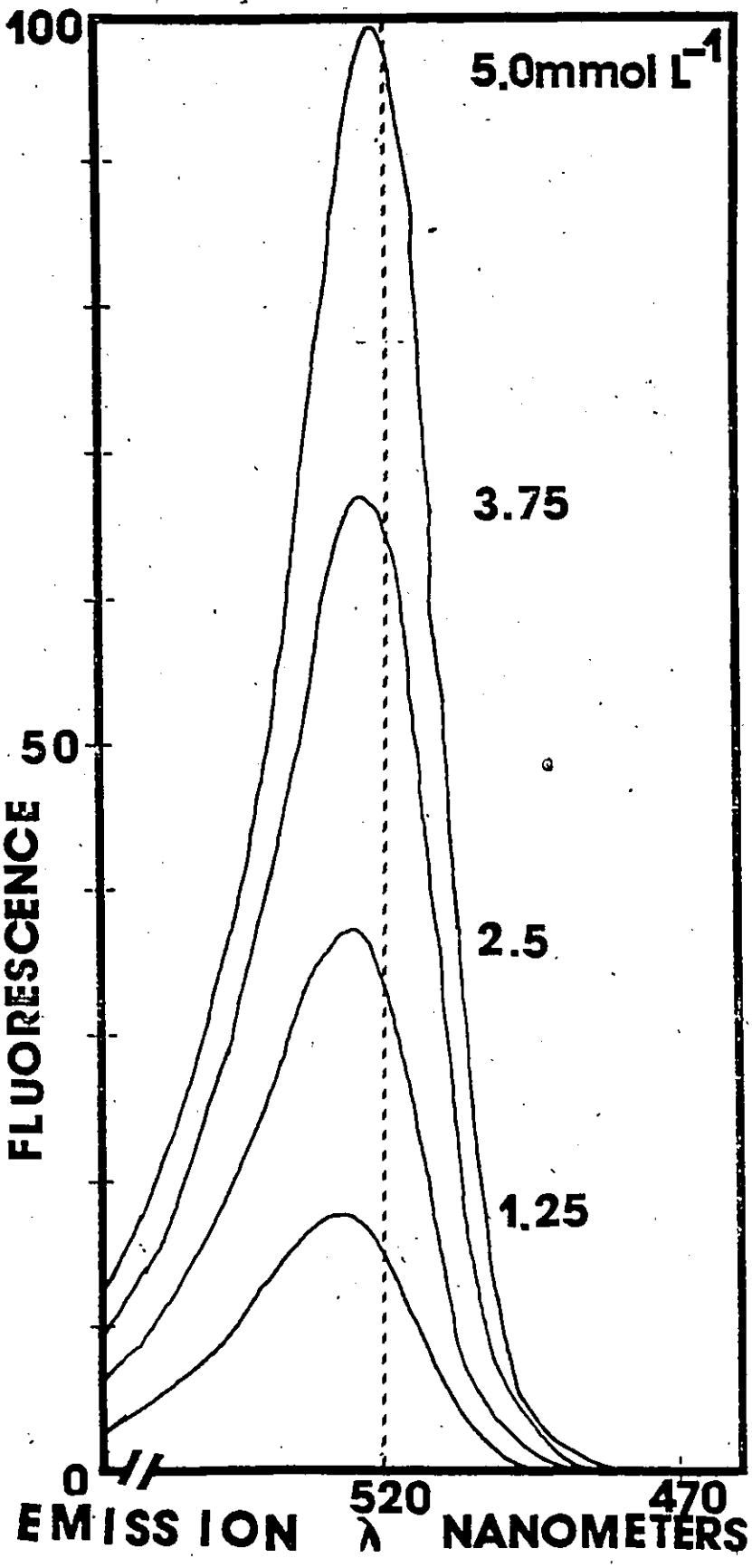

100
50
0
FLUORESCENCE
5.0mmol L-1
3.75
2.5
1.25
520
λ
470
EMISSION
NANOMETERS

FIGURE 24

EXCITATION SPECTRA OF FLUORESCEIN COMPLEXONE SHOWING THE EFFECT OF CALCIUM CONCENTRATION

Legend

The excitation spectra were obtained using the reaction conditions corresponding to the emission spectra of Figure 23. Emission wavelength was 522 nm.

FIGURE 24

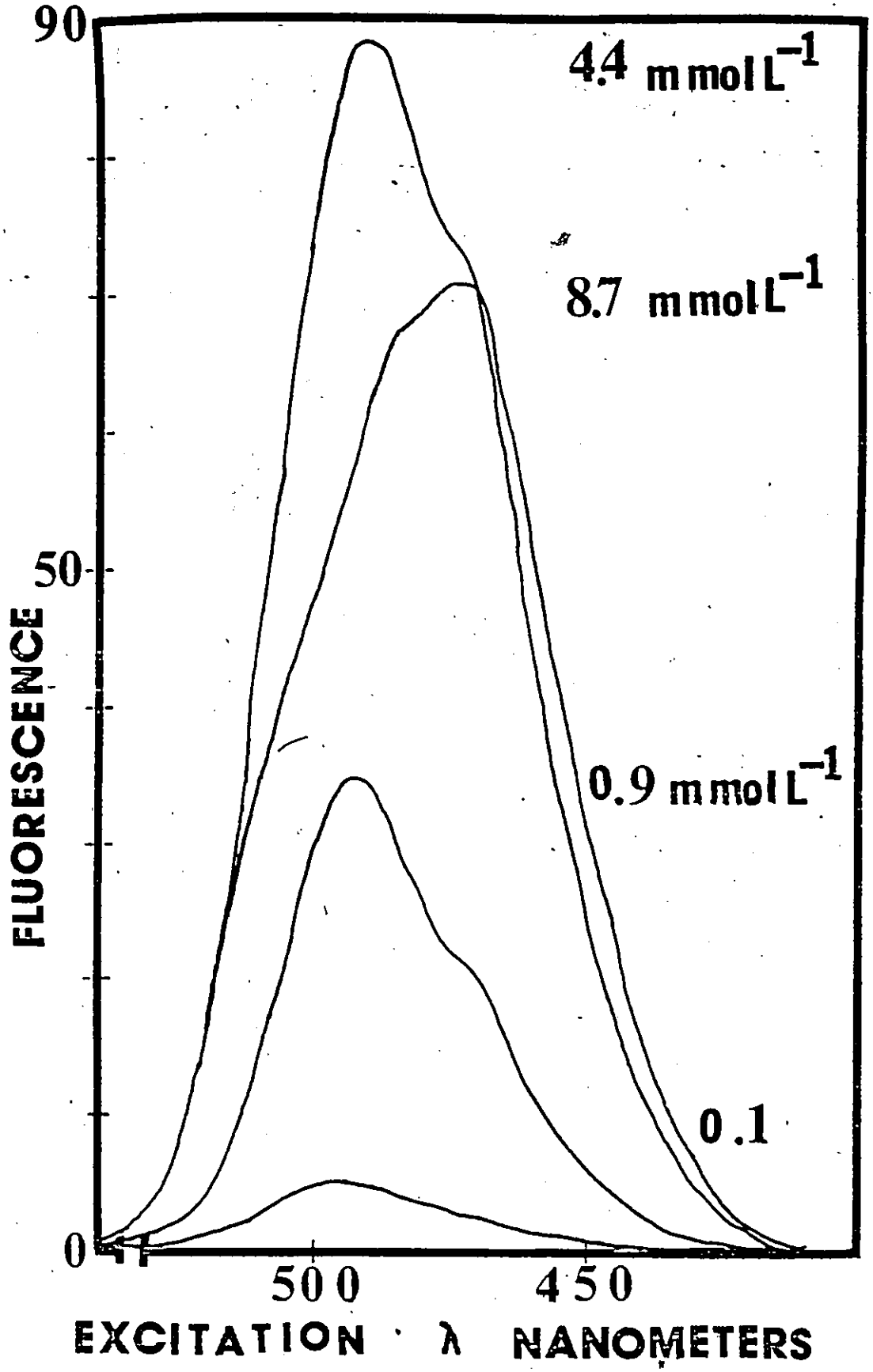

90
50
0
FLUORESCENCE
4.4 mmol L^{-1}
8.7 mmol L^{-1}
0.9 mmol L^{-1}
0.1
500
450
EXCITATION · λ NANOMETERS

FIGURE 25

EXCITATION SPECTRA OF FLUORESCEIN COMPLEXONE SHOWING THE EFFECT OF CALCIUM CONCENTRATION

Legend

This excitation spectra corresponds to the experimental conditions for Figures 23 and 24 except that DEA which had not been distilled after it had become yellow with age was used in the preparation of the reagent.

FIGURE 25

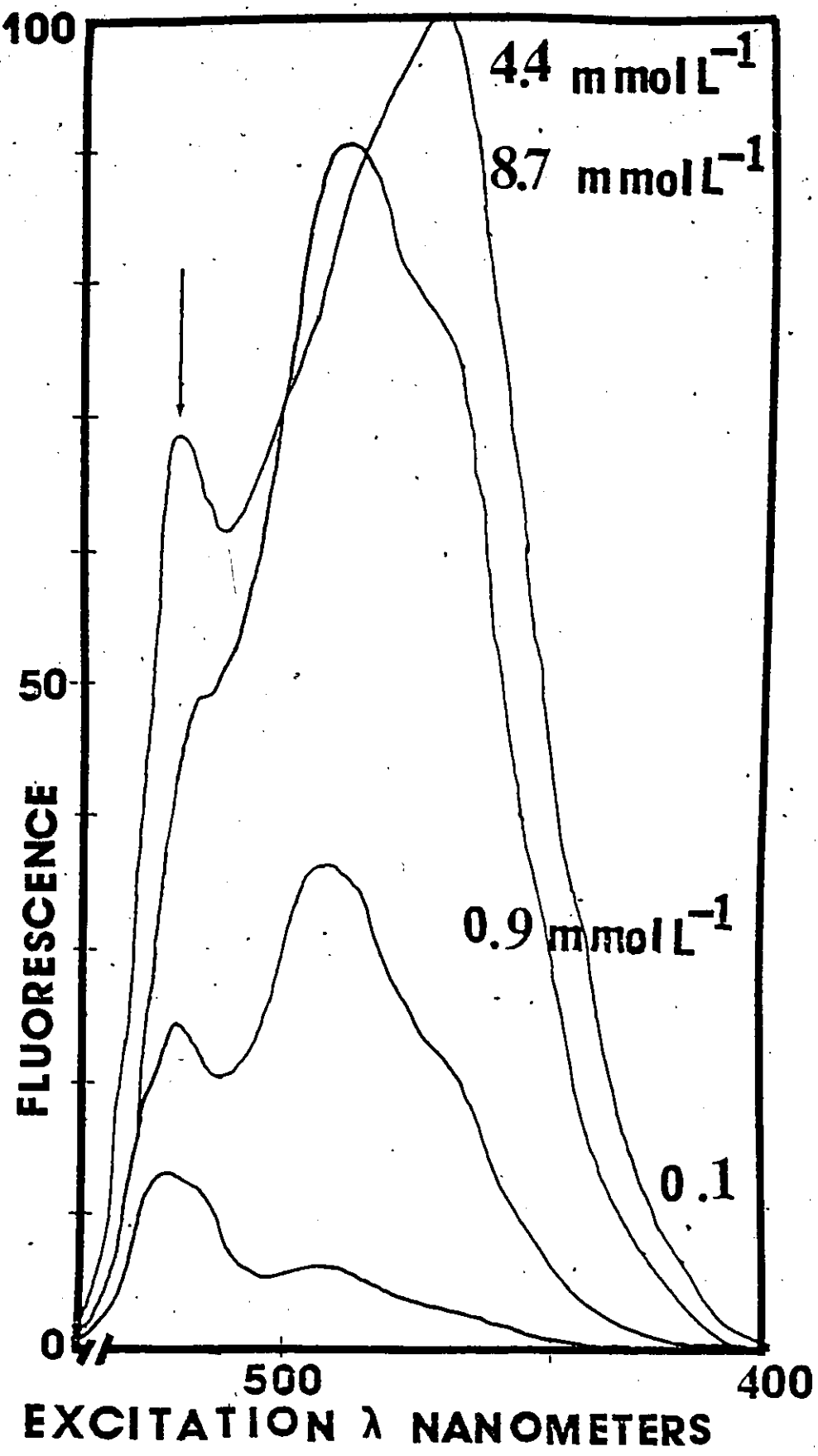

100
50
0
FLUORESCENCE
4.4 mmol L⁻¹
8.7 mmol L⁻¹
0.9 mmol L⁻¹
0.1
500
400
EXCITATION λ NANOMETERS

FIGURE 26

FLUORESCEIN-COMPLEXONE CONCENTRATION AND EXCITATION-EMISSION WAVELENGTH MAXIMA

Legend

Baginski-like reagent containing 10.0, 20.0, 30.0, 40.0, 50.0, and 60.0 mg L^{-1} of fluorescein complexone, respectively, were used with 5 mmol L^{-1} standard calcium samples.

Emission wavelength maxima

Excitation wavelength maxima

FIGURE 26

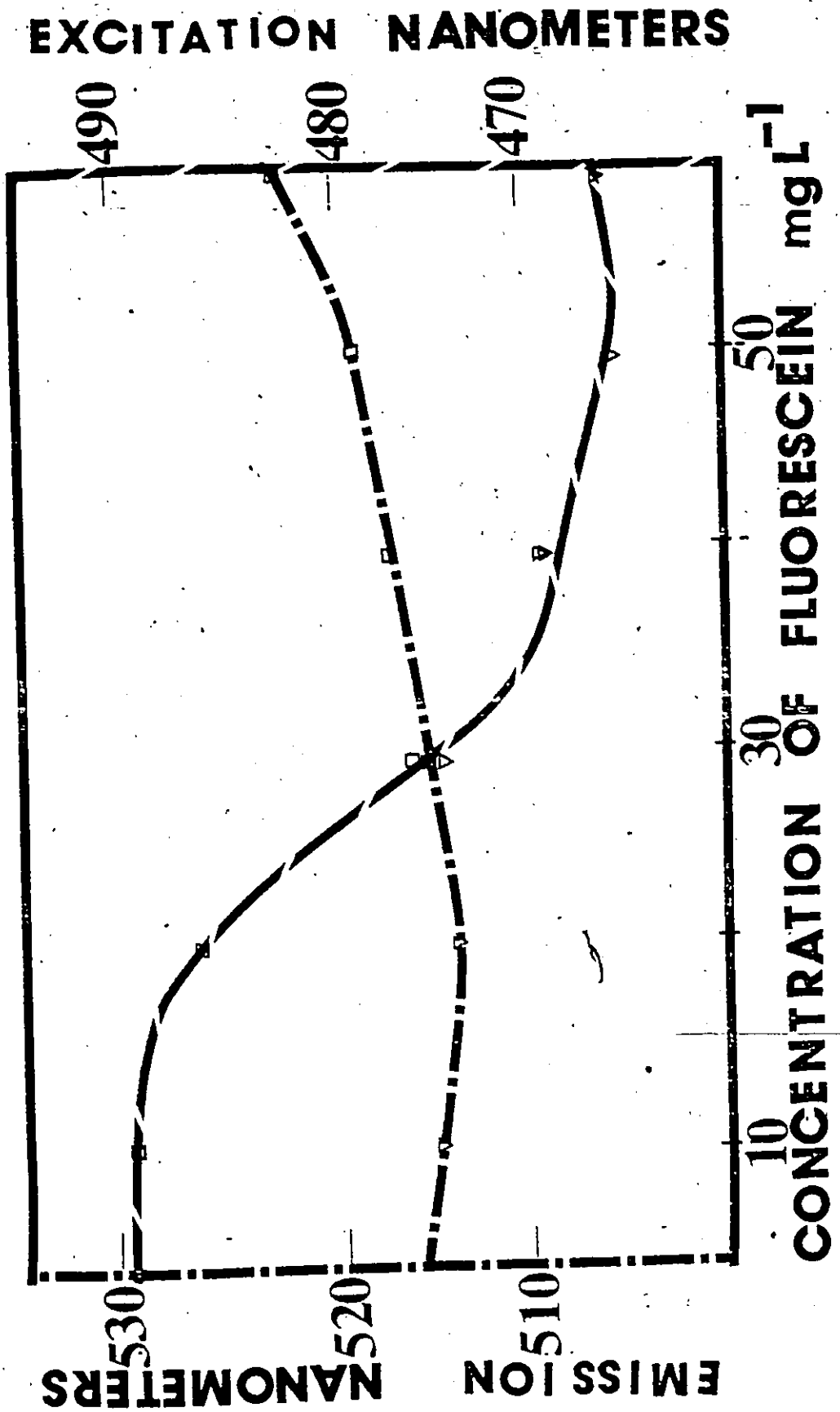

EXCITATION NANOMETERS
490
480
470
50
30
10
CONCENTRATION OF FLUORESCEIN mg L⁻¹
530
520
510
EMISSION NANOMETERS

FIGURE 27

EMISSION FLUORESCENCE WITH VARIOUS FLUORESCEIN-COMPLEXONE CONCENTRATIONS

Legend

Baginski-like reagents containing 10.0, 20.0, 30.0, 40.0, 50.0, and 60.0 mg L^{-1} of fluorescein complexone, respectively, were used with 5.0, 3.75, 2.50, and 1.25 mmol L^{-1} of calcium standards, respectively. An EGTA standard was used as well.

FIGURE 27

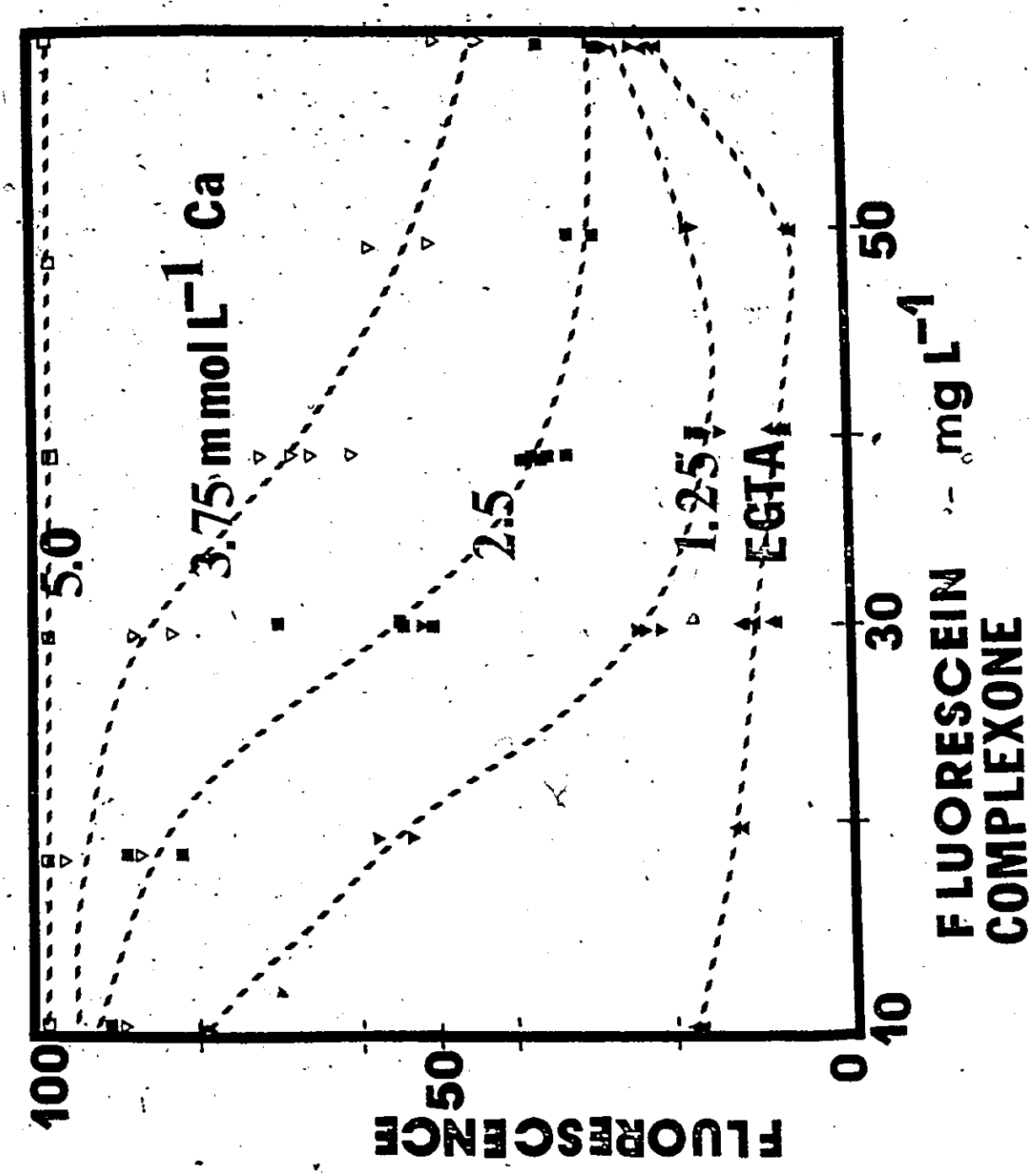

5.0
3.75 mmol L⁻¹ Ca
2.5
1.25
EGTA
100
50
0
FLUORESCENCE
50
30
10
FLUORESCEIN COMPLEXONE - mg L⁻¹

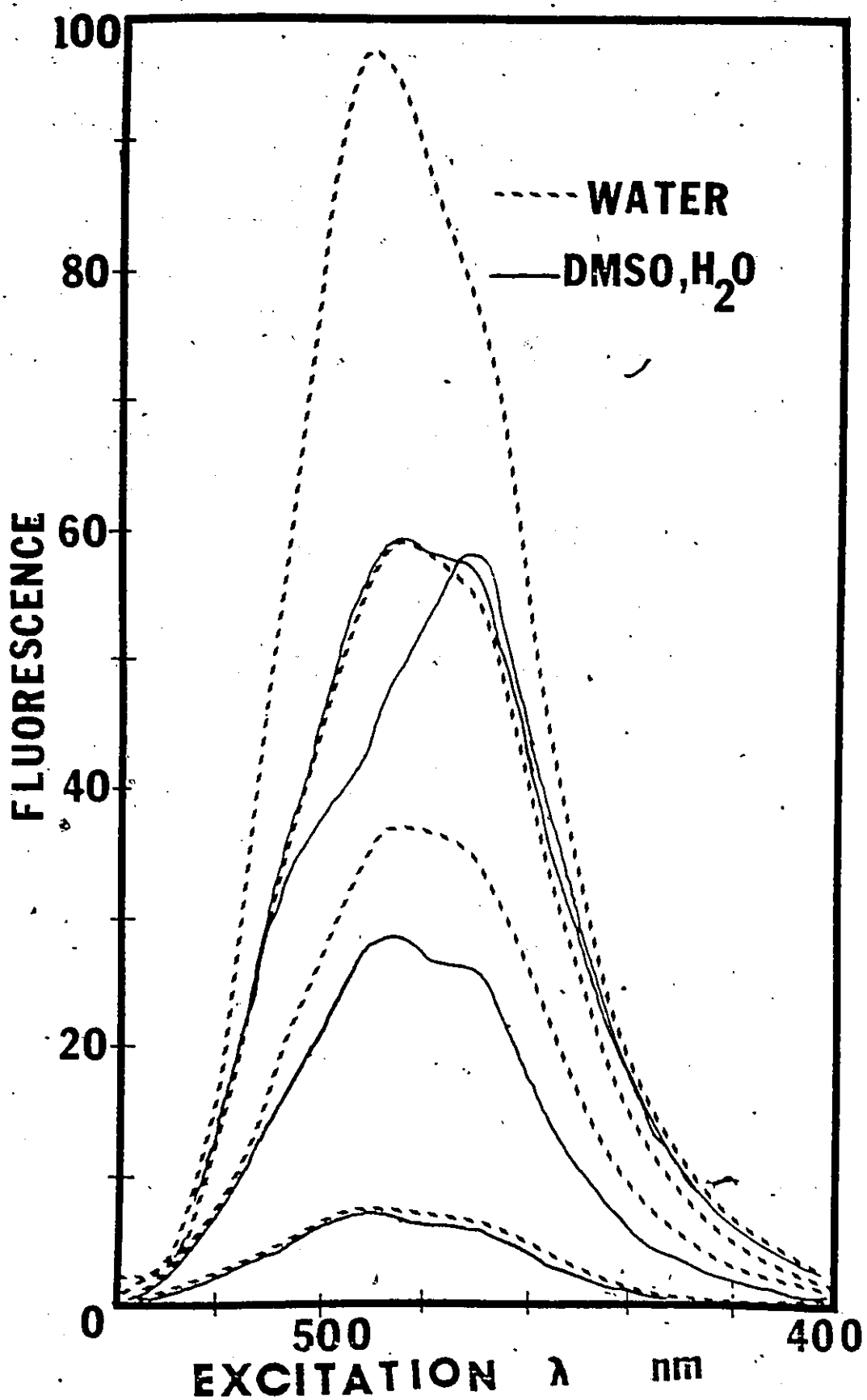
FIGURE 28

EXCITATION SPECTRA OF FLUORESCEIN COMPLEXONE DEMONSTRATING THE EFFECT OF DMSO

Legend

The excitation spectra for the Baginski-like reagent with 5.00, 3.75, 2.50, and 0.00 mmol L^{-1} of calcium, respectively, are presented as a solid line with the corresponding excitation spectra produced by a Baginski-like reagent lacking DMSO represented by the broken line.

FIGURE 28


100
80
60
40
20
0
FLUORESCENCE
WATER
DMSO,H2O
500
400
EXCITATION λ nm

With 60 mg L^{-1} fluorescein-complexone concentration, the excitation wavelength maximum at 466 nm predominated but did not demonstrate linearity with respect to calcium (Figure 29). A situation similar to Figure 27 existed and linearity was improved slightly by increasing the sample sizes to 120 µL rather than 60 µL. The Yoe-Jones' plot (Figure 30) confirmed the suspicion that at this concentration two calcium ions combine with each fluorescein complexone.

At a lower concentration of 17.5 mg L^{-1} of fluorescein complexone, the excitation maximum of 482 nm predominated; but in order to obtain a linear relationship, the sample volume was lowered to 20 µL. This produced the excellent linearity with respect to calcium concentration and wavelength maximum remained constant as shown in Figures 31 and 32. The Yoe-Jones' plot, Figure 33, confirmed the suspicion that the fluorescent species consisted of one calcium ion chelated to one complexone molecule. The statistical analysis as described in APPENDIX A resulted in a correlation coefficient of 0.9010 between the fluorescence intensity and the calcium concentration (mmol L^{-1}). The regression coefficient

FIGURE 29

EXCITATION SPECTRA OF FLUORESCEIN COMPLEXONE WITH VARYING CALCIUM CONCENTRATIONS

Legend

Baginski-like reagent with fluorescein complexone at 60 mg L^{-1} was used to determine 0.00, 1.25, 1.65, 5.00, and 12.50 mmol L^{-1} of calcium, respectively.

FIGURE 29

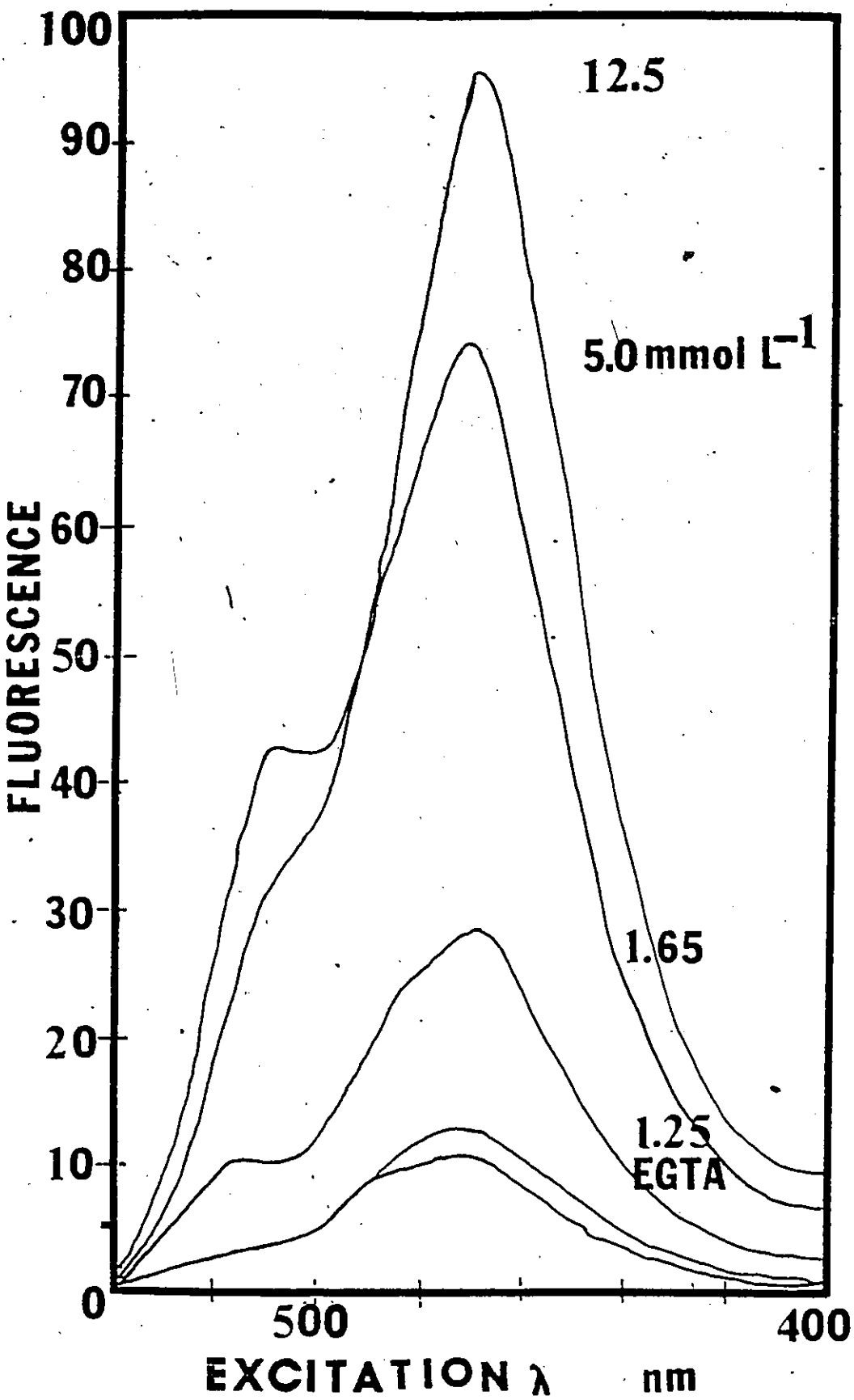

100
90
80
70
60
50
40
30
20
10
0
FLUORESCENCE
12.5
5.0 mmol L⁻¹
1.65
1.25
EGTA
500
400
EXCITATION λ nm

FIGURE 30

YOE-JONES' PLOT WITH FLUORESCEIN COMPLEXONE AND CALCIUM

Legend

Calcium to fluorescein-complexone concentration ratios of 0.30, 0.39, 0.59, 0.59, 1.19, 1.19, 1.78, 2.38, 3.57, 5.95, 11.9, 17.9, and 23.8 were used. The points at a fluorescence of 70 were not used in drawing the lines. This study was carried out with a reagent containing 60 mg L^{-1} of fluorescein complexone. Least squares regression were used to obtain the lines.

FIGURE 30

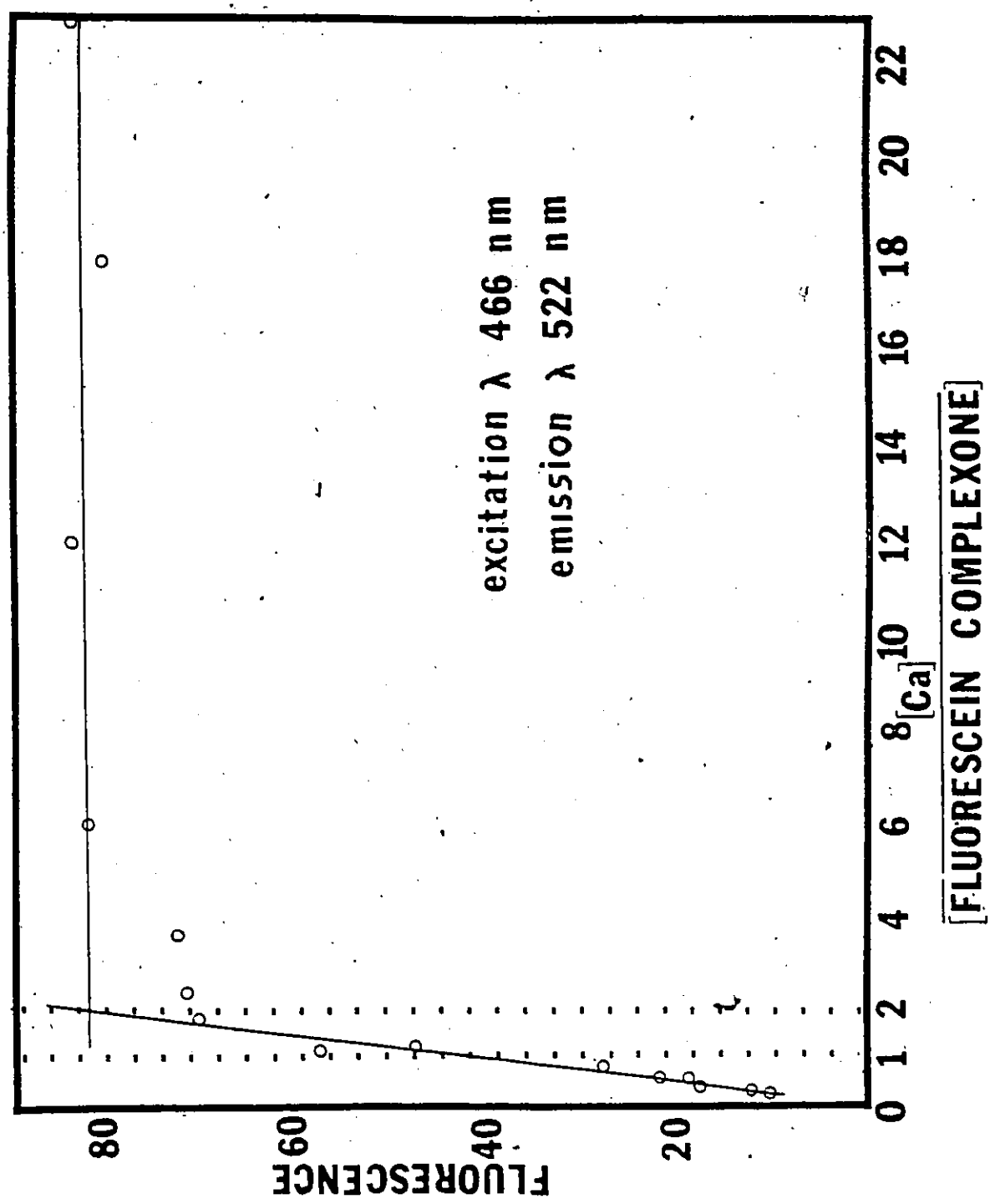

excitation λ 466 nm
emission λ 522 nm
FLUORESCENCE
80
60
40
20
0 1 2 4 6 8 10 12 14 16 18 20 22
[Ca]
[FLUORESCEIN COMPLEXONE]

FIGURE 31

EXCITATION SPECTRA OF FLUORESCEIN COMPLEXONE WITH VARYING CALCIUM CONCENTRATIONS

Legend

A 17.5 mg L^{-1} of fluorescein-complexone-Baginski-like reagent was used with 20 µL of calcium standards of 0.00, 1.25, 2.50, 3.75, and 5.00 mmol L^{-1}. The buffer solution used was 40 mL of DEA L^{-1}. An EGTA blank was used to indicate the degree of calcium contamination in the water, buffer, and fluorescein-complexone reagent.

FIGURE 31

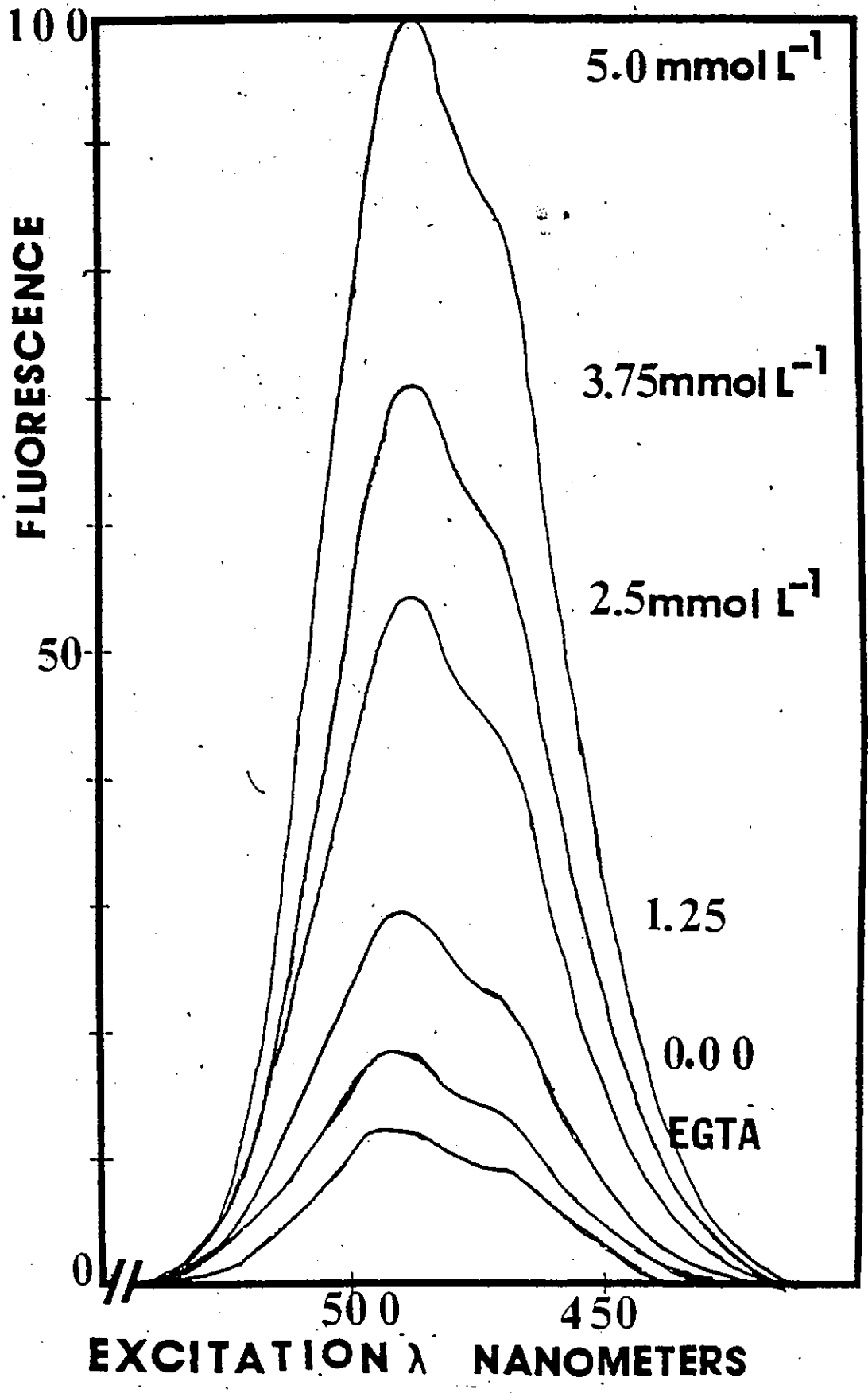

100
50
0
FLUORESCENCE
5.0 mmol L-1
3.75mmol L-1
2.5mmol L-1
1.25
0.00
EGTA
500
450
EXCITATION λ NANOMETERS

FIGURE 32

EMISSION SPECTRA OF FLUORESCEIN COMPLEXONE WITH VARYING CALCIUM CONCENTRATIONS

Legend

These spectra correspond to the excitation spectra of the respective emission spectra shown in Figure 31. An excitation wavelength of 482 nm was used.

FIGURE 32

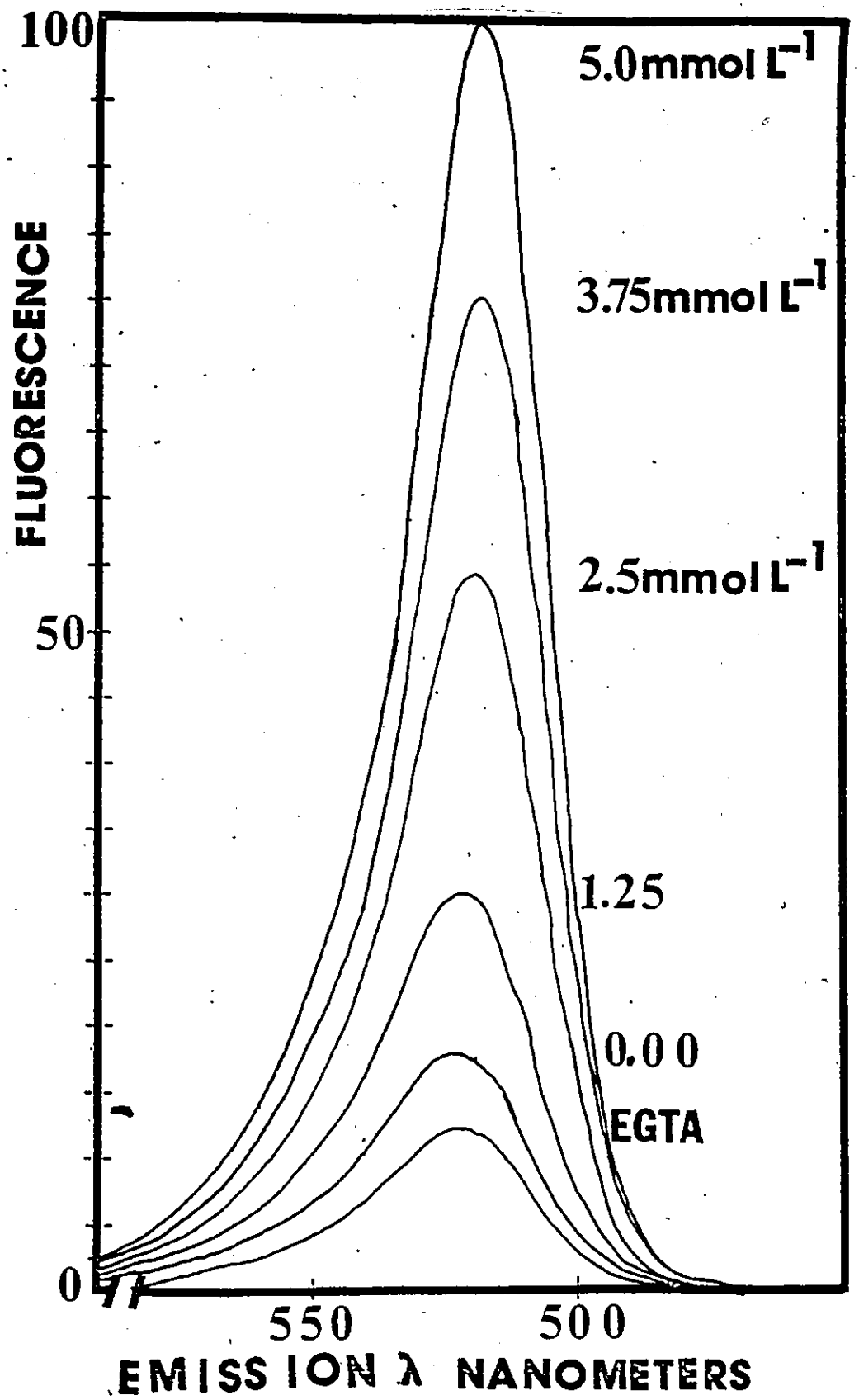

100
50
0
FLUORESCENCE
5.0mmol L⁻¹
3.75mmol L⁻¹
2.5mmol L⁻¹
1.25
0.0 0
EGTA
550
500
EMISS ION λ NANOMETERS

FIGURE 33

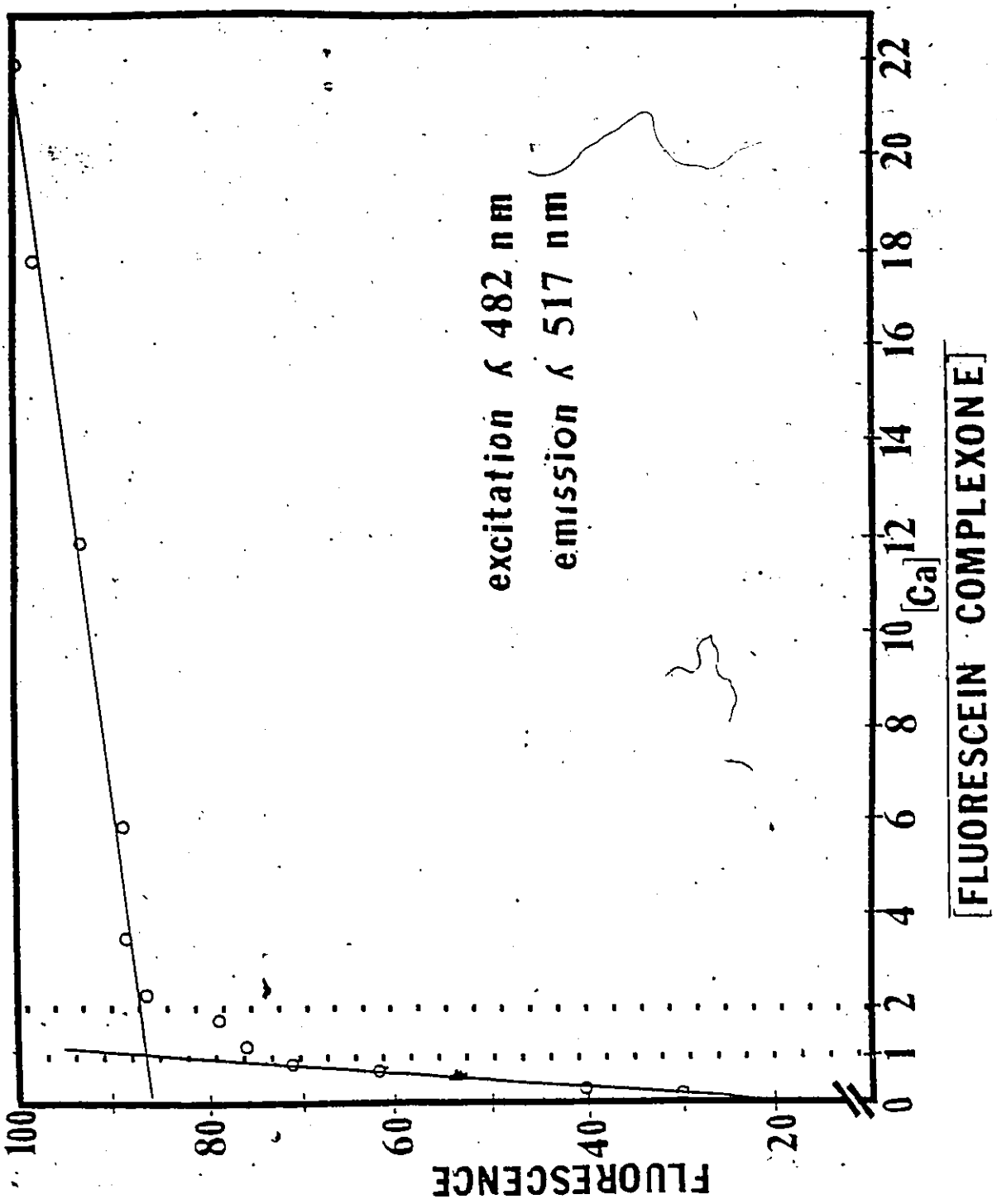
YOE-JONES' PLOT WITH FLUORESCEIN COMPLEXONE AND CALCIUM

Legend

Calcium to fluorescein-complexone concentration ratios of 0.015, 0.29, 0.60, 1.20, 2.38, 3.57, 5.95, 11.90, 17.85, and 21.80 were used to determine the plot. This study was carried out with a reagent containing 17.5 mg L^{-1} of fluorescein complexone.

The data near a fluorescent intensity of 80 was not used to determine the equation of the lines by least-squares regression.

FIGURE 33


excitation λ 482 nm
emission λ 517 nm
FLUORESCENCE
100
80
60
40
20
0
1
2
4
6
8
10
12
14
16
18
20
22
[Ca]
[FLUORESCEIN · COMPLEXONE]

was 20.0 and the intercept was 0.08. A standard deviation of the calcium estimate was 0.004 mmol L^{-1}, n = 30. If this fluorescein-complexone method for calcium were to be automated, a sample size of 7 µL with 1 mL of the above fluorescein-complexone solution and 1 mL of the DEA buffer would be required, thus making this method suitable for application in the neonatal/pediatric situation.

The changes in response due to the varying of the fluorescein-complexone concentration deserve explanation. The observations were consistent with the law of mass action (72) if the ionization constant for the carboxyl group of one of the iminodiacetates of the complexone in this buffer system was much larger for

$$H_4In^{2-} \rightleftharpoons H_3In^{3-}$$

than it was for

$$H_3In^{3-} \rightleftharpoons H_2In^{4-}$$

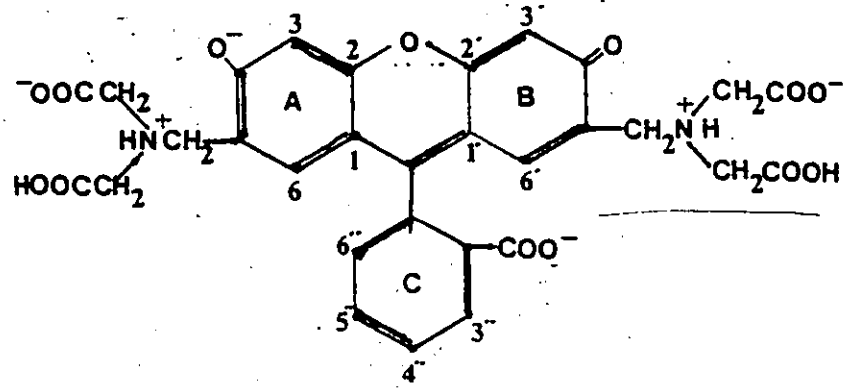
With low concentrations of fluorescein complexone, the H_3In^{3-} species would be favored, and therefore, there would be only one position available to chelate the calcium ion. With high concentrations of fluorescein complexone the equilibrium would be forced

by the law of mass action to favor the H_2In^{4-} species leaving two positions free to chelate calcium ions. Lowering the dielectric constant would have the same effect as increasing the concentration of H_4In^{2-}.

This is consistent with the conclusion that the species forming the excitation peak at 482 nm and emission peak at 517 nm was CaI-fluorescein complexone, and the species forming the excitation peak at 466 nm and emission peak at 522 nm was the CaII-fluorescein complexone.

If the above is indeed the case, the titration curve for 0.01 M fluorescein complexone with 1.0 M NaOH should demonstrate pK_a values for the ionization of the carboxyl group in the 2" position, the hydroxyl group in the 4 position, one carboxyl group of one of the iminodiacetates and then the other (30). Unlike the other complexones examined, fluorescein complexone is not symmetrical (see Figure 2) and therefore it was hoped that the carboxyl groups of the iminodiacetates would not react identically. Instead of the anticipated four-step sodium hydroxide titration curve a smooth S-shaped curvature with one pK_a value near 7.5 was actually obtained. It

is possible that some of the expected titration steps (pK_a's) were not observed due to the relative strength of the sodium hydroxide used.



4. Interference Study

As Figure 34 demonstrates, an unacceptable interference due to albumin was obtained when the samples described in Table I were used with the 1.75 mg L^{-1} Baginski-like fluorescein-complexone reagent. This may be due to a light scattering effect (66) caused by the protein since the excitation and emission wavelengths are close and the slitwidths of the excitation and the emission monochromator are 10 nm (66). The resultant error of about 0.1 mmol L^{-1} of calcium for every 1 g L^{-1} change in serum albumin was totally unacceptable for application to the clinical laboratory.

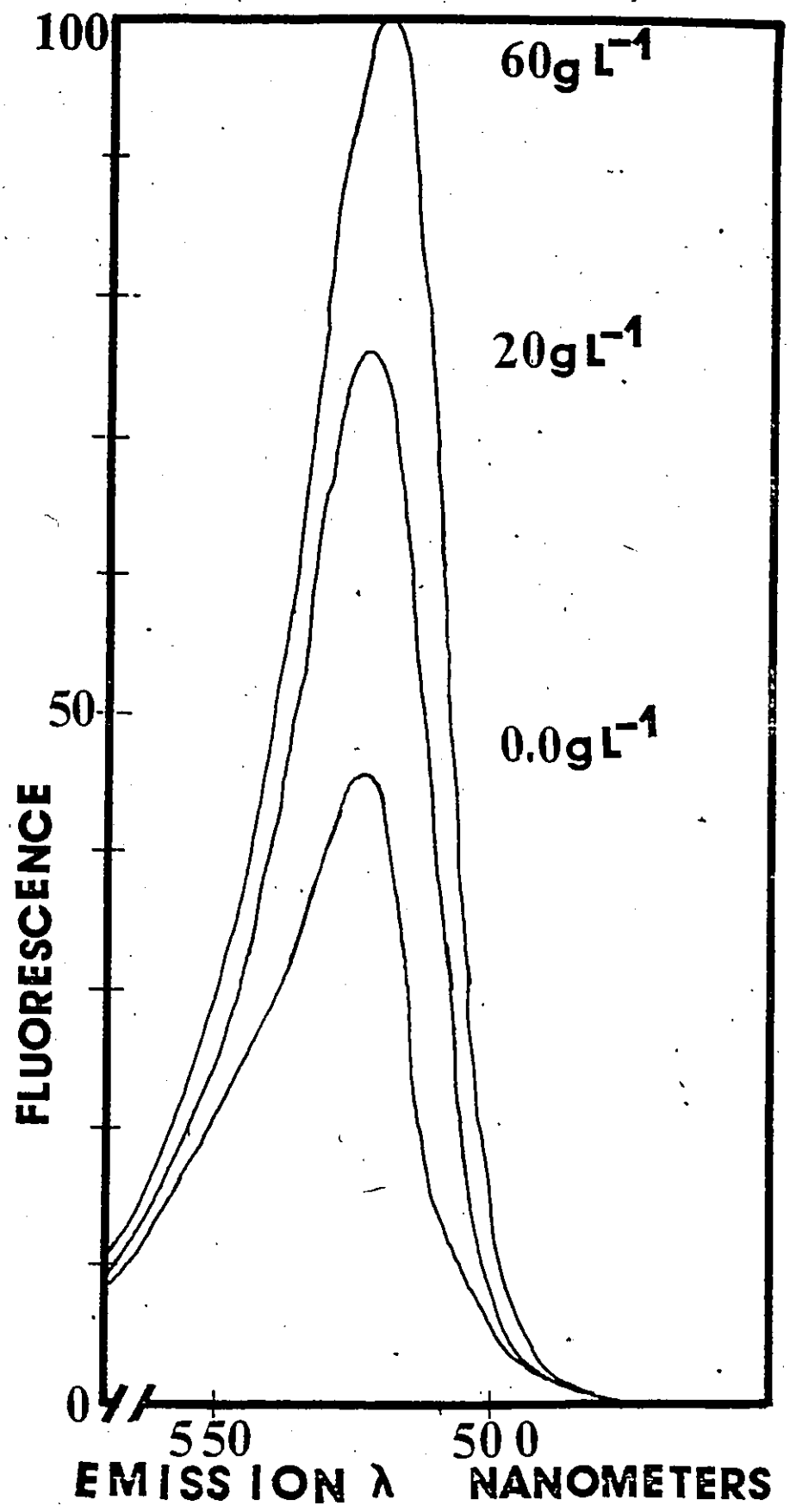
FIGURE 34

EMISSION SPECTRA OF FLUORESCEIN COMPLEXONE SHOWING THE EFFECT OF PROTEIN

Legend

3.75 mmol L^{-1} of calcium were determined with 0.0 , 20 and 60 g L^{-1} of albumin, respectively.

FIGURE 34


100
50
0
60g L-1
20g L-1
0.0g L-1
FLUORESCENCE
550
500
EMISSION λ NANOMETERS

D. CONCLUSION

Due to the interference by protein, a direct method for determining serum total calcium with fluorescein complexone seems unlikely. A precipitation or dialysis step to eliminate the protein with the inherent problems (10, 11) would not be advised. Back titration with ethylenediaminetetraacetic acid (EDTA) or EGTA as described in several papers (62, 65, 69, 70) would result in a loss of precision due to an increase in the number of steps required.

The only advantage the fluorescent method has over the CPC spectrophotometric methods was the greater sensitivity of the method thereby enabling the use of smaller sample sizes useful in neonatal/pediatric patients.

CHAPTER IV

SUMMARY AND RECOMMENDATIONS

A. THE BLANKING HYPOTHESIS

The proposal of Clark (14) that the o-cresolphthalein complexone which absorbs at the same wavelength as the CaIICPC is removed as CaIICPC forms is supported by the shifts in the wavelength maxima of other complexones as calcium was added. These shifts can be explained by the gradual conversion of the complexone which absorbed at one wavelength by the Ca-complexone which absorbed at another wavelength maximum.

B. RECOMMENDED METHOD

o-Cresolphthalein complexone was therefore found to be the ideal complexone for serum calcium determination since no shift in maximum wavelength with the addition of calcium was observed.

The Baginski method (13) is recommended over the EG-AMP-Clark method due to its greater freedom from interference due to magnesium, copper, iron, and zinc. The Baginski reagents used in a manual

method showed greater precision even though they required an extra pipetting step. Therefore, on each automated system, one might expect that a comparison of the Baginski and the EG-AMP-Clark reagents would produce varying results. Also, the temperature of the reaction for all the CPC methods must be kept constant and preferably low.

Some increase in the precision could be obtained if larger sample sizes were used, however, multipurpose automated instruments use sample sizes less than optimal for determining total calcium. It has been suggested or at least implied that to increase the precision required for serum total calcium determinations, duplicate samples should be analyzed on different occasions and an average value used. If four samples were run, the standard deviation of the average would be S.D./$\sqrt{4}$ or one-half that normally expected (73).

A direct method for determining serum total calcium was preferred for it has the added advantage of permitting the subtraction of interference due to lipemic, hemolyzed or otherwise problematic sera. By adding EGTA to a duplicate sample, the degree of interference can be estimated (13).

C. FURTHER RESEARCH

A spectrofluorometer with a bandwidth less than 10 nm may eliminate the albumin interference problem which was encountered with the fluorescein complexone. Also, the temperature effect for the fluorescent method deserves consideration.

APPENDIX

A. MULTIPLE LINEAR REGRESSION SAMPLE PROGRAM

1. *Input* (74)

The following was typed into a WYLBUR terminal.

```
//PARKSIR   JOB (R121XXXXX),'JOHN G.PARKS',CLASS=A
//  EXEC    BIMED,PROG=BMDP1R
//SYSIN DD *
/PROBLEM    TITLE IS 'CRESOLPHTHALEIN CALCIUM DETER-
            MINATIONS EG AMP'.
/INPUT      VARIABLES ARE 3.
            FORMAT IS '(F1.0,F4.1,F5.3)'.
/VARIABLE   NAMES ARE BLANK,CALCIUM,ABSORB.
            GROUPING IS BLANK.
/GROUP      CODES(1) ARE 1.,2.,3..
            NAMES(1) ARE WATER,CPC,EGTA.
/REGRESS    DEPENDENT IS CALCIUM.
            INDEPENDENT IS ABSORB.
/PRINT      DATA.
            CORRELATION.
            COVARIANCE.
?END
1 050 293
1 050 290
2 010 405
```

```
2 010 398
...
//
RUN
```

2. *Output*

```
1.  WATER
VARIABLE    MEAN        S.D.    COEF.VARI.MIN.     MAX.
2 CALCIUM  10.08928   3.69447 0.36618   5.000    1.500
3 ABSORB    0.92271   0.26289 0.28491   0.567    1.336
COVARIANCE MATRIX
                          CALCIUM      ABSORB
                            2            3
CALCIUM 2                 13.6491
ABSORB  3                  0.9658      0.0691
CORRELATION MATRIX
                          CALCIUM      ABSORB
                            2            3
CALCIUM 2                  1.0000
ABSORB  3                  0.9944      1.0000
REGRESSION TITLE.....CRESOLPHTHALEIN CALCIUM DETERMIN
DEPENDENT VARIABLE..... 2 CALCIUM
TOLERANCE..................... 0.0100
MULTIPLE R   0.9944   STD. ERROR OF EST. 0.3963
MULTIPLE R-SQUARE  0.9889
```

ANALYSIS OF VARIANCE

	SUM OF SQUARES	OF	MEAN SQUARE	F RATIO	P
REGRESSION	364.442	1	364.442	2320.85	0.0
RESIDUAL	4.083	26	0.157	0.15	

VARIABLE	COEFF.	STD.ERROR	STD.REG.	T	P(2)
INTERCEPT	-2.806				
ABSORB 3	13.975	0.290	0.994	48.175	00.0

CASE LABEL	RESIDUAL	PREDICTED VALUE	VARIABLES 2CALCIUM	3ABSORB
1	-0.2362	15.2362	15.0000	1.2910
2	-0.2222	15.2222	15.0000	1.2900
3	-0.8651**	15.8651	15.0000	1.3360
4	0.2222	15.2222	15.0000	

...

*The number of standard deviations from the mean.

The program then repeats all calculations for the CPC blank absorbances and then the EGTA blank absorbances.

3. *Variations on the Computer Routine*

The multiple linear regression program has a plotting function as well, and if used will plot the residuals against the predicted, the predicted and observed against the calcium, the normal probability

plot of residuals against expected, and also the detrended normal probability of residuals against the deviations from the expected normal.

The BMDP2V program for the analysis of variance on repeated measures was also employed.

The University computer facility had the capacity to store programs in small personal libraries. With the aid of the WYLBUR, programs could then be recalled weeks later, quickly modified with new absorbance readings and run.

Most important was the print-out copy containing both your input data and the out-put which could then be reviewed for errors.

B. F-TESTS

1. *Goodness of Fit*

Using a Commodore hand calculator and its regression functions the data was entered as absorbance x100 for y and calcium in μmol L^{-1} for x. Woolf's test for linearity assumes the data values are greater than one (54).

Memory 1 ΣXY = 53143750

Memory 2 ΣY^2 = 13610356

Memory 3 ΣY = 207812500

Memory 4 $\Sigma X^2 = 207812500$

Memory 5 $\Sigma X = 72500$

Memory 6 $n = 28$

$$\Sigma x^2 = \Sigma(X - \bar{x})^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{n} = 20089285.72$$

$$\Sigma y^2 = \Sigma(Y - \bar{y})^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n} = 1463664.429$$

$$\Sigma xy = \Sigma(X - \bar{x})(Y - \bar{y}) = \Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{n} = 5392142.857$$

$$\text{Sample variance } S_{yx}^2 = \frac{(Y - \bar{Y})^2}{n-2} - \frac{(\Sigma xy)^2}{n-2} = 629.436$$

$$\text{Total Sum of Squares} = y^2 = 1463664.429$$

$$\text{Error Sum of Squares} = y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2} = 16365.3565$$

$$\text{Regression Sum of Squares} = \frac{(\Sigma xy)^2}{\Sigma x^2} = 1447299.073$$

$$F = \frac{\text{Regression Mean Square}}{\text{Error Mean Square}} = \frac{1447299.073 - 1}{16365.3565 - (28-2)}$$

$$= 2299.36$$

Since $\left[F_{28}^{1}\ 0.05\right] = 4.2$, the null hypothesis of no relationship was rejected. A good fit was presumed.

2. *Linearity*

If repeated measurements of y are made for each value of x, then the error of the method can be estimated from the within - groups sum of squares. The groups sum of squares can be partitioned into the regression sum of squares, the within-groups

sum of squares, and the deviation from linearity sum of squares. Since the first three parameters can be determined, the deviation from linearity sum of squares was obtained by substraction.

Calcium $\mu g L^{-1}$	ΣY	ΣY^2	$\bar{Y}$
3750	5866	5738996	977.6
3125	4840	3905304	806.6
2500	3696	2278219	616.16
1875	2768	1280598	461.33
1250	1271	407239	317.75

$\Sigma\Sigma Y = 18442 \quad \Sigma\Sigma Y^2 = 13610356$

$$\text{Group Sum of Squares} = \frac{5866^2}{6} + \frac{4840^2}{6} + \frac{3697^2}{6} + \frac{2768^2}{6} + \frac{1271^2}{4} - \frac{18442^2}{28} = 1451366.845$$

Within - group sum of squares = 1463664.429 - 13610356

= 12297.5839

Deviation from Linearity Sum of Squares = Group Sum of Squares - Regression Sum of Squares = 4067.7721

$$\text{F-test for Linearity} = \frac{\text{Deviation from Linearity Mean Sq.}}{\text{Within Groups Mean Square}} = \frac{4067.7721 - 3}{12297.5839 - 23} = 2.54$$

$F\left[\begin{smallmatrix}3\\25\end{smallmatrix}\ 0.05\right] = 3.1$

On the basis of the calculated F value, the hypothesis should be accepted that a linear relationship does exist between absorbance and calcium concentration.

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