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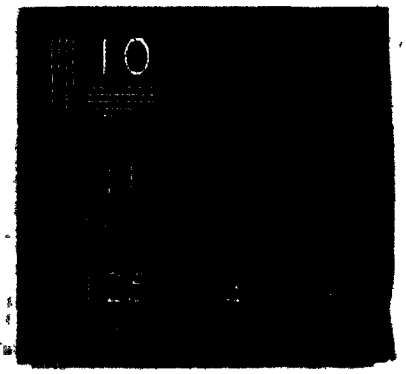

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FLUORIMETRIC DETERMINATION OF ANIONS

by

JIRI HOLZBECHER, M. Sc.

Submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

at Dalhousie University, May 1973

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ABSTRACT

New fluorimetric methods have been developed for the determination of cyanide, sulfide and phosphate at ng/ml concentration levels using ligand-exchange and oxidation-reduction principles. Ligand-exchange reactions are, in general, inferior to oxidation-reduction processes for the determination of anions by fluorescence. Fluorimetric methods based on oxidation-reduction, which allow determination of mercury(II) and copper(II), have also been found. The methods developed are sensitive, the results have a good reproducibility and many interferences may be tolerated in relatively high amounts.

For fluorimetric analysis of anions ligand-exchange reactions require effective quenching of a fluorescent ligand by a metal ion; the electron structure of the metal ion is of primary importance in this quenching process. Good selectivity is achieved in oxidation-reduction reactions when anions do not react directly with the organic reagent; advantage is taken of their complexing properties which results in change of the standard reduction potential.

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JIRI HOLZBECHER

May, 1973

INTRODUCTION

The importance of trace constituents has received increasing recognition. The definition of "traces" is not precise, but usually the term "trace element" means an element present in concentrations below 10^{-3} %. Non-metals make up a significant fraction of trace components and many of these exist as, or can be converted to, anions in solution.

Many examples occur in various systems; in water analysis, for instance, phosphorus[1] (as phosphates) is the key nutrient in algae growth; sulfide[2] is indirectly responsible for corrosion of concrete sewers; fluoride[3] in drinking water provides protection against dental caries; nitrite[4] in raw water supply is an indicator of microbiological activity.

Because it is necessary to monitor (and often control) the concentration of trace constituents, there is an increasing interest in analytical methods for the detection and determination of anions at levels of less than 1 μg/ml. This requires the development of either more sensitive methods or better preconcentration techniques.

More sensitive methods are attractive since they often permit the direct determination of the trace without the necessity for separation or preconcentration of the material; they have, of course, the disadvantage of possibly increased interference and of handling

problems. The application of preconcentration methods has the advantage of permitting subsequent use of well established, even if less sensitive, methods of determination. The obvious disadvantage is the possible loss of the trace constituent to be determined. Focus, in the present work, has been on the establishment of more sensitive methods which could be directly applied to the determination of anions at the nanograms per milliliter (ng/ml) level.

The development of more sensitive methods, of course, imposes some limitations since factors which are not critical at higher concentrations become important in trace analysis. The sensitivity of a method is limited by background[5] since the value of the parameter corresponding to the concentration is given by the difference between the blank and sample. The background, which may be defined as the analytical signal observed with no analyte present, becomes more sensitive to random factors (atmospheric or container contamination) at low concentrations and signal fluctuations result even under apparently constant experimental conditions. Although permanent contributions to background (method, reagent impurities, instrument design) can be compensated by an appropriate blank, an increased background will limit the determinable concentrations.

Another important factor is the experimental error in relation to the sensitivity of the method. Clearly, at concentrations close to the sensitivity limit the experimental errors become very large.

Only if the concentration to be determined exceeds the sensitivity of the method employed by a factor of not less than 10 to 15, does the experimental error become constant and independent of sensitivity.

Some of the phenomena limiting the sensitivity of the method are unique for the nature of the reaction employed, such as, solubility of precipitate in precipitation reactions, sensitivity of color indicator in titrimetric methods, etc. Thus the lower the concentration of the substance to be determined, the smaller becomes the number of methods suitable for its direct determination. The available techniques for direct analysis of trace anions are limited mainly to selective membrane potentiometric, spectrophotometric and fluorimetric methods. The majority of the classical analytical methods, such as gravimetric, titrimetric, emission spectrometric and electrical methods, are less suitable[6]. Fluorimetry is thus one of the methods which holds attractive possibilities for the development of sensitive methods for anions.

Basic concepts in fluorimetry*

Fluorescence methods - which are based on the emission of light by excited molecules or atoms - are among the most sensitive methods

---

* This section is summarized from several monographs dealing with luminescence[7,8,9].

for trace analysis. Molecular luminescence spectra (and atomic fluorescence spectra) arise from radiative electronic transitions between well defined energy levels. A prerequisite of the luminescence process is an absorption of energy. At room temperature most molecules are in the lowest vibrational level of the ground electronic state (S_0 in Fig.1.). By absorption of energy electronic transitions take place populating excited states (S_1, S_2). In most cases internal conversion (IC) (accompanied by vibrational relaxation) to the lowest excited singlet state (S_1) quickly follows. By this process the excited molecule dissipates its energy in the form of heat. From the lowest excited singlet state a number of processes may now occur. The radiative transfer to the ground state (S_o) is called fluorescence. Radiationless processes, internal conversion (IC) and intersystem crossing (IX), may also take place. Intersystem crossing involves vibrational coupling between the excited singlet state and a triplet state resulting in population of triplet state (T_1). The emission from the lowest excited triplet state (T_1) gives rise to phosphorescence. Thus fluorescence arises from a singlet-singlet transition while phosphorescence is a result of a triplet-singlet transition. The multiplicity of the electronic states is given by an expression $(2n + 1)$ where n is the number of unpaired electrons. Since most molecules possess an even number of electrons their ground states have a multiplicity of one and are thus singlets. The electrons

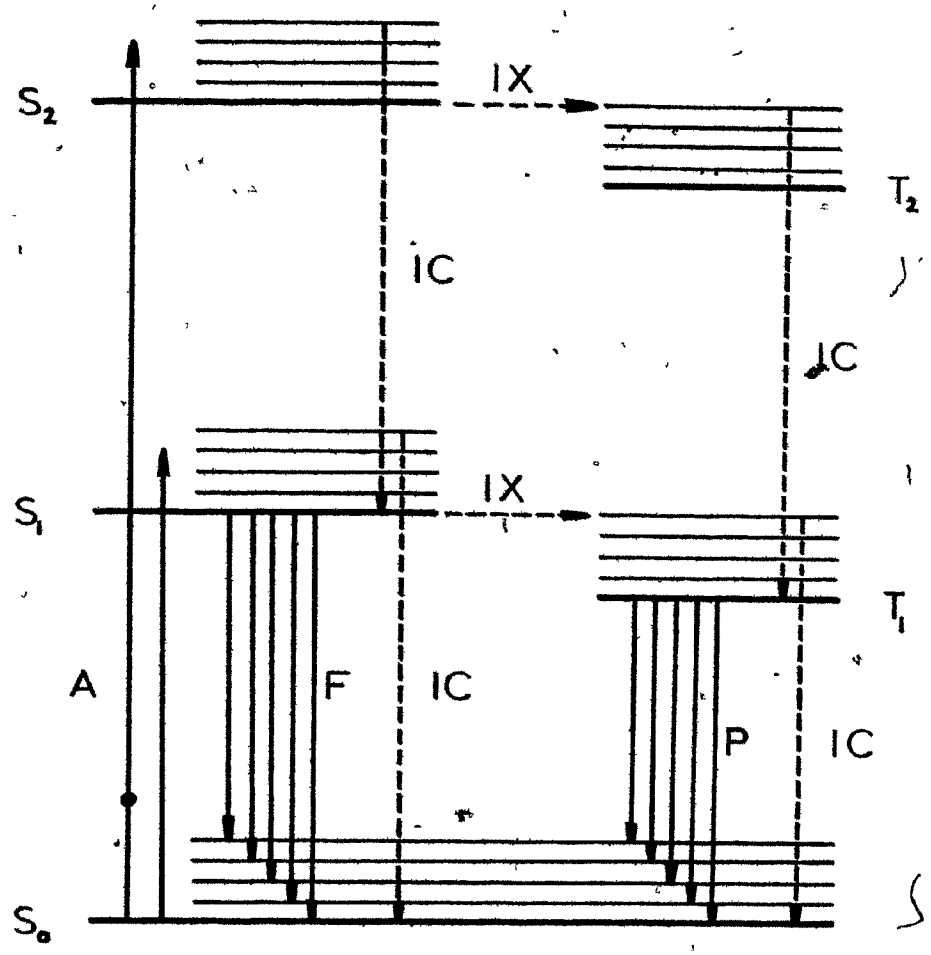
Figure 1. Energy level diagram of radiative (————) and radiationless (--------) transitions in a typical organic molecule.

S_0, S_1, S_2 ... singlet states

T_1, T_2 ... triplet states

A ... absorption F ... fluorescence

P ... phosphorescence IX ... intersystem crossing IC ... internal conversion


S2
IX
T2
IC
IC
S1
IX
T1
A
F
IC
P
IC
S0

in the excited orbitals can have their spins aligned in the same or opposite direction to that of the electron in the original orbital and can thus give rise to singlet or triplet states, respectively.

Radiationless processes compete with fluorescence by diverting the absorbed energy to other channels than fluorescence. The quantum yield (Φ_f) of fluorescence may be expressed as follows:

$$\Phi_f = k_f/(k_f + k_c + k_x)$$

where k_f, k_c and k_x are rate constants for fluorescence, internal conversion and intersystem crossing, respectively. If k_f is much higher than k_c and k_x, the quantum efficiency of fluorescence will approach unity. The rate constants of radiationless processes competing with fluorescence are dependent on the molecular structure and also on the nature of the molecular environment. Other processes competing with fluorescence are radiationless transfer of excitation energy to an appropriate acceptor via either "collisional" or non-collisional mechanisms and a chemical reaction by a molecule in an excited state.

To be able to evaluate fluorescence for analytical use it is necessary to relate it to the structure of the molecule and to possible types of electronic transitions. There are three types of molecular orbitals:

Orbitals which originate from overlap of two atomic orbitals along the line joining the nuclei of bonded atoms are called σ orbitals. Because the electronic charge in a σ bond is localized between two atoms there can be no more than one σ bond between any two atoms in a molecule; these electrons are bound very tightly and a great deal of energy is required to excite them. They are not, therefore, of interest in luminescence spectroscopy.

Overlap of two atomic orbitals at right angles to the nuclei of bonded atoms forms π orbitals. Such bonding is weaker than σ bonding and the distribution of electronic charge is concentrated parallel to and away from the concentration of σ electronic charge; π electrons are freer to move and are frequently distributed over several atoms (delocalized). In aromatic molecules π delocalization extends over the entire molecule and these molecules are of primary interest in molecular luminescence.

Organic molecules can also contain nonbonding electrons (electron pairs on atoms such as nitrogen) which are higher in energy than σ or π electrons and can contribute to the molecular spectral features.

Absorption of energy by an organic molecule may produce various excited states: σ, σ^*, π, π^*, n, π^*, n, σ^*, which may be either singlet or triplet states, triplet levels being lower in energy than corresponding singlet levels. Since aliphatic compounds often do not

strongly absorb in the near UV or visible, they do not usually exhibit fluorescence unless they possess some aldehydic or ketonic groups. Unsubstituted aromatic compounds, in which the lowest energy transition is $\pi \longrightarrow \pi^*$, usually fluoresce in liquid solutions; generally, the greater the number of condensed rings the longer will be the emission wavelength. Substitution with functional groups affects the fluorescence characteristics of the aromatic molecules. While alkyl groups exert only slight effect nitro groups quench strongly the fluorescence as a result of predissociation. Halogens decrease fluorescence intensity, the decrease being more pronounced when passing along the series F, Cl, Br, I (intramolecular heavy-atom effect).[#] Hydroxyl and amino groups, on the other hand, tend to increase the fluorescence output. In aromatic carbonyl compounds as well as in the nitrogen heterocyclics the lowest energy electronic transition is $n \longrightarrow \pi^*$. Such molecules usually do not fluoresce (although they may phosphoresce). The reason is that n, π^* excited states have longer lifetimes than π,π^* states and thus the probability of intersystem crossing is enhanced. If it is possible, however, to tie up the non-bonding electrons and thus remove their influence, fluorescence may occur. For example,

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[#] The presence of a heavy atom either as a substituent or in the molecular environment results in increased spin-orbit coupling and thus in the increase of intersystem crossing which competes with fluorescence.

substitution of groups which can take part in hydrogen bonding (-OH, $-NH_2$) with carbonyl oxygen of aromatic aldehydes, ketones or carboxylic acids leads to fluorescent compounds; a typical example is the non-fluorescence of benzoic acid as compared with the fluorescence of salicylic acid. Similarly, protonation of the lone-pair electrons on nitrogen atom in nitrogen heterocyclics in acid medium or the use of polar solvents leads to fluorescence. Oxygen heterocyclics usually fluoresce when at least one aromatic ring is fused to the oxygen-containing ring.

Molecular geometry also plays an important role in the fluorescence characteristics of organic molecules. Fluorescence is usually observed with molecules which possess highly rigid, planar structures. The rigidity prevents vibrational dissipation of the excitation energy and thus favors fluorescence; planarity of the molecule contributes to effective π-electron delocalization.

In fluorescence spectrofluorimetry the following equation holds for dilute solutions[10]:

$$F = 2.303\ \Phi\ I_o \varepsilon \ell\ c\ p$$

where:

F . . . intensity of the fluorescence generated

Φ . . . quantum efficiency

I_o . . . intensity of the incident light

ε . . . molar absorptivity of the substance at the given wavelength of absorption

ℓ . . . path length

c . . . concentration of absorbing species

p . . . fractional factor (arising from viewing only a fraction of the total fluorescence generated by observing only a small segment of solution in a right angle to the incident radiation)

The fluorescence intensity is thus directly proportional to the concentration of observed species. The sensitivity of the method can be increased either instrumentally (I_o, ℓ, p) or by selecting molecules with high ε and Φ. The reagents suitable for fluorimetry should, therefore, absorb strongly in the near UV or visible and their quantum efficiency should be also high.

Fluorimetry and anions

The common inorganic anions do not themselves fluoresce because they do not possess the unsaturated π - electron system which is the common source of fluorescent properties; they cannot, therefore, be analyzed directly. Reactions must be found which result in fluorescence production, quenching, or shift of excitation and/or emission wavelengths by action of an anion on a suitable organic molecule*.

---

* Typical fluorimetric methods for common inorganic anions reported in the literature are listed in Table 1.

TABLE 1

Typical reactions in fluorimetric determination of common inorganic anions

Anion	Reagent	Type of Reaction	Sensitivity Reported	Major Interferences	Remarks	Ref.
CN^-	2,3-diamino-naphthalene-Se-complex, quenched with Pd	exchange	not reported	Hg(II), S^{2-}	not true ligand-exchange	11
	pyridoxal	catalytic	10^{-9} mole	not reported		12
	luminol, H_2O_2, Cu(II)	retardation of catalytic react.	2.88 µg/ml	SO_3^{2-}, I^-	chemiluminescence	13
	p-benzoquinone & other quinone derivatives	direct	0.2 µg/ml	over 30 ions tested do not interfere		14
	Pd complex of 8-hydroxy-5-quinoline-sulfonic acid	ligand-exchange	0.02 µg/ml	S^{2-}, can also be determined	Mg(II) added to the liberated ligand	15
	chloramine T, nicotinamide	direct	0.3 µg/ml	not reported; CN^- septd. as HCN	ClCN first formed	16
S^{2-}	Hg complex with 2,2'-pyridylbenzimidazole	ligand-exchange	0.3 ng/ml	CN^-, SCN^-		17

TABLE 1 continued

Anion	Reagent	Type of Reaction	Sensitivity Reported	Major Interferences	Remarks	Ref.
S^{2-}	fluorescein tetra-mercury-acetate	quenching	1 ng/ml	SO_3^{2-}, NO_2^-, Br^-, I^- Fe(II), Fe(III), Co(II), Ni(II)	applied to H_2S anal. in the air	18,19, 20,21.
	Cu complex of 2-(o-hydroxy-phenyl)-benzoxa-zole	$S^{2-} + Cu^{2+}$; excess Cu quen. reag.fl.	1 ng/ml	CN^-	can be used for CN^- determination	22
	luminol, I_2	quenching	10 ng/ml	not reported	chemiluminescence	23
SO_4^{2-}	Th(IV) + salicyl-fluorone	$SO_4^{2-} + Th^{4+}$; excess Th quen.reag.fl.	0.05 µg/ml	not reported	Th-reagent complex is non-fluorescent	24,25.
	Th(IV) + morin	quenching	1 µg	F^-, PO_4^{3-}, WO_4^{2-}, MoO_4^{2-} citr., As(V), SeO_4^{2-} VO_3^-, Fe(III), ZrO^{2+}, Al(III)		26
	Zr - calcein blue	fl. enhancement	2 µg/ml	Fe(III), Co(II), F^-, oxalate, PO_4^{3-}, tartrate, WO_4^{2-}	mechanism unknown	27
$S_2O_3^{2-}$	tetramer-curated fluores-cein		0.5×10^{-8} mole	same sensitivity for CN^-	mechanism not given	28

TABLE 1 continued

Anion	Reagent	Type of Reaction	Sensitivity Reported	Major Interferences	Remarks	Ref.
SO_2 as SO_3^{2-}	tetrachloro-mercurate(II) (TCM), formaldehyde, 5-amino-fluorescein	quenching	0.02 µg/ml SO_2 in TCM	Fe(III), K(I), Ca(II), Mg(II), Cu(II), I^-, NO_2^-, OAc^-	Schiff's reaction; SO_2 first trapped in TCM	29
PO_4^{3-}	Al - morin	quenching	0.5 µg	34 interfering ions listed	complexes of Al, Ga, Zr with flavones also studied	30
	quinine	quinine molyb-dophosphate associate formation	0.02 µg P	As(V), As(III), Cr(VI), Ge(IV), Th(IV), W(VI)		31
	rhodamine B	rhodamine B molybdo-phosphate associate formation	0.04 µg P	As(V), As(III), V(V), Cr(VI), Cr(III)		32
	glycogen, triphos-phopyri-dine nucleotide, or nicoti-namideade-nosinedi-phosphate	direct	limited by reagents purity	not reported	enzymatic method	33,34

TABLE 1 continued

Anion	Reagent	Type of Reaction	Sensitivity Reported	Major Interferences	Remarks	Ref.
F^-	Al - PAN (1-(pyridyl-azo)-2-naphtol	quenching	10^{-9} M	PO_4^{3-}. I^-		35
	Zr - 3-hydroxyflavone	quenching	2 ng/ml	Al(III), MoO_4^{2-}, citr., tart., $C_2O_4^{2-}$		36
	Zr - calcein blue	ternary complex formation	10^{-7} M	WO_4^{2-}, OAc^-, tart., PO_4^{3-}, MoO_4^{2-}, SO_4^{2-}, Al(III), Mn(II), Ag(I), As(V) Sb(III), Be(II), Co(II)		37
	Al - morin	quenching	0.1 µg	34 interf. ions listed; c.f.ref 30		38
	Al-dihydroxy-azodyes (eriochrome red B, superchrome-garnet Y)	quenching	0.2 µg	Cu(II), Fe(II), Fe(III), Ni(II), Co(II), Cr(III), Be(II), Zr(IV), Th(IV)		39
NO_3^-	2,3-diamino-naphthalene	direct	0.01 µg/ml	Cu(II), Al(III), Bi(III), Mf(II), Cr(III), Sn(II), Se(IV), Fe(III)	NO_3^- first reduced to NO_2^-	40
	fluorescein	quenching	0.01 µg/ml	NH_4^+, Mg(II), Cu(II), Fe(III), Fe(II), Cl^-, Br^-, I^-, NO_2^-		41

TABLE 1 continued

Anion	Reagent	Type of Reaction	Sensitivity Reported	Major Interferences	Remarks	Ref.
NO_2^-	2,3-diamino-naphthalene	direct	6.5 ng/ml	Cu(II), Al(III), Bi(III), Ni(II), Cr(III), Sn(II), Se(IV), Fe(III).		42
Cl^-	fluoresdein-Na salt, Ag(I)	quenching	2 ng/ml	not reported	AgCl quenches fluorescence of reagent	43
I^-	uranyl-acetate	quenching	2 µg	SCN^-, $C_2O_4^{2-}$, IO_3^-, NO_2^-, F^-, Al(III)		44

The basic principles involved in the fluorimetric analysis of anions can be summarized as:

(1) direct reaction

(2) catalysis

(3) ion associate formation

(4) ligand-exchange

(5) oxidation-reduction

The processes which appear initially most attractive are direct and catalytic reactions since they are often highly selective and sensitive. In these reactions the anion either reacts directly with a suitable organic molecule or it catalyzes (or inhibits) a particular reaction to produce (or quench) fluorescence, respectively. It is, however, difficult to devise such reactions from theoretical considerations and suitable systems are often found by chance.

Some anions may be determined after formation of an ion associate with a fluorescent organic reagent. Solvent extraction of either excess of the reagent or of the ion associate is almost always involved; such procedures are useful in preconcentration processes but are not advantageous when developing direct sensitive methods. Ligand-exchange and oxidation-reduction reactions are more amenable to a systematic approach and such reactions were therefore studied preferentially.

Ligand-exchange reactions leading to fluorescence production involve displacement of a fluorescent ligand from its non-fluorescent complex by means of an anion; the fluorescence intensity is a measure of the amount of anion present. It is therefore necessary, in such reactions, to have both a fluorescent ligand which can be rendered non-fluorescent by reation with particular metal ions and an anion which reacts strongly with the same metal ions. Fluorescing ligands can be quenched by paramagnetic or heavy metal ions and common anions such as fluoride, cyanide, phosphate and sulfide can compete successfully with the complexing molecules.

Various organic compounds exhibit different luminescence behaviour depending on their oxidation state. It is therefore possible to determine oxidizing anions by virtue of their ability to oxidize a non-fluorescent form of a suitable reagent to its fluorescent, oxidized form; similarly, reducing anions can reduce a non-fluorescent reagent into a fluorescent, reduced form.

The present research program has concentrated on a study of the factors affecting ligand-exchange and oxidation-reduction processes for the fluorimetric determination of anions. Generally, oxidation-reduction reactions, even with their limitations, showed the highest analytical potential.

METHODOLOGY

Introduction

Fluorescence is radiation emitted from the lowest excited singlet state by a molecule or an atom after absorption of energy. Thus absorption of radiation is common for both photometric and fluorimetric methods. The theoretical sensitivity of these two methods, however, is not the same. In photometric methods the analytical signal (absorbance) is given by the logarithm of the ratio of intensities of incident and transmitted light while the analytical signal in fluorimetry (fluorescence intensity) is directly proportional to the intensity of the incident radiation. Thus, in photometry an increase in incident radiation intensity or an increase of detector sensitivity will result in no net gain in the analytical signal but it will be increased in fluorimetry. The sensitivity of fluorimetry is, therefore, generally greater than that of absorption photometry.

When evaluating the reaction conditions in direct fluorimetric methods consideration was always given not only to the fluorescence intensity of the "sample" solution (I_S) but also to the fluorescence intensity of the respective blank (I_B). For analytical purposes the reaction conditions must be such as to maintain a constant $I_S - I_B$ difference for the same sample concentration. The ratio I_S/I_B is

also important because it is a measure of the practical sensitivity of the method. (For example, if, for the same sample concentration, I_S increases with a change in pH from 20 to 30 and I_B increases from 10 to 20, $(I_S - I_B)$ remains constant but the I_S/I_B ratio decreases and the sensitivity also decreases.).

Apparatus

The apparatus for both photometric and fluorimetric methods consists of the following basic parts: source of radiation, monochromator or filter system, cell and detector. Since excitation and emission radiation are studied in fluorimetric work, two wavelength selecting systems are necessary.

There are three basic designs for observation of fluorescence[45]. The most advantageous one for dilute solutions (and thus for trace analysis) is the right-angle arrangement which eliminates most of the contamination of fluorescence signal by excitation radiation. Frontal analysis, on the other hand, finds its use in studies of fairly concentrated solutions and a straight-through arrangement is used mainly in phosphorimetry. To correlate spectra obtained by different instruments it is necessary to correct for the output of source as well as the response of detector (usually photomultiplier) which are wavelength dependent; monochromators' transmission characteristics must also be taken into account. However, these corrections are seldom necessary in trace analysis work.

Monochromating instruments are recommended when high resolution and precision, as in the investigation of relatively unknown systems, are required; rather high light losses in such instruments limit their usefulness for very sensitive determinations. Filter instruments, on the other hand, allow higher sensitivity and are thus suitable for routine analytical work.

In the present studies a Hanovia mercury ultraviolet lamp (230 W, type 16106) was used for qualitative preliminary observations and an Aminco-Bowman spectrophotofluorimeter (model 4-8203 with an off-axis ellipsoidal mirror condensing system) with xenon lamp source was used for all quantitative measurements. The slits arrangement was 1, 4, 5, 5, 4, 3, 5 mm for slits Nos. 1, 2, 3, 4, 5, 6, 7, respectively, to give a good combination of resolution and sensitivity. A photomultiplier microphotometer tube was used to measure fluorescence intensity. For fluorescence spectra recordings an XY recorder (Aminco-Bowman part No. 1620 - 827) was employed; an external fluorescence standard solution of 1 μg/ml of quinine sulfate in 0.1 N sulfuric acid was used throughout the experiments to calibrate the instrument.

LIGAND-EXCHANGE STUDIES

Introduction

The ligand-exchange reaction for anion determination resulting in fluorescence production may be schematically represented as follows:

$$ML + A \longrightarrow MA + L$$

where:

ML . . . quenched metal complex

A . . . anion to be determined

MA . . . metal-anion complex or precipitate

L . . . free fluorescent ligand

Thus the basic requirements are a non-fluorescent metal complex whose ligand itself is fluorescent and an anion capable of rapidly displacing the ligand. Consideration must be therefore given to the ligand, the metal ion and the anion to be determined.

For high sensitivity it is essential that the ligand itself be highly fluorescent and the metal ion must be capable - as a result of complexation - of effectively quenching the ligand fluorescence to decrease the background. At the same time the anion must be both kinetically and thermodynamically capable of competing successfully with the ligand for the metal ion.

Organic molecules, to be suitable for these ligand-exchange reactions, must not only fluoresce but must also contain complexing groups which can react with quenching metal ions. Metallo-fluorescent indicators are a good example; these have built into the fluorescent molecule (such as, e.g., fluorescein or β-methyl-umbelliferone) a complexing constituent (such as, methyleneiminodiacetic (half of the EDTA molecule) or a methylene-N-methyl-glycine group) so that the resulting compound possesses both fluorescent and chelating properties.

Metal ions which are known to quench fluorescence include paramagnetic ions, such as, copper(II), cobalt(II), nickel(II) or iron(III) or heavy metal ions, such as, mercury(II).

It is obvious that anions forming strong bonds with particular metal ions (as evidenced by complexation or precipitation) hold the most promise in ligand-exchange reactions. For example, if a fluorimetric method was to be devised for fluoride by ligand-exchange then one might initially consider complexes containing metal ions, such as, Fe(III); for cyanide a number of metal ions [Ni(II), Cu(II), Fe(III)] could be of interest.

The experimental data for the kinetics of ligand-exchange available in the literature involve mostly complexes of the transition metals which are nonlabile because their exchange reactions are slow enough to be accessible to classical kinetic techniques. Only the complexes of Co(III), Pt(II), Cr(III), Rh(III), Au(III) and Pd(II)

have been extensively studied[46]. Since labile rather than nonlabile metal complexes are promising for the development of analytical methods based on ligand-exchange, kinetic considerations cannot be fully employed in the choice of a suitable system because of the insufficient amount of data available.

Choice of ligand

In investigating the potential of ligand-exchange reactions for the fluorimetric determination of anions, the choice of ligand was limited not only to those compounds which meet the above stated requirements but also by their commercial availability or ease of preparation. The initial choice of compounds for study were metallofluorescent indicators. These are fluorescent complexing agents whose metal complexes exhibit fluorescence behaviour different from the ligand molecules and they have had considerable success as indicators in complexometric titrations. The metallofluorescent indicators studied were: calcein (C) - fluorescein-2,7-bis-methyliminodiacetic acid; calcein blue (CB) - β-methylumbelliferonemethyleneiminodiacetic acid; methyl calcein (MC) - fluorescein-di-(methylene-N-methylglycine); methyl calcein blue (MCB) - β-methylumbelliferonemethylene-N-methylglycine; stilbene complexone (SC) - 4,4'-diaminostilbene-2,2'-disulphonic acid-N,N,N',N'-tetraacetic acid-sodium salt and dianisidine

complexone (DAC) - 3,3'-dimethoxybenzidine-N,N,N',N'-tetraacetic acid-tetrasodium salt. A number of fluorescent reagents which do not belong to the class of metallofluorescent indicators were also investigated; these included: 1,10-phenanthroline, carminic acid, 2,2',2"-terpyridine, salicylic acid, 8-mercaptoquinoline, flavonols, riboflavin, α-nitroso-β-naphthol, sulfanilic acid.

Quenching

The action of quenching metal ions on these compounds was studied and, if effective quenching took place, an attempt was made to establish the quenching ratio by means of fluorimetric titration. Quenching ratio is the ratio of metal to ligand required to produce a break in the curve obtained by plotting fluorescence intensity against moles of metal ion added per mole of ligand. It should be emphasized that the quenching ratio does not necessarily indicate the composition of complex formed because the quenched species may not be the normal metal complex. The ligand molecule, for example, may be able to bind more than one metal ion but the first metal ion bound may produce effective quenching. On the other hand, if the stability constant of the complex is not very high then a considerable excess of metal ion may be necessary to quench the fluorescence. Finally, environmental conditions are being changed on the addition of metal ion and the extent of quenching thus influenced.

Quenching ratio is, however, useful from the practical point of view because it indicates the suitable ratio of metal and ligand which should be used in investigation of these reagents in ligand-exchange reactions.

Fig. 2 shows the quenching curves of calcein with Fe(III), Hg(II) and Cu(II); in all three cases the quenching ratio is close to 1:1. From the shape of the curves one may assume that these metals form relatively stable complexes with calcein. Only Cu(II), however, may be said to quench calcein fluorescence effectively since a high fluorescence background remains with Hg(II) and Fe(III). It was observed generally with other ligands that Hg(II) and Fe(III) complexes exhibited higher fluorescence background than those of Cu(II), Ni(II) and Co(II). Thus, it is obvious that the formation of a strong complex is a necessary but not sufficient condition for effective quenching to take place; the nature of metal ion involved is a decisive factor. As an example of weak complex formation the quenching curve of Ni(II) with 3-hydroxyflavone is given in Fig. 3. Although Ni(II) quenches effectively the fluorescence of, e.g., calcein, giving a similar quenching curve as Cu(II), it does not do so with 3-hydroxyflavone; virtually no break in the quenching curve is observed and high fluorescence background remains. Such a complex is, obviously unsuitable for use in ligand-exchange reactions for anions determination. The

reagent solutions which were then prepared for investigation of their usefulness for anions determination contained metal ion and ligand in the ratios determined.

Effective quenching of the fluorescent ligand by metal ion is, obviously, a prerequisite in development of sensitive ligand-exchange methods. It is also necessary, however, for the anion to readily free the ligand from the metal complex. Although some compounds were efficiently quenched by particular metal ions, they did not give reproducible results when reacted with anions for kinetic or thermodynamic reasons (CB complexes and the Co-C complex).

The fluorescence output of the free ligand is, of course, important to sensitivity since the higher the fluorescence output the higher the sensitivity. The relative fluorescence output of the "calcein" metallofluorescent indicators followed the order: C>MC>CB>MCB. MCB was not very stable under UV irradiation and MC showed a high fluorescence background even with Cu(II). For these reasons Cu(II) and Ni(II) complexes of calcein are most promising.

With the exception of DAC all other compounds investigated had limitations. Some compounds, for example, were unstable with time or under UV irradiation (SC, quercetin, sulfanilic acid) or exhibited low fluorescence yield (1,10-phenanthroline, carminic acid, α-nitroso-β-naphthol); other complexes exhibited high fluorescence background

Figure 2 Quenching curves of calcein with Fe(III), Hg(II) and Cu(II).

I ... relative fluorescence intensity

M/L ... metal ot ligand molar ratio

Instrumental settings: meter multiplier (MM): 1.0; sensitivity (S): 0.0; excitation wavelength (λ_{ex}): 486 nm; emission wavelength (λ_{em}): 512 nm; (the overall width of slits was decreased by a factor of 40)

Procedure:

To 2 ml of borate buffer (pH ~ 7.7) 2 ml of 10^{-4} M calcein were added, followed by x ml of 2 x 10^{-4} M $FeCl_3$, $Hg(NO_3)_2$ or $CuCl_2$, respectively; the volume was made up to 10 ml with twice distilled water; x was varied from 0 to 5 ml.

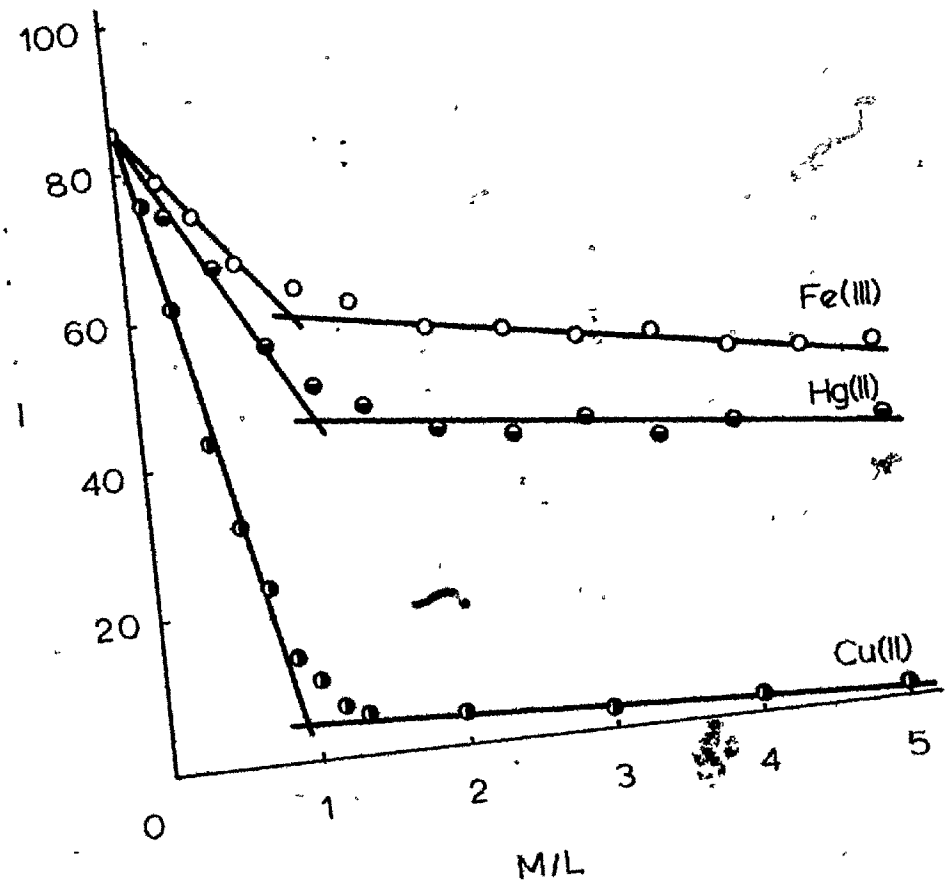
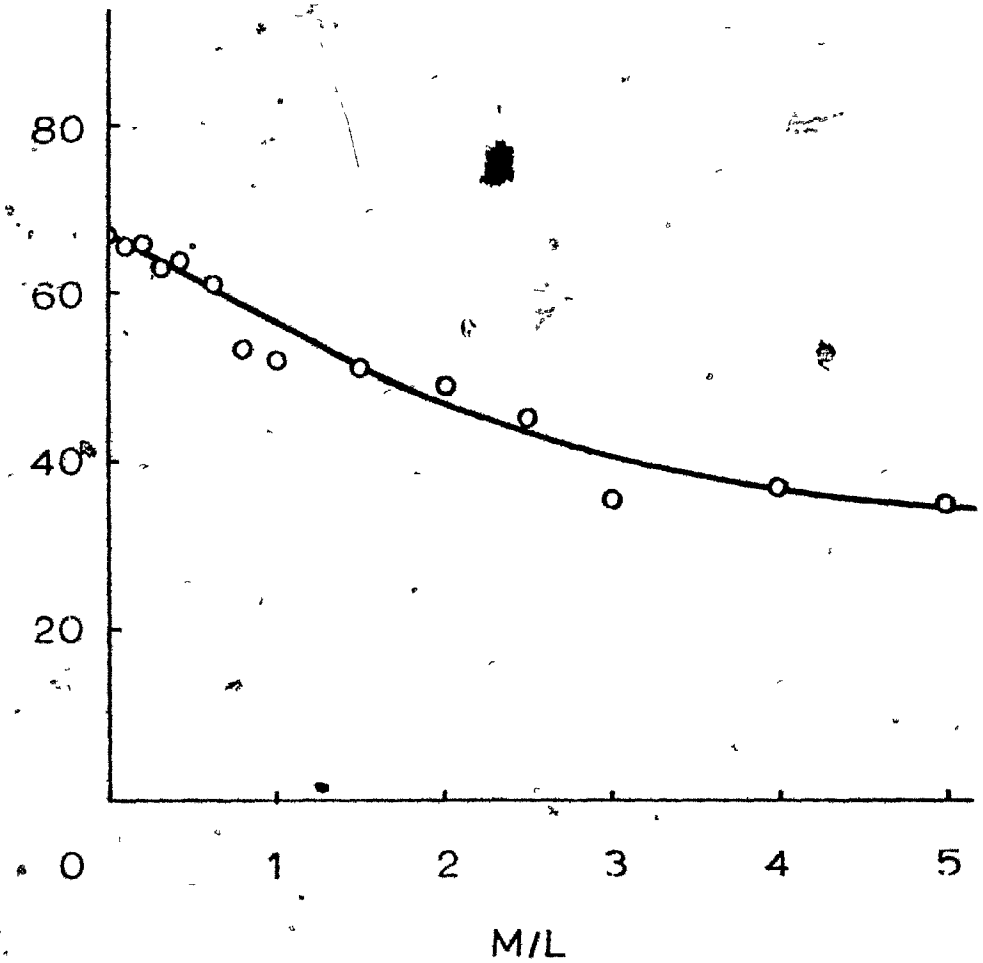

100
80
60
40
20
0
I
1
2
3
4
5
M/L
Fe(III)
Hg(II)
Cu(II)

Figure 3 Quenching curve of 3-hydroxyflavone with Ni(II).

M/L ... metal to ligand molar ratio

MM: 0.1; S: 0.0; λ_{ex}: 350 nm; λ_{em}: 510 nm

Reagent concentration: 1×10^{-5} F


80
60
40
20
0
1
2
3
4
5
M/L

(salicylic acid, riboflavin, 3-hydroxyflavone, morin) or did not react satisfactorily with anions (2,2',2"-terpyridine); 8-mercaptoquinoline required non-polar solvents to fluoresce. Such limitations prevent widespread application of ligand-exchange reactions for anions determination. Practical methods have been developed, however, for cyanide with Cu(II)-calcein and for sulfide with Cu(II)-DAC; these methods are described in the next section.

Determination of Cyanide

Introduction

Cu(II)-calcein appeared to be slightly more sensitive than Ni(II)-calcein towards cyanide and was, therefore, used in development of a ligand-exchange method for ng/ml of cyanide. Substoichiometric amounts of metal complex with respect to cyanide had to be used since higher reagent concentrations gave a relatively high fluorescence background which overshadowed the changes in fluorescence intensity caused by ng/ml of cyanide. In the pH range of 6 to 8.5, where the highest fluorescence intensity of free calcein occurs, the cyanide was not able to compete successfully for copper(II) and a higher pH (9.2) had to be used to obtain satisfactory results.

Reagents

Stock solutions of 10^{-4} F potassium cyanide were freshly prepared for each day's measurement from analytical reagent grade potassium cyanide (BDH) whose purity was checked by titration with standardized silver nitrate.

Reagent stock solutions of 10^{-4} F copper-C were prepared by mixing equimolar volumes of 2×10^{-4} F cupric chloride (Mallinckrodt) and calcein (G.F.Smith) solutions (1 to 2 drops of 1N sodium

hydroxide per 25 ml volume were added to increase the solubility of calcein).

The borate buffer (pH ~9.2) was a 0.1 F solution of borax.

Procedure

To 1 ml of buffer add 5 ml or less of approximately neutral unknown solution (containing 0.1 to 0.25 µg of cyanide), 1 ml of 2×10^{-6} F reagent and make up to 10 ml in a volumetric flask with twice distilled water. Measure the fluorescence at 512 nm using an excitation wavelength of 486 nm. Determine the amount of cyanide from a previously prepared calibration curve.

Results and discussion

Although up to 250 ng/ml of cyanide can be determined, the calibration curve is a straight line only up to 150 ng/ml; the line does not pass through the origin since there is some background fluorescence for the reagent itself (Fig. 4). With the limited number of results (six), the relative standard deviation on the determination of 26 ng/ml of cyanide was 6.5%. Sulfide and phosphate did not interfere at molar ratios of anion to cyanide of 500:1; 1:1 mole ratios of EDTA to cyanide, however, must be absent.

Although milligram quantities of anions which react with copper do produce fluorescence on addition to Cu(II)-C solutions, only cyanide was capable of doing so at the ng/ml level. This may be because, with cyanide, the reaction is not simple ligand-exchange since copper(II) is reduced to copper(I) in its reaction with cyanide.

Determination of Sulfide

Introduction

Fluorescence of dianisidine complexone (DAC) is effectively quenched with paramagnetic metal ions, such as, Cu(II), Ni(II) or Co(II). Fluorescence is readily restored by reaction of these complexes with such anions as EDTA, cyanide or sulfide. The quenching ratio of Cu(II) : DAC is 1:2. The reaction of Cu-DAC complex with sulfide was studied quantitatively.

Reagents

Reagent solution of 10^{-5} F $Cu(DAC)_2$ was prepared by mixing 10 ml of 10^{-4} F $CuCl_2$ (Mallinckrodt) and 20 ml of 10^{-4} F DAC (Fluka) and making up the volume to 100 ml.

Sodium sulfide, $Na_2S \cdot 9\ H_2O$ (J.T.Baker), was chosen as a working standard as recommended by several authors[20,47]; 0.001 F stock solutions were prepared daily from washed and dried crystals. The purity was checked by iodometric titration according to Bethge[48].

The borate buffer (pH ~7.7) was prepared by mixing 47 ml of 0.1 N HCl with 53 ml of 0.05 F borax.

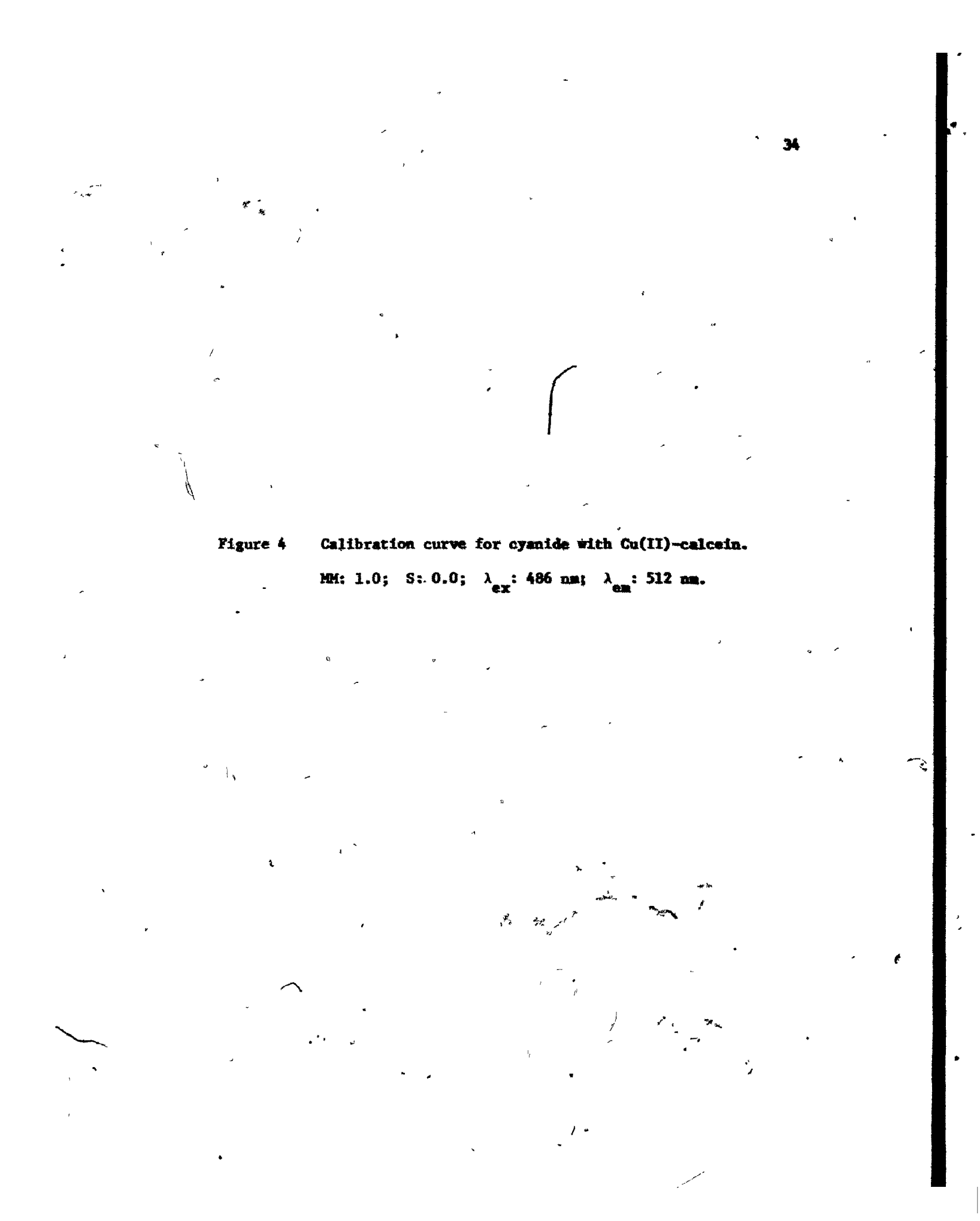
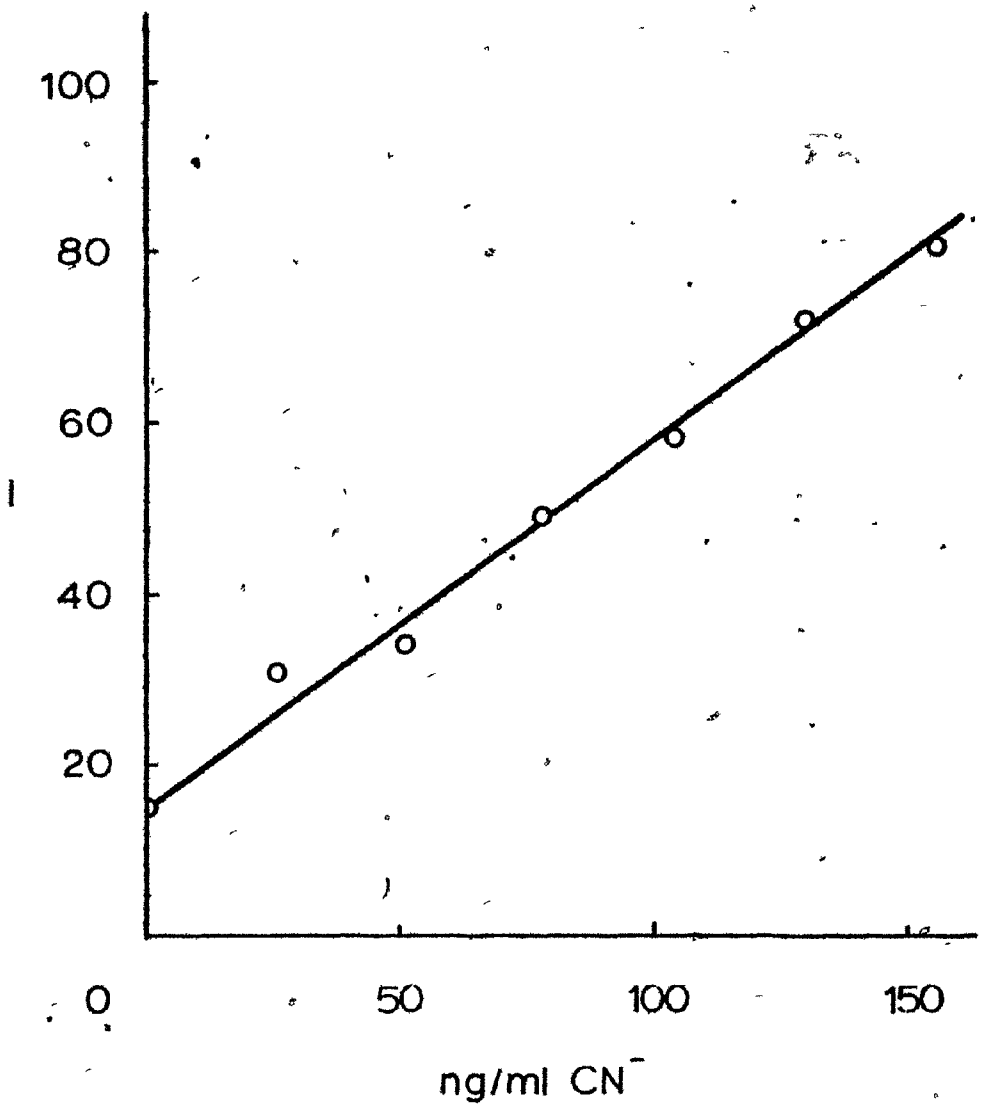


Figure 4 Calibration curve for cyanide with Cu(II)-calcein.

MM: 1.0; S: 0.0; λ_{ex}: 486 nm; λ_{em}: 512 nm.


100
80
60
I
40
20
0
50
100
150
ng/ml CN⁻

Procedure

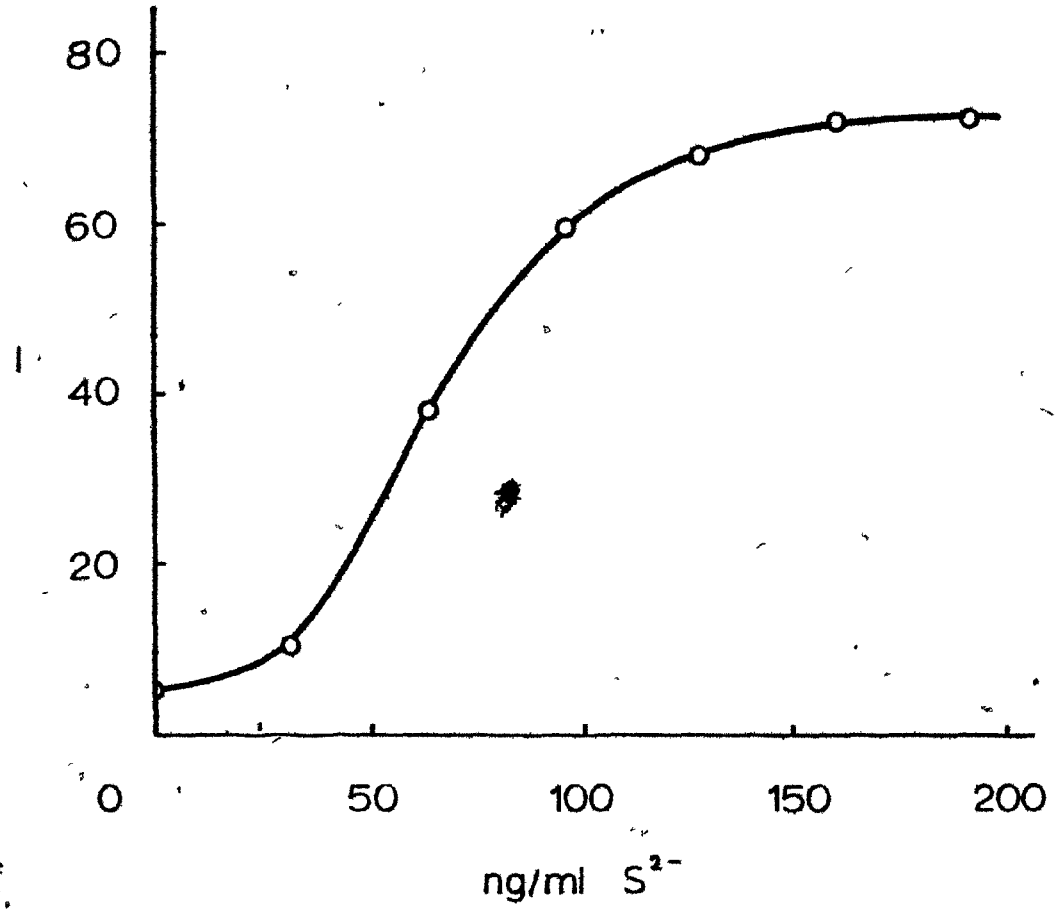
To 2 ml of buffer (pH ~7.7) add 1 ml of 10^{-5} F reagent solution followed by several ml of approximately neutral unknown containing 0.3 to 1 µg of sulfide; make up the volume to 10 ml with twice distilled water; read the fluorescence intensity at 400 nm using an excitation wavelength of 328 nm . Determine the sulfide concentration from a calibration curve.

Results and discussion

Figure 5 shows the calibration curve obtained with 0 to 200 ng/ml of sulfide. Although the method is fast and simple, useful determination is possible only over a limited concentration range (30 to 100 ng/ml). Since the more sensitive ligand-exchange reaction between sulfide and mercury(II)-2,2'-pyridylbenzimidazole[17] appeared in the literature when these studies were being performed, further investigations of the copper-PAC sulfide reaction were abandoned.

Figure 5 Calibration curve for sulfide with Cu(II)-dianisidine complexone.

MM: 0.3; S: 0.0; λ_{ex}: 328 nm; λ_{em}: 400 nm


80
60
40
20
0
50
100
150
200
ng/ml S^{2-}

OXIDATION-REDUCTION STUDIES

Basic requirements for the use of oxidation-reduction for anions determination are an organic reagent exhibiting different fluorescent properties in oxidized and reduced forms and an anion which can either directly or indirectly (as part of a more complex oxidation-reduction system) oxidize or reduce the reagent to produce (or quench) fluorescence.

In the choice of a suitable organic reagent, some additional properties are also desirable: rapid oxidation-reduction reaction, high fluorescence output, stability with time, solubility in water, *etc.* For anions, the obvious choice would seem to be strongly oxidizing (permanganate) or reducing (sulfite) anions. Direct oxidation-reduction reactions of such anions with organic reagents are not, however, usually very specific. When the anion is neither a strong oxidizing or reducing agent and does not react directly with the organic molecule, the complexing properties of the anion may be used to change the oxidation-reduction potential of a suitable system; good selectivity is often possible for such anions because interfering oxidizing or reducing agents can be destroyed prior to complexation.

A detailed study of the various parameters affecting oxidation-reduction reactions has been undertaken. As a result, direct

fluorimetric methods have been developed for the determination of cyanide and phosphate; a quenching method has been devised for sulfide. During these studies direct methods have also been found for the determination of mercury(II) and copper(II).

Determination of Cyanide

Introduction

The leucobase of fluorescein (obtained by reduction of fluorescein in alkaline medium) does not fluoresce but it can be re-oxidized to fluorescein by various oxidizing agents[49,50]. Cyanide (1 μg/ml) has been detected with the fluorescein leucobase in the presence of cupric ion by taking advantage of its complexing ability; although cupric ion itself is not capable of oxidizing the leucobase, the oxidation potential is sufficiently increased when cyanide is present for oxidation to occur. This reaction has been used in the present work to determine cyanide at the ng/ml level.

Reagents

The leucofluorescein reagent solution was prepared as described by Stamm[50]. To a solution containing 0.01 g of fluorescein, 5 ml of ethanol, 2 ml of 33% sodium hydroxide and 5 ml of water add zinc dust in small portions (whilst stirring and heating on the steam bath) until the solution decolorizes. Dilute to 100 ml with water, add 100 ml of ethanol and allow to stand overnight in the dark; remove the solid mixture of zinc oxide and zinc by filtration. The filtrate

keeps in the dark for several months; required concentrations were prepared from this stock solution by dilution with water.

The borate buffer (pH ~7.2) was prepared by mixing 8 ml of 0.05 F borax with 92 ml of a solution containing 12.404 g of boric acid and 2.923 g of sodium chloride per liter.

The cyanide and copper chloride standards were as described previously (p.30).

Procedure

To 2 ml of borate buffer add 1 ml of 10^{-4} F copper chloride, 1 ml of 3×10^{-6} F reagent (stock solution diluted 1:50) and 5 ml (or less) of approximately neutral unknown solution (containing 0.01 to 1 µg of cyanide); dilute to 10 ml with twice distilled water. Measure the fluorescence intensity at 514 nm using an excitation wavelength of 488 nm. Determine the amount of cyanide from a calibration curve.

Results and discussion

The fluorescence intensity is a linear function of concentration at low concentrations of cyanide (0 to 5.2 ng/ml - Fig. 6A); excitation and emission maxima are less than 30 nm apart and accurate determination of still lower amounts of cyanide is not possible because

the scattering effect becomes significant. For higher concentrations of cyanide (where lower instrumental sensitivity is used) there is no measureable background fluorescence and a straight line is obtained up to about 25 ng/ml of cyanide (Fig. 6B); it levels off for still higher concentrations because of an insufficient amount of reagent. The standard deviation of ten determinations of 2.6 ng/ml of cyanide was 10.6%.

Five ng/ml of cyanide was successfully determined in the presence of 500-fold excess of bromide, chloride, perchlorate, fluoride, iodide, nitrate, acetate, thiocyanate and sulfate; a 50-fold excess of phosphate and a 5-fold excess of EDTA did not interfere. Equivalent amounts of sulfide cause a serious decrease in the fluorescence intensity and sulfide must be absent. Persulfate and ferricyanide increase the fluorescence intensity and interfere strongly at ion to cyanide molar ratios of 1:1.

A 500-fold excess of Co(II), Ni(II) and Cd(II) (metal ions reacting strongly with cyanide) did not interfere in the determination of 5 ng/ml of cyanide; a 50-fold excess of Zn(II), Al(III), a 25-fold excess of Mn(II) and a 5-fold excess of Fe(III) also were without interference. Equivalent amounts of Hg(II) and Fe(II) decrease the fluorescence intensity and these ions must be absent.

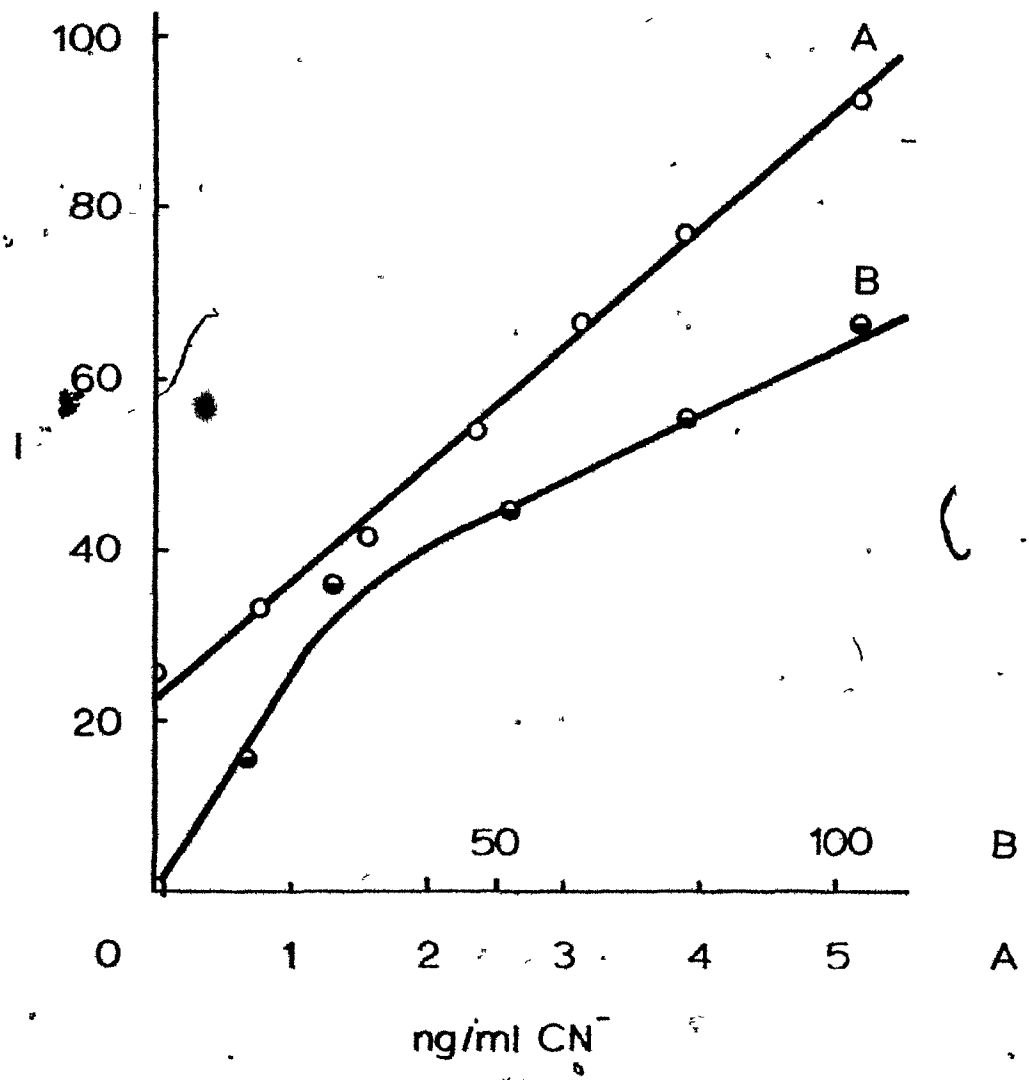
The fluorescence intensity of both sample and blank solutions increased as the pH increased from 6 to 8 but the ratio of fluorescence

Figure 6 Calibration curve for cyanide with leucofluorescein.

Curve A ... MM: 0.3; S: 0.0

Curve B ... MM: 1.0; S: 0.0; (Entr[redacted] slit width was decreased by a factor of 3.3)

λ_{ex}: 488 nm; λ_{em}: 514 nm


100
80
60
40
20
I
A
B
50
100
B
0
1
2
3
4
5
A
ng/ml CN⁻

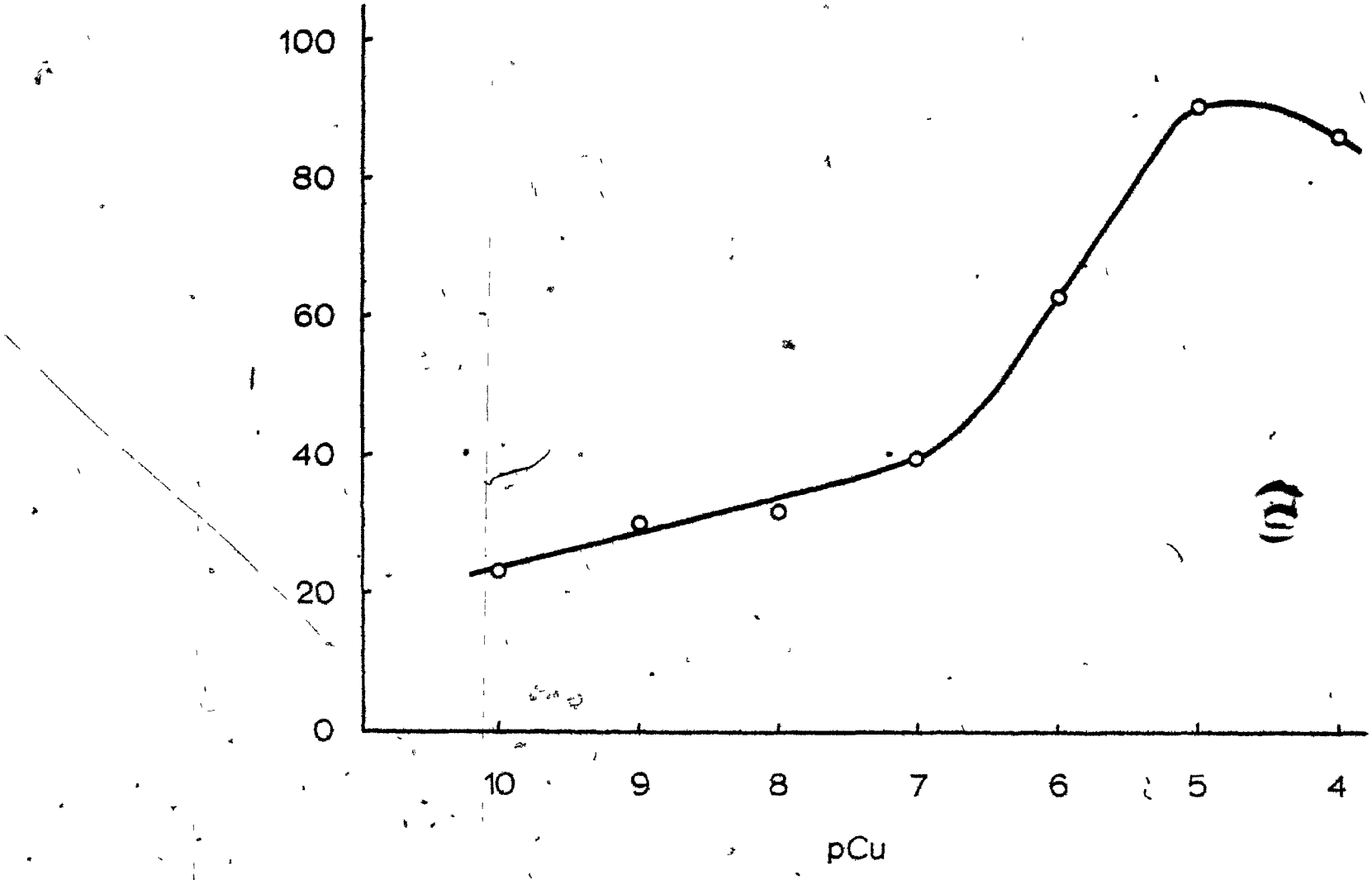
intensities (I_S/I_B) was essentially unchanged; this is in agreement with the observed pH dependence of fluorescein fluorescence intensity. The fluorescence intensity of cyanide containing solutions, however, passed through a maximum at pH 7.5 to 8 whilst the blank fluorescence still increased with a further pH increase; measurements were therefore made in the pH range 7.5 to 8 where the fluorescence is about 10 times higher than at pH 5. The excitation and emission wavelength maxima did not change appreciably over the pH range 4 to 9.

In the concentration range of 1 - 100 ng/ml of cyanide, reagent concentrations of 10^{-7} F are most suitable. Higher reagent concentrations result in decreased sensitivity because of higher background fluorescence and at lower concentrations (10^{-8} F) the scattering effect becomes serious. For example, for 5.2 ng/ml of cyanide and a reagent concentration of 3×10^{-7} F, the I_S/I_B ratio was 3.6 compared to 1.8 at a reagent concentration of 3×10^{-6} F so that a 10-fold increase in reagent concentration results in a 50% decrease in sensitivity.

Fig. 7 shows the effect of copper concentration on the fluorescence intensity obtained for 5.2 ng/ml of cyanide (2×10^{-7} F); the reagent concentration was 3×10^{-7} F. The highest fluorescence intensity occurs in solutions 10^{-4} to 10^{-5} F in cupric ions and a 10^{-5} F final concentration is therefore recommended in the procedure. This concentration is suitable for 1 - 100 ng/ml of cyanide; replacement of copper chloride by copper sulfate does not affect the results.

Figure 7 Effect of copper(II) concentration on fluorescence intensity.

MM: 0.3; S: 0.0; λ_{ex}: 488 nm; λ_{em}: 514 nm


100
80
60
40
20
0
10
9
8
7
6
5
4
pCu

The fluorescence intensity decreased markedly on addition of ethanol. For example, the reading of 69 for 5.2 ng/ml of cyanide in 0.1% ethanol (present in final solution as a result of ethanol in stock reagent solution) was reduced to ~35 in 10% ethanol and to 7 in 50% ethanol.

The fluorescence intensity of solutions containing cyanide remained constant up to 80°C but the background fluorescence increased steadily with temperature; blank readings were 6 at 20°, 14 at 40° and 70 at 80°C whereas for 5.2 ng/ml of cyanide the readings were constant at 34; best results are therefore obtained at a room temperature of 20 - 25°C.

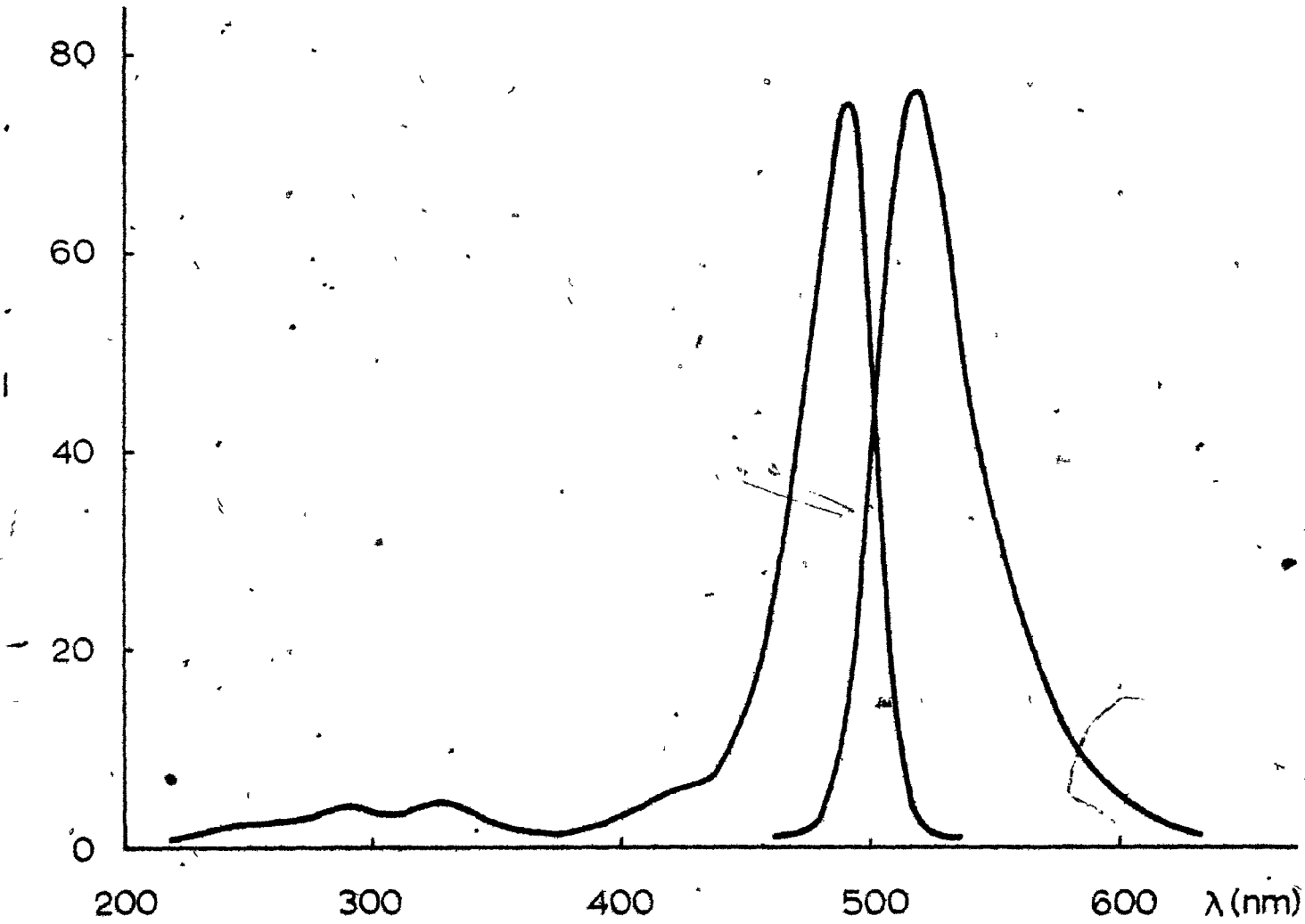
The results obtained for 5.2 ng/ml of cyanide were the same whether measured immediately or 24 hours later; the fluorescence intensity of both sample and blank increased by a factor of about four, on standing, but the intensity ratio increased slightly. Blanks containing no copper (diluted reagent plus buffer) showed an increase in fluorescence intensity of about 40% on 24 hours standing. Measurements can be made, therefore, immediately after mixing the reactants but the reagent solution should be prepared by fresh dilution of the stock reagent solution.

Figure 8 shows the excitation and emission spectra of fluorescein; those obtained from leucofluorescein oxidized by cupric ion in the

Figure 8 Excitation and emission spectra of fluorescein

MM: 1.0; S: 0.0; (the overall width of slits was decreased by a factor of 30)

fluorescein concentration: 3×10^{-6} F


80
60
40
20
0
I
200
300
400
500
600
λ (nm)

presence of cyanide were the same. The quinone type structure (I_a, I_b, Fig. 9) is preferred for fluorescein because of its color[51]; the compound "fluorescin" (II) is colorless and fluorescein is formed on its oxidation[52]. It is probable that "fluorescin" and leuco-fluorescein have the same structure.

Among the fluorimetric methods for cyanide determination reported in the literature (see Table 1) the procedure proposed by Guilbault and Kramer[14] is most selective (over 30 ions tested do not interfere at 0.1 M concentration); the sensitivity limit is 0.2 μg/ml. The catalytic action of cyanide on pyridoxal[12] appears to be most sensitive (10^{-9} mole); its inherent disadvantage is, of course, its time dependence. The method developed compares favorably with these methods for cyanide determination because it is simple, rapid and very low cyanide concentrations (down to 1 ng/ml) can be determined with relatively good reproducibility. Large amounts of common ions do not interfere but sulfide and strongly oxidizing anions must be absent. Since cyanide is usually separated as HCN by distillation from acid medium prior to its determination, only sulfide can be considered a serious interference.

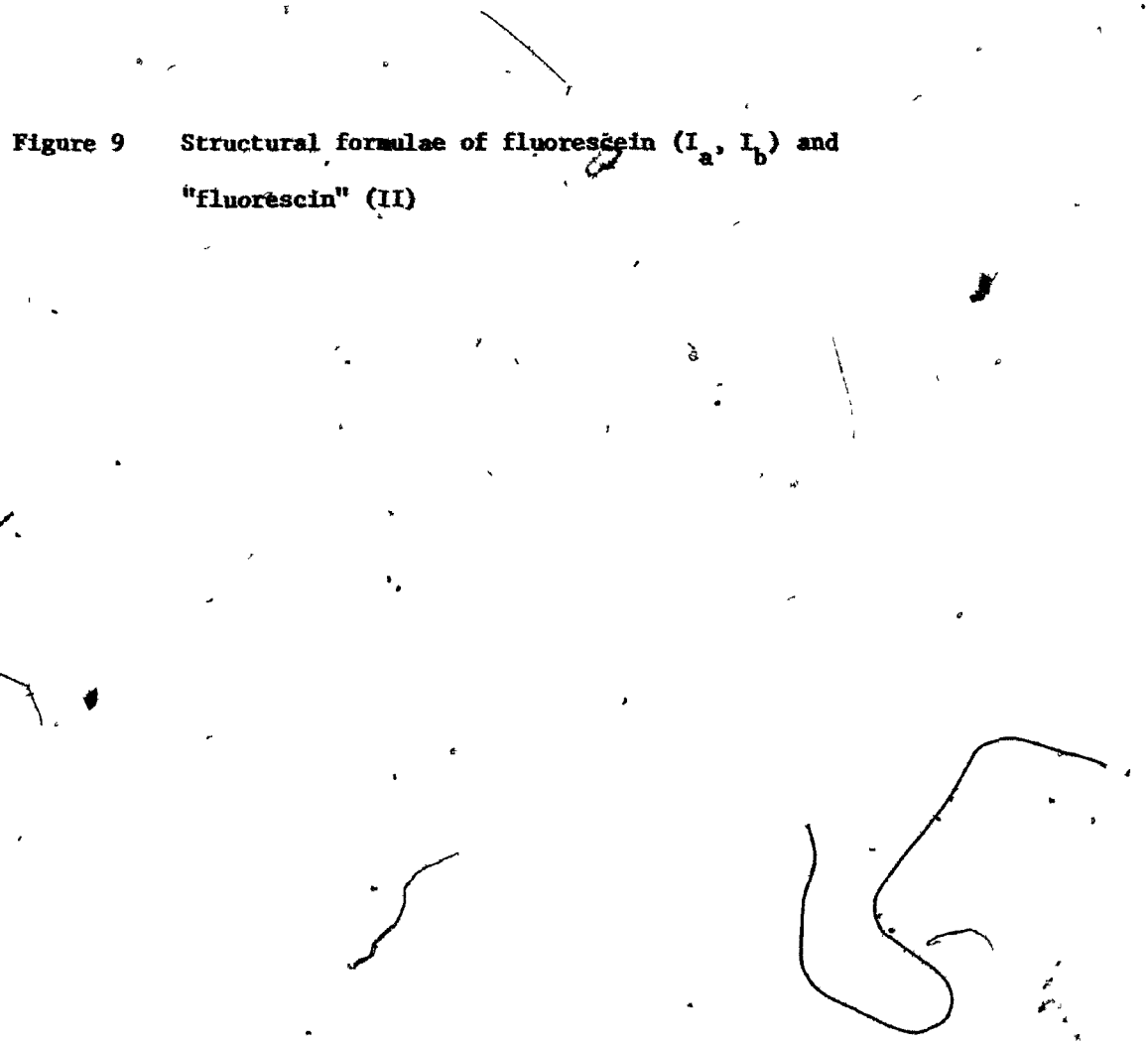
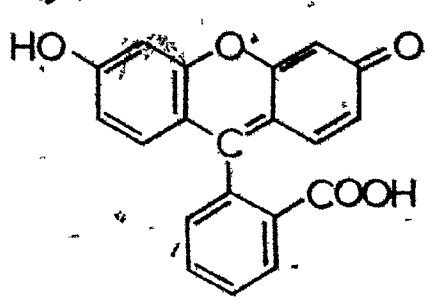
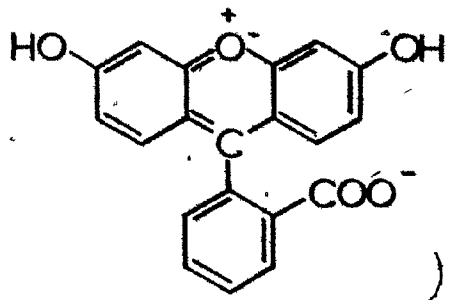


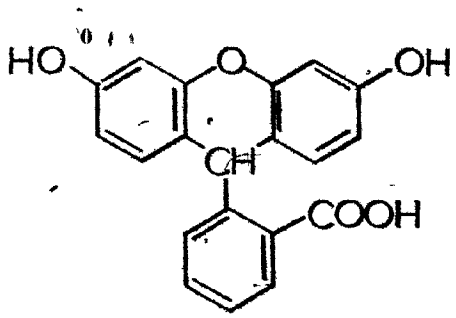
Figure 9 Structural formulae of fluorescein (I_a, I_b) and "fluorescin" (II)



I_a



I_b



II

Determination of Copper(II)

Introduction

In the previous method for the determination of cyanide, the fluorescence intensity was found to be directly proportional to the copper(II) concentration when cyanide and leucofluorescein concentrations were kept constant. Since there are not many methods reported in the literature for the direct fluorimetric determination of copper(II), the reaction conditions of copper determination were evaluated; as a result a very sensitive method has been developed which permits determination of 1 ng/ml of Cu(II). Reproducibility is excellent but some common metal ions interfere seriously.

Reagents

The reagent solutions were prepared in the same manner as in the previous method. The stock solution of 0.01 M $CuCl_2$ (Mallinckrodt) was standardized by EDTA titration[53].

Procedure

To 2 ml of buffer add 1 ml of 3×10^{-5} F KCN, 1 ml of reagent solution (stock diluted 1:100) and 1 to 5 ml of approximately neutral

unknown solution containing 0.01 - 0.20 µg of Cu(II). Make up the volume to 10 ml and read the fluorescence intensity at 514 nm; the excitation wavelength is 488 nm. Determine the concentration from a calibration curve.

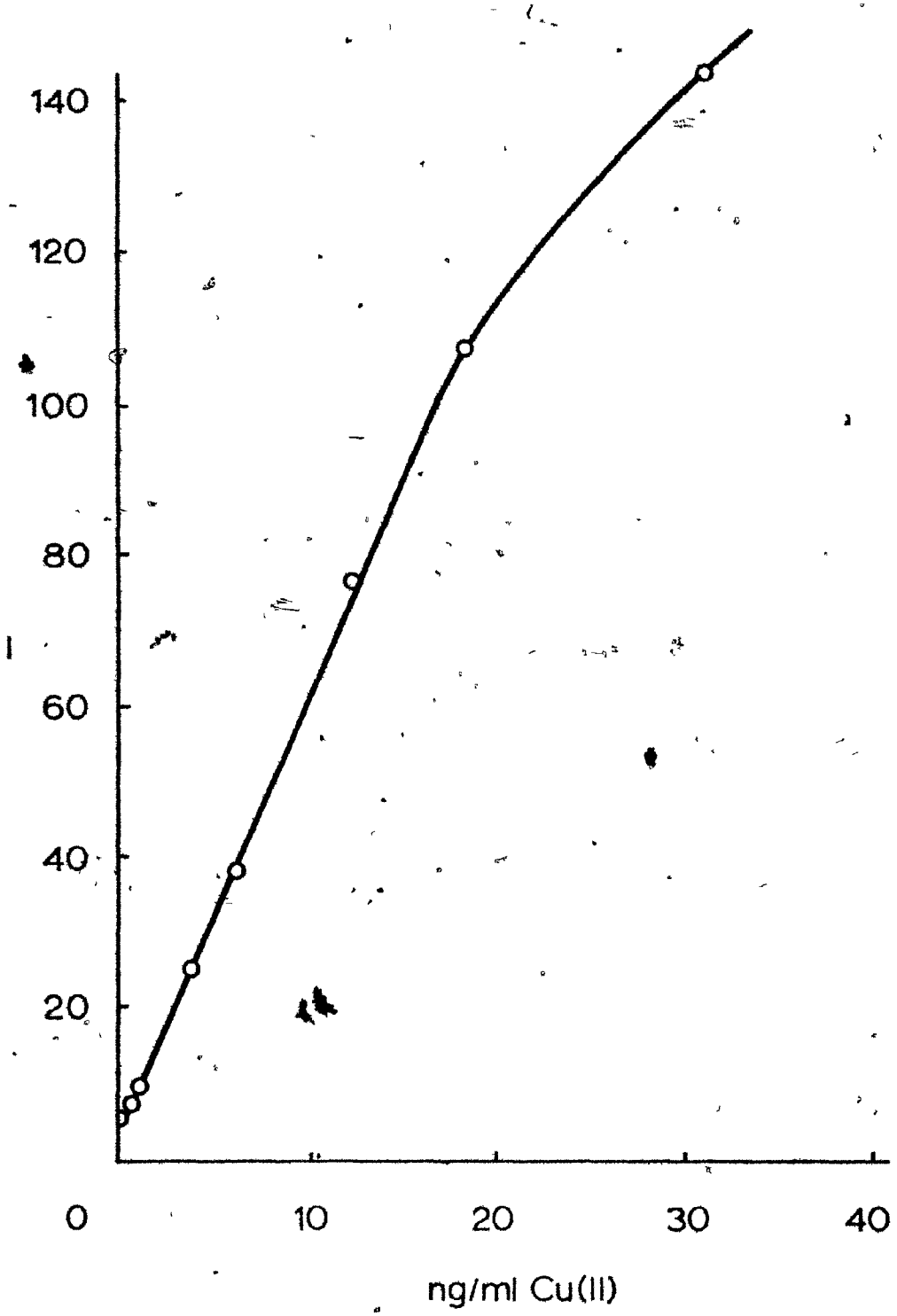
Results and discussion

The calibration curve (Fig.10) is linear from 1 to 20 ng/ml of copper(II); at either higher or lower concentrations the curve flattens slightly. The relative standard deviation of the limited number of determinations (six) of 12.7 ng/ml of Cu(II) (2×10^{-6} mmole) is ~1%.

Copper(II) (12.7 ng/ml) was determined in the presence of 100-fold molar excess of Al(III), Cd(II), Mn(II) and Zn(II), 10-fold excess of Co(II) and Ni(II) and an equimolar amount of Hg(II), Fe(II) and Fe(III). Thus the metal ions which react strongly with cyanide cannot be tolerated at high excess. The use of higher cyanide concentrations does not prevent the interference.

A 10,000-fold excess of acetate, bromide, chloride, nitrate and sulfate, 1,000-fold excess of iodide, perchlorate, phosphate and tartrate and 100-fold excess of fluoride and thiocyanate are without interference. Anions strongly complexing copper, such as, EDTA and citrate decrease fluorescence intensity even at equimolar amounts and must be absent.

Figure 10 Calibration curve for copper(II) with leucofluorescein. MM: 1.0; S: 0.0; (for fluorescence intensity readings higher than 100.0 the width of entrance slit was decreased and readings were recalculated); λ_{ex}: 488 nm; λ_{em}: 514 nm.


140
120
100
80
60
40
20
I
0
10
20
30
40
ng/ml Cu(II)

Evaluation of reaction variables was performed for 12.7 ng/ml of Cu(II). Temperature and pH variations were not studied since the optimum conditions for the reaction had already been established previously for the determination of cyanide; a borate buffer of pH ∿7.2 was used and fluorescence readings were taken at room temperature.

With increasing leucofluorescein concentration both I_S and I_B increase. Their ratio, however, reaches maximum at a final reagent dilution of about 1:1,000.

When increasing the final cyanide concentration from 10^{-7} to 10^{-5} F fluorescence intensity of the sample increases; for still higher cyanide concentrations, however, it begins to decrease; the blank fluorescence intensity follows a similar pattern, its changes are, however, minute. As a result, the highest I_S/I_B ratio is achieved when the final cyanide concentration is in the vicinity of 3×10^{-5} F.

Both sample and blank intensities increase slightly with the progress of time but their ratio remains virtually unchanged and immediate measurement is possible. There is, however, a difference between sample and blank upon irradiation; the intensity for the irradiated blank increases more rapidly with time and the I_S/I_B ratio decreases substantially. For fluorescence measurements it is, therefore, essential to use fresh (previously not irradiated in the instrument) solutions.

Provided that buffer is added as a first component the highest value for I_S is obtained when copper is added last, while I_B is essentially independent of the order of addition of the reactants. When cyanide and copper are present together before addition of the reagent, low I_S values are obtained because the copper is consumed before it can oxidize the reagent. The competing reactions involved can be expressed as follows:

In the analytical reaction copper(II), in the presence of cyanide, oxidizes leucofluorescein (LF) to the fluorescein (F):

$$2\ Cu^{2+} + 8\ CN^- + LF \rightleftharpoons F + 2\ H^+ + 2\ Cu(CN)_4^{3-}$$

When no leucofluorescein is present copper(II) is first reduced to copper(I) by cyanide and then complexed:

$$2\ Cu^{2+} + 4\ CN^- \rightleftharpoons 2\ CuCN + (CN)_2$$

$$+ 6\ CN^- \uparrow\downarrow$$

$$2\ Cu(CN)_4^{3-}$$

Some fluorimetric methods used for copper(II) determination are summarized in Table 2. The main principles used are solid state fluorescence, complex formation and chemiluminescence. Three methods based on complex formation[54, 55, 56, 57] achieve sensitivity in ng/ml range. Except for the luminocupferron method, however, measurement at low temperatures or solvent extraction have to be used.

TABLE 2

Some reactions in fluorimetric determination of copper

Reagent	Type of reaction	Sensitivity Reported	Major Interferences	Remarks	Ref
luminocupferron	complex formation	1 ng/ml	not reported		54,55
aetioporphyrin	complex formation	0.5 ng/ml	Ba, Zn, Cd, Ni, Fe(II), Mg and Ca tolerable in 1000-fold excess	fluorescence measured at -196°C	56
rose bengal, 1,10-phenanthroline	ternary complex formation	1 ng/ml	CN^-	preseparation with neocuproine	57
N-(β-hydroxyethyl)-anabasine	complex formation	0.1 µg/ml	not reported		58
N-(β-hydroxypropyl)-anabasine, H_2O_2	quenching, wavelength shift	0.5 µg/ml	U and Pd tolerable in 100-fold excess		59
1,1,3-tricyano-2-amino-1-propene	complex formation	0.1 µg/ml	15 metal ions do not interfere at 10:1 ratio		60

TABLE 2 continued

Reagent	Type of reaction	Sensitivity Reported	Major Interferences	Remarks	Ref
thiamine	complex formation	0.1 µg/ml	PO_4^{3-}, CN^-, S^{2-}, Ag(I), Hg(II), Co(II) Fe(III)	Cu(II) is first reduced to Cu(I)	61
luminol, H_2O_2	chemiluminescence catalysis	0.5 µg/ml	not reported	traces of other metal ions are known to catalyze also this reaction	62
ZnS-Ag phosphor	solid state fluorescence	0.1 µg/ml	oxidizing agents	selective for Cu(II)	63
CdS-Ag phosphor	quenching of solid state fluorescence	µg range	Ag, Hg, Pt	Cu, Ag, Hg, Pt cannot be distinguished	64

The method developed allows determination of 1 ng/ml of copper(II) at room temperature with a good reproducibility. Interference of some common metal ions, however, decreases its value for practical application unless a suitable preseparation method is employed.

Determination of Phosphate

Introduction

Non-fluorescent thiamine can be readily oxidized by various oxidizing agents to form a strongly fluorescent thiochrome*. Molybdate itself is not able to oxidize thiamine but when phosphate is present hexadimolybdatophosphate is formed and oxidation takes place[65]. A method based on this principle has been developed which allows determination of phosphate at ng/ml level with relatively few interferences.

Reagents

Solutions of the desired concentration were freshly prepared daily by dilution of the following stock solutions: 0.01 F thiamine chloride hydrochloride (Aldrich), 0.01 M (in terms of Mo) molybdate from $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ (Mallinckrodt), and 0.01 F phosphate from KH_2PO_4 (Fisher).

The buffer was a 0.1 F solution of borax.

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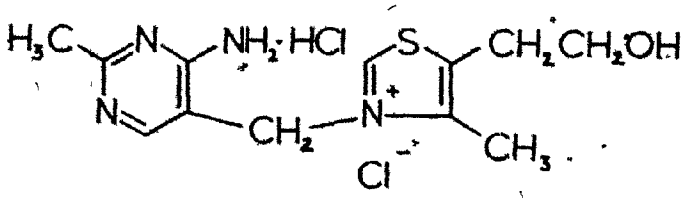
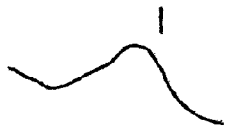
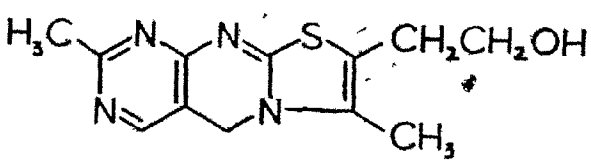
* The structural formulae of the thiamine chloride hydrochloride and of thiochrome are shown in Fig.11.

Figure 11 Structural formulae of thiamine chloride hydrochloride(I) and thiochrome(II).



I





II

Procedure

To 1 ml of 1:100 sulfuric acid add 5 ml (or less) of an approximately neutral unknown solution containing 0.050 to 1.00 µg of phosphate, 0.8 ml of 0.01 M ammonium molybdate, and 0.8 ml of 0.001 F thiamine. Add 2 ml of 0.1 F borax and dilute to 10 ml with twice distilled water. Measure the fluorescence intensity at 440 nm using an excitation wavelength of 375 nm. Determine the amount of phosphate from a calibration curve in preparation of which the volume of phosphate standard used was approximately the same as the unknown.

Results and discussion

Fig. 12A shows a calibration curve obtained without subtraction of the blank; it is a straight line up to 30 ng/ml of phosphate but does not pass through the origin because of background fluorescence. A straight line through the origin is obtained upon subtraction of the blank (Fig. 12B) and even 1 ng/ml of phosphate can be determined at higher instrument sensitivity.

The standard deviation of seven determinations of 50 ng/ml of phosphate was 3%; for 5 ng/ml the deviation was less than 10%.

The method was successfully applied to the determination of phosphate in sodium chloride by standard addition; a sample of reagent

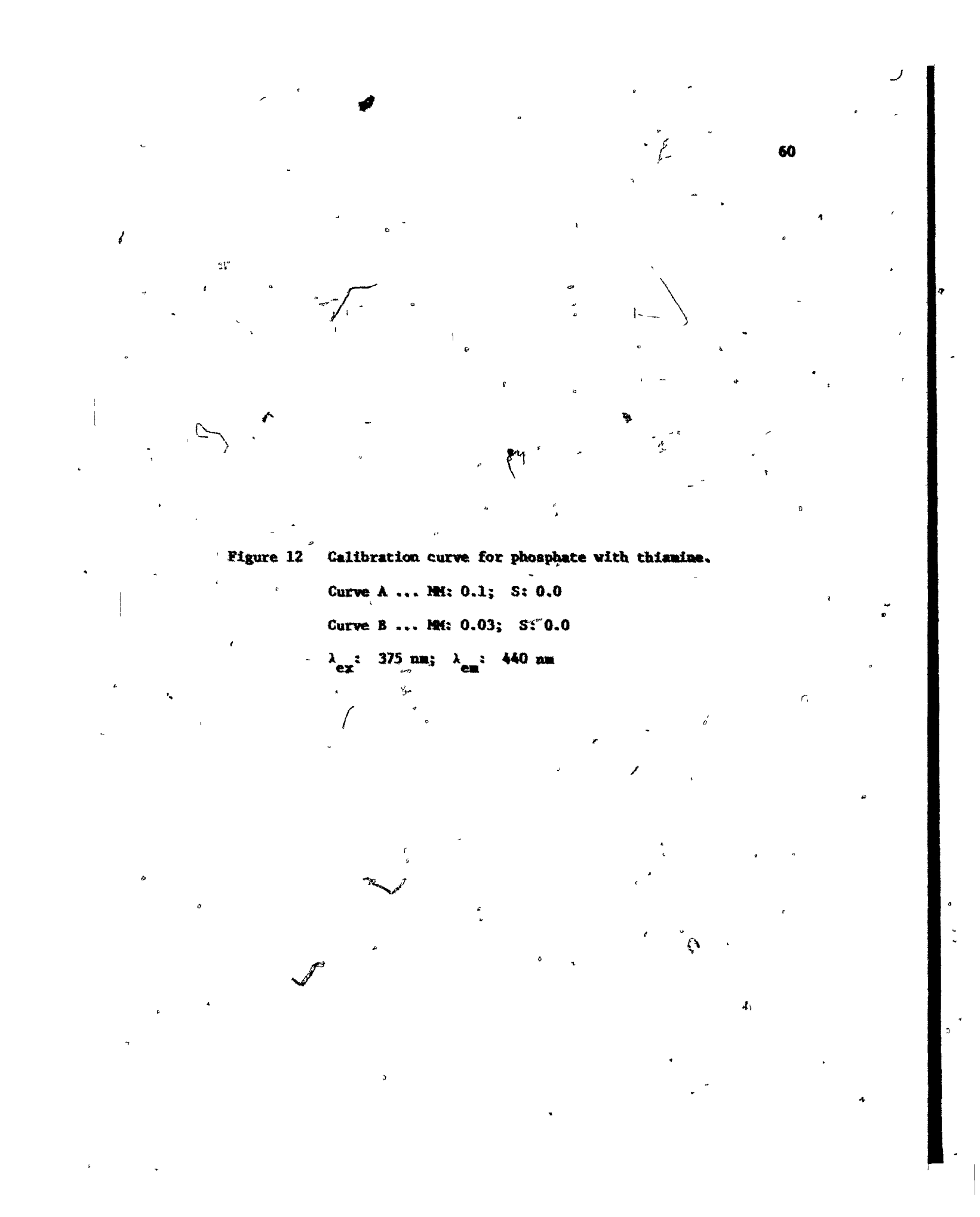
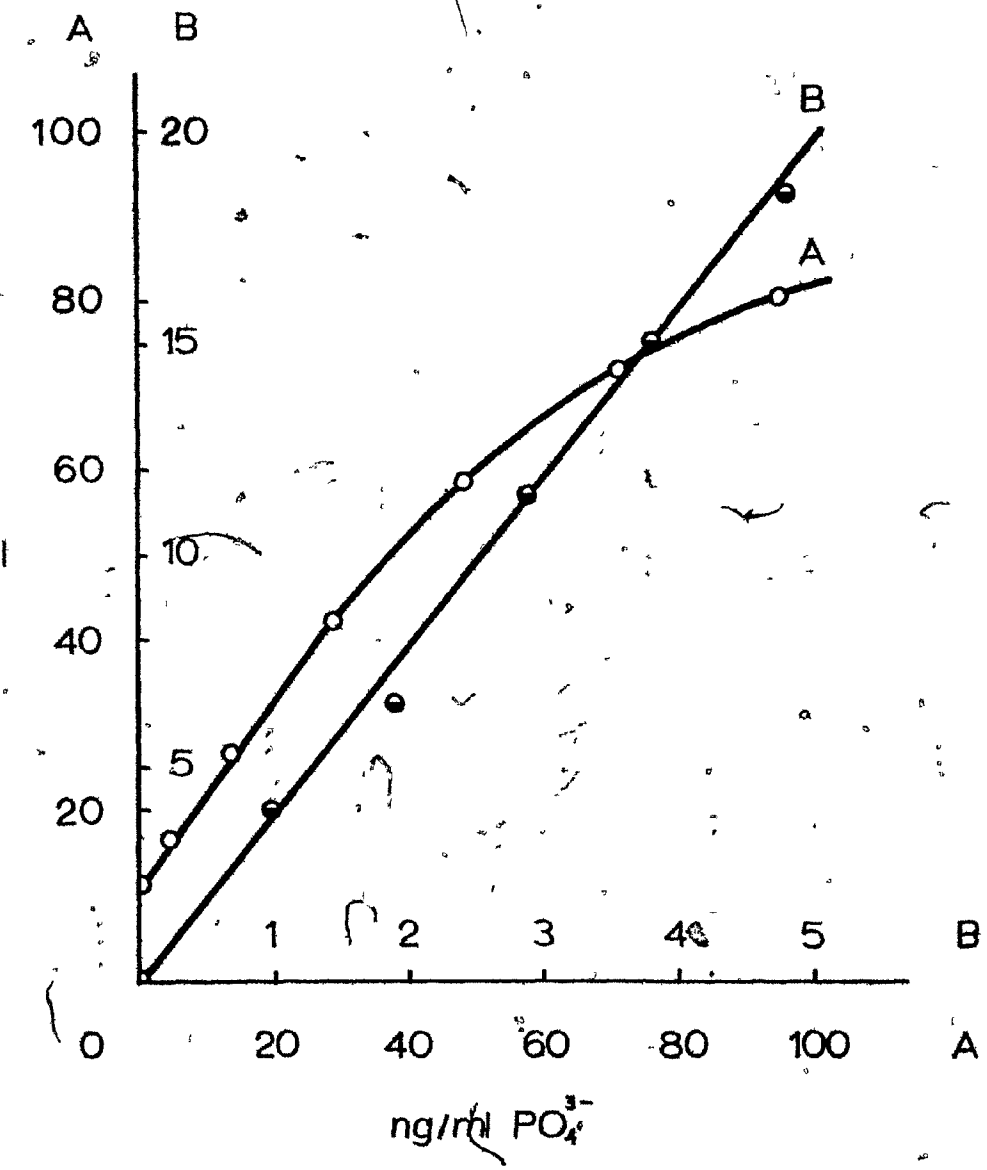


Figure 12 Calibration curve for phosphate with thiamine.

Curve A ... MM: 0.1; S: 0.0

Curve B ... MM: 0.03; S: 0.0

λ_{ex}: 375 nm; λ_{em}: 440 nm


A
B
100
80
60
40
20
0
20
15
10
5
1
2
3
4
5
B
20
40
60
80
100
A
B
A
ng/ml PO_4^{3-}

grade Baker & Adamson salt was found to contain 0.5 μg/ml of phosphate. The well established molybdenum blue photometric method could not be used because no color formed at the sodium chloride concentrations (2%) allowable in the fluorimetric procedure and lower salt concentrations did not have sufficient phosphate present to be determined colorimetrically. The fluorimetric method is more sensitive than the colorimetric and time is not a critical factor in the formation of the product for measurement; except for mercury and iron, most common ions can be tolerated at substantially higher concentrations than in the colorimetric one.

Fifty ng/ml of phosphate was successfully determined in the presence of a 10,000-fold molar excess of acetate, chloride, nitrate, perchlorate and thiocyanate and a 1,000-fold excess of bromide, fluoride and tartrate. Results were also satisfactory with a 100-fold excess of citrate, EDTA, iodide and persulfate. A 10-fold excess of silicate and ferricyanide could be present but larger amounts increased the fluorescence intensity; an equivalent amount of sulfide strongly quenched the fluorescence.

A 10,000-fold molar excess of Ni(II), Mn(II) and Cd(II) and a 1,000-fold excess of Cu(II), Co(II), Ca(II), Zn(II) and Al(III) did not interfere. A 10-fold excess of Fe(III) and an equivalent amount of Fe(II) could be present but an order of magnitude increase in concentration decreased the fluorescence. Mercury(II) and Hg(I) markedly increased the fluorescence even at equimolar ratios.

The fluorescence intensity depends, of course, upon the amount of thiochrome obtained by oxidation of thiamine by the dimolybdato-phosphate formed from phosphate on reaction with molybdate in acid solution. Provided there are no other species in solution capable of oxidizing* thiamine to thiochrome, the same fluorescence intensity would be observed for the same phosphate concentration if there are no interferences in (a) the formation of dimolybdophosphate (b) the oxidation of thiamine (c) the fluorescence output of thiochrome. Thus silicate interferes because it forms a heteropoly acid capable of oxidizing thiamine and ferricyanide also oxidizes thiamine to thiochrome; sulfide interferes with the oxidation of thiamine. Ferrous iron interferes in the oxidation step and the interference of Fe(III) is due to the absorption or scatter of fluorescent light by nuclei of hydrous oxide; mercuric and mercurous ions increase the amount of thiochrome formed either through direct or catalytic oxidation of thiamine.

Important factors in the determination of phosphate with thiamine are the starting acidity, the thiamine concentration, the molybdenum concentration and the final pH.

For phosphate concentrations of 5 to 100 ng/ml the I_S/I_B ratio was a maximum when 1 ml of 1:100 H_2SO_4 was initially present; the amount of molybdophosphate is highly acid dependent[66]. I_B was unchanged

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*Oxidizing agents, prior to formation of molybdophosphate, can be destroyed by treatment with sulfite; excess sulfite must be removed by heating before addition of molybdate.

if more acid was present but I_S decreased rapidly; I_B increased with decreasing acidity whilst I_S decreased slightly. For example, the I_S/I_B ratio was 1.4, 2.1 and 1.6 for 0.25, 1.0 and 2.0 ml of 1:100 H_2SO_4 added respectively; similar results were obtained on replacing sulfuric with hydrochloric or nitric acids.

The I_S/I_B ratio remained unchanged for final thiamine concentrations of 6 to 10 x 10^{-5} F and molybdenum concentrations of 5 to 10 x 10^{-4} M. I_S decreased for higher or lower thiamine concentrations (I_B did not change appreciably over the range studied); for higher molybdenum concentrations I_B increased faster than I_S whilst the reverse was true with lesser amounts of molybdenum present. Molybdenum concentrations of ∿8 x 10^{-4} M and thiamine concentrations of ∿8 x 10^{-5}M are recommended for best sensitivity.

A final pH of ∿8 is necessary for maximum sensitivity. Above pH 8 the absolute increase in I_S and I_B was the same but I_S/I_B decreased; from pH 2 to 8 the blank fluorescence was constant but I_S increased. For example, the I_S/I_B ratio was 1.0, 6.5 and 2.0 for respective pH values of 2.3, 8.0 and 12.0; $I_S - I_B$ was constant from pH 8 up but the sensitivity decreased as a result of the high blank reading.

With increasing temperature both I_S and I_B increase to the same degree. For example, 47.5 ng/ml of phosphate gave I_S and I_B values of 54 and 5 respectively at 24°C and readings of 79 and 27 at 95°C. Again the $I_S - I_B$ figure is essentially unchanged whilst I_S/I_B decreases.

No change in fluorescence intensity was observed for solutions allowed to stand for various time periods before measurement (up to 30 minutes before adjustment to pH 8 and up to 330 minutes after adjustment). The fluorescence measurement can therefore be performed immediately after mixing the reactants and the fluorescence reading is stable for several hours at least.

There are not many fluorimetric methods for phosphate determination reported in the literature (see Table 1). The quenching method[30] suffers from many interferences; ion associate methods[31,32] involve precipitation or solvent extraction, respectively. The enzymatic procedures[33,34] seem to be most sensitive (sensitivity is claimed to be limited only by the purity of reagents used). The most widely used method for practical phosphate determination is still the well-established, colorimetric molybdenum blue method. It was compared with the thiamine method in the practical determination of phosphate in sodium chloride. While the thiamine method gave good results no color was formed in the molybdenum blue procedure because of the high salt concentration. The fluorimetric method is also superior in that it is more sensitive and is not time dependent.

Determination of Mercury(II)

Introduction

In an investigation of the use of thiamine for the fluorimetric determination of phosphate, mercury(II) was found to react with thiamine to give high fluorescence. It appears to be, from the literature survey, the first instance of mercury induced fluorescence. This reaction has been studied in detail and a fluorimetric method has been developed which allows determination of ng/ml of mercury in solutions which have relatively low salt concentrations (<0.02 F). Where digestion with acids is necessary for sample preparation (e.g. organics), samples containing at least 10 µg of mercury are required because the high salt concentration resulting from neutralization must be decreased by dilution.

Reagents

A stock solution of 0.01 F $HgCl_2$ (Merck & Co.) was standardized by EDTA substitution titration[67]. The stock solution of thiamine chloride hydrochloride was the same as in the previous method.

The borate buffer (pH ~7.7) was prepared by mixing 47 ml of 1N HCl with 53 ml of 0.05 F borax.

Procedure

To 2 ml of buffer in a 10 ml volumetric flask add several ml of approximately neutral unknown solution (containing 0.1 to 5 µg of mercury), 1 ml of 3×10^{-5} F reagent and make up to volume with twice distilled water; the resulting solution should be less than 0.02 F in foreign salts. Measure the fluorescence intensity at 440 nm, after one hour or more; the excitation wavelength is 375 nm. A blank should be run concurrently. A typical calibration curve is shown in Fig. 13A.

Factors affecting fluorescence

The determination is based on the oxidative reaction of mercury(II) on thiamine to produce highly fluorescent thiochrome; mercury must, therefore, be present in solution in the divalent state. Mercury(I) solutions give a fluorescence intensity of about one-half that obtained for the same concentration of mercury(II) and organomercury compounds can only be successfully analyzed after destruction of organic matter and conversion to inorganic mercury(II).

The ratio of sample to blank intensity is essentially constant over the pH range of 7 to 8 but decreases rapidly at both lower and higher pH values; no differences are observed, for example, between blank and sample at pH 4.5 and at pH 11.

The fluorescence intensity is both time and reagent concentration dependent. Highest sensitivity is obtained at a reagent concentration of 3×10^{-6} F after approximately 1 hour's standing (Fig. 14). Constant fluorescence intensity can be obtained for higher reagent concentrations within a few minutes but a lower sensitivity is achieved.

Heating increases the reaction rate so that solutions heated to 90°C for ten minutes give essentially the same reading, after cooling, as solutions allowed to stand at room temperature for 1 hour before measurement; the reading for the blank does not change appreciably on heating.

Results and discussion

The fluorescence intensity is a linear function of mercury concentration from 10 to 200 ng/ml; the curve does not pass through the origin because of some background fluorescence. At higher mercury concentrations, where lower instrument sensitivity is necessary, there is no measurable background fluorescence and a straight line is obtained up to 500 ng/ml of mercury (Fig.13B) under the procedural conditions; a linear relationship between fluorescence intensity and concentration is obtained at considerably higher mercury levels if the reagent concentration is increased. The percent standard deviation on the analysis of 50 ng/ml of mercury(II) (seven determinations) was 4.1; for 100 ng/ml (ten determinations) it was 3.2%.

Figure 13 Calibration curve for mercury(II) with thiamine.

Curve A ... MM: 0.03; S: 0.0

Curve B ... MM: 0.3; S: 0.0

λ_{ex}: 375 nm; λ_{em}: 440 nm

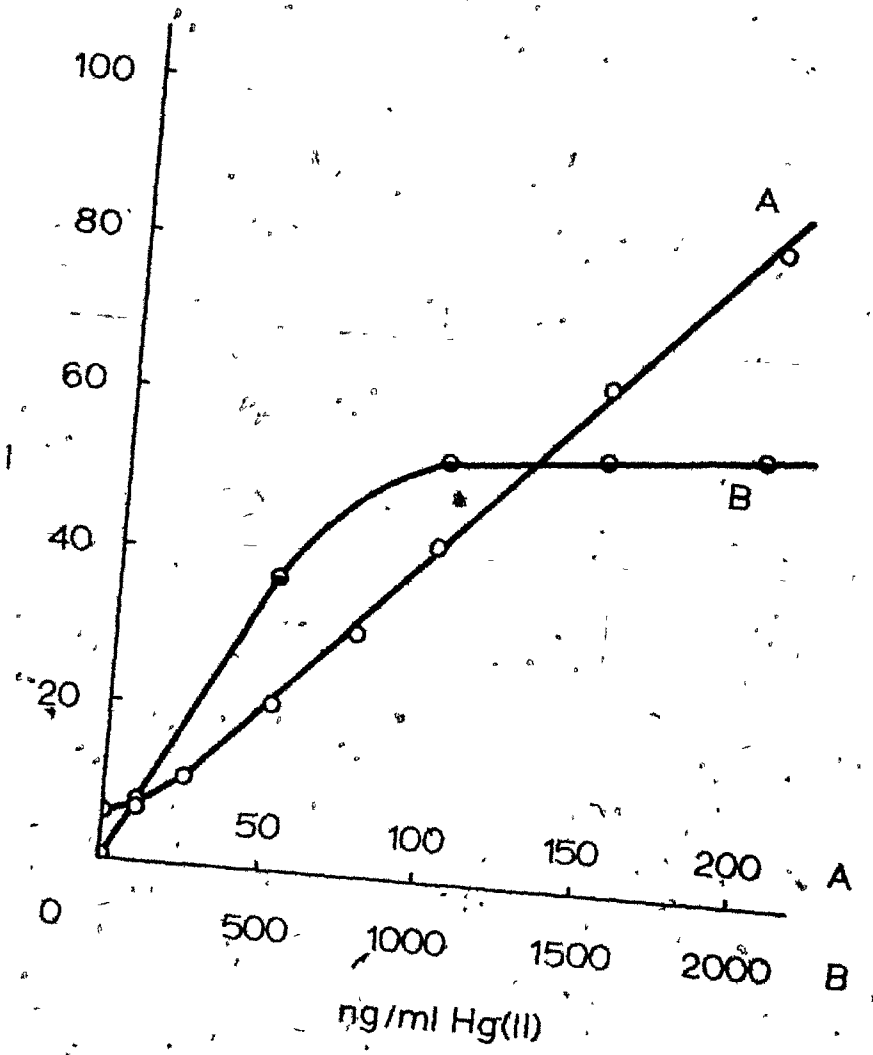
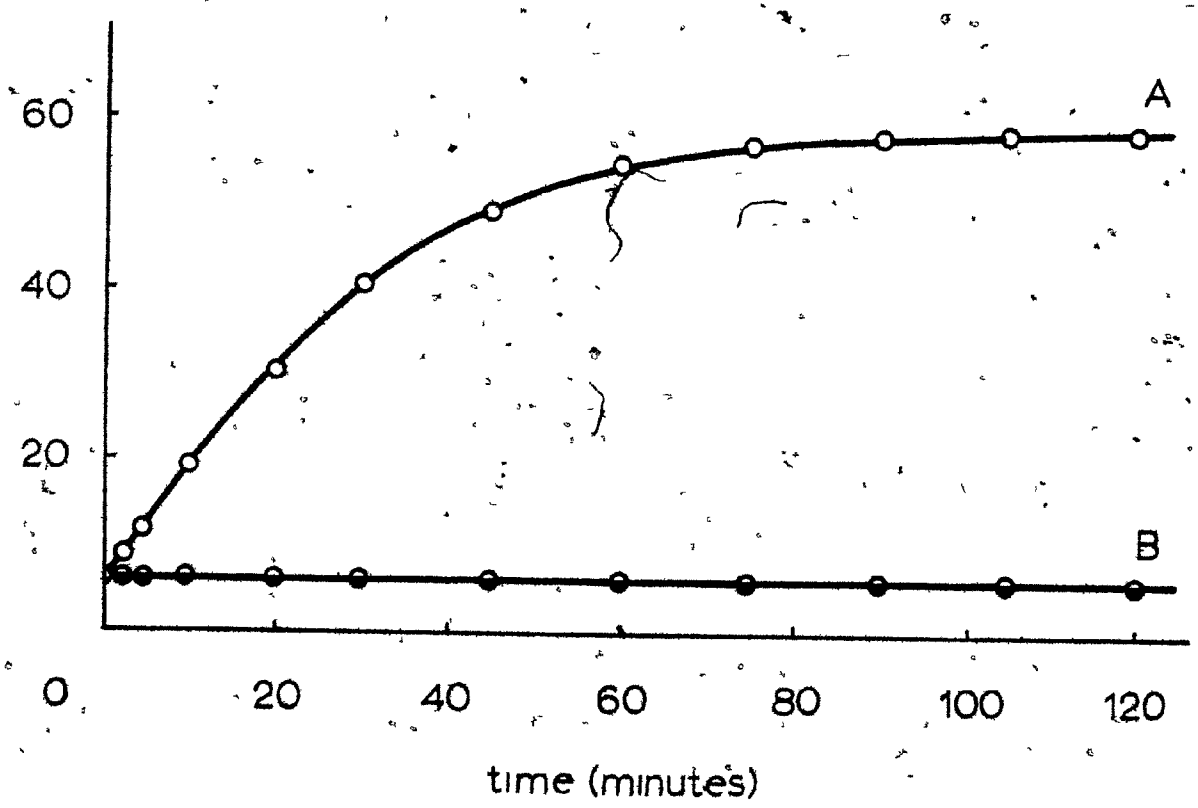

100
80
60
40
20
0
A
B
50
100
150
200
A
500
1000
1500
2000
B
ng/ml Hg(II)

Figure 14 Time dependence of fluorescence intensity.

MM: 0.03; S: 0.0; λ_{ex}: 375 nm; λ_{em}: 440 nm

Curve A ... 100 ng/ml of Hg(II)

Curve B ... respective blank


60
40
20
0
20
40
60
80
100
120
A
B
time (minutes)

Fifty ng/ml (2.5×10^{-6} mmole) of mercury(II) were successfully determined in the presence of a 10,000-fold molar excess of Ni(II), Co(II) and Zn(II), a 1,000-fold excess of Cu(II), Cd(II), Mn(II) and Al(III) and 100-fold excess of Fe(II) and Fe(III). Similarly, a 100,000-fold excess of the sodium or potassium salts of acetate, chloride, citrate, sulfate and tartrate did not interfere. A 10,000-fold excess of fluoride, nitrate, perchlorate and phosphate and a 1,000-fold excess of bromide and thiocyanate could be tolerated; the fluorescence was quenched by equivalent amounts of cyanide, iodide, sulfide and EDTA.

The method can be applied to mercury analysis in various systems. For example, advantage can be taken of the relatively high amounts of foreign ions which can be tolerated and mercury can be determined in, e.g., zinc salts. Zinc (0.2 mmole) in $Zn(ClO_4)_2$ was masked by addition of 2 ml of 0.1 F sodium citrate (to avoid zinc precipitation in basic medium) and 0.1 F borax was added to adjust to the proper pH. Using a standard addition method 10^{-3} % or more of mercury in zinc can be determined. A higher sensitivity cannot be achieved because of the limited allowable concentration of salts in the solution to be analyzed.

Another area of application is the analysis of mercury in systems containing organic material. There are three basic steps involved: (1) organic material has to be mineralized (to remove organic matter, decompose organomercurial compounds and solubilize inorganic mercury

compounds); (2) colorless solution of proper pH must be obtained; (3) the fluorimetric reaction has to be performed.

The digestion technique recommended by Nelson *et al.*[68] was applied to water samples and to organomercurials and was found to be very satisfactory; the method consists of boiling the sample with a mixture of concentrated sulfuric and nitric acids in an Erlenmeyer flask fitted with a shortstemmed funnel with care being taken to prevent charring and resultant mercury loss through carbon reduction. Sample sizes must be taken to provide 10 to 500 µg of mercury. Salt concentrations (formed as result of neutralization after acid digestion) in excess of 0.02 F decrease the fluorescence intensity and it is therefore necessary to dilute digested, neutralized solutions, ordinarily by about two orders of magnitude, to obtain good results.

Tap water samples (25 ml), for example, were spiked with 250 µg of mercuric chloride and were digested with one ml of concentrated sulfuric acid and one ml of concentrated nitric acid. After digestion the solutions were neutralized to pH 7.5 to 8 with 50% sodium hydroxide (some borax was added to provide buffering) and were then made up to the original 25 ml volume. An aliquot of this solution was diluted 100 times to decrease the salt concentration to less than 0.02 F and the regular procedure with thiamine applied. The relative error of determination was less than 5%.

Five mg samples of di-p-tolylmercury (Eastman) were similarly digested with acids and were successfully analyzed for mercury with thiamine. The precision was excellent and the relative error of 6% was satisfactory considering the unknown purity of the sample.

The method developed allows selective and simple determination of mercury(II) at the 10 to 500 ng/ml level in solutions less than 0.02 F in foreign salts by measuring the fluorescence produced on reaction with thiamine. Sample sizes to provide 10 µg or more of mercury are required for samples which necessitate acid digestion prior to analysis (e.g. organics).

The few reported methods for the fluorimetric determination of mercury(II) are listed in Table 3. They involve ion associate extractions or solid state luminescence and are neither very sensitive nor selective. The method developed compares with them favorably in terms of selectivity, sensitivity and simplicity. Its applicability to analysis of practical samples has been demonstrated.

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TABLE 3

Some reactions in fluorimetric determination of mercury

Reagent	Type of reaction	Sensitivity Reported	Major Interferences	Remarks	Ref
rhodamine S, Br^-	ion associate extraction	20 µg/ml	not reported		69
butylrhodamine B, Br^-, crystal violet	ion associate extraction	0.1 µg/ml	Au(III)	preseparation by extraction of crystal violet bromomercurate	70
rhodamine B, I^-	quenching	µM range	Tl(III), Pd(II), Pt(IV), Bi(III), Cd(II), Fe(III), Sb(III)		71
tetracyano-platinate(II)	formation of fluorescent precipitate	5 µg/ml	Y(III), Zr(IV), Ag(I), Zn(II), Cd(II), Al(III), Pb(II), La(III), Th(IV)	interfering metals form also fluorescent precipitates	72
CdS-Ag phosphor	quenching of solid state fluorescence	µg range	Cu, Ag, Pt	Cu, Ag, Hg, Pt cannot be distinguished	64

Determination of Sulfide

Introduction

In the investigation of the use of thiamine for the determination of phosphate and mercury(II) it was found that sulfide inhibited the oxidation of thiamine to fluorescent thiochrome. A quenching fluorimetric method based on this principle has been developed for ng/ml of sulfide. Because of the relatively short reaction time, the high sensitivity, and excellent reproducibility, permanganate was chosen as the most suitable oxidant.

Reagents

A stock solution of ~0.02 M $KMnO_4$ (Shawinigan) was prepared by dissolving 3.2 g of $KMnO_4$ in 1 liter of distilled water and allowing the solution to stand for three days. The MnO_2 formed was then filtered off through a sintered glass crucible and the permanganate solution was kept in the dark.

Thiamine and sulfide standard solutions and borate buffer (pH ~7.7) were prepared in the same manner as described in previous methods.

Procedure

To 2 ml of borate buffer (pH ~7.7) add 1 ml of 10^{-3} F thiamine followed by 5 ml or less of an approximately neutral unknown solution containing 0.03 to 1 µg of sulfide; add 1 ml of 10^{-5} F $KMnO_4$ and make up the volume to 10 ml. After at least 10 min measure the fluorescence intensity at 440 nm using an excitation wavelength of 375 nm. The blank reading should be adjusted to the full scale deflection for high sensitivity. Determine the concentration from a previously prepared calibration curve.

Results and discussion

A typical calibration curve is shown in Fig. 15. The curve is linear up to 20 ng/ml and even 3 ng/ml of sulfide can be determined.

The relative standard deviation of ten determinations of 9.6 ng/ml of sulfide is 1.3%.

Sulfide (9.6 ng/ml, 3×10^{-6} mmole) can be determined in the presence of 100,000-fold molar excess of fluoride, 10,000-fold excess of acetate and chloride, 1,000-fold excess of bromide, iodide, nitrate, nitrite, perchlorate and sulfate, 100-fold excess of phosphate and tartrate and 10-fold excess of thiocyanate and cyanide; equimolar concentrations of citrate do not interfere; sulfite and EDTA interfere strongly and must be absent.

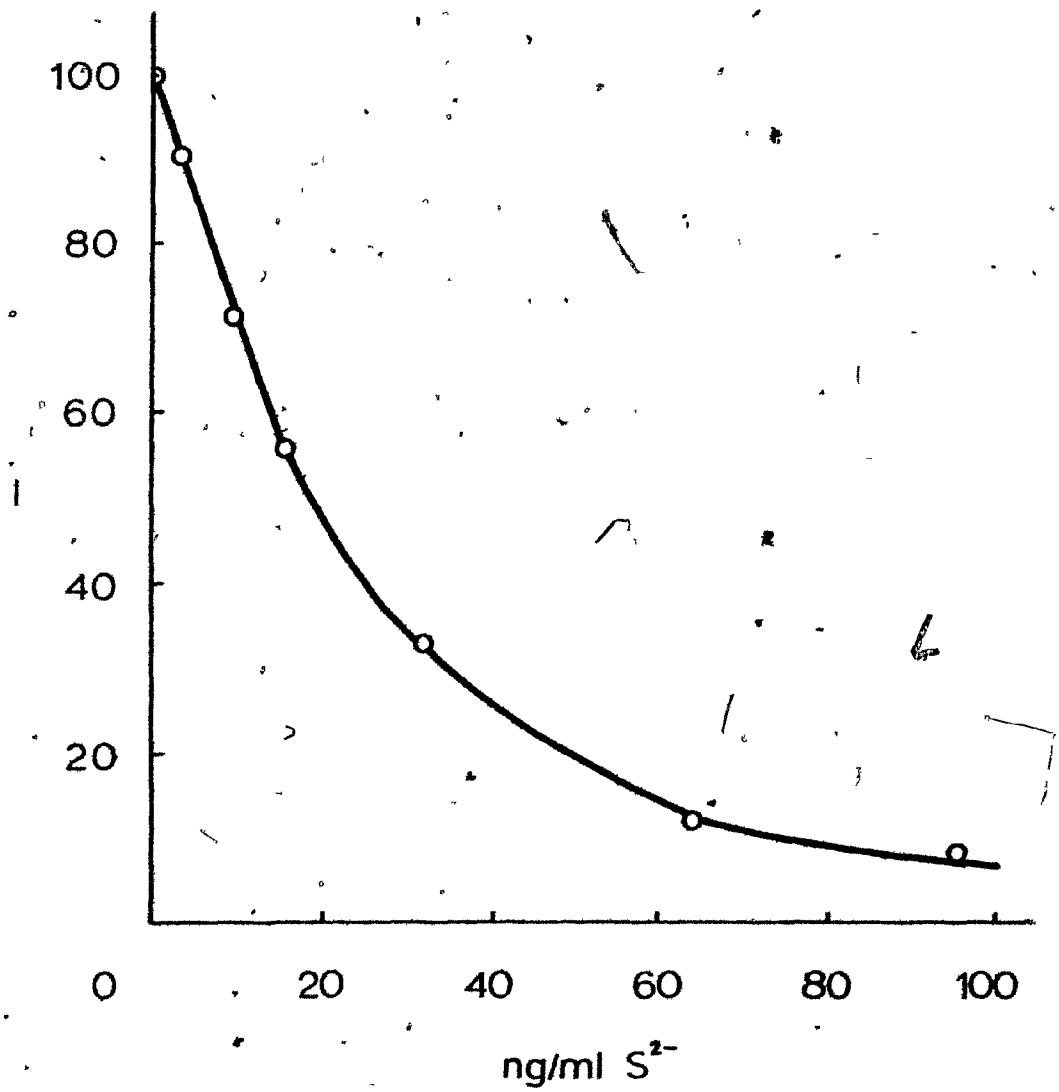
A 10-fold excess of Al(III), Fe(II) and Zn(II) and equimolar concentrations of Fe(III) and Ni(II) can be tolerated. Equimolar concentrations of Cd(II), Co(II), Hg(II), Hg(I) and Mn(II) interfere and must be absent.

The method is based on two competing reactions of permanganate: (1) oxidation of thiamine to thiochrome; (2) oxidation of sulfide. The diverse ions can thus interfere in two ways. They can either react with the sulfide present or they may interfere in the oxidation-reduction reaction between permanganate and thiamine. The permanganate may undergo a number of reduction steps [*e.g.*, to manganate, manganese dioxide and manganese(II)]. The interference may thus occur in any of these steps and to a different degree. Thus the net resulting interference will depend upon which of these processes will be dominant.

For example, Ni(II) and Zn(II) decrease the fluorescence intensity of the sample and they also decrease the fluorescence intensity of the blank alone; since the blank contains permanganate and thiamine (but no sulfide) the dominating factor must be interference in the oxidation-reduction reaction. Phosphate and EDTA, on the other hand, cause an increase in both blank and sample intensity and must, therefore, favor the production of thiochrome.

In practical work, however, most of the interferences may be removed by distillation of sulfide as H_2S from acidic medium and

Figure 15 Calibration curve for sulfide with thiamine.
MM: 0.1; S: 9.0; (blank reading was adjusted to read 100.0)
λ_{ex}: 375 nm; λ_{em}: 440 nm


100
80
60
40
20
I
0
20
40
60
80
100
ng/ml S^{2-}

subsequent recapture in base. One of the main interferences - sulfite - which is carried over in distillation procedure may be eliminated by using filters impregnated with 5% $KHCO_3$[73].

In all subsequent studies of reaction conditions the sulfide ion concentration was 3×10^{-7} F (9.6 ng/ml).

Both I_S and I_B exhibit maxima at pH 7.5 to 8.0. For higher or lower pH values both intensities decrease and so does their difference; for example, at pH 8.6 or 4.2 I_S and I_B are approximately equal.

With increasing permanganate concentration the fluorescence intensities of sample and blank increase to about the same degree. However, when the permanganate concentration is greater than $\sim 2 \times 10^{-6}$ F both blank and sample solutions become yellowish in color and the blank intensity is less than that of the sample. This is due to the formation of dispersed manganese dioxide and changed absorption characteristics of the solutions. The reagent concentration used in the procedure was, therefore, 1×10^{-6} F.

Maximum value of the fluorescence intensity is obtained at a final thiamine concentration in the vicinity of 10^{-4} F. For still higher concentrations there is a slight decrease in the fluorescence intensity probably due to the excessive absorption of either the exciting light or the fluorescence emitted ("inner filter effect").

The fluorescence intensity increases slightly with time; a constant reading is achieved within 10 minutes. That is, therefore, the minimum time period which should be allowed to elapse between making up the solutions and fluorimetric measurement.

While the fluorescence intensity of the blank does not depend on the order of addition of the reagents (provided buffer was added first), the fluorescence of the sample is dependent on the addition sequence. If sulfide is added last no quenching occurs, but if sulfide is added before permanganate, then it does not matter whether thiamine is present from the very beginning or not because quenching takes place. Thus it is probable that sulfide quenches the fluorescence of the system by consuming part of the permanganate.

Both sample and blank intensities increase slightly with increasing temperature while their difference remains virtually constant over the range studied (20 to 95°C). Since their ratio I_B/I_S decreases at higher temperatures the measurement at the room temperature is recommended.

Other reagents may be used instead of permanganate to oxidize thiamine. Using mercury(II) higher sensitivity for sulfide is achieved but the reproducibility is poor. Ferricyanide requires higher pH and reaction is strongly time dependent.

The fluorimetric methods for sulfide published in the literature are all very sensitive (see Table 1). Since sulfide is usually separated

as H_2S by distillation prior to analysis the majority of interferences can be so avoided. The method developed is comparable to the published methods in terms of sensitivity and reproducibility. Its advantage is that it employs common, readily available reagents.

CONCLUSIONS

The object of this work has been the development of new, fluorimetric methods for the determination of anions at the ng/ml concentration levels; as a result direct fluorimetric methods have been developed for cyanide and phosphate and sulfide has been determined by quenching; methods for the determination of mercury(II) and copper(II) have also been found. The use of ligand-exchange and oxidation-reduction reactions for fluorimetric determination of anions has been studied in detail and some useful conclusions may be drawn.

In the search for reagents suitable for direct fluorimetric methods for anions based on ligand-exchange reactions, perhaps the most important aspect to be kept in mind is how efficiently is the fluorescent ligand quenched by a particular metal ion; this factor is critical to the resultant background fluorescence and thus for the sensitivity of the whole method.

It has been stated that reversible electron transfer between members of the complex leading to the depopulation of the excited states is the mechanism for luminescence quenching since the quenching ability of certain substances correlates well with their ionization potentials[74]. The second ionization potentials [third for iron(III)] for some transition metals of interest are given in Table 4 together with the number of unpaired electrons in weak ligand field.

TABLE 4

Ionization potentials of some transition metals[75].

Metal ion	Fe^{3+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}
# of unpaired electrons	5	4	3	2	1	0
ionization potential (eV)	30.64	16.18	17.05	18.15	20.29	17.96

For these transition metal ions, however, the ionization potentials do not correlate with their quenching ability. Zinc, for example, has its second ionization potential close to those of nickel or cobalt and yet zinc(II) is known to form highly fluorescent chelates while nickel(II) and cobalt(II) are rather effective fluorescence quenchers. Iron(III), which in terms of its ionization potential should quench fluorescence effectively, was found to be a poor quencher.

Quenching is a complex phenomenon. The extent of fluorescence quenching of an organic molecule as a result of complexation with a metal ion is primarily influenced by the:

(1) electronic structure of the metal ion

(2) mass of the metal ion

(3) stability of the metal complex formed

Of course, the influence of these factors cannot be completely separated; for example, the stability of the complex is obviously also dependent on the nature of the metal ion involved. Reaction with a metal ion results in a change in the energy of molecular orbitals of the organic molecule and the efficiency of excited state processes which compete with fluorescence (intersystem crossing and internal conversion) may be influenced.*

Diamagnetic complexes of lighter metal ions [Mg(II), Al(III), Zn(II)] fluoresce strongly whereas metal ions which are paramagnetic possess unpaired electrons which create a strong, inhomogeneous magnetic field and spin-orbit coupling occurs. The probability of intersystem crossing is thus greatly increased resulting in population of the triplet state and fluorescence quenching.

Nuclei of heavy metal atoms are less shielded from their valence electrons and their nuclear charge results, once again, in increased spin-orbit coupling and population of the triplet state. Very heavy elements, such as mercury, not only quench fluorescence, but do not increase phosphorescence yield and may even decrease it. This can be

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* Some metal ions may also decrease the fluorescence intensity of the system by their absorption in UV or visible either of excitation or emission radiation of the fluorescent reagent. This process is not, however, considered to be a true quenching. Among the metal ions of interest, nickel(II), iron(II) and iron(III) exhibit appreciable absorption in the near UV and visible.

explained by the contraction of electronic energy levels due to high nuclear charge which increases the probability of internal conversion ("heavy atom effect").

For a fluorescent organic ligand (L), fluorescence of which is quenched on reaction with a metal ion (M) the stability constant for the reaction $M + L \rightleftharpoons ML$ is given by $\frac{[ML]}{[M] \cdot [L]}$. The higher the stability constant of the metal complex the lower will be the amount of free ligand present at equilibrium and quenching will appear to be more efficient.

The experimental results show that copper(II), nickel(II) and cobalt(II) ions (which possess 1, 2 and 3 unpaired electrons, respectively)* are more efficient quenchers than mercury(II) and iron(III). The "heavy atom effect" (mercury) is, apparently, less effective in quenching than unpaired electrons. Iron(III), where 5 unpaired electrons would predict highly effective quenching, however, was the least efficient quencher among the metal ions studied. The possible explanation is that half-filled orbitals [d^5 configuration of iron(III)] which are particularly stable are not as effective as partially filled (d^1 to d^4, d^6 to d^9) orbitals in spin-orbit coupling; it is known, for

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* No pairing of electrons as a result of complexation with EDTA type groups is expected.

example, that light ions with stable p^6 and d^{10} electron configurations give strongly fluorescent complexes.

All metal ions studied form rather stable complexes with EDTA type groups (which are the complexing groups of metallofluorescent indicators). Since the stabilities of metal complexes of metallofluorescent indicators should follow the same sequence as those of respective EDTA complexes, *i.e.*, Fe(III)>Hg(II)>Cu(II)>Ni(II)>Co(II)[76], iron(III) and mercury(II) might be expected to be the most effective quenchers. This was not, however, the case and thus the relative stability of the particular metal complexes was not found to bear any relation to the extent of quenching. For the transition metal complexes of metallofluorescent indicators studied the nature of metal ion is, apparently, the predominant factor in the fluorescence quenching efficiency.

Generally, it should be emphasized that fluorescence quenching is a complex process because of the large number of variables involved, effects of which cannot be completely separated. Nevertheless, knowledge of these phenomena enables some useful decisions to be made in the choice of proper reagents and reaction conditions in the development of ligand-exchange methods.

An effectively quenched ligand is only one prerequisite of a suitable reagent. In addition, the stability constant of the reagent

(metal-ligand complex) must be sufficiently less than that of the metal-anion complex for exchange to occur and kinetics must be also favorable. For new ligand-exchange methods for anions it is, therefore, necessary to find "just right" combination of metal ion, fluorescent ligand and anion. This fact limits the application of the ligand-exchange principle.

Oxidation-reduction reactions have been found to be very useful in the development of fluorimetric methods for anions. In the methods for determination of phosphate and cyanide these anions do not react directly with the organic reagent but advantage has been taken of their complexing properties which resulted in the change of the oxidation-reduction potential of a given system; good selectivity has been so achieved. The methods are highly sensitive and allow determination at ng/ml levels with a relatively good reproducibility; many common ions can be tolerated at high excess. The methods for determination of phosphate and mercury(II) were successfully applied to the analysis of practical samples. The methods for determination of cyanide and sulfide can be also readily applied because of possible use of common separation procedures whereby these anions are separated by distillation from acid medium in the form of HCN and H_2S, respectively. The principle of complexation of the anion to be determined to form species which will then take part in oxidation-reduction reaction has a potential for further applications. For example, in a neutral solution cyanide might

be determined by complexing with iron(III) to form ferricyanide which can then be reacted with, *e.g.*, thiamine or leucofluorescein to give fluorescent species.

Although fluorescence has the attraction of high sensitivity, the present work implies that there are real limitations in its application to anions' analysis. Among the procedures developed only the phosphate method (and mercury(II) method) can be claimed to have distinct advantages over methods already described in the literature. The other procedures, although sensitive and of good reproducibility, are not very selective.

Fluorescence has been shown to have some merit in the determination of low concentrations of anions but other techniques, *e.g.*, selective membrane potentiometry, would seem to hold greater promise, especially in analysis of practical samples.

Reprints of papers on the fluorimetric determination of cyanide and of phosphate are attached. A paper on the determination of mercury(II) is in press.

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SHORT COMMUNICATION

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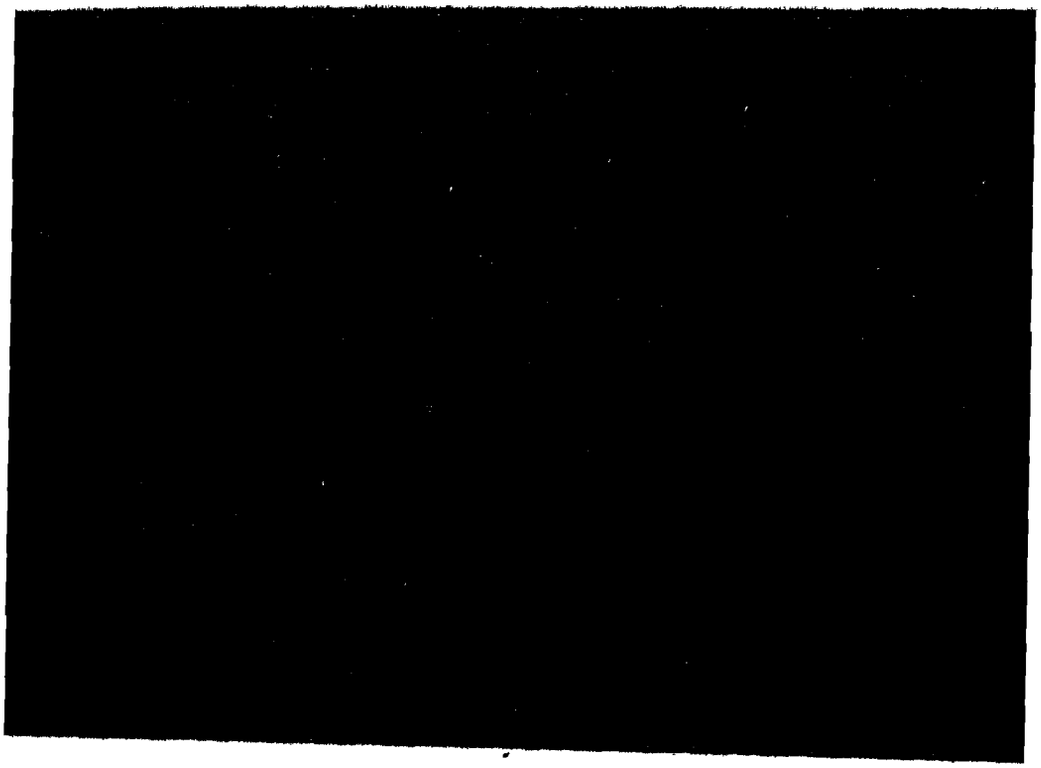

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