# ANALYTICAL APPLICATIONS OF THIN-LAYER PHOSPHORIMETRY

by

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A doctoral thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology, October 1979.

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To my parents

for their faith, guidance, encouragement and support over the years.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. J.N. Miller for his invaluable discussion and guidance during my research.

I would like to thank my friend M. Benaouda for his pleasant company during the period of my research.

I am grateful for the helpful discussion of my colleagues: E.U. Akusoba, C.S. Lim, and H. Sturley.

My thanks must go to the assistant administration officer Mr. C.H.J. Burrough.

My thanks also go to the technicians: Mr. A.F. Bower, Mr. G.S. Dale, Mr. M.K. Patel, Mr. M. Stone, Mr. A.G. Stevens and Mr. J. Swithenbank for their general helpfulness.

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# CHAPTER I

# INTRODUCTION

## 1.1. Luminescence

Hot bodies that are self-luminous solely because of their high temperature are said to emit incandescence. All other forms of light emission are called luminescence.

A system emitting luminescence is losing energy and, if the light emission is to continue indefinitely, some form of energy must be supplied from elsewhere.

Most kinds of luminescence are classified according to their source from which this energy is derived, such as electroluminescence, radioluminescence, bioluminescence, triboluminescence, sonoluminescence, candoluminescence and photoluminescence. For photoluminescence, the energy is provided by the absorption of infra-red, visible or ultra-violet light.

Photoluminescence is the only type with any direct relevance to this work, and itself can be further divided into fluorescence, delayed fluorescence and phosphorescence. The study of most kinds of luminescence can provide some information about the chemical composition of the emitting system and the processes that take place after the absorption of the excitation energy, but photoluminescence

is one of the most informative because it allows a greater degree of experimental control over the excitation process. By choice of the wavelength of the exciting light the energy can be directed to specific components of the system so that the ensuing processes are simpler than if the energy is fed to the system as a whole. The processes leading to the emission of photoluminescence are closely associated with those that result in photochemical reactions and the study of photoluminescence thus forms an integral part of the subject matter of photochemistry.

## 1.2. History of luminescence

Luminescence was first observed and recorded around the 11th century B.C. in the Shih Ching or "Book of Odes", which makes reference to the light from glow-worms and fire-flies. In the 3rd century B.C., Aristotle wrote of light being emitted from decaying fish. The first recorder of a serious interest in luminescence is that of Vincenzo Cascariolo, who lived in the early 17th century; he discovered that when Barite, a form of barium sulphate was heated with coal, the product glowed a purple-blue colour at night, it was assumed that this "sun stone" simply absorbed light and then re-emitted it slowly in the dark. In 1652 Nicolas Zucchi showed that the colour emitted during phosphorescence was the same no matter what the colour of the exciting light.

In 1852 Stokes showed that the wavelength of emitted light was always equal to or greater than the wavelength of the exciting light.

In 1871[1] Becquerel was the first to describe the use of a mechanical device or phosphoroscope, which enabled the delayed emission of phosphorescence to be isolated from the prompt fluorescence emission and from scattered light. In 1888 Weidmann[2] described phosphorescence in organic compounds by exciting solid solutions of a number of dyes. In 1896 Schmidt[3] introduced the use of a rigid solvent for the measurement of phosphorescence, thus the determination of a wide range of organic compounds by phosphorescence was possible.

The use of phosphorescence emission spectra for identification of substances was first suggested by Lewis and Kasha[4] (1944); however very little further work was done until Keirs, Britt and Wentworth[5] in 1957 published a paper which evaluated the possible use of phosphorescence measurements as an analytical technique. It was at that time a new method of analysis. Since then many papers have been published on the analysis of drugs in both blood and urine, and compounds from almost every group of drugs, pesticides and carcinogens have been determined by phosphorimetry.

## 1.3. Phosphorescence and fluorescence Processes.

Absorption of ultra-violet and visible light by molecules of an irradiated sample generates a population of molecules in excited electronic states. As can be seen from (Fig. 1.1) there are various excited electronic energy levels possible and these are termed excited singlet or excited triplet states.

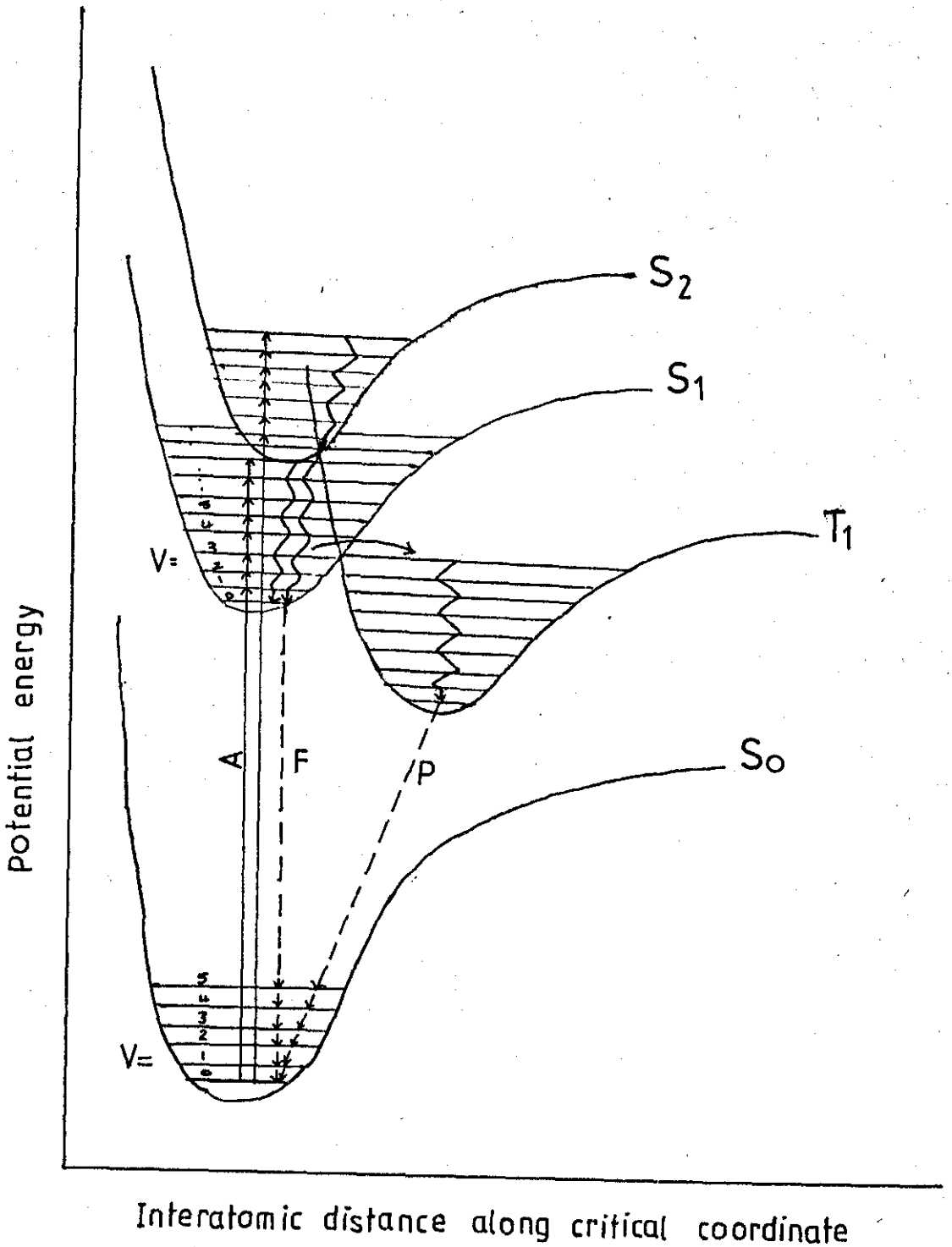



Fig.1.1. Potential Energy Diagram for a Diatomic Molecule
A-is Absorption, F-is Fluorescence ($S_1 \rightarrow S_0$)
P-is Phosphorescence ($T_1 \rightarrow S_0$), (~~~>) Internal Conversion,
(——>) Intersystem Crossing

Each excited electronic state has many different vibrational energy levels, and excited molecules will be distributed in the various vibrational energy levels of an excited state. Most usually this state is a singlet state, i.e., one in which all of the electrons are paired and in each pair the two electrons spin about their own axes in opposite directions (Fig. 1.2).

All electrons have a spin, S, equal to $\pm \frac{1}{2}$ and in a normal molecule in its ground state there is usually an even number of electrons with paired spin. Multiplicity is the term used to describe the orbital angular momentum of a given state and is related to spin as shown below.

$$M = 2s + 1$$

Therefore, when all the electrons are paired, s = 0 and M = 1, which is the state referred to as the singlet state. When there are two electrons with parallel spins, i.e. two unpaired electrons, s will be 1 and M consequently be 3, this state being described as the triplet state. It has been calculated that the average lifetime of a molecule in the singlet excited state is of the order of $10^{-8}$ sec. Molecules at each vibrational level of the excited state could, for example, lose energy by emitting photons and as a result fall to the original ground state. The energy and, therefore, the wavelength of emitted light would then be exactly the same as that absorbed. Such a process is termed Resonance fluorescence. It is an improbable process and is rarely encountered in solution chemistry. Rather, molecules initially undergo a more rapid process, a radiationless

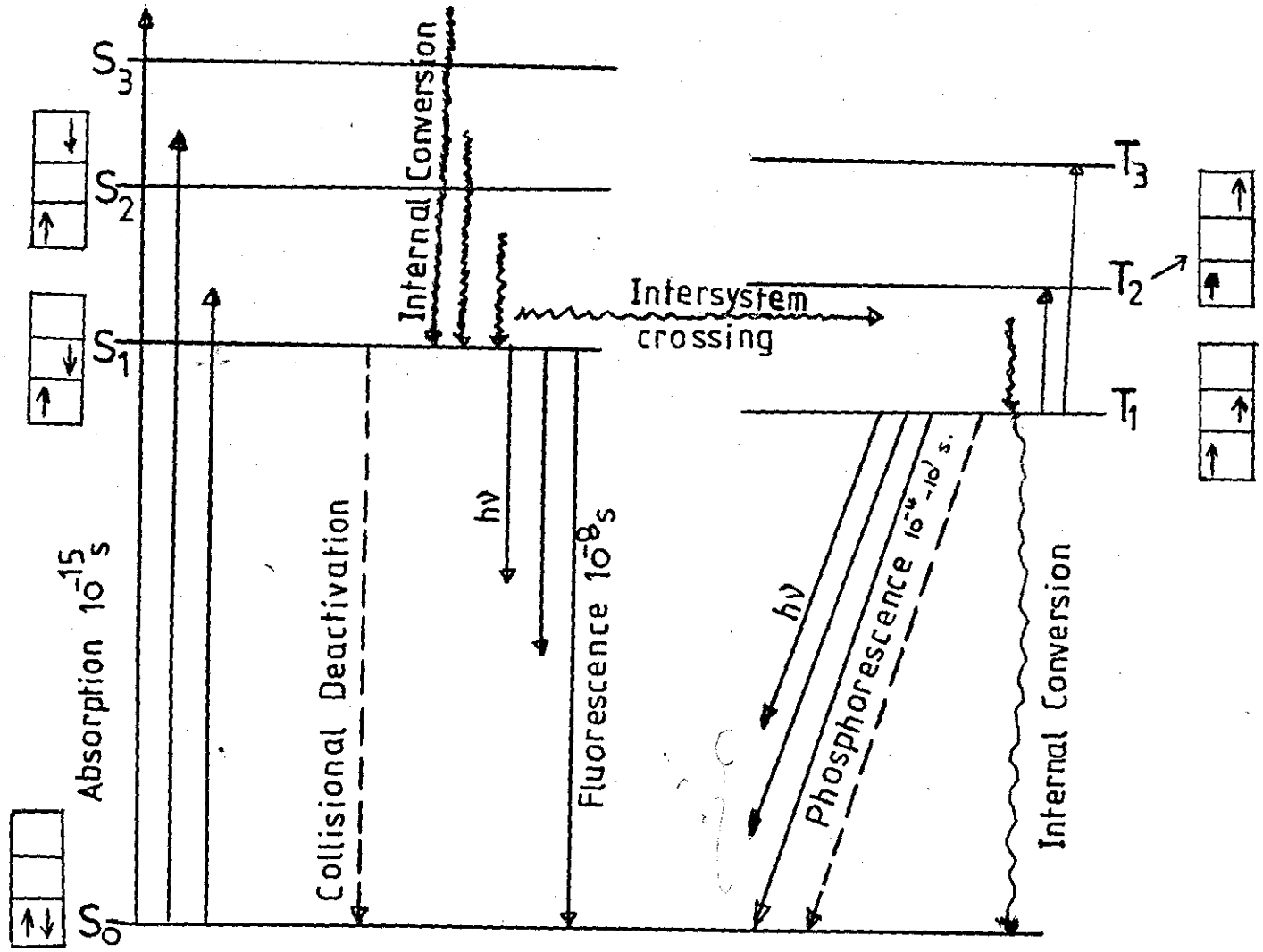


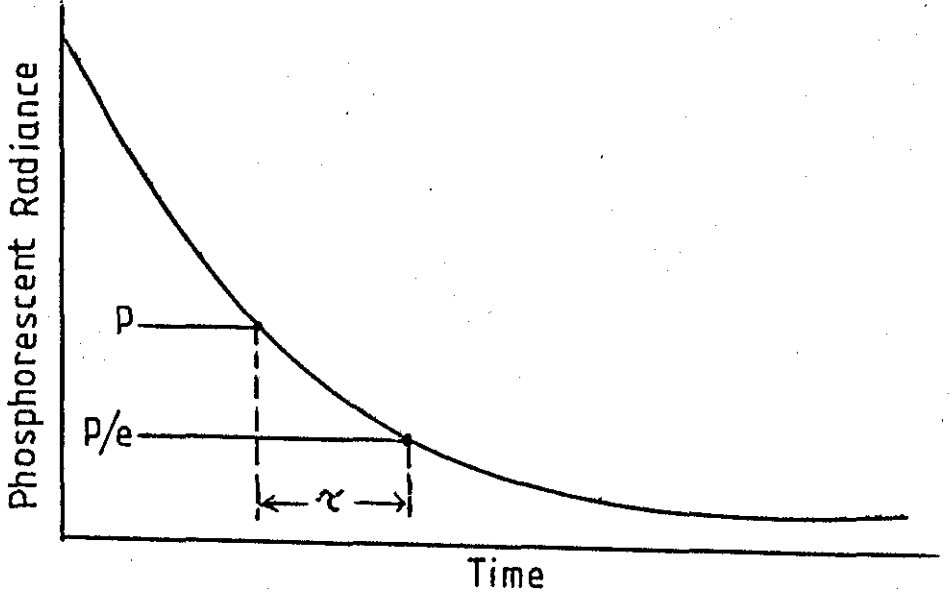

Fig.1.2. Schematic state energy level diagram: S is singlet and T is triplet. The $S_0$ state is the ground state and the subscript numbers identify individual states

loss of vibrational energy, and so quickly fall to the lowest vibrational energy level of the lst excited singlet state $S_1$. The vibrational energy is thought to be lost to solvent molecules. The process is known as vibrational relaxation. From the lowest vibrational level of the excited state, a molecule can either return to the ground state by photoemission or by radiationless processes. If indeed the former occurs, the emission is a type of luminescence referred to as fluorescence. Fluorescence is defined as the radiation emitted in the transition of a molecule from a singlet excited state to a singlet ground state. Because of vibrational relaxation in the excited state and because a molecule may return to a vibrational level in the ground state which is higher than that initially occupied prior to excitation, the radiation emitted as fluorescence is of lower energy and, therefore, of longer wavelength than that originally absorbed.

Other processes involving the exicted state can occur to compete with fluorescence emission, and not all of the absorbed energy will be emitted as fluorescence. A process called internal conversion occurs when an excited molecule can undergo a radiationless loss of energy sufficient to drop to the ground state.With some compounds a process known as intersystem crossing can also occur. Here a molecule in the lowest vibrational level of the excited state converts to a triplet state, a state lying at an energy level intermediate between ground and excited states and characterised by unpairing of two electrons (Fig. 1.2). Once intersystem crossing has occurred, a molecule quickly drops to the lowest vibrational level of the triplet state by vibrational relaxation. The triplet state is much longer lived than the corresponding singlet state with a lifetime of $10^{-4}$ to 10

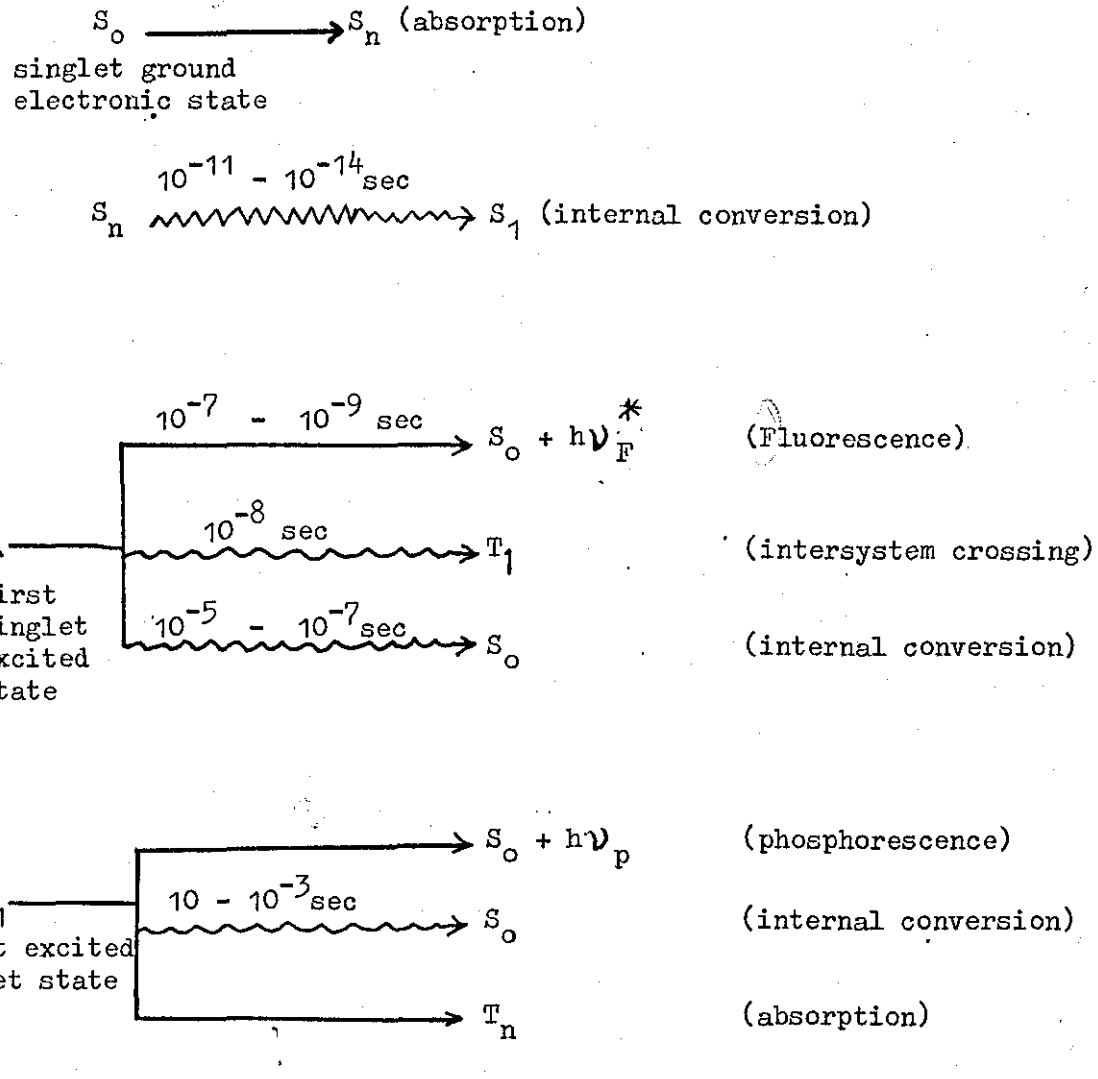
seconds. From the triplet state a molecule can drop to the ground state by emission of radiation. This type of luminescence is termed phosphorescence and is formally defined as emission of radiation resulting from the transition of a molecule from a triplet excited state to a singlet ground state. In order to see phosphorescence, it is normally necessary to dissolve the substance in a rigid medium and usually use low temperature. The wavelength of phosphorescence will be greater than that of the exciting radiation, and of the fluorescence.

Lifetime $\tau$ The lifetime of fluorescence or phosphorescence is defined as the time required for the intensity to fall to $\frac{1}{e}$ of its initial value (i.e. for phosphorescence from P to P/e as shown below). To determine the "phosphorescence lifetime" the intensity of the phosphorescence is plotted logarithmically against the time after cutting off the exciting radiation. The gradient of the resulting straight line is $2.303\tau$ [77].



Phosphorescence Decay Curve

The following is a sequential outline of the processes of concern; see Fig. 1.2, with approximate life-times where appropriate:




The orientations of the spin of the excited electron relative to that of the remaining electron are indicated in Fig. 1.2 in the rectangles adjacent to each level.

* $h\nu_F$ = energy released.

## 1.4. Types of phosphorescence

Two types of phosphorescence can be distinguished depending on the nature of the triplet state. These are $\pi - \pi^*$ ($\pi$ bonding to $\pi$ antibonding orbital) or $n - \pi^*$ (n-non bonding to $\pi$ antibonding) transitions. A series of criteria[6] is available by which $\pi - \pi^*$ and $n - \pi^*$ phosphorescence can be distinguished. Table 1.1. shows several such criteria that can be used to assign the nature of the triplet state from which emission occurs. The phosphorescence spectra of the aromatic hydrocarbons and of most heterocyclics, and of many derivatives of these classes of compounds correspond to $\pi - \pi^*$ transitions, but although this type of excitation is by far the most frequent, there is a series of compounds that shows $n - \pi^*$ phosphorescence.[6]

To this series belong very important families of compounds; the carbonyls (quinones, ketones, etc), the nitro compounds, and a few heterocyclics such as pyrimidine and pyrazine. A knowledge of the type of triplet state produced in a particular molecule allows a better understanding of the basic processes going on in that molecule and should also aid prediction of likely changes in the spectra, as a result of changing molecular substituents.

## 1.5. Measurement of phosphorescence

### Instrumentation

One of the earliest phosphorimeters[7] was a modified fluorimeter with a different sample compartment. Several manufacturers have produced 'custom built' spectrophosphorimeters[7,8,9] which were originally spectrofluorimeters. In 1963 Latz[10] referred to the need for simple filter instruments as well as more sophisticated models. At present, modification of various filter fluorimeters and spectrofluorimeters

TABLE I.1. Criteria for Assignment of Triplet States as Tn-$\pi^*$ or $T_\pi - \pi^*$

| Property | T $\pi-\pi^*$ | T $n-\pi^*$ |
|---|---|---|
| 1. $Q_p/Q_F$ ratio | variable | very high |
| 2. Lifetime of phosphorescence | is strongly dependent on the size of the molecule | $<10^{-1}$ - $10^{-2}$ s only slightly influenced by molecular size |
| 3. effect of substituent | displaced towards longer wavelengths | displaced towards shorter wavelength |
| 4 polarisation of 0-0 phosphorescence bond | out-of-plane polarized | in-plane polarized |
| 5. External heavy atom-effect on | | |
| a) Intensity $T \longleftarrow S_o$ absorption | large increase | very small |
| b) Life time of phosphorescence | decrease | very small |
| 6. Vibrational structure of phosphorescence | variable | Prominent CO, NO or C-N-C progressions |
| 7. Triplet-singlet split | $>3000$ cm$^{-1}$ | $\lesssim 2500$ cm$^{-1}$ |

has been attempted with varying degrees of success[7,8]. Fig. 1.3 is a block diagram of a typical commercial spectrophotofluorimeter fitted with a rotating can phosphoroscope. The instrument consists of a light source, a monochromator, slit, the sample, another slit, another monochromator, a detector, amplifier, and some form of readout (meter or recorder).

Light source: The choice of light source is quite varied and depends partly on financial considerations, but desirable features are a high intensity uniform spectrum which extends well into the u.v. region and good stability in terms of output. The high-pressure xenon-arc lamp is now favoured over mercury lamps for several reasons; the xenon source gives a continuous output that is more amenable to spectral correction and does not suffer from gaps in emission intensity. It has been found that a convection cooled 150W xe-arc lamp with a stabilised pulsed power supply gives a stable output and lasts for several hundred hours.

The monochromators: The excitation and emission monochromators used in a commercial instrumentation are either filters, interference wedges, prisms or diffraction gratings. Their purpose on the excitation side is to provide the most suitable wavelength for excitation of the sample, and on the emission side to separate the sample phosphorescence from light of other sources. Most instruments use a grating or interference filter for the excitation monochromator. The emission monochromator is normally a simple grating in a Czerny-Turner mount or a holographic grating. Both monochromators may be motor driven to facilitate the production of both emission and excitation spectra. They may be linked for producing synchronous spectra for specialised

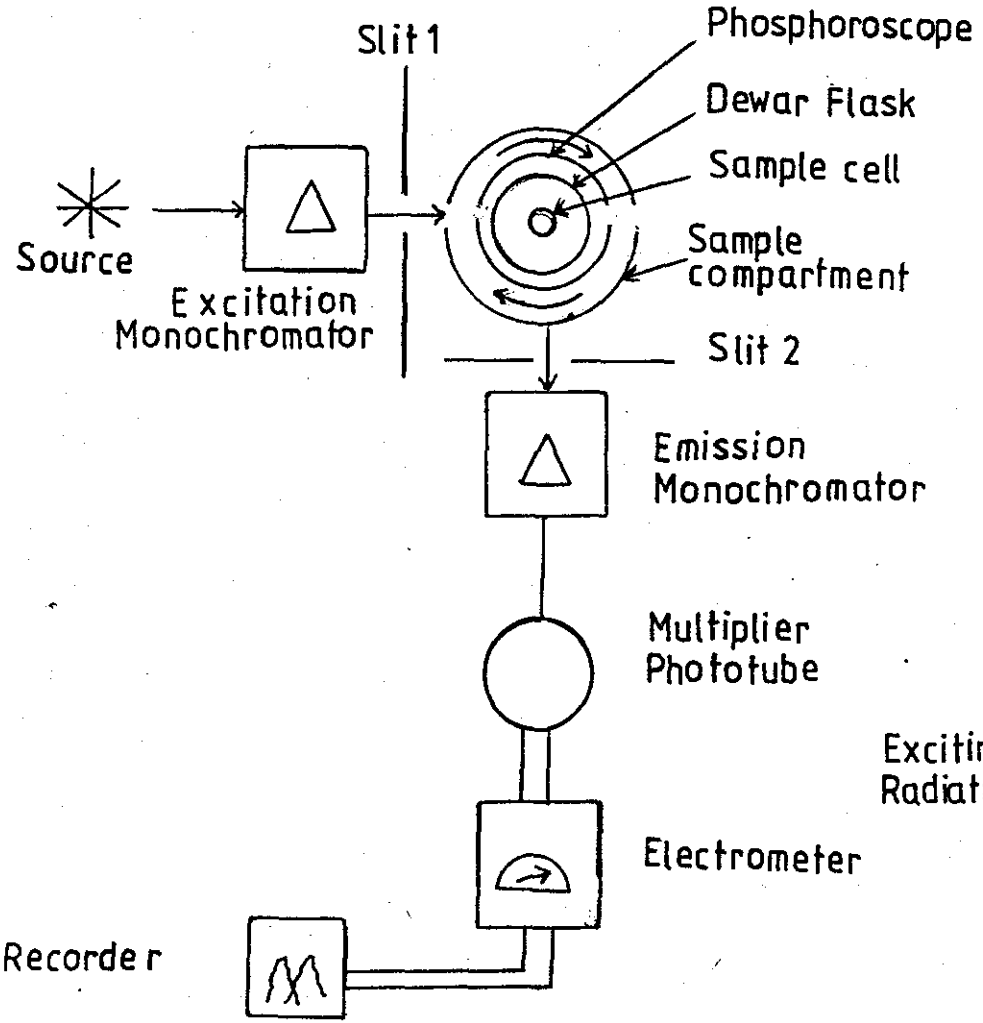
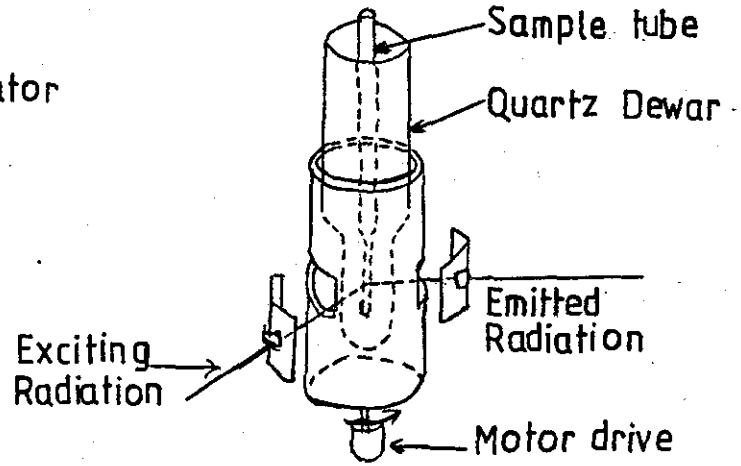



Fig.1.3. Block diagram of a typical commercial spectrophotofluorimeter with phosphoroscope attachment




Schematic diagram of a rotating can phosphoroscope

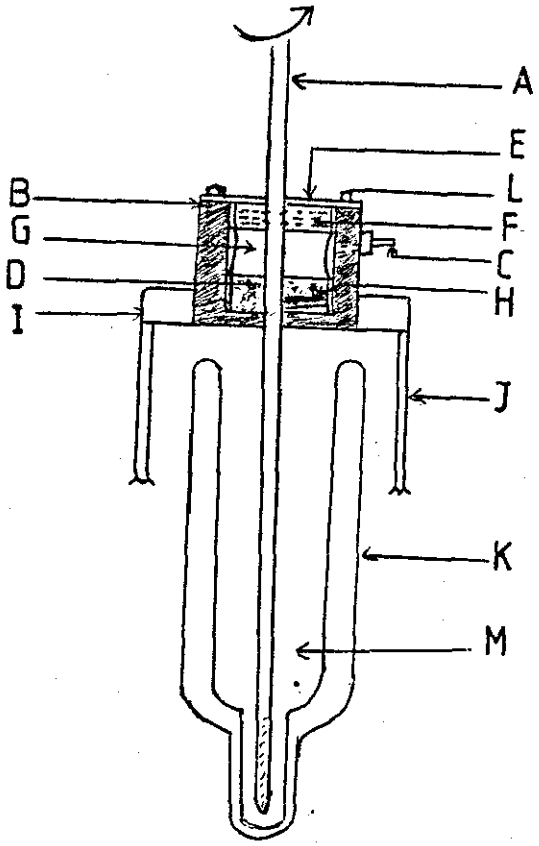
use. The phosphorescence is usually measured at right angles to the incident light, (Fig. 1.3), because its intensity will normally be independent of the direction of measurement, whereas scattered light will be minimised at right angles to the exciting light. Therefore, this optical arrangement gives the best signal to noise ratio.

Slits: In order to enhance the sensitivity and to obtain the best signal to noise ratio the two slits 1 and 2 (Fig. 1.3) should be variable: improvements in limits of detection were noted[11] when slit settings were optimised. A general procedure for obtaining the best results is to use a small excitation slit for recording an excitation spectrum (for good resolution). Conversely when recording an emission spectrum the excitation slit is widened and the emission slit narrowed.

The phosphoroscope: The first phosphoroscope which was described by Becquerel[1] in 1871, enabled the delayed emission of phosphorescence to be isolated from prompt fluorescence emission and scattered light. This type of phosphoroscope is out of use now. There are other types of phosphoroscopes which have since been developed[12] these being the rotating can phosphoroscope Fig. 1.3 and the pulsed source phosphoroscope respectively. The former operates as follows. As the can is turned the radiation from the excitation monochromator is allowed to strike the sample, and alternately the light emitted from the sample is allowed to reach the emission monochromator entrance slit. In the pulsed source phosphoroscope, excitation is achieved by means of a periodically pulsed flash tube and the emission is monitored by means of a photodetector which is operated periodically and out of phase with the flash tube.

The fourth type of phosphoroscope is the single disc one, which has been used in several forms[14,15,16]. The disc shown in Fig. 1.5 is of the type used in the thin layer scanner described previously[14]. The phosphoroscope adds the feature to phosphorimetry of time resolution, by using various rotation speeds the phosphorescence of two components with different decay times can be resolved; this is termed time-resolved phosphorimetry[17].

Sample holder: Most phosphorescence measurements are made at 77K as a rigid medium is essential. The traditional system consists of a Dewar flask which is filled with liquid nitrogen and used to cool a narrow silica tube containing the sample. This has the disadvantage that the sample is radiated and emits through the liquid nitrogen causing scattering and losing some sensitivity. Other tubes of similar design take too long to cool the sample. Hollifield and Winefordner[18] have introduced a rotating sample cell for low temperature phosphorescence measurements (Fig. 1.4). This device reduces some of the sampling problems in phosphorimetry. The first problem which is overcome is solvent "snow" formation. The second problem which is significantly reduced is the sample positioning error, the major contributing factor to poor precision in phosphorimetry. The authors claim that, combined with better solvent clean-up and more stable electronics, a tenfold increase in detection limit is possible as is a precision of 1%. Another recent innovation in sampling tubes is the development of the windowless sample cell[19]. This is particularly useful for studying solid slurries and pastes. The sample is held in a cavity by surface tension in a cell constructed from a copper rod, the other end of the rod being immersed in liquid nitrogen. The limit of




A = Quartz sample cell

B = aluminium sleeve

C = nozzle for air introduction

D = Teflon (Du Pont) Cell holder and rotor

E = Cover with air-exit ports

F = fins for air exit

G = grooves to collect air

H = Nylon set screw

I = ring adaptor

J = standard cover support of the phosphoroscope attachment

K = quartz Dewar flask

L = bolts of hold cover

M = Liquid nitrogen

Fig.14. Rotating sample cell design for phosphorimetry.

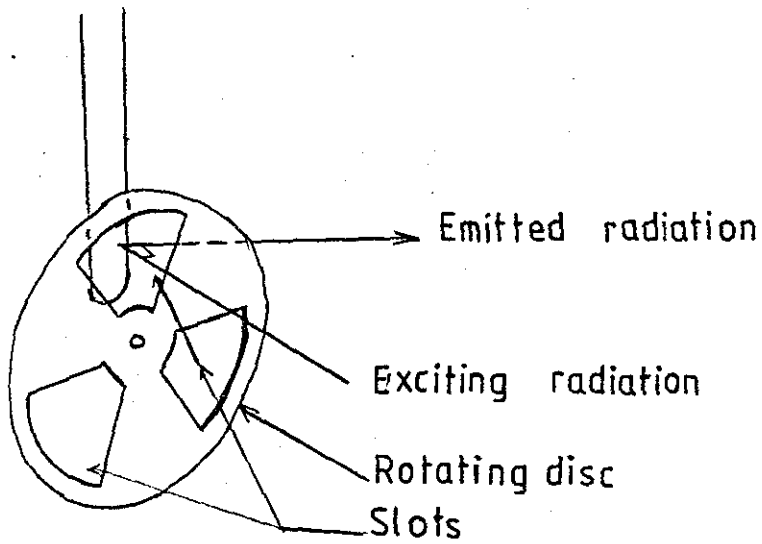



Fig. 1.5. Single disc multi-slot phosporoscope used for Thin-Layer Phosphorimetry

detection thus obtained for several biological molecules was similar to conventional phosphorimetry.

Another recent innovation[14] is a scanning thin-layer device which allows the measurement of samples or portions of chromatograms or other thin layers. The thin layer is wrapped around a hollow copper drum which is cooled by conduction. Excitation and emission is on the front surface of the thin-layer.

Modern Methodology.

In order to see phosphorescence it used to be thought that the sample should be cooled to 77K, and that all solvents had to be capable of forming clear, uncracked glass at 77K. This gave considerable problems in terms of solubility, homogeneity and solvent purity. A different approach to the problem is to measure the phosphorescence at room temperature, having previously adsorbed the sample molecules on to a solid matrix[20]. Little interest was displayed in this method until 1972 when Schulman and Walling[21] described the phosphorescence of ionic organic molecules adsorbed on to paper. Since then several papers[22,23,24] have been published on this application, for further details see Chapter 4.

Detector: A high gain photomultiplier with a low dark current is employed. The RCA IP 28 for response 210-670nm and RCA IP 21 for 310-670 nm are normally used. The detector is nearly always a photomultiplier tube. Recently photon-counters have started to be used in commercial instruments as well as in research built instruments[25]. The read out may be an xy type chart recorder, a galvanometer, or digital recorder.

## 1.6. Applications of phosphorimetry

The analysis of drugs in biological fluids and tissues is important in several aspects of biomedical research, for example, in the study of drug metabolism and the rate of elimination from the body. Phosphorimetry as a means of drug analysis was first applied by Winefordner and Latz[25] (1963) for the determination of aspirin in blood serum and plasma. They also checked that various naturally occurring constituents of the blood were not interfering in the assay. They found that thiamine, riboflavin, tyrosine and tryptophan produced measurable phosphorescence but that interference was negligible at aspirin concentrations of 50 $ug.ml^{-1}$ blood or higher. Extraction was by a single volume of acidified chloroform and this procedure gave recoveries of between 76% and 133%.

The success of the phosphorimetric analysis of aspirin in plasma or serum led to the investigation of the determination of other drugs by means of phosphorimetry.

Winefordner and Tin[27] (1965) also developed procedures for the phosphorimetric determination of procaine, phenobantal, cocaine, and chloropromazine in blood serum, and cocaine and atropine in urine. They gave analytical curves for each compound and percentage recoveries for each drug from "spiked" samples. The phosphorescence characteristics of codeine, morphine and papaverine were described by Hollifield and Winefordner[28]. In 1966 a study of the effects of pH on the phosphorescence of ether extracts of blood and urine was published[29]. For blood it was found that there was very little phosphorescence

after extraction at pH's 0, 6.5 and 14, and maximum phosphorescence at pH's 3 and 12. For urine it was found that the phosphorescence varied widely from sample to sample, even at the same pH value, but the relative intensity at different pH's remained fairly constant, namely high at low pH's falling to a minimum at pH 5 and then steadily increasing with an increase in pH. Phosphorimetry has been found to be of use in biochemistry and pharmacology. Typical samples include pyrimidines, purines, and derivatives, polynucleotides, amino acids and proteins. It was in this field that the first analytical application was produced, namely that of Rybak[30] et al in 1955 for the analysis of amino acids.

Much has been published[31,32] on the phosphorescence characteristics of both amino acids and proteins. Several authors[33-35] have studied the relationships between the phosphorescence of individual amino acids and the phosphorescence of proteins. An investigation of the phosphorescence of adenine compounds was first undertaken by Steele and Szent-Gyorgyi[36] in 1957 with intensive studies of purines, pyrimidines &their nucleoside and nucleotide derivatives. Purine phosphorescence in methanol-water solutions has been shown to give detection limits between 0.1 and 0.0002 $ug.ml^{-1}$[37], the phosphorescence spectra obtained in a methanol-water mixture (v/v 10/90) were found to have fine structure, thus demonstrating the usefulness of predominantly aqueous matrices at 77K for enhancing the vibrational line structure.

Other biologically active or important compounds have also been assayed phosphorimetrically including indoleacetic acid, serotonin[38],

and some of the vitamins[39,40]. The phosphorescence characteristics of 37 antimetabolites have been reported by Sanderset al[41], only 17 antimetabolites displayed analytically useful phosphorescence. The authors indicated that the limits of detection compared favourably with those obtained by colorimetric (0.1 - 1 $ug.ml^{-1}$) and enzymic (0.01 - 0.1 $\mu g.ml^{-1}$) methods of analysis. By suitable preliminary reaction of these conjugated-ring systems phosphorescence may be significantly enhanced and the limits of detection could be appropriately lowered.

During studies on the metabolism of some sulfonamide drugs[42,43] more rapid and sensitive methods of estimating these drugs were sought. Since these compounds contain the aniline moiety which has pronounced luminescence properties[44,45], the fluorescence and phosphorescence characteristics of a series of sulfonamide drugs were investigated. Hollifield and Winefordner[46] have studied fifteen sulfonamides by phosphorimetry, thirteen gave detection limits equal to or better than 1 $ug.ml^{-1}$. The effect of various solvents on the phosphorescence signals[47] and the relationship between structure and luminescence characteristics[48] have been studied thoroughly. When sulfonamides were added <u>in vitro</u> to serum samples, recoveries ranged from 92-105% and there was a relative standard deviation of about 5%.

Several groups of workers have also studied the barbiturates[49-51] and detection limits in ethanediol:water are given. The relationships between molecular structure and luminescence properties under varying conditions of pH have also been studied[51]. The thin layer phosphorimetry of groups of thiobarbiturates have been investigated in this laboratory. Other groups of compounds which have been studied

phosphorimetrically include antihistamines, the properties of which have been described by Schenk and his co-workers[52-53], the anticoagulants[54], griseofulvin[55] and diphenylhydantoin[56]

The characterisation and determination of cannabinols, lysergic acid derivatives and other hallucinogens has been achieved by phosphorimetry as a means of analysis for their minute quantities in the blood[57-59]. Also the effect of external heavy atoms and various pH's on the luminescence signals were studied. Fabrick and Winefordner[59] have described the separation of a mixture of hallucinogens.

Phosphorimetry is also applied to food chemistry and related fields. McCarthy and Winefordner[60] have reported an interesting application of spectrophosphorimetry to food chemistry; the rapid quantitative determination of biphenyl in oranges. It was found that 95-100% of the quantities of biphenyl added were recovered. The accuracy of the method is about $\pm$0.2ppm with 3.8-8.9 ppm of biphenyl in the juice and pulp and about $\pm$ 2ppm with 39-65 ppm of biphenyl in the peel. The determination of pesticides is, from the practical point of view, an important problem in analytical chemistry and also has significance, in food chemistry and forensic medicine. That spectrophotometry can be successfully introduced into this field too has been shown by Moye and Winefordner[61].

Phosphorimetry has been applied to the study of air pollution and the analysis of petroleum products. In the dust of the atmosphere (especially that of industrial towns), numerous polycyclic aromatic hydrocarbons and heterocyclics are to be found. It has been known

for a long time that many of these compounds are carcinogenic. Sawicki[69] pointed out, in a review that appeared in 1964, that phosphorescence spectroscopy should prove of inestimable service for trace analysis, and hence for the investigation of airborne dust samples. Sawicki and Johnson[63] have shown that polycyclic aromatics from the air can easily be rendered visible on thin-layer chromatograms by means of their phosphorescence (in liquid nitrogen). Hood and Winefordner[64] have reported the use of low-temperature fluorescence and phosphorescence measurements in the determination of complex mixtures of carcinogenic hydrocarbons. The authors have combined thin-layer chromatography and luminescence techniques to increase both analytical selectivity and sensitivity of measurements. The authors concluded that 0.1 ug of most carcinogens could be determined with good precision by means of this technique. They suggested that this level makes the procedures well suited for application in environmental studies of hydrocarbon pollution. Since then many papers have appeared on the analysis of hydrocarbons using luminescence techniques[e.g. Refs. 65,66]. Also the phosphorescence of some polyaromatic hydrocarbons was studied at room temperature[67]. The application of phosphorescence spectroscopy to the complex field of petroleum analysis has appeared in the literature. Early work was carried out by Mamedov[68] and Khaluporskii[69], who identified a number of polycyclic aromatic hydrocarbons in the wax distillates from petroleum, and luminescent compounds in lubricating oils.

Other work was carried out in the early 1960's by Sidorov and Rodomakina[70] who showed the various applications of phosphorimetry to the analysis of petroleum products. Drushel and Sommers[71]

carried out an extensive study on 100 compounds and proposed a complete separation and identification scheme for nitrogenous compounds obtained from petroleum[72]. Phillip and Soutar[73] have identified several aromatic hydrocarbons in crude oils by synchronous excitation spectrofluorimetry. In 1978 a wide range of literature[74] was cited for the application of phosphorimetry in environmental chemistry.

It is possible to utilize the sensitivity and selectivity of phosphorimetry in conjunction with thin layer chromatography[75] as a separatory technique. The method has been applied, for example, to the determination of nicotine, nornicotine, and anabasine in tobacco[75], of biphenyl in oranges[60], polycyclic aromatic hydrocarbons[64], p-nitrophenyl in urine[76], and almost any kind of substance which is phosphorescent could be analysed by this method.

## Scope of present work

Luminescence spectrometry, an extremely sensitive analytical tool, has been used to solve many qualitative and quantitative a analytical problems. Phosphorimetry is a sensitive and selective technique capable of being applied in many fields where the determination of small quantities of drugs or organic compounds are required. The greatest use of phosphorimetry in the clinical laboratory will not be for the analysis of very large numbers of samples for one species via automatic instrumentation, but rather will be for the analysis of those molecular species difficult or impossible to measure by conventional methods (colorimetry, fluorimetry, etc).

Despite this advantage, phosphorimetry has disadvantages which affects its applicability. The main reasons for this are that the method is unsuitable for automation, the difficulties in sample handling, and the problems associated with availability and use of liquid nitrogen.

The aim of the present work was to eliminate some of the problems associated with phosphorimetric techniques. Also to extend the use or the application of phosphorescence as an analytical tool to a number of different classes of drugs and determining them in low concentrations. The aim was to extend the application to environmental pollution by studying the characteristics of thirteen polycyclic aromatic hydrocarbons in solutions and on thin layer phosphorimetry.

When this work started thin-layer phosphorimetry was already developed, the aim of this work is to examine the usefulness of TLC phosphorimetry for different groups of drugs and related compounds which have not been studied previously by this technique, and to determine them in very low concentrations. The sensitivity of phosphorescence and thin-layer chromatography could be combined to produce a rapid, sensitive and selective method of analysis capable of resolving small quantities of similar compounds, especially drugs and their metabolites, from complex mixtures.

# CHAPTER 2

# EXPERIMENTAL

## 2.1. Purity Checks

The purity of several groups of compounds was checked by thin-layer chromatography (see below). The compounds studied were (1) 6-mercaptopurine and its metabolites which are shown in Table 2.1: all were obtained from the Sigma Chemical Co. Ltd., (Poole, Dorset, U.K.); (2) phenothiazine and its derivatives are listed in Table 2.2. Compounds I and II were donated by Pharmaceutical Specialities (May and Baker Ltd); compound 3 by Allan and Hanburys, Compound 4 by Hopkin and Williams, compounds 5 and 7 were obtained from the B.P. Commission, and compound 6 from Sandoz Products Ltd.; (3) Thiobarbituric acid and related compounds are listed in Table 2.3. Compound I was obtained from BDH Chemical Ltd., London, compounds 2 and 3 were synthesised by Hewitt[78] according to the method of Morawski[79] 1966, compounds 4, 5, 6 and 7 were synthesised by Rohoman[80] according to the methods of Dickey and Gray[81], and Dox.,[82] Compound 8 was obtained from Boots, Nottingham, England, and compound 9 from ICI Aldenley Park, Cheshire, U.K.

Table 2.4 lists the polycyclic aromatic hydrocarbons that were studied; compounds 1, 3 and 10 were obtained from Aldrich Chemical Company, Inc. U.S.A., compounds 2,4,5,6,7,8,9,11 and 12 were obtained from Aldrich Chemical Company, Ltd, England.

TABLE 2.1.
Mercaptopurines Studied and Source of sample

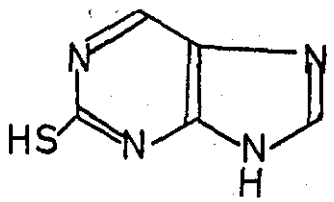
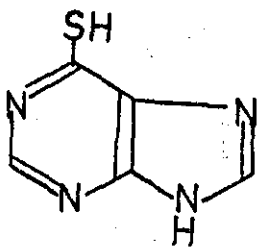
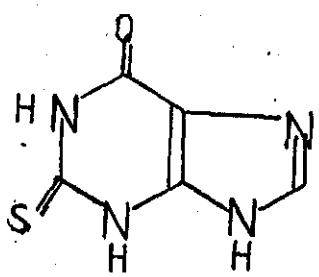
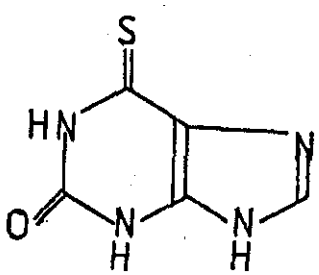
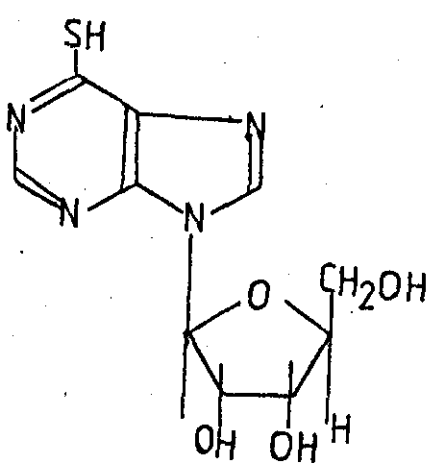
| | Compound | Structure | Source |
|---|---|---|---|
| 1 | 2-mercaptopurine (2-MP) |  | Sigma Chemical Co. |
| 2 | 6-mercaptopurine (6MP) |  | " |
| 3 | 2-thioxanthine (2-thio) |  | " |
| 4 | 6-thioxanthine (6-thio) |  | " |
| 5 | 6-mercaptopurine riboside (6-MPR) |  | " |

TABLE 2.1. Continued

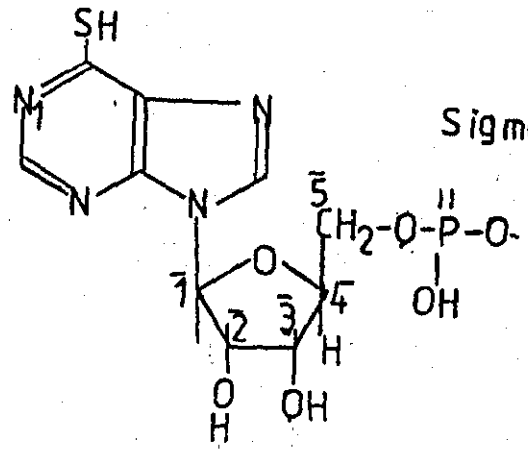
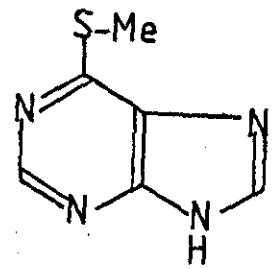
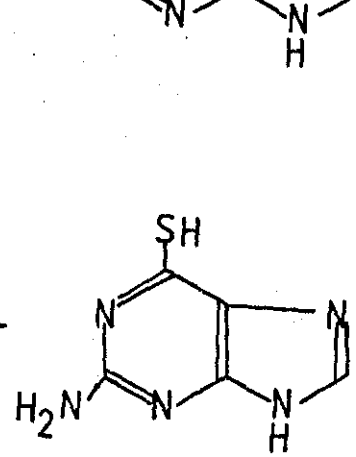
| | Compound | Structure | Source |
|---|---|---|---|
| 6 | 6-mercaptopurine riboside-5-phosphate (6-MPR-5-Phosph) |  | Sigma Chemical Co. |
| 7 | 6-methyl mercapto-purine (6-MeMP) |  | " |
| 8 | 2-amino-6-mercapto-purine (2-amino-6-MP) |  | " |

TABLE 2.1. Continued

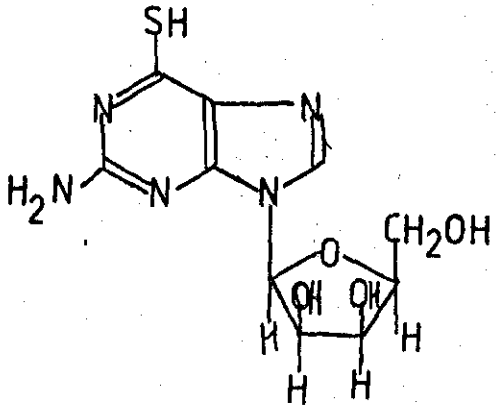
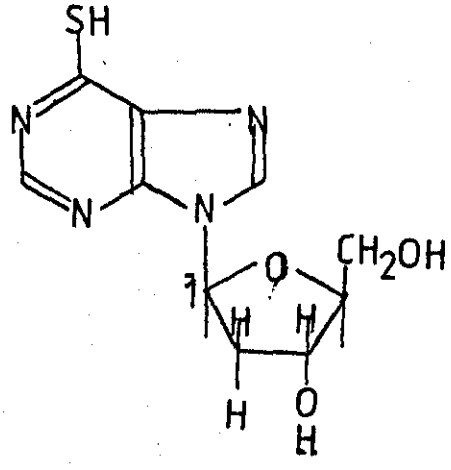
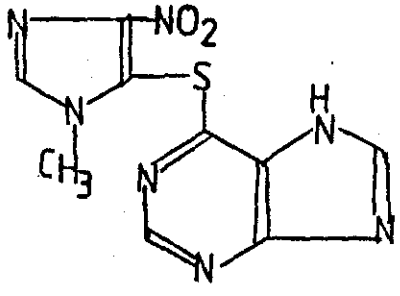
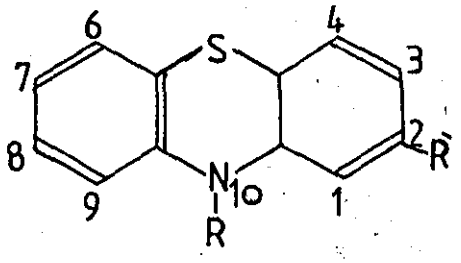
| | Compound | Structure | Source |
|---|---|---|---|
| 9 | 6-mercaptoguanosine (6-MG) |  | Sigma Chemic Co. |
| 10 | 6-mercaptopurine-2-deoxyriboside (6-MP-2-deoxyR) |  | " |
| 11 | Azathioprine (Aza) |  | " |

TABLE 2.2.

Phenothiazines and related compounds investigated



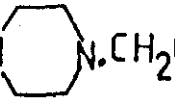
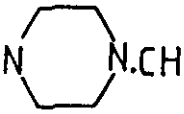
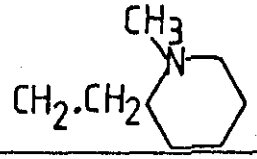

| | Compound | R | R' |
|---|---|---|---|
| 1. | Chlorpromazine HCL | $(CH_2)_3.N(CH_3)_2$ | CL |
| 2. | Methotrimeprazine maleate | $CH_2CHCH_2N(CH_3)_2$ <br> $CH_3$ | $OCH_3$ |
| 3. | Perphenazine | $(CH_2)_3$  $.CH_2CH_2OH$ | CL |
| 4. | Phenothiazine | H | H |
| 5. | Promazine HCL | $(CH_2)_3N(CH_3)_2$ | H |
| 6. | Thiethylperazine maleate | $(CH_2)_3$  $.CH_3$ | $SCH_2CH_3$ |
| 7. | Thioridazine HCL |  | $SCH_3$ |

TABLE 2.3.

Formulae of Thiobarbituric acid and related compounds studied

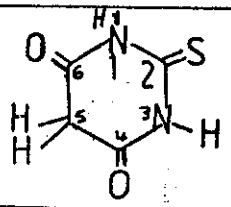
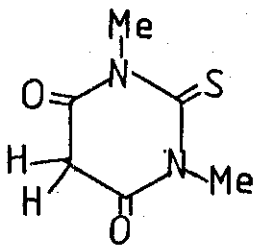
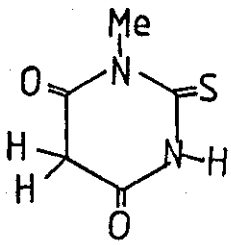
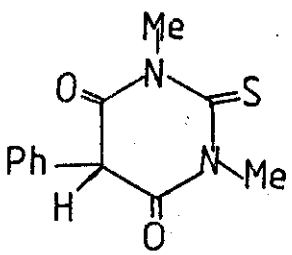
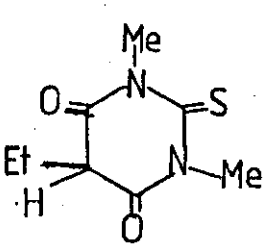
| | Compound | Structure | Source |
|---|---|---|---|
| 1. | 2-Thiobarbituric acid (2-T.B.A.) |  | BDH Chemicals Ltd |
| 2 | 1,3 dimethyl - 2-TBA |  | see text |
| 3. | 1-Methyl 2-TBA |  | " |
| 4 | 5-Phenyl-1,3-dimethyl 2TBA |  | " |
| 5 | 1,3 Dimethyl-5-ethyl 2TBA |  | " |

Table 2.3. Continued

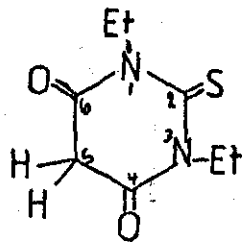
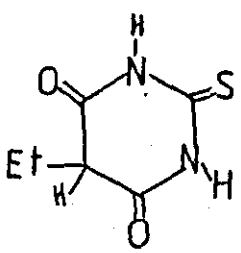
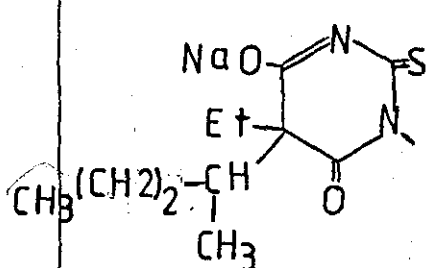
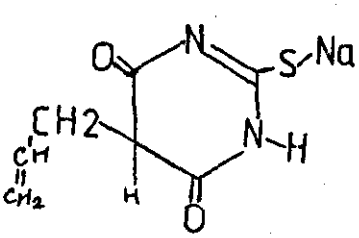
| | Compound | structure | Source |
|---|---|---|---|
| 6. | 1,3 Diethyl-2-TBA |  | see text |
| 7. | 5-Ethyl-2TBA |  | // |
| 8 | Thiopentone Sodium |  | Boots Nottingham |
| 9 | Thiabarbitone Sodium (Kemithal) |  | ICI Alderley park Cheshire |

Table 2.4. Names, Structures, & Suppliers of the polycyclic Aromatic Hydrocarbons Studied

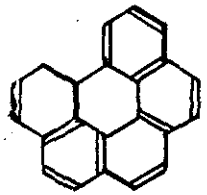
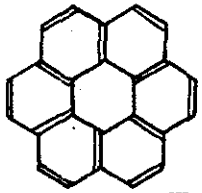
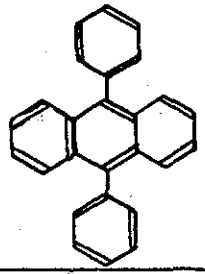
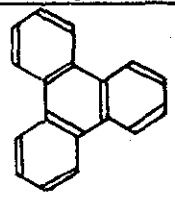
| Compound | Structure | Supplier |
|---|---|---|
| 1. Benzo(ghi)perylene |  | Aldrich Chemical Company, Inc. (A.C.C.) USA |
| 2. Coronene |  | (A.C.C.) Ltd. England |
| 3. 9,10-Diphenylanthracene |  | (A.C.C.) USA. |
| 4. Triphenylene |  | (A.C.C.) Ltd. England |

Table 2.4. (Continued)

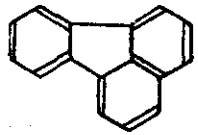
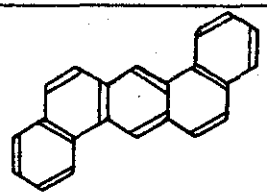
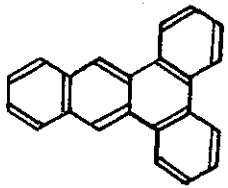
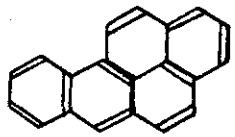
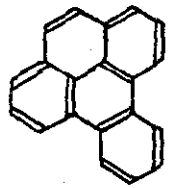
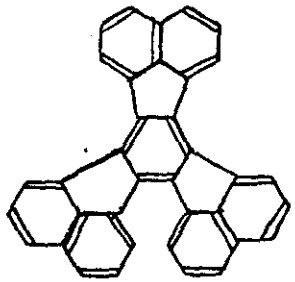
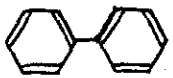
| Compound | Structure | Supplier |
|---|---|---|
| 5- Fluorene |  | (A.C.C.) Ltd. England |
| 6- Fluoranthene |  | " " |
| 7- 1,2,5,6- Dibenzanthracene * |  | " " |
| 8- 1,2,3,4- Dibenzanthracene * |  | " " |
| 9- Benzo(∝)pyrene * |  | " " |

Table 24. (Continued)

| Compound | Structure | Supplier |
|---|---|---|
| 10- Benzo(e)pyrene * |  | (A.C.C.) U S A. |
| 11- Decacylene |  | " " |
| 12- Biphenyl |  | (A. C.C.) Ltd. England |

* Cancer Suspect Agent

Phenytoin has been investigated and was obtained from Sigma Chemical Co. Ltd., (Poole, Dorset, U.K.).

The purity of the above compounds was also checked by using their melting points, which were found to be within $2^{o}$ of literature values.

2.1.1. Safety precautions

All samples were kept in the dark and refrigerated, to avoid decomposition. In all cases the measurements were done on the day solutions were prepared.

Several precautions were taken when handling polycyclic Aromatic Hydrocarbon (pAH). All experiments were carried out in a fume cupboard where possible, disposable gloves, and protective overalls were worn all the time. When skin came into contact with carcinogens by accident it was washed immediately in COLD water (not warm water, to avoid absorption by the skin). Contaminated apparatus, gloves, polythene-covered bench surfaces etc. were washed with cold water and detergent.

2.2. Thin-layer Chromatography

2.2.1. Media

Pre-coated aluminium backed TLC sheets coated with 0.1mm thick layers of cellulose without fluorescent indicator, 20x20cm, (E.G. Merck) were obtained through British Drug Houses Ltd., Poole, Dorset, U.K. Silica gel 60 HPTLC plates with fluorescent indicator, 20x20cm, (E.G. Merck) were obtained through British Drug Houses, Ltd,

Poole, Dorset U.K.) and pre-coated aluminium backed TLC sheets coated with 0.2mm thick layers of silica gel 60 plates without fluorescent indicator, (E.G. Merck) were also used.

2.2.2. Solvents

A suitable chromatographic development system was needed for each drug or group of drugs to be studied, utilising one already available or developing a new method if no suitable ones have been described. Ideally, the methods should give $R_f$ values of 0.2 to 0.8, use only one solvent and take about thirty minutes to develop a 20cm plate.

The solvent systems used are numbered below:

Solvent systems:

1. 0.1M HCl

2. Isopropanol:methanol:water:ammonia (sp.gr. 0.880) (I.M.W.A)
   60 : 20 : 20 : 1 (v/v)

3. Water

4. n-propanol:1N Ammonia
   88 / 12 (v/v)

5. n-propanol:water:Ammonia (sp.gr. 0.880)
   7 : 1 : 2 (v/v)

6. Ammonia:Dioxane:benzene
   5 : 20 : 75 (v/v)

7. Acetone : Chloroform
   1 : 9 (v/v)

8. n-hexane

9. n-hexane:pyridine
   30 : 1 (v/v)

10. Chloroform : methanol
3 : 1 (v/v)

11. Chloroform : acetone : methanol
6 : 1 : 1 (v/v)

12. Toluene : Methanol : Ethyl acetate : Acetic acid : Chloroform
16 : 4 : 3 : 2 : 1

2.2.3. T.L.C. Procedure

Before the samples were applied the plates were completely eluted with ethanol (96%, James Burroughs Ltd., London), and the top 1-2 cm of the plates cut off; the background luminescence of the oven dried plates was thus substantially reduced. The development of plates was carried out in a Shandon chromatographic tank lined with Whatmans NO.1 filter paper and containing enough solvent to fill 5% of the tank volume. The solvents in the tank were allowed to equilibrate for at least an hour before inserting the thin-layer plate.

Thin-layer chromatography (t.l.c.) of the mercaptopurine derivatives was performed on cellulose thin-layers, and on high performance thin-layer chromatography (h.p.t.l.c.) plates coated with silica gel.

A stock solution (10 $\mu g\text{-}ml^{-1}$) was prepared of each drug. 2-MP, 6MPR-5'phosph and 6MPR were initially dissolved in aqueous 0.1M sodium hydroxide, and the solution neutralised with 0.1M HCl. All other samples were dissolved in 0.1M sodium hydroxide followed by an equal volume of 0.2N HCl. 0.1-1 $\mu$l samples were applied to

the plates using disposable micropipettes (ICL Scientific, Fountain Valley, Calif., U.S.A.) and chromatographed using solvent systems 1, 2 and 3 in closed chambers. Solvent system 1 was good for all mercaptopurines except 2-thioxanthine, solvent system 3 was good for all mercaptopurines except 2-thioxanthine and 6-mercaptopurine riboside-5-phosphate, where only solvent system 2 was suitable for all. Solvent system 1 took 70 minutes to develop a 15cm height. The detection was in liquid nitrogen under a well shrouded UV lamp.

For phenothiazine and its derivatives, a stock solution of 10 $\mu g\text{-}ml^{-1}$ was prepared in ethanol. 1 $\mu$l samples were applied on silica gel plates and solvent system 4 was used. The detecting reagent was 40% sulphuric acid.

For thiobarbiturates, the stock solution of 10 $\mu g.ml^{-1}$ was prepared in ethanol. 1 $\mu$l samples were applied to cellulose thin-layers and solvent systems 5, 6 and 7 were used. Solvent system 5 was good for all the members of the group, and solvent systems 6 and 7 were good only for compounds 8 and 9. The detecting reagents were a saturated solution of mercurous nitrate in water, and 1% potassium permanganate in water.

For polycyclic aromatic hydrocarbons, stock solutions of 1 $\mu g.ml^{-1}$ were prepared. 1 $\mu$l samples were applied on to silica gel layers and solvent systems 8 and 9 were used. Solvent system 8 was preferable since it is a one component system and not carcinogenic; solvent system 9 gave good separation but involves pyridine which is carcinogenic.

$R_f$ values were determined using solvent system 8.

For phenytoin, the pure drug (phenytoin) and standard benzophenone ($10\mu g.ml^{-1}$ of ethanol) were checked on silica gel layers using solvent system 11. The location reagent was a saturated solution of mercurous nitrate in water.

When the t.l.c. step was complete for all the compounds, the plates were air-dried and viewed in a polystyrene tray into which liquid nitrogen was poured. The luminescence of the samples was viewed at 254 or 366 nm under a well shrouded u.v. lamp (BLT). Each compound yielded a single spot when analysed by the described procedure.

## 2.3. Preparation of phenytoin samples.

Standards were prepared by either weighing the solid drug, dipphenylhydantoin (DPH) directly into a known volume of plasma or dissolving a known amount in 0.05M sodium hydroxide solution and adding this solution to a known volume of plasma (.3ml of plasma was used). Dill's[83] oxidation procedure was used with the following modifications:

1.5ml of acid phosphate buffer (pH 6.8) was added to plasma in a capped centrifuge tube. This was absolutely essential to precipitate the proteins from the 1,2-dichloroethane layer in the extraction step. The DPH was then oxidised using alkaline $KMnO_4$ and the oxidation product (benzophenone) was extracted into methylcyclohexane (MCH). The MCH layer was separated and tested for purity by T.L.C. A blank was prepared in the same way except that no drug was added.

## Luminescence spectroscopy

### 2.3.1. INSTRUMENTS

Fluorescence measurements at room temperature were performed on a "Fluoricord" spectrofluorimeter (Baird-Atomic Ltd., Braintree, Essex, U.K.) using silica cells of 10mm path-length. Spectra were recorded on a Baird Atomic (model 27000) strip recorder. All measurements were made with slit widths which gave an overall resolution of 10nm.

Phosphorescence studies in rigid glasses at 77K were performed using a "Fluorispec" SF100E spectrofluorimeter (Baird Atomic Ltd.) fitted with a Silica Dewar flask and rotating cylinder phosphoroscope as previously described[14]. Luminescence intensities were measured in arbitary units on an xy chart recorder (Bryans 26000). The monochromators could be motor driven and linked to the x-axis. The photomultiplier output was linked to the Y axis facilitating the automatic production of both emission and excitation spectra. The siting of a mercury lamp (Pen Ray) in the sample compartment was used to check the calibration of the emission monochromator and the scattered light was used to check the excitation monochromator.

### 2.3.2. Thin-Layer phosphorimetry.

Modifications were made to a Baird-Atomic SF Fluoricord spectrofluorimeter to allow a thin-layer strip to be scanned; this device has been described in detail in an earlier paper[84]. Its operation is shown in Fig. 2.1.

After each developed plate had been allowed to dry it was wrapped around the sample holder drum of the thin-layer phosphroimeter and held in place with the elastic bands. It was then sprayed with ethanol until just wet, the drum was inserted into its compartment, and filled with liquid nitrogen. After allowing the drum to cool down for 2 minutes, the sample was scanned and the results recorded on a chart recorder. For room-temperature studies the thin-layer attachment was used in the same way, except that the plate was not sprayed with ethanol and no liquid nitrogen was added to the sample drum. A series of concentrations for each sample was prepared and 0.1 to 1µl samples were applied to t.l.c. plates using disposable micro-pipettes (ICL Scientific, Fountain Valley, Calif., U.S.A.). The plates were sprayed with ethanol. Thin-layer phorphorimetry at 77K was then performed at once: room temperature studies were performed after the plates had been dried overnight.

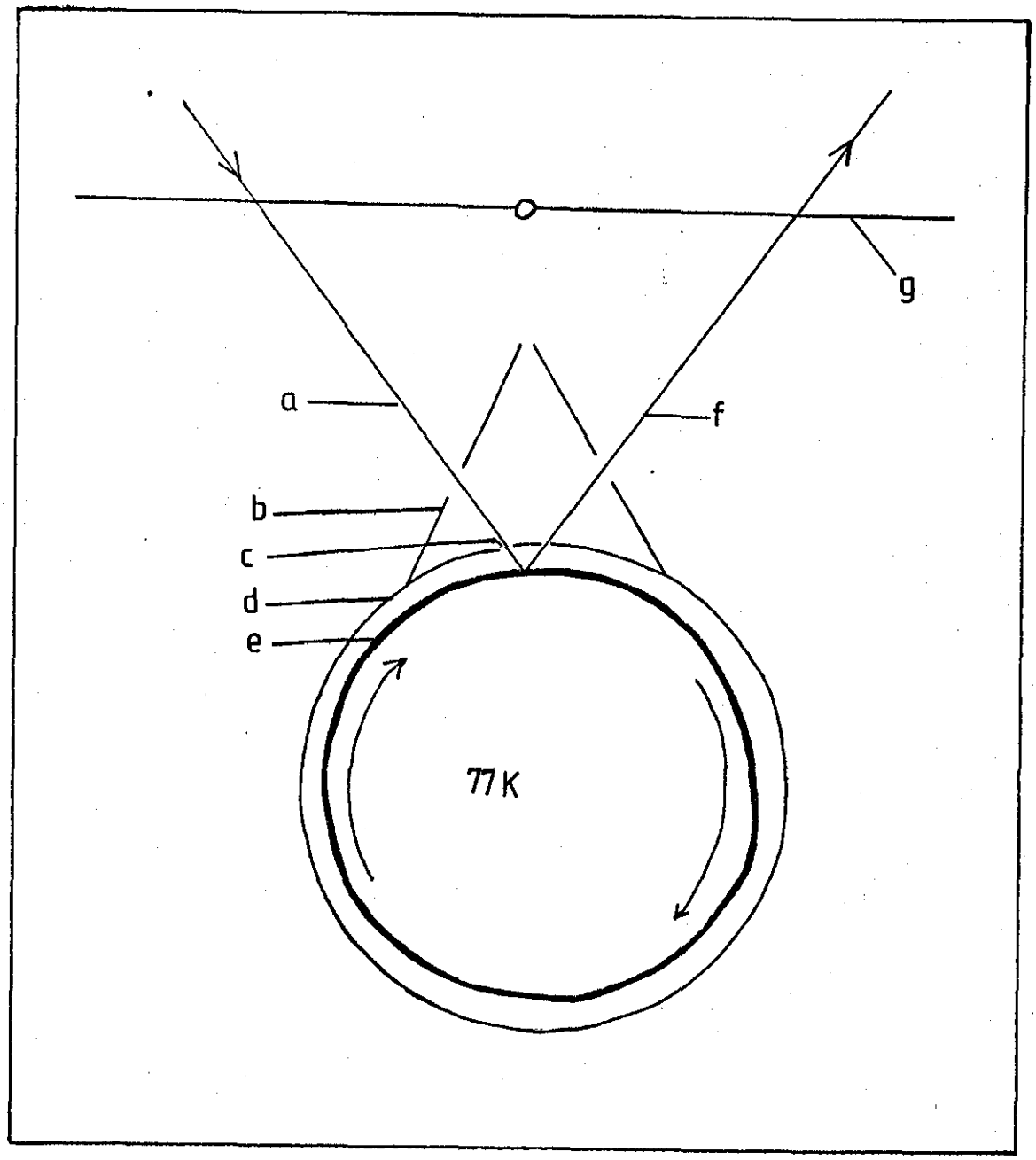



Fig. 2.1. Shows how the instrument works

Legend for Figure 2.1

a is the incident light which strikes the sample at $45^{o}$ to the normal.

b two slits, which help to control the half-band width of the exciting and emitting light.

c two silica windows are fitted to the outer cylindrical container at right-angles to each other.

d outer container painted black to minimise the scattered light.

e the sample holder consists of a hollow copper drum, the top of which is fitted with a narrower cylinder through which the drum can be filled with liquid nitrogen. The base of the hollow drum rests on a motor driven turntable. The turntable is driven by a 12 volt motor. The speed of the motor and hence the rate of excitation of the T.L.C. plate, is controlled by a variable output transformer, and gives a scanning rate from 2-30 cm $min^{-1}$. The drum can be stopped at any position and the spectral characteristics of each component measured.

f the emitted light is measured from the sample at $45^{o}$ to the normal

g single disc phosphoroscope: a thin metal disc 65mm in diameter, having 3 equally spaced slots 13mm wide and 16mm long, cut into it. It is driven by a small electric motor. The disc is mounted in front of the two fixed slits in the sample holder compartment (b). If the

disc is rotated then the sample "sees" alternately the exit slit of the excitation monochromator and the entrance slit of the emission monochromator. That is, in one phase light from the source falls on the sample, the path from the sample to the receiver being closed, and in the next the phosphorescent light can enter the emission monochromator, while the path from the lamp to the sample is closed.

2.3.3. Sample preparation

All samples for direct absorption, fluorescent and phosphorescent measurements were dissolved in 96% ethanol as background emission was not found to be a problem. The ethanolic 0.1N HCl was prepared by diluting 34-37% HCl with ethanol. The level of water in the samples did not produce cracking on cooling. The ethanolic 0.1N NaOH was prepared by warming the ethanol to dissolve the sodium hydroxide.

6-mercaptopurine and its metabolites and thiobarbiturates were dissolved in 0.1N HCl ethanol, neutral ethanol, and 0.1N NaOH ethanol. Phenothiazines and polycyclic aromatic hydrocarbons were dissolved in 96% ethanol.

2.3.4. Analysis of a mixture by thin-layer phosphorimetry.

A TLC plate was prepared as previously described for the purity check. 1 ul samples from 10ug $ml^{-1}$ solutions were applied. After separation and drying the strip was scanned using various excitation and emission wavelengths.

In all luminescence assays the limit of detection of a solute was defined as that concentration yielding a signal two standard deviations above the background signal.

2.3.5. Analysis of Drugs in plasma

Pooled human samples, obtained from Leicester Royal Infirmary, Leicester, U.K., were spiked with the compounds under study. A 0.5ml aliquot of plasma was added to a 10ml glass-stoppered centrifuge tube. A known volume of the drug in ethanol was added, and the volume

was made up to 10ml. The mixture was shaken vigorously for 60 seconds and then centrifuged for 10-15 minutes. The protein was thus precipitated and the clear supernatant was analysed by spotting 1ul samples on to TLC plates. After development the phosphorogram was obtained as previously described (Section 2.3.2.). The background emission was obtained by analysing a number of plasma samples without adding the drug.

2.3.6. The dependence of phosphorescence on the solvent.

The effect of solvents on phosphorescence intensity in thin-layer phosphorimetry was previously described[84]. In this work the TLC plate was prepared (20cm long x 5cm wide) by applying 1ul samples of the drugs under investigation, then dried under infra-red lamp for 30 minutes. The plate was sprayed with different solvents including: Ethanol/iodomethane 4/1 (v/v); 1% lead tetraacetate in methanol; 1%v/v Thallium acetate in ethanol, 1% w/w potassium iodide in ethanol and 0.1M silver nitrate in ethanol, until it had just acquired a wet appearance; i.e. there was a continuous film of solvent on the stationary phase. After spraying, the phosphorogram was determined as previously described (Section 2.3.2).

The "heavy atom" effect was employed to improve the limit of detection for the compounds which were not very strongly phosphorescent.

2.3.7. Effect of Temperature on phosphorescence intensity.

The last part of the experimental work was the investigation of the effect of temperature on the intensity of phosphorescence. The study used promazine HCl as an example.

1ul samples from a 100ug $ml^{-1}$ stock solution were spotted on to silica gel thin layers (10cm x 4cm). After drying, the strip was bound by elastic bands to a hollow copper drum, mounted on a motor driven turntable as shown in Fig. 2.1. The strip was sprayed with ethanol and the drum was inserted in its compartment as described previously[84]. A digital thermometer type CGI range 1 to 280K (supplied by Air Products and Chemicals Inc.) was used to measure the change in temperature. To the thermometer a thermocouple was connected, consisting of a gold negative pole and a silver positive pole. The thermocouple was inserted between the strip and the wall of the drum, then the drum was filled with liquid nitrogen and the reading on the thermometer was taken. Liquid nitrogen was poured into the drum; after 2 minutes, the thermometer read 77K and the phosphorescence intensity was measured, either by reading it on the meter of the instrument or recording the spectrum on the recorder. the excitation and emission wavelengths were 310nm and 495nm respectively.

Simultaneous readings of the change in temperature and the phosphorescence intensity were recorded. Also the change in temperature with time was recorded. A graph of intensity vs temperature was plotted.

The same procedure was carried on for 6-mercaptopurine In this case a cellulose strip of (20cm x 4cm) was used and 1ul samples of different concentrations were applied on the t.l.c. plate. The phosphorogram was obtained by repeated scanning of the drum; the excitation and emission wavelengths were 342nm and 485nm respectively.

## 2.4. Absorption spectroscopy

Absorbance measurements were made with both a Pye-Unicam S.P. 8000 Ultra Violet Recording Spectrophotometer and a Pye-Unicam S.P. 800 Ultra-Violet Spectrophotometer using 10mm path length silica cells. All spectra were recorded at 298K using the same solvents as in the luminescence measurements. Measurements of absorption spectra were made immediately after sample preparation.

## 2.5. General reagents

| | |
|---|---|
| Ethanol: | 96%, James Burroughs Ltd., London. |
| Isopropanol: | (BDH Ltd) |
| n-propanol: | (BDH Ltd) |
| Ammonia s.p. 0.88: | Laporte Industries Ltd., Gin House Lane, Rotherham, Yorks, U.K. |
| Acetone: | Fisons, Loughborough, U.K. |
| Methyl cyclohexane: | Fisons, Loughborough, U.K. |
| Benzophenone: | Fisons, Loughborough, U.K. |
| 1,2 Dichloromethane: | (BDH Ltd) |
| Lead tetraacetate: | 1% w/v lgm was dissolved in 100 ml of methanol |
| Thallous acetate: | 1% w/v lgm was dissolved in 100ml of methanol. (BDH) Poole, England |
| Iodomethane: | Fisons, Loughborough, U.K. |
| 0.1M HCl: | Laporte Industries Ltd, York, U.K. |
| 0.1M NaOH: | Fisons, Loughborough, U.K. |
| Potassium permanganate: | Fisons, Loughborough, U.K. |
| 40% sulphuric acid: | (BDH) Poole, England. |
| n-hexane | Fisons, Loughborough, U.K. |
| Pyridine: | Fisons, Loughborough, U.K. |
| Toluene: | Fisons, Loughborough, U.K. |
| Methyl acetate: | Fisons, Loughborough, U.K. |
| acetic acid: | (Hopkin and Williams) Chadwell Heath, Essex, England. |
| Chloroform: | (BDH) Poole, England |
| mercurous nitrate: | Fisons, Loughborough, U.K. |

# CHAPTER 3

## 3.1. Phenytoin

### INTRODUCTION

Diphenylhydantoin (DPH) and phenobarbital are the two drugs most often used for the control of epileptic seizures; DPH is a commonly prescribed anticonvulsant drug. A need exists for a rapid sensitive and selective chemical procedure capable of determining diphenylhydantoin at therapeutic levels in blood (10 to 30ug.ml$^{-1}$). Anticonvulsant drugs have been determined by a variety of analytical techniques, the most commonly used being spectrophotometric methods (Plaa et al[85] and Wallace et al[86]) and colorimetric methods[87]. These methods are time consuming and subject to interference from phenobarbital and other drugs. Recently, Evans[88] reported a method for the simultaneous determination of diphenylhydantoin and phenobarbital in serum by high-pressure liquid chromatography. However the method required a multi-step sample preparation procedure with critical pH adjustments, and no provision was made for use of an internal standard. Later Pokar et al[89] in 1976 described a sensitive high pressure liquid chromatographic method in which 5-(p-methyl phenyl)-5-phenylhydantoin is used as the internal standard for the simultaneous determination of diphenylhydantoin, phenobarbital and primidone in whole blood and plasma. Gas-liquid chromatographic (GLC) procedures introduced later by Chang[90] and Macgee[91] are highly specific, but generally

require more than 1 ml of plasma and about 20 minutes per sample. A radioimmunoasay method introduced by Booker[92] appears to be highly specific and sensitive, but the prepared reagents are expensive. The oxidation of DPH by heating with alkaline permanganate to yield benzophenone was first described by Wallace et al[93]; this method required reflux operations during the oxidation step, and it lacked sensitivity. Numerous modifications in the extraction and oxidation steps and the development of a sensitive fluorimetric assay procedure for benzophenone were reported by Dill et al:[83,94,95,96] this assay is suitable for clinical use. Determination of diphenylhydantoin by phosphorescence spectrometry has been described by Lee et al[56]; they modified the procedure of Dill[10] by adjusting the pH of 1 ml. of plasma, to 6-7 by acid-phosphate buffer before oxidation. The sensitivity of the method was 50 ng/ml.

The present work describes a sensitive method for the determination of phenytoin by combining thin layer chromatography and phosphorimetry at 77K.

Results and discussion.

3.1.1. Purity check.

Thin layer chromatography was used to check the purity of benzophenone (standard) and phenytoin. The two compounds were pure when silica gel plates, and Chloroform:Methanol:Acetone (6:1:1 v/v) as an eluent were used.

Table3.1.1shows the $R_f$ values of benzophenone and diphenylhydantoin before and after the oxidation procedure at room temperature using mercurous nitrate as a detecting reagent.

Table 3.1.1 $R_f$ values of benzophenone and phenytoin

| Compound | $R_f$ | Colour with mercurous nitrate | |
|---|---|---|---|
| Benzophenone | 0.77 | dark grey | Before oxidation |
| diphenylhydantoin | 0.66 | dark grey | |
| Benzophenone | 0.77 | dark grey | |
| pure drug | 0.77 | dark grey | After oxidation |
| Drug + plasma | 0.77 | dark grey | |

For the detection of the above substances it was possible to use U.V. light, especially in the short-wave region (254nm) where benzophenone absorbs at 77K; this is a non-destructive method of detection. The plate was sprayed with mercurous nitrate and a dark spot for each compound was observed. From this method of detection the compounds were highly pure and no further purification was carried out.

### 3.1.2. Ultra Violet Absorption

The ultraviolet absorption spectra of diphenylhydantoin and its ozidized reaction product are shown in Fig. 3.1.1.

The figure also shows the recovery of the drug from plasma, both curves correspond to 5ug.ml$^{-1}$ and 10ug.ml$^{-1}$ in methycyclohexane in the original solution. The product in methylcyclohexane is well defined and exhibits an absorption maximum at 254nm for both pure drug and drug + plasma (control plasma). The ultra-violet absorption spectrum of benzophenone is identical to that shown for the product in Fig. 3.1.1. The spectra suggest that the two are the same compounds. The figure also shows diphenylhydantoin before the oxidation process. A blank plasma gave zero absorbance reading.

### 3.1.3 Luminescence Spectra at 77K.

The phosphorescence spectra of the oxidation product of DPH (benzophenone) are presented in Fig. 3.1.2. The solid line represents the measurements in a quartz tube (i.e. in solution): it is well resolved and exhibits three phosphorescence maxima at 404, 448 and 488 nm, and a single excitation maximum at 260nm. The broken line represents the spectrum of benzophenone on a thin layer plate, this spectrum is not as well resolved as the solution spectrum, but the maximum at 446nm is well defined. The determination of the limit of detection was done by fixing the emission wavelength at 446nm and the excitation wavelength at 260nm.

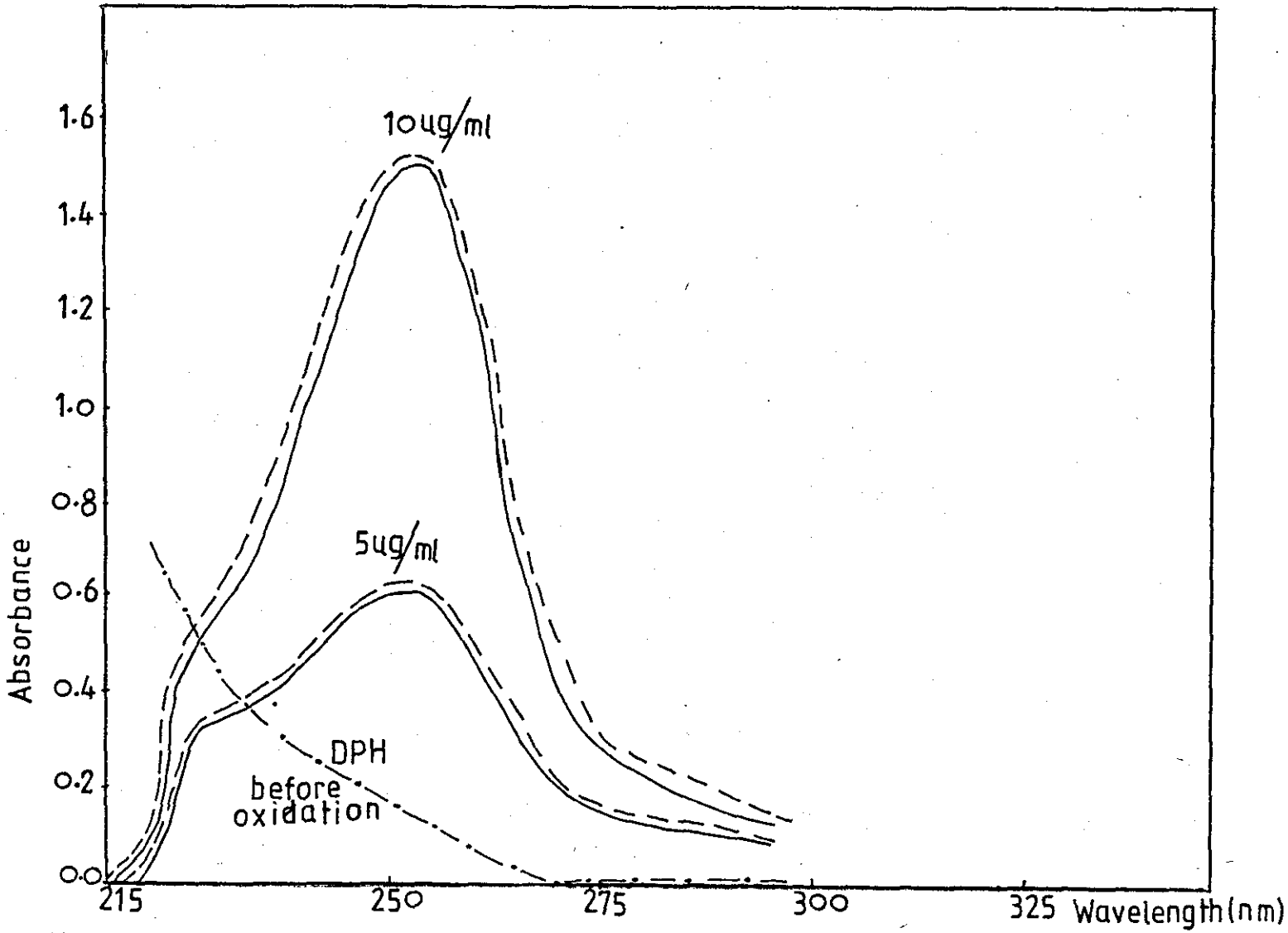



Fig.3.1.1 Ultraviolet absorption spectra of DPH and of the diphenylhydantoin reaction product in methylcyclohexane (– – – – –) pure drug , (————) drug+ plasma

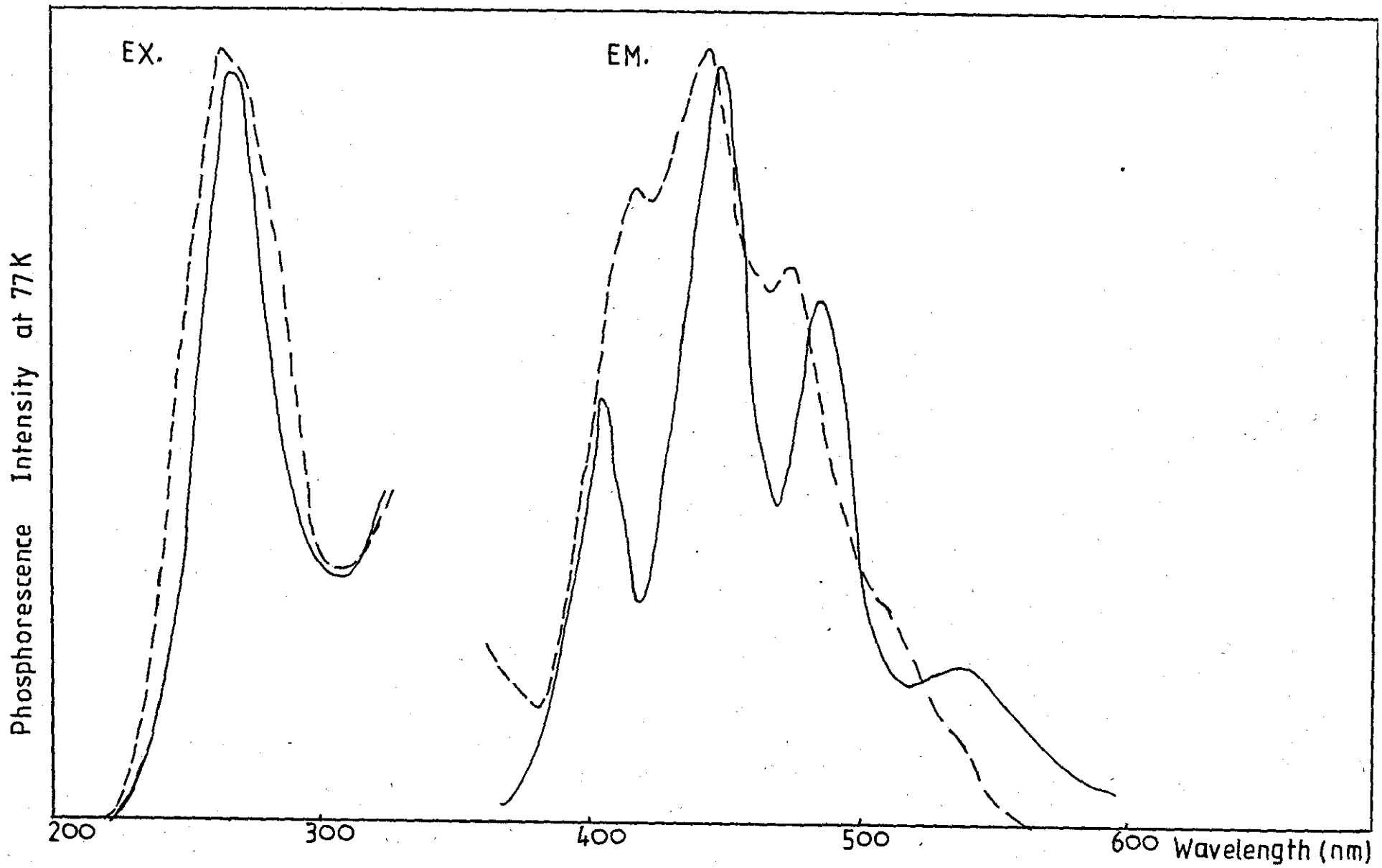



Fig.3.1.2. Phosphorescence spectra of oxidation product (benzophenone) of 5ug/ml DPH in methylcyclohexane
(——) in tube (– – – –) on TLC plate

Table 3.1.2summarizes the recovery of diphenylhydantoin which was added to plasma in known amounts. The recovery was determined by comparing the intensities of the drug and drug + plasma.

Table 3.1.2 Recovery of diphenylhydantoin added to plasma.

| DPH concentration ng/spot | No.of determin-ations | Average intensity in cm. ± std. dev. | Recovery ng/spot (mean ± std.dev | mean % |
|---|---|---|---|---|
| .5 | 5 | .25 ± 0.09 | 0.49 ± 0.09 | 98 |
| .65 | 5 | .35 ± .09 | 0.65 ± 0.09 | 100 |
| 1.0 | 5 | .5 ± 0.1 | 1.0 ± 0.1 | 100 |
| 2.5 | 6 | 1.3 ± 0.1 | 2.5 ± 0.1 | 100 |
| 6.5 | 5 | 3.0 ± 0.2 | 6.0 ± 0.2 | 92 |
| 14 | 6 | 7.5 ± 0.2 | 14 ± 0.2 | 100 |
| 45 | 6 | 25 ± 2.0 | 44 ± 2.0 | 98 |
| 100 | 6 | 30 ± 4.0 | 98 ± 4.0 | 98 |
| Average recovery | | | | 98.3% |

Recovery of diphenylhydantoin 98.3% (Table 3.2.1.) agrees well with Evenson et al[97] and Pippenger et al[98], who reported 90-100% recovery. Figure 3.1.3shows the phosphorograms of the reaction product (benzophenone) on TLC plates. Corresponding to pure drug and (drug + plasma) containing 0.3 ml of plasma and 1.0ng/spot can be seen. Blanks derived from plasma subjected to identical treatment showed virtually no phosphorescence background. Standard curves, prepared with known amounts of DPH added to control plasma, showed good linearity in the range of 0.5 - 45 ng/spot (Fig 3.1.4). The

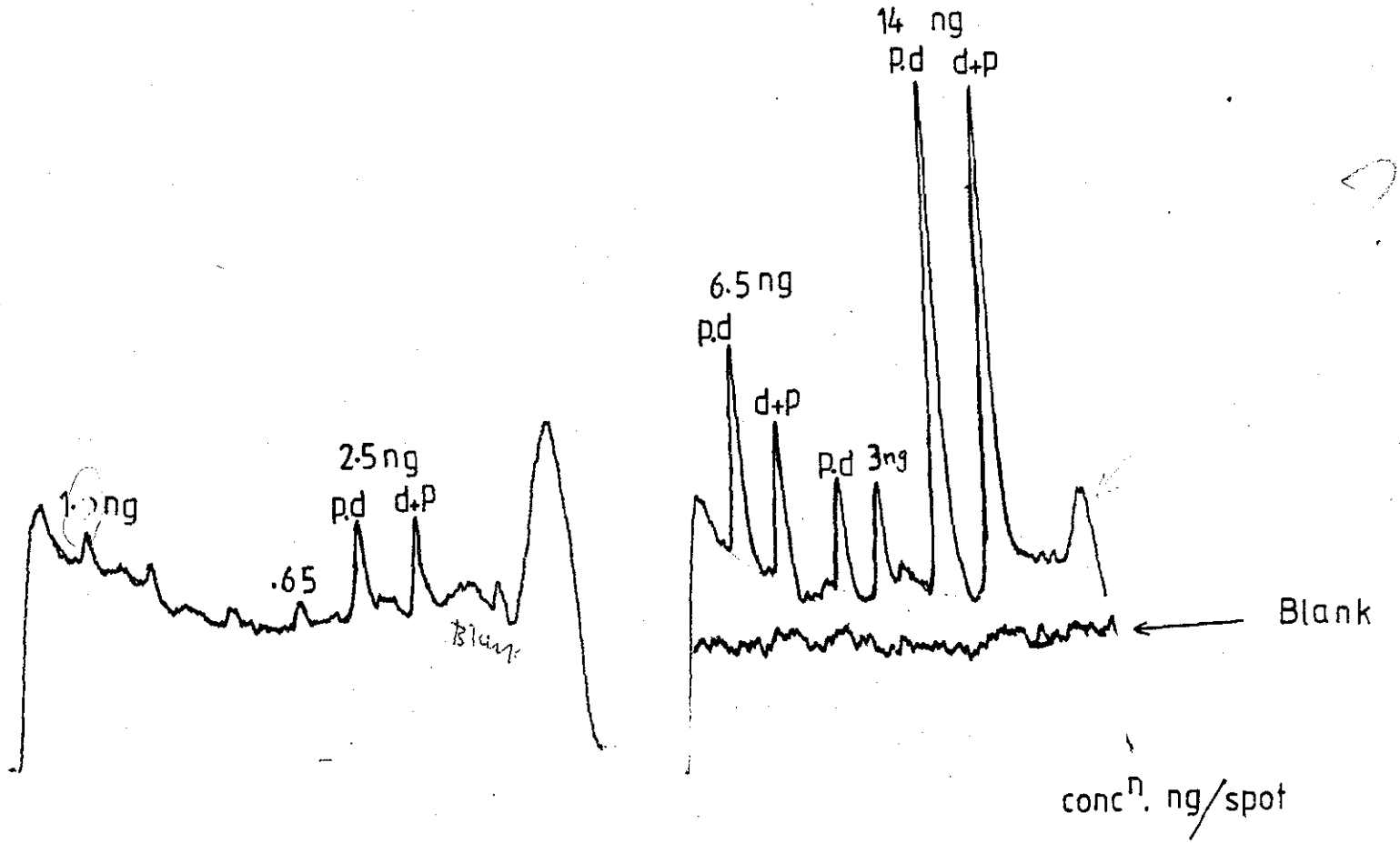



Fig.3.1.3. Phosphorogram of diphenylhydantoin reaction product on TLC plate corresponding to pure drug(p.d) and drug+plasma(d+p) at 77K

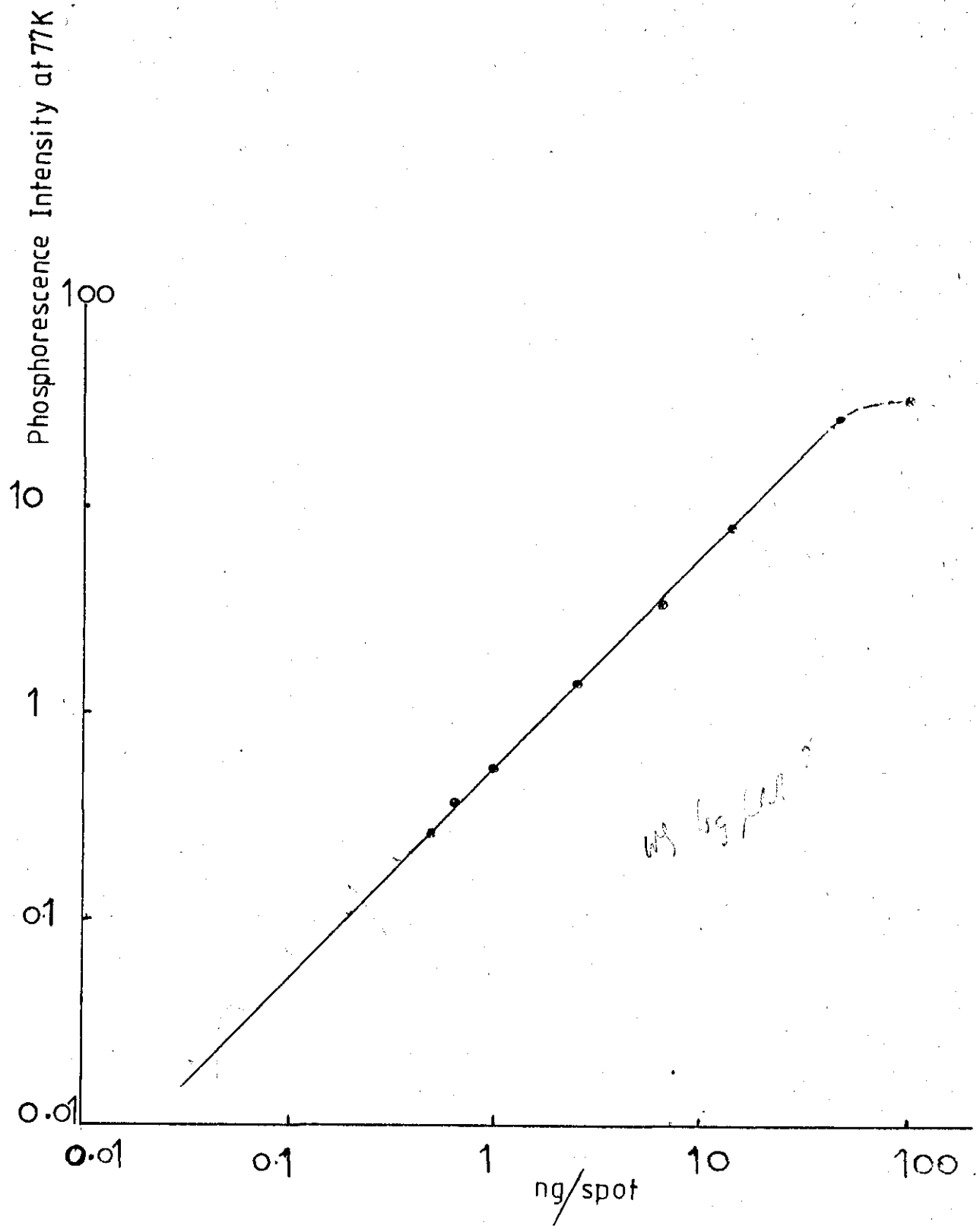



Fig 3.1.4 Analytical growth curve of oxidation product of phenytoin (benzophenone) on HPTLC plate

practical limit of detection for diphenylhydantoin in plasma is 0.4ng per spot using thin-layer phosphorimetry with an error less than 10%. Thin-layer phosphorimetry is thus a very sensitive technique in this case and enables the clinician to monitor nanogram quantities of DPH. The method is thus particularly applicable to pediatric care. The time required to run one scan is only one minutes. A standard curve may be generated in about 15 minutes since many samples may be run in a short period of time. The benzophenone procedure is highly specific for DPH, with no known interference from the major metabolite of DPH, HPPH, and 5-(p-hydroxyphenyl)-5-phenylhydantoin.

In conclusion, thin-layer phosphorimetry demonstrates that derivative analyses are possible.

## 3.2. 6-mercaptopurine and Related Compounds

### INTRODUCTION

6-mercaptopurine (6MP) is used as an antineoplastic agent, and has immunosuppressive properties. The main use of 6MP is in the induction and maintenance of remission in acute leukaemia in children, remission being achieved in a considerable proportion of cases treated. Azathioprine is an immunosuppressive and antineoplastic agent with similar actions to those of 6MP, to which it is slowly converted in the body. It is mainly used as an immunosuppressive agent for facilitating the survival of organ and tissue transplants.

The widespread use of 6-MP and azathioprine as immunosuppressive and antileukaemic agents has created a demand for the selective analysis of these compounds and their metabolites at trace levels in biological fluids. The many methods used include paper[99] and thin layer chromatography[100,101], high voltage[102] and thin-layer electrophoresis[103], gas liquid[104] and high performance liquid chromatography[105] and mass spectrometry[106].

Finkel[107] showed that 6-mercaptopurine could be determined fluorimetrically after oxidising deproteinised plasma samples with potassium permanganate to produce purine-6-sulphonate: the limit of detection was 1 $\mu g.ml^{-1}$. The strong phosphorescence exhibited by many purine derivatives has been frequently reported (Ref. 108), and Aaron and Winefordner showed that 6-methylmercaptopurine and 2-amino-6-methyl-mercaptopurine showed analytically useful phosphorescence[37]. The same compounds were more recently shown to exhibit a

weaker room temperature phosphorescence (R.T.P.) when adsorbed on to chromatography paper from alkaline solution[109].

Maddocks and Davidson[110] reported the detection of picomole quantities of azathioprine, 6-mercaptopurine and seven related compounds after thin-layer chromatography on cellulose, using the luminescence developed when the chromatography plate was cooled to liquid nitrogen temperature. Wong and Maddocks[103] used a similar technique to detect mercaptopurine derivatives after thin-layer electrophoresis on a variety of media. Neither of these papers reported spectroscopic data, and fluorescence and phosphorescence were apparently not distinguished.

The present work describes a detailed study of the luminescence properties of 11 mercaptopurine derivatives. The application of thin layer phosphorimetry[14], both at room temperature and at liquid nitrogen temperature, to their rapid determination at trace levels in deproteinised blood plasma is also described.

## Results and Discussion

### 3.2.1. Purity check.

The compounds studied are listed in Table 2.1. The phosphorescence of the spots could be clearly observed under UV light, in each case a single well defined spot was marked. In Table 3.2.1 lists the solvent systems used and the $R_f$ values.

### 3.2.2. Absorption spectra

The ultra-violet absorption spectra of 6-mercaptopurine derivatives were found to be pH dependent. Table 3.2.2. Spectra obtained in neutral and acidic (0.1M HCl)ethanolic solutions were generally similar, but in ethanol containing 0.1M NaOH shifts of absorption maxima and changes in molar absorbance were sometimes observed. 6-mercaptopurine (Fig. 3.2.1.) for example, exhibited a blue shift of about 18nm, and a slight decrease in molar absorbance in alkaline solution. 6-thioxanthine Fig. 3.2.2.,another example, exhibited a red shift of about 12 nm, and a slight decrease in molar absorbance, in alkaline solution. Such changes, similar to those found by other workers[111], suggested the desirability of performing luminescence studies in acidic, alkaline and neutral solutions.

### 3.2.3. Fluorescence at 298°K

Room temperature studies showed that only one compound, 2-amino-6-mercaptopurine, exhibited significant fluorescence. In alkaline ethanol, with excitation and fluorescence wavelengths of ca. 316 and 402nm respectively, this compound had a limit of detection of 7$\mu g.ml^{-1}$.

Table 3.2.1. Mercaptopurines studied on cellulose thin layers

| | Compound | solvent system 1 0.1M HCL Rf | colour | solvent system 2 I.M.W.A. Rf | solvent system 3 Water Rf |
|---|---|---|---|---|---|
| 1 | 2-mp | 0·39 | yellow | 0·32 | 0·32 |
| 2 | 6-MP | 0·44 | blue | 0·42 | 0·27 |
| 3 | 2-Thioxa | N.D. | N.D. | 0·19 | N.D. |
| 4 | 6-Thioxa | 0·21 | blue | 0·30 | 0·18 |
| 5 | 6-MPR | 0·62 | blue | 0·38 | 0·55 |
| 6 | 6-MPR5P | 0·71 | blue | 0·17 | N.D. |
| 7 | 6-MeMP | 0·63 | blue | 0·73 | 0·35 |
| 8 | 2-$NH_2$-6MP | 0·32 | green | 0·28 | 0·13 |
| 9 | 6-MG | 0·51 | green | 0·23 | 0·3 |
| 10 | 6-MP2deox | 0·41 | blue | 0·45 | 0·27 |
| 11 | Azathioprine | 0·66 | yellow | 0·75 | 0·65 |

Table 3.2.2. The U.V. absorption characteristics of Mercaptopurines in neutral, acid (0.1MHCl) and alkaline (0.1M NaOH) ethanol

| Compounds | Neutral ethanol $\lambda_{max}$ nm | Acidic ethanol $\lambda_{max}$ nm | alkaline ethanol $\lambda_{max}$ nm |
|---|---|---|---|
| 1 | 370, 294, 244 | 295, 244 | 328, 273, 250 |
| 2 | 331, 279 | 329, 225 | 313, 305 |
| 3 | 285, 224 | 285, 229 | 288, 225 |
| 4 | 343, 337, 285 | 340, 276 | 349, 275, 250 |
| 5 | 325, | 330, | 319 |
| 6 | No absorption * | 327, | No absorption * |
| 7 | 289, 289, 284 | 289, 284 | 291, 285 |
| 8 | 349, | 349 | 327 |
| 9 | 349, 339, 275, 259 | 349, 270 | 342, 317, 275, 254 |
| 10 | 325, 229, 213 | 328, 230, 242 | 319, 236 |
| 11 | 278, 216 | 278, 216 | 288, 225 |

* above ~ 250 nm.

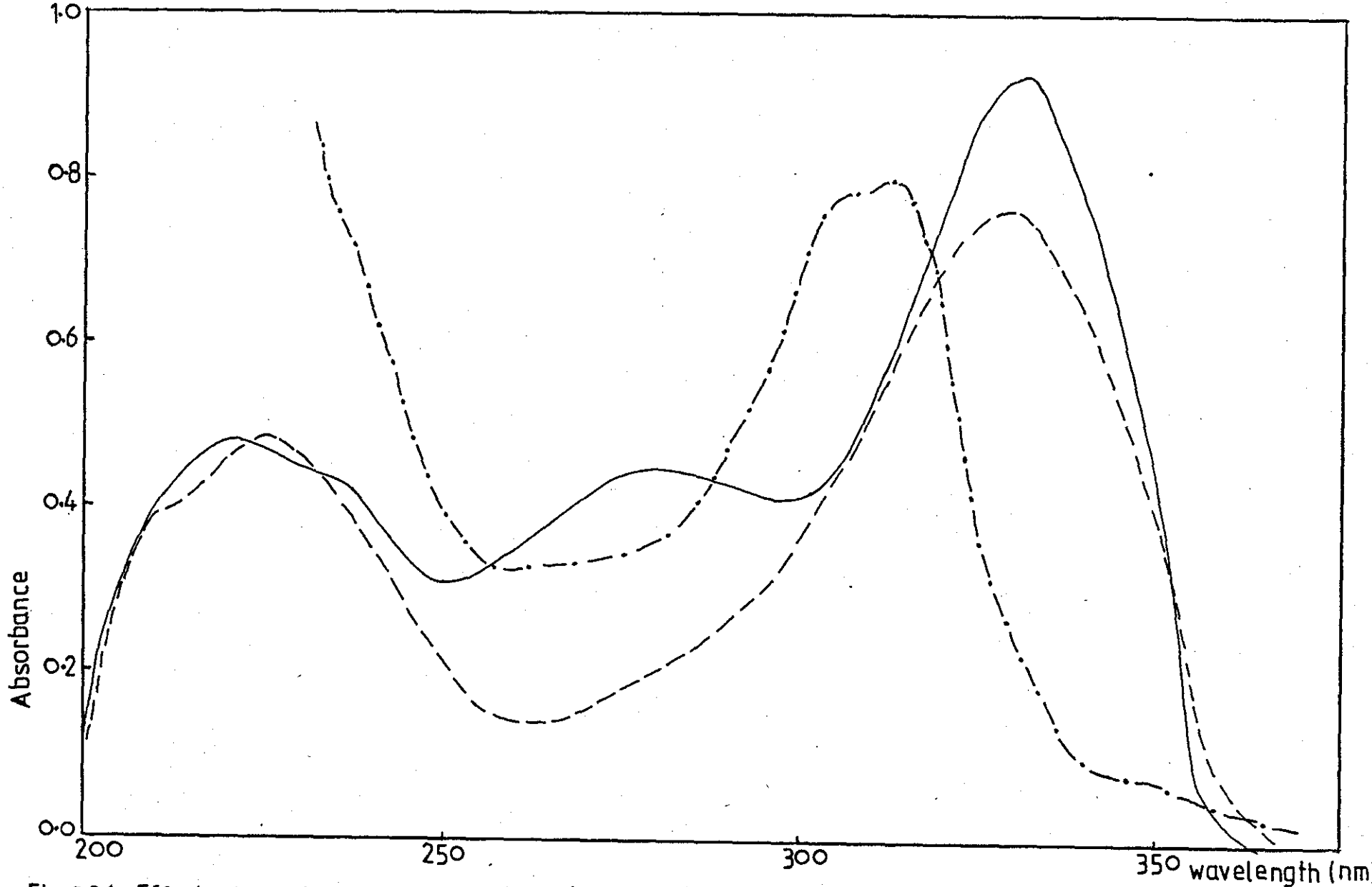



Fig. 3.2.1 Effect of varying PH on the absorption spectra of 6-MP, (——) in neutral ethanol, (– – –) in 0·1M ethanolic HCL & (—·—·—) in 0·1M ethanolic NaOH.

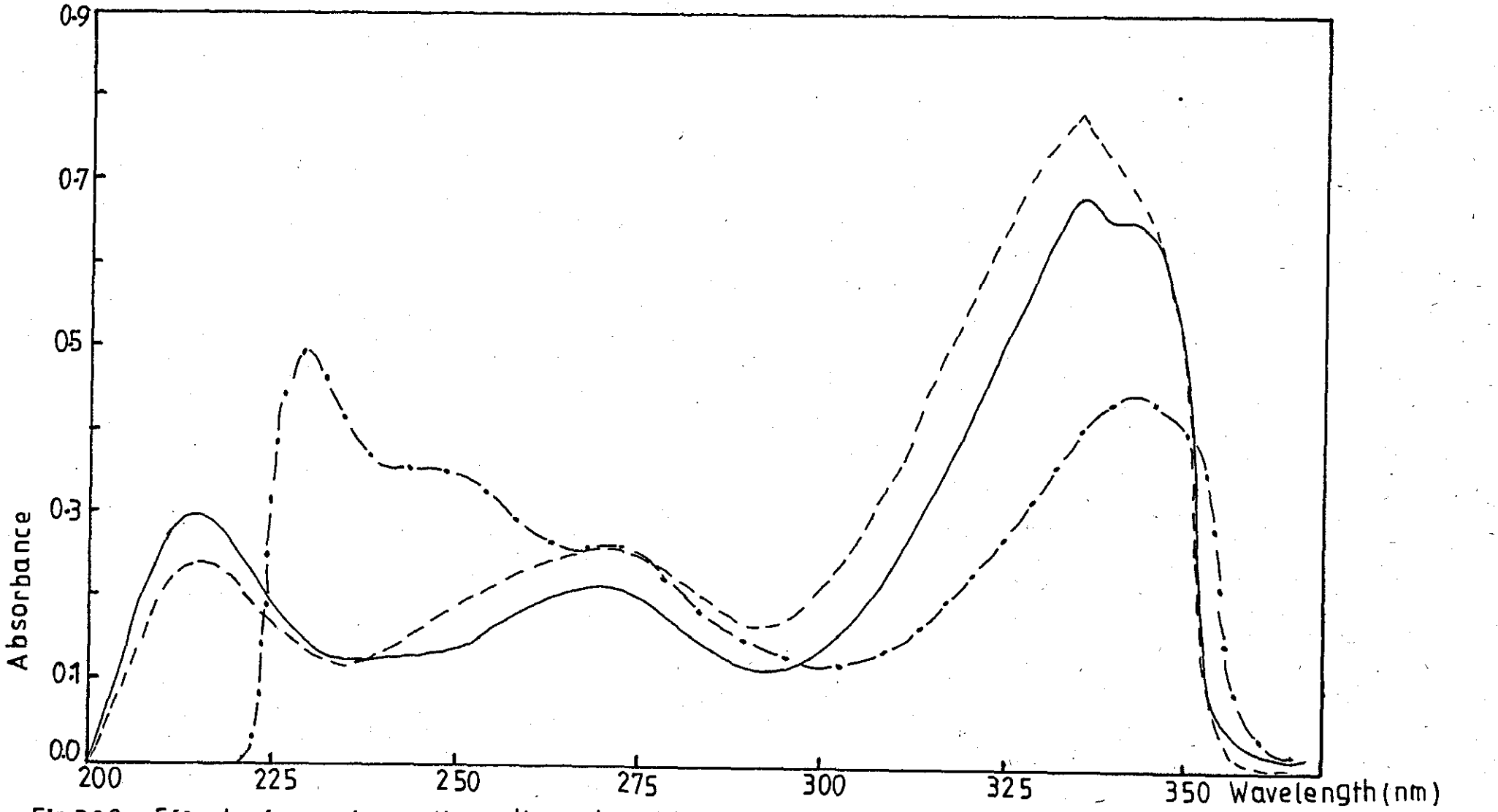



Fig.3.2.2 Effect of varying pH on the absorbtion spectra of 6-thioxanthine, in neutral ethanol (——), in ethanolic 0·1M HCL (– – – –), and in ethanolic 0·1M NaOH (—·—·—·)

Table 3.2.3. Phosphorescence characteristics of Mercaptopurines in Ethanol Glasses at 77K.

| Compounds | Neutral Ethanol | | | Alkaline Ethanol | | |
|---|---|---|---|---|---|---|
| | $\lambda_{ex}$ nm | $\lambda_{p}$ nm | Detection limit $ng.ml^{-1}$ | $\lambda_{ex}$ nm | $\lambda_{p}$ nm | Detection limit $ng.ml^{-1}$ |
| 1 | 342 | 510 | 6 | 330 | 488 | 1 |
| 2 | 340 | 463 | 20 | 316 | 454 | 2 |
| 3 | 295 | 458 | $10^4$ | 295 | 452 | $10^5$ |
| 4 | 344 | 480 | 50 | 349 | 508 | 50 |
| 5 | 325 | 456 | 30 | 323 | 462 | 50 |
| 6 | 337 | 446 | ND | 300 | 430 | $10^5$ |
| 7 | 286 | 436 | 25 | 295 | 446 | 40 |
| 8 | 356 | 484 | 40 | 330 | 479 | 50 |
| 9 | 341 | 466 | 120 | 340 | 461 | 70 |
| 10 | 332 | 476 | 60 | 320 | 460 | 60 |
| 11 | 300 | 442 | $10^4$ | 311 | 451 | 2600 |

ND : Not detected

Table 3.2.4. Limits of detection of Mercaptopurines in Blood Plasma using Thin-layer phosphorimetry at 77K.

| Compound | Limits of detection, ng per spot | | |
|---|---|---|---|
| | Cellulose thin layers | | Silica gel h.p.t.l.c. layers neutral ethanol solvent |
| | neutral ethanol solvent | alkaline ethanol solvent | |
| 1 | 45 | 2 | N.D. |
| 2 | 3 | 0.05 | 0.04 |
| 3 | 50 | 50 | N.D. |
| 4 | 1 | 5 | N.D. |
| 5 | 2 | 5 | N.D. |
| 6 | 30 | 25 | N.D. |
| 7 | 1 | 1 | 1 |
| 8 | 0.1 | 5 | N.D. |
| 9 | 4 | 0.1 | 0.2 |
| 10 | 5 | 3 | N.D. |
| 11 | 150 | 10 | N.D. |

3.2.4. Luminescence measurements at 77K.

The phosphorescence characteristics of all the compounds in ethanolic glasses at 77K are given in Table 3.2.3. [In these conditions, compounds 9, 8 and in alkaline ethanol - 7 exhibited relatively feeble fluorescence signals at 350-400nm (Figs. 3.2.3 and 3.2.4.). The remaining compounds exhibited no fluorescence and could thus be studied without the rotating cylinder phosphoroscope in the light beam]. As expected from the absorption spectra, the phosphorescence spectra in neutral and acidic ethanol solutions were very similar, Fig. 3.2.5 and Table 3.2.3.

It is apparent that $ng.ml^{-1}$ limits of detection can be obtained in many cases. For some compounds, alkaline conditions produced the best detections limits; for others neutral or acidic solutions were preferred.

Analytical curves were found to be linear over large concentration ranges; the curves for 6-mercaptopurine, 6-mercaptopurine-hiboside and 6-mercaptoguanosine are shown in Fig. 3.2.6.

When the compounds were adsorbed on to cellulose T.L.C. plates at 77K, the excitation and emission spectra were generally closely similar to those obtained in ethanolic glasses, and again very low levels of many of the compounds could be detected particularly when the samples were applied as alkaline ethanolic solutions. Table 3.2.4. shows that quantities as low as ten pg could be detected in deproteinised plasma samples. Similar results were obtained when pure solutions of the compounds were studied. The recovery of the solutes

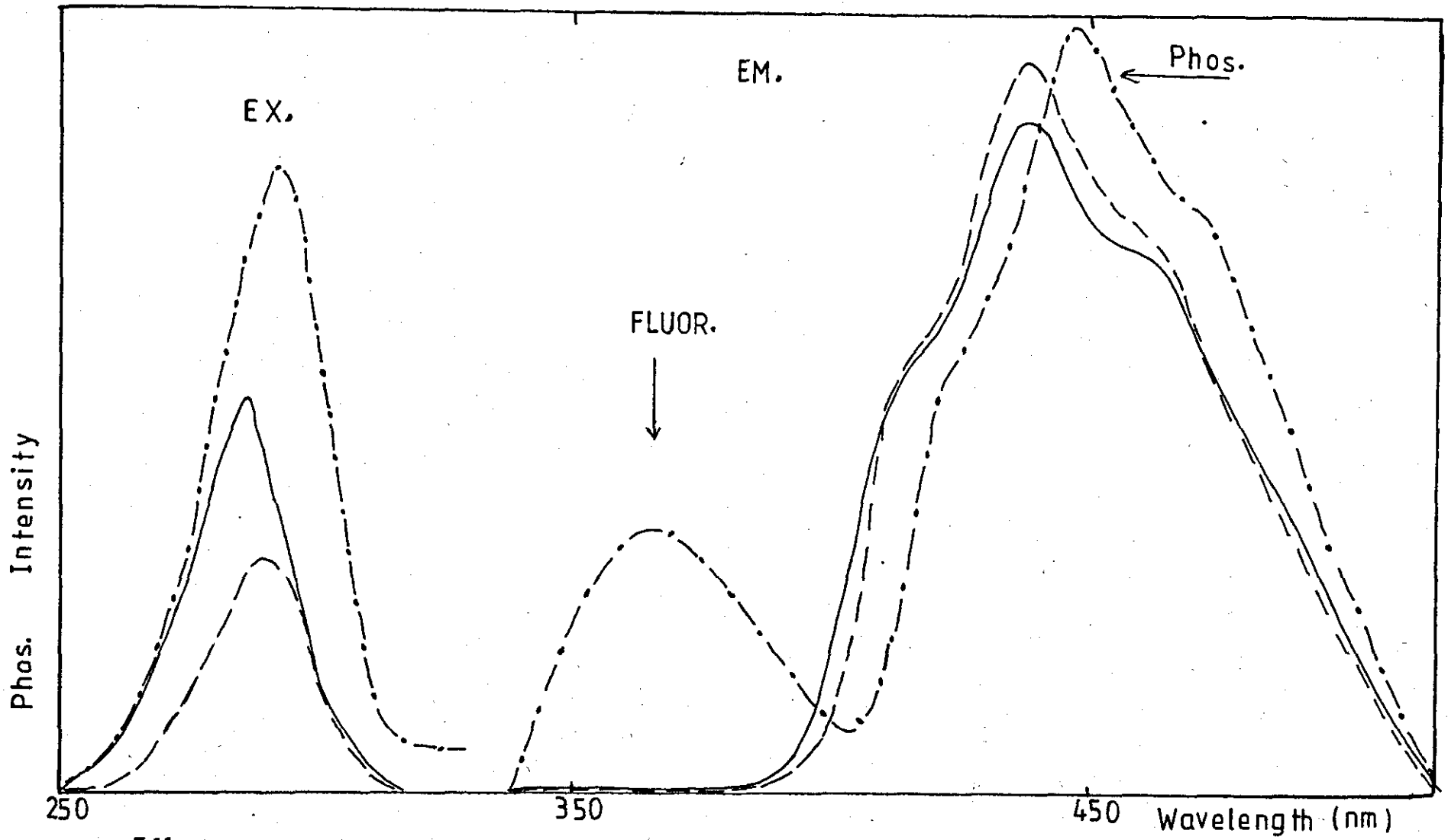



Fig.3.2.3. Effect of varying PH on the Luminscence spectra of 6-MeMP, in neutral ethanol (——), in 0.1M ethanolic HCL (— — — — — —), & in 0.1M ethanolic NaOH (—·—·—·—·—)

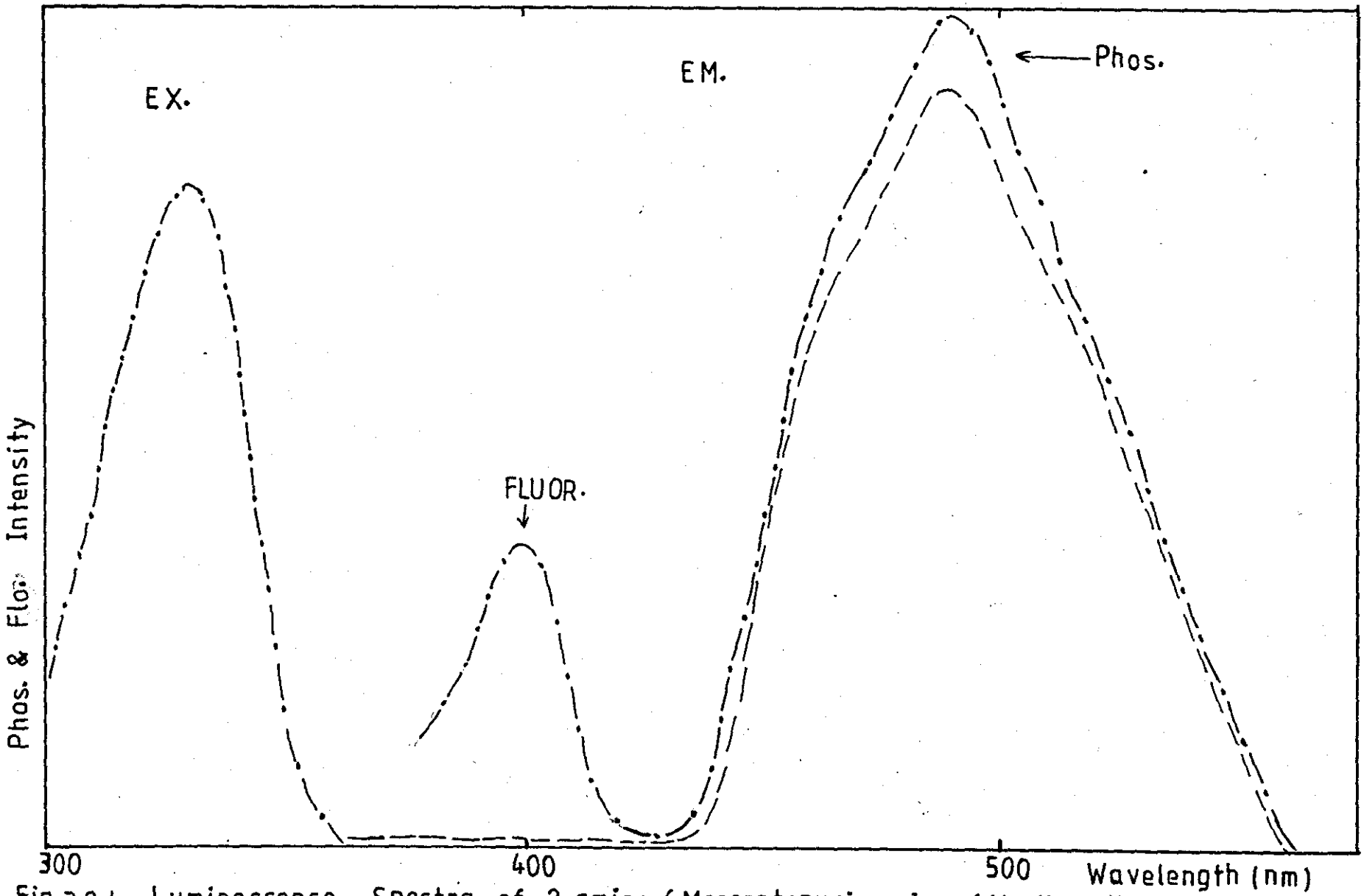



Fig. 3.2.4. Luminescence Spectra of 2-amino-6-Mercaptopurine in 0.1 M ethanolic NaOH

(——————) Total Luminescence, (— — — —) Phosphorescence.

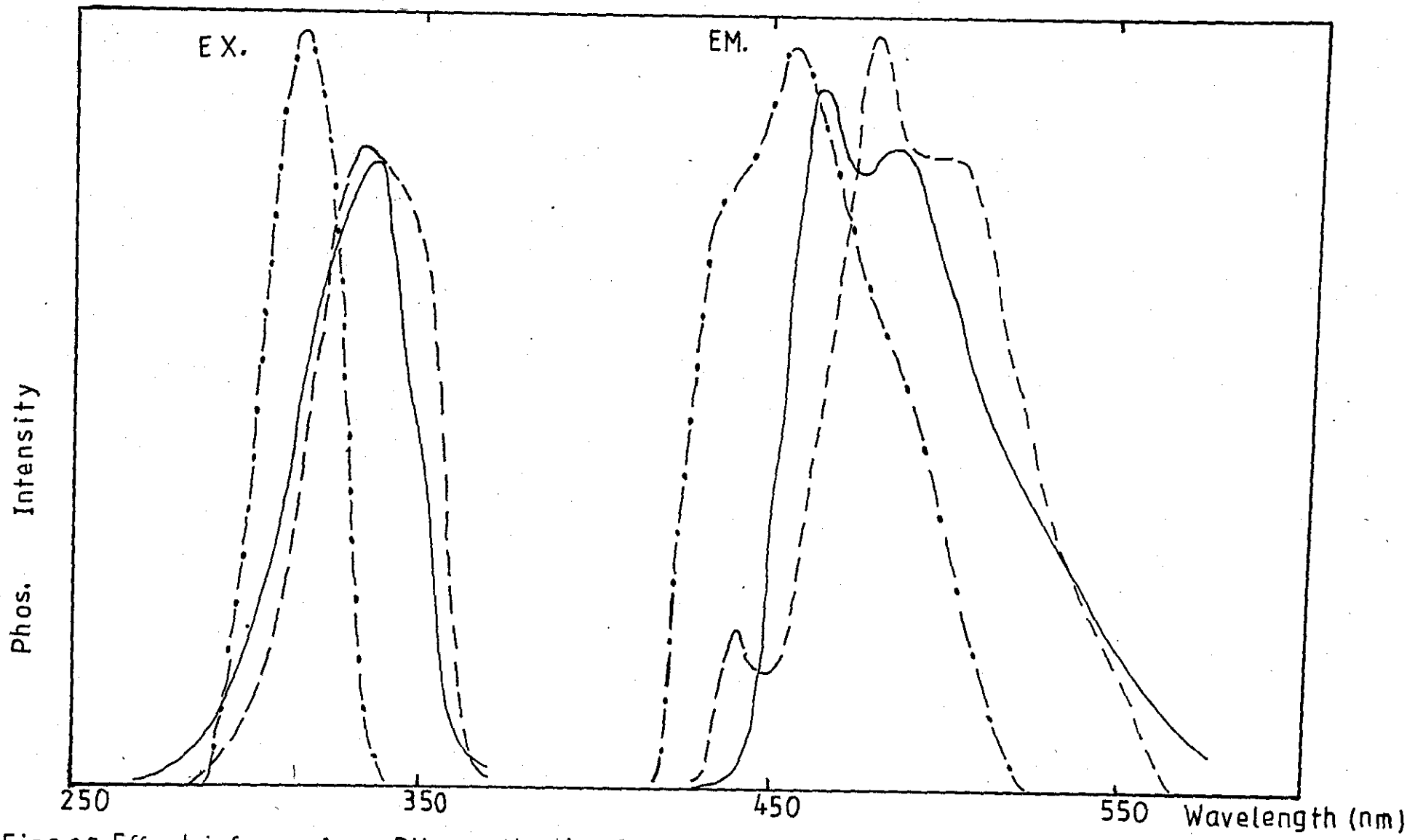



Fig.3.2.5. Effect of varying PH on the Luminscence spectra of 6-Mercaptopurine, in neutral ethanol (_____), in 0.1M ethanolic HCL (_ _ _ _), & in 0.1M ethanolic NaOH (-.-.-)

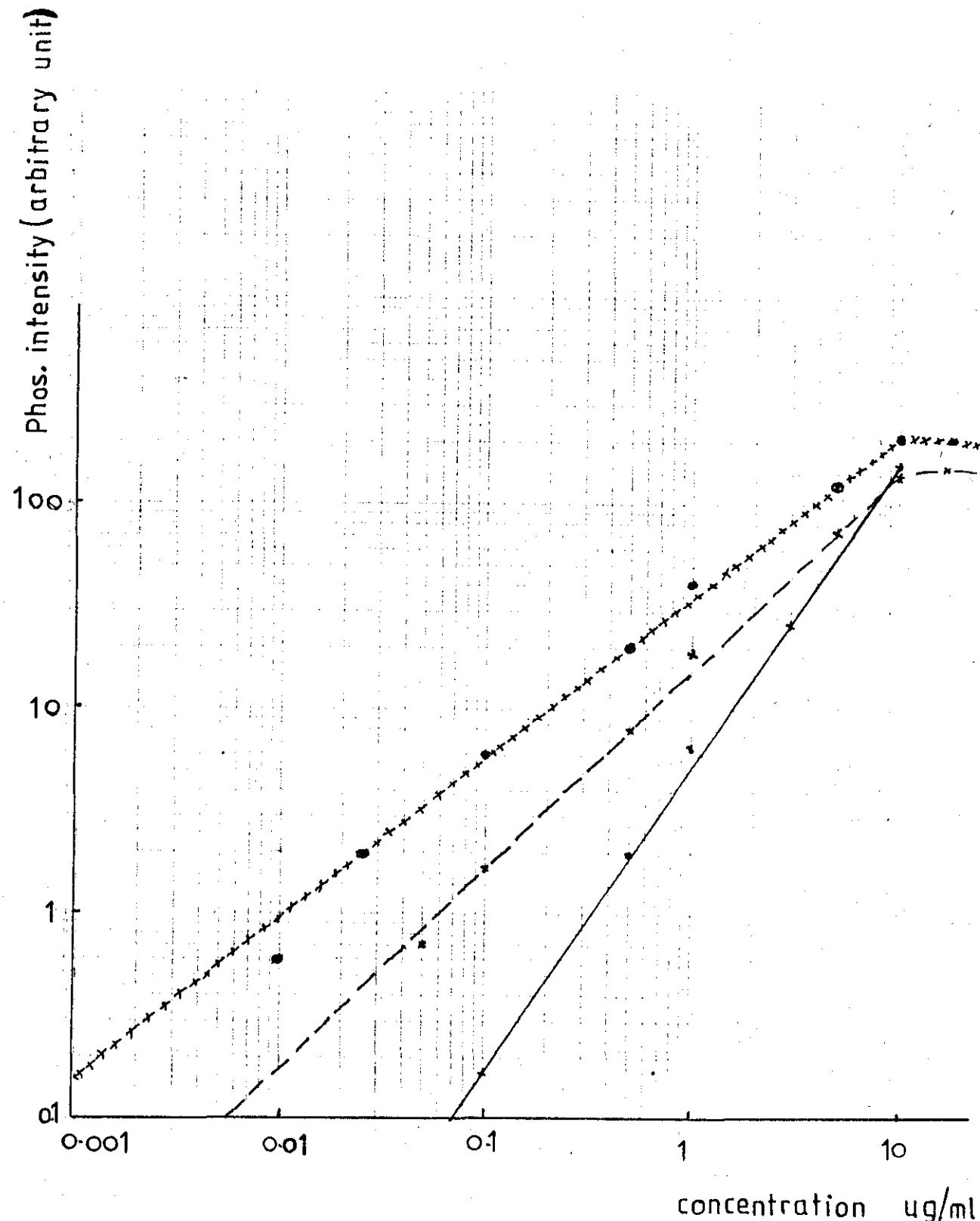



Fig.3.2.6. Calibration curves of 6-mercaptopurine (xxxxxx), 6-mercaptopurine_Riboside (_ _ _ _ _) & 6-mercapto-guanosine (_____). Intensity measured at the emission maximum wavelength of each compound

using the cold ethanol precipitation method was almost 100% : Fig. 3.2.7. shows typical analytical curves for 6-MeMP. Fig. 3.2.8. shows a typical t.l.c. scan for 6-methyl-mercaptopurine at room temperature and 77K, using excitation and emission wavelengths of 296 and 443nm respectively. These detection limits were at least an order of magnitude better than could be obtained by usual observation on the t.l.c. plates. Three of the compounds were also studied using silica gel h.p.t.l.c. plates, and the limits of detection achieved were similar to those obtained using cellulose thin layers. In attempts to enhance the phosphorescence signals still further ethanolic solutions of potassium iodide (1% w/v), lead tetraacetate (10% w/v) and thallium acetate (10% w/v) were investigated as spray reagents: none of these compounds produced a "heavy-atom" enhancement on cellulose or silica gel thin layers and in most cases a quenching of the phosphorescence was observed.

Several mixtures of mercaptopurines were studied in order to test the precision and selectivity of the combined t.l.c. phosphorimetry method: all these measurements were made at 77K using cellulose thin layers. Figure 3.2.9. shows the separation of 4 compounds on a cellulose t.l.c. plate: again, the results obtained were the same whether the compounds were dissolved in ethanol or derived from spiked plasma samples.

The coefficient of variation when the same t.l.c. plate was scanned repeatedly for 30 minutes was found to be 8%.

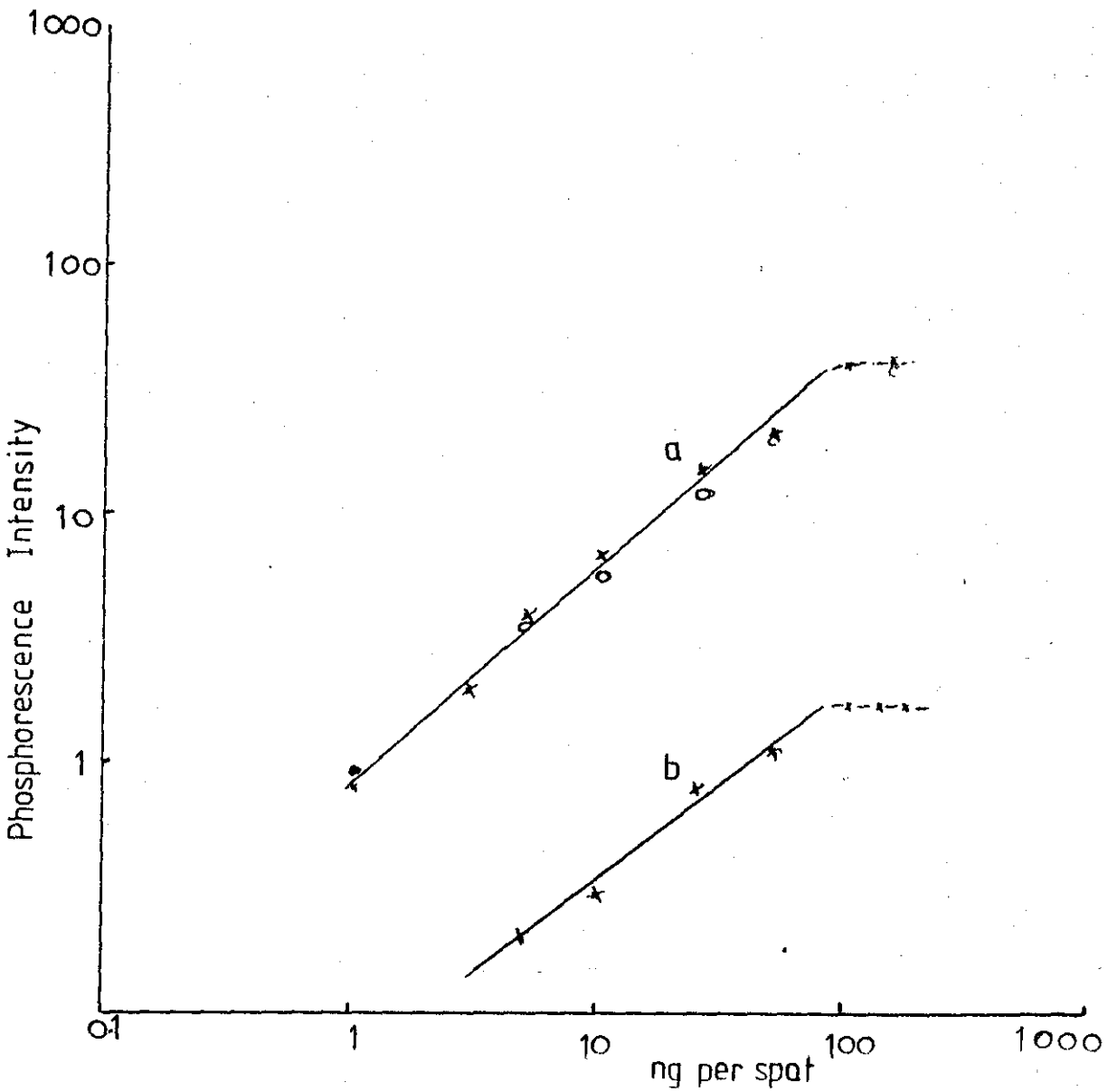



Fig. 3.2.7. Analytical growth curves for the determination of 6-MeMP at (a) 77K & (b) RT. . At 77K the curve was determined using pure solutions of the drug (×), and extracts of spiked blood plasma (○). $\lambda_{ex}$ = 286 nm & $\lambda_{em}$ = 436 nm

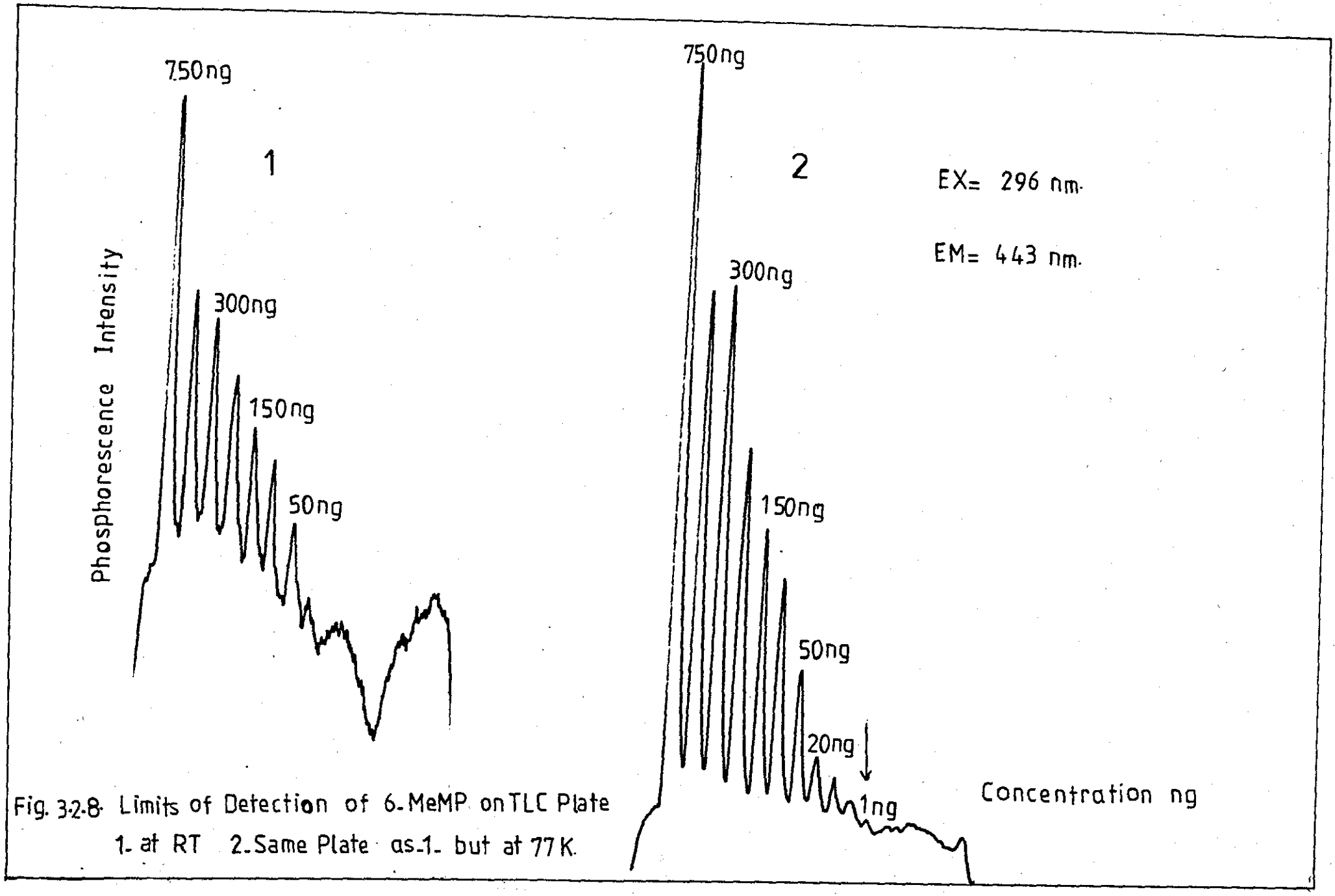



Fig. 3.2.8 Limits of Detection of 6-MeMP on TLC Plate
1. at RT 2. Same Plate as 1. but at 77 K.

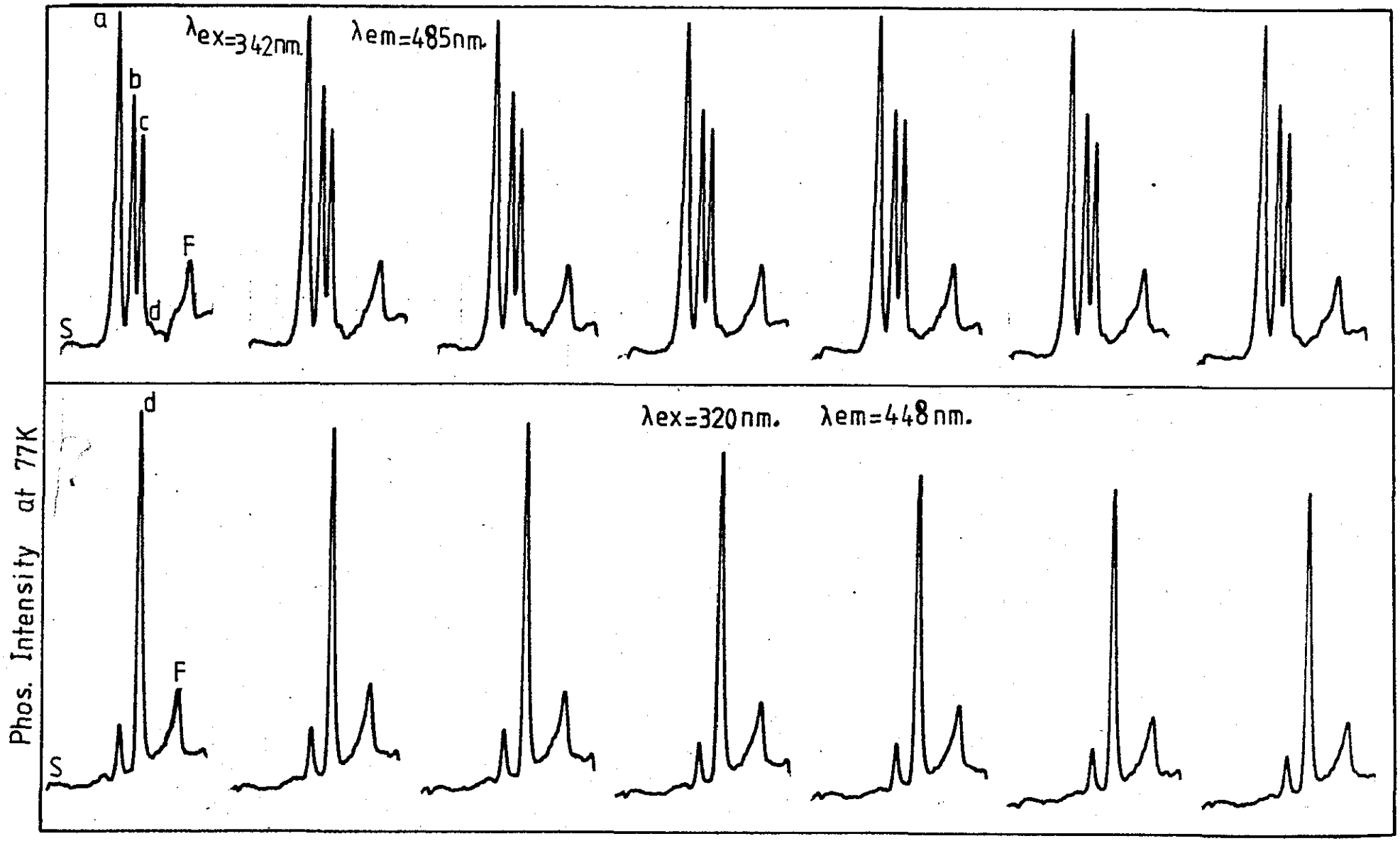



Fig.3.2.9. Repeated scans of separated mixture on TLC Plate (cellulose), solvent system is 0.1M HCL
a= 2-amino-6-MP (Rf=0.32), b= 6-MP (Rf=0.44), c = 6-MG (Rf = 0.51), d = 6-MeMP (Rf=0.63), S = starting point
F = solvent front

The room temperature phosphorescence of all the compounds was studied. Analytically-useful signals were obtained only when cellulose was used as the adsorbent, with the highest intensities generally being obtained when alkaline ethanol was used as the solvent for sample application. Even then, the limits of detection (Table 3.2.5) were at least an order of magnitude inferior to those determined at 77K. Room temperature phosphorescence spectra generally exhibited higher bandwidths and less vibrational fine structure than spectra obtained at 77K: the examples of 6-mercaptopurine (Fig. 3.2.10) and 6MP-2-Deoxyriboside (Fig. 3.2.11) are illustrated.

### 3.2.5. Temperature effect

Fig. (3.2.12) shows a series of spots of 6-mercaptopurine spotted on cellulose thin-layer. As the temperature increases toward room temperature the phosphorescence intensity tends to decrease. A graph of (phosphorescence intensity) versus temperature was plotted (see section 3.4).

### Discussion

The luminescence of purines has been investigated by a number of authors[37,112,113] and it is well established that many purine derivatives exhibit strong phosphorescence signals in the wavelength region 400-550nm. The phosphorescence lifetimes and emission wavelengths indicate that $\pi - \pi^*$ transitions are responsible. Some purines are also fluorescent, although the nature of the lowest singlet state has been a matter of controversy[112]. It has, however, been established that the lowest singlet state of purine itself is $n - \pi^*$ in nature. In alkaline solution purines form anions by loss of a proton at the 9-position:

Table 3.2.5. Limits of Detection of Mercaptopurines in Pure Solution Using Thin Layer Phosphorimetry at Room Temperature.

| Compound | Limits of detection, ng per spot | |
|---|---|---|
| | Neutral ethanol solvent | Alkaline ethanol solvent |
| 1 | N.P. | 25 |
| 2 | 28 | 4 |
| 3 | N.P. | 700 |
| 4 | 40 | 200 |
| 5 | N.P. | 40 |
| 6 | N.P. | 300 |
| 7 | 6 | 5 |
| 8 | 7 | 100 |
| 9 | 20 | 20 |
| 10 | N.P. | 20 |
| 11 | N.P. | 50 |

NP = No phosphorescence detectable on cellulose thin layers.

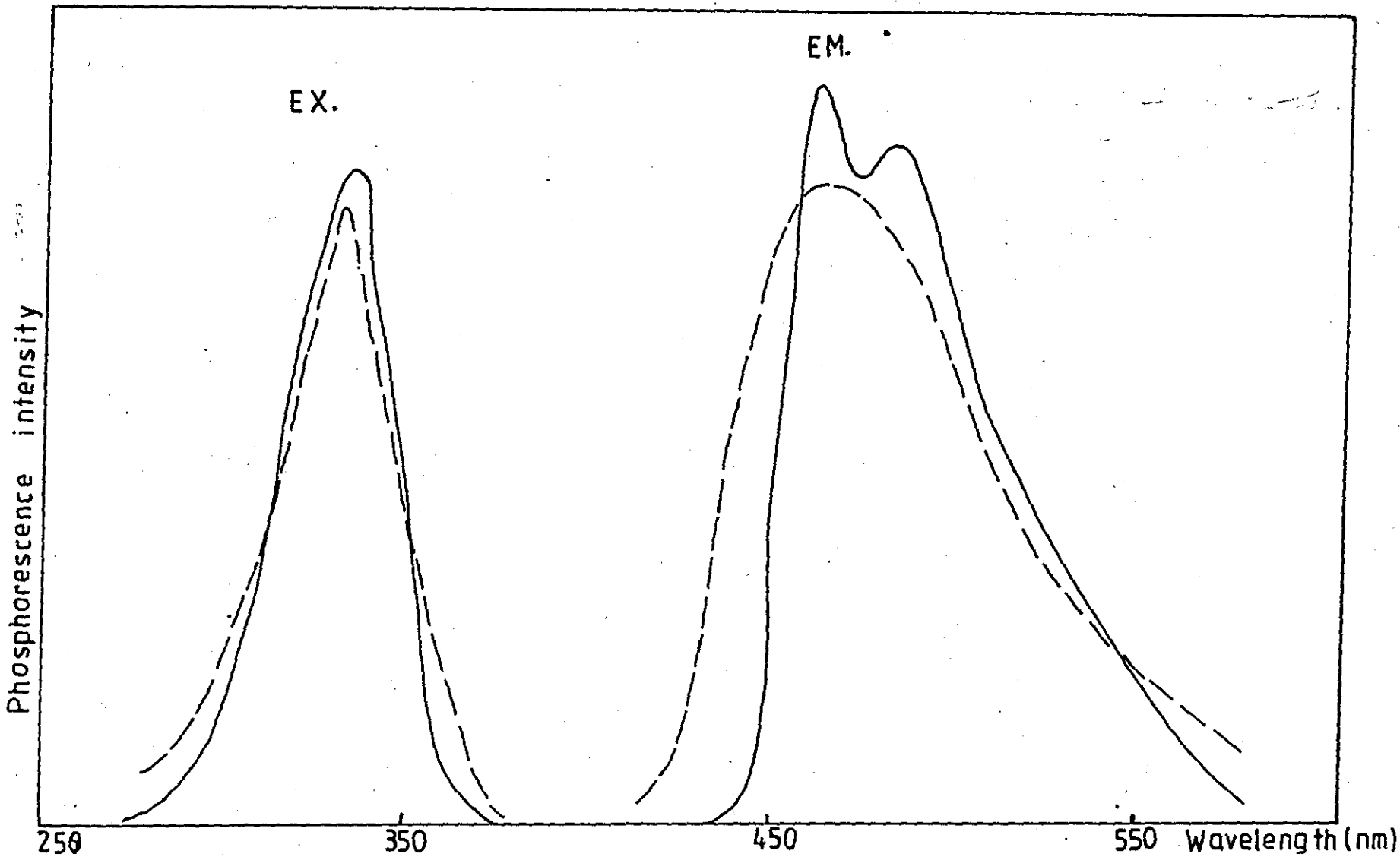



Fig.3-2.10 Phosphorescence spectra of 6-MP in 0.1M ethanolic NaOH solution at 77K (_____) & on TLC plate at R.T. (- - - -)

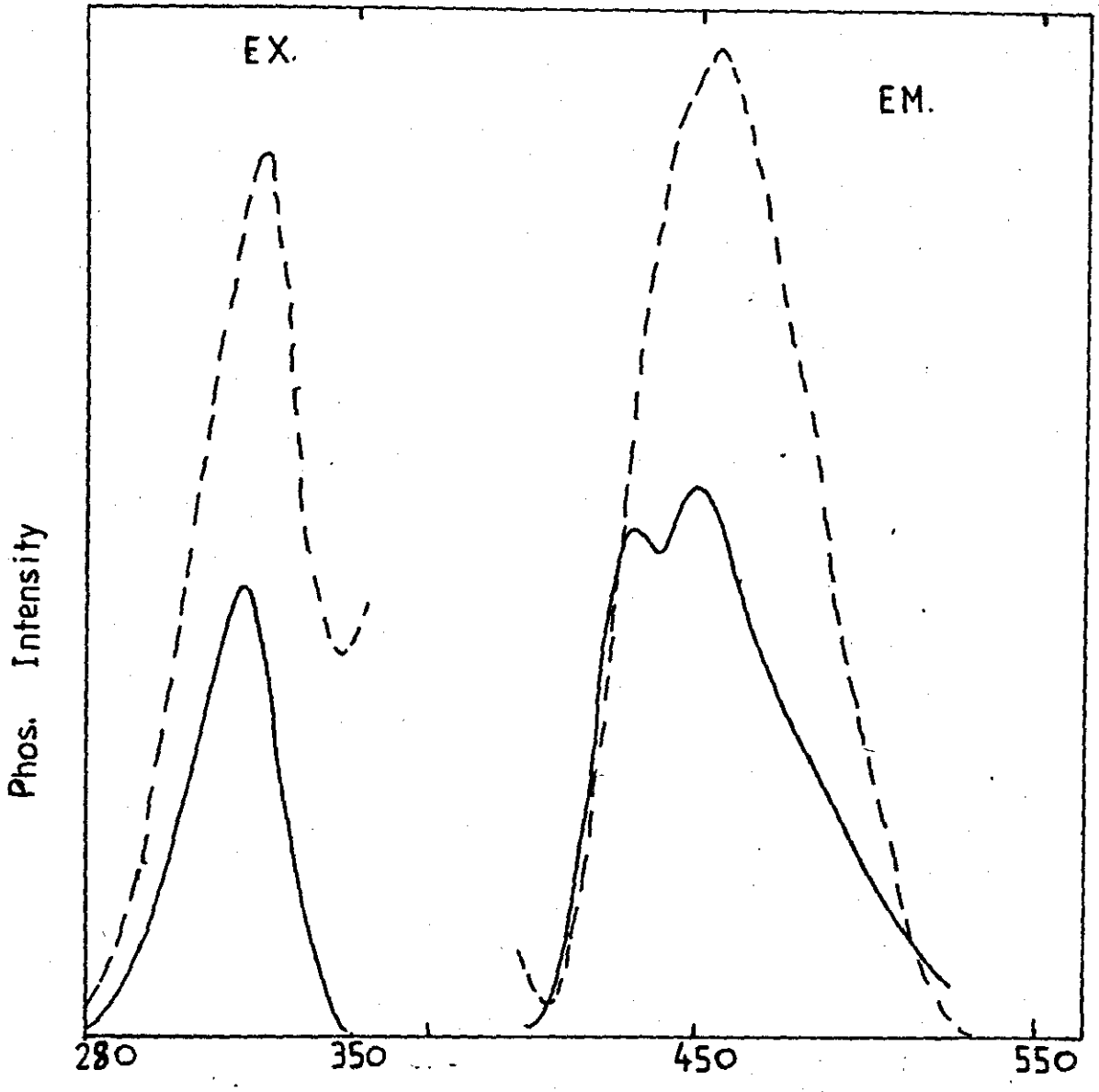



Fig.3.2.11 Phosphorescence Spectra of 6-MP 2'Deoxyriboside on TLC Plate (cellulose)

(-----) at RT (———) at 77 K. concen=90 ug/ml of 0.1 M NaOH Ethanol

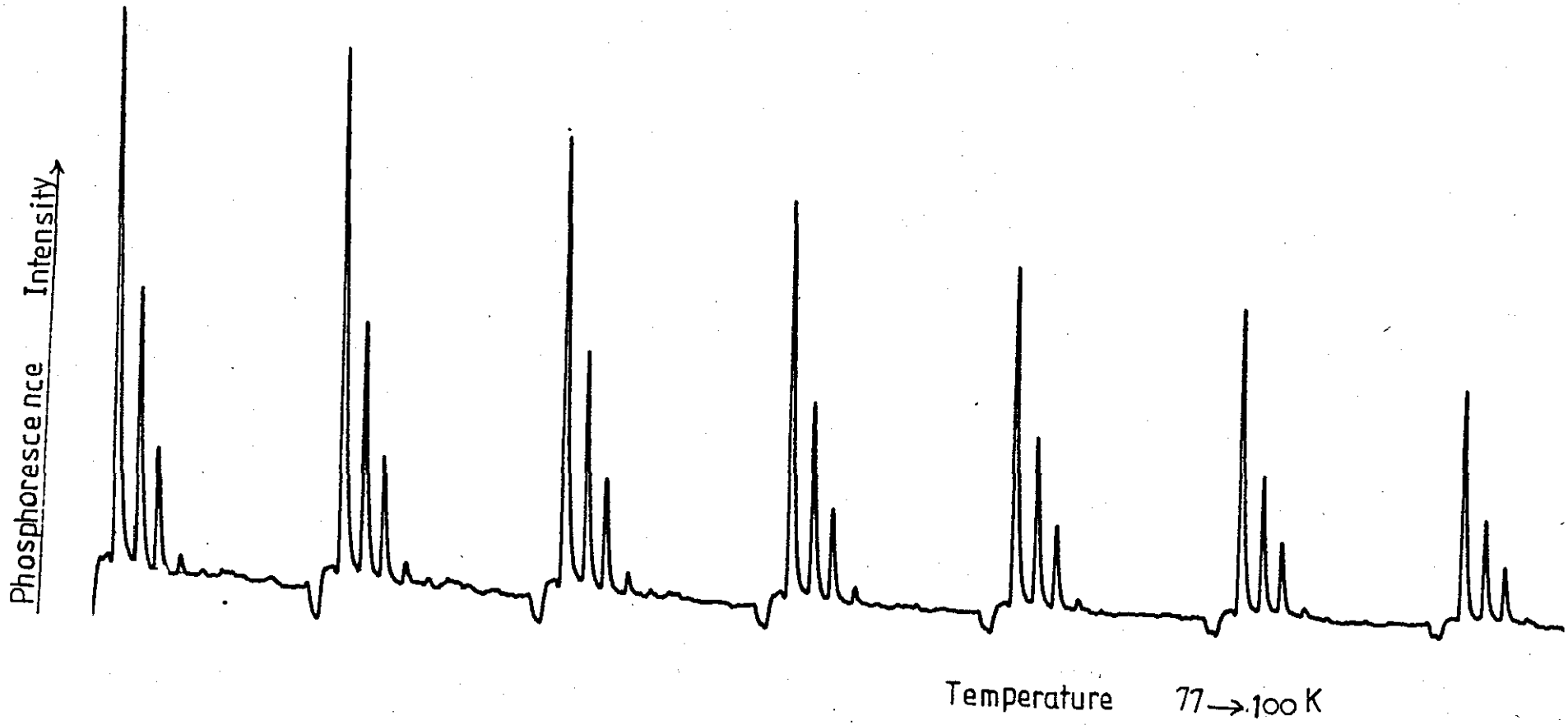
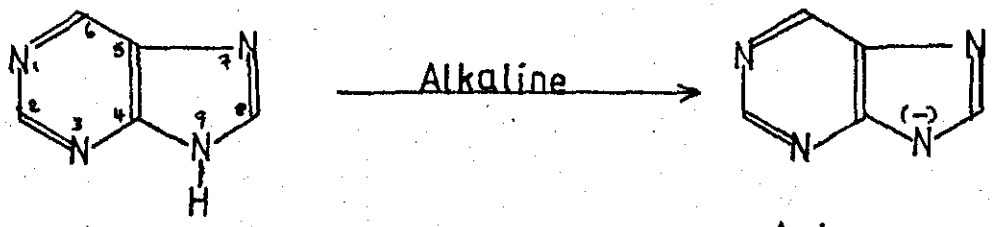



Fig.3.2.12 Phosphorescence Decay of 6-MP on TLC Plate (Cellulose)

EX=342 nm. EM=485nm. different concentration for each peak



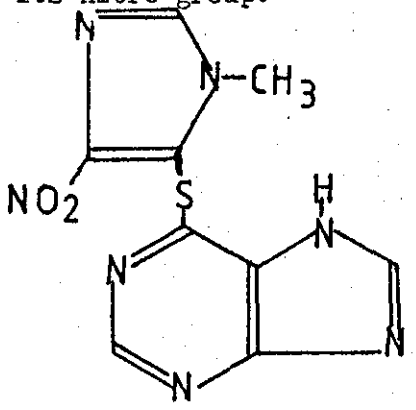

Parent molecule  Anion

the phosphorescence of the purine anion is red-shifted compared with the parent molecule, and fluorescence is also observed (Fig. 3.2.12).

Aaron and Winefordner[37] showed that 6-methylmercaptopurine and its 2-amino derivative could be detected phosphorimetrically at low levels ($< 1 ng.ml^{-1}$), and were more phosphorescent than purines lacking the mercapto group. This may be due to the "heavy atom" effect of the sulphur substituent. Indirect confirmation of this came from the results of Vo-Dinh et al[114] who found that an external heavy-atom perturbant (sodium iodide) did not enhance the phosphorescence of 6-methylmercaptopurine, when it was observed at room temperature adsorbed on filter paper. The limit of detection in these conditions was found to be inferior to that at 77K, but still of analytical value.

In the present work all the compounds studied showed analytically useful phosphorescence, but few were found to be measurably fluorescent, again presumably because of the heavy atom effect on the intersystem crossing rate constant. While the phosphorescence properties of the compounds are generally similar, there are considerable differences in phosphorescence intensity. The position and substitution of the thiol group in the purine system are clearly of importance. Compounds with this group at the 6-position are generally more strongly phosphorescent than 2-substituted purines:6-mercaptopurine and 6-thioxanthine can be detected at lower levels than 2-mercaptopurine and 2-thioxanthine

respectively. 6-methylmercaptopurine and azathioprine both have excitation and phosphorescence maxima blue-shifted compared with the parent compound. Azathioprine(I) is only very weakly phosphorescent, possibly because of its nitro group:



(1)

The presence of a ribose group has, as expected, little effect (compounds 5,9,10), but the phosphate group in 6-mercaptopurine riboside 5-phosphate also has a strong quenching effect. In agreement with earlier studies[114], external heavy-atom effects could not be induced.

It is apparent that thin-layer phosphorimetry at 77K is a powerful method for determining 6-mercaptopurine and its metabolites. In many cases the limits of detection are very low, the drugs may be readily and completely recovered from deproteinised plasma, and the chromatographic and luminescence steps both contribute to the selectivity of the method (Fig. 3.2.9). Furthermore, the t.l.c. step is rapid (especially using h.p.t.l.c. plates) and utilises a simple, non-luminescent eluting solvent. Room temperature phosphorescence (RTP) was, as expected, weaker than the phosphorescence observed at 77K. Nevertheless it may be of some use where higher levels of the mercaptopurines are under study : the thin-layer phosphorimetry method is naturally easier when no liquid nitrogen is required. The finding

that cellulosic thin layers give optimum RTP effects is in agreement with the results of several previous workers.(reviewed in Ref.115).

The present study shows that the combination of thin-layer chromatography and phosphorimetry is a valuable analytical method, especially in cases where several structurally similar phosphorescent compounds (e.g. a drug and its metabolites) are to be analysed. It complements the numerous existing techniques which combine t.l.c. and fluorimetry.

## 3.3. Phenothiazines

### INTRODUCTION

Phenothiazine and its derivatives form the largest and most important groups of major tranquillizers and antihistamines: as tranquillizers they induce a state of calm in a patient in doses which do not produce hypnosis. Chlorpromazine HCl is a central nervous depressant which especially inhibits autonomic nervous activity without appreciable action on the spinal cord.

Phenothiazine and its derivatives have been studied by various analytical techniques including colorimetry[116], gravimetry[117], U.V. absorption[118,119] spectroscopy, I.R. spectroscopy[120], Raman spectroscopy[121], H.P.L.C.[122-124], T.L.C.[125-128], G.L.C.[128,130], square wave polarography[131,132], mass fragmentography[133], and fluorescent derivatisation[134-136]. Blazek[137] has written a review of the analysis of phenothiazine derivatives, describing the physico-chemical properties, methods of identification, purification and determination, biological distribution and stability of 60 phenothiazines. For Chlorpromazine and its metabolites a comparison of methods have been used before 1972 has been given by Usdin[138] and Cimbura[139].

All of the analytical methods mentioned above have some disadvantages in the determination of the normal blood levels of these drugs. For example, T.L.C., U.V., and colorimetric methods are tedious and usually too insensitive to permit measurements of concentrations in serum after administration of therapeutic doses of the drug.

The fluorescent derivatisation method only permits the detection of metabolites containing primary amine or hydroxyl groups. Mass fragmentography is very complicated and expensive.

The determination of phenothiazine and its derivatives by low temperature luminescence is rare, although the technique is not only extremely sensitive and nearly free from interference but also quite rapid and accurate. Winefordner and Tin[27] have described the determination of chlorpromazine HCl in blood plasma using phosphorimetry; they gave only the analytical curve but not the limit of detection. Thiery et al[118] have investigated 13 phenothiazine derivatives by low temperature phosphorescence in solution but did not give the limits of detection and phosphorescence lifetimes. Phillips et al[140] have studied 19 phenothiazine derivatives by phosphorescence at 77K but did not study them by thin-layer phosphorimetry.

The present work provides the limits of detection of phenothiazine derivatives at low temperature, using phosphorimetry combined with thin layer chromatography. The effects of various solvents on the phosphorescence are also described.

## Results and Discussion

### 3.3.1. Purity Check

Thin layer chromatography was used to check the purity of phenothiazine and its derivatives. The phenothiazines were pure when silica gel plates, and n-propanol: 1N ammonia (88:12 v/v) as an eluent were used, (Table 3.3.1). The plate was sprayed with 0.1% sulfuric acid as a detecting reagent. From this method of detection the compounds were highly pure and no further purification was carried out.

Table 3.3.1. $R_f$ values of phenothiazine and its derivative

| Compound | $R_f$ | Colour |
|---|---|---|
| 1 | 0.30 | pink |
| 2 | 0.40 | purple |
| 3 | 0.30 | pink |
| 4 | 0.60 | pink |
| 5 | 0.17 | pink |
| 6 | 0.21 | Light blue |
| 7 | 0.35 | Light blue |

### 3.3.2. Luminescence characteristics at 77K

Table 3.3.2. gives the phosphorescence characteristics (excitation and emission wavelengths and limits of detection) using TLC plates at 77K for the phenothiazines investigated. All the compounds studied have in common the phenothiazine nucleus and differ from one another only in substituents in positions 2 and 10 (Chapter 2). All, with the exception of phenothiazine itself, exhibited broad, single phosphorescence bands at 485-508 nm, and all exhibited double excitation at 250-258 nm and 310-330 nm. Phenothiazine has a pronounced shoulder in the phosphorescence spectrum at 535 nm. This could be due to the substituent at position 10, otherwise a shoulder might be observed in promazine HCl whose substituent at position 2 is the same as for phenothiazine (Fig 3.3.1). In (Fig. 3.3.2), the phosphorescence spectra of chlorpromazine HCl, methotrimeprazine maleate, and perphenazine are shown; the measurements were done on TLC plates.

When the compounds were studied in ethanolic solutions at 77K, the luminescence was almost entirely phosphorescence except for thioridazine HCl, which showed fluorescence at 446 nm (Fig. 3.3.1.); this could be due to the substituent at position 2($-SCH_3$). This group is electron-withdrawing which would suggest that its effect is caused by the lowering of the electron density in the ring, but further studies of compounds with electron-donating substituents would be required to verify that this type of effect is involved.

Table 3.3.2. Luminescence characteristics of phenothiazine at 77K on TLC plate

| Compounds | $\lambda_{ex}$ nm | $\lambda_{em}$ fluo. nm | $\lambda$ em. phosph nm | LOD on cellulose ng/spot | L.O.D. on silica gel (H.P.T.L.C.) ng/spot |
|---|---|---|---|---|---|
| 1. Chlorpromazine HCl | 256, 310 | N.F. | 490 | 1 | 1 |
| 2. Methotrimeprazine amleate | 255, 310 | N.F. | 485 | 5.6 | 3.6. |
| 3. Perphenazine | 250, 312 | N.F. | 495 | 20 | 5 |
| 4. Phenothiazine | 255, 330 | N.F. | 505, 535 | 10 | 5 |
| 5. Promazine HCl | 250, 310 | N.F. | 495 | 5 -10 | 1 - 5 |
| 6. Thiethylperazine maleate | 256, 312 | N.F. | 495 | 24 | 13 |
| 7. Thioridazine HCl | 258, 314 | 446 | 495 | 6 | 4 |

N.F. = Not fluorescent

LOD = limit of detection.

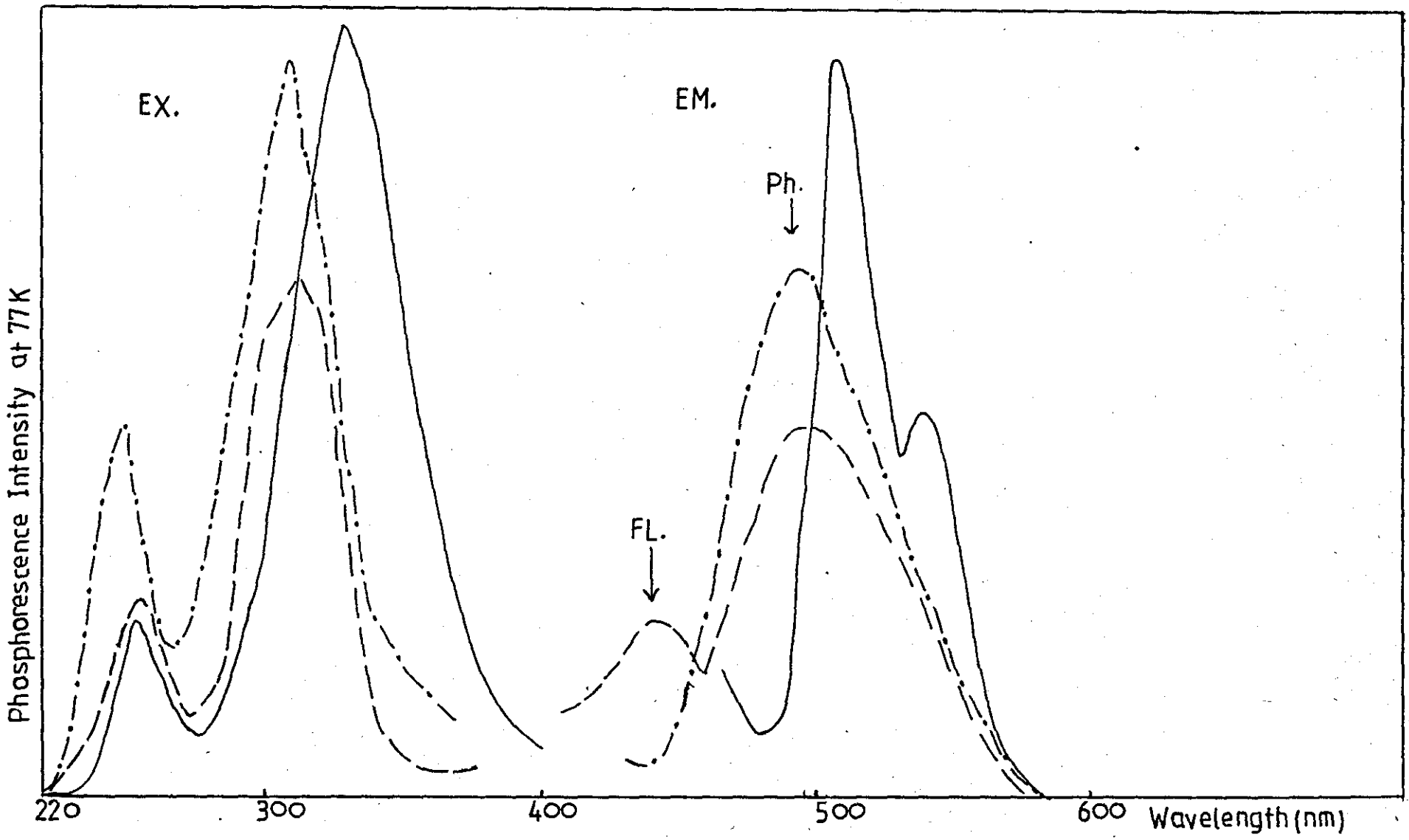



Fig.3.3.1. Phosphorescence spectra of Phenothiazine (——), Promazine HCL (_._._._) on TLC plate (cellulose), and Thioridazine HCL in solution (— — — —)

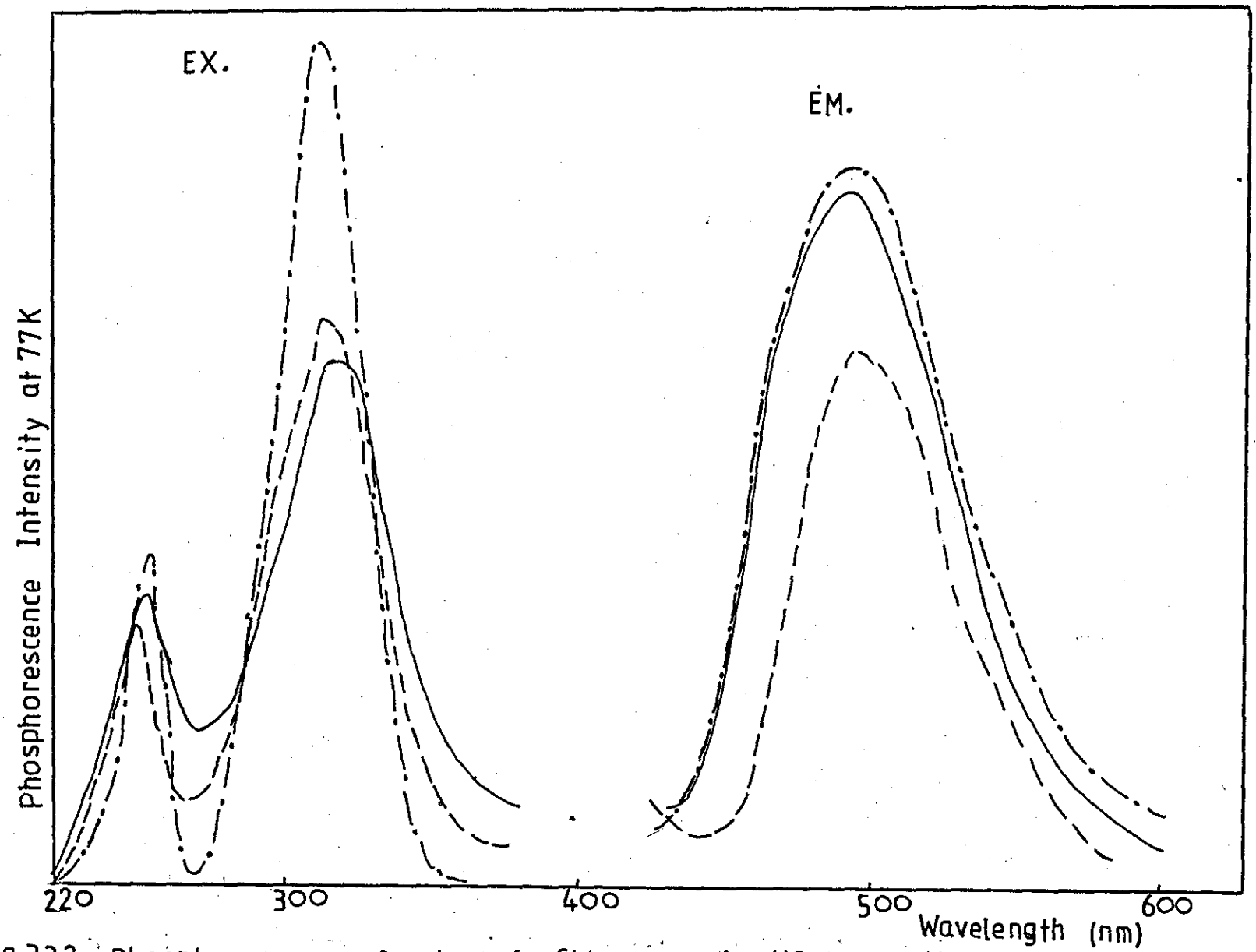



Fig.3.3.2. Phosphorescence spectra of Chlorpromazine HCL(——), Methotrimeprazine maleate(—·—·—), and Perphenazine (– – – –) On TLC plate (cellulose)

### 3.3.3. Thin-layer phosphorimetry

The limits of detection for each compound were determined on T.L.C. plates at 77K, Table 3.3.3. The effects of various solvents on the phosphorescence intensities were studied. A wide range of solvents was used to spray the plate; alcohols in order to study the possible effect of chain length on enhancement, and heavy atom reagents such as ethanol/potassium iodide, lead tetracetate and thallous acetate, to ascertain whether a useful external heavy atom effect could be observed.

Table 3.3.3. gives the limits of detection for chlorpromazine HCl, methotrimeprazine maleate, and thiethylperazine maleate on cellulose layers and high performance thin layer chromatography (H.P.T.L.C. silica gel) layers at 77K. One nanogram quantities of chloropromazine HCl were detected on both cellulose and H.P.T.L.C. layers when the plates were sprayed with ethanol and ethanol/potassium iodide solvents (Figs. 3.3.3 and 3.3.4). Before spraying the plates a very small phosphorescence response was obtained. A series of plates was prepared with the solutions of the same concentrations applied to each, and then sprayed with the solvent under investigation (Fig. 3.3.3 and 3.3.4). It appears from the results that the use of lead tetraacetate and thallous acetate gave poor limits of detection on both layers (cellulose and H.P.T.L.C.). Therefore the best spray reagent solvent for the determination of phenothiazines is either ethanol or ethanol/potassium iodide. As ethanol is one of the best enhancing agents for this particular group of compounds, it is relatively non-toxic and can be obtained with a very low phosphorescence

Table 3.3.3. Limits of detection of phenothiazine derivatives on cellulose and silica gel HPTLC layers at 77K.

| Solvent | Chloropromazine HCl (ng/spot) | | Methotrimeprazine maleate (ng/spot) | | Thiethylperazine maleate (ng/spot) | |
|---|---|---|---|---|---|---|
| | on cellulose | on HPTLC) silica gel | on cellulose | on(HPTLC) silica gel | on cellulose | on (HPTLC) silica gel |
| 1. Ethanol | 1 | 1 | 5.6 | 3.6 | 24 | 13 |
| 2. n-propanol | 1.2 | 1.2 | 15 | 3.8 | 16 | 2 |
| 3. Lead tetraacetate 1% in methanol | 12 | 1.7 | 6.5 | 3.6 | 19 | 1 |
| 4. Thallous acetate 1% in methanol | 25 | 3.5 | 7 | 5 | 60 | 10 |
| 5. Ethanol/potassium iodide 1% | 1.5 | 1 | 5 | 5 | 36 | 13 |
| 6. hexane | 5 | 1 | 5 | 5 | 70 | 10 |

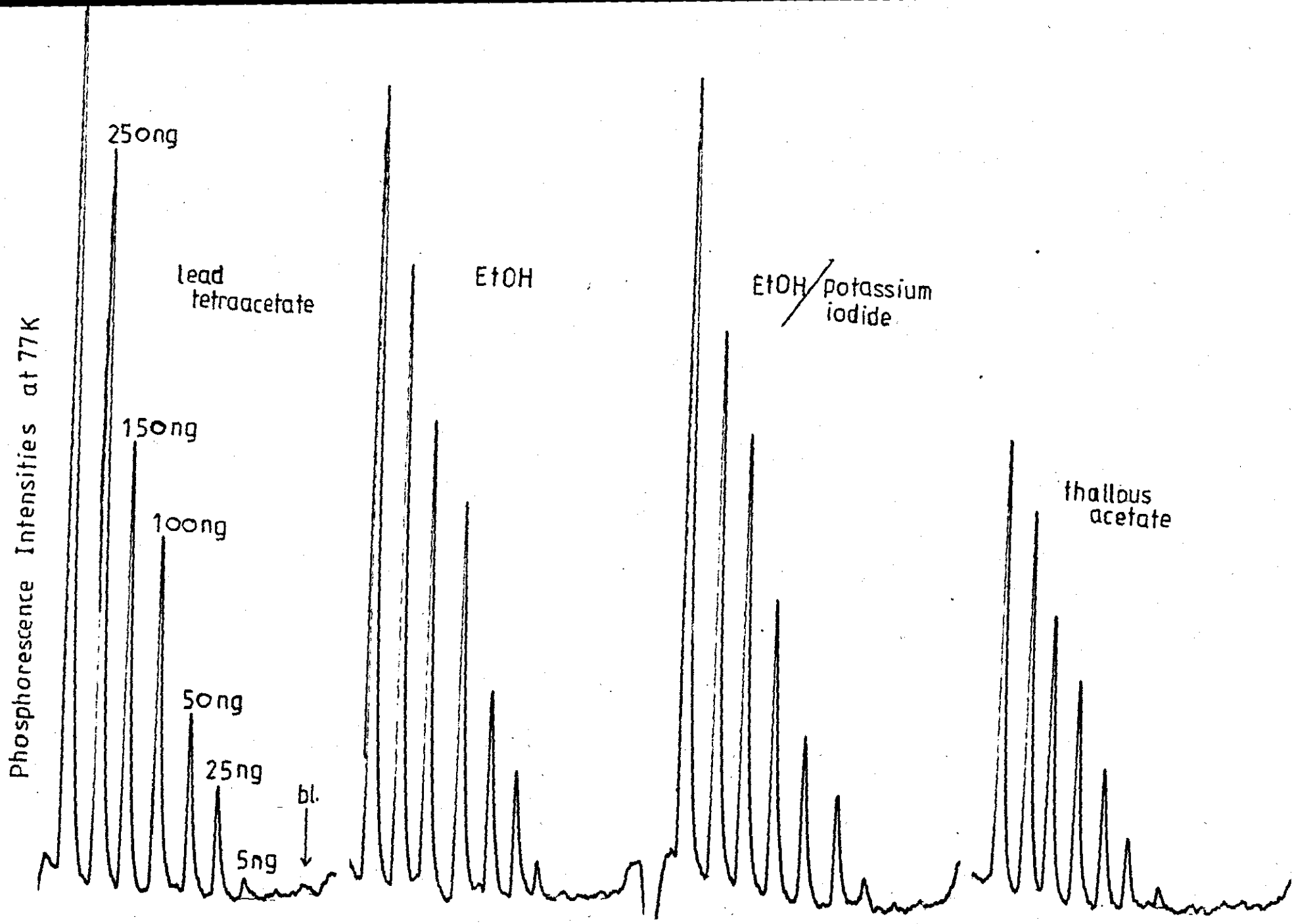



Fig 333. Limits of detection of Chlorpromazine HCL on HPTLC plate sprayed with various solvents
$\lambda ex = 310 nm$. $\lambda em = 490 nm$.

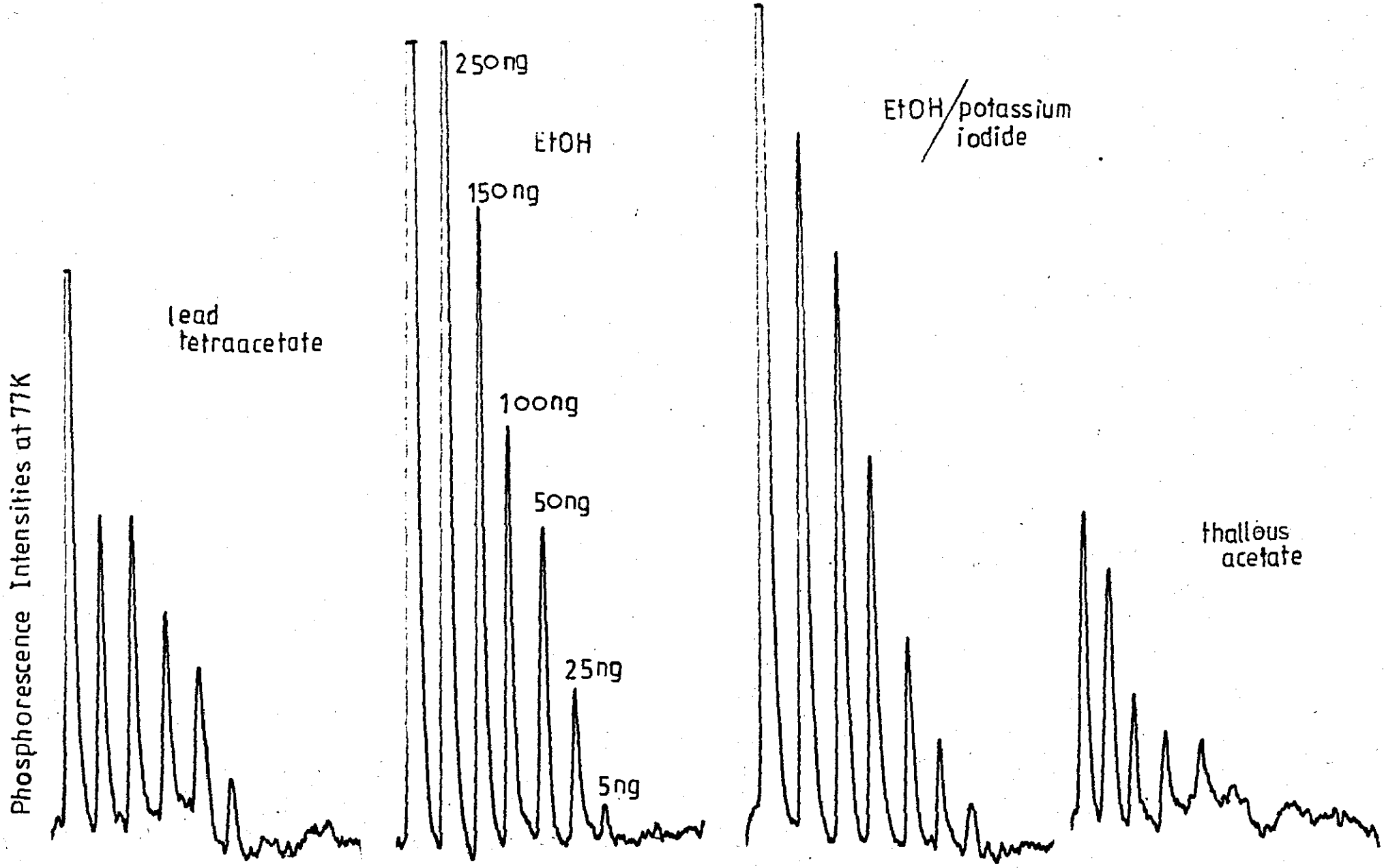



Fig.33.4. Limits of detection of Chlorpromazine HCL on cellulose layers sprayed with various solvents

background, it was decided to use if for the determination of the limits of detection of other compounds.

The effect of different adsorbent layers on the phosphorescence signals of Chlorpromazine HCl, methotrimeprazine maleate, and thiethylperazine maleate, treated with different solvents is shown in Table 3.3.3. From the results obtained the enhancement of phosphorescence seems to depend on the structure of the compound, (related molecules often having similar enhancement factors) the chromotagraphic stationary phase, and the sprayed solvent.

### 3.3.4. Analytical Calibration Curves

Fig. 3.3.5. shows the calibration curves of perphenazine, promazine HCl, and Thioridazine HCl on cellulose thin layers sprayed with ethanol. Linear relationships were observed between phosphorescence intensity and concentration.

### 3.3.5. Separation of mixtures of phenothiazines

A mixture of five phenothiazines was chromatogrammed as in the purity check (see Section 3.3.1). Fig. 3.3.6. shows the phosphorograms of the separated phenothiazine. Using an excitation wavelength of 330nm and emission wavelength of 504nm, three well separated peaks and two shoulders could be distinguished. From the $R_f$ values previously calculated these peaks could be assigned to a) Thiethylperazine maleate, b) Chlorpromazine HCl, c) Thioridazine HCl, d) Methotrimeprazine maleate and e) phenothiazine.

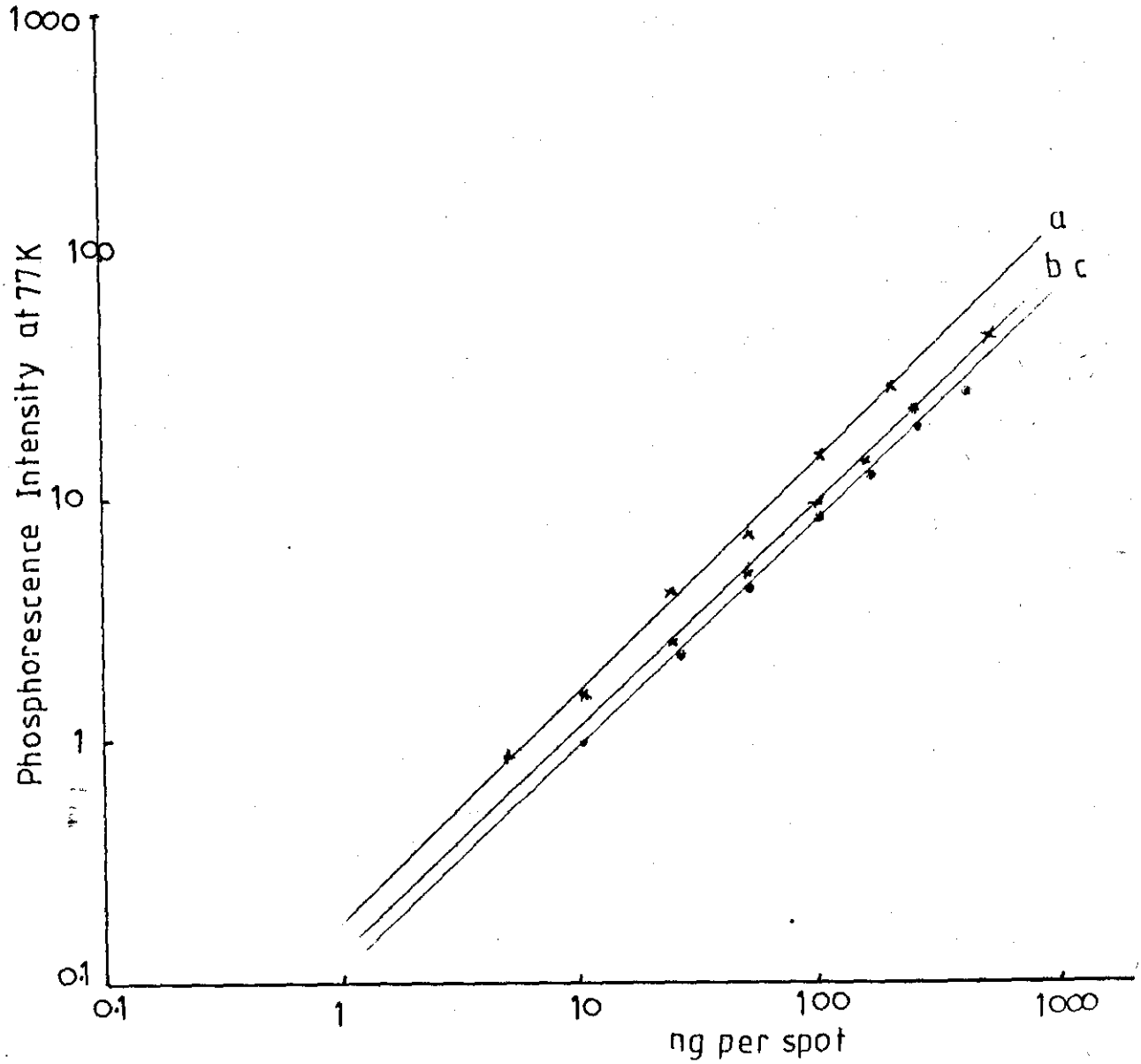



Fig. 3.3.5. Calibration curves of a) Thioridazine HCL b) Promazine HCL & c) perphenazine on T.L.C. plate. Intensity measured at the emission maximum wavelength of each compound

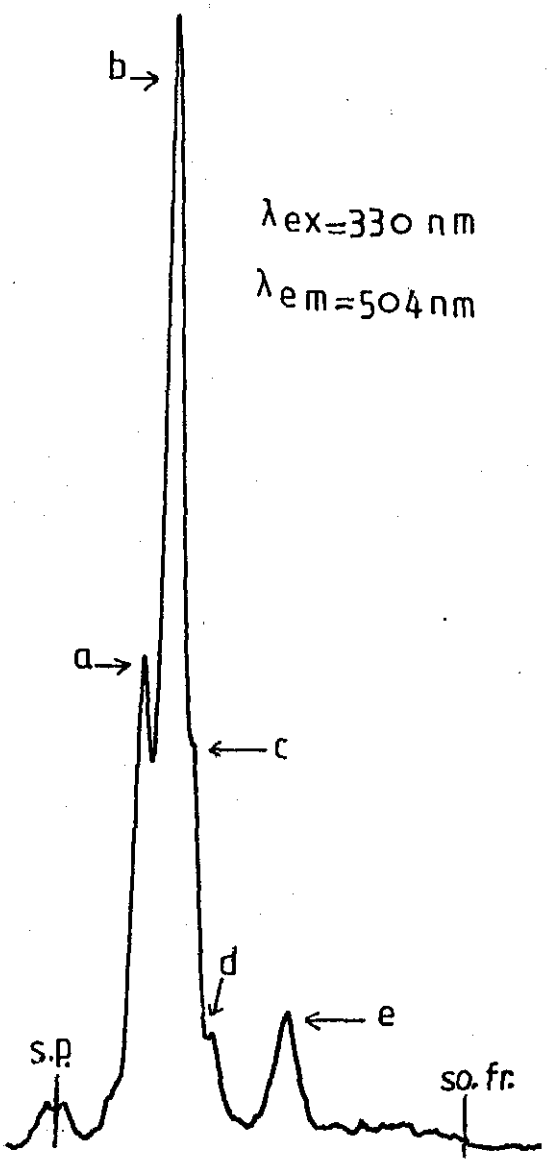



Fig. 3.3.6. Chromatographic separation of mixture of phenothiazines on T.L.C. plate (silica gel)

a = Thiethylperazine maleate

b = Chlorpromazine HCl

c = Thioridazine HCl

d = Methotrimeprazine maleate

e = Phenothiazine

s.p. = starting point

so.fr = solvent front

### 3.3.6. The temperature effects

A graph of phosphorescence intensity against temperature for promazine HCl is shown in Fig. 3.3.7. The figure shows a decrease in the phosphorescence intensity as the temperature increased. The decrease was dramatic (25%) from 77K to 80K. As the temperature rose the phosphorescence intensity rapidly fell and at 208K it was 97% less than at 77K.

The effect is most probably due to an increase in the thermal motion of molecules at higher temperatures. The increased motion favours the probability of intermolecular collisions and subsequent energy loss. Therefore the decrease in phosphorescence intensity passing from a rigid medium at 77K to fluid solution at room temperature is critically dependent on the viscosity of the medium. So the increase in phosphorescence efficiency on lowering the temperature is probably due mainly to the resulting increase in solvent viscosity. The viscosity of the medium is important since it determines the rate of diffusion controlled reactions according to the equation.

$$K_c = 8RT/3000\eta$$

where

$K_c$ = diffusion controlled bimolecular rate constant

$\eta$ = viscosity of the solvent in poises

R = gas constant

T = absolute temperature.

Hence, the phosphorescence of organic molecules is measured in rigid glasses at 77K.

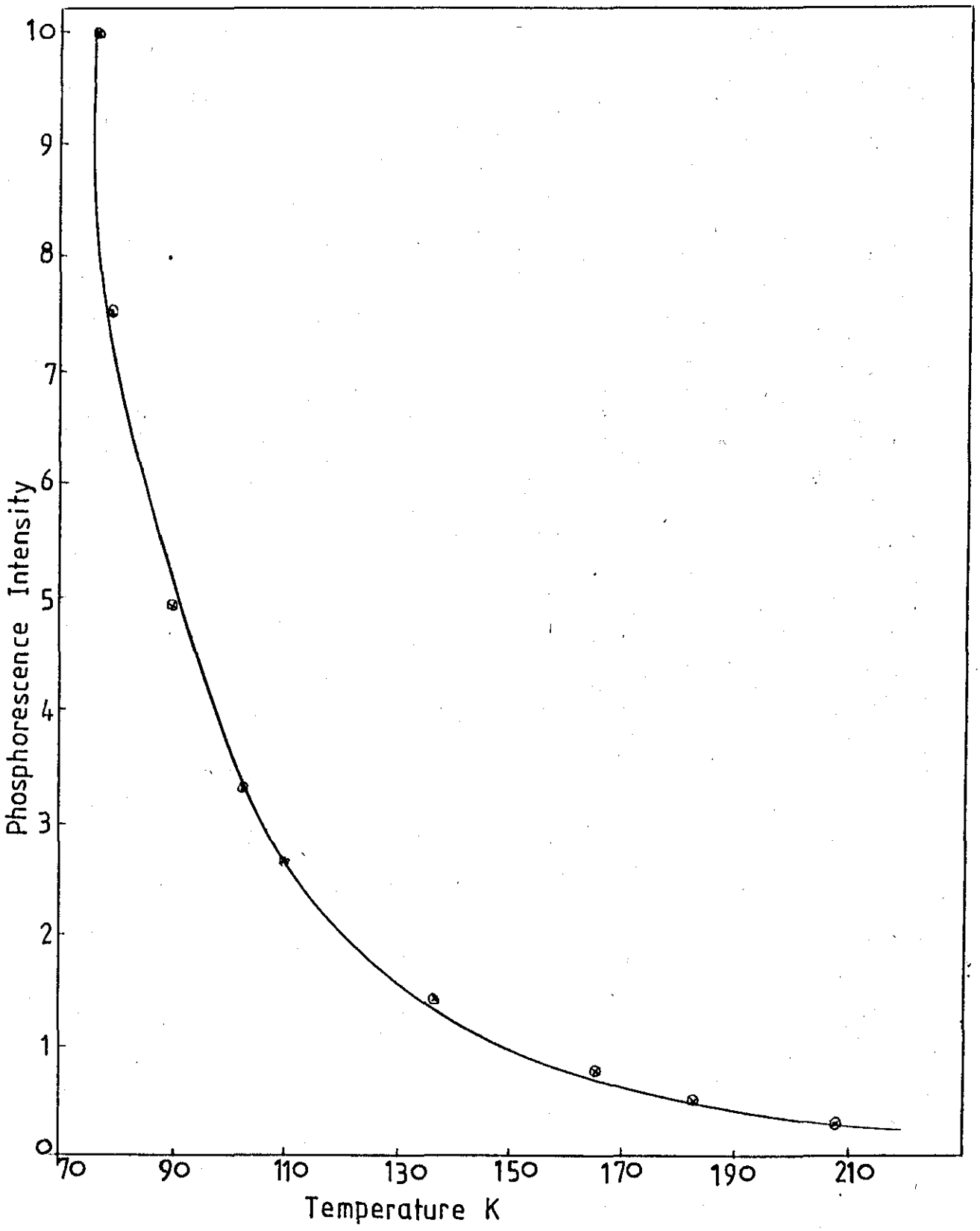



Fig. 3.3.7. Plot shows the decrease of phosphorescence intensity with increasing temperature of Promazine HCL on TLC plate

This work was done to see whether a less vigorous coolant could be used to determine the phosphorescence intensity (e.g. solid carbon dioxide). More work is needed on the effect of temperature on the luminescence intensity e.g. comparison of t.l.c. plates and solutions.

This work indicates that it is possible to detect nanogram quantities of phenothiazines at levels found in the blood. The large number of metabolites found <u>in vivo</u> is a major problem. Turner and Turano[141] identified 35 chlorpromazine metabolites and found another 42 which could not be identified. The phosphorescence coupled with thin layer chromatography[14] to separate and identify metabolites might help to solve the problem.

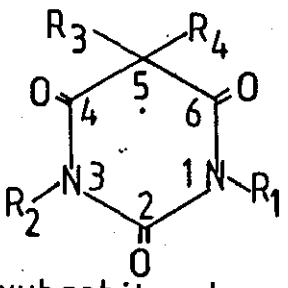
## 3.4. Thiobarbiturates

### INTRODUCTION

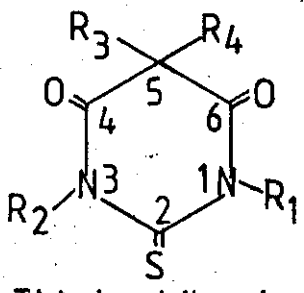
The history of the barbiturates dates from 1903 when Fischer and Von Mering[142] introduced barbitone, or diethylbarbituric acid. This was followed by phenobarbitone and allobarbitone in 1912, and since that date intensive research has resulted in the production of a very large number of barbiturates, some of which have become established in clinical use. In the 1920's and early 1930's intravenous amylobarbitone and pentobarbitone enjoyed a moderate vogue, but their actions by this route were often unpredictable, and their use in this manner was not satisfactory. Two years later Lundy[143] reported the first clinical trials of thiopentone sodium, the first thiobarbiturate to be used clinically (though thiobarbiturates had been synthesised as early as 1911). Since that time numerous thiobarbiturates have been prepared, but only three others - thialbarbitone, thiamylal and buthalitone - have been found to be of clinical use, and thiopentone is still the most widely used drug in this field. The sodium salt of thiopentone is by far the most popular intravenous anaesthetic used to date. It has other uses including an anticonvulsant for eclampsia[144], as a narcotic in psychiatry[145], alcoholism[146] and hysteria, and has been used in criminology for the detection of malingering.

Thiobarbiturates are only slowly metabolised in the body at a rate of about 15% per hour in man[147]. The replacement of the oxygen atom in the 2-position by a less electronegative sulphur atom shortens

the duration of action.



Oxybarbiturate



Thiobarbiturate

The breakdown of thiobarbiturate takes place mainly in the liver. They are cumulative drugs to varying extents, thiamylal[148] being the least cumulative of the thiobarbiturates used clinically.

Analysis of thiobarbiturates.

The oxybarbiturates have been extensively investigated by most analytical techniques, but the thiobarbiturates have received considerably less attention.

Qualitative analysis of thiobarbiturate has been carried out by paper or thin layer chromatography (Cochin[149], Frahm[150], Drost[151], Uhlman[152] and Zecuw[153]; Morvay[154] has qualitatively analysed thiobarbiturates by paper electrophoresis. Some thiobarbiturates have been synthesised by Rohoman[80] and studied by U.V., I.R., NMR and MS. Bruce et al[155] and Repetto[156] have studied thiopentone by gas-liquid chromatography; this method is good for both identification and estimation. A comparison of u.v. absorption spectrophotometry, gas-liquid chromatography and gel-filtration has been made by Repetto and Martinez[156], for the identification of thiobarbiturates in blood. Dusinsky and Faith[157] have studied thiopentone by

oscillopolarography and Smyth[158] has also used polarography. A study using N.M.R. has been done by Avdovich et al[159] for the identification of thiobarbiturates.

Numerous reports have appeared describing the fluorescence analysis of thiobarbiturates extracted from biological fluids and pharmaceutical preparations, although the low temperature luminescence of thiobarbiturates has received little attention[160,162]. Hewitt[78], King and Gifford[163] studied the fluorescence and phosphorescence of some thiobarbiturates. A study was made by Gifford et al[51] on five thiobarbiturates : luminescence structure relationships were investigated.

In the present work nine thiobarbiturates including the parent compound 2-T.B.A. were investigated. Some of the others were 1,3-disubstituted thiobarbiturates.

## 3.4. Thiobaributrates

### Results and Discussion

### 3.4.1. Purity Check

The compounds have been investigated are listed in Table 3 Chapter 2.

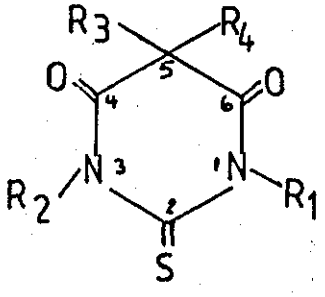
The melting point of each compound was found to be within 2°C of literature values (Table 3.4.1). Attempts were made to test the purity of the compounds by thin-layer chromatography using cellulose layers as stationary phase and n-propanol-water-ammonia (sp.gr. 0.880) (7:1:2 v/v) followed by spraying with mercurous nitrate or potassium permanganate. Different solvent systems were tried including solvent system 5: n-propanol-water-ammonia (.880) (7/1/2 v/v); system 6; ammonia (.880):dioxane:benzene (5:20:75 v/v); and system 7: acetone-chloroform (1:9 v/v).

Solvent systems 6 and 7 were suitable for compounds 8 and 9, but the rest of the compounds did not migrate. Solvent system 5 was suitable for all the compounds but was time-consuming (5-6 hrs to develop 15cm height). The detecting reagent was mercurous nitrate (aqueous saturated solution): it gave dark green spots on a white background. If the plate was kept in the dark, spots produced by compounds 4,8 and 9 retained their colour but the rest faded. When potassium permanganate (1% aqueous) was used yellow spots on a pink background were obtained.

Table 3.4.1. shows the $R_f$ values for each compound.

## TABLE 3.4.1.

## Structures, Melting points, & $R_f$ values of Thiobarbiturates




| Compound | No. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Melting points | $R_f$ |
|---|---|---|---|---|---|---|---|
| 2-Thiobarbituric acid (2-T.B.A.) | 1 | H | H | H | H | 236 (235)* | 0.48 |
| 1,3-dimethyl-2-T.B.A. | 2 | Methyl | Methyl | H | H | 184 (183)* | 0.82 |
| 1-Methyl-2-TBA | 3 | Methyl | H | H | H | 199 (201)* | 0.69 |
| 1,3-dimethyl-5-Phenyl-2-TBA | 4 | Methyl | Methyl | Phenyl | H | 224 (223)* | 0.87 |
| 1,3 dimethyl-5-ethyl-2-T.B.A. | 5 | Methyl | Methyl | Ethyl | H | 105 (107)* | 0.88 |
| 1,3-diethyl-2-TBA | 6 | Ethyl | Ethyl | H | H | 118 (119)* | 0.88 |
| 5-Ethyl-2-TBA | 7 | H | H | Ethyl | H | 189 (188)* | 0.66 |
| Thiopentone sodium | 8 | H | H | Ethyl | 1-Methyl-butyl | 160 (157)* | 0.89 |
| Thiobarbitone sodium (Kemithal) | 9 | H | H | Allyl | Cyclohexenyl | 150 (149)* | 0.88 |

( )* Literature values

### 3.4.2. Absorption spectra

The spectra of the thiobarbiturates studied are summarised in Table 3.4.2. All measurements were made in ethanolic solutions with solute concentrations of 10 $ug.ml^{-1}$ and using 10mm silica cells. The absorption spectra of 5-ethyl-2 T.B.A. and thiopentone sodium in 0.1M HCl in ethanol, neutral ethanol and 0.1M NaOH in ethanol at 25°C are shown in Figs. 3.4.1. and 3.4.2. respectively. From the results obtained, it is seen that the spectra show three absorption maxima in alkaline solution (except that compounds 1 and 8 show two maxima and compound 9 showed one peak), and two peaks in acid solutions.

Solutions were stable during the period of measurements, but when left overnight some changes in the absorption spectra were observed in acid solution (Fig. 3.4.3). both in the position and height of the maximum. The band at 237nm was shifted to 240nm and also the band at 289nm shifted to 290nm. Figure 3.4.4. shows the instability of thiopentone in alkaline solution; the shape of the spectrum is changed completely on standing overnight. Therefore all measurements were done using fresh solutions.

### 3.4.3. Luminescence spectra at Room Temperature

Solutions containing 10-50 $ug.ml^{-1}$ in ethanol (pH range 1-13.5), were investigated for fluorescence at room temperature. It was found that all the thiobarbiturates studied at 20°C were non fluorescent in the three media, 0.1M HCl in ethanol, neutral ethanol and 0.1M NaOH in ethanol. Gifford and King[162] have stated that only 5,5-disubstituted thiobarbituric acids showed significant fluorescence at room temperature in aqueous solution but do not

Table 3.4.2. Absorption spectra of thiobarbiturates in ethanolic solutions.

| No. | Compounds | in 0.1M HCl in ethanol $\lambda$max. nm. | Neutral ethanol $\lambda$ max. nm. | 0.1M NaOH in ethanol $\lambda$ max. nm. |
|---|---|---|---|---|
| 1. | 2 TBA | 237, 260, $309^{p}$ | 233 $288^{p}$ $305^{s}$ | 247, $305^{p}$ |
| 2. | 1,3-dimethyl-2TBA | 237, $280^{p}$, 355 | 240, $275^{p}$ | 240, $267^{p}$, 283 |
| 3. | 1 Methyl 2TBA | 237, $282^{p}$, 360 | 243, $265^{p}$ $282^{s}$ | 237, $282^{p}$, $260^{s}$ |
| 4. | 5 phenyl-1,3-dimethyl 2TBA | 240, $288^{p}$, 360 | 244, $278^{s}$, $306^{p}$ | 243, $278^{s}$, $306^{p}$ |
| 5. | 1,3-dimethyl-5-ethyl 2TBA | 235, $282^{p}$, 368 | 242, $275^{p}$ | 244, $269^{p}$, $283^{s}$ |
| 6. | 1,3 diethyl-2TBA | 238, $287^{p}$, 360 | 242, $272^{p}$ | 263, $285^{p}$ |
| 7. | 5-ethyl-2TBA | 237, $287^{p}$, 360 | 243, $268^{p}$ $292^{s}$ | 235, $263^{s}$, $290^{p}$ |
| 8. | Thiopentone sodium | 238, $289^{p}$, 370 | 238, $289^{p}$ | $255^{s}$, $306^{p}$ |
| 9. | Thiobarbitone sodium | 238, $290^{p}$ | 238, $290^{p}$ | $307^{p}$ |

p = principal peak
s = shoulder

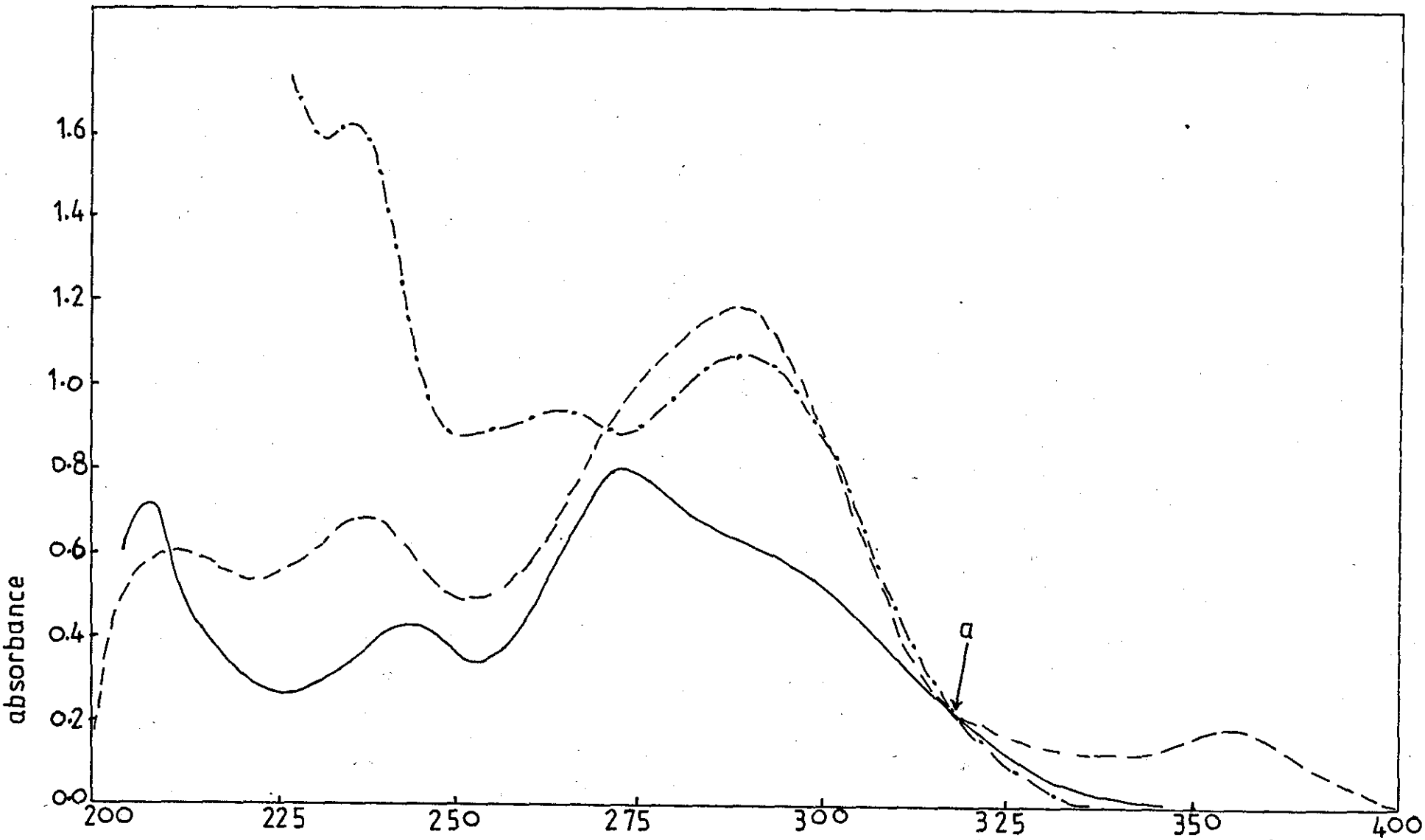



Fig.3.4.1. Absorption spectra of 5-ethyl-2TBA showing variation with PH values

(——) neutral ethanol , (— — —) 0.1M HCL ethanol & (—.—.—.—) 0.1M NaOH ethanol

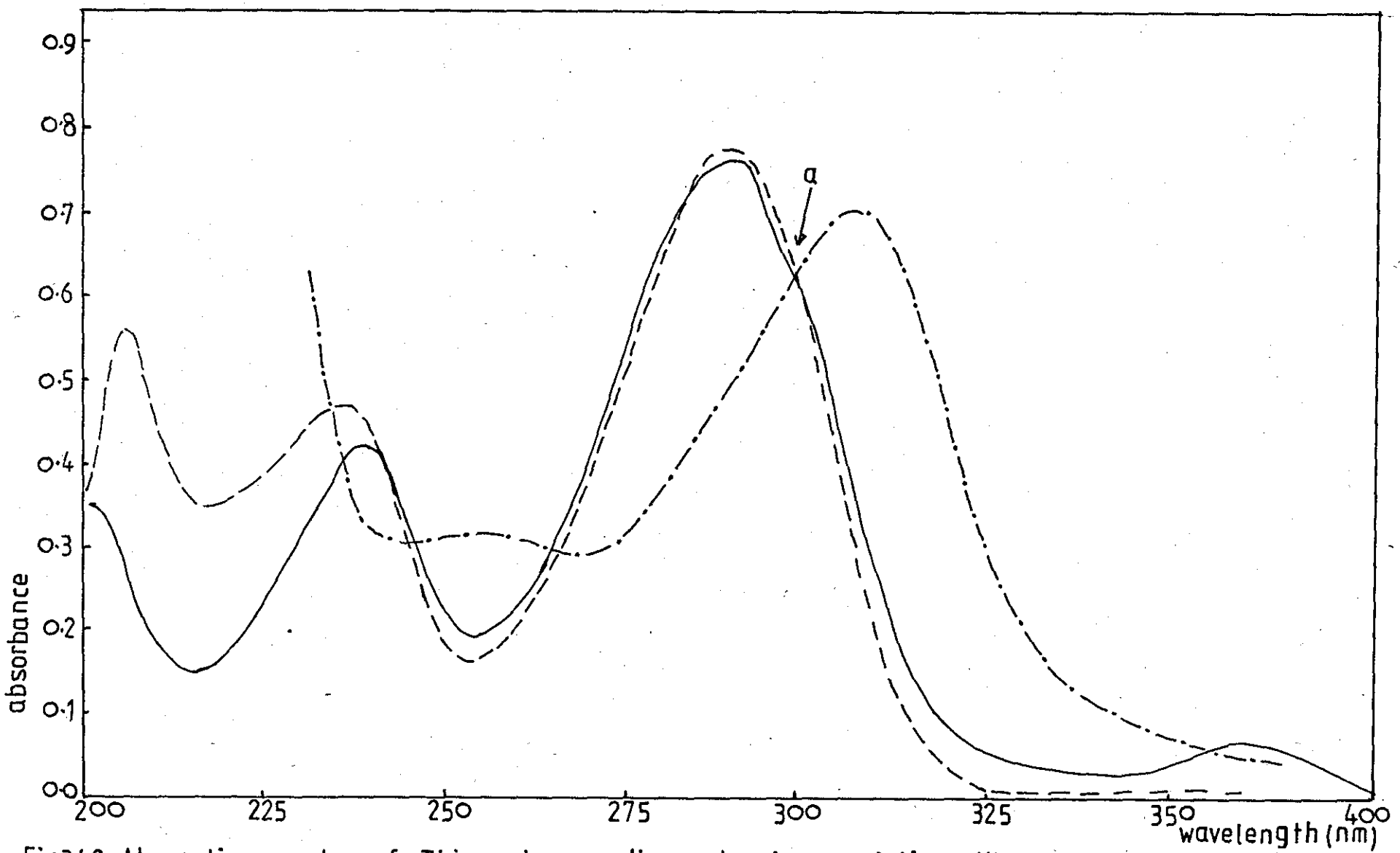



Fig.3.4.2 Absorption spectra of Thiopentone sodium showing variation with PH values

(– – – –) neutral ethanol , (——) 0.1M HCL ethanol & (—.—.—) 0.1M NaOH ethanol

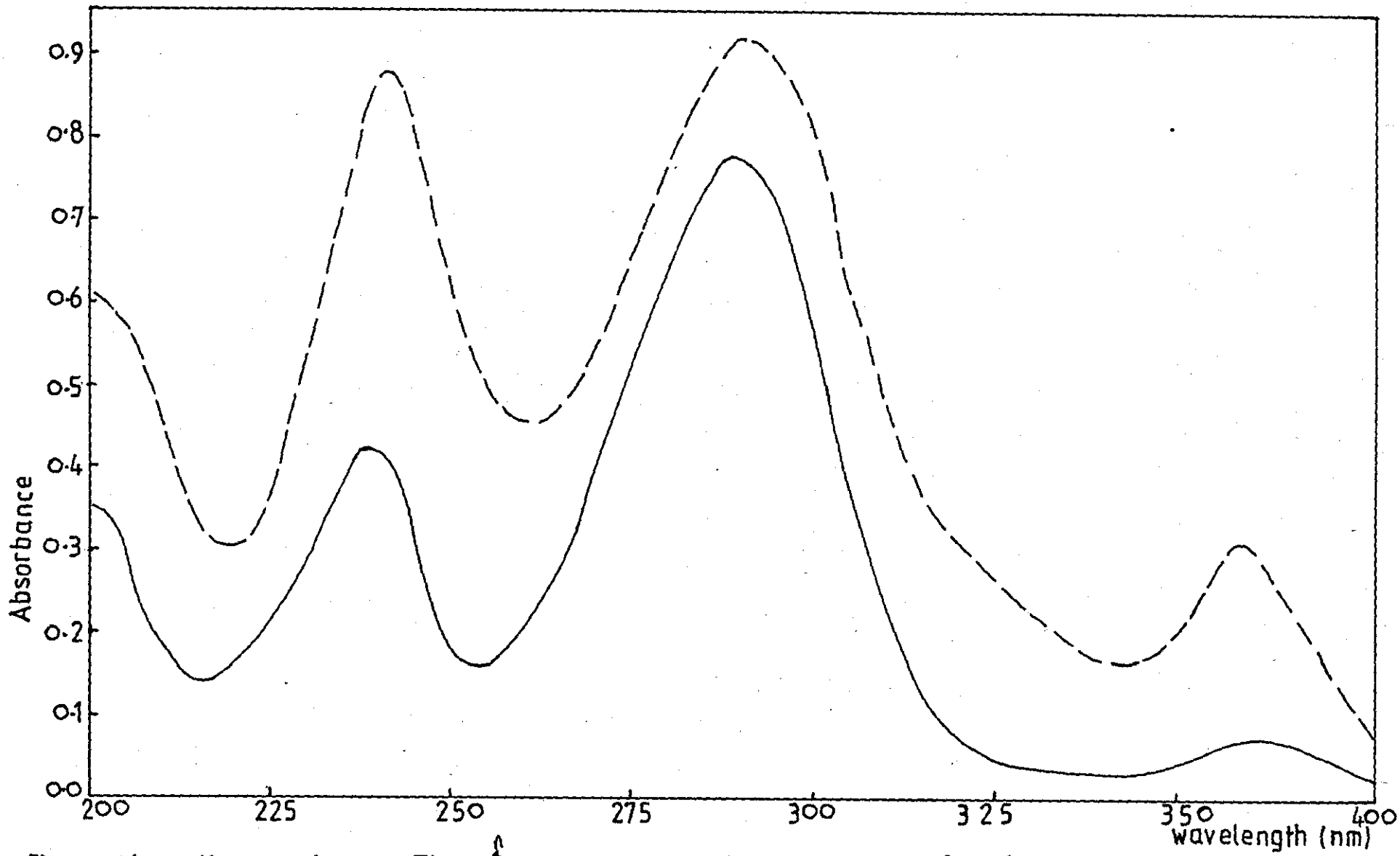



Fig.3.43 Absorption spectra of Thiopetone in 0.1M HCL ethanol : (——) immediately after preparation
(– – – – –) after one day of preparation

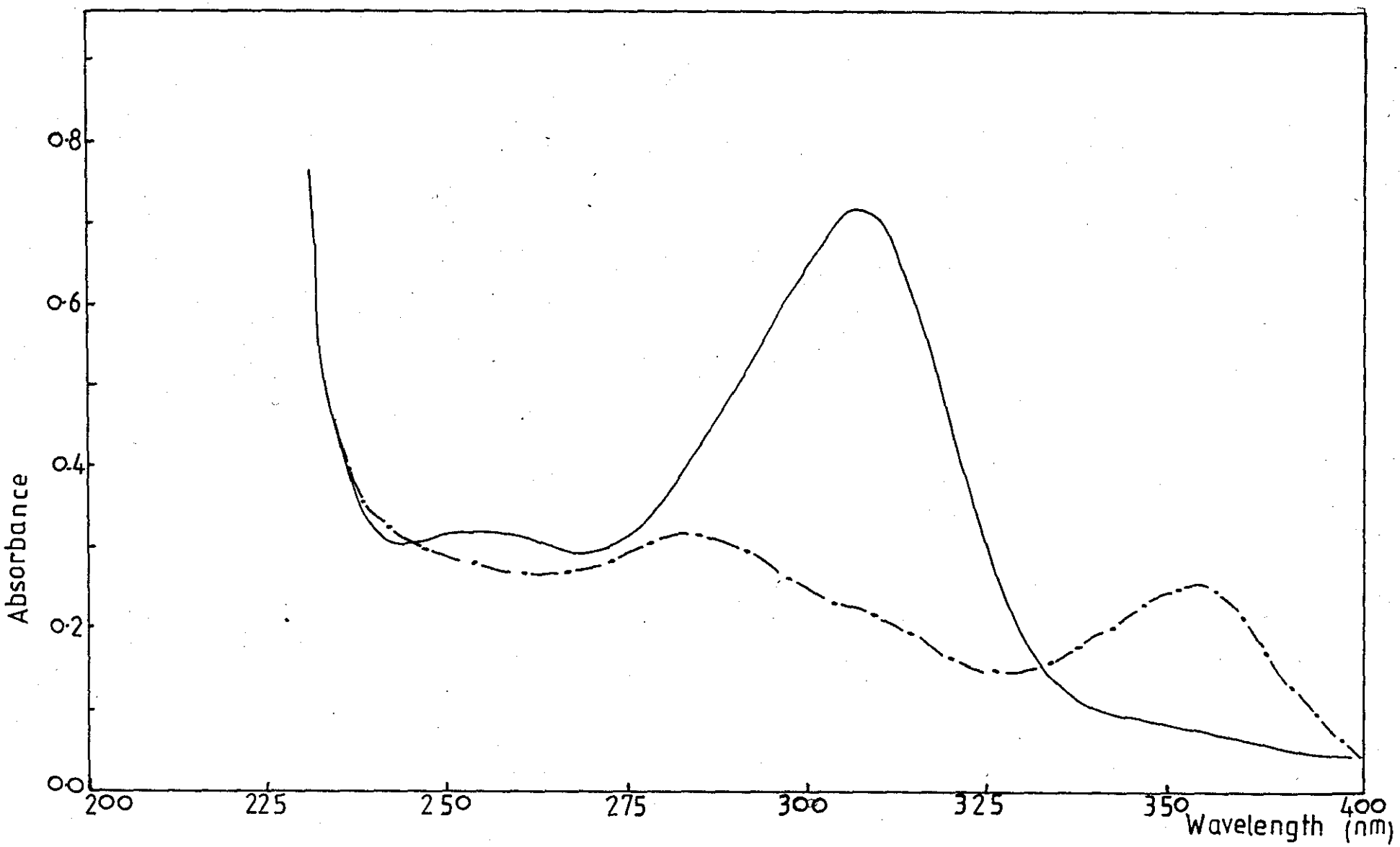



Fig.344. Absorption spectra of Thiopentone in 0.1M NaOH ethanol. as for fig 3

fluoresce in non-aqueous solution. Buddle[163] and Hewitt[20] have reported that of the thiobarbiturates they examined only those having 5,5-disubstitution showed fluorescence at room temperature in aqueous solution, and 1,5,5-trisubstituted compounds showed weaker fluorescence in aqueous solution.

### 3.4.4. Luminescence Spectra at 77K.

All low temperature measurements were made in 0.1M HCl in ethanol, neutral ethanol, and 0.1M NaOH in ethanol. Of the compounds examined the 5,5-disubstituted compounds (8 and 9) showed fluorescence in all three solvents (Fig. 3.4.5.) and compound 5 (1,3-dimethyl-5-ethyl-2 TBA) showed fluorescence in acidic ethanol only (Fig. 3.4.6). Compounds 2 and 6 (1,3-dimethyl and 1,3-diethyl-2TB) showed no luminescence in any conditions. Compound 7 (5-ethyl 2TBA) was fluorescent in acid and neutral solutions and was phosphorescent in 0.1M NaOH in ethanol (Fig. 3.4.7). Compounds 1,2 and 4 were phosphorescent at low temperature in all three solvents (Fig. 3.4.8). Table 3.4.1. gives the structures of the thiobarbiturates studied and Table 3.4.3. demonstrates the luminescence characteristics of the compounds studied in 0.1M HCl in ethanol, neutral ethanol and 0.1M NaOH in ethanol.

TABLE 3.4.3. Low temperature luminescence data for the Thiobarbiturates

| Compound | 0.1M HCL in ethanol | | | | Neutral ethanol | | | | 0.1M NaOH in ethanol | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fluorescence | | Phosphorescence | | Fluor. | | Phosphor. | | Fluor. | | Phosphor. | |
| | λex nm | λem nm | λex | λem | λex | λem | λex | λem | λex | λem | λex | λem |
| 1 | N.F. | | 362 | 442<br>460 | N.F. | | 355 | 445<br>467 | N.F. | | 297 | 446 |
| 2 | N.F | | N.P. | | N.F. | | N.P. | | N.F. | | N.P. | |
| 3 | N.F. | | 358 | 446<br>475 | N.F. | | 358 | 445<br>475 | N.F. | | 362 | 445<br>475 |
| 4 | N.F. | | 330 | 462 | N.F. | | 334 | 466 | N.F. | | 335 | 461 |
| 5 | 300 | 504 | N.P. | | N.F. | | N.P. | | N.F. | | N.P. | |
| 6 | N.F. | | N.P. | | N.F. | | N.P. | | N.F. | | N.P. | |
| 7 | 310 | 485 | N.P. | | 309 | 490 | N.P. | | N.F. | | 300 | 457 |
| 8 | 300 | 490 | N.P. | | 306 | 496 | N.P. | | 325 | 476 | N.P | |
| 9 | 305 | 495 | N.P. | | 306 | 496 | N.P. | | 328 | 476 | N.P. | |

N.F. : Not Fluorescent N.P. : Not Phosphorescent

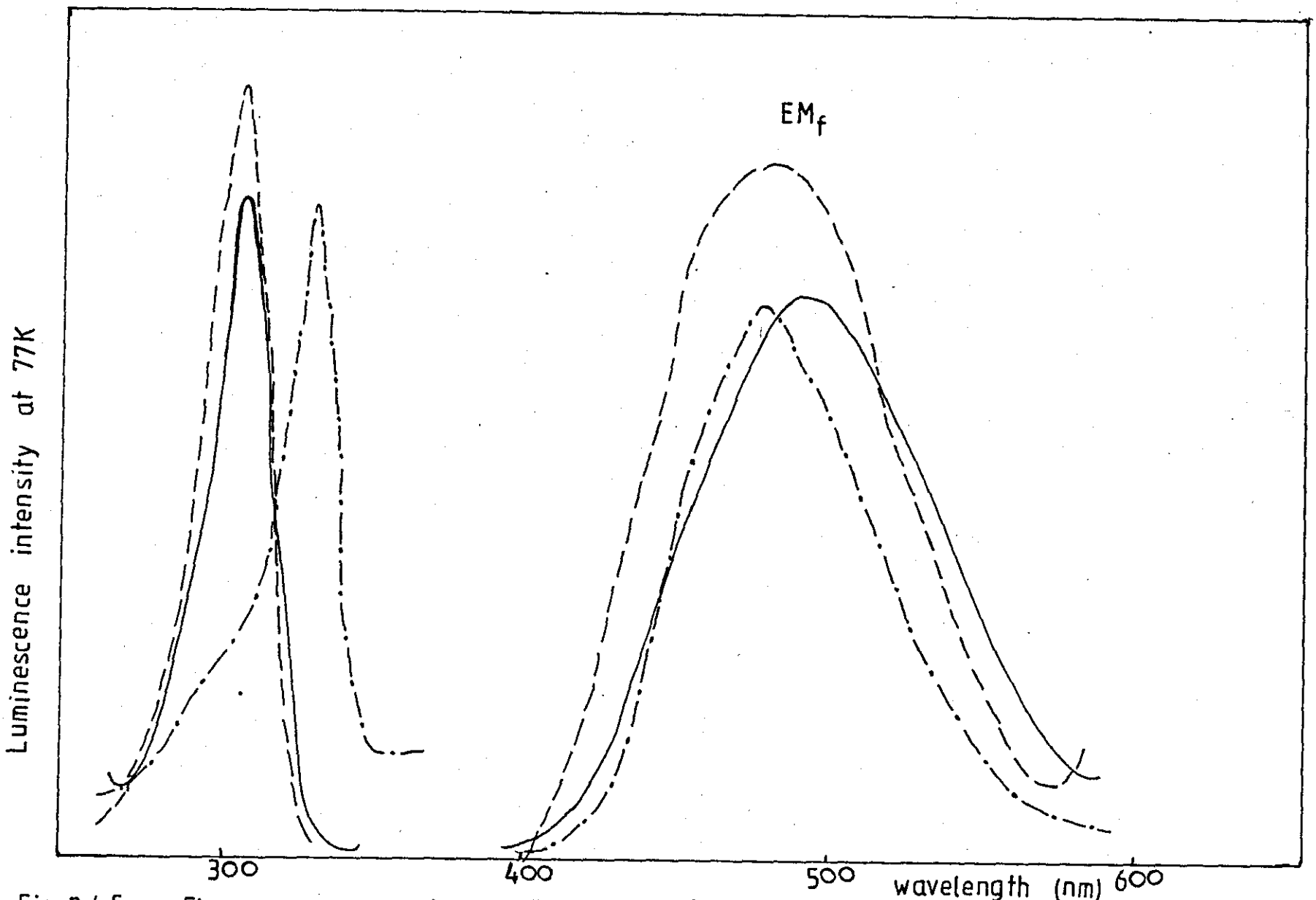



Fig. 3.4.5. Fluorescence spectra of Thialbarbital in 0.1M HCL ethanol (——), (_ _ _) neutral ethanol, & (_._._) 0.1M NaOH ethanol.

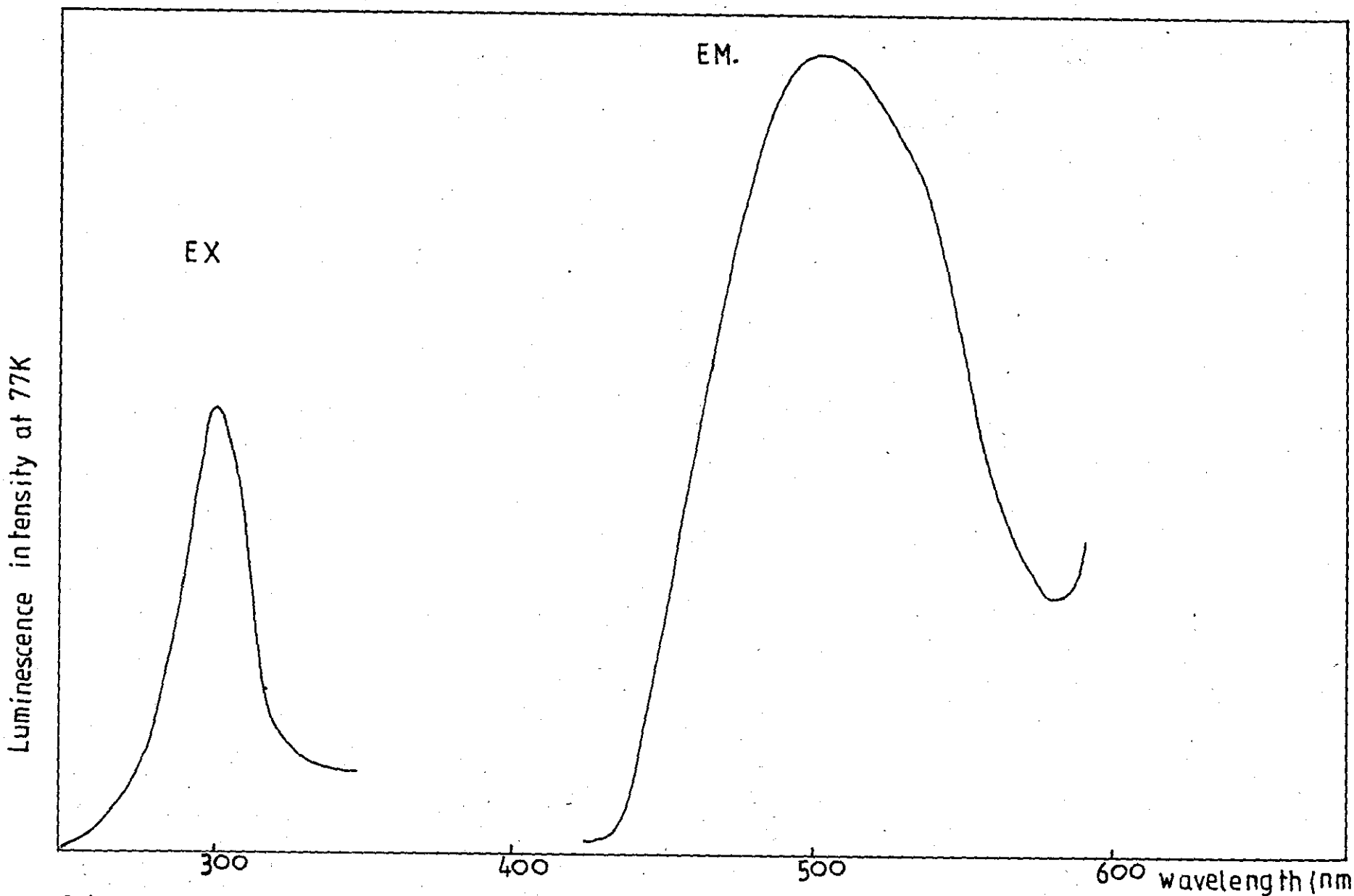



Fig. 3.4.6. Fluorescence spectra of 1,3 dimethyl-5-ethyl-2T.BA in 0.1M HCL ethanol

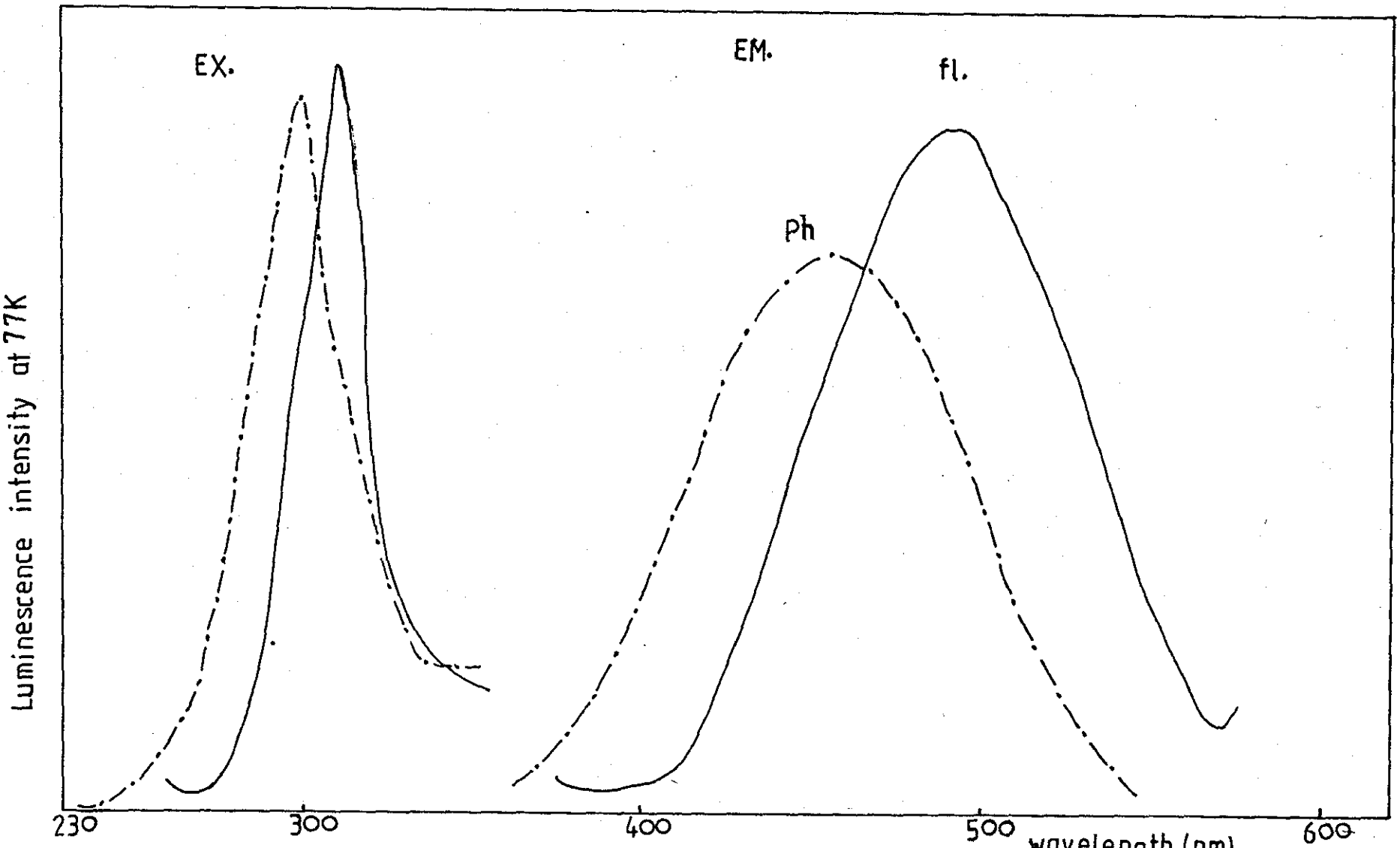



Fig. 3.4.7. Luminescence spectra of 5-ethyl-2TBA (———) fluorescence in neutral ethanol
(—.—.—.—) phosphorescence in 0.1M NaOH ethanol :

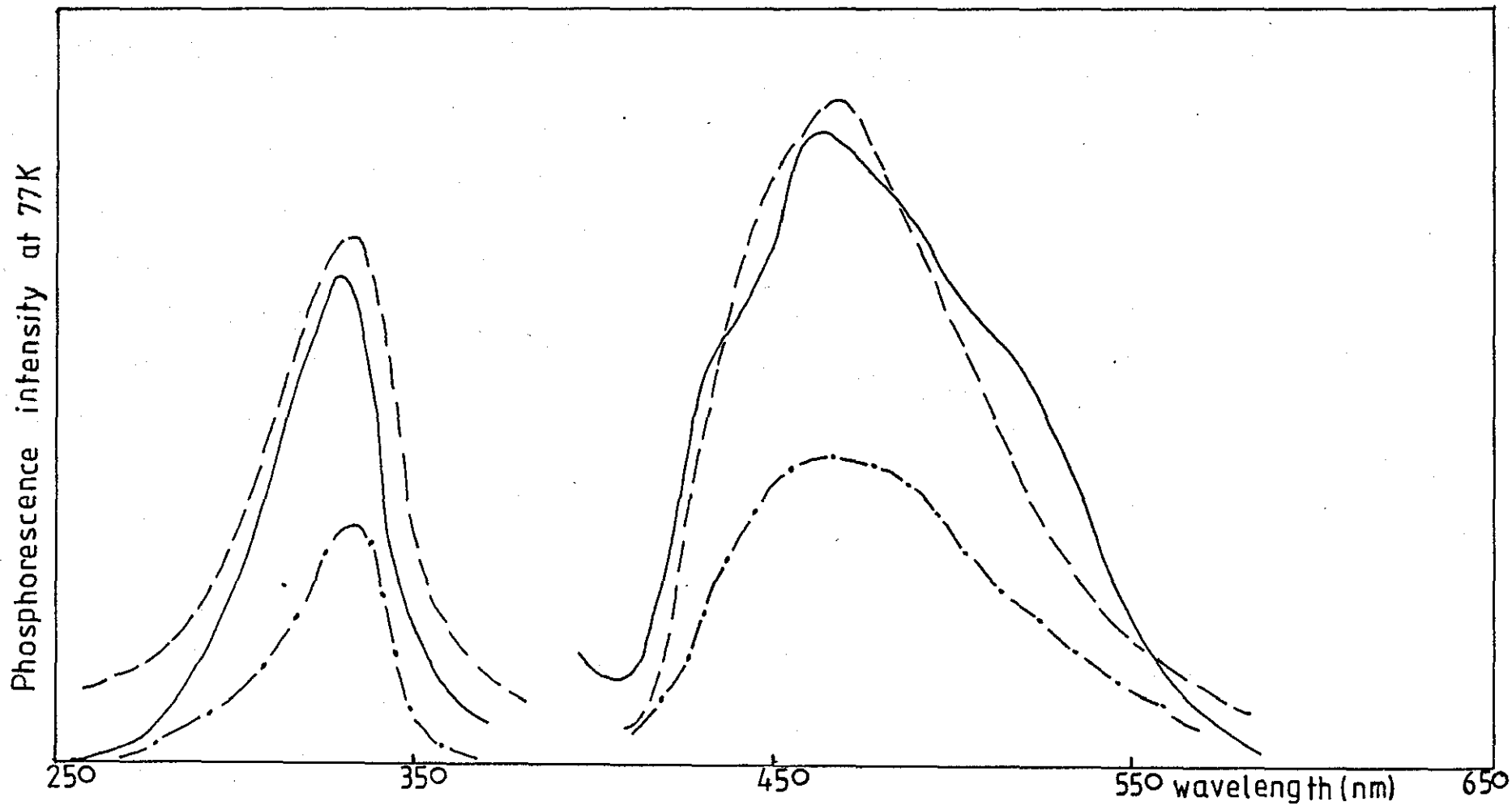



Fig. 3.4.8. Phosphorescence spectra of 1,3-dimethyl-5-phenyl-2TBA (– – –) in 0.1M HCL ethanol :, (——) neutral ethanol, & (– · – · –) 0.1M NaOH ethanol

### 3.4.5. Thin-layer phosphorimetry

From the study of the thiobarbiturates by luminescence it is clear that some of them were phosphorescent at 77K. In this study the limits of detection of the phosphorescent compounds were determined using thin-layer phosphorimetry. An example is illustrated in Fig. 3.4.9. for 1.methyl-2TBA. Heavy-atom solvents were studied in attempts to improve the limits of detection but without success. The best solvent used to spray the plate (cellulose) was ethanol. Table 3.4.4. gives some limits of detection using TLC phosphorimetry.

Table 3.4.4. Limits of Detection of some thiobarbiturates in 0.1M NaOH in Ethanol at 77K on cellulose thin layers

| Compound | Limits of detection $ug.ml^{-1}$ |
|---|---|
| 1 | .06 |
| 3 | .10 |
| 4 | .50 |
| 7 | .10 |

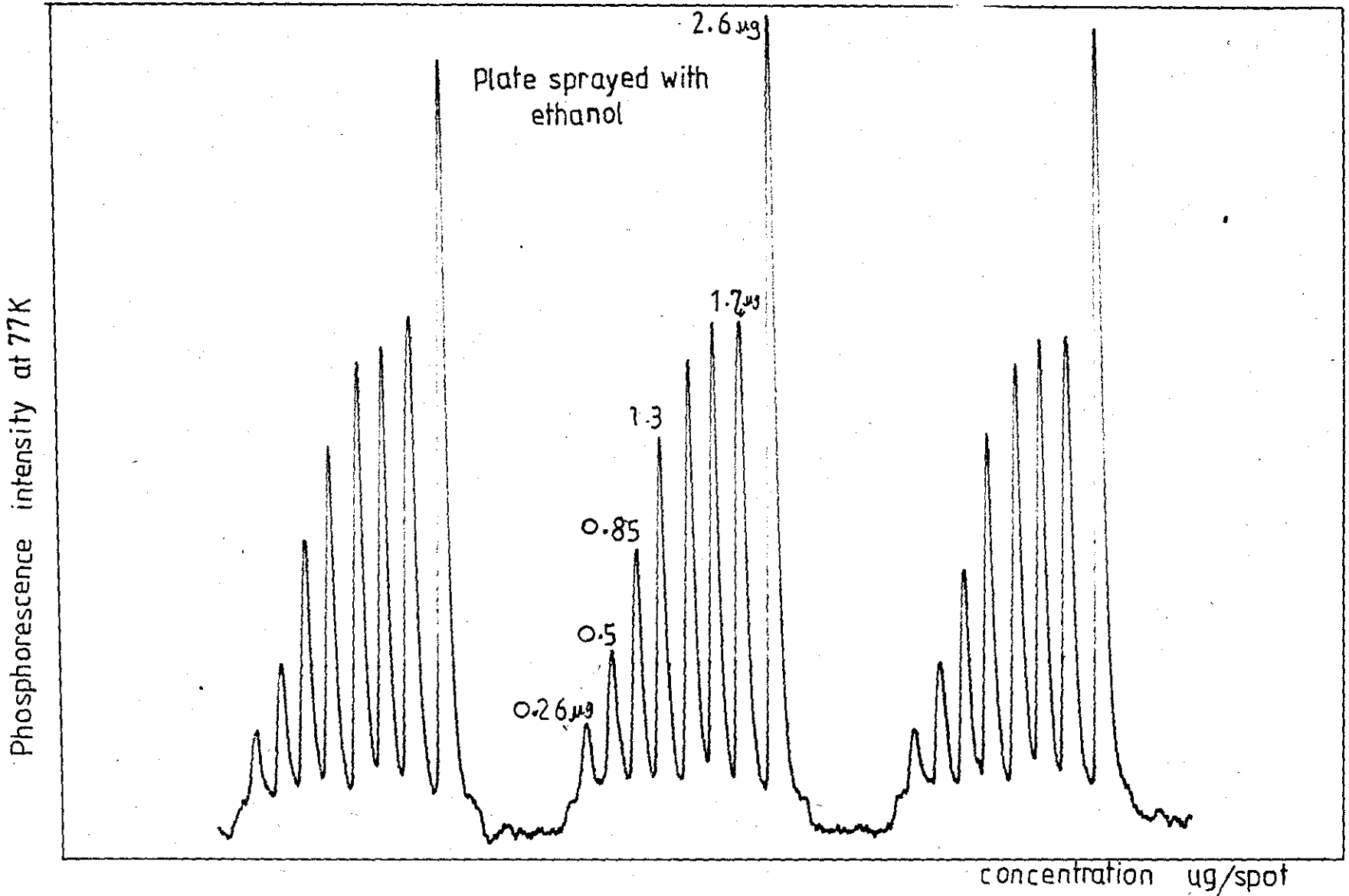


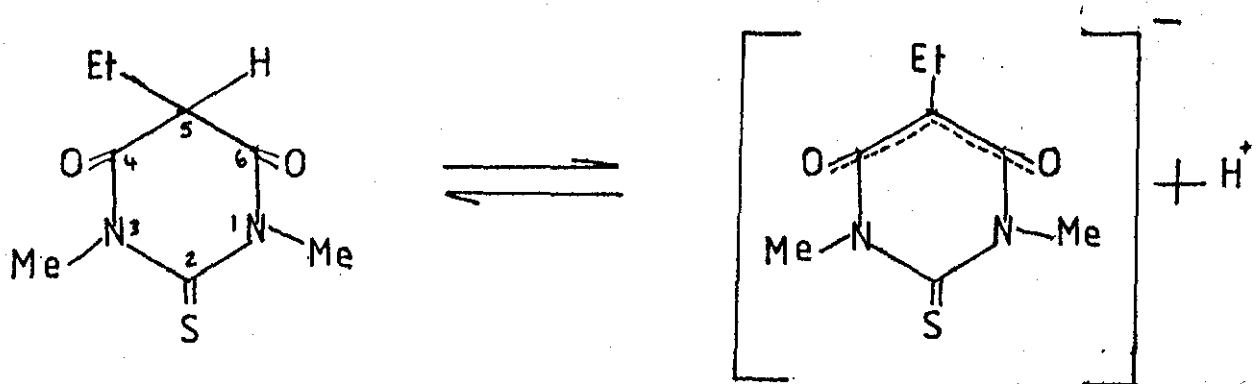

Fig. 3.4.9. TLC scan of 1-methyl-2TBA

Discussion.

The change of spectra with pH is accompanied by an isobestic point (a) observed for all compounds indicating an acid-base equilibrium is involved. As the pH changes to higher values a bathochromic shift in the absorption maximum is observed (Figs. 3.4.1. and 3.4.2). For 1,3-dimethyl-5-phenyl-2TBA, no variation in absorption maxima was observed with change in pH solvents and no isobestic points were observed. Gifford and King[162] found that for 5-phenyl-thiobarbituric acid the wavelength of maximum excitation is constant over the pH range 0-13; it is likely that there is no change in the degree of conjugation with hydrogen inn concentration.

For 1,3-dimethyl-5 ethyl 2TBA an isosbestic point was observed at 246nm. From the work done by Kazimierczuk et al[164], it is suggested that one ionic equilibrium exists over the pH range studied.

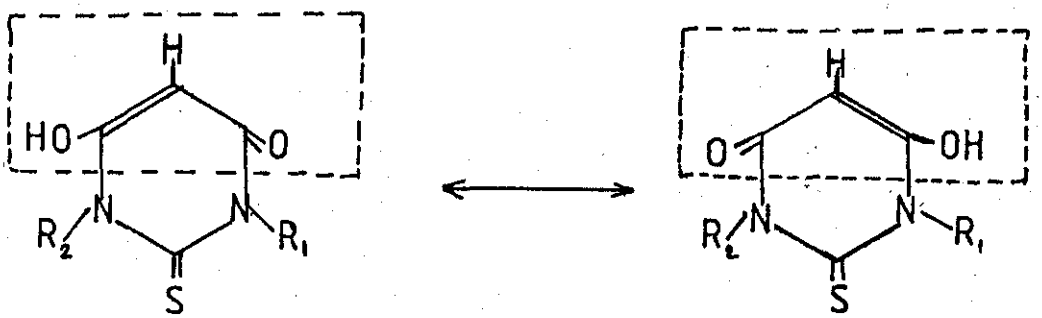
The dissociation pattern is probably as follows:




Et = Ethyl

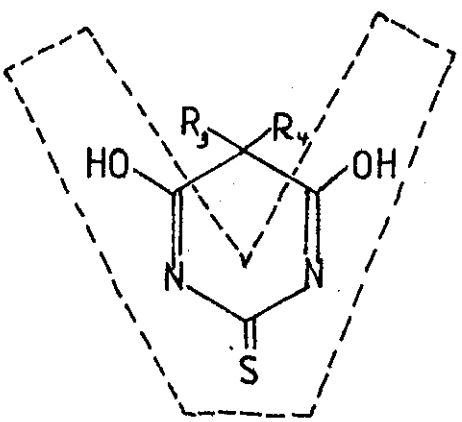
Me = Methyl

For 1,3 disubstituted compounds only one conjugated $\pi$ system can exist, as indicated below;



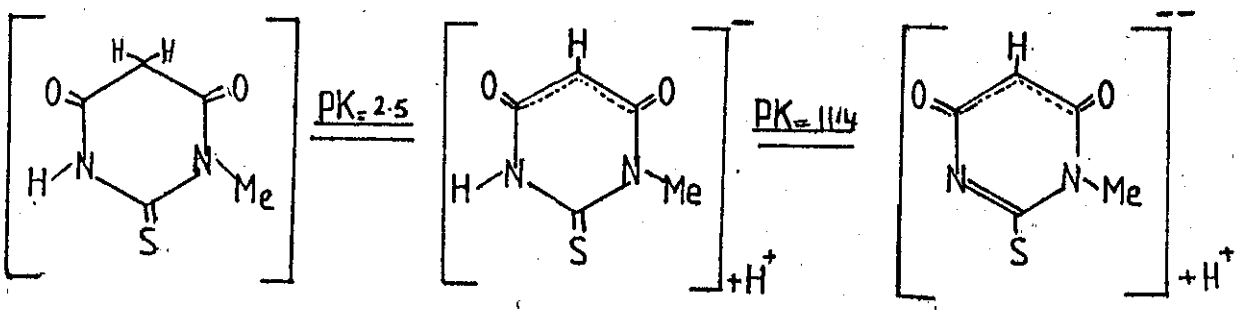

As such compounds are non-luminescent, it suggests the system indicated, by itself, is not responsible for luminescence.

The 5,5-disubstituted thiobarbiturates showed only fluorescence at 77K that is because they exhibit one type of conjugated system and resonance forms and hence, presumably more stability as evidenced by the intense fluorescence observed. In addition they can form a further related structural type, namely the diol or enol-thioenal, as the diagram illustrates below.



Gifford and King[162] state that the magnitude of the deteterium isotope effect as a function of pH suggests the presence of two fluorescent species in 5,5-thiobarbiturates at pH 5, which they explained as the anion and zwitterion forms.

For 1-methyl 2TBA two equilibria were observed and assigned as below; similar conclusions were reached about the ionized forms of the unsubstituted acid




The existence of this particular dianion form was justified by the decrease in extinction coefficient of the long-wavelength absorption band, which would be anticipated from the increase in aromaticity of the heterocyclic ring. These two compounds were phosphorescent as seen from the results.

In 5-phenyl-4,3-dimethyl 2-TBA, the phosphorescence may be due to the mesomeric effect of the phenyl ring which would help to stabilize the structure of the molecule.

This work describes the luminesces characteristics and U.V. absorption spectroscopy of two 5,5-disubstituted thiobarbiturates, two 1,3 disubstituted, 1,N substituted, 5-substituted and

1,3-5 substituted thiobarbiturates. From the results obtained quite different patterns of luminescence were obtained. Some of the compounds are phosphorescent at 77K and this enabled thin layer phosphorimetry to be used to determine them quantitatively. Submicrogram quantities were determined and this is an advantage over U.V. absorption spectrometry. Attempts to improve the limits of detection by using heavy atom affects (KI, or lead tetraacetate) proved unsuccessful. Therefore, fluorescence and phosphorescence may be the preferred method for analysis of special problem dosages.

## 3.5. Polynuclear Aromatic Hydrocarbons (PAH)

INTRODUCTION

Polynuclear Aromatic Hydrocarbons (PAH) occur in diverse sources such as atmospheric particulate matter, tobacco smoke, processed food, high-boiling petroleum distillates, domestic water, and other environmental situations. The potential adverse effect of these chemicals on human health is, therefore, a matter of growing international concern[165]. One hundred and fifty seven polynuclear aromatic compounds of wide structural range have been studied[166] and grouped according to their grade of carcinogenicity. In view of their presence in the above sources PAH have been studied by many analytical techniques in common use including u.v. spectrophotometry[167], gas-liquid chromatography[168] mass spectrometry[168] and new high temperature liquid crystal stationary phases[168], gas-chromatography[169], high performance liquid chromatography[170], differential pulse voltammetry[171], mass spectrometry[172] N.M.R.[172], matrix isolation Fourier Transform infrared spectrometry[173], low temperature fluorescence spectrometric[65,174-5] and phosphorescence[176].

A comparison of methods for the analysis of PAH in marine biota has been given by Gritz[177].

TLC methods for the separation of PAH on silica gel have been described previously[178-83].

The above methods used to analyse mixtures of PAH have some disadvantages; mass fragmentography is far too complex and the equipment is too expensive to be used for general purposes.

Fluorescence measured in solution is not broadly applicable to the quantitative analysis of mixtures of fluorophores, because the fluorescence spectra of aromatic molecules often present severe difficulties with spectral overlap. In addition, quenching and intermolecular energy transfer occur with high efficiency in fluid media; hence the relationship between the observed fluorescence intensity and the concentration for a particular analyte often depends upon the identities and concentrations of other species present in the sample. Consequently, the determination of PAH in complex mixtures can usually be accomplished only in low temperature matrices.

McGlynn et al[184] have discussed the complementry nature and usefulness of low temperature fluorimetry and phosphorimetry for the determination of several hydrocarbons. Winefordner et al[185] have also stressed the analytical utility of combining the two luminescence techniques. Muel and LaCroix[186] have stressed that the extremely sharp and numerous fluorescence bands, which characterise the low temperature fluorescence spectra of PAH, enhance selectivity over room-temperature measurements; the increase in fluorescence intensity with decreasing temperature also enhances sensitivity. Hood et al[64] described the separation of a mixture of carcinogens on thin-layers, but the area of the adsorbent containing the spot was removed from the plate before analysis. The hydrocarbon was eluted from the silica gel by extraction with ethanol at 65°C in a water bath for 15 minutes. Direct luminescence measurements were performed on the resultant solution after centrifugation and decantation.

None of the above authors made use of thin layer phosphorimetry, (i.e. a direct scan of the plate containing the spots) to determine the limits of detection, nor did they study heavy-atom effects, or the different adsorbent materials used in the thin layer chromatography step.

The present work provides such data for twelve polynuclear aromatic hydrocarbons. A mixture of PAH's has also been separated by T.L.C. and HPTLC. Phosphorograms of the separated components were obtained by selecting different excitation and emission wavelengths. The analysis was carried out at low temperature to enhance the selectivity and sensitivity of the method.

## Results and Discussion

### 3.5.1. Fluorescence at room temperature and 77K.

Table3.5.1. shows the room temperature excitation and fluorescence wavelengths, and limits of detection of the 12 hydrocarbons that have been investigated. All the hydrocarbons under study were fluorescent at room temperature and at 77K: an example is coronene Fig. 3.5.1. The aromatic hydrocarbons are well known for their intense fluorescence and highly structured fluorescence maxima at 77K. Most of the hydrocarbons investigated showed a large number of bands in their emission spectra. At 77K, the excitation peaks were similar to those at room temperature, while the fluorescence emission peaks were shifted $\sim$2nm to shorter wavelengths. In addition, an increase in relative fluorescence intensity and in some cases a sharpening of the fluorescence bands were observed. The blue shift on going from room temperature to 77K was explained by the existence of the Franck-Condon excited state at low temperature. At room temperature the solvent molecules can rapidly adjust to the equilibrium excited state of the solutes, and fluorescence then occurs from this state. At low temperature,reorientation of solvent molecules upon excitation requires a longer time, and emission occurs from the Franck-Condon excited state. Because of the energy difference between this state and the equilibrium excited state, shifts in fluorescence emission maxima occur. On the other hand, the energy difference between the equilibrium ground state and the Franck-Condon excited state should be independent of the rigidity of the medium, so that spectral shifts in the absorption spectra are not expected to occur.

Table 3.5.1. Room and Low Temperature Fluorescence data for polynuclear aromatic hydrocarbons in Ethanol.

| Compound (see Chap.2) | Fluorescence at R.T. $\lambda_{ex}$ nm | $\lambda_{em}$ nm | LOD, ug.ml$^{-1}$ | Fluorescence at 77K $\lambda_{ex}$ nm | $\lambda_{em}$ nm | LOD ug.ml$^{-1}$ |
|---|---|---|---|---|---|---|
| 1 | 302,328,362,383 | 422,433*,466 | 0.015 | 303,329,347, 364, 384 | 408,422,484* 449,473 | 0.003 |
| 2 | 305, 340 | 426,446*,450, 472,481 | 0.045 | 304, 340 | 426,446,450, 472,481,503 | 0.0025 |
| 3 | 356,374,393* | 410, 428* | 0.004 | 356,374,393 | 428,450* | 0.001 |
| 4 | 288* | 360, 370 | .09 | 288 | 360, 371,381 | .02 |
| 5 | 302* | 316, 330 | .10 | 302 | 316, 330 | .06 |
| 6 | 288,360 | 440, 470* | .04 | 288, 360 | 438, 466* | .01 |
| 7 | 301,335,350* 364 | 398*,419,442, 473 | .08 | 310,332,350* 365 | 396,419,442, 469,504 | .007 |
| 8 | 303,340*,364 | 376,386,398* | .07 | 300,326,340* 335. | 376,386,398* | 0.006 |
| 9 | 295,368,386* | 404*,427,458,493 | .0003 | 295,368,386 | 404,427,458, 493 | .07x10$^{-3}$ |
| 10 | 291,318,330 | 376,387,394,406 | .02 | 330,340 | 374,386,395, 406,419 | .004 |
| 11 | 338,377*,397, 418 | 438,480*,510 | .08 | 340,378,399 416 | 443,482,512 | .02 |
| 12 | 275 | 320 | .06 | 275 | 320 | .02 |

* The main peak at which the limits of detection were determined.

L.O.D. Limit of detection.

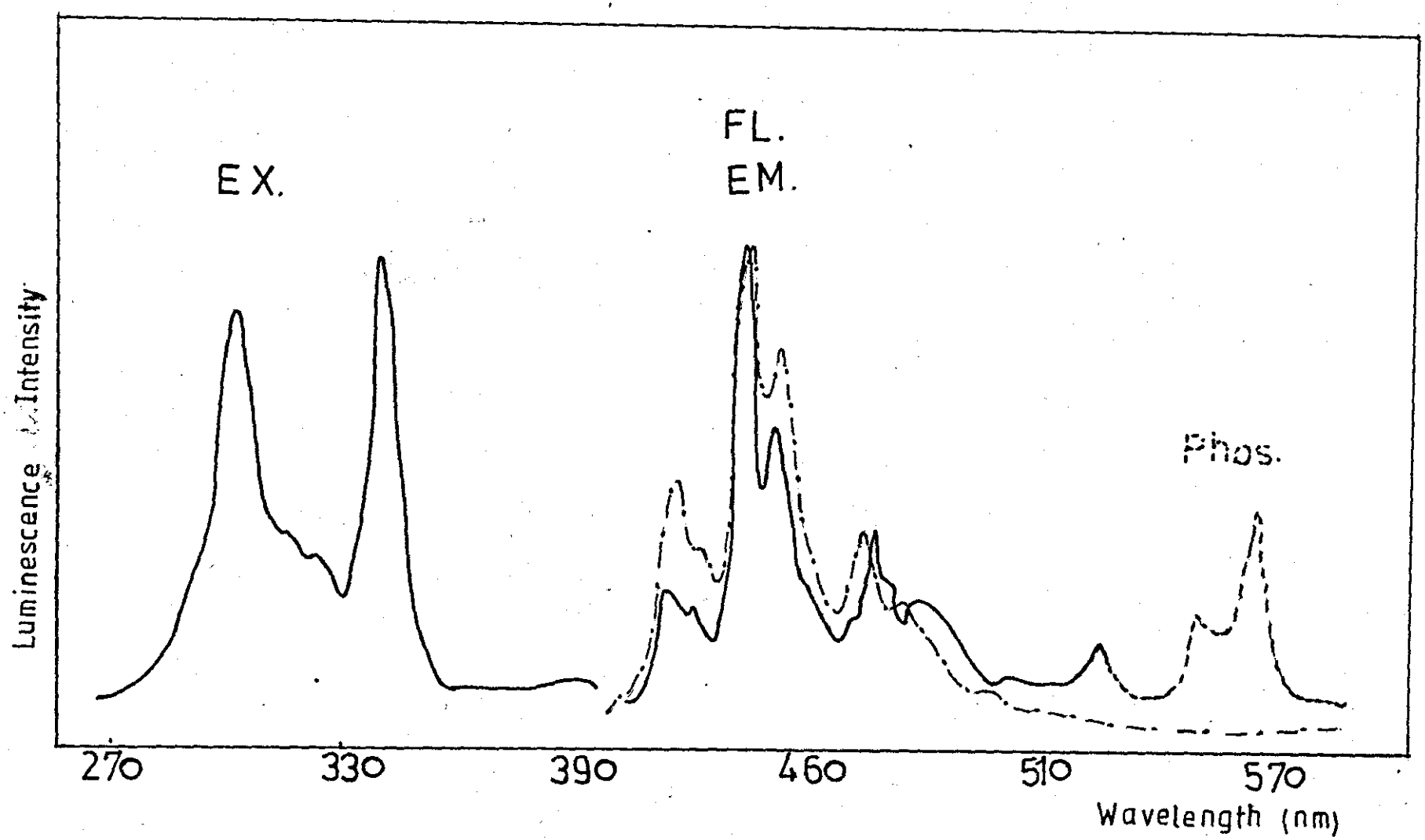



Fig.3.5.1 Total luminescence of Coronene in ethanol at 77 K (———) & at R.T. (–.–.–.–), the Instrumental Amplification is 30 times for Room Temperature (R.T.)

Heavy atom effects on the fluorescence of PAH.

A reduction in the fluorescence intensities of the PAH was observed when a heavy atom was introduced into the immediate molecular environment through the solvent. The heavy-atom reagents tried were iodomethane and silver nitrate in ethanol, the ratio of $C_2H_5OH/CH_3I$ was 4/1 (v/v). A logical explanation for this effect is that heavy atoms increase the rate of intersystem-crossing from $S_1 \longrightarrow T_1$ owing to increased spin-orbit coupling, and hence decrease fluorescence efficiency (Fig. 3.5.3). Further explanation of the heavy-atom effect is given in Chapter 4.

3.5.2. Phosphorescence Characteristics at 77K.

Table 3.5.2. shows the low temperature excitation and phosphorescence wavelengths and limits of detection of the PAH in ethanol. All the PAH studied show fluorescence but not all of them show phosphorescence. Benzo(ghi)perylene and 9-10-Diphenylanthracene are not phosphorescent.

Figs. 3.5.1., 3.5.2 and 3.5.3. show the total luminescence of coronene, 1,2,3,4-dibenzanthracene and Benzo(e)pyrene respectively. The phosphorescence curves show fine structures for both coronene and Benzo(e)pyrene in ethanol.

Heavy atom effects on the phosphorescence spectra.

The introduction of the iodine atom through the solvent enhanced the phosphorescence emission 4-fold except for triphenylene and fluorene, which displayed a negative external heavy-atom effect,

Table 3.5.2. Low Temperature phosphorescence data for Polynuclear Aromatic Hydrocarbons (in ethanol) in solution and on silica gel thin layers

| Compound | $\lambda_{ex}$ | $\lambda_{em}$ | L.O.D. $ug.ml^{-1}$ in ethanol | On T.L.C. sprayed with ethanol ng/spot | On T.L.C. sprayed with $CH_3I$ |
|---|---|---|---|---|---|
| 1 | N.P. | N.P. | N.D. | N.D. | N.D. |
| 2 | 314, 340 | 528, 547, 559 | .003 | .20 | .07 |
| 3 | N.P. | N.P. | N.D. | N.D. | N.D. |
| 4 | 288 | 444,465, 490, 529 | .008 | 11. | 12 |
| 5 | 308 | 428,455, 479 | .15 | 50 | 90 |
| 6 | 360 | 550, 595 | .2 | 200. | 10. |
| 7 | 345 | 545, 579 | .01 | 6 | .6 |
| 8 | 300, 326 340, 350 | 563 | .08 | 7.2 | 1.2 |
| 9 | 370 | 690 | w.s. | w.s. | w.s. |
| 10 | 340 | 541, 551, 583 | .02 | 9 | .5 |
| 11 | 378 | 540, 552 | w.s. | w.s. | w.s. |
| 12 | 276 | 463 | | 10 | 300 |

N.D. Not determined N.P. Not phosphorescent w.s. weak signal

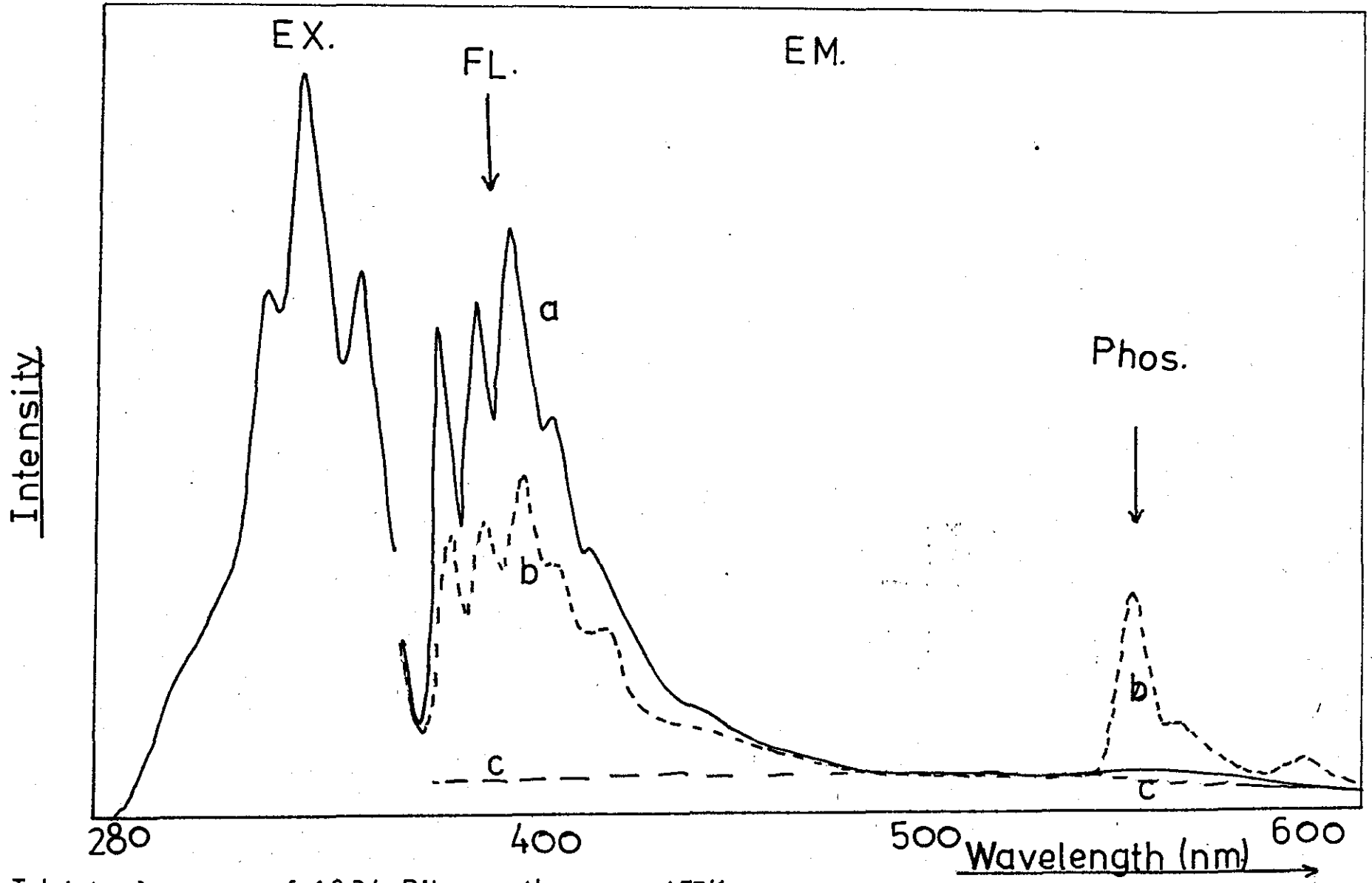



Fig.3.5.2. Total luminescence of 1,2;3,4-Dibenzanthracene at 77K
a) in $C_2H_5OH$ b) $C_2H_5OH/CH_3I$ .. & c) Blank emission

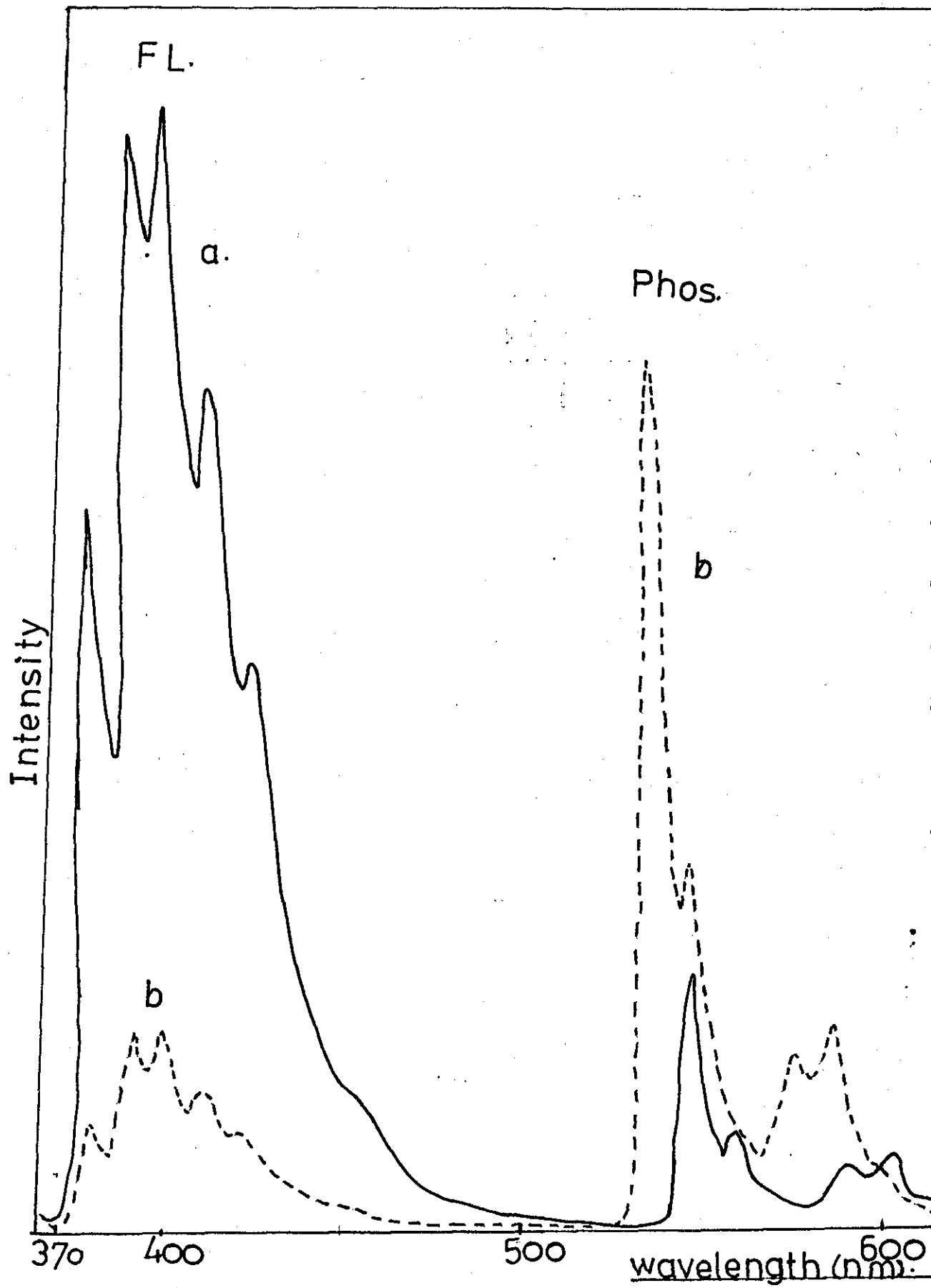



Fig.3.5.3. Total luminescence of Benzo(e)pyrene at 77 K
a) in $C_2H_5OH$ b) in $C_2H_5OH/CH_3I$

i.e. the heavy atom-solvent ($C_2H_5OH/CH_3I$) had a quenching effect on these compounds (for more details see Chapter 4).

The heavy atom increases the rate of intersystem-crossing from $S_1 \longrightarrow T_1$ and sometimes increases the phosphorescence efficiency. Some of the PAH's studied displayed a positive external heavy-atom effect; i.e. the intensity of phosphorescence was greater in the heavy-atom solvent than in the non heavy-atom solvent ($C_2H_5OH$) see Fig. 3.5.3. Fig. 3.5.2. shows the effect of methyl iodide on the total luminescence of 1,2,3,4-dibenzoanthracene, the fluorescence being quenched and the phosphorescence greatly enhanced.

3.5.3. Thin-layer phosphorimetry.

The limits of detection for each phosphorescent hydrocarbon were determined on T.L.C. plates. Table 3.5.3. gives the limits of detection at low temperature (77K), the plates being sprayed with ethanol and then sprayed with ethyl iodide. The external heavy-atom effect obtained with $C_2H_5OH/CH_3I$ was simple to use and resulted in increased sensitivities. For example for coronene the phosphorimetric limit of detection when the plate was sprayed with ethanol was 28ng per spot; 70pg per spot was detectable when the plate was sprayed with pure iodomethane ($CH_3I$). Fluoranthene sprayed with ethanol was detectable at 200 ng per spot, but when sprayed with $CH_3I$ was detectable at 10 ng per spot. For 1,2,5,6-dibenzoanthracene and benzo(e)pyrene sub-nanogram quantities were detected. Fig. 3.5.4. shows the TLC scan for coronene with the two plates sprayed with different solvents. From the above results considerable analytical benefit may occur

Table 3.5.3. Limits of detection of polynuclear Aromatic Hydrocarbons on various thin layers sprayed with various solvents.

| Compound | Phosphorescence $\lambda ex_{nm}$ | Phosphorescence $\lambda em_{nm}$ | L.O.D. ng/spot on silica gel sprayed with 1. Ethanol | L.O.D. ng/spot on silica gel sprayed with 2. $CH_3I$ | L.O.D. ng/spot on HPTLC sprayed with 1. Ethanol | L.O.D. ng/spot on HPTLC sprayed with 2. $CH_3I$ | L.O.D. ng/spot on cellulose sprayed with 1. Ethanol | L.O.D. ng/spot on cellulose sprayed with 2. $CH_3I$ |
|---|---|---|---|---|---|---|---|---|
| Coronene | 340 | 559 | 10 | .07 | 20 | .17 | N.D. | 1 |
| Triphenylene | 288 | 465 | 14 | 28 | 10 | 20 | 70 | N.D. |
| Fluorene | 308 | 455 | 50 | 90 | 50 | 100 | N.D. | N.D. |
| Fluoranthene | 360 | 550 | 200 | 10 | 10 | 5 | N.D. | N.D. |
| 1,2,5,6-Dibenz-anthracene | 345 | 545 | 6 | .6 | 6 | .8 | N.D. | N.D. |
| 1,2,3,4-Dibenz-anthracene | 340 | 563 | 7.2 | 1.2 | 10 | 5 | N.D. | N.D. |
| Benzo(e)pyrene | 340 | 541 | 9 | .5 | 20 | 1.00 | N.D. | N.D. |
| Biphenyl | 276 | 463 | 10 | 300 | 10 | 300 | N.D. | N.D. |

N.D. Not detected.

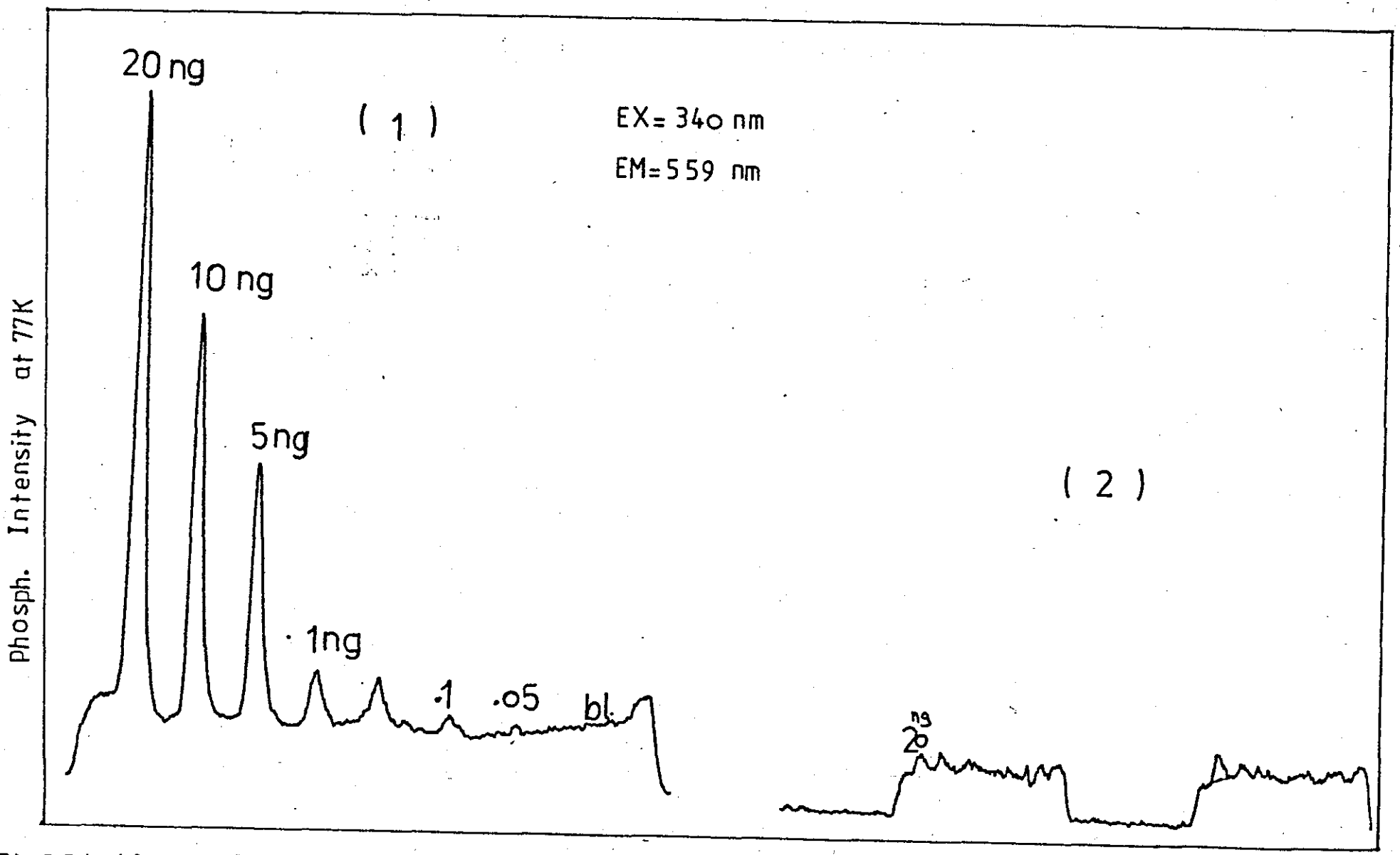



Fig.3.5.4. Limit of Detection of Coronene on TLC plate 1-plate sprayed with $CH_3I$
2- plate sprayed with ethanol

from the use of $C_2H_5OH/CH_3I$ as a solvent.

### 3.5.4. Analytical Calibration Curves.

Fig. 3.5.5. shows the calibration curves of coronene on different adsorbent layers. It can be seen that the best medium for the determination of coronene is silica gel sprayed with $CH_3I$.

Fig. 3.5.6. shows the calibrations curves of 4 hydrocarbons on TLC plates sprayed with $CH_3I$. The range of linearity is approximately 10 - 400 ng.

### 3.5.5. Separation of mixtures of Carcinogens.

A mixture of seven carcinogenic compounds was chromatogrammed using silica gel as the stationary phase and n-hexane:pyridine (30/1, v/v) as the mobile phase. After development the spots were visualised using u.v. light, with the plate immersed in liquid nitrogen. The $R_f$ values are shown in Table 3.5.4.

Table 3.5.4.

$R_f$ values of some hydrocarbons on silica gel layers

| Compound | $R_f$ | Colour of spots when sprayed with $CH_3I$ at 77K |
|---|---|---|
| Coronene | .26 | green-yellow |
| Triphenylene | .46 | blue |
| Fluorene | .70 | blue |
| Fluoranthene | .78 | orange |
| 1,2,3,4-dibenz-anthracene | .27 | orange |
| 1,2,5,6-dibenz-anthracene | .38 | orange |
| Benzo(e)pyrene | .59 | orange |

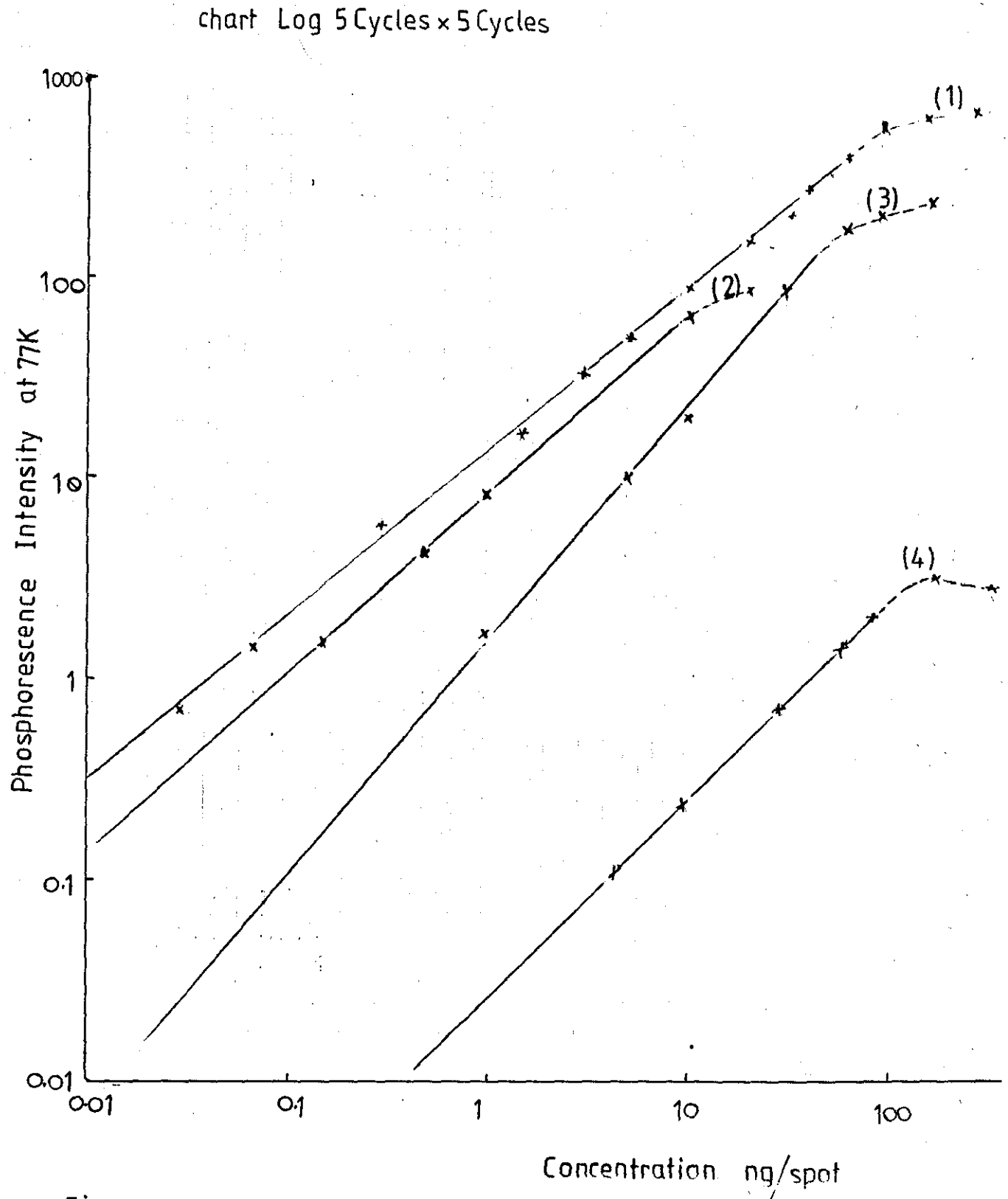



Fig.3.5.5. Analytical growth curves of Coronene on
1-silica gel layers sprayed with ($CH_3I$) 2- HPTLC sprayed with ($CH_3I$)
3- cellulose layers sprayed with ($CH_3I$) 4-silica gel sprayed with ethanol

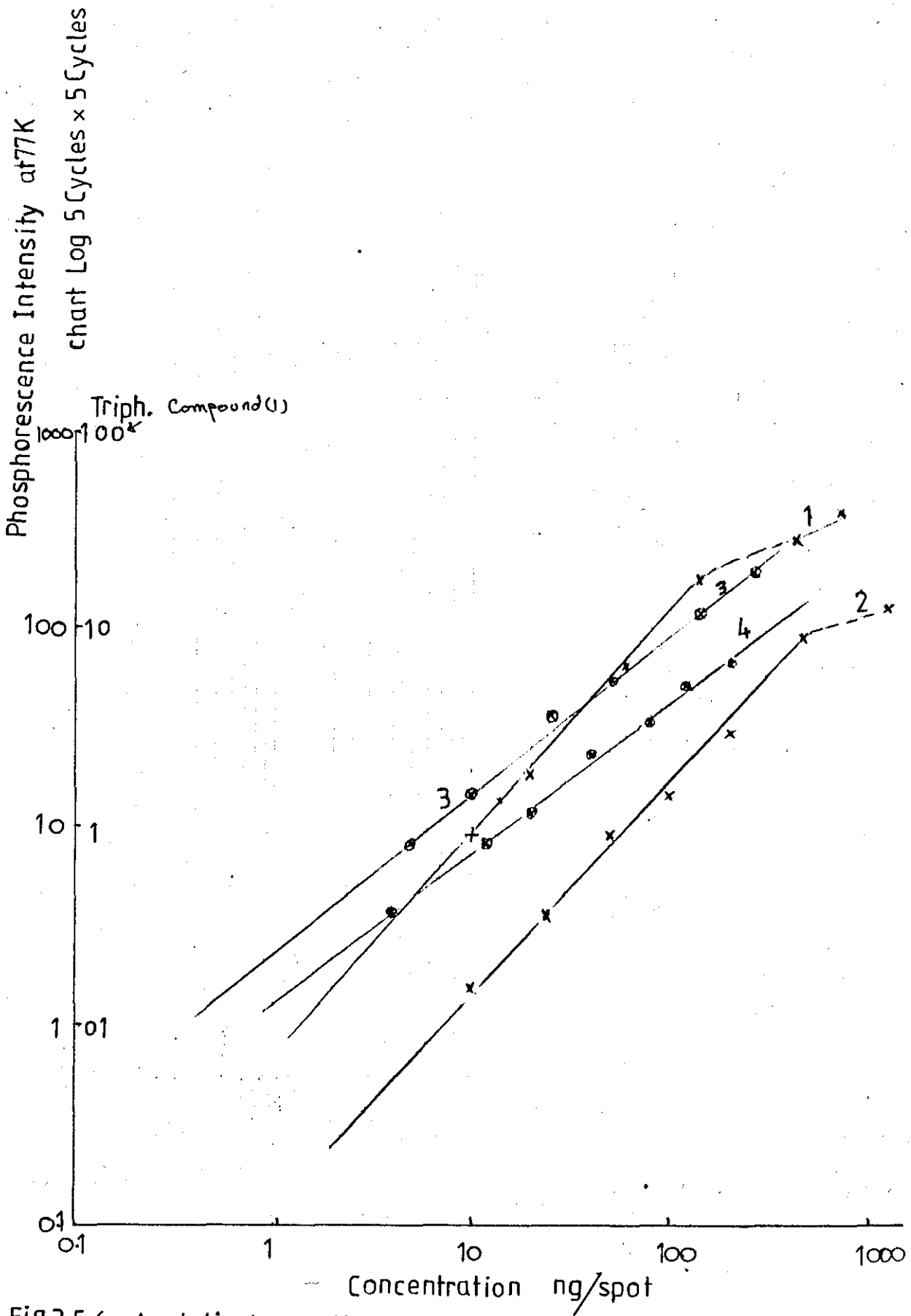



Fig.3.5.6. Analytical growth curves of some polynuclear Aromatic Hydrocarbons on silica gel plates sprayed with $CH_3I$
1- Triphenylene 2-Fluoranthene 3- 1,2;5,6-Dibenzanthracene & 4- 1,2;3,4-Dibenzanthracene

The concentration of each component in the mixture was approximately 50 ng per spot.

Fig. 3.5.7. shows the phosphorgram of the separated hydrocarbons. Using an excitation wavelength of 340nm and emission wavelength of 550nm, four separated peaks could be distinguished. From the $R_f$ values previously calculated these peaks could be assigned to a) Fluoranthene, b) Benzo(e)pyrene, (c) 1,2,5,6-dibenzanthracene, d) coronene. Sample e) was 1,2,3,4-dibenzanthracene which overlapped with coronene. The use of an excitation wavelength of 308nm and an emission wavelength of 455nm produced peaks corresponding to Fluorene and Triphenylene. Wavelengths were chosen for excitation and emission so as to yield the best selectivity, not necessarily the greatest sensitivity. Such separation leads to significantly increased selectivity in routine analyses.

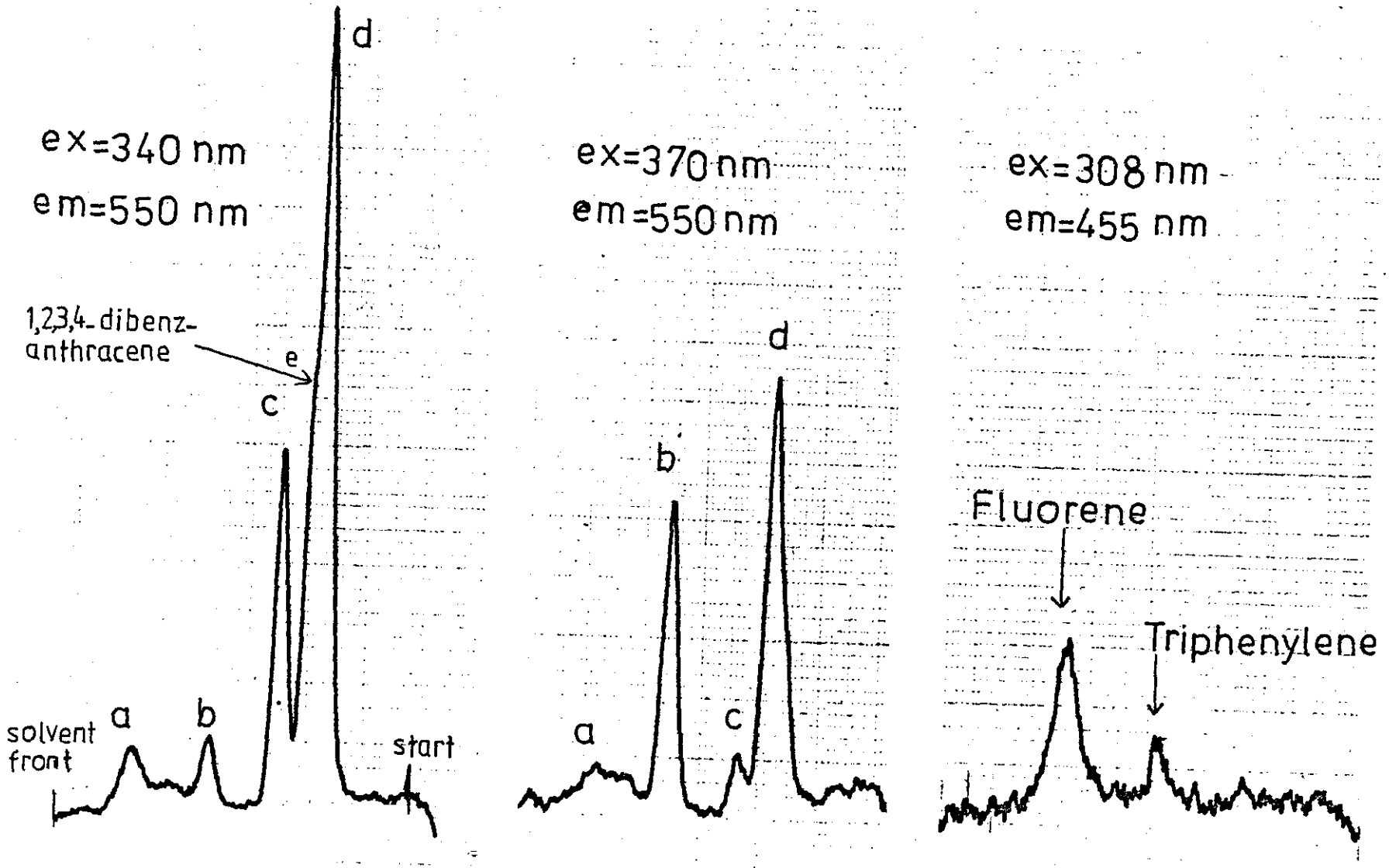



Fig. 3.5.7. Chromatographic Separation of Mixtures of Carcinogens on TLC
a) Fluoranthene b) Benzo(e)pyrene c) 1,2,5,6 Dibenzanthracene d) Coronene

# CHAPTER 4

## GENERAL DISCUSSION AND CONCLUSIONS.

### 4.1. Comparison of spectrophosphorimetry with other spectroscopic methods.

Phosphorimetry is a useful analytical technique for the detection of minute quantities of drugs or drug-like compounds in the body fluids (blood plasma or serum and urine).

The usefulness of the method of analysis may best be judged by comparing it with other related methods. In the case of spectrophosphorimetry the comparison that presents itself is with spectrofluorimetry and with UV absorption spectroscopy. The points of view from which the three methods ought to be compared are:

Application: The overwhelming majority of the unsaturated organic compounds show measurable UV absorption, but not all of them re-emit the absorbed radiation as measurable fluorescence or phosphorescence. Therefore the breadth of application of absorption spectroscopy is, in principle, greater than that of the two luminescence spectroscopic methods. There are many compounds for which all three methods are suitable for identification and quantitative determination. In fluorimetry labels are widely used and in phosphorimetry thin-layers.

Selectivity: In luminescence spectroscopy there are available as analytically realizable parameters not only the emission spectra, but also the excitation spectra. This, as well as the fact that not all compounds that absorb measurably also remit measurably, increases the selectivity of the methods of luminescence spectroscopy, compared

with those of absorption spectroscopy. This means that in complicated mixtures the identification and quantitative determination of individual components is often simpler by a luminescence spectroscopic method than by UV absorption spectroscopy.

TLC phosphorimetry provides additional selectivity by recording complicated mixtures on TLC plates and by scanning the plates at various excitation and emission wavelengths. Different compounds could thus be identified qualitatively, increasing the selectivity of phosphorimetry over other spectroscopic methods. The phosphorescence spectra are usually more characteristic than the fluorescence spectra and therefore frequently better suited for identifying the compounds (e.g. polycyclic aromatic hydrocarbons). Their (PAH) long phosphorescent lifetimes make many analytical applications possible and further increase the selectivity of the method.

Sensitivity: The sensitivity of luminescence methods is frequently from 10 to 1000 times greater than that of absorption methods. In phosphorescence measurements the phosphoroscope involves (compared with fluorescence measurements) a measurable loss of luminescence intensity. On the other hand, in phosphorimetric analyses it is possible to work with considerably wider slits than in fluorimetry because the phosphoroscope completely eliminates scattered (exciting) light. The result is that limits of detection of a compound in phosphorimetry and fluorimetry are still of the same order of magnitude even if the quantum yield of phosphorescence is smaller than that of fluorescence by a factor of 10 or 20. The limits obtained for the same compound by various authors are frequently not exactly comparable

since they depend on apparatus parameters such as excitation intensity, receiver sensitivity, noise, and the background phosphorescence of the solvent. McGlynn[184] and colleagues have compared the phosphorimetric and fluorimetric limits of detection of durene, naphthalene, and phenanthrene. These authors found that for aromatic hydrocarbons spectrophosphorimetry and spectrofluorimetry have comparable sensitivity.

The limits of detection of various compounds can sometimes be further improved in thin-layer phosphorimetry, using appropriate adsorbent layers with the aid of solvent enhancement of phosphorescence intensity[84]. Also the phosphorimetric limits of detection can be improved in some cases if instead of ethanol one uses a solvent that shows an external heavy atom effect (see later).

To summarise, it is clear from this comparison of UV spectral analysis, spectrophosphorimetry and spectrofluorimetry that UV analysis is superior to the methods of luminescence spectroscopy in its breadth of application, but is inferior to them in selectivity and sensitivity. Phosphorimetry and fluorimetry are nearly comparable methods that are not exclusive, but, rather in many cases complement each other very satisfactorily. For the quantitative determination of individual compounds in complicated mixtures, phosphorimetry is frequently to be preferred to fluorimetry because of its greater selectivity.

## 4.2. Heavy-atom effect

A heavy atom may be introduced into the immediate molecular environment through the solvent. The heavy atom introduced in this manner is called an "external" heavy atom, and any change in $\phi_p/\phi_f$ is referred to as the "external heavy-atom effect". McGlynn et al [187] first suggested the use of the external heavy-atom effect for increasing analytical sensitivity. The "heavy-atom effect" will affect the fluorescence and phosphorescence intensities, it will quench the fluorescence intensity and (sometimes) enhance the phosphorescence intensity. The reason is that the spin-orbit coupling increases greatly under the influence of inhomogeneous electric fields such as these present in heavy atoms, i.e., in atoms of high atomic number. With increasing spin-orbit coupling in an organic molecule the probabilities of intersystem crossing ($S_1 \dashrightarrow T_1$), the phosphorescence transitions ($T_1 \longrightarrow S_o$), and of radiationless transitions to the ground state ($T_1 \dashrightarrow S_o$) are all increased. Which process is influenced most must be determined in each particular case. Increased probability of the different processes is revealed in different ways:

1. Enhancement of the $S_1 \longrightarrow T_1$ transition causes an increase of the quantum yield $\phi_p$ of the phosphorescence and, therefore, decrease of the quantum yield $\phi_f$ of fluorescence and increase of the ratio $\phi_p/\phi_f$ (all other things being equal).

2. Enhancement of the $T_1 \longrightarrow S_o$ transition leads to decrease in the lifetime of phosphorescence and increase in the strength of the triplet-singlet absorption.

3. Increase of the radiationless $T_1 \dashrightarrow S_o$ transition causes decrease of both the quantum yield and the lifetime of the phosphorescence.

Hood and Winefordner have undertaken a thorough study of the external heavy-atom effect for 12 polycyclic aromatic hydrocarbons. The heavy-atom solvent system chosen by these workers was ethanol/ethyl iodide in various volume ratios, 19/1, 9/1 and 5/1. Some of the compounds they studied showed positive external heavy-atom effects and some showed negative heavy-atom effects. The positive effect was typical of enhanced intersystem crossing arising from spin-orbit coupling. The negative effect was due to the "quenching" phenomenon which may be due to the transmission characteristics of ethyl iodide (EtI) at phosphorescence excitation wavelengths. It was observed that the phosphorescence excitation spectra appeared to shift toward longer wavelengths as the concentration of EtI increases in the solvent. Excitation maxima were usually between 320 and 330nm in EtOH/EtI solvent system; EtI absorbs strongly below 325nm, and therefore it may be acting as a strong "filter" for excitation radiation. Hood and Winefordner[188] concluded that the external heavy-atom effect obtained with EtOH/EtI is simple to use and frequently results in increased sensitivities and ranges of linearity of analytical curves.

Hood and Winefordner[189] have examined the application of phosphorimetry, and especially the external heavy-atom effect, to the determination of tryptophan metabolites. Using ethanol/ethyl iodide in a 4/1(v/v) mixture (EEI), these authors found significant

enhancement in the phosphorescence emission intensities for several tryptophan metabolites.

Several very interesting analytical applications of the external heavy atom effect have appeared recently in the literature. Boutilier and Winefordner[190] studied the external heavy-atom effect on detection limits and lifetimes of phosphorescence in time resolved laser excited phosphorimetry for several polycyclic aromatic hydrocarbons and drugs at 77K in 10/90(V/v) ethanol/water with no heavy atom, with 0.75M KI, and with 0.1M $AgNO_3$. They also studied the influence of type and concentration of external heavy atoms upon phosphorescence lifetimes for phenanthrene, carbazole, quinine, 7,8-benzoflavone and thiopropazate at 77K. Some effects are still poorly understood.

## 4.3. Room Temperature Phosphorimetry

Jakovljevic[191] described the use of lead or thallium salts as external heavy-atoms as a new technique to enhance room temperature phosphorescence. He claimed that it was possible to quantitate as little as 50pg, of the antibiotic cinoxacin. Thin-layer phosphorimetry can also be used for observations of room-temperature phosphorescence (R.T.P.). This phenomenon was apparently first observed over 30 years ago, but has only recently been used as an analytical technique[192]. Vo Dinh et al[67] have studied the room temperature phosphorescence of several hydrocarbons in suitable experimental conditions. They also studied the external heavy-atom effect and found it very effective in inducing phosphorescence emission. Room temperature phosphorimetry has advantage over low temperature phosphorimetry, in that there is no need for liquid nitrogen to be used as a coolant. With these advantages R.T.P. might become a routine method

of analysis, though many aspects of the phenomenon remain poorly understood.

## Conclusions

The work in this thesis describes the applications of the thin-layer phosphorimetric method for the analysis of drugs, their metabolites and polycyclic aromatic hydrocarbons (PAH). The luminescence properties of several classes of drugs have been studied and efforts have also been made to improve techniques used in phosphorimetric analysis.

Several materials in conventional use in thin layer chromatography were used in the study including paper, and aluminium backed cellulose, silica gel and HPTLC layers. All were found to allow the measurement of phosphorescence from adsorbed organic samples. The background phosphorescence of the supports was found to vary being minimal for cellulose and HPTLC layers. Silica gel layers were found to give a relatively high phosphorescent background, paper was found to have the highest phosphorescent background.

In the study of 6-mercaptopurine and its metabolites it was shown that it was possible to determine some of these compounds at sub-nanogram levels in blood plasma at 77K, and at nanogram levels at room temperature. These detection limits were low enough to enable the method to be considered as a routine means of determining these drugs in blood plasma.

In the study of the phenothiazine family of drugs, the phosphorimetric limits of detection on thin layers were found to be at nanogram levels (on cellulose layers) or sub-nanogram levels (on HPTLC layers). These results were obtained with the aid of the

heavy-atom effect at 77K. Sensitivity was sufficient when low temperature phosphorescence was being measured to allow this to be the basis of an analytical method for the determination of phenothiazines.

In the study of thiobarbiturates it was shown that, some showed fluorescence and some showed phosphorescence in different pH media at 77K. The complementary nature of fluorescence and phosphorescence studies thus become evident.

Thin layer phosphorimetry was useful for the determination of phenylhydantoin after oxidation to benzophenone. The limit of detection was at nanogram quantities on cellulose and HPTLC layers.

In the examination of the phosphorescence and fluorescence characteristics of polycyclic aromatic hydrocarbons at 77K there was a high degree of similarity between individual members of the group in fluorescence emission, but not in phosphorescence emission. Limits of detection were often at subnanogram levels which enabled the method to be considered as a means of determining atmospheric concentrations of these hydrocarbons.

In all cases the use of a solvent to enhance the phosphorescence signal was found to be absolutely necessary in order to obtain low detection limits at 77K.

The application of this phosphorimetric technique in a wide range of fields, particularly in the analysis of small quantities of drugs and their metabolites present in complex mixtures has thus been validated.

## Suggestions for further work

The speed of the technique could be improved by enlarging the drum of the thin layer phosphorimetry to accommodate more samples. Also further investigation on the heavy-atom effect is needed, to find out whether or not it is specific for a particular group of compounds.

Also further investigation to quantify the precision, reliability and detection limits on other thin layers should be made. The thin layers are: reversed-phase plates, plates with concentrated zones and cellulose HPTLC.

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