SOME DEVELOPMENTS IN THE PHOSPHORIMETRIC ANALYSIS OF DRUGS

by

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This is to certify that except where otherwise stated the work described in this thesis is believed to be original. It has not been submitted, in full or in part, to this or any other institution for a degree.

TO MY PARENTS

For their never failing help, guidance and faith in me over the years.

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CHAPTER 1

INTRODUCTION

Luminescence is the term given to any phenomena which pertains to the emission of light other than as a direct result of an increase in temperature (i.e. incandescence). Luminescence can be subdivided into ten or more types depending on the specific cause of emission, such as triboluminescence, bioluminescence, candoluminescence and photoluminescence. The latter is the only one with any direct relevance to this work, and can itself be subdivided into phosphorescence and fluorescence.

A Brief History of Luminescence

The first recorded observation of luminescence appears to have been made in the Shih Ching or 'Book of Odes', which was written around the 11th Century B.C. and makes reference to the light from glow-worms and fireflies. Somewhat later in the 3rd Century B.C., Aristotle wrote of light being emitted from decaying fish.

In our own civilisation, the first record of a serious interest in luminescence is that of Vincenzo Cascariolo, an Alchemist who lived near Bologna 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. As the substance produced was

of a spongy nature, it was originally assumed that this 'sun stone' or lapis solaris simply absorbed light like a sponge and then re-emitted it slowly in the dark. This idea was discredited in 1652 by Nicolas Zucchi, who showed that the colour emitted during phosphorescence was the same no matter what the colour of the exciting light. That appears to be the last important discovery concerning phosphorescence that was made for 200 years until Stokes in 1852 showed that the wavelength of emitted light was always equal to or greater than the wavelength of the exciting light.

In a book published in 1867[1], Becquerel described the use of a mechanical device or phosphoroscope, which enabled the separation of phosphorescence from scattered incident light and fluorescence. Then in 1888 Weidmann[2] demonstrated 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 opening up the possibility of determining the phosphorescence of a wide range of organic compounds.

It was not until 1944, however, that Lewis and Kasha[4] established the now accepted triplet theory of phosphorescence. Then in 1957 Kiers, Britt and Wentworth[5] published a paper which evaluated the possible use of phosphorescence measurements as an analytical technique, and concluded that, although it had a place in analysis, it was not a method of first choice. By the mid 1960's papers were being published on the analysis of drugs in both blood and urine, and now compounds from almost every major group of drugs, pesticides and carcinogens have been determined by phosphorimetry.

The processes of phosphorescence and fluorescence

The processes that can occur when a quantum of light is absorbed by a diatomic molecule are shown in Fig.1; comparable processes occur in more complex molecules. When a molecule absorbs a quantum of light it moves from a lower to a higher electronic energy level, the difference in levels being equivalent to the energy possessed by the light. The energy of a quantum of light can be determined by the application of the Planck-Einstein relationship:

$$E = h\nu = \frac{h\,c}{\lambda}$$

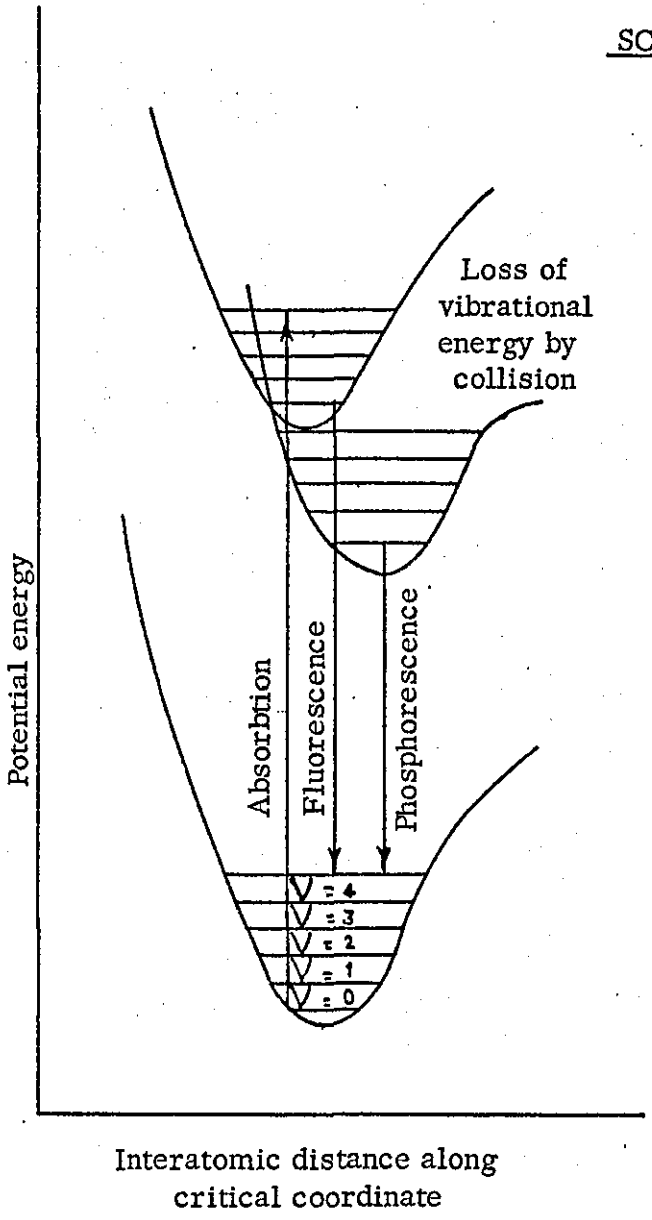
where E is the energy, h is Planck's constant, ν is the frequency, c is the velocity of light and λ is the wavelength.

As can be seen from the diagram there are various excited electronic energy levels possible and these are termed excited singlet or excited triplet states. In addition to this there are various vibrational levels, for each excited state. The difference in the singlet and triplet states are the differences in electron spin. 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

SCHEMATIC ENERGY LEVEL DIAGRAM



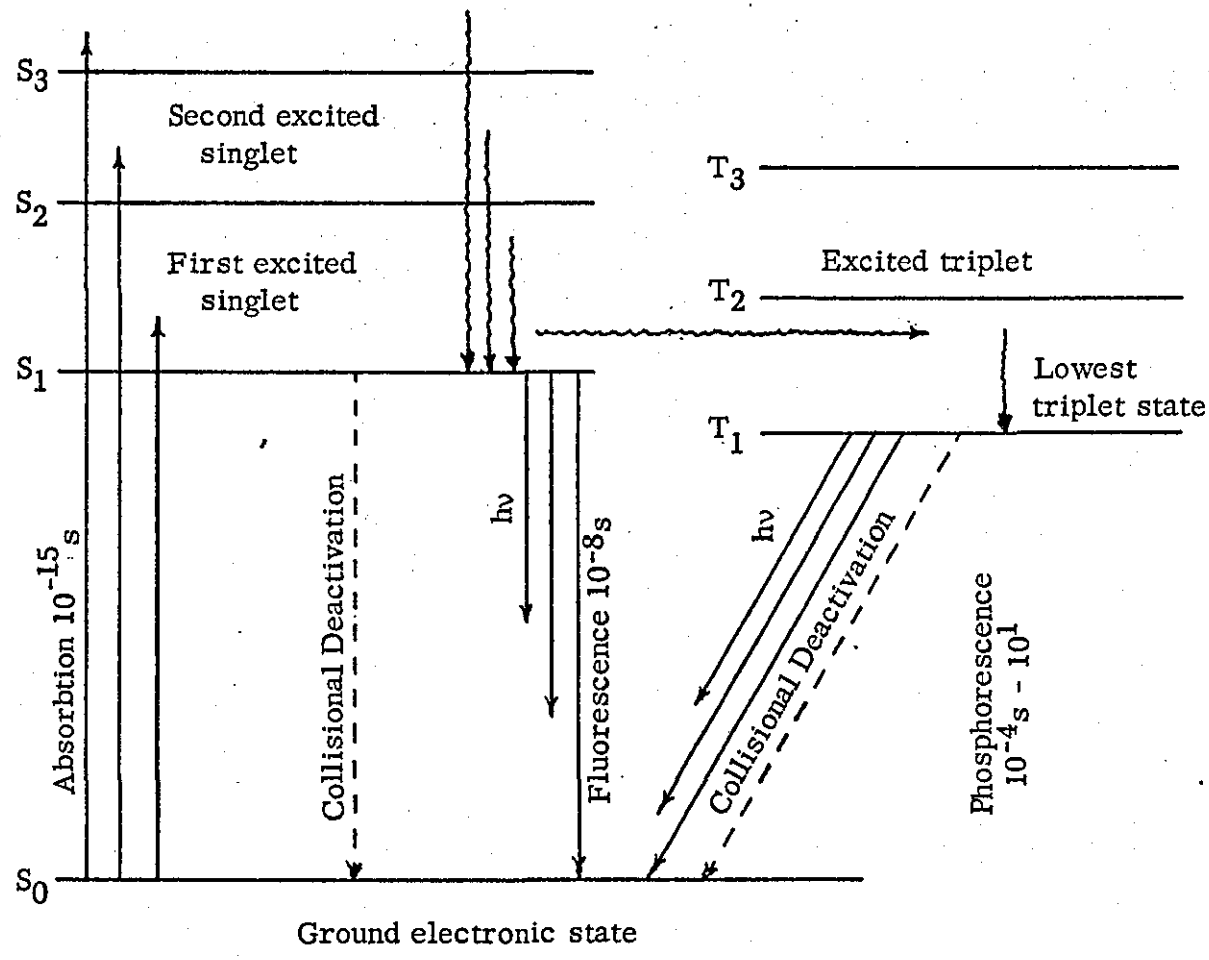


FIG. 1.

electrons with parallel spins i.e. two unpaired electrons, S will be 1 and M will consequently be 3, this state being described as the triplet state.

When a molecule is raised from the ground state to a higher energy level by the absorbtion of a quantum of light, it will then rapidly revert to the lowest vibrational level of the first excited singlet state. From here, one of several things may happen; the molecule may return to the ground state by collisional deactivation, in which case nothing will be observed, or it may return by the emission of energy (fluorescence). A consideration of the process involved will make it clear that the energy of the fluorescence must almost always be lower, and therefore of greater wavelength, than the exciting light. A third possibility is that a process of intersystem crossing occurs and the molecule goes into a triplet state. Deactivation from the triplet state takes place in the same way as from the singlet state i.e. either by collisional deactivation or by the emission of energy (phosphorescence). In the case of phosphorescence though, the time required for the transition to occur is much greater than the time required for collisional deactivation. Therefore, in solutions at room temperature, the predominant means of relaxation will be by collisional deactivation. In order to see phosphorescence, it is normally necessary to dissolve the substance in a rigid media and/or use low temperatures. Again, the wavelength of phosphorescence will be greater than that of the exciting radiation, and it will also be greater than that of the fluorescence.

Types of phosphorescence

It is usual to divide phosphorescence into 2 types, depending on the nature of the triplet state. These are classified as n, π^*. (n or non-bonding to anti π bonding orbital) or π, π^* (π bonding to anti π bonding orbital) transitions. The assignment of a particular triplet state as either n, π^* or π, π^* is, in practise, based on several criteria, but it can still be difficult on occasions. Table 1 shows several such criteria that can be used to aid this assignment.

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.

Measurement of phosphorescence

The basic instrumentation required for the measurement of phosphorescence is very similar to that used for the measurement of fluorescence. It normally consists of a light source, a monochromator, the sample, another monochromator, a detector and some form of readout. The choice of light source is quite varied and depends largely 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. Probably the most commonly used light source in commercial instruments is the high-pressure xenon-arc lamp, which has a high, fairly uniform output well into the U.V. but does suffer somewhat from stability problems.

TABLE 1

Characteristics of π, π^* and n, π^* states

Property	T π, π^*	T n, π^*
Lifetime of phosphorescence	> 1 s	$< 10^{-1} - 10^{-2}$ s
Polarisation of 0-0 phosphorescence bond	Predominantly out of plane	Predominantly in-plane
ϕ_P/ϕ_F ratio	variable	usually very high
Shift in absorbtion spectra from polar $\longrightarrow$ non-polar solvent	small, usually hypsochromic	quite definite, bathochromic
Heavy atom effect on frequency of T $\leftarrow$ S_o transition	increase, sometimes large	very small
Heavy atom effect on phosphorescence lifetime	decrease	very small

Dispersion of the spectra is obtained using 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 from other sources. If the monochromators are capable of providing continuous spectra (e.g. if prisms or diffraction gratings are used) then they can also be used to obtain the phosphorescence absorbtion and emission characteristics of the sample. The phosphorescence is usually measured at right angles to the incident light, because its intensity will normally be independent of the direction of measurement, whereas scattered light will be minimal at right angles to the exciting light. Therefore, this optical arrangement gives the best signal to noise ratio. The detector is nearly always a photomultiplier tube, but recently photon-counters have started to be used in commercial instruments as well as in research built instruments[6]. Readout is normally by meter or chart recorder.

The sampling devices used in phosphorimetry are worth considering in greater detail. Traditional systems consist of a Dewar flask which is filled with liquid nitrogen and used to cool a narrow silica tube containing the sample (Fig.2). Cooling is either by conduction using a copper rod or, more usually, by immersion. In the latter case all parts of the Dewar which are in the light beam have to be made of high quality silica in order to transmit U.V. radiation. Both methods have their disadvantages. With the former, uniform cooling is difficult owing to problems in maintaining good thermal contact between the copper and the sample tube and a long time is required for cooling. With the latter, there are 12 silica-air or

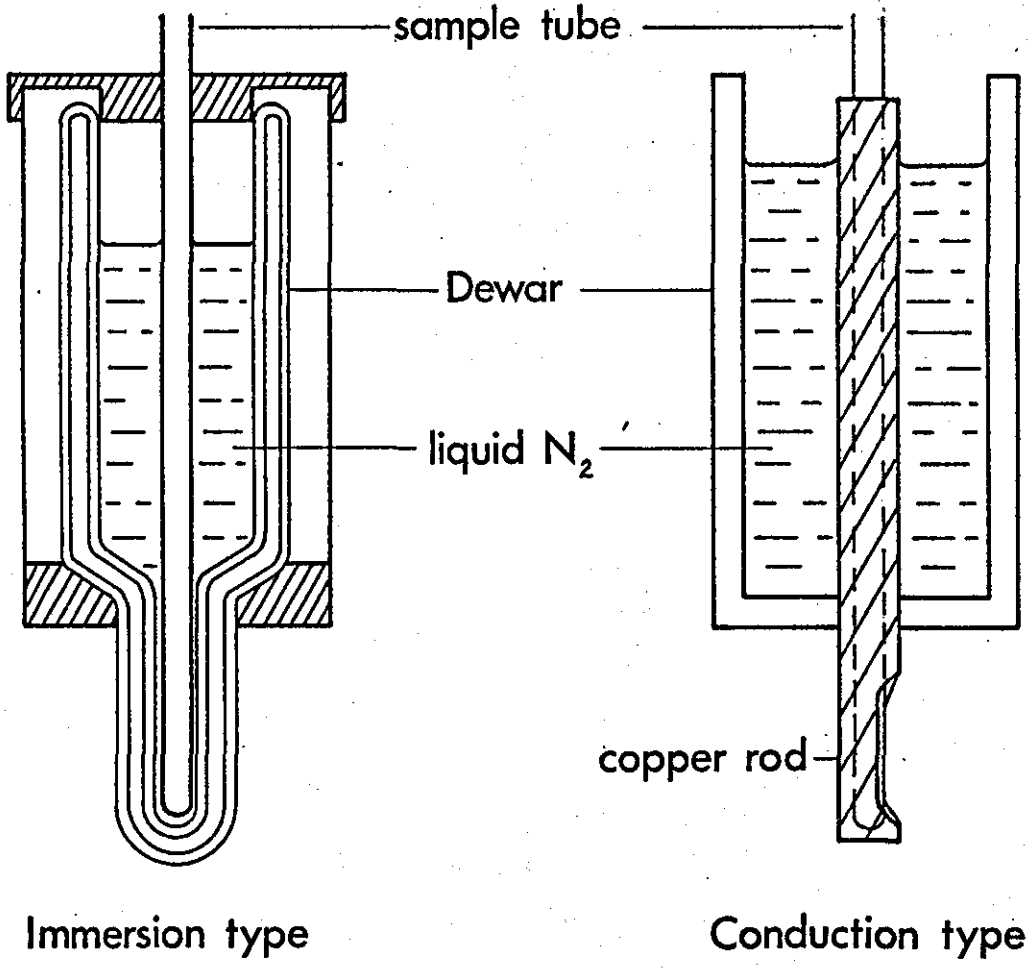


Fig. 2 Types of cooling devices for frozen solutions

silica-vacuum interfaces at which light losses occur, bubbling of the nitrogen can cause high levels of noise and it is difficult to align the sample tube reproducibly. The latter problem has been alleviated to some extent by the introduction of a spinner assembly for the sample tube[7]. 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%.

A more recently described sample cell[8] consists of a copper rod containing a shallow well in which the sample is placed. The rod is cooled by conduction and excitation and emission is on the front surface. Another recent innovation[9] is a scanning thin-layer device which allows the measurement of samples on 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.

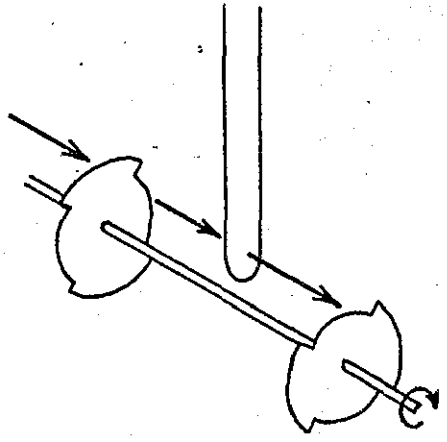
Until the introduction in 1972 of thick-walled capillary tubing, which permits the use of aqueous solvents, [10, 11] all solvents had to be capable of forming clear, uncracked glasses at 77°K. This gave considerable problems in terms of solubility, homogeneity and solvent purity. Many complex mixtures were used to overcome the problem of solubility,[12, 13] but their use can cause as many problems as they solve[14]. On cooling, the various components can separate out at different rates, leading to a non-uniform matrix and a consequent uneven distribution of the sample.

Another approach to the problem is to measure the phosphorescence at room temperature, having previously bound the sample molecules in or on to a solid matrix[15]. There appeared to be little interest in this approach until 1972 when Schulman and Walling described the phosphorescence of ionic organic molecules adsorbed on to paper[16]. Since then

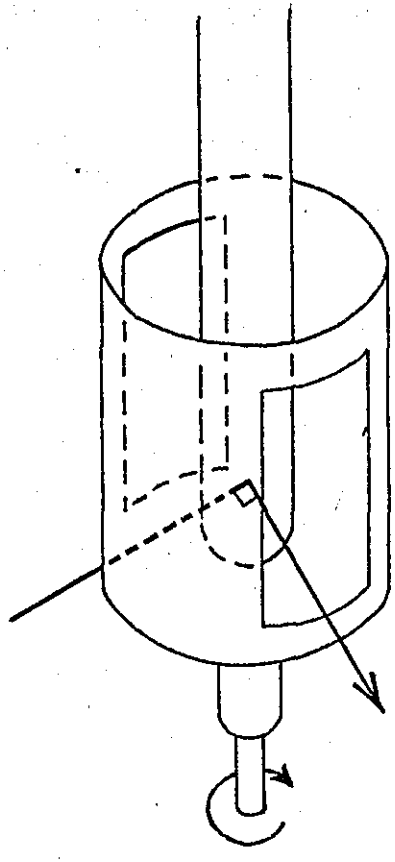
several papers[17-20] have been published on the subject. However, the first reference to room temperate phosphorescence by adsorbtion on to paper appears to have been made by Roth in 1967[21].

In order to measure phosphorescence in the presence of fluorescence and scattered light it is usual to fit a phosphoroscope in the light path. The phosphoroscope may be mechanical or electronic in nature, but all types operate by modulating both the exciting and emitting light in such a way that during one phase the sample is being irradiated but no light reaches the photomultiplier, and during the second phase no light falls on the sample but the long-lived radiation (phosphorescence) can reach the photomultiplier.

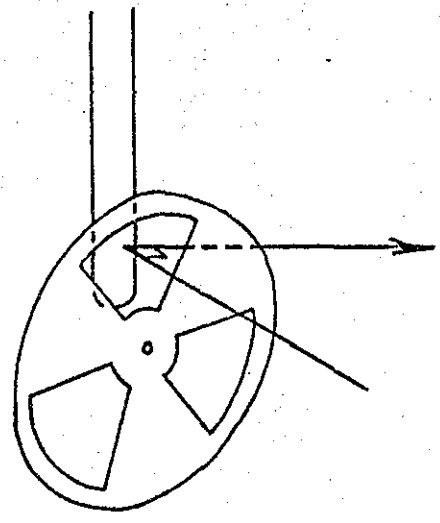
The major types of mechanical phosphoroscope are shown in Fig.3. The earliest was the Becquerel disc phosphoroscope[1] which uses two notched discs rotating on a common axis. A more convenient type from an instrumental design point of view is the rotating-can phosphoroscope, first suggested by Lewis and Kasha[4]. Unfortunately it is not as efficient in terms of the measured phosphorescence signal from a given standard, as was shown by O'Haver and Winefordner[22]. The third type of mechanical phosphoroscope is the single disc one, which has been used in several forms[9, 23, 24]. The disc shown in Fig.3. is of the type used in the thin-layer scanner described previously. The same out-of-phase modulating effect can be obtained electronically, and this has been done either by using a pulsed-source and gated detector[25, 26] or by mechanically 'chopping' the exciting light and using a gated detector[27]. It should also be possible, and probably highly desirable since the introduction of a relatively cheap pulsed-source fluorimeter, to use a pulsed-source and mechanically 'chopped' detector, although nothing appears to have been published yet which describes the use of this combination. Electronic modulation has the advantage of allowing



Becquerel disc
phosphoroscope



Rotating can
phosphoroscope



Single disc multi-slot
phosphorimeter

Fig. 3 Types of Phosphoroscopes

much more of the potentially available phosphorescence to reach the detector, but the disadvantages of being more expensive and not being commercially available.

The range of analytical applications of phosphorimetry

Since a large proportion of all aromatic organic compounds show phosphorescence under suitable conditions, phosphorimetry can be used in most fields that require the trace analysis of organic compounds. It has found use in the coal tar and petroleum industries. The problem there is one of identifying the large numbers of organic compounds present in the various fractions which are produced by distillation, whose identity in many cases are still unknown. Early work was carried out by Mamedov[28] and Khaluporskii[29], who examined aromatic hydrocarbons in wax distillates and luminescent compounds in lubricating oils. Other work was carried out in the early 1960's by Sidorov and Rodomakina [30] who showed the various applications of phosphorimetry to the analysis of petroleum products. More extensive studies were carried out by Drushel and Sommers, who studied in excess of 100 compounds[31] and proposed a complete separation and identification scheme for nitrogenous compounds obtained from petroleum[32]. Zander[33, 34] published methods for the determination of aromatics in coal tar fractions and gave the spectroscopic method of choice for each compound studied.

Another field in which phosphorimetry is of value is the determination of pesticides and other pollutants that may be present in the environment, in man or in other living organisms. Most of the studies of atmospheric pollutants by phosphorescence have been carried out by Sawicki and his various co-workers[35-40], using both conventional

phosphorimeters and by visual observation of the compounds on developed TLC-plates after placing them in liquid nitrogen. Detection limits as low as 1 ng/spot were obtained with suitable compounds. McCarthy and Winefordner[41] described a full analytical procedure for the determination of biphenyl in citrus fruits and Moye and Winefordner[42] gave the phosphorescence characteristics of a large number of pesticides and then went on to apply the method[43] to the determination of p-nitrophenol in urine, and halogenated biphenyls by pulsed source, time resolved phosphorimetry[44]. Martin has described the quantitative determination of several chlorinated organophosphorus[45] and p-nitrophenol based organophosphorus pesticides[46]. Detection limits varied between 4 and 100 $ng.ml^{-1}$ with the exception of morocide and karathane, whose limits were about 1 $\mu g.ml^{-1}$.

A third field in which phosphorimetry has been found to be of great use is that of biochemistry. It was in this field that the first analytical application was produced, namely that of Rybak et al.[47] in 1955 for the analysis of amino acids. Since that first paper a large amount has been published on the phosphorescence characteristics of both amino acids and proteins e.g. (48, 49). Several authors[50-52] have studied the relationship between the phosphorescence of individual amino acids and the phosphorescence of proteins. Other biologically active or important compounds have also been assayed phosphorimetrically, such as indoleacetic acid, serotonin[53] and some of the vitamins[54, 55].

The fourth and by far the largest use of analytical phosphorimetry is for the measurement of drugs and related substances either in body fluids or present in trace amounts elsewhere. The first compound to be analysed in body fluids was aspirin, which was measured in both serum and plasma by Winefordner and Latz[56]. They not only

measured aspirin, but 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 $\mu g.ml^{-1}$ blood or higher. Extraction was by a single volume of acidified chloroform and this procedure gave recoveries of between 76% and 133%. Winefordner and Tin[57] then produced a method for the extraction and measurement of procaine, phenobarbitone, cocaine and chlorpromazine in serum and for cocaine and atropine in urine. They gave analytical curves for each compound and percentage recoveries for each drug from 'spiked' samples. In the same year Hollifield and Winefordner[58] described the phosphorescence characteristics of codeine, morphine, papaverine and a number of other pharmacologically important alkaloids, but did not measure them in body fluids. In 1966 a study of the effect of pH on the phosphorescence of ether extracts of blood and urine was published[59]. 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. Unfortunately, but as might be expected, most compounds including drugs are best extracted at pH's where the phosphorescence is highest.

The Sulphonamide group of drugs have been quite thoroughly

studied, including the effect of various solvents on the phosphorescence signal[60] and the relationship between structure and luminescence characteristics[61]. Of the fifteen sulphonamides studied by Hollifield and Winefordner[62], thirteen gave detection limits equal to or better than 1 $\mu g.ml^{-1}$. When spiked serum samples were used recoveries ranged from 92-105% and there was a relative standard deviation of about 5%. A later paper by Venning et al.[63] using mixed aqueous solvents gave similar limits of detection but used a smaller sample volume.

Several groups of workers have also studied the barbiturates [57, 64-66] and detection limits in ethanediol : water are given. In this laboratory[66] the relationships between molecular structure and luminescence properties under varying conditions of pH have also been studied.

The accurate measurement of minute quantities of cannabinols, lysergic acid derivatives and other hallucinogens has become of great importance over the last few years, owing to an increase in non-medical usage of these drugs and the consequent need for identification in cases of suspected illegal possession. Phosphorimetry is one of the techniques that has been used in an attempt to solve this problem[67-69]. Aaron et al.[68] studied the fluorescence and phosphorescence characteristics of a variety of hallucinogens and gave limits of detection for both types of luminescence. For Bufotenine this was as low as 1 ng ml^{-1}. They also studied the effect of external heavy-atoms and various pH's on the luminescence signals produced. The separation of a mixture of hallucinogens is described by Fabrick and Winefordner[69], but detection limits were much higher than previously described, even though the work

was carried out in the same laboratory in both cases.

Other groups of compounds to have been studied phosphorimetrically include the antihistamines, the properties of which have been described by Schenk and his co-workers[70, 71], the anticoagulants[72], griseofulvin[73] and diphenylhydantoin[74].

Two recent reviews on the subject of phosphorimetry as an analytical technique are 'Phosphorimetry, a spectrochemical method of analysis'[75] and 'The potential of phosphorescence spectrometry in clinical chemistry - the new generation of instrumentation and methodology'[76].

Scope of the present work

When this work was started phosphorimetry had already been shown to be a sensitive and selective technique, capable of being applied in many fields where the determination of small quantities of organic compounds was required. Despite this, phosphorimetry was far less popular as an analytical method than might reasonably be expected from a consideration of its advantages. The main reasons for this are almost certainly the cost of the equipment required, the difficulties in sample handling and the problems associated with availability and use of liquid nitrogen.

The aim of this present work was to reduce or eliminate as many problems as possible associated with current phosphorimetric techniques, and to show further the general applicability of the method. To this end a filter fluorimeter was modified so that it could be used for either fluorimetry or low-temperature luminescence studies. Its potential as a phosphorimeter was then assessed by

comparing its sensitivity to a commercially available spectrophosphorimeter. The thin layer phosphorimeter mentioned earlier[9] was developed to allow it to be used in currently available commercial instruments, to make it easier to use and to increase the precision of results obtained by it.

Finally, groups of drugs and related compounds not previously studied by this technique were examined, to ascertain the potential of phosphorimetry as a means of determining them in low concentrations. Attempts were made to correlate the phosphorescence data obtained with the molecular structure of the compound being studied, in order to increase our knowledge of the basic processes involved in the production of phosphorescence.

CHAPTER 2

Design and evaluation of a filter phosphorimeter

Introduction

The first work to be carried out in analytical phosphorimetry was performed using home-built apparatus, often using white light for excitation[56]. Once the technique became established, manufacturers began to offer accessories which would allow their spectrofluorimeters to be used as phosphorimeters. From then on most of the published work involved the use of either expensive, commercially available equipment, or of highly sophisticated and probably even more expensive, privately developed instruments. One manufacturer (Aminco) has offered phosphorimetric attachments for use on their filter instruments, but Guilbault[77] has reported very poor results when used in his laboratory.

It was the knowledge that almost all phosphorimetric analysis was being carried out using very expensive equipment that led to this attempt to modify a relatively inexpensive filter fluorimeter for phosphorimetry. The aim was to provide a simple to operate instrument which would allow phosphorimetry to be used by people working on a fairly small budget. In order to ascertain how useful the instrument would be, it was compared with a Baird-Atomic 'Fluorispec' using a range of compounds.

Instrumental modifications

The instrument used in this study was a Model 244 Corning-Eel fluorimeter which incorporates tungsten and mercury lamps as alternative light sources, and filters to isolate appropriate excitation wavelengths. Luminescence signals are detected at right angles to the incident beam by a R268 photomultiplier, a movable interference wedge (half-wave bandwidth 13 nm) being used to isolate the luminescence signal. The principle modification to the instrument involved machining a new sample block (Fig.4.) which permitted the location of a rotating cylinder phosphoroscope, and a silica Dewar flask which could accommodate silica sample tubes of up to 6 mm external diameter. The need for a Dewar flask which could accommodate 6 mm sample tubes was to allow aqueous solvents to be used, as suggested by Lukasiewicz et al.[10, 11]. (If the exclusive use of non-aqueous solvents was deemed to be satisfactory, then a commercially-available Dewar flask with narrower stem could be incorporated into the Model 244 fluorimeter by performing only minor modifications to the sample block originally fitted to the instrument). The new block was machined out of aluminium alloy and given a black anodised finish. The tapped holes, used for connecting the block to neighbouring components were put in the same relative positions as in the original block: for the sake of clarity, only the phosphoroscope retaining screw is shown in Fig.4. Because the centre of the exciting light beam was moved approximately 3 mm to the right in the modified instrument, the lamp adjustment slots at the rear of the instrument were extended to facilitate optimal positioning of the light source.

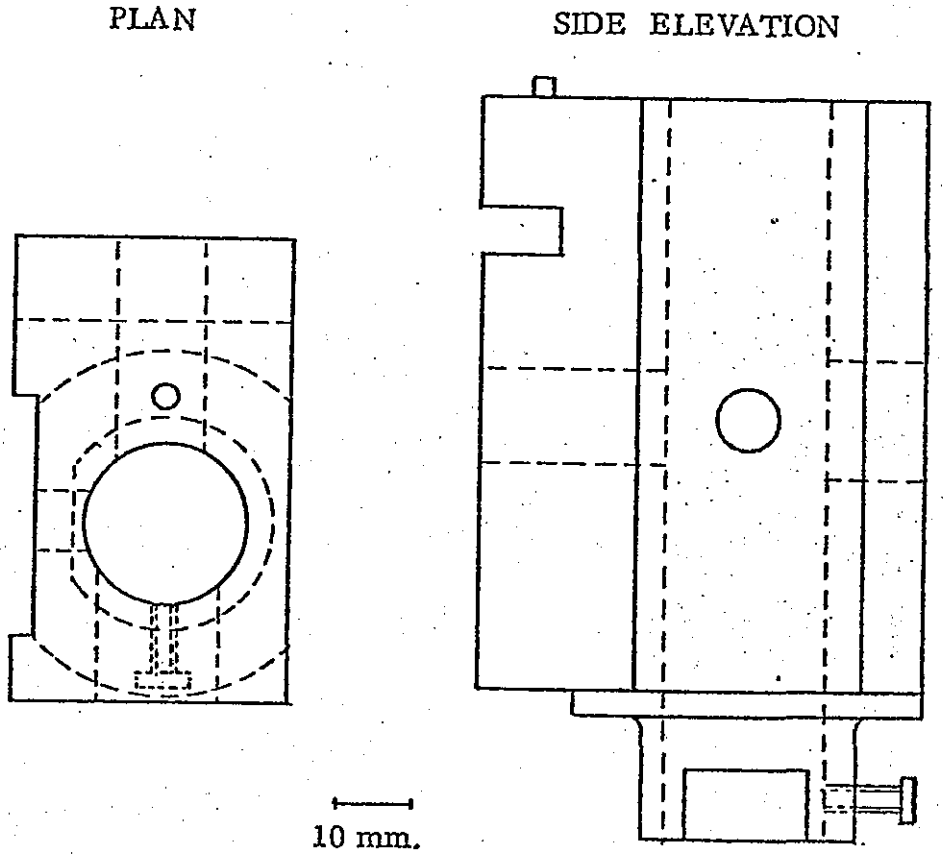


FIG. 4

The new sample block

The anodised phosphoroscope cylinder (Fig.5A) was turned out of a piece of aluminium rod and two slots, each subtending an angle of 45° at the centre, were milled out. The cylinder was fitted to the shaft of a 12 volt, 20,000 rpm motor (Faulhaber motors, Portescap U.K. Ltd.) which could be operated at different speeds using a variable transformer. A 'spectrosil' Dewar flask, (Fig.5B) with its upper half silvered and encased in a light gauge metal case packed with expanded

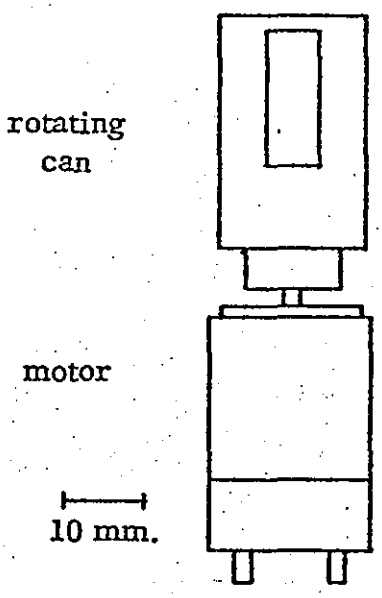


FIG. 5A

Rotating can phosphoroscope

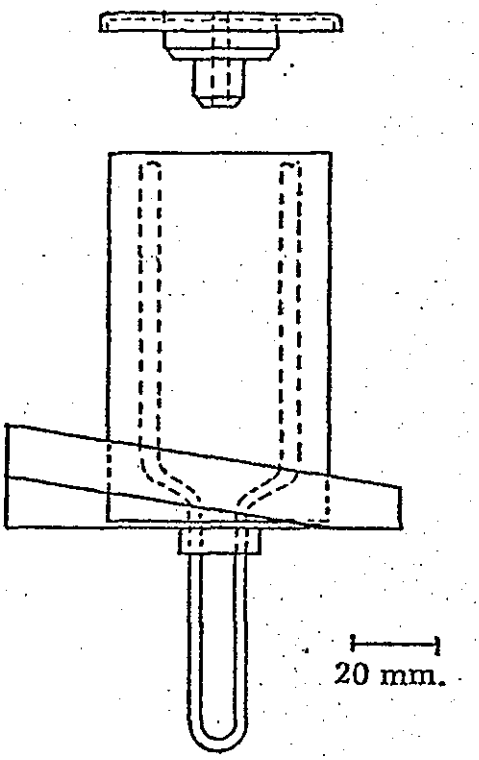


FIG. 5B

Dewar and lid assembly

polyurethane, could be mounted in the sample block. An assembly to permit rotation of the sample tube[7, 8] was not fitted in this instance, although this could easily have been added had it been considered necessary. The sample chamber aperture in the top of the instrument was widened to accommodate the Dewar flask, a flat metal plate with felt on its underside and fitted with two locating pins forming an effective light-tight door.

For fluorescence measurements at room temperature a flanged perspex sleeve was constructed so that the original cylindrical sample tubes could be mounted centrally in the modified block. A blanking plate was also made to cover the space in the top panel normally

MODIFIED RECORDER OUTPUT

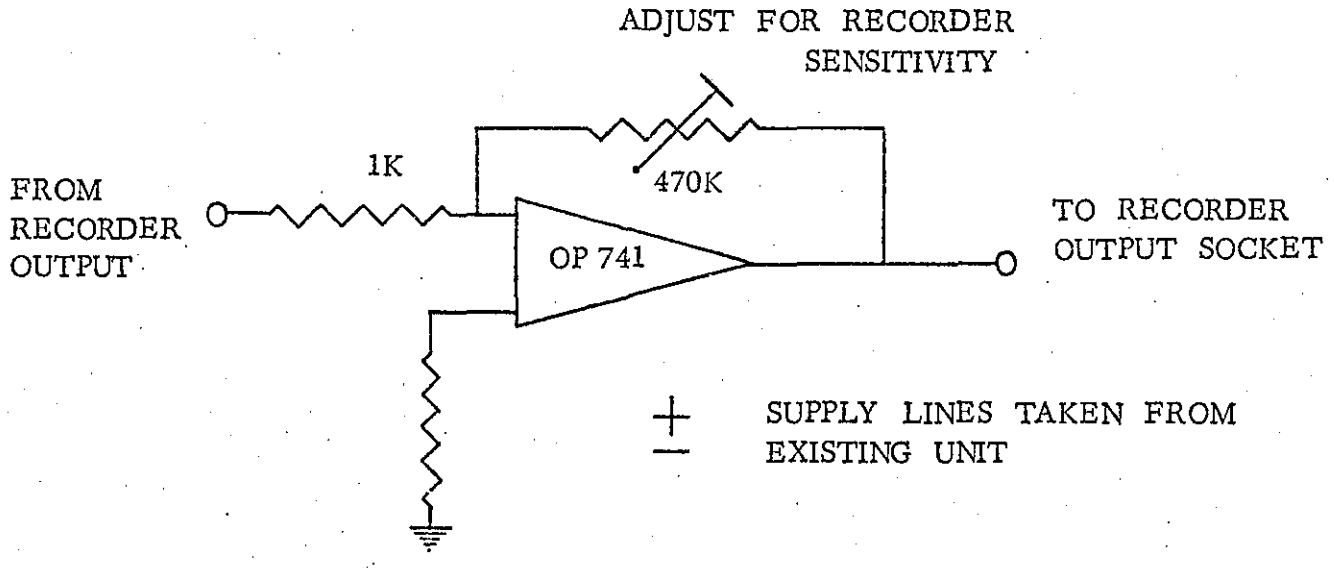


FIG. 6

occupied by the Dewar. The conversion of the instrument from low temperature to room temperature measurements, and vice versa, could be carried out in less than one minute.

The recorder output was modified as shown in Fig.6. The purpose of this was to increase the output to the Bryans 27000 recorder from 10 mV to 1 v, in order to reduce the effect of recorder noise on the trace.

Experimental procedure

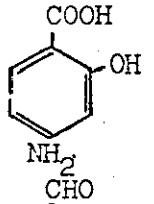
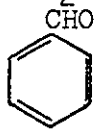
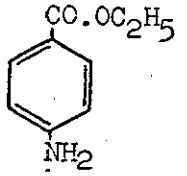
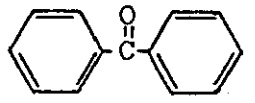
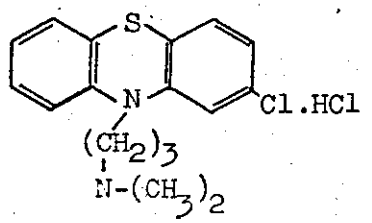
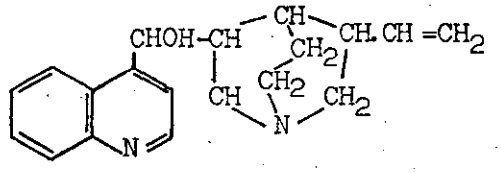
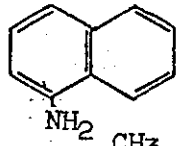
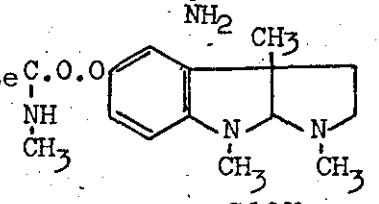
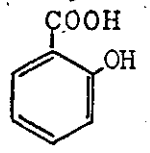
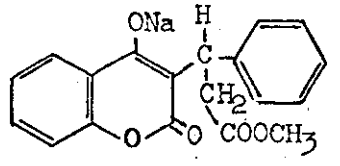
The compounds studied using this instrument are shown in Table 2.

All the compounds were found to melt or boil within 2% of the literature values. A.R. grade ethanol was obtained from James Burroughs Ltd. and used without further purification.

The mercury vapour lamp was used for all the experiments described, as was an excitation filter consisting of an Eel OX1 filter and 1 cm of 50% w/v nickel sulphate in water, contained in a quartz cuvette. This combination had a maximum transmission of 28% at 323 nm, with a half wave bandwidth of 38 nm. The overall stability of the instrument (a combination of light output, photomultiplier sensitivity and amplifier stability) was determined at both room and liquid nitrogen temperatures. For the room temperature determination a solution containing 0.1 $\mu g\ ml^{-1}$ quinine sulpate in 0.1N sulphuric acid was used and the instrument sensitivity was adjusted to give a meter reading of half its full scale deflection. For the low temperature determination A.R. ethanol in a closed end Spectrosil sample tube (4 mm external diameter, 2 mm bore) was used. This represented a

TABLE 2

Compounds studied and source of sample

Compound	Structure	Source
p-aminosalicylic acid		Sigma Chemical Co.
Benzaldehyde		Fisons Scientific Apparatus
Benzocaine		B.D.H. Ltd.
Benzophenone		B.D.H. Ltd.
Chlorpromazine HCl		Gift of Pharmaceutical Specialities (May and Baker) Ltd.
Cinchonidine		Sigma Chemical Co.
1-Naphthylamine		Fisons Scientific Apparatus Ltd.
Physostigmine sulphate		Macfarlan Smith
Salicylic acid		Fisons Scientific Apparatus
Warfarin sodium		Sigma Chemical Co.

'blank' reading as all subsequent determinations were carried out in ethanolic solution using this sample tube. Again, the instrument sensitivity was adjusted to give a 50% meter reading.

The detection limits of a series of compounds were determined phosphorimetrically using this instrument. The phosphoroscope was used in all cases (at a speed of 4,350 rpm) as it had been found that a much higher signal to noise ratio could be obtained with it in place, probably as a result of it reducing the amount of scattered light reaching the photomultiplier tube. Stock solutions were made up containing 100 μg of compound per ml of ethanol, and these were then serially diluted, normally to give final concentrations of 100, 10, 1, 0.1 or 100, 30, 10, 3, 1 μg ml^{-1}. Three readings were taken at each concentration and six 'blank' readings were measured at various times during the determination, in order to detect any long term drift in the instrument. Some fluorimetric detection limits were also determined with this instrument, using a similar technique to that just described. The differences were that a 1 cm diameter cell was used, and the solvent for salicylic acid and p-aminosalicylic acid was triply distilled water and for cinchonidine 0.1N hydrochloric acid. The phosphoroscope was of course taken out of the light path for all these determinations.

The results obtained were compared either with work already published by a variety of authors or with those obtained using a Baird-Atomic SF100 spectrofluorimeter fitted with the manufacturer's phosphorimetric attachments and modified by the addition of a rotating sample tube (Fig.7).

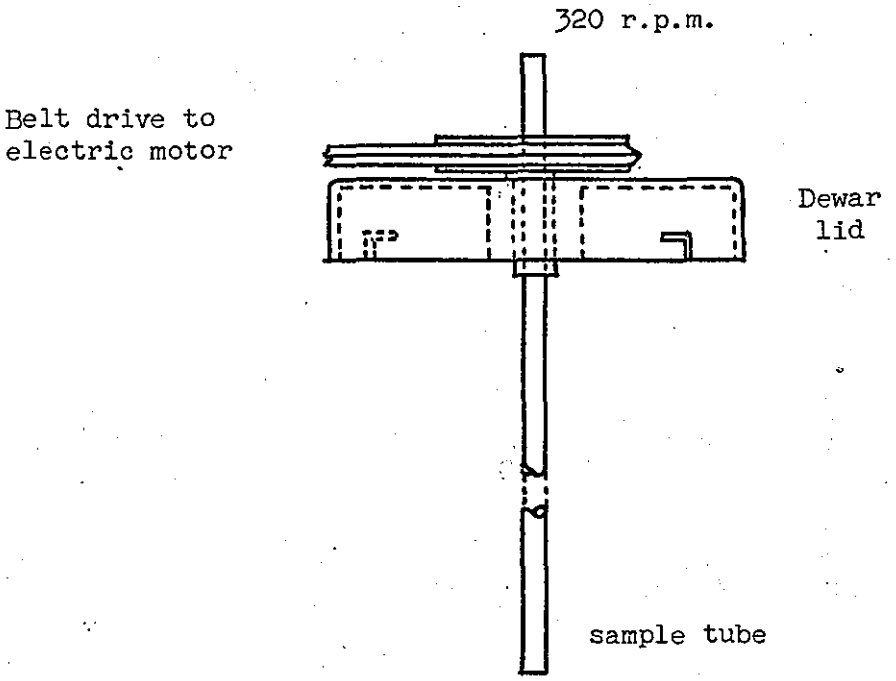


FIG.7 Rotating sample tube attachment .

A half wave bandwidth of 20 nm was used for all readings on the SF100. When this instrument was being used for fluorimetric determinations, the 1 cm diameter cell used with the Eel 244 was fitted, to eliminate any error due to variation in transmission characteristics. A Bryans 26000 A3 X-Y plotter was used to record the results.

Results and discussion

The stability of the instrument was found to be excellent at both room and at low temperatures. At room temperature the output varied by less than ± 3% over a period of 4 hours, once the instrument had achieved stable operating conditions.

At low temperature, the variation was ± 4% over a period of 2 hours and there was no visible signs of condensation or ice on the outer surface of the Dewar flask.

The phosphorimetric detection limits and fluorimetric detection limits obtained using this instrument are shown in Tables 3 and 4 respectively. It is evident from the results that when used as a phosphorimeter the EEL 244 is inferior in sensitivity to grating instruments by at least an order of magnitude, whereas when it is used as a fluorimeter, the difference is somewhat less. The poor phosphorescence detection limit for benzaldehyde is probably a consequence of its excitation wavelength, 33 nm below the optimum transmission wavelength of the primary filter. The warfarin detection limit is better on the filter instrument than on the grating instrument because the latter is a detection limit for the compound after it has been extracted from plasma, which gives it a much higher phosphorescence background.

The superior fluorescence detection limits are harder to explain, but whatever the causes are, they must be related to the Dewar flask, sample tube or solvent, as these are the only significant changes made. One possible reason is that the broader excitation band of the filter instrument could excite more phosphorescent impurities in the ethanol which would lead to a lowering of the signal to background ratio. Another possibility is that it is related to the reduction in available light when the narrow sample tubes are used. The available light is reduced by approximately 80% when a 2 mm diameter tube is used, because the exciting light beam is 12 mm in diameter. The available light is not reduced using a grating instrument because the incident light has already passed through a vertical slit of less than 1 mm width.

One advantage of this instrument which cannot be seen directly

TABLE 3

Phosphorimetric detection limits determined on filter and grating phosphorimeters

Compound	Excitation maximum, nm	Phosphorescence maximum, nm	Phosphorescence lifetime, s	Limits of detection, $\mu g.ml^{-1}$	
				Filter instrument	Grating instrument
Benzaldehyde	290	397	0.002 (79)	3	0.03
Benzocaine	310	430	5.3 (78)	0.05	0.007 (78)
Benzophenone	350	450	0.007 (79)	0.4	0.03
Chlorpromazine-HCl	320	490	0.07	0.6	0.02
Physostigmine-sulphate	315	415	3.6 (78)	0.6	0.03 (78)
Warfarin sodium	305	460	0.8 (72)	2	10 (72)

Except where otherwise stated, excitation and emission wavelengths and lifetimes were determined on the modified Baird-Atomic grating instrument. Numbers in brackets are references.

TABLE 4

Fluorimetric detection limits determined on filter and grating fluorimeters

Compound	Excitation maximum, nm	Fluorescence maximum, nm	Limits of detection, $\mu g.ml^{-1}$	
			filter instrument	grating instrument
p-aminosalicylic acid	330	410	0.05	0.08
cinchonidine	335	425	2	0.07
1-naphthylamine	310	430	0.001	0.0005
salicylic acid	295	410	0.008	0.002

from these results is that the average coefficient of variation of the background using this instrument for phosphorimetry is ± 1.8% as compared with ± 12% for a Baird-Atomic SF100 fitted with the manufacturer's phosphoroscope attachments. There are three reasons for this, the first being that mercury vapour lamps are inherently more stable in their light output than are xenon arc lamps, the second being that the Dewar was specifically designed to give better sample positioning that is possible with commercially available Dewars, and the third being that the broad excitation beam is less susceptible to any errors in sample positioning that may occur.

From these results it can be seen that a modified instrument such as this should be suitable for the determination of a variety of compounds down to a concentration of about 1 μg/ml, thus allowing phosphorimetry to be used on a routine basis at a greatly reduced cost.

CHAPTER 3

The Luminescence Characteristics of the Phenothiazines

Introduction

The phenothiazine derivatives form a large and important family of compounds from a medical point of view. Over 3,000 have been synthesised and at least 100 of them are in clinical use[80], mainly as antihistamines and major tranquillizers. Since their introduction into clinical practice they have been studied by almost every analytical technique in common use, including U.V. adsorption[81, 82] colorimetry[83, 84] densitometry on T.L.C. plates[85-88], polarography[89], I.R. spectroscopy[90], Raman spectroscopy[91], fluorescence[92-96], H.P.L.C.[97-99], mass fragmentography[100], electron capture-G.L.C.[101-103] and fluorescent derivatisation[104-106]. A comparison of the methods used before 1972 has been given by Usdin[107] (for chlorpromazine and its metabolites only) and by Cimbura[108]. All of the methods currently used to determine normal blood levels of these drugs have some disadvantages; mass fragmentography is far too complex and the equipment too expensive to be considered for general purposes, electron capture-G.L.C. can only be used when halogen atoms are present and fluorescent derivatisation only permits the detection of metabolites containing primary amine or hydroxyl groups.

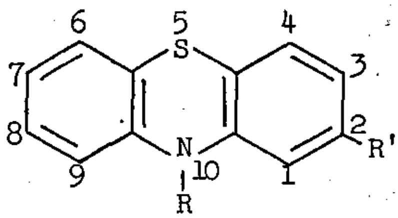
Low temperature luminescence data for medically important phenothiazines are very sparse, which is surprising considering the known sensitivity of the technique. Winefordner and Tin[57] described the determination of an analytical curve for chlorpromazine using a phosphorimeter, but did not give a detection limit. Thiery et al.[81] described the low temperature phosphorescence of 13 phenothiazine derivatives, but less than half of them are in general medical use and neither detection limits nor phosphorescence lifetimes were given. This work provides such data.

Experimental procedure

The phenothiazine derivatives and related compounds which were studied are shown in Table 5. Compounds 1, 3, 4 and 11 were donated by Pharmaceutical Specialities (May and Baker) Ltd., compound 5 by Allan and Hanburys, compound 6 by Hopkin and Williams, compound 9 by Sandoz Products Ltd., compound 10 by G.D. Searle, and compound 14 by E.R. Squibb and Sons. Compounds 2, 7, 8, 12 and 13 were obtained from the B.P. Commission, No. 15 from Koch-Light Labs Ltd., 16 from B.D.H. Ltd., 17 from Fluka AG., 18 from Ralph N.Emanuel Ltd., and 19 from Aldrich Chemical Co. All of the compounds melted within 2°C of the literature value, except for chlorpromazine HCl which was therefore purified by precipitation from ethanol using diethyl ether. After the chlorpromazine HCl was dried in a vacuum oven, its melting point was in agreement with literature values[109]. The solvents used were water, triply distilled from an all glass apparatus, A.R. grade ethanol from James Burroughs Ltd., and n-pentane

TABLE 5

Formulae of Phenothiazines and related compounds studied



Name	R	R'
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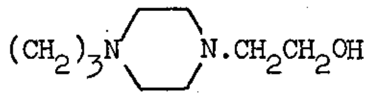
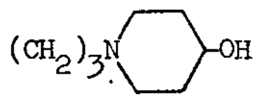
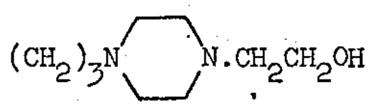
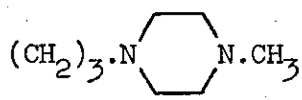
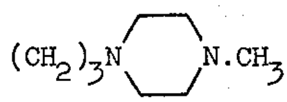
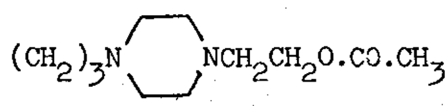
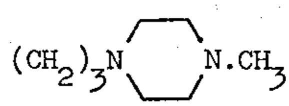
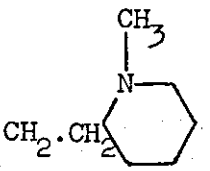
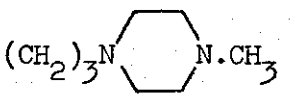
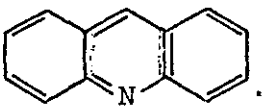
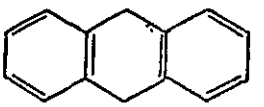
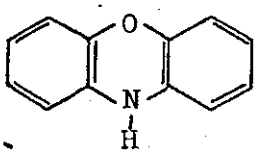
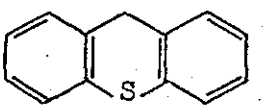
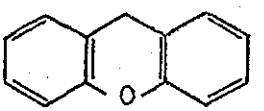
2. Fluphenazine HCl		CF_3
3. Methotrimeprazine maleate	$CH_2CHCH_2N(CH_3)_2$ | CH_3	OCH_3
4. Pericyazine		CN
5. Perphenazine		Cl
6. Phenothiazine	H	H
7. Prochlorperazine maleate		Cl
8. Promazine HCl	$(CH_2)_3N(CH_3)_2$	H
9. Thiethylperazine maleate		SCH_2CH_3
10. Thiopropazate HCl		Cl
11. Thioproperazine mesylate		$SO_2.N(CH_3)_2$

TABLE 5 (Continued)

Name	R	R'
12. Thioridazine HCl		SCH_3
13. Trifluorperazine HCl		CF_3
14. Triflupromazine HCl	$(CH_2)_3N(CH_3)_2$	CF_3
15. Acridine		formulae
16. Anthracene		
17. Phenoxazine		
18. Thioxanthene		
19. Xanthene		

and acetonitrile G.P.R. from B.D.H. Ltd. U.V. absorbtion spectra were determined using a Pye Unicam SP8000 spectrophotometer, and uncorrected luminescence spectra using a Baird-Atomic SF100 spectrofluorimeter fitted with the manufacturer's phosphoroscope attachments and connected to a Bryans 26000 X-Y recorder. The sample tube, a piece of open ended Spectrosil tubing (1 mm i.d. x 4 mm o.d.) was rotated at 320 rpm as previously described. All results were obtained using a slit combination which gave a half-wave bandwidth of 6 nm on both excitation and emission sides of the instrument. Phosphorescence lifetimes of less than 1 second were measured using an Advance O.S.200 oscilloscope in conjunction with the spectrofluorimeter. A micro switch was placed on the light shutter so that when the shutter was raised the oscilloscope was triggered. The trace was recorded on Kodak Tri-X film using a Shackman A.C. 2/25 oscilloscope camera. The negatives were then projected on to graph paper (30 cm x 20 cm) and the lifetimes calculated from these enlargements.

In the case of methotrimeprazine maleate, six lifetime determinations on three separate occasions showed a coefficient of variation of 4%. For compounds with a phosphorescence lifetime of greater than 1 second, the X-Y recorder was used, with the X axis working in the time base mode. The accuracy of the time base calibration was checked using a stop watch and was found to be correct within the limits of the method ($<0.5\%$ at 1 sec cm^{-1}). The fluorimeter monochromators were regularly calibrated with the aid of a Thermal Syndicate low pressure mercury vapour lamp (Thermaline probe). With the instrument switched on, but without the xenon lamp operating, the intense emission lines of the mercury spectrum could be measured by placing

the probe in the sample compartment scanning the emission monochromator, with its slits set to give maximum resolution. Any discrepancy between the observed and published values could be removed by adjusting the scale on the monochromator. The mercury lamp was then removed and the xenon arc struck. By obtaining first order scatter signals at a variety of wavelengths, the settings of the two monochromators could be matched.

In order to obtain 'true' or 'corrected' spectra, the instrument characteristics have to be measured in terms of light output and detection sensitivity. This was done using a procedure based on that of Chen[110]. A solution containing 3 mg of rhodamine.B per ml of ethanediol was used as the fluorescent screen, and a 1 cm cell coated with magnesium oxide was used as the scattering surface. The correction factors are shown in Fig.8. Once the correction factors had been determined, all the spectra requiring correction were obtained before the xenon lamp was switched off, as switching the lamp on and off is known to affect the apparent spectral output.

In studies of the phenothiazines, stock solutions were made up containing 100 μg of compound per ml of either ethanol or water. For determination of the detection limits, the stock solutions were serially diluted to give final concentrations of 100, 10, 1, 0.1, 0.01 μg ml^{-1}. The detection limit for each compound was taken as being the concentration at which the emission was equivalent to two standard deviations of the background. Readings were taken in triplicate and six blank readings were taken for each compound studied. The detection limits were determined directly for methotrimeprazine maleate, thioproperazine mesylate and pericyazine. Since the

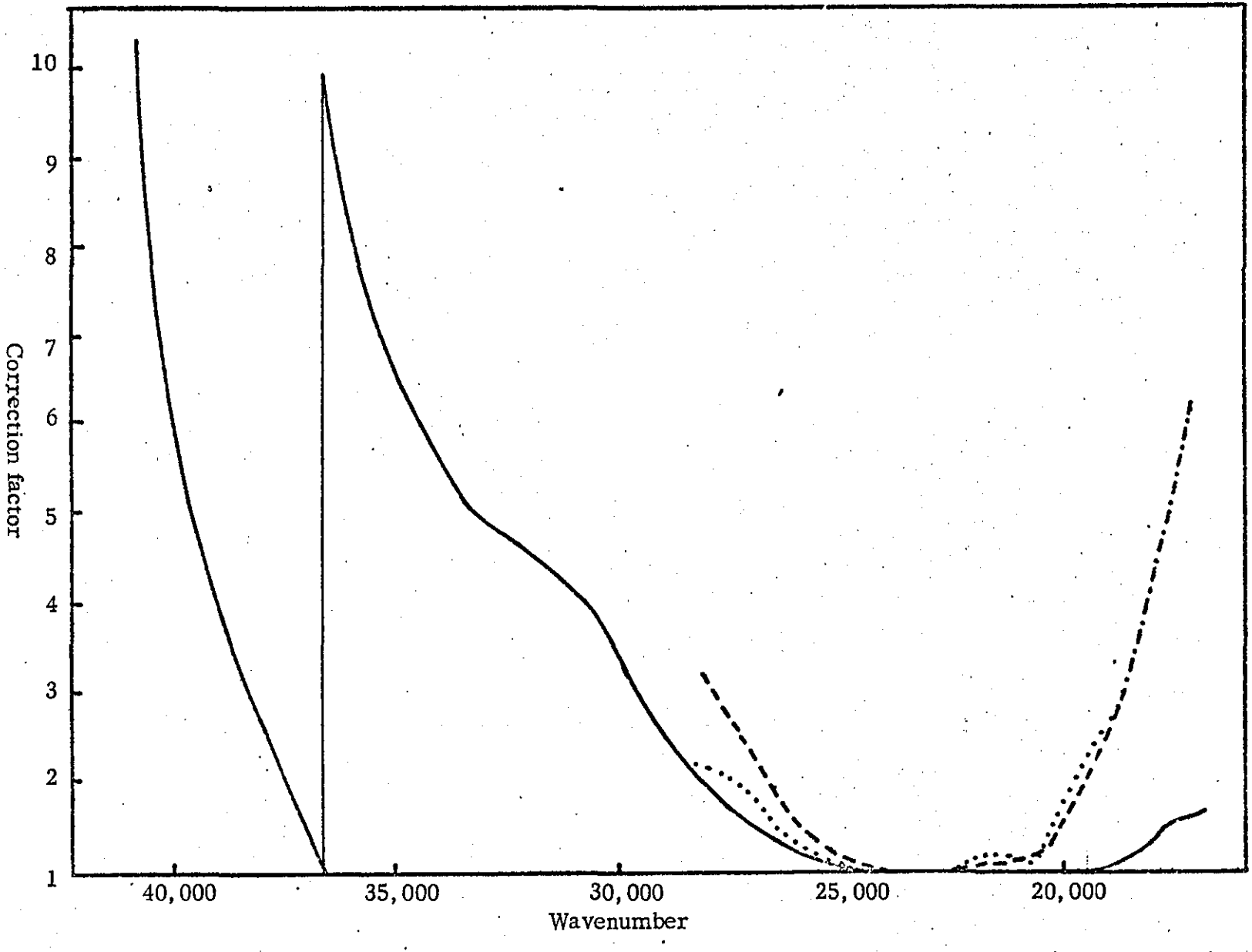


FIG. 8. Instrument correction factors. Rhodamine B correction (——), magnesium oxide correction (. . . . .), final emission correction (-----).

phosphorescence wavelengths (except for pericyazine) and the slopes of the analytical curves were equal, within the limits of experimental error, the detection limits for the other compounds were estimated from their relative phosphorescence intensities at equal weight concentrations.

Results and discussion

It was originally intended to measure the detection limits of the compounds using water as the solvent because, having such a low background luminescence, very good detection limits might be obtained with it[10]. However, it was found that chlorpromazine and several of the other phenothiazines being studied, photo-decomposed in water even at 77°K, although they were stable in ethanol (Fig.9). Flushing the solution with oxygen-free nitrogen (obtained by passing commercial oxygen-free nitrogen through Mercury/Vanadium II solution) for 15 minutes reduced the rate of decomposition but did not prevent it. This suggests that either all the oxygen was not removed by the flushing procedure or that there is more than one route of photo-decomposition. From the studies carried out by Huang and Sands[111,112] on the photo-decomposition of chlorpromazine under both aerobic and anaerobic conditions at room temperature, it would appear most likely that photo-decomposition occurs at 77K under anaerobic conditions. This conclusion is also supported by the work of Grant[113].

It was also intended to study the effect of various concentrations of acid on the phosphorescence spectra of the compounds. A preliminary study showed, however, that the presence of acid increased the rate of photo-decomposition and caused a colour change in the solution. The

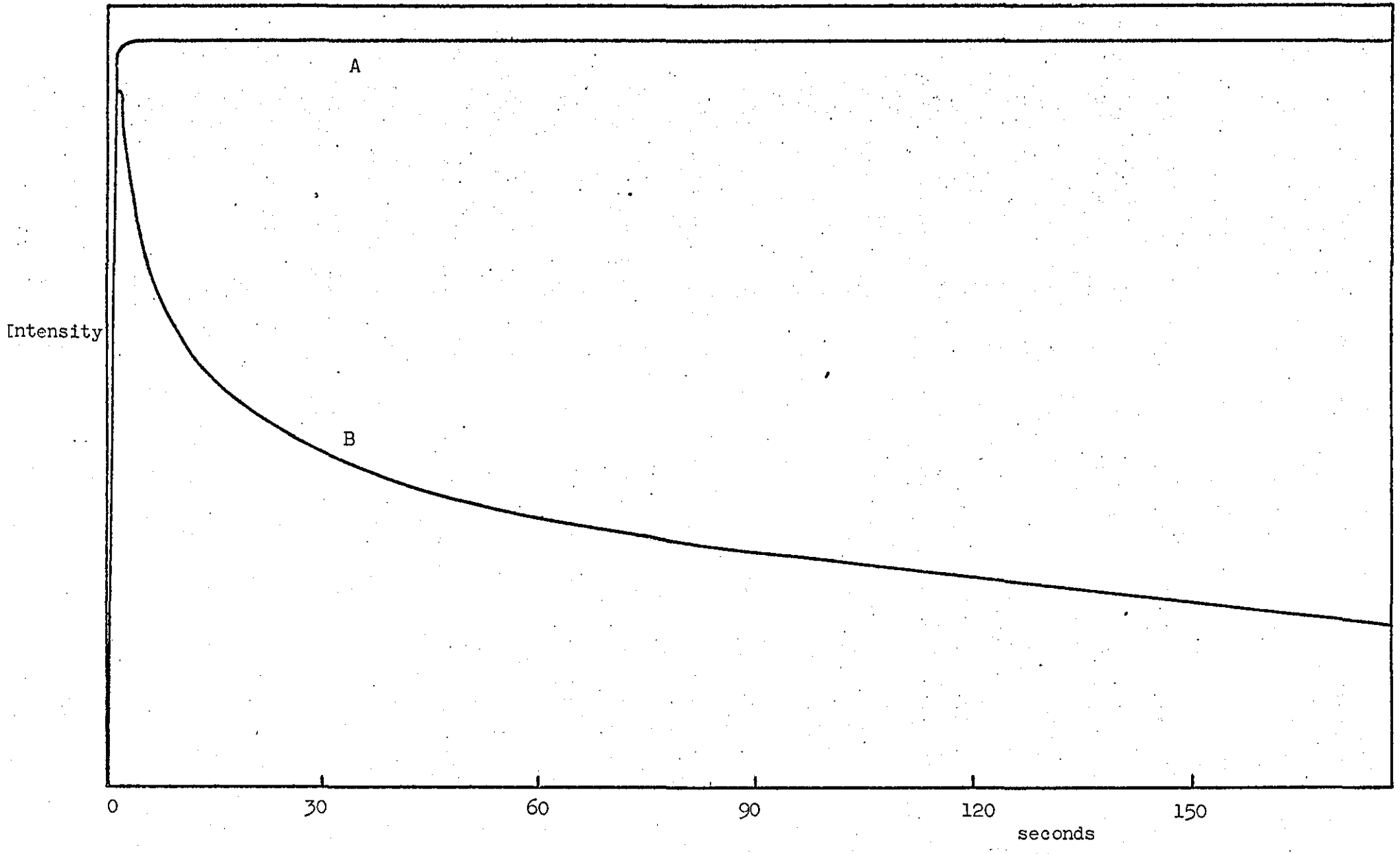


FIG.9. Effect of UV irradiation (320 nm) on the phosphorescence emission of chlorpromazine HCl at 77K, (A) in ethanol (B) in water

changes in the absorbtion spectra that accompanied the irradiation of aqueous solutions of 10 μg ml^{-1} thiopropazate HCl containing different molar concentrations of hydrochloric acid are shown in Fig.10. The visual appearance of the various solutions after exposure to light from a tungsten filament lamp are shown in Table 6.

TABLE 6

Visual appearance of solutions of thiopropazate HCl (10 μg ml^{-1}) after irradiation with light from a tungsten filament lamp for 15 hours.

Solvent	Colour
Water	orange
1M Hydrochloric acid	grey
2M Hydrochloric acid	mauve
4M Hydrochloric acid	cerise
8M Hydrochloric acid	ruby red

An aqueous solution of thiopropazate HCl showed no change in colour when kept in the dark for two weeks, and the coloured solutions showed no change in absorbtion spectra after being stored in the dark for two weeks, thus indicating that the reaction or reactions are photo-dependent and irreversible.

Changes in the colour of solutions of promethazine HCl, perphenazine HCl and chlorpromazine HCl on exposure to light have been reported by Ikeda and Nagahiro[114] but they did not vary the acid concentration or suggest any possible mechanisms for the reaction.

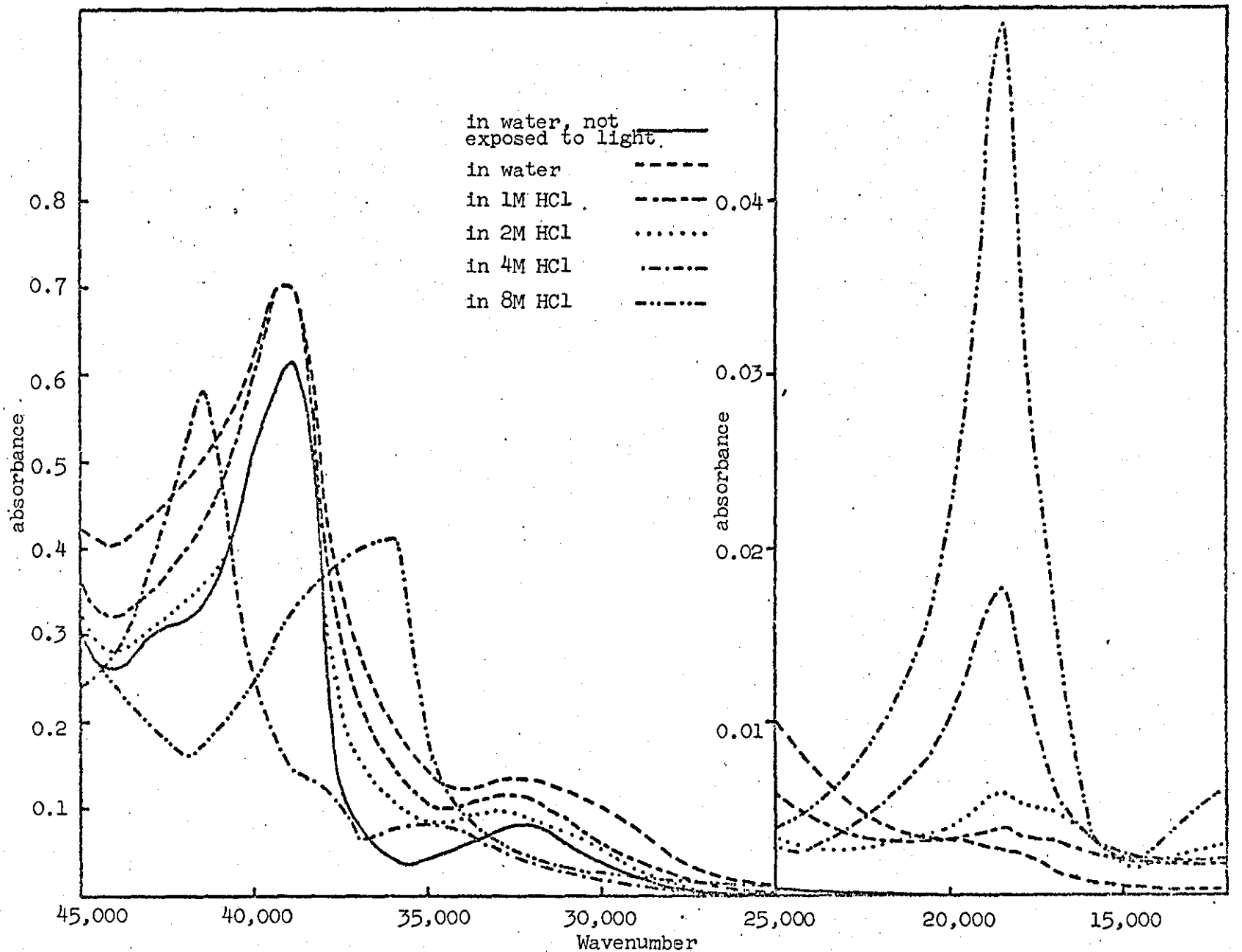


FIG.10. Effect of U.V. radiation on the absorbtion spectra of thiopropazate HCl present in different molar concentrations of hydrochloric acid

These results precluded the measurement of the phosphorescence spectra of phenothiazines in acid solutions, therefore the rest of the results were determined in ethanolic solution.

Table 7 gives the excitation maxima, emission maxima, phosphorescence lifetimes, relative phosphorescence intensities for equimolar and equal weight concentrations and detection limits for phenothiazine and some of its derivatives. Most of the compounds studied show characteristic double excitation and single phosphorescence maxima at 250-265, 305-315, and 485-505 nm (Fig.11). When a fluorescence band is also present, ($\sim$ 450 nm), the phosphorescence undergoes a red shift. Phenothiazine itself has a pronounced shoulder in the phosphorescence spectrum at 535 nm. Pericyazine has an excitation spectrum with three maxima at 270, 315 and 350 nm and a more intense fluorescence (amounting to more than 30% of the total apparent luminescence) than any of the other compounds. The presence or absence of the rotating cylinder phosphoroscope had no significant effect on the wavelengths of any of the phosphorescence maxima. The phosphorescence lifetimes (τ) are all in the range of 60 - 80 ms., except in the case of pericyazine (43 ms), and there is a fivefold variation in the phosphorescence intensities of the compounds. The absence of the shoulder at 535 nm in the phosphorescence spectra of all the compounds except phenothiazine itself, suggests that the substituent at position 10 may cause this effect. Otherwise, the nature of the substituent at this position has little effect on the spectra: this is not surprising since substituents in all the phenothiazines studied have three carbon atoms separating the ring system from the sidechain nitrogen, and any differences further away from the ring system than this will cause very little change in the

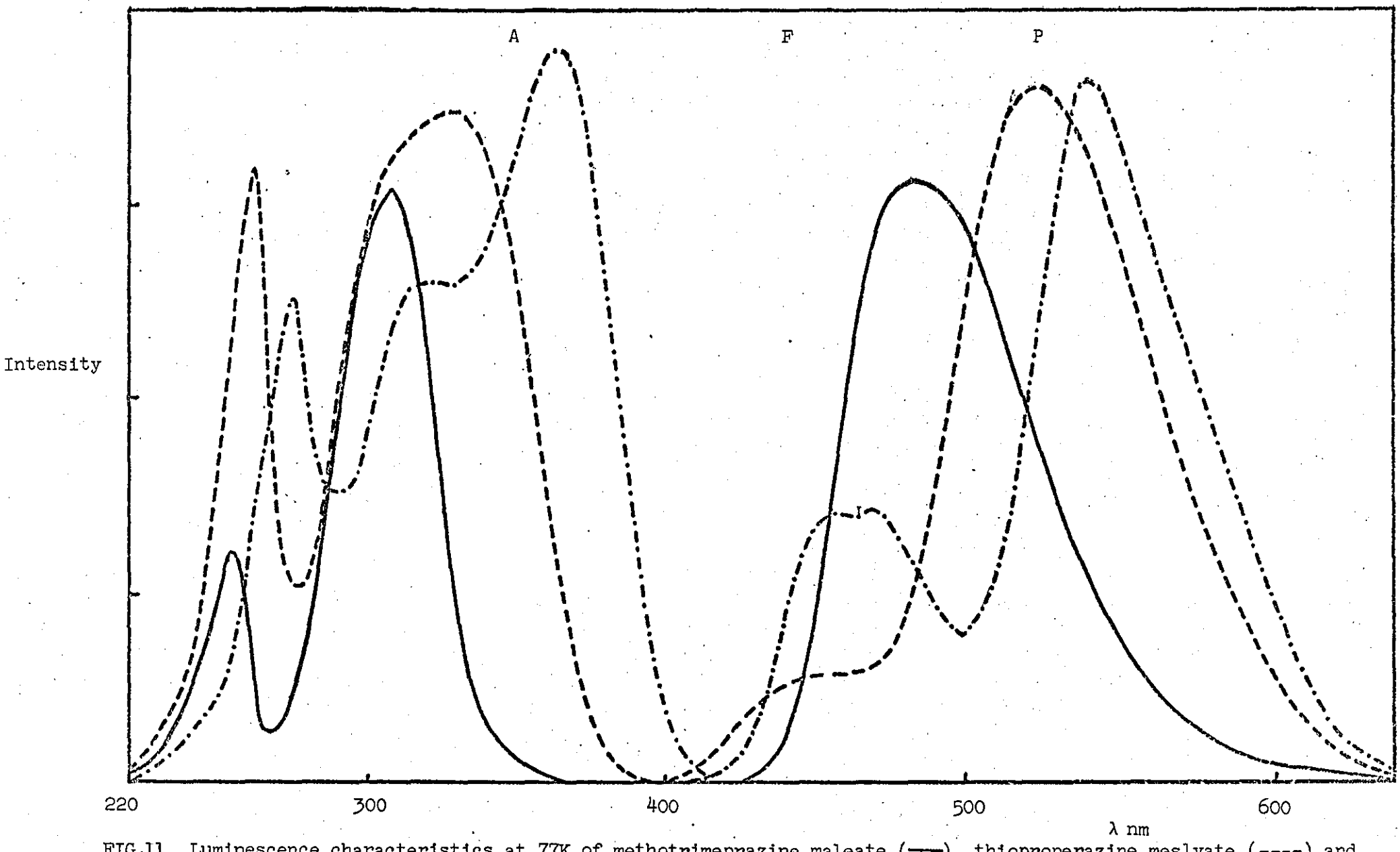


FIG.11 Luminescence characteristics at 77K of methotrimeprazine maleate (——), thioproperazine meslyate (----) and pericyazine (-·---). (A) excitation; (F) fluorescence; and (P) phosphorescence spectra. Fluorescence is

TABLE 7

Low temperature luminescence data for the Phenothiazines

	λ_{Max}, ex, nm	λ_{Max}, f, nm	λ_{Max}, p, nm	τ,ms	Rel. peak height		Detection limit	
					Equimolar	Equi.g/l.	ng/ml	nmol
Chlorpromazine HCl	255, 310	...	490	72	0.61	0.68	30	85
Fluphenazine HCl	260, 315	450	505	59	0.46	0.36	55	110
Methotrimeprazine	255, 305	...	485	58	0.82	1.00[b]	20	60
Pericyazine	270, 315, 350	470	540	43	0.16	0.18	45	125
Perphenazine	255, 310	...	495	77	0.71	0.71	30	75
Phenothiazine	260, 325	...	505, 535	56	0.43	0.87	25	125
Prochlorperazine maleate	250, 305	...	490	78	0.29	0.19	100	165
Promazine HCl	250, 305	...	495	78	0.59	0.74	30	95
Thiethylperazine maleate	255, 310	...	490	75	1.00[a]	0.77	25	50
Thioproperazine mesylate	260, 330		520	57	0.35	0.22	100	155
Thioridazine HCl	265, 315	445	495	66	0.89	0.88	25	60
Trifluorperazine HCl	255, 305	455	505	65	0.47	0.39	50	105
Triflupromazine HCl	255, 305	...	510	68	0.49	0.51	40	105

[a]Assumed to be 1.00 for equimolar concentrations. [b]Assumed to be 1.00 for equal weight concentrations.

Fluorescence maxima only given if peak > 2% of phosphorescence peak.

electron distribution in the ring. The groups at position 2 which have the largest effects are $-CF_3$, $-SO_2N(CH_3)_2$, and - CN, all of which produce a bathochromic shift of phosphorescence (10 - 45 nm compared with promazine) and also cause detectable fluorescence. All these groups are strongly electron-withdrawing which would suggest that their 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.

The detection limit for pericyazine (see above) is lower than would be expected from its relative phosphorescence intensity because the luminescence background of the solvent is greatest at about 390 nm and decreases with increasing wavelength. As pericyazine has a phosphorescence maximum at a greater wavelength than the rest of the compounds, there is a lower background to contend with when it is being studied.

In an attempt to relate the spectrum of phenothiazine to its ring structure, the corrected spectra and lifetimes of various tricyclic compounds were determined. The spectra are shown in Figs. 12 to 14 and other data such as phosphorescence lifetime, relative phosphorescence and fluorescence yields and phosphorescence to fluorescence ratios ($\Phi p/\Phi f$) are given in Table 8.

The U.V. absorbtion spectra agree qualitatively in all cases with the luminescence excitation spectra, but differ quantitatively in the ratio of the 38,000 - 39,000 cm^{-1} absorbtion band compared with the rest of the spectra. This is almost certainly due to the rapidly changing correction factor necessary around 40,000 cm^{-1}, as the fluorimeter reaches the practical limit of its range. Although

TABLE 8

Low temperature luminescence data for various tricyclic compounds

Compound	Excitation maxima (cm^{-1})	Fluorescence maxima (cm^{-1})	Phosphorescence maxima (cm^{-1})	τ(s)	Relative (a) phosphorescence or fluorescence	Φp/ Φf
Acridine	28,200, 29,400 30,700, 31,900 38,200	21,800, 23,000, 24,500 25,500	not visibly phosphorescent		0.67	
Anthracene	28,200, 29,400 30,700, 31,900 38,200	21,800, 23,000, 24,500 25,500	not visibly phosphorescent		1.00	
Phenothiazine	30,500, 37,800	22,200, 23,300	18,200, 19,400	0.055	0.05	50
Phenoxazine	30,000, c.41,000	24,300, 25,200, 26,700	19,400, 20,700	2.16	0.23	4
Thioxanthene	36,500	not visibly fluorescent	19,800, 21,700	0.016	0.05	
Xanthene	33,000, 37,900		22,700, c.26,500	0.74	0.01	

(a) relative to anthracene as 1.00

it is the strongest absorbtion band, it was not used in the determination of the luminescence spectra, because it only appears as a small peak in the uncorrected spectra.

Examination of the spectra of anthracene and acridine (Fig.12) shows that the replacement of a carbon with a nitrogen atom in position 5 of a tricyclic ring, has very little effect, apart from causing a loss of fine structure definition. This contrasts strongly with the effect produced when either oxygen or sulphur is substituted in position 5 (Fig.13). In both cases there is a radical change in the absorbtion spectra, accompanied by a complete loss of fluorescence and the production of phosphorescence. The further substitutions of a nitrogen atom in position 10 of either xanthene or thioxanthene leads to the reappearance of a fluorescence emission spectrum apparently similar to that of acridine but at a slightly higher wavenumber (Fig.14). There are also approximately threefold increases in the phosphorescence lifetimes of both compounds. Assignment of the type of process involved is reasonably straightforward in the case of phenoxazine, as the very long lifetime strongly indicates a $\pi^* \leftarrow \pi$ transition, but the lifetime of phenothiazine indicates neither one type nor the other. A series of six measurements of the apparent phosphorescence maximum of phenothiazine in acetonitrile (dielectric constant 37.5) and n-pentane (dielectric constant 1.8) revealed no significant difference (an average difference of 1 nm, with maxima being read to $\pm$ 1 nm). This would suggest that a $\pi^* \leftarrow \pi$ transition is involved because $\pi^* \leftarrow$ n transitions produce quite pronounced wavelength shifts, depending on the polarity of the solvent in which they are measured.

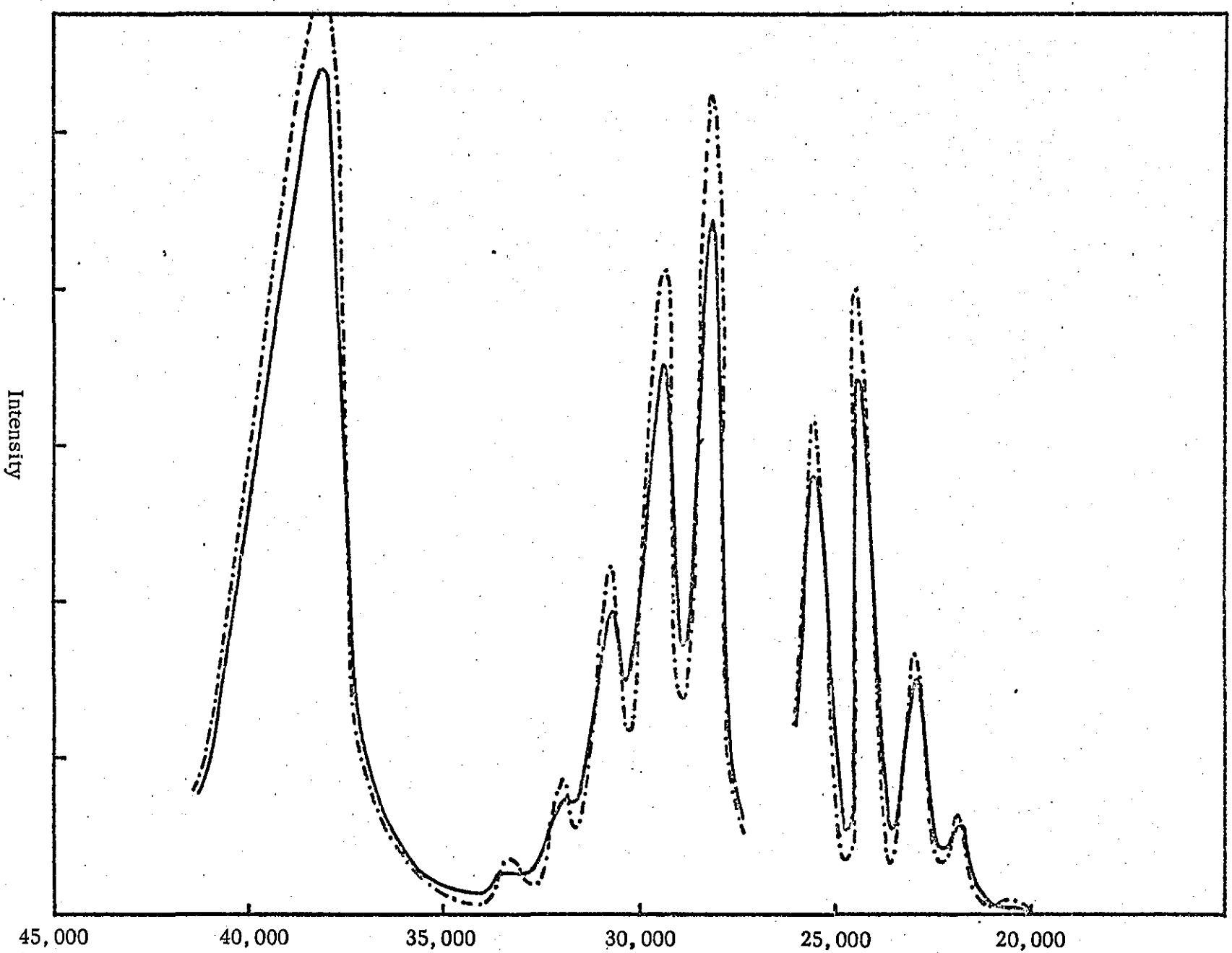


Fig. 12. Corrected luminescence spectra of acridine (——) and anthracene (·-·-·-)

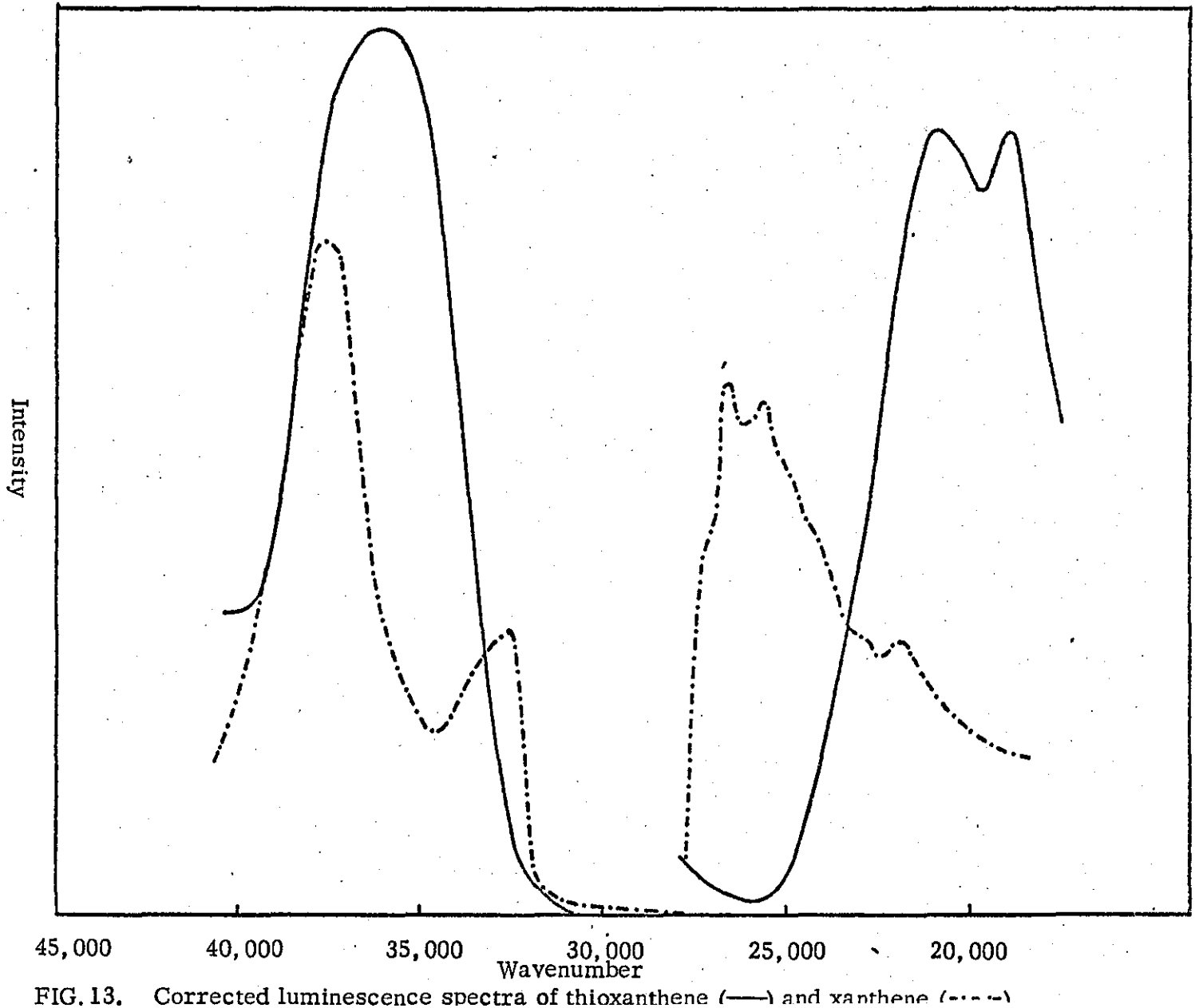


FIG. 13. Corrected luminescence spectra of thioxanthene (——) and xanthene (-·-·-)

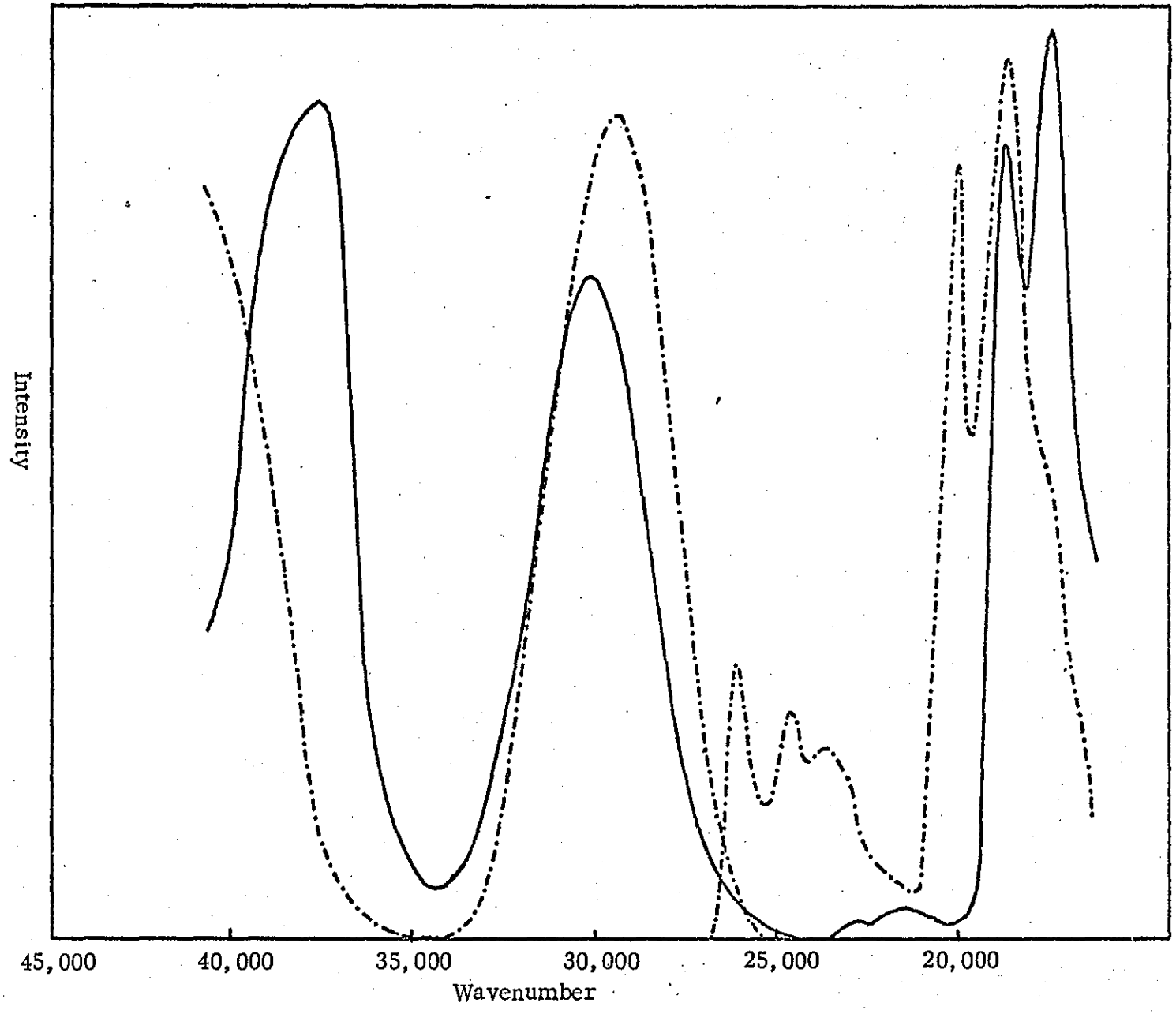


FIG. 14. Corrected luminescence spectra of phenothiazine (——) and phenoxazine (-· ·· -)

The presence or absence of fluorescence in the molecule correlates well with its configuration, anthracene, acridine and phenoxazine are all planar or very nearly planar whereas the other molecules are twisted on the 5 - 10 axis, phenothiazine having a concluded angle of 140 - 160°, xanthene an angle of 154 - 166° and thioxanthene an angle of 127 - 143° (all angles are for the compounds in solution). This is likely to be a significant factor, as it is known empirically that planar molecules with large areas of charge distribution are usually fluorescent.

This work, as well as providing some theoretical basis for the observed phenomena, also indicates that it should be possible to determine phenothiazines at levels found in the blood. A major problem, however, is the large number of metabolites found in vivo. Turner and Turano[115] identified 35 chlorpromazine metabolites and found another 42 which could not be identified. In this respect the use of a scanning thin layer phosphorimeter, as first described by Gifford et al.[9] to separate and identify metabolites, would be advantageous, and further reference is made to this in a later chapter.

CHAPTER 4

The luminescence properties of some anti-inflammatory and antipyretic drugs

Introduction

The antipyretic and anti-inflammatory drugs are composed of several families of chemical compounds classified together only by their ability to lower body temperature in feverish patients (but not in people with 'normal' body temperatures), or their ability to reduce or prevent the inflammatory changes seen in a number of diseases. The salicylates form the most widely used family of compounds of this sort, aspirin being the most popular of all the drugs used in the self-medication of minor pains and fevers. The salicylates have not been included in this study though, because their luminescence properties have already been well described. Weissbach et al.[116] have described a fluorimetric assay for salicylic acid and Moye and Winefordner[42] have described a phosphorimetric assay for aspirin in plasma. As the phosphorescence intensity of salicylic acid is only 0.2% that of aspirin, the former does not interfere in the determination of the latter. The anti-inflammatory drugs have been determined by a variety of analytical techniques, the most commonly used being colorimetry (e.g. 117-119), U.V. spectrophotometry (e.g. 120-122) and G.L.C. (e.g. 123-125). Other methods have included T.L.C.[126, 127], polyamide layer chromatography[128], N.M.R.[129] and both a.c. and d.c. polarography[130-132]. The compound for which the most assays appear to

have been developed is phenylbutazone, due almost certainly to its widespread use combined with the fact that it can cause agranulocytosis, particularly in high doses. The ability to monitor its levels in blood is therefore particularly useful.

Some work has been published on the luminescence characteristics of the anti-inflammatory agents; Dell and Kemp in 1970[133] described a method for the determination of fluphenazine and its derivatives that involved converting them into heterocyclic compounds by the use of either concentrated sulphuric acid or formaldehyde. The products formed were fluorescent and detection limits of better than 1 $\mu g\ ml^{-1}$ could be obtained. Mehta and Schulman[134] found that analytically useful native fluorescence could be obtained for mefenamic and flufenamic acids, by using either dioxan or chloroform as the solvent. The detection limits obtained were as good as those of Dell and Kemp. As there is no chemical reaction involved, the method is quicker and must be at least as precise as the earlier method. Very recently Strojny and de Silva published a paper on the luminescence analysis of anti-inflammatory agents in plasma following separation by T.L.C.[135]. They studied the salicylates, carbazole and tetrahydrocarbazole derivatives, in addition to three compounds covered in this work, namely indomethacin, phenylbutazone and mefenamic acid. A comparison of the two sets of results obtained is given in the results section.

Experimental procedure

The antipyretic and anti-inflammatory compounds and analogues studied are shown in Table 9, along with their suppliers. The melting points of all the drugs studied (compounds 1 - 9) were measured and found to be within 2°C of the literature values. The

TABLE 9

Formulae and Suppliers of the anti-inflammatory drugs and analogues studied

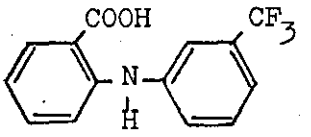
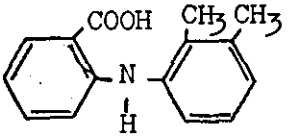
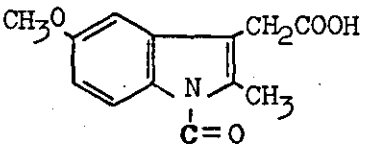
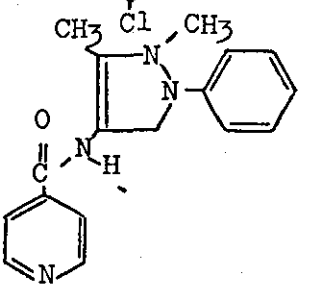
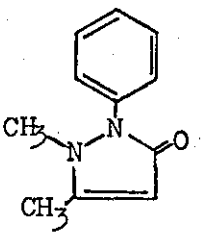
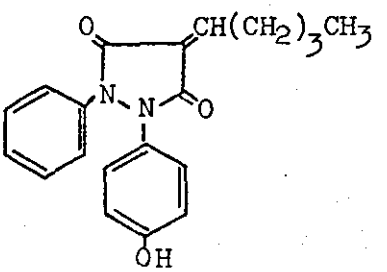
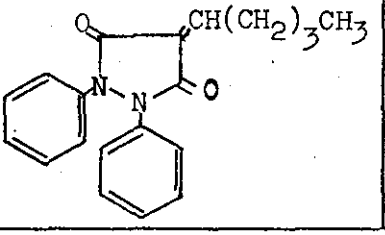
Compound	Formula	Supplier
1. Flufenamic acid		Parke, Davis & Co.
2. Mefenamic acid		Parke, Davis & Co.
3. Indomethacin		Merck, Sharp & Dohme Ltd.
4. Nifenazone		Sinclair Pharmaceuticals Ltd.
5. Phenazone		Fisons Ltd., Pharmaceutical Divn.
6. Oxyphenbutazone		Geigy
7. Phenylbutazone		Geigy Pharmaceuticals

TABLE 9 (Continued)

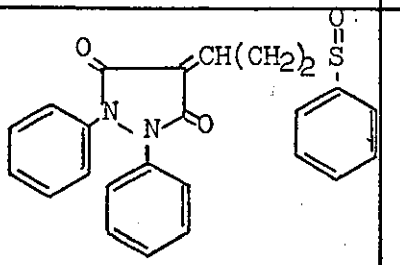
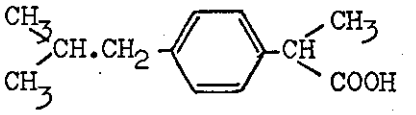
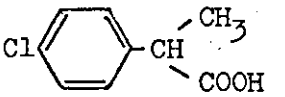
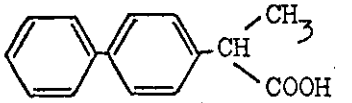
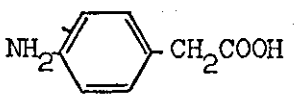
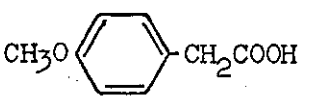
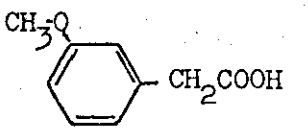
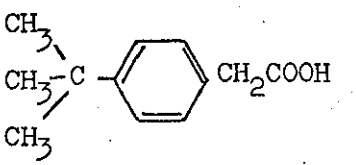
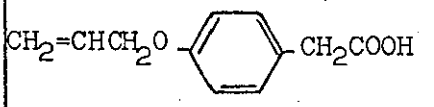
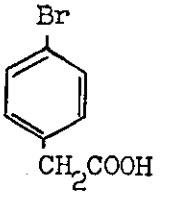
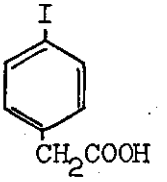
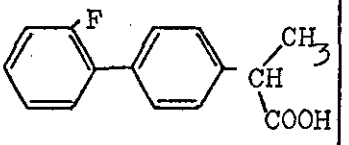
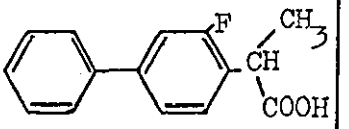
Compound	Formula	Supplier
8. Sulphinpyrazone		Geigy Pharmaceuticals
9. Ibuprofen		The Boots Co.Ltd.
10. 2-(4-chlorophenyl) propionic acid		"
11. 2-(4-biphenyl) propionic acid		"
12. 4-aminophenylacetic acid		"
13. 4-methoxyphenyl-acetic acid		"
14. 3-methoxyphenyl-acetic acid		"
15. 4-t-butylphenyl-acetic acid		"
16. 4-allyloxyphenyl-acetic acid		"

TABLE 9 (Continued)

Compound	Formula	Supplier
17. 4-bromophenylacetic acid		The Boots Co.Ltd.
18. 4-iodophenylacetic acid		"
19. 2-(2'fluoro-4-biphenyl) propionic acid		"
20. 2-(2-fluoro-4-biphenyl) propionic acid		

All compounds were the gifts of the Companies named.

melting points of the propionic and acetic acid derivatives (compounds 10 - 20) were not determined, owing to the very small quantities available in some cases. They were therefore used as obtained. The solvents used were ethanol (A.R. grade from James Burroughs Ltd.), water (triply distilled from an all glass apparatus), and ethanol containing 1M concentrations of either hydrochloric acid or sodium hydroxide. The latter two solvents were made by adding suitable quantities of concentrated hydrochloric acid or 10M sodium hydroxide to the A.R. grade ethanol. All low temperature spectra, lifetimes etc. were obtained using a Baird-Atomic SF100 spectrofluorimeter fitted with the attachments and accessories described in Chapter 3. U.V. absorbtion spectra were obtained using a Pye Unicam SP8000 spectrophotometer and room temperature fluorescence spectra were obtained using a Baird-Atomic Fluoricord spectrofluorimeter, operated at a half-wave bandwidth of 5 nm on both excitation and emission sides of the instrument. All solutions studied contained 100 $\mu g\ ml^{-1}$ of the compound dissolved in the appropriate solvent.

General operating procedures are as described in the previous chapter, and all the results described are uncorrected for instrumental characteristics.

Results and discussion

The room temperature fluorescence characteristics of the compounds are shown in Table 10. Any compound which gave a maximum response of 5% or less on the meter when the amplifier was on maximum sensitivity was classified as non-fluorescent for the purposes of these results. Some compounds showed solvent-dependent emission

TABLE 10

Room temperature fluorescence excitation and emission characteristics

Compound	1M acid		neutral		1M alkali	
	λex	λem	λex	λem	λex	λem
Flufenamic acid	N.F.		N.F.		N.F.	
Mefenamic acid	N.F.		N.F.		N.F.	
Indomethacin	N.F.		N.F.		310	355
Nifenazone	N.F.		N.F.		N.F.	
Phenazone	N.F.		N.F.		N.F.	
Oxyphenbutazone	N.F.		N.F.		N.F.	
Phenylbutazone	N.F.		N.F.		N.F.	
Sulphinpyrazone	290	470	290	470	N.F.	
Ibuprofen	N.F.		N.F.		N.F.	
2-(4-chlorophenyl)propionic acid	N.F.		N.F.		N.F.	
2-(4-biphenyl)propionic acid	282	320	282	320	282	320
4-aminophenylacetic acid	N.F.		300	345	300	345
4-methoxyphenylacetic acid	335	375	335	375	335	375
3-methoxyphenylacetic acid	280	300	280	300	280	300
4-t-butylphenylacetic acid	N.F.		N.F.		N.F.	
4-allyloxyphenylacetic acid	N.F.		N.F.		N.F.	
4-bromophenylacetic acid	N.F.		N.F.		N.F.	
4-iodophenylacetic acid	N.F.		N.F.		N.F.	
2-(2'-fluoro-4-biphenyl) propionic acid	280	310	280	310	280	310
2-(2-fluoro-4 biphenyl propionic acid	280	310	280	310	280	310

N.F. = non-fluorescent

characteristics: table 11 shows the relative intensities obtained for these compounds under varying conditions.

TABLE 11

Relative fluorescence emission intensities under varying solvent conditions at room temperature

Compound	relative emission intensities		
	1M acid	neutral	1M alkali
Indomethacin	0	0	1
Sulphinpyrazone	0.6	1.0	0
3-methoxyphenylacetic acid	0.5	1.0	1.0
4-methoxyphenylacetic acid	0.8	1.0	0.6
4-aminophenylacetic acid	0	1.0	1.0

The U.V. absorbtion spectra of these compounds were determined in order to ascertain whether or not the fluorescent changes were reflected in the absorbtion spectra. Sulphinpyrazone, 3-methoxyphenylacetic acid and 4-methoxyphenylacetic acid showed little or no change in absorbtion spectra when measured in acidic, neutral or alkaline conditions. As the instrument is operating very close to its limit of sensitivity (on all occasions at less than twice the arbitrarily defined operating limit) these differences only represent very small actual changes and are almost certainly due to instrumental variation. 4-aminophenylacetic acid shows very distinct changes in spectrum between acid and neutral solutions as can be seen in Fig.15.

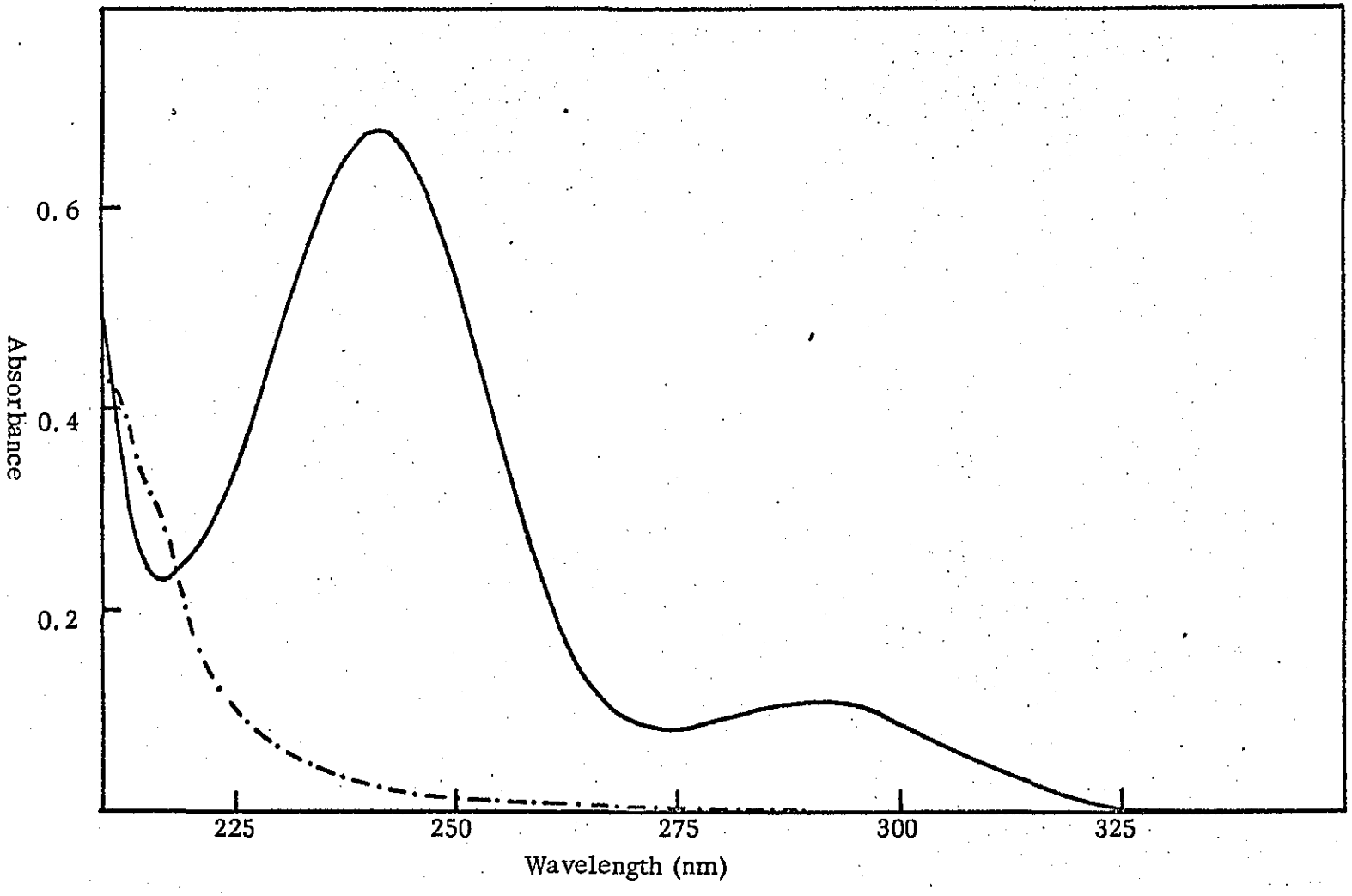
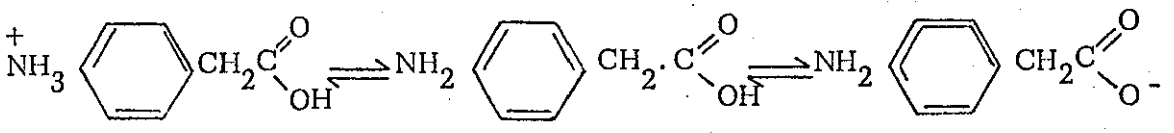


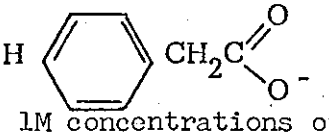
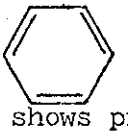
FIG. 15. Effect of varying pH on the absorbtion spectra of 4-aminophenylacetic acid. In neutral (——), and in 1M HCl (·-·-·-)

The absorbtion peaks which are found at 240 nm and 290 nm in neutral conditions are not present when the solvent is acidic ethanol. These observations can be explained in the following terms:

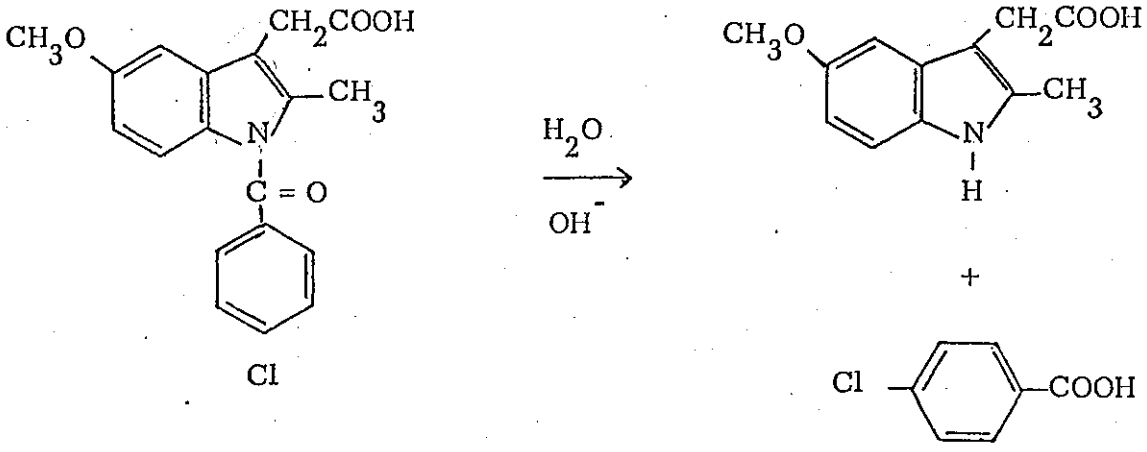


acidic neutral alkaline

NON-FLUORESCENT FLUORESCENT

It would seem probable that the dianion  is not formed to any appreciable extent at 1M concentrations of sodium hydroxide in ethanol, as it would almost certainly be non-fluorescent, like the analinium ion 

Indomethacin also shows pronounced changes in its absorbtion spectrum (Fig.16). The high concentration of hydroxyl ions makes the spectrum obtained in alkaline solutions unreliable below about 240 nm, but the differences are still quite obvious. The only possible cause of such a profound change in spectral characteristics in the presence of hydroxyl ions would appear to be an alkaline hydrolysis.



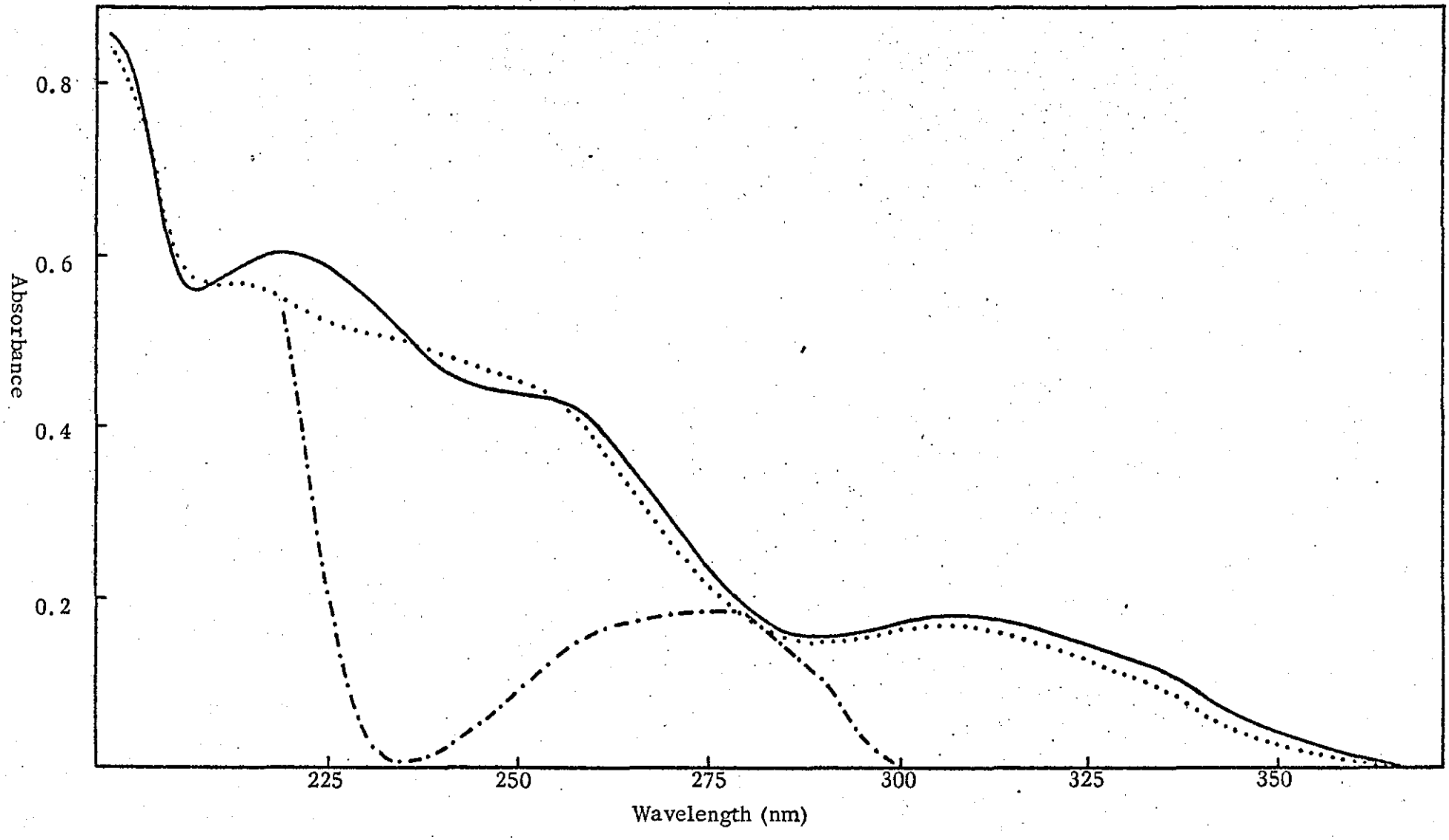


FIG. 16. Effect of varying pH on the absorbtion spectra of Indomethacin, In neutral (——), 1M NaOH (·-·-·) and 1M HCl (.....)

The fact that this reaction occurs was confirmed by Dr. Selby (personal communication) who also provided data which would suggest that the reaction would take place within a matter of seconds at pH 14.

The low temperature luminescence data for ethanolic solutions of the compounds are shown in Table 12. Again, compounds which did not give more than a 5% meter reading at maximum amplification were classified as non-luminescent. The oxyphenbutazone did show a phosphorescence at similar wavelengths to that obtained when the total luminescence was being measured, but at only 30% of the intensity that would be expected (if all the emission was phosphorescence). The oscilloscope trace also showed two distinct decay curves, one very short ($\leq$ 2 ms.) and the other quite long. A sample was therefore run chromatographically, using the method of T.Pomazanska-Kolodziejska[127]. For this procedure silica gel was used as the stationary phase and n-hexane : acetone, 1 : 1 as the mobile phase. After development, the spots were visualised using U.V. light, with the plate immersed in liquid nitrogen. This revealed two spots, as is shown in Fig.17.

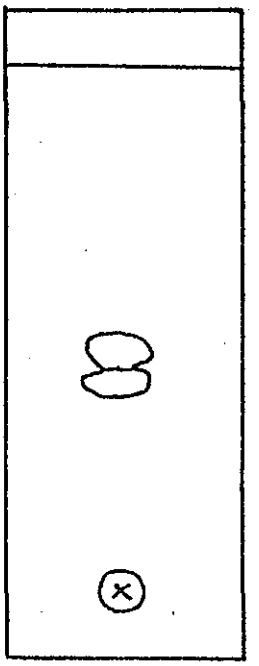


Fig.17
chromatogram of oxyphenbutazone

TABLE 12

Low temperature luminescence characteristics of some anti-inflammatory drugs and related compounds

Compound	λex max(nm)	λem fl (nm)	λem p (nm)	τ (s)
Flufenamic acid	335	(420) 510		
Mefenamic acid	(285),350,360	405		
Indomethacin	330		485	1.0
Nifenazone	300	460		
Phenazone	285	sl. 390	405, 420	rec. 0.58 osc. 0.62
Oxyphenbutazone	280	430	(a)	
Phenylbutazone	285	435		
Sulphinpyrazone	280	440		
Ibuprofen	Non-luminescent			
2-(4-chlorophenyl)propionic acid	(250),(270),275		435	0.035
2-(4-biphenyl)propionic acid	280	(300),320,(332)	(425),465,495	4.4
4-aminophenylacetic acid	(255),295	sl. 350	405	4.0
4-methoxyphenylacetic acid	285		395	3.5
3-methoxyphenylacetic acid	290		400	3.5
4-t-butylphenylacetic acid	Non-luminescent			
4-allyloxyphenylacetic acid	Non-luminescent			
4-bromophenylacetic acid	Non-luminescent			
4-indophenylacetic acid	Non-luminescent			
2-(2'-fluoro-4-biphenyl) propionic acid	290	$\bar{c}$ 310	(430),460,(480)	3.1
2-(2-fluoro-4-biphenyl) propionic acid	280	$\bar{c}$ 310	(430),455,(475)	3.3

(a) see comments in text

One of the spots had a very short luminescence lifetime (probably fluorescence) whereas the other spot had a relatively long lifetime. It would therefore seem certain that the phosphorescence observed in the original material is caused by a trace impurity. As might be expected from the similarity in structure, the emission spectra of phenylbutazone, oxyphenbutazone and sulphinpyrazone are almost identical. 2-(4-biphenyl)propionic acid also has a spectrum fairly similar to those of the two fluoro-substituted biphenyl propionic acid derivatives, but in this case the presence of the fluorine atom has reduced quite considerably the fine structure of the spectrum (Fig.18).

Table 13 shows the luminescence data for indomethacin and 4-aminophenylacetic acid under various solvent conditions.

Indomethacin in acid or neutral conditions shows a single featureless emission band, while in alkaline conditions it shows a certain degree of fine structure, very similar to that of indole, but much less sharply defined. This would suggest that the majority of the phosphorescence seen under these conditions is being produced by the 2-methyl-5-methoxyindole-3-acetic acid which is present, rather than by the 4-chlorobenzoic acid.

Some comment is required to explain the difference in results obtained between Strojny and de Silva's work[135] and the work just described here. For this purpose their relevant data are reproduced in Table 14.

From a comparison of the two sets of results it can be seen that the low temperature fluorescence data for mefenamic acid and phenylbutazone, and the low temperature phosphorescence data for indomethacin are in agreement. The other results disagree; Strojny and de Silva

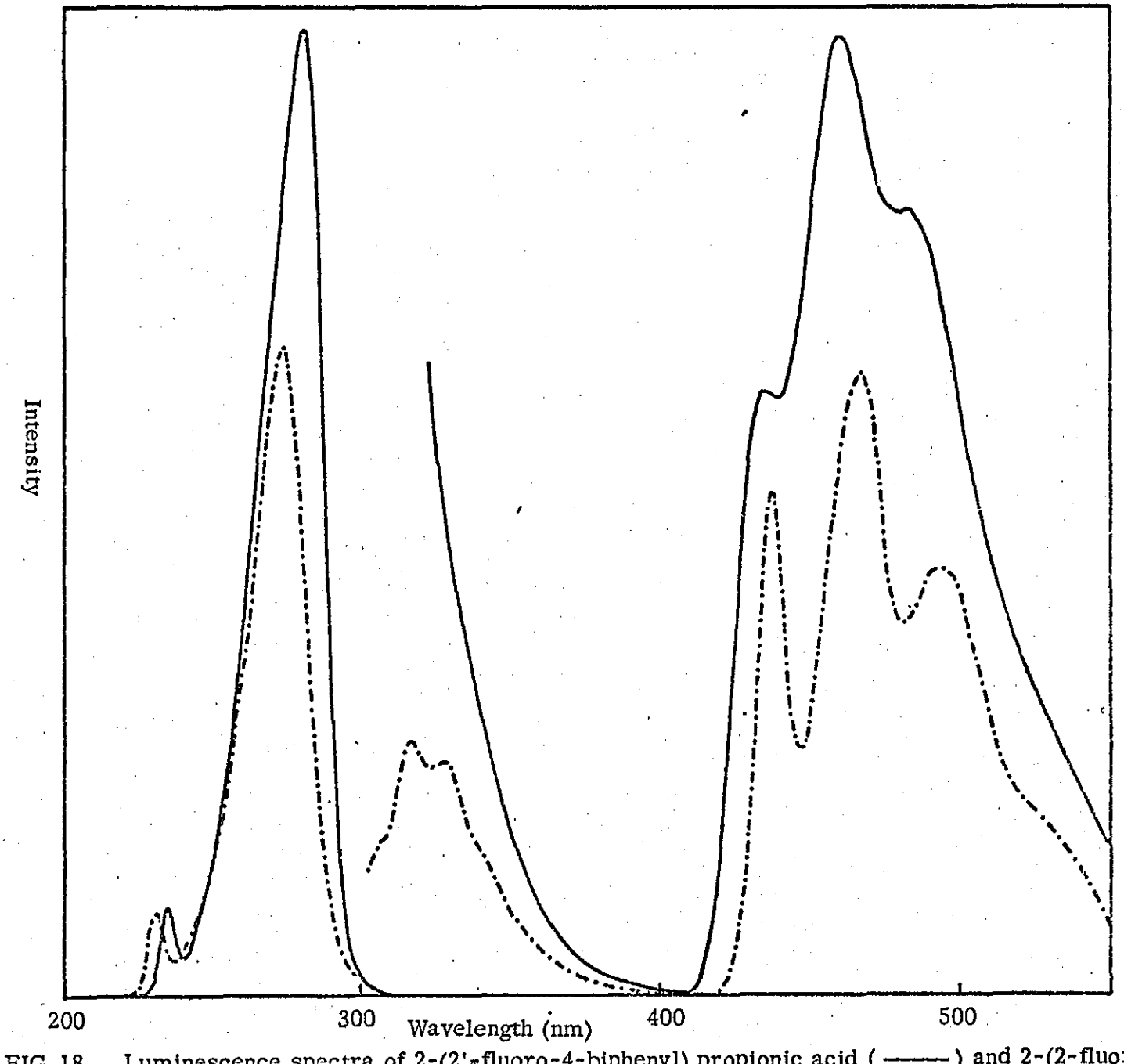


FIG. 18. Luminescence spectra of 2-(2'-fluoro-4-biphenyl) propionic acid (———) and 2-(2-fluoro-4-biphenyl) propionic acid (–·–·–)

TABLE 13

Luminescence characteristics of indomethacin & 4-aminophenylacetic acid

Compound	λex (nm)	λem fl (nm)	λem p (nm)	τ(sec)	relative intensity
4-aminophenylacetic acid					
1M HCl/ethanol	(270)315(365)		(345)(365)390	2.1	0.01
Ethanol	(255)295	350	405	4.0	1.0
1M NaOH/ethanol	(255)295	340	410	4.0	1.0
Indomethacin					
1M HCl/ethanol	330		500	1.1	1.0
Ethanol	330		485	1.0	0.7
1M NaOH/ethanol	(250)310		(405)430(460)	5.6	0.9

TABLE 14

Some of the results shown in Table 1 of Strojny and de Silva J.Chromat.Sci., 13, 583, (1975)

	Indomethacin		Phenylbutazone	Mefenamic acid
25°C Fluorescence	ethanol	0.1N NaOH	ethanol	ethanol
ex max (nm)	328	295 - 300	290	310
em max (nm)	410	405 - 410	460	415
limit of quantitation ($\mu g/ml^{-1}$)	>100	10	10	10
77°K Fluorescence	ethanol		ethanol	ethanol
ex max (nm)	328		275	270,350
em max (nm)	410		430	410
limit of quantitation ($\mu g/ml^{-1}$)	20		0.5	0.1
77K Phosphorescence	ethanol		ethanol	ethanol
ex max (nm)	290,328		275	275,353
em max (nm)	480		420	450
limit of quantitation ($\mu g/ml^{-1}$)	1		5	1
lifetime (s)	1.0		<0.2	<0.2

detecting fluorescence or phosphorescence in cases where none was found in the present work. They do not give any indication of the concentrations they used, but as the limit of quantitation for indomethacin at room temperature is given as >100 $\mu g\ ml^{-1}$, it can only be assumed that very high concentrations were used on occasions. On these occasions the possibility of measuring the luminescence of low concentrations of impurities present in the sample must be quite high, particularly considering the experience with oxyphenbutazone, as previously described. Phenylbutazone did, in fact, show some phosphorescence at the wavelengths quoted by Strojny (and the same as the oxyphenbutazone impurity) but this came to below the 5% on maximum gain that had earlier been set as a limit for reliable results. A second possible cause is that the Farrand Mk.I. spectrofluorimeter is more sensitive than the Baird-Atomic instrument, but as the increase in sensitivity would need to be more than an order of magnitude, this seems unlikely.

These results give the necessary information required to develop analytical techniques for the compounds, based on their luminescence characteristics. The most promising substances are indomethacin, phenazone, mefenamic acid, oxyphenbutazone, sulphinpyrazone and 4-aminophenylacetic acid, all of which should be detectable at submicrogram per ml. concentrations by the use of low temperature fluorescence or phosphorescence.

CHAPTER 5

The development of a phosphorimetric thin-layer scanner

Introduction

Phosphorimetry is a very sensitive and reasonably selective analytical technique, but used with conventional sampling techniques it does require a reasonable degree of skill in order to obtain reproducible results. Even with the required skill a coefficient of variation of ~10% can be expected, although a spinning capillary sample tube is claimed to give a coefficient of variation of ~1%[76]. Also, selectivity is not sufficient to allow the measurement of drugs obtained from samples of blood, a separation must be carried out before readings can be taken. If, therefore, phosphorimetry could be combined with a technique such as thin-layer chromatography, an increase in selectivity and ease of sample handling could be expected. Early combinations of the two techniques consisted either of eluting samples separated on thin-layer plates into a suitable solvent and then determining the sample using the conventional quartz Dewar and sample tube arrangement, or of simply dipping the developed thin-layer plates into liquid nitrogen and relying on the phosphorescence observed with the naked eye using U.V. illumination, as the means of visualisation.

Winefordner and co-workers used the first approach to measure 4-nitrophenol in urine[59], biphenyl in oranges[41] and alkaloids in

tobacco[136], whilst at approximately the same time Sawicki and his fellow workers used the latter approach to study a large series of aromatic compounds of interest in air pollution[36]. The phosphorescence visualisation method has also been used more recently by Mayer et al.[137] to study purines, and by de Silva and Strojny, who studied a wide range of pharmaceuticals[135, 138]. The cutting out of the portion of the thin-layer plate (plastic or aluminium backed) which contains the sample, and then placing this in a phosphorimetry sample tube in order to measure the phosphorescence has also been used by several workers[38,39], but to be able to do this with any success, it must be possible to visualise the separated solute first.

Room temperature phosphorimetry[15-19,21] has been observed on media which could be used chromatographically, (almost always filter paper) but it would appear that nobody has yet taken advantage of this possibility. The advantages of room temperature phosphorimetry over conventional phosphorimetry are considerable, as it does away with the need for liquid nitrogen and the associated problems of maintaining a completely dry atmosphere around the sample holding device. Also oxygen quenching is insignificant using the room temperature method, which removes another problem found using non-polar solvents at low temperatures. Its disadvantages are that filter paper has a much higher phosphorescent background than silica gel, alumina or cellulose acetate, and that the compound being studied must bind strongly to the filter paper before phosphorescence is observed, thus normally requiring the compound to be an anion. This limits the general applicability severely. Also, sensitivities at room temperature are poorer.

It was not until the publication of a paper in 1975 by Gifford et al.[9] that an instrument capable of measuring the phosphor-

escence of samples directly on a T.L.C. plate was described. The device fitted into a Baird-Atomic SF100 spectrofluorimeter and could scan about a 10 cm length of T.L.C. plate over a width equal to the height of the slits (12 mm). The authors derived equations for the characteristics of the single disc multi-slot phosphoroscope that was used and showed the separation of 2 µg of each of three different sulphonamides, using the device. The device described here is a development of that first thin-layer phosphorimeter which incorporates several modifications and improvements.

The basic idea of the device is that the developed thin-layer plate (aluminium backed) is wrapped around the outside of a copper drum, which is then filled with liquid nitrogen. The surface of the T.L.C. plate is placed in the light path of the fluorimeter, and the exciting and emitted light is 'chopped' by a single disc phosphoroscope. The sample drum is enclosed in an outer container to separate it from the atmosphere, and is seated on a turntable in order that the whole length of the strip may be scanned.

The thin-layer phosphorimeter

The present device was designed to fit into a Baird-Atomic 'Fluoricord' spectrofluorimeter, mainly because the older SF100 instrument is no longer commercially available. The larger sample compartment in the newer instrument allows a bigger sample drum to be fitted, which, in turn, permits the use of a full 20 cm long thin-layer strip. Fig.19 shows the complete device assembled outside the spectrofluorimeter, while Fig.20 shows an 'exploded' view of the sample holder, sample holder compartment and single-disc phosphoroscope.

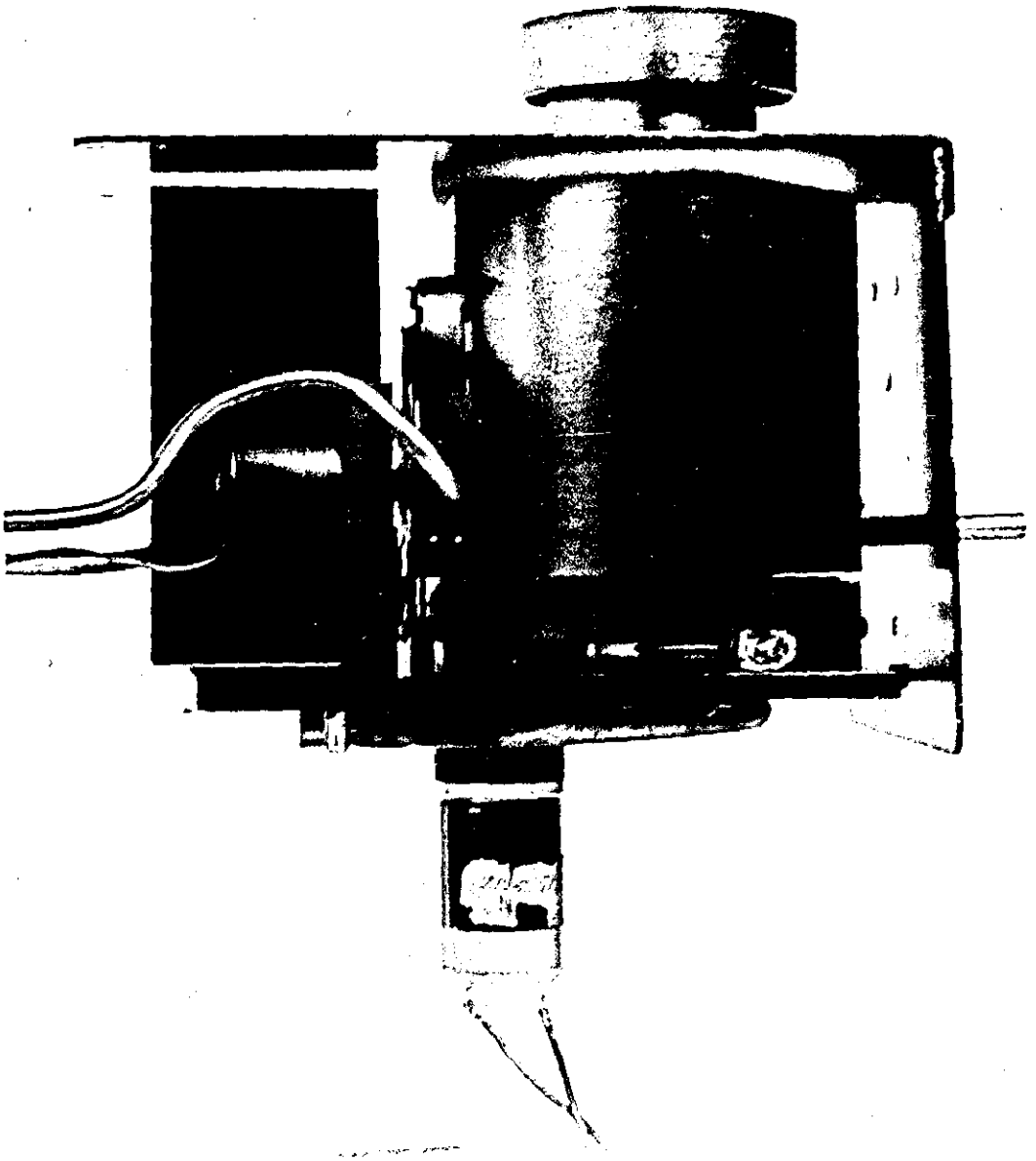


FIG. 19. The thin-layer phosphorimeter

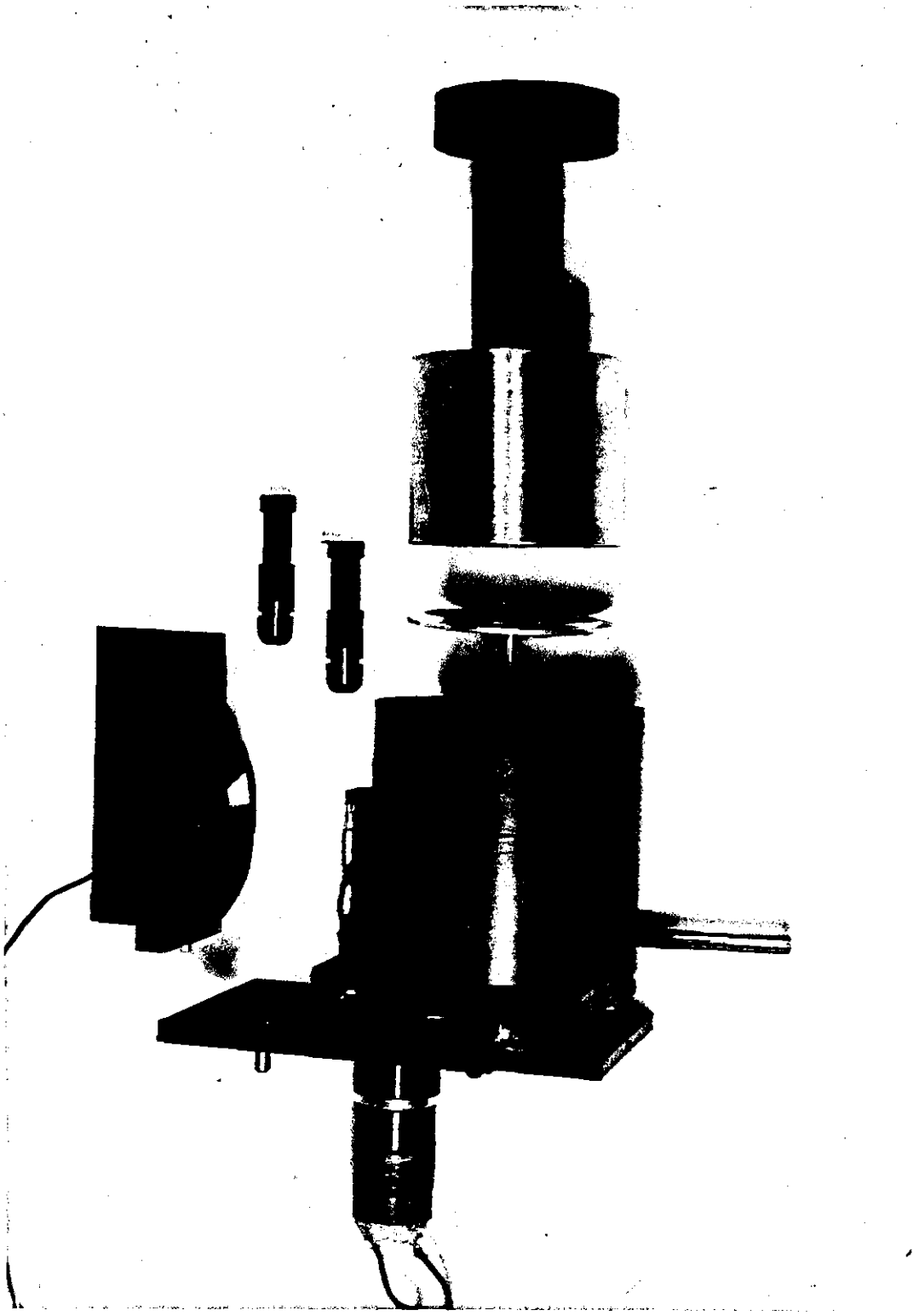


FIG. 20. 'Exploded' view of sample holder, sample holder compartment and phosphoroscope

Fig.21 shows a diagrammatic representation of the sample holder compartment and phosphoroscope assembled.

The sample holder consists of a hollow copper drum of 6.5 cm diameter, the top of which is fitted with a narrower cylinder through which the drum can be filled with liquid nitrogen. The sample strip is attached to the outside of this drum, before the liquid nitrogen is added, by means of two elastic bands. The bottom of the drum is lipped slightly to allow accurate positioning on the turntable. In use, the sample holder fits on the turntable inside the sample holder compartment.

The sample holder compartment is basically a flat steel base-plate fitted with an outer cylinder and a turntable arrangement. The base of the sample drum rests on the turntable, and there is an annular space of 7 mm separating the sample drum from the outer cylinder. The turntable is driven by a 12 v motor, (Maxon 2125-912, Trident Engineering Ltd.) via a reduction gearbox and an intermediate gear. The speed of the motor, and hence the rate of rotation of the T.L.C. plate, is controlled by a variable output transformer, and gives a scanning rate of from 3 to 40 cm min^{-1}. The inside of the cylinder is made effectively airtight by extending it nearly to the lid of the sample compartment, and sealing the gap with an 'O'-ring made out of foam rubber. Dry nitrogen is fed into the instrument by way of a piece of copper tubing, one end of which is brazed on to the cylinder, the other end passing through the new sample compartment door to a supply of dry oxygen-free nitrogen. After passing into the cylinder the nitrogen is allowed to vent to the atmosphere. This prevents oxygen from quenching the excited state sample molecules and also

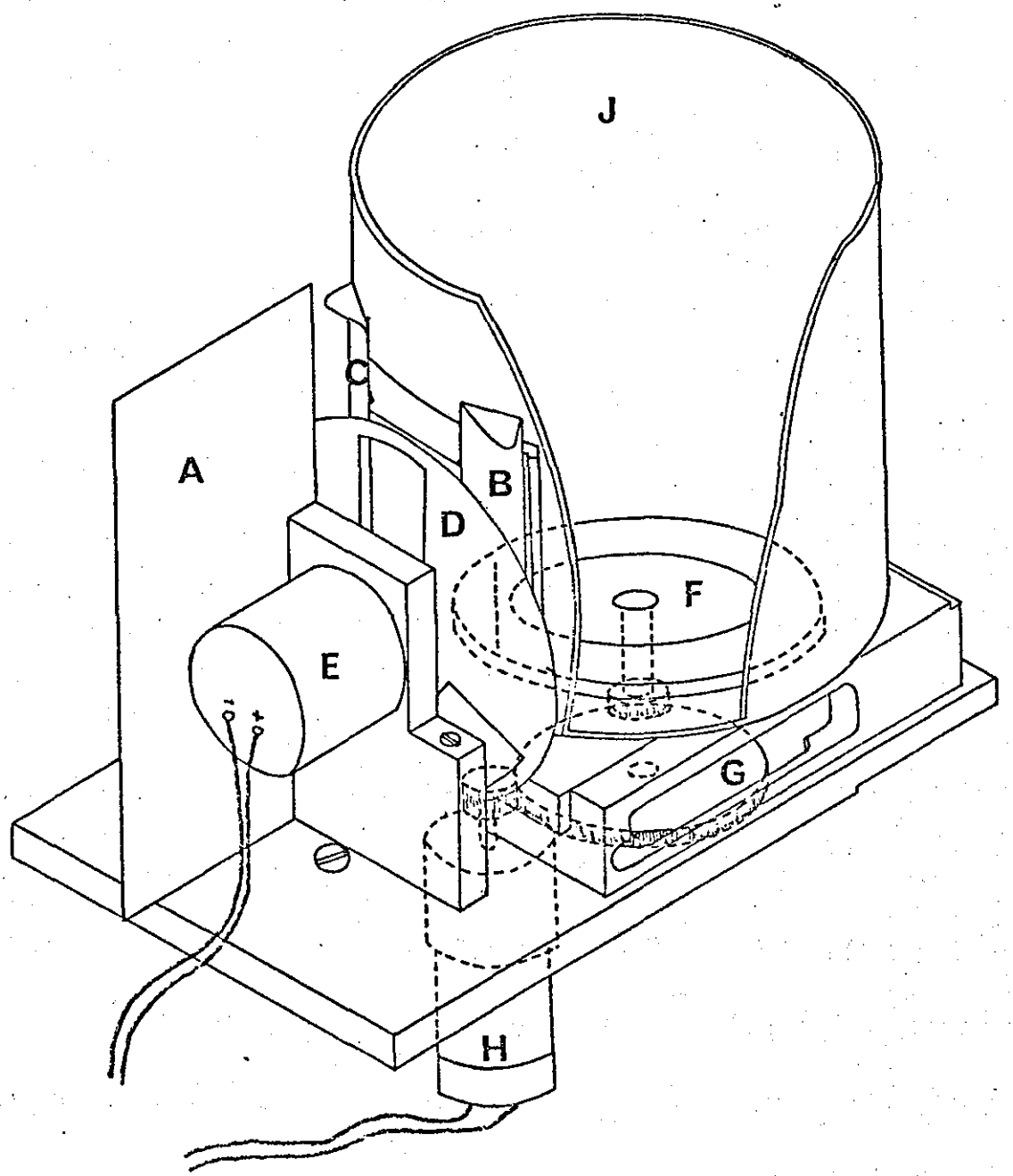


FIG. 21. Sectional diagram of the sample holder compartment and phosphoroscope.

Light baffle (A), slits (B and C), phosphoroscope disc (D), phosphoroscope motor (E), turntable (F), intermediate gear (G), turntable driving motor (H), and outer cylinder (J).

minimises the condensation of water vapour inside the cylinder. In order to allow light to reach the sample, and light from the sample to reach the photomultiplier, two silica windows are fitted to the cylinder at right angles to each other. In front of these are two slits, which help to control the half-bandwidth of the exciting and emitting light. The incident light strikes the sample at 45° to the normal and the emitted light is measured from the same point, also at 45° to the normal. This optical arrangement is theoretically the least desirable of all possible arrangements for surface measurement, because scattered light is at a maximum. The presence of a phosphoroscope however, removes most of the scattered light, and therefore allows the use of such an arrangement.

To prevent ice from forming on the slits and external surfaces of the silica, dry oxygen-free nitrogen is passed over them, via two copper pipes which enter from the rear of the sample compartment.

The phosphoroscope assembly is removable, and is held in place on the baseplate by means of a locating lug and screw. The phosphoroscope is a thin metal disc 65 mm in diameter, having 3 equally spaced slots, 13 mm wide and 16 mm long, cut into it. It is driven by a 12 v electric motor (Faulhaber 26 PC.210, Portescap U.K. Ltd.) at speeds of up to 10,500 rpm. The whole assembly is painted matt black and is fitted with a light baffle to reduce the amount of scattering off the phosphoroscope and other surfaces.

A new lid and front were made for the sample compartment of the fluorimeter. The front is similar to the original, but is located by a small bolt which screws into the baseplate of the thin-layer phosphorimeter and is drilled to accept the front nitrogen-inlet tube.

The lid has a hole cut in it to allow the top of the sample holder to pass through, and around this is fixed a small cylinder. On top of the sample holder fits the mushroom-shaped lid shown in Figs.18 and 19, which was originally intended to prevent stray light from entering the instrument, but was later shown to be unnecessary.

The operation of the instrument is very similar to that of the earlier model. The T.L.C. strip is attached by means of rubber bands to the lower end of the sample drum, and is placed in the sample compartment, which has previously been purged with dry, oxygen-free nitrogen for 5 - 10 minutes. The sample drum is then filled with liquid nitrogen and allowed to cool down (2 minutes), the T.L.C. plate is then ready to be scanned. Scanning is carried out by recording the changes in phosphorescence emission as the T.L.C. plate is rotated, the excitation and emission monochromators having been set to optimal wavelengths for the compounds being investigated.

Method of sample application

Various methods for applying the sample to the plate were attempted, including the use of a hand operated micro-syringe, Corning disposable micro-pipettes (Jobling Ltd., Stone) a 1 cm strip applicator (Burkard, Rickmansworth, Herts.) loaded with disposable micro-pipettes, and an Arnold hand micro-applicator (Burkard, Rickmansworth, Herts.). The latter consists of a 1 ml glass syringe held in an alloy body, the plunger of the syringe being operated by a micrometer screw thread. The barrel of the micrometer is drilled at regular intervals and these holes line up with a spring-loaded ballbearing, giving a positive stop when volumes of 0.25, 0.5, 1, 2 or 5 µl have been delivered.

This study consisted of spotting 5 µl samples of an ethanolic solution of Rhodamine B on to pieces of 250µ thick, pre-coated silica gel T.L.C. plates (E.G. Merck, Darmstedt). Visual observations showed that the Arnold micro-applicator and the Camlab micro-pipettes gave the most reproducible results in terms of spot size and shape. A further study of these two methods of application was carried out. Twenty 5 µl samples of Rhodamine B were applied to another T.L.C. plate using each type of applicator. In the case of the micro-applicator, the needle was placed near the surface of the plate, the micrometer dial was turned rapidly and the needle left in position for 5 seconds before removing. When using the micropipettes the tips were placed just in contact with the plate and the samples allowed to flow out over a period of 5 seconds. When dry the spots were measured using a travelling microscope, the results being shown in Table 15.

TABLE 15

Comparison of spot size using Arnold hand micro-applicator and Corning disposable micropipettes

Diameter of spots (mm) Arnold microapplicator			Diameter of spots (mm) Corning micropipette		
6.19	6.34	6.34	5.74	5.96	5.68
6.33	6.34	6.32	5.73	5.65	5.78
6.28	6.35	6.40	5.94	5.64	5.56
6.28	6.33	6.29	6.07	5.77	5.80
6.38	6.32	6.30	5.79	6.03	5.60
6.32	6.34	6.37	6.04	5.73	5.85
6.31	6.26		5.93	5.83	

Burkard micro-applicator : mean spot dia. 6.32 mm C.V. 0.77%

Camlab micropipette : mean spot dia. 5.81 mm C.V. 2.55%

It can be seen from the results that the micro-applicator gives a more reproducible spot size. This is not to suggest that the micropipettes give a less precise delivery in terms of volume, rather that they cannot apply the liquid at a uniform rate, which the micro-applicator can.

Therefore, the Arnold micro-applicator was used for all quantitative work, except when there were very limited amounts of sample available.

Variation in response of the same sample spot measured several times

The test spots consisted of 5 µl of a 100 µg ml^{-1} ethanolic solution of benzophenone, applied using the Arnold hand micro-applicator. Six spots were applied to both 250µ silica gel coated T.L.C. plates and to 100 µ cellulose acetate electrophoresis strip (Oxoid Ltd. London). The phosphorescence response of each spot was determined five times using the T.L.C.-phosphorimeter, and from these results the coefficient of variation was determined for each type of thin layer. The results are given below:

On cellulose acetate C.V. = 0.7%

On silica gel C.V. = 4.4%

From these results it can be inferred that surface effects of some sort play an important part in determining the reproducibility of sample signals when the sample is adsorbed on to a thin layer. The most likely cause would seem to be physical irregularities in the thin layer itself. From a practical point of view the reproducibility on cellulose is excellent while that on silica gel is quite adequate. In both cases it is superior to conventional phosphorimetric methods[76].

Variation in response of different sample spots measured several times

A similar procedure to that used for determining the variation in response of the same sample spot was adopted, except that the samples were applied to several plates of each type. The results are shown in Tables 16 and 17.

TABLE 16

Instrument response (in mm) to replicate samples of benzophenone on silica gel

Plate Number	Spot	No. of revolutions of sample holder drum					Mean
		1	2	3	4	5	
1	A	141	124	153	158	148	144
	B	133	112	142	141	132	132
	C	64	58	58	60	58	60
	D	49	53	52	50	45	52
	E	57	52	56	53	49	53
2	A	107	136	104	125	100	114
	B	104	141	125	146	110	125
	C	133	127	119	137	105	124
	D	132	101	110	94	102	108
	E	55	53	56	56	56	55
3	A	53	53	48	55	51	52
	B	81	67	76	83	77	77
	C	153	127	140	155	155	146
	D	143	133	160	141	154	146
	E	120	110	131	120	125	121

Mean value of all sample responses 101

Coefficient of variation 36%

TABLE 17

Instrument response (in mm) to replicate samples of benzophenone on cellulose acetate

Plate Number	Spot	No. of rotations of sample holder drum				
		1	2	3	4	5
1	A	127	126	127	126	128
	B	149	148	147	148	149
	C	149	148	148	148	148
	D	149	147	146	148	148
	E	152	149	149	152	153
	F	133	133	131	132	134
2	A	130	135	134	132	132
	B	127	129	128	127	128
	C	131	133	132	133	134
	D	119	121	119	120	122
	E	132	134	134	134	133
	F	130	132	134	132	134
3	A	140	140	144	149	150
	B	154	160	164	165	166
	C	148	153	157	158	158
	D	161	166	170	170	170
	E	144	145	152	154	153
	F	144	148	153	154	154
4	A	106	106	109	108	111
	B	122	121	124	123	124
	C	130	130	130	131	132
	D	128	130	130	130	135
	E	139	137	139	141	143
	F	126	125	128	129	131
5	A	147	148	148	148	146
	B	165	166	163	164	162
	C	152	154	152	152	151
	D	143	144	142	141	141
	E	152	154	148	149	151
	F	125	126	125	125	125

Coefficient of variation of 5 spots on each of 5 different plates = 9.6%

Coefficient of variation of 6 different spots on same plate = 6.2%

Again, they show that reproducibility is far superior when cellulose acetate is used as the thin layer. It would be possible to use the method for quantitative analysis with cellulose acetate, but not with silica gel. In an attempt to obtain better results the silica gel plates were placed in a saturated atmosphere of methanol : acetone (30 : 100) for two hours after spotting. It was hoped that this would allow the spots to equilibrate on the surface of the plate, but no noticeable improvement was observed, the coefficient of variation for this set of results being 44%.

It was not until the solvent enhancement phenomenon (described later in this chapter) was used that it became possible to obtain satisfactorily precise results on silica gel plates. For example, Table 18 shows some results obtained using only 100 ng of each of two sulphonamide drugs, sulphadiazine and sulphanilamide. The plates were developed in a lined chromatographic tank using chloroform : methanol (9 : 1) as the mobile phase. Nine plates were developed separately and each plate was scanned three times. The coefficient of variation was determined for each individual compound and also for the ratio between the two compounds. The precision was still not as good as when cellulose acetate was used, but it was a considerable improvement, and when used with an internal standard the precision would be satisfactory for clinical analysis, particularly considering the small quantities of compound present.

Solvent enhancement of phosphorescence on thin layers

Because several authors[139-142] had reported that various solvents could affect the fluorescence of substances adsorbed on to thin layers,

A, B, C 3 scans
2 sulphonamides + 1 sample, + standard.

TABLE 18

Replicate determinations of sulphadiazine and sulphanilamide on silica gel after spraying with ethanol

Plate No.	Revolution of drum	Response (mm)								Measured ratio of mean	Measured ratio of 'B'
		A Std	A Test	B Std	B Test	C Std	C Test	mean Std	mean test		
1	?	105	140	100	120	94	111	100	124	.81	.83
2		100	112	100	108	94	106	98	109	.90	.92
3		71	84	86	95	70	104	76	95	.80	.90
4	?	69	78	66	78	68	78	67	78	.86	.85
5		85	108	86	117	80	112	84	114	.73	.73
6		70	110	72	97	75	111	72	106	.68	.74
7		74	92	83	112	94	120	84	108	.78	.74
8		84	117	100	120	89	115	91	118	.77	.83
9		100	138	111	146	113	128	108	137	.79	.76
		mean response						88	110		

Coefficient of variation for sulphanilamide (mean of 3 readings)	14.8%
Coefficient of variation for sulphadiazine (mean of 3 readings)	14.6%
Coefficient of variation for the ratio of the two compounds (mean of 3 readings)	8.2%
Coefficient of variation for the ratio of the two compounds (from one reading only, 'B')	8.8%

the effect of ethanol on the phosphorescence of 2.5 µg of chlorpromazine hydrochloride adsorbed on to a 250µ silica gel T.L.C. plate, was determined. The phosphorescence of the silica gel and sample was measured using the T.L.C. phosphorimeter in the normal way, except that the silica gel was sprayed with analar ethanol (Shandon Laboratory spray gun No.2046, London) from a distance of 15-20 cm, prior to cooling the sample drum. The phosphorescence signal of the chlorpromazine was enhanced approximately 100-fold, while the background signal increased less than 10-fold. When a T.L.C. plate with a sample on it was sprayed with ethanol and then allowed to dry before being cooled down, no increase in phosphorescence signal was obtained. This indicates that whatever is responsible for the enhancement, it is totally reversible and dependent only on the presence or absence of the solvent. Table 19 shows the effect of spraying with ethanol for various lengths of time on the measured phosphorescence intensity of chlorpromazine hydrochloride. The term 'wet' is subjective and describes the appearance of the surface of the plate. When a small amount of ethanol is sprayed on the plate it passes into the thin-layer and fills the interstitial spaces. This gives the plate the appearance of being 'damp' but not 'wet'. When all the interstitial spaces have been filled, the ethanol will then form a continuous film on the plate, and this is what is termed 'wet' in this section. Increasing the amount of ethanol on the plate increases the phosphorescence signal, but if the plate is excessively 'wet' i.e. until ethanol is running off it, the integrity of the thin layer is destroyed on cooling, to the extent where parts of the surface may fall off the backing. This is obviously

TABLE 19

Effect of spraying time on the phosphorescence response of chlorpromazine hydrochloride on silica gel

Spray treatment	Corrected response*
None	1
10 seconds, plate not 'wet'	13.5
20 seconds, plate not 'wet'	75
25 seconds, plate just 'wet'	80
until completely 'wet'	135

* 'Not sprayed' result taken as unit response

undesirable as the rough surface causes a very high background signal and noise. It was, therefore, considered best to spray until the plate was just 'wet', a state that can be judged relatively accurately.

A rotating platform and spray holder was built, so that the sample drum could be rotated at 80 r.p.m. and sprayed from a fixed distance. It was found, however, that more reproducible results could be obtained 'by hand' as any unevenness in the spraying could easily be remedied.

A wide range of solvents was then tried, one group consisting of the homologous series of alcohols, in order to study the possible effect of chain length on enhancement, and the second group containing solvents with diverse dielectric constants, to see if this parameter was directly related to the degree of enhancement. Methyl iodide and ethanol/potassium iodide were also used, to ascertain whether a useful external heavy atom effect could be observed. These solvents were used

in conjunction with the drugs sulphadiazine, sulphamethazine, phenobarbitone and methotrimeprazine maleate. Table 20 summarises the results obtained and gives the dielectric constants and viscosity of the solvents used. The enhancement quoted is again a comparison of the phosphorescence response obtained after spraying with the phosphorescence response obtained before spraying. 2-methyl-2-propanol caused severe disruption of the plate, which prevented any results from being obtained for phenobarbitone or methotrimeprazine maleate. With triethylamine, diethylketone methyl iodide and hexane it was not possible to 'wet' the surface of the plate, because all these solvents evaporated too quickly. This would explain the poor enhancement obtained. It was not possible to 'wet' the plate fully with formamide either, the cause in this case being that the cooling effect of the solvent evaporating caused the spray nozzle to ice up completely. It would appear from the results that the best solvent for general use is either one of the lower alcohols or dimethylsulphoxide, although the possible addition of an iodide to produce an external heavy atom effect should be borne in mind. As ethanol is one of the best enhancing agents, is relatively non-toxic and can be obtained with a very low phosphorescence background, it was decided to use it in future work. There is no direct correlation between dielectric constant or viscosity and the enhancement obtained, although compounds with high dielectrics and high viscosity tend to give more enhancement than compounds with low dielectrics and low viscosity.

The enhancing effect of ethanol on a wider range of commonly used drugs is shown in Table 21.

TABLE 20

Enhancement of phosphorescence on silica gel by spraying with various solvents

Solvent	dielectric constant (20°C) (a)	viscosity cp (20°C) (a)	Sulphadiazine	Sulphamethazine	Phenobarbitone	Methotrimeprazine maleate
methanol	32.8	0.60	65	251	15	96
ethanol	24.3	1.20	80	240	20	80
1-propanol	20.1	2.26	120	250	20	75
2-propanol	18.3	2.30 (b)	112	270	34	100
1-butanol	17.1	2.95	100	240	35	100
2-methyl-2-propanol	10.9	(15°C) 4.70	30	60	--	--
1-pentanol	13.9		45	136	16	66
1-hexanol	13.3		21	70	9	50
triethylamine	2.4		5	6	2	18
dimethylformamide	37.6		108	152	9	53
formamide	109.5	(25°C) 3.30	21	18	5	45
diethylketone	17.0	(15°C) 0.49	4	12	--	32
ethanol/potassium iodide	--	--	205	288	30	75
methyl iodide	7.0	0.50	2	8	1	1
dimethylsulphoxide	46.7	2.24 (b)	137	300	13	86
hexane	1.89	0.33	11	31	11	12
water	80.4	1.00	11	19	7	50

(a) from 'Handbook of Chemistry and Physics' 46th ed. 1965

(b) from 'Principles of adsorbtion chromatography', L.R. Sayder, Arnold Ltd., London 1968.

TABLE 21

Enhancing effect of ethanol on the phosphorescence of compounds adsorbed on to silica gel

Compound	enhancement
Methylphenobarbitone	16
Phenobarbitone sodium	20
Phenobarbitone	15
Phenylmethylbarbituric acid	18
Chlorpromazine HCl	52
Sulphadiazine	91
Sulphamerazine	165
Sulphamethazine	309
Methotrimeprazine maleate	80
Pericyazine	55

The degree of enhancement appears to be related in some way to the structure of the compound, related molecules often having similar enhancement factors. Also, the presence or absence of ionic binding to the gel does not seem to have much effect, as is shown by the fact that the phenobarbitone free acid and the sodium salt show similar enhancements.

In an attempt to find out more about the processes producing this enhancement, the phosphorescence of sulphadiazine, sulphamethazine, phenobarbitone sodium and methotrimeprazine maleate were studied on a variety of thin layers. The silica gel and cellulose were commercially produced thin layers (Merck) on aluminium backing, the alumina was a home made thin layer on an aluminium backing and the cellulose acetate was thin electrophoresis sheet (Oxoid Ltd.) The diameter of spot

produced when 5 µl of ethanol was applied to different adsorbants is shown in Table 22, along with the thickness of adsorbant layer.

TABLE 22

Adsorbant layer thickness and diameter of spot produced by 5 µl of ethanol

Type of adsorbant	Thickness (mm)	spot diameter (mm)
Silica gel	0.25	7
Cellulose	0.10	12
Alumina	0.40	5
Cellulose Acetate	0.13	11

This shows that the thicker the layer is on the plate, the smaller the spot size produced, presumably because the sample can diffuse more into the plate instead of having to spread outwards which is what would be expected.

The effect of these different adsorbant layers on the phosphorescence signal of several compounds, either untreated or sprayed with ethanol, is shown in Table 23. The most likely cause of the 'inverted peaks', (i.e. compound signal being smaller than background signal) seen on the alumina thin layer is that the alumina has a high intrinsic luminescence, and the sulphonamides are quenching this to some extent. Apart from this there is a general trend that the thicker the layer is, the lower the original phosphorescence signal and the greater the enhancement obtained by spraying. Although the

TABLE 23

Phosphorescence of samples on various thin layers

Thin layer	Treatment	Corrected response (mean of 3 values)			
		Sulphadiazine	Sulphamethazine	Phenobarbitone sodium	Methotrimeprazine maleate
Silica gel	none	1.6	1.1	N.D. < 0.5	3.6
Silica gel	sprayed	98	260	5.5	400
Alumina	none	inverted peaks		N.D. < 0.5	22
Alumina	sprayed	77	122	7.4	62
Cellulose	none	32	40	1.4	71
Cellulose	sprayed	82	124	1.8	158
Cellulose acetate	none	70	82	8.9	89
Cellulose acetate	sprayed	*	56	1.7	42

N.D. not detectable

* Individual values 5, 93 and 26 so mean not taken

responses for the sprayed plates are not all identical, their range is much smaller than the range of responses obtained on unsprayed thin layers. The results for the cellulose acetate are anomalous, because when the sheets are sprayed there is a large but variable increase in background signal due to the cellulose acetate 'wrinkling', but little or no increase in the phosphorescence signal of the compound. It is this variability of background that gives the appearance of a decrease in phosphorescence emission of the sample compound.

In the final experiment in this series, the phosphorescence lifetimes of a selection of compounds in or on various media were determined. The compounds used were sulphadiazine, sulphamethazine, and phenoxazine, each of the results shown (Table 24) being a mean of three determinations.

TABLE 24

Phosphorescence lifetimes (in seconds) in or on various media

Medium	Sulphadiazine	Sulphamethazine	Phenoxazine
Ethanol glass	0.7	0.8	2.2
Silica gel	0.7	0.8	N.D.
Silica gel (sprayed with ethanol)	0.8	0.8	2.6
Cellulose acetate	0.7	0.7	2.2

N.D. Not determined, signal too weak.

The differences in lifetime shown are within the limits of the experimental method (a chart recorder was used), so no significant changes in lifetime were observed.

To summarise the findings of this work:-

1. The enhancement process is reversible and totally dependent on the presence of a solvent.
2. Little or no diffusion of the sample spot occurs, even if the plate is not cooled immediately after spraying.
3. The physical nature of the solvent has some effect on the enhancement but not a great deal.
4. The degree of enhancement is closely related to the chemical structure of the compound being measured.
5. The quantity of the solvent is important.
6. There is no change in the phosphorescence lifetime, which would suggest that the solvent is not affecting the basic emission processes.
7. The integrity of the plate is not normally destroyed by the solvent, so it is not likely to be increasing the size of the interstitial spaces.

From these results it might be concluded that as spraying with a solvent appears not to affect the T.L.C. adsorbent, the adsorbent-compound interactions or the efficiency of the phosphorescence process, the effect must be an optical one, the adsorbent layer being made transparent to the exciting light, the emitted light or both. This conclusion is supported to some extent by the fact that spraying silica gel with dimethylformamide made the plate completely transparent to the naked eye, so that the 'grain' structure of the aluminium sheet could be seen behind it.

CHAPTER 6

The use of thin-layer phosphorimetry for drug analysis

Experimental method

The method given below was developed mainly from the results described in the last chapter. A suitable chromatographic development system is needed for each drug to be studied, either utilising one already available or developing a new method if no suitable ones have been described. Ideally, the method should give an Rf of approximately 0.3 to 0.7, use only one solvent and take about thirty minutes to develop a 20 cm plate. In practice however, it is unusual to be able to obtain all three features, and with T.L.C. phosphorimetry the former is the most important consideration.

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. The tank and its contents were not thermostated, although if this had been possible, better precision might have been expected[143]. Before applying the samples to the thin layers, the T.L.C. plates were cleaned by developing them in a T.L.C. tank containing ethanol. Most of the impurities were carried to the top of the plate and were removed by cutting the top 2 cm off the plates[144]. All experiments were carried out in the dark to reduce the possibility

of sample photodecomposition. After each developed plate had been allowed to dry it was wrapped around the sample holder drum of the thin-layer phosphorimeter and held in place with two 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 at a rate of 5 cm min^{-1} and the results recorded on a chart recorder.

Detection limits of a variety of drugs in pure solution

The compounds studied, their structure and the suppliers are shown in Table 25. With the exception of N-4-acetylsulphanilamide, all these compounds have also been studied by de Silva and Strojny[138] who observed them on T.L.C. plates either using fluorescamine derivatisation or native phosphorescence at 77K. Quantitation was not possible as they were only observing the spots by eye, but limits of detection were given on this basis. These form a useful comparison with the results obtained in this study.

The solvent systems used for the chromatography of the compounds, the Rf values obtained and the phosphorescence excitation and emission wavelengths used are shown in Table 26. The sulphanilamide T.L.C. system was from Guren and Perkin[145] and the sulphadiazine system was that of Van der Venne and T'Siobbel[146]. The rest of the T.L.C. systems were developed in this work to give Rf values of approximately 0.3 to 0.7.

Table 27 shows the results obtained using the combined technique and also gives the results obtained by de Silva and Strojny. It can be

TABLE 25

Structure and suppliers of compounds studied by T.L.C.-phosphorimetry

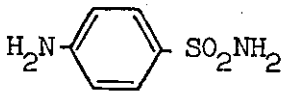
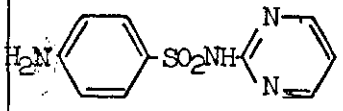
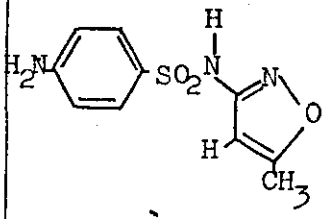
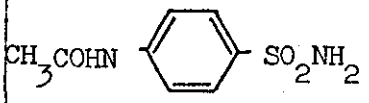
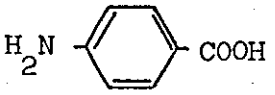
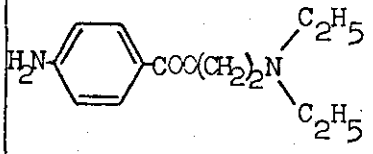
Compound	Structure	Suppliers
Sulphanilamide		Pharmaceutical Specialities (May and Baker) Ltd.
Sulphadiazine		"
Sulphamethoxazole		Dr.Gifford, Loughborough University
N-4-acetylsulphanil-amide		"
4-aminobenzoic acid		Fisons Ltd.
Procaine hydrochloride		B.D.H.Ltd.

TABLE 26

T.L.C. conditions and phosphorescence excitation and emission wavelengths of compounds studied

Compound	solvent system	Rf	λex (nm)	λem (nm)
Sulphanilamide	chloroform : methanol 9 : 1	.28	305	405
Sulphadiazine	ethyl acetate : methanol : 25% NH_3 17 : 6 : 5	.40	300	405
Sulphamethoxazole	chloroform : butanol : acetic acid 15 : 1 : 1	.55	295	410
N-4-acetylsulphanilamide	chloroform : butanol : ethanol 25% NH_3 15 : 5 : 5 : 1	.61	285	410
4-aminobenzoic acid	chloroform : butanol : acetic acid 15 : 1 : 1	.57	310	420
Procaine hydrochloride	methanol	.52	310	420

TABLE 27

Detection limits of compounds on T.L.C. plates (ng/spot)

Compound	T.L.C. phosphorimetry 77K	visual detection limits (a)		
		fluorescamine deriv. 25°C	fluorescamine deriv. 77K	native phosphorescence 77K
Sulphanilamide	0.5	50	50	100
Sulphadiazine	2	200	100	1000
Sulphamethoxazole	3	100	50	1000
N-4-acetylsulphanilamide	7	--	--	--
4-aminobenzoic acid	0.6	200	50	100
Procaine hydrochloride	2	50	50	100

(a) These results from Table 1, J.A.F. de Silva and N.Strojny [138]

seen that the combined technique is between 50 and 500 times more sensitive than visual observations of phosphorescence, as well as being quantitative. It is also about 50 times more sensitive than fluorescamine derivatives observed visually at 77K. The method may also have a wider application than fluorescamine derivatisation, because it does not depend on the presence of a primary amino group in order to achieve its results. This point is illustrated by the inclusion of N-4-acetylsulphanilamide, which could not be determined using fluorescamine. These results show that the sensitivity of the method combined with the established selectivity of T.L.C. gives it great potential in the analysis of *in vivo* drug levels and their metabolites. This is demonstrated in the next section using thioridazine as the example.

Determination of thioridazine and its metabolites *in vitro* and *in vivo*

This work was carried out in collaboration with Dr. R.G.Muusze of Het St.Joris Gasthuis, Delft. Dr. Muusze has over a period of four years developed a T.L.C. quantitation method for thioridazine and five of its metabolites, using fluorescence oxidation for visualisation and measuring the spots with a 'Vitatron'. He became interested in the idea of combining his T.L.C. system with the low temperature phosphorescence measurements as this would obviate the need for an oxidation step, which must introduce additional errors into the procedure. He therefore offered to provide T.L.C. plates containing thioridazine and its metabolites, and also plasma samples of patients undergoing thioridazine therapy. This kind offer was accepted and the results shown below were obtained using T.L.C. plates provided by him. A full description of the procedures he uses is given in chapters

3 and 4 of his doctoral thesis (1975). A brief summary of the parts relevant to this study is given below.

After extraction from plasma with a suitable solvent, the extract is evaporated to dryness and redissolved in 60 µl of the chromatographic solvent system (acetone : 25% NH_3, 96 : 4). The whole extract is placed in a motor driven 100 µl syringe, which when used in conjunction with an 'Autoliner', gives a sample strip of 30 x 3 mm. The sample strip is converted into a sample spot by pre-development in a specially constructed vapour-box: the plate is allowed to become equilibrated with aqueous ammonia vapour, and the strip is then developed with acetone until the whole of it is concentrated in the solvent front as a spot approximately 4 x 3 mm. After drying, the plate is then developed in the conventional way using acetone : 25% aqueous NH_3, 96 : 4. It was at this point that the plates were sent to this laboratory for measurement.

Fig.22 shows the results obtained using the 'Vitatron' and T.L.C.-phosphorimeter, on a developed plate containing 50 ng of thioridazine and 5 of its metabolites, namely: northioridazine (NT), thioridazine-2-sulphone ($T2SO_2$), thioridazine-2-sulphoxide (T2SO), thioridazine-5-sulphoxide (T5SO), and northioridazine-2-sulphoxide (NT2SO). Both methods give similar resolution and baseline stability, even though acetone is a poor solvent for T.L.C. phosphorimetry, owing to the presence of phosphorescence impurities in even the most carefully prepared acetone sample. (The 'Vitatron' results are a mirror image of the originals, in order to make them comparable to the phosphorimetric results).

Fig.23 shows the results obtained from an extract of 1 ml plasma taken from a patient receiving 150 mg of thioridazine daily. Again, it can be seen that both methods give comparable resolution, although

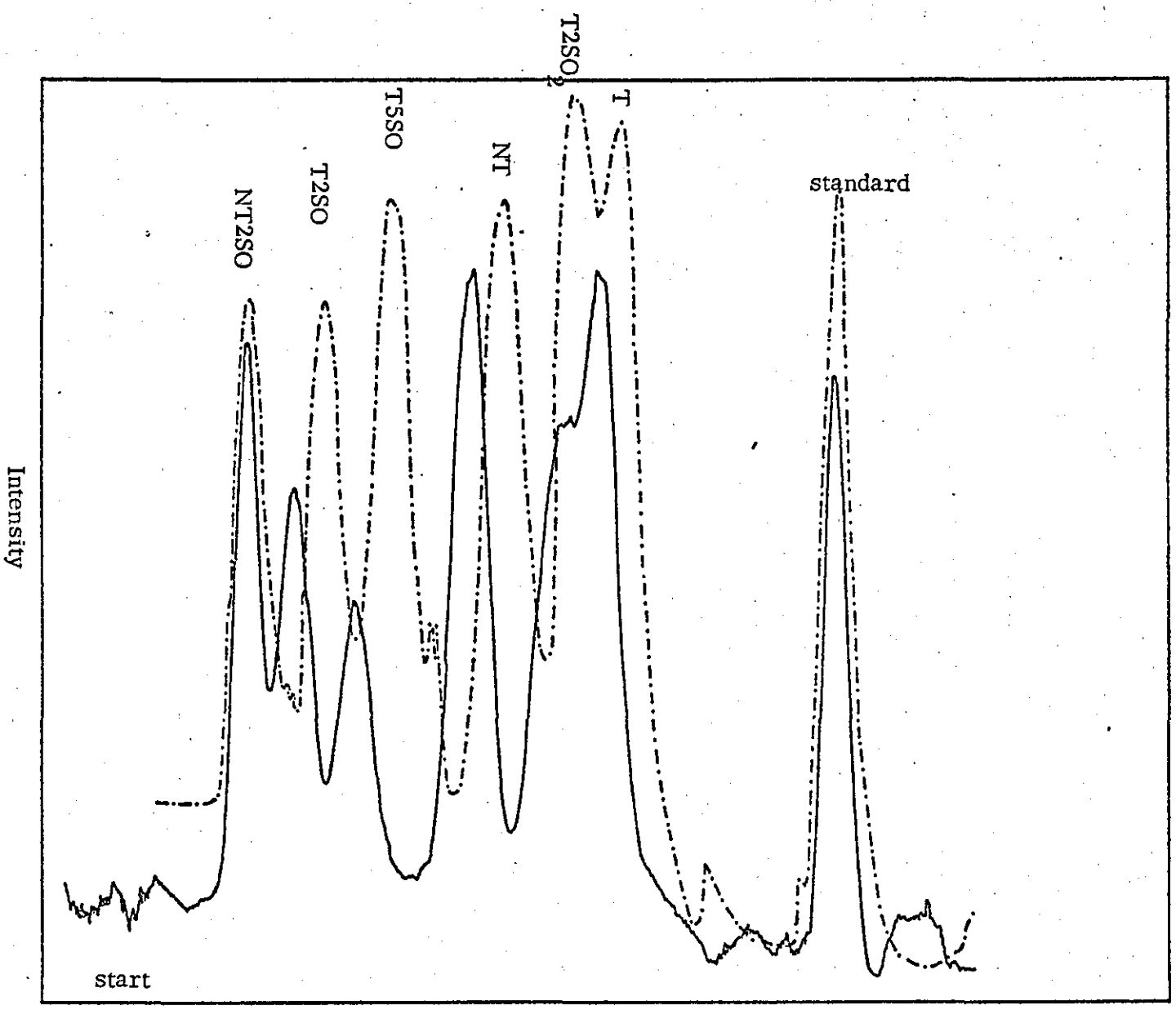


FIG. 22. Comparison of T. L. C. phosphorimeter and densitometer I; scan of a chromatogram containing 50ng of thioridazine and 5 of its metabolites (see text)

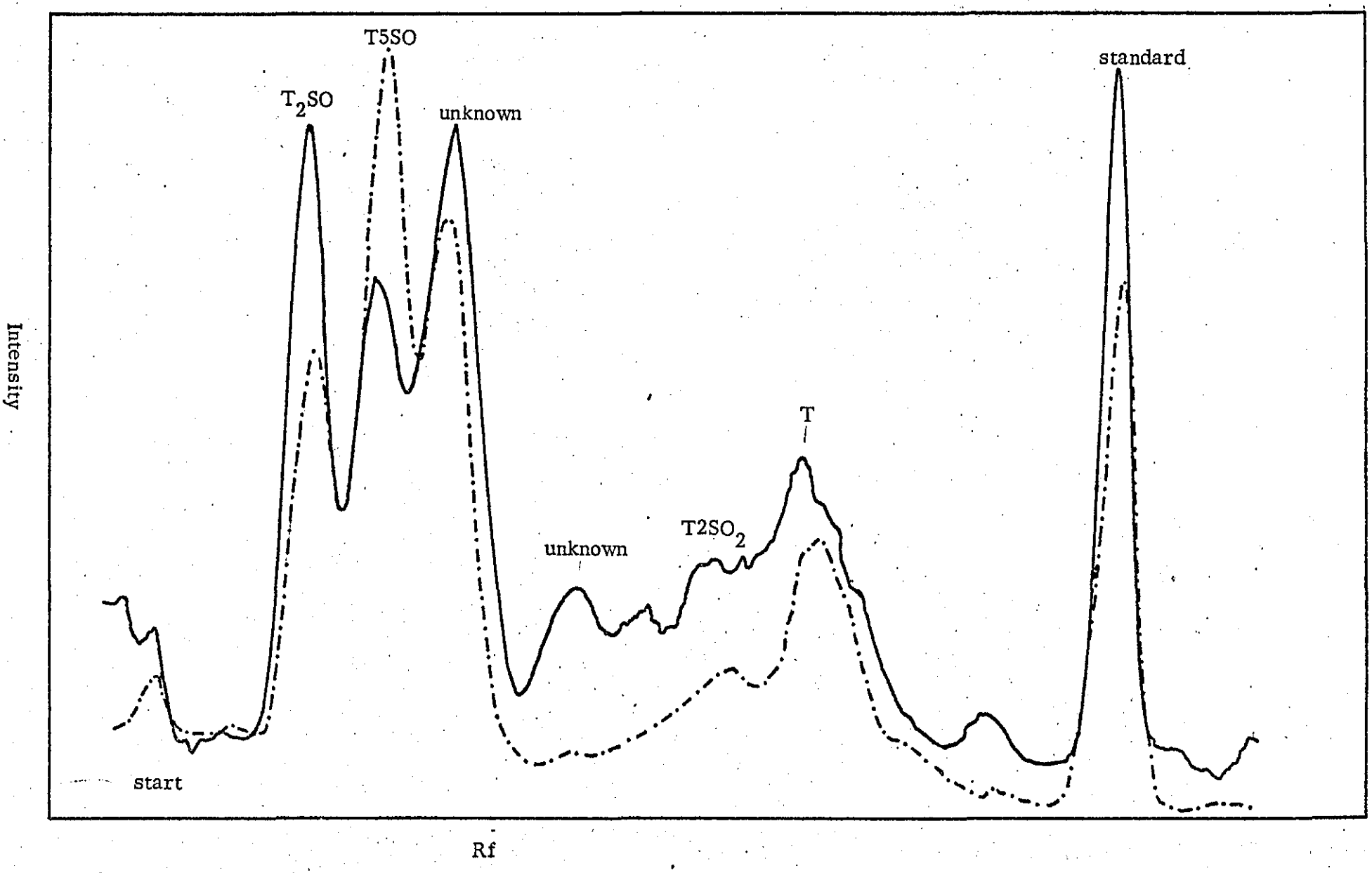


FIG. 23. Comparison of T. L. C. phosphorimeter and densitomer II; scan of a chromatogram containing the extract from 1 ml plasma of a patient on 150 mg thioridazine daily.

the quantitative results do differ quite a lot, as is shown in Table 28.

TABLE 28

Quantity of thioridazine and some of its metabolites found in 1 ml of patient's plasma

compound	Low temp. phosphorescence-T.L.C.	fluorescence oxidation
T	185 ng	153 ng
$T2SO_2$	160 ng	50 ng
T5SO	485 ng	371 ng
T2SO	360 ng	155 ng

The most likely reasons for these differences are twofold. Firstly, in the case of T.L.C. phosphorescence, the ratios of peak response between 50 ng of standard and 50 ng of thioridazine or one of its metabolites were determined from the results obtained using one T.L.C. plate only. Secondly, the peak areas were determined using an integrator on the 'Vitatron' whereas they had to be measured using a planimeter in the case of the phosphorimeter. Both these factors would tend to make the phosphorescence values less accurate, but these problems could easily be overcome were the method to be used on a regular basis.

The practical limit of detection for thioridazine and its metabolites in plasma is around 10 - 20 ng per spot using thin-layer phosphorimetry. Fig.24 shows the results obtained using a 'spiked' plasma sample containing 20 ng of each compound, and all of them can

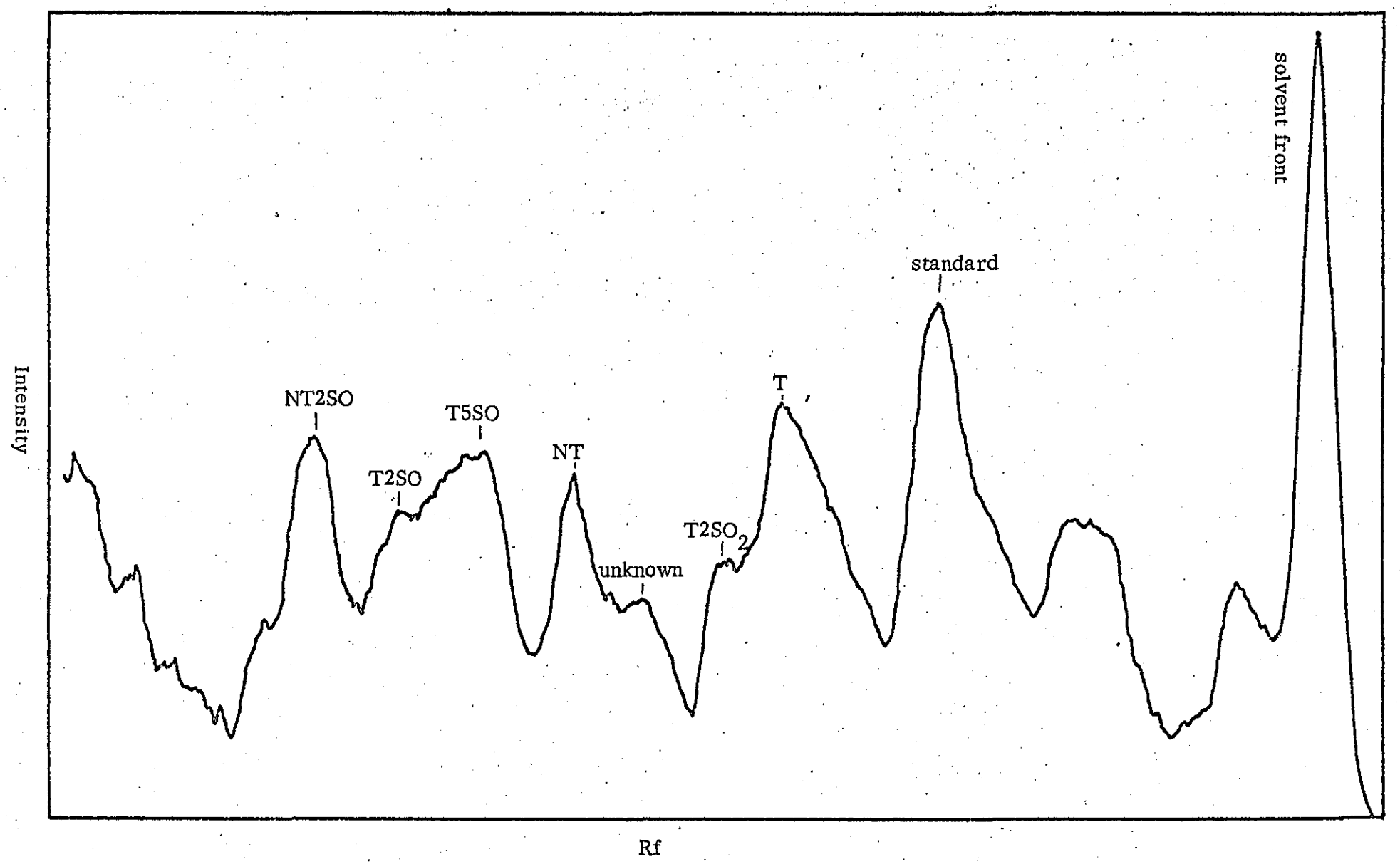


FIG. 24. Comparison of T. L. C. phosphorimeter and densitometer III; scan of a chromatogram containing the extract from 1 ml plasma 'spiked' with 20 ng of thioridazine and 5 of its metabolites.

still be easily seen. This shows very well the great practical potential of the method, which is able to detect low ng/ml^{-1} quantities of drugs present in plasma. With further development of the T.L.C. methods even lower concentrations of drugs might well be detectable.

CHAPTER 7

CONCLUSIONS AND DISCUSSION

Work carried out in this thesis on the phenothiazine family of drugs, showed that it was not possible to use aqueous or aqueous-acid solvents for the phosphorimetric analysis of these compounds. Examination of their phosphorescence characteristics in ethanol at 77K revealed a high degree of similarity between individual members of the group, but enough differences were present to be able to distinguish one compound from another in most cases. Detection limits were low enough to enable the method to be considered as a means of determining the *in vivo* concentrations of these drugs. Attempts to correlate the luminescence spectrum of phenothiazine directly with interatomic distances of π-electron densities were not successful, but the degree of fluorescence present did appear to be related to the configuration of the ring structure.

Some of the antirheumatic and antipyretic drugs were shown to be capable of analysis by either fluorimetry or phosphorimetry. The clinically important drug capable of being determined by fluorimetric analysis was indomethacin, after an alkaline hydrolysis. 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 phenazone, mefenamic acid, oxyphenbutazone, and sulphinpyrazone at sub-microgram per ml. concentrations.

The Corning-Eel 244 filter fluorimeter was shown to be capable, after suitable modifications, of determining drugs and other organic compounds at concentrations of around 1 $\mu g\ ml^{-1}$, by measuring their phosphorescence at 77K. Such an instrument should enable phosphorimetry to be used on a more routine basis, where cost of equipment and ease of use are important considerations. A fluorimeter which seems potentially more suitable for this sort of role has quite recently appeared on the market. It is a Perkin-Elmer model 1000, which has tunable excitation filters, a scanning interference wedge on the emission side and xenon flash tube as a light source. The latter is claimed to give nearly as high a peak intensity as a xenon arc lamp, and to be very stable. This should give the instrument a far greater sensitivity when used as a phosphorimeter, than would be obtainable using a mercury vapour lamp as a light source. As the model 244 has just been discontinued, the latter instrument would seem an ideal substitute, even though it is more expensive.

The combined technique of T.L.C.-phosphorimetry was developed to the point where it could be used as an analytical method under genuine laboratory conditions. It was shown to be capable of measuring low ng quantities of thioridazine and its major metabolites in plasma extracts. The precision of the method was satisfactory for use in the clinical situation, and although improvements could be made by maintaining stricter control over the chromatographic development phase, this would be at the expense of increasing the complexity of the method and the time required to obtain the results. It would therefore depend on the particular needs of the operator whether the better precision or higher throughput was deemed the most important.

The use of a solvent to enhance the phosphorescence signal was found to be absolutely necessary in order to obtain low detection limits, and although the quantity of solvent had some effect on the intensity of the signal, there was no problem of the signal changing whilst in the instrument. This is, of course, due to the solvent being at 77K, but it does obviate a problem which occurs when solvent enhancement is used at room temperature.

This technique appears to have great potential in a wide range of fields, particularly where small quantities of organic compounds are present in complex mixtures.

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