# LOW TEMPERATURE LUMINESCENCE ANALYSIS OF DRUGS AND POISONS

by

LAWRENCE ALBERT GIFFORD, B.Sc., ARIC.

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology. September 1973.

Supervisors: J. N. Miller, M.A., Ph.D.
D. Thorburn-Burns, B.Sc., Ph.D., FRIC., D.Sc.
Department of Chemistry.

T O  M Y  F A T H E R

# A C K N O W L E D G E M E N T S

The author wishes to express sincere thanks to his research supervisors Dr. J. N. Miller, Dr. D. Thorburn Burns, Dr. J. W. Bridges and Dr. W. P. Hayes for their advice and encouragement.

The co-operation of many drug companies in the generous donation of samples is appreciated and financial assistance from the Medical Research Council is gratefully acknowledged.

There are no words to express the deep appreciation that is due to the author's wife and his family for their prayerful support and encouragement.

The author certifies that the work contained in this thesis is original and has not been submitted to this or any other institution for a higher award.

C O N T E N T S

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# C H A P T E R  O N E

## I N T R O D U C T I O N .

### i) Luminescence Processes in Solution.

Fluorescence and phosphorescence are both processes in which radiation is emitted by an excited state molecule following the absorbtion of radiation. The luminescence processes leading to these phenomena are shown in FIG 1.

Prior to the absorbtion of radiation the molecule is usually in the ground electronic state So. Promotion to higher electronic levels is a very rapid process occuring in a time interval of approximately $10^{-15}$ seconds. Electronic excitation produces molecules with additional vibrational as well as electronic energy. The excess vibrational energy is very rapidly removed by collisions, so that the excited molecule becomes thermally equilibrated with the solvent in less than $10^{-12}$ seconds. The excited molecule then returns from the first excited singlet level to an excited vibrational level of the ground state in $10^{-8}$ seconds or longer. The omitted quantum therefore has less energy than the absorbed quantum with the result that the observed emission band is at a longer wavelength than the absorbtion band. In the process described the state from which emission originated and terminated had the same multiplicity, hence the phenomena is termed fluorescence.

Fluorescence from molecules in solution, however, shows a greater separation of absorbtion and fluorescence bands than can be accounted for by vibrational energy losses alone. The separation of the bands is also dependent upon temperature and solvent polarity. Many compounds exhibit a blue shift in the fluorescence spectrum when the temperature is lowered and the solvent becomes more viscous. At the room temperature solvent molecules

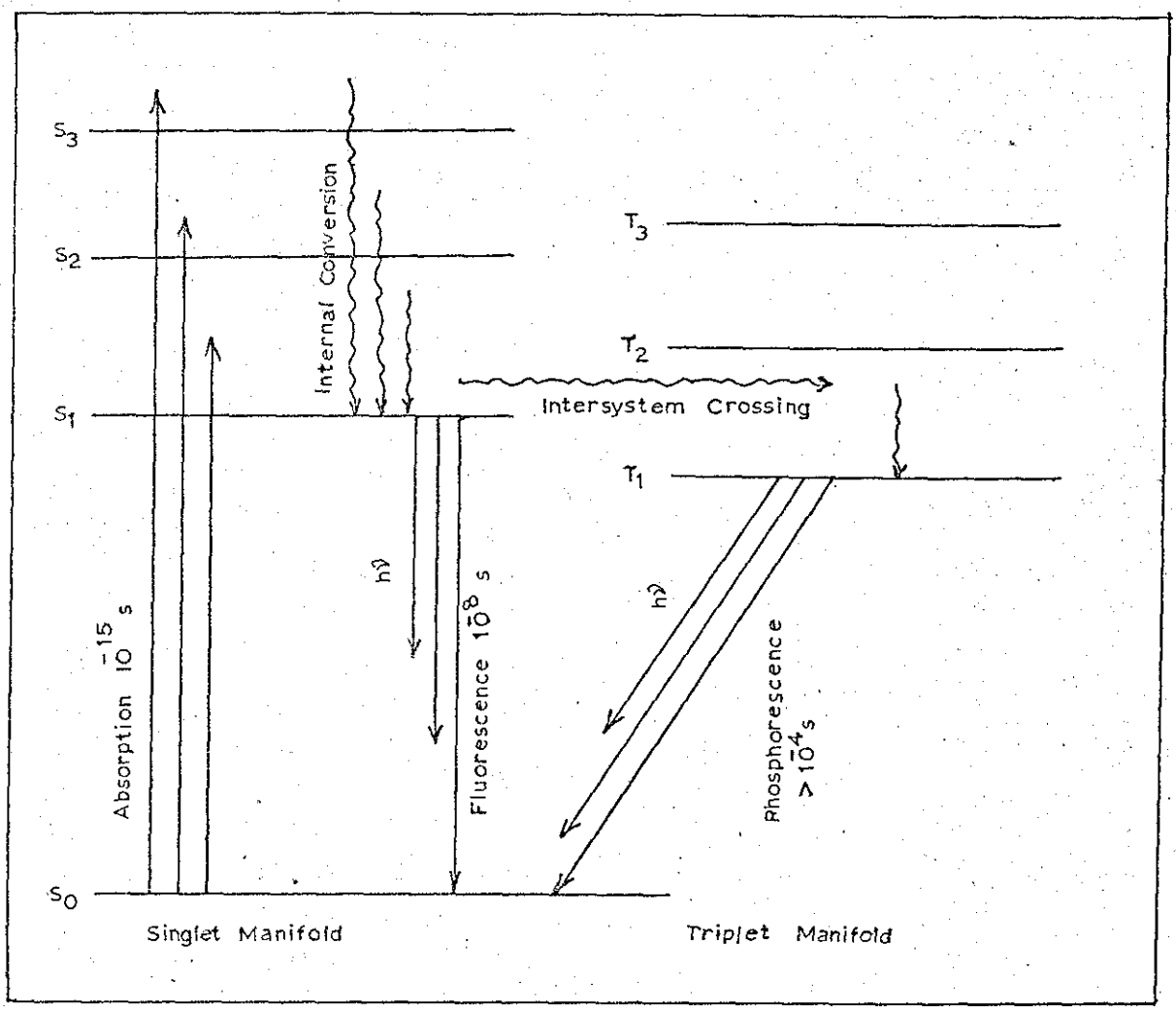



FIG 1. Jablonski diagram representing fluorescence and phosphorescence processes.

reorient themselves around the excited solute molecule in about $10^{-11}$ seconds. Since the lifetimes of the fluorescent states are of the order $10^{-8}$ seconds emission from the Franck-Condon state is not observed at room temperature. If the temperature is lowered sufficiently, the time required for reorientation of the solvent molecules may be longer than the lifetime of the excited state and emission from the Franck-Condon state will be observed.

Molecular fluorescence from solutions has the important feature that only one fluorescence band is observed for any given substance. If shorter wavelength light is used for excitation to produce a higher electronically excited state the same fluorescence band is produced. The excess electronic energy is lost by collisional deactivation in a time interval shorter than that required for fluorescence. Hence the excess energy is dissipated to the solvent as heat before the fluorescence process occurs.

Radiationless transitions between energy levels of the same multiplicity are referred to as internal conversions. Radiationless transitions occuring between energy levels of different multiplicity are referred to as intersystem crossing. Intersystem crossing from the singlet excited levels results in the population of the triplet levels. Emission from the lowest triplet level to the singlet ground level is termed phosphorescence. This is a "forbidden" transition of long radiational lifetime and is susceptible to quenching by collisional deactivation. Phosphorescence can therefore be enhanced by the use of low temperatures and rigid glass-like solvent media. To facilitate the study of phosphorescence, measurements are usually made at 77°K. This can be achieved conveniently by placing a Dewar flask containing liquid nitrogen in the conventional fluorimeter. The presence of heavy atoms

in the molecule or solvent media may also increase the rate of intersystem crossing and lead to a greater phosphorescence signal.

Jablonski[1] suggested a process whereby a reversal of intersystem crossing could take place viz., the $T_1 \rightarrow S_1$ transition. This process involves higher nuclear vibration terms of $T_1$ and requires the addition of thermal energy. Since the final emission is from the $S_1$ energy level it has an identical wavelength to that of fluorescence, however, since the triplet energy levels are involved the lifetime of the delayed fluorescence is comparable to that of normal phosphorescence. Fluorescence produced in this manner is termed "E-type" delayed fluorescence.

Kautsky and Muller[2] postulated a further mechanism to account for the delayed emission encountered in adsorbates of tryptaflavin. The energy necessary for the occupation of the $S_1$ level from the $T_1$ level is obtained when two triplet state molecules react with one another so that one of the molecules takes up the triplet energy of the other and undergoes intersystem crossing into the first excited singlet state. The other molecule undergoes a radiationless transition to the ground state. Delayed

$$T_1 + T_1 \longrightarrow S_1 + S_0$$

fluorescence produced by this mechanism is termed "P-type" delayed fluorescence. "E-type" and "P-type" delayed fluorescence can be distinguished from one another since the former is produced by a mono-photonic mechanism and the latter by a bi-photonic mechanism.

The luminescence mechanisms already outlined account for the majority of those encountered in luminescence analysis. However, there are others such as sensitized luminescence which may be of potential use. Sensitized luminescence occurs by an energy transfer process between an excited donor molecule D and a ground state acceptor molecule A $S_o$. An excited acceptor molecule A is produced which is then free to undergo radiactive deactivation. The three major processes are outlined below:-

Sensitized fluorescence.

$$DS_1 + AS_o \xrightarrow{\text{ENERGY TRANSFER}} AS_1 + DS_o$$

$$AS_1 \xrightarrow{h\nu} AS_o$$

Sensitized phosphorescence.

$$DT_1 + AS_o \xrightarrow{\text{ENERGY TRANSFER}} AT_1 + DS_o$$

$$AT_1 \xrightarrow{h\nu} AS_o$$

Sensitized delayed fluorescence.

i) $DT_1 + AS_o \xrightarrow{\text{ENERGY TRANSFER}} AT_1 + DS_o$

ii) $AT_1 + AT_1 \longrightarrow AS_1 + AS_o$

$$AS_1 \xrightarrow{h\nu} AS_o$$

or

$$AT_1 + DT_1 \longrightarrow AS_1 + DS_o$$

$$AS_1 \xrightarrow{h\nu} AS_o$$

However, these sensitized processes are susceptible to quenching and few examples have been reported.

ii) Phosphorescence Measurements.

The first phosphoroscope, which was described by Becquerel[3] in 1871, enabled the delayed emission of phosphorescence to be isolated from the prompt fluorescence emission. This phosphoroscope consisted of two discs from which notches had been cut at equal intervals. The discs were offset from each other and mounted on a common axis which was turned by a variable speed motor. When the excitation radiation was cut off by the first disc, the fluorescence decayed rapidly and only the long lived luminescence remained. At this point the emission shutter was in the open position and the long lived emission was recorded by the photodetector. A less important function of the phosphoroscope resulted in a minimal measurement of scattered incident light.

Two other types of phosphoroscope have since been developed, these being the rotating can phosphoroscope[4] and the pulsed source phosphoroscope[5]. The rotating cam phosphoroscope consists of a hollow cylinder with one or more slits equally spaced in the circumference. As the can is turned the radiation from the excitation monochromator is allowed to strike the sample, and alternately the light emitted from the sample is allowed to reach the emission monochromator entrance slit. In the pulsed source phosphoroscope, excitation is achieved by means of a periodically pulsed flash tube and the emission is monitored by means of a photodetector which is operated periodically and out of phase with the flash tube. Both of these phosphoroscopes allow the long lived luminiscence to be measured without interference from the short lived radiation.

Phosphorescence is a first order process in solid solution, thus pure compounds containing a single phosphorescent

moiety show a linear relationship between the logarithm of the phosphorescence signal and time after the termination of the exciting radiation. The lifetime of a phosphorescent molecule is defined as the time required for the luminiscence signal to decay to 1/e of its initial value, and is characteristic of a compound at constant temperature and in a given solvent. The lifetime is therefore a useful parameter in the identification of a compound together with the wavelengths of excitation and emission.

Phosphoroscopic resolution[6], based on the variation of shutter delay time, was intoduced to resolve compounds with lifetimes in the millisecond range. Later "time resolved phosphorimetry" was used to resolve compounds with lifetimes in the millisecond range and longer by utilization of differences in the lifetimes of the luminiscent species[7,8]. The exponentially decaying species were resolved using a logarithmic instrument response and the concentration of the components determined from a record of the logarithm of the total phosphorescence vs time, after termination of the excitation radiation. More recently the pulsed source-time phosphorimeter has been used with an increase in sensitivity and selectivity over the previously used phosphorimetric methods of analysis[9,10].

Attempts have been made to observe phosphorescence at room temperature in polymeric matrices[11] and more recently the phosphorescence of adsorbed organic ions at room temperature has been reported[12]. However phosphorescence measurements are almost always performed at 77°K and in solvents or solvent mixtures[13-15] capable of forming clear, rigid glasses at this temperature. Inhomogeneities in the sample glasses and

non-reproducible sample positioning led to difficulties in quantitative phosphorescence measurements. However, the use of high speed sample rotation [16,17] coupled with more stable power supplies and better solvent clean-up procedures has led to a tenfold increase in the precision of results. It was not until recently that quantitative phosphorescence studies in essentially aqueous solutions were possible.[18,19] This was achieved by high speed rotation of open ended quartz capillary tubes used as sample cells. Increased phosphorescence signals from cracked glasses have been achieved by the addition of small amounts of methanol and up to 5% of the alkali metal halides.[20]

iii) Phosphorimetric Determination of Drugs.

As a consequence of the selectivity and sensitivity of phosphorimetry it has found most application in the areas of biology and medicine. Aspirin was the first compound to be determined by this method in blood and plasma at concentrations of 1-100 mg/100ml of plasma.[21] None of the constituents normally present in serum or plasma were found to give serious interferences. The background phosphorescence from the residue of a chloroform extract of acidified whole blood, when measured in EPA, (Ethanol: isopentane: diethyl ether 2:5:5) primarily resulted from the aromatic amino acids tryptophan and tyrosine.[22] The phosphorescence emission of ether extracts of deproteinised whole blood and urine was studied as a function of pH.[23] Maximum background phosphorescence of the ether extract occured on extraction at pH 6-7 for blood and at pH5 for urine extraction.

The use of ethanol as solvent matrix has allowed the determination of twenty-two compounds of pharmacological importance, with a limit of detectability of 10ng/ml of the

final solution for most of the drugs studied.[24] Procaine, cocaine, phenobarbitone and chlorpromazine have been determined in blood serum, and cocaine and atropine in urine.[25] The experimental procedures used for the separation of the specific free drugs from blood and urine were similar to those previously used and in each case the results compared very well with the established methods for these drugs. The complementary nature of phosphorimetry and fluorimetry has been demonstrated in an investigation of blood anticoagulants.[26] The limits of detection by phosphorimetry were 1.0, 1.0, 0.01 and 0.001 $\mu$g/ml for diphenadione, phenindione, tromexan, and dicumarol respectively, but fluorimetry was the optimal method of analysis for Warfarin with a detection limit of 0.001 $\mu$g/ml.

The characterization and determination of the cannabinols and their metabolites has been achieved by a study of the absorption, fluorescence, and phosphorescence spectra.[27] Phosphorescent lifetimes in the range 1.5 - 2.0 seconds were reported and detection limits were found to be of the order 10ng/ml.

Alkaloids of the isoquinoline, morphine and indole groups have been studied in ethanol.[28] Detection limits ranged from 50 $\mu$g/ml for strychnine phosphate to 0.5 $\mu$g/ml for papaverine hydrochloride and lifetimes ranged from 7.4 seconds for yohimbine hydrochloride to 0.25 seconds for morphine alkaloid. The sensitivity to change of pH gives additional selectivity in the identification of alkaloids in this group.

The luminescence characteristics of thirty seven antimetabolites have been reported.[29] Analytically useful phosphorescence was displayed by seventeen antimetabolites and

detection limits compared favourably with those obtained by colorimetric and enzymic methods of analysis. By suitable preliminary reaction of these conjugated ring systems, phosphorescence may be significantly enhanced and the limits of detection appropiately lowered.

Hollifield and Winefordner [30] studied a range of fifteen sulphonamides, whose lifetimes ranged from 0.6 seconds for pthalylsulphacetamide to 1.4 seconds for sodium sulphathiazole. Phosphorimetry was found not to be suitable for the estimation of azosulphamide and succinylsulphathiazole when the limits of detection were considerably greater than 1 $\mu$g/ml. However, for the remaining sulphonamides the detection limits were less than, or equal to 1 $\mu$g/ml. When sulphadiazine, sulphamerazine and sulphacetamide were added in vitro to serum samples, recoveries ranged from 92 - 105 % and the relative standard deviation was about 5 % in all cases. The effect of solvent upon the phosphorescent characteristics of the sulphonamides has also been studied, [31] but no great improvement on the previously reported detection limits was achieved. However, by use of a microsample cell the absolute limits of detection were considerably reduced.

Purine phosphorescence in methanol-water solutions has been shown to give detection limits between 0.1 and 0.0002 $\mu$g/ml. [32] The absolute detection limits for purine and eight of its derivatives were found to be in the picogram range ($3 \times 10^{-9} - 4 \times 10^{-12}$ g). The phosphorescence spectra obtained in methanol-water mixture ( v/v 10/90) were found to have fine structure, thus demonstrating the usefulness of predominantly aqueous matrices at 77°K for enhancing the vibrational fine structure.

It is possible to utilize the sensitivity and selectivity of phosphorimetry in conjunction with thin layer chromatography as a separatory technique. This method has been employed in the determination of p-nitrophenol, a major metabolite of parathion, in urine.[33] After hydrolysis the urine was extracted with ether. The ether extract was then concentrated and chromatographed on a thin layer of silica gel. After developement the portion of the silica gel containing the p-nitrophenol was scraped off the plate and extracted with 0.1M HCl. The aqueous solution was then back extracted into an ether-alcohol mixture and measured phosphorimetrically. The average recovery of p-nitrophenol in the concentration range 0.28 - 142$\mu$g/100ml urine was 88 %.

Phosphorimetry has been appled to the measurement of several tryptophan metabolites following the separation by thin layer chromatography.[34] Ethyl iodide was added to the ethanolic extract to increase the sensitivity of the measurement by the 'heavy atom effect'. Kynurenic acid was measured in this way and at levels between 10 - 200$\mu$g/ml urine recoveries were greater than 91 % Xanthurenic acid was found to cause no interference in the determination of Kynurenic acid by this method.

The use of phosphorimetry has not been confined solely to the determination of drugs in biological fluids; other poisonous anutrients and biologically active compounds have been estimated directly. Phosphorimetry has been used in the determination of several vitamins, [35,36] the $B_1$ and $G_1$ Aflatoxins,[37] biphenyl in oranges, [38] numerous pesticides,[39] hydrocarbon carcinogens,[40] alkaloids in tobacco,[41] and n-alkylcarboxazoles in cigarette smoke[42].

iv) Scope of Present Work.

It is essential to have objective tests to determine the amount of drug or drugs that have been taken by an individual. The main deficiency at the present time is the inability to detect certain drugs in urine, blood and tissues, because the most advanced techniques have not yet been applied.

The complementary nature of low temperature fluorimetry and phosphorimetry coupled with the sensitivity and selectivity of the technique should permit the accurate, rapid analysis of drugs over a wide concentration range. Any mixture of luminescent species can be analysed and quantified by the measurement of excitation, fluorescence and phosphorescence spectra of the pure components. If all the spectra of the components in a mixture were similar then time-resolved phosphorimetry might be used to estimate the single components of such a mixture.

The relationship between structures and fluorescence spectra of many compounds have been elucidated, but the relationships between structure and phosphorescence spectra have as yet been little studied. It is the aim of this work to extend the use of phosphorescence as an analytical technique to a number of different classes of drugs and to study any structure-spectral correlations existing between them. Part of the present work is directed towards developing a technique capable of scanning thin layer chromatograms and measuring the phosphorescence of separated components in situ. The sensitivity of phosphorescence and separatory function of thin layer chromatography could thus be combined to produce a rapid, sensitive method capable of resolving small quantities of similar compounds, especially drugs and their metabolites, from complex mixtures.

# C H A P T E R 2 :

## E X P E R I M E N T A L

### 1) INSTRUMENTATION:

All phosphorimetric measurements were made with the Baird Atomic Fluorispec SF 100 E and rotating can phosphoroscope assembly. Fluorescence measurements were made in a standard 1 $cm^2$ fluorimeter cell after removal of the phosphoroscope assembly. Spectra were recorded on a Bryans X - Y recorder and lifetime measurements greater than 0.2 seconds recorded on a Bryans strip recorder.

### ii) SAMPLE DEVICES:

In order to study low temperature luminescence, samples were placed in quartz tubes (2 mm bore, 1 mm wall thickness) and then inserted into a quartz Dewar flask containing liquid nitrogen. To obtain precise quantitative results, careful alignment of the sample tube in the Dewar was necessary. Improved sample alignment and reproducibility were achieved by rotating the sample at 300 rpm by means of a variable speed motor and pulley assembly designed to fit the Dewar flask. FIG 2. The base of the pulley was extended into the Dewar flask to give better guidance of the sample tube and increased precision in alignment. The sample tube was held firmly in the assembly by means of rubber grommets at the top and bottom of the pulley.

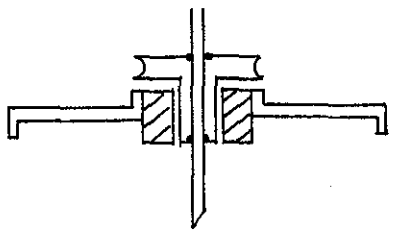


FIG 2.

Sample tubes were filled and emptied by means of long glass pasteur pipettes, connected to a water aspirator. Sample tubes were cleaned by successive rinses with reagent grade nitric acid, distilled water and the sample to be measured.

iii) SOLVENTS.

The choice of solvents available for use at 77°K was limited to those capable of forming clear rigid glasses im addition to favourable solubility characteristics. Most of the phosphorimetric measurements were performed im 50 % ethane-diol water solution or in ethanol. To obtain minimum background luminescence from the solvents, the ethane-diol was stored under nitrogen, and the ethanol distilled under nitrogen and consequently stored under nitrogen. Water was triply distilled from an all glass still.

iv) MEASUREMENT OF SPECTRA.

Excitation spectra were measured by adjusting the emission monochromator to a wavelength where maximum luminescence was observed. The output signal was then recorded as a function of the excitation monochromator wavelength. Similary luminescence emission spectra were measured experimentally by adjusting the excitation monochromator to a wavelength where maximum excitation was observed and the output signal was recorded as a function of the emission wavelength. For normal quantitative estimations spectra corrected for instrumental characteristics were not necessary.

v) QUANTITATIVE ANALYSIS.

Prior to the measurement of spectra it was necessary to calibrate the excitation and emission wavelength drums.

This was carried out using the well established mercury spectrum from a low pressure mercury lamp.

It was necessary to adjust the instrumental sensitivity each time the lamp was turned on since arc lamps rarely strike in the same position. This was achieved by using a standard solution of quinine sulphate for room temperature studies, and a standard solution of toluene in ethanol, for low temperature studies, as reference standards and adjusting the fine gain control to give a constant reading on the photometer, prior to any set of measurements.

Samples were introduced into the sample tube and aligned in the Dewar flask containing liquid nitrogen. In those cases when cracking or snowing occured, the samples were discarded and replaced with a further sample. Analytical curves were prepared by plotting luminescence signal vs concentration for a series of standard solutions. The limiting detectable sample concentration was defined as that concentration producing a signal equivalent to twice the standard deviation of the background signal.

vi) MEASUREMENTS OF LIFETIME.

Phosphorimetric lifetime is defined as the time required for the luminescence signal to decay to $1/e$ of its initial value. Lifetimes greater than one second were measured on a potentiometric recorder after termination of the exciting radiation by means of a shutter. Lifetimes shorter than one second were recorded on the Remscope S01 storage oscilloscope. A microswitch placed close to the excitation shutter produced a potential to the oscilloscope as the shutter was closed thus triggering the scan and producing

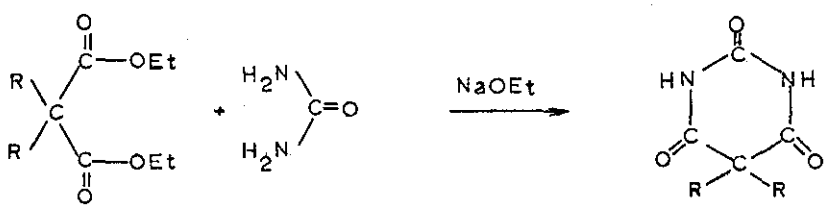
a record of the exponential decay of the luminescence. A square wave of mains frequency was superimposed on the trace to calibrate the time base. The traces were then photographed using Kodak Tri - X panchromatic film and the lifetimes calculated from the processed films.

vii) PURITY OF BARBITURATE SAMPLES.

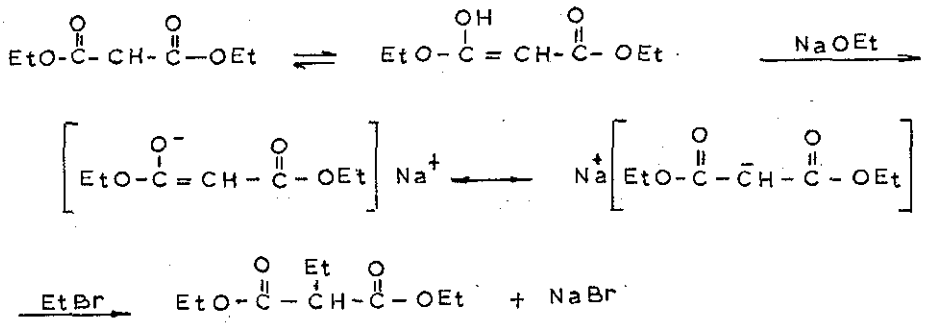
Melting points of the barbiturates studied were found to be within 2° of literature values. Samples were chromatographed using the thin layer technique of Bogan, Rentoul and Smith,[43] in order to establish single component purity. Further evidence of purity was obtained from the gas chromatographic data obtained using Chromasorb 101, narrow bore columns according to the method of Loveland.[44]

viii) BARBITURIC ACID SYNTHESIS.

The 5, substituted barbituric acids were prepared by condensation of the malonic ester with urea in the presence of sodium ethoxide.

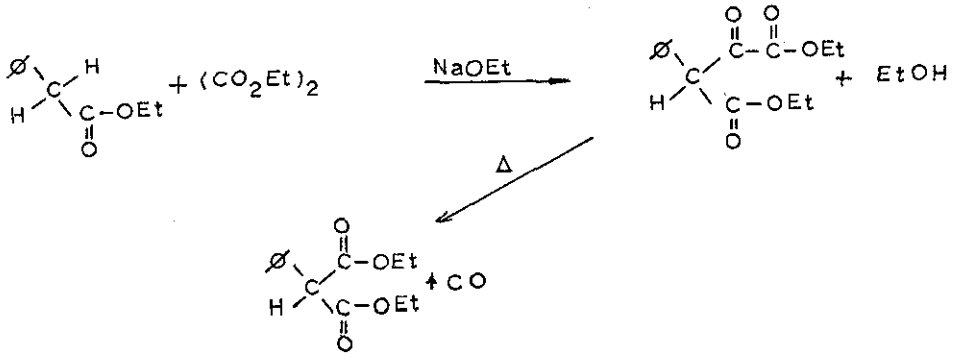


Ethylmalonic ester was prepared by alkylation of diethylmalonate. In strong base diethylmalonate is converted to the enolate anion which then undergoes an $Sn_2$ reaction with ethylbromide.



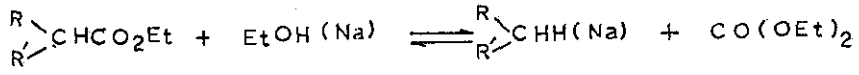
Benzylmalonic ester was prepared in a similar manner using benzylbromide as the alkylating agent.

Phenylmalonic ester was prepared according to the method of Levene and Meye..[45] Ethylphenylacetate and ethyloxalate are condensed in the presence of sodium ethoxide and the condensation product then decarboxylated thermally to produce ethylphenylmalonate.



The synthesis of 5, 5 - ethylbenzylmalonate by the method of Dox and Yoder[46] was attempted, however the yield was so low as to prohibit synthesis of 5,ethyl, 5 benzylbarbituric acid.

This low yield is attributed to the decomposition of the malonic ester by sodium ethoxide.



The success of these syntheses was verified using mass spectroscopy and n.m.r spectroscopy.

ix) MEASUREMENT OF BARBITURATE LUMINESCENCE.

Ultra-violet absorption spectra were measured at room temperatures with a Pye-Unicam SP 8000 spectrophotometer, using 10mm path-length silica cells. Uncorrected excitation, fluorescence and phosphorescence spectra, phosphorescence lifetimes and detection limits were determined as previously described. Solutions containing approximately 30$\mu$g of barbiturate per ml of 0.1M Na OH were used. All solutions had optical densities of $\leqslant 0.02$. The solvents used for low temperature work were obtained by adding equal volumes of ethanediol to the appropiate aqueous solutions.

Acetate buffers were used to obtain solutions with pHs in the range 3.6 - 5.4; phosphate buffers in the range 6.0 - 8.4; and borate buffers in the range 8.4 -10.0. Solutions with pHs below 2.0 and above 10.0 were obtained using dilute hydrochloric acid and sodium hydroxide respectively. All pHs were measured at room temperature with a Pye-Unicam 290 pH-meter.

x) BARBITURATE EXTRACTION

The direct extraction of barbiturates from serum or plasma is feasible for small quantities of fresh blood, but in

putrefying blood and with increased quantities the method is not attractive because of the prevalence of troublesome emulsions.[47] The precipitation of proteins prior to extraction is therefore a recommended procedure. Direct chloroform extraction was studied and compared with methods employing ultrafiltration, trichloroacetic acid precipitation and tungstate precipitation as a deproteinisation step prior to extraction.

The direct chloroform extraction was performed by taking 5 ml of whole blood and extracting into 30 ml of chloroform for two minutes. Three such extractions were performed and the chloroform extracts combined, filtered through a Whatman No. 31 filter paper. The filter paper was finally rinsed with a further 10ml of chloroform. The combined chloroform extracts were back extracted into 10ml 0.45N NaOH by shaking for two minutes. Two extractions were performed and the extracts combined and the final volume adjusted to 25 ml with 0.45N NaOH. Fluorimetric measurements were made using the aqueous extract and phosphorimetric measurements after diluting 1 : 1 with ethanediol.

In the tungstate precipitation technique a 5 ml aliquot of serum or whole blood was mixed with 1.0 ml 10% NaOH, 30.5 ml water and allowed to stand for ten minutes. 10 ml 10% sodium tungstate solution were added with shaking which was then followed by the dropwise addition of 3.5 ml 10% $H_2SO_4$. The mixture was then heated on a boiling water bath for ten minutes cooled and filtered. An aliquot of the aqueous filtrate was then extracted into chloroform as previously described.

To remove the proteins by trichloroacetic acid

precipitation 9 ml 10 % TCA was added to 1 ml plasma, the precipitated proteins were then centrifuged. An aliquot of the resultant solution was then extracted into chloroform as before.

The ultrafiltration technique affords the rapid deproteinization of small samples for biochemical and clinical assay procedures, avoiding acid precipitation. Whole blood was introduced into an Amicon ultrafiltration cell fitted with a P.M 10 ultrafiltration membrane. Nitrogen was applied at 70 p.s.i pressure and the membrane retained all molecules and aggregates of molecular weight greater than 10,000. The plasma water ultrafiltrate was then extracted into chloroform as in the other methods.

xi) SULPHONAMIDE SAMPLES.

Melting points of the sulphonamides were found to be within $2^{\circ}$ of literature values. $N^4$- acetylsulphonamides were synthesised from the corresponding sulphonamide using acetic anhydride and 2- sulphanilamidothiophen was synthesised by the method of Berlin, London and Sjogren.[48] After recrystallization the purity of these compounds was checked chromatographically by the method of Klein and Mader. [49]

xii) PREPARATION OF SULPHONAMIDE SOLUTIONS.

For pH-fluorescence studies stock solutions were prepared by dissolving 10mg of the appropiate compound in 20 ml of ethanol (Spectroscopic grade). Stock solutions were diluted with water (triple distilled) to the desired concentration and pH then estimated immediately. To obtain the desired pH value the aqueous solutions were titrated with 1mM to 11.6 HCl or 1mM to 10M KOH. Solutions of pH 2 - 12 were checked with a pH meter,

solutions of pH 0-2 and 12-14 were made up using the necessary amount of acid or base calculated assuming complete ionization. Since the sulphonamides rapidly undergo photolysis on irradiation with ultraviolet light, all fluorescence intensity measurements were taken within 10 seconds of exposure to the exciting light. Working curves for the sulphonamides were obtained by plotting relative fluorescence intensity vs concentration over the range $10^{-3}$ $\mu$g/ml up to 10 $\mu$g/ml. Linearity was found for sulphonamide concentrations up to 1.0 $\mu$g/ml.

xiii) SULPHONAMIDE FLUORESCENCE.

Spectral data was corrected for variation in xenon lamp output and phototube response by the method of Bridges, Davis and Williams.[50] Absolute fluorescence effeciencies were calculated by the comparative method of Parker and Rees.[51] Quinine bisulphate in 0.05M $H_2SO_4$ (1 $\mu$g/ml) was used as the fluorescence standard which has a quantum efficiency of 55%.[52] Where relative fluorescence intensities are quoted, these were determined by using either Indole in ethanol (4 $\mu$g/ml) or aniline in water pH 8.0 (2 $\mu$g/ml) which were then ascribed an arbitrary fluorescence intensity value of 100.

xiv) FLUORIMETRIC ESTIMATION OF SULPHONAMIDES IN SERUM:

0.2 ml of serum was diluted to 1.0 ml with water and the proteins precipitated by the addition of 1.0 ml 20% trichloroacetic acid. The resultant mixture was then shaken and centrifuged. 1.0 ml of the supernatant was diluted with 50 ml of buffer and the pH adjusted to the required value by the addition of 1 M NaOH. For sulphanilamide, sulphacetamide and sulphaguanidine 0.6 ml 1 M NaOH was added to 0.025 M borate buffer (pH 8.0) and for

sulphamethoxazole 1.0 ml of 1 M NaOH was added to Walpole acetate buffer (pH 4.0). The solutions were then made up to 100 ml with buffer.

The fluorescence intensity of the "unknown" was measured using a strip recorder, at the wavelengths of maximum excitation and emission. Due to the rapid photolysis of dilute sulphonamide solutions it was essential to measure the instantaneous fluorescence, this was achieved by setting the strip recorder running before the irradiation of the sample. The maximum fluorescence signal was then used to estimate the concentration of drug present. Duplicate samples of the blank (Seronorm with no drug) were carried through the same procedure and the mean blank fluorescence signal subtracted from those of the 'unknown' sample, yielding corrected values for the relative fluorescence due to the presence of drug alone. The concentrations of drug present in $\mu$g/ml were taken from the working curve (relating fluorescence intensity to concentration over the range 0.00 - 0.20 $\mu$g/ml). To obtain the concentration of drug present in mg/100 ml, the concentration obtained from the working curve was multiplied by $10^5$.

To estimate the amount of acetylated sulphonamide it was first necessary to split off the $N^4$-acetyl group by hydrolysis. A 1.0 ml portion of the protein free filtrate was added to 50 ml 0.2 M HCl and heated in a boiling water bath for 1 hour. The solution was then cooled and the pH adjusted to that where maximum fluorescence could be observed for the free sulphonamide. The total volume of solution was then made up to 100 ml with buffer. Sulphonamide estimation was performed as previously described. The result thus obtained gives a measure of the total sulphonamide present. The concentration of acetylated drug is calculated by subtracting the amount of free drug from that of the total drug

present.

xv) PSYCHOTROPIC DRUGS.

The phosphorimetric characteristics of the benzodiazepines, phenothiazines, amphetamines, monoamine oxidase inhibitors, and tricydic antidepressants were determined as described in earlier experimental sections.

# C H A P T E R 3 :

## BARBITURATE LUMINESCENCE.

The term 'barbiturate' generally refers to drugs which are derivatives of barbituric acid. Barbitone, the first drug of this class to be synthesised, was introduced into medicine in Germany in 1903. Barbiturates rapidly gained a wide usage as tranquilizers, sedatives and hypnotics which continues to the present time. In the past half century, over 2,000 different barbiturates have been synthesised, although less than a dozen make up the bulk of current use.

Barbiturates are among the most widely used psychoactive drugs and are the toxic agents in thousands of accidental and non-accidental deaths. In addition the barbiturates have considerable potential for producing psychological and physiological dependence, and are probably second only to alcohol in frequency of drug induced debilitation in modern society.

The luminescence properties of the oxybarbiturates have been studied by several groups of workers,[53-57] usually with the intention of developing analytical methods at microgram or sub-microgram levels. In some cases only room temperature fluorescence characteristics have been described, but more recently the development of low temperature ($77^{\circ}K$) techniques has permitted observations of phosphorescence also,[25,57,58].

It has been found that many oxybarbiturates exhibit an analytically-useful fluorescence in alkaline solutions at room temperature. Those containing N-methyl groups, however, are virtually non-fluorescent. Barbiturates containing a phenyl ring at the 5- position may exhibit a fairly strong phosphorescence

even when N-methyl groups are also incorporated.

In an attempt to explain these phenomena, the pH dependence of the absorption and emission spectra of a number of the oxybarbiturates have been studied. The results presented here may enable the luminescence analysis of these molecules to proceed on a sounder theoretical foundation, and provide further information on structure-luminescence relationships in heterocyclic species.

i) ABSORPTION SPECTRA:

The barbiturates studied are shown in Table 1 and Table 2 shows the principal absorption maxima and molar extinction coefficients of several important oxybarbiturates at various pH values. In the spectra of barbituric acid (Fig.3) the curves obtained in the pH range 1.0 - 7.0 apparently passed through a single isosbestic point (a). There were no changes in the spectrum between pH 7.0 and pH 10.0, but above pH 10 there was a bathochromic shift in the absorption maximum and a decrease in the extinction coefficient. All the curves in this alkaline pH region passed through a new isosbestic point (b). Sodium hydroxide concentrations greater than 1M produced no further changes in the spectrum. All the changes described could be reversed by addition of acid to the solution.

Substitution of a single alkyl or aryl group at the 5-position had no marked effect on these phenomena: thus, 5-ethyl, 5-phenyl, and 5-benzylbarbituric acids all had similar properties to those of barbituric acid itself.

The absorption spectra of 5-phenyl 5-ethylbarbituric acid (phenobarbitone) at various pH values are shown in Fig.4. Two isosbestic points were again found but these were distinct from those of barbituric acid. Again a bathochromic shift was

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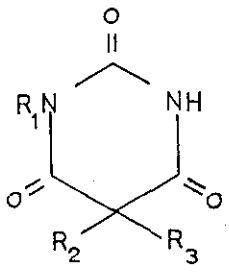


| Compound | Nos. | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| Barbituric acid | 1 | H | H | H |
| 5,-Substituted acids | | | | |
| Ethyl barbituric acid | 2 | H | H | Ethyl |
| Phenyl barbituric acid | 3 | H | H | Phenyl |
| Benzyl barbituric acid | 4 | H | H | Benzyl |
| 5,5-Substituted acids | | | | |
| +Rutonal | 5 | H | Methyl | Phenyl |
| +Phenobarbitone | 6 | H | Ethyl | Phenyl |
| +Harbitone | 7 | H | Ethyl | Ethyl |
| +Butobarbitone | 8 | H | Ethyl | n-Butyl |
| +Butabarbitone | 9 | H | Ethyl | Sec-Butyl |
| +Pentobarbitone | 10 | H | Ethyl | 1-Methyl-butyl |
| +Amylobarbitone | 11 | H | Ethyl | Isoamyl |
| +Nealbarbitone | 12 | H | Neo-pentyl | Allyl |
| +Seconal | 13 | H | 1-Methylbutyl | Allyl |
| +Cyclobarbitone* (Ca) | 14 | H | Ethyl | cyclohex-enyl |
| Heptabarbitone | 15 | H | Ethyl | cyclohept-enyl |
| Diphenyl-barbituric acid | 16 | H | Phenyl | Phenyl |
| 1,5,5-Substituted acids | | | | |
| +Hexobarbitone | 17 | Methyl | Methyl | cyclohex-enyl |
| Mebaral | 18 | Methyl | Ethyl | Phenyl |
| Metharbital | 19 | Methyl | Ethyl | Ethyl |
| Methohexitone** (Na) | 20 | Methyl | Allyl | 1-Methyl-pent-2-ynyl |
| 1,3,5,5-Substituted acids | | | | |
| 1,3 Dimethylphenobarbitone | 21 | N,N'-dimethyl | Ethyl | Phenyl |

Methylphenobarbitone was obtained from Winthrop Laboratories, Methohexitone from Eli Lilly, Diphenylbarbituric acid from ICI and Heptabarbitone from Geigy. Other commercial barbiturates+ were from May & Baker Ltd.

T A B L E 2

| Compound | pH | λ max nm | $\epsilon \times 10^{-3}$ |
|---|---|---|---|
| Barbituric acid | 1 | 257 | 0.6 |
| | 7 - 10 | 257 | 19.8 |
| | 12 | 258 | 18.6 |
| | 13 | 259 | 16.9 |
| | 14 | 259.5 | 15.1 |
| 5-Phenylbarbituric acid | 1 | 262 | 8.0 |
| | 7 - 11 | 267 | 21.6 |
| | 12 | 267 | 19.7 |
| | 13 | 268 | 18.8 |
| | 14 | 269 | 17.9 |
| Benzylbarbituric acid | 1 | 250 | 5.6 |
| | 10 | 263 | 17.1 |
| | 13 | 264 | 13.5 |
| | 14 | 265 | 13.3 |
| 5-Ethyl, 5-Phenyl Barbituric acid | 1 | 240 | 0.7 |
| | 8 | 240 | 8.0 |
| | 11 | 240 | 9.3 |
| | 12 | 242 | 7.5 |
| | 13 | 255 | 6.9 |
| | 14 | 256 | 6.8 |
| 1-Methyl, 5-Ethyl, 5-Phenylbarbituric acid | 1 | 246 | 1.3 |
| | 7 | 246 | 2.6 |
| | 8 | 246 | 5.6 |
| | 10-14 | 246 | 8.5 |

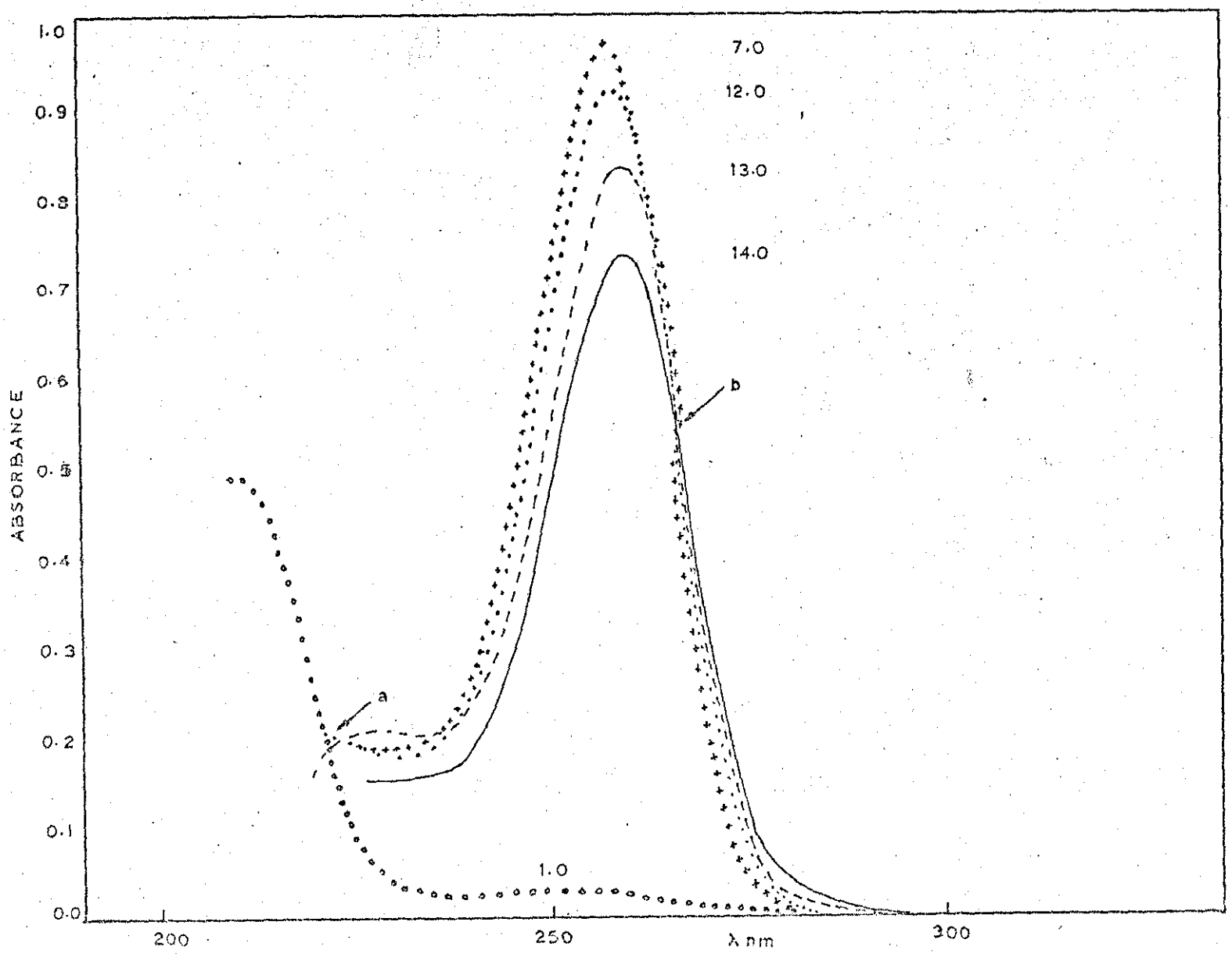



FIG 3. BARBITURIC ACID: Absorption spectra at various pH values.

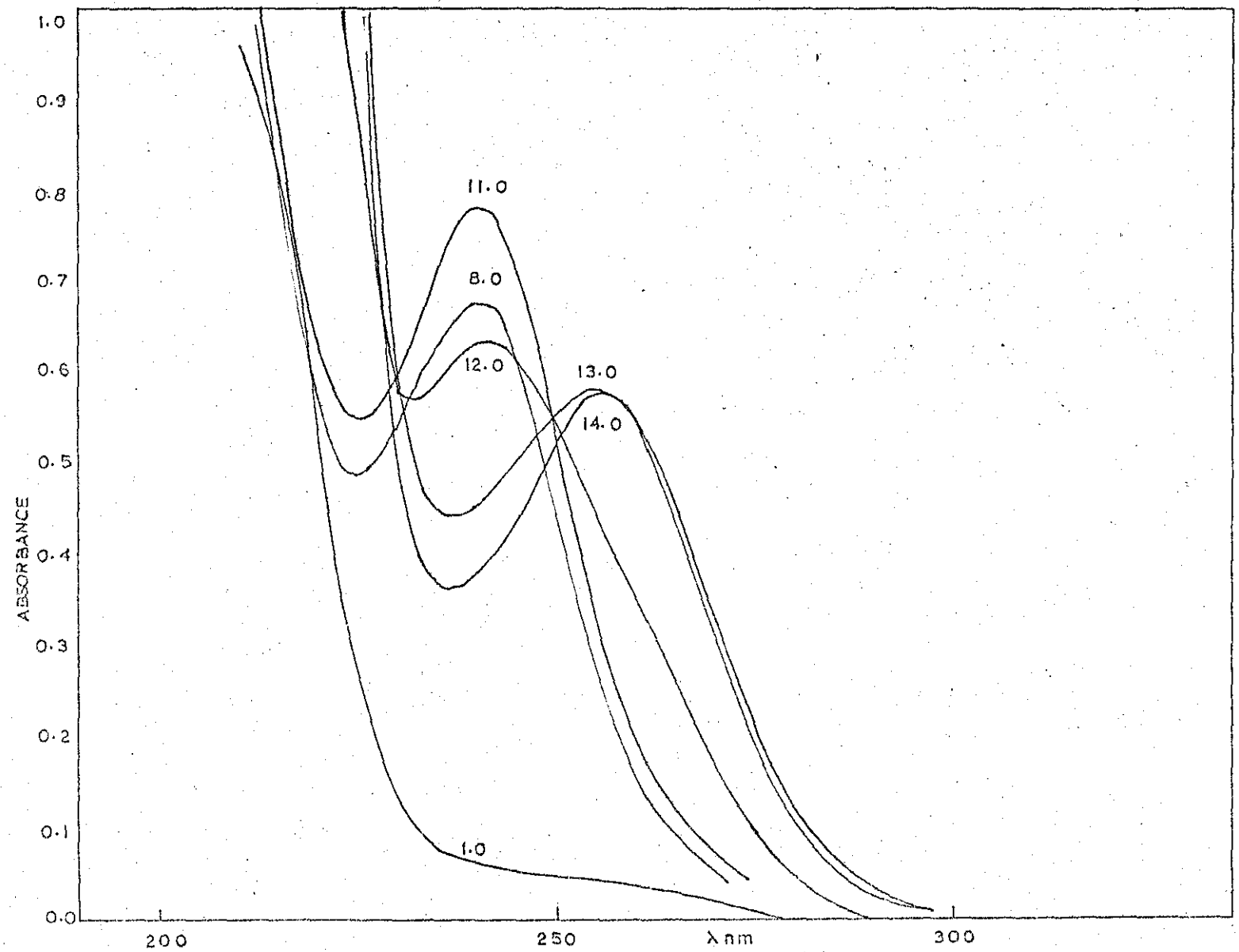



FIG 4. PHENOBARBITONE : Absorption spectra at various pH values.

observed at high pHs, with an accompanying slight fall in extinction coefficient. The properties of 5-phenyl 5-methylbarbituric acid (rutonal) were very similar.

Methylphenobarbitone spectra (Fig.5) showed only a single isosbestic point, which occurred at a similar wavelength to that of the low-pH isosbestic point in phenobarbitone. 1,3-dimethylphenobarbitone, however, had an absorption spectrum which was independent of pH, with a strong absorption below 235 nm.

ii) FLUORESCENCE SPECTRA AT ROOM TEMPERATURE:

At 20°C the fluorescence of barbituric acid, 5-ethylbarbituric acid, and 5-benzylbarbituric acid in 0.1M aqueous NaOH showed a very weak emission at approximately 400 nm: the intensity in each case was 50 % less than the solvent Raman scatter signal. 5-phenylbarbituric acid showed a more intense fluorescence ( $\lambda$ = 300 nm, $\lambda$ = 410 nm) which, however, remained less intense than that of a typical 5, 5 disubstituted barbiturate (Fig.6) In neutral ethanol, 5-phenylbarbituric acid was non-fluorescent, but the fluorescence reappeared on the addition of NaOH to a final concentration of 0.1M.

Most of the 5, 5disubstituted barbiturates were fluorescent in alkaline solution, the excitation and emission spectra of phenobarbitone (Fig.7) being typical. This fluorescence had a maximum intensity at pH 13.0 - 13.5, and was absent at pHs below 10.0. Emission and fluorescence wavelengths and detection limits for these compounds are listed in Table 3.

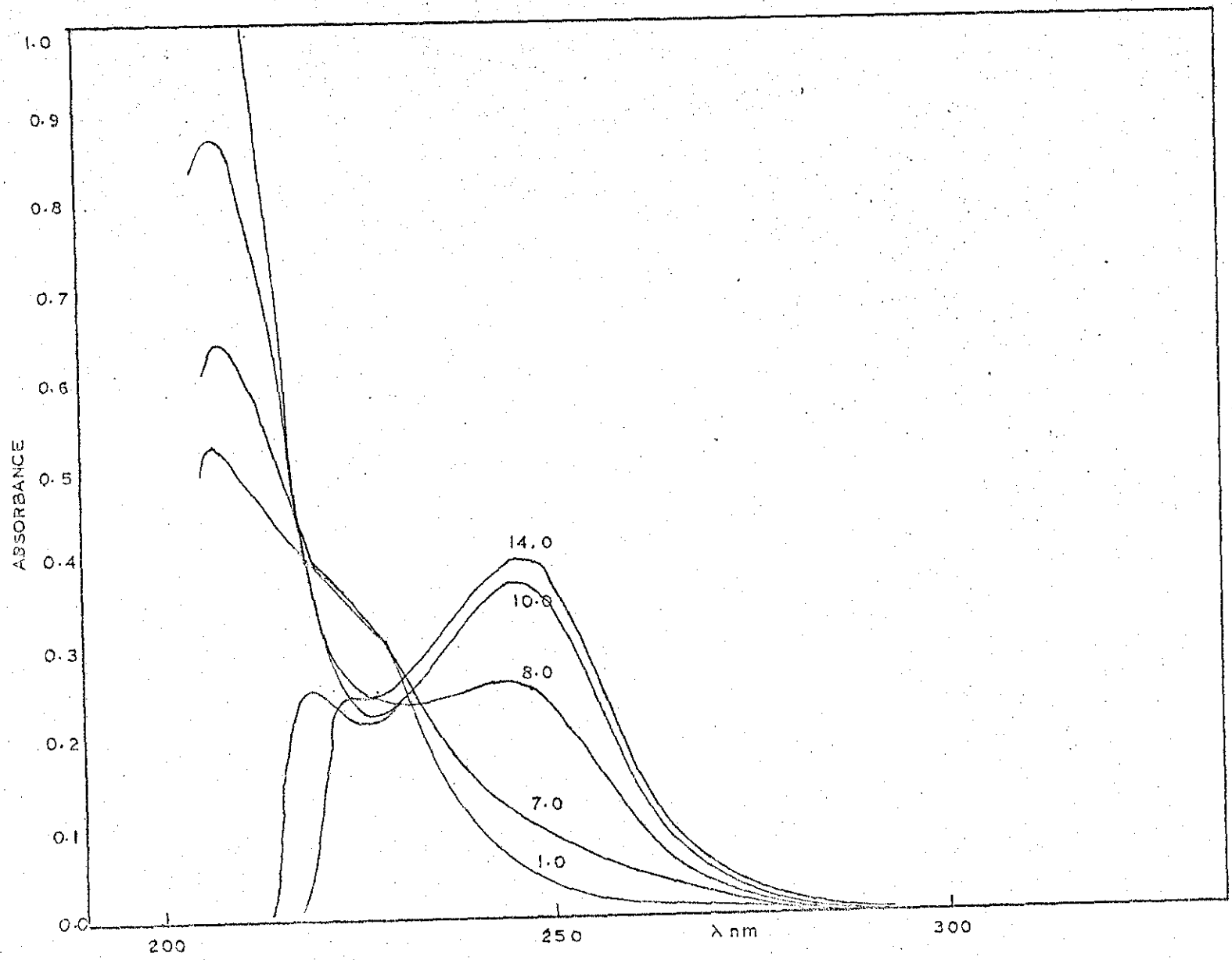



FIG 5. N-METHYLPHENOBARBITONE : Absorption spectra at various pH values.

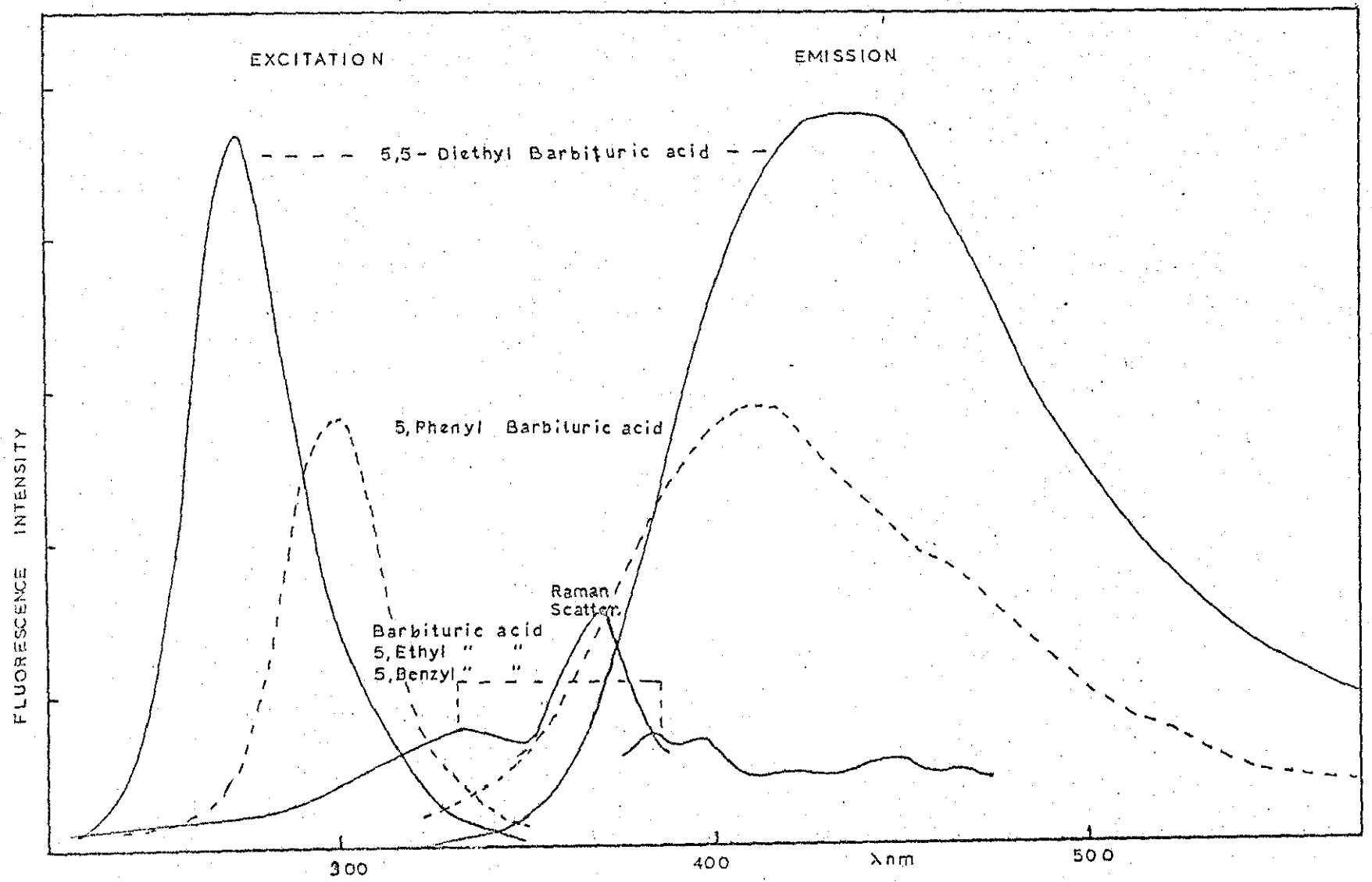



FIG 6. Fluorescence of Barbituric acids in O.IN NaOH.

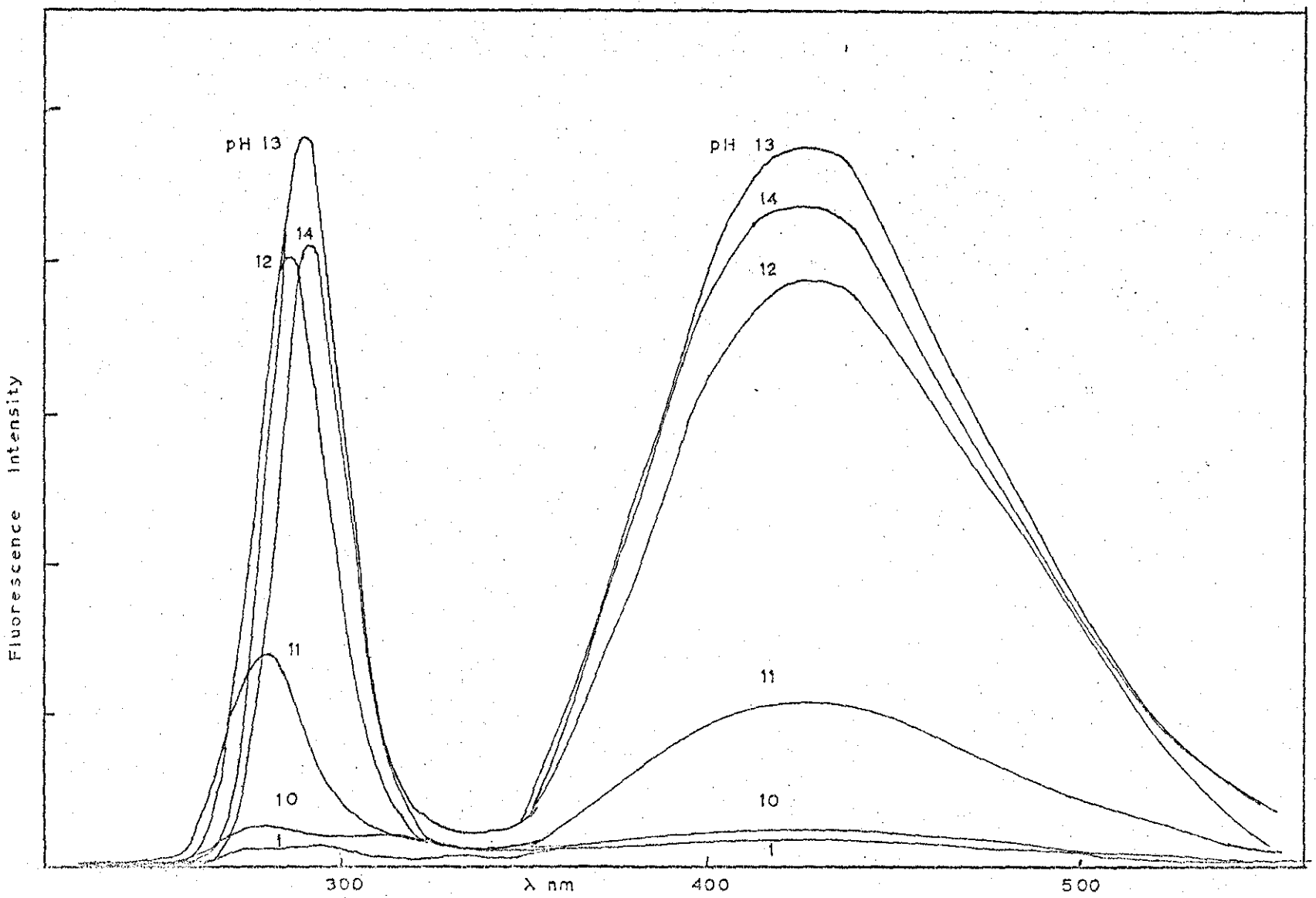



FIG 7. Phenobarbitone Fluorescence vs pH at 298°K.

T A B L E 3

| Compound | λe nm | λf nm | Detection Limit μg/ml |
|---|---|---|---|
| Rutonal | 278 | 420 | 1.5 |
| Phenobarbitone | 278 | 420 | 1.5 |
| Barbitone | 277 | 420 | 0.1 |
| Butobarbitone | 276 | 420 | 0.1 |
| Butabarbitone | 276 | 420 | 0.1 |
| Pentobarbitone | 276 | 420 | 0.05 |
| Amylobarbitone | 276 | 420 | 0.1 |
| Nealbarbitone | 277 | 420 | 1.0 |
| Seconal | 277 | 420 | 1.0 |
| Cyclobarbitone | 277 | 420 | 3.0 |
| Heptabarbitone | 277 | 420 | 3.0 |

Species with aromatic or unsaturated aliphatic substituents (compounds 5, 6, 12-15) were found to have a less intense fluorescence than the others. N-methyl derivatives of 5, 5 disubstituted barbiturates (i.e., compounds 17 - 21) were non-fluorescent at all pHs.

iii) LUMINESCENCE SPECTRA AT 77°K:

In a 1 : 1 v/v water: ethanediol solvent containing 0.1M NaOH, the barbiturates listed in Table 4 were found to have similar fluorescence properties to those exhibited at room temperature, although a slight blue shift was observed in the wavelength maxima of the absorption and emission spectra. Again the fluorescence of these compounds was only observed in alkaline solution, but in the same conditions barbiturates with allyl and

other non-aromatic unsaturated substituents (compounds 12 - 15) were only weakly luminescent.

T A B L E 4

| Compound | λe nm | λ p nm | Detection Limit μg/ml |
|---|---|---|---|
| PHOSPHORESCENCE | | | |
| Rutonal | 266 | 395 | 0.5 |
| Phenobarbitone | 266 | 395 | 0.3 |
| Mebaral | 260 | 395 | 0.5 |
| FLUORESCENCE (TOTAL LUMINESCENCE) | | | |
| | λe nm | λ f nm | |
| Rutonal | 266 | 395 | 0.3 |
| Phenobarbitone | 266 | 395 | 0.2 |
| Barbitone | 265 | 410 | 0.3 |
| Butobarbitone | 265 | 410 | 0.4 |
| Butabarbitone | 266 | 410 | 0.2 |
| Pentobarbitone | 266 | 410 | 0.2 |
| Amylobarbitone | 265 | 410 | 0.1 |
| Nealbarbitone, Seconal, Cyclobarbitone, Heptabarbitone | Weak fluorescence of comparable intensity to that of Raman solvent emission. | | |

All barbiturates with a 5-phenyl substituent showed a delayed luminescence at 77°K, with a lifetime of several seconds. This emission had a maximum intensity at ca 395 nm and showed a considerable degree of fine structure, similar to that found in the phosphorescence spectrum of toluene (Fig.8). Furthermore, except in the case of 5-phenylbarbituric acid, it was maintained over the entire pH range 0-14, although some changes in lifetime did occur (Table 5). Benzylbarbituric was also phosphorescent, but only in alkaline solution.

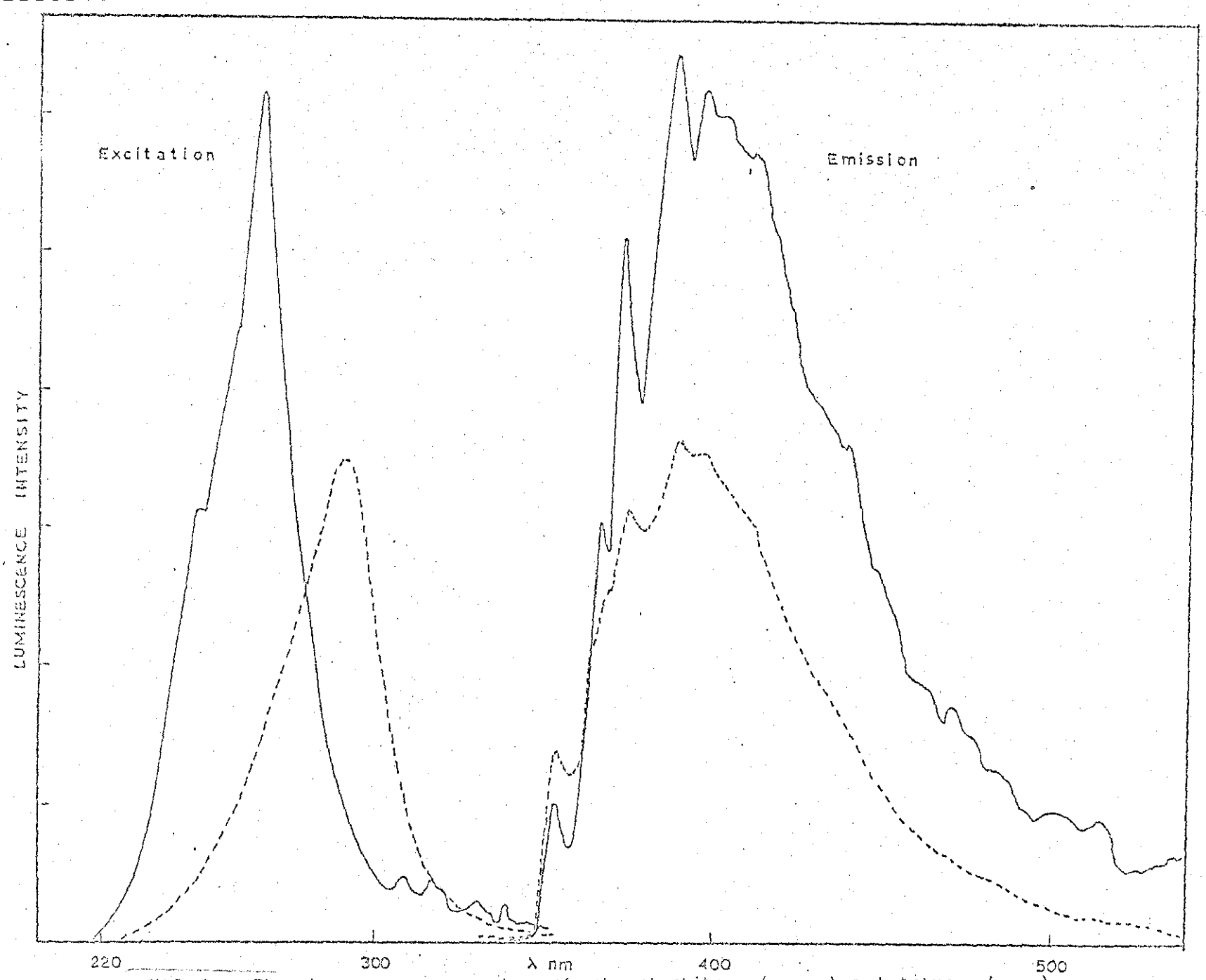



FIG 8. Phosphorescence spectra of phenobarbitone (- - - -) and toluene (——).

T A B L E 5

| Compound | Conditions | | |
|---|---|---|---|
| | 0.1M $H_2SO_4$ | Neutral | 0.1M NaOH |
| 5-Phenylbarbituric acid | N. L. | N. L. | P, $\tau$ = 2.0 |
| Benzylbarbituric acid | F (W) | F (W) | P, $\tau$ = 4.6 |
| Rutonal | P, $\tau$ = 3.0 | P, $\tau$ = 3.0 | P, $\tau$ = 4.1 |
| Phenobarbitone | P, $\tau$ = 2.9 | P, $\tau$ = 4.1 | P, $\tau$ = 5.8 |
| Diphenylbarbituric acid | P, $\tau$ = 2.2 | P, $\tau$ = 2.4 | P, $\tau$ = 2.4 |
| Mebaral | P, $\tau$ = 3.5 | P, $\tau$ = 3.6 | P, $\tau$ = 3.7 |

N. L. = Non-luminescent

F = Fluorescent

P = Phosphorescent

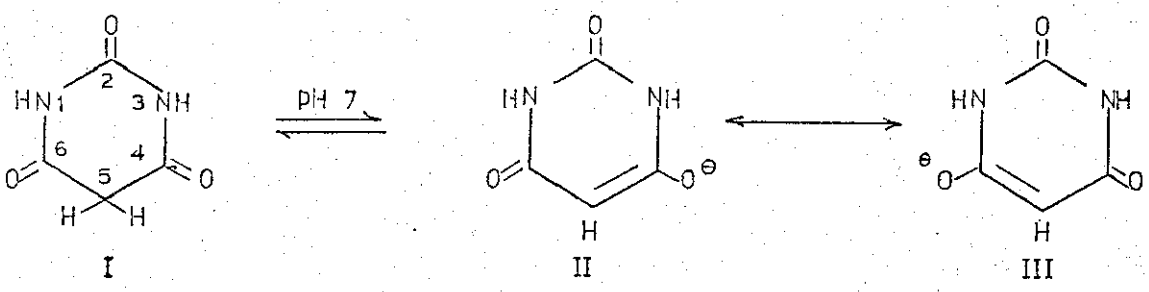
$\tau$ = Phosphorescence lifetime, seconds

W = Weak

iv) INTERPRETATION OF LUMINESCENCE SPECTRA.

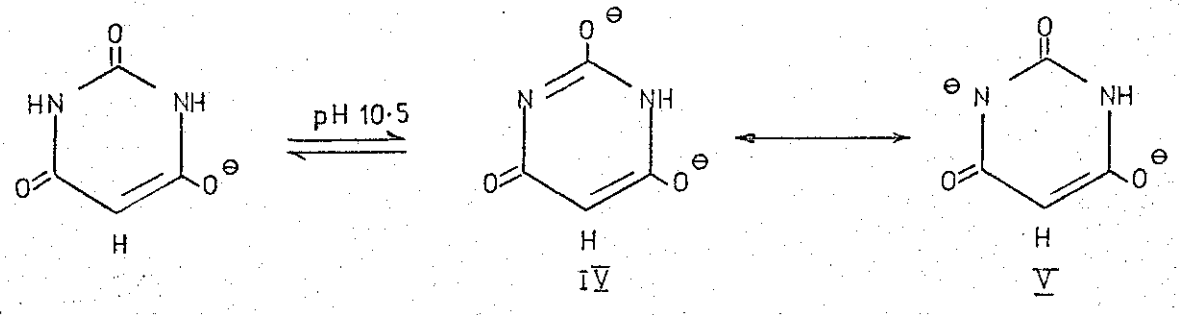
Any attempt to interpret luminescence spectra on the basis of absorption spectroscopy rests upon the assumption that the $pK_a$ values for each substance in the excited state are similar to those of the corresponding ground state molecules, an assumption which is not always well-founded.[59] However, in the species studied here, the major changes in fluorescent intensity occurred at a similar pH to that where the absorption spectra also changed.

The ultra-violet absorption spectra of the oxybarbiturates were first studied in detail by Fox and Shugar,[60] who showed that, in the case of barbituric acid itself, the triketo form (I) was stable at acid pHs. In the pH range 7-10 the mono-anion formed could be stabilised by tautomerism (II and III) -




I II III

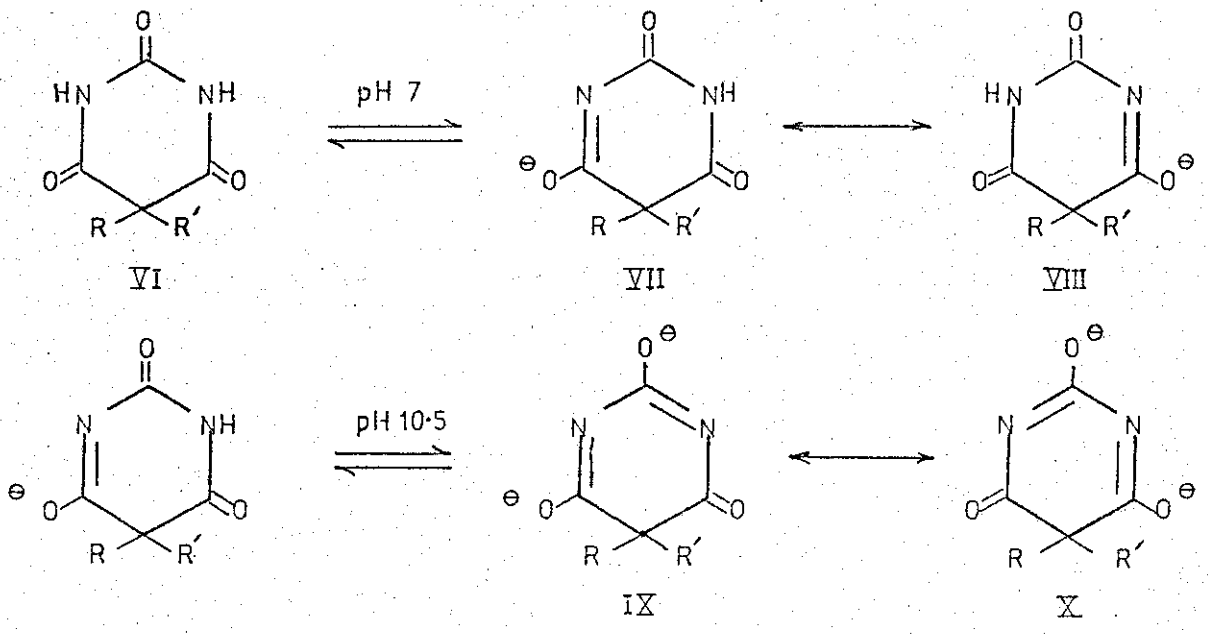
- as could the dianion which was stable at pHs above 11 (IV and V):




IV V

This hypothesis was supported by the result that the spectral properties of Methylbarbituric acid were identical to those of the parent compound. In the present work the spectra obtained for barbituric acid are similar to those of Fox and Shugar, and their hypothesis is further supported by the finding that replacement of a single hydrogen atom at the 5-position (compounds 2-4) has little effect on the spectra.

5,5 disubstituted barbituric acids (compounds 5-15) are also capable of forming mono- and di-anions, but as their absorption spectra are distinct from those of barbituric acid, the anions are presumably formed by a different mechanism:




VI VII VIII

IX X

On this basis, compounds 17-20 (containing an N-methyl group) would be expected to show only the first ionisation, and the spectra of N-methyl phenobarbitone indicate that this is indeed so. Dimethylphenobarbitone (compound 21) is incapable of ionisation.

The pH dependence of the fluorescence of the barbiturates at room temperature shows clearly that the dianions are the fluorescent species. The dianions produced in barbituric acid itself and in other compounds with at least one hydrogen atom at the 5-position exhibit feeble fluorescence, except in the case of 5-phenylbarbituric acid: the enhanced fluorescence of this compound may be due to the ability of the phenyl ring to form a conjugated $\pi$ - bonded system with the heterocyclic ring when the latter is in the dianionic form (IV and V). The dianions derived from 5,5-disubstituted barbiturates, on the other hand, are far more intensely fluorescent, and can be determined fluorimetrically at sub-microgram levels. If one of the substituents at the 5-position is an aromatic or other unsaturated group (compounds 5, 6, 12-16),

the fluorescence intensity is reduced. N-methylated barbiturates are incapable of dianion formation and are thus non-fluorescent at all pHs.

Measurements at 77°K permitted the observation of phosphorescence signals from all the barbiturates containing a 5-phenyl substituent. (It proved impossible to detect the feeble phosphorescence of barbitone or amylobarbitone (compounds 7 and 11) described recently by Miles and Schenk,[61] in spite of the higher light output of the xenon lamp used in the present work). This phosphorescence is unusual, as had been previously noted,[57,61] in that it occurs at a lower wavelength than that of the prompt fluorescence of barbiturates at room temperature. It seems almost certain that this is because the fluorescence is derived from the dianionic form of the heterocyclic ring, whereas the phosphorescence is derived from the aromatic ring. In support of this conclusion, the fine structure of the phosphorescence spectrum is very similar to that of toluene (Fig.8), and, with two exceptions, the phosphorescence can be observed over a wide range of pHs.

It is equally clear, however, that the heterocyclic ring exerts some effects on these phosphorescence phenomena, since phenyl- and benzyl-barbituric acid only phosphoresce in conditions where the molecules are in the dianionic form. This is presumably not because these compounds form monoanions and dianions different from those derived from 5,5 disubstituted barbiturates, since the differences in properties extend to acid solutions, where all barbiturates are in the tri-keto form. It is very possible, however, that steric factors are involved: to minimise steric interference with the oxygen atoms at the 4- and 6-positions, and especially with the other substituent at the 5-position, the

5-phenyl ring may be constrained in a plane nearly perpendicular to that of the heterocyclic ring, and will thus be little affected by the state of ionisation of the latter. In phenylbarbituric acid and benzylbarbituric acid the steric effects will be less severe, because the other 5-substituent is a hydrogen atom, and, in benzylbarbituric acid, because the two rings are separated by a methylene group. In these cases the aromatic ring may thus be more affected by changes in the heterocyclic ring, although the precise nature of the quenching effect of the latter at pH below 10 remains unclear. The present results thus explain a number of apparently anomalous features of oxybarbiturate luminescence, and clarify the nature and interactions of the luminescent moieties involved.

v) DETECTION LIMITS.

Table 3 summarizes the detection limits of the barbiturates at room temperature in 0.1M $NaOH$; similar results were obtained when 0.1M $NaOH$ in ethanediol-water (1 + 1, v/v) was used as solvent. Fluorescence intensity-concentration plots were linear up to solute concentrations of at least 30 $\mu g\ ml^{-1}$ in the final solution.

Table 4 gives the luminescence properties of the barbiturates at 77°K in the ethanediol-water solvent. This solvent was selected because it gave reasonably clear glasses at 77°K and had a low luminescent background. In experiments on blood extracts, pure ethanediol was added to the aqueous extract, thereby avoiding the necessity to change the solvent by an evaporation step.

In the barbiturates exhibiting phosphorescence attempts to increase the intensity of the emission through the "heavy atom" effect, by the addition of small quantities of potassium bromide

and potassium iodide were unsuccessful. However, the presence of up to 5% (w/v) of the halides improved the clarity of the glasses formed at 77°K. Luminescence intensity-concentration plots were linear up to concentrations of at least 40$\mu$ g ml$^{-1}$ of the final solution.

vi) EXTRACTION PROCEDURES

Serum standards containing between 6 and 24$\mu$ g ml$^{-1}$ phenobarbitone were extracted into chloroform and estimated phosphorimetrically following deproteinization by the sodium tungstate method described earlier. A linear calibration curve was produced and the serum blank was found to be similar to the reagent blank. However the overall method was found to be unacceptable since the concentrations of the final solutions were in the range 0-3$\mu$ g ml$^{-1}$ which was too close to the limit of detection for phenobarbitone.

Extraction procedures using trichloroacetic acid as a deproteinization step prior to extraction and estimation were found to be very sensitive to the quality of the TCA used. High variable blanks were often found hence this technique was found unsatisfactory. Serum samples measured fluorimetrically following deproteinization using the ultrafiltration membrane were found to have a reproducible background fluorescence, however barbiturate recoveries were not reproducible. The amount of barbiturate recovered was found to decrease exponentially with the volume of filtrate eluted. This effect is due to a change in the equilibrium between free drug and drug bound to protein. As the concentration of protein builds up in the ultrafiltration cell so the amount of barbiturate retained increases correspondingly. Hence this is a serious drawback to this method as a means of deproteinization in drug plasma studies.

Direct extraction of the barbiturates into chloroform, as described previously, gave recoveries in the range 90-92%. Despite some increase in blank fluorescence values, detection limits remained in the microgram range after making allowance for dilutions. FIG 9, shows the calibration curves for barbitone after extraction from whole blood and distilled water estimated fluorimetrically, together with the calibration curves produced by the direct extraction of phenobarbitone from whole blood and water estimated phosphorimetrically. The detection limit for barbitone measured at room temperature was 0.8 $\mu$g ml$^{-1}$ in the final solution and the detection limit for phenobarbitone measured phosphorimetrically at 77°K was 0.5 $\mu$g ml$^{-1}$. The percentage standard deviation at the 5 $\mu$g ml$^{-1}$ level in the case of barbitone was 6%.

vii) METABOLIC PRODUCTS.

The barbiturates are metabolized by reactions occuring at four different sites of the molecule[62].

a) $C^5$ substituents may undergo oxygenation, oxidation, or complete removal.

b) $N^1$ and $N^3$ alkyl groups may be removed, or unsubstituted N atoms may be methylated.

c) $S^2$ may be replaced by O, converting the thiobarbiturates to the corresponding oxybarbiturates.

d) Scission of the barbiturate ring may occur, at the 1:6 bond to give substituted malonylureas.

From structure-luminescence relationships it is known that the dianionic species alone exhibit fluorescence. Any chemical

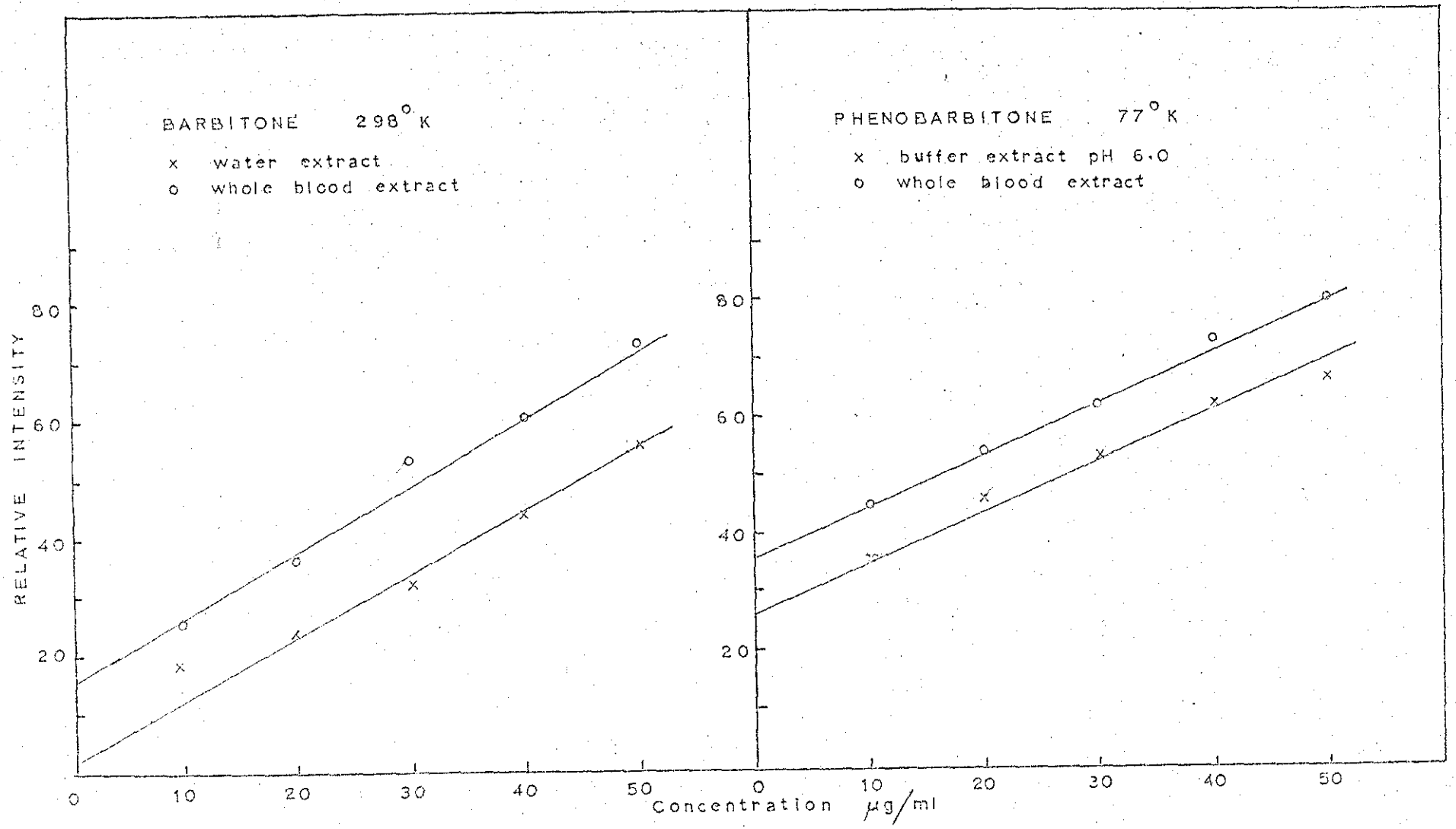



FIG 9. Luminescence calibration curves.

change resulting from metabolism must therefore be considered in terms of the formation or destruction of species capable of dianion formation.

All the therapeutically useful barbiturates have two substituents at the $C^5$ atom, one or both of which may undergo oxygenation to form an alcohol or diol, followed possibly by oxidation to the corresponding Ketone or carboxylic acid. Complete removal of a side chain to produce a monosubstituted barbituric acid, may also occur. No change in the fluorescence characteristics would be expected to occur except in the complete removal of a side chain.

Alkylation at either or both nitrogen atoms in the barbiturate ring would prevent dianion formation and hence fluorescence. De-alkylation of N-substituted barbiturates would produce fluorescent species whose formation could be monitored.

The thiobarbiturates have different fluorescent characteristics than those of the oxybarbiturates. Metabolism producing oxyanalogues could therefore be monitored. Products of ring scission are unlikely to produce fluorescence species and would also have different extraction properties.

viii) INTERFERENCES.

Various molecules which may interfere with the ultraviolet spectrophotometric analysis of oxybarbiturates, e.g., bemegride ( $\beta$ - methyl - $\beta$ - ethylglutarimide), which is sometimes administered in cases of barbiturate intoxication, and diphenylhydantoin, which is often given with phenobarbitone, do not interfere with the luminescence methods.

# C H A P T E R 4:

## LUMINESCENCE PROPERTIES OF THE ANTIBACTERIAL SULPHONAMIDES.

Sulphonamides are amides of p-aminobenzenesulphonic acid and are of considerable importance as bacteriostatic agents. They are much used in the treatment of urinary infections, in bacillary dysentery, and meningococcal meningitis as well as in rheumatic heart disease and nephritis.

The method of Bratton and Marshall[63] has been widely used for the determination of free sulphonamides. The method is based upon diazatisation of the free amine group followed by coupling with N-(1-napthyl)-ethylene diamine dihydrochloride to produce a coloured azo dye which is then measured spectrophotometrically. It is the intention of this study to produce a more rapid and sensitive method of estimation.

Since these compounds contain the aniline moiety, which has pronounced luminescence properties,[64,65] the fluorescence and phosphorescence characteristics of a series of sulphonamides were investigated (Table 6).

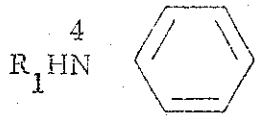
(Credit. The qualitative fluorescence data for the sulphonamides at 298°K was supplied by Dr. J.W.Bridges, - University of Surrey).

### i) INFLUENCE OF SOLVENT ON THE FLUORESCENCE OF SULPHANILAMIDE AND ITS ISOMERS.

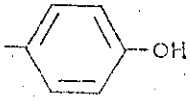
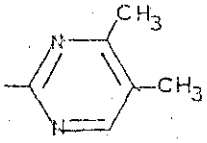
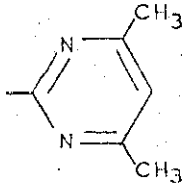
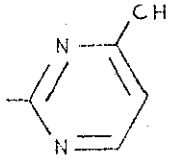
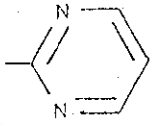
The ultraviolet spectra of sulphanilamide in each of the solvents investigated was similar, showing broad absorption maximum of 259-264 nm. In ethanol and butanol sulphanilamide fluoresced with maximum intensity with a typical aniline fluorescence (345 nm).

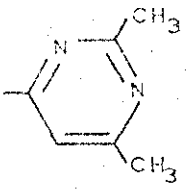
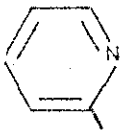
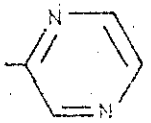
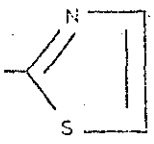
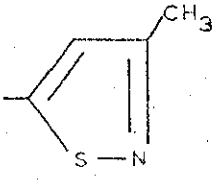
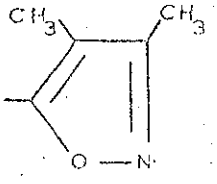
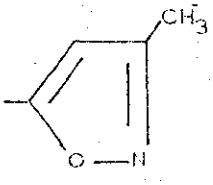
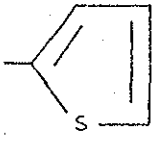
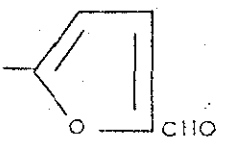
TABLE 6

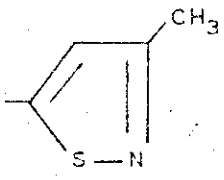
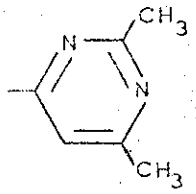
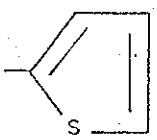
Sulphonamides studied



p-aminobenzenesulphonamide

| No. | Compound | Structure $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| 1 | Sulphanilic acid | p-aminobenzenesulphonic acid | | |
| 2 | Orthanilamide | o-aminobenzenesulphonamide | | |
| 3 | Methanilamide | m-aminobenzenesulphonamide | | |
| 4 | Sulphanilamide | H | H | H |
| 5 | N'-Cetylsulphanilamide | H | $-C_{16}H_{31}$ | H |
| 6 | N'N'-Dimethylsulphanilamide | H | $-CH_3$ | $-CH_3$ |
| 7 | N'N'-Diethylsulphanilamide | H | $-C_2H_5$ | $-C_2H_5$ |
| 8 | N'-Carbethoxysulphanilamide | H | $-CO.OC_2H_5$ | H |
| 10 | p-Hydroxybenzenesulphanilamide | H |  | H |
| 11 | Sulphacetamide | H | $-CO.CH_3$ | H |
| 12 | Sulphaguanidine | H | $-C(:NH)NH_2$ | H |
| 13 | Sulphamethoxine | H |  | H |
| 14 | Sulphamethazine | H |  | H |
| 15 | Sulphamerazine | H |  | H |
| 16 | Sulphadiazine | H |  | H |

| No. | Compound | Structure $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| 17 | Sulphadimethoxine | H |  | H |
| 18 | Sulphapyridine | H |  | H |
| 19 | Sulphapyrazine | H |  | H |
| 20 | Sulphathiazole | H |  | H |
| 21 | Sulphasomizole | H |  | H |
| 22 | Sulphaisoxazole | H |  | H |
| 23 | Sulphamethoxazole | H |  | H |
| 24 | 2-Sulphanilamidothiophen | H |  | H |
| 25 | 2-Sulphanilamidofurfural | H |  | H |
| 26 | $N^1N^4$-Diacetylsulphanilamide | $-CO.CH_3$ | $-CO.CH_3$ | H |
| 27 | $N^4$-Benzylsulphanilamide | $-CH_2C_6H_6$ | H | H |
| 28 | $N^4$-Acetylsulphanilamide | $-CO.CH_3$ | H | H |

| No. | Compound | Structure $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| 29 | $N^4$-Acetylsulphapyridine | $-CO.CH_3$ |  | H |
| 30 | $N^4$-Acetylsulphasomizole | $-CO.CH_3$ |  | H |
| 31 | $N^4$-Acetylsulphadimethoxine | $-CO.CH_3$ |  | H |
| 32 | $N^4$-Acetylsulphaguanidine | $-CO.CH_3$ | $-C(:NH)NH_2$ | H |
| 33 | $N^4$-Acetylsulphathiophen | $-CO.CH_3$ |  | H |

Compounds 1, 4, 11, 12, 14, 16, 18 and 20 were purchased from May and Baker Compounds 17 and 22 were gifts from Roche Products Ltd., Compounds 6, 7, 8, 10, 19, 21, 25 and 27 were gifts from May and Baker Ltd. and Compounds 2 and 3 were the gift of Dr. J. N. Smith.

The fluorescence of sulphanilamide was completely quenched in acetone, but-2-one, nitromethane, chloroform and carbon tetrachloride and partially quenched in water, benzene and cyclohexane (see Table 7). Since the ultraviolet absorption maximum of acetone is at 279 nm it is likely that acetone abolishes the fluorescence of sulphanilamide by an inner filter effect. A similar explanation probably accounts for the quenching by aromatic hydrocarbons and by nitromethane. The quenching by chlorinated hydrocarbons on the other hand is probably the result of Van der Waals type of molecular interaction. There appears to be a bathochromic shift of the fluorescence wavelength with increasing dielectric constant of the solvent in the order cyclohexane (334 nm )<benzene (337 nm )<water (350 nm ). Since the fluorescence wavelength but not the activation wavelength changes with solvent it follows that there must be an interaction between the solvent and sulphanilamide in the excited state. This suggests that polar species of sulphanilamide make an important contribution to the excited state.

ii) OTHER ISOMERS.

Chloroform was also found to completely quench the fluorescence of $N^4$-benzylsulphanilamide and $N^1$, $N^1$- dimethyl-sulphanilamide but only partially quench the fluorescence of orthanilamide (Table 8). Water was also found to partially quench the fluorescence of these isomers. Quenching was particularly marked for orthanilamide, the fluorescence of which in water is only 6% of that in ethanol. A similar result was found for metanilamide. It seems likely that the difference is due to hydrogen bonding between water and the sulphonamide molecule in the excited state.

TABLE 7

Fluorescence of sulphanilamide in various solvents

Concentration of sulphanilamide 4 μg/ml. $N_2$ was bubbled through each solution for 5 min. before a reading was taken.

| Solvent | Excitation max+ mμ | Fluorescence max+ mμ | Relative fl.+ intensity |
|---|---|---|---|
| Water | 269 | 350 | 23.5 |
| Ethanol | 269 | 345 | 36.0 |
| n-butanol | 269 | 343 | 42.0 |
| Acetone | - | - | 0 |
| But-2-one | - | - | 0 |
| Chloroform | - | - | 0 |
| Carbon tetrachloride | - | - | 0 |
| Nitromethane | - | - | 0 |
| Cyclo a-hexane | 269 | 334 | 26.9 |
| Benzene | 269 | 327 | 30.3 |

+ Uncorrected figures. Indole in ethanol taken as 100.

TABLE 8

Fluorescence of some other sulphonamides in various solvents

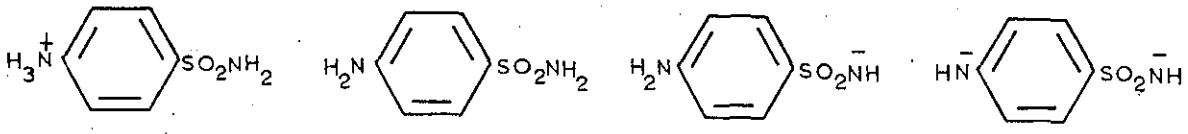
Concentration of sulphonamide 4 µg/ml.

| Compound | Solvent | Excitation max. mµ | Fluorescence max. mµ | Relative fluorescence + |
|---|---|---|---|---|
| Orthanilamide | water | 310 | 380 | 1.4 |
| | ethanol | 313 | 374 | 25.5 |
| | chloroform | 309 | 374 | 11.6 |
| | cyclohexane | 308 | 369 | 32.0 |
| Metanilamide | water | 250, 305 | 377 | 11.4 |
| | ethanol | 254 | 385 | 16.4 |
| | chloroform | 312 | 362 | 19.2 |
| | cyclohexane | 311 | 372 | 37.0 |
| $N^4$-benzylsulphanilamide | water | 282 | 358 | 67.0 |
| | ethanol | 281 | 350 | 95.0 |
| | chloroform | - | - | 0 |
| | cyclohexane | 274 | 347 | 92.0 |
| $N^1$,$N^1$-dimethylsulphanilamide | water | 269 | 350 | 24.1 |
| | ethanol | 272 | 345 | 46.3 |
| | chloroform | - | - | 0 |
| | cyclohexane | 265 | 334 | 40.0 |

+ Indole and aniline taken as 100

iii) EFFECT OF pH ON THE FLUORESCENCE OF SULPHANILAMIDE.

The fluorescence characteristics of sulphanilamide are similar to those of aniline. The effect of pH upon the fluorescence of sulphanilamide is shown in Fig.10. The cation of sulphanilamide (I), pKa 2.36, like that of aniline, is practically non-fluorescent and the increase in fluorescence with pH follows closely the curve for the deprotonation of the cation. The fluorescence reaches a maximum at about pH 3.3 where sulphanilamide is almost entirely (90%) in the molecular form (II) and then remains constant to about pH 9.6. Above this pH the fluorescence diminishes to almost zero at pH 14. At pH 8 sulphanilamide begins to ionize to form an anion (III) (pKa 10.43) and at about pH 12.5 it should be almost entirely in the anionic form. However, at pH 12.5 sulphanilamide still fluoresces with an intensity of about 50 % of that of the molecular form. The fall off of fluorescence at higher pH values is probably due to the formation of the



I II III IV

dianion (IV). Since a similar fall off of fluorescence intensity at pH 11.6-14 is found for aniline and N-methylaniline but not for NN-dimethylaniline.[65]

iv) pH FLUORESCENCE CHARACTERISTICS OF $N^1$ ACYL AND $N^1$ - ALKYL-SUBSTITUTED SULPHANILAMIDES.

The pH-fluorescence characteristics were determined for acyl and alkyl compounds over the pH range -2 to 16 and are summarised in Table 9. None of the compounds show appreciable

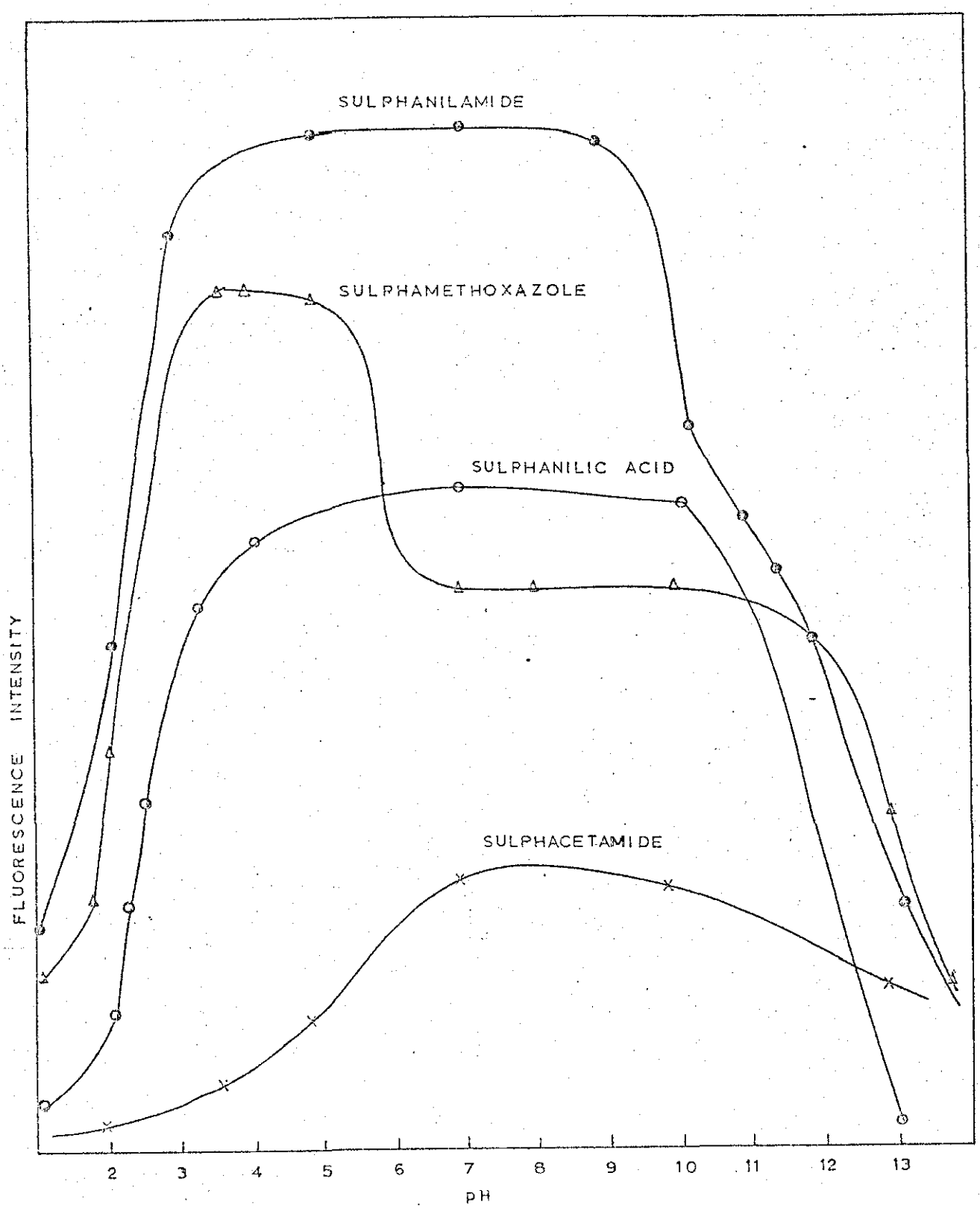



FIG 10 Effect of pH on fluorescence of sulphonamides.

TABLE 9[a]

Influence of pH change on the fluorescence of some sulphonamides

| Compound | pKa | Maximum nm | | | Relative Intensity | pH Range for Max. intensity |
|---|---|---|---|---|---|---|
| | | Absorption | Excitation | Fluorescence* | | |
| Aniline | | | | | | |
| Cation | 4.5 | | | None | 0 | |
| Non-Polar | | 280 | 290 | 350 | 100 | 6- |
| Sulphanilamide | | | | | | |
| Cation | 2.4 | 260 | - | None | 0 | |
| Non-Polar | 10.4 | 259 | 275 | 350 | 480 | 3.3-9.6 |
| Anion | | 249 | 275 | 350 | > 170[+] | |
| N'-Cetylsulphanil-amide | | | | | | |
| Non-Polar | | 266 | 285 | 342 | 410 | 3-13 |
| N'N'-Dimethylsulphan-ilamide | | | | | | |
| Cation | 2.1 | 230 | - | None | 0 | |
| Non-Polar | | 263 | 275 | 350 | 375 | 3.3-11.8 |
| N'N'-Diethylsulphanil-amide | | | | | | |
| Cation | 2.2 | 230 | - | None | 0 | |
| Non-Polar | | 263 | 275 | 350 | 370 | 3.6-11 |
| N'-Carbethoxysulphan-ilamide | | | | | | |
| Cation | | 266 | - | None | 0 | |
| Non-Polar | | 272 | 270 | 350 | 150 | 5.3-9.5 |
| Anion | | 272 | 270 | 350 | 194 | 11.1-11.5 |
| Sulphacetamide | | | | | | |
| Cation | 1.6 | 272 | - | None | 0 | |
| Non-Polar | 5.3 | 272 | 270 | 350 | 25 | 2.4-3.9 |
| Anion | | 257 | 272 | 350 | 250 | 7.2-9.5 |
| Sulphaguanidine | | | | | | |
| Di-Cation | 0.47 | - | - | None | 0 | |
| Mono-Cation | 2.7 | 265 | 285 | 350 | + | |
| Non-Polar | | 258 | 272 | 350 | 250 | 2.6-13.0 |

TABLE 9 (Contd)

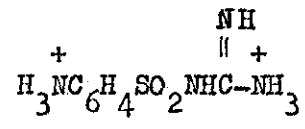
| Compound | pKa | Maximum nm Absorption | Excitation | Fluorescence* | Relative Intensity | pH Range for Max. intensity |
|---|---|---|---|---|---|---|
| Sulphamethazine<br>Sulphadiazine<br>Sulphapyrazine<br>Sulphathiazole<br>Sulphasomizole | | Non-Fluorescent at all pH's | | | | |
| Sulphadimethoxine | | | | | | |
| Cation | 2.0 | 264 | - | None | 0 | |
| Non-Polar | 6.0 | 264 | 288 | 350 | 1.2 | - |
| Anion | | 269 | 290 | 350 | 0.3 | |
| Sulphisoxazole | | | | | | |
| Cation | | 270 | - | None | 0 | |
| Non-Polar | | 266 - 268 | - | 350 | 1.0 | - |
| Anion | | 252 | - | 350 | 0.3 | |
| 2-Sulphanilamido-thiophen | | | | | | |
| Cation | | 236.5 | - | None | 0 | |
| Non-Polar | | 257 | 295 | 350 | 0.8 | - |
| Anion | | 256 | | None | 0 | |
| 2-Sulphanilamido-furfural | | | | | | |
| Cation | | 277 | - | None | 0 | - |
| Non-Polar | | 266 | 275 | 350 | 530 | |

a = Solution concentration 2-4 µg/ml
* = instrumental values
+ = uncertain due to quenching

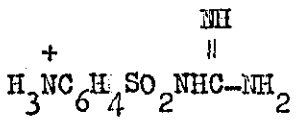
fluorescence as cations ($H_3\overset{+}{N}.C_6H_4SO_2NH.R$). Sulphacetamide and $N^1$-carbethoxysulphanilamide which contain an acyl group, CO.R, are more fluorescent as anions ($H_2N.C_6H_4.SO_2\overset{-}{N}.CO.R$) than in the molecular form.

Sulphaguanidine shows a constant fluorescence intensity from pH 3.6-13, and above pH 5 this must be due to the molecular form. The pKa values of sulphaguanidine are 0.47 and 2.75. At pH 0, sulphaguanidine is non-fluorescent, but fluorescence begins to appear at pH 0.5 rising to a maximum at pH 3.6. The curve for the increase in fluorescence with pH is roughly half-way between the ionization curves of the di-cation (V) and of the monocation (Vla or Vlb). This suggests that the monocation is fluorescent, and, since a positive charge on the aromatic amino group abolishes fluorescence, as seen in the other sulphonamides, the fluorescent cation is probably the form containing the positively charged guanidine group (Vlb).

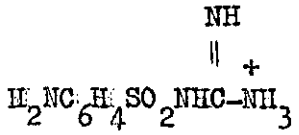
$N^1$, $N^1$-Dimethyl-and-diethyl-sulphanilamide show a similar fluorescence to that of sulphanilamide up to pH 10. However, since, unlike sulphanilamide, they are unable to form an anion corresponding to structure (III) they fluoresce with maximum intensity up to pH 12. Above this pH the fluorescence falls off, probably due to the formation of the monoanion VII. From the pH-fluorescence curves, the pKa of the dimethyl derivative is about 1.8 (Bell and Roblin[66] give 2.1 by titration) and of the diethyl about 2.0-2.1.



V



Vla



Vlb

Sulphacetamide is also fluorescent in the molecular form, though not as a cation. However, it maintains a constant fluorescence up to pH 13.

$$HN\bar{C}_6H_4SO_2N(CH_3)_2 \qquad \text{(VII)}$$

v) FLUORESCENCE OF $N^1$-SUBSTITUTED HETEROCYCLIC SULPHANILAMIDES.

Substitution of a heterocyclic ring into the $N^1$-position has a very pronounced effect on the fluorescence properties of the sulphonamides. All of the nitrogen containing heterocycles studied caused a marked quenching of fluorescence over the entire pH range. Thus sulphamethazine, sulphadiazine, sulphapyrazine, sulphathiazole and sulphasomizole were non-fluorescent while sulphadimethoxine and sulphisoxazole exhibited only a very weak fluorescence. Presumably the preferential light absorption for these compounds is that of an $N \rightarrow \pi$ transition which is known to detract from fluorescence.[64]

Sulphathiophen may be weakly fluorescent for similar reasons. 2-Sulphanilamide-furfural, on the other hand, exhibits a strong fluorescence. It seems probable that in this compound the furfural ring is acting as an electron donating group to the sulphanilamide moiety.

Sulphapyridine differs from the sulphonamides containing the pyrazine, pyrimidine and thiazole rings, since it shows a marked fluorescence at pH 1, (Table 10). The fluorescence (max. 385 nm ) exhibited by this compound, however, is not that of aniline (max. 350 nm ) but of 2-aminopyridine. $N^4$-Acetyl-sulphapyridine was also fluorescent at pH 1 due to its aminopyridine component, but its maximum intensity was only one-sixth of that of sulphapyridine. 2-amino-pyridine is maximally fluorescent (max. 370 nm ) between pH 2.6 and 4.1 at which values it is entirely in the form of a cation. According to Albert[67] the

TABLE 10

Fluorescence of Sulphapyridine and its derivatives

concentrations used, 5 μg/ml.; n.f. = non-fluorescent

| Compound | pKa | Absorption mμ | Excitation mμ | Fluorescence mμ |
|---|---|---|---|---|
| Sulphapyridine | | | | |
| Di-cation | | 308 | 315 | 385 |
| Mono-cation | 1.0* | 308 | 315 | 385 |
| Non-polar | 2.58 | 312 | - | n.f. |
| Anion | 8.43 | 312 | - | n.f. |
| $N^4$-Acetylsulphapyridine | | | | |
| Cation | | 308 | 315 | 385 |
| Non-polar | 8.2 | 260-262-300 | - | n.f. |
| Anion | | 312 | - | n.f. |
| 2-Aminopyridine | | | | |
| Cation | | 307 | 315 | 370 |
| Non-polar | 6.86‡ | 294 | 305 | 355 |

* Bell and Roblin (1944)

‡ Albert (1960)

ionization of 2-aminopyridine involves both nitrogen atoms simultaneously through a resonance hybrid of the cation and presumably this is why the cation of 2-aminopyridine is fluorescent. The pKa for this ionization is 6.86., the fall in fluorescence intensity closely following deprotonation. At pH 9-11 the compound shows a less intense but consistent fluorescence at a new wavelength (max. 355 nm ) due to its molecular form. This fluorescence is near to maximum of aniline (350nm ). In strong acid 2-aminopyridine is non-fluorescent, due to quenching by $Cl^-$, while in strong alkaline the fluorescence is diminished probably by the excited state formation of the anion. Sulphapyridine has two fluorescent peaks, one at pH 0.6-1.1 (max 385 nm ) and one at pH 2.3. The quenching below pH 0 is due to $Cl^-$ ions; the effect of the latter was checked by adding NaCl. The two peaks are probably due to fluorescent cations inter-acting with one another; the one at lower pH values would be the di-cation, $H_3\overset{+}{N}C_6H_4SO_2NHC_5H_4\overset{+}{N}H$, (pKa = 1.0) and the other would be the mono-cation $H_2NC_6H_4SO_2NHC_5H_4\overset{+}{N}H$ (pKa 2.58). This view is supported by the fact that $N^4$-acetylsulphapyridine shows one fluorescent peak st pH 0.8 (max. 385 nm ) and can only form a mono-cation. It is to be noted that neither sulphapyridine nor its $N^4$-acetyl derivative are fluorescent in non-polar or anionic form, since neither of them are fluorescent above pH 4-5. This is to be expected since the pyridine ring is $\pi$ electron-deficient.

vi) $N^4$-SUBSTITUTED SULPHANILAMIDES.

Since acetanilide is non-fluorescent,[68] it could be expected that the $N^4$-acetyl derivatives of the sulphonamides ($CH_3CO.NH.C_6H_4SO_2NH.R$) would also be non-fluorescent. No fluorescence was found with the $N^4$-acetyl derivatives of sulphanilamide sulphacetamide, sulphaguanidine, sulphamethazine, sulphadiazine,

sulphasomizole and $N^1$-methylsulphanilamide. $N^4$-Acetylsulphapyridine is fluorescent only at low pH values and this has already been discussed ( see Table 10)

$N^4$-benzyl sulphanilamide (proseptazine) below pH 9.5 flouresces like N-methylaniline.[65] Below pH 0 it showed no fluorescence. Maximum fluorescence occurred from pH 2.0 to 9.5. Above pH 9.5 the fluorescence fell due to the formation of the less fluorescent mono-anion.

vii) MISCELLANEOUS NON-FLUORESCENT COMPOUNDS.

The following heterocyclic compounds showed no fluorescence at concentrations of 500 $\mu$g/ml solution at any pH value; pyridine, pyrrol, furfural, 2-aminothiazole and 2-amino-5-methylisothiazole.

Nitro compounds are none or very weakly fluorescent, and p-nitrobenzene-sulphonamide, p-nitrobenzenesulphonmethylamine and 4-nitrobenzenesulphonylglutamic acid were found to be non-fluorescent.

viii) FLUORESCENCE OF ISOMERS OF SULPHANILAMIDE.

Both orthanilamide and metanilamide are fluorescent but the fluorescence maxima occur at longer wavelengths (see Table 8) and the intensity is less than sulphanilamide. This is due at least in part to interactions of these compounds with the solvent. pH-Fluorescence studies on these compounds showed that the cations of both were non-fluorescent. The molecular form of metanilamide gave a maximum fluorescence at 385 nm and the appearance of this fluorescence followed the ionization curve of the cation. The anion of metanilamide was about 6 times as fluorescent at the molecular form ( 370 nm ). The pH-fluorescence curve of orthoanilamide was similar to that of metanilamide, but the

fluorescence was only about a third as intense. The anion of orthanilamide was about 5 times as fluorescent as the molecular form (pKa 9.7).

The intensity of fluorescence of the aminobenzenesulphonamides in aqueous solution decreases in the order p,> m,> o, the anions of orthanilamide and metanilamide are, however, more fluorescent than the corresponding molecular forms, whilst the anion of sulphanilamide is probably less fluorescent than the molecular form.

p-Hydroxybenzenesulphonamide is also included in Table 11. This compound could be expected to show the fluorescence of simple phenols which occurs at 310-330 nm.[69] The pH-fluorescence curve of this compound showed two fluorescent maxima, one at 313 nm, of constant maximum intensity from pH 2.5-7.5, and another, twice as intense, at 348 nm, with maximum intensity from pH 9-11. Below pH 2.5 and above 11, the fluorescence diminished and had nearly disappeared at pH 0 and 14. The first fluorescence is probably due to the molecular form and the second to the ion, $HO.C_6H_4SO_2NH^-$. It is likely that the di-anion $^-O.C_6H_4SO_2NH^-$, is only very weakly fluorescent or like the anion of phenol. The absorption spectra of p-hydroxbenzene-sulphonamide at various pH values confirmed the existence of these ions. At pH 0 and 3.6, the compound showed absorption peaks at 236 nm ($E_{max.}$ 13,800) and 279 nm, ($E_{max}$ 900) due to the molecular form but the instrumental excitation peak at 248 nm, corresponds to the intense absorption at the shorter wavelength. At. pH 10 the absorption consists of a single large band at 262 nm, ($E_{max.}$ 10,000) presumably due to the sulphonamide anion. The approximate pKa for the formation of this absorption peak is at 256 nm, ($E_{max.}$9000) presumably due to the di-anion.

TABLE 11

Fluorescence of sulphonamides in ethanol

Concentration of sulphonamide 5 μM.

| Compound | Wavelength mμ of max. absorption | excitation | fluorescence | Relative fluorescence intensity observed | corrected | Absolute fluorescence efficiency % |
|---|---|---|---|---|---|---|
| Sulphanilamide | 259 | 269 | 345 | 5.06 | 6.74 | 1.1 |
| Sulphacetamide | 272 | 272 | 345 | 17.1 | 22.8 | 3.9 |
| $N^1$, $N^1$-dimethylsulphanilamide | 262 | 271 | 345 | 57.8 | 76.9 | 13.6 |
| $N^1$-acetylsulphanilamide | 265 | 274 | 346 | 44.4 | 54.6 | 9.1 |
| Sulphaguanidine | 258 | 270 | 346 | 39.8 | 53.0 | 10.1 |
| Sulphadimethoxine | 273 | 274 | 348 | 1.00 | 1.00 | 0.13 |
| Sulphasomizole | 279 | 285 | 346 | 1.41 | 1.41 | 0.26 |
| Sulphisoxazole | 271 | 280 | 346 | 3.22 | 3.44 | 0.58 |
| Sulphathiophen | 257 | 268 | 346 | 4.06 | 5.41 | 1.01 |
| Sulphafurfural | 266 | 275 | 348 | 63.7 | 78.4 | 9.1 |
| Sulphapyridine | 308 | 308 | 345 | 0.51 | 0.35 | 0.06 |
| Sulphanilic acid | 249 | 262 | 345 | 25.0 | 44.4 | 12.0 |
| Orthanilamide | 302 | 304 | 380 | 2.54 | 3.39 | |
| p-hydroxybenzenesulphonamide | 236 | 246 | 315 | 11.1 | 25.4 | 5.8 |
| Indole | 280 | 283 | 340 | 100 | 100 | 46 |

ix) PHOSPHORESCENCE SPECTRA.

The phosphorescence spectra of aniline and sulphanilamide are shown in Fig. 11. Aniline shows a single assymetric excitation spectrum, $\lambda_e$ 320nm, and a structured emission spectrum, $\lambda_p$ 410 nm. Sulphanilamide has a similar excitation and emission spectra with the appearance of a shoulder peak in the excitation spectrum at 290 nm. Similar excitation and emission spectra are obtained for sulphacetamide, sulphormethoxine, sulphamethazine, sulphamerazine and sulphadiazine. The phosphorescence emission spectrum of sulphanilic acid is also similar to that of aniline; however, the excitation spectrum is composed of three peaks at 275, 310 and 315 nm, and the lifetime of 1.4 seconds is nearer to those found for the sulphonamides in general than that of aniline (5.0 seconds) Table 12. The excitation spectra of sulphathiazole, sulphamethoxazole, and sulphaguanidine show some of the composite nature found in the excitation spectra of sulphanilic acid. The $N^1$ and $N^1N^4$ acetyl derivatives of sulphanilamide have structured excitation spectra with peak maxima at 290 and 310 nm. Sulphapyridine shows two broad excitation bands one at 310 nm corresponding to excitation of the aniline moiety for which emission is observed at 410 nm, and the second band at 350 nm corresponding to the excitation of the 2-aminopyridine nucleus for which emission is observed at 440 nm (Fig. 12.) The phosphorescence emission spectra of the sulphonamides originates from the aniline nucleus, the sulphonic acid group affecting the intersystem crossing rate as seen from the difference in lifetimes of aniline and sulphanilic acid. Substitution at the $N^1$ and $N^4$ positions serves only to change the levels of the singlet state as seen from the change in excitation spectra.

The lone pair electrons of the nitrogen atom in

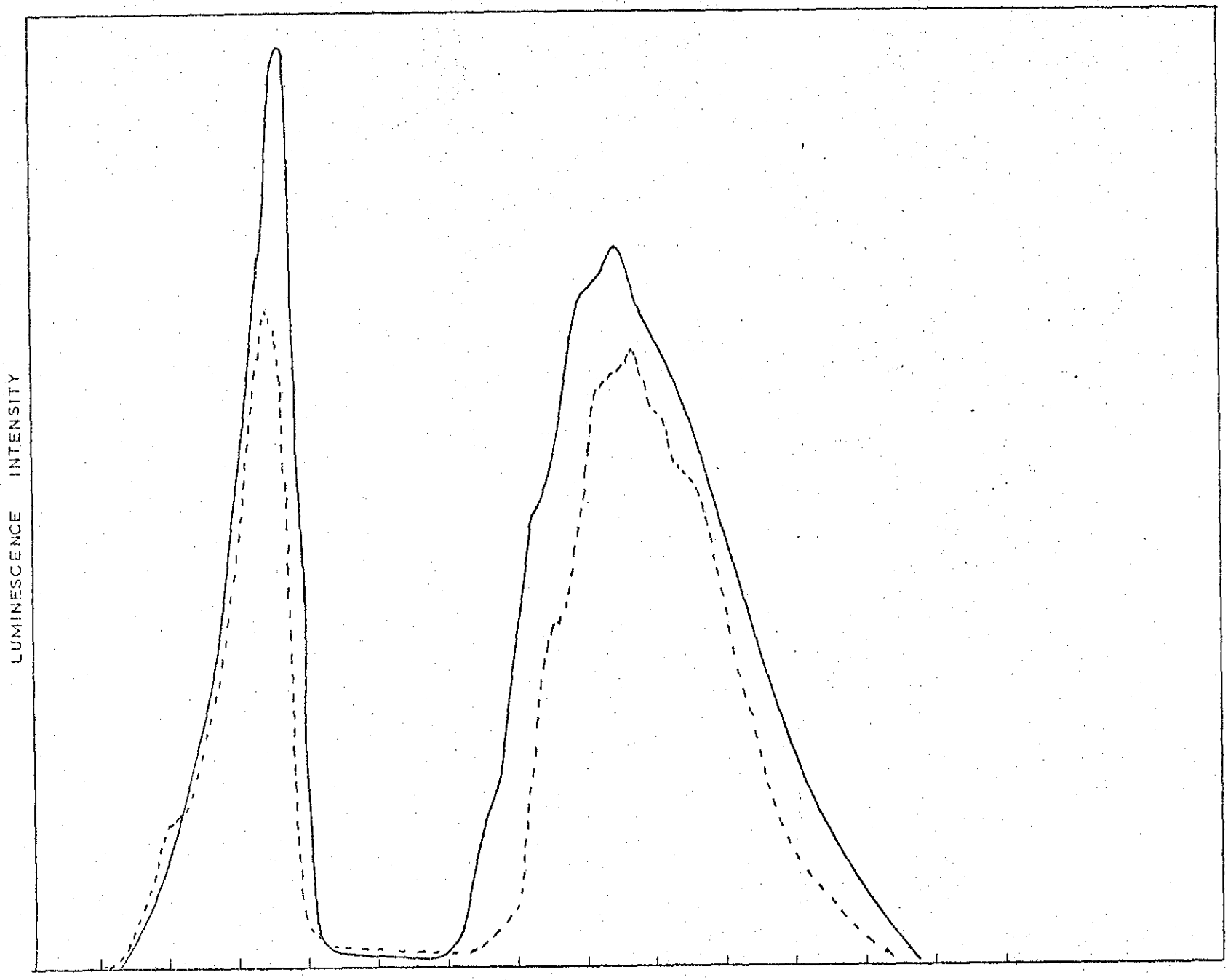



FIG 11. Phosphorescence spectra of Aniline (——) and Sulphanilamide (- - - -).

TABLE 12

Fluorescence and phosphorescence properties of some sulphonamides

| Compound | Fluorescence 298°K | | | | Phosphorescence 77°K | | |
|---|---|---|---|---|---|---|---|
| | pH | λe nm | λf nm | Detection limit μg/ml | λe nm | λp nm | τsecs. |
| Sulphanilamide | 8.0 | 275 | 350 | 0.01 | (280)<br>305 | 405 | 1.2 |
| Sulphacetamide | 8.0 | 272 | 350 | 0.05 | 310 | 410 | 0.9 |
| Sulphaguanidine | 8.0 | 285 | 350 | 0.02 | 310 | 410 | 1.6 |
| Sulphapyridine | 1.0 | 315 | 385 | 0.002 | 310<br>350 | 410<br>440 | 0.6<br>1.3 |
| $N^1N^4$ Diacetylsulphanilamide | - | - | - | - | 290<br>310 | 410 | 1.2 |
| $N^4$-Acetylsulphanilamide | - | - | - | - | 285<br>290<br>310 | 410<br>430 | 1.3 |
| Sulphamethoxazole | 4.0 | 285 | 350 | 0.01 | 310 | 410 | 1.3 |
| Sulphormethoxine | >2.0 | 285 | 350 | N.A.U. | 310 | 412 | 0.6 |
| Sulphathiazole | - | - | - | - | 310 | 420 | 0.9 |
| Sulphadiazine | - | - | - | - | 310 | 410 | 0.7 |
| Sulphamethazine | - | - | - | - | 310 | 410 | 0.8 |
| Sulphamerazine | - | - | - | - | 310 | 412 | 0.8 |
| Aniline | | | | | 320 | 410 | 5.0 |
| Sulphanilic Acid | | | | | 275<br>310<br>315 | 410 | 1.4 |
| 2-Aminopyridine | | | | | 325 | 435 | 2.0 |

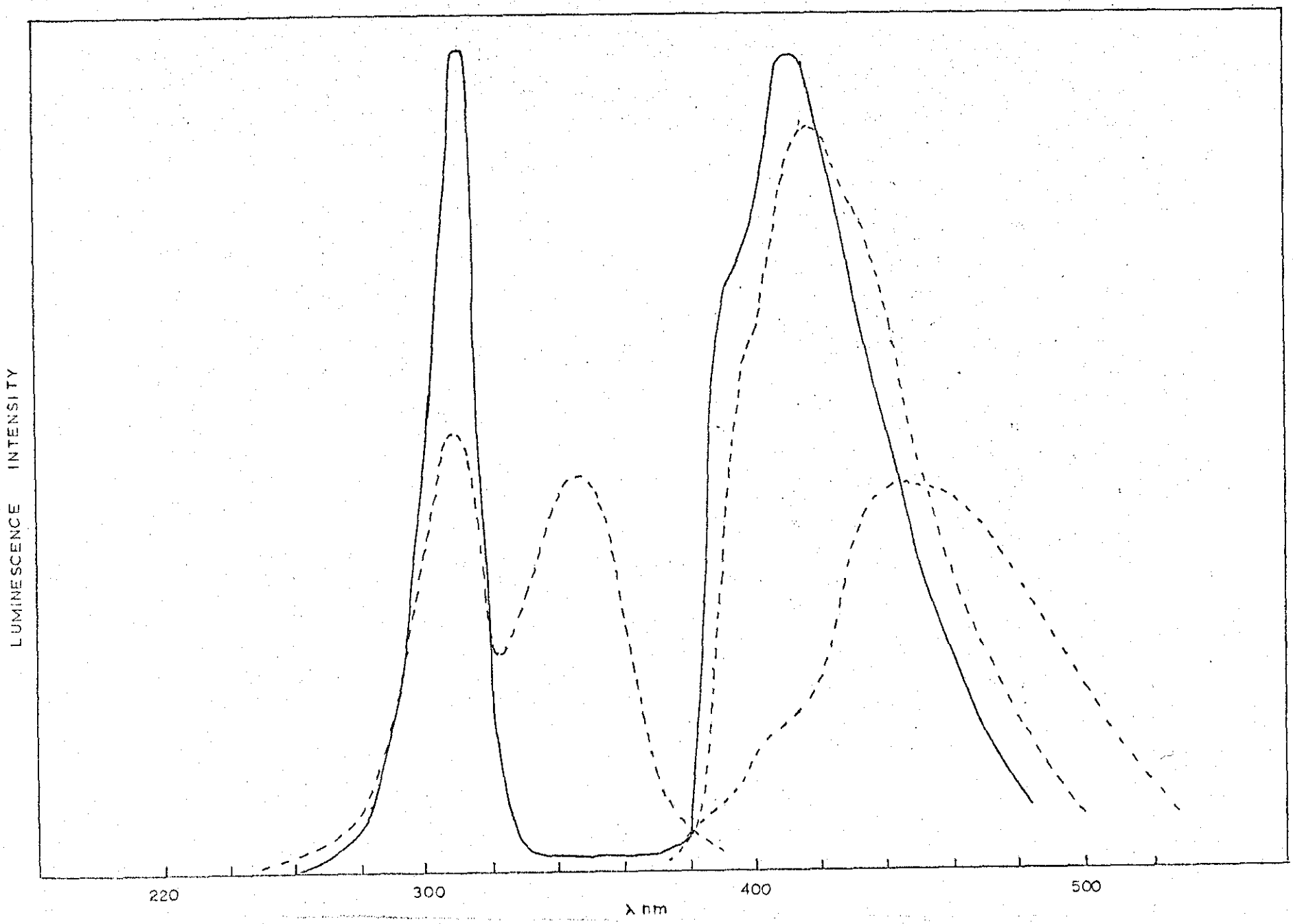



FIG 12 Phosphorescence spectra of Sulphapyridine (——) and 2-aminopyridine(- - - -).

aniline cannot be entirely classified as non-bonding electrons since they are capable of conjugation with the $\pi$-orbital system of the aromatic nucleus. This makes it difficult to assign absorption to the first excited singlet state as an $n-\pi^*$ or $\pi \rightarrow \pi^*$ transition. However it is more easily seen that the emission from the lowest excited triplet is a $\pi^* \rightarrow \pi$ transition from the aromatic nucleus. The phosphorescent lifetimes of the order of seconds, are long compared with the lifetimes observed in transitions originating from $n \rightarrow \pi^*$ triplet states. Furthermore, Venning et al [31] have recently shown that phosphorescent lifetimes of the sulphonamides are susceptible to change in the presence of heavy atoms, which, according to Kearns,[70] is a characteristic of $\pi \rightarrow \pi^*$ triplet states.

## x) QUANTITATIVE ANALYSIS OF SULPHONAMIDES

Many of the sulphonamides exhibit appreciable phosphorescence, even when fluorescence yields are low, and can be determined phosphorimetrically at the submicrogram level as previously demonstrated by Hollifield and Winefordner.[30] The fluorescence and phosphorescence characteristics of the sulphonamides and their fluorimetric limits of detection are given in Table 12. Fluorimetric estimation in the submicrogram range can be used to advantage for the analysis of sulphanilamide, sulphacetamide, sulphaguanidine, sulphamethoxizole, sulphapyridine, $N^1$-cetylsulphanilamide, $N^1N^1$-diethylsulphanilamide, $N^1$-carbethoxysulphanilamide, and sulphanilamidofurfural. The fluorimetric method of analysis is simpler than the phosphorimetric method. Both methods require careful attention to experimental procedure in order to minimise the effects of photolysis during measurement, and the use of freshly prepared standard solutions in the preparation of working curves.

xi) ANALYSIS OF SULPHONAMIDES IN SERUM.

Table 13 shows the results for the analysis of sulphanilamide, suphacetamide, sulphaguanidine and sulphamethoxazole in serum. Each result reported is the average of six determinations. The relative standard deviations of the reults are in the range 2-7%

For the drugs studied the background due to the serum became significant at concentrations below 5mg/100 ml. The lower limit for the estimation of the sulphonamides in serum could conceivably be reduced by using a solvent extraction procedure prior to estimation or by taking a larger sample initially. Since the normal therapeutic levels encountered are within the range 10-20 mg/100 ml a direct fluorimetric estimation is satisfactory. Recoveries using this method ranged from 96-103 % and the time taken to perform an analysis was less than 10 minutes.

TABLE 13

Accuracy of estimation of sulphonamides in serum

| Sulphonamide | Amount Added (mg/100 ml serum) | Amount Found (mg/100 ml serum) | % Recovery | % Std. deviation (6 dtmns.) |
|---|---|---|---|---|
| Sulphanilamide pH 8.0 | 15.0 | 15.05 | 100.3 | 2.0 |
| | 10.0 | 9.58 | 95.8 | 1.4 |
| | 5.0 | 5.00 | 100.0 | 2.5 |
| Sulphacetamide pH 8.0 | 12.4 | 12.6 | 101.6 | 2.9 |
| | 8.2 | 8.1 | 98.7 | 3.7 |
| | 4.1 | 3.95 | 96.3 | 5.9 |
| Sulphaguanidine pH 8.0 | 12.5 | 12.3 | 98.4 | 2.08 |
| | 9.8 | 9.85 | 100.5 | 4.94 |
| | 4.9 | 5.08 | 103.6 | 7.08 |
| Sulphamethoxazole pH 4.0 | 15.0 | 14.7 | 98.3 | 1.86 |
| | 10.0 | 10.2 | 101.6 | 2.56 |
| | 5.0 | 4.9 | 97.9 | 2.27 |

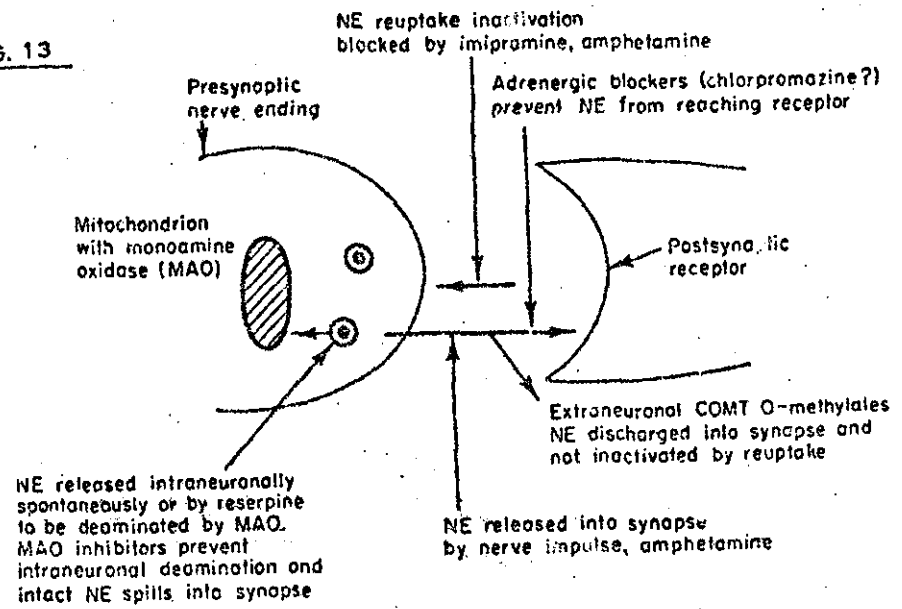
# C H A P T E R 5.

## LUMINESCENCE PROPERTIES OF DRUGS AFFECTING THE CENTRAL NERVOUS SYSTEM.

It has become clear that several important drugs used in psychiatry exert their clinical effects by affecting the synaptic action of the catecholamines (CA) in the brain. It is therefore important to understand the events at the noradrenergic synapses and how the amines are released and subsequently inactivated at brain synapses.

The adrenergic neurone (FIG.13) synthesis noradrenaline from tyrosine. Under resting conditions, in the absence of nerve stimulation, noradrenaline slowly diffuses out of the synaptic vesicles and is deaminated by monoamine oxidase (MAO) within the nerve terminal so that it leaves the neuron in an inactivated form. The arrival of a nerve impulse releases noradrenaline from the nerve ending which diffuses across the gap to activate the receptor. The receptor is cleared ready for further stimulation largely by return and reuptake of noradrenaline by the nerve ending, a small amount being metabolized by the extraneuronal enzyme catechol-o-methyltransferase (COMT).

FIG. 13



The effect of administered drugs on the metabolic pattern of released amines is of great importance. By considering amine synthesis, the intra-and intercellular area of amine release, and the effect of a given drug on noradrenaline inactivation and subsequent metabolism, it is possible to formulate convincing explanations of psychotropic drug action, which may help predict the clinical effects of newly developed drugs.

It is not the purpose of this study to give detailed quantitative information on the luminescence properties of such a wide range of drugs but rather to give a qualitative study which may be used to predict those drugs which might usefully be studied by these techniques.

i) CATECHOLAMINES.

Since the psychotropic drugs affect the synaptic action of the catecholamines it is important that they can be estimated before and after drug administration.

The CA are metabolically degraded by the enzymes MAO and COMT. MAO removes the side chain amine from noradrenaline and through an intermediate aldehyde results in the formation of 3, 4-dihydroxymandelic acid. COMT transfers a methyl group from the methyl donor S-adenosylmethionine to the m-hydroxyl group of the CA. Prior to excretion in the urine, the CA have usually been acted on both by MAO and COMT. The resulting deaminated, O-methylated product is 3-methoxy-4-hydroxymandelic acid, usually referred to as vanillylmandelic acid (VMA).

The CA studied are shown in Table 14 together with their luminescent characteristics.

T A B L E 14

| COMPOUND | $\lambda_e$ nm | $\lambda_p$ nm | $\tau$ seconds |
|---|---|---|---|
| L-Epinephrine | (255) 295 | 435 | 0.5 |
| DL-3,4-Dihydroxymandelic Acid. | (255) 295 | 420 | 0.8 |
| Vanillylmandelic Acid | (255) 290 | 430 | 0.6 |
| Normetanephrine (NE)(b) | 275 | 440 | 0.6 |
| 5-Hydroxytryptamine | (235) 290 315 | (430)450 | 4.4 |
| 5-Hydrotryptophan(b) | 315 | 435 | 6.3 |

(a) - Measured in EtOH 77°K 100μg/ml Resolution 6nm

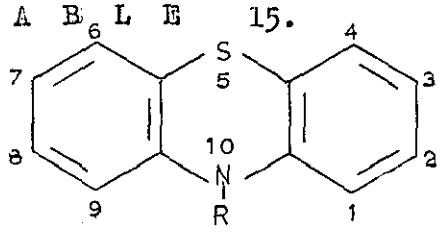
(b) Taken from "Fluorescence Assay in Biology and Medicine" -S. Udenfriend, Vol. II. p.99 (1969).

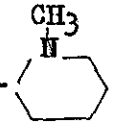
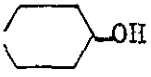
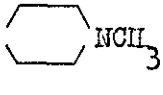
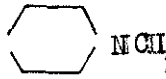
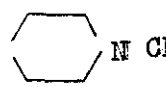
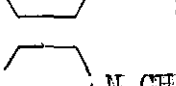
The table also shows the luminescent characteristics of 5-Hydroxytryptamine (5HT) and 5-Hydroxytryptophan. 5HT is a biogenic amine which like the CA may function as a neurohumor in the brain, but whose neurohumoral actions and relationship to psychotropic drug effects is less well established than for the CA. The limits of detection for the catecholamines can be estimated to be in the μg range from the values found for L-Epinephrine (1μg/ml) and normetanephrine (0.1μg/ml).

ii) PHENOTHIAZINES.

This group forms the largest and most important group of major tranquillizers. They are all based on the phenothiazine nucleus, differing only in their substituents at positions 2 and 10. Table 15.

T A B L E 15.




| No | COMPOUND | Substituent at 2 position | Substituent at position 10 |
|---|---|---|---|
| | Aliphatic series | | |
| 1 | Chlorpromazine | Cl | $CH_2CH_2CH_2\overset{H}{N}(CH_3)_2\ Cl^-$ |
| 2 | Triflupromazine | $CF_3$ | $CH_2CH_2CH_2\overset{+}{\underset{H}{N}}(CH_3)_2\ Cl^-$ |
| 3 | Methotrimeprazine | $O\ CH_3$ | $CH_2CHCH_2N(CH_3)_2$ <br> $CH_3$ |
| | Piperidine series | | |
| 4 | Thioridazine | $S\ CH_3$ | $CH_2CH_2-$  |
| 5 | Pericyazine | CN | $CH_2CH_2CH_2-N$  |
| | Piperazine series | | |
| 6 | Prochlorperazine | Cl | $CH_2CH_2CH_2-N$  |
| 7 | Perphenazine | Cl | $CH_2CH_2CH_2-N$  $NCH_2CH_2OH$ |
| 8 | Thiopropazate | Cl | $CH_2CH_2CH_2-N$  $N\ CH_2CH_2O\overset{O}{C}\ CH_3$ |
| 9 | Fluphenazine | $CF_3$ | $CH_2CH_2CH_2N$  $N\ CH_2CH_2OH$ |
| 10 | Trifluoperazine | $CF_3$ | $CH_2CH_2CH_2N$  $N\ CH_3$ |

The above named compounds were the generous gifts of the following

companies;

May & Baker Ltd - cpds 1,3,5 and 6.

E.R.Squibb & Sons Ltd - cpds 2 and 9.

Sandoz Products - cpd 4.

Smith, Kline and French - cpd 10.

Allen and Hanburys - cpd 7.

G.D.Searle - cpd 8.

The phosphorescence characteristics of the phenothiazines are shown in Table 16. All of the phemothiazines studied with the exception of Pericyazine, exhibited luminescence spectra similar to that of Chlorpromazine (Fig. 14.) At 77°K the luminescence is almost entirely phosphorescence and remains unchanged whether measured in acid (0.1N $H_2SO_4$/Et OH) or alkaline (0.1N NaOH /Et OH) solution. Substitution at the 2-position of the phenothiazine nucleus has only minor effects on the lifetime and spectra of the compounds studied, except for Pericyazine (Fig.15) The presence of a cyano group produces a marked effect on the lifetime and spectra, presumably due to a perturbation of the singlet-triplet transition. Phosphorescence lifetimes for the phenothiazines are in the millisecond range indicative of a $N \rightarrow \pi^*$ triplet state involving the interaction of the nitrogen and sulphur lone pair electrons with the $\pi$ electrons of the aromatic nuclei. Table 18 gives the phosphorescence characteristics for xanthene and thioxanthene. It is interesting to note that for thioxanthene the phosphorescence spectra and lifetime are similar to those found in the phenothiazines whilst the lifetime and phosphorescence spectra of xanthene are similar to those of aromatic compounds e.g., toluene. The lifetime for thioxanthene indicates a $N \rightarrow \pi^*$ triplet state whilst the lifetime and spectra of xanthene indicate the presence of a $\pi \rightarrow \pi^*$ triplet. Since nitrogen, oxygen and

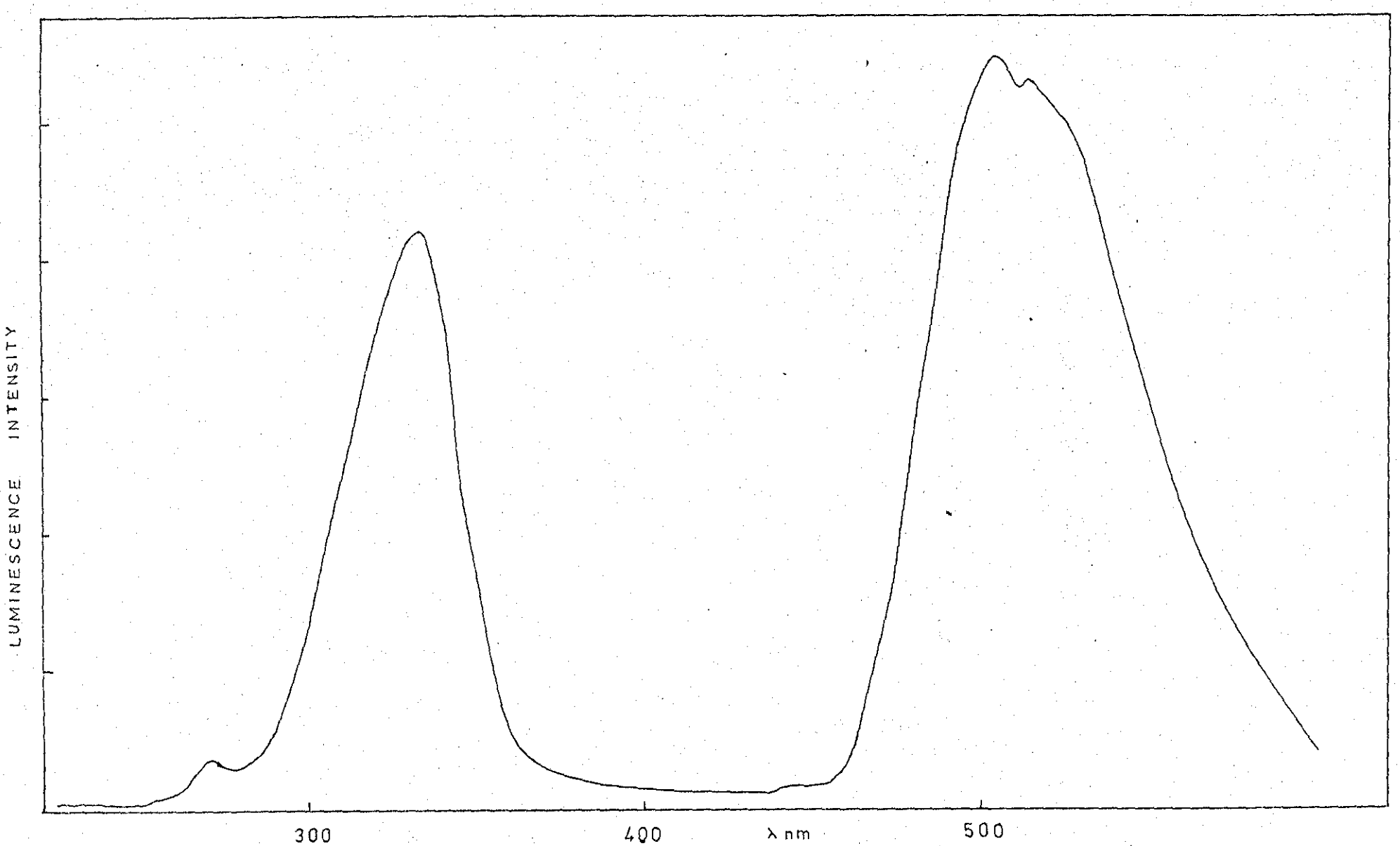



FIG 14. Phosphorescence spectra of chlorpromazine in EtOH at 77 °K.

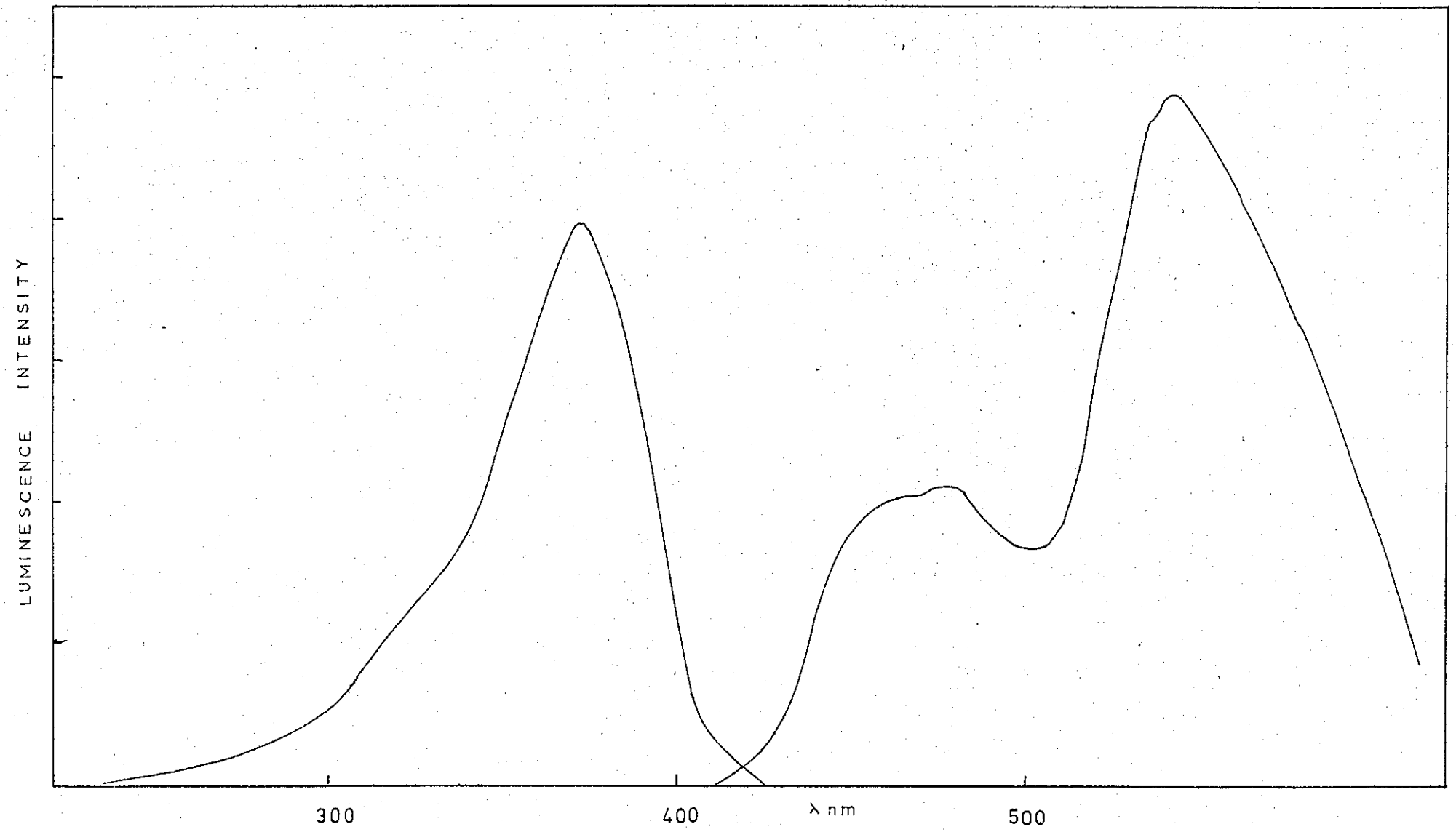



FIG 15. Luminescence spectra of pericyazine in EtOH at 77°K.

sulphur all have lone pair electrons the steric interaction of the lone pairs with the $\pi$ electrons of the aromatic nuclei must be different in xanthene from that of the lone pair electrons of the nitrogen and sulphur atoms in the phenothiazines.

T A B L E 16 [a]

| COMPOUND | $\lambda e$ nm | $\lambda p$ nm | $\tau$ m S |
|---|---|---|---|
| Chlorpromazine | 335 | 495-510 | 72 |
| Triflupromazine | 345 | 510 520 | 72 |
| Methotrimeprazine | 330 | 485 | 66 |
| Thioridazine | 335 | 500 | 66 |
| Pericyazine | (330) 375 | 535 | 46 |
| Prochlorperazine | 330 | 500 | 76 |
| Perphenazine | 330 | 495-510 | 72 |
| Thiopropazate | 330 | 500 | 70 |
| Fluphenazine | 335 | 515 | 65 |
| Trifluoperazine | 340 | 515 | 71 |

(a) Measured in EtOH at 77°K. 100$\mu$g/ml Resolution 6 nm

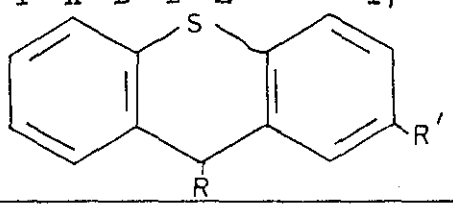
Chlorpromazine is rapidly cleared from blood and excreted in urine in the form of forty or more metabolites. Human urine probably contains less than 0.1 - 0.2 % of the parent drug after oral intake. Not all of the urinary products have been indentified due to the lack of specific standards for reference purposes. Resolution and detection of nanogram quantities from blood samples requires sophisticated techniques which have not been developed for routine use. The luminescence data coupled with thin layer chromatography (see Chapter 6) might well be able to cope with such a problem.

Winefordner and Tin[24] indicate that the phenothiazines may be determined at submicrogram levels and gave the limit of detection for chlorpromazine as 0.03μg/ml.

iii) THIOXANTHENES.

The drugs derived from thioxanthene are chemically related to the phenothiazines and their metabolism is analagous. The compounds studied are shown in Table 17

T A B L E 17




| COMPOUND | R' | R |
|---|---|---|
| Thioxanthene | H | H |
| Thiothixene | $SO_2-N(CH_3)_2$ | C=CH-CH$_2$-CH$_2$-N⟨ ⟩N-CH$_3$ |
| Chlorprothixene | Cl | C=CH-CH$_2$-CH$_2$ N $(CH_3)_2$ |
| Xanthene | Bridging oxygen at position 5. | |

The luminescence characteristics of the thioxanthenes are shown in Table 18. Thioxanthene and Chlorprothixene have phosphorescent lifetimes similar to those of the phenothiazines, however the excitation spectrum of chlorprothixene exhibits three distinct maxima presumably resulting from the double bond at the 10 - position. Xanthene exhibits fine structure in the emission spectrum typical of aromatic species. Thiothixene shows only fluorescence emission presumably due to the - $SO_2-N(CH_3)_2$ group at position 2.

T A B L E 18

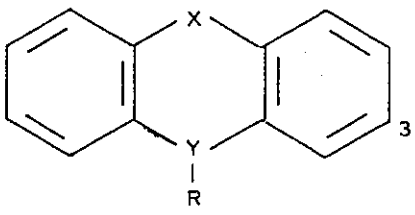
| COMPOUND | $\lambda_e$ nm | $\lambda_f$ nm | $\lambda_p$ nm | $\tau$ seconds |
|---|---|---|---|---|
| Thioxanthene | 280 | - | 460 | < 0.2 |
| Chlorprothixene | (260)(310)385 | - | 470 | < 0.2 |
| Thiothixene | 335 | 385 | - | - |
| Xanthene | (270) 295 | - | 382 | 1.35 |

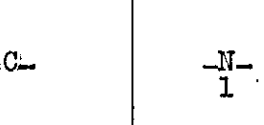
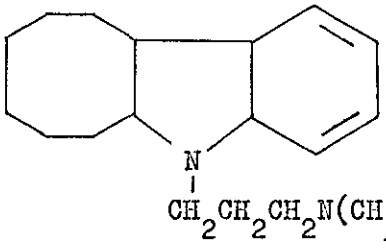
iv) TRICYCLIC ANTIDEPRESSANTS

Imipramine, desimipramine and related compounds such as amitriptyline and nortriptyline appear to be the most effective antidepressant drugs. These drugs are not inhibitors of MAO as were the earliest antidepressants and do not affect the levels of brain CA in animals. However, imipramine is a very potent inhibitor of the reuptake process of inactivation of NE released at the synapse, both in the peripheral sympathetic nerves and in the brain. It therefore potentiates the actions of NE released by nerve discharge.

There are no major structural or chemical differences between the tricyclic antidepressants and the phenothiazine type antipsychotic agents. The two classes differ mainly in the composition of the bridging atoms between the two phenyl rings. The compounds studied are given in Table 19.

T A B L E 19.




| | COMPOUND | SUBSTITUENTS | | | |
|---|---|---|---|---|---|
| | | x | y | R | 3 |
| 1. | Desimipramine | $-CH_2-CH_2-$ | $-\underset{1}{N}-$ | $CH_2CH_2CH_2NH\,CH_3$ | |
| 2 | Chlorimipramine | $-CH_2-CH_2-$ | $-\underset{1}{N}-$ | $CH_2CH_2CH_2N(CH_3)_2$ | Cl |
| 3 | Trimipramine | $-CH_2-CH_2-$ | $-\underset{1}{N}-$ | $CH_2\underset{CH_3}{CH}CH_2N(CH_3)_2$ | |
| 4 | Amitriptyline | $-CH_2-CH_2$ | $>C=C<$ | $-CH_2CH_2N(CH_3)_2$ | |
| 5 | Nortriptyline | $-CH_2-CH_2$ | $>C=C<$ | $CH_2\,CH_2\,\overset{H}{\overset{+}{N}}<\overset{H}{CH_3}$ | |
| 6 | Protriptyline | $-C=C-$ | $-\underset{1}{CH}-$ | $CH_2CH_2CH_2\,NH\,CH_3$ | |
| 7 | Prothiaden | $-C-S-$ | $>C=C<$ | $CH_2\,CH_2\,N(CH_3)_2$ | |
| 8 | Dibenzepin | $-\overset{O}{\overset{\|}{C}}-\underset{1}{\overset{R^1}{N}}-$ | $-\underset{1}{N}-$ | $CH_3$ | |
| 9 | Opiprimol | $-C=C-$ | $-\underset{1}{N}-$ | $(CH_2)_3N$  $N\,CH_2CH_2OH$ | |
| 10 | Doxepin | $-O-C-$ | $>C=C<$ | $CH_2\,CH_2N(CH_3)_2$ | |
| 11 | Iprindole |  $CH_2CH_2CH_2N(CH_3)_2$ | | | |

The compounds listed were generous gifts of the following companies: Geigy Pharmaceuticals Cpds 1,2 & 9., Merck,Sharpe & Dohme, Cpd 6. Roche Products, Cpd 4. Boots Co. Ltd. Cp 7., Eli Lilly - Cpd 5. Sandoz Products Ltd., Cp 8. May & Baker Ltd - Cpd 3, Pfizer Ld.Cpd 10 J. Wyeth, Cpd.11.

The luminescence spectra and lifetimes (Table 20) of desimipramine, chlorimipramine, trimipramine, and dibenzepin show similarities to the phenothiazines. Here, again, the lone pair electrons of the bridging nitrogen atom contribute to produce a $N \rightarrow \pi^*$ triplet state which results in the typical phosphorescence.

No luminescence is observed from amitriptyline, nortriptyline, prothiaden and doxepin, the $\pi$ electrons from the double bond at the 5 position being unable to contribute to the aromatic character of the molecule as a whole. However in the case of protriptyline and opiprimol where the two phenyl rings are conjugated by means of a double bond at the 10-11 position fluorescence alone is observed.

Iprindole, although classed as a tricyclic antidepressant has a significantly different structure to the other compounds studied and this is manifested in the luminescent spectra and lifetime.

T A B L E 20

| COMPOUND | $\lambda e$ nm | $\lambda f$ nm | $\lambda p$ nm | $\tau$ s |
|---|---|---|---|---|
| Desimipramine | 300 | - | 455 | 0.95 |
| Chlorimipramine | 305 | - | 450-470 | 0.12 |
| Trimipramine | 300 | - | 450-470 | 0.7 |
| Opipramol | (300) 365 | 520 | - | - |
| Dibenzepin | 330 | 470 | - | 0.09 |
| Amitriptyline | - | - | - | - |
| Nortriptyline | - | - | - | - |
| Prothiaden | - | - | - | - |
| Doxepin | - | - | - | - |
| Protriptyline | 320 | 355/375 | - | - |
| Iprindole | 310 | 340/350 | (425)450(485) | 5.3 |

The mono-N-demethylated derivatives of imipramine and amitriptyline are active metabolites and are marketed as desimipramine and nortriptyline. Metabolism of the iminodibenzyl nucleus results in side-chain demethylation, ring hydroxylation at the 2-position

and glucuronide conjugation all of which would be expected to maintain luminescent activity.

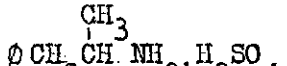
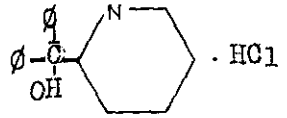
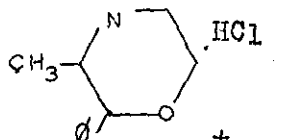
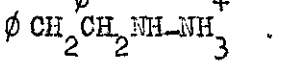
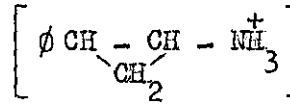
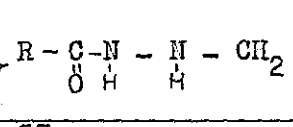
v) CNS STIMULANTS AND MAO INHIBITORS.

Amphetamine and amphetamine derivatives exert powerful direct stimulant action on the CNS. The major mechanism of action is by inhibition of uptake of monoamines by the neuronal membrane, thus lowering the level of NE but not of 5HT.

The antidepressant action of the MAOI is related to the extent to which they inhibit MAO. Normally MAO degrades only NE which leaks out of the vesicles inside the nerve terminal and is therefore not concerned with the actual physiological inactivation of synaptically released NE. However, it is possible that after inhibition of MAO, NE which accumulates within the nerve terminal may flow over the synaptic membrane, across the synaptic cleft and on to postsynaptic receptors.

The compounds studied are shown in Table 21.

T A B L E 21

| No | COMPOUND | STRUCTURE |
|---|---|---|
| | CNS STIMULANTS | |
| 1 | Benzedrine |  |
| 2 | Dexedrine |  (Dextro) |
| 3 | Pipradol |  |
| 4 | Phenmetrazine |  |
| | MAO INHIBITORS | |
| 5 | Phenelzine |  |
| 6 | Tranylcypromine |  |
| 7 | Mebanazine |  |
| 8 | Nialamide |  |
| 9 | Iproniazid | |
| 10 | Isocarboxazid | |

The amphetamines and a number of the MOAI are phenylethylamines incorporating the general structure "1-amino - 2-phenylethane". These compounds have similar luminescent properties which are listed in Table 22. A second group of MOAI incorporate a hydrazine group and these are seen to have different luminescent properties to the phenylethylamines. The phenylethylamines have long phosphorescence lifetimes indicative of a $\pi \rightarrow \pi^*$ triplet transition whilst those compounds incorporating the hydrazine group have short phosphorescent lifetimes indicative of a $N \rightarrow \pi^*$ triplet transition. Miles and Schenk[61] indicate that the phosphorimetric limit of detection for amphetamine (a typical phenylethylamine) is in the range 1 - 2 µg/ml.

T A B L E 22.

| COMPOUND | $\lambda_e$ nm | $\lambda_p$ nm | $\tau$ seconds. |
|---|---|---|---|
| Benzedrine | 270 (355) | 385 | 10.6 |
| Dexedrine | 270 (355) | 385 | 10.4 |
| Pipradol | (235) 265 | 395 | 6.1 |
| Phenmetrazine | 265 | 385 | 7.4 |
| Phenelzine | 265 | 400 | 10.5 |
| Tranylcypromine | 285 | 415 | 7.0 |
| Mebanazine | 270 (285) 370 | 430 | 9.0 |
| Nialamide | 320 | 470 | 0.2 |
| Iproniazid | 300 (370) | 440 | 0.2 |
| Isocarboxazid | 320 (355) | 410-435 | 0.2 |

The compounds were generous gifts of the following companies: - Smith, Kline & French. Cpds 1,2 & 6. Merrell, Cpd 3. Boehringer & Ingleheim, Cpd. 4., Warner, Cpd. 5 I.C.I., Cpd 7. Pfizer, Cpd 8., Roche. Cpd. 9 &, 10.

The major metabolic product of the phenylethylamines is the p-hydroxy derivative, which would be expected to have similar luminescent properties.

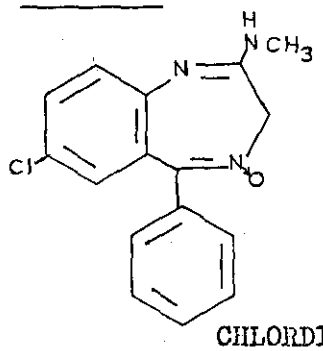
vi) THE 1, 4 - BENZODIAZEPINES

The benzodiazepines are a most important group of minor tranqilisers. While little is known about the biochemical effects of these compounds, they appear to be effective in suppressing the autonomic concomitants of conditioned reflexes.

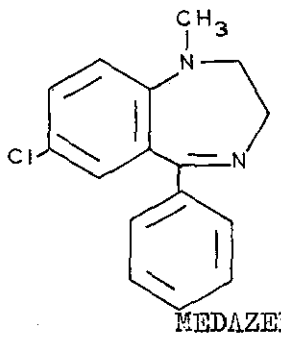
There are two basic types of 1, 4 - benzodiazepines derivatives in wide use. The two types are characterised by differences in the 2 - position of the benzodiazepine nucleus, and by the presence or absence of an N - oxide function in the 4 - position. The compounds studied are given in Table 23.

T A B L E 23

TYPE 1

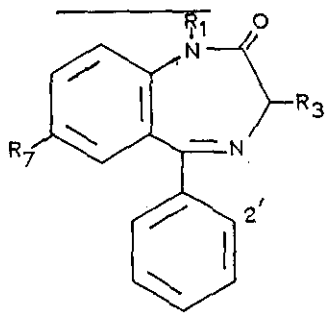


CHLORDIAZEPOXIDE



MEDAZEPAM

TYPE 2



| NAME | $R_1$ | $R_2^1$ | $R_3$ | $R_7$ |
|---|---|---|---|---|
| Oxazepam | H | - | OH | Cl |
| Diazepam | $CH_3$ | - | H | Cl |
| Flurazepam | $CH_2CH_2N(Et)_2$ | F | H | Cl |
| Nitrazepam | H | - | H | $NO_2$ |

The compounds studied were the generous gift of Hoffman La Roche Inc.

The luminescent properties of the 1, 4 - benzodiazepines in neutral, acid and basic solutions are given in Table 24. The effects of pH on the luminescent spectra were complex and with such a small number of compounds studied it would be difficult to state precisely the nature of the processes involved, however, some general conclusions can be drawn.

In those instances where phosphorescence is observed, the short phosphorescence lifetimes indicate an $N \rightarrow \pi^*$ transition occuring to populate the triplet level. Thus the non bonding electrons of the nitrogen atoms and the $\pi$ electrons of the phenyl rings are involved. Hence any changes likely to influence this interaction, such as change in pH, are manifested in the observed luminescence spectra.

T A B L E 24

| COMPOUND | CONDITIONS | $\lambda_e$ nm | $\lambda_f$ nm | $\lambda_p$ nm | ms $\tau$ |
|---|---|---|---|---|---|
| Chlordiazepoxide | Acid | (295) 365 | - | 485 | 102 |
| | Base | 310(320) | - | (450)470 | 164 |
| | Neutral | 360 | 435 | - | - |
| Medazepam | Acid | (318) 364 | 390-490 | 520 | 102 |
| | Base | (280) 370 | 450 | (450)495(530) | 142 |
| | Neutral | 385 | 450 | (450)495(530) | 140 |
| Oxazepam | Acid | (270)(312)370 | - | 490 | 88 |
| | Base | (285) 370 | - | 490 | 126 |
| | Neutral | (295) 335 | - | (445)(510)475 | 76 |
| Diazepam | Acid | (360) 380 | - | 480 | 94 |
| | Base | (290) 325 | - | (445)475(512) | 62 |
| | Neutral | 335 | - | (440)470(510) | 70 |
| Flurazepam | Acid | (320) 370 | - | 475 | 80 |
| | Base | (280)330(370) | - | (450)475(510) | 172 |
| | Neutral | (290) 325 | 370 | (435)470(500) | 56 |
| Nitrazepam | Acid | 378 | - | (525)555 | 310 |
| | Base | 395 | - | (515)535 | 320 |
| | Neutral | 363 | 435 | - | - |

In neutral ethanol chlordiazepoxide exhibits fluorescence only, the phosphorescence excitation and emission maxima are shifted in both acid and basic solution. The spectra of chlordiazepoxide are distinct from those of the other 1, 4 - benzodiazepines presumably the result of the N-oxide function at the 4 - position.

The luminescent spectra for nitrazepam are also distinct from the remaining benzodiazepines presumably due to the presence of the nitro group which is known to perturb singlet-triplet transitions.

In the remaining benzodiazepines certain similarities can be seen between the compounds. A typical phosphorescence spectrum is shown for oxazepam in neutral solution (Fig. 16), which is similar to the phosphorescence spectra obtained for medazepam, diazepam, and flurazepam in neutral and basic solutions. In acid solutions phosphorescence spectra similar to that of oxazepam (Fig. 17) are obtained for medazepam, diazepam, chlordiazepoxide, and flurazepam. This can be attributed to protonation of the basic nitrogen atom at the 4 - position. It is interesting to note that medazepam in acid solution forms a deep orange solution whilst oxazepam, diazepam, and flurazepam form pale yellow solutions on standing. Chlordiazepoxide exhibits no visible colour change in acidic or basic solutions and nitrazepam produces a deep yellow solution in basic media.

From the results it can be seen that the 1, 4 - benzo--diazepines exhibit complex luminescent spectra which are extremely sensitive to pH changes. However, such differentiation can be of advantage in that the selectivity of measurements can be increased. No direct quantitative measurements have been performed but preliminary results indicate that the 1,4-benzodiazepines could be determined at the submicrogram level.

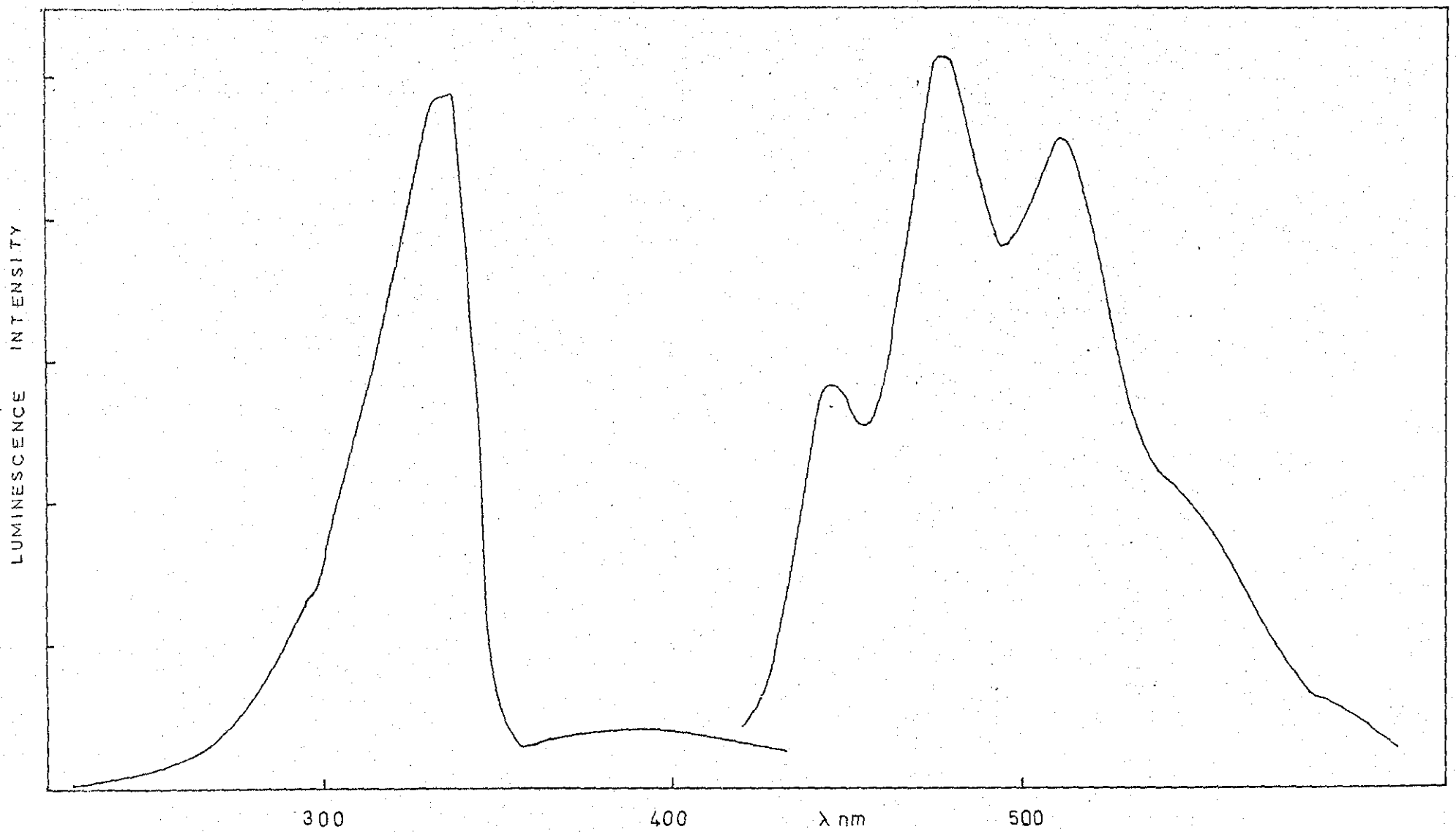



FIG 16. Luminescence spectra of oxazepam in EtOH at 77° K.

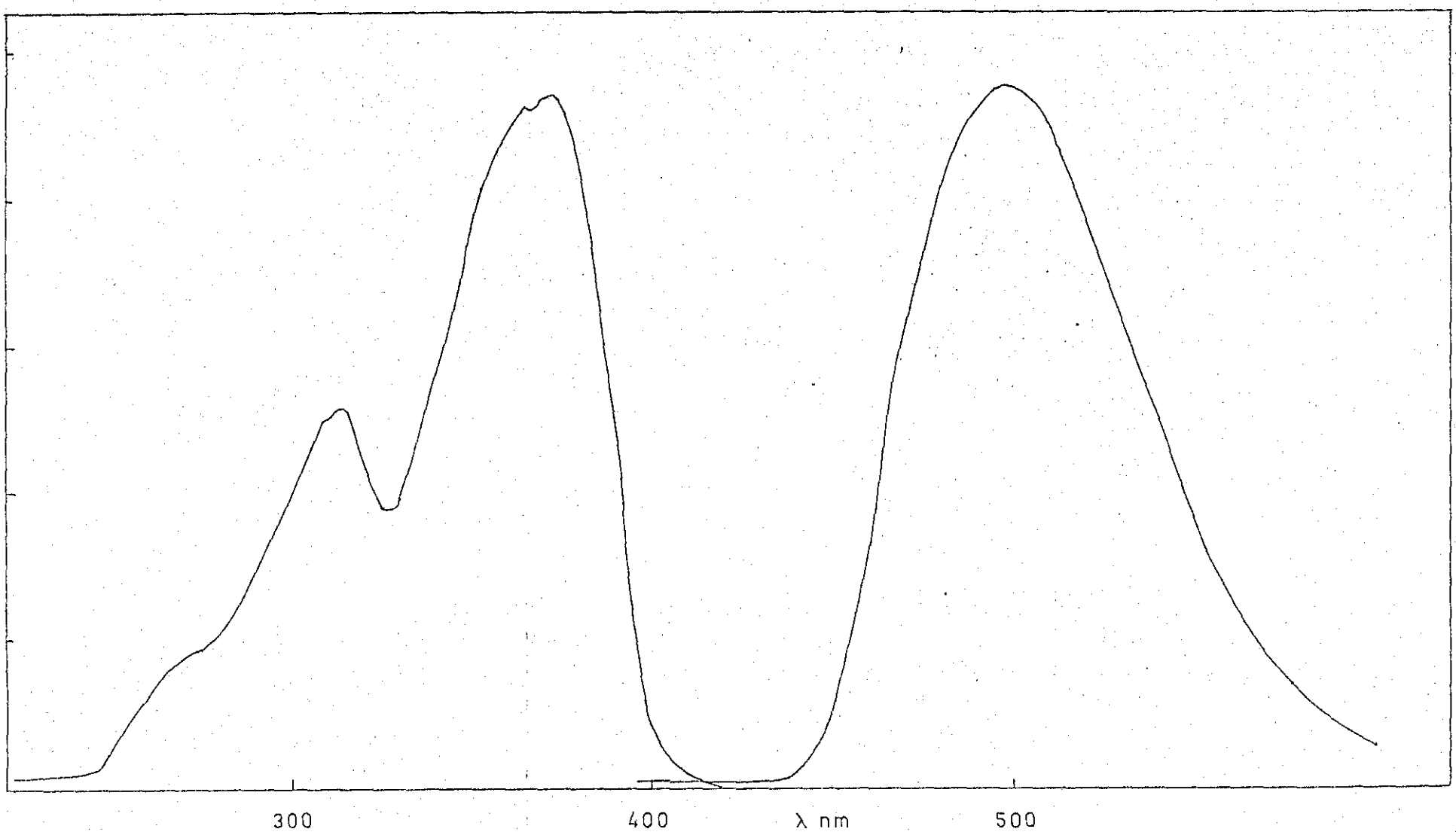



FIG 17. Luminescence spectra of oxazepam in EtOH-0.1N $H_2SO_4$ at 77°K.

# C H A P T E R 6.

## THIN LAYER PHOSPHORIMETRY.

Since the introduction of phosphorimetry as an analytical technique by Kiers, Britt and Wentworth[6] numerous workers have shown phosphorimetric measurements to be extremely sensitive and selective. The selectivity of the technique has been improved further by combination with thin layer chromatography. Applications of the combined technique have included the determination of p-nitrophenol in urine,[23] biphenyl in oranges[38] and alkaloids in tobacco.[41] In each case, however, the sample had to be eluted from the thin layer before measurement. Phosphorescence spectra at 77°K have been observed from materials adsorbed on a variety of supports including silica, alumina, paper, asbestos and glass fibres after immersion in liquid nitrogen in the conventional Dewar flask.[71-74] It was the intention of the present work to develope a device capable of scanning thin layer chromatographic strips and measuring qualitative and quantitative phosphorescence directly.

### i) THIN LAYER PHOSPHORIMETER.

The device developed consists essentially of two discrete parts, a sample holder and a single disc phosphoroscope, Fig.18. The sample holder is constructed from a hollow copper cylinder closed at its lower end. Fixed to the top of the drum is a canopy which acts as a light trap. The thin layer strip is attached to the lower end of the drum by means of elastic bands. The drum is then filled with liquid nitrogen and the sample is cooled by conduction. The copper drum is then inserted into an air-tight box, the function of which is to prevent condensation of

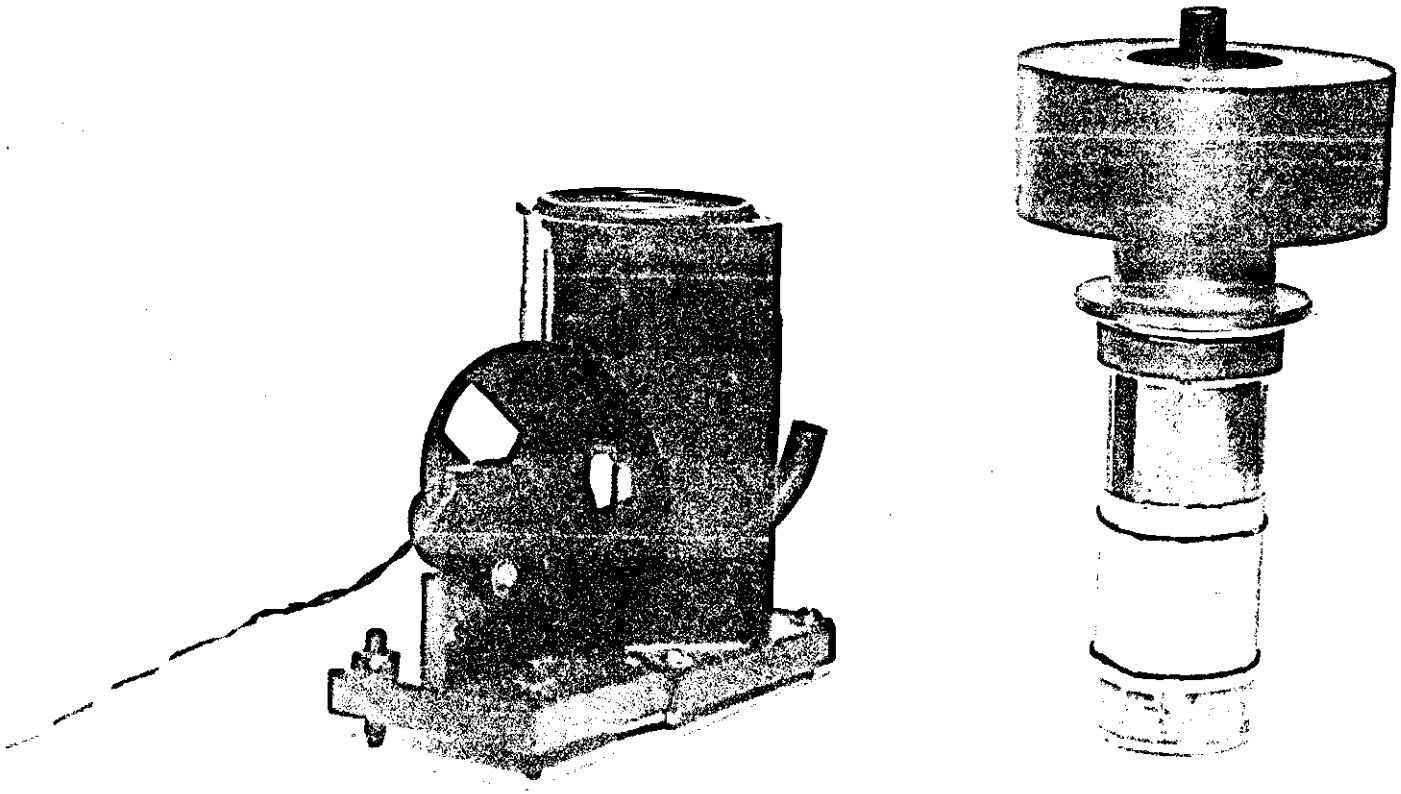


FIG 18. Thin layer phosphorimeter.

water vapour on to the surface of the sample. Two silica windows set at right angles to each other in the front of the box allow the incident radiation to excite the sample and the emitted radiation to pass to the photodetector.

The single disc phosphoroscope[75-78] allows the isolated observation of phosphorescence on the basis of lifetime. A disc with three rectangular slots equally spaced and rotated by means of a variable speed motor, is mounted in front of the two fixed slits in the sample holder compartment. Excitation radiation passes through the rotating disc and fixed excitation slit to irradiate the sample. As the excitation radiation is cut off by the disc, the fluorescence decays rapidly and only the long lived phosphorescence remains. The phosphorescence is then allowed to reach the emission monochromator and photodetector as the slot in the rotating disc opens in front of the emission slit. A less important feature of the phosphoroscope results in the minimal measurement of scattered incident light.

The sample drum is rotated by means of a small variable speed motor mounted outside the light-tight compartment. When a luminescent component on the strip is at the focal point of the optical system a signal is registered in the photodetector circuit. The drum can be stopped at any position and the spectral characteristics of each component measured; the position of the drum is a measure of the $R_f$ value of the component. The amplitude of the signal can be used as a quantitative estimation of the luminescent component. Phosphorescent lifetimes can be measured from the rate of decay of the luminescent signal after cutting off the excitating radiation.

ii) INFLUENCE OF PHOSPHOROSCOPE DESIGN ON PHOSPHORESCENCE INTENSITY.

O'Haver and Winefordner[79] derived a relationship between phosphoroscope design and measured phosphorescence intensity for the conventional rotating cylinder and Becquerel phosphoroscopes. The ability of the phosphoroscope to resolve short and long lived emissions is directly related to three time periods which are a function of phosphoroscope design and speed of rotation. The time periods being;

i) the cycle time, $t_c$, which is the period of time for one complete cycle of excitation and observation.

ii) the exposure time, $t_e$, the time during which the sample is excited or observed, and $t_t$, the transit time, which is the time taken for $t_e$ to reach maximum value.

iii) the delay time, $t_d$, the time between the end of one excitation period and the beginning of the next observation time.

The magnitude of these time periods, relative to the lifetime, $\tau$, of the luminescent species, influences the measured radiant flux of luminescence.

The ratio of $P_p$, the average dc photocurrent observed using a phosphoroscope, to that which would be observed without the phosphoroscope if prompt fluorescence and scattered light were not to interfere, $P_c$, is given by $\alpha$.

$$\alpha = \frac{P_p}{P_c} = \frac{\tau}{t_c} \times \frac{e^{-t_D/\tau}\left[1 - e^{-t_E/\tau}\right]^2}{1 - e^{-t_c/\tau}}$$

It is convenient to calculate times relative to $t_c$ because such value are independent of the speed of rotation, $\theta$, of the phosphoroscope. Relative times are then denoted with a prime superscript. Intensities relative to the absolute

intonsity, $I_{P_0}$, under steady state conditions are also denoted with prime superscripts. Thus the above equation now becomes

$$\alpha' = \frac{P_D'}{P_o'} = \frac{\tau'_o - t_D'/\tau' \left[1 - e^{-t_E'/\tau'}\right]^2}{1 - e^{-1/\tau'}}$$

where

$$t_D' = \frac{t_D}{t_c} = \frac{t_d + t_t}{t_c}$$

$$t_E' = \frac{t_E}{t_c} = \frac{t_o + t_t}{t_c}$$

and

$$\tau' = \frac{\tau}{t_c}$$

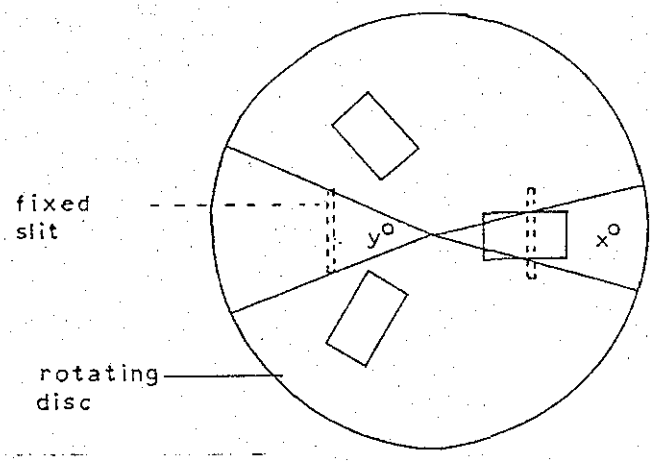
iii) CHARACTERISTICS OF THE SINGLE DISC PHOSPHOROSCOPE.

The dimensions of the slots in the rotating disc and the fixed slits in the cell holder are critical. The slot in the rotating disc is centred on the fixed slit in the excitation or emission system of the spectrofluorimeter (Fig.19 ) If the centre of the slot in the rotating disc and, similarly, the fixed slits in the cell holder subtend angles $x^\circ$ and $y^\circ$ then the dimensions of the disc can be related to the time functions in the equation for the determination of $\alpha'$.

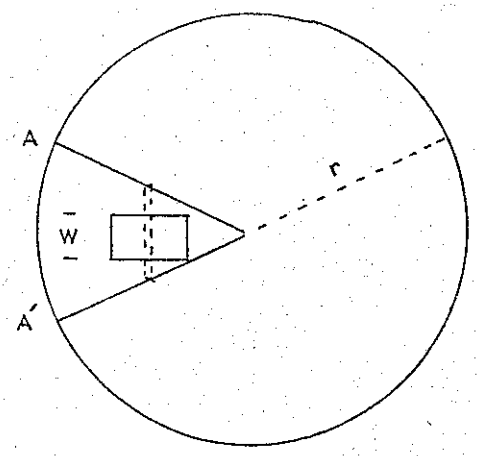
The velocity, v cm/s of a point on the edge of the rotating disc is given by

$$v = \frac{2\pi r\theta}{60} \text{ cm/s}$$

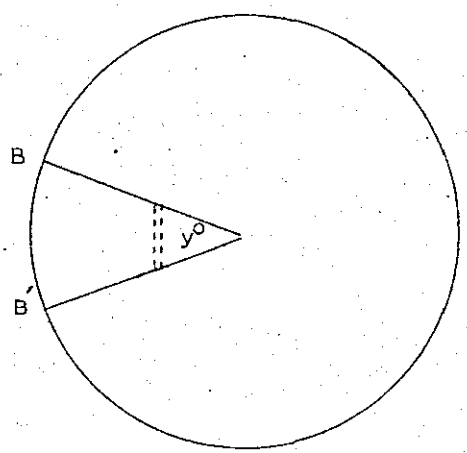
where $\theta$ is the speed of rotation in rpm and r is the radius in cms.



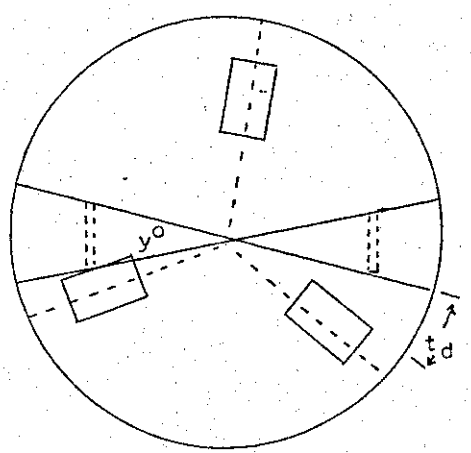

1. General arrangement of single disc phosphoroscope.




2. Determination of transit time $t_t$.




3. Determination of exposure time $t_e$.




4. Determination of dark time $t_d$.

FIG. 19. Schematic representation of time functions of single disc phosphoroscope.

TRANSIT TIME $t_t$. (For the case $y^o > x^o$)

The slit in the rotating disc is centred on the fixed slit in the excitation or emission system (Fig. 19). If the slit in the rotating disc subtends an angle $x^o$ at the centre, then a point, A, on the circumference will travel a distance A - A′ as the slit traverses its own width, W, across the fixed slit. The length of the arc A - A′ is $\frac{x^o}{360^o} \cdot 2\pi r$ cm

The transit time, $t_t$, is the time taken for the fixed slit to become completely illuminated. This is the time required for a fixed point A to move to A′, since it is the time taken for the trailing edge of the slot in the rotating disc to reach the top edge of the fixed slit. The transit time is thus

$$t_t = \frac{x^o}{360^o} \cdot 2\pi r \cdot \frac{60}{2\pi r\, e} = \frac{x}{6e}$$

EXPOSURE TIME $t_e$.

The exposure time is the time taken for the leading edge of the rotating disc to pass both top and bottom edges of the fixed slit (in which time a point, B, on the edge of the disc will have travelled a distance B - B′, (Fig. 19) minus the equivalent distance at the circumference of the width of the slit in the rotating disc (i.e., the transit time)

Thus

$$(B - B') - (A - A') = \frac{y^o}{360^o} \cdot 2\pi r - \frac{x^o \cdot 2\pi r}{360^o} \text{ cm}$$

$$\therefore \quad t_e = \frac{(y - x)}{6e} \text{ seconds.}$$

DARK TIME $t_d$.

The extreme edges of adjacent slits in the

rotating discs (Fig. 19) are separated by a distance

$$\frac{2\pi r}{3} + \frac{x^\circ}{360^\circ} \cdot 2\pi r \quad \text{cm}$$

$$= 2\pi r \left[\frac{120 + x}{360}\right] \quad \text{cm}$$

The centres of the two stationary slits are separated by a distance of $\pi r$ cms. The total dark time is that time between the trailing edge of one slit in the rotating disc leaving the lower edge of the stationary slit and the leading edge of the next slit in the rotating disc reaching the lower edge of the next fixed slit.

The distance between lower edges of the fixed slits is

$$\pi r - \frac{y^\circ}{360^\circ} 2\pi r$$

The total dark time is thus

$$t_d = \left[\left| \pi r - \frac{y^\circ}{360^\circ} 2\pi r \right| - 2\pi r \left| \frac{120 + x^\circ}{360^\circ} \right|\right] \cdot \frac{60}{2\pi r \theta}$$

$$\therefore \quad t_d = \frac{60 - (x + y)}{6\theta}$$

Thus, for phosphorescence alone to be observed then $x^\circ + y^\circ \leq 60^\circ$ since the maximum slit widths for the rotating shutter and fixed slits which will give $t_d = 0$ is given when $x^\circ + y^\circ = 60^\circ$.

CYCLE TIME $t_c$.

The cycle time for a rotating disc with three apertures is given by

$$t_c = \frac{\text{time for 1 complete revolution}}{3} = \frac{2\pi r/v}{3}$$

$$\therefore \quad t_c = \frac{2\pi r}{3} \cdot \frac{60}{2\pi r \theta} = \frac{20}{\theta}.$$

Thus for the case $y > x$ the times relative to $t_c$ are;

$$t_t' = \frac{x}{120} \qquad t_d' = \frac{60 - (x + y)}{120} \qquad t_D' = \frac{60 - y}{120}$$

$$t_c' = \frac{y - x}{120} \qquad t_E' = \frac{y}{120}$$

and for the case $x > y$.

$$t_t' = \frac{y}{120} \qquad t_d' = \frac{60 - (x + y)}{120} \qquad t_D' = \frac{60 - x}{120}$$

$$t_c' = \frac{x - y}{120} \qquad t_E' = \frac{x}{120}$$

In the single disc used in the present experiments $x^o > y^o$. The relative response of the phosphoroscope as a function of sample lifetime was calculated using the equation for $\alpha'$ and plots of $\alpha'$ against $\tau'$ for discs of different dimensions are shown in Fig. 20. In the $\alpha'$- $\tau'$ curve $\alpha'$ is proportional to the reading obtained by an integrating d.c. photometer. For values of $\tau' < 10$ and for a speed of rotation of 7,000 rpm $\tau = \tau' \times t_c = 200/7000 \simeq 0.03$ seconds and the luminescent intensity observed will be minimal. For values $\tau' > 100$ (i.e., $\tau > 0.3$ seconds) then the measured luminescent intensity is independent of speed of rotation of the phosphoroscope. Hence the response will only show variations with speed of rotation for samples with lifetimes in the range 0.3 - 0.03 seconds. However if the response is a function of shutter speed then the lifetime of the sample may be calculated from intensity readings taken at two different shutter speeds. For each shutter speed a value for $\tau'$ is obtained by dividing the unknown value of the sample by $t_c$ as determined for that shutter speed. Each of these values can then be substituted into the equation for $\alpha'$ together with the other relative times and the two resulting equations then solved simultaneously for $\tau$ .

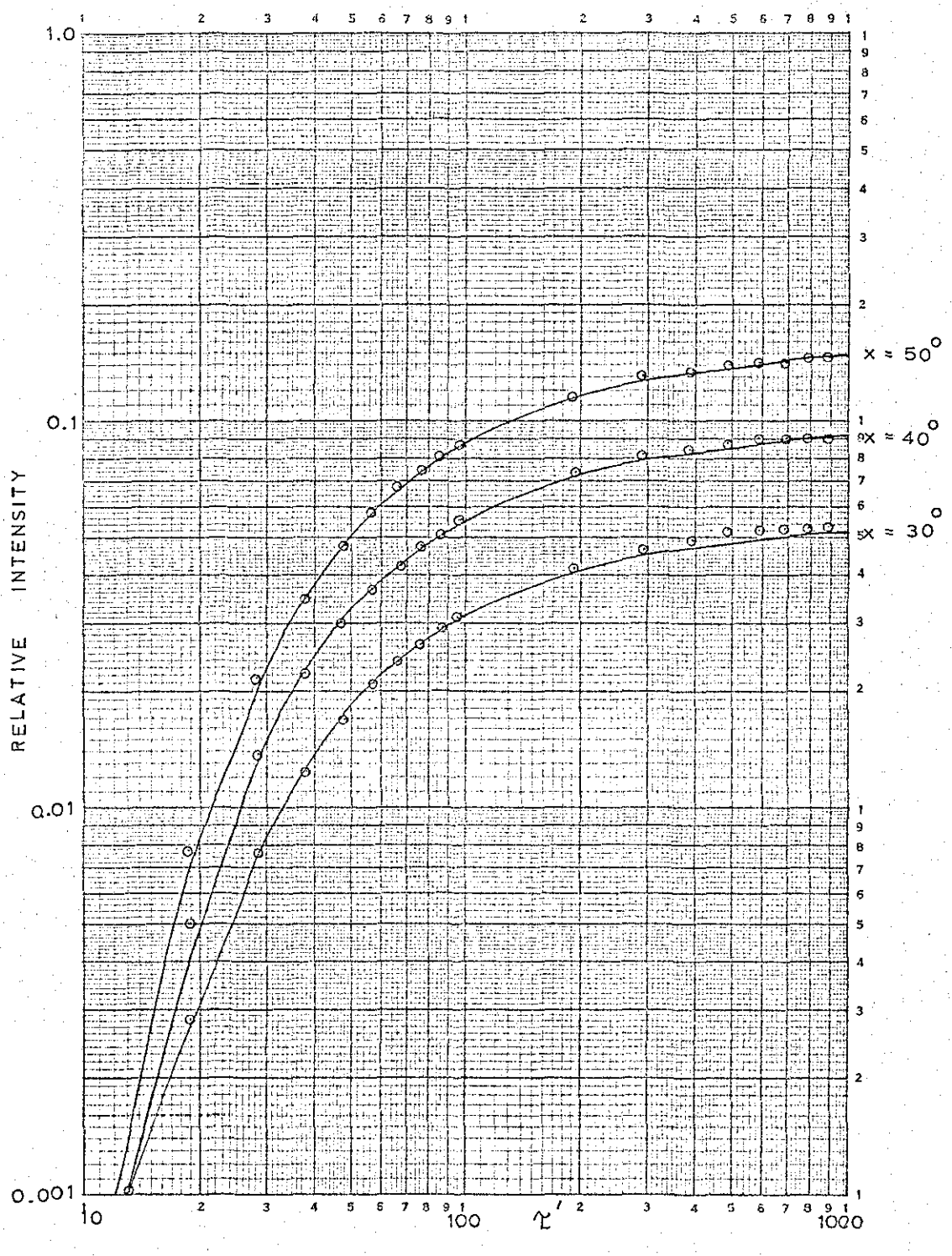



FIG 20. Relative intensity Vs lifetime

iv) QUALITATIVE AND QUANTITATIVE ANALYSIS.

Several materials in conventional use in thin layer chromatography were used in the study including paper, cellulose acetate and aluminium backed aluminium oxide and silica gel. All were found to allow the measurement of phosphorescence from adsorbed organic samples.

The background phosphorescence of the supports was found to vary being minimal for cellulose acetate. Aluminium oxide and silica gel layers were found to give a relatively high phosphorescent background which could be considerably reduced by washing in solvent prior to chromatography. Paper was found to have the highest phosphorescent background probably due to the presence of organic binders and whiteners in the fibres. However the luminescent spectra and lifetimes of samples obtained on these thin layers were very similar to those obtained in conventional solvents.[80]

Fig. 21 shows a chromatographic separation of three sulphonamides, sulphadiazine, sulphamerazine and sulphamethazine. 2 $\mu$l of ethanol containing 2$\mu$g of each of the sulphonamides was separated chromatographically using an aluminium backed silica gel plate in the solvent system[81] $CH\,Cl_3$ (30) / Me OH (12) / $NH_3$ (1). The plate was developed for one hour and the phosphorescence from the separated fractions recorded using the thin layer phosphorimeter. The luminescence characteristics and $R_f$ values for each spot are given in Table 25.

T A B L E 25.

| COMPOUND | $R_f$ | $\lambda e$ nm | $\lambda p$ nm | $\tau$ seconds. |
|---|---|---|---|---|
| Sulphadiazine | 0.41 | 290 | 420 | 0.7 |
| Sulphamerazine | 0.52 | 290 | 420 | 0.8 |
| Sulphamethazine | 0.68 | 290 | 420 | 0.8 |

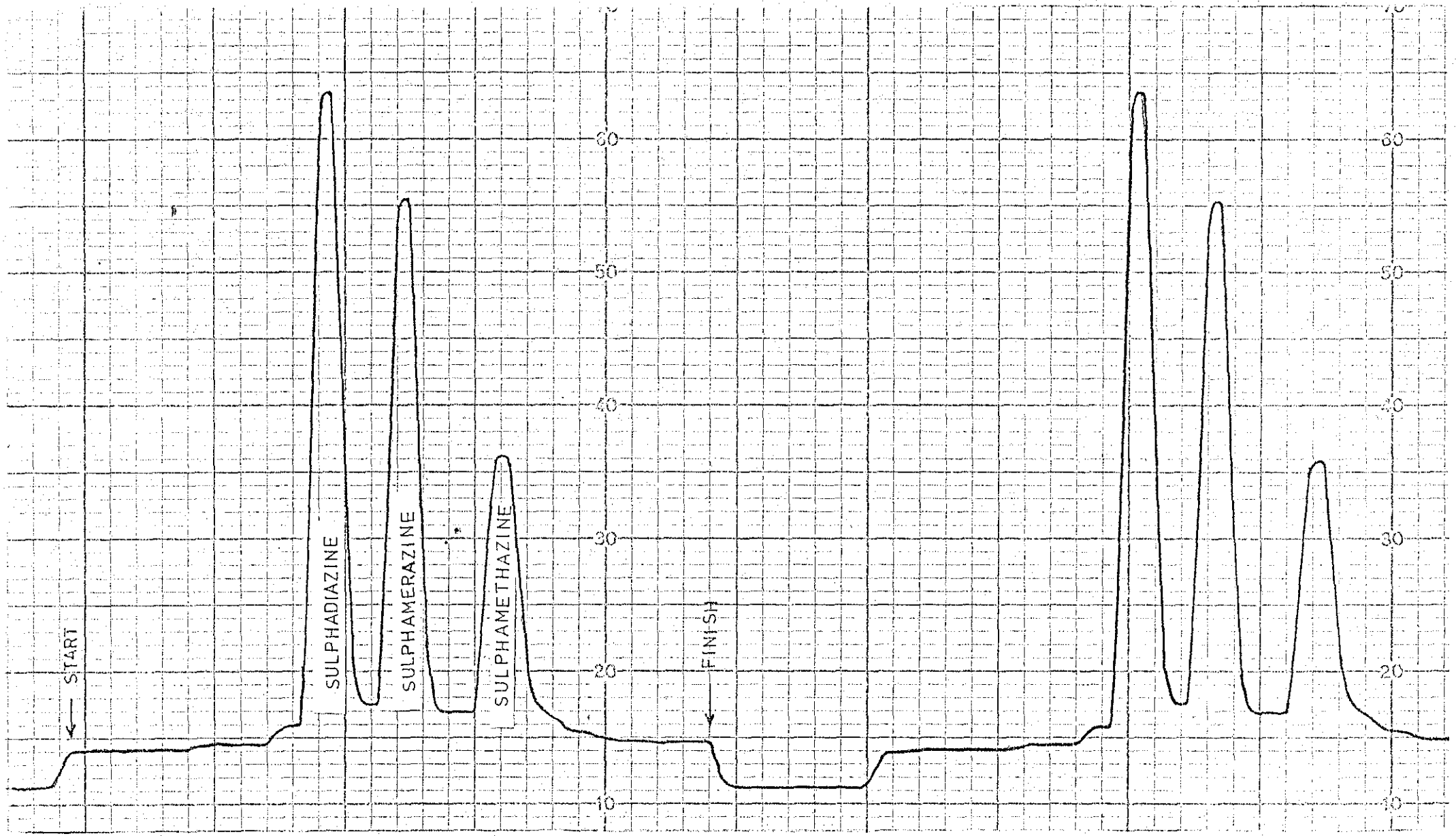



FIG 21. Typical thin layer chromatogram.

The reproducibility of the system is shown from the replicate scans in Fig. 21. The emission from a single spot measured thirty times over a period of one hour was found to give a coefficient of variance of 2 %. However, as with all chromatographic procedures, a series of identical samples measured after a chromatographic step gave coefficients of variance of 7 - 8 %. The application of samples in the form of rectangular zones rather than spots improved quantitation slightly. If the zones are scanned at right angles to the direction of developement then the combined functions of the rotating sample and phosphoroscope disc is similar to the system used in the flying spot technique of chromatographic quantitation.

The application of samples directly on to thin layer plates in order to produce calibration curves was found to be unsatisfactory. This was due to different degrees of spread of the sample on the thin layers and the variation of the baseline between samples, although cellulose acetate was found to give excellent baselines when used for this purpose.

v) FURTHER APPLICATIONS.

Electrophoretic separations can also be examined by this technique, enabling separations of complex mixtures such as plasma and drug-protein binding phenomenon to be studied. Multiple scans can be performed at different wavelengths of excitation and emission. Modification reactions can be performed on chromatographed zones and the amount of data available for the characterization of unknowns can thus be greatly increased.

The combined technique is considered to have great potential for use in pollution control, pharmaceutical and biological analysis and other fields where small quantities are to be determined in complex mixtures.

# C O N C L U S I O N S.

The thesis describes studies on the extent to which phosphorimetric methods are appropriate for the analysis of drugs, their metabolites and other anutrients. The luminescence properties of several classes of drugs have been studied and efforts have also been made to improve techniques used in phosphorimetric analysis.

In the study of the barbiturates it was shown that those barbiturates containing a 5,phenyl substituent were phosphorescent and that those substances could be determined at the submicrogram level. In other cases only prompt fluorescence was observed and its intensity was not substantially improved by working at 77°K. The complementary nature of low temperature and room temperature studies thus became evident. Structure luminescence correlations were studied and the nature of the luminescent species elucidated. A method for the fluorimetric determination of the barbiturates in biological fluids was also devised.

Whereas the barbiturates were only feebly luminescent the sulphonamides were found to have a more intense luminescence. Structure-luminescence studies permitted the prediction of those compounds which might usefully be determined by the fluorescence technique, and a fluorimetric method for the determination of some sulphonamides was devised.

In the general survey of the psychotropic drugs it was clear that many of these drugs could be estimated phosphorimetrically together with the catecholamines whose mode of action they potentiate. Structure-luminescence studies of these compounds indicate that the acridan nucleus is responsible for

the intrinsic luminescence in many of the tricyclic antidepressants and phenothiazines.

The thin layer phosphorimeter has the advantage over the conventional phosphorimeter that low concentrations of substances in complex mixtures can be determined in situ, and the system shows improved reproducibility compared with conventional phosphorimetry.

The utility of the phosphorimetric technique in the analysis of drugs and poisons has thus been validated.

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