Fluorescence-derivatisation of some pharmacologically significant amines

This item was submitted to Loughborough University’s Institutional Repository by the/an author.

Additional Information:


Metadata Record: [https://dspace.lboro.ac.uk/2134/27936](https://dspace.lboro.ac.uk/2134/27936)

Publisher: © Hwang Theng Kian

Rights: This work is made available according to the conditions of the Creative Commons Attribution-NonCommercial-NoDerivatives 2.5 Generic (CC BY-NC-ND 2.5) licence. Full details of this licence are available at: [http://creativecommons.org/licenses/by-nc-nd/2.5/](http://creativecommons.org/licenses/by-nc-nd/2.5/)

Please cite the published version.
This item was submitted to Loughborough University as a PhD thesis by the author and is made available in the Institutional Repository (https://dspace.lboro.ac.uk/) under the following Creative Commons Licence conditions.

For the full text of this licence, please go to: http://creativecommons.org/licenses/by-nc-nd/2.5/
<table>
<thead>
<tr>
<th>VOL. NO.</th>
<th>CLASS MARK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Accession/Copy No.:** 063282/02
- **Loan Copy:**
  - 1 Jul 1988
  - 24 Jul 1991
  - 4 Oct 1996
  - 13 Dec 1996

- **Acquisition Date:**
  - 26 Apr 1986
  - 15 Mar 1986

- **Returns Date:**
  - 2 Jun 1986
  - 21 Mar 1992
  - 13 Dec 1995
  - 6 Feb 1997
FLUORESCENCE DERIVATISATION OF
SOME PHARMACOLOGICALLY SIGNIFICANT AMINES

by
HWANG THENG KIAN, M.Sc.

A Doctoral Thesis

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

Supervisor: Dr J.N. Miller

External Supervisors:
Prof. D. Thorburn Burns,
Dept. of Analytical Chemistry,
Queen's University,
Belfast.

Dr. J.W. Bridges,
Dept. of Biochemistry,
University of Surrey.

© by HWANG THENG KIAN
This is to certify that neither this thesis, nor the original work contained therein has been submitted to this University or any other institution for a degree.

HWANG THENG KIAN
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. J.N. Miller, my external supervisors, Professor D. Thorburn Burns and Dr. J.W. Bridges for their help and guidance during my period of study. My thanks are also due to Dr. W.R. Bowman for discussions on the mechanisms of reactions.

I would also like to thank the technical staff, Mr. J. Swithenbank, Mr. J. Brennan, Mr. I. Katyal, Mr. A. Stevens, Mr. Maurice Coupe and Mr. A.F. Bower for their help and ingenuity.

My thanks are also due to the Department for providing facilities and also the Department of Health and Social Security for financial support for this work.

Last but not least I would also like to thank Mr. D. Phillips for the many fruitful discussions and talks on motor-bikes.
SYNOPSIS

Benzaldehydes and substituted benzaldehydes react with primary amines readily to give Schiff bases whose fluorescence properties have been examined. Only salicylaldehyde shows any potential as a fluorogenic reagent. Some derivatives of this aldehyde have been prepared and their relative fluorescence compared with quinine bisulphate.

By increasing the number of rings in the system the fluorescence of the derivatives might be increased. On this basis the ability of naphthaldehyde, hydroxynaphthaldehyde, anthraldehyde, phenanthrenealdehyde, indolealdehyde, fluorenealdehyde and pyrenealdehyde to generate fluorophores has been investigated. Primary amines used include both aromatic amines e.g. o-aminophenol and aliphatic amines e.g. benzylamine, cyclohexylamine and tyramine. The fluorescence properties of these derivatives have been critically examined. Of the aldehydes studied only a few could be justifiably used as fluorogenic reagents. These include phenanthrenealdehyde, fluorenealdehyde and pyrenealdehyde.

Pyrenealdehyde was chosen for detailed study as an example of a fluorogenic reagent. Some pure derivatives of pyrenealdehyde have been prepared and their relative fluorescence compared with quinine bisulphate. It has been found that some derivatives could be determined at levels as low as 0.1 µg/ml.

Various solvents have been used for the condensation reaction and the fluorescence of the derivatives examined in solution. The detection limits of some amines under these conditions was in the order of 0.01 µg/ml, the sensitivity being reduced considerably due to the fluorescence background of the aldehyde. The scale of the reaction was also reduced to a volume of 40 µl using a specially designed microrefluxing apparatus after which the products
were separated on a thin layer chromatographic (T.L.C.) plate.

In an effort to reduce the fluorescence background of the aldehyde, reactions on T.L.C. plates were attempted. 0.1 ng/spot of some amines could be detected. This method was found to be superior to the microreflux-T.L.C. technique.

Various pharmacologically significant amines have been extracted from biological material and detected after derivatisation on T.L.C. plates. Detection limits are also given.
To my Mother and Father
INDEX

Title Page
Declaration of Originality
Acknowledgement
Synopsis
Dedication
Index

I Introduction

1.1 Introduction to fluorescence

1.1.1 Electronic considerations

1.1.2 Delayed fluorescence

1.1.3 Quantum yield of fluorescence

1.1.4 Fluorescence intensity and concentration

1.1.5 Instrumentation and experimental considerations

1.1.6 Structure and luminescence

1.1.7 Environmental effects

1.1.8 Effect of solvent

1.1.9 Effect of pH

1.1.10 Effect of temperature

1.2 Some aspects of fluorimetry

1.3 Fluorimetry and T.L.C.

1.4 Reactions of Amines

1.4.1 Substitution reaction

1.4.2 Condensation reaction

1.4.3 Hydrolysis of Schiff bases
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>Determination of Amines</td>
<td>22</td>
</tr>
<tr>
<td>1.6</td>
<td>Functional Organic Fluorimetry</td>
<td>23</td>
</tr>
<tr>
<td>1.7</td>
<td>Extraction of drugs</td>
<td>29</td>
</tr>
<tr>
<td>II</td>
<td>Experimental, Results and Discussions</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Derivatives from benzaldehyde and substituted benzaldehydes</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Preparation and identification of Schiff bases</td>
<td></td>
</tr>
<tr>
<td>2.1.1</td>
<td>Purification of reagents</td>
<td>32</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Preparation of Schiff bases</td>
<td>32</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Identification of Schiff bases</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Preparation of sample solutions</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>Measurement of fluorescence</td>
<td>34</td>
</tr>
<tr>
<td>2.4</td>
<td>Relative fluorescence of aldehydes and derivatives</td>
<td>34</td>
</tr>
<tr>
<td>2.5</td>
<td>Discussion</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>o-Hydroxybenzaldehyde, fluorescamine and their derivatives</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Effect of pH on o-hydroxybenzaldehyde and its derivatives</td>
<td>38</td>
</tr>
<tr>
<td>3.2</td>
<td>Detection limit of o-hydroxybenzaldehyde and its derivatives</td>
<td>38</td>
</tr>
<tr>
<td>3.3</td>
<td>Qualitative study on the fluorescence of o-hydroxybenzaldehyde and its benzylamine derivative in methanol</td>
<td>39</td>
</tr>
</tbody>
</table>
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4 Effect of excess aldehyde on the reaction</td>
<td>39</td>
</tr>
<tr>
<td>3.5 To determine the optimum working pH for fluorescamine</td>
<td>39</td>
</tr>
<tr>
<td>3.6 Detection limit of tyramine using fluorescamine</td>
<td>40</td>
</tr>
<tr>
<td>3.7 Results and discussion</td>
<td>40</td>
</tr>
<tr>
<td>4 Relative fluorescence of some aldehydes and their derivatives.</td>
<td>43</td>
</tr>
<tr>
<td>4.1 Preparation and identification of Schiff bases</td>
<td>43</td>
</tr>
<tr>
<td>4.2 Fluorescence of derivatives</td>
<td>44</td>
</tr>
<tr>
<td>4.3 Results and discussion</td>
<td>44</td>
</tr>
<tr>
<td>5 Fluorescence of 2-fluorenealdehyde, 1-pyrenealdehyde and some of their derivatives in various solvents.</td>
<td>50</td>
</tr>
<tr>
<td>5.1 Relative fluorescence of 2-fluorenealdehyde, 1-pyrenealdehyde and their derivatives in various solvents</td>
<td>50</td>
</tr>
<tr>
<td>5.2 Detection limits of some derivatives in 2-fluorenealdehyde and 1-pyrenealdehyde in acidified ethanol</td>
<td>51</td>
</tr>
<tr>
<td>5.3 Discussion</td>
<td>52</td>
</tr>
<tr>
<td>6 Reactions at microlevels</td>
<td>56</td>
</tr>
<tr>
<td>6.1 Reactions at microlevels</td>
<td>56</td>
</tr>
<tr>
<td>6.2 Ethanol as solvent</td>
<td>56</td>
</tr>
<tr>
<td>6.3 1-Butanol as solvent</td>
<td>57</td>
</tr>
<tr>
<td>6.4 Effect of HCl on reaction</td>
<td>58</td>
</tr>
<tr>
<td>6.5 Detection limit of cyclohexylamine</td>
<td>58</td>
</tr>
<tr>
<td>6.6 Effect of excess 2-fluorenealdehyde</td>
<td>58</td>
</tr>
<tr>
<td>6.7 Results and discussion</td>
<td>58</td>
</tr>
</tbody>
</table>
Thin layer chromatography (T.L.C.) of 1-pyrenealdehyde and its derivatives

7.1 Materials for experiment
7.2 Experiments
7.3 Results and discussion

Reaction on T.L.C.

8.1 Effect of HCl and time on the reaction
8.2 Effect of excess aldehyde on the reaction
8.3 Extraction of histamine by XAD-2
8.4 Analysis of amphetamine in serum after deproteination
8.5 Emission spectra of some derivatives of 1-pyrenealdehyde
8.6 Detection limit of some amines
8.7 Results and discussion

Conclusion

References
CHAPTER 1

INTRODUCTION TO LUMINESCENCE

Luminescence is one of the oldest and theoretically well established analytical techniques. The effect having first been observed by Monardes in 1565 from an extract of Lingirium nephiticiem (5). Sir David Brewster noted the red emission from chlorophyll in 1833 and Sir G.G. Stokes described the mechanism of the absorption and emission process in 1852. Stokes also named fluorescence after the mineral fluor spar which exhibits a blue-white fluorescence.

Only in recent years has luminescence analysis become widely used, as analytical chemists have gradually become aware of the selectivity of the method and the high sensitivity obtainable in some instances. It is also only comparatively recently that recording spectrofluorimeters have become commercially available. With increasing requirements for the detailed examination of many chemicals for diverse purposes, luminescence analysis, like many other analytical procedures, has become more widely explored, both in fundamental studies and in the development of routine procedures.

The phenomenon of luminescence includes both fluorescence and phosphorescence. Their similarities and differences will become apparent in the discussion that follows.

1.1. Theory of Fluorescence

1.1.1 Electronic Considerations

When a molecule in the ground state is irradiated with light of the appropriate wavelength, the absorption of this energy results in the promotion of electrons from the ground
state to excited states. There can be several vibrational energy levels in the excited state depending on the nature of the molecule. When the electron returns to the vibrational levels of the ground state from the lowest vibrational level of the first excited singlet state, the energy released may be dissipated in one of several ways. It may be emitted as light, the process which is called fluorescence or radiation-less transitions may occur i.e. no fluorescence is observed. These radiationless transitions may involve conversions into rotational, vibrational or kinetic energy i.e. heat or chemical energy. Photodecomposition may also take place before the electron reaches the lowest vibrational level of the first excited state. Thus the transition from the excited states to the ground state may occur by a number of routes other than fluorescence emission. (see figure 1.1).

Thus molecules that have chromophores and hence can absorb ultra-violet light will have the potential for exhibiting fluorescence: however not all the absorbing compounds exhibit fluorescence, because of the possible radiationless transitions indicated above. (1-5). The electronic states of molecules fall into two categories: singlet and triplet states.

A molecule in an excited singlet state is a highly energetic species with a very short lifetime. Fluorescence usually occurs from the lowest vibrational level of the first excited state and the transitional half life is about $10^{-8}$ sec. It can be seen from figure 1.1 that the emission occurs at a longer wavelength than the absorption. (see section 1.1.8). The separation between absorption and emission spectra varies from one compound to the next. In some instances there is considerable separation, and in others, for example, anthracene, there is an overlap. Some molecules absorb ultra violet-visible radiation of a wavelength
Figure 1.1 Schematic representation of molecular energy levels and transitions. $S_0$, ground state; $S_1$, first excited state (singlet); $S_2$, second excited singlet; $T_1$, first triplet;
1, absorption; 2, vibrational relaxation; 3, internal conversion; 4, intersystem crossing;
5, fluorescence; 6, phosphorescence.
longer than the absorption maxima when in a vibrationally excited state of the ground electronic level. In such a case there is an overlap between the absorption and the emission spectra, referred to as the anti-Stokes Component of the resultant fluorescence emission spectrum. (see section 1.1.8).

Theoretically the fluorescence spectrum will be independent of the excitation wavelength used. Also the entire fluorescence spectrum will be obtained by excitation at any one wavelength, though with various intensities depending on the absorbance of the compound at the different excitation wavelengths used.

When electrons in the excited state migrate from the first excited singlet state to the triplet state and then returning to the ground state, phosphorescence results. The transition from the excited singlet to the triplet level is known as intersystem crossing and the transitional half life is between $10^{-2}$ and $10^{-8}$ sec.

The relatively long lifetime of a triplet allows ample time for the loss of triplet energy by non-radiative interaction with other molecules in the solution at room temperature. Consequently, most organic compounds exhibit phosphorescence only in solvent glasses at liquid nitrogen temperature. Furthermore, the phosphorescent level (triplet) is of a lower energy than the fluorescent level (singlet), and phosphorescence occurs at longer wavelength than fluorescence.

1.1.2 Delayed Fluorescence.

Delayed fluorescence differs from fluorescence and from phosphorescence. The effect has been observed in solutions at various temperatures down to liquid nitrogen temperature and in the vapour state. Delayed fluorescence has been observed by Parker and coworkers for eosin, proflavin, chlorophyll and a series of aromatic hydrocarbons (7-8).
There are several mechanisms proposed for delayed fluorescence. In some cases it involves interactions between two molecules in the triplet state.

One such proposal can be summarised as follows:

\[
\begin{align*}
F_0 & \xrightarrow{h\nu} F^* \quad (1) \\
F^* & \xrightarrow{} F^T \quad (2) \\
F^T + F^T & \xrightarrow{} F^* + F_0 \quad (3) \\
F^* & \xrightarrow{} F_0 + h\nu \quad (4)
\end{align*}
\]

where \( F_0 \) is the molecule in the ground state, \( F^* \) in the excited singlet state, and \( F^T \) in the triplet state. This mechanism accounts for the difference in lifetime between delayed fluorescence and phosphorescence and for the fact that delayed fluorescence occurs at the same wavelength as the normal fluorescence of the molecule.

Delayed fluorescence can occur in aromatic compounds in a variety of physical states by a number of mechanisms. Currently, this is a very active research area, and further clarification of the processes involved in delayed luminescence of organic molecules should be expected.

1.1.3 Quantum yield of Fluorescence

Every molecule possesses a characteristic property that is described by a number called the quantum yield, or quantum efficiency, \( \phi \). This is the energy emitted per quantum of energy absorbed:

\[
\phi = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} = \text{quantum yield}
\]

The higher the value of \( \phi \), the greater the luminescence of a compound. A non-luminescence molecule is one whose quantum efficiency is zero or so close to zero that the luminescence is not measurable. All the energy absorbed by such a molecule is rapidly lost by collisional deactivation.
1.1.4 **Fluorescence Intensity and Concentration**

The basic equation defining the relationship of fluorescence to concentration is:

\[ F = \phi I_0 (1 - e^{-\varepsilon bc}) \]  

where

- \( \phi \) = Quantum efficiency
- \( I_0 \) = Incident radiant power at wavelength of interest
- \( \varepsilon \) = Molar absorptivity
- \( b \) = path length of cell
- \( c \) = molar concentration

From the above equation it becomes apparent that apart from the concentration of the solution there are two other major factors besides \( \phi \) that affect the fluorescence intensity:

1. **The intensity of the incident radiation \( I_0 \):**
   In theory the more intense the source the greater will be the fluorescence but in practice a highly intense source can cause photodecomposition of the sample. Hence one compromises on a source of moderate intensity i.e. mercury or xenon lamp is used.

2. **The molar absorptivity of the sample \( \varepsilon \):**
   In order to emit radiation a molecule must first absorb radiation. Hence the higher the molar absorptivity, the greater will be the fluorescence intensity of the compound.

   It is for this reason that saturated non-aromatic compounds are non-fluorescent.

For very dilute solutions, equation 6 approximates Beer's Law, then

\[ F = K \phi I_0 e^{bc} \]  

At high concentrations self absorption becomes so great that fluorescence intensity decreases (10,11).
1.1.5 Instrumentation and Experimental Considerations

The instrumentation available for the measurement of luminescence has improved considerably over the past decade. Basically there are two main types of fluorimeters, namely the filter fluorimeters and the grating spectrofluorimeters of differing complexities. (see Figures 2 and 3). Instrumentation for fluorescence was reviewed recently (4,5).

In the measurement of luminescence spectra one has to take into account the energy from the source to the detector as well as resolutions and sensitivity of the instrument. As a result of these considerations the sensitivities achieved with various instruments should be compared only when all specifications are known i.e. source intensity, photomultipliers used, noise level and slit widths, since no two instruments are identical.

It is frequently desirable to have available a reference standard to check on the performance of a luminescence spectrophotometer. Quinine sulphate has most frequently been used. This reference standard is stable and once prepared can be used over an extended period of time.(11).

1.1.6 Structure and Luminescence

In order to utilise luminescence as an effective analytical tool it is necessary that the researcher know the basic effects of structure and the environment on the emission process. The researcher may be able to convert a non-fluorescent molecule into a fluorescent species in some cases and will in general have available a wide choice of possible solvent media in which to study the fluorescence of his samples.

To understand how molecular structure affects fluorescence it must be realised that fluorescence competes with a few processes mentioned earlier (see section 1.1.1).
Figure 12 Components of a Filter Fluorimeter

Figure 13 Schematic diagram of a Grating Spectrofluorimeter
<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\phi_F$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>EPA $^a$</td>
<td>0.16</td>
<td>17</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>EPA</td>
<td>0.38</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Ethyl alcohol</td>
<td>0.12</td>
<td>18</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>Ethyl alcohol</td>
<td>0.45</td>
<td>8</td>
</tr>
<tr>
<td>Fluorene</td>
<td>Ethyl alcohol</td>
<td>0.53</td>
<td>19</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>Hexane</td>
<td>0.23</td>
<td>3</td>
</tr>
<tr>
<td>Indole</td>
<td>Water</td>
<td>0.45</td>
<td>18</td>
</tr>
<tr>
<td>Anthracene</td>
<td>Benzene</td>
<td>0.29</td>
<td>18</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Ethyl alcohol</td>
<td>0.10</td>
<td>18</td>
</tr>
<tr>
<td>Triphenylene</td>
<td>Ethyl alcohol</td>
<td>0.065</td>
<td>20</td>
</tr>
<tr>
<td>Chrysene</td>
<td>Ethyl alcohol</td>
<td>0.17</td>
<td>21</td>
</tr>
<tr>
<td>Pyrene</td>
<td>Ethyl alcohol</td>
<td>0.53</td>
<td>20</td>
</tr>
<tr>
<td>Perylene</td>
<td>Benzene</td>
<td>0.99</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Ethyl alcohol</td>
<td>0.94</td>
<td>20</td>
</tr>
<tr>
<td>Coronene</td>
<td>Ethyl alcohol</td>
<td>0.21</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Ethyl ether-isopentane-ethyl alcohol (5:5:2) at 77°K.
Most unsubstituted aromatic compounds exhibit an intense fluorescence in the ultra-violet or visible region. As the degree of conjugation increases, the intensity of fluorescence often increases and a bathochromic shift is observed (15,16). For a given number of aromatic rings it is nearly always observed that linear ring systems fluoresce at longer wavelengths than non-linear systems. (see Table 1)

In addition to the requirement for a conjugated system of double bonds, certain geometrical considerations are also important. Thus planarity of the conjugated system is also essential for maximum fluorescence. When the planarity of a system is destroyed through steric hindrance, the free mobility of the π electrons will be partially inhibited resulting in a loss of fluorescence.

Molecular rigidity is also necessary for maximum fluorescence. In a non-rigid molecule the absorbed energy can be readily dissipated as heat in much the same way that a loosely connected vibrating structure tends to produce heat rather than work. This need for molecular rigidity probably explains the much greater fluorescence of cyclic compared with chain systems with equivalent fluorophors.

Comparison of the fluorescence efficiencies of biphenyl and fluorene provides a good illustration of the need for planarity. These compounds possess the same degree of conjugation, but the benzene rings in fluorene (I) are held rigidly in a planar configuration, whereas those of biphenyl (II) are not. This is reflected in their fluorescence efficiencies (Table 1) (22), although the fluorescence quantum yields were determined in different solvents.
The nature of substituted groups plays an important role in the nature and extent of a molecule's fluorescence. Fluorescence yields and electronic energies of aromatic and heterocyclic hydrocarbons are usually altered by ring substitutions. (2,3,4).

Groups that generally increase fluorescence are OH, OCH$_3$, NH$_2$, NCH$_3$, N(CH$_3$)$_2$ and those that tend to diminish fluorescence include groups like CHO, +NH$_3$, NHCCCH$_3$, COOH, Br, I and a host of others (24). The latter are electron withdrawing groups while the former are electron donating ones.

Intramolecular hydrogen bonding may profoundly affect fluorescence, although the effect is often unpredictable. For example, amide or carbonyl anions may be expected to hydrogen bond with phenolic hydroxy groups. In the case of salicylic acid, such bonding results in the enhancement of fluorescence, whereas in proteins it may quench fluorescence (25).

For most part of the present work, aldehydes would be used to prepare fluorescent Schiff bases. As such it would be of interest to note briefly the properties of some aromatic carboxylic acids. The electronic emission spectroscopy of aromatic ketones, aldehydes and carboxylic acids is quite different from that of most aromatic compounds. In most aromatic carboxylic acids the lowest energy
electronic transition is of \( \pi, \pi^* \) character, whereas that in most unsubstituted aromatics is \( \pi, \pi^* \). Most molecules having low-lying \( \pi, \pi^* \) singlet states usually do not fluoresce, but may exhibit phosphorescence. In fact, such aromatic carbonyls as benzophenone, acetophenone, and anthraquinone exhibit intense phosphorescence but no measurable fluorescence (26).

It should be pointed out at this juncture that the major part of this research work is on Schiff bases. It is thus advantageous to point out the feasibility of aromatic aldehydes as fluorogenic reagents. Schiff bases can be synthesised from carbonyls and amines and it is hoped that non-fluorescent amines could be rendered fluorescent by the process of derivatisation leaving behind an excess of, hopefully, non-fluorescent carbonyl. Aldehydes would thus appear ideal fluorogenic reagents for the assay of amines.

Unfortunately not all aldehydes are non-fluorescent. 1-Naphthaldehyde, 1-phenanthraldehyde show no fluorescence in hydrocarbon or hydroxylic solvents. However 2-naphthaldehyde, 9-anthraldehyde and 3-pyrenealdehyde are fluorescent in ethanol but non-fluorescent in heptane. 4-pyrenealdehyde and 9-tetracenealdehyde are fluorescent in both ethanol and heptane (27).

For 3-pyrenealdehyde, solvents such as alcohols could induce fluorescence because they lower the energy of the \( \pi^*, \pi^* \) state below that of \( \pi, \pi^* \) state. On the other hand solvents like chloroacetic acid could raise the \( \pi, \pi^* \) state above the \( \pi^*, \pi^* \) state, thereby also inducing fluorescence. Apparently phosphorescence occurs at low temperature in a hydrocarbon solvent and is assigned as originating from the lowest \( \pi^*, \pi^* \) triplet state (27).
Figure 1.4 Energy-level diagram showing the "blue shift" in fluorescence spectra at very low temperature.

From Hercules (4).
1.1.7 Environmental Effects

In addition to the structural effects various environmental conditions are also very important in determining the observed fluorescence of a compound. These factors can be summarised under the following headings namely solvent effects, pH effects, temperature and the influence of other solute molecules on fluorescence. (4,5,28).

1.1.8 Effect of Solvent

As in the case of ultraviolet absorption spectra solvent effects are observed in fluorescence measurements. These effects are manifested by wavelength shifts and/or changes in fluorescence emission intensity. The changes in fluorescence phenomena brought about by varying the solvent are in some instances accompanied by comparable changes in the ultraviolet-visible absorption spectra; in some instances, shifts in the position of the fluorescence emission maxima are not accompanied by comparable changes in the absorption spectra. Displacements of the absorption and the fluorescence emission spectra simply imply interaction with the solvent in both the ground state and excited state of the molecule. (see figure 1.4). On the other hand, when the fluorescence emission spectra alone are affected by change of solvent, interaction between the solvent and the excited state, but not the ground state of the molecule is indicated. The interpretation of solvent effects is often complicated because the observed changes may be the result of several quite different forces which may either augment or minimise each other. These effects have been discussed by various authors (26, 29-31).

The significance of hydrogen bonding in solvent effects has been stressed by other workers (32,33). The acridone molecule can function as donor or acceptor in hydrogen bond formation; hence, shifts in maxima and changes in intensity are possible in
various solvents, and this aspect has been studied extensively (34). In proton-donating solvents, such as alcohols and water, the fluorescence intensity is increased; in proton-accepting solvents, for example dioxane, ether and acetone, fluorescence intensity is decreased (34, 35).

Some obvious considerations apply to the choice of a solvent: the solvent should not absorb or fluoresce in the frequency of interest, and it should, of course, dissolve the solutes under study. In addition it is usually desirable to avoid solvent-solute pairs which exhibit pronounced hydrogen-bond tendencies. It is clear that fluorescence data obtained in one solvent should not be assumed to apply to another solvent. Despite a number of fundamental studies correlations between macroscopic properties of solvents and the fluorescence energy and efficiency of a given solute are often tenuous.

Solvents which tend to decompose (even very slightly) upon aging are best avoided in analytical work. The presence of trace impurities, from any source, in the solvent can also lead to anomalous results.

1.1.9 Effect of pH

Many compounds are capable of undergoing ionisation. Almost always the ionic form of a compound exhibits different fluorescent characteristics from those of the unionised one. The effect of pH upon the fluorescence of a compound is therefore of considerable importance, and a knowledge of the changes in fluorescence resulting from altering the pH of a solvent medium can be valuable in a number of ways. In many instances it constitutes the basis of assays for amines, phenols, carboxylic acids and heterocyclic bases.

Some acids like naphthol-sulphonic acids (36) and the amino and hydroxypyrene sulphonic acids (37) show remarkably sharp changes in fluorescence with changes in pH but the application has never become
widely accepted because it requires titrations to be carried out in the dark for visualisation under ultraviolet light. Other compounds which show sharp changes in fluorescence with pH are quinolines, benzquinolines and their derivatives (38), and 1-, and 2-naphthylamines. Forster (39) observed the fluorescence of these compounds over a range of pH values and found that the fluorescence of 2-naphthylamine remains unchanged from pH 2-9 with a maximum at 420nm, which is the same as the fluorescence of this compound in hexane i.e. the fluorescence is due to the neutral molecule. The occurrence of fluorescence of the neutral molecule in acid solution was ascribed to the loss of a proton from the excited naphthylumonium ion, as follows (39):

\[
[\text{RNH}_3^+] \xrightarrow{\text{hv}} [\text{RNH}_3^+]^* \xrightarrow{\text{hv}} [\text{RNH}_2]^* + \text{H}^+ \quad (8)
\]

\[
[\text{RNH}_2]^* \xrightarrow{\text{hv}} \text{RNH}_2 + \text{hv}^* \quad (9)
\]

If the fluorescence of a substance undergoes a sudden change at some specified pH, it is possible to utilise the compound as a fluorescent indicator for acidimetry and alkalimetry (41). Only compounds undergoing very rapid excited-state dissociation would be suitable for this purpose.

The use of fluorescent indicators has never become widespread, but the fluorimetric analysis of dissociable compounds has. Thus, the analytical chemist should be aware the changes in acidity which occur when an organic acid or base absorbs in the ultraviolet region. Since the fluorescent efficiencies of an acid and its conjugate base may be quite different, advantage may be taken of the dissociation to obtain the most strongly-fluorescent species for analytical use. For example, Thommes and Leininger (42) have utilised excited-state dissociation in the fluorimetric determination of mixtures of hydroxybenzoic acids.
1.2 Some Aspects of Fluorimetry

A large number of organic compounds are encountered in the environment and in materials of biological origin; still others are used in agriculture, in the pharmaceutical industry, and in other chemical industries (43). There is therefore a need for the analysis of these compounds from a variety of sources.

Molecular emission (fluorescence and phosphorescence) is a particularly important analytical technique because of its extreme sensitivity and good specificity. Fluorimetric methods can detect concentrations of substances as low as one part in ten billion, a sensitivity 1,000 times greater than that of most spectrophotometric methods. The main reason for this increased sensitivity is that in fluorescence the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy.

The specificity of fluorescence is the result of two main factors: (a) there are fewer fluorescent compounds than absorbing ones because all fluorescent compounds must necessarily absorb radiation, but not all compounds that absorb radiation emit; (b) two wavelengths, namely, excitation and emission, are used in fluorimetry, but only one in absorption spectrophotometry. Two compounds that absorb radiation at the same wavelength will probably not emit at the same wavelength. The difference between the excitation and emission peaks range from 10nm to 280nm.

Materials that possess native fluorescence, those that can be converted to fluorescent compounds and those that diminish the fluorescence of other compounds can all be determined quantitatively by fluorimetry.

As a result of its versatility fluorimetry has found wide applications in clinical and pharmaceutical analysis. In analysis of this nature, it is important that the drug be differentiated from
its metabolites some of which may be active intermediates and others the inactive detoxification products. This differentiation can frequently be accomplished by fluorescence analysis, since the excitation and emission spectra, together with environmental factors provide sensitive and structurally significant information. Usually however, some form of separation is required because this method is so sensitive that the analysis is frequently affected by fluorescence impurities from complex mixtures such as biological samples. Separation can be achieved by several methods such as solvent extraction or thin layer chromatography.

1.3 Fluorimetry and T.L.C.

One of the newer uses of fluorescence has been in the assay of substances directly on solid surfaces such as on paper chromatograms and on thin layer chromatographic (TLC) plates.

T.L.C. is a widely used separation technique and has been the subject of studies by many workers (44-47). Preparation, sample spotting and development of plates have been described in great detail in books by Kirchner (44) and Stahl (47). Of particular relevance to the present work are reactions on plates and their quantitation by fluorimetric means.

Miller and Kirchner (48) in 1953 originated and developed the idea of carrying out chemical reactions directly on thin-layer plates. Using this technique the sample can be spotted onto the plate and then covered with a reagent. After completion of the reaction, development in a suitable solvent separates the products of the reaction. In cases where this technique is not suitable the reagent and compound can be mixed on a microscale in a small test-tube or in capillaries as proposed by Mathis and Ourisson (49). The crude mixture can then be applied directly to the plate. The $R_f$ value of the original compound coupled with the chromatographic results of the reaction often are enough to positively identify a
compound, and in other cases the results can offer valuable clues
to the identity of the compound. As an example, a sample of
citral (an aldehyde in citrus) spotted on a silica gel plate and
covered by a drop of 30% hydrogen peroxide was then exposed to
ultraviolet light for ten minutes in order to catalyse the
oxidation to geranic acid. A second spot of citral was covered
by a drop of 10% solution of lithium aluminium hydride in ether in
order to reduce it to geranol. After chromatographing, the $R_f$
values of the two reaction products along with the $R_f$ value of
the original compound served to establish the identity of the
latter. (1).

In many cases the reactions on the T.L.C. plate do not go to
completion so that there is a mixture of the original compound and
the resulting reaction products. The mixture could be separated
and the products quantified. This could be achieved using
commercially available densitometers.

In the past, the fluorescence of a spot on a thin-layer plate
was obtained by removing the spot from the adsorbent by extraction
or elution and determining the various spectra of the dissolved
components of the spot. This operation is tedious and time-
consuming and the recovery is often not reproducible especially
when the compounds of interest are strongly bound to the adsorbent.
(50).

The direct spectrophotofluorimetric examination of the spots
on a chromatogram is one of the quickest and simplest methods for
identifying and estimating compounds present in complex test
mixtures in nanogram- to microgram amounts. (51). A great deal of
time is saved because a paper or plate can be examined immediately
after separation. Spectral differences between dry and wet spots
can be used for characterisation. In addition, the spectra of the
neutral compound, its salts, its reduced or oxidised forms, and its
derivatives can be obtained directly from the paper or thin-layer
chromatogram. Through the judicious use of various quencher-fluorimetric techniques, interferences can be reduced or eliminated, sensitivity increased and characterisation enhanced, especially when functional group analysis is desired. (52).

Dependent on the functional group of the test substance, a quencher can be used in alkaline or acidic solution to allow maximal use of its selective property. For example, the non-volatile quenchers, e.g. aniline, o-cresol, nitrobenzene and pyrrole are best used for the analysis of the 8-amino fluorenthene and morin types of compounds. With the addition of acid to the quencher the fluorescence of most aromatic amines would be quenched while many of the polynuclearaza heterocyclic compounds would become fluorescent in acidic aniline and nitrobenzene. This technique is useful in detecting air pollutants. (52).

Direct spectrophotometric analysis can be used to analyse not only compounds that are naturally fluorescent in a neutral, acidic or basic state but also those non-fluorescing compounds that can be made to fluoresce on paper or thin-layer chromatograms by reaction with appropriate reagents. (53). Furthermore, by comparison with standards run at the same time and by the use of appropriate formulae separated compounds can be estimated fluorimetrically on the plate. (51).

One possible disadvantage involved in the use of direct spectrofluorimetric analysis of chromatograms is that the excitation and emission spectra of the compound under investigation may be modified. Because the instrument or the adsorbent can modify the wavelength and relative intensity of the fluorescence excitation spectrum, this spectrum must be checked against the absorption spectrum of the pure known compound. The comparison is needed because the absorption spectrum, although less sensitive and selective, is the standard by which one can judge the reliability
of the fluorescence excitation spectra in solution and on adsorbent. This change in fluorescence spectra is easily overcome if all spectral comparisons of unknown and standard are made under the same conditions. (54).

In correcting spectra, one has to consider several factors e.g. characteristics of the source of irradiation, instrumental optics and the response of the photomultiplier to light of various wavelengths. Several ways of spectral correction have been worked out by various workers (14,19).

Another disadvantage is that direct analysis involves more technical skill in that the spots must be fairly well formed. Lack of well defined spots may hinder the assay. A further disadvantage in direct analysis is the increased interference from spectral scatter, the greatest interference being observed when the excitation and emission peaks are close together. This difficulty can be overcome by obtaining the excitation spectrum at a longer emission wavelength or the emission spectrum at a shorter excitation wavelength than the actual maxima. (55). In addition analysis of a plate must often be performed quickly because the separated compounds may be sensitive to air and light and may decompose if assay is delayed. In spite of these disadvantages it is considered that the spectrophotofluorimetric analysis of thin layer and paper chromatograms offer more potential for simplified rapid characterisation of complex mixtures than any other method presently available. (54).

1.4 Reactions of Amines

Some amines possess native fluorescence and can be detected conveniently by fluorimetry. However, several biologically important amines cannot but could be chemically induced to fluoresce. Although most of the present work is concerned with the fluorescence of synthesised Schiff bases it is of interest to note the various
other possibilities in which derivatives that might possess potential fluorescing abilities could be synthesised.

1.4.1 Substitution Reactions

Amines are powerful nucleophiles owing to the presence of a lone pair of electrons on the nitrogen.

All substitutions at this nitrogen atom result from nucleophilic attack by the amine on electrophilic centres in positively charged or neutral species. The general mechanism of substitution is a two-step process shown in equation 10 comprising formation (step a) and decomposition (step b) of a quarternary ammonium ion (I). This is a formal representation of an SN2 reaction.

\[
\begin{align*}
\text{RNH}_2 + E^+ & \rightarrow (a) \rightarrow R - \overset{\text{H}}{\overset{\text{N}}{\overset{\text{E}}{\overset{\text{H}}{\overset{\text{E}}{\text{I}}}}}} \rightarrow (b) \rightarrow R - \overset{\text{H}}{\overset{\text{N}}{\overset{\text{E}}{\text{I}}}} - E^+ \\
& \text{I} \quad 10
\end{align*}
\]

Primary and secondary amines react with many carbonyl compounds to form imines. Tertiary amines are generally unreactive although salt-like addition products have been isolated from reactions with acid chlorides. This acylation reaction leaves a product with a general formula RCONHR. It has been found empirically by Williams (24) that the NHCOR group tend to diminish the fluorescence of a compound. Formylation introduces a similar fluorescence quenching group.
1.4.2 **Condensation Reaction**

Apart from substitution reactions, amines undergo condensation reactions with ketones and aldehydes to give Schiff bases. Various reviews (138, 139) and papers (140, 141) have been published on this topic.

Perhaps the most common method for preparing imines is the reaction of aldehydes and ketones with amines. This reaction was first discovered by Schiff and imines are often referred to as Schiff bases. To simplify the mechanism, the condensation reaction is presented into a simple equation

\[
R'\text{CO} + H_2\text{NR}'' \xrightarrow{\text{OH} H} \left[ \begin{array}{c} \text{OH} \ H \\ R - C - N - R'' \end{array} \right] \\
I \\
\xrightarrow{\text{I}} \\
R' \ C = N - R'' + H_2O
\]

Cordes and Jencks (141) have presented convincing evidence that the formation and hydrolysis of these compounds proceed by a two-step mechanism involving a tetrahedral carbon addition intermediate. Hammett (142) proposed that acids protonate the carbonyl group to give a carbonium ion which adds to the amine in a very fast reaction. The rate determining step then is the deprotonation of this intermediate to give a carbinolamine I, an unstable intermediate which rapidly eliminates water to give the semicarbazones, the final product.

The rates of reaction of carbonyl compounds with nitrogen bases are dependent upon the structure of carbonyl compound, being affected by inductive, resonance and steric effects. It must be remembered that factors governing reactivity are distinct from those governing stability (143).
Both the rate and equilibrium constants for the addition of nitrogen bases to carbonyl compounds will be affected by inductive effects. Electron-withdrawing substituents will favour the addition by increasing the positive character of the carbonyl carbon atom, thereby making it more susceptible to nucleophilic attack. The opposite is also true; for instance, the para-substitution of benzaldehyde with electron-donating groups decreases the reaction rate. This agrees with both the Hammett and Jencks mechanisms.

Substituents capable of electron donation by resonance give a considerable amount of resonance stabilisation to the carbonyl compound. Such substituents cause smaller overall rate and equilibrium constants as well as smaller equilibrium constants for carbinolamine formation than would be predicted from Hammett $\sigma$ values. (142).

The rates of carbonyl addition reactions are strongly dependent on the steric requirements of the carbonyl compound. Bulky groups mean the reaction centre will usually stabilise the carbonyl compound relative to the carbinolamine, thereby decreasing the equilibrium constant for addition compound formation and giving smaller overall rates of product formation.

The thermodynamic stability of the $\text{C} = \text{N}$- linkage increases with the type of amine used in the order $\text{NH}_3 < \text{aliphatic amine} < \text{aromatic amine} < \text{amine containing an adjacent electronegative atom with a free electron pair}$. In contrast to the overall equilibrium constants, the rate and equilibrium constants for addition compound formation appear to be dependent on the basicity of the amine. In studies in which different amines have been reacted with the same carbonyl compound under the same conditions, the following observations have been made. The equilibrium constants for compound formation with p-chlorobenzaldehyde were found (144), to be 21.7, 9.11, 4.14 l/mole for hydroxylamine.
(pKa = 6.0), methoxyamine (pKa = 4.6), and semicarbazide (pKa = 3.6) respectively. Aniline (pKa = 4.6) and its ring-substituted derivatives react more readily than semicarbazide with p-chlorobenzaldehyde. The less basic the amine the slower will be its reaction rate with any given carbonyl compound.

1.4.3 Hydrolysis of Schiff bases

The kinetics of the hydrolysis of Schiff bases derived from strongly basic amines such as aliphatic amines have been studied recently and the mechanism of the reaction has been successfully elucidated (145, 146).

For Schiff bases derived from aromatic amines in basic conditions (pH 9 to 14), the rate of hydrolysis is directly proportional to the concentration of the hydroxyl ions. This suggests that the imine carrying carbon atom of the neutral Schiff base is sufficiently positive to undergo attack by the hydroxyl ion, even without being protonated:

\[
\text{HO}^- \rightarrow \delta^+ \text{C} \equiv \delta^- \text{N} \equiv \text{R}
\]

In the strongly acidic region (Ho < 0), the rate decreases linearly with the acidity. The cationic intermediate decomposes by general base catalysis, as follows:

\[
\text{BH}^+ + \text{OH}^- \rightarrow \text{C} = \text{O} + \text{RNH}_2
\]

The hydrolysis of Schiff bases derived from aliphatic amines has been worked out by Cordes and Jencks (145). Above pH9, the rates of hydrolysis of these unsubstituted bases are independent of pH, and according to experimental findings, strongly suggests that this reaction involves attack of hydroxide ion on the protonated bases. The independence of pH in alkaline conditions
is because the concentrations of the hydroxide ion and the protonated Schiff base vary in an equal and opposite manner as the pH is changed. With decreasing pH the substrate becomes fully protonated so that this compensation no longer occurs and the decrease in the concentration of the hydroxide ion results in a decrease in that part of the observed rate which is due to this reaction. Thus, a decrease in rate is observed for those compounds for which the attack of water on the protonated Schiff base is slow, and an increase in rate is observed for those compounds for which the corresponding reaction is fast.

Under acidic conditions however, the rate of hydrolysis becomes pH-dependent. Schiff bases possessing an electron-withdrawing substituent exhibit either no change or an increase in hydrolysis rate with increasing acidity. On the other hand, Schiff bases possessing an electron-donating substituent exhibit decreased rates of hydrolysis with increasing acidity.

More work along the same lines has been done by various other workers. (159-162).

1.5 Determination of Amines

Amines are widely distributed in nature and are also of industrial and pharmaceutical interest. Practically all existing instrumental analytical methods have been employed in the analysis of amines. Pharmaceutical analysis of a wide variety of amines has been recently reviewed. (56) Spectroscopic techniques of ultraviolet and colorimetric methods (57-61), fluorimetric and phosphorimetric methods (1-6) have been used extensively in the analysis and quantitation of amines. Amines can also be determined by chromatographic methods such as gas chromatography (62-65) and thin layer chromatography. (66-76)

Most of the methods mentioned above suffer from interferences and limitations. Acid-base titrations may fail when bases in
addition to amines are present. Gas chromatography is limited to volatile amines or volatile derivatives. One of the most versatile methods met with in the analysis of drugs and amines is fluorimetry. There are basically two types of analysis involved namely measurements of active fluorescence of the amines or analysis by chemical reaction. This work has been extensively reviewed (5,6,77,78).

In the course of a broad investigation Udenfriend (79) found potential utility for fluorimetry as an analytical tool for both qualitative and quantitative application at the sub-microgram level, useful fluorescence was revealed by many drugs, which have not hitherto been reported to fluoresce. As a result of the intrinsic fluorescence of 5-hydroxytryptamine (5HT) various workers (80-85) have shown that this compound and related compounds could be detected at very low levels. Fluorescence of these compounds are usually observed in acid medium. Amines which are themselves not fluorescent could be made to fluoresce by reaction with suitable reagents.

1.6 Functional Organic Fluorimetry with particular reference to amines

As mentioned earlier not all organic compounds are fluorescent. Some can be rendered fluorescent by using their reactive functional groups present in the species. A reaction can be carried out so that the product formed is a fluorescent species. On this basis an enormous number of reagents have been found which could improve the determination of a wide variety of inherently non-fluorescent species.

7-Chloro-4-nitrobenzoxadiazole (NBD Chloride) which is non-fluorescent, reacts with amino acids and amines in the presence of potassium acetate to form highly fluorescent compounds. Methyamine, diethylamine, benzylamine and ammonia all give highly
non-fluorescent

\[
\text{NR3} \sim \text{HO HO}
\]

highly fluorescent

\[
\text{NR2} \sim \text{BH} + \text{HN R3}
\]

**Figure 1.5**

**Figure 1.6**

**Figure 1.7**
fluorescent derivatives. (86) (see figure 1.5)

Amino acids were shown to react with fluorescein-isothiocyanate (FITC) to form fluorescein-thiocarbamyl amino acid (PTC - amino acids) which could be converted to the thiohydantoin under acidic condition then separated and determined by thin layer chromatography. (87).

Various other fluorimetric methods have been described for nitrogenous bases. The reaction of primary aliphatic, and secondary and tertiary cyclic amines with 3-carboxy-7-hydroxycoumarin results in an increase in fluorescence, accompanied by a change in the excitation wavelength of the mixture, permitting the measurement of the reaction products even in the presence of an excess of fluorescent coumarin reagent (89). Aromatic amines and aromatic heterocycles do not react. (see figure 1.6)

The dye obtained by reacting primary and secondary alkylamines with 1,2 naphthoquinone-4 sulphonic acid is extractable into methylene chloride. (90) Reduction by potassium borohydride yields a fluorescent o-diphenol, allowing the fluorimetric estimation of these nitrogenous bases. (see figure 1.7)

Dombrowski and Pratt (91) described a sensitive fluorimetric method for the determination of primary aromatic amines. The procedure requires diazotisation of the amino group, followed by coupling with 2,6-diamino pyridine (DAP). The resulting azo dye is reacted with copper (II) sulphate to produce an intensely fluorescent derivative (λ ex 360nm, λ em 420nm). As little as 2 to 6 ng/ml of most aromatic primary amines could be determined.

A number of nitrosamines have been reduced to hydrazines and condensed with 9-anthraldehyde and 9-phenanthraldehyde to give highly fluorescent hydrazones which can be separated by thin layer chromatography and identified. (92) A similar reaction had previously been used by Muszik (93) on the determination of primary aromatic amines. The Schiff bases formed fluoresce at room
Primary aliphatic amines can be assayed by reaction with 9-isothiocyanatoacridine to yield the corresponding thiourea derivatives. These derivatives are highly fluorescent in base, allowing the assay of nanogram concentrations of amines (94). An improved procedure (95) was developed for the analysis of primary and secondary aliphatic amines based upon the fluorescence of a cyclised product derived from isothiourea derivatives of amines and 9-isothiocyanatoacridine (see figure 1.8). Initial isothiourea formation as well as its cyclisation was carried out in toluene, which resulted in the formation of highly fluorescent 2-alkylamino-1,3-thiazino acridine. The fluorescence of the derivatives was determined in solution as well as on T.L.C.

Pesez and Partos (96) described a fluorimetric method for primary and secondary amines based on their replacement of the sulphonic acid group in 1,2-naphthquinone-4-sulphonic acid; the resulting products, when treated with KBH₄ yielded a fluorescent material. (see figure 1.9)

Dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride) in acetone-water (3:1) saturated with sodium carbonate normally reacts quantitatively with primary and secondary amines, phenols, (97) and imidazoles (98), as well as with some alcohols e.g. choline (99), Guanidine and guanidine derivatives, aminopyrimidines and aminopurines will also react with dansyl chloride. (100) Ever since it was used to form fluorescent protein conjugates (101) dansyl chloride has been very widely used in the determination of amines. Several reviews have been published on the use of this reagent. (101-103). The reaction is outlined in figure 1.10.

Fluorescamine, 4-phenylspirol[furan-2(3H), 1'-phthalan]-3,3'-dione, is a fluorogenic reagent which reacts with primary aliphatic and aromatic amines to form a fluorophore of high intrinsic fluorescence. It has been widely used in a variety of
**Figure 1.8**

**Figure 1.9**
biophysical-chemical studies for the analysis of amino acids and peptides and in clinical toxicology for the rapid identification of drugs of abuse, such as amphetamine. In the light of its versatility and success it is beneficial to outline how this reagent was developed.

McCaman and Robins (104) introduced a fluorimetric assay for serum phenylalanine which is based on interaction with ninhydrin and peptides. Samejina et al (105, 106) found that phenylalanine was oxidised by ninhydrin to phenylacetaldehyde, which combined with additional ninhydrin and peptides or any other primary amine to yield highly fluorescent ternary products (I). Refer to Scheme I. This ternary product was elucidated by Weigle (107) et al who subsequently synthesised the novel reagent, fluorescamine (108).

It was also noted that 1-dimethylamino-2,4-diphenyl-1-butene-3,4-dione [2] reacts with primary amines to give fluorescent pyrrolinones of structure [3]. However, the use of this reagent is restricted to non-aqueous systems, since it is rapidly destroyed by hydrolysis. When [2] is subjected to alkaline hydrolysis it is converted to 2-hydroxy-2,4-diphenyl-3-(2H)-furanone [4]. Heating [4] in methanol afforded the methyl ether [5]. The methoxyfuranone [5] reacts rapidly with primary amines in non-aqueous solution to yield fluorescent pyrrolinones.

On this basis [3] was modified such that it still retained the structural features for fluorigenicity and also possessed a more reactive leaving group which was anticipated to react with primary amines to yield fluorophores, identical with those of the fluorigenic ninhydrin reaction.

Scheme II outlines the synthesis of the spirolactone [9], which is the improved form of [3]. Alkaline hydrolysis of 3-benzylidene-1,4-isochromanedione [6] gave o-(&-hydroxycinnamoyl) - benzoic acid [7]. Formylation of [8] with tris-(trimethylamino) methane in N,N-dimethylformamide
Figure 1-10

Scheme I (Cont.)
Scheme I

Scheme II
proceeded to the dimethylaminomethylene derivative [8] which upon acidification directly converted to [9].

As expected fluorescamine reacts with primary amines to yield intensely fluorescent substances of general structure [I].

Ever since the development of fluorescamine it has been used extensively in the determination of amino acids and related amines. (109-115). de Silva and Strojny (112) using this reagent could detect sulphonamides and other amines at nanogram levels. It has also been used for determining drugs containing the primary aromatic amino substituent in the presence of drugs containing primary aliphatic amino substituents. (111) Flunitrazepam and other nitro compounds in blood and plasma could be detected at 1-2ng/ml. (116). This was done by spraying fluorescamine onto a T.L.C. plate after separating the drug from its metabolites. Fluorimetric assay of proteins in the nanogram range and picomole range have also been described by Udenfriend et al (113-114).

Fluorescamine has also been used for fluorescence-labelling of antigens and in the course of this study Barger et al (114a) found that MDPF (2-methoxy-2, 4-diphenyl-3-(2H)-furanone) could also be used as a fluorescence label. In fact the conjugate formed had a higher fluorescence yield and more stable than fluorescamine.

All the reagents discussed so far are for analysis of amines in general. Some reagents however could be used for analysis of specific amines. Formaldehyde for instance reacts with tryptamine and after oxidation by H_2O_2 gives rise to fluorescent norharman (117). (see figure 1.11) Lisy and Gerstein (118) showed that the detection limit of tryptamine could be improved by reacting with benzaldehyde followed by acidification by bubbling dry HCl into a solution of the Schiff base to yield a highly fluorescent compound which could be determined down to the nanogram level. (see figure 1.12).
Figure 1:11

Tryptamine

\[ \text{HCHO} \rightarrow \text{Norharman} \]

\[ \text{H}_2\text{O}_2 \]

Figure 1:12

\[ \lambda_{\text{em}} \text{ 360nm} \]
Figure 1.15
Histamine and tyramine are among the few model aliphatic primary amines used in the present course of work. It is thus useful to be acquainted with some of the existing methods of their analyses.

A number of chemical methods have been developed for the determination of histamine and a detailed review of suitable reactions available in 1956 was presented by Code and McIntire (119). At that time, chemical techniques were confined to colorimetric procedures involving coupling of histamine to a diazotised aromatic amine or to dinitrofluorobenzene. These reactions have been superseded by using o-phthalaldehyde for the specific reaction with histamine to give a highly fluorescent product (120). The range of concentrations determinable is 0.005 to 0.5 µg/ml. (see figure 1.13)

Tyramine is assayed by the fluorescent derivatives (see figure 1.14), it forms with 1-nitroso-2-naphthol (128). Dopamine (3,4-dihydroxyphenethylamine) can be converted to the highly fluorescing dihydroxyindole by oxidation with iodine, followed by rearrangement (129). Dopa undergoes a similar reaction to give a product with identical fluorescence characteristics. (see figure 1.15) Obermann et al (130) described a method for determining dopamine in urine based on its reaction with dansyl chloride.

Spatz and Spatz (131) developed a fluorescence method for determination for a large number of amines. Ethylamine, cyclohexylamine, phenylethylamine and amphetamine could be detected at microgram level with p-dimethylamino-cinnamaldehyde. Histamine, serotonin, tryptamine and dopamine did not interfere.

Although amphetamine could be detected by gas chromatography (62) a more sensitive detection could be achieved using fluorescamine. (131,132) As little as 250 µg of amphetamine could
be detected in an extract of amphetamine containing urine after separation by TLC. \(131\). Amphetamine, mescaline, phenylethylamine, benzocaine and procaine could be separated on TLC and determined by the above procedure.

\(\alpha\)-Phthaldehyde, in the presence of 2-mercaptoethanol, has been described by Roth \(134\) as a reagent for the fluorimetric detection of \(\alpha\)-amino acids. However, Roth found that lysine and cysteine showed only about 5% of the fluorescence of other natural amino acids, and that peptides also showed greatly reduced fluorescence values. By increasing the concentration of 2-mercaptoethanol 10-fold and adding Brij (Pierce Chem. Co., Rockford, Ill.) to the reagent mixture, Benson and Hare \(135\) were able to overcome the shortcomings described by Roth. The \(\alpha\)-amino acids, peptides and proteins could then be detected in the picomole range. This method has been critically compared with fluorescamine and the ninhydrin methods. \(135\)

1.7 Extraction of Drugs

Although there now exists a wide variety of very sensitive methods to solve analytical problems they are often unable to differentiate drugs of interest from a variety of exogenous compounds from biological material. Analysts and toxicologists are thus faced with the problem of isolating and identifying drugs from these materials. The complexity of the problem depends upon both the method of identification employed and the biological matrix.

There is no single all-purpose method of isolating all the drugs now available and methods have to be devised or modified to suit the nature of the investigation. There are several standard methods of extraction available and these are extensively reviewed by Sunshine \(147\). In this study we shall only deal with the
extraction of amines.

Serum samples containing amines could be acidified with HCl and extracted with 10 volumes of ether. (147). After discarding the ethereal layer, the aqueous layer is rendered alkaline with concentrated NH₄OH and then extracted with 4 volumes of chloroform which is dried with Na₂SO₄ and then evaporated.

Shore (148) has shown that histamine could be selectively extracted with butanol after precipitation of the serum proteins. By an appropriate choice of solvents or by varying the pH other amines could be extracted e.g. histamine can be extracted by butanol at a high pH (148).

Amberlite XAD-2 has been successfully applied to the extraction of drugs from urine (149-154). Amberlite XAD-2 is a polymeric material adsorbent produced in the form of white insoluble beads (20-50 mesh) designed for use in columns or in batch operations for the adsorption of water soluble organic substances such as amines.

Using this resin, high recoveries of amines are obtainable. Emulsion problems can also be eliminated. Elution time is shorter because only a small volume of eluent is required. Furthermore, water-soluble drugs and metabolites which are not extractable by the usual organic solvents, and hence not detected routinely, are partially adsorbed (150-151) by the resin and may be recovered in part by the elution of the column with methanol. (152,153)

The application of XAD-2 resin as a general method for the extraction of drugs from other biological materials requires a preliminary purification step involving transfer of the drug into an aqueous solution suitable for passage through the resin columns. (155). This solution must be of sufficient dilution so that it possesses the proper flow characteristics, and in
addition it should contain only small amounts of added reagents in order that it be suitable for a general analysis of organic compounds.

Amberlite XAD-2 is now widely used for the extraction of drugs from biological materials. Klein et al (156) found that recovery of 1.0μg and 2.0μg of amphetamine from serum averaged 69% and 67% respectively. Up to 95% of morphine could be extracted using XAD-2 as indicated by Kullberg et al (157). This resin has also been successfully used in urine-screening for primary-amine drugs especially amphetamine. (158)
EXPERIMENTAL, RESULTS AND DISCUSSION

CHAPTER 2

2. Derivatives from benzaldehyde and Substituted benzaldehydes

2.1 Preparation and Identification of Schiff bases

2.1.1 Purification of reagents

Except for benzylamine and o-aminobenzoic acid all the amines had to be purified before preparation of their derivatives. Tyramine hydrochloride was dissolved in water and the free base precipitated out by 0.1N NaOH, then dried out. p-Anisidine was recrystallised from an ethanolic solution warmed with deactivated charcoal. The amine was recrystallised until a sharp melting point, 56.7°C was obtained. Aniline was redistilled under partial vacuum, and kept away from light until required. o-Hydroxybenzaldehyde was purified in a similar way. Except for dimethylaminobenzaldehyde all the other aldehydes had a wide range of melting points and had to be purified. In all cases the aldehydes were recrystallised from a mixture of ethanol and benzene until a sharp melting point was obtained.

2.1.2 Preparation of Schiff bases

The Schiff bases were prepared in the usual way (157, 158, 166).

Between ½ and 1 gm of the primary amine with a stoichiometric amount of the aldehyde were dissolved separately in 10ml benzene. The two solutions were mixed together and refluxed
for 1-2 hours in a 50ml round bottomed flask with a Dean and Stark attachment. The water from the condensation reaction was drained away and the solvent of the reaction partially removed. The flask was then cooled in a trough of ice for sufficient time to permit precipitation of the product. The time required for this was usually about 1/2 hours. The product was filtered through a Buchner funnel and washed with cold dry benzene. After recrystallisation from dry benzene, the product was dried in the oven at about 50°C. This process was repeated until a sharp melting point was obtained.

This method was repeated with different amines and aldehydes.

A different solvent had to be used for the reaction with tyramine and o-aminobenzoic acid which are only partially soluble in benzene. As before about 1/4 to 1/2 gm of the amine was dissolved in 10mls of ethanol. After dissolving a stoichiometric amount of the aldehyde in a similar volume of ethanol the mixture was refluxed in a 50ml round bottomed flask for 1-2 hours. The solvent was partially removed and the product allowed to precipitate by cooling the flask in ice for about 1/2 hours. The product was filtered then washed with cold ethanol and recrystallised with the same solvent until a sharp melting point was obtained.

2.1.3 Identification of Schiff bases

Apart from the melting point, nujol mulls of the amines, aldehydes and the products were made and infra red (I.R.) spectrum of the compounds were taken using Unicam SP 200G Infra Red Spectrophotometer.
2.2 Preparation of Sample Solutions

In each case aldehydes and the derivatives, accurately weighed, were made up to about 5.0 μg/ml in redistilled ethanol. Another sample was made up in ethanol saturated with dry HCl.

2,5-dihydroxybenzaldehyde was dissolved in ethanol and made up to about 5.0 μg/ml, accurately and diluted to 1.0 μg/ml, 0.2 μg/ml and 0.04 μg/ml.

At the same time, 1.0 μg/ml quinine bisulphate in 0.1N H₂SO₄ was prepared.

2.3 Measurement of Fluorescence

Fluorescence of the sample solutions was measured with a Baird Atomic Fluoricord SPF fitted with a 150 watt Xenon Arc lamp and the spectra recorded on Bryan Recorder 2000. The instrument was first calibrated for changes in wavelength and a calibration graph for quinine bisulphate made. This was achieved by measuring the fluorescence of the weakest solution followed by the stronger ones. A blank solution comprising of only 0.1N H₂SO₄ in tridistilled water was also measured. The sample solutions were all measured in a 1 x 1 cm silica cuvette.

Before measuring the fluorescence of the aldehydes and their derivatives, an ultra violet spectrum of each of the solutions was obtained using the Unicam S.P.8000. The same solution of about 5.0 μg/ml was then measured for its fluorescence as above.

2.4 Relative fluorescence of aldehydes and derivatives

Having determined quantitatively the fluorescence of the aldehydes and derivatives, they were then compared with the fluorescence of 2,4-dihydroxybenzaldehyde with the aid of the
**Sources of Reagents**

<table>
<thead>
<tr>
<th>Amines</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylamine</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>Tyramine Hydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>p-Anisidine</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>o-Aminobenzoic acid</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>Aniline</td>
<td>B.D.H.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aldehydes</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>o-Hydroxybenzaldehyde</td>
<td>Sigma</td>
</tr>
<tr>
<td>m-Hydroxybenzaldehyde</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>2,4-Dihydroxybenzaldehyde</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>2,5-Dihydroxybenzaldehyde</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>p-Dimethylaminobenzaldehyde</td>
<td>B.D.H.</td>
</tr>
</tbody>
</table>
Reaction

\[
\text{ArCHO} + R\text{NH}_2 \xrightarrow{\text{benzene, 1-2 Hrs}} \text{ArCH}=NR + H_2O
\]

\[
\text{ArCHO} + \text{Ar'}\text{NH}_2 \xrightarrow{\text{benzene/EtOH, 1-2 Hrs}} \text{ArCH}=\text{NAr'} + H_2O
\]

where,

\[
R = \begin{array}{c}
\text{CH}_2
\end{array}
\]

\[
\text{Ar} = \begin{array}{c}
\text{OH}
\end{array}
\]
Figure 2.1 Calibration graph for 2,5 dihydroxybenzyldehyde in ethanol; $\lambda_{ex}$ 372nm; $\lambda_{em}$ 480nm.
calibration graph. Results of the experiment can be seen in Table 2.1.

2.5 Discussion

Fluorimetry is a highly sensitive method of analysis and traces of fluorescent impurities could interfere with the analysis. Thus in the preparation of derivatives one has to remove as many impurities as possible. Commercial hydroxybenzaldehydes are only 95-98% pure. These impurities were effectively removed by recrystallisation from ethanolic solutions.

Schiff base formation is a straight-forward condensation process which could be acid or base catalysed (157-9) but in practice it has been found that the reaction proceeds best in the neutral condition. In fact the hydrochloride of tyramine had to be neutralised to the free base be removed to help increase the rate of reaction.

It was also found that by removing the water of condensation a better yield could be obtained. A shorter period of time for the reaction was achieved by using the Dean-Stark method of removing water. Since tyramine and o-aminobenzoic acid are only partially soluble in benzene the reaction had to be carried out using ethanol as the solvent. The water of condensation could not then be removed and the reaction was allowed to take an hour longer to ensure sufficient product being formed.

Schiff bases could be identified by observing the $\text{C} = \text{N}$- infra red stretching frequencies. Although n.m.r. and mass spectroscopy could confirm the molecular structure and in the latter case the molecular weight as well, the reaction carried out is a simple one thus by observing the change in the I.R. stretching frequencies from the $\text{C} = \text{O}$ to $\text{C} = \text{N}$- one could obtain sufficient evidence to confirm the formation of a Schiff base.
### TABLE 2.1

Fluorescence of some Schiff bases relative to 2,5 dihydroxybenzaldehyde

<table>
<thead>
<tr>
<th>Compound</th>
<th>m.p.</th>
<th>cm⁻¹</th>
<th>ex</th>
<th>em</th>
<th>Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>liq.</td>
<td>1700</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>tyramine</td>
<td>123-5°</td>
<td>1650</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>p-anisidine</td>
<td>64-5°</td>
<td>1630</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>o-hydroxybenzaldehyde</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liq.</td>
<td>1 liq.</td>
<td>1665</td>
<td>388</td>
<td>488</td>
<td>0.09</td>
</tr>
<tr>
<td>bentylamine</td>
<td>132-3°</td>
<td>1630</td>
<td>388</td>
<td>488</td>
<td>3.16 *</td>
</tr>
<tr>
<td>tyramine</td>
<td>132-3°</td>
<td>1330</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>o-aminobenzoic acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>262-4°</td>
<td>1620</td>
<td>400</td>
</tr>
<tr>
<td><strong>m-hydroxybenzaldehyde</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 4-6°</td>
<td>1680</td>
<td></td>
<td>none</td>
</tr>
<tr>
<td>benzylamine</td>
<td>147-8°</td>
<td>1660</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>p-anisidine</td>
<td>117-8°</td>
<td>1640</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>p-hydroxybenzaldehyde</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>117-8°</td>
<td>1680</td>
<td></td>
<td>none</td>
</tr>
<tr>
<td>benzylamine</td>
<td>195-9°</td>
<td>1645</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>aniline</td>
<td>180-1°</td>
<td>1630</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>2,4 dihydroxybenzaldehyde</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1670</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>372</td>
<td>480</td>
<td></td>
<td>0.50 *</td>
</tr>
<tr>
<td>benzylamine</td>
<td>173-5°</td>
<td>1650</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>173-5°</td>
<td>1640</td>
<td></td>
<td>none</td>
<td>0.00</td>
</tr>
</tbody>
</table>
**TABLE 2.1 - continued**

<table>
<thead>
<tr>
<th>Compound</th>
<th>m.p.</th>
<th>cm$^{-1}$</th>
<th>ex</th>
<th>em</th>
<th>Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5 dihydroxybenzaldehyde</td>
<td></td>
<td>1670</td>
<td>374</td>
<td>480</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>none</td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>benzylamine</td>
<td>1650</td>
<td>-</td>
<td>none</td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>184-5$^\circ$</td>
<td>1640</td>
<td>-</td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>p-dimethylaminebenzaldehyde</td>
<td>73-5$^\circ$</td>
<td>1670</td>
<td>354</td>
<td>380</td>
<td>0.89</td>
</tr>
<tr>
<td>aniline</td>
<td>1620</td>
<td>-</td>
<td>none</td>
<td>none</td>
<td>0.00</td>
</tr>
<tr>
<td>Quinine Bisulphate</td>
<td></td>
<td>350</td>
<td>450</td>
<td>7.40</td>
<td></td>
</tr>
</tbody>
</table>

*in EtOH/KOH.*
Fabian, Legrand and Poivier (167) published an extensive review of the infrared and Raman spectra of imines. They found that the stretching frequencies for $\text{Ar-CH} = \text{N-Alkyl}$ were between 1629-1656 cm$^{-1}$, while those of $\text{AR-CH} = \text{N-AR}$ appeared at a lower frequency between 1626-1637 cm$^{-1}$. The stretching frequency for $\geq \text{C} = \text{O}$ is around 1700-1720 cm$^{-1}$. (168)

The ethanol used for fluorescence measurements was redistilled to get rid of most of the impurities which might affect the analysis. The ultraviolet spectra of each of the compounds were recorded to find the various excitation wavelength to aid fluorescence measurement and also to give an estimate of the fluorescence yield which depends on the molar absorptivity of the compound (see equation 6).

Results show that very few of the aldehydes and derivatives are fluorescent. Benzaldehyde, $m$-hydroxybenzaldehyde, $p$-hydroxybenzaldehydes and the derivatives are not fluorescent. $o$-Hydroxybenzaldehyde is only slightly fluorescent in ethanol while 2,4 dihydroxybenzaldehyde is not fluorescent at all.

Most of the compounds are not affected by ethanol/HCl. However, some aldehydes are affected by ethanol/KOH in a most peculiar way. For instance $o$-hydroxybenzaldehyde is only very slightly fluorescent in ethanol but in alkaline ethanolic solution the fluorescence relative to 2,5 dihydroxybenzaldehyde increases from 0.09 to 3.16 or about forty times more fluorescent. Similarly 2,4 dihydroxybenzaldehyde fluoresce in the alkaline medium but not in neutral solution. However, in the case of 2,5 dihydroxybenzaldehyde, the fluorescence totally disappears in alkaline condition. (see Table 2.1)

It could well be that in alkaline condition the 2,4 dihydroxybenzaldehyde forms an anion having a resonance structure,
Scheme 2.1

(I) $\lambda_{em}^{480\text{nm}}$

Scheme 2.2

(II) $\lambda_{em}^{480\text{nm}}$
(I), shown in Scheme 2.1. This structure is stabilised by the 4-hydroxy group. The o-hydroxybenzaldehyde which also fluoresce at about the same wavelength presumably has the same structural configuration in alkaline medium. The anionic form of 2,5 dihydroxybenzaldehyde does not exist in a form shown in (I) because the 5 hydroxy group destabilised that configuration. Since the aldehydes fluoresce at the same wavelength a more plausible explanation would be to assume a structural similarity. In fact the latter can be represented by a resonance structure as in (II) (see Scheme 2.2). One could then see the structural similarity between (I) and (II).

The fluorescence could not be explained in terms of intramolecular hydrogen bonding for the simple reason that (I) could not stabilise that form of bonding although in structure (II) it is very likely because the electrons could be said to delocalise around the two rings. However, more spectroscopic work has to be done to come to a definite conclusion.

Infra red studies indicate that ethanol cannot be used as the solvent to determine the hydrogen bonding at \(3000 \text{ cm}^{-1}\) since by itself there is considerable bonding and in fact there is a very broad band over that region. This would mask any bond that would otherwise show up. A different solvent would then be required. This would mean that the fluorescence has to be observed in a solvent that could not hydrogen bond on its own e.g. \(\text{CH}_2\text{Cl}_2\) or \(\text{CCl}_4\). The fluorescence data can then be correlated with the infra red values.

Only a few derivatives have been used to compare the relative fluorescence with their corresponding aldehydes. Qualitative work indicated that some derivatives from sulphonamides were slightly fluorescent.
CHAPTER 3
O-HYDROXYBENZALDEHYDE, FLUORESCAMINE
AND THEIR DERIVATIVES

3.1 Effect of pH on o-hydroxybenzaldehyde, and its derivatives

Buffer solutions were prepared to give pH values ranging from pH 5-14.

Varying volumes of M/5 Na₂HPO₄ and M/10 citric acid solutions in tridistilled water were mixed to give pH 5-8, stepwise. A borate buffer was also prepared to give pH 9 and pH 10, and a phosphate buffer for pH 11 and pH 12. pH 13 and pH 14 were prepared by diluting NaOH solution to 0.01 N and 0.1 N respectively.

Appropriate amount of purified o-hydroxybenzaldehyde (see section 2.1.1) accurately weighed out was dissolved in tridistilled water and diluted to 10⁻⁵ M in the various buffers. o-Hydroxybenzylidenebenzylamine and o-hydroxybenzylidenetyramine were also made up to 10⁻⁵ M in the buffers.

The fluorescence of the solutions prepared was observed in the manner described in section 2.3.

3.2 Detection limit of o-hydroxybenzaldehyde and its derivatives

o-Hydroxybenzaldehyde was weighed out accurately and a 10⁻⁵ M solution in phosphate buffer pH 12 was made. This was diluted to 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹ M in the same buffer. The same procedure was repeated with the tyramine derivative.

The derivatives of sulphamethazine and sulphathiazole were prepared in the manner described in section 2.1.2. 10⁻² M of the sulphamethazine was made up in redistilled ethanol and diluted to 10⁻³, 10⁻⁴ and 10⁻⁵. A serial dilution was made for the derivative of sulphathiazole as well.

Blank from the various solvents were measured together with
the sample fluorescence.

3.3 Qualitative study on the fluorescence of o-hydroxybenzaldehyde and its benzylamine derivative in methanol

A $10^{-5}$ M concentration of the aldehyde and the benzylamine derivative were made in redistilled methanol. Fluorescence spectra of the derivative and the aldehyde were recorded at the excitation and emission wavelengths of the derivative. The spectrum of the solvent was also recorded at the stated wavelengths.

3.4 Effect of excess aldehyde on the reaction

Benzylamine was weighed out in a capillary tube and dissolved in redistilled ethanol to give a concentration of $2 \times 10^{-3}$ M. o-Hydroxybenzaldehyde was weighed out to give $1 \times 10^{-3}$ M. 1.0 ml of the amine was added to 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml respectively and the solutions made up to 10 ml in ethanol. Blanks were made at the same time consisting of 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml of the aldehyde diluted to 10.0 ml in ethanol. The fluorescence of the samples and blanks were measured at $\lambda_{\text{ex}} 370$ nm and $\lambda_{\text{em}} 450$ nm.

3.5 To determine the optimum working pH for fluorescamine

Buffer solutions as in section 3.1 were prepared. A 10.0 $\mu$g/ml of tyramine solution was made by dissolving the free base in tridistilled water. To 1.0 ml of the amine was added 0.5 ml of the buffer (pH 5 - 10). The solution was thoroughly mixed with a vortex mixer and 0.5 ml of fluorescamine solution was then transferred into the test tube with the amine. The concentration of the fluorescamine used was 30 mg/100 ml in dry acetone (128).
A blank was also prepared by the same process except that the amine was absent from the tridistilled water. The fluorescence was measured at $\lambda_{ex}$ 395 nm and $\lambda_{em}$ 475 nm.

3.6 Detection limit of tyramine using fluorescamine

1000 $\mu$g/ml of tyramine in tridistilled water was prepared and diluted to 0.01 $\mu$g/ml stepwise. As in the previous experiment 0.5 ml of buffer pH 9 was added to 1.0 ml of the amine followed by 0.5 ml of fluorescamine solution and the mixture thoroughly mixed with the aid of a vortex mixer. A blank was also prepared.

The fluorescence of the solutions was observed at $\lambda_{ex}$ 395 nm and $\lambda_{em}$ 475 nm.

The detection limit of the compound was taken to be the lowest reading that will give twice the standard deviation of the blank. (25).

3.7 Results and Discussion

Although not a highly fluorescent compound, preceding experiments showed that o-hydroxybenzaldehyde could be used as a fluorogenic reagent. The experiments designed here are attempts to illustrate this possibility.

Studies on the effect of pH on fluorescence by Bridges (3) and others (23, 24) have indicated the fluorescence intensity of some compounds are profoundly affected by changes in pH. (see section 1.1.8) In a similar way the fluorescence of the aldehyde increased sharply from pH 7 - 10 and remained on the plateau for pH greater than 10. (see figure 3.1) The pH profiles of the two other derivatives were the same which seem to indicate similar properties under those conditions. It should also be noted that there was a change in excitation and emission wavelength
from a shorter to a longer wavelength as the pH increased from pH 5 to pH 10.

It is rather unfortunate that the derivatives behave in a similar way to the aldehyde under those conditions. It does mean that the aldehyde cannot be used as a fluorogenic reagent here.

However there is also a very likely possibility that the derivatives might have hydrolysed in the process and what in effect was observed were the pH profiles of the aldehyde.

Figure 3.2 shows the working curve for the determination of the aldehyde and the tyramine derivatives. It is not surprising as the results of the above experiments have indicated that the detection limit of the aldehyde and the derivative should be in the same order. (see Table 3.1)

The aldehyde also forms fluorescent Schiff bases with some sulphonamides e.g. sulphathiazole and sulphas methazine. (see figure 3.3)

Under alkaline conditions o-hydroxybenzaldehyde and its derivatives behave in a similar manner thus if the aldehyde is to be used as a fluorogenic reagent at all a solvent has to be found that would affect the reagent and the derivatives in different ways. Figure 3.4 shows the effect of methanol on the fluorescence of the benzylamine derivative and the aldehyde. Although the concentrations of both the compounds were the same the fluorescence of the derivative was much greater than that of the aldehyde. A change in wavelengths of excitation and emission was observed as well, so methanol not only interacted with the excited state but also at the ground state. (see section 1.4.1)

It is quite probable that there is no intramolecular hydrogen bonding in the neutral condition in methanol but nevertheless the derivative was more fluorescent than the aldehyde.
### TABLE 3.1

Limit of detection of some amines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>ex, nm</th>
<th>em, nm</th>
<th>Limit of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Hydroxy-benzaldehyde</td>
<td>pH 12</td>
<td>380</td>
<td>497</td>
<td>0.001 ug/ml</td>
</tr>
<tr>
<td>Tyramine</td>
<td>pH 12</td>
<td>380</td>
<td>497</td>
<td>0.001 ug/ml</td>
</tr>
<tr>
<td>Sulphamethazine</td>
<td>EtOH</td>
<td>420</td>
<td>500</td>
<td>1.0 ug/ml</td>
</tr>
<tr>
<td>Sulphathiazole</td>
<td>EtOH</td>
<td>414</td>
<td>485</td>
<td>0.01 ug/ml</td>
</tr>
<tr>
<td>Tyramine a</td>
<td>pH 8</td>
<td>395</td>
<td>475</td>
<td>0.01 ug/ml</td>
</tr>
<tr>
<td>Dopamine b</td>
<td>pH 8</td>
<td>395</td>
<td>475</td>
<td>1 ug/ml</td>
</tr>
<tr>
<td>Dopa b</td>
<td>pH 8</td>
<td>395</td>
<td>475</td>
<td>1 ug/ml</td>
</tr>
<tr>
<td>Amphetamine b</td>
<td>pH 8</td>
<td>395</td>
<td>475</td>
<td>0.3 ug/ml</td>
</tr>
</tbody>
</table>

a - Tyramine as determined by fluorescamine

b - Data from deSilva and Strojny (ref 131) using fluorescamine
FIGURE 3.1: Effect of pH on fluorescence intensity of -
(a) o-hydroxybenzaldehyde
(b) o-hydroxybenzylidenebenzylamine
(c) o-hydroxybenzylidenetyrmine

Concentration of solutions were $10^{-5}$ M in each case. Measurements were taken at the same wavelengths of ex 380nm; em 497nm and band widths.
Figure 3.2 Fluorescence vs concentration curves for (a) o-hydroxybenzylaldehyde in phosphate buffer pH 12 and (b) its tyramine derivative in the same buffer. Fluorescence was measured at $\lambda_{ex}^{380\text{nm}}$; $\lambda_{em}^{497\text{nm}}$. 
Figure 3.3 Fluorescence vs concentration curve for (a) derivative from sulpha thiazole \( \lambda_{\text{ex}} 414\text{nm}; \lambda_{\text{em}} 485\text{nm} \).

(b) derivative from sulpha methazine
\( \lambda_{\text{ex}} 420\text{nm}; \lambda_{\text{em}} 500\text{nm} \).
FIGURE 3.4: Fluorescence spectra of the benzylamine derivative of o-hydroxybenzaldehyde, the aldehyde excited at the excitation and emission wavelength of the derivative, and the solvent, methanol.
**FIGURE 3.5:** Effect of excess aldehyde on the reaction with benzylamine

$\lambda_{ex} \ 370\text{nm}; \ \lambda_{em} \ 450\text{nm}$
FIGURE 3.6: Fluorescence of a benzylamine derivative in methanol.
FIGURE 3.7: Effect of pH on Fluorescamine.

Conc. of amine: 10 µg/ml

λex 395nm; λem 475nm
Figure 3.8 Detection limit of tyramine by fluorescamine method. Measurement was at $\lambda_{\text{ex}}395\text{nm}$; $\lambda_{\text{em}}475\text{nm}$.
Benzylamine also reacts with the aldehyde in ethanol. Results showed that excess of aldehyde did not increase the fluorescence intensity to any great extent. (see figure 3.5) A four-fold excess of the aldehyde only accounts for 30% increase in the fluorescence. Unlike the previous experiments the fluorescence observed here was stable over a period of one day. The fluorescence background as observed in figure 3.6 is low compared to that of figure 3.4 probably because of the high concentration of $2 \times 10^{-4}$ M aldehyde in the former case.

Under the same conditions aromatic amines like aniline and p-anisidine did not give any positive results thus the method is not only limited to primary aliphatic amines but the quantum efficiencies of the derivatives are rather low. This in itself is a serious drawback. In conclusion, o-hydroxybenzaldehyde does form fluorescent Schiff bases but is ineffective as a fluorogenic reagent.

Fluorescamine (see section 1.6) reacts with primary amines to give highly fluorescent conjugates. Experimental results indicate that the optimum working pH for fluorescence is about pH 8 which is in agreement with literature values (128). The limit of detection for tyramine was found to be 0.01 $\mu$g/ml which is about $10^{-7}$ M. Although not much better than that found with o-hydroxybenzaldehyde as the reagent, the fluorescence of the tyramine was measured in a large excess of fluorescamine, while that in the former was measured in the pure state. While fluorescamine is a very effective fluorogenic reagent it is not the intention here to extend the applications of the reagent. Figures 3.7, 3.8 show the working pH of fluorescamine and the detection limit of tyramine respectively.
4. Relative fluorescence of some aldehydes and their derivatives

Judging from the results of previous experiments with benzaldehyde, substituted benzaldehydes and their derivatives one comes to the conclusion that none of the aldehydes could possibly be used as reagents for preparing fluorescent Schiff bases. Another approach had to be sought to find a family of reagents which could produce fluorescent derivatives. One has to consider several factors when searching for potential reagents of this nature. For instance, structure has a profound effect on the fluorescence yield of a compound. (see Section 1.1.6) Thus by increasing the number of benzene rings on the aldehyde moiety one could effectively increase the fluorescence quantum yield. One also has to pay particular attention to the geometrical (3) arrangement and the effect of substituents (24) on the compound. The environmental effect is also an important factor that should not be ignored. (see Section 1.1.7)

In the experiments that follow a wide variety of aldehydes have been chosen. They range between two and four ring systems. All the aldehydes used are commercially available and are fairly cheap compared to some existing fluorogenic reagents. It must be borne in mind that the search for these aldehydes is by no means exhaustive but merely gives an indication of the potential of such a method.

4.1 Preparation and Identification of Schiff Bases

All the reagents here were purified in the manner described in section 2.1.1 except for 1-pyrenealdehyde. This aldehyde was thrice recrystallised from pet. ether.
<table>
<thead>
<tr>
<th>Amines</th>
<th>Formula</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylamine</td>
<td><img src="image" alt="Formula" /></td>
<td>B.D.H.</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td><img src="image" alt="Formula" /></td>
<td>B.D.H.</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td><img src="image" alt="Formula" /></td>
<td>B.D.H.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aldehydes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthaldehyde</td>
<td><img src="image" alt="Formula" /></td>
<td>Aldrich</td>
</tr>
<tr>
<td>2-Hydroxy-1-naphthaldehyde</td>
<td><img src="image" alt="Formula" /></td>
<td>Aldrich</td>
</tr>
<tr>
<td>3-Indolealdehyde</td>
<td><img src="image" alt="Formula" /></td>
<td>Sigma</td>
</tr>
</tbody>
</table>
9-Anthraldehyde

1-Phenanthraldehyde

2-Fluorene-aldehyde

1-Pyrene-aldehyde
The derivatives were prepared in the way described in section 2.1.2.

Infrared data were recorded for all compounds with Unicam SP 200G. Infrared Spectrophotometer.

4.2 Fluorescence of Derivatives

A stock solution of Quinine bisulphate was made by dissolving an accurately weighed amount of it in 0.1N H₂SO₄ in tridistilled water. The stock solution was then diluted to give 2.4 μg/ml, 1.0 μg/ml, 0.48 μg/ml and 0.10 μg/ml respectively.

In each case the aldehyde and the derivatives were made up to give accurately about 50 μg/ml in redistilled ethanol. This was diluted to about 5.0 μg/ml in ethanol, accurately made and another sample in ethanol saturated with dry HCl. In the case of derivatives obtained from 2-fluorenealdehyde and 1-pyrenealdehyde, the acidified solutions were further diluted to 0.5 μg/ml.

4.3 Results and Discussion

The preparation of the bases was straight-forward and was carried out exactly as in section 2.2. The derivatives were recrystallised from benzene or a mixture of benzene and ethanol until a sharp melting point was obtained. Infrared stretching frequencies of the carbonyl compounds and the imines were similar to those of benzaldehydes and their derivatives and also agree with literature values. (138-139)

An N.M.R. spectrum of 1-phenylethylamine derivative from 1-pyrenealdehyde showed a triplet (2H) at 3.2 τ, a triplet (2H) at 4.1 τ, a multiplet (5H) at 7.35 τ and another multiplet (9H) from 8.05 τ - 9.2 τ. This coupled with a stretching frequency at \( \sim 1635\text{cm}^{-1} \) confirms the imine structure.
A mass spectrum of benzylamine derivative from 1-pyrenealdehyde had $M^+$ of 319 which is the molecular weight of the derivative. Other important fragment ions appeared at 242, 229, 201, 159 and 91. (see figure 4.3)

Although N.M.R. and mass spectroscopy would further confirm the formation of the imine compound sufficient evidence can be obtained by referring to the infra-red stretching frequencies, since the formation of imines is a simple one step synthesis.

1-naphthaldehyde showed very little fluorescence of only 0.002 relative to quinine bisulphate. The excitation and emission wavelengths were 276nm and 345nm respectively and is very close to that of 2-naphthaldehyde having an excitation of 280nm and an emission at 332nm. Crowell and Varsel (163) showed that this aldehyde also had very low fluorescence and was little affected by the presence of acid. The excitation and emission wavelengths remained unchanged as found here. Very little fluorescence was observed with the derivative from an aromatic amine which hydrolysed in the acid medium. The derivative from benzylamine was non-fluorescent.

2-Hydroxynaphthaldehyde showed a little more fluorescence than the unsubstituted aldehyde. As before the fluorescence was unaffected by the presence of acid. The derivatives both from aliphatic and aromatic amine showed little fluorescence and these hydrolysed within minutes in the presence of HCl.

Although 3-indolealdehyde and 9-anthraldehyde were shown to be highly fluorescent the derivatives were virtually non-fluorescent except for the derivative p-aminophenol from 9-anthraldehyde. The emission wavelength of the latter is almost the same as that of the aldehyde. Like the derivatives so far described, these hydrolysed in the acid medium as well.
FIGURE 4.3  Mass spectrum of 1-pyrenealdimine benzylamine
It is interesting to note that the fluorescence of 9-anthraldehyde increased appreciably in the presence of acid. As found by Williams (24), the presence of the aldehyde group quenches the fluorescence of the compound but in acid medium the aldehyde breaks down to form an acetal thereby increasing the fluorescence. A similar observation was made on dihydroxybenzaldehydes but the increase in fluorescence was not appreciable. 9-Phenanthrenealdehyde fluoresces both in ethanol and in the acidified medium for the same reason. A large increase in fluorescence of the acetal was also observed.

Derivatives of 9-phenanthrenealdehyde did not fluoresce in ethanol but in the acid medium. The cyclohexylamine derivative although less fluorescent than the aldehyde emits at a longer wavelength. In fact the excitation of this derivative appeared at a longer wavelength than the emission of the aldehyde.

At this juncture it should be noted that studies on derivatives of the type:

\[ \text{H-N=NCH} \]
\[ \text{H-O} \]

\[ \text{(I)} \]

\[ \text{H-C=N-H} \]
\[ \text{O} \]

\[ \text{(II)} \]
have been made by Voss (104) and Muszik (93). They found that (I) and (II) were non-fluorescent at room temperature but fluoresce at low temperature which is not surprising. In fact is has been shown here that (I) is slightly fluorescent at room temperature.

Derivatives of 2-fluorenealdehyde showed no fluorescence in ethanol while that of 1-pyrenealdehyde showed little fluorescence compared to those in the acidic medium.

On acidification the benzylamine derivative of 2-fluorenealdehyde exhibited an intense fluorescence at \( \lambda_{em} \) 435nm. In the neutral condition it was virtually non-fluorescent. Results showed that the fluorescence of this derivative relative to quinine bisulphate which has almost the same excitation and emission wavelengths was 9.41. However the derivative from an aromatic amine like p-aminophenol showed little fluorescence. An explanation is given in a later chapter.

1-Pyrenealdehyde is fluorescent both in ethanol and acidified ethanol. In the latter it is about twice as fluorescent as the aldehyde in neutral ethanol. Since the excitation and emission wavelengths are different in both cases and also that the instrument is not corrected for optical anomalies, it is difficult to say whether there is any significant change in the fluorescence intensities. (see figure 4.2) However, from studies made by Bredereck et al (39) 3-pyrenealdehyde fluoresces strongly in polar solvents and very weakly in non-polar solvents. An explanation has already been given in section 1.1. The derivative from primary aliphatic amine, viz benzylamine, like the derivative from 2-fluorenealdehyde, also showed intense fluorescence. The emission wavelength observed was also at a much longer wavelength than the emission of the aldehyde in acidified ethanol. Although the derivative is only about five times more fluorescent than the
Figure 4. Fluorescence spectra of 9-anthraldehyde in ethanol. $\lambda_{ex}^{345\text{nm}}$; $\lambda_{em}^{403\text{nm}}$. No change was observed in acid medium.
Figure 42. Fluorescence spectra of 1-pyrenealdehyde.
(A) in ethanol; (B) in ethanol acidified with dry HCl.
aldehyde, it does not give a true picture of the real fluorescence intensities as one emits well below the emission of quinine bisulphate while the other, well above that of the fluorescence standard. The results only indicate roughly their relative fluorescence.

Derivatives of 2-fluorenealdehyde and 1-pyrenealdehyde from a primary aliphatic amine showed intense fluorescence in acidic ethanol, and did not lose appreciable fluorescence on standing for a few weeks. The derivative of 9-phenanthrenealdehyde from cyclohexylamine also showed stable fluorescence on prolonged standing. Derivatives of these three aldehydes from an aromatic amine showed little or no fluorescence at all. Table 4.1 shows that derivatives from 1-naphthaldehyde to 9-anthraldehyde are hydrolysed in acid while those from 9-phenanthreinmaldehyde to 1-pyrenealdehyde are stabilised.

An interesting observation is made on the fluorescence properties of 9-anthraldehyde and 9-phenanthraldehyde and their derivatives from primary aliphatic amines. The two aldehydes have identical molecular weights and similar structures except for the arrangements. 9-Anthraldehyde showed twice the fluorescence of the other. In acidified ethanol structure (II) (see figure 4.4) is non-fluorescent but structure (IV) (see figure 4.5) is reasonably fluorescent. Although the same number of resonance structures (eight in each case) can be drawn for structures (II) and (IV), the former is not stable in acid while the latter is stabilised in acidic ethanol. On closer scrutiny the instability of structure (II) becomes more apparent. In can be seen that there is considerable steric hindrance caused by the hydrogen of the N-H with the H at the 1-position. Thus the $> C-N$ group is forced out of plane with the anthracene moiety thereby losing its
fluorescence. In structure (IV) the hydrogen on the N-H is not hindered and there is more molecular orbital overlap, thus the molecule retains its planarity which is an essential feature in fluorescence properties of organic compounds. This may explain why (IV) is fluorescent and (II) is not.
(I) $\lambda_{em}^{403\text{nm}}$
Rel.Fl. 0.29

(II) non-fluorescent

(III) $\lambda_{em}^{364\text{nm}}$
Rel.Fl. 0.11

(IV) $\lambda_{em}^{495\text{nm}}$
Fluorescent

$R = \text{aliphatic primary amine}$
### TABLE 4.1

Relative fluorescence of Schiff bases measured in ethanol and ethanol acidified with dry HCl

<table>
<thead>
<tr>
<th>Compound</th>
<th>m.p. (°C)</th>
<th>I.R. ( \text{cm}^{-1} )</th>
<th>Ethanol ( \lambda_{ex} - \lambda_{em} )</th>
<th>Ethanol-HCl ( \lambda_{ex} - \lambda_{em} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-naphthaldehyde</td>
<td>liq.</td>
<td>1700</td>
<td>276 - 345, 0.002</td>
<td>276 - 345, 0.002</td>
</tr>
<tr>
<td>benzylamine</td>
<td>51-2°</td>
<td>1645</td>
<td>-</td>
<td>unstable</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>283-4°</td>
<td>1615</td>
<td>280 - 375, 0.001</td>
<td>unstable</td>
</tr>
<tr>
<td>2-hydroxy-1-naphthaldehyde</td>
<td>80-81°</td>
<td>1650</td>
<td>367 - 443, 0.012</td>
<td>367 - 443, 0.010</td>
</tr>
<tr>
<td>benzylamine</td>
<td>98-9°</td>
<td>1625</td>
<td>391 - 451, 0.009</td>
<td>unstable</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>228-30°</td>
<td>1625</td>
<td>467 - 493, 0.014</td>
<td>unstable</td>
</tr>
<tr>
<td>3-Indole-1-aldehyde</td>
<td>192-4°</td>
<td>1640</td>
<td>357 - 441, 0.110</td>
<td>357 - 441, 0.100</td>
</tr>
<tr>
<td>benzylamine</td>
<td>123-4°</td>
<td>1630</td>
<td>430 - 497, 0.001</td>
<td>unstable</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>275-7°</td>
<td>1660</td>
<td>430 - 495, 0.001</td>
<td>unstable</td>
</tr>
<tr>
<td>Compound</td>
<td>I.R. m.p. (°C)</td>
<td>I.R. cm⁻¹</td>
<td>Ethanol λex - λem</td>
<td>Rel.Fl.</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------</td>
<td>-----------</td>
<td>-------------------</td>
<td>---------</td>
</tr>
<tr>
<td>9-Anthraldehyde</td>
<td>99-100°</td>
<td>1680</td>
<td>354 - 403</td>
<td>0.290</td>
</tr>
<tr>
<td>benzylamine</td>
<td>86-7°</td>
<td>1655</td>
<td>362 - 423</td>
<td>0.016</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>183-4°</td>
<td>1620</td>
<td>366 - 412</td>
<td>1.028</td>
</tr>
<tr>
<td>9-phenanthraldehyde</td>
<td>75-6°</td>
<td>1700</td>
<td>290 - 364</td>
<td>0.11</td>
</tr>
<tr>
<td>cyclohexylamine</td>
<td>85-6°</td>
<td>1655</td>
<td>no fl.</td>
<td>-</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>220-2°</td>
<td>1620</td>
<td>no fl.</td>
<td>-</td>
</tr>
<tr>
<td>2-Fluorenealdehyde</td>
<td>85-6°</td>
<td>1700</td>
<td>no fl.</td>
<td>-</td>
</tr>
<tr>
<td>benzylamine</td>
<td>91-92°</td>
<td>1650</td>
<td>no fl.</td>
<td>-</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>245-7°</td>
<td>1630</td>
<td>no fl.</td>
<td>-</td>
</tr>
<tr>
<td>1-pyrenealdehyde</td>
<td>121-2°</td>
<td>1685</td>
<td>394 - 452</td>
<td>0.940</td>
</tr>
<tr>
<td>benzylamine</td>
<td>101-2°</td>
<td>1635</td>
<td>356 - 405</td>
<td>2.780</td>
</tr>
<tr>
<td>p-aminophenol</td>
<td>238-9°</td>
<td>1625</td>
<td>359 - 450</td>
<td>0.24</td>
</tr>
</tbody>
</table>
CHAPTER 5

FLUORESCENCE OF 2-FLUORENEALDEHYDE, 1-PYRENEALDEHYDE AND SOME OF THEIR DERIVATIVES IN VARIOUS SOLVENTS

Having seen the potential of the two aldehydes it is useful to know whether they could be used as fluorigenic reagents. In the analysis of compounds that require some form of reaction before quantitation, a medium has to be found that would give the reaction a maximum yield. In addition, for fluorimetric purposes, the medium sought should preferably be one that would not affect the fluorescence of the resultant product if not to enhance it. The method, if possible, should also be quick and easy to perform. Most reactions of this nature (see section 1.6) are carried out in solutions.

For some procedures e.g. dansyl chloride reactions (101-3), the products must be separated from the excess reagents while in other cases such as the fluorescamine reaction with primary amines (109-115), they could be assayed without any form of separation.

It is the purpose here to examine the effects of various solvents on the fluorescence of derivatives from 2-fluorenealdehyde and 1-pyrenealdehyde. The excitation and emission peaks of compounds can be shifted by appropriate choice of solvents (26, 29-31). Hopefully then, a solvent can be found that would give a big difference in fluorescence maxima between the aldehydes and the derivatives.

5.1 Relative fluorescence of 2-fluorenealdehyde, 1-pyrenealdehyde and their derivatives in various solvents

The fluorescence of the above derivatives relative to 0.1μg/ml quinine bisulphate was observed in redistilled acetone, chloroform,
ethyl acetate, 1-butanol and ethanol. Fluorescence determinations in the solvents acidified with dry HCl were also carried out. The results are summarised in Tables 5.1 and 5.2.

Certain conclusions can be drawn from Tables 5.1 and 5.2. The effects of the solvents on 1-pyrenealdehyde derivatives are similar to those on 2-fluorenealdehyde and its derivatives; it can also be observed that chloroform cannot be used as the aldehydes fluoresce at about the same wavelengths as the derivatives in the acidified form. Acetone tends to turn yellow on acidifying with HCl and is consequently ineffective as a solvent. It should also be borne in mind that the presence of water in the solvent adversely affects the stability of the derivatives. 1-Butanol has less affinity for water than ethanol and thus serves as a better solvent. Ethyl acetate can also be used as a possible solvent for the assay of the derivatives.

5.2 Detection limits of some derivatives of 2-fluorenealdehyde and 1-pyrenealdehyde in acidified ethanol

The reason for performing the experiments in ethanol is because of the high degree of purity in which it can be obtained. Little or no change in the fluorescence of the derivatives in ethanol was observed for the period of time in which the experiments were performed.

Solutions were made in the usual way described in section 2.2. Ultra-violet spectra of the aldehydes and their derivatives were recorded both in the neutral and acidified ethanolic solutions. The molar absorptivities of the compounds were calculated using the Beer-Lambert equation.

Fluorescence of the solutions was measured in the usual way (see section 2.3). Low temperature fluorescence and phosphorescence were measured using Baird Atomic Fluorispec with a recorder attached.
The sample tubes were made of fused silica, 2mm diameter and sealed at one end.

The detection limits of the derivatives from both aldehydes are summarised in Tables 5.3 and 5.4. It can be seen that the detection limits of most of the derivatives are of the same order except that of tyramine which is about 100 times less fluorescent than the others. The derivatives from aromatic amines like p-aminophenol show very little or no measurable fluorescence. This is discussed in the section that follows.

The range of linearity for the derivatives from 1-pyrenealdehyde is over four orders of magnitude while that from 2-fluorenealdehyde is over three orders of magnitude (see fig 5.1). There is considerable self absorption at concentration above 1.0μg/ml especially in the latter. The derivative from both aldehydes are very stable in the acidified solvents and can be kept for weeks with little change in the fluorescence intensity.

5.3 Discussion

Figure 5.2 shows the U.V. spectra of 2-fluorenealdehyde and its derivatives in ethanol and acidified ethanol. It is quite apparent that the derivatives have very similar properties. On acidification there is a change in the maxima to a lower wavelength. A similar observation can be made on the derivatives of 1-pyrenealdehyde.

Figure 5.3 shows that there is also a red shift when the derivatives are measured in acidified ethanol. The change in wavelength is between 87nm and 96nm compared to between 43nm and 45nm for the derivatives from 2-fluorenealdehyde (see fig 5.2). The shift is very large but is to be expected in the protonation of Schiff bases. (169-173)

Tables 5.5 and 5.6 show that there is a slight increase in the molar absorptivity following acidification. This value, $\varepsilon$, directly affects the fluorescence of a compound (see equation 6, section 1.1.1). An increase in $\varepsilon$ would usually enhance the fluorescence of the compound but from the results tabulated in Tables 5.5 and 5.6 it does not appear to be so. For instance, the molar
absorptivity, \( \epsilon \), of 2-fluorenealdehyde in ethanol at 316nm is 31,000 while its value in the acidified solvent at the same wavelength is 12,000. Results show that the aldehyde is not fluorescent in ethanol but the fluorescence in acidified ethanol is 1.075 relative to quinine bisulphate (see Table 5.1). However, in the case of 1-pyrenealdehyde, the value doubles in the acidified solvent (see Table 5.6) and shows a corresponding increase in the relative fluorescence (see Table 5.2).

On the whole the derivatives from primary aliphatic amines of both aldehydes have very high molar absorptivities in acidified solvents and are far more fluorescent than quinine bisulphate except for the tyramine derivatives.

Table 5.3 shows that the excitation and emission wavelengths of the 2-fluorenealdehyde derivatives are about the same. Fig.5.4(a) is a generalisation of the spectral characteristics of the derivatives in ethanol acidified with dry HCl. Derivatives from primary aliphatic amines of 1-pyrenealdehyde also show very similar characteristics. (see Table 5.4 and Fig.5.4(b) ) Thus the fluorescence characteristics are not affected by the amino substituents. It is very likely that on protonation the cation is delocalised around the fluorene moiety (Scheme 5.1) or the pyrene moiety. Thus the derivatives of 2-fluorenealdehyde in the protonated form will have a general structure (I) which fluoresce between 427nm and 435nm while those of 1-pyrenealdehyde will have general structure (II) which fluoresce between 500nm and 505nm.

Qualitative analysis also shows that derivatives from aromatic amines are very weakly fluorescent and hydrolyse quickly. Although the protonated form is very similar to the derivatives from aliphatic primary amines, the charge is not delocalised around the fluorene or pyrene part of the molecule. For instance, in the p-aminophenol derivative, the positive charge tends to be on the -OH group and not on the pyrene. (see Scheme 5.3) Structure III is only very weakly fluorescent.
It should be noted at this juncture that not all derivatives from aliphatic primary amines have fluorescence intensity of the same order.

For instance, the tyramine derivative which is like the 2-phenylethylamine derivative with an extra -OH group at the 4-position on the benzene ring is about 1/100 times as fluorescent as the latter. This is true for derivatives from both the aldehydes. It has already been established that the derivatives have general structure I (Scheme 5.1) or structure II (see Scheme 5.2) and should thus have very similar fluorescence properties. Qualitative studies also showed that the dopa (3-hydroxytyramine) derivatives also have negligible fluorescence presumably for the same reason. It could be that the rotation around the OH group that dissipates the energy of the excited singlet states thereby reducing the fluorescence. If this were the case then the derivatives from tyramine would be just as fluorescent as the ones from 2-phenylethylamine. In fact semi-quantitative studies at 77°C shows that these two derivatives have fluorescence intensities of the same order of magnitude. (see figures 5.7, 5.8)

At 77°C in ethanol, the tyramine derivatives of 1-pyrenealdehyde fluoresces at about the same wavelength as the aldehyde and in fact the spectral characteristics bear great resemblance to the parent compound. (see figures 5.5 and 5.6) However on acidification there is a big change both in the excitation and emission characteristics of the derivatives. There is a blue shift of 35nm compared to only 10nm for the 2-phenylethylamine derivative. At 77°C, there is also a vast difference in the spectral characteristics between the two very similar derivatives which are very similar at room temperature. (see figure 5.7, 5.8) Until more work is done it is difficult to say what the effect of OH group has on the derivatives.

It should be pointed out here at 77°C all the derivatives
including the aldehyde observed were not fluorescent. This could
well be due to the fact that the energy of the \( \pi \rightarrow \pi^* \) state
is lower that that of the \( \pi^* \rightarrow \pi^* \) state in the acidified ethanol.\(^{(27)}\)

It has also been found that derivatives from hydrazines
e.g. phenelzines and mebazines (atomal) form weakly or non-
fluorescent derivatives. This is probably because the proton
attacks the nitrogen next to the imine group and is thus not
delocalised around the pyrene part of the molecule. (Scheme 5.4)

It can be concluded at this point that both aldehydes could be
used as reagents for the quantitation of aliphatic amines.
1-Pyrenealdehyde has an edge over 2-fluorenealdehyde as the
derivatives give a working curve over 4 orders (see figure 5.1).
This aldehyde is also coloured thus easier to work on for practical
reasons.
TABLE 5.1  Relative fluorescence of 2-fluorenealdehyde and some of its derivatives in various solvents. The acidified solvents were made by bubbling dry HCl through the solution. 0.1 µg/ml of quinine bisulphate in 0.1N H₂SO₄ was used as the fluorescence standard. Room Temp.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2-Fluorenealdehyde ( \lambda_{ex-\lambda_{em}} ) Rel.Fl.</th>
<th>2-F/benzylamine ( \lambda_{ex-\lambda_{em}} ) Rel.Fl.</th>
<th>2-F/p-aminophenol ( \lambda_{ex-\lambda_{em}} ) Rel.Fl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>Acetone-HCl</td>
<td>non-fluorescent</td>
<td>359-445 10.080</td>
<td>352-460 0.002</td>
</tr>
<tr>
<td>Chloroform</td>
<td>non-fluorescent</td>
<td>363-420 0.005</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>Chloroform-HCl</td>
<td>340-435 0.048</td>
<td>367-430 11.180</td>
<td>425-517 0.002</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>Ethyl Acetate-HCl</td>
<td>non-fluorescent</td>
<td>360-435 7.40</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>1-Butanol-HCl</td>
<td>303-308 4.225</td>
<td>363-435 6.400</td>
<td>425-517 0.120</td>
</tr>
<tr>
<td>Ethanol</td>
<td>non-fluorescent</td>
<td>356-405 2.778</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>Ethanol-HCl</td>
<td>296-313 1.075</td>
<td>361-435 9.44</td>
<td>425-517 0.100</td>
</tr>
</tbody>
</table>
TABLE 5.2 Relative fluorescence of l-pyrenealdehyde and some of its derivatives in different solvents.

Acidified solvents were made by bubbling dry HCl through the solution. 0.1 μg/ml of quinine bisulphate in 0.1 N H₂SO₄ was used as the fluorescence standard. Room Temp.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>l-Pyrenealdehyde</th>
<th>l-pyr/benzylamine</th>
<th>l-pyr/cyclohexylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λex-λem</td>
<td>Rel.Fl.</td>
<td>λex-λem</td>
</tr>
<tr>
<td>Acetone</td>
<td>348-394</td>
<td>0.074</td>
<td>358-412</td>
</tr>
<tr>
<td>Acetone-HCl</td>
<td>348-394</td>
<td>0.074</td>
<td>438-512</td>
</tr>
<tr>
<td>Chloroform</td>
<td>395-426</td>
<td>0.460</td>
<td>360-402</td>
</tr>
<tr>
<td>Chloroform/HCl</td>
<td>395-426</td>
<td>0.274</td>
<td>455-505</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>348-392</td>
<td>0.250</td>
<td>non-fluorescent</td>
</tr>
<tr>
<td>Ethyl Acetate-HCl</td>
<td>348-392</td>
<td>0.250</td>
<td>436-504</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>340-400</td>
<td>1.790</td>
<td>358-406</td>
</tr>
<tr>
<td>Ethanol</td>
<td>394-452</td>
<td>0.937</td>
<td>355-400</td>
</tr>
</tbody>
</table>
### Table 5.1: Fluorescence of 2-fluorenealdehyde and its derivatives in EtOH acidified with dry HCl.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\varepsilon$</th>
<th>$\lambda_{max}$ nm</th>
<th>$\lambda_{ex}$ nm</th>
<th>$\lambda_{em}$ nm</th>
<th>Rel. Fl.</th>
<th>Linearity</th>
<th>Limit, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Fluorenealdehyde</td>
<td>12,000</td>
<td>316</td>
<td>305</td>
<td>335</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>39,000</td>
<td>355</td>
<td>368</td>
<td>435</td>
<td>1.00</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>2-Phenylethylamine</td>
<td>92,000</td>
<td>355</td>
<td>360</td>
<td>431</td>
<td>0.97</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>48,000</td>
<td>355</td>
<td>358</td>
<td>427</td>
<td>1.08</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>Tyramine</td>
<td>39,000</td>
<td>356</td>
<td>353</td>
<td>392</td>
<td>0.01</td>
<td>1</td>
<td>0.100</td>
</tr>
<tr>
<td>Histamine</td>
<td>54,000</td>
<td>360</td>
<td>360</td>
<td>433</td>
<td>0.49</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>46,000</td>
<td>358</td>
<td>360</td>
<td>428</td>
<td>3.16</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>-</td>
<td>408</td>
<td>356</td>
<td>430</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**a** - Molar absorptivity, $\varepsilon$, measured in EtOH acidified with dry HCl.

**b** - Fluorescence relative to benzylamine derivative of 2-fluorenealdehyde.

**c** - Range over 3 orders of magnitude.
**TABLE 5.4**  
Fluorescence in 1-pyrenealdehyde and its derivatives in EtOH acidified with dry HCl.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \varepsilon ) ( \text{M}^{-1} \text{cm}^{-1} )</th>
<th>( \lambda_{\text{max}} \text{nm} )</th>
<th>( \lambda_{\text{ex}} \text{nm} )</th>
<th>( \lambda_{\text{em}} \text{nm} )</th>
<th>Rel.Fl. Linearity</th>
<th>Detection Limit ug/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Pyrenealdehyde</td>
<td>29,500</td>
<td>343</td>
<td>394</td>
<td>452</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>35,500</td>
<td>445</td>
<td>450</td>
<td>505</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td>2-Phenylethylamine</td>
<td>33,500</td>
<td>443</td>
<td>450</td>
<td>505</td>
<td>0.95</td>
<td>4</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>30,000</td>
<td>440</td>
<td>440</td>
<td>500</td>
<td>1.21</td>
<td>4</td>
</tr>
<tr>
<td>Tyramine</td>
<td>14,00</td>
<td>456</td>
<td>448</td>
<td>500</td>
<td>0.01</td>
<td>3</td>
</tr>
<tr>
<td>Histamine</td>
<td>34,500</td>
<td>443</td>
<td>450</td>
<td>503</td>
<td>0.91</td>
<td>4</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>52,500</td>
<td>443</td>
<td>450</td>
<td>501</td>
<td>1.50</td>
<td>4</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>5,500</td>
<td>380</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(a\) - Molar absorptivity, \( \varepsilon \), measured in EtOH acidified with dry HCl.

\(b\) - Fluorescence relative to benzylamine derivative of 1-pyrenealdehyde.

\(c\) - Range over 4 orders of magnitude.
Figure 51: Analytical curves for (a) Derivatives of Fluorene aldehyde; (b) Derivatives of Pyrene aldehyde. Both (a) and (b) are representative of their derivatives. Sample fluorescence are measured in EtOH-HCl and monitored with the aid of a recorder. The instrument is set at a fixed bandwidth of 8mm and a gain of 10 in both cases.
Figure 5.2: Absorption spectra of (ia) 2-fluorenealdehyde in ethanol; (ib) 2-fluorenealdehyde in ethanol acidified with dry HCl; (ii) Derivatives of 2-fluorenealdehyde in ethanol: (—) benzylamine, (—x—x—) cyclohexylamine, (————) amphetamine (———) histamine; (ii%) derivatives of fluorenealdehyde as in (ii) but solvent acidified with dry HCl.
Figure 5.3: Absorption spectra of (ia) 1-pyrenealdehyde in ethanol; (ib) 1-pyrenealdehyde in ethanol acidified with dry HCl; (ii) derivatives of 1-pyrenealdehyde viz.: (-----) benzylamine, (---x--) cyclohexylamine, (..........) amphetamine, (-----) histamine, (iii) derivatives of 1-pyrenealdehyde as in (ii) but solvent acidified with dry HCl.
<table>
<thead>
<tr>
<th>Sample</th>
<th>( \lambda_{\text{max. nm}} )</th>
<th>( \varepsilon )</th>
<th>( \lambda_{\text{max. nm}} )</th>
<th>( \varepsilon )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Fluorenealdehyde</td>
<td>316</td>
<td>31,000</td>
<td>316</td>
<td>12,000</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>312</td>
<td>30,500</td>
<td>355</td>
<td>39,000</td>
</tr>
<tr>
<td>2-Phenylethylamine</td>
<td>312</td>
<td>32,500</td>
<td>355</td>
<td>92,000</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>313</td>
<td>37,000</td>
<td>355</td>
<td>48,000</td>
</tr>
<tr>
<td>Histamine</td>
<td>315</td>
<td>28,000</td>
<td>360</td>
<td>54,000</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>312</td>
<td>34,000</td>
<td>358</td>
<td>46,000</td>
</tr>
<tr>
<td>Tyramine</td>
<td>312</td>
<td>28,000</td>
<td>356</td>
<td>39,000</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>348</td>
<td>22,000</td>
<td>408</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 5.6  Molar absorptivities, $\varepsilon$, of 1-pyrenealdehyde and its derivatives in ethanol and acidified ethanol. Room Temp.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ethanol</th>
<th>Ethanol-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ nm</td>
<td>$\varepsilon$</td>
</tr>
<tr>
<td>1-Pyrenealdehyde</td>
<td>364</td>
<td>15,500</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>356</td>
<td>32,500</td>
</tr>
<tr>
<td>2-Phenylethylamine</td>
<td>356</td>
<td>34,000</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>360</td>
<td>31,000</td>
</tr>
<tr>
<td>Tyramine</td>
<td>360</td>
<td>26,500</td>
</tr>
<tr>
<td>Histamine</td>
<td>360</td>
<td>22,700</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>356</td>
<td>31,000</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>380</td>
<td>37,000</td>
</tr>
</tbody>
</table>
R = Aliphatic primary amine

2-fluorenealdehyde

\[ \text{H}^+ \text{(dry HCl)} \]

(I) Very stable

Scheme 5.1
Scheme 5.2

Very stable
weakly fluorescent

Scheme 5.3

\[ \text{Scheme 5.4} \]

\[ R = \text{mabanazine or phenelzine} \]
Figure 54: Fluorescence excitation and emission spectra of (a) Derivatives of Fluorone-aldehyde; (b) Derivatives of Pyrene-aldehyde. Both (a) and (b) are representative of their respective derivatives. Sample fluorescence measured in EtOH-HCl at bandwidth of 8 nm.
Figure 5.5 Fluorescence of 1-pyrenealdehyde at 77°K
Concentration of sample: 13.7μg/ml in ethanol. No phosphorescence
was observed. $\lambda_{em} = 345$nm; $\lambda_{em} = 408$nm (Room Temp.)
Figure 5.6 Fluorescence of 1-pyrenealdiminetramine at 77 K. Concentration of sample: 10 µg/ml in ethanol. No phosphorescence was observed. \( \lambda_{ex} \text{375 nm} ; \lambda_{em} \text{379 nm} \).
Figure 5.7 Fluorescence of 1-pyrenealdiminetramine at 77 K. Concentration of sample: 1.0 μg/ml in ethanol-HCl. (a) λ<sub>ex</sub> at 385nm; (b) λ<sub>em</sub> at 235nm. No phosphorescence was observed.
Figure 5.8 Fluorescence of 1-pyrenealdimine-2-phenylethylamine. Concentration of sample: 1.0 μg/ml in ethanol-HCl. (a) λ<sub>ex</sub> at 460nm; (b) λ<sub>ex</sub> at 248nm. No phosphorescence was observed.
CHAPTER 6

6.1 Reactions at Microlevels

In the quantitation of organic compounds by a chemical reaction, it is desirable to take it to completion which might be hours. For a routine analytical method of quantitation it is also desirable that the reaction be carried out with simplicity and in the shortest possible time.

Since a condensation reaction leaves traces of water in the solvent, the amount of water originally present in a solvent would naturally affect the yield of a reaction (138, 139). The rate of a reaction can be increased by increasing the concentration of one of the reactants. In this case, the reaction rate could be increased by increasing the concentration of the aldehyde as it is the amine that is to be determined.

It is the purpose in this chapter to describe experiments that were performed to find the best solvent and the optimum conditions for carrying out the reaction for the determination of amines. The model amine used throughout is cyclohexylamine.

6.2 Ethanol as solvent

About 40 µg/ml of cyclohexylamine in ethanol was prepared by diluting a stock solution of 400 µg/ml. At the same time 1-pyrenealdehyde ethanolic solution was prepared, having a concentration 5-fold molar excess stronger than the amine. This was diluted to 1, 2, 3 and 4-fold molar excess of the amine. 2.0ml of the amine and the aldehyde were pipetted into a 10ml round bottomed flask previously scrupulously cleaned and with a specially made miniature condenser, 3" long, fitted to it. The five flasks were then warmed up for one hour in a water bath at 60°C.
The solutions were then bubbled with dry HCl before the fluorescence was measured.

This experiment was repeated with 4, 8, 12, 16 and 20-fold molar excess and another set at 100-fold molar excess stronger than the amine.

Another experiment was carried out, this time with a constant 20-fold molar excess of the aldehyde. The flasks were kept at 60°C in a water bath for 20, 40, 60, 80 and 120 minutes respectively. After acidification with dry HCl, the fluorescence was measured in the usual way.

The experiment was repeated at 70°C with varying times.

6.3 1-Butanol as solvent

As in 6.2, about 1.0 µg/ml of the cyclohexylamine solution was prepared by diluting a stock solution with redistilled 1-butanol dried with molecular sieves. 1-Pyrenealdehyde was dissolved in 1-butanol and made up to 100-fold molar excess relative to the amine and subsequently diluted to 5, 10, 15, 20, 30, 40, 60 and 80-fold molar excess. 2.0ml of aldehyde and the amine were pipetted into 10ml round bottomed flasks and refluxed over a sand bath.

The experiment was repeated with different amounts of aldehyde, one with 300-fold molar excess and another at 1000-fold molar excess relative to the amine.

A further experiment was carried out; this time the concentration of the cyclohexylamine was 0.1 µg/ml and that of the aldehyde was 800-fold molar excess relative to the amine. 2.0ml of each was pipetted into a flask and refluxed for different lengths of time. The fluorescence was measured after acidification with dry HCl.
6.4 Effect of HCl on reaction

The experiment above was repeated except that with one set the solution was acidified before refluxing and with the other the solutions were acidified after the reaction.

The reaction was first carried out using 1-butanol as the solvent and then repeated with ethanol.

6.5 Detection limit of Cyclohexylamine

A solution of the amine was made up in 1-butanol and diluted to 1.0, 0.5, 0.1, 0.05, 0.01 and 0.005 µg/ml respectively. A solution of 1-pyrenealdehyde in 1-butanol was made such that its concentration was 800-fold molar excess relative to the strongest amine solution. 2.0ml of each was pipetted into a 10ml round bottomed flask and the solutions refluxed for two hours. The fluorescence was then measured after acidification.

6.6 Effect of excess 2-fluorenealdehyde

About 1.0 µg/ml of cyclohexylamine was made up in ethanol by diluting from a stock solution. At the same time 2-fluorenealdehyde was made with increasing concentration.

The reaction was then carried out in the usual way. The fluorescence was determined after acidification of the solution.

6.7 Results and Discussion

Figure 6.1(a) shows that the yield indicated by the first of the reactions increases sharply as the concentration of the aldehyde is increased. This tends to level off at concentration greater than 20-fold molar in excess of the amine (figure 6.1(b)). These two reactions were carried out at 60°C for two hours. It should be noted at this juncture that the yield of the reaction is
indicated by the fluorescence intensity. A further reaction was carried out; this time it was allowed to reflux for two hours and as can be seen in figure 6.1(C) no further increase in fluorescence was observed at an aldehyde concentration 60-fold molar in excess of the amine.

At a constant excess of the 1-pyrenealdehyde i.e. 20-fold molar in excess of the amine, the yield increases linearly with respect to time when the reaction is carried out at 60°C. Figure 6.2(a) shows the linearity up to 120 minutes. The reaction did not reach a maximum by extending the reaction time to 240 minutes.

Figure 6.3(a) shows that the fluorescence intensity responds linearly to the concentration of 1-pyrenealdehyde in 1-butanol. Even at 300-fold molar in excess of the amine, the response is still linear. A further experiment indicates that the fluorescence levels off at a concentration beyond 500-fold molar in excess of the amine (see figure 6.3(C)).

Unlike the reaction in ethanol, equilibrium is reached quickly after refluxing in 1-butanol for 2 - 3 hours. (see figure 6.4). It is difficult to decide on the optimum conditions of the reaction for the determination in 1-butanol. Figure 6.3(c) shows that although at 800-fold molar concentration excess of the amine, the fluorescence intensity seems to be at the plateau but it should be borne in mind that the intensity is on a logarithmic scale and that in fact the fluorescence is slowly increasing with increasing concentration of the aldehyde. However, on the time scale (figure 6.4) it appears that 2-3 hours refluxing might be sufficient for the reaction as the fluorescence intensity increases only marginally by doubling the refluxing time. The best possible choice then is to reflux for 2-3 hours at an aldehyde
concentration of 800-fold molar in excess of the amine, although this is still not the optimum.

Figure 6.5 shows the analytical curve for cyclohexylamine using the above conditions. The limit of detection is around 0.01 µg/ml. It should be noted that although 1.0 µg/ml of the amine is not the upper limit of detection, the aldehyde concentration used was 800-fold molar in excess of the higher concentration of the amine. Figure 6.6 shows that at 0.5 µg/ml cyclohexylamine, the blank was about 5% of the total fluorescence measured.

A further experiment showed that the limit of detection of an amine e.g. histamine could be improved by using the aldehyde purified by a chromatographic column. The aldehyde was eluted by dry ethyl acetate through a column of silica gel. The limit of detection for histamine was about 0.005 µg/ml. The reagent blank at the lower concentration was about 27%. Although the limit of detection could still be improved, it should be noted that the histamine is in the pure form.

Although a condensation reaction can be catalysed by acid or base it has been found here that by acidifying the solution before refluxing inhibits the rate of reaction. No fluorescence was measured even after refluxing for 2-3 hours. Table 6.1 shows the effect of dry HCl on the fluorescence intensity of the derivative in 1-butanol and ethanol.

It has just been established that although 1-pyrenealdehyde could be used for the determination of primary amines, the detection limit is not very good considering that the amine is in the pure state. A similar trial was made using 2-fluorene-aldehyde but an initial experiment indicated that a great excess of the aldehyde is also required. (see figure 6.7)
### TABLE 6.1

Effect of solvents on the fluorescence intensity of the cyclohexylamine derivative of 1-pyrenealdehyde. Instrumental settings were identical in both cases. Solutions were acidified with dry HCl before fluorescence was measured.

<table>
<thead>
<tr>
<th></th>
<th>1-Butanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of amine</td>
<td>0.97 µg/ml</td>
<td>1.00 µg/ml</td>
</tr>
<tr>
<td>refluxing time</td>
<td>1 1/2 hours</td>
<td>1 1/2 hours</td>
</tr>
<tr>
<td>boiling point</td>
<td>116-118°C</td>
<td>80°C</td>
</tr>
<tr>
<td>no. of readings</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>std dev.</td>
<td>2.04</td>
<td>2.40</td>
</tr>
<tr>
<td>*fluorescence int.</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*Fluorescence intensity in arbitrary units measured under the same experimental conditions.*
There are a few basic problems using this method for the determination of amines. Ethanol could be used as a solvent for the reaction but is too volatile. Thus the volume after the reaction might not be consistent and carrying out the reaction at a lower temperature in a water bath poses a practical problem in that water might seep through the joints on condensation thereby hydrolysing the product added to the fact that the reaction time would inevitably be lengthened.

1-Butanol is better in that it has a higher boiling point (see Table 6.1). Thus the reaction time could be reduced by refluxing at a higher temperature.

It has been found that at 100-fold molar concentration of aldehyde in excess of the amine the intensity was three times better in 1-butanol than in ethanol, and that the blank was about 1.5% of the total fluorescence intensity while in the latter, the blank was 15%.

Although a better yield can be obtained by increasing the concentration of the aldehyde, one would inevitably put up the background fluorescence due to the aldehyde. At the same time, the risk of absorption of the fluorescence by the aldehyde is greater added to the fact that there is to a certain extent, competition for the incident light energy.

The problems of self absorption and high background can be overcome by a separation technique. High pressure liquid chromatography would undoubtedly be ideal for work of this nature but unfortunately due to lack of instrumentation, this method was not tried out.

The separation and quantitation is faster than most conventional methods like T.L.C. (Thin Layer Chromatography) which would be dealt with in a later chapter.
A method was also attempted using very small volume for the reaction. The idea behind this was to find out whether amines in minute biological samples e.g. serum could be assayed. 20 µl of each was pipetted into a specially made microreaction vessel with matching microcondenser. After refluxing for 2-3 hours, the product was spotted on a T.L.C. plate and developed. Results showed that this was no better than a reaction on the plate itself. In fact the latter was considerably better than the former.
Figure 6.1. Effect of excess aldehyde on the reaction. Conc. of amine: 40 μg/ml. in ethanol. Reaction at 60°C for 2 hours. \( \lambda_{em} 500 \text{nm} \).
Figure 6.1 (a),(b). Effect of excess aldehyde on the reaction. Conc. of amine: 4.35 μg/ml in ethanol. (a) Reaction at 60°C for 2 hours. (b) Reaction refluxed for 2 hours. λem 500 nm.
Figure 6.2 (a) Effect of time on reaction. Conc of amine: 4.35 µg/ml. Conc. of aldehyde: 206 µg/ml in ethanol. Reaction carried out at 60 C. λem 500nm.
Figure 6.2 (b) Effect of time on reaction. Conc. of amine: 1.8 μg/ml
Conc. of aldehyde: 85 μg/ml in ethanol. Reaction carried out at
70 C for 2 hours. λem 505 nm.
Figure 6.3 (a) Effect of excess aldehyde on the reaction.
Conc. of amine: 0.96 μg/ml. in 1-butanol. Reaction time: 1½ hr.
λ_em 500nm
Figure 6.3 (b) Effect of excess aldehyde on the reaction. Conc. of amine: 0.96 μg/ml in 1-butanol. Reaction time: 1½ hr. λem 500nm.
Figure 6.3 (c) Effect of excess on the reaction. Conc. of amine: 1.05 µg/ml in 1-butanol. Reaction time: 1.5 hr. λem 500nm.

Figure 6.4 Effect of time on reaction. Conc. of amine: 0.1 µg/ml in 1-butanol. Conc. of aldehyde: 800-fold molar in excess of amine. λem 500nm.
Figure 5.5 Detection of cyclohexylamine in a large excess of 1-pyrenealdehyde. Reaction time: 2hr. $\lambda_{em}$ 500nm.
Figure 6.5(b). Analytical curve for histamine.
Reaction time: 2 hours. $\lambda_{em} = 503$ nm. Slope: 0.55
Linear regression: 0.99
Figure 6.6 Fluorescence spectra of the cyclohexylamine of 1-pyrenealdehyde in a large excess of the aldehyde. Blank shows the fluorescence of the pure aldehyde of the same concentration as that used in the reaction. Conc. of amine: 0.50 µg/ml in 1-butanol. λem 500nm.
Figure 6.7 Effect of excess 1-fluorenealdehyde on the reaction. Conc. of amine: 1.14 μg/ml in ethanol. Reaction time: 3½ hr. λ<sub>em</sub> 427nm.
It has been established in the previous chapter that although aliphatic primary amines could be determined by reacting the amine with the aldehyde in solution, there are also drawbacks in the method. Since the advent of the fluorescence T.L.C. scanner, T.L.C. has been widely used for quantitative work on fluorescence. (51-54)

T.L.C. has its advantages over the method described in Chapter 6 chiefly because of the ability to separate the fluorescence derivatives from the reagents thereby reducing the signal due to the background. It is the purpose in this chapter to investigate the feasibility of this technique for the quantitation of derivatives formed from the condensation reaction.

Frequently drugs in plasma samples have to be assayed and it is not unusual to find fluorescence impurities in these samples. The method just mentioned is thus suitable for the assay of such samples.

7.1 Materials for experiments

Silica gel G60, 100-200 mesh, 2x50cm column, Merck Silica gel plates G60, 250µm thick, chromatographic tanks, ethanol, 1-butanol, acetic acid, benzene, dimethylformamide (DMF), chloroform, dichloroethane and acetone.

Apart from acetic acid and DMF, all the other solvents were redistilled to remove impurities as well as being dried in molecular sieves.

7.2 Experiments

Experiments were carried out to find out the $R_f$ values of the 1-pyrenealdehyde and its derivatives in the usual way. (44-47)
Experiments were also carried out to check qualitatively the purity of the aldehyde by the T.L.C., column chromatography and high pressure liquid chromatography (H.P.L.C.).

7.3 Results and Discussion

H.P.L.C. showed that there were four peaks in the twice re-crystallised aldehyde although the peak height is not indicative of the quantity present. This is because the compounds are excited at 254nm which is not necessarily the λ_max of the compounds.

1-Pyrenealdehyde was eluted with benzene and the column resolved at least four distinct fractions. The various fractions were collected and the UV absorption spectra recorded. The results together with fluorescence measurements indicated there was no difference in them. The various fluorescent fractions observed was probably because of the large quantity of aldehyde going through the column. However the column was able to separate some impurities that were left behind at the top of the column. It was probably these impurities that showed up in the H.P.L.C.

It is usually better to perform a T.L.C. separation using single solvents but Table 7.1 shows that with the derivatives used, the separation was very poor. It was found that the reason for the poor separation was because the aldehyde and the derivatives had very similar physical properties e.g. solubility in the neutral condition. By acidifying the aldehyde and the derivatives the separation was slightly improved.

Plates have to be evenly sprayed with ethanol saturated with dry HCl to obtain good separation. This was achieved by spraying from a distance of between 6" to 8" away from the plate for about
5 to 10 seconds. (See Table 7.2)

The only derivative that did not separate well was the histamine derivative, perhaps because of its poor solubility. Table 7.3 shows that although a mixture of a 1:1 ratio of DMF:B-tOH/HCl resulted in a fairly good separation it had its drawbacks. The DMF has an adverse effect on the silica gel plate. Although 1-butanol saturated with dry HCl also has a similar effect, it is to a lesser extent, and that is it leaves behind a second solvent front which overlaps the spots where the derivative occurs. (See Plate 1.) Judging from the table it seems that a 1:5 ratio of ethyl acetate:butanol/HCl is the only developing solvent that could be used.

Tailing of the samples is another problem that is met with here. This usually arises from the fact that the atmosphere in the developing tank has not been given sufficient time to equilibrate or from gross overloading of the sample. Having taken these precautions tailing was still observed in some plates. (see Table 7.1)
<table>
<thead>
<tr>
<th>Developing Solvents</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; Values</th>
<th>1-Pyrenealdehyde</th>
<th>1-Pyrenealdimine cyclohexylamine</th>
<th>1-Pyrenealdimine tyramine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene e 2.3</td>
<td></td>
<td>0.50</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Chloroform e 4.8</td>
<td></td>
<td>0.65</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Dichloroethane e 10.6</td>
<td></td>
<td>0.85</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>1-Butanol e 17.8</td>
<td></td>
<td>0.85</td>
<td>0.85</td>
<td>0.80</td>
</tr>
<tr>
<td>Acetone e 20.7</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ethanol e 24.3</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>EtOH:C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt; 1:3</td>
<td></td>
<td>0.80</td>
<td>-tailing</td>
<td>-tailing</td>
</tr>
<tr>
<td>EtOH:C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt; 1:25</td>
<td></td>
<td>0.80</td>
<td>-tailing</td>
<td>-tailing</td>
</tr>
<tr>
<td>C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;:Ac&lt;sub&gt;2&lt;/sub&gt;O 9:1</td>
<td></td>
<td>0.85</td>
<td>-tailing</td>
<td>-tailing</td>
</tr>
<tr>
<td>EtOH:Et&lt;sub&gt;Ac&lt;/sub&gt; 1:4</td>
<td></td>
<td>0.40</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>EtOH:Et&lt;sub&gt;Ac&lt;/sub&gt; 1:9</td>
<td></td>
<td>0.50</td>
<td>0.10</td>
<td>0.06</td>
</tr>
</tbody>
</table>
TABLE 7.2

Effect of acid on the \( R_f \) values

<table>
<thead>
<tr>
<th>Sample</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Pyrenaldehyde</td>
<td>0.50</td>
<td>0.63</td>
<td>0.65</td>
<td>0.60</td>
<td>0.71</td>
<td>0.6</td>
<td>0.60</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.50</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.50</td>
<td>0.27</td>
<td>0.00</td>
<td>0.13</td>
<td>0.21</td>
<td>0.30</td>
<td>0.15</td>
</tr>
<tr>
<td>2-Phenylethylamine</td>
<td>0.50</td>
<td>0.27</td>
<td>0.28</td>
<td>0.13</td>
<td>0.21</td>
<td>0.45</td>
<td>0.20</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>0.50</td>
<td>0.27</td>
<td>0.28</td>
<td>0.13</td>
<td>0.21</td>
<td>0.45</td>
<td>0.30</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>0.50</td>
<td>0.25</td>
<td>0.30</td>
<td>0.15</td>
<td>0.20</td>
<td>0.45</td>
<td>0.30</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>0.50</td>
<td>0.25</td>
<td>0.30</td>
<td>0.15</td>
<td>0.20</td>
<td>0.45</td>
<td>0.30</td>
</tr>
</tbody>
</table>

A: Benzene
B: EtOH:EtAc/HCl; 1:6
C: EtOH: EtAc/HCl; 1:6
D: EtOH:C_6H_6/HCl; 1:15
E: EtOH/HCl:EtAc; 1:5
F: EtOH/HCl:C_6H_6; 3:2
G: EtOH/HCl: C_6H_6; 1:3
TABLE 7.3

$R_f$ value of 1-pyrenealdehyde and its histamine derivative in various solvent systems

<table>
<thead>
<tr>
<th>Developing Solvents</th>
<th>1-Pyrenealdehyde</th>
<th>Pyr/histamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_6H_6$:EtOH/HCl 4:1</td>
<td>0.65</td>
<td>0.00</td>
</tr>
<tr>
<td>$C_6H_6$:EtOH/HCl 2:3</td>
<td>0.80</td>
<td>0.10</td>
</tr>
<tr>
<td>EtOH/HCl</td>
<td>0.60</td>
<td>0.12</td>
</tr>
<tr>
<td>AcOH:$C_6H_6$ 1:9</td>
<td>0.43</td>
<td>0.00</td>
</tr>
<tr>
<td>AcOH:$C_6H_6$ 1:3</td>
<td>0.60</td>
<td>0.00</td>
</tr>
<tr>
<td>AcOH:$C_6H_6$:DMP 5:3:2</td>
<td>0.75</td>
<td>tailing</td>
</tr>
<tr>
<td>$Et^0Ac$:EtOH/HCl 1:5</td>
<td>0.90</td>
<td>0.10</td>
</tr>
<tr>
<td>$Et^0Ac$:EtOH/HCl 1:3</td>
<td>0.80</td>
<td>0.00</td>
</tr>
<tr>
<td>DMF:EtOH/HCl 1:1</td>
<td>0.80</td>
<td>0.50</td>
</tr>
<tr>
<td>DMF:$C_6H_6$:EtOH/HCl 1:1:1</td>
<td>0.80</td>
<td>0.30</td>
</tr>
<tr>
<td>DMF:$C_6H_6$:EtOH/HCl</td>
<td>0.95</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Studies on 1-pyrenealdehyde and its derivatives showed that it is possible to quantify them following development on T.L.C. As with other techniques, difficulties arise in the determination of amines in biological material. Serum proteins have to be removed prior to reaction on the plate but this has been reviewed in Section 1.7.

For obvious reasons, reactions on T.L.C. plates has its advantages. Not only is time saved but the compound of interest is also separated from the reagent thereby reducing the background. In the previous method (see Chapter 7) several steps were involved before the fluorescence was eventually measured. Reaction on T.L.C. plate is more direct and reduces the inaccuracies caused by the transferance of solutions from one vessel to another.

8.1 Effect of HCl and Time on the Reaction

A stock solution of 2-phenylethylamine was prepared in ethanol and diluted to about 10 µg/ml. A similar stock solution of 1-pyrenealdehyde in ethanol was also prepared at a 0.1 gm/5 ml concentration. This is about 1000-fold molar excess of the amine. 10 µl of each reagent were then spotted onto the plate at twenty minute intervals. The experiment was repeated with cyclohexylamine.

For another set of experiments, the plate was sprayed with 1-butanol saturated with dry HCl before spotting the reagent.

After reaction at 70°C for an hour the plates were developed in 1:5 1-butanol: ethylacetate/HCl.
8.2 Effect of excess aldehyde on reaction

The solutions were prepared in the same manner as in Section 6.1 except that the aldehyde was diluted to 500, 100, 50, 10 and 5 fold molar excess of the amine. The plate was sprayed with butanol/HCl before application of the samples. 10μg quantities of the amine were spotted on the plate followed by an equal amount of the aldehyde in falling dilutions, on top of the amine. The plate was then left in the oven at 70° for an hour, then developed in the same developer as before.

8.3 Extraction of histamine by XAD-2

The column was made up in the way described and washed with 75 ml of tridistilled water followed by 25 ml of 0.01N NaOH. 1.0ml of 100 μg/ml histamine solution was passed through the column and then eluted with acidified ethanol. The first 0.5ml of the eluate was discarded and 5.0ml subsequently collected. 10μl of the eluate was then spotted on the T.L.C. plate and 1-pyrene-aldehyde spotted on the amine. At the same time, an equivalent amount of the pure histamine was spotted next to the extract. Aldehyde blanks were also done. This was repeated with 10μg/ml and 1.0μg/ml of the histamine.

After spraying with acidified butanol, the reaction was carried out at 70°C for an hour then developed in butanol saturated with dry HCl. Fluorescence was subsequently determined and the percentage extraction calculated.

8.4 Analysis of amphetamine in serum after deproteination

100μg/ml amphetamine was prepared by diluting 0.5ml of serum with 0.5ml of 200μg/ml stock solution of amphetamine in tridistilled water. 0.1ml of the serum containing the amine was mixed with 0.9ml of absolute ethanol and warmed at 60°C for ten minutes to
flocculate the proteins. It was then centrifuged and 10μl of the resulting solution spotted out on a T.L.C. plate. 10μl of 0.04μg/ml of 1-pyrenealdehyde in benzene was then spotted on the extract. An equivalent amount of pure amphetamine and an aldehyde blank was also spotted out on the plate which was sprayed with acidified butanol and the reaction carried out as before. The fluorescence was measured at 510nm using an interference filter on a commercially available T.L.C. densitometer, Vitatron TLC 100. (see figure 8.9) The light source used here is the mercury lamp and the instrument switched to the fluorescence mode. The signal obtained was plotted on a chart recorder.

The experiment was repeated using amphetamine concentrations of 10 μg/ml and 1.0 μg/ml respectively. The percentage extractions were worked out accordingly.

8.5 Emission spectra of some derivatives of 1-pyrenealdehyde

Some derivatives of 1-pyrenealdehyde were dissolved in butanol saturated with dry HCl. The derivatives were then spotted on a strip of T.L.C. plates; each about 500 μg/spot and two cm apart. The strip was mounted on a specially constructed cylinder fitted on a stage with a motor incorporated. The strip was then aligned such that light could be seen to fall on to the spots. The emission spectra of the derivatives were then recorded. (See figure 8)

8.6 Detection limit of some amines

25.0, 10.0, 7.5, 5.0, 2.5 and 1.0 μg/ml of amphetamine in serum was prepared in the fashion described in section 8.4. After deproteination in the usual way (section 8.4) 10 μl of each solution was spotted on a T.L.C. plate 1.5cm apart. 10 μl of 0.04μg/ml aldehyde in benzene was spotted on top of the amine. Three aldehyde blanks were also made. The reaction was carried
out at 80°C for an hour. After development the fluorescence was measured at 510 nm.

This was repeated with 2-phenylethylamine, tyramine and histamine. The developing solvent used was 1:4 1-butanol: ethyl acetate saturated with dry HCl, except for histamine where the developing solvent used was solely 1-butanol/HCl.

This experiment was repeated with the pure amines without the serum.

8.7 Results and discussion

It has been found that the condensation reaction takes place only in neutral condition. However this is not the case in solid surfaces e.g. T.L.C. Figure 8.1 shows that the progress of the reaction is independent of time in the neutral condition. In the presence of a large excess of aldehyde and under acidic conditions the reaction quickly reaches a maximum after half an hour at 70°C (see figure 8.2).

Figure 8.3 shows that the intensity of fluorescence responds linearly with increasing amounts of aldehyde used in the reaction up to 1000 times the concentration of the amine. (cf reaction in solution). Although a higher concentration of the aldehyde could be used, this creates tailing effects due to overloading which prevents the quantitation of the amine. Thus there is an upper limit to which the aldehyde could be used without producing an unduly high background. It has been worked out that this concentration is about 0.04 µg/ml.

The fluorescent Schiff bases are more susceptible to hydrolysis on T.L.C. than in solution. Under normal laboratory conditions the derivatives hydrolysed within half an hour. This was due to the plate absorbing the water vapour from the air. Further, the presence of HCl accelerates the rate of hydrolysis which could be
reduced considerably by drying the solvents with molecular sieves and also by keeping the developed plates in a chamber with dessicating reagent viz. silica gel. By observing the above conditions, it was found that the fluorescent derivatives were stable over a period of a few weeks.

Having found the optimum conditions for the reaction on T.L.C. a suitable method for the extraction of the amines from serum had to be worked out. Recently (see section 1.7) Amberlite XAD-2, a non-ionic polymeric material has been widely used for the extraction of organic molecules from various solutions including biological material such as serum and other tissues. A general survey of extraction procedures (see section 1.7) indicated that the recovery of simple amine using XAD-2 was between 60-80%. The present results indicate figures well below literature values. (see Table 8.1 and Plate 8.1). This was due to the small volume (5ml) of the eluant collected. The accuracy could be improved using a larger volume of eluant but a longer time was required to evaporate the solvent away. It was also found that the extraction was more effective if the column was rendered alkaline by washing it with 25ml of 0.01 N NaOH. Preliminary studies showed that less than 1% of the amine was extracted from a neutral column. Eluting the amine from the column with acidified ethanol had its problems. Though more effective, the acidified eluent tended to emulsify on evaporation.

Although more straightforward than most methods, the above method was also time consuming. The percentage extraction was also not consistent for varying concentrations (see Table 8.2). A more direct method was subsequently attempted.

Serum proteins could be precipitated by absolute ethanol (see Section 1.7). This is a more efficient way of separating the amines although there is a risk that some amines might be
### TABLE 8.1

Recovery of histamine from serum samples using Amberlite XAD-2

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Int. in arb. units</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histamine</td>
<td>Standard</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>8.9</td>
<td>15.1</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>6.6</td>
<td>13.1</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>1.3</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Note: Extractions were done in quadruplet. Blanks were also made.

### TABLE 8.2

Recovery of amphetamine from serum sample by direct precipitation of serum proteins using absolute ethanol.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Int. in arb. units</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amphetamine</td>
<td>Standard</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>139.2</td>
<td>138.0</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>135.0</td>
<td>140.5</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>52.0</td>
<td>55.0</td>
</tr>
</tbody>
</table>
bound to the precipitated proteins but the amines which were tried out were unaffected by the presence of the proteins. The advantage here is the rapidity and a reduction in the number of steps involved compared to the previous methods.

The precipitation is never 100% efficient and tends to leave traces of amino acids and albumin in the sample used for analysis. However, as the reaction is performed on T.L.C., the impurities could be separated and the fluorescence of the derivative measured. Table 8.3 shows percentage recovery of amphetamine is greater than 90% for a 1 µg/ml concentration in serum. (see Plate 8.2)

**TABLE 8.3**

Detection limit of some amines. Fluorescence measured at 510nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pure amine (μg/spot)</th>
<th>Serum Sample (μg/spot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>2-phenylethylamine</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyramine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histamine</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>3-phenylpropylamine</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

The detection limits of some pharmaceutically significant amines are summarised in Table 8.3. Although the histamine derivative of 1-pyrenealdehyde is more fluorescent than that of tyramine the detection is poorer because of interference from
the background. The histamine derivative moves up the T.L.C. plate with great difficulty even with DMF as a developer and the interference is caused by the overlap with the impurities left on the origin (see Plate 8.1). There is also a slight interference from the second solvent front.

A few other drugs were determined but were found to yield non-fluorescent derivatives. These drugs include actamal, dopamine, dopa and phenelzine. Dopamine is very much like tyramine but has an extra -OH group and it has been found (Chapter 5) that the -OH group on tyramine reduces the fluorescence of the compound. An additional -OH on the compound (dopamine) almost totally quenches the fluorescence of the derivative. Actamol and phenelzine, both hydrazines, were found to produce non-fluorescent derivatives. Although the serotonin creatine sulphate derivative was found to be fluorescent, it was difficult to determine whether that was attributed to the serotonin part of the molecule or to the creatine sulphate as both have an amino group on them. Serotonin on its own is unstable in solution and a separation was attempted without much success.

Figure 8.4 shows the analytical curve for the determination of histamine and 2-phenylethylamine. This was obtained by reacting the amines with the aldehyde in solution and spotting them out on T.L.C. Fluorescence was then measured in the normal way. (See Section 8.4) Although the detection limits of the

\[
\begin{align*}
\text{HO} & \quad \text{CH}_2\text{CH}_2\text{NH}_2 & \text{NH}_2 & \quad \text{CH}_3 \\
\text{N} & \quad \text{CH}_2\text{CO}_2\text{H} & \text{H}_2\text{SO}_4
\end{align*}
\]

**Serotonin Creatine Sulphate**
pure amines are comparable with those reactions performed on T.L.C. plate, the quantity of material e.g. 2ml amine with 2ml aldehyde, are both much larger than those of the latter. The former method is also more time consuming and involves more steps before the fluorescence is eventually monitored.

At this juncture it should be noted that the fluorescence of all the derivatives were measured using a 510nm filter and that this is not the emission maxima of the derivatives. Figure 8.10 shows that the emission maxima vary between a few nanometers of each other and since the interference filters are expensive, this small wavelength difference does not justify the expense. Suffice it to say then that the sensitivity can be further improved using filters at the appropriate wavelengths. The spectra were obtained by adapting the fluorimeter for T.L.C. measurements. The main disadvantage of this adaptation is that there is an unduly high background caused by the right-angled optics designed for measurements of solution fluorescence. However there are commercially available instruments that could eliminate this disadvantage.

The optical layout of the Vitatron TLD 100 is shown in Figure 8.8, it is designed solely for qualitative and quantitative work on T.L.C. but lacks a device for scanning emission spectra of fluorescent compounds. As can be seen, background signal due to scatter is almost totally eliminated. Nevertheless different filters could be used.
Figure 8.1 Effect of acid on the reaction. The plate was sprayed with ethanol saturated with dry HCl after completion of reaction. $\lambda_{em}$ 510nm.
Figure 8.2 Effect of time on reaction. The plate was sprayed with 1-butanol saturated with dry HCl before reaction was carried out. Reaction temp. 70°C. $\lambda_{em}$ 510nm.
Figure 8.3 Effect of excess aldehyde on the reaction. The plate was sprayed before sample application. Amine used: 10 µg/ml cyclohexylamine. Time of reaction: 1 Hr. λ<sub>em</sub> 510nm.
Figure 8.4 Detection limit of histamine and 2-phenylethylamine Reaction carried out in solution and \(10 \mu l\) spotted out on TLC plate. Fluorescence measured at 510nm. cf. Reaction on TLC plate.
Figure 8.5 Detection limit of 2-phenylethylamine and amphetamine in the pure state. Reaction on TLC carried out at 80°C for an hour. $\lambda_{em}$ 510nm
Figure 8.6 Detection limit of pure histamine. Reaction on TLC carried out at 80°C for an hour. $\lambda_{em}$ 510nm.
Figure 8.7 Detection limit of some amines in serum. Reaction carried out at 80°C for an hour. $\lambda_{em}$ 510nm.
Figure 8.8 Fluorimeter modified to do measurements on TLC.

Figure 8.9 Vitatron TLD 100, TLC scanner.
Figure 8.10. Emission spectra of some derivatives of 1-pyrenealdehyde on TLC plate (silica gel G60).

The derivatives were: (-----) Amphetamine, $\lambda_{em} 495$nm;
(------------) Benzylamine, $\lambda_{em} 502$nm; (-----) Histamine, $\lambda_{em} 498$nm; (-----) 2-Phenylethylamine, $\lambda_{em} 497$nm;
(-----) Cyclohexylamine, $\lambda_{em} 493$nm.
CONCLUSION

Fluorimetry has long been known as a sensitive instrumental technique for the determination of organic molecules. Although amines can be detected by various classical methods and other instrumental techniques most of these are either not sufficiently sensitive or indirect and tedious (see introduction).

The main aim of this work was to develop a sensitive routine method for the assay of some pharmacologically significant drugs with a primary amino group. Some which are inherently fluorescent (e.g. acridine) can be determined directly. Those which do not exhibit native fluorescence could be rendered fluorescent by reacting them with a fluorogenic reagent. It was the aim here to find such a reagent.

In developing an assay for a compound one has to consider various practical aspects like simplicity, accuracy and the speed with which the assay is carried out. For an assay of non-fluorescent amines by fluorescence derivatisation, the ideal condition would be to carry out the reaction in solution and measure the resultant fluorescence. Though this can be achieved, it is only applicable to pure solutions.

Of the various aldehydes examined, a few showed potential as fluorogenic reagents. Derivatives of these aldehydes were prepared and subsequent determination showed that levels as low as 0.1 ng/ml could be detected. (see Table 5.4) Experiments also show (see Chapter 6) that amines could be detected by reacting them with an excess of aldehyde e.g. 1-pyrenealdehyde. An excess of aldehyde is used so as to cut down the reaction time. The method can be improved considerably by reducing the
amount of aldehyde but would increase the time of reaction.

Derivatives from 1-pyrenealdehyde are so fluorescent that trace amounts of amines apart from the amine of interest would interfere with the analysis if present. There are various methods of separating complex mixtures of organic molecules. Amberlite XAD-2 has been widely used by separation procedures of this nature usually leaves traces of water which adversely affects the condensation reaction. This problem can be easily overcome by evaporating the extract to dryness, then dissolving it again in an appropriate solvent before carrying out the reaction. However this unduly prolongs the analysis time. T.L.C. would both be reasonably fast and performs separation at the same time.

The derivatives are so fluorescent that even on a T.L.C. plate nanogram amounts could be detected. This technique is at least as good as methods using fluorescamine and dansyl chloride performed in a similar manner. In the method described, 9.0ml of ethanol is added to 1.0ml serum to precipitate the unwanted proteins and from this only 10μl of the solution is spotted out on the T.L.C. plate - a 1000 fold dilution factor before the derivatisation reaction. This is however a universal problem but the method can be further improved by evaporating the extract to a managably small volume.

The method described (see Chapter 8) is simple and straightforward and if carried out in a dry atmosphere, reproducible. The derivatives are also stable for weeks if kept in a tank which is dried with silica gel.

2-Fluorenealdehyde is almost non-fluorescent in ethanol while 1-pyrenealdehyde fluoresces at 452nm. In the acidified form the derivative from the former aldehyde fluoresces at 335nm.
while the latter fluoresces at 500nm. The separation in wavelength between the aldehyde and derivative is smaller for 2-fluorenealdehyde than for 1-pyrenealdehyde. Table 1.1 shows that the quantum yield of fluorene and pyrene are both 0.53 in ethanol and that of perylene is 0.94 in the same solvent. Although this does not imply that their derivatives would possess corresponding fluorescence quantum yield, judging from the experiments carried out so far, there is a possibility that perylenealdehyde might form derivatives with higher quantum yields than derivatives from other aldehydes e.g. 1-pyrenealdehyde.

In conclusion, 1-pyrenealdehyde is as good a reagent as most existing ones for the quantitation of non-fluorescent amines by fluorimetric means. This reagent is economical and readily obtained and can be purified without difficulty. The method described is also sufficiently sensitive, simple, straightforward and can be performed within a reasonably short time.
REFERENCES

(5) Guilbault, G.G., Practical Fluorescence, Theory, Method and Techniques, Dekker, N.Y. 1973
(6) Van Duuren, E.L.; Chan, T.L., Chap. VII
(9) Chen, R.F., Anal Biochem, 19, 374, 1967
(10) Fluorescence News, 4(4), 9, 1969
(12) Fluorescence News, 4(4), 1, 1969
(14) Parker, C.A., Photoluminescence of Solutions, Elsevier, Amsterdam, 1968
(15) Birks, J.B., Photophysics of Aromatic Molecules, Wiley, New York, 1970
(22) Bowen, E.J., Advances in Photochemistry, p32
Interscience N.Y. 1963


(26) Kasha, M., Radiation Res. Suppl. 2, 243, 1960

(27) Bredereck, D.K., Forster, T., Oesterlin, H., Luminescence of Organic and Inorganic Materials,

(28) Van Duuren, B.L., Chem. Rev., 63, 325, 1963

(29) Bayliss, N.S., McRae, E.G. J.Phys. Chem. 58, 1006, 1954


(33) Pimentel, G.C., McCollam, A.L., The Hydrogen Bond,
W.H. Freeman, San Francisco, 1960


(37) Forster, T., Z. Elektrochem., 54, 42, 1950

(38) Van Duuren, B.L., Anal. Chem., 32, 1436, 1960

(39) Forster T., in Heidt L.J., Livingston, R.S.,
Rabinowitch, E., Daniels, F., eds.,
Photochemistry in the Liquid and Solid States,
Wiley N.Y., 1960, p.12

(40) Forster, T., Z. Elektrochem., 54, 42, 1950

(41) Kolthoff, I.M., Stenger, V.A., Volumetric Analysis,


(44) Kirchner, J.G., Thin-Layer Chromatography, ed by
Perry, E.S., Weissberger, A. in Techniques of
Organic Chemistry, Interscience, 1967

(45) Touchstone, J.C., Quantitative Thin-Layer Chromatography,
Wiley Interscience, 1973

(47) Stahl, E., Thin-Layer Chromatography Springer-Verlag, N.Y. 1969


(49) Mathis, C., Ourisson, G., J. Chromatog., 12, 94, 1963


(52) Sawicki, E., Johnson, H., J. Chromatog. 23, 142-8, 1966


(55) Sawicki, E., Stanley, T.W., Johnson, H., Microchem J. 8, 257-284, 1964


(57) Danick, A., Pogonowska-Walg, E., Diss-Pharm. Pharmacol. 22, 67, 1970, CA 72, 114902A


(64) Ervik, M., Acta Pharm Sencia, 6, 393, 1969


(67) Teichert, K., Mutschler, E., Rochelmeyer, H., Dent. Apotheker-Ztg. 100, 283, 1960

(68) Jones, G.R.N., J. Chromatog., 77, 357-367, 1973
(69) Moffat, A.C., Clarac, B., J. Pharm. Pharmas., 26, 665-70, 1974
(70) Goodall, R.R., J. Chromatog., 103(2), 265078, 1975
(73) Tomaka, H., Miyake, Y.; Bunseki Kagaka, 22(3), 335-6, 1973
(74) Macek, K., Pharm. Appl. Thin Layer Paper Chromat., 143-8, 1972
(75) Macek, K., Pharm. Appl. Thin Layer Paper Chromat., 155-274, 1972
(78) Bartoz & Peser, Talanta, Vol.19, 92-124, 1972
(85) Quay, W.B., Fluorescence News, 4(5), 5, 1969
(87) Kawanchi, H., Tuzimura, K., Maeda, H., Khida, N., J. Biochem., 65(6), 783-788, 1969
(88) Pasez, M., Bartos, J., Talanta, 14, 1097, 1967

(94) Sinsheimer, J., Hong, D., Stewart, J., Fink, W., Burckhalter, J., J. Pharm. Sci., 60, 141, 1971

(95) DeLennhecr, A., Sinsheimer, J., Burckhalter, J., J. Pharm. Sci., 62(8), 1370, 1973


(105) Samejina, K., Dairman, W., Udenfriend, S., Anal. Biochem. 42, 222, 1971

(106) ibid, 42, 237, 1971


(108) ibid, 94, 5927, 1972


(110) Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Science, 178, 871-872, 1972


(113) Bohlen, P., Stein, S., Dairman, W., Udenfriend, S., Arch. Biochem. Biophys., 155(1), 203, 1973

(114) ibid, 155(1), 213, 1973


Haefelfinger, P., J. Chromatog., 323-9, 1975

Hess, S.M., Udenfriend, S., J. Pharmacol Exptl Therap., 127, 175, 1959


Jason, J., & Stevens, B., Nature, 172, 772, 1953

Quay, W., J. Pharm. Sci., 57, 1568, 1968

Vanable, J., Anal. Biochem., 6, 393, 1963


Cox, R.H., Perhach, J.L., Neurochem., 20(6), 1737, 1973


J. Oates in Methods in Medical Research, J. Quastel ed., Vol. IX, Year Book, Chicago, 1961, P169


Sprung, M.M., Chem. Rev., 26, 297, 1940


Kullberg, M.P., Gorodetsky, G.W., Clin, Chem. 20(2), 177-183, 1974


(165)


