New aromatic dialdehyde labels for analytical fluorimetry

PLEASE CITE THE PUBLISHED VERSION

PUBLISHER

PUBLISHER STATEMENT

LICENCE

REPOSITORY RECORD

NEW AROMATIC DIALDEHYDE LABELS
FOR
ANALYTICAL FLUORIMETRY

by

Mohammad Aminuddin

B.Sc.(Hons.), M.Sc., C.Chem. M.R.S.C.

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for

the award of

DOCTOR OF PHILOSOPHY

of the Loughborough University of Technology

Supervisor: Professor J. N. Miller

May 1987

IN THE NAME OF ALLAH,

THE BENEFICIENT, THE MERCIFUL

ACKNOWLEDGEMENTS

CONTENTS

SYNOPSIS

Fluorigenic aromatic molecules have found wide application in Fluorescence Spectroscopy. Commercially available fluorigenic reagents have been used to detect amines, amino acids, peptides and proteins. Ortho-phthalaldehyde (OPA) is an aromatic dialdehyde which is specific for a primary amino group. Three polyaromatic dialdehyde molecules similar to this compound have been synthesised and investigated for their analytical applications. They are (1) naphthalene-2,3-dicarboxaldehyde (NDA), (2) 1-phenylnaphthalene-2,3-dicarboxaldehyde (ØNDA) and (3) anthracene-2,3-dicarboxaldehyde (ADA).

NDA, ØNDA and ADA react with primary amino groups in the presence of thiols to produce fluorescent isoindoles. The products are very stable and they have excitation and emission wavelengths (emission 520, 520, 640nm and excitation 462, 462 and 470nm respectively) different to that of OPA derivative which has emission at 450 nm when excited at 350 nm.

The fluorescence intensity and stability of the reaction products are found to have been greatly influenced
either
by the presence of β-cyclodextrin, a nonionic surfactant, a branched chain thiol and the pH of the medium. Inorganic salts and organic solvents also affect the fluorescence properties. Naphthalene-2,3-dicarboxaldehyde (NDA) has successfully been used as a pre-labelling reagent prior to reversed phase HPLC for qualitative and quantitative amino acid analysis. NDA-glycine and ØNDA-glycine derivatisation

reactions have been carried out using flow injection analysis. The effect of flow rate and tube length on fluorescence intensity and dependence of fluorescence signal on the amount of thiol and reaction time studies have been made.

The effect of certain absorbers such as cytochrome C and ATP on the fluorescent isoindoles has also been studied.

NDA, øNDA and ADA have been shown to be suitable labels for proteins. The reaction time and stability of the fluorophores are comparable to those of low molecular weight fluorophores.

CHAPTER 1   INTRODUCTION

1.1   Fluorimetric Analysis

1.1.1   Importance and Merits

Fluorimetry as an analytical technique is useful in both qualitative and quantitative [1] analysis. The technique is not only sensitive and selective but also accurate with good stability, limits of detection and speed of analysis. Spectrofluorimetric methods, either direct or in combination with high performance liquid chromatography (HPLC) or paper and thin layer chromatography, have provided the opportunity to (a) study and investigate peptides [2] and proteins including rare proteins for gene cloning in genetic manipulation, (b) measure amino acid constituents in nutritional foods, the free amino acid content of tissue, tissue extracts and physiological fluids [3] (e.g. blood and urine) and (c) analyse biogenic amines [4] which play an important role in the hormonal control, etiology and pathogenesis of disease.

Fluorescence methods have been very useful for the detection of carcinogenic material, benzo[a]pyrene, in trace quantities present in smoked food [5], beer [6], cigarette smoke [7] and paraffin wax [8] for food. The most difficult complex samples of polycyclic aromatic hydrocarbons (PAH) and organic pollutants have been dealt with by synchronous luminescence spectrometry which provides a high degree of selectivity by simultaneous scanning of both excitation and emission wavelengths,

keeping a constant wavelength interval, $\Delta\lambda$, between them
[9, 10]. A substantial number of drugs with polycyclic or
polyheterocyclic rings possessing native fluorescence may
be estimated with increased sensitivity by the variable
angle synchronous scanning (v.a.s.s.) technique. In this
method, the wavelength separation between the excitation
and emission monochromators is continuously varied [11] by
varying the relative scan speeds of each monochromator
[12].

Fluorescence detection is sensitive [13] and has a
number of merits, the most important of these being
selectivity [14] by virtue of the fluorescence measurement
being made at a wavelength different from the excitation
wavelength. By contrast, low wavelength UV detection
offers the advantage of almost universal detection at
200nm, but suffers from the disadvantage that any
impurities present in buffers or solvents used may be
detected, obscuring the peaks of interest. Also the
detection at extremely low concentrations by absorption
(UV-visible detection) is not suitable, since it measures
the difference between two large numbers which is
difficult to obtain accurately.

1.1.2 Areas of application

The rapidly increasing demand for speed, sensitivity
and accuracy in the analysis of a wide variety of
compounds at the subnanomole or even femtomole level has
generated a great interest in fluorescence. Applications
span many areas such as bio-research and bio-technology

for useful information concerning peptides and proteins.
Other areas of interest are pharmacology, medicine,
neurobiology, diagnosis, quality control and environmental
science.

1.1.3 Basic principles and mechanisms of fluorescence [15,16]

Fluorescence is the emission of radiation by certain
molecules at a wavelength that is longer than the
wavelength of excitation. The process is dependent on a
series of photophysical events such as radiative and
radiationless molecular transitions. The absorption of
radiation is of prime importance in the whole process.
Radiation consists of photons and can interact with
matter. The light (photon) may either collide or be
absorbed. The collision is called elastic when the
impinging and dispersed radiation are of the same
wavelength (Rayleigh scattering) and is somewhat random.
However, the scattering of light may be at a different
wavelength from the impinging radiation (Raman
scattering). This is due to nonelastic collisions where
an energy loss occurs by mixing of the rotational and
vibrational energy of the colliding molecules. Raman
scattering appears at a longer wavelength than the
excitation wavelength.

When a molecule absorbs energy, it is said to become
excited and the electrons in the molecule are promoted to
a higher electronic orbital in which there are no unpaired
electrons. The change from the ground state $[S_0]$ to the

excited state by process of absorption, which occurs in about 10^{-15}s, is essentially instantaneous compared to changes in the molecular co-ordinates. This is in accordance with the Franck-Condon principle. The energy is rapidly lost (Figure 1.1) in about $10^{-12}$to 10^{-11}s by being converted to internal vibrational and rotational energy, a radiationless transition in which no photon is emitted in the visible or UV regions and the molecule returns to the lowest excited state $[S_1]$. This is called a metastable state and loss of energy from this state to the ground state proceeds either by emission of a photon (fluorescence), the reverse of the normal singlet-singlet absorption process; by internal conversion; by the reverse of a strongly forbidden singlet-triplet absorption process. These transitions are all competing processes and only the $[S_1] \rightarrow [S_0]$ transition leads to useful fluorescence. The life time of the molecule in $[S_1]$ is about 10^{-8}s.

From the triplet state $[T_1]$ the molecule can return to $[S_0]$ by radiative (phosphorescence) or non-radiative processes. However, the former process occurs typically on a timescale of 10^{-5}-10s.

Due to the large energy gap between $[S_0]$ and $[S_1]$ or $[T_1]$ the internal conversion (radiationless transition) is often fast enough [17] to compete with the slower rate of the $[S_1] \rightarrow [S_0]$ and $[T_1] \rightarrow [S_0]$ transitions.

The energy transfer from both singlet and triplet states may occur over a large distance (10 to 50A) to another molecule by a process referred to as resonance energy transfer.

Figure 1.1:   Simplified energy level diagram of a
polyatomic molecule.

1.2 Measurement of fluorescence

1.2.1 Relationship between fluorescence and concentration

Fluorescence intensity is directly related to concentration. The following equation can be used to calculate the fluorescence intensity

$$I_f = \phi . I_o [1 - e^{-(\epsilon_{\lambda_{ex}} + \epsilon_{\lambda_f})c.l.}]$$

where $\epsilon_{\lambda_{ex}}$ is the extinction coefficient at the exciting wavelength, ϵ_{λ_f} is the extinction coefficient at the wavelength of emission, c is the concentration, I_o is the power of the incident light, ϕ is the fluorescence quantum efficiency and l is the optical volume determined by the perpendicular pathways of the excitation and emission slits, rather than the cell dimension as in absorption spectrometry.

1.2.2 Derivative spectroscopy

In the case of multicomponent mixtures peak overlapping is very common and the assay of the individual components can be difficult. The technique of derivative spectroscopy seems to reduce or overcome such problems. The following differential quotients give rise to a derivative spectrum.

$\dfrac{dF}{d\lambda}$ for 1st derivative, $\dfrac{d^2F}{d\lambda^2}$ for 2nd derivative and so on -

where λ = wavelength and F = fluorescence, over the wavelength range of interest.   Relating the derivative

signal to time (t) the quotient is related to wavelength (λ) as follows[18]:

$$\frac{dF}{dt} = \frac{dF}{d\lambda} \times \frac{d\lambda}{dt} \quad \text{where} \quad \frac{d\lambda}{dt} = S = \text{scan speed.}$$

The scan speed (S) is normally constant, thus the expressions above become:

$$\frac{dF}{d\lambda} = \frac{dF}{dt} \times \frac{1}{S} \quad \text{for the 1st derivative}$$

$$\frac{d^2F}{d\lambda} = \frac{d^2F}{dt^2} \times \frac{1}{S^2} \quad \text{for the 2nd derivative}$$

Increasing the scanning speed will gradually improve the signal to noise ratio.

1.2.3 Fluorescence quantum efficiency

The ratio of the number of photons emitted to the number of photons absorbed is referred to as the quantum efficiency (ϕ). In practice the fluorescence quantum efficiency of an unknown, ϕ_x, is usually determined by the relative method and is obtained by the following equation:

$$\phi_x = \phi_R \cdot \frac{A_R}{A_x} \cdot \frac{I_x}{I_R} \cdot \frac{n_x^2}{n_R^2}$$

where if the quantum efficiency of the standard, ϕ_R, is known the value of the sample may easily be obtained, A is the absorbance of the solution, I is the corrected

emission intensity, n is the average refractive index of
the solution. The above relationship holds good for the
measurement of fluorescence emission spectra of the two
solutions with the same apparatus and a constant
excitation light intensity, I_o.

1.3 Structural effects on fluorescence of Organic
                    Compounds [19,20]

1.3.1 General consideration

Fluorescent organic compounds are usually aromatic
and heteroaromatic molecules. Organic molecules which are
saturated and heterocyclic do not usually exhibit
fluorescence and phosphorence. Aliphatic aldehydes and
ketones, however, do fluoresce, but the fluorescence of
the aliphatic carbonyl has no analytical significance.
The fluorescence yield of most unsubstituted hydrocarbons
increases with the length of the conjugated system,
multiple bonding π electrons further increases the
fluorescence and the emission shifts to longer wavelength.
A rigid and planar structure is highly desirable for a
molecule to be fluorescent. This will reduce vibrational
amplitudes and so the efficiency of the $[S_1] \rightarrow [T_1]$
transition and internal conversion will be reduced.

1.3.2 Nature of the lowest excited singlet state

The lowest states $(\pi^* \rightarrow \pi)$ in the case of an
unsubstituted aromatic hydrocarbon are responsible for
some fluorescence, often very intense, and also
phosphorescence in rigid media. Substituent groups or

hetero-atoms in the ring system introduce $\overset{*}{\pi} \to$ n states, which show very low fluorescence efficiencies, but high phosphorescence efficiencies [19] in rigid media.
Pyridine (I) which contains a hetero-atom, for example, is non-fluorescent due to little overlap between the n and π orbitals compared to benzene which is both fluorescent and phosphorescent.

Compounds containing pyrrole rings, e.g. indole (II) are again both fluorescent and phosphorescent due to $\overset{*}{\pi} \to \pi$

I

Pyridine

II

Indole

$\overset{*}{\pi} \to \pi$

states since the lone pair of electrons in indole conjugates with the aromatic π electrons in the ground state, forbidding $\overset{*}{\pi} \to$ n transitions.

1.3.3 Substituent effects [20]

Fluorescence in substituted benzenes depends on the freedom of the π electrons. The greater the freedom of these electrons, the more fluorescent the molecule.

Ring substitution alters the fluorescence yields and energies of aromatic hydrocarbons. Electron donating

substituents increase the $[S_0] \rightarrow [S_1]$ transition and vice versa, so the emission process competes more effectively with radiationless deactivation. The groups which generally enhance fluorescence, with the exception of CN and O-Ph, belong to the classical ortho, para-directing group of substituents (e.g., OH, OCH_3, NH_2, NCH_3, $N(CH_3)_2$ and F). The most strongly electron-withdrawing substituent, the NO_2 group completely quenches the fluorescence unless the lowest excited state $[S_1]$ is replenished by absorption of relatively low-energy radiation.

Usually, monosubstituted benzenes having meta-directing groups, e.g. NO_2, COOH, CH_2COOH, $\overset{+}{N}(CH_3)_2$ and $NHCOCH_3$ are non-fluorescent. However, the influence of meta-directing groups decreases as the size of the aromatic or heterocyclic system increases.

Fluorescence frequently occurs when inductive and mesomeric effects are toward the ring. Toluene is more fluorescent than benzene due to hyperconjugation of the methyl group with the aromatic ring. Similarly, ethylbenzene is less fluorescent than toluene because the carbon atom next to the ring has only two carbon-hydrogen bonds and thus there is less hyperconjugation. Methylated amino groups when present as ring substituents are found to enhance fluorescence, e.g. dimethylaniline is more fluorescent than monomethylaniline. This is thought to be due to the greater mesomeric contribution of the methylated group. Mesomerism of the amino group is weakened by acetylation and acetanilide is almost non-

fluorescent. Similarly, the inductive effect where
electrons are drawn away from the ring decreases the
fluorescence or quenches it completely. Substitution of a
flexible saturated side group is found to increase the
vibrational and rotational degrees of freedom without
preventing fluorescence. Vibrational resolution, however,
is enhanced by the formation of an additional ring system,
increasing the rigidity of the molecule.

1.3.4 "Heavy atom" effect

Heavy halogen substituents increase the extent of
spin-orbit coupling [21] in aromatic systems. The
electron spins are affected and the singlet states appear
to have a certain amount of triplet character, increasing
the rate of intersystem crossing from $[S_1] \rightarrow [T_1]$.

1.3.5 Effect of metal complexation

Complexation of the heterocyclic organic molecules
with a metal ion stabilizes the nonbonding electrons of a
hetero-atom. The energy required to promote the non-
bonding electrons (n-electrons) to π^* is high and the
lowest excited singlet state changes from $\pi^* - n$ to $\pi^* - \pi$,
causing a weakly or non-fluorescent molecule to become
strongly fluorescent. This allows the determination of
both metal and non-fluorescent organics. This is only
true when the metal is not paramagnetic and/or has a low
atomic number to avoid change in spin i.e. decrease in the
rate of intersystem crossing.

1.4   Fluorescent labelling reactions

1.4.1 Importance and use

Fluorescent labelling is an analytical approach to the detection of non-fluorescent species and the determination of specific compounds in a complex mixture. The technique is simple in concept. Many compounds that are devoid of natural fluorescence or are weakly fluorescent contain reactive functional groups which may be derivatised by a chemical reaction in the presence of a fluorigenic reagent. Fluorescent labelling of target-specific molecules, especially certain drugs, insecticides, antibiotics, amino acids, peptides and other biogenic amines in physiological fluids have proven extremely valuable for quantitative trace analysis.

A reversed-phase high performance liquid chromatography separation of polar amino acids after pre-column derivatisation is the most attractive feature of the fluorigenic labelling technique. The position of the analyte of interest may shift to a unique position in the elution profile.

Fluorescent derivatives formed may exhibit emission at the same wavelength but the absorption spectra may not be totally overlapping, allowing the components to be selectively excited and the fluorescence measured.

1.4.2   Ideal label properties

1.   Fluorescent labelling reactions should be rapid, stoichiometric and preferably in an aqueous medium under mild conditions.

2. Fluorescent derivatives should be non-polar, so that they can be separated and concentrated by organic solvents.

3. The reaction must be with a specific funtional group so that any interference from contaminants is eliminated.

4. The reagent (fluorescent label) itself must be non-fluorescent and the hydrolysis of the excess of reagent in a reaction should not yield any fluorescent products.

5. The Stokes shift i.e. the difference in energy between the absorption and fluorescence maxima must be large. This will avoid any interference from the solvent.

6. The derivatives must be stable so that fluorescence detection may be used in high performance liquid chromatography and flow injection analysis, and anodic electrochemical detection in Polarography.

7. The derivative must strongly absorb radiation of a suitable wavelength and have a high molar extinction coefficient.

1.5 Labels for amines and amino acids

1.5.1 5-Dimethylamino-1-naphthalenesulphonyl chloride

(DNS-Cl)

Dansyl chloride (DNS-Cl) was first used by Weber [22] for the formation of fluorescent conjugates of albumin. Coons and his co-workers [23] applied DNS-Cl successfully

in the fluorescent antibody technique for histochemical localisation of antigens in immunochemistry, virology, and bacteriology [24 - 31].

Dansylation is particularly useful for the analysis of trace compounds based on a fluorigenic reaction with the free and N-terminal amino acids [32].

The reaction with proteins and peptides proceeds under mild conditions. The derivatives formed are stable, resistant to acid hydrolysis and intensly fluorescent. The dansylation reaction can easily be adapted to allow chromatographic and electrophoretic separations to be performed. Dansyl chloride is very reactive to primary and secondary amines, imidazoles and phenols. The reactivity of the specific group towards DNS-Cl is influenced by pH. Labelling of most amino acids, amines and phenols is optimal at pH 9.0 - 10.5. Hydrolysis of the reagent is faster at high pH.

Side reactions: The reaction with DNS-Cl is not free from side reactions. The typical products formed include DNS-NH$_2$, CO, and the aldehyde or ketone molecules [33] (Scheme 1.1). The large excess of DNS-Cl as well as increased pH values in the reaction mixture accelerates the formation of DNS-NH$_2$.

1.5.2 5-Di-n-butylamino-naphthalene-1-sulphonyl chloride (BNS-Cl)

Bansyl chloride (BNS-Cl) has the same structural skeleton as DNS-Cl (Table 1.1) with a di-n-butylamino group replacing the dimethylamino group. The reaction

Scheme 1.1: Proposed side reactions or effect of
increased amount of DNS-Cl and increased pH
values (Scheme from Methods of Biochemical
Analysis, Glick, D. (Ed.), Vol. 18, John
Wiley & Sons, New York/London, 1970, 265.

5-Di-n-butylamino-naphthalene-
1-sulphonyl chloride (BNS-Cl)

4-Chloro-7-nitrobenzo-2-
oxa-1,3-diazole (NBD-Cl)

5-Dimethylamino-1-naphthalene-
sulphonyl chloride (DNS-Cl)

4-Fluoro-7-nitrobenzo-2-
oxa-1,3-diazole (NBD-F)

4-Phenylspiro[furan-2(3H),
1'-phthalan]-3,3'-dione
(Fluorescamine)

o-Phthalaldehyde (OPA)

usually gives fluorescent products that are less polar and more easily extracted from the reaction mixutre [34, 35]. The quantum efficiencies of BNS-derivatives in ethyl acetate are approximately 15% higher than the corresponding DNS-derivatives. BNS-Cl has been of great importance in the formation of suitable derivatives for the identification of biogenic amines by mass spectrometry [34, 35]. BNS-Cl and DNS-Cl both give a mixture of fluorescent products from the derivatisation reaction and are not specific. The reaction is slow.

1.5.3 4-Phenylspiro[furan-2(3H),1'-phthalan]3,3'-dione (Fluorescamine)

Fluorescamine [36] was introduced by Weigele et al· in 1972. This reagent is virtually insoluble in water and is prepared in a nonhydroxylic solvent i.e. acetone. Fluorescamine reacts with compounds that contain nucleophilic functional groups, primary and secondary amines, alcohols and water, but only the reaction with primary amino groups yields fluorescent products [37].

The reaction with primary amines is carried out in an aqueous system at an appropriate pH 8 - 9 using a large excess of the reagent. The excess fluorescamine is hydrolysed [38] to give water soluble products that are non-fluorescent. The intensity of the fluorescence is not influenced by pH changes within the range 4 - 10 but can be affected by the solvent composition.

The mechanism [39] of the reaction involves the rapid, reversible addition of the primary amine across the

double bond of fluorescamine (scheme 1.2), giving a non-
fluorescent intermediate, which rearranges via several

Fluorescamine
non-fluorescent

+ RNH₂

R-NH

non-fluorescent
intermediate

fluorescent derivative

Scheme 1.2: Fluorigenic reaction of fluorescamine with a
primary amine

steps to the desired fluorescent product.

With α and β amino acids the derivative produced
exists in two forms, the hydroxy acid form and the lactone
which are in equilibrium [40] with each other (scheme
1.3). The lactone form is, however, not obtained with
peptides and γ-amino acids which may be due to the
thermodynamically unstable state of larger ring

structures.

Scheme 1.3: Equilibrium between the hydroxy acid and
lactone forms of a fluorescamine α-amino acid
derivative.

1.5.4 <u>4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole</u>

<u>(NBD-F)</u>

NBD-F forms fluorescent derivatives with primary and secondary amines (scheme 1.4). The fluorescence is measured at 530nm by exciting the NBD adducts at 470nm.

Scheme 1.4:   Fluorigenic derivatisation of a amine with
              NBD-F

The reaction of amino and imino acids in 50% ethanol and 0.1M borate buffer at pH 8.0 is usually complete within one minute by heating with NBD-F at 60°C in the dark. The reaction is terminated by the addition of hydrochloric acid. The reaction rate is faster in organic solvents such as ethanol and acetonitrile. The quantum efficiencies of the adduct, NBD-hydroxyproline, varies

from 0.01 in water (pH 9.0) to 0.80 in isobutylketone
[41]. The fluorescent derivative is quite stable. The
fluorescence emission at long wavelengths has made NBD-F a
suitable reagent for biological samples. For example, the
labelled amino acids and imino acids can be separated and
detected at the picomole level of blood platelets [42].

1.5.5   4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole

(NBD-Cl)

NBD-Cl was introduced in 1968 by Ghosh and Whitehouse
[43].   It reacts with both primary and secondary amines.
The reaction takes place by a nucleophilic aromatic
substitution of the 4-chloro substituent, activated by the

NBD-Cl

+   RNH$_2$

Amine

+   HCl

Fluorescent

Derivative

Scheme 1.5:   Fluorigenic reaction of NBD-Cl with a primary
amine.

7-nitro group (scheme 1.5).   The reaction is performed in
a mildly basic aqueous/organic solvent system which is
heated (55 - 80°C) for 30 - 60 minutes [44, 45].   The
derivative formed with the secondary amino acid, proline,

is highly fluorescent. The spectra with λ_{ex} = 475nm and λ_f = 540nm are such that the excitation and emission bands are very broad and appear to overlap to a large extent. The fluorescence quantum efficiency is influenced greatly by the composition of the solvent, being greater in polar organic solvents than in aqueous media [44]. The NBD-amino acid derivatives are susceptible to light induced decomposition [41]. NBD-adducts with aniline, phenol and thiols are, however, weakly fluorescent (or non-fluorescent. The hydrolysis product of NBD-Cl is non-fluorescent.

1.6 Review of the Ortho-phthalaldehyde (OPA) reaction.

1.6.1 History

Roth [46] first showed in 1971 that OPA formed a fluorescent derivative with amino acids in the presence of 2-mercaptoethanol (2-MERC) in an aqueous alkaline medium (pH 8 - 10). This was later confirmed in 1973 by Taylor

III

X = OH for 2-mercaptoethanol

X = H for ethanethiol

(Structure of the derivative)

and Tappel [47]. In 1976, Simons and Johnson [48] presented the first study on the structure of the derivative formed in the preparative reaction of a primary amine with OPA and 2-mercaptoethanol. Based upon the proton NMR spectrum of the OPA-n-butylamine derivative they came to the conclusion that the structure of the product was the 1-alkylthio-2-alkyl-substituted isoindole (III) [48, 49].

In a later investigation Simons and Johnson [50] obtained UV and mass spectral (MS) analysis results of various amines derivatised to confirm the presence of the isoindole ring system.

1.6.2 Mechanism of the reaction of OPA and thiol with a primary amine

The mechanism involves protonation of the imine intermediate followed by a "partially S_N1-like intramolecular reaction" to give a protonated isoindole and finally (IV).

This mechanism accounts for the lack of fluorigenic reaction with secondary amines and primary amines in low pH solutions [46, 51, 52].

1.6.3 Degradation mechanism [50] for the OPA-MERC adduct

The isoindole product of the OPA fluorigenic reaction has been observed to undergo decomposition under the conditions used [53 - 57]. The isoindole, formed with 2-MERC as the thiol compound, decomposes to give an ethylene sulphide polymer and a non-fluorescent 2,3-dihydro-1-H-

Scheme 1.6:   Proposed mechanism [50] of isoindole

formation in the fluorigenic reaction of an

amine with OPA.

Scheme 1.7:   Proposed degradation mechanism for 1-(2-
              hydroxyethylthio)-2-alkylisoindole

isoindole-1-one (V).

This decomposition pattern suggests that the 2-MERC adduct had undergone an intramolecular sulphur-to-oxygen rearrangement [50, 57].

1.6.4 Stability of OPA-derivatives

The stability of the OPA-derivatives has been found to be affected by both the thiol and the amine involved. Glycine, lysine and ornithine derivatives are exceptionally unstable [56]. The stability also seems to depend on the structure of the thiol used in the reaction. The stability is greatly increased [57] by substitution of ethanethiol (ET) for 2-mercaptoethanol (2-MERC). The OPA-alanine derivative [57] which has shown severe degradation when mercaptoethanol is used is stabilised by the use of 3-mercaptopropionic acid. The OPA-alanine adduct with ethanethiol seems to fluoresce, the fluorescence increasing with time.

The instability [58, 59] of the OPA-based derivative according to some workers has been derived from the OPA. A suitable ratio (2 or 3) of 2-MERC to OPA is found to lessen the degree of instability [60]. An excess of 2-MERC seems to enhance the decomposition of the product.

1.7 Applications of OPA in Analysis

1.7.1 General

OPA is suitable for amines [51], amino acids [46, 61] and proteins [48 - 50, 62 - 66] but not for proline and cysteine. The reagent is applicable to compounds

containing an amino group, such as histidine, histamine and various peptides which contain either an N-terminal histidine [46, 51, 61, 63, 67, 68] or a free amino group [47, 52, 65, 69, 70].

Compounds can be detected at the nanogram level by measuring the fluorescence emission of the OPA-derivatives at 450nm using an excitation wavelength of 350nm.

Since OPA gives a fluorescent spot it can be used for developing chromatograms but is limited by the instability of the fluorescence of the spot [70]. The OPA reagent is one of the very few stains for proteins which is water soluble.

Proteins [52, 65, 71] show intense fluorescence when labelled with this reagent despite the presence of peptide bonds. Sulphhydryl groups need to be amino-ethylated [63, 68, 70, 72] before OPA application to peptides and proteins.

OPA reacts with proteins in the presence of 2-MERC to give fluorescent products [52, 73]. The high sensitivity of this reagent for peptides is of much greater importance for studies in protein chemistry where the availability of sample is limited. Membrane proteins and viral proteins can be partially characterised even if they are only available in quantities as small as a picomole. Weidekamm et al· [52] reported the successful detection of as little as 10ng of protein in gel electrophoresis experiments.

Peptidase activity [47, 74] on physiological peptide substrates has been determined based on the OPA reaction. This method is appealing since (a) OPA is soluble and

stable in aqueous solution, (b) the reaction proceeds to completion [50] within several seconds at room temperature [60, 75] and (c) the difference in fluorescence between peptides and amino acids is quite large [46, 47, 60]. The formation of fluorescent derivatives by the OPA reaction with peptides has provided a sensitive assay for such compounds [69, 76]. Proteins of molecular weight greater than 10,000 are generally broken into peptides of a convenient size. The separation and detection of peptides, derived from digestion with trypsin, is described [2] which is simple, sensitive and repeatable.

1.7.2 High performance liquid chromatography

The OPA application in high performance liquid chromatography (HPLC) is another area where it has been of much importance. HPLC with fluorescence detection is probably one of the most suitable means for the analysis of complex mixtures, especially biological materials, since it provides both efficient separation and selective detection. For the fluorimetric determination of amines, amino acids and thiols by HPLC, the procedure followed is either pre- or post-column derivatisation with the fluorigenic reagent.

(a) Post-column labelling procedure

The detection of amino acids separated from a complex mixture poses a great problem, especially for biological samples. There is a growing need to separate and quantitate amino acids for the characterisation and

structural elucidation of peptides and proteins. This is normally achieved by separating amino acid mixtures using ion-exchange chromatography [77] followed by post-column derivatisation with a fluorigenic reagent. If a fluorigenic reagent is used as the post-column derivatising reagent, most amino acids can be resolved with good detection limits.

OPA/2-MERC has recently become popular for the post-column fluorigenic detection of amino acids in conventional amino acid analysers [63, 78] and as a reagent for the estimation of total amino acids in natural waters [79]. The sensitive fluorescence reaction of OPA for α-amino acids [46] has led to the detection of amino acids in column effluents following ion-exchange separation.

Aliphatic amines by nature are not easily detected since they do not fluoresce, nor do they possess chromophores absorbing in the UV region. Fluorophoric labelling of the molecule prior to detection is the best solution to the problem. In one of the investigations [80] aliphatic amines are first eluted from an HPLC column and then subjected to a post-column derivatisation reaction with OPA to allow sensitive fluorescence detection. Various post-column reaction systems have been described for biogenic amines [4, 81] and are found to give good limits of detection.

Various HPLC methods have been developed [82 - 84] for the fluorimetric determination of thiol compounds which play an important physiological role in living cells

[85]. In a recent study L-cystein (CYSH), glutathione, coenzymes etc. are separated by anion-exchange chromatography with gradient elution. After separation they are derivatised with OPA and taurine and pH 10.0 to produce highly fluorescent derivatives. In a similar study with modified post-column derivatisation, HPLC combined with fluorescence detection [86] has been reported as sensitive and suitable for simultaneous determination of thiols and disulphides.

However, the post-column procedure requires specialised equipment and is time consuming. In contrast, the pre-column technique has been developed to improve the efficiency and decrease the analysis time for the resolution of amino acid mixtures.

(b) Pre-column labelling procedure

The application of high performance liquid chromatography to the analysis of biogenic amines has been used for the separation [87] of catecholamines and their metabolites on a reversed-phase column (octadecylsilyl silica) with aqueous, isocratic elution. This approach, however, may not be suitable for measuring biogenic amines unless coupled with fluorescence detection to offer the needed specificity and sensitivity.

An investigation [88] into the use of o-phthal-aldehyde (OPA) for pre-column derivatisation of biogenic amines in samples of plasma, tissue or urine has confirmed the method to be rapid, sensitive and simple for measuring nanogram amounts of histamine, norepinephrine,

octapamine, normetanephrine, dopamine, serotenine and tyramine. Catecholamines and their metabolites in urine and brain tissue [54] gave highly fluorescent products with OPA.

The stability of the OPA-histamine complex and the method of derivatisation have been the subject of numerous studies. Pre-column derivation followed by separation on a reversed-phase HPLC column has been described. One of the derivatisation methods [89] is reported to give a stable and fluorescent OPA-histamine complex, extractable with ethyl acetate.

A very sensitive analytical techniquie is needed to determine aliphatic diamines and polyamines in biological materials. Although there are a large number of methods available, as reviewed by Bachrach [90], including those using HPLC [91], all have some limitations when applied to biological samples. An automated column chromatographic method has been reported for polyamine measurement [92] with fluorescamine, but appears to be inadequate for biological samples. In another automated HPLC technique [93] the fluorigenic OPA reaction with polyamines in physiological fluids has been described to give a 6 to 10 fold increase in sensitivity when compared to ninhydrin.

The importance of amino acids is in diverse and physiologically important areas of biology and medicinal chemistry. There is an essential requirement for an analytical technique to separate and quantitate individual amino acids reliably and rapidly. HPLC using pre-column derivatisation seems to be suitable for amino acid

analysis.

Clinically important amino acids in serum or urine
have been estimated using pre-column derivatisation
followed by reversed-phase HPLC [94]. The separation is
reported to be simple and easy as the derivatives are less
polar than the free amino acids. A rapid and sensitive
method for the quantitative determination of up to 30
amino acids has been described [95] in another
investigation. The method employs pre-column
derivatisation with OPA and separation by reversed-phase
HPLC. The most abundant plasma amino acid, glutamine and
the amide, asparagine, which are not easy to separate from
other amino acids by ion-exchange chromatography, are
found to be well separated by this method. The plasma
imino acids, proline and hydroxyproline, are secondary
amines and do not give fluorescent products. However,
these imino acids have also been converted to suitable
fluorescent products [96] The method involves oxidation
of the imino acid with Chloramine-T to pyrroline which is
finally converted to 4-amino-1-butanol by treatment with
sodium borohydride. The end product is allowed to react
with OPA and then separated by reversed-phase HPLC.
Turnell and Cooper [94] have recently reported the pre-
column OPA/2MERC derivatisation to determine taurine as
well as 30 of the amino acids commonly found in human
serum and urine. These workers used a C-18 column and
included acetonitrile in their solvent system. Using the
pre-column OPA/2-MERC derivatisation of 26 amino acid
standards, a separation has been reported [55] in which

amino acid values were found to be reproducible /̲ with an

average relative deviation of ±1.4%, with a detection

limit of approximately 50 femtomoles.

(c)   Comments

There continues to be a need for the accurate and

sensitive measurement of amines, peptides and proteins in

a variety of samples, especially biological fluids.   It is

possible to determine these using the OPA-based

fluorigenic reaction.   By selecting a suitably buffered

eluent, post-column derivatisation allows fluorescence

detection at low levels.   Using pre-column OPA/2MERC

derivatisation and separation by reversed-phase high

performance liquid chromatography, most amino acids can be

determined in the nano-to-picomole range.

1.8   Modification of o-phthalaldehyde (OPA)

It has been revealed by previous studies that the OPA

reaction product is not very stable.   The wavelength of

emission of the derivatives is at 450nm where there is a

high background emission from biological samples.

There have been attempts to modify the OPA reaction

by altering the mole ratio [60] of 2-MERC to OPA or

replacing 2-MERC by ethanethiol [50, 57] in the

fluorigenic reaction with amines or amino acids.   The

success in this respect has not been as good as was

expected.

The best solution to the problems seems to be in the

modification of the structure of OPA itself.   In recent

years and also during present studies [97] the OPA
structure has been modified by the addition of another
condensed benzene ring system giving a new reagent
naphthalene-2,3-dicarboxaldehyde (NDA). This reagent
reacts in a similar fashion to OPA to give a 1-alkylthio-
2-alkyl-substituted isoindole:

Cyanobenzoisoindole(CBI)

Scheme 1.8:   Fluorigenic reaction of NDA/CN with a primary
               amine.

More recently the NDA fluorigenic reaction has been
modified [98] by replacing the thiol with sodium cyanide
in the fluorigenic derivatisation reaction with an amine.
The end product is a 1-cyano-2-substituted benzo-isoindole
(CBI) (Scheme 1.8). This reaction has successfully been
applied at pH 9, in a borate buffer, to determine
catecholamine [99] using pre-column reversed-phase high
performance liquid chromatography (HPLC). The derivative

exhibits fluorescence at 483nm with excitation at 420nm
and is suitable for both fluorescence and
chemiluminescence detection.

The reagent system, naphthalene-2,3-dicarboxaldehyde
(NDA)/cyanide is useful for the in vivo trace
determination of bioactive peptides and proteins [100].
The NDA/CN method has been found to be effective and
sensitive for lysyl-peptide [101] detection coupled with
pre-column HPLC derivatisation. These studies claimed
that relative to the OPA/thiol system, the NDA method
offered improved fluorescent product stability, enhanced
sensitivity and the absence of apparent fluorescence
quenching for primary amines containing α-amido functional
groups.

1.9 Aims of the present work

The present work is aimed at producing some new
fluorigenic reagents suitable for use as end group reagent
in peptides and proteins, amines and amino acid analysis
and the determination of enzyme activities in different
tissues.

A large number of fluorigenic reagents are available
but suffer from some significant drawbacks relating to
specificity, stability and spectral interferences.

Ortho-phthalaldehyde (OPA) is by far the most
commonly used label for fluorescence detection and is
specific in its reaction with the primary amino group.
Its disadvantages include low stability and its excitation
and emission wavelengths ($\lambda_{Ex} = 340nm$ and $\lambda_{Em} = 450nm$)

which overlap those of serum [102].

In the present study, the aim is to synthesise polyaromatic dialdehyde label molecules. These labels are expected to have greater stability, high quantum yields, absorb and fluoresce at appropriate wavelengths because of specific structural criteria. Since the conjugation of the molecule will be increased, the labels will absorb in the visible region of the spectrum, which will not only avoid the use of ultraviolet sources, a cause of instrumental instability, but will also decrease the interference from impurities. Additionally the emission of fluorescence at higher wavelengths is expected, thus making the labels suitable for biological systems especially in the case of fluorescence immunoassays where background interference would be avoided.

CHAPTER 2   MATERIALS, INSTRUMENTATION,

&

GENERAL EXPERIMENTAL PROCEDURES

2.1   <u>Materials</u>   <u>Source of Supply</u>

<u>Hydrocarbons</u>

Anthracene	BDH Chemicals Ltd.
2,3-Benzanthracene	Aldrich Chemical Co. Ltd.
9-Phenylanthracene	Aldrich Chemical Co. Ltd.

<u>Oxidising agents</u>

Osmium tetroxide	Aldrich Chemical Co. Ltd.
Periodic acid	Aldrich Chemical Co. Ltd.

<u>Fluorescent labels</u>

Ortho-phthalaldehyde	Aldrich Chemical Co. Ltd.
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	Aldrich Chemical Co. Ltd.
Fluorescamine (Fluram)	Aldrich Chemical Co. Ltd.
5-Dimethylamino-1-naphthalene sulphonyl chloride	Sigma Chemical Co. Ltd.
4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole	Sigma Chemical Co. Ltd.

<u>Amines, Drugs and Amino acids</u>

L-Proline	Sigma Chemical Co. Ltd.
L-Threonine	Calbiochem Ltd.
L-Leucine	Calbiochem Ltd.
L-Glutamic acid	Calbiochem Ltd.
L-Isoleucine	Calbiochem Ltd.
L-Methionine	Calbiochem Ltd.
L-Cysteine	Calbiochem Ltd.
L-Aspartic acid	Calbiochem Ltd.
Glycine	Fisons Ltd.
Histamine dihydrochloride	Aldrich Chemical Co. Ltd.
n-Propylamine	Aldrich Chemical Co. Ltd.
L-Alanine	Aldrich Chemical Co. Ltd.
Taurine	Aldrich Chemical Co. Ltd.

Materials	Source of Supply
L-(+)-Arginine	Aldrich Chemical Co. Ltd.
Spermidine	Aldrich Chemical Co. Ltd.
L-Serine	Sigma Chemical Co. Ltd.
L-Tryptophan	Sigma Chemical Co. Ltd.
Tyrosine	Sigma Chemical Co. Ltd.
L-β-Phenylalanine	Sigma Chemical Co. Ltd.
Amino acid calibration standards	Calbiochem Ltd.

Proteins

Protein standard solution	Sigma Chemical Co. Ltd.
Bovine albumin	Sigma Chemical Co. Ltd.
Avidin	Sigma Chemical Co. Ltd.
Cytochrome C	Sigma Chemical Co. Ltd.
Myoglobin	Sigma Chemical Co. Ltd.
Albumin (human serum)	Calbiochem Ltd.

Rabbit immunoglobulins
to human serum

Human albumin economy	DAKO-immunoglobulins Ltd.

Solvents

Benzene	Aldrich Chemical Co. Ltd.
Chloroform	Aldrich Chemical Co. Ltd.
Ethyl acetate (spectrophotometric grade)	Aldrich Chemical Co. Ltd.
1,4-Dioxane	Aldrich Chemical Co. Ltd.
Absolute alcohol	Aldrich Chemical Co. Ltd.
N,N-Dimethylformamide	Aldrich Chemical Co. Ltd.
Dimethylsulphoxide	Aldrich Chemical Co. Ltd.
Acetone	Aldrich Chemical Co. Ltd.
Methanol (HPLC Grade)	Fisons Ltd.
Pyridine	Fisons Ltd.
Acetonitrile	Fisons Ltd.
Petroleum ether (40-60°)	Carless Solvents Ltd.
Petroleum ether (60-80°)	Carless Solvents Ltd.

Chromatographic Media

Sephadex G-25	Pharmacia Ltd.

Materials	Source of Supply
Silica gel, 100 - 200 mesh (Chromatographic grade)	Aldrich Chemical Co. Ltd.

Thiols

Dithiothreitol	Aldrich Chemical Co. Ltd.
Ethanethiol	Aldrich Chemical Co. Ltd.
2-Methyl-2-propanethiol	Aldrich Chemical Co. Ltd.
1-Methyl-1-propanethiol	Aldrich Chemical Co. Ltd.
3-Mercaptopropionic acid	Sigma Chemical Co. Ltd.
2-Mercaptoethanol	Sigma Chemical Co. Ltd.

Other chemicals, reagents etc.

α-Cyclodextrin	Sigma Chemical Co. Ltd.
β-Cyclodextrin	Sigma Chemical Co. Ltd.
ATP (Adenosine 5'-Triphosphate disodium salt)	Sigma Chemical Co. Ltd.
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co. Ltd.
γ-Cyclodextrin	Aldrich Chemical Co. Ltd.
d-Biotin (Vit. H)	Aldrich Chemical Co. Ltd.
Brij-35 (Polyoxy-ethylene lauryl ether)	East Anglia Chemicals
Sodium sulphite, A.R. (hydrated, $Na_2SO_3 \cdot 7H_2O$)	Fisons Ltd.
Disodium tetraborate	Fisons Ltd.
Sodium hydroxide	Fisons Ltd.
Disodium hydrogen ortho-phosphate (dihydrate)	Fisons Ltd.
Mono-sodium dihydrogen ortho-phosphate	Fisons Ltd.
Citric acid	Fisons Ltd.
Quinine Sulphate	BDH Chemicals Ltd.
Perchloric acid 60% (spectrosol)	BDH Chemicals Ltd.

All other reagents and buffer salts used were of Analar or equivalent grade.

2.2 Instrumentation

2.2.1 Absorbance measurements

A Pye-Unicam SP8-100 and Shimadzu UV-160 spectrophotometers were used to record absorption spectra of the solutions. Another Pye-Unicam instrument, a PU-8600, a UV-visible single beam spectrophotometer was also used to measure absorbance. This instrument was fitted with an automatic light source (tungsten-halogen and deuterium arc sources) and filter change. Microprocessor control and data handling facilities, with the provision for programme storage, are features of the instrument.

For fixed wavelength measurements using the PU-8600 the instrument was zeroed with a reference blank solution. The reference solution was then replaced by the sample and the absorbance measured.

2.2.2 Fluorescence measurements

Fluorescence measurements were generally made using an MPF-44B spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, Bucks). The spectrofluorimeter was fitted with a differential corrected spectra unit (DSCU-2) and an R928 photomultiplier making it suitable for recording corrected excitation and emission spectra. The DSCU-2 unit was calibrated using a Rhodamine B counter (as per the instructions in the operating manual supplied by the manufacturer).

Synchronous spectra and second derivative spectra were obtained using the DSCU-2 unit.

Fluorescence and phosphorescence measurments were

made using a Baird-Atomic model SFR 100 recording spectrofluorimeter. This fluorimeter had been adapted for phosphorescence measurements using a rotating phosphoroscope with provision for cooling of the sample with liquid nitrogen. An Apple II microcomputer was interfaced to the fluorimeter to collect data and record fluorescence and phosphorescence spectra. Excitation and emission correction factors were collected to allow calculation of corrected spectra.

A second Baird Atomic fluorimeter was available for the measurement of variable angle synchronous excitation spectra. The instrument had been modified to allow variation of the relative scan speeds of the excitation and emission monochromators.

Quantum yields were measured using an LS-5 luminescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks.). The instrument was fitted with a R928 red sensitive photomultiplier and data were recorded using PECLS II software and a Perkin-Elmer model 3600 data station. The PECLS II software allowed the determination of second derivative spectra, measurement of fluorescence lifetime and three dimensional emission and excitation spectra. All spectra were obtained using a 1cm silica cuvette (thermostatted at 25 ±1°C). Measurement of fluorescence at fixed wavelengths was achieved by integrating the signal over a period of several seconds.

Fluorescence measurements for all qualitative and quantitative purposes were made either on a Perkin-Elmer model MPF-44B spectrofluorimeter or a Baird Atomic model

SFR100 using 1cm path length silica cuvettes (thermostatted, if required).

2.2.3 Measurement of pH

A Corning digital pH meter model PT1-5 combined with a glass electrode was used for all the pH measurements. The pH meter was always standardised with buffers of known pH before measurement of the pH of a sample solution. The range 0-14 pH units could be measured with an accuracy of ±0.001.

2.3 Experimental

Fluorescence Studies, General Procedure:

2.3.1 Preparation of solutions

(a) Fluorigenic reagent solutions

A suitable quantity of the fluorigenic reagents, naphthalene-2,3-dicarboxaldehyde (NDA), 1-phenylnaphthalene-2,3-dicarboxaldehyde (∅NDA), anthracene-2,3-dicarboxaldehyde (ADA) or o-phthalaldehyde (OPA) was dissolved in a minimum volume of ethanol and made up to the required volume with either distilled water or 0.025M sodium borate buffer, pH 10 to obtain a solution of known molarity.

(b) Cyclodextrin solutions

An appropriate quantity of the cyclodextrin was dissolved in distilled water using ultra sound and made up to the required volume with water to obtain a solution of known concentration.

(c)   Thiol solution

Different thiol solutions (0.1ml/100ml solution) were freshly prepared either in ethanol or distilled water before use.

(d)   Borate buffer (pH 10)

Borax (4.8g) and sodium hydroxide (0.80g) were dissolved in distilled water, stirred well to obtain a clear solution and made up to 1 litre with distilled water.

2.3.2   Fluorescent labelling of amines, amino acids and proteins

(a)   Procedure (Labels: NDA, øNDA and ADA)

A dilute solution of the sample/ (0.2-0.3ml) was treated with 1ml of 10.9mM β-cyclodextrin (β-CD). Borax-sodium hydroxide buffer (0.6ml, pH 10.0), 0.1ml aqueous thiol solution (0.1%v/v) were then added, followed by the addition of the suitable fluorigenic reagent in slight excess over the sample. The final volume was then made up to 5ml with water. The derivative was then excited at a suitable wavelength for fluorescence emission measurement.

(b)   Procedure (Label: OPA)

The procedure was the same as in section 2.3.2(a) but omitting β-CD from the reaction solutions.

CHAPTER 3   SYNTHESIS OF POLYAROMATIC DIALDEHYDES

3.1   Introduction

There are only a few methods available for synthesis
of polyaromatic dialdehydes and are mainly for
naphthalene-2,3-dicarboxaldehyde (NDA). F. Weygand et al·
[104], in 1950, and K. Yagi [105], in 1951, reported the
synthesis of NDA using naphthalene-2,3-dicarboxylic acid
as the starting material. However, the synthesis of NDA
from the dicarboxylic acid is said to be time consuming
and gives a poor yield [106]. In an investigation in 1956
                H.
by W. Ried and/Bodem [106], NDA was synthesised by the
bromination of 2,3-dimethylnaphthalene to 2,3-di-(dibromo-
methyl)-naphthalene followed by its hydrolysis   with
either sodium acetate or potassium oxalate to give the
desired dialdehyde.

In the present work for the synthesis of polyaromatic
dialdehydes, (i) 1-phenylnaphthalene-2,3-dicarboxaldehyde
(∅NDA), (ii) anthracene-2,3-dicarboxaldehyde (ADA)
and (iii) naphthalene-2,3-dicarboxaldehyde (NDA), we
envisaged using the method of Cook and Schoental [97]·For NDA
 this involves periodate cleavage of 1,2,3,4-tetrahydroxy-
1,2,3,4-tetrahydroanthracene, obtainable from anthracene
by its oxidation with osmium tetroxide and hydrolysis of
the osmate complex [103].

3.2   Reaction Scheme

Polyaromatic hydrocarbons in a non-aqueous solvent
containing a little pyridine react readily with osmium

tetroxide with the formation of a dark brown, pyridine-
osmic ester complex [103, 107] shown in structure (vi) for
anthracene.   This osmate complex, on hydrolysis with
sodium sulphite, yields 1,2,3,4-tetrahydroxy-1,2,3,4-
tetrahydro derivative, (vii) (tetrol) of the original

vi

vii

hydrocarbon.   Reaction of the tetrol with periodic acid
gives the desired dialdehyde, (viii) [97].

3.3   Discussion

3.3.1   Synthesis of Naphthalene-2,3-dicarboxaldehyde
(NDA)

It was anticipated that the compound 1,2,3,4-
tetrahydroxy-1,2,3,4-tetrahydroanthracene, tetrol, (vii)
would be available from anthracene by its oxidation with
osmium tetroxide and the hydrolysis of the osmate complex
(vi).   The formation of the tetrol was supported by the
determination of its melting point 241°C, decomposition
(literature value 241°C, decomposition) [103] and
elemental analysis.

The cleavage of tetrol by periodate was very straight
forward and the product (viii) with melting point 131°C
(literature 131 - 132°C) [97] was very easy to obtain.

viii

The elemental composition agreed well with the molecular
formula of NDA (viii), $C_{12}H_8O_2$.

The compound (viii) was supported by further
spectroscopic studies.   The IR spectrum showed a band at
$2860cm^{-1}$ which is assigned to the C-H absorption of an
aldehyde.   In addition, there is a band at $1690cm^{-1}$ (C=O
group) and bands at $1625cm^{-1}$ and $1450cm^{-1}$ (aromatic C=C).

The ^{1}H NMR spectrum gave an important singlet at $\delta 10.75$ equivalent to 2H, indicating the presence of two aldehyde groups. The 2,3-positions of the aldehyde are supported by the appearance of a 2H singlet at $\delta 8.55$ assigned to the 1- and 4-H protons. The other protons gave rise to a multiplet around $\delta 7.55-8.10$ suggesting a condensed benzene system. The mass spectrum showed a molecular ion, M^+ at m/z 184 and fragment ions at m/z 155 and 126 corresponding to M-CHO$^+$ and M-2CHO$^+$ respectively. This data supports the assignment of the structure as (viii).

3.3.2   Synthesis of 1-Phenylnaphthalene-2,3-
        dicarboxaldehyde (ϕNDA)

(i)   Tetrol formation

The formation of 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydro-9-phenylanthracene, tetrol (x), was first examined. It was found that (x) was formed by the hydrolysis of an osmic ester complex of a tetrol (ix) with sodium sulphite. The product (x) was crystallised and had m.p. 214°C with decomposition. The elemental analysis of (x) confirmed the product was tetrol.

(ii)   Periodate cleavage of tetrol to ϕNDA

Already in the present work, in section 3.3.1, the periodic acid treatment of a 'tetrol' has been proved to be satisfactory. The tetrol (x) was oxidised under similar conditions to those previously employed and gave the final product (xi).

The identity of this dialdehyde with m.p. 104-105°C was proved by its elemental analysis and supported by the

following spectroscopic studies. The IR spectrum of the 1-phenylnaphthalene-2,3-dicarboxaldehyde (xi) exhibited an absorption band for C=O at $1700 cm^{-1}$ and a C-H band at $2880 cm^{-1}$. Bands at $1620 cm^{-1}$ and $1460 cm^{-1}$ indicate the aromatic character. The ^1H NMR spectrum further

ix

x

xi

supported this finding. A singlet at $\delta 9.95$ is assigned to the two protons from the two aldehyde groups. The group of protons giving rise to signals at $\delta 7.25-8.15$ are aromatic and is composed of four protons from the condensed ring and five from the phenyl group attached directly to the benzene ring. A singlet at $\delta 8.4$ is due to 4-H proton. Mass spectral analysis further supported the structure. A molecular ion peak at m/z 260 is due to

compound (xi) with molecular formula $C_{18}H_{12}O_2$. The other

fragment ions at m/z 231, 202 and 125 corresponding to

$M-CHO^+$, $M-2CHO^+$, and $M-2CHO-C_6H_5^+$ respectively confirmed

the presence of two aldehyde groups and a phenyl group

substituent. This data supports the assignment of the

structure as (xi).

3.3.3 Synthesis of Anthracene-2,3-dicarboxaldehyde (ADA)

The preparation of the dialdehyde (xii) involved

'tetrol' formation and its periodate cleavage, as

described in the previous section 3.3.1. The tetrol

(xiii) obtained from an osmic ester complex (xiv) was

found to have a m.p. 250°C with decomposition and the

elemental analysis was in agreement with the molecular

formula, $C_{18}H_{16}O_4$. This was utilised for the synthesis of

anthracene-2,3-dicarboxaldehyde (ADA) (xii). Since the

tetrol (xiii) did not dissolve completely in water, water-

alcohol (9:1 parts by volume) was necessary to dissolve it

xii

before reaction with periodic acid. The final product

after periodate cleavage was the anthracene-2,3-

dicarboxaldehyde (xii) which was confirmed by the IR, [1] H

NMR, and MS studies and elemental analysis.

The IR spectrum showed the aromatic C-H absorptions

xiii

xiv

of an aldehyde at 2890cm^{-1}, a C=O absorption band at
1670cm^{-1}, and C=C skeletal absorption bands (condensed
benzene system) at 1580cm^{-1} and 1530cm^{-1}. The ^{1}H NMR
spectrum further supported the presence of aldehyde groups
in the compound since a 2H singlet appeared at δ10.80.
The 2,3-positions of the aldehyde are supported by the
presence of a 2H singlet at δ8.65 assigned to the 1- and

- 51 -

4-H protons. The other protons of the condensed benzene system yielded a multiplet around $\delta 7.85$-8.35 equivalent to 6H. The mass spectrum showed a peak at m/z 235 which may be assigned to the M+1^{+} ion. Other ions of relative significance are at m/z 206 (M+1-CHO) and m/z 177 (M+1-2CHO) which corroborate the presence of two aldehyde groups which may be lost during fragmentation. Elemental analysis further confirmed it to be the aldehyde (xii).

3.4 Experimental

Solvents used were distilled following the standard procedures. Solutions were dried over magnesium sulphate and evaporated under vacuum. Infrared (IR) spectra were obtained using Perkin-Elmer models 457 and 1310 spectrophotometers. The spectra were obtained as solid dispersions in potassium bromide pellets or as suspensions in NUJOL between sodium chloride plates. ^{1}H NMR spectra were recorded using a 60MHz Varian EM 360A spectrometer or 90MHz Perkin-Elmer R32 spectrometer in solutions of deuterochloroform and/or DMSO d$_6$ with tetramethylsilane (TMS) as the internal reference.

The following abbreviations are used in the presentation of these spectra:
s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad.

Mass spectra (MS) were recorded on a Kratos MS80 mass spectrometer using a DS-55 data system.

Melting points were measured on a Gallenkamp melting point apparatus MF-370 and are uncorrected. The elemental

analyses were carried out by the Microanalytical
Laboratory, Department of Chemistry, University of
Manchester.

Synthesis of Naphthalene-2,3-dicarboxaldehyde, NDA (viii)

(i)   Formation of 1,2,3,4-tetrahydroxy-1,2,3,4-tetra-
        hydroanthracene (tetrol)

A solution of anthracene (0.445g, 0.0025mol), osmium
tetroxide (1.270g, 0.005mol) and pyridine (0.79g, 0.01mol)
in benzene (25ml) was kept at room temperature for 15-20
days with occasional shaking. A brown osmic ester complex
(a solid matter) formed and was separated by filtering.
The product was washed with benzene and air dried (2.1g).

The complex (1.0g) was hydrolysed by heating on a
water-bath for 1 hour with a solution of crystalline
sodium sulphite (5g) in water (25ml) and ethanol (25ml).
Sufficient ethanol was then added to precipitate out the
excess sodium sulphite in solution. This was then
filtered and the filtrate stored. The black residue on
the filter paper was extracted several times with hot
ethanol, the extracts combined with the original filtrate,
and the combined liquors were then allowed to stand at
room temperature overnight. The excess sodium sulphite
separated out from the solution as a solid at the bottom
of the container. This was then filtered using a Whatman
filter No. 40. The filtrate was concentrated under
reduced pressure.

A solid separated out after cooling. This was re-
crystallised from water to yield almost colourless fine

solid particles (80mg) of 1,2,3,4-tetrahydroxy-1,2,3,4-
tetrahydroanthracene (vii) m.p. 241°C with decomposition
(lit. 241°C decomposition).

Found,   C, 68.2; H, 5.6%, $C_{14}H_{14}O_4$
Requires C, 68.3; H, 5.7%.

(ii) <u>Periodate cleavage of 1,2,3,4-tetrahydroxy-1,2,3,4-
      tetrahydroanthracene to naphthalene-2,3-dicarbox-
      aldehyde, NDA</u>

To a hot solution of the tetrol (vii) (80mg) was
added a solution of periodic acid (1.2g) in water (8ml).
Crystalline dialdehyde (viii) (50mg) was obtained as the
reaction product after separating by filtration and drying
in a desiccator. Recrystallisation from light petroleum
(b.p. 60-80°C) gave colourless needles, m.p. 131-2°C (lit.
131-2°C).

IR (KBr) 2860, 1690, 1625, 1470, 1450, 1325, 1285, 1260,
         1185, 1160, 1100, 940, 860, 790, 770, 750cm^{-1}.

^{1}H NMR (CDCl$_3$) δ7.75-8.25 (m, 4H), 8.55 (s, 2H),
10.75 (s, 2H)

MS (EI) 184 (M$^+$, 65.5%), 155 (M-CHO, 100%),
        126 (M-2CHO, 21.7%), 125 (M-2CHO-H, 2.2%),
        101 (M-2CHO-H-2C, 7.8%), 87 (C$_7$H$_3$, 4.2%),
        77 (C$_6$H$_5$, 10.6%), 63 (C$_5$H$_3$, 8.5%),
        51, (C$_4$H$_3$, 16.4%).

Found   C, 77.4; H, 4.3%, $C_{12}H_8O_2$

Requires C, 78.2; H, 4.4%

Synthesis of 1-Phenylnaphthalene-2,3-dicarboxaldehyde, ∅NDA

(i) Formation of 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydro-
     9-phenylanthracene (tetrol)

A solution of 9-phenylanthracene (0.635g, 0.0025mol),
osmium tetroxide (1.27g, 0.005mol) and pyridine (0.79g,
0.01mol) in benzene (30ml) was kept at room temperature
for 15-20 days with occasional shaking. The solid osmic
ester complex separated out and was filtered, washed with
benzene and air dried (2.4g).

The complex (1.0g) was hydrolysed with a solution of
crystalline sodium sulphite (5g) in water (30ml) and
ethanol (30ml) by heating on a water-bath for 1½ hours.

For the recovery of 1,2,3,4-tetrahydroxy-1,2,3,4-
tetrahydro-9-phenylanthracene (tetrol) from the hydrolysed
mass, the procedure followed was the same as discussed for
NDA. The tetrol (x) was recrystallised from water and was
obtained as a deep yellow solid (90mg), m.p. 214°C with
decomposition

Found   C, 76.2; H, 4.4%, $C_{20}H_{18}O_4$

Requires C, 74.5; H, 5.59%

(ii) Periodate cleavage of 1,2,3,4-tetrahydroxy-1,2,3,4-
     tetrahydro-9-phenylanthracene (tetrol) to 1-phenyl-
     naphthalene-2,3-dicarboxaldehyde (∅NDA)

To a hot solution of the tetrol, (x) (70mg) in water

(20ml) a solution of periodic acid (1.2g) in water (5ml)
was added. After cooling the mixture was kept overnight
in the fridge. A sticky solid was obtained. A little
chloroform was added to this until it dissolved. The
solution was then transferred to a clean porcelain dish
and allowed to stand in the open air. After evaporation
of the chloroform an orange solid (xi) (43mg) was
obtained, m.p. 105°C.

IR (KBr) 2880, 1800, 1700, 1620, 1460, 1325, 750, 700cm^{-1}

^{1}H NMR (CDCl$_3$) δ7.25-8.15 (m, 9H), 8.4 (s, 1H),
9.95 (s, 2H)

MS (EI) 260 (M^{+}, 41.58%), 231 (M^{+}-CHO, 100%),

      202 (M^{+}-2CHO, 94.3%), 125 (M^{+}-2CHO-C$_6$H$_5$, 1.75%),

      101 (C$_8$H$_5$, 45.1%), 88 (C$_7$H$_4$, 18.9%),

      77 (C$_6$H$_5$, 6.08%), 76 (C$_6$H$_4$, 5.68%),

      51 (C$_4$H$_3$, 4.96%)

Found   C, 83.6; H, 3.8%, C$_{18}$H$_{12}$O$_2$
Requires C, 83.0; H, 4.6%

Synthesis of anthracene-2,3-dicarboxaldehyde, ADA (xii)

(i)   Formation of 1,2,3,4-tetrahydroxy-1,2,3,4-tetra

      hydrobenzanthracene (Tetrol)

     A solution of 2,3-benzanthracene (0.570g, 0.0025mol),
osmium tetroxide (1.270g, 0.005mol) and pyridine (0.79g,
0.01mol) in benzene (25ml) was kept at room temperature

for 15-20 days with occasional shaking. The osmate
complex (solid) (xiv) was filtered, washed with benzene
and then air dried (2.5g).

The osmate complex (1.0g) was hydrolysed by heating
on a water-bath for 1½ hours with a solution of
crystalline sodium sulphite (5g) in water (25ml) and
ethanol (25ml).

For the recovery of 1,2,3,4-tetrahydroxy-1,2,3,4-
tetrahydrobenzanthracene, tetrol (xiii), the procedure
followed was the same as described previously (section
3.4). The tetrol obtained (70mg) was recrystallised
from water as an orange solid, m.p. 250°C, decomposition.

Found   C, 72.3; H, 6.2%, $C_{18}H_{16}O_4$
Requires C, 72.9; H, 5.4%

(ii) Periodate cleavage of 1,2,3,4-tetrahydroxy-1,2,3,4-
     tetrahydrobenzanthracene to anthracene-2,3-
     dicarboxaldehyde (ADA)

To a hot solution of 1,2,3,4-tetrahydroxy-1,2,3,4-
tetrahydrobenzanthracene, tetrol (xiii) (70mg) in a
mixture of water (20ml) and ethyl alcohol (5ml) was added
a solution of periodic acid (1.2g) in water (5ml). The
periodic treated solution was concentrated to a minimum
volume under reduced pressure and was placed in the fridge
overnight. A light yellow solid separated out and was
recrystallised from water to a light yellow product (xii)
(40mg), m.p. 195°C

IR (KBr) 2890, 1670, 1580, 1535, 1450, 1320, 1290, 1210,
         1130, 1080, 950, 790, 710, 580cm^{-1}.

^{1}H NMR (CDCl$_3$) δ7.85-8.35 (m, 6H), 8.65 (s, 2H),

10.8 (s, 2H)

MS (EI) 235 (M+1, 100%), 234 (M$^+$, 27.26%),
        206 (M+1-CHO, 49.2%), 177 (M+1-2CHO, 10.9%),
        152 (C$_{12}$H$_8$, 30.0%), 151 (C$_{12}$H$_7$, 67.9%)
        125 (C$_{10}$H$_5$, 6.4%), 99 (C$_8$H$_3$, 6.9%),
        77 (C$_6$H$_5$, 7.4%), 75 (C$_6$H$_3$, 32.9%),
        74 (C$_6$H$_2$, 19.3%), 63 (C$_5$H$_3$, 4.8%),

Found   C, 81.0; H, 4.5%, C$_{16}$H$_{10}$O$_2$
Requires C, 82.0; H, 4.3%

CHAPTER 4 POLYAROMATIC DIALDEHYDE COMPOUNDS AS NOVEL FLUORESCENT LABELS

4.1 Introduction

There are at present commercially available two universal fluorigenic reagents which have attracted much attention. These are o-phthalaldehyde (OPA) [46] and fluorescamine [36] which are non-fluorescent until they are reacted with a primary amino group. Of these two OPA has been reported to be more sensitive [63] than fluorescamine but its application, especially in energy transfer or fluorescent probe studies, has been limited by the instability of the fluorescent products.

In the present work the newly synthesised fluorigenic reagents, naphthalene-2,3-dicarboxaldehyde (NDA), 1-phenylnaphthalene-2,3-dicarboxaldehyde (∅NDA) and anthracene-2,3-dicarboxaldehyde (ADA) have been studied to evaluate the fluorescent properties of the derivatives formed with simple amines, amino acids and proteins. The studies include the effect of pH, solvents, inorganic salts, detergent, cyclodextrins and various thiols on the fluorescence intensity and stability of their products. The fluorescence quantum efficiency of the OPA, NDA, ∅NDA, and ADA-amino acid derivatives have also been measured. Also some studies have been undertaken to determine the possibility of using NDA, ∅NDA, and ADA as fluorescent labels (fluorigenic reagents) for proteins.

4.2 Experimental

(a) Stock solutions and General procedure

Stock solutions (10^{-3}, 10^{-4} or 10^{-5}M) of amino acids were prepared in double or triply distilled water and stored in the fridge. The amino acids and proteins were labelled as described in section 2.3.2. 2-methyl-2-propanethiol (2MPT) was used unless stated otherwise for NDA, ϕNDA, and ADA-derivatives. For labelling with OPA the thiol used was usually ethanethiol except in cases where it is mentioned. The fluorescence quantum efficiency (ϕ) of the NDA, ϕNDA, and ADA-derivatives with glycine (10^{-4}M) were determined by the comparative method, with quinine sulphate as a standard ($\phi_R = 0.59$). The absorbance values were close to 0.02 for both the standard quinine sulphate and sample solutions. For the effect of cyclodextrins (α, β and γ) on the fluorescence intensity of the derivatives, the quantity of dextrin varied from 1.0 to 1.38mol/ml of glycine or any other amino acid.

The background fluorescence arising especially from the reagents and buffer was taken into account for use in correcting all fluorescence measurements.

(b) Amount of β-cyclodextrin for a fluorigenic reaction

0.3ml of 10^{-4}M glycine solution was put into each of a number of 5ml volumetric flasks. To each of these was added 10.9mM solution of β-cyclodextrin in varying amounts. 0.6ml borate buffer, pH 10.0 was then added, followed by 0.1ml (0.1%v/v) of 2-methyl-2-propanethiol,

and 0.4ml of 10^{-4}M naphthalene-2,3-dicarboxaldehyde.
Finally, the volume was made up to the mark with distilled
water and shaken well. Fluorescence measurements were
then made on all of the derivatives using a Perkin-Elmer
MPF-44B spectrofluorimeter.

(c) Effect of detergent, organic solvents and inorganic
     salts on the fluorescence properties of the
     derivatives

The derivatisation method was as described in section
2.3.2 except that detergent, Brij-35 (5.18 x 10^{-4}mole/l),
organic solvents (38-40%) and inorganic salts (10^{-2}mole/l)
were introduced into separate derivatisation reactions.
NDA, ∅NDA and OPA were used as fluorigenic reagents for
the derivatisation of glycine (10^{-4}M).

(d) Stability of the fluorescent derivatives

Fluorescent derivatives of glycine (10^{-4}M solution)
were formed with various fluorigenic reagents (NDA, ∅NDA,
and ADA) according to the procedure in section 2.3.2. The
fluorescence intensities of these derivatives were then
measured at regular time intervals until the fluorescence
diminished to its minimum value.

(e) Effect of thiol on the fluorescence intensity and
     stability of the derivatives

Following the standard general procedure (section
2.3.2) glycine (10^{-4}M) was derivatised with NDA, ∅NDA, ADA
or OPA in the presence of different thiols, 2-mercapto-

ethanol, ethanethiol, 3-mercaptopropionic acid or 2-
methyl-2-propanethiol. These derivatives were then used
for obtaining spectra and measuring fluorescence
intensity.

4.3 Results and Discussion

4.3.1 Fluorescent derivatives of amino acids using NDA, øNDA, and ADA as labels

(a) Reaction

Naphthalene-2,3-dicarboxaldehyde (NDA), 1-phenyl-
naphthalene-2,3-dicarboxaldehyde (øNDA), and anthracene-
2,3-dicarboxaldehyde (ADA) were observed to form intensely
fluorescent products, isoindoles, after reaction with
amino acids or primary amines in alkaline solution and in
the presence of strong reducing agent, such as ethanethiol
or 2-methyl-2-propanethiol. Since these labels are
dialdehydes like OPA the reaction products are assumed to
be the isoindoles (xv-xvii). The reaction of NDA with
arginine and glycine was found to take place in 1:1 molar
ratio (Figure 4.1(a)) at pH 10.0. The fluorescence of the
NDA, øNDA and ADA-amino acid derivatives was found rising
to a maximum intensity after 5-8 minutes (Figure 4.1(b))
with the excitation maximum occurring at 462nm (or 470nm
for ADA) and maximum emission at 520nm, 520nm and 640nm
respectively. Figure 4.2 shows the excitation and
emission spectra of the NDA-glycine derivative. The
maximum emission occurs at 550nm which is due to the
effect of the thiol structure (see section 4.3.2.3).

Figure 4.1(a): Amino acid:NDA mole ratio curve

(Amino acid, glycine and arginine)

Figure 4.1(b):   Reaction time curves: NDA-glycine,

ØNDA-glycine and ADA-glycine.

Figure 4.2: Excitation and emission spectra of NDA-glycine derivative obtained with ethane-thiol.

xv   xvi   xvii

(b) Linearity and limit of detection

The derivatives of a number of amino acids with NDA
indicated that their fluorescence response was linear with
increasing concentration (Figure 4.3). The limit of
detection was 0.63nmol/ml (3.72 x 10^{-8}g/ml) glycine,
obtained from the experiment shown in Figure 4.4. The
reaction is thus quantitative and can be applied to
complex samples coupled with a suitable separation method.

(c) Quantum efficiency

A number of amino acid derivatives were formed in
solution using the NDA, ∮NDA and ADA labels; their quantum
efficiencies were measured and compared to the OPA
derivative.   The results are presented in Table 4.1.
Since the sample solutions were dilute with an absorbance
value of 0.02, any effect from the re-absorption of the

Table 4.1: Fluorescence quantum efficiencies

(Abbreviation: QS, Quinine sulphate)

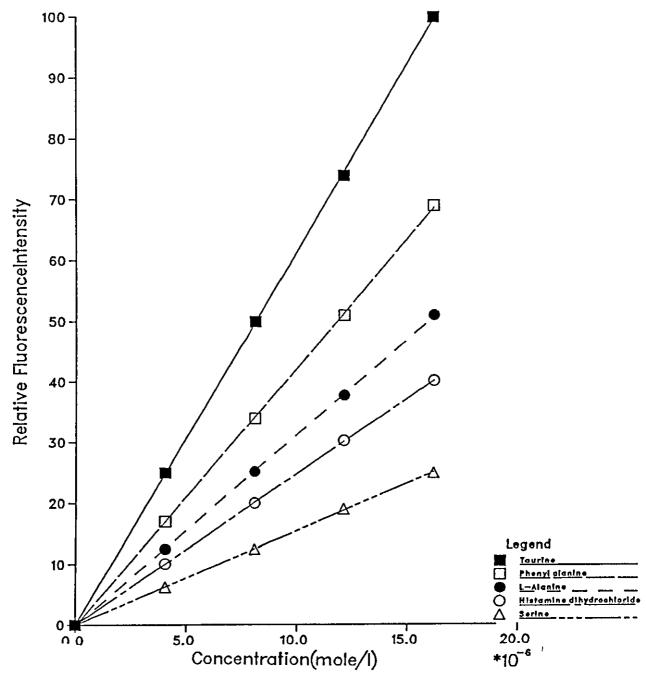| Fluorescent derivative	Solvent system	Excitation (nm)	Emission (nm)	Quantum efficiency (ϕ)	Standard (ϕ_R)	Lit. Values	Ref.
NDA-Gly	water	462	520	0.38		–	–
ϕNDA-Gly	water	462	520	0.36	QS	–	–
ADA-Gly	water	462	640	0.01	(0.59)	–	–
OPA-Gly	water	340	450	0.33		0.39	60

Figure 4.3:   Standard curves obtained with various amino
acids using NDA as fluorigenic reagent.
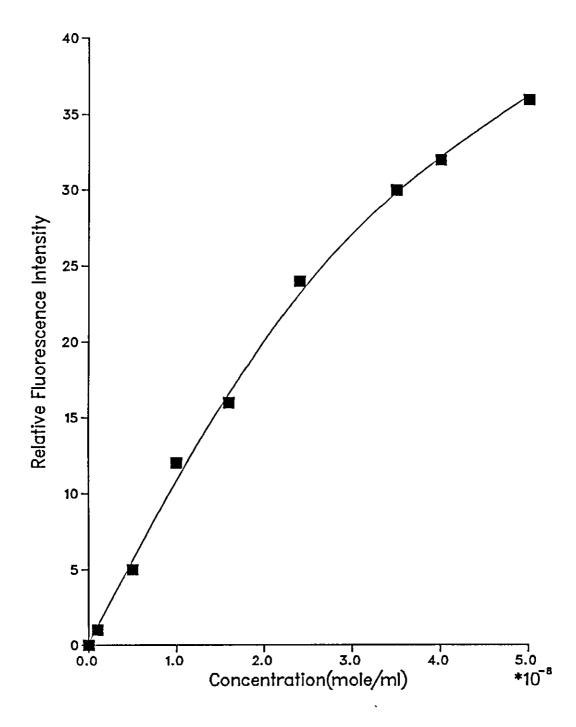
Figure 4.4:   Fluorescence intensity of NDA-glycine
as a function of the glycine concentration

emission should be minimal. The quantum efficiencies of all the fluorescent derivatives (0.33-0.38) are comparable to that of the OPA derivative (0.33) except the ADA derivative (0.01). The reason for such a low quantum efficiency for the ADA-derivative is not known.

4.3.2 Factors affecting the fluorescence intensity and stability of the fluorescent derivatives

4.3.2.1 Effect of pH on fluorescence intensity of reaction products

The optimum pH for the labelling reaction was shown to be alkaline similar to that of the OPA fluorigenic reaction [46]. The measured fluorescence intensities of the NDA-glycine derivatives were plotted against pH in Figure 4.5. The fluorescence was at a maximum in the range pH 10.0 to 10.5 after which it gradually decreased as the pH increased. The alkaline medium suitable for reaction points to the fact that the unprotonated amino group is the species involved in the derivatisation. This assumption is supported by the fact that the reaction rates are faster [60] .at high pH.

4.3.2.2 Effect of cyclodextrins and detergent on fluorescence

(a) Enhancement of fluorescence intensity by β-cyclodextrin

Cyclodextrins have been reported to form complexes in aqueous solution with various substrates [108]. The cyclic sugar molecules of cyclodextrin form a cavity of

Figure 4.5: Effect of pH on the fluorescence development in the reaction of glycine with NDA in the presence of a thiol

15.3A

7.8A

The 'lining' of the cavity, the glycosidic oxygen bridges, high electron density

Edge of secondary hydroxyls

7.8A

Figure 4.6(a):   Geometry of β-cyclodextrin.

Ortho-   Meta-   Para-

Figure 4.6(b):   Simplified comparisons of how ortho-, meta- and para-disubstituted benzenes are accommodated into the cyclodextrin cavity. (Figures reprinted from International Laboratory, Vol. 10, No. 9, November/December 1985, pp.34).

doughnut shaped assemblies (Figures 4.6(a) and 4.6(b)).
The internal diameter of the cavity depends on the number
of glucose residues in the molecule. The larger end of
the cyclodextrin assemblies is ringed with secondary
hydroxyl groups that form a key-like structure through
which guest molecules can enter. The smaller end of the
assemblies has a rim of primary hydroxyl groups. Due to
the high electron density of glucosidic oxygens a
hydrophobic trap is created inside the cavity.
Cyclodextrins had long been used to improve the stability
and increase fluorescence of many compounds by inclusion
complexing.

In the present study the addition of cyclodextrins
in the fluorigenic reaction of glycine, 10^{-4}M, with NDA
has produced results of some interest. Of the three
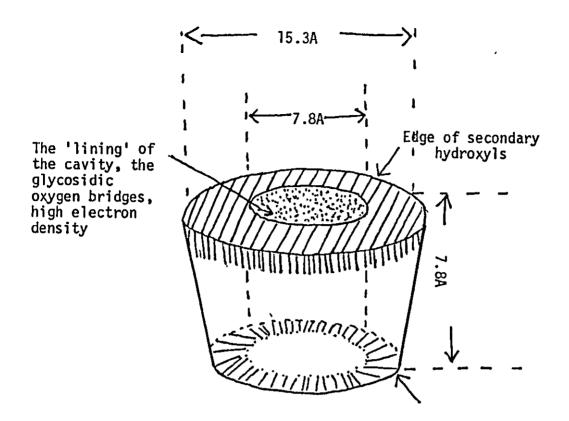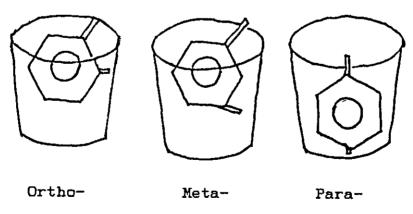cyclodextrins (α, β and γ), β-cyclodextrin was found to
enhance the fluorescence intensity of the NDA-derivative
by nearly eight times, compared with the NDA-derivative
without β-cyclodextrin (Figure 4.7). Similar results were
obtained by incorporating β-CD into reactions using other
fluorigenic reagents, ϕNDA and ADA (section 4.3.2.6).

However, the fluorescence enhancement was maximal for
an addition of a little over 2.5mmol/l of β-cyclodextrin
to the reaction (Figure 4.8). In contrast, α and γ-
cyclodextrins were found to have very little effect on the
fluorescence intensity.

The complexes may be regarded as inclusion [109]
compounds in which hydrogen bonding [110], van der Waals
forces [111], and hydrophobic interactions [112-114] are
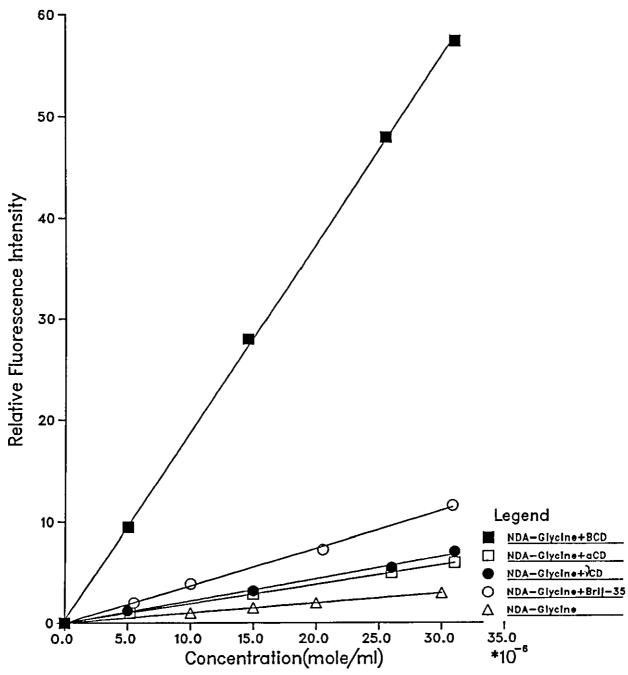
Figure 4.7:   Effect of cyclodextrins (α, ß and γ) and
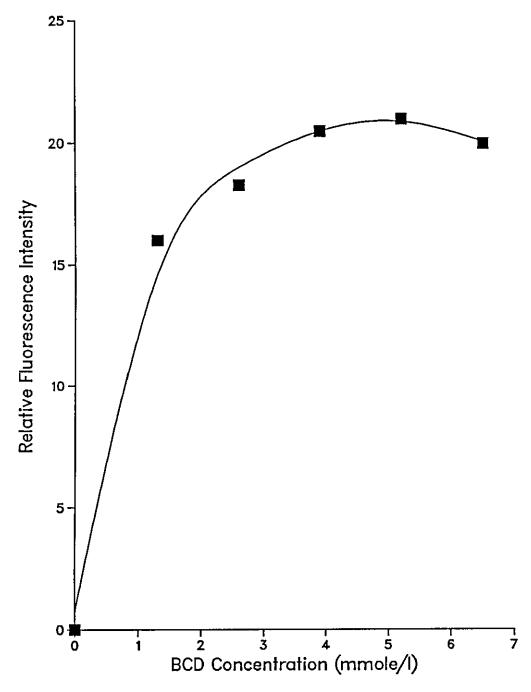   detergent (Brij-35) on fluorescence
               intensity.

Figure 4.8: Amount of β—cyclodextrin to obtain maximum
fluorescence intensity in a fluorigenic
reaction.

the main binding forces.

The increase in the fluorescence of the NDA-glycine derivative in Figure 4.7 may be due to the formation of an inclusion complex between the derivative and cyclodextrin. In this way the NDA-derivative molecule may have been transported into a partially hydrophobic surrounding leading to enhancement of fluorescence. β-cyclodextrin with a hydrophobic interior of 7.5Å internal diameter seems to include the whole of the isoindole molecule and gives increased fluorescence compared to α- and γ- cyclodextrins with 6 and 9-10Å internal diameter respectively, which are either too small or too big to enclose the NDA-derivative (isoindole). The other possible explanation for the very insignificant increase in fluorescence of the derivatives in the presence of α- and γ-cyclodextrins may be that the side chain functional group of the derivative has found a passage into the cavity while the rest of the molecule remains outside.

The mechanism for the formation of the inclusion complex is divided into several steps, for example, breakdown of the water structure inside the cyclodextrin ring and removal of some water molecules from the ring; breakdown of the water structure around part of the substrate molecule; interaction of the substituents of the substrate molecules with groups on the rim or on the inside of the cyclodextrin. Any of these steps may have an important effect on the formation of inclusion compounds leading to enhanced fluorescence and stability.

(b) Effect of detergent on fluorescence intensity

In the presence of Brij-35 the NDA-glycine derivative formed with 2-methyl-2-propanethiol (2-MPT) was found to give enhanced fluorescence (Figure 4.7). The increase in fluorescence, however, was very smll compared to β-cyclo-dextrin (discussed above). A very small improvement in the fluorescence intensity by the detergent suggests that (i) shielding of the NDA-glycine fluorophore from vibrational quenching by the hydrogen-bond structure of water (ii) alteration of the quantum efficiency [115-117] or (iii) increased local viscosity about the fluorophore-micelle binding sites may have not been so effective as the deactivation modes.

4.3.2.3 Effect of the thiol compound on the spectra of
          NDA, ∅NDA, and ADA-glycine derivatives

In a fluorigenic reaction where amines or amino acids react with a labelling reagent, the use of a thiol is a must since it becomes a part of the fluorescent product [48]. In the present study the difference in the thiol structure was shown to affect the fluorescence property of the final product.

(i) Dependence of the emission wavelength on thiol
     structure

The thiol structure has very little effect on the excitation wavelength of isoindoles. The wavelength of emission in contrast was more sensitive to structural changes of the thiol. The largest effect was noticeable

when the thiol contained the bulky, hydrophobic 2-methyl-2-propyl group. Studies showed that the structure of the thiol used had a profound effect on the wavelength of emission. Where the NDA, OPA and ADA-amino acid derivatives made with ethanethiol previously exhibited fluorescence at 550nm, 450nm and 680nm they have maxima at 520nm, 420nm and 640nm respectively when the ethanethiol is replaced with 2-methyl-2-propanethiol (2MPT) (Figures 4.9 and 4.10). Similar results are expected for the ϕNDA-adduct as well. By replacing ethanethiol with 2-methyl-2-propanethiol the emission wavelength of the ϕNDA-glycine derivative would shift from 550nm to 520nm. It appears that as the hydrophobicity of the molecule increases, the dipole-dipole interaction of the derivative with the solvent decreases and the emission moves towards the blue.

(ii) Influence of the thiol structure on stability

The stability of the amino acid (glycine, 10^{-4}M) derivatives has been examined in the presence of the active NDA, ϕNDA or ADA/thiol reagent. When the amino acid derivatives were formed with different fluorigenic reagents the resulting derivatives not only exhibited varying fluorescent responses but also varying degrees of stability (Figure 4.11). It is interesting to note that all of these derivatives in the presence of 2-methyl-2-propanethiol (2MPT) were stable over a period of 15-20 hours. The fluorescence of the NDA and ϕNDA adducts was reduced by only a half in 6 hours whereas the ADA-
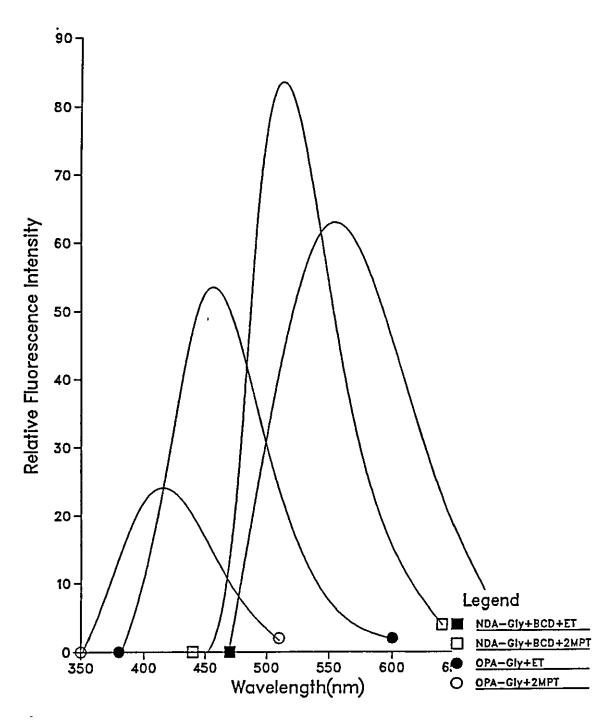
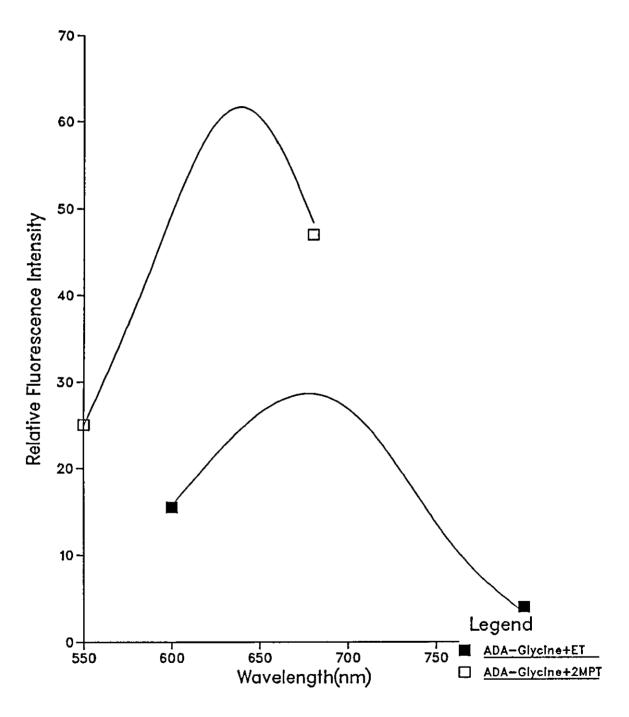Figure 4.9:   Emission spectra of NDA and OPA-glycine

derivatives.

- 79 -

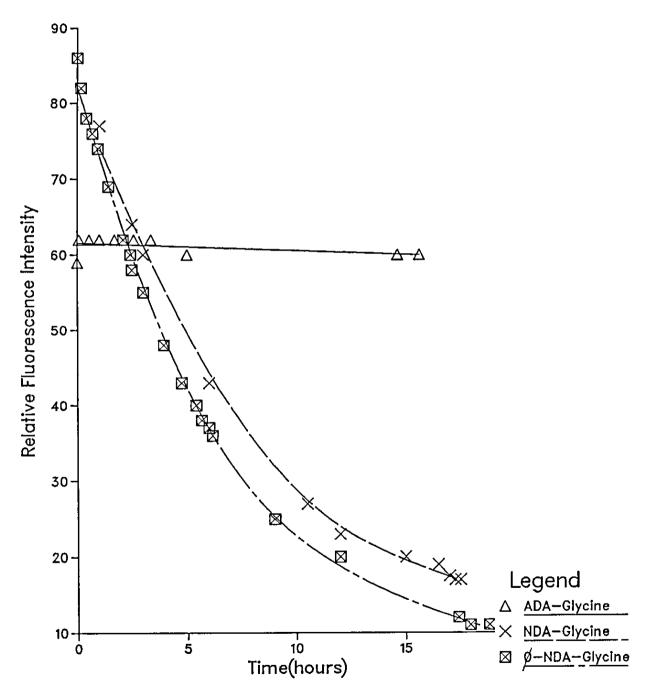Figure 4.10:   Emission spectra of ADA-glycine derivative

Figure 4.11: Stability studies of NDA, ØNDA and ADA-glycine derivatives.

derivative showed no sign of decay in fluorescence during
this period. However, the stability was found to be much
more affected by either using ethanethiol (ET), 2-
mercaptoethanol (2-MERC) or 3-mercaptopropionic acid in
the reaction (Figure 4.12). Of these three, only
3-mercaptopropionic acid and ethanethiol were found to
produce stable products of NDA-glycine with nearly total
decay of the fluorescence over a period of three hours and
seven hours respectively. These results are in agreement
with those for OPA-derivatives which show similar
instability [57, 62] in the presence of 2-mercaptoethanol
and ethanethiol.

In conclusion the highly branched thiol, 2-methyl-2-
propanethiol produced more stable products.

4.3.2.4 <u>Effect of Inorganic salts on fluorescence</u>
          <u>intensity</u>

A distinct quenching effect (decreasing fluorescence)
was observed for the glycine derivatives of NDA (Figures
4.13 and 4.14) by incorporating inorganic salts into the
labelling reaction. The decreasing order of quenching of
fluorescence was as follows, fluorescence in a water
system without any salt being the control: (potassium
bromide, potassium sulphate)>(sodium chloride, sodium
acetate)>sodium nitrate. The extent of the decrease in
fluorescence in each case was very little. However, the
addition of sodium carbonate resulted in the increase of
fluorescence intensity which may be due to the alkalinity
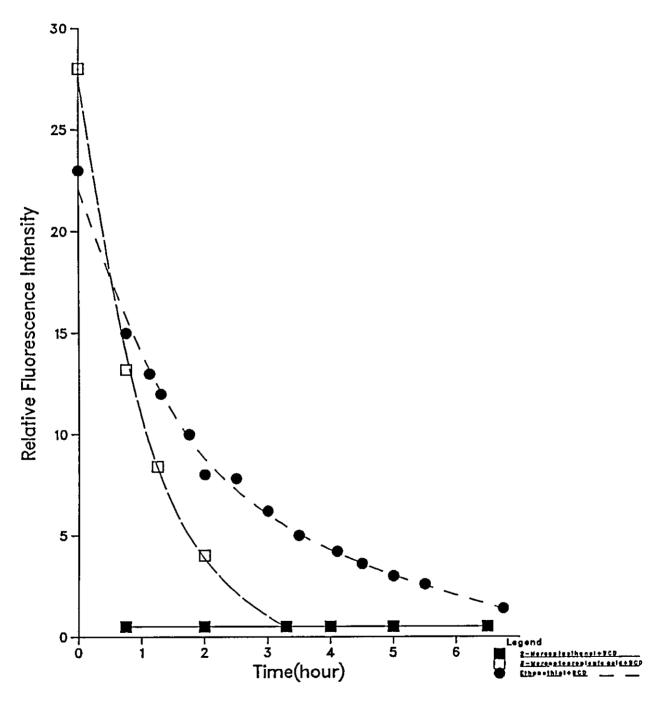of the salt solution helping or correcting the pH of the

Figure 4.12: Fluorescence decay of NDA-glycine
derivatives obtained in the presence of
different thiols.

Figure 4.13:   Quenching of fluorescence of NDA-glycine

derivatives in presence of inorganic salts.

Figure 4.14: Spectra of NDA-glycine derivatives showing effect of inorganic salts of fluorescence intensity.

reaction medium.

Diluting the derivatives 2,4 and 8-fold the decrease
of fluorescence as shown in the bar chart (Figure 4.15)
was linear as expected.

The quenching effects of different salts on the
fluorescence may be expected on the basis of the
enhancement of the transition from singlet to triplet
state (intersystem crossing) with simultaneous decrease in
fluorescence. With bromide ions the quenching was
relatively greater because of the increased probability of
intersystem crossing with an element of higher atomic
weight and relative position in the periodic table. A
similar quenching of the OPA-spermidine fluorescence by
halogen ions has also been reported [118].

4.3.2.5 Effect of solvents on fluorescence intensity and
         emission wavelength

(a) Wavelength of emission in different solvents

A study of the effects of organic solvents on
fluorescence intensity and wavelength of emission has led
to some intersting results.Figures 4.16, 4.17 and 4.18
show the NDA, ∅NDA and OPA-glycine derivatives to
fluoresce at 510nm, 530nm and 460nm respectively in water
whereas in organic solvents the maxima are shifted to
shorter wavelengths. Interestingly, the extent of
wavelength shift from water to organic solvents for all of
these derivatives is not very large (5-10nm) except for
the OPA-derivative when it is 10-20nm. The emission peaks
were found to be at shorter wavelengths in solvents with

Figure 4.15: Bar chart showing the effect of different inorganic salts on the fluorescence intensity of NDA-glycine derivatives.

Figure 4.16: Spectra of NDA-glycine derivative showing
the effect of organic solvents on
fluorescence properties.

Figure 4.17:   Spectra of ∅NDA-glycine derivative obtained

in presence of organic solvents.

Figure 4.18: Spectra of OPA-glycine derivative containing organic solvents.

low dielectric constant. The correlation between the
amount of blue shift and the dielectric constant by itself
does not explain some of the other obsevations. The
wavelength of maximum emission for all the above
fluorescent derivatives remained unaffected although the
solvent with a high dielectric constant (dimethyl-
formamide) was replaced by those ones with low dielectric
constants (acetone or dioxane) (Table 4.2).

However, the higher wavelengths of maximum emission
in water may be explained on the basis of dipole-dipole
interaction of the fluorescent derivatives with the
solvent molecule. This interaction is increased in water
and the emission moves further towards the red, i.e.,the
emitted photons possess less energy [119].

(b) Fluorescence intensity in different solvents
        The effects of solvents on the fluorescence intensity
of the NDA, ϕNDA, and OPA-adducts seemed to be moderate.
A fluorescence enhancement of these derivatives was
observed as the solvent polarity changed from polar to
non-polar ones (Figures 4.16, 4.17 and 4.18) with a few
exceptions. The exceptions were the fluorescence
intensity of NDA and OPA-adducts being more intense in
water and dimethylformamide respectively, although the
solvent polarities were high. The other solvents,
acetonitrile, methanol, ethanol and dimethylsulphoxide,
however, seemed to offer the expected enhanced
fluorescence for these two derivatives with decrease in
solvent polarity.

Table 4.2:   Fluorescence data for isoindoles, showing
             solvent effect on wavelength of emission.

Solvent dielectric constant (debyes)*	Solvent	Emission, λ (nm)$_{max}$		
		NDA-Gly	⌀NDA-Gly	OPA-Gly
78.5	Water	510	530	460
31.2	Methanol	510	525	460
25.8	Ethanol	505	520	450
36.7	Dimethyl formamide	505	520	440
21.5	Acetone	505	520	440
3.0	Dioxane	505	520	440
46.7	Dimethyl sulphoxide	520	–	440
37.5	Acetonitrile	505	520	450

* Solvent dielectric constant (Reference 57, 62).

A few other solvents like acetone and dioxane present
in the solvent system, about 38%v/v and 40%v/v
respectively had a very severe effect on the fluorescence
intensity. Dioxane was found to quench the fluorescence
completely while acetone showed roughly 3-4 times
reduction in the fluorescence intensity of all three
derivatives relative to water as a solvent. The reason
for this quenching may be due to the carbonyl group in the
acetone and oxygen atoms in dioxane.

4.4 Fluorescence assay of proteins by the application of
NDA, øNDA and ADA as fluorigenic reagents

4.4.1 Introduction

Most proteins absorb in the region 270-300nm and
possess some degree of fluorescence due mainly to aromatic
amino acid residues, tyrosine and tryptophan. However,
other fluorescent structures bound to the protein other
than by peptide linkages may also contribute to
fluorescence. Proteins can also be combined chemically
with the fluorigenic reagents to yield fluorescent protein
derivatives. In the combined form the fluorescence
properties such as intensity, the excitation and
fluorescence spectra, and the degree of fluorescence
polarisation may alter/shift to higher or lower values.
Measurement of these factors with a fair degree of
precision is possible and the information obtained is
useful to ascertain the size and shape of macromolecules
and their interaction with other molecules.

The fluorigenic reagents may find application as an

end group reagent [120]. The constituent of amino acids may be determined by identifying the fluorescent fragments [121] following degradation of the fluorescent derivatives. In addition the fluorescent labelling technique may be used as protein tracers and in immunofluorescence.

In the present study an effort has been made to investigate the fluorescence properties of the NDA, ⌀NDA and ADA-protein derivatives with special references to their stability and ease of formation. Such investigations would be useful to determine the applicability of these labels in fields of immunology and protein structure study.

4.4.2 Experimental
(a) Purification of fluorescent derivatives of proteins
A Sephadex G-25 column was first equilibrated with 25ml of borate buffer (pH 10.0). 2.5ml of the fluorescent derivative was then loaded at the top of the column and the column eluted with borate buffer (3.5-4.5ml) in an aliquot of 1ml of the buffer for each collection.

(b) Fluorescent labelling of proteins, their reaction time and stability, including inner filter effects and biotin assay
The proteins were labelled as described in section 2.3.2. Human albumin economy, RIG, (antibody) was derivatised with NDA, ⌀NDA and ADA separately. These derivatives were used for stability studies. For inner

- 94 -

filter effect studies, absorbers such as cytochrome C (0.006mM), myoglobin (2mg%) and ATP (2mg%) were used unless otherwise stated. For biotin assay, avidin (4mg%) was labelled with NDA according to the procedure described in section 2.3.2. The fluorescent-labelled avidin was then treated with a gradually increasing amount of d-biotin (10^{-6}mol/ml) and the fluorescence intensities were measured.

4.4.3   Results and Discussion

4.4.3.1   Fluorescent labelling of proteins

(a)   Reaction time

The success of a fluorescent labelling method is due mainly to the ease of reaction and the time required to give a fluorescent product of measurable and reproducible intensity. In this study the fluorigenic reagents NDA, ∅NDA and ADA have been employed to label proteins in order to determine the reaction time for successful labelling. Figure 4.19 which is a plot of fluorescent intensity versus time showed that the fluorescent intensities of these derivatives were at their maxima within 3-9 minutes. The reaction times were 3 minutes for NDA-antibody, 6 minutes for ∅NDA-antibody and 8 minutes for ADA-antibody derivatives. The reaction time with proteins seemed to be comparable to that of the simple amino acid reactions (see section 4.3.1(a)).

(b)   Stability

The NDA, ∅NDA and ADA fluorescent protein derivatives

Figure 4.19:   Reaction time, NDA, ⌀NDA and ADA-antibody

derivatives.

Figure 4.19:   Reaction time, NDA, øNDA and ADA-antibody

derivatives.

have been studied to determine their stability. The
fluorescence intensities of the derivatives were measured
at regular time intervals. Figure 4.20(a) and 4.20(b)
indicated that the fluorescence decay of the NDA, φNDA and
ADA-antibody was found to extend over a period of 10, 4
and 8 hours respectively. The stability of protein
derivatives in comparison with simple amino acid
derivatives (see section 4.3.2.3 (ii)) was a little less,
the fluorescence intensity was reduced to its minimum in
less than half of the time of simple amino acid
derivatives. The increase in instability may be due to
the unfolding of the labelled protein leading to
conformational alteration of the protein macromolecule.

(c) Inner filter effects

        An insignificant decrease in the fluorescence
intensity of the fluorescent species may result by the
addition of an absorbing molecule to it. The absorption
of light by the molecule at the wavelength of emission is
called secondary absorption and this inner filter effect
needs to be compensated for before carrying out a
quantitative analysis of the results. The present study
attempts to investigate such an effect on the NDA-Human
albumin (NDA-HAlb) fluorophores using cytochrome C,
myoglobin and ATP as absorbers.

        In Figure 4.21 the inner filter effect resulting from
the addition of cytochrome C, myoglobin and ATP to the
NDA-HAlb fluorophores is shown by the three curves. These
curves represented the uncorrected fluorescence which may

Figure 4.20(a): Stability curves of NDA, ⌀NDA
and ADA-antibody.

Figure 4.20(b):   Spectra of ADA-antibody, showing
                   fluorescence decay of the derivative.

be corrected by applying the equation of Geren and Millett
[122]:

$$F_c = F_o \times \text{antilog}_e (A_1 + A_2/2) \qquad (4.1)$$

where F_c is the corrected fluorescence, F_o is the observed
fluorescence corrected for dilution, and A_1 and A_2 are the
primary and secondary absorbances respectively.

The drivatives of HAlb formed with other fluorigenic
reagents ϕNDA and ADA showed a similar quenching of
fluorescence (Figure 4.22) by cytochrome C.

For an addition of 0.4ml of the absorbers, the
fluorescence was quenched roughly by 70, 24 and 58% for
addition of cytochrome C, myoglobin and ATP respectively
(Figure 4.21). However, the inner filter effect in the
case of ϕNDA and ADA by cytochrome C was found to be
small.

The selection of these absorbers for the study was
due to the fact that all these are non-fluorescent.
Cytochrome C and myoglobin are note worthy in that in
spite of these being proteins they are non-fluorescent due
to the quenching effect of the heme group present [123].

(d) d-Biotin assay

Biotin-induced changes in the fluorescence [124]
properties of avidin form the basis of biotin assays.
Figure 4.23 showed that the fluorescence of NDA-labelled
avidin was quenched upon the addition of biotin. The
fluorescence was reduced by half on the addition of

Figure 4.21: Inner filter effect, resulting from the

addition of different absorbers to the

NDA-Human albumin fluorophore
(different initial concentration)

Figure 4.22:   Inner filter effects, due to addition of
              absorber, cytochrome C, to ∅NDA and ADA-
              Human albumin fluorophores.

Figure 4.23: Curve showing the effect of biotin on the
fluorescence intensity of NDA-labelled
Avidin.

4×10^{-6} mol/ml biotin to the system. The relatively large change in NDA-labelled avidin fluorescence was unexpected since biotin on binding brings no gross change [125] in the structure of avidin. However, a reduction in intensity of up to 40% occurs when biotin binds to dansyl avidin [125]. The biotin-induced decrease in fluorescence of NDA groups associated to some sites on avidin may be explained on the basis of local displacement of the NDA groups into a more aqueous environment where they have greater rotational freedom and less interaction with the protein structure, as described applicable to dansyl groups [126].

In conclusion the NDA label can be employed for a sensitive detection of d-biotin on a quantitative basis in a complex sample.

CHAPTER 5   A STUDY OF POTENTIAL LABELS FOR FLUORESCENCE

DETECTION OF AMINO ACIDS USING FLOW

INJECTION ANALYSIS

5.1   Introduction

The technique of flow injection analysis (FIA),

originally developed by Ruzicka and Hansen [127] in 1975,

is a rapid and precise method for screening large numbers

of samples. In FIA a small volume of liquid sample is

reproducibly introduced via an injection valve into a

moving, non-segmented continuous carrier stream containing

the reagent. The injected sample zone disperses in the

reaction coil and is carried by the stream to an

appropriate detector. The method has very many attractive

features, the most important of which are flexibility,

small sample volume, high sampling rates and simple

instrumentation. A controlled dispersion of the sample

zone allows the FIA system to be suitably automated [128]

for a given analytical procedure. The dispersion D has

been determined as the ratio of concentration (or maximum

peak height) of an undiluted element of fluid (C_o) that

gives the analytical signal (readout) over the

concentration (C_p) (or maximum preak height) of a well-

defined volume of the same element of fluid injected into

an inert carrier stream.

$$D = \frac{C_o}{C_p} = \frac{H_o}{H_p}$$

In most FIA methods the dispersion varies between two extremes (1.5<D>10). A lower dispersion value i.e. lower dilution of the sample solution is usually preferred in the case of trace analysis to obtain maximum sensitivity. The method is versatile and may incorporate spectrophotometric, electrochemical, conductometric or fluorescence detection. Since fluorescence detection is sensitive and allows detection even at the pgml^{-1} level, it has been preferred to other detection systems, especially in automatic analyses [129].

For an analytical scheme that requires two or more reagent solutions, manifolds with two or more lines can be employed since the theory of dispersion confirms that the sample and reagent solutions do not have to be completely mixed [130].

Several workers have found FIA a suitable tool for the determination of glycine and albumin [131] and of primary amines in sea-water [131]. They have used o-phthalaldehyde (OPA) as a fluorigenic reagent for the derivatisation of the substance of interest.

In the present study the FIA system has successfully been employed to carry out a fluorigenic reaction of an amino acid (glycine) using the newly synthesised fluorescent label molecules, naphthalene-2,3-dicarboxaldehyde (NDA) and 1-phenylnaphthalene-2,3-dicarboxaldehyde (∅NDA). The results have been compared with OPA as a labelling reagent for amino acids. In order to economise in the use of expensive fluorigenic reagents, NDA and ∅NDA, a "reverse flow injection analysis" system

Figure 5.1:   Schematic diagram for reverse flow injection
analysis.

has been employed (Figure 5.1).   In this method the sample

(glycine) is pre-mixed with thiol, borate buffer and

β-cyclodextrin and is continuously pumped through the

connector T piece to the detector.   The fluorigenic

reagent is injected into the non-segmented continuous

carrier stream containing the buffer.   The labelling

reagent meets the sample in a connector T and the reaction

product then passes on to the detector and the

fluorescence signal is recorded.

Using the reverse flow injection analysis the effect

of various parameters, i.e., pumping rate, tube length,

sample size etc.on the derivatisation reaction of glycine

with the fluorigenic reagents have been investigated.

5.2   Experimental

(a)   Apparatus

The flow injection analysis (FIA) manifold shown in
Figure 5.1 was used.   'L' is the length of 0.58mm i.d.
Teflon tubing (R.S. Components Ltd.), which was 10cm for
the investigation of reaction time.   The FIA manifold was
directly attached to a Perkin-Elmer model 2000
fluorimeter, fitted with a flow cell.   Injections were
made using a Rheodyne 5020 valve fitted with a 50μl loop.
The selection of a 50μl loop offered a dispersion value of
2.54.   Reagents and buffers were pumped using a Gilson
Minipuls 2, 4-channel peristaltic pump.   A model 28000
recorder (Bryans Ltd., Mitcham, Surrey) was used to record
the FIA fluorescence data.

(b)   Detection

Fluorescence detection was carried out at the
appropriate wavelengths, 520nm for NDA and $\emptyset$NDA-amino acid
derivatives and 450nm for OPA-derivatives with excitation
at 460nm and 350nm respectively.

(c)   Solutions

(i)   Glycine sample solution

10^{-4}M glycine solution (20ml) was mixed with 10.9mM
β-cyclodextrin, β-CD ((4.8ml) and 0.1%v/v 2-methyl-2-
propanethiol (1.7ml) and borate buffer, pH 10.0 (1ml).

(ii) Fluorigenic reagent solutions

A suitable quantity of the fluorigenic reagent, NDA,

ɸNDA or OPA, was dissolved in a minimum volume of ethanol
(5-10ml) and the final volume made up to 25ml with either
distilled water or 0.025M sodium borate buffer, pH 10.0 to
give a solution of the required concentration.

(d)   Procedure

     The glycine sample solution, pre-mixed with β-CD,
thiol and borate buffer, was continuously pumped
(1.0ml/min) through one of the two lines of the manifold
(Figure 5.1) for reaction time investigation. A carrier
stream containing the borate buffer was flowing through
the other line (1.0ml/min, for reaction time study) into
which the fluorigenic reagents, NDA, ɸNDA or OPA was
introduced. The fluorigenic reagent and glycine entered
the merging point T via a connector before passing on to
the detector. The pump was stopped five seconds after the
fluorigenic reagent had been injected into the carrier
stream. For reaction time studies the reactants were kept
in the reaction coil for 5, 10, 15, 30 and up to 300
seconds, after which the pump was restarted. For all
other experiments the conditions remained the same unless
stated otherwise.

5.3   Results and Discussion

5.3.1   Investigation of Reaction Time

     Fluorescent derivatives of glycine formed separately
with NDA, ɸNDA or OPA using FIA were suspended temporarily
in the reaction coil by halting the carrier flow and then
allowed to pass on to the detector. Thus, by switching

the pump 'off' and 'on', the fluorescence signal
corresponding to the time lapse was obtained on a
recorder. In order to minimise dispersion, the length of
0.58mm i.d. Teflon tubing between the connector 'T' and
the detector was 10cm. It appears from the results as
shown in Figure 5.2, that as the time lapse between the
pump switching 'off' and 'on' increases the fluorescence
of the derivatives also seems to increase to a plateau.
For OPA-glycine the plateau was reached in about 3
minutes, whereas both NDA and ∅NDA-glycine took 5 minutes
for the fluorescence to become constant. The plateau
obtained in each case seems to suggest that the reaction
was complete within this time.

By applying the same stopped-flow mode in FIA, the
fluorigenic reaction of NDA with glycine in the absence of
β-cyclodextrin showed the fluorescent derivatisation
reaction to be complete in about the same time (Figure
5.2) as that with β-cyclodextrin. This shows that the
reaction time remains unaffected by β-cyclodextrin. The
use of β-cyclodextrin in the fluorigenic reaction is for
enhancement of the fluorescence intensity of the
derivative, discussed already in section 4.3.2.2.

The NDA and ∅NDA seem to be equally efficient
labelling reagents for glycine as OPA, using FIA.

5.3.2   Dependence of the fluorescence signal on the amount
        of thiol

For the fluorigenic reaction of OPA, NDA or ∅NDA with
glycine, a thiol is required, without which the

Figure 5.2: Reaction time: derivatisation of glycine using NDA, ∅NDA and OPA as fluorigenic reagents.

fluorescent derivative, an isoindole, would not be formed. However, the quantity of thiol used for a static system may not be the same a that required in FIA. This needs to be confirmed.

Using FIA, a study has been made to determine the thiol:glycine mole ratio required for fluorescent derivatisation of the glycine with OPA and NDA. The fluorescence intensity of both the OPA and NDA derivatives were measured by changing the tube length between the connector T and the detector from 10 to 20cm for convenience. As the amount of thiol, 2-methyl-2-propanethiol for the NDA-glycine derivative and ethanethiol for the OPA-glycine derivative, was increased the fluorescence intensity of the derivatives also increased gradually (Figure 5.3). The fluorescence intensity, however, was found to level off as the ratio of thiol:glycine was around 10mmol for both OPA and NDA-derivatives.

The addition of thiol to glycine in this ratio may be necessary to obtain the maximum fluorescence signal for a fluorescent product.

5.3.3 Effect of flow rate on fluorescence intensity

Flow injeciton analysis of glycine $(10^{-4}M)$ based on the fluorigenic reaction with the labelling molecules seems to be influenced by the flow rates (Figure 5.4). The fluorescence intensity (peak height) of the OPA and NDA-glycine derivatives decreased with the increase of flow rate. The fluorescence intensity for both the

-112-

Figure 5.3:   Thiol:amino acid ratio curve (OPA and NDA-

glycine derivatives).

Figure 5.4: Variation of peak height with flow rate.

derivatives was almost halved when the flow rate was altered from 1.0 to 1.5ml/min. The fluorescence intensity was further adversely affected as the flow rate was changed to 2.0ml/min reducing the signal to virtually zero. Such results may seem to arise from the shorter time available for the reaction and may possibly be a combined effect of high dispersion [132] with increasing flow rate.

A suitable flow rate may thus be necessary to obtain a fluorescence signal of appreciable intensity.

5.3.4 Effect of tube length on fluorescence peak height

Changing the tube length 'L' in between the merging point (T) and the detector in flow injection analysis affects the fluorescence signal monitored (Figure 5.5). The peak heights corresponding to the signals for all of these derivatives, OPA and NDA-glycine, were at their minimum value when the shorter length of tube, 10cm, was used. The peak heights kept on increasing as the length of 0.58mm i.d. Teflon tubing was increased from 10cm to 20, 30 and up to 200cm. By observing the trend of this change in terms of peak height, the progress of the fluorimetric reaction between glycine and the labelling reagents has been studied. The yield of the fluorescent product is very very low when the reactants/products travel through only 10cm of tubing. In contrast the yield of the fluorescent derivative constantly rises as the residence time increases by increasing the tube length. Increasing manifold length helps mixing and promotes

1. Quinine Sulphate

2. NDA-glycine + β-CD

3. OPA-glycine

4. NDA-glycine – β-CD

Figure 5.5:   Effect of tube length on peak height of the fluorescing molecules.

chemical reaction but leads to increased dispersion and loss of sensitivity. The increasing peak height with increasing tube length seems to indicate that more fluorescent product is being formed overriding the effect of the dispersion process. Since the reaction is not complete and may take longer than 20 seconds, it would be difficult to accommodate such a long reaction time in an FIA system.

However, in the case of standard quinine sulphate there is a decrease in peak height with increasing tube length. This is due to the increased dispersion as expected.

5.3.5 Calibration curve

Glycine solutions at different concentrations (0.94 x 10^{-8} mol/ml to 7.54 x 10^{-8} mol/ml) were found to form fluorescent derivatives using reverse flow injection analysis. The fluorescent derivatisation of glycine using NDA was carried out maintaining a flow rate of 1.0ml/min. The fluorescence signal peaks were recorded as traces on a chart (Figure 5.6). The fluorescence signal peak height versus concentration relationship was linear as expected (Figure 5.7). In the present investigation it became clear that the fluorigenic reaction may successfully be carried out with very dilute solutions, as low as 0.9425 x 10^{-8} mol/ml of glycine. The flow injection analysis may equally be applicable to other primary amino acids, giving the same linear relationship as obtained with glycine.

Figure 5.6: Traces obtained due to fluorescent derivatisation of glycine with NDA using reverse flow injection analysis.

Figure 5.7: Calibration curve showing fluorescence signal

versus concentration relationship.
(from Figure 5.6)

5.4 High performance liquid chromatographic determination of amino acids by pre-column fluorescence derivatisation with naphthalene-2,3-dicarboxaldehyde (NDA)

5.4.1 Introduction

In many diverse areas such as biology and medicinal chemistry where amino acids play a vital role, there is a need for sensitive and specific analytical methods for separation and quantification of the individual amino acid species. Several approaches to high performance (HPLC) analysis of amino acids are possible. Separations can be achieved with an ion-exchange column or a reversed-phase column.

In recent years many modifications in terms of resin characteristic, column sizes, column temperature, buffer pH and ionic strength have been made to improve resolution of amino acid mixtures and to achieve specific separation.

The detection of the separated components poses a special problem for the high performance liquid chromatography separation of simple amino acids. UV detection is not specific and may complicate the analysis. However, fluorescence detection is both sensitive and specific, making it superior to UV or visible detection [133]. Many compounds which do not contain fluorophores may be derivatised by pre-or-post-column reactions with fluorescent label molecules.

There are several fluorigenic reagents [46, 63, 134-135] which have been employed for post-or-pre-column derivatisation. Of these reagents, the most widely used

are fluorescamine, dansyl chloride and o-phthalaldehyde
(OPA). OPA is a reagent which is specific for the primary
amino group. Recent studies demonstrated that OPA can
successfully be used for pre-column derivatisation and
separation by reversed phase HPLC [56, 136-139]. In this
present work, the OPA has been replaced by a new reagent,
naphthalene-2,3-dicarboxaldehyde (NDA), which, like OPA,
is specific for the primary amino group. Its
applicability for amino acid analysis in a mixture is
investigated.

5.4.2 Experimental

(a) Apparatus

The HPLC system used comprised a Pye-Unicam X-PS pump
and a 100mm x 5mm i.d. column pre-packed with 5μm diameter
ODS-Hypersil, a Pye-Unicam PU4020 UV-detector and/or
Perkin-Elmer 2000 fluorescence spectrophotometer with a
flow cell. The analytical column was fitted with a silica
pre-column before the injection valve and the injections
were made using a Rheodyne 7125 valve fitted with a 20μl
loop.

(b) Detection

Detection was carried out at 254nm by UV combined
with fluorescence at 520nm (excitation 460nm).

(c) Flow rate

The flow rate of the carrier stream was maintained at
1.0ml/min at a pressure of 3000psi.

(d)   Mobile phase

Mixture of acetonitrile and water (65:35%v/v).

(e)   Reagents and standards

(i)   Preparation of amino acid standards

Amino acid standard solutions (10^{-5}mol/l) were

prepared by dissolving a suitable quantity of the amino

acids in distilled water.

(ii) Preparation of the fluorigenic reagent solution

(Derivatising solution)

A suitable quantity of naphthalene-2,3-dicarbox-

aldehyde (NDA) was dissolved in a minimum volume of

ethanol (5-10ml) and made up to 25ml with either

distilled water or 0.025M sodium borate buffer, pH 10.0 to

give a solution of 10^{-5}mol/l concentration.

(f)   Derivatisation Procedure

The general procedure for derivatisation of amino

acids present singly or in a mixture was as described in a

previous section 2.3.2.

5.4.3   Results and Discussion

Preliminary separations of the individual

naphthalene-2,3-dicarboxaldehyde (NDA)/2-methyl-2-

propanethiol derivatives of primary amino acid were

performed using a mixture of acetonitrile and water

(65:35%v/v) as the mobile phase.   The NDA-amino acids

derivatives in a mixture were satisfactorily resolved.

Figure 5.8   shows the chromatogram which illustrates

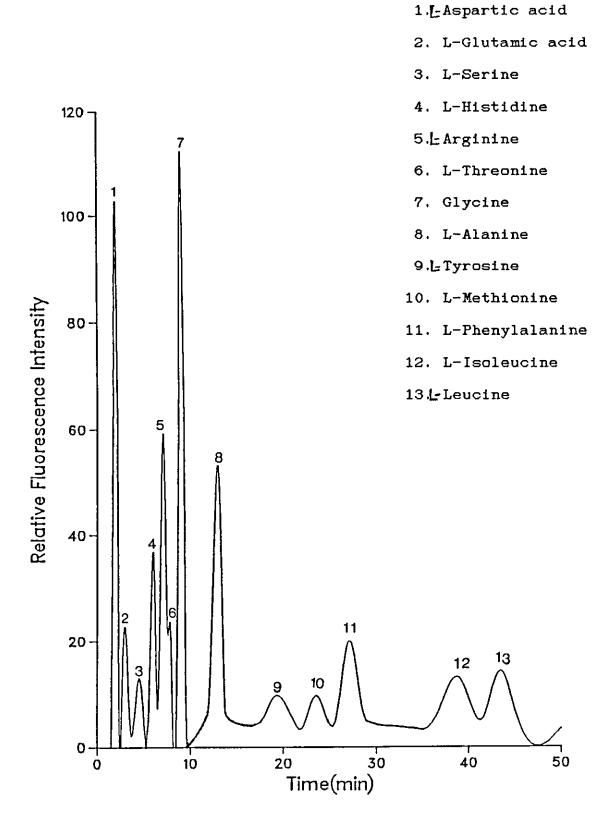Figure 5.8: Elution chromatogram of standard amino acid mixture

Legend:
1. L-Aspartic acid
2. L-Glutamic acid
3. L-Serine
4. L-Histidine
5. L-Arginine
6. L-Threonine
7. Glycine
8. L-Alanine
9. L-Tyrosine
10. L-Methionine
11. L-Phenylalanine
12. L-Isoleucine
13. L-Leucine

the resolution obtained under isocratic conditions for a

mixture of 13 amino acids; this method may be extended to

resolve a larger number of amino acids in a complex

mixture by using gradient elution. For the separation of

the NDA-amino acid derivatives in a mixture the

concentration of acetonitrile in the mobile phase was 65%

which proved to be suitable in terms of achieving

resolution of the amino acid derivatives. The analysis

time varied from about 2-45 minutes. This variation in

retention time of the various derivatives may be due to

the wide range of polarities. The NDA-amino acid

drivatives separated by reversed-phase HPLC seemed to be

affected by the balance between the hydrophobic and

hydrophilic parts of the·fluorescent derivatives. The

hydrophobic parts of the molecules may be squeezed [56]

out of the mobile aqueous-acetonitrile phase onto the

hydrocarbon stationary phase. This, however, needs

confirmation.

However, the elution order of aspartic acid followed

by L-glutamic acid, L-serine, L-histidine, L-threonine,

glycine, L-alanine, tyrosine, L-methionine, L-phenyl-

alanine, L-isoleucine, leucine is in agreement with the

order of the OPA-derivatives [55, 56, 94]. Arginine is

the only acid which was eluted unexpectedly before

threonine in the elution order series.

The linearity of response was also examined in this

study in the concentration range 1-24nmol/ml for a number

of amino acid derivatives with NDA (Figure 5.9). The

fluorescence response decreased in the order glycine,

Figure 5.9: Quantitative separation of amino acids in a
mixture.

taurine, L-alanine, L-methionine to arginine.   However, a
quantitative analysis of five different amino acids in a
mixture was achieved.

In conclusion naphthalene-2,3-dicarboxaldehyde (NDA)
has successfully been applied to the pre-column
derivatisation of a number of amino acids in a mixture and
their separation by reversed-phase HPLC.   The HPLC method
coupled with fluorescence detection has been confirmed for
a rapid and quantitative analysis of amino acids in a
mixture at the nmole level.

CHAPTER 6   GENERAL SUMMARY AND CONCLUSION

The synthesis of the fluorigenic reagents (labels),
naphthalene-2,3-dicarboxaldehyde (NDA), 1-phenyl-
naphthalene-2,3-dicarboxaldehyde (∮NDA) and anthracene-
2,3-dicarboxaldehyde (ADA) involves a number of steps,
i.e.,oxidation, hydrolysis, cleavage etc., yet it forms a
suitable basis for the preparation of compounds containing
the required functional groups.

The studies of these labels for analytical
fluorimetry have demonstrated that they can enter into a
fluorigenic reaction with primary amines or amino acids in
the presence of an alkyl or hydroxyalkylthiol. The end
product is an isoindole, the stability of which is heavily
dependent upon the pH of the reaction medium and the
structures of the labels, thiols and primary amines. The
stability of the products is increased by replacing
hydroxyalkylthiols with alkylthiols, and increases
dramatically as the thiol becomes highly branched. The
steric bulk of the isoindole N-substituent when branched
at the carbon atom adjacent to the ring nitrogen may
provide additional stability to the products: this
requires further confirmation.

The drivatives of amino acids formed with NDA, ∮NDA
and ADA in the presence of 2-methyl-2-propanethiol (2MPT)
are very stable and fluoresce intensely at longer
wavelengths (520nm, 520nm and 640nm respectively) than the
OPA-adduct with an emission wavelength of 450nm. The OPA-
derivative is unstable [140] in the presence of an excess

of the reagent.

The wavelength of emission of NDA, ∮NDA and ADA-derivatives shift to even longer wavelengths as the solvent polarity increases and the effect is more pronounced when 2-methyl-2-propanethiol (2MPT) is replaced by ethanethiol in the reaction.

The stoichiometric nature of the reaction facilitates the quantitation of free amino groups and the raction has provided the basis for a convenient, sensitive assay for amino acids. The reaction is complete within 5-10 minutes using the new labels.

The fluorescence intensities of the NDA, ∮NDA and ADA-adducts have been considerably enhanced by the presence of β-cyclodextrin. α- and γ-cyclodextrins have very little effect on the fluorescence intensity of the products. With the exception of the bromide ion, inorganic salts seem to have very little effect in terms of quenching the fluorescence of the amino acid derivatives.

Unlike OPA, the newly developed labels seem to offer background-interference-free fluorimetric analysis of biological samples due to measurements being made at longer wavelengths. Furthermore, these labels have demonstrated their applicability as derivatising reagents for glycine using reverse flow injection analysis. NDA has also successfully been used for pre-column derivatisation of a number of amino acids in a mixture and separation by reversed-phase high performance liquid chromatography (HPLC). The result is excellent and 13

different amino acids may be separated, five of which can also be quantified, using isocratic elution. This procedure may be adapted for separating complex amino acid mixtures with the help of gradient elution.

These labels are also found to form stable fluorescent derivatives with proteins. The fluorigenic reaction is rapid and the general fluorescence properties of the derivatives are the same as those of the simple amino acid derivatives. Labelling of proteins has enabled the study of inner filter effects in the presence of ATP, cytochrome C and myoglobin as absorbers. Biotin has been assayed based on biotin-induced changes in the fluorescence properties of the labelled protein.

Finally, in conclusion it is worth mentioning that the NDA, ∮NDA and ADA labels may be further modified by the introduction of an electron donating substituent at a suitable position in the benzene ring, especially in NDA and ADA. This will not only enhance the fluorescence quantum efficiencies of the fluorophores but will also increase their stability in the aqueous system.

REFERENCES

1. Skoog, D. A., and West, D. M., Principles of
    Instrumental Analysis, Holt, Reinehart and Winston,
    New York, 1971, Chapter 6.

2. Creaser, E.H., and Hughes, G. J., J. Chromatogr.,
    144 (1977) 69.

3. Wassner, S. J., and Li, J. B., J. Chromatogr., 227
    (1982) 497.

4. Simpson, R.C., Mohammad, H. Y., and Veening, H., J.
    Liq. Chromatogr. 5 (1982) 245.

5. Howard, J. W., Fazio, T., White, R. H., J. Ass. Off.
    Anal. Chem., 51 (1968) 544.

6. Narziss, L., Keininger, H., Reichender, E.,
    Brauwissenschaft, 19 (1966) 284.

7. Davis, H. J., Lee, L. A., Davidson, T. R.,, Anal.
    Chem., 38 (1966) 1752.

8   Rudakova, N. Y., Kvyatkovskaya, T. A., Sheremeta, B.
    K., Garmash, E. A., Neftepererab Neftekhim (Moscow)
    9 (1968) 23.

9. Lloyd, J. B. F., Nature, 231 (1971) 64.

10.   Lloyd, J. B. F., J. Forensic Sci. Soc., 12 (1972) 83; 11 (1971) 153; 11 (1971) 235.

11.   Eastwood, D. L.., in Modern Fluorescence Spectroscopy, Wehry, E. L. (Ed.), Vol. 4, Plenum Press, New York, 1981 pp 251.

12.   Miller, J. N., Analyst, 109 (1984) 191.

13.   Guilbault, G. G., Practical Fluorescence Theory, Methods and Techniques, Marcel Dekker, New York, 1973, Chapter 1.

14.   Seiler, N., and Demirsch, L., in Handbook of Derivatives for Chromatography, Blau, K., and King. G. S. (Eds.), Heyden, London, 1977, Chapter 9.

15.   Juan, Y., in Methods in Enzymology, Hirs, C. H. W., and Timasheff, S. N. (Eds), Vol. 26C, Acad. Press, New York/London, 1972, pp 498.

16.   Turro, N. J., Molecular Photochemistry, Benjamin, W. A., Inc., New York, 1967.

17.   Kasha, M., Disc. Farad. Soc., 9 (1950) 14.

18.   Cottrell, C. T., and Masters, V. M., Pye-Unicam Applications Booklet, 1985.

19. Robinson, G. W., and Frosch, R. P., J. Chem. Phys. 38 (1963) 1187.

20. Guilbault, G. G., Fluorescence Theory, Instrumentation and Practice, Marcel Dekker, New York, 1967.

21. Parlopoulos, T., and El-Sayed, M. A., J. Chem. Phys., 41 (1964) 1082.

22. Weber, G., Biochem. J., 51 (1952) 155.

23. Coons, A. H., Creech, H. J., and Jones, R. N., Proc. Soc. Exptl. Biol. Med., 47 (1941) 200.

24. Clayton, R. M., Nature, 174 (1954) 1059.

25. Mayersbach, H., Acta Histochem., 5 (1958) 351.

26. Redetzki, M., J. Biol. Med., 98 (1958) 120.

27. Uehleke, Z., Naturforsch, 136 (1958) 722.

28. Ito, M., and Nishioka, K., Japan J. Mikrobiol., 3 (1959) 71.

29. Wolochow, H., J. Bacteriol., 77 (1959) 164.

30.   Rinderknecht, H., Experentia, 16 (1960) 430.

31.   Sokol, F., Hána, L., and Albrecht, P., Folia
      Microbiologia, 6 (1961) 145.

32.   Chen, R. F., Arch. Biochem. Biophys., 120 (1967)
      607.

33.   Neadle, D. J., and Pollitt, R. J., Biochem. J., 97
      (1965) 607.

34.   Seiler, N., Schmidt-Glenewinkel, T., Schneider, H.
      H., J. Chromatogr., 84 (1973) 95.

35.   Seiler, N., and Knodgen, B., Org. Mass Spectrom., 7
      (1974) 97.

36.   Weigele, M., De Bernardo, S. L., Tengi, J. P., and
      Leimgruber, W., J. Am. Chem. Soc., 94 (1972) 5927.

37.   De Bernardo, S., Weigele, M., Toome, V., Manhart,
      K., Leimgruber, W., Bohlen, P., Stein, S., and
      Udenfriend, S., Arch. Biochem. Biophys., 163 (1974)
      390.

38.   Udenfriend. S., Stein, S., Bohlen, P., and Dairman,
      W., Science, 178 (1972) 871.

39. Stein, S., Bohlen, P., and Udenfriend, S., Arch.
    Biochem. Biophys., 163 (1974) 400.

40. McHugh, W., Sandman, R. A., Haney, W. G., Sood, J.
    P., and Wittmer, D. P., J. Chromatogr., 124 (1974)
    376.

41. Ahnoff, M., Grundevik, I., Arfwidsson, A.,
    Fonselius, J., and Persson, B. A., Anal. Chem., 53
    (1981) 485.

42. Watanabe, Y., and Imai, K., Anal. Chem., 55 (1983)
    1786.

43. Ghosh, P. B., and Whitehouse, M. W., Biochem. J.,
    108 (1968) 155.

44. Lawrence, J. F., and Frei, R. W., Anal. Chem., 44
    (1972) 2046.

45. Fages, R. S., Kurina, C. B., and Abrahamson, E.
    W., Anal. Biochem., 53 (1973) 290.

46. Roth, M., Anal. Biochem., 43 (1971) 880.

47. Taylor, S., and Tappel, A. L., Anal. Biochem., 56
    (1973) 140.

48. Simons, S. S. Jr., and Johnson, D. F., J. Am. Chem.
     Soc., 98 (1976) 7098.

49. Simons, S. S. Jr., and Johnson, D. F., J. Chem. Soc.
     Chem. Commun., (1977) 374.

50. Simons, S. S. Jr., and Johnson, D. F., J. Org.
     Chem., 43 (1978) 2886.

51. Roth, M., and Hampai, A., J. Chromatogr., 83 (1973)
     353.

52. Weidekamm, E., Wallach, D. F. H., and Fluckiger, R.,
     Anal. Biochem., 54 (1973) 102.

53. Kagan, J., Tetrahedron Lett., (1966) 8097.

54. Mell, L. D., Dasler, A. R., and Gustafsun, A. B., J.
     Liq. Chromatogr., 1 (1978) 261.

55. Jones, B. N., Pääbo, S., and Stein, S., J. Liq.
     Chromatogr., 4 (1981) 565.

56. Lindroth, P., and Mopper, K., Anal. Chem., 51 (1979)
     1667.

57.   Simons, S. S. Jr., and Johnson, D. F., Anal.
Biochem., 82 (1977) 250.
Rucera, P., and Umagat, H., J. Chromatogr., 255
(1983) 563.

58.   Reader, D. M., Sneigoski, L. T., and Schaffer, R.,
Anal. Biochem., 86 (1978) 490.

59.   Fourche, J., Jensen, H., and Neuzil, E., Anal.
Chem., 48 (1976) 155.

60   Chen, R. F., Scott, C., and Trepman, E., Biochim.
Biophys. Acta, 576 (1979) 440.

61.   Cronin, J. R., and Hare, P. E., Anal. Biochem., 81
(1977) 151.

62.   Simons, S. S. Jr., and Johnson, D. F., Anal.
Biochem., 90 (1978) 705.

63.   Benson, J. R., and Hare, P. E., Proc. Nat. Acad.
Sci., USA, 72 (1975) 619.

64.   Hare, P. E., in Methods in Enzymology, Hirs, C. H.
W., (Ed.), Vol. 47E, Acad. Press, New York, 1977
pp. 3.

65.   Torres, A. R., Alvarez, V. L., and Sandberg, L. B.,
Biochim. Biophys. Acta, 434 (1976) 209.

66.   Viets, J. W., Deen, W. M., Troy, J. L., and Brenner,
       B. M., Anal. Biochem., 88 (1978) 513.

67.   Schwabe, C., and Catlin, J. C., Anal. Biochem., 61
       (1974) 302.

68.   Schiltz, E., Schnackerz, K. D., and Gracy, R. W.,
       Anal. Biochem., 79 (1977) 33.

69.   Benson, J. R., Anal. Biochem., 71 (1976) 459.

70   Mendes, E., and Gavilanes, J. G., Anal. Biochem., 72
       (1976) 473.

71.   Kutchai, H., and Geddis, L. M., Anal. Biochem., 77
       (1977) 315.

72.   Butcher, E. C., and Lowry, O. H., Anal. Biochem., 76
       (1976) 502.

73.   Jocelyn, P. C., and Kamminya, A., Anal. Biochem., 37
       (1970) 417.

74.   Porter, D. H., Swaisgood, H. E., and Catignani, G.
       L., Anal. Biochem., 123 (1982) 41.

75.   Trepman, E., and Chen, R. F., Arch. Biochem.
       Biophys., 204 (1980) 524.

76.　Winspear, M. J., and Oaks, A., J. Chromatogr.,
270 (1983) 378.

77.　Spackman, D. H., Moore, S., and Stein, W. H., Anal.
Chem., 30 (1958) 1190.

78.　Roth, M., J. Clin. Chem. Clin. Biochem., 14 (976)
361.

79.　Josefsson, B., Lindroth, P., and Ostling, G., Anal.
Chim. Acta, 89 (1977) 21.

80.　Simpson, R. C., Spriggle, J. E., and Veening, H., J.
Chromatogr., 261 (1983) 407.

81.　Schwedt, G., Anal. Chim. Acta, 92 (1977) 337.

82.　Takahashi, H., Nara, Y., Meguro, H., Tuzimura, K.,
Agric. Biol. Chem., 43 (1979) 1439.

83.　Jarrot, B., Anderson, A., Hooper, R., and Louis, W.
J., J. Pharm. Sci., 70 (1981) 665.

84.　Newton, G. L., Dorian, R., and Fahey, R. C., Anal.
Biochem., 114 (1981) 383.

85.　Jocelyn, P. C., Biochemistry of the SH group, Acad.
Press, New York, 1972.

86.   Nakamura, H., and Tamura, Z., Anal. Chem., 54 (1982)
       1951.

87.   Molnár, I., and Horváth, C., Clin. Chem., 22
       (1976) 1497.

88.   Davis, T. P., Gehrke, C. W., Gehrke, C. W. Jr.,
       Cunningham, T. D., Kuo, K. C., Gerhardt, K. O.,
       Johnson, H. D., and Williams, C. H., Clin. Chem.,
       24 (1978) 1317.

89.   Buteau, C., and Duitschaever, C. L., J. Chromatogr.,
       212 (1981) 23.

90.   Bachrach, U., Function of Naturally Occuring
       Polyamines, Acad. Press, New York/London, 1973.

91.   Sugiura, T., Hayashi, T., Kawai, S., and Ohno, T.,
       J. Chromatogr., 110 (1975) 385.

92.   Veening, H., Pitt, W. W. Jr., and Jones, G. Jr., J.
       Chromatogr., 90 (1974) 129.

93.   Lawrence, J. M., and Lee, L. Y., Clin. Chem., 21
       (1975) 1721.

94.   Turnell, D. C., and Cooper, D. H., Clin. Chem., 28
       (1982) 527.

95. Griffin, M., Price, S. J., and Palmer, T., Clin. Chim. Acta, 125 (1982) 89.

96. Cooper, J. D. H., Lewis, M. T., and Turnell, D. C., J. Chromatogr., 285 (1984) 484.

97. Cook, J. W., Hunter, L., and Schoental, R., J. Chem. Soc., Suppl. (1949) 228.

98. Matuszewski, B., Givens, R., Srinivasachar, K., and Carlson, R. G., in Abstracts, 10th International Symposium on Column Liquid Chromatography, San Francisco, California, 1986 pp. 1303.

99. Kawasaki, T., Wong, O., Imai, K., Higuchi, T., Givens, R. S., Stobugh, J.,and Kuwana, T., in Abstracts, 10th International Symposium on Column Liquid Chromatography, San Francisco, California, 1986 pp. 3511.

100. Kristjansson, F., and Stobaugh, J. F., in Abstracts, 10th International Symposium on Column Liquid Chromatography, San Francisco, California, 1986 pp. 3805.

101. Kuwana, T., and Imai, K., in Abstracts, 10th International Symposium on Column Liquid Chromatography, San Francisco, California, 1986 pp. 3515.

102. Soini, E., and Hemmila, I., Clin. Chem., 25 (1979) 353.

103. Cook, J. W., and Schoental, R., Nature, 161 (1948) 237.

104. Weygand, F., Kinkel, K. G., and Tietjen, D., Chem. Ber., 83 (1950) 394.

105. Yagi, K., Mem. Inst. Sci. Ind. Research, Osaka, Univ. 8 (1951) 200; in C. A. 46 (1952) 7086f.

106. Ried, W., and Bodem, H., Chem. Ber., 89 (1956) 708

107. Criegee, R., Marchand, B., and Winnowius, H., Ann., 550 (1942) 99.

108. Cramer, F., "Einschlussverbindungen", Springer-Verlag, Heidelberg, 1954.

109. Cramer, F., Saenger, W., and Spatz, H., J. Am. Chem. Soc., 89 (1967) 14.

110. Cramer, F., and Kampe, W., J. Am. Chem. Soc., 87 (1965) 1115.

111. French, D., Advan. Carbohydrate Chem., 12 (1957) 250.

112. Cramer, F., Angew. Chem., 73 (1961) 49.

113. Nemethy, G., and Scheraga, H. A., J. Chem. Phys.,
     36 (1962) 3401.

114. Zahn, H., Kolloid, Z., 197 (1964) 14.

115. Heinze, W. I., in Solution Chemistry of Surfactants,
     Mittal, K. L., (Ed.), Vol. 1, Plenum Press, New
     York, 1979 pp.79.

116. Turro, N. J., Gratzel, M., and Braun, A. M., Angew.
     Chem. Int. Ed. Engl., 19 (1980) 675.

117. Singer, L. A., in Solution Behaviour of Surfactants,
     Mittal, K. L., and Fendler, E. J., (Eds.), Vol. 1,
     Plenum Press, New York, 1982 pp.73.

118. Michaelson, I. A., and Smithson, H. P., Anal. Chem.,
     43 (1971) 1300.

119. Lippert, E., Z. Electrochem., 61 (1957) 962.

120. Hartley, B. S., Biochem. J., 80 (1961) 36P.

121. Hartley, B. S., and Massey, V., Biochim. Biophys.
     Acta, 21 (1956) 58.

122. Geren, I. M., and Millett, F., J. Biol. Chem., 256 (1981) 10485.

123. Weber, G., and Teale, F. J. W., Disc. Farad. Soc., 27 (1959) 134.

124. Lin, H. J., and Kirsch, J. F., Anal. Biochem., 81 (1977) 442.

125. Geren, N. M., Advan. Protein Chem., 29 (1975) 85.

126. Al-Hakiem, M. H. H., Landon, J., Smith, D. S., and Nargessi, R. D., Anal. Biochem., 116 (1981) 264.

127. Ruzicka, J., and Hansen, E. H., Anal. Chim. Acta, 78 (1975) 145.

128. Stewart, K. K., Brown, J. F., and Golden, B. M., Anal. Chim. Acta, 114 (1980) 119.

129. Froehlich, in Modern Fluorescence Spectroscopy, Wehry, E. L. (Ed.), Vol. 2, Heyden, London, 1976.

130. Ruzicka, J., and Hansen, E. H.,Anal. Chim. Acta, 99 (1978) 37.

131. Braithwaite, J. I., and Miller, J. N., Anal. Chim. Acta, 106 (1979) 395.
Petty, R. L., Michel, Snow, J. P., and Johnson, K. S., Anal. Chim. Acta, 142 (1982) 299.

132. Ruzicka, J., and Hansen, E. H., Flow Injection Analysis, John Wiley and Sons, New York, 1981 pp.37.

133. Johnson, E., Abu-Shumays, A., and Abbott, S. R., J. Chromatogr., 134 (1977) 107.

134. Stein, S., Böhlen, P., Stone, J., Dairman, W., and Udenfriend, S., Arch. Biochim. Biophys., 115 (1973) 203.

135. Bohlen, P., and Miller, M., Anal. Biochem., 94 (1979) 313.

136. Hill, D. W., Walters, F. H., Wilson, T. D., and Stuart J. D., Anal. Chem., 51 (1979) 1338.

137. Stuart, J. D., Wilson, T. D., Hill, D. W., Walters, F. H., and Feng, S. Y., J. Liq. Chromatogr., 2 (1979) 809.

138. Hodgin, J. C., J. Liq. Chromatogr., 2 (1979) 1047.

139. Gardner, W. S., and Miller, W. R., Anal. Biochem., 101 (1980) 61.

140. Stobaugh, J. F., Ph.D. Thesis, The University of
      Kansas, 1982.