New luminescence-linked immunoassay techniques

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NEW LUMINESCENCE-LINKED IMMUNOASSAY TECHNIQUES

by

Hasumita Thakrar M.Sc.

A Doctoral Thesis
submitted in partial fulfilment of the requirements for
the award of
DOCTOR OF PHILOSOPHY
of the Loughborough University of Technology

Supervisor:
Professor J. N. Miller

September, 1982

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

I wish to take this opportunity to express my deepest gratitude to my supervisor, Professor J. N. Miller, for his kind help, encouragement, advice and esteemed guidance which provided inspiration not only during this project but for a life-time of research work. The valuable discussions, suggestions and constructive criticism from him have all contributed to the production of this thesis.

I would also like to thank the Science and Engineering Research Council for their financial support.

Thanks are due to the technical staff, in particular J. J. Swithenbank and A. G. Stevens; and the kind help of my colleagues, Dr. A. Mallard and Mr. R. E. Simmons. I am most appreciative of the continuous support and excellent typing by Carolyn Newton, and also of the valuable assistance from the administrative staff.

Lastly, to my dear father who can no longer witness this thesis, my ultimate gratitude. To my dear mother for her patience, prayerful blessings for my welfare and success, my most sincere gratitude.
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Several fluorescent labels were studied to develop simple luminescence immunoassay methods for both macromolecules and low molecular weight analytes.

Initially, the 'Fluram (Fluorescamine) enhancement phenomenon' was investigated by extending its use for assay of the thyroid hormone, triiodothyronine (T3). The observed enhanced fluorescence permitted the development of a homogeneous assay capable of detecting nanogram concentrations of the hormone in pure solution and hormone depleted serum. The technique was however, complicated by the overlapping Raman scatter peak and attempts to overcome this problem included the use of derivative spectroscopy.

Ortho-phthalaldehyde (OPT), a potential fluorescent label was studied for developing a fluorescence immunoassay for thyroxine (T4). The work involved the preparation and study of fluorescence properties of OPT derivatives of several iodinated amino acids.

An energy-transfer immunoassay was developed for human serum albumin where a "triplet state label" eosin was the acceptor and fluorescein the donor label. To overcome some of the shortcomings of the assay, two further donor labels, quinacrine and fluorescamine were investigated, with eosin as the acceptor in each case. The assay seemed sufficiently sensitive for determining for example, urinary albumin levels.

Most fluorescence homogeneous immunoassays have been largely limited to the ng. ml$^{-1}$ range by background fluorescence from biological samples (blood serum, urine etc.). A detailed study was thus performed to examine (a) the nature of serum interference, (b) variation from one serum sample to another (serum samples included both from patients and commercial sources), (c) simple
ways to reduce/remove the background signals, these included chemical treatment and abstraction of serum constituents/fractions giving rise to endogeneous fluorescence and possibly scattering by adsorbents such as hydroxyapatite.

In efforts to improve luminescence-linked immunoassay development, another approach was investigated where the fluorescent label (conjugate) was excited by a chemical reaction between hydrogen peroxide and a bis-oxalate ester. This technique was applied to the Fluram-T3 assay to compare and overcome some problems encountered with the photo-excitation method.

Flow injection analysis was used in conjunction with chemiluminescence detection.
CHAPTER 1

INTRODUCTION: LUMINESCENCE-LINKED IMMUNOASSAYS

1.1 General

1.1.1 Principles of Immunoassay

Since its realization by Berson and Yalow (1959), the technique of immunoassay has provided a powerful means of determining a broad spectrum of substances of interest. The power of immunoassay lies in its combination of specificity, versatility, practicability and sensitivity.

The principles of an immunoassay are straightforward. If the substance of interest is foreign to an animal (typically a rabbit or sheep) the injection of the substance into that animal will cause the animal to produce a glycoprotein, known as an antibody (Ab) that will specifically bind to that substance. In this case, the substance is known as an antigen (Ag). Antigens are generally naturally occurring macromolecules (proteins, polysaccharides, nucleic acids etc.) or micro-organisms that can be regarded as assemblies of such molecules. Smaller molecules such as peptides or drugs do not cause antibody production on their own but can be made to do so by coupling them to macromolecular carriers (proteins or synthetic polypeptides) before injecting them into experimental animals. The resulting antibodies react with the small molecule alone as well as with the small molecule-carrier conjugate. Such small molecules are known as haptens.

Immunoassay is based on the antigen (or hapten)-antibody binding reaction which is reversible and noncovalent.

\[ Ag + Ab \rightarrow Ag - Ab \]
If a label is covalently attached to the antigen such that the label does not block the antigen region recognised by antibody, the presence of label will not significantly perturb the binding of labelled antigen (Ag*) to antibody; analogously,

\[ \text{Ag}^* + \text{Ab} \rightleftharpoons \text{Ag}^* - \text{Ab} \]
can be written. Finally, a situation is considered in which labelled antigen, unlabelled antigen and antibody are present,

\[ \text{Ag} + \text{Ag}^* + \text{Ab} \rightleftharpoons \text{Ag} - \text{Ab} + \text{Ag}^* - \text{Ab} \]
the labelled antigen will compete with unlabelled antigen for antibody binding sites. The reaction (known as competitive binding reaction) is normally allowed to attain equilibrium and the relative concentration of labelled antigen-antibody complex will decrease as a function of increasing unlabelled antigen concentration in the reaction mixture. If some property proportional to the concentration of the labelled antigen-antibody complex is measured, standard curves can be constructed where the measured value of the property is plotted as a function of unlabelled antigen concentration. The unknown antigen concentration can then be determined by merely reacting the sample in a mixture which contains a known amount of labelled antigen and a known amount of antibody and allowing the competitive binding to occur; the antigen content can be inferred from the standard curve.

Immunoassays are of two basic types: homogeneous assays, in which no separation step is required, and heterogeneous ones where a separation step is necessary to separate antibody-bound from unbound materials.
The specificity of an immunoassay is obtained through the use of the antibody; the versatility of immunoassay stems from the fact that antibodies may be produced against a wide range of substances, and the sensitivity is obtained through a judicious choice of the label. Immunoassays are most commonly used to quantitate antigens, but also have been employed to detect the presence of circulating antibodies. Immunoassays for the quantitation of antigen can employ labelled antibodies (immunometric assays) or labelled antigens.

1.1.2 Radiolabels: Advantages and Disadvantages

The type of label employed is a major distinguishing factor between immunoassays. The label originally used was a radioisotope (Yalow and Berson, 1960), and this radioimmunoassay (RIA) method remains the most common form. The conventional label is $^{125}$I or $^{131}$I. Radioimmunoassays have been widely used for the determination of hormones (especially thyroid hormones), drugs (therapeutic and drugs of abuse) and macromolecules (e.g., α-fetoprotein, ferritin, thyroglobulin) whose normal concentrations are too low. The principal advantages of RIA are the selectivity conferred by the specific immunological reaction, and its extreme sensitivity, the latter derives from the absence of background radiation in biological samples as well as the very low levels at which radioactivity can be detected. The major disadvantages of RIA are (i) the specialised equipment (and associated safety measures) required is not available in many laboratories, (ii) the labelled antigens (hapten)s are intrinsically unstable and often costly, (iii) the properties of the radiolabel are the same whether the labelled molecule is bound to antibody or not, hence a separation is needed before the distribution of labelled antigens (hapten)s.
between bound and free fractions can be determined. The separation methods usually used are: (i) adsorption of the antigen (hapten) on charcoal or dextran-coated charcoal, (ii) use of solid phase antibodies e.g. antibodies adsorbed on the inside surfaces of polystyrene tubes, and (iii) the use of second, precipitating antibodies which form insoluble complexes with the anti-antigen (hapten) antibodies. The separation step inevitably complicates the assay and renders its automation difficult. It would be advantageous to develop an alternative method, one with sensitivity and specificity of radioimmunoassay which would avoid the associated drawbacks.

Miles and Hales (1968) introduced an immunoassay based on radiolabelled specific antibodies, termed immunoradiometric assay (IRMA). In this type of assay, quantitation is possible if either the amount of antibody that becomes bound to antigen or the remaining amount of free antibody, can be determined i.e. it is necessary to separate the antibody in the bound fraction from that in the free fraction and the counts in the bound fraction are related directly to the total amount of antigen present. A practical drawback of IRMA (in addition to the other disadvantages as mentioned for RIA) is that immunospecifically purified antibodies must normally be used. The recent advent of monoclonal antibodies (Koprowski et al., 1977; Catty et al., 1981) should overcome this problem.

Despite the apparently favourable sensitivity offered by RIA and of the theoretically distinct but practically similar immunoradiometric method, the last few years have seen a rapid upsurge of methods in which alternative (non-isotopic) labels are used.
1.1.3 Alternatives (non-isotopic labels): Merits in general

In order to overcome the problems and inconvenience posed by RIA, substantial research efforts have been committed to the field of non-isotopic immunoassay i.e. assays in which the labels used in RIA (³H, ¹⁴C, ¹²⁵I etc.) are replaced by less hazardous and more conveniently detected labels. Non-isotopic labels may also confer the additional advantage of permitting homogeneous assays, i.e. methods in which separation steps are not necessary. Furthermore, such labels often provide stable reagents. (These and other advantages offered especially by luminescent labels are discussed in Section 1.2.) The applicability of alternative labels has rapidly increased; several reviews on non-isotopic immunoassay techniques have been recently published (Table 1.1). Among the alternative labels that have been used or investigated have been enzymes and fluorescent and chemiluminescent groups: see Table 1.2. An important test of such labels of course is the extent to which they can match the sensitivity of detection of radiolabels. It can be seen from Table 1.2 that it is the label and the end-point detection which differentiates the various types of non-isotopic immunoassays.
Table 1.1

Some published reviews on non-isotopic immunoassay techniques

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<tr>
<th>Immunoassay Type</th>
<th>References (recent)</th>
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<td>Antibodies: Ideal reagents for analysts</td>
<td>Miller, 1981</td>
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<td>Enzyme-Immunoassay</td>
<td>Schuurs and van Weeman, 1980; Ishikawa et al., 1981*</td>
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<tr>
<td>Luminescence Immunoassay</td>
<td></td>
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<tr>
<td>Fluorescence</td>
<td>O'Donnell and Suffin, 1979; Soini and Hemmila, 1979; Maggio, 1980; Ullman, 1979, 1981; Smith et al., 1981 (a) and (b); Visor and Schulman, 1981; Quatrone et al., 1981; Van der Plas et al., 1981</td>
</tr>
<tr>
<td>(Also see Section 1.2)</td>
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</tr>
<tr>
<td>Chemiluminescence and Bioluminescence</td>
<td>Whitehead et al., 1979; Gorus and Schram, 1979; Olsson and Thore, 1981; Wilson, 1980; Seitz, 1981; Schram and Stanley, 1979; Deluca and McElroy, 1981*</td>
</tr>
<tr>
<td>(Also see Section 1.3)</td>
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* Published in the form of books (reviews and the references therein).
<table>
<thead>
<tr>
<th>Label (Imunoassay Technique)</th>
<th>Detection System (Common)</th>
<th>Comments</th>
<th>Reference (Examples)</th>
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<tr>
<td>Enzymes</td>
<td>i  spectrophotometry</td>
<td>simple</td>
<td>Van Weeman and Schuurs, 1971</td>
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<td></td>
<td>ii fluorometry</td>
<td></td>
<td>Rubenstein et al., 1972</td>
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<td></td>
<td>iii photometry</td>
<td></td>
<td>Ishikawa and Kato, 1978</td>
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<td></td>
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<td></td>
<td>Velan and Halman, 1978</td>
</tr>
<tr>
<td>Coenzymes</td>
<td>spectrophotometry</td>
<td>complicated</td>
<td>Carrico et al., 1976</td>
</tr>
<tr>
<td>NAD</td>
<td></td>
<td></td>
<td>Carrico et al., 1976(a)</td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
<td>Several - see Section 1.2</td>
</tr>
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<td>Luminescent molecules</td>
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<td></td>
<td>Schroeder et al., 1978(a)</td>
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<tr>
<td>Fluorophore</td>
<td>fluorimetry</td>
<td>simple</td>
<td></td>
</tr>
<tr>
<td>(Fluorescence Immunoassay)</td>
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<td>Chemiluminescent groups</td>
<td>photometry</td>
<td>complicated</td>
<td>simple in principle but time dependence presents difficulties in practice</td>
</tr>
<tr>
<td>(Chemiluminescence &amp; Bioluminescence Immunoassay)</td>
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</tr>
<tr>
<td>Label (Imunoassay Technique)</td>
<td>Detection System (Common)</td>
<td>Comments</td>
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<tr>
<td>Metal atoms (Metalloimmunoassay)</td>
<td>i atomic absorption spectrometry</td>
<td>complicated</td>
<td>requires complex instrumentation</td>
</tr>
<tr>
<td></td>
<td>ii fluorimetry</td>
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<tr>
<td>Metal sols (Sol particle immunoassay)</td>
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<td></td>
<td>ii visible response</td>
<td></td>
<td></td>
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<td>i photometry</td>
<td>simple</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ii particle counting</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>iii visible response</td>
<td></td>
<td></td>
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<tr>
<td>Virus/bacteriophage (Viroimmunoassay)</td>
<td>bacterial culture</td>
<td>difficult to retain viability of bacteriophage</td>
<td>complicated and tedious</td>
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<tr>
<td>Erythrocytes</td>
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<td>simple</td>
<td></td>
</tr>
<tr>
<td>Free radicals (Spin immunoassay)</td>
<td>electron spin resonance spectrophotometry</td>
<td>complicated</td>
<td>requires complex instrumentation</td>
</tr>
<tr>
<td>Label (Immunoassay Technique)</td>
<td>Detection System (Common)</td>
<td>Comments</td>
<td>Reference (Examples)</td>
</tr>
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<tr>
<td>Electroactive group (Voltammetric immunoassay)</td>
<td>voltammetry</td>
<td></td>
<td>Heineman et al., 1979</td>
</tr>
<tr>
<td>Photoelectro-chemically active label</td>
<td>electrochemistry</td>
<td></td>
<td>Weber, 1981</td>
</tr>
<tr>
<td>Dispersed dyes (water-dispersed hydrophobic dyes)</td>
<td>spectrophotometry</td>
<td></td>
<td>Gribnau et al., 1981</td>
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The future choice of the label will be based on the end-point detection, Table 1.3 indicates criteria for an ideal end-point detection.

Table 1.3
Criteria for an ideal end-point detection in a non-isotopic immunoassay

1. The cost, availability and stability of the label.
2. The cost and availability of simple appropriate instrumentation.
3. Precision, accuracy and sensitivity of end-point detection.
4. No influence of biological samples.
5. Non-hazardous.

In several of the non-isotopic immunoassays (Table 1.2), it appears that the complexity (procedures and instrumentation), the time taken and/or insensitivity of the end-point detection makes extensive use at their present stage of development difficult. These include the use of viruses, free radicals and metals. Some enzyme immunoassays are believed to be comparable to radioimmunoassays in as far as sensitivity is concerned (Schuurs and van Weeman, 1980).

One of initial practical alternatives to RIA was the homogeneous enzyme immunoassay (Rubenstein et al., 1972). Recently, Rubenstein (1981) has adapted the EMITR homogeneous enzyme immunoassay for proteins. The applications of enzyme immunoassay has continued to expand dramatically and a large number of heterogeneous enzyme
immunoassay kits are also commercially available (Schall and Tenoso, 1980). Enzyme immunoassays using fluorimetric detection have been described. Ishikawa and Kato (1978) developed an assay for macromolecular analytes with a sensitivity of $10^{-18}$ moles.

During the last few years, a lot of interest has been directed towards luminescent immunoassays. Luminescent labels meet almost all of the criteria mentioned in Table 1.3 and the number of reviews published (see Table 1.1) clearly indicates the interest that many investigators have expressed in luminescence-linked immunoassays, the degree of success achieved and the substantial potential for improvement over the present approaches. The potential advantages of luminescence immunoassays (referred largely to fluorescence and chemiluminescence in this chapter) are clear. Firstly, in principle they will be capable of extreme sensitivity. A strongly fluorescent molecule can routinely be detected at pg ml$^{-1}$ levels in pure solution. Secondly the instrumentation and methodology required are neither costly nor complex; fluorescence spectrometers may in any case be used in clinical laboratories for other assays. Finally homogeneous (and hazard-free) assays can be developed. Chemiluminescent molecules can be detected at < pg ml$^{-1}$ levels in a few seconds.

In this chapter, immunoassays based on luminescent techniques are described. Substantial research and development of fluorescence immunoassays has been concentrated in the area of homogeneous assay, as a result only homogeneous immunoassays are discussed; heterogeneous assays, which offer some special advantages are only briefly described. Chemiluminescence-linked immunoassays, especially in homogeneous formats, are in the early stages of development, thus the discussion is largely limited to heterogeneous assays.
1.2 Luminescent Labels

1.2.1 Luminescence: Photoluminescence and Chemiluminescence

Luminescence may be defined as the emission of light from a molecule following its electronic excitation through the absorption of any form of energy. It is the source of the energy responsible for molecular excitation that distinguishes the several categories of luminescent phenomena. Fluorescence and phosphorescence are two forms of luminescence (photoluminescence) in which the excitation is achieved using an external light source. These latter two forms are defined on the basis of the time interval between excitation and photon emission. In the case of fluorescence this time interval is small, being on the order of $10^{-12}$ to $10^{-3}$ seconds, whereas in phosphorescence the interval between excitation and emission may extend from $10^{-3}$ seconds to $10^2$ seconds in duration. Photon emission from a molecule that has undergone excitation as the result of absorbing energy derived from a chemical reaction is defined Chemiluminescence (CL). One special form of chemiluminescence that is operative in a number of biological systems is referred to as Bioluminescence (BL). Bioluminescent systems utilize highly specialised proteins as catalysts in the luminescent reaction (see Whitehead et al., 1979) i.e. BL $\equiv$ CL, but with high quantum yield. (Luminescence quantum yield is defined as the fraction of molecules that emit a photon after excitation by a light (energy) source.)

1.2.2 Fluorescence Immunoassay (FIA)

1.2.2.1 Fluorescent labels criteria and availability

Fluorescent groups have a number of advantages. They may be cheap, stable, safe and easy to introduce into the antigen or
hapten to be labelled, they can be detected at very low concentra-
tions, fluorimeters are available in many laboratories - they may
be cheap and simple, yet still capable of great sensitivity; and
in many cases the fluorescence properties of the label group may
change when the labelled molecule is bound to an antibody, giving
rise to an easily-automated homogeneous assay.

The choice of the fluorescent label or probe is critical in
designing a fluorescence immunoassay (FIA). In principle it should
be stable, the fluorescence emission will be clearly distinguishable
from the background, it should have a high quantum yield and the
label should not adversely affect the binding of the antibody or
antigen/hapten. Also, the label will only be suitable provided
(i) means are available to link the label to the antigen and
(ii) the antibody does not distinguish significantly between
labelled and unlabelled antigen. A large number of fluorescent
labels (e.g. of reference Kanoaka, 1977) and probes (Beddard and
West, 1981) are reported in literature; due to the rather stringent
requirements, only a few fluorophores have given satisfactory
results. Fluorescein isothiocyanate has been the label of choice
in FIA (Smith et al., 1981(a)) because of its high quantum yield,
chemical stability and its insignificant photolability under normal
fluorimetric conditions. However, fluorescein has a small Stoke's
shift (ca. 30 nm) and some fluorescein-labelled haptens bind non-
specifically to albumin (e.g. of reference Andersson et al., 1971).
The other labels used include various rhodamines, dansyl chloride,
fluorescamine and recently lanthanide metal chelates of europium
and terbium (see Fig. 1.1). Although their quantum yield and
hence the intrinsic fluorescence is lower than some organic probes,
their advantage is that they exhibit very long life times (see
Table 1.4) and would allow sensitive time resolved determinations
suitable in fluorescence immunoassay (see Sections 1.2.2.2 and 1.2.2.3).

Table 1.4

Fluorescent life times of some fluorescent labels

<table>
<thead>
<tr>
<th>Label</th>
<th>Life Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-specific background</td>
<td>10 ns</td>
</tr>
<tr>
<td>Fluorescein Isothiocyanate</td>
<td>4.5 ns</td>
</tr>
<tr>
<td>Dansyl Chloride</td>
<td>14 ns</td>
</tr>
<tr>
<td>N-3-pyrene maleimide</td>
<td>100 ns</td>
</tr>
<tr>
<td>Lanthanide metal chelates</td>
<td>1 µs - 1 ms</td>
</tr>
</tbody>
</table>

Table 1.5 shows the properties of some fluorophores suitable for fluorescence immunoassay.

Figure 1.1

Structure of Europium thenoyl trifluoroacetate (EuTTFA)
(An example of lanthanide metal chelate).

1.2.2.2 Types of Fluorescence Immunoassay (FIA)

Fluorescence immunoassays may be classified into two major groups: heterogeneous (separation step necessary) and homogeneous (separation step not required). The separation step is a serious
### Table 1.5

Properties of some Fluorescence Labels

<table>
<thead>
<tr>
<th>Label</th>
<th>λEx.</th>
<th>λFl.</th>
<th>Quantum yield ϕ</th>
<th>c</th>
<th>Life time, τ ns</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>492</td>
<td>~520</td>
<td>0.85</td>
<td>$7.2 \times 10^4$</td>
<td>4.5</td>
<td>Rinderknecht, 1962; Chen, 1969</td>
</tr>
<tr>
<td>RBITC</td>
<td>550</td>
<td>585</td>
<td>0.70</td>
<td>$1.23 \times 10^4$</td>
<td>3</td>
<td>Chen, 1969; Brandtzaeg, 1975</td>
</tr>
<tr>
<td>RB200SC</td>
<td>~565</td>
<td>~590</td>
<td>0.04</td>
<td>$7.3 \times 10^4$</td>
<td>~1</td>
<td>Fothergill, 1969; Brandtzaeg, 1973</td>
</tr>
<tr>
<td>DNS-C1</td>
<td>340</td>
<td>480</td>
<td>0.30</td>
<td>$3.4 \times 10^3$</td>
<td>14</td>
<td>Hartley et al., 1956; Chen, 1968</td>
</tr>
<tr>
<td>Fluorescamine</td>
<td>~390</td>
<td>~490</td>
<td>0.10</td>
<td>$6.3 \times 10^3$</td>
<td>7</td>
<td>Katsch et al., 1974; Handschin et al., 1976</td>
</tr>
<tr>
<td>MDPF</td>
<td>~390</td>
<td>~490</td>
<td>0.10</td>
<td>$6.4 \times 10^2$</td>
<td></td>
<td>Handschin et al., 1976</td>
</tr>
<tr>
<td>QM</td>
<td>~350</td>
<td>~520</td>
<td>0.1-0.3</td>
<td></td>
<td>~10</td>
<td>Chen, 1976; Andreoni et al., 1980</td>
</tr>
<tr>
<td>ANS</td>
<td>385</td>
<td>471</td>
<td>0.80</td>
<td></td>
<td>16</td>
<td>Kanaoka, 1977</td>
</tr>
<tr>
<td>EITC</td>
<td>~520</td>
<td>~550</td>
<td>0.19</td>
<td>$8.3 \times 10^4$</td>
<td>0.9</td>
<td>Cherry et al., 1976</td>
</tr>
<tr>
<td>Erythrosin ITC</td>
<td>~530</td>
<td>~560</td>
<td>0.02</td>
<td></td>
<td>0.1</td>
<td>Garland et al., 1978</td>
</tr>
<tr>
<td>SITS</td>
<td>~320</td>
<td>430</td>
<td></td>
<td>$3.7 \times 10^4$</td>
<td>~1</td>
<td>Chen, 1969</td>
</tr>
<tr>
<td>Anthracene ITC</td>
<td>~355</td>
<td>460</td>
<td>0.6</td>
<td>$3.04 \times 10^4$</td>
<td></td>
<td>Chen, 1969</td>
</tr>
<tr>
<td>NPM</td>
<td>340</td>
<td>~380</td>
<td></td>
<td></td>
<td>100</td>
<td>Weltman et al., 1973</td>
</tr>
<tr>
<td>NBD-C1</td>
<td>~470</td>
<td>530</td>
<td></td>
<td>$1.29 \times 10^4$</td>
<td></td>
<td>Ghosh et al., 1969; Huang, 1975</td>
</tr>
<tr>
<td>SBD-C1</td>
<td>~390</td>
<td>510</td>
<td></td>
<td></td>
<td></td>
<td>Andrews et al., 1981</td>
</tr>
<tr>
<td>Lucifer Yellow CH (derivatives)</td>
<td>~430</td>
<td>540</td>
<td>0.25</td>
<td></td>
<td></td>
<td>Stewart, 1978</td>
</tr>
</tbody>
</table>

† delayed fluorescence

λEx. Excitation wavelength

λFl. Fluorescence wavelength

c Molar absorption coefficient  

(See page 16 for abbreviations)
Fluorescence Labels in Table 1.5

<table>
<thead>
<tr>
<th>Label</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>RBITCH</td>
<td>Rhodamine-β-isothiocyanate</td>
</tr>
<tr>
<td>RB200SC</td>
<td>Lissamine rhodamine-B200 sulphonyl chloride</td>
</tr>
<tr>
<td>DNS-CL</td>
<td>Dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride)</td>
</tr>
<tr>
<td>Fluorescamine</td>
<td>4-Phenylspiro[furan-2(3),1'-phthalan-3,3'dione] (Fluram)</td>
</tr>
<tr>
<td>MDPF</td>
<td>2-Methoxy-2,4-diphenyl-3(2H)-furanone</td>
</tr>
<tr>
<td>QM</td>
<td>9-(4-diethylamino-1-methyl-buty1amino)-6-chloro-2-methoxy acridine mustard</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilino-1-naphthalenesulphonic acid</td>
</tr>
<tr>
<td>EITC</td>
<td>Eosin isothiocyanate</td>
</tr>
<tr>
<td>Erythrosin ITC</td>
<td>Erythrosin isothiocyanate</td>
</tr>
<tr>
<td>SITS</td>
<td>4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid</td>
</tr>
<tr>
<td>Anthracene ITC</td>
<td>Anthracence-2-isothiocyanate</td>
</tr>
<tr>
<td>NPM</td>
<td>N-(3-pyrene)maleimide</td>
</tr>
<tr>
<td>NBD-CL</td>
<td>7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole</td>
</tr>
<tr>
<td>SBD-CL</td>
<td>7-Chloro-4-sulphonbenzofurazan</td>
</tr>
<tr>
<td>Lucifer Yellow CH</td>
<td>3,6-disulphonate-4-aminonaphthalimide</td>
</tr>
</tbody>
</table>
concern in an immunoassay. This is largely due to the complexity of separation techniques (cf. RIA, see Section 1.1.2) which poses problems in developing fully automated systems.

At the present time, homogeneous techniques are somewhat more limited in sensitivity than heterogeneous techniques. Heterogeneous procedures offer a different approach with both advantages and disadvantages. Heterogeneous fluorescence immunoassays may be analogous to radioimmunoassays and are only briefly described here. Table 1.6 shows the classification of FIA.

<table>
<thead>
<tr>
<th>Table 1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classification of fluorescence immunoassay (FIA)</strong></td>
</tr>
</tbody>
</table>

A  Heterogeneous
1. Solid phase Antigen assay
2. Solid phase Antibody assay

B  Homogeneous
1. Fluorescence Polarisation
2. Quenching and Enhancement
3. Energy Transfer
   (a) Direct antigen label
   (b) Indirect antigen label
4. Fluorescence Protection
5. Fluorescence Internal Reflectance
6. Fluorescence Lifetime
7. Substrate labelled FIA
8. Phase resolved FIA?
9. Photodecomposition FIA?
10. Others
The separation step in heterogeneous fluorescence immunoassays serves two main functions: firstly it separates the antibody bound and free fractions and secondly any interfering substances present in the sample are removed, this enables improvement in sensitivity provided there are adequate wash steps. A large number of heterogeneous assays have been based on solid phase techniques; the commonly used solid-phase FIA techniques include the (i) competitive, (ii) sandwich, (iii) indirect, and (iv) fluorooimmunometric assays. Many heterogeneous FIAs have involved antibodies covalently bound to polyacrylamide beads (Curry et al., 1979), to magnetisable particles (Nargessi et al., 1978(a); Pourfarzanch et al., 1980) or immobilised on a special surface (Hull, 1978).

At least two solid-phase fluorescence immunoassays are commercially available. The first utilizes dipsticks to which are attached flat discs of a polymeric matrix, such as cellulose (the FIAx/StiQ method, IDT, Santa Clara, California, U.S.A.) and the second utilizes suspendable polyacrylamide microbeads having a refractive index equal to the assay medium making them transparent in the assay medium (the Fluoromatic System, BioRad, Richmond, California, U.S.A.). A fluorescent dye bound to the microbeads is measured in a dedicated automated fluorimeter. This instrument employs photon counting techniques for increased sensitivity (Simonsen, 1981).

Heterogeneous methods are generally more complicated and require specialised or dedicated instrumentation; however heterogeneous assays do offer advantages over homogeneous techniques (see Section 1.2.2.3).
A homogeneous immunoassay is made possible by changes in fluorescence properties resulting from antibody binding, these may include enhancement or quenching of fluorescence; energy transfer effects, changes in fluorescence wavelength and/or fluorescence polarization, increased or decreased resistance to photodecomposition etc. (see Table 1.6). The major advantage of homogeneous assays lie in speed, convenience, ease of automation and the enhanced precision that may be obtained with such assays.

The changes in properties induced by antibody binding of the fluorophore labelled analyte, as listed in Table 1.6 have been used to monitor several homogeneous fluorescence immunoassays for proteins, haptens and drugs. Table 1.7 shows the sensitivity comparison of some of the homogeneous FIAs. The assay techniques are described below.

<table>
<thead>
<tr>
<th>Assay Technique</th>
<th>Analyte (Example)</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Polarization</td>
<td>Gentamicin</td>
<td>2 μg ml⁻¹</td>
<td>Watson et al., 1976</td>
</tr>
<tr>
<td>2. Quenching</td>
<td>Gentamicin</td>
<td>1 μg ml⁻¹</td>
<td>Shaw et al., 1977</td>
</tr>
<tr>
<td>3. Enhancement</td>
<td>Thyroxine</td>
<td>10⁻⁹M</td>
<td>Smith, 1977</td>
</tr>
<tr>
<td>4. Energy Transfer</td>
<td>Morphine</td>
<td>10⁻¹⁰M</td>
<td>Ullman et al., 1976</td>
</tr>
</tbody>
</table>
1. **Polarisation FIA**

The fluorescence from a small molecule is normally unpolarised, or very nearly so, since the molecule undergoes many random changes in orientation during the lifetime of the excited singlet state. Small molecules bound to macromolecules may show appreciably polarised fluorescence, since molecular motions are much slower in such cases. The increase in fluorescence polarisation that accompanies binding of a fluorescent-labelled small molecule to an antibody can thus be used in the development of a homogeneous immunoassay. The polarisation FIA technique was first described by Dandliker et al., (1973, and recently reviewed 1980), and Spencer et al., (1973) who described a simple continuous flow automation of polarisation FIA. Most of the published fluorescence polarisation methods involve labelled antigen (Dandliker et al., 1980).

Polarisation FIA is quick and precise, however it has some disadvantages. Specialised instrumentation is required for polarisation measurements, in many cases the change in polarisation that occurs is small and the sensitivity of the assay limited (μ mol l⁻¹ to upper nmol l⁻¹ range): see Section 1.2.2.3.

2. **Quenching and Enhancement FIA**

A decrease or increase in the fluorescence of a labelled antigen upon binding to antibody has provided a basis for a homogeneous FIA. In fluorescence quenching immunoassay, the quenching induced by antibody binding to the labelled antigen is relieved by unlabelled antigen which will compete with labelled antigen for binding sites on antibody. An example of such assays is the
quenching of fluorescein-gentamicin conjugate on binding to anti-gentamicin antibodies (Shaw et al., 1977, also used in conjunction with continuous flow technique, Shaw et al., 1979). This phenomenon provides access to extremely simple homogeneous FIA, however the method is not general, antibodies from different sources are likely to produce variable quenching effects which may be too small to provide a satisfactory signal. The mechanisms of fluorescence quenching are apparently not fully understood - and may not be the same in all assays - but this does not prevent the use of the method.

In some cases, antibody binding to fluorescent antigen produces enhancement (termed Fluorescence enhancement assay, see Chapter 5) and an example includes the enhanced fluorescence of a fluorescein-thyroxine conjugate on binding to anti-T4 antibodies (Smith, 1977). This observation has been attributed to inhibition of intramolecular quenching due to the iodine atoms of the thyroxine moiety when it becomes bound to the antibody. Such assays especially with fluorescein-iodothyronine conjugates will be unreliable because serum also relieves quenching through non-specific effects, e.g. serum proteins, particularly albumin, cause non-specific enhancement of fluorescein-thyroxine conjugates.

Recently, other fluorescence enhancement immunoassays have been developed and are based on a 'Fluram enhancement phenomenon' (Handley, 1979; Lim, 1980). A fluorescent enhancement assay capable of determining nanogram quantities of thyroxine in blood serum has been developed (Handley et al., 1979). This assay uses fluorescamine as the label. Subsequent work showed that the fluorescence enhancement phenomenon found in the case of fluorescamine-labelled T4 is a general phenomenon with this label (and with the related label methoxydiphenylfuranone, MDPF). Any
molecule labelled with fluorescamine exhibits enhanced fluorescence (normally 2-5 fold enhancement) on binding to an appropriate antibody. No exceptions to this phenomenon have been found so far amongst a considerable number of low- and high-molecular weight species.

It seems that this technique will find more use since the equipment cost is very low compared with radioimmunoassays and polarisation FIA, however, a very stable fluorescence spectrometer would be required. (Also see Section 1.2.2.3 and Chapter 5.)

3. **Energy Transfer FIA**

This is a general immunochemical method for assay of haptens and proteins, first described by Ullman and co-workers (1976) and most recently reviewed by Ullman and Khanna, 1981. It involves labelling both antigen (hapten) and antibody. The principles of the assay are shown in Fig. 1.2. The fluorescent labels are so chosen that antigen-antibody combination permits non-radiative energy transfer from one label to another: the fluorescence of the donor label is thus quenched, and that of the acceptor label possibly enhanced, thus separation of reactants is not required. Energy transfer FIAs have been applied to a variety of analytes (e.g. of references, Ullman et al., 1976; Van Derwerf and Chang, 1980). These assays have utilized fluorescein and rhodamine as donor and acceptor labels respectively. Lim (1980) made a detailed study of energy transfer assays, both in homogeneous solution and with the aid of solid phase and found that the fluorescein and rhodamine label pair is far from ideal (see Chapter 7). New pairs of donor and acceptor labels have been studied, fluorescamine-fluorescein (donor-acceptor) pair was found
to be highly satisfactory (Miller et al., 1980) and sensitive assays were developed for both small (tricyclic antidepressants) and large molecules (transferrin) (Lim, 1980).

A modification of the technique has been described (Ullman et al., 1976) in which separate antibody samples (portions) can be labelled with donor and acceptor groups: sample antigen causes aggregation of the separately labelled components and the effects of energy transfer are again observed. In this version of the method (also called Indirect antigen labelling), which is confined to the assay of antigens with two or more determinant groups, a purified antigen is not essential, however purified antibodies are required so as to avoid excessive fluorescence background due to non-specific fluorescently labelled proteins. This problem can be circumvented with the advent of monoclonal antibodies, but the tedium of two labellings involved is a disadvantage of energy transfer FIAs. (For further comments see Section 1.2.2.3 and Chapter 7.)

4. **Fluorescence Protection Immunoassay**

Immunoassays upon the principle of fluorescence protection (Zuk et al., 1979) are of some interest because they are homogeneous techniques. The fluorescence protection assay is based on the formation of an immune complex which incorporates a fluorescent label. The binding of the fluorescent label to a macromolecular solute capable of modulating the fluorescence efficiency is then sterically inhibited. In its simplest form the fluorescence protection assay (see Fig. 1.3) consists of an antigen with a fluorescent tag, and antibody to the particular antigen and an antibody to the fluorescer. In the protected assay,
Figure 1.2  Principles of Energy Transfer Immunoassay

Sample X reverses these effects

Figure 1.3  Principles of Fluorescence Protection Immunoassay (Zuk et al. 1979)
the labelled antigen reacts with antigen specific antibody, and the subsequent binding to the fluorescent label by anti-fluorescent label antibody is sterically prevented by the immune complex. The quenching reaction occurs when there is sufficient unlabelled antigen to sequester the antibody, leaving the fluorescent labelled antigen free to be bound by anti-fluorescent label antibody, resulting in a quenched fluorescence signal. This homogeneous assay technique has been variously termed in literature, viz, double-receptor fluorescence immunoassay (Ullman, 1973), indirect quenching FIA (Nargessi et al., 1978(a)) and recently with some modifications in the technique, double antibody FIA (Zuk et al., 1981), and fluorescence immunoassay using mixed binding reagent (Smith, 1981).

Although fluorescence protection assays have been reported to overcome some of the problems encountered in energy transfer assays (Ullman et al., 1980), this technique does not seem to offer any particular advantages, and moreover utilizes two types of antibodies.

5. **Fluorescence Internal Reflectance**

This immunoassay technique uses a solid phase but does not require a separation of bound and free fluorophore has been described by Kronick and Little (1975). Here total internal reflection of light excites a fluorophore bound to the surface of an immunologically reactive quartz plate. The fluorescence at the surface of such a plate which is in contact with a solution containing the analyte and fluorophore-analyte conjugate is determined, only the fluorophores bound at the surface are excited, while those remaining in solution (free phase) are not.
Morphine has been assayed by this method, this technique has certain disadvantages (e.g., Scatter of light) and has not found many applications.

6. **Fluorescence Lifetime (Time-Resolved FIA)**

The enhanced selectivity of luminescence spectroscopy derives from the increased number of photophysical parameters available for study. Of these parameters the lifetime of fluorescence (or delayed fluorescence or phosphorescence) has been little used. Fluorescence lifetimes can only be measured with specially designed equipment incorporating pulsed light sources. Pulsed xenon lamps are now available and have simplified such measurements. Fig. 1.4 shows the principle of time-resolved fluorimetry and how it can be exploited to remove signals (background fluorescence, see Table 1.4) with short decay times.

The sensitivity of fluorescence immunoassays can be improved by use of time resolved measurements, since the high background fluorescence due to serum proteins (see Section 1.2.2.3) necessarily present in immunoassay systems which has a decay time of about 10 ns can be removed. Two types of labels can be employed for time resolved assays. Long-lived organic fluorophors e.g. those based on pyrene [N-(3-pyrene)-maleimide] (Weltman et al., 1973; Lux and Dominique, 1981) will be the easier to use in forms of labelling technique and may be preferable also on grounds of stability. However, they require more costly nanosecond spectrometers, since their lifetimes are ca. 100 ns. The second possibility is to utilize lanthanide ions as labels in the form of stable chelates that can also be covalently bound to the sample molecule of interest.
Figure 1.4

Diagram of time-resolved fluorescence measurements.

$\tau_{\text{Ex}} = \text{Excitation pulse time (< 1 ns)}$

$\tau_{\text{D}} = \text{Delay time (during this time short decay background is reduced to almost zero)}$

$\Delta\tau = \text{Counting time interval (fluorescence of a label with long lifetime is measured at certain intervals)}$
Europium and terbium chelates (with various α-diketones and salicylates) appear promising since they fluoresce at high wavelengths with very long lived emissions (ca. 0.3 ms) compared with the fluorescence lifetimes of compounds responsible for background fluorescence in serum samples. A number of organometallic complexes which may serve as labels (Cais, 1980) and fluorescent labels comprising rare earth chelates (Frank and Sundberg, 1981) have been recently reported.

Most recently, two time-resolved pulsed light FIAs have been developed where a europium chelate was utilized (Marshall et al., 1981; Hemmila et al., 1982).

7. Substrate-labelled FIA

The principles of this assay are schematically shown in Fig. 1.5. The conjugate contains an enzyme substrate that produces a fluorescent product after reaction. The enzyme reaction is inhibited if the conjugate is bound to the antibody. This inhibition is relieved by the presence of unlabelled analyte in the competitive binding reaction. Hence, the rate of production of fluorescence will be related to the analyte (sample) concentration. Since the assay utilizes a change in the physicochemical properties of the substrate labelled analyte upon binding to antibody, no separation step is required in the procedure. This substrate-labelled fluorescence assay has also been termed as homogeneous reactant-labelled fluorescence immunoassay (Burd, 1977) and release fluorescence immunoassay (Smith et al., 1981(a) and (b)).

Homogeneous substrate-labelled FIAs have been described for proteins (e.g. Ngo et al., 1981) and drugs (Burd et al., 1977). Recently a substrate labelled FIA utilizing dry reagents in a paper matrix has been developed (Greenquist et al., 1981).
Homogeneous substrate-labelled fluorescence immunoassays are also commercially available (Miles Laboratories, Inc., Richmond, California, U.S.A.).

Figure 1.5

\[
\begin{align*}
L^* \xrightarrow{\text{enzyme}} & \text{fluorescent products} \\
L^* + Ab & \leftrightarrow L^*Ab \xrightarrow{\text{enzyme}} \text{no reaction} \\
& \quad \text{(no fluorescence)} \\
L^*Ab + L & \rightarrow AbL + L^* \rightarrow \text{fluorescence} \\
& \text{displacement} \\
& \text{of } L^* \text{ by } L \\
& \text{in competitive} \\
& \text{binding} \\
& \text{reactions} \\
& (L = \text{analyte})
\end{align*}
\]

The most attractive substrate so far described appears to be umbelliferyl-β-galactoside which is non-fluorescent under assay conditions. The relatively short wavelengths required to excite this dye also excite common endogeneous substances in biological samples which fluoresce sufficiently strongly to reduce assay sensitivity. Nevertheless the method itself appears viable provided satisfactory substrates can be prepared.

Other Types of Fluorescence Immunoassay

A variety of other immunoassay techniques employing fluorescence detection has been described (Ullman, 1979). Most of these are not strictly fluorescence immunoassays because the labels are not simple fluorescent labels. These include liposome lysis assays (Kinsky, 1974) and vesicle fusion immunoassay (Van Derwerf and Ullman, 1980). Another potential concept that is likely to be
useful in FIA is the phase sensitive detection method (Lakowicz and Cherek, 1981).

Some Developments in Fluorescence Immunoassay

(1) Combined h.p.l.c. Immunoassay

This type of immunoassay has been described by Lidofsky (1980). The immunoassay is carried out first, and high performance liquid chromatography, h.p.l.c., used to separate free and antibody-bound analyte and/or remove fluorescent contaminants. A number of successful laser-induced fluorescence immunoassays of insulin have been developed using this route.

(2) Fluorescence Autocorrelation Immunoassay

This recently described method (Nicoli et al., 1980) depends on an immunological reaction taking place at the surface of a large (ca. 5 micron diameter) carrier particle. Studies of the number fluctuations and diffusion of these particles in a small (10^{-6} ml.) volume of solution discriminate between the desired signal and the fluorescence from soluble contaminants. Preliminary experiments used fluorescein labelled antibodies excited by an argon laser; the vibrating cell needed for diffusion studies was controlled by a microcomputer.

(3) Fluorescence Immunoassay with Flow Cytometric Detector

This is a 'sandwich' homogeneous FIA very recently described by Lisi and co-workers (1982). Antibody coated microspheres, sample and fluorescent antibody are reacted together in a conventional 'sandwich' immunoassay except that separation and washing steps are omitted. After the reaction is completed, the suspension
is introduced directly into a flow cytometer equipped with a laser light source and both fluorescent and scattered light detector capabilities. By gating fluorescence light on scattered light pulses, particle associated fluorescence may be selectively measured. This system was evaluated in an immunoassay of human IgG, is applicable to both small and large molecules.

1.2.2.3 Comments on Fluorescence Immunoassays

Homogeneous fluorescence immunoassays have the distinct advantage of eliminating the separation step and are easily adaptable to automation. Automation of a homogeneous fluorescence immunoassay has been undertaken (Lim et al., 1980(b)) using flow injection analysis with merging zone principles. Despite the apparent advantages, fluorescence immunoassays have not been utilized nearly so often as for example enzyme immunoassays. In addition, fluorescence immunoassays that have become commercially available have been based on heterogeneous procedures.

A potential problem with fluorescence immunoassay is that biological samples (blood serum, urine etc.) exhibit considerable background fluorescence, this explains why such assays have not yet attained the sensitivity comparable to RIA. Most of the homogeneous assays described above (especially for haptens) are currently limited to the $\mu$mol $1^{-1}$ or nmol $1^{-1}$ range. Further comments on background fluorescence arising from serum samples and affecting the otherwise simple homogeneous fluorescence enhancement and energy transfer assays are made in Chapters 5 and 7 respectively.

Heterogeneous procedures relieve much of the problem associated with sample background fluorescence. The separation step markedly improves the sensitivity by removing the interfering contaminants;
larger volumes of serum samples can thus be employed for the assay of compounds present at low levels. The possible disadvantages with this approach include the complexity of some of the separation techniques which require more time and multiple washes causing imprecision. Fluorescence time-resolution methods appear attractive in that they can overcome the background fluorescence problems on a life time basis and improve the sensitivity. But the complexity and cost of instrumentation involved (especially for nanosecond assays) is a likely disadvantage for routine work in many laboratories.

The newer approaches described above, viz, combined h.p.l.c. and autocorrelation immunoassays have been developed presumably to reduce/remove the background fluorescence from biological samples, these methods though viable are apparently too complicated from a user point of view.
1.2.3 Chemiluminescence Immunoassay (CLIA)

Several problems underlie fluorimetric analytical techniques. Limits of detection are usually set by Rayleigh and Raman scattering of the solvent and the native fluorescence of biological samples (blood serum etc.). A considerable reduction in background, in particular stray and scattered light interference should be secured via the use of chemiluminescence, since by definition, chemiluminescence (CL) does not involve a primary light source. CL can be measured in essentially any instrument sensitive to emission of visible light. CL measurements can usually be made in a few seconds since the light is often emitted as a short flash immediately following the mixing of reactants.

1.2.3.1 Chemiluminescent Labels

The use of CL labels in immunoassays has increased in the last few years. The principle of this application has been to label antigen or antibody with substances active in CL reactions, using CL as the final detection step. The labelling procedures are analogous to those for more common types of labelling such as fluorescence or radioisotope. Because of its low detection limits, CL labelling is a possible alternative to radioisotopes in a large number of assays. The potential advantages of CL-linked assays include sensitivity and simplicity of apparatus. Luminescence photometers capable of detecting picomole quantities of solutes are commercially available and simple fluorimeters can also be modified to measure CL. An additional advantage of using CL measurements in immunoassays is the non-toxicity of CL reagents, the use of CL labels thus avoids biohazards associated with isotope handling.
The CL labels have usually been various reactants from the CL systems. The substance to be used as the label in the CL reaction should fulfil certain requirements, viz, it should (i) be capable of taking part in the CL reaction, (ii) be possible to attach it to substance (analyte) of interest (not normally chemiluminescent) to form a stable conjugate, (iii) not significantly alter the properties of the substance to which it is attached, (iv) be able to participate in the CL reaction after being coupled to form the conjugate.

CL labels in general are characterised by a marked stability in solution and are less expensive as compared with radiolabels. Many of the published assays use derivatives of luminol or isoluminol and pyrogallol as labels, though acridine derivatives and bioluminescent procedures have also been used. Another efficient way of achieving CL is via the oxidation of diaryl oxalate esters by hydrogen peroxide in the presence of fluorescent species under study (peroxyoxalate chemiluminescence, Rauhut, 1969). Although discovered some ten years ago this approach has only very recently been proposed as CL immunoassay procedure (Boguslaski and Carrico, 1980).

The type of CL labels used in immunoassay as mentioned above are briefly discussed (see Fig. 1.6 for structures of some CL compounds).

(1) Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)

One of the best known chemiluminescent compounds is luminol which produces light when treated with a strong oxidizing agent in the presence of a catalyst. The catalysts are any of several oxidants, metal complexes or enzymes containing haemin, such as
Structures of some reagents which may be used for labelling in CL immunoassays (e.g. based on aryl hydrazide chemiluminescent reactions).

Approaches to coupling chemiluminescent aryl hydrazides to potential analytes (e.g. antigens or antibodies).
For coupling diazonium salt of luminol to antibodies see Simpson et al., 1979.
For coupling to isoluminol derivative see Schroeder et al., 1978(a).
peroxidase or microperoxidase. The quantum yield of luminol is low and the maximum light emission occurs at ca. 425 nm. The reaction of luminol with an oxidising agent is summarised in Fig. 1.7.

![Diagram of luminol reaction](image)

**Figure 1.7**

Luminol appears to be the common label for immunoassay work, it is readily available. Various chemical methods have been used to covalently attach proteins to luminol and its derivatives (see Fig. 1.6) by activation of -NH$_2$, -OH or -COOH groups, the labelling procedures are well established (Whitehead et al., 1979). Labelling with luminol presents special problems, since derivatisation often or totally abolishes luminescent activity (Schroeder and Yeager, 1978(b)). Coupling to the -NH$_2$ group results in a strong decrease of luminescent activity possibly due to steric hindrance. However such conjugates have been successfully and extensively used in immunoassays (see Tables 1.11 and 1.12). The reaction to yield CL is not unique for luminol, a large number of cyclic hydrazides (Schroeder and Buckler, 1980(a),(b)) react similarly to luminol to yield CL, but with less efficiency. The use of isoluminol (Fig. 1.6) which itself emits light may be an alternative label. With isoluminol the conjugates may even have increased luminescent activity compared to that of free substance,
possibly owing to the more favourable position of the -NH₂ group. Applications using isoluminol derivatives have also been performed (see Tables 1.11 and 1.12). Chemiluminescent-labelled antibodies have been prepared using luminol and found to be stable for extended periods of time (Simpson et al., 1979), other workers (Pratt et al., 1978) have reported poor stability of luminol conjugates.

(2) **Acridinium esters**

Acridinium esters are considerably luminescent than luminol (McCapra et al., 1977). A bromide derivative of an acridinium ester has been recently synthesised which was subsequently used to develop a CL assay for human α-fetoprotein with a limit of detection comparable to RIA (Simpson et al., 1981).

The possibility of using 1,2-dioxetanes as CL probes or labels in immunoassay has been suggested (Wynberg et al., 1981) but any application of such labels has not yet been described.

(3) **Bioluminescent systems**

Bioluminescence is considered to be a special case of chemiluminescence which involves enzyme-mediated light production. The two most common bioluminescent reactions involving (i) fireflies (catalysed by the enzyme firefly luciferase) and (ii) certain bacteria (catalysed by bacterial luciferase) are shown below:

**Firefly reaction**

\[
\text{ATP} + \text{luciferin} + \text{O}_2 + \text{luciferase} \rightarrow \text{AMP} + \text{PP} + \text{oxyluciferin} + \text{CO}_2 + \text{H}_2\text{O} + \text{light}
\]

(Quantum yield ca. 0.9, maximum intensity at 562 nm).
Bacterial reaction

$$\text{FMNH}_2 + \text{R-CHO} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light}$$

(R-CHO is a long chain aldehyde)

(Quantum yield ca. 0.1, maximum intensity at 490 nm).

The firefly luciferase system has found to be very useful for adenosine triphosphate (ATP) determinations and the bacterial luciferase has been used for analysis of reduced flavin mononucleotide (FMNH) or reduced nicotinamide adenine dinucleotides (NADH, NADPH): see Table 1.8. (The applications of these systems have been reviewed by Seitz, 1981.)

<table>
<thead>
<tr>
<th>Table 1.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioluminescent Systems</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound detected</th>
<th>Detection limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firefly luciferase</td>
<td>ATP</td>
</tr>
<tr>
<td>Bacterial luciferase</td>
<td>NADH</td>
</tr>
</tbody>
</table>

Due to the extreme sensitivity of bioluminescence reactions, the bioluminescent systems have a potential for replacing conventional labels (e.g. radionuclides) in immunoassays. In principle, any of the reagents used in the firefly reaction (or the bacterial reaction) can be used for 'labelling' antigen or antibody, the luminescent reaction then initiated by the addition of the other reagents. Recently, the considerable sensitivity of a bioluminescent immunoassay has been demonstrated by the results obtained in an assay for
the drug methotrexate where firefly luciferase served as a 'label' (Wannlund et al., 1980). The reagents for the bioluminescent systems are commercially available though as yet there are no commercial sources of bioluminescently labelled antigens or antibodies.

(4) **Diaryl oxalate esters** (peroxyoxalate CL)

(a) **General**

The oxidation of diaryl oxalate esters by hydrogen peroxide in the presence of a fluorescent compound produces very efficient chemiluminescence. Systems of the oxalate ester type in which the fluorescent material is an additive are termed sensitised in contrast with direct chemiluminescent systems in which the fluorescer is a reaction product. The reaction of an oxalate ester with hydrogen peroxide generates a high energy dioxetane intermediate which transfers its energy to any fluorescent species in solution to produce CL (Fig. 1.8). Some aspects of the mechanism are still obscure, but the generated fluorescence is in the form of a brief pulse of light; typical quantum yields are as high as ca. 0.25 (cf. luminol, $\phi \approx 0.01$, Isaccson and Wettermark, 1974). The usual esters are 2,4,6-bis(trichlorophenyl)oxalate (TCPO) and bis(2,4-dinitrophenyl)oxalate (DNPO), (Fig. 1.8) both being characterised by Rauhut (1969) and Mohan and Turro (1974) and also Kobayashi and Imai (1980). Very recently, bis-(1,4,5-trichloro-6-carbopentoxyphenyl)oxalate (CPPO) has been studied (Cole and Seitz, 1981). As far as is known, none of the oxalate esters are commercially available but TCPO and DNPO can be readily synthesised (Mohan and Turro, 1974). The qualitative CL from other less common oxalate esters has been disclosed (Bollyky and Rauhut, 1971).
Peroxyoxalate chemiluminescence refers to a large class of reactions summarised below (e.g. Curtiz and Seitz, 1977).

\[
\text{oxalate or oxalate derivative} + \text{H}_2\text{O}_2 \rightarrow \begin{array}{c}
\text{1,2-dioxetane} \\
\text{(intermediate)}
\end{array}
\]

\[
\begin{array}{c}
\text{fluorophore} \\
\text{fluorophore*}
\end{array} \rightarrow \text{light} + \text{fluorophore}
\]

(chemiluminescence)

**Structures of common oxalate esters**

2,4,6-bis(trichlorophenyl)oxalate [TCPO]

bis(2,4-dinitrophenyl)oxalate [DNPO]
Use of oxalate systems in conjunction with conventional labels is surprisingly limited; however it has been reported that the most efficiently excited fluorophore are those with low energy (high wavelength) excitation bands (Sherman et al., 1978).

Peroxyoxalate CL has been used to determine fluorophores. The initial study involved detection of dansylated amino acids separated on a thin-layer chromatographic plate (Curtis and Seitz, 1977) and more recently peroxyoxalate CL was used to detect dansylated amino acids (Kobayashi and Imai, 1980) and fluorescamine labelled catecholamines (Imai et al., 1980; Kobayashi et al., 1981) separated by liquid chromatography.

(b) Advantages of peroxyoxalate CL in immunoassay

Advantages of this approach to immunoassay development would include:-

(i) Extreme sensitivity, deriving partly from the high quantum yield and partly from the simplicity of instrumentation.

(ii) The light emission reactions are fast thus would reduce assay times substantially.

(iii) The possibility of devising new homogeneous immunoassays by studying the kinetics of light emission process e.g. antibody-bound labels may emit more slowly than unbound ones.

(iv) The kinetics of light emission can also be regulated by the addition of acids and bases (Biffin and Paul, 1973).

(v) More importantly, the emission wavelengths can be varied by changing the fluorescent labels, and

(vi). Peroxyoxalate CL can be used in flow systems (cf. luminol) permitting a reduction in time/or reagent required (see Chapter 6).

Possible problems with this procedure will include (i) limited
shelf-life and solubility of some of the reagents, (ii) some existing types of assay might not be feasible (e.g. conventional energy transfer immunoassay will not be viable because both donor and acceptor will be directly excited).

A number of other chemiluminescent reactions appear to be related to peroxyoxalate chemiluminescence although their mechanistic details may vary. For example, various chlorinated esters and ethers react with H₂O₂ and a fluororescer to emit light (Maulding and Roberts, 1972).

A major difference between the more common luminol system and the oxalate ester system is the requirement that oxalates have an additional fluororescer to absorb the chemical energy generated in the reaction and then convert that energy to visible light. The oxalate system is generally employed in an organic solvent and this requirement has made its use in immunoassay methods less desirable than other CL materials, which are soluble in aqueous media.

Table 1.9 shows a comparison of the two methods:

(I) A CL compound which generates CL energy and itself emits light, such as luminol, or

(II) A fluorophore which absorbs chemical energy and emits light, which can be used to develop CL-linked immunoassay.
### Table 1.9
Comparison of methods for using components of chemiluminescence reaction for immunoassay

<table>
<thead>
<tr>
<th></th>
<th>Method I</th>
<th>Method II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Object of detection</strong></td>
<td>Luminol (conjugation of luminol)</td>
<td>Peroxyoxalate CL (conjugation of fluorophore)</td>
</tr>
<tr>
<td><strong>Label example</strong></td>
<td>antigen</td>
<td>antigen</td>
</tr>
<tr>
<td><strong>Label example</strong></td>
<td>Luminol and derivatives</td>
<td>fluorescent conjugate(s)</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>Pratt, 1978; Hersch, 1979; Simpson et al., 1979; Schroeder et al., 1979; Olsson, 1979; Also see Table 1.11</td>
<td>Boguslaski and Carrico, 1980; Grayeski and Seitz, 1982?; Mandie, 1981</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Reagents readily available</td>
<td>(1) Immunological reaction separate and distinct from CL production.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) Highest level of light intensity obtainable.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) Sample may be retested with additional oxalate ester.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4) Label is usually stable for coupling and storage.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) Most desirable functional group may be used for attachment to clinical analyte, minimising destruction of label, specificity and activity.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6) Fluorescent label inexpensive.</td>
</tr>
</tbody>
</table>
Table 1.9 continued

<table>
<thead>
<tr>
<th>Disadvantages</th>
<th>Method I</th>
<th>Method II</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) CL label consumed in reaction, thus destroyed.</td>
<td>(1) Oxalate esters not generally available.</td>
<td></td>
</tr>
<tr>
<td>(2) Poor quantum yield of light.</td>
<td>(2) Extreme sensitivity of system may cause light emission from traces of fluorescent impurities.</td>
<td></td>
</tr>
<tr>
<td>(3) Reaction susceptible to other catalysts and quenchers.</td>
<td>(3) Possibility of direct quenching of the excited molecules.</td>
<td></td>
</tr>
</tbody>
</table>
1.2.3.2 Types of Chemiluminescence Immunoassays (CLIA)

The general design of chemiluminescence immunoassay systems is the same as used in other immunoassays. Several assay types have been described (Whitehead et al., 1979 and Olsson and Thore, 1981) viz. conventional heterogeneous assays (cf. RIA), in most cases solid-phase or double-antibody precipitation techniques have been used; homogeneous assays based on luminescence enhancement or quenching effects (Kohen et al., 1980(a)) or on delayed light emission effects (Kohen et al., 1980(b)) and enzyme immunoassays with CL detection methods (Arakawa et al., 1977). Table 1.10 shows the classification of chemiluminescence immunoassays.

Table 1.10

Classification of Chemiluminescence Immunoassays

Competitive binding assays based on luminescent measurements are variously known as:-

- (1) Luminescent Immunoassay (LIA)  
  Example of Reference: See Table 1.11 & 1.12
- (2) Luminescent Enzyme Immunoassay (LEIA)  
  Tsuji et al., 1978
- (3) Luminescent Enzyme-Multiplied Immunoassay (LEMIT)  
  Stanley, 1978
- (4) Luminescent Cofactor Immunoassay (LCIA)  
  Schroeder et al., 1976

The principles of heterogeneous and homogeneous CLIA are described below.

(a) Heterogeneous

Heterogeneous immunoassays monitored by CL are based on the same principles as RIAs. The analyte of interest is covalently
bound to a substrate which participates in a light producing reaction. This conjugate replaces the radiolabelled compound in radioassay formats. Upon completion of the procedure, the amount of label (substrate) in the isolated bound or free fraction is determined by the amount of light produced when the fraction is treated with the remaining components of the light generating system. The intensity of light produced is proportional to the concentration of label which in turn, is related to the quantity of analyte present in the sample.

(b) Homogeneous

Homogeneous assays monitored by CL are similar to the heterogeneous assays, except that the step which separates the bound and free fractions is omitted. Since the light producing abilities of the bound and free forms of the label can be quite distinct, it is possible to measure one in the presence of the other. Thus the two forms of the label do not necessarily have to be separated in order to determine the distribution of the label present in the assay mixture. As in heterogeneous assays, the amount of label present in either fraction is related to the quantity of analyte present in the sample.

Heterogeneous CLIA's have been reported for a number of biologically important analytes, examples of these assays are listed in Table 1.11. In many cases, the assays have been reported to be of comparable sensitivity to the corresponding radioimmunoassay and/or enzyme immunoassay. Homogeneous CLIA's have apparently been found to be suitable only for low molecular weight solutes, e.g. steroids and drugs, Table 1.12 shows the assays developed using largely luminol or isoluminol derivatives. Recently a CLIA based on peroxyoxalate CL has been reported (Boguslaski and Carrico, 1980).
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroxine</td>
<td>isoluminol</td>
<td>Schroeder et al., 1978(a), 1979</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&quot;</td>
<td>Pratt et al., 1978</td>
</tr>
<tr>
<td>Human IgG</td>
<td>&quot;</td>
<td>Schroeder et al., 1980, 1981;</td>
</tr>
<tr>
<td></td>
<td>luminol</td>
<td>Hersh et al., 1979</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>&quot;</td>
<td>Simpson et al., 1979*</td>
</tr>
<tr>
<td>Insulin</td>
<td>&quot;</td>
<td>Maier, 1978</td>
</tr>
<tr>
<td>Mumps virus antibody</td>
<td>&quot;</td>
<td>Konishi et al., 1980*</td>
</tr>
<tr>
<td>Pregnane diol-3α- glucuronide</td>
<td>isoluminol</td>
<td>Barnard et al., 1981</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&quot;</td>
<td>Kohen et al., 1981</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&quot;</td>
<td>Pazzagali et al., 1981</td>
</tr>
<tr>
<td>Staphylococcal enterotoxin B</td>
<td>peroxidase</td>
<td>Velan and Halmann, 1978</td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
<td>luminol &amp;</td>
<td>Mandle, 1981*</td>
</tr>
<tr>
<td></td>
<td>TCPO/H₂O₂ solid phase system</td>
<td></td>
</tr>
</tbody>
</table>

* Sensitivity comparable with RIA
Table 1.12

Homogeneous Chemiluminescence Immunoassays

(Some examples)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>isoluminol</td>
<td>Kohen et al., 1979</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&quot;</td>
<td>Kohen et al., 1980(b)</td>
</tr>
<tr>
<td>Estriol-16α-glucuronide</td>
<td>&quot;</td>
<td>Kohen et al., 1980(a)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>luminol</td>
<td>Maier, 1978</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>DNPO-H₂O₂ System</td>
<td>Boguslaski and Carrico, 1980</td>
</tr>
</tbody>
</table>

Recent Developments in Chemiluminescence Immunoassay

Some novel approaches to CLIA development have been reported:

(1) Cambell et al., 1982, have developed an energy transfer assay utilising luminol and fluorescein as labels: the energy from the luminol reaction excites the fluorescent label emitting light of different wavelengths. In the described assay for cyclic AMP, the antigen was labelled with fluorescein and the antibody with luminol and measurements made at 540 nm, the emission at this wavelength deriving from the fluorescein labelled component which is bound to antibody.

(2) Mandle (1981) has developed a heterogeneous assay utilising peroxoxygenate chemiluminescence (described in Section 1.2.3.1) with a fluorescer/glass sample. In the described assay for Hepatitis B Surface (HBs) Antigen, perylene was employed as the fluoroscer and the reaction (involving 'sandwich technique') used was:
Glass. Ab . Ag . Ab . Perylene + TCPO + H$_2$O$_2$ + light

Ab = anti HB$_2$ (goat)

(3) Oberhardt and Wotherspoon (1981) have described a solid-phase electrochemiluminescence immunoassay system which uses a pair of electrodes to oxidise a chemiluminescent label; the antibodies being immobilised on the anode and the cathode constructed of a transparent material such that the light produced can be detected by a photomultiplier.

1.2.3.3 Comments on Chemiluminescence-Linked Immunoassay

The application of chemiluminescence techniques to immunoassay is increasing; a large number of heterogeneous chemiluminescence immunoassays using luminol or its derivatives have been developed with sensitivities equal to or even better than that obtained in radioimmunoassays. Since homogeneous chemiluminescence immunoassays have only rarely been described, the well known problem of background interference (from biological samples) inherent in fluorescence immunoassays seems likely. Serum interference has only briefly been mentioned, none of the publications on CLIA provide much information on the background CL from serum, though a review (Gorus and Schram, 1979) implies that CL signal will be a less formidable problem than the fluorescence background. Kohen and co-workers have reported luminescence from human urine, which had to be removed using an XAD-2 column (1980(a)) and they also developed an assay for cortisol (1980(b)) where a plasma extract was used as a sample. The more recent application of the oxalate ester system (Boguslaski and Carrico, 1980) indicated that considerable background signals interfered with a sisomicin assay.

- 49 -
As in fluorescence immunoassays (see Section 1.2.2.3) direct background interferences will not be the only sources of concern in chemiluminescence immunoassays. Of equal importance will be the possibility that CL signals might be quenched (or enhanced) by species present in samples or reagents. An important survey of this type of interference as it affects the ATP bioluminescence assay indicates the magnitude of the problems involved (Thore, 1979). Some of the listed interferences might arise from enzyme inhibition phenomena that would be absent in CL assay, but most seem likely to involve direct quenching of the excited molecules. Light absorption is another potential problem. It has been suggested that the extreme sensitivity of CL techniques will allow substantial dilutions of samples and hence minimal interferences, such dilutions may limit the number of analytes that can be profitably studied.

Another potential problem arising in CL assays is poor precision, this is due to the fact that chemiluminescence (light) is emitted as a brief flash immediately following the mixing of reactants. Flow injection analysis (Ruzicka, 1981), which is simple yet very precise, may play a major role. (Also see the review by Rocks and Riley, 1982.)
1.3 Aim of Present Work

The aim of the thesis was to make a thorough investigation of serum fluorescence and its interference in fluorescence immunoassays, to evaluate simple procedures of background reduction/removal and compare these with other instrumental approaches.

A variety of fluorescent labels was studied to develop homogeneous fluorescence immunoassays for both small and large molecules. The possibility of using chemically excited fluorescence for developing immunoassays was investigated, the peroxyoxalate chemiluminescence system utilizing (TCPO), 2,4,6-bis(trichlorophenyl) oxalate was evaluated in conjunction with flow injection analysis.
2.1 Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Source of Supply</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Albumin, lyophilised and 100%</td>
<td>Hoechst (U.K.) Ltd., Hounslow, Middlesex</td>
</tr>
<tr>
<td>electrophoretic purity</td>
<td></td>
</tr>
<tr>
<td>Transferrin, lyophilised and 99%</td>
<td>Sigma (London) Chemical Co. Ltd.,</td>
</tr>
<tr>
<td>pure (5% Fe)</td>
<td>Poole, Dorset</td>
</tr>
<tr>
<td>Fatty acid free albumin</td>
<td>Miles Laboratories Ltd.,</td>
</tr>
<tr>
<td>Fraction V, lyophilised</td>
<td>Stoke Poges, Slough</td>
</tr>
<tr>
<td>Immunoglobulin, G, lyophilised</td>
<td></td>
</tr>
<tr>
<td>Bovine albumin, Fraction V,</td>
<td>Sigma (London) Chemical Co. Ltd.</td>
</tr>
<tr>
<td>powder, 96-99% purity</td>
<td></td>
</tr>
<tr>
<td>Porcine thyroglobulin</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Sigma (London) Chemical Co. Ltd.</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Glycine</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Material</td>
<td>Source of Supply</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Iodinated amino acids and related samples</strong></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Sigma (London) Chemical Co. Ltd.</td>
</tr>
<tr>
<td>Thyronine</td>
<td>&quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>3-Iodotyrosine</td>
<td>&quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>3,5-Diiodotyrosine</td>
<td>&quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>3,5-Diiodothyronine</td>
<td>&quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>3,5,3'-Triiodo-L-thyronine (T3) Grade A</td>
<td>Calbiochem. Ltd., Bishops Stortford</td>
</tr>
<tr>
<td>3,3',5'-Triiodothyronine (reverse T3) Grade A</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>3,3',5,5'-Tetraiodo-L-thyronine (L-Thyroxine, T4) free acid</td>
<td>Sigma (London Chemical Co. Ltd.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum samples</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>From lung cancer patient</td>
<td>Nottingham University Hospital, Nottingham. (Sodium azide was added and the serum samples were stored frozen.)</td>
</tr>
<tr>
<td>From bronchus cancer patient</td>
<td></td>
</tr>
<tr>
<td>Whole human serum (sterilised)</td>
<td>Miles Laboratories Ltd., Stoke Pages, Slough. (Pooled serum from normal fasted donors, stored frozen.)</td>
</tr>
</tbody>
</table>
Material

Serum samples continued

T3/T4 depleted serum
Advanced Laboratory Techniques,
Tonbridge Wells, Kent

Plasma
Donated by a healthy laboratory
worker (male)

Standard human serum (Type ORDT)
Hoechst (U.K.) Ltd.

Monospecific rabbit antisera
to the following human proteins
(partially purified)

Albumin
DAKO - immunoglobulins Ltd.,
Mercia Brocades Ltd.,
Weybridge, Surrey

Immunoglobulin G, specific for
γ-Chains

Transferrin

Rabbit whole antisera to
Triiodothyronine
RIA (U.K.) Ltd., Sunderland

Thyroxine
(i) " " " "
(ii) Gift from University of Surrey

Monoclonal antibodies to
Triiodothyronine
Miles-Yeda Ltd.,
Rehovot, Israel
<table>
<thead>
<tr>
<th>Material</th>
<th>Source of Supply</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescent dyes</strong></td>
<td></td>
</tr>
<tr>
<td>1-Dimethylaminonaphthalene-5-</td>
<td>BDH Chemicals Ltd., Poole, Dorset</td>
</tr>
<tr>
<td>Sulphonyl chloride</td>
<td></td>
</tr>
<tr>
<td>Eosin isothiocyanate</td>
<td>Polysciences Inc., Warrington, PA, U.S.A. (obtained through International Enzymes Ltd., Windsor, Berks.)</td>
</tr>
<tr>
<td>Fluorescamine (Fluram)</td>
<td>Roche Diagnostics Ltd., Welwyn Garden City</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate (10% Isomer I on celite)</td>
<td>Calbiochem. Ltd.</td>
</tr>
<tr>
<td>Lissamine Rhodamine-B 200 chloride (on celite)</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Rhodamine-B isothiocyanate</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Ortho-phthaldehyde</td>
<td>Pierce Chemical Co., Rockford, IL, U.S.A.</td>
</tr>
<tr>
<td>Lucifer yellow CH (lithium salt)</td>
<td>Sigma (London) Chemical Co. Ltd.</td>
</tr>
<tr>
<td>Quinacrine mustard diHCl</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>8-Anilino-1-naphthalene-sulphonic acid</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>7-Chloro-4-nitrobenzo-2-oxa-1,3, diazole</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Material</td>
<td>Source of Supply</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Chromatographic media</td>
<td></td>
</tr>
<tr>
<td>Sephacryl S-300 (superfine)</td>
<td>Pharmacia Ltd., Hounslow, Middlesex</td>
</tr>
<tr>
<td>Sephadex G-25 (medium grade)</td>
<td></td>
</tr>
<tr>
<td>PD-10 disposable columns containing</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td></td>
</tr>
<tr>
<td>&quot;Blue Sepharose&quot; (Sepharose-Cibacron Blue 3GA conjugate)</td>
<td></td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>Bio-Rad Laboratories, Watford, Hertfordshire</td>
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<tr>
<td>Bio-Gel HTP</td>
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<td>Bio-Gel P10</td>
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</tr>
<tr>
<td>Florisil</td>
<td>Sigma (London) Chemical Co. Ltd.,</td>
</tr>
<tr>
<td>Dowex 50X₄</td>
<td></td>
</tr>
</tbody>
</table>

All other reagents were of Analar or equivalent grade.

Buffers and solutions of other reagents/samples were normally made up in triply distilled water; in one specified case ultrafiltered water was used.
2.2 Instrumentation

2.2.1 Absorbance measurements

The measurements of absorbances of solutions at fixed wavelengths were routinely made on Pye-Unicam SP-600 and Shimadzu UV-240 (Shimadzu Corporation, Kyoto, Japan) spectrophotometers. A Pye-Unicam SP8-100 (and Shimadzu UV-240) was used to record absorption spectra.

2.2.2 Fluorescence measurements

An MPF-44B spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, Bucks.) fitted with a DSCU-2 correction accessory and a R 928 photomultiplier (range 200-950 nm) was used to record all corrected excitation and emission spectra. The DCSU-2 unit was regularly 'calibrated' using a Rhodamine B counter (as directed by the manufacturer). In addition, a fluoricord spectrofluorimeter (Baird Atomic Ltd., Braintree, Essex) also fitted with an R 928 side-on photomultiplier (Hakuto International, Leigh-On-Sea, Essex) for improved high wavelength sensitivity, was used to record other uncorrected spectra. Also, an LS-5 Luminescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks.) was used to record some fluorescence spectra. Quantitative fluorescence measurements were made using a Perkin-Elmer Model 2000 filter fluorimeter and the MPF-44B spectrofluorimeter. 10 mm path length silica cuvettes (thermostatted in specific cases) were used in all fluorimetric measurements.

2.2.3 Chemiluminescence measurements

A Fluorimet (Baird Atomic Ltd., Braintree, Essex) filter fluorimeter with the light source removed and an LKB (Croydon, Surrey) Model 1250 luminometer were used for chemiluminescence measurements.
MPF-44B Spectrofluorimeter
The fluorimeter was modified to accommodate a low volume silica flow-cell close to the photomultiplier and also to allow injection via a septum directly into the flow-cell. The luminometer was also adapted for continuous-flow analysis: a teflon rotary Rheodyne valve (Type 50, Anachem Ltd., Luton, Bedfordshire) aided sample injection into a coiled Teflon tubing (44 cm in length, 0.76 mm internal diameter) flow-cell.

Further details where necessary will be given in the appropriate chapters.
2.3 Experimental

FLUORESCENCE STUDIES

2.3.1 Human serum and plasma examination

2.3.1.1 Fractionation procedure

A K26/40 column (Pharmacia, GB) was packed with Sephacryl S-300 superfine, bed volume 125 ml. (using the manufacturer's procedure) and equilibrated with 0.1M Tris-HCl buffer, pH 8, containing 0.5M NaCl. A 1 ml. whole human serum sample diluted (1:1) in the same buffer was applied to the column and eluted at a flow rate of 0.25 ml. min⁻¹. The absorbance of each 2.5 ml. fraction was measured at 280 nm. After identifying four major peaks (manufacturer's reference; see Fig. 3.2) the fractions were pooled and stored at 4°C in the dark.

2.3.1.2 Background reduction/removal procedures

(a) Chemical treatment

Peracid/sulphite

The serum was chemically treated by a modification of the procedure of Kam and Yoshida (1981). The method consisted of treating a whole human serum sample in 0.01M phosphate buffer, pH 7.4 with 5mM sodium persulphate. After an incubation period of 10 minutes in the dark, excess sodium sulphite (10mM) was added. The mixture was shaken on a vortex mixer for 1 minute and absorbance and fluorescence measurements made immediately.

(b) Adsorbents

Hydroxyapatite

A 4 ml. column of hydroxyapatite (Brooks, 1981) was equilibrated with 0.01M phosphate buffer, pH 7.0, and a 100 µl whole human serum applied.
The column was eluted with 10 ml. of the same buffer. The bound fluorescent species were subsequently eluted with a 0.2M phosphate buffer, pH 7.4 containing 0.145M NaCl. A variation of the method involved batch addition of hydroxyapatite to the serum sample, followed by incubation and centrifugation. 0.01M phosphate buffer, pH 7.0 was employed in this experiment.

Florisil

In some preliminary work, a short column of florisil (1 g.) equilibrated with 0.01M phosphate buffer, pH 7.0, was employed. The column was also eluted with 2mM and 5mM phosphate buffer, pH 7.0, in separate experiments.

Blue Sepharose (immobilised Cibacron Blue 3GA)

A 2.5 ml. column of the immobilised dye was equilibrated with 50mM Tris-HCl buffer, pH 7.0 and a 100 ul plasma sample applied. The column was eluted with 10 ml. of the same buffer. The eluent was then changed to 0.2M sodium thiocyanate and the fluorescence was quantitatively recovered. The procedure was repeated for human serum albumin and defatted serum albumin samples. (The Blue Sepharose procedure was adapted from the manufacturer's instructions (Pharmacia Ltd., U.K.).)
2.3.2 Labelled protein conjugates

2.3.2.1 Some general practical considerations

Most proteins can be labelled by direct reaction with the fluorescent label under mild conditions. The labelling reaction is largely affected by and optimised with respect to the following experimental parameters:

(a) pH

The influence of pH has been investigated especially well in the case of labelling with fluorescein isothiocyanate (McKinney et al., 1964). The choice of pH is a compromise between the rate of the labelling reaction (favoured by increasing alkalinity) and the avoidance of any possible denaturation of the protein at higher pH levels.

(b) Buffer concentration

Mildly alkaline aqueous media, generally, carbonate or phosphate buffers are employed for all commonly used labels. The buffer concentration is dependent on the functional group of the label involved in the conjugation reaction. For example, 0.05M buffer may be adequate for isothiocyanate, but 0.5M buffer may be required for a sulphonyl chloride group.

(c) Temperature

It has been shown by McKinney et al., (1964) and Klugerman (1965) that labelling at room temperature is several times faster than at 0°C, and has no obvious disadvantages (e.g. protein denaturation). In normal practice, room temperature conditions are employed. If the label or the conjugate shows photolability, protection against light becomes a necessary precaution.
(d) **Time**

It has been reported (Goldman, 1968) that the degree of labelling in the case of fluorescein isothiocyanate is affected by shortening or extending of reaction times.

Use of labels dispersed on inert materials such as celite afford a considerable saving in reaction time (Rinderknecht, 1962). However, many fluorescent labels are not available in this form.

(e) **Concentration of label and protein**

At moderate molar ratios of label to protein the reaction will proceed essentially to completion, and the extent of labelling may be controlled by appropriate adjustment of concentrations in the reaction mixture. However, in certain cases, molar excess of the label is desirable, e.g. fluorescamine (fluorescamine is rapidly hydrolysed in aqueous conditions).

The labelled protein may be rapidly and completely separated from the small amounts of unreacted or degraded label using gel filtration chromatography. Further purification, if necessary, may be achieved by ion-exchange chromatography.

**Characterisation of fluorescent protein conjugates**

Spectrophotometric methods are generally used to characterise labelled conjugates with respect to protein content, label concentration and the fluorochrome to protein (F:P) ratio.

A suitable colorimetric technique for protein determination in all conjugates is the Folin-copper method of Lowry et al. (1951). Protein concentration can also be obtained from known absorption coefficients (see Table 2.1 for proteins used here) provided that due attention is paid to the absorption characteristics of the label.
The label is assayed spectrophotometrically assuming that the molar absorption coefficients of the bound and free forms of the labels are equivalent.

The F:P ratio can be obtained by calculation or found from nomographs available in certain cases. [For fluorescein labelled immunoglobulins refer to the nomograph of Well et al., 1966 or The and Feltkemp 1970. The nomograph of Handschin and Ritschard, 1976 can be used for fluorescamine labelled immunoglobulins.]

Other specific methods will be mentioned where appropriate in Section 2.3.2.2.

Table 2.1

Absorption coefficient data for some commonly used proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\varepsilon_{1 \text{ cm}}$ [280 nm]</th>
<th>Ref.</th>
<th>MW</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human albumin</td>
<td>5.94</td>
<td>Berrens (1965)</td>
<td>66241</td>
<td>Putnan (1975)</td>
</tr>
<tr>
<td>Bovine albumin</td>
<td>6.14</td>
<td>&quot;</td>
<td>69000</td>
<td>Longworth (1971)</td>
</tr>
<tr>
<td>Human IgG</td>
<td>13.4</td>
<td>Grey (1972)</td>
<td>150000</td>
<td>White et al. (1978)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>11.4</td>
<td>Feeny and Komatsu (1966)</td>
<td>76500</td>
<td>Putnan (1975)</td>
</tr>
</tbody>
</table>
2.3.2.2 Fluorescent labelling of proteins

(a) Label: Fluorescein isothiocyanate (10% Isomer I on celite)
Protein: 100% pure human serum albumin
Procedure: The method of Rinderknecht (1962) was used. The mixture was centrifuged to separate the celite, and separation of the labelled conjugate from the unreacted dye was performed on a short column of Sephadex G-25 equilibrated with 0.01M phosphate buffer, pH 7.4. The conjugate was characterised (F:P ratio) by determining the protein concentration by the Lowry (1951) method, and using a value of 7.2 x 10^4 M\(^{-1}\)cm\(^{-1}\) for the molar absorption coefficient of the fluorescein thiocarbamide group at 493 nm (Chen, 1969).

(b) Label: Eosin isothiocyanate (see page 67 for synthesis)
Protein: Mono specific rabbit immunoglobulin to human serum albumin (partially purified)
Procedure: Eosin conjugates were prepared by using the procedure of Cherry et al., (1976) with some modifications. It involved the addition of eosin isothiocyanate in 0.1M NaHCO\(_3\) buffer, pH 8, containing 0.5M NaCl to the protein on a vortex mixer. The reaction was subsequently allowed to proceed at room temperature in the dark for 1-3 hours, depending on the degree of labelling required. The labelled protein was separated from the unreacted dye on a Bio-Gel P10 column equilibrated with 0.01M phosphate buffer, pH 7.4, (precautions being taken against light). The protein content of the conjugate was determined by the method of Lowry (1951) and the bound eosin assayed spectrophotometrically, assuming the molar absorption coefficient to be 8.3 x 10^4 M\(^{-1}\)cm\(^{-1}\) at 522 nm (Cherry et al., 1976).
(c)
Label: **Fluorescamine (Fluram)**
Protein: 100% pure human serum albumin
Procedure: This consisted of rapid addition of 0.5 ml. of a 0.03% solution of the label in acetone (Böhlen et al., 1973) to 2 ml. of the protein solution (20-30 mg.) in 0.05M phosphate buffer, pH 9, that was being mixed on a vortex mixer.

After vigorous shaking for 2 minutes in the dark, the mixture was applied to a Sephadex G-25 column (protected from light), equilibrated with 0.01M phosphate buffer, pH 7.4, containing 0.145M NaCl, to remove any hydrolysed products and acetone.

In determining the F:P ratios, a value of 6300 ± 100 M⁻¹cm⁻¹ was used for the molar absorption coefficient of fluorescamine group at 385 nm (Handschin and Ritschard, 1976), and the protein concentration was obtained by the Lowry method.

(d)
Label: **Quinacrine mustard hydrochloride**
Protein: 100% pure human serum albumin
Procedure: The preparation and characterisation of quinacrine conjugates was based on the procedure of Chen (1976). Varying amounts of the dye in tridistilled water were added to the protein solution (1% w/v) in 0.1M NaHCO₃ buffer. The mixture was agitated on a flask shaker for 1 hour at room temperature. Unreacted dye was removed using a short column of Sephadex G-25 equilibrated with 0.01M phosphate buffer, pH 7.4.
(e)  
Label: **Lissamine Rhodamine-B200 Sulphonyl chloride** (on celite)  
Protein: Human serum transferrin  
Procedure: The same method of Rinderknecht (1962) for the preparation of the fluorescein conjugates in (a) above was employed. The degree of conjugation was controlled by using different amounts of the dye. In determining the F:P ratios, the rhodamine group was assumed to have a molar absorption coefficient of $7.3 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ at 565 nm (Fothergill, 1969).

2.3.2.3 **Fluorescent labelling of iodinated amino acids**

(a)  
Label: **Ortho-phthalaldehyde (OPT)**  
Amino acids: glycine, tyrosine, thyronine, 3-Iodotyrosine, 3,5-Diiodotyrosine, 3,5-Diiodothyronine, 3,5,3'-Triiodothyronine (T3), 3,5',3'-Triiodothyronine (reverse T3) and Thyroxine (T4)  
Procedure: Amino acids were labelled by a modification of the procedure of Butcher and Lowry (1976). Equal volumes (100 μl) of the amino acid stock solution and the OPT/thiol reagent were mixed, allowed to react for 2 minutes at room temperature in the dark, and then diluted to a final volume of 5 ml. with 0.5M NaOH.

(b)  
Label: **Fluorescamine (Fluram)**  
Amino acids: 3,5,3'-Triiodothyronine (T3) 3,5',3'-Triiodothyronine (reverse T3) and Thyroxine (T4)  
Procedure: 0.5 ml. of 0.03% fluorescamine in acetone was rapidly added to 2 ml. of the amino acids (10 μM) in 0.05M phosphate buffer, pH 8.6, and vigorously mixed in the dark on a vortex mixer for 2 minutes. No further purification was deemed necessary as neither the label nor the hydrolysis products of the label are fluorescent.
Label: Dansyl chloride
Amino acids: Glycine, T3 and T4
Procedure: The dansylation was performed by adding the reagent solution (10mM) in dry acetone, with stirring to a solution of the amino acid maintained at pH 9.5 (Felgner and Wilson, 1977). After shaking for a further hour at room temperature, excess dansyl chloride was hydrolysed with 0.1M NaOH, and the solution acidified with 6M HCl. The product was purified using a Dowex 50 X4 column (Zanetta et al., 1970) equilibrated with 0.01M acetic acid. The dansylated amino acid was eluted with water-acetone-25% ammonia (80:20:4), subjected to evaporation, lyophilised and stored in the dark at 4°C. The entire procedure was carried out under conditions permitting minimal exposure to light.

Synthesis of Eosin Isothiocyanate

The synthesis of eosin isothiocyanate was based on the methodology of Cherry et al., (1976) involving bromination of fluorescein isothiocyanate (FITC).

Bromine solution (230 mg.) was added dropwise with vigorous stirring to FITC (50 mg.) in 0.5 ml. ethanol. The mixture was left for 2 hours (with moderate shaking) at room temperature. After filtering, the insoluble material was solubilised in 0.01M phosphate buffer, pH 7.4, and reprecipitated at 4°C with 1M phosphoric acid. The washed (triply distilled water) precipitate was lyophilised and stored desiccated in the dark at -20°C.

The product was examined by thin layer chromatography (Kieselgel F254, eluted with benzene-methanol 2:1), the elemental analytical data (Microanalytical Laboratory, Manchester University) and NMR spectra of the product and FITC were also obtained.
2.3.3 Chemiluminescence analysis

2.3.3.1 Synthesis of bis(2,4,6-trichlorophenyl)oxalate (TCPO)

TCPO was synthesised by the method of Mohan and Turro (1974). Dry 2,4,6-trichlorophenol (10 g.) in sodium dried benzene (100 ml.) was introduced into a vacuum dried flask under nitrogen. After cooling the solution to 10°C, freshly distilled triethylamine (5 g.) was added and then oxalyl chloride (3.5 g.) in dry benzene (25 ml.) was added dropwise with stirring over 30 minutes. The suspension was allowed to warm to room temperature and left overnight (with continuous stirring). Removal of the solvent was followed by the addition of 50 ml. of petroleum ether with stirring for 15 minutes. The white insoluble residue was collected, dried under vacuum for 1 hour and recrystallised from dry chloroform. The crystalline oxalate was stored in a light-proof bottle with a screw-on lid in a vacuum desiccator.
CHAPTER 3

BACKGROUND FLUORESCENCE FROM BLOOD SERUM (PLASMA)

3.1 Introduction

In setting out to design fluorescence methods, one is struck by the concerns frequently expressed about background problems in fluorescence measurements. Background signals in fluorescence spectrometry arise from a number of sources, namely, (i) scattered and stray light; (ii) endogenous fluorescence of the sample; (iii) fluorescence impurities in solvents, sample cells etc.; (iv) poor experimental technique. Amongst these sources, (iii) and (iv) can be minimised by the use of careful experimental methods and good quality materials. The major remaining sources of background interference arise from the intrinsic (fluorescence and absorbance) properties of the sample and light scattering. The fact that the extreme sensitivity of fluorescence spectrometry demands a very high standard of experimental work and the desirable precautions is well described by Miller (1981(b)).

Immmunoassays are usually performed directly on serum/plasma or urine samples, and unquestionably the principal difficulty in the development of viable homogeneous assays has been the substantial fluorescence background signals from these biological samples. The fluorescence background problem has the effect of drastically worsening the limits of detection of many otherwise excellent fluorescence immunoassays, and in many cases renders the assays valueless in practical terms. The problem is not, of course, confined to immunoassays: many if not most other fluorescence analyses are in practice background limited.
It follows that any method capable of reducing background signals in fluorescence immunoassays may be of general value in the application of fluorimetric methods to biological problems. Most of the fluorescent labels used (and further labels currently studied for immunoassay development) in fluorescence immunoassay methods have their maximum emission wavelengths above 400 nm (Fig. 3.1), so background reduction in the wavelength region above 400 nm rather than the ultra-violet region of the spectrum is of most importance. However, since many organic fluorophors have very broad fluorescence spectra, fluorescence bands with maxima below 400 nm may make substantial contributions to the background at higher wavelengths.

Several possible instrumental approaches to the elimination of serum background and light scattering have been reported. Amongst these, is time-resolved fluorimetry where use is made of lifetime measurements, the concept first described by Weider (1977, 1979). Information on the lifetimes of serum/plasma fluorescence background seems to be sparse though Weider (1978) states without citing wavelengths that serum background lifetime is mostly in the lifetime domain <50 ns. Alternatively, phase modulation spectroscopy (Kronick and Little, 1975; Lakowicz and Cherek, 1981) or bleaching lifetime (Hirschfeld, 1979), with appropriate time gating of fluorescence detection may be employed to discriminate against the endogeneous background.

Another common approach is sample pretreatment by chemical methods. The effects of ethanol and trichloroacetic acid deproteinisation methods and of ultrafiltration have been described (Abdullahi, 1979). Very recently Kam and Yoshida (1981) proposed a chemical treatment method that produced considerable reduction of serum fluorescence; however, any benefit at wavelengths other
1. A Fluorescence spectrum of a Fluorescamine-albumin conjugate (F:P ~ 2), Excitation λ 390 nm (A₃₉₀ < 0.01)

B Fluorescence spectrum of Quinacrine mustard (ca. 10⁻⁸M), Excitation λ 350 nm (A₃₅₀ ~ 0.02)

2. A Fluorescence spectrum of an Albumin-dansyl conjugate (F:P ~ 11), Excitation λ 340 nm (A₃₄₀ ~ 0.04)

B Fluorescence spectrum of an NBD-Cl-albumin conjugate, Excitation λ 470 nm (A₄₇₀ < 0.01)

[All dilutions were made in 0.01M phosphate buffer, pH 7.4. The spectra were obtained on the LS-5 Luminescence Spectrometer (with Data Station); bandwidths 5 nm.]
Figure 3.1
1. A Fluorescence spectrum of Fluorescein isothiocyanate (ca. $10^{-8}$M), Excitation $\lambda$ 490 nm ($A_{490} < 0.01$)

   B Fluorescence spectrum of Lucifer Yellow (lithium salt, ca. $10^{-8}$M), Excitation $\lambda$ 430 nm ($A_{430} < 0.01$)

2. A Fluorescence spectrum of Eosin isothiocyanate (ca. $10^{-8}$M), Excitation $\lambda$ 550 nm ($A_{550} < 0.01$)

   B Fluorescence spectrum of Rhodamine isothiocyanate (ca. $10^{-8}$M), Excitation $\lambda$ 550 nm ($A_{550} < 0.01$)

[All dilutions were made in 0.01M phosphate buffer, pH 7.4. The spectra were obtained on the LS-5 Luminescence Spectrometer (with Data Station); bandwidths 5 nm.]
Figure 3.1 continued
than the excitation/emission wavelength combination, 490/520 nm have not been evaluated.

Other procedures for tackling background problems have been mooted. These include (a) Automatic blank subtraction; (b) Kinetic measurements (Ullman et al., 1980); (c) Combined hplc immunoassays (Lidofsky, 1980); (d) Chemiluminescence excitation (f) Fluorescence autocorrelation immunoassay (Nicoli et al., 1980).

Light scattering (Rayleigh in particular) is most serious when a fluorophore with a small Stokes shift is being used (e.g. fluorescein $\lambda_{\text{Ex.}} \approx 490 \text{ nm}$, $\lambda_{\text{Fl.}} \approx 520 \text{ nm}$). Suitable cut-off filters may help to minimise its effects. Removal of suspended particles etc. from the sample and background reduction procedures that remove part or whole of the protein content of a sample will also reduce scattered light signals. Additionally several optical methods are available to minimise this interference, viz, (a) Derivative spectroscopy (Green and O'Haver, 1974); (b) Synchronous scanning (Lloyd, 1971); (c) Polarised (horizontal) exciting light (Lim et al., 1978); and/or possibly laser excitation.

The topic of background fluorescence from blood serum/plasma has been reviewed at least once before (Soini and Hemmila, 1979) and recently Smith et al.,(1984a)) have discussed the limitations and interfering factors in fluorescence immunoassays with particular reference to the interference arising from any of the many components of plasma or serum.

This chapter attempts to describe and discuss the nature of the background fluorescence signals from blood serum/plasma and compares the various possible approaches to their reduction or removal. The knowledge of the solutes responsible is sparse;
and in efforts to identify the spurious and any artifactual fluorescence, serum/plasma samples were subjected to fractionation (gel chromatography). Young (1973) performed a detailed study of the fluorescence signals from urine samples of 26 male and female volunteers. There was considerable variation in the intensity of fluorescence signals from person to person. Similar information on serum was desirable; serum samples from different sources were thus examined.
3.2 Experimental

3.2.1 General

All serum samples were stored in the dark at -20°C. Aliquots were thawed and allowed to reach room temperature before analysis. The four major Sephacryl S-300 fractions (Fig. 3.2) of whole human serum and plasma (Section 2.3.1.1) and other proteins were stored at 4°C and equilibrated to room temperature prior to fluorescence analysis. All the dilutions were made in 0.01M Tris-HCl buffer, pH 7.4, unless otherwise specified. The fluorescence spectra and measurements were obtained at room temperature on the MPF-44B spectrofluorimeter and corrections were made for the background fluorescence contributed by the buffer.

3.2.2 Background reduction/removal methods

3.2.2.1 Chemical treatment

Fluorescence and absorbance measurements were made at a number of wavelengths on the peracid/sulphite treated serum sample (Section 2.3.1.2(a)) diluted to a final volume of 5 ml. (giving a hundred-fold dilution) in 0.01M phosphate buffer, pH 7.4 and compared with those of an untreated serum sample of same dilution.

3.2.2.2 Removal using adsorbents

Hydroxyapatite (HA)

The fluorescence and absorbance intensities of the serum sample eluted from the HA column with 0.01M phosphate buffer, pH 7.0 (Section 2.3.1.2(b)) were compared with those of an untreated but similarly diluted aliquot of the same serum (in the same buffer passed alone through an identical HA column).
Figure 3.2

Fractionation of whole human serum on Sephacryl S-300 superfine. Peak 1 was eluted at the void volume of the column; Peak 2 taken to be the 'plateau region' containing traces of IgM, α₂-macroglobulin and IgA; Peak 3 contained mainly IgG; and Peak 4 contained mainly albumin.

Reference from
Pharmacia booklet
Sephacryl S-300

Gel filtration of serum proteins on Sephacryl S-300 Superfine. Pharmacia Column K 26/100; bed height: 94 cm; eluent: Tris-HCl buffer solution (I = 0.1, pH 8.0) containing NaCl (0.5 M); flow rate: 2.4 ml · cm⁻² · h⁻¹. (Work from Pharmacia Fine Chemicals).
The bound fluorescent species were subsequently eluted with a 0.2M phosphate buffer, pH 7.4, containing 0.145M NaCl.

In order to check the suitability of this approach in continuous flow systems, experiments were performed where the same HA column was used several times without desorbing the bound species. Fluorescence measurements were continued (\(\lambda_{\text{Ex.}} 285 \text{ nm}\)) until a significant increase in fluorescence was observed i.e. until proteinaceous material was no longer adsorbed on HA when small aliquots (50 \(\mu\)l) of serum were eluted (each of 100-fold dilution) continuously with 0.01M phosphate buffer, pH 7.0.

**Florisil**

The procedure was very similar to that for hydroxyapatite (Section 2.3.1.2 (b)).

**Blue Sepharose (immobilised Cibacron Blue-3GA)**

Fluorescence and absorbance measurements were obtained at a number of wavelengths of a Blue Sepharose-treated plasma sample (Section 2.3.1.2(b)) and compared with those of a similarly diluted untreated plasma sample. In control experiments the eluant was changed (0.2M sodium thiocyanate) and the bound fluorescence was quantitatively recovered.
3.3 Results

3.3.1 Ultra-violet and visible spectra

Three dilutions of different serum and plasma samples were studied (Fig. 3.3). Table 3.1 indicates the absorbance values of these samples at a number of excitation wavelengths. The 10-fold diluted serum samples absorbed intensely at 285 nm, the absorbances were apparently very similar due to instrumental limitations. The 1:100 dilutions showed significant absorption at 285 nm, and because of high concentration (of protein) deviation from the Beer-Lambert Law was observed. At other wavelengths the absorbance values for 1:100 dilutions were small, showing some non-linearity in the absorbance-concentration (dilution) relationship.

3.3.2 Fluorescence spectra

Plasma and serum samples showed similar emissions. With the above dilutions, four fluorescence bands were found (Fig. 3.5, A, B, C; see Fig. 3.4 for excitation spectra). The fluorescence spectra of a whole human serum sample are described here. One, with an excitation wavelength of 285 nm, yielded maximum fluorescence at ca. 335 nm. The fluorescence from a 100-fold dilution was very intense; the intensity of this signal was equivalent to that of a 2.5 ppm solution of tryptophan. Excitation at 285 nm also generated a much weaker fluorescence signal in the 640-700 nm range. This band (from 1:100 dilution) was at least 60 times less intense than the 285/335 nm band. Use of a 430 nm cut-off filter in the emission beam removed this signal (Fig. 3.6). The second fluorescence band occurred with excitation and emission at ca. 350 nm and 450 nm respectively, and a comparison of the signals from a 100-fold dilution showed that this band was over a hundred
Figure 3.3

Ultra-violet spectra of serum (--- 100-fold dilution, 
-.-.-. 50-fold dilution, -- -- 10-fold dilution) and 
plasma — 100-fold dilution, --- 10-fold dilution samples.

A  Cancer patient serum sample

B  Whole human serum sample (commercial)

C  Male plasma sample (donated by a healthy laboratory worker)
Table 3.1

Absorbance and Fluorescence intensities (arbitrary units) of various serum samples (1:100 and 1:10 fold dilutions in Tris-HCl buffer)

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>Excitation λ nm</th>
<th>285</th>
<th>350</th>
<th>401</th>
<th>425</th>
<th>470</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Emission λ nm)</td>
<td>(335)</td>
<td>(∼450)</td>
<td>(∼470)</td>
<td>(∼490)</td>
<td>(∼620)</td>
</tr>
<tr>
<td></td>
<td>285</td>
<td>350</td>
<td>401</td>
<td>425</td>
<td>470</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>*0.92</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
<td>Not</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†3.42</td>
<td>0.25</td>
<td>0.21</td>
<td>0.16</td>
<td>Available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*88</td>
<td>8</td>
<td>6.5</td>
<td>3.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†10</td>
<td>44</td>
<td>21</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>Not</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.37</td>
<td>0.28</td>
<td>0.18</td>
<td>0.10</td>
<td>Available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>39</td>
<td>19</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.80</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>Not</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.39</td>
<td>0.23</td>
<td>0.20</td>
<td>0.16</td>
<td>Available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>9.5</td>
<td>5</td>
<td>3.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>55</td>
<td>18</td>
<td>16</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.02</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.42</td>
<td>0.28</td>
<td>0.24</td>
<td>0.21</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>42.5</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.98</td>
<td>0.06</td>
<td>0.04</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.49</td>
<td>0.37</td>
<td>0.28</td>
<td>0.24</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>11.5</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>66</td>
<td>31</td>
<td>18</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.96</td>
<td>0.07</td>
<td>0.035</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.37</td>
<td>0.32</td>
<td>0.25</td>
<td>0.22</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>13.5</td>
<td>6</td>
<td>4</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>83</td>
<td>42</td>
<td>25</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.03</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.41</td>
<td>0.31</td>
<td>0.20</td>
<td>0.16</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Plasma)</td>
<td>87</td>
<td>23</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>79</td>
<td>26</td>
<td>17</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Columns represent 1:100 dilution; † Columns represent 1:10 dilution
θ 3 pooled lung cancer patient samples (exhibited turbidity)

Serum samples: 1. Bronchus cancer patient; 2. 3. 4. Lung cancer patient; 5. Whole human serum (commerical); 6. T3/T4 depleted serum (commerical)

Fluorimetersensitivity at Excitation Wavelength 285 nm, = x 1.
Fluorimetersensitivity at all other Excitation Wavelengths, = x 15.
Corrected Excitation spectra of whole human serum sample (100-fold dilution): 1, Fluorescence (Fl.) λ 335 nm; 2, Fl. λ 450 nm; 3, Fl. λ 470 nm; 4, Fl. λ 495 nm.

Instrument sensitivity at Fl. λ 335 nm = x 1;
Instrument sensitivity at other Fl. λ = x 30;
Emission bandwidth 12 nm and excitation bandwidth 8 nm.
Corrected fluorescence spectra of two of the serum samples
(--- lung cancer patient, —— whole human serum, commercial)
at excitation λs, 1, 285; 2, 350; 3, 401; 4, 425 nm.
Instrument sensitivity at Ex. λ 285 nm = x 1;
" " Ex. λ 350 nm = x 15;
" " Ex. λ 401, 425 nm = x 50
Emission bandwidth 8 nm; Excitation bandwidth 12 nm.
Corrected fluorescence spectra of --- lung cancer patient serum sample and —— whole human serum, commercial sample at the excitation λs and instrument conditions given below.

Corrected fluorescence spectra of two serum samples (—— T3/T4 depleted serum, commercial and --- bronchus cancer patient) at excitation λs, 1, 285; 2, 350; 3, 401; 4, 425 nm.

Instrument sensitivity at Ex. λ 285 nm = x 1;
Instrument sensitivity at other Ex. λs = x 15;
Emission bandwidth 8 nm, Excitation bandwidth 12 nm.
times less intense as the first band. The apparent intensity of
the signal from a 10-fold dilution was comparable with a 0.02 ppm
solution of quinine sulphate. In addition, two weaker bands at
excitation/emission wavelength combination of 401/470 nm and
425/500 nm were observed, with the intensity of the former being
two-thirds and of the latter ca. half the intensity of the
350/450 nm band. These bands were rather broad and the use of the
subtraction facility furnished in the MPF-44B (DCSU unit) allowed
the removal of any Raman scatter contribution (Figs. 3.7, 3.8).
The relative fluorescence intensities of six different serum samples
(100-fold dilution, Table 3.2) were obtained. Some variation
between the samples was observed and the magnitude of the 285/335 nm
band seemed larger when compared with the other signals.

3.3.3 Serum fractions - Fluorescence spectra

Each of the four major Sephacryl S-300 fractions of whole
human serum and plasma was diluted over a hundred times and the
fluorescence spectra obtained at excitation wavelengths 285, 350,
401 and 425 nm (Figs. 3.9, 3.10).

The figures showed that the 285/335 nm band was more intense
in the albumin fraction. The 350/450 nm band apparently weaker
in other fractions was more prominent in the albumin fraction.
Also, the albumin fraction and to a lesser extent, the IgG fraction,
exhibited fluorescence at ca. 470 nm and 490-500 nm when excited
at 401 nm and 425 nm respectively. (This description was based on
the absorption information of the fractions.)

Fluorescence spectra were also recorded of commercial prepara-
tions of human serum albumin (100% pure), defatted human serum
albumin and human IgG; all of equal concentration, 50 µg ml⁻¹
(Fig. 3.11).
Figure 3.6

Fluorescence spectrum (---) of a 100-fold diluted whole human serum sample at excitation λ 285 nm.
-.--.-- shows the signal obtained when a 430 nm cut-off filter (emission beam) was used;
---- shows the buffer signal.
Instrument sensitivity = x 15.

Figure 3.7

Fluorescence spectrum (---) of a 10-fold diluted serum sample at excitation λ 425 nm.
-x-x shows the DCSU fluorescence spectrum obtained when the background contribution from the buffer is subtracted;
---- shows the buffer signal.
Instrument sensitivity = x 15.
Figure 3.8A

Fluorescence spectrum (-----) of a 10-fold diluted serum sample at excitation λ 401 nm.
-x-x shows the DCSU fluorescence spectrum obtained when the buffer contribution is subtracted;
---- shows the buffer signal.
Instrument sensitivity = x 15.

Figure 3.8B

[Shifting of excitation λ to check for Raman scatter (solvent) signal]
As above except excitation λ 381 nm used.
Figure 3.8
Table 3.2

Relative Fluorescence Intensities of Serum/Plasma Samples
(100-Fold Dilution)

<table>
<thead>
<tr>
<th>Excitation Wavelength, nm</th>
<th>285</th>
<th>350</th>
<th>401</th>
<th>425</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emission Wavelength, nm</td>
<td>335</td>
<td>~440-450</td>
<td>~470</td>
<td>~490</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>1 (Bronchus Cancer Patient)</th>
<th>2 (Lung Cancer Patient)</th>
<th>3 (Lung Cancer Patient)</th>
<th>4 (Lung Cancer Patient)</th>
<th>5 (Whole Human Serum, Commercial)</th>
<th>6 (T3/T4 Depleted Serum, Commercial)</th>
<th>7 (A Male Plasma Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100*</td>
<td>0.68</td>
<td>0.49</td>
<td>0.26</td>
<td>68</td>
<td>0.87</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.53</td>
<td>0.45</td>
<td>0.26</td>
<td>93</td>
<td>0.71</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>0.58</td>
<td>0.45</td>
<td>0.24</td>
<td>99</td>
<td>0.58</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>0.87</td>
<td>0.38</td>
<td>0.19</td>
<td>77</td>
<td>0.81</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>0.81</td>
<td>0.41</td>
<td>0.21</td>
<td>77</td>
<td>0.81</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>0.98</td>
<td>0.30</td>
<td>0.18</td>
<td>99</td>
<td>0.98</td>
<td>0.30</td>
</tr>
</tbody>
</table>

* Reference arbitrary number taken to be 100 for this sample.

Instrument sensitivity at Excitation Wavelength 285 nm, $= x 1$.

Instrument sensitivity at all other Excitation Wavelengths, $= x 15$.

In all cases the emission bandwidth was 8 nm and the excitation bandwidth 12 nm.

The following information was available on the patient specimen:

Sample 3: Protein content $63 \, \text{gL}^{-1}$, Albumin $37 \, \text{gL}^{-1}$, Bilirubin $18 \, \mu\text{molL}^{-1}$;

Sample 4: Protein content $67 \, \text{gL}^{-1}$, Albumin $38 \, \text{gL}^{-1}$, Bilirubin $13 \, \mu\text{molL}^{-1}$, and the commercial samples 5 and 6 were sterilised with the use of membrane filtration.
Figure 3.9

Fluorescence spectra of Sephacryl S-300 serum fractions (1-5) at excitation λs, —— 285, ---- 350, -.-.-. 401 and -x-x 425 nm.
Fraction 1 eluted at void volume of column,
Fraction 2 contained the 'plateau' region proteins,
Fraction 3 contained mainly IgG,
Fraction 4 contained mainly albumin,
Fraction 5 contained mainly 'non-specific proteins'.
[Also see Fig. 3.2]
6 shows the buffer signals at above Ex. λs.
Instrument sensitivity at Ex. λ 285 nm x x 1;
Instrument sensitivity at other Ex. λs = x 15.
Figure 3.9

Fraction 1

Fraction 2

Fraction 3

Fraction 4

Fraction 5

Buffer (Tris-HCl pH 8)
Figure 3.10

Fluorescence spectra of Sephacryl S-300 plasma fractions (2, 3, 4) at excitation λs —— 285 nm, ---- 350 nm, 401 nm and -x-x 425 nm.

Instrument sensitivity at Ex. λ285 = x 1,
Instrument sensitivity at other Ex. λs = x 15.
Fraction 2
('Plateau' region proteins)

Fraction 3
(IgG)

Fraction 4
(Albumin)

Figure 3.10
Figure 3.11

Fluorescence spectra of a commercial human serum albumin sample (---), defatted human serum albumin, commercial (-----) and human Immunoglobulin G (-----), [all of concentration = 50 µg ml⁻¹] at excitation λ 1, 285 nm; 2, 350 nm; 3, 401 nm; 4, 425 nm. Instrument sensitivity at Ex. λ 285 nm = x 1; Instrument sensitivity at other Ex. λs = x 15.
There were some differences between the normal and the defatted albumin preparations, the former showed a fluorescence band centred at ca. 460 nm, the latter exhibited fluorescence at ca. 450 nm when excited at 350 nm. Quantitatively, defatted albumin showed a stronger 285/335 nm band, at other wavelengths the fluorescence intensities were 30-50% less as compared to those from the normal albumin preparation. The fluorescence signals from the human IgG preparation were generally very weak.

Additionally, a brief fluorimetric examination was made of pyridoxine, and bilirubin in the presence of albumin. Pyridoxine (0.5 μg ml⁻¹) showed fluorescence at ca. 390 nm at an excitation wavelength of 320 nm. Addition of increasing amounts (1-15 μg ml⁻¹) of pyridoxine to a whole human serum sample (100-fold dilution) showed no significant changes in the intensity of the 350/450 nm band, however, the fluorescence in the 390 nm region of the spectrum became more intense (Fig. 3.12). The excitation at 366 nm of a 100-fold diluted serum containing >0.5 μg ml⁻¹ pyridoxine produced a fluorescence spectrum no different from the one without any pyridoxine.

The fluorescence spectra of human serum albumin (50 μg ml⁻¹) in the presence of bilirubin (20 μg ml⁻¹) were obtained at excitation wavelengths 285 nm, 350 nm, 401 nm, 425 nm and 470 nm (Fig. 3.13). When compared with the fluorescence spectra of human serum albumin, bilirubin was seen to quench the 285/335 nm signal. Some enhancement was observed in the 350/450 nm and 401/470 nm emissions, the 425/490-500 nm band seemed to be considerably amplified, and an additional peak appeared at ca. 540 nm in all cases. Excitation at 470 nm showed the characteristic (intense) bilirubin-albumin fluorescence at ca. 530 nm (Beaven et al., 1973), pure albumin did not fluoresce at this excitation wavelength.
Figure 3.12

C  Addition of pyridoxine (0.75 μg ml$^{-1}$) to whole human serum sample (final dilution 100-fold)

---  Fluorescence spectrum of serum and pyridoxine

——  Fluorescence spectrum of 100-fold diluted serum

----  Fluorescence spectrum of pyridoxine, and

....  buffer signal, all at excitation $\lambda$ 366 nm.

B  Fluorescence spectra (----) obtained when increasing amounts of pyridoxine ($i = 1$, $ii = 3$, $iii = 10$, $iv = 15$ μg ml$^{-1}$) added to whole human serum sample (final dilution 100-fold) at excitation $\lambda$ 350 nm.

—— shows the fluorescence spectrum of 100-fold diluted serum (alone) at excitation $\lambda$ 350 nm.

A  Fluorescence spectra of pyridoxine at excitation $\lambda$ 320 nm (——) and 350 nm (-----, $i = 1$, $ii = 3$, and $iii = 10$ μg ml$^{-1}$ pyridoxine; Absorbance at 350 nm for $ii$ and $iii$ was >0.05).
Figure 3.13

Fluorescence spectra of human serum albumin (50 µg ml\(^{-1}\)) ----, and bilirubin (20 µg ml\(^{-1}\)) + human serum albumin (50 µg ml\(^{-1}\)) —— at excitation \(\lambda_s\) 1, 285; 2, 350; 3, 401; 4, 425; and 5, 470 nm.

Instrument sensitivity at Ex. \(\lambda\) 285 nm = x 1;
Instrument sensitivity at other Ex. \(\lambda_s\) = x 15.
(Bilirubin + albumin mixture was incubated for over 4 hours before spectra were obtained.)
3.3.4 Chemical background reduction

Table 3.3 compares the fluorescence and absorbance intensities of a treated and subsequently diluted whole human serum sample (Section 2.3.1.2(a)) with those of an untreated sample of the same dilution at a number of wavelengths. The treatment reduced the background fluorescence by ca. 66% at all wavelengths except at excitation wavelength 350 nm where a reduction of <50% was seen.

3.3.5 Use of adsorbents

The results of the background removal procedure using hydroxyapatite and Blue Sepharose, immobilised Cibacron Blue 3GA (Section 2.3.1.2(b)) are shown in Tables 3.4 and 3.5 respectively. Using hydroxyapatite, the fluorescence background was found to be reduced to ca.15% of its original value at all wavelengths (using 0.01M phosphate buffer, pH 7.0). Blue Sepharose appeared to reduce serum background fluorescence by ca. 40-70% according to the wavelength studied.
Table 3.3

Effect of Peracid/Sulphite Treatment on a Whole Human
Serum Sample (100-Fold Dilution)

<table>
<thead>
<tr>
<th>Excitation/ Emission λ, nm</th>
<th>Fluorescence Intensity Before Treatment</th>
<th>Fluorescence Intensity After Treatment</th>
<th>Absorbance Before Treatment</th>
<th>Absorbance After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>*285/335</td>
<td>†62 (3)</td>
<td>22</td>
<td>0.94</td>
<td>0.48</td>
</tr>
<tr>
<td>350/440</td>
<td>27 (5)</td>
<td>15</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>401/470</td>
<td>21 (3)</td>
<td>7</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>425/490</td>
<td>9.5 (2)</td>
<td>4</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>470/520</td>
<td>7 (2)</td>
<td>2</td>
<td>0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

* At Excitation 285 nm Instrument sensitivity = x 1. At all other excitation wavelengths Instrument sensitivity = x 50.

† Uncorrected fluorescence intensities (average of four experiments) in arbitrary units, background from buffer (figures in brackets) subtracted.
### Table 3.4

Effect of Hydroxyapatite Treatment on a Whole Human Serum Sample (100-Fold Dilution)

<table>
<thead>
<tr>
<th>Excitation/Emission λ, nm</th>
<th>Fluorescence Intensity Before Treatment</th>
<th>Fluorescence Intensity After Treatment</th>
<th>Recovered Fluorescence With 0.2M Phosphate</th>
<th>Absorbance Before Treatment</th>
<th>Absorbance After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>285/335</td>
<td>60 (3)</td>
<td>5</td>
<td>53</td>
<td>0.90</td>
<td>0.20</td>
</tr>
<tr>
<td>350/440</td>
<td>28 (5)</td>
<td>5</td>
<td>21</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>401/470</td>
<td>25 (4)</td>
<td>4</td>
<td>19</td>
<td>0.045</td>
<td>0.01</td>
</tr>
<tr>
<td>425/490</td>
<td>11 (3)</td>
<td>2</td>
<td>7</td>
<td>0.035</td>
<td>0.02</td>
</tr>
</tbody>
</table>

### Table 3.5

Effect of Cibacron Blue 3GA (Blue Sepharose) Treatment on a Normal Plasma Sample (100-Fold Dilution)

<table>
<thead>
<tr>
<th>Excitation/Emission λ, nm</th>
<th>Fluorescence Intensity Before Treatment</th>
<th>Fluorescence Intensity After Treatment</th>
<th>Recovered Fluorescence With 0.2M Sodium Thiocyanate</th>
<th>Absorbance Before Treatment</th>
<th>Absorbance After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>285/335</td>
<td>70</td>
<td>20</td>
<td>50</td>
<td>0.88</td>
<td>0.18</td>
</tr>
<tr>
<td>350/440</td>
<td>37</td>
<td>9</td>
<td>26</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>401/470</td>
<td>22</td>
<td>10</td>
<td>10</td>
<td>0.05</td>
<td>0.009</td>
</tr>
<tr>
<td>425/490</td>
<td>8</td>
<td>4.5</td>
<td>3</td>
<td>0.04</td>
<td>0.003</td>
</tr>
</tbody>
</table>
3.4 Discussion

3.4.1 Nature of Background

It was apparent from earlier work (e.g. Soini and Hemmila, 1979) that a good deal remained to be established on the background fluorescence of serum, particularly the origins of some of the bands which have been obscure. Several deductions can be made from the present studies.

The fluorescence with an excitation wavelength of 285 nm and an emission maximum of ca. 335 nm was certainly from proteins, overwhelmingly due to their tryptophan residues. This fluorescence extended beyond 440 nm but could probably be neglected at >500 nm. Another weak signal was observed in the 640-700 nm region when the serum was excited at 285 nm. Use of a cut-off filter showed that this band was due to the second order protein signal i.e. an instrumental artifact. The occurrence of this signal indicated the size and strength of the 335 nm band. Since not all fluorescence spectrometers are equipped with extra cut-off filters, it is important to beware of this second order phenomenon. Equally, it should be borne in mind that certain components of serum, e.g. porphyrins and hemolysed sera do exhibit fluorescence around 600 nm.

A second band had excitation and emission wavelengths of ca. 350 and 450 nm respectively. The apparent intensity of a 10-fold diluted serum sample was found to be equivalent to that of a 0.02 ppm solution of quinine sulphate excited at the same wavelength: although this was purely an arbitrary comparison, it indicated the seriousness of background fluorescence problems. Soini and Hemmila (1979) detected but did not identify this emission, though they indicated that it was apparently not
associated with the protein fraction: it generated appreciable fluorescence at wavelengths as high as 580 nm. The origin of this band could not be unequivocally identified, but it seemed very likely that this fluorescence arises from a combination of some variable sources.

The existence of this emission (340-350/440-460 nm) from the serum of renal disease patients has been reported (Coolen et al., 1978; Schwertner et al., 1980, and Digenis et al., 1981). There seems to be no general agreement on the origin of this fluorescence. The study of Coolen and co-workers (1978) established that the artifact responsible for this band was non-protein in nature, bound to albumin and possibly occurring in normal serum in low concentrations. There is the possibility that aromatic amino acids and proteins which have been exposed to daylight may undergo photo-oxidation reactions yielding products (e.g. N-formyl-kynurenine, λEx. ca. 360, λFl. ca. 460 nm, Duggan et al., 1957) with higher wavelength fluorescence. The study of Lunec and Dormandy (1979, Lunec 1981) showed that lipid peroxides, phagocytosing white cells or ultra-violet light generate free radicals which damage human γ-globulin, this is related to changes in tryptophan and other constituent aromatic amino acids and the formation of fluorescent complexes (λEx. 360 nm, λFl. 454 nm). It has been reported that human serum albumin is likely to be susceptible to development of fluorescent modification by lipid peroxidation, the resulting fluorescent chromophores having excitation and emission maxima at ca. 350 and 440 nm respectively (Fletcher and Tappel, 1971). There has also been some speculation that the endogeneous fluorescent material(s) may be as a result of vitamin B6 (pyridoxine) supplementation (Coolen et al., 1978).
However, from the fluorimetric study of pyridoxine it was seen that the fluorescence spectrum differed clearly from that due to the 'unidentified' material and addition of the vitamin to normal human serum did not indicate any well defined emission at excitation wavelength 350 nm (Fig. 3.12).

Two further emissions observed from the serum/plasma samples, 401/470 nm and 425/490-500 nm band were rather broad. In normal circumstances these bands could easily be regarded as Raman bands. Raman peaks have frequently been confused with fluorescence maxima thus, the classical method of shifting incident light was employed to distinguish the phenomena (Fig. 3.8). Since the bands were broad, use was made of the blank subtraction facility available on the MPF-44B to remove the substantial Raman scatter overlap (Figs. 3.7, 3.8); this allowed to see serum fluorescence at ca. 470 nm when excited at 401 nm and ca. 490-500 nm when an excitation wavelength of 425 nm was used. The origins of these bands are not quite clear, however, the 425/490-500 nm emission resembles the bilirubin-albumin fluorescence (Fig. 3.13; cf. Smith et al., 1981(a)).

In efforts to identify the endogenous fluorescence, in particular the 350/450 nm band (emission extending to ca. 580 nm), serum was subjected to fractionation by gel chromatography. Comparison with the manufacturer's results indicated that peak 4 (Fig. 3.2) contained mainly albumin. The fluorescence spectra of the four major fractions (Figs. 3.9, 3.10) showed intense protein emission from the albumin fraction and the 350/450-460 nm band though somewhat weaker in the other fractions was prominent in the albumin fraction. The fluorescence intensity of the two commercial human serum albumin preparations varied but the excitation and emission were very similar, \( \lambda_{\text{Ex.}} \) 350, \( \lambda_{\text{Fl.}} \) 450-460 nm.
From these observations, it was evident that a good deal of background fluorescence from serum/plasma samples resides in/with albumin, though the effect of exogeneous factors has not been ruled out.

3.4.2 **Background Elimination**

The simple de-proteinising approaches (Abdullahi, 1979) would be less suitable since the resulting solutions will require further treatment for immunoassay work. The recent chemical method (Kam and Yoshida, 1981) claimed to eliminate 99% of the serum background, although only its effects at 490/520 nm were described. Table 3.3 shows the results of further work done for the evaluation of its beneficial effects at other wavelengths. The apparent intention of this method was to minimise the contribution of bilirubin fluorescence, the results, in particular at 470/520 nm, indicated that this was largely achieved. The treatment had variable effects on serum fluorescence at other wavelengths; some selective removal of fluorescent materials was observed (285/335 nm). This method could hardly be classed as convenient, and it has been reported (Ullman, 1981) that not all analytes can survive this treatment.

A second possible approach to the reduction of background signals involved the use of adsorbents to abstract from serum/plasma (etc.) samples the fractions giving rise to endogeneous fluorescence and possibly scattering. Such an approach will normally involve treating the sample with the adsorbent either in the form of a small column, or as a batch procedure. The column procedure will inevitably involve dilution of the sample; however this may be necessary in any event to minimise inner filter effects (see Section 3.4.4).
Hydroxyapatite (Tiselius et al., 1956) and Blue Sepharose (Travis et al., 1976) are two of the possible adsorbents which suggested themselves. Hydroxyapatite, HA (also known as hydroxylapatite, reviewed by Brooks, 1981) is a form of hydrated calcium phosphate and following its early use in protein fractionation it has found many applications in analytical and preparative biochemistry. It has been used to separate antibody-bound and free steroids in radioimmunoassays (Trafford et al., 1976) on the basis that most macromolecules are strongly adsorbed to it, whereas low molecular weight solutes are generally not bound. There is also good evidence that important low molecular weight analytes such as T3 and T4 do not bind to HA. (Trafford and Makin, 1980).

The possibility of HA to remove proteins and protein-bound material from serum was thus apparent.

A column procedure was mostly used (Section 2.3.1.2(b)) and elution with 0.01M phosphate buffer, pH 7.0, was found to reduce the fluorescence background by ca. 80-90% at all wavelengths (Table 3.4). The reduction in absorbance (e.g. at 285 nm) and the quantitative recovery of fluorescence clearly indicated substantial adsorption of protein material on HA. The serum background could be reduced further by changing the buffer conditions, generally speaking, lower the buffer (phosphate) concentration, the more strongly the proteins are adsorbed. This background removal procedure is thus relatively simple and very efficient. A 2 ml. hydroxyapatite column could satisfactorily be used more than once for a relatively small serum sample without any change of experimental conditions. This result indicated a potential use of HA (e.g. packed flow cell) in continuous flow systems. It has been suggested that fine suspensions of HA may be suitable for continuous flow systems (Trafford et al., 1976).
There is now available HA-ultrogel (LKB, S. Croydon; hydroxyapatite crystals are individualised and immobilised within cross-linked agarose beads) in which fine particle production is prevented; this would perhaps enable the same column to be used several times more for smaller serum samples without repacking.

The development of a batch procedure proved less satisfactory, this was due to increased scattering caused by fine HA particles.

Some experiments with florisil (a coarser but chemically different adsorbent) using the same experimental conditions as with HA, did not give any conclusive results, however, very recently the use of florisil as a separating agent in an immunoassay for thyroid stimulating hormone has been reported (Derfler et al., 1982); further study with appropriate modifications in the experimental conditions is worthwhile to see if the cheaper florisil can be employed for removing background fluorescence from serum.

Substantial interest has been generated in recent years by the binding properties of various active dyes (Amicon Corporation, 1980). These dyes, immobilised via triazine linkages to polyhydroxy matrices, are capable of non-covalent binding to a number of proteins, in particular dehydrogenase enzymes and human serum albumin. Since it is likely that a good deal of serum fluorescence is associated with the albumin fraction (Section 3.4.1) the use of such dyes to remove background fluorescence appeared attractive.

Blue Sepharose (immobilised dye, Cibacron Blue 3GA) has a superior adsorption capacity for human serum albumin (ca. 50 mg. ml⁻¹ adsorbent, Harvey, 1980). The results of the experiments with Blue Sepharose (Table 3.5) showed considerable reduction in background fluorescence from plasma proteins, mainly albumin, at 285/335 and 350/450 nm.
Separate experiments with normal human serum albumin and defatted albumin demonstrated substantial specific adsorption of albumin (Tables 3.6 and 3.7). Control experiments confirmed that bound fluorescence could all be released by a change of eluant (0.2M sodium thiocyanate, as opposed to the original 50mM Tris-HCl buffer, pH 7.0).

Comparison of the results of the two (sample pretreatment) methods, indicate that although, as expected, a good deal of serum fluorescence resides in the albumin fraction, there are also other substantial sources of fluorescence.

Both the adsorbents have had varying but substantial effect on the fluorescence signals arising from serum/plasma samples. The 285/335 and 350/450-460 nm bands were dramatically reduced with HA treatment and the corresponding reduction in the absorbances meant that proteins were strongly adsorbed, the efficiency dependent on buffer conditions: a reduction in background fluorescence to ca. <5% of its original value could be achieved with 2mM phosphate buffer, pH 7.0. Blue Sepharose brought a respectable reduction in the background fluorescence at 285/335 and 350/450-460 nm, demonstrating albumin adsorption and the related drop in fluorescence intensities at the wavelength of protein fluorescence. Since Blue Sepharose has a superior adsorption capacity for human serum albumin, it can be assumed from the results that the 350/450-460 emission is associated substantially with the albumin fraction and that the remaining observed fluorescence could have developed from photo-oxidation or lipid peroxidation of other co-existing solutes (probably with albumin).

Both the adsorbents allowed the acquisition of the fundamental information on the serum/plasma background fluorescence and their possible use for its removal.
Table 3.6

Effect of Cibacron Blue 3GA Treatment on Human Serum Albumin (0.5 mg)

<table>
<thead>
<tr>
<th>Excitation/Emission ( \lambda ), nm</th>
<th>Fluorescence Intensity Before Treatment</th>
<th>Fluorescence Intensity After Treatment</th>
<th>Recovered Fluorescence</th>
<th>Absorbance Before Treatment</th>
<th>Absorbance After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>285/335</td>
<td>95</td>
<td>2</td>
<td>93</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td>350/460</td>
<td>39</td>
<td>2</td>
<td>33</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>401/470</td>
<td>13</td>
<td>2</td>
<td>9</td>
<td>0.03</td>
<td>-</td>
</tr>
<tr>
<td>425/490</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td>Not Available</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7

Effect of Cibacron Blue 3GA Treatment on Defatted Human Serum Albumin (0.5 mg)

<table>
<thead>
<tr>
<th>Excitation/Emission ( \lambda ), nm</th>
<th>Fluorescence Intensity Before Treatment</th>
<th>Fluorescence Intensity After Treatment</th>
<th>Recovered Fluorescence</th>
<th>Absorbance Before Treatment</th>
<th>Absorbance After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>285/335</td>
<td>97</td>
<td>3</td>
<td>90</td>
<td>0.75</td>
<td>0.12</td>
</tr>
<tr>
<td>350/450</td>
<td>19</td>
<td>1</td>
<td>17</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>401/470</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>425/490</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>Not Available</td>
<td></td>
</tr>
</tbody>
</table>

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HA would appear more suitable for sample pretreatment in the immunoassay for low molecular weight solutes since a significant reduction was also achieved at other higher wavelengths; and the potential for automation is high.

3.4.3 Comments on the Background reduction procedures

As far as is known, adsorbents have not been studied in connection with the reduction of serum fluorescence. The approaches adopted in this work involved sample treatment using the adsorbent in the column mode. Such operations, though open to criticism on the grounds that they complicate assays and inhibit automation are not uncommon in clinical assays.

Hydroxyapatite is seen to remove protein material from serum/plasma samples, hence bringing in a dramatic reduction of the fluorescence background and scattered light signals. Of the two possible approaches that can be adopted, the sample pretreatment procedure seems promising for immunoassay work (for low molecular weight solutes). Practically non-fluorescent 'protein-free' solutions can be obtained by HA adsorption, and after a relatively simple change in the buffer conditions (ionic strength), these solutions would be suitable for use in immunoassays without further treatment. The second approach where HA can act as a separating agent in immunoassay appears less attractive. It cannot be applicable for macromolecules (it may appear that for macromolecular analytes, simple dilution will in any case reduce/overcome the background problem, this is not absolutely true since certain proteins e.g. a-fetoprotein are present in low concentration; thus for such analytes neither HA treatment nor dilution would be suitable) and for each low molecular weight solute (e.g. T3, T4) binding properties and equilibria must be studied.
Adsorption of particularly albumin from serum/plasma may well interfere with the binding of antibody-antigen complexes on HA; other proteins will be involved in specific instances e.g. thyroxine-binding globulin will cause interference in the immunoassay for T3 and T4. The possibility of using a blocking agent to resolve this problem in the development of a fluorescence enhancement immunoassay for serum triiodothyronine proved unsatisfactory (Chapter 5). The interference of some component present in plasma has been reported to prevent adsorption of antibody-bound steroid by hydroxyapatite; this approach is thus claimed to be impractical for radioimmunoassay of steroids in unextracted plasma (Trafford and Makin, 1980).

Blue Sepharose has shown to be useful in indicating/removing background fluorescence associated with albumin. However, the application of this procedure to "real immunoassay" would be unsuitable on the grounds of inefficiency when used for sample pretreatment; since Blue Sepharose does not bind albumins from other species so avidly as human albumin, the use of this adsorbent as a separating agent would be complex if not impractical.

Other immunoadsorbents, immobilised lectins, or a suitable combination of the two types might well be capable of removing most of the proteins from serum, but this approach will probably be more costly and complex than the use of the apparently efficient hydroxyapatite method.

One innovative approach using lifetime measurements to remove fluorescence background signals is based on the hypothesis that a characteristic label can be used, whose lifetime is much longer than that of the endogeneous background.
Although the more recent advent of pulsed xenon lamps has simplified measurements, in practice this use of fluorescence lifetimes to develop a homogenous assay will be problematical: even a single fluorophor does not always yield a single exponential decay of fluorescence, and it seems likely that this problem would greatly complicate the deconvolution of decay curves in an immunoassay. The second, and so far more enthusiastically explored possibility is to utilise lanthanide ions as labels (Weider, 1978; Soini and Hemmila, 1979, 1982; Leif et al., 1977; and Frank and Sundberg, 1981), e.g. terbium and europium chelates. Not only do they have suitable lifetimes (ca. 0.3 ms); they also emit intense fluorescence at wavelengths where serum background is generally low in any case, and have narrow spectral bandwidths, which would also aid background rejection. Nonetheless, difficulties in the practical application of lanthanide ions in fluorescence immunoassay remain in the preparation of labelled reagents and in their stability in assay systems. (Very recently, a time resolved fluorescence immunoassay utilising a europium chelate for human IgG has been described (Marshall et al., 1981).) A third approach, which would seem ideal, involves use of a "triplet state" organic label (reactive) having lifetime in the millisecond range. Such "triplet state immunoassays" appear feasible (Chapter 7) and worthy of further study since they are likely to present a relatively straightforward solution to the fluorescence background problem.

A further method of minimising background signals is via the use of chemically-excited fluorescence, since the instrumentation involves no primary light source, stray and scattered light problems should be absent.
However, in chemiluminescence immunoassays (homogeneous) other indirect sources of background interference also exist (survey of Thore, 1979) and identification of any interfering compounds may present some special problems because of the difficulty in obtaining "Chemiluminescence spectra" (transient).

Despite the general value of derivative and synchronous spectroscopy, this approach would suffer from the disadvantage that by no means all instruments provide the necessary facilities and that a spectrum needs to be scanned for each sample; automation would thus be difficult. Preliminary work on serum samples also points out that in practice the $\Delta \lambda$ values used would be small for removing scattered light peaks and with larger $\Delta \lambda$ values, the high wavelength (350/450 nm) interference is noticeable, and a possible combination of two $\Delta \lambda$ values would even further complicate the approach for background elimination in immunoassay.

The other methods for tackling background problems as mentioned in Section 3.1 are in some ways beset with difficulties, particularly with the complexity and cost of instrumentation involved.

### 3.4.4 Other types of interference

It is important to realise that the presence of direct, positive background signals imposing an inferior limit of detection is only one problem liable to arise in homogeneous assays. At least two other types of interference may occur, (i) inner filter effects and (ii) quenching and enhancement effects of exogeneous components, buffer salts etc.

Inner filter effects are extremely common in routine fluorimetry largely because many workers take insufficient precautions against them. These effects are optical artifacts and the principal precaution to be observed if the conventional right-angled optics
are to be used involves keeping the absorbance of the sample as low as possible (Parker, 1968). The spurious reduction in observed fluorescence due to inner filter effects was examined on serum samples of 10 and 100-fold dilution (Table 3.1). These observations suggest that, as a rough guide, an average sample of normal serum should be present at a dilution of ca. 50-fold for total inner filter errors in the fluorimetry of commonly used labels to be negligible.

The inner filter effects can be reduced by removing the absorbing impurities using hydroxyapatite. (This treatment reduces the absorbance of normal serum by ca. 60% over a wide wavelength range (see Fig. 3.14). Alternatively, the sample may be diluted but at some cost in terms of sensitivity. Very strongly absorbing solutions may be studied using an alternative optical arrangement—frontal illumination (see Parker, 1968) but this method also has disadvantages and is not widely used even though most fluorimeters will provide the necessary facilities with minimum modification.

Besides these optical artifacts, other phenomena of quenching and enhancement are well known in fluorescence spectroscopy. The effects can be general (e.g. halide ions are quenchers of many fluorescence signals), while others are quite specific (e.g. quenching of tyrosine fluorescence by phosphate ions).

Another type of interference arises from non-specific interactions with exogenous components causing partial fluorescence enhancement. In practice it seems impossible to cater for all such possibilities, except by minimising the number of solutes present when the fluorescence measurements are made.
Figure 3.14

Serum pretreatment using hydroxyapatite: effect on absorbance.

---- shows absorption spectrum of a 50-fold diluted T3/T4 depleted serum sample.

----- shows the absorption spectrum of a similarly diluted T3/T4 depleted serum sample eluted through a 2.5 ml. HA column.
3.5 Conclusion and Comments

The studies enabled to see the nature and indicated the extent of background fluorescence interference arising from serum (plasma) samples. Four emissions were observed; the 285/335 nm band ascribed to proteins extended to ca. 440 nm but is less likely to cause severe interference in the fluorimetry of the commonly used fluorescent labels. The second, 350/440-460 nm band extended to ca. 580 nm, this emission was of major concern since it would interfere with most of the fluorescent labels used in fluorescence immunoassay methods. This finding was noted with all the serum/plasma samples examined, and its occurrence was seen to be largely associated with albumin (gel chromatography of serum/plasma on Sephacryl S-300 provided stronger evidence), though other combined sources deemed possible for this signal. Commercial human serum albumin showed a similar emission, defatted albumin signal (350/450 nm) was half as intense; it thus seemed that peroxidation of albumin bound solutes (e.g. lipids) or other co-existing solutes could further (additionally) be responsible for this high wavelength fluorescence. The other two higher wavelength bands, 401/470 and 425/490-500 nm were also observed, substantial overlapping Raman scatter from the solvent was also present; such "combined" signals can cause severe interference in homogeneous fluorescence assay utilising labels such as fluorescamine (see Chapter 5). The fluorophors responsible for these emissions are not known, however, the magnitude of the 425/490-500 nm interference would be comparable with elevated bilirubin.
The adsorbents, hydroxyapatite and Blue Sepharose work allowed to 'see' remove background signals; hydroxyapatite treatment brought a substantial reduction of fluorescence background at all the wavelengths tested. Blue Sepharose preferentially removed albumin fluorescence, this observation strongly suggesting that the 350/450 nm interference was caused by solutes protein in nature, the other remaining fluorescence could be from covalently bound solutes to albumin or photo-oxidation products etc. It is certain that differences will exist between serum samples, mainly due to dietary factors, drug administration in disease states, vitamin supplementation, bilirubin, protein binding of other low molecular solutes in the sample, enzymes and co-factors, as a result the unequivocal identification of especially the 350/450 nm emission with a single pure compound would be difficult.

In summary, it seems very likely that homogeneous fluorescence immunoassays at levels below ca. 10nM will only succeed if (a) labels are chosen so that absorbance interference, scattered light and fluorescence background are all minimised and possibly also (b) as many co-existing solutes as possible are removed by sample pretreatment.

An 'ideal' label for fluorescence immunoassay work needs yet to be identified or developed. On the basis and consideration of the above findings (observations) a suitable label would be that which absorbs at a wavelength ca. 500 nm (inner filter effects negligible: see Table 3.1) and fluorescence wavelength ca. 600 nm (background fluorescence signals from sample will be absent) although a limitation may be imposed by the photomultiplier sensitivity of the fluorimeter.
This label would have a larger Stokes' shift thus removing the scattered light interference. One 'recent' label, Lucifer yellow (or its derivatives, Stewart, 1978) with λEx. ca. 430 nm and λFl. ca. 530 nm possesses this property but endogeneous serum interference might still set a sensitivity limitation at the 430/530 nm emission. The ideal label is of course expected to be suitable in other respects for immunoassay work; there should then be no need for sample pretreatment (if at all necessary, the hydroxyapatite adsorption technique is simple and efficient in removing background signals over a wide wavelength range, though specific - but see Chapter 5) and the limit of detection achievable by homogeneous fluorescence assay would be comparable with the radioimmunoassay technique. Also, in the longer term, technical developments such as the use of laser light sources and lanthanide ion containing labelling reagents may produce additional benefits.
4.1 Introduction

The determination of iodinated amino acids (tyrosine and thyronine derivatives) is of great importance because of the use of these compounds as indicators of thyroid function in clinical chemistry. Determinations of thyroxine (3,3',5,5'-tetraiodothyronine, T4) and 3,3',5-triiodothyronine (T3) are particularly frequently required in blood serum at ng. ml⁻¹ and lower levels. The need for analytical methods combining sensitivity and specificity has restricted the number of useful techniques: in practice either high performance liquid chromatography (h.p.l.c.) or competitive binding immunoassays have been used almost exclusively. Fluorescent derivatives of the analyte molecules are of value in conjunction with both these techniques. In recent years two unusual fluorogenic reagents have been introduced for the determination of amino acids and other primary amines; ortho-phthaldehyde (Roth, 1971) and fluorescamine (Wiegele et al., 1972). Fluorescent labels (Soini and Hemmila, 1979; O'Donnell and Suffin, 1979; Williams, 1980) have been studied in several laboratories and a number of simple systems for determining proteins and hormones have appeared, and in many cases the fluorescence properties of the label group has changed when the labelled molecule is bound to an antibody, so giving rise to an easily-automated homogeneous assay. Fluorescence immunoassays for T4 (Smith, 1977; Technicon, [Assignee], 1978; Handley et al., 1979) have been described using fluorescein and fluorescamine as the fluorescent labels.
Smith found that the fluorescence of the fluorescein moiety of the fluorescein-labelled thyroxine derivative was substantially quenched by the iodine atoms of T4, presumably through the "heavy atom" effect (Zander, 1967). This quenching was reversed when the labelled T4 was bound to anti-T4 antibodies, the fluorescence being enhanced approximately 3-fold. Handley (1979) has shown that a similar assay for T4 could be developed using the two closely related fluorogenic labels fluorescamine (Weigele et al., 1972) and methoxydiphenyl furanone (MDPF, Weigele et al., 1973). The mechanisms of such enhancement effects are described in the next chapter.

In the light of these results it was apparent that the fluorescence properties of o-phthaldehyde (OPT) derivatives of iodinated amino acids would be of great interest. This reagent forms highly fluorescent derivatives with primary amines and amino acids in the presence of thiols (Roth, 1971). OPT has received less attention as compared with fluorescamine, and its use in fluorimetric studies e.g. fluorescent probe applications has been inhibited by the lack of knowledge of the chemical nature of the reaction and the instability of the fluorescent products (Chen, 1979). However, the structures of the products have recently been elucidated (Simons and Johnson, 1978).

\[
\text{OPT + HS-CH}_2\text{-CH}_2\text{-OH + H}_2\text{N-CH}_2\text{-OH} \rightarrow \text{amino acid}
\]

\[
\text{0-phthaldehyde 2-mercaptoethanol}
\]

\[
\text{postulated product}
\]
OPT has been used in the determination of non-iodinated amino acids by h.p.l.c.-fluorimetry (Hill et al., 1979; Lindroth, 1979) and the properties of OPT derivatives of such amino acids have been studied (Chen, 1979); the fluorimetric applications of amino acid analysis with OPT have also been described (Lee and Drescher, 1978). It is claimed that the chemical yields in OPT-amino acid reactions are higher than those of fluorescamine-amino acid reactions, and that the OPT products offer up to ten times more fluorimetric sensitivity (Benson and Hare, 1975).

This chapter describes the preparation and properties of OPT derivatives of several iodoamino acids and a comparison with those of glycine and tyrosine. The feasibility of general fluorescence enhancement (or quenching) immunoassays of the types developed with fluorescence and fluorescamine as labels were investigated for OPT derivatives of T3 and T4.

Some studies were made to examine the possibility of using dansyl chloride as a fluorescent label for assaying T3 and T4. Also the suitability of Lissamine rhodamine B chloride as a potential labelling agent was briefly investigated for human serum transferrin in these studies.

4.2 Experimental

Stock solutions (1mM) of amino acids were prepared in nitrogen purged 0.1M borate buffer (of highest grade and made up in water triply distilled from silica and passed through a 0.2 µm filter (Amicon, High Wycombe), pH 10, and stored at 4°C. OPT/ethanethiol and OPT/2-mercaptoethanol solutions were made up afresh whenever possible, but could be stored for short periods in the dark at 0°C.
Figure 4.1

Structures of some iodinated amino acids

Tyrosine

3-iodo-L-tyrosine

3,5-diiodo-L-tyrosine

3,5-diiodo-L-thyronine

3,3',5-triiodo-L-thyronine (T3)

3,3',5,5'-tetraiodo-L-thyronine (T4)
they contained 1mM OPT and 2mM thiol in borate buffer, in some experiments the OPT/thiol reagent was made up in absolute (spectro-grade) ethanol. The amino acids were labelled as described in Section 2.3.2.3. The fluorescence quantum yields ($\Phi_f$) of the OPT-amino acids were determined by the comparative method, with the OPT derivative of glycine as standard ($\Phi_f = 0.39$, Chen, 1979). The reaction mixtures were kept on ice to minimise the destruction of the thiol reagents.

The dansyl-T3 and T4 (Section 2.3.2.1) and the Lissamine rhodamine transferrin conjugates were prepared as in Section 2.3.2.2; the dansyl derivatives were freeze dried and the transferrin conjugates were stored in the dark at 4°C. All dilutions were made in 0.01M phosphate buffer, pH 7.4, containing 0.145M NaCl.

In all fluorimetric measurements, corrections were made for the background fluorescence from the buffer, added antibodies, and from OPT/thiol reagent and 0.5M NaOH in the OPT work.

4.3 Results and Discussion

4.3.1 OPT-iodinated amino acid derivatives

The reaction of the amino acids with o-phthalaldehyde was rapid, the fluorescence rising to a maximum intensity after ca. 120 seconds, and decreasing slowly thereafter (Fig. 4.2). As previously reported (Simons and Johnson, 1977), ethanethiol (ET) yielded more stable derivatives than 2-mercaptoethanol (MERC): see Fig. 4.2. Several workers have noted the instability and the background fluorescence problems with the OPT reagent (e.g. references: Reeder et al., 1978; Fourche et al., 1976). Ammonium ions seem to react with OPT and have been suggested as a source of interference with reagents (Mendez and Gavilanes, 1976), this observation was largely confirmed by Shute (1980)
Figure 4.2

Reaction of OPT with some iodinated amino acids

![Graph showing reaction of OPT with some iodinated amino acids.]

- X OPT-glycine
- O OPT-MER glycine
- ▲ OPT-tyrosine
- □ OPT-3-iodotyrosine
- ◊ OPT-3,5-diiodothyronine
- ■ OPT-T4

(Final dilution was in 0.5M NaOH; see Fig. 4.3)

ET, ethanethiol was used as the thiol reagent unless otherwise specified.

MERC, 2-mercaptoethanol
who found that ammonium ions and also Decon detergents react with the fluorogenic reagent giving fluorescence spectra similar to that of OPT-glycine derivatives. It was apparent from Fig. 4.3 that the instability of the OPT reagent was associated and depended on the solvent. Purity of water was an important requisite, however, the background was significant and appeared to be erratic (with time) when the OPT-thiol reagents were made up in ultra-filtered water and borate (or phosphate) buffer, pH 9.2. The use of ethanolic reagents reduced the background considerably but such conditions were found not to be ideal for the fluorogenic reaction with the iodinated amino acids, particularly, T3 and T4. The stability and fluorescence background problems were minimised by treating the reaction mixture with alkali. Sodium hydroxide (0.5M) evidently reduced the blank contribution from the reagent as well as from any ammonium ions that may be present (in the borate salts); this observation has been reported by Butcher and Lowry (1976). Moreover, the fluorescence due to the reagents was stabilised over longer periods (Fig. 4.3).

It is noteworthy that very recently "Fluoraldehyde", an o-phthaldehyde reagent preparation stable for one year, has been formulated and is available commercially (Pierce and Warriner (U.K.) Ltd., Chester).

While the excitation (maximum at 339 nm) and fluorescence (maximum at 454 nm) spectra of all the OPT derivatives were very similar (Fig. 4.4), their fluorescence intensities varied substantially. The quantum yield values in Table 4.1 showed clearly that iodine atoms strongly quench the fluorescence. A single iodine atom exerted a major quenching effect, with further iodine atoms producing additional, though smaller reductions in quantum yield. This behaviour was in complete contrast to that of fluorescamine derivatives of the iodo-amino acids, in which the quenching effect of the iodine atoms was small (Reiterer et al., 1978).
The effect of solvents on the stability and background fluorescence from OPT-thiol reagents

- OPT-ET in borate buffer, pH 9.2, diluted in 0.5M NaOH
- OPT-MERC in borate buffer, pH 9.2, diluted in 0.5M NaOH
- OPT-ET in absolute ethanol (Spectrograde)
- OPT-ET in borate buffer, pH 9.2, nitrogen purged
- OPT-ET in ultrafiltered tridistilled water
- OPT-ET in borate buffer, pH 9.2

The borate buffer and sodium hydroxide were made up in ultrafiltered tridistilled water.
A corrected excitation and B fluorescence spectra of several OPT-amino acid derivatives (2 x 10^{-5} M in 0.5M NaOH).

Excitation bandwidth 8 nm, emission bandwidth 12 nm.

B  Fluorescence spectra
1  OPT-glycine
2  OPT-tyrosine
3  OPT-thyronine
4  OPT-3,iodo-tyrosine
5  OPT-3,5-diiodo-tyrosine
6  OPT-3,5-diiodo-thyronine
7  OPT-T3
8  OPT-reverse T3
9  OPT-T4
10 Blank OPT-Ethanethiol

A  Excitation spectra
1  OPT-glycine
2  OPT-tyrosine
3  OPT-thyronine
4  OPT-3,iodo-tyrosine
5  OPT-T3
6  OPT-T4
Figure 4.4
Table 4.1

Fluorescence quantum yields of OPT derivatives with ethanethiol as the thiol reagent

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantum yield (φf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.340 ± 0.015 (3)</td>
</tr>
<tr>
<td>Thyronine</td>
<td>0.230 ± 0.007 (2)</td>
</tr>
<tr>
<td>3-Iodotyrosine</td>
<td>0.060 ± 0.005 (3)</td>
</tr>
<tr>
<td>3,5-Diiodotyrosine</td>
<td>0.050 ± 0.002 (3)</td>
</tr>
<tr>
<td>3,5-Diiodothyronine</td>
<td>0.030 ± 0.002 (3)</td>
</tr>
<tr>
<td>3,5,3'-Triiodothyronine (T3)</td>
<td>0.023 ± 0.002 (3)</td>
</tr>
<tr>
<td>3,3',5'-Triiodothyronine (reverse T3)</td>
<td>0.020 ± 0.001 (3)</td>
</tr>
<tr>
<td>3,3',5,5'-Tetraiodothyronine (T4)</td>
<td>0.014 ± 0.001 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference value (Chen, 1979). Figures in parentheses indicates the number of determinations.
It was also noticeable that the hydroxylated aromatic ring in thyroxine exerted a separate quenching effect: OPT-thyronine had a lower quantum yield than OPT-tyrosine, and 3,5-diiodothyronine showed a similar reduction in quantum yield compared with 3,5-diiodo-tyrosine. The effects of the hydroxylated ring and its substituents on the quantum yields of the OPT derivatives were indeed at least as great as those of the tyrosine ring: thus OPT-"reverse T3" and OPT-T3 had similar quantum yields which were lower than that of OPT-diiodothyronine. Model-building studies (Orbit molecular models: Fig. 4.5) showed that OPT-thyronines may adopt conformations in which the hydroxylated aromatic ring was close to the isoindole moiety of the fluorescent group: such conformations would permit π-electron interactions that might be responsible for the observed effects of the hydroxylated ring. Indirect evidence for the short-range nature of the iodine (heavy atom) quenching effect was revealed by a comparison of the OPT derivatives of the proteins human serum albumin and porcine thyroglobulin. These derivatives showed similar fluorescence intensities, (Fig. 4.6), even though thyroglobulin contains >1% by weight of iodine: the iodine atoms are presumably not close to the amino groups at which OPT derivatives are formed. However, one qualitative difference was apparent, OPT-albumin and OPT-thyroglobulin showed maximum emission at ca. 440 nm as compared with 454 nm for amino acid derivatives.

When anti-T3 antibodies were added to the OPT-T3 derivative at pH 9 (neutralisation of 0.5M NaOH carried out prior to the addition of antibodies using 1M phosphoric acid) showed fluctuating increases (though small) in the fluorescence intensity at 454 nm. The observations were somewhat difficult to interpret: it appeared that there could be unreacted OPT present which might be interacting with the antibodies producing spurious fluorescence.
Figure 4.5
Corrected fluorescence spectra of OPT derivatives of (a) human albumin and (b) thyroglobulin, both 10 μg. ml\(^{-1}\) in 0.5M NaOH. Excitation wavelength 339 nm.
In the present studies, equimolar quantities of the reagents (1mM OPT, 1mM amino acids) were used, this does not necessarily ensure a complete reaction, (the stoichiometry of the reaction was unknown) and it has been reported that any excess (or unused) OPT remaining in solution is potentially active (Chen, 1979); further knowledge of the reaction mechanism was desirable. The purification of the OPT-T3 (or OPT-T4) conjugates was apparently difficult, since small molecules were dealt with gel-filtration separation techniques would have been unsuccessful. Furthermore, the OPT-amino acids were stable for shorter periods, thus removal of unused label would not be worthwhile in real practice. Similar observations were noted when anti-T4 antiserum was mixed with OPT-T4, and since the increases were small (ca. 5-10%) and the background from the OPT reagents and the antiserum was assumed to be additive, it was 'concluded' that under the conditions of the reaction, the enhancement of the fluorescence intensity was not significant.

4.3.2 Dansyl derivatives of T3 and T4

The fluorescence spectra of dansyl labelled T3 and T4 were similar (Fig. 4.7), however, the dansyl-T3 derivative appeared to have a higher quantum yield. It was not clear whether the presence of iodine atoms was playing a part, it may be possible that the bulky iodine atoms were causing some sort of steric hindrance.

Addition of appropriate antibodies to either conjugate showed no appreciable change in the fluorescence intensity. The labelled T3 and T4 (of concentrations 50-100 ng. ml⁻¹) were incubated with the specific antibodies for varying periods; no spectral distortion was evident and there was no shift in the emission spectrum maximum. The freeze dried labelled T3 and T4 were stable for over three months, after reconstitution, the samples were found not to
B Corrected Fluorescence Spectra of Dansyl Labelled T3 (---) and Dansyl Labelled T4 (---)  $4 \times 10^{-8}$ M.

A Corrected Excitation Spectra of Dansyl Labelled T3 (---) and Dansyl Labelled T4 (---) $4 \times 10^{-8}$ M.
have suffered any significant loss in the luminescence properties.

4.3.3 Lissamine-rhodamine (LR)-transferrin conjugates

The fluorescence and excitation spectra of a Lissamine-rhodamine-transferrin conjugate are shown in Fig. 4.8. It has been reported that the excitation wavelength of LR conjugates vary according to the degree of labelling (Chen, 1969), however such observations were not found with the various LR-transferrin conjugates (Fig. 4.9). Due to a substantial overlap of the excitation and emission spectra, the changes in fluorescence intensity in the presence of antibodies were monitored at 585 nm using an excitation wavelength of 550 nm. Several conjugates, both heavily and lightly labelled were incubated with anti-transferrin antibodies, a relatively small enhancement in fluorescence was observed. Blank experiments with antibody dilutions alone did not show significant background fluorescence.

Since the magnitude of enhancement was small, it appeared that such increases could have arisen from light scattering or possibly from some non-specific environmental effects. Brief work using synchronous scanning indicated that the former was largely responsible for the spurious fluorescence (Fig. 4.10).

For the stability studies, the Lissamine-rhodamine conjugates were kept in the dark at 4°C. After four weeks in storage conditions the LR-transferrin conjugates were found to be stable. Furthermore, no significant difference in the fluorescence properties between the conjugates was evident. A very slight precipitation of the conjugates was noted with time, and after six weeks, the heavily-labelled conjugate showed some signs of dissociation, i.e., an increase in fluorescence was noticeable.
Figure 4.8

Corrected excitation (Ex) —— and fluorescence (Fl) —— spectra of a Lissamine rhodamine-Transferrin conjugate (F:P 5, 2.2 x 10^{-8} M).

(a) and (b) show the fluorescence spectra of the same conjugate at Ex. 550 and Ex. 530 nm respectively.

Excitation and emission bandwidths were 8 nm throughout.
Figure 4.9

B Corrected Excitation Spectra of LR-Transferrin Conjugates
-.--. F:P 9, ---- F:P 6, —— F:P 2.5

A Ultra-Violet Absorption Spectra of LR-Transferrin Conjugates
--- F:P 3, —— F:P 7.5
A Effect of the addition of anti-transferrin antibodies to Lissamine rhodamine-transferrin conjugate (F:P 5, 2.2 x 10^{-8} M)

--- conjugate + antibodies

--- conjugate alone

--- background from antibodies

B Synchronous scanning of the above solutions using a $\Delta \lambda$ value of 20 nm. Excitation and emission bandwidths of 8 nm used throughout.

(The figure shows only one example, experiments using other Lissamine rhodamine-transferrin conjugates with various antibody dilutions, and also use of different $\Delta \lambda$ values for synchronous scanning showed similar results).
4.4 Conclusion and Comments

It was seen that incorporation of successive iodine atoms in the tyrosine and thyronine structures produced increasing quenching of the fluorescence of the OPT derivatives, presumably by the internal heavy atom effect, without significant changes in the spectral distribution. Addition of anti-T4 antiserum to OPT-T4 (and anti-T3 antiserum to OPT-T3) did not show any change in the fluorescence intensity; similar observation was noted with dansyl-T4 (and dansyl-T3) derivative. The studies demonstrated that o-phthalaldehyde and dansyl chloride cannot be used to develop homogeneous fluorescence immunoassays for T4 and T3. Use of these labels for fluorescence immunoassay work would be difficult in any case, because of the fact that their excitation and emission wavelengths are rather close to serum background fluorescence (see Chapter 3). Lissamine rhodamine-B chloride exhibits high wavelength fluorescence (585 nm) where serum background is negligible, however addition of anti-transferrin antibodies to Lissamine rhodamine-transferrin conjugates produced no significant changes in the fluorescence intensity (using fluorescence emission at 585 nm and excitation wavelengths of both 550 and 535 nm) indicating that this label too is not suitable for fluorescence immunoassay development.
CHAPTER 5

FLUORESCENCE ENHANCEMENT IMMUNOASSAY:
Development of a Homogeneous Immunoassay for Triiodothyronine in Serum based on "Fluram Enhancement" Method

5.1 Introduction

Thyroxine (T4) and triiodothyronine (T3) are important thyroid hormones and their levels in serum have been recognised as important indicators of thyroid status (Evered, 1974). The therapeutic T4 and T3 concentrations in serum are ca. 100 ng. ml⁻¹ and 1 ng. ml⁻¹ respectively. Highly sensitive techniques are required for determining such low levels of the hormones. A direct serum assay is normally subject to interference by the presence of T3 and T4 binding proteins (e.g. thyroxine-binding globulin, albumin) in serum making T4 and T3 determination difficult. At present radioimmunoassay procedures are used for thyroid hormone assay. This work evaluates the sensitivity of a fluorescence immunoassay for T3 determination in serum.

"Fluram Enhancement Phenomenon"

In recent years a number of homogeneous fluorescence immunoassays have been described (O'Donell and Suffin, 1979; Soini and Hemmila, 1979; and Smith et al., 1981). Such assays utilise a change in the fluorescence properties of a labelled antigen or hapten that occurs when it combines with an appropriate antibody. Methods of this type include fluorescence enhancement (Smith, 1977) and quenching (Shaw et al., 1977) assays, energy transfer assays (Ullman et al., 1976), and fluorescence polarisation (Watson et al., 1976) and protection (Nargessi et al., 1979) assays.
Since such analyses do not require the separation step that is characteristic of radioimmunoassays, they are readily automated, for example by flow injection analysis (Lim et al., 1980(b)). The first fluorescence enhancement assay was described by Smith (1977) who showed that thyroxine (T4) could be determined at nanogram levels with the aid of a fluorescein-labelled thyroxine derivative. It was found that the fluorescein derivative of thyroxine was markedly less fluorescent than other fluorescein-amino acid conjugates (Kawauchi et al., 1971); the fluorescence of fluorescein-T4 was, however, enhanced on binding to anti-T4 antibodies. It was suggested that the iodine atoms of T4 quenched the fluorescence of the fluorescein conjugate via the heavy-atom effect, the effect being in some way reduced or abolished in the presence of specific antibody.

The studies of Handley (1979) have shown that a similar assay for T4 could be developed using the more convenient fluorogenic label, fluorescamine (Fluram: {4-phenylspiro[furan.2(3) -l'-phthalan]-3,3'-dione}) (Neigele et al., 1972). The resulting fluorescent T4 derivative, which is more readily prepared than fluorescein derivatives, showed 4-fold enhancement of fluorescence on combination with anti-T4 antibodies. It was first assumed, by analogy with the results of Smith (1977), that the reversal of a heavy atom effect was also responsible for these enhancements. Later work, however, indicated that a different mechanism possibly involving an environmental effect on the fluorophore is operating. A more likely explanation (Froehlich and Murphy, 1977) is that fluorescamine derivatives form exciplexes in aqueous solution, with a consequent fluorescence quenching effect. When the derivatives are in partly organic media or bound to antibody combining sites, such
exciplex formation is reduced or abolished, so the observed fluorescence is enhanced. (See Handley, 1979.) Moreover, this mechanism is valid for a wide variety of fluorescamine and also its closely related analogue, [2-methoxy-2,4-diphenyl-3(2H)-furanone] MOPF (Weigele et al., 1973) derivatives, thus permitting fluorescence enhancement immunoassays to be developed for a large number of high- and low-molecular weight species. In addition to T4, enhancement effects of similar magnitude have been found using transferrin, nortriptyline, human serum albumin, human IgG and human IgA as antigens (Lim, 1980): no fluorescamine derivatives that do not exhibit the enhancement effect have yet been identified.

Both fluorescamine and MOPF react rapidly with primary amino groups to yield fluorescent products, any excess label is readily hydrolysed to non-fluorescent products so the separation to remove unreacted hydrolysed products from the labelled conjugates is optional (Handschin, 1976).
This chapter attempts to extend the utility of the "Fluram enhancement phenomenon" for the determination of T3 in serum using fluorescamine as the fluorogenic label. Some fluorescence properties of the Fluram-T3 derivative were also studied. The development of the homogeneous fluorescence enhancement assay is described; the detection limit was affected by the overlapping Raman scattering band and efforts to overcome the problem included the use of derivative spectroscopy and the blank subtraction facility available on the spectrofluorimeter. Some preliminary studies were made where the hydroxyapatite procedure (for background elimination: see Chapter 3) was also used in the Fluram-T3 immunoassay.

5.2 Experimental

Quantitative fluorimetric measurements were made in thermostatted 10 mm. silica cuvettes using the MPF-44B spectrofluorimeter. All fluorescamine derivatives of the amino acids were made up fresh when necessary (for method see Section 2.3.2.3(b)), fluorescamine-T3 conjugate was stored in the dark at 4°C for stability monitoring. All the dilutions were made in 0.075M barbital buffer, pH 8.6, for studies in T3/T4 depleted serum, the serum was diluted 50-fold in barbital buffer containing known concentrations of T3. The antibody dilution graph was constructed by adding varying dilutions of anti-T3 antiserum to fluorescamine-T3 (150 ng. ml⁻¹) and incubation in the dark for 20 minutes, and for standard analytical curves, the anti-T3 antiserum was added to the mixture of labelled and unlabelled T3 followed by incubation in the dark. All the spectral measurements were performed in the corrected mode, the "(M-S)" blank subtraction facility incorporated in the DCSU-2 unit was used to subtract the background signals from the buffer, antiserum and serum samples.
The subtraction procedure was as directed by the manufacturer (Perkin-Elmer Ltd., Beaconsfield).

5.3 Results and Discussion

The fluorescamine(Fluram)-T3 derivative was readily prepared by the sequential addition of molar excess of the fluorigenic reagent to T3. The corrected excitation and fluorescence spectra of Fluram-T3 are shown in Fig. 5.1. Fig. 5.2 shows the spectra obtained for the Fluram conjugates of thyronine, reverse T3 and T4, all of equal concentration. Apart from very small differences in the fluorescence intensity, these Fluram derivatives showed identical spectra with an emission maximum of ca. 490 nm. It was apparent that the quantum yield of Fluram-T4 was not significantly lower than the quantum yields of Fluram derivatives of the above amino acids. Reiterer et al. (1978) likewise found that the Fluram derivatives of both T3 and T4 were not quenched by a heavy atom effect. Handley (1979) obtained indirect confirmation of the absence of a heavy atom effect by studying the effects of potassium iodide on equimolar solutions of Fluram, fluorescein and rhodamine derivatives of glutamic acid. The fluorescence of the fluorescein derivative was found to be quenched by 80% and that of the rhodamine derivative by 40% but the Fluram-glutamic acid fluorescence was not significantly affected.

The fluorescence intensity of the Fluram-T3 derivative was found to depend on environmental factors such as temperature and light. The fluorescence decreased approximately in linear fashion by ca. 55% over a temperature range 4°-40°C. The effect of light on the Fluram-T3 conjugate is shown in Fig. 5.3. The fluorescence declined with time; exposure to excitation radiation caused considerable deterioration of the conjugate, and daylight also had some effect, the
Figure 5.1

A - Corrected Excitation and B - Fluorescence spectra of a fluorescamine-T3 derivative (500 ng. ml$^{-1}$)

Bandwidths 8 nm
Figure 5.2

Fluorescence spectra of Fluram derivatives of: —— T3, -.-. reverse T3, —— T4, -x-x DL-thyronine (all of 150 ng. ml⁻¹).

Excitation λ 390 nm; bandwidths 8 nm

Figure 5.3

Effect of light on the fluorescence intensity of Fluram-T3

○ Exposure to excitation radiation (390 nm)

Δ Exposure to daylight

(The Fluram-T3 derivative had an initial arbitrary fluorescence intensity of 100).

Excitation λ 390 nm, Fluorescence λ 490 nm; bandwidths 8 nm
Figure 5.2

Figure 5.3
fluorescence falling by ca. 20% in over 60 minutes. The instability would preclude the use of Fluram derivatives in assays requiring a long incubation period. As observed by previous workers (Handschin et al., 1976; Handley, 1979) Fluram conjugates are also of limited stability in solution, but it was found that the Fluram-T3 conjugate (undiluted) when stored for a week in the dark at 4°C, lost ca. 20% of its original fluorescence. The instability, however, was not a serious problem since Fluram-T3 conjugate could be rapidly and simply prepared.

A limitation was imposed on the detection sensitivity of Fluram-T3 by the overlapping solvent Raman band, and additional background contribution from buffer in the 470-500 nm region, such interference being largely dependent on the buffer used, barbital buffer, 0.075M, pH 8.6, was generally found to give small background signals. A dilute solution of Fluram-T3 (<100 ng. ml⁻¹) exhibited a broad fluorescence band with a sharp Raman scattering band superimposed on it (Fig. 5.4). Attempts to use the derivative spectroscopic technique (Green and O'Haver, 1974) were unsuccessful in resolving the overlapping bands. The second derivative spectrum showed the Raman band clearly but the fluorescence band was scarcely detectable (Fig. 5.4A), because the effectiveness of derivative spectroscopy is an inverse function of the bandwidth of the zero-order spectrum. Better detection limits were achieved by blank subtraction (see Experimental Section), Fig. 5.4B, 10 ng. ml⁻¹ Fluram-T3 could just be determined in pure solution i.e. barbital buffer (a concentration of 50 ng. ml⁻¹ was used in the subsequent work).

The effects of addition of a series of anti-T3 antiserum dilutions to the Fluram-T3 conjugate are shown in Fig. 5.5. The enhancement effect for the Fluram-T3 derivative was substantial, being ca. 3-fold in the presence of a large excess of antibody.
Use of subtraction facility (on DCSU-2) to remove overlapping Raman peak from solvent.

Fluram-T3 spectrum is overlapped by Raman peak

Fluram-T3 spectrum with the Raman overlapping removed (i.e. subtracted)

Background signal from solvent (i.e. barbital buffer)

Excitation $\lambda$ 390 nm; bandwidths 8 nm

Zero-order spectrum of Fluram-T3 ($\lambda_{FL} = 490$ nm) is overlapped by a Raman peak ($\lambda = \text{ca.} 440$ nm). In the second-derivative (DCSU-2) spectrum, only the Raman signal is clearly defined.

Excitation $\lambda$ 390 nm
Figure 5.6

Standard graphs of fluorescence enhancement assay of T3 in (○) barbital buffer and (●) T3/T4 depleted serum. Reagent mixture without added unlabelled T3 has arbitrary fluorescence intensity of 100 (labelled T3 50 ng. ml⁻¹). Error bars represent standard deviation. Ex. λ 390 nm, F1. λ 490 nm

Figure 5.5

Antiserum dilution graph

(Pooled results of four experiments)
Figure 5.5

Antiserum Dilution

Figure 5.6

Triiodothyronine Concentration
No significant shift in the emission maxima at 490 nm was evident. When a 100-fold diluted standard human serum (in barbital buffer) was added to the Fluram-T3 conjugate a small increase (<3%) in the fluorescence intensity was observed. This might be attributed to the increased viscosity. Non-specific binding of T3 to thyroxine binding globulin (TBG) was likely to be small in barbital buffer (Ratcliffe and Ratcliffe, 1977). The addition of a human immunoglobulin preparation (ca. 10^{-8} M) produced a negligible enhancement effect (<1%), and anti-T4 antiserum similarly produced little change in the fluorescence intensity (i.e. negligible cross-reactivity).

The results confirmed that the Fluram enhancement phenomenon is a consequence of specific antigen-antibody combinations, (cf. Handley, 1979; Lim, 1980) and not of spurious environmental effects on the fluorescence intensity. The incubation period was examined, a maximum fluorescence intensity could be attained within 20 minutes, a slow decrease being observed thereafter.

On the basis of the data obtained from the dilution curve, an antiserum dilution of 100-fold was chosen to construct standard curves. The addition of unlabelled T3 caused the fluorescamine T3-antibody complex to dissociate, and consequently the Fluram enhancement effect to be reversed. Fig. 5.6 shows the standard analytical curves for the determination of T3 in serum and aqueous solutions. It was seen that concentration of T3 at a level of ca. 50 ng. ml^{-1} could be detected (with the aid of the blank subtraction facility), the mean coefficient of variation in aqueous solution being 2% at 50-100 ng. ml^{-1}. The coefficient of variation in the T3/T4 depleted serum (1:50 dilution) was somewhat higher, ca. 6%. Serum itself showed background fluorescence in the 450-500 nm region (see below and Chapter 3).
The sample and the blank could not be observed simultaneously (i.e. the T3/T4 depleted serum + antiserum blank spectrum had to be stored in the microprocessor memory first), small variations in the experimental conditions can make subtraction programs imprecise, this possibly accounts for the high coefficient of variation when the assay was performed in the T3/T4 depleted serum sample.

In serum, the reversed changes were smaller, this effect may be due to residual T3 binding to TBG, although the affinity of T3 for TBG is reported to be small (Gharib and Mayberry, 1972; Robbins, 1975). Non-specific binding of the Fluram-T3 conjugate to albumin is also possible. Thyroxine-binding globulin and albumin interference is also common in thyroid hormone radioimmunoassays (reviewed by Ratcliffe and Ratcliffe, 1977). Such non-specific binding has been effectively inhibited by blocking reagents such as 8-aminoo-l-naphthalene sulphonic acid (ANS) (Eastman et al., 1973), merthiolate (Hufner and Hesch, 1973), or by using alkaline buffers like barbital, pH 8.6.

It is interesting to note that up to 8-fold enhancement of the fluorescence of fluorescein-T4 and T3 conjugates has been produced by appropriate antibody binding. It seemed that part of the increase in fluorescence was due to non-specific protein binding in serum (Ullman, 1981)! It is noteworthy that the uniform behaviour of Fluram derivatives contrasts with the properties of fluorescein-labelled molecules, whose fluorescence signals are sometimes enhanced (Smith, 1977), and sometimes quenched (Shaw et al., 1977) in the presence of antibodies. Recently, Ullman et al. (1980) have developed a fluorescence protection immunoassay for serum thyroxine where non-specific binding of serum components was apparently prevented, and a similar assay for T3 appears promising.
In this study, efforts were made to improve the limit of detection of T3 in serum by using the hydroxyapatite procedure (see Chapter 3), whereby background signals due to serum could be eliminated. It was found that ca. 95% Fluram-labelled T3 in barbital buffer could be recovered after hydroxyapatite treatment, however, problems arose when Fluram-T3 in serum was studied. Apparently, serum proteins were binding some of the T3 and subsequently being adsorbed on HA. Preliminary experiments where ANS was used as a blocking agent appeared unsatisfactory. This possibility was only briefly (indeed) investigated on the belief that any ANS-protein complex will be adsorbed on HA, apparently a broad fluorescence band was observed after HA treatment, in the 450-520 nm region and further work was not pursued.

5.4 Conclusion and Comments

It was seen that T3 labelled with fluorescamine exhibited enhanced fluorescence on binding to the appropriate antibody, the magnitude of enhancement comparable to that found with T4 (Handley, 1979). A homogeneous assay was developed with the limit of detection of T3 being ca. 50 ng. ml\(^{-1}\) in pure solution and ca. 100 ng. ml\(^{-1}\) in serum. Such a sensitivity does not compare favourably with the more common radioimmunoassay method (50 pg. ml\(^{-1}\), Gharib and Mayberry, 1972). The limit of detection was set by background fluorescence (see Chapter 3) and possible serum protein interferences e.g. thyroxine-binding globulin (i.e. T3 binding to TBG). It is possible that the latter effect may be eliminated by the inclusion of merthiolate in the assay medium. The sensitivity of the assay could further be improved by using the hydroxyapatite procedure (Chapter 3) whereby the serum sample will be pretreated and it would be possible to incorporate merthiolate in the assay medium to inhibit the binding
of T3 to TBG; the certainty of complete T3 recovery from HA treated serum samples yet remains to be investigated. An alternative method of overcoming the serum interference would be to use another label which fluoresces well outside the range of serum fluorescence.

Indeed the Fluram enhancement technique is extremely simple (only one straightforward labelling step is required) and would permit the rapid and convenient assay of compounds present in large amounts in the blood/plasma, currently determined by radioimmunoassay and other techniques. This homogeneous assay is amenable to automation, for example by flow injection analysis with merging zone principles (Ruzicka and Hansen, 1978).
CHAPTER 6

CHEMILUMINESCENCE-LINKED IMMUNOASSAY (CLIA):

Development of a homogeneous immunoassay for thyroid hormones using a fluorescent label and chemical excitation via an oxalate ester

6.1 Introduction

Recently, several chemiluminescence immunoassays have been developed; the potential advantages of such assays include sensitivity and simplicity of apparatus. Most chemiluminescence assays have been based on a limited number of chemiluminescence reactions, namely the two reactions based on oxidation of luminol or pyrogallol by peroxide (see Chapter 1) or the enzyme catalysed bioluminescent reactions (Whitehead et al., 1979).

Another more efficient way of achieving chemiluminescence, CL, (as already described in Section 1.2.3, Chapter 1) is via the oxidation of diaryl oxalate esters by hydrogen peroxide in the presence of fluorescent species under study (peroxyoxalate chemiluminescence, Rauhut, 1969). Among the advantages of using this approach in immunoassay (see Chapter 1) is the possibility of developing a homogeneous assay utilising fluorescent labels with a substantial improvement in sensitivity and avoiding background problems arising from biological samples; furthermore, fluorescent labels can be easily coupled to various analytes and the emission wavelengths can be varied by changing the fluorophore. However, all CL assays at the present stage of development are affected by environmental interferences, largely the quenching of CL signals.
The peroxyoxalate CL approach has only recently been applied to immunoassay development (Boguslaski and Carrico, 1980).

Oxalate esters used in peroxyoxalate CL are not soluble in water so the use of a small percentage of organic solvent is inevitable. The use of mixed solvents (giving rise to mixing problems) and the fast reaction kinetics has affected high precision in many CL measurements (Seitz, 1981). Because flow injection methods (Ruzicka and Hansen, 1975, 1978, 1981) provide for rapid reproducible mixing they are well suited for CL measurements (Rule and Seitz, 1979; Burguera et al., 1980) and permit the detection of 'initial light flashes'.

This Chapter describes the utility of peroxyoxalate chemiluminescence (using bis(2,4,6-trichlorophenyl)oxalate, TCPO as the oxalate ester) in immunoassay development. Studies were made to establish whether the effects of specific monoclonal antibodies on CL efficiencies could be used for a homogeneous assay for thyroxine and triiodothyronine labelled with fluorescamine. (Fluorescamine conjugates were used to compare the results with the photoexcitation method, (Chapter 5), and also because they are readily prepared.) All experiments were performed using a combined flow-injection-chemiluminescence system. A luminometer and a fluorimeter (Fluorimet) were both modified for continuous CL measurements. The luminometer system was optimised for good precision with respect to the determination of the limit of detection of fluorescamine labelled T4 and T3. The fluorimeter was largely used for the investigation of its chemiluminescence detection sensitivity using fluorescein. A brief comparison was also made of the limits of detection of fluorescamine labelled T4 in the "photoluminescence and chemiluminescence modes" on the fluorimeter using a static system.
Finally, a preliminary absorption study was carried out to investigate (i) the stability of the reactants (TCPO) in the mixed solvent medium and (ii) any interference attributable to inner filter effects.

6.2 Experimental

6.2.1 Modification of equipment

In all experiments the carrier stream (Hydrogen peroxide) was pumped through polyethylene tubing (2.5 mm i.d.: Altec Ltd., Alton, Hampshire) by a four-channel peristaltic pump (Ismatec Minipump-S-840, Zurich, Switzerland).

A Fluorimet filter fluorimeter (Baird Atomic Ltd., Braintree, Essex) was adapted to make continuous flow chemiluminescence measurements. A standard silica flow cell of 100 μl capacity was positioned close to the detectors; samples (100 μl) were injected manually from disposable syringes with standard hypodermic needles (thickness 0.5 mm, length 10 mm, B-D Plastipac) via a silica septum immediately above the flow cell. The light source of the fluorimeter was removed and all the openings were sealed. (See Fig. 6.1 for a general lay-out of equipment.) An LKB Model 1250 luminometer (Croydon, Surrey) in which the sample cell is very close to the photocathode, (and equipped with a reflector), was also modified to carry out combined chemiluminescence-flow injection procedures. Teflon tubing (0.76 mm i.d., 44 cm in length) flow cell in the form of a coil (supported by an ordinary luminometer cuvette) was constructed in this work. A Rheodyne valve (6-port and 100 μl fixed volume) was fitted above the flow-cell compartment for sample injection. An interference filter (maximum transmission ca. 500 nm) was placed between the flow-cell and the detector to cut out any background light.
Figure 6.1

Experimental layout for the combined flow injection-chemiluminescence measurements. (Only single channel flowing stream used in this work.)

[For a given sample volume, the peak height (i.e. maximum CL) on the recorder trace was proportional to the solute concentration.]
The light emission (CL) was recorded by connecting the fluorimeter and the luminometer to a chart recorder.

The flow rate of the carrier stream and all other mixing times were measured with a stop watch.

6.2.2 Procedures

Sample and reagent preparation:

Fluorescamine labelled T3 and T4 were prepared as in Section 2.3.2.3(b). The TCPO solution was made up afresh (hourly) in acetone (HPLC grade), the dissolution being aided by an ultrasonic bath. Hydrogen peroxide (0.5M) was prepared in tridistilled water and stored in a Teflon container.

Assay procedure:

The assay was performed by mixing the fluorescent conjugate(s) (150 ng. ml⁻¹ in 0.01M phosphate buffer, pH 7.4) with TCPO (5-10 mM in acetone) in standard luminometer glass cuvettes. After rapid, thorough mixing on a vortex mixer and appropriate dilution with the phosphate buffer (2-3 ml.), 100 µl aliquots of the mixture were injected with a syringe in the fixed volume loop of a Rheodyne valve and carried into the flow cell by a hydrogen peroxide flowing stream. The light production was initiated within 2 seconds of mixing and the entire course of light emission was generally recorded within 1-5 seconds interval on a chart recorder. The procedure was optimised with respect to the solvent system (i.e., percentage of acetone in the reaction mixture), pH of the buffer, the concentration of TCPO and also hydrogen peroxide (0.1M - 0.5M), and the flow rate of the hydrogen peroxide stream. Work with proteins involved the addition and incubation of antibodies (etc.) with the fluorescent conjugates before TCPO was added.
Blank experiments without the fluorescent conjugates were carried out to allow for any background signals. All measurements were made at room temperature.

Absorbance measurements of mixtures containing a fixed concentration of TCPO (as used in the assay) and varying amounts of acetone and/or buffer were made at three different wavelengths immediately and at certain time intervals using the Shimadzu 240 spectrophotometer.

6.3 Results and Discussion

Initial work was done with the modified fluorimeter largely to evaluate its sensitivity when used for chemiluminescence measurements. Fluorescein served as the fluorophor. Direct injection into the flow cell permitted as little as $10^{-11}$M fluorescein (5 mM TCPO used) to be detected: Fig. 6.2 shows the characteristic 'flow injection peaks' obtained. When fluorescein-labelled albumin ($F:P = 4$) was injected, a reduction in CL intensity was noted and with a lightly labelled conjugate ($F:P = 1.5$) light was diminished substantially, about $10^{-7}$M fluorescein-albumin ($F:P = 1.5$) could be detected. Background measurements in the absence of fluorescent-conjugates were performed [and was found, for example, to be ca. 40% of the signal obtained for $10^{-7}$M fluorescein-albumin conjugate ($F:P = 1.5$): see Fig. 6.2], it was not clear whether the reagents (TCPO + $H_2O_2$) and/or scattered/stray light was responsible for the background signals. With fluorescamine-labelled thyroxine a limit of detection of ca. 75 ng. ml$^{-1}$ was obtained. With all these measurements the precision was unsatisfactory, the coefficient of variation ranging from 10-15%.
Figure 6.2

Showing the chart trace obtained for fluorescein and fluorescein-albumin (using modified Fluorimet-flow injection system, FFS).

1 Signals from fluorescein (10^-8M)/TCPO (5 mM).
2 Signals from fluorescein (10^-10M)/TCPO.
3 Signals from fluorescein (10^-9M)/TCPO.
4 Signals from fluorescein (10^-11M)/TCPO.
5 Signals from fluorescein (10^-7M, F:P = 4)/TCPO.
6 Signals from fluorescein-albumin (10^-7M, F:P = 1.5)/TCPO.
7 Signals from TCPO/H_2O_2 (background signals).

Samples for 3-7 contained 7% acetone.
Flow rate of H_2O_2 = 35 ml min^{-1}

I_{CL} = Chemiluminescence intensity, arbitrary units,
1 cm = Ca. 30 seconds (horizontal axis).
The manual injection technique used may explain such observations; it is quite possible that the force of injection and the exact positioning of the syringe needle into the septum above the flow cell will vary from measurement to measurement, thus affecting the precision. Moreover, since the reaction kinetics were fast, a controlled mixing of the reactants was desirable but apparently could not be achieved with the system. In a static system, ca. 50 ng. ml\(^{-1}\) fluorescamine-labelled thyroxine could be determined again with poor reproducibility (coefficient of variation ca. 8%). For comparison purposes, the fluorimetric determination of fluorescamine-T4 was performed. Scattered light problems set the detection limit to ca. 150 ng. ml\(^{-1}\). These experiments clearly demonstrated that the CL system was more sensitive and with improvements in the injection technique even better results can be expected.

The luminometer was used for subsequent work (to obtain better sensitivity) to develop homogeneous immunoassays for T4 and T3. In the earlier parts of this work good precision could not be attained despite the use of a fixed volume (100 \(\mu\)l) valve injection (Fig. 6.3). It appeared that the mixing times of the reagents (i.e. addition of TCPO in acetone to the fluorescent conjugate in the buffer and the time taken to inject this mixture into the hydrogen peroxide stream: see later) were responsible for poor reproducibility. As a result, the timing of mixing was controlled by a stop watch which yielded some improvement in precision.

The percentage of acetone (the solvent used to dissolve TCPO in this work) in the final mixture was then investigated. It was seen that the use of 7% acetone produced maximum CL and there was also a dramatic improvement in reproducibility (coefficient of variation...
±4%, Fig. 6.4). The limit of detection of fluorescamine labelled T3 and T4 was ca. 1 ng. ml⁻¹. The reaction kinetics also seemed to vary with different amounts of acetone in the mixture, with the use of ca. 10% acetone, the CL signals (peaks) were displaced in ca. 1.5 seconds as compared to ca. 2.5 seconds with 7% acetone. Also, with 10% or greater use of acetone, the reproducibility was affected and a drop in the CL intensity (by ca. 20%) was observed. However, it should be pointed out that since 'fixed' system conditions are employed, the time elapsing before observation affects intensity (i.e. the peak maximum displayed may not be the true 'reaction maximum').

The system was then optimised with respect to TCPO and hydrogen peroxide concentrations; 5 mM TCPO and 0.5M H₂O₂ appeared optimum. The reported work in peroxyoxalate CL has suggested that increased TCPO concentrations in the reactant mixtures show higher CL yields, but it was found that the use of concentrations > 5 mM quenched the signals and caused larger variations in the CL measurements. It has been noted that excess concentration of TCPO causes precipitation in the flowing stream of H₂O₂ and disturbs the flow rate (Kobayashi and Imai, 1980). The pH of the buffer (only phosphate buffer, 0.01M used in this work for diluting the fluorescent conjugates and TCPO mixtures) was varied and it was found that pH >8 reduced the CL intensity. The effect of the pH and nature of the buffer on the CL intensity and reaction kinetics has been studied by Williamset al. 1976 and Kobayashi and Imai 1980, and Puget et al. (1977) has reported that CL intensity can be increased by organic bases and inhibited by organic acids. High concentrations of various salts and buffers are known to interfere in CL reaction (Thore, 1979).
Figure 6.3

Showing the chart trace obtained for fluorescamine-labelled T4 (using Luminometer-flow injection system, LFS).

1. Signals from TCPO/H₂O₂
   (5 mM)(0.5 M)

2. Signals from TCPO/fluorescamine-T4/H₂O₂
   (5 mM) (13 ng ml⁻¹) (0.5 M)

Flow rate of H₂O₂ stream 5 ml min⁻¹.

Figure 6.4

(LFS used)

1. Signals from TCPO/fluorescamine-T3/H₂O₂
2. Signals from TCPO/fluorescamine-T4/H₂O₂

In both cases the mixtures contained 7% acetone, concentrations of the components as in Fig. 6.3

ICL = Chemiluminescence intensity, arbitrary units
1 cm ≡ ca. 20 seconds (horizontal axis)
(Applicable to all subsequent figures where LFS has been used.)
In this work phosphate buffer of pH 7.4 was found to give satisfactory results. A flow rate of 5 ml min\(^{-1}\) (H\(_2\)O\(_2\) stream) seemed satisfactory.

The final system (i.e. one with which maximum CL intensity could be achieved) consisted of rapid and timed mixing of 5 mM TCPO (volume adjusted to give 7% acetone in the final mixture) to fluorescamine labelled T3 or T4 and immediate injection (within 30 seconds) into the H\(_2\)O\(_2\) stream. If the fluorescamine T4 (or T3) TCPO mixture was allowed to stand for two minutes and then injected into the H\(_2\)O\(_2\) stream, there was a reduction in the CL intensity. On further standing, the intensity of the signal decreased considerably and within ten minutes the background level was approached (Fig. 6.5). It appeared that TCPO was hydrolysing, experiments were performed where TCPO (5 mM in acetone) diluted in buffer was allowed to stand for varying intervals followed by addition of fluorescamine labelled T4 (or T3) and immediate injection into the H\(_2\)O\(_2\) stream. The CL intensity had dropped (Fig. 6.5) and absorbance studies (see Table 6.1) also provided some indication of TCPO hydrolysis. It was also noted that the absorbances at 490 nm (fluorescence maximum for fluorescamine conjugates) of the TCPO and fluorescamine T3 or T4 mixtures in buffer were appreciable. These high absorbances could be due to turbidity; with time the absorption of the product(s) dropped. This observation can be associated with the hydrolysis product which is more soluble in water thus reducing turbidity or alternatively, the particles (of precipitation) were settling at the bottom of the measurement cell, the resultant solutions showing lower absorbance values. It was also not clear whether any inner filter effects were reducing the chemiluminescence signals. Ambiguities due to inner filter effects have been demonstrated in several CL systems (Wampler et al., 1979; Van Dyke et al., 1979, and Lee and Seliger, 1970).
Figure 6.5

Showing the changes in CL intensities from fluorescamine-T4/TCPO mixtures with time.

1 Signals obtained from fresh TCPO/fluorescamine-T4 (~ 26 ng ml\(^{-1}\)) mixture.

2 Signals from TCPO/fluorescamine-T4 mixture allowed to stand for 2 minutes before injection.

3 Signals from TCPO/fluorescamine-T4 mixture in which TCPO in buffer was allowed to stand for 2 minutes followed by addition of fluorescamine-T4 and immediate injection.

4 Signals from TCPO/fluorescamine-T4 mixture allowed to stand for 10 minutes before injection.

5 Signals from TCPO/H\(_2\)O\(_2\) (i.e. background signals).

TCPO concentration 5mM; H\(_2\)O\(_2\) (0.5M) flow rate 5 ml min\(^{-1}\).
Table 6.1

Absorbance values of TCPO and TCPO-fluorescamine T4 mixtures at 490 nm at various time intervals. (Stock TCPO solution was made up in acetone.)

<table>
<thead>
<tr>
<th>Time</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5 mM TCPO in Acetone</td>
</tr>
<tr>
<td>Straight</td>
<td>0.055</td>
</tr>
<tr>
<td>After 2 minutes</td>
<td>0.04</td>
</tr>
<tr>
<td>&quot; 5 &quot;</td>
<td>0.04</td>
</tr>
<tr>
<td>&quot; 10 &quot;</td>
<td>0.05</td>
</tr>
<tr>
<td>&quot; ½ hour</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Samples 3 and 4 contained 7% acetone in the final mixture.
At present, no remedy for inner filter effects has been reported, though Stieg and Nieman (1978) has described an expression for CL in terms of absorbance and pathlength (it must be noted that with flow cells used in this work such expressions would not be applicable), most recently Ratzlaff and Crouch (1982) have developed a correction method for inner filter effects in CL presumably based on static measurements. Thore (1979) has reported that light-absorbing or fluorescent solutes in the sample may affect measurements. Light absorption has been mentioned as a potential cause of diminished light emission in bioluminescence (Brolin et al., 1971).

The possibility of immunoassay development was considered using careful timed conditions. Fig. 6.6 shows the addition of anti T4 antiserum to fluorescamine-labelled T4 subsequently "excited by TCPO and the flowing H\textsubscript{2}O\textsubscript{2} stream". The signals were quenched, the magnitude of quenching depended on the antiserum dilution. When increasing concentrations of unlabelled T4 were added, there was no appreciable change in CL intensity except at very high concentration (ca. 500 ng. ml\textsuperscript{-1} T4) additional quenching was observed. Similar quenching was seen when monoclonal anti T3 antibodies were added to fluorescamine-labelled T3. The CL intensities from fluorescamine-T4 were also quenched by other non-specific proteins (Fig. 6.7) e.g. bovine serum albumin, human serum albumin, various other antibody preparations. A hundred fold dilution of normal human serum produced ca. 50-60% reduction in the CL measurements. Experiments were also performed where various proteins were added to fluorescein, again the CL signals were quenched (Fig. 6.8).

These results demonstrated that the presence of any protein was affecting the peroxyoxalate chemiluminescence where TCPO was used as the required oxalate ester.
Figure 6.6

Addition of anti-T4 antiserum to fluorescamine-T4 followed by TCPO/H$_2$O$_2$ excitation.

1 Signals from ~ 26 ng ml$^{-1}$ fluorescamine-T4/TCPO.
2 Addition of 1:1000 dilution anti-T4 antiserum.
3 Addition of 1:500 " " "
4 Addition of 1:300 " " "
5 Addition of 1:100 " " "

TCPO concentration 5 mM; H$_2$O$_2$ (0.5M) flow rate 5 ml min$^{-1}$.

[In one experiment where anti-T4 antiserum was added to a fluorescamine-T4-TCPO mixture, similar quenching effect was observed.]
Figure 6.7

Addition of various proteins to fluorescamine-T4 followed by TCPO/H₂O₂ excitation.

1 Signals from fluorescamine-T4 (ca. 39 ng ml⁻¹).
2 Addition of 0.1 µg ml⁻¹ human serum albumin.
3 Addition of 0.1 µg ml⁻¹ bovine serum albumin.
4 Addition of 1:200 dilution anti-albumin antiserum.
5 Addition of 1:200 dilution anti-IgG antiserum.
6 Addition of 0.1 µg ml⁻¹ human IgG.
7 Addition of 1:100 diluted standard human serum.

TCPO concentration 5mM; H₂O₂ (0.5M) flow rate 5 ml min⁻¹.
Addition of various proteins to fluorescein followed by TCPO/H₂O₂ excitation.

1. Signals from fluorescein (10⁻⁹M).
2. Addition of 0.1 µg ml⁻¹ human serum albumin.
3. Addition of 0.1 µg ml⁻¹ bovine serum albumin.
4. Addition of 0.1 µg ml⁻¹ human IgG.
5. Addition of 1:100 diluted standard human serum.

TCPO concentration 5mM; H₂O₂ (0.5M) flow rate 5 ml min⁻¹.
It was clear that the quenching effects would vary from one serum sample to another; also use of urine samples would show similar observations although urine contains much smaller amounts of proteins as compared with serum/plasma.

Although the problem of serum interference (largely quenching effects) in CL assays has been reported (Olsson and Thore, 1981) the mechanism of quenching is obscure. The knowledge is limited, but it appears that since a chemical reaction (rate reaction) is used to generate light, any factors that will affect the reaction might affect the light emission. It has been reported (Thore, 1979) that the presence of salts, certain buffers, organic solvents, detergents etc. cause considerable interference (inhibiting effects) in Bioluminescence reactions. CL assays based on luminol have encountered quenching problems (Hersh et al., 1979; Kohen et al., 1980(b)). In peroxyoxalate CL since an additional step of energy transfer from TCPO to the fluorescent species under study is involved (for light generation) further problems are likely. It appears from this work that energy transfer to proteins by TCPO might be causing the reduction in CL intensity. It was originally believed that since proteins absorb at lower wavelengths this interference will be smaller and TCPO will only excite the high wavelength fluorescent labels used in the peroxyoxalate CL reaction. Sherman and Holtzbecker (1978) have reported that the most efficiently excited fluorophors are those with low energy (high wavelength) excitation bands. Since serum largely contains proteins the quenching effect became significant. Alternatively, high absorption (inner filter effects) may be responsible for the reduced CL signals in the presence of serum (or protein material; cf. photoluminescence). Further study on the quenching phenomenon is required (see Section 1.2.3.3 and Chapter 8), at present, the
development of a homogeneous CL immunoassay using the peroxyoxalate approach is not feasible.

6.4 Conclusion and Comments

The chemically excited fluorescence was found to be more sensitive than the conventional photoluminescence approach for the detection of fluorescamine labelled T3 and T4. Fluorescamine conjugates have a considerably low fluorescence quantum yield limiting the assay sensitivity (ca. 50 ng. ml⁻¹ in pure solution) for T3 and T4. In the case of chemiluminescence, < 1 ng. ml⁻¹ fluorescamine labelled T3 or T4 could be determined. Since the reaction kinetics are fast, the precision is affected significantly if the reactants are not mixed in a controlled (i.e. timed) manner. A further improvement in precision can be achieved by using a simultaneous mixing of TCPO and the sample: viz,

This approach would also protect TCPO from hydrolysis.

From the immunoassay development point of view, the homogeneous approach is not feasible because of protein quenching effects. Other esters may not give rise to such interference and a further study is required (see Chapter 8).
CHAPTER 7

FLUORESCENCE ENERGY TRANSFER IMMUNOASSAY:
Evaluation of potential donor-acceptor pair(s)

7.1 Introduction

The phenomenon of energy transfer offers excellent prospects for the development of immunoassays. This effect (Forster, 1948) involves a non-radiative transfer of energy (also known as resonance energy transfer) from one excited fluorescent group (donor) to the ground state of a neighbouring group (acceptor). (It is singlet-singlet energy transfer which is commonly observed, triplet-singlet energy transfer is less common and because of the spin-forbidden transition from the ground to the triplet state of an acceptor, singlet-triplet and triplet-triplet (Dexter, 1953) resonance energy transfer resulting respectively in singlet- and triplet sensitized phosphorescence have very low probabilities and such phenomena are generally not observed.) The applications of fluorescence energy transfer have been reviewed by Steinberg (1971) and Stryer (1978), and Fairclough and Cantor (1978) reviewed the uses of singlet-singlet energy transfer. The donor and acceptor are required to meet several spectroscopic criteria important in a study of energy transfer (Stryer and Haugland, 1967); amongst the necessary conditions are that the fluorescence spectrum of the donor should overlap the excitation spectrum of the acceptor, and that the two groups are very close together: the efficiency of the energy transfer effect varies as the inverse sixth power of the distance between the donor and acceptor groups. In general this means that measurable effects only occur when the donor and acceptor are less than ~5 nm apart, though in favourable cases
energy transfer may occur over somewhat larger distances.

In the simplest form of immunoassay the donor and acceptor labels are attached to the antigen and antibody respectively, and when the labelled molecules are in specific combination, short-range energy transfer effects take place, leading to a reduction in the fluorescence of the donor label and possibly an enhanced fluorescence of the acceptor label. These effects are reversed when unlabelled (sample) analyte competes successfully for the labelled antibody. This principle was first applied by Ullman and co-workers (1976) to the assay for both low and high molecular weight antigens. One main advantageous feature of energy transfer assay is that it is homogenous i.e. no separation step is involved, and it can thus be more easily automated. One practical limitation of the assay, however, is the requirement of the acceptor-labelled antibody reagent to be relatively immunospecifically pure so as to minimise spurious fluorescence quenching due to the inner filter effect of labelled non-specific antibodies.

Most work thus far has utilised fluorescein as the donor group and rhodamine as the acceptor (Ullman et al., 1980; Ullman, 1981). The application of this pair was assessed in a detailed study of energy transfer assays (Lim, 1980) and the disadvantages of these labels enumerated. One other donor-acceptor pair, fluorescamine-fluorescein has been found valuable and highly sensitive assays for both small and large molecules have been developed (Miller et al., 1980). This chapter describes studies of further pairs of labels, in particular involving eosin (2'·4'·5'·7'-tetrabromofluorescein) as an acceptor group (Fig. 7.1).
This group might be expected to have two advantages over rhodamine, the bromine atoms in the eosin nucleus might produce large quenching effects (by the heavy-atom mechanism) on any neighbouring fluorophors, and the eosin group itself may be expected to show delayed fluorescence and phosphorescence (Cherry et al., 1976). [Spectroscopic phenomena associated with the triplet state are well discussed in review forms: Lower et al., 1966; Parker et al., 1980.] Early studies showed the triplet state of eosin is protected from oxygen when the dye is non-covalently bound to albumin (Youtsey and Grossweiner, 1967). More recent work shows that eosin and erythrosin (2',4',5',7'-tetraiodofluorescein) triplet state emissions can be detected with satisfactory sensitivity, especially using laser excitation and that both delayed fluorescence and phosphorescence can be seen; covalently bound eosin also exhibits triplet state phenomena and binding to proteins furnishes at least some protection against oxygen quenching (Garland and Moore, 1979; Jovin et al., 1981). These triplet state phenomena, which have millisecond lifetimes, may facilitate the removal of the short-lived (nanosecond) background fluorescence of blood serum and other biological samples (cf. Chapter 3). Currently eosin isothiocyanate, eosin-5-iodoacetamide, eosin-3-maleimide and eosin-5-thiosemicarbazide are commercially available and other derivatives can be readily prepared (Haugland, 1980). "Triplet state labels" based on eosin and erythrosin derivatives (e.g. erythrosin isothiocyanate, Moore and Garland, 1979) if suitable for covalent labelling and valid in other respects would thus be useful in "triplet state immunoassay".
Eosin Isothiocyanate (acceptor)
\[ C_{21}H_7Br_4NO_5S = 704.6 \]
\[ \lambda_{\text{Ex.}} \sim 520 \text{ nm} \]
\[ \lambda_{\text{Fl.}} \sim 545 \text{ nm} \]

Fluorescein Isothiocyanate
\[ C_{21}H_{11}NO_5S = 389.35 \]
\[ \lambda_{\text{Ex.}} \sim 490 \text{ nm} \text{ (470 nm was used)} \]
\[ \lambda_{\text{Fl.}} \sim 525 \text{ nm} \]

Fluorescamine
\[ C_{17}H_{10}O_4 = 278.27 \]
\[ \lambda_{\text{Ex.}} \sim 390 \text{ nm} \]
\[ \lambda_{\text{Fl.}} \sim 490 \text{ nm} \]

Quinacrine Mustard Dihydrochloride
\[ C_{23}H_{28}Cl_3N_3O.2\text{HCl} = 541.76 \]
\[ \lambda_{\text{Ex.}} \sim 420 \text{ nm} \]
\[ \lambda_{\text{Fl.}} \sim 500 \text{ nm} \]

Figure 7.1 Structures of acceptor and donor labels used.
This work describes the suitability and evaluation of fluorescein and eosin as donor-acceptor labels in an energy transfer assay for human serum albumin. In a search for suitable donor labels, preliminary investigations were also carried out with fluorescamine and quinacrine as donor, eosin being the acceptor in each case. Finally some experiments were performed to examine delayed emissions from eosin-albumin conjugates.

7.2 Experimental

Rabbit anti-human albumin antibodies were labelled with eosin isothiocyanate and human serum albumin with fluorescein isothiocyanate, fluorescamine and quinacrine mustard as described in Section 2.3.2.2. The MPF-44B spectrofluorimeter was used to evaluate the donor-acceptor pair(s); all measurements were made at 25°C after preincubation of the reagents for 20 minutes in the dark. Corrections were made for the background fluorescence contributed by the buffer (0.01M phosphate containing 0.145M NaCl, pH 7.4) and added proteins including labelled antibodies. Absorbances were also measured of individual labelled solutions and of mixtures of labelled antigen and labelled antibody solutions at 520 nm.

For stability studies, the labelled protein solutions were stored in the dark at 4°C and examined for their fluorescent and immunological characteristics over a period of several months.

7.3 Results and Discussion

Substantial overlap of the fluorescence spectra of fluorescein conjugates and the excitation spectra of eosin conjugates was verified (Fig. 7.2).
Figure 7.2

Corrected excitation (Ex.) and fluorescence (Fl.) spectra of albumin-fluorescein, F:P = 1.5:1 (---), and of eosin-antibody conjugate, F:P = 5.5:1 (----).
This is one requirement for efficient fluorescein-eosin energy transfer. Such energy transfer was demonstrated by adding eosin-labelled antibodies to a fixed concentration of albumin-fluorescein conjugate (1.31 nM, F:P = 1.5).

Fig. 7.3 shows the enhancement of eosin at 545 nm and the quenching of fluorescein at 520 nm using an excitation wavelength of 470 nm throughout; the changes of fluorescence intensity were governed largely by the degree of labelling of the antibody molecules. The addition of increasing amounts of lightly labelled antibodies to the albumin-fluorescein conjugate produced increases in the fluorescence intensity at 545 nm and approached a maximum value with excess antibodies. A simultaneous quenching of fluorescein emission at 520 nm though feasible in such assay (Lim, 1980) could not be observed. In control (blank) experiments where unlabelled anti-albumin antibodies were added to the albumin-fluorescein conjugate, a non-specific enhancement (ca. 10%) at 520 nm occurred; addition of unlabelled albumin to the antibody-eosin conjugates showed no significant change in the eosin emission at 545 nm, and the presence of unlabelled antibodies in mixtures containing the labelled antigen-labelled antibodies had very little effect on the fluorescence intensity at either 520 nm or 545 nm.

It appeared from these experiments that the small non-specific enhancement of fluorescein-albumin may be related to and partially offset the quenching of fluorescein at 520 nm due to energy transfer effects. Similar findings were reported by Lim et al. (1980(a)) and Ullman and co-workers (1976) also noted the non-specific enhancement of fluorescein conjugates caused by unlabelled antibodies.
Enhancement of eosin fluorescence (545 nm) and quenching of fluorescein fluorescence (520 nm) when increasing amounts of eosin-labelled antibodies are added to an albumin-fluorescein conjugate (F:P = 1.5:1). The degree of labelling of the antibody is indicated by the n values: \( n = \) moles of eosin per mole of antibody. Pure solutions of fluorescent conjugates have arbitrary fluorescence intensities of 100. Excitation wavelength: 470 nm, excitation and emission bandwidths 12 nm and 8 nm respectively.
Figure 7.3

Relative Intensities

\[ \frac{A_b-E_r}{A_{lb-F_{1.5}}} \]
Enhancements of up to 40% could be seen at 545 nm with lightly labelled antibodies (F:P ~ 4). Addition of heavily labelled antibodies to the albumin-fluorescein conjugate showed a substantial reduction in the fluorescence intensity at 520 nm. These results demonstrated that energy transfer from fluorescein-eosin took place within the labelled antibody labelled antigen complex and that the magnitude of the transfer effects was strongly dependent on the number of molecules of eosin bound to the antibody molecules, with heavily labelled conjugates producing the largest effects especially at high antibody:high antigen (Ab:Ag) ratios. A quenching of <60% at 520 nm was obtained with eosin-antibodies, F:P ~ 10, and Ab:Ag molar ratio ca. 12. More heavily labelled antibodies F:P >12 showed increase in fluorescence background and also any inner filter effects could diminish the magnitude of the energy transfer effects. The quenching of albumin-fluorescein fluorescence by excess labelled antibodies can be related to the greater probability of one or more eosin groups being attached close to antigen binding site: energy transfer is thus more efficient because of the lower mean distance between the fluorescein and eosin groups.

In some experiments where an albumin-fluorescein conjugate with label: protein ratio of 3.5 was used, addition of excess lightly labelled antibodies did not produce any significant changes in the fluorescence intensities at 520 or 545 nm, and with heavily labelled antibodies the changes due to energy transfer were generally low. A possible explanation for these observations is the intramolecular fluorescein-to-fluorescein energy transfer (Pringsheim, 1949, and Chen, 1969) competing with the fluorescein-to-eosin energy transfer effects: since
the corrected excitation and emission spectra of an albumin-fluorescein conjugate (Fig. 7.2) overlap considerably, the fluorescein-to-fluorescein energy transfer is likely to be efficient (Gennis and Cantor, 1972).

It was apparent from these results that an energy transfer immunoassay for albumin based on the fluorescein-eosin pair would have to make use of the enhancement effects because (a) the fluorescence background from lightly labelled antibodies would be small, (b) the background interference from biological samples would be less at a higher wavelength of 545 nm (measurements could be made at ca. 555 nm without too much loss in sensitivity), and (c) the scattered light problems would be less severe as compared to 470/520 nm fluorimetry. Fig. 7.4 shows that as little as $10^{-10}$ M albumin could be determined in pure solution (ca. $10^{-9}$ M in diluted serum) using an antibody conjugate with F:P = 4, and an Ab:Ag molar ratio 6. Addition of human immunoglobulin G to a mixture of eosin labelled antibodies and fluorescein-labelled albumin had no effect on the fluorescence intensities at either wavelength, thus showing the specificity of the assay. Both eosin and fluorescein conjugates were found to be stable in aqueous solution with respect to fluorescence and immunological characteristics under both measurement conditions and when stored in the dark at 4°C over a period of several months.

These results demonstrated that the fluorescein-eosin pair behaved similarly to the fluorescein-rhodamine pair in a conventional energy transfer assay. The magnitudes of the quenching and enhancement were similar, i.e. the presence of the four bromine atoms in the structure (eosin) did not make any appreciable difference; and similar shortcomings (small Stokes' shift leading to direct excitation of eosin acceptor groups, bandwidth effects
Figure 7.4

Changes in the fluorescence intensity at 545 nm when unlabelled albumin (a) in pure solution (-x-) and (b) in standard serum (-I-) is added to mixtures containing $2.4 \times 10^{-9}$ M albumin-fluorescein (F:P = 1.5) and $1.7 \times 10^{-8}$ M antibody-eosin (F:P = 4) λex. 470 nm; bandwidths 10 nm. Error bars are standard deviations for 3 measurements.
(see below) etc.) were apparent (Lim et al., 1980(a)). In efforts to relieve these problems, two further donor labels were investigated, with eosin as the acceptor in each case. Fluorescamine was an obvious choice since (a) its emission spectrum matched the spectral requirement, Fig. 7.5; (b) it has a large Stokes' shift; (c) fluorescamine conjugates are relatively easy to prepare (Chapter 5), and (d) it is possible to compare the efficiencies of the fluorescamine-fluorescein (Miller et al., 1980) and fluorescamine-eosin pairs in energy transfer assay. The efficiency of energy transfer in the fluorescamine-eosin system was assessed by adding eosin-labelled antibody conjugates (F:P = 3 ~ 4) to a fixed concentration of fluorescamine-albumin conjugate (F:P = 5 ~ 6) and measuring the increase in the eosin emission intensity at 545 nm and the relatively small decrease in the fluorescamine fluorescence at 490 nm (excitation wavelength, 390 nm). See Fig. 7.6. The eosin-labelled antibodies showed a 40% enhanced fluorescence signal at 545 nm, little corresponding fluorescamine quenching was observed, probably because of a compensating enhancement effect observed with all fluorescamine-labelled antigens (see Chapter 5). The sensitivity offered by this pair was evaluated by adding unlabelled albumin; the eosin enhancement effect could detect $10^{-9}$ M albumin in pure solution.

The overlap of quinacrine (Chen, 1976) emission and eosin excitation spectra is shown in Fig. 7.7. The efficiency of quinacrine-eosin energy transfer was investigated by addition of eosin-labelled anti-albumin antibodies to a fixed concentration of albumin-quinacrine conjugate. The resultant energy transfer effects were assessed by measuring the quenching of the quinacrine emission at 490 nm and any enhancement of eosin fluorescence at
Figure 7.5

Corrected excitation (Ex.) and fluorescence (Fl.) spectra of an albumin-fluorescamine conjugate, F:P = 6:1 (----) and of eosin-labelled antibody conjugate, F:P = 5 (----).
Figure 7.6

Emission spectra showing the changes in fluorescence intensity of mixtures containing a fixed concentration of albumin-fluorescamine conjugate (4.8 x 10^{-8} M, F:P = 5) and increasing amounts of antibody-eosin conjugate (F:P = 3). 

R = Emission spectrum of albumin-fluorescamine conjugate alone. Emission spectra of antibody-eosin conjugates alone are indicated by numerals within circles.

Antibody:Antigen ratios:
1, (Ab:Ag = 3.4:1); 2, (Ab:Ag = 6.6:1); 3, (Ab:Ag = 9.7:1)

Excitation wavelength, 390 nm; bandwidths, 10 nm.

(Blank experiments with (i) albumin-fluorescamine and (ii) mixture of labelled albumin and labelled antibody, in the presence of unlabelled antibodies showed an enhancement in the fluorescence intensity at 490 nm.)
Figure 7.7

Corrected excitation (Ex.) and fluorescence (Fl.) spectra of quinacrine-albumin conjugate, F:P = 2 (——) and of eosin-antibody conjugate, F:P = 5 (----).
Figure 7.8

Emission spectra showing the changes in fluorescence intensity of mixtures containing a fixed concentration of albumin-quinacrine conjugate (1.8 x 10^{-8} M, F:P = 2) and increasing amounts of antibody-eosin conjugate (F:P = 4.5). R = Emission spectrum of albumin-quinacrine conjugate alone. Emission spectra of antibody-eosin conjugates alone are indicated by numerals within circles.

Antibody:Antigen ratios:-
1, (Ab:Ag = 3.1:1); 2, (Ab:Ag = 6.3:1); 3, (Ab:Ag = 10:1)

Excitation wavelength, 420 nm; bandwidths, 8 nm.

(Blank experiments with (i) albumin-quinacrine and (ii) mixture of labelled albumin and labelled antibody in the presence of unlabelled antibodies did not show any appreciable changes in the fluorescence intensities at ca. 500 or 545 nm.)
545 nm using an excitation of 420 nm (Fig. 7.8). The quinacrine fluorescence at 490 nm was reduced by ca. 60% and the reversal of this effect could be used to detect ca. $10^{-9}$ M albumin in pure solution (quinacrine-labelled albumin F:P = 2 and eosin-labelled antibody F:P = 6 were used). A further advantage of using quinacrine or fluorescamine as the donor dye was that eosin fluorescence at 490 nm was negligible in the experimental conditions used, so the fluorescence background due to the reagents was minimised; unfortunately, endogeneous serum fluorescence is higher at 490 nm than at higher wavelengths. These pairs had practical limitations and are discussed below.

The conditions of maximum spectral overlap is an important prerequisite for efficient energy transfer by the resonance mechanism. In a given donor-acceptor system where this condition amongst others is fulfilled, the transfer efficiency can be expected to be high; at the same time the observation of energy transfer effects can be complicated by other factors related to the spectral characteristics of both the donor and acceptor labels. Too much spectral overlap was clearly the largest problem with these studies, in particular with the fluorescein-eosin pair and this is exemplified in Fig. 7.2. The required spectral overlap was excellent but in addition undesirable overlaps occurred e.g. the excitation spectrum of eosin overlaps that of the fluorescein label, the excitation and emission spectra of fluorescein overlap to a considerable extent. Such overlaps can cause other phenomena like the direct excitation of the acceptor label (at the wavelength of light required to excite the donor) and also intramolecular energy transfer to occur in
addition to and in competition with the donor-acceptor energy transfer. Furthermore, the acceptor label, eosin showed significant emission at 520 nm which was the optimum wavelength for the donor emission (excitation 470 nm) and for both the labels the small Stokes' shift make the fluorimetric measurements at the emission maximum very liable to interferences from Rayleigh (and possibly Raman) scatter especially in assays of biological samples.

The considerable spectral overlapping made the energy transfer studies with the fluorescein-eosin pair complicated; detection of the simultaneous quenching and enhancement effects was difficult because of the overlap problems mentioned above. A limitation was thus imposed on the fluorimeter bandwidth, maximum changes in the fluorescence intensity could only be observed with narrow bandwidths, this problem has been previously noted during other studies of energy transfer phenomena (Schiller, 1975) and in energy transfer assays (Lim, 1980).

With the other donor labels, the spectral overlap problem was not as severe but large enough to cause some interference. The desirable spectral overlap condition for the observation of energy transfer between quinacrine and eosin was offset by the broad spectral bandwidth of the quinacrine fluorescence spectrum, which also overlapped the eosin emission spectrum to a considerable degree. One practical limitation of this observation was the difficulty in measuring accurately the enhancement of eosin intensity (Fig. 7.8). With fluorescamine as the donor label, a rather different type of problem (interference) existed. The Fluram enhancement phenomenon (Chapter 5) was presumably operating and offset the quenching of the fluorescamine emission
by energy transfer causing some disproportionality between the quenching and enhancement effects.

The results may further be complicated due to random labelling of the antibodies, i.e. a labelling procedure usually produces a heterogeneous population of labelled conjugates; (the $n$ values were averages of several experiments in these studies). The conjugates should be fractionated to ensure uniform labelling. In addition, antibody preparations are heterogeneous anyway. So, unless monoclonal antibodies are used, reagents are inevitably heterogeneous, hence it may possibly be difficult to interpret all observed phenomena.

Preliminary work was done to investigate delayed fluorescence from eosin isothiocyanate-albumin conjugates (using a conventional mechanical phosphoroscope, O'Haver and Winefordner, 1966), the results were unsatisfactory and it seemed that measurable delayed emissions could be observed only from thoroughly deoxygenated samples, contrary to literature (Cherry et al., 1976; Moore and Garland, 1979, and Jovin et al., 1981).

7.4 Conclusion and Comments

The feasibility of an energy transfer assay utilising fluorescein as the donor and eosin as the acceptor was investigated. The excitation spectrum of eosin closely matched the emission spectrum of fluorescein but the system was beset with an undesirable spectral overlap problem. The evaluation of eosin as a possible acceptor label was thus difficult, with a very careful and selective choice of chemical and instrumental parameters, an assay was developed for human serum albumin,
the sensitivity (ca. $10^{-9}$ M) was comparable with the fluorescein-rhodamine pair. Eosin protein conjugates were found to be stable in aqueous buffers over considerable periods. This observation suggests a search for a suitable donor, one label very similar to fluorescein isothiocyanate, Lucifer yellow V (or its derivatives, Stewart, 1978, 1981) with an excitation wavelength 430 nm and emission wavelength ca. 530 nm appears an attractive possibility. If suitable in other respects for immunoassay work, the validity of this label as a donor and eosin as the acceptor remains to be tested.

It was apparent that the labels studied were not ideal for the development of sensitive energy transfer assays; other pairs that might be useful in energy transfer measurements have been suggested (Fairclough and Cantor, 1978): see Table 7.1. The special requirement of a fluorescent label for use in fluorescence immunoassay (Visor and Schulman, 1981) and further specific requirements in energy transfer immunoassay severely limit the choice to only a few fluorophors. It seems that new fluorescent labels must be developed before the energy transfer immunoassay technique can be optimised, the development of new donor-acceptor pairs has indeed attracted attention (Khanna and Ullman, 1980; Ullman, 1981; Rubenstein, 1981). Fig. 7.9 shows the spectra of an 'ideal' donor-acceptor pair. Ideally, the energy donor must be fluorescent (high fluorescence quantum yield) with emission wavelength above 550 nm (but less than 600 nm due to photomultiplier sensitivity restrictions) to reduce matrix interference. The wavelength of excitation should be in the visible region to minimise inner filter effects due to high absorption in the ultraviolet region and with a large Stokes' shift to minimise spectral contamination of fluorescence emission by scattered light.
Table 7.1

Donor-Acceptor Pairs useful for energy-transfer measurements

<table>
<thead>
<tr>
<th>Donor</th>
<th>$\phi_D$</th>
<th>$\lambda_{Ex.}$ nm</th>
<th>$\lambda_{Fl.}$ nm</th>
<th>Acceptor</th>
<th>$\varepsilon \times 10^4$ M$^{-1}$ cm$^{-1}$</th>
<th>$\lambda_{Ex.}$ nm</th>
<th>$\lambda_{Fl.}$ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DNS-cl</td>
<td>0.1-0.2</td>
<td>340</td>
<td>480-520</td>
<td>FITC</td>
<td>4.2-8.5</td>
<td>~490</td>
<td>520</td>
</tr>
<tr>
<td>2 DNS-cl</td>
<td>0.1-0.2</td>
<td>340</td>
<td>480-520</td>
<td>RITC</td>
<td>1.2</td>
<td>550</td>
<td>585</td>
</tr>
<tr>
<td>3 AEDANS</td>
<td>0.1-0.5</td>
<td>350</td>
<td>495</td>
<td>FITC</td>
<td>4.2-8.5</td>
<td>~490</td>
<td>520</td>
</tr>
<tr>
<td>4 AEDANS</td>
<td>0.1-0.5</td>
<td>350</td>
<td>495</td>
<td>NBD-cl</td>
<td>2</td>
<td>470</td>
<td>530</td>
</tr>
<tr>
<td>5 NBD-cl</td>
<td>0.1-0.5</td>
<td>470</td>
<td>530</td>
<td>RITC</td>
<td>1.2</td>
<td>550</td>
<td>585</td>
</tr>
<tr>
<td>6 QM</td>
<td>0.1-0.3</td>
<td>420</td>
<td>~500 (broad)</td>
<td>FITC</td>
<td>4.2-8.5</td>
<td>~490</td>
<td>520</td>
</tr>
<tr>
<td>7 QM</td>
<td>0.1-0.3</td>
<td>420</td>
<td>~500 (broad)</td>
<td>RITC</td>
<td>1.2</td>
<td>550</td>
<td>585</td>
</tr>
<tr>
<td>8 MDPF</td>
<td>0.1</td>
<td>~395</td>
<td>~485</td>
<td>FITC</td>
<td>4.2-8.5</td>
<td>~490</td>
<td>520</td>
</tr>
</tbody>
</table>

2, 6, 7, 8 - see Lim, 1980;

$\phi_D$ = quantum yield of bound fluorophore (donor label);

$\varepsilon$ = molar extinction coefficient of bound acceptor label at $\lambda$ of absorption.

DNS-Cl  Dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride)
FITC    Fluorescein isothiocyanate
RITC    Rhodamine-B-isothiocyanate
AEDANS  Acetyl N'-5-sulphonic-1-naphthyl)ethylene diamine
NBD-cl  7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole
QM      Quinacrine[9-(4-diethylamino-1-methyl-butylamino)-6-chloro-2-methoxy acridine] mustard
MDPF   2-Methoxy-2,4-diphenyl-3(2H)-furanone
Figure 7.9

'Ideal' spectra of a donor (---)-acceptor (----) pair in energy transfer assay.

* If acceptor is fluorescent.
In addition, there must be a good overlap of the donor emission spectrum with the acceptor excitation spectrum. A non-fluorescent acceptor is preferable (to minimise fluorescence background) with a large absorption coefficient and excitation spectrum free of donor contamination. If the donor and acceptor are both fluorescent, the emission spectra of the both labels should be well resolved. Such an ideal situation is yet to be identified, most recently, Syva Research Institute (Palo Alto, California, U.S.A.) have developed new fluorescein derivatives (Khanna and Ullman, 1982) and with the aid of chemical modifications seem to have achieved considerable success approaching an ideal situation for a donor-acceptor pair in an energy transfer immunoassay.

Energy transfer immunoassays have an advantageous feature in that they are homogeneous and can be automated (Lim et al., 1980(b)). The recent advent of monoclonal antibodies should overcome some of the difficulties and tedium of labelling and if a suitable pair of labels applicable to the widest possible range of materials is identified, this elegant fluorescence energy transfer immunoassay technique can be expected to be routinely applicable. Perhaps no single all round 'best' pair will be identified but the search should continue.

Recently fluorescein iodoacetamide and eosin iodoacetamide were used as donor-acceptor labels respectively in the detection of active assembly by fluorescence energy transfer (Taylor et al., 1981). Eosin was claimed to be a better acceptor than rhodamine.

Eosin, erythrosin and their derivatives appear to be potential fluorescent labels, in particular erythrosin isothiocyanate which is characterised by a high triplet yield (phosphorescence and delayed fluorescence) and a corresponding
weak prompt fluorescence; thus erythrosin would be a better energy acceptor. The triplet state emissions of these labels are worthy of further study, such phenomena can be distinguished from background sample fluorescence on a lifetime basis; the necessary precaution of oxygen removal, though irritating is not intractable, it is routine in other techniques (e.g. polarography) and fluorimeter cells that allow continual deoxygenation can readily be fabricated. Thus, the 'triplet state labels' in conjunction with moderate-cost commercially available equipment are likely to facilitate the development of time-resolved fluorescence immunoassays.
CHAPTER 8

PERSPECTIVES

This thesis describes the development of homogeneous luminescence-linked immunoassays.

The main problem inherent in most homogeneous luminescence immunoassays is the interference arising from the biological samples. It frequently happens in fluorescence immunoassays that a solute that can be detected at pg ml\(^{-1}\) in pure solution can only be detected at ng ml\(^{-1}\) or µg ml\(^{-1}\) level in 'real' samples because of high background signals. All the homogeneous fluorescence immunoassays thus far reported have been limited to nanomolar range, very little has been done to evaluate and overcome the background fluorescence problems affecting these otherwise simple assays.

In this work, the background interference arising from serum in two types of homogeneous fluorescence immunoassays was demonstrated; one utilised the 'Fluram enhancement phenomenon' for serum triiodothyronine determination and the second assay exploited the phenomenon of fluorescence energy transfer using a new acceptor label, eosin, fluorescein as the donor and human serum albumin as the model analyte. Another type of luminescence immunoassay, based on chemically excited fluorescence, peroxoxalate chemiluminescence was studied. This approach was affected by the presence of any protein in the system, the chemiluminescence signals being quenched.

The detailed investigation of background fluorescence from serum enabled the identification of the wavelength range affected and the likely components responsible for the spurious signals.
Simpler ways of abstracting the interfering components involved the use of adsorption techniques with small columns, these proved highly satisfactory for removing the background fluorescence at most wavelengths. Such techniques are however open to criticism in that additional steps of this kind may inhibit automation of assays in clinical laboratories.

In summary, it seems that homogeneous luminescence-linked immunoassays are simple, require only the mixing of reagents followed by a measurement, they offer the possibilities of increased speed, simple instrumentation, and ready automation. Sensitivities comparable to radioimmunoassays (or even better!) could be attained by overcoming the interferences posed by the biological samples. One of the ways of achieving this is to develop a new fluorescent label whose excitation and emission wavelengths are as far removed as possible from those background regions where interferences from the biological samples (e.g. serum) are significant (see Chapter 3, Section 3.5). An 'ideal' label would be the one with high extinction coefficient and high quantum yield; upon weighing the various properties of commercially available dyes (see Chapter 1, Table 1.5), it appears that fluorescein isothiocyanate is probably the most practical despite the serum background interference. This label has most recently been chemically modified (Khanna and Ullman, 1982; see Chapter 7, Section 7.4). Another powerful method, as yet only at the development stage makes use of fluorescence lifetime measurements to discriminate against background fluorescence. If this technique can be incorporated into the homogeneous assays, then sensitivities better than the radioimmunoassay method will be achieved. Yet another possibility is the use of chemiluminescence
measurements which can be made by very simple equipment. There remains the development of a new oxalate ester (peroxyoxalate chemiluminescence system) which would overcome the quenching effects, the outcome will then produce a very sensitive method with further advantages in that a fluorophor may be selected to achieve the desired compromise between emission wavelength, chemical stability and quantum efficiency. Two further recent technical developments will have a substantial impact on homogeneous luminescence immunoassay. Firstly, the use of monoclonal antibodies produced by cell fusion techniques which will improve quality control; in terms of routine immunoassays the possible advantages of these homogeneous antibody reagents have not yet been properly explored. Secondly, the use of continuous flow injection analysis in non-segmented streams promises to be cheaper and offers convenience and flexibility of portable apparatus. Flow injection analysis has already been applied to a homogeneous fluorescence energy transfer assay (Lim et al., 1980(b)) and its use in conjunction with chemiluminescence detection will then certainly provide very simple homogeneous assays with sensitivities competing with RIA and complementary to the existing heterogeneous assays which have already entered the commercial world.
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