The development of fluorescence immunoassay methods

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THE DEVELOPMENT OF FLUORESCENCE IMMUNOASSAY METHODS

BY

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SUPERVISOR: J. N. MILLER, Ph.D.

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TO MY PARENTS

for their constant support and encouragement
The contents of this thesis are original and have not been submitted previously for any award.
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SUMMARY

Fluorescent immunoassay methods are being developed in an attempt to overcome some of the disadvantages of radioimmunoassay (R.I.A.) when the extreme sensitivity of R.I.A. is not required. This work was an investigation into the possibility of producing a viable fluorescent immunoassay technique.

At the start, the techniques of solid phase immunoassay and a double antibody technique, originally developed for radioimmunoassay, were investigated to see if they could be adapted for a fluorescent assay of thyroxine (T\(_4\)) and immunoglobulin G (IgG). In these and most subsequent studies "fluorescamine" (4-phenyl spiro [furan - 2 (3H), 1 - phthalan] - 3,3 dione), a compound which reacts readily with primary amine groups, was used as the fluorescent label.

All these systems require an obligatory separation step and there is need for a rapid, homogeneous technique such as fluorescence polarization. The somewhat unproductive investigation into this technique led to the discovery that the fluorescence of labelled T\(_4\) is greatly increased on binding with antibody.

This enhancement phenomenon permitted the development of a rapid, homogeneous assay capable of detecting nanogram concentrations of T\(_4\). In serum this enhancement technique was complicated by the fluorescence of serum itself. Thus, the possibility of using Rhodamine, a compound which fluoresces outside the fluorescent spectrum of serum, as a fluorescent label in the enhancement technique was investigated. Also investigated was the nature of the enhancement phenomenon and the possibility of using it in the assay of other compounds.
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CHAPTER 1
INTRODUCTION

PART 1 LUMINESCE

Luminescence, the emission of light other than as a direct result of an increase in temperature, was first observed by the ancient Greeks and Chinese in the form of bioluminescence. They wrote of the light emitted by decaying fish, and by glow-worms and fireflies.

In the 1600's Viscenzo Cascariolo discovered that when Barite was heated with coal the resultant substance glowed a purple-blue colour at night. As this "lapis solaris" (sun stone) was spongy in appearance it was assumed that it simply absorbed light, as a sponge would absorb water, and slowly re-emitted it in the dark. This theory was refuted by Nicholas Zucchi who discovered that the emitted light was the same no matter what the colour of the exciting light.

Although there were these and other discoveries it was not until the mid-nineteenth century that the subject was studied systematically. In 1852 G.C. Stokes described the mechanism of the absorption and emission process and formulated the first law of luminescence (Stokes' rule), namely that the wavelength of luminescence is always equal to or greater than the wavelength of the exciting radiation. He also named fluorescence after the mineral fluorspar which produces a blue-white luminescence.

A little later (1867) Bequerel laid the foundations for the experimental investigation of the efficiency of excitation and emission spectra and the duration of phosphorescence by his experiments with a "phosphoroscope". In 1889 Wiedmann demonstrated phosphorescence in organic compounds, introduced the term luminescence and defined this phenomenon as the excess emission over and above the thermal emission background.

Today luminescence is well established and widely used as an analytical technique and there is a great deal of research into the phenomenon.
For this purpose luminescence is subdivided into at least ten types depending on the specific means of excitation, such as chemiluminescence, triboluminescence, bioluminescence and photoluminescence. The latter type, where the excitation of molecules is by interaction with photons of electromagnetic radiation, can itself be further subdivided. When the emission of electromagnetic radiation is "immediate" the process is called Fluorescence, but when the release of energy is delayed the process is termed Phosphorescence. Fluorescence is the only process with direct relevance to this work.

THE PROCESS OF PHOTOLUMINESCENCE

When light strikes a sample solution, one of a number of things can happen, it passes through the sample, it is scattered or it is absorbed. If absorption takes place energy is transferred to the molecules in the sample.

This absorption must take place in discrete units called quanta, the energy of which can be determined by the Planck-Einstein equation:

$$E = h\nu = \frac{hc}{\lambda}$$

when $E$ is the energy, $h$ is Planck's constant ($6.62 \times 10^{-27}$ erg-sec), $\nu$ is the frequency, $c$ the velocity of light and $\lambda$ is the wavelength.

In absorbing a quantum of light the molecule is raised from a lower to a higher electronic energy level. This process can be seen in Fig.1, which illustrates the various potential energy levels of a diatomic molecule; a comparable process occurs in more complex molecules. The difference in energy levels is equivalent to the energy possessed by the quantum of light. In addition to the main energy states, excited singlet and excited triplet states, there are various vibrational levels represented by $0, 1, 2, 3, 4$. The difference in singlet and triplet states being a difference in electron spin, $S$. All electrons have a spin, $S$, equal to $\frac{1}{2}$ and in a
FIG.1 Schematic Energy Level Diagram
normal polyatomic molecule in the ground state there is usually an even number of electrons with paired spin (i.e., usually one electron with \( S = + \frac{1}{2} \) and one with \( S = - \frac{1}{2} \)). Multiplicity is a term given to express the orbital angular momentum of a given state and is related to spin by the equation below:

\[
m = 2s + 1
\]

Therefore when all the electrons are paired, \( s = 0 \) and \( m = 1 \), and is referred to as the singlet electronic state. When the spin of a single electron is reversed to produce two electrons with parallel spins (i.e., two unpaired electrons), \( s = 1 \) and \( m = 3 \). This state being described as the triplet state.

When a molecule absorbs a quantum of light it is raised to an excited singlet state, \( s_1, s_2 \), etc. In the time the molecule can spend in the excited state (up to \( 10^{-4} \) sec.) some energy is dissipated and the molecule reverts to the lowest vibrational level of the first excited singlet state. If all excess energy is not further dissipated by collision, the molecule can return to the ground state with the emission of energy, fluorescence. As energy is lost prior to emission, the fluorescence must of necessity be of a longer wavelength (lower energy) than the light absorbed.

Alternatively an intersystem crossing may take place producing a transition from singlet to the triplet state. For the molecule then to return to the ground state energy may be dissipated by collision or emitted as phosphorescence. Consideration of the process will make it clear that the energy emitted will be lower than that of the absorbed light and of fluorescence, consequently the wavelength of phosphorescence will be greater than either. Since transition from singlet to triplet states requires a greater time than deactivation by collision, this latter will be the preferred means of relaxation in solutions at room temperature. This time lag in the appearance of phosphorescence also accounts for its characteristic
FIG. 2

Fluorescence spectra of Quinine

Sulphate in 0.1N sulphuric acid (excitation wavelength = 320 nm) The peaks are:-

1. 320 nm, Rayleigh scatter
2. 360 nm, Raman scatter of water
3. 450 nm, Quinine fluorescence
4. 640 nm, Second order Rayleigh scatter
feature of continuing after the exciting source is removed.

**OBSERVED SPECTROSCOPIC PHENOMENA**

The fluorescence normally observed in solution and that which is the most important form (at least to this work) is referred to as Stokes' fluorescence. This is the re-emission of energy at a longer wavelength than that of the absorbed light.

However, there are several other types of fluorescence that are possible, such as anti-Stokes' fluorescence and resonance fluorescence. Anti-Stokes' fluorescence is the emission of light at a shorter wavelength than the exciting photons and is due to the addition of thermal energy to an excited state of a molecule. Usually, it is seen only in dilute gases at high temperatures. Resonance fluorescence is the re-emission of photons at the same energy as the absorbed light. It is never observed in solutions because of solvent interactions, but it does occur in gases and crystals and is the basis of atomic fluorescence.

When an electron is excited to a higher vibrational level with no electronic transition, energy is entirely conserved and a photon of the same energy as the exciting photon is emitted as the electron returns to the ground state. This is termed Rayleigh scatter and occurs at all wavelengths. As its intensity varies as the inverse fourth power of the wavelength its effect can be minimised by working at long wavelengths and only becomes a problem when either the intensity of fluorescence is low in comparison with the exciting wavelength or when absorption and fluorescence spectra are close together.

Raman scatter is related to Rayleigh scatter and is normally due to vibrational energy being taken from the excitation photon. This effect is much weaker than the Rayleigh effect and is not normally observed unless high-intensity light sources are used (e.g. lasers). It is usually associated with the solvent as the solute is normally too dilute for any noticeable
Fig. 3 Schematic Diagram of a Typical Fluorimeter
effect. The relationship between fluorescence, Rayleigh scatter and Raman scatter is shown in Fig. 2.

MEASUREMENT OF FLUORESCENCE.

Fluorescence is measured in a device whose basic components are shown in Fig. 3, and which consist of a light source, wavelength selectors, sample compartment and detector system. This is similar to instruments used in spectrophotometry with two exceptions: (i) the detector is at 90° to the incident light path and (ii) a second wavelength selector is placed in front of the detector.

The light source can be of two basic types, continuum or line source, the choice depending on the desired features and financial considerations. It is more usual, however, for the source to be of the high-pressure xenon-arc type which gives a line spectrum superimposed onto a broad background continuum and has a high, fairly uniform output extending well into the U.V.

The desired narrow band of wavelengths of exciting light is selected by the use of a filter or monochromator (prism, interference wedge, diffraction grating) placed between the sample and light source. The resultant fluorescent radiation is studied by a detection system at 90° to the incident optical path. This is because its intensity will normally be independent of the direction of measurement, whereas scattered light will be at a minimum at right angles to the exciting light.

A second filter or monochromator placed between the sample and photodetector selects the wavelength of fluorescence to be measured. Radiation falling on the photodetector, usually a photomultiplier, is converted into an electrical signal, proportional to the intensity of the fluorescence energy, and is displayed after suitable amplification on a meter or recorder.

USES, ADVANTAGES AND LIMITATIONS OF FLUORESCENCE

Fluorescence is now a well established technique, with applications
from medicine to inorganic analysis, for qualitative and quantitative analysis of both naturally fluorescent compounds and compounds which have been labelled with a fluorescent moiety.

The basic equation which defines the relationship of fluorescence to concentration is:

$$F = \Phi I_o \left( 1 - e^{-\varepsilon b c} \right)$$

(2)

For very dilute solutions this equation reduces to one comparable to Beer's law in spectrophotometry:

$$F = K \Phi I_o \varepsilon b c$$

(3)

Where $F$ is the fluorescence, $\Phi$ the quantum efficiency, $I_o$ the intensity of the incident light, $\varepsilon$ is the molar absorptivity, $b$ the path length of the cell and $c$ is the molar concentration.

A plot of fluorescence versus concentration should therefore be linear at low concentrations.

The most important factors in favour of fluorescent techniques are its sensitivity and specificity. It is possible to measure concentrations as low as one part per ten billion, which is due mainly to the fact the emitted radiation is measured directly. The specificity of fluorescence relies on the facts that not all compounds that absorb light exhibit fluorescence and that if two fluorescent compounds absorb at the same wavelength they will probably not fluoresce at the same wavelength.

There are, however, some serious limitations to fluorescence because of its dependence on the environment (temperature, pH, viscosity etc.). Some compounds are photochemically decomposed on prolonged exposure to U.V. light and the quenching of fluorescence can occur with high concentrations (inner-cell effect), increased temperature, dissolved oxygen and with the presence of impurities. The impurities can cause quenching in several different ways, such as; absorption of the incident light in preference to the fluorescent compound, fluorescing at or near to the fluorescent wavelength.
of the compound to be studied, causing relaxation of the excited compound by collision or energy transfer, or by the inner-cell effect.
The concept of immunity, if not its mechanism, was known to the Chinese of the eleventh century who observed the inhalation of smallpox scabs prevented the later onset of the disease. This practice of immunisation eventually reached Britain, in the eighteenth century, via the Middle East where the process had been changed to introducing an intradermal application of powdered smallpox scabs (variolation). In the latter part of that century Jenner discovered that inoculation with cowpox had a similar effect in preventing the onset of smallpox.

The work of Pasteur (1881), who coined the phrase vaccine, led to further preventative immunisation and the development of the germ theory of disease. In 1885 Roux and Yersin, following the isolation of the diphtheria bacillus, demonstrated the existence of an exotoxin. This substance was used by von Behring and Kitasato to inoculate animals which produced in their serum a toxin-neutralizing substance called anti-toxin.

At the turn of the century (1906) Paul Ehrlich (4) proposed the humoral theory of antibody production. His side chain hypothesis proposed the pre-existence of receptors on the living cell surface which reacted with toxins. Excess of these receptors could be released into the circulation as antibodies.

Subsequently the major research in immunology was directed into the identification, characterisation and function of the humoral factors. Thus, it has been elucidated that the antibodies are protein molecules of a particular group (immunoglobulins) that have a specific binding reaction to the particular toxin or antigen which elicited their formation by lymphocytes.

However, it was not until the 1950's that Yalow and Berson (5) observed that low concentrations of antibodies to the antigenic hormone insulin,
could be detected by their ability to bind radiolabelled insulin. This
discovery was the basis of all subsequent work on radioimmunoassay (R.I.A.).

Today work is being carried out in the hope of replacing the radioactive label with other moieties, such as: enzymes, in such assays as

BASIC CONCEPTS OF IMMUNOASSAY

Immunoassays, of no matter what type, are all based on the ability of
an immunological protein, the antibody to bind a specific antigen, the
compound to be measured. Antibodies are proteins which are produced by
specialized lymphoid cells (plasma cells) and circulate in the blood of
animals. They are produced in response to a foreign substance (an antigen)
and have the ability to bind specifically to that substance in order to
facilitate its removal or destruction. Antigen is a term applied to a
substance able to stimulate antibody production.

Both the specificity and sensitivity of an immunoassay depend predomi-
nantly on the avidity (ability of antibody to bind antigen) and specificity
of the reaction between antigen and antibody. The specificity is dependent
on the interaction of sites on the antigen surface with specific areas of
the antibody molecule, the binding occurring through ionic and van der Waal's
forces. Thus, the most important factor of an immunoassay is the use of a
suitable antiserum.

In order to elicit the production of antibodies, a saline solution of
antigen, which is sometimes coupled to a protein or straight chain polymer
in order to increase its immunogenicity, is injected into a suitable animal.
The antigen is usually injected in the presence of an adjuvant (6) which
also increases the immunogenic response by releasing the antigen slowly,
increasing phagocytosis (7) and stimulating the reticuloendothelial system.
When sufficient antibodies of a high avidity have been produced by the
FIG. 4 Schematic Representation of the Principles of Immunonsay
plasma cells (8), (this is after several injections of antigen, the schedule of which is determined empirically) blood samples are taken and the serum, now called antiserum, is collected and stored. This antiserum can be used without further treatment or after purification of the antibodies in an immunoassay.

When labelled (radioisotope, fluorescent compound etc.) and unlabelled antigen are mixed with antibody they will compete for binding sites on the antibody. If the concentration of antibody and labelled antigen is fixed, less labelled antigen will be able to bind to the limited binding sites of the antibody as increasing amounts of unlabelled antigen are added to the system. Thus, the amount of labelled antigen binding to antibody will be inversely proportional to the amount of unlabelled antigen present. Therefore, if a means of measuring the amount of labelled antigen bound to antibody is available, such a system can be used as the basis for an assay for the antigen. A schematic diagram in Fig.4. illustrates this system.

Such an assay requires that the affinity of both unknown and standard unlabelled antigens be identical. The amount of unknown antigen in a sample is determined by the comparison of the amount of labelled antigen able to bind to antibody with the amount of labelled antigen able to bind to antibody when a series of standards of known concentration are treated in an identical manner. So, if the binding abilities of standard and unknown are not identical no such comparison can be made as the result would be meaningless. For example, if the affinity of antibody for the unknown antigen was greater than for the standard, in a given set of circumstances (an assay mixture containing antibody labelled antigen, and unknown or standard-unlabelled antigen) the amount of labelled antigen able to bind to antibody would be less when unknown was present than when the same amount of standard was present, unknown binding more readily and displacing more labelled antigen than the standard. However, the assay does not require that the
behaviour of unlabelled and labelled antigen be identical because the assay compares the results of standard and unknown samples behaving identically in the competition with labelled antigen for binding sites on the antibody. Thus, as long as the behaviour of labelled antigen is constant it need not be the same as unlabelled antigen.

It is also usual in such an assay to arrange that the antibody is in a dilution that allows approximately 50% of the labelled antigen to be bound in the absence of standard or unknown unlabelled antigen.

The exact nature of the antibody-antigen interaction remains unknown, but it is accepted that the reaction reaches an equilibrium during the incubation period and which can be described by the equation:

\[
[Ag] + [Ab] \xrightarrow{k_+} [Ag-Ab]
\]

This is for a simple bimolecular reaction, the interactions usually being more complex, where Ag represents antigen, Ab represents antibody, the square brackets molar concentration and \( k_+ \) are constants.

A plot of the kinetics of this reaction gives a hyperbola (9), the study of which shows the ratio of antibody bound to free labelled antigen \((B/F)\) decreases as the concentration of unlabelled antigen increases. Therefore a change in \(B/F\) is greatest when the antigen concentration is small compared to the antibody concentration.

**TYPES OF IMMUNOCASSAY**

Immunocassays can be subdivided into several classes, dependent on the type of label employed (enzyme, isotopic, fluorescent etc.) However, all these can be further subdivided into Heterogeneous assays, where antigen bound to antibody is separated from free antigen before measurement, and Homogeneous assays, where no separation step is required.

The Heterogeneous assay can employ a wide range of separation techniques, which can best be described by reference to R.I.A. for which, a
separation step being obligatory, many were pioneered. Some of these techniques are illustrated below:

a) Electrophoresis. This method was limited in that it would only handle about 200μl of incubate. It was however, one of the earlier techniques and included paperchromato-electrophoresis (10) and cellulose acetate electrophoresis (11).

b) Gel filtration. The fact that antibody bound antigen is a much larger particle than free antigen has been utilised to separate the two by filtration on sephadex columns (12,13).

c) Precipitation. Methods of precipitation fall into two categories. The first utilises neutral salts and organic solvents, and depends on the fact that at a critical concentration of the precipitant used, gamma globulins are insoluble while the free antigen remains in solution. Many variations have been used, including the use of ammonium sulphate (14,15), sodium sulphate (16), ethanol (17) and dioxane (18). The second system uses a second or precipitating antibody (19–21). This second antibody is produced in a different, usually larger, animal than that which is used to elicit the first antibody and against whose immunoglobulins it is induced.

d) Adsorption. The technique of adsorption has, among other, employed the use of talc (22), ion exchange resin (23) activated charcoal (24) and dextran coated charcoal (25). The idea behind coating charcoal with dextran was that the polysaccharide formed a matrix, allowing only the smaller molecular weight particles (unbound antigen) through to the charcoal.

e) Solid phase. In solid phase assays the antibody (or more rarely, the antigen) is bound to an insoluble support that
can be easily separated from the incubate. The supports used have varied in form from fine powders (26-28), beads (30,31), thin discs (32,33) to the inside surface of plastic tubes (34,35). Materials used have included sepharose (29), sephadex (26,27), enzacryl AA (36), polystyrene (30,34), polypropylene (35) and cellulose (32); and the attachment to the support has utilized both covalent bonding, such as by the use of CNBr, and physical adsorption. A description of some types of solid phase assay are shown in Fig.5. As can be seen the "sandwich" type of assay uses a labelled antibody as opposed to a labelled antigen (32,52). Where the "sandwich" type of assay utilizes solid phase bound antigen and labelled antibody, it is termed an immunoradiometric assay (50).

Homogeneous assays are unable to use isotopic labels and utilise such moieties as enzymes or fluorescent compounds. As with R.I.A. it is usual for the label to be attached to the antigen, however this is not true in all instances. In the enzyme assays the binding of antigen to antibody can either inhibit or stimulate its activity. To the incubate is added a substrate for the enzyme, the reaction of which can be followed by either spectrophotometric or fluorimetric means. This type of assay was first developed by the Syva corporation and was given the trade name E.M.I.T., but there is now a great deal of research in the area.

The use of fluorescent labels is also expanding but the types of homogeneous assay now being investigated are described in the chapter on Fluorescence Immunoassay (F.I.A.).

USES, ADVANTAGES AND LIMITATIONS

Immunoassays, mainly in the form of R.I.A., have been developed to assay for a wide range of compounds. These include both peptide hormones
FIG. 5

Schematic Representation of some Solid-phase Assay Techniques

1. Competitive inhibition system

Antigen + Labelled Antigen → Solid phase coupled antibody

2. "Sandwich" assay system

Solid phase coupled antibody + Antigen → Labelled antibody

3. Immunoradiometric assay system

Antigen + Labelled antibody → Solid phase coupled antigen
(e.g. insulin, A.C.T.H., L.H., bradykinin) and nonpeptidal hormones (e.g. thyroxine, prostaglandins, triiodothyronine), and substances such as digoxin, morphine, cyclic nucleotides (e.g. cAMP) immunoglobulins and fructose-1,6-diphosphatase.

Two of the great advantages of immunoassay are their specificity and sensitivity. The specificity is due to the fact that the compound to be measured is used to elicit the antibodies utilised in the assay. With R.I.A. it is possible to detect picogram levels (in some instances femtogram levels) of antigen, due mainly to the ability to detect minute amounts of radioactive material with gamma and scintillation counters. The sensitivity of other types of assay is not so great with the determining factor being the detection system. For example, it is possible to detect fluorescent compounds down to parts per ten billion (48) and the current limit for P.I.A. is in the nanogram range. However, it is possible that this could be improved by the use of lasers as the energy source and photon counters as the detection system.

The storage life of isotopically labelled antigens is dependent on the half life of the isotope (60 days for $^{125}$I; 8 days for $^{131}$I), whose decay may cause disruption of the antigen (49), and the loss of immunoreactivity with time. With enzyme and fluorescent labels the first two limitations do not occur and storage when lyophilized and in the dark at $-20\,^\circ C$ is of a much longer period. The isotopic label also has the drawback that there are many regulations for its handling and storage as it is a potentially hazardous material. Neither enzymes nor fluorescent materials suffer from this, although it is thought that some fluorescent compounds may be carcinogenic.

The heterogeneous assay has a separation step, obligatory in R.I.A., that is often inconvenient, is time consuming and needs a long incubation period as the reaction must be allowed to reach equilibrium. On the other hand, homogeneous assays have no separation step and as the rate of reaction
is measured there is no need for the reaction to be allowed to reach equilibrium. These points mean that whereas the homogeneous type of assay lends itself to automation the heterogeneous assay does not.

The expensive instruments needed for R.I.A. (scintillation and gamma counters) have little other use in most laboratories which normally possess a spectrophotometer and often a fluorimeter. These commonly used instruments can be adapted for use in fluorescence and enzyme linked immunoassays.
Isotopic labels produce problems in handling and are expensive, thus research is being carried out into the possible alternative of fluorescent labels.

The first assays to be investigated were of the heterogeneous type such as the solid phase “sandwich” type assay of Aalberse (32). This utilises both cellulose coupled anti-IgG antibodies and fluorescently labelled anti-IgG antibodies (labelled with fluorescein isothyocyanate). An outline of this type of assay is shown in Fig. 5 of the previous chapter.

Other heterogeneous assays have been developed by Bio-Rad laboratories and International Diagnostic Technology. The Bio-Rad system is an assay for immunoglobulins (IgG, IgM, IgA), is of the sandwich type and utilises small hydrophilic, derivatized polyacrylamide beads as the stable immunoadsorbent. As in the previous method the sample is first incubated with an excess of antibodies coupled to the solid phase and then with fluorescently labelled antibodies. However, unlike the method of Aalberse, the “sandwich” (of antibody-antigen-fluorescent antibody) is not cleaved from the solid phase before the measurement of fluorescence but the beads with the “sandwich” still attached are suspended in buffer and the fluorescence of this mixture is measured.

The International Diagnostic Technology method utilises a “FIAx/STIQ” system. Here the immunoadsorbent of a solidphase assay is deposited on one face of the paddle of a paddle shaped stick (STIQ). Being attached to a plastic support means the immunoadsorbent can be easily moved from one incubation mixture to the next without the need for centrifugation and the decanting of supernatant. When the reactions are complete, the stick is inserted into a specially designed “FIAx” fluorimeter which is of fixed wavelengths (fluorescein is used as the fluorescent label) and so can be of high sensitivity. The system can be adapted to both sandwich and competitive
antigen assays.

Several other solidphase assays have been developed, such as the ones by Burgett (51) and Skurkovich (52). The assay by Burgett for human C3 is similar to the Bio-Rad technique employing the same polyacrylamide beads and being carried out in a similar fashion. The assays developed by Skurkovich employ two techniques, the first is analogous to an immunoradiometric assay (see Fig. 5 in the previous section) and the other is a sandwich type assay. Both methods are assays for interferon and use interferon bound (via Con A) to sepharose 4B. The first assay uses a fluorescein labelled anti-interferon and the sandwich type assay uses a fluorescein labelled second antibody which was raised to the anti-interferon. In both assays the fluorescence is measured by a microfluorimetric technique where the immunoadsorbent is smeared onto slides which are placed on a microscope fitted with a photometric device.

However, because there is often a change in physical properties of the fluorescent compound when the labelled antigen binds to antibody, fluorescent labels lend themselves to the homogeneous type of assay. The research into this field is now growing with the utilisation of various effects, some of which are illustrated below.

One of the more highly investigated areas of homogeneous fluorescence immunoassay has been the utilisation of Fluorescence Polarization. In order to understand the principles of fluorescence polarization in immunoassay, polarization itself must be understood. In classical terms the emission from a single molecule can be regarded as radiation from a single oscillating dipole; this radiation has an oscillating electric field parallel to the direction of oscillation of the dipole and is said to be polarized in the same direction.

It can be assumed that the direction of the absorption and emission oscillators in a single molecule are the same and that they are rigidly fixed with respect to the geometric axis of the molecule. If the molecule was rigidly fixed in position between absorption and emission (10^-8 sec) the probability of absorbing light is proportional to the angle between the
absorption oscillator and the incident electric field, the possibility decreasing with an increase in the angle. As the absorption and emission oscillators are parallel, the emitted light will be partially polarized with a degree of polarization $P$. This is defined in terms of the intensity of the emitted light ($I$), polarized either parallel ($I||$) or perpendicular ($I\perp$) to the incident light and is given by:

$$P = \frac{I|| - I\perp}{I|| + I\perp}$$

The maximum value for $P$ has been shown (38) to be $\frac{1}{2}$.

As molecules in solution are subjected to Brownian motion, then molecular rotation will take place between absorption and emission, producing values of $P$ between 0 and $\frac{1}{2}$. Fluorescence polarization is normally employed when the antigen is small, these will consequently rotate rapidly producing small values of $P$ when fluorescently labelled. On binding with antibody the size of the molecule is greatly increased, the amount of movement decreased and consequently the degree of polarization increased.

Fluorescence polarization has been used to measure trace environmental contaminants (37,38) gentamicin (39), insulin (53) and H.C.G. (54).

Fluorescence Quenching and Fluorescence Enhancement are two related but opposite effects. The nomenclature for these two effects is somewhat confusing as, for example, in Fluorescence Quenching the fluorescence of a labelled antigen (e.g. Gentamicin (40)) is quenched on binding with antibody. However, the effect that is seen in an assay is an increase in fluorescence with increasing amounts of unlabelled antigen. The direct opposite occurs with the technique of Fluorescence Enhancement (46). The mechanism for Fluorescence Quenching is not yet understood but may be due to a negation of solvent effects when the antigen moves from the incubation medium to the environment of the antibody binding site. It is also postulated, when fluorescein is used as the label, that interaction of the ionised groups of
fluorescein with ionic groups on the antibody produces the relatively unfluorescent unionised form of fluorescein (54). The mechanism of Fluorescence Enhancement is not yet fully understood but several theories have been put forward, some of which are discussed later in this work.

A technique that is now being investigated uses the effect of Energy Transfer. This technique involves the use of two fluorescent labels, the emission spectrum of one overlapping the absorption spectrum of the other. One label is attached to the antibody the other to the antigen. Thus, if measurement is carried out at the absorption and emission maxima of the label which fluoresces at a lower wavelength, there will be a quenching of fluorescence on formation of the antibody-antigen complex, due to the transfer of energy (41-44). If, however, the measurements are taken at the absorption maximum of the compound which fluoresces at a lower wavelength and the emission maximum of the other compound, an enhancement of fluorescence should be observed.

As well as being involved with the development of assays based on energy transfer Ullman has also developed a Double Receptor fluorescent immunoassay (45). In this two antibodies are employed, one to the antigen (X) and the other to the fluorescent label (Y), and a fluorescently labelled antigen. Binding of Y to the label quenches the fluorescence but Y is prevented from interacting with the label when the antigen is bound to X. Thus, increasing amounts of unlabelled antigen will cause increasing quenching as less antibody (X) will be available to bind the labelled antigen.

A novel but rather esoteric homogeneous solid phase assay was suggested by Kronick et al (55). In this, antigen is bound to the flat surface of an optically transparent sheet, which serves as one wall of a cell containing assay medium. The material of the transparent sheet is chosen such that its refractive index will cause total internal reflection when irradiated at a certain angle. This is so excitation of fluorescent groups only takes place within a few hundred Angstroms of the surface. The assay medium consists of
sample antigen and fluorescently labelled antibodies. The amount of antibodies binding the antigen on the surface and being excited will be inversely proportional to the amount of antigen present in the medium. However, this method is more suited to an heterogeneous assay, a slide with fixed antigen incubated with assay medium and then removed, washed and placed, with a drop of cyclohexanol, on the dry surface of a prism in the sample compartment of a fluorimeter.

For completion the enzyme linked immunoassays should be mentioned. These are not strictly fluorescence immunoassays in the described sense as the antigen is not labelled with a fluorescent moiety but a fluorophor or fluorescent precursor (47). In the incubate is included an enzyme that is able to cleave the fluorophor-antigen bond to produce a fluorescent product. However, binding of the labelled antigen to antibody prevents the enzyme from reacting with its substrate. If unlabelled antigen is added less labelled antigen is able to bind to antibody and the fluorescence increases. It should also be noted that it ought to be possible to modify most enzyme immunoassays (e.g. EMIT (Syva Company) or CELIA (56)) where the label is an enzyme to a fluorescence assay by choosing an enzyme which produces a fluorescent product from a precursor or a non fluorescent product from a fluorescent compound.

A type of assay that is related to fluorescence immunoassay is the chemiluminescence linked immunoassay which is exemplified by the assay devised by Pratt (57). In this a derivative of luminol was used to label testosterone (via ovalbumin). A second or precipitating antibody was used to separate antibody bound from free testosterone, and the bound fraction placed with an H₂O₂ / cupric acetate mixture in a scintillation counter to measure the chemiluminescence. The advantages with such a technique (if a special instrument is designed) are that single molecules are counted, all the light produced is from the chemiluminescence of the luminol derivative, and no chemiluminescent products occur in natural biological samples. This means that a high sensitivity could be obtained.
At the beginning of this work Fluorescence immunoassay was still in its infancy with only a few papers published in the field. Thus the basic brief was to investigate immunoassay methods with a view to producing a viable fluorescent technique. After initial work on solid phase, double antibody and fluorescence polarization techniques the work concentrated on the phenomenon of fluorescence enhancement.

Initially the technique of solid phase immunoassay, originally developed for R.I.A., was investigated in order to see if it could be adapted for a fluorescent assay of thyroxine ($T_4$) and immunoglobulin G (IgG). These two compounds were chosen as they are widely different in terms of molecular weight, and antibodies and pure antigen are commercially available. In this and most subsequent studies fluorescamine ($4$-phenyl spiro [furan -2(3) 1'- phthalan] - 3,3' dione) was used as a fluorescent label. The solid phases looked at included sepharose, sephadex and polystyrene.

Subsequent to this a double antibody technique for the assay of IgG was attempted after the assay conditions had been elucidated by the use of an isotopic label.

As there is a need for a rapid, homogeneous (i.e., no separation step) assay, the possibility of using fluorescence polarization was investigated. Although this proved to be a dead end for the two compounds ($T_4$ and IgG) it led to the discovery that the fluorescence of the fluorescamine labelled compounds is greatly increased on binding with antibody. Thus, finally, the development of an assay for $T_4$, based on this fluorescence enhancement phenomenon, was investigated along with the nature of the response and the possibility of using rhodamine as a fluorescent label. This later possibility was investigated as the fluorescence maximum of fluorescamine is at a wavelength where serum is also fluorescent, whereas that of rhodamine is not.
CHAPTER 2
MATERIALS AND METHODS

MATERIALS

All distilled water used was tridistilled with the final two distillations in silica. Thyrone derivatives such as Thyrone (T₄; see Fig.7) and triiodothyronine (T₃) were obtained from Sigma; glutamic acid was also from Sigma; human IgG was obtained from Miles Laboratories. Antibodies to T₄ and T₃, unless otherwise stated, were obtained from Calbiochem; rabbit anti-human IgG and swine anti-rabbit IgG were obtained from Dakopatts A/S, Denmark. The fluorescent labels fluorescamine (trade name Fluram) and M.D.P.F. were obtained from Roche Products Ltd.; fluorescein isothiocyanate and rhodamine isothiocyanate were from Sigma. Sephadex was from Pharmacia and disposable columns from Bio-Rad.

All glass ware was washed in nitric acid and rinsed with copious quantities of distilled water. The use of detergents was avoided.

FLUORIMETRY

The measurement of fluorescence intensity was carried out using a Baird Atomic "Fluoripoint" fluorimeter. This instrument uses grating monochromators and a high-pressure xenon arc lamp as the light source. When a fluorescent spectrum was required the "Fluoricord" variation was used, the only differences between the two instruments being a recorder mode, motor monochromators and variable slits; a) between the lamp and the excitation monochromator and b) between the emission monochromator and the photomultiplier. On both instruments the sample slits normally used were such as to give an 8nm bandwidth.

The photomultiplier (RCA 1P28 or its EMI equivalent) normally fitted to these instruments has a maximum sensitivity at wavelengths between 300 and 400 nm. The sensitivity then falls off and becomes extremely low at higher wavelengths. Thus, for experiments where rhodamine was used (an emission maximum at about 600 nm) the photomultiplier was exchanged.
a) Fluorescamine

Non fluorescent

+ H₂O

Non fluorescent

Fluorescent

b) M.D.P.F.

Non fluorescent

+ H₂O

Non fluorescent

fluorescent

FIG. 6 Diagram of the structure of a) fluorescamine and b) M.D.P.F. and their reactions with primary amines and water.
a) Fluorescein isothiocyanate

![Fluorescein isothiocyanate structure]

b) Rhodamine B isothiocyanate

![Rhodamine B isothiocyanate structure]

c) Thyroxine ($T_4$)

![Thyroxine ($T_4$) structure]

**FIG. 7** Diagram of the structure of
a) Fluorescein isothiocyanate
b) Rhodamine isothiocyanate and c) Thyroxine ($T_4$)
for an Hamamatsu model (R928) which has a fairly sensitive and level response up to a wavelength of 800 nm.

The sample cells were either of 1 cm path length, where sample volumes of 2.5 mls or greater were employed, or 3 mm in cases when the sample volumes were lower than 2.5 mls. The instrument was used at its highest sensitivity with a 0.3 sec time constant.

As conditions vary the amplifier of the machine was usually adjusted to give a constant reading with a 5ppm quinine sulphate solution in 0.1N sulphuric acid. In this work no correction of spectra for instrumental characteristics was deemed necessary.

Absorption spectra, where shown, were produced on a Pye Unicam SP800 spectrophotometer and absorption measurements carried out using a Pye Unicam SP600 spectrophotometer.

CONJUGATION OF FLUORESCAMINE AND M.D.P.F.

The most commonly used fluorescent label in this work was fluorescamine (4-phenyl spiro [furan - 2\(^{(3H)}\), 1' - phthalan] -3,3' - dione), a compound which reacts readily with primary amine groups (see Fig.6a). This compound was first introduced by Wiesle et al. (58) and has been used as a reagent to detect picomole levels of amino acids (59,60) and nanomole levels of proteins (61). Other uses for this compound have included the staining of cells (62) and cell membranes (63,64). It has been shown that the reaction of fluorescamine with primary amines at alkaline pH (pH 6-9 but pH 8 is usually chosen due to the greater buffering capacity of phosphate buffer at this pH) is in the order of millisecond, whereas the hydrolysis reaction takes several seconds (65). Stein (65) also proposed that the reaction with primary amines proceeds via a nonfluorescent secondary amine intermediate.

Another recently synthesised (58) compound used in this work was M.D.P.F. (2-methoxy -2, 4-diphenyl -3 (2H) - furanone). This compound (see Fig.6b) is similar to fluorescamine in its structure, reactions, and
excitation and emission spectra. However, the optimal labelling conditions are higher at pH9.5, the fluorescent products are more stable and the fluorescent spectra are pH independent. (66).

Both fluorescamine and M.D.P.F. have similar fluorescent spectra with excitation and emission maxima at wavelengths of about 390 nm and 490 nm respectively. Neither fluorescamine or M.D.P.F. nor their hydrolysis products are fluorescent and therefore it is not usually necessary to remove them from the fluorescent product. Only when present in high concentrations do they interfere with fluorescence by the inner filter effect as they still absorb light at 390 nm.

Compounds (IpG, thyronine derivatives and glutamic acid) were labelled with fluorescamine by one of two methods. The first followed the method of Böhlen (61) and involved the addition of 0.5 mls of a 0.03% solution of the fluorophor in acetone to 2 mls of a solution containing 2-50 nanomoles of the compound to be labelled in phosphate buffer (0.1M, pH8.0), which was being mixed on a vortex mixer. The second technique involved the incorporation of the fluorophor into a cycloheptaarylose (Sigma) complex. This was done by the method of Nakaya (65). To 33 mls of a 2% aqueous cycloheptaarylose solution (w/v) was added dropwise 1.6 mls of an acetone solution containing 41.5 mg fluorescamine. After 15 mins. at room temperature the mixture was stood in an ice bath for 30 mins. and the precipitate formed collected by centrifugation. This precipitate was then dried over P₂O₅ at reduced pressure. An excess of this stable complex (about 0.5 mg) was then added to 2 mls of a solution of the compound to be labelled (2-50 nanomoles) in phosphate buffer (0.1M, pH8.0) and the mixture incubated at 37°C for 45 mins. Residual cycloheptaarylose was removed by centrifugation.

M.D.P.F. labelled thyroxine was prepared by the second method only, a cycloheptaarylose complex of M.D.P.F. being formed in the same way as for fluorescamine. In the labelling with fluorescamine the second method was
used for preference, the first method being used only in the earliest work, as no violent agitation during labelling is required and no acetone, now found to enhance fluorescence due to the reduction in polarity of the solution (67), is present. It was not considered necessary to purify the labelled compounds further as neither fluorescamine or M.D.P.F. nor their hydrolysis products are fluorescent.

CONJUGATION OF RHODAMINE AND FLUORESCEIN

Thyroxine was labelled with rhodamine and fluorescein by the method of Smith (46). To a solution of thyroxine (10 mg) in 0.5 mls of a pyridine-water - triethylamine mixture (in a ratio of 9 : 1.5 : 0.1, V/V/V) was added 1 ml of a solution of the same mixture containing either 25 mg Rhodamine - isothiocyanate (Sigma) or 20 mg Fluorescein isothiocyanate (Sigma). The structure of both these compounds is shown in Fig. 7. After gentle mixing at room temperature for 2 hours the conjugated thyroxine was crudely separated by precipitation with the addition of 10 mls of ammonium acetate buffer (0.2 M, pH 4.0) and collected by centrifugation. The precipitate was washed with 10 mls of distilled water and then redissolved, after collection by centrifugation, in 8 mls of 0.05 M ammonium bicarbonate solution with the aid of a minimal amount of ammonia solution. Trace amounts of excess fluorescent label were removed by two methods. In the first aliquots (1 ml) were placed on columns of sephadex G-25 (24 x 0.9 cm) equilibrated with 0.05 M ammonium bicarbonate solution. The labelled T4 becomes adsorbed to the Sephadex gel allowing the removal of small amounts of fluorescent impurities by washing with the ammonium bicarbonate solution. The exact nature of the binding of T4 to Sephadex is unknown but the reaction has been shown to take place in alkaline solutions (68). The purified product was then eluted with distilled water.

The other method employed was ultrafiltration using "Diallo" ultrafiltration membranes (Amicon Corp.) UM2 with a molecular weight cut off of 1,000.
Aliquots were put into the ultrafiltration apparatus (Amicon) and the impurities washed away by 0.05 M ammonium bicarbonate solution. After washing the residual conjugate was dissolved in the bicarbonate solution. Products from this and the previous method were freeze dried and stored at -20°C.

Glutamic acid was conjugated with fluorescein and rhodamine by the following method. Glutamate (2 mg) was dissolved in a little dilute HCl the pH adjusted to approximately 9.5 by the addition of dilute NaOH and the solution made up to 2.5 ml with carbonate buffer (pH9.5, 0.1M). To this was added 0.5 ml of rhodamine isothiocyanate (15.5 mg) or fluorescein isothiocyanate (12 mg) in a pyridine-water-triethylamine mixture (9:1.5:0.8). After gentle mixing at room temperature for 2 hours aliquots (0.5 ml) were placed on a Sephadex G-15 column (24 x 0.9 cm) and eluted with Barbital buffer (0.075 M, pH 8.6) to separate the glutamate from unconjugated fluorescent material.

POLARIZATION

As was seen in the introduction (chapter 1 part 2) the immunoassay of compounds by fluorescence polarization is dependent on the motion of the fluorescent moiety. A molecule labelled with a fluorescent group rotates in solution due to Brownian motion; this means that in between the absorption and emission of light by the fluorescent group the molecule will have moved randomly, causing a degree of partial polarization. On binding with antibody the molecular size of the particle is increased, the motion is decreased and the degree of polarization consequently increased.

In order to measure the polarization of samples rectangular strips of polarizing film (MN38, HOE Maskin) were cut to a size of 12 mm x 70 mm. These could then be easily inserted and removed from behind the excitation and emission slits of the sample block of the fluorimeter. By this means it was possible to polarize the excitation and emission light both vertically.
and horizontally by insertion of the correct strip of polarizing film in the light path.

Normally the degree of polarization is measured by using the following formula:

\[ P = \frac{I(vv) - I(vh)}{I(vv) + I(vh)} \]

Where \( I \) is the intensity of fluorescence; \( v \) is the orientation of the polarizing film to produce vertically polarized light, and \( h \) is the orientation of the polarizing film to produce horizontally polarized light. The first figure in the brackets refers to the film in the excitation light beam and the second to the film in the emission light beam.

However, the monochromator of a fluorimeter imposes a degree of polarization on the light beam and a correction factor \( G \) is usually used to neutralize the parallel diffraction anomalies. This correction factor is found by using horizontally polarized excitation light as opposed to the normally used vertical polarization and is found by the following formula:

\[ G = \frac{I(hz)}{I(hh)} \]

This factor will also rectify any anisotropy introduced by the photomultiplier and the walls of the cuvette. It need only be found once for a particular set of results measured at the same wavelength and slit setting.

Thus, the degree of polarization can be more accurately represented by the following formula:

\[ P = \frac{I(vv) - GI(vh)}{I(vv) + GI(vh)} \]

**IMMUNODIFFUSION**

Reactions between large antigens and antibody can be observed by means of a precipitation reaction which was first described by Heidelberger (70).
In his work purified capsular polysaccharide of pneumococci, which is devoid of nitrogen, was used as an antigen and the reaction followed by the presence of nitrogen, from the antibody, in the precipitate. The results are shown schematically in Fig.8. At low antigen concentration, when antibody concentration is constant, precipitation increases with antigen and excess antibody can be found in the supernatant. A point is eventually reached where there is no excess of antibody or antigen and this is called the equivalence zone. Beyond this point the amount of precipitate decreases and excess antigen is found in the supernatant. This precipitation reaction depends on the antigen being multivalent and the antibody at least bivalent.

It has been proposed (71) that at the equivalence zone optimal proportions of antigen and antibody are present and a stable antigen-antibody lattice is formed. When excess antigen is present solubilization of the lattice occurs as a result of free antigen competing for binding sites on the antibody.

Early observations of the precipitation reaction were made in liquid media but now most observations are carried out using semi-solid gels. Many qualitative and quantitative techniques have been developed where the reaction is observed in a gel through the formation of a precipitate at the equivalence point (72). One of the earliest methods of qualitative analysis was devised by Ouchterlony (73) and is termed double diffusion in two dimensions. In this method diffusion of both antigen and antibody is necessary before precipitation is possible. It is possible from the precipitin lines that are formed to ascertain some indication of the relationship between antigens in adjacent wells. The technique employs a central well, containing antibody, which is symmetrically surrounded by a number of wells containing antigen. The antibodies and antigens diffuse radially from the wells and as antigen diffuses outwards it will encounter an increasing concentration of antibody as it approaches the antibody well.
Precipitation will occur at the equivalence point, occurring closer to the origin of the weaker reactant, forming a precipitation line that will then be reinforced as antigen and antibody diffuse into the region at equivalent rates. It is normal to choose proportions of antigen and antibody which are equivalent but small differences will be compensated for.

The quantitative assays are illustrated by the single radial immunodiffusion technique of Mancini (74). Here, antigen is placed in a well in a gel containing a suitable concentration of antibody. The antigen will diffuse radially outwards into the gel and react with antibody to form a precipitate where the antigen has diffused far enough to be sufficiently dilute to be equivalent to the antibody concentration. More antigen will, however, diffuse outwards towards the precipitate causing it to dissolve and causing precipitation to occur further from the well. Precipitation will be complete when all the antigen has been reacted with antibody and the diameter of the precipitation region will then be indicative of the amount of antigen present.

Both the Mancini and Ouchterlony techniques were used in this work. In the Mancini technique LC - Partigen plates (Behring) were used to test the antigenic competence of fluorescently labelled IgG. The Ouchterlony method was carried out using plates prepared by pouring 6.2 uls of a 1% (W/V) agarose (1' Industrie Biologique Francaise, SA) in barbital buffer (pH 8.5, 0.02M) containing 0.1% sodium azide (W/V) onto glass plates (7.5 x 5.5 cm) and allowing it to solidify before cutting sample wells. This was carried out using a cutter prepared in the Chemistry Department of the University. The sample wells so cut, the unwanted agarose being removed by suction, were 3.5 mm in diameter and the pattern consisted of six sample wells equidistant from a central antiserum well with centres of the wells 6 mm apart. Samples of both antigen and antibody were used in 10 ul aliquots. Plates were left in a humid box for 24 hours until precipitate formed. After this period the plates were washed with 0.9% (W/V) saline solution.
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<td>Ag in supernatant</td>
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Antibody excess zone  | Equivalence zone | Antigen excess zone

Increasing antigen concentration →

**Fig. 3** The quantitative precipitin reaction first described by Hildesheimer (73)
to remove all nonprecipitated material. The protein was then stained using a solution of coomassie brilliant blue R (0.5% w/v) in an ethanol-water-glacial acetic acid mixture (4:5:4.5:1) and background stain removed by washing with the same solvent.

The antiserum, rabbit anti-human IgG and swine anti-rabbit IgG, used in the Ouchterlony technique were both obtained from Dakopatts A/S, Denmark. When samples had to be concentrated this was carried out using a Minicon concentrator from Amicon.

**SOLID PHASE IMMUNOASSAY - PREPARATION OF IMMUNOADSORBENT.**

The classical system of immunoassay is based upon the system originally described by Yalow and Berson (10) and is based upon the ability of an antibody to bind its labelled antigen (in the system of Yalow et al the label is a radioactive isotope) and the competitive inhibition of this reaction by unlabelled antigen. In this method the antibody bound labelled antigen is then separated from the unbound and the amount of label in one or both of these fractions is measured. This separation procedure was simplified by the use of solid phase coupled antibody (75, 28). The antibody bound antigen was then attached to the solid phase while the unbound antigen remained in the solution and the two phases could then be separated with ease. As this separation procedure was independent of the size of antigen (unlike the use of charcoal etc.) it could be used to assay antigens with physico-chemical properties similar to those of antibodies, i.e. immunoglobulins (76). It was also found that systems other than ones based on the principles described by Yalow (10) could be developed by the use of solid phase coupled antibodies or antigens (see Fig. 5).

The form of the solid phase has been varied in many ways from polysaccharides to various plastics (Chapter 1, part 2) and consequently the coupling reaction has also varied from covalent binding to physical adsorption. In this work micro polysaccharide particles (the cross-linked dextrans
sephadex G25 and sepharose 4B) and the inside surface of a polystyrene tube (Jobling, 1.1 x 4.4 cm) have been used with the antibody coupled by covalent bonding and physical adsorption respectively. The covalent bonding of antibody to the cross-linked dextrans was by using cyanogen bromide activated sephadex and sepharose.

The cyanogen bromide activation of both the sepharose and sephadex was carried out by a modification of the method of Paterly et al (27). Approximately 20 mls of gel were washed with distilled water and placed in an ice bath. The pH of the gel was then set to 11 by the addition, with stirring, of 2.5 N NaOH. A 1% solution of cyanogen bromide (about 95 mls.) was quickly added to the gel and the pH kept at about 11 by the addition of 2.5 N NaOH. After 1 hour the gel was washed with, first, cold distilled water and then cold 0.001 N HCl. The pH of the gel was then set to 7.4 by addition of phosphate buffer (0.1M).

To 6 mls of this gel, or 4 mls of cyanogen bromide activated sepharose 4B obtained from Pharmacia, was added with stirring 1.0 mls of rabbit anti-human IgG (Dakopatts S/1). The mixture was then left overnight at 4°C after which it was filtered through a glass filter and washed copiously with phosphate buffered saline (0.1M, pH 7.4; 0.5M NaCl). The immunosorbent was then washed twice with acetate buffer (0.1M, pH 4.0) and twice with phosphate buffered saline (0.1M, pH 8.0; 0.5M NaCl). To the gel suspension was added enough sodium azide to produce a concentration of about 0.02% and the material stored at 4°C in such way as to prevent the immunosorbent from drying out.

For the adsorption of the anti-IgG antibody onto plastic tubes both the methods of Foti et al (35) and Cunningham et al (34) were used.

In the first method 1.5 mls of antiserum (anti-IgG) diluted with carbonate buffer (pH 9.6, 0.05 M) or buffer was added to polystyrene tubes. After 1 1/2 hours the tubes were aspirated and washed twice with 2 mls phosphate buffered saline (0.1M, pH 7.5; NaCl 0.15M) and then 1.5 mls of 0.5% BSA
(Bovine serum albumin) in the same buffer was added and incubated for one hour. The albumin solution was then aspirated and an assay medium consisting of 0.1 ml labelled IgG and 1.4 ml of the phosphate buffered saline. Following a four hour incubation the fluorescent intensity of a) the supernatant and b) the polystyrene tubes was measured. In the latter case the tubes were first washed twice with 2 ml of the phosphate buffer and then inserted, containing 1.5 ml phosphate buffer, into an adaptor for the fluorimeter which held the tubes in the sample position of the fluorimeter.

In the second method, 1.5 ml antibody (anti-IgG) diluted in Tris buffer (pH 8.6, 0.05M) was added to the tubes. After four hours at room temperature the tubes were aspirated and washed 3X with Tris buffer (pH 7.5, 0.05M) followed by one wash of 1% BSA containing 0.02% (7/7) Na$_3$N$_5$ in the same buffer. Each wash was removed immediately after its addition. To each tube was added an assay mixture of 0.1 ml labelled IgG and 1.4 ml phosphate buffered saline (0.1M, pH 7.5). After incubation overnight fluorescent measurements were taken as for the first method.

In addition, for the attempted assay of fluorescently labelled thyroxine (T$_4$), commercially available plastic tubes coated with anti-T$_4$ were used (Mallinckrodt, SPAC T$_4$ RIA kit). A range of labelled T$_4$ concentrations in Barbitol buffer (0.075M, pH 8.6) were used and the fluorescent intensity of the supernatant measured after one hour incubation at 37°C.

DOUBLE ANTIBODY IMMUNOASSAY - PREPARATION OF 125I-LABELLED IgG

The technique of double antibody separation was first introduced by Morgan and Lazarow (21) and uses a second antibody to precipitate the antibody which is able to bind the antigen to be measured. The system relies on the fact that the antigenic sites of the gamma-globulin of the first antibody are distinct from those involved in the antibody activity of the molecule.
In this work in order to look at a fluorescence immunoassay using a double antibody technique a RIA was first set up to ascertain the assay conditions. In order to do this human IgG was labelled with $^{125}\text{I}$.

The use of $^{125}\text{I}$ to detect biological material is well established and picogram quantities of labelled protein are readily detected. Incorporation of a single iodine atom usually has little effect on the structure and activity of a protein molecule. Originally, labelling of organic materials was by the use of strong oxidising agents such as chloramine T (77) or iodine monochloride (78). These methods however are suspect as nonspecific side reactions can cause alteration of protein structure and activity (79) or low efficiency of iodine incorporation (80). Recently there has been the development of enzymic methods such as the lactoperoxidase method (81), which employs low levels of relatively weak oxidising agent and can yield products with high specific activity. The only drawback is the possible inclusion of some impurities and the labelling of products not of specific interest. However, it has been assumed that low levels of enzyme result in only the major components being labelled (82). Consequently, IgG was labelled by the lactoperoxidase method described below.

To a solution containing 5$\mu$ Ci $^{125}\text{I}$ (50$\mu$L of a 0.1 mCi per mL solution), 400$\mu$g IgG (1 mg/mL in phosphate buffered saline of 0.1M, pH 7.5 and 0.15 M NaCl), 8$\mu$L $\text{H}_{2}$ $\text{O}_{2}$ ($2 \times 10^{-6}\text{M}$) and 10$\mu$L KI ($2 \times 10^{-3}\text{M}$) was added 2$\mu$L of a lactoperoxidase (Calbiochem) solution (10$\mu$g in phosphate buffered saline containing $10^{-5}\text{M}$ mercaptoethanol). After gentle shaking and incubation at room temperature for 20 minutes all further reaction was stopped by the addition of 50$\mu$L of a Thiosulphate solution ($10^{-3}\text{M}$). In order to determine that the IgG had been labelled the mixture was put on a column (24 x 0.9 cm) of G-25 and eluted with phosphate buffer (pH 7.5). Otherwise the mixture was dialysed against 4 x 100 mL phosphate buffered saline at 4°C for 18 hours using dialysis tubing (Visking). The solution was then diluted to give an IgG concentration of about 0.5$\mu$g/mL.
The double antibody method finally used was a modification of the technique of Gleich et al. (19) for the assay of IgG.
CHAPTER 3
RESULTS AND CONCLUSIONS

PART I PRELIMINARY WORK

a) Absorption and Fluorescent Spectra

Originally the fluorescence spectra of thyroxine ($T_4$) and IgG Conjugates of fluorescamine were determined. Both conjugates were formed by the method of Bohlen (61), that is the addition of a fluorescamine solution in acetone to a solution of IgG or $T_4$ which was being rapidly agitated on a vortex mixer. The solutions were then diluted to give a concentration of about 5μg/ml and the fluorescence spectra measured using a Baird Atomic "Fluoricord" fluorimeter, this model having a recorder output and motorized grating. The results can be seen in Fig.9. These spectra are typical of all fluorescamine conjugates, the excitation and emission maxima being about 385 nm and about 495 nm respectively. As can be seen the absorption and emission spectra are well separated, although the slight overlap permits the possibility of energy transfer between fluorophor groups. The slight differences in maxima of the conjugates could possibly be attributed to the use of uncorrected spectra or slight differences in the micro-environment of the label.

Later in the work it became necessary to determine the U.V. absorption spectrum of $T_4$. This is shown in Fig.10 along with that of IgG. The $T_4$ was at a concentration of 10μg/ml in carbonate buffer (0.05 M, pH 10.3) and the IgG at a concentration of 0.5mg/ml in phosphate buffer (0.1 M, pH 8.0).

b) Detection of Fluorescamine Conjugates.

The detection limits of $T_4$ and IgG conjugates of fluorescamine, which was defined as the smallest amount of fluorescamine conjugate to give a fluorescence reading of more than twice the background level of fluorescence, were found by the sequential dilution of a solution prepared by the method of Bohlen (61). In the case of IgG the solution was first passed through a Sephadex G-25 column (0.9 x 24 cm) and lyophilised before the dilutions.
Fig. 9  The fluorescence spectra (uncorrected) of a) Fluorescamine conjugated IgG and b) Fluorescamine conjugated T₄ in phosphate buffer (pH 8.0, 0.1M)
Fig. 10  U.V. Absorbance spectra of a) IgG in phosphate buffer (pH 8.0, 0.1m) and b) T₄ in carbonate buffer (0.05M, pH 10.3)
However, similar results were obtained when no such treatment was carried out but when smaller amounts of reactants were used (i.e., 25 μg IgG and a 0.003% solution of fluorescamine). The results in Fig.11 show the response was linear over the range covered, each point being the mean of 4 results. A limit of about 5 ng/ml for IgG compares favourably with the results obtained by Bohlen (61). The limit for T₄ appears to be about 15 ng/ml.

In these results some reduction of fluorescence by the hydrolysis products of fluorescamine will take place but this should be negligible as DeBernardo (83) only observed significant reduction at about 1 μM/ml of fluorescamine. This reduction is due to the hydrolysis products still absorbing light at 385 nm, even though they are non-fluorescent, and producing an inner filter effect.

STABILITY OF CONJUGATES

The graphs in Fig.12 show the stability of fluorescamine conjugates as a function of time. The effect at room temperature is demonstrated by a conjugate of IgG (12a), whereas in 12b the effect at 37°C is demonstrated by a conjugate of T₄. Both graphs show that there is a decline of fluorescence with time; and an increase in temperature appears to increase the loss of fluorescence. A comparison of the fluorescence after 5 hours shows that at room temperature there has been approximately a 25% loss of fluorescence whereas at 37°C this has increased to about 38%. In both 12a and 12b the mean of 4 results is shown. The loss of fluorescence of the IgG conjugate is comparable with the results obtained by Bohlen for a variety of proteins.

DYE/PROTEIN RATIO

In order to estimate the molar ratio of fluorescamine to IgG the method of Handschin (85) was employed. Handschin derived the following formula for measuring the molar dye/protein ratio:

\[
\frac{\text{Dye/Protein}}{\text{molar}} = \frac{c \times d}{a} \times \frac{A_{385}}{(A_{280} - b \times A_{385})}
\]
Fig.11 Relationship between fluorescence intensity and concentration of the fluorescamine conjugates of a) IgG and b) T₄
Fig. 12 Variation of fluorescence intensity with time of
fluorescamine conjugates of a) IgG at room temperature
and b) T₄ at 37°C
Where \( A \) is the absorbance at 280nm and 385nm; and \( a \) to \( d \) are coefficients.

These coefficients are found from the following equations devised by Wells (86).

1. \( [F] = a \times A_{P280} \) thus \( a = [F] / A_{P280} \)
2. \( A_{P280} = A_p + A_{P280} - A_{P280} = b \times A_{F385} \) thus \( b = A_{P280} / A_{F385} \)
   then \( [P] = c \times (A_p + A_{P280} - b \times A_{F385}) \)
3. \( [F] = c \times A_{F385} \) thus \( c = [F] / A_{F385} \)
4. \( d = Mw_p / Mw_f \times 10^3 \)

Where \( [P] \) is the concentration of protein in mg/ml, \([F]\) is the concentration of bound fluorescamine in \( \mu g/ml \), \( Mw \) is molecular weight and the suffixes \( P \) and \( F \) refer to protein and fluorogen.

Hendrich found these coefficients to be as follows:

\[
\begin{align*}
a &= 0.811 \\
b &= 2.42 \\
c &= 43.86 \\
d &= 0.58
\end{align*}
\]

The value of \( d \) is based on the mean molecular weight for gammaglobulins being 160,000.

Thus using the above information the molar ratio of fluorescamine to IgG can be found from the following equation:

\[
\text{Dye/Protein} = \frac{43.86 \times 0.58 \times A_{385}}{0.81 \times (A_{280} - 242 \times A_{385})}
\]

IgG labelled by the Bohlen method was placed on a sephadex G-50 column (24 x 0.9 cm) equilibrated with phosphate buffer (pH 8.0, 0.1M). The eluted IgG fractions were pooled and the absorbance at 280 and 385 nm measured.

The results from three separate labellings are shown below in Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Labelling</th>
<th>Dye/Protein Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>10.2</td>
</tr>
<tr>
<td>Mean</td>
<td>9.7</td>
</tr>
</tbody>
</table>
The results are within Handschin's determination of a ratio of between 4 and 14 for rabbit and goat gammaglobulin.

The estimation of the fluorescamine / T₄ ratio was complicated by fluorescamine absorbing light over the same waveband that T₄ absorbs light; to devise a formula similar to that for IgG would have required large amounts of work in determining the necessary coefficients. However, as the molar absorption coefficient of the fluorescamine hydrolysis product has been determined by Handschin (22,400 M⁻¹ cm⁻¹ at 280 nm), the amount of fluorescamine bound to T₄ can be determined if the amount of fluorescamine before reaction is known, and if the T₄-bound fluorescamine can be separated from the hydrolysis products of fluorescamine. This separation was possible as it has been shown that, at alkaline pH, sephadex will adsorb thyroxine (68).

Thus, a known quantity of T₄ was labelled with fluorescamine by the method of Nakaya and an aliquot (0.5 ml) put on a sephadex G-25 column (0.9 x 12 cm) equilibrated with 0.05 M ammonium bicarbonate solution. The eluted fractions containing hydrolysis products of fluorescamine were pooled and after suitable dilution the absorbance at 280 nm estimated. To determine the amount of fluorescamine before reaction a blank was prepared at the same time by addition of an equal amount of cycloheptaamylose complex to a blank solution of phosphate buffer (i.e. no T₄ present). An aliquot (0.5 ml) of this mixture was diluted with the ammonium bicarbonate buffer and the absorbance at 280 n.m. estimated. Using the molar absorption coefficient and allowing for a 4% loss on the column, (found by measuring the absorbance of aliquots of hydrolysed fluorescamine before and after passage through the column) the amount of fluorescamine bound to T₄ can be determined. From this the molar ratio of dye to T₄ can be found by using the following equation:

\[
\frac{Dye}{T₄} = \frac{(A_B - A_R)}{22.4} \times \frac{MW\ T₄}{[T₄]}
\]

Where A is the absorption at 280 n.m.; the suffixes B and R stand for the blank and the solution after reaction and passage through the column.
respectively; \( M \) is the molecular weight; and \([T_4]\) is the concentration of \( T_4 \) in \( \mu g/ml \).

The results of 3 labellings are shown below in Table 2.

**Table 2**
Molar ratio of fluorescamine bound to \( T_4 \) after labelling by the method of Nakaya.

<table>
<thead>
<tr>
<th>Labelling</th>
<th>( \text{Dye/T}_4 \text{ Ratio} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>0.91</td>
</tr>
<tr>
<td>3</td>
<td>0.91</td>
</tr>
<tr>
<td>Mean</td>
<td>0.91</td>
</tr>
</tbody>
</table>

The results show that the ratio is almost unity. They are, however, slightly higher than the ratio quoted by De Bernardo (83) for amino acids labelled by the method of Böhler.

**Immunogenicity**

To test that the immunogenic properties of IgG were not lost when labelled with fluorescamine both Mancini and Ouchterlony techniques were used. In the Mancini technique "Tri-partigen" plates (Behring) were employed and 5 \( \mu l \) samples of IgG – conjugate (IgG-F) and IgG were placed in the sample wells. The results are shown in Fig.13a with the well contents being:

<table>
<thead>
<tr>
<th>Plate 1</th>
<th>Well No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>200 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>150 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>150 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>200 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>200 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>250 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>250 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>250 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>200 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>200 ( \mu g/ml ) IgG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 2</th>
<th>Well No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>200 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>150 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>200 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>150 ( \mu g/ml ) IgG-F</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>200 ( \mu g/ml ) IgG</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>150 ( \mu g/ml ) IgG-F</td>
</tr>
</tbody>
</table>
The results of Ouchterlony technique to show the reaction between b. rabbit anti-human IgG (Ab1) and fluorescamine labelled/unlabelled IgG, and c. Ab1 and swine anti-rabbit IgG (Ab2)
Clearer results were obtained from the Ouchterlony technique, an example of the results being seen in Fig. 13b. The centre well contains rabbit anti-human IgG (Ab1) in a dilution of 1:10 and the outer wells contain:

1. 250 µg/ml IgG
2. 250 µg/ml IgG-F
3. 250 µg/ml IgG-F
4. 150 µg/ml IgG-F
5. 150 µg/ml IgG-F
6. 250 µg/ml IgG

Both techniques however, indicate that there is no loss of immunogenic properties when labelled with fluorescamine. Better results would have been obtained in the Mancini technique if more concentrated solutions had been used. This would have also allowed a quantitative assessment.

In Fig. 13c the centre well contains swine anti-rabbit IgG (Ab2) in a dilution of 1:10; the outer wells contain rabbit anti-human IgG (Ab1) in dilutions of:

1. 1:100
2. 1:200
3. 1:400
4. 1:800
5. 1:1600
6. 1:100

As can be seen precipitin lines were obtained when the rabbit anti-human IgG was in a dilution of 1:100. When the outer wells contained human IgG no reaction was obtained.

In order to see if T₄ labelled with fluorescamine also retains its immunogenic properties known dilutions were taken and estimated by a commercially available T₄ R.I.A. kit. (Radiochemical Centre). The results are
shown below in Table 3.

Table 3

Estimation of T₄ labelled fluorescamine by R.I.A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts/100sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard T₄</td>
<td></td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>33,300</td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>33,384</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>26,074</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>26,423</td>
</tr>
<tr>
<td>Labelled T₄</td>
<td></td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>32,800</td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>33,059</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>25,252</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>25,690</td>
</tr>
</tbody>
</table>

The results show a slight but insignificant loss of the immunogenic properties.
PART 2  UNIVERSAL LABEL

The possibility of producing a 'universal' label that increased the sensitivity of fluorimetric detection was investigated by the use of poly-L-lysine (Mol.Wt. 3,400). If this peptide, with a number of primary amine groups, was labelled with fluorescamine it was thought possible that this could then be used to label proteins and other molecules by a carbodiimide reaction. This might produce a label capable of increasing fluorimetric sensitivity by providing several fluorescamine molecules at each labelling locus.

**DYE/PEPTIDE RATIO**

To ascertain how many fluorescamine molecules were bound to each poly-L-lysine molecule it was not possible to use the method of Handschin (85) as poly-L-lysine does not absorb light at 280 nm. However, it is possible to use the molar absorption coefficient of the primary amine conjugate of fluorescamine ($6,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 385 nm (85)) if the hydrolysis products of fluorescamine are removed.

Thus, poly-L-lysine was labelled with fluorescamine by the method of Böhlen (61) (the molar ratio of fluorescamine to poly-L-lysine was 800 to 1) and an aliquot (0.5 ml) placed on a sephadex G-50 column (0.9 x 24 cm) equilibrated with phosphate buffer (0.1M, pH 8.0). The eluted poly-L-lysine fractions were pooled and the absorbance at 385 nm estimated. The amount of poly-L-lysine lost on the column was previously determined as 8% by measuring the fluorescence of an aliquot of labelled peptide before and after being passed through the column.

Using this information the molar dye/peptide ratio was estimated by the following equation:

$$\text{Dye/peptide} = \frac{A}{6.4} \times \frac{MW_P}{[P]}$$

Where $A$ is the absorbance at 385 nm; $MW$ is molecular weight; the suffix $P$ refers to the peptide; and $[P]$ is the concentration of poly-L-lysine in ug/ml.
The results obtained are shown in Table 4.

**Table 4**

The molar ratio of fluorescamine bound to poly-L-lysine.

<table>
<thead>
<tr>
<th>Labelling</th>
<th>Dye/Peptide Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>Mean</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The results are rather lower than expected but this may have partly been a result of steric hindrance, due to the flexibility of the peptide molecule, preventing binding of fluorescamine at all the primary amine groups. The figure of 8% loss on the column was possibly an exaggeration as some fluorescence would have been lost because of the somewhat unstable nature of the conjugate (see Part 1 of this chapter). However, the effects of this on the above calculation would have been slight.

**BINDING OF THE LABEL TO IgG**

Poly-L-lysine (2 mg) was labelled with fluorescamine by the method of Böhlen and the resulting solution adjusted to pH 6.0 by the addition of dilute HCl. To this solution was added 2 mg IgG in 4 mls phosphate buffer (pH 5.8, 0.1M) and 5 µg N,N'-dicyclohexyl carbodiimide in acetone (0.5 mls). The mixture was incubated at room temperature for 4 hours after which time 1 ml of 0.1 N acetic acid was added to remove excess carbodiimide by precipitation and filtration.

In order to remove excess labelled poly-L-lysine a 1 ml aliquot of the resulting mixture was put on a Sephadex G-50 column (0.9 x 24 cm) equilibrated with phosphate buffer (pH 8.0, 0.1M). The IgG fractions were pooled and the effects of the label on the immunogenic properties of IgG and on the limit of detection investigated.
Prior to this however, two aliquots were taken, one was stored and the other concentrated to 0.5 ml using an Amicon concentrator. This concentrated fraction was diluted to 1 ml with phosphate buffer containing 6M urea and placed on a Sephadex G-50 column (0.9 x 24 cm) equilibrated with the same buffer.

After measuring the volume of the pooled IgG fractions the volume of the stored aliquot was adjusted so that the two were equal. The fluorescence and absorbance at 280 nm of the two aliquots was then compared, the results being shown in Table 5.

Table 5
Effect of phosphate-urea buffer (0.1M, pH 8.0 and 6M urea) on IgG labelled with poly-L-lysine-fluorescamine conjugate. The figures are the mean of two results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at 280 nm</th>
<th>Fluorescence at 390 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.200</td>
<td>87</td>
</tr>
<tr>
<td>Treated with urea</td>
<td>0.177</td>
<td>73</td>
</tr>
</tbody>
</table>

These results indicate that most of the fluorescence associated with the labelled IgG is due to covalently bound poly-L-lysine-fluorescamine conjugate. The difference between the treated and untreated samples was probably due to loss of material on the column (an 8% loss of poly-L-lysine was recorded earlier) and non specific adsorption to the concentrator; with only a small proportion being due to removal of non covalently bound fluorescent material.

To investigate the immunogenic properties of the labelled IgG the Ouchterlony technique was employed: the results are shown in Fig. 14. In both Fig. 14a and 14b the centre wells contained a 1:10 dilution of rabbit anti-human IgG. The outer wells of Fig. 14a contained (in a clockwise direction): -
The outer wells of Fig. 14a contained the same dilutions of human IgG.

The results in Fig. 14 indicate that there is no loss of the ability of IgG to bind with its antibody when labelled with the poly-lysine - fluorescamine conjugate.

To follow the relationship between fluorescence intensity and concentration of the labelled IgG, a series of sequential dilutions were taken and the fluorescence intensity estimated. The results shown in Table 6 would indicate that the detection limit of IgG labelled with poly-lysine - fluorescamine conjugate (approximately 5 ng/ml) is no improvement on that of IgG.
labelled with fluoresceamine. The relationship appeared to be linear over the range indicated.

Table 6

Relation between fluorescence intensity and concentration of the IgG labelled with poly-lysine - fluoresceamine conjugate.

The figures are the mean of 4 results.

<table>
<thead>
<tr>
<th>IgG concentration (ng/ml)</th>
<th>Fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>1</td>
<td>6.0</td>
</tr>
<tr>
<td>5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>10.7</td>
</tr>
<tr>
<td>50</td>
<td>45.0</td>
</tr>
<tr>
<td>100</td>
<td>82.5</td>
</tr>
</tbody>
</table>

This result of no improvement on the detection limit was somewhat disappointing and was thought to be due to the poor binding of poly-lysine-fluoresceamine conjugate to IgG even when the carbodiimide reaction was carried out with a 100 x Molar excess of poly-lysine conjugate. However, when equimolar concentrations of lysine - fluoresceamine conjugate and poly-lysine - fluoresceamine conjugate were compared the expected increase in fluorescence intensity (see Table 7.) is non existent. It would be expected that there would be about a three fold increase in intensity as each poly-lysine molecule is labelled with 3 molecules of fluoresceamine. Here it appears that the fluorescence decreases.

Table 7

Comparison of fluorescence intensity of equimolar concentrations (100µl) of the fluoresceamine conjugates of lysine and poly-L-lysine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lysine</th>
<th>Poly-L-Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.0</td>
<td>32.0</td>
</tr>
<tr>
<td>2</td>
<td>44.5</td>
<td>33.0</td>
</tr>
<tr>
<td>3</td>
<td>44.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Mean</td>
<td>43.8</td>
<td>33.7</td>
</tr>
</tbody>
</table>
Solid phase assays were originally developed for radioimmunoassays in order to provide a simple, quick and effective separation step (see chapter 1, Part 2). Thus, as the investigation of heterogeneous fluorescence immunoassays was contemplated, it was thought possible that this technique might be adapted for use with a fluorescent label.

The majority of this section (a to o) deals with the description of attempts to adapt established, for radioimmunoassay, solid phase assays for use with a fluorescent label. This involved the use of Sepharose, Sephadex and polystyrene tubes as the solid phase. The last method investigated (d), an adaption of the fluorimetric assay of Alberse (32), was unlike the other methods in that it was not a competitive type of assay and involved the use of fluorescently labelled antibodies. This means that by the established nomenclature it was not a fluorescence immunoassay but an immuno fluorimetric assay (see chapter 1, Part 2).

a. Sepharose and Sephadex as the Solid Phase.

In all cases the assay followed the following scheme. To a glass tube containing 0.5 mls of a standard solution (0.1 - 2 µg/ml IgG) in phosphate buffered saline (0.1 M, pH 7.5 with 0.15 M NaCl) containing 0.2% B.S.A. (bovine serum albumin) or 0.5 mls of the phosphate buffer, was added 0.1 mls of a solution containing fluoresceamine labelled IgG (50 ng), followed by 0.5 mls or 0.2 mls of immunosorbent (solid phase with bound antibodies) or an equal amount of solid phase with no coupled antibodies. In all cases the final volume was 1.1 mls, made up to this with the phosphate buffer when 0.2 mls of immunosorbent was used. After 2 hours incubation at room temperature with gentle agitation the tubes were centrifuged for 5 minutes on a benchtop centrifuge. This was sufficient to sediment the solid phase. The supernatant was decanted and its fluorescence intensity determined.

1. Originally no standard IgG solution was used and the effect of 0.2 and
0.5 mls of Sephadex immunosorbent on 50 ng of labelled IgG was investigated. The figure of 50 ng was arrived at as a compromise of the need to use the smallest amount of labelled antigen possible and the amount needed to give a reasonable fluorescence intensity. The results in Table 8 show that the fluorescence of the supernatant was reduced by the immunosorbent, showing that binding to the immobilised antibody was removing labelled IgG. The 0.5 mls of immunosorbent removed about 40% of the IgG but the 0.2 mls only 10%. The high background was probably due to the intrinsic fluorescence of, and the scatter produced by BSA (see section e).

Table 8

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence Intensity (Arbitrary Units) at 390 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 mls Is</td>
</tr>
<tr>
<td>Solid phase (no Ab)</td>
<td>80</td>
</tr>
<tr>
<td>Immunosorbent</td>
<td>74</td>
</tr>
<tr>
<td>Background</td>
<td>22</td>
</tr>
</tbody>
</table>

11. In order to ascertain that the solid phase without coupled antibody was not removing any labelled IgG by adsorption, the effect of 0.5 mls of Sephadex and 0.5 mls of alpha-1-antitrypsin (an available non specific antiserum) bound to Sephadex on 50 ng labelled IgG were compared. The results in Table 9 show that very little labelled IgG was removed by adsorption or other effects, and both immunosorbent and Sephadex alone removed comparable amounts. The fluorescence values for labelled IgG were obtained by correcting the fluorescence intensity of labelled IgG in 1.1 mls of the phosphate buffer for the presence of Sephadex and are the theoretical values for the fluorescence intensity of the supernatant.
iii. After this, construction of a standard curve for the assay of unlabelled IgG was attempted. In order to do this unlabelled IgG, in a series of dilutions (0-2 µg/ml), was added to the incubation medium. The results were not always good, the IgG having no effect and failing to displace labelled IgG from the immunosorbert. However, a typical example of a standard curve produced when the unlabelled IgG was effective in displacing labelled IgG is shown in Table 10.

Table 10

Standard curve for IgG estimation using a solid phase technique with Sephadex as the solid phase. Where the sample is "Sephadex", the immunosorbert was replaced by Sephadex (i.e. no antibodies present).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence (Arbitrary Units) at 390 nm</th>
<th>% Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex</td>
<td>84.0</td>
<td>0</td>
</tr>
<tr>
<td>0µg/ml IgG</td>
<td>64.0</td>
<td>100</td>
</tr>
<tr>
<td>100µg/ml IgG</td>
<td>64.0</td>
<td>100</td>
</tr>
<tr>
<td>500µg/ml IgG</td>
<td>71.0</td>
<td>65</td>
</tr>
<tr>
<td>1µg/ml IgG</td>
<td>75.0</td>
<td>45</td>
</tr>
<tr>
<td>2µg/ml IgG</td>
<td>76.0</td>
<td>40</td>
</tr>
<tr>
<td>Background</td>
<td>22.0</td>
<td></td>
</tr>
</tbody>
</table>

The construction of a standard curve was also attempted, with similar results, using Sepharose bound anti-IgG. A typical example can be seen in Table 11.
Table 11

Standard curve for IgG estimation using a solid phase technique with Sepharose as the solid phase. Where the sample is "Sepharose" the immunosorbent was replaced by Sepharose (i.e. no antibodies present.)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence (Arbitrary Units) at 390 nm</th>
<th>% Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose</td>
<td>88.0</td>
<td>0</td>
</tr>
<tr>
<td>0ng/ml IgG</td>
<td>64.0</td>
<td>100</td>
</tr>
<tr>
<td>100ng/ml IgG</td>
<td>64.5</td>
<td>93</td>
</tr>
<tr>
<td>500ng/ml IgG</td>
<td>66.0</td>
<td>91</td>
</tr>
<tr>
<td>1ug/ml IgG</td>
<td>72.0</td>
<td>68</td>
</tr>
<tr>
<td>2ug/ml IgG</td>
<td>73.5</td>
<td>57</td>
</tr>
<tr>
<td>Background</td>
<td>22.0</td>
<td>-</td>
</tr>
</tbody>
</table>

b Polystyrene Tubes as the Solid Phase

Initially a polystyrene tube was placed in a spectrophotometer and its absorption spectrum determined. The resultant spectrum indicated that the polystyrene tubes are optically transparent over the wavelengths 300 - 650 nm, that is over the fluorescence spectrum of fluorescamine.

In the methods of Foti (35) and Cunningham (34) a range of dilutions of labelled IgG, between 0 and 100ng/ml, was used (see Chapter 2). However, the results indicated that the antibody coated tube had failed to bind any fluorescamine labelled IgG. The fluorescence of the supernatant was not reduced and no increase in the fluorescence associated with the polystyrene tubes was observed after incubation.

It would have been helpful if a figure for the amount of antiserum adsorbed to the polystyrene tubes had been obtained. This would have shown if the antibodies had failed to become attached to the inside of the tubes. In fact, no values for the amount absorbed appear to have been published for similar techniques (e.g. Foti (35) and Cunningham (34)).
c. **SPAC Assay for Thyroxine (T₄)**

Using the SPAC (Malinckrodt) assay kit for T₄, the antibody coated tubes were taken and to them was added 1ml barbital buffer (0.075 M, pH 8.6) containing a range of concentrations of fluoresceamine labelled T₄ (0-100ng/ml). After incubation at 37°C for 1 hour the supernatant was desanted and its fluorescence intensity measured. This value was compared with the fluorescence intensity of an equal concentration of labelled T₄ which had been kept at 37°C for 1 hour but had not been added to antibody coated tubes.

Unfortunately, the results indicated that no labelled T₄ was binding to the adsorbed antibodies as no significant decrease in fluorescence intensity of the supernatant was observed.

d. **Cellulose as the Solid Phase**

This followed the technique of Aalberse, but with some modifications. Discs (5mm) were punched from filter paper (Whatman – 20) and 250 mg of these discs were CNBr-activated by the method used for activation of Sephadex (see Chapter 2). After washing the discs were suspended in a solution of 20 ml 0.12 M NaHCO₃ (pH 8.4) containing 1.5 mls of anti-human IgG antiserum (about 3.4 mg of antibody by single radial immunodiffusion according to Dakopatts) and incubated overnight at room temperature. After blocking of any residual binding sites with ethanolamine and washing with saline (0.9%), the discs were washed with water, air dried and stored at -4°C.

In order to label the anti-human IgG with fluoresceamine 0.5 mls of the antiserum was taken and diluted to 2 mls with phosphate buffer (pH 8.0, 0.1M). To this solution was added an excess of the cyclophateglycosyllose complex and the labelling carried out by the method of Nakaya. The resultant solution was diluted to 25 mls. This preparation was carried out just before it was needed so that the labelled anti-IgG would be fresh.

In glass tubes were placed 3 of the antibody coated discs and 0.5 ml of a buffered solution containing a range of IgG dilutions (0-200ng/ml). The
buffer contained 0.1% Tween - 20, 0.1% B.S.A. and 0.1% Na \( \text{N}_3 \) in phosphate buffered saline (pH 7.4, 0.1 M and 0.15 M NaCl).

After incubation over night at room temperature under gentle agitation the disc were washed with 0.3 M NaCl (3 x 2 mls). The discs were then incubated with 0.5 mls of the labelled anti-human IgG for 5 hours. The cellulose bound fluorescamine was eluted by incubation for 10 minutes with 0.1 N NaOH and the fluorescence intensity measured.

The results for one such experiment are shown in Table 12 and were obtained two days after preparation of the antibody coated discs. Experiments after 6/7 days failed to produce any positive results, possibly due to loss of immunological properties by the anti-human IgG.

Table 12

Solid phase assay for IgG using cellulose as the solid phase and the method of Aalbers et al. The figures are the mean of 3 results.

<table>
<thead>
<tr>
<th>IgG Concentration (ng/ml)</th>
<th>Fluorescence (Arbitrary Units) at 390 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>10</td>
<td>18.0</td>
</tr>
<tr>
<td>25</td>
<td>19.5</td>
</tr>
<tr>
<td>50</td>
<td>22.0</td>
</tr>
<tr>
<td>100</td>
<td>25.0</td>
</tr>
<tr>
<td>200</td>
<td>29.3</td>
</tr>
</tbody>
</table>

Fluorescence of Bovine Serum Albumin (BSA)

As BSA was known to possess an intrinsic fluorescence and as it was used in the preceding assays, it was decided that an investigation of the effects of BSA on the fluorescence of fluorescamine labelled IgG was necessary. Thus, to 0.5 mls of phosphate buffer (0.1 M, pH 7.5) or to 0.5 mls of phosphate buffer containing fluorescamine labelled IgG (approximately 100ng/ml) was added 0.5 mls of a BSA solution (0.1% - 1.0%) in the phosphate buffer. The fluorescence intensity of the resultant mixture was then
The results are shown in Table 13.

**Table 13**

The effects of BSA on the fluorescence of fluorescamine labelled IgG, showing the mean of 4 results.

<table>
<thead>
<tr>
<th>BSA Concentration in %</th>
<th>Fluorescence Intensity (x 10)</th>
<th>(a) with labelled IgG</th>
<th>(b) without labelled IgG</th>
<th>of the labelled IgG (a-b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>7.2</td>
<td>0.8</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>9.7</td>
<td>1.6</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>19.0</td>
<td>3.5</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>28.7</td>
<td>6.6</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>37.0</td>
<td>9.7</td>
<td>27.3</td>
<td></td>
</tr>
</tbody>
</table>

The results indicate that even though the BSA has only a slight fluorescence it produces a substantial increase in the fluorescence of the labelled IgG. This effect was noted by Tengerdy (87) who found that the presence of normal rabbit serum produced a considerable increase in the fluorescence of fluorescein - labelled IgG. Strangely he found that the presence of the specific antibody to IgG quenched the fluorescence of the fluorescein labelled IgG.
After initial unsuccessful work it was decided to establish the conditions for a double antibody technique by using radioactively labelled IgG. Thus, IgG was labelled with $^{125}$I (see Chapter 2) and the reaction mixture separated on a Sephadex G-25 column (Fig.15). It was deduced that the labelling of the IgG had been successful and 53% of the added $^{125}$I had become incorporated with the IgG.

The resultant labelled IgG was used in an assay based on the technique described by Glioch et al. (19). All the reagents were dissolved in phosphate-albumin buffer (0.1 M, pH 7.5 containing 0.1% BSA). To a glass tube were added the following reagents: 1) 0.5 mls of buffer, 2) 0.1 mls of rabbit anti-human IgG in a suitable dilution, and 3) 0.1 mls of radiolabelled IgG (approximately 50ng). After incubation with occasional agitation at 37°C for 2 hours, the following were added: 1) swine anti-rabbit IgG in a dilution of 1:10 (0.15 mls) and 2) a 1:20 dilution of normal rabbit serum (0.15 mls). This mixture was then incubated for a further 2 hours after which the tubes were centrifuged at 3,500 r.p.m. for 30 minutes, the supernatant decanted and the radioactivity of the residue measured.

In order to ascertain the optimum first antibody dilution for these assay conditions about 50ng of $^{125}$I labelled IgG and a series of dilutions of rabbit anti-human IgG was used. The results are shown in Fig.16a. The percentage binding was obtained by the following equation:

$$\% \text{ Binding} = \frac{A - B}{A} \times 100$$

Where A was the c.p.m. (counts per minute) of the added labelled IgG and B the c.p.m. of the residue from the assay mixture. In both cases the background, found by measuring the radioactivity produced by an assay mixture containing no labelled IgG, was first subtracted from the value obtained.

From these results it was decided to use a dilution of first antibody of 1:200 in producing a standard curve in a fluorescence immunoassay.
Fig. 15 Separation of $^{125}$I labelled IgG from excess $^{125}$I by elution from a sephadex column (G-25, 0.9 x 24 cm)
In this assay a range of dilutions of unlabelled IgG was added to the assay mixture instead of 0.5 mls of buffer and 50ng of fluoresceamine labelled IgG replaced the radio labelled IgG. The results are shown in Fig.16b. The % Binding was obtained by using the following equation:

\[
\% \text{ Binding} = \frac{I_0 - I}{I_0 - I_{100}} \times 100
\]

Where \( I_{100} \) is the fluorescence intensity when the assay mixture contained no unlabelled IgG (i.e., 100% binding); \( I_0 \) is the fluorescence intensity when no first antibody was present (i.e., 0% binding) and \( I \) is the fluorescence intensity where unlabelled IgG was present.

The results form a typical sigmoid curve, obtained for the standard curve of most immunoassays. However, the actual changes in fluorescence were only small as the fluorescence intensity at 0% binding was 52 units and the fluorescence intensity at 100% binding 44 units. The results were the mean of two experiments.
Fig. 16  

a) Antibody dilution curve using approximately 50ng labelled IgG in a double antibody assay. 

b) Standard curve for estimation of IgG in a double antibody assay.
As there is a need for quick and simple homogeneous assays, it was decided to investigate the possibility of using fluorescence polarization. In order to become conversant with the technique some of the work by Chen (90) was repeated: parts of the polarization spectra of fluorescamine labelled glutamate and BSA were investigated. Both compounds were labelled by the method of Bühlen and the solution diluted to obtain a concentration of $10^{-5} \text{M}$ with a solution consisting of 90% glycerol and 10% phosphate buffer (pH 7.4, 0.1 M). The fluorescence polarization spectra were then determined by the method described in Chapter 2 and with the emission wavelength set at 490 nm.

The results in Table 14 show that the degree of polarization increases with increasing excitation wavelength; they also compare favourably with the results obtained by Chen (90). The values for BSA are somewhat higher than those Chen obtained but these results may be attributed to a lower dye/protein ratio, causing less energy transfer in the labelled BSA. The figures for glutamate are slightly lower than those of Chen.

**Table 14**

Fluorescence polarization of fluorescamine labelled BSA and glutamate in a 90% glycerol - 10% phosphate buffer (0.1 M, pH 7.4 solution). Emission wavelength of 490 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Excitation Wavelength</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA -</td>
<td>390 nm</td>
<td>0.2824</td>
</tr>
<tr>
<td>Fluorescamine</td>
<td>400 nm</td>
<td>0.2910</td>
</tr>
<tr>
<td></td>
<td>410 nm</td>
<td>0.3078</td>
</tr>
<tr>
<td>Glutamate -</td>
<td>390 nm</td>
<td>0.3021</td>
</tr>
<tr>
<td>Fluorescamine</td>
<td>400 nm</td>
<td>0.3243</td>
</tr>
<tr>
<td></td>
<td>410 nm</td>
<td>0.3601</td>
</tr>
</tbody>
</table>

Following this, the effect of antibody on the degree of polarization of fluorescence of fluorescamine labelled $T_4$ and IgG was investigated. In
the case of IgG, to 0.5 ml of a phosphate buffer solution (pH 7.5, 0.1 M) of fluorescamine labelled IgG (5 µg) was added 0.5 ml of antiserum (1:5 - 1:20 dilutions of anti-human IgG) and the mixture made up to 2.5 ml with the phosphate buffer. After 10 minutes incubation at room temperature the fluorescence and hence the degree of polarization of the solutions was measured. In the case of T4, to 0.5 ml of fluorescamine labelled T4 (200 ng/ml) in barbital buffer (pH 8.6, 0.075 M) was added 0.5 ml of antiserum (1:10 - 1:40 dilutions of anti-T4) and the mixture made up to 2.5 ml with barbital buffer. The polarization of the solutions was measured after 2 and after 10 minutes incubation at room temperature.

The results for IgG (Table 15) are what were expected; IgG is a large molecule and will thus have a large degree of polarization because of its slow rotation due to Brownian motion. Binding with antibody will double its molecular size but as it is a large molecule the decrease in rotation will be relatively small and any increase in polarization slight, as is demonstrated.

Table 15

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antibody Dilution</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td></td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>0.309</td>
</tr>
<tr>
<td>T4 after</td>
<td></td>
<td>0.417</td>
</tr>
<tr>
<td>2 minutes</td>
<td>1:40</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>0.426</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>0.428</td>
</tr>
<tr>
<td>T4 after</td>
<td></td>
<td>0.417</td>
</tr>
<tr>
<td>10 minutes</td>
<td>1:40</td>
<td>0.425</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>0.428</td>
</tr>
</tbody>
</table>
It is possible that if a longer incubation period had been used the increase in the degree of polarization would have been larger. As the incubation time is increased the nearer equilibrium would be the antibody - antigen binding reaction and therefore more labelled IgG would be bound to antibody.

The results obtained for T₄ were, however, completely unexpected. The degree of polarization was initially very high, higher than that of IgG, and binding with antibody produced only slight increases in polarization. For comparison, the degree of polarization of fluorescein labelled gentamicin (a compound of about half the molecular weight of T₄) is 0.08, which rises to a maximum of 0.2 on binding with antibody.

In addition, the results for thyroxine were very variable the figures in Table 18 being an example only, and in one instance the degree of polarization appeared to decrease on addition of antibody (from 0.414 to 0.401). Some of these problems may have been a consequence of the low concentrations of labelled T₄ used, causing low fluorescence intensities to be observed. However, in all cases the fluorescence of both vv and vh results (see chapter 2) appeared to increase on the addition of antibody. It was thus decided to repeat the experiments with T₄ but without the presence of polarizing film in the fluorimeter. The results, corrected for antibody fluorescence, in Table 16 show that there is a fluorescence enhancement effect on the binding of labelled T₄ to antibody which can not be attributed to either antibody fluorescence or an increase in viscosity. This enhancement is in opposition to the findings of Tengerdy (87), who found the fluorescence of fluorescein labelled human gamma globulin (HGG) was decreased by addition of a specific anti - HGG antibody.

58
Table 16

The enhancement of fluorescence of fluorescamine labelled T₄ by the addition of anti-T₄ antibody.

<table>
<thead>
<tr>
<th>Antibody Dilution</th>
<th>Fluorescence (Arbitrary Units) at 390 nm (λex.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>1:40</td>
<td>68</td>
</tr>
<tr>
<td>1:20</td>
<td>79</td>
</tr>
<tr>
<td>1:10</td>
<td>84</td>
</tr>
<tr>
<td>Anti IgG (1:10)</td>
<td>50</td>
</tr>
</tbody>
</table>
An antibody dilution curve was constructed by the addition of a series of 1 ml antiserum dilutions to 1 ml of a fluorescamine labelled T₄ solution (about 10ng/ml). Both reagents were diluted in barbital buffer (0.075 M, pH 8.6). After incubation at room temperature for 15 minutes the fluorescence of each mixture was measured with the coarse gain of the fluorimeter set at ×1K. In order to correct for the background contribution of the antiserum, the fluorescence of the antiserum dilutions were also measured separately in the absence of T₄. These corrected results are shown in Fig.17. From this curve it can be seen that the fluorescence of labelled T₄ is enhanced on binding with antibody and a maximal enhancement, of about 4 fold, is obtained at an antibody dilution of 1:100. The data obtained from this curve, which is of a typical sigmoid shape, allowed the antiserum dilution for a standard curve to be determined.

Initially a standard curve was constructed using 0.5 ml aliquots of T₄ diluted to a known concentration in the barbital buffer. These were added to 0.5 mls of fluorescamine labelled T₄ (20ng/ml), followed by 0.5 mls of anti-T₄ serum. The anti-serum was used in an initial dilution of 1:200 (1:600 final dilution) on the basis of information gained from the antibody dilution curve. After incubation at room temperature for 15 minutes, the fluorescence of the mixtures was measured (at a fluorimeter gain setting of ×1K) and corrected for the fluorescence of the antiserum. The results in Fig.18a show that unlabelled T₄ competes for antibody binding sites, causing a decrease in observed fluorescence with increasing concentration of added T₄.

It should be noted that other antisera (e.g. anti-T₂) did not produce an enhancement of fluorescence and anti-T₂ did not affect the fluorescence of either fluorescamine labelled Triiodothyronine (T₃) or Diiodothyronine (T₂). Also, addition of 100 ng of T₃ to the assay medium of T₄ had no effect on the standard curve.
Fig 17 Antibody dilution curve for a fluorescence enhancement assay of T₄ showing mean of 6 results
**Fig. 18**

**a)**

Fluorescence (arbitrary units) vs. T4 Concentration (ng/ml)

**b)**

Fluorescence (arbitrary units) vs. T4 Concentration (ng/ml)
FIG. 10

a) Standard curve of $T_h$ from Barbital buffer (pH 8.6, 0.1M), showing the mean and S.E.M. of 6 results.

b) Standard curve of $T_h$ from serum, showing mean and S.E.M. of 6 results.
These findings of fluorescence enhancement are in accordance with the results of Smith, who discovered that the fluorescence of fluorescein labelled $T_4$ was enhanced by antiserum (46), but contradict the findings of Tengordy (87).

**ASSAY OF THYROXINE IN SERUM**

It is normal to wish to measure $T_4$ concentrations in serum. Thus, a series of $T_4$ solutions of known concentration were prepared in pooled $T_4/T_3$ depleted serum. Aliquots (0.25 ml) of these solutions were diluted to 0.5ml with barbital buffer to which was added 0.5 ml of labelled $T_4$ solution (20ng/ml) and 0.5 ml of anti-$T_4$ serum (in a 1:200 dilution). After incubation at room temperature for 15 minutes, the fluorescence of the solutions was determined (the fluorimeter course gain was set at x 100). In order to correct for the background contributed by the serum, the fluorescence of a serum sample containing no labelled $T_4$ but made up to 1.5 ml with the antiserum and barbital buffer was measured. The results, shown in Fig.18b, show that the effect of unlabelled $T_4$ on the enhancement of fluorescence of labelled $T_4$ is still observed. The results however do not indicate the size of the background "fluorescence" produced by serum, which is about 30 x that produced by barbital buffer.

The high background of serum may be caused by either the fluorescence of species present in the serum or by the scattering of light. If the latter, the high background would be greatly reduced by the presence of an horizontal polarizer in the incident beam of the fluorimeter. This is because it has been shown that horizontally polarized light reduces the effect of scattered light and improves the sensitivity of standard fluorescence curves (88). Therefore, the previous experiment was repeated and the fluorescence measured (the course gain set at x300) with a strip of polarizing film present in the incident beam of the fluorimeter to produce an horizontally polarized beam of light. The results, Fig.19a, show that the effect of unlabelled $T_4$ on the
FIG. 19
Fig. 19

a) Standard curve of T₄ from serum using horizontally polarized incident light, showing mean and S.E.M. of 6 results.

b) Standard curve of T₄ from serum using M.D.P.F. as the fluorescent label, showing mean and S.E.M. of 6 results.
Fig. 20 Antibody dilution curve for T₃ using fluorescence enhancement and showing the mean of 6 results.
enhancement of labelled T₄ is still observed, but the background (not shown) was only reduced by approximately ¾. This indicated that the background was due mainly to fluorescence. Also, as polarizing film reduces the intensity of light passing through it, some sensitivity must be lost.

It is interesting to note that when the results are adjusted to be comparable with those obtained with barbital buffer (when the fluorimeter course gain was set at x1K), the serum appears to produce an overall increase in fluorescence as well as a high background. This would agree with other findings that serum increased the fluorescence of fluorescently labelled compounds (87).

ASSAY OF THYROXINE USING MDPF AS A FLUORESCENT LABEL

Using the protocol as described in the previous experiment, a standard curve for the assay of thyroxine in serum was constructed using T₄ labelled with MDPF. That is, to aliquots of serum containing T₄ was added 0.5 ml of MDPF labelled T₄ and 0.5 ml of antiserum. After incubation at room temperature for 15 minutes the fluorescence of the solutions was determined with the excitation and emission wavelengths set at 390 nm and 490 nm respectively as when fluorescamine was used as the label (the course gain of the fluorimeter was set at x100). The results, shown in Fig.19b, are very similar to those obtained using fluorescamine as the fluorescent label.

CONSTRUCTION OF AN ANTIBODY DILUTION CURVE FOR T₃

Using the same techniques as for T₄ an antibody dilution curve for the assay of T₃ was prepared using anti-T₃ serum and fluorescamine labelled T₃ (20ng/ml). The results Fig.20, indicate that a similar phenomenon occurs in the case of labelled T₃ as occurs for labelled T₄. That is, the binding of fluorescamine – labelled T₃ to its antibody enhances the fluorescence. However, the enhancement does not appear to be as great as was the case for T₄, being only about two fold.

It should also be noted that no enhancement effect was observed when
other antisera (e.g. anti-\(T_4\)) were used in place of anti-\(T_3\) serum.

**THE USE OF RHODAMINE IN THE ASSAY OF THYROXINE**

As serum fluoresces in the same region as fluorescamine (Fig. 21a), a property which would vary in samples from different sources, it was thought that thyroxine labelled with a fluorescent compound which fluoresced at a longer wavelength than fluorescamine would be an advantage. Rhodamine, which has an excitation and emission maximum at 560 and 594 nm respectively (see Fig. 22a), well above the fluorescence of serum, was thought to be ideal. Thus, thyroxine was labelled with rhodamine as described in Chapter 2. The purity of the resultant complex was monitored by chromatography (Fig. 21b) on Whatman No. 1 paper developed by a solution consisting of butanol, glacial acetic acid and water in a ratio of 6:2:2. The rhodamine was visualised by its red colour and the iodothyronine function of \(T_4\) by a diazotised sulphoanilic acid spray (Pauly reagent).

The absorbance of the resultant complex was determined and this is shown in Fig. 22b. As can be seen the complex absorbs in the regions of rhodamine and \(T_4\), indicating that each moiety behaves independently in this respect. This enabled the concentration of rhodamine and \(T_4\) in a solution of the complex to be determined by comparison with standard curves of rhodamine and \(T_4\) prepared independently. From this it was discovered that the complex purified by ultrafiltration had a molar ratio of rhodamine to \(T_4\) of about 0.9 and by gel chromatography of about 1.1. The latter possibly indicates that some rhodamine impurity may have become adsorbed to the Sephadex and been removed with the rhodamine - labelled thyroxine.

When the rhodamine labelled \(T_4\) was used to attempt to construct an antibody dilution curve and a standard curve no enhancement of fluorescence was observed. This means that either the immunogenic properties of the labelled \(T_4\) have been destroyed or that the enhancement effect shown on binding with antibody by fluorescamine labelled \(T_4\) or fluorescein labelled
a) Fluorescence spectrum of serum diluted 1:50 in barbital buffer (pH 8.6, 0.075M)

b) Chromatogram of Rhodamine/Rhodamine labelled $T_4/T_4^*$, developed by butanol-glacial acetic acid-water (6:2:2).
FIG 22

a) Fluorescence spectrum of Rhodamine labelled T4,
10μg/ml in barbital buffer (pH 8.6, 0.075M).

b) Absorbance spectrum of Rhodamine labelled T4,
10μg/ml in water.
Fig. 22
T4 (4.6) does not occur. Therefore, a known dilution of thyroxine, as the complex, was estimated by R.I.A. (Radiocchemical Centre).

The results are shown below in Table 17.

**Table 17**

Estimation of thyroxine labelled with rhodamine by R.I.A. Shown are the mean of 4 results.

<table>
<thead>
<tr>
<th>Sample ng T4/ml</th>
<th>Radioactivity Counts/100s.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards:</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29,000</td>
</tr>
<tr>
<td>40</td>
<td>19,159</td>
</tr>
<tr>
<td>114</td>
<td>12,093</td>
</tr>
<tr>
<td>212</td>
<td>9,062</td>
</tr>
<tr>
<td>Complex purified by ultrafiltration:</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>32,2027</td>
</tr>
<tr>
<td>Complex purified by chromatography:</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>31,032</td>
</tr>
</tbody>
</table>

The conclusion to be drawn from these results, is that the T4 has lost its ability to bind to antibody on being labelled with rhodamine.

**BASIS OF THE ENHANCEMENT EFFECT OF FLUORESCAMINE AND MDPF**

Originally it was thought that the phenomenon of fluorescence enhancement was linked to an "heavy atom" effect (89) of the iodine atoms of the iodothyronine moiety of T4 and T3. If this was true, comparison of the fluorescence of equimolar concentrations of fluorescamine labelled T4 and another amino acid (e.g. glutamic acid) would show the fluorescence of the labelled T4 to be lower than that of the labelled amino acid. Also, the addition of KI to a solution of the labelled glutamic acid might have the
effect of quenching the fluorescence. Two such experiments were carried out and compared with the results obtained with rhodamine/fluorescein isothiocyanate labelled T₄ and glutamic acid. The first compared solutions of 2 μM of labelled T₄ and glutamic acid in phosphate buffer (pH 8.0, 0.1M). The second was carried out by adding KI solutions of varying concentration to a solution of labelled glutamic acid in phosphate buffer (pH 8.0, 0.1M). The final volume of the solutions was 2.5 mls with a final concentration of labelled glutamic acid of 0.1 μM.

Table 18

Comparison of the fluorescence of equimolar (2 μM) concentrations of fluorescently labelled T₄ and glutamic acid.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescane Fluorescin Rhodamine</th>
<th>arbitrary units</th>
<th>Label</th>
<th>Label</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>23</td>
<td>83</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroxine</td>
<td>23</td>
<td>42</td>
<td>78</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results in Tables 18 and 19 would indicate that the heavy atom effect is involved in the enhancement of fluorescein labelled T₄, as found by Smith (46). However, it would appear that some other mechanism is involved in the case of fluorescamine, as KI does not effect the fluorescence of fluorescamine labelled glutamic acid, which has the same fluorescence intensity as fluorescamine labelled T₄. In the case of the rhodamine label, the KI does effect the fluorescence of labelled glutamic acid but there is no difference in the fluorescence of rhodamine labelled T₄ and glutamic acid.
Table 12
Effect of KI on the fluorescence of glutamic acid (0.1 μM) labelled with fluorescamine, fluorescein and rhodamine.

<table>
<thead>
<tr>
<th>Final KI concentration (nM)</th>
<th>Fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescamine</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>15.6</td>
<td>80</td>
</tr>
<tr>
<td>31.3</td>
<td>79</td>
</tr>
<tr>
<td>62.5</td>
<td>80</td>
</tr>
<tr>
<td>125</td>
<td>78</td>
</tr>
<tr>
<td>250</td>
<td>78</td>
</tr>
</tbody>
</table>

The quantum yield of fluorescamine labelled T₄ was also investigated, along with that of T₃ and T₂. This was carried out using a modification of the formula:

$$\bar{E}_u = \bar{E}_s \times \frac{F_u}{F_s} \times \frac{q_u}{q_s} \times \frac{A_u}{A_s}$$

Where $\bar{E}$ is quantum yield; $F$ relative fluorescence determined by integrating the area beneath the corrected fluorescence spectrum; $q$ is the relative photon output of the source of the excitation wavelength; $A$ is the absorbance; and the suffixes $u$ and $s$ refer to the unknown and standard.

However, as a comparison (standard) the quantum yield of glutamic acid as determined by Chon (92) was used. Then, as the same excitation wavelength was used for detecting the fluorescence of both labelled glutamic acid and thyronine derivative the $q$ values will cancel out. Also as the same label was being compared it was thought permissible to use the arbitrary fluorescence intensities (i.e. peak heights) at the fluorescence maxima instead of $F$. Thus the equation will reduce to:

$$\bar{E} = 0.111 \times \frac{F_u}{F_s} \times \frac{A}{A_u}$$

66
Where }A\text{ is the absorbance at 385 nm; }F\text{ is the fluorescence at the emission maximum; 0.11 is the quantum yield of fluorescamine labelled glutamic acid (standard); and the suffixes }u\text{ and }s\text{ refer to unknown and standard.}

To find the quantum yields fluorescamine derivatives were prepared by the method of Nakaya and the hydrolysis products of fluorescamine removed by gel filtration. In the case of glutamic acid this was done using a sephadex G-15 column (0.9 x 12 cm) equilibrated with distilled water. For thyronine derivatives a sephadex G-25 column (0.9 x 12 cm) equilibrated with ammonium bicarbonate (see Chapter 2 for preparation of rhodamine labelled }T_4\text{) was used, the thyronine derivatives being removed from the column by distilled water. After dilution with phosphate buffer the absorbance and fluorescence of the labelled compounds were determined.

The results in Table 20 are within the values obtained by Chen for most fluorescamine labelled amino acids. This further demonstrates that in the case of fluorescamine as a fluorescent label the iodine atoms are not significantly affecting the fluorescence. If they were the quantum yields of thyronine derivatives would have been found to be much lower. Thus, the enhancement of fluorescence must be by some action not involving the heavy atom effect.

### Table 20

The quantum yield of fluorescamine labelled thyronine derivatives.

<table>
<thead>
<tr>
<th>Sample</th>
<th>}A\text{</th>
<th>}F\text{</th>
<th>}F\text{</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>0.025</td>
<td>40</td>
<td>0.11</td>
</tr>
<tr>
<td>}T_2\text{</td>
<td>0.044</td>
<td>99</td>
<td>0.155</td>
</tr>
<tr>
<td>}T_3\text{</td>
<td>0.035</td>
<td>65</td>
<td>0.13</td>
</tr>
<tr>
<td>}T_4\text{</td>
<td>0.038</td>
<td>52</td>
<td>0.095</td>
</tr>
</tbody>
</table>
As the enhancement effect did not appear to be associated with the iodine atoms it was thought possible for the phenomenon to occur with other fluorescamine labelled compounds (e.g. IgG and albumin). In the case of IgG, an antibody dilution curve was constructed by adding 0.5 mls of a series of anti-IgG antiserum dilutions to 1.0 mls of fluorescamine labelled IgG solutions (500ng/ml). Both reagents were diluted in barbital buffer (pH 8.6, 0.075 M). After incubation at room temperature for 15 minutes the fluorescence of the solutions was measured and corrected for the fluorescence of the antibody. The results in Table 21 show that fluorescence enhancement occurs on the binding of IgG with its antibody. A maximum increase of about 3 fold is obtained with an antiserum dilution of 1:10.

Table 21

The effect of antibody on the fluorescence of fluorescamine labelled IgG; showing the mean of 3 results.

<table>
<thead>
<tr>
<th>Antibody Dilution</th>
<th>Fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>1:5</td>
<td>75</td>
</tr>
<tr>
<td>1:10</td>
<td>76</td>
</tr>
<tr>
<td>1:20</td>
<td>64</td>
</tr>
<tr>
<td>1:40</td>
<td>58</td>
</tr>
<tr>
<td>1:80</td>
<td>43</td>
</tr>
</tbody>
</table>

On the basis of the data obtained from these results an antiserum dilution of 1:20 was chosen to construct a standard curve. To 0.5 mls of labelled IgG (1pg/ml) was added 0.5 mls of unlabelled IgG solution (1-16pg/ml) and 0.5 mls of antiserum. As previously all dilutions were in barbital buffer. After incubation for 15 minutes at room temperature the fluorescence of the solutions was determined and corrected for antibody fluorescence. The results in Table 22 show that unlabelled IgG competes for antibody binding sites.
decreasing observed fluorescence with increasing IgG.

Table 22
The effect of unlabelled IgG on the fluorescence enhancement of fluorescamine labelled IgG by antibody; showing the mean of 2 results.

<table>
<thead>
<tr>
<th>Added IgG (µg/ml)</th>
<th>Fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
</tr>
<tr>
<td>16</td>
<td>38</td>
</tr>
</tbody>
</table>

In the case of albumin labelled with fluorescamine the results were kindly supplied by Mr. C.S. Lim who was working with albumin and its specific antibody at the time. The results in Table 23 show that an increase in fluorescence is obtained on binding of the labelled albumin with its specific antibody. These two results would further indicate that the enhancement phenomenon is a property of the fluorescamine label, occurring when it is associated with an antibody molecule. It also indicates that the technique of fluorescence enhancement could possibly be adapted for the assay of a wide variety of compounds that could be labelled with fluorescamine.

Table 23
Enhancement of the fluorescence of fluorescamine labelled albumin (albumin - F) by a specific antibody; results supplied by Mr. C.S. Lim. The results are corrected for antibody fluorescence and are at a concentration of albumin - F of 2.3 x 10^{-5} M.

<table>
<thead>
<tr>
<th>Antibody/Albumin - F Molar Ratio</th>
<th>% Increase in Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.107</td>
<td>2.4</td>
</tr>
<tr>
<td>1.07</td>
<td>8.3</td>
</tr>
<tr>
<td>10.7</td>
<td>88.0</td>
</tr>
</tbody>
</table>
CHAPTER 4

DISCUSSION

The choice of the compounds $T_4$ and IgG for this work was due to a variety of reasons. They are widely different in molecular weight, so affording a contrast; they both possess primary amine groups, so are easily labelled with fluorescamine; and antisera for both compounds are commercially available. It is also interesting to note that $T_4$ determination is currently one of the most requested clinical assays, its level giving an indication of thyroid function and overall metabolic condition.

Labeling with fluorescamine is quick and simple. It is not usually necessary to separate unconjugated label from the labeled compound as the hydrolysis products of fluorescamine are nonfluorescent. Only in high concentration do these hydrolysis products quench fluorescence by the inner filter effect (52). The spectra of all fluorescamine derivatives are in the same region, having excitation and emission maxima of about 385 nm and 495 nm respectively.

The detection limits of $T_4$ and IgG are comparable with those obtained in previous work but are inferior to those obtained in the detection of compounds with radio isotope labels (61). Also, the fluorescent products are not stable, the fluorescence declining over a number of hours. Indeed, Reiterer (67) has observed that fluorescamine labelled products are light sensitive and obtained a 50% loss of fluorescence over 30 minutes, a much greater loss than found in this work. Because of this it would normally be necessary, in carrying out a fluorescence immunoassay, to prepare fluorescamine derivatives immediately before use or, if stored in a lyophilised form, reconstitute them immediately before use. The possibility that fluorescamine derivatives may be stored without loss whenfreeze-dried and kept at $-20^\circ C$ is suggested by the detection limit of IgG being approximately the same when the conjugate is prepared fresh as when it is freeze-dried immediately after preparation. More work would be necessary to
determine how long the lyophilised conjugates could be stored before they began to deteriorate. The instability would also preclude the use of fluorescamine derivatives being used in assays requiring a long incubation period.

In order to obtain a high chemical yield in the labelling reaction (e.g. a dye to IgG and T₄ ratio of 10 and 0.9 respectively) a great excess of fluorescamine (100-500 fold) is needed. This finding appears to be at odds with the kinetics of the reaction. In order to explain this anomaly, and the findings that the reaction rate differs with different amines (66), Chen suggested the following reaction scheme (92) for the conjugation of fluorescamine with primary amines:

1. A + Fl ↔ [Fl - A] →→ FP
2. Fl + [Fl-A] \(\overset{H_2O}{\longrightarrow}\) NP + A
3. B + Fl ↔ [Fl - B] \(\overset{H_2O}{\longrightarrow}\) NP + B

Where A is the amine; Fl is fluorescamine; FP, fluorescent product; NP, nonfluorescent product; B, base; Fl - A, a secondary amine intermediate, and Fl - B an adduct of fluorescamine with base.

The secondary amine intermediate of reaction 1 was postulated by Stein (65) and is non-fluorescent as all adducts of secondary amines with fluorescamine have been shown to be non-fluorescent (93).

It is thought that the formation of the secondary amine intermediate is followed by several steps leading to the fluorescent product. Reaction 2. seems likely as fluorescamine reacts rapidly with secondary amines and though it is not certain what products would be formed the regeneration of A is probable. It would also explain the low yields often attained and why amino acids enhance the inactivation of fluorescamine (92). Reaction 3. represents hydrolytic deactivation of fluorescamine and accounts for the different hydrolysis rates of different buffers at the same pH(92). Water itself only deactivates fluorescamine slowly and it is stable for days when
in a 50% solution of acetone-water or when in a cycloheptamylose complex (64).

The scheme also explains why it has been found that sequential addition of fluorescamine is more effective than the addition of the same total amount in a single aliquot as reactions 2. and 3. are dependent on fluorescamine concentration. This would indicate that the Nakaya method of labelling amines (64) (see Chapter 2) would be more efficient than the method of Böhlen (61), as the cycloheptamylose complex presumably only releases fluorescamine slowly. Chen has also shown that the fluorescent product may be in equilibrium with free amine as the fluorescamine products are almost as strongly ninhydrin positive as free amines. This would go some way to explain the instability of fluorescamine-labelled compounds, the free fluorescamine in the equilibrium being available for deactivation by base.

An immunoassay relies on the avidity of the antibody and on having the smallest possible amount of labelled antigen present. A fluorescence assay thus depends on the extent of labelling possible without loss of immunogenic properties. Handschin (85) has stated that though it is usual in the case of immunoglobulins to obtain a molar dye-to-protein ratio of from 4 to 14, in exceptional circumstances this ratio could be increased to 20 without loss of antigenic properties. If the label has overlapping excitation and emission spectra, as does fluorescamine, there is the possibility of energy transfer between fluorescent moieties, thus introducing another factor in obtaining optimum labelling. Too high a dye/protein ratio and energy is transferred between neighbouring fluorescamine molecules, producing a quenching effect; too low a ratio and the required detection limit is not reached. It would appear from the results that both IgG and T4 are labelled close to the optimum limit as both retained their immunogenic properties and the detection limit of IgG was about the value reported by other workers. It may be possible for the detection limit to be improved by improvement of the
fluorimeter with such devices as a laser light source and a photon counter as the detection device. These changes may also improve the very variable sensitivities encountered in the use of conventional fluorimeters.

As can be seen from Part 2 of Chapter 3 fluorescamine labelled poly-L-lysine can be coupled to IgG (and hence other proteins) by a carbodiimide reaction resulting in a fluorescent labelled IgG molecule which retains its immunogenic properties. However, no advantage was apparent from having more than one fluorescamine molecule at each locus as the detection limit from IgG was not improved. Indeed, equimolar concentrations of fluorescamine labelled lysine and poly-L-lysine (having a molar ratio of dye to peptide of approximately 3) show little difference in fluorescence intensity. This was most likely due to energy transfer between fluorophore groups on the poly-L-lysine. Loss of the slightly higher fluorescence of the fluorescamine labelled poly-L-lysine may have occurred by deactivation of some of the fluorescamine labels in the conditions necessary for the carbodiimide reaction. If of no advantage for labelling proteins the poly-L-lysine conjugate may be of use in the labelling of suitable compounds which do not posses primary amine groups but which do have carboxyl groups; coupling of the carboxyl group to a primary amine group on the poly-L-lysine by a carbodiimide reaction.

In Part 3 of Chapter 3 the work with Sepharose and Sephadex indicates that a solid phase assay could be devised with the utilisation of a fluorescent label. The immunosorbent (consisting of antibody covalently bound to the solid phase) was able to bind fluorescamine labelled IgG, and when unlabelled IgG was present competition for the available binding sites occurred with the result that less labelled IgG was bound to the immunosorbent. Unfortunately the changes in fluorescence intensity obtained were only small and further research is needed to rectify these short-comings. Some of these failures may have been caused by poor binding of antibody to the solid phase. In the use of polystyrene as a solid phase, for both IgG and T4, the failure to detect any changes in fluorescence intensity may have
been because only very small amounts of antibody were bound to the polystyrene. This fact is unimportant in radioimmunoassay (R.I.A.), for which such techniques were first developed, because of the ability to detect very small amounts of radio labelled compounds (picogram and even femtogram levels) and hence detect small changes in the concentration of radio labelled compounds in solution. It is, however, an obvious shortcoming when dealing with fluorescent labelled compounds with their much inferior detection limits and the consequent inability to detect small differences in concentration.

The technique of Aalberse, which is properly an immunofluorimetric technique, appears to work with a fluorescamine label as well as a fluorescein label. However, again the changes in fluorescence intensity are small and investigation is needed into the efficiency of binding of antibody to cellulose. Also, higher concentrations of reactants may be helpful.

The double antibody assay was based on the work of Gleich et al (19) who developed a double antibody assay for IgE. The second antibody dilution was chosen because of the work of Gleich and because it had been shown (Fig.13) that a dilution of 1:10 of the second antibody, which is needed in excess for the assay, was approximately equivalent to a dilution of 1:100 of the first antibody. The results show a sigmoid curve for the plot of percentage binding against the logarithm of the antibody concentration, when radio labelled IgG was used. Most studies of antibody-antigen binding reactions produce such a curve. When fluorescamine labelled IgG was used, the presence of unlabelled antigen was seen to compete for binding sites and the amount of labelled IgG bound to antibody was reduced with increasing IgG concentration. However, the changes in fluorescence intensity were not great, there was only a 22% difference in fluorescence intensity values obtained for 0% binding and 100% binding. Some improvement might be obtained by using different concentrations of normal rabbit serum (N.R.S.)
and different concentrations of second antibody. The N.R.S. was used in the assay as a carrier for the second antibody and affects the precipitation of the first antibody and thus the sensitivity of the assay to a large extent (94).

In the work on fluorescence polarization some of the results were expected but others were not. It was to be expected that fluorescamine labelled IgG possessed a high initial degree of polarization which did not increase by a great deal on binding with antibody. This was because IgG is a large molecule with a high molecular weight and therefore only has a slow rotation due to Brownian motion. On binding with antibody the molecular size is only increased by a small factor (doubled) in comparison with the increase in molecular size occurring when a molecule such as gentamicin (39) binds with its antibody (about a 300 fold increase). Thus, the reduction in the rate of rotation, and consequently the increase in the degree of polarization, will be relatively small.

What was completely unexpected was the initial high degree of polarization of T₄. This is a relatively small molecule and it would be expected to have a rapid rotation with a consequently low degree of polarization. At the moment no theory can be advanced to explain this phenomenon. On binding with antibody the degree of polarization increased only slightly; this was a time dependent effect, as would be expected. Indeed, fluorescence polarization assays do not allow the reaction to proceed to equilibrium and use either the rate of change of polarization (37) or the polarization after a standard time interval (39). The binding of antibody with labelled antigen will proceed at a rate which is reflected in the rate of change of polarization. On addition of unlabelled antigen to the system the labelled and unlabelled antigens will compete for binding sites on the antibody, thus altering the rate at which antibody can bind labelled antigen; consequently altering the rate of change of polarization. As this type of assay does not require equilibrium to be reached, only a short incubation period is
required, resulting in a quick homogeneous assay. However, the experiments in this work indicated that unless a specially modified fluorimeter is used the measurement of polarization is time consuming. Two fluorimeter readings had to be taken for every sample with a change of polarizing film between each reading. This delay in taking the two readings must have introduced some inaccuracy as the degree of polarization must have changed slightly between the two readings.

A second, unexpected result to emerge from the work on fluorescence polarization was the enhancement in fluorescence obtained on the binding of fluorescamine labelled $T_4$ with its specific antibody. This enhancement could not have been as a result of either an increase in viscosity of the solution or of antibody fluorescence, as the enhancement did not occur in the presence of non-specific antisera such as anti-IgG and anti $T_3$. The results obtained from experiments on fluorescence enhancement indicate this phenomenon could be used to assay thyronine derivatives in serum. The enhancement in fluorescence obtained when labelled $T_4$ was bound by its antibody was reduced by the presence of increasing amounts of unlabelled $T_4$. An assay such as this would be quick as only a short incubation period is required, homogeneous as no separation step is needed, and cheap as the assay is followed by conventional fluorimetry. Unfortunately, serum has a high intrinsic fluorescence and effects the fluorescence of labelled $T_4$. This background fluorescence will vary from sample to sample. This could be overcome by either extracting the $T_4$ from serum prior to the assay, which would negate the attempt to provide a simple, one step, homogeneous assay, or by using the rate of change of fluorescence. Presumably the fluorescence enhancement produced by a specific amount of labelled $T_4$ in the presence of a fixed amount of antibody would proceed at a fixed rate no matter what the background. The presence of unlabelled $T_4$ would then predictably affect the rate of fluorescence increase.
An alternative method of overcoming the serum effects would be to use a label which fluoresces well outside the range of serum fluorescence. Such a compound is rhodamine and after some work on labelling and separation a rhodamine-T₄ complex was produced. Unfortunately this compound was unable to bind with anti-T₄ antibodies, the labelled T₄ appearing to have lost its immunogenic properties. This was possibly due to steric hindrance, rhodamine with its ethyl groups (see Fig. 7) being a larger molecule than even fluorescein, which has been demonstrated to have no effect on the antigenic properties of T₄ when used as a fluorescent label (46). It may also be possible that the positive charge of the nitrogen atoms in the rhodamine molecule may have had an inhibitory effect.

From the results of the fluorescence enhancement experiments, an assay using this phenomenon could be extended to the determination of other thyronine compounds such as T₃. However, more work is needed for the development of such assays in order to obtain a larger enhancement effect. It would be normal in these assays to incorporate a compound such as Merthiolate (95) in the assay medium in order, along with the high pH, to inhibit the binding of thyronine molecules to thyroxine-binding globulin (T.B.G.). This compound, unlike A.N.S. (anilino naphthalene sulphonio acid), is nonfluorescent and has been shown not to interfere with the binding of T₃ to its specific antibody (95), a property that is presumably also applicable to the case of T₄ and T₄ antibody. Also, it may be possible to treat the serum samples initially with Na OH, in order to produce a high pH (12-13), to remove the thyroxine molecules already bound to T.B.G. and so obtain an estimate of total T₄ or T₃ concentration (68,13).

There did not appear to be any cross reaction between T₄ and T₃ in the assays as anti-T₃ serum did not enhance the fluorescence of labelled T₄, anti-T₄ serum did not enhance the fluorescence of labelled T₃ and the presence of T₃ did not effect the standard curve for the assay of T₄. If cross reaction did take place it was at a level which was insignificant in the
conditions used in this work. However, the ability for an antiserum to bind with analogues of the compound to which it was raised will depend on the particular immunoglobulin molecules that it contains and will vary with antisera from different sources.

When methoxy diphenyl-furanone (M.D.P.F.) was used as the fluorescent label for T4 in the fluorescence enhancement experiments, a standard curve was produced which was similar to that obtained when fluorescamine was the fluorescent label. This is understandable when it is noted how similar the structure of the two compounds is. The only advantage in its use as a fluorescent label in place of fluorescamine would be the reportedly greater stability of its conjugates (96). However it has recently been reported (oral communication by C.S. Lim) that solid M.D.P.F. is unstable, breaking down after lengthy storage at room temperature in a desiccator.

It was thought originally that the observed enhancement effects were a result of the iodine atoms of the iodothyronine derivatives. Iodine is well known for its heavy atom effect (89) in quenching the fluorescence of fluorescent compounds. Thus it would be possible for the fluorescence of fluorescamine labelled T4 and T3 to be normally quenched by the iodine atoms of the iodothyronine moiety. If so, the enhancement of fluorescence on binding with antibody might be explained in terms of inhibition of the quenching. This quenching may be inter- or intra-molecular in nature. One theory put forward (98) was that the label, on a flexible side chain, was capable of rotation and able to come into close proximity with the iodine atoms of its own iodothyronine moiety. Binding with antibody would prevent movement of the side chain moving it away from the iodine atoms and eliminating the quenching (see Fig.23)

If the iodine atoms were involved the effect could be either intramolecular (e.g. as above) or intermolecular, the quenching effect being induced by iodine atoms associated with a molecule other than the one to which the quenched fluorescent label is conjugated. Thus if the effect
was due to intermolecular interactions, the addition of KI to a solution of an amino acid not having any iodine substitution (i.e., glutamic acid) but labelled with a fluorescent molecule might have the effect of quenching the fluorescence. Quenching of the fluorescence occurred when the fluorescent label was fluorescein or rhodamine but not when it was fluorescamine. This indicates that there was no intermolecular heavy atom effect quenching the fluorescence of fluorescamine labelled thyronine derivatives.

If intramolecular quenching was occurring, it would be expected that a comparison of the fluorescence intensity of fluorescently labelled glutamic acid with the fluorescence intensity of an equimolar concentration of fluorescently labelled T₄ would show a significant difference. This proved to be the case when a fluorescein label was used but not when fluorescamine or rhodamine were used as the fluorescent label. In addition, the quantum yields of the thyronine molecules (T₄, T₃, and T₂) [3, 5]
diodothyronine) labelled with fluorescamine were not significantly different from the quantum yields obtained with other amino acids (92). However, even though the iodine atoms appear to have little intramolecular effect on the fluorescence of fluorescamine labelled thyronine derivatives there is some effect, demonstrated by the fact that the quantum yields for the three derivatives differed in the following manner: \( T_2 > T_3 > T_4 \).

All this would indicate that the iodine atoms are not involved in the enhancement effect when the fluorescent label of \( T_4 \) or \( T_3 \) is fluorescamine. This is confirmed by Reiterer (67), who has stated that there is no heavy atom effect on the fluorescence of fluorescamine labelled \( T_4 \) and \( T_3 \). It does not preclude the involvement of iodine in the enhancement effect obtained by Smith (46) when the \( T_4 \) was labelled with fluorescein.

It has recently been shown that the fluorescence of fluorescamine labelled compounds is affected by the presence of organic, non-polar solvents. Froehlich (97) obtained a 277% enhancement of the fluorescence of fluorescamine-labelled tryptophan in a 50% dimethylsulphoxide-water mixture and Chen (92) reported fluorescence increases for fluorescamine-labelled amino acids with the addition of acetone and ethanol. Chen noted that a maximum increase in fluorescence was usually obtained with an optimum solvent/water ratio; above this figure the fluorescence declined. In order to explain this phenomenon he postulated that the organic solvent diminished dipole interactions and inter system crossing normally occurring in aqueous solution and which quench the fluorescence. It may also be possible that excited state charge transfer (89), exciplex, is also involved in the normal quenching of fluorescence in aqueous solution. However, high concentrations of organic solvent increase intramolecular interaction of the fluorescamine moiety with polar quenching groups, such as the carbonyl group.

Polar fluorescamine derivatives will show a maximum fluorescence at a low solvent level and quenching at higher concentrations; whereas less polar derivatives, such as tryptophan, will have little intramolecular interaction
occurring at high solvent levels and will consequently exhibit no fluorescence quenching.

Thus, the binding of fluorescamine derivatives with their antibody could be envisaged as the removal of these compounds from the polar aqueous solution into the less polar environment of the antibody binding site. This would reduce the dipole interactions and the excited state charge transfer, therefore reducing the quenching of fluorescence of the fluorescamine derivatives and producing an apparent enhancement of fluorescence. Because of the similarity in structure of fluorescamine and M.D.P.P. (see Chapter 2) a similar explanation could be postulated for the enhancement effect occurring with M.D.P.P. derivatives. However, as M.D.P.P. derivatives are less polar than those of fluorescamine they would be expected to exhibit a higher initial fluorescence and a lower enhancement on binding with their antibody.

This explanation along with the fact that the enhancement effect was found to occur with fluorescamine labelled IgG and albumin in the presence of their specific antibodies, would indicate that the phenomenon could be used for the analysis of a wide variety of compounds.

In this work it was seen that serum (normal rabbit serum, N.R.S., and human serum) and bovine serum albumin not only possessed an intrinsic fluorescence by virtue of their constituents (albumin, bilirubin, etc.) but were able to produce an enhancement in fluorescence. This effect could be due to two factors; the first being an increase in viscosity of the solution; and the second may be related to the above explanation. That is specific and non-specific adsorption of the derivatives on to T.B.G. and serum albumins may reduce the possibility of dipole interactions and excited state charge transfer reactions with the aqueous solvent. It is possible that this effect may be eliminated by the inclusion of merthiolate in the assay medium to help prevent binding to T.B.G.
In conclusion, it would appear that it is possible to develop fluorescent immunoassays (F.I.A.) utilising a variety of techniques to produce both heterogeneous and homogeneous types of assay. The most useful are the homogeneous techniques that provide a fast, simple, one step assay. In this area the fluorescence enhancement technique described in this work could be further developed to produce a viable routine assay for several compounds. There are some problems with the use of fluorescent labels, such as naturally occurring background fluorescence. This can be overcome by judicial selection of the assay conditions; e.g. using a solid phase heterogeneous assay or a homogeneous assay with a suitable label and following reaction rates. At the moment the detection limit is only in the nanogram range for most F.I.A. systems and so they are limited to the assay of only a certain range of compounds which are present in relatively large amounts in the blood, such as drugs and IgG. This limited the detection of $T_4$ by the enhancement assay described to the normal and hyperthyroid levels. However, improvements must be possible, especially with the introduction of more sensitive fluorimeters. If standard fluorimetry is used to follow F.I.A., the equipment is cheap compared with the scintillation and gamma counters needed for R.I.A., although with improvement and automation this factor may be negligible. On the plus side for F.I.A., fluorescent labels are cheap, compared with isotopes; non radioactive; and except for a few cases, nontoxic. In the case of fluoroscan the labelling of derivatives or the reconstitution of freeze-dried derivatives would have to take place immediately before use. As labelling only takes 5-30 minutes, depending on the method, it is of little inconvenience. All this would mean that F.I.A. must have advantages over R.I.A. when extreme sensitivity is not required, in that they can be homogeneous (one step), rapid, cheap, non-hazardous assays. Therefore F.I.A. has a place in the analysis of drugs and other compounds alongside R.I.A.
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