THE USE OF NON-RADIOACTIVE IODINE AS A LABEL IN IMMUNOASSAYS

A thesis submitted for the degree of Ph.D.

by

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September 1995

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Mary Butler

Date: 21/9/95

ACKNOWLEDGEMENTS.

I would like to thank Prof. Richard O'Kennedy for the opportunity to do a PhD, for his advice, support and enthusiasm throughout the course of the work, and for his help in the preparation of this thesis.

I am grateful to my fellow biology postgraduates for their assistance in the lab and their help with the preparation of this thesis, for their frequent and much needed encouragement over the years, and for their friendship.

Thanks to Gary for carrying out the work on the BIAcore. Thanks also to Frank Clarke of St. James' Hospital for the donation of anti-T4 antibody and serum samples.

I acknowledge the financial support of BioResearch Ireland.

CONTENTS

ABSTRACT.

The research described in this thesis has centered on the development of a non-radioactive iodine label for use in immunoassays. A protein can be iodinated with non-radioactive iodine, used in an immunoassay, and the iodine label detected by a chemical method.

A microassay was developed to measure iodine by means of its catalytic effect on the oxidation of antimony(III) by cerium(IV). The reaction was monitored both spectrophotometrically, by measuring the absorbance of cerium(IV), and fluorimetrically, by measuring the fluorescence of cerium(III) or by measuring the fluorescence produced by the oxidation of 8-hydroxyquinoline-5-sulphonic acid with cerium(IV). The detection of potassium iodide, iodine-containing organic compounds and iodinated proteins was possible using the microassay. Iodinated Bolton-Hunter reagent, an iodine-containing hapten which is used to iodinate proteins, could also be detected using the microassay. The catalytic activity of iodide is greater than that of iodine in iodocompounds, therefore, it was not possible to achieve the same sensitivity using IBHR as with potassium iodide.

A two-site immunoassay for the measurement of human IgG was set up and the effectiveness of non-radioactive iodine as a label in the immunoassay was illustrated using several assay formats. Initially, IBHR-labelled antibodies were used and directly compared to enzyme-labelled antibodies. The cerium(IV) - antimony(III) reaction was successfully used to detect the iodine label. The results obtained were comparable to those obtained using an enzyme label, with respect to accuracy and precision of the standards and with respect to the results obtained for serum samples. However, the use of enzyme-labelled antibodies enabled measurement of lower concentrations of human IgG.

In order to improve the detection limit when using the iodine label, the use of the avidin-biotin system and of bispecific $F(ab')_2$ antibodies was investigated. IBHR-labelled avidin and IBHR-labelled biotin were prepared, and used in a labelled avidin-biotin (LAB) immunoassay and bridged avidin-biotin (BRAB) immunoassay, respectively. Bispecific $F(ab')_2$ antibodies were prepared by a chemical method, and were used in an immunoassay as bridging agents between human IgG and iodinated BSA. An improvement in the detection limit was achieved using IBHR-labelled avidin in the LAB immunoassay.

An immunoassay for the measurement of thyroxine, which eliminates the necessity to prepare labelled derivatives of antigen or antibody, was developed. In this assay format, anti-thyroxine antibodies were used to capture thyroxine. Thyroxine contains four iodine atoms which served as the label, and these were monitored using the cerium(IV) - antimony(III) reaction.

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
BCA	Bicinchoninic acid
BHR	Bolton Hunter reagent
BRAB	Bridged avidin-biotin
BSA	Bovine serum albumin
CV	Coefficient of variation
Da	Dalton
Di-IBHR	Di-iodinated Bolton Hunter reagent
DMF	Dimethylformamide
EDTA	Ethylene diamine tetra-acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
Fab	Antigen binding fragment of antibody
Fc	Crystallisable fragment of antibody
FIA	Fluoroimmunoassay
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBHR	Iodinated Bolton Hunter reagent
ICl	Iodine monochloride
IgG	Immunoglobulin G
IRMA	Immunoradiometric assay
LAB	Labelled avidin-biotin
ME	Mercaptoethanol
NHS	N-hydroxysuccinimide
OPDA	o-phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
r	Correlation coefficient
RIA	Radioimmunoassay
RT	Room temperature

SD	Standard deviation
SDS	Sodium dodecyl sulphate
T_3	3',3,5-tri-iodothyronine
T_4	3',5',3,5-tetra-iodothyronine
TEMED	Tetra-methyl-ethylene diamine
TLC	Thin layer chromatography
v/v	Volume per volume
w/v	Weight per volume

1. INTRODUCTION.

Immunoassays enable the measurement of the concentration of an analyte by means of the antibody - antigen reaction. Immunoassays can be divided into unlabelled and labelled assays (Porstmann and Kiessig, 1992). Unlabelled assays are based on secondary immune reactions, for example, precipitation and agglutination, and are monitored by light scattering or particle counting methods. Labelled assays are based on the primary immune reaction, and are monitored by attachment of a label to either the antibody or antigen. Detection of the label is by physicochemical means, and depends on the assessment of the fraction of the labelled reagent that is present in either the bound form (i.e. in an antibody - antigen complex) or the free form (i.e. uncomplexed) for heterogeneous assays. For homogeneous assays the activity of the label must be altered by the immune reaction in such a way as to make separation of the bound and free forms unnecessary.

Immunoassays are a valuable and widely used analytical technique for the measurement of biological compounds, such as peptide hormones, steroid hormones, serum proteins, enzymes and drugs, to femtomole levels (Porstmann and Kiessig, 1992). Their strength lies in the combination of versatility, specificity and sensitivity. Immunoassays can, in theory, be developed for the measurement of any compound provided an antibody specific for the compound is available. The specificity of an antibody for an antigen enables analysis of an analyte that is present even in complex sample matrix, such as serum, with little or no pretreatment of the sample. The high sensitivity is dependent on the affinity of the antibody - antigen binding reaction, and on the detection system for measurement of the label.

Several types of label have been employed in immunoassays. The first immunoassays introduced in the 1950s and 1960s used radioactive compounds as labels. However, problems associated with the use of radioactive materials led to the development in the 1970s of non-isotopic labels, such as enzymes, fluorescent compounds and luminescent compounds. In principle, any material which can be accurately determined at low levels and can be attached to the antibody or antigen can be used as a label.

This thesis describes the use of non-radioactive iodine as a label. Using the methodology that already exists for radioiodination, a compound can be labelled with non-radioactive iodine, used in an immunoassay, and the iodine label detected using a chemical method.

1.1 Antibodies.

Antibodies belong to a group of proteins known as immunoglobulins. Immunoglobulins comprise part of the immune system of vertebrates, and bind strongly and specifically to foreign molecules (antigens). The antibody - antigen binding reaction is reversible and non-covalent. The sites on foreign molecules that are recognised by antibodies are called antigenic determinants, and may consist, for example, of a sequence of a few amino acids in the structure of a protein. Substances that are not immunogenic, but are antigenic, are known as haptens.

The basic structure of immunoglobulins comprises four polypeptide chains; two identical light chains and two identical heavy chains (Turner, 1989). The light chain is about 212 amino acid residues in length and the heavy chain is about 450 amino acid residues in length. The heavy chains are linked by disulphide bonds. The light chains are also attached to the heavy chains by disulphide bonds, so that one light chain associates with one heavy chain. There are two types of light chain, κ and λ. There five types of heavy chain, γ, μ, α, δ and ε, that result in five classes of immunoglobulins, IgG, IgM, IgA, IgD and IgE. These differ in molecular weight, number of units, available antigen binding sites and distribution in the blood (Turner, 1989). Subclasses of immunoglobulins also exist. For example, there are four human IgG subclasses: IgG1, IgG2, IgG3 and IgG4, which have heavy chains γ_1, γ_2, γ_3 and γ_4. The differences between subclasses are less than the differences between different classes, i.e., IgG1 is more closely related to IgG2, IgG3 or IgG4 than to IgM, IgA, IgD or IgE.

IgG is the major type of immunoglobulin in normal human serum. The structure of IgG is illustrated in Fig 1.1, and will be discussed here.

The light and heavy chains of IgG are divided into domains of approximately 110 amino acid residues. These domains are constant or variable between different IgG molecules. The light chain is made up of one amino-terminal variable domain (V_L) and one carboxy-terminal constant domain (C_L). The heavy chain is made up of one amino-terminal variable domain (V_H) and three constant domains (C_{H1}, C_{H2}, C_{H3}). Regardless of the subclass, IgG molecules always have carbohydrate moieties attached to the C_{H2} domains.

The variable domains are not uniformly variable throughout their length; there are three hypervariable regions or complementarity determining regions (CDR1, CDR2, CDR3) that show much more variability than the rest of the chain. These regions vary in size and in sequence between different immunoglobulins and are the regions that determine

the specificity of the antibody - antigen interaction. The remaining parts of the variable domain are known as framework regions (FR1, FR2, FR3, FR4). The function of these regions is to place the hypervariable regions into the right position for contact with antigen, and to bring stability to the three dimensional structure of the variable domains.

The region between the C_{H1} and C_{H2} of the heavy chain is called the hinge region. The two predominant amino acids in this region are cysteine and proline. The cysteine residues form the disulphide bonds that link the heavy chains. The number of interchain disulphide bonds in the hinge region varies, for example, human IgG1 has two, while IgG3 has fifteen. Proline residues are frequently found in flexible regions of proteins. Flexibility is important for the function of immunoglobulins as it contributes to the ability to bind antigen.

Treatment of IgG with the proteolytic enzymes papain or pepsin cleaves the molecule into fragments, as illustrated in Fig 1.2. Papain cleaves the heavy chains at the hinge region above the disulphide bonds, to yield two identical Fab (or antigen binding) fragments and one Fc (or crystallisable) fragment. The Fab fragment comprises a light chain, linked by disulphide bonds to a fragment of the heavy chain consisting of the V_H and C_{H1} domains. The Fc fragment comprises the C_{H2} and C_{H3} domains from both heavy chains, linked by disulphide bonds. Treatment with pepsin cleaves IgG below the disulphide bonds of the hinge region, to yield an $F(ab')_2$ fragment, some low molecular weight peptides and a portion of the Fc fragment, a pFc' fragment. The $F(ab')_2$ fragment consists of the two Fab fragments linked by the hinge region. The pFc' fragment comprises the C_{H3} regions from both heavy chains.

IgG is the immunoglobulin exploited for use in immunoassays. Properties of antibodies, such as affinity, specificity and titre, are important as the sensitivity and specificity of immunoassays depends on the characteristics of the antibody. Polyclonal antiserum is serum from an immunised animal, and contains a heterogeneous population of antibodies that differ in their properties. Monoclonal antibodies are derived from the fusion of antibody-producing cells with myeloma cells to produce a hybridoma, a technique which was developed by Kohler and Milstein (1975). The myeloma cells confer immortality to the hybridoma, and it secretes monoclonal antibodies that are homogeneous in affinity and titre, and are specific to a single antigenic determinant of the immunogen. Monoclonal antibodies can also be produced by genetic engineering (Owens and Young, 1994). A comparison of the properties of polyclonal antiserum and monoclonal antibodies is shown in Table 1.1.

Antibody affinity is a thermodynamic measurement of the energy of the interaction between a single binding site of the antibody and its corresponding antigenic determinant. Avidity is a term used to express the ability of antiserum to bind antigens, and depends on the affinity and the number of binding sites (Tijssen, 1985). Polyclonal antiserum generally contains antibodies to all the determinants of an antigen, which contributes to the avidity. This is not obtained with monoclonal antibodies which react with one antigenic determinant. Mixing monoclonal antibodies which react with two or more different determinants on an antigen may result in an "affinity bonus" (Tijssen, 1985).

Non-specificity of an immunoassay can occur due to cross-reactivity (Tijssen, 1985). An antibody binding site is specific for a particular antigenic determinant, but cross-reactivity can sometimes occur with antigens of similar molecular structure. Cross-reactivity can also occur with different antigens that share the same epitope (shared reactivity). For monoclonal antibodies, problems due to shared reactivity can generally be avoided by selection during cloning against the appropriate antigenic determinant. Production of monoclonal antibodies is expensive and time-consuming, but the major advantage compared with polyclonal antiserum is that the very specific properties of monoclonal antibodies allows standardisation of immunoassays with respect to sensitivity and specificity.

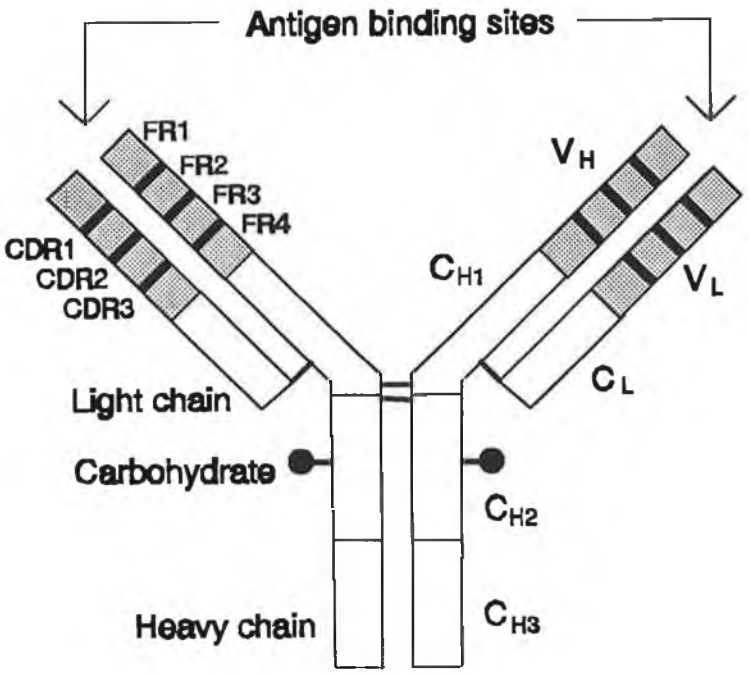


Figure 1.1 The structure of IgG.

The light chains are made up of a variable domain (V_L) and a constant domain (C_L). The heavy chains are made up of a variable domain (V_H) and three constant domains (C_{H1}, C_{H2} and C_{H3}). The variable domains are divided into three complementarity determining regions (CDR1, CDR2, CDR3) and four framework regions (FR1, FR2, FR3, FR4). The antigen binding sites are located at the end of the variable regions. Disulphide bonds link the four chains.

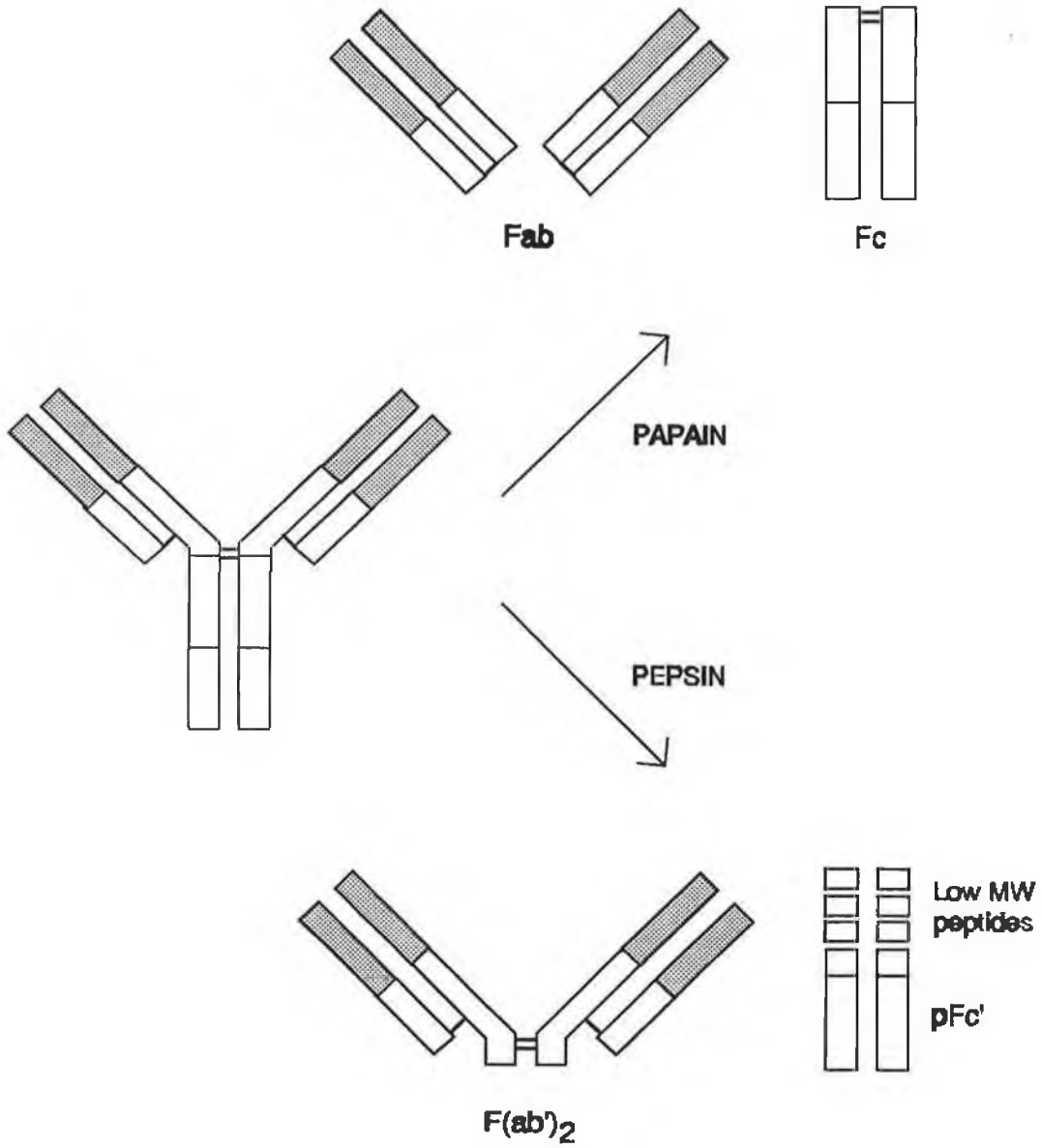


Figure 1.2 Treatment of IgG with proteolytic enzymes.

Papain cleaves the molecule yielding two Fab fragments and a Fc fragment. Pepsin cleaves the molecule yielding a $F(ab')_2$ fragment and a pFc' fragment.

	Polyclonal antiserum	Monoclonal antibodies
Antigenic determinant	Several	Single
Specificity	Variable	Standard
Affinity	Variable	May be selected during cloning
Yield of useful antibody	Up to 1mg/ml	Up to 100µg/ml in tissue culture. Up to 20mg/ml in ascitic fluid.
Relative cost	Low	High

Table 1.1 Comparison of polyclonal antiserum and monoclonal antibodies. (Data taken from Campbell, 1984).

1.2 Labels used in immunoassays.

The label in immunoassays is employed to assist in the observation of the distribution of antibody or antigen in the bound or free fractions. The label used in an immunoassay is often the most distinguishing feature, and is the basis of a system of classification. Thus, radioimmunoassay (RIA) represents an immunoassay using radiolabelled antigen, and immunoradiometric assay (IRMA) represents an immunoassay using radiolabelled antibody. Similarly, enzyme immunoassay (EIA), immunoenzymometric assay (IEMA), fluoroimmunoassay (FIA), immunofluorometric assay (IFMA), etc. are terms which represent immunoassays using alternative labels. Radiolabels are robust, and the disintegrations are unaffected by physical or chemical factors. In contrast, the majority of non-isotopic labels are vulnerable to the effects of pH, ionic strength and temperature, or the presence of inhibitors or quenchers. As a result, immunoassays using radiolabels are always heterogeneous and require separation of antibody-bound and free fractions. The separation technique must effectively remove the free fraction without disruption of the antibody - antigen complexes. It must be simple to perform, applicable to a wide range of analytes and be readily automated (Porstmann and Kiessig, 1992). The use of non-isotopic labels provides the potential for the development of homogeneous immunoassays, which do not require a separation step.

1.2.1 Radiolabels.

The main isotopes used as radioactive labels for immunoassays are ^{14}C, ^{3}H, ^{125}I and ^{131}I. Some properties of these isotopes are listed in Table 1.2. There are two types of labelled reagent preparation: intrinsic and extrinsic labelling. With intrinsic labelling, the radiolabel replaces a non-radioactive element which is present in the molecule. Usually ^{14}C or ^{3}H are used to intrinsically label a compound by substitution of a carbon or hydrogen, although radioactive iodine can be substituted for non-radioactive iodine in the thyroid hormones. With extrinsic labelling the radiolabel is introduced into an already complete molecule of the compound.

The sensitivity of immunoassays depends, among other factors, on the specific activity of the tracer. Labelling with ^{14}C and ^{3}H provides tracers which have a long shelf-life, but which have lower specific activities than tracers obtained using ^{125}I and ^{131}I. With a radioactive label of low specific activity, relatively high concentrations are required to obtain a practical level of radioactivity in the assay (Parker, 1976). ^{125}I and ^{131}I can be counted by scintillation crystals using well type gamma counters, and transfer of

samples from the test tubes before counting is not necessary. Counting by scintillation crystals is, therefore, simpler and faster than liquid scintillation counting. Also, iodination can be carried out using simple chemical methods. Thus, iodination is the more widely used labelling technique, although the short half-life of ^{131}I makes ^{125}I the isotope of choice.

Yalow and Berson (1959) first described the use of RIA for the assay of human insulin. RIA is based on the reaction of radiolabelled antigen with its specific antibody, in the presence of unlabelled antigen. Concentrations of labelled antigen (tracer) and antibody are constant, and the concentrations are such that the tracer is in excess. In the absence of unlabelled antigen, the tracer binds quantitatively to the antibody. On addition of unlabelled antigen, competition occurs between labelled and unlabelled antigen for the available binding sites, and the binding of tracer to antibody is decreased. Thus, the ratio of antibody-bound to free tracer decreases with increasing concentration of unlabelled antigen. After an incubation period, free tracer is separated from antibody-bound tracer, and the radioactivity in the free or bound fraction is measured. A schematic representation of RIA is illustrated in Fig 1.3.

The use of IRMA was first described by Miles and Hales (1968). They used radio-labelled antibody, rather than antigen, in a two-step non-competitive assay for measurement of insulin. Unknown antigen, in standards or samples, was first reacted with labelled antibody, in excess. Antigen attached to a solid phase matrix was then added. Any unreacted antibody bound to this, and was subsequently removed from the reaction. This is illustrated in Fig 1.4. The radioactivity of the antibody-unknown antigen complexes provides a direct measure of the amount of unknown antigen. This immunoassay scheme overcomes problems that may be encountered with the labelling of antigen, for example, some antigens may be susceptible to damage due to labelling or may lack residues that can be labelled. Antibodies, on the other hand, contain numerous residues for labelling.

Alternative formats for IRMA are two-site assays and assays using indirect labelling (Hales and Woodhead, 1980). Two-site assays are suitable for compounds with more than one antigenic determinant. Antigen is reacted with antibody attached to a solid phase matrix (antibody-solid phase). A second labelled antibody is then added; this antibody binds to a second antigenic determinant on the antigen. After a separation step, the radioactivity of the binding complex (labelled antibody:antigen:antibody-solid phase) can be measured, and is directly related to the antigen concentration. This binding complex is illustrated in Fig 1.5a. For assays using indirect labelling, the

antibody used for measuring the antigen (primary antibody) is not labelled, instead a labelled second antibody, which is generally an anti-immunoglobulin antibody, is used. The primary antibody acts as the antigen for the labelled second antibody. Indirect labelling can be used in different IRMA formats; its use in a two-site assay is illustrated in Fig 1.5b. The two-site assay is carried out as outlined, but the primary antibody is not labelled. Labelled second antibody is added, and binds to the primary antibody. After a separation step, the radioactivity of the binding complex is again a direct measure of the concentration of the antigen. An important requirement for this assay format is that the antibody attached to the solid phase matrix must be from a different animal species to the primary antibody, to avoid binding of the labelled second antibody to it. The advantage of indirect labelling is that one labelled antibody can be used for IRMA procedures for many different antigens.

The use of radioactive labels in immunoassays has several advantages. Radioactivity can be detected with great sensitivity, and the signal is unaffected by environmental factors. Radiolabels are small so they do not normally affect the kinetics of the antibody - antigen reaction, and radiolabelling procedures are relatively simple and well characterised. However, there are certain disadvantages to the use of radiolabels. As the signal produced is unaffected by environmental factors and is unaltered on incorporation of the labelled reagent into the antibody - antigen complex, these assays are always heterogeneous and are difficult and expensive to automate. Radioiodinated reagents only have a short shelf-life due to the short half-life of the isotopes. ^{125}I has a half-life of just 60 days, therefore, labelled reagents must be regularly prepared for routine assays. There are hazards associated with the handling of radioisotopes due to the exposure to ionising radiation, especially in the use of raw isotopes for the preparation of tracer. These hazards have resulted in restrictive laws for the use and disposal of radioisotopes. The ionising radiation can cause damage to protein molecules during labelling and storage. The signal must be detected against a background signal due to external radiation sources, which limits the sensitivity of an immunoassay. Detection is impractical when the signal approaches the background level. These disadvantages led to the development of non-isotopic labels as an alternative to radiolabels.

Isotope	Half-Life (#)	Specific Activity (#) (Ci/mmol)	Counting method
^{14}C	5730yr	0.07	Liquid scintillation
^{3}H	12.3yr	33	Liquid scintillation
^{125}I	60d	2560	Scintillation crystal
^{131}I	8d	19250	Scintillation crystal

(#) Taken from Parker (1976)

Table 1.2 Properties of the main isotopes used for radiolabelling.

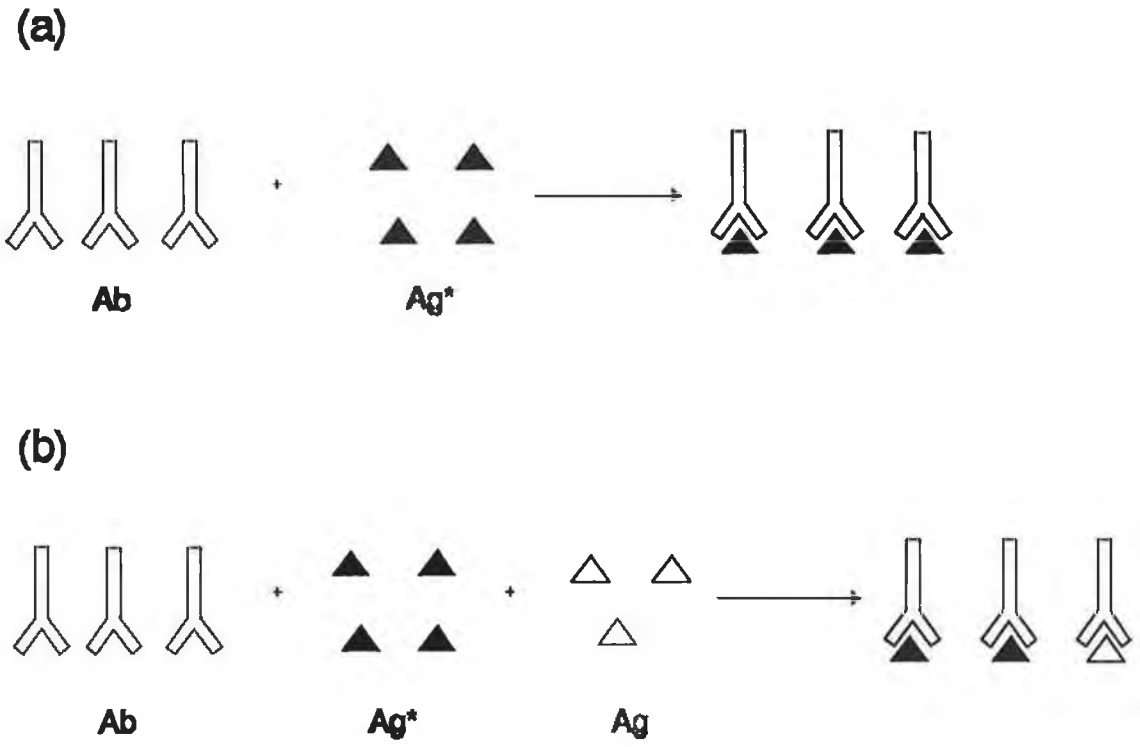


Figure 1.3 Schematic diagram of RIA.

(a) Reaction in the absence of unlabelled antigen: labelled antigen (Ag^*) binds to antibody (Ab).

(b) Reaction in the presence of unlabelled antigen: competition occurs between labelled antigen (Ag^*) and unlabelled antigen (Ag) for binding to antibody (Ab).

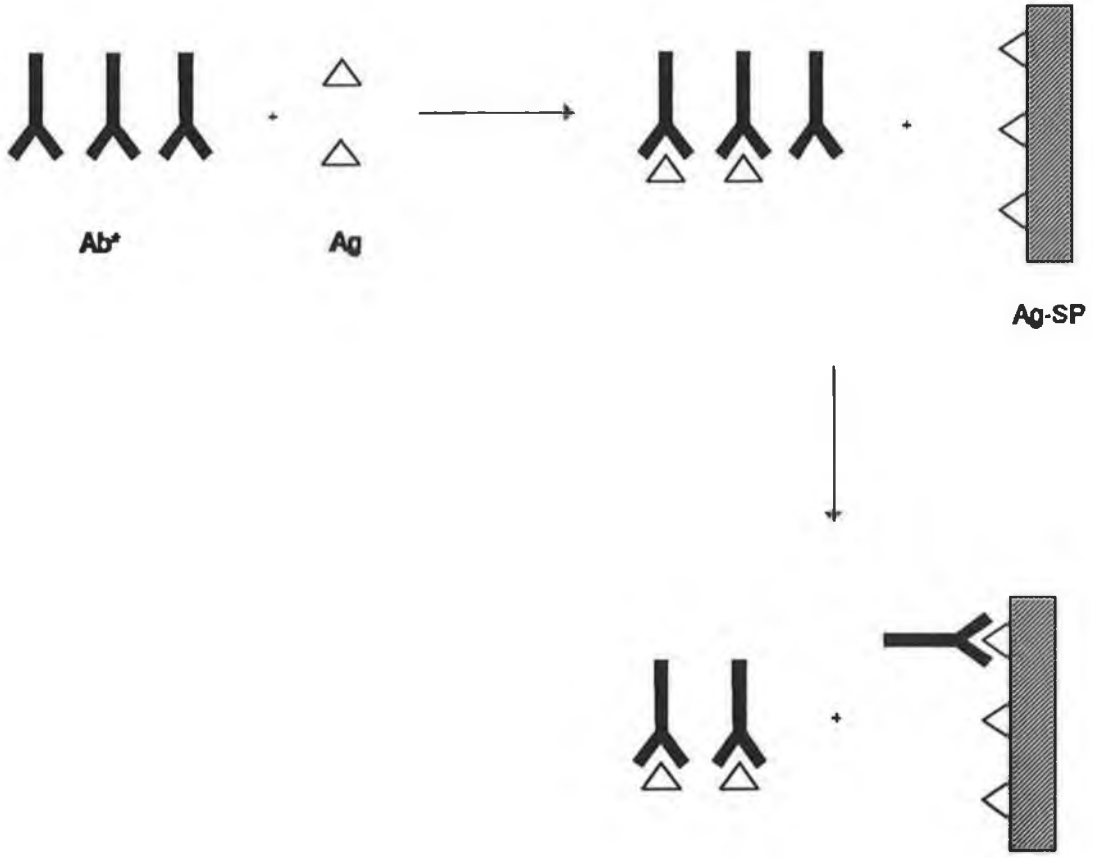


Figure 1.4 Schematic diagram of IRMA.

Labelled antibody (Ab^*) binds to antigen (Ag). Free antibody is removed on addition of antigen attached to a solid phase matrix (Ag-SP).

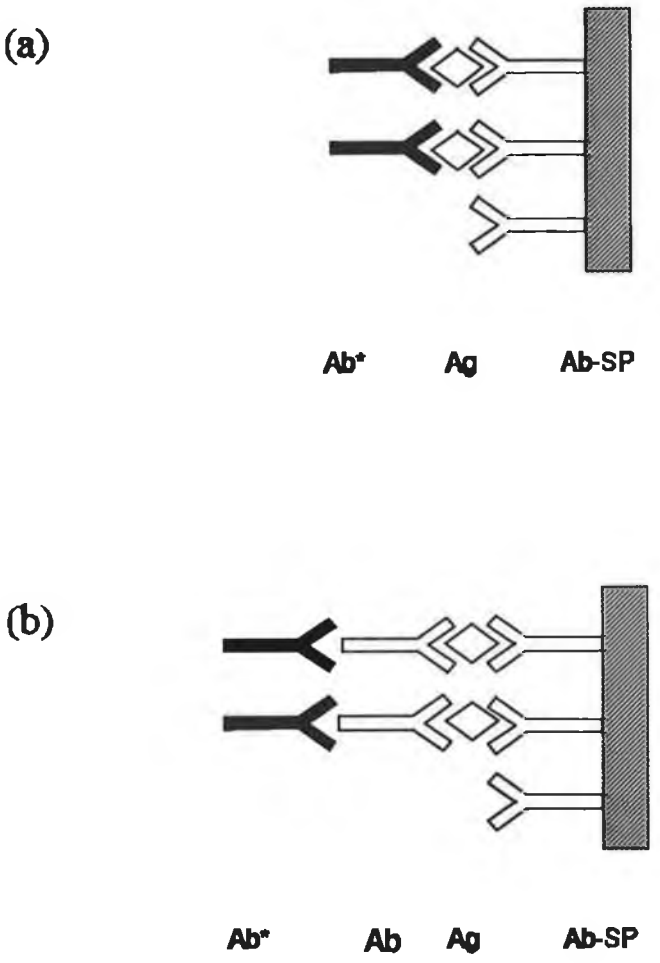


Figure 1.5

(a) Schematic diagram of two-site IRMA.

Antigen (Ag) binds to antibody attached to a solid phase matrix (Ab-SP). Labelled antibody (Ab^*) is added, and binds to a second antigenic determinant on the antigen (Ag).

(b) Schematic diagram of the use of indirect labelling in a two-site IRMA.

Antigen (Ag) binds to antibody attached to a solid phase matrix (Ab-SP). Primary antibody (Ab) is added, and binds to a second antigenic determinant on the antigen (Ag). Labelled second antibody (Ab^*) is then added, and binds to primary antibody.

1.2.2 Enzyme labels.

The use of alkaline phosphatase as a label in an immunoassay for IgG was described by Engvall and Perlmann (1971). Since then, enzymes have found widespread use in immunoassays. The enzyme label is detected by addition of the appropriate substrate, and detection of the resulting product by spectrophotometry, fluorimetry, luminescence or electrochemical techniques. Detection by spectrophotometry is most frequently used. Enzymes are sensitive labels because they are very efficient catalysts; they act repeatedly on a substrate to produce an amplified response. The sensitivity depends on their efficiency as catalysts, and on the detection of the product with high sensitivity.

Since enzymes are affected by environmental factors, heterogeneous and homogeneous EIAs can be developed (Engvall, 1980). Heterogeneous EIAs are based on the same principles as RIA and IRMA, and separation of the bound and free fractions is required before detection of the enzyme label. Enzyme-linked immunosorbent assay (ELISA) is an example of heterogenous EIA, with immobilisation of antibody or antigen onto a solid phase. Enzyme multiplied immunoassay technique (EMIT) is a homogeneous EIA system, in which no separation step is required. Homogeneous assays are rapid and simple; there is one incubation and no separation step. The enzyme label used in EMIT shows a change in catalytic activity on incorporation of the labelled reagent into the immune complex. However, EMIT is generally only applicable to the assay of low molecular weight haptens.

The enzymes commonly used as labels are shown in Table 1.3. The choice of enzyme is governed by several factors, such as sensitivity, practicality, availability, shelf-life and cost (Porstmann and Kiessig, 1992). The enzyme should have a high specific activity, and the resulting product should be detectable with high sensitivity. The enzyme chosen, or its substrate, should not be present in test samples, and samples should not contain substances that could interfere with the activity of the enzyme, for example, activators or inhibitors. This is important in heterogeneous assays where there may be an incubation of enzyme-labelled compound with sample, and is of prime importance in homogeneous assays where there is no separation of reactants from sample before measurement of enzyme activity. The enzyme must be stable in the free and conjugated form, and possess reactive groups for covalent linkage to antibody or antigen. Pure enzyme must be available at low cost, and the substrate must also be inexpensive, stable and non-toxic.

The choice of enzyme in EMIT is restricted to a small group of enzymes whose activity is altered after interaction with antibody. Lysozyme acts on a high molecular weight substrate, and its activity can be sterically hindered by binding of antibody. With glucose-6-phosphate dehydrogenase and malate dehydrogenase, when residues in the enzyme molecule are substituted with hapten the enzyme becomes susceptible to conformational changes on binding of hapten to antibody, which leads to inhibition of enzyme activity (Engvall, 1980). Homogeneous assays have also been developed using enzyme co-factors, substrates and inhibitors as labels (Blake and Gould, 1984). In these assays the enzyme is not labelled as in EMIT. The binding of antibody to hapten-labelled compound causes steric hindrance and alters the enzyme activity by alteration of the natural function of the compound with the enzyme.

The use of enzyme labels offers several advantages. Enzyme-labelled compounds have a shelf-life of one year or longer. The equipment for detection of the reaction product can be inexpensive and is generally available, especially when detection is by spectrophotometry. Rapid measurement can be made, and the reaction can be stopped, for example, by addition of acid, resulting in a stable product. The time required to carry out homogeneous assays is very short, and these assays can be easily automated. However, there are disadvantages also, the major one being the possible presence in the sample of substances that interfere with the enzyme activity. This is most important for homogeneous assays which have, at present, limited sensitivity and are generally only applicable to measurement of haptens. Environmental factors must be carefully controlled as these affect the enzyme activity. The enzyme label is bulky, and labelling with a macromolecule can alter the antibody - antigen binding reaction kinetics. An additional incubation step with a substrate is required, and if the product is determined spectrophotometrically the dynamic range of the EIA is limited due to the narrow dynamic range of spectrophotometric measurement.

Assay type	Enzyme	Source	Specific activity* (U/mg at 37°C)
Heterogeneous	Alkaline phosphatase	Calf intestine	1000
	β-galactosidase	*E. coli*	600
	Peroxidase	Horseradish	4500
	Urease	Jack beans	10000
	Glucose oxidase	*A. niger*	200
Homogeneous	Glucose-6-phosphate dehydrogenase	*L. mesenteroides*	400
	Lysozyme	Egg white	-
	Malate dehydrogenase	Pig heart	1000

* Taken from Porstmann and Kiessig (1992)

Table 1.3 Enzymes commonly used as labels in EIA.

1.2.3 Fluorescent labels.

Luminescent molecules are capable of absorbing energy and emitting it as photons. The absorbed energy excites electrons of the molecule from the ground singlet state to a higher electronic state. With photoluminescence, excitation is effected by infra-red, visible or ultra-violet radiation. Fluorescence and phosphorescence are types of photoluminescence. The excitation energy can be lost by non-radiative conversion, for example, as heat, or by radiative transition to the ground state (fluorescence) or through a semistable triplet state (phosphorescence). The principle of fluorescence and phosphorescence is illustrated in Fig 1.6.

Fluorescent molecules were used to label antibodies for qualitative staining techniques as early as the 1940s. However, it was not until the 1970s that fluorescent labels were used for quantitative immunoassays.

Fluorescent molecules can be characterised by the absorption and emission spectra, the Stokes shift, the quantum yield and the fluorescent life-time (decay time). The emission wavelength is always longer than the excitation wavelength because of energy losses prior to emission. This difference between the wavelengths is known as the Stokes shift. The ratio between the absorbed and emitted light is defined as the quantum yield. The ideal quantum yield is one, but this is rarely attained due to the energy losses before emission. The properties of some compounds used as fluorescent labels in FIA are listed in Table 1.4. There are several requirements of compounds used as fluorescent labels (Wood, 1991). Ideally, the compound should have long excitation and emission wavelengths far from the background due to, for example, serum. The excitation wavelength should be greater than 360nm, and the emission wavelength should be greater than 500nm. The compound should also have a high quantum yield and a large Stokes shift of greater than 50nm.

Fluorescein and rhodamine are widely used as fluorescent labels. Fluorescein derivatives, for example, fluorescein isothiocyanate (FITC), have a high fluorescence intensity and a good quantum yield approaching unity. The fluorescence intensity of fluorescein is sensitive to pH and is reduced in acidic solutions, unlike the fluorescence intensity of rhodamines. Rhodamine B isothiocyanate, tetramethyl rhodamine isothiocyanate and lissamine rhodamine B sulphonyl chloride are rhodamine derivatives used for labelling of antibodies. Rhodamines have longer excitation and emission wavelengths than fluorescein, but the quantum yield is lower. Both fluorescein and rhodamine have narrow Stokes shifts of only 28nm and 35nm, respectively. Umbelliferone, a 7-hydroxycoumarin, and aminomethyl coumarin acetic acid have the

advantage of large Stokes shifts. However, they also have short emission wavelengths which overlap with the serum background.

In principle, fluorescent measurement is very sensitive. Fluorescent molecules can be excited many times within one measuring period, and the resulting emission integrated with a sensitivity of a few photons. In practice, however, the sensitivity is limited due to scattering, background fluorescence and quenching (Hemmila, 1985). Light scattering can arise as a result of dissolved molecules or particles in the matrix or the solid-phase material. It results in high background values in solutions containing a high concentration of protein or small colloidal particles as found in serum. Background fluorescence can occur due to the solid phase, the presence of fluorescent compounds in the sample or the presence of impurities in the reagents. Serum has a very high background fluorescence, which extends over a wide range of wavelengths. Quenching occurs due to the sensitivity of fluorescent compounds to environmental factors. The close proximity of two fluorescent molecules on a labelled compound can cause self-quenching, which precludes the use of multiple labelling to increase the sensitivity.

These limitations to the sensitivity mean that conventional FIA methods are not as sensitive as RIA, and so have not found widespread use. However, the development of new fluorescent labels and new instrumentation has reduced the background and has resulted in FIAs which have a sensitivity comparable to RIA and IRMA. The new labels are lanthanide ions, for example, europium and terbium, which form highly fluorescent chelates with suitable organic ligands (Wood, 1991). These can be detected using time-resolved fluorescence (Diamandis and Christopoulos, 1990). This technique exploits the difference between the fluorescent life-time of the specific signal and the non-specific background. The specific signal of europium and terbium chelates has a decay time longer than the average background decay, so that measurements can be taken after the background signal has decayed. The specific signal can, therefore, be measured with very high sensitivity.

As fluorescence intensity is dependent on environmental factors the development of homogeneous FIAs is possible. Homogeneous FIAs are, in general, competitive assays and depend on the antibody binding causing some change in the fluorescence properties of the label. Homogeneous FIAs are based on the exploitation of, for example, polarisation, enhancement of fluorescence, quenching of fluorescence, or excitation transfer, i.e. energy transfer from an excited fluorescent dye to an acceptor dye molecule (Price and Newman, 1991a). These assays are rapid and simple. However,

the sensitivity is limited by interferences. Samples, especially serum samples, cause high background and a high signal-to-noise ratio. The means by which fluorescence is modulated on antibody - antigen binding may only lead to a small degree of fluorescent change, and involves close reactant proximity or large changes in molecular mass which means that homogeneous assays are applicable only to the quantitation of haptens. Careful engineering of labelled molecules must be carried out in order to produce modulation on antibody - antigen binding.

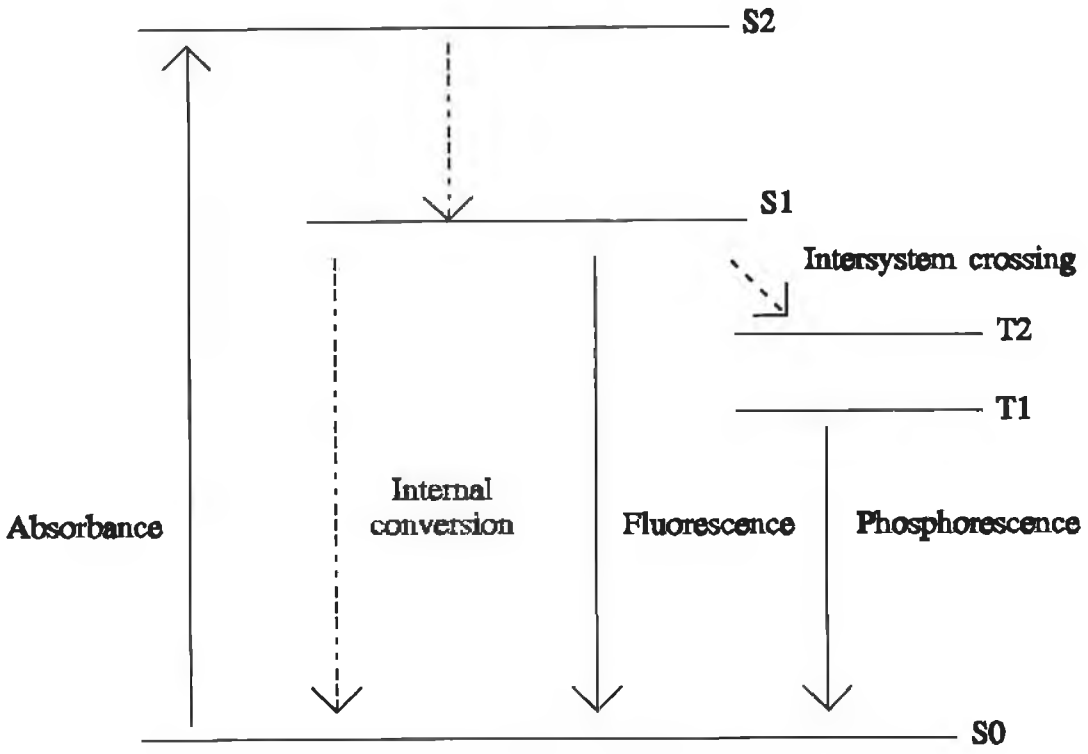


Figure 1.6 The principle of fluorescence and phosphorescence.
Electrons are excited from the ground state singlet (So) to excited singlets (S1, S2). The excitation energy can be emitted by non-radiative energy transfer (broken line) or radiative transition (solid line) to the ground state or through semi-stable triplet states (T1, T2).

Compound	Excitation max (nm)	Emission max (nm)	Quantum yield*	Decay time (ns)*
Fluorescein isothiocyanate	492	520	0.85	4.5
Rhodamine B isothiocyanate	550	585	0.7	3.0
Umbelliferone	380	450	-	-
Aminomethyl coumarin acetic acid	350	440-460	-	-
Anilino-naphthalene sulphonic acid	385	471	0.8	16.0
Eu-(β-naphthoyltri-fluoroacetone)$_3$	340	590,613	-	500,000
Tb-EDTA-sulpho-salicyclic acid	300	490,545	-	~150,000

* Taken from Hemmila (1985)

Table 1.4 Properties of some compounds used as fluorescent labels.

1.2.4 Luminescent labels.

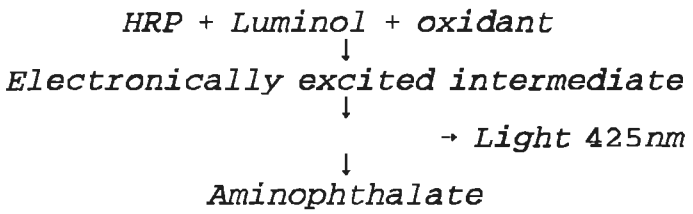
Chemiluminescence is light emission that arises due to excitation of a luminescent molecule with the exciting energy provided by a chemical reaction, usually an irreversible oxidation reaction. In contrast to fluorescence no external excitation light source is required. Bioluminescence is a type of chemiluminescence found in biological systems, in which an enzyme increases the efficiency of the chemiluminescent reaction. Components of both chemiluminescent and bioluminescent reactions have been used as labels in immunoassays.

The first chemiluminescent immunoassays were introduced in the mid-1970s, and were based on the use of luminol, its isomer, isoluminol, or its substituted derivatives (Kricka *et al.*, 1991). These compounds emit blue light, at a wavelength of 425nm, when oxidised. The light emission from luminol and its derivatives is relatively slow; emission may last more than 25 seconds after initiation of the chemiluminescent reaction. The quantum efficiency of these compounds is low, and the quantum yield of luminol decreases on covalent coupling to proteins or steroid hormones. Other organic compounds which are chemiluminescent on oxidation have also been used as labels, for examples, acridinium esters, oxalate esters and dioxetanes. Acridinium esters produce light emission in a very rapid flash; the maximum intensity is reached 0.4 seconds after initiation. Fully automatic luminometers, with automated pipetting devices and integration of the light emission from each tube, are therefore required. Acridinium esters are unaffected by covalent coupling, but they are difficult to use as labels as synthesis of these molecules is complex and there are no commercial sources of labelled compounds. Oxalates are efficient labels as they have high quantum yields. The compounds themselves are not chemiluminescent, therefore, addition of a fluorescent molecule is required and the energy of the reaction is transferred to this. Dioxetanes are small molecules, which makes them ideal as labels. However, most dioxetanes are thermally unstable, and decompose on emission of light. Recently, stable 1,2-dioxetane derivatives have been designed which can generate light with high quantum yield (Beck and Koster, 1990).

Chemiluminescent labels are very sensitive. As no external light source is required, all the light reaching the detector is due to the chemical reaction and there is no background signal due to scattered excitation radiation. The labels are chemically stable and have high specific activity. However, there are limitations to the use of chemiluminescent labels. Light emission can be of very low intensity, and can decay rapidly. In most cases, emission is over in less than 30 seconds. Signal measurement

must, therefore, be rapid and be carried out in a fully automatic luminometer. Reactions are usually performed on one sample at a time. There is no second chance of recording signals, as once the reagents are mixed the label is consumed.

An alternative approach to improve the performance of chemiluminescent reactions is to use an enzyme, such as horseradish peroxidase (HRP), xanthine oxidase or alkaline phosphatase (AP) as the label (Jansen, 1993). HRP catalyses the oxidation of luminol as follows:



Hydrogen peroxide is generally used as oxidant, although its replacement with another oxidant, for example, urea peroxide, is possible. Luminol can be replaced by one of its derivatives. Enhancement of this reaction is possible using appropriate compounds, for example, substituted phenols and naphthols which increase the chemiluminescent signal by a factor of 100. The half-life time of the signal is one hour. Xanthine oxidase can be used in conjunction with luminol and hypoxanthine. This label system is superior to almost all existing chemiluminescent labels as the detection limit is extremely low and the signal has a half-life time of 30 hours. AP is used with 1,2-dioxetanes as substrate. Light emission occurs at 470nm, and the half-life time of the signal is approximately 5 hours. Macromolecules, such as bovine serum albumin (BSA), can be used to enhance the signal.

The light emission from these reactions is intense and decays slowly. Batches of reactions can, therefore, be started and measurements made some minutes later using relatively simple instrumentation. Immunoassays using enzymes as labels are widespread, and can be easily modified for enhanced chemiluminescent detection. The use of an enzyme label provides the advantage of an amplified signal, however, the disadvantages associated with the use of enzyme labels in EIAs will also apply to these assays.

Bioluminescence occurs in biological systems. Bioluminescent systems consist of an enzyme, luciferase, a substrate, luciferin, and co-factors, for example, ATP or NADH (Barnard and Collins, 1987). The most extensively studied bioluminescent system is that of the firefly, although bioluminescence is also observed in marine bacteria.

Immunoassays using firefly or bacterial luciferase as a label, or using the co-factor as a label, have been developed. As the substrate is unstable, and complex chemistry would be required for the preparation of substrate-labelled reagents, it has not been exploited as a label. Bioluminescent reagents have high quantum yields and stable emission of light which eliminates the necessity for automated luminometers.

Homogeneous immunoassays using luminescent labels are not widespread. Most of the assays are based on the alteration of the total light emission or the kinetics of the light emission from the reaction on binding of the labelled analyte to antibody. These effects have been used to develop homogeneous assays for biotin and some steroids hormones (Kricka *et al.*, 1991). However, as with other homogeneous assays, the fundamental problem is the susceptibility to interference from biological samples.

1.2.5 Other labels.

Many other labels have been used for immunoassays, including particle labels, metal atoms, free radicals, and bacteriophages.

Particle labels are solid particles, for example, sols, latexes and red cells. Red cells can be used as particle labels in qualitative and semi-quantitative assays (Coombs, 1987). Their detection is based on haemagglutination on formation of the immune complex. The red cells are randomly distributed in the test sample in the unagglutinated state. Once agglutination occurs large aggregates form due to lattice formation. Treatment of the cells with enzyme increases the agglutinability and, therefore, the sensitivity of these labels. The main drawback is that the haemagglutination signal is read microscopically or by eye, which is not suitable for quantitating small concentration differences, as is possible with the precise linear recording of signals from other labels which allows construction of a standard curve.

Fully quantitative immunoassays using particle labels can be developed by using light scattering for detection of the label (Price and Newman, 1991b). Detection depends on changes in light scattering on formation of the antibody - antigen complex. Quantitation is performed using turbidimetry, nephelometry, particle counting or photon correlation spectroscopy. There are three styles of assay using particle labels. Antibody can be coupled to the particle, and addition of antigen results in formation of the immune complexes. Standard antigen can be coupled to the particle, and addition of antibody results in formation of the immune complexes. Detection of unknown antigen in samples is by observation of inhibition of the immune complex formation. Both antibody and standard antigen can be independently coupled to

particles, and detection of unknown antigen is by inhibition of immune complex formation. This third approach yields highly scattering immune complexes and results in greater sensitivity.

Metal sols, for example, gold, silver, silver iodide and barium sulphate sols, have all been used as labels. Gold has superior optical properties and, therefore, is the most popular metal sol.

Latex particles can be synthesised using one of three techniques: emulsion polymerisation, suspension polymerisation or swollen emulsion polymerisation (Price and Newman, 1991b). Emulsion polymerisation is the most suitable technique for production of small particles (40-1000nm). The particle size can be determined using electron microscopy or photon correlation spectroscopy. Properties of the particle, such as density, refractive index and surface charge are important. The particle density should, ideally, be equal to that of the suspension buffer as this eliminates the need for regular agitation of reagents during the assay. The refractive index of the particle influences the light scatter, and the surface charge influences the non-specific aggregation of the particles. The use of an appropriate monomer for the polymerisation reaction enables introduction of active groups to the particle surface. For example, carboxyl groups can be introduced by the use of acrylic acid and amino groups can be introduced by the use of acrylamide. Antibody or antigen can thus be coated onto the particle using simple coupling chemistry, and the coated particles used to produce rapid, simple and inexpensive assays.

The use of metal atoms as labels for immunoassays was first reported by Cais *et al.* (1977). The label can be detected using a suitable analytical method, such as emission, absorption or fluorescence spectroscopy, electrochemistry or neutron activation. Metals that have been used as labels include iron, mercury, chromium, copper, gold, manganese, platinum and cobalt (Schall and Tenoso, 1981). However, the use of metal atoms is not widespread as the measuring equipment is expensive and is not generally available.

The use of free radicals, or spin labels, has also been reported (Schall and Tenoso, 1981). Electrons possess a rotational property called spin. When held in a magnetic field of appropriate intensity and excited by radiowaves of the right frequency, unpaired electrons absorb and scatter energy by resonance. The resonant absorption is altered by changes in environment, such as the binding of antibody to antigen, thus enabling development of homogeneous assays. Detection is by electron spin resonance (ESR) spectroscopy. Assays using spin-labelled compounds are generally only suitable for

haptens present in micromolar concentrations. Also, the labels are rather insoluble, and the ESR spectrometer is expensive.

Bacteriophages, viruses which cause the lysis and death of bacterial cells, can be used as labels (Schall and Tenoso, 1981). Bacteria grown on culture medium in a petri dish form a dense carpet. In a culture infected with bacteriophages, plaques appear where the bacteria have been killed. The number of plaques is proportional to the severity of the infection by bacteriophages. Bacteriophages can be neutralised by chemically coupling a molecule to their surface and reacting the resulting product with an antibody specific for the coupled molecule. The number of plaques obtained on infection of a bacterial culture by bacteriophages is altered in the presence of antibody that can neutralise the bacteriophages. Concentrations of haptens can be measured by competitive neutralisation of bacteriophage-hapten conjugates with antibodies to hapten.

Non-radioactive iodine can also be used as a label in immunoassays. Using the methodology that already exists for radioiodination, a substance can be labelled with non-radioactive iodine. The label can be detected using a chemical method, for example, a kinetic reaction catalysed by iodine which can be monitored spectrophotometrically.

1.3 Non-radioactive iodine as a label.

O'Kennedy and Keating (1991) first reported the use of non-radioactive iodine as a label in an immunoassay. The assay was based on the use of iodinated Bolton Hunter reagent (IBHR), an iodine-containing hapten, to label antibodies and detection of the iodine label using the Sandell-Kolthoff reaction, a kinetic reaction between cerium(IV) and arsenic(III) which is catalysed by iodine (Section 1.3.2.1). Mouse IgG was detected in both a two-site assay and a direct assay using IBHR-labelled anti-mouse IgG antibodies. The two-site immunoassay was used to measure mouse IgG in serum, ascitic fluid and cell culture supernatants. The results obtained using IBHR-labelled antibodies compared favourably with those obtained using a conventional ELISA with HRP-labelled antibodies. Non-radioactive iodine was also used as a label in a two-site immunoassay for detection of human IgG in standards and human serum samples (O'Kennedy and Keating, 1993).

The use of the non-radioactive iodine label has several advantages. Iodination of compounds with non-radioactive iodine can be easily achieved using one of the well established iodination techniques available for radioiodination. The label can be detected using a chemical reaction catalysed by iodine, which provides an amplified

response. The Sandell-Kolthoff reaction was used by O'Kennedy and Keating (1991), but there are many other kinetic catalytic reactions that can also be used for the detection of iodine (Section 1.3.2.2).

1.3.1 Iodination techniques.

There are a variety of techniques available for the iodination of proteins which can be divided into substitution techniques and conjugation techniques. Several techniques for the direct substitution of iodine into proteins are available. Initially, iodine monochloride was used as a direct iodination reagent. Subsequent methods have involved the oxidation, at alkaline pH, of sodium iodide to form cationic iodine (I^+). Oxidising agents, such as chloramine T, iodogen, iodo-beads and peroxidases have been used. Cationic iodine reacts with aromatic moieties present in the protein. In a study by Knight and Welch (1978), proteins were labelled using chloramine T, lactoperoxidase or chloroperoxidase with variation of the reaction pH in the range 2.2 to 9.3. The proteins were degraded enzymatically, and the labelled amino acids were analysed by chromatography. The pH of the labelling reaction determined the site of labelling. At pH 7, predominantly tyrosine was labelled. At pH 4.5 to 6, predominantly cysteine was labelled, although iodination at cysteine residues is unstable and iodide was released on enzymatic degradation. Yields of iodinated histidine and of di-iodinated tyrosine were low at the pH range tested. Bolton (1986) reported that labelling of histidine is favoured using conditions of greater alkalinity.

Iodination using substitution techniques is straightforward. The oxidising agent is added to the protein, and the iodination reaction is subsequently stopped by addition of a reducing agent. Purification of the labelled protein is carried out immediately in order to remove any unconjugated oxidation products, free iodine and reducing agent. Purification can be achieved by gel filtration, ion exchange or dialysis. Direct iodination methods result in high yields of incorporation of iodine into a protein, producing a tracer with high specific activity. The main drawback of these methods is the exposure of the protein to ^{125}I, oxidising and reducing solutions which may damage the protein.

In conjugation techniques a reactive precursor is labelled by one of the substitution methods, and the labelled product is reacted with the protein. This is more gentle than direct iodination techniques as the protein never comes into contact with the ^{125}I, oxidising or reducing solutions. The first such method to be described used the Bolton Hunter reagent for iodination of protein (Bolton and Hunter, 1973). This reagent reacts

with free amino groups in the protein to form amides. In the study by Knight and Welch (1978), protein conjugated to IBHR had predominantly lysine residues labelled, although labelled histidine and tyrosine were also present. Conjugation techniques can, therefore, be used to label different residues to those labelled using substitution techniques. This is valuable for iodination of proteins that do not contain tyrosine residues, or where iodination of tyrosine residues renders a protein unsuitable for use in RIA. However, conjugation techniques tend to yield tracers of lower specific activity than direct iodination as two reaction steps are involved resulting in lower yield of iodine incorporated into the protein (Bolton, 1986).

Iodination of a protein may result in alteration of its biological or immunological reactivity compared to that observed in its native form. Denaturation can occur due to radiation damage and chemical damage caused by reagents used in the iodination reaction, manipulation of the protein in dilute solutions during iodination and purification, and introduction of iodine into the protein. (These effects are discussed in detail in Section 4.2.2.1). Any loss in immunological reactivity will result in a loss of affinity in antibody-antigen binding, and a subsequent loss of sensitivity in the immunoassay. The susceptibility of individual proteins to damage during an iodination may vary. One method may damage the protein, while another method may cause no damage.

There are many examples in the literature of comparison of iodination techniques. McCarthy and Markowitz (1983) compared the immunoprecipitation, in the presence of excess antibody, of prostatic acid phosphatase labelled by the chloramine T, lactoperoxidase and Bolton Hunter reagent methods. The Bolton Hunter reagent produced a tracer with optimal precipitation characteristics suitable for use in RIA, while the tracer produced using chloramine T and lactoperoxidase produced tracers with suboptimal precipitation characteristics. A comparison of the chloramine T and Iodo-Bead methods for iodination of human α-fetoprotein (hAFP) was performed by Lee and Griffiths (1984). The chloramine T method yielded a tracer of higher specific activity than that obtained using Iodo-Beads, but the Iodo-Bead method was a more controllable, milder method and the tracer produced by this method was more stable on storage at -20°C. Thean (1990) iodinated human α-lactalbumin using the chloramine T, lactoperoxidase and Iodogen methods. Iodination with Iodogen resulted in a tracer with the highest specific activity and immunoreactivity. A comparison of the iodination of epidermal growth factor (EGF) using chloramine T, Iodogen, Iodo-Beads, ICl and lactoperoxidase-glucose oxidase-coupled beads (Enzymobeads) was

carried out by Kienhuis *et al.* (1992). Iodination using chloramine T, Iodogen and Iodo-Beads caused oxidation of amino acid residues of EGF, and the resulting tracer had a decreased receptor binding affinity compared with native EGF. Iodination using ICl and Enzymobeads caused insignificant oxidation, and did not affect the ligand-receptor interaction.

The most suitable iodination technique must be assessed for the particular protein. The choice of iodination procedure will depend on several factors. The stability of the protein during a particular labelling procedure, the desired specific activity of the labelled product and its performance in the system for which it is required, and the simplicity and cost of the labelling procedure are factors which must be considered.

1.3.1.1 Iodine monochloride.

The use of iodine monochloride (ICl) to iodinate proteins was first introduced by McFarlane (1958). Radioactive iodine in the form of iodide ions is mixed with a solution of the protein to be iodinated, buffered to a pH of approximately 8. ICl is added, and two reactions take place. Radioactive iodine is converted to radioactive ICl by isotope exchange with nonradioactive ICl, and iodination of tyrosine residues of the protein occurs as a result of conversion of ICl to hypoiodite. The iodination reaction can be represented as follows:

$$ICl + NaOH \rightarrow HOI + NaCl$$

$$HOI + tyrosine\ residue \rightarrow iodinated\ protein + H_2O$$

(Angeles Contreras *et al.*, 1983).

The reaction is very rapid, so after just one minute a protective protein can be added, and the iodinated protein isolated from the reaction mixture. The only oxidising agent present during the iodination is ICl, so no damage can occur to the protein that may occur with other oxidising agents.

It is also possible to carry out an oxidative ICl iodination technique which can be used to label to high specific activity, and has the advantage that mild oxidation conditions associated with the original technique are maintained (Helmkamp *et al.*, 1960). In this technique radioiodine is directly oxidised to radioactive ICl, as follows:

$$2KI + KIO_3 + 6HCl \rightarrow 3ICl + 3KCl + 3H_2O$$

Iodination of the protein then occurs, without the need for an isotope exchange reaction.

1.3.1.2 Chloramine T.

Hunter and Greenwood (1962) first showed that low concentrations of chloramine T promoted incorporation of inorganic iodide into protein. Chloramine T, the N-chloro-derivative of p-methylbenzenesulphonamide, is an oxidising agent. The structure is shown in Fig 1.7.

The iodination reaction is initiated on addition of chloramine T to a mixture of protein and sodium iodide, and is allowed to proceed for approximately one minute. The reaction is stopped by addition of sodium metabisulphite, which reduces any unreacted chloramine T. Sodium metabisulphite can cause protein denaturation, so addition of tyrosine may be preferable. The level of protein iodination is affected by the concentration and duration of exposure to chloramine T; both should be kept to a minimum to avoid non-specific chloramine T damage to the protein.

The chloramine T method is rapid, and does not require the use of organic solvents or extremes of pH that might denature the protein. However, chloramine T is a powerful oxidising agent which may cause denaturation and optimal reaction conditions need to be established for each protein, as parameters such as tyrosine reactivity and susceptibility to iodination and chloramine T damage have to be assessed.

1.3.1.3 Iodogen.

The use of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) as an iodination reagent was first described by Fraker and Speck (1978). The structure of Iodogen is shown in Fig 1.7. The reagent is water-insoluble, but soluble in organic solvents such as chloroform and dichloromethane. It can be used as a solid phase oxidising agent for iodination by plating aliquots in a thin film on the walls of the reaction vessel. On addition of sodium iodide and an aqueous solution of protein the iodination reaction proceeds rapidly, and is stopped by decanting the reaction solution away from the Iodogen reagent. Thus, no reducing agent is required. However, the method requires evaporation of organic solvent for plating of Iodogen on the reaction vessel which is tedious and hazardous.

1.3.1.4 Iodo-Beads.

Markwell (1982) first reported the use of Iodo-Beads for protein iodination. Iodo-Beads consist of N-chlorobenzenesulphonamide immobilised on non-porous polystyrene beads (Fig 1.7). The iodination procedure is simple. Iodo-Beads are added to a solution of sodium iodide and protein, and iodination is allowed to proceed for 15

minutes. The reaction is stopped by removal of the Iodo-Beads with a tweezers or by removal of the reaction solution using a pipette. As with Iodogen, the need to use a reducing agent is eliminated.

1.3.1.5 Lactoperoxidase.

Lactoperoxidase is an oxidising enzyme that can be used for enzymatic iodination of a protein (Marchalonis, 1969). Iodination is carried out in the presence of low concentrations of hydrogen peroxide; the quantity of peroxide can be used to control the level of iodination. The iodination reaction is stopped by the addition of a reducing agent, such as mercaptoethanol. Lactoperoxidase iodination is a gentle method and is important for proteins that are subject to damage using chemical methods. The lactoperoxidase method avoids exposure of the protein to excessive quantities of strong oxidising agents, and for some proteins the method is superior in maintaining the structural integrity of the protein.

The reaction conditions for iodination using lactoperoxidase should be carefully controlled. Lactoperoxidase is sensitive to inhibition by sodium azide and high salt concentrations. The use of too high a concentration of peroxide, or partially inactive peroxide or enzyme, can cause problems.

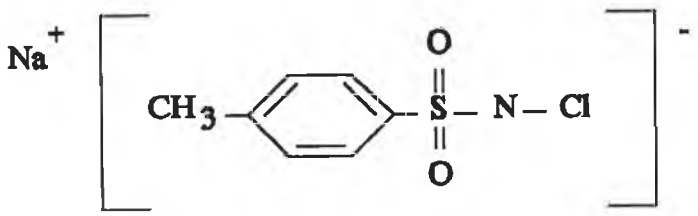
Other peroxidases can be used for enzymatic iodination, for example, HRP, thyroid peroxidase and chloroperoxidase, but these have a lower iodination efficiency than lactoperoxidase. Marchalonis (1969) reported that HRP was at least an order of magnitude less effective than lactoperoxidase for iodination of proteins.

1.3.1.6 Bolton Hunter reagent.

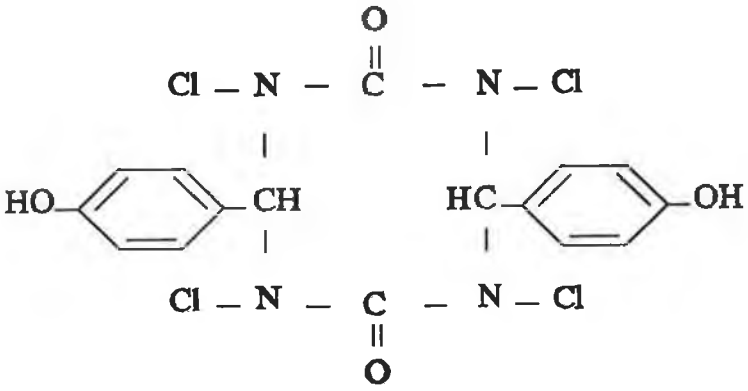
The first conjugation reagent to be used for iodination of proteins was 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester, which is known as Bolton Hunter reagent (Bolton and Hunter, 1973). Bolton Hunter reagent (BHR) is iodinated by the chloramine T method, and the iodinated product is extracted into benzene and evaporated to dryness. Mono- or di-iodinated BHR can be produced, depending on the reaction conditions used. The reaction should be carried out as rapidly as possible due to the susceptibility of the N-hydroxysuccinimide ester to hydrolysis in aqueous solution. In order to iodinate proteins, the iodinated reagent is reacted with protein in borate buffer, pH 8.5. The reaction scheme is shown in Fig 1.8. Since IBHR was first introduced it has been used to label a wide variety of compounds, such as proteins and peptides, antibodies, haptens, drugs, viruses and cell lysates (Langone, 1980).

The use of other conjugation reagents has also been reported. Aniline can be iodinated by the chloramine T method, diazotized and reacted with the protein under basic conditions (Hayes and Goldstein, 1975). This method is suitable for proteins that have reactive thiol groups. The reagent is presumed to react with the phenol moiety of tyrosine residues, producing an iodinated protein that is analogous to that obtained by direct iodination (Langone, 1980). The use of the iodinated derivative of methyl p-hydroxybenzimidate HCL (MPHBIM) was reported by Wood *et al.* (1975). MPHBIM was iodinated using chloramine T, and the iodinated product was isolated by acid precipitation. This reagent reacts with amino groups of the protein. However, the positive charge of the amino groups may be preserved with this reagent whereas the positive charge is probably lost on reaction with IBHR. Garg *et al.* (1989) used iodinated N-succinimidyl (tri-n-butylstannyl) benzoate (ATE) for labelling of antibodies. They reported that the labelling efficiency using ATE was twice that observed using IBHR. This was possibly due to the inertness to hydrolysis of the N-succinimidyl group of ATE, due to a lack of a methylene spacer between this group and the aromatic ring.

(a)



(b)



(c)

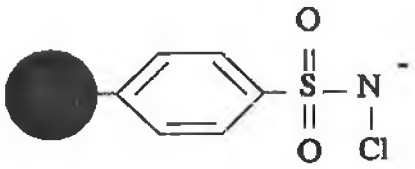


Figure 1.7 The structures of some oxidising agents used for iodination of protein.
(a) Chloramine T (b) Iodogen (c) Iodo-beads

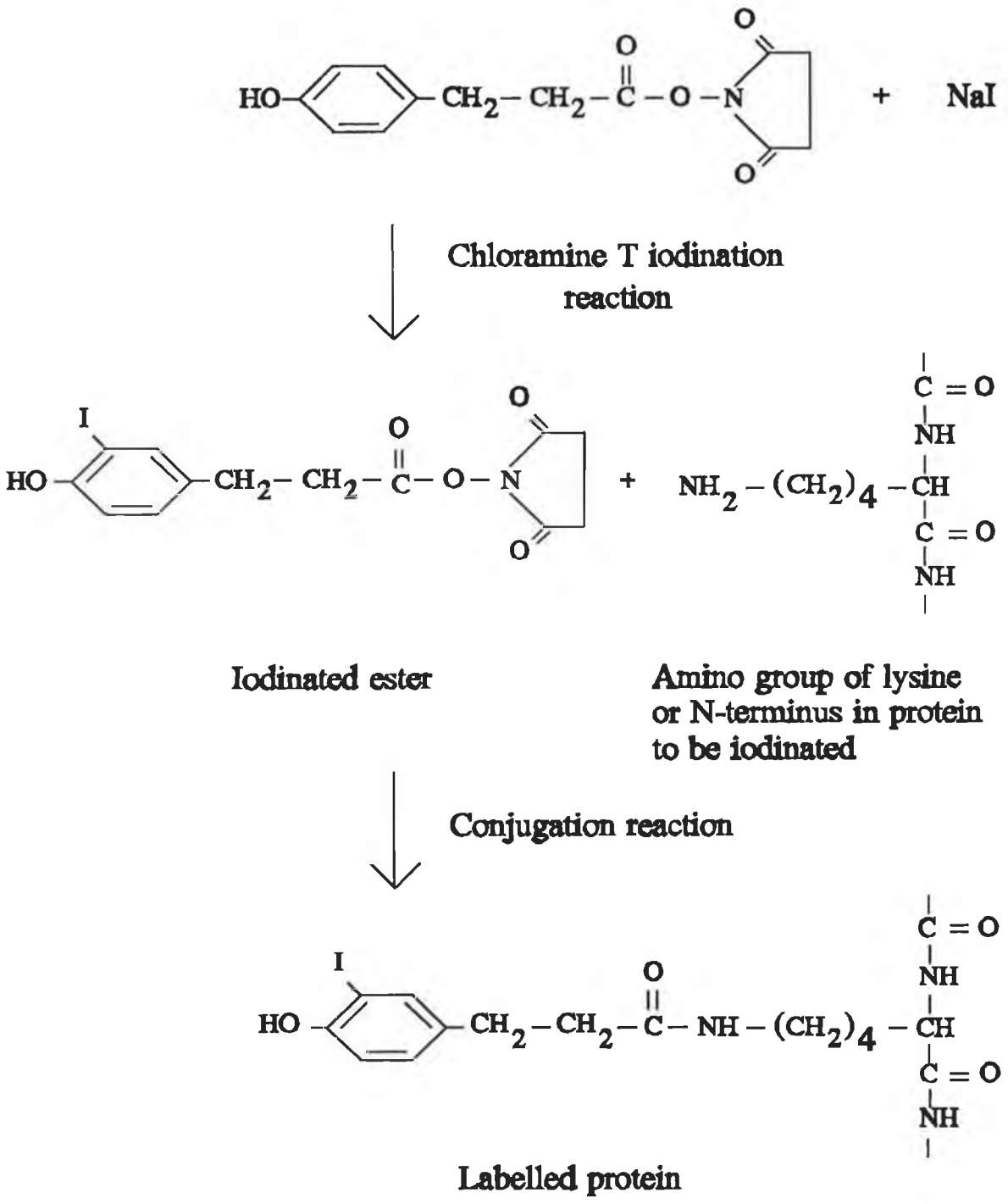


Figure 1.8 The reaction scheme for iodination of protein using the Bolton Hunter reagent.

1.3.2 Kinetic catalytic methods for determination of iodine.

The use of non-radioactive iodine as a label in immunoassays depends on the availability of a suitable detection method for iodine. Kinetic catalytic methods are suitable for the sensitive determination of iodine. Iodide ion is one of the most effective inorganic catalysts (Fritz and Schenk, 1966); it can be determined at ultratrace level in the presence of many other ions.

For kinetic catalytic reactions, the presence of the catalyst increases the rate of reaction. The rate is, therefore, directly proportional to the concentration of catalyst. Catalytic reactions have low limits of detection. They can be used for the determination of reactants, catalysts and substances reacting with catalysts (activators, inhibitors).

Several experimental methods can be applied to the measurement of the rate of a reaction. To determine the rate, the change with time of the concentration of at least one of the reactants or products must be studied using a suitable method of analysis. Methods that measure the change of some physical property of the system, proportional to the concentration, as a function of time are suitable. Spectrophotometric methods are most widely used. Electrochemical methods, polarimetry, fluorescence, and luminescence are also used. Determination of the catalyst concentration can be made by plotting the rate of the reaction against the concentration of the catalyst. Alternatively, the absorbance of a reactant or product after a fixed time, or the time required to reach a fixed absorbance, can be plotted against the concentration of the catalyst.

Spectrophotometric methods are of interest for the detection of the non-radioactive iodine label in immunoassays, therefore, only kinetic catalytic reactions that can be monitored spectrophotometrically will be discussed here. Many kinetic catalytic reactions have been recommended for the determination of iodine (Zak, 1978; Williams, 1979). The most frequently used method is the Sandell-Kolthoff reaction.

1.3.2.1 The Sandell-Kolthoff reaction.

The determination of iodide by measurement of its catalytic action on the reaction between cerium(IV) and arsenic(III), in acidic solution, was first described by Sandell and Kolthoff (1934). The reaction proceeds as follows:

$$\underset{\text{(Yellow)}}{2Ce^{4+}} + As^{3+} \rightarrow \underset{\text{(Colourless)}}{2Ce^{3+}} + As^{5+}$$

In the absence of catalyst the reaction requires many hours to go to completion. In the presence of catalyst the reaction rate increases, the rate being proportional to the

catalyst concentration. The reaction was originally monitored chronometrically which involved waiting until the yellow colour due to cerium(IV) ions had disappeared, and noting the time. This was too time-consuming for routine determinations. Therefore, methods based on the employment of an empirical absorbance-concentration standard curve under controlled conditions of time, temperature, acidity, and concentration and order of reagent addition were developed (Zak, 1978).

The Sandell-Kolthoff reaction is catalysed not only by inorganic iodide, but also by iodine-containing organic compounds (Bowden *et al.*, 1955). It is routinely used in a large number of laboratories for the determination of iodine in many types of samples and of iodine-containing proteins, hormones and drugs. The measurement of iodine in biological samples and foods is of interest, as absence of iodide in the diet produces several diseases. Iodine deficiency is a major health problem in Asia, Africa and South America, and can result in goitre, irreversible mental retardation and decreased survival among children (Dunn *et al.*, 1993). Urinary iodine excretion is used to assess iodine deficiency in communities in these countries. In the developed world studies have indicated that the intake of iodine is several times greater than the Recommended Nutrient Intake (RNI) (Fischer *et al.*, 1986). There are regulations that govern the amount of iodine in certain foods, for example, infant formula and table salt, and in order to enforce these regulations the iodine content of foods must be determined. Determination of thyroid hormones is also of major importance. The primary action of thyroid hormones is to increase the basal metabolic rate in many tissues. Hypothyroidism, a deficiency in thyroxine (T_4) is characteristic of the disease myxoedema. Hyperthyroidism, excessive secretion of T_4, is responsible for Graves' disease or exophthalmic goitre.

Many authors have reported the use of the Sandell-Kolthoff reaction for the measurement of iodine in biological samples. Soon after the introduction of the reaction, Chaney (1940) used it for the determination of iodine in blood. Samples were subjected to chromic acid oxidation followed by distillation of the iodine. The iodine content of the distillate was determined by reading the percentage transmittance of the reaction solution exactly 5 minutes after the addition of ceric sulphate. Percentage transmittance was also used by Mougey and Mason (1963) in the determination of inorganic iodide, iodotyrosines and thyroid hormones in serum or plasma, following separation by gel filtration. Knapp and Leopold (1974) separated the thyroid hormones 3',3,5-tri-iodothyronine (T_3) and T_4 by ion-exchange chromatography, and determined the hormone concentration by measurement of the absorbance of the Sandell-Kolthoff

reaction at 365nm. Nachtmann *et al.* (1978) separated the thyroid hormones by HPLC, and used the reaction for post-column detection as UV detection was not sufficiently sensitive. Iodide, iodotyrosine, T_3 and T_4 were also determined by Palumbo *et al.* (1982) by monitoring the absorbance at 410nm. Their method was modified by Saboori *et al.* (1993), and used for the determination of iodine in thyroglobulin. Plasma inorganic iodine (PII) was determined by Aumont and Tressol (1987) after separation of iodide from organically bound iodine by ion-exchange chromatography. Following alkaline ashing, iodide was determined by the Sandell-Kolthoff reaction. The reaction was arrested by the addition of brucine, which causes the reduction of the remaining cerium(IV) to colourless cerium(III), with oxidation of brucine to an orange-coloured product. The absorbance of the orange-coloured product was measured. Mezzetti *et al.* (1988) determined T_4 and iodine-containing drugs in plasma following separation by ion-exchange chromatography. The Sandell-Kolthoff reaction was arrested by addition of mercuric ions, which inhibit the catalytic action of iodide.

The iodine content of many other biological samples has also been determined using the Sandell-Kolthoff reaction. Fischer *et al.* (1986) determined iodine in samples of dairy products, meat, eggs, fish, cereal and potatoes after acid digestion of the organic material. Iodine in urine and milk was determined by Aumont and Tressol (1986) after alkaline ashing. They used brucine to arrest the reaction. Alkaline ashing, and measurement of the absorbance of the Sandell-Kolthoff reaction at 410nm or 365nm, was used by Ayiannidis and Voulgaropoulos (1988) and Gutierrez *et al.* (1989) to measure iodine in samples such as blood, milk, water, salt and alfalfa meal. Urinary iodine was measured after alkaline ashing by May *et al.* (1990) and after acid digestion by Dunn *et al.* (1993). The detection limit achieved by some of these authors is shown in Table 1.5, and illustrates that the Sandell-Kolthoff reaction is a sensitive method for measurement of iodide and iodine-containing compounds at nanogramme levels in biological samples.

A microassay system based on the Sandell-Kolthoff reaction was developed by O'Kennedy *et al.* (1989). The Sandell-Kolthoff reaction was modified for use with microtitre plates, and the absorbance was monitored using a microplate reader. The use of a microassay system has several advantages. The microassay has good sensitivity and reproducibility, small quantities of reagents are required and many samples can be tested simultaneously. O'Kennedy *et al.* (1989) used the microassay system to measure iodide at nanogramme levels, and to monitor the conjugation of IBHR to ovalbumin and human serum albumin. Their system thus eliminated the need

to use radioactivity to monitor iodination. The microassay was applied to the detection of non-radioactive iodine-labelled antibodies in immunoassays (Section 1.3). Iodine-labelled antibodies were prepared using the IBHR iodination technique, but without the need to use hazardous radioactive materials, and the iodine label was detected using the Sandell-Kolthoff microassay. This immunoassay system showed favourable comparison with ELISA. The main drawback of the system is that as it is based on the use of the Sandell-Kolthoff reaction it requires the use of arsenic, a known carcinogen. Therefore, the replacement of the Sandell-Kolthoff reaction with another kinetic catalytic reaction for detection of the iodine label would be favourable.

1.3.2.2 Other kinetic catalytic reactions.

There are many alternative kinetic catalytic reactions which have detection limits equivalent to that achieved using the Sandell-Kolthoff reaction. The detection limits for iodide obtained using some of these reactions are reported in Table 1.6 and Table 1.7.

Several of these reactions involve the use of hydrogen peroxide as oxidising agent. Fuchs *et al.* (1964) used p-aminophenol and hydrogen peroxide for catalytic determination of iodide. Iodide is oxidised by hydrogen peroxide to iodate, which in turn oxidises p-aminophenol to a blue indamine dye. The absorbance of the blue dye was measured at 540nm. The oxidation of benzidine and o-tolidine by hydrogen peroxide was used by Yasinskene and Umbrazhyunaite (1975a). The oxidation occurs in two stages: during the first stage the absorbance increases due to the formation of a coloured reaction product, while during the second stage the absorbance decreases due to decolouration of the product. Iodide catalyses both stages, but the first stage of the reaction was monitored as this was the more sensitive.

Yasinskene and Umbrazhyunaite (1973) also investigated the oxidation of the organic dyes pyrogallol red, bromopyrogallol red and stilbazo by hydrogen peroxide. The reactions were based on the decolouration of the dyes, and the absorbance was monitored at 440nm. The use of bromate, iodate and periodate as alternative oxidising agents was examined, but these gave reduced sensitivity. The measurement of iodide using the oxidation of pyrocatechol violet by hydrogen peroxide was also investigated by Yasinskene and Umbrazhyunaite (1973), a reaction which was used by Yonehara *et al.* (1989) for the simultaneous determination of iodide and bromide. The catalytic effect of bromide is relevant only in the early stages of the reaction, and ceases after less than a minute. Iodide acts as a catalyst throughout the reaction, thus enabling the

ions to be determined simultaneously. Kreingold *et al.* (1978) studied the oxidation of o-phenylenediamine (OPDA), diphenylcarbazide and variamine blue by hydrogen peroxide. These reactions involve the oxidation of colourless organic reagents to intensely coloured products. The OPDA-hydrogen peroxide reaction was the most sensitive of these reactions.

Other reactions involving oxidation by hydrogen peroxide are the reaction of 3,3'-dimethylnaphthidine with hydrogen peroxide (Bognar and Nagy, 1969), and the reaction of sodium 2-thiosemicarbazone-1,2-naphthoquinone-4-sulphonate (TNHS) with hydrogen peroxide (Igov *et al.*, 1979).

The reaction of iron thiocyanate with nitrite ions is the most frequently used reaction, after the Sandell-Kolthoff reaction, for the determination of iodide. Iodide catalyses the oxidation of thiocyanate by nitrite, and the decrease in the orange colour of the iron(III) thiocyanate can be used to follow the reaction. The reaction can be represented by:

$$NO_2^- + 2SCN^- + 4H^+ \rightarrow 2NO + (SCN)_2 + 2H_2O$$

(Williams, 1979).

The method was first proposed for the determination of iodide by Iwasaki *et al.* (1953). However, Ottaway *et al.* (1969) reported that with this method two widely different iodide concentrations can lead to the same value of the measured parameter. The method can be used if it is known that the iodide concentration in a sample will fall within 1ng/ml to 10µg/ml, as accurate results are obtained within these limits. The reaction has been used for the determination of iodine in foods (Moxon and Dixon, 1980), of total and free iodide in water samples (Moxon, 1984), and of inorganic iodine in rain (Fournier-Bidoz *et al.*, 1992). Iodide and nitrite have been determined simultaneously using this reaction (Zhu and Gu, 1993). The reaction is characterised by an induction period. The length of the induction period depends on the concentration of nitrite, and is independent of iodide. The rate of the reaction depends on the concentration of iodide, and is independent of nitrite. Therefore, nitrite and iodide can be determined simultaneously by measurement of the induction period and the reaction rate.

Oxidations involving chloramine T or chloramine B also provide sensitive methods of iodide determination. The use of the reaction between chloramine T and N,N'-tetramethyl-diaminodiphenylmethane (tetrabase acetate) was reported by Jungreis and Gedalia (1960). Tetrabase acetate is oxidised to an unstable blue quinoidal product,

which decomposes within a few minutes to produce a yellow-green product. The reaction was monitored by measurement of the absorbance of the blue compound at 600nm, as the absorbance of the final product was low. Oxidation of organic dyes by chloramine B was used by Bunikene and Ramanauskas (1968) and Yasinskene and Umbrazhyunaite (1975b). The oxidation of 11 triphenylmethane dyes was studied by Bunikene and Ramanauskas (1968). The most sensitive dyes for iodide determination were brilliant green, acid green G and acid bright blue. Yasinskene and Umbrazhyunaite (1975b) examined the oxidation of pyrocatechol violet and bromopyrogallol red by chloramine B. The use of chloramine B as an oxidising agent for bromopyrogallol red proved to be more sensitive than the use of hydrogen peroxide. The determination of iodide using the oxidation of chlorpromazine, a tranquillizing drug, by bromate was reported by Vinas *et al.* (1987). The reaction was followed by measuring the increase in absorbance at 525nm due to the formation of a red semiquinone product. The use of this reaction, with hydrogen peroxide as oxidising agent, was reported by Liang *et al.* (1993) for the determination of iodide and by Tomiyasu *et al.* (1994) for the differential determination of iodate and iodide.

The use of several other kinetic catalytic reactions has also been reported. Bognar and Sarosi (1965) used the reaction between cerium(IV) and antimony(III) for the determination of traces of iodine. The reaction was monitored chronometrically using ferroin as indicator for determination of the end point. The catalytic effect of iodide on the reaction between iodate and arsenic(III) was used by Weisz and Rothmaier (1974). The reaction scheme is as follows:

$$3AsO_3^- + IO_3^- \rightarrow 3AsO_4^- + I^-$$

The oxidation of arsenic(III) by hexacyanoferrate(III) was used by Sriramam *et al.* (1987). The reaction was monitored by measurement of the absorbance of unreacted hexacyanoferrate at 415nm.

Many of these reactions have low limits of detection for iodide, and would be suitable for the development of an alternative to the Sandell-Kolthoff microassay described by O'Kennedy *et al.* (1989). The aim of this project is to develop a microassay for measurement of iodine using one of these reactions, and to apply this to the detection of the non-radioactive iodine label in immunoassays.

This thesis describes the development and optimisation of an alternative microassay, based on the reaction between cerium(IV) and antimony(III) (Bognar and Sarosi, 1965). The use of the cerium(IV) - antimony(III) reaction allows the replacement of

arsenic(III) with antimony(III), which is advantageous for safety reasons, while allowing the continued use of cerium(IV). Cerium(IV) oxidises iodine-containing organic compounds liberating iodine; the bound iodine must be liberated for the detection of these compounds by a catalytic reaction (Section 3.2.3). The catalytic activity of iodide and iodine-containing organic compounds on this new microassay is studied, and compared with the catalytic activity on the Sandell-Kolthoff microassay. The feasibility of using the new microassay for the detection of the non-radioactive iodine label in an immunoassay using labelled antibodies is assessed by comparison with an immunoassay using the Sandell-Kolthoff microassay for detection of the iodine label, and comparison with an ELISA. The model system used is an immunoassay for the measurement of human IgG. As human IgG has more than one antigenic determinant it is suitable for use as an analyte in a two-site immunoassay using iodinated antibodies for detection of the analyte. The results for the model system can therefore be extrapolated to illustrate how iodinated antibodies would perform in immunoassays for the detection of other antigens. Alternative immunoassays using non-radioactive iodine-labelled reagents in conjunction with the avidin-biotin system or bispecific antibodies are reported. An immunoassay for T_4, which uses the four iodine atoms present on T_4 as the label, is also evaluated.

Compound	Detection limit (ng/ml)	Reference
T_3	0.2	Knapp and Leopold, 1974
T_4	2	
Iodide	0.1	Fischer *et al.*, 1986
	3	Aumont and Tressol, 1987
	<10	Mezzetti *et al.*, 1988
	1	Gutierrez *et al.*, 1989
	1	O'Kennedy *et al.*, 1989
	10	Dunn *et al.*, 1993

Table 1.5 The detection limit for thyroid hormones or iodide obtained using the Sandell-Kolthoff reaction.

Reaction	Detection limit	Reference
p-Aminophenol - H_2O_2	2.5µg/ml	Fuchs *et al.*, 1964
Pyrogallol red - H_2O_2	1.1µg/ml	Yasinskene and Umbrazhyunaite, 1973
Bromopyrogallol red - H_2O_2	11ng/ml	
Stilbazo - H_2O_2	55ng/ml	
Pyrocatechol violet - H_2O_2	2ng/ml	
	5ng/ml	Yonehara *et al.*, 1989
Benzidine - H_2O_2	4ng/ml	Yasinskene and Umbrazhyunaite, 1975a
o-Tolidine - H_2O_2	2ng/ml	
OPDA - H_2O_2	2ng/ml	Kreingold *et al.*, 1978
Diphenylcarbazide - H_2O_2	11ng/ml	
Variamine blue - H_2O_2	20ng/ml	
3,3'-dimethylnaphthidine - H_2O_2	2ng/ml	Bognar and Nagy, 1969
TNHS - H_2O_2	0.5µg/ml	Igov *et al.*, 1979

Table 1.6 The detection limit for iodide obtained using several kinetic catalytic reactions with hydrogen peroxide as oxidising agent.

Reaction	Detection limit	Reference
Iron thiocyanate - nitrite ions	1ng/ml	Iwasaki *et al.*, 1953
	0.4ng/ml	Fournier-Bidoz *et al.*, 1992
	5µg/ml	Zhu and Gu, 1993
Tetrabase acetate - chloramine T	1.8ng/ml	Jungreis and Gedalia, 1960
Brilliant green - chloramine B	0.6ng/ml	Bunikene and Ramanauskas, 1968
Acid green G - chloramine B	2.1ng/ml	
Acid bright blue - chloramine B	2.9ng/ml	
Pyrocatechol violet - chloramine B	1.3ng/ml	Yasinskene and Umbrazhyunaite, 1975b
Bromopyrogallol red - chloramine B	1.3ng/ml	
Chlorpromazine - bromate	5ng/ml	Vinas *et al.*, 1987
Cerium(IV) - antimony(III)	1ng/ml	Bognar and Sarosi, 1965
Iodate - arsenic(III)	0.1µg/ml	Weisz and Rothmaier, 1974

Table 1.7 The detection limit for iodide obtained using several kinetic catalytic reactions.

2. MATERIALS AND METHODS.

2.1 MATERIALS.

All chemicals used in the experimental work were analytical grade, and were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) except for the following:

3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester, 3-iodophenol, and benzene were obtained from Aldrich Chemical Co. Ltd. (Dorset, England).

L(+)-tartaric acid, antimony potassium oxide(+)tartrate 0.5-hydrate, potassium iodide, sodium periodate, boric acid, di-sodium tetraborate, acetic acid and nitric acid were obtained from BDH Chemicals Ltd. (Poole, England).

L-thyroxine, ethanol, 2-iodophenol and silica gel 60 for column chromatography were obtained from Merck Chemical Co. Ltd. (Darmstadt, Germany).

Ammonium persulphate, Tween 20, sulphuric acid, hydrochloric acid, toluene, glycerol, bromophenol blue, 2-mercaptoethanol and TLC plates (10 x 20cm; silica gel 60; 0.2mm layer thickness) were obtained from Riedel-de Haen (Hannover, Germany).

Ethyl acetate and methanol were obtained from LabScan Analytical Sciences (Dublin).

Avidin (from hen egg white), biotin-LC-hydrazide, N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB) and BCA reagents were obtained from Pierce Chemical Co. (Rockford, Il., USA).

BioRad dye reagent concentrate was obtained from BioRad Laboratories (Munchen, Germany).

Sephadex G-25 (medium) was obtained from Pharmacia LKB (Uppsala, Sweden).

Ethanolamine-HCl, N-hydroxysuccinimide - coupling solution and N-ethyl-N'-(dimethylamino-propyl)carbodiimide - coupling solution were obtained from Pharmacia Biosensor Ltd. (Uppsala, Sweden).

PBS tablets were obtained from Oxoid (Hampshire, England).

8-Hydroxyquinoline-5-sulphonic acid monohydrate was obtained from MTM Research Chemicals Ltd. (Lancashire, England).

Microtitre plates (Maxisorb, 96-well, flat-bottomed) were obtained from Nunc (Roskilde, Denmark).

Dynatech MicroFluor plates (white) were obtained from Dynatech (West Sussex, UK).

Human serum samples were obtained from St. James' Hospital (Dublin).

The antibodies used, and the companies from which they were obtained, were as follows: Human IgG, goat anti-human IgG antibodies, rabbit anti-BSA antibodies, HRP-labelled rabbit anti-goat IgG antibodies, HRP-labelled goat anti-rabbit IgG antibodies and rabbit anti-thyroxine serum were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).
Sheep anti-thyroxine serum was obtained from Scottish Antibody Production Unit (Lanarkshire, Scotland).
Rabbit anti-thyroxine antibodies were obtained from Dako (Glostrup, Denmark).

The following instrumentation was used:
Titertek Twinreader plus (Flow Laboratories, Lugano, Switzerland).
Perkin Elmer LS50 Luminescence Spectrophotometer (Perkin Elmer Ltd., Buckinghamshire, England).
Shimadzu UV 160A recording spectrophotometer (Shimadzu Corporation, Tokyo, Japan).
BIAcore (Pharmacia Biosensor, Uppsala, Sweden).
Atto mini-gel electrophoresis unit (Atto Corporation, Tokyo, Japan).

2.2 Microassays for the measurement of iodine.

2.2.1 Cerium(IV)-Antimony(III) spectrophotometric microassay.

Ceric ammonium sulphate (0.02M) was prepared in 6M H_2SO_4. Concentrated H_2SO_4 (334ml) was added slowly to 500ml of ultrapure water, followed by 12.65g of ceric ammonium sulphate. The solution was stirred until the ceric ammonium sulphate had dissolved, cooled to room temperature and made up to 1 litre with ultrapure water. Antimony potassium tartrate (0.03M) was prepared in 1% (w/v) tartaric acid.
Standards, or samples, (100µl) were added to the wells of a microtitre plate. Antimony potassium tartrate (50µl) was added using an eight-channel multipipette, followed by 50µl of ceric ammonium sulphate. The reactants were mixed thoroughly, and after incubation at room temperature for 1hr the absorbance was measured at 380nm using the Titertek plate reader.

2.2.2 Cerium(III) fluorimetric microassay.

The procedure was carried out as for the cerium(IV)-antimony(III) spectrophotometric

microassay, except antimony potassium tartrate was used at a concentration of 0.04M. After incubation at room temperature for 1hr the fluorescence was measured at excitation and emission wavelengths of 260nm and 360nm, respectively.

2.2.3 Cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric microassay.

The procedure was identical to the cerium(IV)-antimony(III) spectrophotometric microassay, except that after incubation at room temperature for 1hr, 50µl of 1.5mM 8-hydroxyquinoline-5-sulphonic acid was added to the wells. The fluorescence was measured at excitation and emission wavelengths of 370nm and 480nm, respectively.

2.2.4 Sandell-Kolthoff microassay.

The microassay was carried out as reported by O'Kennedy and Keating (1991). Ceric ammonium sulphate (0.1M) was prepared in 2.5M H_2SO_4, using the method outlined in Section 2.2.1. This solution was diluted to a working concentration of 0.028M in 10% (v/v) H_2SO_4. Arsenious acid (0.075M) was prepared by dissolving 14.84g of arsenious trioxide in 700ml of ultrapure water containing 28ml of concentrated H_2SO_4. (Care should be taken in the preparation of this reagent, as arsenious trioxide is a carcinogen.) The solution was heated to near-boiling, stirring constantly, until the arsenious trioxide had dissolved. Some precipitation of arsenious trioxide may occur. After reaching room temperature, the solution was made up to 1 litre with ultrapure water.

Standards, or samples, (100µl) were added to the wells of a microtitre plate. Arsenious acid (60µl) was added using an eight-channel multipipette, followed by 25µl of ceric ammonium sulphate. The plate was mixed well, and after incubation at room temperature for 5min the absorbance was measured at 414nm using the Titertek plate reader.

2.3 Preparation of iodinated Bolton Hunter reagent.

2.3.1 Preparation of mono-iodinated Bolton Hunter reagent (IBHR).

IBHR was prepared as reported by O'Kennedy *et al.* (1989). A 0.1M solution of 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (Bolton Hunter reagent) and a 2M solution of iodine monochloride (ICl) were prepared in ethyl acetate. ICl was added to Bolton Hunter reagent at a 2:1 molar ratio. The reaction was allowed to proceed at 4°C overnight. IBHR, which forms as a white precipitate, was recovered by centrifugation at

400g for 10 min. The precipitate was washed several times in ice-cold ethyl acetate. The precipitate was dried under nitrogen, and stored at -20°C.

The purity was tested by TLC on silica gel plates with a fluorescent indicator, and a solvent system of ethyl acetate : toluene (1:1, v/v). IBHR had an Rf value of 0.50.

2.3.2 Preparation of di-iodinated Bolton Hunter reagent (di-IBHR).

This was carried out according to the method of Bolton and Hunter (1973). Bolton Hunter reagent (0.1mg) was reacted with 0.68mg of NaI and 25mg of chloramine-T in 5ml of 0.25M phosphate buffer, pH 7.5 (iodide : Bolton Hunter reagent molar ratio of 10:1). After approximately 30 seconds the reaction was stopped by the addition of 60mg of sodium metabisulphite in 5ml of 0.05M phosphate buffer, pH 7.5. DMF (1ml) was immediately added, and the iodinated products were extracted into benzene and evaporated to dryness under nitrogen. The products were identified by TLC on silica gel plates with a fluorescent indicator, and a solvent system of ethyl acetate : toluene (1:1, v/v). Di-IBHR had an Rf value of 0.68.

Di-IBHR was purified by chromatography on a silica column with a solvent system of ethyl acetate : toluene (1:2, v/v). The reaction products were dissolved in ethyl acetate : toluene (1:2, v/v), and applied to the top of a silica column (particle size 0.063mm) in a 75ml separation funnel (12 x 3cm). Fractions (0.5ml) were collected, and the purity of each fraction was tested by TLC. Fractions found to contain di-IBHR were pooled, and evaporated to dryness under nitrogen.

2.4 Preparation of IBHR-labelled antibodies.

The method was carried out as reported by O'Kennedy *et al.* (1989). IBHR was dissolved at a concentration of 4mg/ml in benzene, containing 4% (v/v) DMF. One milligramme was dried under nitrogen, and reconstituted with 200µl of DMF. Antibody (1mg in 1ml of 0.1M borate buffer, pH 8.5) was added. The reaction was allowed to proceed for 2hr at 4°C. The reaction mixture was centrifuged at 400g for 10 min, to remove any precipitate that may have formed during the reaction. Any unreacted IBHR was removed by dialysis at 4°C for 24hr against 0.03M phosphate buffer, pH 7.3.

2.5 Preparation and assessment of horseradish peroxidase (HRP) - labelled antibodies.

2.5.1 Preparation of HRP-labelled antibodies.

The procedure was carried out using a modification of the method described by Tijssen and Kurstak (1984). There are three steps involved: (i) activation of HRP (ii) conjugation of HRP to antibodies (iii) purification of the conjugate.

(i) Activation of HRP.

HRP (0.5mg) was weighed into an eppendorf tube, and was dissolved in 0.05ml of freshly-prepared 0.1M sodium bicarbonate. Following addition of 0.05ml of 8mM $NaIO_4$, the eppendorf was sealed, and stored in the dark for 2hr at room temperature.

(ii) Conjugation of HRP to antibodies.

Antibody (1mg) was dissolved in 0.2ml of 0.1M sodium carbonate, pH 9.2. This was added to the activated HRP, followed by dry Sephadex G-25 (approximately 0.1g). The eppendorf tube was sealed and stored in the dark for 3hr at room temperature. The reaction mixture was eluted from the Sephadex by centrifugation at 8000g for 15min, and 1/20 volume of freshly-prepared $NaBH_4$ (5mg/ml in 0.1mM NaOH) was added. After incubation for 30min at room temperature, 3/20 volume of another freshly-prepared $NaBH_4$ solution was added. This was incubated for 1hr at room temperature.

(iii) Purification of the conjugate.

An equal volume of saturated ammonium sulphate was added gradually to the reaction mixture to precipitate the free and conjugated antibody, and incubated for 1hr at 4°C. After centrifugation at 8000g for 15min, the precipitate was washed with 50% (w/v) ammonium sulphate, and recentrifuged. The precipitate was resuspended in a minimum volume of 0.1M PBS, pH 7.3, and dialysed against 0.1M PBS, pH 7.3, for 24hr at 4°C.

2.5.2 Measurement of the enzyme activity of HRP-labelled antibodies.

The enzyme activity was measured using the method described by Catty and Raykundalia (1989). The conjugate was diluted to a concentration of 1µg/ml IgG in 0.1M PBS, pH 7.3; 20µl was added to 3ml of freshly-prepared substrate solution (10mg of OPDA in 25ml of 0.15M citrate-phosphate buffer, pH 5.0, with 5µl of 30% (w/v) H_2O_2). The tube was wrapped in foil, and incubated at room temperature. Aliquots (200µl) were removed at 0, 5, 10, 15, 20, 25 and 30 min intervals, and transferred to the wells of a microtitre plate. The reaction was stopped by the addition of 25µl of 2.5M HCl. The absorbance was read

at 492nm using the Titertek plate reader.

2.6 Immunoassay for the measurement of human IgG using labelled antibodies.

2.6.1 Immunoassay for the measurement of human IgG.

Microtitre plates were coated with 100µl of anti-human IgG antibodies (10µg/ml) diluted in 0.03M phosphate buffer, pH 7.3, for 2hr at 37°C. They were washed (5 times) in 0.03M phosphate buffer, pH 7.3, containing 0.05% (v/v) Tween 20, and once in phosphate buffer alone. The plates were blocked by incubation with 0.5% (w/v) gelatin in 0.03M phosphate buffer, pH 7.3, (200µl) for 1hr at 37°C. The plates were washed as before. Human IgG standards (range 0 - 2µg/ml) were prepared in blocking solution; 100µl of each standard or diluted human serum sample was added to the appropriate wells, and incubated for 1hr at 37°C. The plates were washed as before. Dilutions of IBHR-labelled anti-human IgG antibodies (1/20) and HRP-labelled anti-human IgG antibodies (1/160) were prepared in blocking solution. The labelled antibodies (100µl) were added to the wells, and incubated for 30 min at 37°C. The plates were washed as before. Appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.6.2 Detection of IBHR-labelled antibodies.

For the detection of IBHR-labelled antibodies by the cerium(IV)-antimony(III) reaction, 50 µl of 0.03M antimony potassium tartrate in 1% (w/v) tartaric acid was added to the plate, followed by 50µl of 0.02M ceric ammonium sulphate in 6M H_2SO_4. The plate was mixed well, and the absorbance measured at 380nm.

For detection by the Sandell-Kolthoff reaction, 60µl of 0.075M arsenious acid was added to the plate, followed by 25µl of 0.028M ceric ammonium sulphate in 10% H_2SO_4. The plate was mixed well, and the absorbance measured at 414nm.

2.6.3 Detection of HRP-labelled antibodies.

For the detection of HRP-labelled antibodies, 100µl of substrate solution (Section 2.5.2) was added to the plate. The absorbance was measured at 405nm.

2.6.4 Immunoassay for determination of optimum dilution of labelled antibodies.

Microtitre plates were coated, washed and blocked as described in Section 2.6.1. Human

IgG standards (range 0 - 1 µg/ml) were prepared in blocking solution; 100µl of each standard was added to the appropriate wells, and incubated for 1hr at 37°C. The plates were washed as before. A range of dilutions of IBHR-labelled or HRP-labelled anti-human IgG antibodies, diluted in blocking solution (100µl) were added to the wells, and incubated for 30 min at 37°C. The plates were washed as before. Appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.7 Preparation and use of labelled avidin.

2.7.1 Preparation of IBHR-labelled avidin.

IBHR was dissolved at a concentration of 4mg/ml in benzene, containing 4% (v/v) DMF; 0.15mg was dried under nitrogen, and reconstituted with 50µl of DMF. Avidin (1mg in 1ml of 0.1M borate buffer, pH 8.5) was added. The reaction was carried out as described in Section 2.4.

2.7.2 Preparation of biotinylated antibodies.

N-hydroxysuccinimidobiotin (0.4mg) was dissolved in 200µl of DMF. Antibody (1mg in 1ml of 0.1M borate buffer, pH 8.5) was added. The reaction was carried out as described in Section 2.4.

2.7.3 Immunoassay for the measurement of human IgG using IBHR-labelled avidin.

Microtitre plates were coated and washed as described in Section 2.6.1. The plates were blocked by incubation with 1.5% (w/v) BSA in 0.03M phosphate buffer, pH 7.3, (200µl) for 1hr at 37°C. The plates were washed as before. Human IgG standards (range 0 - 0.5µg/ml) were prepared in blocking solution; 100µl of each standard or diluted human serum sample was added to the appropriate wells, and incubated for 1hr at 37°C. The plates were washed as before. Biotinylated anti-human IgG antibodies, diluted 1/200 in blocking solution, (100µl) were added to the wells, and incubated for 1hr at 37°C. The plates were washed as before. IBHR-labelled avidin, diluted 1/16 in blocking solution, (100µl) was added, and incubated for 20min at room temperature. After washing, appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.7.4 Immunoassay for determination of optimum dilution of biotinylated antibodies.

Microtitre plates were coated, washed and blocked, and human IgG standards were added, as described in Section 2.7.3. A range of dilutions of biotinylated anti-human IgG antibodies, diluted in blocking solution, (100µl) were added to the appropriate wells, and incubated for 1hr at 37°C. IBHR-labelled avidin, diluted in blocking solution, (100µl) was added, and incubated for 20min at room temperature. After washing, appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.7.5 Immunoassay for determination of optimum dilution of IBHR-labelled avidin.

Microtitre plates were coated, washed and blocked, and human IgG standards and biotinylated anti-human IgG antibodies were added, as described in Section 2.7.3. A range of dilutions of IBHR-labelled avidin, diluted in blocking solution, (100µl) were added to the appropriate wells, and incubated for 20min at room temperature. After washing, appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.8 Preparation and use of labelled biotin.

2.8.1 Preparation of IBHR-labelled biotin.

IBHR was dissolved at a concentration of 4mg/ml in benzene, containing 4% (v/v) DMF. One milligramme was dried under nitrogen, and reconstituted with 100µl of DMF. Biotin hydrazide was dissolved at a concentration of 1.5mg/ml in 0.1M borate buffer, pH 8.5. A 0.25ml aliquot of biotin hydrazide was added to the IBHR. The reaction was carried out as described in Section 2.4.

The reaction of biotin-LC-hydrazide with IBHR was carried out as above, except that 0.36ml of a 1.5mg/ml solution of biotin-LC-hydrazide was added to the IBHR.

IBHR-labelled biotin was purified using the TLC method described by Evangelatos *et al.* (1991). TLC was carried out on silica gel plates (0.2mm layer thickness) with a fluorescent indicator, and a solvent system of butanol : 2M ammonia : ethanol (3:1:1, v/v). IBHR-containing spots were visualised under a UV lamp. Biotin hydrazide or biotin-LC-hydrazide-containing spots were visualised as reported by McCormick and Roth (1970), using a spray reagent consisting of equal volumes of 2% (v/v) H_2SO_4 in ethanol and 0.2% (w/v) p-dimethylaminocinnamaldehyde in ethanol. The reaction products had the following

Rf values: IBHR, 0.24; biotin hydrazide, 0.46; biotin-LC-hydrazide, 0.50; IBHR-labelled biotin hydrazide, 0.81; and IBHR-labelled biotin-LC-hydrazide, 0.84. The IBHR-labelled biotin was recovered by scraping off the appropriate spot area, and eluting with 50%(v/v) ethanol. The solution was centrifuged at 400g for 10min to remove the silica gel; the supernatant was evaporated to 100µl under nitrogen, and made up to 1ml with 0.03M phosphate buffer, pH 7.3.

2.8.2 Use of avidin coated plates to detect IBHR-labelled biotin.

Microtitre plates were coated with 100µl of avidin (10µg/ml) diluted in 0.03M phosphate buffer, pH 7.3, for 2hr at 37°C. The plates were washed and blocked as described in Section 2.7.3. Samples (100µl) diluted in 0.03M phosphate buffer, pH 7.3, were added to the appropriate wells, and incubated for 20min at room temperature. The plates were washed as before. Appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.8.3 Colorimetric assay for biotin.

The colorimetric assay was carried out using a modification of the method described by McCormick and Roth (1970). Standards were prepared by dissolving biotin hydrazide or biotin-LC-hydrazide in 0.03M phosphate buffer, pH 7.3. Appropriate aliquots were taken, dried under nitrogen, and reconstituted with 1ml of 2% (v/v) H_2SO_4 in ethanol. IBHR-labelled biotin (100µl) was dried under nitrogen and reconstituted with 1ml of 2% (v/v) H_2SO_4 in ethanol. To the standards and samples was added 1ml of 0.2% (w/v) p-dimethylaminocinnamaldehyde in ethanol. The tubes were mixed, and the absorbance was measured at 533nm after 1hr.

2.8.4 Immunoassay for the measurement of human IgG using IBHR-labelled biotin or biotinylated HRP.

The immunoassay was carried out as outlined in Section 2.7.3, except that after incubation with biotinylated anti-human IgG antibodies, the plates were washed, and 100µl of avidin (diluted in blocking solution) was added. After incubation for 20min at room temperature, the plates were washed as before. IBHR-labelled biotin or biotinylated HRP, diluted in blocking solution, (100µl) was added, and incubated for 20min at room temperature. After

washing, appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.8.5 Immunoassay for determination of optimum dilution of avidin.

Microtitre plates were coated, washed and blocked, and human IgG standards and biotinylated anti-human IgG antibodies were added, as described in Section 2.7.3. A range of dilutions of avidin, diluted in blocking solution, (100µl) were added to the appropriate wells, and incubated for 20min at room temperature. After washing, biotinylated HRP, diluted in blocking solution, (100µl) was added, and incubated for 20min at room temperature. The plates were washed, appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.9 Preparation and use of bispecific $F(ab')_2$ antibodies.

2.9.1 Preparation of bispecific $F(ab')_2$ antibodies.

Affinity-purified antibodies were used for the production of bispecific antibodies. The preparation was carried out using a modification of the method of Glennie *et al.* (1987). Goat anti-human IgG antibodies and rabbit anti-BSA antibodies were subjected to pepsin degradation to yield $F(ab')_2$ fragments. Antibodies (5mg/ml in 0.1M sodium acetate, pH 4.2) were incubated at 37°C for 18hr with 0.1mg/ml pepsin. The samples were dialysed at 4°C for 24hr, against 0.2M Tris-HCl, pH 8.0, containing 10mM EDTA. Fab'_{SH} fragments were produced by reduction of the $F(ab')_2$ fragments with 2-mercaptoethanol (2ME) at 30°C for 30min. The Fab'_{SH} fragments were cooled to 4°C. This temperature was maintained throughout the remainder of the preparation procedure. They were passed through a Sephadex G-25 column equilibrated with 50mM sodium acetate, pH 5.3, containing 0.5mM EDTA. A half volume of 12mM o-phenylenedimaleimide, dissolved in chilled DMF, was added to the rabbit Fab'_{SH} component and incubated for 30min. The maleimidated Fab' (Fab'_{MAL}) were passed through a Sephadex G-25 column equilibrated with 50mM sodium acetate, pH 5.3, containing 0.5mM EDTA. The Fab' were mixed, and incubated for 18hr. After incubation, the pH was adjusted to 8.0 using 1M Tris-HCl, pH 8.0, and the solution treated for 30min at 30°C with 20mM 2ME and 25mM iodoacetamide to remove unwanted products. Excess reagents were removed by dialysis at 4°C for 24hr against 0.1M PBS, pH 7.3, and the bispecific $F(ab')_2$ was purified by affinity

chromatography.

2.9.2 Immunoassay for the detection of goat and rabbit Fab' fragments of the bispecific $F(ab')_2$ antibodies.

Microtitre plates were coated with 100µl of human IgG or BSA (10µg/ml) diluted in 0.03M phosphate buffer, pH 7.3, for 2hr at 37°C. The plates were washed and blocked as described in Section 2.6.1. Dilutions of bispecific $F(ab')_2$, anti-human IgG antibodies and anti-BSA antibodies were prepared in 0.03M phospate buffer, pH 7.3, and 100µl of each were added to the appropriate wells. The plates were incubated for 1hr at 37°C, and washed as before. HRP-labelled anti-rabbit IgG antibodies or anti-goat IgG antibodies (100µl) were added to the appropriate wells, and incubated for 30min at 37°C. The plates were washed as before, substrates were added, and the absorbance measured using the Titertek plate reader.

2.9.3 Immunoassay for the measurement of human IgG using bispecific $F(ab')_2$ antibodies.

Microtitre plates were coated, blocked, and washed as described in Section 2.6.1. Human IgG standards (range 0 - 2µg/ml) were prepared in blocking solution; 100µl was added to the appropriate wells and incubated for 1hr at 37°C. The plates were washed as before. Bispecific $F(ab')_2$ antibodies diluted in blocking solution were added to the wells (100µl) and incubated for 1hr at 37°C. The plates were washed as before. Iodinated BSA or HRP-labelled BSA (100µl) were added to the appropriate wells, and incubated for 30min at 37°C. The plates were washed as before, substrates added, and the absorbance measured using the Titertek plate reader.

2.9.4 Preparation of BSA, iodinated by the IBHR and chloramine T iodination methods.

IBHR-labelled BSA was prepared as outlined in Section 2.4. To 1ml of IBHR-labelled BSA (3mg/ml) was added 0.25ml of sodium iodide (15mM in 0.05M phosphate buffer, pH 7.5) and 0.1ml of chloramine T (2mg/ml in 0.05M phosphate buffer, pH 7.5). After 1min at room temperature the reaction was stopped by the addition of 0.35ml of sodium metabisulphite (2mg/ml in 0.05M phosphate buffer, pH 7.5). Excess reagents were

removed by dialysis at 4°C for 24hr against 0.03M phosphate buffer, pH 7.3.

2.10 Immunoassay for the measurement of thyroxine.

Microtitre plates were coated with 100µl of anti-thyroxine (T_4) antibodies (10µg/ml) diluted in 0.03M phosphate buffer, pH 7.3, for 2hr at 37°C. They were washed and blocked as outlined in Section 2.6.1. A 50µM stock solution was prepared by dissolving T_4 in 0.1M NaOH; standards (0 - 300nM) were prepared from this by dilution in 0.03M phosphate buffer, pH 7.3. Standards (100µl) were added to the appropriate wells and incubated for 1hr at 37°C. The plates were washed as before. Appropriate substrates were added, and the absorbance measured using the Titertek plate reader.

2.11 Protein assays.

2.11.1 BioRad assay.

Standards, in the range 0 - 25µg/ml, were prepared. To 0.8ml of standards or samples was added 0.2ml of BioRad dye reagent concentrate. The tubes were mixed gently, and the absorbance measured at 595nm after a period from 5 min to 1hr.

2.11.2 Bicinchoninic acid (BCA) assay.

The reagents for this assay were purchased in kit form, and consisted of two reagents:
Reagent A: sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2M sodium hydroxide.
Reagent B: 4% (w/v) copper sulphate solution.
The working reagent was prepared by combining 50 parts reagent A with 1 part reagent B. Standards, in the range 0 - 1mg/ml, were prepared. Standards or samples (10µl) were added to the wells of a microtitre plate, and 200µl of working reagent was added. The plate was incubated at 37°C for 30min, and the absorbance measured at 560nm.

2.12 Antibody purification.

2.12.1 Ammonium sulphate precipitation.

Serum was cooled to 4°C, and an equal volume of saturated ammonium sulphate was added dropwise. The solution was incubated for 1hr at 4°C with gentle stirring, and then centrifuged at 400g for 30min. The precipitated antibody was washed with 50% (v/v)

ammonium sulphate, and centrifuged as before. The pellet was resuspended in a minimum volume of 0.1M PBS, pH 7.3, and dialysed at 4°C for 24hr against 0.1M PBS, pH 7.3.

2.12.2 Affinity chromatography.

(i) Preparation of affinity columns.

CNBr-activated Sepharose (0.4g) was suspended in 3ml of 0.001M HCl, and transferred to a sintered glass funnel, where it was washed over 15min with 80ml of 0.001M HCl, followed by 2ml of coupling buffer (0.1M $NaHCO_3$ containing 0.5M NaCl, pH 8.3). Antigen (6mg in 2.8ml of coupling buffer) was added to the washed gel and mixed in a shaking waterbath for 3hr at room temperature. The gel was returned to the funnel and washed with 5ml of coupling buffer. The gel was transferred to a flask containing 3ml of blocking buffer (0.2M glycine in coupling buffer, pH 8.3) and incubated for 1hr at room temperature. To remove non-covalently bound protein, the gel was washed with 3 cycles of alternating pH, each cycle consisting of 5ml each of 0.1M sodium acetate containing 0.5M NaCl, pH 4.5, and 0.1M Tris-HCl containing 0.5M NaCl, pH 8.5. The gel was suspended in 0.1M PBS, pH 7.3, and used to pour a 1ml column.

(ii) Affinity purification.

The column was washed with 0.1M PBS, pH 7.3, to remove any protein that may have leaked from the gel during storage. The ammonium sulphate-purified antibody sample was applied slowly to the column, followed by washing with 0.1M PBS, pH 7.3. Fractions (1ml) were collected, and the presence of protein was detected by measurement of the absorbance at 280nm. When no more protein could be detected, dissociating buffer (0.1M glycine/HCl, pH 2.5) was added, the column was clamped off and left for 5min before elution of the specific antibody. To 1ml of antibody-containing fractions was added 100µl of 1M Tris, pH 10.5; these fractions were pooled and dialysed at 4°C for 24hr against 0.1M PBS, pH 7.3.

The column was regenerated by washing with 5ml each of 0.1M Tris-HCl containing 0.5M NaCl, pH 8.5; 0.1M sodium acetate containing 0.5M NaCl, pH 4.5; and PBS. The column was stored at 4°C in 0.1M PBS, pH 7.3, containing 0.02% (w/v) sodium azide.

2.13 SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a modification

of the procedure described by Laemmli (1970). Proteins were separated on a resolving gel containing 10% (w/v) acrylamide, 0.26% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate, 0.1% (v/v) TEMED and 0.375M Tris-HCl, pH 8.8. A stacking gel containing 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.075% (w/v) ammonium persulphate, 0.1% (v/v) TEMED and 0.125M Tris-HCl, pH 6.8, was used. Protein samples (1mg/ml) were diluted 1:1 in solubilisation buffer consisting of 2% (w/v) SDS, 10% (w/v) glycerol, 0.05% (w/v) bromophenol blue and 0.08M Tris, pH 6.8, and were incubated for 2min at 100°C. The samples (20µl) were applied to the gel. The gels were run at 18mA/gel in electrode buffer containing 0.1% (w/v) SDS, 0.192M glycine and 0.025M Tris, pH 8.3. Proteins were visualised by staining the gels for 15min in 0.5% (w/v) Coomassie brilliant blue in acetic acid:water:methanol (1:10:8 v/v/v), and destaining in the same solvent system.

2.14 Extraction procedure for the measurement of iodine.

The iodine-containing compound was dissolved in 200µl DMF. One millilitre of ceric ammonium sulphate (0.02M in 6M H_2SO_4) was added, and the solution mixed thoroughly for 10min. The liberated iodine was extracted into 3x1ml volumes of toluene. The aqueous and organic layers were separated by centrifugation for 5min at 400g, and the toluene layer was removed; the toluene extracts were combined and the volume made up to 3ml. The absorbance was read at 497nm.

2.15 Real-time biospecific interaction analysis (BIAcore).

Analyses on the BIAcore were carried out by Mr. Gary Keating, Biology Department, D.C.U.

2.15.1 Procedure for studying the relative binding pattern of iodinated antibodies.

The carboxymethylated dextran surface of the sensor chip was activated by derivatisation with a 1:1 mixture of N-hydroxysuccinimide (NHS)-coupling solution and N-ethyl-N'-(dimethylamino-propyl)carbodiimide (EDC)-coupling solution (35µl at a flow rate of 5µl/min) forming reactive NHS-ester groups. Human IgG (200µg/ml in 10mM sodium acetate, pH 5.5) was immobilised onto the chip (35µl at 5µl/min). Any remaining NHS-ester groups on the chip were deactivated with 1M ethanolamine, pH 8.5 (35µl at 5µl/min). Anti-human IgG antibodies, labelled with various amounts of IBHR, (100µg/ml in 0.03M

phosphate buffer, pH 7.3) were passed over the chip (50µl at 2µl/min). The human IgG surface on the chip was regenerated between runs with 6µl pulses of 10mM HCl.

2.15.2 Procedure for studying the binding of iodinated avidin to biotinylated antibodies.

The dextran surface was activated as in Section 2.15.1. Protein A (100µg/ml in 10mM sodium acetate, pH 4.0) was immobilised onto the chip (40µl at 2µl/min). Any remaining NHS-ester groups were deactivated as in Section 2.15.1. Biotinylated anti-human IgG antibodies (0.25mg/ml in PBS) were passed over the chip (20µl at 10µl/min), followed by avidin or IBHR-labelled avidin (100µg/ml in 0.03M phosphate buffer, pH 7.3). The protein A surface was regenerated between runs with 6µl pulses of 50mM HCl.

2.15.3 Procedure for studying the binding of iodinated BSA to anti-BSA antibodies.

The dextran surface was activated as in Section 2.15.1. Anti-BSA antibodies (100µg/ml in 10mM sodium acetate, pH 5.0) were immobilised onto the sensor chip (40µl at 2µl/min). Any remaining NHS-ester groups were deactivated as in Section 2.15.1. BSA samples (100µg/ml in 0.03M phosphate buffer, pH 7.3) were passed over the chip (20µl at 5µl/min). The antibody surface was regenerated between runs with 6µl pulses of 20mM HCl.

2.15.4 Procedure for studying the binding of anti-T_4 antibodies to T_4.

The dextran surface was activated as in Section 2.15.1. T_4 (500µg/ml in ethanol:10mM sodium acetate, pH 5.0 (1:1,v/v)) was immobilised onto the sensor chip (50µl at 10µl/min). Any remaining NHS-ester groups were deactivated as in Section 2.15.1. Anti-T4 antibodies (100µg/ml in 0.03M phosphate buffer, pH 7.3) were passed over the chip (25µl at 5µl/min). The T_4 surface was regenerated between runs with 6µl pulses of 20mM HCl.

3. DETERMINATION OF IODINE USING THE CERIUM(IV) - ANTIMONY(III) REACTION.

3.1 Introduction.

Bognar and Sarosi (1965) determined iodine concentrations by measuring its catalytic action on the reaction between cerium(IV) and antimony(III):

$$2Ce^{4+} + Sb^{3+} \rightarrow 2Ce^{3+} + Sb^{5+}$$

(yellow) (colourless)

The reaction conditions were as follows: to 0.01ml of potassium iodide solution was added 1ml of 0.05M antimony potassium tartrate in 1% (w/v) tartaric acid, 1ml of 7M H_2SO_4, and 1ml of 0.001M ferroin. The volume was adjusted to 4ml with H_2O, and the reaction was started by the addition of 1ml of 0.01M ceric ammonium sulphate in 0.5M H_2SO_4. The time, t, for formation of a red colour due to the reduction of the ferroin indicator, was recorded, and a graph of 1/t plotted against iodide concentration was prepared. The reaction was used to measure iodide concentrations of 1ng/ml.

This chapter describes major modifications to the assay conditions reported by Bognar and Sarosi (1965). The assay was adapted for use in a microassay format, and was monitored spectrophotometrically by measuring the absorbance of the yellow cerium(IV) solution. The microassay was optimised with respect to reagent concentration and temperature, and was evaluated as an alternative to the Sandell-Kolthoff microassay (O'Kennedy *et al.*, 1989). Several compounds have been reported to interfere in the Sandell-Kolthoff reaction (Sandell and Kolthoff, 1937), and the effect of three compounds (chloride, bromide and azide) on the cerium(IV) - antimony(III) microassay was studied.

The Sandell-Kolthoff reaction is catalysed by iodide and by iodine-containing organic compounds; the catalytic activity of iodide is greater than that of iodine in iodo-compounds (Bowden *et al.*, 1955). The catalytic activity of some iodo-compounds was determined in the cerium(IV) - antimony(III) microassay, and compared to the catalytic activity in the Sandell-Kolthoff reaction.

The use of fluorimetric monitoring of the cerium(IV) - antimony(III) microassay, by fluorimetric detection of cerium(III) or cerium(IV), was investigated. The measurement of the fluorescence of cerium(III) produced by the reduction of cerium(IV) with arsenic(III) was first reported by Kirkbright *et al.* (1966), while the measurement of the

fluorescence produced by the oxidation of 8-hydroxyquinoline-5-sulphonic acid with cerium(IV) was reported by Pal *et al.* (1977). The reaction conditions for the fluorimetric microassays were optimised, and the methods were evaluated as alternatives to spectrophotometric detection of the cerium(IV) - antimony(III) microassay.

3.2 Results and discussion.

3.2.1 Optimisation of reaction variables for the spectrophotometric microassay.

The cerium(IV) - antimony(III) microassay was optimised with respect to concentration of ceric ammonium sulphate, H_2SO_4 and antimony potassium tartrate, and incubation temperature. The assay was optimised for the measurement of potassium iodide standards, in the range 2 to 10ng/ml, by altering each reaction variable in turn, while keeping the others constant. The optimum conditions chosen were those that yielded good linearity (correlation coefficient, r) and a large difference in absorbance values over the range of standards tested (slope) for graphs of absorbance plotted against potassium iodide concentration. The results shown are the mean of at least four determinations. Error bars have not been plotted in the graphs as this was beyond the capacity of the computer system available; standard deviation values have been included in the text.

The microassay was monitored by measuring the absorbance of ceric ammonium sulphate; its absorbance spectrum in 6M H_2SO_4 is illustrated in Fig 3.1. The absorbance maximum for cerium(IV) is 320nm; the molar absorptivity at this wavelength is 5.6×10^3, while it is only one-sixth of this at 400nm (Marczenko, 1976). The Sandell-Kolthoff microassay was monitored at 414nm (O'Kennedy *et al.*, 1989), as filters for this wavelength are widely available on microplate readers since it is used to monitor ELISA. O'Kennedy and Keating (1993) reported the use of 340nm to monitor the Sandell-Kolthoff microassay. Potassium iodide levels of 1ng/ml could be detected using 3.5×10^{-3}M ceric ammonium sulphate in 10% (v/v) H_2SO_4, and an incubation period of 30min. However, monitoring at 340nm may produce absorbance readings that are not reliable, as many plate readers lack a proper filter for this wavelength, or there may be background absorbance due to the plate material. It was decided, therefore, to monitor the absorbance of cerium(IV) for the cerium(IV) - antimony(III) microassay at 380nm.

The effect of increasing the ceric ammonium sulphate concentration from 0.008M to 0.03M were studied in the microassay (Fig 3.2). As the cerium(IV) concentration was

increased, there was an increase in the slope from a value of 0.021(±0.002) using 0.008M cerium(IV) to 0.059(±0.005) using 0.03M cerium(IV). The correlation coefficient obtained using 0.02M cerium(IV) (r=0.994) was better than those obtained using 0.025M or 0.03M cerium(IV) (r=0.988 and 0.989, respectively). A ceric ammonium sulphate concentration of 0.02M was chosen as the optimum concentration.

The sulphuric acid concentration was varied from 4M to 8M, as shown in Fig 3.3. The slope of the calibration graph increased slightly from a value of 0.043(±0.006) using 4M to a value of 0.045(±0.002) using 6M H_2SO_4, and then decreased slightly for higher concentrations of H_2SO_4. All concentrations of H_2SO_4 tested gave correlation coefficients of 0.99. A concentration of 6M H_2SO_4 was chosen as the optimum. The reaction was also studied in two other acids: $HClO_4$ and HNO_3. Solutions of cerium(IV) in $HClO_4$ and HNO_3 are much stronger oxidising agents than in H_2SO_4 (Kolthoff *et al.*, 1969). Fukasawa *et al.* (1973) reported that the sensitivity of the Sandell-Kolthoff reaction was better in $HClO_4$ - HCl medium than in H_2SO_4. A twenty-fold increase in the catalytic activity of iodine measured in the Sandell-Kolthoff reaction was reported by Knapp and Leopold (1974) when HNO_3 was used instead of H_2SO_4, and O'Kennedy *et al.* (1989) reported a ten-fold improvement in the sensitivity of the Sandell-Kolthoff microassay using 10% (v/v) HNO_3 instead of H_2SO_4. The use of $HClO_4$ and HNO_3 in the cerium(IV) - antimony(III) microassay resulted in a very rapid reaction and did not lead to any improvement in sensitivity. Mishra and Gupta (1968) studied the chloride catalysed cerium(IV) - antimony(III) reaction and reported that on addition of $HClO_4$ the rate increased, due to the increase in hydrogen ion concentration. On addition of H_2SO_4, the rate decreased as the increase in hydrogen ion concentration led to a corresponding increase in the concentration of sulphate ion, which decreases the rate in cerium(IV) oxidations.

The concentration of antimony potassium tartrate was varied from 0.02M to 0.05M (Fig 3.4). For the antimony(III) concentrations tested, the slope of the calibration graph decreased with increasing concentration, from a value of 0.054(±0.007) using 0.02M antimony(III) to a value of 0.046(±0.002) using 0.05M antimony(III). The correlation coefficient obtained using 0.02M antimony(III) was 0.983, for all other antimony(III) concentrations tested the correlation coefficient was 0.99. An antimony(III) concentration of 0.03M was chosen as the optimum.

The microassay was studied at incubation temperatures of room temperature (20°C), 25°C,

30°C and 37°C (Fig 3.5). The slope of the calibration graph increased with increasing temperature. A value of 0.051(±0.004) was obtained for the slope at 20°C, while a value of 0.068(±0.005) was obtained at 37°C. However, a correlation coefficient of 0.995 was obtained at 20°C, compared with 0.988 at 37°C. Incubation at 20°C was chosen as the optimum. The sensitivity of the Sandell-Kolthoff reaction is reported to increase as the temperature is increased (Knapp and Leopold, 1974), but this was not observed under the conditions used here for the cerium(IV) - antimony(III) microassay.

Potassium iodide standards were measured in the optimised microassay and a graph of absorbance plotted against concentration was prepared (Fig 3.6). The assay shows excellent linearity (r=0.995) for potassium iodide standards in the range 2 to 10ng/ml. The intra-assay accuracy and precision (Table 3.1) and inter-assay accuracy and precision (Table 3.2) were calculated for the standards. The accuracy of the assay is determined by the closeness of the mean of the analytical result to the true value; the mean value should fall within 90 to 110% of the true value. The intra- and inter-assay mean values for the potassium iodide standards were within 96 to 107% of the true values. The precision of the assay is determined by the percentage coefficient of variation (%CV), which should be less than 10%. The intra- and inter-assay %CV values for the potassium iodide standards were less than 10%. The detection of potassium iodide levels of 2ng/ml in the cerium(IV) - antimony(III) microassay compares well with the levels detected in the Sandell-Kolthoff microassay. O'Kennedy *et al.* (1989) detected 0.01 to 0.1µg/ml KI using 0.075M arsenious acid, 0.028M ceric ammonium sulphate diluted in 10% (v/v) H_2SO_4 and an incubation period of 5min. Potassium iodide levels of 1ng/ml could be detected using 0.014M ceric ammonium sulphate diluted in 10% (v/v) HNO_3, and an incubation period of 15 min. O'Kennedy and Keating (1993) detected 1ng/ml potassium iodide by monitoring the absorbance at 340nm.

The optimised microassay was used for the measurement of IBHR standards. A graph of absorbance plotted against concentration for IBHR standards is shown in Fig 3.7. The linear range is 0.02 to 0.1µg/ml (r=0.991). Intra-assay accuracy and precision results are shown in Table 3.3; inter-assay accuracy and precision results are shown in Table 3.4. The intra- and inter-assay mean values for the standards were within 95 to 110% of the true value, and the %CV values were less than 10%, with the exception of the intra-assay %CV for 0.02µg/ml which was 10.1%.

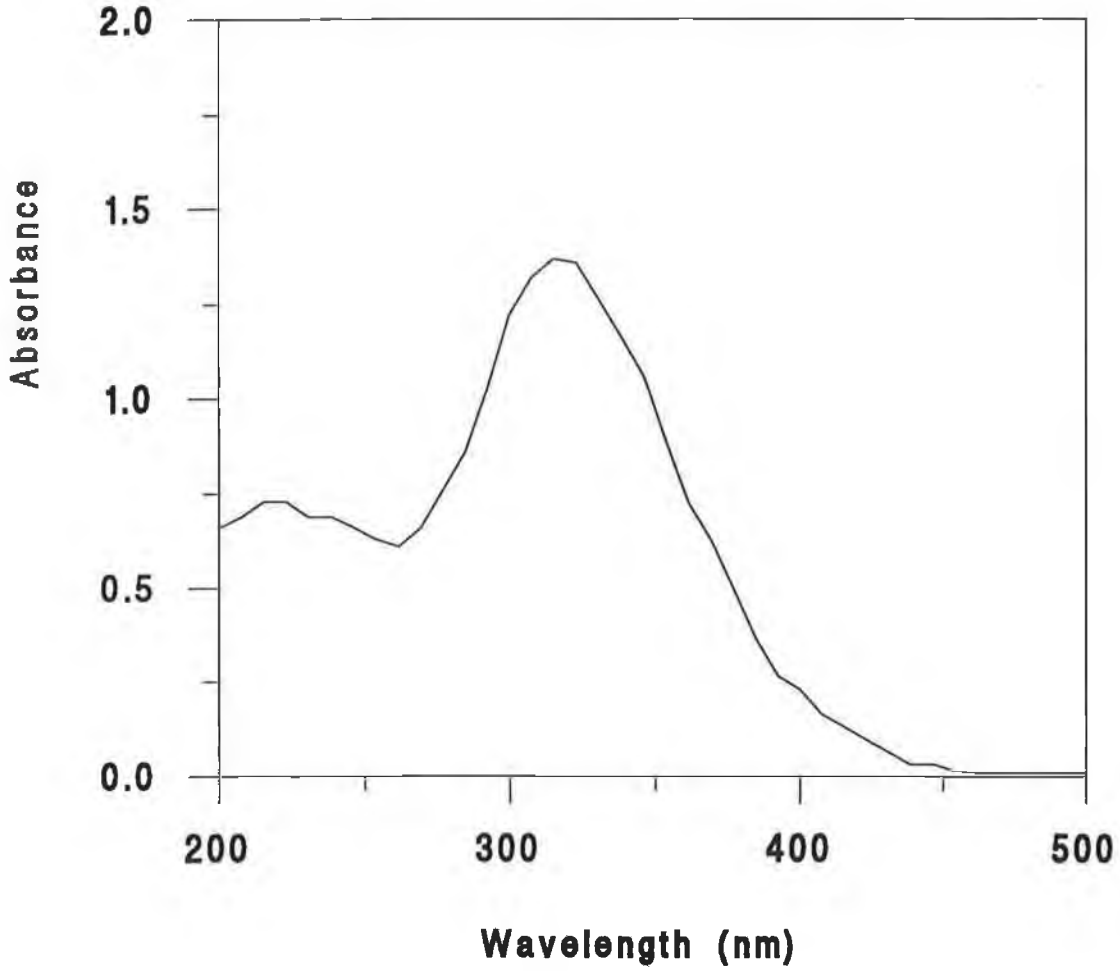


Figure 3.1 Absorbance spectrum of ceric ammonium sulphate in 6M H_2SO_4.

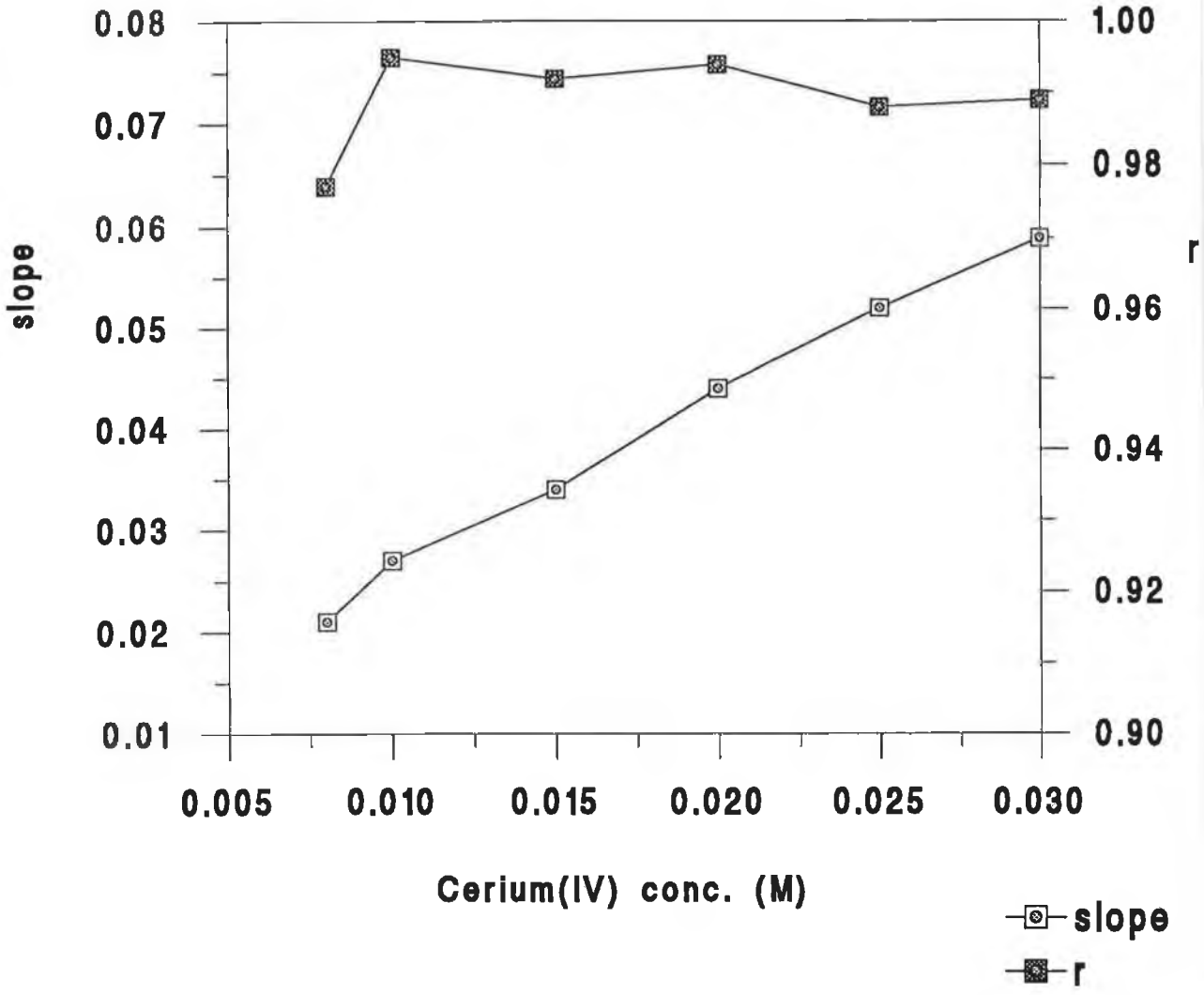


Figure 3.2 Effect of cerium(IV) concentration on the slope and correlation coefficient (r) of the graph of absorbance plotted against concentration for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay.

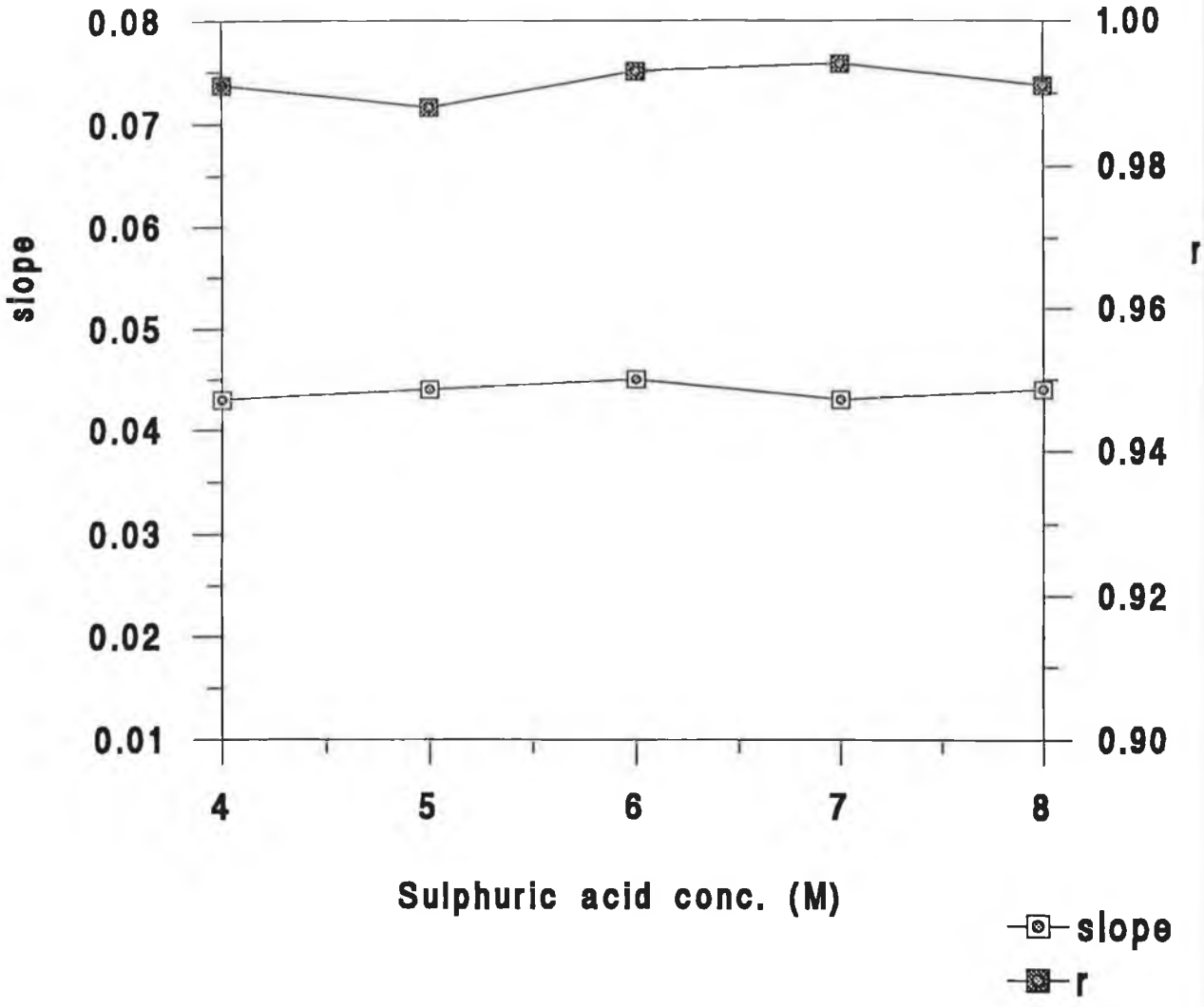


Figure 3.3 Effect of sulphuric acid concentration on the slope and correlation coefficient (r) of the graph of absorbance plotted against concentration for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay.

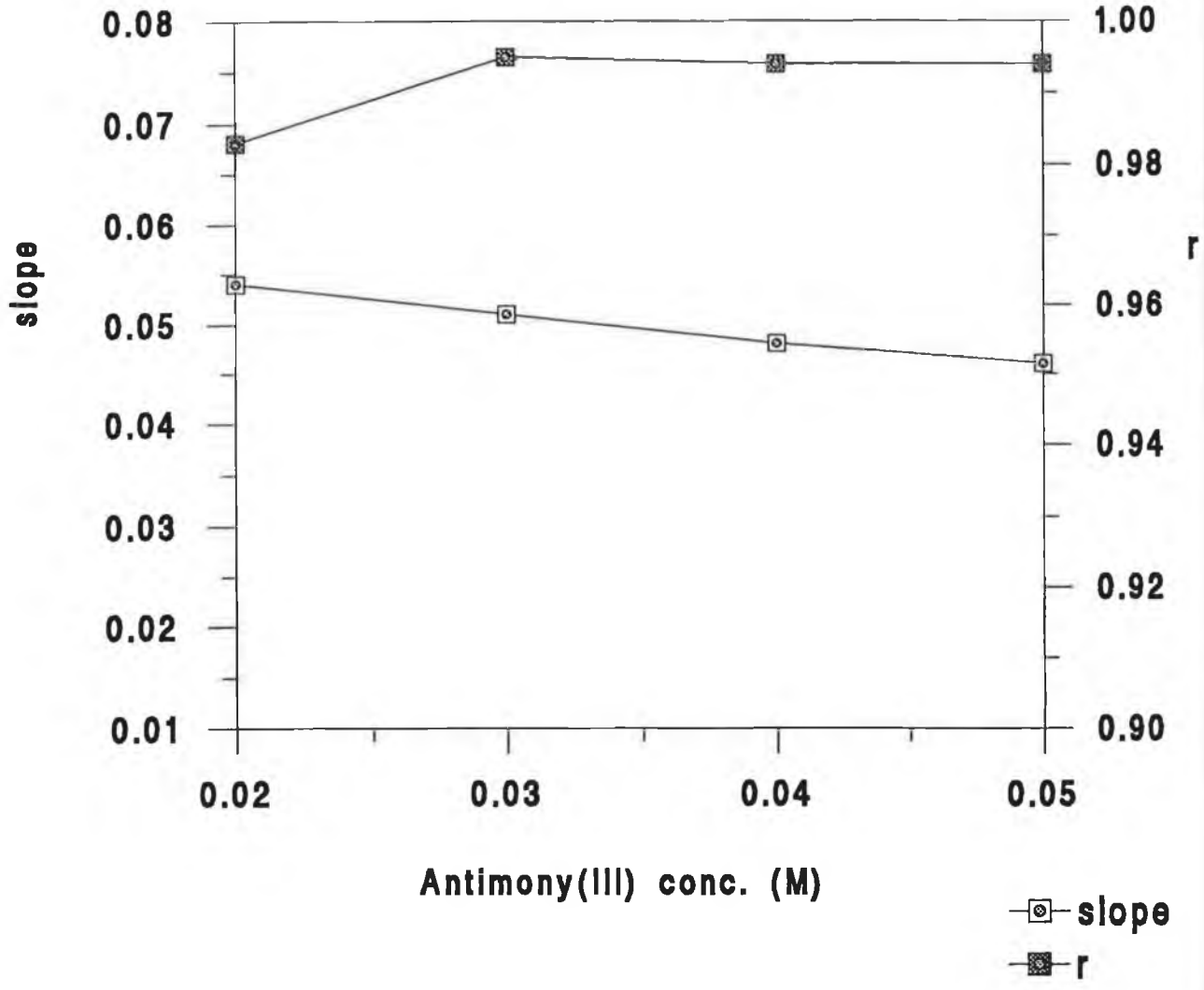


Figure 3.4 Effect of antimony(III) concentration on the slope and correlation coefficient (r) of the graph of absorbance plotted against concentration for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay.

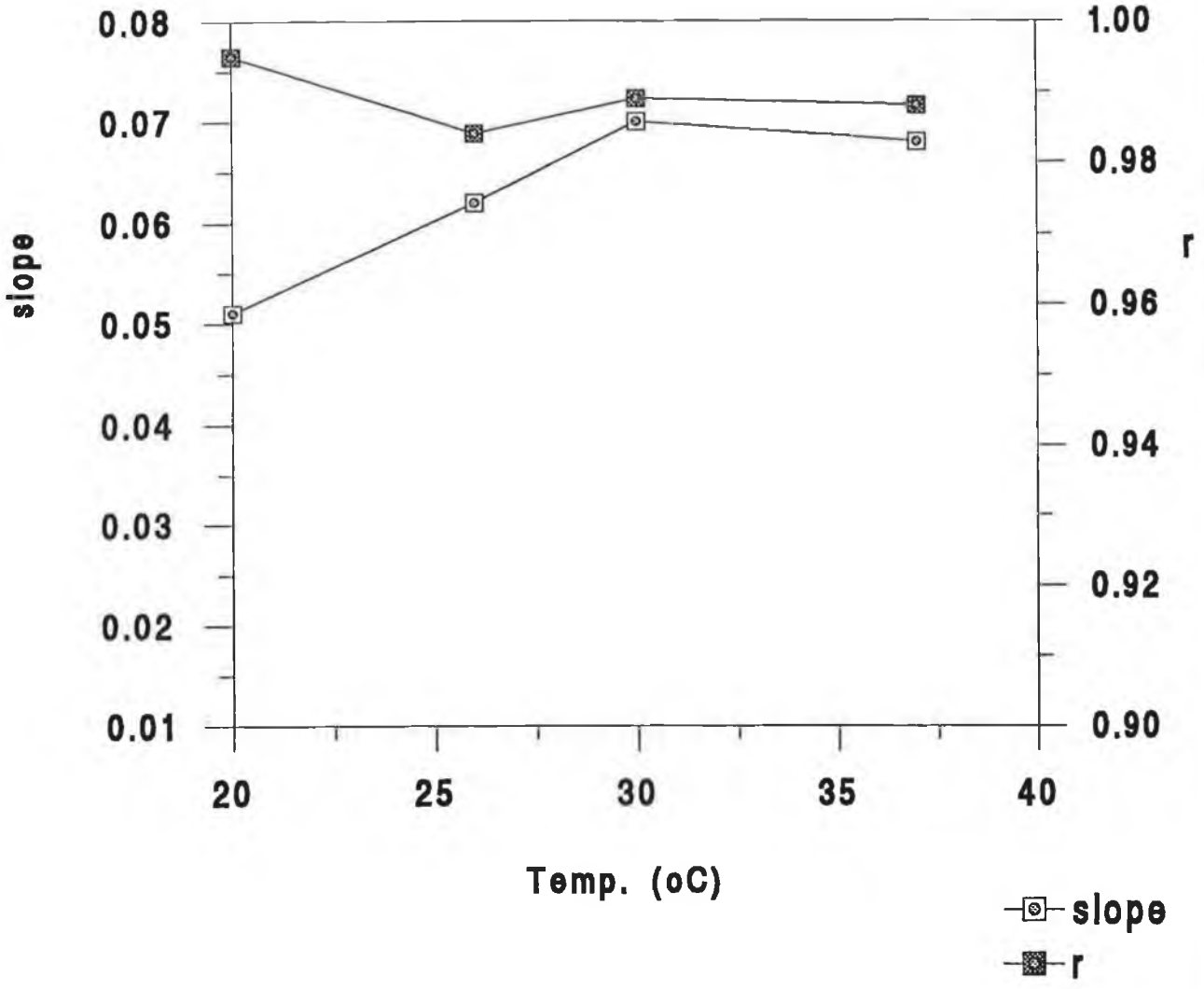


Figure 3.5 Effect of temperature on the slope and correlation coefficient (r) of the graph of absorbance plotted against concentration for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay.

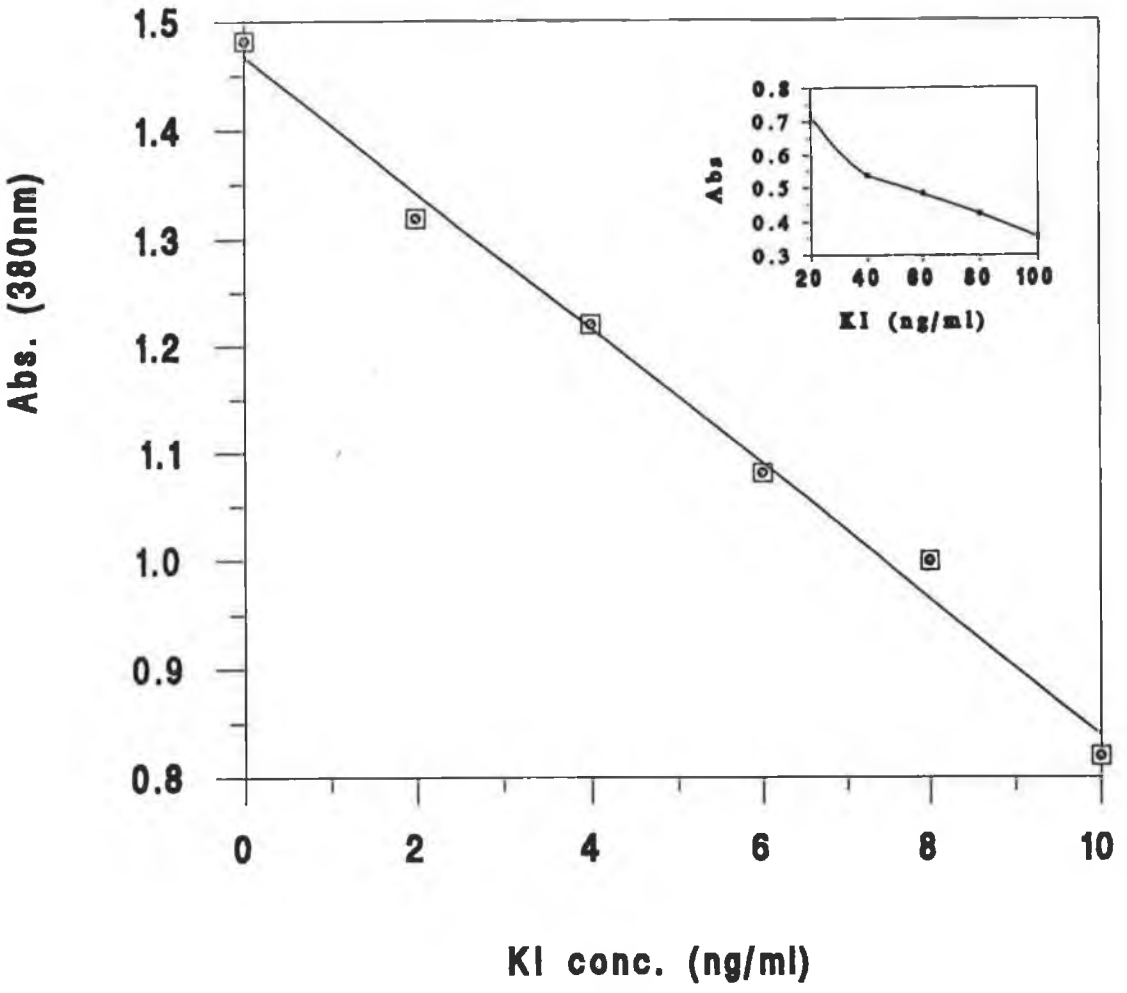


Figure 3.6 Graph of absorbance at 380nm plotted against concentration of potassium iodide standards (0 to 10ng/ml) assayed in the optimised cerium(IV) - antimony(III) spectrophotometric microassay.

The insert displays the graph of absorbance at 380nm plotted against concentration of potassium iodide standards in the range 20 to 100ng/ml.

KI conc. (ng/ml)	Mean ± SD	%CV
2	1.95 ± 0.19	9.9
4	4.03 ± 0.31	7.8
6	5.88 ± 0.55	9.4
8	8.59 ± 0.62	7.2
10	9.57 ± 0.88	9.2

Table 3.1 Intra-assay accuracy and precision results for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay (n=8).

KI conc. (ng/ml)	Mean ± SD	%CV
2	2.08 ± 0.15	7.3
4	4.18 ± 0.37	8.9
6	6.05 ± 0.45	7.4
8	7.87 ± 0.34	4.3
10	9.89 ± 0.27	2.7

Table 3.2 Inter-assay accuracy and precision results for potassium iodide standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay (n=7).

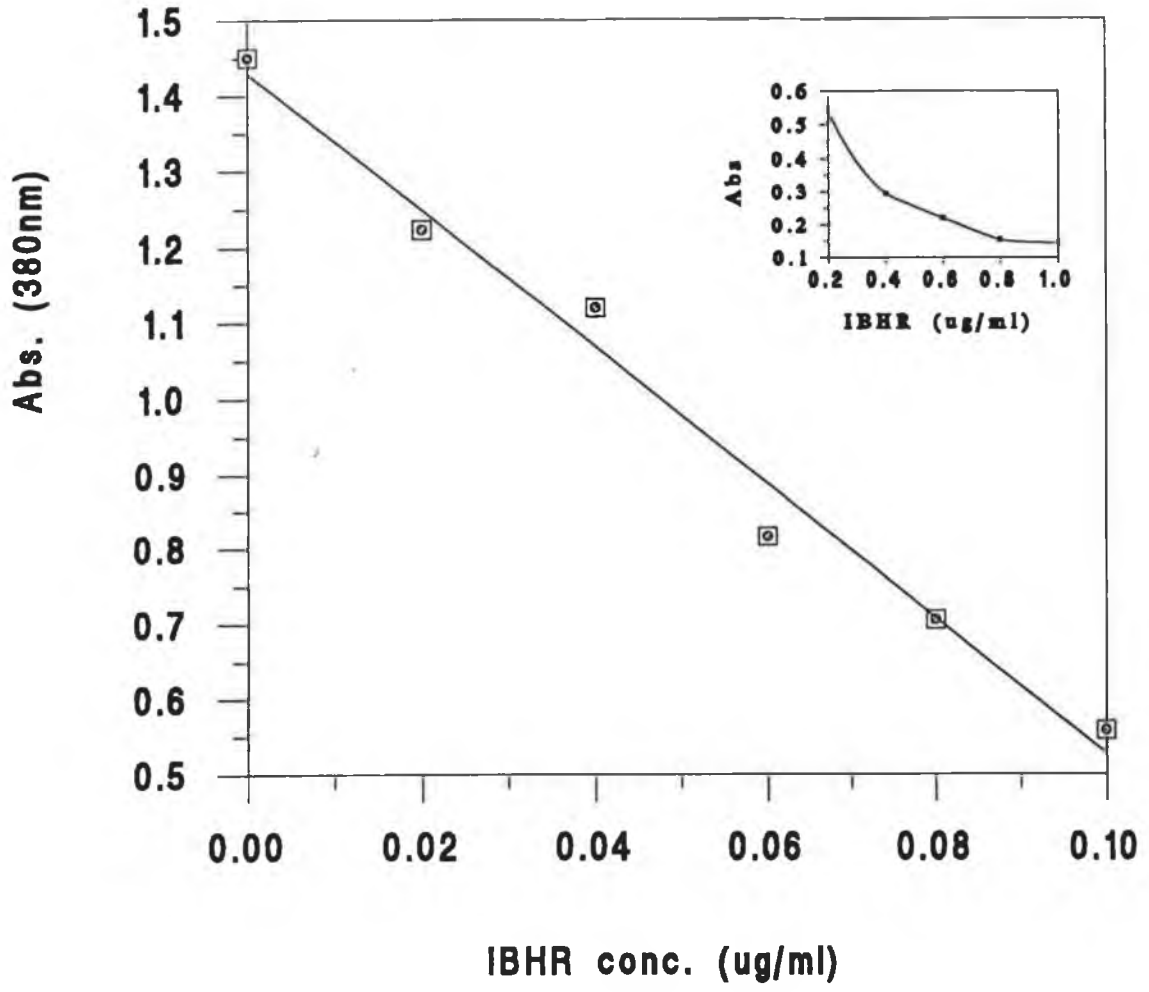


Figure 3.7 Graph of absorbance at 380nm plotted against concentration of IBHR standards assayed in the optimised cerium(IV) - antimony(III) spectrophotometric microassay. The insert displays the graph of absorbance at 380nm plotted against concentration of IBHR standards in the range 0.2 to 1ug/ml.

IBHR conc. (µg/ml)	Mean ± SD	%CV
0.02	0.022 ± 0.002	10.1
0.04	0.044 ± 0.003	6.9
0.06	0.062 ± 0.003	5.4
0.08	0.081 ± 0.004	4.8
0.1	0.095 ± 0.004	3.8

Table 3.3 Intra-assay accuracy and precision results for IBHR standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay (n=8).

IBHR conc. (µg/ml)	Mean ± SD	%CV
0.02	0.021 ± 0.002	9.9
0.04	0.044 ± 0.003	7.1
0.06	0.061 ± 0.004	6.1
0.08	0.078 ± 0.004	4.5
0.1	0.099 ± 0.004	4.0

Table 3.4 Inter-assay accuracy and precision results for IBHR standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay (n=8).

3.2.2 Interferent species.

The effect of chloride, bromide and azide in the cerium(IV) - antimony(III) and Sandell-Kolthoff microassays was studied; the results are shown in Tables 3.5 and 3.6. The interferent species were measured in the microassays, and the absorbance of wells to which the interferent species was added was compared to the absorbance of control wells.

The addition of chloride led to only a slight enhancement of the Sandell-Kolthoff microassay. A chloride concentration of 2mg/ml resulted in absorbance values that were 94% of the control, in the absence of iodide, and 92% of the control in the presence of 10ng/ml iodide. Small amounts of chloride are reported to enhance the Sandell-Kolthoff reaction, and the addition of NaCl can be used to obtain linear calibration graphs (Sandell and Kolthoff, 1937). The addition of chloride led to a much greater enhancement of the reaction in the cerium(IV) - antimony(III) microassay. The absorbance values on addition of 2mg/ml chloride were just 42% of the control, in the absence of iodide, and 51% of the control, in the presence of 10ng/ml iodide. Chloride acts as a catalyst for the cerium(IV) - antimony(III) reaction, although Mishra and Gupta (1968) reported that the catalytic action of iodide in the reaction was greater than chloride. They obtained a rate constant of 10 in the presence of 248µg/ml chloride, and a rate constant of 12 in the presence of only 0.127µg/ml iodide. Bognar and Sarosi (1965) reported that, in the absence of iodide, the addition of 20µg/ml chloride enhanced the cerium(IV) - antimony(III) reaction, while in the presence of 0.1µg/ml iodide the addition of 200µg/ml chloride had no effect on the rate of the reaction. The addition of 0.1M PBS, which contains 0.14M NaCl and 0.003M KCl, was found to interfere in the cerium(IV) - antimony(III) microassay. In the absence of iodide, the addition of PBS gave absorbance values that were only 19% of the control, and values that were 24% of the control in the presence of 10ng/ml iodide. PBS is widely used as a diluent for proteins, and as a buffer in the washing steps of immunoassays, but its use to dilute iodinated proteins to be assayed in the cerium(IV) - antimony(III) microassay or as a washing buffer for an immunoassay using iodinated antibodies would lead to inaccurate results. It was, therefore, decided to use 0.03M phosphate buffer, pH 7.3, as an alternative to PBS. On addition of higher molar phosphate buffers (0.04M, 0.05M, 0.1M tested) to the microassay the cerium(IV) solution became cloudy.

Bromide is reported to enhance the Sandell-Kolthoff reaction to a greater extent than chloride (Sandell and Kolthoff, 1937). In the Sandell-Kolthoff microassay, 10µg/ml

bromide caused greater enhancement of the reaction, giving an absorbance value 73% of the control, in the absence of iodide, than 2mg/ml chloride, which gave an absorbance value 94% of the control. This was also observed for the cerium(IV) - antimony(III) microassay, but to a smaller extent. In the absence of iodide, the addition of 10µg/ml bromide gave an absorbance value 39% of the control, while the addition of 2mg/ml chloride gave an absorbance value 42% of the control. Mishra and Gupta (1968) reported a rate constant of 12 for the cerium(IV) - antimony(III) reaction in the presence of 32µg/ml bromide, and a rate constant of 10 in the presence of 248µg/ml chloride. In the presence of iodide, the microassay was enhanced to a smaller extent by bromide; 10µg/ml bromide gave an absorbance value only 39% of the control, in the absence of iodide, but an absorbance 70% of the control in the presence of 10ng/ml iodide. A similar trend was observed by Bognar and Sarosi (1965), who reported enhancement of the cerium(IV) - antimony(III) reaction by 10µg/ml bromide, in the absence of iodide, while in the presence of 0.1µg/ml iodide the addition of 0.2µg/ml bromide inhibited the reaction.

The interference of azide in the Sandell-Kolthoff microassay was reported by O'Kennedy *et al.* (1989). Azide, a reducing agent, caused decolouration of ceric ammonium sulphate, independent of the presence of iodine or arsenic(III). They reported a 35% decrease in the absorbance of ceric ammonium sulphate at 414nm in the presence of 0.02% (w/v) azide, and a 95% decrease in the absorbance in the presence of 0.2% (w/v) azide. When tested here in the Sandell-Kolthoff microassay, 0.02% (w/v) azide gave an absorbance value that was 16% of the control, in the absence of iodide, and a 7% of the control in the presence of 10ng/ml iodide; 0.2% (w/v) azide gave absorbance values 4% of the control in both the absence and presence of iodide. The observations for the cerium(IV) - antimony(III) microassay were similar; 0.02% (w/v) azide gave an absorbance value 19% of the control, in the absence of iodide, and 23% of the control, in the presence of iodide; 0.2% (w/v) azide gave an absorbance value 4% of the control, in the absence of iodide, and 3% of the control, in the presence of iodide. Samples stored in buffer containing 0.02% (w/v) sodium azide, which is widely used as a preservative for protein solutions, cannot be assayed in the microassay without taking this interference into account.

Other ions that have been reported to interfere in the cerium(IV) - antimony(III) reaction include vanadium, tungtsen, selenium, tellurium, osmium, manganese, silver, mercury and fluoride (Bognar and Sarosi, 1965). In addition, Zak (1978) reported interference in the

Sandell-Kolthoff reaction by cyanide, thiocyanate and citrate. Oxidising or reducing agents (for example, bromate, nitrite or ferrous ion) that react with antimony(III) or cerium(IV) will also cause interference.

Interferent species	Abs (380nm) expressed as a% of the control wells	
	No iodide	10ng/ml iodide
20µg/ml chloride	66	70
2mg/ml chloride	42	51
PBS	19	24
10µg/ml bromide	39	70
1mg/ml bromide	4	11
0.02% (w/v) azide	19	23
0.2% (w/v) azide	4	3

Table 3.5 Effect of three interferent species in the cerium (IV) - antimony (III) spectrophotometric microassay.

Interferent species	Abs (414nm) expressed as a % of the control wells	
	No iodide	10ng/ml iodide
20µg/ml chloride	92	86
2mg/ml chloride	94	92
PBS	93	93
10µg/ml bromide	73	81
1mg/ml bromide	78	80
0.02% (w/v) azide	16	7
0.2% (w/v) azide	4	4

Table 3.6 Effect of three interferent species in the Sandell-Kolthoff microassay.

3.2.3 Catalytic activity of iodine in iodine-containing organic compounds.

It has been reported that the catalytic activity of iodine in organic compounds is inferior to that of inorganic iodide (Bowden *et al.*, 1955; Pantel and Weisz, 1977). In order to compare the catalytic activity of iodo-compounds to that of iodide, Pantel (1982) introduced a "relative molar coefficient of catalytic activity", F:

$$F=\frac{C_I}{C_O}$$

where C_I is the molar concentration of iodide and C_O is the molar concentration of organic compound that have exactly the same catalytic activity. Coefficient F is suitable for iodo-compounds containing one iodine atom, but for compounds containing more than one iodine Timotheou-Potamia (1988) introduced coefficient K, the "catalytic activity per iodine atom of the compound". Coefficient K is calculated as follows:

$$K=100\frac{C_I}{mC_O}$$

Where C_I and C_O are defined as for coefficient F, and m is the number of iodine atoms per mole of organic compound.

Several iodine-containing organic compounds were assayed in the cerium(IV) - antimony(III) microassay along with potassium iodide. The catalytic activity was directly compared to that of potassium iodide in order to calculate the K values. The K values obtained are reported in Table 3.7; also reported are the number of moles of organic compound that are required to give the same catalytic activity as one mole of iodide. The structures of some iodine-containing compounds are illustrated in Fig 3.8 and 3.9.

The K value for IBHR was calculated as 33.7%, while for di-IBHR K was calculated as 25.5%. O'Kennedy *et al.* (1989) reported a K value of 33.1% for IBHR assayed in the Sandell-Kolthoff microassay, which is similar to the value obtained in the cerium(IV) - antimony(III) microassay.

The K values calculated for T_3 and T_4 were 37.0% and 47.0%, respectively. Higher K values have been reported for these compounds assayed using the Sandell-Kolthoff reaction. Values of 42% and 57% were obtained for T_3 and T_4, respectively, by Timotheou-Potamia (1988), and a value of 62% was obtained for T_4 by O'Kennedy *et al.*

(1989).

The K values obtained for the iodophenol compounds were 13.0% for 2-iodophenol, 1.3% for 3-iodophenol and 50.0% for 4-iodophenol. Pantel (1982) reported F values of 0.09 for 2-iodophenol, 0.0015 for 3-iodophenol and 0.42 for 4-iodophenol in the Sandell-Kolthoff reaction using a potentiometric stat-method (an F value of 1 is equivalent to a K value of 100 for iodo-compounds containing one iodine atom). The K values obtained in the cerium(IV) - antimony(III) microassay were slightly higher than those obtained by Pantel (1982), but the same pattern of catalytic activity was observed, i.e. 4-iodophenol > 2-iodophenol > 3-iodophenol. O'Kennedy *et al.* (1989) reported a K value of 40.0% for 4-iodophenol.

N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB) was tested in the cerium(IV) - antimony(III) microassay, in order to compare the catalytic activity of aliphatic iodine-containing compounds with aromatic iodine-containing compounds. The K value of SIAB was calculated as 7.5%; 13.3 moles of SIAB are required to give the same catalytic activity as one mole of iodide in the cerium(IV) - antimony(III) microassay. All of the aromatic iodine-containing compounds, with the exception of 3-iodophenol, displayed higher catalytic activities than SIAB. Bowden *et al.* (1955) reported that the Sandell-Kolthoff reaction was relatively insensitive to aliphatic iodine-containing compounds.

The catalytic activity of iodine in iodo-compounds is complex, and has been investigated by several authors. Bowden *et al.* (1955) determined the amount of iodine liberated from iodo-compounds following treatment with excess cerium(IV). The iodine-containing compound was mixed thoroughly with a ceric sulphate solution; the liberated iodine was extracted with benzene, which was in turn extracted with sodium thiosulphate, followed by back titration with I_2. They found that there was a correlation between the percentage of iodine liberated, and the amount of iodo-compound detectable on paper chromatography using a cerium(IV) - arsenic(III) reagent for detection. One of their conclusions was that the detection of iodine-containing compounds depends on the liberation of iodine by oxidation with cerium(IV). Cerium(IV) has wide application in the oxidation of organic compounds (Ho, 1986). Oxidations utilising cerium(IV) are very rapid, as it is a very powerful oxidising agent which removes one electron at a time from the substrate. The limitations to its use are the fact that acidic conditions must be used, and large amounts of cerium(IV) are required as it is a one equivalent oxidant.

An experiment was carried out to study the liberation of iodine from iodo-compounds by cerium(IV). IBHR, T_3, T_4 and SIAB were treated with ceric ammonium sulphate, and the liberated iodine was extracted into toluene. Iodine forms a violet-coloured solution in organic solvents, and this can be used for its colorimetric determination (Marczenko, 1976). Potassium iodide was used to prepare a standard curve (Fig 3.10); the milligrammes of iodine liberated from the iodo-compound was determined from the standard curve, and from this the percentage of the total iodine liberated was calculated. The results obtained are reported in Table 3.8. The percentage of total iodine liberated from T_3 and T_4 was calculated as 32.8% and 23.2%, respectively. Bowden *et al.* (1955) reported that 10.9% of the iodine in T_3 and 7.9% of the iodine in T_4 was liberated, which is considerably lower than the results obtained here. For IBHR, 15.5% of the total iodine was liberated. It is not possible to achieve the same sensitivity in the cerium(IV) - antimony(III) microassay using IBHR standards as with potassium iodide standards (Section 3.2.1). The sensitivity is limited by the fact that only a percentage of the iodine in IBHR is liberated to catalyse the reaction. Di-iodinated BHR, which has a higher catalytic activity than IBHR (Table 3.7), was tested in the microassay to see if any improvement in sensitivity could be obtained. Fig 3.11 shows the graph of absorbance plotted against concentration that was obtained for both compounds. The same range of standards (0.052 to 0.258µM) could be assayed for both compounds, thus the use of di-IBHR offered no advantage with respect to the sensitivity.

From their experiments, Bowden *et al.* (1955) also concluded that the catalytic activity of iodine in an iodobenzene ring is strongly dependent on the nature and position of the second substituent of the ring. Of the compounds they tested, an electron-donating group, for example, a hydroxyl or amino group, in a position ortho to the iodine atom was necessary for liberation of significant amounts of iodine. There was no liberation of iodine when, for example, a methyl ether group was in a position ortho to the iodine. Similar observations were made by Morreale de Escobar and Rios (1958) and by Barker (1964). Morreale de Escobar and Rios (1958) studied the catalytic activity in the Sandell-Kolthoff reaction of 3,5-di-iodotyrosine, 3,5-di-iodothyronine, T_3 and T_4. They found that the catalytic activity of the compounds depended not only on the number of iodine atoms but on their position in the molecule. Iodine atoms on the outer ring, with a hydroxyl group ortho to the iodine, showed greater catalytic activity than iodine atoms on the inner ring,

with an ether oxygen ortho to the iodine. Barker (1964) studied the deiodination of thyroxine analogues by measuring the decolouration of cerium(IV) - arsenic(III) reagents on paper chromatography; 3,5-di-iodothyronine was used as the reference compound. Removal of the 4'-hydroxyl group of 3,5-di-iodothyronine resulted in a complete loss of reactivity; replacement with a 4'-amino group caused a return of reactivity, while replacement with a 4'-methyl ether yielded a compound with low reactivity. Pantel (1982) reported that 2-, 3-, and 4-iodoaniline showed similar catalytic activity in the Sandell-Kolthoff reaction to 2-, 3-, and 4-iodophenol, while substitution in the 4- position with alkyl or methyl ether groups resulted in iodobenzene compounds that had significantly lower catalytic activity than 4-iodophenol or 4-iodoaniline.

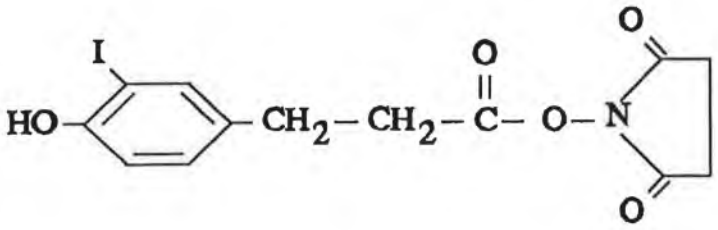
Bowden *et al.* (1955) suggested that the aniline side chain of 3,5-di-iodotyrosine and 3,5-di-iodothyronine may be partly responsible for liberation of iodine, as replacement of the aniline side chain of 3,5-di-iodotyrosine with, for example, a carboxyl or nitro group yielded compounds which liberated less iodine, and replacement of the aniline side chain of 3,5-di-iodothyronine with a cyano group yielded a compound which did not liberate iodine. Barker (1964), however, found that alterations to the aniline side chain of 3,5-di-iodothyronine did not alter the reactivity. As removal of the ether oxygen to yield 4'-hydroxy-diphenyl analogues resulted in compounds that did not liberate iodine, Barker (1964) suggested that liberation of the iodine from 3,5-di-iodothyronine may involve splitting of the diphenyl ether leading to formation of a hydroxyl group in the 4- position.

Compound	n	K Mean ± SD	mol. organic compound*
IBHR	8	33.7 ± 3.7	2.9
di-IBHR	5	25.5 ± 3.1	2.0
T_3	6	37.0 ± 3.7	0.9
T_4	4	47.0 ± 6.0	0.5
2-iodophenol	3	13.0 ± 0.5	7.7
3-iodophenol	3	1.3 ± 0.2	76.9
4-iodophenol	3	50.0 ± 6.2	2.0
SIAB	4	7.5 ± 1.0	13.3

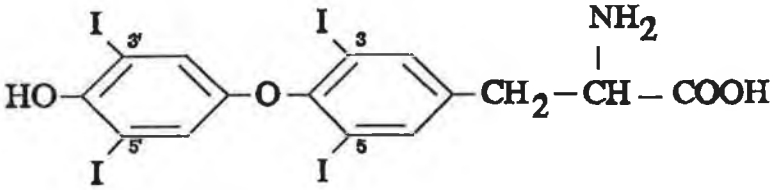
*mol. organic compound is the number of moles of organic compound that have the same catalytic activity as one mole of iodide.

Table 3.7 Catalytic activity of some iodine-containing organic compounds in the cerium(IV) - antimony(III) spectrophotometric microassay.

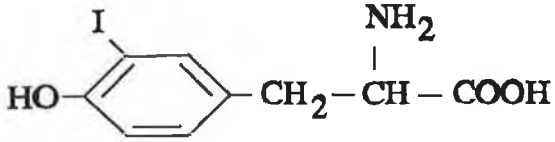
(a)



(b)



(c)



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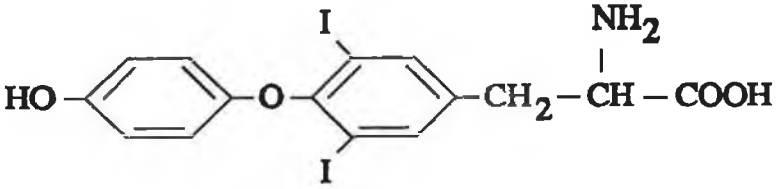

(e)

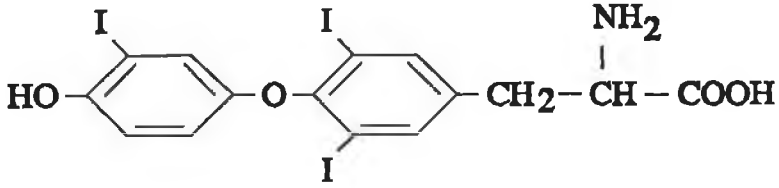

Figure 3.8 The structures of some iodine-containing organic compounds.
(a) IBHR (b) 3',5',3,5-tetraiodothyronine (T_4) (c) 3-iodotyrosine
(d) 3,5-di-iodothyronine (e) 3',3,5-tri-iodothyronine (T_3).

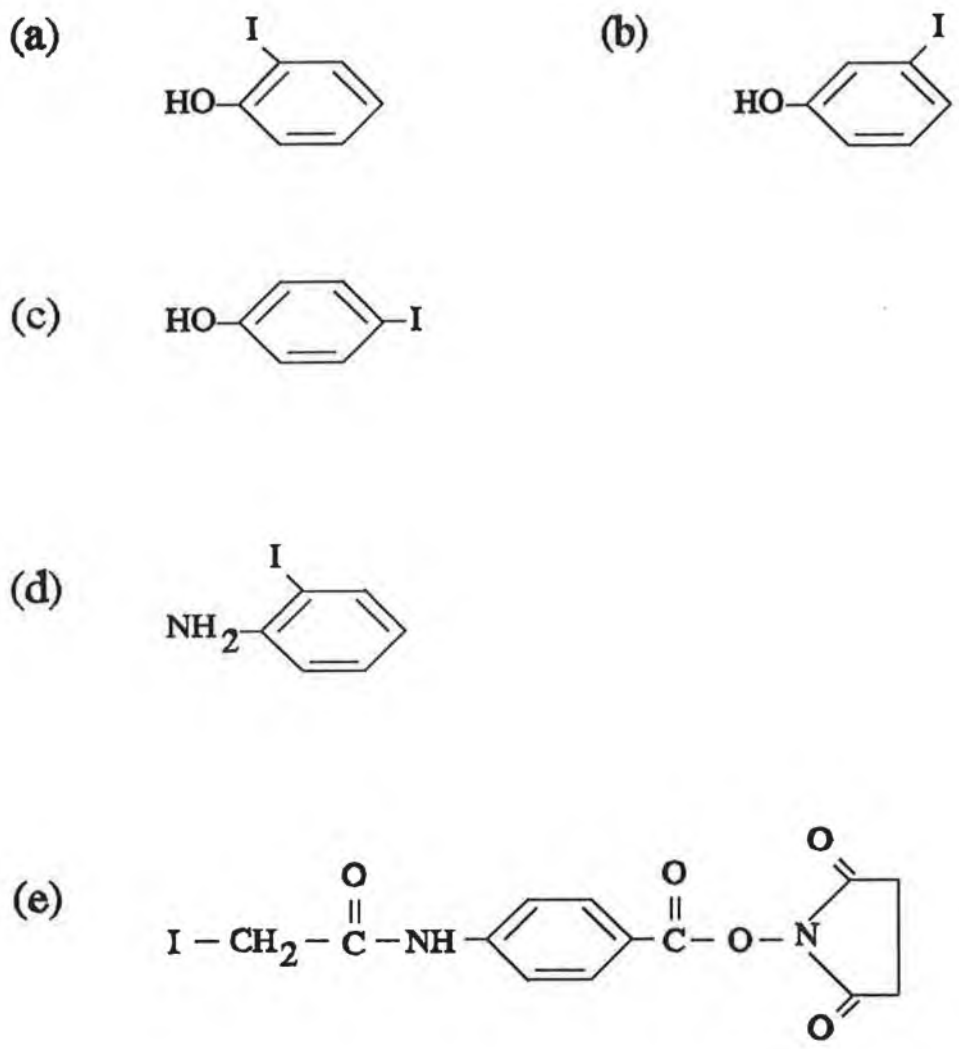

Figure 3.9 The structures of some iodine-containing organic compounds.
(a) 2-iodophenol (b) 3-iodophenol (c) 4-iodophenol (d) 2-iodoaniline
(e) N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB).

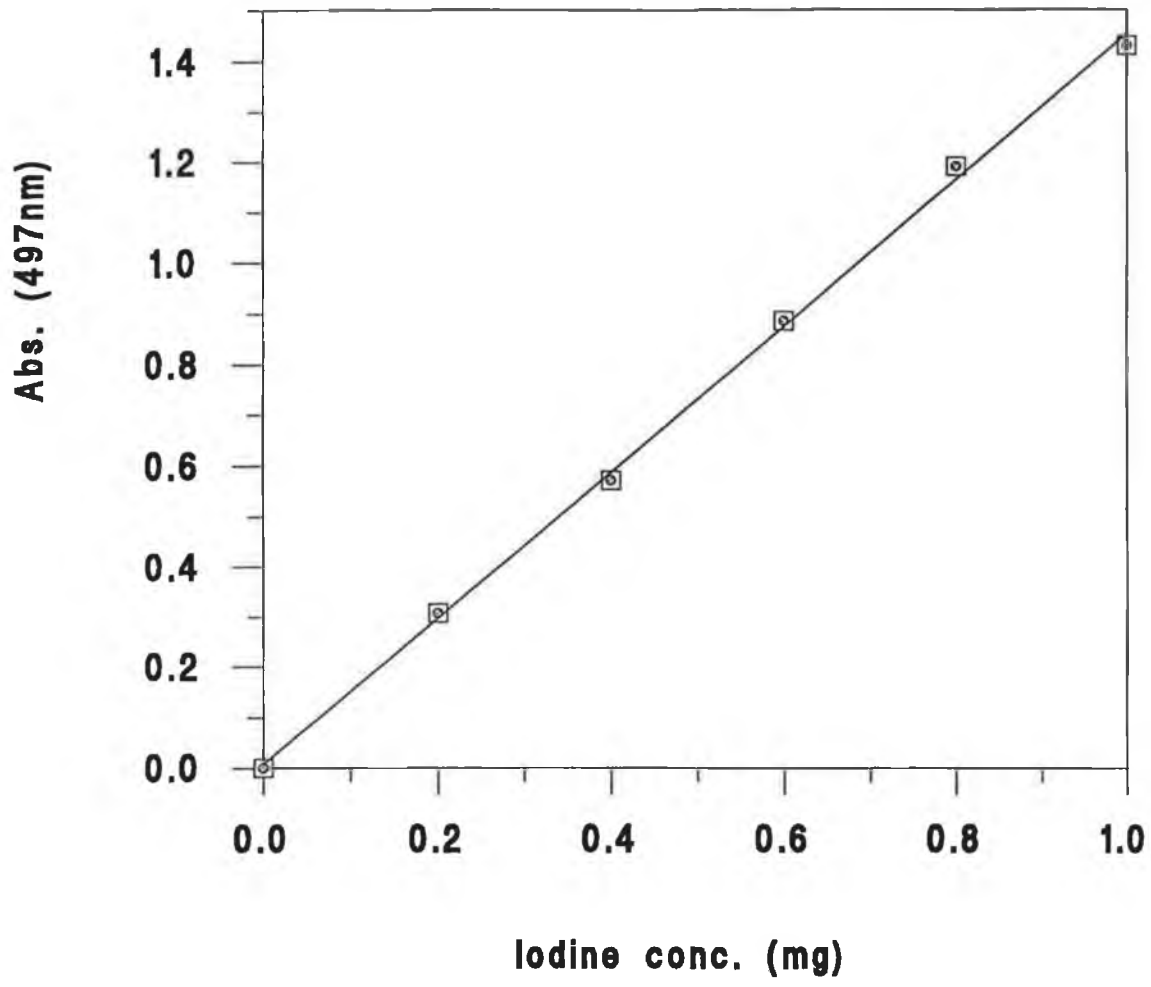

Figure 3.10 Graph of absorbance at 497nm plotted against iodine concentration, following treatment of potassium iodide standards with cerium(IV) and extraction of the liberated iodine into toluene.

Compound	**Iodine liberated (mg/10mg compound)**	**% of total iodine**
IBHR	0.51	15.5
T_3	1.92	32.8
T_4	1.52	23.2
SIAB	0.01	0.2

Table 3.8 Iodine liberated by iodine-containing organic compounds, following treatment with cerium(IV) and extraction of the liberated iodine into toluene (n=2).

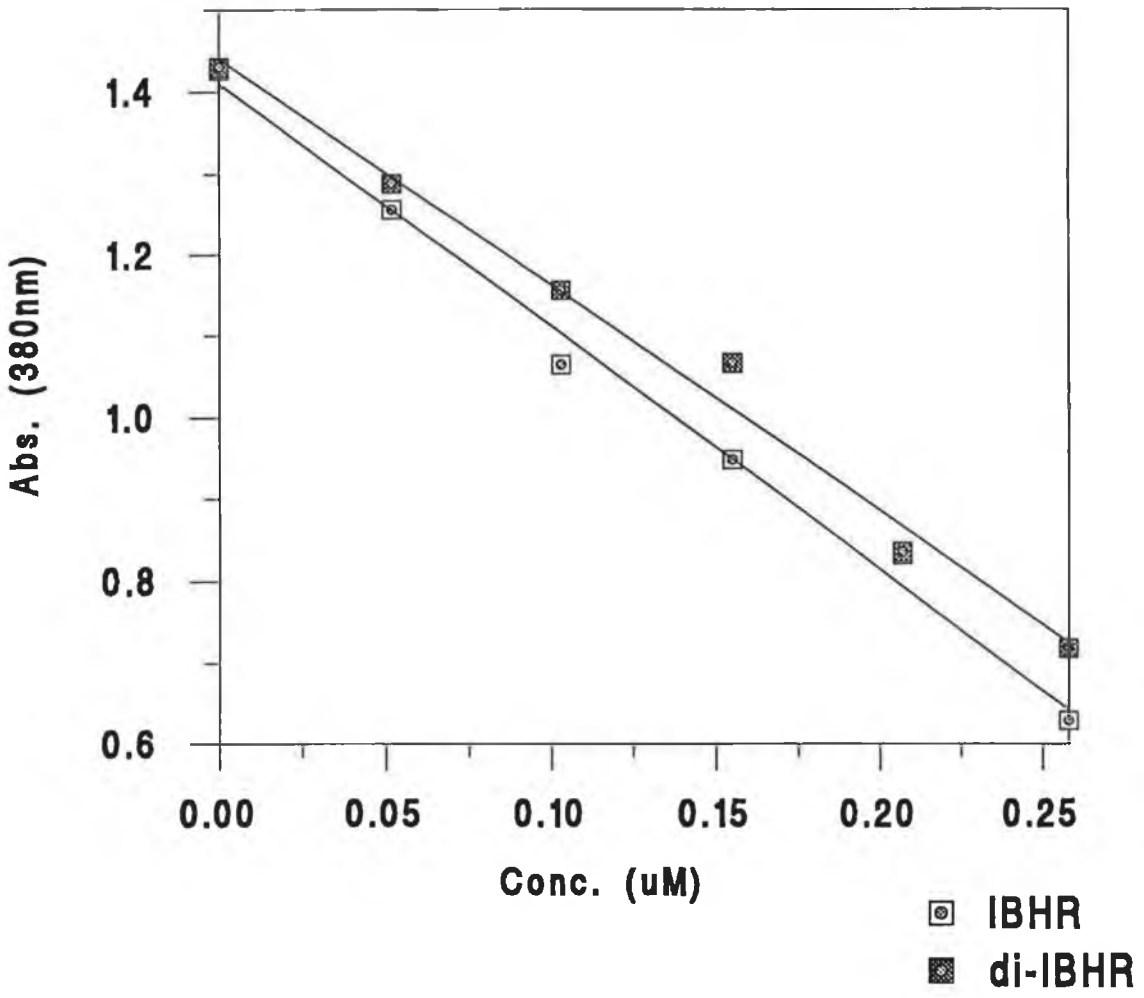

Figure 3.11 Graph of absorbance at 380nm plotted against concentration for IBHR and di-IBHR standards assayed in the cerium(IV) - antimony(III) spectrophotometric microassay.

3.2.4 Optimisation of reaction variables for the cerium(III) fluorimetric microassay.

The cerium(IV) - antimony(III) microassay can be monitored by measuring the fluorescence of the cerium(III) formed during the reaction. The excitation and emission spectra of cerium(III) are shown in Fig 3.12; excitation and emission wavelengths of 260nm and 360nm, respectively, were used in the experimental work. The microassay was optimised with respect to incubation period and reagent concentration, using the same procedure as for the spectrophotometric microassay (Section 3.2.1), except that potassium iodide standards in the range 2 to 8ng/ml were used as the reaction became non-linear above a concentration of 8ng/ml. The optimum conditions chosen were those that yielded good linearity (correlation coefficient, r) and a large difference in absorbance values over the range of standards tested (slope) for graphs of absorbance plotted against potassium iodide concentration.

The fluorescence intensity was measured every 10min, from 10 to 60min (Fig 3.13). Incubation periods of 10min and 20min resulted in graphs with correlation coefficients of less than 0.94. Increasing the incubation period resulted in better correlation coefficients (r=0.985 for 30min, r=0.993 for 60min). The slope increased as the incubation period was increased; a value of 0.693(±0.102) was obtained using a 10min incubation, while a value of 22.495(±0.522) was obtained using a 60min incubation. An incubation period of 60min was chosen as optimum.

The effect of increasing ceric ammonium sulphate concentration is shown in Fig 3.14. Increasing the concentration from 0.01M to 0.03M did not cause any significant change in the slope. A value of 15.495(±0.509) was obtained using 0.01M cerium(IV) and a value of 15.810(±1.200) was obtained using 0.03M cerium(IV). Correlation coefficients of 0.98 were obtained using 0.01M, 0.015M and 0.02M cerium(IV), which were better than those obtained using 0.025M and 0.03M cerium(IV) (r=0.951 and 0.889, respectively). A ceric ammonium sulphate concentration of 0.02M was chosen as optimum. Kirkbright *et al.* (1966) studied the variation in fluorescence intensity produced by increasing the cerium(IV) concentration, while keeping the arsenic(III) concentration constant. The fluorescence intensity increased up to a 2:1 molar ratio of cerium(IV):arsenic(III), and then decreased for higher ratios. It was suggested that the decrease was due to an inner- filter effect caused by absorption of the emission radiation by the excess cerium(IV).

The sulphuric acid concentration was increased from 5M to 8M (Fig 3.15). The use of 6M

H_2SO_4 resulted in the highest slope value (13.900±0.183). All H_2SO_4 concentrations tested gave similar correlation coefficients (r=0.98). A concentration of 6M H_2SO_4 was chosen as optimum. The use of H_2SO_4 was reported by Kirkbright *et al.* (1966), and by Tanaka *et al.* (1986) who used flow injection analysis for fluorimetric determination of the Sandell-Kolthoff reaction. Toledano *et al.* (1989) used a medium of HCl for the fluorimetric stopped flow determination of the Sandell-Kolthoff reaction. They studied the use of H_2SO_4, $HClO_4$ and HCl, and found that the catalytic action of iodide was greatest in HCl. HNO_3 could not be used, as nitrate ions had an inhibitory effect on the fluorescence of cerium(III).

Antimony potassium tartrate concentrations in the range 0.02M to 0.05M were tested in the microassay (Fig 3.16). Concentrations of 0.02M and 0.03M antimony(III) gave lower slope values, of 9.305(±0.938) and 15.950(±1.145), respectively, than 0.04M antimony(III) which gave a slope value of 24.455(±1.049). A concentration of 0.05M antimony(III) gave a slope value of 21.085(±1.876). Similar correlation coefficients (r=0.98) were obtained for all the concentrations tested. An antimony(III) concentration of 0.04M was chosen as optimum.

The conditions for the optimised cerium(III) fluorimetric microassay were similar to those for the spectrophotometric microassay, except that a higher concentration of antimony potassium tartrate was used for the fluorimetric assay. Potassium iodide standards were assayed in the optimised fluorimetric microassay; an example of a graph of fluorescence intensity plotted against concentration is shown in Fig 3.17. The linear range for the graph is 2 to 8ng/ml potassium iodide (r=0.991). The intra-assay accuracy and precision (Table 3.9) and inter-assay accuracy and precision (Table 3.10) were calculated. The intra- and inter-assay mean values obtained for the standards were within 93-107% of the true values, but the %CV for some of the standards was greater than 10%.

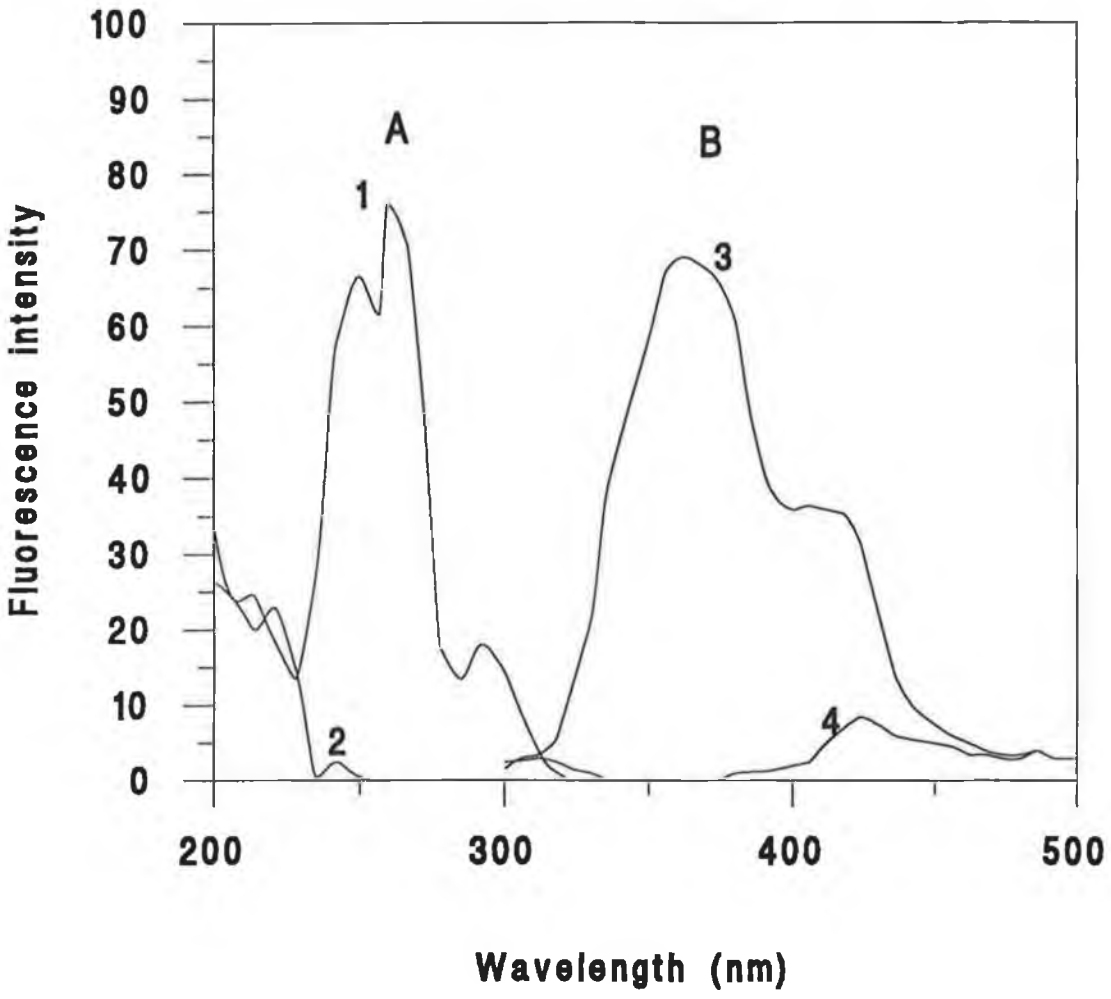

Figure 3.12 Fluorescence spectra for cerium(III): A, excitation and B, emission.

1 and 3: 10ng/ml potassium iodide, 0.03M antimony potassium tartrate, 0.02M ceric ammonium sulphate in 6M H_2SO_4.

2 and 4: 10ng/ml potassium iodide, 0.03M antimony potassium tartrate.

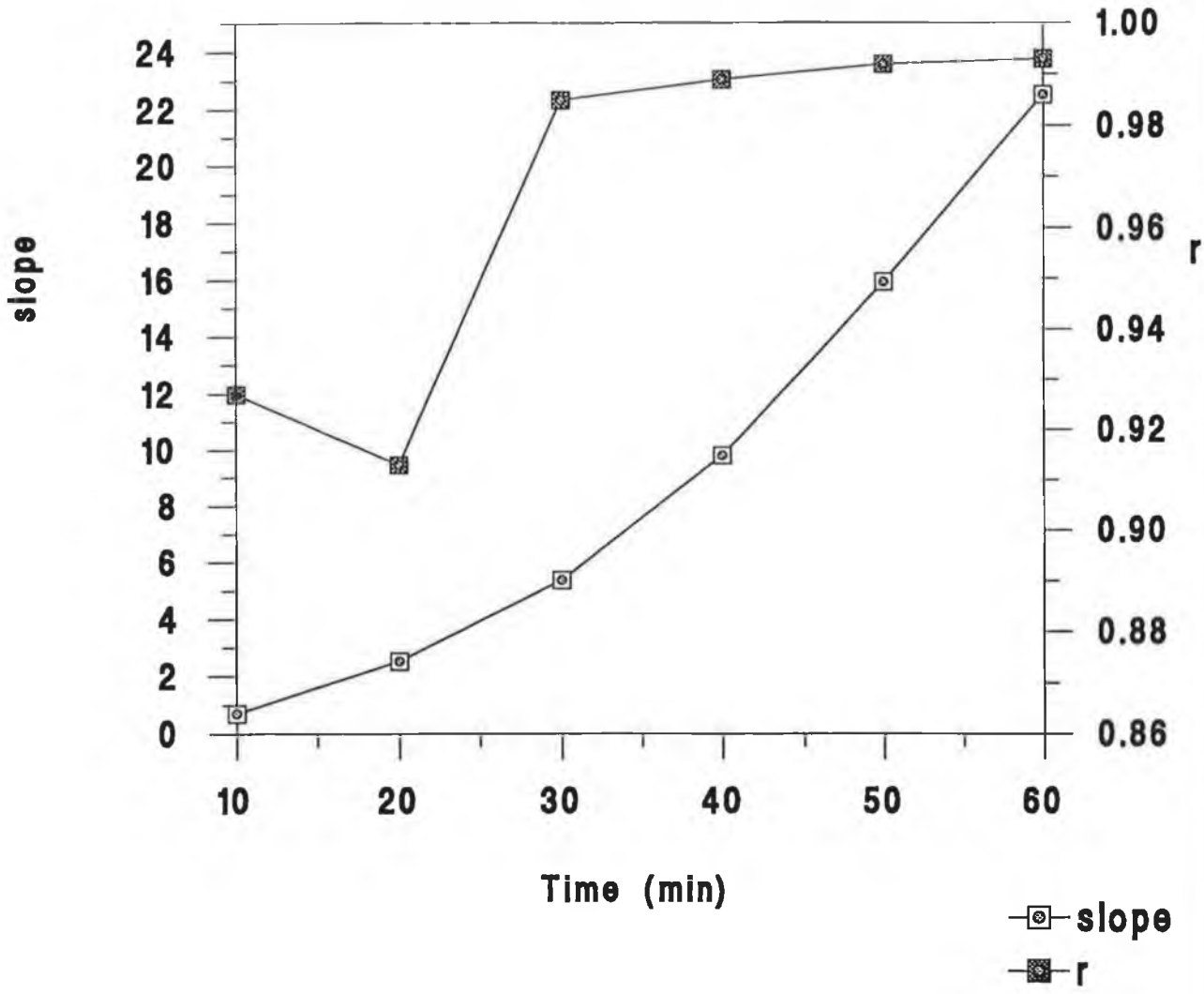

Figure 3.13 Effect of incubation period on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(III) fluorimetric microassay.

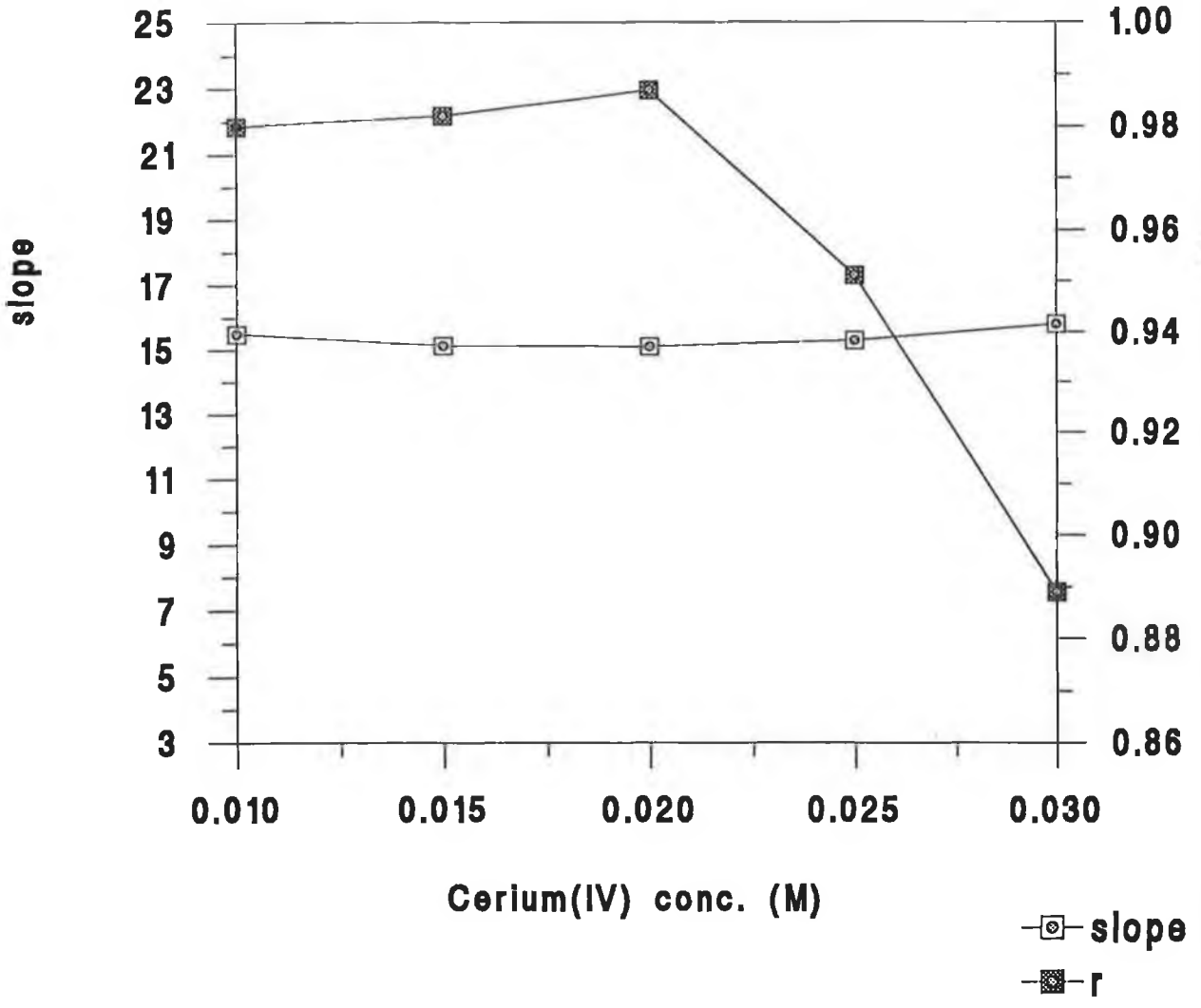

Figure 3.14 Effect of cerium(IV) concentration on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(III) fluorimetric microassay.

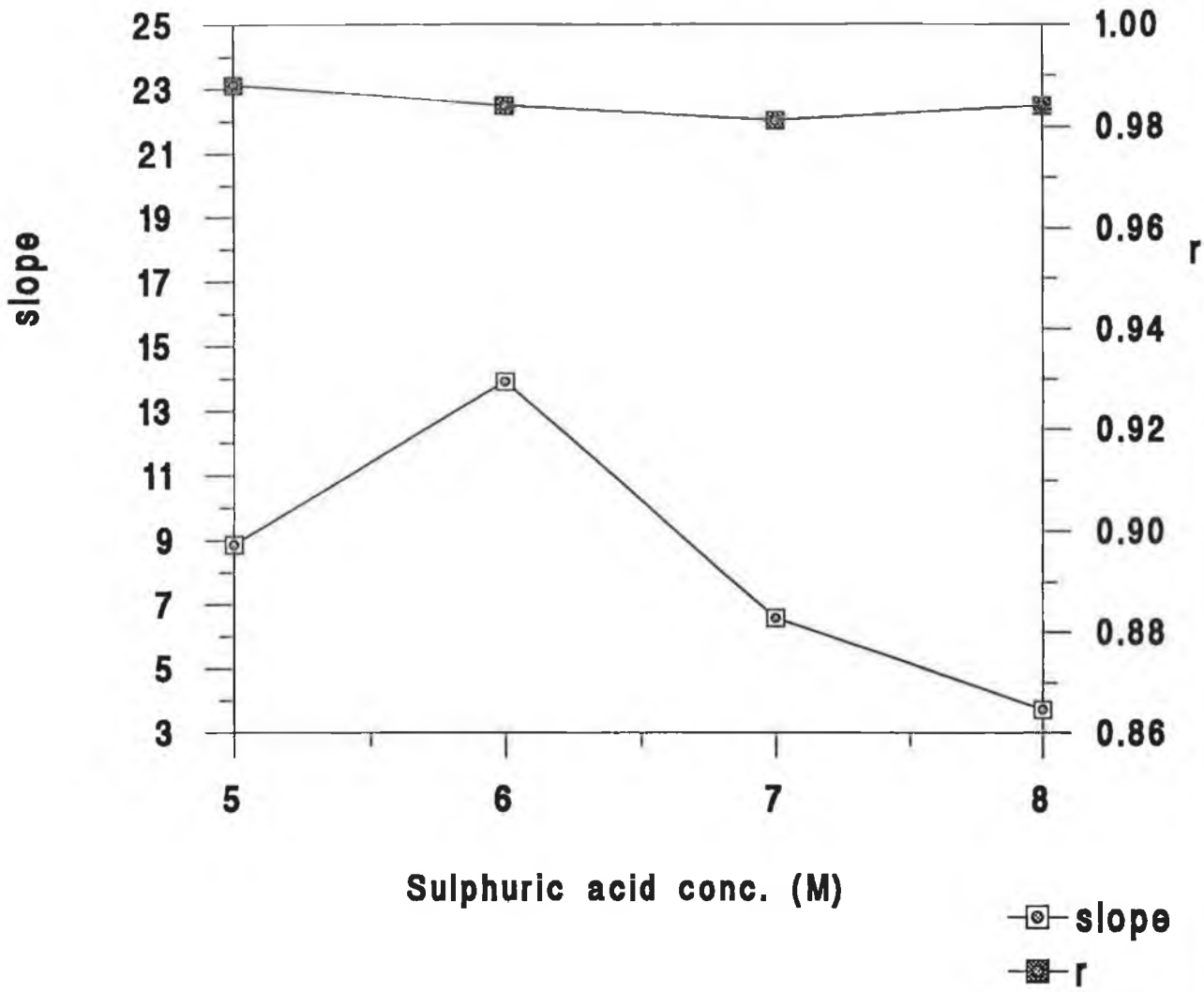

Figure 3.15 Effect of sulphuric acid concentration on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(III) fluorimetric microassay.

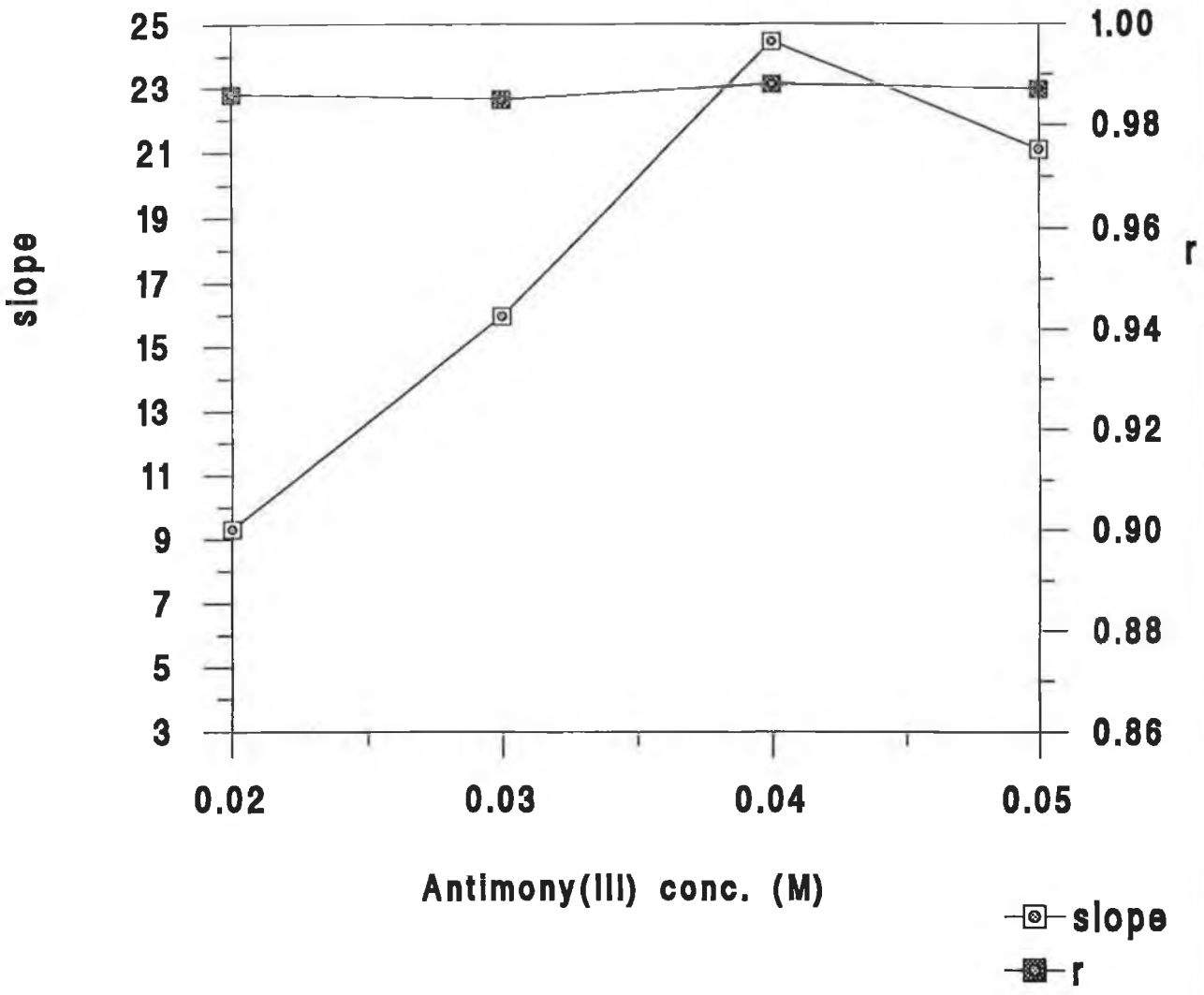

Figure 3.16 Effect of antimony(III) concentration on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(III) fluorimetric microassay.

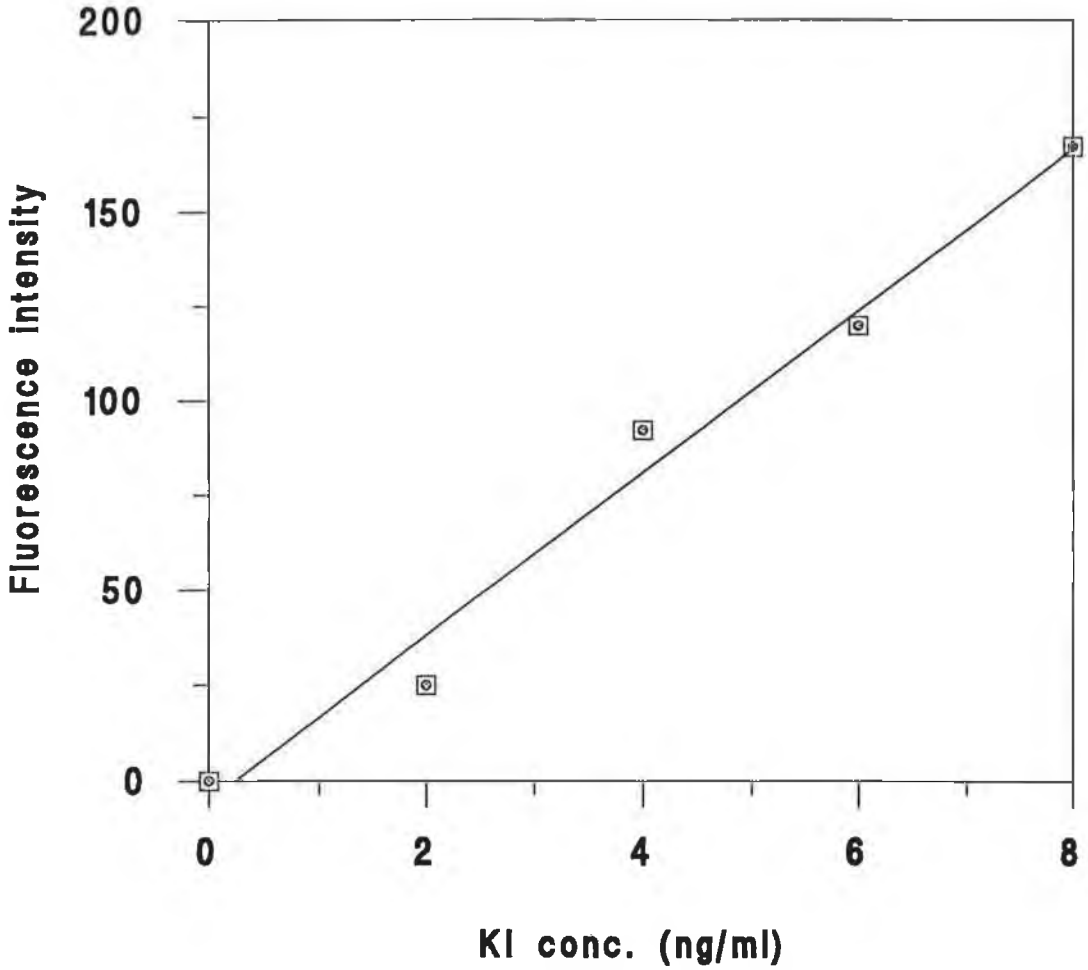

Figure 3.17 Graph of fluorescence intensity plotted against concentration of potassium iodide standards assayed in the optimised cerium(III) fluorimetric microassay.

KI conc. (ng/ml)	Mean ± SD	%CV
2	1.87 ± 0.31	16.7
4	4.12 ± 0.32	7.7
6	5.69 ± 0.66	11.7
8	8.50 ± 0.43	5.0

Table 3.9 Intra-assay accuracy and precision results for potassium iodide standards assayed in the cerium(III) fluorimetric microassay (n=8).

KI conc. (ng/ml)	Mean ± SD	%CV
2	1.85 ± 0.24	13.2
4	4.01 ± 0.53	13.3
6	6.39 ± 0.52	8.1
8	7.88 ± 0.69	8.8

Table 3.10 Inter-assay accuracy and precision results for potassium iodide standards assayed in the cerium(III) fluorimetric microassay (n=7).

3.2.5 Optimisation of reaction variables for the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric microassay.

Cerium(IV) can oxidise 8-hydroxyquinoline-5-sulphonic acid to give a fluorescent species. The cerium(IV) - antimony(III) reaction is allowed to proceed as usual, and after a fixed time 8-hydroxyquinoline-5-sulphonic acid is added and the fluorescence produced is measured. The excitation and emission spectra of the fluorescent species are shown in Fig 3.18; excitation and emission wavelengths of 370nm and 480nm, respectively, were used in the experimental work. The microassay was optimised with respect to incubation period before addition of 8-hydroxyquinoline-5-sulphonic acid, time of reading the fluorescence intensity and volume of 8-hydroxyquinoline-5-sulphonic acid.

Incubation periods of 45min, 60min, 75min and 90min were tested (Fig 3.19). Incubation periods of 60min and 75min gave slope values of 7.330(±0.823) and 7.555(±0.776), respectively, and correlation coefficients of 0.995 and 0.993, respectively. These values were better than those obtained for 45min or 90min, which gave slope values of 4.195(±0.376) and 2.847(±0.455), respectively, and correlation coefficients of 0.957 and 0.856, respectively. An incubation period of 60min was chosen as optimum.

The fluorescence intensity produced was monitored from 1min to 25min after the addition of 8-hydroxyquinoline-5-sulphonic acid (Fig 3.20). The fluorescence reached a maximum after 5min, after which there was a slight decrease from a fluorescence intensity of 125 at 5min to 119 at 25min. All fluorescence readings were taken within 5 to 15min after the addition of 8-hydroxyquinoline-5-sulphonic acid. Pal *et al.* (1977) studied the reaction between cerium(IV) and 8-hydroxyquinoline-5-sulphonic acid, and reported that the fluorescence intensity of the oxidation product reached a maximum after 30 seconds and remained constant for 35 to 40min.

The effect of increasing the volume of 8-hydroxyquinoline-5-sulphonic acid is shown in Fig 3.21. (Due to the poor solubility of 8-hydroxyquinoline-5-sulphonic acid, the highest concentration that could be prepared was 1.5mM, therefore the concentration in the reaction was increased by increasing the volume added to the reaction.) Increasing the volume from 30µl to 50µl caused an increase in the slope from a value of 4.450(±0.059) to 6.495(±0.581). Further increases in the volume to 60µl or 70µl caused a decrease in the slope; a value of 5.880(±0.350) was obtained using 60µl and a value of 5.335(±0.451) was obtained using 70µl. Correlation coefficients of 0.99 were obtained for all volumes tested.

A volume of 50µl was chosen as optimum.

A graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the optimised microassay is shown in Fig 3.22. The linear range for the assay is 2 to 8ng/ml potassium iodide (r=0.990). The intra-assay accuracy and precision (Table 3.11) and inter-assay accuracy and precision (Table 3.12) were calculated. The mean values for the standards were within 95 to 107% of the true values, except for the inter-assay result for 2ng/ml potassium iodide which gave a mean value that was 112% of the true value. The %CV for some of the standards was greater than 10%.

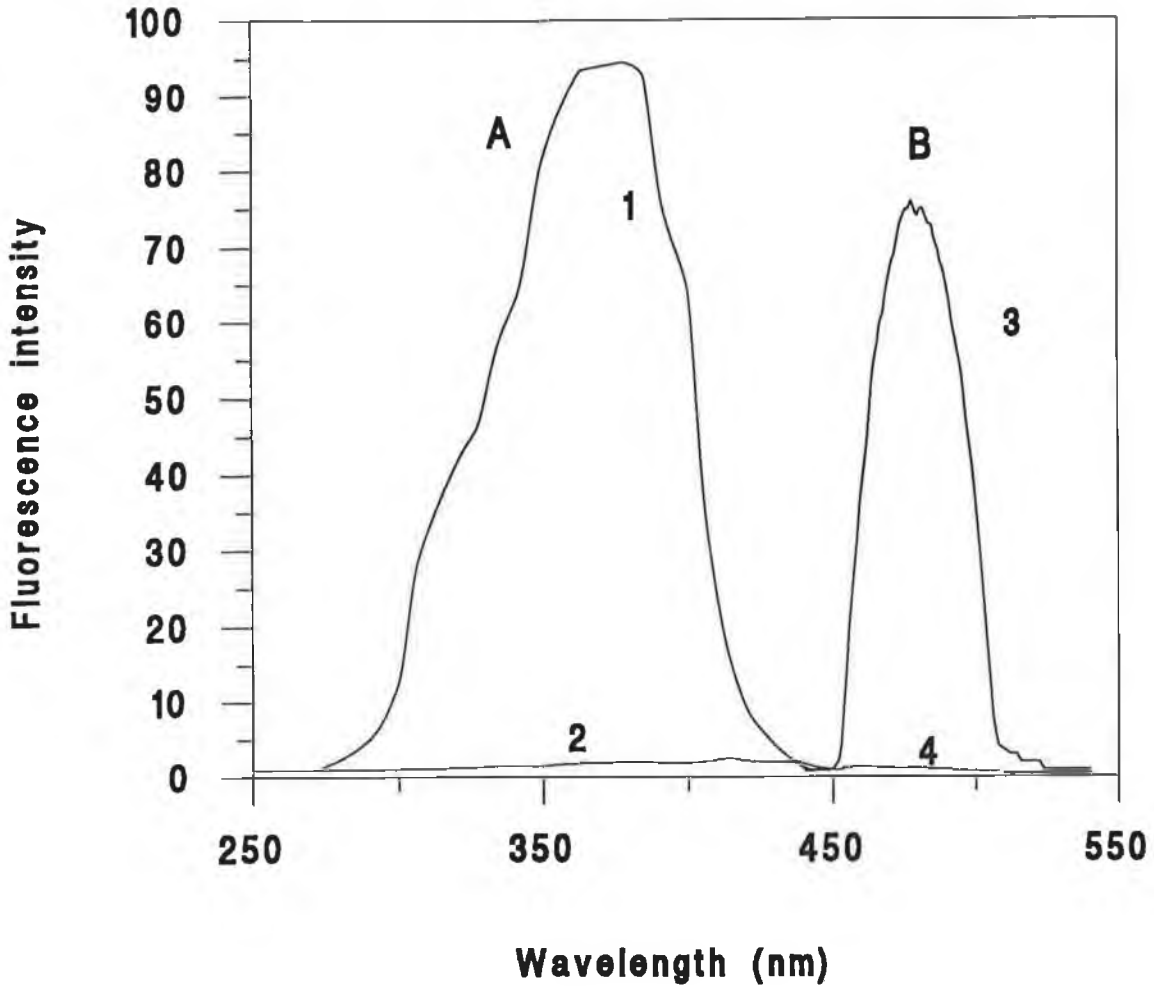

Figure 3.18 Fluorescence spectra for cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid (8OHQ): A, excitation and B, emission.

1 and 3: 10ng/ml potassium iodide, 0.03M antimony potassium tartrate, 0.02M ceric ammonium sulphate in 6M H_2SO_4, 1.5mM 8OHQ.

2 and 4: 10ng/ml potassium iodide, 0.03M antimony potassium tartrate, 1.5mM 8OHQ.

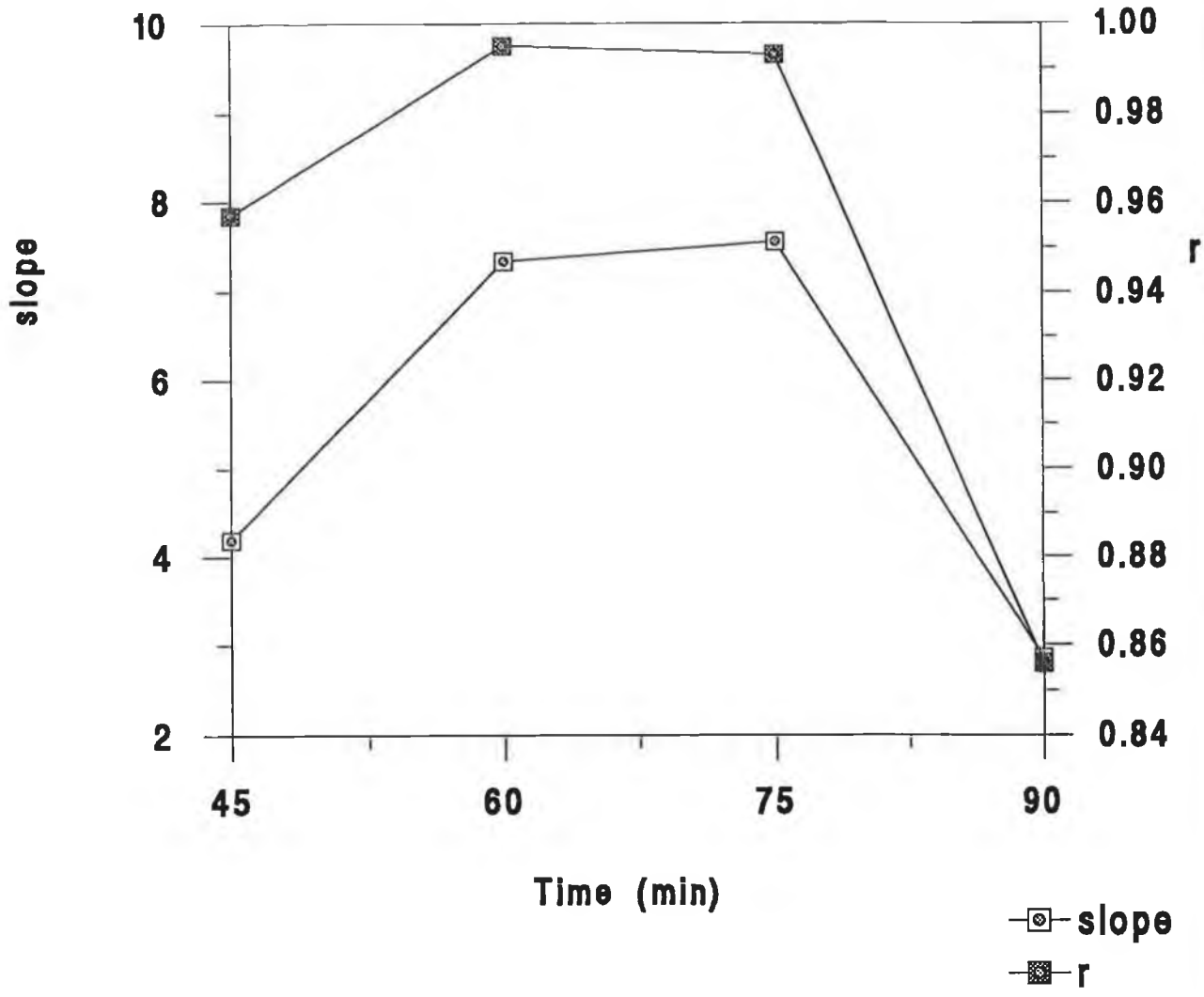

Figure 3.19 Effect of incubation period on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric microassay.

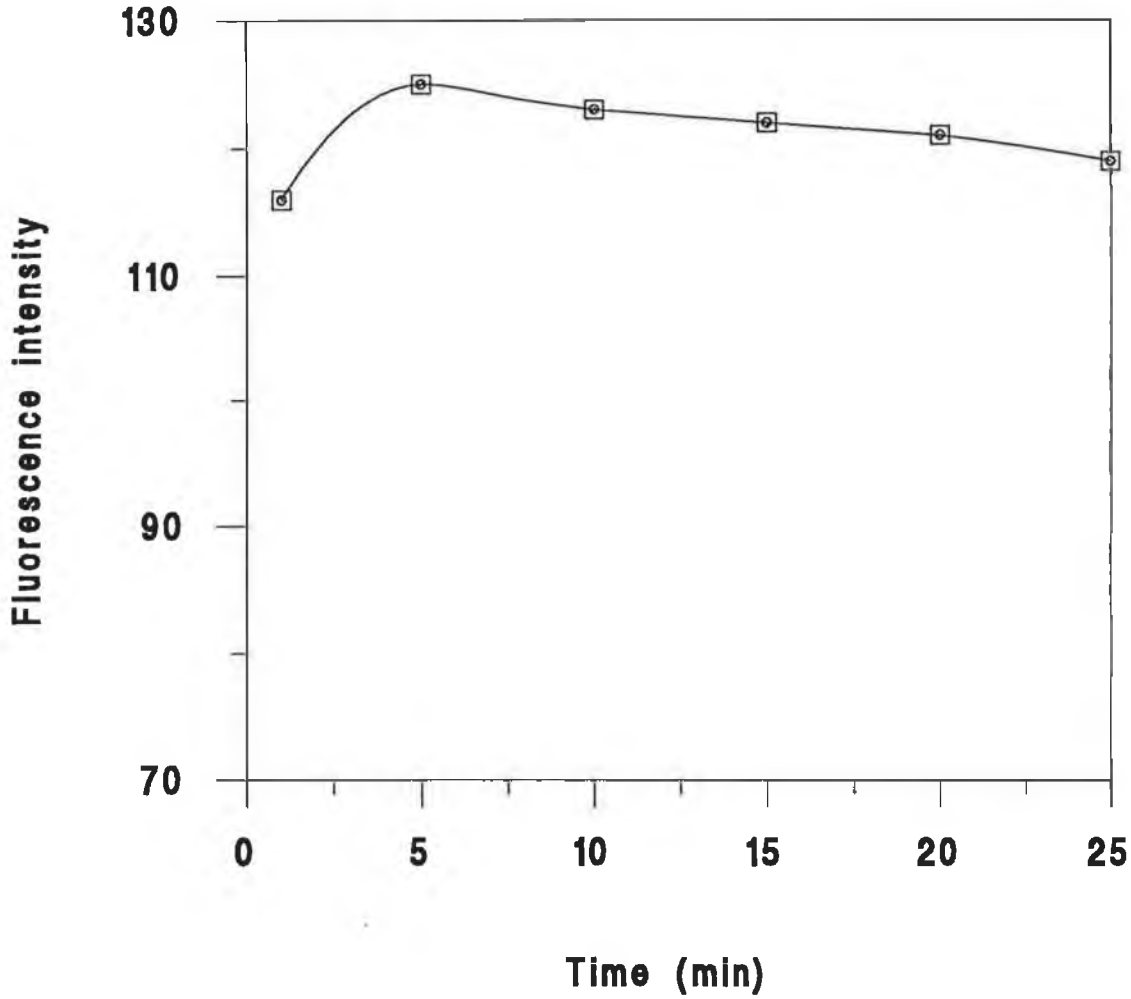

Figure 3.20 The fluorescence intensity of the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric microassay, read at various time points after the addition of 8-hydroxyquinoline-5-sulphonic acid.

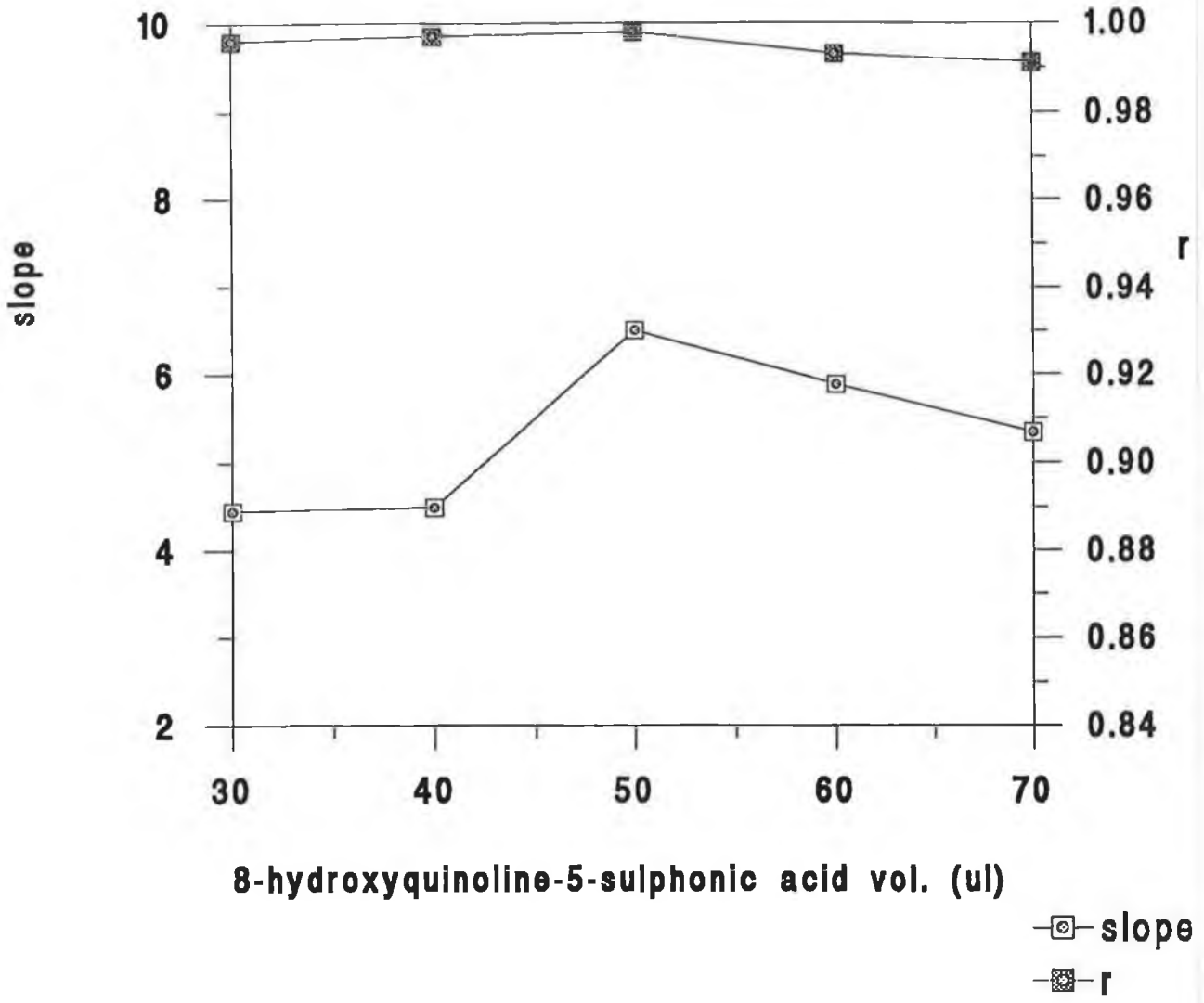

Figure 3.21 Effect of 8-hydroxyquinoline-5-sulphonic acid volume on the slope and correlation coefficient (r) of the graph of fluorescence intensity plotted against concentration for potassium iodide standards assayed in the cerium(IV) - 8-hydroxy-quinoline-5-sulphonic acid fluorimetric microassay.

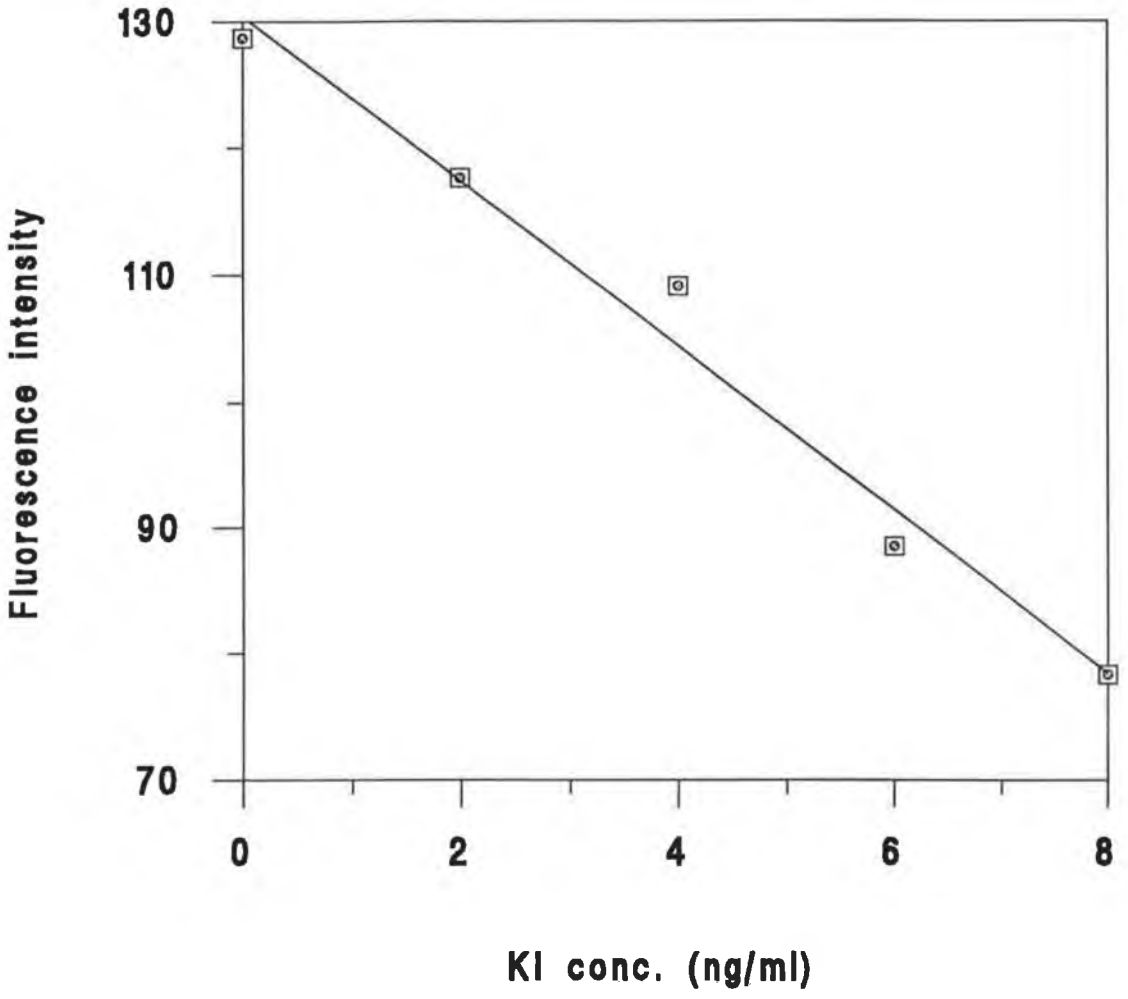

Figure 3.22 Graph of fluorescence intensity plotted against concentration of potassium iodide standards assayed in the optimised cerium(IV) - 8-hydroxy-quinoline-5-sulphonic acid fluorimetric microassay.

KI conc. (ng/ml)	Mean ± SD	%CV
2	2.14 ± 0.31	14.5
4	3.80 ± 0.45	11.9
6	6.30 ± 0.44	7.0
8	7.73 ± 0.66	8.6

Table 3.11 Intra-assay accuracy and precision results for potassium iodide standards assayed in the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric microassay (n=8).

KI conc. (ng/ml)	Mean ± SD	%CV
2	2.25 ± 0.20	9.0
4	3.94 ± 0.52	13.2
6	6.30 ± 0.10	1.5
8	7.72 ± 0.23	3.0

Table 3.12 Inter-assay accuracy and precision results for potassium iodide standards assayed in the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric assay (n=5).

3.2.6 Comparison of the spectrophotometric microassay with the fluorimetric microassays.

The cerium(IV) - antimony(III) microassay has been monitored by both spectrophotometric and fluorimetric methods. In the cerium(III) fluorimetric method the increase in fluorescence intensity was measured against a low blank value, while in both the spectrophotometric and the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid methods the decrease in absorbance or fluorescence intensity was measured against a high blank value. The linear range for the fluorimetric microassays for potassium iodide standards was 2 to 8ng/ml; the spectrophotometric microassay had a wider linear range of 2 to 10ng/ml for potassium iodide standards. The relationship between fluorescence intensity and fluorophore concentration is non-linear at higher fluorophore concentrations (Ingle and Ryan, 1981). This is not a problem in trace analysis where the concentration of the monitored species is less than 1μM, but for these experiments the concentration of cerium(IV) is in the millimolar range.

Tanaka *et al.* (1985) reported a detection limit of 0.8μM iodide using spectrophotometric monitoring of the Sandell-Kolthoff reaction. The detection limit was improved by the use of fluorimetric monitoring (Tanaka *et al.*, 1986). A detection limit of 50nM iodide was obtained using fluorimetric monitoring of the fluorescent species produced by cerium(IV) oxidation of 8-hydroxyquinoline-5-sulphonic acid, and a detection limit of 3nM iodide was obtained using fluorimetric monitoring of cerium(III). In the work carried out here, no improvement in sensitivity was obtained using fluorimetric monitoring of the cerium(IV) - antimony(III) microassay. Fluorimetric monitoring is, in principle, more sensitive than spectrophotometric monitoring, but in practice the sensitivity is restricted by several factors (Hemmila, 1985). Background fluorescence from, for example, plastics or impurities in the reagents and quenching of the fluorescence intensity due to the sensitivity of fluorescent compounds to their environment can limit the sensitivity of fluorescent techniques. The instrumentation is also expensive and complex, as excitation and emission optics must be incorporated, and low light levels are measured by the detector. The Perkin Elmer Luminescence Spectrophotometer used to monitor fluorescence intensity for these experiments is capable of reading both cuvettes and 96 well plates. However, there is a 40-fold reduction in fluorescence intensity when reading from plates compared to cuvettes. Also, in order to read plates the optics had to be altered manually. Alignment of the

optics is critical, and manual alteration introduces the potential for errors in the alignment and damage of the optical fibres over time.

The intra- and inter-assay accuracy and precision values obtained for the spectrophotometric assay were better than those obtained for the fluorimetric assays. The mean values for potassium iodide standards were within 96 to 107% of the true values when assayed in the spectrophotometric assay; the mean values were within 93 to 107% and 95 to 112% of the true values when assayed in the cerium(III) fluorimetric assay and the cerium(IV) - 8-hydroxyquinoline-5-sulphonic acid fluorimetric assay, respectively. The %CV values for all standards were less than 10% when assayed in the spectrophotometric assay, but values of greater than 10% were obtained for some standards in both of the fluorimetric assays. Since assay parameters such as linear range, accuracy and precision were better for the spectrophotometric microassay compared with the fluorimetric microassays, it was decided to use only the spectrophotometric microassay for future work.

3.3 Summary.

The reaction between cerium(IV) and antimony(III), reported by Bognar and Sarosi (1965), has been successfully adapted for use in a microassay format, and used to determine iodide and iodine-containing compounds. The microassay can be used as a replacement for the Sandell-Kolthoff microassay developed by O'Kennedy *et al.* (1989). The spectrophotometric cerium(IV) - antimony(III) microassay has a linear range of 2 to 10ng/ml for potassium iodide standards, and 0.02 to 0.1µg/ml for IBHR standards. It was not possible to achieve the same sensitivity using IBHR standards as with potassium iodide standards. The sensitivity of the microassay for IBHR is limited by the fact that only a percentage of the iodine in IBHR is liberated to catalyse the reaction, thus the catalytic activity is inferior to that of potassium iodide. The use of di-IBHR did not lead to any improvement in the sensitivity.

Fluorimetric monitoring of the reaction was investigated in an attempt to improve the sensitivity. Two fluorimetric methods were investigated. One was based on the direct measurement of cerium(III), and the other was based on the measurement of the fluorescence produced by the cerium(IV) oxidation of 8-hydroxyquinoline-5-sulphonic acid. The linear range for the fluorimetric microassays was 2 to 8ng/ml for potassium iodide standards; the spectrophotometric microassay can be used for measurement of a wider

range of potassium iodide standards and no improvement in sensitivity was obtained using fluorimetric monitoring of the microassay. The intra- and inter-assay accuracy and precision values obtained for potassium iodide standards measured in the spectrophotometric assay were better than the values obtained in the fluorimetric assays.

4. USE OF IBHR - LABELLED ANTIBODIES.

4.1 Introduction.

The use of the Sandell-Kolthoff microassay for the detection of IBHR-labelled antibodies in immunoassays has been described. O'Kennedy and Keating determined mouse IgG levels (O'Kennedy and Keating, 1991) and human IgG levels (O'Kennedy and Keating, 1993) using a two-site immunoassay and the Sandell-Kolthoff reaction for detection of the IBHR label. This system was compared with the use of HRP-labelled antibodies in an ELISA. In this chapter, the use of IBHR-labelled antibodies in an immunoassay for human IgG levels and their detection using the cerium(IV) - antimony(III) microassay is described. The results were compared with those obtained using the Sandell-Kolthoff microassay, and with an ELISA.

The level of iodination of antibodies was studied by labelling with different amounts of IBHR. The immunoreactivity of the iodinated antibodies was assessed in the two-site immunoassay. The relative binding pattern of native and iodinated antibodies was studied using real-time biospecific interaction analysis (BIAcore).

The principle of BIAcore is described in detail by Jonsson and Malmquist (1992). BIAcore uses surface plasmon resonance (SPR) to investigate biospecific interactions at the surface of a sensor chip. The SPR response is correlated to changes in the refractive index at the chip surface, which are caused by mass changes on the surface due to components binding to the chip, for example, on immobilisation of ligand onto the chip or on binding of analyte to the immobilised ligand. The sensor chip is held in place in the instrument with a microfluidic cartridge which controls delivery of samples into a buffer that passes continuously over the chip surface. The chip consists of a glass slide with a gold film, to which is linked a carboxymethylated dextran. The dextran is activated by derivatisation with N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylamino-propyl)carbodiimide (EDC), forming reactive NHS-esters. The ligand of interest is immobilised by formation of a covalent bond between amino groups on the ligand and the NHS-esters. Unreacted NHS-esters remaining on the chip are deactivated by reaction with ethanolamine. The ligand bound to the chip surface is freely accessible to three-dimensional antibody-antigen interactions. Interactions between the ligand and the analyte are directly detected by measuring changes in the SPR response, which is presented through the BIAcore software as resonance units (RU). A sensorgram (a plot of RU versus time) is obtained for each interaction. The ligand matrix on the chip is regenerated after each interaction using either acidic or

basic solution, which removes the non-covalently bound analyte but not covalently attached ligand. In this way, multiple antibody-antigen interactions can be studied using the same ligand matrix.

4.2 Results and discussion.

4.2.1 Two-site immunoassays for human IgG.

Antibodies were labelled with IBHR, and free and conjugated IBHR were separated by dialysis. The cerium(IV) - antimony(III) microassay was used to measure the amount of IBHR in the conjugate, assuming that the catalytic activity of IBHR is not altered on conjugation. Since the catalytic activity of the iodine atom in IBHR is dependent on the presence of the hydroxyl group ortho to it (Section 3.2.3), and this group is not involved in the conjugation reaction, the catalytic activity of free and conjugated IBHR should be similar. The BioRad assay was used to determine the amount of protein in the conjugate. IBHR can introduce groups into a protein that interfere in the measurement of its concentration. The methods frequently used for measurement of protein concentration are absorbance at 280nm, the BCA assay and the BioRad assay. The structure of IBHR is similar to tyrosine, thus conjugation of IBHR to a protein introduces groups which absorb at 280nm (O'Kennedy *et al.*, 1989), and the use of absorbance at 280nm is no longer an accurate measure of protein concentration. Knight and Welch (1978) reported that the main sites of labelling on conjugation of IBHR to a protein are lysine residues, with small amounts of tyrosine and histidine residues being labelled. The BCA assay is based on the use of Biuret reagent, which interacts mainly with cysteine, cystine, tryptophan and tyrosine, and the peptide bond (Wiechelman *et al.*, 1988). Increasing IBHR concentration was found to be linearily related to increasing colour intensity in the BCA assay (O'Kennedy and Keating, 1993). This interference may be due to the structural similarity of IBHR to tyrosine. The BioRad assay is based on the use of the Bradford reagent, Coomassie brilliant blue G-250, which interacts mainly with arginine residues, while other basic resides (lysine and histidine) and aromatic residues (tyrosine, tryptophan and phenylalanine) give a slight response (Compton and Jones, 1985). Thus, IBHR may interfere in the BioRad assay due to modification of lysine residues, or IBHR itself may interfere in the assay (O'Kennedy *et al.*, 1989). To test for interference in the assay, a set of IBHR standards was assayed using the BioRad reagent. The absorbance values obtained were compared to those obtained for IgG standards in the same range (Fig 4.1); the absorbance due to IBHR was negligible. Therefore, it was decided to use the BioRad

assay for the determination of protein concentration of IBHR-labelled proteins. On average, 19 moles of IBHR were conjugated per mole of antibody. Conjugation levels of 17 moles of IBHR (O'Kennedy and Keating, 1991) and up to 23 moles of IBHR (O'Kennedy and Keating, 1993) per mole of antibody have been reported.

Antibodies were labelled with HRP also. The enzyme-antibody absorbance ratio was determined by measuring the absorbance of the conjugate at 403nm and 280nm. This ratio provides a guide to the amount of HRP conjugated, which influences conjugate performance. A ratio in the range 0.3 to 0.5 indicates a conjugate with high enzyme activity without adverse effect on the antibody titre, and low background readings (Catty and Raykundalia, 1989). On average, a ratio of 0.36 was obtained for the conjugates prepared for these experiments. The enzyme activity of the conjugate was measured by addition of substrate to the conjugate, diluted to 1µg/ml IgG, and measurement of the absorbance at intervals. A graph of absorbance plotted against time is shown in Fig 4.2. Conjugates that have an absorbance of 1.0 in 10 to 15min, and an absorbance of 1.5 or greater in 25 to 30min have suitable enzyme activity for ELISA (Catty and Raykundalia, 1989); these criteria were met by the conjugate prepared for these experiments.

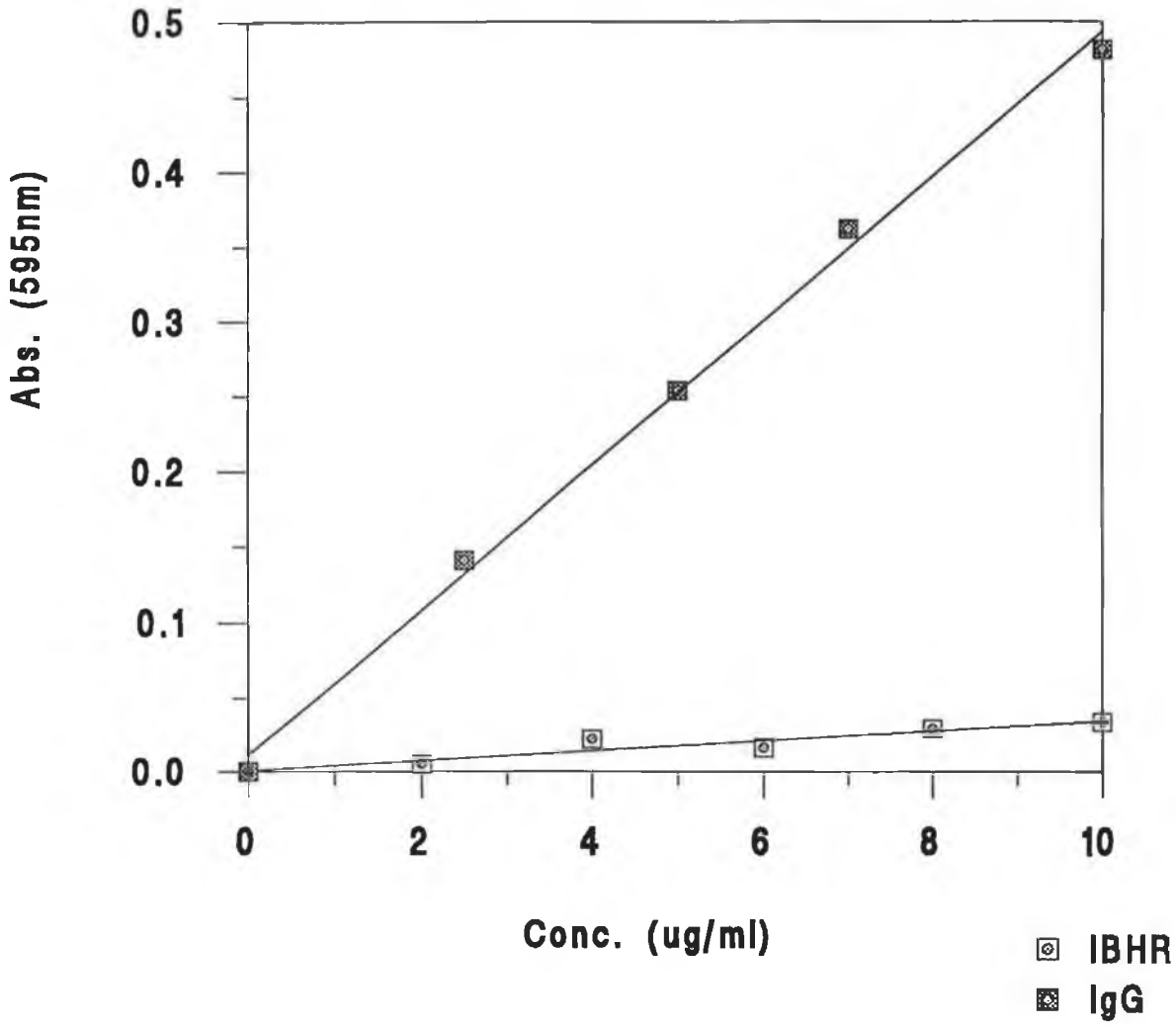

Figure 4.1 Graph of absorbance at 595nm plotted against concentration of IBHR standards and IgG standards assayed in the BioRad protein assay.

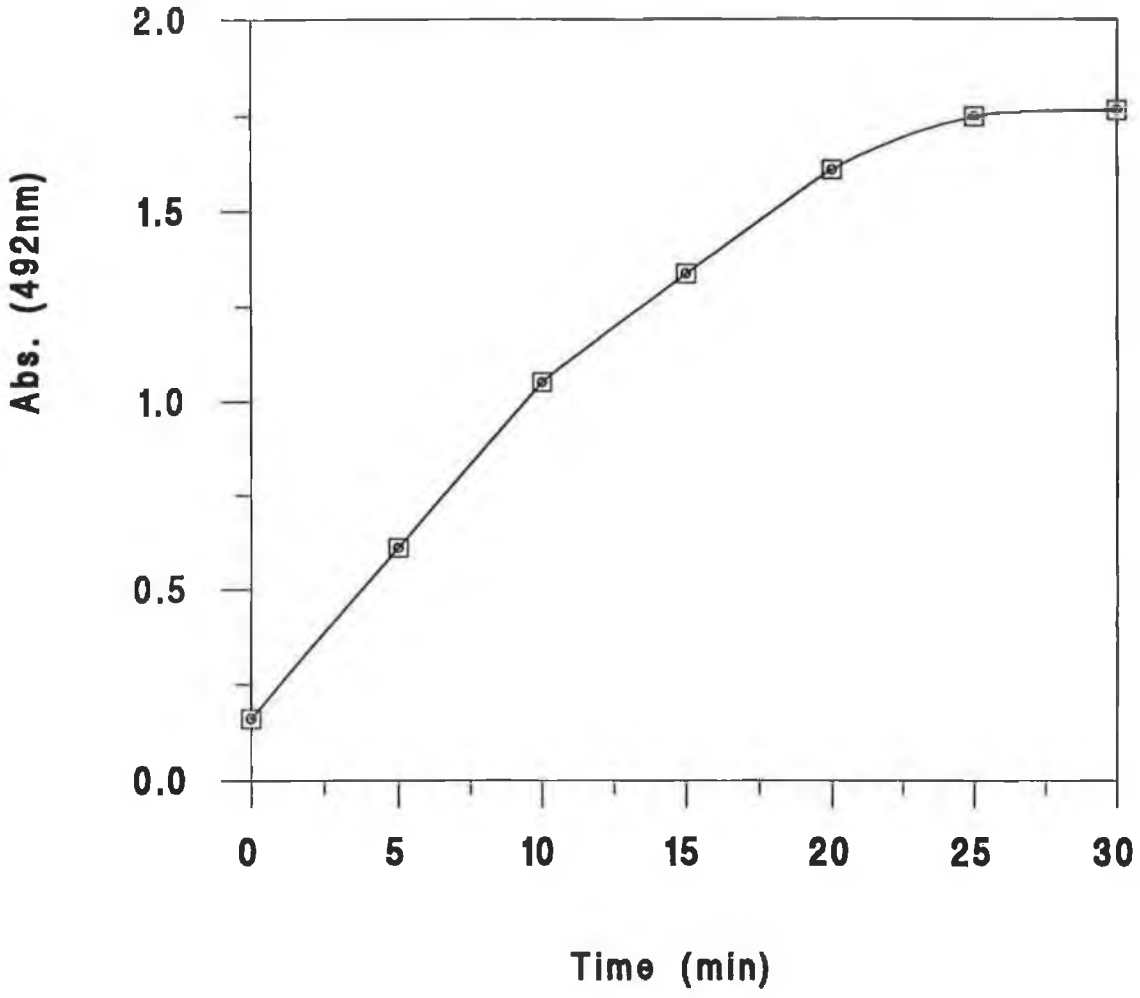

Figure 4.2 Graph of absorbance at 492nm plotted against time, for measurement of the enzyme activity of HRP-labelled antibodies.

Human IgG levels in standards and human serum samples were determined in a two-site immunoassay using IBHR-labelled antibodies, with detection of the IBHR label by either the cerium(IV) - antimony(III) microassay or the Sandell-Kolthoff microassay, and using HRP-labelled antibodies. The protocol for the immunoassay is outlined in a flow diagram in Fig 4.3. In order to ensure the reliability of the comparison between the three assay systems, the same batch of anti-human IgG antibodies were used in each system.

The optimum dilution of the conjugated antibodies was determined in the immunoassay. IBHR-labelled antibodies were diluted in the range 1/10 to 1/80; the results are shown in Table 4.1. Dilutions of 1/10 and 1/20 gave the largest change in absorbance over the range of IgG standards tested; a dilution of 1/20 was chosen as optimum, since this was less wasteful of antibody. On average, this corresponded to an antibody concentration of 22µg/ml. HRP-labelled antibodies were diluted in the range 1/80 to 1/640; the results are shown in Table 4.2. A dilution of 1/160 was chosen as optimum. On average, this corresponded to an antibody concentration of 6µg/ml.

The linear range of the immunoassays was determined from standard curves of absorbance plotted against human IgG concentration, on a log scale. The standard curves obtained using IBHR-labelled antibodies, with detection by the cerium(IV) - antimony(III) microassay and the Sandell-Kolthoff microassay are shown in Fig 4.4 and Fig 4.5, respectively. The standard curve obtained using HRP-labelled antibodies is shown in Fig 4.6. Human IgG standards, in the range 0.05 to 2µg/ml, could be determined using IBHR-labelled antibodies with detection by the cerium(IV) - antimony(III) microassay (r=0.994) or the Sandell-Kolthoff microassay (r=0.973). Standards in the range 0.0064 to 0.25µg/ml could be determined using HRP-labelled antibodies (r=0.998); the use of an HRP label allowed measurement of lower concentrations of human IgG. The use of an HRP label also resulted in a greater difference in absorbance values over the range of standards tested. The difference in absorbance values between the zero standard and the highest standard was 0.666 using IBHR-labelled antibodies, with detection by the cerium(IV) - antimony(III) reaction, while it was 1.163 using HRP-labelled antibodies.

O'Kennedy and Keating (1993) measured 5 to 40ng/well (which corresponds to 0.05 to 0.4µg/ml) of human IgG using IBHR-labelled antibodies with detection by the Sandell-Kolthoff microassay, and HRP-labelled antibodies. They measured the absorbance for both systems after 1hr, and the absorbance using HRP-labelled

antibodies became non-linear above 25ng/well. For the experiments reported here, the absorbance of each system was measured at different times in order to determine the linear range of each system. The absorbance for the cerium(IV) - antimony(III) microassay was measured after 2hr, for the Sandell-Kolthoff microassay the absorbance was measured after 1hr, and for HRP-labelled antibodies the absorbance was measured after 15min.

The intra- and inter-assay accuracy and precision were calculated for the standards. The results obtained using IBHR-labelled antibodies, with detection by the cerium(IV) - antimony(III) microassay are shown in Table 4.3 and Table 4.4. The results obtained using the Sandell-Kolthoff microassay are shown in Table 4.5 and Table 4.6. The results obtained using HRP-labelled antibodies are shown in Table 4.7 and Table 4.8. The intra-assay mean values for all standards were within 90 to 110% of the true values, with the exception of the 0.01µg/ml standard for the immunoassay using the Sandell-Kolthoff reaction for detection of antibodies which had a mean value 115% of the true value. The inter-assay mean values were within 83 to 115% of the true values; each system had standards that fell outside the acceptable range of 90-110%. Each of the systems had intra- and inter-assay %CV values greater than 10% for some standards.

The IgG concentration in five human serum samples was measured in each of the assay systems; the results are shown in Table 4.9. The concentration of IgG in serum from healthy individuals varies over a wide range, falling between 7 and 14 mg/ml. The results obtained for the samples using the cerium(IV) - antimony(III) microassay differed from the results using the Sandell-Kolthoff microassay by 19% (range 3 to 51%), and from the results using HRP-labelled antibodies by 15% (range 1 to 42%). The results for the Sandell-Kolthoff microassay differed from the results obtained using HRP-labelled antibodies by 24% (range 1 to 50%). The standard error obtained using IBHR-labelled antibodies was lower than that obtained using HRP-labelled antibodies, an observation that was also reported by O'Kennedy and Keating (1993).

The possibility of using IBHR-labelled antibodies in an immunoassay, and the favourable comparison of this to an ELISA, was previously reported by O'Kennedy and Keating (1991 and 1993). The results reported here indicate that the Sandell-Kolthoff reaction can be successfully replaced by the cerium(IV) - antimony(III) reaction for detection of the IBHR label. The use of IBHR-labelled antibodies to measure human IgG concentration shows good agreement with the use of HRP-labelled antibodies, with respect to accuracy and precision of the standards, and with respect to the results

obtained for serum samples. However, the use of HRP-labelled antibodies does offer the advantage that lower concentrations of human IgG can be measured. The detection limit of immunoassays is determined by several factors (Tijssen, 1985). The adsorption of ligand to the solid phase, the stability of this interaction, the ratio of the surface of the solid phase to the volume of the sample, the avidity of the antibody - antigen reaction, the quality of the conjugate and the detection system for the label are all factors which determine the detection limit. The systems compared here are identical with respect to the first three factors, but differ with respect to the last three factors. For example, the colorimetric reaction used for detection of the HRP label gives an increase in absorbance against a low blank value, while the microassays used for detection of the IBHR label give a decrease in absorbance against a high blank value. The sensitivity of the cerium(IV) - antimony(III) detection system is limited by the fact that only a percentage of the iodine in IBHR is liberated to catalyse the reaction (Section 3.2.3). The use of fluorimetric monitoring, instead of spectrophotometric monitoring, did not improve the sensitivity (Section 3.2.6). The same batch of anti-human IgG antibodies were used in all systems, but the antibodies were labelled with either IBHR or HRP producing conjugates with very different characteristics. This is illustrated, for example, by the optimum dilution of the conjugates since a higher dilution of the HRP-labelled antibodies could be used compared with the IBHR-labelled antibodies. The HRP conjugation procedure was carried out according to the periodate method, which was investigated and optimised by Tijssen and Kurstack (1984). The conjugates produced by this optimised method gave higher titre in an ELISA compared with conjugates produced using a previous periodate method (Nakane and Kawaoi, 1974). Iodination procedures using radioactive iodine have been widely used, and are thus well investigated and optimised. However, iodination using non-radioactive iodine has not been widely used. Therefore, the iodination of antibodies with various levels of non-radioactive IBHR was investigated.

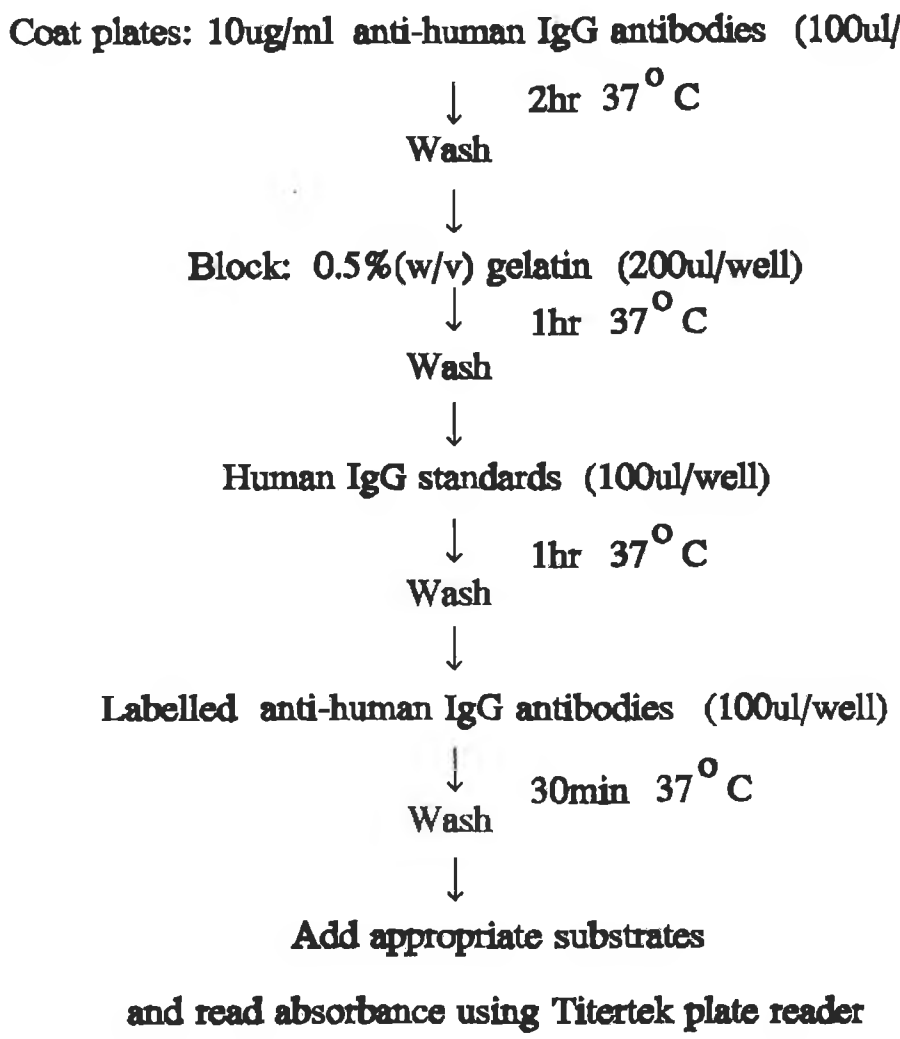

Figure 4.3 Flow diagram of the two-site immunoassay using IBHR- or HRP-labelled antibodies. (A detailed description of the protocol is given in Section 2.6.1)

Human IgG conc. (µg/ml)	Dilution of conjugated antibody			
	1/10	1/20	1/40	1/80
0	1.343	1.345	1.366	1.364
0.05	1.296	1.285	1.298	1.316
0.1	1.257	1.266	1.248	1.298
0.25	1.118	1.186	1.210	1.254
0.5	1.089	1.143	1.175	1.221
1	0.960	1.067	1.112	1.224

Table 4.1 Determination of the optimum dilution of IBHR-labelled antibodies to use in the two-site immunoassay. The results shown are absorbance values at 380nm.

Human IgG conc. (µg/ml)	Dilution of conjugated antibody			
	1/80	1/160	1/320	1/640
0	0.199	0.174	0.139	0.126
0.05	0.720	0.642	0.523	0.358
0.1	0.837	0.760	0.596	0.438
0.25	1.020	0.906	0.743	0.542
0.5	1.096	1.012	0.819	0.610
1	1.153	1.042	0.907	0.698

Table 4.2 Determination of the optimum dilution of HRP-labelled antibodies to use in the two-site immunoassay. The results shown are absorbance values at 405nm.

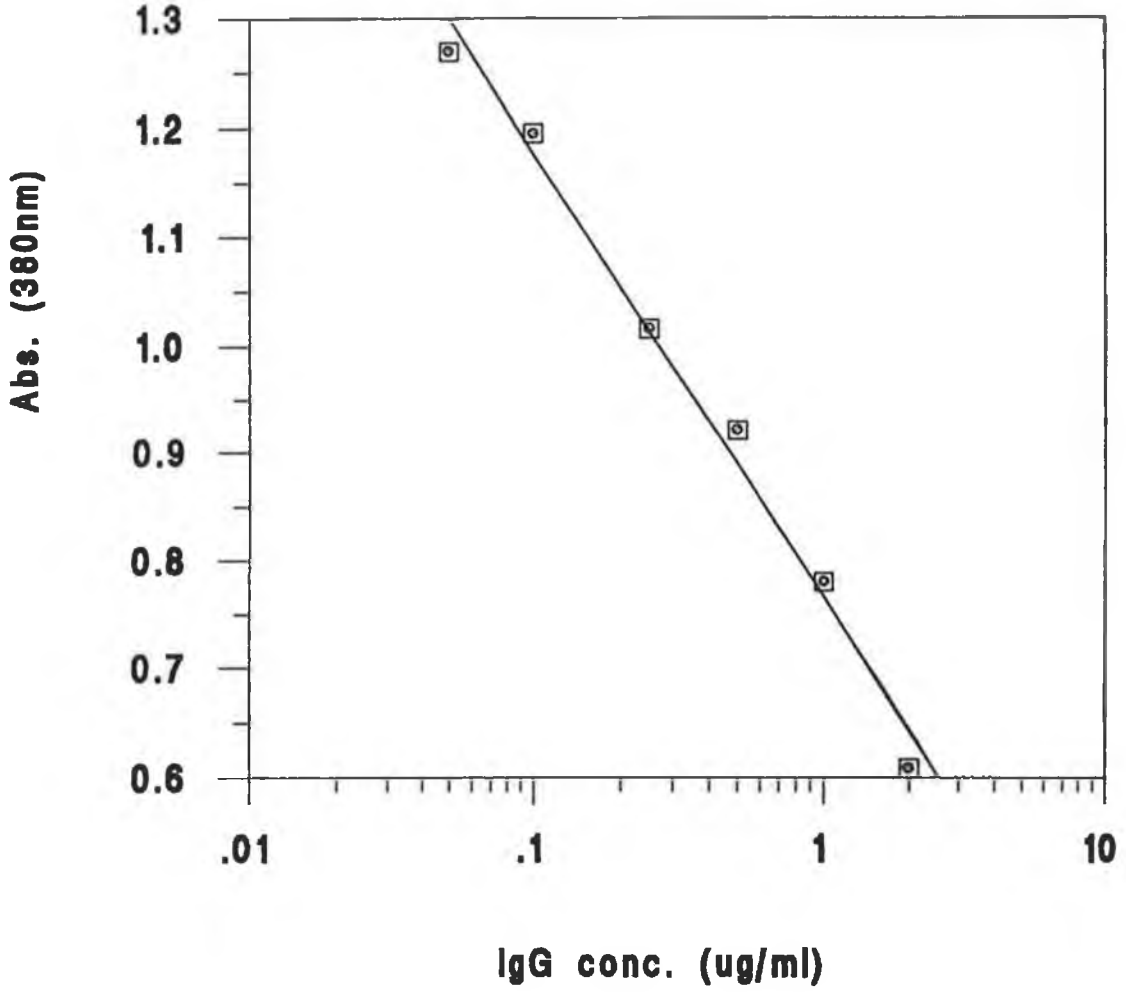

Figure 4.4 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in a two-site immunoassay, using IBHR-labelled antibodies detected by the cerium(IV) - antimony(III) reaction.
The absorbance value for the zero standard was 1.294.

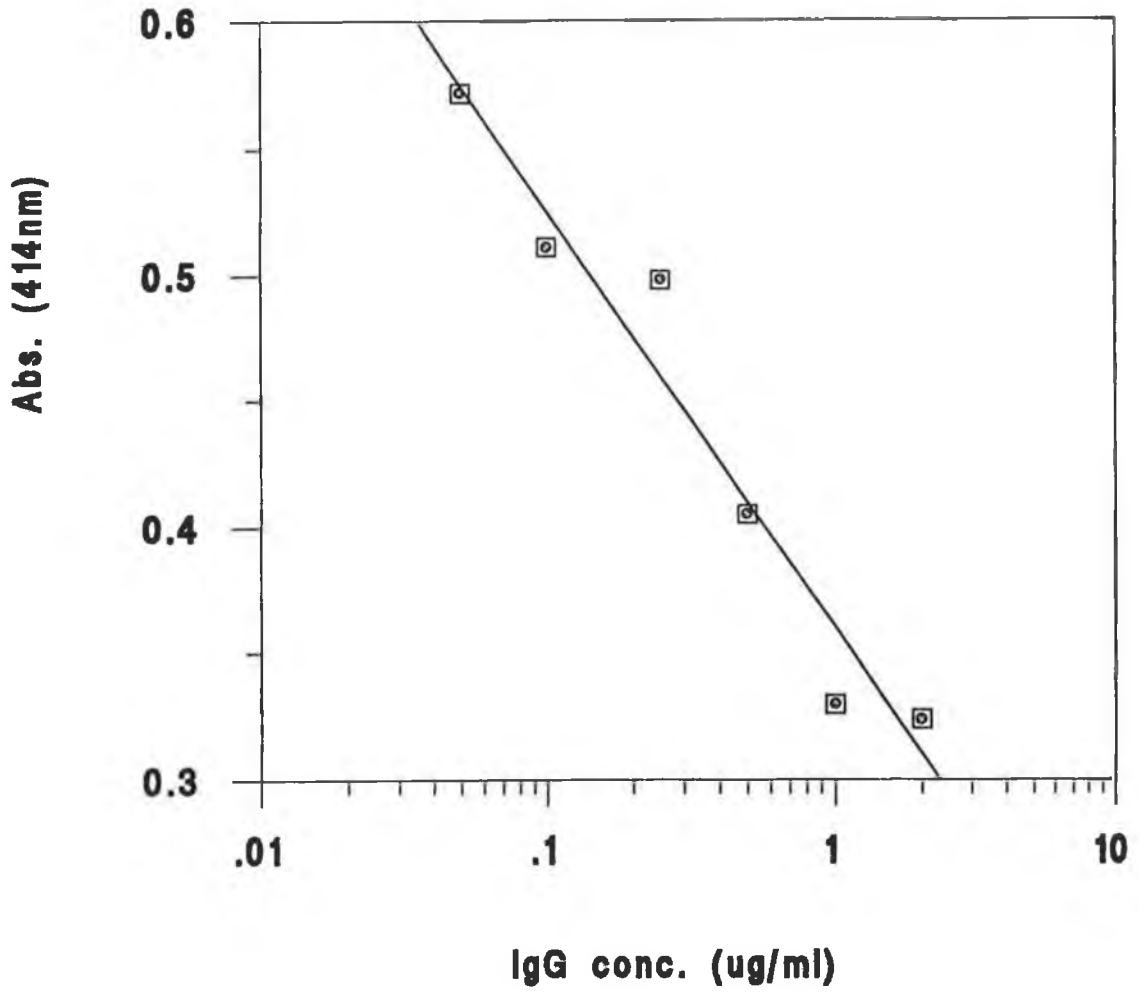

Figure 4.5 Graph of absorbance at 414nm plotted against concentration of human IgG standards assayed in a two-site immunoassay, using IBHR-labelled antibodies detected by the Sandell-Kolthoff reaction.

The absorbance value for the zero standard was 0.620.

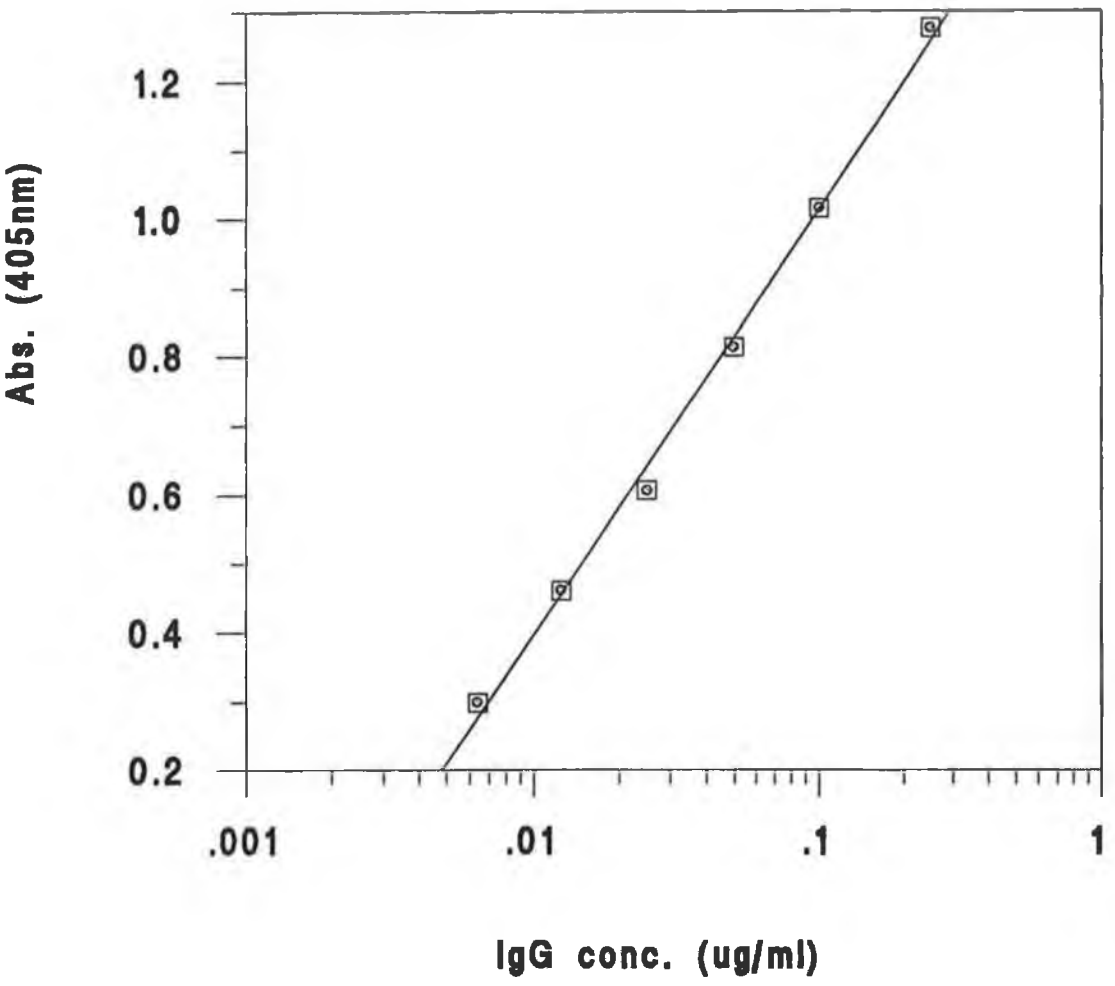

Figure 4.6 Graph of absorbance at 405nm plotted against concentration of human IgG standards assayed in a two-site immunoassay, using HRP-labelled antibodies.
The absorbance value for the zero standard was 0.114.

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.05 ± 0.01	9.4
0.1	0.10 ± 0.02	17.9
0.25	0.23 ± 0.04	16.9
0.5	0.53 ± 0.07	12.5
1	1.06 ± 0.12	11.2
2	1.99 ± 0.13	6.4

Table 4.3 Intra-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using IBHR-labelled antibodies detected by the cerium(IV) - antimony(III) microassay (n=8).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.06 ± 0.004	7.2
0.1	0.09 ± 0.01	9.8
0.25	0.23 ± 0.03	12.5
0.5	0.50 ± 0.05	9.5
1	1.06 ± 0.10	9.5
2	2.29 ± 0.34	15.0

Table 4.4 Inter-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using IBHR-labelled antibodies detected by the cerium(IV) - antimony(III) microassay (n=6).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.05 ± 0.01	14.6
0.1	0.12 ± 0.02	17.3
0.25	0.22 ± 0.04	17.6
0.5	0.47 ± 0.09	20.0
1	1.08 ± 0.15	14.4
2	1.94 ± 0.27	14.0

Table 4.5 Intra-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using IBHR-labelled antibodies detected by the Sandell-Kolthoff microassay (n=8).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.05 ± 0.004	6.9
0.1	0.11 ± 0.002	1.8
0.25	0.25 ± 0.05	19.4
0.5	0.42 ± 0.06	14.3
1	1.08 ± 0.18	16.6
2	1.84 ± 0.27	14.6

Table 4.6 Inter-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using IBHR-labelled antibodies detected by the Sandell-Kolthoff microassay (n=3).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.0064	0.007 ± 0.001	9.2
0.0125	0.012 ± 0.001	9.1
0.025	0.023 ± 0.003	11.3
0.05	0.050 ± 0.005	9.6
0.1	0.110 ± 0.013	11.4
0.25	0.238 ± 0.031	12.9

Table 4.7 Intra-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using HRP-labelled antibodies (n=8).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.0064	0.006 ± 0.001	11.4
0.0125	0.011 ± 0.001	9.6
0.025	0.026 ± 0.005	17.7
0.05	0.057 ± 0.008	13.5
0.1	0.113 ± 0.008	7.4
0.25	0.233 ± 0.022	9.6

Table 4.8 Inter-assay accuracy and precision results for human IgG standards assayed in a two-site immunoassay using HRP-labelled antibodies (n=5).

Sample	A (n=5) Mean ± SD	B (n=3) Mean ± SD	C (n=5) Mean ± SD
1	5.5 ± 2.0	4.5 ± 0.6	4.9 ± 0.7
2	8.3 ± 1.9	7.7 ± 2.3	7.6 ± 2.4
3	13.9 ± 4.7	13.5 ± 2.9	9.8 ± 2.8
4	15.4 ± 0.8	23.3 ± 0.6	15.5 ± 4.3
5	24.0 ± 2.3	26.6 ± 0.9	21.9 ± 10.2
SE	2.3	1.5	4.1

SE is standard error.

Table 4.9 IgG concentration (mg/ml) in human serum samples assayed in a two-site immunoassay using:

(A) IBHR-labelled antibodies detected by the cerium(IV) - antimony(III) microassay

(B) IBHR-labelled antibodies detected by the Sandell-Kolthoff microassay

(C) HRP-labelled antibodies.

4.2.2 Iodination of antibodies with various levels of IBHR.

Anti-human IgG antibodies (1mg) were reacted with 0.1, 0.25, 0.5, 1, 1.5 and 2mg of IBHR. The molar ratio of IBHR reacted to free amino groups was calculated using an estimate of 90 free amino groups per goat IgG (Kendall *et al.*, 1983). This is shown in Table 4.10. In Table 4.10 the moles of IBHR conjugated to antibody for each conjugate are also shown.

4.2.2.1 Assessment of the relative binding pattern using the BIAcore.

The relative binding pattern of the conjugates was assessed using the BIAcore, i.e. how do antibodies labelled with increasing amounts of IBHR bind relative to each other and relative to native antibodies. Human IgG was immobilised onto a sensor chip. An example of a sensorgram obtained for the immobilisation of human IgG onto a sensor chip is shown in Fig 4.7. Native anti-human IgG antibodies were passed over the surface of the chip, and the response obtained was taken to represent maximum binding (i.e. 100%). An example of a sensorgram obtained for the interaction between the immobilised human IgG and native anti-human IgG antibodies is shown in Fig 4.8. The IBHR-labelled anti-human IgG antibodies were passed over the surface of the chip; the response obtained was expressed as a percentage of the maximum binding. A control, which consisted of antibodies that were taken through the iodination reaction in the absence of IBHR, was included. The BIAcore results are shown in Table 4.11. The binding of IBHR-labelled antibodies to immobilised antigen was decreased relative to the binding of native antibodies; the decrease in binding was directly related to the amount of IBHR conjugated to the antibodies. For example, antibodies labelled with 21 moles of IBHR per mole of antibody gave only 14% of the maximum binding obtained for native antibodies, while antibodies labelled with 6 moles of IBHR per mole of antibody gave 68% of the maximum binding.

This decrease in binding upon iodination may be due to several factors. The mere manipulation of antibodies in dilute solutions during the iodination reaction and purification can cause denaturation (Parker, 1976), as can exposure to chemicals used in the iodination reaction. This is illustrated by the control antibodies which were taken through the iodination process in the absence of IBHR, and gave only 84% of the maximum binding. The introduction of IBHR into antibodies can cause conformational and charge changes. Use of the chloramine T method, with introduction of an iodine atom into tyrosine residues, causes conformational changes as iodine is a large atom, similar in size to a benzene ring, and causes a reduction of

the pK of the tyrosine residues (Bolton and Hunter, 1973). IBHR is larger than iodine, and conjugation of IBHR to a protein is by substitution of an amino group with an iodophenol group. Introduction of IBHR molecules may, therefore, cause greater alteration in conformation and charge of a protein than observed with introduction of iodine atoms (Bolton and Hunter, 1973). The number of IBHR molecules conjugated to the antibodies is also a factor. Heavy iodination may be detrimental. Introduction of iodine into a protein depends on the distribution, reactivity and accessibility of the target amino acids to iodination. As the level of iodination is increased, the likelihood of introduction of iodine into a critical amino acid is increased, and this can lead to losses in reactivity (Parker, 1976). Iodination with chloramine T to produce heavily iodinated proteins is reported to decrease the specific binding activity, and increase the tendency of the protein to aggregate and bind non-specifically, although these effects may be due to the direct reaction of chloramine T with the protein, in addition to the introduction of iodine atoms (Parker, 1976). It is difficult to compare the levels of incorporation of IBHR into antibodies reported in these experiments, with the levels of incorporation of radioactive iodine reported in the literature. With radioiodination, the incorporation of iodine is usually kept to one atom of ^{125}I per IgG, as incorporation of levels higher than this may lead to "decay catastrophe" (Hales and Woodhead, 1980), where reduction in protein reactivity occurs during iodination and storage due to damage caused by exposure to high levels of radioactivity.

Mg of IBHR#	Molar ratio IBHR:amino group*	moles of IBHR conjugated per mole of Ab
Control	-	-
0.1	0.5 : 1	6
0.25	1.1 : 1	10
0.5	2.3 : 1	18
1	4.6 : 1	21
1.5	6.9 : 1	26
2	9.1 : 1	30

Milligrammes of IBHR reacted with 1mg of goat anti-human IgG antibodies.

* The molar ratio of IBHR reacted to free amino groups, using an estimate of 90 free amino groups per goat IgG molecule (Kendall et al., 1983).

Table 4.10 Conjugates prepared by reaction of anti-human IgG antibodies with increasing amounts of IBHR.

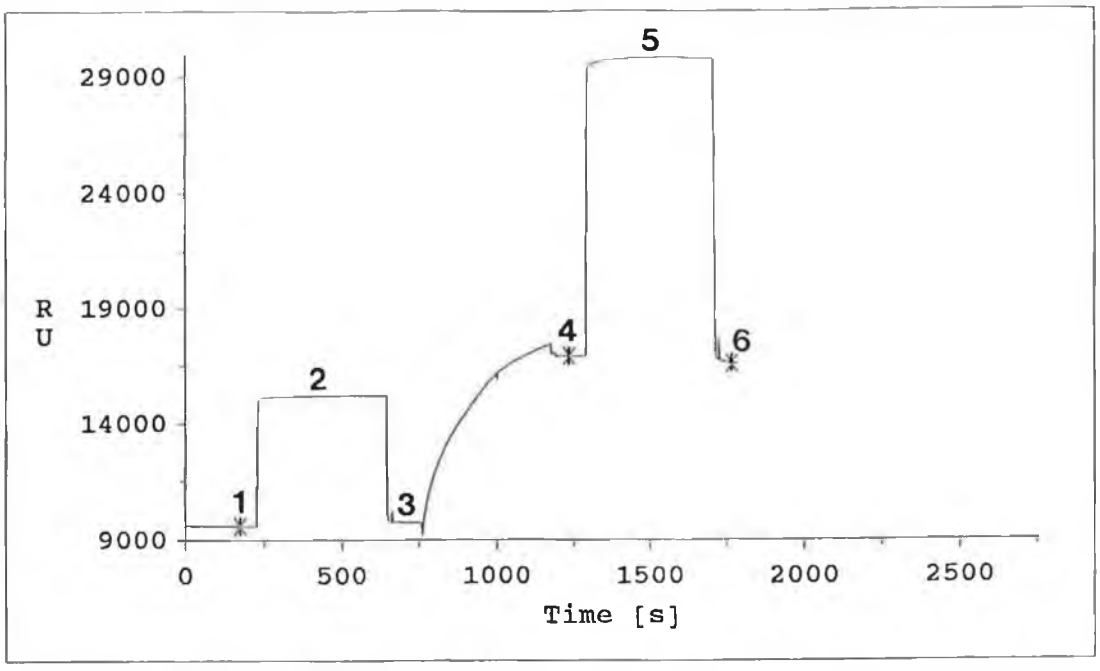

Figure 4.7 An example of a sensorgram showing the immobilisation of human IgG onto the activated surface of a sensor chip.

(1) Baseline for the unmodified sensor chip surface

(2) Activation of the chip surface causes an increase in the SPR signal due to changes in the refractive index

(3) Baseline after activation

(4) Immobilised human IgG before deactivation

(5) Deactivation of unreacted NHS-esters

(6) Immobilised human IgG after deactivation

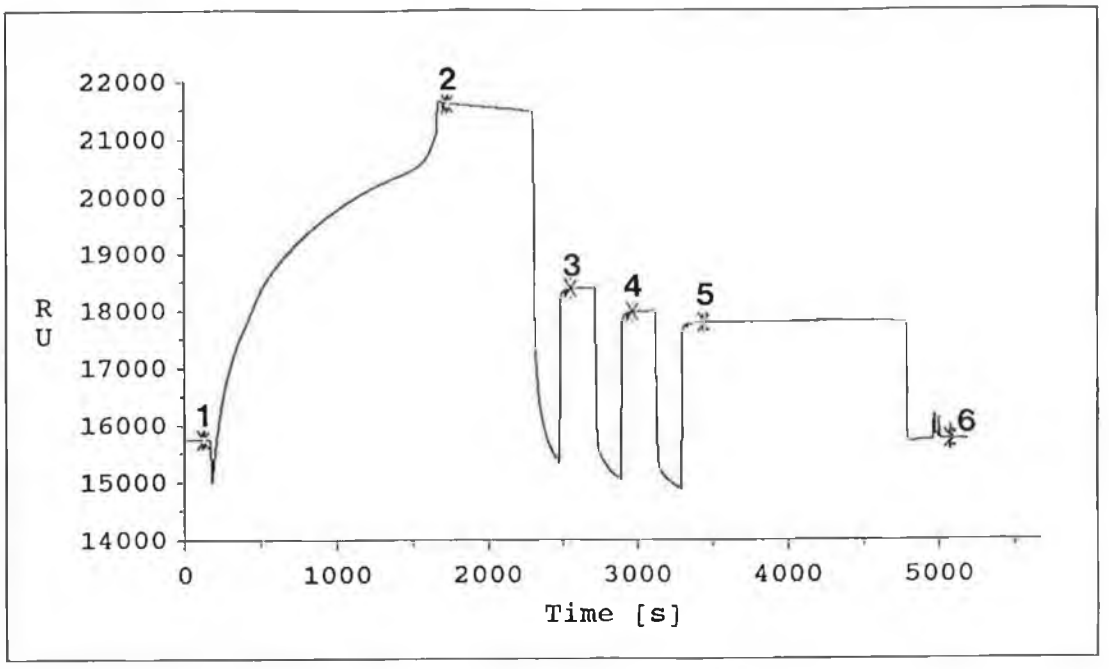

Figure 4.8 An example of a sensorgram showing the interaction between human IgG immobilised on the surface of a sensor chip, and native anti-human IgG antibodies.
(1) Baseline for the human IgG matrix on the chip surface
(2) Anti-human IgG antibodies bound to the human IgG matrix
(3,4,5) Regeneration of the human IgG matrix with pulses of 10mM HCl
(6) Regenerated human IgG matrix

Molar ratio IBHR:amino group*	% of maximum binding
Control	84
0.5 : 1	68
1.1 : 1	44
2.3 : 1	22
4.6 : 1	14
6.9 : 1	10

*The molar ratio of IBHR reacted to free amino groups, using an estimate of 90 free amino groups per goat IgG molecule (Kendall et al., 1983).

Table 4.11 BIAcore results (n=2) for the binding of anti-human IgG antibodies, labelled with increasing amounts of IBHR, to human IgG. The results are expressed as a percentage of the maximum binding, where the binding of native anti-human IgG antibodies is taken as 100%. The control is anti-human IgG antibodies that have been through the iodination process in the absence of IBHR.

4.2.2.2 Assessment of immunoreactivity in an immunoassay.

The immunoreactivity of the conjugates was assessed in the two-site immunoassay for human IgG (Section 4.2.1). Human IgG standards were measured using each of the conjugates; the graphs of absorbance plotted against IgG concentration, on a log scale, are shown in Fig 4.9. A decrease in the amount of IBHR conjugated to the antibodies did not lead to any improvement in the immunoassay results.

The results for each conjugate can be compared to the results for the conjugate obtained using a 4.6:1 molar ratio, as this is the conjugation ratio used for the experiments in Section 4.2.1. The graph obtained using this conjugate was linear in the range 0.05 to 2µg/ml human IgG (r=0.994), as were the graphs for the conjugates produced using a 1.1:1 molar ratio (r=0.988) and a 2.3:1 molar ratio (r=0.989). The conjugate produced using a 0.5:1 molar ratio resulted in a loss of sensitivity; standards in the range 0.1 to 2µg/ml human IgG were measured (r=0.965). The slope of the graphs decreased as the level of iodination was decreased. A slope of 0.349 was obtained using the conjugate produced at a 4.6:1 molar ratio, while a slope of 0.116 was obtained using the conjugate produced at a 0.5:1 molar ratio. The BIAcore results indicated that an increase in the binding of antibody to antigen is obtained when the amount of IBHR conjugated to the antibody is decreased. No benefit due to increased binding was observed in these immunoassays using conjugates labelled with decreasing amounts of IBHR. Immunoassay performance is based not just on the antibody - antigen reaction, but on several parameters including the detection reaction. The sensitivity of the cerium(IV) - antimony(III) reaction for IBHR is limited as only a percentage of the iodine in IBHR is liberated to catalyse the reaction (Section 3.2.3), thus for conjugates containing low levels of IBHR the reaction may not be sensitive enough to detect the small amount of label present in the immunoassay.

Increasing the level of iodination did not adversely affect the immunoassay results. Human IgG standards in the range 0.05 to 2µg/ml could be measured using conjugates produced using a 6.9:1 molar ratio (r=0.993) and a 9.1:1 molar ratio (r=0.988). The disadvantage of the use of higher levels of iodination was that the non-specific binding increased. For example, the absorbance for wells containing iodinated antibody, but no antigen, was 1.499 using a 0.5:1 molar ratio, 1.423 using a 4.6:1 molar ratio and 1.319 using a 9.1:1 molar ratio.

It is difficult to compare the immunoreactivity of these conjugates with conjugates prepared using radioactive iodine because, as previously discussed, the level of conjugation with radioactive iodine is considerably lower. Bolton and Hunter (1973)

reported that conjugation levels of up to 3 moles of radioactive IBHR per mole of insulin, 1.5 moles of IBHR per mole of human growth hormone, and 0.8 moles of IBHR per mole of human thyroid stimulating hormone did not adversely affect the immunoreactivity of the tracers; but these levels of conjugation are considerably lower than those used for non-radioactive IBHR. Alfthan (1986) labelled monoclonal anti-human chorionic gonadotropin antibodies with two moles of ^{125}I per mole of IgG. Preparation of labelled antibodies with higher specific activities resulted in a tracer with lower immunoreactivity and lower sensitivity. For comparison purposes, labelling of antibodies with NHS-biotin could be considered. NHS-biotin is similar in size to IBHR, and the same chemistry is used for the labelling reaction for both molecules. Guesdon *et al.* (1979) prepared biotinylated sheep anti-rabbit IgG antibodies by reaction of various molar ratios of NHS-biotin to free amino groups of the antibody. NHS-biotin was dissolved in DMF, and reacted with a 10mg/ml solution of antibody in 0.1M $NaHCO_3$. Conjugates were prepared using NHS-biotin : amino group ratios of 0.1:1, 1:1, 4:1 and 10:1. The binding capacity of these biotinylated antibodies was not altered compared with native antibodies. Kendall *et al.* (1983) prepared biotinylated goat anti-mouse IgG antibodies using the same procedure as Guesdon *et al.* (1979). They assessed the immunoreactivity of the conjugates in an ELISA. Conjugation at NHS-biotin : amino group ratios of 0.25:1 and 0.5:1 resulted in a loss of sensitivity; ratios of 1:1, 2:1, and 4:1 produced usable reagents, with the 1:1 reagent being the most sensitive; a ratio of 8:1 produced an undesirable product.

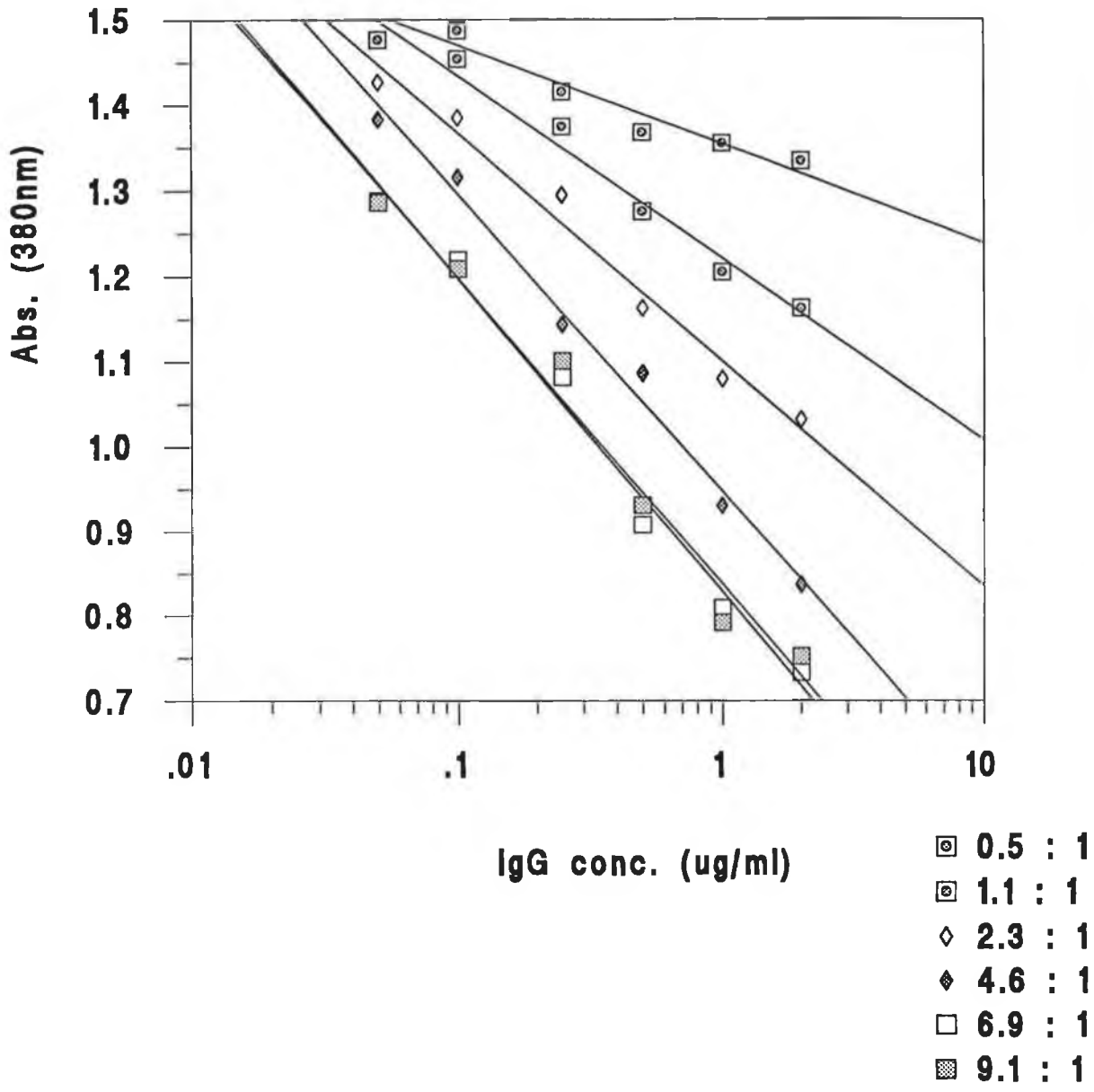

Figure 4.9 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in a two-site immunoassay, using antibodies labelled with various amounts of IBHR.

4.3 Summary.

The feasibility of the use of the cerium(IV) - antimony(III) reaction for the detection of IBHR-labelled antibodies in an immunoassay for human IgG has been illustrated, and the results obtained have been shown to be comparable to those obtained using the Sandell-Kolthoff reaction for detection of the IBHR label, or using HRP-labelled antibodies. The three assay systems show good agreement with respect to accuracy and precision of the standards, and with respect to the results obtained for serum samples measured in each assay. The use of HRP-labelled antibodies does, however, enable the use of a higher working dilution of the conjugated antibody and measurement of lower concentrations of human IgG.

Antibodies were labelled with various levels of IBHR. The relative binding pattern of the labelled antibodies was assessed using the BIAcore. The results indicated that reduction of the amount of IBHR conjugated to the antibodies led to an increase in the binding to antigen, relative to the binding of native antibodies. The immunoreactivity of the labelled antibodies was assessed in an immunoassay. Standard curves of absorbance plotted against human IgG concentration were prepared for each conjugate. Using antibodies labelled with high levels of IBHR (10 to 30 moles of IBHR per mole of antibody) human IgG in the range 0.05 to 2µg/ml could be measured. Using antibodies labelled with low levels of IBHR (6 moles of IBHR per mole of antibody) resulted in a loss of sensitivity, as standards in the range 0.1 to 2µg/ml could be measured. The slope of the standard curves increased as the level of iodination was increased, but so also did the non-specific binding. A molar ratio of IBHR to free amino groups of the antibody of 2.3:1 or 4.6:1 produced conjugates which contained 18 and 21 moles of IBHR per mole of antibody, respectively, and which could be used to measure human IgG in the range 0.05 to 2µg/ml producing standard curves with good slope values and low non-specific binding.

5. USE OF THE AVIDIN-BIOTIN SYSTEM AND OF BISPECIFIC $F(ab')_2$ ANTIBODIES.

5.1 Introduction.

In an immunoassay for human IgG, the linear range using IBHR-labelled antibodies was 0.05 to 2µg/ml, while using HRP-labelled antibodies the linear range was 0.0064 to 0.25µg/ml. The HRP-labelled antibodies could be used to measure lower concentrations of human IgG. The detection limit of immunoassays is determined by several factors, including the quality of the conjugate and the detection system for the label (Tijssen, 1985). The preparation of IBHR-antibody conjugates was previously optimised (Section 4.2.2), and so also was the detection system for the IBHR label (Section 3.2). Therefore, improvement of the detection limit by modification of the design of the immunoassay was investigated. The immunoassay was modified using procedures that lead to a high accumulation of label per antigen molecule (Tijssen, 1985). This chapter describes the use of two such procedures: the avidin-biotin system and bispecific $F(ab')_2$ antibodies.

Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia. Avidin is a tetramer, consisting of four identical subunits, each of which has a binding site for biotin. Biotin, one of the B complex vitamins found in tissue and blood, is a co-enzyme for enzymes involved in carboxylation reactions. Avidin binds non-covalently to biotin with high affinity (10^{15} M^{-1}), and the resulting complex is extremely stable (Tijssen, 1985). Due to the high affinity and stability, the use of avidin-biotin technology has been exploited for many applications, such as affinity chromatography, affinity cytochemistry, immunoassays and gene probes (Wilchek and Bayer, 1988). The avidin-biotin system can be applied in several ways to immunoassays (Avrameas, 1992). In the labelled avidin-biotin (LAB) system, antigen is detected by addition of biotinylated antibody, followed by addition of labelled avidin. In the bridged avidin-biotin (BRAB) system, antigen is detected by addition of biotinylated antibody, followed by addition of native avidin and, finally, addition of labelled biotin. This system is based on the fact that due to steric hindrance all four binding sites on the avidin are not involved in the interaction with the biotinylated antibody. Thus, the remaining sites are free to bind the labelled biotin. The BRAB system can be simplified by formation of soluble avidin-labelled biotin complexes, by preincubation of labelled biotin with avidin. These complexes are subsequently used to analyse biotinylated antibody.

Immunoassays based on the avidin-biotin system are reported to be in the order of 2 to 100 fold more sensitive than conventional immunoassays (Avrameas, 1992). This is due to several factors. The high affinity of the avidin-biotin binding means that the avidin-biotin complex is stable in washing and incubation steps. Biotinylation of antibodies, and of some enzymes, has no effect on the biological activity (Guesdon, 1979). The avidin-biotin system is very versatile. One biotinylated antibody can be used as a second antibody in many different immunoassays, and detected using LAB or BRAB systems. The high isoelectric point of avidin (pI 10.5) can contribute to non-specific binding and high background (Avrameas, 1992). This can be overcome by the use of streptavidin, a biotin binding protein isolated from *Streptomyces avidinii* which has an isoelectric point of 5.

The use of LAB and BRAB immunoassays for the measurement of human IgG was investigated. IBHR-labelled avidin and IBHR-labelled biotin were prepared, and used in the LAB and BRAB immunoassays, respectively.

Bispecific $F(ab)_2$ antibodies were also used in an immunoassay to measure human IgG. Bispecific antibodies are hybrid antibodies that possess two different Fab' fragments, which are each specific for a different antigen. They may be produced by biological or chemical means, and have a variety of potential uses. Bispecific antibodies binding to both a target cell and a therapeutic drug can be used in chemotherapy to localise large quantities of the drug at the target site, while bispecific antibodies binding to, for example, an enzyme and an antigen can be used for enzyme immobilisation in immunocytochemistry and EIA (Nolan and O'Kennedy, 1990).

Biological production involves the use of cell fusion techniques. Two hybridomas, producing monoclonal antibodies to different antigens, can be fused producing a quadroma, or, a hybridoma, producing antibodies to one antigen, can be fused with a spleen cell from an animal immunised with the second antigen, producing a trioma (Nolan and O'Kennedy, 1990). Both of these methods produce cell lines which secrete a mixed population of parental and bispecific antibodies, from which the bispecific antibodies can be purified. Milstein and Cuello (1984) reported the use of cell fusion techniques to produce hybrid hybridomas secreting bispecific antibodies that could be used in immunoassays and immunohistochemistry.

Chemical production of bispecific antibodies involves separation of parental antibodies into fragments, and reconstitution of the fragments to produce the bispecific antibody. Nisonoff and Mandy (1962) prepared bispecific $F(ab')_2$ antibodies by chemical means. Parental antibodies were treated with pepsin to yield $F(ab')_2$ fragments, which were

reduced to Fab' fragments by the addition of mercaptoethylamine hydrochloride. Fab' fragments from the two parental antibodies were mixed in equal quantities, and random reoxidation of the disulphide bonds was achieved in the presence of oxygen. The product contained both homodimers and heterodimers of $F(ab')_2$, although heterodimers of dual specificity made up a large proportion of the product.

Other chemical methods have been reported. Brennan *et al.* (1985) subjected antibodies to digestion with pepsin, followed by reduction with 2-mercaptoethylamine, in the presence of the dithiol complexing agent sodium arsenite, to yield Fab' fragments. Free thiols on the Fab' fragments were activated using 5,5'-di-thiobis(2-nitrobenzoic acid) to yield Fab'- thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives was reconverted to Fab'-thiol by reduction with 2-mercaptoethylamine, and mixed with the other Fab'-TNB derivative to form the bispecific $F(ab')_2$ antibody. To determine whether the chemical manipulations caused alterations in the antigen binding site, anti-β-galactosidase $F(ab')_2$ was prepared, and was carried through the procedure. The original and reconstituted $F(ab')_2$ were tested by ELISA, and no significant difference in the performance was observed.

Glennie *et al.* (1987) also produced $F(ab')_2$ fragments by digestion with pepsin. These were reduced with 2-mercaptoethanol (2-ME) to produce Fab' fragments (Fab'_{SH}). One of the Fab'_{SH} fragments was reacted with *o*-phenylenedimaleimide, producing maleimidated Fab' (Fab'_{MAL}). The two Fab' species were mixed at a molar ratio of Fab'_{MAL} : Fab'_{SH} of 1:1.3, using low pH and the presence of EDTA, which favours thiol and maleimide reaction and minimises the reoxidation of thiol groups. The product was reduced with 2-ME and alkylated with iodoacetamide to remove unwanted products, such as $F(ab')_2$ homodimers. The bispecific $F(ab')_2$ antibodies formed were linked by thioether bonds; the proposed structure is shown in Fig 5.1. The bispecific $F(ab')_2$ antibodies were tested in three systems to confirm that the antigen binding sites were not altered by the chemical manipulations: they were used to stain cells with the fluorescent compound, phycoerythrin; to deliver the plant ribosome inactivating protein, saporin, to cells; and to target cells for lysis by cellular effectors.

The work reported in this chapter describes the preparation of bispecific $F(ab')_2$ antibodies from goat anti-human IgG antibodies and rabbit anti-BSA antibodies using the method of Glennie *et al.* (1987), and their use as bridging agents between human IgG and iodinated BSA in an immunoassay for human IgG.

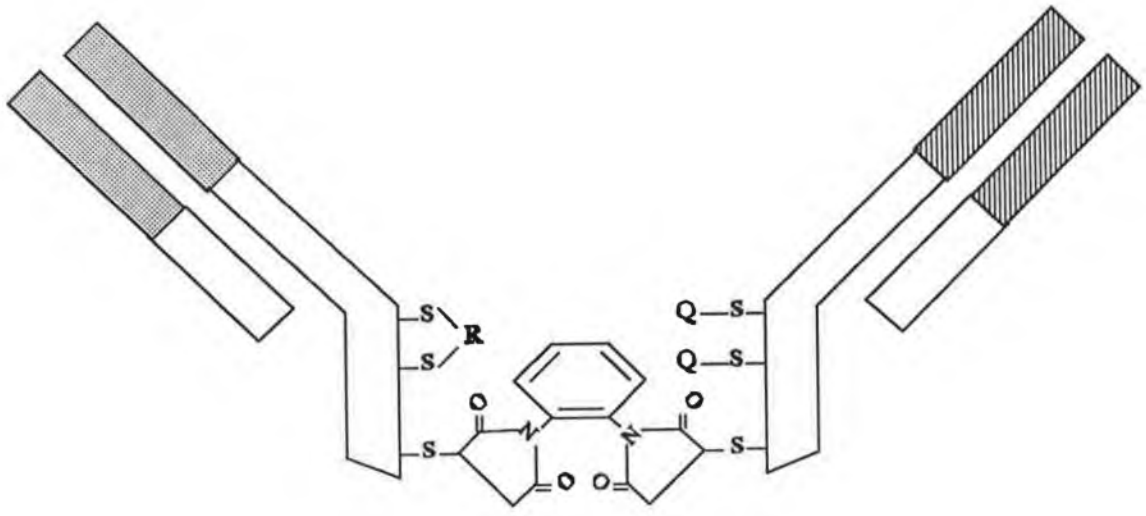

Figure 5.1 The proposed structure of the bispecific F(ab')$_2$ antibody produced by the method of Glennie *et al.* (1987). The structure shown is for a bispecific F(ab')$_2$ antibody produced by recombination of Fab' fragments from mouse antibodies. The cysteinyl sulphurs of one fragment are joined to carboxyamidomethyl (Q), while the cysteinyl sulphurs of the other fragment are joined to o-phenylenedisuccinimide (R) which also links the two Fc' fragments.

5.2 Results and discussion.

5.2.1 The avidin - biotin system.

5.2.1.1 Preparation of IBHR-labelled avidin.

IBHR-labelled avidin was prepared using reaction ratios of 0.15, 0.25, 0.35, 0.45 and 1mg of IBHR to 1mg of avidin. The conjugates were purified and characterised using the same procedure as for IBHR-labelled antibodies (Section 4.2.1). The use of 1mg of IBHR produced an unsuitable conjugate, as the conjugate precipitated on storage overnight at 4°C. The amount of IBHR conjugated at each reaction ratio is shown in Table 5.1.

The conjugates were tested in a LAB immunoassay for human IgG to determine the reaction ratio which would yield a conjugate with suitable activity. The protocol for the immunoassay is outlined in a flow diagram in Fig 5.2. BSA was used as the blocking protein for this immunoassay, as non-specific binding of avidin to gelatin was observed. On addition of IBHR-labelled avidin to wells precoated with 0.5%(w/v) gelatin or 1.5%(w/v) BSA, washing, followed by addition of the reagents for the cerium(IV) - antimony(III) reaction, average absorbance values of 0.258 and 1.506 were obtained for the wells precoated with gelatin and BSA, respectively. An absorbance value of 1.496 was obtained for control wells.

Biotinylated anti-human IgG antibodies were prepared by reaction of N-hydroxy-succinimidobiotin (NHS-biotin) with antibodies at a 2:1 molar ratio, using an estimate of 90 free amino groups per goat IgG (Kendall *et al.*, 1983). The free and conjugated NHS-biotin were separated by dialysis.

The standard curves of absorbance plotted against human IgG concentration, on a log scale, obtained for the conjugates are shown in Fig 5.3. Each of the conjugates could be used to measure human IgG in the range 0.05 to 0.5µg/ml. The slope of the graph increased as the level of iodination was decreased, from a value of 0.238 using avidin labelled with 21 moles of IBHR, to a value of 0.470 using avidin labelled with 12 moles of IBHR. Since a low amount of IBHR conjugated to avidin led to improved performance in the immunoassay, a reaction ratio of 0.15mg IBHR reacted with 1mg avidin was chosen for use in further work.

The binding of IBHR-labelled avidin to biotinylated antibodies was compared to the binding of native avidin, using the BIAcore. Biotinylated antibodies were immobilised onto the surface of a sensor chip, and native avidin was passed over the surface. The response obtained was taken to represent maximum binding (i.e. 100%). IBHR-labelled avidin was passed over the surface; the response was 54% of the maximum binding.

The reduction in binding activity of labelled avidin may be due to the conjugation of IBHR to lysine residues. Gitlin *et al.* (1987) reported that the lysine residues of avidin are involved in the biotin binding site. They modified lysine residues with 1-fluoro-2,4-dinitrobenzene, and the biotin binding activity decreased with increasing modification. Modification of just one lysine residue per subunit was sufficient to destroy biotin binding. Nevertheless, iodination of avidin does not preclude its use in assay systems. Cuatrecasas and Hollenberg (1976) prepared radiolabelled avidin using IBHR, and reported that the biotin binding activity of the labelled avidin was not impaired compared with native avidin. Kulomaa *et al.* (1979) used avidin iodinated by the chloramine T method as a tracer in a radioimmunoassay for chicken avidin and Mock and DuBois (1986) used IBHR-labelled avidin to measure biotin concentration in plasma and urine samples.

mg IBHR *	moles of IBHR conjugated per mole of avidin
0.15	12 (n=3)
0.25	14 (n=3)
0.35	18 (n=1)
0.45	21 (n=1)

* Milligrammes of IBHR reacted with 1mg of avidin.

Table 5.1 Conjugates prepared by reaction of avidin with increasing amounts of IBHR.

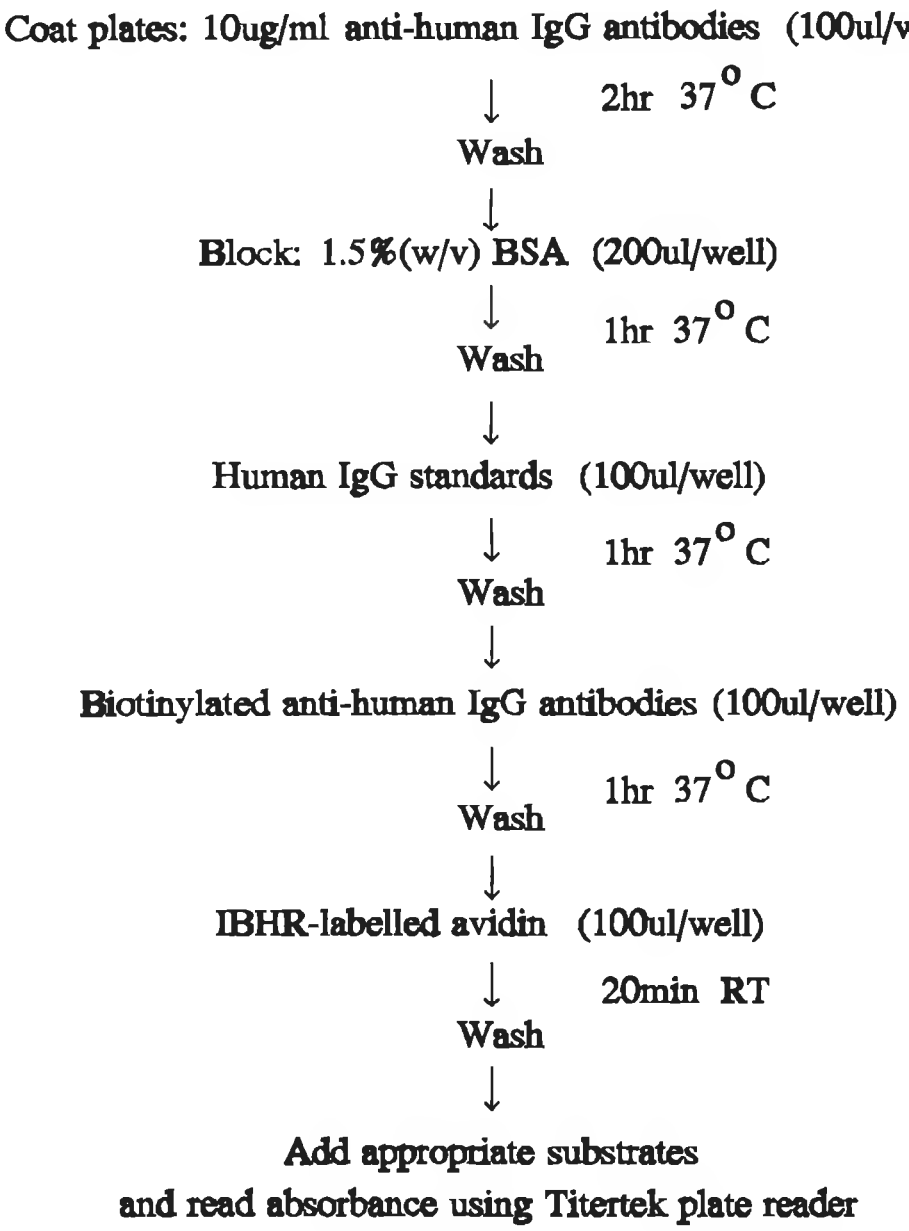

Figure 5.2 Flow diagram of the immunoassay using IBHR-labelled avidin. (A detailed protocol is given in Section 2.7.3).

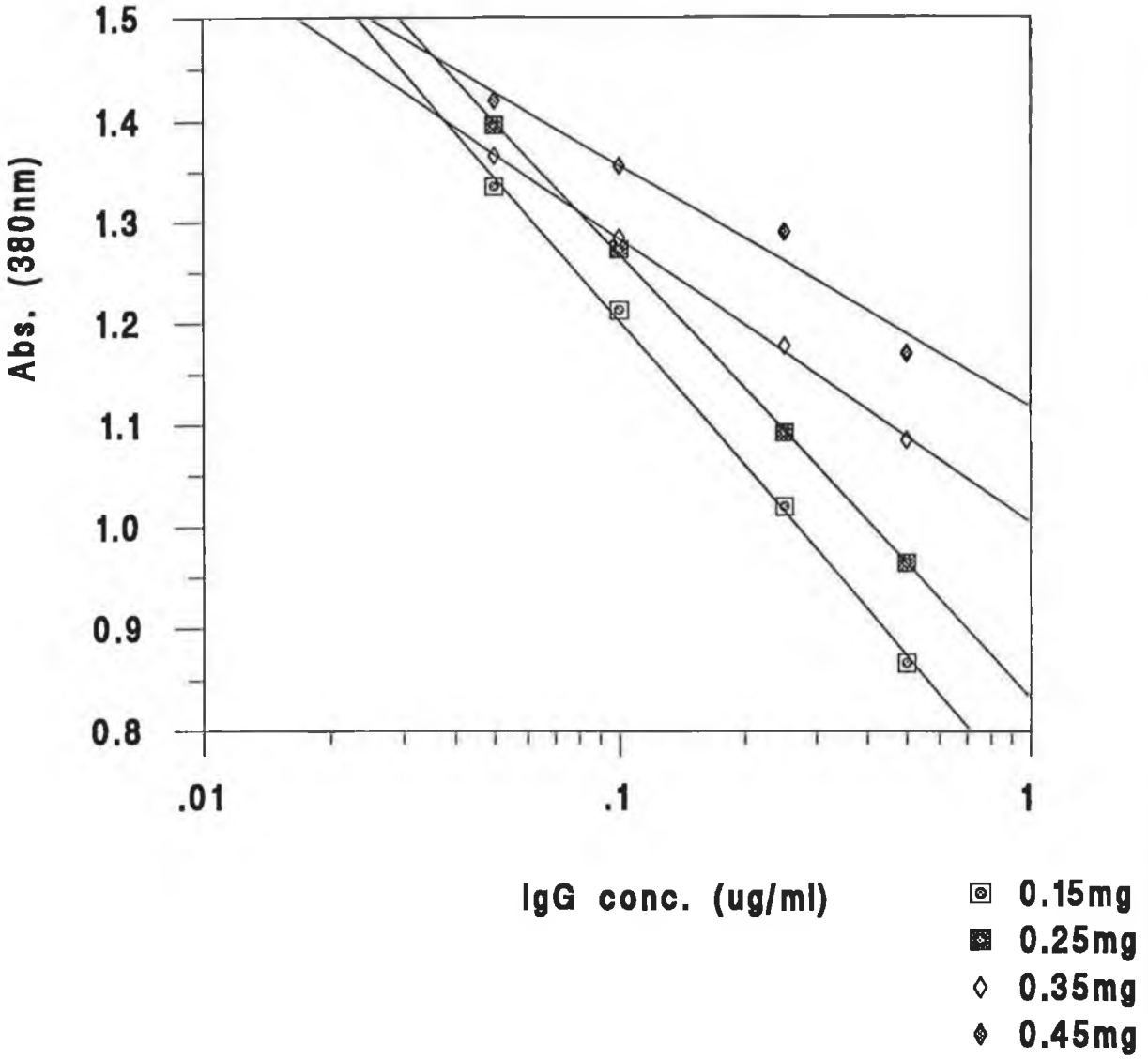

Figure 5.3 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in an immunoassay using avidin labelled with various amounts of IBHR.

5.2.1.2 Labelled avidin-biotin (LAB) immunoassay for human IgG.

Human IgG levels in standards and human serum samples were measured in a LAB immunoassay using biotinylated anti-human IgG antibodies and IBHR-labelled avidin. The same batch of anti-human IgG antibodies was used for these experiments as for the immunoassays using labelled antibodies (Section 4.2.1). The optimum dilution of biotinylated anti-human IgG antibodies and IBHR-labelled avidin was determined in the immunoassay. Biotinylated antibodies were diluted in the range 1/50 to 1/400. The results are shown in Table 5.2. A dilution of 1/200 was chosen as optimum, as this gave a large change in absorbance values over the range of IgG standards tested. IBHR-labelled avidin was diluted in the range 1/8 to 1/64. The results are shown in Table 5.3. A dilution of 1/16 was chosen as optimum.

The standard curve of absorbance plotted against human IgG concentration, on a log scale, is shown in Fig 5.4. The linear range of the immunoassay was 0.025 to 0.5µg/ml human IgG (r=0.993). The intra- and inter-assay accuracy and precision were calculated for the standards. The results are shown in Table 5.4 and Table 5.5. The intra-assay mean values were within 91 to 112% of the true values. The inter-assay mean values were within 84 to 118% of the true values. The %CV values for all standards were less than 10%, with the exception of the intra-assay %CV for the 0.1µg/ml standard, and the inter-assay %CV for the 0.025µg/ml and 0.05µg/ml standards.

The results for the LAB immunoassay can be compared with the results obtained for the immunoassays using labelled antibodies (Section 4.2.1). Human IgG standards in the range 0.05 to 2µg/ml could be measured using IBHR-labelled antibodies. Thus, the assay was improved by the use of IBHR-labelled avidin as lower concentrations of human IgG could be measured. However, standards in the range 0.0064 to 0.25µg/ml could be measured using HRP-labelled antibodies, and the difference in absorbance values between the zero standard and the highest standard was 1.163, which is greater than the difference in absorbance values using either IBHR-labelled antibodies (0.666) or IBHR-labelled avidin (0.597). The intra- and inter-assay accuracy results were better using labelled antibodies than with labelled avidin.

The IgG concentration in five human serum samples was measured in the LAB immunoassay, and in immunoassays using IBHR-labelled antibodies and HRP-labelled antibodies. The results are shown in Table 5.6. There is good agreement between the results obtained in the different immunoassay systems. The results for the samples using IBHR-labelled avidin differed from the results using IBHR-labelled antibodies

by 12% (range 2 to 22%), and from the results using HRP-labelled antibodies by 8% (range 1 to 29%). The results obtained using IBHR-labelled antibodies differed from the results using HRP-labelled antibodies by 18% (range 6 to 27%). The standard error obtained using an HRP label was lower than that obtained using an IBHR label, in contrast to the results reported previously for labelled antibodies (Section 4.2.1). Different preparations of HRP and IBHR conjugates were used for the previous experiments and these experiments, which may account for the difference in the standard error.

Human IgG conc. (µg/ml)	Dilution of antibody			
	1/50	1/100	1/200	1/400
0	1.448	1.495	1.505	1.492
0.05	1.286	1.353	1.336	1.335
0.1	1.194	1.278	1.257	1.248
0.25	1.093	1.186	1.146	1.214
0.5	1.039	1.104	1.110	1.129

Table 5.2 Determination of the optimum dilution of biotinylated antibody. The results shown are absorbance values at 380nm.

Human IgG conc. (µg/ml)	Dilution of avidin			
	1/8	1/16	1/32	1/64
0	1.490	1.517	1.501	1.509
0.05	1.359	1.372	1.351	1.362
0.1	1.268	1.243	1.248	1.283
0.25	1.136	1.128	1.186	1.196
0.5	1.006	0.985	1.043	1.099

Table 5.3 Determination of the optimum dilution of IBHR-labelled avidin. The results shown are absorbance values at 380nm.

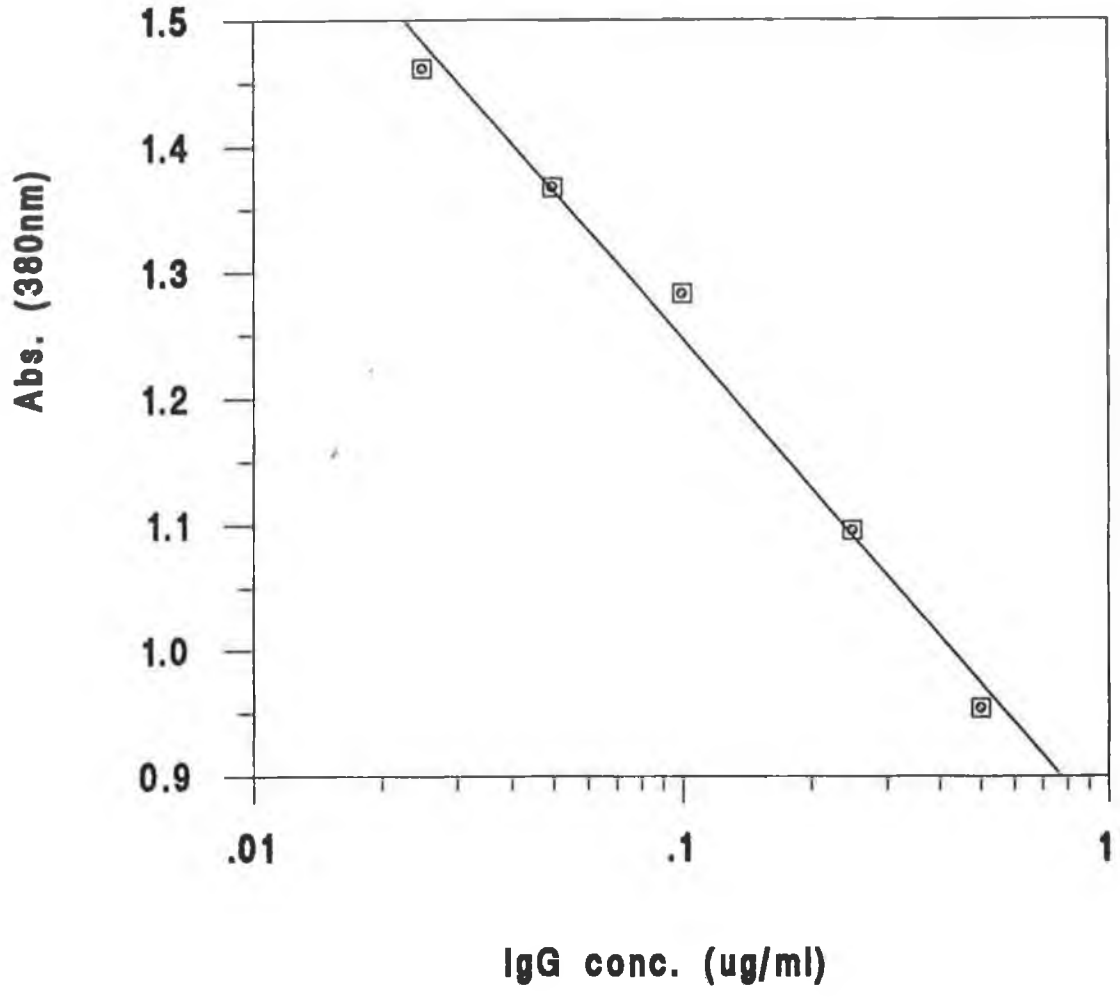

Figure 5.4 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in an immunoassay using IBHR-labelled avidin.
The absorbance value for the zero standard was 1.551.

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.025	0.028 ± 0.003	9.1
0.05	0.046 ± 0.004	9.5
0.1	0.091 ± 0.013	14.3
0.25	0.240 ± 0.009	3.9
0.5	0.533 ± 0.035	6.6

Table 5.4 Intra-assay accuracy and precision results for human IgG standards assayed in an immunoassay using IBHR-labelled avidin (n=6).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.025	0.029 ± 0.003	10.6
0.05	0.044 ± 0.005	12.0
0.1	0.080 ± 0.014	6.1
0.25	0.245 ± 0.013	8.1
0.5	0.591 ± 0.030	4.9

Table 5.5 Inter-assay accuracy and precision results for human IgG standards assayed in an immunoassay using IBHR-labelled avidin (n=7).

Sample	A (n=6) Mean ± SD	B (n=5) Mean ± SD	C (n=5) Mean ± SD
6	11.5 ± 2.5	9.4 ± 1.8	11.3 ± 1.1
7	13.3 ± 1.9	12.6 ± 3.3	13.4 ± 0.7
8	13.7 ± 2.3	13.5 ± 2.3	10.6 ± 1.4
9	15.1 ± 4.8	13.1 ± 2.8	15.7 ± 1.7
10	16.3 ± 1.9	12.8 ± 1.0	16.6 ± 1.9
SE	2.7	2.2	1.4

SE is standard error.

Table 5.6 IgG concentration (mg/ml) in human serum samples assayed in immunoassays using:

(A) IBHR-labelled avidin

(B) IBHR-labelled antibodies

(C) HRP-labelled antibodies.

5.2.1.3 Preparation of IBHR-labelled biotin.

The LAB immunoassay, using IBHR-labelled avidin, can be directly compared with a BRAB immunoassay, using IBHR-labelled biotin. IBHR-labelled biotin was prepared using two biotin compounds: biotin hydrazide and biotin-LC-hydrazide. The structures of some biotin compounds are shown in Fig 5.5. Initially, the reaction of biotin hydrazide with IBHR was carried out using a reaction ratio of 1mg of IBHR to either 1mg or 0.75mg of biotin hydrazide, but using these ratios a large amount of precipitate was obtained soon after the addition of biotin hydrazide to IBHR. A reaction ratio of 1mg of IBHR to 0.38mg of biotin hydrazide was therefore used; this corresponds to a molar ratio of 1.7:1. The conjugate was purified by TLC. Two TLC methods, reported by Horsburgh and Gompertz (1978) and by Evangelatos *et al.* (1991) for the purification of iodinated biotin, were examined to see if they were suitable to use for the purification of the IBHR-biotin conjugates prepared for these experiments.

Horsburgh and Gompertz (1978) prepared N-(6-aminohexyl)-biotinamide, and radioiodinated this using IBHR. The iodinated biotin conjugate (IBC) was purified using two TLC systems. In the first system a mobile phase of ethyl acetate:glacial acetic acid (100:3, v/v) was used; N-(6-aminohexyl)-biotinamide and IBC remained at the origin, while IBHR migrated. The biotin compounds were eluted using 50%(v/v) ethanol, and applied to the second TLC system in order to separate them. A mobile phase of butanol:glacial acetic acid:water (93:5:5, v/v/v) was used. IBC migrated with an Rf of 0.38. The area of the chromatogram corresponding to this was removed, and the conjugate eluted as before. The conjugate prepared using biotin hydrazide and IBHR was applied to these TLC systems. In the first system, IBHR migrated with an Rf of 0.21, while biotin hydrazide and the conjugate remained at the origin. In the second system, IBHR remained at the origin, while biotin hydrazide and the conjugate migrated with Rf's of 0.11 and 0.16, respectively. This purification procedure was considered inconvenient as two TLC systems were required, and poor separation of biotin hydrazide and the conjugate was obtained.

Evangelatos *et al.* (1991) prepared iodinated biotinamide, by reaction of radioiodinated tyramine with NHS-biotin, and purified the conjugate by TLC using a mobile phase of butanol:2M ammonia:ethanol (3:1:1, v/v/v). Iodinated biotinamide migrated with an Rf of 0.67. The area of the chromatogram corresponding to this was scraped off, and the conjugate eluted with 50%(v/v) ethanol. The conjugate prepared using biotin hydrazide and IBHR was applied to this TLC system. IBHR migrated with an Rf of 0.24, biotin hydrazide migrated with an Rf of 0.46 and the conjugate migrated with an

Rf of 0.81. The use of this purification method was preferred as only one TLC procedure had to be carried out, and good separation of the conjugate from the reactants was obtained.

A conjugate was also prepared by reaction of biotin-LC-hydrazide with IBHR. Biotin-LC-hydrazide has a "spacer arm" between the bicyclic ring system and the amino group of the side chain. The biotin binding site is deep in avidin, and suboptimal results can be obtained in immunoassays if this is not taken into account (Tijssen, 1985). The IBHR label is introduced into biotin hydrazide via the amino group, thus any reduction in sensitivity due to steric hinderance or engulfment of the iodine label in the binding site will be eliminated using biotin-LC-hydrazide. O'Shannessy *et al.* (1987) used biotin-LC-hydrazide for quantification of glycoproteins on electroblots. Glycoproteins were labelled with biotin-LC-hydrazide, and after PAGE were transferred onto nitrocellulose membranes and detected using HRP-labelled streptavidin. The use of biotin-LC-hydrazide proved twice as sensitive as the use of biotin hydrazide for the detection of α_1-acid glycoprotein.

The reaction of IBHR and biotin-LC-hydrazide was carried out using a reaction ratio of 1mg of IBHR to 0.55mg of biotin-LC-hydrazide, which corresponds to a molar ratio of 1.7:1. The conjugate was purified by TLC; IBHR migrated with an Rf of 0.24, biotin-LC-hydrazide migrated with an Rf of 0.50 and the conjugate migrated with an Rf of 0.84.

The area of the TLC corresponding to the appropriate compounds was scraped off, and eluted with 50%(v/v) ethanol. The solutions were evaporated to 100µl, and made up to 1ml with 0.03M phosphate buffer, pH 7.3. Appropriate dilutions were prepared and added to plates that had been precoated with avidin. After washing, the presence of IBHR was detected using the cerium(IV) - antimony(III) reaction. The results for biotin hydrazide and biotin-LC-hydrazide are shown in Table 5.7 and Table 5.8, respectively. In both cases, only the area corresponding to the conjugate displayed catalytic activity, confirming that it is IBHR-labelled biotin as it contains biotin that can bind to avidin and IBHR that can catalyse the cerium(IV) - antimony(III) reaction. The concentration of the purified conjugates was measured in the cerium(IV) - antimony(III) microassay and the colorimetric assay for biotin, and the percentage yield of conjugate was calculated. Low yields were obtained: the average yield of conjugate was 15% using biotin hydrazide and 11% using biotin-LC-hydrazide. The solubility of the biotin compounds in borate buffer was poor; the highest concentration that would stay in solution was 1.5mg/ml. This limited the amount of biotin that could be

reacted with IBHR, and therefore the amount of conjugate produced. The volume of the two reactants for the conjugation reaction had to be low, as the purification by TLC could be cumbersome if carried out on large volumes.

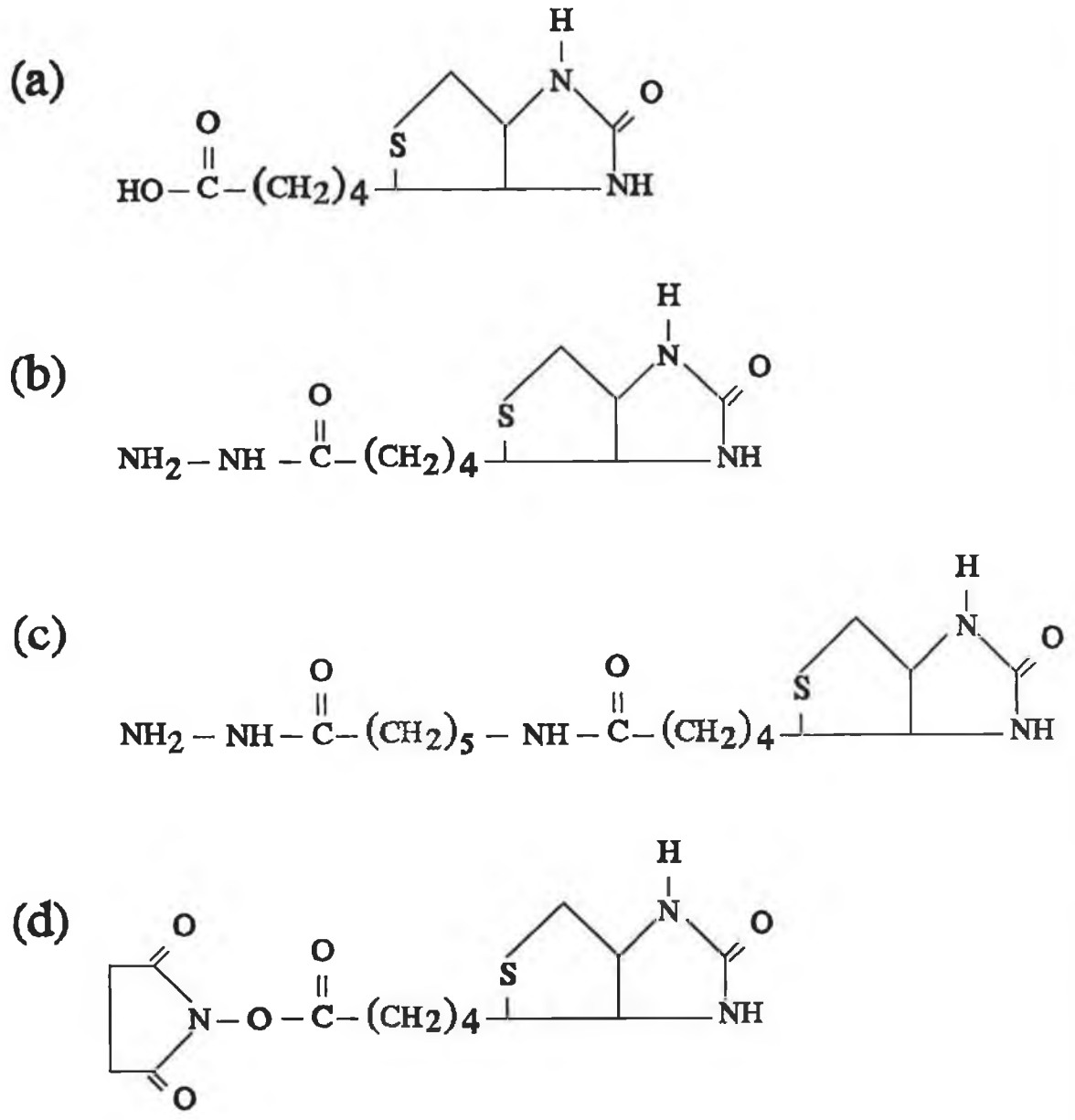

Figure 5.5 The structures of some biotin compounds (a) biotin (b) biotin hydrazide (c) biotin-LC-hydrazide (biotin aminocaproyl hydrazide) (d) NHS-biotin.

Sample	Dilution	Abs. (380nm)
Control	-	1.258
IBHR	1/10	1.246
Biotin hydrazide	1/10	1.236
Conjugate	1/10	0.852
	1/20	0.986
	1/40	1.202

Table 5.7 Spots from a TLC plate, corresponding to IBHR, biotin hydrazide and IBHR-labelled biotin, were added to an avidin coated plate. After incubation and washing, the cerium(IV) - antimony(III) microassay was used to detect the presence of IBHR.

The control is wells to which 0.03M phosphate buffer, pH 7.3, was added.

Sample	Dilution	Abs. (380nm)
Control	-	1.254
IBHR	1/10	1.249
Biotin-LC-hydrazide	1/10	1.255
Conjugate	1/10	1.039
	1/20	1.172
	1/40	1.244

Table 5.8 Spots from a TLC plate, corresponding to IBHR, biotin-LC-hydrazide and IBHR-labelled biotin, were added to an avidin coated plate. After incubation and washing, the cerium(IV) - antimony(III) microassay was used to detect the presence of IBHR.

The control is wells to which 0.03M phosphate buffer, pH 7.3, was added.

5.2.1.4 Bridged avidin-biotin (BRAB) immunoassay for human IgG.

Human IgG levels were measured in a BRAB immunoassay, using biotinylated anti-human IgG antibodies, avidin and IBHR-labelled or HRP-labelled biotin. A flow diagram of the protocol is shown in Fig 5.6. The optimum dilution of biotinylated antibodies was determined previously (Section 5.2.1.2). The optimum dilution of avidin was determined using avidin diluted in the range 1/50 to 1/400, and biotinylated HRP. The results are shown in Table 5.9. A dilution of 1/50 was chosen as optimum. The standard curves of absorbance plotted against human IgG concentration, on a log scale, obtained for IBHR-biotin conjugates prepared using biotin hydrazide and biotin-LC-hydrazide are shown in Fig 5.7. The graphs were linear in the range 0.05 to 2µg/ml for both conjugates (r=0.993 for the conjugate prepared using biotin hydrazide, r=0.995 for the conjugate prepared using biotin-LC-hydrazide). However, the difference in absorbance values between the zero standard and the 2µg/ml standard was only 0.096 for the conjugate prepared using biotin hydrazide, and 0.101 for the conjugate prepared using biotin-LC-hydrazide. Lower concentrations of human IgG could be measured using the LAB immunoassay (0.025 to 0.5µg/ml), and the difference in absorbance values between the zero standard and the 0.5µg/ml standard was 0.597. The immunoassay using IBHR-labelled antibodies had the same linear range as the BRAB immunoassay, but the difference in absorbance values between the zero standard and the 2µg/ml standard was 0.666.

As a check on the BRAB immunoassay procedure, the immunoassay was carried out using biotinylated HRP. A standard curve of absorbance plotted against human IgG concentration, on a log scale, is shown in Fig 5.8. The graph was linear in the range 0.05 to 2µg/ml (r=0.989), thus the measuring range of the immunoassay was the same using either an HRP or an IBHR label. The difference in absorbance values between the zero standard and the 2µg/ml standard was 1.030. The intra-assay and inter-assay accuracy and precision results for the standards are shown in Table 5.10 and Table 5.11, respectively. The intra-assay mean values were within 81 to 114% of the true values; the inter-assay mean values were within 88 to 128% of the true values. The intra-assay %CV values for all standards were less than 10%, while the inter-assay %CV values were greater than 10% for the 0.25µg/ml, 1µg/ml and 2µg/ml standards. The intra-assay and inter-assay accuracy results obtained using IBHR-labelled avidin in the LAB immunoassay were better than those obtained using biotinylated HRP in the BRAB immunoassay.

The poor results obtained using IBHR-labelled biotin may be improved by increasing the amount of label present. A biotin-thyroxine conjugate could be used, which would contain four times the amount of iodine compared with IBHR-labelled biotin. Smith-Palmer *et al.* (1993), reported the preparation of a biotin-thyroxine conjugate by reaction of NHS-biotin with thyroxine methyl ester. Purification of IBHR-labelled biotin by TLC meant that only small reaction volumes could be used, resulting in low yields of conjugate from any single conjugation reaction. Alternative purification methods could be used. For example, Wai Chan and Bartlett (1986) used gel filtration to purify a conjugate prepared by reaction of N-(6-aminohexyl)-biotinamide and IBHR, and Smith *et al.* (1987) used HPLC to purify iodinated biotinylglycyltyrosine, prepared by reaction of NHS-biotin with glycyltyrosine which had been radioiodinated using iodobeads.

5.2.1.5 Comparison of LAB and BRAB immunoassays.

LAB and BRAB immunoassays are reported to be in the order of 2 to 100 fold more sensitive than conventional immunoassays (Avrameas, 1992). For these experiments, the use of IBHR-labelled avidin in the LAB immunoassay enabled measurement of lower concentrations of human IgG than the use of IBHR-labelled antibodies in a two-site immunoassay, but the use of IBHR-labelled biotin in the BRAB immunoassay did not. The use of biotinylated HRP did not improve the measuring range of the BRAB immunoassay, but did give a greater difference in absorbance values between the zero standard and the highest standard than any of the immunoassays using an IBHR label. Reports in the literature of the sensitivity of LAB systems compared to BRAB systems differ. Kendall *et al.* (1983) compared a standard ELISA using alkaline phosphatase (AP) labelled antibodies for detection of mouse antibody to hepatitis B surface antigen, with a LAB ELISA using AP-labelled avidin and a BRAB ELISA using biotinylated AP. The LAB ELISA was 82 times more sensitive than the standard ELISA, while the BRAB ELISA was 23 times more sensitive than the standard ELISA. Vilja *et al.* (1985) used the avidin-biotin system in an ELISA for lactoferrin. The use of HRP-labelled avidin in the LAB ELISA enabled measurement of 0.1ng/ml lactoferrin. The use of biotinylated HRP in the BRAB ELISA enabled measurement of 0.2ng/ml lactoferrin. Both the LAB and BRAB systems were more sensitive than RIA. However, Yolken *et al.* (1983) found that the BRAB system was more sensitive than the LAB system, when used for the detection of bacterial antigens. For example, a LAB ELISA using AP-labelled avidin for measurement of antigen derived from

Haemophilus influenzae had a detection limit of 0.64ng/ml, while a BRAB ELISA using biotinylated HRP had a detection limit of 0.16ng/ml.

The BRAB system has greater potential for increased sensitivity than the LAB system. Biotinylation of many proteins has no effect on the activity of the protein (Guesdon *et al.*, 1979), and a second amplification step is included in the BRAB assay. Nevertheless, there are several factors which may have led to increased sensitivity in LAB immunoassays compared with BRAB immunoassays. The high affinity of the avidin-biotin binding means that the complex is stable in the extra wash and incubation steps required in BRAB immunoassays, but some reversal of antibody-antigen binding may occur (Kendall *et al.*, 1983). The formation of large lattices in BRAB immunoassays on the binding of avidin-labelled biotin complexes to biotinylated antibody may cause steric hindrance (Vilja *et al.*, 1985). Also, the biotinylation of AP adversely affects the enzyme activity (Guesdon *et al.*, 1979).

For the experiments reported here, iodination of avidin was shown to decrease the biotin binding activity, therefore, it would be expected that the results for the BRAB immunoassay would be better than the LAB immunoassay. However, in the BRAB immunoassay only one IBHR molecule would be bound per avidin molecule, while in the LAB immunoassay more than this would be bound as, on average, 12 moles of IBHR were conjugated per mole of avidin.

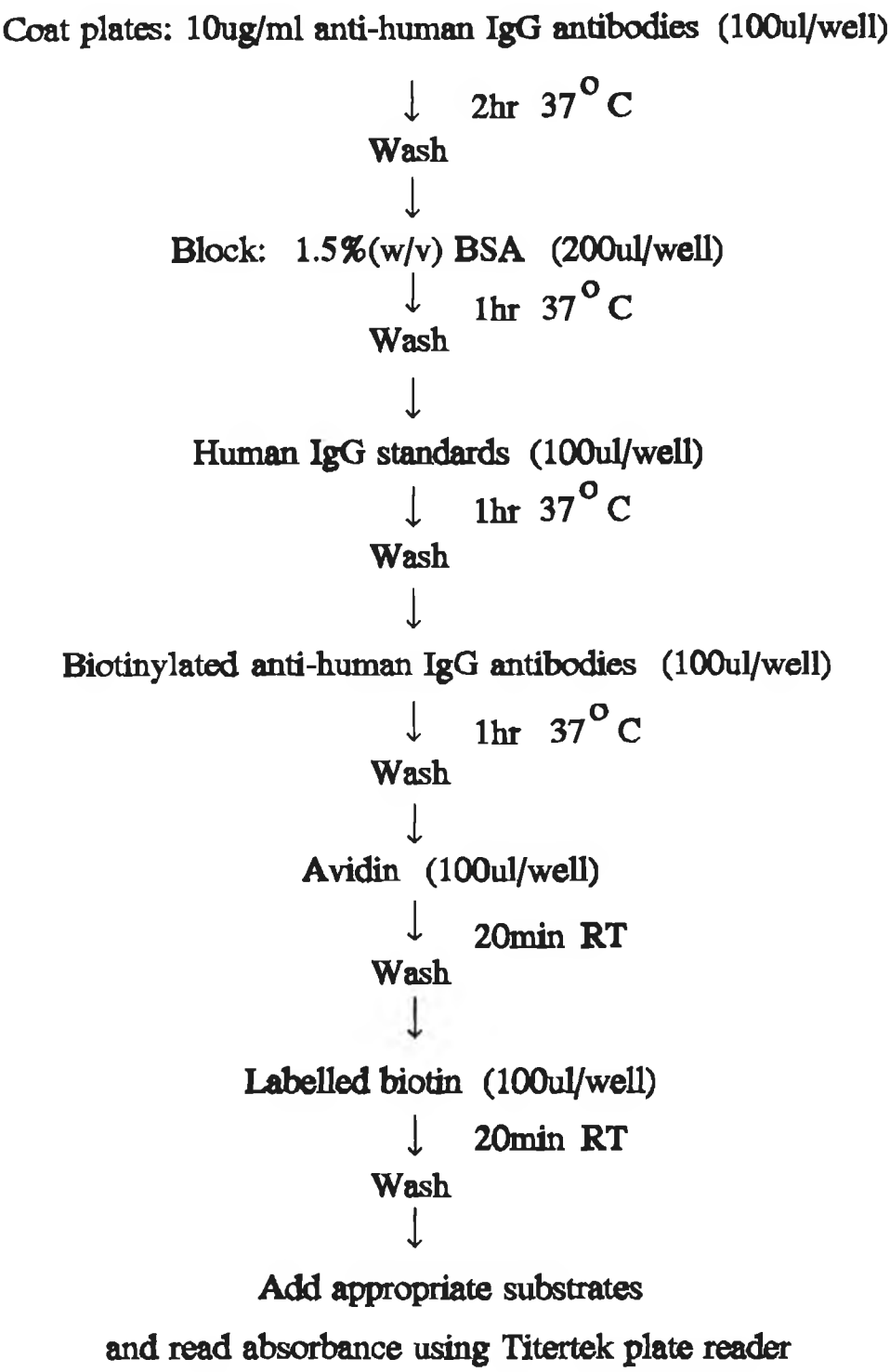

Figure 5.6 Flow diagram of the immunoassay using IBHR-labelled biotin and biotinylated HRP. (A detailed protocol is given in Section 2.8.4).

Human IgG conc. (µg/ml)	Dilution of avidin			
	1/50	**1/100**	**1/200**	**1/400**
0	0.108	0.078	0.079	0.073
0.05	0.154	0.130	0.125	0.125
0.1	0.186	0.166	0.160	0.155
0.25	0.404	0.314	0.270	0.263
0.5	0.665	0.566	0.477	0.388
1	0.994	0.827	0.683	0.491
2	1.108	0.956	0.794	0.521

Table 5.9 Determination of the optimum dilution of avidin. The results shown are absorbance values at 405nm.

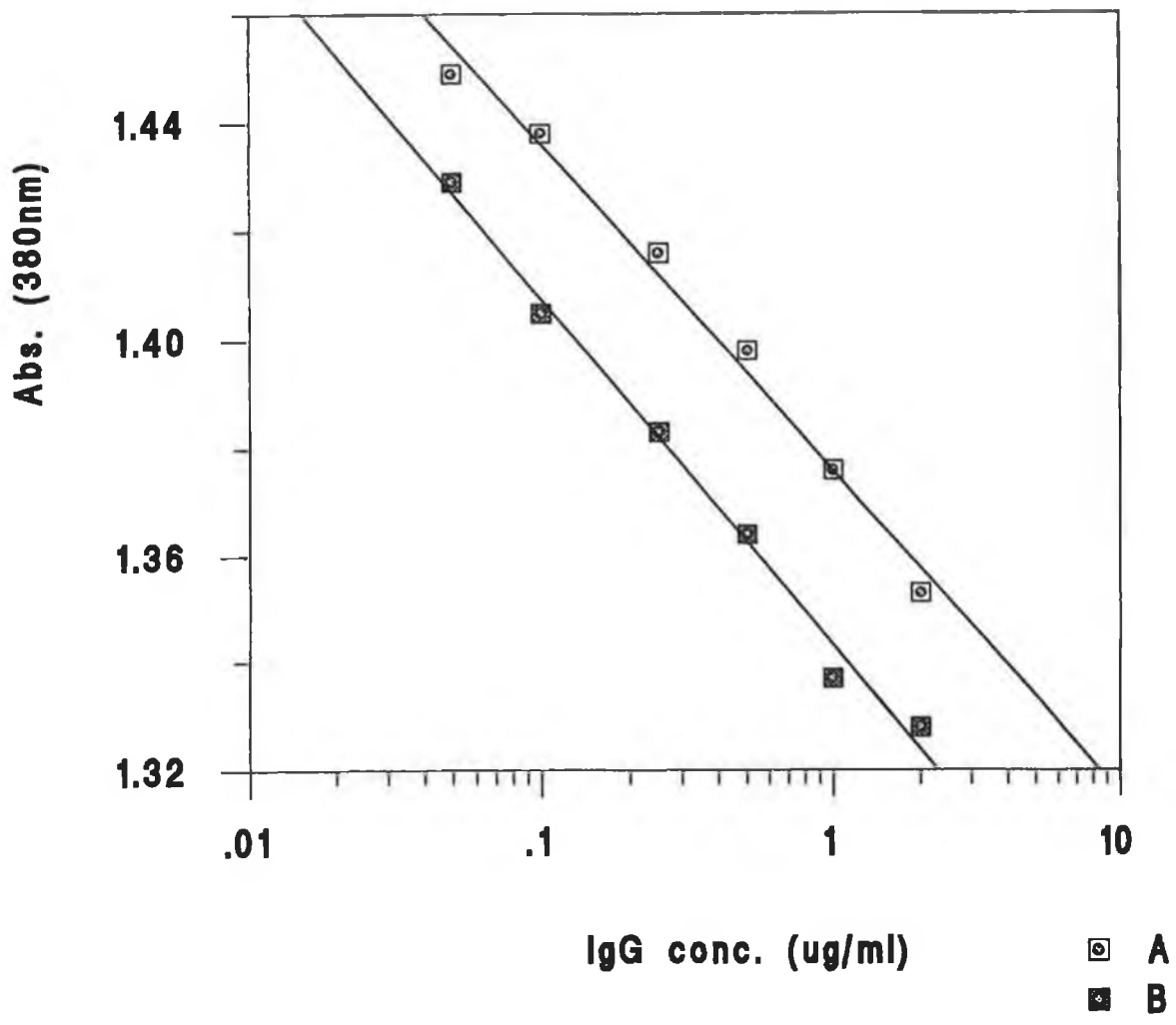

Figure 5.7 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in an immunoassay using IBHR-biotin conjugate.

(A) Conjugate prepared using biotin hydrazide.

(B) Conjugate prepared using biotin-LC-hydrazide.

The absorbance values for the zero standards were 1.464 using the conjugate prepared using biotin hydrazide and 1.460 using the conjugate prepared using biotin-LC-hydrazide.

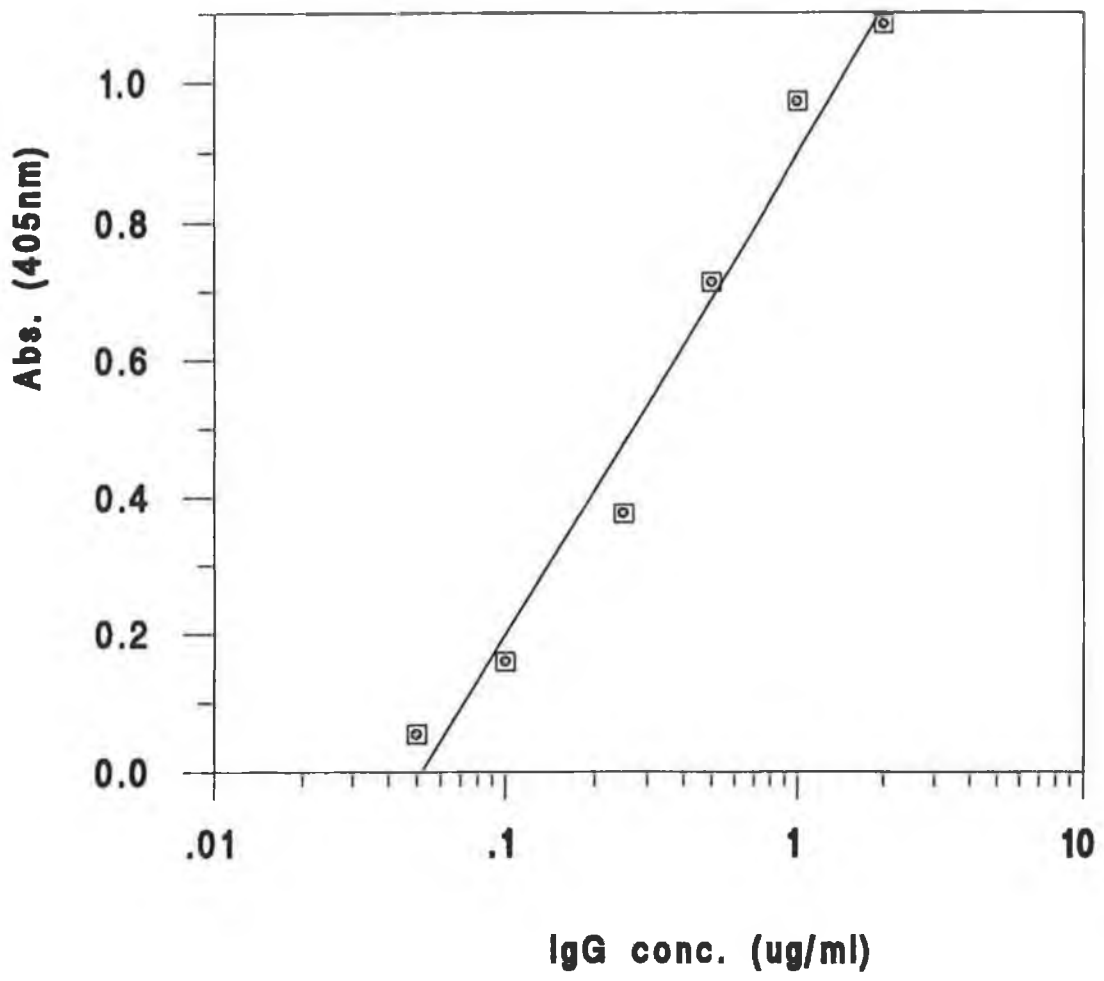

Figure 5.8 Graph of absorbance at 405nm plotted against concentration of human IgG standards assayed in an immunoassay using biotinylated HRP.
The absorbance value for the zero standard was 0.017.

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.06 ± 0.004	6.4
0.1	0.11 ± 0.01	7.1
0.25	0.20 ± 0.01	5.6
0.5	0.50 ± 0.04	8.3
1	1.14 ± 0.08	7.4
2	1.90 ± 0.07	3.5

Table 5.10 Intra-assay accuracy and precision results for human IgG standards assayed in an immunoassay using biotinylated HRP (n=8).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.05	0.05 ± 0.01	9.3
0.1	0.11 ± 0.01	8.4
0.25	0.22 ± 0.02	10.2
0.5	0.51 ± 0.04	7.5
1	1.28 ± 0.15	12.0
2	1.85 ± 0.20	10.6

Table 5.11 Inter-assay accuracy and precision results for human IgG standards assayed in an immunoassay using biotinylated HRP (n=5).

5.2.2 Bispecific F(ab')$_2$ antibodies.

5.2.2.1 Preparation of bispecific F(ab')$_2$ antibodies.

Bispecific F(ab')$_2$ antibodies, with the capability to bind to human IgG and iodinated BSA, were produced using goat anti-human IgG and rabbit anti-BSA antibodies. Goat anti-human IgG antibodies were purchased as affinity isolated antibodies. Rabbit anti-BSA antibodies were purified from rabbit antiserum by ammonium sulphate precipitation, followed by affinity chromatography using CNBr-activated Sepharose to which BSA had been coupled. The purity of goat and rabbit antibodies was assessed using SDS-PAGE (Fig 5.9a). A single band was obtained for both antibodies, with an apparent molecular weight of 195kDa.

Bispecific F(ab')$_2$ antibodies were prepared by the method of Glennie *et al.* (1987), and were purified by affinity chromatography. The final performance of bispecific antibodies in an immunoassay depends on the absence of parental antibodies, as these may interfere in the binding of the bispecific antibodies to the antigen by displacement of the bispecific antibodies (Bugari *et al.*, 1990). Takahashi *et al.* (1991) used bispecific antibodies, produced from hybridomas secreting antibodies to hCG and urease, in a two-site immunoassay for hCG. Using unpurified bispecific antibodies (i.e. parental antibodies were still present) the sensitivity was equivalent to that obtained in an immunoassay using anti-hCG antibodies conjugated to urease; the sensitivity was enhanced using bispecific antibodies purified by HPLC.

The yield of bispecific F(ab')$_2$ antibodies was low. The procedure was initially carried out using 1.5mg of parental antibodies, resulting in a yield of only 1.2%. The procedure was repeated using 5mg of parental antibodies, resulting in a yield of 11.1%. Glennie *et al.* (1987) used 10 to 15mg of parental antibodies, and obtained bispecific F(ab')$_2$ of between 50 to 70% of the final product. However, Cook and Wood (1994) used the method of Glennie *et al.* (1987) and reported a yield of only 7%. The low yield could be due to the different susceptibilities of antibodies to digestion by pepsin. Glennie *et al.* (1987) carried out pepsin digestion on two different mouse monoclonal antibodies; one antibody yielded greater than 70% of F(ab')$_2$, while the other yielded 50%. Cook and Wood (1994) carried out pepsin digestion on four mouse monoclonal antibodies; only two of these produced sufficient F(ab')$_2$ for production of bispecific F(ab')$_2$. The number of purification steps required during the production of the bispecific antibodies may also contribute to the low yield, as protein will be lost during dialysis and chromatography steps.

The purity and molecular weight of the bispecific $F(ab')_2$ antibodies were checked by SDS-PAGE (Fig 5.9b). A single band was obtained, with an apparent molecular weight of 84kDa. The approximate molecular weight of $F(ab')_2$ would be 100kDa. The bispecific $F(ab')_2$ may have a lower molecular weight than expected due to dissociation of the goat light chain, yielding a bispecific $F(ab')_2$ antibody composed of rabbit light chain - rabbit heavy chain - goat heavy chain. Dissociation of the rabbit light chain is unlikely to occur, as the maleimidation reaction may lead to intra-molecular cross-linking of the light chain to the heavy chain by a thioether bond (Glennie *et al.*, 1987).

The presence of goat Fab' fragments and rabbit Fab' fragments of the bispecific $F(ab')_2$ was checked by ELISA (Table 5.12). The ELISA confirms the presence of goat Fab' and rabbit Fab' fragments, and demonstrates that both Fab' fragments have retained antigen binding capacity.

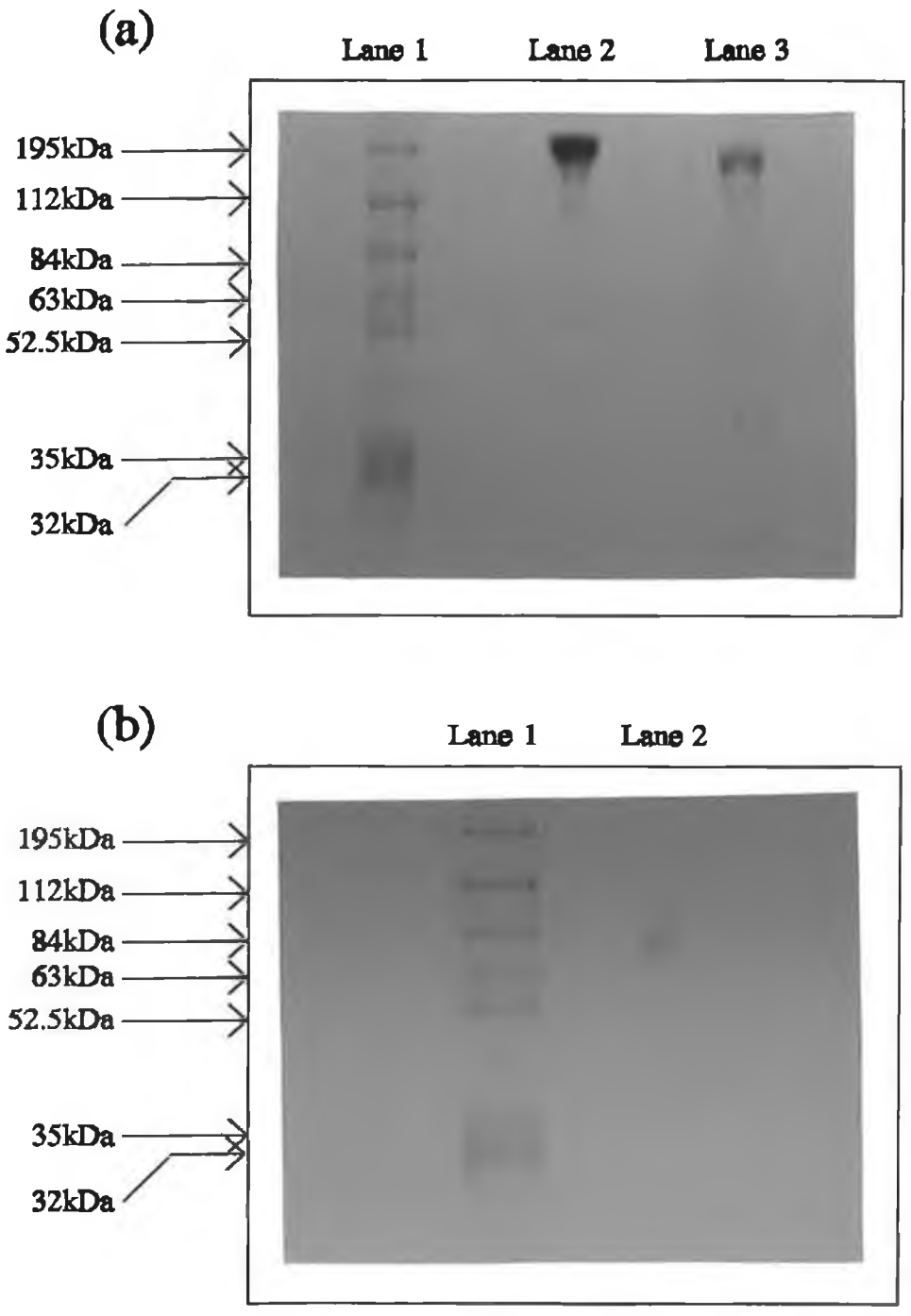

Figure 5.9 SDS-PAGE gels obtained for:

(a) molecular weight marker (lane 1), affinity purified goat anti-human IgG antibodies (lane 2), and affinity purified rabbit anti-BSA antibodies (lane 3).

(b) molecular weight marker (lane 1), and bispecific $F(ab')_2$ antibodies (lane 2).

Sample	Abs. (405nm)	
	(a)	(b)
Control	0.154	0.184
Bispecific $F(ab')_2$ antibodies	1.185	1.056
Goat anti-human IgG antibodies	0.217	0.089
Rabbit anti-BSA antibodies	0.208	0.197

Table 5.12 ELISA to detect the presence of goat Fab' and rabbit Fab' fragments of the bispecific $F(ab')_2$ antibodies.

(a) Microtitre plates were coated with human IgG. After washing and blocking, antibody samples were added. After washing, HRP-labelled anti-rabbit IgG antibodies were added.

(b) Microtitre plates were coated with BSA. After washing and blocking, antibody samples were added. After washing, HRP-labelled anti-goat IgG antibodies were added.

The control is wells to which 0.03M phosphate buffer, pH 7.3, was added.

5.2.2.2 Immunoassay for human IgG using bispecific F(ab')$_2$ antibodies.

Bispecific F(ab')$_2$ antibodies were used in an immunoassay for the measurement of human IgG concentration. The protocol for the immunoassay is outlined in a flow diagram in Fig 5.10. The immunoassay was initially performed using IBHR-labelled BSA; the standard curve of absorbance plotted against human IgG concentration, on a log scale, is shown in Fig 5.11. The graph was linear in the range 0.25 to 2μg/ml human IgG (r=0.990), but the difference in absorbance values between the zero standard and the 2μg/ml standard was only 0.059. This may be due to the low amount of IBHR label present, as on average just 15 moles of IBHR were conjugated per mole of BSA. BSA contains 19 free amino groups (Schipper *et al.*, 1991) to which IBHR can be conjugated, which is low compared with goat antibodies which have 90 free amino groups. In order to introduce more iodine label into BSA, IBHR-labelled BSA was subjected to iodination by the chloramine T method to introduce iodine into the tyrosine residues. Iodinated BSA prepared in this way had, on average, 40 moles of iodine conjugated per mole of BSA.

The binding of rabbit anti-BSA antibodies to iodinated BSA was assessed using the BIAcore. Anti-BSA antibodies were immobilised onto a sensor chip. Native BSA was passed over the surface of the chip, and the response obtained was taken to represent maximum binding (i.e. 100%). IBHR-labelled BSA and BSA, iodinated by the IBHR and chloramine T iodination methods, were passed over the surface of the chip, and the response obtained was expressed as a percentage of the maximum binding. IBHR-labelled BSA gave 104% of the maximum binding; BSA, iodinated by the IBHR and chloramine T iodination methods, gave 112% of the maximum binding. Native BSA was reapplied at the end of the experiment; 97% of the maximum binding was obtained. Iodination of BSA does not have an adverse effect on the binding to anti-BSA antibodies.

BSA, iodinated by the IBHR and chloramine T iodination methods, was used in the immunoassay for measurement of human IgG. The standard curve of absorbance plotted against human IgG concentration, on a log scale, is shown in Fig 5.11. The graph was linear in the range 0.1 to 2μg/ml human IgG (r=0.997), thus the use of BSA, iodinated by the IBHR and chloramine T iodination methods, enabled measurement of slightly lower concentrations of human IgG compared with the use of IBHR-labelled BSA. However, the use of IBHR-labelled antibodies (linear range 0.05 to 2μg/ml) and IBHR-labelled avidin (linear range 0.025 to 0.5μg/ml) enabled measurement of lower concentrations of human IgG. Also, using BSA, iodinated by the IBHR and

chloramine T iodination methods, the difference in absorbance values between the zero standard and the 2µg/ml standard was only 0.123.

In order to check the performance of the bispecific $F(ab')_2$ antibodies, the immunoassay was carried out using HRP-labelled BSA. The standard curve of absorbance plotted against concentration, on a log scale, is shown in Fig 5.12. The graph was linear in the range 0.0125 to 1µg/ml (r=0.995), thus enabling measurement of lower concentrations of human IgG compared with the use of IBHR-labelled antibodies and IBHR-labelled avidin. The intra-assay and inter-assay accuracy and precision results for the standards are shown in Table 5.13 and Table 5.14. The intra-assay mean values were within 92 to 110% of the true values, while the inter-assay mean values were within 82 to 110% of the true values. The intra- assay %CV values were greater than 10% for the 0.05µg/ml and the 0.25µg/ml standards, while the inter-assay %CV values were greater than 10% for the 0.0125µg/ml and the 0.1µg/ml standards. Overall, the intra- and inter-assay accuracy results were better than those obtained using labelled antibodies or labelled avidin. The linear range obtained for the bispecific $F(ab')_2$ antibodies using HRP-labelled BSA shows good comparison with that obtained using HRP-labelled antibodies (linear range 0.0064 to 0.25µg/ml). This illustrates that the bispecific $F(ab')_2$ antibodies can be successfully applied to the immunoassay, and indicates that the poor results obtained using iodinated BSA are possibly due to the iodine label. The cerium(IV) - antimony(III) reaction can only detect a percentage of the iodine present in iodinated compounds (Section 3.2.3), which limits the sensitivity of the immunoassay using iodinated BSA. Better results may be obtained using a protein which could be more heavily iodinated than BSA. For example, thyroglobulin which has 90 free amino groups (Schipper *et al.*, 1991) could be heavily labelled with IBHR, and used in the immunoassay with bispecific $F(ab')_2$ antibodies produced from anti-human IgG and anti-thyroglobulin antibodies.

There are several advantages to the use, in immunoassays, of bispecific $F(ab')_2$ antibodies with one binding site for the antigen and one binding site for the label. Immobilisation of the label is achieved without the need for chemical modification of the antibody by covalent attachment of the label. Labelling antibodies with enzymes can alter the activity of both the antibody and the enzyme, and antibody-enzyme conjugates are often less stable than the unconjugated species (Takahashi *et al.*, 1991). Iodination of antibodies has been shown to alter the antigen binding activity, and the performance in an immunoassay (Section 4.2.2). Production of bispecific $F(ab')_2$ antibodies requires chemical modification of antibodies, but these modifications are site

specific. Using the method of Glennie *et al.* (1987) modification is carried out at the hinge region of the antibodies away from the antigen binding sites. Therefore, the antigen binding capacity should be unaffected.

There are several examples in the literature of the successful use of bispecific antibodies for immobilisation of the label in immunoassays. Bispecific antibodies were produced by fusion of hybridomas and by chemical means, by Gorog *et al.* (1989). The bispecific antibodies reacted with carcinoembryonic antigens (CEA) and with β-galactosidase, and were used in a homogeneous immunoassay for measurement of CEA. Bispecific antibodies produced by fusion of hybridomas have been used by several workers. Bugari *et al.* (1990) used bispecific antibodies to human lutropin and β-galactosidase in a two-site immunoassay for the measurement of lutropin, which had a sensitivity comparable to commercial RIA and IRMA kits. Takahashi *et al.* (1991) detected 15mIU/ml of hCG using bispecific antibodies reacting with hCG and urease. The detection limit was 25mIU/ml using urease-labelled anti-hCG antibodies. Bispecific antibodies reacting with human interferon-α-2 and HRP were used by Kontsekova *et al.* (1992) in a two-site ELISA to measure interferon-α-2 levels of 0.1ng/ml, which was almost the same sensitivity as an ELISA using HRP-labelled antibodies. Stratieva-Taneeva *et al.* (1993) used bispecific antibodies binding both interleukin-2 (IL2) and HRP for the analysis of recombinant IL2 in both direct and competitive EIAs. The experiments reported in this chapter illustrated that bispecific $F(ab')_2$ antibodies, with one binding site for the analyte and one binding site for a protein conjugated to the label, could be successfully used to measure human IgG. The use of HRP-labelled BSA illustrated that the bispecific antibodies could produce comparable results to a conventional immunoassay using HRP-labelled antibodies, and could improve the results using IBHR-labelled antibodies. The poor results obtained using IBHR-labelled BSA were possibly due to problems with detection of the IBHR label.

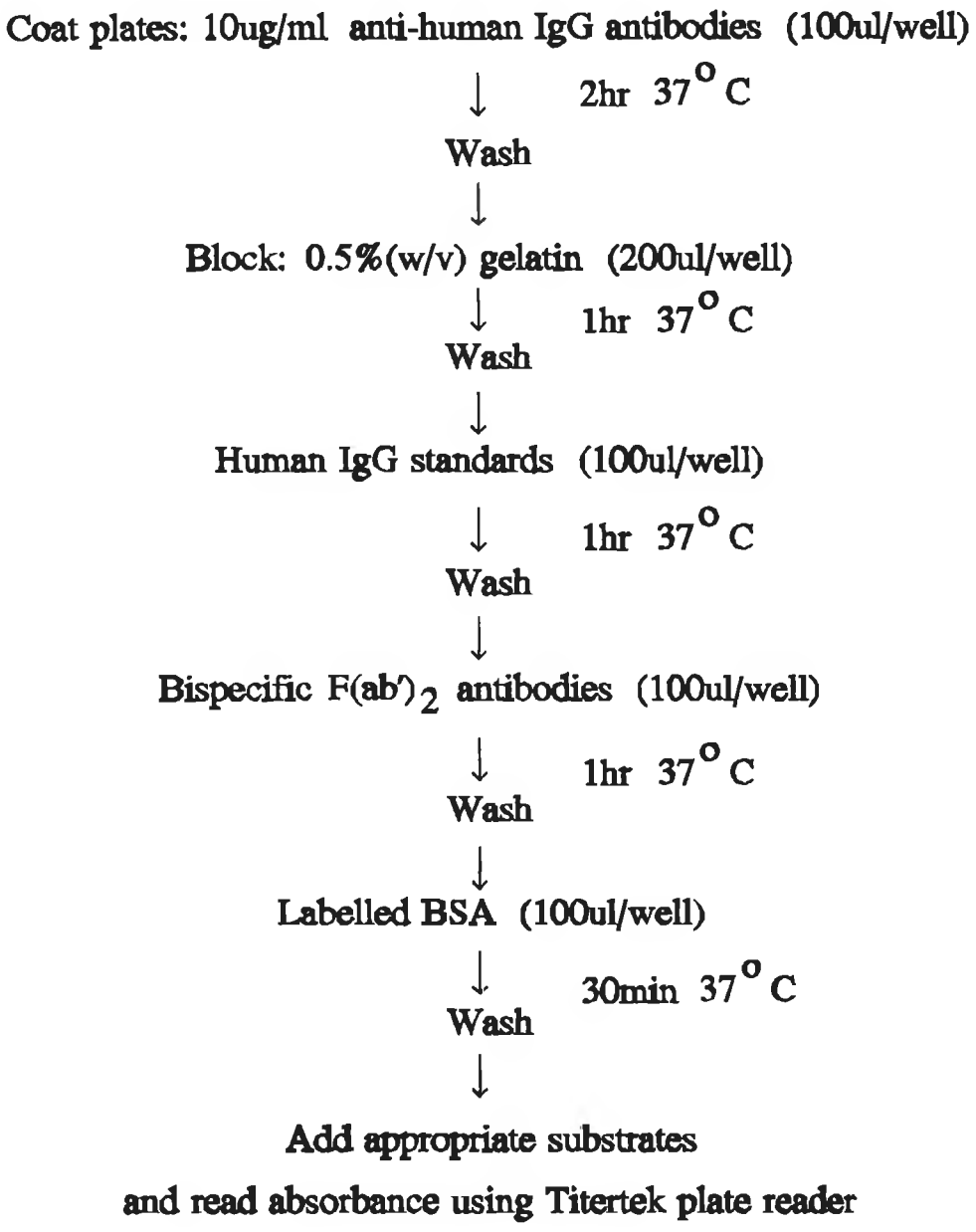

Figure 5.10 Flow diagram of the immunoassay using bispecific $F(ab')_2$ antibodies. (A detailed protocol is given in Section 2.9.3).

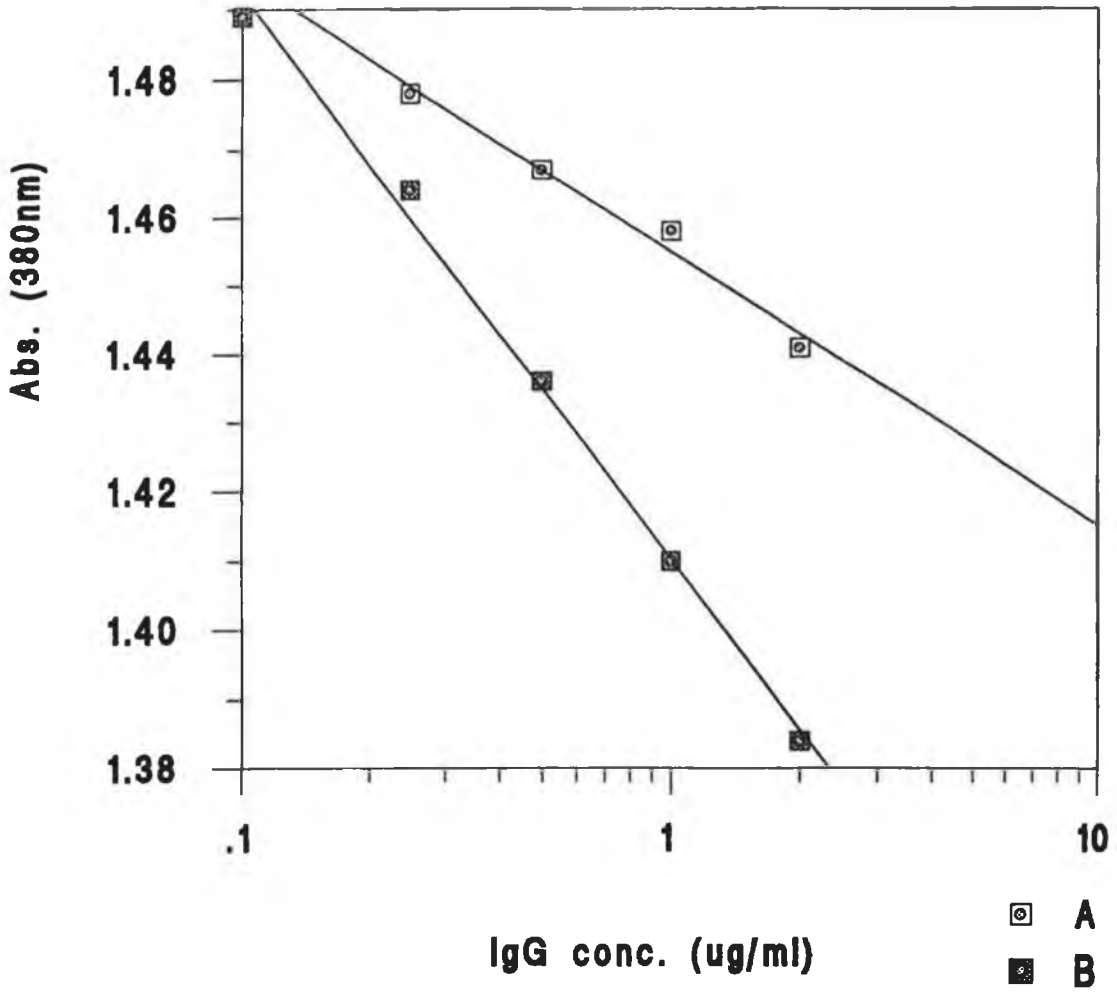

Figure 5.11 Graph of absorbance at 380nm plotted against concentration of human IgG standards assayed in an immunoassay using bispecific $F(ab')_2$ antibodies. The antibodies were detected using:

(A) IBHR-labelled BSA

(B) BSA, iodinated using the IBHR and chloramine T methods of iodination.

The absorbance values for the zero standards were 1.500 using IBHR-labelled BSA and 1.502 using BSA, iodinated using the IBHR and chloramine T methods of iodination.

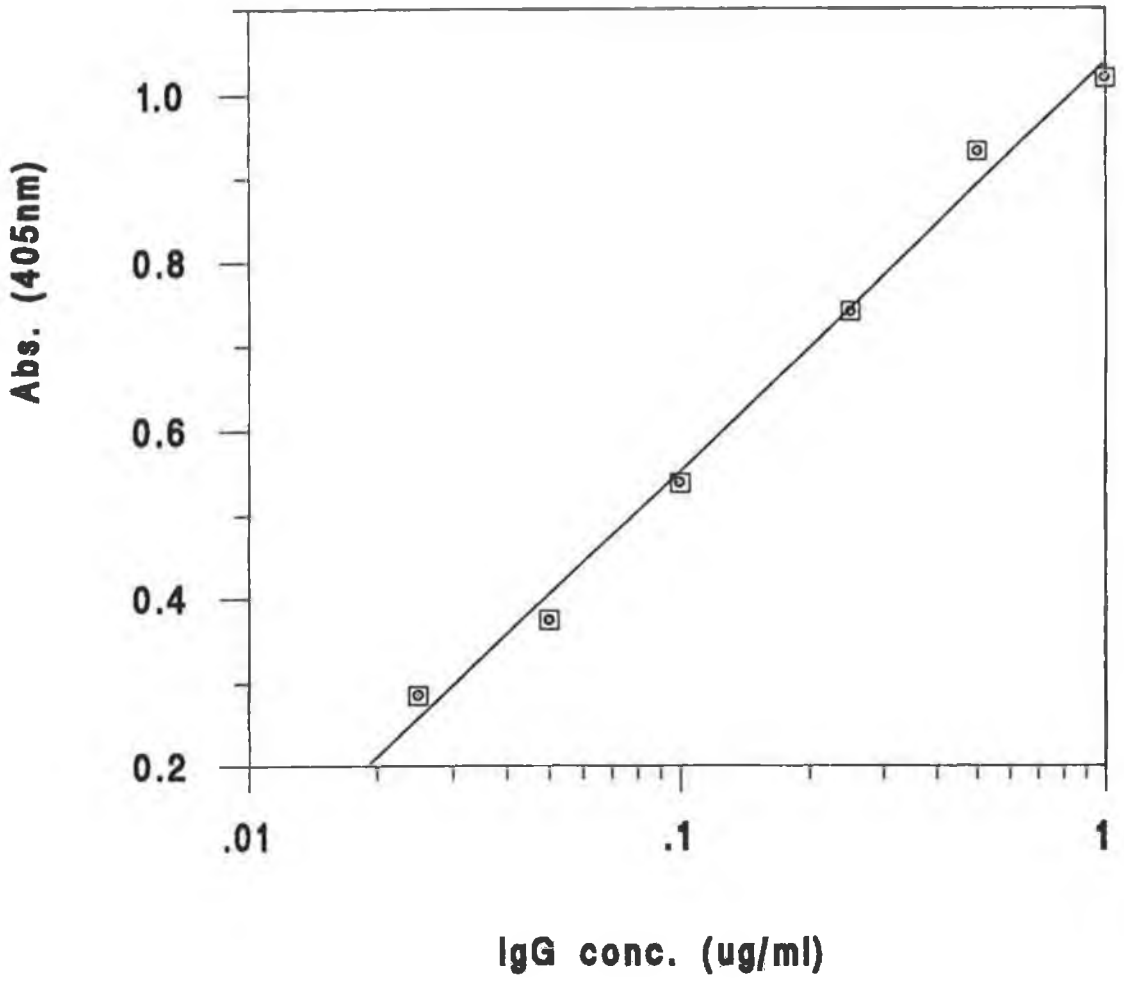

Figure 5.12 Graph of absorbance at 405nm plotted against concentration of human IgG standards assayed in an immunoassay using bispecific $F(ab')_2$ antibodies and HRP-labelled BSA.

The absorbance value for the zero standard was 0.165.

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.0125	0.014 ± 0.001	7.3
0.025	0.023 ± 0.001	5.7
0.05	0.047 ± 0.008	17.0
0.1	0.095 ± 0.003	3.4
0.25	0.243 ± 0.038	15.5
0.5	0.535 ± 0.002	0.4
1	1.014 ± 0.081	8.0

Table 5.13 Intra-assay accuracy and precision results for human IgG standards assayed in an immunoassay using bispecific $F(ab')_2$ antibodies and HRP-labelled BSA (n=8).

Human IgG conc. (µg/ml)	Mean ± SD	%CV
0.0125	0.013 ± 0.002	11.3
0.025	0.021 ± 0.001	2.9
0.05	0.049 ± 0.005	10.0
0.1	0.110 ± 0.013	12.0
0.25	0.262 ± 0.018	6.8
0.5	0.528 ± 0.036	6.9
1	0.986 ± 0.028	2.8

Table 5.14 Inter-assay accuracy and precision results for human IgG standards assayed in an immunoassay using bispecific $F(ab')_2$ antibodies and HRP-labelled BSA (n=4).

5.3 Summary.

The avidin-biotin system and bispecific $F(ab')_2$ antibodies were used in immunoassays for the measurement of human IgG. A LAB immunoassay, using biotinylated anti-human IgG antibodies and IBHR-labelled avidin, was set up. Avidin was labelled with various levels of IBHR; a reduction in the amount of IBHR conjugated to avidin led to improved performance of the labelled avidin in the immunoassay. Human IgG standards in the range 0.025 to 0.5µg/ml were measured using this immunoassay, which is an improvement to the measuring range for the immunoassay using IBHR-labelled antibodies (0.05 to 2µg/ml). A BRAB immunoassay, using biotinylated anti-human IgG antibodies, avidin and IBHR-labelled biotin, was also set up. IBHR was conjugated to two biotin compounds; biotin hydrazide and biotin-LC-hydrazide. The conjugates were used in the BRAB immunoassay to measure human IgG standards in the range 0.05 to 2µg/ml, but a very small difference in absorbance values between the zero standard and the 2µg/ml standard was obtained. The performance of the BRAB immunoassay was checked using biotinylated HRP. The same range of human IgG standards could be measured using biotinylated HRP as could be measured using IBHR-labelled biotin, but a greater difference in absorbance values between the zero standard and the 2µg/ml standard was obtained with biotinylated HRP. The use of the LAB immunoassay enabled measurement of lower concentrations of human IgG than was possible using either IBHR-labelled antibodies in a conventional immunoassay or using the BRAB immunoassay.

Bispecific $F(ab')_2$ antibodies, reacting with human IgG and BSA, were prepared by a chemical method. These antibodies were used as bridging agents between human IgG and iodinated BSA, in an immunoassay for human IgG. Using IBHR-labelled BSA, human IgG standards in the range 0.25 to 2µg/ml were measured. In order to increase the amount of label, the tyrosine residues on IBHR-labelled BSA were iodinated by the chloramine T method. Iodinated BSA prepared in this way could be used to measure standards in the range 0.1 to 2µg/ml. In both cases, a very small difference in absorbance values was obtained between the zero standard and the 2µg/ml standard. The performance of the bispecific $F(ab')_2$ antibodies was checked using HRP-labelled BSA; human IgG standards in the range 0.0125 to 1µg/ml could be measured, which is lower than the measuring range of the immunoassay using IBHR-labelled antibodies. This indicates that the bispecific $F(ab')_2$ antibodies functioned effectively as bridging agents between human IgG and BSA. The poor results obtained using iodinated BSA were possibly due to the detection of the iodine label. The cerium(IV) - antimony(III)

reaction can only detect a percentage of the iodine present in iodinated compounds, which limits the sensitivity of the detection of iodinated BSA. The use of a protein that could be more heavily iodinated than BSA could possibly lead to an improvement in the immunoassay results.

6. MEASUREMENT OF THYROXINE.

6.1 Introduction.

The thyroid gland secretes the hormones thyroxine (T_4) and 3',3,5-tri-iodothyronine (T_3). (The structures of the hormones are shown in Fig 3.8) The hormones are synthesised by iodination of tyrosine residues of thyroglobulin, a glycoprotein in the follicles of the thyroid gland. Thyroglobulin is not biologically active, but on proteolysis releases the hormones into the blood. T_4 is produced in much larger quantities than T_3. The mean T_4 concentration in normal human serum is 111nM, while the mean T_3 concentration is just 1.82nM (Wiersinga and Chopra, 1982). However, T_3 is the more potent hormone and is five to ten times more active than T_4. The primary function of the hormones is to increase the basal metabolic rate and oxygen consumption in many tissues, and they play an important role in the maturation of brain and bone. Hypothyroidism, a deficiency in T_4, is characteristic of the disease myxedema. A deficiency of T_4 during development results in retarded mental development and bone growth. Hyperthyroidism, excessive secretion of T_4, is responsible for Graves' disease or exophthalmic goitre. In hypothyroidism, serum T_3 levels are decreased to a lesser extent relative to the decrease in T_4 levels, while in hyperthyroidism, T_3 levels are increased to a greater extent relative to the increase in T_4 (Wiersinga and Chopra, 1982).

A large proportion of the T_4 in the blood circulates tightly bound to the serum proteins thyroxine-binding globulin (TBG), thyroxine-binding pre-albumin (TBPA) and albumin. The unbound T_4 (free T_4) represents only a small amount (<0.03%) of the total T_4. Free T_4 is the physiologically active component, whereas the protein bound fraction represents a reservoir of the hormone with which the free fraction is in dynamic equilibrium (Liewendahl, 1990). In order to measure the total T_4 concentration in serum the binding of T_4 to serum proteins can be blocked using several agents, for example, 8-anilino-1-naphthalenesulphonic acid, thiomerosal or sodium salicylate (Wiersinga and Chopra, 1982). Mean free T_4 concentrations in the region of 25pM have been reported (Liewendahl *et al.*, 1990). Total T_4 concentration falls within the range 50 to 150nM in euthyroid patients; T_4 serum concentrations are lower than 50nM in hypothyroid patients and higher than 150nM in hyperthyroid patients.

Historically, T_4 concentration was estimated by measurement of the iodine content in a protein precipitate of serum (serum protein bound iodine) and by *in vivo* radioiodine tracer tests, but these methods were subject to interference from iodine-containing

drugs and non-hormonal iodine (Liewendahl, 1990). In the 1960s competitive saturation analysis, based on the use of TBG, was introduced for measurement of T_4. The assay was based on competition between labelled and unlabelled T_4 for binding to TBG, similar to RIA, but as the separation of bound and free fractions was by electrophoresis the method was unsuitable for routine use (Liewendahl, 1990). It was not until the 1970s that RIAs for T_4 were developed, because of the problems in raising polyclonal antibodies to small haptens. Since then, RIA has been widely used for the measurement of T_4. ^{125}I can be used as an internal label in the T_4 molecule, providing a tracer with high specific activity. In the 1980s nonisotopic T_4 immunoassays using labels such as enzymes, fluorescent compounds and chemiluminescent compounds, were introduced. As non-isotopic methods can be automated, their use for measurement of T_4 will probably become widespread in the future.

This chapter describes an immunoassay for T_4 which uses the four iodine atoms present in T_4 as the label. Anti-T_4 antibodies immobilised onto microtitre plates are used to capture T_4, and the iodine is detected using the cerium(IV) - antimony(III) reaction.

6.2 Results and discussion.

6.2.1 Determination of T_4 using the cerium(IV) - antimony(III) microassay.

T_4 standards were measured using the cerium(IV) - antimony(III) microassay. A graph of absorbance plotted against concentration of T_4 is shown in Fig 6.1. The graph was linear in the range 20 to 100nM T_4 (r=0.995). The intra-assay accuracy and precision (Table 6.1) and inter-assay accuracy and precision (Table 6.2) were calculated for the T_4 standards. The intra- and inter-assay mean values were within 94 to 108% of the true values, with the exception of the 40nM standard which had an inter-assay mean value of 114% of the true value. The %CV values for all standards were less than 10%. Clarke (1991) measured T_4 in the range 32 to 1000nM in the Sandell-Kolthoff reaction, using 0.075M arsenious acid and 0.02M ceric ammonium sulphate diluted in 10% (v/v) HNO_3 with measurement of the absorbance at 405nm. The detection of lower concentrations of T_4 using the cerium(IV) - antimony(III) reaction may be due to the measurement of the absorbance at 380nm, a wavelength closer to the absorbance maximum for cerium(IV) than 405nm (Section 3.2.1).

The catalytic activity of iodine in organic compounds is complex, and is inferior to the catalytic activity of inorganic iodide. The catalytic activity of iodine in T_4 was directly compared to that of potassium iodide, and the K value (the catalytic activity per iodine atom of the compound) was calculated (Section 3.2.3). The K value for T_4 was

calculated as 47.0%; 0.5 moles of T_4 have the same catalytic activity as one mole of iodide. K values of 57% and 62% were obtained for T_4 by Timotheou-Potamia (1988) and O'Kennedy *et al.* (1989), using the Sandell-Kolthoff reaction. The sensitivity of the cerium(IV) - antimony(III) reaction for measurement of T_4 is limited by the fact that only a percentage of the iodine in T_4 is liberated by cerium(IV) to catalyse the reaction. Following treatment with cerium(IV), just 23.2% of the total iodine in T_4 was liberated.

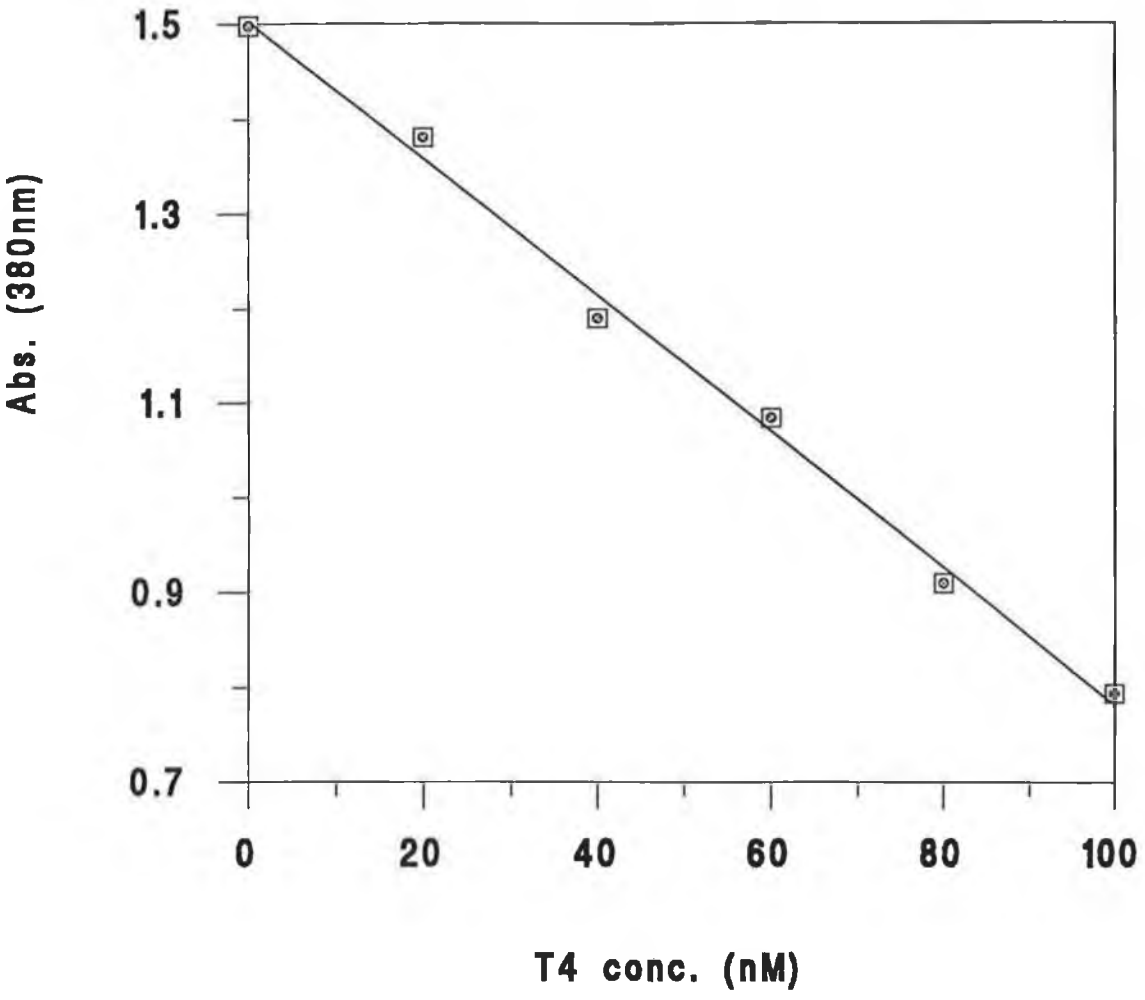

Figure 6.1 Graph of absorbance at 380nm plotted against concentration of T_4 standards assayed in the cerium(IV) - antimony(III) microassay.

T_4 conc. (nM)	Mean ± SD	%CV
20	20.9 ± 1.5	7.3
40	43.2 ± 2.5	5.9
60	57.4 ± 1.6	2.8
80	79.6 ± 7.9	9.9
100	104.0 ± 8.6	8.3

Table 6.1 Intra-assay accuracy and precision results for T_4 standards assayed in the cerium(IV) - antimony(III) microassay (n=8).

T_4 conc. (nM)	Mean ± SD	%CV
20	18.7 ± 1.8	9.8
40	45.5 ± 2.2	4.8
60	62.2 ± 6.1	9.9
80	80.2 ± 4.7	5.8
100	98.1 ± 2.0	2.0

Table 6.2 Inter-assay accuracy and precision results for T_4 standards assayed in the cerium(IV) - antimony(III) microassay (n=6).

6.2.2 Immunoassay for T_4.

T_4 standards were measured in an immunoassay using anti-T_4 antibodies to capture the T_4, and the four iodine atoms in T_4 as the label. The protocol for the immunoassay is outlined in a flow diagram in Fig 6.2. Three anti-T_4 antibodies were available: Dako rabbit anti-T_4 antibodies which were purchased as the purified immunoglobulin fraction of rabbit antiserum, Sigma rabbit anti-T_4 antiserum and SAPU sheep anti-T_4 antiserum. The IgG fraction was purified from rabbit and sheep antiserum by ammonium sulphate precipitation. The binding of the antibodies to T_4 was studied using the BIAcore. T_4 was immobilised onto the surface of a sensor chip, and anti-T_4 antibodies were passed over the surface. The response obtained for SAPU sheep anti-T_4 antibodies was taken to represent maximum binding (i.e. 100%). The response for the other antibodies was expressed as a percentage of the maximum binding. Dako rabbit anti-T_4 antibodies gave 20% of the maximum binding, and Sigma rabbit anti-T_4 antibodies gave 37% of the maximum binding. The anti-T_4 antibodies obtained from SAPU were, therefore, most suitable for use in the T_4 immunoassay. The SAPU antiserum to T_4 is produced using a T_4 - human serum albumin conjugate, which is prepared using a water soluble carbodiimide to form a peptide bond between T_4 and albumin. In this way, T_4 is oriented such that the outer ring, which is the portion of the molecule required to produce the most specific antibodies, projects outwards from the framework of the carrier protein (Munro *et al.*, 1983).

The ammonium sulphate-purified SAPU anti-T_4 antibodies were used in an immunoassay to measure T_4 standards. A graph of absorbance plotted against concentration of T_4 is shown in Fig 6.3. The graph was linear in the range 75 to 300nM (r=0.990); but a difference of only 0.163 was obtained between the absorbance values for the zero standard and the 300nM standard. Clarke (1991), using the same batch of sheep anti-T_4 antibodies and the same immunoassay format, but with the Sandell-Kolthoff reaction for detection of the iodine, achieved a detection limit of 125nM. The lower detection limit using the cerium(IV) - antimony(III) reaction may be due to measurement of the absorbance at a wavelength closer to the absorbance maximum for cerium(IV) (Section 6.2.1).

The normal range for T_4 in human serum is 50 to 150nM; samples from hypothyroid patients have T_4 concentrations lower than 50nM. Immunoassays for the measurement of T_4 must, therefore, be able to measure lower than 50nM. This has been achieved using radioactive iodine and non-isotopic labels. A competitive RIA for total T_4, using anti-T_4 antibodies and ^{125}I-T_4, was reported by Wiersinga and Chopra (1982). The

detection limit was 6nM, and the measuring range was 15 to 515nM. Immunoassays using T_4 conjugated to enzymes such as HRP, AP or β-galactosidase have been reported. For example, Gonzalez *et al.* (1991) developed a competitive immunoassay using a T_4 -β-galactosidase conjugate and anti-T_4 antibodies immobilised onto microtitre plates. The label was detected using 4-methyl-umbelliferyl-β-D-galactopyranoside as substrate. The sensitivity was 2.5nM, and the measuring range was 24 to 386nM. A chemiluminescent immunoassay which does not require the addition of substrate or secondary signal substances was developed by Sturgess *et al.* (1986). Anti-T_4 antibodies labelled with a chemiluminescent acridinium ester were incubated with T_4 and T_4-rabbit IgG conjugate. Separation of bound and free fractions was achieved using anti-rabbit IgG antibodies bound to magnetic particles. The sensitivity was 1nM, and the measuring range was 20 to 190nM. Other methods include the use of europium chelators for a time-resolved fluorimetric immunoassay (Diamandis, 1992), and a homogeneous assay based on phase-modulation fluorescence spectroscopy (Ozinskas *et al.*, 1993). These assays are suitable for measurement of total T_4 concentration in serum from hypothyroid, euthyroid and hyperthyroid patients. Immunoassays for measurement of free T_4 require greater sensitivity. For example, Christofides *et al.* (1992) developed a one-step EIA for free T_4 using ^{125}I-labelled anti-T_4 antibodies. The measuring range of the assay was 2 to 130pM.

The immunoassay for T_4 described in this chapter has the advantage that there is no necessity to prepare labelled derivatives of either T_4 or antibodies, as the iodine atoms present in T_4 serve as the label. In one step free T_4 analogue methods, labelled derivatives of T_4 compete with T_4 for binding to immobilised anti-T_4 antibodies. The methods are based on the assumption that T_4 analogues will bind to antibodies but not to serum proteins. However, several reports have demonstrated that albumin does bind to T_4 analogues, leading to inaccurate measurements of free T_4 in serum containing abnormal concentrations of albumin (Liewendahl, 1990). The immunoassay described here would eliminate this problem if it was sufficiently sensitive for measurement of free T_4. The assay format is simple; all that is required is the incubation of T_4 with anti-T_4 antibodies immobilised onto a microtitre plate, and detection of the iodine using the cerium(IV) - antimony(III) reaction. T_4 concentrations in the range 75 to 300nM can be measured in this assay. However, human serum samples were not assayed as the assay was not sufficiently sensitive to measure euthyroid and hypothyroid samples. Alterations to the assay design could extend the measuring range to lower concentrations of T_4. Antibodies could be immobilised onto a particulate solid phase,

such as agarose, cellulose, Sephadex or dextran. Particulate solid phases are more efficient as the ratio of the surface of the solid phase to the volume of the sample is high, the solid phase can be dispersed throughout the reaction mixture and the rate of antibody-antigen reactions is faster than with antibodies immobilised onto microtitre plates (Tijssen, 1985). The orientation of solid phase binding of the antibody via the Fc portion could also be used to improve the antigen binding capacity and, therefore, the measuring range of the assay (Porstmann and Kiessig, 1992). This can be achieved in two ways. The solid phase can be precoated with protein A, a protein isolated from the cell wall of *Staphylococcus aureus* which has the ability to bind the Fc portion of IgG, or antibodies can be immobilised, after periodate oxidation, to amino groups on the solid phase via the carbohydrate moiety of the Fc portion. Alternatively, T_4 could be isolated from samples using organic extraction or chromatography, and the isolated T_4 measured directly in the cerium(IV) - antimony(III) microassay. Knapp *et al.* (1974) separated T_4 and T_3 on ion-exchange columns, and measured the concentrations of the hormones using the Sandell-Kolthoff reaction. The measuring range for T_4 was 2.6 to 386nM, while for T_3 the measuring range was 0.3 to 46nM.

6.3 Summary.

The cerium(IV) - antimony(III) microassay can be used to measure T_4 standards in the range 20 to 100nM. The catalytic activity of the iodine atoms in T_4 is inferior to that of inorganic iodide, which limits the sensitivity of the assay for measurement of T_4. An immunoassay for measurement of T_4 was developed. Anti-T_4 antibodies were immobilised onto microtitre plates, and T_4 standards were added. The iodine atoms in T_4 served as the label, and were detected using the cerium(IV) - antimony(III) reaction. T_4 standards in the range 75 to 300nM could be measured in the immunoassay.

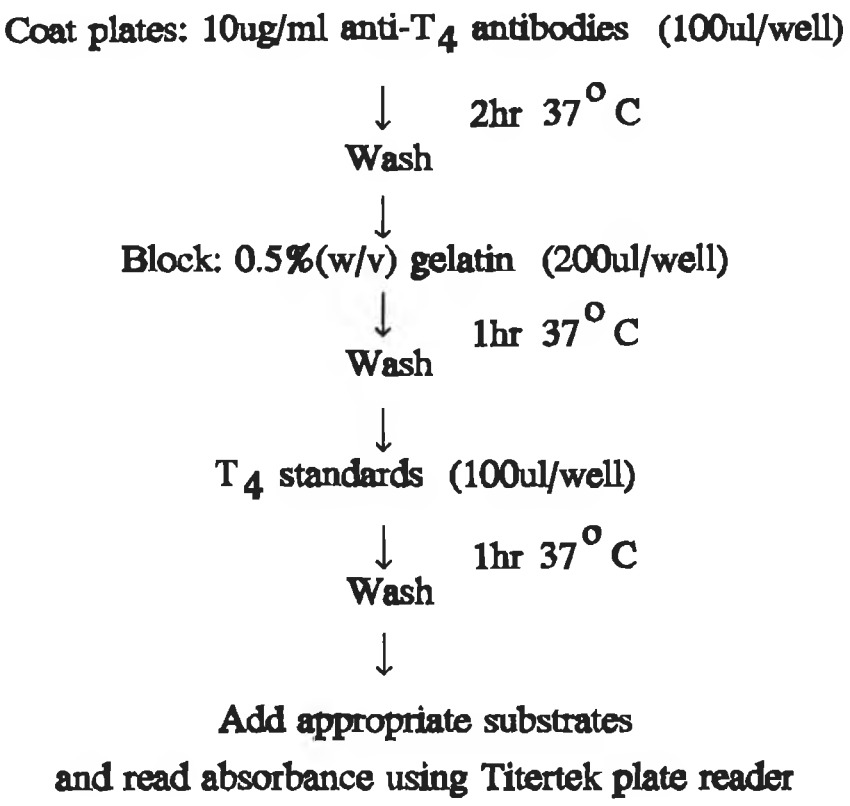

Figure 6.2 Flow diagram of the immunoassay for measurement of T_4. (A detailed description of the protocol is given in Section 2.10).

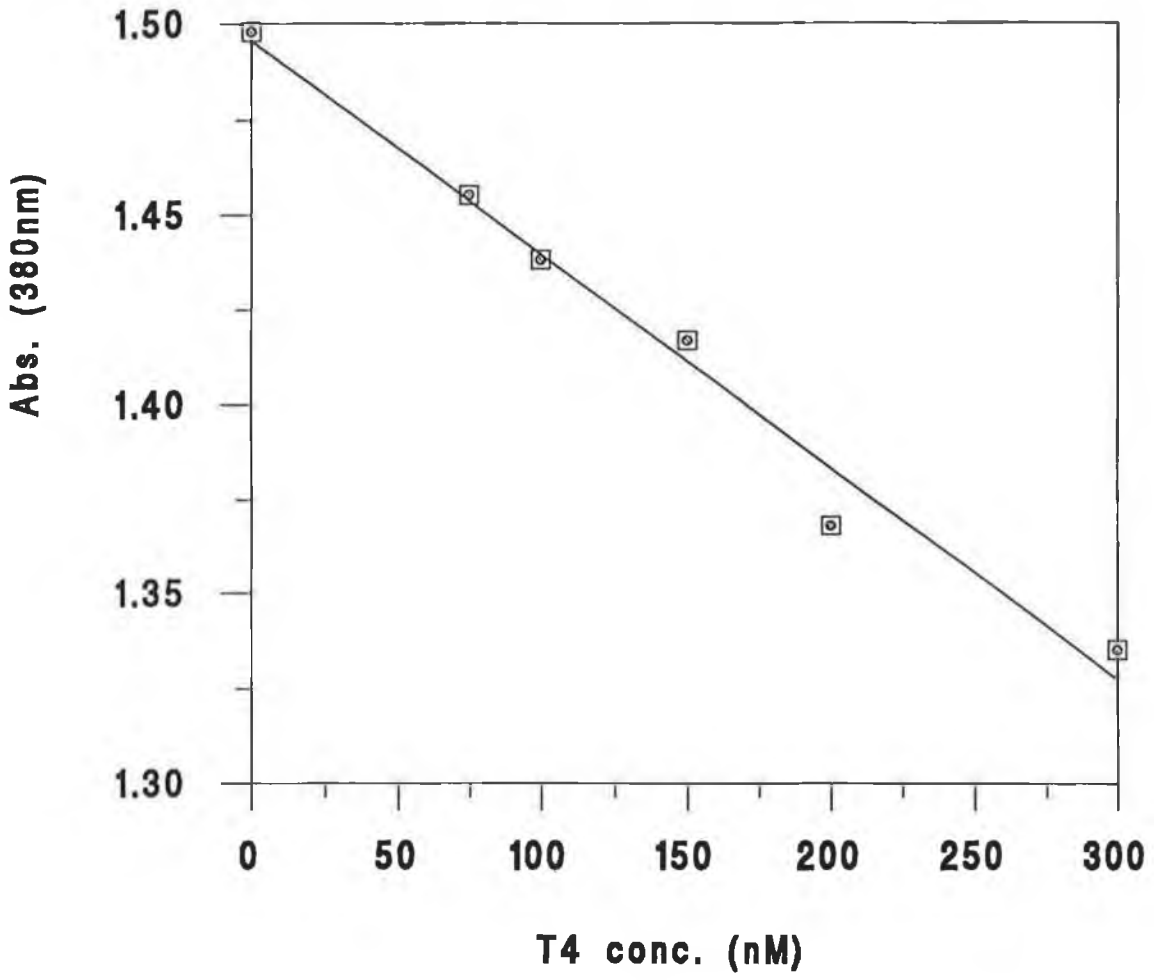

Figure 6.3 Graph of absorbance at 380nm plotted against concentration for T_4 standards assayed in the T_4 immunoassay.

7. CONCLUSION.

Historically, radioactive iodine has been used as a label for the development of sensitive immunoassays, but there are hazards associated with the use of radioisotopes. Non-radioactive iodine can be used as an alternative label in immunoassays. Iodination techniques are widely used and well characterised, and these can be applied to the labelling of proteins with non-radioactive iodine for use as a tracer in immunoassays. The iodine label can be detected using a chemical method.

The reaction between cerium(IV) and arsenic(III), the Sandell-Kolthoff reaction, has been used for the detection of iodine. O'Kennedy *et al.* (1989) developed a microassay for iodine based on the Sandell-Kolthoff reaction. This was later used to detect IBHR-labelled antibodies in immunoassays for mouse IgG (O'Kennedy and Keating, 1991) and human IgG (O'Kennedy and Keating, 1993).

A microassay to measure iodine, based on the reaction between cerium(IV) and antimony(III), was developed. This assay was optimised, and was proved to be an alternative to the Sandell-Kolthoff microassay. The microassay was monitored both spectrophotometrically, by measuring the absorbance of the cerium(IV), and fluorimetrically, by measuring the fluorescence of cerium(III) or measuring the fluorescence produced by the oxidation of 8-hydroxyquinoline-5-sulphonic acid by cerium(IV). Potassium iodide standards in the range 2 to 10ng/ml were measured in the spectrophotometric microassay; standards in the range 2 to 8ng/ml were measured in the fluorimetric microassays. The spectrophotometric assay had a wider linear range, and better accuracy and precision.

The microassay was used to measure IBHR standards in the range 0.02 to 0.1µg/ml. The catalytic activity of iodine in iodo-compounds depends on the liberation of the iodine by cerium(IV), and is inferior to that of inorganic iodide (Bowden *et al.*, 1955). On treatment with cerium(IV), 15.5% of the iodine in IBHR was liberated. The catalytic activity of IBHR on the cerium(IV) - antimony(III) reaction is limited by the fact that only a percentage of the iodine is liberated to catalyse the reaction. Thus, it was not possible to achieve the same sensitivity using IBHR standards as with potassium iodide standards.

IBHR-labelled antibodies were prepared, and were used in an immunoassay for human IgG, with detection of the IBHR label by the cerium(IV) - antimony(III) reaction or the Sandell-Kolthoff reaction. Both of these systems could be used to measure human IgG standards in the range 0.05 to 2µg/ml. The results obtained illustrated that the

cerium(IV) - antimony(III) reaction could be successfully used as a replacement to the Sandell-Kolthoff reaction, for the detection of the IBHR label in immunoassays. The immunoassay was also carried out using HRP-labelled antibodies. Human IgG standards in the range 0.0064 to 0.25µg/ml were measured using HRP-labelled antibodies. The systems using the IBHR label and the HRP label were comparable with respect to accuracy and precision of the standards, and with respect to the results obtained for serum samples. However, lower concentrations of human IgG could be measured using the HRP label, and the difference in absorbance values between the zero standard and the highest standard was greater using the HRP label (1.163) compared with the IBHR label (0.666).

The iodination of antibodies with non-radioactive IBHR was examined. Iodination techniques using radioactive iodine are well characterised, but the use of non-radioactive iodine is not. The iodination of antibodies with various levels of IBHR was investigated, using molar ratios of IBHR to free amino groups of the antibody of 0.5:1, 1.1:1, 2.3:1, 4.6:1, 6.9:1 and 9.1:1. (A molar ratio of 4.6:1 was used for the production of IBHR-labelled antibodies for use in the immunoassay for human IgG). Greater conjugation of IBHR was achieved using higher molar ratios. The relative binding pattern of the iodinated antibodies was assessed using the BIAcore. The binding to antigen decreased, relative to the binding of native antibodies, as the level of conjugation of IBHR increased. In an immunoassay for human IgG, the use of the conjugate prepared using a 0.5:1 ratio resulted in a loss of sensitivity, as the cerium(IV) - antimony(III) reaction could not detect the low concentrations of label present in the immunoassay, and the resulting standard curve had a low slope value. Using conjugates prepared at higher molar ratios resulted in greater sensitivity. The slopes of the standard curves increased with increasing conjugation of IBHR, but so too did non-specific binding. Conjugates produced using 2.3:1 and 4.6:1 ratios gave the best performance in the immunoassay.

A 4.6:1 molar ratio was the ratio already in use. Thus, changing the level of iodination of the antibody would not result in any significant improvement to the immunoassay performance. Therefore, procedures that lead to a high accumulation of label per antigen molecule were examined in an effort to improve the immunoassay. Two methods were investigated: the avidin-biotin system and bispecific $F(ab')_2$ antibodies. A LAB immunoassay, using biotinylated anti-human IgG antibodies and IBHR-labelled avidin, was used to measure human IgG standards in the range 0.025 to 0.5µg/ml. A BRAB immunoassay, using biotinylated anti-human IgG antibodies, avidin and labelled

biotin, was used to measure standards in the range 0.05 to 2µg/ml. The measuring range of the BRAB immunoassay was the same using IBHR-labelled biotin or biotinylated HRP, but the use of the HRP label produced a difference in absorbance values between the zero standard and the 2µg/ml standard of 1.030, while the difference was, on average, only 0.099 using the IBHR label. The LAB immunoassay could be used to measure lower concentrations of human IgG than the BRAB immunoassay and was an improvement on the conventional immunoassay using IBHR-labelled antibodies.

Bispecific $F(ab')_2$ antibodies, reacting with human IgG and BSA, were used in an immunoassay as bridging agents between human IgG and labelled BSA. Using BSA, iodinated by the IBHR and chloramine T methods of iodination, human IgG standards in the range 0.1 to 2µg/ml could be measured, while using HRP-labelled BSA standards in the range 0.0125 to 1µg/ml could be measured. As before, the use of the HRP label produced a greater difference in absorbance values between the zero standard and the highest standard; the difference was 0.123 using iodinated BSA, and 0.854 using HRP-labelled BSA.

An immunoassay for T_4, which used the four iodine atoms on T_4 as the label, was developed. Anti-T_4 antibodies, immobilised onto microtitre plates, were used to capture the T_4, and the iodine was detected by the cerium(IV) - antimony(III) reaction. The assay could be used to measure T_4 standards in the range 75 to 300nM. The format of this immunoassay is simple and there is no requirement to prepare labelled derivatives of either antigen or antibody. However, in order to be used to measure T_4 in patient samples a detection limit of less than 50nM would be required.

The detection limit in immunoassays is determined by several factors, including the detection system for the label. In all the immunoassay systems tested here, the use of HRP-labelled reagents resulted in detection of lower concentrations of analytes and a greater difference in absorbance values over the range of the standard curves than the use of IBHR-labelled reagents. Since only a percentage of the iodine in IBHR is catalytically active, only a percentage of the iodine label present can be detected by the cerium(IV) - antimony(III) reaction. An improvement in the detection of the iodine label would lead to an improvement in the performance of immunoassays using IBHR-labelled reagents.

Fluorimetric monitoring of reactions is, in general, more sensitive than spectrophotometric monitoring. However, for the cerium(IV) - antimony(III) microassay the detection limit was not improved by the use of fluorimetric monitoring.

This may have been due to the spectrophotometer used to measure the fluorescence intensity. This was capable of reading cuvettes and plates, but with a 40 fold reduction in fluorescence intensity when reading from plates. Manual alteration of the optics had to be carried out in order to read from plates, which was not ideal as the alignment of the optics is critical and manual alteration could lead to damage of the optics. The detection limit of the microassay may, therefore, have been improved if a dedicated fluorimetric plate reader had been used to measure the fluorescence intensity. The cerium(IV) - antimony(III) reaction is just one of many kinetic reactions which are catalysed by iodide. One of these reactions could be used as an alternative detection system for the IBHR label. Since cerium(IV) is a one electron oxidising agent that oxidises organic compounds, an alternative reaction would only be suitable if it involved a reactant that could also oxidise iodine-containing organic compounds with subsequent liberation of the iodine. Alternatives to IBHR could be used as labelling reagents. The use of di-IBHR was considered as an alternative label which would increase the amount of iodine present per label molecule. However, the use of di-IBHR did not improve the detection limit of the cerium(IV) - antimony(III) reaction, as the same range of standards could be measured for IBHR and di-IBHR.

The use of non-radioactive iodine, and of the cerium(IV) - antimony(III) reaction for detection of the iodine, has several advantages. The Sandell-Kolthoff reaction has been used to measure iodine in samples such as milk, urine, salt and pharmaceutical samples. The cerium(IV) - antimony(III) reaction could be used as a replacement for the Sandell-Kolthoff reaction, as it has a comparable measuring range, and the requirement to use arsenious acid, a carcinogen, would be eliminated. Non-radioactive iodine can be used as a replacement for ^{125}I as a label in assays where an iodine label is required, but sensitivity is not critical. There are several examples of this. The immunoassays described here for human IgG illustrate that non-radioactive iodine and the cerium(IV) - antimony(III) reaction are suitable for measurement of analytes which are present in milligramme amounts in serum. Keating *et al.* (1991) used non-radioactive iodine-labelled BSA, with the Sandell-Kolthoff reaction for detection, for the determination of proteolytic activity. Microtitre plates were coated with IBHR-labelled BSA, protease was added, and after washing the amount of iodinated substrate remaining was determined and used to calculate the proteolytic activity. The enzymes trypsin, pepsin, papain and collagenase were tested. Nolan (1994) used non-radioactive iodine-labelled IgG, $F(ab')_2$, Fab' and bispecific $F(ab')_2$ antibodies to determine relative affinity values. Microtitre plates were coated with the appropriate antigen, and the

iodinated antibodies were added. After washing the iodine label was measured using the Sandell-Kolthoff reaction, and used to calculate the relative binding of the antibody fragments. A laboratory that does not have facilities for using radioactive compounds but needs to use a protein purification protocol that requires the use of ^{125}I to "track" the protein during the purification steps could use the non-radioactive iodine label instead of ^{125}I.

The major drawback to the use of the non-radioactive iodine label in immunoassays is the poor sensitivity compared to an enzyme label. The sensitivity is limited by several factors. Iodine must be liberated from iodine-containing organic compounds in order to be catalytically active, but only a percentage of the total iodine is liberated by cerium(IV). Detection of the cerium(IV) - antimony(III) reaction is not performed at the absorbance maximum for cerium(IV), the dynamic range of the spectrophotometric measurement of this reaction is narrower than that of the reaction for detection of the HRP label, and the decrease in absorbance is measured against a high blank value. Due to the poor sensitivity the non-radioactive iodine label would not be suitable for detection of analytes where sensitivity is critical.

The work reported in this thesis illustrates the effectiveness of the cerium(IV) - antimony(III) microassay as a replacement for the Sandell-Kolthoff reaction for detection of iodide and iodine-containing organic compounds. It also illustrates the effectiveness of non-radioactive iodine as a label in immunoassay systems using iodinated antibodies, the avidin-biotin system and bispecific $F(ab')_2$ antibodies.

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