THE DEVELOPMENT AND USE OF A NON-RADIOACTIVE IODINE-LABELLING SYSTEM FOR MICROASSAYS

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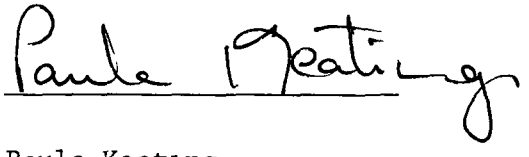
December 1990

I hereby declare that the research described within this thesis is based entirely upon my own work



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PUBLICATIONS

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2 Keating, P , and O'Kennedy, R (1990) "The development and use of a non-radioactive iodine labelling and detection system for microassays " BIOTECH'90 Trans (USA)

3 Keating, P , and O'Kennedy, R (1991) "Use and detection of non-radioactive iodine-labelled antibodies for immunoassay " Anal Biochem (in press)

CONTENTS

ABBREVIATIONS

Ab	Antibody
ACTH	Adrenocoticotrophic hormone
Ag	Antigen
hAFP	Human alpha fetoprotein
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
ϵ	Extinction coefficient
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
ESR	Electron spin resonance
Fab	Antigen binding fragment of antibody
Fc	Crystallizable fragment of antibody
FIA	Flourescence immunoassay
FRIAT	Free-radical immunoassay technique
IBHR	Iodinated Bolton Hunter reagent
IgG	Immunoglobulin G
IRMA	Immunoradiometric assay
KI	Potassium iodide
mw	Molecular weight
NAD(P)H	Nicotinamide adenine (phosphate) dinucleotide
PBS	Phosphate buffered saline
RIA	Radioimmunoassay
SD	Standard deviation
SE	Standard error
SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate

ABSTRACT

The research described in this thesis has centred on the development, detection and evaluation of a non-radioactive iodine-label for assay purposes Traditionally, radioactively-labelled iodine has been used as a reporter group for the detection of analytes in test samples

The catalytic effect of iodide on the oxidation of arsenic III by cerium IV was used to measure iodine-containing compounds The reaction can be followed spectrophotometrically by measuring the intensity of the cerium yellow colour after a defined time period The assay procedure was optimised with respect to temperature, incubation time, detection wavelength, concentration of reagents and acidity of reacting medium

The iodine labelling system was then successfully applied to the detection of immunoglobulin G levels in biological samples from human and mouse sources using a sandwich type immunoassay The performance of the iodine label was then evaluated against an enzyme label Attempts were made to increase the sensitivity of the iodide immunoassay through the conjugation of poly-L-lysine to second antibody This procedure should increase the number of iodide binding sites available The successful application of the iodide assay to the detection of proteolytic activity is also described

The assay system described uses small quantities of reagents, is suitable for use with a photometric microplate reader, can test many samples simultaneously, eliminates problems associated with the use of radioisotopes and has good sensitivity and reproducibility

1 INTRODUCTION

There are many well described methods using tracers in the measurement of analytes One of the most widely used of such systems involve the use of radioactive labels Radioactivity can be detected with great sensitivity by simple methods that are virtually unaffected by the reaction milieu and independent of other environmental factors However, radiolabels have drawbacks associated with health risks and, in recent years, the disposal of radioactive waste has become a major concern

Stemming from these problems much research has focused on finding alternatives Some of these alternatives include the use of enzymes, flourescence, red cells, luminescence and free radicals as labels The objective of this project was to develop and evaluate a novel non-radioactive iodide labelling system

From the wealth of iodination techniques described in the literature proteins, cell membranes and nucleic acids can be labelled with iodine The catalytic effect of iodide on the oxidation of arsenic III by cerium IV is then used to measure iodine-containing compounds The applicability of non-radioactive iodine as a label is evaluated in an immunoassay The use of an iodine label is also applied to a proteinase assay

1 1 IMMUNOASSAYS AND THE USE OF LABELS

Immunoassays use the specific interaction of antibody with antigen to provide quantitative information about antigen (or antibody) concentration in samples This technique exploits the

extraordinary discriminatory powers of antibodies, based on the ability of the immune system of vertebrates to produce a virtually unlimited variety of antibodies, each with an affinity for a specific foreign compound (antigen or hapten) This immunochemical reaction is one of the most selective chemical reactions known, its high selectivity permitting assays in complex biological media with little interference The sensitivity of an immunoassay depends primarily on the type of tracer or label used to tag the antigen or antibody for detection Any material which can be accurately determined at low levels, and which can be firmly attached to the ligand molecule without grossly altering its properties, may serve as a tracer

Immunoassays can be very sensitive and specific and, therefore, are commonly used for a great variety of measurements both in research and analytical laboratories

1 1 1 RADIOISOTOPIC LABELS

The use of radiolabelled ligands as a means of quantifying small molecules came about from the demonstrations of *Yalow and Berson* (1959) who measured the binding of trace amounts of radioiodinated insulin to an insulin specific antibody Subsequent to these initial studies radiolabels came to predominate in their use in immunoassay procedures

Radiolabelled ligands can be prepared with radioisotopes substituted for the chemically similar, but non-radioactive stable isotopes Molecules labeled with ^{3}H and ^{14}C are examples of this Such labels require extensive sample preparation and are quite expensive They have been replaced increasingly with chemically substituted radioactive atoms, radioiodine being the usual choice

^{125}I is chemically very reactive and can be incorporated into a wide range of biological molecules (to be described in section 1 2 2)

The use of radioactive tracers in immunoassays can be divided into radioimmunoassays (RIA) where the analyte is labeled, and immunoradiometric assays (IRMA) where the antibody is labeled An outline of the reactions is given below

Radioimmunoassay

$$[^{125}I]Ag + Ag + Ab \longrightarrow AgAb + [^{125}I]AgAb$$

labelled antigen	free antigen	antibody	antibody antigen complex	labelled antigen antibody complex

Immunoradiometric assay

$$Ag + [^{125}I]Ab \longrightarrow Ag[^{125}I]Ab$$

free antigen	labelled antibody	labelled antigen-antibody complex

Radioimmunoassays depend upon competition between a fixed amount of labelled antigen and a variable amount of unlabelled test antigen, for the specific antigen-binding sites of a limited number of antibody molecules The more unlabelled antigen added to the system, the less tracer will be bound The amount of labelled antigen bound

by a fixed level of antibody is inversely proportional to the amount of unlabelled antigen present This can be measured after separation of antibody-bound tracer from the free tracer and counting one or other or both fractions The standard curve allows a precise determination of the amount of unlabelled antigen present in the solution

The immunoradiometric assays were first developed by *Miles and Hales* (1968) IRMA's use an excess but constant amount of labelled antibody The excess radiolabelled antibody is allowed to react with the antigen in the sample Once equilibrium has been reached, a solid-phase antigen preparation is added to the reaction tube where it binds and precipitates the excess radiolabelled antibody The radioactivity in the supernatant is left as a direct measure of antigen concentration In the "two-site" immunometric assay, the complex of radiolabelled antibody and antigen is precipitated from the reaction by the addition of excess solid-phase antibody directed towards the second antigenic binding site on the antigen Another variation, involves the indirect labelling of the antibody using a radiolabelled second antibody

Radioactive labels have proved very advantageous in their use in immunoassays Radioactivity can be detected with great sensitivity by simple methods that are virtually unaffected by the reaction milieu and independent of other environmental factors Radiolabelling procedures are relatively simple to perform with many radiolabelled compounds commercially available As radiolabels are comparatively small they do not normally cause steric hindrance However, use of radiolabels has the associated problem of radioactive waste disposal Other disadvantages include the large expense of

counting instrumentation and licensing, the radioactive half-life, radiolysis of the labelled reagent which limits assay sensitivity and imposes a time constraint, and the cumulative real risk in preparation of radiolabels The disadvantages of radiolabels encouraged the search for alternate strategies

1 1 2 ENZYME LABELS

The enzyme is the most widely used of alternative labels to radiolabels There are many reviews of the technique (*Blake and Gould*,1984, *Monroe*,1984, *Tijssen*,1985, and *O'Kennedy*,1989) Enzyme labels are particularily useful in immunoassay in that their catalytic activity on their substrate to product reaction provides the chemical amplification that allows the detection of extremely low concentrations of analyte Enzyme labelling actually became practical when *Avrameas* (1969) used glutaraldehyde to couple peroxidase to antibodies Once this method became available, antibodies conjugated with enzymes could easily be used for locating and identifying specific tissue antigens

The use of enzyme labels in immunoassays is analogous to the use of radioisotopes Enzymes may be linked to antigen for use in immunoassays or linked to antibody for immunometric assay systems In EIA's, after separation of bound from free fractions, the quantity of labelled immunological reagent is monitored by measuring the absorbance change after addition of enzyme substrate Detection techniques applied to quantify the enzymatic reaction include the use of fluorimetry, luminescence and electrochemical techniques while the most common method of detection is spectrophotometry

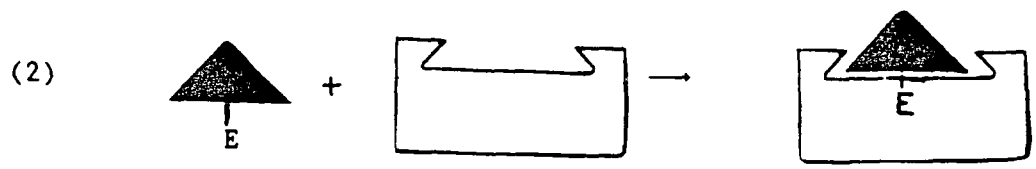
Enzyme immunoassays may be classified as heterogenous or

homogenous assays Heterogenous EIA technique originated with the work of *Van Weemen and Schuurs* (1971) and *Engvall and Perlmann* (1971) In the heterogenous assay antigen-antibody complexes are physically separated from free antigen and antibody using some type of solid phase system The use of solid supports was given the name Enzyme-Linked ImmunoSorbent Assay (ELISA) by *Engvall and Perlmann* (1971) The homogenous assay technique was introduced by *Rubenstein et al* (1972) No separation step is required with this assay technique and it depends on a change in the enzyme activity when antibody binds to enzyme-labelled antigen The activity of the unseparated assay mixture corresponds to the proportion of enzyme-labelled antigen to which antibody is bound Enzyme multiplied immunoassay technique (EMIT) is an example of a homogenous assay system (Fig 1 1)

Both heterogenous and homogenous assays can be further subdivided into two main divisions, i e competitive and non-competitive assays The former is based on the competitive equilibrium between an excess of labelled and unlabelled antigen for a limited amount of highly specific antibody In a non-competitive assay, the antigen to be measured reacts with the antibody bound to a solid phase and this binding is detected using enzyme-labelled second antibody The sandwich ELISA technique illustrated in Figure 1 2 is an example of such an assay A comparison of both ELISA and EMIT is outlined in Table 1 1

Figure 1.1 A typical enzyme multiplied immunoassay technique (EMIT)

Enzyme Hapten Hapten-Enzyme Conjugate



Active enzyme antibody antibody-bound hapten-enzyme conjugate (inactive enzyme)

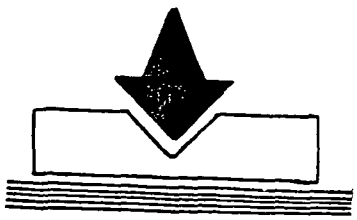
- hapten

E - enzyme

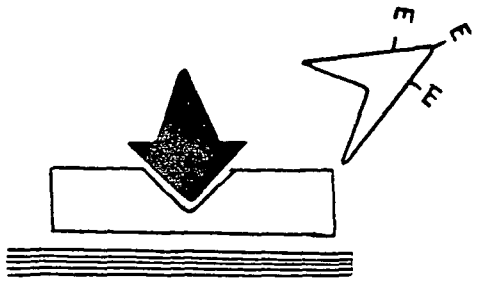
- antibody

Figure 1 2 A typical enzyme-immunosorbent assay technique (ELISA)

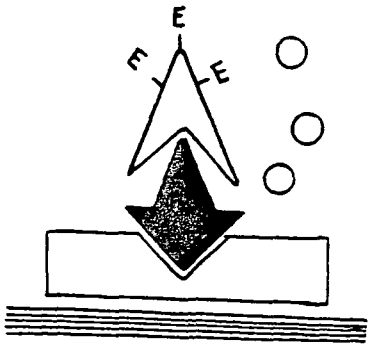
(1) Antibody bound to polystyrene well plus antigen to be measured



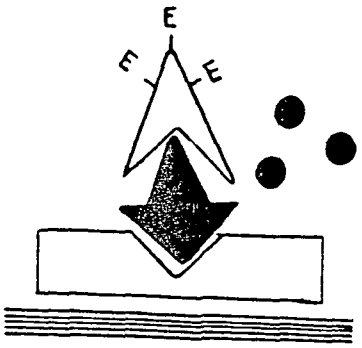
(2) Enzyme conjugate added to well with bound antigen-antibody immune complex

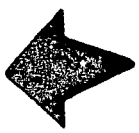


(3) Substrate added to enzyme-conjugate bound to immune complex

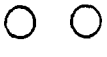


(4) Positive test or colour change denoting changed substrate due to presence of enzyme conjugate bound to immune complex



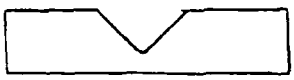


- hapten



- substrate

- chemically changed substrate



- antibody

- enzyme-conjugate

There are numerous enzymes available for use as labels in immunoassay, but none can be classified as the ideal label A suitable enzyme must be selected for each assay application The enzyme labels most commonly used in heterogenous EIA's together with their substrate systems are listed in Table 1 2 The most popular are peroxidase and alkaline phosphatase Several substrates are available for both of these enzymes which give soluble or insoluble products Fluorescent and luminescent substrate products are also available thus increasing the utility of the assay system

If samples do not posses endogenous peroxidase activity then peroxidase is the cheapest of these enzymes Of the several substrate systems available to peroxidase most have been shown to be carcinogenic or mutagenic in test systems (*Voogd et al* ,1980) However, peroxidase is an easy and useful enzyme for conjugation and the resulting conjugates are very stable

Alkaline phosphatase and its conjugates are stable However, the procedure for its conjugation to antibodies or antigens is more difficult as polymerisation often occurs The substrates used with alkaline phosphatase are generally safe chemicals with no reported toxic properties The main drawback with the use of alkaline phosphatase is its expense which is due to the steps involved in its purification from calf intestine, the preferred source

Since EMIT does not require a separation of immune reactants from the sample before measuring enzyme activity the choice of enzyme is more critical This choice is also limited by the fact that the enzyme must be capable of being conjugated near the active site without significant loss of activity Binding of the antigen to the antibody then affects the activity of the enzyme by preventing access

of the substrate to the active site The enzymes most commonly used in homogenous EIA's include glucose-6-phosphate dehydrogenase and malate dehydrogenase Both enzymes are measured by monitoring the conversion of the cofactor NAD to NADH in a spectrophotometer using absorbance measurements at 340nm

Conjugation of Enzymes

The ease with which enzymes can be chemically bound to antibodies and antigens has made possible the production of protein conjugates with high enzymatic and immunological activity An ideal conjugation procedure should yield 100% conjugate of well defined composition without inactivation of the enzyme or the ligand, produce a stable link, be cheap and be easy to perform The original methods whereby chemical labelling was accomplished are still the most popular The development and application of new conjugation methods eliminates most of the problems of the original procedure

For the conjugation of glycoproteins (e g peroxidase) the periodate oxidation method is very useful (*Nakane and Kawaoi*,1974) Periodate oxidizes carbohydrates to form aldehyde groups which react with free amino groups on the antibody The original method has been optimised by *Tijssen*(1985) and gives better results in terms of ease and extent of conjugation and activity of conjugates

Proteins can be readily cross-linked by reaction with the homobifunctional reagent glutaraldehyde, mainly through the amino groups of lysyl residues of proteins (Avrameas,1969) This glutaraldehyde crosslinking may be performed by two methods (1) the one-step procedure, in which the enzyme, the protein carrier and glutaraldehyde are reacted in a single step, (2) a two-step procedure

in which enzyme and glutaraldehyde are first reacted, then after removal of excess glutaraldehyde the protein is coupled in a second step (*Schuurs and Van Weemen*,1977) One of the drawbacks with this procedure includes polymerisation effects

Other bifunctional reagents that can be used for conjugation include N,N'-o-phenylenediamalemide (*Kato et al* ,1975), and bis-succinic acid N-hydroxysuccinimide ester (*Ryan et al* ,1976) The use of the heterobifunctional reagent N-succinimidyl-3-(2 pyridyldithio)propionate may be used for the production of intermolecular conjugates, although results obtained were not always satisfactory due to instability problems(*Tijssen*,1985) Other heterbifunctional reagents include esters of maliemide derivatives which have been found to be more successful (*O'Sullivan et al* ,1978)

Numerous cross-linking procedures have been used to form hapten-enzyme conjugates and most of them are similar to those used for the preparation of hapten-immunogens Due to their low molecular weight and great variety, haptens have few and various attachment sites and it may often be necessary to introduce a reactive group to facilitate the cross-linking reaction

Enzyme labels have proved favourable alternatives to radioisotopes as is evident by the ever increasing number of applications for which they are used The formation of a coloured product by the enzyme reaction allows simple visual assessment of the results Another important advantage is the relatively long shelf-life (1 year) of enzyme labels However, enzymes are subject to activators or inhibitors in biological samples This is a particular problem for homogenous assays Activity of enzymes may also be affected by substrate and coenzyme concentration, pH,

temperature and ionic strength All of these factors require accurate control if serious errors are to be avoided

Enzyme labels possess great potential because their use not only eliminates the problems in the use of radioisotopes, but allows the development of a diverse range of assay protocols For example, *Bowden et al* (1986), have developed a liposome-based assay The assay measures complement activity It is a rapid homogenous assay using liposome-encapsulated enzyme which has covalently attached artificial antigen When these bind antibody they initiate complement fixation leading to lysis of the membrane and release of enzyme Enzyme activity may be detected using a suitable substrate and is a measure of complement activity

Systems using enzyme electrodes, enzyme thermistors and rapid automated flow-through thermometric systems are widespread Enzyme immunoassay can also be linked to electrochemical detection (*Jenkins et al* ,1988) and luminescent detection (*Thorpe et al* ,1985) Enzymes offer significant advantages in a wide range of situations Like radioisotopes there are also problems associated with the usage of enzyme labels, for example, some of the enzyme substrates are toxic, enzymes require controlled environmental conditions, there are limitations in the methods available for their linkage to antibodies and also, there is a period of time required for colour development

Table 1 1 A comparison of EIA procedures

ELISA	EMIT
Heterogenous assay	Homogenous assay
Separation step required	Separation step not required
Washing steps required	Washing steps not required
Slower than EMIT	Faster than ELISA
Greater sensitivity than EMIT	Lesser sensitivity than ELISA
Measures large molecules	Measures small molecules
Solid phase assay	Liquid phase assay

Table 1 2 Common enzymes and their substrate systems for EIA

ENZYME	SOLUBLE SUBSTRATE	DETECTION WAVELENGTH (nm)
Peroxidase	O-Phenylenediamine dihydrochloride	492
	Tetramethylbenzidine	450
	5-Aminosalicyclic acid	450
Alkaline phosphatase	N-p-nitrophenyl phosphate	410
β-galactosidase	o-Nitrophenyl-β-D-galactoside	420
Urease	Bromocresol purple	588

1.1.3 FREE-RADICAL LABELS

Free-radical immunoassay techniques (FRIAT) utilize the determination of free radicals linked to antigens or antibodies for the detection of antibody-antigen interaction. The determination of the free radical is by an electron spin resonance (ESR) spectrometer (*Leute et al.*,1972).It is possible to label haptens with a nitrogen-radical. The nitroxide-labelled hapten has a simple spectrum, but the binding of the antibody or antigen causes a broadening of the electron spin resonance signal and a decrease in intensity of the signal. Thus, the assay of the intensity of the ESR signal reflects the concentration of free nitroxide labelled-hapten. In the FRIAT immunoassay the labelled hapten and the unlabelled hapten in the sample compete for binding to antibody. The application of the FRIAT method is limited by the expense of the ESR-spectrometer.

1.1.4 FLUORESCENT LABELS

The possibility of covalently attaching fluorescent substances (fluorophores) to antibody molecules without significantly affecting their ability to react with antigen was demonstrated in 1950 by *Coons and Kaplan*. Fluorophores are chemicals, which, when activated by light of a given wavelength release energy in the form of light at a longer wavelength - this emitted radiation is called "fluorescence". The difference between the activation and emission wavelengths is defined as the Stoke's shift. The ratio between the adsorbed and the emitted light is defined as the quantum efficiency - the theoretical quantum efficiency being one but, as some energy is lost before

emission of radiation occurs, this is rarely attained

In selecting a fluorophore the following characteristics should be selected for

(1) a high quantum yield of flourescence

(2) a high Stoke's shift

(3) an ability of the fluorophore to be excited by a common light source

(4) the emission should be detectable by conventional photomultipliers

and (5) stability and immunological activity

As with other immunoassays the options for fluorescent labelling can be divided into fluorescence immunoassay (FIA) where a labelled antigen is used, and immunofluorometric assay using labelled antibodies Each assay type includes both heterogenous and homogenous approaches

The homogenous assays include the enhancement FIA and direct quenching FIA where fluorescence is enhanced or quenched, respectively, upon the formation of antigen-antibody complex Sensitivity of non-separation assays is limited by interference from fluorescent compounds in the sample Most of the applications of non-separation FIA are designed for analytes present in concentrations greater than 1ng/ml (*Barnard and Collins*,1987)

Heterogenous assays include simultaneous addition FIA analogous to convential RIA and sequential addition FIA where excess antibody, immobilised on a solid-phase, is added After a series of washing steps, fluorescence in the bound fraction is measured

Since 1984, the use of long-lived fluorophores has become possible The emission from long-lived fluorophores can be

distinguished from the background fluorescence with a short decay time using time-resolved fluorometer with appropriate delay, counting and cycle times (*Hemmila et al* 1984) In 1987 the use of a Europium chelator was described and a commercial immunoassay kit using this label (Cyber-Fluor) was made available (*Khosravi and Diamandis*,1987)

Immunofluorescence has been exploited in many areas of biological sciences The advantage of a fluorescent label are those of all non-isotopic labels ie its long shelf-life and the abscence of radiation damage or hazard It is useful to compare immunofluorescence with the alternative immunochemical method which employs enzymes as labels and provides comparable sensitivity Similar procedures are used in both techniques but the enzyme method requires an extra step to produce a coloured reaction product Dual detection using two antibodies to give different colours on reaction is available with FIA's due to the development of specific fluorescent filter systems which enable the individual contribution of each antibody to be separately assessed Enzyme conjugates have an advantage over fluorescent conjugates in that, theoretically, the reaction with the enzyme can be run as long as necessary to detect whatever binding occurs, whilst fluorescence is a transient signal

Most biological fluids, e g serum, have endogenous fluorophores yielding a background noise level interfering with the measurement of the specific signal reducing sensitivity and precision There are continued developments in instrumentation and the increasing of assay sensitivity The application of fluorophores in fluorescence activated cell sorters (FACS) offers further potential for their usage

1 1 5 RED CELLS AS LABELS

Red cell-labelled antibodies are excellent reagents in a whole series of immunoassays, measuring either antigen or antibody itself In the latter case anti-globulins are linked to the red cells On formation of an immune complex haemagglutination occurs which serves as the signal in these assays The red cells are first treated with enzyme to increase their agglutinability Chromic chloride is used for the coupling of antibody to the red cell Chromic chloride essentially cross-links the carboxyl groups on the antibody with the carboxyl groups of the red cell membrane

Red cell immunoassays can also be divided into homogenous or heterogenous assays The homogenous assay is exemplified by the reverse passive haemagglutination test To achieve haemagglutination or cross-linking of antibody-coupled cells, the antigen must have multiple epitopes Specificity is checked by simply showing that the reaction is specifically inhibited if free antibody is added *Scott et al* (1982), using affinity purified anti-IgE-coupled red cells, found this assay method compared very favourably with a double antibody radioimmunoassay for serum IgE in terms of sensitivity

In the heterogenous assay system antibody is adsorbed onto microtitre wells which capture the antigen from the samples Red cells coupled to the specific antibody to antigen are then added and the resulting haemagglutination is a measure of the presence of antigen (*Bradbourne et al* ,1979)

A major advantage of red cell immunoassays is that they can be performed directly on samples, are very rapid and require little instrumentation The most limiting factor of the assay is that it is

very difficult to quantitate As the reaction signal, haemagglutination, can only be detected microscopically or by the naked eye, on serial dilutions of the analyte Thus, it is not suitable for quantifying analytes However, it is a sensitive and inexpensive qualitative method

1 1 6 LUMINESCENT LABELS

Luminescence is the emission of light resulting from the dissipation of energy from a substance in an electronically-excited state The exciting energy in luminescence is provided by a chemical reaction whereas it is in the form of light in fluorescence Luminescence is classed namely as chemiluminescence and bioluminescence

Many organic compounds are chemiluminescent upon oxidation e g isoluminol and its derivatives The limitation to the use of chemiluminescent molecules as probes is that they have a low quantum yield Consequently the light emission upon oxidation is invariably short-lived producing a flash of light However, where chemiluminescence has become very useful is in amplification systems for enzyme labels attached to antibodies or antigen

Thorpe et al ,(1985), discovered that many compounds e g phenols, enhance the light emission from the horseradish peroxidase-catalysed oxidation of luminol The enhancement effect produces a light emission that decays slowly The light output is directly related to the amount of horseradish peroxidase-conjugated antibody or antigen A similar system, using firefly luciferin as enhancer, is commercially available from Amersham

Bioluminescence is a naturally occurring chemiluminescence Here

enzyme-mediated oxidations occur in which the free energy changes are utilised to excite a molecule to a higher energy state The de-excitation process is accompanied by the emission of light In addition to the enzyme (luciferase) and substrate (luciferin), bioluminescent systems require co-factors The firefly luciferase system is specific for ATP whilst the bacterial luciferase system is specific for NADH/NADPH Because of the problems encountered with specific components e g loss of enzyme activity, loss of cofactor specificity on conjugation and instability of substrate, an alternative approach to the use of the bioluminescent system is to use non-specific enzymes as labels that produce cofactors For example, antigens labelled with malate dehydrogenase which produces NADH upon incubation, can then be measured with bacterial luciferase (*Stanley*,1978) Also, a pyruvate kinase antibody-conjugate was used to generate ATP from added ADP and phosphenyl pyruvate, with ATP production monitored by the firefly luciferin reaction(*Fricke et al* ,1982)

Luminescence has potential for use in analytical systems but it is a detection system prone to interference For example, a change in pH can not only reduce the quantum yield obtained, but it can shift the light emission spectrum leading to a change in the maximum wavelength In common with all enzyme-related reactions, the bioluminescent systems will also be susceptible to interference from endogenous enzyme substrates/inhibitors, or endogenous nucleotides where the concentrations of ATP and NAD(P)H are critical

1 2 THE APPLICATION OF NON-RADIOACTIVE IODINE AS A LABEL

1 2 1 THE SANDELL-KOLTHOFF REACTION AND ITS APPLICATIONS

Many methods based on the reaction in which a trace of iodide accelerates the reduction of cerium (IV) by arsenic (III) have been developed and are commonly employed in the analysis of biological samples *Sandell and Kolthoff* (1934), first reported that a minute quantity of iodide could be detected by measuring its catalytic effect on the slow cerium (IV)-arsenic (III) reaction

$$2Ce^{4+} + As^{3+} \longrightarrow 2Ce^{3+} + As^{5+}$$

(yellow) (colourless)

As can be seen from Table 1 2 1 the course of this reaction has been followed by different analytical techniques, photometry affording the lowest detection limit

Table 1 2 1 Determination of iodine with the Ce (iv)/ As (III) system by different techniques

Detection technique	Sensitivity	Reference
Photometric (400nm)	10 - 70 ng/ml	*Mougey and Mason*,1963
Absorptiostat (415nm)	11 - 110 ng/ml	*Weisz and Rothmaier*,1975
Potentiostat	6 - 60 ng/ml	*Weisz et al* ,1974
Biamperostat	ug level	*Pantel and Weisz*,1974

The oxidation of arsenic by cerium is catalysed not only by inorganic iodide but also by iodine-containing organic compounds such as thyroxine , proteins, hormones and drugs (*Knapp and Leopold*,1974, *Mougey and Mason*,1963, *Pantel and Wiesz*,1977, *Pantel*,1982 and Timotheou-Potamia et al ,1985) The Sandell-Kolthoff reaction is ,therefore, often used to determine micro amounts of these species by kinetic methods It has been found that the catalytic activity of iodide in organic compounds is inferior to that of inorganic iodide (*Pantel and Wiesz*,1977) This is due to the nature of the side groups attached to the iodine atom on the parent molecule This has resulted in the concept of a "relative molar coefficient of catalytic activity" F (*Pantel*,1982) This is a measure of catalytic activity of iodo compounds in comparison to that of inorganic iodide *Timotheou-Potamia* (1988) introduced the K coefficient which is defined as the "catalytic activity per iodine atom of the compound"

$$K = \frac{100\,[C_i]}{m\,[C_o]}$$

where $[C_i]$ and $[C_o]$ are the molar concentrations of iodide and organic compound, respectively, which have exactly the same catalytic activity and m is the number of iodine atoms per mole of organic compound The K values of several compounds are given in table 1 2 2

Table 1 2 2 Catalytic activity (K) of iodine atoms in iodo compounds

COMPOUND	K VALUE %
Iodide	100
Thyroxine	62
N-Iodosuccinimide	94
3-Iodotyrosine	77
3,5-Diiodotyrosine	72
IBHR	33
3,5-Diiodothyronine	52
3,5,3-Triiodothyronine	42
3,5,3-Triiodothyroacetic acid	35
4-Iodophenol	40

Catalysed reactions can be applied in different ways in analytical chemistry Methods of analysis can be divided into two main groups

(a) methods based on thermodynamic equilibrium (gravimetry, titrimetry, photometry, etc) and

(b) kinetic methods

In the former, measurements are made in a system which is in a stable (equilibrium) state and time is not a variable, in the latter, it is a temporal course of the chemical reaction which is observed, ie the characteristic quantity measured varies with time

Kinetic methods themselves can be divided into two groups: those based on uncatalysed reactions and those based on catalysed reactions. Both types can be used for the determination of reactants. In catalysed reactions determination of catalyst and substances reacting with them (activators, inhibitors, re-activators) are possible. As the Sandell-Kolthoff reaction is an example of the latter grouping only this class will be described.

An important group of catalytic-kinetic analytical methods use "open systems" in which, during the course of the reaction a reactant is added or a product is removed, or even both may be removed, as opposed to a closed system where no further operations (addition/subtraction) occur after the start of the reaction. The so-called "stat" methods belong to this group. In these "stat" methods , a preset stationery state within the catalysed system is kept constant by adding a suitable reagent so that any change of concentration is just compensated. The concentration of catalyst can be derived from the rate of addition which is just necessary to keep constant a particular concentration required.

In measuring iodide in the iodide catalysed reaction between cerium IV and arsenic III some of the properties used in keeping constant a certain preset state of the system include, the polarization of electrodes (biamperostat), spectrophotometric absorption (absorptiostat) or the potentiostatic methods.

Biamperostat

This method requires two polarised electrodes so that, when both the oxidizable and reducible forms of a species are electroactive, the current flowing at steady state will depend on the species

present at lower concentration With this method a preset biamperometric current, corresponding to a definite concentration of a biamperometrically active substance, is kept just constant The rate of addition of the appropriate reagent provides a measure of the concentration of the catalyst being determined

A biamperometric system has been used to determine a range of iodine-containing organic compounds, because it is not only sensitive but is unaffected by side reactions The system has been used to determine 4-iodophenol (0 16-1 6 mol/ml), 2-iodophenol (0 8-8 nmol/ml) and 4-iodo-N,N-dimethylaniline (0 12-1 2 nmol/ml) (*Pantel*,1982) as well as L-thyroxine (13-130 ng/ml), 5-chloroo-7-iodoquinolin-8-ol (13-130 ng/ml, *Pantel and Weisz*,1977) and iodide (80-800 ng/ml, *Pantel and Weisz*,1974) The biamperometric system has been used for the systematic investigation of substituent effects in monosubstituted iodobenzenes in aqueous and 20% ethanolic solutions (*Pantel*,1982)

Absorptiostat

To a solution containing iodide and an excess of arsenic, a standard cerium IV solution (indicator substance) is added until, after a certain phase of development, a preset absorbance value is reached (*Weisz and Rothmaier*,1975) Each deflection from this preset value, caused by the course of the catalysed reaction, is compensated by adding the necessary volume of cerium IV solution The rate of addition of the cerium IV solution is proportional to the iodide concentration and consequently a measure for its determination Iodide-containing organic compounds such as thyroxine, proteins, hormones and drugs cannot be measured by this technique because of

the yellow iodo complexes formed in solution with cerium IV (*Pantel*,1982)

Potentiometric

Another widely applicable method for the regulation of a stat apparatus is potentiometry Potentiometry involves a reversible exchange of a charged species across an electrode/solution interface In the CeIV/AsIII reaction the charged species are electrons The reaction is monitored with a platinum indicator electrode, and the time required for a fixed change in potential is measured and related to the catalyst concentration The method has been applied successfully for the measurement of thyroxine (*Timotheou-Potamia et al* ,1985) and organic iodine-containing compounds (*Timotheou-Potamia*,1982)

The following methods for the determination of iodine-containing compounds involve the use of a closed system (spectrophotometry) whereby no operations are needed after the start of the reaction *Chaney* (1940), used the Sandell-Kolthoff reaction to measure iodine in blood spectrophotometrically measuring the intensity of the yellow colour of the cerium IV at various time periods The percentage transmittance is linearly related to concentration of iodine content *Mougey and Mason*(1963), and *Knapp and Leopold*(1974) have also measured iodide by recording the change in percentage transmittance over a defined time period A stopped flow method coupled to a spectrophotometer for the determination of iodide has recently been developed which is fast and suitable for routine analysis (*Gutierrez et al* ,1989) *O'Kennedy et al* ,(1989), applied

the cerium/arsenious reaction to a microassay format to measure iodine-containing compounds The microassay was suitable for use with a photometric microplate reader, allowing for detection of many samples simultaneously

Other spectrophotometric applications include the measurement of iodide in conjunction with a separation step *Nachtmann et al* ,(1978), described a detection system for high-performance liquid chromatography in which the separation column is coupled to a catalytic detection system for iodide based on the cerium/arsenic reaction The decolouration of cerium IV by tetraiodothyroxine and triiodothyronine was measured spectrophotometrically at 365nm *Bowden et al* ,(1954) describe the detection of inorganic and organic iodine-containing compounds on paper chromatograms using the cerium/arsenic reaction The mixed ceric sulphate-arsenious acid reagent was applied evenly to a sheet of chromatograph paper of the same size as the chromatogram on which the iodocompounds were separated The wetted paper was placed on the dry chromatogram and left for 30 minutes Paper then dried in an iodine free atmosphere The positions of iodine-containing compounds were indicated by white spots on a yellow background

The microassay developed by *O'Kennedy et al* (1989), was also applied to the measurement of iodinated ovalbumin and human serum albumin, demonstrating the use of iodine as an inorganic catalyst in biological systems In this thesis the applicability of non-radioactive iodine as a catalyst in applications using antibodies (immunoassays) and radioiodinated compounds (proteinase assays) for detection is demonstrated

1 2 2 IODINATION TECHNIQUES

To further apply the Sandell-Kolthoff reaction to the measurement of analytes in biological samples, proteins can be labelled with iodine for detection with this reaction The following section reviews iodination techniques The chemistry involved in the iodination of proteins and other molecules is well characterized and a considerable wealth of experience in labelling methodology exists Iodination of proteins may be accomplished by a variety of techniques, each with its own set of conditions for producing maximum labelling efficiencies These differences in labelling conditions may result in differences in the properties of the resulting labelled proteins There are broadly two techniques described by which iodine can be introduced into the functional groups present in proteins

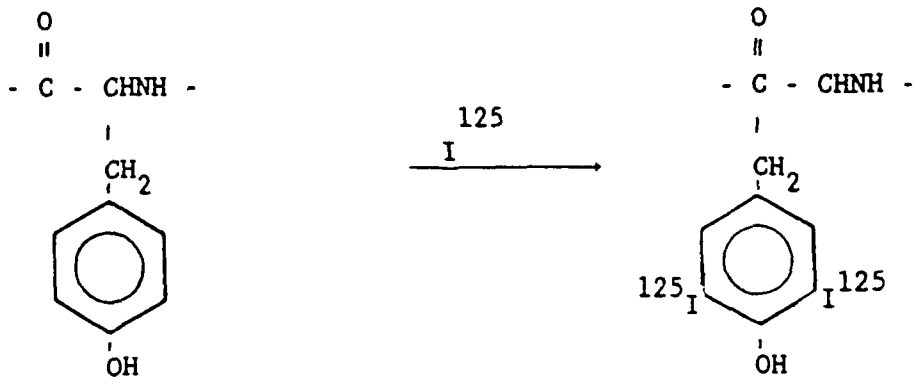
(A) substitution (chemical, electrolytic and enzymatic)

and (B) conjugation

1 2 2 1 SUBSTITUTION

The reaction consists of the electrolphilic substitution of a hydrogen atom by iodine into the tyrosyl or histydl moiety of proteins (*Hughes*,1957) Between pH 7 and pH 8, the ortho position in the aromatic ring of tyrosine of peptides and proteins is activated for electrophilic attack, owing to the electron donating effect of the neighbouring hydroxyl group Similarily, at pH 9, the two nitrogen atoms in the imidazole ring of histidine have an electron donating effect on their mutual neighbouring carbon atom Iodine in the form of iodous ion (I^+) acts as the electrophilic agent to give the labelled tracer The reaction is illustrated in Figure 1 4 1

Figure 1 4 1 Tyrosine iodination



The substitution with iodine in tyrosine residues may result in monoiodotyrosine or diiodotyrosine, as the reactivity of the individual tyrosine residues depends on availability during the iodination reaction Tyrosine residues in a protein molecule may attain specific positions allowing better exposure for labelling depending on buffer strength, pH value or the presence of unfolding reagents such as urea The molar amount of iodine relative to the molar amount of protein and the efficiency of stirring during the reaction will also influence the final iodine distribution among

tyrosine residues and the formation of mono- and diiodotyrosine

The advantages of direct iodination methods are their manipulative simplicity and the high yields of incorporation of iodine which are routinely obtained However, this approach has certain drawbacks, particularly when it involves exposure of the peptide to both oxidizing and reducing agents during the labelling procedure

Substitution methods include

(i) Chemical (a) Chloramine-T

(b) Iodine monochloride

(c) Iodogen

(d) Iodobead

(ii) Enzymatic

(iii) Electrolytic

(i) CHEMICAL

(a) CHLORAMINE-T

Information gained through the use of labelled proteins can be used with confidence if it is established that minimal if any, alteration occurs in the reactivity of the protein compared with that observed in the native form following labelling Iodination employing a modification of the chloramine-T labelling method, originally described by *Greenwood et al* (1964), meets this criterion as has been demonstrated in many laboratories with a wide spectrum of proteins

I^+ is needed for electrophilic substitution of a proton in the benzene ring of tyrosine, whereas the iodine present in NaI is present as anion Therefore, the catalysts used in iodinating

tyrosine are oxidizers which are capable of subtracting two electrons from iodine anion to transform it to the cation i e to perform the transformation $I^- \longrightarrow I^+$

N-chlorotoluenesulphamide under the trade name Chloramine-T is utilized as one such catalyst (Fig 1 4 2a) The reaction has been widely used for obtaining radiolabelled preparations with high specific activity The reaction is initiated on addition of chloramine-T to the mixture of protein and sodium iodide Iodination is generally complete within one minute at room temperature The reaction is then stopped by adding sodium metabisulphite to reduce excess chloramine-T The addition of tyrosine is often favoured as sodium metabisulphite can cause protein denaturation (*Benade and Ihle* ,1982) The labelled protein is then separated from any free label by gel filtration or dialysis

As the iodination is carried out in the presence of a powerful oxidising agent, protein denaturation is an ever present danger This denaturation results from the oxidation of labile residues such as methionine (*Marchalonis*,1968) and can manifest itself in an altered electrophoretic mobility and the loss of biological activity (*Landon et al* ,1968) These changes may result in altered affinity of labelled antigen for antibody causing decrease in immunoassay sensitivity In such cases alternative methods of iodination must be employed Furthermore, it has been noted by *McConahey and Dixon* (1980) that proteins with a high specific reducing capacity may be poorly iodinated by this method Chloramine-T, being an oxidising agent is itself reduced, leaving insufficient chloramine-T to complete the reaction In general, it has been found that the yield of iodinated proteins with chloramine-T is variable and

unpredictable However the chloramine-T method is a technically straightforward method requiring inexpensive, readily available reagents and has a conveniently short completion time for the reaction

(b) IODOGEN

Another iodination system, involving a similar approach, is the introduction of a water insoluble oxidizer instead of chloramine-T This may be achieved with the agent IODOGEN (produced by the Pierce Chemical Co) (Fig 1 4 2b) This is 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril and was proposed by *Fraker and Speck*, (1978) It is soluble in chloroform and methylene chloride and can be layered onto the walls of test tubes from these solvents Thus, the oxidizer comes into less contact with the compound to be iodinated than with chloramine-T This substrate is not soluble in water and acts as a solid oxidizer for iodination of the protein To stop the reaction the solution is poured out of the test tube The incidence of side reactions involving sulphydryl groups with this method is negligible

Fraker and Speck, (1978) used IODOGEN to radiolabel rabbit IgG, chicken lysozyme and a membrane from a living cell Their results demonstrated the mildness of the reagent as enzyme activity was preserved and greater than 80% viability of cells was obtained Similarly, *Markwell and Fox* (1978) used the reagent for the surface specific iodination of membrane proteins of viruses and eukaryotic cells while maintaining viral integrity and cell viability It has also been used to label human growth hormone and human luetinizing

hormone obtaining maximum theoretical iodine incorporation with minimal chemical damage (*Salacinski et al*, 1981) In a comparative study *Salacinski et al* (1981) found a higher iodine incorporation with less iodination damage using Iodogen, than caused by Chloramine-T, with the majority of proteins and peptides they had tested

(c) IODO-BEADS

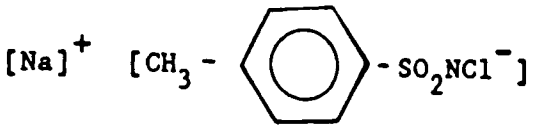
Markwell (1982) reported the use of IODO-BEADS as an iodination reagent The Iodo-bead reagent (Pierce Chemical Co) consists of immobilized chloramine-T molecules on non-porous polystyrene beads (Fig 1 4 2c) The Iodo-bead iodination reagent provides a more gentle method for iodination than soluble chloramine-T since there is no direct contact between the protein and the immobilized oxidizing agent The Iodo-bead iodination reagent can be easily removed from the reaction mixture with tweezers or a pasteur pipet This feature allows one to have more control over the incubation time, since the addition of a reducing agent is not required to stop the reaction The beads are simple to use and store are inexpensive and have a long shelf-life (about one year)

In a comparative study *Lee and Griffiths* (1984) found that the electrophoretic mobilities of [^{125}I]h-AFP iodinated by both Iodo-bead and chloramine-T are comparable This is suggestive of a similar oxidative effect of these reagents However, it was found that the chloramine-T radiolabelled h-AFP was less stable when stored at 20^{o}C The chloramine-T method gave consistently higher (10%) net binding activity with anti-h-AFP than its counterpart from the Iodo-bead method Also, the chloramine-T method caused conformational changes

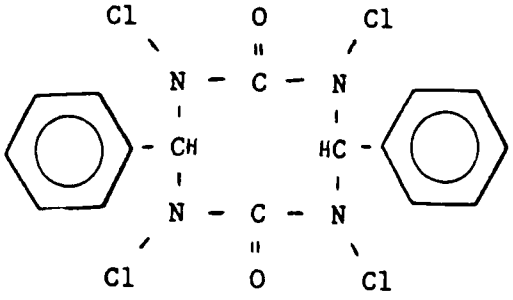
(or other structural damage) to h-AFP so that the immunoreactivity of labelled and non-labelled h-AFP are not identical While the chloramine-T method yielded a labelled protein with higher specific activity than than obtained by Iodo-bead method, the latter was a simpler more controllable and milder method giving rise to a more stable labelled protein

The Iodo-bead method offers several important unique advantages in protein iodination Among these are the flexibilities in labelling conditions e g time (2-3mins), medium (0 1M Tris or PBS, pH 5 5-5 8), presence or absence of azide, detergents, urea or high salt, temperature (4^{o}C to room temperature), level of activity required and, in addition, the simplicity of the procedure

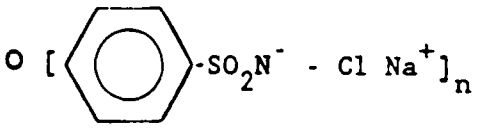
Figure 1 4 2 Chemical structures



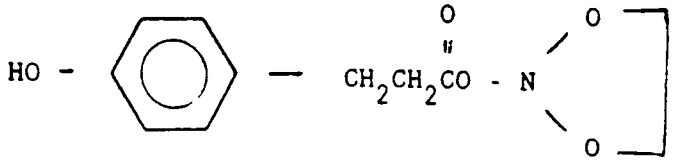
(a) Chloramine-T



(b) Iodogen



(c) Iodobeads



(d) Bolton Hunter Reagent

(d) IODINE MONOCHLORIDE

McFarlane (1958) introduced the use of iodine monochloride The method was further improved by *Helmkamp et al* ,(1960) and by *Samols and Williams* (1961) The final result of the reaction is the same as with the other methods so far mentioned i e iodination of the protein tyrosine moieties The basic method is as follows radioactive iodide ($Na^{125}I$) is mixed with the protein to be iodinated, buffered to pH 8 and an appropriate amount of ICl, in a weakly acidic NaCl solution, is added As a result of ICl addition two reactions occur Firstly, radioactive iodine present as iodide ($Na^{125}I$) is converted to ^{125}I by very rapid chemical exchange with non-radioactive ICl Iodination of protein then takes place as a result of the following reaction

$$ICl + NaOH \longrightarrow HOI + NaCl$$

$$HOI + \text{tyrosine residue} \longrightarrow \text{iodinated tyrosine residue} + H_2O$$

This latter reaction is also rapid, so that after one minute a protective protein may be added and the labeled preparation removed from the vial This method was found efficient in its use of radioiodine when used to radioiodinate proteins to high specific activities

Helmkamp et al , (1960) made further modifications in the method eliminating inefficiencies in labelling to high specific activities associated with the original method while still retaining mild iodination conditions *Doran and Spar* (1980) modified the oxidative method of *Helmkamp et al* , such that microgram amounts of protein

could be efficiently iodinated

The chemical basis of the oxidative ICl technique is the oxidation of radioiodide ($Na^{125}I$) and stable iodide (KIO_3) to iodine monochloride which then iodinates the protein The reaction generating the ICl moiety has been summarized by *Helmkamp* as

$$2I^- + IO_3^- + 6HCl \longrightarrow 3ICl + 3H_2O$$

Through this oxidation step, up to two thirds of the iodine monochloride can contain radioiodine as against half in the original method Thus, proteins can be labelled to higher specific activity

An important advantage of the ICl method is the total amount of iodine incorporated into the iodinated material is known and can be controlled, simply by limiting the amount of the ICl available for iodination The importance of the degree of the iodination as measured by the iodine to protein ratio is well documented For example, iodination of the tyrosine residues of the active sites may result in loss of activity of enzymes Thus, with the ICl technique one can maximise the iodine to protein ratio *Johnson et al* ,(1960) report no loss of precipitin activity with an iodine to protein ratio of 2 with IgG *Doran and Spar* (1980) report no detectable loss of antigenic activity with an iodine to protein ratio of one with human serum albumin

Additional advantages include the possibility of preparing proteins with different specific activities, the lack of exposure of proteins to oxidising and reducing agents, the convenience of use and the fact that no special reagents are required

(ii) ENZYMATIC IODINATION

The disadvantages of the chloramine-T procedure gave impetus to

the search for a more gentle means of iodinating proteins to high specific activity The selectivity of the lactoperoxidase-catalyzed iodination of tyrosine provides the basis for labelling methods that avoid exposure to excessive quantities of strong oxidizing agents (*Marchalonis,* 1969) Although an oxidising agent (H_2O_2) must still be used in this procedure, very small quantities need to be used This method has also the potential advantage that the susceptibility of a given tyrosyl residue of a protein to iodination will be expected to vary greatly depending on its environment, exposure to solvent and pK Therefore, it is possible to avoid introducing the very large and bulky iodine molecule, into crevices of the protein Such sites may be accessible to free I_2 in solution but are not accessible to the lactoperoxidase molecule (*Osheroff et al,* 1977) The tyrosine residues on the outside surface of the protein are usually not directly involve the active centre of enzymes, lactoperoxidase labelling is thus especially good in not destroying biological activity

In a study carried out by *Miyachi et al* (1972) with human chorionic gonadotropin (HCG) and human leutenizing hormone (HLH)it was found that conditions for radioiodinating varied for each hormone To attain high specific activity sufficient for use in RIA, HLH required relatively larger amounts of H_2O_2 and lactoperoxidase than did HCG This was attributed to differences in accessibility of tyrosyl groups in the hormones *Marchalonis* (1969) showed different rates of iodination among immunoglobulins and postulated that rates depended on relative accessibility of reactive tyrosines in immunoglobulins It is necessary to determine optimal conditions for each preparation to be labelled Most success has been claimed for

methods in which H_2O_2 is generated by a glucose-glucose oxidase system (*Miyachi et al*, 1973) Immobilized lactoperoxidase and glucose oxidase are conveniently available as ready prepared Enzymobeads (Bio Rad Laboratories) The use of such insoluble enzyme derivatives permits the rapid separation of the enzyme from protein and reactants

Thorell and Johannson, (1971) have described the use of lactoperoxidase for the labelling of polypeptides to high specific activity The specific activities achieved compared favourably with those attained with the chloramine-T method However, no substantial improvement could be demonstrated for the iodination of insulin, plant lecitins, or ACTH (*Cuatrecasas and Holenberg,* 1976) The residue which will be iodinated by lactoperoxidase must have the proper geometric position, while other methods of halogenation are influenced by reactivity Enzymatic methods are slow and sensitive to inhibitors such as azide, cyanide and high salt Lactoperoxidase has a very high turnover and little enzyme is required for the labelling procedure

(iii) ELECTROLYTIC IODINATION

This method shares with the iodine monochloride method the advantage that no oxidizing agents are required to liberate iodine from iodide, thus avoiding one potential hazard to the protein molecule However, in electrolytic iodination it is possible to use very little iodine so that tagging of small quantities of protein is easier than in the iodine monochloride technique

Electrolytic iodination as described by *Rosa et al* (1964) uses an electric cell consisting of a glass beaker of a smooth platinum

sheet as anode, and a platinum wire placed in a glass tube whose bottom is closed by a cellophane membrane as the cathode A known weight of protein in 0 9% (w/v) NaCl is placed in the electrode vessel, and the cathode tube is filled with NaCl of the same molarity The radioactivity (a selected quantity) is placed in the vessel together with sufficient KI to give the required level of iodine substitution Electrolysis is carried out at a constant current for about half-an-hour After iodination is completed, non-protein-bound activity is removed by standard procedures

The electrolytic method has not been widely used, perhaps because it is difficult to adapt the method to the small volumes required for labelling to high specific activities and may also be due to inconvenience in setting up the electrolysis apparatus The method is complex and time consuming and, thus, inappropriate for routine laboratory use

1 2 2 2 CONJUGATION

The first of such methods to be described used radioiodinated N-succinimidyl 3-(4-hydroxyphenyl)proprionate as the labelling agent (*Bolton and Hunter*, 1973) Similar reagents derived from methyl p-hydroxybenzimidate (*Wood et al* , 1975) have been synthesized and used to iodinate proteins but not specifically for immunoassay This approach is suitable for peptides that lack tyrosine, for example secretin (*Mutt et al* , 1970) and porcine parathyroid hormone (*Woodhead et al* , 1971) It also has the advantage that the antigen or hapten never comes in contact with potentially damaging oxidising or reducing agents

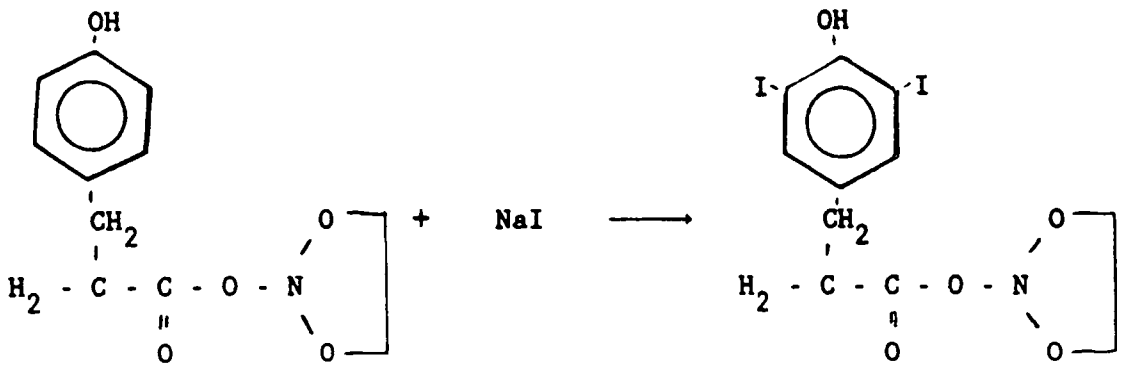
BOLTON-HUNTER REAGENT

This procedure involves reacting the protein under mild conditions with N-succinimidyl 3-(4-hydroxyphenyl) proprionate (Bolton-Hunter reagent, Fig 1 4 2d) that has been previously labelled with iodine and separated from the products of iodination reaction by solvent extraction The Bolton-Hunter reagent is a phenol and can be iodinated under the same conditions as the tyrosine in proteins The iodinated reagent reacts with the lysyl residues of the amino groups present in most proteins and peptides to form amide bonds The reaction scheme is shown in Fig 1 4 3 Although the unlabelled proprionate can be labelled "in house", the labelled material is available commercially in purified mono- or diiodonated form as iodinated Bolton-Hunter reagent

Knight and Welch, (1978) reported detailed studies in which human serum albumin was iodinated by several methods including the Bolton-Hunter method The protein was degraded enzymatically and labelled amino acids were analysed by HPLC Lysine residues were labelled predominantly but iodinated histidine and tyrosine were also produced

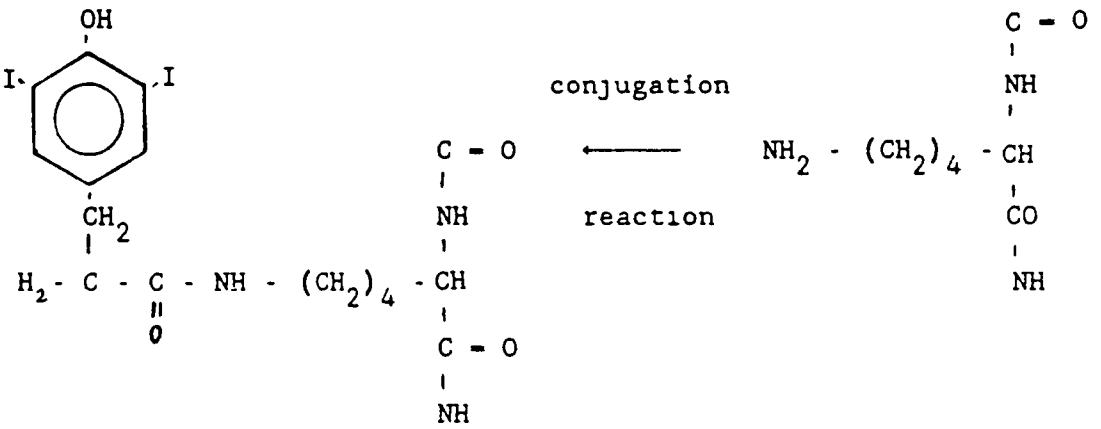
Since the Bolton-Hunter reagent was introduced it has been used to label a wide variety of targets including viruses and cell lysates as well as pure compounds Undoubtedly one of the most important applications has been the preparation of iodinated antigens and hapten derivatives of high specific activity for

Figure 1 4 3 Bolton Hunter Iodination



radioiodinated ester

+



I-labelled protein

amine group of lysine

radioimmunoassay The structure of proteins determines the reactivity of potential conjugation sites Steric effects may be the most limiting factors due to the large size of the Bolton-Hunter reagent

This method has the theoretical advantage that the introduction of the bulky iodine will occur at the surface lysyl moieties and, in large proteins this should occur randomly Such modifications at the protein surface may be less likely to lead to adverse effects on the conformation of the protein This procedure involves a condensation with a moiety that is larger than I and results in a greater alteration in charge This facilitates subsequent purification of monoiodinated derivatives The principal disadvantage may be that the method requires more manipulation than other direct methods Nevertheless, it offers a very important major alternative for the introduction of iodine in cases where, for whatever reason, the substitution procedures result in an unsatisfactory product

Cuatrecacas et al ,(1976) have used this method successfully to label derivatives of nerve growth factor which are far superior, with respect to receptor-binding studies, to those obtained by chloramine-T or lactoperoxidase methods The best success has been achieved with larger proteins, such as immunoglobulins, plant lecitins and avidin (*Cuatrecasas,* 1976) This is possibly due to the larger number of potentially reactive lysyl amino groups on large proteins With peptides and proteins having few amino groups such as insulin, ACTH and MSH, very poor incorporation can be expected

Since the reagent is highly susceptible to hydrolysis in aqueous media, it is to be expected that low concentrations of protein would lead to low efficiences of substitution

OTHER CONJUGATION METHODS

Aniline can be iodinated by NaI in the presence of chloramine-T Under the action of sodium nitrite the aniline amino group transforms into a diazo group, which can be attached to the protein in alkaline conditions It is presumed to couple to the phenol moiety of tyrosine residues (*Langone*,1980) and the product is analogous to that obtained by direct iodination As well as aniline, methyl-paraphydroxybenzimidate was described as an intermediate, which can be iodinated and then attached to a protein (*Wood et al* ,1975) Iodination is performed similarily with chloramine-T Addition to the protein occurs due to the elimination of the reagents methyl ester It is of note that the protein retains a positive charge The phenol OH group adjacent to the two iodine molecules readily dissociates at neutral and alkaline pH leading to the formation of an additional negative charge This approach has been used by *Wood et al* (1975) to radioiodinate 1mg protein to a level of 10^7dpm The reagent is commercially available The disadvantage of this method is that it proceeds rather slowly the labelling of protein continuing after 100 hours of incubation at room temperature The duration of iodination can be reduced to 33 hours by increasing the temperature to 37°C

Conjugation labelling has become an important addition to the available methods of introducing iodine into antigens, haptens and antibodies for use in immunoassay and other immunochemical studies The Bolton-Hunter reagent is widely used, especially to label compounds that lack tyrosine groups or are labile under the reaction conditions of substitution methods.

1 2 2 3 SELECTION OF IODINATION METHOD

When choosing an iodination technique, many factors must be examined These factors include the simplicity of the procedure, relative cost as well as the quality of the iodinated protein desired The ultimate criterion is the production of a labelled protein which performs satisfactorially in the experimental system being used

The iodine ion is as large as the phenolic ring of tyrosine (*Cuatrecasas and Hollenberg*,1976) which is the primary site of iodine incorporation via substitution methods Tyrosine residues are often associated with biological activity and hence the blocking of an active residue with one or two iodine ions may completely eliminate this activity *Johnson et al* (1960), reported a decrease in activity of antibody with increasingly levels of iodination of3 to 10 atoms per protein molecules This effect was attributed to the iodination of tyrosines within the antigen binding site Similarly, *Levy and Dawson* (1976), showed that human isoantisera lost part of its cytotoxic activity when iodinated The loss in activity was postulated to be due to interference with complement binding to the Fc region of antibody molecule

The overall yield of "good" tracer may be relatively low in the use of oxidation reactions However, this must be considered in relation to the overall lower yield of incorporation of iodine into peptides using chemically more gentle conjugation methods The effect of the iodination method on biological properties of calmodulin has been studied in detail It was found that iodination with chloramine-T resulted in complete inactivation The lactoperoxidase method decreased the protein's activity significantly

and only after iodination with Bolton-Hunter reagent was the biological activity of this protein completely retained (*Chafouleas et al* ,1979)

Such points need to be investigated experimentally for each new peptide or new application of a peptide tracer

1 2 2 4 IODINATION OF NUCLEIC ACIDS

NaI can also be used to label nucleic acids The iodine is attached to the pyrimidine ring at the C5 position The bond with cytosine is markedly more stable than that with uracil in nucleic acids Therefore, it would be worthwhile removing iodine from uracil after iodination in order to have a sample of stable specific activity The addition of iodine results from the same electrophilic attack of the I^+ cation The oxidation of cation to anion can be performed by chloramine-T *Shasposhnikov et al* ,(1976) have radioiodinated rat liver total RNA using chloramine-T to 7 $X10^{10}$ dpm per mg of RNA Thallium trichloride ($TLCl_3$) is more often used as a catalyst in this reaction (*Commerford*,1971) The reaction is rapid and simple It results in the formation of a stable covalent bond with the C_5 atom of cytosine The reaction is ideally carried out at pH 5 The incorporation of iodine at pH 6 is about 8% of that at pH 5 Labelling with NaI is markedly more difficult for double stranded nucleic acid (*Scherberg et al* ,1974)

Such methods of labelling nucleic acids involve chemical modification of nucleic acid Taking into account the template function of the majority of nucleic acids which is strictly determined by its chemical composition, such modifications should result as a rule in distortion of this function

1 2 2 5 IODINATION OF CELL MEMBRANES

There are presently available several reagents, including diazotized iodosulphanilic acid (ISA) and lactoperoxidase, that have been used to label cell membranes The lactoperoxidase-catalysed NaI reaction has been shown, in addition to labelling the tyrosine residues of plasma membrane proteins , to label a variety of cell surface lipids (*Schlager*,1979) *Schlager* attributes the incorporation of iodine to substitution or exchange reactions with the cell surface lipids The hallmarks of the reaction are that it labels all lipids regardless of class (i e phospholipids, neutral lipids and free fatty acids) In addition the iodine-lipid bond is stable in face of lytic or cytotoxic stimuli

Iodosulphanilic acid (ISA) is an advantageous iodinizing agent in that it does not enter into the interior of cells, and only the exposed membranes are labelled (*Berg*,1969, *Sears et al* ,1971) When ISA used to label erythrocyte and platelet membrane proteins *in vivo* neither morphology nor subsequent *in vivo* behaviour of these cells was affected (*George et al* ,1976) The principal platelet membrane components which were labelled were glycoproteins which reflect their prominence on the exterior of the membrane Diazonium compounds principally label tyrosine, histidine and lysine residues (*Higgins and Harrington*,1959) *Young and Alpers*(1981), describe the successful use of ^{125}I iodosulphanilic acid to label intestinal brush border proteins *in vivo* by intraluminal injection Such labelling was achieved without damage to cellular morphology or enzyme activity Cell labelling is a useful and accurate means of studying turnover rates of membrane proteins in various physiological and

pathophysiological states

1 2 3 AIMS OF PROJECT

Following from this review of the various types of labels used in the measurement of analytes, the objective of this project was to develop and evaluate a novel non-radioactive iodide labelling system It is possible to label proteins, cell membranes and nucleic acids with iodide from the wealth of iodination techniques described Detection of the iodine-containing compounds is possible with the Sandell-Kolthoff reaction With this objective in mind the following problems had to be addressed

(1) the optimisation of an iodide assay with respect to temperature, reagent concentration, detection wavelength, sensitivity and acidity of reaction medium,

(2) the application of the optimised procedure for the detection of mouse immunoglobulin (IgG) levels by immunoassay,

(3) the development of an immunoassay to detect human IgG levels and an evaluation of its performance in comparison to that of an enzyme immunoassay,

(4) the optimisation of the assay to increase on sensitivity, and finally,

(5) the application of the iodide assay to detect proteolytic activity

2 MATERIALS AND METHODS

2 1 MATERIALS

Ceric ammonium sulphate, arsenic trioxide (As_2O_3), N,N-dimethylformamide, sodium periodate, o-phenylenediamine (OPD), magnesium chloride, methyl α-D-mannopyranoside, pepsin (from porcine stomach mucosa) E C 3 4 23 1 , trypsin (from bovine pancreas) E C 3 4 21 4 , bovine serum albumin, horseradish peroxidase (type VI-A), human IgG, mouse IgG, goat anti-human IgG, goat anti-mouse IgG, horseradish peroxidase-conjugated goat anti-mouse IgG, N-ethylmaleimide (NEM), dithiothreitol (DTT), poly-L-lysine (PLL) mw 22000, Ponceau S and trichloroacetic acid were obtained from Sigma Chemical Company, St Louis, Mo , USA

Sodium borohydride, sodium chloride, sodium hydrogen carbonate, potassium iodide, sodium periodate, potassium chloride, boric acid, potassium dihydrogen phosphate, disodium tetraborate, sulphuric acid, poly (ethylene glycol) mw6000, nitric acid,hydrogen peroxide (30% w/v) and sodium carbonate were obtained from BDH, Poole, England

Citric acid, ammonium sulphate, sodium carbonate, sodium phosphate, sodium dihydrogen phosphate, sodium acetate, disodium hydrogen phosphate, methanol, Tween 20 and potassium hydrogen phosphate were obtained from Riedel de Haen, Germany

Collagenase (from *Clostridium histolyticum)* E C 3 4 24 3 and Dispase (from*Bacillus polymyxa*) E C 3 4 24 1 were obtained from Boehringer Mannheim, Germany

Microtitre plates were obtained from Nunc, Denmark and Sterilin, Poole, England

Ultrogel AcA44 was obtained from IBF Biotechnics,

Villeneuve-La-Garenne, France Sephadex G-25 (fine) and Con A-Sepharose were obtained from Pharmacia, Sweden

Papain (from *Papaya latex*) E C 3 4 22 2 was obtained from Chemcon Ltd , Cork, Ireland

Cerium sulphate tetrahydrate was obtained from Merck, Germany BCA protein assay reagent and SPDP were obtained from Pierce Chemical Co , Illinois, USA Benzene was obtained from Lab-Scan, Dublin, Ireland

Monoiodinated Bolton Hunter Reagent (IBHR) was obtained as a gift from Dr C Reading and Ms J Bator, M D Anderson Cancer Centre, Houston, Texas

Mouse serum was obtained from a pool of mouse blood collected by conventional techniques Antibody-rich ascites fluid from the *in vivo* growth in Balb/C mice of the H12 hybridoma was used This hybridoma secretes an IgG2a antibody which reacts with human leukaemic lympocytes (*Lannon et al* ,1988) Cell Culture supernatants were obtained from the *in vitro* growth of the H9 hybridoma This IgG2a antibody reacts with normal peripheral blood and leukaemic lymphocytes (*Lannon et al* ,1988)

Human blood serum samples were obtained from St Vincents Hospital, Dublin

Instrumentation

A Titertek Twinreader Plus supplied by Flow, a Philips PU8625 UV/VIS spectrophotometer, a Shimadzu UV-160A uv-visible recordingspectrophotometer, a Heraeus Christ bench-top centrifuge, a Pharmacia peristaltic pump ,an LKB (2112 redirac) fraction collector and Brand automated pipettes were used

2 2 METHODS

2 2 1 IODIDE MICROASSAY

Reagent solution for iodide assay Ceric ammonium sulphate and ceric sulphate tetrahydrate (0 1M) were made up in 2 5M H_2SO_4 Dilutions of these stock solutions were made up fresh daily in 1M H_2SO_4 or HNO_3 as the intensity of the cerium IV yellow colour is a function of acid concentration Arsenious acid (0 075M) was made up by dissolving 14 84g arsenious trioxide (As_2O_3) in 700ml distilled water containing 28ml concentrated H_2SO_4 Care was taken in the preparation of this reagent as As_2O_3 is a potential carcinogen The mixture was heated to near boiling with constant stirring until the As_2O_3 was dissolved, some precipitation of arsenious oxide may occur After reaching room temperature it was made up to a final volume of 1L with distilled water

Standards Potassium iodide was made up in ultrapure water to a concentration of lug/ml Two millilitres of 0 1M NaOH was added to prevent air oxidation It was stored at $4^{o}C$ in an opaque container and stored stable for several months Dilutions of this stock solution were made in distilled water Standards of IBHR were made by dissolving 1mg in 200ul DMF and diluting with dH_2O/PBS

Microassay procedure Standards in duplicate were added to the wells of microtitre plates, to a final volume of 100ul Samples for analysis, in duplicate, were added to remaining wells with a final volume of 100ul Arsenious acid (60ul) was added to each well using a multichannel pipette Ceric ammonium sulphate solution (25ul) was added to wells, the plate mixed and absorbance readings taken at precisely timed intervals Wells incubated with water/0 01M PBS

served as zero controls These wells will have the highest absorbance values as no iodide is present to catalyse the Sandell-Kolthoff reaction

2 2 2 IMMUNOASSAYS

Conjugation of IBHR to proteins IBHR was dissolved at a concentration of 4mg/ml in benzene, containing approximately 4% (v/v) DMF One mg of IBHR was dried under nitrogen and dissolved in 200ul DMF One ml of a 1mg/ml or a 2mg/ml solution of protein in 0 1M borate buffer (pH 8 5) was added to the IBHR solution with mixing The reaction was allowed to proceed on ice for two hours The solution was then centrifuged to remove any precipitate formed (2 min at 13,000g) The conjugate was purified by gel filtration on Ultrogel AcA44 Human IgG, goat anti-human IgG, goat anti-mouse IgG and BSA were conjugated using this method

Gel filtration A 16mm i d X 360mm column of Ultrogel AcA44 was used and eluted with 0 01M PBS, pH 9, at a flow rate of 1 ml/min One ml fractions were collected Protein was determined by measuring absorbance of fractions at 280nm and by the BCA assay

IBHR incorporation was measured with the iodide microassay

Conjugation of horseradish peroxidase (HRP) to goat anti-humanIgG The procedure of *Tijssen and Kurstak*,(1984) was followed There are three steps involved (a) activation of peroxidase, (b) conjugation of the activated peroxidase to the immunoglobulin and (c) purification of the conjugate

(a) Activation of peroxidase 0 6mg horseradish peroxidase was dissolved in 0 5 mls 100mM $NaHCO_3$ To this was added 0 5 mls 8mM

$NaIO_3$ The tube was sealed and stored in the dark for two hours at room temperature

(b) Conjugation Dissolved 2 mg goat anti-mouse IgG in 1 ml 0 1M sodium carbonate buffer, pH 9 2 This solution was then added to the activated horseradish peroxidase The mixture was then transferred to a glass wool-plugged pasteur pipette (tip closed with a flame) and dry Sephadex G-25F (5 mg) was added The mixture was covered with tinfoil and left to incubate at room temperature for 16 hours The reaction mixture was eluted off the Sephadex and incubated with 1/20 volume of freshly prepared $NaBH_4$ (5 mg/ml in 0 1mM NaOH) 30 minutes later another volume (1/10) of freshly prepared $NaBH_4$ solution was added The mixture was incubated at 4^oC for 1 hour

(c) Purification of conjugate free peroxidase is soluble in solutions of $(NH_4)_2SO_4$ up to 70-80% saturation Therefore the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution to the preparation precipitates free and conjugated IgG but not free peroxidase After an equilibrium period of one hour the precipitate was collected by centrifugation (15 min, 6000g), washed with a half-saturated $(NH_4)_2SO_4$ solution and recentrifuged

The precipitate was resuspended in PBS and dialysed in PBS (0 1 M, pH 7 2) overnight Free IgG was separated from the IgG-Peroxidase conjugates by affinity chromatography on a Con A-Sepharose column (1 ml bed volume) Once the sample was applied the column was eluted in 10 mls of a buffer containing 100mM sodium phosphate, 100mM NaCl, 1mM $MgCl_2$ and 1m $CaCl_2$, pH 7 2 Peroxidase complexes were desorbed with methyl α-D-mannopyranoside, (10mM in elution buffer) 1 ml fractions were collected and monitored at 280nm and 403nmMeasurement of enzyme activity of HRP conjugate The conjugate was diluted in PBS (0 15M)

to give a concentration of 0 6 ug/ml (an absorbance reading at 280nm of approximately 1 45 is equivalent to 1 mg/ml IgG, *Catty*,1989) 3 ml of freshly prepared OPD substrate solution was added to 20ul of diluted conjugate, incubated and wrapped in foil, at room temperature 200ul aliquots were serially removed at 0, 5, 10, 15, 20, 25 and 30 minutes and transfered to wells of a microtitre plate, and 25ul of 2 5M HCl stopping solution was added immediately at each time point The plate was placed in the dark between sample additions, and, when the series was complete the absorbance values of each well were read at 492nm To measure enzyme activity of the HRP conjugate the absorbance readings were related to time

MOUSE IgG ASSAY (sandwich assay)

Assay procedure for measuring mouse IgG levels 96 well microtitre plates were coated with 100ul goat anti-mouse IgG (10ug/ml) diluted in 50mM sodium bicarbonate buffer, pH 9 6, incubated overnight at $4^{0}C$ They were washed (3X) in PBS, pH 7 2 The plates were blocked by incubation with 2% (w/v) BSA in 0 01M PBS,pH 9, for 1 hour at room temperature The plates were washed (3X) in wash solution [0 1% (v/v) Tween 20, 0 1% (w/v) BSA in 0 15M PBS, pH 7 2] Dilutions of mouse IgG were then made up (range 40 - 400 ng/ml in PBS, pH 7 2) and incubated in the wells (100ul/well) in triplicate for 60 minutes at room temperature The plates were washed 6 times in wash solution 100ul HRP-conjugated goat anti-mouse antibody (diluted 1 1000 in wash solution as recommended by Sigma) or 100ul IBH-conjugated goat anti-mouse antibody (diluted to approximately 10ug/ml in wash solution) were added and incubated for 60 minutes at room temperature The plates were rewashed in wash solution (5X) and once

in PBS, pH 7 2 Appropriate substrates were added and absorbances read at 414nm at intervals over 60 minutes

For IBHR determination, 60 ul arsenious acid (0 075M) and 25 ul cerium ammonium sulphate (0 028M) was added per well The absorbance values were converted to % transmittance using the Beer-Lambert Law (*Montgomery and Swenson*,1969) The % transmittance was plotted against concentration for the range of concentrations of IgG

100ul of the substrate for HRP (10mg of o-phenylenediamine and 5ul of 30% (v/v) H_2O_2 in 25 mls 0 1M citrate buffer, pH 4 5) was added to the wells Absorbance values were plotted against concentrations of IgG

IgG levels in serum, ascites fluid and cell culture supernatants of mouse were determined using both IBHR-conjugated second antibody and Sigma's HRP-conjugated second antibody

HUMAN IgG ASSAYS

Sandwich assay procedure for measuring human IgG levels The same procedure as for mouse IgG was followed with the following exception a 0 9ug/ml dilution of prepared HRP-conjugate was used This assay procedure was used to measure human IgG levels in human serum samples

Determination of conjugate dilutions to use in sandwich assay Microtitre plates were coated with appropriate IgG in the range of 0 - 400 ng/ml, washed, blocked and then IBHR or HRP-conjugate titred out against IgG levels

Competitive assay procedure for measuring human IgG levels

Microtitre plates were coated with goat anti-human IgG as before, washed, blocked and then incubated for 30 minutes with a range of human IgG dilutions (0 25 - 10 ug/ml) and 1 5000 dilutions of unknown serum samples The plates were washed and then incubated for a further 30 minutes with a dilution of IBHR labelled human IgG (30 ug/ml) After washing the reagents for the iodide assay were added and absorbance read at 414nm

Conjugation of Poly-l-lysine to proteins

The procedure of *Canfi et al* (1989), was followed In this procedure the heterobifunctional cross-linking agent SPDP was used to try to conjugate PLL to proteins Human IgG (3 mg/ml) was dissolved in 0 1M PBS, pH 7 2 A 10mM solution of SPDP in methanol was prepared and added to the protein solution to give a 10 1 molar ratio of SPDP to protein The mixture was stirred at room temperature for 2 hours and dialysed overnight against 0 05M PBS, pH 7,2 containing 5% PEG to speed up the dialysis

Poly-L-lysine in a molar amount five times that of the protein was dissolved in 0 1M PBS, pH 7 2 A methanolic solution of SPDP containing a molar ratio of 10 1 to the PLL was added The mixture was stirred at room temperature for 2 hours and dialysed overnight as described above

Following dialysis, the number of bound dithiopyridyl groups was determined by reacting 100 ul of the protein-SPDP with 900 ul dithiotreitol (DTT) and measuring the absorbance of the pyridinedithione product at 343nm, (ϵ 8080 I/mol/cm, *Carlsson et al* ,1978) PLL does not absorb light above 230nm, and the number of

SPDP moities bound per PLL was determined by measuring the absorbance at 280nm using ϵ 5100L/mol/cm (*Carlsson et al* ,1978)

The pH of the PLL-SPDP solution was adjusted to 4 5 with 0 1M HCl, DTT was added to a final concentration of 50mM and the mixture allowed to react for 30 minutes at room temperature The low pH prevents free SH groups from interacting The resulting thiopropyl derivative of PLL was purified on Sephadex G-25 (fine) column (1 X 20 cm), eluted in 0 1M sodium acetate buffer, pH4 5 and 1 5ml fractions collected

20ul was transferred into polyethylene tubes 800ul of Ponceau S solution (76 mg/ml in 30 g/L trichloroacetic acid) was added to the fractions, and the contents centrifuged for 10 minutes Supernatants were removed and precipitates resuspended in 1 4ml of sodium hydroxide (8 g/L) The absorbances were then measured at 560nm, and the amount of purified PLL-SPDP determined from a calibration graph in the range 0 06 - 2 5 mg/ml The fractions containing PLL-SPDP were combined and pH adjusted to 7 4 with IM NaOH The protein-SPDP was added immediately and the solution incubated at room temperature for 20 hours Excess NEM was added to block unreacted SH groups After 30 minutes at room temperature, the solution was concentrated against solid PEG The concentrated solution was purified on a column of Ultrogel AcA44, eluting with 0 1M PBS, pH 7 2 Fractions of 1 ml were collected, and the protein content for each fraction was determined from the absorbance at 280nm and the total polypeptide content by the Ponceau S procedure previously described

2 2 3 PROTEINASE ASSAY

Microtitre plates were coated with 100ul per well IBHR conjugated

BSA (10ug/ml) diluted in 0 01M PBS, pH9 and incubated at 37°C for two hours Wells were washed three times for ten minutes with PBS, pH 7 2, incubated at 37°C for two hours with PBS, and then washed a further three times Duplicate 100ul samples of enzyme, at a range of dilutions (1mg/ml - 1pg/ml), were added to wells and incubated for 16 hours at 37°C All enzymes were diluted in PBS, pH 7 2 Wells incubated with 100ul PBS served as zero controls Supernatants were aspirated from the plate Iodide remaining was detected by the addition of 60ul 0 075M arsenious acid and 25ul 0 028M ceric ammonium sulphate made up in 10% H_2SO_4 Absorbance readings at 414nm were taken after ten minutes incubation With increasing enzyme concentration the amount of IBHR conjugated BSA remaining bound to wells is reduced Absorbance at 414nm was plotted against enzyme concentration to determine enzyme activity

2 2 4 PROTEIN DETERMINATION

Modified BCA protein assay

Standards of BSA/IgG in the range 0 -1 0 mg/ml were prepared in PBS, pH 7 2 The assay was performed using a microtitre plate, 200ul of a working BCA reagent (prepared by adding 1 part reagent B to 50 parts reagent A) was first dispensed into a series of wells 20ul of the standards or sample was then added, and mixed in the Titretek After 30 minutes incubation at 30°C the absorbance readings were taken at 620nm

Micro-BCA assay (*Smith et al* ,1985)

The following reagents were prepared Micro-Reagent A (MA) was made up from an aqueous solution of 8% (w/v) Na_2CO_3, 1 6% (w/v) NaOH, 0 16% (w/v) Na_2tartrate and sufficient $NaHCO_3$ to adjust the pH to 11 25

Micro-Reagent B (MB) was made up of 4% BCA-Na_2 in deionized water Micro-Reagent C (MC) ts of 4 volumes of 4% (aq) $CuSO_4$ $5H_2O$ plus 100 volumes of Micro-Reagent B Micro-Working Reagent (M-WR) consists of 1 volume of MC plus 1 volume of MA MA and MB are stable indefinetly at room temperature, but MC and M-WR are freshly prepared

One volume of sample or standard (range 0 5 - 10 ug/ml) and 1 volume of M-WR were mixed and incubated in a waterbath at 60°C for 1 hour Samples and standards were assayed in duplicate Absorbance at 562nm were recorded

3 OPTIMISATION OF THE IODIDE MICROASSAY

3 1 Introduction

The catalytic effect of iodide on the reduction of ceric sulphate by arsenic acid, first described by *Sandell and Kolthoff* (1934) has the advantage of great sensitivity The method described by *O'Kennedy et al* (1989) for the determination of iodide and iodine-containing compounds based on the Sandell-Kolthoff reaction partially optimised the assay for a microplate format This microassay follows the course of the reaction spectrophotometrically measuring the intensity of the cerium IV yellow colour remaining after a defined time period In the latter stages of the reduction the amounts of cerium IV present are very small, and thus, it is more convenient and accurate to determine the cerium IV remaining after a given period of time

In this chapter, further optimisations on the microassay method developed are described In order to find the optimum conditions for the assay various factors controlling catalytic activity were studied These included concentration of reagents, acidity of reacting medium, temperature, detection wavelength, chemical form of the reactant cerium (IV) and the incubation time Using a microplate reader with temperature control, automatic reagent dispenser and kinetic function it was possible to overcome limitations on the previously described assay The incubation time was shortened, sensitivity of the assay was increased and excellent linear responses were obtained using absorbance units rather than conversion to percentage transmittance

3 2 Results

The microassay was firstly optimised with respect to incubation time The microassay originally described converted absorbance units to % transmittance to achieve linear responses with incubation periods of 5 - 30 minutes In Fig 3 1 the linear relationship of absorbance units against concentration of KI are graphed for 1 - 5 minutes For incubation periods greater than 5 minutes the relationship between absorbance units and concentration of KI was found to be non-linear The best linear relationship was found at 30°C with an incubation of 2 minutes using 0 028M ceric ammonium sulphate (r=0 99, where r is the regression coefficient)

Fig 3 2 shows the linear responses when temperature was varied Using an incubation period of 2 minutes at 40°C there is a greater difference in absorbance units compared to 30°C At 50°C the reaction proceeds very quickly and the linear response is lost However, at 50°C and an incubation period of 1 minute a good linear response was achieved at concentrations of KI up to 0 07 ug/ml (Fig 3 3) The optimal concentration of ceric ammonium sulphate was found to be 0 028M which gave absorbance units within the linear range of the microplate reader at 414nm and in comparison to 0 007M and 0 014M ceric ammonium sulphate 0 028M gave a good range in absorbance values (Fig 3 4) Using equimolar concentrations of both ceric ammonium sulphate and ceric sulphate tetrahydrate the former was found to be more suitable as a greater range in absorbance values was obtained (Fig 3 5)

The absorbance spectrum of ceric ammonium sulphate has an absorbance maximum at 320nm (Fig 3 6) In the assay previously

described 414nm was the chosen wavelength because of its suitability with microplate readers as this wavelength is used in enzyme-linked immunosorbent assays With a 340nm option it was possible to measure the reaction using 3 5 X 10^{-3}M ceric ammonium sulphate The use of 340nm resulted in far greater sensitivity in the detection of KI It was possible to detect KI levels as low as 10pg/100ul by reading at 340nm using 3 5X10^{-3}M ceric ammonium sulphate, 9 37X10^{-3}M arsenious acid and an incubation time of 30 minutes When nitric acid was used in the dilution of the ceric ammonium sulphate there was no improved sensitivity of the assay (Fig 3 7)

With IBHR a good linearity was achieved when absorbance values were plotted against a concentration range of 0 1 - 1 0 ug/ml, when using 0 028M ceric ammonium sulphate with a 3 minutes incubation period A typical curve is shown in Fig 3 8 Lower concentrations (0 01ug/ml) can be detected by extending the incubation time to 30 minutes Reading at 340nm using 3 5X10^{-3}M ceric ammonium sulphate and 9 37X10^{-3}M arsenious acid did not lead to improved sensitivity in IBHR detection

Figure 3 1 Standard graph of absorbance values at 414nm (A_{414}) against concentration of KI (0 01 - 0 10 ug/ml) using 0 028M ceric ammonium sulphate incubated at 30°C and measured at 1 minute intervals over 5 minutes (t_1= the values at 1 minute, t_2= the values at 2 minutes, etc) as indicated (t_1, r=0 99, t_2, r=0 99 and t_3, r=0 98)

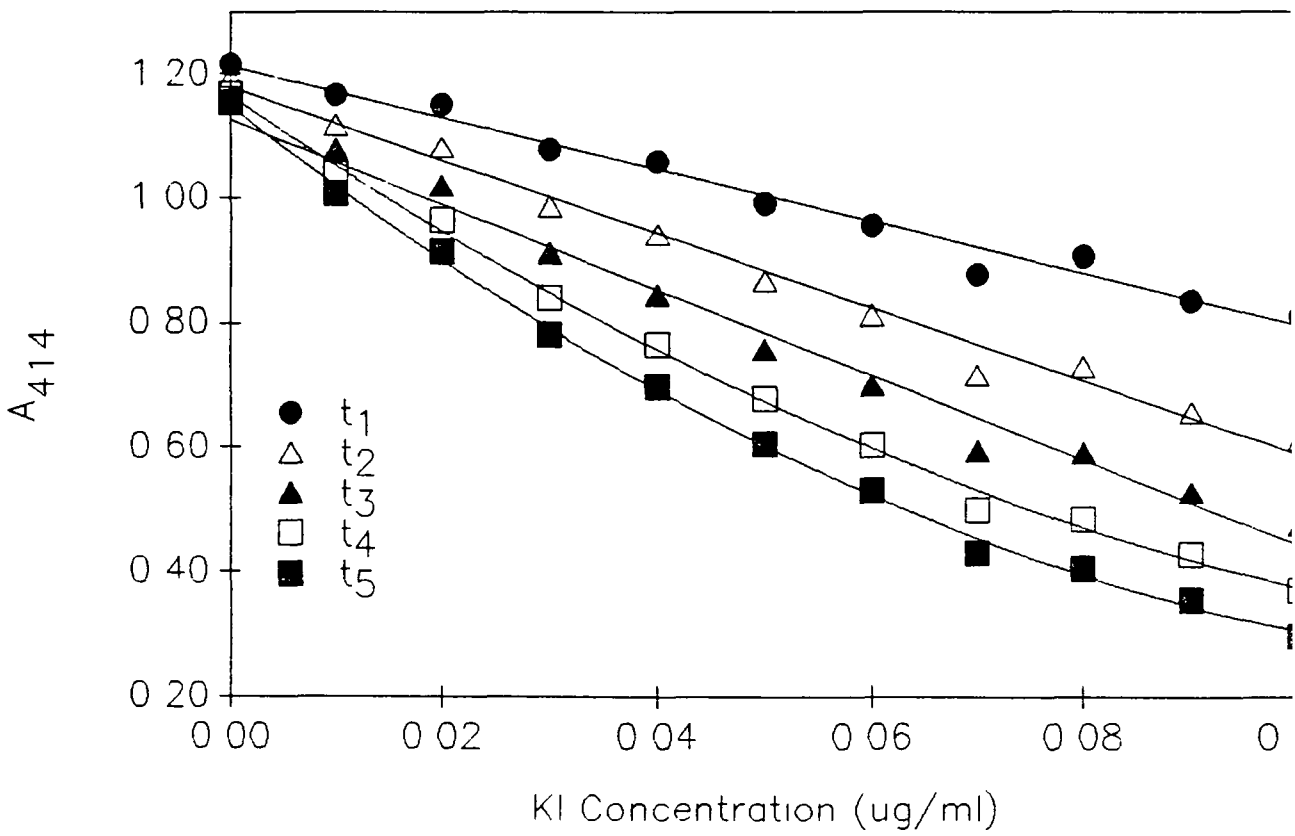


Figure 3 2 Standard graph obtained by plotting absorbance at 414nm (A_{414}) against concentration of KI using 0 028M ceric ammonium sulphate and an incubation period of 2 minutes at 30°C, 40°C and 50°C, as indicated

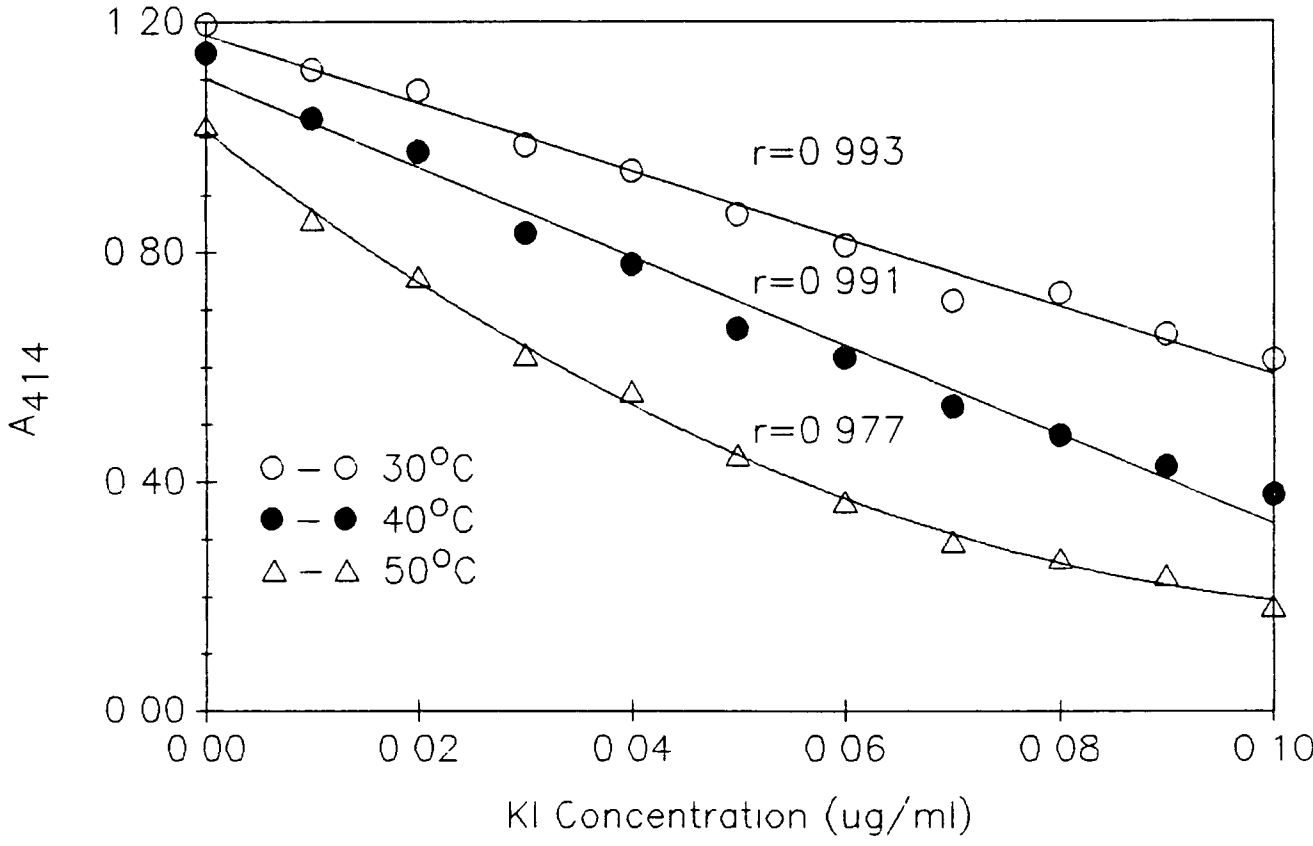


Figure 3 3 Standard graph obtained by plotting absorbance at 414nm (A_{414}) against concentration of KI using 0 028M ceric ammonium sulphate and an incubation temperature of 50°C for 1 minute

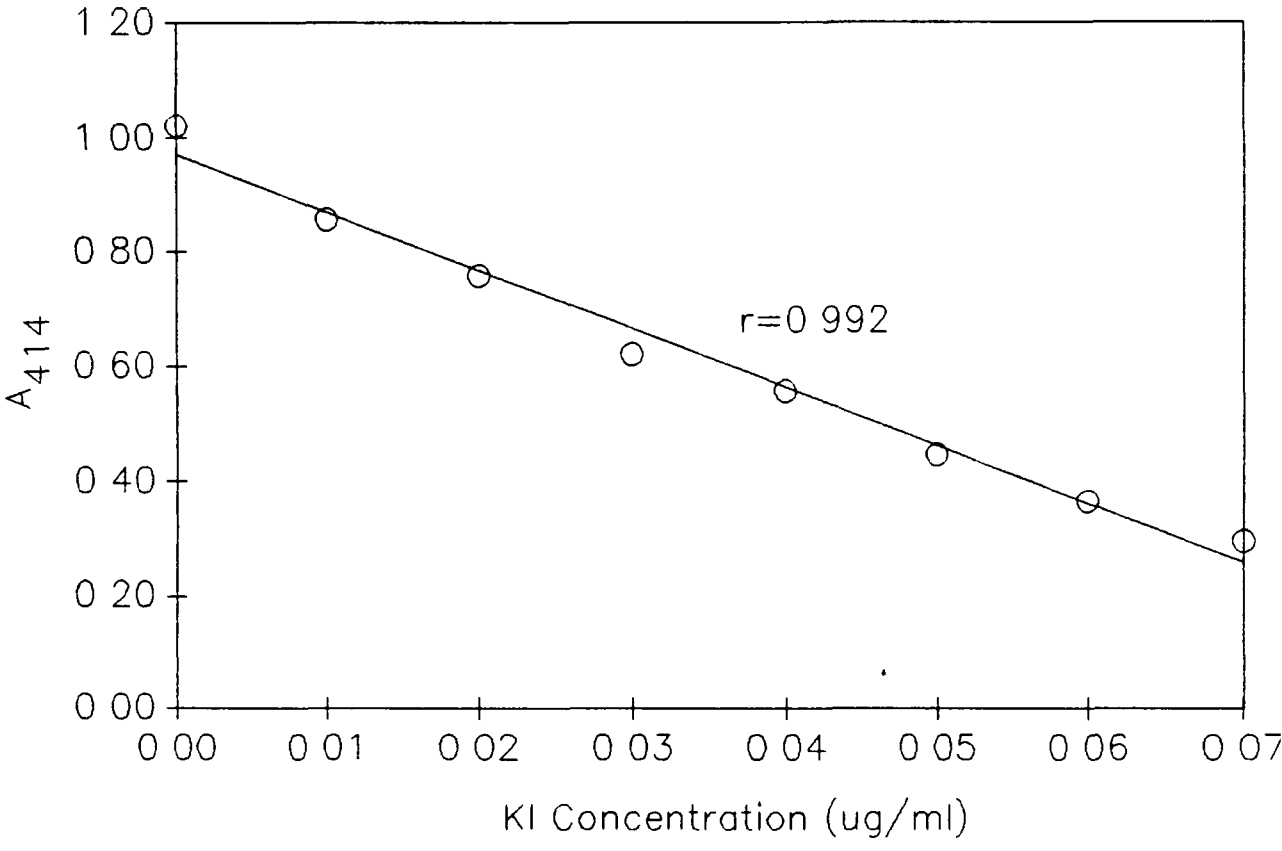


Figure 3 4 Determination of optimum concentration of ceric ammonium sulphate to use in the assay to measure iodide levels 0 007M, 0 014M and 0 028M ceric ammonium sulphate concentrations, 0 01 - 0 10 ug/ml KI standards and an incubation time of 2 minutes at 30°C were used A_{414} is the absorbance at 414nm

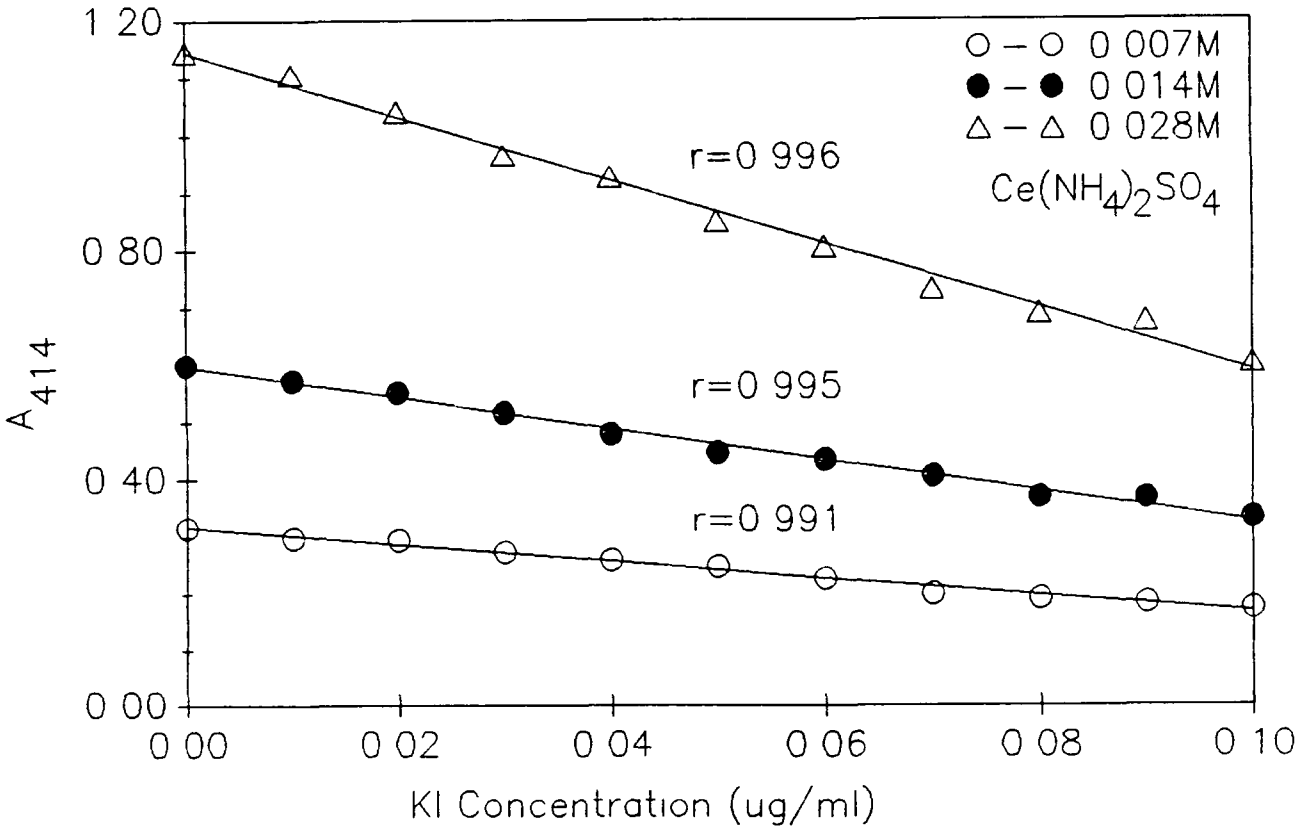


Figure 3 5 Standard graph obtained by plotting absorbance values at 414nm (A_{414}) against concentration of KI using 0 028M ceric ammonium sulphate (0) and 0 028M ceric sulphate tetrahydrate (●)

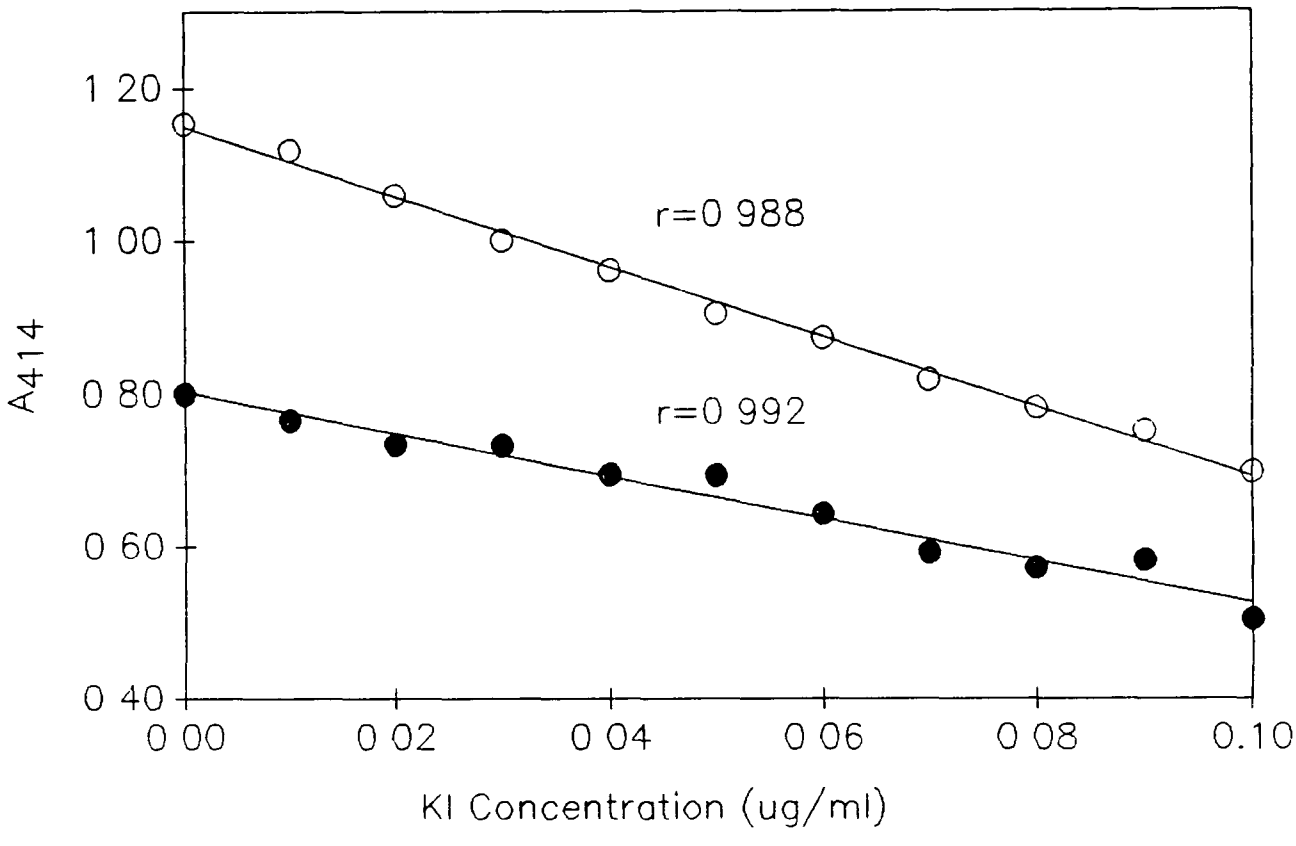


Figure 3 6 Absorption spectrum of ceric ammonium sulphate

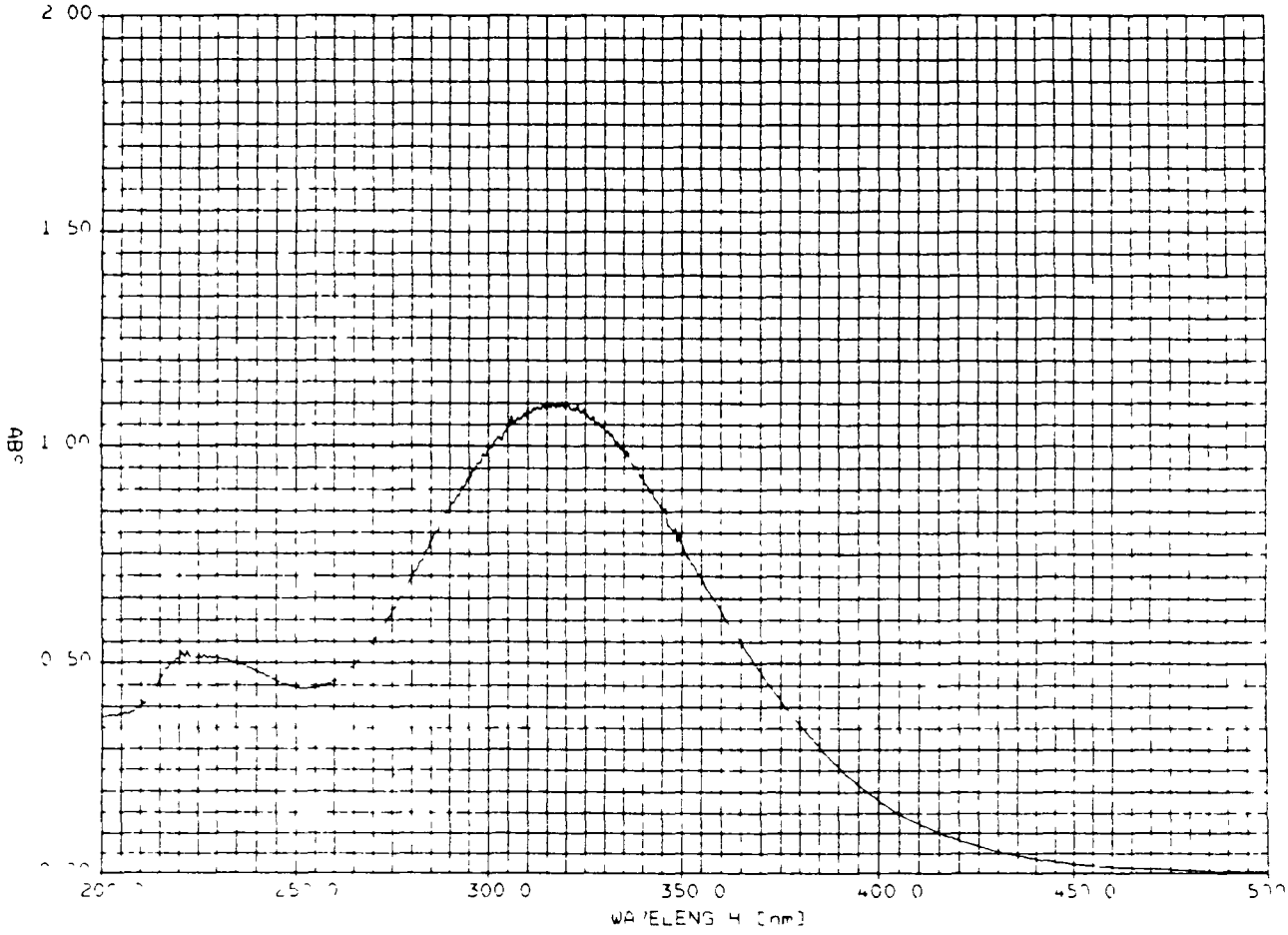


Figure 3 7 Standard graph of absorbance at 340nm (A_{340}) against concentration of KI (0 001 - 0 01ug/ml) using 3 5X10^{-3}M ceric ammonium sulphate diluted in 10% H_2SO_4 and 10% HNO_3, incubated for 30 minutes at 30^{o}C

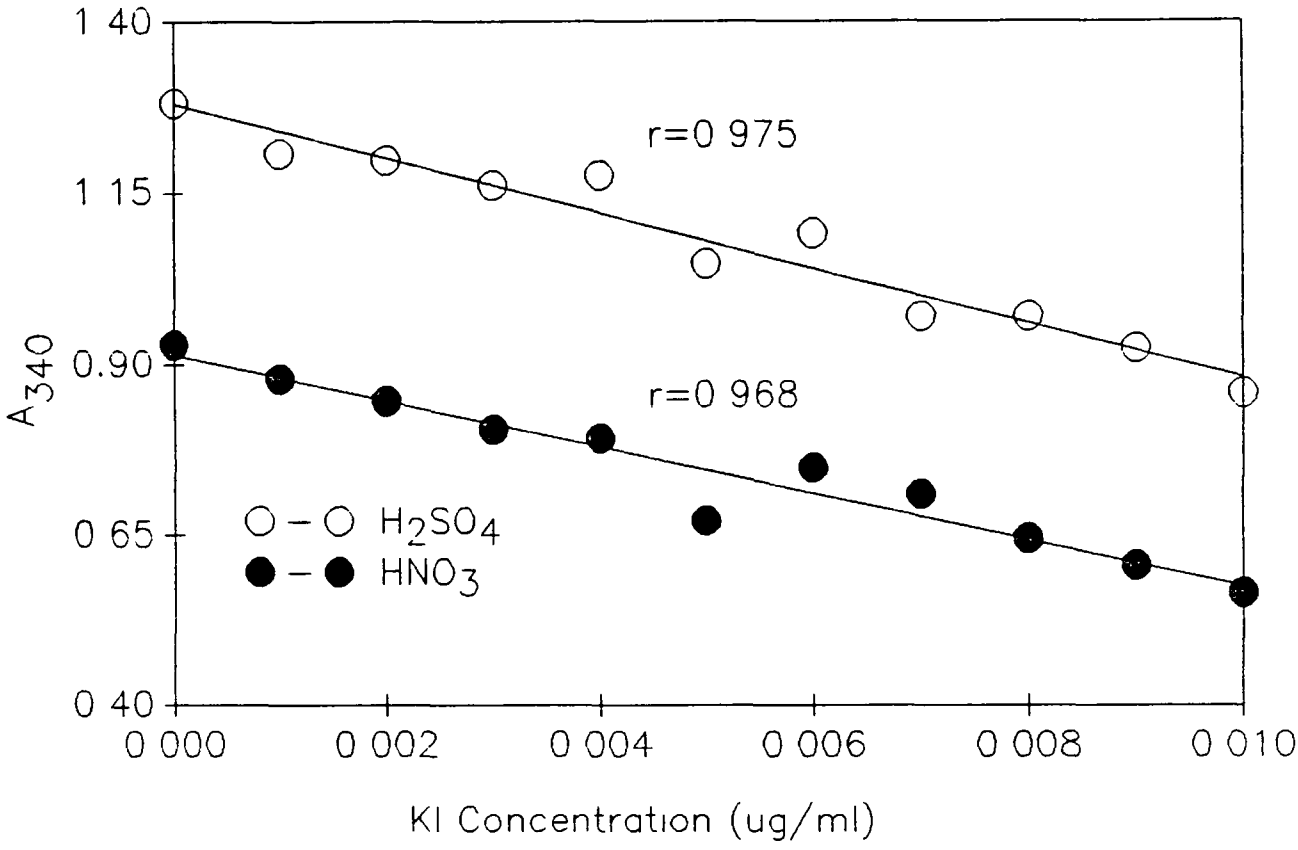
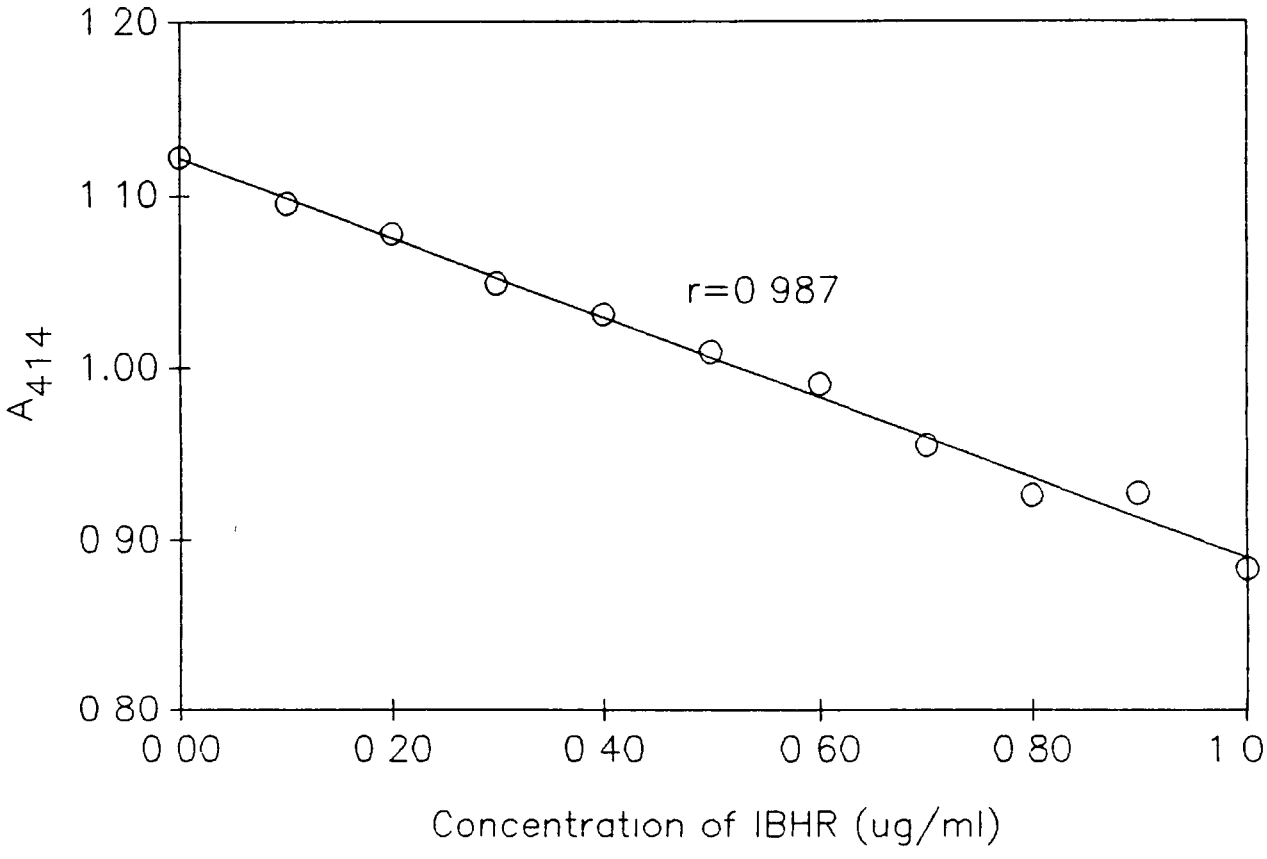


Figure 3 8 Standard graph of absorbance at 414nm (A_{414}) against IBHR concentrations (0 10 - 1 00 ug/ml) using 0 028M ceric ammonium sulphate incubated at 30°C for 2 minutes



3 3 Discussion

In the absence of iodide the Sandell-Kolthoff reaction proceeds with extreme slowness The difference in the reaction rates (Δ v) between reaction with and without catalyst can be expressed as $\Delta v = C_c - C_o$, where C_c is the test value of a reaction with the catalyst and C_o is the test value without the catalyst As the temperature is increased so also does the sensitivity of the reaction (*Knapp and Leopold*,1974) However, with increasing temperature there is a corresponding increase in the rate of reaction In these experiments a good sensitivity was achieved measuring absorbance values after 2 minutes at 30°C At increased temperatures the reaction proceeded too quickly Incubation at 30°C for 2 minutes was selected in order to keep the absorbance values within the linear range of the microplate reader, with a constant C_o value To that effect use of the incubator and kinetic function of the Titertek platereader proved very advantageous in keeping a constant C_o value

Percentage transmittance was used in determining concentrations of KI (*Sandell and Kolthoff*,1934, *Mougey and Mason*,1963, *O'Kennedy et al* ,1989) at incubation periods greater than 5 minutes The relationship of absorbance values and KI concentration at incubation periods less than 5 minutes was investigated and it was found that at incubation periods less than 5 minutes absorbance was linearly related to increasing KI concentration Standards were prepared in duplicate and triplicate and excellent standard curves were obtained (r=0 98 - 0 99) For incubation periods greater than five minutes this relationship was non-linear However, percentage transmittance may be used with longer incubation periods

A range of concentrations of ceric ammonium sulphate were used

for the assay, but 0 028M was selected as it gave increased sensitivity within the linear photometric range of the instrument Use of an alternate cerium compound, ceric sulphate tetrahydrate, for the preparation of cerium IV solution did not to lead to improved sensitivity as had been suggested (*O'Kennedy et al* ,1989) Previously, it has been reported that use of nitric acid increased the catalytic effect of iodide (*Knapp and Leopold*,1974, *Timotheou-Potamia et al* ,1985 and *O'Kennedy et al* ,1989,) on the Sandell-Kolthoff reaction Under the conditions of the assay described in this report the use of nitric acid enhanced the rate of the reaction but no increase in sensitivity was observed Similarily, sodium chloride was found to enhance the reaction rate It was possible to detect picogram levels of KI (equivalent of 7 6 pg/100ul iodide) when measurements were taken at 340nm rather than 414nm The absorbance spectrum absorbs maximally at 340nm and with the 340nm option available on the microplate reader this wavelength proved more advantageous However at 340nm, a lower concentration of ceric ammonium sulphate was used Otherwise, the relationship between KI concentration and the absorbance values were non-linear Increased IBHR sensitivity was not attained when reading at 340nm

Several studies have reported that not all iodine-containing compounds catalyse the Sandell-Kolthoff reaction equally (*Pantel*,1987, *Timotheou-Potamia*, 1988 and *O'Kennedy et al* ,1989) The presence of side groups adjacent to the iodine atom on the parent molecule affect the catalytic activity of the iodide atom Using IBHR it was not possible to achieve the same sensitivity as with KI The assay was successfully applied to the detection of IBHR-conjugated proteins based on the assumption that conjugation

does not alter the catalytic ability of the iodide

The advantageous features of this optimised assay are the short incubation times, use of absorbance values as against conversion to percentage transmittance and increased sensitivity when measuring at 340nm

4 THE APPLICATION OF THE IODIDE ASSAY IN IMMUNOASSAYS

4 1 Introduction

Chemical measurements based on immune reactions have been used by investigators for many years to accurately and precisely determine analytes in test samples as discussed in Section 1 Traditionally radioisotopes have been used as reporter groups for the detection of antibodies in immunoassay Due to the many associated problems in the use of radioactive compounds, enzymes have come to supercede the use of radioisotopic labels The specificity and sensitivity of enzymes has given rise to an array of simple and complex assay systems

In this section the use and detection of non-radioactive iodine-labelled antibodies for immunoassay is described From the wealth of iodination techniques described in the literature, the Bolton Hunter method of iodination was selected to label antibodies with non-radioactive iodine The iodinated antibodies were detected by the iodide assay already described Determination of IgG levels in both mouse and human serum samples was therefore possible

The performance of this system was then evaluated against an enzyme immunoassay Determinations of mouse IgG levels were made with an IBHR-conjugated goat anti-mouse antibody and compared with that of a commercially available peroxidase goat anti-mouse conjugate Determinations of human IgG levels were directly compared by conjugating the same batch of goat anti-human IgG with both horseradish peroxidase (HRP) and IBHR Human IgG levels were measured also, using both a sandwich and a competitive iodide-based immunoassay

4 2 Results and Discussion

4 2 1 Degree of hapten conjugation

Immunoglobulin G was labelled with IBHR Free IBHR was separated from bound IBHR by gel filtration The iodide assay was used to determine the IBHR content of the conjugate, assuming that the catalytic activity of the iodine atom was not greatly altered on conjugation The catalytic activity of the iodine atom is greatly affected by the nature of side groups attached to the benzene ring (*Pantel*,1987, *Timotheou-Potamia*,1988) Since these side groups do not change appreciably when a protein is conjugated using IBHR it is expected that the catalytic activity of the iodine atom attached to the protein should be similar to IBHR (*O,Kennedy et al* ,1989)

Hapten conjugation introduces groups into the the protein (*Knight and Welch*,1978) that absorb at 280nm so that use of absorbance at 280nm as a measure of protein concentration is no longer accurate (Fig 4 1) Similarily, IBHR was found to affect the BCA assay of protein determination Increasing IBHR concentration was found to be linearly related to increasing colour intensity with the BCA assay (Fig 4 2) determinations of protein concentration was made using an IBHR concentration equivalent to that determined in the conjugate as a zero control in the assay The protein concentration could then be directly read from the standard curve As many as 23 moles of IBHR were conjugated per mole of antibody

Figure 4 1 Isolation of IBHR-labelled immunoglobulin G by chromatography on Ultrogel AcA44 The eluant was PBS, pH 9 The flow rate was 1ml/min and 1ml fractions were collected The absorbance readings of every fifth fraction was monitored at 280nm (A_{280}) The first peak eluting represents iodinated immunoglobulin (fractions 20-40) The protein content was measured by the BCA protein assay No protein was present in the second peak The second peak represents unconjugated IBHR

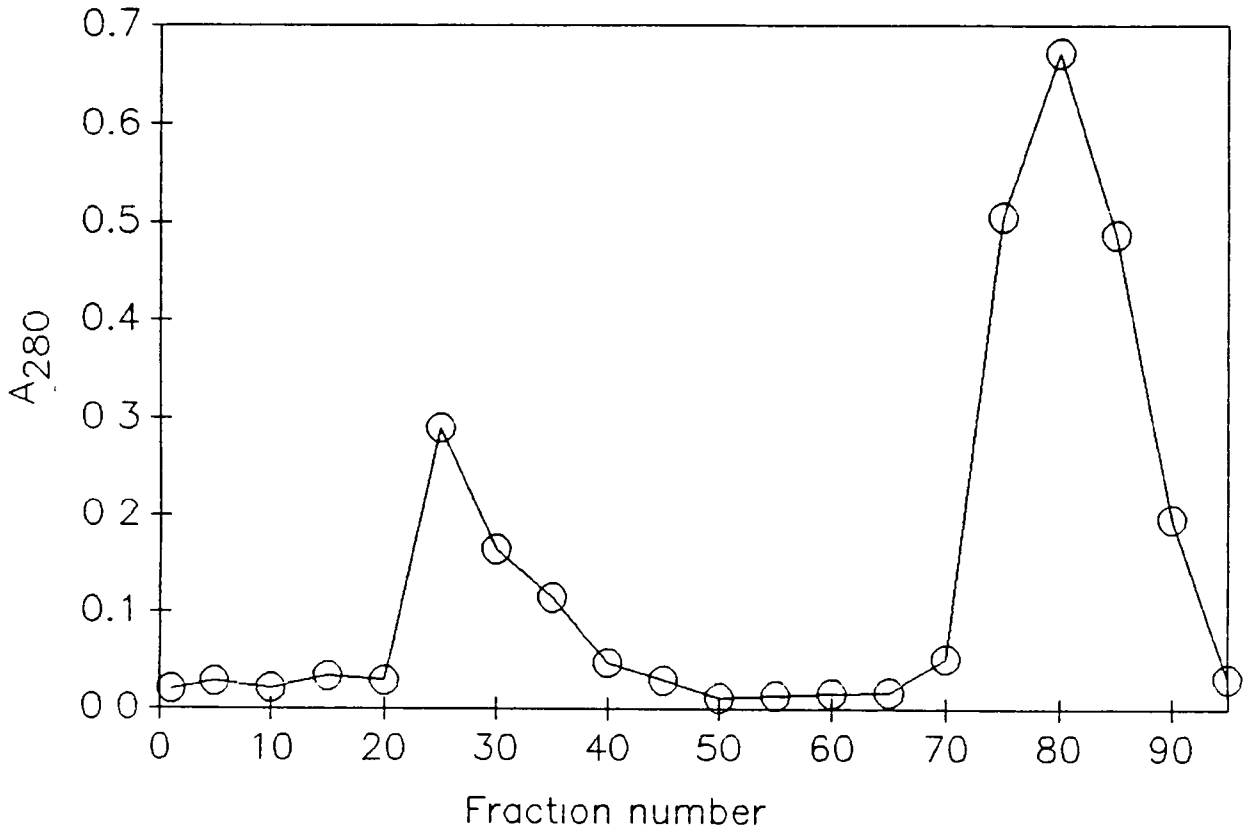
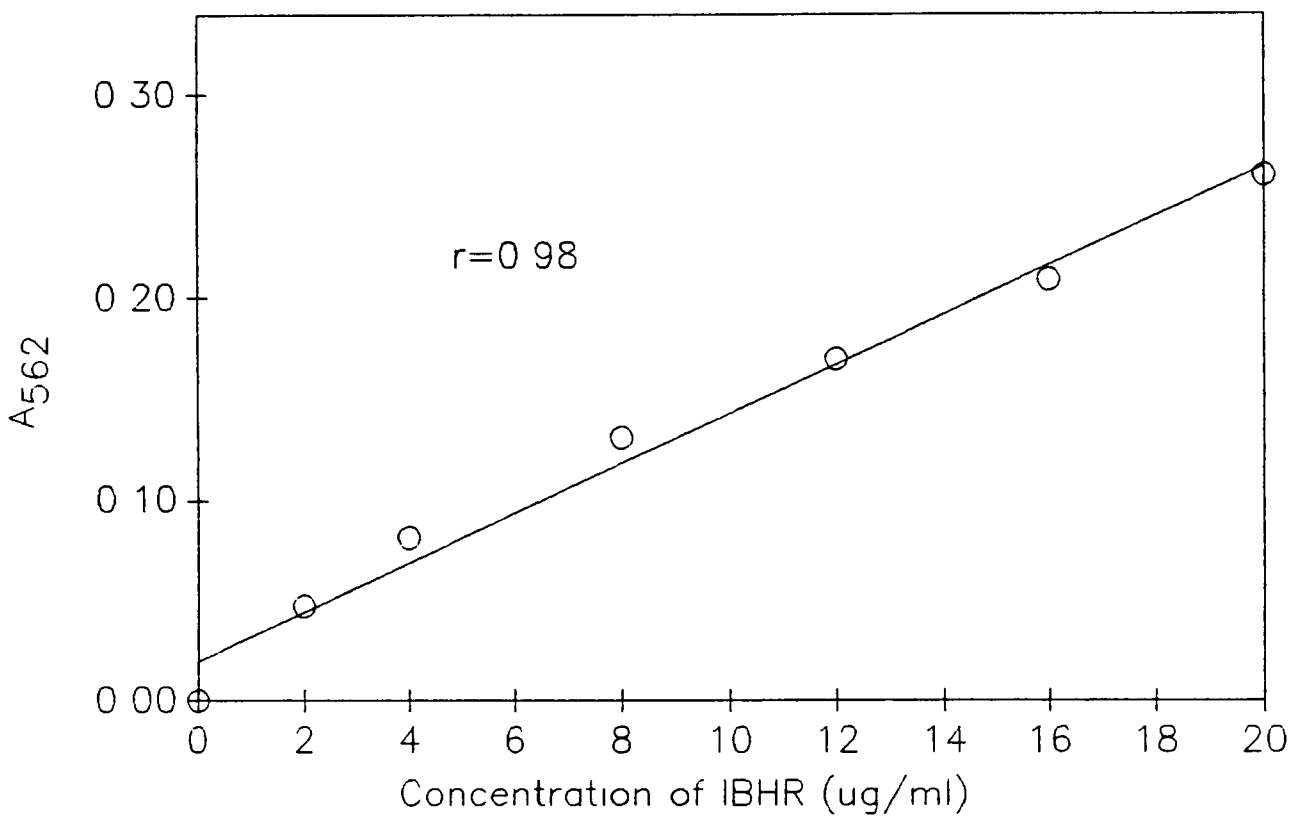


Figure 4 2 Standard curve demonstrating the effect of IBHR on the BCA protein assay Concentrations of IBHR are plotted against absorbance values at 562nm (A_{562})



4 2 2 Determination of mouse IgG levels in biological samples

In Fig 4 3, the binding of IBHR conjugated goat anti-mouse antibody to wells precoated with goat anti-mouse IgG followed by 0 - 40 ng/well mouse IgG is plotted It can be seen that when % transmittance, after 60 minutes, is plotted against mouse IgG concentration a straight line is obtained Thus, it was used to determine concentrations of mouse IgG over this concentration range in serum, ascites and hybridoma supernatants Plots of % transmittance rather than absorbance values were used in constructing standard curves, as the level of iodide present in wells was low Increasing the incubation temperature had little effect in reducing the time required for the assay to reach completion

Possible interference effects due to the presence of iodine in biological fluids are minimised in the assay due to the use of multiple washings and a range of controls

Fig 4 4 shows the binding of HRP-conjugated goat anti-mouse to wells coated as described for the IBHR-conjugated goat anti-mouse antibody At concentrations of mouse IgG higher than 20 ng/well the assay is non-linear It was possible to measure mouse IgG concentrations from the standard curves obtained after 30 minutes colour development with the HRP-labelled second antibody However 60 minutes incubation was chosen to keep this assay uniform with that of the iodide immunoassay

The values of mouse IgG levels in serum, ascites and hybridoma supernatants, obtained using both HRP- and IBHR-conjugated second antibody were compared (Table 4 1) to evaluate the performance of the iodide immunoassay with an enzyme immunoassay The measurement of

precision is based upon comparing results of replicate tests of the same sample between assays (i e on different days) While the results indicate that the method can be used to measure mouse IgG levels in biological fluids there are significant differences in IgG levels detected by both conjugates The differences observed have been attributed to the use two different batches of second antibodies Apart from the differences in titre and protein concentration between both conjugates, differences in antibody binding characteristics are possible as these are polyclonal antibodies raised in different goats (Sigma, *personal communications*) These observations highlight the importance of quality control in immunoassays To ensure that uniformity of a test exists between assay methods it is necessary to use external positive and negative reference reagents which can act as an external standard To overcome possible differences in binding between different batches of antibody it was decided that identical antibodies should be conjugated with both IBHR and peroxidase and the results compared This approach is described in the next subsection (4 2 3)

Figure 4 3 The plate was coated with 100ul/well of 10ug/ml goat anti-mouse immunoglobulin Mouse IgG (0 4ug/ml) was then added to give a concentration range of 0 - 40ng/well IBHR-conjugated goat anti-mouse IgG was then added, followed by the appropriate reagents and the % transmittance (%T) at 414nm was determined after 60 minutes

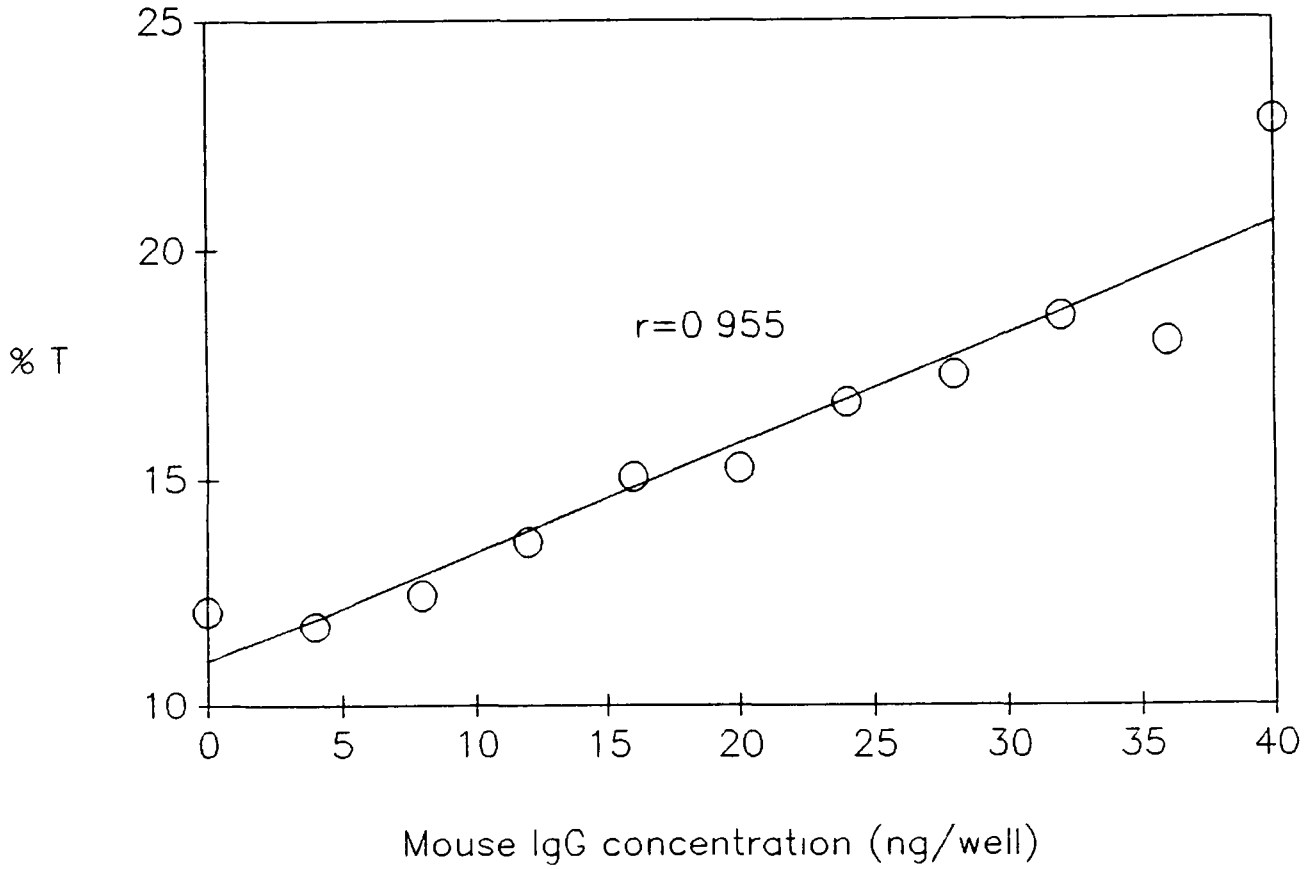


Figure 4 4 The plate was coated as described for Figure 4 3 HRP-conjugated goat anti-mouse IgG was then added followed by its substrate and the absorbance at 414nm determined after 30 and 60 minutes as indicated

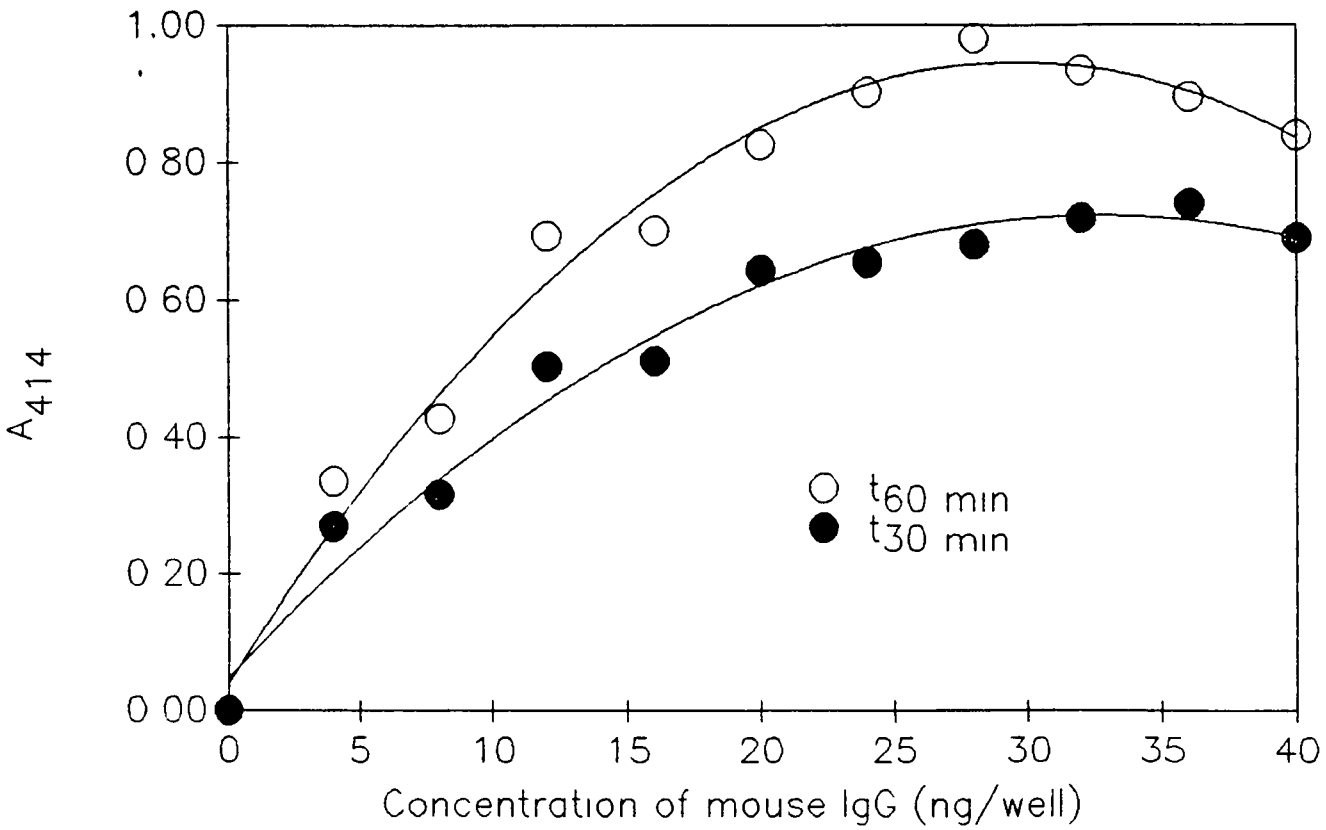


Table 4 1 Determination of mouse immunoglobulin levels in serum, ascites and hybridoma supernatant using both IBHR-conjugated and an HRP-conjugated goat anti-mouse antibody

SAMPLE	IBHR-CONJUGATE MIgG (ng/ml)	HRP-CONJUGATE MIgG (ng/ml)
Supernatant (neat)	264 0 ± 37 7 (5)	127 4 ± 34 0 (5)
Serum (1 20,000 dilution)	258 3 ± 65 0 (6)	136 6 ± 28 2 (5)
Ascites (1 100,000 dilution)	132 0 ± 58 0 (5)	37 8 ± 8 9 (6)

The results are the mean ± SD The number of determinations are given in parenthesis

4 2 3 Determination of human IgG levels (sandwich method)

Before the prepared peroxidase conjugate was used in assays its composition and properties were tested A dilution of the conjugate was measured for absorbance at both 403nm and 280nm, the adsorption maxima for HRP and IgG, respectively The ratio of these two readings offers a guide to the amount of enzyme conjugated which is an important element in determining conjugate performance An absorbance ratio (403 280nm) of 0 5 was observed, which is within the generally accepted range 0 3 - 0 5 for high enzyme activity without prejudice to antibody titre, while having low antibody background characteristics (*Catty*,1989)

Catty,(1989) recommends that when measuring enzyme activity an absorbance of 1 0 in 10 - 15 minutes in the test (*cf* Materials and Methods section 2 2 2), and a final absorbance of 1 5 or greater within 25 - 30 minutes is suitable for enzyme activity in ELISA tests Table 4 2 shows the results achieved with the conjugation of goat anti-mouse antibody with HRP which prove favourable for ELISA tests

The dry Sephadex used during the conjugation of HRP to antibody served to increase the HRP and IgG concentrations through the uptake of liquid by the beads and to decrease the relative periodate concentrations In the procedure a slightly higher molecular ratio than one was used for the formation of monomeric couplets of IgG and enzyme At a HRP/IgG molecular ratio of about one a maximum close to 40% of the IgG will be conjugated with one HRP only (*Tijssen and Kurstak*,1984) Increasing the ratio beyond this optimum will lower the free IgG fraction but increase the fraction of IgG conjugated

with two enzyme molecules which may lead to IgG inactivation The slightly higher ratio used was to allow for the fraction of HRP which would not become involved in binding

Both the IBHR and HRP conjugates were directly compared with the iodide immunoassay and ELISA, respectively The standard curves obtained with both conjugates are shown in Fig 4 5 The straight line obtained with the IBHR-conjugate can be used to determine human IgG over the concentration range 0 - 40 ng/well However, with the HRP conjugate absorbance values were non-linear with 25 ng/well human IgG and above

Table 4 3 shows the data of both IBHR-labelled antibody and HRP-labelled antibody methods to detect human IgG levels in serum samples The standard error value obtained using both the IBHR-conjugate antibody was lower than that obtained with the HRP-conjugated second antibody Thus, these results clearly indicate that the iodide immunoassay method compares favourably with existing enzyme immunoassays The values given are ng of IgG per well and when multiplied up by the dilution factor represent a range of 7 - 27 mg/ml IgG These values are within the range of IgG levels in normal human serum

Table 4 2 Measurements of enzyme activity of the HRP-conjugate Absorbance readings at 492nm are tabulated against time

TIME (minutes)	ABSORBANCE (492nm)
0	0 000
5	0 038
10	0 088
15	0 143
20	0 175
25	0 218
30	0 239

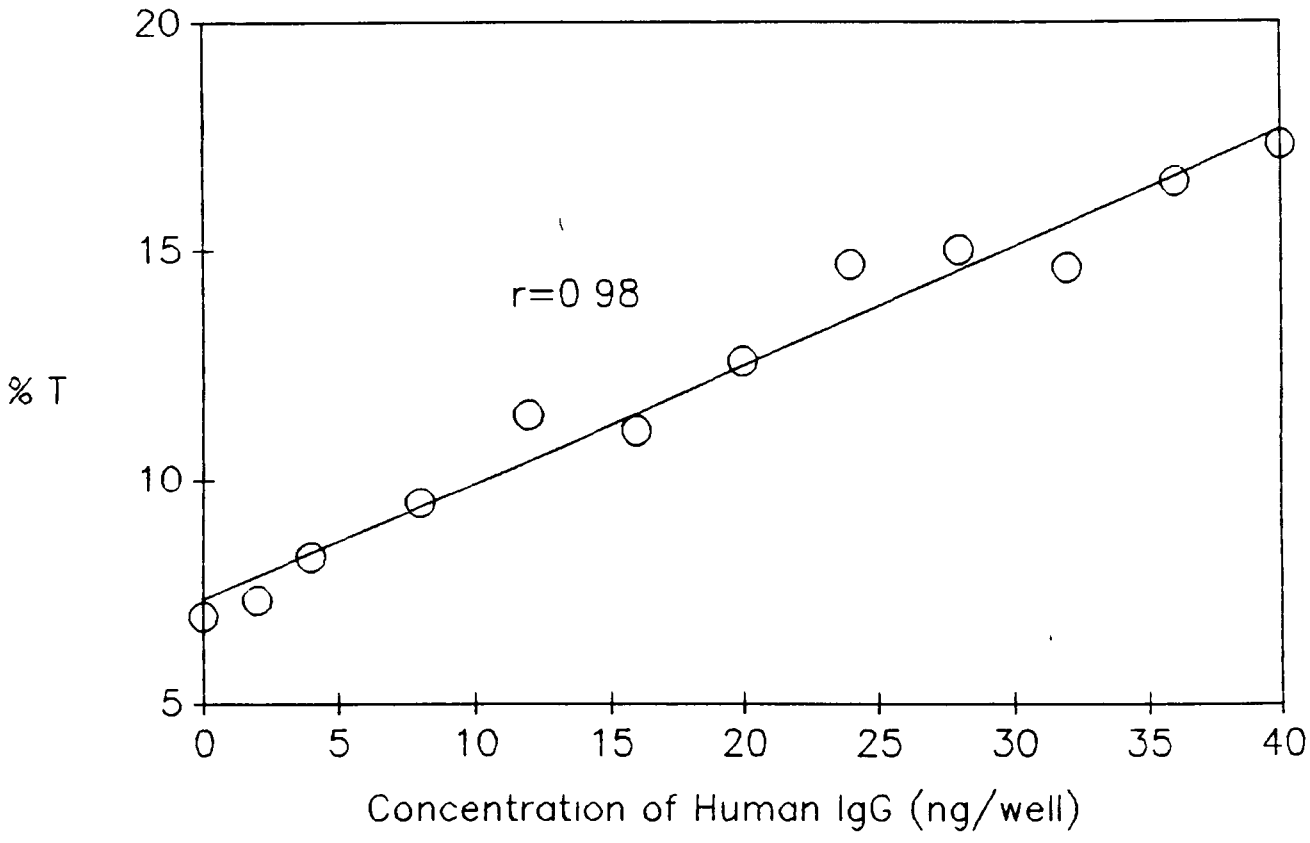
Table 4 3 Determinations of IgG levels in human serum samples diluted 1 100,000 using both the HRP-conjugated goat anti-human antibody and IBHR-conjugated goat anti-human antibody

SAMPLE	HRP-CONJUGATE IgG (ng/ml)	IBHR-CONJUGATE IgG (ng/ml)
1	180 6 ± 59 4 (8)	179 2 ± 24 5 (7)
2	126 8 ± 20 4 (8)	147 8 ± 24 3 (7)
3	76 2 ± 23 2 (8)	105 0 ± 32 9 (7)
4	101 2 ± 17 4 (8)	122 1 ± 31 9 (7)
5	180 0 ± 43 5 (7)	241 4 ± 33 3 (7)
6	212 0 ± 59 3 (5)	275 0 ± 45 0 (6)
SE	37 2	31 9

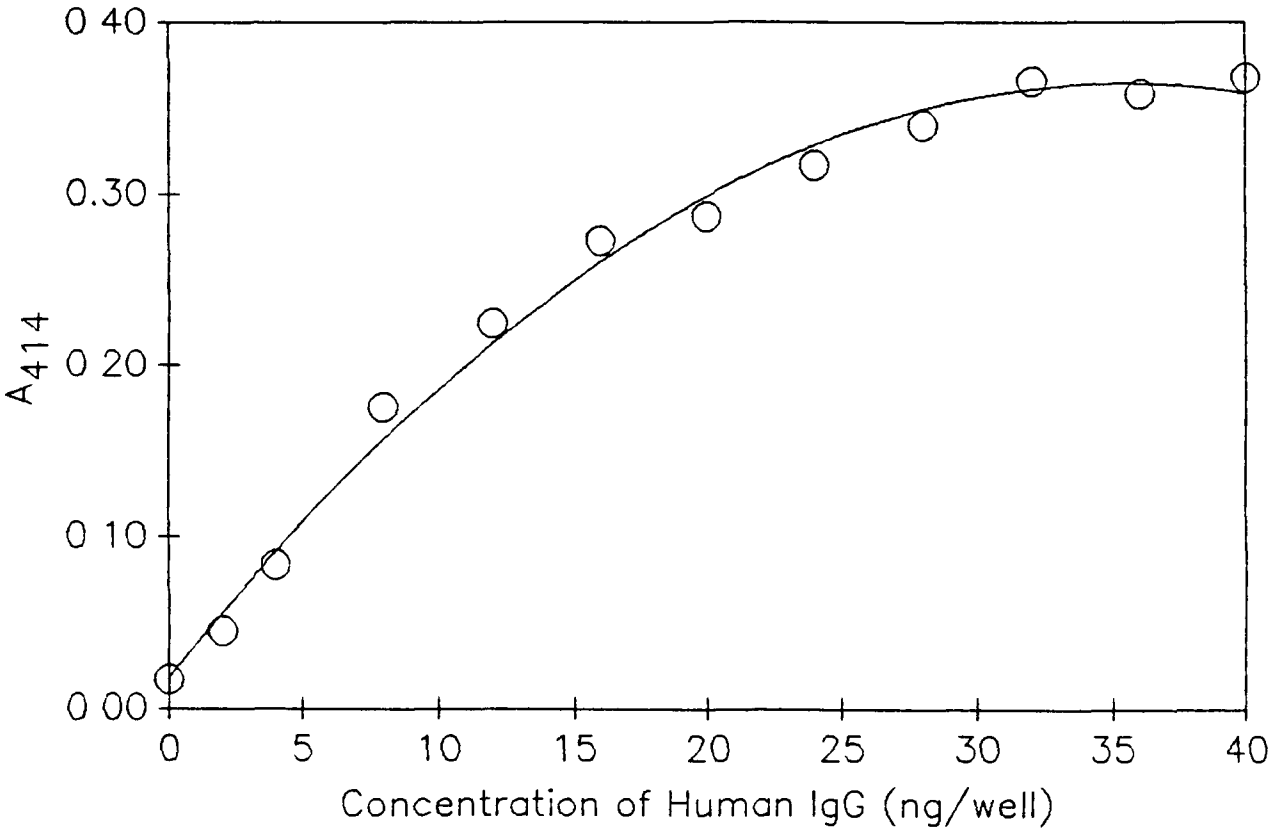
The results are the mean ± SD The number of determinations are given in parenthesis

Figure 4 5 (a and b)· The plates were coated with 100ul/well goat anti-human IgG (10ug/ml). Human IgG was then added to give a concentration range of 0 - 40 ng/well In (a) IBHR-conjugated goat anti-human IgG was then added, followed by appropriate reagents and the % transmittance at 414nm was determined after 60 minutes. In (b) HRP-conjugated goat anti-human IgG was added followed by its substrate and the absorbance at 414nm (A_{414}) determined after 60 minutes

(a)



(b)


0 40
0.30
0 20
0 10
0 00
A_{414}
0
5
10
15
20
25
30
35
40
Concentration of Human IgG (ng/well)

4 2 4 Determination of human IgG levels (competitive assay)

Human IgG was labelled with IBHR A standard curve was obtained when plates were coated with goat anti-human IgG, followed by incubating with 0 025 - 1 000 ug/ml human IgG, washing and then incubating with IBHR-conjugated human IgG With this assay absorbance values could be directly related to human IgG concentrations after 30 minutes incubation (Fig 4 6) The concentration range used in this competitive assay is higher than that of the sandwich assay This resulted in more IBHR-labelled human IgG binding, which allowed for the use of absorbance values in constructing a standard curve and a shorter incubation time

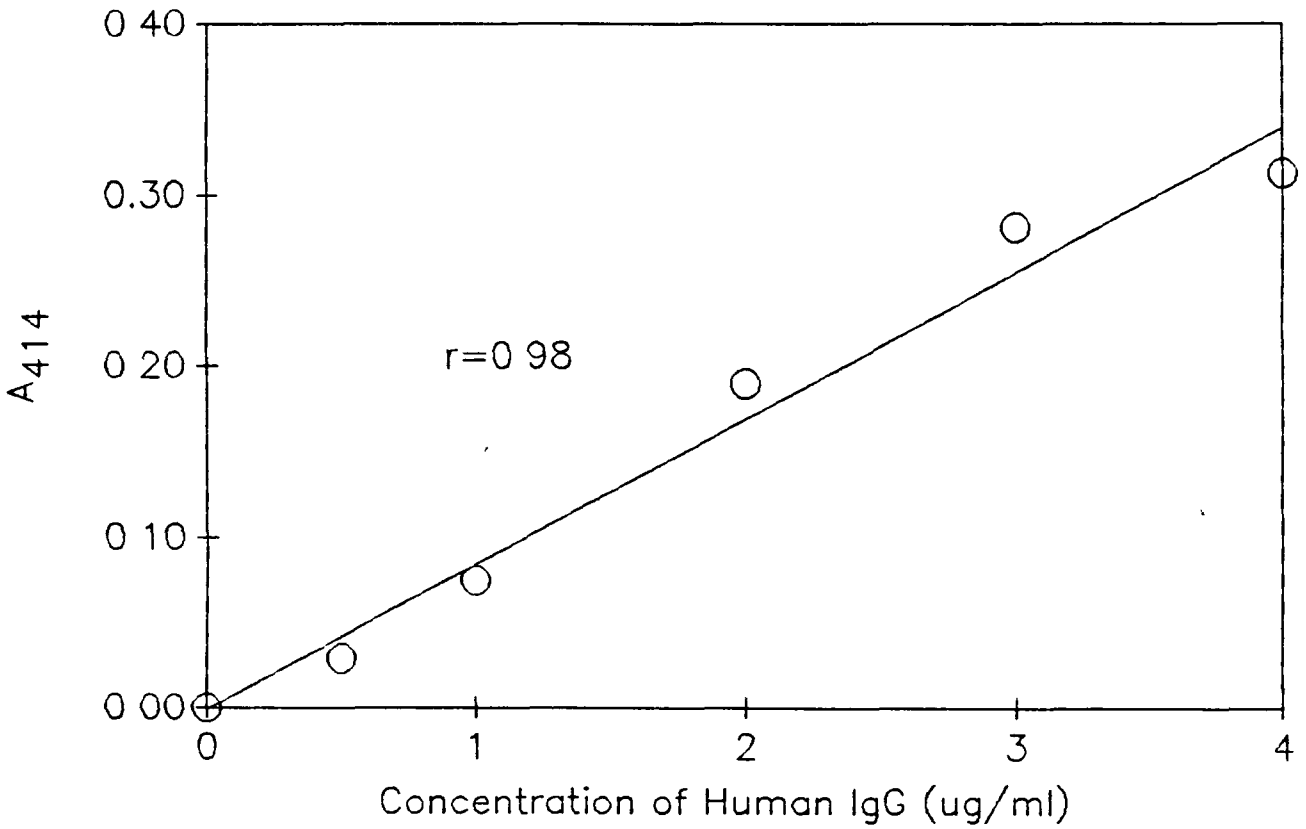
Using the same serum samples as used in the sandwich type assay, the levels of IgG were evaluated with this assay The results are listed in Table 4 4 The values when multiplied up by the dilution factor fall within a lower range (3 - 9 mg/ml) to that obtained with the sandwich assay The variability in detection is also greater Thus demonstrating the greater sensitivity of the sandwich assay over the competitive assay

Table 4 4 Determinations of IgG levels in serum samples diluted 1 25,000 using the competitive assay with IBHR-conjugated human IgG Values are expressed as ug/ml

SAMPLE	IgG DETERMINATION (ug/ml)
1	1 92 ± 0 80 (8)
4	1 25 ± 0 97 (8)
5	1 90 ± 0 52 (7)
8	3 32 ± 0 27 (8)
9	1 57 ± 0 44 (6)

The results are expressed as the mean ± SD The number of determinations are in parenthesis

Figure 4 6· The plate was coated with 100ul/well goat anti-human IgG (10ug/ml), followed by human IgG standards (0 5 -10ug/ml) and incubated for 30 minutes The wells were then incubated for 30 minutes with IBHR-conjugated human IgG followed by the appropriate reagents and absorbance at 414nm plotted against IgG concentration



4 3 Concluding remarks

From these results it is apparent that the iodide immunoassay is a feasible alternative to enzyme immunoassays With iodine-labelled second antibodies it was possible to detect IgG levels of mouse and human biological samples at ng quantities The IBHR labelled antibodies proved to be more sensitive over the concentration range used than the HRP-labelled antibody With the sandwich immunoassay the standard curve with the IBHR-conjugate was sensitive over the range 0 - 40 ng/well, whereas the HRP-conjugate only achieved linearity in the range 0 - 25 ng/well

With the attachment of a label of low relative molecular mass, such as iodine, it would be expected to influence the reactivity of the molecule to a lesser extent than the presence of the larger HRP However, the observed sensitivity with the iodide immunoassay was not greater than that seen in ELISA Enzymes are very efficient catalysts A single molecule of enzyme typically converts 10^3 - 10^4 molecules of substrate into product per minute, for some enzymes this number may be higher Horseradish peroxidase in these studies proved more efficient as a catalyst than iodine Peroxidase converted substrate to product in half the incubation time it took for iodide to catalyse the Sandell-Kolthoff reaction

In the following section a method is described to increase the number of iodine labelled to antibodies, in an attempt to increase on the sensitivity of the assay, thereby allowing for the use of absorbance values in determining levels of IgG

5 IODIDE IMMUNOASSAY AMPLIFICATION

5 1 Introduction

In an effort to increase on the sensitivity of the iodıde immunoassay, conjugation of poly-L-lysıne (PLL) to antıbody was attempted IBHR binds to lysine groups, so that conjugatıng antıbodies wıth poly-L-lysine followed by IBHR labellıng would ıncrease on the number of bound IBHR molecules per antıbody molecule and, thereby increase assay sensitivity

A number of methods exıst for the modification of functıonal groups ın proteıns Many of them ınvolve bıfunctıonal reagents ie reagents wıth two reactıve groups that are capable of reactıng wıth and formıng brıdges between the sıde chains of certaın amino acids ın proteıns The cross-links may be eıther of the ıntra- or inter-molecular type In thıs work the synthesis of an inter molecular link between PLL and ımmunoglobulın usıng N-succinımidyl 3-(2-pyrıdyldithıo)propıonate (SPDP) was attempted

2-Pyridyl disulphıde groups were introduced ınto the ımmunoglobulın and ın the other (PLL) thıol groups are introduced by pyrıdylthiolatıon and subsequent reduction wıth dithıothreitol On mıxıng, the two substituted proteins react to form a conjugate ın which the lınkage ıs a disulphıde bond The expected conjugate should have the general structure

$$PLL - NH \quad COCH_2CH_2 \quad SS \quad CH_2CH_2CO \quad NH - IgG$$

5 2 Results

SPDP is a heterobifunctional reagent The method of protein conjugation consists of three steps (i) the separate derivatisation of both IgG and PLL with SPDP, (ii) the reduction of the SPDP group on PLL to its thiol form with dithiothreitol, and, (iii) the reaction of the SPDP group on IgG with the reduced SPDP group of PLL when the derivatized IgG and PLL are brought into contact

The reaction can be followed quantitatively by measuring the dithiopyridyl released Absorbance measurements showed that the number of dithiopyridyl groups per polypeptide was between 1 and 4 at a 10 1 molar ratio of SPDP to PLL These values are less than those reported by *Canfi et al* (1989) who achieved between 5 and 8 dithiopyridyl groups per PLL of the same molecular weight At the same molar ratio, the degree of substitution with IgG was 1 7 moles of dithiopyridyl groups per mole of IgG which correlate well with that attained by *Carlsson et al* (1978) Under the experimental conditions used only a small fraction of both the protein and polypeptide amino groups reacted with the reagent At higher concentrations of SPDP (20 1 molar ratio SPDP IgG) the modified IgG resulted in a turbid solution The increased hydrophobicity as a result of the introduction of several aromatic groups may be responsible for the turbidity Using a 20 1 molar ratio of SPDP PLL their was no increase in the number of dithiopyridyl groups incorporated

The PLL-bound dithiopyridyl structure was converted into a PLL-bound thiol compound by reduction with dithiothreitol PLL, rather than IgG was selected to introduce the sulphydryl group into

this group to avoid exposing the latter to reducing conditions that could result in the alteration of its immunoreactivity The PLL-2-pyridyl disulphide was reduced to PLL-thiol derivative at acidic pH values

When both the dithiopyridyl-IgG and thiol-PLL group react, their reaction with each other should lead to the formation of a PLL-IgG conjugate Following the purification of the protein-PLL solution by gel filtration, fractions were monitored for protein content from the absorbance at 280nm and polypeptide content by the Ponceau S procedure On analysis no conjugate formation occurred, two peaks were observed the first was an IgG peak and the second was free PLL

5 3 Discussion

Clearly, more work needs to be done to perform the successful conjugation of PLL to IgG Attempts at increasing the number of dithiopyridyl groups incorporated into the immunoglobulin lead to precipitation of protein Perhaps in further experiments the immunoglobulin should be thiolated rather than the PLL, as homogenous aggregates of PLL may have formed before reaction with the dithiopyridyl derivative of immunoglobulin Alternatively Fab' fragments of immunoglobulin could be used in the final reaction step as they contain thiol groups Use of Fab' fragments would eliminate the thiolation step and only require the incorporation of dithiopyridyl into the PLL chain This method would be limited, in that attachment of only a single PLL chain could be conjugated per Fab' fragment There is only one available thiol group per Fab' fragment

6 PROTEINASE ASSAY

6 1 Introduction

Proteinases have an important function in the regulation of certain physiological events (*Neurath*,1984) A variety of methods for the quantitative measurements of proteinase activity have been developed These include spectrophotometric assays using flourochrome (*Tsiljar and Denker*,1986) or chromogenic (*Chavira et al* ,1984, *Church et al* ,1985) indicators conjugated to substrate molecules in a variety of forms Soluble substrates, for example o-phthalaladehyde, require the removal of undegraded material (*Church et al* ,1984) unless they have been attached to a solid phase support Insoluble substrates such as Azocoll (*Chavira et al* ,1984) can be removed by centrifugation Proteolytic activity is quantified by measuring the amount of indicator released into the supernatant Alternatively, indicators may be added to the supernatant to detect digestion products, by measuring primary amine groups The most sensitive of these spectrophotometric assays can detect trypsin, collagenase and elastase at 50ng/ml (*Twining*,1984)

As an alternative to the spectrophotometric assays radiolabelled substrates have been described These assays involve the radioisotopic labelling of either the substrate for which a particular enzyme is specific eg ^{125}I-labelled fibronectin (*Unkless et al* ,1973) or the labelling of a substrate which is susceptible to a broad spectrum of enzymes eg ^{125}I-labelled gelatin (*Robertson et al* ,1988)

The many hazards associated with the use of radioisotopes were outlined in section 1 Therefore, it was decided to develop an assay

to detect proteolytic activity by means of the non-radioactive iodide assay described in earlier sections This might also demonstrate the wide applicability of the iodide assay In the assay developed iodinated BSA is adsorbed onto the bottom of the wells of 96-well microtitre plates Following addition of protease, the amount of iodinated substrate remaining, can be determined and, thus, the level of proteolytic activity measured

6 2 Results and Discussion

The BSA used in this study was labelled with IBHR Gel filtration on Ultrogel AcA44 showed that the labelled product was free of unbound IBHR (Fig 6 1) The degree of hapten conjugation was found to be approximately 27 moles IBHR/ mole of BSA This is a considerable improvement on the value of 13 moles IBHR/ mole HSA reported by *O'Kennedy et al* (1989) This increase may be attributed to the use of a borate buffer during the conjugation procedure, maintaining a constant pH

Non-ionic detergents, such as Tween 20 and Triton X-100 are commonly used in washing buffers for many immunological and biochemical assays However, they were excluded from this assay because of the possible effects of such detergents on the action of proteolytic enzymes Hence, a stringent washing procedure using PBS was adopted based on the report of *Robertson et al* ,(1988)

A range of commercially available enzymes were tested to establish the sensitivity of the assay and to ascertain the classes of enzyme to which the substrate was susceptible Enzymes were tested at a range of dilutions from 1mg/ml to 1pg/ml The amount of iodinated BSA remaining adsorbed to the wells was then determined by

measuring the absorbance of each well at 414nm following the addition of the reagents for the Ce(IV)/As(III) reaction This data is represented in Figures 6 2(a,b and c) With this assay it was possible to detect each enzyme at concentrations above background in the range 1-100 ng/ml with papain and pepsin detected at concentrations as low as 100 pg/ml An assay using radioiodinated gelatin as substrate reports detection of trypsin and collagenase at 100pg/ml and papain at 100ng/ml (*Robertson et al* ,1988)

The endolytic enzymes trypsin and papain both cleave next to Lys and Arg and these residues are widely distributed in BSA Collagenase has a relatively restricted substrate specificity, cleaving only at proline residues Pepsin cleaves adjacent to aromatic or dicarboxylic L-amino acid residues and dispase cleaves adjacent to hydrophobic amino acid residues The ability of each enzyme (except dispase) to degrade the substrate reflects its sensitivity and susceptibility to a broad range of enzymes The Bolton Hunter reagent labels lysine residues, and BSA with 58 lysine residues was chosen to achieve a high conjugation level Gelatin radioiodinated with the Bolton Hunter reagent, has also been used due to its susceptibility to a broad range of enzymes (*Robertson et al* ,1988) The IBHR-labelled BSA proved to be an unsatisfatory substrate for the measurement of dispase activity An alternative substrate needs to be selected for this enzyme The use of microtitre plates allows large numbers of samples to be measured rapidly using small volumes of reagents which is a considerable advantage over previous methods described This assay thus further demonstrates the applicability of the iodide microassay

Figure 6 1 Isolation of bovine serum albumin by chromatography on Ultrogel AcA44 The eluent was PBS pH 9 The flow rate was 1ml/min and 1ml fractions were collected The absorbance of each fraction was monitored at 280nm (A_{280}) The first peak eluting represents iodinated BSA The protein content was measured by the BCA assay No protein was present in the second peak as measured with the BCA protein assay This peak is due to non-conjugated IBHR

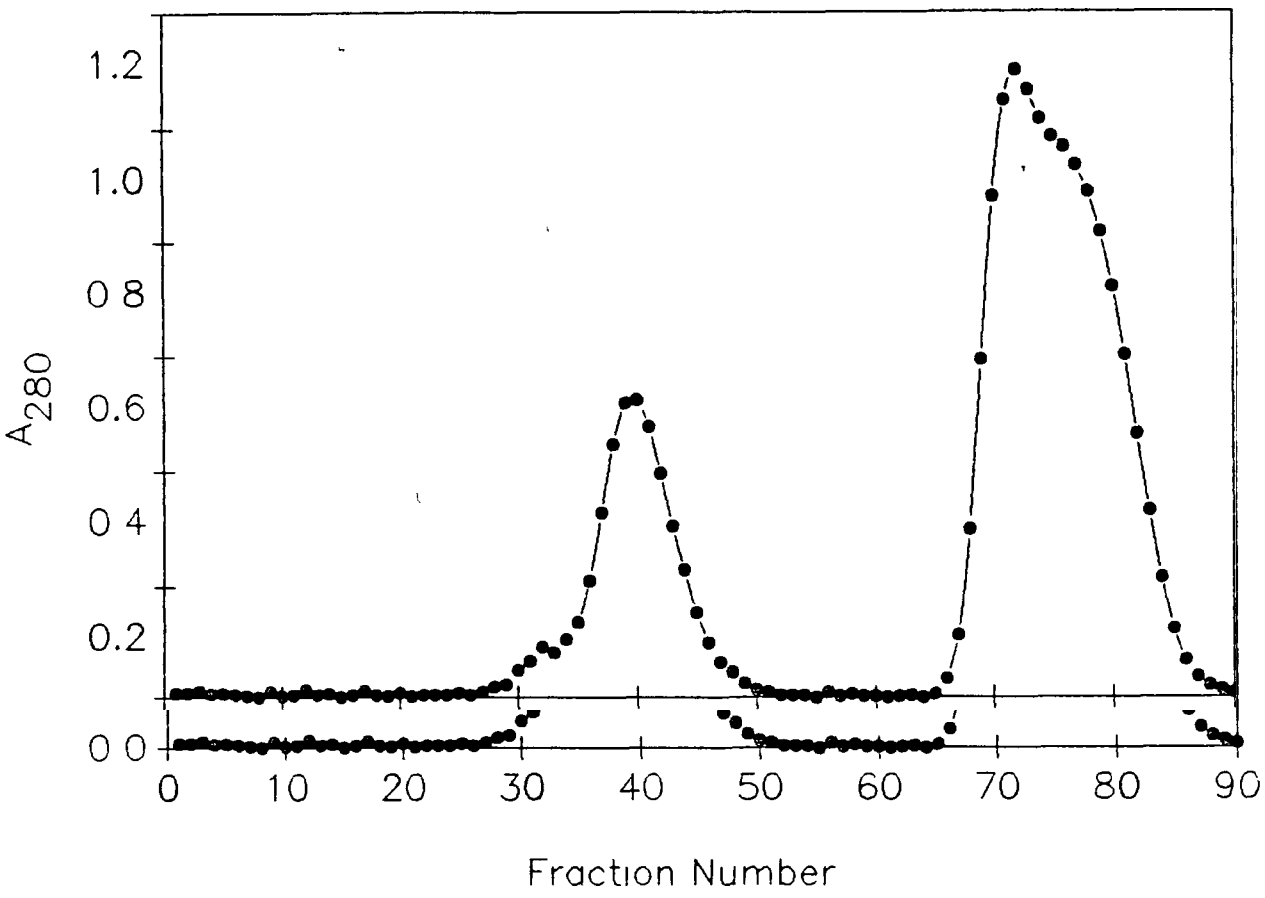
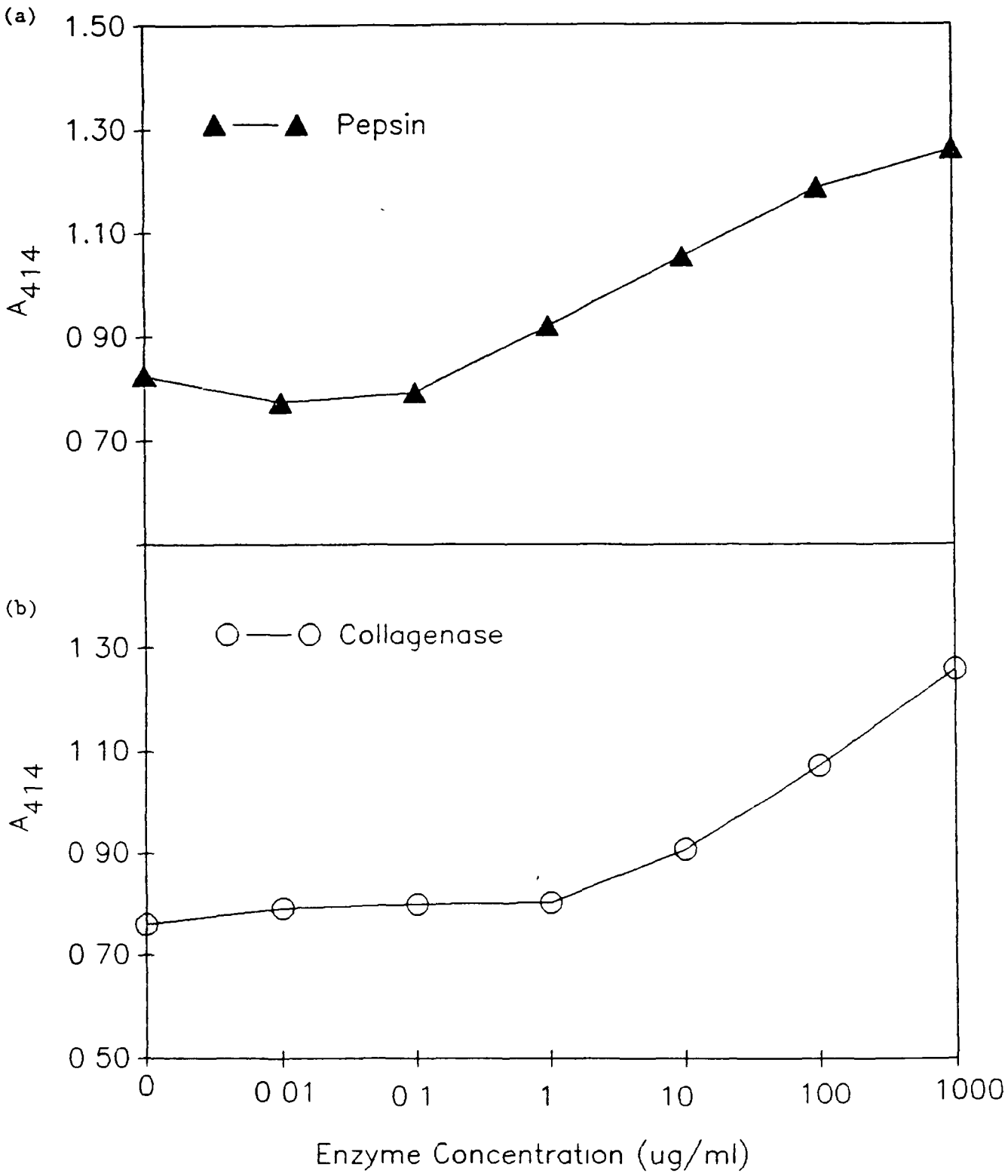
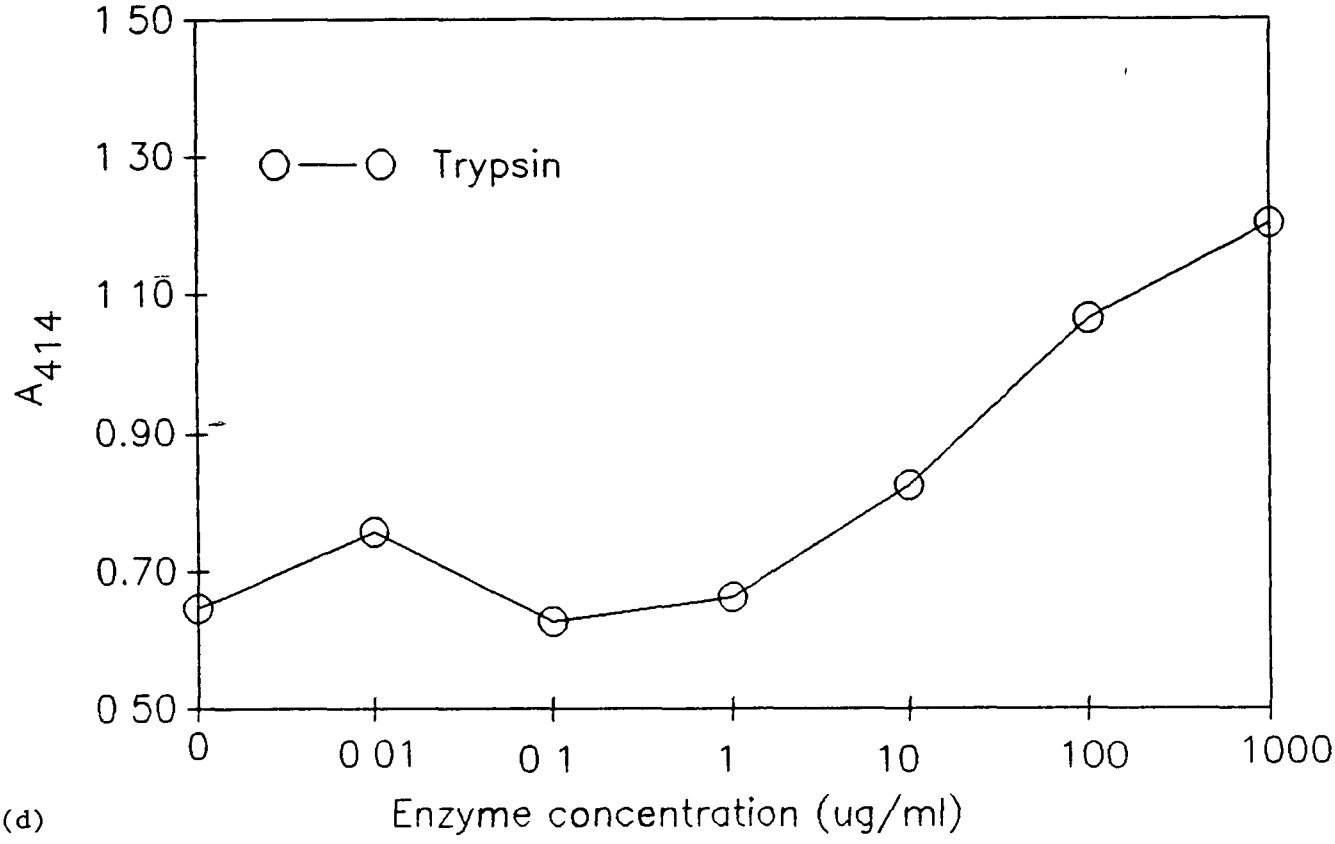


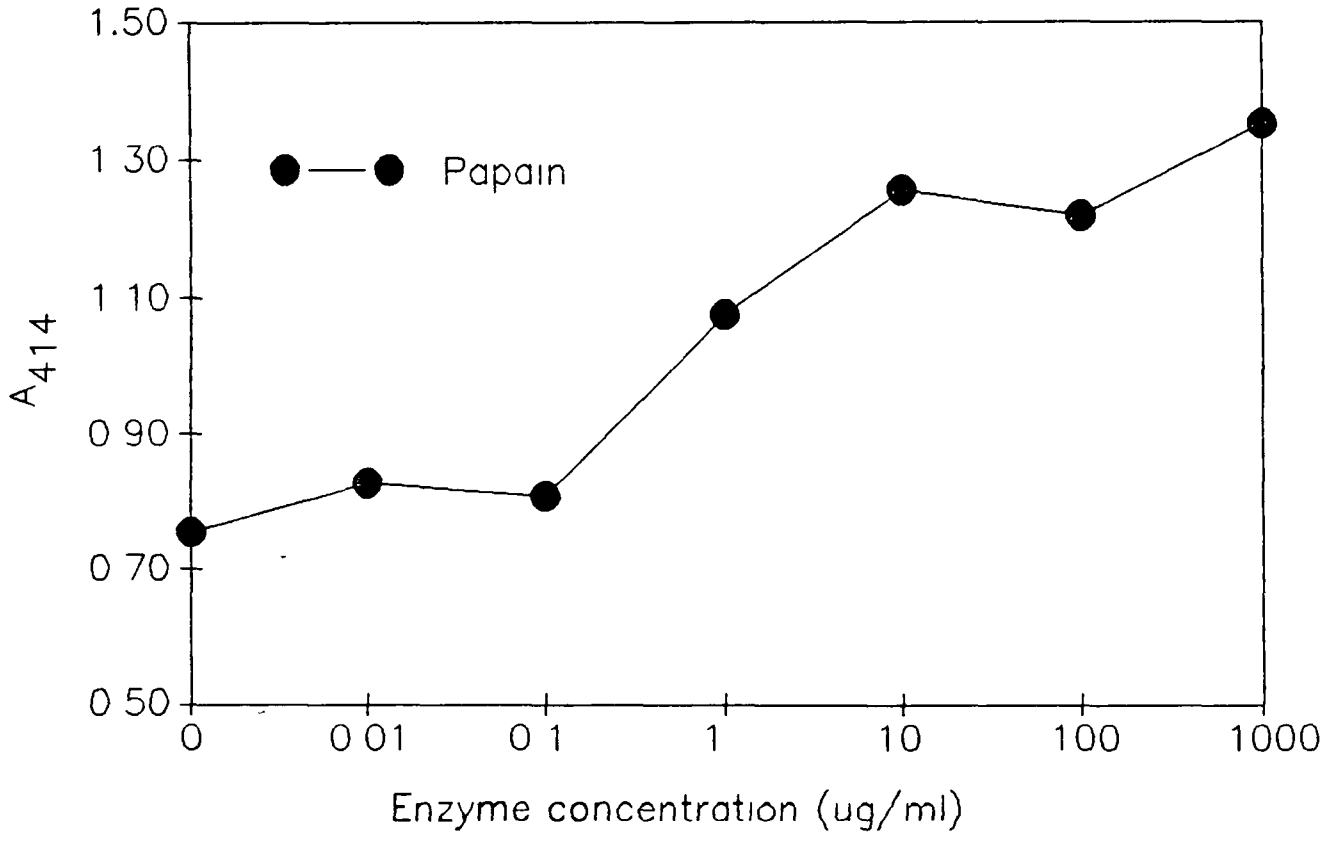
Figure 6 2 (a, b c,d and e) Absorbance at 414nm (A_{414}) plotted against concentration of enzyme incubated in microtitre wells with iodinated BSA Each enzyme was assayed in duplicate at 10-fold dilutions



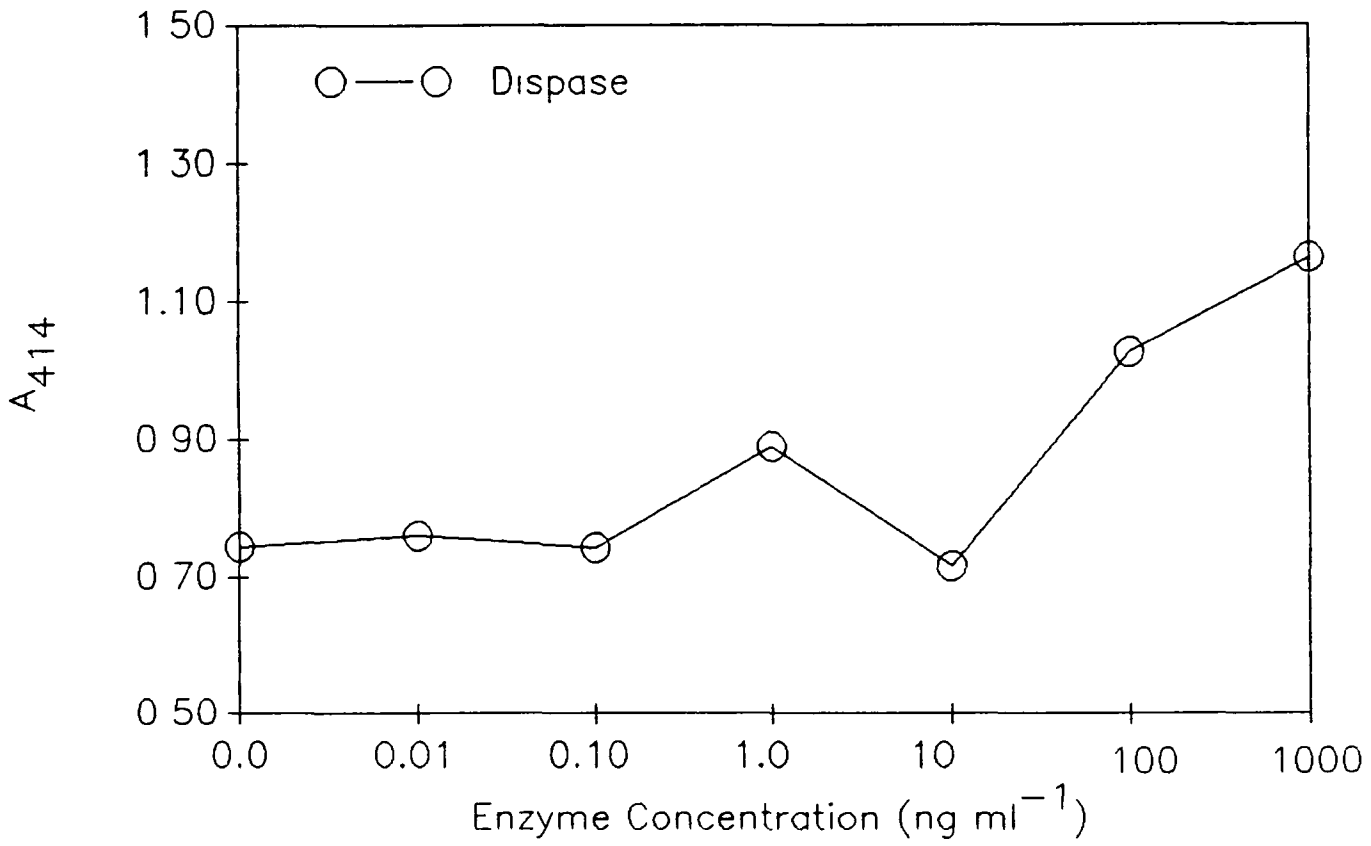
(c)


1 50
1 30
1 10
0.90
0.70
0 50
A414
Trypsin
0
0 01
0 1
1
10
100
1000
Enzyme concentration (ug/ml)

(d)


1.50
1 30
1 10
0 90
0 70
0 50
A414
Papain
0
0 01
0 1
1
10
100
1000
Enzyme concentration (ug/ml)

(e)


1 50
1 30
1.10
0 90
0 70
0 50
A_{414}
Dispase
0.0
0.01
0.10
1.0
10
100
1000
Enzyme Concentration (ng ml^{-1})

7 CONCLUSION

Non-radioactively labelled-iodine as a label in the measurement of analytes is described The Sandell-Kolthoff reaction was optimised for the measurement of iodide containing compounds The optimised iodide assay was then successfully applied in an immunoassay format for the measurement of IgG levels in biological samples The performance of the iodide immunoassay was evaluated directly against that of an enzyme immunoassay and the results compared favourably An iodide assay for the determination of proteolytic activity was also developed

The developed iodide assays overcome the problems associated with radioactivity, use small quantities of reagents, are suitable for use in photometric microplate readers and can test many samples simultaneously However, the iodide assay system does not offer the same sensitivity as achieved with the use of radioactive labels or even enzyme labels Increased sensitivity may be achieved with the iodide assay by increasing the number of iodide molecules labelled per protein molecule

The described assay system offers potential for further developments and applications One such area which may prove exciting is its application in biosensor technology

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