Flow injection immunoassays using solid phase immunoreactors and fluorescence detection

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FLOW INJECTION IMMUNOASSAYS USING SOLID PHASE IMMUNOREACTORS AND FLUORESCENCE DETECTION

by

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B.Sc; M.Sc; M.Phil.

A Doctoral Thesis Submitted in Partial Fulfilment of the Requirements for the award of

DOCTOR OF PHILOSOPHY

of
Loughborough University of Technology
1993

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Department of Chemistry
Loughborough University of Technology
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"To my late brother Abdur-Rashid"
Acknowledgements

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I would also like to thank the Ministry of Education, Government of Pakistan, for their financial help and Bahauddin Zakariya University, Multan, for giving me study leave to carry out this work.

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Aims of the Project

In the past flow injection immunoassays with solid phase immunoreactors have received little attention. Different solid phases, such as controlled-pore glass, Sepharose, agarose, immunodyne membrane, etc. have been included and monitored on-line with electrochemical, fluorescence, chemiluminescence and spectrophotometric detection. The reagents and washing buffers are pumped consecutively through the immunoreactor in a flow injection manifold. Antigen, antibody and labelled antigen are mixed, incubated and then injected into the buffer stream onto the immunoreactor. After washing, the bound fraction is eluted and measured with a detector, such as fluorescence or chemiluminescence.

The aim of the project is to investigate a reusable solid phase immunoreactor in the flow injection immunoassays with fluorescent detection.

The research will be carried out in three phases:

1) A simple flow injection analysis (FIA) system will be used to evaluate FIA with fluorescence detection.
2) Labelled analytes will be prepared using a series of fluorophores for example insulin-fluorophores.
3) Solid phase immunoreactors in flow injection immunoassays will be investigated.
ABSTRACT

The use of flow injection analysis with fluorescence detection was evaluated using the host-guest phenomenon between the cyclodextrins and DL-lysine and L-serine. Fluorescence enhancement, kinetic and equilibrium studies were recorded and the effect of pH and time on fluorescence were also observed.

Rhodamine isothiocyanate was conjugated to insulin. Insulin and dye were mixed in different ratios, and the dye : insulin ratio was determined for each conjugate. These conjugates were checked for immunoreactivity. Insulin-biotin and antibody-iminobiotin conjugates were also prepared. Insulin : biotin ratio was also determined. An insulin-biotin-avidin-Texas Red complex was also prepared. Each was checked for immunoreactivity.

Protein G-agarose, protein A-controlled pore glass(CPG), streptavidin-agarose, and avidin D-agarose-biotin-antibody solid phase immunoreactors were used in flow injection immunoassay of insulin. In these immunoassays, antibody, insulin and labelled insulin were incubated in vitro and then injected onto the immunoreactor. A binding buffer carried the sample through the immunoreactor and a fluorescent detector. An acidic buffer then eluted the components of the sample bound to the immunoreactor, which were then measured. An assay range for insulin was developed in each solid phase assay.
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CHAPTER ONE

GENERAL INTRODUCTION
1.1 Fluorescence

Fluorescence is the emission of radiation at longer wavelength than the excitation wavelength of some molecules, atoms, or ions. If the emission of light is short lived (usually $10^{-8}$ second), then the phenomenon is called fluorescence, but if delayed, the process is called phosphorescence.

When a molecule absorbs light energy of proper wavelength, i.e. a photon having energy equal to the difference of the electronic energy levels, it moves to the excited state. After staying in the excited state for $10^{-7}$ to $10^{-8}$ s, it may emit a photon equal in energy to the difference between the two energy levels. As can be seen in fig. 1.1 fluorescence involves transitions from the lowest vibrational level of the first excited singlet to various vibrational levels of the ground state. Due to this, fluorescence photons have lower energies (i.e. longer wavelengths) than absorbed photons.

The basic equation used to relate the fluorescence intensity with concentration is:

$$I_f = \phi I_0 \left(1-e^{-ebc}\right)$$

Where $\phi$ is the quantum efficiency, $I_0$ is the incident radiant power, $e$ is the molar absorptivity, $b$ is the path length of the cell, and $c$ is the molar concentration.

There are several structural factors that affect fluorescence behaviour. Increase of the $\pi$-electron system increases both absorption and fluorescence. Structural rigidity reduces the rate of internal conversion and hence tends to increase the luminescence. While the presence of internal heavy atoms and increase of temperature decreases the fluorescence intensity. Meta-directing groups like -NO$_2$, halides, etc. reduce the fluorescence because they reduce the $\pi$-electron densities. While para-directing groups like -NH$_2$, -OH, etc; enhance the fluorescence. Changes in the pH may change the electronic environment of
Fig. 1.1 Simplified energy level diagram of a polyatomic molecule.
the fluorophore and hence a change in absorption and fluorescence spectra may be observed, especially for molecules with charged or ionisable groups.

1.2 Flow Injection Analysis:

Flow injection analysis (FIA) was developed by Ruzicka and Hansen (1). It is based on the injection of a liquid sample into a moving, non segmented continuous carrier stream of liquid. The sample is transported towards a detector in order to measure some physical property like, fluorescence, absorbance, etc.

FIA is based on three principles: sample injection, controlled dispersion of the injected sample, and reproducible timing of its movement from the injection point toward and into the detector. The detector signal may be measured in the form of peak height or area. Peak height (H) and area (A) are related to the conc. of analyte as:

\[ H \text{ (or A)} = KC \]

Peak width measurements are less precise and gives the time difference (\(\Delta t\)) between the rising and falling edges of the peak. Such measurements have special applications.

For kinetic studies, peak height in continuous flow systems or fixed time reaction rate measurement in stopped flow systems are the commonest FIA approaches. Rigorous mixing, very reproducible timing of all operations, and protection of the flowing liquid from contamination and ambient conditions, are all advantages in the determination of rate constant for fast reactions.
1.3 Cyclodextrin:

Cyclodextrins are cyclic, water soluble receptor compounds. The most common are \( \alpha \), \( \beta \), and \( \gamma \)-cyclodextrins. (Fig. 1.2)

Cyclodextrin forms inclusion complexes, with the characteristic structure of an adduct and compounds of a size compatible with the dimensions of the cavity. The interaction between substrate and cyclodextrin depends on solute size and geometry. Van der Waals interactions, hydrogen bonding, release of strain energy in the cyclodextrin ring and release of high energy water molecules from the cyclodextrin cavity are also involved. Various influences of cyclodextrin inclusion complexes on guest molecules have been reported, for example, sheltering of hidden parts and enhanced reactivity of exposed parts of the guest molecules, and strong interactions within a guest pair caged in the cyclodextrin cavity. Inclusion complexes alter many properties of the included molecule, for example, chemical reactivity, volatility, and absorption, fluorescence and circular dichroism. These changes in chemical and physical properties are of both theoretical and practical interest.

1.3.1 Fluorescence Spectra:

Many workers have observed enhancement of the fluorescence signal with the addition of cyclodextrin, for example, Nakamura and Tamura (2) in the reaction of coenzyme A and 3-dephosphocoenzyme A with OPA-taurine, Miura et al (3) with \( \beta \)-cyclodextrin with the adduct of arginine-2,3-naphthalenedicarbaldehyde. Also cyclodextrin has been used with proteins (4) and hallucinogenic drugs (5). Patonay et al (6) have reported methods for enhanced fluorescence with cyclodextrin and micelles. Also, the inhibition of oxygen quenching by complexing with cyclodextrin has been studied elsewhere (7).
Fig. 1.2 Structure and the approximative geometrical dimensions of the α-, β-, and γ-cyclodextrins.
Scypinski and Love (8) discussed enhancement of luminescence spectroscopy with the addition of cyclodextrin and also studied the room-temperature phosphorescence and fluorescence of polynuclear aromatic hydrocarbons, biphenyls and nitrogen heterocycles induced into the cyclodextrin (9-11). The chemiluminescence of Lucigenin (12) and aqueous peroxoxyxalate (13), and room-temperature phosphorescence of anthracene (14) are examples of luminescence found to increase in the presence of cyclodextrin. The effect of cyclodextrin on the chemiluminescence of 10,10-dimethyl-9,9-biacridinium nitrate (15) and luminated compounds (16) has also been observed by some workers.

Fluorescence techniques have been used by many workers to study the effect of cyclodextrin, for example, β-cyclodextrin on para-amino acids (17), arenes and amphiphilic molecules (18), diphenylphosphate in aqueous solution (19), polycyclic aromatic hydrocarbons and densylated amino acids (20), steroids (21), and europium, ion-diketone complexes (22).

Fluorescence spectra have been used to investigate the complex formation of cyclodextrin with some substances, for example, β-cyclodextrin with pyrene (23), indole (24), 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP) (25), and α-, β- and γ-cyclodextrin with naphthalenes (26).

The formation of excimer by complexing γ-cyclodextrin with α-naphthylacetate (27), and pyrenes (28); β-cyclodextrin with phenyl propionate (29); and cyclodextrins with pyrenes (30) and related compounds (31), has been shown by different workers.

Determination of some substances have been improved by reaction with some reagents in the presence of cyclodextrin, for example, hydrogen peroxide and lucigen (32), beryllium in biological samples (33), scandium (34) and gallium (35).
In the literature some references are mentioned for fluorescence quenching. β-cyclodextrin quenches the fluorescence of methyl-2-aminobenzoate (36), sodium 1-pyrenesulfonate-aniline (37), 4,6,8(14)-trieneone steroids (38), while cyclodextrin quenches the fluorescence of pyrene and naphthalene in aqueous solutions (39).

1.3.2 UV-Visible Spectra:

Changes in the UV-Visible spectra are reported when a cyclodextrin is added to a substance, for example, cyclodextrins in copper (II) alkaline solution (40), KI-I₂, I₃-I₂, I₂ systems (41), 1,8-dihydroxyanthraquinone (42), and β-Cyd in pyrene (22), indole (23), heptyliviolgen (43), diflunisal anion (44), phenalpthalein (45).

Cyclodextrins have been determined in very small amounts spectrophotometrically (46,47) and colorimetrically (48) by various authors. While other substances when complexed with β-cyclodextrin acts as a good ligand for metals such as β-CyD-1,2-diaminoanthraquinone for palladium (49), β-CyD-triphenylmethane and xanthene for metal ions (50). Cyclodextrins has been used in many colour producing reactions in order to increase the sensitivity and selectivity of method, for example, PAR, PAN, 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol, 2-(3,5-dibromo-2- yridylazo)-5-(diethylamino)phenol with Zn²⁺, Cd²⁺, and Hg²⁺, etc (51). Hanada and Yamanish (52) removed interfering substances in the colorimetric determination of ammonia.

1.3.3 Kinetic and Equilibrium Studies:

There are a few examples in literature of the kinetic and equilibrium studies of reactions involving cyclodextrin as host. Turro et al (53) studied the kinetics of holonaphthalenes with β-cyd via time correlated phosphorescence. Methyl orange with γ-cyclodextrin (54), vitamin B6 compounds with cyclodextrins
(55), pyronine B with β- and γ-Cyclodextrin (56), pyronine B and pyronine Y with β- and γ-CyD (57), and rhodamine B with β-CyD (58) are examples of kinetics and equilibrium studies in the literature. The equilibrium constant has also been determined between pyrene and cyclodextrins (59), and naphthalene and its 1- and 2-methyl and 1,4-,1,5-,and 1,8-di-methyl derivatives (60) with α-, β-, and γ-cyclodextrins.

1.4 Ortho-Phthalaldehyde Reactions:

Roth (61) in 1971 formed fluorescent products by the reaction of ortho-phthalaldehyde (OPA), 2-mercaptoethanol (2-ME) and amino acids in aqueous alkaline solution (pH 8-10). Later Simon and Johnson (62,63) established the structure of the fluorescent product, (1-alkylthio-2-alkyl-substituted isoindole) with the help of UV, NMR, and mass spectroscopy.

The isoindole formed undergoes decomposition to an ethylene sulphide polymer and a non-fluorescent 2,3-dihydro-1-H-isoindole-1-one (63-71). A suitable ratio (2 or 3) of 2-ME to OPA is found to lessen the degree of instability (72).

Amino acids, proteins, and amines can be analysed with the OPA-thiol reaction (62-63,73-78) with the exception of proline and cysteine. Proteins (65,75,77,79,80-82) show intense fluorescence when labelled with this reagent. Membrane and viral proteins can be partially characterised at picomole levels (65). Peptides also formed a fluorescent product with OPA (83,84). The method was especially useful in studies of peptides obtained by fermentation of proteins (85).

Ortho-phthalaldehyde can be used for both pre- and post-column high pressure liquid chromatography (HPLC) derivatisation. Examples of post-column derivatisation include the fluorogenic detection of amino acids in
conventional amino acid analysers (75-84,86-91), analysis of total amino acids in natural waters (92), biogenic amines (93,94), and catecholamines and other metabolites in urine and brain tissues (67). Examples of pre-column derivatisation include the formation of an OPA-histamine complex extractable with ethyl acetate, amino acids in serum and urine (95), and separation of 30 (96) and 26 (68) amino acids. OPA pre-column derivatisation of polyamines in physiological fluids gave 6 to 10 fold increase in sensitivity (97) when compared to ninhydrin.

1.5 Affinity Chromatography:

In affinity chromatography, a specific ligand is immobilised on a support matrix in such a way that its specific binding affinity for another molecule is retained. Samples containing this second molecule are applied to a column of the immobilised ligand. After washing away the non-interacting solutes, the desired molecule is eluted by adjusting the elution conditions, for example, by using low pH. A schematic diagram for affinity chromatography is shown in fig. 1.3.

1.6 Immunoassays:

This involves the binding of antigen to antibody, followed by physical separation of the bound from unbound antigen/antibody in a heterogeneous assay (fig. 1.4), or a separation-free (homogeneous) assay where the interaction is detected directly. In competitive immunoassays a labelled antigen competes with the unlabelled (i.e. sample) antigen to bind to a limited quantity of antibody. In non-competitive immunoassay the antigen to be determined binds to an excess of antibody.
Fig. 1. A schematic diagram for affinity chromatography showing separation of antibody on protein A.
Fig. 1.4 A schematic diagram for heterogeneous immunoassay.
1.6.1 Antigen:

An antigen is defined as any substance that will stimulate the production of a specific antibody and subsequently bind to that specific antibody in vitro or vivo. Proteins and their glyco-, lipo, nucleo-conjugates, polysaccharides, and haptens i.e. small molecules bound to the lysine, tyrosine or histidine residues of proteins or other large carrier molecules, are all antigenic. In vitro, it is the hapten alone and not the conjugate which reacts specifically to the antibodies.

Antigens have epitope(s) which are enfolded by the corresponding antibodies. In proteins epitopes are mostly located on the hydrophilic parts of the immunogen, each of them being recognised by a specific antibody. The epitopes may have different shapes

![Antigen and carrier protein showing epitopes and haptens.](image)

... and there may be several on the same molecule. An epitope can reach the size of four to five linear lysine residues. In polysaccharides a typical epitope size is five to six monosaccharides and are usually identical.

1.6.2 Antibodies:

Antibodies are group of serum proteins called immunoglobulins and are produced by an animal's response to foreign substances. Antibodies bind to the antigen/immunogen that stimulate their formation. Usually in human blood serum they occur at a concentration of 12-15 mg/ml. Their isoelectric point varies between 4.5 to 9.5, and their molecular mass between 150,000 and 970,000 Daltons. They can be purified by salt precipitation, gel filtration, ion-exchange chromatography, or immunoaffinity fractionation.
There are two types of antibodies, polyclonal and monoclonal. Antibodies produced directly by immunising the animal with antigens are called polyclonal antibodies and are often lightly heterogeneous even when purified antigens are used. Identical antibodies monospecific to a single antigen epitope are called monoclonal antibodies. By fusing two types of cells, a splenic antibody secreting cell from the immune system and a long lived cancerous cell, a hybrid cell or hybridoma is produced. Such hybridoma cells secrete a specific antibody from the immune donor. The hybridoma cell lines can be stored at -80°C and thawed for a new production of the ascitic fluid.

Human immunoglobulins are divided into five main classes, IgG, IgM, IgA, IgD, and IgE. There are four IgG subclasses (IgG1, IgG2, IgG3, IgG4) and two IgA (IgA1, IgA2). IgG constitutes ca. 70% of the serum immunoglobulin.

The IgG structure resembles a letter Y (fig. 1.6). Each molecule has two identical heavy polypeptide chains (H) and two identical light chains (L) linked by interchain and intrachain disulphide bonds. The carbohydrates are attached to the constant parts of heavy and light chains i.e. the parts with an invariant amino-acid sequence. Light chains are of type kappa or lambda and have two domains—one with a constant sequence and one with a variable one. The heavy chains have four domains—three constant and one variable. IgE and IgM heavy chains have five domains, i.e. one domain more than IgG, IgA, and IgD. The Fc portion of an immunoglobulin is the stem of the Y-shaped molecule-dimer of the two last heavy chain domains, and the F(ab')2 is the remaining upper part. A fragment containing only one light chain and two upper heavy domains is called Fab.
There are structural dissimilarities among Ig classes. IgG is a Y-shaped structure, while IgM molecules are pentamers of an IgG like structure in a star-shaped cluster. IgA is found as a dimer of the Ig structure.

Paratopes or determinants are portions of antibody that fit to the specific portion of the antigen-epitope in antigen-antibody interactions. The two N-terminal domains, one from each H and L chain facing each other in a tip of an immunoglobulin's Y-arm, constitute a paratope, whose structure varies from antibody to antibody. A paratope is made of six "fingers" or polypeptide loops, three belonging to the H chain and three to the L chain that enfold the specific epitope.

1.6.3 Antigen and Antibody Reaction:

Antibodies (Ab) bind specifically to antigen (Ag). Antigen-antibody complexes are linked by hydrogen bonds, electrostatic forces, Van der Waals forces, hydrophobic bonds but not by covalent bonds. The formation of the
antigen-antibody complex (Ag-Ab) is reversible:

\[
\begin{align*}
Ag + Ab & \rightleftharpoons \text{Ag-Ab Complex} \\
Ka &= \frac{\{Ag\} \{Ab\}}{\{Ag-Ab\}}
\end{align*}
\]

where \(Ka\) is the affinity constant, usually \(10^8\)-\(10^{11}\) L/M. Affinity represents the force existing between one epitope and one paratope, while the binding force that exists between a polyvalent antigen and an antibody is called avidity. Antigen-antibody complex formation is affected by pH, temperature, ionic strength of the solution, time, and the ratio of the antibody to antigen.

1.6.4 Classification of Immunoassays:

Immunoassays can be divided according to the type of the analysis, test sample, assay system, assay conditions, etc. The assay system broadly can be divided into labelled and non-labelled (direct) methods. The labelled methods can be sub-divided according to the type of marker, i.e. radioisotopic and non-radioisotopic immunoassays, the reaction design (limited or excess reagent, competitive or non-competitive, heterogeneous or homogeneous, etc.), and methods for detection. The division of immunoassays according to the assay conditions includes automatic or manual, equilibrium or non-equilibrium, and liquid or solid phase immunoassays.

1.6.5 Radio Labelled Immunoassays:

These immunoassays use reagents incorporating radioisotopes as tracers to monitor the distribution of free and bound antigen in radioimmuno assay (RIA) or free and bound antibody in immunoradiometric assays (IRMA). Iodine-125 and tritium (\(^3\)H) are the most commonly used radioisotopes in radioimmunoassays.
The general assay range is between $10^{-6}$ M and $10^{-14}$ M. The good limits of detection found in radio labelled immunoassays are related to the negligible background in biological samples. However, RIAs are expensive as they involve the use of short lived radio iodinated tracers, gamma counters, the liquid scintillation counting and subsequent disposal of waste scintillant associated with tritiated tracers. There are also health hazards and safety problems for analysts and others working nearby.

1.6.6 Enzyme Immunoassays:

An enzyme labelled analyte or antibody reacts with an antibody or analyte, and the concentration of analyte or antibody is determined either by separating the excess labelled material from the antibody-bound material (heterogeneous assays) prior to the measurement or in separation-free (homogeneous) assays. The enzymes usually used in homogeneous immunoassays are glucose 6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lysozyme, and β-galactosidase. In heterogeneous immunoassays, horseradish peroxidase, alkaline phosphatase, β-galactosidase and urease are used. Methods of detection employed are UV-Visible spectrometry, fluorescence, chemiluminescence, electrochemical and thermometric.

1.6.7 Fluorescence Immunoassays:

In this technique a fluorophore labelled analyte is used instead of a radio labelled or enzyme one. Different types of fluorophore are found in the literature and some are available commercially. A fluorophore having a high fluorescent intensity, molar absorptivity, quantum yield, and emission wavelength, is useful in immunoassays. It must have a group to be conjugated with analyte, and show stability towards pH and temperature changes.
1.7 Solid Phase Immunoreactors:

For many years immunoassay methods have been used in clinics, agriculture and process and quality control plants. Flow injection immunoassays with solid phase immunoreactors have received relatively little attention. Until now different solid phases in immunoreactors have included controlled-pore glass (CPG) (98), Sepharose (99), Agarose (100), Trisacryl GF 2000 (101), Pall Immunodyne membrane (102-104), Biomag 4100 beads (104), etc. Immunoreactors in flow injection analysis have high potential for the development of analytical protocols with improved speed and simplicity.

In recent years on-line monitoring of substances using solid phase immunoreactors with electrochemical, fluorescence; chemiluminescence, and spectrophotometric detectors has gained momentum. The reagents and washing buffers are pumped consecutively through the immunoreactor in a flow injection manifold. Antigen, antibody and labelled antigen are mixed, incubated and then injected into the buffer stream onto the immunoreactor. After washing an enzyme substrate is injected via a second valve and the fluorescence or electrochemical signal of the product is detected.

1.7.1 Immunoreactors in Enzyme Immunoassays:

A number of solid phase immunoreactors has been developed for flow injection enzyme immunoassays with electrochemical detection. Fab' fragments of antibody coupled to Trisacryl GF-2000 support for the determination of human IgG was used by Alwis, D. E; et al (101). Similarly, immobilised IgG was used for the determination of second antibody using glucose oxidase (GOD)(105) and adenosine daminase as tracer. Immobilised monoclonal antibodies (MAb) of human α-interferon (IFN) on nylon membrane and sepharose bead were used for the determination of α-interferon using a second anti-IFN antibody labelled with GOD(106). Pinella, et al (107) used EZ-BEAD

There are quite a few references to flow injection enzyme immunoassays using solid phase reactors and fluorescence detection. Mouse IgG or anti-mouse IgG attached to immunodyne membranes or magnetic particles, were used in flow injection immunoassays of analytes with various antibody-peroxidase conjugates and fluorescence detection (102-104). Herbicides of the triazine group (108-111), atrazine and aminohexylatrazine, were assayed using antibodies attached to a membrane. The product of the peroxidase substrate hydroxyphenyl propionic acid was measured by a fluorescence detector.

Many workers have reported flow injection enzyme immunoassays with solid phase reactors and chemiluminescent detection. Arefyew, et al (112) reported the determination of thyroxin using immobilised antigen and horseradish peroxidase (HRP)-labelled antibodies. Insulin and 17-α-hydroxyprogesterone were determined using glucose oxidase (GOD) as the label and an immobilised second antibody reactor (113). The enzyme activity was measured by the chemiluminescence produced by luminol and hydrogen peroxide, catalysed by potassium hexacyanoferrate (III) after incubation with glucose. A flow injection immunoassay for human IgG using an immobilised anti-IgG reactor and stopped-flow chemiluminescent detection of the HRP product was reported by Osipov et al (114). The enhanced luminescent reaction using luminol with p-iodophenol was used to detect the peroxidase label. Liu et al (115) reported a flow injection chemiluminescent sandwich immunoassay using bovine IgG
immobilised on a polyvinylidene difluoride polymer membrane. The analyte, mouse monoclonal anti-bovine IgG, and goat anti-mouse IgG-HRP conjugate were injected into the flowing stream. The HRP catalysed the luminol reaction, which has detected directly on the immunoreactor.

Enzyme immunoassays using solid phase reactors and spectrophotometric detection were described by some researchers. Larsson, et al (116) determined transferrin in human plasma by mixing it with HRP-labelled transferrin and passing them through an immobilised anti-human transferrin reactor in an FIA system. A fully automated computer controlled system using a protein A-Sepharose immunoreactor and photometric detection at 280 nm for the determination of rabbit IgG was reported by Nilsson et al (117). Rabbit IgG mixed with HRP-labelled rabbit anti-human IgG competed for limited binding sites available on protein A in the flow injection system. Lee and Meyerhoff (118) measured insulin using immobilised anti-guinea pig whole serum, HRP-labelled insulin, guinea pig anti-insulin antibodies, and photometric detection in flow injection analysis.

A number of researchers worked on a variety of solid phase reactors in immunoassays, such as open tubular wall reactors, packed bed reactors and single bead string reactors. Among those who used open tubular wall reactors were Hornby et al (119,120) who immobilised enzymes on inner walls of open polystyrene and nylon tubes, Horvath and co-workers (121,122) used a thick, porous enzymatic matrix attached to the inner wall of nylon tubes, and Iob and Mottola (123) immobilised uricase enzyme on coiled borosilicate glass tubing. Onuska et al (124) modified the inner walls of a glass tube by the growth of "whiskers" to increase the surface area and then immobilised the enzyme by glutaraldehyde coupling. Packed bed reactors were used by many workers: Iob and Mottola (125) used a uricase-controlled-pore glass (CPG) reactor, Emneus
et al (126) an amyloglucosidase-CPG reactor, Mascini et al (127) a protease-CPG reactor, Bradberry et al (128) an ascorbic acid oxidase-Sepharose reactor, and Worsfold (129) a firefly luciferose-Sepharose 4B or CPG reactor. An immobilised glucose reactor has been used for the analysis of D-glucose, and the product, H$_2$O$_2$, was monitored colorimetrically (130), electrochemically (131,132), and by luminescence (133). An example of the single bead string reactor is the use of immobilised penicillinase on acid-etched glass beads for the determination of penicillin (134).

1.7.2 Immunoreactors in Liposome Immunoassays:

Fluorescence detection and signal amplification using liposome labels are described by Locascio-Brown and co-workers (135-138). They developed a novel FIA system that contained an immunospecific reactor containing antibody covalently bonded to a solid support, liposome-conjugated antigen, and a fluorescence detector (135-137). Free antigen and liposome-conjugated antigen were injected together to compete for the binding sites on the immobilised antibody reactor. Bound and/or unbound liposomes were lysed, and the fluorescent dye released and measured in the detector. By this method, both theophylline and anti-theophylline antibodies were determined using immobilised anti-theophylline antibodies, liposomes containing theophylline-phosphatidylethanolamine in their membranes, and a fluorescence detector in flow injection immunoassays (138). For the detection of anti-theophylline, the analyte competes with immobilised anti-theophylline for sites on the theophylline-labelled liposomes. In the detection of theophylline, the analyte competes with the theophylline-labelled liposomes for immobilised antibody sites.

There are a few references in which liposomes immunoassays with electrochemical detection are described. A flow injection immunoassay of
theophylline using liposome-encapsulated peroxidase enzyme molecules, a packed-bed antibody reactor column, and a potentiometric detector was reported by Wu (139,140). The release of liposome-encapsulated peroxidase in a competitive immunological reaction produced fluoride ions from p-fluorophenol which were then measured potentiometrically. The competition between the analyte and theophylline-liposomes for immobilised antibody sites in flow through reactor columns results in unbound liposomes being carried down-stream where they were ruptured in the presence of $\text{H}_2\text{O}_2$ and p-fluorophenol. Katoaka et al (141) used multilamellar liposomes composed of dipalmitoylphosphatidylcholine, cholesterol, and GA1 antigen and entrapped molybdate as marker ions, to measure anti-asialo-GM1 (GA1) antibodies. Molybdate was released by a complement-mediated immunoreaction, and acted as a catalyst in the $\text{H}_2\text{O}_2$-iodide ion redox reaction. After mixing and incubating the antibody sample, liposomes and complement, they were injected into a flow injection system. The decrease in the number of the iodide ions caused by the molybdate ion-catalysed reaction was monitored using an iodide ion-selective electrode.

1.7.3 Immunoreactors in Fluoroimmunoassays:

Reusable protein A reactors utilising agarose (142) and controlled-pore glass (143) have been reported in assays of goat and rabbit antibodies, and cyclosporin A. Another researcher (144) carried out the detection of the mouse IgG using fluorescein isothiocyanate-conjugated anti-mouse IgG immobilised on bacterial magnetites.
1.8 Protein G

1.8.1 Introduction:

Protein G is a bacterial cell surface protein of Streptococcus and is an analogue of protein A-Staphylococcus aureus. It was first isolated by Reis et al (145) and Björck and Kronvall (146). Later others (147-151) also isolated the protein G. The gene was cloned and expressed in Escherichia Coli by Fahnestock et al. (152) and others (153,154). Fahnestock (155) also genetically engineered a plasmid from which the sequences coding for albumin binding were deleted without loss of the immunoglobulin binding activity of the protein G. Protein G is stable to heating at 100°C and to extremes of pH (pH 1.5 to 11.0) (156). It binds mostly to the Fc region of immunoglobulins G by a non-immune mechanism leaving the antigen binding site free. The binding between protein G and various polyclonal and monoclonal IgG's is pH dependent, it is strongest at pH 4 and 5, and weakest at pH 10.0 (157).

Protein G binds to some IgG's to which protein A binds poorly or does not bind at all. Goat and sheep IgG are not bound by protein A, but bind well to protein G (158-160). Rat IgG binds strongly to protein G and poorly to protein A (158). Furthermore, protein G has been shown to bind more strongly to many mouse monoclonal IgGs than to protein A. Protein G binds to all subclasses of human IgG strongly while protein A does not bind to IgG3. Protein G only binds IgG subclasses, while protein A crossreacts with some IgM, IgA and IgE antibodies (158).

1.8.2 Structure and Biochemistry of Protein G:

Protein G largely consists of three sets of repeated amino acid sequences. The two A repeats are 37 amino acids long, separated by 38 unique residues, and are responsible for albumin binding (152,158). The two B repeats are 55 amino acids long, separated by 15 unique residues, the IgG-binding activity is
Table: 1.1 Comparison of binding of protein A and G to Ig of different animals

<table>
<thead>
<tr>
<th>IgG</th>
<th>Protein G</th>
<th>Protein A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Goat</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Rat</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Bovine</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Chicken</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Pig</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Horse</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Dog</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Human IgG1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IgG2</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IgG3</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>IgG4</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Human IgA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IgM</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IgD</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

++ = strong binding, + = weak binding, - = no binding

Localised in these repeats (152,158). The third repeats-C are homologous, consisting of pentapeptide sequence AspAspAlaLysLys, repeated five times.
Other features include a typical secretion signal sequence (S) at the N-terminus, an alanine rich region before the A repeats, a proline rich region after the B repeats, and a sequence at the C-terminus which resembles an inverted signal sequence (M). Protein A and G have no similarity in amino acid sequence except at the C-terminus which is suggested to mediate the binding of the proteins to the cell wall (161): the proline-rich region, and the inverted signal (M), which has been referred to as a membrane anchor (158,162).

![Diagram of protein structure](image)

Fig. 1.7 A schematic representation of the structure of protein G

The structure of the IgG-binding domains of protein G have been studied both by NMR (163,164) and X-ray diffraction (165). The NMR studies of the B1 IgG-binding domain of protein G shows that it comprises of 56 residues (163). The structure has no disulphide bridges (163,166) and is composed of a four-stranded $\beta$ sheet on top of which lies a long helix. The central two strands ($\beta$1 and $\beta$4), comprising the NH$_2$- and COOH-termini, are parallel and the outer two strands ($\beta$2 and $\beta$3) are connected by the helix in a +3x cross-over and have overall topology of -1, +3x, -1 (163). The X-ray structure of the B2 IgG-binding domain of protein G showed 13 residues preceding the 57-residue Ig-binding domain and 13 additional residues following it, a total of 83 residues (165). Like the B1 domain, the B2 domain consists of four $\beta$-strands and a single helix lying diagonally across the $\beta$-sheet, with a -1, +3X, -1 topology. The overall fold of the B2-domain is similar to the B1-type domain except for amide proton exchange rates and hydrophobic packing which are observed in the B2-
domain (164). The secondary structure of protein G consists of a central $\alpha$-helix flanked by two portions of $\beta$-sheet (167) and is different from protein A which is made up of three consecutive $\alpha$-helices in free solution (168).

These bacterial proteins, protein A and G, have binding domains for IgG. The Fc-binding of protein A has four or five homologous domains with affinity to IgG (169). Whereas the protein G has a binding affinity to both albumin and IgG at different sites on its molecule (170, 171). The protein G molecule carries three homologous albumin binding domains located in the NH$_2$-terminal half of the protein and three homologous IgG-binding domains located in the COOH-terminal half of the molecule (161). Von Mering and Boyle (172) examined the size of protein G produced by group C and G streptococci. The smallest size structure has 2A + 2B, the largest 3A + 3B and the intermediate 2A + 3B or 3A +2B (albumin (A) and IgG (B) binding domains). Otten et al (173) identified five structurally and functionally distinct forms of protein G, i.e. 3 albumin and 3 IgG binding domains; 3 albumin and 2 IgG binding domains; 2 albumin and 2 IgG binding domains; 0 albumin and 3 IgG binding domains; and 0 albumin and 2 IgG binding domains.

Faulman et al (174) observed that the "wild type" protein G from Calbiochem has the largest size, i.e. Mr=52,000 and the Sigma has the smallest size, i.e. Mr=18,000 approximately. Two recombinant forms of protein G have a molecular size 35,000 (Genex and Perstorp) and 28,000 (Pharmacia). Calbiochem protein G showed the most reactivity towards human, rabbit and goat IgG than of all products. Sigma protein G showed minimal reactivity. Recombinant protein G products showed good reactivity towards all IgG's. Pharmacia recombinant protein G showed a low level of reactivity with human, rabbit and goat IgG.
Protein G not only binds to the Fc but also to the Fab and F(ab')2 regions of IgG. Björck and Kronvall (146) showed the binding of protein G to Fab regions of IgG. Eliasson, M; et al (175) demonstrated that the C-terminal C domains of the protein G have both IgG-Fc- and IgG-Fab-binding capacities, whereas the N-terminal AB region is responsible for human serum albumin (HSA)-binding only. The results of the experiments carried out by Eternal et al (176) indicate two independent and separate binding sites for Fab- and Fc-fragments on protein G. The lower molecular weight protein G (28K) showed only affinity for Fc-fragments, while the higher molecular weight bound both IgG Fab- and Fc-fragments. Later Erntell et al (177,178) observed that the heavy chains of IgG inhibited the binding of F(ab')2 fragments to protein G and A. The light chains failed to inhibit the binding implying that the reactive site is located on the heavy immunoglobulin chain. Schröder, et al; (179,180) and others (181) showed that protein G binds the Cy2-Cy3 interface region of the IgG. The interaction of IgG Fab with protein G is analogous to protein A but not identical in reactivity (182,183).

The different forms of protein G showed different reactivities towards albumin (153,170,174,184,185). Nygren et al (184) studied the affinity of protein G to various albumins the highest affinity was for rat, man, and mouse, medium for rabbit, cow, hen, and horse, and little or no binding for sheep. Akerstrom, B; et al (185) showed that 28 KDa protein G, produced by mild acid hydrolysis of 35 KDa protein G, retained its binding to IgG but lost its albumin binding capacity. While a 65 KDa protein G, isolated after cloning and expression of the protein G gene in E. Coli, had comparable affinity to IgG, but a much higher affinity to albumin than the 35- and 28 KDa protein G fragments. Faulman et al (174) noticed that the wild type protein G from Calbiochem showed significant binding to human, baboon, and guinea pig albumin and
weaker binding to mouse and horse albumin with no binding to goat, cow, and sheep albumin. Neither source of recombinant protein G showed any significant reactivity with serum albumin from any species. However, it has been observed that the efficiency of measuring IgG with wild type protein G is not affected by the presence of excess human serum albumin (HSA) in a competitive assays (174). This confirms the early findings that the albumin and IgG binding sites on wild type protein G are distinct domains (186).

1.8.3 Affinity Chromatography:

Antibodies have been isolated, and purified using protein G (187-193). McGuire, J; (187) isolated the IgG from serum, ascites fluids, and hybridoma clones using a protein G polymer coated silica bead column. Jungbauer, et al; (188) isolated the antibodies of HIV 1 using 0.1 M glycine pH 3.0 buffer in order to elute the major fraction of antibodies and avoid harsh elution conditions. The two monoclonal antibodies of rat RAM11 and RAM12, raised against mouse IgM, were purified from the supernatants on protein G-agarose and not on protein A-agarose (190). Another group of workers (189) linked protein G to three different support matrices and separated IgG from a variety of animal species, these were compared with protein A. Cassulis, et al; (194) purified IgG from human serum and Ohlson, et al; (195,196) from the crude samples of mouse and rat by high pressure ligand affinity chromatography (HPLAC) and compared the results with those obtained using protein A. Niss and Ohlson (197) used the SiO2 and TiO2 to covalently bond the protein G for the separation of the antibodies by HPLAC.

IgGs have been removed from sera using protein G affinity chromatography and the eluant was tested for IgA and IgM for rubella virus (198) and early infection of HIV (199). Haun, et al; (200) isolated and purified the IgA from pooled normal human sera using antibody and protein G affinity
chromatography and gel filtration-HPLC. Backer, et al. (201) separated the IgG-creatine Kinase (IgG-CK) and IgA-CK complex by binding with immobilised protein G and jacalin columns respectively, leaving noncomplexed CK in the solution. Kanamaru, et al. (202) specifically adsorbed both the bovine IgG1 and IgG2 among the whole IgG's onto protein G and achieved separation in a single affinity chromatography.

In addition to the separation of the antibodies, many workers have used different approaches to purify proteins and substances with protein G. Eliasson et al (203) and Nygren et al (204) separated the 64 amino-acid albumin binding region of the protein G and used it to purify heterologous proteins by affinity chromatography in a single step. While Higuchi (205) prepared an immunoaffinity matrix, comprising immobilised protein G cross-linked to a specific antibody of the substance to be separated, for the separation of a particular substances.

1.8.4 Immunoassays:

Protein G binds to the IgG of human, rabbit, mouse, rat, cow, pig, etc. and may be used to detect and estimate antibodies. Björck and Blomberg (153) used $^{125}$I labelled protein G to detect antibodies instead of biotin-avidin in the DU point Anti-HIV immunoassay test kit. Radio labelled protein G has also been used in Western blots for the detection of the monoclonal antibody of rat IgG2a and proteins in human urine with whole rabbit or goat antisera (206). Clarke, G. N; (207) developed a radioimmunoassay for sperm antibodies using $^{125}$I-protein G. A solid-phase radioimmunoassay for alpha1-micro-globulin and rat IgG was developed by Nilson et al; (208) based on $^{125}$I-protein G. Rabbit antibodies against the rat adipose tissue hormone-sensitive lipase were detected by protein G-based solid-phase RIA (209).
As protein G binds to IgG and not IgA and IgM, it allows an efficient quantification of sperm antibodies. For the assay of anti-MP70 antibodies (210), biotinylated protein G was used to detect it in enzyme linked immunosorbent assay (ELISA). Protein G-avidin biotin conjugate was used in the enzyme-linked immunosorbent assay of caprine arthritis-encephalitis virus for the detection of its antibodies (211). Another researcher used a protein G-alkaline phosphatase conjugate to detect the antibodies in ELISA for human alpha-1-m (212). Schramm et al; (213) determined the progesterone alkaloid by adsorbing protein G onto micro wells and using progesterone-horseradish peroxidase conjugate and 3,3',5,5'-tetramethylbenzidine (TMB) substrate in a competitive immunoassay. Antibodies were reacted with the protein G-β-lactamase conjugate. After washing, the assay activity of the marker enzyme was observed by adding the substrate (214). Larsson (215) developed an ELISA procedure to determine the antibodies in serum, hybridoma clones, etc. by immobilising the protein G on microtitre plates.

To improve the immunoassay procedure protein G was used in different strategies. Vinten and Tranum-Jensen (216) covalently linked protein G to a polymer-coated noble metal (Au particles) and reacted it with antibodies of HepG2/erythroid glucose transporter in order to determine these transporter proteins by transmission electron microscopy. Uthemann (217) used the protein G coated plate to identify the anti-erythrocyte antibody-erythrocyte-polyspecific anti-human globulin complex. Toledano (218) prepared micro spheres comprising aluminium and magnetite filings, acrylic floc, and hydroxylapatite, activated to bind the protein G, A, or IgG for bioassays. The cancer-specific antibodies were determined by reacting the serum with protein G and cancer-specific antigen or cancer-related antigen (219). Kariwa et al (220) reacted the sera with hantavirus-infected cells, followed by incubation of biotinylated
protein G and amplification with the avidin-biotinylated peroxidase complex. Many other workers have also detected antibodies of interest using protein G (221-223).

High performance ligand affinity chromatography (HPLAC) using protein G has been used to quantify antibodies and antigens by UV and fluorescence methods. Janis et al. (224) and Riggin et al. (225,226) used both HPLAC and reverse phase column chromatography (RPC) to isolate and determine antibodies. Antibodies to human growth Hormone (hGH) (225,226) and transferrin (224) were adsorbed on an immobilised protein G column and then desorbed onto the RPC column, where a second pump was used to generate the gradient elution conditions for RPC. Antibodies were separated and quantified using UV (224-226) and fluorescence (225,226) detectors. Transferrin has also been determined using HPLAC and RPC column with a UV detector (224). Later Blank and Vetterlein (196) quantified the monoclonal antibodies of murine IgG in various complex mixtures by HPLAC using protein G immobilised on silica in just 15 min compared to the ELISA which takes 5 h.

1.9 Avidin/Streptavidin and Biotin:

1.9.1 Avidin:

This is a basic glycoprotein found in the egg-white and tissues of birds, reptiles and amphibia. It has four identical subunits of total molecular mass of 67,000 daltons. Each subunit has a sequence of 128 amino acids. Avidin is very soluble in water and salt solutions. Its isoelectric point is at pH 10. Chemical modification of avidin has little or no effect on its affinity towards biotin. Avidin is stable over a wide range of pH and temperatures.

The properties of avidin are shown in table 1.2. Avidin is a stable tetramer with two-fold symmetry, the binding sites being in two pairs on opposed faces
Table 1.2

Properties of avidins and streptavidin (229)

<table>
<thead>
<tr>
<th>Property</th>
<th>Avidin egg</th>
<th>Streptavidin Unpro</th>
<th>Streptavidin Proced</th>
<th>Avidin yolk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid Residues</td>
<td>128</td>
<td>159</td>
<td>125</td>
<td>-</td>
</tr>
<tr>
<td>Subunits size from sequence</td>
<td>15600</td>
<td>16473</td>
<td>13400</td>
<td>-</td>
</tr>
<tr>
<td>SDS gels</td>
<td>16400</td>
<td>19000</td>
<td>14500</td>
<td>19000</td>
</tr>
<tr>
<td>Subunits</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Isoelectric pH</td>
<td>10</td>
<td>-</td>
<td>5-6</td>
<td>4.6</td>
</tr>
<tr>
<td>$\varepsilon_{280}$</td>
<td>24000</td>
<td>-</td>
<td>34000</td>
<td>-</td>
</tr>
<tr>
<td>$\Delta \varepsilon_{233} (+Biotin)$</td>
<td>24000</td>
<td>-</td>
<td>8000</td>
<td>7000</td>
</tr>
<tr>
<td>Fluorescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{max} (nm)$</td>
<td>338</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\tau$ (nsec)</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*Binding of HABA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kd (µM)</td>
<td>6</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>$\varepsilon_{500}$</td>
<td>35000</td>
<td>-</td>
<td>35000</td>
<td>7000</td>
</tr>
<tr>
<td>Kd biotin (M) (pH 7.25°)</td>
<td>0.6 x</td>
<td>-</td>
<td>4 x</td>
<td>1.7 x</td>
</tr>
<tr>
<td>$t_{1/2}$ (days)</td>
<td>200</td>
<td>-</td>
<td>2.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>
of the molecule. Tryptophan and an amino group of the avidin are considered to be involved in the binding site for biotin (227). The stability is greatly enhanced by the binding of the biotin. The dissociation constant for biotin is very low. The binding is accompanied by a red shift of the tryptophan spectrum and by a decrease in fluorescence. In avidin, the four tryptophans of each subunit are protected when biotin is bound, but fluorescence quenching by oxygen is still observed. The binding of biotin can be blocked by oxidation of any of the several tryptophan residues (228) or by the dinitrophenylation of a single lysine residue. Dinitrophenylation at any one of two or three different lysines or tryptophans led to inactivation and blocked further reactions on avidin.

Table: 1.3
Dissociation rates of avidin-biotin complexes (229)

<table>
<thead>
<tr>
<th>Rate and</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>half-life</td>
<td>1.7</td>
</tr>
<tr>
<td>Avidin</td>
<td></td>
</tr>
<tr>
<td>k(sec⁻¹ x10⁷)</td>
<td>200</td>
</tr>
<tr>
<td>t₅ (days)</td>
<td>0.4</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>35</td>
</tr>
<tr>
<td>k(sec⁻¹ x10⁷)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

33
The protein information resource (PIR) database showed no significant similarity of proteins to avidin (229). Potential similarity is with β-structured proteins. Proteins which have anti parallel β-structure fall into two main classes; a) β sandwiches of 6-10 strands like transthyretin (pre albumin) and immunoglobulin and b) orthogonal β-barrels like serum retinal-binding protein and β-lactoglobulin.

Dissociation rates for avidin/streptavidin-biotin complexes are shown in table 1.3. The rate constant for avidin decreases with increase of pH, and the reverse is true for streptavidin. This may be due to different isoelectric points. Streptavidin shows maximum stability near pH 5 and hence releases its ligand more readily at the extremes of pH, where the net charge on the protein increases. It is opposite to avidin where the net charge falls with increase of pH up to 10.5.

1.9.2 Nonglycosylated Avidin:

Usually commercial avidin prepared from egg-white contains 30% of the nonglycosylated form of the tetrameric protein (230). Hiller, et al; (231) separated the nonglycosylated form from the glycosylated one. The nonglycosylated avidin does not have sugar but still has the same biotin-binding properties as the fully glycosylated form. It has less non-specific binding than avidin (glycosylated) but still more than the streptavidin. It is because streptavidin does not have a sugar moiety and also has a low pI. The commercially available avidins have a A280 nm/A260 nm ratio of about 0.8, which shows that they might contain some nucleic acid. Vector Laboratories (30 Ingold Road, Burlingame, CA 94010, U.S.A.) isolated an avidin which had a A280 nm/A260 nm ratio between 1.6-1.9 and named it avidin D. It possesses very low non-specific binding properties and shows homogeneity in nature by SDS-polyacrylamide gel electrophoresis.
1.9.3 Streptavidin:

The properties of the streptavidin are listed in tables 1.2 and 1.3. It has an acidic isoelectric point (pH 5-6) and contains no carbohydrates, this minimises its non-specific binding. Streptavidin has less non-specific binding than avidin, but the biotin-binding properties of streptavidin are still the same as that of avidin, i.e. 4 moles of biotin per mole of subunit of streptavidin. Its molecular weight is 60,000 daltons. Streptavidin can be crystallised from water or 50% isopropanol. It is more resistant than avidin to guanidinium hydrochloride (232).

Unprocessed streptavidin has a 159 amino acid sequence, some 30 residues longer than avidin. It has both low and high molecular weight subunits. The lower molecular weight subunit is used in most commercial preparations and has 125-127 residues. This subunit has higher water solubility than its precursor but still has four tryptophans residues involved in the biotin-binding site. It also resembles avidin in its predicted secondary structure.

Chemical modification studies show that tryptophan residues are involved in biotin binding like egg-white avidin. Some scientists suggest that tyrosines residues are also important for binding in both proteins. Unlike egg-white avidin, the role of the lysines in streptavidin is not clear. Streptavidin is very stable at high temperatures. Above 60°C and in the presence of SDS, the octamers and tetramers begin to break up into monomers and dimers but they are still capable of binding to biotin (233).

1.9.4 Biotin:

Biotin is also known as vitamin H and is present in minute amounts in every living cell. It has a molecular mass of 244 daltons. It acts as a co-factor for carboxylating enzymes. The carboxyl group of biotin is the site of attachment of the molecule ε-amino groups of lysine residues through an amide bond. It binds
with very high affinity to avidin. The bond formed between the bicyclic ring of biotin and binding site of avidin can withstand harsh reaction conditions like extremes of pH, organic solvents, and other denaturing agents. Binding can be broken by using 8 M guanidine HCl or autoclaving. The avidin-biotin is the strongest known non covalent biological recognition (Ka=10^{15} M) between protein and ligand. The molecular structure of d-biotin and NHS-LC-biotin is shown in fig. 1.8.

The biotin binding site of avidin, is reported to be 9 Å below the surface of the avidin molecule. It can be sterically hindered when certain amino acids or glycosylated residues are present near the lysine necessary for binding (234).

Antigen binding to avidin can be optimised using a biotin derivative having an extended spacer arm (fig. 1.8) and thus reducing the steric hindrance. This spacer arm improves the complex formation of biotin with the deep biotin binding site of avidin.

The introduction of biotin into proteins must be controlled and reproducible. The functional capacities of an antibody will most likely be reduced if it is maximally biotinylated. As monoclonal antibodies can be damaged by modification of functionally important lysine groups, it is necessary to find the optimal efficiency of biotin binding for retention of antigen binding capabilities.

A molar ratio of 2.2:1 to 52:1 between biotin and protein has been used to biotinylate antibodies. A molar ratio of 22:1 yielded biotin substitution levels of 8-14 M biotin per 1 M of monoclonal antibody. This substitution level gives 100% derivatised molecule bound to streptavidin agarose. Higher biotinylation of antibody decreased its binding capabilities. Guesdon et al (235) described a standard procedure for the biotinylation of monoclonal and polyclonal antibodies by reacting Biotinyl-N-hydroxysuccinimide (BNHS) with them in solution.
Fig. 1.8 Structure of biotin.
1.9.5 Iminobiotin:

Iminobiotin is the guanido analogue of biotin. Iminobiotin-avidin complexes require mild dissociation conditions compared to the biotin-avidin complex which needs 8 M guanidine.HCl, and pH 1.5, a condition too harsh for most of the proteins. The dissociation of the iminobiotin-avidin complex occurs at pH 4.0. Tight binding of Iminobiotin to avidin occurs at pH 9.5 or higher and is due to two factors. One factor is the ionising character of the cyclic guanido group of iminobiotin. With increasing pH, the affinity for avidin increases. Since the cyclic guanido group of 2-iminobiotin has a pKa of 11.5 to 12, the avidin may bind iminobiotin only as the deprotonated, or base form. The second factor comes from the observation that the binding affinity of iminobiotin-avidin complex is reduced at pH values below 6, which suggests that an ionisable group on avidin may also be involved. (Fig. 1.9)

1.9.6 Elution Conditions For Affinity Purifications of

Avidin and Biotinyl Proteins:

The avidin-biotin complex possesses a very low dissociation constant (Kd=4x10^-14). The strength of this interaction prevented the isolation of biotinylated or avidin derivatives to be isolated. Cuatrecases and Wilcheck (236) isolated avidin on a biocytin (biotin-ε-N-lysyl)-sepharose 4B column at low pH (1.5) with 6M guanidine.HCl. It is clear that in these harsh elution conditions the biological activity of the avidin derivatives could not be retained. Green (228) found that the free base form of 2-iminobiotin forms a stable complex with avidin but the salt form interacts poorly with its binding protein. (Fig.1.9)

Orr (237) showed that the decrease in affinity observed at neutral and acidic pH values was due to the combined protonation of the cyclic guanidino group of the 2-iminobiotin and the ionisation of some residue on the avidin. He
Fig. 1.9 a) Structure of 2-iminobiotin and NHS-iminobiotin·HBr
b) 2-iminobiotin showing ionization of the cyclic guanidino group.
isolated avidin from eggs using a 2-iminobiotin-6-aminohexyl-sepharose 4B column. The column was equilibrated/washed with 50 mM sodium carbonate pH 11 and eluted with 50 mM ammonium acetate pH 4.0. Others (239,240) also used pH 11.0 and 4.0 for binding and eluting avidin/streptavidin from a 2-iminobiotin-AH-Sepharose 4B column. Bayer et al (241) reported that the iminobiotin AH-(Chloroformate-activated)-CL-sepharose resin is quite stable for repetitive use.

The Hofmann group (242,243) made iminobiotinyl derivatives of Hormones to allow efficient elution of Hormone receptors, but affinities were too low to be useful. The dissociation constants for the aminohexanoate and lysine derivatives of iminobiotin at pH 6.8 (Kd=10^-5 M) were 5- to 10-times greater than the parent iminobiotin. He also studied the effects of dethiobiotin and incorporation of a spacer arm. Hofmann et al; (244) retrieved Streptavidin from a culture broth of Streptomyces avidini on an iminobiotin Sepharose 4B column. This column binds Streptavidin at pH 11.0 and releases the protein at pH 4.0.

Heney (245) and Engelhardt (246) reported a detailed procedure for the isolation of avidin from egg whites and streptavidin from streptavidin avidini using the iminobiotin column. Homogenised egg whites were diluted with water and brought to 70% saturation with ammonium sulphate at 4°C. After centrifuging and filtration, the pellet was dissolved and dialysed against water. The dialysate was then applied to a 2-iminobiotin-6-aminohexyl-Sepharose 4B column which was already equilibrated with 50 mM sodium carbonate-1 M NaCl pH 11.0. The column was washed with the same buffer and then eluted with 50 mM ammonium acetate-0.5 M NaCl pH 4.0 buffer. Engelhardt (246) centrifuged the crude extract of streptavidin (from Streptomyces avidini, absorbed on DEAE-cellulose), filtered and washed it with 20 mM Tris-HCl (pH
It was then eluted with 20 mM Tris-HCl (pH 7.2) containing 0.5 M NaCl. Ammonium sulphate precipitation was used to further concentrate the Streptavidin. This protein extract was dissolved in 50 mM sodium carbonate and 1 M NaCl (pH 11) and applied to an iminobiotin column. The column was washed with the same buffer and eluted with 50 mM ammonium acetate pH 4.0 containing 0.5 M NaCl.

Shimazaki et al.; (247) isolated the binding protein of JSTX-3, spider toxin, by iminobiotinylating the JSTX, and then reacting protein with the synaptic membrane fraction derived from the rat hippocampus. Iminobiotin-JSTX-protein extract was then passed through an avidin D agarose column. The column was equilibrated with 0.1 M borate buffer pH 8.5 and then eluted with 0.1 M acetate buffer pH 4.0.

0.1% (w/v) Triton X-100, Tween 80, Ammonyx-LO, and deoxycholate had no effect on the affinity isolation, reported by Heney and Orr (245). Sodium dodecyl sulphate completely destroyed all binding whereas Lubrol PX resulted in only partial binding of the derivatised fetuin. Columns have been used in the presence of Triton X-100 and Ammonyx-LO without loss of activity. Zeheb et al (248) found that 300 mM NaCl prevents non-specific recovery of counts in the immunoprecipitate, while having little effect on specifically recovered counts. At higher salt concentrations, interference with specific immunorecovery occurs, probably as result of reduced antigen-antibody affinity. Additional blockage of non-specific binding of proteins was provided by maintaining 1 mg/ml BSA in the reaction buffers.

Zeheb et al (248,249) reported the disruption of the immune complex in different ways. Incubation of the immunoprecipitate in a solution containing 1 mM biotin released 80% of the specifically bound counts back into the solution.
The same percentage of counts was released by boiling the immunoprecipitated pellet for 2 min in sample application buffer containing 5% 2-mercaptoethanol or by incubation of the pellet at pH 4.0 for 15 min.

1.10 Insulin:

1.10.1 Introduction:

Insulin is an amphoteric pancreatic protein. It is secreted by the cells of the islets of Langerhans in the pancreas and stimulates the absorption of glucose by the tissues. The main use of insulin is in the treatment of type 1 diabetes mellitus. Many forms of insulin including Bovine and porcine, and preparations containing zinc or portamine to control the rate of release of the insulin, have been used therapeutically. In 1980, human insulin became commercially available after the development of new methods for example the enzymatic conversion of porcine insulin to human insulin and the use of recombinant DNA technology. Porcine insulin differs from human insulin by the presence of an alanine C-terminal in the B-chain in place of threonine.

1.10.2 Structure:

The synthesis of insulin consists of three steps (250,251). Firstly, the 108 amino acid preproinsulin is produced on the ribosomes. This then undergoes post-translational enzymatic cleavage in the endoplasmic reticulum of the first 23 hydrophobic aminoacids of the N-terminal to produce pro insulin. Finally, pro insulin is enzymatically cleaved in the Golgi vesicles to give insulin, a 34-amino acid connecting peptide and two dipeptides, Arg-Arg and Arg-Lys. The final form of the insulin has a molecular weight of 6000Da and consists of two polypeptide chains A and B, containing 21 and 30 amino acids respectively. The two chains, A and B are linked together by two disulphide bridges at positions A7-B7 and A20-B19, and a third disulphide bridge at the A6-B11 position. The
amino acid sequence of bovine insulin was determined by Ryle, et al; (252) in 1955 and since then many variants have been described in literature. In all mammalian insulins, 43 of the 51 amino acids are invariant (253) with the exception of guinea pig insulin which varies in 17 amino acid residues from porcine insulin (254). It is suggested that the tertiary structure of all mammalian insulins is similar to that of the porcine insulin (255,256). In most animals the presence of zinc crystallises the insulin into hexamers except the guinea pig, the casivagua, and the primitive cyclostome of the hagfish which is due to lack of zinc-binding histidine at position B10 (257).

The three-dimensional structure of porcine insulin has been determined by X-ray diffraction (255-256,258-259). It showed that each monomer within the crystal has a three-dimensional structure and is roughly spherical in shape. Only the amino and carboxyl termini of the B-chain extend away from the core of the molecule. The insulin monomer possesses a hydrophobic core and two predominantly hydrophobic surfaces which are important for dimer and, in the presence of zinc, hexamer formation. The hydrophobic core and residues involved in dimer formation are generally invariants in most mammalian insulins. Most of the hydrophobic residues are located on the surface of the hexamer. The rhombohedral unit cell of zinc insulin contains six molecules of the Hormone. The hexamer is organised as three equivalent dimers which in turn consist of two crystallographically independent molecules. Each hexamer contains two zinc ions which are situated 16°A apart and each zinc is coordinated by three equivalent B10 histidines. It is believed that the monomer form of the insulin is the active one and not the hexamer form of insulin. It has also been established that the structure of the insulin in crystal and solution is very similar.
1.10.3 Immunoassays:

Insulin has been determined by sandwich enzyme immunoassay using anti-insulin coated beads. The second antibody is conjugated with enzymes like horseradish peroxidase (260-271), glucose oxidase (272), alkaline phosphatase (273). Yoshioka et al; (260) used the 5-aminosalicylic acid substrate and recorded the absorbance at 500 nm. While Imagawa et al; (261) and Ruan et al; (262-264) used 3-(p-Hydroxyphenyl) propionic acid and hydrogen peroxide as substrate and measured the fluorescence intensity ($\lambda_{ex/em}=320/405$ nm). Others (267-268) used a non fluorescent substrate-p-hydroxyphenylacetic acid, which upon catalysis is converted to a fluorescent product- 6,6'-dihydroxy-[1,1'-biphenyl]-3,3'-diacetic acid (DBDA), and was measured by an HPLC technique. Imasaka et al; (269) used substrate-indocyanine green (ICG) and hydrogen peroxide to measure the fluorescence intensity at 810 nm by a semiconductor laser fluorometer. The enzyme activity is determined by measuring the fluorescence quenching effect of ICG by an OH radical produced by peroxidase from $\text{H}_2\text{O}_2$. Another researcher measured the enzyme activity of the glucose oxidase by the chemiluminescence of the luminol and hydrogen peroxide catalysed by potassium hexacyanoferrate (III) in a flow injection system (272). Tsuji et al; (270) and Matsuoka et al; (271) determined insulin by incubating insulin-horseradish peroxidase conjugated to Sepharose 4B anti-guinea pig IgG rabbit IgG with insulin standards, and anti-insulin serum. The peroxidase activity was measured by adding tyramine, hydrogen peroxide, potassium cyanide and potassium hydroxide and recording the fluorescence intensity of the supernatant.

Monoclonal antibodies have also been used in the enzyme immunoassay of insulin. Franken, et al; (265,266) and Comitti, et al; (273) used two monoclonal antibodies directed against two different epitopes of insulin. One antibody immobilised to plastic tubes (265,266) or microtitre plates (273) was incubated
with serum sample or insulin standards and the second monoclonal antibody was conjugated to horseradish peroxidase (265, 266) or alkaline phosphatase enzyme (273). After washing, Franken, et al; added chromogen 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate (ABTS) and water and recorded absorbance at 405 nm. While Comitti, et al; used phenolphthalein-monophosphate in diethanolamine as substrate and measured absorbance at 540 nm.

A competitive homogeneous immunoassay of insulin is described in the literature (274). Coenzyme labelled insulin-nicotinamide-adeninedinucleotide (NAD)-insulin, competes with unlabelled insulin for a limited amount of antibody. The NAD-insulin conjugate on binding to antibody loses its co-enzymatic activity due to steric inaccessibility of the coenzyme. The activity of the unbound NAD-insulin in the reaction is measured by adding NDMA(p-nitroso-N,N-dimethylaniline) in cyclohexanol and ADH (horse liver alcoholdehydrogenase) and recording the absorbance at 340 nm.

An analytical method using micro capsules comprises encapsulating a labelling substance-carboxyfluorescein, and a precursor there of in micro capsules, measuring the amount of the labelling substance released by destruction of the liposomes by antigen-antibody reaction, and thereby determining the concentration of a substance to be assayed (275). Micro capsules are composed of small particles made up of lipid thin membrane and an antigen-bovine insulin, is attached to the surface of the micro capsules. Micro capsules are then incubated with anti-human insulin antibody and removed by centrifugation. After that they are incubated with serum sample or insulin standards and a complement solution, i.e. guinea pig serum. The insulin concentration in the serum is determined by measuring the intensity of fluorescence of carboxyfluorescein which has flowed out of the micro capsules owing to the destruction of the micro capsules by antigen-antibody reaction.
Insulin labelled fluorescence metal chelates have been used for the immunoassay of insulin. Insulin-EDTA-Eu conjugate (labelled insulin), insulin serum or its standards, anti-insulin serum and a solution β-diketone (β-NTA) and a synergetic compound like trioctylphosphine oxide (TOPO) were incubated (276). The fluorescence of each sample was measured in 10 μsec at different times after excitation. As the change of the intensity of the signal follows the kinetics of a first order reaction, the half-life of the fluorescence intensity for the insulin concentrations used was determined. Spallholz (277) used selenium labelled insulin and incubated it with first and second insulin complexing agents and insulin standards at ambient temperature. Following centrifugation, the precipitated insulin complexes were quantified by a fluorometric technique.

Ullman (278,279) used another approach to determine the insulin in serum. He incubated fluorescein-insulin, insulin and anti-insulin serum for one hour. At the end of this time he added anti-fluorescein in phosphate buffer pH 8.0 and the resulting emission was recorded. The assay was relied upon the ability to observe quenching of fluorescence, when antibody bound. In the presence of ligand, the amount of anti-ligand available for binding to the conjugate was diminished, so that enhanced fluorescence was observed.

Yamaguchi, et al; (280) carried out a fluorescence polarisation immunoassay of insulin. They incubated the FITC-insulin, insulin serum or insulin standards and antiserum for insulin and measured the polarisation of the solution.

Immuoassay of insulin has been carried out by HPLC column and laser fluorometer. Lidofsky, et al; (281) incubated unlabelled bovine insulin with FITC-insulin, guinea pig antiserum to bovine insulin and bovine serum albumin in phosphate buffer pH 7.4 and then injected the incubation samples into a gel filtration column. An increase in insulin concentration decreases the FITC-insulin-antibody peak in laser fluorometry. Others (267,268) incubated anti-
insulin coated plastic disks with serum samples or insulin standards and antibody-horseradish peroxidase conjugate. After washing the plastic disks, a non fluorescent substrate- p-hydroxyphenylacetic acid was added, which upon catalysis was converted to a fluorescent product- 6,6'-dihydroxy-[1,1'-biphenyl]-3,3'-diacetic acid (DBDA) and measured by HPLC equipped with a laser fluorescence detection system.
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CHAPTER TWO

MATERIALS AND METHODS
2.1 Materials:

Bovine Insulin  
Rhodamine Isothiocyanate  
Polyclonal Antibody for Bovine Insulin, Guinea Pig  
Texas Red  
Protein G-agarose (Wild Type)  
Avidin D-agarose  
Avidin-Texas Red  
Avidin-Allophycocyanin  
NHS-LC-Biotin  
NHS-Iminobiotin  
Streptavidin-agarose  
di-Sodium Hydrogen Orthophosphate  
Sodium di-Hydrogen Orthophosphate  
Potassium di-Hydrogen Orthophosphate  
Sodium Carbonate  
Sodium Hydrogen Carbonate  
[Tris(hydroxymethyl)aminomethane Hydrochloride](Tris)  
Sodium Chloride  
Sodium Hydrochloride  
Citric Acid

Sigma  
Sigma  
Biogenesis  
Molecular Probes  
Calbiochem  
Vector Laboratories  
Molecular Probes  
Calbiochem  
Pierce  
Pierce  
Pierce  
BDH  
BDH  
BDH  
BDH  
Sigma  
BDH  
Sigma  
BDH
Normal Human Serum, Sodium Azide, PD-10 column containing Sephadex G-25, Glass microcolumn 50 mm X 3 mm I.D. fitted with polyethylene endpieces with 25 or 40 micron teflon frits, Injection Valve Rheodyne 5020, 2132 Microperpex Peristaltic Pump, All Organic Solvents-HPLC grade, Triton X-100, Ortho-phthaldehyde (OPA), 2-Mercaptoethanol, Ethanethiol, 2-Methyl, 2-propanethiol, Sodium Tetraborate, L-Serine, Glycine, L-Tyrosine, L-Glutamic Acid, DL-Aspartic Acid, L-Alanine, \( \alpha \)-Cyclodextrin, \( \beta \)-Cyclodextrin, \( \gamma \)-Cyclodextrin.

Sigma, Sigma, Pharmacia, Omnifit, Omnifit, LKB, Fisons, BDH, Sigma, Sigma, Aldrich, Aldrich, Hopkin & Williams, Aldrich, Fisons, Calbiochem, Calbiochem, Sigma, Aldrich, Sigma, Sigma, Sigma.
2.2 Methods:

2.2.1 Fluorescence Measurements:

Fluorescence spectra (uncorrected) were recorded at room temperature using a Perkin-Elmer LS-50 Luminescence Spectrometer. The spectrometer was also used as a detector in the flow injection experiments (chapter six). The spectrometer was interfaced with an Epson AX3 personal computer for data manipulation and processing.

All the flow injection fluorescence measurements in chapters three, four and five were carried out on a Perkin-Elmer LS-2B Filter Fluorimeter fitted with a flow cell of 7 µl illuminated volume. An integrator (SP4290, Spectra-Physics) was connected to the fluorimeter for recording the time drive and calculating peak areas.

2.2.2 Absorption Measurements:

Absorption measurements were carried out on a Shimadzu UV-160 double beam spectrophotometer, an LKB Ultraspec II and a Philips Pye-Unicam PU8600 UV/Vis single beam spectrophotometer.

2.2.3 pH Measurements:

pH measurements were made on a Philips PW9420 and a Corning 140 meter fitted with a KCl reference electrode. The pH meter was always calibrated before use with buffers at pH 4.00, 7.00 and 10.00.

2.2.4 Pipetting:

Gilson Pipetman and Finnpipettes (variable volume automatic pipettes) were used throughout the whole project.
2.2.5 Water Purification:

All solutions were prepared in water (0.05 μS/cm) purified using a Liquipure Modulab System.

2.2.6 Size Exclusion Chromatography:

PD-10 columns (Sephadex G-25) from Pharmacia were used as directed for size exclusion chromatography.

2.2.7 Buffers:

2.2.7.1 Binding Buffer (Tris):

50 mM Tris.HCl, 0.5 M NaCl, 0.1% Triton X-100, pH 8.8 with 5 M NaOH

2.2.7.2 Elution Buffer:

0.1 M Citric acid, 0.5 M NaCl, 0.1% Triton X-100, pH 2.5 with 2 M di-Sodium hydrogen orthophosphate.

2.2.7.3 Phosphate Buffered Saline (PBS):

8.0 mM di-Sodium hydrogen orthophosphate
1.4 mM Potassium di-hydrogen orthophosphate
130 mM NaCl
2.6 mM KCl

2.2.7.4 Carbonate Buffer pH 8.0:

0.1 M Sodium carbonate
0.1 M Sodium hydrogen carbonate

2.2.7.5 Carbonate Buffer pH 7.5 or 9.5:

50 mM Sodium carbonate
50 mM Sodium hydrogen carbonate
1 M NaCl with 1 M NaOH
2.2.7.6 Borate Buffer pH 9.26:

9.53425 g of Na₂B₄O₇.10H₂O was dissolved in distilled water, stirred, filtered and the final volume was made up to one litre with distilled water.

2.2.8 Fluorogenic Reagents.

Ortho-phthaldehyde (OPA) (0.2 g) was dissolved in 2.00 ml of ethanol. Then 50 μl of thiol, i.e. 2-mercaptoethanol or ethanethiol, was added and the total volume was made up to 250 ml with 0.025 M Na₂B₄O₇ pH 9.26.

2.2.9 Cyclodextrin Solutions:

A stock solution was prepared every day by dissolving an appropriate quantity of cyclodextrin in the fluorogenic reagent solution using ultrasonication. The standard and other cyclodextrin solutions were prepared from the stock solution by taking an appropriate volume of stock solution and diluting it with fluorogenic reagent solution.

2.2.10 Amino Acid Solutions.

An appropriate quantity of amino acid was dissolved in distilled water.

2.2.11 Bovine Insulin:

Stock solutions of insulin were prepared in 50 mM carbonate buffer pH 7.5 or PBS buffer, a few drops of 1 M HCl were added to dissolution.

2.2.12 Synthesis of Insulin-Rhodamine Isothiocyanate Conjugates:

Bovine insulin was brought into the solution with 1 ml of PBS pH 7.4 buffer and a minimum amount of dil HCl to dissolve it (pH 7.0). 1.5 ml of carbonate buffer pH 9.5 was added to the resulting solution, followed by the addition of solid rhodamine.
isothiocyanate. The flask was then placed in a rocking shaker and agitated for 24 h at 4°C. The contents of the flask were centrifuged and the filtrate separated on a Sephadex G-25 PD 10 column.

The following conjugates of insulin-Rhodamine isothiocyanate (RITC) in different combining ratios of insulin and RITC were prepared and separated on PD 10 columns.

Conjugate 1) Insulin : 6.1027 × 10^{-3} g
   
   RITC : 0.00184 g

   \[ \frac{C_{\text{RITC}}}{C_{\text{Insulin}}} = 3.45 \]

Conjugate 2) Insulin : 0.00263 g
   
   RITC : 0.00131 g

   \[ \frac{C_{\text{RITC}}}{C_{\text{Insulin}}} = 5.7 \]

Conjugate 3) Insulin : 0.00221 g
   
   RITC : 0.00205 g

   \[ \frac{C_{\text{RITC}}}{C_{\text{Insulin}}} = 10.62 \]

Conjugate 4) Insulin : 1.6429 × 10^{-3} g
   
   RITC : 0.00294 g

   \[ \frac{C_{\text{RITC}}}{C_{\text{Insulin}}} = 20.48 \]
2.2.13 Purification of the Insulin-Rhodamine Isothiocyanate (RITC) Conjugates:

The insulin-RITC conjugate was purified by gel filtration on a Sephadex G-25 PD 10 column to remove the excess rhodamine isothiocyanate from the mixture. Before separating the conjugate the PD 10 columns were equilibrated with PBS buffer pH 7.4 by passing through three column volumes of the buffer. 2.5 ml volumes of conjugate were loaded onto the column and eluted with a 3.5 ml volume of PBS buffer, according to the instructions of the PD 10 column's manufacturer (Pharmacia). Fractions were collected and stored in the refrigerator.

2.2.14 Determination of Rhodamine Isothiocyanate (RITC) and Insulin in the Insulin-Rhodamine Isothiocyanate Conjugates:

Calibration curves for rhodamine isothiocyanate and insulin were plotted at 554 and 275 nm respectively. As the RITC absorbs at 275 nm as well as at 554 nm (fig. 2.1 and table 2.1), a calibration curve for RITC at 275 nm was also constructed. The insulin did not have a significant absorbance at 554 nm (fig. 2.1), hence the RITC was determined at this wavelength without any interference. The absorbance of RITC at 275 nm was subtracted from the total absorbance of the insulin-RITC conjugate in order to find out the absorbance of insulin and hence its concentration in the conjugate. The concentration of the insulin and RITC determined in each conjugate is shown in table 2.2.
Fig. 2.1 Absorbance Vs wavelength of insulin rhodamine isothiocyanate (RITC) and insulin-rhodamine isothiocyanate conjugate in PBS buffer.
Table: 2.1

Maximum absorbance wavelength of compounds and conjugates:

<table>
<thead>
<tr>
<th>Compound/Conjugate</th>
<th>Absorbance λmax nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>-</td>
</tr>
<tr>
<td>RITC</td>
<td>554 402</td>
</tr>
<tr>
<td>Insulin-RITC</td>
<td>554 -</td>
</tr>
<tr>
<td>d-Biotin</td>
<td>-</td>
</tr>
<tr>
<td>Insulin-Biotin</td>
<td>-</td>
</tr>
</tbody>
</table>

Results and Discussion:

The amounts of insulin and RITC added and found after separation on Pharmacia's Sephadex G-25 PD 10 column for the synthesis of various insulin-RITC conjugates are shown in table 2.2. Table 2.2 shows that in most cases 50% of the insulin is found in the insulin-RITC conjugate. According to the instructions of the manufacturer, Pharmacia, 2.5 ml of protein applied to a Sephadex G-25 PD 10 column will be eluted in 3.5 ml. Proteins usually have a molecular weight higher than 6000, however bovine insulin has a molecular weight of 5733 amu. Thus, insulin-RITC conjugates eluted in 3.5 ml volume have less insulin than expected.

Mono, di, and tri-substituted derivatives of insulin have been reported in the literature (2,5). Tietze et al (5) reacted insulin with fluorescein isothiocyanate and obtained mono, and di-substituted derivatives at the terminal amino group of the phenylalanyl (B) chain and the terminal glycyl residue of the insulin. May, et al (2); reacted biotin-NHS with three -NH₂ groups of the insulin, the two α-amino groups of
the polypeptide chains and the e-amino group of the lysine B29. Thus it was expected

Table: 2.2

The quantity of analyte added and found in the conjugates after separation on Sephadex G-25 PD 10 column:

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Name of the Analyte</th>
<th>Analyte added (g)</th>
<th>Analyte found (g)</th>
<th>C_{RITC} / C_{Insulin}</th>
<th>% Recovery of insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin-RITC</td>
<td>Insulin</td>
<td>6.1027 x 10^{-3}</td>
<td>3.2849</td>
<td></td>
<td>53.83</td>
</tr>
<tr>
<td>Conjugate 1</td>
<td>RITC</td>
<td>0.00184</td>
<td>2.15 x 10^{-4}</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Conjugate 2</td>
<td>Insulin</td>
<td>0.00263</td>
<td>1.4977 x 10^{-3}</td>
<td>56.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RITC</td>
<td>0.00131</td>
<td>2.4654 x 10^{-4}</td>
<td>1.88</td>
<td></td>
</tr>
<tr>
<td>Conjugate 3</td>
<td>Insulin</td>
<td>0.00221</td>
<td>8.5315 x 10^{-4}</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RITC</td>
<td>0.00205</td>
<td>1.7216 x 10^{-4}</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>Conjugate 4</td>
<td>Insulin</td>
<td>1.6429 x 10^{-3}</td>
<td>7.7595 x 10^{-4}</td>
<td>47.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RITC</td>
<td>0.00294</td>
<td>1.7471 x 10^{-4}</td>
<td>2.58</td>
<td></td>
</tr>
<tr>
<td>Insulin-Biotin</td>
<td>Insulin</td>
<td>5.2 x 10^{-5}</td>
<td>1.9146 x 10^{-5}</td>
<td>36.82</td>
<td></td>
</tr>
</tbody>
</table>

that up to three RITC groups could be attached to each insulin molecule. The average molar RITC: insulin ratios in the third and fourth conjugates were between 2 & 3 (table 2.2). The labelling efficiency of RITC reported by its manufacturer (Sigma Chemical Co.) was >70%. Conjugates three and four were used in chapters four and five respectively as the labelled analyte in the flow injection immunoassay of insulin using solid phase immunoreactors.
2.2.15 Fluorescence Spectra of the Insulin-Rhodamine Isothiocyanate (RITC) Conjugate:

Conjugate 3 was reconstituted in PBS buffer and its fluorescence spectra were obtained and are shown in fig. 2.2

2.2.16 Synthesis of Insulin-Biotin Conjugate:

An insulin-biotin conjugate was synthesized according to Pang and Shafer's (1) method with some modifications. Bovine insulin and biotin-LC-NHS with a longer spacer arm were used instead of porcine insulin and biotin-NHS.

From a stock solution of biotin-LC-NHS (0.00246g/500 μl of DMF), 102.6 μl of biotin-LC-NHS (5.0479 X10-5g) was added to 50 μl of insulin (5.2 X10-5 g taken from the stock solution of insulin, 0.00208 g/2 ml carbonate buffer pH7.5) in 2.4 ml of 50 mM carbonate buffer pH 7.5. The addition was made while stirring of the insulin solution with a magnetic stirrer over a period of 15 minutes. The conjugate was then kept in the refrigerator overnight at 4°C. The next day it was separated on a Pharmacia PD 10 Sephadex G-25 column with PBS buffer according to the instructions of the manufacturer.

2.2.17 Determination of Insulin and Biotin in the Insulin-Biotin Conjugate:

As insulin has an absorbance maximum at 275 nm and d-biotin does not absorb at this wavelength (fig. 2.3), a calibration curve for insulin was constructed at 275 nm and used to determine the insulin-biotin conjugate without any biotin interference (table 2.2).
Fig. 2.2 Excitation (-) and emission (---) spectra of Insulin-rhodamine isothiocyanate conjugate.
Fig. 2.3 Absorbance Vs wavelength of insulin, d-biotin and insulin-biotin conjugate in PBS buffer.
The determination of biotin in insulin-biotin conjugates has been reported in the literature (1-2). Spectrophotometric (3) and fluorimetric (4) methods have been used for the determination of biotin in biotin-substrate conjugates. May et al (2) reacted insulin with NHS-biotin and determined biotin in the insulin-biotin conjugates by the spectrophotometric method. They found a mixture of mono, di, and triacylated insulin derivatives due to reaction with the α-amino groups of the two polypeptide chains and the ε-amino group of lysine B29. While Pang and Shafer (1) isolated seven derivatives of insulin-biotin by HPLC (table 2.3). Since the random biotinylation procedure carried out by Pang (1) and May (2) was used, no attempt was made to determine the biotin in the insulin-biotin derivatives. As a large number of different insulin-biotin derivatives were expected, they were used without further characterisation.

Table: 2.3

The position of biotin in the seven derivatives of insulin-biotin: (1)

<table>
<thead>
<tr>
<th>Derivative No.</th>
<th>Gly-A1</th>
<th>Phe-B1</th>
<th>Lys-B29</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Biotinylated, - = Not biotinylated
2.2.18 Synthesis of Insulin-Biotin-Avidin-Texas Red Complex:

684 μl of insulin-biotin (containing 0.00374 mg of insulin prepared in section 2.2.16) was mixed with 50 μl of avidin-Texas Red (0.1125 mg of avidin) in 1.8 ml of 50 mM carbonate buffer pH 7.5 while stirring over a period of 10 minutes at room temperature. The mixture was further stirred for 15 minutes and then left overnight in the refrigerator at 4°C. The following day the insulin-biotin-avidin-Texas Red complex was purified and separated on a PD 10 column containing Sephadex G-25 in PBS buffer. The volume of the complex was 3.5 ml and was used as a labelled analyte in the flow injection immunoassay of insulin using protein G as solid phase immunoreactor in chapter six.

2.2.19 Synthesis of Insulin-Biotin-Avidin-Allophycocyanin Complex:

The procedure for synthesis was the same as described in section 2.2.18 except that 50 μl of avidin-allophycocyanin containing 0.05 mg of avidin was used instead of avidin-Texas Red.

2.2.20 Fluorescence Spectra of Insulin-Biotin-Avidin-Texas Red and Insulin-Biotin-Avidin-Allophycocyanin Complexes:

Fluorescence spectra of the insulin-biotin-avidin-Texas Red and insulin-biotin-avidin-allophycocyanin complexes in PBS buffer were recorded using a Perkin-Elmer LS-50 Luminescence Spectrometer and are shown in fig. 2.4 & 2.5 respectively.

2.2.21 Influence of pH on Fluorescence Intensity of the Insulin-Rhodamine Isothiocyanate (RITC), Avidin-Texas Red and Avidin-Allophycocyanin Conjugates:

With some fluorophores, e.g; fluorescein, the fluorescence intensity decreases with a decrease of pH. The purpose of this investigation was to check the influence of pH on
Fig. 2.4 Excitation (-) and emission (...) spectra of Insulin-biotin-avidin-Texas Red in PBS buffer.
Fig. 2.5 Excitation (-) and emission (..) spectra of insulin-biotin-avidin-allophycocyanin complex
the fluorescence intensity of the conjugates used in this work: If low pH quenches the fluorescence, then the derivative might not be suitable for flow injection immunoassays.

Citrate and tris buffers were prepared as described in an earlier section of this chapter (2.2.7.1 and 2.2.7.2). Tris and citrate buffers were adjusted to different pH values in the basic and acidic region respectively. A PBS buffer pH 7.4 having 0.1% Triton X-100 was also prepared. For each conjugate a series of solutions of fixed concentration were prepared at various values. The fluorescence intensity was measured and plotted against the pH as shown in fig. 2.6 and 2.7.

Results and Discussion:

Generally, it can be seen from fig. 2.6 that the insulin-RITC and avidin-Texas Red conjugates are unaffected by low and high pH except at pH 7.4 where a small decrease in fluorescence intensity is observed. In insulin-RITC the fluorescence intensity at low pH is slightly higher than at high pH. However, the avidin-allophycocyanin conjugate suffers considerable fluorescence intensity quenching, both at low and high pH comparative to pH 7.4 (fig. 2.7). Therefore, its use in flow injection immunoassays appears uncertain. Both the insulin-RITC and avidin-Texas Red have the potential to be used in the development of immunoassays.
Fig. 2.6 Fluorescence intensity Vs pH of insulin-rhodamine isothiocyanate and avidin-Texas Red conjugates.

For insulin-RITC, \( \lambda_{ex}=550\text{nm}, \lambda_{em}=585\text{nm} \)

and for avidin-TR, \( \lambda_{ex}=595\text{nm}, \lambda_{em}=620\text{nm} \)
Fig. 2.7 Fluorescence intensity Vs pH of avidin-allophycocyanin.

$\lambda_{ex} = 650 \text{ nm, } \lambda_{em} = 660 \text{ nm, } \Delta \lambda_{slit} = 5/5 \text{ nm}$
2.3 References:

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CHAPTER THREE

FLOW INJECTION ANALYSIS OF DL-LYSINE
AND L-SERINE USING OPA
Introduction:

The aim of the work carried out in this chapter was to gain familiarity of flow injection technique with fluorescence detection, which would later be helpful in immunoassays. Amino acids were injected into a flowing stream of fluorogenic reagent containing ortho-phthaldehyde (OPA) and a thiol. The addition of cyclodextrin (CyD) to the fluorogenic reagent allowed the investigation of fluorescence enhancement. The kinetic and association constants of these host-guest interactions were determined in order to understand this phenomenon.

3.1 Procedure:

The fluorogenic reagent with or without cyclodextrin was continuously pumped (3.4 ml/min) through the manifold (Fig. 3.1).

![Flow Injection manifold for the fluorescence enhancement of OPA-Thiol-DL-Lysine or L-serine with cyclodextrin.](image)

The amino acid solutions were injected into the carrier stream using an injection valve (injected volume 75 µl). The amino acid solutions mixed and reacted with the carrier stream while moving towards the detector, where fluorescence was continuously recorded. UV and fluorescence spectra were measured statically in 3 ml fluorescence cells. The mechanism of the reaction is shown in fig. 3.1B.
Fig. 3.1B Formation of isoindole.
3.2 RESULTS AND DISCUSSION:

3.2.1 OPA-Thiol-DL-Lysine-Cyclodextrin Complex:

3.2.1.1 Effect of Cyclodextrins on Fluorescence Intensity:

In the past cyclodextrins have been used to enhance the fluorescence intensity and improve the stability of many compounds by forming inclusion complexes. The addition of β-cyclodextrin to fluorogenic reagent and DL-lysine enhances the fluorescence intensity in two steps (Figs. 3.2, 3.3). Enhancement was first observed with a β-cyclodextrin concentration of $10^{-4}$ M and continued enhancement occurred up to $3 \times 10^{-4}$ M β-CyD. While the second fluorescence enhancement was observed from $10^{-3}$ to $3 \times 10^{-3}$ M β-CyD concentration. The molecule DL-lysine is too large to be completely included into a single β-CyD cavity. It can accommodate only one isoindole group leaving the other exposed to solvent molecules (Fig. 3.4). At higher concentrations, additional cyclodextrin molecules associate together to form a 2:1 complex as shown in fig. 3.4. γ-cyclodextrin has a larger cavity. It appears that one of the isoindole group incorporates into the γ-cyclodextrin cavity and shows enhancement as the concentration of γ-cyclodextrin was increased from $10^{-4}$ M to $3 \times 10^{-4}$ M. Further addition of γ-CyD did not enhance the fluorescence intensity. It can be said that the second isoindole of DL-lysine does not fit into the γ-cyclodextrin cavity. Hence no complexation formed with the second isoindole group and therefore, no increase in fluorescence intensity was observed with further addition of γ-cyclodextrin (Fig. 3.3).

α-cyclodextrin did not give any fluorescence enhancement. The fluorescence of the isoindoles was quenched by an increased concentration of α-CyD (Figs. 3.2, 3.3). The cavity of α-cyclodextrin is too small to form an inclusion complex with the isoindole of DL-lysine, hence no fluorescent enhancement was observed.
Fig. 3.2 Fluorescence intensity vs conc. of cyclodextrin of opa + 2-ME + DL-lysine in a FIA system. (1 = α, 2 = β, 3 = γ-cyclodextrin)
Fig. 3.3 Fluorescence intensity (P.H) vs concentration of cyclodextrin of OPA + ethanethiol + DL-lysine in FIA system.
The mechanism for the formation of an inclusion complex was explained by Bender et al. (1, 2) and Bergeron et al. (3, 4) using thermodynamic parameters in terms of the release of high energy water from the cyclodextrin cavity. The water molecules associated with the cavity are enthalpy rich, as they cannot have a full compliment of hydrogen bonds, due to interference from the glycopyranose rings of cyclodextrin. The inclusion of substrate results in the expulsion of these water molecules into the surrounding system. Saenger (5, 8) found using X-ray crystallographic data that the molecules of α-CyD have an unstrained hexagonal geometry in most α-CyD adducts, except for the water-α-CyD adduct where the macrocyclic conformation of α-CyD is unsymmetrically distorted and energetically unstable. Tabushi (9) showed that strain release, release of high energy water molecules, Van der Waals interactions and
breaking of water clusters around an apolar guest molecule are the important forces stabilising the inclusion complex.

3.2.1.2 Effect of Thiol:

A thiol was essential for the reaction between OPA and amino acids. The two step fluorescence enhancement observed for β-CyD was found with 2-mercaptoethanol and ethanethiol (Figs. 3.2, 3.2), but not with 2-methyl-2-propanethiol (Fig. 3.5). With γ-CyD ethanethiol gave a single step enhancement (Fig. 3.2). The 2-methyl-2-propanethiol is a large molecule compared to 2-mercaptoethanol and ethanethiol. As a result of its size the isoindole could not enter the β- or γ-CyD cavity and hence no enhancement was observed. The observed enhancements were by factors of 2- to 10-fold. These results point to a combination of factors being responsible for the fluorescence enhancement, with specific interaction effects as well as the size compatibility of the cyclodextrin cavity and the isoindole influencing the outcome.

3.2.1.3 Dependence of Absorption and Emission Wavelength:

As shown in Tables 3.1 & 3.2 the emission wavelength shifts toward a shorter wavelength and the absorption wavelength shifts toward a longer wavelength, with the addition of cyclodextrins. A six to ten nanometer blue shift was observed in the fluorescence spectra, while only a one to four nanometer red shift was observed in the UV spectra. The blue shift in fluorescence spectra and red shift in absorption spectra is a characteristic feature of inclusion complexes (Figs. 3.6, 3.7).
Fig. 3.5 Fluorescence intensity vs concentration of cyclodextrin of OPA + 2-methyl, 2-propanethiol + DL-lysine in FIA system. Excitation wavelength filter=337nm λem = 450 nm.
Fig. 3.6 Fluorescence intensity Vs wavelength of OPA + ethanethiol + DL-lysine with (2,3,4) and without (1) β-cyclodextrin.
Fig. 3.7 Absorbance Vs wavelength of OPA + ethanethiol + DL-lysine with (2,3,4) and without β-cyclodextrin.
Table: 3.1

Maximum Emission Wavelength of OPA-Thiol-DL-lysine-Cyclodextrin Complex at λex=337 nm

<table>
<thead>
<tr>
<th>Thiol</th>
<th>α-CyD</th>
<th>β-CyD</th>
<th>γ-CyD</th>
<th>λem(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>454</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>456</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>460</td>
</tr>
<tr>
<td>Ethanethiol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>452</td>
</tr>
</tbody>
</table>

Table: 3.2

Maximum Absorption Wavelength of OPA-Thiol-dl-Lysine with and without Cyclodextrin (CyD).

<table>
<thead>
<tr>
<th>Thiol</th>
<th>No CyD</th>
<th>β-CyD</th>
<th>γ-CyD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>291</td>
<td>293 at 3X10^{-4} M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>333</td>
<td>290 at 3X10^{-3} M</td>
<td>-</td>
</tr>
<tr>
<td>Ethanethiol</td>
<td>291</td>
<td>293 at 3X10^{-4} M</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>333</td>
<td>290 at 3X10^{-3} M</td>
<td>336</td>
</tr>
</tbody>
</table>
3.2.1.4 Influence of the Thiol Structure on Stability:

The stability curves for OPA-2-mercaptoethanol-DL-lysine and OPA-ethanethiol-DL-lysine with \( \beta \)-cyclodextrin are shown in figs. 3.8 & 3.9. The figures show that the complexes are not stable. The OPA-ethanethiol-\( \gamma \)-cyclodextrin complex is also not stable (Fig. 3.10).

3.2.1.5 Effect of pH:

The optimum pH for the OPA-thiol-amino acid reaction has been shown to be alkaline (10). The fluorescence intensity of the OPA-2-mercaptoethanol-\( \beta \)-CyD-lysine complex was plotted against pH (Fig. 3.11). It was found to have maximum fluorescence intensity at pH 9.26.

3.2.1.6 Rate and Formation Constants:

Rate and formation constants with and without cyclodextrin are shown in Table 3.3. The rate constants observed with 2-mercaptoethanol (\( K'_1 \)) were found to be lower than with ethanethiol (\( K'_1 \)). A graphical method was used to calculate the rate constants. All reactions with and without cyclodextrin were observed to be first order (e.g; see fig. 3.11B,C,D,E).

The formation constants for cyclodextrin complexes were calculated using the Benesi and Hildebrand equation (11).

\[
\frac{Ca}{\Delta I} = \frac{1}{K\Delta i.1/Cd + 1/\Delta i}
\]

where \( Ca \) represents the concentration of amino acid, \( Cd \) = concentration of cyclodextrin, \( \Delta I \) = increment of fluorescence of OPA-thiol-lysine on the addition of the cyclodextrin, and \( \Delta i = \Delta I / \text{conc. of OPA-thiol-lysine-CyD complex} \).
Fig. 3.8 Fluorescence intensity Vs time of OPA +

2-mercaptoethanol + β-cyclodextrin + DL-lysine

Excitation wavelength filter = 337nm,

λem = 450nm.
Fig. 3.9 Fluorescence intensity vs time of OPA + ethanethiol + DL-lysine with (1) and without (2) β-cyclodextrin. Excitation wavelength filter = 337 nm, λ em = 450 nm.
Fig. 3.10 Fluorescence intensity Vs time of OPA + ethanethiol + γ-cyclodextrin + DL-lysine

Excitation wavelength filter=337nm, λ_em=450nm
Fig. 3.11 Fluorescence intensity vs pH of OPA + 2-mercaptoethanol + β-cyclodextrin + DL-lysine in FIA system. Excitation wavelength filter = 337 nm, λem = 450 nm.
Table: 3.3
Association and Rate Constants of OPA-Thiol-DL-Lysine with and without Cyclodextrin (CyD).

<table>
<thead>
<tr>
<th>Host</th>
<th>Guest</th>
<th>Association Constants</th>
<th>Rate Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K'</td>
<td>K''</td>
</tr>
<tr>
<td>~-CyD</td>
<td>OPA-Ethanethiol-DL-Lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>~-CyD</td>
<td>OPA-2-ME-DL-Lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-CyD</td>
<td>OPA-2-ME-DL-Lysine</td>
<td>3.072 \times 10^3</td>
<td>4.673 \times 10^2</td>
</tr>
<tr>
<td>β-CyD</td>
<td>Ethanethiol-DL-Lysine</td>
<td>3.265 \times 10^3</td>
<td>1.548 \times 10^3</td>
</tr>
<tr>
<td>β-CyD</td>
<td>2-propanethiol-DL-Lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ-CyD</td>
<td>OPA-2-ME-DL-Lysine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ-CyD</td>
<td>OPA-Ethanethiol-DL-Lysine</td>
<td>3.7015 \times 10^3</td>
<td>-</td>
</tr>
<tr>
<td>γ-CyD</td>
<td>2-propanethiol-DL-Lysine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 3.11B Calibration curve between fluorescence intensity and conc. of complex, i.e. OPA + 2-ME + β-CyD (..x 10^{-4} M) + DL-lysine (..x 10^{-5} M) determined by FIA.
FIG. 3.11C Determination of kinetic ($K_1$) of OPA + 2-ME + $\beta$-CyD($x10^{-4}$M) + DL-lysine($x10^{-5}$ M) complex by FIA method.
Fig. 3.11D Calibration curve between fluorescence intensity and conc. of complex, i.e. OPA + 2-ME + β-CyD (.x10^{-3} M) + DL-lysine (.x 10^{-5} M) determined by FIA.
Fig. 3.11E Determination of $K_2$ of OPA

+ 2-ME + $\beta$-CyD ($\ldots \times 10^{-3} \text{ M}$)

+ DL-lysine ($\ldots \times 10^{-5} \text{ M}$)

complex by FIA.
3.2.2 OPA-Thiol-L-Serine-Cyclodextrin:

3.2.2.1 Effect of Cyclodextrins on Fluorescence Intensity:

The addition of γ-cyclodextrin to the fluorogenic reagent, i.e.; OPA+2-mercaptoethanol+L-serine, enhances the fluorescence intensity as shown in fig. 3.12. Increased fluorescence was observed from $10^{-6}$ M to $3 \times 10^{-6}$ M γ-cyclodextrin. The addition of α or β-cyclodextrin did not cause enhancement. This suggests that the isoindole formed by the reaction of OPA+2-mercaptoethanol and L-serine does not fit inside the cavity of α or β-cyclodextrin.

3.2.2.2 Effect Of Thiol:

Increased fluorescence was observed with 2-mercaptoethanol (i.e.; OPA+2-ME+L-serine-γ-cyclodextrin) but not with ethanethiol (i.e.; OPA+ethanethiol+L-serine-γ-cyclodextrin) as shown in fig. 3.13. It is probable that hydrogen bonding inside the cavity of γ-cyclodextrin is more favourable for 2-mercaptoethanol than for the ethanethiol complex, as the former has an -OH group available to participate in hydrogen bonding.

3.2.2.3 Dependence of Absorption and Emission Spectra:

The absorption and emission spectra of OPA-2-mercaptoethanol-L-serine, with and without γ-cyclodextrin, are shown in figs. 3.14 and 3.15. The main peaks along with their shifts are shown in Tables 3.4 & 3.5. It can be seen from the absorption table that the peak at 332 nm shifts to 335 nm with the addition of γ-cyclodextrin while the other peak at 312 nm remains unchanged. No change was
Fig. 3.12 Fluorescence intensity (P.H) vs the concentration of cyclodextrin of OPA + 2-mercaptoethanol + L-serine FIA system.

Excitation wavelength filter = 337nm, λem = 450nm.
Fig. 3.13 Fluorescence intensity of OPA + Thiol + L-serine in FIA system.

Excitation wavelength filter = 337nm,

$\lambda_{em} = 450$nm.
Fig. 3.14 Absorbance Vs wavelength of OPA + 2-mercaptoethanol + L-serine with (2) and without (1) γ-cyclodextrin.
Fig. 3.15 Fluorescence intensity Vs wavelength of OPA + 2-mercaptoethanol + L-serine with (2,3,4) and without (1)\(\gamma\) -cyclodextrin.
observed in the fluorescence spectra. Fluorescence enhancement (fig. 3.15) due to the addition of γ-cyclodextrin was more than the absorption one (fig. 3.14).

### Table: 3.4

Maximum Emission Wavelength (nm) of OPA-Thiol-L-serine with and without Cyclodextrin (CyD). Excitation wavelength = 337 nm

<table>
<thead>
<tr>
<th>Thiol</th>
<th>No CyD</th>
<th>β-CyD</th>
<th>γ-CyD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>449</td>
<td>-</td>
<td>451</td>
</tr>
</tbody>
</table>

### Table: 3.5

Maximum Absorption Wavelength (nm) of OPA-Thiol-L-serine with and without Cyclodextrin (CyD).

<table>
<thead>
<tr>
<th>Thiol</th>
<th>No CyD</th>
<th>γ-CyD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>282</td>
<td>282</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>332</td>
<td>335</td>
</tr>
</tbody>
</table>

#### 3.2.2.4 Influence of Thiol Structure on Stability:

The stability curve of OPA-2-mercaptoethanol-L-serine with γ-cyclodextrin showed (Fig. 3.16) that the complex was not stable. It is possible that the bonding inside the cavity of γ-cyclodextrin was not strong enough to stabilise the complex.
Fig. 3.16 Fluorescence intensity Vs time of OPA + 2-mercaptoethanol + \( \gamma \)-cyclodextrin + L-serine

Excitation wavelength filter = 337nm,

\( \lambda_{em} = 450 \text{nm} \).
3.2.2.5 Effect of pH:

Fluorescence increased with increasing pH up to pH 9.26 (fig. 3.17). At very low pH the fluorescence was quenched entirely. In alkaline media an unprotonated amino group formed the derivatization. This is supported by the fact that the reaction rates are faster at higher pH (12).

3.2.2.6 Rate and Formation Constants:

Table 3.6 shows the rate and formation constants of OPA-2ME-L-serine with and without γ-cyclodextrin. The rate constant with γ-cyclodextrin was found to be higher than without it. Generally rates are higher in an apolar medium than polar. The γ-Cyclodextrin cavity provides an apolar environment to its guest by expelling the water from its cavity. OPA-2-ME-L-serine reactions with and without γ-cyclodextrin were found to be first order. The formation constant was calculated from the Benesi and Hildebrand equation (11)

Table: 3.6
Association and Rate Constants of OPA-Thiol-L-serine with and without Cyclodextrin (CyD).

<table>
<thead>
<tr>
<th>Host</th>
<th>Guest</th>
<th>Association Constant</th>
<th>Rate Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K'</td>
<td>Klmin-1</td>
</tr>
<tr>
<td>-</td>
<td>OPA-2-ME-L-serine</td>
<td>-</td>
<td>0.7816</td>
</tr>
<tr>
<td>γ-CyD</td>
<td>OPA-2-ME-L-serine</td>
<td>8.2559 105</td>
<td>1.336</td>
</tr>
</tbody>
</table>
Fig. 3.17 Fluorescence intensity Vs pH of

OPA + 2-mercaptoethanol + γ-cyclodextrin +

L-serine in FIA system.

Excitation wavelength filter=337nm,

\( \lambda_{em}=450\text{nm} \).
3.3 Other Isoindole-Cyclodextrin Complexation:

Enhancement of the fluorescence signals of other OPA-thiol-amino acid products with cyclodextrin was also investigated. Glycine, aspartic acid, tyrosine, glutamic acid, and alanine were investigated. Initially, fluorescence enhancement was observed for glycine, aspartic acid and tyrosine, but not for glutamic acid and alanine. Later experiments showed no enhancement for glycine, aspartic acid and tyrosine as well. Experimental conditions like pH, flow rate, etc. were varied to achieve the fluorescence enhancement with cyclodextrins, but in vain. It seemed that the isoindoles of these amino acids do not fit properly inside the cyclodextrin cavities.

3.4 Conclusion:

The host-guest inclusion phenomenon was successfully observed for DL-lysine and L-serine with cyclodextrins. Fluorescence enhancement was in agreement with expectations. Equilibrium and kinetic studies were made by flow injection fluorescence analysis. Effect of pH and time on fluorescence intensity were recorded. But failure to observe the enhanced fluorescence signal for other amino acids with cyclodextrins prevent us from a generalisation.
3.5 References:


2) VanEtten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L.;

3) Bergeron, R. J.; Channing, M. A.; Gibeily, G. J.; Pillor, D. M.;


CHAPTER FOUR

STREPTAVIDIN-AGAROSE AND AVIDIN D-BIOTIN-
ANTIBODY SOLID PHASE IMMUNOREACTORS
AND INSULIN-RITC LABELLED ANALYTE IN
FLOW INJECTION IMMUNOASSAYS
Introduction:

The aim of this chapter is to investigate the use of biotin-avidin system in flow injection immunoassays. In the first part of this chapter, a streptavidin-agarose solid phase immunoreactor was used in the flow injection manifold (fig. 4.1). Iminobiotin-antibody and insulin-RITC conjugates were used for the competitive immunoassay of insulin. In the second half of this chapter an avidin D-biotin-antibody immunoreactor was used along with the insulin-RITC for the flow injection immunoassay of insulin. The solid phase immunoreactor formed the basis of the separation step by initially binding selected components and then subsequent elution following the removal of interferences.

4.1 Iminobiotinylation of Anti-Insulin Antibodies:

Iminobiotinylation of polyclonal anti-insulin antibodies was performed according to a standard procedure (1) except that NHS-iminobiotin.HBr was used instead of NHS-biotin. 0.00087 g of NHS-iminobiotin.HBr in 50 μl of dimethyl formamide (DMF) was added to 50 μl of polyclonal anti-insulin antibody (1.581 mg) in 2.5 ml of 0.1 M carbonate buffer pH 8.0. The mixture was shaken and left for two hours at room temperature. The iminobiotinylated antibody was separated on a Sephadex G-25 PD 10 column, equilibrated with PBS buffer. The reaction mixture was made up to 2.5 ml with carbonate buffer pH 8.0 and poured onto the Sephadex G-25 PD 10 column. The column was eluted with 3.5 ml of PBS buffer pH 7.4, according to the instructions of the manufacturer (Pharmacia). The separated iminobiotinylated antibodies were stored at -20°C.

4.2 Packing of Streptavidin-agarose Column:

A glass micro column (0.39 ml) was filled with streptavidin-agarose using a pasteur pipette and then PBS was pumped through it to compact the column. The process was repeated in order to pack the column fully.
4.3 Design of the Flow Injection Manifold:

The flow injection manifold used is shown in fig. 4.1. Two channels for the tris and citrate buffers passed through a peristaltic pump, to the immunoreactor, and to the detector. The injection valve, 125 µl volume, and the flow cell, 7 µl illuminating volume, were incorporated into the manifold.

4.4 Flow rate through the Column:

At any one time the flow through the column was provided by either tris or citrate buffer. A constant flow rate was maintained throughout the whole experiment. The binding buffer (tris) and elution buffer (citrate) flow rates were maintained at 0.28 ml/min.

4.5 Binding and Elution of Iminobiotin-Antibodies to Streptavidin-agarose Immunoreactor:

125 µl of iminobiotin-antibodies (1:420) were injected into a stream of tris buffer pH 8.8, without Triton X-100. As the fluorescence of the non-bound peak at 337 nm returned to the base line the flow was switched to citrate buffer pH 2.5, also without triton, to elute the bound fraction. The column was then re-equilibrated with tris buffer before the next sample application.

Results and Discussion:

The binding and elution profile for the iminobiotin-antibody on the streptavidin-agarose column is shown in fig. 4.2. Excess iminobiotin-antibodies and some proteins e.g. albumin constitute the non binding peak. The elution peak consists of the iminobiotin-antibody previously bound onto the streptavidin-agarose column.
Tris pH 8.8
buffer

Peristaltic Pump

Injection valve
Immunoreactor

Switch valve

Waste

Citrate pH 2.5
buffer

Fluorescence Detector

Integrator or data handling software

Fig. 4.1 Flow injection manifold of insulin immunoassay using solid phase immunoreactor and fluorescence detector.
Fig. 4.2 Binding and elution of iminobiotin-antibody on streptavidin-agarose immunoreactor in FIA system.

\[ \lambda_{ex} = 280 \text{ nm}, \quad \lambda_{em} = 337 \text{ nm}. \]
4.6 Binding and Elution of Iminobiotin-Antibody-Insulin-Rhodamine Isothiocyanate (RITC) Complex to Streptavidin-agarose Column:

5 μl of insulin-RITC, containing 1.2188 μg of insulin, was added to 50 μl of iminobiotin-anti-insulin antibody (1:70) and mixed with 170 μl of tris buffer pH 8.8, with or without 0.1% Triton X-100 or bovine serum albumin (BSA). The mixtures were incubated for 25 min and then injected onto the streptavidin-agarose column in a flowing system using the binding buffer, tris pH 8.8, again with or without 0.1% Triton X-100 or BSA. As the fluorescence signal from the non-bound peak at 585 nm returned to the base line the flowing stream was switched to citrate buffer pH 2.5, with or without 0.1% Triton X-100 or BSA, to elute the bound fraction. The column was then re-equilibrated with tris buffer before the next application of the sample.

Results and Discussion:

The binding and elution profiles of sample (iminobiotin-antibody-insulin-rhodamine isothiocyanate) and blank (insulin-rhodamine isothiocyanate (RITC)) on the streptavidin-agarose column are shown in fig. 4.3, 4.4 and 4.5. Excess sample or blank constitutes the non binding peak while the elution peak is represented by either the binding of the sample or blank onto the streptavidin-agarose column.

It can be seen from fig. 4.3 when tris buffer pH 8.8 and citrate buffer pH 2.5, without Triton X-100 or BSA, were used the elution profile was very poor. In this case when the flowing stream was switched back to tris buffer in order to re-equilibrate the column for the next application, another binding peak was observed both for the sample and blank. This shows clearly the inability of the citrate buffer to remove completely the binding component from the streptavidin-agarose column.

The use of 0.1% Triton X-100 or BSA improved the elution pattern and reduced the non-specific binding to the streptavidin-agarose column. The
Fig. 4.3 Fluorescence Intensity Vs Time of sample (Inminobiotin-Ab, Insulin-RITC) and blank (Insulin-RITC) on Streptavidin-agarose Column in FIA system.

*Note:* Binding & Elution buffers are without Triton X-100.

$\lambda_{ex}=550\text{nm}, \lambda_{em}=585\text{nm}, \text{Slit}=10/15\ \text{nm}$
Fig. 4.4 Fluorescence Intensity Vs Time of sample (Iminobiotin-Ab-Insulin RITC) and blank (Insulin-RITC) on streptavidin-agarose column in FIA system.

Note: Binding & Elution buffers contains 0.1%BSA. No Triton X-100
λ_ex=550 nm, λ_em=585 nm, Slit=10/15 nm.
Fig. 4.5 Fluorescence Intensity Vs Time of Sample

(Iminobiotin-Antibody, Insulin-RITC) and Blank

(Inulin-RITC) on streptavidin-agarose column
in FIA system.

Binding & Elution buffers contains 0.1% Triton X-100

$\lambda_{ex}=550\text{nm}, \lambda_{em}=585\text{nm}, \text{Slit}=10/15\text{ nm}.$
binding and elution profile of the sample and blank using tris and citrate buffers with Triton X-100 or BSA are shown in figs. 4.4 and 4.5 respectively. It can be seen from fig. 4.4 that when 0.1% BSA is used some non-specific binding of insulin-RITC to the streptavidin-agarose column still occurs, while in fig. 4.5 when 0.1% Triton X-100 is used no such non-specific binding is observed. Therefore, it was decided to use 0.1% Triton X-100, in both the tris pH 8.8 and citrate buffer pH 2.5, in all steps needed to develop the flow injection immunoassay for insulin.

4.7 Immunoassay of Insulin using Streptavidin-agarose Column:

Attempts were made to develop an immunoassay for insulin using the streptavidin-agarose column and iminobiotin-antibody-insulin-RITC complex. For this purpose, 30 μl of iminobiotin-anti-insulin antibody (1:70) was mixed with 3 μl of insulin-RITC, containing 7.3127 X 10^-7 g of insulin, insulin standard and tris buffer pH 8.8. The mixture was incubated for ten minutes and then injected onto the streptavidin-agarose column using binding buffer, tris pH 8.8. When the fluorescence of the non-bound peak at 585 nm returned to the base line the flow through the column was switched to citrate buffer to elute the bound fraction. After the elution of bound fraction the flow through the column was switched back to tris buffer to re-equilibrate the column for the next application of sample.

Results and Discussion:

The therapeutic range for insulin is between 0.21 to 4.167 ng/ml. An assay in this range was attempted but it failed due to lack of displacement of the labelled insulin with increasing concentration of unlabelled insulin in a competitive immunoassay. The reason was the incomplete removal of the iminobiotin-antibody-insulin-RITC complex from the streptavidin-agarose column. The binding between streptavidin and biotin is very strong (Kd = 4
while the binding between streptavidin and iminobiotin is less strong (\(K_d = 3.5 \times 10^{-11}\) M), it is still sufficiently strong not to be reversed completely at pH 2.5. It is therefore, very difficult to remove completely the iminobiotin-antibody-insulin-RITC complex from the streptavidin-agarose column, although not impossible. It may be removed by denaturing the streptavidin with 6 M guanidine·HCl pH 1.5 (2).

Binding and elution profiles of sample (iminobiotin-antibody-insulin-RITC) and blank (insulin-RITC) on a new streptavidin-agarose column (Fig. 4.5) and on an older column (Fig. 4.6) show that the non-specific binding of the blank (insulin-RITC) increases as the number of runs on the streptavidin-agarose column increases (Fig. 4.7). This is because the citrate buffer pH 2.5 removes most of the sample complex (iminobiotin-antibody-insulin-RITC) from the column, but leaves some of the iminobiotin-anti-insulin antibody matrices on the column. It is these streptavidin-iminobiotin-anti-insulin antibody complexes on the column which give increasing blank signals as the number of runs increases. Therefore, it was impossible to develop a flow injection immunoassay for insulin on the streptavidin-agarose column using the iminobiotin-antibody-insulin-RITC complex.

The failure to develop a flow injection immunoassay of insulin using the streptavidin-agarose column and iminobiotin-antibody-insulin-RITC complex led to the preparation of a streptavidin/avidin-biotin-anti-insulin antibody matrix as a reversible immunoreactor and use of the insulin-RITC conjugate as labelled analyte in a flow injection competitive immunoassay for insulin. The streptavidin-agarose was replaced by avidin D-agarose, which is a nonglycosylated avidin and thus has less non-specific binding than avidin. The rest of this chapter deals with the biotinylation of the antibodies, the preparation of the avidin D-agarose-biotin-antibody matrix, and the development of a flow injection immunoassay for insulin.
Fig. 4.6 Fluorescence Intensity Vs time of sample (Laminobiotin-Antibody..Insulin-RITC) and blank (Insulin-RITC) on old (after 15 runs) streptavidin-agarose column in FIA system. Binding & Elution buffers contain 0.1% Triton X-100 
\( \lambda_{\text{ex}} = 550\text{nm} \) \( \lambda_{\text{em}} = 585\text{nm} \)
Fig. 4.7 Peak Area Vs No. of days of Blank (Insulin-RITC) on streptavidin-agarose column in FIA system.

Note: Blanks were run after running samples on the same column.

$\lambda_{ex}=550\text{nm}, \lambda_{em}=585\text{nm}$
4.8 Biotinylation of Anti-Insulin Antibodies:

Polyclonal anti-insulin antibodies were biotinylated according to a published procedure (1) except that NHS-LC-Biotin with longer spacer arm was used instead of NHS-biotin. 0.00042g of NHS-LC-biotin in 50 μl of dimethyl formamide (DMF) was added to 20 μl of polyclonal anti-insulin antibodies (0.6324 mg) in 2 ml of 0.1 M carbonate buffer pH 8.0. The mixture was shaken and left for two hours at room temperature. The biotinylated anti-insulin antibodies were then separated on a Sephadex G-25 PD 10 column already equilibrated with PBS buffer. The reaction mixture was made up to 2.5 ml with carbonate buffer pH 8.0 and poured onto the Sephadex G-25 PD 10 column. The column was eluted with 3.5 ml of PBS pH 7.4 buffer, according to the instructions of the manufacturer (Pharmacia). The separated biotinylated antibodies were stored at -20°C.

4.9 Preparation of Avidin D-agarose-Biotin-Antibody Matrix:

0.7 ml of the settled gel, avidin D-agarose, was washed thoroughly with 50 mM carbonate pH 7.5 and 0.1 M carbonate pH 8.0 buffers alternately three times each with 100 ml to remove any preservatives added to the gel. It was then suspended in 15 ml of carbonate buffer pH 7.5 and stirred. The biotinylated antibodies (section 4.8), were defrosted and diluted to 15 ml with carbonate buffer pH 7.5, and added to the gel drop wise with stirring over a period of one hour and the mixture was then stirred for a further fifteen minutes. The gel was then packed into a column and washed thoroughly with 500 ml of carbonate buffer pH 7.5, containing 0.1% Triton X-100, to remove the uncoupled biotinylated antibodies from the gel column. The gel was then stored in PBS buffer pH 7.4 containing 0.05% w/v sodium azide.
4.10 Packing of the Avidin D-agarose-Biotin-Antibody Matrix into the Column:

The avidin D-agarose-biotin-antibody matrix was packed into a column according to the procedure described in section 4.2.

4.11 Design of the Flow Injection Manifold:

The design of the flow injection manifold used in this immunoassay is shown in fig. 4.1 and described in section 4.3.

4.12 Flow Rate through the Column:

The flow rate through the column for binding buffer, tris pH 8.8, and elution buffer, citrate pH 2.5, was maintained constant throughout the whole experiment at 0.28 ml/min.

4.13 Binding and Elution of Insulin-RITC on the Avidin D-agarose-Biotin-Antibody Column:

5 μl of insulin-RITC, containing 1.2188 μg of insulin, in 220 μl of tris buffer pH 8.8 was loaded into the injection valve and injected onto the avidin D-agarose-biotin-antibody column. When the fluorescence from the non-bound peak at 585 nm returned to the base line the flow through the column was switched to citrate buffer to elute the bound fraction. After the elution of bound fractions the flow through the column was switched back to tris buffer to re-equilibrate the column for the next application of the sample.

Results and Discussion:

The binding and elution profile of insulin-RITC on the avidin D-agarose-biotin-antibody column is shown in fig. 4.8. Excess insulin-RITC constitutes the non binding peak, while the elution peak shows the fraction of insulin-RITC bound to the column.
Fig. 4.8 Peak Height (F.I.) Vs Time of insulin-RITC on Avidin D-agarose-Biotin-Antibody column in FIA system.

\[ \lambda_{ex} \text{filter}=546\text{nm}, \lambda_{em}=585\text{nm} \]
4.14 Immunoassay of Insulin using Avidin D-agarose-Biotin-Antibody Immunoreactor:

A calibration curve for insulin was obtained using the following protocol:

Insulin (sample or standard) between 1.1-10.6 pg/ml was mixed with 5 µl of insulin-RITC, containing 1.2188 µg of insulin, and tris buffer. The mixture was shaken and then loaded into the injection valve. It was then injected onto the avidin D-agarose-biotin-antibody column in the flow injection system using the binding buffer, tris pH 8.8. When the fluorescence from the non-bound peak at 585 nm returned to the base-line, the flow through the column was switched to citrate buffer in order to elute the bound fraction and then again switched back to tris buffer to re-equilibrate the column for the next injection. The total time, including re-equilibrium of the column (five min.), from injection to injection was 20 minutes. The fluorescence intensity of the elution peak was used to calculate the \( \frac{B/B_0}{B} \times 100 \) values and these were plotted against the concentration of insulin.

Results and Discussion:

The calibration curve (fig. 4.9) for insulin was constructed by plotting \( \frac{B/B_0}{B} \times 100 \) versus insulin concentration in the standard, where

\[
\frac{B}{B_0} \times 100 = \frac{\text{F.I. of the bound peak at stated insulin conc.}}{\text{F.I. of the bound peak at zero insulin conc.}}
\]

The assay range of insulin is 1.1 to 10.6 pg/ml. As shown in figure. 4.9, sufficient displacement of the insulin-RITC is achieved in this assay. The assay works at the lower end of the therapeutic range of the insulin, i.e. 0.21-4.167 ng/ml.
Fig. 4.9 A calibration curve of insulin using Avidin D-agarose-Biotin-Ab column and insulin-RITC as labelled analyte in FIA system.

$\lambda_{ex}$ filter = 546nm, $\lambda_{em}$ = 585nm.
4.15 Reproducibility of the Standard Curves on the Same Avidin D-agarose-Biotin-Antibody Column:

Day to day calibration curves of insulin on the same avidin D-agarose-biotin-antibody column are shown in fig. 4.10. The procedure for the assay is the same as described in section 4.14.

Results and Discussion:

From fig. 4.10 it is quite clear that the reproducibility of the calibration curve on the same avidin D-agarose-biotin-antibody column on different days is poor. The column is useful for one day working only. It appears that the anti-insulin antibody on the column loses its binding to antigen, bovine insulin, after one day. Throughout the day 50 mM tris buffer pH 8.8 and 0.1 M citrate buffer pH 2.5 were passed alternately through the avidin D-agarose-biotin-antibody immunoreactor to bind the insulin-RITC to the anti-insulin antibody and then to elute it for the determination of insulin. It is possible that this treatment dampens the binding activity of the antibody. Therefore it was difficult to construct calibration curves in the following days on the same column.

4.16 Reproducibility of the Standard Curves on Different Protein G-agarose Column:

Three calibration curves for three separate avidin D-agarose-biotin-antibody column were constructed following the procedure described in section 4.14. Each of the column matrices, i.e. avidin D-agarose-B-Ab, was prepared separately and individually. The calibration curves are shown in fig. 4.11.

Results and Discussion:

Although batch to batch variations are significant (Fig. 4.11), the assay range is same, i.e. 1.1 to 10.6 pg/ml. The variation in displacement may be explained due to errors involved in synthesising the separate batch of biotin-
Fig. 4.10 Calibration curves of insulin on three days on the same column of Avidin D-agarose-Biotin-Ab in FIA system

$\lambda_{ex} = 546\text{nm}$, $\lambda_{em} = 585\text{nm}$.
Fig. 4.11 Three calibration curves of insulin on different batches of agarose-
Avidin D-Biotin-Ab in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm.
antibody and avidin D-agarose-biotin-antibody matrices, error in aliquating the small amounts of liquids and an instrumental error in day to day running. However the results for column prepared from the same batch, show good reproducibility (fig. 4.12).

4.17 Reproducibility of the Column with Time:

5 µl of insulin-RITC (1.2188 µg of insulin) was mixed with 30 µl of insulin (4.775X10^-3 ng/ml) and 170 µl of tris pH 8.8 buffer. The mixture was then injected onto the avidin D-agarose-biotin-antibody column using tris buffer at a flow rate of 0.28 ml/min. When the fluorescence from the non-bound peak at 585 nm returned to the baseline the flow through the column was switched to citrate buffer pH 2.5 for the elution of bound fraction and then switched back to tris buffer to re-equilibrate the column for the next injection of the sample.

The above procedure was repeated for one whole day and also on the following days, on the same column. The same concentration of insulin-RITC was used in order to check the working reproducibility and efficiency of the column.

Results and Discussion:

Fig. 4.13 and 4.14 show the variation of the zero dose response binding of the insulin-RITC complex to avidin D-agarose-biotin-antibody immunoreactor. The coefficient of variation within the day for 10 runs was 10.66% and between days for 11 runs was 61.75%.

4.18 Insulin Assay in Serum Sample:

Human serum samples containing insulin were assayed to check the validity of this flow injection immunoassay. The human serum was diluted one hundred fold with tris buffer pH 8.8 and standard insulin samples were prepared in this buffer between 1.1 and 21.2 pg/ml. The procedure for the immunoassay was the same as described in section 4.14.
Fig. 4.12 Calibration curves of insulin on different avidin D-agarose-Biotin-Antibody column but same batch in FIA system.

λex filter=546 nm, λem=585 nm
Fig. 4.13 Reproducibility of the agarose-
Avidin D-Biotin-Antibody Immunoreactor
within-day in FIA system.

λex filter=546nm, λem=585nm
Fig.4.14 Reproducibility of the blank (Insulin-RITC) on the same column (Avidin D-agarose-Biotin-Antibody) and different days in FIA system.

$\lambda_{ex}$ filter=546nm, $\lambda_{em}$=585nm
Results and Discussion:

The assay for insulin between 1.1 and 21.2 pg/ml was attempted but failed due to lack of displacement of the fluorescence labelled insulin with increasing concentration of unlabelled insulin. The lack of displacement was suggested to be due to non-specific binding of proteins and IgGs in the serum to the antibody-biotin-avidin D-agarose matrix column. The therapeutic range of insulin is between 0.21 and 4.167 ng/ml and the assay range developed by this method described in section 4.14 is 1.1 to 10.6 pg/ml, i.e.; much lower than the therapeutic range. Therefore, it seemed that at such low concentrations of insulin the non-specific binding was so effective that few binding sites for insulin were left on the immunoreactor to allow an effective immunoassay.
4.19 References:

1) Guesdon, J; Ternynck, T; Avrameas, S; *J. Histochem. Cytochem.* 27, 1131(1979)

2) Green, N.M; *Biochem J.* 101, 774(1966)
CHAPTER FIVE

PROTEIN G-AGAROSE IMMUNOREACTOR AND INSULIN-RITC LABELLED ANALYTE IN FLOW INJECTION IMMUNOASSAYS
Introduction:

The work in this chapter describes a flow injection immunoassay of bovine insulin using protein G-agarose as a solid phase immunoreactor and insulin-rhodamine isothiocyanate (RITC) as the labelled analyte.

Although a large number of homogeneous and heterogeneous immunoassay methods for insulin are described in the literature (chapter 1), the use of flow injection with protein G-agarose as a solid phase immunoreactor has a lot of potential in heterogeneous immunoassays. Therefore, it was decided to investigate the potential of a flow injection immunoassay based on protein G-agarose as the immunoreactor, a polyclonal anti-insulin antibody and an insulin-RITC derivative. Preliminary experiments were performed with protein A-controlled pore glass (CPG) and protein G-agarose to assess the binding characteristics of the guinea pig anti-insulin antibody in flow injection immunoassay.

5.1 Packing of the Immunoreactor:

Protein A-CPG and protein G-agarose were packed into the columns according to the procedure described in the section 4.2.

5.2 Design of the Flow Injection Manifold:

The design of the flow injection manifold used in this immunoassay is shown in fig. 4.1 and described in section 4.3.

5.3 Flow Rate through the Column:

The flow rate through the column for binding buffer, tris pH 8.8, and elution buffer, citrate pH 2.5, were the same, i.e.; 0.28 ml/min, as described in section 4.12.
5.4 Binding and Elution of Anti-Insulin Antibody to the Protein A-Controlled Pore Glass (CPG) Column:

125 µl of polyclonal anti-insulin antibody (1:100) was injected onto the Protein A-controlled pore Glass (CPG) column using the binding buffer without Triton X-100. As the fluorescence from the non-bound peak at 337 nm returned to the base line, the flow through the column was switched to citrate buffer, also without Triton X-100, to elute the bound fraction. The buffer was then switched back to tris to re-equilibrate the column for the next sample application.

Results and Discussion:

The binding and elution profile for the reversible immobilisation of antibody on the protein A-CPG column is shown in fig. 5.1. Proteins e.g. albumin and excess Ig constitute the non binding peak. It can be seen from figure 5.2 that there was no elution peak at pH 2.5 which indicates either very strong binding between the guinea pig IgG and protein A, or no binding between them. According to the literature some researchers have shown that there is a strong binding between guinea pig IgG antibody and protein A (1-2). The absence of the elution peak may be due to this.

5.5 Binding and Elution Profile of Antibody..Insulin-Rhodamine Isothiocyanate Complex to Protein A-CPG:

50 µl of anti-insulin antibody (1:50) was mixed with 25 µl of insulin-RITC conjugate, containing 1.1085 µg of insulin, and 170 µl of tris buffer pH 8.8. The mixture was incubated for two minutes and injected onto the protein A-CPG column using the binding buffer. When the fluorescence from the non-bound peak at 585 nm returned to the baseline the flow through the column was switched to citrate buffer and then back to tris buffer to re-equilibrate the column for the next sample.
Fig. 5.1 Fluorescence intensity vs time of anti-insulin antibody on protein A-CPG column in FIA system.

$\lambda_{\text{ex}}=280\text{nm}$, $\lambda_{\text{em}}=337\text{nm}$, Slit=10/15 nm
Results and Discussion:

It can be seen from fig. 5.2 that the antibody (Ab).insulin-RITC complex is not eluted with citrate buffer pH 2.5. This indicates strong binding between protein A and guinea pig antibody which is not broken even at pH 2.5.

5.6 Binding and Elution of Anti-Insulin Antibody to the Protein G Column:

The anti-insulin antibody was diluted 1:100 and 125 µl of it was injected onto the protein G column using the binding buffer, tris pH 8.8, without Triton X-100. As the fluorescence from the non-bound peak at 337 nm returned to the base line the flow through the column was switched to citrate buffer, also without Triton X-100, to elute the bound fraction, and then switched back to the tris buffer to re-equilibrate the column for the next sample application.

Results and Discussion:

The binding and elution profile for the reversible immobilisation of anti-insulin antibody on the protein G-agarose column is shown in fig. 5.3. Proteins e.g. albumin, and some immunoglobulins such as, IgA, IgM and IgD constitute the non binding peak. It can be seen from figure 5.3. that there was no elution peak which indicates either strong binding between the guinea pig antibodies and protein G or no binding between them. Some workers have suggested strong binding between these antibodies and protein G (3). This may explain the absence of the elution peak.

5.7 Binding and Elution Profile of Antibody..Insulin Rhodamine Isothiocyanate Complex to Protein G-agarose Column:

25 µl of Insulin-RITC conjugate, containing 1.1085 µg of insulin, was added to 50 µl of anti-insulin antibody (1:50) and 170 µl of tris buffer. The mixture was incubated for two minutes, then loaded into the injection valve and
Fig. 5.2 Elution profile of sample (Ab-insulin-RITC) on protein A-CPG immunoreactor column in FIA system.

$\lambda_{\text{ex}}$ filter = 546 nm, $\lambda_{\text{em}}$ = 585 nm
Fig. 5.3 Fluorescence intensity vs time of anti-insulin antibody in FIA system on Protein G-agarose immunoreactor column.

$\lambda_{ex}=280\text{nm}, \lambda_{em}=337\text{nm}, \text{Slit}=10/15 \text{ nm}$
injected onto the protein G-agarose immunoreactor using tris buffer, with 0.1% Triton X-100, at a flow rate of 0.28 ml/min. When the fluorescence from the non-bound peak at 585 nm returned to the baseline the flow through the column was switched to citrate buffer, also with 0.1% Triton X-100, and then switched back to tris buffer to re-equilibrate the column for the next sample.

Results and Discussion:

Fig. 5.4 shows that the antibody-insulin-RITC complex is eluted with citrate buffer pH 2.5. As 0.1% Triton X-100 was used in the elution buffer, it may be concluded that it helps in the dissociation of protein G and antibody-insulin-RITC complex along with the low pH. Therefore, 0.1% Triton X-100 was used in both binding and elution buffers.

5.8 Reproducibility of the Column with Time:

25 μl of insulin-RITC (1.1088 μg of insulin) was added to 20 μl of anti-insulin antibody (1:50) and mixed with 180 μl of tris buffer pH 8.8. The mixture was incubated for two minutes and then injected onto the protein G-agarose column using tris buffer at a flow rate of 0.28 ml/min. When the fluorescence from the non-bound peak at 585 nm returned to the baseline the flow through the column was switched to citrate buffer pH 2.5 to elute the bound fraction and then switched back to tris buffer to re-equilibrate the column for the next sample.

The above procedure was repeated during the whole day and also the following days, on the same column, using the same concentration of insulin-RITC and anti-insulin antibody in order to check the working reproducibility of the column.

Results and Discussion:

Fig. 5.5 and 5.6 show the variation of the zero dose response binding of the antibody-insulin-RITC complex to the protein G-agarose immunoreactor on the
Fig. 5.4 Elution profile of blank (insulin-RITC) and sample (Ab-insulin-RITC) on protein G-agarose immunoreactor column in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm
Fig. 5.5 Reproducibility of the binding of the same sample (Ab-insulin-RITC) to protein G-agarose immunoreactor column on the same day in FIA system.

λ<sub>ex</sub> filter = 546 nm, λ<sub>em</sub> = 585 nm
Fig. 5.6 Reproducibility of the binding of the same sample (Ab-insulin-RITC) to the same protein G-agarose immunoreactor column on different days in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em} = 585$ nm
same day and different days respectively. The coefficient of variation within the day for 14 runs was 3.77 % and between days for 22 runs was 4.10 %.

5.9 Optimisation of Assay Conditions:

5.9.1 Concentration of Anti-Insulin Antibody (Ab):

The anti-insulin antibody concentration was varied while keeping the insulin-RITC concentration constant, i. e; 30 µl of insulin-RITC containing 1.3302 µg of insulin. The mixtures were incubated and injected onto the protein G-agarose column and the fluorescence intensity of the eluted peak measured and plotted Vs antibody concentration (dilution).

Results and Discussion:

30 µl of anti-insulin antibody (1:25) was chosen as it gave sufficient sensitivity for use in the construction of the immunoassay calibration curve. From fig. 5.7, it can be seen that maximum displacement can be achieved at this antibody dilution (1:25).

5.9.2 Effect of pH on the Binding of Blank (Insulin-RITC Conjugate) and Sample (Ab..Insulin-RITC Complex) on the Protein G-agarose Immunoreactor:

To study the effect of pH on the binding of sample (Ab..Insulin-RITC complex) to the protein G-agarose column, 20 µl of insulin-RITC containing 8.868 x 10⁻⁷ g of insulin, was added to 20 µl of anti-insulin antibody (1:100) and 185 µl of tris buffer and the pH varied from 7.5 to 10.0. For the blank study 20 µl of insulin-RITC was mixed with 205 µl of tris buffer at various pH values. The mixtures were incubated for two minutes and then injected onto the protein G-agarose column using the same tris buffer as used in the incubation mixtures. When the fluorescence from the non-bound peak at 585 nm returned to the base line the flow through the column was switched to citrate buffer pH
Fig. 5.7 Effect of antibody dilution on fluorescence intensity. (Antibody was incubated with insulin-RITC and passed through the protein G-agarose immunoreactor column in FIA system. Elution buffer: 0.1 M citrate pH 2.5.

λex filter = 546 nm, λem = 585 nm
2.5 for the elution of the bound fraction and then switched back to tris buffer to re-equilibrate the column for the next sample.

**Results and Discussion:**

From fig. 5.8 it is clear that an increase in the pH of binding buffer decreases the fluorescence intensity of the bound peak, both for the sample and the blank. However, the decrease of fluorescence intensity for the sample (Ab-insulin-RITC) is more than for the blank (Insulin-RITC). From this observation it may be concluded that the binding of insulin-RITC is small or there is no non-specific binding at higher pH. Therefore, the binding buffer, tris pH 8.8, was chosen.

**5.9.3 Effect of pH on the Elution of Ab..Insulin-RITC Complex from the Protein G-agarose Column:**

25 μl of insulin-RITC, containing 1.1085 μg of insulin, was added to 75 μl of anti-insulin antibody (1:50) and 125 μl of tris buffer. The mixture was incubated for two minutes and then injected onto the protein G-agarose column using tris buffer at a flow rate of 0.28 ml/min. When the fluorescence from the non-bound peak at 585 nm returned to the base line the flow through the column was switched to the elution buffer. The elution buffer pH was varied from 2.5 to 10.0, using citrate from pH 2.5 to 4.5 and 0.1 M glycine pH 10.0. Finally the column was cleaned in each case with citrate buffer pH 2.5 before the application of the next sample and then switched back to tris buffer to re-equilibrate the column.

**Results and Discussion:**

From fig. 5.9 it is clear that the maximum fluorescence intensity of the elution peak was achieved at pH 2.5. At other pH's, i.e. 3.5 and 4.5, some of the IgG fractions were eluted and others were still bound to the protein G. Therefore, for the complete removal of the IgG..insulin-RITC complex from the protein G-agarose column citrate buffer pH 2.5 is essential. Although some industrial manufacturers (Pharmacia) of the protein G-agarose matrix described
Fig. 5.8 Blank (insulin-RITC) and sample
(Antibody..insulin-RITC) binding to
protein G-agarose immunoreactor
at different pH and having
elution at the same pH (2.5)
λex filter = 546 nm, λem = 585 nm
Fig. 5.9 Fluorescence intensity (F.I) of bound peak vs pH of elution of antibody-insulin-RITC complex on protein G-agarose immunoreactor in FIA system.

λex filter = 546 nm, λem = 585 nm.
the removal of IgG from protein G at pH 10.0, fig. 5.9 and 5.10 show that only a very small quantity of the IgG could be eluted at this pH. Different classes of IgG may have different affinities for protein G and hence different pH values may be required to elute them separately. But in the immunoassay where no separation of IgG is required and the removal of the all IgG is essential for a successful assay, the use of the citrate pH 2.5 buffer is therefore necessary.

5.9.4 Incubation Time:

20 μl of insulin-RITC, containing 8.868 x 10\(^{-7}\) g of insulin, and 40 μl of anti-insulin antibody (1:50) and 165 μl of binding buffer, tris, were mixed and incubated at room temperature. After incubation, the mixture was loaded into the injection loop and injected onto the protein G-agarose column using tris buffer. When the fluorescence from the non-bound peak at 585 nm returned to the base line the flow through the column was switched to citrate buffer to record the elution profile of the peak. The times of incubation for the above mixture were 0.30, 0.45, 1.30, 3.05, 5, 10, 15, 24, and 30 minutes. The fluorescence intensity was plotted against incubation time as shown in fig. 5.11.

Results and Discussion:

Fig. 5.11 shows the fluorescence intensity Vs incubation time for the anti-insulin antibody and insulin-RITC conjugate. It shows that after 1.30 minutes any further increase of incubation time does not increase the formation of antibody..insulin-RITC complex and hence the fluorescence intensity of the bound peak.

5.9.5 Immunoassay of Insulin:

A calibration curve using the optimised assay conditions with the following protocol was established as follows;

Insulin sample or standards between 0.05 to 2.2 ng/ml was added to 30 μl of anti-insulin antibody (1:25), and shaken for 45 seconds, binding buffer, tris,
Fig. 5.10 Elution of antibody-insulin-RITC with 0.1 M glycine buffer pH 10.00 from protein G-agarose immunoreactor in FIA system.

$\lambda_{ex} = 546 \text{ nm}, \lambda_{em} = 585 \text{ nm}$.
Fig. 5.11 Fluorescence intensity of bound peak vs incubation time of antibody and Insulin-RITC conjugate on protein G-agarose immunoreactor in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm
and 30 µl of insulin-RITC containing 1.3302 µg of insulin, were then added and the mixture incubated for two minutes. The mixture was then injected onto the protein G-agarose column using tris as the binding buffer. When the fluorescence from the non-bound peak at 585 nm (six minutes) returned to the base-line the flow through the column was switched to citrate buffer (four minutes) in order to elute the bound fraction and then again switched back to tris buffer to re-equilibrate the column (three minutes) for the next injection of the sample. The total time from injection to injection was sixteen minutes. The fluorescence intensity of the elution peak was used to calculate the B/B% values and plotted against the concentration of insulin.

Results and Discussion:

The calibration curve, shown in fig. 5.12, for insulin is between 0.05 to 2.2 ng/ml for 30 µl of anti-insulin antibody (1:25). It was constructed by plotting B/B% versus the insulin concentration, where

$$\text{B/B}_b\% = \frac{\text{F.I. of the bound peak at stated insulin conc.}}{\text{F.I. of the bound peak at zero insulin conc.}} \times 100$$

As shown in the figure 5.12, a 20% displacement of the insulin-RITC was achieved in this assay, which covers the lower end of the therapeutic range of insulin, i.e. from 0.21- 4.167 ng/ml. A concentration of insulin as low as 0.05 ng/ml can be detected by this method.

5.9.6 Reproducibility of the Standard Curves on the Same Protein G-agarose Column:

Day to day calibration curves of insulin on the same protein G-agarose column are shown in fig. 5.13. The procedure for the assay is the same as described in section 5.9.5 except that the pH of the tris and citrate buffers used in these assays were 8.93 and 2.63 respectively.
Fig. 5.12 Calibration curve of bovine insulin on protein G-agarose immunoreactor in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm.
Fig. 5.13 Calibration curves of bovine insulin on the same protein G-agarose immunoreactor and different days in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm.
Results and Discussion:

As can be seen from fig. 5.13 there is quite good reproducibility between the two calibration curves for insulin on two consecutive days using the same protein G-agarose column. The small variations in part may be due to poor reproducibility in aliquoting small volumes and instrumental variations.

The calibration curve, shown in fig. 5.13 shows only 10% displacement in the immunoassay of insulin compared to the 20% displacement as shown in fig. 13. The probable reason for this is the use of the tris and citrate buffers which were adjusted to pH 8.93 and 2.63 instead of 8.8 and 2.5 respectively. The higher pH of the tris buffer allowed less binding between the anti-insulin antibody (Ab) and insulin or insulin-RITC and also between protein G and antibody, which resulted in a smaller fluorescence intensity of the binding peak (Fig. 5.8). Also, as shown in fig. 5.9 the use of a higher pH for the elution buffer, i.e. citrate pH 2.63 instead of pH 2.5, for the removal of Ab-insulin-RITC from protein G reduced the efficiency of elution and hence the fluorescence intensity of the bound peak.

A single protein G column reproduces the calibration curve for two days only and does not work to produce a third calibration curve. One reason may be the use of the higher pH citrate buffer, i.e. pH 2.63 instead of 2.5, for the elution of IgG's from the protein G column. As shown in fig. 5.9 that at higher elution pH less and less IgG's are eluted. Therefore, it may be possible that some IgG's left on each run on the column may gradually stop further binding of the IgG's to the column. Another reason may be that the use of the alternating high and low pH for long periods of time destroys the activity of protein G. Therefore, after performing the immunoassay for insulin on the same protein G column, on two consecutive days, and using the high pH binding and elution buffers, tris pH 8.93 and citrate pH 2.63, the same column does not work to produce a third calibration curve due to the damage to the protein G.
5.9.7 Reproducibility of the Standard Curves on Different Protein G-agarose Column:

Three calibration curves on three separate protein G-agarose immunoreactor columns were constructed following the procedure described in section 5.9.5. They are shown in fig. 5.14.

Results and Discussion:

It can be seen from fig. 5.14 that there is quite good reproducibility between calibration curves, 1 & 2, both showing the same assay range, i.e. 0.05-2.2 ng/ml and approximately the same displacement, i.e. 20%. In the third calibration curve, although the assay range is the same, the displacement is only 10%. The small displacement in the third calibration curve was probably due to the accidental use of high pH tris and citrate buffers, i.e. 8.93 and 2.63 respectively (see section 5.9.6).

5.9.8 Insulin Assay in Serum Sample:

In order to check whether the assay would work for serum, samples containing bovine insulin were analysed. The standard insulin samples were made up in tris pH 8.8 buffer between 0.05 and 2.2 ng/ml. Human serum was diluted one hundred fold with tris buffer and 115 µl of it was added to every insulin standard. These solutions were used in the flow injection immunoassay of insulin using the protein G-agarose immunoreactor as described in section 5.9.5.

Results and Discussion:

The calibration curve for insulin in serum between 0.05 and 2.2 ng/ml is shown in fig. 5.15. A sample containing the 0.1122 ng/ml of insulin was prepared by adding known amounts of insulin to the blank serum. The mean recovery was 120.08%.
Fig. 5.14 Calibration curves of insulin on different protein G-agarose immunoreactor and days in FIA.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm.
Fig. 5.15 Calibration curve of insulin in serum on protein G-agarose immunoreactor in FIA system.

$\lambda_{ex}$ filter = 546 nm, $\lambda_{em}$ = 585 nm.
5.10 References:

1) Eliasson, M; Olsson, A; Palmerantz, E; Wiberg, K; Inganas, M; Guss, B; Lindberg, M; Uhlen, M; *J Biol Chem.* 263, 4323-4327 (1988)

2) Guss, B; Eliasson, M; Olsson, A; Uhlen, M; Frej, A.-K; Jörnvall, H; Flock, J.-I; Lindberg, M; *EMBO J.* 5, 1567-1575 (1986)

CHAPTER SIX

PROTEIN G-AGAROSE IMMUNOREACTOR AND INSULIN-BIOTIN-AVIDIN-TEXAS RED LABELLED ANALYTE IN FLOW INJECTION IMMUNOASSAYS
Introduction:

In this chapter the use of a protein G-agarose solid phase immunoreactor and insulin-biotin-avidin-Texas Red labelled analyte in the flow injection immunoassay of insulin is described. Insulin-biotin-avidin-allophycocyanin labelled analyte will also be used.

6.1 Design of the Flow Injection Manifold:

The design of the flow injection manifold used in this immunoassay is shown in fig. 4.1 and described in section 4.3.

6.2 Flow Rate through the Immunoreactor:

The flow rate through the immunoreactor for the binding buffer, tris pH 8.8, and the elution buffer, citrate pH 2.5, were the same as described in section 4.4.

6.3 Binding and Elution Profile of Blank (Insulin-Biotin-Avidin-Allophycocyanin) and Sample (Antibody . Insulin-Biotin-Avidin-Allophycocyanin) on Protein G Immunoreactor.

The sample was prepared by mixing 100 µl of insulin-biotin-avidin-allophycocyanin with 40 µl of antibody (1:25) and 85 µl of PBS buffer pH 7.4. The mixture was incubated for two minutes. A blank was prepared by mixing 100 µl of insulin-biotin-avidin-allophycocyanin with 125 µl of PBS buffer. The mixtures were injected onto the protein G-agarose immunoreactor using the binding buffer, tris pH 8.8. When the fluorescence from the non-bound peak at 660 nm returned to the baseline the flow through the immunoreactor was switched to citrate buffer pH 2.5 in order to elute the bound fraction and then switched back to tris buffer to re-equilibrate the immunoreactor for the next sample.

Results and Discussion:

The binding and elution profile for blank and sample are shown in fig. 6.1. The lack of elution peak for the sample indicates that the insulin-biotin-avidin-
Fig. 6.1. Fluorescence intensity Vs time of sample (Antibody..Insulin biotin-avidin-allophycocyanin) and blank (Insulin-biotin-avidin-allophycocyanin) on protein G-agarose column in FIA system.

$\lambda_{ex}=650 \text{ nm} \quad \lambda_{em}=660 \text{ nm}, \quad \text{Slit}=10/15 \text{ nm}$
allophycocyanin may not react with antibody. This may be due to the large size of the allophycocyanin fluorophore. It is possible that it creates hindrance in the immunoreactivity of the insulin-biotin-avidin-allophycocyanin with antibody. Also, as fig. 6.2 shows the avidin-allophycocyanin is a highly unstable fluorophore conjugate and fluorescence decreases very sharply within one minutes. As we know that a single flow injection sample analysis takes thirteen minutes and the elution peak comes off from the protein G immunoreactor after eight or nine minutes. Therefore, due to the highly unstable nature of the fluorophore conjugate it is very difficult to detect the elution peak with this span of time.

Therefore, by observing the lack of the immunoreactivity of the insulin-biotin-avidin-allophycocyanin complex with antibody and the highly unstable nature of the fluorophore it was decided not to use this complex as labelled analyte in the further investigation of the immunoreactivity of the insulin using protein G immunoreactor.

6.4 Binding and Elution Profile of Sample (Antibody..Insulin-Biotin-Avidin-Texas Red) and Blank (Insulin-Biotin-Avidin-Texas Red) to the Protein G-agarose Immunoreactor in FIA System.

For the study of the binding and elution profile of the sample (Antibody..Insulin-Biotin-Avidin-Texas Red) to a protein G-agarose immunoreactor, 50 µl of insulin-biotin-avidin-Texas Red was added to 20 µl of antibody (1:25) and 155 µl of PBS buffer pH 7.4. The mixture was incubated for two minutes. A blank was prepared by mixing 50 µl of insulin-biotin-avidin-Texas Red with 175 µl of PBS buffer pH 7.4. The mixtures were injected onto the protein G-agarose immunoreactor using the binding buffer, tris pH 8.8. When the fluorescence from the non-bound peak at 620 nm returned to the baseline the flow through the immunoreactor was switched to citrate buffer, with Triton, in order to elute the bound fraction and then switched back to tris buffer to re-equilibrate the column for the next sample.
Fig. 6.2 Fluorescence intensity Vs time of avidin-allophycocyanin in continuous light source.

$\lambda_{ex}=650 \text{ nm, } \lambda_{em}=660 \text{ nm, Slit}=5/10 \text{ nm}$
Results and Discussion:

Fig. 6.3 shows the binding and elution profile of the sample and blank. The absence of an elution peak for the blank indicates negligible non-specific binding of the insulin-biotin-avidin-Texas Red complex onto the protein G-agarose immunoreactor. In the case of the sample the elution peak is represented by the binding of antibody..insulin-biotin-avidin-Texas Red complex onto the protein G-agarose immunoreactor. The non binding peak comprises the excess insulin-biotin-avidin-Texas Red complex in the flow injection system.

6.5 Concentration of Antibody (Ab):

The antibody concentration was varied while keeping the insulin-biotin-avidin-Texas Red conjugate concentration constant. 50 μl of insulin-biotin-avidin-Texas Red complex was incubated with increasing concentration of antibody and injected onto the protein G-agarose immunoreactor. The fluorescence intensity of the eluted peak was measured and plotted against the antibody concentration (dilution).

Results and Discussion:

20 μl of antibody dilution 1:25 was chosen as it gave sufficient sensitivity for use in the construction of a calibration curve. From fig. 6.4 it can be seen that sufficient displacement can be achieved at this antibody dilution (1:25).

6.6 Incubation Time:

A series of solution containing 50 μl of insulin-biotin-avidin-Texas Red complex, 20 μl of antibody (1:25) and 155 μl of the PBS buffer, pH 7.4, were prepared (mixed). After incubation times of 0.30, 1.00, 2.00, 3.00, 5, 10, and 15 minutes, the mixture was loaded into the injection loop and injected onto the protein G-agarose immunoreactor using tris buffer pH 8.8. When the fluorescence from the non-bound peak at 620 nm returned to the base line the flow through the
Fig. 6.3 Fluorescence intensity Vs time of sample (Ab...insulin-biotin-avidin-Texas Red) and blank (insulin-biotin-avidin-Texas Red) on protein G-agarose column in FIA system.

$\lambda_{ex}=595\text{nm, } \lambda_{em}=620\text{nm, Slit}=10/15\text{nm}$
Fig. 6.4 Fluorescence intensity Vs antibody dilution using protein G-agarose as column and insulin-biotin-avidin-Texas Red as labelled analyte in FIA system.

\[ \lambda_{\text{ex}}=595\text{nm}, \lambda_{\text{em}}=620\text{nm}, \text{Slit}=10/15\text{nm} \]
protein G column was switched to citrate buffer pH 2.5 in order to elute the bound fraction. Fluorescence intensity was plotted against incubation time in fig. 6.5.

Results and Discussion:

Fig. 6.5 shows the relationship between the fluorescence intensity and the incubation time for the antibody and insulin-biotin-avidin-Texas Red complex. The graph levels off after two minutes and further increases in incubation time do not increase the antibody...insulin-biotin-avidin-Texas Red binding and hence the fluorescence intensity of the bound peak.

6.7 Immunoassay of Insulin:

A calibration curve using the following protocol was established.

An insulin sample or standard between 0.04 and 1.03 ng/ml was added to 20 μl of antibody (1:25), and PBS buffer pH 7.4. The mixture was shaken for one minute, and then 50 μl of insulin-biotin-avidin-Texas Red was added and incubated for two minutes. The mixture was then injected onto the protein G-agarose immunoreactor using the binding buffer, tris pH 8.8. When the fluorescence from the non-bound peak at 620 nm (six minutes) returned to the base-line the flow through the immunoreactor was switched to citrate buffer pH 2.5 (four minutes) in order to elute the bound fraction and then again switched back to the tris buffer to re-equilibrate the immunoreactor (three minutes). The total time from injection to injection was sixteen minutes. The fluorescence intensity of the elution peak was used to calculate the B/B₀% value and was plotted against the concentration of the insulin.

Results and Discussion:

The calibration curve, shown in fig. 6.6, for insulin is applicable between 0.04 and 0.12 ng/ml for 20 μl of antibody (1:25). It was constructed by plotting B/B₀%
Fig. 6.5 Fluorescence intensity Vs incubation time of insulin-biotin-avidin-Texas Red and antibody on protein G-agarose column in FIA system.

$\lambda_{ex}=595\text{nm}, \lambda_{em}=620\text{nm}, \text{Slit}=10/15\text{nm}$
Fig. 6.6 Calibration curve of insulin in standard solutions using protein G-agarose as column and insulin-biotin avidin-Texas Red as labelled analyte in FIA system.

$\lambda_{ex}=595\text{nm}, \lambda_{em}=620\text{nm}, \text{Slit}=10/15\text{nm}$
versus the insulin concentration in the standards. Where

\[
\frac{B}{B_0} \times 100 = \frac{\text{F.I. of the bound peak at stated insulin conc.}}{\text{F.I. of the bound peak at zero insulin conc.}}
\]

As shown in figure 6.6, a 20% displacement of the insulin-biotin-avidin-Texas Red was achieved in this assay and covers the lower end of the therapeutic range of insulin, i.e. from 0.21 to 4.107 ng/ml.

6.8 Insulin Assay in Serum Sample:

A human serum sample containing bovine insulin was analysed by the flow injection immunoassay. The human serum was diluted one hundred fold with PBS pH 7.4 and standard insulin samples were prepared in this buffer. The procedure for the flow injection immunoassay of insulin was the same as described in section 6.7 of this chapter.

Results and Discussion:

The calibration curve for insulin in serum between 0.04 and 0.2 ng/ml is shown in fig. 6.7. A sample containing the 0.1231 ng/ml of insulin was prepared by adding known amounts of insulin to the blank serum. The mean recovery was 117.62%.
Fig. 6.7 Calibration curve of insulin in serum using protein G-agarose as column and insulin-biotin-avidin-Texas Red as labelled analyte in FIA system.

$\lambda_{ex}=595\text{nm, } \lambda_{em}=620\text{nm, } Slit=10/15\text{nm}$
CHAPTER SEVEN

CONCLUSION
7.1 Conclusion:

Radioimmunoassays have been widely used in medicine and biology and have unique specificity and sensitivity characteristics. However, interest has now been focused on non-isotopic immunoassay methods using enzyme, fluorescence or chemiluminescence labels. The reasons for this change are environmental, legal, economic and practical: the limited shelf life of isotopically labelled reagents, the problems of radioactive waste disposal, the cost and complexity of radioisotope counting equipment, and the demand for simple diagnostic kits for home use. Non-isotopic methods address all these problems, and therefore are increasingly replacing radioimmunoassays.

Immunoassay methods can be heterogeneous (separative) or homogeneous (non-separative). The heterogeneous methods are slower compared to homogeneous ones as they require an extra step for separation of antibody-bound from unbound molecules. But this separation has the advantage of minimising the background signal of the biological compounds in the immunoassays. Possible separation steps include fractional precipitation by ammonium sulphate or polyethylene glycol, precipitating the bound fraction with a second antibody (second antibody method), binding of the labelled antibody or antigen to solid phases such as paper strip or plastic strips, magnetic beads, microtitre plates, tubes, etc. All these separation steps, along with multiple pipetting and prolonged equilibration periods, make it difficult to automate heterogeneous immunoassays. Therefore, the automation of heterogeneous immunoassay methods using an immunoreactor in a flow injection system would be potentially advantageous if it could be done successfully. A review of heterogeneous immunoassay methods using a solid phase immunoreactor in a flow injection system is in section 1.7.
The investigation of a reusable immunoreactor in a flow injection immunoassay was further investigated in this project. The following aims of the project were established.

1) To run a simple system of flow injection in order to understand this technique.

2) To synthesise and characterise different fluorophore labelled analytes, such as insulin.

3) To investigate the use of a number of immunoreactors in the flow injection technique for the immunoassay of insulin.

In the first part of this project, the work concentrated on running a simple flow injection system with a fluorescence detector in order to understand this technique (chapter three). Amino acid solutions, such as DL-lysine and L-serine, were separately injected into a flowing stream of reagents, i.e. ortho-phthaldehyde and a thiol. The addition of cyclodextrin into the fluorogenic reagent as host to the isoindole (guest) formed by the reaction allowed the investigation of fluorescence enhancement. In this flow injection system, kinetic and association constants (tables 3.3 & 3.6) of these host-guest interactions were determined to understand this phenomenon. Fluorescence enhancement of isoindoles from many amino acids, such as glycine, aspartic acid, glutamic acid, tyrosine and alanine, was not observed. It seemed that these isoindoles did not fit properly inside the cavities of cyclodextrins. Therefore, and rather unusually, no general fluorescent enhancement phenomenon could be observed for OPA-amino acids.

Insulin was labelled with rhodamine isothiocyanate (insulin-RITC), Texas Red via biotin-avidin (insulin-biotin-avidin-Texas Red) and allophycocyanin also via biotin-avidin (insulin-biotin-avidin-allophycocyanin). The rhodamine isothiocyanate (RITC)
was reacted directly with insulin, and insulin-RITC conjugates were separated on columns containing Sephadex G-25. The complex conjugates, insulin-biotin-avidin-Texas Red and insulin-biotin-avidin-allophycocyanin, were synthesised separately in two steps. In the first step NHS-LC-biotin was reacted with insulin and separated. In the second step the insulin-biotin conjugate was reacted separately with avidin-Texas Red or avidin-allophycocyanin at room temperature. These conjugates were used as labelled analytes in the flow injection immunoassay of insulin using protein G-agarose as solid phase immunoreactor (chapter six). The insulin-RITC was used along with solid phase immunoreactors such as streptavidin-agarose and avidin D-agarose-biotin-antibody, (chapter four) and protein G-agarose (chapter five) in the flow injection immunoassay of insulin.

The first attempt was to use streptavidin-agarose as the solid phase immunoreactor in the flow injection immunoassay of insulin (chapter four). Streptavidin was chosen over avidin as the former has no carbohydrate and also lower pI (Ca. 4) than the latter (pI= 10). Hence the streptavidin exhibits less non-specific binding than avidin. The objective was to synthesise biotinylated antibodies and used them to bind reversibly to a streptavidin-agarose column, thus performing a competitive immunoassay of insulin using insulin-RITC as the labelled analyte. But the streptavidin-biotin binding was too strong ($K_d = 4 \times 10^{-14} \text{M}$). This made the task of regeneration of the streptavidin-agarose column impossible. Therefore, it was decided to use iminobiotin instead of biotin to biotinylate the antibodies. Iminobiotin has a weaker interaction with streptavidin than biotin, and its dissociation occurs at pH 4.0 (1-4). However, as shown in sections 4.6 & 4.7, the dissociation was not 100%. Some of the iminobiotinylated antibodies were left on the immunoreactor and thus progressively reduced its capacity and produced highly non-specific binding effect in consecutive assays. This made the task of developing a flow injection immunoassay for insulin using the streptavidin-agarose immunoreactor impossible. This led to the preparation
of a streptavidin/avidin-biotin-antibody matrix as a reusable immunoreactor and insulin-RITC conjugate as a labelled analyte in the flow injection competitive immunoassay of insulin. The streptavidin-agarose was replaced by avidin D-agarose purely for reasons of cost. Avidin D is a nonglycosylated avidin and has less nonspecific binding than avidin.

The development of an immunoassay for insulin using an avidin D-agarose-biotin-antibody solid phase immunoreactor, and insulin-RITC as labelled analyte in the flow injection system was very successful (section 4.14 & fig. 4.9). But the system failed with real serum samples (section 4.18). This may be due to non-specific binding of IgGs and other serum proteins to the avidin D-agarose-biotin-antibody solid phase immunoreactor. As the therapeutic range of insulin is between 0.21 and 4.167 ng/ml and the assay range developed by this method was 1 and 10 pg/ml, it seemed that the non-specific binding was leaving few binding sites for insulin on the immunoreactor.

The other problem with the avidin D-agarose-biotin-antibody solid phase immunoreactor was its reproducibility. The reproducibility of the immunoreactor within the day was not bad, i.e. coefficient of variation (CV) for 10 runs was 10.66%, but the between day variation was very poor, i.e. for 11 runs 61.75%. Similarly, the reproducibility of the calibration curve on the same avidin D-agarose-biotin-antibody solid phase immunoreactor on different days was very poor (fig. 4.10). It appears that each column really works well for only one day and then the antibody in the immunoreactor (avidin D-agarose-biotin-antibody) loses its ability to bind to antigen. It is possible that the repeated cycling of the immunoreactor between pH 8.8 (binding buffer) and 2.5 (elution buffer) damages its binding activity.

The reproducibility of the standard curves on different avidin D-agarose-biotin-antibody immunoreactor is shown in fig. 4.11. Although, batch to batch variations are significant the assay range is the same, i.e. 1 to 10 pg/ml. Batch to batch variations can
arise in synthesis of separate batches of biotin-antibody and avidin D-agarose-biotin-antibody as well as errors in aliquoting the small amounts of liquids and in day to day running of the instrument.

Other solid phase immunoreactors, tried for the flow injection immunoassay of insulin, were protein A-controlled pore glass (CPG) and protein G-agarose. The absence of an elution peak in the case of protein A-CPG (section 5.4 and 5.2) led to its abandonment. The reason was the excessively strong binding of the guinea pig anti-insulin antibody to the protein A-CPG (5,6) immunoreactor. Some researchers have used a protein A-CPG immunoreactor in flow injection immunoassays using sheep (7) antisera and mouse monoclonal antibodies (8). These IgGs have weaker binding to protein A-CPG (5,6), and hence are easily dissociated. In contrast, protein G-agarose was successfully applied to the flow injection immunoassay of insulin. Protein G has weaker binding with guinea pig antibodies of insulin (5,6). Therefore, insulin-RITC and insulin-biotin-avidin-Texas Red were both used successfully in immunoassays for insulin using protein G-agarose, as described in Chapters five and six respectively. The use of insulin-biotin-avidin-allophycocyanin as labelled analyte failed, perhaps because of the very large allophycocyanin fluorophore, which may block the antibody access to the insulin.

Chapter five shows that the use of a protein G-agarose immunoreactor was quite successful. Its reproducibility was quite good. The coefficient of variation within the day for 14 runs was 3.77% and the between day variation for 22 runs was 4.10%. The reproducibility of the standard curves on different columns (section 5.9.7) and on the same column (section 5.9.6) was also quite good.

The immunoassay of insulin using a protein G-agarose immunoreactor and insulin-RITC as labelled analyte in the flow injection system works very well in buffers
(section 5.9.5) and in serum (section 5.9.8). The assay range developed was 0.05 to 2.24 ng/ml of insulin. The percentage mean recovery of the insulin in the serum was 120.08%.

The use of insulin-biotin-avidin-Texas Red as the labelled analyte and the protein G-agarose immunoreactor was also successfully applied to the flow injection immunoassay of insulin (chapter six). The assay range of insulin was 0.041 - 0.21 ng/ml. The percentage recovery of the insulin was 117.62%.

The use of insulin-biotin-avidin-Texas Red as labelled analyte was another approach to label the analytes in the immunoassays. This approach has a lot of analytical potential. Firstly a single fluorophore could be used for almost every analyte, for example, Texas Red in avidin-Texas Red. By synthesising different biotinylated analytes, one can then combine each of them separately to avidin-Texas Red and thus prepare fluorophore label analytes. Therefore, using the same excitation and emission wavelengths, the flow injection immunoassay of different analytes could be carried out easily and successfully. This is especially useful in filter fluorimeters where single excitation and emission filters could be used for all the analysis of the analytes. A variety of activated biotins are commercially available for all sorts of functional groups such as -NH₂, -OH, -COOH, -COR, etc. Therefore, biotinylation of analytes is not a problem and can be carried out quickly and efficiently. Secondly, by buying or synthesising the bulk quantities of the avidin-fluorophore, activated biotins, and the use of a cheap fluorimeter such as a filter fluorimeter, one can cut down the price of the analysis. Thirdly, fluorescent labelling of avidin, a protein, and its subsequent purification on a Sephadex G-25 column, is much easier than the fluorescent labelling of many small molecules.

The table 7.1 summarises the solid phase immunoreactors used in this project, and their failure or success in the flow injection immunoassay of insulin.
Table: 7.1 A comparison of different solid phase immunoreactors used in this project.

<table>
<thead>
<tr>
<th>Solid Phase Immunoreactor</th>
<th>Failure (F) or Successful (S)</th>
<th>Assay Range</th>
<th>Time of Assay (min)*</th>
<th>Reproducibility</th>
<th>% Recovery of insulin</th>
<th>Reference Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin-agarose</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Avidin</td>
<td>S</td>
<td>0.05-2.24</td>
<td>13</td>
<td>Good</td>
<td>120.08</td>
<td>5</td>
</tr>
<tr>
<td>Biotin-Avidin-TR</td>
<td>S</td>
<td>0.04-0.12</td>
<td>13</td>
<td>-</td>
<td>117.62</td>
<td>6</td>
</tr>
<tr>
<td>Protein A-CPG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein G-agarose with Labelled Analyte</td>
<td>F</td>
<td>1.1-10.6 pg/ml</td>
<td>15</td>
<td>Poor</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>1)Insulin-RITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2)Insulin-Biotin-Avidin-TR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Excluding the re-equilibration time.

Table 7.2 shows a few enzyme immunoassay methods reported in the literature for the determination of insulin. All the methods cover the therapeutic range of insulin 5-100 μu/ml. The difference between these methods and the flow injection methods developed in this project are the speed and the cost of the analysis. The methods reported in table 7.2 take hours to complete. The cost of the analysis is also high as
solid phase, and other materials are consumed for every sample. These disadvantages are lessened in the flow injection immunoassay of insulin reported in this project. As the table 7.1 shows that the time taken for each sample assay in the flow injection immunoassay using an avidin D-agarose-biotin-antibody reactor is ca. 15 minutes and for protein G-agarose ca. 13 minutes. Flow injection immunoassay methods are thus much faster than these earlier methods. Also, the flow injection methods employ reusable immunoreactors, thus reducing the unit cost of the assay.

Table: 7.2 Some immunoassay methods for insulin.

<table>
<thead>
<tr>
<th>Name of the Method</th>
<th>Assay Range</th>
<th>Mode of Detection</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandwich Enzyme Immunoassay</td>
<td>0-250 μu/ml</td>
<td>Absorbance</td>
<td>9</td>
</tr>
<tr>
<td>Two-Site Enzyme Immunoassay</td>
<td>2-200 μu/ml</td>
<td>Absorbance</td>
<td>10</td>
</tr>
<tr>
<td>Sandwich Enzyme Immunoassay</td>
<td>6.00μu/ml-</td>
<td>Fluorescence</td>
<td>11</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0.6 μu/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme Immunoassay</td>
<td>2.5-160 μu/ml</td>
<td>Fluorescence</td>
<td>12</td>
</tr>
<tr>
<td>Enzyme Immunoassay</td>
<td>0-400 μu/ml</td>
<td>Fluorescence</td>
<td>13</td>
</tr>
<tr>
<td>Enzyme Immunoassay</td>
<td>7.5x10^{-17}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoassay</td>
<td>150x10^{-17} mole per assay tube</td>
<td>Chemiluminescence</td>
<td>14</td>
</tr>
</tbody>
</table>
The literature methods operate over the therapeutic range of insulin. The flow injection immunoassay methods using solid phase immunoreactor-protein G-agarose, developed in this project cover the lower end of the therapeutic range of insulin, hence allowing dilution of the samples and having similar performance characteristics. All determined insulin with varying degrees of success.

7.2 Recommendations for Future Work:

The present work demonstrated the use of a flow injection immunoassay using solid phase immunoreactors and fluorescence detection. As the objectives of the project have been successfully demonstrated, further work has to be carried out to improve performance, e.g. shortened assay times and better percentage recoveries.

The time of analysis for a single sample using flow injection immunoassay with protein G-agarose immunoreactor developed in this project was 13 min. This may be shortened significantly by increasing the flow rate. Work should be carried out to see the effect of flow rate on sensitivity, perhaps leading to shortened assay times. Also, the use of perfusion chromatography, i.e. columns with polymeric beads of large surface area, could be incorporated into the flow injection system. This will increase the flow rate and may thus shorten the assay time to couple of minutes (15). Further, using a number of immunoreactors in parallel could shorten the assay time, if practical problems can be overcome. If all these factors are explored, a throughput of hundreds of samples per day may be possible.

The reproducibility of the solid phase immunoreactors such as avidin D-agarose-biotin-antibody and protein G-agarose can be increased if the immunoreactors are prepared in a single batch and packed under identical conditions. Columns prepared in this way would be expected to have similar lifetimes and binding characteristics.
The immunoreactors used in this study were 50 mm in length and 3 mm internal diameter. A shorter column could be used as all the binding sites on the column were not used in the flow injection immunoassay. For example, the binding capacity of the protein G-agarose immunoreactor was ca. 7 mg of antibody and the amounts of antibody injected 0.02-0.14 mg. Therefore, a shorter column will not only be economically beneficial, but it may give a better reproducibility, while retaining the essential feature of having excess antibody binding capacity.

Flow injection immunoassay systems using solid phase can be fully automated. Manually operated injection and switching valves could be replaced by computer controlled valves. Sampling, addition of marker analyte, washing, monitoring, evaluation, dissociation, reconditioning, etc. all can be controlled by a simple personal computer. Nilsson et al (16) have already described such a flow injection system using a protein A-Sepharose immunoreactor and a UV detector for the determination of rabbit IgG: this could act as a model for future work.
7.3 References:

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