Sensing of theophylline by enzyme immunoassay coupled with electrochemical detection

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SENSING OF THEOPHYLLINE BY ENZYME IMMUNOASSAY COUPLED WITH ELECTROCHEMICAL DETECTION.

By

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B.Sc., M.Sc., C. Chem., M.R.S.C.

A Doctoral Thesis Submitted in partial fulfilment of the requirements for the award of

DOCTOR OF PHILOSOPHY

Of

Loughborough University of Technology

1991

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Loughborough University of Technology

CERTIFICATE OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own except as specified in acknowledgements or in footnotes, and that neither the thesis nor the original work contained therein has been submitted to this or any other institution for a higher degree.

........................... (Signed)

........................... (Date)
To my parents
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. T. E. Edmonds, Dr. N. J. Seare and Professor J. N. Miller for their help and encouragement during the course of this project, and to the technical staff of the Chemistry Department for their cooperation and advice, especially John Swithenbank.

I would like to thank the Science and Engineering Research Council for provision of the research grant.

Finally, I would like to thank Rashmi and Ravinder Hindocha for the time and effort put into the typing of this thesis, Inutu Mutti for the encouragement and support, my colleagues in the department; Martin French, Mohammed Khokar, Simon Cocks, Marc Brown, David Riley and Steve Summerfield for their help and support and my parents especially my mother for her constant encouragement.
AIM OF THE PROJECT

Theophylline is a member of the xanthine family of drugs, which are central nervous system stimulants. The main action of theophylline is to alleviate the symptoms of chronic asthma. The therapeutic range for theophylline is generally between 10-20μg ml⁻¹. Toxic side effects are commonly encountered with theophylline levels greater than 40μg ml⁻¹. The metabolism of theophylline varies between individuals and hence the use of the drug as a therapeutic agent necessitates close monitoring.

The aim of the research is to develop a rapid flow injection electrochemical enzyme immunoassay for theophylline.

The research will be carried out in four phases:

a. Voltammetric analysis of alkaline phosphatase substrates and their hydrolysis products.


c. Investigations into the immobilisation of anti-theophylline IgG on controlled pore glass.

d. Incorporation of parts a-c into a flow injection electrochemical enzyme immunoassay for theophylline.
ABSTRACT.

The voltammetry of several substrates and their hydrolysis products for the enzyme alkaline phosphatase (E.C. 3.1.3.1.) were investigated and theophylline-alkaline phosphatase (TH-AP) conjugates were synthesized in order to develop a flow injection electrochemical enzyme immunoassay (FIEEIA) for the antiasthmatic therapeutic drug theophylline.

Cyclic voltammetry was used to determine the oxidation potentials for the substrates and their hydrolysis products and to investigate electrode poisoning by the hydrolysis products. The p-aminophenyl phosphate (PAPP) and p-aminophenol (PAP) system was found to be best suited for incorporation into a FIEEIA.

The PAPP/PAP system was optimised for use in an amperometric flow injection system. This included (i) construction of hydrodynamic voltammograms for PAPP and PAP; (ii) investigations of the stability of PAP in different buffer solutions at various pH values and (iii) determination of the limits of detection for PAP using gold, platinum and glassy carbon electrodes.

TH-AP conjugates were synthesized by the mixed anhydride and carbodiimide methods using theophylline-7-acetic acid and by a non-activation procedure using theophylline-8-butyric acid lactam. The conjugates were purified and characterized by determining (i) the residual enzymatic activity; (ii) the degree of conjugation of theophylline; (iii) the immunoreactivity; (iv) the enzymatic and immunoreactive stability over a three month period and (v) the kinetic parameters of the native enzyme and the theophylline-enzyme conjugate.

Preliminary investigations into the immobilisation of theophylline antisera and anti-theophylline antisera on controlled pore glass were performed by the aminopropyltriethoxysilane immobilisation procedure using dimethylpilimidate (DMP) and the carbonylimidazole immobilisation procedure. The loading and activity of the immobilised antibodies by the above methods were determined and
were found to be unsatisfactory for use in a flow injection immunoassay.

On-line and off-line FIEELA were developed for the determination of serum theophylline levels using reversibly immobilised theophylline antisera on controlled pore glass protein A in a microreactor.

Studies into the development of strip sensors were performed. The studies included immobilising antibodies on six different types of membranes and assessing the effect of different blocking agents on the specific protein binding to the membrane with the best antibody uptake characteristics.

Competitive and displacement enzyme immunoassays were developed for a model system.
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<td>αNP</td>
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</tr>
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<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ab*</td>
<td>Labelled Antibody</td>
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<td>Ag</td>
<td>Antigen</td>
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GOD  Glucose Oxidase
HCG  Human Chorionic Gonadotropin
HPIC High Performance Immunoaffinity Chromatography
HSA  Human Serum Albumin
IV   Injection Valve
LC   Liquid Chromatography
LC-ECD Liquid Chromatography with Electrochemical Detection
MBSE m-Maleimidobenzoic Acid N-Hydroxysuccinimide Ester
MPGS N-(3-Maleimidopropionylglycylolyl) Succinimide
NAD  Nicotinamide Adenine Dinucleotide
NADH Reduced Nicotinamide Adenine Dinucleotide
NP   Normal Phase
NSB  Non Specific Binding
PACIA Particle Counting Immunoassay
PAP  p-Aminophenol
PAPP p-Aminophenyl Phosphate
PBS  Phosphate Buffered Saline
PCTFE Polychlorotetrafluoroethylene
PDP  Phenolphthalein Diphosphate
PETINA Latex Patricle-Enhanced-Turbidimetric Inhibition Immunoassay
PNP  p-Nitrophenol
PNPP p-Nitrophenyl Phosphate
PP   Phenyl Phosphate
PTFE Polytetrafluoroethylene
PVC  Polyvinyl Chloride
RIA  Radioimmunoassay
RP   Reversed Phase
SPA  Staphylococcal Protein A
TLC  Thin Layer Chromatography
TPA⁺ Tripentylammonium ion
TMPA⁺ Trimethylphenylammonium ion
PUBLICATIONS.

1. Flow Injection Immunoassays Using a Protein A Immunoreactor.
   J.N. Miller, D.A. Palmer and M.T. French.
   Conference Proceedings Journal of Pharmaceutical and Biomedical
   Analysis (submitted).

2. Electrochemical Flow Injection Immunoassay for Theophylline Using a
   Protein A Immunoreactor.
   Analytical Proceedings (submitted).
PRESENTATION OF WORK.

1. November 1989; Link programme on molecular sensors sponsored by the Department of Trade and Industry (DTI), Science and Engineering Research Council (SERC) and the Agricultural and Food Research Council (AFRC). The main objective of the programme was to promote the transfer of technology from the U.K. science base to U.K. industry in the area of chemical and biochemical sensing.

Heathrow Park Hotel, London. (*Poster Presentation*).

2. November 1989; Young scientist meeting, Royal Society of Chemistry (RSC).

Liverpool Polytechnic. (*Lecture*).

3. July 1990; R & D topics in Analytical Chemistry, Royal Society of Chemistry (RSC).

ICI, Runcorn. (*Poster presentation*).

4. August 1990; Euroanalysis VII

Vienna, Austria. (*Poster presentation*).

5. April 1991; Third International Symposium on Pharmaceutical and Biomedical Analysis.

Boston, Massachusetts, USA. (*Poster presentation*).


University of Aberdeen. (*Lecture*).
1.1 Drug Therapy

1.1.1 Serum Drug Concentration as Therapeutic Guides.

Dosages of any individual drug needed for optimal therapeutic effects may differ widely among patients. The "usual dose" of many potent drugs accomplishes little in some persons, causes serious toxicity in others, and is fully satisfactory in few. The inability of standard dosage schedules to exert a sufficiently intense pharmacologic effect in many patients is often misinterpreted; and drug toxicity often arises through a failure to reduce the usual dosage appropriately in other patients. More than half these reactions are dose-related - that is, they result from dosages that are too high for the patient affected\(^1\). The habitual administration of the conventional dose can be satisfied only when a drug's therapeutic margin is very large and when its full therapeutic potential is not required. Therapy with potent drugs becomes safer and more effective when the dosage is adjusted to the need and tolerance of each patient.

How can one determine the optimal dose of a drug for each patient? Dosage adjustments are easy when the intensity of a drug's pharmacologic effects can be quickly and accurately gauged during its clinical use. For example dosage requirements for guanithdine or warfarin can vary by a factor of 50 from one patient to another. Nevertheless, these drugs are useful therapeutic agents, because their optimal individual dosage is readily determined by measuring the upright arterial pressure or the prothrombin time. Unfortunately, such simple and exact indices of the intensity of therapeutic activity are the exception; and the dosage of most drugs cannot be estimated in this fashion.

With many drugs, ascertainment of the best dosage for individual patients is difficult because the pharmacologic response is not quantifiable in the usual clinical situation. The physician may not even know whether the prescribed dosage is producing the expected therapeutic benefit, particularly when the
drug is given for prophylactic purposes. The practice of increasing drug dosage until mild toxic manifestations appear, and then decreasing it slightly, is feasible only for some drugs; and can be uncomfortable, if not dangerous, to the patient. In this quandary determinations of drug concentrations in blood plasma and serum have become absolutely vital.

1.1.2 Relationship of Dosage, Serum Concentration, and Effect.
The enormous individual differences in the relation between the dosage of a drug and the intensity of its pharmacologic action are not surprising when one considers the many factors that may influence the biological effects of an orally administered drug (Fig 1.1). It is not generally appreciated that the variability of the dose-effect relation among patients is very commonly due to individual differences in the serum concentration achieved with a given dosage schedule rather than to a different intensity of action associated with the same serum concentration. For many drugs the relationship between the amount administered and the resulting concentration in the body is quite unpredictable. In contrast, the intensity of the pharmacologic action generally correlates rather well with the concentration of the free drug in the serum. Changes in the serum concentration of most drugs are probably accompanied by similar changes in their concentration at the site of action and in the number of drug-receptor complexes. This is true only for drugs that act reversibly - i.e., whose action does not outlast their presence at the receptor site.

Why is the relation between the dosage of a drug and its concentration in the serum so variable? It is influenced by the bioavailability of the dosage form used, the co-administration of other substances (food, drugs etc.), by host factors that affect the completeness of gastro-intestinal absorption, by body size and composition, by distribution through fluid compartments, by binding to inactive sites, and by rates of metabolism and excretion. All these determinants are subject to much individual variation. There are large genetically controlled differences in the rate of bio-transformation of many
drugs. Superimposed on these are differences due to induction or inhibition of drug metabolizing enzyme systems by environmental factors and by administration of other drugs.

Individual and temporal differences may also exist however in the processes that determine the relationship between serum drug level, concentration at the site of drug action, and intensity of action (Fig 1.1). Thus, serum concentrations of drugs are not perfect indices of the degree of pharmacological response. Knowing the serum concentration of a drug, one cannot necessarily predict exactly how intense the therapeutic or toxic action will be in individual patients. However, a more accurate prediction can usually be made from the serum level than from the dosage. For this reason dosage adjustments can often be guided by knowledge of serum levels.

There has been particular concern about the value of serum level determinations of reversibly acting drugs whose ratios of serum to tissue concentration are very low because they are extensively bound in tissues. This concern appears to be unjustified as long as the serum concentration of unbound drug can be reliably measured. The serum concentration of such drugs remain in dynamic equilibrium with their concentration at pharmacologically inactive tissue sites and at their receptors. Some individual and temporal variations in the ratio of serum to tissue concentration do occur, but these are usually not large enough to invalidate the meaning of serum concentrations. This has been well demonstrated with digoxin, where the ratio of myocardial to serum concentration is 40 to 50 in most persons and does not vary greatly with time, over a wide range of serum levels. Regardless of the degree of tissue binding, serum concentrations of most drugs generally serve as a useful idea of the degree of receptor occupancy. In fact, drug concentration at the receptors may correlate better with the concentration in the tissue on which the drug exerts its action because a large proportion of tissue binding is often to "non-receptor" sites.
Fig 1.1: Dose-Effect Relation of Drugs in Man and the Factors the Influence it.
Even when the ratio of the serum concentration of a drug to its concentration at the site of action varies appreciably among individual patients and with time, serum levels usually remains a better index of drug concentration at the receptor site than dosage.

Measurements of serum levels of a drug become useful guides for dosage adjustments only when the therapeutically effective range of serum concentration has been defined by careful clinical studies. At present this has been accomplished for quite a few important drugs (Table 1.1). Compared to the large individual variations in the optimal dosages of these drugs, the width of their therapeutic serum concentration ranges is relatively narrow. Concentrations of these drugs below the accepted therapeutic range may exert beneficial effects in a few subjects but are likely to be inadequate in most patients. Within the therapeutic range the intensity of the desired effect of the drug increases with its serum concentration.

Occasionally patient concentrations in the upper therapeutic range may have toxic actions. As serum concentrations rise above the therapeutic range, the frequency and severity of toxic effects increase progressively. Some patients can tolerate serum concentrations considerably above the usual therapeutic levels, and a few may even require them for a fully satisfactory response. The overlap between therapeutic and toxic serum concentrations is considerable for certain drugs, such as the digitalis glycosides. Whenever concentrations above the usual therapeutic ranges are produced for therapeutic purposes, patients must be closely monitored against the appearance of serious toxicity.
Table 1.1 Ranges of Serum Concentrations of Various Drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Usual Range of Therapeutic Serum Concentrations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitoxin</td>
<td>14-30 ug l⁻¹</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.9-2 ug l⁻¹</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>10-20 mg l⁻¹</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1-10 mg l⁻¹</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>1.5-4 mg l⁻¹</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.5-1.3 mEq l⁻¹</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>50-140 ug l⁻¹</td>
</tr>
<tr>
<td>Procainamide</td>
<td>4-8 mg l⁻¹</td>
</tr>
<tr>
<td>Propanolol</td>
<td>20-50 ug l⁻¹</td>
</tr>
<tr>
<td>Quinidine</td>
<td>2-5 mg l⁻¹</td>
</tr>
<tr>
<td>Salicylates</td>
<td>150-300 mg l⁻¹</td>
</tr>
<tr>
<td>Theophylline</td>
<td>10-20 ug l⁻¹</td>
</tr>
<tr>
<td>Warfarin</td>
<td>1-10 ug l⁻¹</td>
</tr>
</tbody>
</table>
1.1.3 Clinical Usefulness of Determining Serum Drug Concentrations.

During the last decade the value of clinical practice of determination of serum concentrations has been convincingly demonstrated for several important drugs. Such assays are generally unnecessary during treatment with drugs of limited effectiveness and potency, and in patients who respond well to the usual dosage regime of any drug. They are superfluous for drugs whose intensity of action can be judged accurately from their effects on the patient and whose dosage can be adjusted on that basis. Nevertheless, a broad area of clinical usefulness remains. Measurement of serum concentrations generally clarifies the picture when usual doses of a drug fail to produce therapeutic benefits or results in unexpected toxicity. It is particularly helpful in patients with gastrointestinal, cardiovascular, hepatic, or renal disease, in whom relation between dosage and plasma concentration may be grossly abnormal. It is also useful when many drugs are being administered together and may be altering each other's metabolic fate. By monitoring serum concentrations of potent drugs with narrow margins of safety, we are able to individualize dosages and increase the efficacy and safety of pharmacotherapy. The basic pharmacological concepts of drugs have been extensively discussed7,8,9.

1.1.4 Methods of Measurement.

During the last two decades serum concentrations of many drugs have been measured during their therapeutic use. This has become possible through the development of methods of analysis that permit determination of very low concentrations. Methods for drug measurements have been extensively reviewed7,10,11,12.
1.2 Therapeutic Use and General Consideration of Theophylline

1.2.1 Chemistry and Physical Properties of Theophylline.

Structure:

![Structure of Theophylline]

Other names: 3,7 dihydro 1,3-dimethyl-1Hpurine-2,6 dione; 1,3 dimethylxanthine; theocin; armophylline.

Molecular weight (M.Wt) of theophylline : 180.2

Chemical composition: C46.66% H 4.48% N 31.10% O 17.76%

Description : White anhydrous, crystalline powder

Solubility: One gram dissolves in 120ml of water, 80ml of alcohol, about 110ml of chloroform, soluble in hot water, alkali hydroxides, ammonia, dilute hydrochloric acid or nitric acid, and sparingly soluble in ether.
1.2.2 Clinical use.

The main action of theophylline is to relax bronchospasm, it may thus be used to prevent or alleviate asthma and acute exacerbation of obstructive airways disease. In adults, theophylline may be a useful adjunct in the treatment of acute pulmonary oedema and congestive cardiac failure and as a respiratory stimulant to treat cheyne-stokes respiration. The third major use of the drug is to reduce recurrent neonatal apnoea.

Clinical studies have shown that the therapeutic control of bronchospasm is related to the maintenance of adequate serum theophylline concentration\textsuperscript{13,14}. This can only be achieved reliably by measuring the serum drug concentration either routinely during chronic therapy or during acute intravenous infusion\textsuperscript{15}.

1.2.3 Metabolism and therapeutic concentration.

Theophylline is metabolised in the liver mainly by N-demethylation and 8-hydroxylation to methylxanthines and methylurates (Fig 1.2). In children there is a close relationship between plasma profiles of the main metabolites, with peak 3-methylxanthines concentrations occurring rapidly as a result of N-demethylation. Conversion of theophylline to caffeine (1,3,7 trimethylxanthine) occurs in neonates and children and in adults with hepatic failure, but not to a significant extent in normal adults\textsuperscript{16,17}. Approximately 10% of theophylline is excreted unchanged in the urine.

The rate of metabolism of theophylline may be altered by factors such as diet, age, smoking habits or administration of other drugs (Table 1.2). For effective control of neonatal apnoea a plasma concentration of between 28-44 \( \mu \text{mol} \text{ l}^{-1} \) (5-8 mg l\(^{-1}\)) is usually necessary. Adequate control of adult asthma is generally achieved with plasma concentrations of 44-110 \( \mu \text{mol} \text{ l}^{-1} \) (8-20mg l\(^{-1}\)). The minimum effective therapeutic range for use in pulmonary oedema and congestive cardiac failure has not been established.
Fig 1.2 Metabolism of Theophylline and Caffeine in Man.
Table 1.2 Factors Associated with Variation in Theophylline Elimination

<table>
<thead>
<tr>
<th>Factors</th>
<th>Age</th>
<th>No. of Patients</th>
<th>Clearance mean ± SD (ml/min/kg)</th>
<th>Half-life mean ± SD (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infants under 12 weeks</td>
<td>12 ± 4 weeks</td>
<td>8</td>
<td>Incomplete data</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>6 months</td>
<td>18 ± 2 weeks</td>
<td>3</td>
<td>0.8 ± 0.1</td>
<td>6.9 ± 1</td>
</tr>
<tr>
<td>6 to 11 months</td>
<td>34 ± 10 weeks</td>
<td>4</td>
<td>2.0 ± 0.5</td>
<td>4.6 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>34 ± 7 weeks</td>
<td>5</td>
<td>Incomplete data</td>
<td>3.7 ± 1</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-12 years</td>
<td>9.4 ± 3 years</td>
<td>17</td>
<td>1.5 ± 0.4</td>
<td>not measured</td>
</tr>
<tr>
<td>6-17 years</td>
<td>10.7 ± 2.6 years</td>
<td>30</td>
<td>1.4 ± 0.6</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>Adults (otherwise healthy, non-smoking asthmatic)</td>
<td>31 ± 10 years</td>
<td>16</td>
<td>0.65 ± 0.19</td>
<td>8.7 ± 2.2</td>
</tr>
<tr>
<td>Healthy (non-smoking volunteers)</td>
<td>22 - 35 years</td>
<td>19</td>
<td>0.86 ± 0.35</td>
<td>8.1 ± 2.4</td>
</tr>
<tr>
<td>Abnormal Physiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever associated with acute viral upper respiratory track illness</td>
<td>9 - 15 (during illness)</td>
<td>6</td>
<td>not measured</td>
<td>7.0 ± 3.0</td>
</tr>
<tr>
<td>Hepatic cirrhosis</td>
<td>52 ± 8.2</td>
<td>9</td>
<td>0.43(0.13-3.3)b</td>
<td>14.1(7.1-59.1)b</td>
</tr>
<tr>
<td>Smoking History</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marijuana alone</td>
<td>20 - 25a</td>
<td>7</td>
<td>1.2 ± 0.5</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>Marijuana and cigarettes</td>
<td>19 - 27a</td>
<td>7</td>
<td>1.5 ± 0.4</td>
<td>4.3 ± 1</td>
</tr>
<tr>
<td>Ex-smokers (for at least 2 years)</td>
<td>22 - 39a</td>
<td>6</td>
<td>0.85 ± 0.2</td>
<td>6.4 ± 1</td>
</tr>
<tr>
<td>Concurrent drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>22 - 32</td>
<td>6</td>
<td>0.75 ± 0.35</td>
<td>not measured</td>
</tr>
<tr>
<td></td>
<td>(before)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(after 4 weeks)</td>
<td>1.0 ± 0.5</td>
<td>not measured</td>
<td></td>
</tr>
<tr>
<td>Aberrant diets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low carbohydrate</td>
<td>22 - 29</td>
<td>6</td>
<td>8.1 ± 2.4</td>
<td>5.2 ± 1</td>
</tr>
<tr>
<td>high protein</td>
<td>(before)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(after 2 weeks)</td>
<td>5.2 ± 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Age range  
b median & range
1.2.4 Pharmacokinetics and Distribution.

Following oral administration, theophylline is rapidly and completely absorbed. Peak plasma levels are usually attained within 2-3 hours. In normal individuals, theophylline is about 40% protein-bound, principally to albumin. Small changes in the degree of protein binding do not, therefore, significantly affect the concentration of free theophylline.

The drug is distributed rapidly into peripheral tissues, except fat, with an average volume of distribution (\( \text{vd} \)) of 0.5 l kg\(^{-1} \) (range 0.3 - 0.7 l kg\(^{-1} \)) at steady state concentrations. The volume of distribution (\( \text{vd} \)) is higher in neonates and in disease states associated with a low plasma albumin concentration (e.g., liver disease).

1.2.5 Mode of Action.

It is still not clear how theophylline exerts its effects. Methylxanthines are potent inhibitors of phosphodiesterases in vitro, and cause increased intracellular concentrations of cyclic AMP. However, it is unlikely that inhibition of phosphodiesterases explains the action of theophylline, as therapeutic concentrations of the drug produce only 10% inhibition of phosphodiesterase in human lung tissue preparations. Furthermore, the phosphodiesterase inhibitor diprymidole has no bronchodilator activity. Other proposed mechanisms of drug action include mediation through intercellular calcium and prostaglandin inhibition or by antagonising the actions of endogenous adenosine. Adenosine has been shown to increase the secretion of histamine from stimulated mast cells.

1.2.6 Toxicity.

The side effects commonly encountered with theophylline and its derivatives irrespective of the route of administration, are gastro-intestinal irritations and
stimulation of the central nervous system.

Theophylline may cause nausea, vomiting, abdominal pain, gastro-intestinal bleeding, insomnia, headache, anxiety, restlessness, vertigo and palpitations. Severe overdosage or idiosyncrasy may also lead to maniacal behaviour, diuresis and repeated vomiting with extreme thirst, tremor, delirium, hyperthermia, tachycardia, electrolyte disturbances, convulsions and death (Table 1.3).

1.2.7 Pharmaceutical Preparation and Dosing.

Theophylline has a half life in adults of 3 - 8 hr, and is rapidly absorbed from solution or plain coated tablets. Short dosing intervals are therefore required to maintain steady-state plasma concentrations. Solutions are also well absorbed rectally although administration through this route results in more variable absorption patterns and is therefore not generally recommended. Intramuscular injections are not used as the alkaline pH of the preparation results in theophylline precipitating at the injection site causing tissue damage and delay in absorption.

Sustained release oral theophylline preparations are, therefore, the accepted method of administering the drug, except for intravenous infusion for the relief of acute bronchial spasm. The usual dosing interval for slow release compounds is usually 8-12 hours and such regimens have shown to result in steady state plasma concentration.

The aim of an effective dosing regimen is to produce a constant plasma concentration of theophylline. Nonetheless, the relationship of plasma concentration to time after dose may still vary significantly in one patient and between individuals. Samples for analysis should, therefore, be taken just before the next dose. The sampling time after single doses should take account of the formulation (for further details refer to 18). In all cases, adequate
laboratory interpretation of an analytical result is possible only if full information is supplied with the serum sample.

Table 1.3 Toxic Side-Effects Associated with Theophylline Treatment.

<table>
<thead>
<tr>
<th>Serum concentration range μmol l⁻¹ (mg l⁻¹)</th>
<th>Symptoms</th>
<th>Frequency</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>28-110 (5-20)</td>
<td>Nausea, cramps, insomnia, headache</td>
<td>Rare - if dose is slowly titrated over 2 weeks</td>
<td>Transient</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Common - if therapeutic serum concentrations are rapidly attained (loading dose)</td>
<td></td>
</tr>
<tr>
<td>Tremors</td>
<td></td>
<td>Rare - with concurrent administration of oral beta-2 antagonists</td>
<td>Unknown</td>
</tr>
<tr>
<td>Excessive gastric</td>
<td></td>
<td>Rare - may produce symptoms in patients with an active gastric ulcer</td>
<td></td>
</tr>
<tr>
<td>83-193 (15-35)</td>
<td>nausea, vomiting, diarrhoea, stomach ache, headaches, irritability, nervousness, insomnia, sinus tachycardia hyperglycaemia</td>
<td>Common - at serum concentrations &gt; 110 μmol l⁻¹ (20mg l⁻¹)</td>
<td>Persistent</td>
</tr>
<tr>
<td>&gt; 193 (&gt;35)</td>
<td>Scizure, which may be intractable and unresponsive to anti-convulsants, cerebral hypoxia, cardiac arrhythmias, cardio-respiratory arrest, death</td>
<td>Common</td>
<td>Persistent</td>
</tr>
</tbody>
</table>
1.3 Measurement of Theophylline.

Since theophylline is not extensively metabolised, but also structurally related to caffeine which is present from dietary sources in many samples, it is important that any theophylline assay is free from interference by caffeine and other metabolites. As caffeine is a pharmacologically active metabolite of theophylline in neonates, an assay procedure able to determine both compounds simultaneously has obvious advantages for these patients. Immunoassay methods offer speed and convenience for routine measurements of theophylline, but may suffer some degree of interference depending on the specificity of the antibody. Chromatographic methods are more labour intensive, but offer the ability to measure metabolites including caffeine. Such assays may be rapid and can be used for an emergency service.

Various methods are available for the measurement of theophylline in body fluids and have been reviewed\textsuperscript{28,29}. These include:

a. Ultraviolet spectrophotometry
b. Enzyme inhibition methods
c. Chromatographic methods
d. Immunoassays.

The first two methods cannot be recommended as reliable routine methods and are outlined below for completeness.

In the method of Jatlow\textsuperscript{30}, modified from that of Schack and Waxler\textsuperscript{31}, theophylline was back-extracted from chloroform/isopropanol into sodium hydroxide solution and the absorbance measured at 275nm. These methods are subject to interferences from other ultraviolet absorbing compounds and use relatively large amounts of sample.
In the enzyme-inhibition method of Vinet and Zizian\textsuperscript{32}, theophylline was back-extracted into sodium hydroxide solution from chloroform/isopropanol. The theophylline inhibited beef liver alkaline phosphatase (p-nitrophenyl phosphate substrate) in 2-amino-2-methyl-1-propanol buffer at pH 9.5 in inverse proportion to drug concentration. The method is technically complex. Foulds \textit{et al}.\textsuperscript{33} have recently used this technique to develop a rapid electrochemical assay for theophylline employing a disposable electrochemical test strip.

\subsection*{1.3.1 Chromatographic Methods.}

\textbf{Sample preparation.}

Sample preparation serves a number of purposes;
\begin{itemize}
  \item a. to isolate the drug
  \item b. to decrease interference in the assay
  \item c. to remove protein and possibly to concentrate the sample.
\end{itemize}

Inadequate or inappropriate sample preparation is the area most likely to contribute to overall assay imprecision. Extraction of the xanthine derivatives from the sample before chromatography offers many advantages, particularly with regard to method specificity. The most widely used techniques have been simple solvent extractions with optimisation of the pH and extracting solvent. Solvent extraction methods produce a less complicated chromatogram, extend analytical column life and enhance sensitivity since they introduce a concentration step in the analytical procedure.

Precipitation with acetonitrile or methanol has been used to remove serum or plasma proteins although some proteins, chiefly albumin, will remain in solution and cause high back pressure and blockages in liquid chromatographic (LC) systems even if zinc sulphite precipitation is used\textsuperscript{34}. Sub-optimal chromatography due to on-column partitioning of solvents may occur if a solvent incompatible with the LC eluant is used for protein precipitation\textsuperscript{35}.  

Ultrafiltration has proved successful for removing protein\textsuperscript{36}, but is time consuming.

Liquid/Liquid extraction is usually performed with a halohydrocarbon-alcohol solvent mixture from a buffered aqueous phase at an acidic pH. Good absolute recoveries may be achieved. Chloroform/isopropanol is commonly used although dichloromethane may be substituted to decrease possible carcinogenicity and to facilitate solvent evaporation; lower boiling point alcohols are also suitable for this reason. Ethyl acetate or diethylether may substitute for chloroform.

In more recent studies, theophylline in blood samples dried onto Guthrie filter paper cards was extracted by shaking for at least 90 min with aqueous sulphosalicylic acid solution. Measurements of the eluted drug was carried out using the Ames TDA fluorescence immunoassay system. Theophylline was shown to be stable in the dried blood spot and there was no effect of changes in haematocrit on the measured drug concentration. The correlation between capillary dried blood spot theophylline and venous plasma theophylline was high\textsuperscript{37}.

Solid phase extraction may be carried out using commercially available materials such as Bond Elut\textsuperscript{TM} and Clin Elut\textsuperscript{TM}. There is little advantage over liquid/liquid extraction except to undertake specialised assay of polar metabolites, such as 3-methylxanthine, which can be efficiently extracted by solid phase, but not by liquid/liquid methods.

Internal standardisation.

Internal standards should compensate reproducibility for analyte losses during sample pretreatment and, therefore, must be added before the addition of the extraction solvent. They are required for methods using protein precipitations and solvent extraction, but are not essential for direct injection methods and
possibly not for plasma ultrafiltration methods provided that binding of the drug to the ultrafiltration membrane is low and reproducible.

It is generally accepted that an internal standard should be chemically similar to the analyte. This need not, however, be the case; the only requirements are that the internal standard should be non ionised in the aqueous phase, efficiently extracted and elute close to the analyte of interest.

A number of theophylline analogues and barbiturates have been used as internal standards in LC and gas chromatography (GC) assays. Tritiated theophylline has been used as an internal standard for GC-mass spectrometry. β-hydroxyethyl-theophylline has been used for reverse phase (RP) and normal phase (NP) systems whiles 3-isobutyl-1-methylxanthine has been used for GC systems.

**Gas Chromatography.**

Gas chromatography has been utilized for the determination of theophylline. In most gas chromatographic procedures theophylline is derivatized to minimize adsorption and thermal degradation.

Theophylline has been determined without derivatisation on columns containing OV-17, and OV-1. However, low sensitivity, poor separation, long retention times and peak tailing are persistent problems and most GC procedures employ derivatisation. In particular, separation of theophylline from caffeine and its metabolite 1,7, dimethylxanthine (1,7-DMX) may be difficult.

Butylation before injection of sample is the usual derivatisation technique, although a column derivatisation has been used with good precision. Alkylolation prior to injection has been reported: ethyl, butyl and pentyl derivatives appear to give acceptable chromatographic performances.
Interferences have not been adequately characterised in many GC procedures. In particular, 1,7-DMX can co-chromatograph with theophylline if the selectivity of the method is inadequate.

A variety of GC detectors have been used, including flame ionisation, and electron capture.

In conclusion, derivatisation should be used to resolve theophylline and its metabolites on GC systems. In general, GC methods are more imprecise than either HPLC or a commercial immunoassay (EMIT) at sub-therapeutic concentrations.

Thin Layer Chromatography (TLC).

There have been occasional reports of quantitative TLC procedures for theophylline using either liquid/liquid extractions before chromatography or by direct application either with sample precipitation on the plate but with the disadvantage of a 20min drying time before chromatography. In all cases, plates were developed linearly, dried and then scanned with a flying spot densitometer.

A maximum sensitivity of 5 µmol l⁻¹ (1mg ml⁻¹) was achieved using dual wavelength zig-zag reflectance densitometric scanning and slow scan speeds. Although no interference is claimed for all the above TLC procedures, only one excluded all endogenous xanthine metabolites and caffeine.

The capital cost of the equipment for TLC is comparable to that for LC and GC techniques. TLC allows batch analysis, is simple and although unfashionable, may be a promising technique.
Liquid Chromatography (LC).

A large number of LC procedures have been described for the determination of theophylline in serum. These include ion-exchange\textsuperscript{54}, normal phase\textsuperscript{55-57} and reversed phase\textsuperscript{36,58-61}.

Reversed phase procedures divide into those with or without control of pH or ion-pair. One of the major problems in the determination of theophylline by LC has been the interference by caffeine metabolites, particularly 1,7 dimethylxanthine(1,7-DMX)\textsuperscript{34,38,62}. Early reverse phase methods used eluant mixtures of a dilute aqueous acid and an organic modifier. This ensures reproducible ionisation of theophylline but 1,7-DMX interferes\textsuperscript{63,64} and such methods cannot be recommended even when extensively validated as a selected method\textsuperscript{65}.

Beta-lactam antibiotics, have been reported to interfere with the assay of theophylline by LC. This may give rise to difficulties in the investigation of serum samples from patients suffering from acute bronchitis receiving antibiotic treatment. Acetazolamide has also been shown to interfere in one LC assay\textsuperscript{66}.

Acetonitrile buffer mixtures do not adequately separate theophylline from 1,7-DMX. Methanol must be a constituent of the mobile phase. Resolution of theophylline and 1,7-DMX requires both optimal eluant composition and a high resolution column. Eluants using buffers between pH 3.6 and 5.2 give partial resolution of theophylline and 1,7-DMX\textsuperscript{40,62} and in combination with an organic modifier such as tetrahydrofuran can completely separate the two compounds\textsuperscript{67,68}.

Reverse-phase assays using tetraethyl or tetrabutyl ammonium ion-pairs in an acidic eluant give adequate selectivity and separation of 1,7-DMX and theophylline\textsuperscript{35,62,66}. Normal-phase LC has better selectivity than reverse phase
LC and has been used to completely resolve theophylline and 1,7-DMX. The eluants were halohydrocarbons with traces of organic acids and a polarity modifier. No interfering compounds were found\textsuperscript{69,70,53}.

### 1.3.2 Immunoassay Methods.

For the theory and principles of immunoassay techniques refer to Chapter 2. In this section commercial immunoassay systems and some ‘in-house’ immunoassay procedures for the determination of theophylline are reviewed.

**Substrate Labelled-Fluorescent Immunoassay:**\textsuperscript{71}

This system is marketed for use on the Ames Fluorostat\textsuperscript{TM} and Optimate systems (Miles laboratories, Slough, Berks, U.K.) and relies upon competition for antibody between free theophylline and the drug conjugated to umbelliferyl-β-D-galactoside. This compound is non-fluorescent under the reaction conditions, but hydrolysis by β-galactosidase yields a fluorescent product. When antibody to theophylline reacts with the conjugate it is inactive as an enzyme substrate. Competition is set up between theophylline in the test sample and a constant amount of conjugated drug for a limited concentration of antibody. Unbound conjugated drug is hydrolysed by the enzyme and fluorescence is, therefore, directly proportional to drug concentration. It is now established that 1,3-dimethyluric acid cross-reacts and can increase the measured theophylline concentration\textsuperscript{72,73}.

**Monoclonal Antibody Apoenzyme Reactivation Immunoassay:**\textsuperscript{74,74}

This system developed by Miles Laboratories, Slough, Berks, U.K. is marketed as a solid-state assay using reflectance photometry on the Ames Seralyser\textsuperscript{TM}. Free theophylline competes with theophylline conjugated to flavin-adenine-dinucleotide (FAD) for limiting amounts of antibody. FAD is the prosthetic group for glucose oxidase which is activated by free FAD-theophylline in the
test mixture. The activity of the glucose oxidase is, therefore, proportional to
the theophylline concentration of the solution. Positive interference by high
concentrations of 1,3-dimethyluric acid in serum from uremic patients has
been reported.\textsuperscript{73}

**Fluorescence Polarisation Immunoassay.\textsuperscript{76}**

For this method using the Abbott TDX system (Abbott Laboratories, Wokingham, Berks, U.K.) theophylline is labelled with fluorescein as the tracer. Upon binding to antibody, the rotation of the tracer assumes that of
the antibody molecule. The polarisation of the emitted light increases when
the tracer is bound. Free theophylline competes with the tracer for antibody.
If the test sample contains a high concentration of the drug, the observed
polarisation value is close to that of the tracer alone.

By sequentially exciting the reaction mixture with vertically and then
horizontally polarised light and analysing only the vertical component of the
emitted light, the degree of polarisation of fluorescence in the reaction
mixture can be determined accurately and is proportional to the concentration
of drug.

Caffeine has been reported to interfere positively with the measurement of
theophylline using this system. At therapeutic caffeine levels (5-20 mg l\textsuperscript{-1}, 25-
100 \(\mu\)mol l\textsuperscript{-1}) this interference was approximately 10% and has been used to
assay plasma caffeine concentrations in neonates with apnoea.\textsuperscript{77}

**Enzyme Multiplied Immunoassay (EMIT).\textsuperscript{78}**

For the Syva EMIT assay (Syva U.K. Ltd., Maidenhead, Berks) free
theophylline in the sample is mixed with antibody, NAD and substrate for
glucose-6-phosphate dehydrogenase. A second reagent containing drug-enzyme
conjugate is added and competes for antibody. If this conjugate binds to the
antibody the enzyme activity is reduced. Residual enzyme activity correlates directly with unbound drug concentrations and is monitored by the appearance of NADH at 340nm.

The EMIT system has been available for over 10 years in the U.K. and can be easily adapted to run on other laboratory equipment. The system has also been issued as the Syva Advance™ with monitoring of the NADH fluorescence at 470nm.

In a further modification (EMIT-QST™), the assay reagents have been combined and lyophilised into a single vial and are automatically reconstituted by the addition of sample.

Positive interference in the EMIT assay has been reported with serum from uraemic patients.

Enzyme Linked Immunoabsorbent Immunoassay (ELISA).

Immunotech Corp., Boston, Massachusetts has recently commercialised a heterogeneous enzyme immunoassay method for theophylline, the EZ-BEAD™ kit. This heterogeneous solid phase competitive-binding enzyme immunoassay is based on competition between theophylline in the patient's sample and a theophylline-alkaline phosphatase (EC.3.1.3.1) conjugate in the reagent for a limited number of binding sites on a bead coated with a highly specific anti-theophylline antibody. The amount of theophylline in the patient's serum is inversely proportional to the amount of conjugate bound to the bead, which is determined by the amount of phenol enzymatically generated from phenyl phosphate. As marketed, the phenol produced is determined spectrophotometrically by the coloured complex formed when potassium ferricyanide (stopping reagent) is added.
Enzyme Immunochromatography\textsuperscript{81,82}.

The recently introduced Acculevel\textsuperscript{TM} method allows the quantitative measurement of whole blood theophylline without instrumentation. The assay range is equivalent to a plasma assay range of 16.5-193 μmol l\textsuperscript{-1} (3-35 mg l\textsuperscript{-1}). Blood is mixed with a constant concentration of theophylline conjugated to peroxidase and the components migrate up a chromatography paper strip containing immobilised monoclonal anti-theophylline antibodies. The concentration of free theophylline, therefore, determines the migration distance. Bound peroxidase-conjugated theophylline then forms part of a reaction mixture with a glucose oxidase system and 4-chloro-1-napthol. The height of the visible colour is proportional to theophylline concentration and is converted to concentration units from a chart.

Correlation coefficients between 0.93 and 0.97 were reported between this method and enzyme-immunoassay, fluorescence polarisation immunoassay and high-performance liquid chromatography\textsuperscript{82}. Fifteen related compounds and metabolites were tested for interference in the assay. The greatest positive effect was with β-chlorotheophylline at more than 116 μmol l\textsuperscript{-1} (25 mg l\textsuperscript{-1}) increasing the measured theophylline concentration by 30%.

Enzyme Immunosensor.

An enzyme immunoelectrode system for monitoring theophylline has been developed\textsuperscript{83}. The electrode is composed of an oxygen electrode and an antibody coupled membrane. Anti-theophylline antibody is covalently immobilised on a nylon net with dimethylsulphate,1,-6-hexadiamine and glutaraldehyde. The assay procedure involves the competitive immunochemical reaction of the membrane bound antibody with catalase-labelled and non labelled theophylline. The amount of labelled theophylline bound specifically on the membrane was determined electrochemically from the reducing current of the oxygen generated enzymatically. In order to enable the repeated use of
the same antibody-bound membrane, it is necessary to dissociate the antigen-antibody complexes. This procedure causes denaturation of the antibodies, which results in the necessity of exchanging the membrane. A modification of the above immunosensor has been reported\textsuperscript{84} using a theophylline hapten bound membrane and \( F_{ab} \) antibody-enzyme conjugate to improve reproducibility and capability of multi-cyclic operations.

A simple sensitive liposome immunosensor has been developed for theophylline by combining the advantages of liposome immunoassays and enzyme immunosensors\textsuperscript{85,86}.

**Latex Particle-Enhanced-Turbidimetric Inhibition Immunoassay (PETINA)\textsuperscript{87}**.

The method is incorporated into the DuPont ACA\textsuperscript{TM} (DuPont U.K., Stevenage, Herts., U.K.) test pack system in which theophylline linked to latex particles forms aggregates with a theophylline-specific monoclonal antibody. Free theophylline competes and, therefore, decreases the rate of aggregation and the turbidity is measured kinetically at 340nm. Marked overestimation of theophylline in serum from uraemic patients has been noticed\textsuperscript{19}.

**Latex Inhibition Immunoassay\textsuperscript{88}**.

In the system prepared for use on the Technian RA-1000 (Technicon Ltd., Basingstoke, Hants, U.K.), free theophylline prevents agglutination by competing with a theophylline-Ficoll\textsuperscript{TM} conjugate for monoclonal antibody bound to latex particles. The concentration of theophylline is inversely proportional to the rate of agglutination which is estimated by means of measuring the rate of increase in absorbance at 600nm. The assay is stated to have within-batch imprecision levels of 4.2 and 2.8\% at 27.5 and 222 \( \mu \)mol l\(^{-1}\) (5 and 40 mg l\(^{-1}\)) respectively.
Radioimmunoassay.

An in-house assay was described in which the antibody was raised against an 8-carboxytheophylline-bovine serum conjugate\textsuperscript{99}. Incubation time was 1 h at 4°C. Bound and unbound counts were separated by ammonium sulphate/calcium sulphate precipitation.

Fluorimmunoassay\textsuperscript{90}.

In a recently described fluorimmunoassay, fluorescein labelled theophylline was used as the tracer and phase separation was achieved using antibodies coupled to magnetisable solid phase particles. The assay required 10μl sample and results were obtained within 30 min. The assay was reported to be highly specific for theophylline with a maximum cross reactivity of approximately 3% for 1-methylxanthine\textsuperscript{90}.

A comparison of an in-house fluorescent immunoassay for theophylline with HPLC, radioimmunoassay and enzyme immunoassay showed good agreement between all the methods\textsuperscript{91}.

Nephelometric Inhibition Immunoassay\textsuperscript{92}.

The rate of reaction of drug-conjugated antibody is measured nephelometrically using the Beckman ICS analyser (Beckman Analytical Instruments, High Wycombe, Bucks, U.K.). The reaction is inhibited by the presence of free drug and results are obtained over 30-60s. The reaction is stated to be unaffected by non-specific side reactions or matrix composition.
Radial Partition Immunoassay\textsuperscript{93}.

Theophylline and alkaline phosphatase labelled drug compete for binding to monoclonal anti-theophylline antibody immobilised on a glass fibre disc (Stratus, American Dade; Dade Division of American Hospital Supply, Miami, Florida, U.S.A.). Unbound drug and general contaminants are washed from the centre of the disc. The enzymatic reaction rate of the bound fraction is then measured fluorimetrically. The analytical speed is 70 samples/h. Samples require dilution before analysis. The manufacturer's information indicates little cross-reaction between metabolites and theophylline although possible interaction with 1,3-dimethyluric acid was not reported.

Flow Injection Immunoassays.

Flow injection immunoassays have been developed for theophylline using potentiometric\textsuperscript{94,95}, amperometric\textsuperscript{96}, fluorescence\textsuperscript{97} and spectrophotometric\textsuperscript{98} detection systems. For further details of this technique see chapters 2.3 and 6.1.1.

1.3.3 Miscellaneous Methods.

In the mid seventies Munson et al\textsuperscript{99} described a method for the determination of plasma theophylline based on the electrochemical oxidation of theophylline at a stationary carbon paste electrode.

1.3.4 Endpoint Detection of Theophylline Assays.

Various endpoint detection systems have been employed in the assays of theophylline. The most commonly used system is UV detection at 254nm. Other detection systems are given in Table 1.4.
Table 1.4 Endpoint Detection Systems for Theophylline Assays.

<table>
<thead>
<tr>
<th>Endpoint Detection System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet Spectrophotometric</td>
<td>55-61</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>83-86</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>90</td>
</tr>
<tr>
<td>Nephelometric</td>
<td>92</td>
</tr>
<tr>
<td>Colorimetric</td>
<td>80</td>
</tr>
<tr>
<td>Turbimetric</td>
<td>87</td>
</tr>
<tr>
<td>Radiochemical</td>
<td>89</td>
</tr>
</tbody>
</table>


44. Sheeham, M., and Haythorn, P., *J. Chromatogr.*, (1976), 117, 393


80. Manufacturers Information EZ-BEAD™ Kit (Immunotech Corp., Boston, MA 02134)


CHAPTER TWO

IMMUNOASSAY TECHNIQUES
2.1.1 Principles of Immunoassay.

Immunoassay techniques have over the past 30 years allowed biochemists to understand many physiological, pathological and pharmacological processes. Since its realization by Yalow and Berson in 1959, the technique has provided a powerful means of determining a broad spectrum of substances of interest. The power of immunoassay lies in its combination of specificity, versatility, practicability and sensitivity.

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

Immunoassays are fundamentally simple and are based on the reversible and non-covalent interaction of the antigen (or hapten) with its specific binding partner or antibody:

\[ Ag + Ab \rightarrow Ag - Ab \]  
Equation 2.1

If a label is covalently attached to the antigen such that it does not block the antigen region recognised by antibody, the presence of the label will not significantly perturb the binding of labelled antigen (Ag') to antibody. Therefore in a situation in which a mixture of labelled (Ag') and unlabelled
(Ag) antigen react with antibody, competition for the antibody binding site occurs:

\[ Ag + Ag^* + Ab \rightarrow Ag - Ab + Ag^* - Ab \]  

Equation 2.2

Using labelled antigen (Ag*) it is possible to determine an unknown amount of antigen in a given solution. The above reaction (known as a competitive binding reaction or immunoassay) is normally allowed to attain equilibrium. At equilibrium, the binding sites of the antibody are saturated with Ag* and Ag in proportion to their relative concentration in the assay solution. The antibody bound and unbound (free) forms of the antigen are separated and the amount present in the bound fraction plotted as a function of the unlabelled antigen concentration. The concentration of Ag in an unknown sample may thus be determined from a competitive binding reaction with known amounts of labelled antigen and antibody, the antigen content subsequently being determined from a calibration curve.

Another form of the competitive immunoassay is the immunoradiometric approach first described over 20 years ago. In this type of assay the labelled components is a specific antibody (Ab*), the process again being based on the antigen-antibody binding reaction:

\[ Ag + Ab^* + Ab \rightarrow Ag - Ab^* + Ag - Ab \]  

Equation 2.3

Imunoassay are of two basic types: homogeneous assays, in which no separation step is required, and heterogeneous ones where a separation step is necessary to separate antibody-bound from unbound materials.

The major advantage of immunoassays, are its specificity, versatility and sensitivity. Specificity can be attributed to the selective and specific nature of the antigen-antibody binding reaction, versatility stems from the large number of molecules of interest that themselves, or coupled to a carrier (i.e., haptens) may be used to initiate antibody production and sensitivity is obtained through the wide choice of labels available. Typical radioimmunoassay procedures have
limits of detection in the pmol 1⁻¹ range³ while limits of detection in the attomole (10⁻¹⁸M) range have been reported using enzyme immunoassays⁴,⁵.

2.1.2 Isotopic Labels: Advantages and Disadvantages.

The label originally used was a radioisotope¹ and this radioimmunoassay (RIA) method remains the most common form. The conventional label is¹²⁵I or ¹³¹I. The widespread and diversified applications of radioimmunological methods in the biomedical field may be related to the following advantages:

a. High sensitivity (10⁻¹² - 10⁻¹⁵ M) and selectivity; the former derived from the absence of background radiation in biological samples as well as the very low levels at which radioactivity can be detected and the latter due to the specificity of immunological reactions.

b. Wide applicability; radioimmunoassays have been widely used for the determination of peptide and steroid hormones, drugs (therapeutic and drugs of abuse) and various macromolecules of clinical interest (e.g. α-fetoprotein and thyroglobulin) whose normal concentrations are too low.

c. Small sample size, usually from 10-100 µl.

The major disadvantages of RIA are:

a. The radiation hazards which impose several restrictions such as regulation of production, transport, handling and disposal of radioactive materials.
b. The labelled antigens (haptens) are intrinsically unstable and often costly.

c. The specialised equipment required is not available in many laboratories.

d. The properties of the radio label are the same whether the labelled molecule is bound to antibody or not hence, a separation is needed before the distribution of labelled antigen (haptens) between bound and free fractions can be determined. The separation methods usually are:

(i) adsorption of the antigen (hapten) on charcoal or dextran coated charcoal,

(ii) use of solid phase antibodies e.g. antibodies adsorbed on the inside surfaces of polystyrene tubes, and

(iii) the use of second, precipitating antibodies which form insoluble complexes with the anti-antigen (hapten) antibodies. The separation step inevitably complicates the assay and renders its automation difficult.

e. The radiation damage to the immune reactants.

Despite the apparently favourable sensitivity offered by RIA, the last decade has seen a rapid upsurge of methods in which alternative (nonisotopic) labels are used which may be measured with similar sensitivity.
2.1.3 Non-Isotopic Immunoassays.

To overcome some of the limitations of radioimmunoassay methods, alternative immunological methods have been developed which do not require the use of radio labelled materials. These non-isotopic immunoassay methods take full advantage of the specificity and sensitivity that result from the application of antibodies but avoid the use of radioisotopes.

Non-isotopic immunoassay are divided into two groups: those using labelled and those using non labelled methodology. In the former, the antigen-antibody reaction is detected using marked labelled substances. In the latter, the antigen-antibody complex is detected, directly without markers.

Imunoassays employing no labels: several immunoassay methods do not require a labelled reagent, rather they depend upon the precipitation line which forms in a gel support when an antigen comes into contact with its specific antibody, or upon the increase in light scattering produced by the formation of antibody-antigen complexes. Such assays are well established in clinical chemistry laboratories, but their use is restricted to a few proteins and other large molecules which are present in relatively high concentrations (>10^-7M) since only in such circumstances are the resultant antibody-antigen complexes sufficiently large to form a precipitation line to scatter light.

Attempts have been made to improve the sensitivity of these assays; the most interesting approach is that adopted by Technicon in their particle counting immunoassay (PACIA). The antibody is coated on to fine latex particles which agglutinate in the presence of antigen. Thus, the number of particles passing through a flow cell is inversely proportional to the antigen concentration. The full potential of this approach has been limited by non-specific agglutination caused by other factors.
Non isotopic labels for immunoassay: the function of a label in an immunoassay is to act as a marker of the relative distribution of antigen (saturation analysis) or antibody (immunometric analysis) between bound and free fractions. In order to assess the suitability of a particular non-isotopic label for use in an immunoassay the label and end point detection system must be considered. Table 2.1 indicates the criteria for ideal label and method for a non-isotopic immunoassay. Consequently, many non-isotopic methods have been developed using different labels (Table 2.2). These labels include enzymes, fluorescent or luminescent compounds, metals, viruses, free radicals etc. Table 2.2 lists non-isotopic immunoassays with examples.

Table 2.1 Criteria for an Ideal Label and Method for Non-Isotopic Immunoassay.

1. Ease of introduction into sample molecules.
2. Freedom from hazards
3. Cost and availability of appropriate instrumentation
4. No effect on Ag-Ab reaction
5. Free from interference from biological samples
6. Suitable for homogeneous assay and automation
7. Possibility of combination with another label to develop a multicomponent analysis.
Table 2.2 Some Non-Isotopic Immunoassay methods.

<table>
<thead>
<tr>
<th>Type of Immunoassay</th>
<th>Label Used</th>
<th>Detection Methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Immunoassay</td>
<td>Enzymes</td>
<td>i) Spectrophotometry</td>
<td>8-11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Fluorimetry</td>
<td>12-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii) Visual assessment</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv) Thermometry</td>
<td>16, 17</td>
</tr>
<tr>
<td>Co-Enzyme Immunoassay</td>
<td>NAD,FAD,ATP</td>
<td>i) Spectrophotometry</td>
<td>18, 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Luminometry</td>
<td>20, 21</td>
</tr>
<tr>
<td>Chemiluminescence and Bioluminescence Immunoassay</td>
<td>Luminescent Compounds</td>
<td>Luminometry</td>
<td>22-24</td>
</tr>
<tr>
<td>Fluorescence Immunoassay</td>
<td>Fluorophores</td>
<td>Fluorimetry</td>
<td>25-30</td>
</tr>
<tr>
<td>Metallo-Immunoassay</td>
<td>Metal atoms</td>
<td>i) Colorimetry</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Atomic absorption spectroscopy</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii) Voltammetry</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv) Fluorimetry</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v) Luminometry</td>
<td>35, 36</td>
</tr>
<tr>
<td>Sol-Particle Immunoassay</td>
<td>Metal Sols</td>
<td>i) Visual assessment</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Colorimetry</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii) Atomic absorption spectroscopy</td>
<td>39</td>
</tr>
<tr>
<td>Particle Counting Immunoassay</td>
<td>i) Latexes</td>
<td>i) Visual assessment</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>ii) Particles</td>
<td>ii) Particle counting</td>
<td>7, 40, 41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii) Nephelometry</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv) Turbidimetry</td>
<td>41</td>
</tr>
<tr>
<td>Spin Immunoassay</td>
<td>Free radicals</td>
<td>Electron spin resonance (E.S.R)</td>
<td>42-45</td>
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<tr>
<td>Viro Immunoassay</td>
<td>i) Virus</td>
<td>Bacterial Culture</td>
<td>46-49</td>
</tr>
<tr>
<td></td>
<td>ii) Bacteriophage</td>
<td></td>
<td></td>
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<tr>
<td>Substrate Immunoassay</td>
<td>Fluorophores</td>
<td>Fluorimetry</td>
<td>50-53</td>
</tr>
<tr>
<td>Erythrocyto Immunoassay</td>
<td>Red blood cells</td>
<td>i) Visual assessment</td>
<td>54-56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Spectrophotometry</td>
<td></td>
</tr>
<tr>
<td>Voltammetric Immunoassay</td>
<td>Metal chelates</td>
<td>Voltammetry</td>
<td>57-60</td>
</tr>
<tr>
<td>[see section 2.3]</td>
<td></td>
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</tr>
</tbody>
</table>
2.2 Enzymes as Labels in Immunoassay.

2.2.1 Enzyme Immunoassays (EIA).

In the past decade many informative books and reviews on enzyme immunoassays have been published\(^9,61-69\). In this section only a brief review of some aspects of enzyme immunoassays are presented.

Enzyme immunoassay using enzyme as labels have a number of advantages:

a. Detection limits for enzymes are very low\(^4,5\), because enzyme reactions can be amplified as catalytic reactions.

b. The enzyme-labelled antigen and antibody are stable.

c. Simple inexpensive equipment may be used to determine the enzyme activity.

Since the first report on enzyme immunoassay appeared in 1971\(^69,70\) relevant techniques have been developed and applications are increasing. There are many reports on coupling agents for preparing conjugates of enzymes and antigens or antibodies [refer to chapter 5]. The sensitivity of determination of enzyme activity can be improved by using fluorescent or luminescent substrates\(^71\). To simplify the assay procedure, extensive studies have been directed toward the development of non separation or homogeneous methods\(^50,72-79\).
2.2.2 Choice of Enzyme Label.

Enzymes function as useful labels because they are very efficient catalysts. A single molecule of enzyme converts $10^3$-$10^4$ molecules of substrate into product per minute, but for some enzymes this figure can be as high as $10^5$-$10^6$. Ideally the enzymes label should have the following properties:

- a. High turnover number
- b. Purity of the enzyme preparation
- c. Sensitivity of its detection
- d. Simplicity and speed of detection of the enzyme reaction
- e. Absence of interfering factors or enzyme-like activity in the test fluid.
- g. Presence of a potentially reactive group in the enzyme which allows linkage to other molecules while retaining a substantial part of the enzyme activity.
- h. Suitability of the enzyme for homogenous enzymes immunoassay (if applicable)

The first three criteria determine the sensitivity of an eventual assay. Generally speaking, the smaller the amount of enzyme label which can be detected the more sensitive the resulting assay will be.

The absence in the test fluid of factors interfering with the enzymes activity measurement is not an absolute requirement, since problems can usually be avoided by adopting an appropriate protocol.

Most of the enzymes that have been used in enzymes immunoassay are given in Table 2.3; refer to chapter five for the preparation of enzyme labels.
Table 2.3 Commonly Used Enzyme Labels.

<table>
<thead>
<tr>
<th>Enzyme Label</th>
<th>EC Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterogeneous EIA</strong></td>
<td></td>
</tr>
<tr>
<td>Horse-radish peroxidase</td>
<td>(EC. 1. 11. 1. 7)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>(EC. 3. 1. 1. 1)</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>(EC. 3. 2. 1. 23)</td>
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<tr>
<td>Glucoamylase</td>
<td>(EC. 3. 2. 1. 3)</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>(EC. 4. 2. 1. 1)</td>
</tr>
<tr>
<td>Catalase</td>
<td>(EC. 1. 11. 1. 6)</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>(EC. 3. 1. 1. 7)</td>
</tr>
<tr>
<td>Penicillninase</td>
<td>(EC. 3. 5. 2. 6)</td>
</tr>
<tr>
<td><strong>Homogeneous EIA</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose-6-Phosphate dehydrogenase</td>
<td>(EC. 1. 1. 1. 49)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>(EC. 3. 2. 1. 17)</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>(EC. 3. 2. 1. 23)</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>(EC. 1. 1. 1. 37)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>(EC. 1. 1. 1. 27)</td>
</tr>
<tr>
<td>Lipoanide dehydrogenase</td>
<td>(EC. 1. 6. 4. 3)</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>(EC. 2. 7. 1. 1)</td>
</tr>
<tr>
<td>Glucosephosphate isomerase</td>
<td>(EC. 5. 3. 1. 9)</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
<td>(EC. 1. 1. 3. 4)</td>
</tr>
</tbody>
</table>
2.2.3 Detection of Enzyme Labels.

Colorimetric\textsuperscript{80,81} and spectrophotometric\textsuperscript{82} assays are the most commonly used end point detection systems for enzyme immunoassays. Other end point detection systems are possible and include visual assessment\textsuperscript{15}, thermometric\textsuperscript{16,17}, fluorescent\textsuperscript{50,83}, electrochemical [potentiometric\textsuperscript{84-86} and amperometric\textsuperscript{87-89}], luminescent\textsuperscript{90-92} and radiometric\textsuperscript{93} assays. Fluorescence, luminescence and electrochemical end points have gained popularity because of their sensitivity. The limits of detection of some enzyme immunoassays using various end point detection systems are listed in Table 2.4.

Table 2.4 Enzyme Immunoassays with Fluorometric, Thermometric, Potentiometric/Amperometric, Luminometric and Radiometric end point detection.

<table>
<thead>
<tr>
<th>Analyte Label</th>
<th>Detection Limit</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin Catalase</td>
<td>Thermometric 0.1nM</td>
<td>16</td>
</tr>
<tr>
<td>Gentamicin Catalase</td>
<td>Thermometric 1ng ml(^{-1})</td>
<td>17</td>
</tr>
<tr>
<td>Gentamicin β - Galactosidase</td>
<td>Fluorescence 0.8ng ml(^{-1})</td>
<td>50</td>
</tr>
<tr>
<td>Biotin Porcine esterase</td>
<td>Fluorescence 10nM</td>
<td>83</td>
</tr>
<tr>
<td>Estradiol-17β Peroxidase</td>
<td>Potentiometric 50pmol l(^{-1})</td>
<td>84</td>
</tr>
<tr>
<td>Factor VIII related antigen Alkaline phosphatase</td>
<td>Amperometric 1.6ng</td>
<td>85</td>
</tr>
<tr>
<td>Insulin Glucose oxidase</td>
<td>Potentiometric 1μM</td>
<td>86</td>
</tr>
<tr>
<td>Digoxin Alkaline phosphatase</td>
<td>Amperometric 50pg ml(^{-1})</td>
<td>87</td>
</tr>
<tr>
<td>Mouse IgG Alkaline Phosphatase</td>
<td>Amperometric 0.8pg ml(^{-1})</td>
<td>88</td>
</tr>
<tr>
<td>Theophylline Alkaline phosphatase</td>
<td>Amperometric 80ng l(^{-1})</td>
<td>89</td>
</tr>
<tr>
<td>Cortisol Peroxidase</td>
<td>Luminometric 10pg</td>
<td>90</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone Glucose oxidase</td>
<td>Luminometric 0.5pg</td>
<td>91</td>
</tr>
<tr>
<td>Methotrexate Firefly luciferase</td>
<td>Luminometric 2.5pmol</td>
<td>92</td>
</tr>
<tr>
<td>Adenosine Alkaline phosphatase</td>
<td>Radiometric 1 nM</td>
<td>93</td>
</tr>
</tbody>
</table>
2.2.4 Types of Enzyme Immunoassays.

**Heterogeneous Enzyme Immunoassays.**


   This technique involves competition between enzyme labelled and unlabelled antigen for a limited quantity of antigen-specific antibody. Antibody-bound antigen is separated from unbound antigen and the enzyme activity in either phase is measured. The activity in the bound phase is inversely proportional to the concentration of unlabelled antigen in the assay. This type of enzyme immunoassay is analogous to the classical radioimmunoassay method.

2. Immunoenzymometric Assay for Antigen.

   In this technique the antigen reacts with excess labelled antibody and after incubation, excess solid-phase antigen is added. The solid-phase antigen reacts with the free labelled antibody remaining and, after separation of the solid phase, the enzyme activity associated with soluble antigen is measured and related to the concentration of antigen. This assay is analogous to the immunoradiometric assay of Miles and Hales.

3. Sandwich EIA for Antigen

   This procedure requires the antigen to have at least two binding sites. The antigen reacts with excess solid-phase antibody, and, after incubation followed by washing, the bound antigen is treated with excess labelled antibody. After further washing the bound label is assayed, and this provides a direct measure of the amount of antigen present. This assay is analogous to the "two-site" immunoradiometric procedure. A variation of this method ("double sandwich" EIA) involves a third antibody. This antibody carries the
label and reacts with unlabelled second antibody already bound to the antigen. As before, the amount of antigen is found by measuring the amount of bound label.

4. EIA for Antibody

Antibody binds to excess solid-phase antigen; and after incubation followed by washing, labelled second antibody is added. The bound label is assayed after further washing and it provides a direct measurement of the amount of specific antibody present. This assay is analogous to the radioallergosorbent technique. This system may also be used to assay antigens.

Homogeneous Enzyme Immunoassay.

A number of novel EIAs have been developed which do not require a separation phase. These assays are based upon a variety of ingenious mechanisms.

1. Assays Based on Enzyme - Antigen Conjugates.

This technique has been used to quantitate haptens, the key reagent being an enzyme-hapten conjugate. Binding of hapten-specific antibody to this conjugate inhibits the activity of the enzyme. The antibody is believed either to hinder access of substrate sterically, to the active site of the enzyme or induce conformational changes in the enzyme which prevent the enzyme from acting upon its substrate. Free hapten competes with the hapten-enzyme conjugate for a limited quantity of antibody and so reduces this inhibition. The enzyme activity of the conjugate is proportional to the concentration of free hapten in the sample or standards. This assay has been termed the enzyme multiplied immunoassay technique (EMIT).
The EMIT thyroxine assay is an exception to this mechanism. In this instance the thyroxine malate dehydrogenase conjugate is enzymatically inactive. However, binding of thyroxine-specific antibody to the conjugate actually activates the enzyme. The enzyme activity of this label is inversely proportional to the concentration of unlabelled thyroxine in the assay.

A homogeneous EIA for quantitating proteins has been reported. This assay is based upon a galactosidase-IgG conjugate and a synthetic high-molecular-weight substrate. Binding of IgG specific antibodies to this conjugate inhibited the activity of the enzyme, provided the high molecular-weight substrate was used to quantitate the activity of the enzyme. The assay was reported to have a sensitivity of 25 ng ml⁻¹ for human IgG. A homogeneous EIA for polyvalent ligands and antibodies, which avoids the need for a labelled antigen, has been reported by Gibbons and co-workers.

2. Assay Based on Antigen-substrate conjugates

The key reagent in this assay is the antigen-substrate conjugate. One example of such a conjugate consisted of gentamicin linked to an umbelliferone-galactoside derivative. β-Galactosidase was able to hydrolyze this derivative, even when the derivative was conjugated to gentamicin. However, when bound by gentamicin-specific antibody, the derivative did not function as a substrate for the enzyme. This inhibition was relieved by unlabelled gentamicin; the amount of substrate hydrolyzed was proportional to the concentration of unlabelled gentamicin in the assay. In this type of assay the amplification property of the enzyme is not used. This effect is minimized as a highly fluorescent product is formed when the substrate is hydrolyzed by the enzyme. This type of assay has mainly been used to quantitate haptens, but has been adapted to the
determination of high-molecular-weight compounds\textsuperscript{77}.

3. Assays Based on Antigen-Modulator Conjugates

These assays employ a modulator which act to alter the activity of an indicator enzyme. The key reagent is a antigen-modulator conjugate: binding of antigen-specific antibody to this conjugate neutralizes the activity of the modulator.

Modulators such as cofactors\textsuperscript{18} or prosthetic groups\textsuperscript{77} are necessary for enzyme activity. In this situation the presence of antigen-specific antibody results in inhibition of the enzyme. Unlabelled antigen, by competing for antigen-specific antibody, reduces this inhibition. Enzyme activity is proportional to the concentration of free ligand in the assay. Where the modulator acts to inhibit the enzyme, the reverse is true. Such modulators have included enzyme inhibitors\textsuperscript{78} or inhibitory enzyme-specific antibodies\textsuperscript{79}.

4. Assay Based on Enzyme Channelling

These assays share features of both homogeneous and heterogeneous assays. The assay is based upon the finding that the overall rate of two consecutive reactions is enhanced when the enzymes catalyzing these reactions are co-immobilized on the same support. In the assay, an antigen - enzyme conjugate competes with free antigen for a limited quantity of antigen-specific antibody co-immobilised on a solid support with a second enzyme. The product of the first enzyme acts as substrate for the second. When the antigen-enzyme conjugate is bound to the solid support, the two enzymes are brought into close proximity and the rate of the overall reaction is enhanced. This rate is inversely proportional to the concentration of unlabelled antigen in the assay. In practice the system would appear to be rather
complicated, particularly as a third enzyme is required to minimize background reactions in the assay.

2.2.5 Applications of Enzyme Immunoassays.

EIAs have been developed for wide variety of compounds and extensive lists of applications have been published\(^3\),\(^7\),\(^7\),\(^8\),\(^8\),\(^10\). The number of applications have expanded enormously in the last decade. In this section a brief review of compounds measured by EIA is presented.

EIAs for Antigens and Haptens

The antigens and haptens that have been measured by EIAs are listed in Table 2.5 together with details of the enzymes used and the sensitivity obtained. Molecules that have been measured include macromolecular hormones, other serum proteins, bacterial toxins, drugs and steroids. Most of the assays have been applied to serum, but there are also assays for components of cerebrospinal fluid\(^1\) and urine\(^1\).

EIA for Antibodies

Antibodies against macromolecules, viruses, bacterial products and eucaryotic parasites have been identified and measured using EIA. These are listed in Table 2.6 together with the enzyme used. The antibody EIA may be used in a simplified form for screening large numbers of samples and for obtaining qualitative results without the use of spectrophotometry.

Homogeneous EIA for Haptens

The homogeneous EIAs for haptens are listed in Table 2.7, together with the enzymes used as labels and the sensitivity of each assay. Kits are commercially available (the "EMIT" system) for assay of drugs of abuse in urine,
Table 2.5 Enzyme Immunoassays for Antigens and Haptens.

<table>
<thead>
<tr>
<th>Antigen or Hapten</th>
<th>Enzyme</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>AP</td>
<td>1μg l⁻¹</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.2mg l⁻¹</td>
</tr>
<tr>
<td></td>
<td>GO</td>
<td>50μg l⁻¹</td>
</tr>
<tr>
<td></td>
<td>BG</td>
<td>50pg</td>
</tr>
<tr>
<td>IgE</td>
<td>AP</td>
<td>10μg l⁻¹</td>
</tr>
<tr>
<td>α-Fetoprotein (human)</td>
<td>AP</td>
<td>1μg l⁻¹</td>
</tr>
<tr>
<td></td>
<td>GO</td>
<td>0.7μg l⁻¹</td>
</tr>
<tr>
<td>α₂ H-Globulin</td>
<td>P</td>
<td>50μg l⁻¹</td>
</tr>
<tr>
<td>Carcinoembryonic antigen</td>
<td>AP</td>
<td>20μg l⁻¹</td>
</tr>
<tr>
<td>Choriongonadotropin</td>
<td>P</td>
<td>0.1KIU l⁻¹</td>
</tr>
<tr>
<td>Hepatitis surface antigen</td>
<td>P</td>
<td>3μg l⁻¹</td>
</tr>
<tr>
<td>Ferritin</td>
<td>AP</td>
<td>8pmol l⁻¹</td>
</tr>
<tr>
<td>Insulin</td>
<td>AP</td>
<td>75U l⁻¹</td>
</tr>
<tr>
<td></td>
<td>GO</td>
<td>0.3μU l⁻¹</td>
</tr>
<tr>
<td></td>
<td>BG</td>
<td>0.5μU</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>5μU l⁻¹</td>
</tr>
<tr>
<td>Thyrotropin</td>
<td>AP</td>
<td>10μlU l⁻¹</td>
</tr>
<tr>
<td>Cortisol</td>
<td>AP</td>
<td>10μg l⁻¹</td>
</tr>
<tr>
<td>Oestriol</td>
<td>P</td>
<td>200pmol l⁻¹</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>P</td>
<td>3pg</td>
</tr>
<tr>
<td>Progesterone</td>
<td>BG</td>
<td>500pmol l⁻¹</td>
</tr>
<tr>
<td>Testosterone</td>
<td>P</td>
<td>100pg</td>
</tr>
<tr>
<td>Exotoxin</td>
<td>AP</td>
<td>90μg l⁻¹</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>AP</td>
<td>1.3mg l⁻¹</td>
</tr>
<tr>
<td>Arabis Mosaic Virus</td>
<td>AP</td>
<td>0.9pmol l⁻¹</td>
</tr>
<tr>
<td>Plum pox virus</td>
<td>AP</td>
<td>0.06pmol l⁻¹</td>
</tr>
<tr>
<td>Adenosine</td>
<td>BG</td>
<td>0.5mmol l⁻¹</td>
</tr>
<tr>
<td>DNP-lysine</td>
<td>AP</td>
<td>10nmol l⁻¹</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>AP</td>
<td>50μg l⁻¹</td>
</tr>
<tr>
<td></td>
<td>BG</td>
<td>10μg l⁻¹</td>
</tr>
<tr>
<td>Penicillin</td>
<td>BG</td>
<td>10μg l⁻¹</td>
</tr>
<tr>
<td>Viomycin</td>
<td>BG</td>
<td>0.3μg l⁻¹</td>
</tr>
</tbody>
</table>

AP, Alkaline Phosphatase; BG, β-D-Galactosidase; P, Peroxidase; GO, Glucose oxidase
Table 2.6 Enzyme Immunoassays for Antibodies\(^{101}\).

<table>
<thead>
<tr>
<th>Antibody against</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoeba, strain HK-9</td>
<td>P</td>
</tr>
<tr>
<td>Albumin, serum</td>
<td>AP</td>
</tr>
<tr>
<td>Allergens, Various</td>
<td></td>
</tr>
<tr>
<td>Carcinoembryonic antigen</td>
<td>AP</td>
</tr>
<tr>
<td>Choriogonadotropin</td>
<td>P</td>
</tr>
<tr>
<td>DNA</td>
<td>P</td>
</tr>
<tr>
<td>Dextran</td>
<td>AP</td>
</tr>
<tr>
<td>Echinococcus granulosus</td>
<td>P</td>
</tr>
<tr>
<td>Escherichia Coli enterotoxin</td>
<td>AP</td>
</tr>
<tr>
<td>$\alpha$ - Fetoprotein</td>
<td>AP</td>
</tr>
<tr>
<td>$\alpha$ - Globulin</td>
<td>P</td>
</tr>
<tr>
<td>IgG myeloma protein</td>
<td>AP</td>
</tr>
<tr>
<td>Immunoglobulin light chains</td>
<td>AP</td>
</tr>
<tr>
<td>Hog cholera Virus</td>
<td>P</td>
</tr>
<tr>
<td>Rubella Virus</td>
<td>AP</td>
</tr>
<tr>
<td>Salmonella species, O antigens</td>
<td>AP</td>
</tr>
<tr>
<td>Schistosoma Mansoni</td>
<td>P</td>
</tr>
</tbody>
</table>

P, Peroxidase; AP, Alkaline Phosphatase.
Table 2.7 Homogeneous Enzyme Immunoassay for Haptens

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Enzyme</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug of abuse:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine and other opiates</td>
<td>L</td>
<td>0.5mg l⁻¹</td>
</tr>
<tr>
<td>Barbiturates (phenobarbital sodium)</td>
<td>G6PD</td>
<td>4-20μmol l⁻¹</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>L</td>
<td>1.7nmol l⁻¹</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>L</td>
<td>1mg l⁻¹</td>
</tr>
<tr>
<td>Methadone</td>
<td>L</td>
<td>0.3mg l⁻¹</td>
</tr>
<tr>
<td>Benzoyl ecgonine (cocaine metabolite)</td>
<td>L</td>
<td>1mg l⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-epileptic drugs in serum:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphenylhydratoin</td>
<td>G6PD</td>
<td>2.5-30mg l⁻¹</td>
</tr>
<tr>
<td>Primidone</td>
<td>G6PD</td>
<td>2.5-30mg l⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>G6PD</td>
<td>0.5-40μg l⁻¹</td>
</tr>
<tr>
<td>Morphine and codeine</td>
<td>MD</td>
<td>20μg l⁻¹</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>MD</td>
<td>20μg l⁻¹</td>
</tr>
</tbody>
</table>

L. Lysozyme; MD, Malate Dehydrogenase; G6PD, Glucose-6-phosphate Dehydrogenase;
anti-epileptic drugs, cardiovascular drugs, anti-depressants, chemotherapeutics and hormones in serum.

The principle of EIA can be applied to all antigens, haptens and antibody systems. EIAs have so far been developed for serum proteins, tumour antigens hormones, drugs antigens derived from various microorganisms and antibodies directed to them. These applications are listed in Table 2.5-2.7. They constitute a most important contribution to the solution of diagnostic problems in many situations.

2.3 Electrochemical Immunoassays.

The growing trend away from radioimmunoassays and the increasing number of immunoassays, coupled with the wide range of low detection limits of electroanalytical methods, has resulted in a proliferation of papers in the last decade trying to link immunoassays to electrochemical means of detection. Amperometric and potentiometric methods have both been used with varying degrees of success. The fundamental difference between the two electrochemical techniques is that amperometric assays measure current and a linear relationship exists between the current and the concentration of an electroactive species that is either oxidised or reduced at the electrode whiles potentiometric assays measure the change in potential and a logarithmic relation exists between potential and concentration with an idealised change in potential of $59/n$ mV per decade of activity.
Excellent reviews on this topic have been published covering a wide range of methodologies\textsuperscript{104-108}. Current published electrochemical immunoassays can be classified into the following groups:

a. Amperometric immunoassays developed around the Clark oxygen electrode. These employ enzymes that can either consume or produce oxygen in the presence of suitable substrates\textsuperscript{85,109-114}.

b. Amperometric enzyme immunoassays that employ an enzyme and electrochemically detect product of the enzyme\textsuperscript{87,89,115-131}.

c. Amperometric immunoassays that do not employ enzymes but utilise either an antibody or antigen that is labelled with an electroactive species\textsuperscript{57,58,132-139}.

d. Potentiometric immunoassays that are based on a change in potential that occurs when either an antibody or antigen is immobilised on an electrode and its specific binding partner binds to it\textsuperscript{140-142}.

e. Potentiometric immunoassays where the method of detection is a more conventional, potentiometric electrode, e.g. ion selective electrode, carbon dioxide electrode, ammonia electrode, or pH electrode\textsuperscript{84,143-159}.
a. Amperometric Immunoassay Based on the Clark Oxygen Electrode.

Glucose oxidase and catalase are the two most commonly used enzyme labels in this type of assay. The use of a Clark Oxygen electrode in an immunoassay is illustrated by an assay for human chorionic gonadotropin (HCG), where an antibody to HCG was immobilised onto a membrane\textsuperscript{109}. The membrane was then placed over the electrode and competitively reacted with HCG and HCG-labelled with catalase. The probe was then washed and exposed to hydrogen peroxide. If any catalase-HCG was bound to the membrane, it would cause the hydrogen peroxide to disproportionate and result in an increase in the cathodic current due to increase in the oxygen. The rate of increase in oxygen tension is monitored, a calibration plot suggested that the sensor can monitor between 0.02 and 100 Uml\textsuperscript{-1} of HCG. This approach has been demonstrated for $\alpha$-fetoprotein\textsuperscript{110}, theophylline\textsuperscript{111} and human serum albumin\textsuperscript{112,113}. Renneberg and co-workers\textsuperscript{85} have concentrated on the consumption of oxygen rather than its production, using a less than simple enzyme linked immunoassay for Factor VIII related antigen. They have adapted a glucose oxidase (GOD) electrode\textsuperscript{114} to monitor glucose produced by an antibody labelled with alkaline phosphatase (AP):

\begin{align*}
\text{AP} \\
\text{Glucose-6-Phosphate} + H_2O & \rightarrow \text{Glucose} + \text{Phosphate}. \quad \text{Equation 2.4}
\end{align*}

\begin{align*}
\text{GOD} \\
\text{Glucose} + O_2 & \rightarrow \text{Glucolactone} + H_2O_2.
\end{align*}

This assay appears somewhat cumbersome to perform and requires expensive reagents.
b. Amperometric Enzyme Linked Immunoassay.

The strategy for amperometric enzyme linked immunoassay is based on labelling the antigen with an enzyme that catalyses the production of an electrochemically detectable product. The rate at which the product is formed is related to the concentration of the analyte in the sample. Enzyme immunoassays rely on the antibody for specificity and the enzyme label for sensitivity through chemical amplification. Chemical amplification refers to the passing of a substance through a catalytic, cycling, or multiplication mechanism to generate a relatively large amount of product. In this way, a trace concentration of an analyte may result in significantly higher product concentrations, which for analytical purposes, can be more easily measured than the analyte itself.

Heterogeneous Immunoassay

Heterogeneous enzyme immunoassay with electrochemical detection include both competitive and sandwich assay formats.

Alkaline phosphatase (AP) has been one of the more commonly used labels in this type of assay. The use of this enzyme in amperometric immunoassays with phenyl phosphate as the substrate is widespread. Phenyl phosphate is electrochemically inactive, but its hydrolysis product, phenol, can be oxidised electrochemically:

Several heterogeneous enzyme immunoassays with electrochemical detection (ECD) have been demonstrated. They are based on the enzyme-linked immunoabsorbent assay (ELISA) technique in which antibody is typically immobilised on the walls of small volume (500μl) plastic cuvettes. The general procedure is outlined in Fig 2.1 for the determination of antigen. A competitive enzyme immunoassay has been demonstrated for digoxin, a
stimulant commonly used for the treatment of chronic heart disease. The therapeutic range is both low and very narrow (0.8-2.0 ng ml\(^{-1}\)) in the blood. Lower concentrations are not efficacious, whereas higher concentrations are toxic. Alkaline phosphatase, which catalyses the hydrolysis of the phenyl phosphate ester to phenol and phosphate, is used as the enzyme label. The enzyme generated phenol is easily detectable by either flow injection analysis with electrochemical detection (FIA-ECD) or liquid chromatography with electrochemical detection (LC-ECD), whereas phenyl phosphate is electroinactive and hence non interfering\(^{57}\). A schematic diagram of the instrumentation for these two methods is shown below in Fig 2.2. A detection limit of 50pg ml\(^{-1}\) has been achieved for digoxin which is well below the therapeutic range for the drug and illustrates the low detection capability of the technique. Another competitive assay has been developed for \(\alpha\)-acid glycoprotein\(^{115}\).

Recently a new substrate p-aminophenyl phosphate (PAPP) has been developed\(^{128}\) for use with alkaline phosphatase in amperometric enzyme linked immunoassays. Several immunoassays have been developed using this substrate by detecting its hydrolysis product p-aminophenol (PAP) at ca. +0.1V vs Ag/AgCl. At this very low potential there is no interference from protein oxidation.

The sandwich assay another widely used format Fig 2.1 in which the analyte is ‘sandwiched’ between two different antibodies, one of which is enzyme labelled has been demonstrated for IgG. Alkaline phosphatase is the enzyme label and the rate of phenol production is measured by LC-ECD\(^{116}\). A detection limit of 10pg ml\(^{-1}\) was achieved. Another sandwich type enzyme immunoassay has been described by Robinson et al\(^{118,119}\) and Gyss\(^{120}\).
Fig 2.1 General protocol for heterogeneous enzyme immunoassay
Fig 2.2 Schematic diagram of instrumentation for (a) Flow Injection Assay with Electrochemical detection (FIA-ECD) and (b) liquid chromatography assay with Electrochemical detection (LC-ECD).
Electrochemical immunoassays can also be based on high performance immunoaffinity chromatography with electrochemical detection of the column effluent. In this technique antibody is immobilised on a chromatography column. Antigen labelled with enzyme (Ag') is mixed with the sample (Ag) and injected into the immunoaffinity column where competitive binding occurs with antibody (Ab), as shown in Fig 2.3. Enzyme substrate is then injected. The amount of electroactive product formed is proportional to the amount of Ag in the sample. The electroactive product flows through a thin-layer detector where it is oxidised or reduced and quantitated from the area of the resulting current peak. A switching valve is then used to pass an acidic buffer through the column to displace bound Ag and Ag' in preparation for the next sample. The Ab-Ag reaction is sufficiently fast that reactions are more than 90% complete in 8-10s. The flow injection chromatography mode enables carefully controlled conditioning of the immunoabsorbents (e.g.,washing), which is essential to good experimental precision. It has been demonstrated that the affinity column can be used to perform a sandwich assay for IgG with a detection limit of 1 fmol in less than 30min. The second antibody is a glucose oxidase-goat anti IgG conjugate, and peroxidase is detected following injection of glucose into the affinity column. Other on line flow injection electrochemical enzyme immunoassays have been demonstrated for theophylline and human IgG using potentiometric detection.

The heterogeneous enzyme immunoassay with electrochemical detection has several advantages. The detection limit, as demonstrated for digoxin and IgG, is typically in the pg ml range and is a function of the antigen-antibody binding constant rather than the ability to detect phenol by LC-ECD. Consequently, even lower limits should be attainable.
Fig 2.3 Schematic diagram of Instrumentation for high performance Immunoaffinity Chromatography (HPIC)
Homogeneous Immunoassays.

A homogeneous enzyme immunoassay with the electrochemical detection of NADH has been developed for phenytoin, an antiepileptic drug. The phenytoin is labelled with glucose-6-phosphate dehydrogenase, which catalyses the reduction of NAD to NADH. The concentration of NADH is monitored by oxidation at +0.75V vs Ag/AgCl at a glassy carbon electrode in a flow-through thin layer cell. Antibody and proteins in the sample are removed by a reverse-phase C-18 LC precolumn to prevent adsorption on the electrode and consequent fouling. Assays of serum samples from patients on phenytoin treatment gave results that were in good agreement with a standard spectrophotometric procedure at therapeutic levels (10-20μg ml⁻¹).

A homogeneous enzyme immunoassay based on antibody inhibition of enzyme conversion from apo- to halo- form has been developed for 2,4-dinitrophenol-aminocaproic acid (DNP-ACA). A competitive equilibrium between the analyte (DNP-ACA) and the DNP-conjugate apoglucose oxidase (DNP-CAGO), which is the labelled hapten takes place. Flavin adenine dinucleotide, FAD, added to the mixture binds to free DNP-CAGO to give DNP-CAGO:FAD, which is enzymatically active and catalyses the production of hydrogen peroxide from oxygen. Because FAD cannot bind Ab:DNP-CAGO, the rate of hydrogen peroxide generation is a measure of the concentration of free DNP-CAGO, which in turn reflects the concentration of DNP-ACA in the sample. The rate of production of hydrogen peroxide is measured amperometrically by oxidation. This assay has a range of 2-40μg ml⁻¹.

The main advantage of homogeneous immunoassays, compared with heterogeneous assays, is the absence of a step for separating the antibody-bound antigen from the free antigen. Homogeneous assays are restricted to higher detection levels than are heterogeneous assays and are more susceptible to interferences from other sample constituents that would
otherwise be removed in a separation step.

c. Amperometric Immunoassay Utilizing Antigens Labelled with Electroactive Species.

This group of electrochemical immunoassays involve labelling an antigen with a group that renders the antigen electroactive. The labelled antigen (Ag⁺) would be reducible or oxidisable in a potential range over which Ag is electroinactive, thereby enabling Ag⁺ to be electrochemically distinguished from Ag.

Homogeneous Assays.

The homogeneous assay is based on competitive equilibrium between Ag and Ag⁺ for a limited amount of antibody, and relies on a decrease in the current signal for reduction-oxidation of Ag⁺ when bound to Ab.

This format has been demonstrated with estriol, an estrogen whose plasma and urine levels are related to pregnancy. Estriol is made electroactive by labelling with nitro groups in the 2 and 4 positions to give estriol-(NO₂)₂. A differential pulse polarogram of estriol-(NO₂)₂ gives distinctive reduction peaks in a potential window in which estriol is electroinactive. The addition of Ab causes peak height to decrease, as estriol-(NO₂)₂ is bound to Ab. Consequently the magnitude of the peak height can be used to monitor the binding, because it is determined by the distribution of estriol-(NO₂)₂ between the free and antibody-bound forms. In the assay the peak current grows in proportion to estriol as estriol-(NO₂)₂ is displaced from Ab, as predicted from the competitive equilibria.

This labelling strategy has also been used with other systems, such as morphine labelled ferrocene, which was detected by FIA-ECD at a glassy
carbon electrode\textsuperscript{57}, ovalbumin labelled with electroactive diazotized p-aminobenzoic acid, which was detected polarographically by reduction at a conventional dropping mercury electrode\textsuperscript{132,133}, p-(p-aminophenylazo)-phenyl arsonic acid, which was monitored by cathode ray polarography\textsuperscript{134}, and estriol labelled with mercuric acetate, which was detected by differential pulse polarography\textsuperscript{135}.

Immunoassays for human serum albumin (HSA) have been based on HSA labelled with Pb\textsuperscript{2+}\textsuperscript{136}, Co\textsuperscript{2+}\textsuperscript{137} and Zn\textsuperscript{2+}\textsuperscript{138}. The metal ion bound to the HSA can be detected with differential pulse polarography by reduction at a mercury electrode. The peak current for the labelled HSA decreases on binding with Ab, which is the basis for the immunoassays.

Heterogeneous Assays.

A heterogeneous format has been devised by Doyle and co-workers\textsuperscript{139} for larger molecules using human serum albumin modified by diethylenetriaminepentaacetic acid (DTPA) and labelled with indium (In\textsuperscript{3+}). Indium has not yet been found in human tissue, and, hence in clinically relevant analyses, interferences from the sample itself is avoided. This assay is a competitive assay using a fixed amount of antibody. The free and bound antigens are separated and the indium label is released from the DTPA complex by lowering the pH. The metal is detected using anodic pulse stripping voltammetry. This method is potentially a very sensitive method.

d. Potentiometric Immunoassays.

Proteins, being polyelectrolytes, have a net electrical charge except at their isoelectric point. Hence, in general, if both antibody and antigen have a net electrical charge and antigen-antibody binding is by Van der Waals hydrogen bonding and electrostatic forces\textsuperscript{140}, the electric charge of the resulting complex will be different from that of the antibody alone. The principle was
demonstrated by Janata\textsuperscript{141} by covalently binding concanavalin A to a polyvinyl chloride membrane deposited on a platinum wire. A potential change was observed when a polysaccharide, yeast mannan, was added to the solution, unfortunately a similar response was observed using a platinum wire without concanavalin A. The response of an ovalbumin electrode to its antibody was of the order of 2mV with reference to an electrode which contained immobilised serum proteins. Unfortunately, these potentiometric electrodes are rather prone to non specific binding and little success has been reported on their use.

A similar system was examined using a titanium wire onto which human chronic gonadotropin (HCG) or antibody against HCG were immobilised\textsuperscript{142}. These also showed small (<5mV) changes in the potential on addition of either the respective antibody or antigen. In general, these electrodes as yet have insufficient sensitivity for most practical uses.

e. Potentiometric Electrode Linked Immunoassays.

Conventional ion-selective electrodes have been used as detectors for immunoassays. Antibody binding measurements can be made with hapten-selective electrodes such as the trimethylphenylammonium ion (TMPA\textsuperscript{+}) electrode\textsuperscript{143}. Enzyme immunoassays in which the enzyme label catalyses the production of a product that is detected by an ion-selective or gas-sensing electrodes take advantage of the amplification effect of enzyme catalysis in order to reach lower detection limits. Systems for hepatitis B surface antigen\textsuperscript{144,145} and estriol\textsuperscript{84} use horse radish peroxidase as the enzyme label and an iodide electrode as the detector. Biotin\textsuperscript{146} and cyclic AMP\textsuperscript{147} have been determined using lysozyme and urease labels. The immunoreaction between human antibody IgG and peroxidase-labelled anti-human IgG antibody can be detected with a fluoride electrode\textsuperscript{148}. Adenosine deaminase, asparaginase and urease have been used as enzyme labels for immunoassays using potentiometric detectors with the ammonium gas-sensing membrane
electrode\textsuperscript{149}, and adenosine deaminase with the ammonium ion electrode\textsuperscript{150}. Carbon dioxide gas-sensing electrodes have been used for the determination of human IgG\textsuperscript{151} and digoxin\textsuperscript{152}.

A potentiometric ionophore - modulation immunoassay which is applicable to antigens of low molecular mass has been described by Keating and Rechnitz\textsuperscript{152,153}. The model antigen digoxin was covalently coupled to a potassium ionophore e.g., cis-dibenzo-18-crown-6- or benzo-15-crown-5- which was included in a polyvinyl chloride membrane fixed onto a conventional ion-selective electrode. The electrode was exposed to a constant concentration of potassium and a stable background signal was observed. When a suitable antibody was added, it bound reversibly to the antigen-ionophore conjugate molecules present at the membrane-solution interface, resulting in a potential change that was proportional to the antibody concentration. A competitive assay could be performed, if free antigen was present in solution.

An electrode of this type can also be based on a proton carrier\textsuperscript{154}. Immobilising an antigen in a membrane without benefit of an ion carrier also gives a potential response to specific antibody as demonstrated with a complex of cardiolipin antigen and Wassermann antibody\textsuperscript{155}.

Immunoassays have been based on the potentiometric measurement of marker ions such as tetrapentylammonium ion (TPA\textsuperscript{+}) that are loaded in phospholipid liposomes\textsuperscript{156-158}. Complement mediated immunolysis of these loaded vesicles is caused by the presence of appropriate antibodies. The amount of marker released is related to the antibody concentration. Similarly, complement and antibody levels have been measured using marker trimethylphenylammonium (TMPA\textsuperscript{+}) loaded sheep red blood cell ghosts in which released TMPA\textsuperscript{+} is measured with a TMPA\textsuperscript{+} ion selective electrode\textsuperscript{159}.

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2.4 Separation Systems in Heterogeneous Assays.

Classically, the separation of bound label from free in radioimmunoassay was achieved by adsorbing the free label with a suspension of charcoal\textsuperscript{160} or by precipitating the label bound to antibody with a second antibody and polyethylene glycol as precipitation accelerator\textsuperscript{162}. In each case the actual separation was achieved by centrifugation, and the amount of the bound fraction of label was generally determined. Though such procedures are still commonly used, solid-phase antibodies or antigens/haptens predominate in new assays because of their convenience and efficiency. A list of separation methods used in immunoassays is given Table 2.8.
Table 2.8 Some Separation Methods Used in Immunoassays\textsuperscript{163}

<table>
<thead>
<tr>
<th>Type</th>
<th>Regent/Solid -phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid-phase adsorption</td>
<td>Anion resin</td>
</tr>
<tr>
<td></td>
<td>Dextran-coated charcoal</td>
</tr>
<tr>
<td></td>
<td>Fluorosil</td>
</tr>
<tr>
<td>Liquid-phase precipitation</td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td></td>
<td>Polyethylene glycol;</td>
</tr>
<tr>
<td></td>
<td>Second antibody</td>
</tr>
<tr>
<td></td>
<td>Second antibody with polyethylene glycol</td>
</tr>
<tr>
<td>Solid-phase adsorption</td>
<td>Glass fibre membrane</td>
</tr>
<tr>
<td></td>
<td>Glass fibre membrane-latex</td>
</tr>
<tr>
<td></td>
<td>Large bead</td>
</tr>
<tr>
<td></td>
<td>Microtitre plate</td>
</tr>
<tr>
<td></td>
<td>Nylon membrane</td>
</tr>
<tr>
<td>Solid-phase indirect adsorption</td>
<td>Biotin-antibody, solid-phase -avidin</td>
</tr>
<tr>
<td></td>
<td>FITC-antibody, solid-phase anti-FITC</td>
</tr>
<tr>
<td></td>
<td>Microtitre plate-second antibody</td>
</tr>
<tr>
<td>Solid-phase precipitation</td>
<td>Magnetizable bead-antibody</td>
</tr>
<tr>
<td></td>
<td>&quot;Micro beads&quot;- antibody</td>
</tr>
<tr>
<td></td>
<td>&quot;Micro beads&quot; - second antibody</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus protein A</td>
</tr>
</tbody>
</table>


CHAPTER THREE.

VOLTAMMETRY, AMPEROMETRY AND FLOW INJECTION ANALYSIS
3.1 Voltammetry.

3.1.1 Introduction to Voltammetry.

Voltammetry may be defined generally as the measurement of current-voltage relationships at an electrode immersed in a solution containing electroactive species\(^1\). More specifically, it is the determination of the current at the working electrode, due to an electron transfer reaction at the electrode surface, while a potential is applied. Polarography is a branch of voltammetry which employs a dropping mercury electrode as the working electrode. Other branches of voltammetry employ stationary or solid working electrodes.

Rapid development of voltammetry occurred after 1922 when Heyrovsky discovered the technique of polarography\(^2\). Polarography, however, suffered from a number of shortcomings which made it less than ideal for routine analysis purposes until the late 1950's when modifications were introduced which helped to overcome some of the various problems associated with it\(^3\).

Between 1955 and 1965 a considerable number of polarographic techniques were developed e.g. alternating current and square wave polarography, normal and differential pulse polarography. Since the late 1960's there has been a lot of interest in the use of voltammetric techniques. This is because of the demands of environmental scientists for a large number of heavy metal analyses, and because of the appearance of greatly improved commercially available instruments at a relatively low cost\(^4\). In addition, because of the growing realization of its applicability in organic analysis, especially in the pharmaceutical industry, voltammetry has come to be more widely used again. The revival of the technique has mainly occurred in three areas:
a. Polarographic and related methods for use in biological systems especially the introduction of differential pulse polarography which offers greater sensitivity and resolution compared with conventional DC polarography. This technique has recently been applied to a vast number of problems involving the analysis of biological fluids.

b. The development of stripping voltammetric techniques which makes possible the determination of small amounts of both organic and inorganic substances at the $10^{-8}$M to $10^{-9}$M levels, and recently the development of adsorptive stripping voltammetry which reduces the detection limit to about the $10^{-11}$M level.

c. The development of electrochemical detectors, particularly for use with high performance liquid chromatography.

3.2 Theory of Voltammetry.

3.2.1 Fundamental Principles.

When a slowly changing potential ramp is applied to an electrode that is immersed in an electrolyte solution containing a redox species, a current will be observed to flow, rise rapidly to a peak and then gradually decay. The current arises from a heterogeneous electron transfer between the electrode and the redox species, resulting in either an oxidation or a reduction of the electroactive species. For a redox couple to be reversible in voltammetry the rate of electron transfer between the redox species and the electrode, must be rapid compared with the rate of mass transfer. Under these circumstances the ratio of concentrations of the oxidised and reduced forms of the couple at the electrode surface at a given potential (E) is described by the Nernst equation:
\[ E = E^o' + \frac{RT}{nF} \ln \left( \frac{[Ox]}{[Red]} \right) \]

Equation 3.1

where:

- \( n \) is the number of electrons transferred per molecule.
- \( E^o' \) is the formal reduction potential of the couple.
- \( \text{Red} \) is the reduced form of species.
- \( \text{Ox} \) is the oxidised form of species.
- \( R \) is the universal gas constant.
- \( T \) is the absolute temperature.
- \( F \) is the Faraday constant.

If the rate of electron transfer between the redox species and the electrode is very slow, relative to the mass transport of solution species to the electrode, then, irrespective of the electrode potential, the observed current will not be a function of mass transport. In this case, the low rate of electron transfer results in a concentration ratio of the two forms of the redox couple at the electrode surface that does not follow the Nernst equation. Current-voltage curves for these so-called irreversible processes are of limited analytical use. In some cases the current-voltage curve does not reach a peak or limiting value and so cannot be used for qualitative estimations of analyte concentrations. A reversible reaction may be rendered irreversible, simply by changing the rate of the mass transport to the electrode, and vice versa. An intermediate situation arises when the electron transfer and mass transport rates are comparable. These semi or quasi-reversible electrode reactions are quite common, and, their analytical use depends to a large extent on the careful control of the mass transfer rate of the electroanalytical method used for their study.

Electrode reactions are complex affairs. The dissolved redox form must move from the bulk solution to the electrode surface region. This step is dominated
by the kinetics of mass transport. Once in the electrode surface region, the redox form may be subject to a wide range of homogeneous or heterogeneous chemical reactions that may precede, follow, or run parallel to the electron transfer. Typical homogeneous reactions include protonation and dissociation of complexes. Heterogeneous chemical reactions include catalytic decompositions on the electrode surface, or the simpler cases of adsorption and desorption. The rates of the steps that take place in the electrode surface region will be determined by the kinetics of the chemical processes. Finally, after electron transfer, the electro-generated form of the redox couple, or the product of its participation in a chemical reaction must then diffuse back again into the bulk solution. Once again mass transport will determine the rate of this step.

Diffusion is one of the most important mass transfer mechanisms for bringing fresh electroactive species to the electrode. Diffusion occurs when there is a concentration gradient in a solution; in electrochemical measurements this gradient occurs at the electrode surface. When the potential of an electrode is pulsed from a quiescent region to one in which the electroactive species undergoes a reversible electrode reaction, the surface concentration of the electroactive form of the redox species drops to zero, whereas in the bulk solution it is finite. The layer of solution between the electrode surface and the point in solution at which the redox species concentration is unchanged is known as the diffusion layer. At first, the diffusion layer is thin, the concentration gradient is steep, and the current observed reaches a high value. As the time interval from the imposition of the pulse increases, the diffusion layer moves further out into the bulk solution, the concentration gradient decreases, and the current decays. Time is an important function in an electroanalytical measurement, both from the point of view of the reversibility of an electrode reaction, and with respect to the value of the observed current.

The current arising from the heterogeneous electron transfer (the faradaic current) is not the only current to flow at a voltammetric electrode. Changing
the potential of an electrode involves changing the charge density at its surface. The layer of solution immediately adjacent to the electrode behaves as a capacitor because, in response to the electrode's surface charge, charged species in this solution zone are orientated in an organised fashion. The orientation of charge in this region (known as the double layer) follows any variations in the electrode's charge density. Consequently, when the potential is scanned, the double layer responds, and this movement of charges in solution gives rise to a current in the electrochemical cell (the capacitance current). This current decays exponentially with time, as the double layer becomes fully re-orientated (charged).

The decay of the faradaic current with time is described by the Cottrell equation:

\[ I_0 = n F A D^{1/2} C (\pi t)^{-1/2} \]  

Equation 3.2

where:

- \( n \) is the number of electrons involved in the redox reaction
- \( F \) is the Faraday constant
- \( A \) is the electrode area
- \( D \) is the diffusion coefficient of the electroactive species
- \( C \) is the bulk concentration
- \( t \) is the time in seconds elapsed from the application of the potential pulse.

Although the current is a complicated function of \( t^{1/2} \), it is still directly proportional to the analyte concentration. Despite two types of current being generated by the voltage pulse, all that is actually measured is the total current. In many cases, the charging current is significantly larger than the faradaic current and therefore represents background noise against which the smaller faradaic current must be detected. Fortunately the charging current decays with time much more rapidly than the faradaic current.
3.2.2 Instrumentation.

The instrumentation of voltammetry and amperometry can be divided into three areas:

a. Waveform generation;

b. Potential control;

c. Electrochemical cell.

A schematic diagram of the function and inter-relationships of the above three primary elements is shown in Fig 3.1.

---

Fig 3.1 Instrumentation for Voltammetry and Amperometry
The simplest electrochemical cells consist of two electrodes immersed in a suitable electrolyte. In these cells the potential is applied across the entire cell, rather than across the working electrode-solution interface, and thus yields data which are considerably in error if solution resistances, and the resultant ohmic drop across the system are significant.

Modern instruments incorporate a potentiostat which controls the potential at the working electrode-solution interface, by making use of a three electrode system as shown in Fig 3.2.

![Fig 3.2 Schematic Diagram of a Potentiostat](image)

1 and 2 Operational amplifiers
R Resistance

Fig 3.2 Schematic Diagram of a Potentiostat
The three electrode system minimizes the effect of solution resistance and provides greater flexibility in the location of the reference and working electrode. The electrodes used in a three electrode control system are the working electrode, the reference electrode and the auxiliary electrode. The working electrode is the electrode at which the electrochemical phenomena being investigated takes place. The reference electrode, known also as the unpolarised electrode, is the electrode whose potential is constant enough to be taken as the reference standard, against which the potential of the working electrode in the cell can be measured. The auxiliary electrode, also known as the counter electrode, is the electrode that serves as a source or sink for electrons so that the cell current passes between it and the working electrode and no current passes through the reference electrode.

3.3 Voltammetric Techniques

3.3.1 Linear Sweep Voltammetry.

Nicholson and Shain have given a comprehensive treatment of single sweep voltammetry for simple systems and those with various chemical reactions coupled to reversible and irreversible charge transfers. The theoretical and analytical applications are reviewed by Bond and Bard and Faulkner.

Due to the rapid scanning of the potential in linear sweep voltammetry, the shape of the current-voltage curve is in the form of a peak. This peak occurs because the diffusion process is too slow to supply electroactive material to the electrode at a rate sufficient to keep up with the rapidly increasing potential. The waveform used and the signal produced in linear sweep voltammetry are shown in Fig 3.3.
The diffusion problem to a plane electrode for a reversible reaction was first solved independently by Randles\textsuperscript{12} and Sevick\textsuperscript{13}. The peak current is given by the following equation:

\[
i_p = 2.687 \times 10^5 \ n^{3/2} A \ D^{1/2} C \nu^{1/2}
\]

Equation 3.3

where:

- \( i_p \) is the peak current
- \( \nu \) is the potential scan rate.

The other variables are as given in equation 3.2
Matsuda and Ayabe\textsuperscript{14} determined the relationship between the peak potential ($E_p$) and half peak potential ($E_{p/2}$) in linear sweep voltammetry for the cases of reversible, quasi-reversible and totally irreversible systems.

For a reversible system:

\[ E_p - E_{p/2} = \frac{0.057}{n} \ V \text{ at } 25^\circ C \]  \hspace{1cm} \text{Equation 3.4}

For an irreversible system the corresponding equations are as follows:

\[ i_p = 2.985 \times 10^5 \ n(\alpha n_a)^{1/2}AD^{1/2}C_v^{1/2} \]  \hspace{1cm} \text{Equation 3.5}

and

\[ E_p - E_{p/2} = \frac{0.048}{\alpha n_a} \ V \text{ at } 25^\circ C \]

where:

- $\alpha$ is the electron transfer coefficient,
- $n_a$ is the number of electrons involved in the rate determining step

The other variables are as given in equations 3.2 and 3.3

As $(\alpha n_a)$ decreases the peak voltammograms become more spread out and the peaks tend to be rounded. This situation is frequently met in organic oxidations. The peak current is significantly less than the reversible electrode process, and the whole curve is more drawn out as $n_a$ decreases.

$E_p$ and $E_{p/2}$ for irreversible processes vary with potential sweep rate. This differentiates reversible from the irreversible processes, but the variation is only about $0.03 \alpha n_a$ volt per tenfold change in sweep rate.
3.3.2 Cyclic Voltammetry.

Cyclic voltammetry was first practised by Sevick\textsuperscript{13}. It is usually performed by varying the potential of the working electrode linearly with time until a switching potential is reached at which time the direction of potential sweep is reversed and the potential is reversed to the original value.

As in linear sweep voltammetry the forward scan in cyclic voltammetry causes a depletion of reactant species at the electrode surface, concomitant with a significant increase in the concentration of product near the electrode surface. Also the current behaves in the same way. When the scan direction is reversed, if the forward scan was cathodic then the product is oxidised back to the original starting material (for a simple redox reaction) and the current for the reverse process is recorded. The waveform used and the signal produced are shown in Fig 3.4.

\[ E \]

\[ i_{pa} \]

\[ i_{pc} \]

\[ R \rightarrow O \]

\[ O \rightarrow R \]

\( i_{pa} \) = Anodic Current  
\( i_{pc} \) = Cathodic current  
\( O \) = Oxidised species  
\( R \) = Reduced species

Fig 3.4 Potential Waveform and Measured Signal for Cyclic Voltammetry.
For a completely reversible system, the magnitude of the anodic peak current $i_{pa}$ is equal to the magnitude of the cathodic peak current $i_{pc}$. The potential difference between anodic and cathodic peaks for a reversible system is:

$$E_{pa} - E_{pc} = \frac{0.058}{n} \nu$$  \hspace{1cm} \text{Equation 3.6}

A quasi-reversible system shows a greater separation in $E_p$ values. The voltammograms are more drawn out and the peaks are more rounded.

For an irreversible system, a complete separation of anodic and cathodic peak potentials is observed. Cyclic voltammetry is a very useful technique for studying charge transfer rates$^{15}$ and for investigating the overall process which may occur in a complete electrode reaction$^{10}$.

3.3.3 Pulse Voltammetry.

Pulse voltammetric techniques tend to give better signal-to-noise ratios compared with linear sweep voltammetry because the faradaic current is larger and there is better discrimination against charging currents. When a potential pulse is applied to an electrode the capacitative current that flows is proportional to the magnitude of the pulse, and decays exponentially with time. The faradaic current, on the other hand, decays according to the square root of time. By measuring the current towards the end of the pulse the signal-to-noise ratio may be improved. This is done in normal pulse voltammetry.
The waveform used and the signal produced in normal pulse voltammetry are shown in Fig 3.5. The excitation waveform consists of successive pulses of gradually changing amplitude between which a constant base potential \( (E_b) \) is applied. The initial potential is usually chosen to lie in a region where none of the sample components are electroactive. The delay between pulses needs to be sufficient to allow the concentration profile near to the electrode surface to decay.

![Waveform and Measured Signal](image)

- \( \bullet \) = starting point for current measurements
- \( E_b \) = base potential

**Fig 3.5 Potential Waveform and Measured Signal for Normal Pulse Voltammetry.**

In differential pulse voltammetry, the current is measured just before the application of the pulse and towards the end of the pulse, giving a current which is the difference between the two measurements. The waveform used and the signal produced in differential pulse voltammetry are shown in Fig 3.6. The pulse width is often fixed at 50ms. For most cases a pulse amplitude
of 50mV or less is used. Large amplitudes provide an increase in response which must be balanced against the loss in resolution and the increase in charging current.

\[ E_b \]

\[ t \]

\[ E \]

\[ i \]

(a) Waveform  
(b) Measured signal

- = starting point for current measurements  
\( E_b \) = base potential

Fig 3.6 Potential Waveform and Measured Signal for Differential Pulse Voltammetry

3.3.4 Square Wave Voltammetry.

The major advantage of square wave voltammetry over other pulse techniques is the speed of analysis (especially useful for the dropping mercury electrode). The analytical signal in this technique is the difference between the current for the forward pulse and the current for the reverse pulse. Because of the large amplitude of the square wave, for a reversible reduction, the reduced electroactive species formed at the electrode during
the forward pulse is re-oxidised by the reverse pulse. Consequently, the sensitivity of this method is enhanced when compared to differential pulse voltammetry. Another advantage of square wave voltammetry is the discrimination against a wide range of background currents, and this ought to be advantageous when applied to solid electrodes.

3.3.5 Hydrodynamic Voltammetry.

Hydrodynamic voltammetry is the voltammetric technique where the mass transfer to the electrode surface occurs by forced convection rather than solely by diffusion. This is generally achieved by moving the electrode through the solution or by letting the sample solution flow past a stationary electrode. The recording of a voltammogram under these conditions is referred to as hydrodynamic voltammetry. The use of hydrodynamic voltammetry and more specifically hydrodynamic amperometry in continuously flowing solutions has seen a remarkable increase in interest largely due to the widespread use of electrochemical detectors in high performance liquid chromatography and flow injection analysis. Flow-through solid electrodes based on various configurations have been introduced in recent years\(^1\). The most popular cells are those in which the solution flows through a thin layer channel\(^1\), through an open tubular electrode\(^1\) or onto a wall jet electrode\(^1\).

Theoretical treatment of hydrodynamic voltammetry has been excellently covered in the monograph by Stulik and Pacakova\(^2\), while the principles of current distribution and mass transport in flowing systems have been reviewed by Newman\(^3\). Pungor et al\(^4\) comprehensively reviewed the theory, practice and applications of hydrodynamic voltammetry. Hydrodynamic voltammetry offers a better sensitivity under steady-state conditions than does classical voltammetry because of the increased mass transport to the electrode surface.
3.4 Polarographic Techniques.

3.4.1 Direct Current (DC) Polarography.

In classical DC polarography the potential-time waveform which is applied to a dropping mercury electrode (DME) is a linear increasing DC potential ramp similar to linear sweep voltammetry. The resulting current-voltage (i-E) curve obtained when an electroactive species is oxidised or reduced, takes the form of an S shape. When oxidation occurs, electrons are accepted by the DME and the current is anodic in nature. When reduction takes place, electrons are donated by the electrode and the current is cathodic. The basic principles of this technique have been well established by Heyrovsky and Kuta\textsuperscript{23}.

3.4.2 Normal Pulse Polarography (NPP).

Pulse polarography was originally developed by Barker\textsuperscript{24} in 1960 and became widespread with the development of practical instrumentations in about 1970. An extensive review of the theory and application of normal pulse polarography was made by Osteryoung and Hasebe\textsuperscript{25} and Bond\textsuperscript{9}.

3.4.3 Differential Pulse Polarography (DPP).

DPP is the most widely used form of pulse polarography. The principles and theory of DPP have been covered by Parry and Osteryoung\textsuperscript{26} and Barker and Gardner\textsuperscript{24}.
3.5 Theory of Amperometry.

3.5.1 Fundamental Principles.

Amperometry is the measurement of the current at an electrode immersed in a solution containing an electroactive species at a fixed single applied potential.

The applied potential at which the current measurement is made is usually selected to correspond to the mass transport limited portion of the voltammetric scan. The current obtained at conventional electrodes under such circumstances gradually decays to zero according to the Cottrell equation already described (equation 3.2). This decrease in current is due to the slow spread of the diffusion layer out into the bulk solution, with a concomitant decrease in the concentration gradient. In practice, this process continues for about 100s or so, after which time random convection processes in the solution take over, and put an end to further movement of the diffusion layer.

The advantage of amperometric measurements is that the faradaic currents are observed at fixed electrode potentials. In these circumstances, capacitative currents no longer contribute to the overall cell current, and much lower detection limits are obtainable compared to linear sweep voltammetry. However, some variants of amperometry do involve pulsing the electrode potential to the active region; measurements in these cases need to be made carefully to produce optimum signal-to-noise ratios.

3.5.2 Hydrodynamic Amperometry.

Mass transport in amperometric systems in which the reagent stream is forced to flow along the surface of the electrode may be described in terms of convective diffusion. Effectively this means that at sufficiently high values of
Pe, the Peclet number, the liquid above an electrode may be divided into two distinct zones. In one zone, far away from the electrode surface, convection is important, and the concentration profile is substantially flat. In the other zone, adjacent to the electroactive surface, there is a sharp concentration gradient: here diffusion is the predominant mass transport process. The Peclet number is given by \( v_m l / D \), where \( v_m \) is the main stream fluid velocity, and \( l \) is the length of the electrode (measured in the direction of fluid flow). Under these conditions, the mass transport limited current \( i_L \) for a reversible electrode couple (i.e. the concentration of the electroactive form is zero at the electrode surface) is given by,

\[
i_L = n F A D^{1/3} v_k^{-1/6} x^{-1/2} (v_m)^{1/2} C
\]

Equation 3.7

where:

\( v_k \) is the kinematic velocity,
\( x \) is the distance along the electrode.

The other variables are as given in equation 3.2.

This current is time dependent, but in the convective diffusion case, the current rapidly reaches a stationary value. The speed with which the current plateau is reached arises from the establishment of a well defined steady state diffusion layer.

3.5.3 Pulsed Amperometry.

The application of potential pulses of fixed amplitude to a constant base potential is the amperometric equivalent of pulse voltammetry. The current is measured in a similar way to differential or normal pulse voltammetry. The pulse waveforms have been used to improve the selectivity of amperometric detection in flowing systems and in some cases to overcome adsorption of electrochemical reaction products.
The detection limits using these pulse techniques are consequently poorer than for DC amperometry\textsuperscript{27,28}. The improved selectivity\textsuperscript{29,30} is therefore only useful where the analyte is present at sufficiently high concentrations. The decrease in adsorption of reaction products and impurities present in the carrier have been reported for pulse amperometry applied to glassy carbon\textsuperscript{27}, platinum\textsuperscript{31}, and mercury film electrodes\textsuperscript{30}.

3.5.4 Pulsed Amperometric Detection. (PAD).

Pulsed amperometric detection (PAD) is a relatively new technique, and has made possible the direct amperometric determination of many compounds that were at one time unsuitable for this type of measurement. These include carbohydrates, amino acids, halides and sulphur compounds\textsuperscript{32,33}.

The application of the PAD waveform to platinum and gold electrodes enables compounds to be detected without the loss of electrode activity due to the electrochemical cleaning of the electrode\textsuperscript{34}.

The method usually relies on the adsorption of analyte onto the electrode surface, where catalysis of the analyte oxidation occurs, by metal oxides. These compounds need not be electroactive in order to be detected, the suppression of surface oxide formation enables the analyte to be detected as a negative peak.

3.6 Electrodes.

A variety of electrode materials have been employed as voltammetric electrodes and their utility is determined mainly by their useful potential range and by the size and reproducibility of their currents in the medium used in the experiment.
The electrodes used in voltammetry can be divided into three types:

a. mercury type electrodes;
b. noble metal electrodes;
c. carbon electrodes;
d. modified electrodes.

Voltammetric studies of solid electrodes are playing an increasing part in the interest of the chemical industry in organic oxidation-reduction processes. In studying the mechanism of electrode reactions, the use of stationary electrodes with a cyclic potential scan makes it possible to investigate the products of the electrode reaction and detect electroactive intermediates. Furthermore, the time scale for the method can be varied over an extremely wide range, and both relatively slow and fairly rapid reactions can be studied with a single technique.

The types, utility and applications of solid electrodes together with the relevant theory are reviewed by Adams¹ and Murray³⁵.

3.7 Flow Injection Analysis.

3.7.1 Introduction.

The term flow injection analysis (FIA) was first used by Ruzika and Hansen³⁶ in 1975 to describe the use of sample injection into an unsegmented flowing stream for rapid continuous-flow analysis. FIA is now widely used and well established and has the following advantages; its simple basis, relatively inexpensive equipment, handy operation and great capacity for achieving results that are excellent in view of the rapidity, accuracy and precision with which they are obtained. FIA is based on four main principles:
a. unsegmented flow  
b. direct injection  
c. controlled partial dispersion  
d. reproducible operational timing.

The basic scheme of an FIA system is shown in Fig 3.7. An FIA system usually consists of the following components:

a. A propelling unit which should produce a steady pulse-less flow of one or more solutions. The solutions may be of dissolved reagents or merely a carrier for the sample plug. The most common method of propulsion is a peristaltic pump, other methods include gas pressure systems and gravity feed reservoirs.

b. An injection system which allows the reproducible introduction of a volume of sample solution into the flow without stopping it.

c. A length of tubing along which the transport operation takes place, this is referred to as the reactor. Dispersion of the sample plug into the carrier or reagent takes place as the plug passes along the tube, also there may be chemical reactions occurring.

d. A flow cell, accommodated in a detector (e.g., colorimeter or photometer) which transduces some property of the analyte into a continuous signal to a recorder or a microcomputer.
The flow emerging from the sensing system usually goes to waste, although it is sometimes recirculated through the peristaltic pump to achieve greater constancy of flow rate.

The signal obtained from FIA techniques is transient. A typical FIA peak is shown in Fig 3.8. The parameters affecting the peak shape are:

a. the flow rate of the carrier;
b. the volume injected along with the length and bore of the sample loop;
c. the length and bore of the manifold;
d. the viscosity of the sample and carrier;
e. the detector and recording system time constant.
3.7.2 Theory of Flow Injection Analysis.

The theory and applications of FIA are covered by review articles$^{37-41}$ and books$^{42,43}$.

In FIA a sample injected into a carrier stream flowing through a narrow bore straight section of tube initially exists as a well defined plug. As the plug travels downstream it disperses and mixes with the carrier stream. A well defined concentration gradient is formed. If the sample dispersion is due to
convection, the flow profile is characterised by a parabolic head and tail. This type of flow is called laminar flow, and is characteristic of most flow injection systems. If this were the only mass transport process operating, the peak would have an infinitely long tail, as the velocity at the walls of the conduit is zero. Two additional mass transport processes are operational though; molecular diffusion in the longitudinal direction (parallel to the direction of flow) and molecular diffusion in the radial direction (perpendicular to the direction of flow). The longitudinal diffusion is small compared with the dispersion due to the flow velocity and can be ignored under the conditions of most flow injection experiments. Radial diffusion moves sample molecules to and from conduit walls where the flow velocity profile is zero towards the centre of the tube, where the flow velocity is at a maximum.

The net result of radial diffusion is that a peak with a finite peak width is obtained. Radial diffusion becomes more important as the residence time of the sample increases. At long residence times, the dispersion product process is controlled primarily by the diffusion process and the peak shape assumes a symmetrical Gaussian shape.

The dispersion or dilution of a sample in FIA is given by the following equation:

\[ \Sigma = \frac{C_0}{C} \]  

Equation 3.8

where:
\( \Sigma \) is the dispersion  
\( C_0 \) is the concentration of sample  
\( C \) is the concentration of sample at the peak maximum calculated from the peak response of the detector due to the analyte.
The degree of dispersion of the sample zone depends on the tube length and radius, the flow rate, the sample volume injected and the molecular diffusion coefficient of the species concerned.

By changing the flow parameter, the dispersion can be easily manipulated to suit the requirements of a particular analytical procedure so that optimum response is obtained at minimum time and reagent expense.

The dispersion types fall into three categories: limited, medium and large. The peak shape varies from sharp and asymmetric for limited dispersion, Gaussian for medium dispersion and broad with exponential peak shape for large dispersion.

3.7.3 Flow Injection Analysis with Electrochemical Detection.

The combination of amperometric detectors with FIA is a very convenient method of detecting electroactive species provided that the sample matrix is fairly simple.

In recent years amperometric detectors have gained popularity because they are sensitive, their response is generally rapid, and they can be applied to a relatively broad range of organic and inorganic compounds. The theoretical and practical aspects of amperometric detectors used in flow injection systems have been discussed by Pungor et al. Kissinger has listed several electrochemical liquid chromatography detectors that could be adapted for FIA. The construction of amperometric detectors has been discussed by Stulik and Pacakova and Fleet and Little. Reviews of electrochemical detection in flowing systems, including voltammetric, polarographic and amperometric detectors have been published.
The measuring technique in flow injection amperometry involves monitoring the limiting current at a constant electrode potential. This technique has many advantages when used in flowing systems if compared with the linear sweep voltammetric technique when used in static systems. These advantages are:

a. The current is increased because of increased mass transport to the electrode, the diffusion layer being thinner in flowing streams.

b. The background current is decreased because at constant potential no current is needed to change the double layer and the oxidation states of the functional groups in the electrode surface are in equilibrium.

The requirements of the measuring cell in a flowing system are that its dead volume is as small as possible with hydrodynamic conditions permitting the highest reproducibility and sensitivity, and the impedance between the electrodes is minimal.

The most popular flow through cells which have been used as amperometric detectors in flowing systems are tubular, wall-jet and thin layer flow cells. The wall-jet in particular offers the advantages of very high sensitivity, extremely small dead volume and relative freedom from surface adsorption. In this configuration, which is used in this project, a jet of fluid strikes a wall (electrode surface) perpendicularly and then spreads radially over the surface of the wall.

Yamada and Matsuda described the construction and the electrochemical response of a wall-jet cell in terms of the hydrodynamic conditions. The equation they derived defines the performance of the wall-jet electrode in terms of the volume flow rate, diameter of the nozzle, and radius of the electrode. The equation is given by:
\[ i_L = 1.38 \, n \, F \, C \, D^{2/3} \, v_k^{-5/12} \, V_m^{3/4} \, a^{-1/2} \, r^{3/4} \] \quad \text{Equation 3.9}

where:

- \( i_L \) is the limited current
- \( v_k \) is the kinematic velocity
- \( V_m \) is the flow rate
- \( a \) is the inlet diameter
- \( r \) is the radius of the disk electrode.

The other variables are as given in equation 3.2
REFERENCES


CHAPTER FOUR

VOLTAMMETRIC AND AMPEROMETRIC STUDIES OF THEOPHYLLINE AND RELATED COMPOUNDS AND ALKALINE PHOSPHATASE SUBSTRATES AND THEIR HYDROLYSIS PRODUCTS.
4.1 Instrumentation, Equipment and Chemicals.

4.1.1. Instrumentation.

Metrohm E611 VA-Detector.

This instrument is a potentiostat, suitable for amperometric methods. Voltammetric methods were used with this instrument connected to a Metrohm E612 VA-Scanner unit. The Metrohm E611 VA detector is capable of performing the following operations:

a. DC Amperometry;
b. DC Tast Amperometry;
c. Differential Pulse Amperometry DP1 (DP1: duration of pulses superimposed on the base potential = 60ms; integrated current measurement for 20ms immediately before and at the end of the pulses).
d. Differential Pulse Amperometry DP2 (DP2: duration of pulses superimposed on base potential = 160ms; Current integration as far as DP1).

Metrohm E612 VA-Scanner.

When connected to the above potentiostat the Metrohm E612 VA-scanner is capable of performing the following methods:

a. Single sweep voltammetry;
b. Triangular-wave voltammetry;
c. Cyclic wave voltammetry.
Metrohm E506 Polarecord.

This instrument was used for polarographic measurements. The Metrohm E506 Polarecord comprises four main functional subunits:

a. Voltage generator;
b. Potentiostat;
c. Current evaluator;
d. Current recorder.

and can perform the following operations:

a. DC polarography;
b. Phase-selective AC polarography;
c. Pulse polarography;
d. Kalousek polarography;
e. Inverse voltammetry;
f. Potentiostatic coulometry.

The polarography stand is a piece of apparatus for polarographic measurements with both free-dropping and controlled dropping mercury electrodes. The electronic circuitry for controlled dropping is incorporated in the control unit of the E506 Polarecord.

Dionex Ionochrom™ / Pulsed Amperometric Detector.

The Pulsed Amperometric Detector consisted of a potentiostat which applies a repeating sequence of up to three different, selectable applied potentials ($E_1$, $E_2$, $E_3$) to the cell working electrode. Each potential is applied for a selectable period of time or pulse duration ($t_1$, $t_2$ and $t_3$ respectively).
In all modes of operation the potentiostat measures current only during the $E_1$ pulse. Sampling of the current occurs 20ms before the end of the $E_1$ pulse. Use of a 1/50 second (20ms) sampling period cancels the 50Hz line noise. The sampled current is amplified and held by a sample-and-hold amplifier which returns the value to the recorder. The following modes of operation were available:

a. DC amperometry ($t_1>0$, $t_2=0$ and $t_3=0$);

b. Double pulse amperometry ($t_1>0$, $t_2>0$ and $t_3=0$);

c. Triple pulse amperometry ($t_1>0$, $t_2>0$ and $t_3>0$).

Chart Recorder.

Gould HR 2000
Linseis L6512

4.1.2. Equipment.

Peristaltic pump.

An LKB peristaltic pump was employed to propel the eluent in the flow injection system. The pump was capable of propelling three channels if three pump heads were fitted. The pump head consisted of ten rollers. Pump tubes of PVC were used throughout the project. Different pump flow rates were achieved either by changing the diameter of the pump tubes or by increasing the speed of the pump head by means of a switch. The pump generated amplitude pulsing which produced a signal noise at the detector at high current sensitivities.
Injection Valve.

The injection valve used in the flow injection system was a low pressure Teflon rotary valve Rheodyne 5020. All parts of the valve which are in contact with the eluent are of Teflon construction, the remainder are stainless steel and polypropylene. The valve has six ports, two ports to form the loop, one port for injection of the sample, one for excess of injected sample and the last two are for the inlet and the outlet of the eluent towards the detector.

Electrochemical Cell.

The wall jet cell used was designed by Taylor\textsuperscript{1} based on the recommendations of Yamada and Matsuda\textsuperscript{2} and Gunasingham and Fleet\textsuperscript{3}. The cell was constructed from PCTFE because of its chemical inertness and good machining properties. For details of its construction refer to reference \textsuperscript{1}.

Electrodes.

The following Metrohm electrodes were used:

Metrohm glassy carbon electrode; diameter of active zone: $4.8\pm0.05\text{mm}$, o.d. 7mm.

Metrohm gold electrode diameter; of active zone as above.

Metrohm platinum electrode diameter; of active zone: $3.0\pm0.5\text{mm}$, o.d. 7mm.

Pulse Damper.

A pulse damper was constructed from glass with a platinum wire ground connection (\textit{Fig 4.1}). This platinum wire eliminated the static electricity pulses generated by the peristaltic pump.
Polishing equipment.

The electrodes were cleaned using coarse and fine alumina powder and Bhuler polishing pads.

The alumina powder was supplied by Dionex Corporation, CA, USA and the Bhuler pads obtained from Anachem.

pH meter.

The pH values of solutions used in the experimental work were measured on a Philips PW9240 pH meter fitted with a combined glass electrode. The instrument response was checked before use with standard buffer solutions of pH 4.00 ± 0.01, 7.00 ± 0.01 and 10.00 ± 0.01.

Miscellaneous.

PTFE transmission tubes, flow injection connectors and peristaltic pump tubing were obtained from Omnifit Ltd.
### 4.1.3 Chemicals.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>Sigma</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Catechol</td>
<td>Fisons</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>Fisons</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate</td>
<td>BDH</td>
</tr>
<tr>
<td>Ethylaminoethanol</td>
<td>Aldrich</td>
</tr>
<tr>
<td>α-Naphthol</td>
<td>Sigma</td>
</tr>
<tr>
<td>α-Naphthyl Phosphate Disodium</td>
<td>Edward Guur Ltd.</td>
</tr>
<tr>
<td>Nitrogen Gas</td>
<td>BOC</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>Sigma</td>
</tr>
<tr>
<td>p-Nitrophenyl Phosphate Disodium</td>
<td>Sigma</td>
</tr>
<tr>
<td>Palladium/Charcoal Catalyst</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Phenol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phenolphthalein</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phenolphthalein Diphosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium Dihydrogen Orthophosphate</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium Hydrogen Carbonate</td>
<td>BDH</td>
</tr>
<tr>
<td>Theobromine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
4.1.4 Buffer Solutions.

Acetate Buffer (0.25M, pH 4.6)

This buffer was prepared by mixing equal volumes of 0.5M acetic acid and 0.5M sodium acetate solutions. The pH was checked with a calibrated pH meter and adjusted accordingly.

Phosphate Buffer (0.1M, pH 7.0).

This buffer was prepared by making up 95ml of a 0.2M solution of sodium dihydrogen orthophosphate to 500ml with a 0.2M solution of disodium hydrogen orthophosphate then diluting to 1 litre with distilled water. The pH was checked with a calibrated pH meter and adjusted accordingly.

Carbonate Buffer (0.05M, pH 9.0 and pH 9.6).

These buffers were prepared by dissolving 1.59g of sodium carbonate and 2.92g of sodium hydrogen carbonate in 1 litre of distilled water. The pH was checked with a calibrated pH meter and adjusted accordingly.

Diethanolamine Buffer (1M, pH 9.8).

This buffer was prepared by adding 880ml of distilled water to 120ml of diethanolamine and adjusting the pH accordingly.

Ethylaminoethanol Buffer (1M, pH 9.8).

This was prepared by adding 900ml of distilled water to 100ml of ethylaminoethanol and adjusting the pH accordingly. The pH of all buffer solutions in this and following chapters were adjusted with either 5M hydrochloric acid or 5M sodium hydroxide.
4.2 Voltammetry of Theophylline and Related Compounds.

4.2.1. Introduction.

Over the past two decades extensive studies have been carried out into the electrochemical behaviour of biologically important compounds and their precursors. One such group includes biologically important N-methylxanthines.

The mechanism for the electrochemical oxidation of xanthine and several other naturally occurring N-methylxanthines including theophylline, caffeine and theobromine was elucidated by Hansen and Dryhurst⁴⁵ using linear and fast sweep cyclic voltammetry.

The electrochemical oxidation of xanthine and N-methylxanthines proceeds by a two electron oxidation of the \(-N_9=C_\beta\) double bond to give the appropriately substituted uric acid (II, Fig 4.2), which being more readily oxidised than the parent compound (I, Fig 4.2), is immediately further oxidised in a two electron process to a uric acid-4,5 diol (III, Fig 4.2). This primary electrochemical product (III) is extremely unstable as a result of steric factors so it rapidly fragments to give the appropriately substituted alloxan (VI), allatoin (VII) and urea (V) along with carbon dioxide and ammonia (Fig 4.2). A very small amount of the uric acid-4,5 diol intermediate (III) is further oxidised to a parabanic acid.
Fig 4.2: The Pathways for the Electrochemical Oxidation of N-methyl Xanthines at a Pyrolytic Graphite Electrode.

(I) Methyl xanthine; (II) Methyl Uric Acid; (III) Methyl Uric Acid-4,5 diol; (IV) Methyl Parahemic; (V) Methyl Urea; (VI) Methyl Alloxan; (VII) Methyl Allatoin.
4.2.2 Linear Sweep Voltammetry of Theophylline and Related Compounds.

Experimental

The linear sweep apparatus consisted of the Metrohm E611 VA-detector, Metrohm E612 VA-Scanner, a Gould HR 2000 XY recorder and an electrochemical cell (working electrode, stainless steel auxiliary electrode and a saturated calomel reference electrode i.e. SCE).

Linear sweep voltammograms were obtained for theophylline, theobromine and caffeine in aqueous solutions over the pH range 4.6 to 9.0. Sodium acetate buffer of ionic strength 0.25M, pH 4.6; phosphate buffer of ionic strength 0.1M, pH 7.0 and carbonate buffer of ionic strength 0.05M, pH 9.0 were used as the background electrolyte solutions in this study and were prepared as described in section 4.1.4. 1 mM solutions of theophylline, theobromine and caffeine were prepared using the above buffer solutions which had been degassed before use. A glassy carbon electrode (Section 4.1.2) was used in this study. The electrode was polished using a slurry of alumina and rinsed with distilled water before placing it in the buffer solution. The electrode was conditioned in the background electrolyte solution by scanning three times between the potential range to be used in the experiment, starting from the lower potential. The scan being a linear sweep voltammogram, scan rate 5mV/s. The background scan was recorded and then the sample to be investigated was placed in the cell. The electrode was taken out of the cell and repolished as before and the process repeated for each compound in each buffer solution in turn.

Results and Discussion.

The voltammograms for the oxidation of theophylline, theobromine and caffeine in acetate buffer pH 4.6 are shown in Figs 4.3 - 4.5. Table 4.1 shows the effect of pH on the oxidation potentials of these compounds.
Fig 4.3 Linear Sweep Voltammogram of a 1mM Solution of Theophylline in 0.25M Acetate Buffer pH 4.6

----- = Background Electrolyte: Acetate Buffer pH 4.6

Fig 4.4 Linear Sweep Voltammogram of a 1mM Solution of Theobromine in 0.25M Acetate Buffer pH 4.6

----- = Background Electrolyte: Acetate Buffer pH 4.6
Table 4.1: The effect of pH on the Oxidation Potentials of Theophylline, Theobromine and Caffeine.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Peak Oxidative Potential vs. SCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Theophylline</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.6</td>
<td>+1.11</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.0</td>
<td>+1.02</td>
</tr>
<tr>
<td>Carbonate</td>
<td>9.0</td>
<td>+0.85</td>
</tr>
</tbody>
</table>

One of the main aims of this project was to investigate the determination of theophylline by electrochemical detection. The peak oxidation potential for theophylline was found to be greater than +1.0V vs SCE. Voltammetry at these potentials tend to produce more noise, electrode failure and extensive...
interference mainly from other organic compounds. The results illustrate the interference of theophylline determination by theobromine and caffeine present in diet. It has also been reported that 3-methyl xanthine a theophylline metabolite interferes with theophylline determination by electrochemical determination. The oxidation potentials of the three compounds were found to be dependent upon the pH. Increasing the pH did not improve the resolution of the oxidation potential of theophylline.

The disadvantages of the direct determination of theophylline by electrochemical detection are:

a. The high oxidation potential of theophylline;
b. The severe interference of theophylline metabolites and structurally similar compounds.

Based on these findings this mode of determination of theophylline was not further pursued.

4.3 Voltammetry of Alkaline Phosphatase Substrates and Hydrolysis Products.

4.3.1 Introduction.

Alkaline phosphatase (EC.3.1.3.1. orthophosphoric monoester phosphohydrolase) is a commonly used enzyme label. This enzyme hydrolyses orthophosphoric monoesters yielding inorganic phosphate and the corresponding alcohol, phenol, etc.

There are four main types of substrates available for alkaline phosphatase assays; the first group includes β-glycerophosphates and hexose phosphates, the hydrolysis products of which can, for all practical purposes, be estimated only by the measurement of the liberated phosphates.

A second group of substrates is represented by phenyl phosphate and
β-naphthyl phosphate\textsuperscript{11}. Phenol and β-naphthol resemble inorganic phosphates in requiring chromogens to make them measurable by colorimetric means; however, the blank values of the hydrolysis products liberated from these substrates are quite low.

The third group of substrates is typified by p-nitrophenyl phosphate\textsuperscript{12}, phenolphthalein diphosphate\textsuperscript{13,14} and 4-methylumbelliferyl phosphate\textsuperscript{15}. These substrates liberate "self-indicating" products that are visible or fluorescent, although some may require the addition of alkali for full color development.

The fourth group is represented by phosphoenolpyruvate\textsuperscript{16}. Alkaline phosphatase hydrolyses this compound with the release of free pyruvate. A second enzyme (lactic acid dehydrogenase) converts the pyruvate to lactate with the coupled disappearance of NADH. This second reaction is monitored spectrophotometrically.

Enzyme products are commonly detected by spectroscopic techniques\textsuperscript{17,18}, however absorbance measurements lack high sensitivity while fluorescence detection often suffers from endogenous interferences. The wide dynamic limits of electroanalytical techniques offers an attractive alternative to spectroscopic methods.

In the following sections, the electrochemical characteristics of several substrates for alkaline phosphatase [ p-nitrophenyl phosphate (PNPP), p-aminophenyl phosphate (PAPP), phenyl phosphate (PP), α-naphthyl phosphate (αNP), phenolphthalein diphosphate (PDP)] and their hydrolysis products are investigated.
4.3.2 Polarography of Phenolphthalein Diphosphate and Phenolphthalein.

Experimental.

Polarography was carried out using a Metrohm E506 Polarecord with polarographic stand. A three electrode system was employed with the polarograph using a conventional dropping mercury working electrode, a platinum counter and a saturated calomel reference electrode. The polarographic cell was a double walled glass vessel with a perspex top cover. The top had an O-ring fitted tightly onto the cell. There were a few holes on the perspex top to enable the three electrodes and the deoxygenation train to be placed in the solution in the cell. The polarography stand contained a two way tap which was connected at the end of the deoxygenation train to keep nitrogen gas over the solution throughout the duration of the polarographic analysis.

Differential pulse polarograms were recorded for phenolphthalein and phenolphthalein diphosphate (5mM) dissolved in 0.05M Carbonate buffer pH 9.6, between -0.6 and -1.6V.

Results and Discussion.

Reduction peaks for phenolphthalein were observed at -0.98V and -1.3V (Fig 4.6). According to Koltchoff and Lehmicke\textsuperscript{19} phenolphthalein in solution exists in different forms which are in equilibrium (Fig 4.7). At pH values of about 10 the red form predominates. The reduction of the red form occurs in two, 1e\textsuperscript{−} steps, resulting in the formation of the trivalent anion of phenolphthalein (Fig 4.8).

120
Fig 4.6 Differential Pulse Polarogram of:

(a) 5mM Solution of Phenolphthalein in 0.05M Carbonate Buffer pH 9.6
(b) Background Electrolyte 0.05M Carbonate Buffer pH 9.6
Fig 4.7 Different forms of Phenolphthalein in Solution\textsuperscript{19}
IV = Red form of Phenolphthalein
VI VII = reduction intermediates
VIII = Trivalent anion of Phenolphthalein

Fig 4.8 Reduction Mechanism of Phenolphthalein in Alkaline Medium$^{19}$
A differential pulse polarogram of phenolphthalein diphosphate (Fig 4.9) showed that it was reduced at a potential similar to that for the reduction of phenolphthalein. From these results the phenolphthalein/phenolphthalein diphosphate system is not suitable for the construction of an electrochemical enzyme immunoassay since both substrate and product are electroactive in the same region.

4.3.3 Voltammetry of Phenyl Phosphates and Their Hydrolysis Products.

Experimental.

Cyclic voltammetry apparatus was the same as that used for linear sweep voltammetry (see section 4.2.2). The glassy carbon working electrode was cleaned and conditioned in the same manner as in section 4.2.2. and the solutions were degassed before use.

1mM solutions of the substrates (PNPP, PAPP*, PP, αNP) and the products (p-nitrophenol, p-aminophenol, phenol, α-naphthol and catechol) were prepared in 0.05M carbonate buffer pH 9.6 and cyclic voltammograms obtained. The buffer was prepared as previously described (4.1.4). * refer to section 4.3.5 for the synthesis of PAPP.

Results and Discussion.

Typical voltammograms of the products are shown in Figs 4.10-4.14. The oxidation potentials of the substrates and products are given in Table 4.2. o-Hydroxyphenyl phosphate could not be prepared by the prescribed method\(^{20}\), therefore, its oxidation potential could not be determined. The voltammograms illustrate that the products can be detected independently without any interference from the substrates. This is an important criterion for the development of an electrochemical enzyme immunoassay.
Fig 4.9 Differential Pulse Polarogram of:

(a) 5mM Solution of Phenolphthalein Diphosphate in 0.05M Carbonate Buffer pH 9.6

(b) Background Electrolyte 0.05M Carbonate Buffer pH 9.6
From the voltammograms of the products Figs 4.10-4.14 only p-aminophenol and probably catechol show electrochemical reversibility. p-Aminophenol, catechol and α-naphthol are much easier to oxidise than phenol and p-nitrophenol. This is advantageous because background noise generally increases with applied potential for electrochemical detection, therefore, detection of these compounds would be more selective and lower limits of detection would be achieved.

Table 4.2:- The Peak Oxidation Potentials of Alkaline Phosphatase Substrates and their Hydrolysis Products.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxidation potential (V) vs SCE</th>
<th>Product</th>
<th>Oxidation Potential (V) vs SCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminophenyl phosphate</td>
<td>+0.52</td>
<td>p-Aminophenol</td>
<td>+0.03</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>+1.46</td>
<td>p-Nitrophenol</td>
<td>+0.82</td>
</tr>
<tr>
<td>Phenyl phosphate</td>
<td>+1.45</td>
<td>Phenol</td>
<td>+0.50</td>
</tr>
<tr>
<td>α-Naphthyl phosphate</td>
<td>+1.11</td>
<td>α-Naphthol</td>
<td>+0.35</td>
</tr>
<tr>
<td>α-Hydroxyphenyl phosphate</td>
<td>-,-</td>
<td>Catechol</td>
<td>+0.17</td>
</tr>
</tbody>
</table>

The effect of repetitive cyclic voltammetry scans on the electrode performance was investigated. Fig 4.15 shows that there was very little fouling of the electrode by the oxidation products of p-aminophenol. Greater fouling was observed for catechol, p-nitrophenol, and phenol. Fouling of the electrode by phenol was found to be dependent on the concentration. Two consecutive cyclic voltammograms of a 1mM solution of phenol resulted in a total loss of the oxidative signal (Fig 4.16a) while the same number of scans for a 0.1mM solution resulted in a 50% decrease of the oxidative signal (Fig 4.16b). A similar pattern was observed for α-naphthol. The effect of p-aminophenol, catechol and p-nitrophenol concentration on the electrode fouling was not investigated.
Fig 4.10 Cyclic Voltammogram of a 1mM Solution of p-Aminophenol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.11 Cyclic Voltammogram of a 1mM Solution of Phenol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.12 Cyclic Voltammogram of a 1mM Solution of \( \alpha \) Naphthol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.13 Cyclic Voltammogram of a 1mM Solution of p-Nitrophenol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.14 Cyclic Voltammogram of a 1mM Solution of Catechol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.15 Relationship Between the Peak Oxidative Current and Number of Cyclic Voltammetric Scans of:

- 1mM Solution of p-Aminophenol
- 1mM Solution of Catechol
- 1mM Solution of p-Nitrophenol
- 0.1mM Solution of Phenol

in 0.05M Carbonate Buffer pH 9.6
Fig 4.16(a) Repetitive Cyclic Voltammograms of a 1mM Solution of Phenol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
Fig 4.16(b) Repetitive Cyclic Voltammograms of a 0.1mM Solution of Phenol in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
The fouling of the electrode by phenol and its analogues occurs as a result of adsorption of the oxidation products onto the electrode. The formation of the oxidation product is as follows\textsuperscript{21,22}:

\[
\text{Phenol} \xrightarrow{\text{SOL}} \text{Phenoxy radical} + \text{H}^+ + \text{e}^{-}
\]

Equation 4.1

The phenoxy radical formed from the oxidation of phenol polymerises and forms a film on the electrode thereby preventing further oxidation.

From these investigations the p-aminophenyl phosphate/ p-aminophenol system is best suited for an electrochemical enzyme immunoassay because:

a. p-aminophenol is easily oxidised ca. +0.05V;
b. p-aminophenol gives good "clean" electrochemistry;
c. the oxidation potential of p-aminophenyl phosphate and p-aminophenol is separated by ca. 0.4V.
4.3.4 Voltammetric Studies of p-Aminophenol.

Experimental.

The following experiments were performed using cyclic voltammetry to further investigate the oxidation mechanism of p-aminophenol:

- a. The effect of the potential scan rate on the peak oxidative and reductive current of p-aminophenol.
- b. The effect of the potential scan rate on the peak oxidative potential of p-aminophenol.

The cyclic voltammetry apparatus used was the same as in section 4.3.3. In both experiments a 1mM solution of p-aminophenol in 0.05M carbonate buffer pH 9.6 was used and the potential scan rates were varied from 5 to 80 mV/s. The potential scanning range was between -0.5 to +0.6V.

Results and Discussion.

The peak oxidative and reductive current and potentials of p-aminophenol were found to be dependent on the potential scan rate (Table 4.3). The peak oxidative current was found to be linearly proportional to the square root of the potential scan rate (Fig 4.17). The oxidative reaction could not be confirmed as either reversible or irreversible because the above proportionality holds true for the forward sweep of the first cycle for both reversible\textsuperscript{23} and irreversible\textsuperscript{24} systems.

One difference between a quasi-reversible and reversible system is the variation of the peak potentials of the quasi-reversible reaction with the potential scan rate. In this study the peak potentials of p-aminophenol was found to vary very slightly with the potential scan rate (Table 4.3) suggesting a quasi-reversible reaction.
Fig 4.17 Relationship of (Potential Scan Rate)¹⁄² and Peak Oxidative Current for p-Aminophenol
The ratio of the peak oxidative current to peak reductive current was found to be ca. 1 V (Table 4.3) suggesting a completely reversible system. The number of electrons involved in the oxidation of p-aminophenol based on the separation of the peak oxidation and reduction potentials (equation 4.2) was found to be 0.5.

Table 4.3 The Effect of Potential Scan Rate on the Peak Oxidative Current and Potential.

<table>
<thead>
<tr>
<th>Potential Scan Rate (mV/s)</th>
<th>Peak Oxidative Current ($i_{pa}$) ($\mu$A)</th>
<th>Peak Reductive Current ($i_{pe}$) ($\mu$A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>20</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>50</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>70</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>44</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Potential Scan Rate (mV/s)</th>
<th>Peak Oxidative Potential (V)</th>
<th>Peak Reductive Potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-0.04</td>
<td>-0.11</td>
</tr>
<tr>
<td>10</td>
<td>-0.03</td>
<td>-0.12</td>
</tr>
<tr>
<td>20</td>
<td>-0.01</td>
<td>-0.13</td>
</tr>
<tr>
<td>50</td>
<td>+0.01</td>
<td>-0.14</td>
</tr>
<tr>
<td>70</td>
<td>+0.02</td>
<td>-0.15</td>
</tr>
<tr>
<td>80</td>
<td>+0.03</td>
<td>-0.16</td>
</tr>
</tbody>
</table>
\[ \Delta E_p = E_{pa} - E_{pc} = \frac{0.059}{n} \]  

Equation 4.2

where:-
- \( E_{pa} \) is the Oxidative peak potential.
- \( E_{pc} \) is the Reductive peak potential.
- \( n \) is the number of electrons in the redox reaction.

According to Hawley and Adams\(^{25} \) the oxidation of p-aminophenol in acidic and alkali conditions exhibits the classical properties of a 2e\(^{-} \) reversible electrochemical process followed by a chemical reaction (an EC mechanism), with the hydrolysis of the first formed quinoneimine generating p-benzoquinone (equation 4.3)

![Reaction Equations](image)

Equation 4.3

Hawley and Adams also reported that at higher pH values the oxidation of p-aminophenol was complicated by 1,4-additions of p-aminophenol to benzoquinone, generating polymeric products, which gave rise to electrode fouling. The studies performed in section 4.3.3 showed that there was very little fouling of the electrode by p-aminophenol at high concentrations.
4.3.5 Synthesis of p-Aminophenyl Phosphate.

Experimental.

p-Aminophenyl phosphate was synthesised from p-nitrophenyl phosphate according to the published method\textsuperscript{26}.

Disodium p-nitrophenyl phosphate (0.4627g) was dissolved in 130ml of distilled water and the pH adjusted to 6.5 by the addition of 1M hydrochloric acid. A 10\% Pd/C catalyst (10mg) was then added to the solution. The solution was then placed under 1atm of hydrogen and reduction was allowed to proceed until hydrogen consumption had ceased. The catalyst was then removed by filtration and the filtrate was taken to dryness under reduced pressure. The product was then characterised.

Results and Discussion.

The synthesis of p-aminophenyl phosphate from p-nitrophenyl phosphate was a straight forward reduction reaction (equation 4.4).

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{P} & \quad \text{H}_2 \\
\text{O} & \quad \text{P} \\
\text{I} & \quad \text{H}_2\text{O}
\end{align*}
\]

\[\text{p-Nitrophenyl Phosphate} \quad \xrightarrow{\text{Pd/C}} \quad \text{p-Aminophenyl phosphate}\]

\text{ Equation 4.4}\]

The percentage yield of p-aminophenyl phosphate was 73.1\% and it was characterised by cyclic voltammetry and nmr spectroscopy. The oxidation potential of p-aminophenyl phosphate was +0.52V (Fig 4.18) which was in
Fig 4.18 Cyclic Voltammogram of a 1mM Solution of p-Aminophenyl Phosphate in 0.05M Carbonate Buffer pH 9.6. Scan Rate 50mV/s
accordance with the published value. Addition of 9U of the enzyme alkaline phosphatase to a 1mM solution of p-aminophenyl phosphate followed by a 5 minute incubation resulted in the production of p-aminophenol as noted by the oxidation potential at ca. +0.05V.

p-Aminophenyl phosphate was insoluble in the organic solvents; DMSO, trichloromethane and tetrachloromethane. A 250MHz nmr analysis using D$_2$O in which the p-aminophenyl phosphate protons were exchanged with deuterium gave a nmr spectra with an AB pattern with peaks at 6.6, 6.7, 6.9, and 7.2ppm. This was in accordance with the published data.

4.4 Analysis of p-Aminophenol and p-Aminophenyl Phosphate by Flow Injection Analysis with Amperometric Detection.

4.4.1 Hydrodynamic Voltammetry of p-Aminophenol and p-Aminophenyl Phosphate.

Experimental.

Hydrodynamic voltammetry was used to find the most suitable potential for hydrodynamic amperometric measurements of p-aminophenol. The flow injection analysis manifold used is shown in Fig 4.19. The background electrolyte i.e. the carrier stream (carbonate buffer pH 9.6) and the samples (p-aminophenol and p-aminophenyl phosphate) were propelled by a peristaltic pump. A Rheodyne 5020 injection valve with a 25μl loop was used to introduce the samples into the carrier stream and a wall jet cell incorporating a platinum working electrode was used as the electrochemical cell. A pulse damper (Fig 4.1) incorporating a piece of platinum wire to earth the solution, was placed between the pump and the injection valve. The transmission tubing was made of PTFE and had an i.d. of 0.8mm. The injection valve was connected to the electrochemical cell by a 50mm length of this tubing.
potentiostat used was the Dionex PAD Detector in a DC mode. The results were recorded on a Linseis chart recorder.

After a steady base line signal was obtained with the chart recorder by flowing the background electrolyte through the flow injection system, the injection valve was turned to introduce 25μl of the sample (p-aminophenol or p-aminophenyl phosphate) into the system. The oxidation of the samples in the electrochemical cell was recorded as flow injection analysis peaks on the chart recorder. This was repeated for potentials of -0.2V to +0.5V for p-aminophenol and +0.1 to +0.8V for p-aminophenyl phosphate in increments of 0.1V. The readings at each potential were obtained in triplicate. The flow rate of the carrier stream was 2ml min⁻¹ and was measured by collecting the waste eluent in a weighed beaker for a measured time.

Fig 4.19 Schematic Diagram of the Flow Injection Manifold Used.
Results and Discussion.

Hydrodynamic voltammetry was used to determine the optimum potential for the detection of p-aminophenol because the p-aminophenyl phosphate/p-aminophenol system was to be used in a flow injection electrochemical enzyme immunoassay. The hydrodynamic voltammograms for the oxidation of p-aminophenyl phosphate and p-aminophenol are shown in Fig. 4.20. The oxidation signals of p-Aminophenyl phosphate and p-aminophenol show good separation in their electrochemical activity in a flowing stream, this is essential if they are to be used in a flow injection immunoassay. p-Aminophenol was electroactive from -0.1 V while p-aminophenyl phosphate was electroactive from ca. +0.3 V, therefore p-aminophenol can be easily detected without interference from p-aminophenyl phosphate. The hydrodynamic voltammogram for p-aminophenol indicates the limiting oxidation potential starts at potentials greater than ca. +0.2 V. A potential of +0.2 V was used to detect p-aminophenol in all further experiments.

4.4.2 Stability of p-Aminophenol and p-Aminophenyl Phosphate.

Solutions of p-aminophenol made in carbonate buffer pH 9.6 were found to go black after standing for about 10 minutes. This is probably due to:

a. Air oxidation of p-aminophenol to p-benzoquinoneimine,

or

b. The hydrolysis of the oxidation product of p-aminophenol, p-benzoquinoneimine to p-benzoquinone.

In order to obtain reliable analytical results a solution of the indicating analyte must be stable and not decay otherwise the results obtained would be low thereby increasing the margin of error in the determination.
Normalised Peak Oxidative Current.

Fig 4.20 Hydrodynamic Voltammograms for 1mM Solutions of p-aminophenol and p-aminophenyl Phosphate.
Experiments were performed to assess the stability of both p-aminophenol and p-aminophenyl phosphate.

Experimental.

The apparatus and manifold was the same as used previously Fig 4.19. The flow rate was 2.0ml min$^{-1}$.

The following experiments were performed:

a. 0.1mM solutions of p-aminophenol made in a carbonate buffer pH 9.6 (either non-deaerated or deaerated for 30 minutes) were injected into the carrier stream over a period of twenty minutes at 1minute intervals and the oxidative current at a potential of +0.2V recorded.

b. A volumetric flask containing a 0.1mM solution of p-aminophenol made in deaerated carbonate buffer pH 9.6 was wrapped in aluminium foil and injections made into the carrier stream over a period of 20 minutes at 5minute intervals. The oxidative current at +0.2V was recorded.

c. Experiment (a) was repeated using solutions of p-aminophenol made in 0.05M carbonate buffer pH 9.0, 1M diethanolamine (DEA) pH 9.8 and 1M ethylaminoethanol (EAE) pH 9.8. Cyclic voltammograms for diethanolamine and ethylaminoethanol were obtained between -0.5 and +0.6V in order to assess their electroactivity in this region.

d. The stability of a 0.1mM solution of p-aminophenyl phosphate was investigated over a 3 day period. This was done by injecting the same solution of p-aminophenyl
phosphate into the carrier stream over this period and measuring the oxidative current at a potential of +0.2V.

Results and Discussion.

The loss of the oxidative signal for p-aminophenol in non-deaerated and deaerated carbonate buffer pH 9.6 over a period of 20 minutes is given in Table 4.4. Despite deaeration of the buffer solution there was still an unacceptable loss in the oxidative signal ca. 90%. This was not due to fouling of the electrode (section 4.3.3). The absence of light from the p-aminophenol solution did not have any effect on the decay of the oxidative signal. Hence, a photochemical contribution can be ignored.

Table 4.4: The Loss of p-Aminophenol Oxidative Signal in Deaerated and Non-deaerated Carbonate Buffer pH 9.6.

<table>
<thead>
<tr>
<th>Relative Time (mins)</th>
<th>Percentage Loss of Oxidative Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Deaerated</td>
</tr>
<tr>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td>20</td>
<td>97</td>
</tr>
</tbody>
</table>

Xu et al found that p-aminophenol solutions made in 0.1M Tris buffer pH 9.0 at concentrations greater than 0.1mM were rapidly oxidised by air (O₂) and light (UV). However, the air oxidation was minimised by deoxygenating the solution and wrapping the container with aluminum foil. Xu et al suggested
that in an immunoassay, the problem of p-aminophenol decay can be circumvented by adjusting the substrate (p-aminophenyl phosphate) incubation time so that the concentration of the enzyme generated p-aminophenol does not exceed 0.1mM.

The reduced decay of p-aminophenol in the work of Xu et al\textsuperscript{28} is probably due to the concentration of p-aminophenol used (1\textmu M) and the pH of the buffer used (pH 9.0).

If equation 4.3 is considered it can be seen that a high p-aminophenol concentration would favour the forward reaction (i.e. the air oxidation of p-aminophenol) according to Le Chatelier's principle, resulting in the formation of p-benzoquinoneimine which is then hydrolysed to p-benzoquinone. At low p-aminophenol concentrations the air oxidation of p-aminophenol and the reduction of p-benzoquinoneimine would be balanced resulting in less hydrolysis of p-benzoquinoneimine to p-benzoquinone.

The effect of lowering the pH of the buffer solution was investigated (Table 4.5); there was only a 35\% decrease in the p-aminophenol oxidative signal after 20 minutes at pH 9.0 as compared to 95\% at pH 9.6 this could be explained by considering equation 4.3. Increasing the H\textsuperscript{+} ion concentration i.e. decreasing the pH results in the equilibrium being forced to the right according to Le Chatelier's principle which results in less air oxidation of p-aminophenol to p-benzoquinoneimine.

The stability of p-aminophenol in 1M EAE and DEA buffers was investigated over a period of 20 minutes. EAE and DEA are the most commonly used buffers in alkaline phosphatase assays and are particulary important because of their phosphate-acceptor ability, making them two of the best buffers for enhancing alkaline phosphatase activity\textsuperscript{29}. There was a 20\% and 5\% decrease in the peak oxidative current of p-aminophenol over a 20 minute period in EAE (1M, pH 9.8) and DEA (1M, pH 9.8) respectively (Fig 4.21).
Fig 4.21 The Decay of p-Aminophenol Oxidative Signal in Different Buffers

(a) Diethanolamine (1M, pH 9.8)
(b) Ethylaminoethanol (1M, pH 9.8)
(c) Carbonate Buffer (0.05M, pH 9.0)
(d) Carbonate Buffer (0.05M, pH 9.6)
EAE and DEA minimises the air oxidation of p-aminophenol (Fig 4.21) as well as producing an ideal environment for alkaline phosphatase activity.

Table 4.5: The Effect of the pH of Carbonate Buffer on the Decay of p-Aminophenol Oxidative Signal.

<table>
<thead>
<tr>
<th>Relative Time (mins)</th>
<th>Percentage Loss of Oxidative Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 9.6</td>
</tr>
<tr>
<td>5</td>
<td>12.4</td>
</tr>
<tr>
<td>10</td>
<td>46.1</td>
</tr>
<tr>
<td>15</td>
<td>73.0</td>
</tr>
<tr>
<td>20</td>
<td>83.0</td>
</tr>
<tr>
<td>25</td>
<td>89.8</td>
</tr>
<tr>
<td>30</td>
<td>93.3</td>
</tr>
</tbody>
</table>


Experimental.

Concentrations of p-aminophenol in the range 1 to 10μM were prepared in 0.05M carbonate buffer pH 9.0. The apparatus and manifold was the same as used previously (Fig 4.19). The carrier stream was 0.05M carbonate buffer pH 9.0, the flow rate and measuring potential were 2.0ml min⁻¹ and +0.2V respectively. Each concentration was measured in triplicates.
Results and Discussion.

An important phenomenon in flow injection with electrochemical detection is the blank current response that occurs when the sample is injected into the carrier stream. Since the blank response is obtained in the absence of electroactive material in the sample, it is attributed to a mixture of a capacitative response, i.e. non-faradaic charging current, caused by ionic changes in the electrical double layer associated with the electrode and reactions of organic functional groups such as protonation-deprotonation, and a faradaic response caused by reduction-oxidation of these surface functional groups on the electrode. The magnitude of the capacitance current is dependent on the differences in ionic strength, pH and dielectric constant between the sample and the carrier stream in the flow injection system. Since, the capacitance current defines the detection limit it is important in optimising this phenomenon in the system. The capacitance current was minimised by matching the sample matrix with the carrier stream (Fig 4.22). For this reason DEA and EAE were not used as the sample matrix for p-aminophenol. Alternatively these buffers could have been used as the carrier streams and sample matrix. However, high concentrations of these buffers as used in section 4.4.2 are toxic, therefore they were not used as the carrier stream. Fig 4.22 shows how the capacitance current increased due to differences in pH between the sample and carrier stream. The response was typically a complicated pattern of both anodic and cathodic peaks.

A typical set of flow injection peaks from this study are shown in Fig 4.23. A standard calibration curve for p-aminophenol in carbonate buffer pH 9.0 using platinum, gold and glassy carbon electrodes are given in Figs 4.24-4.26 respectively. In each case the linear range for the detection of p-aminophenol was between 1 and 10μM. The limits of detection for the platinum, gold and glassy carbon electrodes as defined by the concentration corresponding to twice the baseline noise were 1.5μM, 0.46μM and 0.73μM respectively.
Fig 4.22: Typical Capacitance Peak for Unmatched Blank Sample and Carrier Stream.

a. Blank Sample 0.05M Carbonate Buffer, pH 9.0, Carrier Stream 0.05M Carbonate Buffer, pH 9.0.

b. Blank Sample 0.05M Carbonate Buffer, pH 9.6, carrier stream 0.05M Carbonate Buffer, pH 9.0.
Fig 4.23 Flow Injection Peaks for the Oxidation of p-aminophenol using a Glassy Carbon Electrode: a) 2, b) 4, c) 6μM.
Fig 4.24 Calibration Curve for the Determination of p-Aminophenol by FIA using a Platinum Electrode
Fig 4.25 Calibration Curve for the Determination of p-Aminophenol by FIA using a Gold Electrode
Fig 4.26 Calibration Curve for the Determination of p-Aminophenol by FIA using a Glassy Carbon Electrode
REFERENCES.


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CHAPTER FIVE.

SYNTHESIS OF THEOPHYLLINE-ALKALINE PHOSPHATASE CONJUGATES.
5.1 Introduction.

5.1.1 Methods for Coupling Enzymes to Haptens.

The methods for coupling haptens to enzymes are usually different from those for coupling proteins to enzymes because the small hapten molecules may differ greatly in the number and nature of their functional groups, in solubility etc. These features and the precisely known structures of the hapten, influence the choice of the hapten to which the enzyme is attached. This may have an important influence on both the sensitivity and specificity of the resulting enzyme immunoassay.

a. Mixed anhydride method.

The mixed anhydrides of acids e.g. alkylchloroformates, are formed at low temperatures and in inert organic solvents and then slowly added to cooled enzyme solutions. Under these conditions only protein lysyl and tyrosyl residues react. This method has been used with many haptens including oestradiol, cortisol\(^1\), oestriol\(^2\), morphine\(^3\) and methotrexate\(^4\). Normally, a ten to twenty fold molar excess of mixed anhydride is used and the yields are generally of the order 20-30%. The reaction scheme is shown in Fig 5.1.

![Figure 5.1: Formation of Hapten-Protein Conjugates Using the Mixed Anhydride Method.](attachment:fig5.1.png)
b. Carbodiimide Method.

The water soluble carbodiimides, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 1-cyclohexyl-3-(2-morpholino-4-ethyl) carbodiimide methyl-p-toluenesulphonate have been used to couple haptens, including testosterone, cortisol and progesterone to a number of enzymes. The coupling reaction is generally carried out at pH 5.5-6 and the amino groups on the proteins are involved. The reaction scheme is shown in Fig 5.2.

![Chemical Reaction Diagram]

Fig 5.2: Formation of Hapten-Protein Conjugates using the Carbodiimide Condensation Method.

One problem with the carbodiimide method is that significant intra- and inter-molecular cross-linking of the enzymes occur. A two-step conjugation has been used for the conjugation of hippuric acid to IgG that should be generally applicable to hapten-enzyme conjugate formation where the hapten lacks amino groups. The acid was activated for 2min with carbodiimide at room temperature and then added to the protein.
in strong phosphate buffer at pH 8 to minimise the activation of the carboxyl groups of the protein. This reduced protein cross-linking from 70-80% to less than 10%.

Both the anhydride and carbodiimide methods produce peptide bonds. The carboxylic acid group can be introduced into the hapten if necessary. Oxygen and nitrogen substituents can be alkylated with halo esters followed by ester hydrolysis. Reactions of hydroxy groups with succinic anhydride and ketones or aldehydes with carboxy methyl oxime have also been widely used.

c. Periodate Method.

Carbohydrate residues are cleaved with periodate to generate dialdehydes that can be coupled to amines. This method was used to prepare a digoxin immunogen and an adenosine-β-galactosidase conjugate for EIA. In theory, any amino containing hapten can be conjugated with a glycoprotein after periodate cleavage. However, the result of coupling thyroxine with peroxidase was not efficient.

d. m-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBSE) and Related Methods.

The heterobifunctional reagent MBSE has been used in a two stage reaction to couple haptens such as viomycin, which contains an amino group, to β-galactosidase, which contains about 10 free thiol residues. The same enzyme has been conjugated with viomycin, gentamicin and penicillin using N-(3-maleimidopropionylglycyloxy) succinimide (MPGS). The use of these two reagents, one to produce the immunogen and the other the enzyme label, incorporates different binding groups and improves sensitivity in the resulting EIA. The reaction schemes using these two reagents are shown in Fig 5.3 and 5.4. In the preparation of the immunogen the protein used, bovine serum
albumin lacks thiol groups. These have been introduced by the reduction of disulphide bridges with dithiothreitol. An important advantage of the conjugates made using the above methods was that of retention of high enzyme activity and immunoreactivity.

Fig 5.3: Formation of Hapten-Protein Conjugate using MBSE.

e. Methods using Bifunctional Imidates.

Dimethyl adipimidate reacts with amino groups under alkaline conditions. Using this reagent, conjugates have been formed between β-galactosidase and desmethylnortriptyline and triiodothyronine. The reaction was carried out in two stages. Initially the hapten and dimethyladipimidate were reacted under
anhydrous conditions before being allowed to react with the enzyme in aqueous conditions. The desmethylnortriptyline conjugate retained about 80% enzyme activity and immunoreactivity.

Fig 5.4: Formation of Hapten-Protein Conjugate using MPGS.
5.1.2 Characteristics of Enzyme-Hapten Conjugates.

The important features of an enzyme-hapten conjugate are its enzymatic activity and immunoreactivity. These are affected by the ratio of hapten to enzyme, and in homogeneous EIA by the location of hapten relative to the active sites. In heterogeneous EIA insufficient work has been carried out to clarify the importance of the hapten to enzyme ratio. A sensitive assay for oestradiol-17β was developed using oestradiol-β-galactosidase with 100% immunoreactivity and a steroid to enzyme ratio of only 1:1.7\(^{18}\) whilst Comoglio and Celeda\(^1\) found that 10 cortisol molecules per molecule of β-galactosidase were required to give 100% immunoreactivity.

Comment has been made on the lack of attention paid to poor characterisation of conjugates, most authors being content to report the development of a working assay\(^{19}\). Given that conjugation can interfere with the properties of the analyte and the catalytic activity of the enzyme, more care should be taken in the characterization of conjugates. The following parameters should be carefully assessed:

a. Enzyme Activity.

The effect of conjugation on the Michaelis constant (\(k_m\)) and maximum velocity of reaction (\(V_{max}\)) of the enzyme should be determined. Significant alterations in these parameters can lead to loss of enzyme activity and, hence, an insensitive assay requiring lengthy incubation times to develop a measurable colour. For EMIT assays a high level of activity is essential, as measuring times are short.

b. Immunoreactivity.

Conjugation can mask binding sites, antigenic determinants or sterically inhibit binding by the antibody. The reaction may also damage the molecule. The best test of the conjugate
immunoreactivity is the use of the conjugate in a working assay, and by performing an antibody dilution curve.

5.2 Instrumentation, Equipment and Chemicals.

5.2.1 Instrumentation.

Perkin-Elmer LS 50 Fluorescence Spectrometer.

This instrument was used for fluorescence measurements and consisted of a spectrometer unit connected to a personal computer (PC) Epson AX-3. Data obtained from this instrument was processed using the Perkin-Elmer FL Data Manager (FLDM) software. The light source of the spectrometer was a xenon discharge lamp and the wavelength ranges of the Monk-Gillieson excitation and emission monochromators were 200-800nm and 200-900nm respectively.

LKB Ultrospec II UV/Vis Spectrometer.

This instrument was used for absorbance measurements. The light sources were a deuterium lamp (200-325nm) and a tungsten halogen lamp (325-900nm). The type of monochromator was a Czerny-Turner conjugated with a holographic diffraction grating (1200 lines mm⁻¹). The accuracy of this instrument was quoted as ± 1nm with a bandwidth of 5nm.

5.2.2 Equipment.

LKB Peristaltic Pump;
Water bath;
Centrifuge;
Philips PW9240 pH meter.
### 5.2.3 Chemicals.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (7.5U/mg)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alkaline Phosphatase (EIA grade, &gt;2500U/mg)</td>
<td>Boehringer Mannhein</td>
</tr>
<tr>
<td>Anti-Sheep IgG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma</td>
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<tr>
<td>Diethanolamine</td>
<td>Sigma</td>
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<tr>
<td>Dimethylformamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide Hydrochloric Acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Isobutyl Chloroformate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Potassium Dihydrogen Orthophosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>PD-10 Sephadex G-25</td>
<td>Sigma</td>
</tr>
<tr>
<td>p-Nitrophenyl Phosphate Disodium Salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>Sigma</td>
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<td>Sodium Chloride</td>
<td>Sigma</td>
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<tr>
<td>Sodium Dihydrogen Orthophosphate</td>
<td>Sigma</td>
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<tr>
<td>Sodium Hydrogen Carbonate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Theophylline-7-Acetic Acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Theophylline Antisera (sheep)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Theophylline-8-butyric Acid Lactam</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tributylamine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tris(hydroxymethyl) Aminoethane Hydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>International Laboratory Services (ILS)</em></td>
<td></td>
</tr>
<tr>
<td>Theophylline-8-butyric Acid Lactam</td>
<td>Novabiochem</td>
</tr>
<tr>
<td>Tributylamine</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Tris(hydroxymethyl) Aminoethane Hydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>International Laboratory Services (ILS)</em></td>
<td></td>
</tr>
</tbody>
</table>

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5.2.4 Buffer Solutions.

Phosphate Buffered Saline (PBS, 0.15M, pH 7.4).

This buffer was prepared by dissolving sodium chloride (8.0g), potassium dihydrogen orthophosphate (0.2g), disodium hydrogen orthophosphate (1.13g) in 1 litre of distilled water and adjusting the pH accordingly.

Tris-HCl Buffer (0.05M, pH 7.5).

This buffer was prepared by dissolving Tris (7.88g), magnesium chloride (2.03g) and sodium chloride (5.85g) in 1 litre of distilled water and adjusting the pH accordingly.

Carbonate buffer (0.05M, pH 9.0, pH 9.6) and Diethanolamine buffer (1M, pH 9.8).
These were prepared as described in section 4.1.4.

5.3 Synthesis of Theophylline-Alkaline Phosphatase Conjugates Using the Mixed Anhydride and Carbodiimide Method.

5.3.1 Synthesis of Theophylline-Alkaline Phosphatase Conjugates.

Experimental.


Theophylline-7-acetic acid (3.6mg) and tri-n-butylamine (5μl) were dissolved sequentially in dimethylformamide (0.2ml) and cooled to 10°C. Isobutylchloroformate (5μl) was added and the mixture stirred for 30min at
10°C before mixing with a solution of alkaline phosphatase (0.6mg, 4.5U) in 2ml of 0.05M carbonate buffer pH 9.6. The reaction was allowed to proceed for 4 hours at 10°C. The solution was left overnight at 4°C. The ratio of theophylline-7-acetic acid to alkaline phosphatase was 2500:1.

The above procedure was repeated using theophylline to alkaline phosphatase ratios of 400:1, 200:1, 100:1 and 25:1.

b. Carbodiimide Method 1 (CDI 1).

Theophylline-7-acetic acid (1.2mg) in 250μl of 0.15M PBS buffer pH 7.4 was added to 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-hydrochloric acid (EDC) (2mg) also in 250μl of PBS buffer pH 7.4. The resulting mixture was added to 2ml of PBS buffer pH 7.4 containing alkaline phosphatase (1.2mg, 9U) and allowed to react for 2 hours at room temperature followed by 4 hours at 4°C.

c. Carbodiimide Method 2 (CDI 2).

Theophylline-7-acetic acid (1.2mg) and EDC (2mg) were dissolved in 2ml of deionised water. The pH of the solution was then adjusted to pH 5 with dilute hydrochloric acid and allowed to react at room temperature for 15 min. The solution was then added to 2ml of PBS buffer, pH 7.4 containing alkaline phosphatase (1.2mg, 9U) and the pH of the solution was adjusted to 5 with dilute hydrochloric acid. The mixture was then incubated for 3 hours at 4°C.
5.3.2 Purification of Theophylline-Alkaline Phosphatase Conjugates.

Experimental.

The theophylline-alkaline phosphatase conjugate mixtures were subjected to gel filtration on PD-10 Sephadex G-25 columns to remove excess unlabelled theophylline-7-acetic acid. Before separation of the conjugate mixtures the PD-10 columns were equilibrated with Tris-HCl buffer, pH 7.5, containing 5mM MgCl₂ by passing 25ml of the buffer through the column. Samples of 2.5ml volumes were loaded onto the column and eluted with Tris-HCl buffer. Fractions (1ml) were collected and assayed for alkaline phosphatase activity using p-nitrophenyl phosphate (PNPP) as the substrate. The fractions containing enzyme activity were pooled and bovine serum albumin and sodium azide were added to produce 0.05% w/v and 0.02% w/v solutions respectively.

Estimation of Enzyme Activity.

Alkaline phosphatase catalyses the conversion of PNPP to p-nitrophenol (PNP). The ultraviolet absorption spectra of both PNPP and PNP are sufficiently resolved to allow PNP to be monitored without interference at 415nm.

Eluted fractions (50µl) were added to 2ml of freshly prepared PNPP (1mM in 0.05M carbonate buffer pH 9.0) and incubated for 10min at 30°C. To stop the reaction the incubation mixture was removed from the water bath and 5M NaOH (0.5ml) added. The amount of hydrolysed substrate was estimated by measuring the resulting absorbance at 415nm using a spectrometer.

Results and Discussion.

The manufacturers elution profile for the PD-10 column is given in Fig 5.5 the theophylline-alkaline phosphatase conjugate should be collected between fractions 2 and 6. In the above procedure the enzyme activity was observed in
Fig 5.5 Removal of NaCl from Albumin Solution. Yield of Albumin (Between Arrows) 95.3%
fractions 3 to 5 for some columns and in fractions 4 to 6 for other columns. The observed elution profiles for alkaline phosphatase and theophylline-7-acetic acid are given in Fig 5.6. The alkaline phosphatase activity was determined using the substrate PNPP and measuring its hydrolysis product PNP at 415nm. Theophylline-7-acetic acid was monitored by spectrophotometry at 278nm. From the elution profile (Fig 5.6) most of the alkaline phosphatase is eluted between fractions 3 and 5 with very little contamination from unlabelled theophylline-7-acetic acid.

5.3.3 Characterisation of the Theophylline-Alkaline Phosphatase Conjugates.

Determination of the Residual Enzymatic Activity of the Conjugates.

The activity of the native enzyme and the conjugates was determined as described in section 5.3.2. 50μl of the native enzyme and conjugates (0.15U) were used in the assay. The residual enzymatic activity of each conjugate was expressed as the ratio of the enzymatic activity of the conjugate to the enzymatic activity of the native enzyme.

Determination of the Immunoreactivity of the Conjugates.

The proportion of alkaline phosphatase bound to immunologically reactive theophylline was determined by incubating an aliquot of the conjugate (50μl, 0.15U) with excess theophylline antiserum (100μl of a 1:50 dilution with 0.15M PBS buffer, pH 7.4) for 2hrs. The enzyme conjugate bound by theophylline antibodies was precipitated by the addition of rabbit anti-sheep second antibody (100μl, 2.5mg/ml). The precipitate formed was spun down at 3000 rpm for 15min using a centrifuge and washed twice with PBS buffer pH 7.4, containing 0.1% w/v bovine serum albumin (1 ml) and once with PBS buffer pH 7.4 (1 ml). The enzyme activity in the precipitate was measured using the PNPP assay as described in section 5.3.2. The immunoreactivity of the
Fig 5.6 Elution Profile for:

(a) Alkaline Phosphatase

(b) Theophylline-7-Acetic Acid

using a PD-10 Sephadex G-25 Column
conjugates was expressed as the ratio of the enzyme activity bound to antibody (B) to the total enzyme activity (T).

**Determination of the Degree of Incorporation of Theophylline to Alkaline Phosphatase in the Conjugates.**

The theophylline to alkaline phosphatase ratio in the conjugate was determined by the differential spectrophotometric method of Erlanger. This method employed two simultaneous equations to estimate the concentration of both the theophylline groups (TH) and the enzyme (E) (alkaline phosphatase).

\[
A_{278} = a_{TH(278)}[TH] + a_{E(278)}[E]
\]

\[
A_{220} = a_{TH(220)}[TH] + a_{E(220)}[E]
\]

The molar absorptivities, \(a\), of theophylline-7-acetic acid and alkaline phosphatase were obtained at both 220 and 278nm. The theophylline to alkaline phosphatase (TH/E) ratios were obtained from the calculated concentrations. The molecular weight of the conjugates were taken as 160,000.

**Results and Discussion.**

The characteristics of the theophylline-alkaline phosphatase conjugates prepared using the mixed anhydride and carbodiimide procedures are given in Table 5.1. Unfortunately all the conjugates showed an unacceptable loss in enzymatic activity upon coupling to theophylline. Alkaline phosphatase is known to be susceptible to partial denaturation by organic solvents which may account for the poor performance of the mixed anhydride conjugates. Attempts were made to remove the solvent dimethylformamide which was
used to dissolve theophylline-7-acetic acid and tributylamine but this was not possible as its boiling point was 152°C. Other solvents with lower boiling points were used to dissolve theophylline-7-acetic acid and tributylamine without any success.

Table 5.1: Characteristics of Theophylline-7-Acetic Acid/Alkaline Phosphatase Conjugates.

<table>
<thead>
<tr>
<th>Enzyme Conjugate</th>
<th>Initial Ratio of Theophylline/Alkaline Phosphatase</th>
<th>Degree of Conjugation</th>
<th>% Residual Enzymatic Activity</th>
<th>% Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA (1)</td>
<td>2500</td>
<td>12.8</td>
<td>25.3</td>
<td>28.4</td>
</tr>
<tr>
<td>MA (2)</td>
<td>400</td>
<td>3.8</td>
<td>20.3</td>
<td>25.8</td>
</tr>
<tr>
<td>MA (3)</td>
<td>200</td>
<td>4.1</td>
<td>18.3</td>
<td>19.2</td>
</tr>
<tr>
<td>MA (4)</td>
<td>100</td>
<td>2.8</td>
<td>25.6</td>
<td>24.0</td>
</tr>
<tr>
<td>MA (5)</td>
<td>25</td>
<td>1.6</td>
<td>32.8</td>
<td>9.9</td>
</tr>
<tr>
<td>CDI (1)</td>
<td>400</td>
<td>0.4</td>
<td>93.4</td>
<td>14.7</td>
</tr>
<tr>
<td>CDI (2)</td>
<td>400</td>
<td>1.1</td>
<td>76.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*MA and CDI denotes the mixed anhydride and carbodiimide conjugate methods respectively.

b Refers to molar ratios of theophylline-7-acetic acid and alkaline phosphatase in the conjugation mixture.

Only a small loss in enzymatic activity was observed with the coupling procedure using the water soluble carbodiimide. The CDI (2) conjugates showed a greater loss in enzymatic activity after coupling compared to the CDI (1) conjugates. This was probably due to the more rigorous conditions used in the CDI (2) procedure i.e. the reaction was carried out at pH 5.

The theophylline to alkaline phosphatase ratios (TH/E) are the average number of theophylline molecules per molecule of alkaline phosphatase. For the mixed anhydride method this ratio was dependent on the ratio of theophylline to alkaline phosphatase in the reaction mixture (Table 5.1). There was a greater degree of incorporation of theophylline in the theophylline-
alkaline phosphatase conjugate for the CDI (2) procedure compared to the CDI (1) procedure. This was expected as the carbodiimide reaction is an acid catalysed reaction.

The immunoreactivity of the theophylline-alkaline phosphatase conjugates using excess theophylline antiserum was very poor (Table 5.1) with a maximum binding of 28.4% for the MA (1) conjugate. There were several attempts to improve on these results by varying the second antibody concentration and incubation time but none of these steps altered the degree of binding obtained. From the above results the conjugation procedures used were not suitable for the synthesis of conjugates with high residual enzymatic activity and immunoreactivity for use in an EIA.

In the following section vastly improved theophylline-alkaline phosphatase conjugates were synthesised by a non-activation procedure in aqueous media using theophylline-8-butyric acid lactam and EIA grade alkaline phosphatase.

5.4 Synthesis of Theophylline-Alkaline Phosphatase Conjugates Using a Non Activated Procedure.

5.4.1 Synthesis of Theophylline-Alkaline Phosphatase Conjugates Using Theophylline-8-Butyric Acid Lactam.

Theophylline-8-butyric acid lactam was used in this synthesis. Theophylline-8-butyric acid lactam, a derivative of theophylline contains a carboxyl group that is naturally cyclised onto the imidazole nitrogen (Fig 5.7). It is therefore an acyl imidazole analog that will react rapidly with free amino groups of proteins in aqueous media, producing a stable amide linkage.
Theophylline-8-butyric acid lactam

\[
\text{AP} \quad \text{pH 9, } 4^\circ\text{C}
\]

\[
\begin{align*}
\text{X} &= \text{NH(AP)}\frac{1}{n} \\
\text{AP} &= \text{Alkaline phosphatase}
\end{align*}
\]

**Fig 5.7: Reaction Scheme for the Conjugation of Theophylline-8-butyric Acid Lactam to Alkaline Phosphatase.**

**Experimental.**
Alkaline phosphatase (10μl, 250U) was made up to 190μl with 0.05M carbonate buffer pH 9.0. With stirring and cooling by means of an ice bath theophylline-8-butyric acid lactam (310μl of a 2mg/ml solution in carbonate buffer, pH 9.0) was added in one portion. The mixture was stirred for 4hr at 4°C and left in the fridge overnight. The ratio of theophylline-8-butyric acid lactam to alkaline phosphatase in the reaction mixture was 2500:1. The above procedure was repeated using ratios of 500:1 and 25:1. The reaction scheme of the synthesis is given in **Fig 5.7.**
5.4.2 Purification of the Theophylline-Alkaline Phosphatase Conjugates.

Experimental.

The conjugates were purified using PD-10 Sephadex G-25 columns. The columns were equilibrated with Tris-HCl buffer as previously described in section 5.3.2. The PD-10 columns were connected to the flow cell of a Perkin-Elmer LS-50 spectrofluorimeter using Omnifit connectors. Samples of 0.5ml volumes were loaded onto the column and eluted with Tris-HCl buffer pH 7.5 at a flow rate of 0.4ml min\(^{-1}\) using a peristaltic pump. The elution profile of the conjugates was monitored at an excitation wavelength of 280nm and an emission wavelength of 335nm and the purified conjugates were collected between approximately 300 and 800s after application onto the column.

Results and Discussion.

The on-line elution profile for alkaline phosphatase and theophylline-8-butyric acid lactam using PD-10 columns with fluorescence detection is shown in Fig 5.8. The elution volumes of alkaline phosphatase and theophylline-8-butyric acid lactam were 4.9; 7.8 and 10.2 ml respectively. At high theophylline-8-butyric acid lactam concentrations two elution peaks were observed. Probably at these concentrations there is an interaction either between the frit in the column or the Sephadex gel itself with theophylline-8-butyric acid lactam which results in a delay of elution of some of the theophylline-8-butyric acid lactam (Fig 5.8). The elution profiles of the conjugate reaction mixtures 2500:1, 500:1 and 25:1 shown in Fig 5.9; shows that the on-line conjugate purification procedure using a PD-10 Sephadex G-25 column with fluorescence detection produces very pure conjugates. There was a five fold dilution of the sample i.e. 0.5ml of the reaction mixture was introduced onto the column and approximately 2.5ml of the purified conjugates were collected.
Fig 5.8 On-line Elution Profile for Alkaline Phosphatase and Theophylline-8-Butyric Acid using a PD-10 Sephadex G-25 Column With Fluorescence Detection (EX 280nm; EM 335nm)
Fig 5.9 On-line Elution Profiles for Theophylline-Alkaline Phosphatase Conjugates
5.4.3 Characterisation of the Theophylline-Alkaline Phosphatase Conjugates.

Determination of the Residual Enzymatic Activity.

The activity of the native enzyme and the conjugates was determined as described in section 5.3.2 with the following exceptions; 50µl of the native enzyme and conjugates (0.025U) and 2ml of a 10mM PNPP stock solution made in 1M diethanolamine buffer pH 9.8 were used in the assay. The enzyme and substrate incubation time was 15min.

Determination of the Immunoreactivity of the Conjugates.

The immunoreactivity of the conjugates was determined as described in section 5.3.3.

Determination of the Degree of Incorporation of Theophylline to Alkaline Phosphatase in the Conjugates.

The theophylline to alkaline phosphatase ratio in the conjugates was determined as previously described in section 5.3.3.

Determination of the Kinetic Parameters of the Native Alkaline Phosphatase and the Theophylline Alkaline Phosphatase Conjugates.

Classical Michaelis Menten kinetic analyses were performed to test the viability of the enzyme following conjugation by measuring the Michaelis Menten constant (k_m) and the maximum velocity (V_max). The kinetic
parameters were investigated using the following:

a. Lineweaver-Burke plot \((1/v \text{ versus } 1/s)\);

b. Hanes plot \((s/v \text{ versus } s)\);

c. Eadie-Hofstee plot \((v \text{ versus } v/s)\).

where \(v\) = reaction velocity \((\Delta \text{AU min}^{-1})\).

\(s\) = substrate concentration (mmol).

The native alkaline phosphatase and conjugates were assayed using PNPP (2mM stock solution made in 1M diethanolamine buffer, pH 9.8) at concentrations varying between 0.08mM and 1.96mM. The incubation time and temperature were 10min and 30°C respectively.

**Results and Discussion.**

The residual enzymatic activity of the conjugate decreased with increasing incorporation of theophylline in the conjugate (Table 5.2). Excessive substitution of theophylline on the free amino group of lysyl residues which play a major role in the catalytic function of the enzyme, may cause loss of activity directly, (by hindering the access of substrate if the lysyl residues are at or near the active sites), or indirectly by masking the positive charges associated with the free amino groups leading to conformational changes. The immunoreactivity of the conjugates increased with increasing incorporation of theophylline in the conjugates (Table 5.2). Conjugate-1 with a theophylline/enzyme ratio of 8.8 had close to 100% immunoreactivity while conjugate-3 with a theophylline/enzyme ratio of 4.2 was only 17% immunoreactive.
Table 5.2: Characteristics of Theophylline-8-butyric acid Lactam/Alkaline Phosphatase Conjugates.

<table>
<thead>
<tr>
<th>Enzyme Conjugate</th>
<th>Initial Ratio of Theophylline/Alkaline Phosphatase</th>
<th>Degree of Conjugation</th>
<th>% Residual Enzymatic Activity</th>
<th>% Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate-1</td>
<td>2500</td>
<td>8.8</td>
<td>32.2</td>
<td>93.2</td>
</tr>
<tr>
<td>Conjugate-2</td>
<td>500</td>
<td>7.6</td>
<td>66.6</td>
<td>35.5</td>
</tr>
<tr>
<td>Conjugate-3</td>
<td>25</td>
<td>4.2</td>
<td>91.6</td>
<td>17.1</td>
</tr>
</tbody>
</table>

The conjugates were stable upon storage for 3 months at 4°C in Tris-HCl buffer pH 7.5 containing 0.05% w/v bovine serum albumin and 0.02% w/v sodium azide. Conjugates-1 and 3 showed less than a 10% reduction in enzymatic activity and immunoreactivity while conjugate-2 showed an unexpected 10% increase in immunoreactivity and approximately 20% decrease in enzymatic activity (Fig 5.10).

There was little difference in the average $k_m$ and $V_{max}$ values between alkaline phosphatase and the conjugates (Table 5.3). From these results physical modification of alkaline phosphatase upon coupling to theophylline did not significantly alter its activity or prevent substrate diffusion into its active sites.

Table 5.3: Kinetic Parameters of Native Alkaline Phosphatase and Theophylline-Alkaline Phosphatase Conjugates.

<table>
<thead>
<tr>
<th>Method and Plot</th>
<th>$k_m$ (μM/min)</th>
<th>$V_{max}$ (μM/min)</th>
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<tr>
<td></td>
<td>AP</td>
<td>Conj-1</td>
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<tr>
<td>Lineweaver-Burke 1/v vs 1/s</td>
<td>0.65</td>
<td>0.79</td>
</tr>
<tr>
<td>Hanes s/v vs s</td>
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<td>0.67</td>
</tr>
<tr>
<td>Eadie-Hofstee v vs v/s</td>
<td>0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>Average Values</td>
<td>0.63±</td>
<td>0.71±</td>
</tr>
</tbody>
</table>

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Fig 5.10 Stability of Theophylline-Alkaline Phosphatase Conjugates

IMMR: Immunoreactivity
ENZA: Residual Enzymatic Activity
REFERENCES.


DEVELOPMENT OF A FLOW INJECTION ELECTROCHEMICAL ENZYME IMMUNOASSAY FOR THEOPHYLLINE USING A PROTEIN A IMMUNOREACTOR.
6.1 Introduction.

6.1.1 Flow Injection Solid Phase Immunoassays.

Currently most solid phase immunoassays are performed with 96 well microtitre plates in which samples can be processed simultaneously. This technique has proven to be sensitive, but it is only semiquantitative and is difficult to automate. Flow injection analysis (FIA) on the other hand is an easily automated technique that can be adapted to accommodate many immunoassay formats.

During the past decade flow injection immunoassays making use of small immunoaffinity columns, referred to as immunoreactors, where the antibody or ligand binder is covalently coupled to a rigid support have been developed\textsuperscript{1-3}.

Sepharose\textsuperscript{4}, non porous silica\textsuperscript{3-5}, Trisacryl GF 2000\textsuperscript{6}, Pall Immunodyne membrane\textsuperscript{7}, Biomag 4100 beads\textsuperscript{7} have all been used as solid phases in immunoreactors in flow injection immunoassays. Controlled pore glass (CPG) exhibits physical properties which make it ideal as a solid support for flow injection immunoassay and displays none of the limitations usually associated with soft gels such as agarose or sepharose i.e. compression and attrition of gel in a flowing system\textsuperscript{8}. The unique features of controlled pore glass are:

a. High mechanical strength.
b. High flow rate capability i.e. non compressible.
c. Stable in solvents.
d. Thermally stable.
e. Stable bead size in changing environment.
f. Low non specific protein adsorption.

A number of solid phase immunoassays have been developed employing a range of binders\textsuperscript{4,9,10}, a binder is any molecule which exhibits molecular recognition for another molecule (ligand). Antibodies are the binders most commonly employed
in ligand binder assays, both polyclonal and monoclonal antibodies have been used. Other binders include cell-surface receptors, carrier or transport proteins, such as thyroxine binding globulin, riboflavin binding protein, avidin, concanavalin A, protein A, DNA and protein G.

De Alwis and Wilson reported the first application of FIA in a solid-phase sandwich enzyme immunoassay. In their system, a reversible immunoreactor containing an immobilised antibody was used on-line with a FIA system. The system was capable of continuously carrying out each of the steps involved in solid-phase immunoassays, including the immune reaction, the washing, the sandwich reaction, and the enzymatic reaction in about 10 min. De Alwis and Wilson have also described a FIA based solid-phase competitive immunoassay. More recently, Mattiasson and Lee and Meyerhoff have reported their work in the field of FIA solid-phase enzyme immunoassay. Table 6.1 gives a list of some FIA solid-phase immunoassays currently developed.

Antibodies are by far the most common ligand binders used in solid phase assays that utilise flow injection techniques, however the antibodies are coupled to the support matrix in a random fashion limiting the antigen binding capacity, although attempts have been made to overcome this problem. In addition, the specificity of the immobilised antibody means that only a very limited range of antigens can be bound by each immunoaffinity column. These problems can be largely overcome by the use of protein A, most commonly immobilised on Sepharose and controlled pore glass. Because protein A binds the Fc region of antibodies the antigen receptors are orientated away from the support material and into the mobile phase maximising potential binding sites. In addition, a whole range of antibodies with different specificities can be bound to and eluted from the affinity column ensuring that the matrix not only has high antibody binding efficiency but it also is extremely flexible in its use.

A number of methods have been developed employing immobilised protein A in a flowing system, the majority of which are used for isolating and purifying
antibodies\textsuperscript{19,22} and labelled antibody conjugates\textsuperscript{23}. Surprisingly, the use of protein A in flow injection immunoassays appears limited\textsuperscript{4}.

FIA-based solid phase immunoassays generally offer better speed and precision than conventional assays such as the ELISA using microtitre plates or plastic tubes. This improvement is due to the much better reproducibility associated with reactions of solid-phase components and soluble species.

Table 6.1: Review of FIA Solid Phase Immunoassay.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ligand Binder</th>
<th>Label</th>
<th>Ligand Binder Support</th>
<th>Detection System</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mouse IgG</td>
<td>Antibody</td>
<td>Glucose Oxidase</td>
<td>Reactigel-6X</td>
<td>Amperometric</td>
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</tr>
<tr>
<td>Human IgG</td>
<td>Antibody</td>
<td>Glucose Oxidase</td>
<td>Tresyl Activated Trisacryl GF 2000</td>
<td>Amperometric</td>
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<tr>
<td>Mouse IgG</td>
<td>Antibody</td>
<td>Acridinium</td>
<td>Tresyl Activated Trisacryl GF 2000</td>
<td>Chemiluminescence</td>
<td>6</td>
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<td>Human IgG</td>
<td>Antibody</td>
<td>Adenosine Deaminase</td>
<td>Controlled Pore Glass (CPG)</td>
<td>Potentiometric</td>
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<tr>
<td>Theophylline</td>
<td>Antibody</td>
<td>Alkaline Phosphatase</td>
<td>Controlled Glass Pore (CPG)</td>
<td>Potentiometric</td>
<td>3</td>
</tr>
<tr>
<td>Insulin</td>
<td>Antibody</td>
<td>Horse Radish Peroxidase</td>
<td>Controlled Glass Pore (CPG)</td>
<td>Potentiometric</td>
<td>3</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Protein A/</td>
<td></td>
<td>Oxiare Acrylic Beads</td>
<td>Fluorimetric</td>
<td>24</td>
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<tr>
<td>Antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Antibody</td>
<td>Horse Radish Peroxidase</td>
<td>Immunoodyne Membrane, BioMag 4100 Beads</td>
<td>Fluorimetric</td>
<td>7</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Antibody</td>
<td>Carboxyfluorescein</td>
<td>Controlled Glass Pore (CPG)</td>
<td>Fluorimetric</td>
<td>25</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Antibody</td>
<td>Liposome-Encapsulated Horse Radish Peroxidase</td>
<td>Controlled Glass Pore (CPG)</td>
<td>Potentiometric</td>
<td>26</td>
</tr>
<tr>
<td>Glucosides</td>
<td>Concanavalin A</td>
<td>Horse Radish Peroxidase</td>
<td>Sepharose CL 4B</td>
<td>Spectrophotometric</td>
<td>18</td>
</tr>
<tr>
<td>Human IgG</td>
<td>Antibody</td>
<td>Horse Radish Peroxidase</td>
<td>Polystyrene Beads (6mm diameter)</td>
<td>Chemiluminescence</td>
<td>2</td>
</tr>
<tr>
<td>17-α-Hydroxy Progesterone, Insulin, α-Fetoprotein</td>
<td>Antibody</td>
<td>Glucose Oxidase</td>
<td>Double Antibody Second Phase (DASP) Beads</td>
<td>Chemiluminescence</td>
<td>27</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>Antibody</td>
<td>Horse Radish Peroxidase</td>
<td>Cyanogen Bromide-Activated Sepharose</td>
<td>Chemiluminescence</td>
<td>28</td>
</tr>
<tr>
<td>Bovine IgG</td>
<td>Antibody</td>
<td>Horse Radish Peroxidase</td>
<td>Polyvinylidene Difluoride Polymer Membrane</td>
<td>Chemiluminescence</td>
<td>29</td>
</tr>
</tbody>
</table>
6.1.2 Protein A.

Introduction.

Staphylococcal protein A (SPA) a protein component of the cell wall of *Staphylococcus aureus*, binds the Fc portion of many immunoglobulins of most mammalian species. Verwey first described SPA in 1940 as a protein antigen present in type A staphyloccoci (coagulate-positive, α-toxin-positive, mannitol-fermenting, and pathogenic i.e., *S.aureus*), but not in type B Staphylococcus (those lacking these characteristics). The systematic study of antigen A did not begin in earnest until the 1960's when Grov and co-workers proposed the designation "protein A " to avoid confusion with the antigenic polysaccharide A.

Biochemical Characterization of SPA.

Staphylococcal protein A is a highly stable 42,000 molecular weight protein which retains its activity following exposure to 4M urea, 4M thiocyanate, 6M guanidine hydrochloride, pH or temperature extremes. It also retains its activity following conjugation to a number of molecules e.g. erythrocytes, ^125^I, fluorescein, alkaline phosphatase and gold. It has an extended rather than globular shape as determined by its frictional ratio of 2.1-2.2 and its intrinsic viscosity of 29 ml g^-1^, and it contains few if any sugar residues. SPA contains α-helical regions that comprise about 50% of the molecule. Analysis of tryptic digests reveals four highly homologous Fc-binding regions, each consisting of a nearly identical sequence of approximately 60 amino acids. These Fc-binding regions are arranged in tandem in the N-terminal portion of the protein. The C-terminal end of the molecule is bound to the bacterial cell wall and consists of the remaining 150 amino acids that are not associated with the repetitive Fc-binding sequences. The contact domain by crystallography, is hydrophobic.
Reactivity of SPA with Immunoglobulins.

SPA reacts almost exclusively with the sera of mammals. Kronvall and co-workers\textsuperscript{34} showed essentially no reactivity of SPA with sera of fishes, amphibia, reptiles and birds, with the single exception of a primitive flightless bird, \textit{Rhea americana}. In contrast, SPA binds to serum immunoglobulins of almost all mammalian species\textsuperscript{34}.

In both humans and animals there are marked differences in the affinity of different classes and sub classes of immunoglobulins for SPA. For example, human IgG\textsubscript{1}, IgG\textsubscript{2}, and IgG\textsubscript{4} bind strongly. IgG\textsubscript{3}, which does not bind to SPA, comprises only 1% to 3% of total human immunoglobulin. IgA\textsubscript{2} and some IgM proteins also bind well. In addition, 6% to 9% of IgE proteins bind to SPA, but probably in a manner different from that of the other immunoglobulins. These IgE molecules apparently bind via their F\textsubscript{ab} portion, rather than their F\textsubscript{c} portion. Several other studies of the subclass binding specificities of the sera from various animal species have been published\textsuperscript{35}.

Since, SPA binds to the F\textsubscript{c} portion of the immunoglobulin molecule\textsuperscript{26}, an important practical consequence of this binding site is that the attachment of SPA to an immunoglobulin molecule does not interfere with antigen binding by the F\textsubscript{ab} portion. Conversely, antigen binding probably does not induce a conformational change in the F\textsubscript{c} portion of the immunoglobulin molecule and thus does not appear to modify SPA binding, however enhanced interaction between antigen, antibody and SPA may occur if complexes are generated with polyvalent antigens\textsuperscript{37}.

Potential Advantage of SPA over Anti-Immunoglobulins.

SPA offers several potential advantages over anti-immunoglobulins (Table 6.2). SPA is a well-defined molecule that is readily available in pure form, either commercially or as a result of relatively simple laboratory preparations.
Table 6.2: Potential Advantages of SPA over Anti-Immunoglobulins\textsuperscript{21}.

1. SPA is readily available in pure form.
2. SPA is a highly stable molecule under a variety of conditions.
3. SPA reacts with the immunoglobulins of most mammalian species.
4. SPA can be conjugated with a number of marker molecules with little or no loss of immunoglobulin-binding activity.
5. SPA generally exhibits low non specific binding to materials and reagents.

SPA is a highly stable molecule that can be conjugated to a number of marker molecules with little or no loss of activity (Table 6.3).

Table 6.3: SPA Conjugates Used in Immunoassays\textsuperscript{21}.

<table>
<thead>
<tr>
<th>SPA Conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
</tr>
<tr>
<td>$^{125}$I</td>
</tr>
<tr>
<td>$^{3}$H</td>
</tr>
<tr>
<td>Fluorescein</td>
</tr>
<tr>
<td>Ferritin</td>
</tr>
<tr>
<td>Gold</td>
</tr>
<tr>
<td>Invertase</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td>Peroxidase</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>Biotin</td>
</tr>
</tbody>
</table>

SPA reacts with the immunoglobulins of most mammalian species\textsuperscript{34}. This permits indirect antibody assays with sera for which anti-globulins are not readily available\textsuperscript{38}. The tendency of SPA to bind non specifically to materials
such as plastic, filters, cells and uncomplexed antibody appears to be lower than that of anti-globulins\textsuperscript{21}.

Anti-immunoglobulins possess several potential advantages over SPA. The former may react with certain classes and subclasses of immunoglobulins such as IgG\textsubscript{3} or IgM which may be important in a particular assay. Moreover, serum immunoglobulins of certain animals (e.g. goat or birds) may react very poorly with SPA. A specific anti-immunoglobulin of particularly high affinity may provide greater sensitivity than SPA.

Applications of SPA for Immunoassays.

SPA can be used advantageously in many ways in the development and performance of immunoassays (Table 6.4). Affinity chromatography with SPA-conjugated sepharose represents one of the most simple and effective methods of purifying and concentrating immunoglobulins\textsuperscript{19}. Purifying antibody prior to conjugation with labelling molecules increases the specific activity of the conjugates and reduces non-specific labelling. Affinity chromatography is also an effective and easy method for purifying F\textsubscript{ab} fragments from F\textsubscript{c} fragments and intact immunoglobulins molecules\textsuperscript{19}.

Table 6.4: Applications of SPA for Immunoassays\textsuperscript{21}.

| 1. Purification and concentration, by affinity chromatography, of immunoglobulins from antisera or from hybridoma cultures. |
| 2. Separation by affinity chromatography of Fab and F(ab')\textsubscript{2} fragments or whole immunoglobulin molecules. |
| 3. Immunoadsorption for antigen detection or purification. |
| 4. Detection by indirect immunoassay of specific antigens or antibodies, using liquid phase or solid phase assay techniques |

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Another application of SPA as an immunoadsorbent is antigen separation and purification using SPA in pure form or bound to sepharose beads or to the intact staphylococcal cell\textsuperscript{39-42}. SPA has been used as a convenient and practical method for screening hybridomas for antibody production\textsuperscript{43}. The advantage of such a selection technique is that the monoclonal antibodies that result from such a process are easy to purify, manipulate, and detect in immunoassays that employ SPA.

Finally, SPA has found many applications in indirect immunoassays as a conjugate with various molecules (Table 6.3). One distinct advantage of radiolabelled or enzyme labelled SPA in competitive immunoassays is the ability to use a single tracer molecule regardless of the nature of the antigen or specificity of the antibody. This has allowed the development of immunoassays for some drugs and prostaglandin derivatives that were unstable using conventional labelling conditions and for which classical radioimmunoassays could not be developed\textsuperscript{44-46}.

6.1.3 Aim of Work.

The aim of the work in this chapter was to develop a flow injection electrochemical enzyme immunoassay for the determination of serum theophylline using the p-aminophenyl phosphate/p-aminophenol detection system described in chapter 4 and the theophylline-alkaline phosphatase conjugates synthesised in chapter 5.4.

Investigations were carried out into the immobilisation of anti-theophylline antisera on controlled pore glass for use in a flow injection system. The loading and activity of the immobilised antibodies were determined.

Assessment of the binding characteristics of theophylline antisera to controlled pore glass protein A (CPG-PrA) in a flow injection system was also performed.
Based on its superior binding characteristics to theophylline antisera CPG-PrA was used in the development of the flow injection electrochemical enzyme immunosensor.

6.2 Instrumentation and Chemicals.

6.2.1 Instrumentation.

Flow Injection Binding Studies of Theophylline Antisera.

The flow injection manifold used is shown in Fig 6.1a.

The peristaltic pump, pulse damper and injection valve were identical to those used in section 4.4.1.

The immunoreactor a glass microcolumn (50mm x 3mm i.d.) and the 3-way switching valves were obtained from Omnifit Ltd, U.K.

The detector was the Perkin-Elmer LS-50 fluorescence spectrometer incorporating a 100μl flow cell (Hellma, U.K.) and the data handling system an Epson AX-3 personal computer.

Flow Injection Electrochemical Enzyme Immunoassay for Theophylline.

The flow injection manifold used is shown in Fig 6.1b.

The peristaltic pump, pulse damper, immunoreactor, injection and switching valves were the same as used above.
Fig 6.1a: Flow Injection Manifold for the Binding Studies of Theophylline Antisera.


BB - Binding Buffer; EB - Elution Buffer; P - Peristaltic Pump; PD - Pulse Damper; IV - Injection Valve; SV1 - SV4 - Switching Valves; IR - Immunoreactor; D - Detector; W - Waste; DHS - Data Handling System.
The electrochemical cell and potentiostat were identical to those used in section 4.4.1. The data handling system was a Spectra Physics SP 4290 integrator.

6.2.2 Chemicals.

The theophylline-alkaline phosphatase conjugates used in this chapter in the development of the assay were those whose synthesis are described in chapter 5.4.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>Fisons</td>
</tr>
<tr>
<td>Anti-Sheep IgG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Carbonylimidazole</td>
<td>Sigma</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Controlled Pore Glass (CPG)</td>
<td>Waters Associates Inc.</td>
</tr>
<tr>
<td>Controlled Pore Glass (CPG glyophase)</td>
<td>Pierce</td>
</tr>
<tr>
<td>Controlled Pore Glass Protein A (CPG-Pr A)</td>
<td>Oros Ltd.</td>
</tr>
<tr>
<td>Dimethylpilimidate</td>
<td>Pierce</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate</td>
<td>BDH</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>Fisons</td>
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<tr>
<td>Magnesium Chloride</td>
<td>BDH</td>
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<tr>
<td>Phosphoric Acid</td>
<td>Fisons</td>
</tr>
<tr>
<td>Potassium Dihydrogen Orthophosphate</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium Dihydrogen Orthophosphate</td>
<td>BDH</td>
</tr>
<tr>
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<td>Sigma</td>
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<td>Theophylline Antisera</td>
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<td>Theophylline Standards in Human Serum</td>
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<tr>
<td>Triethanolamine</td>
<td>Fisons</td>
</tr>
<tr>
<td>Tris(hydroxymethyl) aminoethane</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

* ILS - International Laboratory Services.
Manufacturers Data Information for Controlled Pore Glass Protein A (CPG-Pr-A).

The matrix consists of staphylococcal protein A immobilised onto controlled pore glass. The matrix has been designed to maximise IgG binding, particularly to murine IgG subclasses that do not bind well to traditional affinity matrices.

Matrix Characteristics:

Maximum Binding Capacity: Approximately 40-45mg mouse IgG, per ml of settled gel.
Protein A loading: 5.5mg per ml of settled gel.
Particle Size: 75-125 micron.
pH range: 1.5-9.0.
Temperature Stability: 4°C-37°C.

Storage and Handling.

The controlled pore glass matrix is supplied packed in 0.05M acetate buffer pH 5.0 containing 0.5M NaCl and 0.01% thimerosal. After packing it is recommended that the matrix is washed with at least 5 column volumes of equilibration buffer, to remove the preservative.

Flow Characteristics.

Controlled pore glass protein A matrix can withstand high flow rates because the porous glass beads are not compressible. Relatively low pressures can be used to achieve high flow rates.
Cleaning in Place.

The matrix can be sanitised in place by cleaning with 6.0M guanidine hydrochloride. Guanidine hydrochloride unfolds the protein A and ensures that any contaminants are effectively removed. The matrix should be flushed thoroughly with equilibration buffer to make sure all traces of guanidine hydrochloride have been removed.

Recommended Operating Procedure.

The following buffers and protocol are recommended by the manufacturers for use with the controlled pore glass protein A matrix.

Buffers.

Equilibration Buffer: 50mM Tris-HCl pH 8.8 containing 0.5M NaCl.
Elution Buffer : 0.1M Citric Acid pH 2.5 containing 2.0M di-sodium hydrogen orthophosphate and 0.5M NaCl.
Cleaning Solution : 6.0M Guanidine Hydrochloride.

Method of Immobilisation and Elution of Sample.

1. Equilibrate the matrix with equilibration buffer (5 column volumes).

2. Load the sample. Column direction upwards.

3. After loading the sample it is recommended that the matrix is washed with approximately 10 column volumes of equilibration buffer.

4. The sample can then be eluted from the matrix either by running a citrate-phosphate gradient from pH 9.0 to 2.5 rate of change of pH, 0.05
units per minute or by stepping the pH to pH value at which the antibody is eluted.

5. The column must then be re-equilibrated with Tris-HCl buffer before the next assay. It is recommended that the matrix is periodically sanitised by cleaning with 1-2 column volumes of 6.0M guanidine hydrochloride. It is important that the matrix is flushed thoroughly with equilibration buffer to ensure that all traces of guanidine hydrochloride have been removed.

6.2.3 Buffer Solutions.

Ethanolamine (0.2M, pH 8.2). This buffer was prepared by making up 12.2ml of ethanolamine to 1 litre with distilled water and adjusting the pH accordingly.

Triethanolamine (0.2M, pH 8.2). This buffer was prepared by making up 26.5ml of triethanolamine to 1 litre with distilled water and adjusting the pH accordingly.

Phosphate Buffered Saline (PBS) (0.15M, pH 7.4) was prepared as described in section 5.2.4.

Tris-HCl equilibration buffer (50mM, pH 8.8) and citric acid buffer (0.1M, pH 2.5) were prepared as recommended in the manufacturers data information (section 6.2.2).
6.3 Outline of the Flow Injection Electrochemical Enzyme Immunoassay for Theophylline Using a Protein A Immunoreactor.

One characteristic of this kind of assay is the need for reuse of the immunoreactor. The general assay cycle for off-line and on-line immunoassays are given in Figs 6.2 and 6.3 respectively. In the off-line assay the antibody, antigen and enzyme labelled antigen are incubated in a vessel for a fixed interval then injected onto the protein A immunoreactor while in the on-line assay the actual competitive immunoassay takes place on the immunoreactor. For the on-line assay the antibody is first injected onto the column followed by a mixture of the enzyme labelled antigen and unlabelled antigen.

It is important to investigate the duration of each of the phases of the assay cycle in order to obtain an assay that fulfils the demands concerning specificity, accuracy and reproducibility. Optimisation of the steps in order to minimise the duration is of importance since the shorter the time for an assay cycle, the more useful is the analysis.

6.4 Immobilisation and Binding Studies of Theophylline Antisera onto Controlled Pore Glass (CPG) and Controlled Pore Glass Protein A (CPG-Pr A).

6.4.1 Immobilisation of Anti-Theophylline IgG on CPG.

Introduction.

In this section theophylline secondary antibodies were immobilised on CPG and its activity i.e. ability to bind theophylline primary antibody assessed.

There are numerous methods for immobilising proteins to solid supports such as agarose, cellulose and silica. Two linking reagents dimethylpilimidate (DMP) and 1',1-carbonyliimidazole (CDIZ) were chosen for the
Fig 6.2: Schematic Diagram of an Off-line Electrochemical Enzyme Immunoassay Reaction Cycle. The Arrows Indicate Changes in Perfusing Medium. The Schematic Peak Illustrates the Response from the Bound Enzyme.

Pr A - Protein A, Ab - Antibody, Ag-E - Enzyme Labelled Antigen, Ag - Unlabelled Antigen, S - Enzyme Substrate, P - Substrate Hydrolysis Product.
Fig 6.3: Schematic Diagram of an On-line Electrochemical Enzyme Immunoassay Reaction Cycle. The Arrows Indicate Changes in Perfusing Medium. The Schematic Peak Illustrates the Response from the Bound Enzyme.

immobilisation of anti-theophylline antisera onto CPG. Silica beads were used as the solid support because of their excellent flow characteristics and their availability in a variety of controlled diameters and pore sizes.

Alkylamine derivatised beads were used for the DMP method and glycophase diol beads for the CDIZ method.

The evaluation of the immobilisation procedures was based on:

1. the amount of antibody loaded onto the beads;
2. the degree of non-specific binding; and
3. the retention of antibody "activity" (i.e. the ability to recognise its specific antigen) after immobilisation.

Experimental.

DMP Immobilisation Procedure.

Preparation of Alkylamine CPG Beads.

The alkylamine derivatisation of the CPG beads (100-150 mesh) was carried out according to the published method

Immobilisation of Anti-theophylline IgG onto Alkylamine Derivatised CPG.

0.3ml of anti-theophylline IgG(2.5mg ml⁻¹) made up to 0.6ml with 0.2M triethanolamine buffer pH 8.2 was added to 200mg of alkylamine beads in 0.9ml of the same buffer. 2ml of DMP(10mg ml⁻¹) was then added to the slurry and the mixture was shaken for 1 hour at room temperature. After this period the reaction was stopped by the addition of 2ml of 0.2M ethanolamine pH 8.2. The beads were then washed four times(2ml aliquots) with 0.15M PBS buffer pH 7.4. Each washing was saved for fluorimetric measurements.
CDIZ Immobilisation Procedure.

200mg of glycophase CPG was suspended in 4ml of acetonitrile with 640mg of CDIZ. The mixture was degassed for 15min by ultrasonication, then shaken for 30min at room temperature. The activated beads were then filtered, washed with acetonitrile and dried. The beads were then washed with 0.15M PBS, pH 7.4 after drying. 0.3ml of anti-theophylline IgG(2.5mg ml⁻¹) was added to the beads in a vial and the volume made up to 1ml with distilled water. The antibody bead mixture was then incubated on a shaker at 4°C for 3days. After this period the immunobeads were washed four times (1ml aliquots) with PBS buffer and suspended overnight in 0.05M Tris-buffer, pH 8.2 to hydrolyse any unreacted sites after the incubation step. Each wash after the incubation step was saved for fluorescence measurements.

Determination of Loading.

The loading of antibody onto the CPG was determined by measuring the fluorescence of the antibody solution prior to and after incubation (pre and post fluorescence) on a Perkin-Elmer LS50 spectrofluorimeter. The fluorescence measurements were converted to antibody concentration using a calibration curve of antibody versus fluorescence intensity. The excitation wavelength was 280nm with the emission wavelength set at 335nm. Slit widths were optimised for each set of antibody measurements.

The same quantity of anti theophylline IgG was used in the two immobilisation procedures (i.e. 0.3ml of a 2.5mg ml⁻¹ solution).

Determination of the Activity.

The activity of the immobilised antibodies was determined by FIA using the manifold in Fig 6.1a (see section 6.2.1).
The Omnifit column i.e. the immunoreactor was tightly packed with the anti theophylline IgG immobilised beads using a peristaltic pump and incorporated into the manifold shown in Fig 6.1a. 25μl of theophylline antisera (1:50 dilution in 0.15M PBS buffer, pH 7.4) was injected onto the immunoreactor using PBS buffer pH 7.4 as the binding buffer. After the peak of the non bound fraction of antisera returned to the baseline the flow through the immunoreactor was switched to phosphoric acid, pH 2.0 to elute the bound sample and switched back to PBS to re-equilibrate the column. The binding and elution flow rates were 0.4ml min⁻¹.

The above procedure was repeated with a blank immunoreactor (i.e. CPG without immobilised anti-theophylline IgG) to assess the degree of non specific binding.

Results and Discussion.

The reaction schemes of DMP and CDIZ immobilisation procedures are given in Figs 6.4 and 6.5 respectively. DMP is a bifunctional imidoester and has been widely used in successfully crosslinking proteins and compounds with primary amino groups. Similar success has been achieved with the CDIZ procedure.

![Fig 6.4: Reaction Scheme for the Dimethylpilimidate (DMP) Immobilisation Procedure.](image-url)
Fig 6.5: Reaction Scheme for the Carbodiimidazole (CDIZ) Immobilisation Procedure.

The antibody loading for the DMP and CDIZ procedures was 3.61 and 3.20mg of antibody per g of beads respectively. The binding and elution profiles of the primary theophylline antibody to the DMP and CDIZ immobilised secondary antibody immunoreactors are shown in Figs 6.6 and 6.7 respectively. The non specific binding for the blank column of the alkylamine CPG was very high (Fig 6.6). The signal to noise ratio for the alkylamine and glycophas CPG beads based on the elution peak area measurements was 1.4 and 10.2 respectively. The glycophas CPG incorporate a hydrophillic, non-ionic layer which is covalently bonded. This "glycerol" coating effectively covers the active sites on the glass to minimise their influence on the materials being separated. The alkylamine beads are not treated in this manner which probably accounts for the very high non specific binding.

Theoretically, the difference in the non binding peak area of the CDIZ immobilised and blank immunoreactors must be equal to the difference in the elution peak area. In the experiment the difference in the non binding peak area was much greater than that of the elution peak area suggesting
Fig 6.6 The Binding and Elution Profile of Theophylline Antisera on Controlled Pore Glass Immobilised Anti-Theophylline IgG (Dimethylpilimidate Procedure) Using Fluorescence Detection (Ex Wavelength 280nm; EM Wavelength 335nm)
Fig 6.7 The Binding and Elution Profile of Theophylline Antisera on Controlled Pore Glass Immobilised Anti-Theophylline IgG (Carbodiimide Procedure) Using Fluorescence Detection (Ex Wavelength 280nm; EM Wavelength 335nm)
incomplete elution of the primary theophylline antibody from the secondary antibody immobilised column. The theophylline antisera (i.e. primary theophylline antisera) used was a heterogeneous mixture of immunoglobulins with different binding affinities. It is therefore possible that some fractions bind very tightly and cannot be eluted with the phosphoric acid at pH 2.5. Chaotropic agents such as 6M potassium thiocyanate, 6M guanidine hydrochloride or 8M urea which disrupt the tertiary structure of proteins, can be used to dissociate antigen-antibody complexes. These agents were not used because they destroy the activity of antigens or antibodies.

Based on the above results the immobilised antibody on the glycophase CPG using the CDIZ immobilisation procedure shows favourable loading and activity characteristics for use in flow injection immunoassays.

6.4.2 Binding and Elution Studies of Theophylline Antisera using CPG-Pr A.

Experimental.

The instrumentation and manifold in Fig 6.1a were used in this study. The Omnifit column i.e. the immunoreactor was tightly packed with CPG-Pr A using a peristaltic pump and incorporated in the manifold as shown in Fig 6.1a. 25μl of theophylline antisera (1:50 dilution in 0.15M PBS buffer, pH 7.4) was injected onto the immunoreactor using the 0.05M Tris-HCl binding buffer, pH 8.8. After 500s the peak of the non-bound fraction of antisera returned to the baseline and the flow through the immunoreactor was switched to citric acid, pH 2.5 to elute the bound sample. After 800s the flow through the immunoreactor was switched back to Tris-HCl buffer to re-equilibrate the column. The binding and elution flow rates were maintained at 0.5 and 0.8 ml min⁻¹ respectively.
Results and Discussion.

The binding and elution profile for the reversible immobilisation of theophylline sheep antisera on CPG-Pr A is shown in Fig 6.8. Albumin, transferrin and other non IgG proteins constitute the non-binding peak while the IgG bound fraction is represented by the elution peak.

Goudswaard et al. investigated the binding of sheep IgG subclasses to Sepharose protein A. When serum was applied to the column at pH 7.5, 76% of the IgG1 eluted in the void volume and only 2% was eluted with 0.1M glycine at pH 2.5. In contrast, approximately 6% of IgG2 eluted at pH 7.5, and 33% with acid. It was therefore concluded that sheep IgG2 had a much higher affinity than IgG1, for protein A. These results were consistent with experiments of Kessler, who also found selective adsorption of IgG2.

The elution peak (Fig 6.8) therefore primarily consists of the IgG2 fraction.

CPG-Pr A was used as the stationary phase in the immunoreactor for the development of a flow injection electrochemical enzyme immunoassay for theophylline. Protein A immobilised on CPG was chosen over antibody immobilised on CPG using the CDIZ procedure because of the ease of antibody binding and its superior reversible binding characteristics.

6.5 Optimisation of Conditions for the Flow Injection Immunoassay.

In these studies conjugate-1 was used (see section 5.4).
Fig 6.8 The Binding and Elution Profile of Theophylline Antisera on the Controlled Pore Glass Protein A Immunoreactor Using Fluorescence Detection (Ex Wavelength 280nm; EM Wavelength 335nm)
6.5.1 The Effect of the Substrate Injection Loop Volume on the Peak Oxidative Signal.

Experimental.

The instrumentation and manifold used are shown in Fig 6.1b. Preliminary investigation into the effect of the substrate injection loop (IV2) volumes on the FIA peak shape were carried out.

100µl of enzyme-labelled theophylline (0.5U) and 100µl of 0.05M Tris-HCl equilibration buffer pH 8.8 were mixed with 50µl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10min, loaded into IV1 (25µl injection loop volume) and injected onto the CPG-Pr A immunoreactor using the Tris-HCl equilibration buffer at a flow rate of 0.4ml min^{-1}. The actual exposure time for this mixture within the immunoreactor was very short (ca. 20s) after which the carrier buffer washed off the unbound species for a few minutes. When IV2 was opened the substrate solution (1mM p-aminophenyl phosphate) passed through the column at a flow rate of 0.6ml min^{-1}. The product of the enzymatic reaction p-aminophenol was measured down stream by the electrochemical cell in a wall jet configuration. Results (i.e. the oxidative peak area of p-aminophenol) were recorded on a Linseis recorder. The immunoreactor was regenerated by washing with citric acid elution buffer, pH 2.5 for 3mins to dissociate the complex between theophylline antisera and the immobilised protein A. The immunoreactor was then re-equilibrated for 2mins with the Tris-HCl buffer, after which the system was ready for another sample. The above procedure was performed for IV2 with injection loop volumes of 500, 100 and 50µl.

Results and Discussion.

The p-aminophenol oxidative peaks for IV2 with injection loop volumes of 500, 100 and 50µl are shown in Fig 6.9. The larger injection loops produced
Fig 6.9 FIA Peaks for Substrate Injection Loop Volumes

(a) 500µl  (b) 100µl  (c) 50µl
broad peaks due to dispersion of the substrate in the system. Based on the peak shapes obtained (Fig 6.9) a 50μl injection loop volume was chosen for IV2 and was used in all subsequent investigations.

6.5.2 The Effect of the Contact Time Between the Elution Buffer and the Protein A Immunoreactor on Dissociating the Theophylline Antisera/Theophylline-Alkaline Phosphatase Complex.

Experimental.

50μl of enzyme labelled theophylline (5U) and 100μl Tris-HCl buffer, pH 8.8 were mixed with 50μl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10mins and loaded into IV1 (25μl injection loop volume) and injected into the CPG-Pr A immunoreactor using the Tris-HCl equilibration buffer at a flow rate of 0.4 ml min⁻¹. The flow through the immunoreactor was then switched back to Tris-HCl buffer. IV2 was then opened and the substrate solution (1mM p-aminophenyl phosphate) passed through the immunoreactor at a flow rate of 0.6ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded on a Spectra Physics SP 4290 integrator and the immunoreactor regenerated as described in section 6.5.1. The above procedure was repeated for citric acid flow times of 0, 20, 30, 60, 90 and 120s.

Results and Discussion.

From Fig 6.10 it can be seen that the response signal generated from the oxidation of p-amino phenol goes to less than 2% of its initial value after only 60s of washing the reactor with the citric acid elution buffer. Such treatment, repeated for every sample analysis is not detrimental to the performance of the CPA-Pr A column according to the manufacturers information (see section 6.2.2). The life time of each CPG-Pr A immunoreactor was approximately
Fig 6.10 Effect of Contact Time Between Citric Acid Buffer and Protein A Immunoreactor on Dissociating the Antisera-Conjugate Complex
between 80 and 100 runs. Such results do not imply that the absolute binding ability of the CPG-Pr A matrix is maintained after repeated treatment with pH 2.5 buffer. However, since the amount of CPG-Pr A was in great excess to the amount of theophylline antisera binding sites used in the assays, substantial loss in the binding ability was not apparent in the immunoreactor for less than 60 runs. To reduce the total time of an assay cycle it was decided to use a citric acid regeneration time of 2 mins as compared to 3 min used in the previous experiments.

6.5.3 The Effect of the Incubation Time of the Theophylline-Alkaline Phosphatase Conjugate and Theophylline Antisera on the Peak Oxidative Signal.

Experimental.

50\(\mu\)l of enzyme labelled theophylline (5U) and 100\(\mu\)l of 0.05M Tris-HCl equilibration buffer, pH 8.8 were mixed with 50\(\mu\)l of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10 mins, loaded into IV1 (25\(\mu\)l injection loop volume) and injected onto the CPG-Pr A immunoreactor using the Tris-HCl buffer at a flow rate of 0.4 ml min\(^{-1}\). When IV2 was opened, the substrate (1mM p-aminophenyl phosphate) passed through the column at a flow rate of 0.6 ml min\(^{-1}\). The product of the enzymatic reaction was measured as before. The results were recorded on a Spectra Physics SP 4290 integrator and the immunoreactor was regenerated as described in section 6.5.1. The above procedure was repeated for enzyme labelled theophylline and theophylline antisera incubation times of 5, 15, 20 and 30 mins.
Results and Discussion.

Fig 6.11 shows that the peak oxidative signal of p-aminophenol increases rapidly for conjugate and antisera incubation times between 5 and 15 mins after which it levels off. In this assay the antibody binds the conjugate, the degree of which depends on a variety of parameters including the incubation time. After the incubation period the antibody conjugate complex is injected onto the CPG-Pr A immunoreactor. The antibody then binds the protein A by the F\text{c} region, leaving the conjugate exposed on the F\text{ab} region. The substrate is then introduced into the immunoreactor and is hydrolysed by the enzyme portion of the conjugate to form the product (p-aminophenol) which is oxidised down stream in the electrochemical cell. The measured signal i.e. the peak oxidative area of p-aminophenol is directly proportional to the quantity of antibody-bound conjugate.

Ideally, an incubation time on the flat section of the curve (Fig 6.11) i.e. 30 min or greater should be chosen to reduce the error in measurement which would be greater if the steeper section of the curve is used. In order to reduce the total time of the assay, an incubation time of 10 min was chosen. This period of time despite being on the steep section of curve (Fig 6.11) can be accurately measured in a controlled manner.

The following experiments were performed in order to optimise flow rate conditions for the development of off-line and on-line flow injection immunoassays for theophylline.
Fig 6.11 Effect of Conjugate and Antisera Incubation Time on the Response Signal for the Theophylline Assay System
6.5.4 The Effect of the Theophylline Antisera/Theophylline-Alkaline Phosphatase Complex Binding Flow Rate on the Peak Oxidative Signal.

Experimental.
50μl of enzyme-labelled theophylline (5U) and 100μl of 0.05M Tris-HCl equilibration buffer pH 8.8 were mixed with 50μl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10min, loaded into IV1 (25μl injection loop volume) and injected onto the CPG-Pr A immunoreactor using the Tris-HCl buffer at a flow rate of 0.19ml min⁻¹. When IV2 was opened the substrate solution passed through the column at a flow rate of 0.6ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded on a Spectra Physics SP 4280 integrator and the immunoreactor regenerated as described in section 6.5.1. The above procedure was repeated with the incubation mixture flow rates varied between 0.38 and 1.04 ml min⁻¹ while maintaining the other flow rates constant.

Results and Discussion.

Fig 6.12 shows that the peak oxidative signal decreases in a geometric fashion as the antibody/theophylline–alkaline phosphatase complex flow rate is increased. This result indicates that under the flow conditions studied, the association reaction between the complex and the immobilised protein A does not reach equilibrium. This should not be the case as the immobilised protein A is in excess. The reason for the inefficient binding of the complex to protein A at high flow rates probably lies in the fact that the association binding rate of the antibody complex and the immobilised protein A is altered. The theophylline-alkaline phosphatase molecule is bulky and when incorporated into the antibody/theophylline-alkaline phosphatase complex probably alters the Fc binding site of the antibody for protein A or causes steric hinderance of the antibody for Protein A thereby decreasing the binding affinity of the
Fig 6.12 Effect of Antibody-Conjugate Complex Flow Rate on the Response Signal for the Theophylline Assay System
antibody for protein A. A flow rate of 0.4 ml min\(^{-1}\) was chosen as the optimum value. This flow rate represents a compromise between the assay throughput time and the efficiency of the complex binding to the CPG-Pr A immunoreactor.

6.5.5 The Effect of Theophylline Antiserum Binding Flow Rate on the Peak Oxidative Signal.

**Experimental.**

50 µl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4) was loaded into IV1 (25 µl injection loop volume) and injected onto the CPG-Pr A immunoreactor using Tris-HCl buffer, pH 8.8 at a flow rate of 0.19 ml min\(^{-1}\). A mixture of 50 µl of enzyme labelled theophylline (5 U) and 100 µl of 0.05M Tris-HCl equilibrating buffer pH 8.8 was loaded into IV1 (25 µl injection loop volume) and injected onto the CPG-Pr A immunoreactor at a flow rate of 0.19 ml min\(^{-1}\). When IV2 was opened the substrate solution passed through the immunoreactor at a flow rate of 0.6 ml min\(^{-1}\). The product of the enzymatic reaction was measured as before. The results were recorded on a Spectra Physics SP4290 integrator and the immunoreactor regenerated as described in section 6.5.1. The above procedure was repeated at antisera loading flow rates between 0.38 and 1.04 ml min\(^{-1}\) while maintaining the other flow rates constant.

**Results and Discussion.**

*Fig 6.13* shows that the peak oxidative signal decreases in a linear manner as the antibody flow rate was increased. There was only a 30% decrease in the signal on increasing the flow rate from ca 0.2 ml min\(^{-1}\) to 1.0 ml min\(^{-1}\). This was mainly due to the presence of an excess of immobilised protein A in the immunoreactor compared to the injected antibody. In addition, it is likely that the association binding rate of the antibody alone is faster than that of the
Fig 6.13 Effect of Antibody Flow Rate on the Response Signal for the Theophylline Assay System
bulkier antibody enzyme conjugate complex. Thus, even with a high flow rate (e.g. 0.9 ml min⁻¹), the injected antibody can react nearly completely with the immobilised protein A during the short period that it spends in the immunoreactor. A flow rate of 0.6 ml min⁻¹ was chosen as the optimum value in order to prevent the breakup of the immunoreactor matrix i.e. CPG-PrA.

6.5.6 The Effect of the Theophylline-Alkaline Phosphatase Conjugate Binding Flow Rate on the Peak Oxidative Signal.

Experimental.

The procedure was identical to that in section 6.5.5 except that the flow rate for Tris-HCl buffer/enzyme-labelled theophylline mixture was varied between 0.19 and 1.04 ml min⁻¹ while maintaining the other flow rates constant.

Results and Discussion.

Fig 6.14 shows that the peak oxidative signal decreases in a geometrical manner as the enzyme-labelled theophylline flow rate was increased. Fig 6.14 gives a similar profile to that observed for the antibody/conjugate complex. The reason for the above profile probably lies in the fact that the enzyme portion of the conjugate being a large globular molecule sterically hinders the interaction of the analyte theophylline with the antibody already bound onto the immobilised protein A. Also the antibody affinity (for its antigen) probably decreases on binding to protein A. A flow rate of 0.5 ml min⁻¹ was chosen for all subsequent measurements. This flow rate compromised the assay through put time and the efficiency of the enzyme labelled conjugate binding to antibody on the CPG-Pr A immunoreactor.
Fig 6.14 Effect of Conjugate Flow Rate on the Response Signal for the Theophylline Assay System
6.5.7 The Effect of the Theophylline-Alkaline Phosphatase Conjugate Substrate Flow Rate on the Peak Oxidative Signal.

Experimental.

50μl of enzyme labelled theophylline (5U) and 100μl of 0.05M Tris-HCl equilibrating buffer, pH 8.8 were mixed with 50μl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10min, loaded into IV1 (25μl injection loop volume) and injected onto the CPG-Pr A immunoreactor using the buffer at a flow rate of 0.4ml min⁻¹. When IV2 was opened the substrate solution passed through the column at a flow rate of 0.19ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded on a Spectra Physics SP4290 integrator and the immunoreactor regenerated as described in section 6.5.1. The above procedure was repeated for substrate flow rates between 0.38 and 1.04ml min⁻¹.

Results and Discussion.

Fig 6.15 shows that the peak oxidative signal of p-aminophenol decreases very rapidly as the substrate flow rate was increased from approximately 0.2 to 1.0ml min⁻¹. This was expected as the extent of the enzyme reaction (i.e. hydrolysis of the substrate by the antibody immobilised conjugate) is directly related to the residence time of the substrate in the immunoreactor. Fig 6.15 also shows that there is very little difference in the oxidative peak area for flow rates approximately between 0.5 and 1.0ml min⁻¹. The zero dose response oxidation peak signal obtained at 0.5ml min⁻¹ was large enough to perform a competitive immunoassay. Typical oxidation peaks are shown in Fig 6.16. In order to increase the through put time of the assay the substrate flow rate was chosen to be 0.6ml min⁻¹.
Fig 6.15 Effect of Substrate Flow Rate on the Response Signal for the Theophylline Assay System
Fig 6.16 Typical Oxidation Peaks for p-aminophenol at Varying Flow Rates;

(a) 0.18 ml min$^{-1}$
(b) 0.36 ml min$^{-1}$
(c) 0.69 ml min$^{-1}$
(d) 1.03 ml min$^{-1}$
6.5.8 Assessment of the Binding Activity of Theophylline-Alkaline Phosphatase Conjugates and Theophylline Antisera using the Protein A Immunoreactor.

Experimental.

The binding reaction of theophylline antisera and the three theophylline-alkaline phosphatase conjugates prepared in section 5.4 were assessed by following the procedure in section 6.5.4 except that the complex flow rate was 0.4 ml min⁻¹ instead of 0.19 ml min⁻¹ as used in section 6.5.4.

Results and Discussion.

The binding activities of the three conjugates (conjugates 1-3) are given in Fig 6.17 together with the blank values. The blank values or non-specific binding values (NSB) were determined by replacing 50 µl of the theophylline antisera used with Tris-HCl buffer. The NSB values for the three conjugates 1, 2 and 3 were 9.3%, 15.9% and 14.7% respectively. This shows that the antisera and not the conjugates themselves bind to the protein A immunoreactor. The binding activity of conjugate-1 to antisera was greater than that of conjugates 2 and 3. This is due to the higher incorporation of theophylline molecules in conjugate-1 which results in a higher immunoreactivity.

6.6 Flow Injection Immunoassay for Theophylline.

6.6.1 Antisera Dilution Curve using the Protein A Immunoreactor.

Off-Line Procedure.

50 µl of enzyme-labelled theophylline (5U) and 100 µl of Tris-HCl equilibration buffer, pH 8.8 were mixed with 50 µl of theophylline antisera (1:50 dilution in
Fig 6.17 Binding Activity of the Theophylline-Alkaline Phosphatase Conjugates With Theophylline Antisera
0.15M PBS, pH 7.4). The mixture was incubated for 10 mins, loaded onto IV1 (25μl injection loop volume) and injected onto the CPG-Pr A immunoreactor using Tris-HCl at a flow rate of 0.4 ml min⁻¹. When IV2 was opened, the substrate solution passed through the immunoreactor at a flow rate of 0.6 ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded and the immunoreactor regenerated as described in section 6.5.1. The above procedure was performed using conjugates 1 and 3 with antisera dilutions of 1:50, 1:100, 1:200, 1:500, and 1:1000.

**On-Line Procedure.**

50μl of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4) was loaded into IV1 (25μl injection loop volume) and injected onto the CPG-Pr A immunoreactor using Tris-HCl buffer pH 8.8 at a flow rate of 0.6 ml min⁻¹. A mixture of 50μl of enzyme labelled theophylline (5U) and 100μl of 0.05M Tris-HCL equilibrating buffer, pH 8.8 was loaded into IV1 (25μl injection loop volume) and injected onto the CPG-Pr A immunoreactor at a flow rate of 0.5 ml min⁻¹. When IV2 was opened the substrate solution passed through the immunoreactor at a flow rate of 0.6 ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded and the immunoreactor regenerated as described in section 6.5.1. The above procedure was performed using conjugates 1 and 3 with antisera dilutions of 1:100, 1:200, 1:400, 1:600, 1:800 and 1:1000.

**Results and Discussion.**

The investigation of the binding capacities of the enzyme labelled theophylline conjugate with varying antisera concentrations is important in determining the antisera concentration that would give a reasonable displacement of the conjugate by the unlabelled antigen in an immunoassay. Fig 6.18 shows how the peak oxidative signal for p-aminophenol varies with the concentration of antisera for conjugates 1 and 3 using the off-line incubation format. Over an
Fig 6.18 Antisera Dilution Curves for Conjugates-1 and 3 Using the Off-line Assay Format
antisera dilution range of 200-1000, the peak oxidative signal of conjugate 1 decreases by approximately 80% while the equivalent decrease for conjugate 3 is approximately 40%. This suggests that the binding affinity of conjugate 1 is more dependent on the antibody concentration than conjugate 3. Conjugate-1 has a very high immunoreactivity for a 1:50 antisera dilution i.e. ca 94% (see section 5.4.3) compared to 17% for conjugate-3 at the same antisera dilution. This is due to the high incorporation of theophylline into conjugate-1. Nearly, all of conjugate-1 ca. 94% is bound by the antisera at a 1:50 dilution. Reducing the antisera concentration results in less conjugate been bound as the antisera concentration becomes the limiting factor (i.e. fewer antibody binding sites are available). This is an important requirement for a competitive immunoassay. Therefore, only a two fold dilution of the original 1:50 antisera dilution results in a significant decrease in the peak oxidative signal of p-aminophenol.

The antisera dilution curve constructed using the on-line incubation format (Fig 6.19) shows a similar profile to Fig 6.18 i.e. off-line format. The decrease in the peak oxidation signal for conjugate-1 and 3 (Fig 6.18) with increasing antisera dilution is greater than in Fig 6.19. The most probable reason for this apparent difference is due to the binding hinderance of the antibody-conjugate complex to immobilised protein A in the case of the off-line assay format due to steric factors as already discussed in section 6.5.4. This phenomenon probably causes only a proportion of the complex injected onto the column to bind to protein A hence the signal measured on introduction of the substrate solution through the immunoreactor is reduced.

From the above results it was decided to use an initial antibody dilution of 1:50 in the construction of the flow injection immunoassay. The antibody dilution would be increased if the percentage displacement of the conjugate by the unlabelled antigen is not significant within the assay range.
Fig 6.19 Antisera Dilution Curves for Conjugates-1 and 3 Using the On-line Assay Format
6.6.2 Off-Line Immunoassay for Theophylline.

Experimental.

Off-Line Immunoassay using Conjugate-1.

50\(\mu\)l of enzyme-labelled theophylline (5U) and 100\(\mu\)l of 0.05M Tris-HCl equilibration buffer, pH 8.8 were mixed with 50\(\mu\)l of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4). The mixture was incubated for 10min, loaded into IV1 (25\(\mu\)l injection loop volume) and injected onto the CPG-Pr A immunoreactor using the Tris-HCl buffered at a flow rate of 0.4ml min\(^{-1}\). When IV2 was opened the substrate solution passed through the column at a flow rate of 0.6ml min\(^{-1}\). The product of the enzymatic reaction was measured as before. The results were recorded and the immunoreactor regenerated as described in section 6.5.1. The above procedure was for the zero dose response signal for theophylline. For the competitive immunoassay the above procedure was slightly modified i.e. 50\(\mu\)l of enzyme labelled theophylline (5U), 50\(\mu\)l of 0.05M Tris-HCl buffer, pH 8.8 and 50\(\mu\)l of sample (unlabelled theophylline) were mixed with 50\(\mu\)l of theophylline antisera (1:50 dilution in 0.15M PBS, pH 7.4).

The above procedure was repeated using a theophylline antisera dilution of 1:100. Calibration curves were then constructed for theophylline concentrations between the sub-therapeutic and toxic levels.

Off-Line Immunoassay using Conjugates 1, 2 and 3.

The above procedure was repeated for conjugates 1, 2 and 3 using a theophylline antisera dilution of 1:100 and 100\(\mu\)l of sample (unlabelled theophylline) instead of 50\(\mu\)l Tris-HCl buffer and 50\(\mu\)l sample (unlabelled theophylline).
Results and Discussion.

Calibration curves for theophylline between 2.5 and 35μg ml⁻¹ for antisera dilutions of 1:50 and 1:100 are given in Figs 6.20 and 6.21 respectively. The calibration curves were constructed by plotting \((B/B_0)\%\) versus the theophylline concentration in the standards, where:

\[
\begin{align*}
B &= \text{peak oxidative area of p-aminophenol at the stated drug concentration} \\
B_0 &= \text{peak oxidative area of p-aminophenol at zero drug concentration}
\end{align*}
\]

The calibration curve constructed using the 1:50 antisera dilution only gave ca. 20% displacement of enzyme labelled theophylline conjugate at the top end of the assay range while the 1:100 antisera dilution calibration curve gave approximately 45% displacement of the conjugate at the same range. At lower antisera concentrations the antibody binding sites are limiting and facilitates the competition between the conjugate and the unlabelled analyte. The percentage displacement obtained using conjugate-1 and antisera dilutions of 1:50 and 1:100 at theophylline concentrations between 2.5 and 35μg ml⁻¹ were not large enough to accurately monitor theophylline levels between the therapeutic range of 10-20μg ml⁻¹. Calibration curves (Fig 6.22) were therefore constructed for conjugates-1, 2 and 3 using a 1:100 antisera dilution and 100μl of unlabelled theophylline instead of 50μl of theophylline standard. Fig 6.22 shows calibration curves for the three theophylline alkaline phosphatase conjugates 1, 2 and 3 with relatively high, medium and low degrees of incorporation of theophylline. Fig 6.22 also shows that decreasing the degree of incorporation of theophylline in the conjugate results in an increase in the steepness of the calibration curve and an increase in the sensitivity of the assay.

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Fig 6.20 Calibration Curve for the Off-line Assay of Theophylline (Aqueous Media) Using Conjugate-1, (Antisera Dilution 1:50)
Fig 6.21 Calibration Curve for the Off-line Assay of Theophylline (Aqueous Media) Using Conjugate-1, (Antisera Dilution 1:100)
Fig 6.22 Calibration Curve for the Off-line Assay of Theophylline (Aqueous Media) Using Conjugate-1, 2, 3 (Antisera Dilution 1:100)
6.6.3 On Line Immunoassay for Theophylline.

Experimental.

50μl of theophylline antisera (1:100 dilution in 0.15M PBS, pH 7.4) was loaded into IV1 (25μl injection loop volume) and injected onto the CPG-PrA immunoreactor using Tris-HCl buffer pH 8.8 at a flow rate of 0.6ml min⁻¹. A mixture of 50μl enzyme-labelled theophylline (5U) and 100μl of 0.05M Tris-HCl equilibration buffer, pH 8.8 was loaded into IV1 and injected onto the CPG-PrA immunoreactor at a flow rate of 0.5ml min⁻¹. The product of the enzymatic reaction was measured as before. The results were recorded and immunoreactor regenerated as described in section 6.5.1. The above procedure was for the zero dose response signal for theophylline. For the competitive immunoassay the above procedure was slightly modified i.e. a mixture of 50μl enzyme labelled theophylline (5U) and 100μl of sample (unlabelled theophylline) was injected into IV1 instead of 50μl of enzyme labelled theophylline and 100μl Tris-HCl buffer.

Results and Discussion.

The on-line calibration curve for theophylline using conjugates 1, 2 and 3 shown in Fig 6.23 gave similar profiles and trends as the off-line assay format calibration curves (Fig 6.22) but smaller displacements were observed. In the off-line assay format the incubation time of the enzyme labelled theophylline conjugate, sample (unlabelled theophylline) and antisera was 10 minutes while the incubation time for the on-line assay was less than 1 minute, therefore in the off-line assay there was more time for a competition reaction to be set up between the conjugate and the sample for the antibody sites.

The time for one complete cycle of the on-line assay including the regeneration step was 11 minutes. The corresponding time for the off-line assay was 18 minutes. Based on the results in this section (i.e. section 6.6) the
Fig 6.23 Calibration Curve for the On-line Assay of Theophylline (Aqueous Media) Using Conjugate-1, 2, 3 (Antisera Dilution 1:100)
following conclusions have been reached:

a. To increase the sensitivity of the flow injection immunoassays the antibody concentration must be reduced sufficiently to facilitate competition between the conjugate and the sample for the antibody binding sites.

b. Using conjugates with low degrees of incorporation of antigen results in a more sensitive assay.

c. The off-line assay format gives greater conjugate displacement than the on-line assay format but the assay time is approximately 7 minutes longer.

6.7 Improved Flow Injection Immunoassay for Theophylline.

6.7.1 Sensitive Immunoassay for Theophylline.

Experimental.


Off-line and on-line competitive assays between conjugate-3 and unlabelled theophylline were performed at different antisera dilutions. The procedure was identical to that described in section 6.6.1 except that the antisera dilutions were 1:100, 1:500 and 1:1000 for the off-line assay and 1:100, 1:200, 1:400 and 1:1200 for the on-line assay. Also 100μl of Tris-HCl buffer, pH 8.8 was used for the B₀ value and 100μl of theophylline standards (125 and 400ng ml⁻¹) were used for the B values.
The antisera dilution that gave the greatest displacement of conjugate was used in the development of a sensitive immunoassay for theophylline see (b).

b. Immunoassay using Conjugate-3.

Off-line and on-line immunoassays were performed for theophylline using antisera dilutions of 1:1000 and 1:200 respectively. The procedures were as described in section 6.6.2 and 6.6.3. The assay range investigated was between 0 and 500ng ml⁻¹.

Results and Discussion.

The displacements of conjugate-3 by unlabelled theophylline at different antibody concentrations using the off-line and on-line assay formats are shown in Figs 6.24 and 6.25 respectively. Increasing the antisera dilution at a given standard concentration results in a greater displacement of the conjugate (i.e. less conjugate is being bound by the antibody). In order, to develop a sensitive immunoassay for theophylline, antisera dilutions of 1:200 and 1:1000 were chosen for the on-line and off-line formats respectively. Calibration curves using these antibody dilutions are given in Figs 6.26 and 6.27. In the off-line and on-line formats (Figs 6.26 and 6.27) approximately 85% and 90% of the conjugate was bound respectively at the lower assay limit (i.e. standard theophylline concentration 12.5ng ml⁻¹). At the upper assay limit (standard theophylline concentration 500ng ml⁻¹) only approximately 30% and 60% of the conjugate were bound respectively.
Fig 6.24 Displacement of Conjugate-3 by Unlabelled Theophylline at Different Antisera Dilutions Using the Off-line Assay Format
Fig 6.25 Displacement of Conjugate-3 by Unlabelled Theophylline at Different Antisera Dilutions Using the On-line Assay Format
Fig 6.26 Calibration Curve for the Off-line Assay of Theophylline (Aqueous Media) Using Conjugate- 3 (Antisera Dilution 1:1000)
Fig 6.27 Calibration Curve for the On-line Assay of Theophylline (Aqueous Media) Using Conjugate-3 (Antisera Dilution 1:200)
The therapeutic range for serum theophylline levels is generally between 10 and 20 μg ml\(^{-1}\). The flow injection assay developed in section 6.6 can measure theophylline levels between 2.5 and 35μg ml\(^{-1}\) in aqueous media i.e. buffer. Undiluted serum samples cannot be used in the assay as the IgG present in the serum would bind to the immobilised protein A in the immunoreactor. This would drastically reduce the lifetime of the column as most of the protein A sites would be saturated. To prevent the saturation of the protein A immunoreactor with IgG present in the serum it was decided to dilute the serum samples one hundred fold with buffer before injecting onto the immunoreactor (see section 6.7.2).

6.7.2 Immunoassay for Theophylline using Serum Samples.

Standard theophylline samples made up in serum between 2.5 and 40μg ml\(^{-1}\) were used. A one hundred fold dilution in a calibration range of 25-400ng ml\(^{-1}\) (which is roughly equivalent to the calibration range in section 6.7.1b. [Figs 6.26 and 6.27]).

* results

Experimental.

Theophylline standard solutions made in human serum (2.5, 5.0, 10.0, 20.0 and 40 μg ml\(^{-1}\)) were diluted one hundred fold with equilibration buffer pH 8.8 to give standard solutions of concentration 25ng ml\(^{-1}\) to 400ng ml\(^{-1}\). These solutions were used in the immunoassay. The off-line and on-line assays were performed for serum theophylline levels using conjugate-3. The off-line and on-line procedures were the same as described in section 6.6.2 and 6.6.3.
Results and Discussion.

Calibration curves for the off-line and on-line assay formats for serum theophylline levels between 25 and 400 ng ml\(^{-1}\) are shown in Fig 6.28 and 6.29 respectively. The detection limits for the off-line and on-line assay formats were found to be less than 25 ng ml\(^{-1}\). Samples containing 60, 140 and 300 ng ml\(^{-1}\) of theophylline (i.e. low, middle, and high levels) were prepared by adding known amounts of theophylline to the blank serum. The mean recovery for the off-line format was 123.3%. Figs 6.30 and 6.31 show calibration curves for the measurement of theophylline levels in serum for both assay formats using the same column on consecutive days. The reproducibility of the calibration curves on two consecutive days (Figs 6.30 and 6.31) was poor. The reason for this was mainly due to the column design and packing. The column design was not ideal for these assays as only approximately 20% of the total column volume was used. Most of the binding on the column took place within the first 10 mm of the CPG-Pr A. Also screw-on caps were used to seal the columns. When the columns were packed there was always a gap between the screw-on cap and the top of the CPG-Pr A bed. This was usually topped up with more matrix and tightly packed using a peristaltic pump at approximately twice the flow rate to be used in the assay. On storing the column in the fridge overnight, the matrix packed more tightly and left a gap between the top of the bed and the screw-on cap. This led to mixing of the CPG-Pr A when it was incorporated back into the flow injection system resulting in non-identical binding conditions for assays performed on consecutive days. The on-line assay format was particularly prone to these variations as the competition between the conjugate and the sample for antibody sites was dependent on reproducible binding of the antisera to the protein A column. A different column design was investigated in section 6.8 to solve the problem of the binding variation.
Fig 6.28 Calibration Curve for the Off-line Assay of Theophylline (Serum) Using Conjugate-3 (Antisera Dilution 1:1000)
Fig 6.29 Calibration Curve for the On-line Assay of Theophylline (Serum) Using Conjugate-3 (Antisera Dilution 1:200)
Fig 6.30 Calibration Curve for the Off-line Assay of Serum Theophylline on Consecutive Days Using Conjugate-3 (Antisera Dilution 1:1000)
Fig 6.31 Calibration Curve for the On-line Assay of Serum Theophylline on Consecutive Days Using Conjugate-3 (Antisera Dilution 1:200)
6.8 Comparison of the Performance of Two CPG-Pr A Immunoreactors of Different Design.

Experimental.

The off-line assay format was used in this investigation. The procedure was as described in section 6.6.2 using the zero dose response signal for theophylline.

The immunoreactors used were the Omnifit column (see section 6.2.1) and the Drew column (Figs 6.32a and b).

Results and Discussion.

Diagrams of the two immunoreactors used in this investigation are shown in Figs 6.32a and b. Fig 6.33 shows the variation of the zero dose response binding of the antibody-conjugate-3 complex to protein A for the Omnifit and Drew columns. The coefficient of variation for 10 runs for the Omnifit and Drew columns were 18.4 and 21.2% respectively. Based on the above results the Drew column has no advantage over the Omnifit column. Other column designs must be further investigated.

6.9 Final Discussion and Conclusion.

Over the past two decades radioimmunoassays have had widespread use in clinical practice. However, radiolabelling is not ideal, because radioactive decay does not change significantly when antibody and antigen combine (i.e. only heterogeneous assays are possible), radiolabelled materials may have rather short half life times and health hazards are potentially involved in their production and use. Therefore, many other labels have been studied in recent years, including enzymes, fluorescent or chemiluminescence molecules, viruses and free radicals. Enzyme labelling has been the subject of a considerable amount of work and evidence is increasingly accumulating that enzyme
Fig 6.32 Immunoreactor Columns

(a) Omnifit Column  (b) Drew Column
Fig 6.33 Assessment of the reproducibility of Binding of Conjugate-3 to Protein A Using Omnifit and Drew Microcolumns.
immunoassays provide a feasible alternative to radioimmunoassays.

In recent years, newer enzyme immunoassay methods have essentially replaced classical radioimmunoassay procedures for the determination of a wide variety of biomolecules at trace levels. Although some lower molecular weight species (e.g. drugs and hormones) can be determined using the rapid homogeneous enzyme immunoassay techniques (e.g. EMIT assays), the selective determination of others require the use of much slower heterogeneous (i.e., solid-phase) approaches (e.g., ELISAs). The prolonged equilibration periods coupled with multiple pipetting and washing steps involved makes it difficult to fully automate such solid-phase methods. A potentially more rapid approach involving the use of a reusable immunoreactor in a flow-through arrangement has been reported by De Alwis et al\textsuperscript{1} and Lee et al\textsuperscript{2} for the determination of human IgG.

In this work, this general concept was further investigated by developing a flow injection electrochemical enzyme immunoassay for the anti-asthmatic drug theophylline. The project consisted of four sections:

a. the voltammetric analysis of several alkaline phosphatase substrates and their hydrolysis products.

b. the synthesis of theophylline alkaline phosphatase conjugates.

c. investigations into the immobilisation of anti-theophylline IgG and theophylline antisera on controlled pore glass and controlled pore glass protein A.

d. the incorporation of sections (a)-(c) into flow injection electrochemical enzyme immunoassay for theophylline.

Of the alkaline phosphatase substrates and hydrolysis products investigated the p-aminophenyl phosphate/p-aminophenol system was best suited for
incorporation into a flow injection electrochemical enzyme immunoassay because:

a. p-Aminophenol was easily oxidised (ca. +0.05V vs SCE) therefore very little or no interference from other oxidisable species.

b. p-Aminophenol gave good "clean" electrochemistry i.e. there was very little fouling of the electrode on successive oxidations.

c. The oxidation potential of p-aminophenyl phosphate and p-aminophenol was separated by ca. 0.4V therefore p-aminophenol could be measured without any interference from p-aminophenyl phosphate.

The mixed anhydride and carbodiimide conjugation procedures produced theophylline-alkaline phosphatase conjugates with low residual enzymatic activity and immunoreactivity (section 5.3). Vastly improved conjugates with high residual enzymatic activity and immunoreactivity were synthesised by a non activation procedure using an acyl imidazole derivative of theophylline, theophylline-8-butyric acid lactam (section 5.4). These conjugates were then used in the development of the flow injection electrochemical enzyme immunoassay. The conjugates were stable upon storage for 3 months at 4°C in Tris buffer, pH 7.5 containing 0.05% w/v bovine serum albumin and 0.02% w/v sodium azide. The kinetic parameters of the conjugates and the native alkaline phosphatase were investigated and there was little difference in the average Michaelis Menten constant ($k_m$) and the maximum reaction velocity ($V_{max}$) alkaline values suggesting that the physical modification of the native phosphatase upon coupling did not significantly alter its activity.

The controlled pore glass immobilised anti-theophylline IgG prepared using the dimethylpilimidate and carbonylimidazole procedures retained very little activity and therefore was not suitable for use in the flow injection immunoassay.
Protein A from *Staphylococcus aureus* binds the IgG subgroups of most mammalian species via their F\(\text{c}\) region, leaving the antigenic F\(\text{ab}\) region free\(^{35}\). Controlled pore glass protein A was used to successfully immobilise theophylline antisera. The controlled pore glass Protein A was used as the immobilising matrix in the immunoreactor.

In the assay developed, enzyme immunoassay grade alkaline phosphatase was utilized as the label. p-Aminophenol generated from the enzymatic reaction of the conjugate with p-aminophenyl phosphate in the immunoreactor was detected by a simple wall jet electrochemical cell. The major advantage of this flow injection system is the improvement in the assay speed compared to microtitre based immunoassays. This was achieved by adapting non-equilibrium flow rates to deliver the sample and reagent solutions. In addition, the system did not require separate washing steps since all unbound species and interferences were washed continuously by the stream of the flow injection carrier buffer. The advantage of using a flow-through immunoreactor packed with immobilised antibody on controlled pore glass beads over a tubular reactor (i.e. antibody immobilised on the inner walls of tubing) or a conventional stationary device (e.g. microtitre plates) is the efficient on-line mixing of the reagent stream while flowing through the beads. This minimises reagent diffusion barriers and thus increases the rate of mass transport between the analytes/reagents and immunoadsorbents. Furthermore, the mechanical strength of the controlled pore glass beads enables the assay system to employ high flow rates.

The total assay times of the off-line and on-line assay formats developed were 18 mins and 11 mins respectively. The life time of each controlled pore glass protein A immunoreactor was between 80 and 100 runs. The reproducibility of the assay on two consecutive days using the same immunoreactor was poor. This was due to (a) poor column packing which was performed manually using a peristaltic pump and (b) poor column design of the immunoreactor.
In order to increase the reproducibility of the assays, the controlled pore glass protein A immunoreactors must be prepared in a single batch and packed under identical conditions. Columns prepared in this way would be expected to have similar lifetimes and gave similar binding characteristics. The design of the immunoreactor column must also be investigated. The immunoreactor used in this study was of the dimensions 50mm length and 3mm internal diameter. Probably a short wider column would give better reproducibility. The flexibility of protein A binding to various mammalian IgG and its use in an immunoreactor offers the potential for the development of flow injection immunoassays for other drugs using different detection systems. The assay developed in this work used sheep antisera with electrochemical detection. Another flow injection immunoassay has been developed in our laboratory for the immunosuppressive drug cyclosporin A using mouse monoclonal antibodies with fluorescence detection.

The work presented in this thesis has been a preliminary investigation designed to demonstrate the feasibility of a flow injection electrochemical enzyme immunoassay system employing a protein A immunoreactor. While the objective has been successfully achieved, additional work has to be carried out to further optimise the performance of this system, e.g., increase sensitivity, lower detection limit, reduce assay time and increase reproducibility of the protein A column. In addition, to further enhance the operation of this system, the manually operated injection and switching valves can be replaced by computer-controlled valves thereby providing a semi-automated assay system. Finally, the sample throughput of the assay can be further increased by staggering the assay times and using a number of immunoreactors in parallel.
REFERENCES.


CHAPTER SEVEN

IMMOBILISATION STUDIES OF ANTIBODIES FOR USE IN THE DEVELOPMENT OF IMMUNOSENSORS.
7.1 Introduction

Recent advances in analytical methodology in clinical chemistry make use of reagents in a dry format for quantitative analysis. Providing a reagent in a dry format for rapid use is not a new concept, a familiar example is litmus paper, which dates back to the 19th century. By introducing litmus, a coloured extract from several lichens into a paper matrix the inventor provided a dry reagent chemistry for testing the alkalinity or acidity of a solution.

The first major impact dry format chemistry had on clinical testing was the appearance in the 1950s of Ames Clinistix urine reagent strips for testing urinary glucose. By comparing the colour developed on the reagent strip with a colour chart provided on the product labels, the user got a rapid qualitative glucose analysis that otherwise would require a laboratory and skilled personnel. Clinistix reagent strips provided the ground work for the reagent strip developed by Ames and other manufacturers. In the 1970s, more sophisticated dry chemistries emerged for quantitative analysis of blood analytes. These included serum metabolites e.g. blood urea nitrogen\textsuperscript{1} serum electrolytes e.g. potassium\textsuperscript{2} and serum enzymes e.g. acid phosphatase\textsuperscript{3}.

A more recent advance in dry chemistries has been the development of carriers for the analysis of therapeutic drugs in serum e.g. theophylline\textsuperscript{4} lidocaine\textsuperscript{5} and digoxin\textsuperscript{6}. The chemistry for the theophylline dry reagent strip is a competitive protein-binding assay based on substrate labelled fluorescence immunoassay\textsuperscript{7}. Upon placing the sample on the carrier, a competition for antibody-binding sites is established between the serum theophylline and the theophylline conjugate. The unbound conjugate remaining is proportional to the theophylline in the sample. The conjugate is monitored by fluorescence after the removal of the galactose moiety by the action of $\beta$-galactosidase. The conjugate, antibody, and enzyme are introduced in the paper matrix by differential solubility techniques. This prevents the premature interaction of the conjugate with the antibody or enzyme, yet provides a single step,
homogeneous assay. Similar dry reagent chemistries have been described for the detection of the antibiotic gentamicin\textsuperscript{4} and tobramycin\textsuperscript{4} and the anticonvulsant drug carbamazepine\textsuperscript{4}.

The dry reagent strips contain all the reagents required to conduct an analysis. Separation steps required by conventional analysis are integrated into the analytical procedure. This offers several advantages to the user. The small sample volumes needed to conduct an analysis make these chemistries suitable for clinical analysis of sera from geriatric and neonatal patients. Most of the dry reagent chemistries do not require more than 10\(\mu l\) of serum or 30\(\mu l\) of whole blood. Since infants have a small total blood volume, it is undesirable and difficult to extract a sufficient quantity of sample to conduct serum analysis by conventional methods (0.5-2.0\text{ml} serum). Dry reagent chemistries are custom made for conducting total serum profiles on a minimal sample volume.

The simplicity of using dry reagent chemistries and small instrumentation favours more decentralized clinical testing. Traditionally, laboratories for clinical analysis have been centralized in hospitals to serve the routine needs of wards and the specialized needs of intensive care ward and outpatients clinics. Establishing and maintaining a central laboratory requires a variety of often expensive automated instrumentation, reagents and skilled operators. To make testing efficient and cost effective, central laboratories depend on high volume testing. This in conjunction with the low cost of small instruments that have the capability of analyzing for a variety of chemistries, encourages decentralized clinical testing. For example, physicians could run in the office all the routine analysis that they would otherwise send to a laboratory. The advantage is a rapid analysis extracted from freshly drawn blood sample. Further, dry reagent chemistries, by virtue of instrumentation, can be used for high volume testing in central laboratories, and they are significantly flexible to meet the needs of emergency and intensive care wards. Probably the ultimate in decentralized testing would be the home care front. With reliable,
inexpensive, hand held instruments, dry reagent chemistries would allow patients to monitor and control their own medication. These patients would therefore need fewer visits to the doctor and could lead more normal productive lives.

7.2 Aim of Chapter.

The aim of the work in this chapter is two fold:

(a) The investigation of the antibody binding characteristics of six different types of membrane for use in the development of immunosensors.

(b) The incorporation of the best suited membrane into competitive and displacement enzyme immunoassays using rabbit IgG/anti-rabbit IgG and anti-rabbit IgG-alkaline phosphatase as a model system.

7.3 Instrumentation and Chemicals.

7.3.1 Instrumentation

LKB Ultrospec II UV/VIS Spectrometer
Water Bath
Orbital Shaker
Philips PW9240 pH Meter
### 7.3.2 Chemicals and Materials

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit IgG</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-Rabbit IgG-Alkaline Phosphatase</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Casein</td>
<td>BDH</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>Fisons</td>
</tr>
<tr>
<td>Horse Serum</td>
<td>Sigma</td>
</tr>
<tr>
<td>p-Nitrophenyl Phosphate Disodium salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween - 20</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodyne A Membrane</td>
<td>Pall Biosupport</td>
</tr>
<tr>
<td>Biodyne B Membrane</td>
<td>Pall Biosupport</td>
</tr>
<tr>
<td>Biodyne C Membrane</td>
<td>Pall Biosupport</td>
</tr>
<tr>
<td>Immunodyne Membrane</td>
<td>Pall Biosupport</td>
</tr>
<tr>
<td>Ultrabind Membrane</td>
<td>Gelman Sciences</td>
</tr>
</tbody>
</table>

### 7.3.3 Buffer Solutions

Phosphate buffered saline (PBS, 0.15M pH 7.4) and Diethanolamine buffer (1M, pH 9.8) were prepared as described in section 5.2.4.
7.4 Investigation of the Antibody Binding Characteristics of Six Different Types of Membrane.

7.4.1 Immobilisation of Rabbit IgG on the Membranes by the Membrane Immersion Technique.

Experimental

Immobilisation Procedure

The Pall immunodyne membrane (50mm x 30mm) was incubated for 2 hours on an orbital shaker with 2mg of rabbit IgG in 10ml of 0.15M PBS pH 7.4 (0.2 mg ml⁻¹). After this period the residual binding sites on the membrane were blocked by treatment with 10ml of 0.5% w/v Casein (made in PBS buffer) for 2 hours. The membrane was then washed four times with PBS buffer (20ml aliquots) for 5mins, and dried on filter paper for 1 hour. The antibody immobilised strips were then cut into 6mm circular discs with a hole puncher and stored in a dessicator.

For the assessment of non specific binding (NSB) a piece of blank immunodyne membrane (50mm x 30mm) (without immobilised IgG) was blocked with 0.5% w/v casein, washed with PBS buffer and stored as described above.

The above procedures were repeated for the other membranes (Biodyne A, B, C, Nitrocellulose and Ultrabind membranes).

The amount of antibody (IgG) immobilised on the membranes was assessed by determining the protein contents of the antibody solution before and after the immobilisation procedure using the bicinchoninic acid protein assay.

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Assay of the Antibody Immobilised and Blank Membrane Discs.

The membrane discs were incubated with 10ml of anti-rabbit IgG - alkaline phosphatase (1:5000 dilution in 0.15M PBS buffer pH 7.4, 2.9U) for 1 hour on an orbital shaker. After this period the discs were washed four times with PBS buffer (20ml aliquots) for 5 mins. Each membrane disc was then incubated in 1ml of alkaline phosphatase substrate (10mM p-nitrophenyl phosphate in 1M diethanolamine buffer pH 9.8) for 30 mins. The reaction was stopped by the addition of 0.5ml of 5M Sodium hydroxide. The absorbance of the supernatant solution was read at 415nm.

Results and Discussion.

The uptake of the rabbit IgG by the six membranes is given in Table 7.1.

Table 7.1 Uptake of Rabbit IgG by the Membranes

<table>
<thead>
<tr>
<th>Membrane</th>
<th>IgG Uptake(µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodyne A</td>
<td>87.5</td>
</tr>
<tr>
<td>Biodyne B</td>
<td>68.7</td>
</tr>
<tr>
<td>Biodyne C</td>
<td>40.9</td>
</tr>
<tr>
<td>Immunodyne</td>
<td>52.7</td>
</tr>
<tr>
<td>Nitrocellulose</td>
<td>127.1</td>
</tr>
<tr>
<td>Ultrabind</td>
<td>22.6</td>
</tr>
</tbody>
</table>

The Nitrocellulose membrane had the highest uptake of IgG and the Ultrabind membrane the lowest uptake. The binding characteristic of the membranes were different due to variations in their surface chemistry. The Biodyne A membrane comprised of 50% amine and 50% carboxyl groups i.e amphoteric, the Biodyne B membrane surface was populated by a high
number of quaternary ammonium groups making it strongly cationic i.e positively charged. The Biodyne C membrane surface comprised of a high concentration of carboxyl groups, which created an anionic surface suitable for the stable binding of basic proteins and the immunodyne membrane surface featured a chemically pre-activated surface offering a high density of covalent binding sites that would permanently immobilise proteins on contact without loss of biological activity. The surface chemistries of the two other membranes i.e the Nitrocellulose and Ultrabind membranes were similar to the Biodyne A and Immunodyne membranes respectively.

The immobilisation of the IgG on to the Biodyne A, B, C and the traditional Nitrocellulose membranes was by adsorption whereas the immobilisation on to the Immunodyne and Ultrabind membrane was by covalent interactions. The degree of uptake of IgG by the Ultrabind was small, this was probably due to very few pre-activated sites present on the membrane surface thereby resulting in the controlled immobilisation of the antibody.

The non specific binding of the labelled antigen (anti-rabbit IgG-alkaline phosphatase) to the six membranes was investigated using casein (0.5%w/v) as the blocking agent. The signal to noise ratios for the membranes i.e.

<table>
<thead>
<tr>
<th>Signal due to the interactions between labelled antigen and antibody immobilised membrane</th>
<th>Signal due to the interactions between labelled antigen and blank membrane</th>
</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

are given in Table 7.2.

The Ultrabind membrane gave the highest signal to noise ratio i.e 7.4 and was used in all further experiments.
Table 7.2 Signal to Noise Ratio for the Membranes Using Caesin as the Blocking Agent

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Signal to Noise Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodyne A</td>
<td>5.0</td>
</tr>
<tr>
<td>Biodyne B</td>
<td>4.2</td>
</tr>
<tr>
<td>Biodyne C</td>
<td>5.8</td>
</tr>
<tr>
<td>Immunodyne</td>
<td>4.6</td>
</tr>
<tr>
<td>Nitrocellulose</td>
<td>5.7</td>
</tr>
<tr>
<td>Ultrabind</td>
<td>7.4</td>
</tr>
</tbody>
</table>

7.4.2 The Effect of Different Blocking Agents on the Non-Specific Protein Binding to the Ultrabind Membrane.

Experimental

Rabbit IgG was immobilised onto the Ultrabind Membrane (50mm x 30mm) as described in section 7.4.1 and residual binding sites blocked with 5% v/v horse serum, 2.5% w/v bovine serum albumin and 0.1% v/v Tween 20. Blank Ultrabind membranes (50mm x 30mm) were also treated with the above blocking agents. The antibody immobilised and blank discs were then assayed as in section 7.4.1.

Results and Discussion

In order to increase the signal to noise ratio for the Ultrabind membrane different blocking agents were investigated. The signal to noise ratio for horse serum, bovine serum albumin and Tween 20 were 6.2, 5.4 and 1.2 respectively. Therefore casein (0.5% w/v) which gave a signal to noise ratio
of 7.4 was used in all further experiments.

7.4.3 The Effect of Rabbit IgG Concentration on the Ultrabind Membrane Uptake.

Experimental.

The immobilisation procedure in section 7.4.1 was performed for the Ultrabind membrane using rabbit IgG concentrations of 0, 0.025, 0.05, 0.1, 0.2 and 0.4 mg ml\textsuperscript{-1}. The antibody immobilised and blank membranes were blocked with caesin and the discs were assayed as described in section 7.4.1.

Results and Discussion.

In section 7.4.1 and 7.4.2 a total of 2mg of IgG was used in the immobilisation procedure. The effect of increasing the IgG concentrations was investigated. Fig 7.1 shows that increasing the IgG concentrations above 2mg did not significantly increase the antibody uptake by the Ultrabind membrane. Therefore 2 mg of IgG in 10ml of PBS buffer i.e a 0.2mg ml\textsuperscript{-1} solution was used in all further experiments.

7.5 Enzyme Immunoassay using Rabbit IgG, Anti-Rabbit IgG and Anti-Rabbit IgG- Alkaline Phosphatase as a Model System.

7.5.1 Competitive Enzyme Immunoassay

Experimental

A competitive enzyme immunoassay for anti-rabbit IgG was set up by incubating rabbit IgG immobilised Ultrabind membrane discs with a mixture of 5ml of anti-rabbit IgG-alkaline phosphatase (1.45U) and 5ml of
Absorbance at 415nm

Fig 7.1 Effect of Antibody Content on the Ultrabind Membrane Uptake
anti-rabbit IgG (0-1000μg) for 1 hour. The discs were then washed twice with PBS buffer (10ml aliquot) and assayed as discussed in section 7.4.1

7.5.2 Displacement Enzyme Immunoassay

Experimental

The displacement enzyme immunoassay for anti-rabbit IgG was set up by incubating rabbit IgG immobilised Ultrabind discs with 10ml of anti-rabbit IgG-alkaline phosphatase (2.9U) for 1 hour. The discs were then washed twice with PBS (10ml aliquots) and incubated with anti-rabbit IgG (0-1000μg) for 1 hour. The discs were then washed again with PBS assayed as in section 7.4.1.

Results and Discussion

Competitive and displacement assay formats were configured for an IgG model system. Typical calibration curves for the competitive and displacement assays between 0 - 1000μg of anti-rabbit IgG are shown in Fig 7.2 and Fig 7.3 respectively. In the above assays the enzyme labelled antigen and immobilised antibody were incubated for 1 hour. Ideally this incubation time must be approximately 10 mins in order to develop a rapid assay. The effect of decreasing the antibody, antigen and labelled antigen incubation time for the competitive assay format at the 1000μg antigen level was investigated. Fig 7.4 shows that decreasing the incubation time results in a decrease in the signal to noise ratio. In order to develop a rapid assay based on the above assay formats a compromise must be made between the signal to noise ratio and the antibody, antigen and labelled antigen incubation time.
Fig 7.2 Calibration Curve for Anti-Rabbit IgG Using a Competitive Enzyme Immunoassay Format
Fig 7.3 Calibration Curve for Anti-Rabbit IgG Using a Displacement Enzyme Immunoassay Format
Fig 7.4 Effect of Antigen and Antibody Incubation Time on the Signal to Noise Ratio for the Competitive Enzyme Immunoassay.
From the work presented in this chapter using rabbit IgG as a model system it has been shown that antibodies immobilised onto various membranes can be used to develop solid-phase immunoassays systems. The advantages of using membranes in immunoassays are their high surface area and in the case of pre-activated membranes, their high binding capacity which can significantly increase the sensitivity for a wide range of optimised assay formats. These membranes can also be incorporated into disposable strip sensors with various detection systems for use in the immunodiagnostic field.
REFERENCES


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