

**THE USE OF NON-RADIOACTIVE IODINE
AS A LABEL IN BIOLOGICAL ASSAYS**

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DECLARATION

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

Elizabeth Creed

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7.3.'94

Date

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ABSTRACT

A microassay for the determination of iodide and iodine-containing compounds based on the Sandell-Kolthoff reaction was developed by O'Kennedy *et al.*, (1989). Non-radioactive iodine was then used to label antibodies for immunoassay. The sensitivity of this immunoassay has been shown to be comparable to that obtained in an ELISA when the enzyme used was horseradish peroxidase. This study was carried out to investigate the feasibility of labelling DNA probes with non-radioactive iodine. Nucleic acids have previously been labelled with ¹²⁵Iodine and these iodination procedures were adopted.

Labelling of nucleic acids was carried out both chemically using the oxidizer *Iodogen*, and enzymatically via the incorporation of 5-iodo-2'-deoxycytidine 5'-triphosphate by random priming. 2% and 38% incorporation was observed for these methods, respectively. The degree of iodination achieved was determined using the iodide microassay. HPLC of digested nucleic acids was also carried out to measure the incorporation of iodine.

The iodide microassay was modified and then validated in the range 1-10ng/ml potassium iodide. The catalytic effect of 5-iodocytidine, and related compounds, in the iodide microassay was also considered. The iodide assay was performed on nitrocellulose paper to investigate the feasibility of applying this assay in a dot blot format. The limit of quantification for iodide in this format was 1.0µg/ml.

To use cold-iodine as a direct label for DNA probes a more sensitive iodide microassay would be required. For this reason a number of chemiluminescent systems, which were quenched by iodide, were investigated. However, the lowest limit of quantification obtained for the microassays considered was 10ng/ml iodide.

The use of iodine as an indirect label for DNA probes was also examined and polyclonal antibodies to 5-iodocytidine were produced.

ABBREVIATIONS

AAIF	7-iodo-N-acetoxy-N-2-acetylamine fluorene
AMP	adenosine monophosphate
AMPPD	adamantyl 1,2-dioxetane phosphate [or 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxetane]
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCPIP	5-bromo-4-chloro-3-indoyl phosphate
BHR	Bolton-Hunter reagent
BSA	bovine serum albumin
cDNA	complementary DNA
CPS	counts per second
(d)ATP	(2'-deoxy)adenosine 5'-triphosphate
(d)CTP	(2'-deoxy)cytidine 5'-triphosphate
(d)GTP	(2'-deoxy)guanosine 5'-triphosphate
DNA	2'-deoxyribonucleic acid
DNCB	2,4-dinitro-1-chlorobenzene
DNP	2,4-dinitrophenyl
dNTP	2'-deoxyribonucleotide 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ECL	enhanced chemiluminescence (trade name for Amersham's luminol/ hydrogen peroxide enhanced system)
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
HIV-I	human immunodeficiency virus Type I
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
IBHR	iodinated Bolton-Hunter reagent
IgG	immunoglobulins: class G

LOD	limit of detection
mRNA	messenger ribonucleic acid
NADP	β -nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NHS	N-hydroxysuccinimide
OPDA	ortho-phenylenediamine
PBS	phosphate buffered saline (0.1M, pH 7.4)
PBS-Tween	0.05% (v/v) Tween-20 in PBS (0.1M, pH 7.4)
PCR	polymerase chain reaction
PEG	polyethylene glycol
RIA	radio-immunoassay
RLU	relative light units
RNA	ribonucleic acid
RPM	revolutions per minute
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
TNBS	2,4,6-trinitrobenzene sulphonic acid
TRF	time resolved fluorescence
UTP	uridine 5'triphosphate
v/v	volume per volume
w/v	weight per volume

Chapter 1

INTRODUCTION

SECTION I

1.11 THE APPLICATION OF NON-RADIOACTIVE IODINE AS A LABEL

Radioactive labels are widely used as tracers in bio-assays, immunoassays and in DNA probe assays. These radiolabels include ^{125}I , ^{32}P , ^{35}S and tritium. Radioactivity can be detected with great sensitivity by simple methods such as scintillation counting or by autoradiography. However, working with radiolabels has major disadvantages including potential health risks to the worker and the disposal of the radioactive waste. A further disadvantage is the short shelf-life of the label (e.g., ^{125}I has a half-life of 60 days and ^{32}P has a half-life of only 14 days). For these reasons alternatives to radiolabels have been sought. These include the use of enzyme, fluorescent and chemiluminescent labels (Keller and Manak, 1989). A novel non-radioactive labelling system which uses non-radioactive iodine has been described by O'Kennedy *et al.*, (1989). Though this system eliminates the need for the use of radioactivity, iodine-labelling methodologies previously developed may be used.

The catalytic effect of iodide on the reduction of ceric sulphate by arsenious acid was first described by Sandell and Kolthoff (1934). This reaction has been followed by different analytical techniques and has been used to measure iodine and iodide levels in biomedical, food and environmental studies (outlined in Section 1.12.2). A microassay for the determination of iodide and iodine-containing compounds based on this assay was developed by O'Kennedy *et al.*, (1989). Optimization of this microassay and its applications were later described (O'Kennedy and Keating, 1991). The microassay for iodide was successfully applied for the detection of mouse IgG using a second antibody labelled with iodinated Bolton-Hunter reagent (IBHR). The

Bolton-Hunter reagent (3-(4-hydroxyphenyl)-propionic acid N-hydroxysuccinimide ester) has been widely used to radioiodinate a range of biological molecules, including antibodies (Bolton and Hunter, 1973; Langone, 1981). This immunoassay compared favourably with a more conventional Enzyme-Linked ImmunoSorbent Assay (ELISA), using horseradish peroxidase-labelled second antibody. Thus, it has been shown that cold-iodine, when used as a catalyst to a chemical reaction, can act as the reporter group for the detection of antibodies in an immunoassay.

This novel immunoassay was also used to measure human antibody levels in serum. Anti-human IgG antibody was labelled with iodine using IBHR and used in a sandwich assay to detect antibody levels in serum samples. The performance of this iodide-based immunoassay was comparable to that of an ELISA using horseradish peroxidase linked to the same antibody (O'Kennedy and Keating, 1993). A method has also been described for the determination of proteolytic activity based on the use of IBHR-labelled bovine serum albumin (BSA) as the substrate. 100ng/ml of proteinase (trypsin, pepsin, and papain) could be detected (Keating *et al.*, 1991).

One aim of this project was to investigate the feasibility of using non-radioactive iodine as a label for DNA probe assays. Initially, the iodide microassay developed (O'Kennedy *et al.*, 1989) was optimized and validated so that iodide in the range 1-10ng/ml could be detected. This modified iodide microassay was used to detect nucleic acids labelled with iodine. The development of an alternative, more sensitive, microassay for iodide was also undertaken. Chemiluminescent assays were considered as such assays have been shown to offer sensitivity greater than colorimetric assays and a wider linear range (see Section 1.13).

1.12 CATALYTIC-KINETIC ASSAYS TO DETECT IODIDE

1.12.1 Introduction

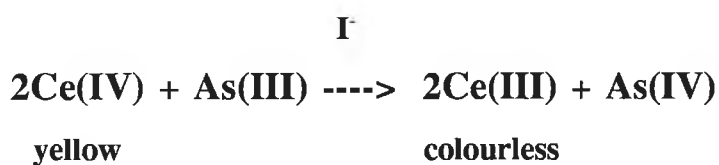
Iodine has been the focus of many biomedical, food and environmental studies. For this reason many analytical methods are available for the determination of trace amounts in different matrices. In this project iodide was measured using a catalytic-kinetic method and using a range of chemiluminescent assays.

Catalysed reactions can be applied in different ways in analytical chemistry. Methods of analysis can be divided into two main groups: methods based on thermodynamic equilibrium and kinetic methods. In the former, measurements are made in a system which is in a stable (equilibrium) state and time is not a variable. Gravimetric, titrimetric and photometric analyses fall into this category. In kinetic methods a temporal course of the chemical reaction is observed, that is, the variable changes with time. Kinetic methods can be based on catalysed and uncatalysed reactions and the reactants may be determined. In catalysed reactions it is also possible to measure the catalyst and substances which may react with them, such as inhibitors and activators (Mottola *et al.* 1988).

An important group of catalytic-kinetic analytical methods use "open systems" in which, during the course of the reaction a reactant is added or a product is removed, as opposed to the closed system where no further operations occur after the start of the reaction. The "stat methods" belong to the former group. In these "stat methods" a preset stationary state within the catalysed system is kept constant by adding a suitable reagent so that any change in concentration is compensated (Pantel, 1987). The concentration of a catalyst can be derived from the rate of addition which is necessary to keep the system constant.

1.12.2 The Sandell-Kolthoff reaction

The most commonly used catalytic-kinetic method for the estimation of iodide is the measurement of its catalytic action on the reaction which occurs between cerium(IV) and arsenic(III), described by Sandell and Kolthoff (1934). They used a colorimetric method (measuring absorbance at 420nm) and detected microgram quantities of iodide with 2-4% accuracy. This is a closed system.



THE SANDELL-KOLTHOFF REACTION

The oxidation of arsenic by cerium is catalyzed not only by iodide but also by iodine-containing organic compounds. Several studies have reported that not all iodine-containing compounds catalyze the Sandell-Kolthoff reaction equally (Pantell, 1982; Timotheou-Potamia, 1988; O'Kennedy *et al.*, 1989). The presence of side-groups adjacent to the iodine atom on the parent molecule effect the catalytic activity of the iodide atom. Pantell (1982) introduced the term F factor. This was defined as the molar concentration of iodide (C_I) and the respective iodo-compound (C_O) which have exactly the same catalytic activity.

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

The K value introduced by Timotheou-Potamia (1988) was also a measure of the catalytic activity of iodo-compounds in comparison to that of inorganic iodide.

$$K = \frac{100 (C_I)}{n (C_O)}$$

C_I and C_O are the molar concentrations of iodide and the organic compound, respectively, that have identical catalytic activity and n is the number of iodine atoms per mole of compound. Pantel (1982) found that the catalytic activity of the substituted iodobenzenes of the general formula $X-C_6H_4-I$ is strongly dependent on the nature of X and the relative position of substituents in the benzene ring. Timotheou-Potamia (1988) showed that the catalytic activity of iodine-containing compounds in the Sandell-Kolthoff reaction was related to the structure of the compound. The K value was shown to depend on the presence of electron donors and on steric effects. Table 1.1 shows the K values which have been determined for a range of iodo-compounds.

Reference	Compound	K value, %
	iodide	100.0
Pantell, (1982)	2-iodophenol	9.0
	3-iodophenol	1.5
	4-iodophenol	42.0
	2-iodoaniline	0.5
	3-iodoaniline	1.2
	4-iodoaniline	48.0
Timotheou-Potamia, (1988)	thyroxine	57.0
	3-iodotyrosine	77.0
	N-iodosuccinimide	94.0
O'Kennedy <i>et al.</i> , (1989)	thyroxine	62.0
	4-iodophenol	40.0
	iodinated Bolton-Hunter reagent	33.0

Table 1.1
Catalytic activities of some organic iodine-containing compounds.

The closed system, for catalytic-kinetic reactions, has also been used in the following determinations of iodide and iodo-compounds using the Sandell-Kolthoff reaction. Chaney (1940) adapted the above method for the analysis of iodide in blood, the limit of detection was 0.04 μ g iodide. The percentage transmittance was linearly related to the concentration of iodide. Mougey and Mason (1963) and Knapp and Leopold (1974) have also measured iodide by recording the percentage transmittance over time. The automated system described by Knapp and Leopold allowed detection of 0.2ng tri-iodothyronine (T3) and 0.1ng thyroxine (T4). These thyroid hormones were separated from blood serum by column chromatography prior to analysis.

Bowden *et al.*, (1955) described the detection of inorganic and organic iodine-containing compounds on paper chromatography using the cerium/ arsenic reaction. The ceric sulphate-arsenious acid reagent was applied evenly to a

sheet of chromatography paper the same size as the chromatogram on which the iodo-compounds were separated. The wetted paper was placed on the dry chromatogram and left for 30 minutes. The paper was dried and white spots on a yellow background indicated the position of the iodo-compounds. 0.01 μ g of potassium iodide and 0.1 μ g of thyroxine were detected.

O'Kennedy *et al.*, (1989) adapted the Sandell-Kolthoff assay to a microplate format to measure iodide (outlined in Section 1.11). A novel immunoassay using non-radioactive iodine-labelled antibodies was described (O'Kennedy and Keating, 1991). Fischer *et al.*, (1986) described the colorimetric determination of iodide in foods and Aumont and Tressol (1987) adapted the Sandell-Kolthoff reaction to the routine analysis of iodine in biological fluids. A stopped flow method, coupled to a spectrophotometer, was developed by Gutierrez *et al.*, (1989) for the routine analysis of iodide in pharmaceutical and food samples.

Hadjiannou (1964) developed an automatic ultramicro system for determining iodine in natural waters. The Sandell-Kolthoff reaction was stopped after a given time by the addition of iron(III). After the addition of thiocyanate ions the resulting red colour of the iron-thiocyanate complex could be measured. The system was simple and rapid and samples containing 1-15ng/ml iodide could be determined.

Stat methods for the analysis of catalytic-kinetic reactions have also been used to measure iodide in the Sandell-Kolthoff reaction. These include biamperostat, absorptiostat and potentiostatic methods. The biamperostat method requires two polarized electrodes so that, when both the oxidizable and reducible forms of a species are electroactive, the current flowing at steady state will depend on the species present at lower concentration. With this method a preset biamperometric current, corresponding to a definite concentration of a biamperometrically active substance (Ce(IV) in the Sandell-Kolthoff reaction), is kept just constant. The rate of addition of the appropriate reagent provides a measure of the catalyst being determined. Pantel and Weisz (1974, 1977)

used the biamperostat method to determine iodide in the range 80-800ng/ml and subsequently to determine the iodine-containing compounds L-throxine (13-130ng/ml) and 5-chloro-7-iodoquinolin-8-ol (13-130ng/ml). The substituent effects in monosubstituted (both phenolic and amino groups) iodobenzenes in aqueous and 20% ethanolic solutions were also investigated (Pantel, 1982).

Weisz and Rothmaier (1975) described an absorptiostat method (where a preset absorbance is maintained by the constant addition of a coloured reagent) to determine iodide in the range 11-110ng/ml using the Sandell-Kolthoff reaction. To a solution containing the iodide and an excess of As(III), a standard Ce(IV) solution was added until the preset absorbance was reached. Each deflection from this value, caused by the course of the catalysed reaction, was compensated by addition of Ce(IV). The rate of addition of the Ce(IV) is proportional to the iodide concentration. Pantel (1982) reported that this method could not be used for the determination of iodo-compounds because yellow iodo-compounds formed in solution with the Ce(IV).

A potentiostat method has also been used to measure iodide in the Sandell-Kolthoff reaction. Potentiometry involves a reversible exchange of a charged species across an electrode/solution interface. The Ce(IV)/ As(III) reaction was monitored with a platinum indicator electrode and the time required for a fixed change in potential was measured. This was proportional to the iodide concentration (Weisz and Rothmaier, 1974). The method has been applied to the measurement of thyroxine in the range 0.4-4.0µg/ml (Timotheou-Potamia *et al.*, 1985). Timotheou-Potamia (1988) also used the potentiostat method for the determination of various iodine-containing compounds.

A new method of catalytic determination was described by Lòpez-Cueto and Cueto-Rejòn (1987). Experimental assessment was made by examining the behaviour of the iodide catalysed Ce(IV)/ As(III) reaction. Amperometric measurements were taken. The method involved the comparison of the kinetic curve for the sample solution with the one for the reference solution to which

a standard solution of catalyst was added at a constant rate. In this way an "intersection time" may be determined. Using the value for the "intersection time" the concentration of the catalyst may be obtained by either a direct calculation or by a calibration plot. Iodide was read in the range 8.8-58.9 ng/ml.

1.12.3 Other catalytic-kinetic methods to detect iodide

Other catalytic-kinetic systems have been used for the determination of iodide and these are shown in Table 1.2

REACTION	LOD g/ml	REFERENCE
$\text{NO}_2^- + 2\text{SCN}^- + 4\text{H}^+ = 2\text{NO} + (\text{SCN})_2 + \text{H}_2\text{O}$	2×10^{-9}	Proskuryakova, 1967
3,3'-dimethylnaphthidine + H_2O_2	1×10^{-9}	Bognar & Nagy, 1969
p,p'-tetramethyldiaminodiphenylmethane + chloramine T	1×10^{-14}	Balczko, 1969
Hg(II)-PAR + 1,2-cyclo-hexane-diamine N,N,N',N' tetraacetic acid	1×10^{-9}	Funahashi <i>et al.</i> , 1971
$2\text{Ce}^{4+} + \text{Sb}^{3+} = 2\text{Ce}^{3+} + \text{Sb}^{5+}$	1×10^{-9}	Bognar & Sarosi, 1965

Table 1.2
Catalytic-kinetic methods for the determination of iodide. [LOD is the limit of detection].

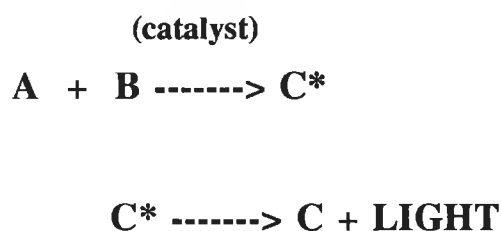
The Sandell-Kolthoff reaction uses an arsenic derivative, a known carcinogen. It would be advantageous if an alternative reaction with less toxic reagents could be used in an iodide microassay. The reactions listed in Table 1.2, though they show good sensitivity, only offer one alternative. Thiocyanate is extremely toxic and is also a suspected tetratogen. 3'3'-dimethylnaphthidine is extremely toxic and is also thought to cause heritable genetic damage. p,p'Tetramethyldiamino-diphenylmethane is a suspected carcinogen and a

mutagenic compound and the use of mercuric compounds is to be avoided. Thus, the cerium-antimony reaction is the only one of the listed reactions which offers a safer alternative to the Sandell-Kolthoff reaction. Butler and O'Kennedy (1994) have developed a microassay using this reaction for the determination of iodide (2-10ng/ml), IBHR (0.02-0.10µg/ml) and IgG in an immunoassay (0.05-2.00µg/ml).

1.13 CHEMILUMINESCENT ASSAYS TO DETECT IODIDE

1.13.1 Introduction

Chemiluminescence is the emission of light from chemical reactions at normal temperatures. It is observed when an electronically excited product or intermediate formed during a chemical reaction decays to the ground state by emitting a photon. If the reaction occurs in a living system, or derived from one, the process is called bioluminescence.



The above depicts a generalised chemiluminescent reaction and any one of the components, including the catalyst if one is used, can be measured as the analyte. The reaction conditions are adjusted so that the light measured is a function of the level of the analyte to be determined. The signal is transient and, thus, the measurement of the chemiluminescence intensity is time-dependent. The chemiluminescent intensity (I_{CL}) is defined as follows (Grayeski, 1987):

$$I_{CL} = \phi_{CL} \text{ (photons/ molecules reacted)} \times \delta c/\delta t \text{ (molecules reacted/second)}$$

ϕ_{CL} is the chemiluminescent efficiency and is equal to the efficiency of production of excited state molecules reacted times the emission efficiency (number of photons emitted per number of molecules reacted) and $\delta c/\delta t$ is the number of molecules reacting per unit of time.

In chemiluminescent reactions the photon efficiency is usually less than 1%. This is because only some of the excited molecules lead to the production of photons while most molecules lose their excitation energy through "dark reactions" as heat. In bioluminescence, however, the catalyzing enzyme directs

the reaction through the path leading to the production of light, and photon efficiency for bioluminescent systems is between 10-90%.

The light may be observed with photography, by exposure to X-ray film or by using a photomultiplier tube. A light source is not required. Thus, source noise and light scatter are eliminated. Luminescence detection is often more sensitive than conventional fluorescence detection for this reason (Cambell, 1988)

Three basic types of formats can be used for the detection of chemiluminescence (Grayeski, 1987). The first, used for making static measurements in solution, involves mixing reagents in front of a detector. Mixing is often achieved by the force of injection of a final reagent that is added to a tube or well of a microtitre plate containing the other reagents. Flow systems can be used to mix the reagents when the analyte is injected into a stream of luminescent reagents. Analytes on solid surfaces such as filter paper can be measured by saturating the surface with chemiluminescent reagents and recording the light emitted with a microplate reader, by contact printing with photographic detection or exposure to X-ray film.

Luminescence offers several potential advantages for analytical applications (Grayeski, 1987). Low detection levels are possible, often in the femto/atto mole range and for many analytes the linear dynamic range is several orders of magnitude. In addition, methods are often rapid and instrumentation is relatively simple and easily automated.

Kricka and Thorpe (1983) described the practical considerations necessary when carrying out luminescent assays so as to avoid high background levels and irreproducible results. These considerations included the criteria of reagent purity and the importance of thorough rapid mixing. Klopff and Nieman (1983) also reported that the results of their experiments indicated the importance of the exact conditions under which a luminescent reaction is carried out. They considered the effect of iron(II), cobalt(II), copper(II) and manganese(II) on the chemiluminescence of luminol in the absence of hydrogen peroxide. The

pH of the system, the oxygen content of solutions and the presence of other metal ions in the system all had significant effects on a chemiluminescent reaction. Thus, the conditions must be carefully determined for a chemiluminescent assay.

Two typical luminescent reactions will be described. The firefly reaction is an example of a bioluminescent system. In the transformation of firefly luciferin to an excited state of luciferin, a dioxetanone is formed by the action of the luciferase enzyme using oxygen and adenosine triphosphate (ATP) (see Figure 1.1). Many applications of this bioluminescent reaction have been described including the following: a rapid and sensitive method for measuring cell numbers and the effects of toxic or stimulating substances on somatic or microbial cells, and sensitive assays to measure ATP-specific enzymes and their substrates (Kolehmainen, 1979).

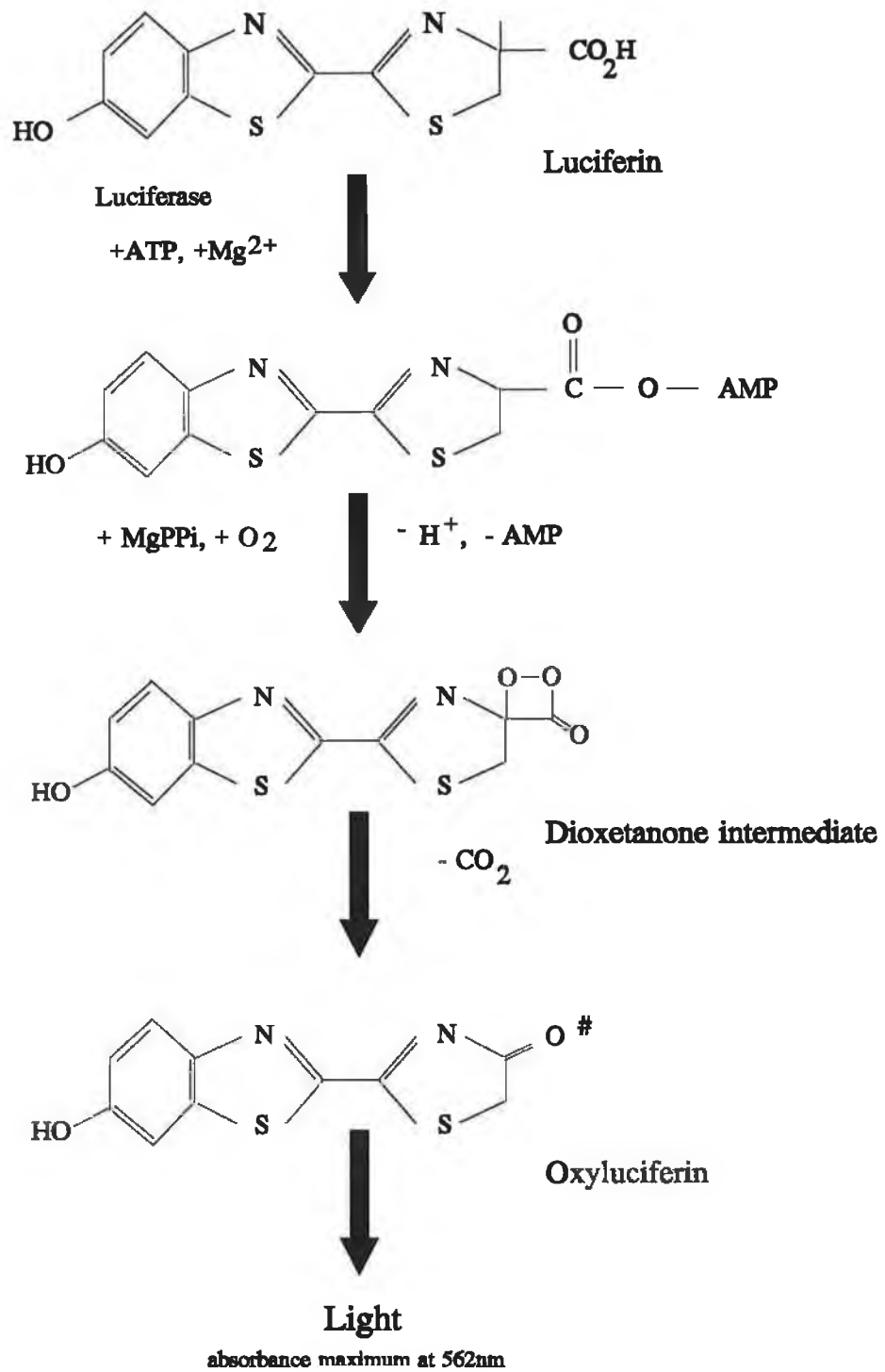


Figure 1.1
 Firefly bioluminescence (PPi = inorganic phosphate).

Luminol when oxidized by most strong oxidants (for example, hypochlorite, iodine or hydrogen peroxide with an associated catalyst or cooxidant), in alkaline solution, gives rise to a characteristic blue luminescence due to the electronically excited 3-amino-phthalate dianion (Roswell and White, 1978). The luminol luminescent reaction is illustrated in Figure 1.2. This reaction has been used to measure luminol, the oxidant, and metal and enzyme catalysts. The reaction has also been used to determine compounds which interact with the oxidant (Townshend, 1990).

The luminol system has been applied for automatic measurement of glucose in blood and urine. Other analytes which have been determined include uric acid, xanthine aldehydes, amino acids, trace elements, haemoglobins and myoglobins and the enzymes, peroxidase, superoxide dismutase oxidase and catalase (Kolehmainen, 1979).

The luminol/ hydrogen peroxide system, catalyzed by hydrogen peroxidase, has been enhanced by the presence of certain substituted phenols (e.g., *p*-iodophenol), naphthols and amine compound (Thorpe and Kricka, 1986). Enhanced reactions are easily measured as prolonged, relatively constant and intense light emission is achieved. These enhanced reactions have allowed sensitive detection of hydrogen peroxidase which has facilitated the use of this enzyme as a label in immunoassays and in DNA probe assays.

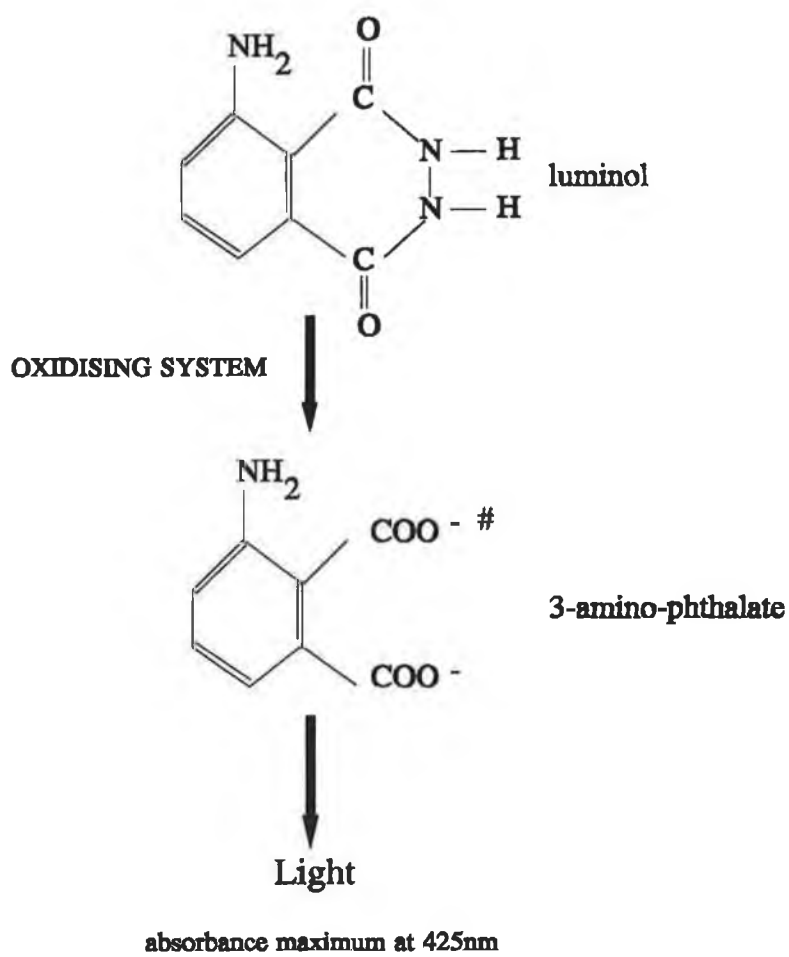


Figure 1.2
Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) chemiluminescence.

1.13.2 Detection of iodine and iodide

The iodine/ luminol system was first studied by Babko *et al.*, (1968). Seitz and Hercules (1974) made a quantitative study of this reaction. If the reaction pH is properly adjusted the chemiluminescence is linearly proportional to the iodine concentration from 5×10^{-10} to 3×10^{-7} M. They proposed an azoquinone intermediate. Since halides and halates do not cause luminol chemiluminescence, it is necessary to turn them into a form active in the chemiluminescent reaction with subsequent separation from the excess oxidant.

The generation of chemiluminescence upon reaction of iodine with luminol in reversed micelles was investigated by Fujiwara *et al.*, (1989). The detection limit was 2×10^{-10} M iodine. The method was found potentially useful for the selective chemiluminescent determination of iodide where oxidation of iodide to iodine and iodine extraction were simultaneously carried out prior to chemiluminescent analysis. The extraction procedure separates the iodine from the oxidant which would interfere with the iodine determination by the luminol method. A calibration curve of 0.1ng/ml - 1.0µg/ml iodine was reported and a similar calibration curve for iodide was obtained.

Micelle aggregates are used to manipulate the solubility and microenvironment of analytes and reagents and to control the reactivity, equilibrium and pathway of chemical processes. Hoshino and Hinze (1987) used reversed micelles as a medium in analytical chemiluminescent measurement with application to the determination of hydrogen peroxide using luminol. The advantages listed for this type of assay included improved sensitivity, improved selectivity, better precision and a relaxation of the strict pH requirements for observation of efficient chemiluminescence. They demonstrated that it was possible to obtain analytically useful chemiluminescent emission from the luminol/ hydrogen peroxide reaction in the pH 7.8-9.0 region in a CTAC (hexadecyltrimethylammonium chloride) reversed micellar medium without the addition of any added catalyst or co-oxidant.

Pilipenko *et al.*, (1986) described a chemiluminescent method for the determination of trace amounts of iodide. Iodide was oxidised using bromine water to iodine, and then converted to cyanogen iodide which reacted with luminol to give luminescence. The oxidant bromine water does not interfere in the luminescent reaction because it is converted to bromide cyanogen which does not react with luminol. A linear function of the iodide concentration in the range 5-90ng/ml was obtained.

Iodide and bromide have also been determined by a chemiluminescent method coupled with dynamic gas extraction. (Pilipenko *et al.*, 1989). Iodide or bromide was selectively oxidised and the halogen was swept by nitrogen or argon into an alkaline luminol solution. The luminescence was recorded. The limit of detection was 3ng/ml iodide and 1.3ng/ml bromide. The method was applied in the analysis of water.

Chemiluminescent systems which do not use luminol have also been described. Burguera and Burguera, (1982) used flow-injection analysis to determine iodine (the limit of detection was 0.3ng/ml). Iodine was determined by measuring the chemiluminescent emission peak generated when an iodine solution was injected into a premixed hydrogen peroxide/ sodium hypochlorite flow system. Lu *et al.*, (1989) described the chemiluminescent system acetone/ hydrogen peroxide/ hypochlorite/ iodide. The reagents were injected into a cell and the luminescence was read for 8 seconds. The system was catalyzed by the iodide ion and the calibration curve was linear from 0.4ng/ml - 0.3µg/ml iodide.

Several analytes such as the inorganic anions bromide, iodide, sulphite and nitrate, and organic compounds such as substituted anilines, cause quenching of peroxyoxalate chemiluminescence. A detection method for HPLC based on this quenching phenomenon has been developed (Gooijer *et al.*, 1989). It makes use of an immobilized luminophore, 3-aminofluoranthene, covalently bound via an alkyl-spacer on controlled pore-glass, packed in the detector cell. It was proposed that most probably the quencher destroys the radical ion-pair

produced after electron transfer in the last stage of the chemically-induced electron exchange luminescence (CIEEL) reaction scheme, thus preventing the formation of electronically excited luminescence. The detection limit for iodide was 0.3ng.

We wished to develop a chemiluminescent microassay for iodide and the systems described by Lu *et al.*, (1989) and Burguera and Burguera (1982) were investigated in a microplate format. The other luminescent systems investigated involved the luminescent substrate luminol. To analyze iodide directly using luminol chemiluminescence it is necessary to turn it into a form active in the chemiluminescent reaction, i.e., oxidation of iodide to iodine, with subsequent separation from the excess oxidant (e.g., the methods described by Fujiwara *et al.*, 1989; Pilipenko *et al.*, 1986; Pilipenko *et al.*, 1989). The work described in this thesis investigated the indirect effect of iodide, that is, whether iodide enhanced or suppressed a number of luminol luminescent systems. These systems have a simpler format and are thus more suitable for a microassay format. An outline of these systems is given in Section 4.1.

SYSTEM	Linear range LOD	REFERENCE
Luminol/ iodine	5×10^{-10} - 3×10^{-7} M	Seitz & Hercules, 1974
Luminol/ iodine or iodide in reversed micelles	0.1ng/ml - 1.0 μ g/ml 2×10^{-10} M	Fujiwara <i>et al.</i> , 1989
Luminol/ iodide--> iodine --> cyanogen iodide	5ng/ml - 90ng/ml 5ng/ml	Pilipenko <i>et al.</i> , 1986
Luminol/ iodide--> iodine and gas extraction	6ng/ml - 500ng/ml 3ng/ml	Pilipenko <i>et al.</i> , 1989
H ₂ O ₂ / ClO ⁻ / iodine flow injection analysis	0.5ng/ml - 0.25 μ g/ml 0.3ng/ml	Burguera & Burguera, 1982
Acetone/ H ₂ O ₂ / ClO ⁻ / I ⁻	0.4ng/ml - 0.3 μ g/ml	Lu <i>et al.</i> , 1989
Peroxyoxalate quenching by iodide, HPLC	- 0.3ng	Gooijer <i>et al.</i> , 1989

Table 1.3
Chemiluminescent methods for the detection of iodine and iodide. [LOD is the limit of detection.]

SECTION II

1.21 DNA PROBE ASSAYS

1.21.1 INTRODUCTION

A probe is a molecule having a strong interaction with a specific target and having a means of being detected following the interaction. Nucleic acids interact with their complement primarily through hydrogen bonding. [Complementary DNA or RNA strands are those with complementary base sequences: the base pairs adenine and thymine (or uracil in RNA) and guanosine and cytosine are complementary]. Under conditions of appropriate stringency the complementary strands will bind with high affinity and specificity. Thus, if one of the strands is labelled by some means, a specific DNA sequence may be detected. Basepairing is possible for nucleotides modified with single atoms, functional groups or long side-chains depending on the site of attachment and the nature of the side-chain. Potential base modification sites are those which do not interfere with hydrogen-bonding (Keller and Manak, 1989).

Hybridization methods have proved powerful tools for the detection of specific genes and the analysis of gene function, and these DNA probe based assays show a rapidly growing number of applications (Matthews and Kricka, 1988) including:

1. Research: DNA probes have been used for many research applications including, gene analysis, quantification of mRNA, and localization of nucleic acid sequences in cells (by *in situ* hybridization).
2. Identification of infectious agents: Much work has been carried out to clone and synthesis specific DNA probes for the detection of pathogenic organisms for use in clinical diagnosis, in veterinary diagnosis, and in food and environmental sciences.
3. Screening for genetic susceptibility to disease: DNA probes can be used to test for the presence of gene sequences associated with genetic diseases,

whether they are alterations to the specific gene involved (e.g., sickle cell anemia; Geever *et al.*, 1981) or by analysis of restriction length polymorphisms, (e.g., adult polycystic kidney disease; Reeders *et al.*, 1985). Such tests can be applied to prenatal diagnosis or used for carrier detection and genetic counseling.

4. Oncology: Hybridization techniques have been used in the study of the mutations, activation and expression of oncogenes.

5. Forensic science: Jeffreys *et al.*, (1985) described the cloning of minisatellite sequences (or hypervariable regions) of the human genome. By using such sequences as probes it is possible to produce a unique genetic fingerprint of an individual. Such a discovery has obvious corollaries in forensic science. Genetic fingerprints are also of use in cases of disputed parentage as the pattern of the hypervariable regions is subject to Mendelian inheritance.

7. Plant breeding: Plant viruses cause high losses in a number of agriculturally important crops. Screening of crops with appropriate DNA probes can be of great value in breeding programs and in preventing crop losses.

Improving the methodology of DNA probe use will allow the technique to be more widely applied in both routine and research settings. Considerations include the overall sensitivity, specificity, convenience and cost effectiveness of the assay. The methodology can be divided into four main sections:

- a) Sample preparation
- b) Labelling procedures
- c) Hybridization formats

and d) Detection schemes

At present key goals in the field of hybridization technology include : improvement of non-radioactive probe labels; amplification of target DNA and development of simplified hybridization formats. Hybridization formats, labelling procedures and detection schemes which are available will be considered.

1.21.2 HYBRIDIZATION FORMATS

Formats for the analysis of nucleic acid sequences can be divided broadly in two: membrane-immobilized target sequences (e.g., Southern blots) and detection of target sequences in solution using either hydroxylapatite, which preferentially binds double-stranded nucleic acids, or by having the probe attached to solid-support which then captures target sequences from solution (competition type assay or sandwich-type format).

Nucleic acid hybridization technology began with the work of Hall and Spiegelman (1961). Probe and target were hybridized in solution and hybrids were isolated from non-hybrids by equilibrium-density centrifugation. This process was slow, labour-intensive and inaccurate. Britten and Kohne (1968) used hydroxylapatite which specifically binds double-stranded DNA under low salt conditions. A modification of this approach is currently in use in some Gen-Probe products. The DNA probe is labelled with an acridinium ester, which provides for sensitive chemiluminescence based detection (Grancto and Franz, 1989). The probe:target hybrids are captured on magnetized beads which have the DNA binding properties of hydroxyapatite. The beads are removed from solution by magnets and the bead-bound probe is measured by chemiluminescence.

Solution hybridizations offer the fastest hybridization rates of the commonly used formats, but the difficulties in separating the free and hybridized probe have limited routine use of this format. It is more common to immobilize one of the reacting nucleic acids on a solid support while the others remain free in solution. Such mixed-phase hybridizations offer convenient formats for the detection of nucleic acid hybridization since unreacted molecules can be easily washed away after hybridization. Solid supports used include: nitrocellulose (Nygaard and Hall, 1964, Denhardt, 1966); nylon filters (Cannon *et al.*, 1985) and microtitre plates (Nagata *et al.*, 1985, Keller *et al.*, 1989, Sören *et al.*, 1991). Immobilization has an additional advantage as it prevents self-annealing of the target molecule. The classical hybridization formats will be described.

In 1975, Southern described the use of DNA probes in gene analysis on a solid phase. This technique, now known as Southern blotting, allows the mapping of the position of a DNA sequence relative to restriction enzyme sites. The protocol involves the immobilization of electrophoretically separated fragments of DNA onto a filter (nitrocellulose or nylon) followed by hybridization to a specific DNA probe sequence. Mapping of sequences in relation to restriction enzyme cleavage sites can be achieved by measuring the sizes of the restriction fragments binding to the probe. Southern blotting has been used for the following: analysis of genomic organisation; study of genetic disease; DNA fingerprinting and analysis of PCR products. Northern blotting for separating RNA fragments, is similar in principle (Alwine *et al.*, 1977).

Quantitation of the amount of a specific DNA or RNA sequence in a sample can be achieved by using the related technique of dot or slot blotting (Kafatos *et al.*, 1979). In this case, the sample is immobilized on a nitrocellulose or nylon filter as a dot or a slot shape, and the intensity of hybridization of a specific DNA probe to the sample is a measure of the amount of that specific sequence in the sample. Dot blotting is less complex than conventional Southern blotting because no electrophoretic step is required, and it is less demanding on DNA quality because no digestion with restriction enzymes is necessary.

Membrane hybridizations are also useful for screening bacterial colonies, for example, selection of recombinant molecules (Grunstein and Hogness, 1975). Bacterial colonies to be screened are lifted from agarose plates onto nitrocellulose filters making an exact replica of the distribution of colonies on the original plate. The replica is processed for hybridization while the original is retained as a reference plate.

DNA sequences immobilized on filters by the Southern blot or slot blot are hybridized in basically the same way. The filters are first prehybridized in hybrid buffer minus probe. This means that non-specific DNA binding sites on the filter are thus saturated with carrier DNA and synthetic polymers.

Prehybridization buffer is then replaced with hybridization buffer containing the labelled probe and incubated to allow hybridization of this probe to the target nucleic acid. Following hybridization, unhybridized probe is removed by a series of washing steps (note stringency of the washes and sensitivity of the assay go hand-in-hand). Low stringency conditions (i.e., higher salt and lower temperature) may lead to non-specific hybrid signals and, thus, background noise.

Sandwich hybridization is a composite format utilizing two probes, one for capture and another for detection. The detection probe is soluble, the capture probe may or may not be immobilized during hybridization but is always immobilized during detection. The procedure was described by Ranki *et al.* (1983). The use of capture DNA sequences immobilized in microtitre wells potentially allows the simultaneous analysis of a large number of biological samples, (Keller *et al.*, 1989).

Localization of nucleic acid sequences within cells and tissues can be achieved by using *in situ* hybridization. In this technique, biological tissue is fixed on a microscope slide and hybridized to a labeled DNA probe, and the results recorded microscopically (Gall and Pardue, 1969). In this case the cells themselves are the solid support. *In situ* hybridization can be used to identify the viral sequences concentrated within infected cells. The microscopic examination of the specimen allows detection of small amounts of hybridization signals in a well defined area and identification of the intracellular distribution of given sequences.

Alternative strategies to these classical methods have been developed. The choice of format can be made after consideration of the resources, the number of samples, and the signal to be generated. Some novel formats which have been described for solution hybridization assays are considered.

Yehle *et al.*, (1987) described a novel solution hybridization format for RNA detection involving affinity capture of DNA:RNA hybrids and

immunochemical detection. A biotinylated DNA probe was hybridized in solution to its RNA target. The hybrids as well as the unreacted probe were captured by an immobilized anti-biotin antibody. Hybridization was distinguished from unhybridized probe by binding of an enzyme-labelled anti-hybrid antibody and use of a fluorescent enzyme substrate. There was a rapid detection of RNA with sensitivity of about 10^7 copies. The format was modified by Viscidi *et al.* (1989) to detect human immunodeficiency virus Type I (HIV-1) RNA in cultured cells. The hybrids of biotinylated probe and RNA targets were captured in microtitre wells coated with goat anti-biotin antibody.

Homogeneous solution hybridization assays have also been described, that is, where there is a single-step hybridization reaction, washing and immobilization steps are not required. Heller and Morrison (1985) used non-radioactive energy transfer. Two adjacent probes were required, one labelled with a chemiluminescent group (donor) and the other labelled with a moiety capable of fluorescence only when both probes are in proximity. The two probes and two detectors were in proximity only when both are hybridized to the target nucleic acid. The practical sensitivity of this method has been questioned.

Solution-phase sandwich hybridization was first described by Syvänen *et al.*, (1986). Urdea *et al.*, (1987) have developed the most sensitive variation on solution-phase hybridization. They used multiple synthetic probes and these multiple oligomer capture and detection probes were hybridized to the target nucleic acid in solution. [The primary probes (unlabelled detection probes/soluble capture probes) were 50 bases long containing 30 bases of organism-specific sequence and 20 bases of single-stranded tail used to capture the probe-target complex from solution]. The complex was then captured onto a solid support by hybridization of the soluble capture probes to immobilized capture probes. Cross-linked secondary probes were hybridized to the primary probes to amplify the number of binding sites for the tertiary enzyme-labelled detection probes.

Morrissey and Collins (1989) described nucleic acid hybridization assays employing a dA-tailed capture probe and a detector probe. Capture of the targets on the poly(dT) support was used to remove excess labelled probe and sample impurities prior to radioisotopic or non-radioisotopic detection.

A "universal probe system", based on the principle of sandwich hybridization, was described by Nakagami *et al.*, (1991). The primary probe was prepared from a chimeric phage-plasmid vector containing the complementary sequence to the primary gene. The secondary probe has a sequence complementary to the vector portion of the primary probe and is labelled with biotin via the transamination reaction. Thus, it can be used with any primary probe prepared with the same vector. Membrane blotted DNA was treated with the primary probe and then with the secondary probe. The biotinylated secondary probe hybridized to the primary probe, which was already attached to the target DNA. This format was sensitive enough to detect single-copy human genes on Southern blots.

Dattagupta *et al.*, (1989) described a "reversed hybridization" procedure. A simple method of non-isotopically labelling sample nucleic acids was developed (photochemical labelling by monoadduct-forming isoprosoralen). The sample nucleic acids were then hybridized simultaneously to an array of unlabelled immobilized probes.

With the development of *Taq* polymerase it was possible to use the polymerase chain reaction in routine diagnostic techniques (Erlich *et al.*, 1988) where the target DNA is amplified before detection, thus achieving greater sensitivity. One example described is for the detection of HIV-I. Whetsell *et al.*, (1992) compared three non-radioisotopic PCR based methods for the detection of the virus. The required sensitivity could be achieved using non-radioactive detection schemes when the target DNA was amplified first.

1.21.3 LABELLING PROCEDURES

1.21.3a Introduction

Probe labelling and a suitable detection method are the most important parts of a DNA probe assay. Characteristics of a probe label include the following (Kricka, 1992):

1. It should be easily attached to DNA.
2. No measurable effects on hybridization quality or kinetics should be observed.
3. Detection at very low concentrations using simple instrumentation is paramount. [Consider the problem of detecting a single copy gene within the human genome. On average this may represent 1000 bases from a total of 3×10^9 bases. Since the samples of DNA available are often small (10ug) this can constitute levels of DNA between 1-5pg. Amplification systems, such as the polymerase chain reaction may be used to amplify the target nucleic acid resulting in improved sensitivity (Saiki *et al.*, 1985).] A highly sensitive detection system removes the requirement for target amplification.
4. Ideally it would produce a signal which was modulated when the labelled DNA probe hybridized to its complementary DNA sequence, thus facilitating the development of non-separation DNA probe assays.
5. The labelled probe should also be stable at the elevated temperatures used in hybridization.

Labelling can be carried out enzymatically or chemically. Radioisotopes are generally incorporated into DNA probes by enzyme action, although chemical methods have been used (e.g., Commerford (1971) chemically incorporated ^{125}I into nucleic acids). Enzymes used as reporters can be coupled to nucleic acids either by direct chemical cross-linking to derivatized synthetic oligonucleotides or by conjugation to avidin or strepavidin and affinity interaction of the conjugate with biotin-containing probe. Fluorescent organic molecules and metal-ion chelates may be incorporated into DNA by either chemical means or by enzyme action on appropriately modified nucleotide triphosphates. The

range of labelling methods will be outlined below.

1.21.3b Radioactive Labelling Strategies

Radioactive probes, coupled with autoradiography detection, provide the highest degree of sensitivity and resolution currently available in hybridization assays. ^{32}P is the most commonly used isotope to label nucleic acids (others include tritium, ^{125}I and ^{35}S). This is because probes prepared with ^{32}P provide maximum sensitivity in most autoradiographic detection procedures. Detection limits down to 1-10 femtograms target DNA can be achieved. The radioactive labelling procedure relies upon enzymatic incorporation of the labelled moiety into the probe DNA. All forms of nucleic acid, double-stranded and single-stranded DNA, RNA and oligonucleotides, can be labelled with radioactive isotopes. The traditional method involved the enzymatic process of nick translation (Rigby *et al.*, 1977). Nicks are introduced into double-stranded DNA by DNase I, and then the enzyme DNA polymerase I (from *Escherichia coli*) digests away from the nick and replaces the strand as it proceeds. Incorporation of labelled nucleotides in the reaction mixture allows labelling of the DNA to high specific activity.

Radiolabelling can also be achieved using random priming of single-stranded DNA with mixed hexanucleotides (Feinburg and Vogelstein, 1983; 1984) or by copying DNA cloned into a single-stranded vector such as phage M13 (Hu and Messing, 1982). DNA fragments to be cloned are first cut with restriction enzymes and ligated into the corresponding sites of the multiple cloning site in M13, using standard cloning techniques. The purified single-stranded phage DNA is isolated and used for generating probes. A synthetic oligonucleotide primer complementary to the region of the M13 just upstream of the insert (5' side) is annealed to the template and incubated in the presence of *E. coli* DNA polymerase 1 Klenow fragment and dNTPs including at least one [^{32}P]dNTP. The product of this reaction is a labelled single-stranded DNA complementary to the sequence in the phage DNA. The latter method produces single-stranded probes, thus abolishing the problem of complementary probe

strands competing with the hybridization of probe to the sample DNA.

The generation of radiolabelled DNA and RNA probes by polymerase chain reaction (PCR) has also been reported (Schowalter and Sommer, 1989). By including a radioactive triphosphate during PCR, probes of very high specific activity can be generated.

The preparation of tailed oligonucleotide probes involves the enzyme terminal deoxynucleotidyl transferase. This will add deoxyribonucleoside triphosphates, one of which can be radiolabeled, to the free 3'hydroxyl end of a DNA molecule (Bollum, 1974). The addition of deoxynucleotides results in the formation of an elongated 'tail' at the 3' end of the probe. Tailed oligonucleotide probes are suitable for the identification of cloned sequences in gene banks, detection of point mutations in genomic DNA samples and for *in situ* hybridization.

However, just as radio-immunoassay (RIA) has been steadily replaced by its non-radioactive counterpart, Enzyme-Linked ImmunoSorbent Assay (ELISA), so too are radioactively labelled DNA probes being replaced by non-radioactive versions. Problems such as health hazards, disposal of radioactive waste, short shelf-life of radioactive probes are among the reasons for investigating useful alternatives to radioactive labels.

1.21.3c Non-Radioactive Labelling Strategies

Labelling strategies can be broadly divided as direct or indirect. A direct label is attached via a covalent bond to DNA while an indirect labelling procedure would involve, either attaching a hapten to the DNA and detecting by using a labelled binding protein with specificity for the hapten, or by detecting hybrid formation by using a binding protein with specificity for double-stranded DNA. The assay detection limit of direct labels depends largely on the detection limit of the label. Radiolabels, which are direct labels, have detection limits which make them suitable probes for say, a single-copy gene

in the human genome. For other direct labels in use those probe assays based on labels which provide signal amplification (ie. enzymes) are likely to be more sensitive than assays using labels which provide only a single signal per label (e.g. fluorescein).

Renz and Kurz (1984) described a general method which allows crosslinks to be produced between proteins and single-stranded DNA. A synthetic polymer carrying many primary amino groups is fused with a protein. The modified protein (now able to bind electrostatically to any polyanion due to its positively charged tail) is allowed to interact with DNA. The ionic binding between the 2 macromolecular components is essential for a successful crosslinking reaction leading to covalent bonds. Single-stranded DNA-protein probes hybridize with their complementary sequences and can be detected by searching for the protein (e.g. with an antibody) and if the protein moiety of the probe is an enzyme molecule which can convert a soluble colourless substrate into an insoluble coloured product the hybridization sites can be visualized directly. Alkaline phosphatase-labeled oligonucleotides can also be made. Labelling is via the terminal amino group of a linker arm attached to a deoxyuridine base using the heterobifunctional coupling reagent disuccinimidyl suberimidate. This procedure produced 1:1 oligomeric:enzyme labels which retained full enzymatic activity (Jablonski *et al.*, 1986). A frequent criticism of these directly cross-linked probes is their instability at temperatures greater than 45°C and interference of the protein with hybridization (AL-Hakim and Hull, 1986)

Microperoxidase-II, a catalyst for luminol chemiluminescence, has been crosslinked to a guanosine terminally labelled polyadenylic acid, using the bifunctional coupling reagent azidophenyl glyoxal. This model system had the sensitivity required for a DNA probe assay (Heller and Morrison, 1985). Pollard-Knight *et al.*, (1990i) used glutaraldehyde as a linker to conjugate horseradish peroxidase to the amino groups of purine and pyrimidines.

Syvänen *et al.*, (1985) complexed single-stranded DNA and single-strand

binding protein (SBB) of *E. coli* in the mass ratio of 30:1. The protein moiety of this complex can be labelled by a number of methods, radioiodination and biotinylation being taken as examples. It was shown that more label can be complexed to the M13 DNA by mediation of the SBB protein, without severely affecting the hybridization properties of the molecule, than by direct methods.

The simplest indirect procedure makes use of a specific antibody to a DNA:DNA duplex or DNA:RNA duplex. The duplex:antibody is then detected using a labelled second antibody (Huang *et al.*, 1985). Alternatively, a histone or a radiolabeled histone is used to bind to the duplex and the duplex:histone complex is detected colorimetrically or by counting the radioactivity (Bulow and Link, 1986; Renz, 1982). A variant of the indirect procedure involves a DNA probe labeled with a hapten. Various haptens can be used but the most popular is biotin. This was the first non-radioactive DNA probe system of a practical design, and was described by Langer *et al.* (1981).

This early probe-labelling scheme employed biotin-labeled deoxyribonucleotide triphosphates, which were incorporated into the probe DNA by enzymatic polymerization. The most widely used modified nucleotide is biotin-11-dUTP. The molecule incorporates the following features: modification at the C-5 position where it will not interfere with hydrogen bonding; a double bond to minimize flexing of the linker arm and a linker arm long enough to ensure access of detection reagents to the biotin. This and other modified nucleotides can be incorporated into DNA by nick-translation (Leary *et al.*, 1983) or onto the ends of DNA by tailing (Riley *et al.*, 1986). Biotin may also be introduced by use of a photoactivable analogue, photobiotin, which crosslinks to DNA on irradiation (Forster *et al.*, 1985). Photobiotin has a linker arm, with biotin coupled to one end and an aryl azide coupled to the other end. In the presence of visible light the aryl azide is converted into a reactive aryl nitrene moiety which reacts readily with DNA or RNA. The reaction is relatively specific for adenines at the N7 position. After hybridization, these biotin-labeled probes are detected using avidin or streptavidin-enzyme conjugates. Note that streptavidin

is superior to avidin for DNA detection because it exhibits far less non-specific binding.

Conjugates with fluorescent groups, and electron-dense particles or molecules (for *in situ* hybridization) for these biotin-labeled probes have also been described. When combined with a precipitating substrate the probe-immobilized target hybrid is visualized as a coloured band or spot on nitrocellulose, (Leary *et al.*, 1983) or as cellular staining following *in situ* hybridization (Brigati *et al.*, 1983). Enzyme labels [enzymes which have been used include horseradish peroxidase, acid phosphatase, alkaline phosphatase and β -galactosidase] with colorimetric, fluorescent or chemiluminescent endpoints are now in use and these various detection schemes are discussed in Section 1.21.4.

Labelling DNA directly with alternative haptens to biotin and detection with enzyme-labelled anti-hapten antibodies have been described. Sulphonation of cytosine residues by bisulphite, (Poverenny *et al.*, 1979), is an extremely easy and inexpensive labelling procedure because it relies on simple inorganic reagents. After hybridization the probe is detected using a monoclonal anti-sulphonate first antibody followed by an alkaline phosphatase conjugated anti-mouse IgG second antibody. A kit, using such a labelling procedure is available from FMC Bioproducts.

Another chemical labelling scheme employing the carcinogen AAIF (7-iodo-N-acetoxy-N-2-acetylamine fluorene), which covalently binds to guanosine residues in DNA, has been described (Tchen *et al.*, 1984). Detection was by means of a rabbit anti-AAIF first antibody followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody. This method had many disadvantages including the toxicity of AAIF and long incubation times. Nevertheless, it established that a DNA probe labelled with a different hapten was essentially as useful as a biotin-labelled probe, that is, 4×10^6 copies of target nucleic acid could be detected after hybridization.

Scippo *et al.*, (1989) described a non-radioactive method for probing RNA or DNA on dot and Northern blots using a synthetic oligonucleotide with bromodeoxyuridine free ends. The probe was detected with a monoclonal antibody against bromodeoxyuridine and immunogold silverstaining.

A system using a digoxigenin-labelled nucleic acid probe was described by Martin *et al.*, (1990) and is available from Boehringer Mannheim. Hybrids are detected with an immunoassay using an anti-digoxigenin antibody conjugate. The system allows the detection of less than 0.1pg of DNA. Several methods have been optimized for labelling with digoxigenin-11-dUTP, including the random priming method, nick translation, oligonucleotide tailing and cDNA synthesis. The polymerase chain reaction can also be used to make digoxigenin-labelled probes. Numerous applications of the digoxigenin-labelled probes have been reported. For example, Suzuki *et al.*, (1992) describe a chemiluminescent microtitre method for detecting PCR amplified HIV-I using digoxigenin-labelled probes.

Tables 1.4 and 1.5 show examples of indirect labels used for nucleic acid hybridization assays.

Hapten	Binding protein	Label	Reference
Biotin	Avidin/streptavidin	AP, HRP, Fluorescein	Langer, (1981)
Digoxigenin	Anti-digoxigenin	AP, HRP, Fluorescein	Martin <i>et al.</i> , (1990)
Bromodeoxy- -uridine	Anti- bromodeoxyuridine	Immunogold staining	Scippo <i>et al.</i> , (1989)

Table 1.4

*Examples of indirect labels used for nucleic acid hybridization assays.
[AP= alkaline phosphatase; HRP= horseradish peroxidase]*

Label	Binding Protein	Binding protein Label	Reference
Sulphone	Anti-sulphone	Anti-species IgG-AP	Poverenny <i>et al.</i> , 1979
AAIF	Anti-AAIF	Anti-species IgG-AP	Tchen <i>et al.</i> , 1984
5-bromodeoxy- uridine	Anti-5-bromodeoxy- uridine	Anti-species IgG-AP	Sakamoto <i>et al.</i> , 1987
-	Anti-double- stranded DNA	Anti-species IgG-AP	Huang <i>et al.</i> , 1985

Table 1.5

*Examples of indirect labels used for nucleic acid hybridization assay that use
as intermediate binding protein.
[AP= alkaline phosphatase; AAIF= N-acetoxy-N-2-acetylamine fluorene]*

Modification of DNA with non-radioactive detectable groups is more difficult than, say antibody modification, because DNA contains none of the reactive groups common to proteins such as amines, sulphhydryl groups or carboxylic acid groups. Alternative chemical labelling approaches to facilitate non-radioactive detection have been designed. The modification of nucleotide triphosphates is limited by what can actually be incorporated into DNA via nick translation and random priming.

Non-covalent labelling of DNA is principally via the formation of intercalation complexes with fluorescent organic molecules. Covalent labelling is much more popular. Labels functionalized with azido groups can be activated photochemically to produce a highly reactive nitrene intermediate that reacts readily and non-specifically with many chemical bonds. This type of procedure is applicable to many labels, and has been utilized for ethidium and biotin labels (photobiotin, as described previously).

Amino groups on purine and pyrimidine bases serve as convenient sites for the attachment of labels. Thus, labelling can be achieved using bifunctional coupling reagents such as 3-(4-bromo-3-oxobutane-1-sulphonyl)propionate-N-hydroxysuccinimide (Al-Hakim and Hull, 1986) or glutaraldehyde, for the direct coupling of enzymes or biotin to basic molecules, e.g., Histone H1 (Pollard-Knight *et al.*, 1990i).

Labelling via amino groups on DNA or RNA can also be undertaken using labels containing aldehyde groups. For example, the carbohydrate moiety in horseradish peroxidase is oxidized by periodate to produce aldehyde groups and these will react with amino groups on deoxythymidine of a poly-deoxythymidine-tailed DNA (Woodhead and Malcolm, 1984). An amine substitution reaction can also be used to attach biotin to single-stranded DNA by the use of biotin hydrazide (Reisfeld *et al.*, 1987).

Linker arm-modified DNA can be labelled with a variety of detectable groups or it can be covalently attached to solid supports (ie. styrene beads or wells)

which would not covalently bind unmodified DNA. Viscidi *et al.*, (1986) describe a method in which the cytosines of DNA or RNA are modified at the N4 position with a linker arm of ethylenediamine in the presence of bisulphite. Following attachment of the ethylenediamine linker arm, detectable groups such as N-hydroxysuccinimide-biotin (NHS-biotin) can be added by reaction with the primary amine.

N⁴-alkylamino deoxycytidine has been introduced into polynucleotides by nick translation with N⁴-(6-aminohexyl) dCTP (Gillam and Tener, 1986), by bisulphite catalyzed transamination (Draper and Gold, 1980), and by the coupling of fully protected N⁴-alkylamino cytidine 3'-phosphoramidite during automated synthesis (Urdea *et al.*, 1988). There are significant advantages to the incorporation of the cytidine analog through chemical DNA synthesis. The modified nucleotide can be added in any number and at any position within the probe. Large scale synthesis of a variety of non-isotopically labeled derivatives can be conducted readily from commercially available or easily synthesized amine-specific labelling reagents.

DNA may be reacted with N-bromo-succinimide at alkaline pH resulting in bromination of a fraction of the thymine, guanine and cytosine residues (Keller *et al.*, 1988). The bromine is subsequently displaced by a primary amino group of the linker arm. The other end of the linker arm may be a free amino or it may have a detectable group attached to it. Such a scheme involves modification of a site not involved in hydrogen-bonding and the labelling is rapid.

There are no naturally occurring thiol groups in polynucleotides, but thiol groups can be introduced via the reaction of amino groups on purines and pyrimidines with reagents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Thiolated DNA will then react with an SPDP-derivatized label to form a disulphide bond, linking the label to the DNA (Malcolm and Nicolas, 1984).

The enzymatic methods which have already been discussed for the

incorporation of radiolabels, that is the processes of nick translation and random priming of single-stranded DNA with mixed hexanucleotides can also be applied to the incorporation of say a biotinylated nucleotide or any other modified nucleotide. Polymerase chain reaction can also be used to incorporate the modified nucleotide. One approach is that the modification group is incorporated into the PCR product by including the modified deoxynucleoside triphosphates in the reaction (Lo *et al.*, 1988). In general, the enzymatic non-radioactive techniques yield the most sensitive probes but these techniques are not as suitable as the chemical methods for labelling large quantities (greater than 50µg) of probe.

Tailing with a modified dNTP is the most efficient enzymatic method for labelling oligonucleotide probes with non-radioactive moieties. The modified nucleotides are added onto the 3'-end of the probe in the presence of terminal transferase. Using a modified oligonucleotide alone about 10-20 residues are added to the probe. The resulting hybrid signal is not as strong as expected because only a portion of the detectable groups will be available for detection because of steric hindrance. Chemical attachment of non-radioactive labels to synthetic oligonucleotide probes requires procedures somewhat different to those used by cloned probes. Non-radioactive labels are difficult to incorporate directly during the chemical synthesis of oligonucleotides since they are not stable in the reagents employed for synthesis and/or deprotection. As a result, appropriately blocked nucleophilic "handles" such as alkyl- sulphhydryls or - amines have been incorporated during the solid-supported synthesis of DNA. Subsequent to deprotection and purification of the probes, these sites can be used to direct the introduction of nucleophilic-specific labelling reagents. Urdea *et al.* (1988), reported the synthesis of a fully protected 3'-phosphoramidite of an alkylamine derivative of deoxycytidine and its use in the synthesis of oligodeoxyribonucleotides. These alkylamine-containing oligonucleotides have been modified with fluorescent, chemiluminescent and enzyme moieties.

In designing a new labelling system we wish to increase speed, convenience, safety and sensitivity. The choice of signal is dictated by factors such as the

sensitivity in terms of the amount of the target required to give a positive result, the equipment which is required to identify the signal, and the time required for the signal to be generated. Further considerations which may be made include: are qualitative or quantitative results required and can the system be reprobated?

1.21.4 DETECTION SCHEMES

The effectiveness of any labelling method goes hand in hand with the detection method employed. Hybridization assays using ^{32}P labeled probes can detect down to 1-10 femtograms of nucleic acid. However, a lengthy autoradiography step (minimum of 24 hours but can be weeks) is required. Enzyme labels provide the most sensitive detection to date for the non-radioactive schemes. Although many enzymes have been suggested as labels the most commonly used are calf-intestine alkaline phosphatase (AP) and horseradish peroxidase (HRP) [β -galactosidase from *Escherichia coli* is used to a lesser extent]. These enzymes offer a choice of detection schemes: colorimetric, fluorescent and luminescent. Enzymes are used as direct labels but they are more often used as a secondary label conjugated to the anti-hapten protein. Fluorescent groups and chemiluminescent groups have also been used as labels.

For visual, qualitative results then colorimetric detection may be a good choice. Quantification of such an image requires access to an image analysis system such as a charge couple device (CCD) camera. The most sensitive detection which leads to the formation of insoluble precipitates is obtained using alkaline phosphatase and a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue-tetrazolium salt (NBT) (McGadey, 1970).

Colorimetric assays are not often sensitive enough for the requirements of a hybridization assay. However, the highly sensitive method based on enzyme amplification described by Self (1985) has been applied to a DNA probe assay by Gatley (1985). In this method alkaline phosphatase is used as a label. The substrate nicotinamide adenine dinucleotide phosphate (NADP^+) is dephosphorylated by phosphatase to form NAD^+ . NAD^+ , in turn, activates a secondary enzyme system which comprises a redox cycle driven by two enzymes, alcohol dehydrogenase and diaphorase. During each round of this cycle one molecule of a tetrazolium salt, idonitrotetrazolium violet, is reduced to an intensely coloured soluble formazan dye. This detection method is capable of measuring as little as 0.01attomol of alkaline phosphatase

(Johannsson *et al.*, 1986).

Fluorogenic substrates have been used in enzyme immunoassay to measure peroxidase, alkaline phosphatase and β -galactosidase. The best signal amplification is observed when using β -galactosidase and its fluorogenic substrate 4-methylumbelliferyl- β -D-galactopyranoside (MUG). Nagata *et al.*, (1985) and Yokota *et al.*, (1986) have detected picogram levels of specific DNA using MUG as the substrate for β -galactosidase. Fluorescence-based detection schemes must compensate for the problem of non-specific background fluorescence, which is present in all biological samples. This problem can be overcome with time-resolved fluorescence (TRF) detection using lanthanide chelates as the light-emitting species. The detection of alkaline phosphatase has been combined with TRF detection of a lanthanide chelate reagent. This procedure is called enzyme-amplified lanthanide luminescence (EALL) (Evangelista *et al.*, 1991) and is reviewed in relation to DNA probe assays by Templeton *et al.*, (1992).

Luminescent detection techniques have become available to detect small amounts of enzyme. In these techniques the light emission from the reaction is measured in a luminometer or recorded on instant photographic or X-ray film.

The chemiluminescent method described by Matthews *et al.*, (1985) involves biotin-streptavidin-HRP complexes. The HRP label was detected using a luminol/ hydrogen peroxide/ enhancer system. The enhancers used were *p*-iodophenol and *p*-hydroxycinnamic acid. The probes could be washed off and re-examined with a second probe. This chemiluminescent system was used to quantify specific gene sequences and it offered sensitivity comparable with other methods using radioisotopes and alkaline phosphatase as labels.

A horseradish peroxidase/luminol enhanced chemiluminescent (ECL) range is available from Amersham. This non-isotopic labelling and detection system can detect low levels of immobilized nucleic acids. DNA or RNA probes of greater

than 50 base pairs are directly labelled with the enzyme, HRP, in 20 minutes. In the presence of the ECL gene detection reagents an enhanced light signal is produced within 1 minute and lasts for 4 hours. The advantages of such a system are that it is easy to use and no special equipment is required. Labelling, hybridization, and detection on nylon or nitrocellulose membranes is possible in less than 7 hours, reprobing can be carried out without stripping the blot and the probes are stable for six months. Such a system would be suitable for detecting PCR-amplified targets and single-copy human genes can be detected by overnight hybridizations. Amersham also provide an ECL oligonucleotide labelling and detection system. The oligonucleotide probes are coupled via the 5' end to HRP and these HRP-labelled probes are used to detect nucleic acids immobilized on membranes in hybridization experiments. The specificity and efficiency of hybridization is equivalent to ^{32}P . However, 20 attomol of nucleic acid is the lower detection limit. Hence, the sensitivity is not equivalent to that of ^{32}P . Urdea *et al.*, (1987) described the detection of 0.2pg of hepatitis B viral DNA in human serum samples in 4 hours using an amplification scheme based on chemically crosslinked oligodeoxyribonucleotides coupled with a horseradish peroxidase label and the hydrogen peroxidase/luminol chemiluminescent detection scheme.

Pollard-Knight (1990) used nucleic acid probes directly labelled with horseradish peroxidase for detection of single-copy sequences on Southern blots of human genomic DNA by enhanced chemiluminescence. 3pg of the target sequences were detected on blue-sensitive film using exposures of up to 60 minutes and 0.3 -5.1kb probes. The enzyme has no significant effect on the stability of a DNA/RNA hybrid formed with a 3571 base probe and the target as determined by increasing the stringency of post-hybridization washes by decreasing the concentration of a monovalent cation and by a melting-temperature analysis (measurement of the T_m values of probe directly labelled with HRP was comparable to those labelled with ^{32}P). However, the method can only be used to label probes of size greater than 50 bases. The system can be reprobed.

AMPPD, (adamantyl 1,2-dioxetane phosphate or 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane) is a substrate for alkaline phosphatase and was developed by Bronstein and Voyta (1989). Cleaving off of a phosphate group from the substrate leads to the formation of an unstable intermediate and the emission of light at 477nm. The light emission is recorded on camera film.

Bronstein *et al.*, (1989) compared the sensitivity of the chemiluminescent substrate, AMPPD, and a chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) for detection of an alkaline phosphatase label in a hepatitis B virus "core antigens" (HBV_c) DNA probe hybridization assay. The chemiluminescent assay detected 1.18×10^6 copies of HBV_c plasmid DNA in 30 minutes. By comparison, 9.8×10^7 copies of DNA could be measured using chromogenic BCIP/NBT substrate within the incubation time. After further development, the chemiluminescent endpoint permitted detection of 4.39×10^4 copies of HBV_c plasmid DNA in 2 hours. Bronstein concluded that AMPPD makes ultrasensitive detection of alkaline phosphatase possible.

Lumingen Inc. also provide a kit for alkaline phosphatase detection which was a modification of the AMPPD system (Schapp *et al.*, 1989). Phenylphosphate substituted dioxetane is used in the presence of fluorescent micelles formed from cetyltrimethyl ammonium bromide (CTAB) and 5-(N-tetradecanoyl-aminofluorescein in a buffer at pH 9.6. Enzymatic dephosphorylation of the dioxetane produces the more hydrophobic hydroxyl-substituted dioxetane which is sequestered in the micelle. Chemiluminescence is enhanced in the decomposition of the hydroxyl-substituted dioxetane in the micelle to give a singlet-excited intermediate, with subsequent efficient energy transfer to the co-micellized fluorescer. Detection is 0.001 attomol, or less than 1000 molecules, of alkaline phosphatase. Pollard-Knight *et al.*, (1990ii) applied the system to DNA detection on Southern blots. 180fg of target DNA was detected in a model system.

Lanzillo (1991) prepared digoxigenin-labelled nucleic acid probes for angiotensin-converting enzyme. In a model system these probes were used to optimize conditions for target detection by direct chemiluminescence (the alkaline phosphatase/ AMPPD system was used). After dot blot or Southern hybridizations digoxigenin-labelled probe detected 23fg of target consistently. This makes this the most sensitive non-isotopic system available.

A bioluminescence assay for alkaline phosphatase has been described using a firefly D-luciferin-O-phosphate substrate. The enzyme cleaves the phosphate group to generate firefly-D-luciferin which reacts with the firefly luciferase to produce light. The bioluminescence detection system has been used in hybridization experiments enabling the detection of 19ng of target DNA (Hauber and Geiger, 1988).

Acridinium esters are direct labels. Methods have been developed for labelling DNA probes with acridinium esters at high specific activity and for stabilizing the label under the relatively harsh conditions of hybridization reactions. The label emits light upon exposure to alkaline hydrogen peroxide. A property of acridinium esters allows chemical destruction of the label, when it is present within a deoxyoligonucleotide probe. The label is stable to this process when the probe is hybridized. This implies a minimization of background signals and is also a suitable scheme for the format of homogeneous assay without separation step. Tenover *et al.*, (1990) used the Accu Probe assay system (Gen-Probe Inc.), which is based on an acridinium ester-labelled probe, to carry out DNA probe culture confirmation assays for the identification of thermophilic *Campylobacter* species. A comparison of the acridinium ester and dioxetane detection schemes was carried out by Nelson and Kacian (1990). They concluded that the dioxetane system offered inherent sensitivity due to signal amplification, however, a simpler and faster format could be used with acridinium esters as indirect labels, such as dioxetane, require incubation steps for the attachment of the second binding protein.

Non-radioisotopic hybridization assay methods using fluorescent,

chemiluminescent and enzyme-labelled synthetic oligodeoxyribonucleotide probes were compared (Urdea *et al.*, 1988). Detection limits of the labels and the labeled probes were assessed. The enzyme-modified oligonucleotides were found to be significantly better labelling materials than the fluorescent or chemiluminescent derivatives. It was observed that, in contrast to the fluorescent and chemiluminescent labels tested, little loss in sensitivity is noted upon attachment of enzymes to probes, or as the result of hybridization of the labeled probes to the solid supported target fragments. Their results suggest that enzymes such as HRP and AP are the superior non-radioisotopic reporter groups and provide sensitivities nearly equivalent to ^{32}P .

The use of alternative fluorescent reporters or chemiluminescent moieties may alleviate the shortcomings of the labels used by Urdea and co-workers (1988). Appropriate molecular design that takes into consideration electron density changes upon attachment, hydrophobicity and hydrophilicity, quenching and orientation may eventually lead to adequate small molecular labels. However, many of the best molecular reporting functions for direct labelling (e.g., isoluminol) have also been tailored to serve as enzyme substrates (e.g., luminol oxidation by HRP). This coupled with the intrinsic amplification provided by enzymes with high substrate turnovers significantly favours enzyme labelling methods.

Thus, many of the non-radioactive labelling techniques employing colorimetric or fluorescent detection lack the sensitivity required (e.g. for single-copy gene detection in human genomic DNA) in the absence of the PCR amplification technique or high levels of target sequence. Alternative non-radioactive systems need to be considered. We proposed to label the DNA probe with cold-iodine. [Iodination of nucleic acids is detailed in the Section 1.22.] A microassay for the colorimetric detection of iodide has been developed by O'Kennedy *et al.*, (1989) and was considered as a detection scheme. The development of a more sensitive microassay for iodide was also undertaken. The incorporated iodine could also be detected indirectly if an antibody against 5-iodocytidine was available and if the anti-hapten antibody was conjugated

to a signalling molecule for detection. Both monoclonal and polyclonal antibodies have been used to detect haptens on DNA probe assays. Anti-5-iodocytidine polyclonal antibodies were produced during the course of this work.

Table 1.6 summarizes the detection limits for nucleic acid labels and Table 1.7 shows examples of model DNA probe assay systems, and their sensitivity, which have been reported.

	LABEL	CL	BL	FLUOR	COL	RL
Radiolabels	³² P	-	-	-	-	50
	AP	.001	.01	.10	#.01	-
Enzymes	HRP	25	0.4	10	100	-
	β-gal	-	5	-	-	-
CL labels	Acridinum esters	0.5	-	-	-	-
Fluor. Labels	Europium chelate	-	-	10	-	-

Table 1.6

Detection limits in attomoles for nucleic acid labels (from Kricka, 1992; # from Johannsson et al., 1986).

[CL= chemiluminescence; BL= bioluminescence; Fluor= fluorescence; Col= colorimetric; RL= radioluminescence; AP= alkaline phosphatase; HRP= horseradish peroxidase; β-gal= β-galactosidase]

Label	Detection	Sensitivity	Reference
HRP	Colorimetric (anisidine + H ₂ O ₂) with PEG in the hybridization mixture	20pg for 2kb DNA fragment 1-5pg for 2kb DNA fragment	Renz & Kurz, 1984
HRP	ECL (Amersham)	3pg for probes of 0.45-2.75 kb	Pollard-Knight <i>et al.</i> , 1990i
Biotin	Streptavidin-HRP ECL (Amersham)	1pg of plasmid PBR322	Matthews <i>et al.</i> , 1985
Biotin	Streptavidin-AP Lumingen PPD (Lumingen Inc.)	180fg of <i>Hind</i> III digested DNA	Pollard-Knight <i>et al.</i> , 1990ii
Digoxigenin	Anti-digoxigenin-AP Boehringer Mannheim	23fg of target DNA, probing for angiotension converting enzyme	Lanzillo, (1990)

Table 1.7

Examples of model DNA probe assays described in the literature.

[HRP= horseradish peroxidase; AP= alkaline phosphatase; PEG= polyethylene glycol; ECL= enhanced chemiluminescence]

1.22 IODINATION OF NUCLEIC ACIDS

Substitution and conjugation methods have been described for the radioiodination of proteins. Substitution reactions consist of an electrophilic substitution of a hydrogen atom by iodine, in the form of the iodous ion (I^+), into the tyrosyl or histidyl moiety of proteins (Hughes, 1957). The substitution reaction may be carried out chemically (Mcfarlane (1958); Greenwood *et al.*, (1964)), enzymatically (Marchalonis (1969)) and electrolytically (Rosa *et al.*, (1964)). The procedure described by Bolton and Hunter (1973) is an example of a conjugation method to label proteins. This procedure involves reacting the protein under mild conditions with Bolton-Hunter reagent that has previously been labeled with iodine. The Bolton-Hunter reagent is a phenol and can be iodinated under the same conditions as the tyrosine in proteins. The iodinated reagent reacts with the lysyl residues of the amino groups present in most proteins and peptides to form amide bonds.

Nucleic acids have been labeled with ^{125}I using chemical substitution reactions and these radioiodination methods shall now be considered. The advantages of direct iodination methods include manipulative simplicity and high incorporation of iodine. However, these direct methods often involve exposure of the sample to strong oxidizing and reducing agents.

Iodination of nucleic acids was first considered by Brammer in 1963. It was found that iodine does not react with nucleic acids or their constituent bases in aqueous solution, but formation of small amounts of an iodinated product was demonstrated when viral RNA was exposed to *n*-iodosuccinimide. In 1966, Ascoli and Kahan showed that iodine monochloride may be used to introduce substantial amounts of iodine into the constituent nucleotides of polyuridylylate, polycytidylylate, soluble RNA and DNA, when these polymers were dissolved in anhydrous solvents. However, the conversion of nucleic acids into a salt, soluble in organic solvents also leads to their degradation.

The first successful method to chemically incorporate a radiolabel into nucleic acids was described by Commerford in 1971. He demonstrated that polynucleotides could be iodinated *in vitro* by heating in an aqueous solution of thallic trichloride plus iodide at pH 5. Iodine was incorporated into DNA (the reaction favours single-stranded DNA) through a stable covalent bond as 5-iodocytosine. Commerford proposed that an addition reaction occurred, that is, the addition of IOH across the 5,6, double bond of the pyrimidine to form the 5-iodo-6-hydroxy dihydro pyrimidine, which is similar to the mechanism proposed by Brammer (1963) for pyrimidine bromination. The reaction is pH-dependent and is carried out at pH 5.0-5.5. The pKa of 5-iodo-6-hydroxydihydro cytidine is somewhat below pH 5.5. The proposed reaction mechanism is illustrated in Figure 1.3.

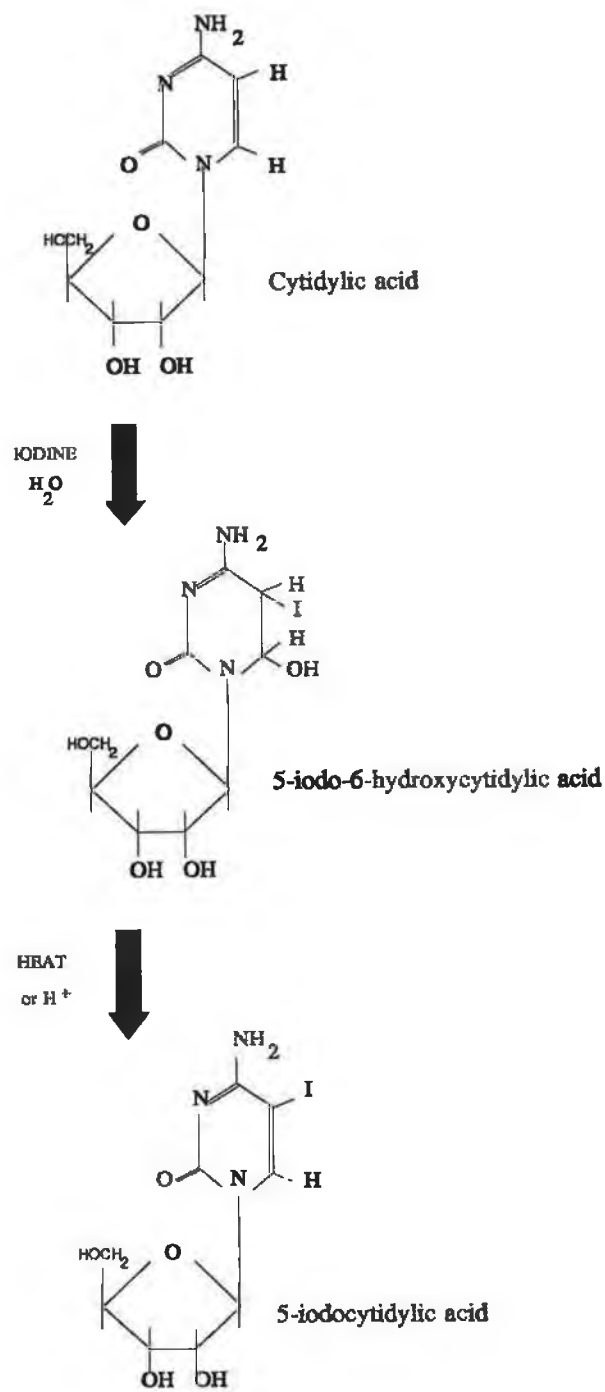


Figure 1.3
 Iodination of cytidylic acid (Brammer, 1963).

Chromatography of enzymatic digests of heavily iodinated DNA, prepared with venom phosphodiesterase, showed that 5-iodocytosine is the only stable base modification. Commerford also considered the iodination of cytidylic acid to investigate the nature of the chemical changes which occur in the macromolecule. He found that it was necessary to remove an unstable form of bound iodine by heating. The iodine that was bound covalently was not removed by heating at 100°C, pH 5-9. Thus, labeling by this method means iodine is bound to pyrimidine in two forms, one stable and associated with the appearance of UV absorption at 310nm and the other relatively unstable.

Iodination is carried out in the presence of a strong oxidising agent and it is necessary to show that degradation of the DNA or chain scission does not occur. This may be verified by electrophoretic mobility. Further considerations are, the thermolability of the product, the change in melting temperature of the hybrid (this is a sensitive probe for mismatch) and the influence of iodinated bases on the biological activity of nucleic acids.

Commerford observed that iodination of native DNA had little or no effect on the melting profile, ability to renature and sedimentation rate. However, extensive iodination of single-stranded DNA produced significant changes in physical properties. Some of these, for example, alteration of the UV absorption and increase in density can be attributed to the formation of 5-iodocytosine. Single-stranded *Clostridium perfringens* DNA containing 7% of its cytosine as 5-iodocytosine renatured normally and exhibited a sharp thermal transition when remelted which was identical with that of native *C. perfringens* DNA except that it was shifted 1.4°C toward a lower temperature. However, single-stranded *C. perfringens* DNA containing 24% 5-iodocytosine no longer fully renatured and when remelted the thermal transition was relatively broad. The loss of ability to renature is difficult to explain, especially since iodine occupies a position on cytosine equivalent to the methyl group on thymidine and hence should present no steric hindrance to double-stranded formation.

Orosz and Wetmur, (1974) optimized Commerford's method. They maximized iodination while minimizing degradation of the nucleic acid. They replaced the sulphite treatment, used by Commerford to reduce excess thallic chloride, because it causes deamination of cytosine and hydrolysis of the *n*-glycosidic linkages. The labelled DNA was sufficient for separation via buoyant density shifts and did not show the melting point transition which Commerford had observed.

Chan *et al.*, (1976) investigated the problem of chain scission which seemed to occur during the iodination reaction. The reaction which resulted in chain scission was shown to require the formation of iodocytosine or an intermediate in the reaction leading to iodocytosine. Chan proposed that, when the iodine to cytosine ratio is 0.02 or less, lambda DNA may be labelled without significant chain-scission.

A detailed characterization of the iodination of RNA using ^{125}I was carried out by Tereba and McCarthy (1973). They found that the iodinated RNA retained its specificity in DNA:RNA hybridization with only a negligible decrease in the stability of the DNA:RNA hybrids. Though iodination reduced the ability of cytosine to base-pair this did not seriously impair the specificity or efficiency of hybridization unless a high degree of cytosine is iodinated. The fidelity of pairing in DNA:RNA hybrids was assessed by determination of their thermal dissociation behaviour and 1.4-2.0% mismatching was within the experimental error of the estimated 1% of the bases which were labeled with iodide. With about 1% 5-iodocytosine thermal stability of chicken 28s rRNA hybrids was found to be within 2°C of expected rate. Thus, the degree of mismatching observed was attributed to the iodinated bases themselves rather than unknown damage introduced during the labeling procedure.

A similar study was carried out by Scherberg and Retetoff (1974) and their conclusion was also that neither incorporation of iodine into a small portion of the RNA nucleotides, nor the treatment used for incorporation of the isotope alter the hybridization capacity of the polymer. Their results also showed that

treatment of the pyrimidine reaction product at pH above neutrality for 20 minutes at 60°C would be sufficient to eliminate the unstable form of the bound iodine. Instability of the iodine adduct in RNA could contribute to background levels in hybridization. Iodination of RNA and single-stranded DNA are quite similar. The yield of iodination versus pH showed a sharp dependence between pH 5-5.5. This is consistent with Commerford's assessment that the pKa of the intermediate 5-iodo-6-hydroxydihydrocytidylic acid is somewhat below pH 5.5.

Shaposhnikov (1976) described an alternative method of *in-vitro* iodination. This was a modification of the technique, described by Greenwood *et al.*, (1964), for labeling proteins using chloramine T (*n*-chlorotoluenesulphamide, see Figure 1.4). The reaction was initiated by the addition of Chloramine T and was stopped by the addition of sodium metabisulphite. The preparations were uniformly labeled and non-degraded and conditions were milder than those used by Commerford (1971).

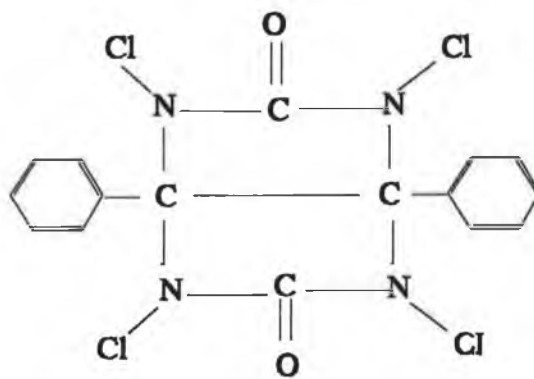
Shaposhnikov showed that iodocytosine was the only stable product of RNA iodination. The procedure described was simple, convenient and easily reproducible. Using Commerford's method some of the product formed between ¹²⁵I and RNA was thermolabile and had to be removed or degraded by heating in mild alkaline or neutral medium. Practically all the product was thermostable when labeled via Chloramine T. Also, a previously indicated specific activity of RNA (Tereba and McCarthy, 1973) could be obtained using twenty times less radioiodine. Finally, to consider the extent of RNA degradation, radioiodination of 18s and 28s RNA was carried out. There was no significant shift in electrophoretic mobility and it was concluded that there was practically no RNA degradation during the iodination procedure.

Iodogen-mediated radioiodination of nucleic acids was described by Piatyszek *et al.*, (1988). *Iodogen* (1,3,4,6, tetrachloro-3 α ,6 α -diphenylglycouril, see Figure 1.4) is supplied by the Pierce Chemical Company and it is chemically similar to four chloramine T molecules joined together. It is soluble in

chloroform and methylene chloride and can be layered onto the walls of test-tubes from these solvents. Thus, the oxidizer comes in less contact with the compound to be iodinated than with Chloramine T. It acts as a solid oxidizer. Therefore, no strong reducing agent is required to stop the reaction. *Iodogen* is widely used as an oxidizing agent for iodination of proteins (described initially by Fracker and Speck, 1978). [For protein iodination Salacinski *et al.*, (1981) describe a higher iodine incorporation with less iodination damage using *Iodogen* than with chloramine T]. Chromatograms of the hydrolysates of the radioiodinated tRNA showed mainly one spot co-migrating with iodocytosine indicating that there is only one stable product for the iodination reaction as before. Radioiodinated DNA was used as a probe in homologous hybridization and it was shown that DNA iodinated by this procedure was a good probe in hybridization experiments and gave low background levels.



Chloramine T



Iodogen

Figure 1.4
The structures of chloramine T (n-chlorotoluenesulphamide) and Iodogen (1,3,4,6, tetrachloro-3 α ,6 α -diphenylglycoluril).

The considerations in choosing a method for the iodination of nucleic acids are simplicity, cost and quality. Shaposhnikov's method involves milder conditions than the method of Commerford and a higher specific activity can be achieved. However, a strong reducing agent is required at the end of the iodination reaction to remove excess chloramine T, whereas this is not necessary if *Iodogen* is used. The chemical substitution methods described above are usually less expensive and probably more simple than the application of an enzymatic substitution method and these methods are usually used when large quantities of DNA have to be labelled.

Iodination by the enzymatic incorporation of the iodinated nucleotide has been carried out. Lewis *et al.*, (1986) described a system of high-resolution *in situ* hybridization histochemistry with radioiodinated synthetic oligonucleotides. Synthetic oligonucleotides were labelled to high specific activity with ^{125}I using either the primer extension method with the Klenow fragment of DNA polymerase I or the 3'-tailing method with terminal deoxynucleotidyl transferase. Allen *et al.*, (1987) evaluated the use of ^{125}I -cytosine triphosphate in the generation of complementary RNA probes using two RNA polymerases (T7 and SP6). It was possible to generate full length cRNA transcripts from the DNA template in the presence of ^{125}I -CTP.

The generation of non-radioactive bromodeoxyuridine labelled probes by polymerase chain reaction (PCR) was carried out by Tabibzadeh *et al.*, (1991). dTTP was substituted with bromodeoxyuridine during PCR. *Taq* polymerase incorporated bromodeoxyuridine as efficiently as dTTP into the synthesized strands of amplified DNA without a need to alter the concentration of any of the reagents in the PCR mixture or the conditions of the amplification. It is probably possible to incorporate 5-iododeoxycytidine by a similar protocol.

Iodination methodologies which were available were used during the course of this work. However, alternative ways to determine the extent of iodination had to be considered. Neither polycytosine nor DNA absorb at 310nm. It was observed by Commerford that absorption, at this wavelength, of iodinated

polynucleotides was linearly related to the concentration of 5-iodocytosine. Orosz and Wetmur (1974) also examined the change in spectrum of DNA upon iodination. Renatured iodinated samples in phosphate buffer, pH 6.8, showed a linear relationship between the absorbance at 310nm and the molar concentration of 5-iodocytidine. An extinction coefficient was obtained of 3200 +/- 300, providing a straightforward estimation of the percent iodination.

Buoyant density shifts also occur between the labeled and unlabeled nucleic acids and it is possible to separate them using gradient density centrifugation. Orosz and Wetmur (1974) reported that there was a linear relationship between the degree of substitution and buoyant densities. However, both these methods are most suitable in determining the extent of iodination if high incorporation has taken place. For example, at 1% incorporation the shift in absorbance at 310nm is not detectable. However, the iodide microassay (O'Kennedy *et al.*, 1989) can be used to determine the extent of iodination even at low incorporation levels.

In this study labelling of nucleic acids was carried out both chemically using the oxidizer *Iodogen*, and enzymatically via the incorporation of 5-iodo-2'-deoxycytidine 5'-triphosphate by random priming. The degree of iodination achieved was determined using the iodide microassay. Also, a method using High Performance Liquid Chromatography (HPLC) has been described to measure 5-iododeoxyuridine (Berlanger *et al.*, 1986) and labelled nucleic acids were analysed using this HPLC method to measure 5-iododeoxycytidine.

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

Reagents used were of analytical grade and were purchased from: Sigma Chemical Co., St Louis, Mo, USA; BDH Chemicals Ltd., Poole, England and Riedel de Haën, Hannover, Germany.

The following were also obtained from Sigma: cytidine; cytidine 5'-monophosphate; N α -acetyl-L-lysine; anti-rabbit IgG peroxidase conjugate; DNase I (Type II from bovine pancreas); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); Freund's complete and incomplete adjuvant; 5-iodocytidine; 5-iodocytidine 5'-monophosphate; 5-iodo-2'-deoxycytidine 5'-triphosphate; luminol (5-amino-2,3-dihydro-1,4-phthalazinedione); ortho-phenylenediamine (OPDA); phosphodiesterase I (Type VII from *Crotalus atrox* venom); polydeoxycytidylic acid; polylysine (70,000-150,000); single-stranded calf thymus DNA and 2-4-6-trinitrobenzene sulphonic acid (TNBS).

2-4-dinitro-1-chloro-benzene (DNCB) and tetrachloroauric acid were obtained from BDH. Acetone, acetonitrile and chloroform were provided by Labscan, Analytical Sciences (Dublin, Ireland) and absolute ethanol was supplied by the Merck Chemical Co. (Darmstadt, Germany). The Pierce Chemical Co. (Rockford, IL, USA) supplied: the BCA (bicinchoninic acid) Protein Assay kit; *Iodogen* (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) and sulpho-N-hydroxysuccinimide (sulpho-NHS).

Deoxynucleotides, lambda DNA and the *Prime-a-Gene* kit were obtained from Promega (Madison, WI, USA) and *Hind* III was bought from Gibco BRL (Gaithersburg, MD, USA). PBS tablets were provided by Oxoid (Hampshire, UK) and Sephadex-G25 was supplied by Pharmacia (Uppsala, Sweden).

The oligoprobe, V₆ primer + 10 C, was a gift from The National Diagnostic Centre, Galway (Barry *et al.*, 1990), plasmid PAAH5 was a gift from Margaret Duffy, Dublin City University (DCU), Dublin, and iodinated Bolton-Hunter reagent (IBHR), prepared as described by O'Kennedy *et al.*, (1989), was a gift from Dolores Cahill, DCU.

The microtitre plates used were supplied by Greiner (Frickerhausen, Germany) [ELISA plates], Dynatech (West Sussex, UK) [white *Microfluor* plates] and Nunc (Roskilde, Denmark) [*Covalink*-NH plates].

2.2 DETECTION OF IODIDE

2.21 Colorimetric microassay for iodide (O'Kennedy *et al.*, 1989)

Reagents for assay: Ceric ammonium sulphate (0.1M) was made up in 2.5M H₂SO₄. It was necessary to filter this solution before it was made up to volume. Dilutions of this stock solution were made up daily in 1M HNO₃. Arsenious acid (0.075M) was made up by dissolving 14.84g arsenious trioxide (As₂O₃) in 700ml ultrapure water containing 28ml concentrated H₂SO₄. Care was taken in the preparation of this reagent as As₂O₃ is a carcinogen. The mixture was heated to near boiling with constant stirring until the As₂O₃ was dissolved. Some precipitation of arsenious oxide may occur and filtration may be necessary before making up to 1l.

Standards: 1µg/ml potassium iodide was made up in ultrapure water (2ml of 0.1M NaOH was added to prevent air oxidation). It was stored at 4°C and was stable for several months. Dilutions of this stock solution were made in ultrapure water. 1mg/ml stock solutions of IBHR, 5-iodocytidine, 5-iodocytidine 5'-monophosphate and 5-iodo-2'-deoxycytidine 5'-triphosphate were made up in ultrapure water and stored at 4°C. Dilutions of the stock solutions were made in ultrapure water.

Microassay procedure: 100µl of standards were added in duplicate to a microtitre plate (Greiner). Samples for analysis (100µl) were then added in duplicate. 0.075M arsenious acid (60µl) was added to each well using a multichannel pipette, followed by 25µl of 0.028M ceric ammonium sulphate solution. The plate was shaken and incubated at 30°C. Absorbance readings at 414nm were taken after 5 minutes (iodide standards 0.01-0.10 µg/ml) or after 20 minutes (iodide standards 0.001-0.010 µg/ml) using a Titertek Twinreader Plus microplate reader. Ultrapure water is used as a zero control and will give the highest absorbance value in the assay, as no iodide is present to catalyze the Sandell-Kolthoff reaction.

2.22 Luminescent assays for the detection of iodide

A number of luminometers were used in the assay development involving luminescence.

- | | |
|-------------------------------------|---|
| i) Berthold Chemilumat LB9502: | Test-tube (75 x 5mm) reader, with injection facility |
| ii) Labsystems Luminoskan 5300017: | Microplate reader, with injection facility |
| iii) Amersham Amerlite Luminometer: | Microplate reader |
| iv) Flow-injection luminometer: | Designed and built by Simon McCabe at the Physics Dept., Dublin City University |

The chemiluminescent systems investigated were as follows:

- Acetone/ hydrogen peroxide/ hypochlorite/ iodide (Lu *et al.*, 1989)
- Hydrogen peroxide/ hypochlorite/ iodide
(Modification of the system described by Burguera and Burguera, 1982)
- Luminol/ hydrogen peroxide/ iodide (Bause *et al.*, 1979)
- Luminol/ hypochlorite/ iodide
- Luminol/ hydrogen peroxide/ tetrachloroauric acid/ iodide
(Lin *et al.*, 1989)
- Luminol/ hydrogen peroxide/ 2-4-dinitro-1-chlorobenzene/ iodide

Reagents for luminescent assays: 30% (w/v) hydrogen peroxide was diluted daily, with ultrapure water, to the molarity required for a given assay. 0.02M ClO^- , pH 13.5, was prepared from a sodium hypochlorite (NaOCl) solution (14% (w/v) available chlorine). This 4M solution of chlorine was diluted 200 fold, with ultrapure water, to give 0.02M ClO^- , pH 13.5. A stock solution of 0.05M luminol was prepared as follows: 0.913g luminol (97% pure) was dissolved in 10ml of 0.1M NaOH and brought to 100ml with ultrapure water. To prepare the 4.0mM working solution required for system d and for system e (flow-injection analysis), 2.0ml of the 0.05M luminol stock solution was taken. 5.0ml of 0.1M Na_2HPO_4 solution was added and the pH adjusted to 11.5. The solution was then brought to 25ml with ultrapure water. To prepare a stock solution of $1 \times 10^{-3}\text{M}$ tetrachloroauric acid ($\text{AuCl}_3\text{HCl} \cdot 4\text{H}_2\text{O}$), 0.2048g

of the salt was dissolved in 10ml 10% HCl. 0.25g of KCl was then added and the volume was brought to 100ml with ultrapure water. This stock solution was diluted daily to the required working concentration. 1mg/ml solution of 2,4-dinitro-1-chlorobenzene (DNCB) was prepared in ultrapure water. Heating was required to dissolve the DNCB.

Standards: Potassium iodide standards were prepared daily from a 1µg/ml or a 1mg/ml iodide stock solution as appropriate. The stock solution was made up in ultrapure water and stored at 4°C. Dilutions of this stock solution were made in ultrapure water.

System a: Acetone/ hypochlorite/ hydrogen peroxide/ iodide (Lu *et al.*, 1989)

1. Berthold Chemilumat LB9502

Assay procedure: The assay was followed as described by Lu *et al.*, (1989). 0.5ml of potassium iodide standard (50-1000µg/ml) or ultrapure water (zero control) was added to the test-tube (75 X 5mm) followed by 1.0ml of 49.7% acetone and 0.5ml 1.45M H₂O₂. The test-tube was then inserted into the luminometer and 300µl of 0.02M ClO⁻ was injected into the above reaction mixture. Luminescence was read for 10 seconds and the highest reading for counts per second (CPS), over this time interval, were recorded.

2. Labsystems Luminoskan 5300017

Microassay procedure: 100µl of potassium iodide standard (0.1-100.0µg/ml) or ultrapure water (zero control) was added in duplicate to the wells of the white Dynatech *Microfluor* plates. These plates were used for all the chemiluminescence microassays. 50µl of acetone was then added. [The microtitre plates used are polystyrene which acetone will dissolve. However, if the acetone is diluted or added to an aqueous solution in the well no damage to the plate is observed.] The plate was inserted into the luminometer. 20µl of 0.1M H₂O₂ was injected into the well being read, followed by 20µl 0.02M ClO⁻. The luminescence (relative light units) was read immediately, as only a flash of luminescence occurs when the hypochlorite is added.

System b: Hypochlorite/ hydrogen peroxide/ iodide

1. Labsystems Luminoskan 5300017

Microassay procedure: 100µl of potassium iodide standard (1-100µg/ml) or ultrapure water (zero control) was added to the wells of the microtitre plate in triplicate. The plate was then inserted into the luminometer. 20µl of 0.05M H₂O₂ was injected into the well being read, followed by 20µl 0.02M ClO⁻. The luminescence was read immediately.

System c: Luminol/ hydrogen peroxide/ iodide (Bause *et al.*, 1979)

1. Labsystems Luminoskan 5300017

Microassay procedure: 100µl of potassium iodide standard (>100µg/ml) or ultrapure water (zero control) was added in triplicate to the wells of a microtitre plate. 100µl of 2.5mM luminol was added to each well. The plate was inserted into the luminometer, and 50µl of 0.01M H₂O₂ was injected into each well. The flash of luminescence was recorded.

System d: Luminol/ hypochlorite/ iodide

1. Labsystems Luminoskan 5300017

Microassay procedure: 150µl of potassium iodide standard (10ng/ml-100µg/ml) or ultrapure water (zero control) was added in triplicate to the wells of a microtitre plate. The plate was then inserted into the luminometer. 30µl 4.0mM luminol was injected into the well being read, followed by 20µl 0.02M ClO⁻. Relative light units (RLU) were read immediately after this addition.

System e: Luminol/ hydrogen peroxide/ Au (III)/ iodide (Lin *et al.*, 1989)

1. Flow-injection luminometer

Flow-injection procedure: Iodide standards were made up in the range 10pg to 10.0µg/ml. Before bringing each standard to a final volume of 100ml, 0.2ml of 500µg/ml AuCl₃HCl₄H₂O and 1.0ml of 5M H₂O₂ were added. These reagents were added immediately prior to reading the sample in the flow system. Ultrapure water served as a zero control, to which, the above reagents were added and brought to the same volume as in the standards. The iodide standards, or the zero control, flowed through the system and three injections

of 4mM luminol, pH 11.5, were made at 10 second intervals. Continuous measurement of photon count (every 50ms) was made over this time.

Stopped-flow procedure: 4mM luminol was injected into the flow system (iodide standard, or zero control, as above) and the flow stopped. The photon count was read for 250 milliseconds, every second, until the readings levelled off.

2. Amersham Amerlite Luminometer

Microassay procedure: The following reagents were added to the wells of a microtitre plate, in the order listed, using a multichannel pipette: 50µl of potassium iodide standard (1-100µg/ml) or ultrapure water (zero control); 50µl 0.1mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$; 50µl 0.01M luminol (diluted from the 0.05M storage solution with ultrapure water); 50µl 0.25M H_2O_2 . The microtitre plate was inserted into the luminometer and relative light units (RLU) were read at 5 minutes.

System f: Luminol/ hydrogen peroxide/ DNCB/ iodide

1. Amersham Amerlite Luminometer

Microassay procedure: The following reagents were added to the wells of a microtitre plate, in the order listed, using a multichannel pipette: 50µl of potassium iodide standard (1-100µg/ml) or ultrapure water (zero control); 50µl 1.0mg/ml DNCB; 50µl 0.01M luminol (diluted from the 0.05M storage solution with ultrapure water); 50µl 0.25M H_2O_2 . The microtitre plate was inserted into the luminometer and RLU were read at 5 minutes.

2.3 IODINATION OF NUCLEIC ACIDS

Nucleic acids were iodinated by the following methods:

- 1) Chemical substitution of iodide using the solid oxidizer *Iodogen* (Pierce Chemical Co.) as described by Piatysek *et al.*, (1988).
- 2) Enzymatic incorporation of 5-iodo-2'-deoxycytidine 5'-triphosphate by random priming using the *Prime-a-Gene* kit supplied by Promega.

2.31 *Iodogen*-mediated iodination of nucleic acids

Method 1 (Piatysek *et al.*, 1988)

To prepare the iodination vessels 100 μ l of chloroform containing 14 μ g of *Iodogen* (1,3,4,6,-tetrachloro-3 α ,6 α -diphenylglycouril) was placed in a glass test tube (8 x 120mm), and the solvent was thoroughly evaporated to dryness under nitrogen (Salacinski *et al.*, 1981). Large numbers of these test tubes were prepared and stored in a desiccator until required.

Prior to use the test tube was rinsed with 0.1M sodium acetate, pH 4.0, to remove any flakes of *Iodogen* not sticking to the glass. Typically, 100 μ g of DNA was labelled at any one time and the reaction conditions in each test-tube were as follows:

- 2 μ g of single-stranded DNA
- 30 μ l 0.35M sodium acetate, pH 4.0
- 600pmol NaI in 20 μ l in ultrapure water
- Volume was adjusted to 100 μ l with sterile water

The capped reaction vessels were incubated at 40°C for 30 minutes with occasional shaking. After chilling on ice, 10 μ l of 1.0M NaOH was added to each of the reaction vessels and the mixture was transferred to Eppendorf tubes.

Unbound iodide was removed by ethanol precipitation of the DNA (Sambrook *et al.*, 1989). Twice the volume (2X volume) in each Eppendorf of absolute ethanol and 0.1X volume of 3.0M sodium acetate, pH 5.2, was added to each Eppendorf and left at room temperature for 20 minutes. DNA was pelleted by centrifugation at 13,000g for 20 minutes, using a Heraeus Sepatech Biofuge 13. This pellet was then washed with 1X volume 70% ethanol, and pelleted as above. The final pellet was resuspended in 10mM Tris, pH 8.0,. A control reaction was also carried out where no *Iodogen* was attached to the reaction vessel. Absorbance at 260nm was used for the estimation of DNA concentration.

Method 2

Method 2 proved cumbersome as large quantities of DNA (100µg) were labelled which resulted in 50 test-tubes being used. An alternative procedure was devised where a glass petri-dish (90mm diameter) was used as the reaction vessel. 1.4mg of *Iodogen* was dissolved in 5ml of chloroform, and placed in the petri-dish. The chloroform was left to evaporate in the fumehood while the petri-dish was rotated using a Blood Tube Rotator SB1 (Stuart Scientific). Thus *Iodogen* was evenly coated onto the surface of the dish.

The reaction conditions were the same as for Method 1, except that the reaction mixture was added to the petri-dish instead of to the test-tubes. The reaction vessel was incubated at 37°C for 1 hour, while rotating. After chilling on ice, NaOH was added and the mixture was transferred to Eppendorf tubes. The unbound iodide was removed by ethanol precipitation as before. A control reaction was also set up where no *Iodogen* was coated onto the surface of the petri-dish.

2.32 Enzymatic incorporation of 5-iodo-2'-deoxycytidine 5'-triphosphate by random priming (Feinburg and Vogelstein, 1983;1984)

The *Prime-a-Gene* kit from Promega was used and the protocol was followed as described in the insert. Plasmid PAAH5 (12.5KB) was linearized using *Hind* III and then labelled. The reaction conditions were as follows:

2 μ l plasmid DNA (1 μ g/ μ l)
1 μ l (10 units) *Hind* III
10 μ l Buffer X10,
[The buffer is provided with restriction enzyme from
Gibco]
77 μ l sterile water

The reaction was incubated at 37°C for 3 hours and the linearized plasmid was ethanol precipitated (Sambrook *et al.*, 1989). 2X volume absolute ethanol and 0.1X volume 3.0M sodium acetate, pH 5.2, were added to the reaction mixture, and left at room temperature for 20 minutes. DNA was pelleted by centrifugation at 13,000g for 20 minutes, using a Heraeus Sepatech Biofuge 13. This pellet was then washed with 1X volume 70% ethanol, and pelleted as above. The pellet was resuspended in 100 μ l 10mM Tris-HCl, pH 8.0.

Prior to incorporation in the random priming reaction, the sample was denatured by heating in an Eppendorf tube at 95-100°C for 2 minutes followed by rapid chilling of the tube on an ice-bath. The following reaction was set up in an Eppendorf tube on ice. The reagents were added as listed.

10 μ l sterile water
40 μ l 5X labelling buffer

20 μ l of the mixture of dNTPs [1.5mM 5-iodo-2-deoxycytidine 5'-triphosphate was included in the mixture instead of dCTP. All the nucleotide solutions were at a concentration of 1.5mM 10 μ l of each were taken and 20 μ l of this mixture of dNTPs was added to the reaction.]

100 μ l (500ng) of denatured DNA template
20 μ l nuclease-free BSA
10 μ l (50 units) Klenow enzyme

The contents of the Eppendorf were mixed gently and incubated at room

temperature for 60 minutes. The reaction was stopped by placing the tube in a waterbath at 95-100°C for 2 minutes. The tube was transferred directly onto ice.

Selective precipitation of labelled DNA, with ammonium acetate and ethanol, was followed as described as in the Promega insert. This method results in the precipitation of DNA greater than 20 nucleotides in length, while free dNTPs remain in the supernatant. One volume of 4M ammonium acetate, pH 4.5, was added to the Eppendorf tube and vortexed. Two volumes (where 1 volume was the total volume after adding ammonium acetate) of ethanol were added. The tube was mixed and chilled on an icebath for 15 minutes. The tube was then heated at 37°C for 2 minutes with occasional gentle mixing. This step redissolved any free deoxyribonucleotides which had precipitated. The DNA was pelleted at 12000g, using a Heraeus Sepatech Bifuge 13, for 15 minutes and the supernatant was carefully aspirated. The pellet was washed by adding the following to the Eppendorf: 100µl 4M ammonium acetate, pH 4.5, 400µl absolute ethanol and 100µl sterile water, and then gently shaking the tube at room temperature. The DNA was pelleted and the supernatant removed as before. The pellet was finally washed in 90% ethanol and dried under vacuum. The pellet was redissolved in 50µl 10mM Tris, pH 8.0.

2.4 HPLC ANALYSIS OF LABELLED DNA

Belanger *et al.*, (1986) describe a method for the HPLC analysis of DNA nucleosides, after hydrolysis of the DNA. Labelled polydeoxycytidylic acid and labelled oligoprobe (V_6 primer, + 10C) were digested by incubating at 37°C for 18 hours with phosphodiesterase I (type VII) from *Crotalus atrox* venom and DNase I (type II) from bovine pancreas in potassium phosphate (KPi) buffer (0.05M, pH 7.45). The initial digestion was set up as follows: 4µg polydeoxycytidylic acid or 20µg oligoprobe in KPi buffer, pH 7.45, (both labelled and control samples of polydeoxycytidylic acid and oligoprobe) were hydrolysed with 40 units DNase I and 0.1 unit phosphodiesterase I in a total volume of 200µl.

The following standards were also incubated under identical conditions: the four dNTPs (32.5ng of each was taken); cytidine and 5-iodocytidine (50µg); cytidine 5'-monophosphate and 5-iodocytidine 5'-monophosphate (50µg). 20µl of each hydrolyzed sample was analyzed by HPLC. Conditions were as follows:

HPLC: System Gold, Beckman
Column: µ Bondapak C18, 125Å, 10µm, 3.9 X 300mm, (with guard column). Waters Chromatography Division, Millipore Corporation.
Mobile Phase: 0.1M acetate buffer, pH 5.4, containing 4% acetonitrile
Flow Rate: 2.0ml/minute
Detection: Absorbance was read at 260nm and 300nm

2.5 PRODUCTION AND CHARACTERIZATION OF ANTISERA TO 5-IODOCYTIDINE

2.51 Production of 5-iodocytidine and cytidine conjugates

The method described by Erlanger and Beiser (1964) for the conjugation of nucleosides to proteins was followed. 50mg of 5-iodocytidine or cytidine was oxidized in 2.5ml 0.1M sodium periodate at room temperature for 20 minutes. Excess sodium periodate was decomposed by the addition of 150µl 1M ethylene glycol, followed by 5 minutes at room temperature. The reaction mixture was then added, with gentle stirring to an aqueous solution of 5ml of 100mg polylysine (molecular weight: 70,000-150,000) or bovine serum albumin (BSA), adjusted to pH 9.0-9.5 with 5% (w/v) potassium carbonate. A solution of 75mg of sodium borohydride in 5ml H₂O was added, and the reaction mixture was set aside for 18 hours. 2.5ml of 1M formic acid was then added, followed one hour later by adjustment of the pH to 8.5 using 1M NH₄OH. The preparation was dialyzed for 36 hours, against several changes of distilled water, at 4°C and freeze-dried (Hetosicc, Heto Lab. Equipment, Denmark). BSA and polylysine conjugation controls were also prepared, by carrying out an identical reaction but without cytidine or 5-iodocytidine.

2.52 Characterization of the conjugates

2.52.1 Bicinchoninic acid protein assay

The bicinchoninic acid (BCA) protein assay from Pierce Chemical Co. was used. Protein reacts with Cu⁺⁺ in an alkaline medium yielding Cu⁺. Bicinchoninic acid is a sensitive detection reagent for the cuprous ion. The protocol for the microplate format was followed, as described in the insert with the kit. 10µl of standard (0.1-2.0 mg/ml BSA or 0.1-2.0 mg/ml polylysine) or sample was added to the wells of a microtitre plate. 200µl of the working reagent (50 parts reagent A with 1 part reagent B) was then added to each well. The plate was shaken and incubated at 37°C for 30 minutes for BSA standards and samples. The polylysine standards and samples were incubated

for one hour at 37°C. For both sets of standards and samples absorbance was read at 562nm on a Titretek Twinreader Plus microplate reader. Unknowns were determined from a standard curve.

2.52.2 Determination of free amino groups in BSA and polylysine after conjugation to the haptens cytidine and 5-iodocytidine, by the 2-4-6-trinitrobenzenesulphonic acid (TNBS) assay

The method used was described by Habeeb (1964). The following standards were prepared: 0.05-0.10M N α -acetyl-L-lysine; 10-100 μ g/ml polylysine and 100-1000 μ g/ml BSA. 1ml of standard or sample was taken. 1ml of 4% (w/v) NaHCO₃, pH 8.75 and 1ml of 0.1% (w/v) TNBS were added. The reaction mixture was incubated at 37°C for 90 minutes. 1ml of 10% (w/v) SDS was added to solubilize the protein and prevent its precipitation with the addition of 0.5ml 1M HCl. Absorbance was read at 335nm on a dual-beam spectrophotometer (UV-160A, UV Visible recording spectrophotometer, Shimadzu).

2.52.3 Gel exclusion chromatography and spectral analysis of the BSA and polylysine conjugates prepared

The conjugates were eluted through a Sephadex G-25 column, to ensure separation from any free iodocytidine or cytidine remaining after dialysis, before examining their UV spectra. Conditions for the chromatography of the polylysine conjugates were as follows:

Column size:	7 X 1 cm
Sample:	200 μ g/200 μ l
Mobile phase:	0.1M sodium phosphate buffer, pH 5.5
Fraction size:	1ml

Conditions for the BSA conjugates were as follows:

Column size:	17 X 1 cm
Sample:	200 μ g/200 μ l
Mobile phase:	0.1M sodium phosphate buffer, pH 7.0
Fraction size:	0.5ml

Absorbance spectra (200-320nm) were taken of the fractions on a dual-beam spectrophotometer (UV-160A, UV Visible recording spectrophotometer, Shimadzu).

2.52.4 High Performance Liquid Chromatography (HPLC) of the conjugates

HPLC was used to analyse the purity of the conjugates after dialysis, and to investigate if a change in molecular weight due to the conjugation of the hapten could be observed. Two different systems were used. To analyse the polylysine conjugates the following system was used:

HPLC: System Gold, Beckman
Column: Protein-Pak 300 sw 10 μ m, 7.8 x300mm (with guard column) Waters, Chromatography Division, Millipore Corporation.
Mobile Phase: 0.1M sodium phosphate buffer, pH 5.5
Flow rate: 0.5ml/minute
Detection: Absorbance was read at 220nm and 293nm

To analyze the BSA conjugates the system used was as follows:

HPLC: System Gold, Beckman
Column: Biosep-sec-S400, 300 x 7.8mm, (with Biosep-sec-s guard column) Phenomex.
Mobile phase: 0.1M sodium phosphate buffer, pH 7.0
Flow rate: 0.75ml/minute
Detection: Absorbance was read at 280nm and 293nm

2.53 Immunization protocol

A 3mg/750µl solution of 5-iodocytidine-polylysine was prepared in 0.1M PBS, pH 7.4. This was added to 750µl of Freund's complete adjuvant (first immunization) or Freund's incomplete adjuvant. The solution was vortexed for 2 minutes. A 1ml sample was injected at four sites intradermally. A serum sample was taken to serve as control serum before the initial immunization. The animal (white male New Zealand rabbit) was boosted on day 14, and every two weeks, thereafter. The animal was bled from the marginal vein in the ear, 11-14 days after the third and subsequent injections. The antibody titre did not increase over a period of 3 months, so the carrier protein was changed to BSA.

A 3mg/750µl solution of 5-iodocytidine-BSA was prepared in 0.1M PBS, pH 7.4. This was added to 750µl of Freud's incomplete adjuvant. The solution was vortexed for 2 minutes, and a 1ml sample was injected at four sites intradermally. The animal was boosted every two weeks and bled after 11-14 days, until the antibody titre was sufficiently high.

2.54 Screening for antibody production

Non-competitive ELISA was used to determine the serum titre. The antigen, 5-iodocytidine or cytidine, was bound directly to Nunc *Covalink-NH* plates (Parker and Halloran, 1968). 1mg of cytidine-monophosphate and 1mg 5-iodocytidine-monophosphate were each dissolved in 2ml of ultrapure water, to which 0.7mg of sulpho-N-hydroxysuccinimide (sulpho-NHS) had been added. 50µl of these solutions were added to the appropriate wells of the Nunc *Covalink-NH* plate. To each of these wells was added 50µl of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) solution (5.8mg/ml). The reagents were left to react for 90 minutes at room temperature. The plate was washed three times with *covabuffer* (116.8g NaCl, 10g MgSO₄·7H₂O and 0.5ml Tween 20 made up to 1 litre with PBS (0.1M, pH 7.4)). Controls wells with no antigen bound were also included.

The plate was blocked with 0.2% (w/v) gelatin in 0.1M PBS, pH 7.4. 200µl of the gelatin solution was added to the wells, and incubated at 37°C for 1 hour. The plate was washed twice with PBS-Tween (0.05% (v/v) Tween in 0.1M PBS, pH 7.4). and once with PBS (0.1M, pH 7.4). After washing, 100µl of serum diluted in blocking solution were added to the appropriate wells and incubated at 37°C for 2 hours. The plate was washed five times with PBS-Tween and once with PBS.

100µl of the working dilution of anti-rabbit IgG peroxidase conjugate (antibody developed in goat, IgG fraction of antiserum, Sigma Immunochemicals) was added to each well and incubated at 37°C for 30 minutes. The plate was washed five times with PBS-Tween and once with PBS. The horseradish peroxidase substrate, ortho-phenylenediamine (OPDA), was used. 10mg of OPDA was added to 25ml of 0.1M citrate-phosphate buffer, pH 5.0, to which 5µl of H₂O₂ had already been added. 100µl of this substrate solution was added to each well of the plate. The colour was allowed to develop at 37°C for 30 minutes, and the absorbance was read at 405nm using a Titretrek Twinreader Plus microplate reader.

2.55 Antibody purification: ammonium sulphate precipitation

Antibodies were precipitated from rabbit serum according to the method described by Hudson and Hay (1980). The rabbit serum was cooled to 4°C, and the pH adjusted to 4.4-5.1 using HCl. The serum was then incubated at 4°C for 1 hour, after which it was centrifuged at 3000g [5000 RPM on the Sorvall-RC-5B refrigerated superspeed centrifuge (DuPont instruments), using the SS-34 KSB rotar] for 20 minutes at 4°C. The supernatant was removed and the pH adjusted to between 7.0 - 7.2 with NaOH. An equal volume of cold 100% (w/v) saturated ammonium sulphate solution was added dropwise while stirring. The solution was incubated at 4°C for 1 hour, and then centrifuged as above. The precipitated immunoglobulin was washed in 50% (w/v) saturated ammonium sulphate. The pellet was resuspended in a minimum volume of 0.1M PBS, pH 7.4, and dialysed at 4°C against several changes of PBS. The

total protein concentration of the pellet was estimated by the BCA protein assay as outlined in section 2.52.1.

2.56 High Performance Liquid Chromatography of partially-purified antibody

The partially purified antibody (ammonium sulphate precipitation, Section 2.55) was analysed by HPLC to assess its purity. 20 μ l of a 1mg/ml solution was injected onto the column. Conditions for HPLC were as follows:

HPLC: System Gold, Beckman
Column: Biosep-sec-S400, 300 x 7.8mm, (with Biosep-sec-s guard column), Phenomex.
Mobile phase: PBS, pH 7.4
Flow rate: 0.5ml/minute
Detection: Absorbance was read at 280nm

Gel filtration molecular weight markers (Sigma Chemical Co.) were also run on this system. These markers were: cytochrome C (12,400); carbonic anhydrase (29,000); BSA (66,000); alcohol dehydrogenase (150,000) and β -amylase (200,000).

2.57 Competitive ELISA for 5-iodocytidine

Antigen (5-iodocytidine or cytidine) was bound to the Nunc *Covalink*-NH plate and the plate was blocked as outlined in Section 2.54. 25 μ l of 5-iodocytidine or cytidine standard, as appropriate, and 75 μ l of antibody (the working dilution of the partially-purified antibody) were added to each well and incubated at 37°C for 2 hours. The plate was washed, labelled-second antibody was added and the label was detected as described in Section 2.54.

Chapter 3

OPTIMIZATION AND VALIDATION OF THE COLORIMETRIC IODIDE MICROASSAY

3.1 INTRODUCTION

The catalytic effect of iodide on the reduction of ceric sulphate by arsenious acid, in sulphuric acid, was first described by Sandell and Kolthoff (1934). A microassay for the determination of iodide, and iodine-containing compounds, based on this assay was developed by O'Kennedy *et al.* (1989). Optimization of this microassay and its applications were later described by O'Kennedy and Keating (1991, 1993). The method has been used to quantify mouse antibody levels, human antibody levels in serum and the proteolytic activity of trypsin, pepsin and papain.

The various factors controlling catalytic activity have been studied. These include temperature, incubation time, concentration of reagents, acidity of reacting medium and chemical form of the reacting cerium(IV). The following conditions gave good linearity for the range 0.01-0.10µg/ml potassium iodide (O'Kennedy and Keating, 1991): absorbance was read at 414nm, 2 minutes after the addition of 0.028M ceric ammonium sulphate (diluted in 1M H₂SO₄, from a stock 0.1M solution). 0.075M arsenious acid was used. The incubation temperature was 30°C.

A ten-fold increase in sensitivity was reported when absorbance was read at 340nm (Keating, 1991). A standard curve in the range 1-10ng/ml potassium iodide was obtained with the following assay conditions: 3.5mM ceric ammonium sulphate (diluted with 1M H₂SO₄ or 1M HNO₃ from a 0.1M stock solution) and 9.37mM arsenious acid were used. The plate was incubated at 30°C for 30 minutes, and absorbance was read at 340nm. Interference which may occur because absorbance is read at a wavelength close to the ultra-violet region is a disadvantage of this assay.

Incorporation of nitric acid has been reported to increase the catalytic effect of iodide (Pantel, 1987; O'Kennedy *et al.*, 1989). However, Keating (1991) found that though nitric acid inclusion enhanced the rate of reaction, no significant increase in sensitivity was observed. These observations were investigated further. It was found that by using 1M HNO₃, rather than 1M H₂SO₄, to dilute the stock ceric ammonium sulphate solution, a standard curve in the range 1-10ng/ml potassium iodide (KI) was obtained. This work is described below.

Percentage transmittance, rather than absorbance readings, have been plotted against KI concentration to achieve linearity for incubation times greater than 5 minutes (O'Kennedy *et al.*, 1989). It has been found, during the course of this work, that improved correlation coefficients could be obtained using an exponential fit.

Several studies have reported that not all iodine containing compounds catalyze the Sandell-Kolthoff reaction equally (Pantell, 1982; Timotheou-Potamia, 1988; O'Kennedy *et al.*, 1989). [Discussed in Section 1.12.2]. When measuring iodinated Bolton-Hunter reagent (IBHR) it is not possible to achieve the same sensitivity as with iodide. O'Kennedy and Keating (1991) obtained a linear range for IBHR for the concentration range 0.1-1.0 µg/ml. During the course of this work conditions for the assay were modified as for the KI standard curve described above, and a ten-fold increase in sensitivity was achieved.

In the work described in this thesis the catalytic activities in the Sandell-Kolthoff reaction of iodocytidine and the related compounds, 5-iodocytidine 5'-monophosphate and 5-iodo-2'-deoxycytidine 5'-triphosphate were also examined.

3.2 RESULTS

3.21 Optimization and validation of the Sandell-Kolthoff microassay: potassium iodide standards

Two concentration ranges of KI were considered: 0.01-0.10 $\mu\text{g/ml}$ and 0.001-0.010 $\mu\text{g/ml}$. Acidity of the reacting medium and incubation time were the conditions optimized. Figure 3.1 illustrates two standard curves for KI in the range 0.01-0.10 $\mu\text{g/ml}$. The first of these standard curves was obtained using the assay optimized by O'Kennedy and Keating (1991) [see Section 3.1] while for the second the assay was changed as follows: 0.1M $\text{Ce}(\text{NH}_4)_2\text{SO}_4$ was diluted to 0.028M using 1M HNO_3 rather than 1M H_2SO_4 , and incubation time was increased from 2 minutes to 5 minutes. Figure 3.1a shows the linear plots of KI concentration against absorbance for both these assays while Figure 3.1b shows the exponential plots obtained for the same data. An exponential fit of the data, rather than a linear fit, gives a correlation coefficient closer to 1.000.

Validation of the modified assay was carried out. Table 3.1 shows the correlation coefficients (r value) obtained for the intra- and inter-day validation assays. Five intra-day and 5 inter-day assays were carried out, and controls were included with each assay. Table 3.2 shows the relative standard deviation (RSD%), and the percentage recovery, for the controls included in the intra-day validation assays. A relative standard deviation of less than 5% implies that the modified assay is precise. The percentage recovery is a measure of the accuracy of the assay; a value between 95-105% is acceptable. The limit of detection (LOD) is the concentration which gives a value greater than the mean for the zero control plus three times the standard deviation, for ten replicates of zero. The LOD of the assay is 0.01 $\mu\text{g/ml}$ KI.

Absorbance at 414nm

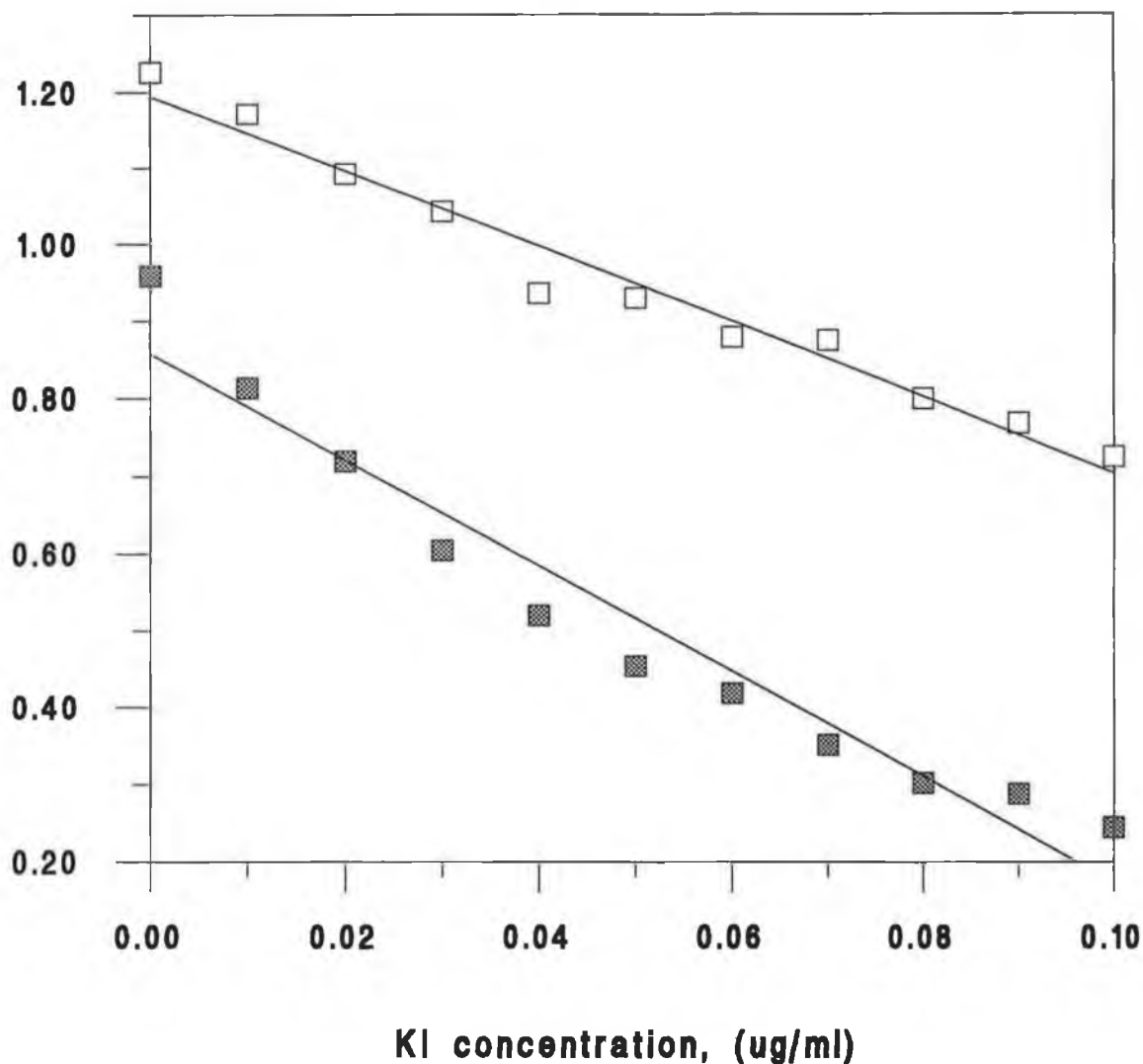


Figure 3.1a

Linear graphs of absorbance at 414nm plotted against concentration of KI (0.01-0.10 μ g/ml).

(□) Ceric ammonium sulphate was diluted in 1M H_2SO_4 . The reaction was incubated for 2 minutes at 30°C. [$r = 0.986$]

(■) Ceric ammonium sulphate was diluted in 1M HNO_3 . The reaction was incubated for 5 minutes at 30°C. [$r = 0.972$]

Absorbance at 414nm

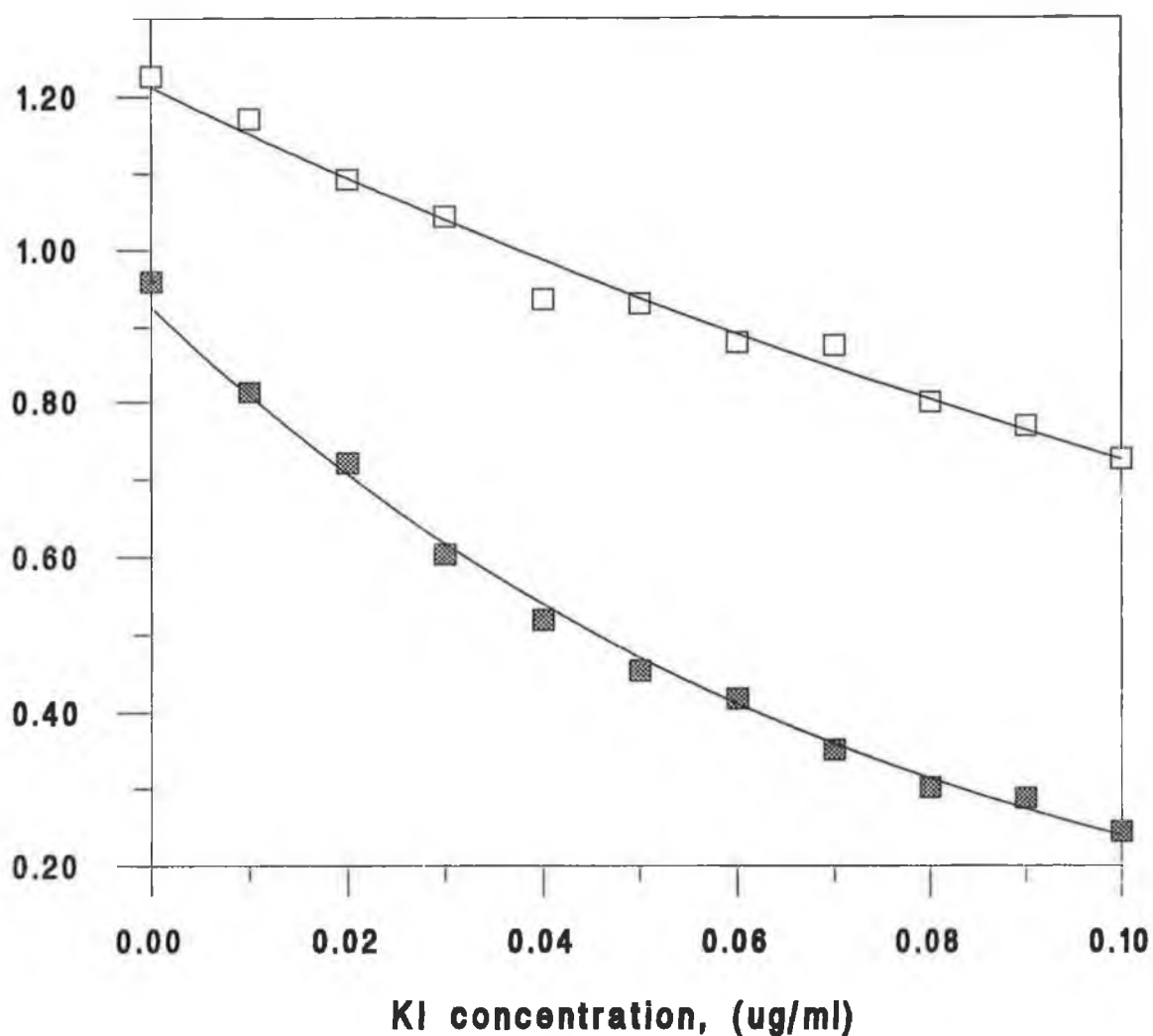


Figure 3.1b

Exponential graphs of absorbance at 414nm plotted against concentration of KI (0.01-0.10 μ g/ml).

(\square) Ceric ammonium sulphate was diluted in 1M H₂SO₄. The reaction was incubated for 2 minutes at 30°C. [$r = 0.992$]

(\blacksquare) Ceric ammonium sulphate was diluted in 1M HNO₃. The reaction was incubated for 5 minutes at 30°C. [$r = 0.997$]

Assay number	Correlation coefficient	
	Intra-day validation	Inter-day validation
1	0.994	0.997
2	0.992	0.993
3	0.996	0.999
4	0.999	0.998
5	0.991	0.997

Table 3.1

Correlation coefficients obtained for intra- and inter-day validation assays for the modified iodide microassay (KI concentration range: 0.01-0.10 μ g/ml). Absorbance at 414nm was plotted against KI concentration. The data was fitted exponentially.

(a)

Assay number	KI concentration, (µg/ml)	Percentage recovery
1	0.0247	98.8
2	0.0270	108.0
3	0.0259	103.6
4	0.0258	103.2
5	0.0245	98.0
Mean	0.0256	102.3
Standard deviation	0.0010	4.05
RSD%	3.91	

(b)

Assay number	KI concentration, (µg/ml)	Percentage recovery
1	0.0760	101.3
2	0.0749	99.9
3	0.0770	102.7
4	0.0751	100.1
5	0.0787	104.9
Mean	0.0763	101.8
Standard deviation	0.0016	2.07
RSD%	2.07	

Table 3.2

The controls 0.0250µg/ml (a) and 0.0750µg/ml (b) KI were included when carrying out the 5 intraday validation assays. The concentration determined for the controls for each assay is shown, as is the percentage recovery. [RSD = relative standard deviation]

The sensitivity of the microassay was increased ten-fold by increasing the incubation time to 20 minutes, when nitric acid is used, instead of sulphuric acid, to dilute the stock ceric ammonium sulphate. Table 3.3 shows the correlation coefficients obtained for the standard curves of absorbance at 414nm against potassium iodide concentration (0.001-0.010µg/ml), under different conditions. Both 1M H₂SO₄ and 1M HNO₃ were used to dilute the 0.1M stock ceric ammonium sulphate solution, and the absorbance was read over time. Linear and exponential graphs were plotted for absorbance against KI concentration. Linear graphs for percentage transmittance against KI concentration were also plotted. The best correlation coefficients could be achieved by plotting an exponential curve of absorbance against KI concentration and by using HNO₃ to dilute the stock ceric ammonium sulphate.

Time, minutes	r value					
	H ₂ SO ₄			HNO ₃		
	Abs Linear	Abs Exp	% Trans Linear	Abs Linear	Abs Exp	% Trans Linear
15	0.971	0.971	0.967	0.993	0.991	0.987
20	0.977	0.973	0.967	0.991	0.995	0.993
25	0.976	0.976	0.975	0.985	0.995	0.995

Table 3.3

Correlation coefficients (r value) obtained for standard curves for absorbance (abs) at 414nm against KI concentration (0.001-0.010µg/ml), and percentage transmittance (% Trans) at 414nm against KI concentration (0.001-0.010µg/ml), under different conditions in the iodide microassay:

- a) *The 0.1M ceric ammonium sulphate was diluted with either H₂SO₄ or HNO₃*
- b) *Absorbance was read over a range of incubation times*

[Linear = linear plot ; Exp = exponential plot]

After considering the results in Table 3.4 the following assay was validated. 0.1M ceric ammonium sulphate was diluted to 0.028M using 1M HNO₃, and absorbance values at 414nm were read after a 20 minute incubation at 30°C. An exponential graph was plotted of absorbance at 414nm against KI concentration (0.001-0.010µg/ml). A sample graph obtained is shown in Figure 3.2. Table 3.4 shows the correlation coefficients obtained for the intra- and inter-day validation assays, and Table 3.5 shows the RSD% and the percentage recovery for the controls included in the intra-day validation assays. The limit of detection for the assay is 0.001µg/ml.

Absorbance at 414nm

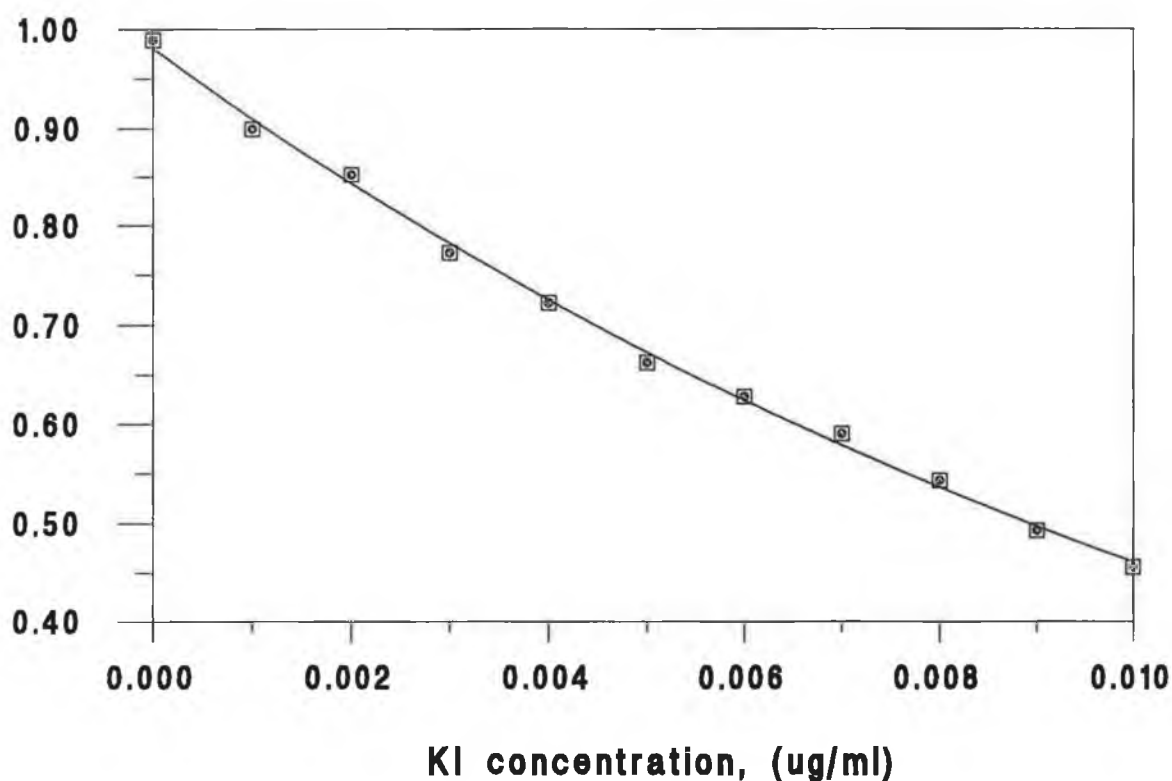


Figure 3.2

Exponential graph of absorbance at 414nm plotted against KI concentration (0.001-0.010 µg/ml) using ceric ammonium sulphate diluted in 1M HNO₃. Absorbance was read after a 20 minute incubation at 30°C. [r = 0.999]

Assay number	Correlation coefficient	
	Intra-day validation	Inter-day validation
1	0.993	0.986
2	0.995	0.996
3	0.999	0.996
4	0.993	0.994
5	0.996	0.995

Table 3.4

Correlation coefficients obtained for intra- and inter-day validation assays for the modified iodide microassay (KI concentration range: 0.001-0.010 μ g/ml). Absorbance at 414nm was plotted against KI concentration. The data was fitted exponentially.

(a)

Assay number	KI concentration, (µg/ml)	Percentage recovery
1	0.0025	100.0
2	0.0024	96.0
3	0.0026	104.0
4	0.0026	104.0
5	0.0024	96.0
Mean	0.0025	100.0
Standard deviation	0.0001	4.00
RSD%	4.00	

(b)

Assay number	KI concentration, (µg/ml)	Percentage recovery
1	0.0073	97.3
2	0.0073	97.3
3	0.0075	99.3
4	0.0075	99.9
5	0.0076	101.3
Mean	0.0074	99.0
Standard deviation	0.0001	1.73
RSD%	1.76	

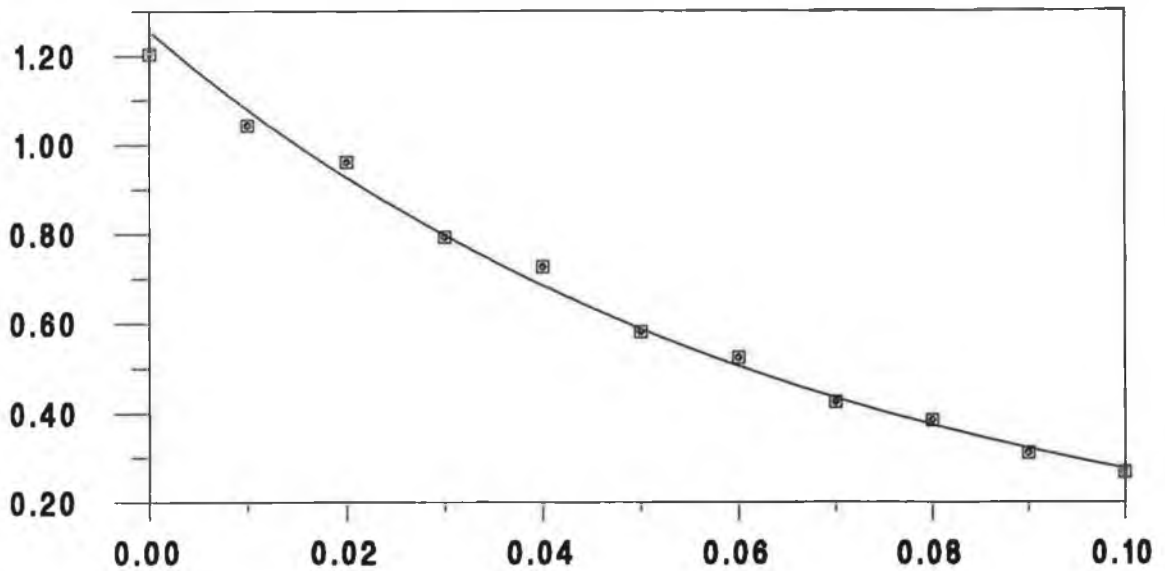
Table 3.5

The controls 0.0025µg/ml (a) and 0.00750µg/ml (b) potassium iodide were included when carrying out the 5 intra-day validation assays. The concentration determined for the control for each assay is shown, as is the percentage recovery. [RSD = relative standard deviation]

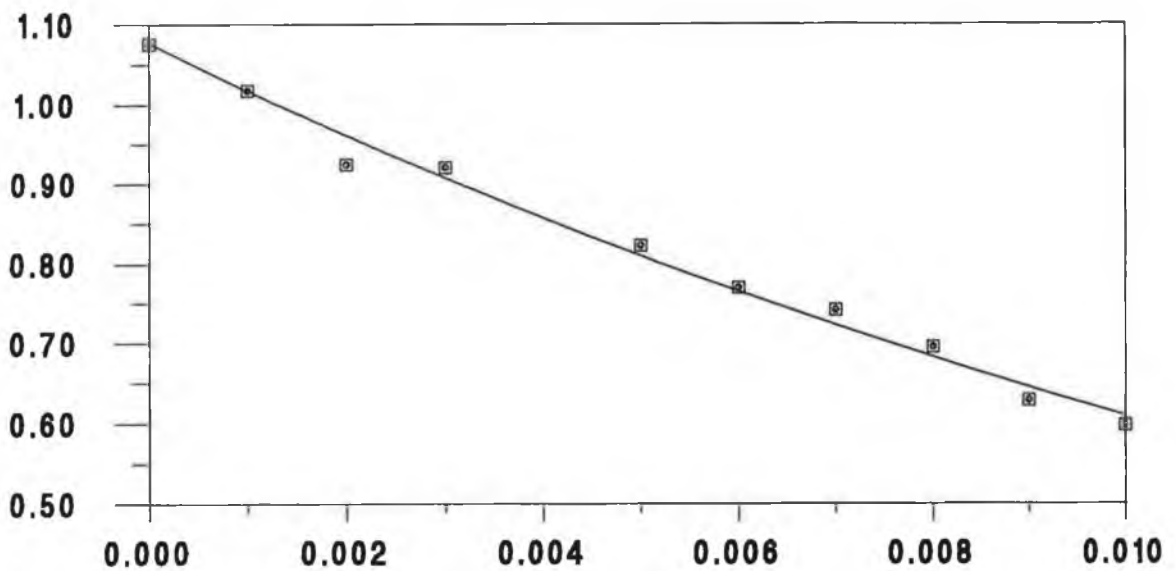
3.22 Reading absorbance at 405 and 380nm in the modified iodide microassay

The use of detecting absorbance at 405 and 380nm, instead of 414nm, in the modified iodide microassay was considered. The absorbance spectrum of ceric ammonium sulphate has an absorbance maximum at 320nm in sulphuric acid. It was therefore investigated whether reading at lower wavelengths could improve the sensitivity of the assay. It was necessary to use a lower concentration of ceric ammonium sulphate (0.014M) when reading at 380nm, as the zero control and low iodide standards gave too high absorbance values when 0.028M ceric ammonium sulphate was used. The concentration of arsenious acid was kept at 0.075M. Standard curves obtained at wavelengths of 405 and 380nm, are shown in Figures 3.3 and 3.4. No increase in sensitivity was obtained and it was decided to continue reading absorbance at 414nm as this is a filter routinely available with microplate readers.

Absorbance at 405nm



(a) KI concentration, (ug/ml)



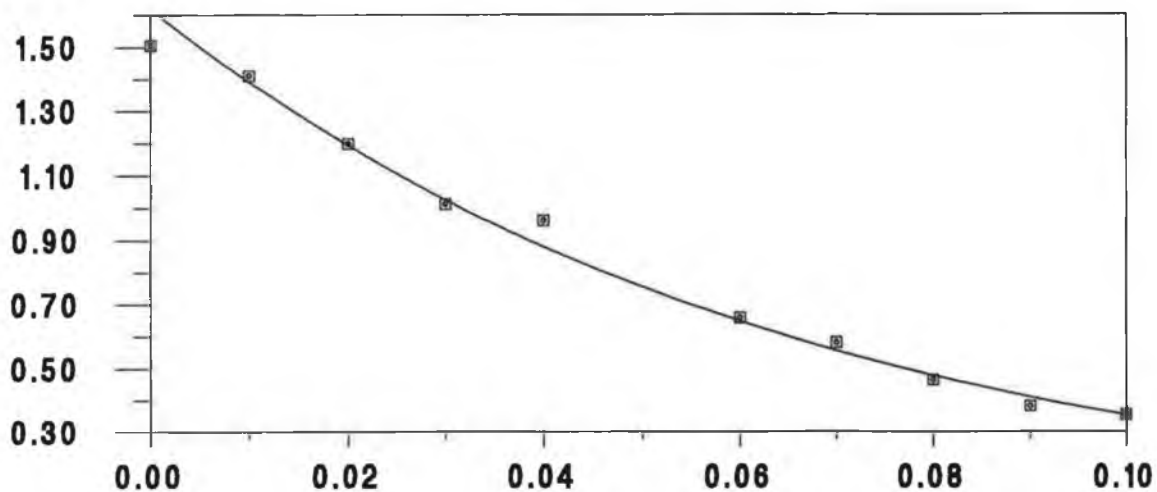
(b) KI concentration, (ug/ml)

Figure 3.3

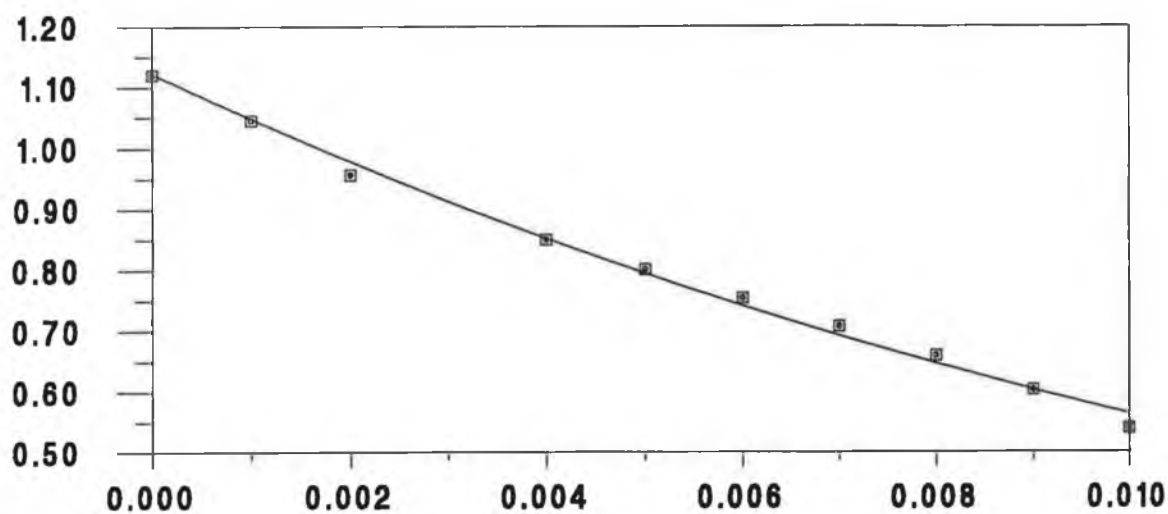
Exponential graphs of absorbance at 405nm plotted against KI concentration. 0.028M ceric ammonium sulphate was diluted from 0.1M stock solution with 1M HNO₃:

- 0.01-0.10 ug/ml potassium iodide: Absorbance was read after a 5 minute incubation at 30°C [$r = 0.997$]
- 0.001-0.010 ug/ml potassium iodide: Absorbance was read after a 20 minute incubation at 30°C [$r = 0.995$]

Absorbance at 380nm



(a) KI concentration, (ug/ml)



(b) KI concentration, (ug/ml)

Figure 3.4

Exponential graphs for absorbance at 380nm plotted against KI concentration. 0.014M ceric ammonium sulphate was diluted from 0.1M stock solution with 1M HNO₃:

- 0.01-0.10 ug/ml potassium iodide: Absorbance was read after a 5 minute incubation at 37°C [$r = 0.996$].
- 0.001-0.010 ug/ml potassium iodide: Absorbance was read after a 20 minute incubation at 30°C [$r = 0.996$].

3.23 Standard curve for iodinated Bolton-Hunter reagent using the modified iodide microassay

Figure 3.5 shows the standard curve obtained for the iodinated Bolton-Hunter reagent (IBHR) standards, 0.1-1.0 $\mu\text{g/ml}$, when the assay optimized by Keating (1991) was used, that is, diluting the ceric ammonium sulphate in 1M H_2SO_4 . The assay was incubated for 2 minutes at 30°C. A linear graph was obtained. For the range of standards 0.01-0.10 $\mu\text{g/ml}$ IBHR, when absorbance at 414nm was read, after an incubation at 30°C for 20 minutes, the assay was not linear, nor could it be fitted exponentially,

The modified assay, that is, diluting ceric ammonium sulphate with 1M HNO_3 and reading absorbance at 414nm after 5 minutes, was used to measure IBHR in the range 0.1-1.0 $\mu\text{g/ml}$. The standard curve obtained is shown in Figure 3.6.

To improve the sensitivity of the modified assay, such that IBHR standards in the range 0.01-0.10 $\mu\text{g/ml}$ would give a good correlation coefficient, it was necessary to increase the concentration of ceric ammonium sulphate to 0.05M. Figure 3.7 shows the set of standard curves obtained when incubation time is varied. It is still possible to obtain an exponential response for the concentration range 0.1-1.0 $\mu\text{g/ml}$ IBHR using 0.05M ceric ammonium sulphate (results not shown). This modified assay offers a ten-fold increase in sensitivity when measuring IBHR standards.

Absorbance at 414nm

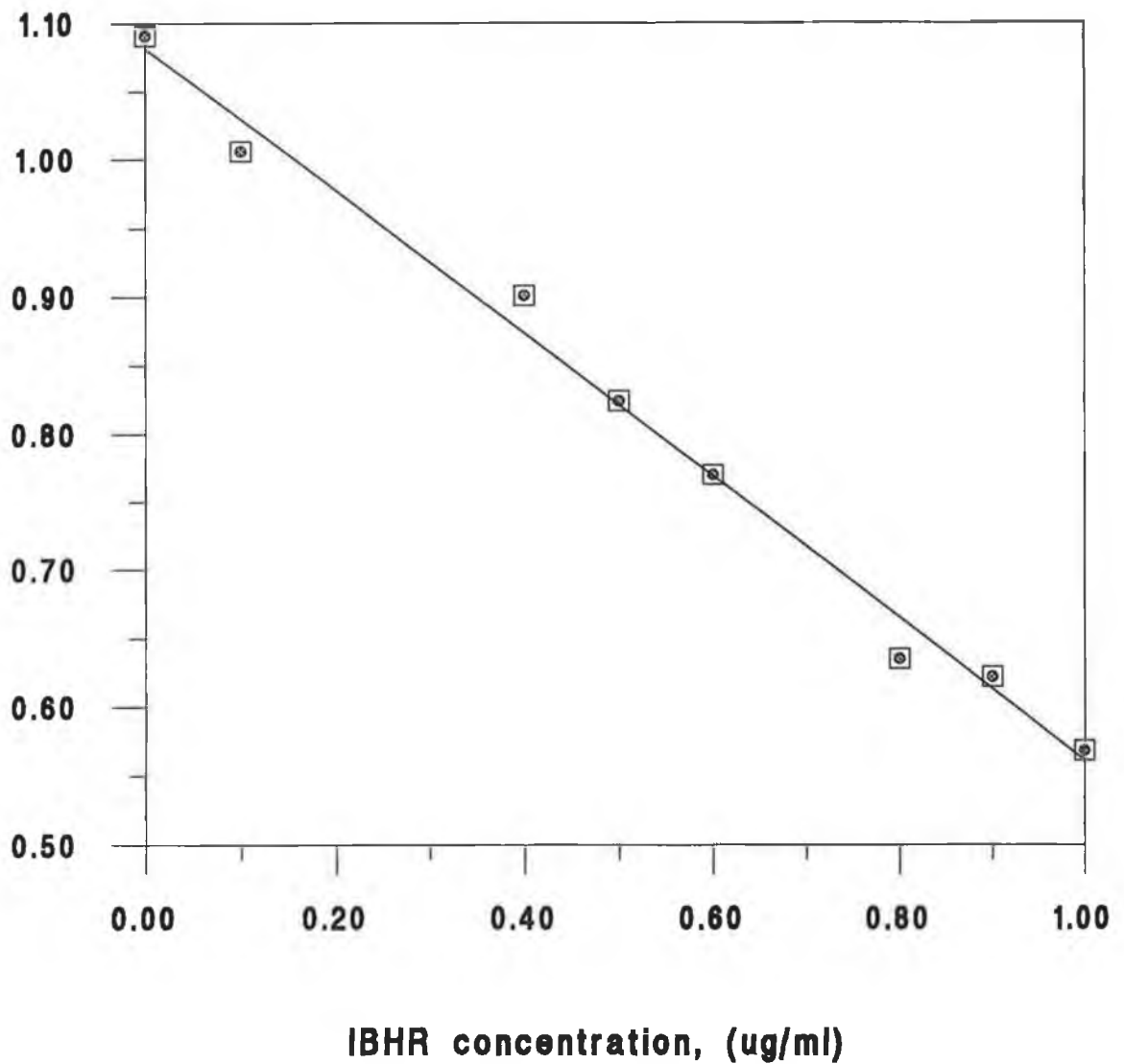


Figure 3.5

Linear graph of absorbance at 414nm plotted against IBHR concentration (0.1-1.0 μ g/ml). Ceric ammonium sulphate was diluted with 1M H_2SO_4 and absorbance was read after a 2 minute incubation at 30°C. [$r = 0.995$]

Absorbance at 414nm

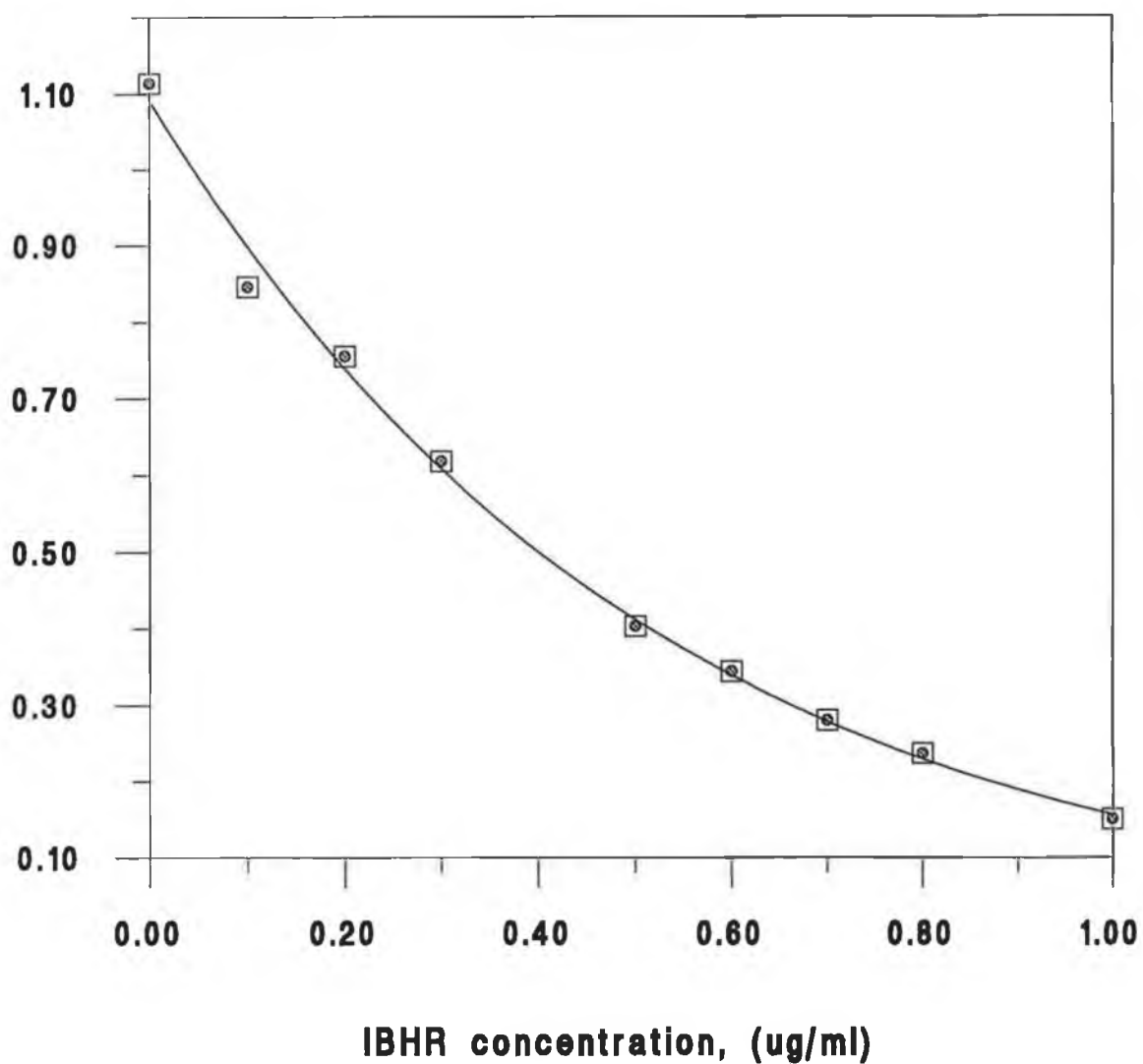


Figure 3.6

Exponential graph of absorbance at 414nm plotted against IBHR concentration (0.1-1.0 μ g/ml). Ceric ammonium sulphate was diluted with 1M HNO₃ and absorbance was read after a 5 minute incubation at 30°C. [r = 0.999]

Absorbance at 414nm

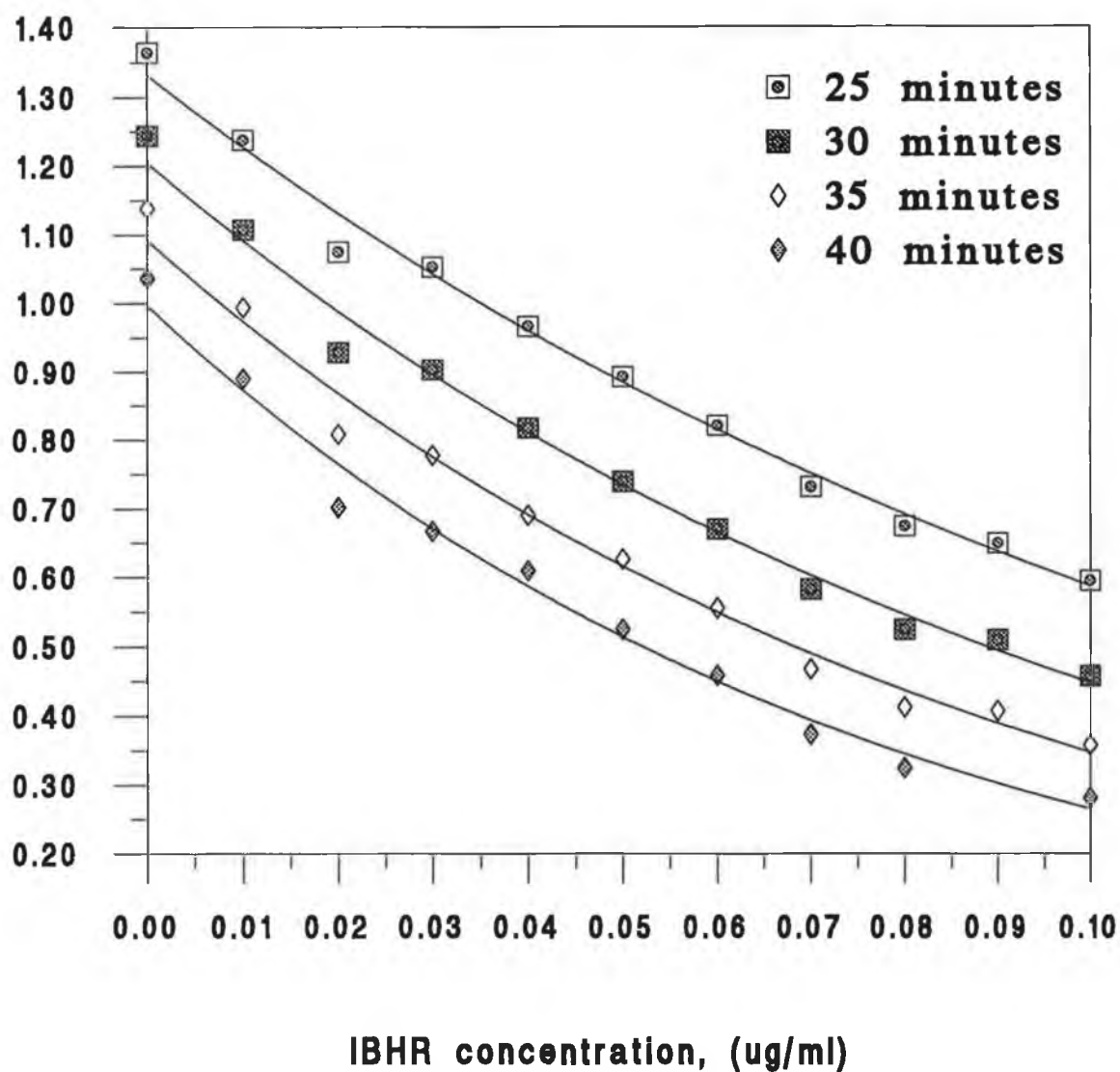


Figure 3.7

Exponential graphs of absorbance at 414nm plotted against IBHR concentration (0.01-0.10 μ g/ml) over a range of incubation times. Incubation temperature was 30°C. Ceric ammonium sulphate was diluted to 0.05M with 1M HNO₃. [25 minutes, $r = 0.996$: 30 minutes, $r = 0.996$: 35 minutes, $r = 0.995$: 40 minutes, $r = 0.993$]

3.24 Standard curves for 5-iodocytidine and related compounds using the modified iodide microassay

Using the modified iodide microassay a standard curve was obtained for absorbance at 414nm plotted against 10-100 µg/ml 5-iodocytidine. The reaction was incubated at 30°C for 20 minutes (see Figure 3.8). Standard curves were also plotted for 1-10 µg/ml 5-iodocytidine 5'-monophosphate (5 minute incubation) and the iodinated nucleotide, 5-iodo-2'-deoxycytidine 5'-triphosphate (10-100 µg/ml; 20 minute incubation). These graphs are shown in Figures 3.9 and 3.10, respectively.

To obtain a good correlation coefficient for the concentration range 1-10 µg/ml 5-iodocytidine it was necessary, as described in section 3.23 for the IBHR standards, to increase the concentration of ceric ammonium sulphate to 0.05M. Figure 3.11 shows the set of standard curves obtained for absorbance at 414nm plotted against 5-iodocytidine concentration, when incubation time is varied. Figure 3.12 shows that it is still possible to obtain an exponential response for the concentration range 10-100 µg/ml 5-iodocytidine using 0.05M ceric ammonium sulphate rather than 0.028M.

The K value, discussed in Section 1.12.2, for each of these compounds was calculated and these values are tabulated in Table 3.7 (see also Table 1.1).

COMPOUND	K VALUE (%)
5-iodocytidine	0.02
5-iodocytidine 5'-monophosphate	1.50
5-iododeoxycytidine 5'-triphosphate	0.04

Table 3.7

K values calculated for 5-iodocytidine and related compounds. These are relative to KI.

Absorbance at 414nm

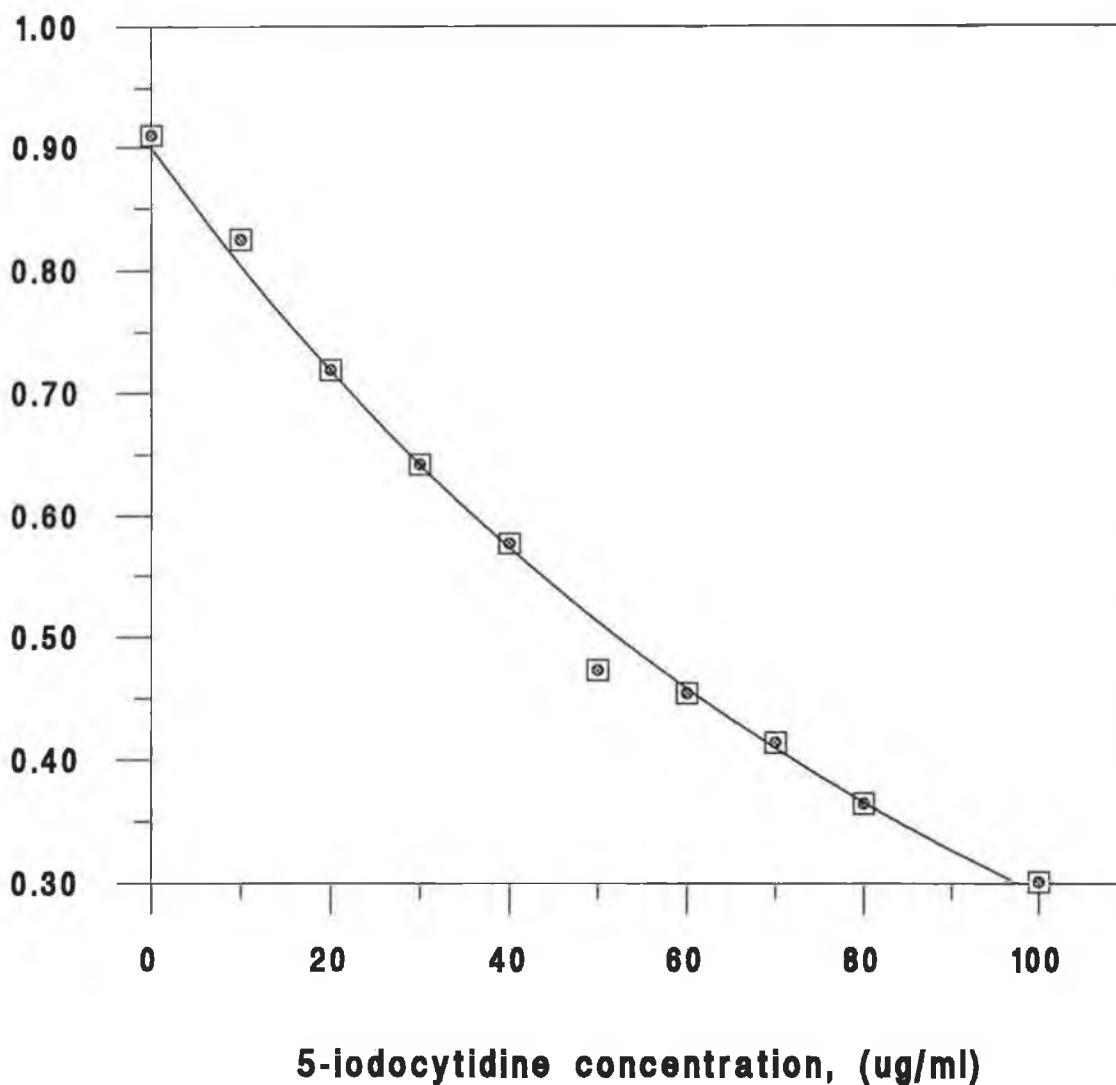


Figure 3.8

Exponential curve of absorbance at 414nm plotted against concentration of 5-iodocytidine (10-100 µg/ml). Ceric ammonium sulphate was diluted with 1M HNO₃. The reaction was incubated at 30°C for 20 minutes. [r = 0.996]

Absorbance at 414nm

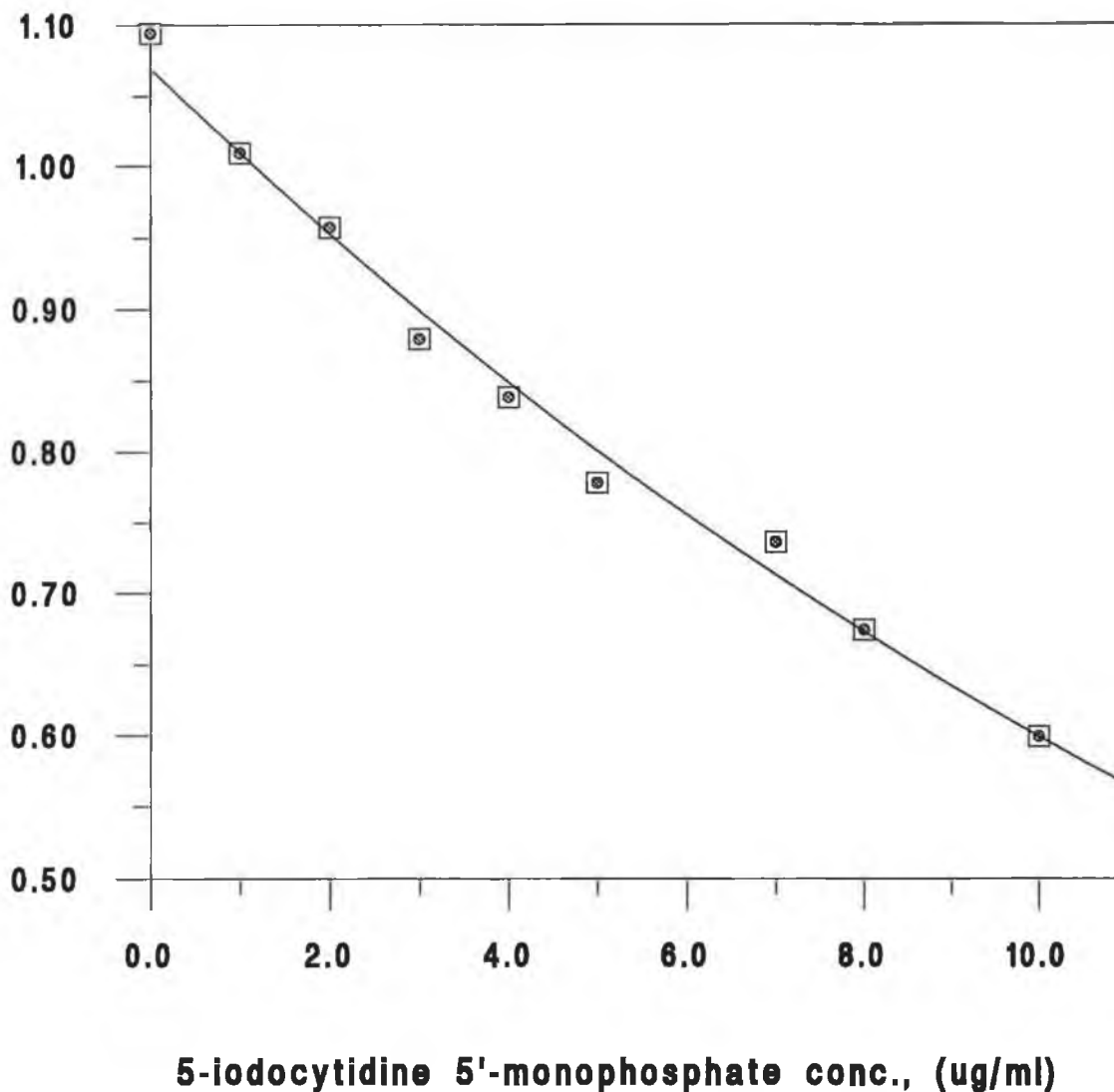


Figure 3.9

Exponential curve of absorbance at 414nm plotted against concentration of 5-iodocytidine 5'-monophosphate (1-10 $\mu\text{g/ml}$). Ceric ammonium sulphate was diluted with 1M HNO_3 . The reaction was incubated at 30°C for 5 minutes. [$r = 0.995$]

Absorbance at 414nm

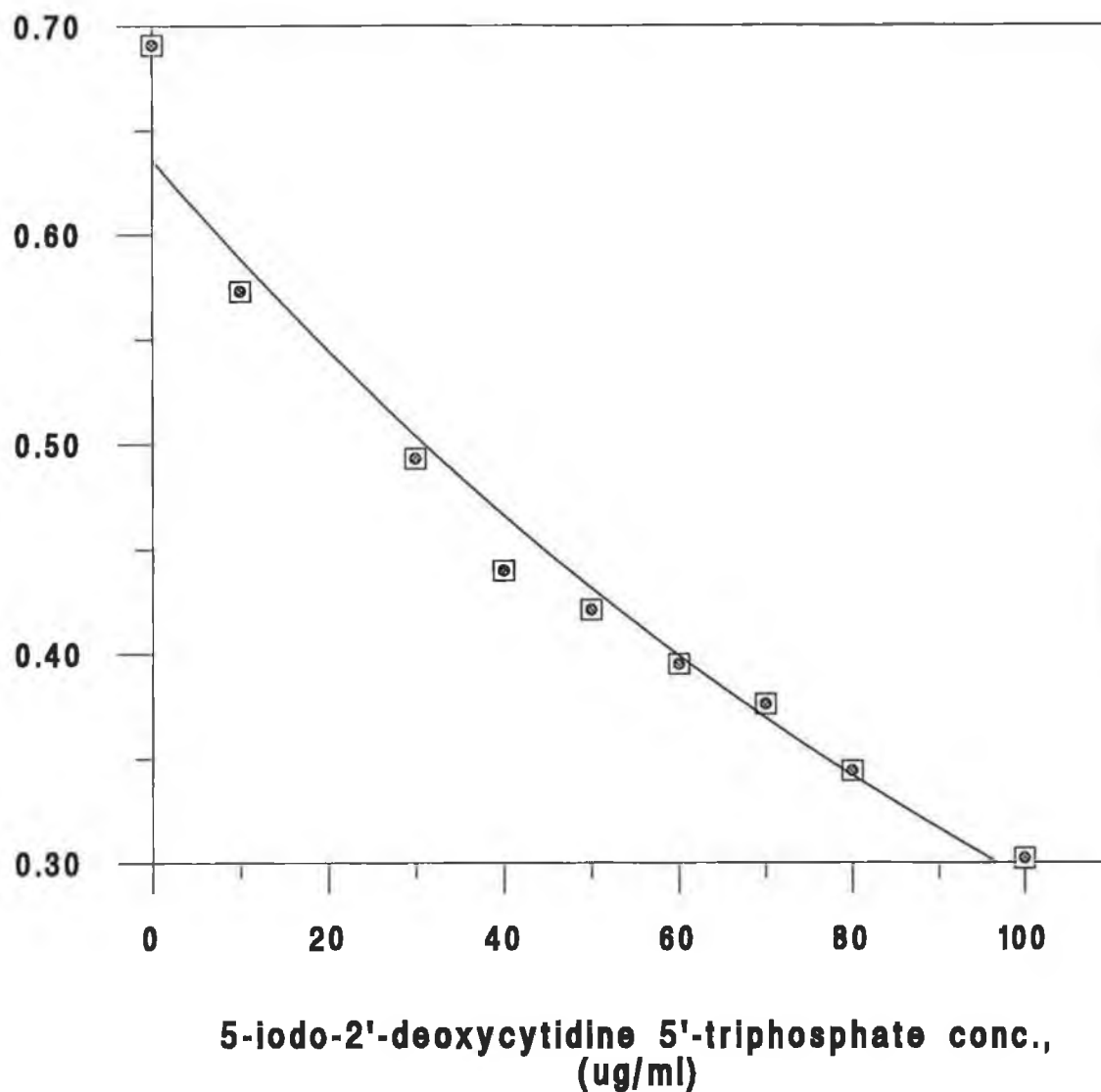


Figure 3.10

Exponential curve of absorbance at 414nm plotted against concentration of 5-iodo-2'-deoxycytidine 5'-triphosphate (10-100 $\mu\text{g/ml}$). Ceric ammonium sulphate was diluted with 1M HNO_3 . The reaction was incubated at 30°C for 20 minutes. [$r = 0.987$]

Absorbance at 414nm

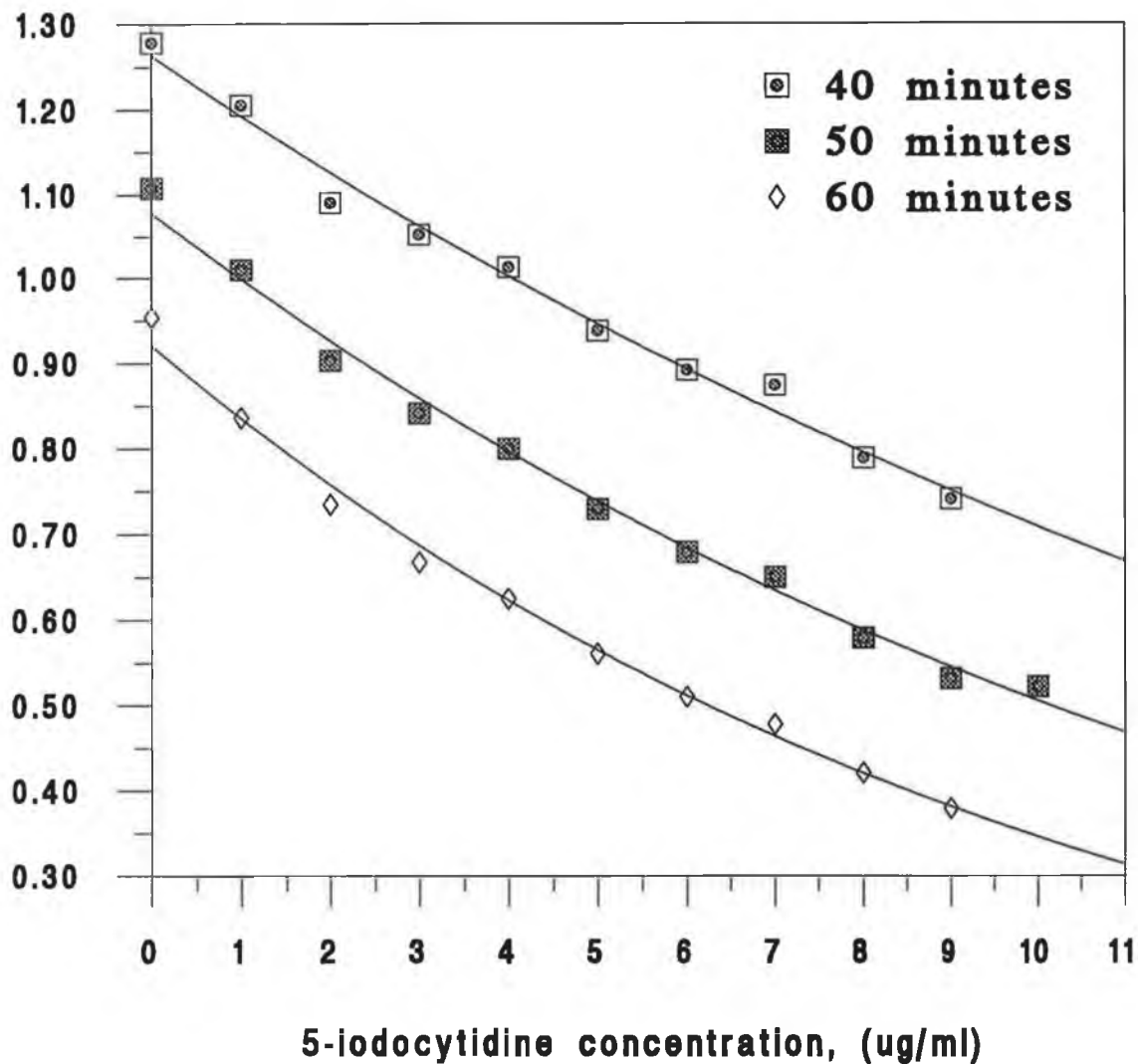


Figure 3.11

Exponential curves of absorbance at 414nm plotted against concentration of 5-iodocytidine (1-10 $\mu\text{g/ml}$) over a range of incubation times. Incubation temperature was 30°C . 0.1M ceric ammonium sulphate was diluted with 1M HNO_3 to a concentration of 0.05M. [40 minutes: $r = 0.995$, 50 minutes: $r = 0.996$, 60 minutes: $r = 0.997$]

Absorbance at 414nm

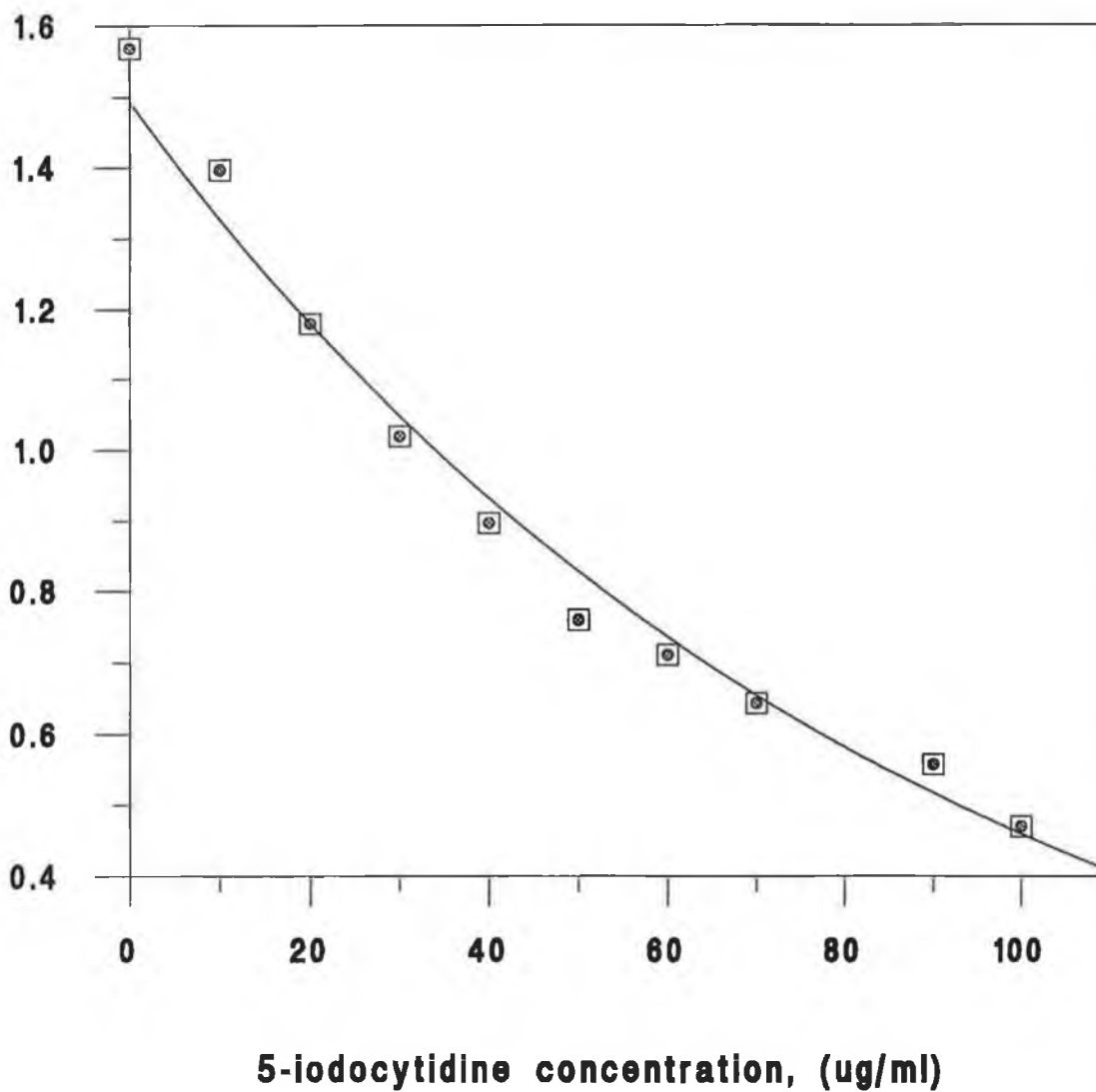


Figure 3.12

Exponential curve of absorbance at 414nm plotted against concentration of 5-iodocytidine (10-100ug/ml). 0.1M ceric ammonium sulphate was diluted with 1M HNO₃ to a concentration of 0.05M. The reaction was incubated at 30°C for 20 minutes. [r = 0.992]

3.25 Interference in the iodide microassay

The study being undertaken involved the use of the iodide microassay to read DNA samples labelled with cold-iodine. DNA is normally stored in tris-EDTA (TE) buffer [10mM Tris/ 1mM EDTA, pH 8.0]. EDTA is included to chelate magnesium which is required by DNases. However, it was found that this buffer was not suitable for samples which are going to be read in this iodide microassay as EDTA interferes in the Sandell-Kolthoff reaction. The addition of EDTA serves only a secondary precautionary function, as all reagents, plasticware and glassware are autoclaved. Thus, in the following study DNA was stored in 10mM Tris, pH 8.0. Figure 3.13 illustrates the effect of TE in the modified iodide microassay. A linear response is obtained for absorbance plotted against volume of TE in 100µl ultrapure water. Work by Shah and Aust (1993) has shown that iodide can function as a free radical mediator for the oxidation of EDTA to EDTA radicals (a reductant). This may be the reason EDTA affects the Sandell-Kolthoff reaction.

Table 3.7 lists other interferants in the Sandell-Kolthoff reaction which have been reported (Zak, 1978).

INTERFERING ION	EFFECT
Cyanide	Iodocyanide is formed which does not catalyze the reaction.
Mercury	Insoluble mercuric iodide is formed which inhibits the catalysis completely.
Bromide	Enhances the rate of the reaction but at a lower rate.
Chloride	Enhances the rate of the reaction but at a lower rate than bromide.
Osmium	Catalyzes the reaction in the same way as iodide.

Table 3.7

Ions which interfere in the Sandell-Kolthoff reaction and their effect. [Zak, 1978]

Gutiérrez *et al.*, (1989) reported interferences (see Table 3.8) in the determination of iodide in pharmaceutical and food samples using a stopped-flow method based on the Sandell-Kolthoff reaction.

Tolerance ratio Interferent species:iodide	species assayed
100:1	B ₄ O ₇ ²⁻ , Cl ⁻ , ClO ₄ ⁻ , CN ⁻ , F ⁻ , CO ₃ ⁻ , NO ₃ ⁻ , SO ₄ ⁻ , PO ₄ ⁻ , Ca ²⁺ , acetate, glycine.
50:1	SCN ⁻ , citrate.

Table 3.8
Interferent species in the Sandell-Kolthoff reaction as reported by Gutiérrez et al. (1989).

Absorbance at 414nm

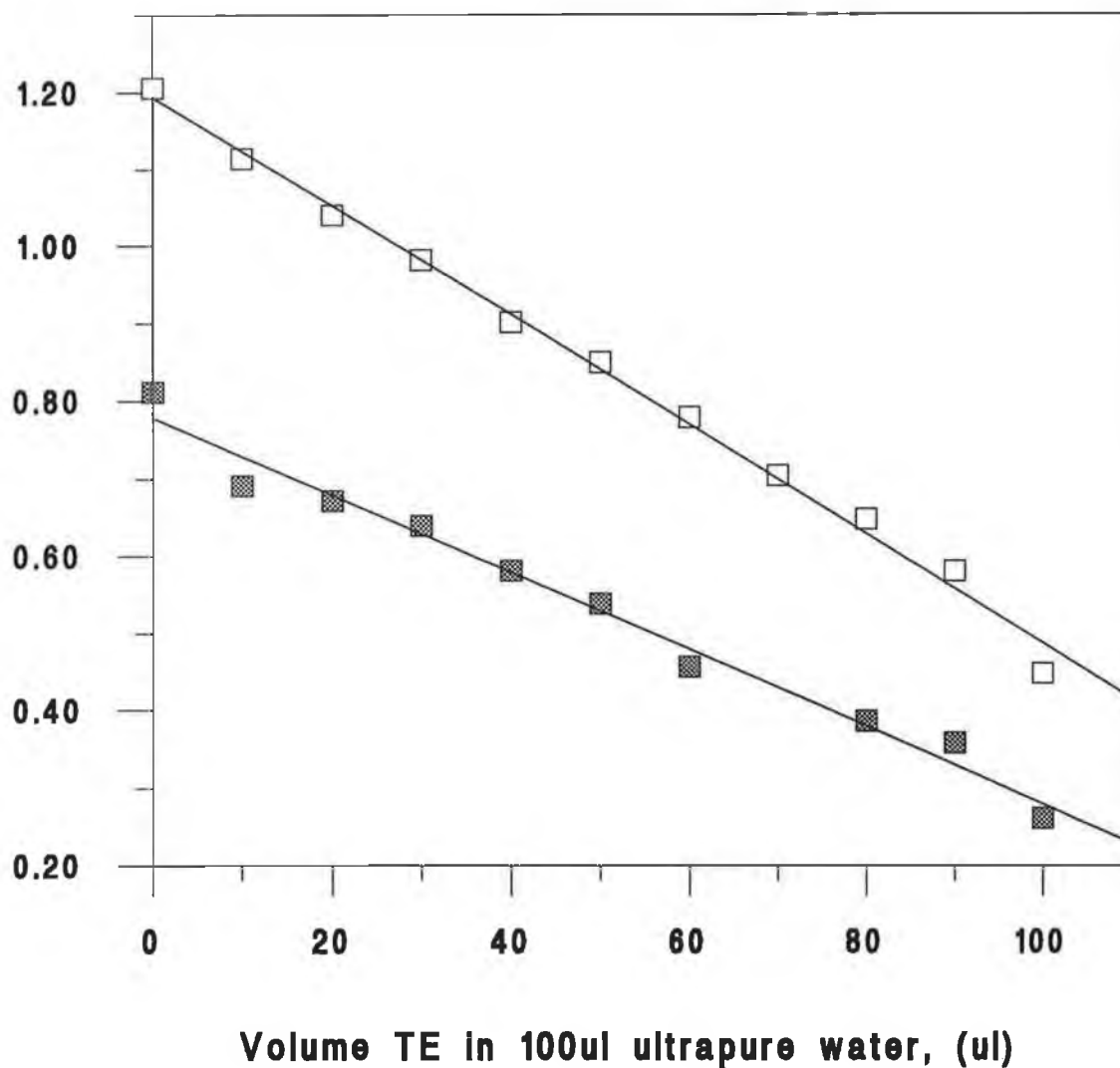


Figure 3.13

Linear graphs obtained for absorbance at 414nm plotted against volume Tris-EDTA buffer in the modified iodide microassay, after incubation for 5 minutes [(□) $r = 0.997$] and 20 minutes [(■) $r = 0.991$] at 30°C.

3.3 DISCUSSION

The modified iodide microassay described in Section 2.21 has been validated for the KI concentration range 1-10ng/ml. This is a ten-fold increase in the sensitivity of the assay. It was investigated whether reading absorbance at 405 and 380nm would further increase the sensitivity of the assay, as ceric ammonium sulphate has an absorbance maximum at 320nm in sulphuric acid. No increase in sensitivity was obtained.

A ten-fold increase in sensitivity was also obtained for IBHR standards. The concentration range 0.01-0.10µg/ml IBHR could be detected using the modified microassay. However, it was necessary to increase the concentration of the ceric ammonium sulphate solution.

The K values for 5-iodocytidine, 5-iodocytidine 5'-monophosphate and 5-iodo-2'-deoxycytidine 5'-triphosphate were determined. These values were found to be very low compared to IBHR. It was also noted that the K value for 5-iodocytidine 5'-monophosphate was one hundred-fold higher than for 5-iodocytidine. This is possibly due to the steric effects of the phosphate group.

It is unclear how the iodide in iodo-compounds catalyzes the Sandell-Kolthoff reaction, i.e. is it necessary that the iodide is removed from the organic compound so that the iodide is available for catalysis or can the iodide catalyze the reaction as part of the organic compound? Work carried out by Barker (1964) indicated the former to be the case. Work by Butler (personal communication, 1993) would support this contention.

Pantel's work, in 1982, showed that 2-iodophenol had a K value of 9.0% while 4-iodophenol had a K value of 42.0%. However, the phenolic group is ortho/para directing and in IBHR the iodine is ortho to an hydroxyl group in a benzene ring and has a K value of 33.0%. Thus, for iodo-containing organic compounds to catalyze the Sandell-Kolthoff reaction an electron donor group must be present on the ring (Pantel, 1982).

However, pyrimidine is a heterocyclic compound, and the chemistry of such compounds is quite different to that of aromatic compounds. Research, such as that reported by Barker (1964) and Pantel (1982) to investigate the behaviour of iodinated aromatic compounds in the Sandell-Kolthoff reaction, would also need to be carried out for iodinated pyrimidines, such as 5-iodocytidine. In any case the low K values found would suggest that the feasibility of using iodinated nucleotides as direct labels for DNA would be limited by the sensitivity of the reaction available for iodine measurement.

Chapter 4

DEVELOPMENT OF LUMINESCENT ASSAYS TO DETECT IODIDE

4.1 INTRODUCTION

The aim of the work carried out was to develop a sensitive chemiluminescent microassay for iodide. The chemiluminescent systems investigated were as follows:

- a) Acetone/ hydrogen peroxide/ hypochlorite/ iodide (Lu *et al.*, 1989)
- b) Hydrogen peroxide/ hypochlorite/ iodide (Modification of the method described by Burguera and Burguera, 1982)
- c) Luminol/ hydrogen peroxide/ iodide (Bause *et al.*, 1979)
- d) Luminol/ hypochlorite/ iodide
- e) Luminol/ hydrogen peroxide/ tetrachloroauric acid/ iodide (Lin *et al.*, 1989)
- f) Luminol/ hydrogen peroxide/ 2-4-dinitro-1-chlorobenzene/ iodide

The chemiluminescence in systems a-d is of a transient nature, that is, there is a flash of light when the reactants come together, whereas with systems e and f the luminescence can be measured over a period of time, as the light intensity drops off slowly.

Lu *et al.*, (1989) described the chemiluminescent system acetone/ hydrogen peroxide/ hypochlorite/ iodide. The reagents were injected into a cell, and the luminescence was read for 8 seconds. The system was catalyzed by the iodide ion, and the calibration curve was linear from 0.4ng/ml - 0.3µg/ml iodide. This system (System a) was considered both in test-tube and microplate formats, and the results are reported below. Iodide was found to quench the chemiluminescence.

Burguera and Burguera (1982) used a chemiluminescent system similar to the system described by Lu *et al.*, (1989). They determined the concentration of iodine, rather than iodide, by measuring the chemiluminescence emission peak generated when an iodine solution was injected into a premixed hydrogen peroxide/ sodium hypochlorite flow system. The limit of quantification for iodine was 0.5 ng/ml. The effect of iodide, rather than iodine, in a hydrogen peroxide/ hypochlorite system was investigated here in microplate format (System b).

The other luminescent systems investigated all involved the luminescent substrate luminol. Luminol, when oxidised by most strong oxidants [e.g., H₂O₂, ClO⁻, Ce (IV) and I₂] in alkaline conditions gives rise to a characteristic blue chemiluminescence (Roswell and White, 1978). This reaction has been used to determine oxidants, but it has also been used to determine compounds which interact with the oxidant. The reaction is catalysed by a number of metal ions, which include iron (II), copper (II) and cobalt (II). Determination of metal ions by their catalysis of the luminol reaction has been reported (Townshend, 1990). Many different enhanced luminol reactions have been described. For example, Thorpe *et al.*, (1985) investigated the effect of phenols on the luminol reaction when it is catalyzed by horseradish peroxidase.

A quantitative study of chemiluminescence from the iodine-luminol reaction was described by Seitz and Hercules, (1974). If the reaction pH is properly adjusted, chemiluminescence is linearly proportional to the iodine concentration from 5×10^{-10} - 3×10^{-7} M. However, halides and halites do not cause luminol chemiluminescence. Thus, to analyze iodide directly using luminol chemiluminescence it is necessary to turn it into a form active in the chemiluminescent reaction, that is, to oxidise iodide to iodine, with subsequent separation from the excess oxidant. Such methods are described in the Introduction (Section 1.13.2). The work described in this thesis investigated the indirect effect of iodide, that is, whether or not iodide enhanced or suppressed a number of luminol luminescent systems.

Bause *et al.*, (1979) investigated the enhancement of luminol chemiluminescence with halide ions. Chromium (III) catalyzes the oxidation of luminol by H_2O_2 , at a basic pH in aqueous solution. Intensity of the light emission is proportional to the free chromium (III) concentration, if the luminol and H_2O_2 are present in excess. They also reported that a chemiluminescent reaction occurred in the absence of Cr (III), when iodide or thiocyanate were present. Zhang (1988) used this luminol/ hydrogen peroxide/ iodide system to determine trace amounts of molybdenum (VI). This system was investigated in microplate format and the results are described below (System c).

Seitz *et al.*, (1972) used a luminol/ hypochlorite system to determine trace amounts of Cr (III). This is similar to the system described by Bause *et al.*, (1979), except that a different oxidising agent is used. Thus, a luminol/ hypochlorite/ iodide system was investigated (System d).

Finally, the chemiluminescent system described by Lin *et al.*, (1989), where iodide was determined by its suppression of the chemiluminescent system luminol/ hydrogen peroxide/ tetrachloroauric acid was considered. The detection limit reported was 50pg/ml. A number of formats for this assay were considered (System e). This system was also investigated using 2-4-dinitro-1-chlorobenzene (DNCB) rather than the metal salt, because Yurow and Sass (1977) described DNCB as an enhancer of the luminol/ hydrogen peroxide system (System f).

4.2 RESULTS

4.21 Iodide detection using the chemiluminescent system acetone/ hydrogen peroxide/ hypochlorite

The luminescent system acetone/ hydrogen peroxide/ hypochlorite (System a) was first investigated with a Berthold Chemilumat LB9502. Using similar conditions to those described by Lu *et al.*, (1989) the linear concentration range was found to be 50-1000 μ g/ml potassium iodide. Quenching of the luminescence was observed rather than catalysis. Relative light units (RLU) are a measure of luminescence and Figure 4.1 illustrates the linear graph obtained when $\log [RLU_{\text{BLANK}} - RLU_{\text{STANDARD}}]$ was plotted against $\log [\text{KI concentration}]$.

This system was also adapted for microplate format and RLU were read on the Labsystems Luminoskan 5300017. Conditions for the assay are described in Section 2.22. A linear graph was obtained for the concentration range 0.1-100 μ g/ml potassium iodide (see Figure 4.2). Quenching of the chemiluminescence was observed.

Log [RLU (blank) - RLU (standard)]

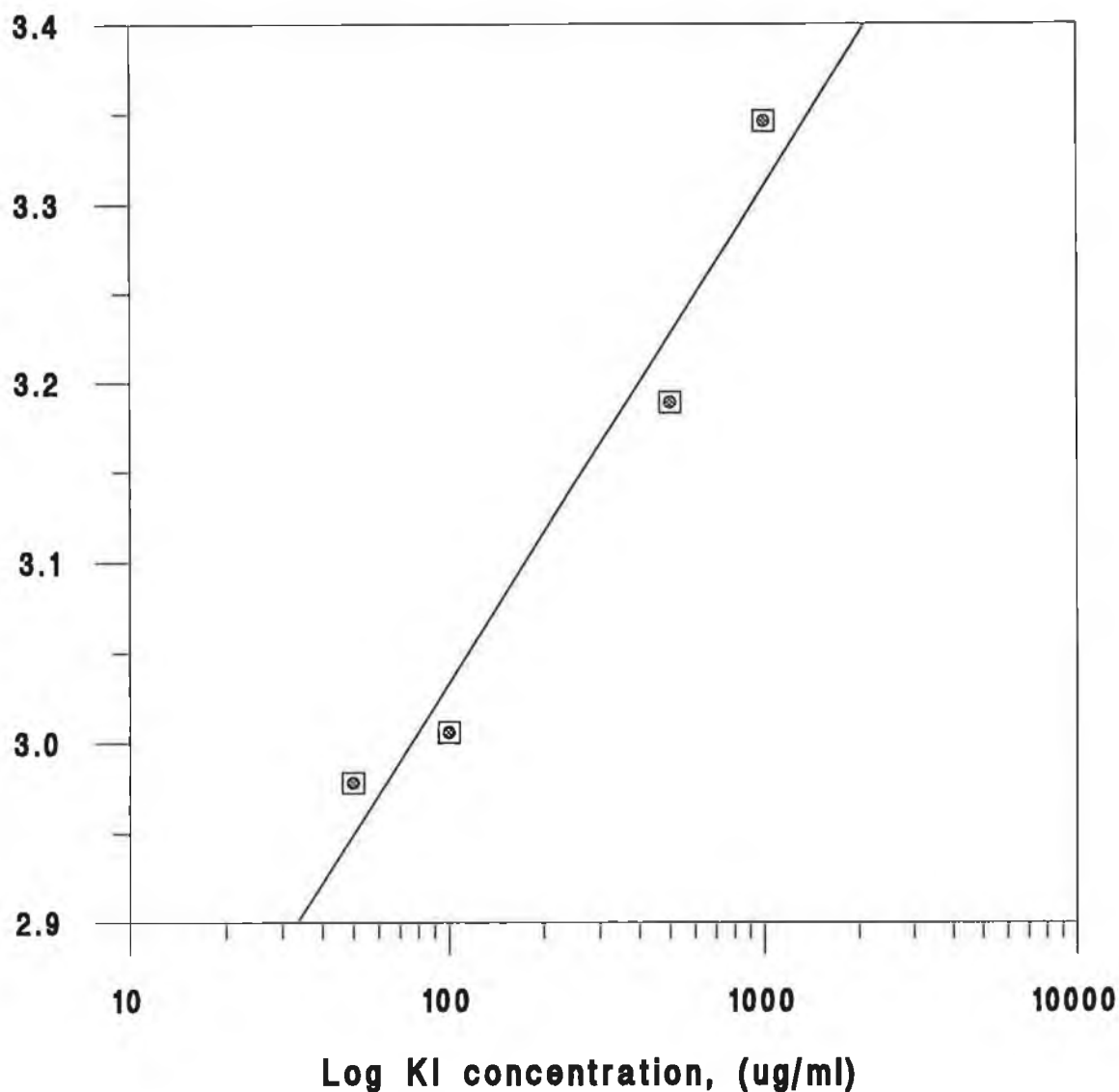


Figure 4.1

The relationship of luminescence ($\log [RLU_{BLANK} - RLU_{STANDARD}]$) and KI concentration ($\log [KI \text{ concentration}]$), obtained for the chemiluminescent system acetone/ hydrogen peroxide/ hypochlorite, is shown. Luminescence was measured on the Berthold Chemilumat LB9502. This is a straight line graph ($r = 0.98$).

Log [RLU (blank) - RLU (standard)]

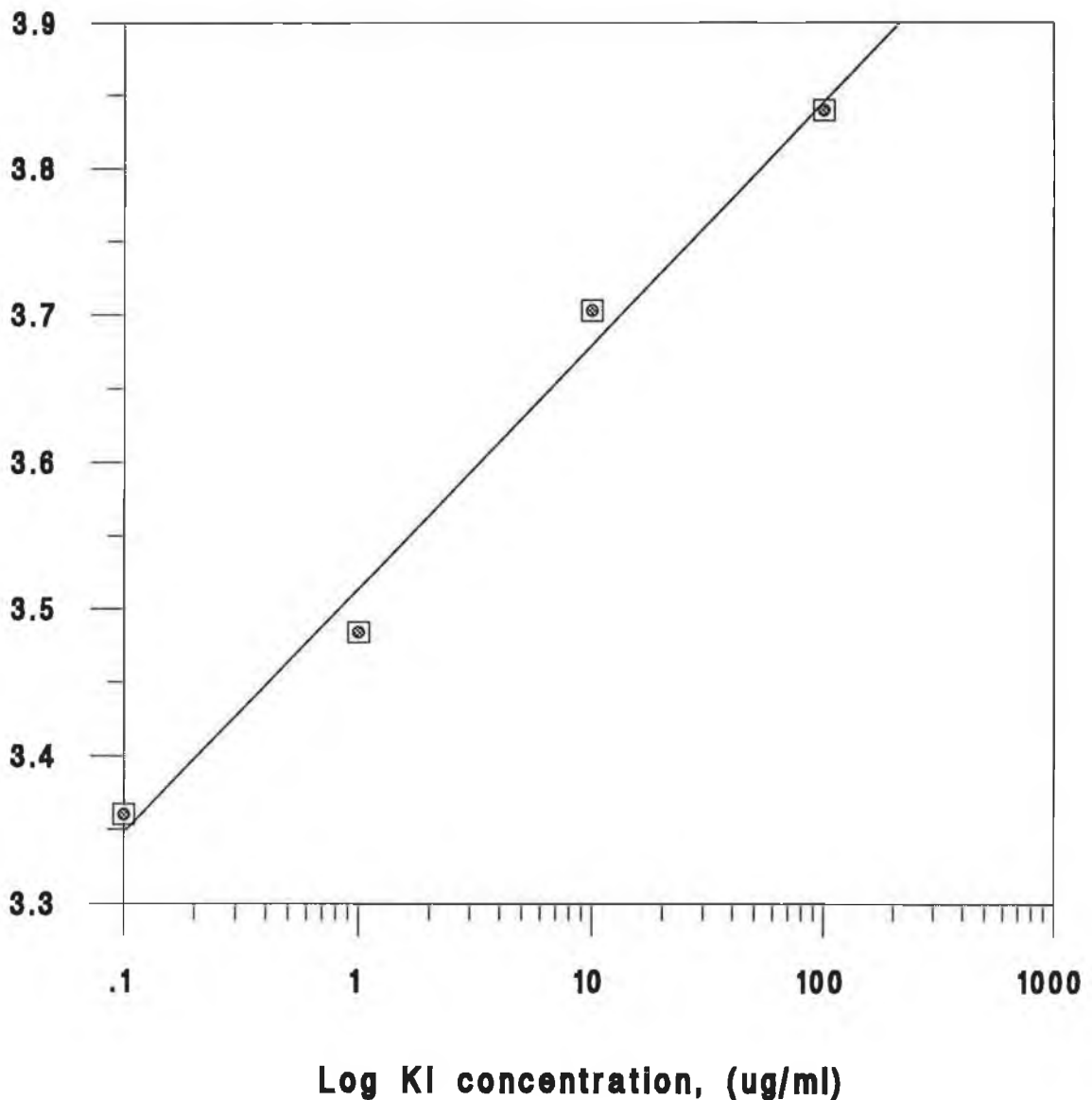


Figure 4.2

The relationship of luminescence ($\log [RLU_{BLANK} - RLU_{STANDARD}]$) and KI concentration ($\log [KI \text{ concentration}]$), obtained for the chemiluminescent system acetone/ hydrogen peroxide/ hypochlorite, is shown. Luminescence was measured on the Labsystems Luminoskan 5300017. This is a straight line graph ($r = 0.99$).

4.22 Iodide detection using the chemiluminescent system hydrogen peroxide/ hypochlorite

This system, described by Burguera and Burguera (1982) to measure iodine in a flow-system, was adapted for microplate format, and the effect of iodide investigated (System b). Conditions for the assay were described in Section 2.22. A linear graph was obtained for the concentration range 1-100 μ g/ml potassium iodide.

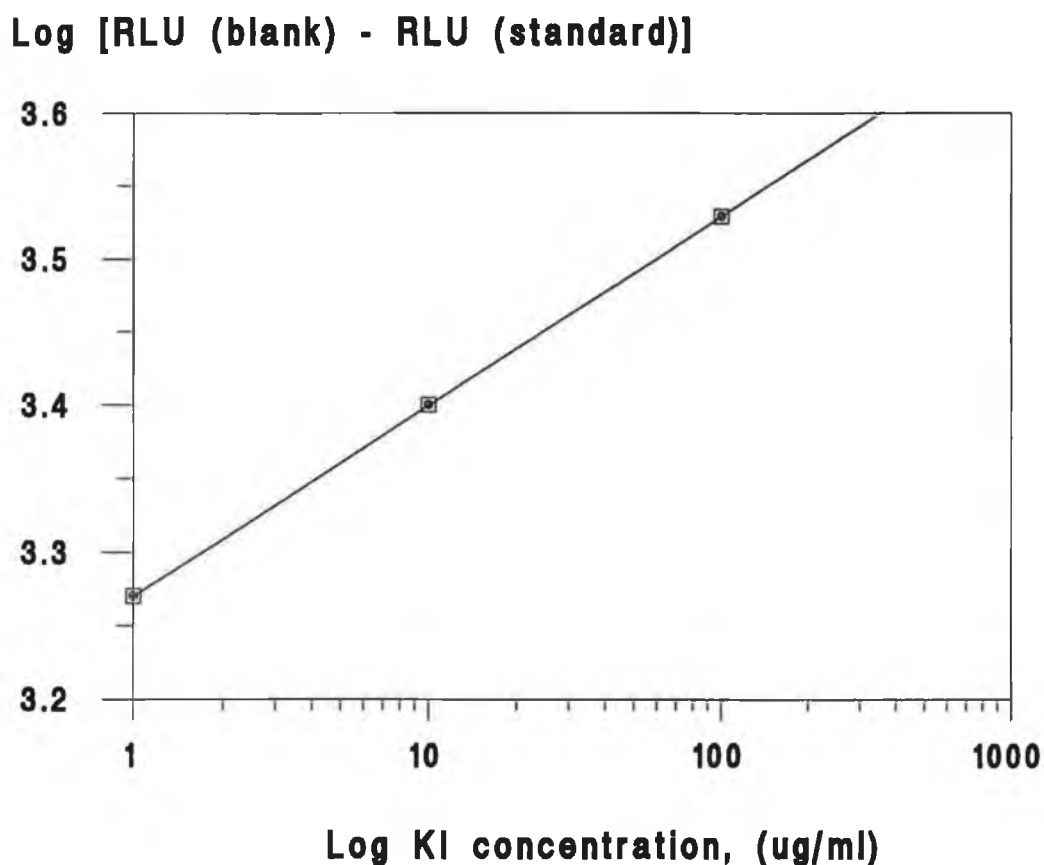


Figure 4.3

The relationship of luminescence ($\log [RLU_{BLANK} - RLU_{STANDARD}]$) and concentration of KI ($\log [KI \text{ concentration}]$) obtained for the chemiluminescent system hydrogen peroxide/ hypochlorite is shown. RLU were measured on the Labsystems Luminoskan 5300017. This is a straight line graph ($r = 1.00$).

4.23 Iodide detection using the chemiluminescent system luminol/ hydrogen peroxide

The luminescent system luminol/ hydrogen peroxide/ iodide was investigated in microplate format on the Labsystems Luminoskan 5300017 (System c). Conditions were described in Section 2.22. Table 4.1 illustrates the luminescence observed in the presence of 100µg/ml potassium iodide. No luminescence was observed at 50µg/ml and 10µg/ml potassium iodide.

	Zero Control (RLU)	100µg/ml potassium iodide		
		mean, n=4 (RLU)	standard deviation	RSD%
A	5.0	835.0	9.8	1.2
B	7.1	686.6	13.4	2.0
C	8.0	671.9	12.4	1.0

Table 4.1
Relative light units (RLU) measured for the luminescent system luminol/ hydrogen peroxide in the presence and absence of potassium iodide. A, B and C are three separate determinations which were performed. Luminescence was read on the Labsystems Luminoskan 5300017.

4.24 Iodide detection using the chemiluminescent system luminol/hypochlorite

The luminescent system luminol/ hypochlorite/ iodide was adapted for microplate format, and read on the Labsystems Luminoskan 5300017 (System d). Conditions for the assay were described in Section 2.22. A linear graph (see Figure 4.4) was obtained for the concentration range 10ng/ml-100µg/ml potassium iodide.

[RLU (blank) - RLU (standard)]

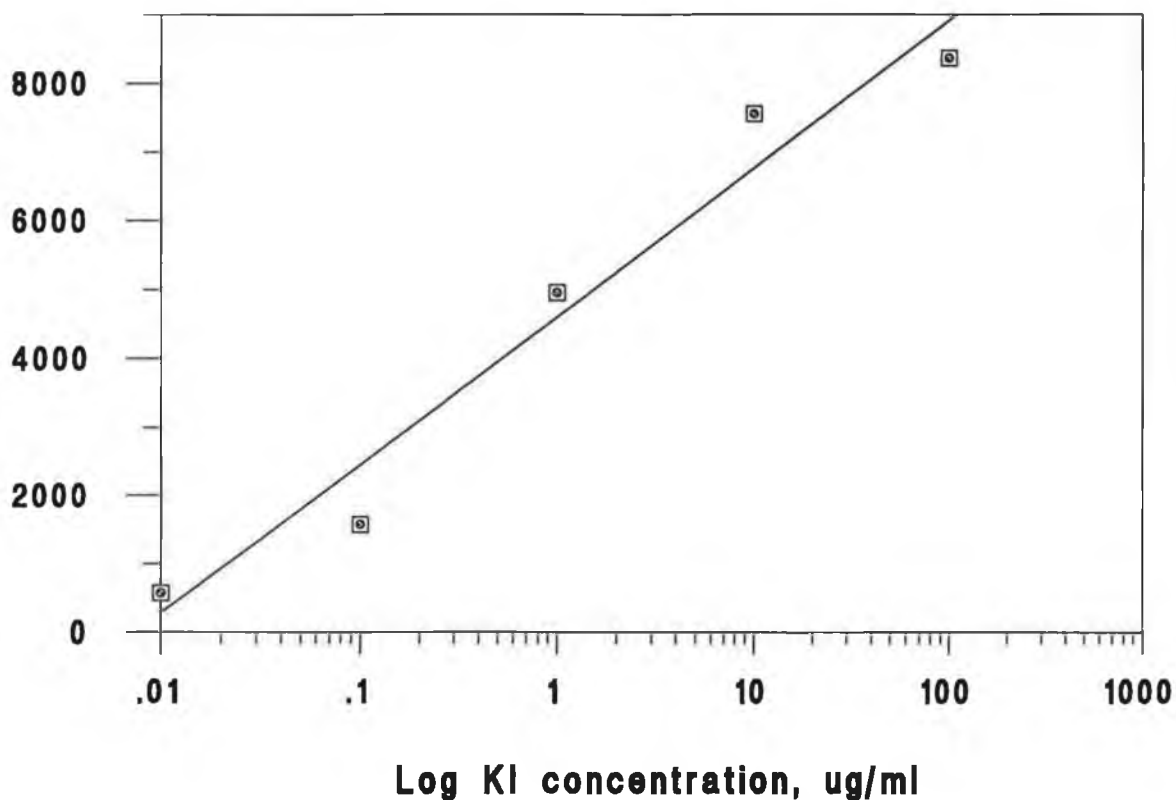


Figure 4.4

The relationship of luminescence ($RLU_{BLANK} - RLU_{STANDARD}$) and concentration of KI ($\log [KI \text{ concentration}]$) obtained for the chemiluminescent system luminol/ hypochlorite is shown. RLU were read on the Labsystems Luminoskan 5300017. This is a straight line graph ($r = 0.98$).

4.25 Iodide detection using the chemiluminescent system luminol/ hydrogen peroxide/ tetrachloroauric acid

Iodide was determined by its suppression of the chemiluminescence in the system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ (System e). Flow injection analysis and a microplate format were considered.

Using the flow injection system described in Section 2.22, the concentration range 10pg/ml-10 μ g/ml potassium iodide was investigated. Table 4.2 illustrates that iodide had no effect on the luminescence observed. High relative standard deviations (RSD%) were obtained for the determinations (between 11-25%). The high value obtained for relative light units (RLU) for some samples may be due to residue in the flow system, from the previous run. Figure 4.5 illustrates a sample plot of photon counts against time which shows the peaks in photon counts which were obtained when luminol was injected into the flow system.

KI concentration	Mean RLU n=6	Standard deviation	RSD%
zero	7797	1212	16
10pg/ml	8772	1512	17
100pg/ml	5951	1517	25
1ng/ml	5960	801	13
10ng/ml	6336	666	11
100ng/ml	5703	773	14
1 μ g/ml	8116	1737	21
10 μ g/ml	9582	1729	18

Table 4.2
Effect of potassium iodide in the flow-injection luminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ (Section 2.21). [RSD% = relative standard deviation]

Stopped flow analysis was also carried out on the chemiluminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ / iodide. Figure 4.6 shows the plot of photon counts against time for both the zero control and the standard $100\mu\text{g/ml}$ potassium iodide. Photon counts were read for 250 milliseconds, every second. No difference between the two plots was observed.

Photon counts

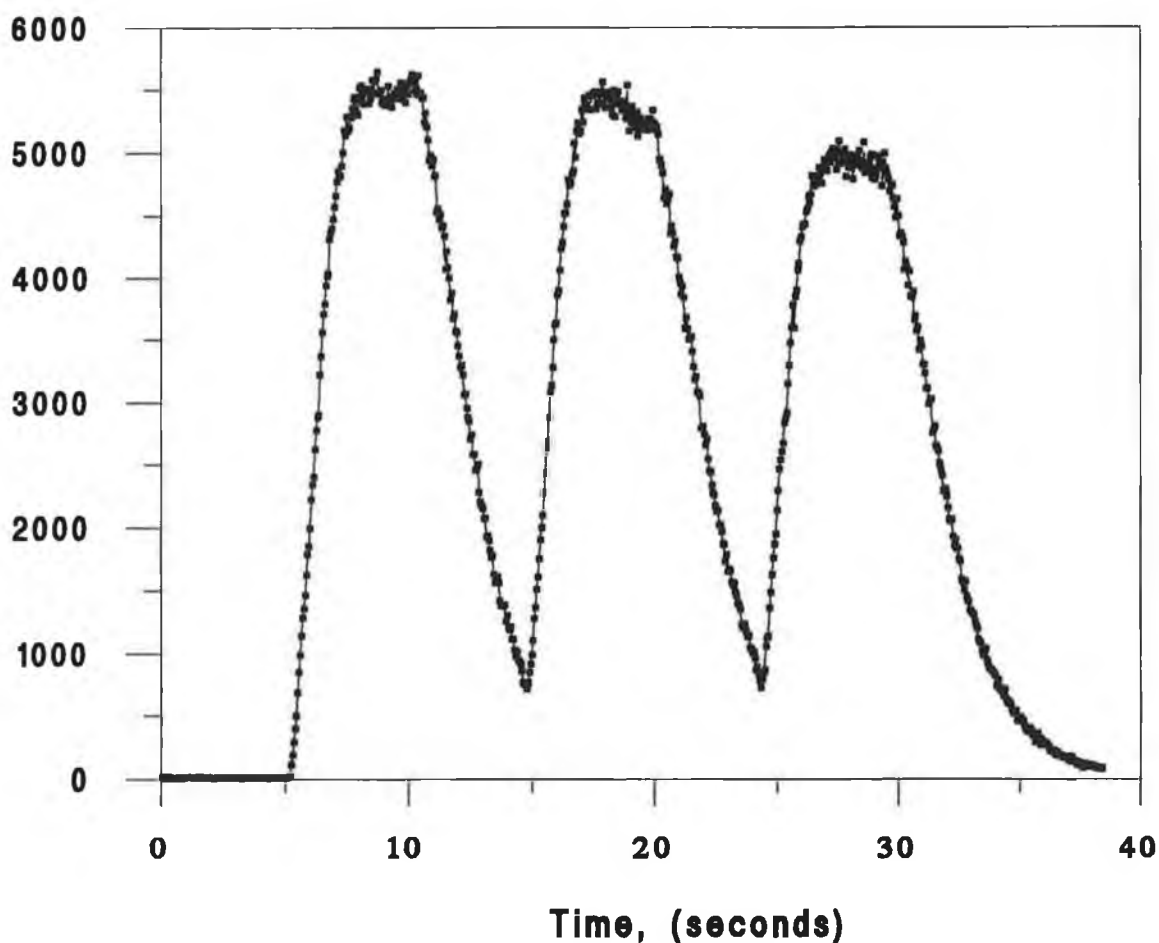


Figure 4.5

Sample plot of photon counts against time for the flow injection system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ / 10ng/ml KI standard. Three injections of luminol were made, at 10 second intervals. Photon counts were measured every 50 milliseconds.

Photon counts

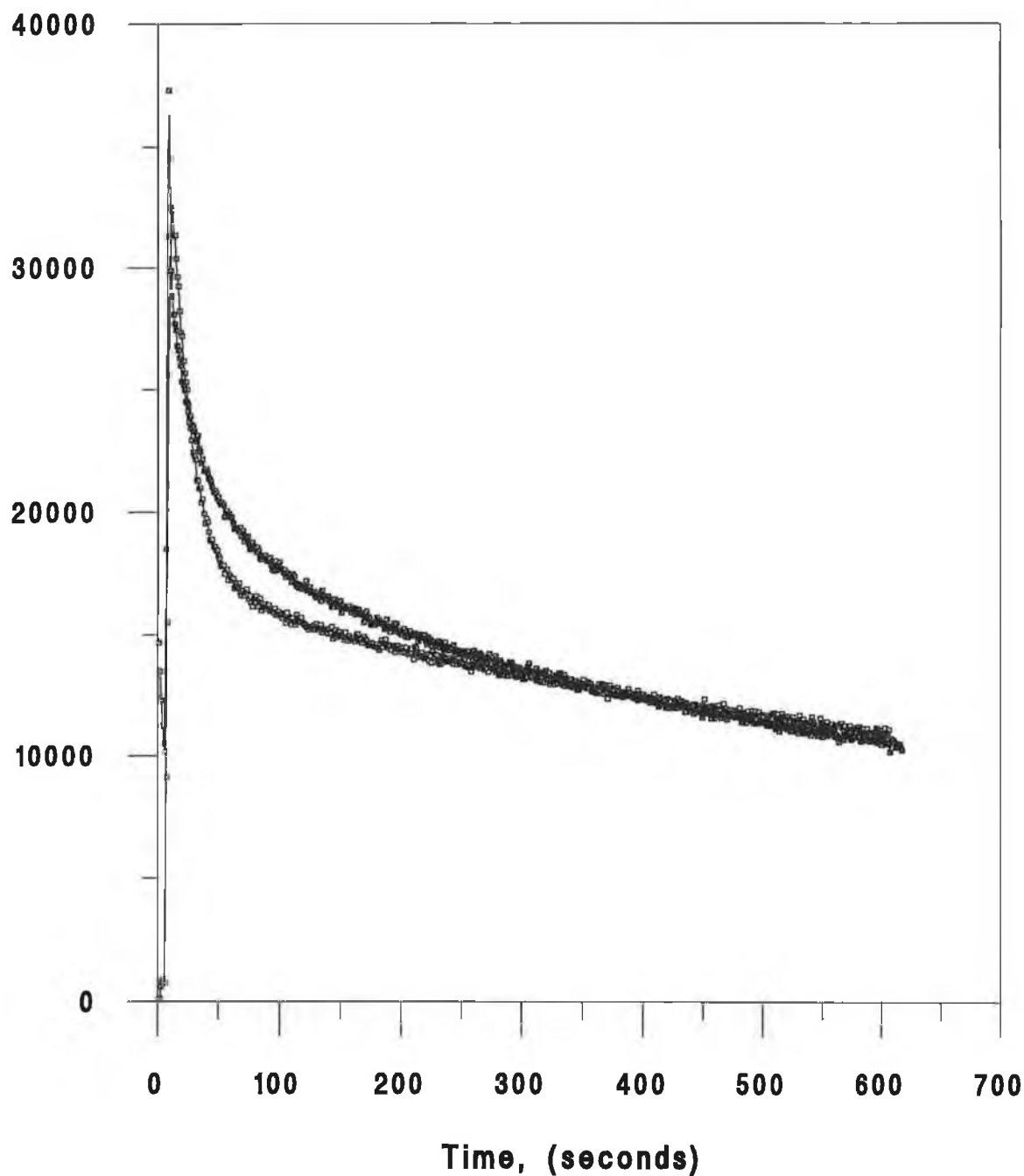


Figure 4.6

Plot of photon counts against time for the stopped flow analysis of the luminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ for both the zero control (□) and the 100 µg/ml potassium iodide standard (■). Photon counts were read for 250 milliseconds, every second.

A microplate format was also considered for System e. This work was carried out on the Amersham Amerlite Luminometer which is only capable of reading the luminescent response from enhanced systems, as no injection facility is available. Conditions for the assay are described in Section 2.22. The luminescence was read over a twenty minute time interval, for the KI standards 10-100 $\mu\text{g/ml}$. Figure 4.7 shows the linear graphs obtained when $1/\text{RLU}$ was plotted against KI concentration. A linear response is observed for an incubation period between 5 and 20 minutes.

The optimum concentration of reagents to be used was determined. The relationship between luminescence and KI concentration (10-100 $\mu\text{g/ml}$) was linear over the concentration range 0.05-0.10mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ (see Figure 4.8). 0.075mM and 0.100mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ gave the best correlation coefficient. The latter concentration was used for further work. A linear response was not obtained for 0.025mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$, and a concentration of 0.25mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ inhibited the luminescent reaction.

The concentration range 0.10-0.50M H_2O_2 gave a linear response in this assay. At concentrations greater than this good correlation coefficients were not obtained. The best correlation coefficient was obtained for 0.25M H_2O_2 (results not shown). The concentration of luminol was also considered. A linear response was obtained for the concentration range 10-15mM luminol (see Figure 4.9). As expected the luminol concentration does not effect the luminescent response to the same extent as the concentration of the catalyst $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ does. At concentrations lower than 7.5mM luminol a linear response was not observed.

This chemiluminescent assay (0.10mM $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$, 0.25M H_2O_2 and 10mM luminol) was also used to determine a lower concentration range of iodide. A linear response was obtained for 1-10 $\mu\text{g/ml}$ KI, if the luminescence was measured after 30 minutes (see Figure 4.10).

(1/ RLU) x 10,000

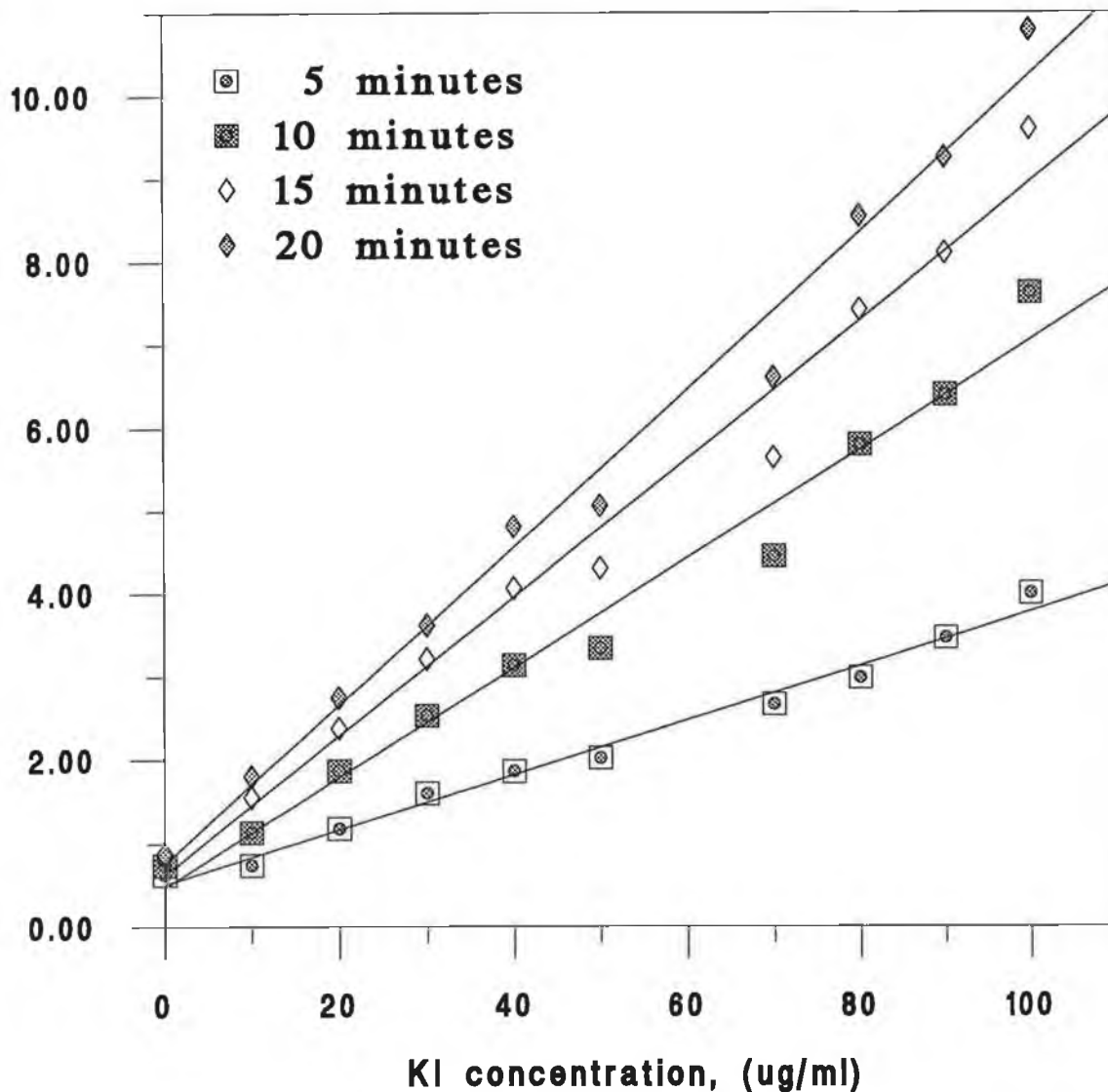


Figure 4.7

The relationship of luminescence [1/ RLU] and KI concentration, obtained for the chemiluminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$, is shown. RLU were read on the Amersham Amerlite Luminometer over a time interval of 20 minutes. These straight line graphs have the following r values: 5 minutes, $r = 0.994$; 10 minutes, $r = 0.990$; 15 minutes, $r = 0.991$; 20 minutes, $r = 0.994$.

(1/ RLU) x 10,000

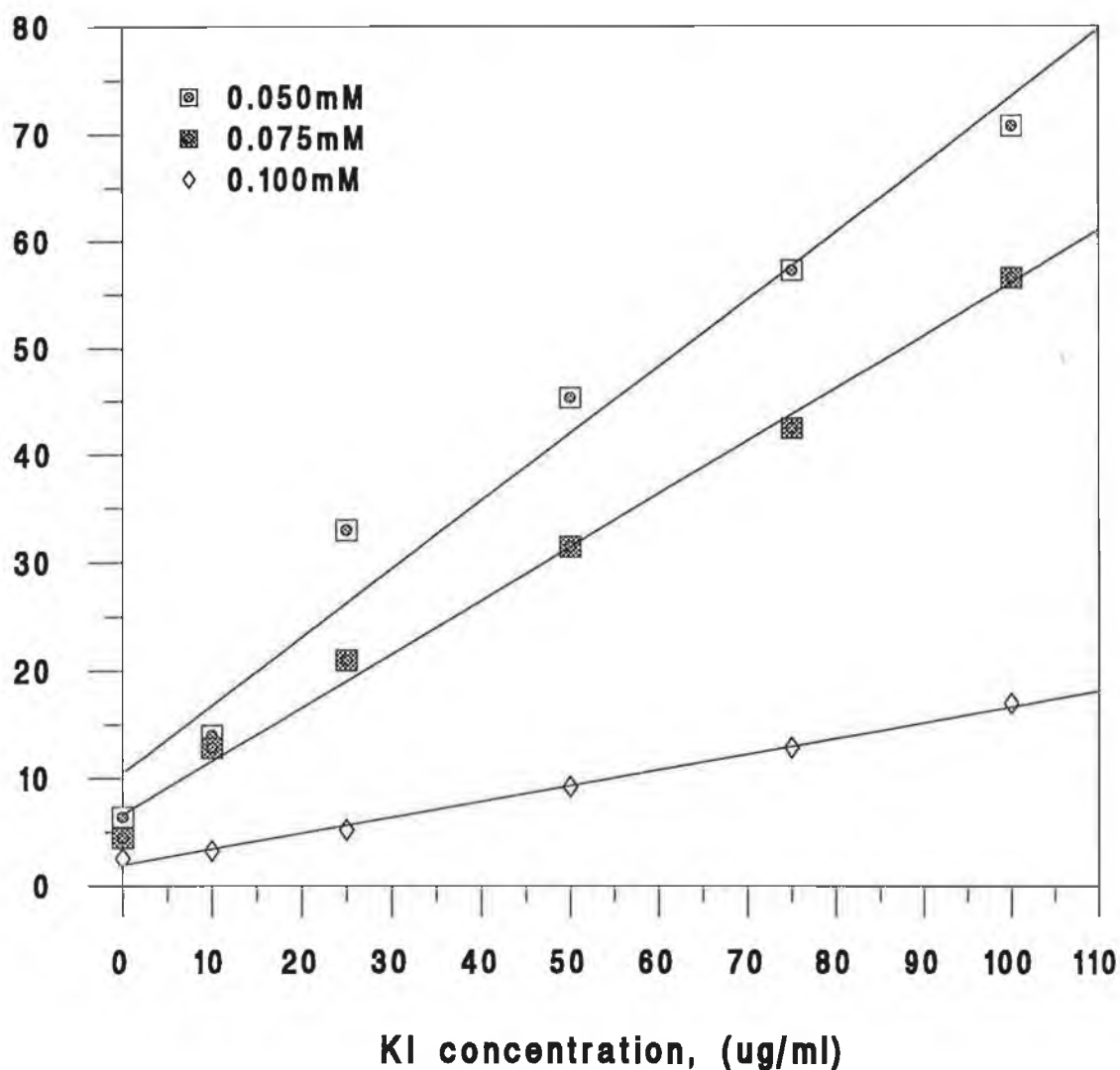


Figure 4.8

The relationship of luminescence (1/ RLU) and KI concentration, obtained for the chemiluminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$, is shown for a concentration range of $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$. RLU were read, at five minutes, on the Amersham Amerlite Luminometer. The r values for the straight line graphs obtained were: 0.050mM, $r = 0.986$; 0.075mM, $r = 0.997$; 0.100mM, $r = 0.998$.

(1/ RLU) x 10,000

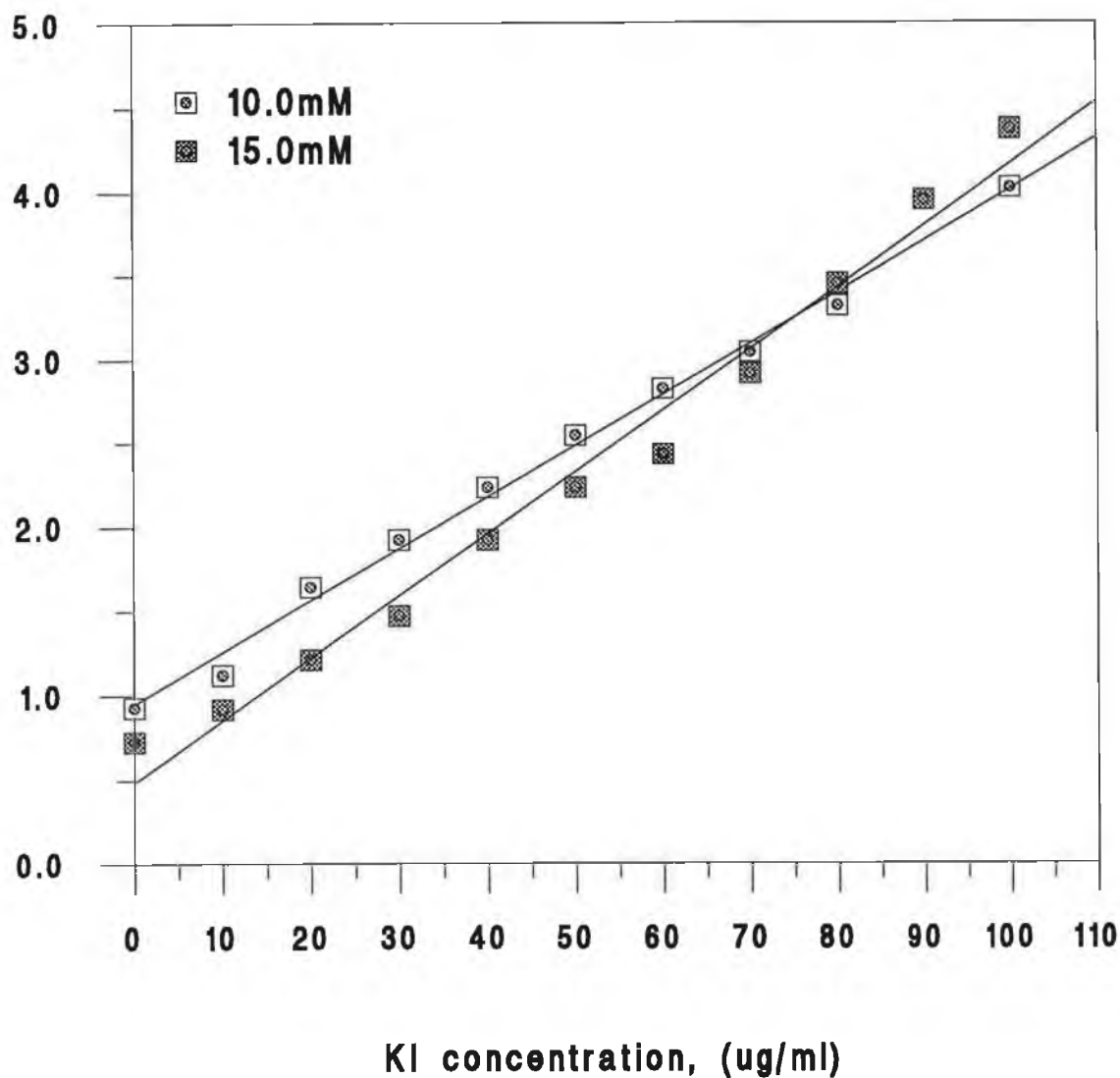


Figure 4.9

The relationship of luminescence (1/ RLU) and KI concentration, obtained for the chemiluminescent system luminol/ hydrogen peroxide/ AuCl₃HCl.4H₂O, is shown for a concentration range of luminol. RLU were read, at five minutes, on the Amersham Amerlite Luminometer. These straight line graphs have the following r values: 10.0mM, r = 0.997; 15.0mM, r = 0.992.

[RLU (blank) - RLU (standard)]

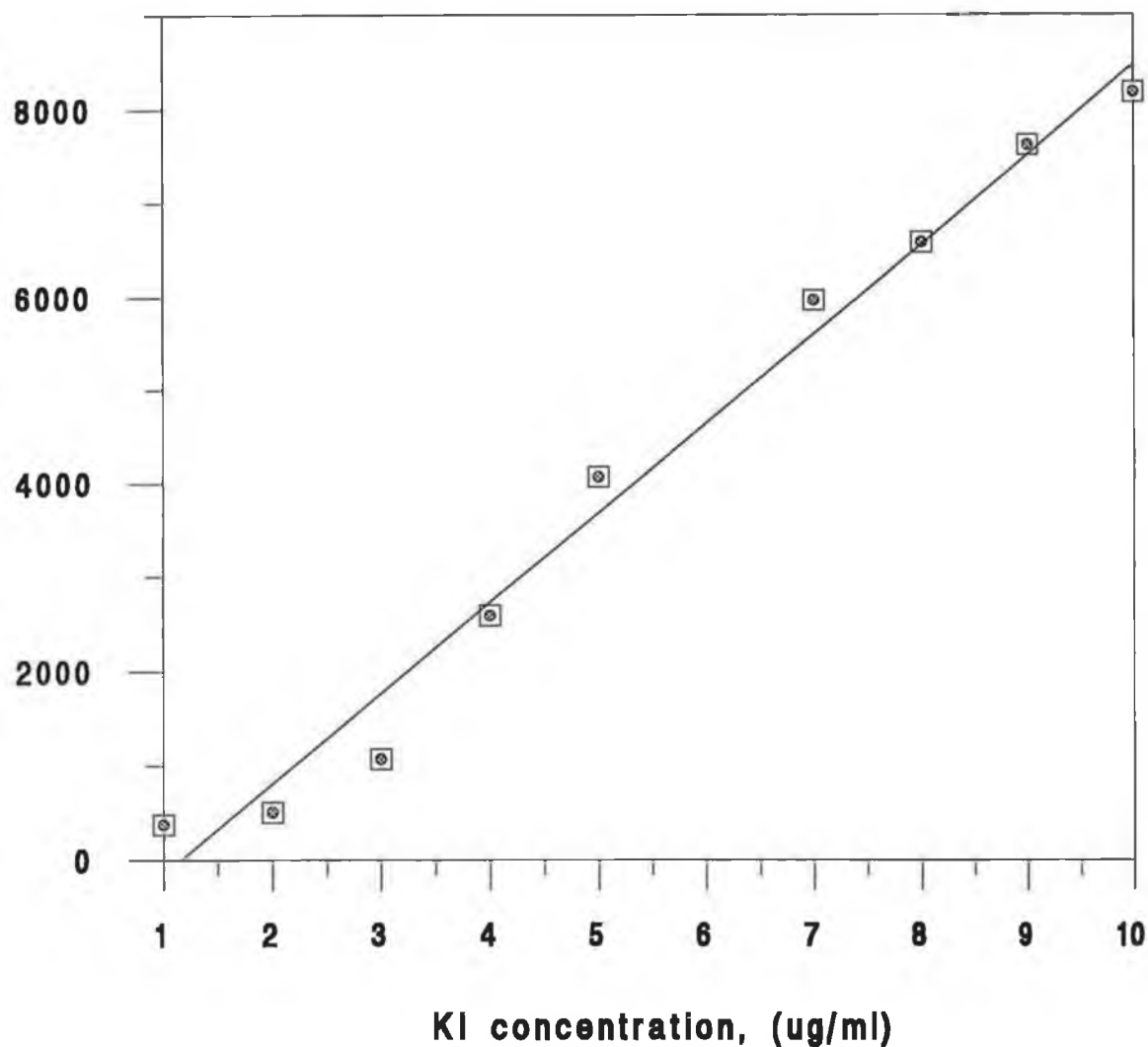


Figure 4.10

The relationship of luminescence (1/ RLU) and KI concentration (1-10 μ g/ml), obtained for the chemiluminescent system luminol/ hydrogen peroxide/ $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ is shown. RLU were read, after 30 minutes, on the Amersham Amerlite Luminometer. [$r = 0.992$]

The absorbance, instead of the chemiluminescence, could be read for the assay described in Section 2.22 (System e, microplate format). A linear relationship was obtained for the plot of absorbance at 540nm (read after an hour incubation using a Titertek Twinreader Plus microplate reader) against 10-90µg/ml KI (see Figure 4.11)

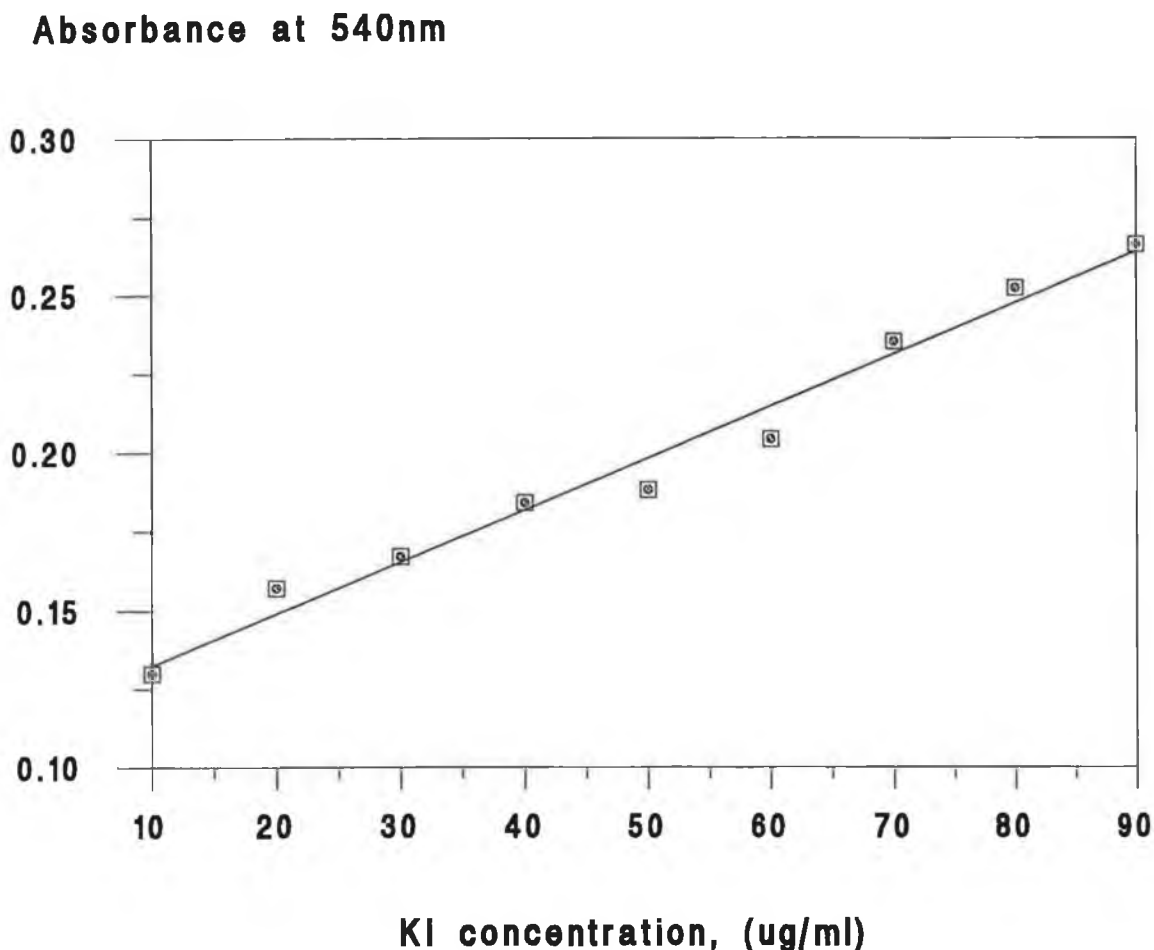


Figure 4.11
Linear graph of absorbance at 540nm [$ABS_{BLANK} - ABS_{STANDARD}$] plotted against iodide concentration for the luminescent system luminol/ hydrogen peroxide/ $AuCl_3 \cdot HCl \cdot 4H_2O$. Absorbance was read after one hour. [$r = 0.991$]

4.26 Iodide detection using the chemiluminescent system luminol/ hydrogen peroxide/ DNCB.

The luminescent system luminol/ hydrogen peroxide/ DNCB/ iodide was investigated in a microplate format on the Amersham Amerlite Luminometer (System f). Conditions were described in Section 2.22. Figure 4.12 illustrates the linear graph obtained for the concentration range 1-100 μ g/ml potassium iodide.

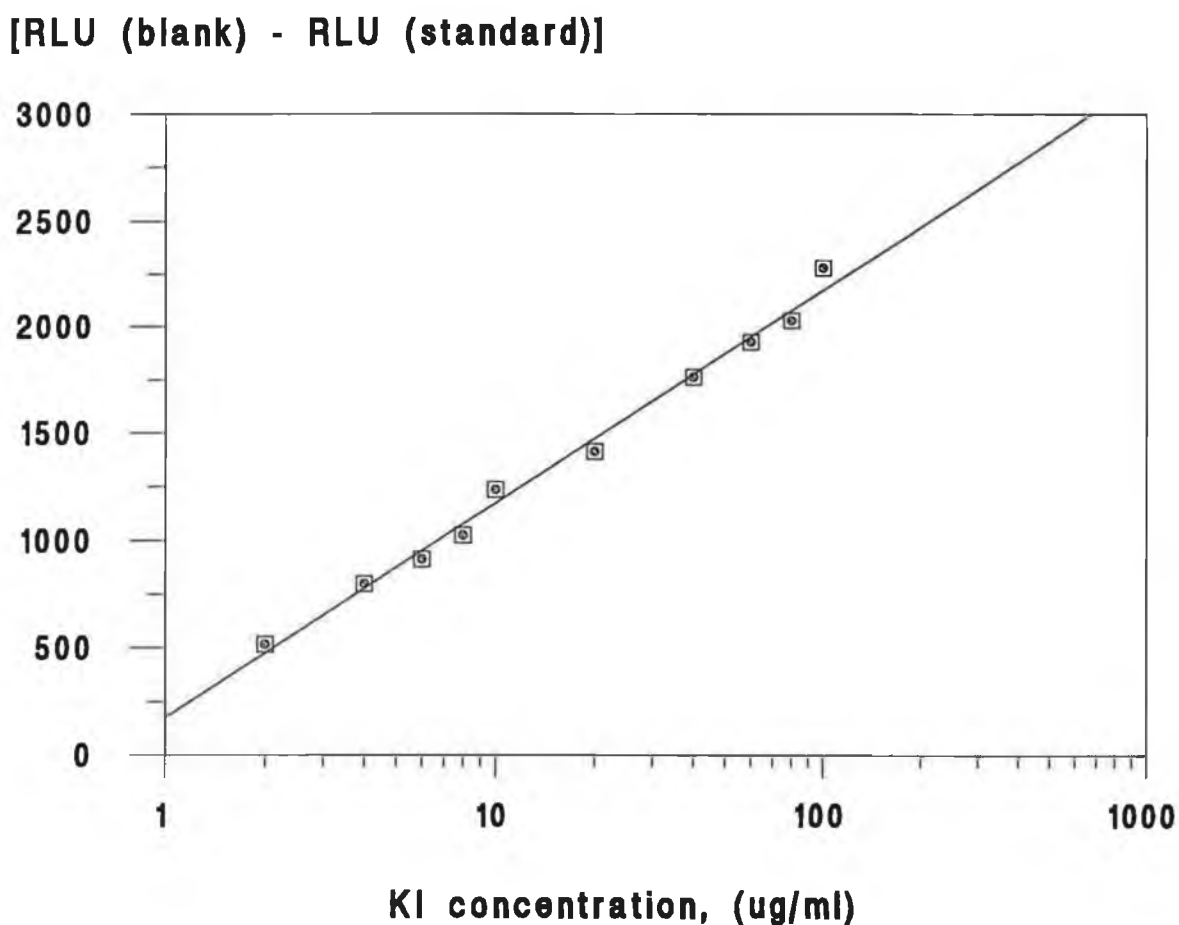


Figure 4.12

The relationship between luminescence ($RLU_{BLANK} - RLU_{STANDARD}$) and KI concentration ($\log [KI \text{ concentration}]$), obtained for the chemiluminescent system luminol/ hydrogen peroxide/ DNCB, is shown. RLU were read after 5 minutes on the Amersham Amerlite Luminometer. This is a straight line graph ($r = 0.995$).

4.3 DISCUSSION

One aim of this work was to develop an alternative, more sensitive, assay for iodide to the colorimetric microassay based on the Sandell-Kolthoff reaction (see Chapter 3). As outlined in Section 1.13.1 such assays offer several advantages for analytical applications including low detection levels and a wide linear dynamic range for many analytes (Grayeski, 1987).

Luminometers capable of measuring luminescence in microplates are now commercially available. The Amersham Amerlite luminometer used during the course of this work is limited in its use as only enhanced chemiluminescent reactions can be monitored because the instrument has no injection facility. The Labsystems Luminoskan 5300017, which has an injection facility, permitted the investigation of a much wider range of iodide assays.

A number of chemiluminescent systems were considered and the assays developed are tabulated in Table 4.3.

System	Luminometer used	Linear KI conc. range $\mu\text{g/ml}$
a	(i)	50 - 1000
a	(ii)	0.1 - 100
b	(ii)	1 - 100
c	(ii)	> 100
d	(ii)	0.01 - 100
e	(iii)	1 - 100
f	(iii)	1 - 100

- (i) Berthold Chemilumat LB9502
(ii) Labsystems Luminoskan 5300017
(iii) Amersham Amerlite Luminometer

- (a) Acetone/ hydrogen peroxide/ hypochlorite/ iodide
(b) Hydrogen peroxide/ hypochlorite/ iodide
(c) Luminol/ hydrogen peroxide/ iodide
(d) Luminol/ hypochlorite/ iodide
(e) Luminol/ hydrogen peroxide/ tetrachloroauric acid/ iodide
(f) Luminol/ hydrogen peroxide/ DNCB/ iodide

Table 4.3

The linear range for KI concentration obtained for the chemiluminescent assays developed. In each assay quenching of the chemiluminescence was observed.

The assay described by Lu *et al.*, (1989) was repeated. They reported a linear range from 0.4ng/ml-0.3 $\mu\text{g/ml}$ iodide and that iodide had a catalytic effect on the luminescence from the acetone/ hypochlorite/ hydrogen peroxide system (System a). However, the data they reported would seem to indicate a limit of detection greater than 10ng/ml. Using the Berthold Chemilumat LB9502 (test-tube format) a linear range was obtained for 50-1000 $\mu\text{g/ml}$ KI. The assay was adapted for microplate format and a linear range was obtained for 0.1-100.0 $\mu\text{g/ml}$ KI. In both assay formats quenching of the luminescence, rather than catalysis, was observed. Iodide also quenched the luminescence from the hydrogen peroxide/ hypochlorite system (System b). The linear range for the microassay developed was 1-100 $\mu\text{g/ml}$. This system was used by Burguera and

Burguera (1982) to measure iodine in water. Iodine induced a chemiluminescent emission peak when injected into a flow-system and the limit of detection was 0.5ng/ml. Iodine is a strong oxidising agent and iodide would not be expected to have the same effect on a chemiluminescent system.

The indirect effect of iodide on a number of luminol luminescent systems (i.e. whether iodide enhanced or suppressed the luminescence), rather than the direct effect of iodine on luminol, was investigated. Initially, the luminol/ hydrogen peroxide/ iodide system was investigated in a microplate format (System c). The limit of detection was high (100µg/ml). However, when the oxidising agent was changed to hypochlorite a linear response was obtained for 10ng/ml-100µg/ml KI (System d). This assay offered the best sensitivity and linear range of the assays developed.

Lin *et al.*, (1989) obtained a limit of detection of 50pg/ml for iodide using the luminescent system luminol/ hydrogen peroxide/ tetrachloroauric acid (System e). The reagents were mixed together by injection in front of the detector. This assay was adapted for microplate format. However, the Amersham Amerlite Luminometer microplate reader, used for this development work had no injection facility. The loss in sensitivity observed was probably due to the fact that the initial burst of luminescence could not be monitored. System e was also investigated by flow-injection. The results observed would seem to indicate that iodide had no effect on the luminescence of this system. However, it is worth noting that no optimization of reagent concentration was carried out for the flow injection assay. The concentration of tetrachloroauric acid and luminol were critical in observing a linear response for iodide quenching when this system was optimized in microplate format. The enhancer for the system, tetrachloroauric acid, was replaced by DNCB and a similar linear range was obtained (System f).

None of the assays developed were more sensitive than the colorimetric iodide microassay (O'Kennedy *et al.*, 1989). System d (luminol/ hypochlorite/ iodide) offers the best sensitivity of the chemiluminescent microassays for iodide

investigated and optimization of the assay conditions could lead to increased sensitivity. Further developmental work on System e (luminol/ hydrogen peroxide/ tetrachloroauric acid/ iodide) using a microplate reader with injection facility could also be considered.

Chapter 5

IODINATION OF NUCLEIC ACIDS

5.1 INTRODUCTION

The extent of iodination of the nucleic acids was determined by two methods: using the microassay for cold iodine and HPLC analysis of nucleosides.

The microassay for iodide (O'Kennedy *et al.*, 1989) is based on the Sandell-Kolthoff reaction and can detect picogram quantities of iodide (see Chapter 3). Thus, microgram quantities of DNA can be detected using this microassay if 1% of the cytosines incorporate iodine.

Berlanger *et al.*, (1986) determined the incorporation of 5-iododeoxyuridine into DNA of granulocytes in patients by extracting the DNA, hydrolysing it and then separating and measuring thymidine and iododeoxyuridine by HPLC. Labelled nucleic acids were analysed using this HPLC method.

DNA was labelled initially during this work using the method described by Piatysek *et al.*, (1988) where iodide is incorporated into cytosine by chemical substitution. Polydeoxycytidylic acid, lambda DNA and single-stranded calf thymus DNA were labelled. Unbound iodide was removed by ethanol precipitation of the nucleic acids. This resulted in the loss of some nucleic acid, which was often more than half the starting material.

An enzymatic system of labelling was also considered. The modified nucleotide, 5-iodo-2'-deoxycytidine 5'-triphosphate, was incorporated via random priming (Feinburg and Vogelstein, 1983, 1984) using the *Prime-a-Gene* kit from Promega. Plasmid PAAH5 (12.5 kb) was labelled by this procedure, after linearization using *Hind* III.

5.2 RESULTS

5.21 *Iodogen*-mediated iodination of nucleic acids

The results obtained for the iodination of nucleic acids, by chemical substitution, are shown in Table 5.1. The concentration of nucleic acids was estimated by measuring the absorbance at 260nm and the iodide concentration was determined using the iodide microassay described in Section 2.21. Polydeoxycytidylic acid was labelled initially. Less than 1% of the cytosines were iodinated. Similarly, when lambda DNA was labelled less than 1% incorporation was observed. Slightly higher incorporation (greater than 2%) was observed when single-stranded calf thymus DNA was labelled.

The concentration of iodide was increased ten-fold in the labelling reaction but no increase in incorporation was obtained when Method 1 (Piatyskey *et al.*, (1988)) was used. When Method 2 (Section 2.31) was used, that is, the reaction was carried out in a rotating petri-dish, rather than in test-tubes, an increase in incorporation was observed with increased iodide concentration. However, the labelling reaction was not always successful when Method 2 was used. This was probably due to unsuccessful layering of the *Iodogen* onto the petri-dish. When Method 2 was used the chloroform was evaporated in a fumehood, without using nitrogen. For further work the chloroform should be evaporated with nitrogen, to ensure thorough dryness of the petri-dish.

	SAMPLE	ng I/ μg DNA
METHOD 1	polydeoxycytidylic acid	1.60
	Lambda DNA	0.12
		0.52
	Calf thymus DNA (single-stranded)	2.26
		2.46
		#2.01
METHOD 2	Calf thymus DNA	0.40
	(single-stranded)	#9.00

Iodide concentration increased ten-fold

Table 5.1

Results for the Iodogen mediated iodination of nucleic acids. The concentration of iodide (ng I) was determined using the iodide microassay (Section 2.21) and the value is given per microgram of DNA. DNA concentration was estimated by measuring the absorbance at 260nm. Method 1 and Method 2 are described in Section 2.31.

5.22 Enzymatic incorporation of 5-iodo-2'-deoxycytidine 5'-triphosphate by random priming

After the random priming reaction unincorporated nucleotides were removed by three successive ethanol/ ammonium acetate precipitations of the DNA. Precipitation of the DNA also ensures removal of EDTA (present in the reaction mixture) which interferes in the Sandell-Kolthoff reaction (see Section 3.25). It should be noted that the values recorded using the KI microassay (Table 5.2) result from the presence of unincorporated 5-iodo-2'-deoxycytidine 5'-triphosphate and other interfering substances, such as EDTA , in the supernatant following the precipitation of DNA. The successive supernatants were evaluated in the the iodide microassay.

Supernatant following DNA precipitation	Value read in the KI microassay	
	Labelled	Control
First	0.014	0.014
Second	0.002	0.003
Third	<0.001	<0.001

Table 5.2
Removal of unincorporated 5-iodo-2'-deoxycytidine 5'-triphosphate and other interfering substances by successive ethanol/ ammonium acetate precipitation of the labelled plasmid. The values read in the iodide microassay for the first, second and third supernatants are shown.

The final pellets for the labelled and control plasmid PAAH5 samples were resuspended in 10mM Tris, pH 8.0, and an estimation of DNA concentration was made. [500ng of plasmid DNA was labelled and after the ethanol/ ammonium acetate precipitation step it was assumed that 50% of the DNA

remained as noted in the insert with the *Prime-a-Gene* kit]. As before, iodide concentration was determined using the iodide microassay (O'Kennedy *et al.*, 1989). The labelled plasmid was found to have 40.0 ng iodide/ μ g DNA. Thus, this would infer that 38% incorporation of 5-iodo-2'-deoxycytidine 5'triphosphate was achieved. However, further work would be needed to optimize the conditions for this system.

5.23 HPLC analysis of labelled nucleic acids

Hydrolysed nucleotides (dATP, dCTP, dGTP and dTTP) were run as standards, as were cytidine 5'-monophosphate, 5-iodocytidine 5'-monophosphate, and cytidine and 5-iodocytidine. The latter served as controls for the peaks of interest, and the hydrolyzed dNTPs were run to ensure that the peaks due to the other nucleosides were separated from the cytidine and 5-iodocytidine peaks. Table 5.3 illustrates the retention times observed when these standards were run on the HPLC system described in Section 2.4. 5-iodocytidine has a maximum absorbance at 293nm whereas the 4 nucleosides, adenosine, cytidine, guanosine and 2-deoxythymidine, show maximum absorbance between 253 and 271nm. Thus, the ratio (percentage) of absorbance at 300nm to absorbance at 260nm is over 100% for the iodinated nucleoside and less than 5.2% for the unlabelled nucleosides.

5-iodocytidine has a retention time of 7.43 minutes, while the hydrolyzed 5-iodocytidine 5'-monophosphate has a retention time of 2.45 minutes. This would seem to indicate that the phosphate has not been removed by hydrolysis as shorter retention times were observed for the unhydrolysed nucleotides, i.e., when phosphate groups are present, than for the nucleosides. [It has not been determined whether the activity and specificity of the DNase and phosphodiesterase are affected because the nucleic acids have been iodinated]. Thus, when considering the chromatograms of hydrolysed nucleic acid samples good separation of the iodinated nucleotide and the unlabelled nucleotides may not be achieved as many of these have retention times under 3 minutes. However, the ratio of absorbance at 300nm to absorbance at 260nm would be a clear indication that the nucleotide is labelled.

The chromatograms for the hydrolyzed polydeoxycytidylic acid samples, both labelled and control (labelled using the *Iodogen* chemical substitution method), are shown in Figure 5.1. The labelled polydeoxycytidylic acid sample has a peak with a retention time 2.95 minutes, not found in the control sample. It was not possible to obtain the absorbance ratio, $\text{absorbance}_{300\text{nm}}:\text{absorbance}_{260\text{nm}}$,

as an equivalent peak was not observed when the absorbance was read at 260nm. This is probably due to the fact that the peak height at 300nm is 0.001 absorbance units thus, at 260nm, absorbance would be too low to be detected. The peak with a retention time of 1.47 minutes is due to the buffer.

However, on the chromatogram obtained for the labelled oligoprobe sample there was no evidence for an iodinated nucleotide peak. This would seem to indicate that the oligoprobe was not iodinated using the *Iodogen* method.

SAMPLE	Retention Time, (min.)		abs ₃₀₀ :abs ₂₆₀	X100
	Peak I	Peak II		
dCTP	1.77		4.7%	
dGTP	2.69	5.88	3.1%	4.0%
dTTP	2.53	6.38	2.6%	4.1%
dATP	4.24	13.42	-	-
5-Iodocyt-1-P	2.45		125.0%	
cytidine-1-P	2.30		5.2%	
5-iodocyt	7.43		146.8%	
cytidine	2.30		5.2%	

Table 5.3

Retention times and absorbance ratio (absorbance_{300nm}: absorbance_{260nm}) for the standards run on the HPLC system described in Section 2.4 for the analysis of hydrolysed labelled nucleic acids. Standards, which were hydrolysed under the same conditions as the nucleic acids, were as follows: 2'-deoxycytidine 5'-triphosphate (dCTP); 2'-deoxyguanosine 5'-triphosphate (dGTP); 2'-deoxythymidine 5'-triphosphate (dTTP); 2-deoxyadenosine 5'-triphosphate (dATP); 5-iodocytidine 5'-monophosphate (5-iodocyt-1-P); cytidine 5'-monophosphate (cytidine-1-P); 5-iodocytidine (5-iodocyt) and cytidine.

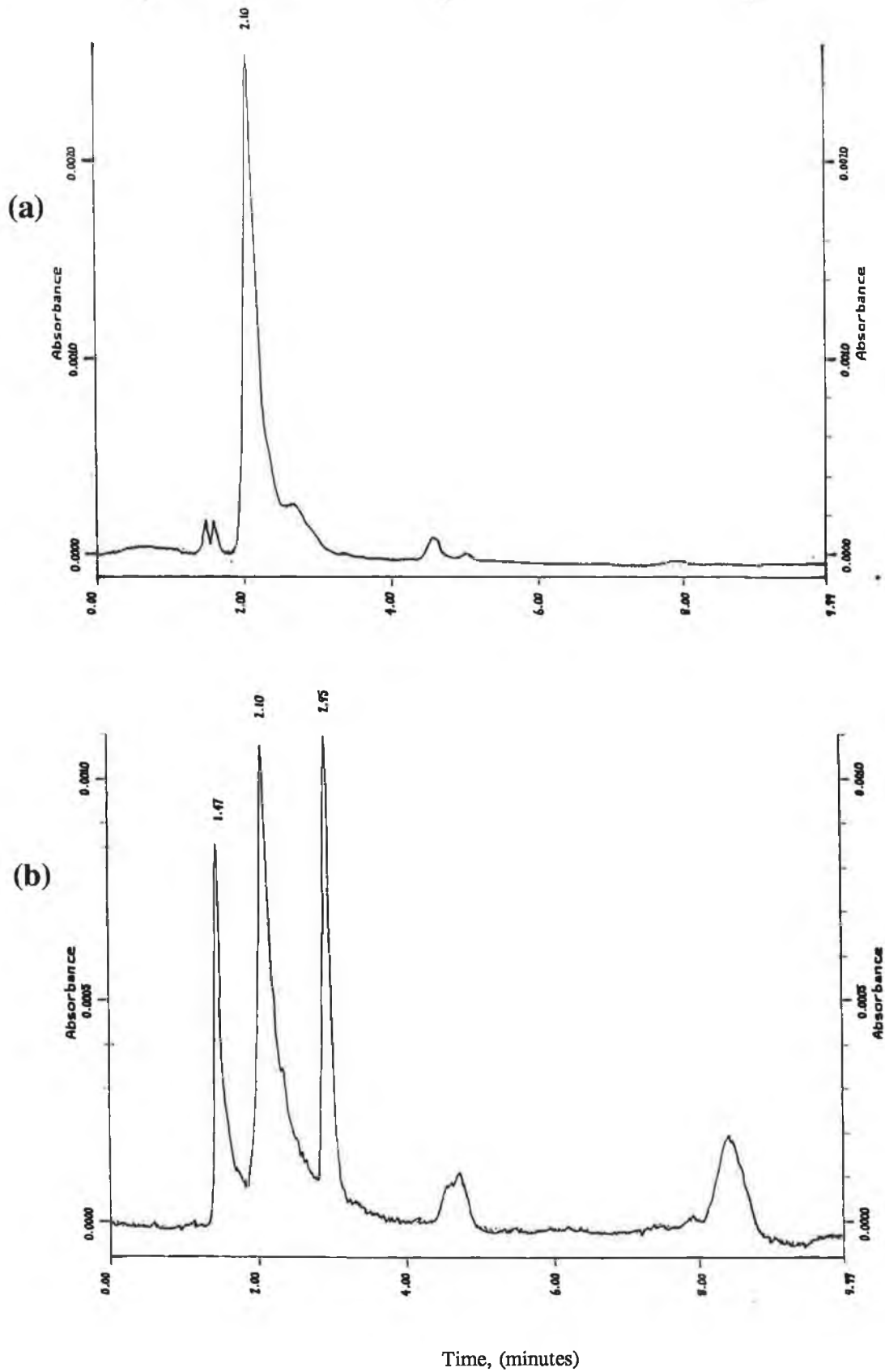


Figure 5.1

Chromatograms for both the control (a) and labelled (b) hydrolysed polydeoxycytidylic acid samples. 20 μ l of sample was injected onto a μ Bondapak C18, 125 \AA , 10 μ m, 3.9 x 300mm column (Waters Chromatography division), the mobile phase was 0.1M acetate buffer, pH 5.4, containing 4% acetonitrile and the flow rate was 2ml/minute. Absorbance was read at 300nm.

5.3 DISCUSSION

Both the chemical substitution and the random priming methods can be used to label DNA. Higher incorporation was obtained using the latter method but for labelling large quantities of DNA the chemical substitution method would be more suitable. However, conditions for the iodination reaction using *Iodogen* would have to be optimized so that higher incorporation is achieved. Greater than 50% incorporation can be achieved using the random priming method (Keller and Manak, 1989). Lower incorporation of the modified nucleotide was probably achieved during this work because the conditions for the assay were not optimized, though the quantity of DNA in the reaction mixture was greater than that recommended in the insert with the *Prime-a-Gene* kit. Efforts to label the oligoprobe (V₆ primer +10C) with iodine by chemical substitution were not successful. Incorporation of the modified nucleotide, (5'-iodo-2'-deoxycytidine 5'triphosphate) during synthesis of the oligoprobe, or by tailing using terminal deoxynucleotidyl transferase, are more suitable methods of iodination.

For further work using the HPLC method described the following considerations should be made: hydrolysis should be carried out with more phosphodiesterase to achieve complete breakdown to the component nucleosides and larger quantities of DNA should be injected onto the HPLC column. For the above work 5-10µg of hydrolysed nucleic acid was injected while Berlinger *et al.*, (1986) injected 10-50µg.

The iodide microassay is a suitable means of measuring the levels of iodination and more convenient than the HPLC method. The microassay can detect 141 picograms of iodide (1ng/ml KI can be detected in 185µl). Table 5.4 illustrates the theoretical limit of detection for DNA which would be obtained at various levels of iodine incorporation. [e.g., for 1% incorporation the ratio of part iodide to part DNA is 1:945, thus, to detect 1µg of DNA it must be possible to detect approximately 1ng of iodide. Thus, at the limit of detection of the iodide microassay it is possible to detect 133ng of DNA].

Percentage cytosines with iodine incorporated	LOD for DNA which could be determined in the iodide microassay
1%	133.0ng
10%	13.3ng
20%	6.7ng
50%	2.7ng

Table 5.4

Theoretical limit of detection (LOD) for DNA which could be measured in the iodide microassay for a range of incorporation levels for iodine.

The iodide microassay is not sensitive enough to measure iodine as a direct label in a DNA probe assay because it is necessary to detect DNA in the picogram range (discussed in Section 1.21.4). If a more sensitive iodide microassay was developed, be it colorimetric, fluorimetric or chemiluminescent, the feasibility of using cold-iodine as a direct label for DNA probes could be investigated more fully, either in dot blot or microplate format. The feasibility of using iodine as an indirect label for DNA probes is considered in Section 6.3.

Chapter 6

AN INVESTIGATION OF MODEL PROBE SYSTEMS

6.1 INTRODUCTION

Labels for DNA probes can be classified as direct or indirect (see Section 1.21.3). The feasibility of using cold-iodine as a direct label for DNA probes was considered. The Sandell-Kolthoff reaction was used in a blot format, to detect iodide and iodo-compounds, by Bowden (1955). This assay could detect 0.2µg/ml potassium iodide. The sensitivity of the Sandell-Kolthoff assay on nitrocellulose paper blotted onto Whatman chromatography paper was investigated.

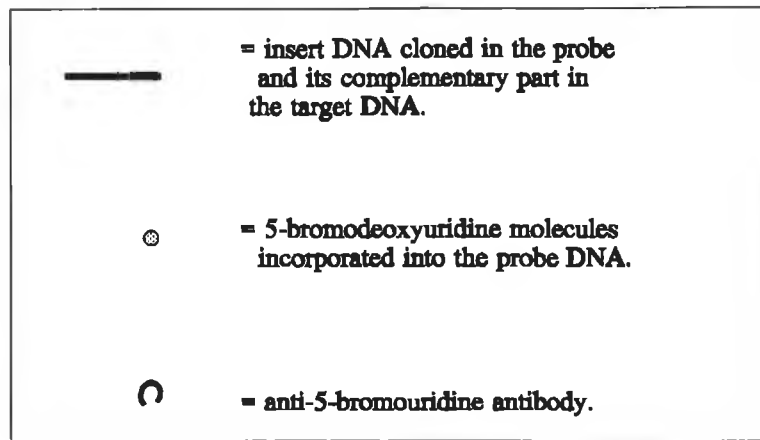
The feasibility of using iodine as an indirect label for DNA probes was also considered. The label is the iodinated nucleotide 5-iodo-2'-deoxycytidine. Anti-5-iodocytidine antibodies would bind to the hapten and could then be detected using a labelled antispecies IgG second antibody or by using a labelled primary antibody.

Erlanger and Beiser (1964) described a general method for the preparation of purine- and pyrimidine-containing antigens. The periodate method they used required that a ribonucleoside or a ribonucleotide derivative of the base was available as the deoxyribonucleotides do not react with periodate. Rabbits immunized with the purine or pyrimidine BSA conjugates yielded antisera that precipitated the homologous antigen. Nucleoside-protein conjugates yield antibodies against nucleosides. Subsequent characterization of these antibodies has revealed that they possess a high degree of specificity toward the nucleoside hapten, its homologous base and nucleotide component, yet minimal or no cross-reactivity with other bases, nucleosides and nucleotides. For modified nucleosides, the modified site must be immunodominant. [The subject of antibodies specific for modified nucleosides was reviewed by Munns and Liszewski (1980).]

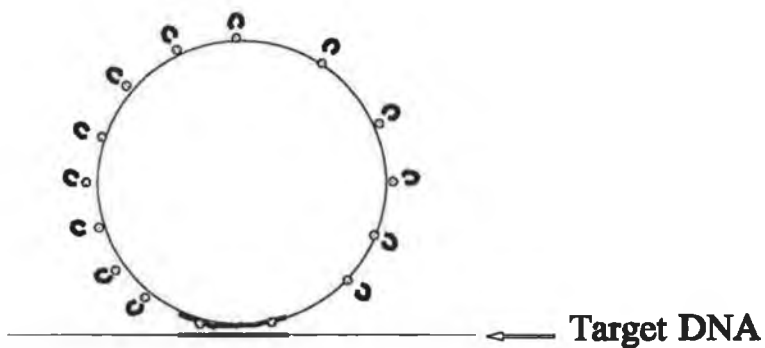
Polyclonal antibodies to cytosine modified at the 5 position and halogenated nucleosides have been produced. Lubit *et al.*, (1976) employed anti-5-methyl cytosine to examine the abundance and location of 5-methylcytosine residues in human metaphase chromosomes by both light and immunoelectron microscopy techniques. Gratzner *et al.*, (1975) produced polyclonal antibodies which reacted with bromodeoxyuridine alone, or when incorporated into single-stranded DNA. Later, Gratzner (1982) produced monoclonal antibodies to 5-bromodeoxyuridine and 5-iododeoxyuridine. The Balb/C mice were immunized with a conjugate of iodouridine and ovalbumin. The polyclonal and monoclonal antibodies produced by Gratzner were only reactive with the modified nucleotide in single-stranded DNA. In 1985, Gonchoroff *et al.*, produced a monoclonal antibody reactive with 5-bromo-2'-deoxyuridine that did not require DNA denaturation.

For an indirect labelling system the binding protein should be able to bind to double-stranded DNA. However, if the polyclonal antibodies produced are only reactive with 5-iodo-2'-deoxycytidine incorporated in single-stranded DNA, the antibodies could still be useful for a DNA probe assay using a format similar to that described by Sakamoto *et al.*, (1987). Traincard *et al.*, (1983) described a DNA probe assay detection scheme based on an immunoenzymatic procedure. The modified base, 5-bromo-2'-deoxyuridine, was incorporated into the DNA probe, either by nick translation or by *in vivo* labelling of double-stranded DNA. The detection limit was high (50pg of target DNA) because the antibody recognises the halogenated bases mainly in single-stranded DNA, and therefore poorly in the hybridized probe. Sakamoto and his co-workers then incorporated 5-bromo-2'-deoxyuridine into single-stranded phage M13 DNA probe produced *in vivo*. These labelled M13 single-stranded probes were used in dot-hybridization experiments. Sensitivity was improved because about 80% of the hybridized probe was single-stranded (see Figure 6.1). Using the plasmid PCP10, which carries the hepatitis B virus (HBV) genome, and the probe mHBV4 they were able to detect 1pg of target DNA in a model dot-hybridization experiment.

Polyclonal antibodies to 5-iodocytidine were produced and the production and characterization of the conjugates used for their production is described.



(a)



(b)

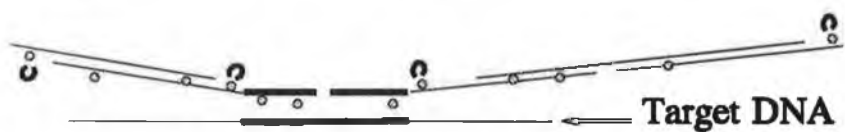


Figure 6.1

Hybridization scheme with (a) 5-bromdeoxyuridine *in vivo* labelled M13 single-stranded DNA and (b) 5-bromodeoxyuridine nick translated probe (Sakamoto *et al.*, 1987).

(a) Because the cloning vector is bigger than the cloned insert about 80% of the hybridized probe is in the single-stranded state when the probe is hybridized to the target DNA.

(b) Most of the hybridized probe is in the double-stranded state when the insert hybridizes with the target. The hybridization network which forms when two complementary strands of the probe hybridize with each other is illustrated.

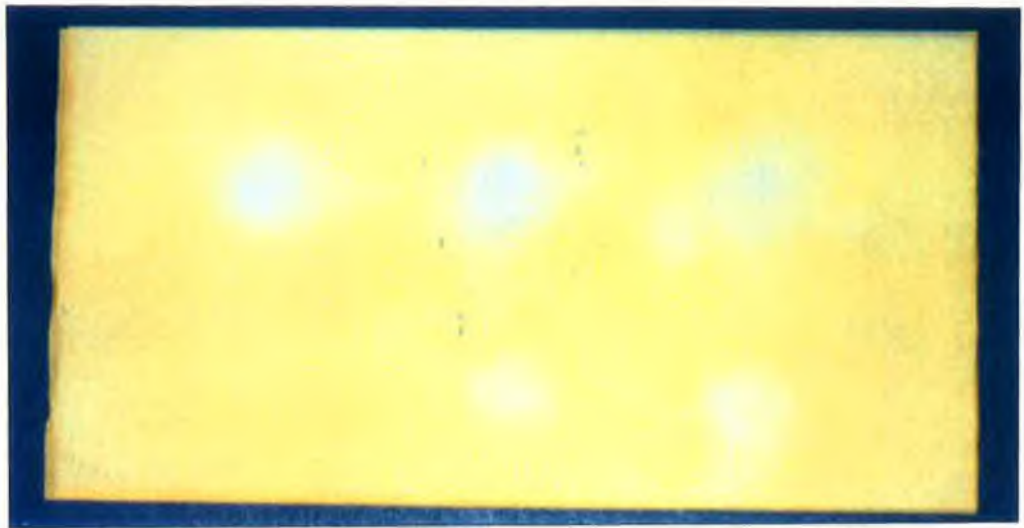
6.2 THE SANDELL-KOLTHOFF ASSAY FOR IODIDE IN BLOT FORMAT

Procedure: 10 μ l of standards (potassium iodide and 5-iodocytidine) were spotted onto nitrocellulose paper. The reagent mixture (1:1 solution of 20% ceric ammonium sulphate and 0.075M arsenious acid) was applied evenly to a sheet of chromatographic paper, the same size as the nitrocellulose paper, by laying it flat on a sloping clean glass plate and allowing the reagent to flow down the paper so that it was evenly wetted and free from wrinkles and air bubbles. The glass plate was then placed on a flat surface and the wetted paper covered by the dry nitrocellulose paper. A second glass sheet was placed on top and firmly pressed down so that the nitrocellulose paper was also evenly wetted. A weight (approx. 2kg) was placed on the glass plates. The blot for the standards, 10-100 μ g/ml potassium iodide, was photographed after 10 minutes while the blot for the lower range of iodide standards and the blot for the iodocytidine standards were photographed after 1 hour.

Results: The blots obtained for the standards 1-10 μ g/ml potassium iodide and 10-100 μ g/ml potassium iodide are shown in Figure 6.2 and the blot for the iodocytidine standards, 100-1000 μ g/ml, is shown in Figure 6.3. The photographs show the white spots on a yellow background obtained for the KI and iodocytidine standards due to the catalytic effect of iodide on the Sandell-Kolthoff reaction in which yellow Ce(IV) is reduced to colourless Ce(III) by As(III). A comparison of the spots for the following: 1.0 and 7.5 μ g/ml KI; 10 and 75 μ g/ml KI and 100 and 750 μ g/ml 5-iodocytidine illustrate that spot size varies with concentration. The radius of the spot can be measured to obtain quantitative rather than qualitative results.

Discussion: This format is one thousand-fold less sensitive than the microplate format and, thus, not suitable for the detection of DNA labelled with cold-iodine.

(a)



(b)

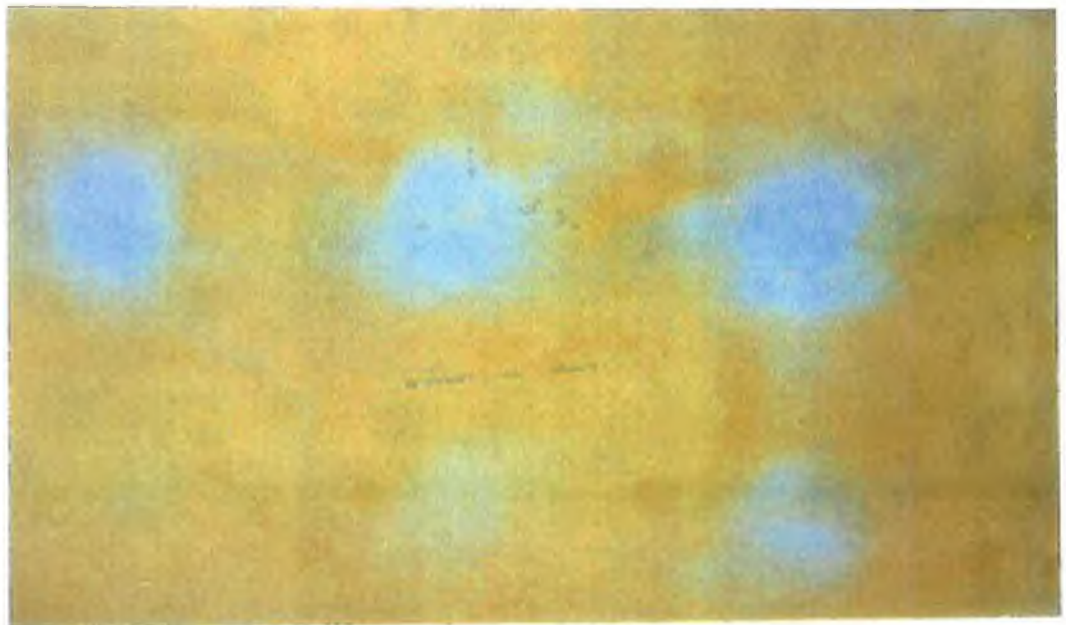


Figure 6.2

Photographs of the nitrocellulose dot blots obtained for the iodide standards, 1-10 μ g/ml and 10-100 μ g/ml using the Sandell-Kolthoff assay. Layout is as follows:

(a) 1-10 μ g/ml

5.0 μ g/ml 7.5 μ g/ml 10.0 μ g/ml

zero 1.0 μ g/ml 2.5 μ g/ml

(b) 10-100 μ g/ml

50 μ g/ml 75 μ g/ml 100 μ g/ml

zero 10 μ g/ml 25 μ g/ml

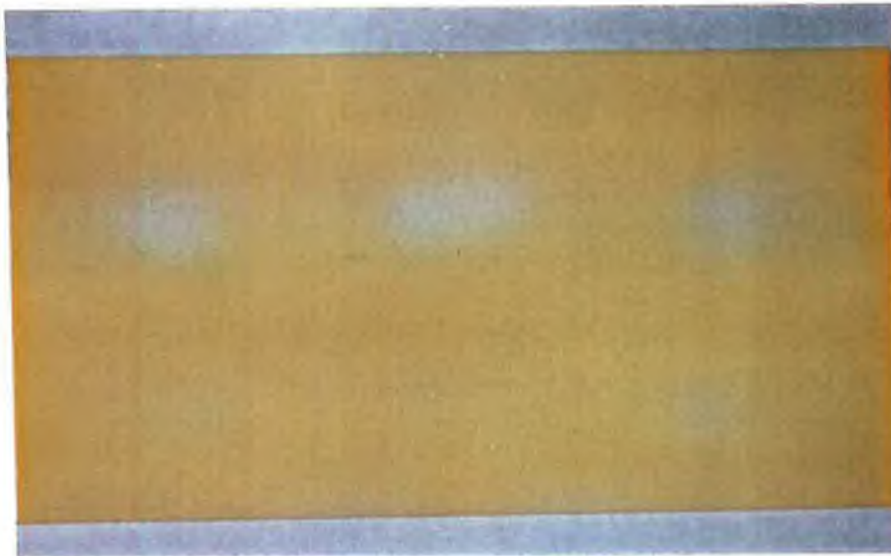


Figure 6.3
Photograph of nitrocellulose dot blot obtained for the iodocytidine standards, 100-1000 μ g/ml, using the Sandell-Kolthoff assay. Layout is as follows:

500 μ g/ml	750 μ g/ml	1000 μ g/ml
zero	100 μ g/ml	250 μ g/ml

6.3 PRODUCTION AND CHARACTERIZATION OF ANTISERA TO 5-IODOCYTIDINE

6.31 Introduction

6.31.1 Conjugate production and characterization

Antibodies can be produced against large molecular weight proteins quite easily. However, low molecular weight compounds, such as 5-iodocytidine, are too small to elicit an immune response and therefore, to produce antibodies against them they must be conjugated to large proteins or synthetic polypeptides (Erlanger, 1980). The proteins which are most often chosen as carriers for these haptens are bovine serum albumin (BSA), ovalbumin, thyroglobulin, keyhole limpet haemocyanin and fibrinogen and the polypeptide, polylysine (Findlay, 1987).

Levine *et al.*, (1968) investigated the genetic control of the immune response to hapten-polylysine conjugates in guinea pigs. It was found that when random-bred Hartley guinea pigs were immunized with 2,4,-dinitrophenyl-poly-L-lysine (DNP-PLL) conjugates in complete Freund's adjuvant only 25-35% produced immune responses. The "all or nothing" character of the immune response to hapten-polylysine showed Mendelian patterns of inheritance. It was shown that the response (either positive or negative) was carried on a single gene and that the trait depended on the poly-L-lysine carrier and not on the hapten, since virtually 100% of Hartley guinea pigs can respond to DNP coupled to foreign protein. Maurer and Pinchuck (1968) reported that α -L-homopolymers were not immunogenic in rabbits. However, Sela *et al.*, (1964) reported that antibodies with specificity towards uridine may be obtained in rabbits upon injection of synthetic molecules in which uridine 5'carboxylic acid is covalently bound, through an amide bond, to the amino-terminal groups of the poly-L-alanyl side-chain, of a multichain synthetic polypeptide. They reported that the attachment of the uridine 5'carboxylic acid residues converted the non-antigenic multichain poly-L-alanine into an immunogen. Other work using poly-L-lysine as a carrier has been described. Beachy (1986) produced

antibodies to a synthetic peptide of Streptococcal M protein conjugated to polylysine without adjuvant in rabbits. Cyanoginosin-LA was conjugated to polylysine and muramyl dipeptide to form a high molecular weight complex, consisting of a hapten, a carrier and a built-in adjuvant for the production of monoclonal antibodies to cyanoginosin-LA (Kfir *et al.*, 1985).

5-Iodocytidine-polylysine conjugates were initially used to immunize a white New Zealand male rabbit. However a very low titre of antibody was obtained after 3 months (less than 1:50) and therefore, BSA was subsequently used as a carrier.

Chemical crosslinking agents, such as glutaraldehyde and carbodiimides, have been used to link haptens to carrier molecules (Briand *et al.*, 1985). The hapten may have a number of reactive groups already present, or it may require chemical derivatisation in order to introduce a group which can be covalently linked to the carrier protein. The use of existing groups in the hapten for linkage may effect the specificity of the anti-hapten antibodies (Tijssen, 1985). It has been found that the antibodies tend to be directed primarily to the groups furthest away from the point of linkage to the carrier (Erlanger, 1980). Haptens with carboxyl, amino, and reducible nitro groups, sulphhydryl, hydroxyl, aldehyde and ketone groups can be conjugated directly to a carrier molecule (Tijssen, 1985). The sites on the carrier protein that are most often targeted are the carboxyl groups of the C-terminal and of the aspartic and glutamic acid residues, the amino groups of the N-terminal and of the lysine residues, the imidazo and phenolic functions of the histidine and tyrosine residues respectively, and the sulphhydryl groups of cysteine residues (Erlanger, 1980).

The method of Erlanger and Beiser (1964) was used for the conjugation of 5-iodocytidine to polylysine and to BSA. The reaction utilises the vicinal hydroxyl groups contained in the ribose moiety of both nucleosides and nucleotides. The oxidation of these hydroxyl groups with periodate yields a nucleoside-dialdehyde that readily undergoes condensation with primary amino

groups, for example, the ϵ -amino group of lysine present in the carrier protein. After removal of the excess periodate by the addition of ethylene glycol the reaction product was coupled to BSA or polylysine at pH 9.0-9.5. Subsequent reduction with sodium borohydride stabilizes the linkage between the haptenic group and BSA or polylysine. The reaction scheme is shown in Figure 6.4. The position of the iodine in the conjugate is at a point furthest away from the linkage to the carrier, which, as pointed out above, means the iodo-part of the compound is immunodominant.

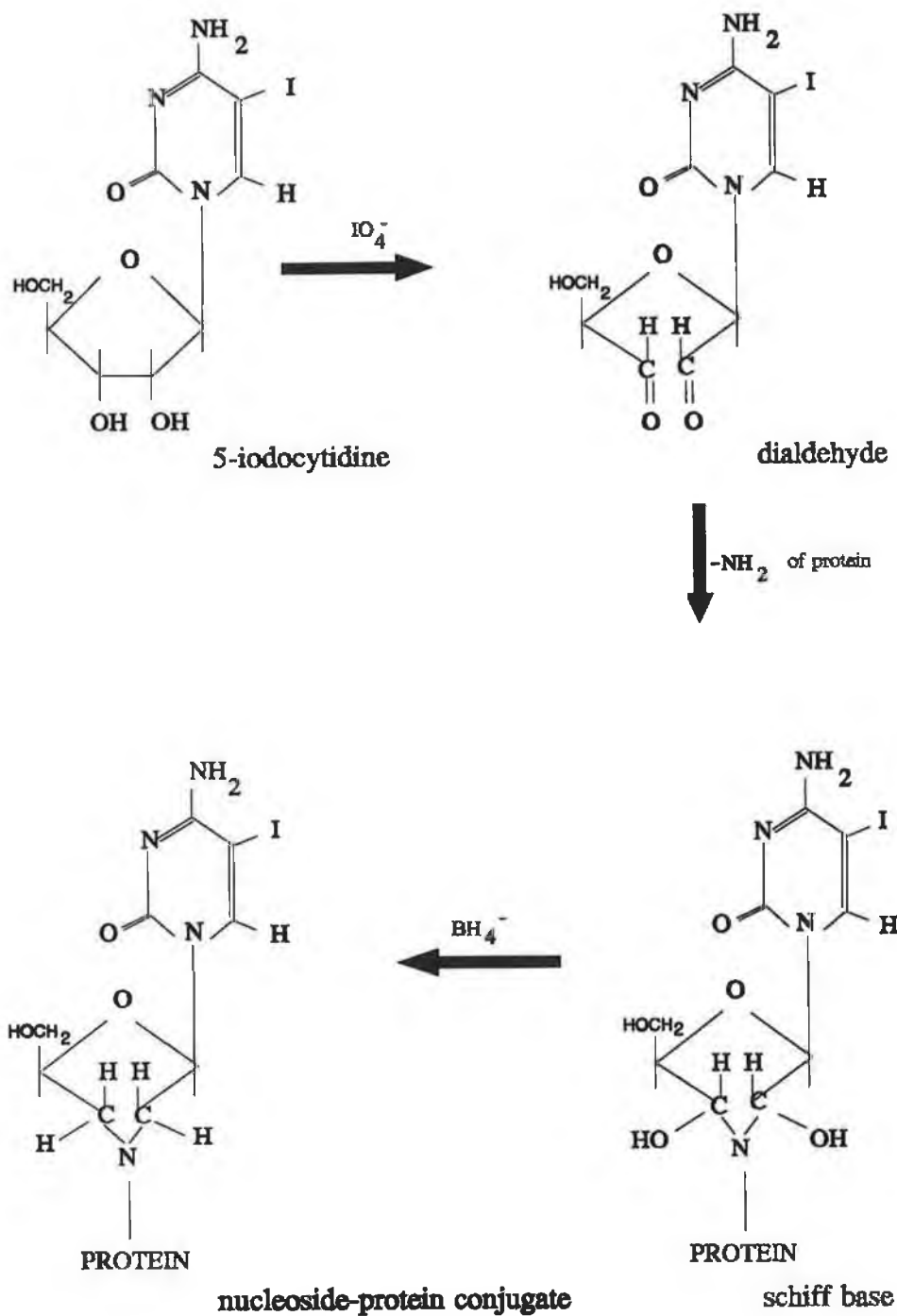


Figure 6.4
Schematic representation of the conjugation reaction whereby nucleosides (5-iodocytidine is the nucleoside illustrated here) are coupled to protein carriers (Erlanger and Beiser, 1964).

When the hapten-carrier conjugate has been synthesized it is necessary to remove all of the unconjugated hapten molecules. The most commonly chosen methods are exhaustive dialysis and affinity or gel chromatography. Dialysis was carried out on the 5-iodocytidine conjugates prepared.

The conjugate should be characterized in order to determine the number of hapten molecules per molecule of carrier protein. 5-Iodocytidine was conjugated to the carrier protein through its amino groups, therefore it is possible to determine the number of free amino groups before and after substitution. The method is based on the formation of a coloured product between the free amino groups and 2-4-6-trinitrobenzenesulphonic acid (TNBS) (Habeeb, 1964).

The conjugate may have a different absorption spectrum to the carrier protein due to the presence of the hapten and such information is useful in determining the degree of nucleoside conjugation (Erlanger *et al.*, 1957). The absorbance spectra of the conjugates prepared were examined after they were purified using a Sephadex G-25 column.

The molecular weight of the carrier protein before and after conjugation can be determined. If the hapten is sufficiently large only a small number of hapten molecules will be sufficient to cause a significant increase in the molecular weight. HPLC with a gel filtration column (Carty and O'Kennedy, 1988) can be used to determine an increase in molecular weight of the conjugate. Both the polylysine and BSA conjugates were eluted through a gel filtration column using HPLC to observe if there was a change in molecular weight.

6.31.2 Immunization

Although hapten molecules are conjugated to carrier proteins, adjuvants are also used in order to maximize the immune response. Adjuvants are immunopotentiators which activate the immune response. Freund's complete adjuvant, which contains heat-killed *Mycobacterium tuberculosis*, was used for the initial immunization, while incomplete Freund's adjuvant was used for

subsequent booster immunizations. The antigen was suspended in 0.1M PBS, pH 7.4, and vortexed with the adjuvant to form a stable oil-in-water emulsion before immunization.

The animals that are commonly used for polyclonal antibody production are rabbit, guinea pig, goat, sheep, donkey and horse. Rabbits are the most commonly used as they are relatively cheap, easy to handle and can yield large volumes of serum (Cahill *et al.*, 1994). A New Zealand white male rabbit was used in this work and the animal was immunized intradermally at four sites.

6.31.3 Antibody Purification

Polyclonal antibodies can be purified using techniques such as ammonium sulphate precipitation and affinity chromatography. Ammonium sulphate precipitation is a crude method which is used as an initial purification step. Antibodies may be further purified using affinity chromatography. Protein A and G affinity chromatography columns can be used to selectively remove immunoglobulins on the basis of their class. Affinity chromatography, using the antigen bound to activated sepharose, serves to remove those antibodies specific for the antigen. Gratzner (1975) used affinity chromatography to purify the polyclonal antibodies produced against bromouridine. Bromouridine was coupled onto AH Sepharose 4B (Pharmacia) and thus anti-bromouridine was removed by the column while non-specific IgGs or proteins were eluted. Ammonium sulphate precipitation was used as a initial purification step for the polyclonal antibodies produced in this work.

6.31.4 Development of ELISA

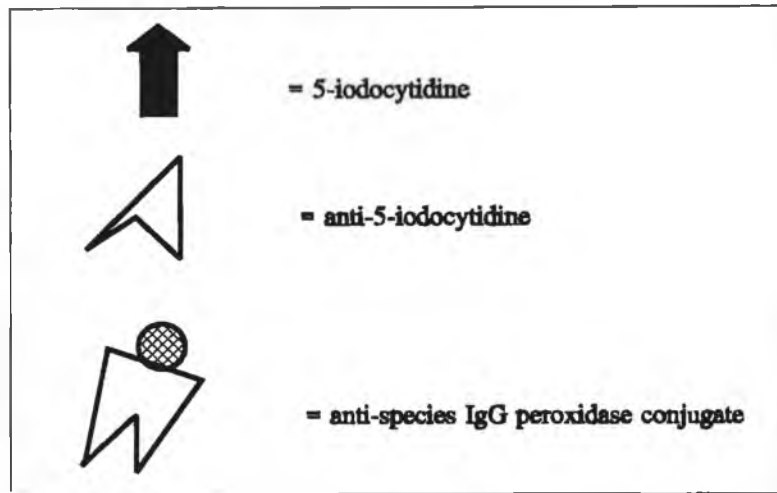
All immunoassays are based on antigen-antibody interactions. The ability to immobilize both antigens and antibodies onto solid supports lead to the development of Enzyme-Linked ImmunoSorbent Assays (ELISA). In an ELISA the antigen-antibody complex is separated from the free antigen and antibody and can be either a competitive or a non-competitive format (reviewed by O'Kennedy, 1989).

In a competitive ELISA, sample or test antigen competes with labelled antigen for a limited amount of immobilized antibody. Alternatively antigen can be immobilized and sample or test antigen competes for binding to the labelled antibody. In a non-competitive ELISA system antigen or antibody can be immobilised. If the antigen is immobilized the antibody binds to it and this bound antibody is detected by using a second labelled antibody. The enzyme label is detected by adding a substrate for the enzyme and detecting the coloured, fluorescent or luminescent product. If the antibody is immobilized the antigen is allowed to bind and a second labelled antibody is used to detect the bound antigen (Sandwich ELISA).

A non-competitive ELISA system was used here for screening serum for antibody production. [The scheme for the non-competitive ELISA used is shown in Figure 6.5]. The antigen was immobilized onto the solid support and dilutions of serum were added. Bound antibody was detected using a second enzyme-labelled antispecies IgG antibody. Substrate was then added.

Antigen, iodocytidine, was coated directly onto Nunc *Covalink-NH* plates. *Covalink-NH* is a polystyrene surface grafted with secondary amino groups which can be used to covalently couple molecules to the plate. Parker and Halloran (1968) described a method for the preparation of nucleotide-protein conjugates. Water soluble carbodiimides, such as EDC, were found to promote conjugation between proteins and mononucleotides and they suggested that the predominant bond formed is a phosphoramidate bond between amino groups and the phosphate moiety of the nucleotide. This reaction was carried out in the well of a Nunc *Covalink-NH* plate and the bond formed is that between the amino groups grafted to the plate and the phosphate moiety of the nucleotide (see Figure 6.6).

A competitive ELISA was also carried out. Antigen was bound to the plate as described for the non-competitive ELISA, but in this format free antigen competed with bound antigen for the partially-purified antibody.



1) The antigen 5-iodocytidine was covalently bound to the Nunc Covalink-NH plate.

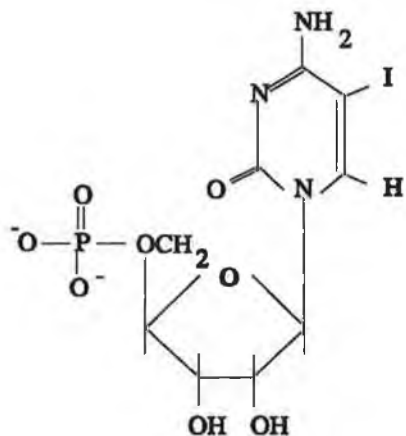


2) Dilutions of anti-serum were added after the plate was blocked.

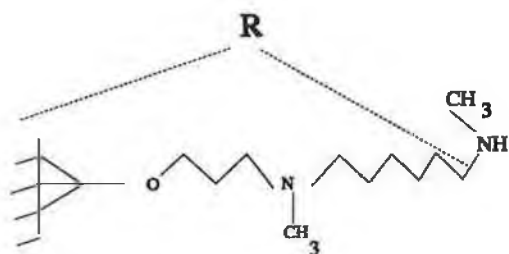


3) Anti-species IgG peroxidase conjugate was added and bound antibody was detected.

Figure 6.5
Scheme for non-competitive ELISA.



5-iodocytidine 5'-monophosphate



amino group on Covalink-NH plate



EDC

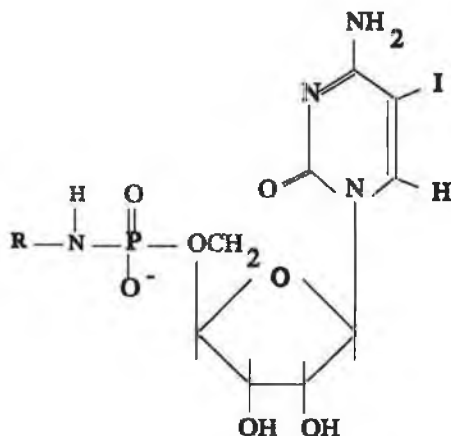


Figure 6.6

Phosphoramidate bond formed between amino groups and the phosphate moiety of the nucleotide (Parker and Halloran, 1968) in the presence of EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide). The amino group on the surface of the Nunc Covalink-NH plate is also illustrated.

6.32 Results

The conjugates which were produced by the method of Erlanger and Beiser (1964) [cytidine-BSA and 5-iodocytidine-BSA; cytidine-polylysine and 5-iodocytidine-polylysine] were characterized using the following methods.

6.32.1 Determination of free amino groups in BSA and polylysine after conjugation to the haptens cytidine and 5-iodocytidine, by the 2-4-6-trinitrobenzenesulphonic acid (TNBS) assay

The TNBS assay was used to determine free amino groups in the conjugates. Figure 6.7 shows the standard curve obtained for absorbance at 335nm (wavelength at which the coloured product formed between the free amino group and TNBS absorbs) plotted against lysine concentration. Lysine concentration is proportional to free amino group concentration.

The results shown in Table 6.1 indicate that both BSA and polylysine conjugates have fewer free amino groups than unconjugated BSA and unconjugated polylysine. BSA has 57 lysines, assuming they all react with TNBS it is possible to estimate that 22% of these have reacted with 5-iodocytidine, thus, thirteen 5-iodocytidine molecules are conjugated to 1 molecule of BSA. 34% of the total lysines have reacted with cytidine. Hence, nineteen cytidine molecules are conjugated to BSA. The results obtained for the polylysine conjugates can be interpreted qualitatively. The values obtained indicate that approximately 60% of the amino groups have been used in the conjugation of hapten to polylysine. However, a control-polylysine was run thus, these results were not interpreted quantitatively.

SAMPLE	mM Lysine per mg/ml of sample	% Free Amino groups
BSA	0.688	100
CONTROL-BSA	0.693	101
CYTIDINE-BSA	0.463	67
IODOCYTIDINE-BSA	0.538	78
POLYLYSINE	8.377	100
CYTIDINE-POLYLYSINE	3.079	37
IODOCYTIDINE-POLYLYSINE	3.399	41

Table 6.1

The determination of free amino groups, by the TNBS assay (Section 2.52.2), in BSA and polylysine and in the cytidine and 5-iodocytidine conjugates produced. The concentration of lysine (mM) in 1 mg/ml concentration of sample, as calculated from a lysine standard curve, is shown. [Lysine concentration is used as a measure of free amino groups]. It is assumed that BSA and polylysine have 100% free amino groups and the values for the controls and the conjugates are given relative to these.

Absorbance at 335nm

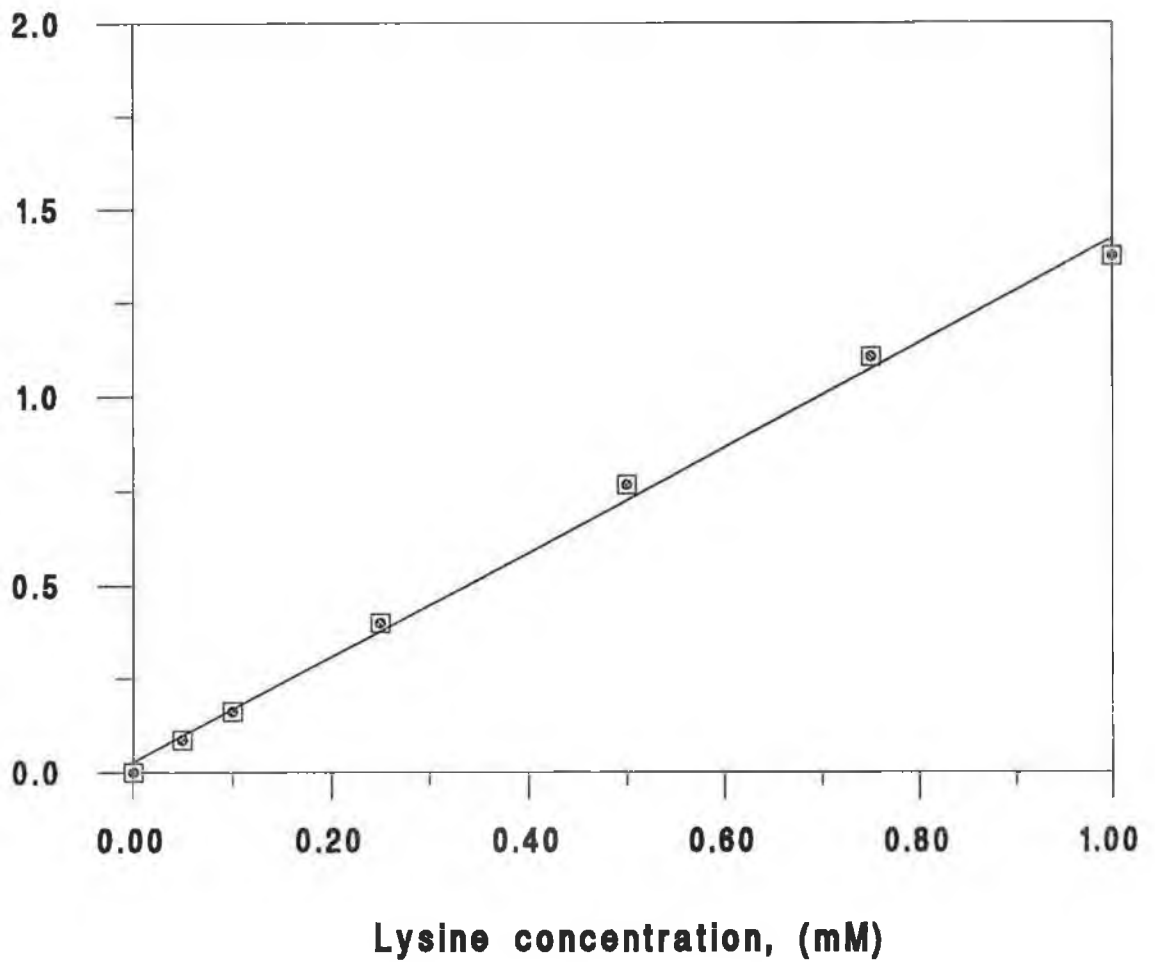


Figure 6.7

Standard curve of absorbance at 335nm plotted against lysine concentration (mM), for the TNBS assay [$r = 0.998$]. Lysine concentration is a measure of free amino group concentration.

6.32.2 Gel exclusion chromatography and analysis of absorbance spectra

The conjugates were eluted through a Sephadex G-25 column, after dialysis, to ensure that any free iodocytidine or cytidine was removed before reading the UV absorption spectra. The elution profiles are shown in Tables 6.2 and 6.3. These elution profiles indicate that no free iodocytidine is present in the conjugate fractions.

Absorbance at given wavelength (nm)

Fraction number	Polylysine		5-Iodocytidine		Polylysine/5-iodocytidine mixed		Polylysine/5-iodocytidine conjugate	
	220	293	220	293	220	293	220	293
1								
2					+			
3	+				+		+	+
4	+				+		+	+
5							+	+
6			+	+	+	+		
7			+	+	+	+		
8			+	+	+	+		
9			+	+	+	+		
10								

Table 6.2

Elution profiles through Sephadex G-25 (conditions described in Section 2.52.3) obtained for the following: polylysine; the hapten, 5-iodocytidine; polylysine and 5-iodocytidine mixed and the conjugate 5-iodocytidine-polylysine. The 5-iodocytidine-polylysine conjugate elutes in fractions corresponding to polylysine and these fractions also absorb at 293nm (the absorbance maximum of 5-iodocytidine). This absorbance is due to conjugated 5-iodocytidine as free iodocytidine elutes in higher fractions.

Absorbance at given wavelength (nm)
BSA 5-Iodocytidine 5-Iodocytidine-BSA

Fraction number	BSA		5-Iodocytidine		5-Iodocytidine-BSA	
	280	293	280	293	280	293
13	+					
14	+					
15	+				+	+
16					+	+
17					+	+
18					+	+
19						
20						
21						
22						
23						
24						
25						
26			+	+		
27			+	+		
28			+	+		

Table 6.3

Elution profiles through Sephadex G-25 (conditions as described in Section 2.42.3) obtained for the following: BSA; the hapten, 5-iodocytidine and the conjugate 5-iodocytidine-BSA. The elution profiles illustrate that no free 5-iodocytidine is present in the 5-iodocytidine-BSA conjugate sample.

Absorption spectra of the fractions containing the conjugates were taken and these are shown in Figure 6.8 and 6.9. Figure 6.8 shows the spectrum overlays obtained for the BSA conjugates while Figure 6.9 shows similar spectra for the polylysine conjugates prepared. The change in the UV absorption spectrum for the polylysine conjugates is more apparent than for the BSA conjugates. This is because the wavelength of maximum absorbance for polylysine (220nm) and for 5-iodocytidine (293nm) and cytidine (271nm) are further apart than for BSA (280nm). Table 6.4 gives the wavelengths of the absorbance peaks, as given by the Peak-Pick facility on the Shimadzu UV visible recording spectrophotometer. The wavelength of maximum absorbance for the polylysine conjugates at 287 and 271nm for the 5-iodocytidine and cytidine conjugates respectively indicate that these haptens have been conjugated to polylysine.

SAMPLE	Peaks in Abs. spectrum (200-320nm), wavelength, (nm)	
BSA	233	278
control-BSA	232	278
iodocytidine-BSA	236	276
cytidine-BSA	230	271
Polylysine	221	
control-polylysine	211	
iodocytidine-polylysine	232	287
cytidine-polylysine	233	271

Table 6.4

UV absorption spectrum (200nm-320nm) were read for the samples above on the Shimadzu UV visible recording spectrophotometer (UV-160A) and the wavelengths of the absorbance peaks recorded using the peak-pick facility of the instrument. The shift in absorbance peaks indicates that conjugation has occurred.

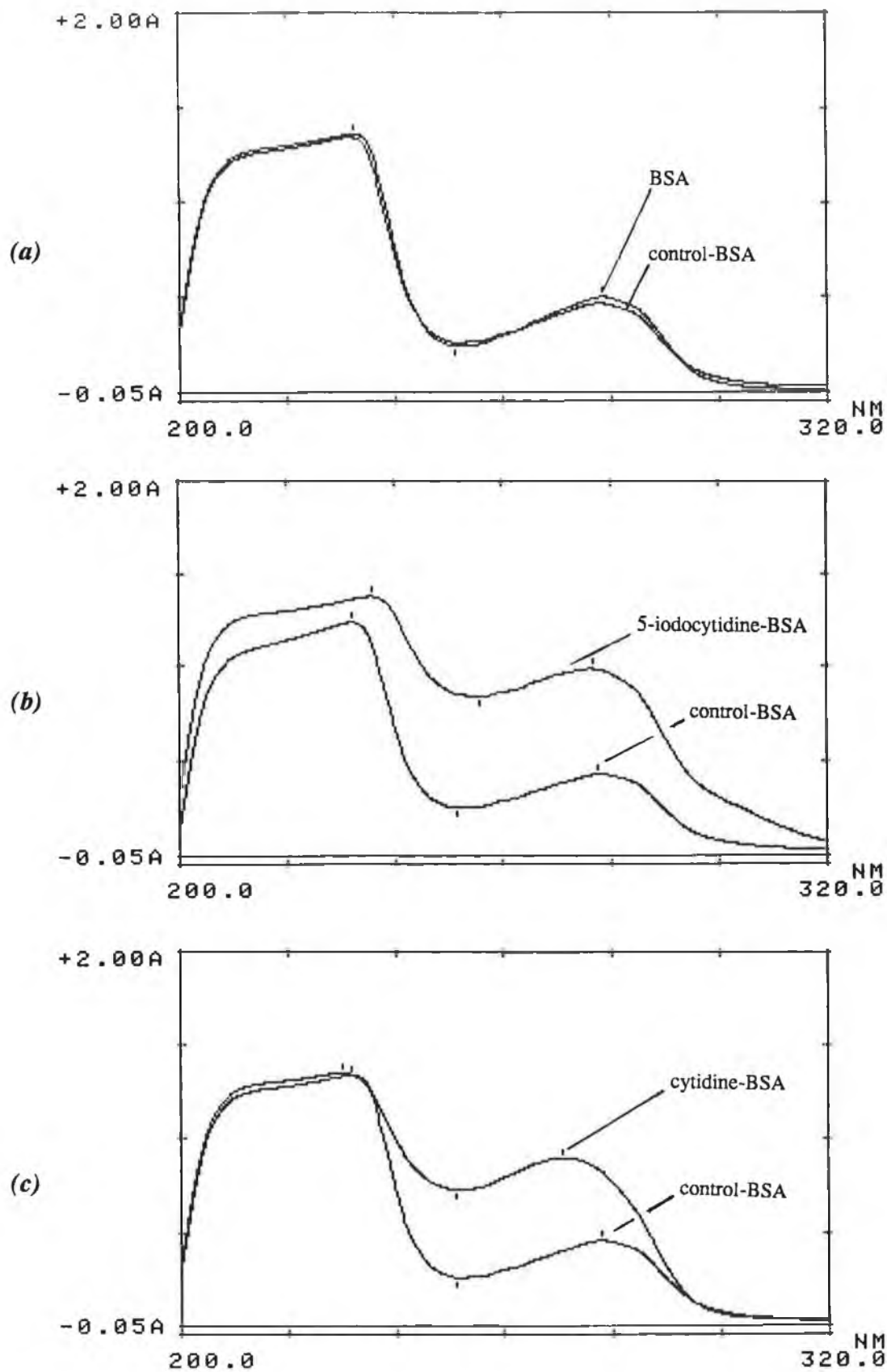


Figure 6.8
 UV absorption spectra (200nm-320nm), recorded on a dual UV-visible spectrophotometer (UV-160A, Shimadzu), for the following:
 (a) BSA and control-BSA
 (b) control-BSA and 5-iodocytidine-BSA
 (c) control-BSA and cytidine-BSA

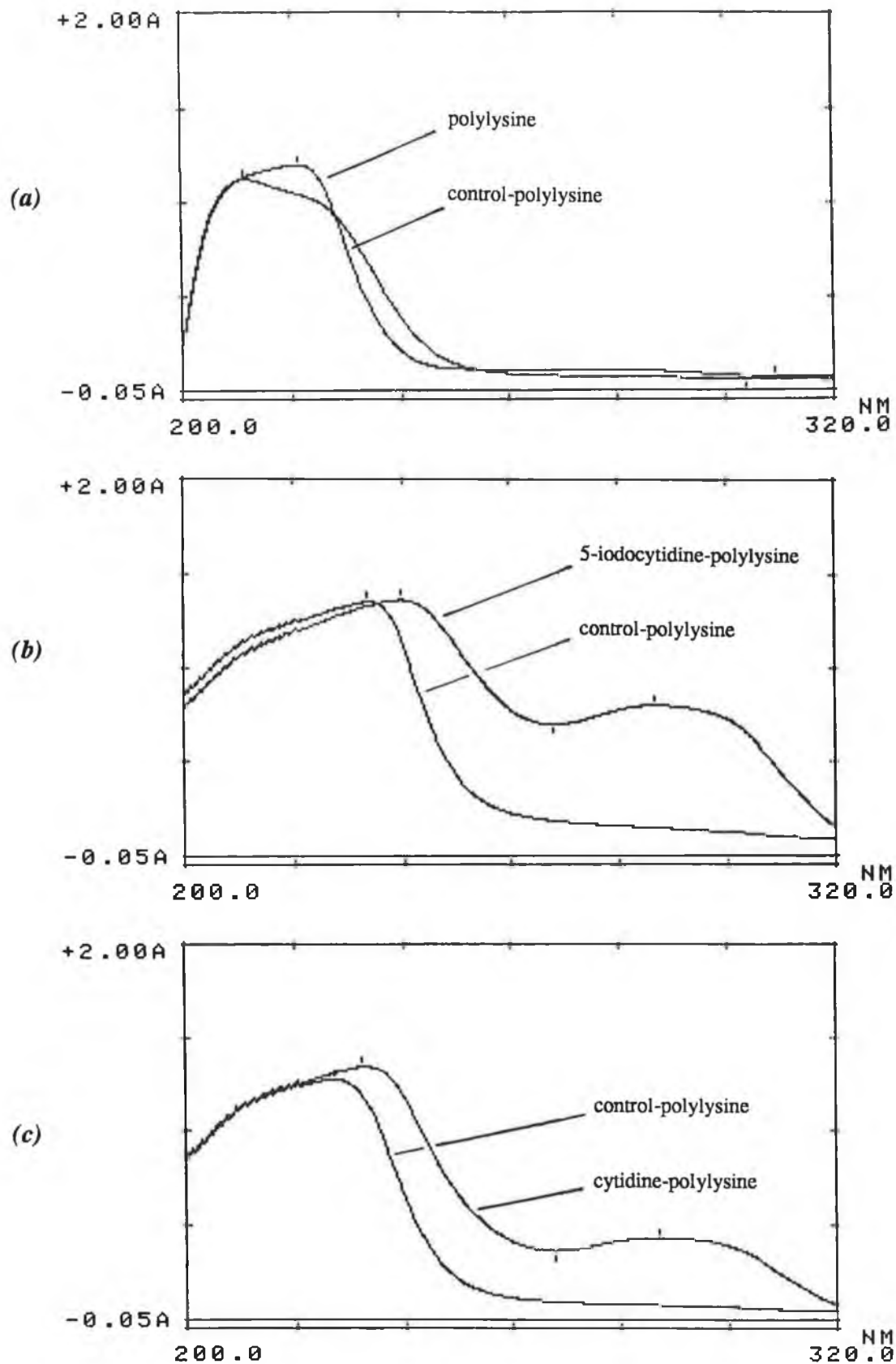


Figure 6.9
 UV absorption spectra (200nm-320nm), recorded on a dual UV-visible spectrophotometer (UV-160A, Shimadzu), for the following:
 (a) polylysine and control-polylysine
 (b) control-polylysine and 5-iodocytidine-polylysine
 (c) control-polylysine and cytidine-polylysine

The absorbance ratios determined for the conjugates are further evidence that conjugation of the haptens, 5-iodocytidine (λ_{max} is 293nm) and cytidine (λ_{max} is 271nm), to polylysine and BSA has taken place. These absorbance ratios are shown in Table 6.5.

SAMPLE	ABSORBANCE RATIO	
control-polylysine	220:271	7.06
cytidine-polylysine	220:271	1.01
control-polylysine	220:293	9.00
iodocytidine-polylysine	220:293	1.60
control-BSA	280:271	1.10
cytidine-BSA	280:271	0.85
control-BSA	280:310	12.56
iodocytidine-BSA	280:310	5.77

Table 6.5
Absorbance ratios of the conjugates prepared in comparison to control-BSA and control-polylysine. The lower ratios obtained for the conjugates indicate that hapten conjugation has occurred.

Using absorbance values for the 5-iodocytidine-polylysine conjugate it is possible to determine the mole to mole ratio of hapten to carrier. A standard curve of absorbance at 293nm against 5-iodocytidine concentration was plotted (see Figure 6.10). From this curve the concentration of 5-iodocytidine in the conjugate was determined. Absorbance at 287nm was used as there was a shift in the wavelength of maximum absorbance with conjugation to polylysine. It was estimated that there were 18 moles of 5-iodocytidine to every mole of polylysine. The ratio of moles hapten to moles BSA could not be calculated in this manner because the absorbance at 280nm for BSA is too close to the absorbance at 293nm for 5-iodocytidine.

Absorbance at 293nm

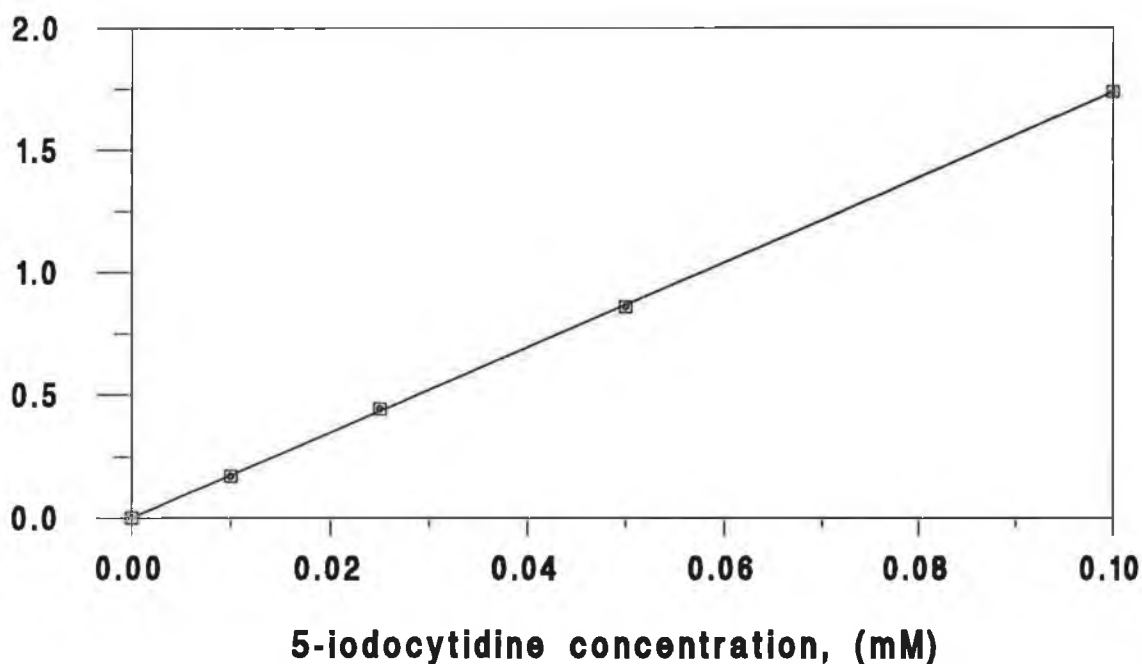


Figure 6.10

Standard curve of absorbance at 293nm plotted against 5-iodocytidine concentration [$r = 1.000$] which was used to determine the mole:mole ratio for 5-iodocytidine and polylysine in the 5-iodocytidine-polylysine conjugate.

6.32.3 High Performance Liquid Chromatography (HPLC) of the conjugates

HPLC was used to assess the purity of the conjugates, that is, the absence of free hapten. The technique was also used to determine if there was any significant difference in the retention time between BSA and polylysine and their respective conjugates. The gel filtration column separates proteins on the basis of size and, thus, one would expect the conjugate, as it would have a slightly higher molecular weight, to elute first. This is not the case and this could be for two reasons: although the molecular weight of the conjugate is greater its tertiary conformation might be smaller than BSA or polylysine or due to polarity changes on the surface of the protein molecule or polypeptide elution through the column is affected.

The following chromatograms are shown: Figure 6.11 shows an overlay of the chromatograms obtained for polylysine and for 5-iodocytidine-polylysine. Absorbance was read at 220nm. The retention time for the conjugate was slightly longer than for polylysine. No peak is observed at 25 minutes, the retention time for the hapten 5-iodocytidine, indicating that there is no free 5-iodocytidine or cytidine in the preparation after dialysis. Figure 6.12 is the chromatogram obtained for the conjugate when absorbance was read at 293nm. The retention time indicates that this peak is due to the conjugate and not free iodocytidine. Polylysine does not absorb at this wavelength thus, the absorbance observed is due to 5-iodocytidine conjugated to polylysine.

Figures 6.13-6-15 show the following chromatogram overlays: BSA and control-BSA [Control-BSA is the BSA conjugation control prepared by carrying out a conjugation reaction but without cytidine or 5-iodocytidine]; control-BSA and iodocytidine-BSA; and control-BSA and cytidine-BSA. A comparison of these chromatograms seems to indicate that there is no difference between the control-BSA and the actual conjugates when eluted on the Biosep-sec-S400 column. Thus, from these chromatograms the only

conclusion that can be made is, that there is no free 5-iodocytidine or cytidine in the preparation after dialysis. There is no apparent shift in molecular weight.

Retention times for the conjugates and both BSA and polylysine are shown in Table 6.6. Because the shift in retention time for the conjugation blanks are similar to that for the iodocytidine-conjugates it is not possible to conclude that there is an increase in molecular weight due to conjugation of the hapten using this method.

SAMPLE	Mean Retention Time	n	Standard Deviation	CV%
5-iodocytidine	25.9	6	0.123	0.47
cytidine	24.3	6	0.075	0.31
polylysine	23.4	6	0.047	0.20
control-polylysine	23.8	3	0.091	0.38
iodocytidine-polylysine	23.9	5	0.036	0.15
cytidine-polylysine	24.0	4	0.072	0.30
BSA	15.9	3	0.061	0.38
control-BSA	16.1	3	0.012	0.07
iodocytidine-BSA	16.0	3	0.009	0.05
cytidine-BSA	17.1	3	0.009	0.05

Table 6.6

Retention times (minutes) for polylysine and the polylysine conjugates obtained using the gel filtration column, Protein-pak (Waters Chromatography Division) and for BSA and its conjugates using the gel filtration column Biosep-sec-S400 (Phenomex) [Section 2.52.4].

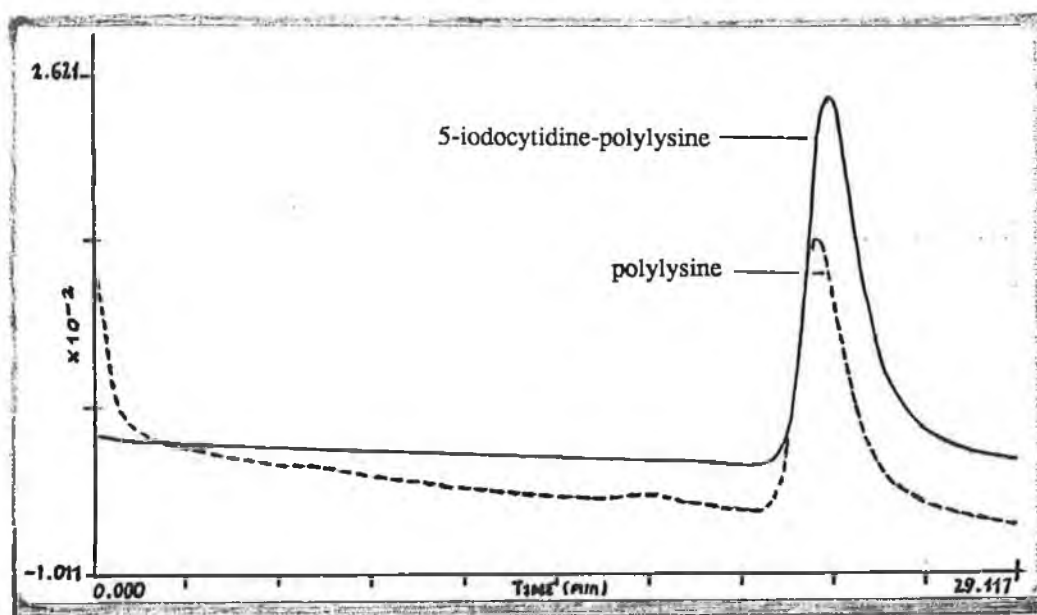


Figure 6.11

Overlay of the chromatograms obtained for polylysine (1mg/ml) and the conjugate 5-iodocytidine-polylysine (1mg/ml) eluted through a gel filtration column (Protein-Pak 300 sw 10 μ m, 7.8x300mm) by HPLC. The mobile phase was 0.1M sodium phosphate, pH 5.5, and the flow rate was 0.5ml/min. Absorbance was read at 220nm.

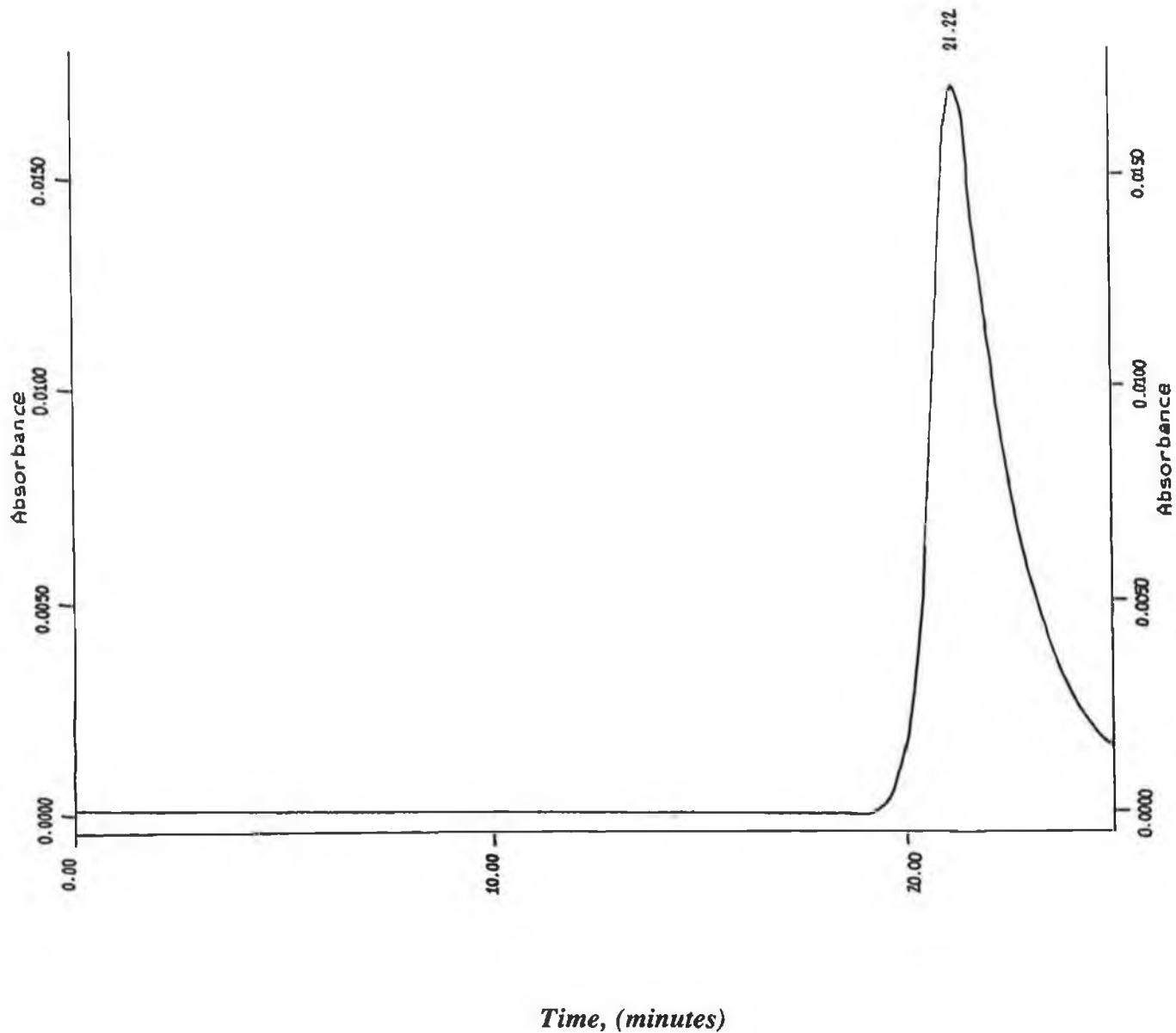


Figure 6.12

Chromatogram obtained for the conjugate 5-iodocytidine-polylysine (1mg/ml) eluted through a gel filtration column (Protein-Pak 300 sw 10 μ m, 7.8x300mm) by HPLC. The mobile phase was 0.1M sodium phosphate, pH 5.5, and the flow rate was 0.5ml/min. Absorbance was read at 293nm.

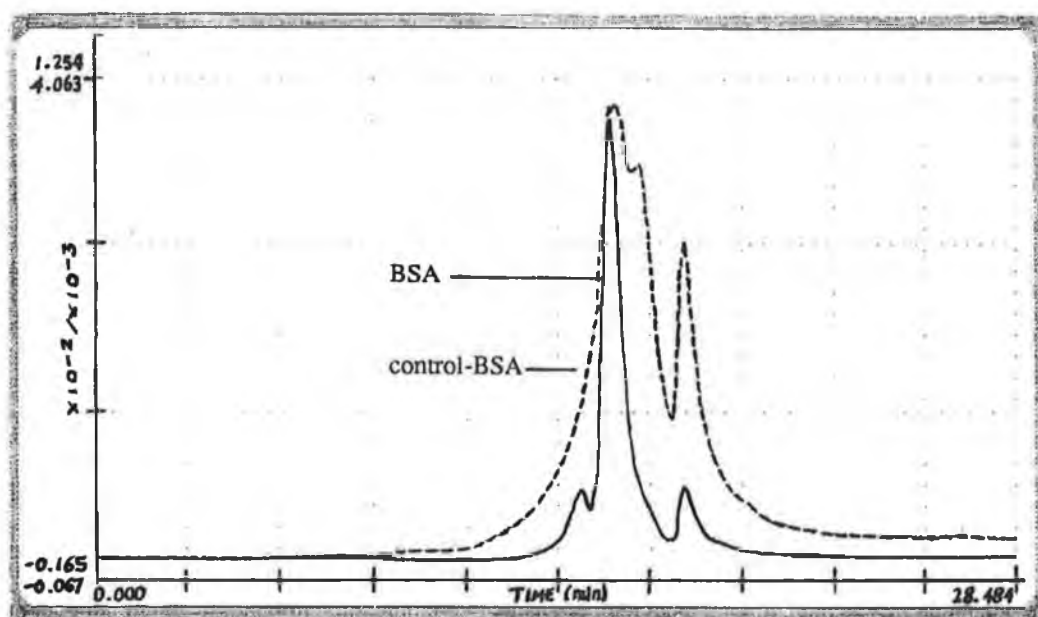


Figure 6.13

Overlay of the chromatograms obtained for BSA and control-BSA eluted through a gel filtration column (Biosep-sec-S400, 7.8x300mm) by HPLC. The mobile phase was 0.1M sodium phosphate, pH 7.0, and the flow rate was 0.75ml/min. Absorbance was read at 280nm.

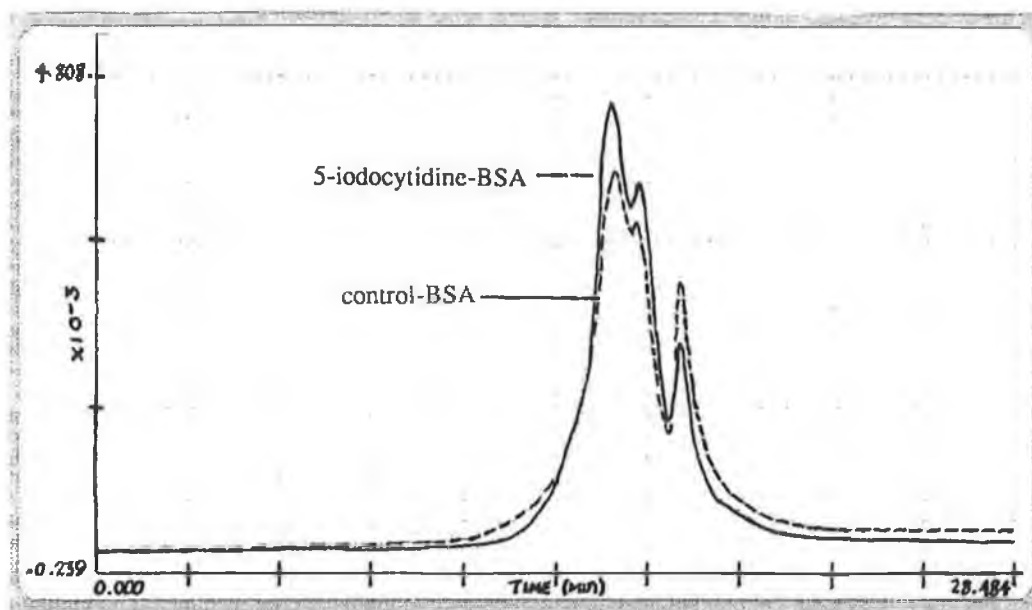


Figure 6.14

Overlay of the chromatograms obtained for control-BSA and 5-iodocytidine-BSA eluted through a gel filtration column (Biosep-sec-S400, 7.8x300mm) by HPLC. The mobile phase was 0.1M sodium phosphate, pH 7.0, and the flow rate was 0.75ml/min. Absorbance was read at 280nm.

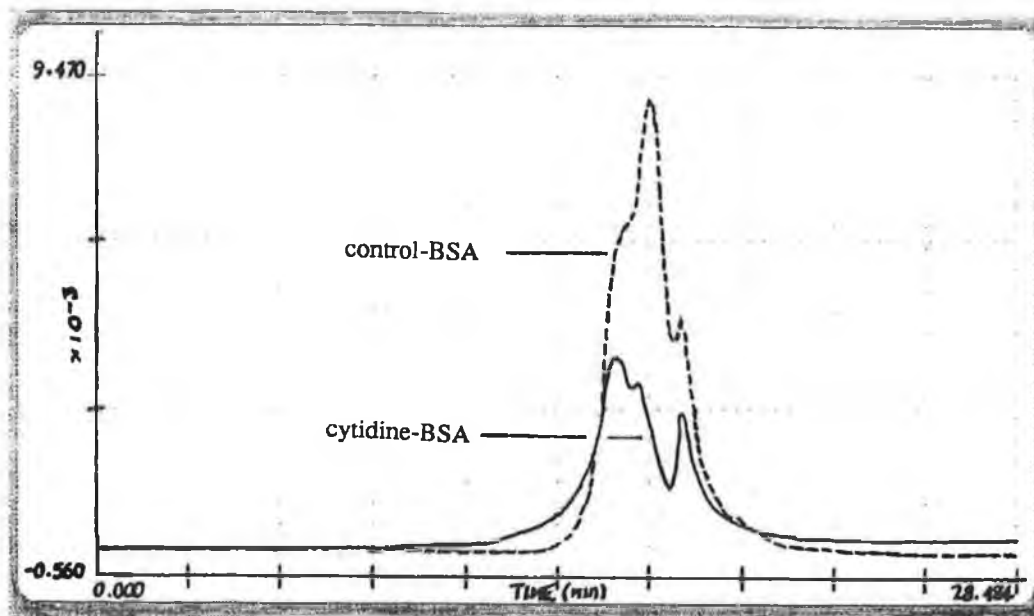


Figure 6.15

Overlay of the chromatograms obtained for control-BSA and cytidine-BSA eluted through a gel filtration column (Biosep-sec-S400, 7.8x300mm) by HPLC. The mobile phase was 0.1M sodium phosphate, pH 7.0, and the flow rate was 0.75ml/min. Absorbance was read at 280nm.

6.32.4 Characterization of conjugates: summary

The results obtained for the characterization of the conjugates (cytidine- and 5-iodocytidine-BSA and cytidine- and 5-iodocytidine-polylysine) prepared are summarised in Table 6.7.

METHOD	BSA conjugates	Polylysine conjugates
Determination of free amino groups in conjugates by the TNBS assay	13 moles iodocytidine: mole BSA 19 moles cytidine: mole BSA	Reduced number of free amino groups in conjugates \Rightarrow conjugation has occurred
UV spectrum analysis	Observed altered UV spectra for the conjugates	Spectra of conjugates altered: possible to determine that there were 18 moles of 5-iodocytidine per mole of polylysine
HPLC: gel filtration	No free 5-iodocytidine or cytidine present in dialysed preparation	No free 5-iodocytidine or cytidine present in dialysed preparation

Table 6.7
Summary of the results obtained for the characterization of the conjugates prepared.

6.32.5 Production and purification of 5-iodocytidine antibodies

6.32.5a Production of 5-iodocytidine antibodies

The 5-iodocytidine-BSA conjugate was used to raise polyclonal antibodies as described in Section 2.54. The highest antibody titre obtained was 1:5000 dilution of serum. The non-competitive ELISA used to determine the titre was outlined in Section 2.56. However, antibodies to cytidine were also obtained at the same dilution. Figure 6.16 shows the absorbance obtained in the ELISA when the bound antigen was either 5-iodocytidine or cytidine at a range of dilutions of the serum.

Absorbance at 405nm

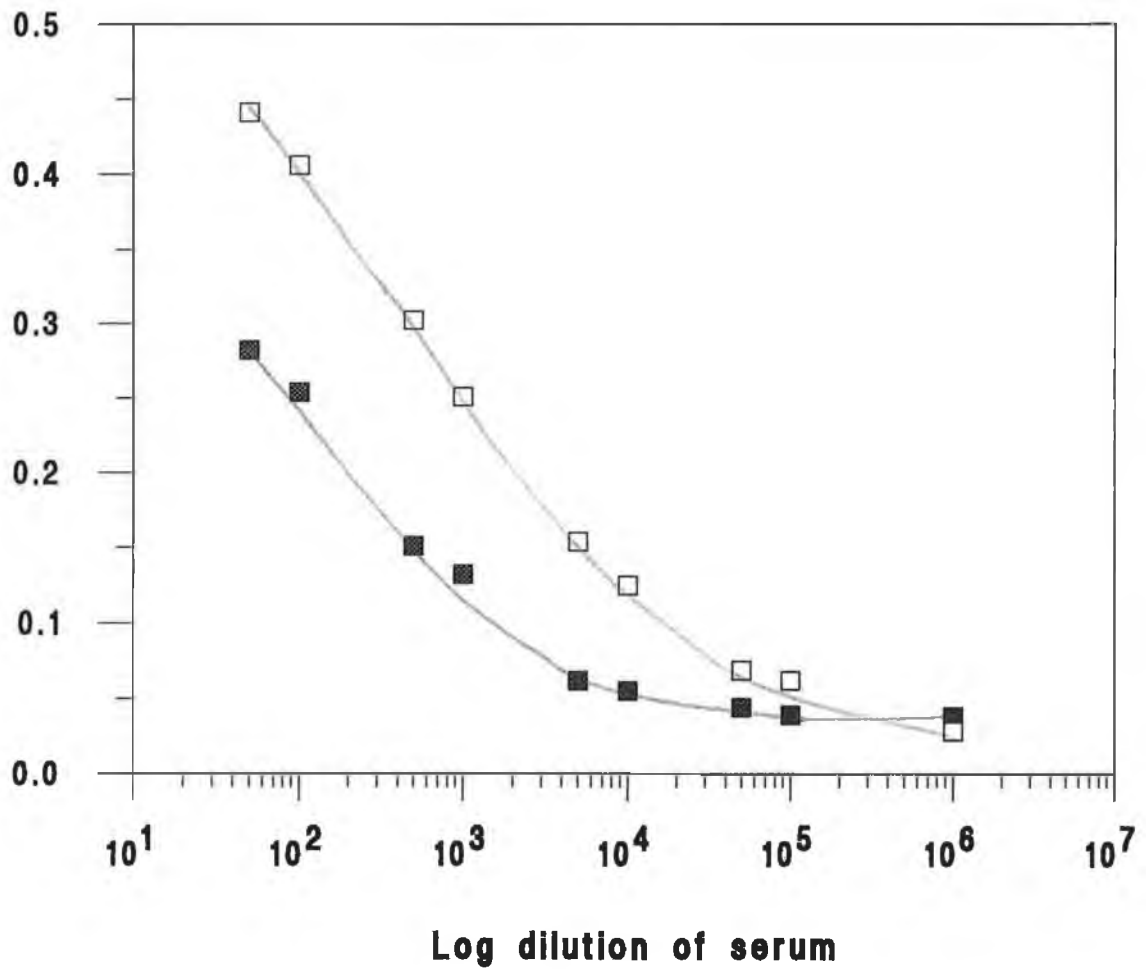


Figure 6.16

Absorbance plotted against Log [dilution of serum] for the non-competitive ELISA described in Section 2.56.

□) shows the curve obtained when the bound antigen was 5-iodocytidine

■) shows the curve obtained when the bound antigen was cytidine

6.32.5b Antibody purification: ammonium sulphate precipitation

10 ml of anti-serum (titre of 1/ 5000) was taken and two ammonium sulphate precipitations (50% w/v) were carried out as described in Section 2.55. The protein concentration of the resuspended antibody pellet was determined using the bicinchoninic acid protein assay (Section 2.52.1). 20 μ l of a 1mg/ml solution was injected onto the Biosep-sec-S400 (Phenomex) gel filtration column (see Section 2.56). One peak was obtained and the chromatogram is shown in Figure 6.17. The molecular weight corresponding to this retention time was calculated from a standard curve of retention time plotted against Log [molecular weight] using gel filtration molecular weight markers (Sigma). Molecular weight estimated for the peak obtained was 170,000 (+/- 8500). IgG has a molecular weight of approximately 160,000. Thus, it can be assumed that the one peak obtained on the chromatogram is IgG (see Figure 6.18).

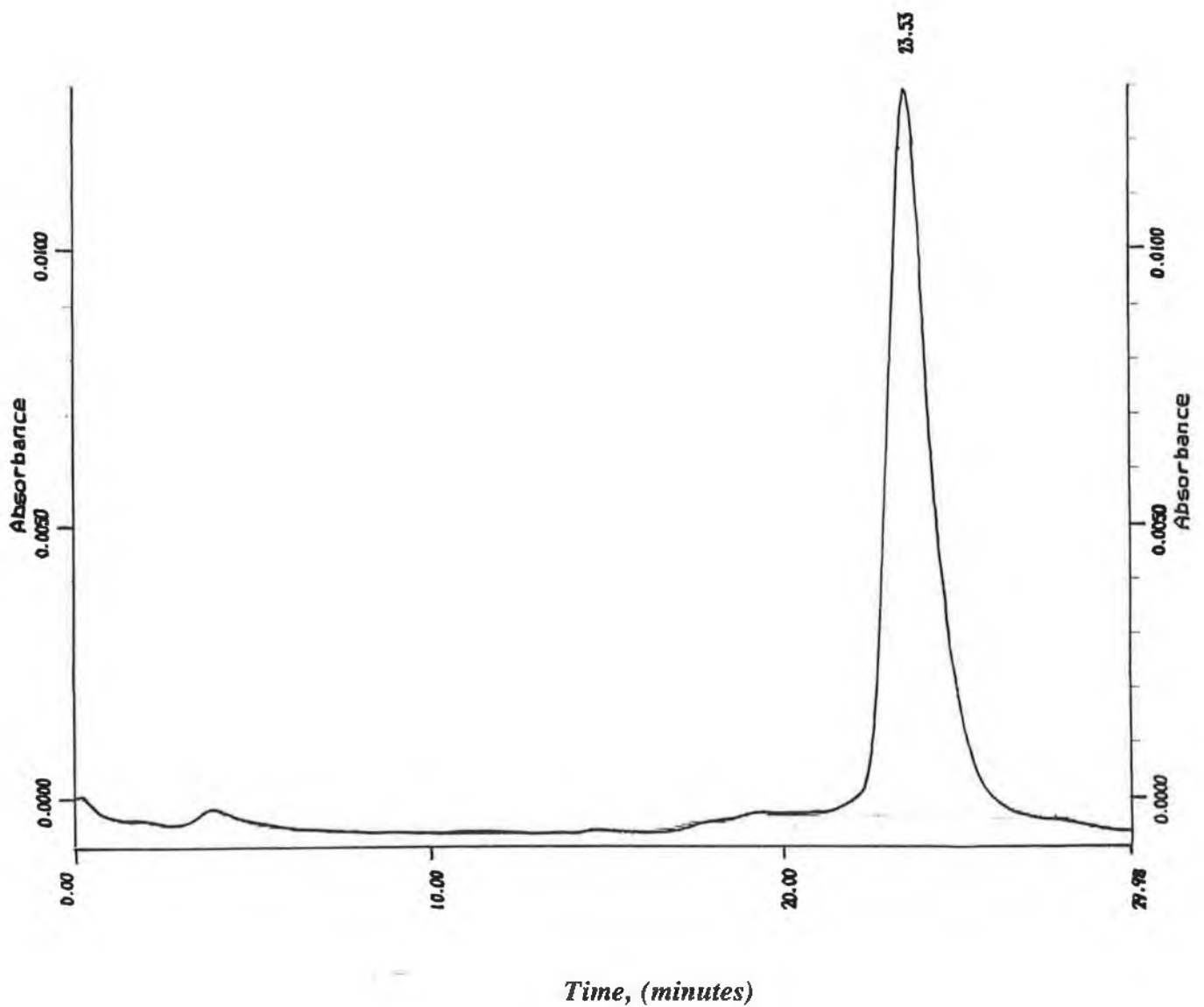


Figure 6.17

Chromatogram obtained for the partially purified antibody (after ammonium sulphate precipitation) on the Biosep-sec-S400, (7.8x300mm) gel filtration column. The mobile phase was 0.1M PBS, pH 7.4, and the flow rate was 0.5ml/minute. Absorbance was read at 280nm.

Retention time, (minutes)

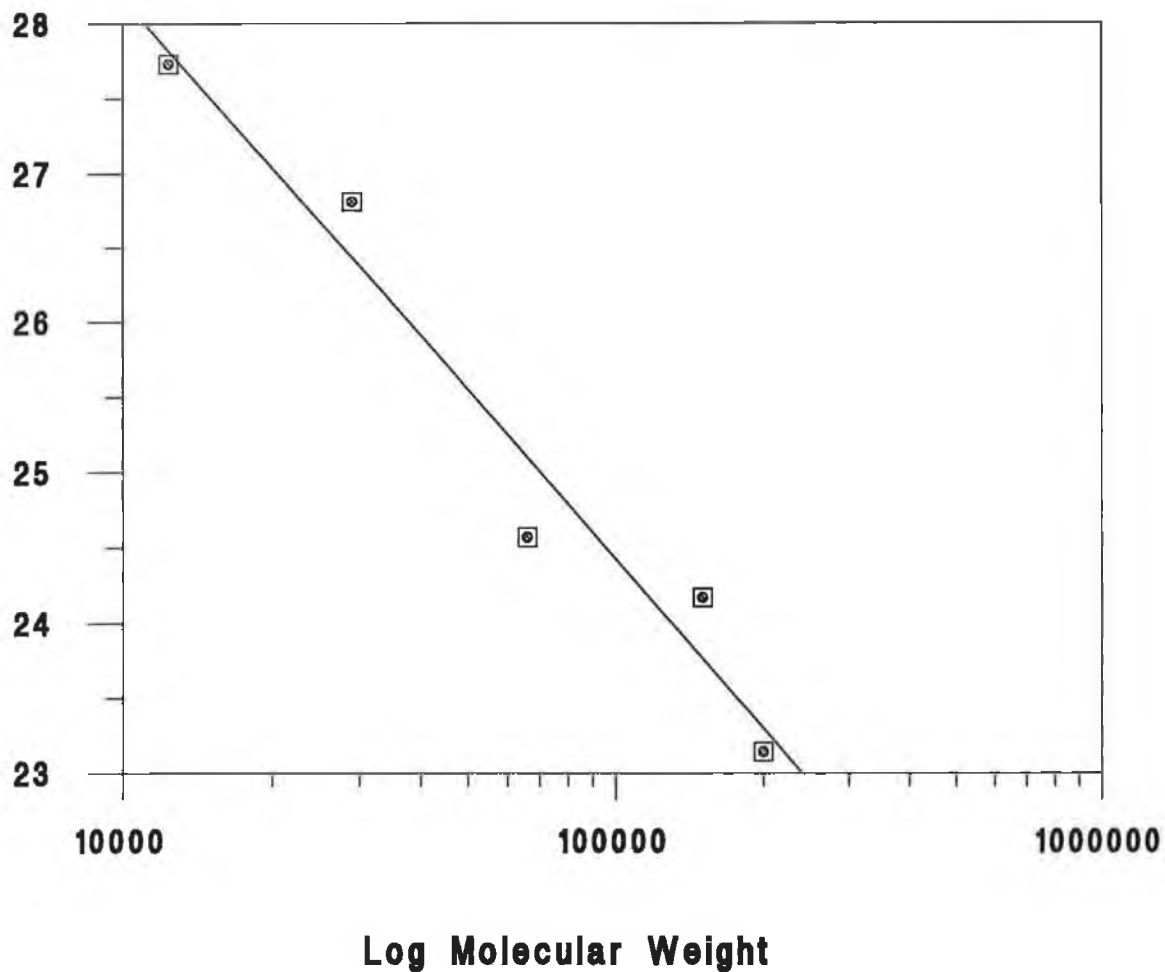


Figure 6.18

Retention time (minutes) plotted against Log [molecular weight] for a series of protein standards: cytochrome C (12,400); carbonic anhydrase (29,000); bovine serum albumin (66,000); alcohol dehydrogenase (150,000) and β -amylase (200,000) run on the Biosep gel filtration column (Section 2.56). The r value for this straight line graph is 0.98.

6.32.6 Development of a competitive ELISA for 5-iodocytidine

The working dilution of the partially-purified antibody was determined. The results for the checker-board ELISA are shown in Table 6.8. A working dilution of 1:2500 was chosen. The competitive ELISA was carried out as described in Section 2.57. A linear response was obtained for the concentration range 1-10 μ g/ml 5-iodocytidine using a semi-log plot (see Figure 6.19). However, a similar response was obtained when the bound antigen was cytidine (see Figure 6.20).

Dilution of partially-purified antibody

5-iodocytidine conc., μg/ml	0	2500	5000	7500	10000
0	0.100	0.455	0.312	0.281	0.254
5	0.101	0.444	0.344	0.332	0.259
10	.092	0.386	0.257	0.225	0.202
25	0.095	0.473	0.281	0.351	0.225
100	0.109	0.440	0.307	0.256	0.199

Table 6.8

Determination of working dilution of partially-purified anti-5-iodocytidine antibodies by competitive ELISA (Section 2.57) using a range of 5-iodocytidine standards (0-100 μ g/ml) and dilutions of the antibody. 1:2500 dilution was chosen.

Absorbance at 405nm

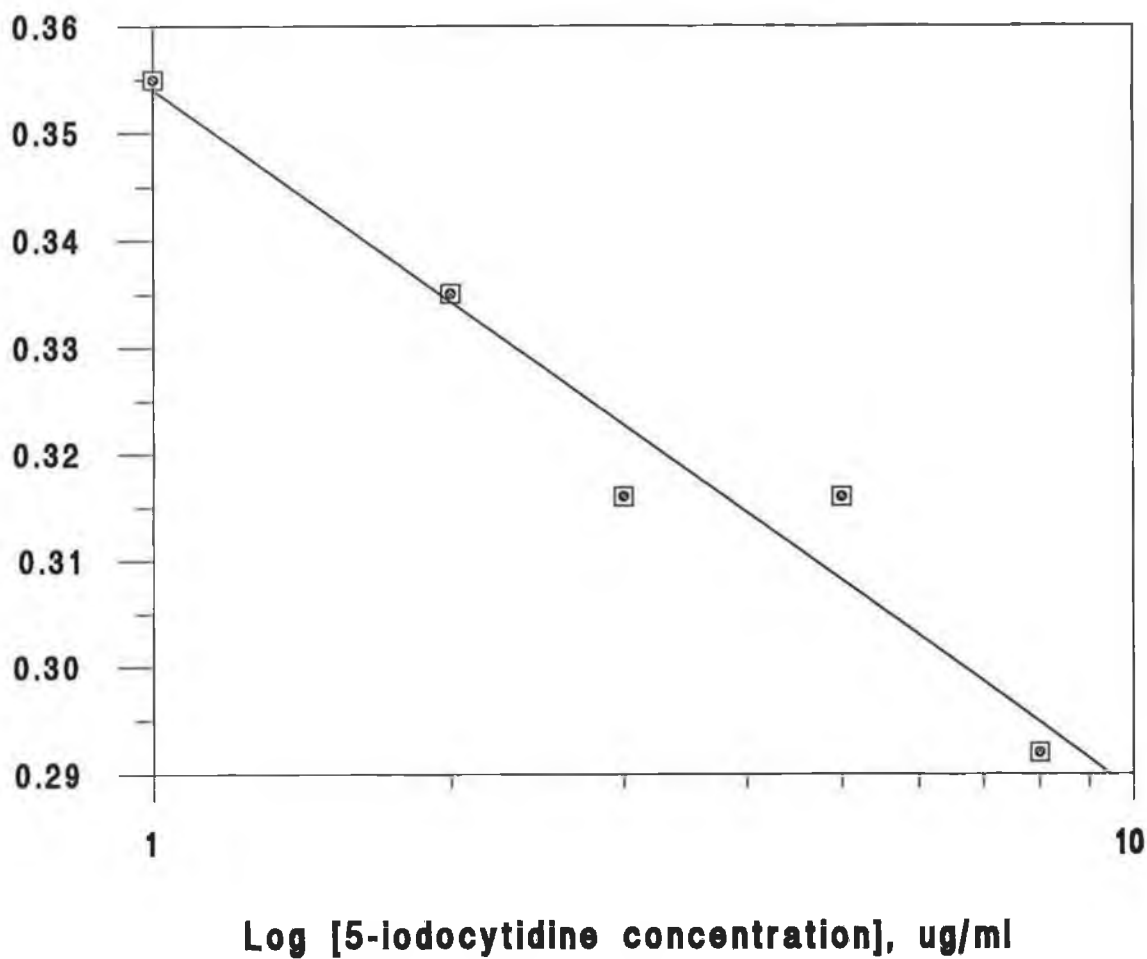


Figure 6.19

Determination of the linear response of 5-iodocytidine (bound antigen was 5-iodocytidine) in the competitive ELISA described in Section 2.57. The absorbance value was plotted against Log [5-iodocytidine concentration]. The linear range was 1-10 μ g/ml and the r value was 0.974.

Absorbance at 405nm

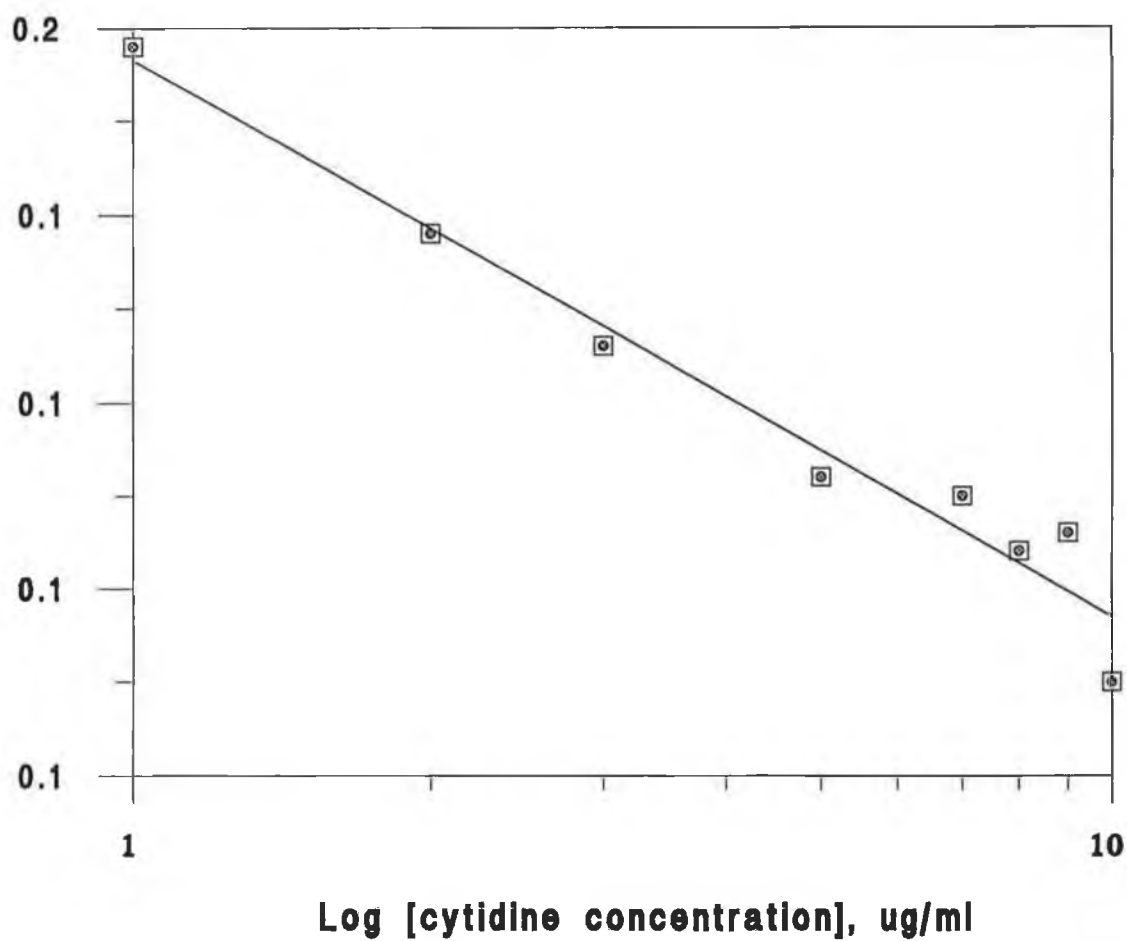


Figure 6.20

Determination of the linear response of cytidine (bound antigen was cytidine) in the competitive ELISA described in Section 2.57. The absorbance value was plotted against Log [cytidine concentration]. The linear range was 1-10 μ g/ml and the r value was 0.982.

6.33 Discussion

A suitable detection scheme was not available to detect iodine acting as a direct label for DNA probes and an indirect labelling approach was considered (see Section 1.21.3c). This system could be similar to either the digoxigenin indirect labelling scheme, where digoxigenin is detected using labelled anti-digoxigenin antibody (Martin *et al.*, 1990) or the system described by Sakamoto *et al.*, (1987) where anti-5-bromodeoxyuridine antibody binds to the label 5-bromodeoxyuridine, and the anti-hapten antibody is detected using a labelled anti-species IgG antibody.

The rabbit was immunized initially using 5-iodocytidine-polylysine conjugates. Polylysine was chosen as a carrier because of the availability of ϵ -amino groups for the periodate reaction used to prepare the conjugates, thus increasing the hapten:carrier ratio. Antibody titre obtained after a number of months was low (less than 1:50) using this conjugate and 5-iodocytidine-BSA conjugates were subsequently used to immunize the rabbit.

However, the polyclonal antibodies raised were non-specific for 5-iodocytidine, thus, it would seem that that the iodo-portion of the hapten was not immunodominant. It is possible that if further antibody production was carried out using different rabbits more specific antibodies would be produced, since even with genetically identical animals, a single preparation of antigen stimulates different B-lymphocytes producing different antibodies to it (Catty and Raykundalia, 1988).

Gratzner *et al.*, (1975) raised polyclonal antibodies to 5-bromouridine and an affinity column was used to purify the antibodies specific for 5-bromouridine. The hapten was covalently bound to a Sepharose column. If the polyclonal antibodies produced here were purified further using a similar method, such that the specificity of the antibodies is improved, a competitive ELISA giving a true standard-curve for 5-iodocytidine could be developed and the purified

5-iodocytidine could also be tested against 5-iodo-2'-deoxycytidine incorporated into single- and double-stranded DNA.

If the polyclonal antibodies produced were reactive against the modified nucleotide incorporated into DNA a model probe system could be set up to test the feasibility of the indirect labelling system proposed i.e., anti-5-iodocytidine antibody binds to 5-iodo-2'-deoxycytidine and the primary antibody is labelled or is detected using a labelled anti-species IgG antibody.

Chapter 7

CONCLUSION

The aims of this project were three-fold: to optimize and validate the iodide microassay developed by O'Kennedy *et al.*, (1989); to develop an alternative more sensitive microassay for iodide and to investigate the feasibility of using non-radioactive iodine as a label in DNA probe assays.

The sensitivity of the colorimetric microassay for iodide was increased ten-fold by the incorporation of nitric acid and the assay was validated in the range 1-100ng/ml. A number of chemiluminescent assays were considered. Though none of the assays developed were more sensitive than the colorimetric iodide microassay two of the systems investigated, System d (luminol/ hypochlorite/ iodide) and System e (luminol/ hydrogen peroxide/ tetrachloroauric acid/ iodide), warrant further developmental work.

Both the chemical substitution and the random priming methods used to iodinate nucleic acids were successful. However, for further work optimization of the conditions for the *Iodogen*-mediated iodination reaction should be carried out so that higher incorporation of iodine would be achieved. Iodination of the nucleic acids was detected using the modified iodide microassay. This assay is not sensitive enough to detect iodine acting as a direct label in a DNA probe assay and the results described in this thesis lead to the conclusion that the use of iodinated nucleotides as direct labels in the detection of DNA is not yet feasible given the methods available for the detection of iodide.

An indirect labelling system was also proposed where the iodinated nucleotide would be detected using an anti-5-iodo-2'-deoxycytidine antibody. This label could be detected either with a secondary antibody (labelled anti-species IgG antibody) or the primary antibody could be labelled itself. Polyclonal antibodies were raised against 5-iodocytidine. However, these antibodies were not specific for 5-iodocytidine (i.e. the iodo-portion of the hapten was not

immunodominant), and further purification work using an affinity column with bound 5-iodocytidine is necessary to isolate the required specific antibody, before a model probe system can be set up to investigate the feasibility of using the iodinated nucleotide as an indirect label in DNA probe assays.

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