THE USE OF Fab' ENZYME CONJUGATES FOR THE MEASUREMENT OF URINARY GROWTH HORMONE

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DECLARATION

The work presented in this thesis was performed solely by the author, except where otherwise acknowledged.

^{125}I	radioisotope of iodine
^{131}I	radioisotope of iodine
α MSH	alpha melanocyte stimulating hormone
Ab	antibody
ABTS	2,2-azino-bis(3 ethyl thiazoline-6-sulphonic acid)
acetyl co-A	acetyl co-enzyme A
ACTH	adrenocorticotrophic hormone
Ag	antigen
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	concentration
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
DELFIA	dissociation enhanced lanthanide fluoroimmunoassay
DNA	deoxyribonucleic acid
ε	extinction coefficient
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Epps	n-(2-(2 hydroxy ethyl)-piperazine-n-[y-propane-sulphonic acid]
Fab'	antibody fragment of IgG with antigen binding region
Fc	crystallisable fragment of IgG with no antigen binding region
FEIA	fluorescence enzyme immunoassay
g	gram
GABA	gamma amino butyric acid
GHRH	growth hormone releasing hormone
GnRH	gonadotrophin releasing hormone
H_2O_2	hydrogen peroxide
HGH	human growth hormone
HPPA	3-(4-hydroxy phenyl) propionic acid
HRP	horse radish peroxidase
ICMA	immunochemiluminometric assay
IGF-I	insulin like growth factor one
IgG	immunoglobulin G
IRMA	immunoradiometric assay
kD	kilo daltons
λ	lambda (wavelength)

M	molarity
µ	micro
mRNA	messenger ribonucleic acid
MW	molecular weight
NETRIA	North East Thames Radioimmunoassay
OPD	ortho phenylenediamine
p	pico (10^{-12})
pFc	terminal region of Fc fragment
RIA	radioimmunoassay
SAPU	Scottish Antibody Production Unit
TMB	3,3',5,5'-tetramethyl-benzidine dihydrochloride
TRH	thyrotrophin releasing hormone
Tris	Tris (hydroxy methyl) aminomethane hydrochloride
TSH	thyroid stimulating hormone
uv	ultraviolet
Å	absorbance
°C	degree Celsius

SUMMARY

This project was initiated following work performed within this Institute to produce antibodies against human growth hormone for use in a serum immunoradiometric assay (IRMA) and some work to investigate the use of insulin like growth factor I (IGF-I) as a diagnostic and management tool for patients with particular growth disorders. Following initial attempts by other workers within the Institute to measure urinary growth hormone, it was concluded that an IRMA was unlikely to be capable of providing adequate sensitivity for this application. This project was intended to investigate the possibility of using the available antibodies in a fluorimetric assay, using similar methodologies to those of Ishikawa et. al. (1987), (1988), to provide a sensitive assay for measuring urinary growth hormone on a routine basis.

The production of a suitable antibody fragment from sheep polyclonal IgG was the first major hurdle in the project and it was not until this problem was successfully traversed, by the use of an alternative method to that of Ishikawa et. al. (1988), that it was possible to progress onto production of a peroxidase conjugate or to develop a working assay.

In the resulting assay, microtitration wells were used as an alternative separation system to polystyrene beads, for reasons of practicability. Initially a number of colourimetric peroxidase substrates were used in the optimization of the assay, and later when appropriate measuring equipment became available, fluorimetric and luminometric substrates were used in the hope of achieving enhanced sensitivity. The luminometric substrate,which was only investigated briefly at the last stage of the project, provided an assay with sensitivity in the range necessary for a urine growth hormone assay.

A number of problems were encountered when attempting to measure growth hormone in urine due to the presence of matrix effects, and attempts were made to eliminate these as far as possible from the assay. Hence a significant proportion of the work carried out involved investigating these matrix effects and showed that dialysis of the urine samples produced unsatisfactory results.

An assay was developed with the sensitivity required to measure growth hormone in urine but the matrix effects of urine samples on the assay could not be overcome adequately. Perhaps the realistic conclusion to be drawn is that a successful assay of this type requires investment in state of the art microtitre plate equipment, detection systems and the best available antibodies and enzyme labels. These are most likely to be provided by a commercial company with an international market for the product and the necessary financial backing.

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CHAPTER 1 : INTRODUCTION

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1.1 BACKGROUND TO THE PROJECT

Growth hormone has been measured in serum, at the Institute of Biochemistry, Glasgow Royal Infirmary, for more than fifteen years. More recently insulin-like growth factor I (IGF-I) has been added to the list of available tests for the assessment of patients with abnormal secretion of growth hormone.

Recent publications have shown that measurement of growth hormone in urine is possible, despite the exceedingly low levels present. (Hashida et. al. 1985), (Girard et. al. 1990) There is accumulating evidence that measurement of growth hormone in urine may be useful in the diagnosis and management of acromegaly and Sheehans syndrome (Hashida et. al.1987) and in the investigation of children with short stature (Tanaka et. al. 1990), (Okuno et. al. 1987), (Hibi et. al. 1987).

A number of monoclonal antibodies have been raised against human growth hormone, by the staff of the Institute of Biochemistry. Two of these antibodies have been successfully incorporated into the 'in house' immunoradiometric assay for serum growth hormone, which is in routine use in the Institute (Perry 1992).

This project was initiated with the intention of utilising these available antibodies to produce an assay for growth hormone in urine, based on the published method of Ishikawa et. al. (1988). The project would thus involve producing an enzyme-antibody fragment conjugate as an alternative label to radioisotopic antibody. This conjugate could then be used with either colourimetric or fluorimetric enzyme substrates in an attempt to maximise the signal to noise ratio, and so improve the minimum detection limit of the immunometric assay.

At the outset of this project, the production of antibody fragments and enzyme conjugates was a relatively unexplored technique within the Institute.

1.2 GROWTH HORMONE

1.2.1 Biosynthesis and structure

Growth hormone (somatotropin) is a single chain 191 amino acid polypeptide hormone (MW 21,500) which is synthesized and secreted by the somatotroph cells of the anterior pituitary. Growth hormone is produced by cleavage of the larger, physiologically inactive precursor, pre-growth hormone (MW 28,000) (Baulieu and Kelly 1990). There is heterogeneity of growth hormone structure in plasma (section 1.2.6)

Growth hormone and human placental lactogen have similar structures, having the same number of amino acids (161 of which are identical), and both having two disulphide bonds. Prolactin has some structural similarities but has an additional sequence of amino acids, being 199 amino acids in length, and has a third disulphide bond (Baulieu and Kelly 1990).

The genes corresponding to growth hormone, human placental lactogen and prolactin are thought to have evolved from a single precursor which subsequently diverged producing differences in structure and function of the hormones. Each of the genes contains distinct regulatory sequences. The genes for growth hormone and human placental lactogen exist as multiple copies which are closely linked on chromosome 17, while prolactin exists as a single copy on chromosome 6.

1.2.2 Function

Growth hormone has two major functions. The primary function is to promote linear growth while the secondary function is to influence carbohydrate metabolism.

The carbohydrate and growth functions operate by different mechanisms and the majority of the growth promoting effects are mediated by a group of small peptides called somatomedins, which have molecular weights of around 7.5 kD. Somatomedins are produced predominantly by the liver, but there is some production by cells from other sites. These peptides have structural similarities to proinsulin and are now known as insulin-like growth factors (IGF-I and II have been accepted). The insulin like growth factors circulate in plasma bound to specific high molecular weight binding proteins which may protect them from proteolysis, thus extending their half lives in plasma .

1.2.3 Mechanism of action

The long term effects of growth hormone on the growth of muscle and skeletal tissues are anabolic, and are mainly mediated by IGF-I, while the effects of growth hormone on carbohydrate metabolism and lipolysis are catabolic and opposed to those of insulin, these direct effects are mediated by receptors within the target tissue (Table 1.2.1) (Baulieu and Kelly 1990).

The action of growth hormone on its target organ involves the binding of the hormone to specific receptors located on the plasma membrane of the target cell. The existence of receptors in humans for growth hormone has been shown in a number of tissues including the liver and lymphocytes (Hughes et. al. 1985).

The growth hormone receptor was thought to be a monomeric glycoprotein with a molecular weight of around 130 kD, which spans the plasma membrane. More recently the structure of the rabbit growth hormone receptor has been determined by purification of the receptor from liver and partial amino acid sequencing (Leung et.al. 1987). The receptor consists of 638 amino acids divided almost equally into intracellular and extracellular domains by 24 hydrophobic amino acids which are likely to provide the transmembrane domain. The extracellular domain contains an identical

Table 1.2.1 Effects of growth hormone on the major sites of action. (Adapted from Baulieu and Kelly 1990.)

Action of Growth Hormone	Tissue
Direct action	
IGF-I production	Liver and fibroblasts (in culture)
Protein synthesis	Liver
Amino acid transport	Liver, muscle, and adipose tissue
Lipolysis	Adipose tissue
Carbohydrate metabolism (hyperglycaemia)	Liver
Indirect action (via IGF)	
Chondrogenesis	Cartilage
Skeletal growth	Bone
Protein synthesis	Soft tissues
General cell growth	

N terminal amino acid sequence to that of growth hormone binding protein isolated from serum. The 18 amino acids which precede the N terminal sequence form the signal peptide (Thorner et. al. 1993). Rabbit cDNA clones have been used to examine a human cDNA library to identify similar clones of the human growth hormone receptor. The rabbit and human receptors have 84% amino acid homology. The receptor has a molecular size of 70 kD, smaller than the 130 kD receptor originally isolated, mainly due to glycosylation. The amino acid sequence of the receptor for prolactin has been shown to have homology with the growth hormone receptor (Boutin et. al.1988), (Baulieu and Kelly 1990), (Thorner et. al. 1993). The number of these receptors increase following puberty, during pregnancy and lactation (Kelly et. al. 1974). The receptors may be up or down regulated depending on the levels of hormone acting on the receptors. The binding of growth hormone to the receptor stimulates protein synthesis.

Receptors have been identified for IGF-I and II, the first preferentially binds IGF-I and has weak affinity for insulin, while the second preferentially binds IGF-II and has no affinity for insulin (Baulieu and Kelly 1990). The IGF-I receptor is structurally similar to the insulin receptor. Administration of IGF-I to hypophysectomised rats has a direct effect on stimulation of body weight increase, hypophyseal cartilage width, and DNA synthesis in cartilage (Schoenle et. al. 1982).

Serum concentrations of IGF-I rise steadily during childhood, from a low point at birth to the start of puberty, at which time there is a large increase in concentration which then falls gradually during adulthood (Baulieu and Kelly 1990).

In the liver cell growth hormone stimulates IGF-I production and directly increases the rate of cell replication and the mechanisms of protein synthesis including increased uptake of amino acids and acceleration of transcription and translation of mRNA (Baulieu and Kelly 1990).

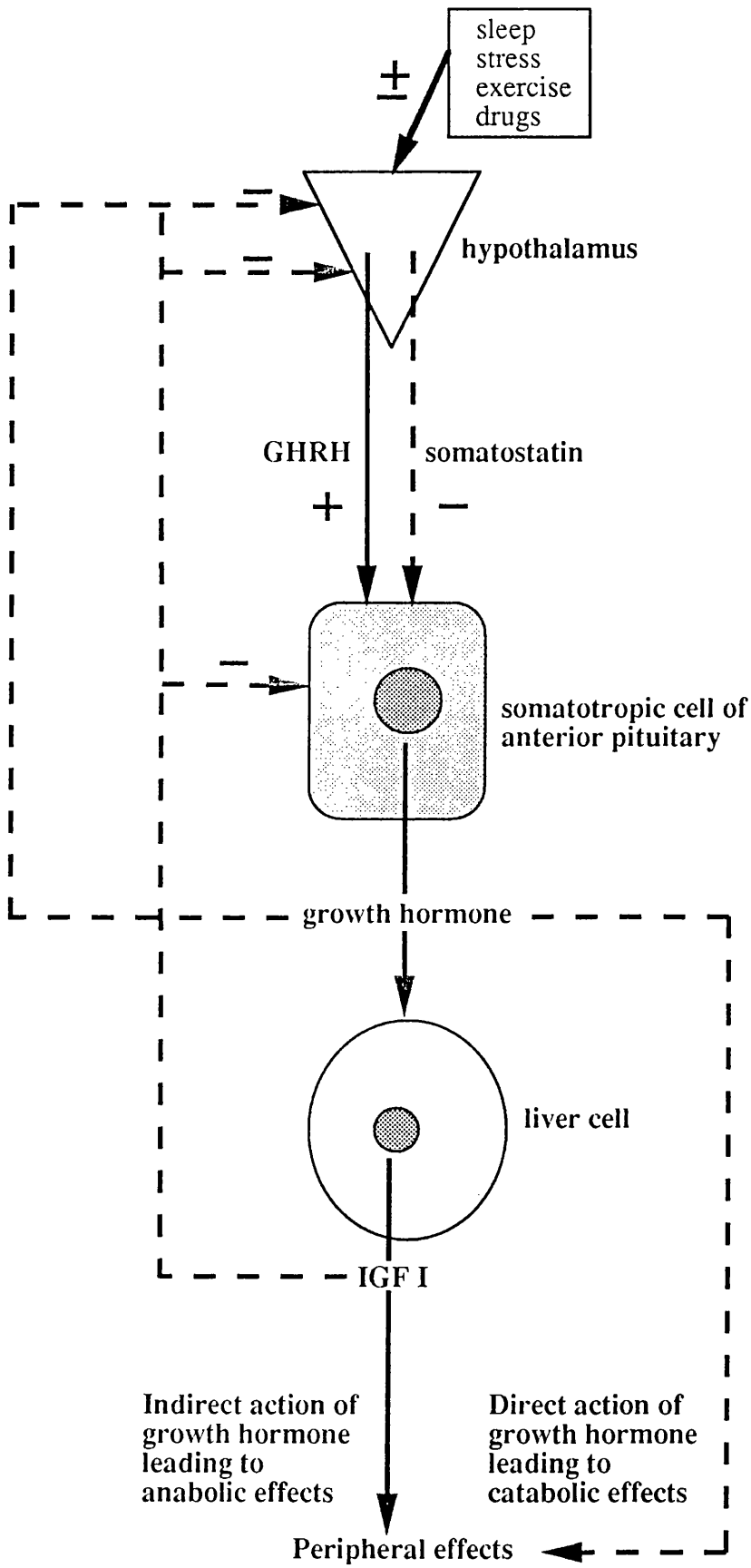
The rate of protein catabolism is also reduced in response to growth hormone by stimulating lipolysis and therefore utilising fat as an alternative source of energy. Fatty acids are released from adipose tissue and converted by β oxidation to acetyl-CoA which is then available as a source of energy via the Krebs Cycle .

Carbohydrate metabolism is moderated in response to growth hormone release, with reduced glucose uptake by cells due to impairment of insulin action. There is a resulting increase in insulin secretion in response to the increased plasma glucose concentration.

1.2.4 Control of secretion

There are two neurohormones produced by the hypothalamus which continuously adjust the level of growth hormone secretion. Growth hormone releasing hormone (GHRH) is a 44 amino acid peptide which specifically stimulates growth hormone secretion. Somatostatin specifically inhibits growth hormone secretion. (Figure 1.2.1) Somatostatin exists as both a 14 amino acid form and as a 28 amino acid form, with the additional amino acids on the N terminal end (Baulieu and Kelly 1990).

Figure 1.2.1
Primary mechanism of control of growth hormone secretion
(Adapted from Baulieu and Kelly 1990)



There are specific receptors for GHRH on the somatotropic cells of the anterior pituitary. These respond to GHRH by increasing production of cyclic adenosine monophosphate (cAMP). Somatostatin acts on the somatotropic cells inhibiting cAMP production, but it is likely that there are other modes of inhibiting growth hormone secretion, which are also affected by somatostatin (Baulieu and Kelly 1990).

A variety of other factors also affect growth hormone secretion (Table 1.2.2). These include several hormones; thyroid hormones, glucocorticosteroids, oestrogens, and androgens (Baulieu and Kelly 1990), (Thorner et. al. 1993).

The secretion of growth hormone over a 24 hour period is pulsatile (Veldhuis et. al.1988). This pattern of secretion is mainly due to the influences of somatostatin and GHRH, under the control of higher centres. The circadian rhythms of release of these two hormones are out of phase. Therefore when the level of somatostatin falls, and GHRH rises, there is a peak in growth hormone secretion.

One of the major growth hormone peaks occurs during the night (Rudd 1991). There are additional minor peaks associated with sleep. In children and young adults a growth hormone pulse occurs after around one hour of deep sleep followed by subsequent peaks during the sleep cycle. The increased secretion of growth hormone during sleep may be due to stimulation by falling levels of free fatty acids. This may also account for the decreased levels of growth hormone found in obese patients (Baulieu and Kelly 1990) (Griffen 1991).

IGF-I has an inhibitory effect on growth hormone production by means of a negative feedback loop which acts on the anterior pituitary and on the hypothalamus (Baulieu and Kelly 1990).

Table 1.2.2 Factors affecting growth hormone secretion.
(Adapted from Frohman 1981)

Increase in growth hormone	Decrease in growth hormone
Physiological	
sleep exercise stress (physical or psychological) postprandial: hyperaminoacidaemia hypoglycaemia (relative)	postprandial hyperglycaemia elevated free fatty acids
Pharmacological	
hypoglycaemia absolute: post glucagon or 2-deoxyglucose relative: post glucagon hormones: GHRH peptides (ACTH, α-MSH, vasopressin) oestrogen neurotransmitters, etc: α adrenergic agonists (clonidine) β adrenergic antagonists (propanolol) serotonin precursors dopamine agonists (levodopa, apomorphine, bromocriptine) GABA agonists (muscimol) pyrogens (*Pseudomonas* endotoxin)	hormones: somatostatin growth hormone progesterone glucocorticoids neurotransmitters, etc.: α adrenergic antagonists(phentolamine) β adrenergic agonists (isoproterenol) serotonin antagonists (methysergide) dopamine antagonists (phenothiazines)
Pathological	
protein depletion and starvation anorexia nervosa ectopic GHRH production chronic renal failure acromegaly TRH GnRH	obesity acromegaly: dopamine agonists hypo and hyperthyroidism

Growth hormone secretion is stimulated by stress and exercise, and is subject to changes in nutritional status. Hyperglycaemia prevents the growth hormone release due to stress in normal individuals, but is not effective in diabetics (Buchanan 1987).

Thus in summary it is clear that the pattern of growth hormone release is complex due to a variety of different influences. The basic circadian rhythm of release is enhanced during the night by the effects of sleep, reduced during the day following meals and increased following periods of exercise (Figure 1.2.2)

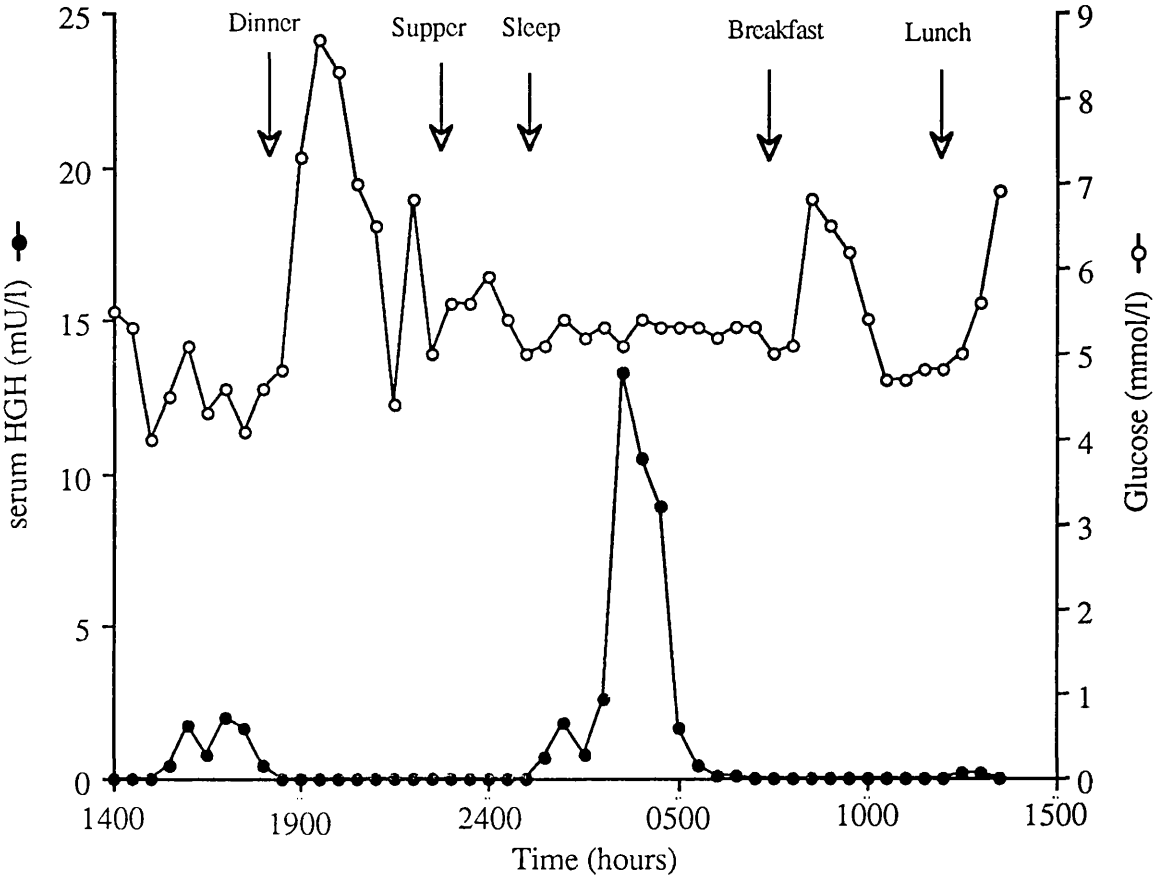
1.2.5 Distribution and peripheral metabolism

Following secretion from the pituitary growth hormone is distributed within a compartment whose volume is intermediate between the intravascular space and the intracellular space.

A binding protein for growth hormone has recently been identified which complexes a major portion of circulating growth hormone. The biological function of this protein is as yet unknown. The complexed growth hormone is restricted to the intravascular space to a greater extent than growth hormone, and the volume of distribution is more limited, this reduces the clearance of bound growth hormone by the kidneys, and may prolong the plasma half life (Baumann et. al. 1986), (Baumann et. al. 1987) (Baumann 1988).

The confinement of complexed growth hormone to the intravascular space may prevent glomerular filtration by the kidneys and explain why growth hormone, which is readily filtered by the glomerulus, does not appear in large quantities in urine (Baumann et. al. 1987).

Figure 1.2.2
Typical adult 24 hour growth hormone profile
(Griffin 1991)



A concentration gradient for growth hormone exists between plasma and tissue fluid. There is a resulting increase in the volume of distribution of growth hormone in conditions which cause increased capillary permeability such as hepatic failure, renal failure, and uncontrolled diabetes (Owens et. al. 1973).

The metabolism of growth hormone is carried out by the liver and the kidneys in approximately equal proportions (McCormick et. al. 1969). The fractional clearance of growth hormone from these sites is unaffected by differences in plasma concentrations (Owens et. al. 1973). Metabolism of growth hormone by the liver may be due to receptor mediated cellular uptake and degradation by lysosomes (Baumann et. al. 1987).

Growth hormone metabolism is reduced in renal failure and myxodema but is little affected in liver disease (Owens et. al. 1973).

1.2.6 Heterogeneity of human growth hormone

Growth hormone exists in plasma in more than one form and this heterogeneity may cause discrepancies between assays using different antibodies and create problems of interpretation (Table 1.2.3). The physiological significance of these different forms of growth hormone is unclear but they may be relevant where the levels of growth hormone, measured by immunoassay, do not agree with the clinical picture due to the presence of circulating immunoreactive but bio-inactive forms (Smith et. al. 1990).

Table 1.2.3 Major forms of growth hormone found in serum. (Smith et. al. 1990).

Variant	Approx. MW (kd)	Comments
Monomeric pituitary HGH	22	Approx 75% total immunoreactive HGH
HGH_{20kd}	20	Approx 16% total immunoreactive HGH
'Big' HGH	40-50	Dimers
'Big-big' HGH	>60	(a) Aggregates
		(b) HGH binding protein complex
Fragments	<20	Present in serum in basal state
		May be formed in tissues.
Placental HGH	22	Secreted by placenta
" "	24,26	Glycosylated forms of placental HGH

Molecular heterogeneity may affect immunoassays for growth hormone since the sites to which the antibodies bind may be on different regions of the molecule to the physiological binding site. It is therefore possible that any immunoassay may detect inactive fragments or aggregates of the hormone. In addition the purified or synthetic hormone used to raise the antibodies is generally in an unmodified monomeric form and therefore an assay may not detect modified forms which have biological activity (Smith et. al. 1990) (Rudd 1991).

1.2.7 Excretion of growth hormone into urine

Despite the fact that the kidneys account for half the growth hormone metabolism, the majority of this undergoes proteolysis and the amount of growth hormone excreted unchanged by the kidneys is very small (<0.001 % of production), the concentrations in urine are therefore normally very low (Girard et. al. 1990) (Okuno et. al. 1987).

1.3 INDICATIONS FOR MEASUREMENT OF GROWTH HORMONE

1.3.1 Usefulness of basal serum growth hormone measurement

Due to the pulsatile nature of growth hormone release and its short plasma half life growth hormone concentrations in adults and in children may be very low and random determination of the serum growth hormone level is of little value in the assessment of growth hormone status (Hashida et. al. 1985).

A number of provocation tests have been used to determine the ability to produce growth hormone in response to physiological or pharmacological stimuli (Table 1.3.1). The exercise test, the nocturnal profile, oral clonidine and the insulin tolerance test, are the most commonly used in Great Britain at present. Great care is required in performing these tests to ensure standard conditions are maintained. The nocturnal profile is particularly difficult and labour intensive to perform (Buchanan 1987).

1.3.2 Growth hormone deficiency

There are a variety of conditions which cause short stature most of which are growth hormone independent (Table 1.3.2). It is therefore important to measure growth hormone as part of the assessment of short stature to distinguish those children who may be aided by growth hormone therapy from those who are not growth hormone deficient (Table 1.3.3). There are a number of causes of growth hormone deficiency which may be congenital acquired or which may only be transient sequelae of another condition (Table 1.3.4).

Table 1.3.1 Growth hormone provocation tests

(Adapted from Buchanan 1987)

Test	Protocol
Post-prandial	Samples 3 and 4 hours post ingestion of glucose (1.4 mg / kg) or high carbohydrate meal
Post exercise	Samples pre and 30 minutes post strenuous exercise (15 minute walk or running up and down stairs for 5 minutes)
Nocturnal profile	regular samples prior to and after the onset of sleep
Arginine infusion	Intravenous arginine HCl 10 % administered for 30 minutes and samples taken at times 0, 30 ,60 ,90, 120 minutes
Glucagon	Intramuscular glucagon (100 mg / M^2 max 1 mg) administered and samples collected at 30 minute intervals for 3 hours
Oral clonidine	Oral clonidine (0.1 mg / M^2) and samples are taken at 0, 30, 60, 90,120 minutes
Insulin tolerance test	Intravenous insulin (0.1-0.15 U/kg) administered and samples for glucose and growth hormone taken at 0, 30, 45, 60, 90, 120 minutes

Table 1.3.2 Causes of short stature

(Adapted from Buchanan 1987)

Congenital
Hereditary Laron dwarfism Turner's syndrome Primary hypothyroidism Congenital cardiac disease Growth hormone deficiency **Development defects** Constitutional delay in growth and puberty Familial short stature Chronic gastro intestinal disease eg.Crohns disease, coeliac disease Intrauterine growth retardation eg. foetal alcohol syndrome Skeletal dysplasia
Acquired
Chronic renal disease Diabetes mellitus Chronic systemic disorders Glucocorticoid excess eg. Cushings syndrome, adrenal tumour
Transient
Nutritional insufficiency eg. protein malnutrition Psychosocial deprivation

Table 1.3.3 Assessment of child with short stature

(Adapted fom Buchanan 1987)

Medical and social history
Anthropometry
Child's height and weight Estimation of child's rate of growth Parental height
Physical examination
Bone age
Karyotype to exclude Turners syndrome in females
Other relevant investigations
Haematological Biochemical Endocrine Cardiac Renal Gastrointestinal Skeletal

Table 1.3.4 Causes of growth hormone deficiency

(Adapted from Buchanan 1987)

Congenital
Hereditary Autosomal recessive Autosomal dominant Idiopathic growth hormone releasing hormone deficiency **Development defects** Pituitary aplasia Pituitary hypoplasia Midline anomalies
Acquired
Tumour Hypothalamic Pituitary Other intracranial **Irradiation** **Infection** Meningitis Encephalitis **Infiltration** Histiocytosis Haemachromatosis **Injury** Perinatal insult Head injury
Transient
Peripubertal Psychosocial Primary hypothyroidism

1.3.3 Growth hormone excess

Hypersecretion of growth hormone from a pituitary adenoma in adults produces acromegaly, whereas in adolescents if hypersecretion occurs before the closure of the epiphyses of the long bones, the result is acromegalo-gigantism. In addition there are rare examples of ectopic growth hormone production and pancreatic GHRH releasing tumours (Baulieu and Kelly 1990).

IGF-I is raised in acromegaly and it may be a more useful marker of excessive growth hormone production than growth hormone itself since growth hormone concentrations can fall within the upper part of the reference range, whereas IGF-I is almost always raised (Baulieu and Kelly 1990), (Thorner et. al. 1993). Glucose tolerance is reduced by around 50% in acromegaly and approximately 10% of patients are diabetic. Acromegaly can be treated by using neurosurgery to remove microadenomas by irradiation of the tumour or with bromocriptine or pergolide which are dopamine agonists. An alternative medical treatment for patients who do not respond to the above therapies is to use long acting synthetic analogs of somatostatin, such as Octreotide, to suppress growth hormone production by the pituitary (Harris 1990), (Liuzzi et. al. 1990), (Sassolas et. al. 1990).

1.3.4 Urine growth hormone measurement

It has been shown that concentrations of growth hormone in nocturnal urine, when corrected for renal function, correlate with the corresponding mean serum growth hormone, serum IGF-I and urine IGF-I measurements over the period of urine collection (Tanaka et. al. 1990) (Okuna et. al. 1987) (Sukegawa et. al. 1988) (Quattrin et. al. 1988). This can be a useful tool in distinguishing excessive from normal growth hormone production in acromegaly although the overlap between normal reduced and deficient production is more difficult to resolve (Kohno et. al. 1990). Urine growth

hormone measurement is useful for monitoring growth hormone secretion but cannot be used to follow the pattern of pulsatile release (Girard et. al. 1990).

There could be considerable benefits to be gained from the use of a simple urine screening test in preference to the more invasive stimulation tests described above which carry their own inherent risks to the child (Table 1.3.1). Not the least of these is the advantage of not having to subject children to such testing where a simple early morning urine or timed specimen can be collected.

1.3.5 Treatment with recombinant growth hormone

Growth hormone is a species specific hormone and this prevents the use of animal derived hormone in treatment. Since 1958 (Raben 1958) cadaveric human growth hormone has been used in limited amounts in replacement therapy. This treatment ceased in the UK following the occurance of Creutzfeldt-Jakob disease in some patients receiving treatment with the relatively impure preparations of cadaveric growth hormone (Buchanan 1987).

The first biosynthetic growth hormone, Somatrem, was given a product licence in 1985, this and other synthetic preparations have since been used in treatment of growth hormone deficiency (Buchanan 1987). The greater availability of growth hormone has now led to it's contentious use in short children who are not necessarily growth hormone deficient and in adults who are growth hormone deficient or are elderly.

1.4 METHODS FOR GROWTH HORMONE MEASUREMENT

1.4.1 Bioassay for growth hormone

Early assays for growth hormone depended on quantification of its biological activity using for example the effect of injected growth hormone on increasing the width of the epiphyseal tibia cartilage of rats (Greenspan et. al. 1949).

Subsequently specific radioreceptor assays were developed where ^{125}I labelled human growth hormone competes with the unlabelled growth hormone for binding to human cultured lymphocytes (Lesniak et.al. 1973) (Gavin et. al. 1982) (Tanaka et. al. 1983).

With the introduction of radioimmunoassays the use of antibodies allowed measurements which were dependent on the three dimensional structure of the hormone. The presence of heterogeneous forms of growth hormone with different activities as well as inactive fragments, can provide misleading information in structure specific assays. However although it is the biological activity of growth hormone which is of clinical interest, function specific assays are time consuming and unsuitable for large scale laboratory use and immunoassays have become firmly established.

1.4.2 Early radioimmunoassay for growth hormone

A radioimmunoelectrophoretic assay for human growth hormone using ^{131}I was first published in 1964 by Hunter and Greenwood. In this method 10 ml of plasma was first extracted with acetic acid-acetone and then a radioimmunoassay was performed using rabbit antibody with an 8 to 12 day incubation followed by separation of bound from free growth hormone using electrophoresis. Due to the 8 day half life of ^{131}I, growth hormone was iodinated using chloramine T each week (Hunter and Greenwood 1962), (Greenwood and Hunter 1963).

During the following two decades improvements in reagents, coupled with a greater understanding of immunoassay design led to the evolution of radioimmunoassays for serum growth hormone based on much smaller specimen volumes (typically 100 µl) and on a much shorter turn around time (typically 36 hours). Assays for serum growth hormone became firmly established in most regional endocrine laboratories. By preparing and distributing carefully matched reagents the Edinburgh group were able to demonstrate dramatic improvements in inter-laboratory performance of growth hormone radioimmunoassays which in turn led to consensus action limits for clinical practice (Hunter et. al. 1978).

1.4.3 Two site immunometric assays for growth hormone

A method of labelling anti-growth hormone antibody with ^{125}I was published by Miles and Hales in 1968 and this was used in a one site immunometric assay. Growth hormone was attached to a cellulose-based immunoabsorbent and incubated with labelled antibody and free growth hormone. After separation, the amount of labelled antibody which was bound to the immobilised growth hormone provided a measure of the amount of free growth hormone in the sample.

A two site IRMA or sandwich assay was developed by Addison and Hales in 1971. In this method the antigen is sandwiched between a labelled antibody and a solid phase coupled antibody.

The most sensitive systems being those where labelled antibody is added first:

$^{*}Ab+Ag \rightleftharpoons {}^{*}Ab\text{-}Ag$

followed by addition of solid phase coupled antibody:

$^{*}Ab\text{-}Ag+Ab_{solid} \rightleftharpoons {}^{*}Ab\text{-}Ag\text{-}Ab_{solid}$

(Hunter 1982)

1.4.4 Current status of two site immunometric assays for serum growth hormone

Unlike TSH there has been only a limited number of attempts to develop immunoassays for growth hormone and growth hormone IRMA's have been relatively slow to appear. This may be due to the smaller demand for growth hormone kits. From the UK external quality assessment scheme reports of December 1991 there were three suppliers of serum growth hormone IRMA's with five or more users: IDS OMNIA, NETRIA IRMA (North East Thames Radio Immunoassays), and Pharmacia IRMA. The IDS assay was developed within this Institute and is based on a labelled monoclonal antibody and polyclonal antibody linked solid phase. The NETRIA assays similarly uses a monoclonal label and a polyclonal linked solid phase.

1.4.5 Methods used for urinary growth hormone

Since the assay of growth hormone in urine requires sensitivity in excess of one hundred times that of the corresponding serum assay, non-specific interference from pH, osmolarity and urea concentration must be minimised. In addition high avidity antisera are essential as is the sensitivity provided by antibody excess immunometric assays (Girard et. al. 1990).

Sample collection and preparation must be carefully considered, some assays use albumin to prevent adsorption of growth hormone onto the surface of the urine collection container. Other assays involve dialysis of the urine to remove interfering salts while less sensitive assays concentrate urine samples before analysis (Table 1.4.1).

The incubation times of antibodies with the antigen are generally extended to allow maximum binding and the highest sensitivity possible, for example one assay uses a 44 hour incubation with the secondary antibody (Girard et. al. 1990).

Table 1.4.1
Methods for urinary growth hormone
(Adapted from Girard et. al. 1990)

	Girard et.al. 1987	Hashida et.al. 1985	Fehskens et.al. 1989	Price et. al. 1989	Bullen Wilkin 1989	Torresani et.al. (in press)	Daco (Novo) (unpublished)
Sample							
24 hr	yes	yes	yes	no	no	yes	yes
early morning	yes	yes	yes	yes	yes	no	yes
timed	yes	yes	no	yes	yes	yes	yes
Collection							
Albumin	no	yes	no	no	yes	yes	
Preservative	no	azide	no	no	yes	no	
Pre-treatment							
Dialysis	no	yes	no	yes	yes	yes	no
Concentration	no	no	no	no	no	yes	no
Assay type	Radiometric solid phase	sandwich enzyme immunoassay coated beads	enzyme linked immunosorbent assay ELISA plates	solid phase IRMA	RIA	ELISA plates	Elisa plate Kinetic enz. cycling
Incubation times	1) overnight 2) overnight	1) 6 hrs 37°C 2) 16 hrs/ON room temp.	1) 12-16 hrs 2) 44 hrs	1) 24 hrs 4°C 2) 24 hrs 4°C with mixing	1) 20 hrs 2) 5 hrs	1) 24 hrs 4°C 2) 1 hr RT 3) 16 hrs 4°C	
Antibody	sheep polyclonal	mouse monoclonal	goat polyclonal	NETRIA	Novo Biolab	guinea-pig polyclonal	
Label	monoclonal 125 I	rabbit Fab' peroxidase conjugate	goat anti-hGH-HRP conjugate	NETRIA	Novo Biolab	HRP-labelled guinea-pig polyclonal	
Substrate	----------	HPPA	OPD	----------	----------	TMB	Alkaline phosphatase
Sensitivity pg per ml urine	0.16	0.3	4	0.8	0.25	1.0	0.5 ng/l

Since growth hormone excretion may be affected by decreased renal function, growth hormone concentrations are generally expressed with reference to the corresponding creatinine concentration.

1.5 DESIGN OF AN ASSAY FOR URINARY GROWTH HORMONE

1.5.1 Objectives

Any assay for urinary growth hormone faces two major difficulties from the outset. Firstly growth hormone is only present in urine in quantities below the minimum detection limit of most immunoradiometric assays. This means that either a highly sensitive non-isotopic assay is required (sensitivity of 0.006 mU/l (3 ng/l)), or that the samples must be concentrated prior to analysis (Hashida et. al. 1986). Concentration of large numbers of urine samples by a quantitative method is time consuming and would be unsuitable for routine use. The second major hurdle in measuring growth hormone in urine is the interference from salts which may be present at widely varying concentrations in urine. In a number of the published assays for urinary growth hormone, this problem has been solved by dialysing the urine samples in buffer, before analysis (Evans and Wood 1989) (Girard et. al. 1990). Whilst successful, this is a laborious task and unworkable on a large scale.

With these facts in mind, the objectives in designing an assay for growth hormone in urine were:

a) The assay should be capable of measuring growth hormone with a sensitivity of 0.006 mU/l. If possible sample concentration should be avoided.

b) The assay should not be subject to interference from different urine matrices.

c) The assay should be suitable for routine use in a busy department, and should be robust.

1.5.2 Immunoassay types and components

Immunoassays can be classified according to whether they involve:

1 Limited reagent or reagent excess

2 Separation of antigen bound to antibody from free antigen or non separation

3 Isotopic or non-isotopic labels

1.5.2.1 Limited reagent and reagent excess assays

The assay design chosen for an application depends on the particular nature of the analyte. Small molecular weight antigens are unlikely to be large enough to have more than one distinct antibody binding site and therefore a limited reagent (competitive) assay is most suitable (Hunter 1982). Where a minimal detection limit is essential, a reagent excess assay (sandwich assay) is more suitable as this is less dependant on the precision of reagent addition (Price et. al. 1991).

In these immunometric assays, one of the antibodies can be coated or attached chemically to the surface of microtitre wells to provide the separation system. The second antibody may be labelled with ^{125}I, an enzyme, or with a lanthanide chelate such as europium, and provides the detection system for the assay. These reagent excess assays use quantities of antibodies which do not act as a limiting factor on antigen binding.

1.5.2.2 Separation and non-separation assays

Heterogeneous assays are assays which include a separation stage to remove the antibody bound antigen from the unbound antigen. In the case of homogeneous assays, the detection system allows the presence of both the bound and free fractions without interference. Heterogeneous assays tend to have lower detection limits and be subject to less interference from sample components. (Price et. al. 1991) These are

desirable factors in the analysis of growth hormone in urine. Homogeneous assays generally have less steps and are simpler to perform and to automate than heterogeneous assays and are used in for example therapeutic drug analysis.

In microtitre plate assays, separation is achieved by coating one antibody onto the surface of the wells, to which the antigen binds, allowing the removal of the unwanted sample components by repeated washing using an automated washing device.

1.5.2.3 Isotopic and non-isotopic labels

Isotopic labels have been used extensively, as in most cases incorporation of ^{125}I into small molecules and into antibodies is relatively simple. There are disadvantages in the limitations on the handling and disposal of radio-isotopes and the short half life of some of the isotopes. The limited signal to noise ratio of ^{125}I is unlikely to provide a detection limit which is adequate for use in a urinary growth hormone assay.

An immunochemiluminometric assay for serum growth hormone using antibodies produced within this institute has been published by Weeks and Woodhead (1986). This assay uses acridium ester labelled monoclonal antibodies and a polyclonal antibody attached to magnetisable solid phase particles. The reported sensitivity is 0.12 mU/l and the working range is 0.88 to over 100 mU/l.

A lanthanide chelate of europium has been used in this institute as an alternative isotope for measuring human growth hormone. This DELFIA (Dissociation-Enhanced Lanthanide Fluoroimmunoassay) assay may potentially have a signal to noise ratio large enough to produce the low detection limit required for measuring growth hormone in urine.

The use of an enzyme-antibody fragment conjugate as an alternative label, coupled with an appropriate fluorimetric substrate, may provide a large signal amplification and

low background counts, and an assay suitable for urinary growth hormone measurement (Hashida et. al. 1986).

1.5.3 Production of antibody fragments

A single IgG molecule is composed of two heavy chains and two light chains which are linked by the thiol groups of the amino acid cystine (Figure 1.5.1). These bonds can be broken by reduction with compounds such as mercaptoethanol, mercaptoethylamine, iodoacetamide, or dithiothreitol.

IgG can also be cleaved by using the protease pepsin, leaving the fragments $F(ab')_2$, Fc and, pFc (Figure 1.5.1). The $F(ab')_2$ fragments produced can be reduced to Fab' fragments, as above. The Fab' fragments, thus produced, retain the hinge region and the thiol groups. In contrast papain cleaves the IgG molecule above the hinge region producing two Fab' fragments, which lack thiol groups, and an Fc fragment.

1.5.4 Preparation of Fab'-enzyme conjugates

As mentioned in the previous paragraph pepsin digestion of IgG followed by reduction with mercaptoethanol leaves an Fab' fragment which retains the hinge region and the thiol groups of cysteine. This allows an enzyme molecule to be linked, by heterobifunctional reagents, to an Fab' fragment (Figure 1.5.2). This process requires two steps and uses a linking group such as maleimide with two different arms, one of which attaches to the enzyme, and one of which attaches to the thiol group of the Fab' fragment (Figure 1.5.3).

In step one the enzyme is mixed with excess linking reagent, allowing maximum binding to the enzyme. In step two the excess linking reagent is removed and Fab' fragment is added to allow conjugate formation. Finally the conjugates are separated

Figure 1.5.1
Illustration of IgG showing the cleavage points of pepsin, papain and mercaptoethylamine.

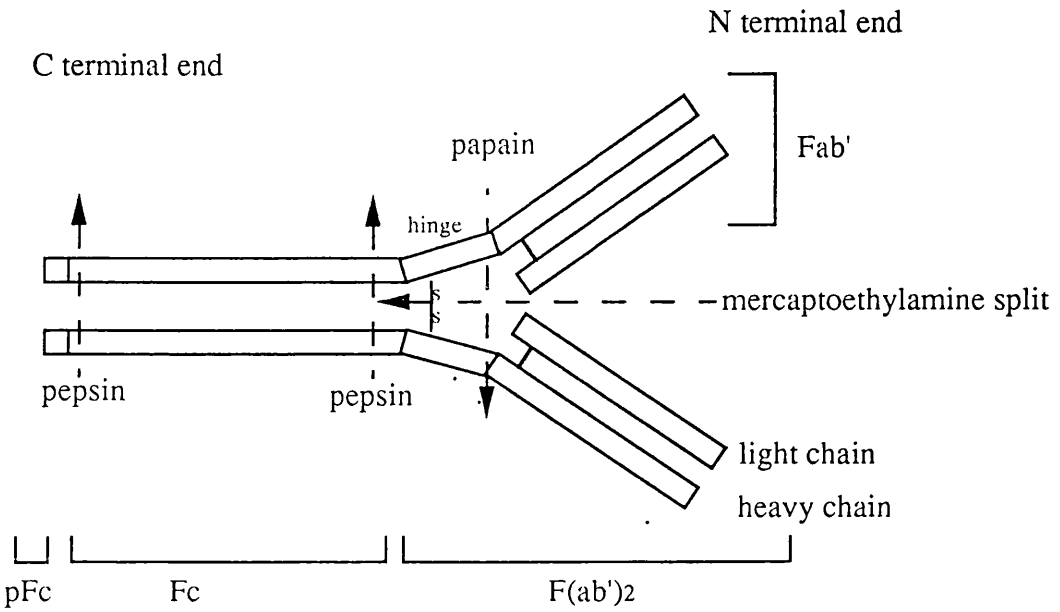
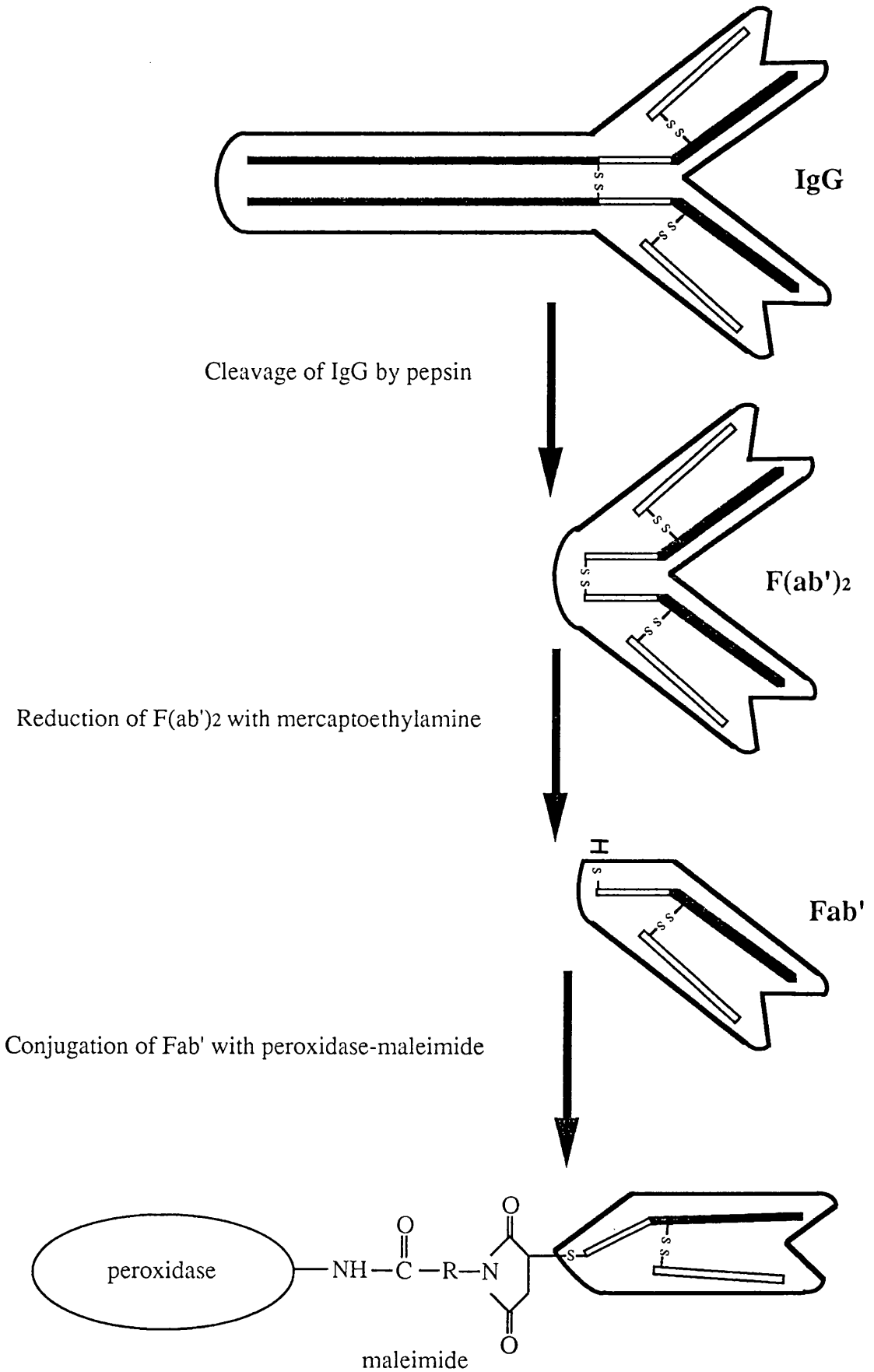


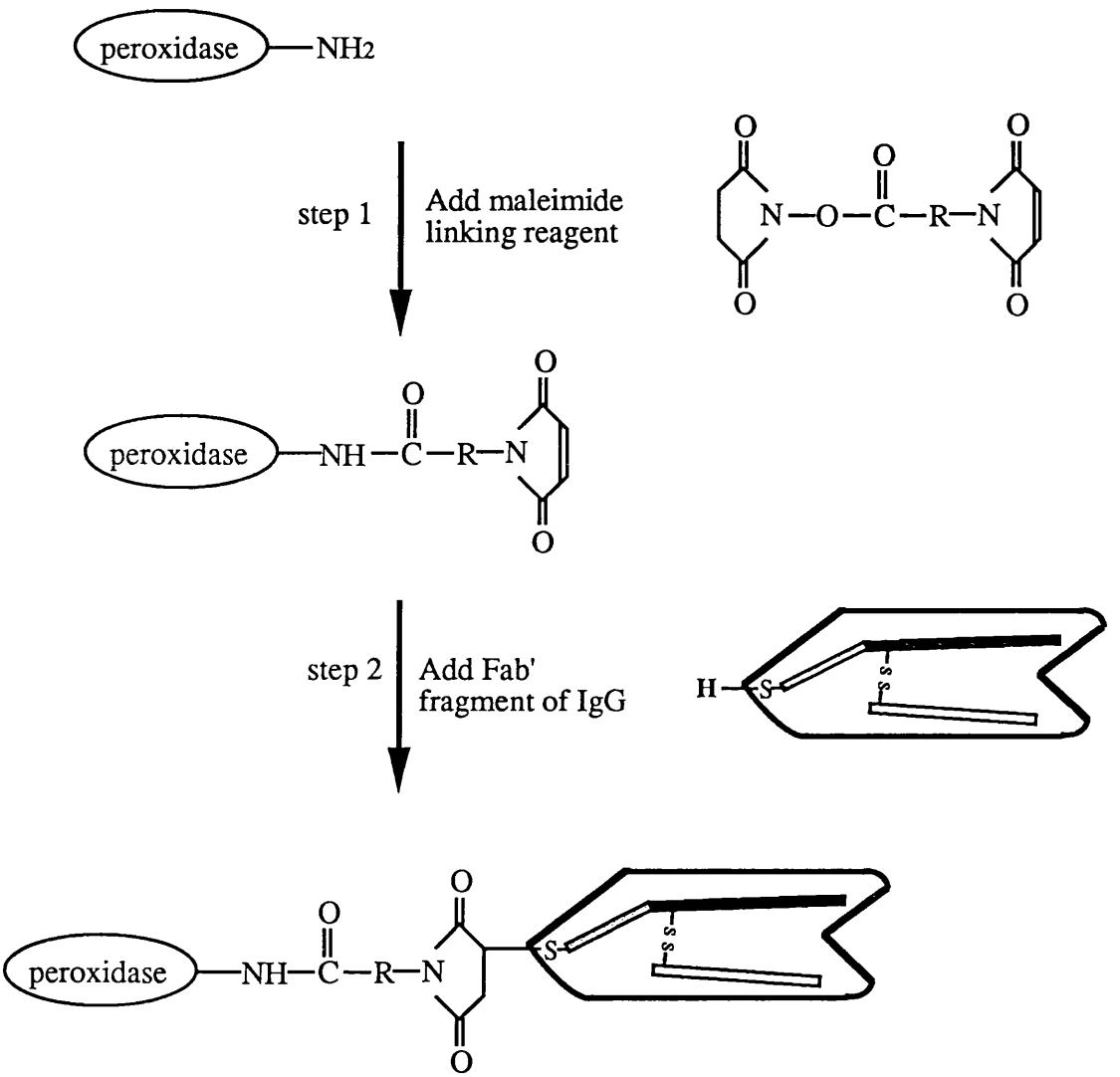
Figure 1.5.2
Production of an enzyme-Fab' conjugate by the hinge method



Peroxidase Fab' conjugate.

Figure 1.5.3
The two step maleimide method for enzyme labelling Fab' fragments. (hinge method)

Using the two step maleimide method for enzyme labelling of Fab' fragments the reaction products are more predictable than one step methods and the conditions for each step may be optimised to improve the yield of conjugate. (Adapted from Ishikawa et. al. 1988)



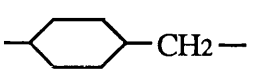
Peroxidase -Fab' conjugate.

The R group of the linking reagent may be either:

-$(CH_2)_5$-

N-succinimidyl-6-maleimidohexanoate

or



N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate

from any unbound Fab' fragments. Reaction conditions, at each step, can be adjusted to optimize linking (Ishikawa et. al. 1983).

It is also possible to link Fab fragments from papain digestion of IgG, to enzymes such as peroxidase, β-D-galactosidase, or alkaline phosphatase, using homobifunctional linking reagents such as glutaraldehyde (Avrameas 1969), (Avrameas et al. 1978). This conjugation may be performed as a one step process by incubating glutaraldehyde with Fab fragment and enzyme. (Figure 1.5.4) However since the rates of reaction of the functional groups on the Fab fragment and enzyme are likely to be different, the reaction may favour polymerisation of either the Fab fragment or the enzyme in preference to Fab-enzyme conjugate formation.

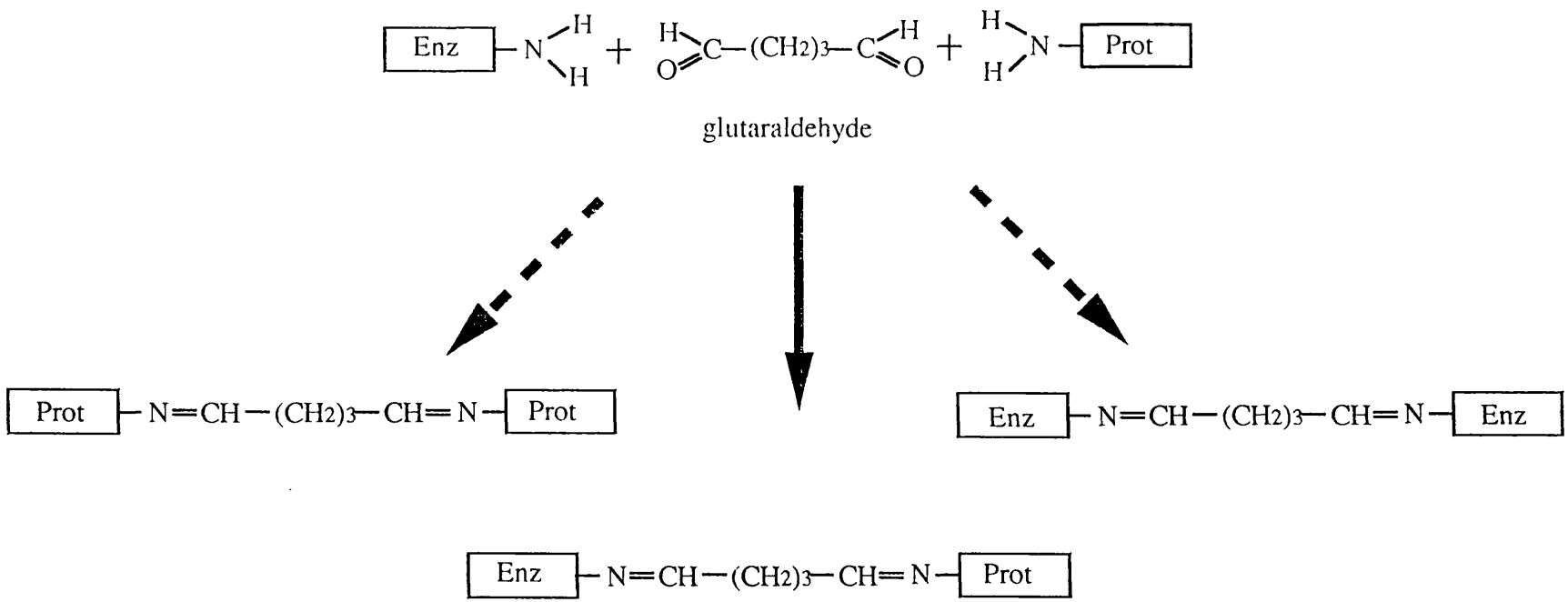
1.5.5 Enzymes as labels in immunoassays

Enzymes have a number of advantages over radioactive labels in immunoassays (Price et. al. 1991). In the particular application of urine growth hormone, the stability of the reagents is an important advantage since monthly iodinations are acceptable, where the assay is carried out several times a week, but where the workload is lower, and the assay is only being performed once or twice a month, the time required for iodination and purification of the label is unacceptable. The time taken to produce an enzyme conjugate is longer than for an iodine label, but producing an enzyme label once a year saves time and gives a more consistent reagent. The use of enzymes as labels can allow improved sensitivity by providing a higher signal to noise ratio than radioisotopes. The other useful advantage is that using coated plates and enzyme labels provides a semi-automated assay which is simple to perform.

There is also the advantage that there is no radiation hazard involved, but since production of the label involves the use of mutagenic chemicals and some of the enzyme substrates are toxic, this is a marginal benefit.

Figure 1.5.4
Crosslinking of an enzyme to a protein using glutaraldehyde.

Crosslinking of an enzyme to a protein using glutaraldehyde as a homobifunctional linking reagent in a one step process may produce more than one reaction product. Some of the possible reaction products are shown. (Adapted from Avrameas et al . 1978).



There are three main enzymes which are used as labels in immunoassays: alkaline phosphatase, β-D-galactosidase, and horseradish peroxidase (Ishikawa et. al. 1983). Assays employing alkaline phosphatase and conventional substrates do not give adequate detection limits for this particular application without extended incubations and will therefore not be considered further (Ishikawa et.al. 1983).

β-D-galactosidase was initially considered for use in this assay, since it is reported to provide a detection limit similar to and possibly lower than that of horseradish peroxidase, and there is a fluorimetric substrate available (4-methylumbelliferyl-β-D-galactoside) (Yoshitaki et. al. 1982) (Imagawa et. al. 1984). However β-D-galactosidase is a larger enzyme than peroxidase, (465 kD and 44 kD respectively) and it was thought that it may possibly cause steric hindrance problems in the assay. Since it had no major advantages over horseradish peroxidase it was considered to be 'first reserve' and peroxidase was the enzyme of choice.

1.5.6 The choice of substrate for horseradish peroxidase

Horseradish peroxidase has a number of colourimetric substrates: OPD (o-phenylene diamine), ABTS (2,2-azino-bis(3 ethyl thiazoline-6-sulphonic acid)) and TMB (3,3',5,5'-tetramethyl-benzidine dihydrochloride) and also a fluorimetric substrate HPPA (3-(4-hydroxy phenyl) propionic acid). It was hoped that one or more of these substrates would provide the sensitivity required for the assay of growth hormone in urine. (Ishikawa et. al. 1983). It should be noted that the actual substrate of horseradish peroxidase in each case is H_2O_2 and that the listed compounds are chromogenic indicators of H_2O_2 reduction (Bergmeyer 1986).

The H_2O_2 can be added to the substrate solution immediately before the assay, or where the substrates are supplied in tablet form, it is generated on dissolution of the substrate.

ABTS is a safe substrate which produces a blue green colour in the presence of peroxidase which can be read at 405 nm, in timed and stopped assays, without a wavelength shift. The colour formation is due to a reversible redox system in which single electron transfers connect two different oxidation products (Figure 1.5.5), (Bergmeyer 1986) (Sigma Immuno Notes 1991).

There is a potential problem with TMB as a substrate in that oxidation in the presence of hydrogen peroxide produces two colour forms in sequence. A proposed reaction sequence is shown over (Figure 1.5.6), in which the blue primary product is in rapid equilibrium with the radical cation (Bergmeyer 1986). The use of 2 M H_2SO_4 as a stop solution produces a yellow colour which can be read at 450 nm. TMB is not as light sensitive as other substrates and can be developed in daylight, although there may be an effect on the blank values (Sigma Immuno Notes 1991).

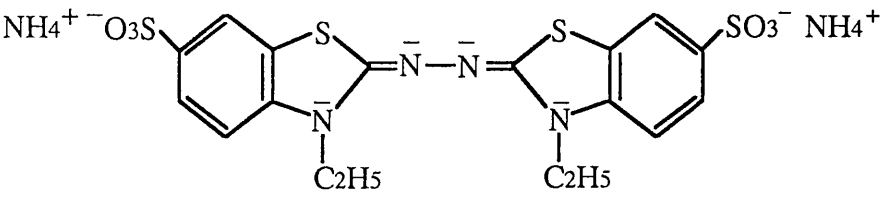
HPPA is currently in limited use as a fluorimetric substrate of horseradish peroxidase and although it was previously measured using single cell fluorescence spectrometers, filters are now available for use with fluorescence platereaders. This allows assays using HPPA to be semi-automated. Unfortunately fluorescence plate readers are more costly than conventional colourimetric readers and are less commonly available within laboratories. In theory fluorescence substrates should provide a wider working range and greater sensitivity than their colourimetric equivalents.

1.5.7 Basic theory of fluorescence

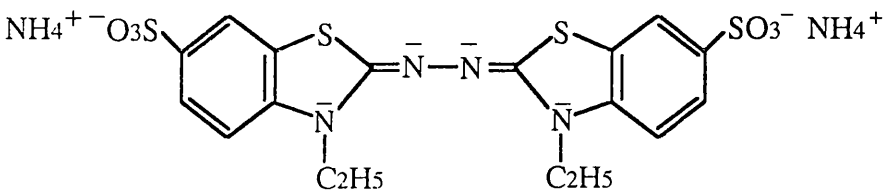
Some organic molecules are capable of absorbing radiation and emitting this energy in the form of photons of light which have longer wavelength and therefore less energy than the excitation radiation (Figure 1.5.7). The difference between the excitation wavelength and the emission wavelength is known as the Stokes shift. In general organic molecules have a Stokes shift of 30-50 nm, whereas the lanthanide chelates such as samarium or europium have Stokes shifts of over 200 nm. This means that

Figure 1.5.5
Oxidation products of ABTS

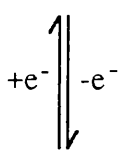
Structure of ABTS showing the reversible formation of the two different oxidation products by single electron transfers. In the presence of peroxidase the blue green radical cation is formed which is stable if disproportionation is inhibited by a 100 fold molar excess of ABTS. (Bergmeyer et. al. 1986).

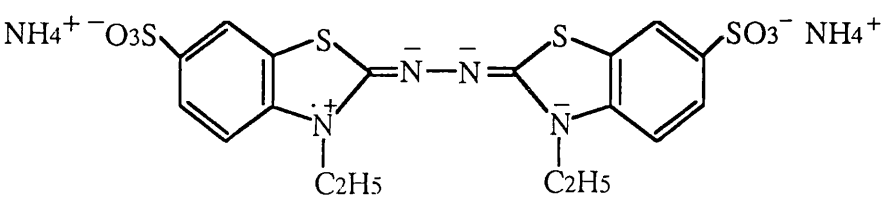


ABTS

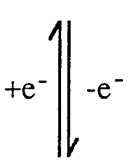


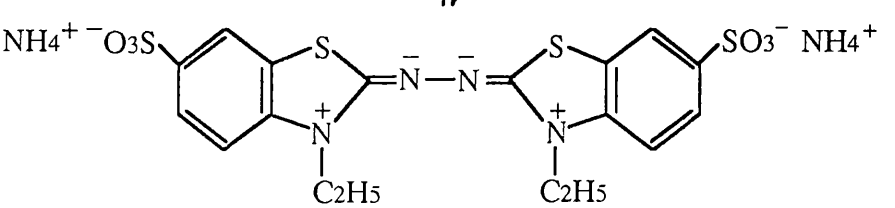
azine





radical cation
(blue green)





azo di-cation

Figure 1.5.6
Oxidation products of TMB

Oxidation of TMB, in the presence of peroxidase, produces two colour forms in sequence, the first being blue and the second brown. (Bergmeyer et. al.1986)

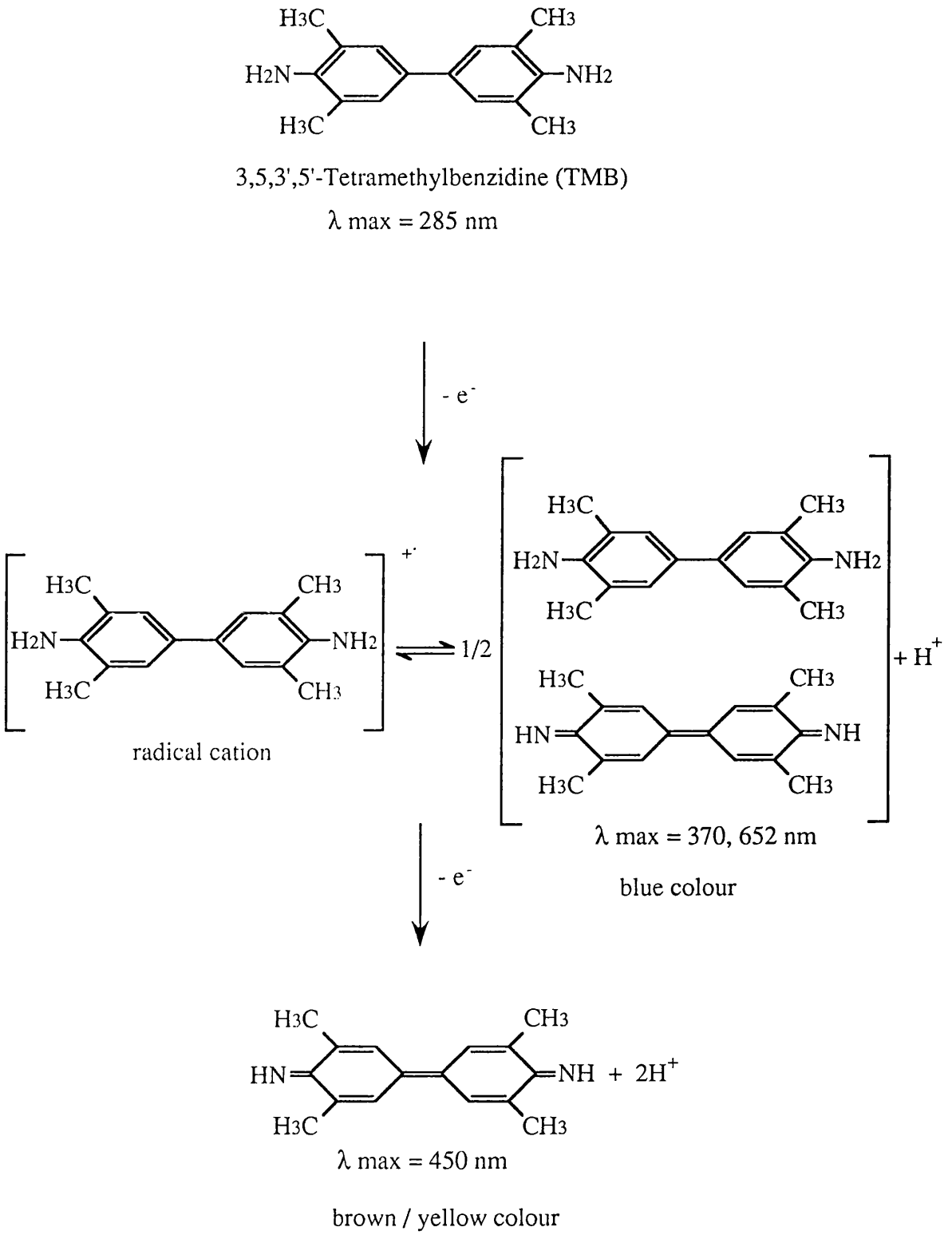
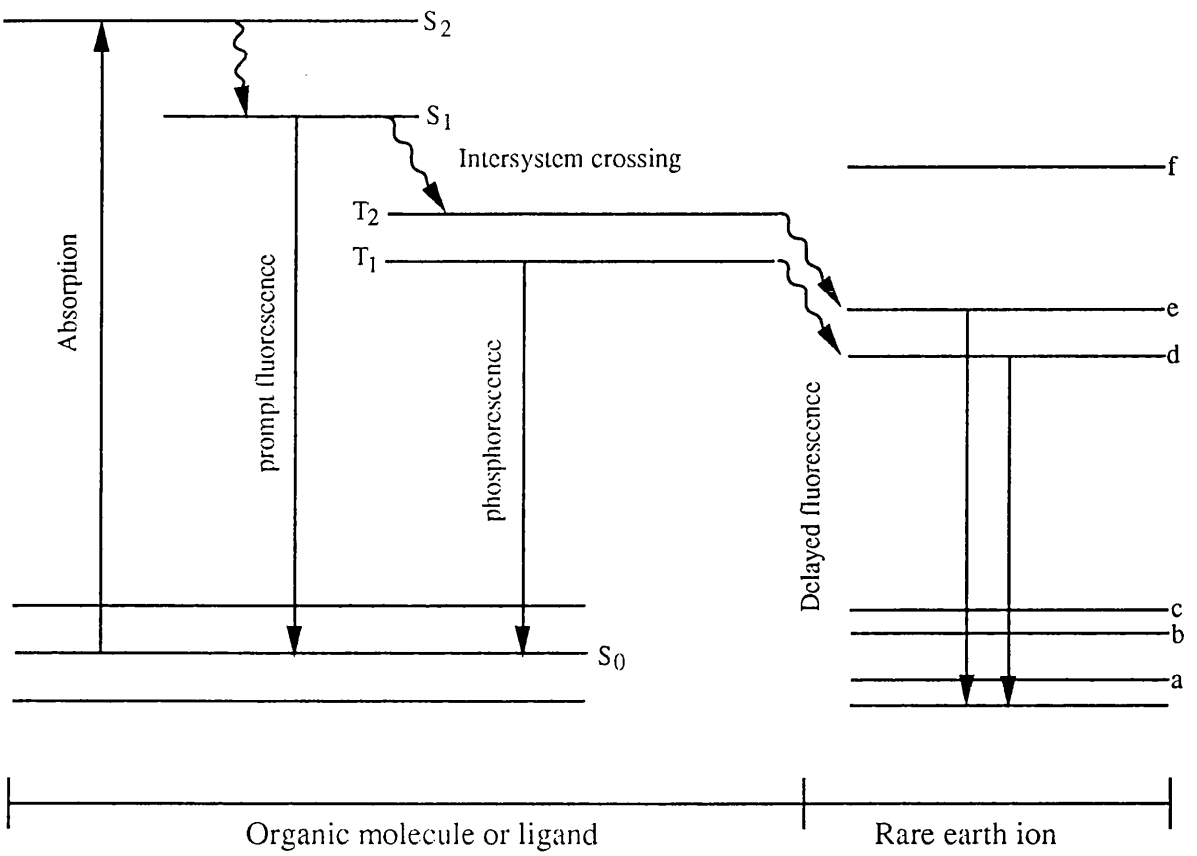


Figure 1.5.7
Principle of fluorescence
(adapted from Price and Newman 1991)



the lanthanide chelates are less likely to present problems of distinguishing between excitation and emission wavelengths when selecting suitable filters to be used in the design of detection equipment. In addition when using pulsed excitation lanthanide chelates continue to fluoresce long after the interfering background fluorescence has decayed. These properties are used in time resolved fluorescence (Price and Newman 1991).

Fluorescence enzyme immunoassays (FEIA) make use of enzymes which convert a non-fluorescent organic molecule into a fluorescent form and this fluorescent substrate is used simply as an alternative to colourimetric detection of enzymes. The advantages of fluorescent substrates are wider measuring ranges and in theory greater sensitivity.

1.6 AIMS FOR THIS THESIS

The original primary aim for this thesis was to produce an assay to measure growth hormone in urine. It quickly became apparent however that there were intermediate aims, namely to investigate the production of antibody fragments and their use in producing enzyme conjugates; and to select the substrate capable of yielding optimal assay sensitivity.

The aims for the project were therefore:-

1) To produce an Fab' antibody fragment of anti-growth hormone IgG and to conjugate this to either horseradish peroxidase or β-D-galactosidase.
2) To produce an assay for urine growth hormone, using this conjugate as label, and a colourimetric or fluorimetric substrate.
3) To measure urinary growth hormone in relevant groups of patient samples, including children with short stature and acromegalic patients.

CHAPTER 2 : MATERIALS AND METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

	Supplier
ABTS [2,2-azino-bis (3 ethyl thiazoline-6-sulphonic acid)]	Sigma Chemical Co.
Anti-human growth hormone monoclonal antibody G2	SAPU
Anti-human growth hormone monoclonal antibody G3	SAPU
Anti-human growth hormone sheep polyclonal IgG	SAPU
Avidchrome $F(ab')_2$ kit	Bio Probe International Inc.
Bovine albumin fraction V	ICN Biomedicals Ltd.
Casein	Presto
Centricon 30 microconcentrators	Amicon Corporation
DEAE cellulose DE 52	Whatman Biochemicals Ltd.
Enhanced luminescence substrate	Kodak diagnostics Ltd.
Etched polystyrene beads	Northumbria Biochemicals
$F(ab')_2$ Sheep IgG	Jackson Immuno Research
Fab' sheep IgG	Jackson Immuno Research
Horseradish peroxidase	Sigma Chemical Co.
Horseradish peroxidase	Boehringer Mannheim GmbH
HPPA (3-(p-Hydroxyphenyl)propionic acid)	Aldrich Chemical Co. Ltd.
Human growth hormone standard UK 9 (IRP 80/505)	UK NEQAS, Edinburgh Royal Infirmary
4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid	Sigma Chemical Co.
2-Mercaptoethylamine	Sigma Chemical Co.

Microtitre plates	Dynatech
Microtitre plates	Labsystems Flow Laboratories
Microtitre plates	NUNC
N-Ethyl maleimide	Sigma Chemical Co.
N-Hydroxy succinamide ester	Sigma Chemical Co.
OPD (o-phenylene diamine)	Sigma Chemical Co.
Phosphate citrate buffer capsules	Sigma Chemical Co.
Porcine pepsin	Boehringer Mannheim GmbH
Porcine pepsin	Sigma Chemical Co.
Rabbit IgG	Sigma Chemical Co.
Sephadex G50	Pharmacia
Sheep IgG	Sigma Chemical Co.
TMB (3,3',5,5'-tetramethyl-benzidine dihydrochloride)	Sigma Chemical Co.
Ultrogel ACA 44	IBF Biotechnics

All other reagents and chemicals were Analar grade from BDH (British Drug Houses)

2.2 BUFFERS AND REAGENTS

2.2.1 Buffers

A) Sodium Phosphate Buffer 0.1 M, pH 8.0

A NaH_2PO_4 $2H_2O$ M.W. 156.01	15.6 g dissolved in 1 litre deionised water
B Na_2HPO_4 M.W. 141.96	14.2 g dissolved in 1 litre deionised water

Add B to A to adjust to required pH.

B) Sodium Phosphate Buffer 0.1M, pH 7.0

A NaH_2PO_4 $2H_2O$ M.W. 156.01	15.6 g dissolved in 1 litre deionised water
B Na_2HPO_4 M.W. 141.96	14.2 g dissolved in 1 litre deionised water

Add B to A to adjust to required pH.

C) Sodium Phosphate Buffer 0.1 M, pH 6.0

A NaH_2PO_4 $2H_2O$ M.W. 156.01	15.6 g
B Na_2HPO_4 M.W. 141.96	14.2 g dissolved in 1 litre deionised water
C Na_2 EDTA $2H_2O$ 5 mM	
M.W. 372.24	1.86 g

Dissolve A and C in 1 litre deionised water and add B to adjust pH to 6.0.

(filter before use)

D) Sodium Phosphate Buffer 17.5 mM pH 6.3

A NaH_2PO_4 $2H_2O$ M.W. 156.01	2.73 g dissolved in 1 litre deionised water
B Na_2HPO_4 M.W. 141.96	2.484 g dissolved in 1 litre deionised water

Add B to A to adjust to required pH.

E) Pepsin Digestion Buffer

Sodium Acetate Buffer 0.1M pH 4.5

$CH_3COO.Na$ (anhydrous) M.W. 82	8.2 g dissolved in 1 litre deionised water
Glacial acetic acid	Add drop wise to adjust pH to 4.5

F) Tris HCl Buffer 2.0 M, pH 8.0

Tris HCl M.W. 157.6	17.76 g
Tris (hydroxy methyl) aminomethane hydrochloride	
Tris base M.W. 121.1	10.6 g dissolved in 100 ml deionised water

G) Tris HCl Buffer 2.0 M, pH 8.0

Tris base M.W. 121.1	24.2 g dissolved in 100 ml deionised water
HCl	10 % add drop wise to adjust pH to 8.0

H) OPD Peroxidase Substrate Assay Buffer

Sodium acetate buffer 50 mM, pH 5.0	4.1g dissolved in 1litre deionised water
Bovine albumin	0.25 g

I) Avidchrom Pepsin Digestion Buffer and Dialysis Buffer

Sodium acetate buffer 25 mM, pH 4.5 Supplied with kit

J) Avidchrom Hi Yield Binding Buffer Supplied with kit

K) Avidchrom Hi Yield Elution Buffer Supplied with kit

L) Avidchrom Hi Yield Regeneration Buffer Supplied with kit

M) Avidchrom Phosphate Buffered Saline pH 7.2

Sodium phosphate buffer 0.02 M	2ml 0.5 M PO_4 to 50 ml
Sodium chloride 0.15 M M.W. 58.44	0.44 g
Sodium azide 0.01 %	0.005g

(azide is only added for long term storage of chromatography columns)

N) Conjugation Buffer

Sodium phosphate buffer 0.1 M, pH 6.5

A NaH_2PO_4 $2H_2O$ M.W. 156.01	15.6 g dissolved in 1 litre deionised water
B Na_2HPO_4 M.W. 141.96	14.2 g dissolved in 1 litre deionised water

Add B to A to adjust to required pH.

O) TMB and ABTS Peroxidase Substrate Assay Buffer

Phosphate citrate buffer pH 5.0	1 capsule in 100 ml deionised water

P)Peroxidase Fluorimetric Substrate Assay Buffer

Sodium phosphate buffer 10 mM, pH 7.0 Dilute 0.1 M Sodium Phosphate Buffer B

Sodium chloride 0.1 M	0.58 g per 100 ml
Bovine albumin 0.5 g/litre	0.5 g per 100 ml

Q) Growth Hormone Plate Assay Buffer

Sodium phosphate buffer 0.01 M, pH 7.0

Sodium chloride 0.1 M	0.58 g per 100 ml
Bovine albumin 1g/litre	0.1 g per 100 ml

Dilute 1 in 10 the 0.1 M buffer B which contains NaCl 1 M and add the BSA before use.

R) EPPS Buffer

EPPS 0.1 M, pH 8.0 M.W. 252.3	10.1g in 500 ml
(N-2 (2-Hydroxy ethyl)-piperazine-N-[3-propane-sulfonic acid]	
NaOH	Add approximately 1g to adjust pH to 8.0

S) Microtitre plate Coating Buffer

K_2HPO_4 $2H_2O$ 0.05 M, pH 9.0	
K_2HPO_4 $3H_2O$ M.W. 228.23	Dissolve 11.4 g in 1 litre deionised water

2.2.2 Reagents

A) Peroxidase Fluorimetric Substrate

Tris buffer 0.1 M pH 7.8	20 ml
HPPA 7.5 g/litre	0.18 g or 1 tablet
(dissolving HPPA adjusts pH of Tris to 7.0)	
H_2O_2	4 ml

HPPA /Tris solution can be stored at 4° C for two months. The HPPA and H_2O_2 should be allowed to reach room temperature and the H_2O_2 is added immediately before use.

B) Saline / Saline and Tween

Sodium chloride	9g dissolved in 1 litre deionised H_2O
Tween 20	5 ml per litre

C) HPPA stop reagent

1.5 M glycine (amino acetic acid)	
pH 10.3 M.W. 75.07	Dissolve 11.3 g in 100 ml deionised H_2O
NaOH	Add 2 M NaOH dropwise to adjust pH to 10.3

2.3 PRODUCTION OF Fab' FRAGMENTS

2.3.1 Purification of IgG from polyclonal antiserum

IgG was precipitated from sheep polyclonal anti-growth hormone serum by the addition of Na_2SO_4 followed by purification of the IgG by gel filtration using DEAE cellulose.

3.6 g of Na_2SO_4 was added slowly to 20 ml of neat sheep antiserum in a 50 ml conical Sarstedt tube. This was mixed for 30 minutes to ensure complete dissolution of the sodium sulphate. The mixture was spun at 2000 rpm for 10-15 minutes. The supernatant was discarded and the precipitate re-dissolved in 20 ml buffer A.

This purified IgG was then dialysed overnight against 600 ml of buffer A. The resulting dialysate was divided into 2 ml aliquots for further processing.

Mini columns of DEAE cellulose DE 52 were poured using 10 ml syringes with glass wool plugs and 3 way taps. The bed volume of each syringe was around 8 ml.

A 2 ml aliquot of IgG dialysate was added to a mini column and washed through with buffer A. Twenty 1 ml fractions were collected using a fraction collector. The absorbance at 280 nm was determined for each fraction and the peak fractions were pooled. The pooled fractions were then concentrated to by centrifugation at 4000 rpm in Amicon Centricon 30 microconcentrators until a final volume of 2ml was obtained. A fixed angle rotor was used to ensure that some liquid remained in the concentrators.

The amount of IgG present in the resulting concentrate was calculated using the extinction coefficient at 280 nm and the molecular weight of sheep IgG.

2.3.2 Pepsin Digestion of IgG to $F(ab')_2$

An aliquot of purified IgG (approx 5 mg/0.5ml) was dialysed overnight at 4 °C against 200 ml of pepsin digestion buffer E.

0.2 mg of porcine pepsin was added per 5 mg of IgG. The pepsin solution was then incubated for 24 hours at 37 °C in an incubator (or water bath). The pH of the digest was adjusted to 7.0 using NaOH (or tris HCl buffer F). The digest was centrifuged briefly to remove the precipitate which forms.

The digest was then added to an ACA 44 column (45cm x 1.5 cm) and eluted with buffer D. Ninety 2 ml fractions were collected and the peak fractions were identified by using a UV recorder (280 nm). The peak fractions containing $F(ab')_2$ were pooled and concentrated to 0.45 ml using Amicon 30 microconcentrators. The amount of $F(ab')_2$ produced was calculated using the extinction coefficient and molecular weight of $F(ab')_2$.

In order to help interpret the resulting protein elution profile the column had previously been calibrated by running markers of bovine albumin, pepsin, sheep IgG, purified IgG, sheep $F(ab')_2$, and sheep Fab'.

2.3.3 Avidchrome kit for $F(ab')_2$ production

The kit is designed to digest 10 mg of purified IgG to produce $F(ab')_2$.

A 10 mg/ml solution of partially purified IgG (SAPU sheep polyclonal anti-growth hormone) was prepared by adjusting the volume of a 25 mg/ml dialysed preparation with Avidchrom pepsin digestion buffer I. Solutions (10 mg/ml) of rabbit and sheep IgG (Sigma) were prepared by weighing out 10 mg of lyophilised IgG and adding 1.0 ml of pepsin digestion buffer I.

One microcentrifuge tube of pepsin, as supplied in the kit, was dissolved in 100 ul of pepsin digestion buffer I. To this was added 1 ml of IgG followed by mixing and incubation for 6 hours or overnight at 37 °C in a water bath. The solution at this stage was turbid.

The sample was split into two 550 µl aliquots to each of which was added 600 µl of the Hi Yield binding buffer J, followed by mixing. 75 µl of anti-pepsin antibody was added to each microcentrifuge tube, which were vortex mixed and left to stand at room temperature for 15 minutes. The digest was then spun at 2000 g for 15 minutes to remove the turbid precipitate which forms. The clear supernatant was drawn up into a 5 ml syringe with a needle.

Separation of the $F(ab')_2$ fragments was achieved using a protein A cartridge supplied with the kit. The protein A cartridge was regenerated, before use, by passing 5 ml of Hi Yield regeneration buffer L through the cartridge at a flow rate of about 20 drops per minute (1 ml / min) keeping the cartridge in a vertical position.

The cartridge was equilibrated with 4 ml of Hi Yield binding buffer J at a flow rate of about 20 drops per minute.

The IgG digest was passed through the column at 10 drops per minute (0.5 ml / min). The effluent was collected into a 15 ml Henley tube. The cartridge was rinsed with 1 ml of Hi Yield binding buffer J, using the same syringe and this effluent was collected into the Henley tube.

The combined 3.45 ml of flow through fraction, containing the $F(ab')_2$ fragments, was dialysed using 10 cm of 50,000 MW cut-off dialysis tubing against phosphate buffered saline M to remove the smaller peptides. The $F(ab')_2$ fragments were then concentrated to 1 ml using Amicon 30 miniconcentrators.

The cartridge was rinsed with 5 ml of Hi Yield binding buffer J and the effluent discarded. The Fc fragments and undigested IgG were eluted from the cartridge with 5 ml Hi Yield elution buffer K. The cartridge was washed with 10 ml of phosphate buffered saline M containing 0.01 % sodium azide and stored at 4 °C.

2.3.4 Reduction of $F(ab')_2$ to Fab'

The $F(ab')_2$ fragment of sheep IgG (2-5mg / 0.45ml) was prepared using either the Avidchrom kit (Section 3.3), or the method of Ishikawa et. al. (1988) (Section 3.2), in a Sarstedt microcentrifuge tube. 50 ul of 2-mercaptoethylamine (0.1 M) in sodium phosphate buffer D was added and incubated for 1.5 hours at 37 °C in a water bath.

The reaction mixture was then added to a column (1.5 x 90 cm) of Ultrogel ACA 44 at a flow rate of 20 ml per hour using buffer D which contains EDTA to prevent re-association of the Fab' fragments. The method of Ishikawa et. al. (1988) shows the use of a 45 cm column of ACA 44 but this did not give adequate separation and a 90 cm was used. Ninety 6 minute fractions (2 ml) were collected and a 280nm UV detector used to monitor the peak fractions from the column. This confirmed the complete split

of the $F(ab')_2$ fragment to Fab' fragments. The peak fractions were pooled and concentrated using Amicon Centricon-30 concentrators.

The amount of Fab' produced was calculated from the extinction coefficient at 280 nm and the molecular weight.

2.4 PRODUCTION OF PEROXIDASE Fab' CONJUGATE

2.4.1 Introduction of maleimide groups into horseradish peroxidase

Buffer D (0.3 ml) was added to 2 mg of horseradish peroxidase in a Sarstedt micro centrifuge tube, and vortex mixed. Maleimide (0.7 mg) (n-succinimidyl-4-(n-maleimidomethyl) cyclohexane-1-carboxylate was weighed into another micro-centrifuge tube and dissolved in 0.03 ml of n,n dimethyl formamide. This process was performed in a fume cupboard. The maleimide-formamide mixture was warmed to 30 °C in a water bath to prevent precipitation of the peroxidase. The maleimide was then transferred into the peroxidase tube. The mixture was incubated at 30 °C in a water bath for 1 hour. The maleimide could be replaced with n-succinimidyl-6-maleimidohexanoate which is more soluble in the reaction mixture and does not require the toxic solvent dimethyl formamide (Hashida et. al. 1984).

The mixture was centrifuged briefly to remove the white precipitate which formed and the brown supernatant was added to a 5 ml Sephadex G 50 fine mini column, made up in a 10 ml syringe with a glass wool plug.

Twelve 1 ml fractions were collected and the absorbance at 280 and 403 nm of each was measured. The peroxidase activity in each fraction was measured using OPD as substrate.

2.4.2 Peroxidase assay of column fractions

In order to identify which column fragments showed peroxidase activity, a quick screening assay protocol was developed using OPD as substrate.

The column fractions were initially screened using 10 µl of column fraction and 200 µl of buffer. The dilution was then increased for the peak fractions which gave absorbance values above 3.0. The assay was set up in microtitre wells and read using a plate reader. The high levels of enzyme present mean that the assay should be read within five minutes of adding the hydrogen peroxide.

The assay protocol was as follows. To 2 µl diluted column fractions was added 200 µl OPD (one 10 mg tablet dissolved in 25 ml buffer H). The enzyme reaction was started by addition with a repeating pipette of 10 µl of H_2O_2 (1/6 dilution of 30 % stock). The assay was mixed on a plate mixer briefly and read immediately (unstopped) at 450 nm on a plate reader.

2.4.3 Conjugation of Fab' fragment to maleimide peroxidase

Maleimide-peroxidase (1.8 mg, 45 nmol) was incubated with Fab' fragment (2.0 mg 43 nmol) in 1 ml of sodium phosphate buffer G at 4 °C for 20 hours or at 30 °C for one hour. The final concentrations of the maleimide peroxidase and Fab' fragment should be 0.01-0.05 mM.

The mixture was then added to a 90 cm column of ACA 44 at a flow rate of 20 ml/hr using conjugation buffer N. Ninety 6 minute fractions of 2 ml were collected.

The absorbance of each fraction at 280 and 403 nm was determined, and the peroxidase activity measured as described in section 2.4.2. The binding of the Fab'-peroxidase was tested for the two pooled peak fractions and the intercept fraction as in section 2.4.5. The Fab'-peroxidase was concentrated by a factor of 10 using Amicon Centricon 30 concentrators and stored at 4 °C. Thiomersal (2-5 g/l) was added at 1/98 volume as a preservative. Sodium azide is unsuitable as a preservative as it inactivates peroxidase. Bovine albumin (100 g/l) was added at 1/98 volume as required.

2.4.4 Coating of microtitre plate wells

Anti-growth hormone G2 monoclonal antibody (5µg / 200 µl) diluted in plate coating buffer S was added to microtitre wells and the plate was incubated overnight at room temperature covered with adhesive tape. The G2 was recovered for re-use and the wells were washed 3 times in saline reagent B. Bovine albumin (300 µl of 0.5 % BSA) diluted in plate coating buffer S was then added to overcoat the wells followed by overnight incubation at room temperature. The plate was washed 3 times in saline regent B prior to assay.

2.4.5 Assay to test binding of label fractions

Growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give standards of: 0, 0.1, 1.0, 10.0 mU/l. Standards (200 µl) were added to wells coated with G2 (methods section 2.4.4) and the plate incubated overnight at 4 °C covered with adhesive tape.

The wells were washed three times in saline and blotted dry. Fab'-peroxidase peak fractions (Section 2.4.3) were diluted 1 in 50 in assay buffer and 200 ul was added to each of the wells. The plates were again incubated washed and blotted dry.

Immediately before use one OPD substrate tablet was dissolved in 50 ml buffer H and 200 µl was added to the wells. H_2O_2 (10 µl of 1/6 dilution of 30 % stock) was added to start the reaction. The plate was incubated on a plate shaker for 30 minutes wrapped in a paper towel to exclude light. The colour developed in each well was determined at 450 nm using a plate reader. The standard curves were plotted for the two peaks and the intercept region of the Fab'-peroxidase purification.

2.5 GROWTH HORMONE ELISA

2.5.1 Coating microtitre wells

Partially purified G2 or G 3 monoclonal anti-growth hormone antibody was diluted in plate coating buffer S to a concentration of 5 µg per 200 µl. This antibody was added to microtitre wells (200 µl per well). The plates were covered with clear adhesive tape and left at room temperature from Friday to Monday morning (approximately 65 hours).

The antibody was recovered for re-use and the plates were washed 6 times in saline reagent B using a plate washer. The plates were left for a few minutes inverted on paper towels to drain off the remaining wash solution. Overcoating material (300 µl of BSA or casein) was then added at a concentration of 0.05%, diluted in plate coating buffer S. The plates were covered and incubated overnight at room temperature.

The plates were washed 6 times in saline and Tween reagent B inverted and left to drain for a few minutes. The plates were then ready for immediate use or for storage at 4 °C in a sealed polythene bag containing moist filter paper.

2.5.2 ELISA using OPD substrate

G2 or G3 coated plates (5µg / 200µl) were prepared as detailed in the methods section 2.5.1.

Human growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give concentrations of: 0.0, 0.001 , 0.01, 0.1, 1.0, 10.0 mU/l. Standard, buffer, or sample (200 µl) was added to wells in duplicate using the plate layout shown in Figure 2.5.1 and the plate was incubated overnight at 4 °C covered with adhesive tape.

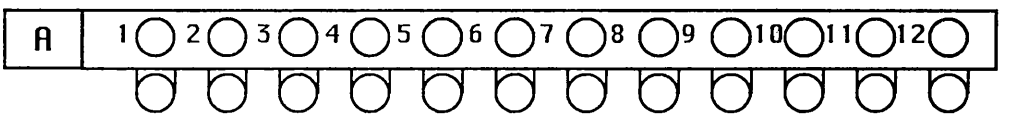
The plate was washed 6 times in saline and Tween reagent B using an automatic plate washer and blotted dry on paper towels. Fab' peroxidase label was diluted 1/500 in Assay Buffer Q and 200 µl was added to each well. The plate was covered and incubated overnight at 4 °C.

The plate was washed 6 times and blotted dry.

Immediately before use one 10 mg tablet of OPD was dissolved in 50 ml of growth hormone plate assay buffer Q. H_2O_2 (30% stock) was diluted 1/6 in distilled H_2O.

OPD substrate was added with a repeating pipette (200 µl per well) followed by H_2O_2 (10 µl) to start the reaction. The plate was then covered with a paper towel to exclude light and incubated on a plate mixer at room temperature for up to 2 hours. The optical density of the wells was measured at 450 nm on a plate reader.

Figure 2.5.1
Layout of growth hormone standard replicates on a microtitre strip.



Wells 1, 7	standard	0.0 mU/l
2, 8	"	0.001
3, 9	"	0.01
4, 10	"	0.1
5, 11	"	1.0
6, 12	"	10.0

2.5.3 ELISA using ABTS substrate

G2 or G3 coated plates (5µg / 200µl) were prepared as detailed in the methods section 2.5.1.

Human growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give concentrations of: 0.0, 0.001 , 0.01, 0.1, 1.0, 10.0 mU/l. Standard, buffer, or sample (200 µl) was added to wells in duplicate using the plate layout in Figure 2.5.1, and the plate was incubated overnight at 4 °C covered with adhesive tape.

The plate was washed 6 times in saline and Tween reagent B using an automatic plate washer and blotted dry on paper towels.

Fab' peroxidase label (200 µl) diluted 1/500 in Assay Buffer Q was added to each well. The plate was covered and incubated overnight at 4 °C.

The plate was washed 6 times and blotted dry as above.

Immediately before use a 10 mg tablet of ABTS was dissolved in 100 ml of ABTS substrate assay buffer O.

ABTS substrate was added to each well with a repeating pipette (200 µl per well). The plate was then incubated on a plate mixer at room temperature for up to 2.5 hours wrapped in a paper towel to exclude light. The optical density of the wells was measured at 405 nm on a plate reader.

2.5.4 ELISA using TMB substrate

G2 or G3 coated plates (5µg / 200µl) were prepared as detailed in the methods section 2.5.1.

Human growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give concentrations of: 0.0, 0.001 , 0.01, 0.1, 1.0, 10.0 mU/l. Standard, buffer, or sample (200 µl) was added to wells in duplicate using the plate layout in Figure 2.5.1 and the plate was incubated overnight at 4 °C covered with adhesive tape.

The plate was washed 6 times in saline and Tween reagent B using an automatic plate washer and blotted dry on paper towels.

Fab' peroxidase label (200 µl) diluted 1/500 in Assay Buffer Q was added to each well. The plate was covered and incubated overnight at 4 °C.

The plate was washed 6 times and blotted dry.

Immediately before use a 1 mg TMB substrate tablet was dissolved in 10 ml TMB substrate assay buffer O in a Henley tube. The Henley tube was spun briefly to remove the small amount of undissolved substrate and the substrate was transferred to a clean container. As TMB is toxic the substrate tablets were not handled and inhalation of dust from the tablets was avoided.

TMB substrate (200 µl) was added to each well with a repeating pipette. The plate was covered with a paper towel, to exclude light, and incubated for 30 minutes on a plate shaker at room temperature.

Stop reagent (50 µl H_2SO_4, 2 M) was added to each well, causing a colour change from blue to yellow. The plates were read immediately at 450 nm on the plate reader. Where the absorbance of the well exceeded 3.0 absorbance units the well contents were diluted x 2 in TMB substrate assay buffer O and the measurement repeated.

2.5.5 TMB transfer assay

Fab' Peroxidase label (200 µl) was added to 200 µl urine, dialysed urine and buffer standards in a blank microtitre plate and incubated overnight at 4 °C. From each well 200 µl was then transferred to G2 coated plates (5µg/200µl) prepared as detailed in the methods section 2.5.1. The plate was then incubated overnight at 4 °C covered with adhesive tape. The plate was then washed with saline, 200 µl of TMB added followed by incubation and measurement of the optical density as in section 2.5.4.

2.5.6 Fluorescence enzyme immunoassay (FEIA) using HPPA substrate

G2 or G3 coated plates (5µg / 200µl) were prepared as detailed in the methods section 2.5.1.

Human growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give concentrations of: 0.0, 0.001 , 0.01, 0.1, 1.0, 10.0 mU/l. Standard, buffer, or urine (200 µl) was added to wells in duplicate and the plate incubated overnight at 4 °C covered with adhesive tape.

The plate was washed 6 times in saline and Tween reagent B using an automatic plate washer and blotted dry on paper towels.

Fab' peroxidase label (200 µl) diluted 1/1000 in Assay Buffer Q was added to each well. The plate was covered and incubated overnight at 4 °C.

The plate was washed 6 times and blotted dry as above.

HPPA was dissolved in 0.05 M Tris buffer pH 7.8 (7.5g per litre). Immediately before use five volumes of HPPA were mixed with one volume of 0.06% H_2O_2. The HPPA solution and H_2O_2 were allowed to come to room temperature in the dark before use.

HPPA substrate (200 µl) was added to each well with a repeating pipette. The plate was incubated for 60 minutes on a plate mixer at room temperature. Stop reagent (100 µl of 1.5 M glycine buffer pH 10.3) was added to each well. The fluorescence was read on a Fluoroscan II plate reader, the excitation and emission wavelengths being 320 and 405 nm respectively.

2.5.6.1 Recrystalisation of HPPA fluorimetric substrate

The following protocol was followed unsuccessfully in an attempt to purify the HPPA substrate supplied by Sigma. The HPPA used in the fluorescence assay (methods section 2.5.6) was supplied by Labsystems Finland and had been purified by Helsinki University Department of Chemistry.

The HPPA available commercially was recrystalised before use to provide the best possible purity.

2g of HPPA was added to 50 ml of distilled water and heated to 50-60 °C on a hot plate with a magnetic stirrer until the substrate dissolved. The minimum amount of distilled water was used to dissolve the substrate.

The substrate was removed from the hot plate and allowed to cool to room temperature. It was then cooled further in the fridge to 4 °C to allow the crystals to form. The substrate was allowed to cool slowly to maximise the purity of the crystals at the expense of a high yield of substrate.

The crystals were recovered by filtering under vacuum through a Whatman filtration system with microfibre glass filter pads. The crystals were rinsed with ice cold distilled water.

The crystals were allowed to dry on filter paper and then transferred to a desiccator. Once dry the crystals were weighed and the recovery calculated.

2.5.7 Enhanced Luminescence assay

Human growth hormone standard (UK 9) was diluted in growth hormone plate assay buffer Q to give concentrations of: 0.0, 0.001 , 0.01, 0.1, 1.0, 10.0 mU/l. Standard (200 µl) was added in duplicate to wells of NUNC G2 coated plates (5 µg / 200 µl) prepared as in section 2.5.1. The plates were incubated and washed as in the methods section 2.5.6. Kodak Amerlite enhanced luminescence signal reagent was prepared by adding one tablet A and one tablet B to a bottle of signal reagent buffer. Immediately after the plate was washed the signal reagent was added (250 µl per well) and the plate was read in the luminescence plate reader five minutes later (2-20 minutes).

2.6 URINE ASSAY

2.6.1 Dialysis of urine

Fresh urine was collected and a measured volume was dialysed overnight at 4 °C using dialysis tubing with a 12 to 14 000 molecular weight cut off and 600 ml of growth hormone plate assay buffer Q which contains 1 g/l BSA. The volume of the urine after dialysis was checked to ensure that there was no significant loss of urine during the dialysis procedure.

2.6.2 Spiked urines

Growth hormone standard UK 9 was diluted in growth hormone plate assay buffer Q and added to measured volumes of urine and dialysed urine.

CHAPTER 3 : EVALUATION OF REAGENTS AND RESULTS

CHAPTER 3 : EVALUATION OF REAGENTS AND RESULTS

3.1 PRODUCTION OF ENZYME CONJUGATE

3.1.1 Purification of sheep anti growth hormone antiserum

Growth hormone antiserum was purified as detailed in the methods section 2.3.1. The peak produced was monitored by measuring the Å 280 nm of each fraction. A typical column chromatography profile is shown (Figure 3.1.1). The IgG produced was concentrated and the amount of protein present was estimated from the Å 280 nm according to the formula .

$$Å = \varepsilon CL$$

Where Å is the absorbance of a pure protein at 280 nm, C is the concentration of the protein in moles per litre, L is the path length of the cuvette, usually 1 cm, and ε is the molar extinction coefficient for the protein

$$\text{Hence } C = Å/\varepsilon$$

The extinction coefficient for sheep IgG is 1.5 $l^{-1}cm^{-1}$ and the molecular weight is 150 KD. The stock IgG for use in fragment production was calculated to contain 23.7 g/l.

3.1.2 Pepsin digestion of IgG to $F(ab')_2$

Initially albumin was used as a molecular weight marker to characterise the fragments obtained from the column purifications of IgG digestion. Unpurified sheep antiserum, purified sheep antiserum and sheep $F(ab')_2$ were then all run on the column to indicate in which fractions they eluted (Figure 3.1.2). Pepsin was also run to check that it did not co-elute with any other major fragment. Pepsin was shown to be a minor peak which elutes before IgG and was therefore not considered further. The sheep IgG profile had two peaks the second of which was considered to be IgG and the first was thought to be polymers of IgG.

Figure 3.1.1
Elution profile of IgG purification
(8ml DEAE cellulose column)

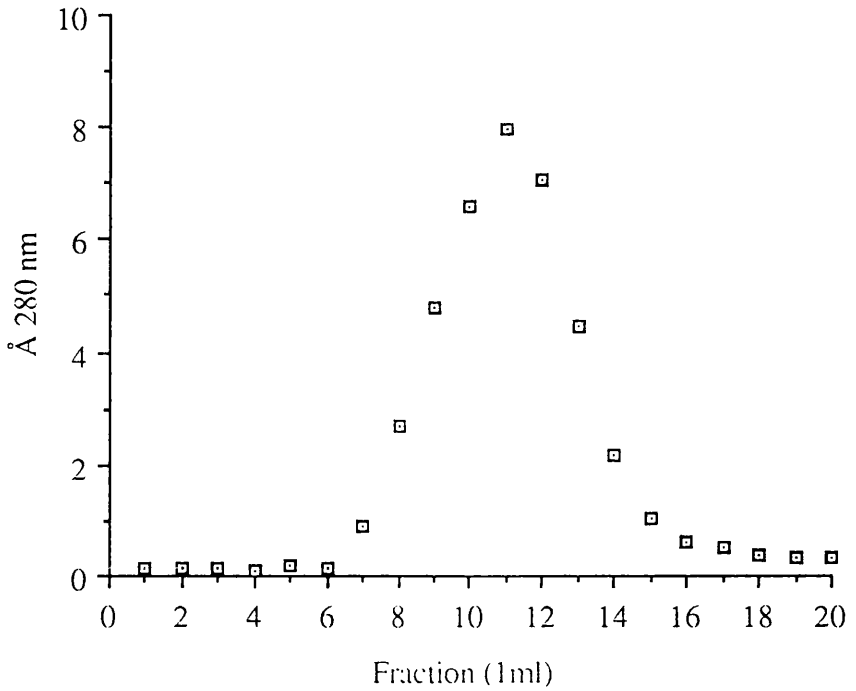
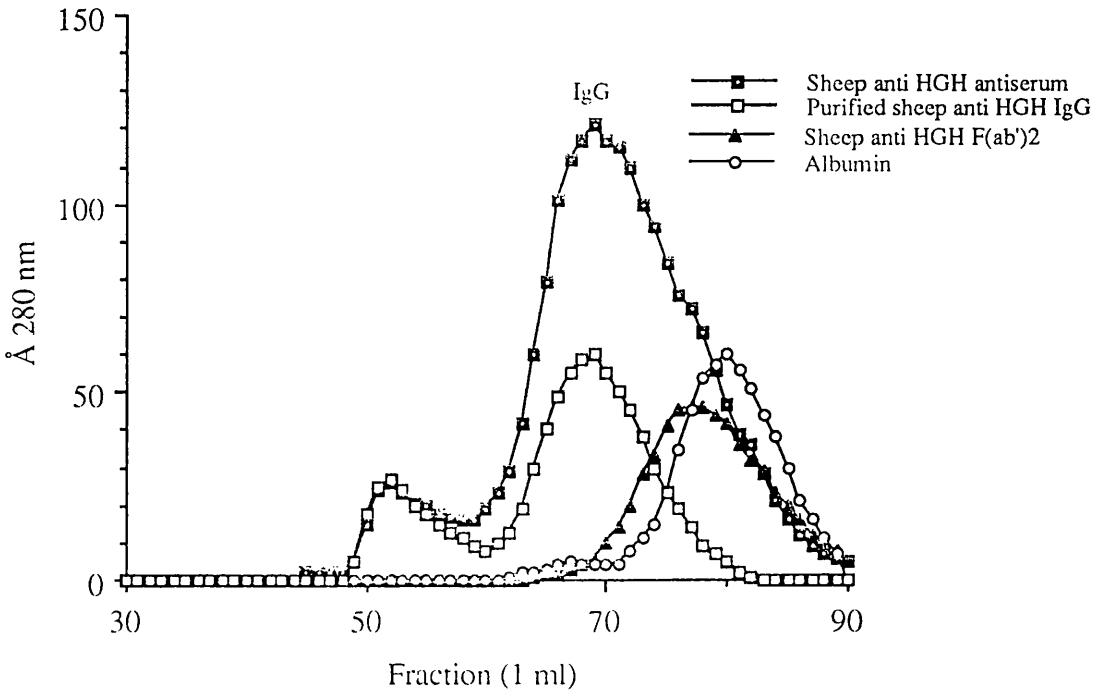


Figure 3.1.2
Elution profiles from a 45 cm ACA 44 column



3.1.3 Reduction of F(ab')$_2$ to Fab'

Many attempts were made to produce Fab' from F(ab')$_2$, as detailed in the Methods Section Chapter 2.3.4. It was not possible to determine whether Fab' had in fact been produced since run to run variations in the elution fractions suggested that the 45 cm column was not capable of resolving Fab' from F(ab')$_2$. Markers of sheep Fab' and F(ab')$_2$ were used individually and mixed together in equal proportions to determine if they could be resolved by the column and these showed that there was considerable overlap of the peaks using this column length (Figure 3.1.3). A 100 cm column was then prepared and when the mixture of markers was run, adequate separation was achieved (Figure 3.1.4). This longer column was adopted for all further experiments.

Use of the longer column showed that the F(ab')$_2$ produced was not being reduced to Fab' successfully. An alternative kit method of producing F(ab')$_2$ was examined using Sigma Sheep IgG and sigma rabbit IgG as sources of IgG to preserve the stocks of anti-growth hormone antiserum. F(ab')$_2$ was successfully produced from both of these and subsequently Fab' was obtained from them using mercapto-ethylamine reduction as detailed in the methods section 2.3.4. (Figure 3.1.5), (Figure 3.1.6), (Figure 3.1.7). The kit was used to produce F(ab')$_2$ from the SAPU anti HGH antiserum and from this Fab' was obtained using the method detailed in Chapter 2.3.4.

There are several possible theories to explain why the F(ab')$_2$ could not be reduced to Fab' using the original method of Hashida et. al. (1985). Firstly, the material produced may have been altered or damaged such that it could not be further reduced. This is unlikely since the methods of production were very similar, although different concentrations or acidity of the buffers may have led to conformational changes in the F(ab')$_2$. Secondly, one of the steps may not have been optimized sufficiently for sheep IgG and, for example, the pepsin digestion may have been incomplete or excessive. Thirdly, the Fab' produced was being immediately reconverted to F(ab')$_2$. This is

Figure 3.1.3
Fab' and F(ab')2 markers on a 45 cm ACA 44 column

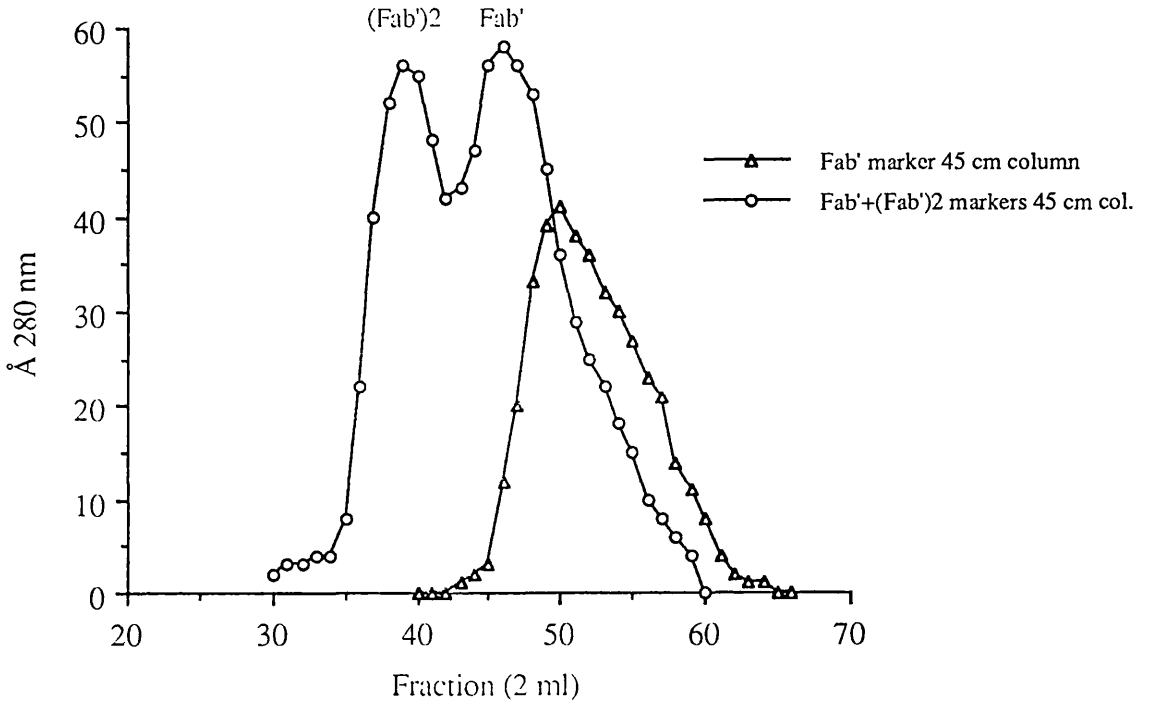


Figure 3.1.4
Comparison of separation of sheep Fab' and F(ab')2 using two different column lengths

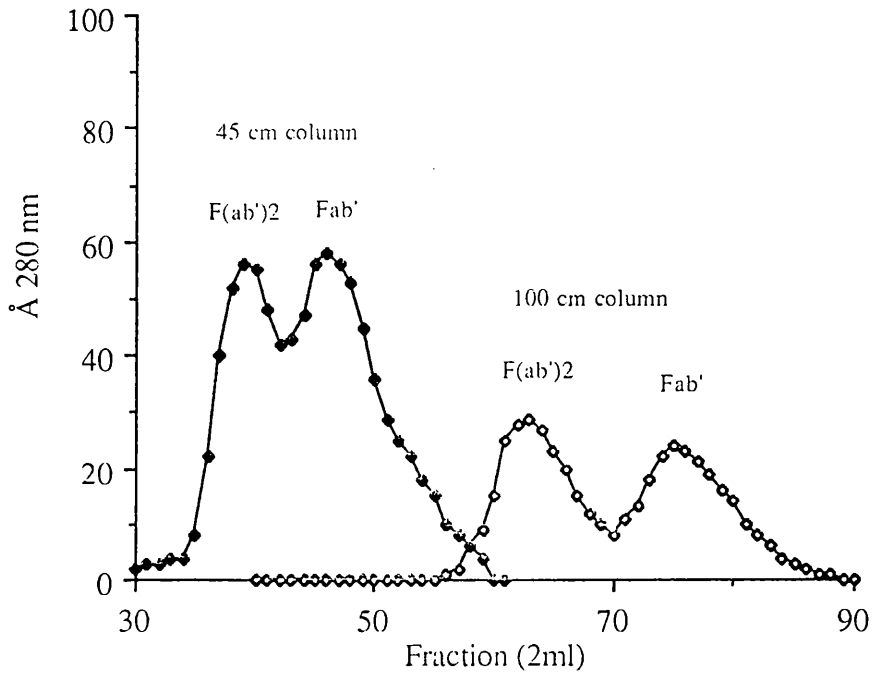


Figure 3.1.5
Elution profiles of rabbit IgG fragments on a 100 cm ACA 44 Column (Pearce method)

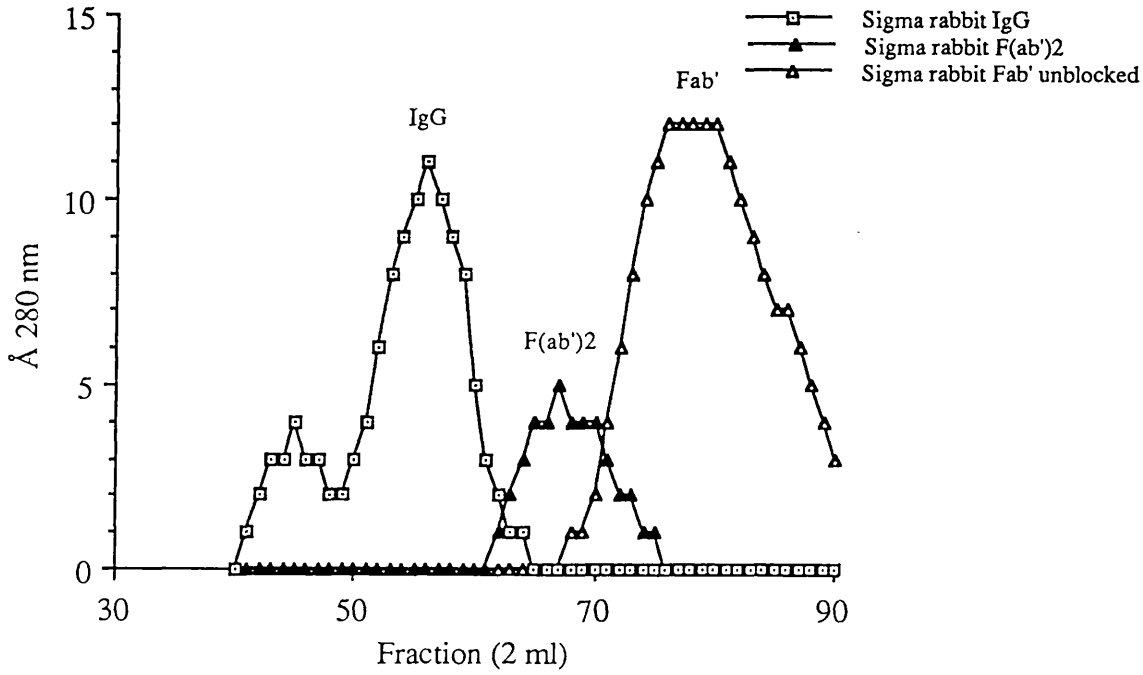


Figure 3.1.6
Elution profiles of Sigma rabbit fragments and Jackson column markers (100 cm ACA 44 column Pearce method)

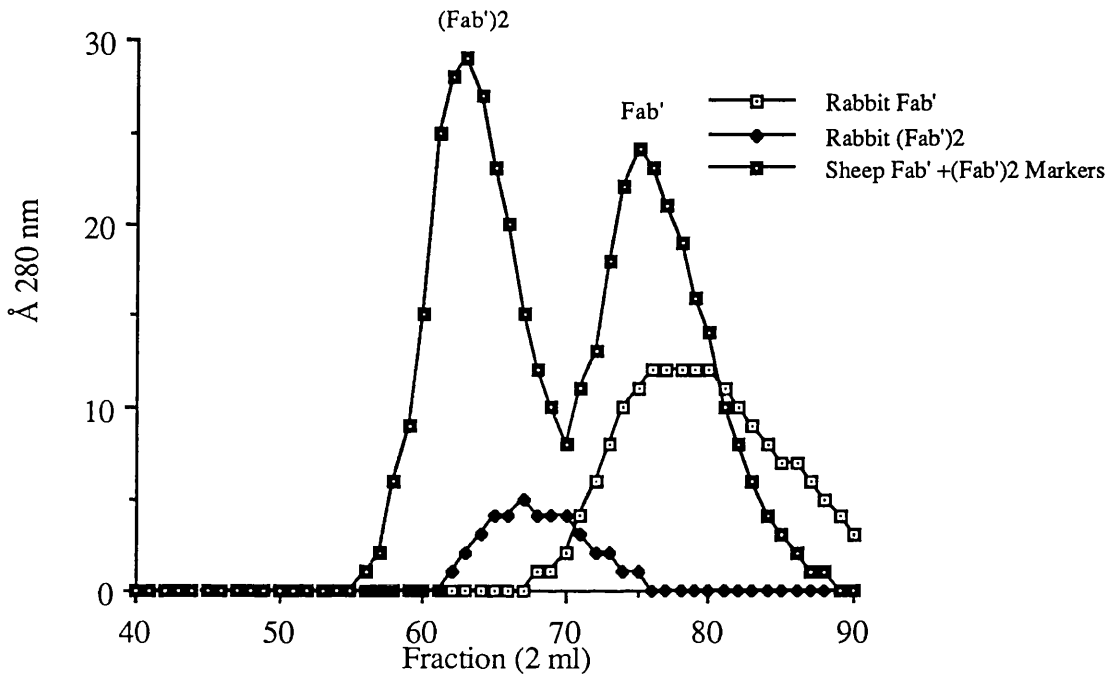
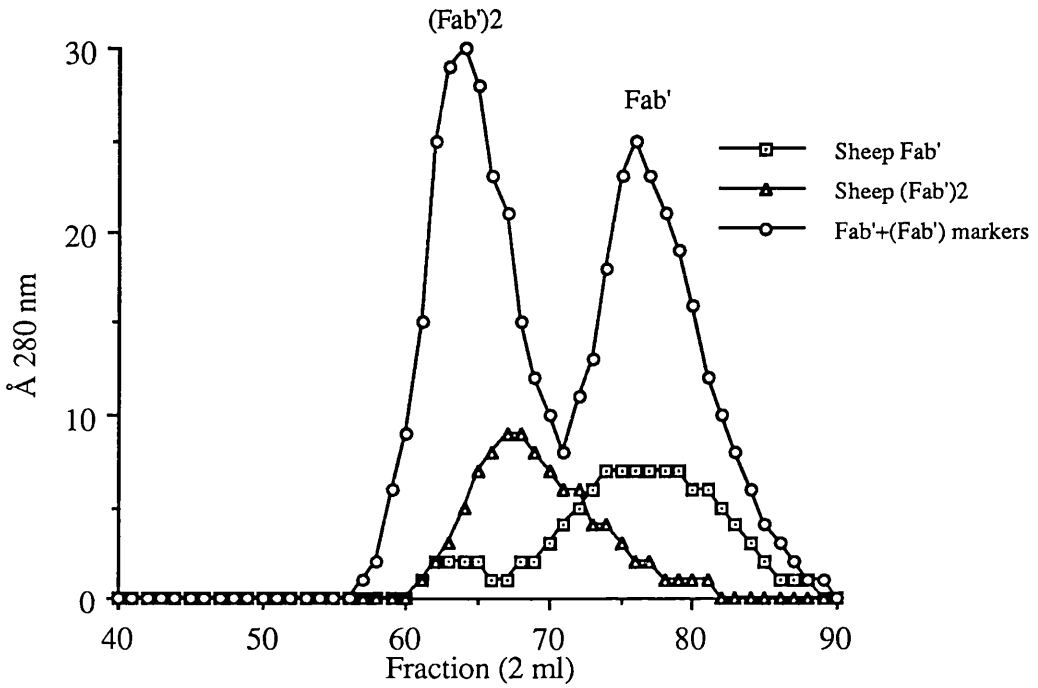


Figure 3.1.7
Elution profiles of Sigma sheep fragments and Jackson column markers (100 cm ACA 44 Column Pearce method)



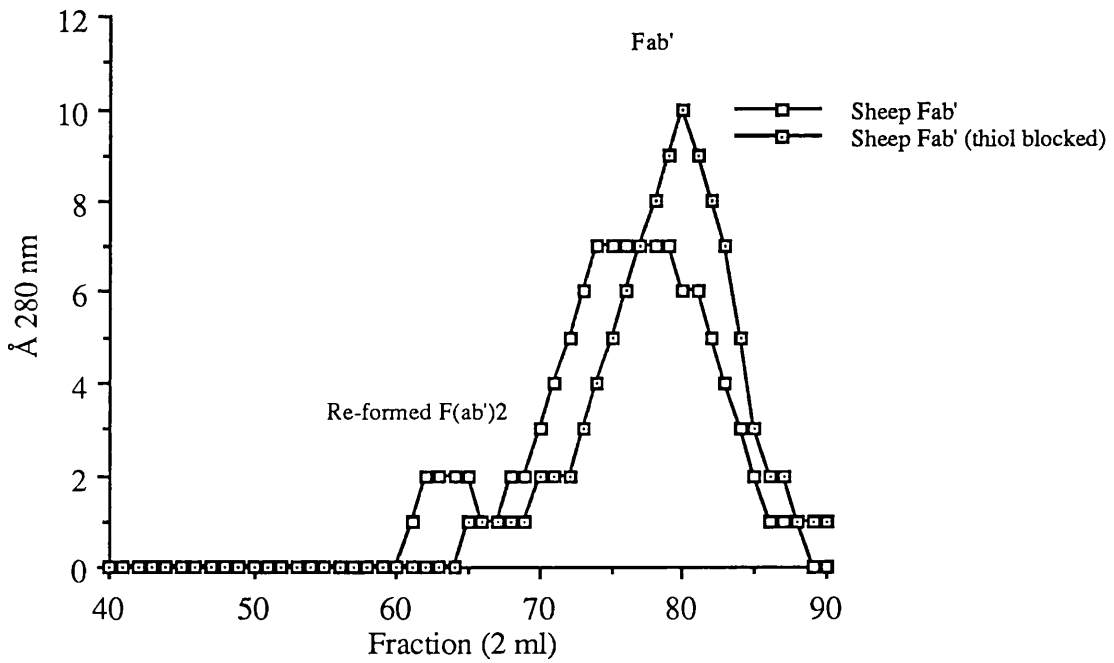
unlikely as the $F(ab')_2$ produced using the Pearce kit was successfully reduced to Fab' using the same method. Attempts were made to investigate this problem, including the use of thiol blocking to prevent the reformation of $F(ab')_2$ from Fab', but the results were inconclusive and due to time limitations the Pearce method was adopted as it was much more simple and quicker to carry out.

An attempt was made to monitor the pepsin digestion of IgG by removing 20 ml of digest and adding 17 ml of NaOH to adjust the pH to 7.0 hence stopping the digestion. This was repeated at a series of steps from one hour to 24 hours and the products were run on a protein electrophoresis gel. The IgG band on these strips gradually disappeared over the 24 hour time course forming a transient digestion product band which appeared at around one hour of digestion and disappeared after four hours, the production of $F(ab')_2$ could not be shown but a diffuse pre-origin band increased in intensity during the incubation period. Markers of Fab' and $F(ab')_2$ were also examined using electrophoresis and the sheep markers formed a similar pre-origin band while the rabbit markers formed an immediately post-origin band. Since the Fab' could not be distinguished from the $F(ab')_2$ the technique was considered of little use and was abandoned. The alternative of Poly Acrylamide Gel Electrophoresis (PAGE) could not be undertaken within the constraints of the project.

3.1.4 Thiol blocking of Fab'

The thiol groups of Fab' were blocked with N-ethyl maleimide to check whether the Fab' was re-forming $F(ab')_2$. There appeared to be some re-formation of $F(ab')_2$ and it was suggested that if the Fab' was not to be used immediately then it should be thiol blocked (Figure 3.1.8). The Fab' prepared for conjugation was used immediately after preparation. It is not clear from the publications of Ishikawa whether or not thiol blocking affects the conjugate formation.

Figure 3.1.8
Profiles of Sigma sheep Fab' and thiol blocked Fab' (100 cm ACA 44 column Pearce method)



3.1.5 Production of horseradish peroxidase maleimide

The purification of horseradish peroxidase-maleimide produced two peaks when the Å 403 nm was measured for each fraction, but only one peak at Å 280 nm (Figure 3.1.9). The majority of the horseradish peroxidase activity was to be found in the first peak (Figure 3.1.10) and several experiments were devised to identify the second peak. These experiments included applying the various components individually and in combination to columns and collecting fractions. The components included: horseradish peroxidase (Figure 3.1.11), maleimide (Figure 3.1.12), horseradish peroxidase in formamide (Figure 3.1.13), and horseradish peroxidase which had been incubated at 30 °C for one hour to replicate the horseradish peroxidase maleimide conjugation procedure (Figure 3.1.14). The maleimide on its own gave a small peak in the region of the second peak but its peak height was much lower than in the horseradish peroxidase-maleimide purification. This second peak appeared to be a reaction product which had little or no horseradish peroxidase activity. The peak was not the result of damage to horseradish peroxidase by formamide or due to formamide itself.

The relative quantities of maleimide and horseradish peroxidase were adjusted to optimize the linking and the effect of extending the incubation time from one to two hours was examined (Figure 3.1.15) and it was shown that there was no significant advantage in increasing the amount of maleimide or in extending the incubation time to two hours.

Figure 3.1.9
Elution profile from purification of horseradish peroxidase-maleimide (5 ml sephadex G50 fine column)

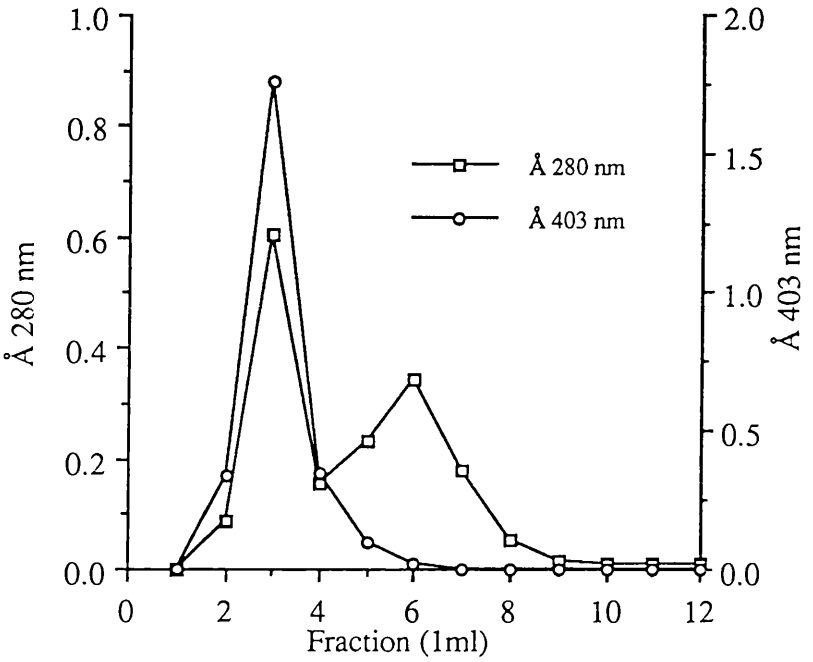


Figure 3.1.10
Elution profile from purification of horseradish peroxidase-maleimide (5ml Sephadex G50 fine column)

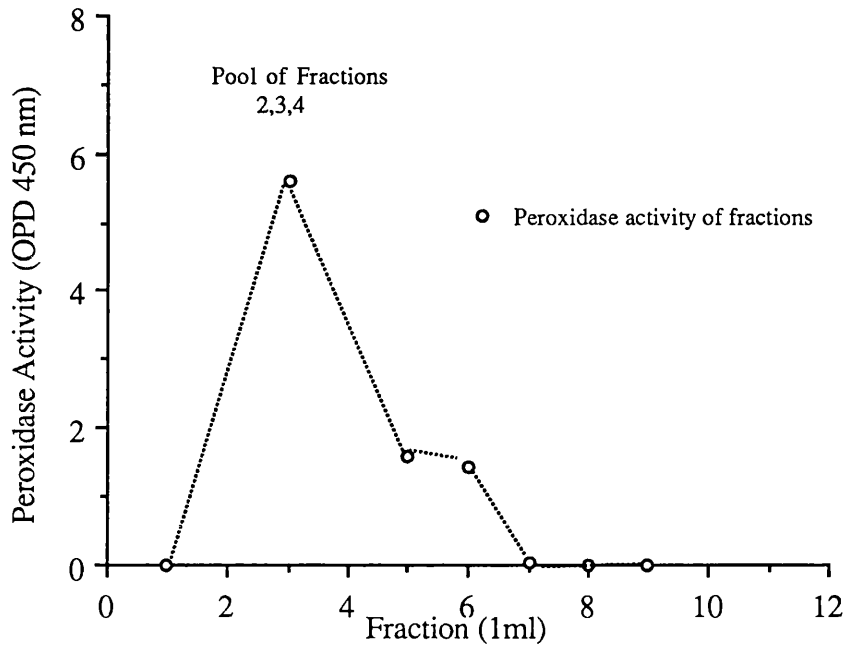


Figure 3.1.11
Elution profile of horseradish peroxidase purification (5 ml Sephadex G50 fine column)

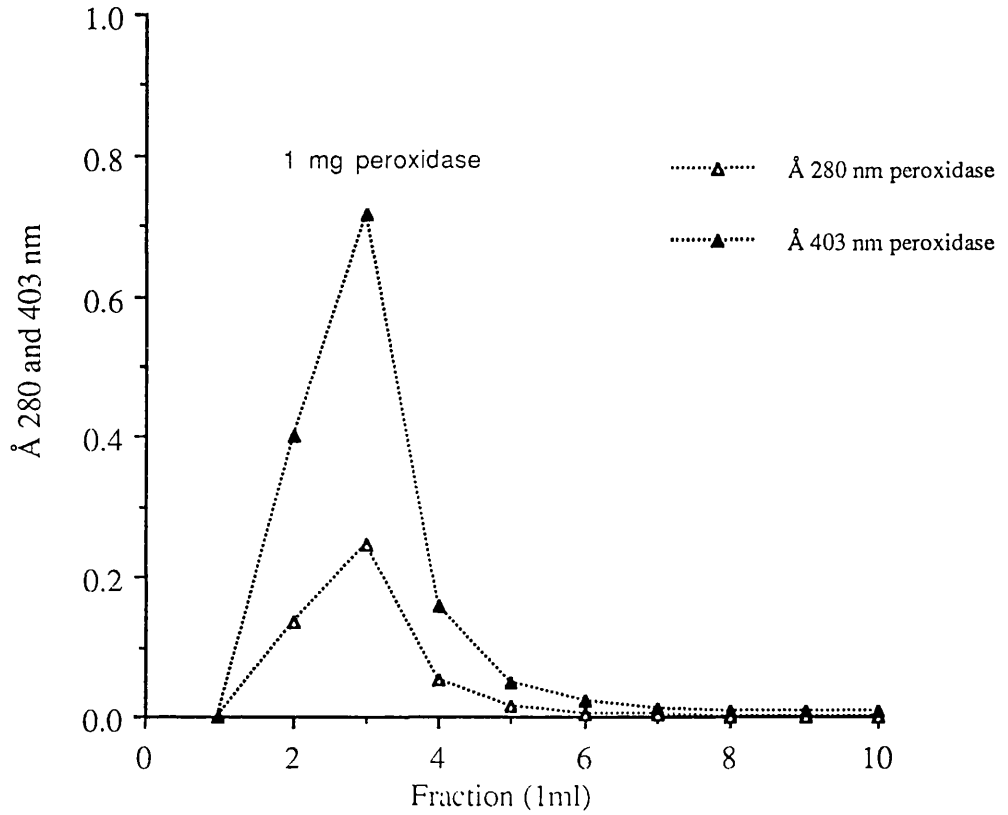


Figure 3.1.12
Elution profile of maleimide marker
(5ml Sephadex G50 fine column)

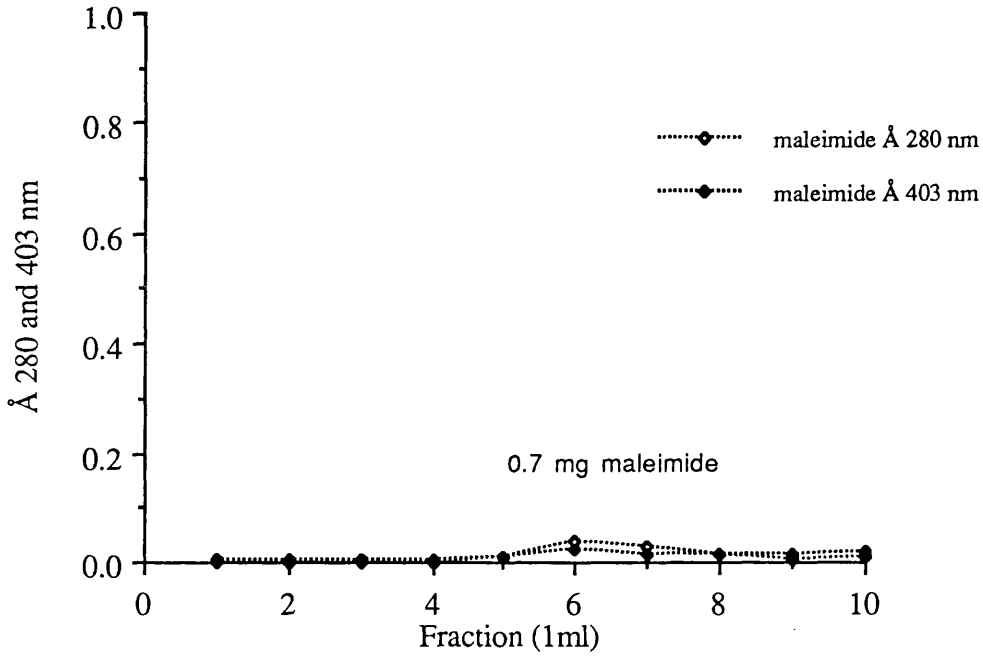


Figure 3.1.13
Elution profile of horseradish peroxidase+formamide purification (5 ml Sephadex G50 fine column)

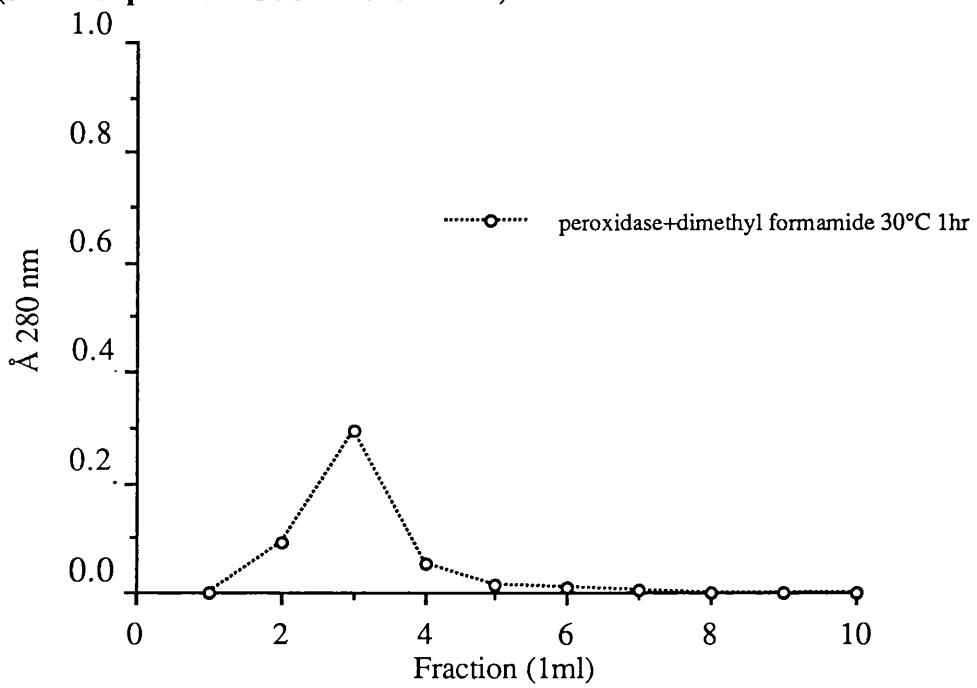


Figure 3.1.14
Elution profile of horseradish peroxidase purification (5 ml Sephadex G50 fine column)

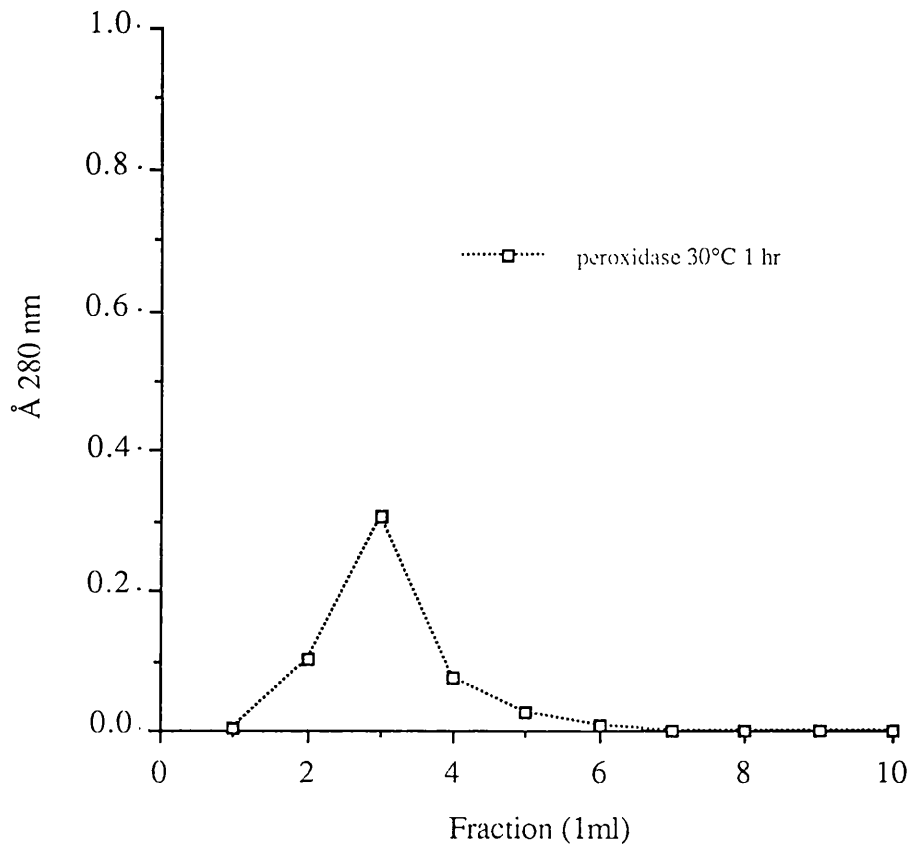


Figure 3.1.15 a
Elution profiles of horseradish peroxidase-maleimide purification with varying maleimide concentrations and incubation times (5 ml Sephadex G50 fine column)

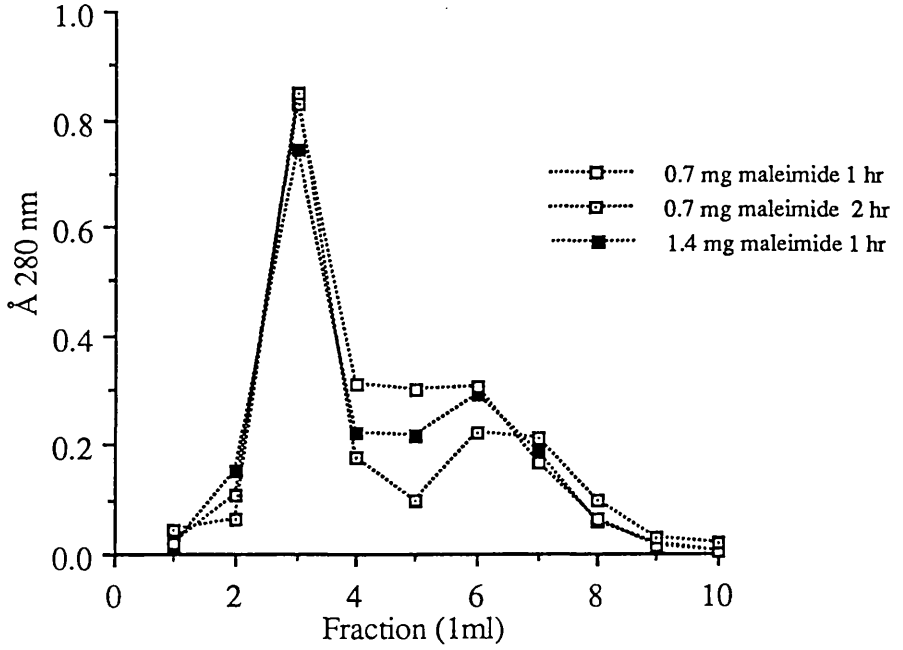


Figure 3.1.15 b

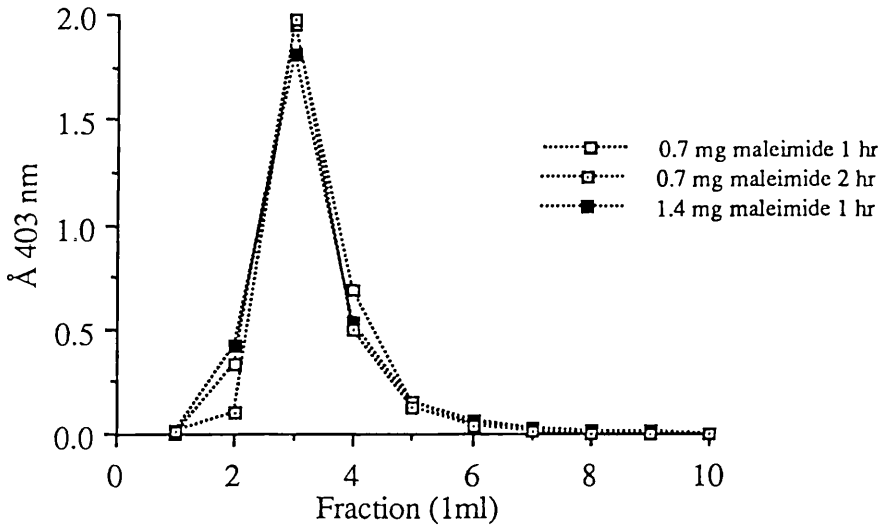
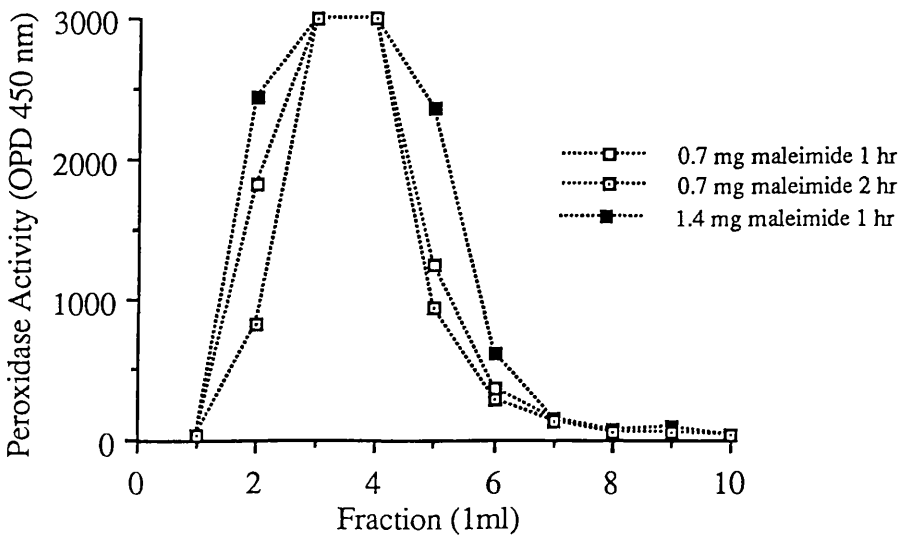


Figure 3.1.15 c



3.1.6 Conjugation of Fab' with horseradish peroxidase-maleimide

The product of the horseradish peroxidase-maleimide conjugation was characterised by purification on an ACA 44 column (100 cm) with each of the fractions produced being examined for their absorbance at 280 and 450 nm and for their horseradish peroxidase activity (Figure 3.1.16). These all showed two large peaks with a cross-over area. Since the first peak was of larger molecular weight, having the shortest retention time, it was anticipated that this would prove to be the conjugate.

3.1.7 Test of binding of label fractions

The two peaks obtained from the purification of the product from the Fab' peroxidase-maleimide conjugation were examined for growth hormone binding by setting up a simple ELISA using plates coated with G2 anti growth hormone antiserum and OPD substrate. Fractions around both peaks were tested and in addition a fraction at the cross-over point of the two peaks was tested. Peak one showed most binding, the cross-over showed less binding, and peak two showed no significant binding (Figure 3.1.17). It was concluded that peak one contained the conjugate of peroxidase and Fab', peak two may consist of unconjugated Fab' and unconjugated horseradish peroxidase and the cross-over contained a mixture of both. Since the second peak had considerable peroxidase activity it was considered to be mainly unconjugated horseradish peroxidase. The Fab' should be present in excess to prevent linkage of Fab' with more than one peroxidase molecule but it would appear from the amount of peroxidase activity remaining in the second peak that in this case the horseradish peroxidase was in slight excess.

The recovery of fragments at each stage of the conjugate production was low and the overall recovery of Fab'in the label was calculated as 36%. However since only small

Figure 3.1.16 a
Elution profiles from peroxidase-Fab' conjugate purification (ACA 44 100 cm column)

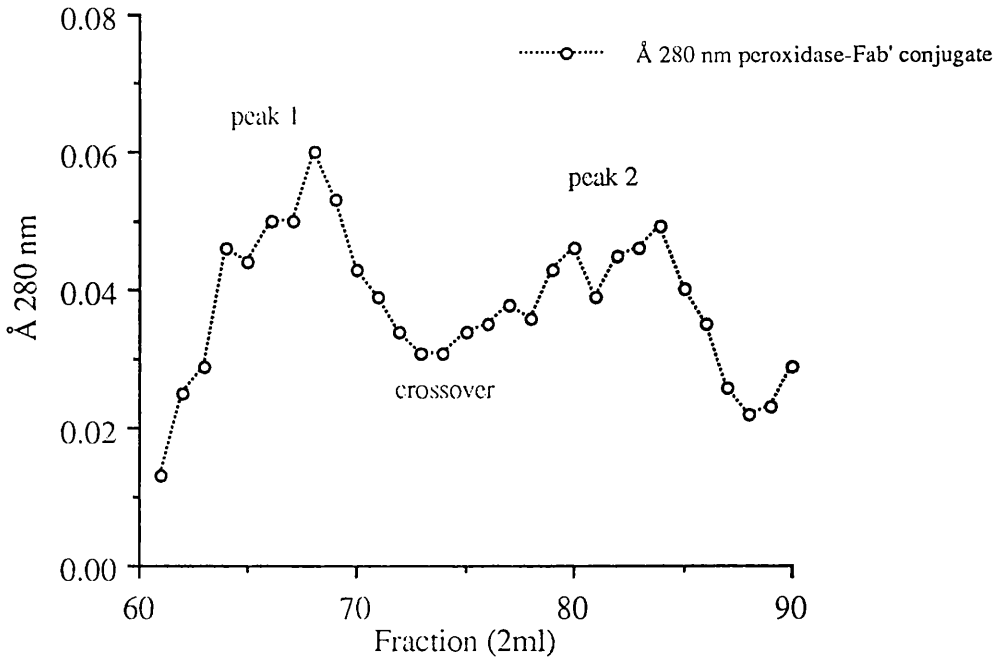


Figure 3.1.16 b

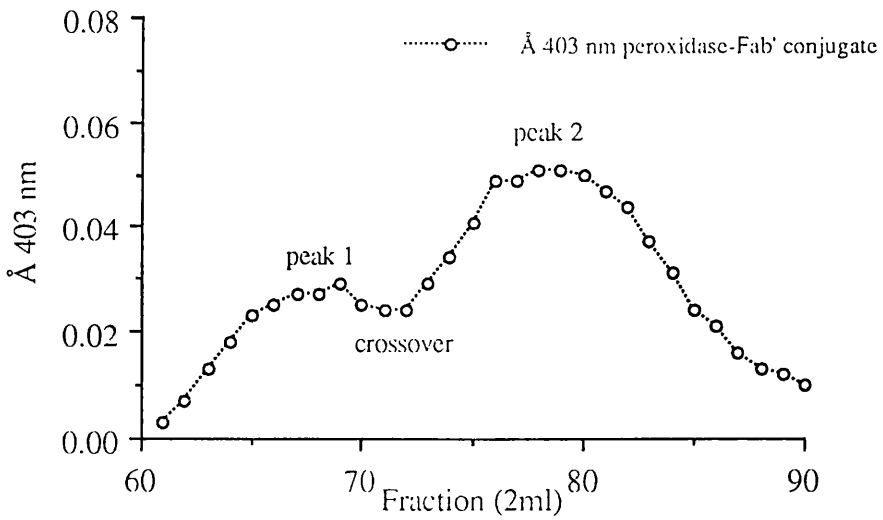


Figure 3.1.16 c

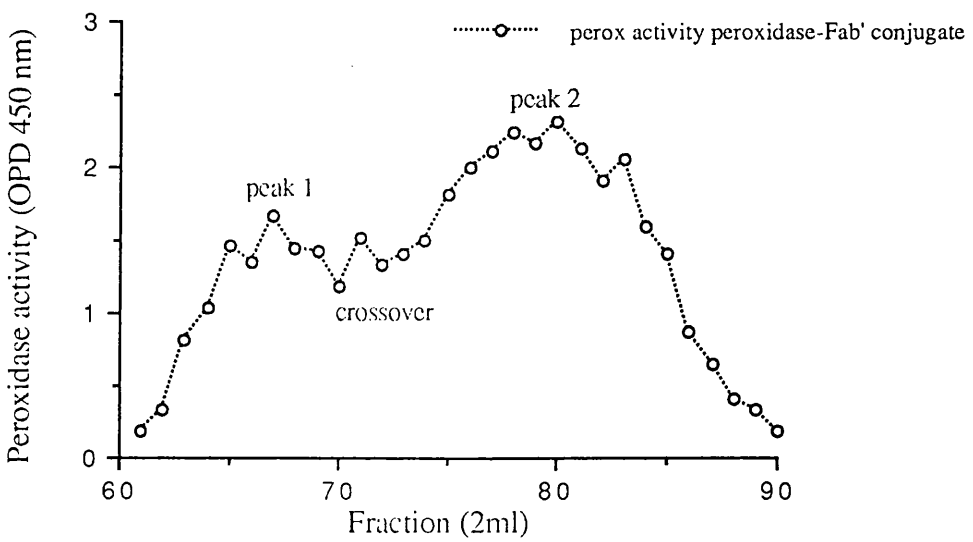
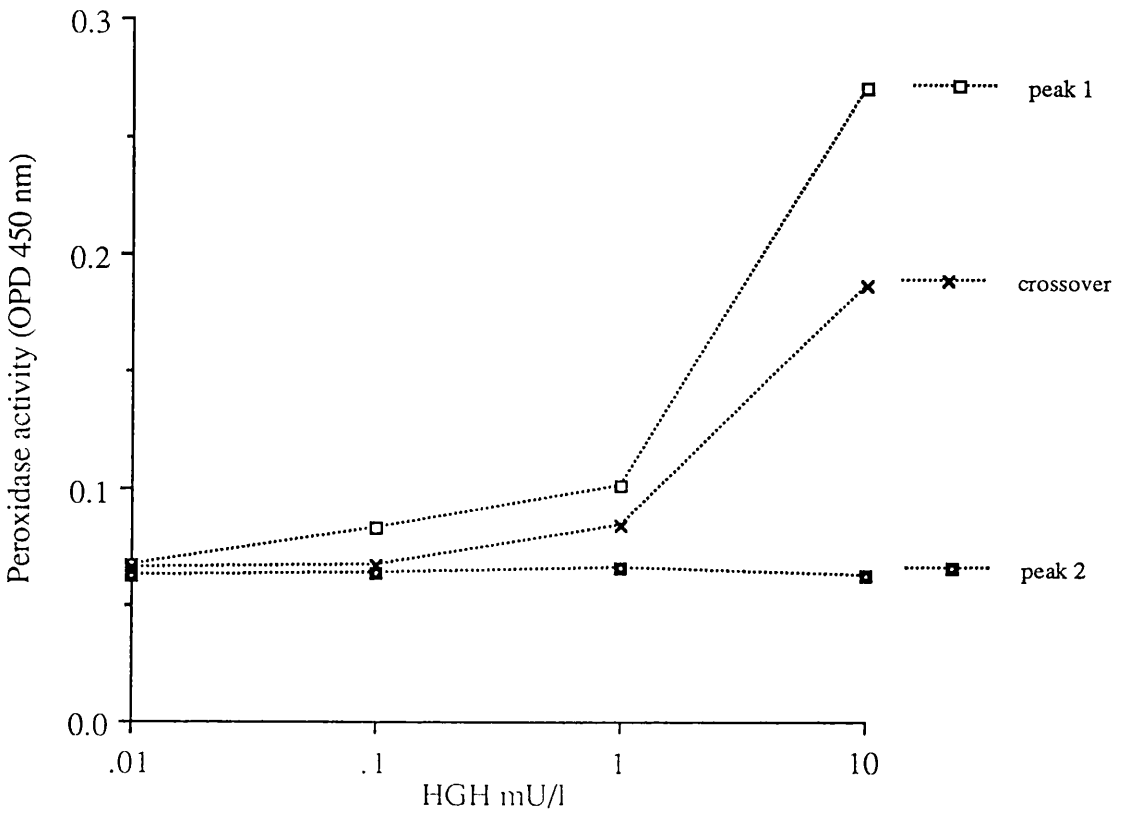


Figure 3.1.17
Test of column fractions from peroxidase label purification in a growth hormone assay (OPD substrate)



amounts of label were required this was not of immediate concern, although further investigations would be advisable if the method were to be used frequently.

3.2 DEVELOPMENT OF A PRELIMINARY ASSAY ON PLATES

Initially an assay was set up using OPD as a substrate but since the colour changes produced were so minimal the conjugate production was almost considered a failure until alternative substrates were examined. These substrates showed much better colour development than OPD making it possible to proceed with the assay development.

Using the preliminary OPD assay the concentration of antibody coated to the plates, the label dilution, incubation times and temperatures were all optimised. These experiments provided general guide-lines which were later re-checked as the TMB assay was improved.

3.2.1 Colourimetric substrates: ABTS,TMB,OPD

A variety of different substrates and incubation times were investigated to determine which gave the highest signal to noise ratio. ABTS and TMB proved to be useful substrates while OPD gave a very small change in colour and was not used further (Figure 3.2.1). TMB and ABTS can be read either unstopped after a set time or more conveniently by addition of a strongly acidic or alkaline solution which stops the enzyme reaction. In the case of TMB a colour change from blue to yellow results providing a larger change from the blank value and improved sensitivity. Optimizations of the incubation times for ABTS and TMB using G2 and G3 plates are shown (Figure 3.2.2), (Figure 3.2.3), (Figure 3.2.4). TMB was found to be the better substrate and was selected for use in later experiments.

Figure 3.2.1
Comparison of different peroxidase substrates in growth hormone assays using G2 and G3 antibody

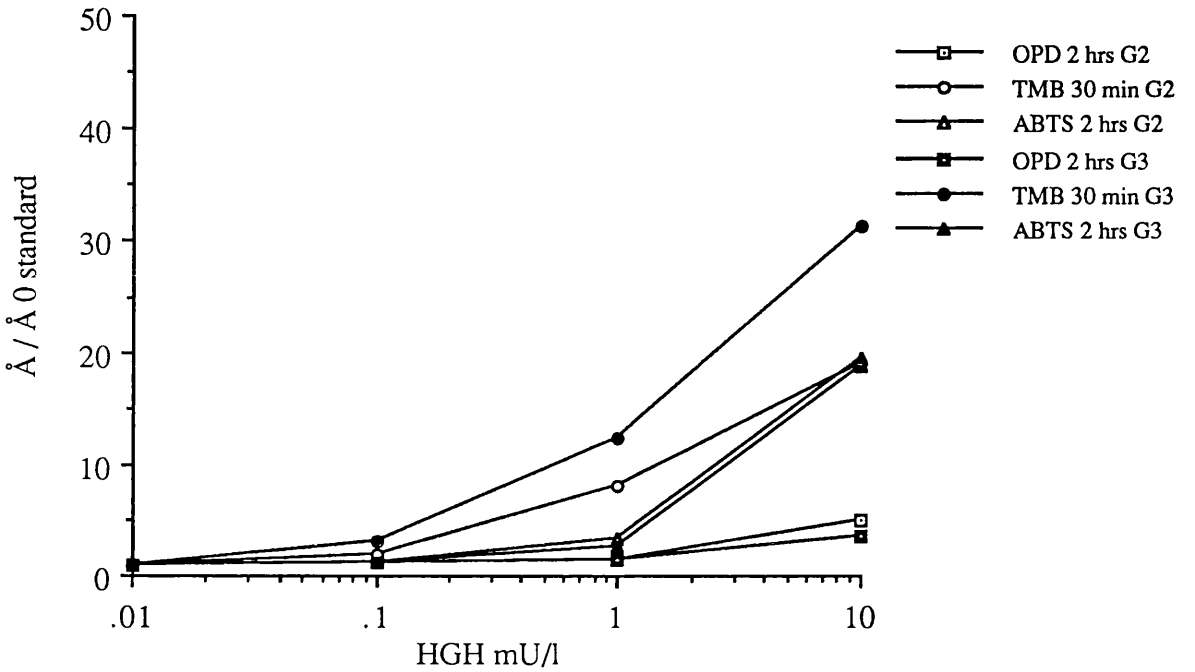


Figure 3.2.2
Comparison of incubation times for ABTS substrate in a growth hormone assay based on antibody G2 coated microtitre plates

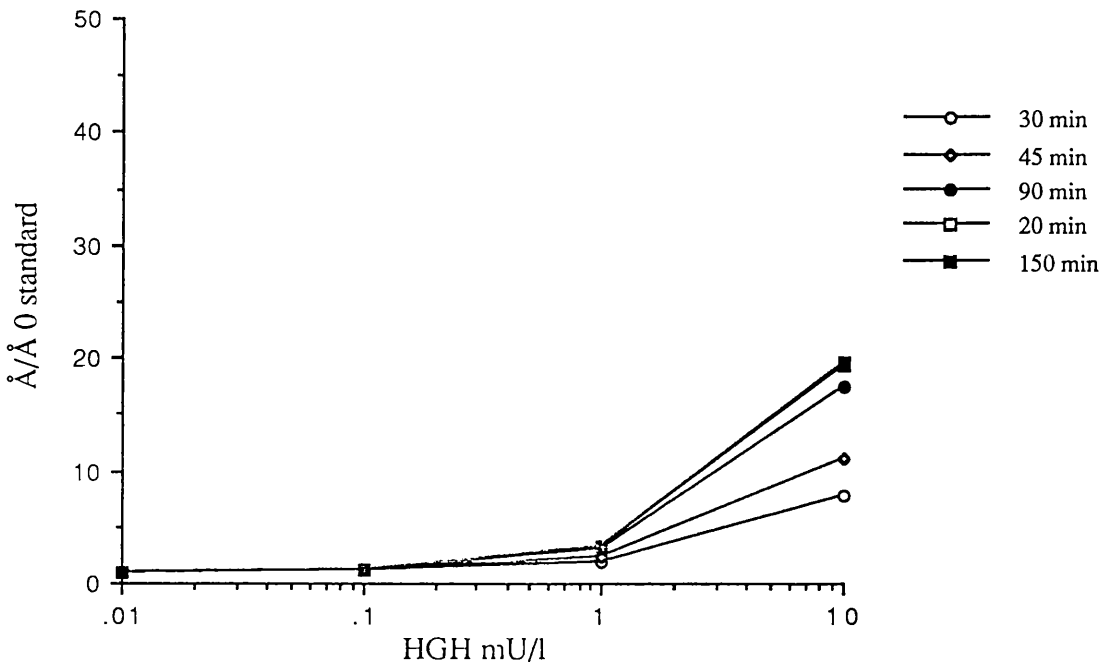


Figure 3.2.3
Comparison of incubation times for TMB substrate in a growth hormone assay based on antibody G2 coated microtitre plates

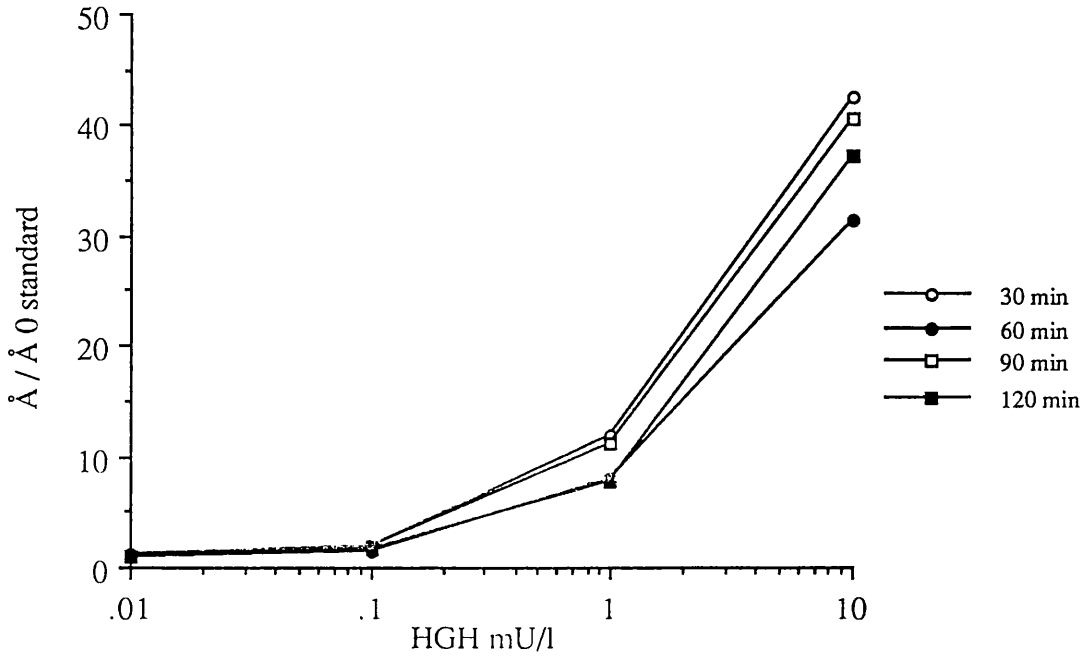
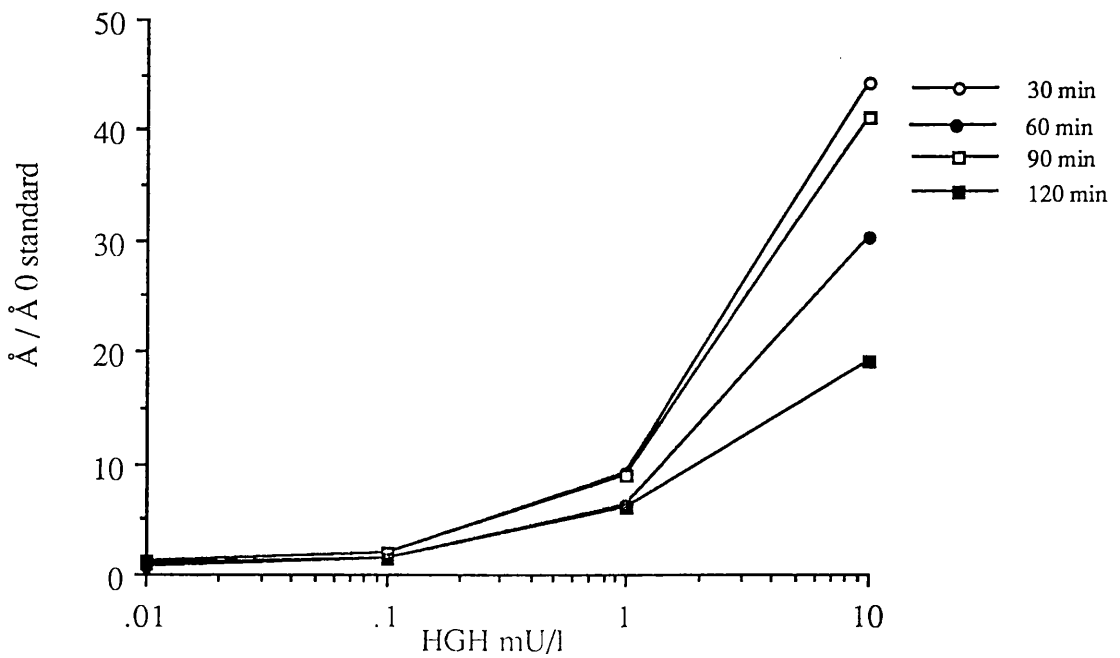


Figure 3.2.4
Comparison of incubation times for TMB substrate in a growth hormone assay based on antibody G3 coated microtitre plates



3.2.2 Coating of microtitre plates

Different coating concentrations were investigated and two different monoclonal antibodies were used for coating known as HGH G2 and G3. The optimal coating concentration for G2, judged as the signal to noise ratio (Å/Å 0 standard) was between 2.5 and 5 mg per 200 ml (Figure 3.2.5 a). The optimal coating concentration for G3 was similar to that of G2 although the plateau was not so evident (Figure 3.2.6 a). Similar standard curves were obtained using G2 (Figure 3.2.5 b) and G3 (Figure 3.2.6 b) and there was little to choose between the two antibodies. Due to the availability of antisera antibody G2 was used for the the majority of further experiments although antibody G3 was not rejected.

Different coating temperatures were also studied showing, as expected, that room temperature provided better binding than 4 °C (Figure 3.2.7). Investigation of the time of coating showed that extending the coating time improved the binding (Figure 3.2.7). In order to provide a protocol which could include coating, over-coating and an assay in one week while minimising plate storage time, the plates were coated on Friday, over-coated on Monday, the assays were set up on Tuesday and completed on Thursday.

3.2.3 Label concentration

A range of peroxidase-Fab' label dilutions was evaluated to show which gave the best signal to noise ratio (Å / Å 0 standard) across the standard curve while using as little label as possible. The optimal dilutions were 1 in 500 and 1 in 1000 of the stock label (Figure 3.2.8). In the preliminary assays 1 in 500 had been used but subsequent to this optimization 1 in 1000 was used to conserve the label as both dilutions were found to perform adequately.

Figure 3.2.5 a
Optimisation of coating of microtitre plates with antibody G2

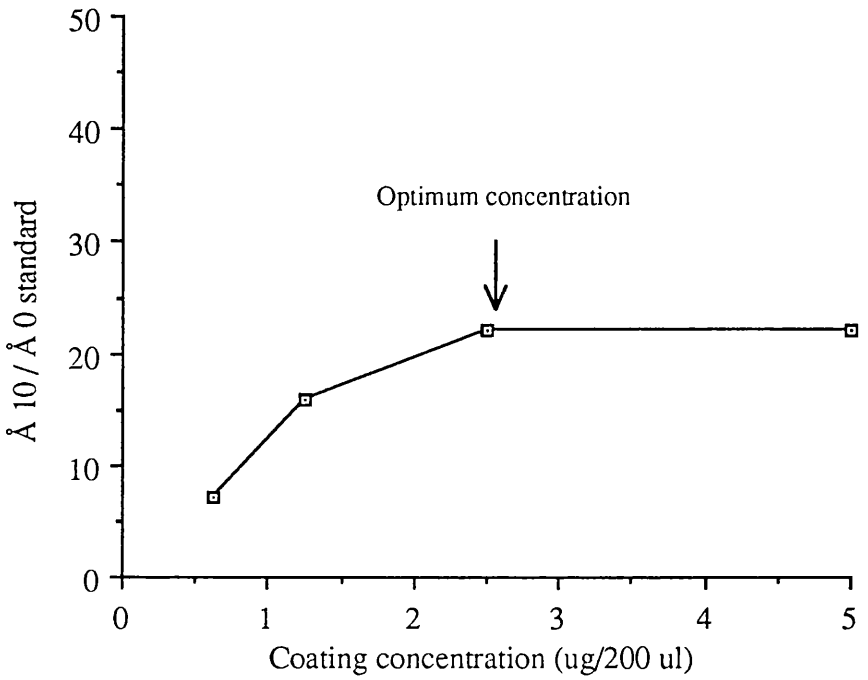


Figure 3.2.5 b
The effect of varation in coating concentration on a growth hormone assay (antibody G2)

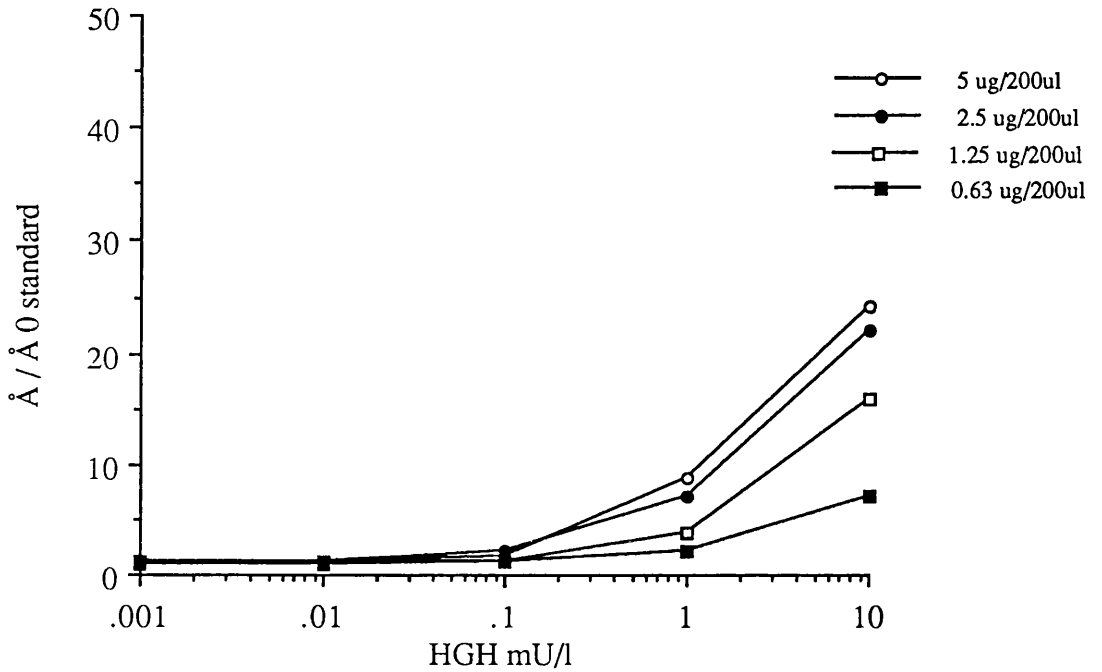


Figure 3.2.6 a
Optimisation of coating of microtitre plates with antibody G3

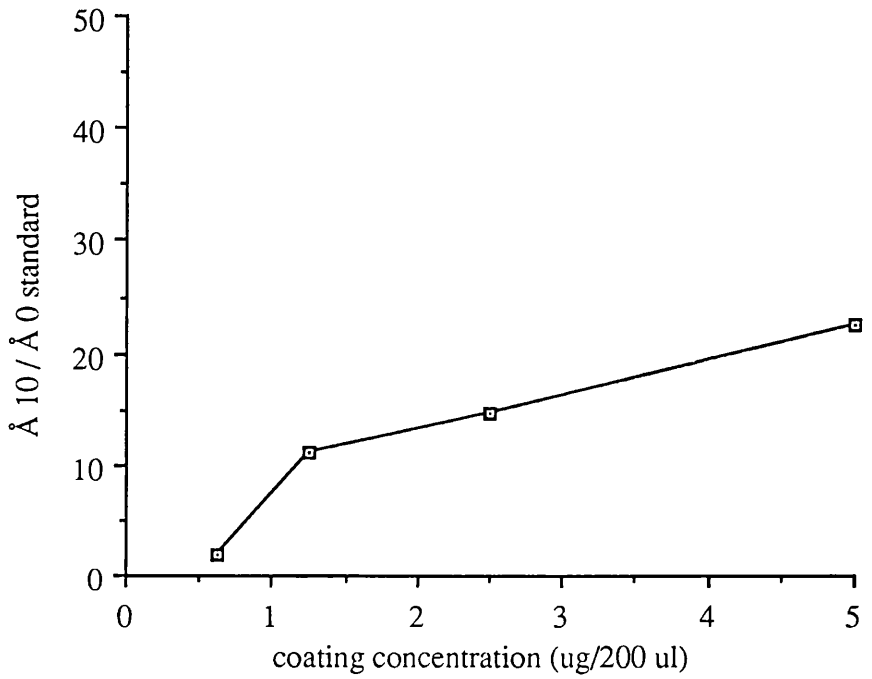


Figure 3.2.6 b
The effect of variation in coating concentration on a growth hormone assay (antibody G3)

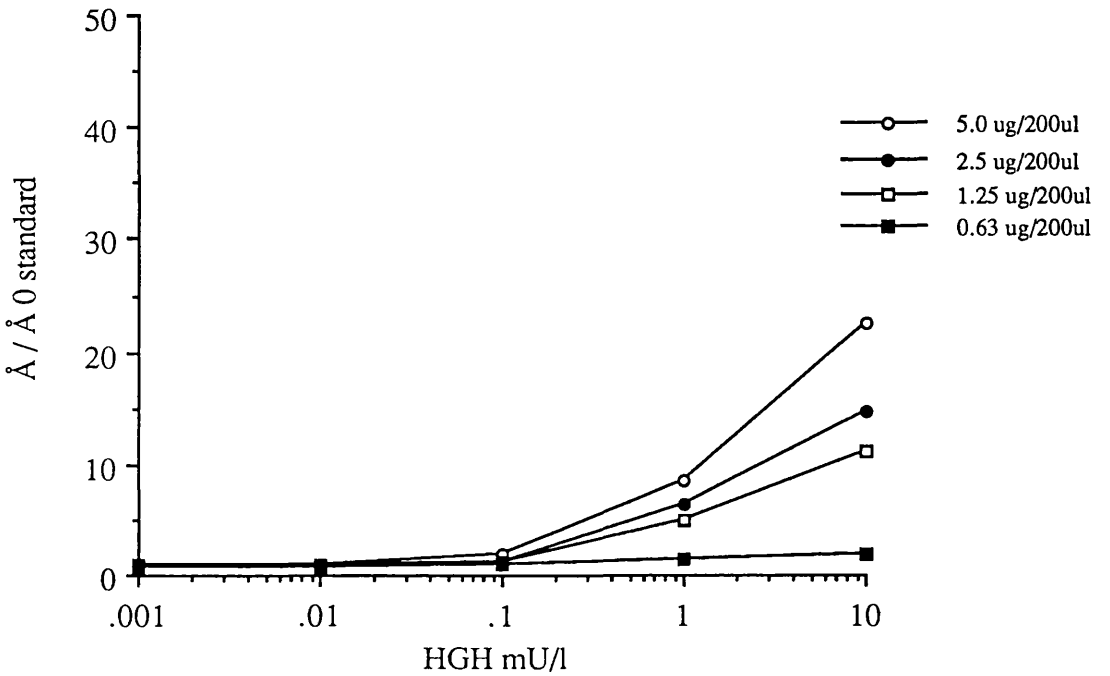


Figure 3.2.7
Optimisation of plate coating conditions
(antibody G2 coated microtitre plates overcoated with casein)

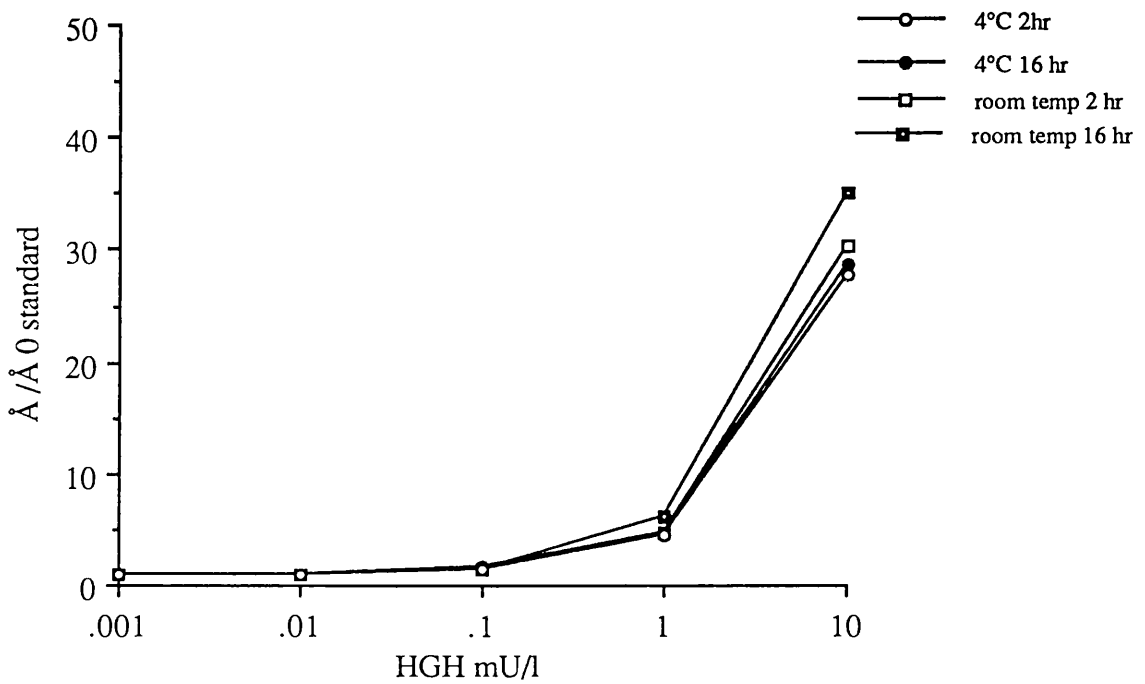
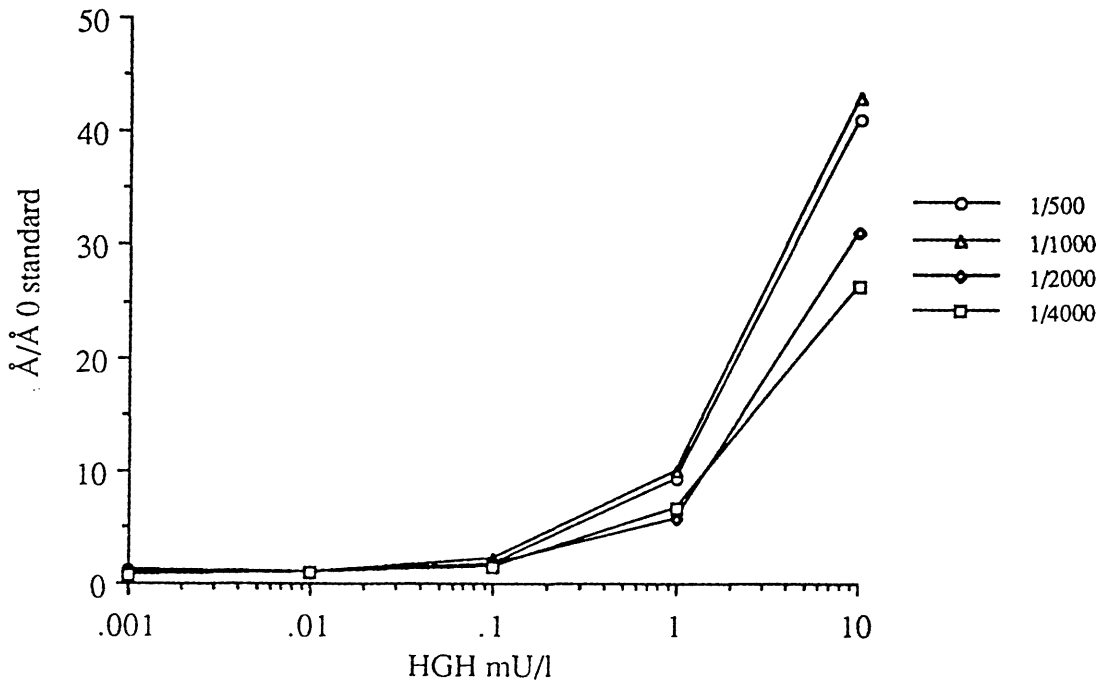


Figure 3.2.8
Optimisation of the Fab' peroxidase label dilution in a growth hormone assay (antibody G2 coated microtitre plates overcoated with casein)



3.2.4 Over-coating of microtitre plates

The concentration of BSA used for over-coating or blocking the antibody coated plates was optimised over a range from 0.05 % to 1 %, 0.5 % being commonly recommended. For this application the optimal concentration was found to be 0.05 % for G2 plates (Figure 3.2.9) and 0.5 % for G3 plates (Figure 3.2.10). Increasing the concentration of BSA appeared to increase the blank values in both G2 and G3 coated plates. The optimum over-coating concentration of BSA differed when using G2 and G3 coated plates and decreasing the BSA concentration appeared to increase the signal and the signal to noise ratio in G2 plates but it had the reverse effect in G3 plates.

Casein from dried milk powder was examined as an alternative over-coating material to BSA and this was found to be marginally better than BSA and was adopted for further use at 0.05% (Figure 3.2.11) (Livesey and Donald 1982). There may be useful information to be gained from investigating the use of alternative over-coating reagents such as $F(ab')_2$ as recommended by Hashida et. al. (1985).

3.2.5 Assay buffers

Epps buffer pH 8 was examined as an alternative to phosphate buffer pH 7. Surprisingly Epps buffer was found to perform badly in this application despite the fact that it is used routinely in the IRMA and DELFIA growth hormone assays used in this Department (Figure 3.2.12).

Figure 3.2.9
Optimisation of the BSA overcoating concentration in a growth hormone assay (antibody G2 coated microtitre plates)

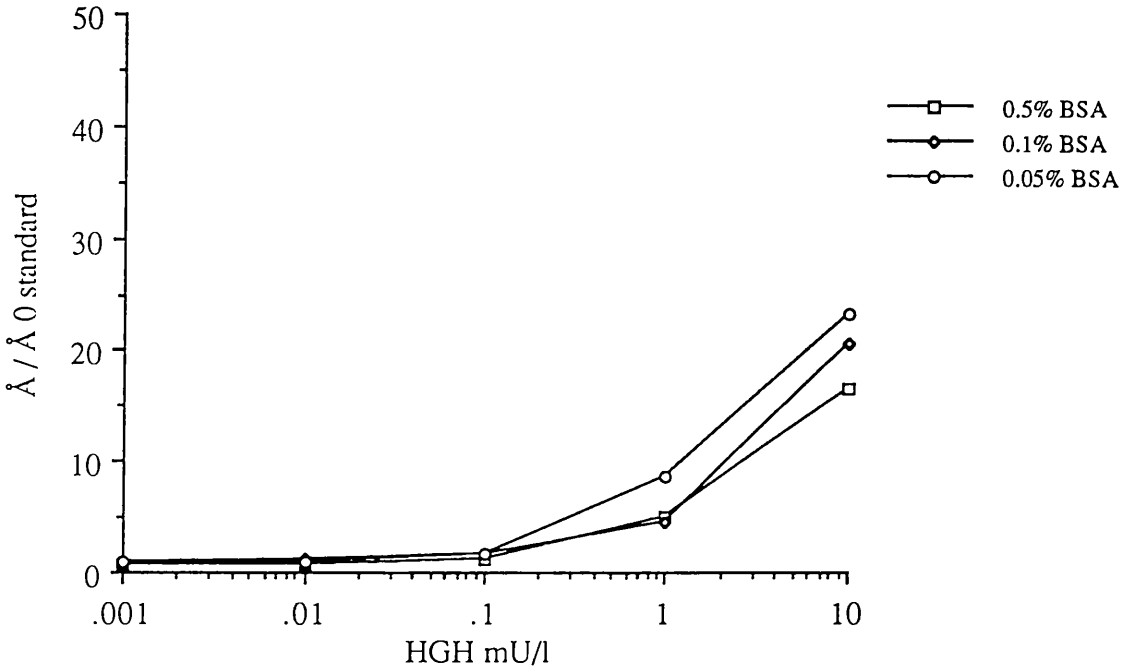


Figure 3.2.10
Optimisation of the BSA overcoating concentration in a growth hormone assay (antibody G3 coated microtitre plates)

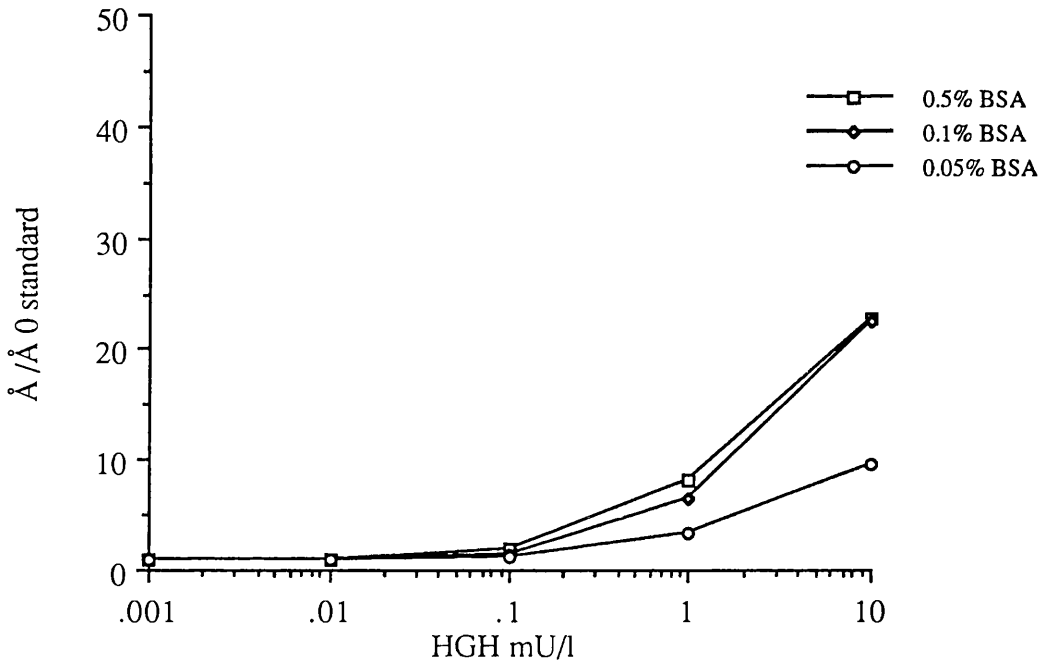


Figure 3.2.11
Comparison of BSA with casein for overcoating in a growth hormone assay (antibody G2 coated microtitre plates)

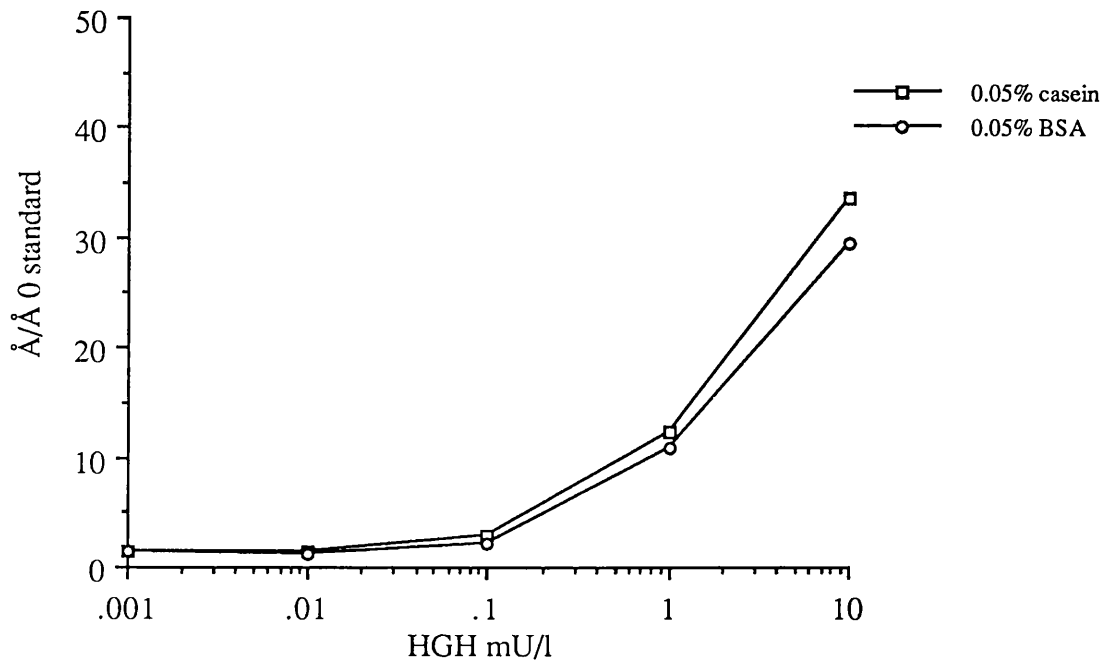
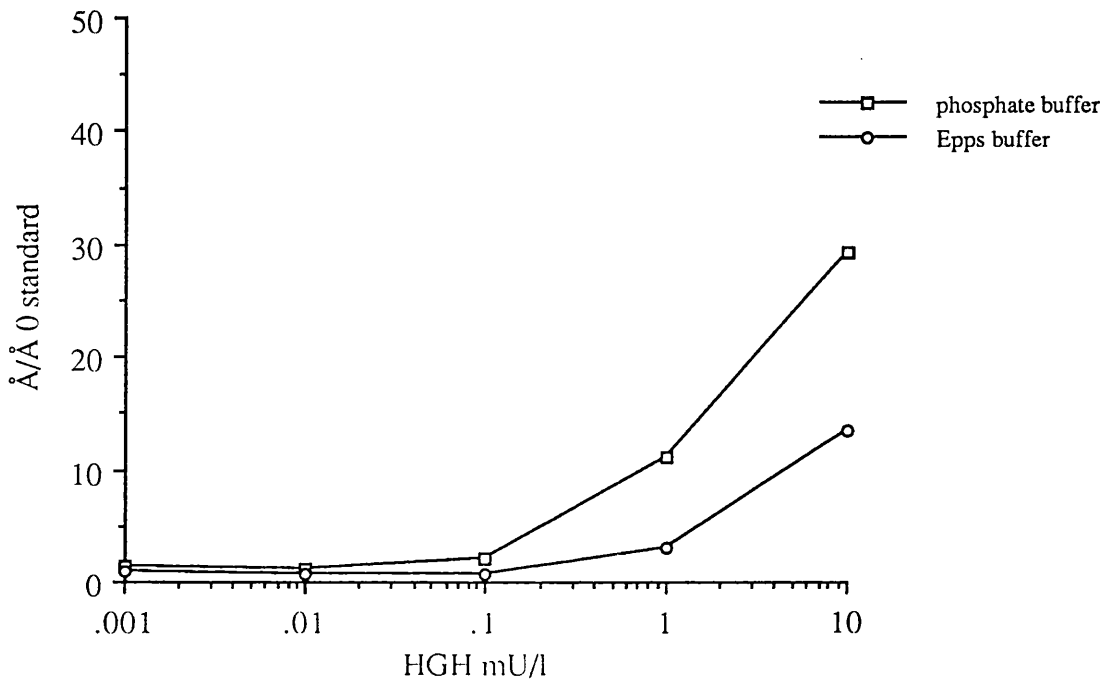


Figure 3.2.12
Comparison of Epps with phosphate buffer in a growth hormone assay (antibody G2 coated microtitre plates overcoated with casein)



3.2.6 Precision study and derivation of theoretical sensitivity

Ten sets of standards were assayed in parallel to examine the theoretical sensitivity for the TMB colourimetric assay Figure (3.2.13 a). Theoretical sensitivity for the colourimetric assay was 0.004 mU/l and was calculated as follows.

$S_t = 2\,SD_{Å0} \times C_{st} / (Å_{st} - Å_0)$, where

S_t - theoretical sensitivity

$SD_{Å0}$ - standard deviation of the fluorescence of the zero standard

$Å_{st}$ - mean absorbance of the lowest standard giving coefficient of variation of less than 10 %

$Å_0$ - mean absorbance of the zero standard

C_{ST} - concentration of the standard giving $Å_{ST}$

Due to time limitations less data was available for the luminometric assay but a mean standard curve of 4 sets of luminometric standards is shown (Figure 3.2.13 b) a sensitivity of 0.001 mU/l is not unrealistic. The fluorimetric assay (Figure 3.2.13 c) suffers from reagent blank problems and the sensitivity is around 0.1 mU/l .

3.2.7 Optimisation of the washing step

The number of washes used between the steps of the reagent additions was increased from three to six to test whether the blank value was dependant on washing. The use of Tween as an additive to the saline wash was investigated as generally Tween is considered to provide more efficient washing but for this application this was not found to be the case (Figure 3.2.14). Six washes were used between reagent additions as it was considered that this should be more than adequate. Tween was not used between coating and over-coating since it was considered that this may reduce the binding of the

Figure 3.2.13 a
Theoretical sensitivity of growth hormone assay (TMB substrate)

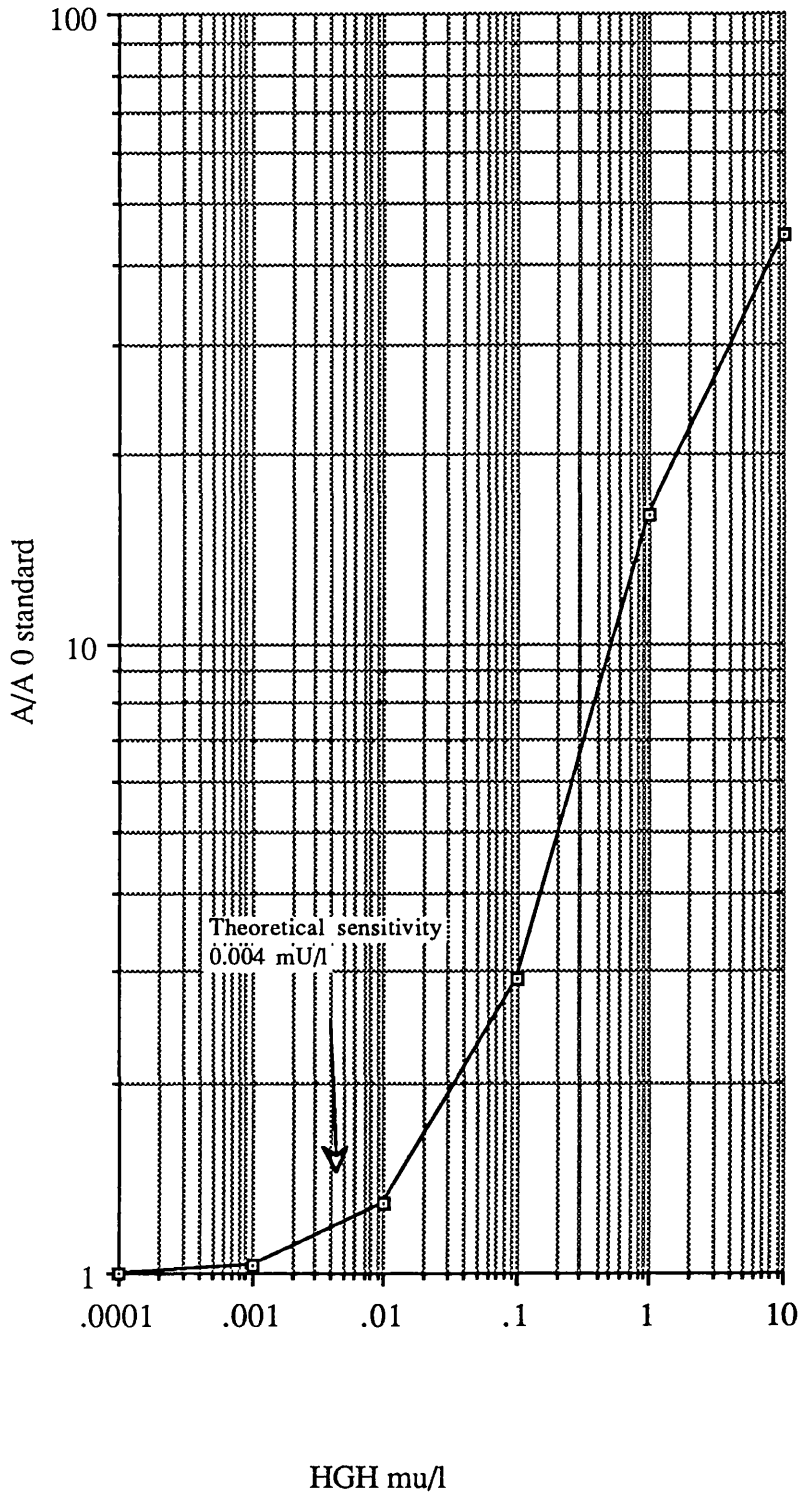


Figure 3.2.13 b
Theoretical sensitivity of growth hormone assay (Luminescence substrate)

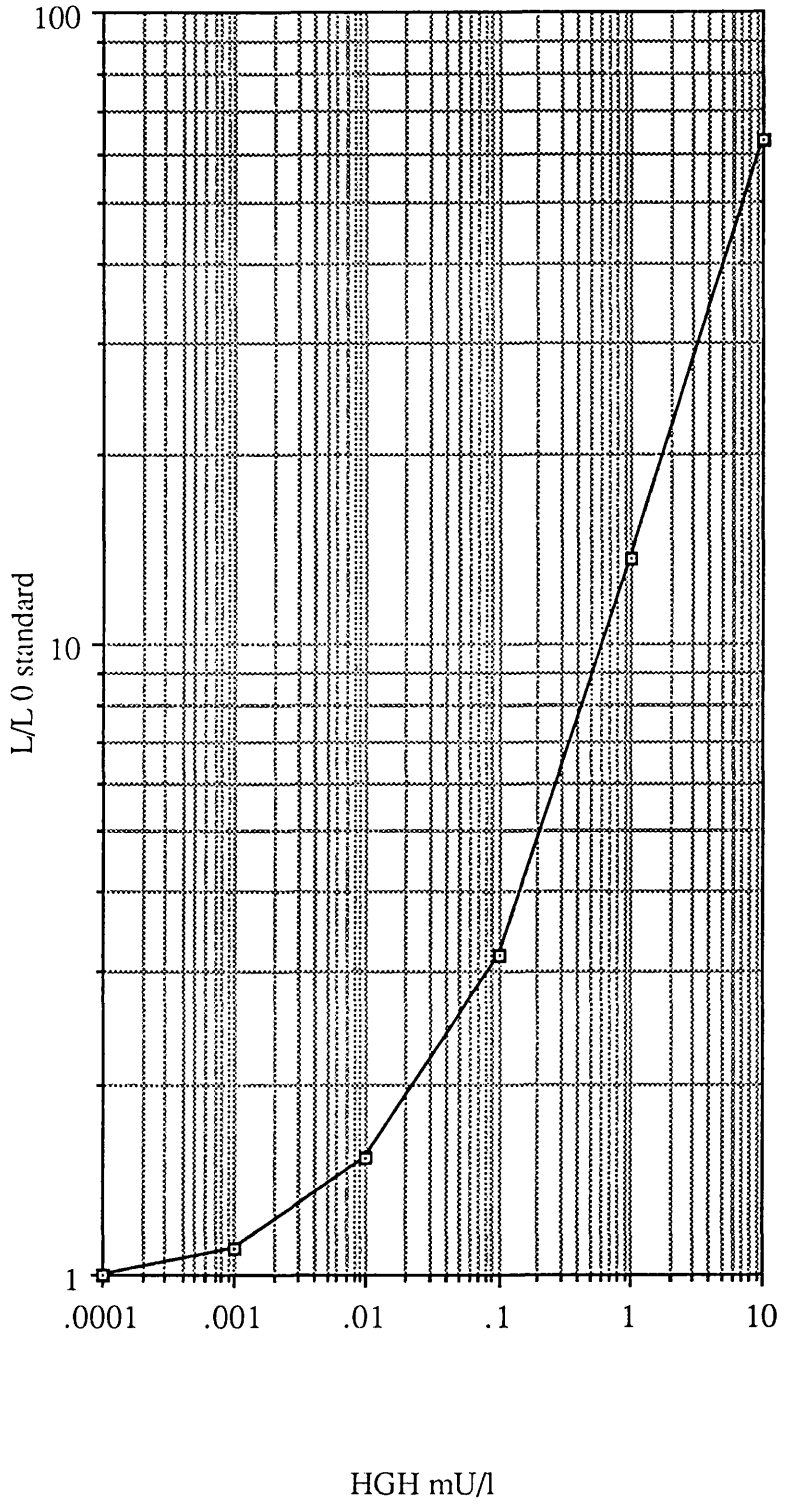


Figure 3.2.13 c
Theoretical sensitivity of growth hormone assay (HPPA fluorescence substrate)

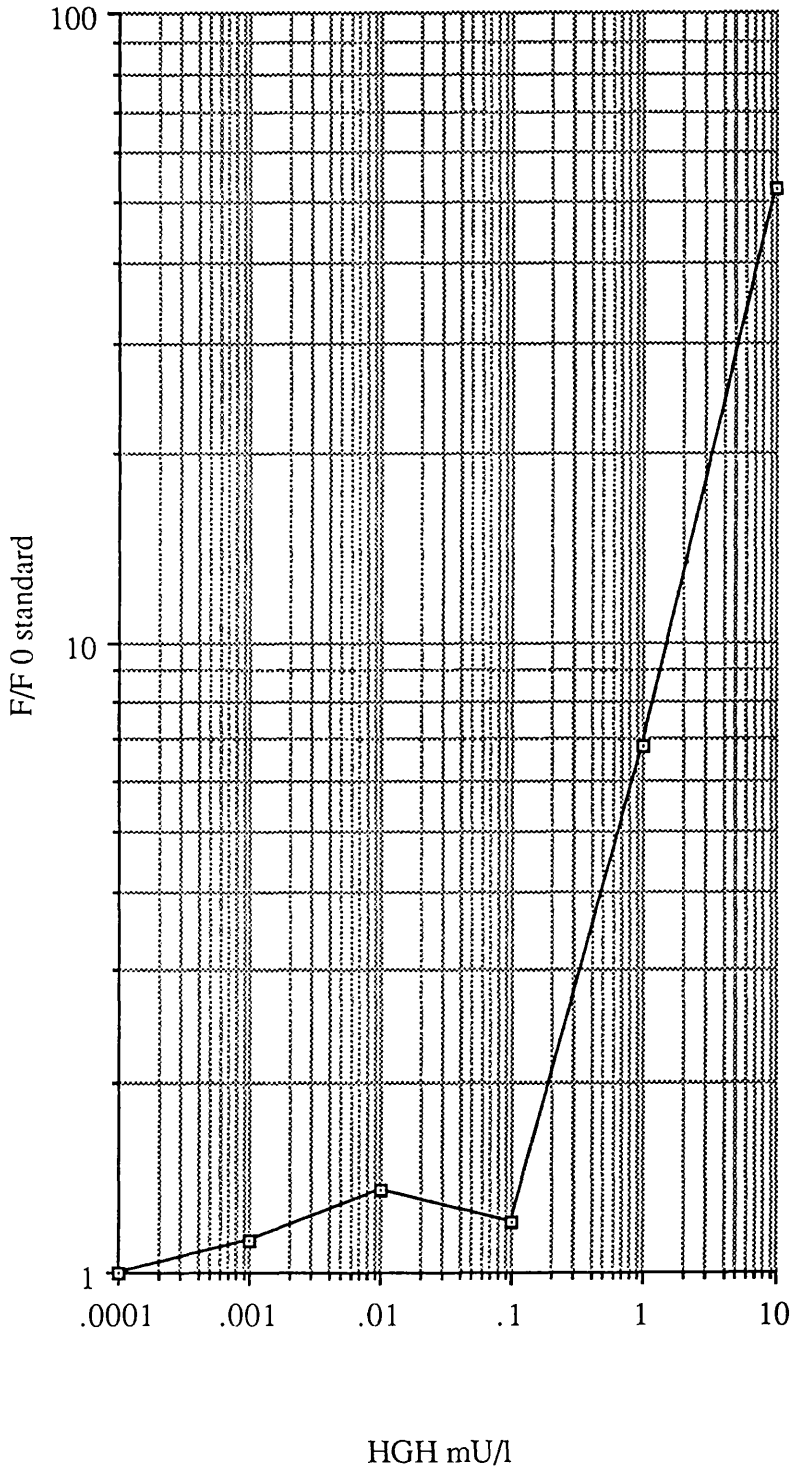
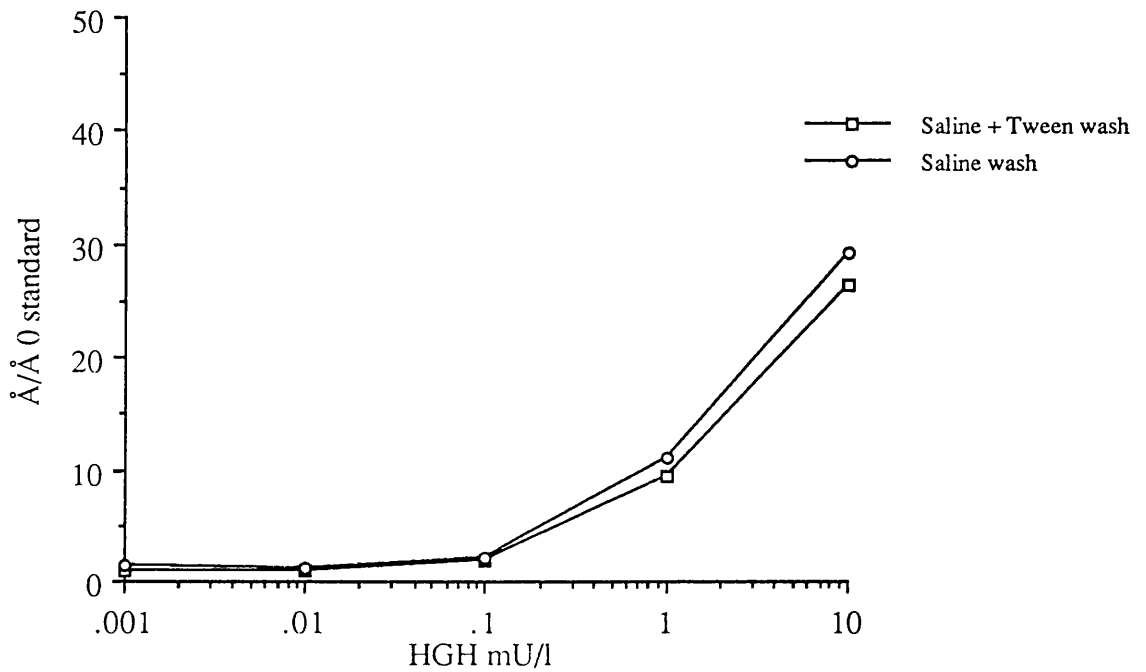


Figure 3.2.14
Comparison of saline and saline and Tween as wash solutions in a growth hormone assay (antibody G2 coated microtitre plates overcoated with casein)



BSA or casein to the plate. Azide was not used as a preservative in any of the buffers or wash solutions as it inhibits peroxidase activity.

Considerable care was required to ensure that the plates were completely dry between washing and reagent additions as the plate washers did not leave the plates dry. The plates were 'bashed' dry on paper towels and allowed to stand, inverted, for a few minutes after washing.

3.2.8 Sensitivity of TMB to light

The substrate reaction for TMB was carried out in daylight and with light excluded by covering the plate with a paper towel to investigate whether the blank values increased due to any light sensitive reaction of the substrate (Figure 3.2.15). The substrate reaction was carried out in the dark thereafter since it was felt that there was a slight increase in blank readings when the plates were incubated in daylight. The TMB substrate was made up immediately before addition as it changes colour from clear to blue when exposed to light for short periods of time.

3.2.9 Different manufactures of microtitre plate

A variety of different manufacturers microtitre plates were used to examine for any differences in binding and reproducibility of coating (Figure 3.2.16). NUNC plates were used for the majority of the experiments of the project and none of the other types tried were found to be significantly better. It was felt that reproducibility of coating of the plates was a major problem leading to imprecision in the assay and unexplained outliers. It was also felt that increasing the binding of the growth hormone antibody to the plates may improve the signal obtained but this appeared not to be the case. Covalent linking of the antibody to the plates was not attempted and this may improve the binding.

Figure 3.2.15
The effect of daylight on TMB substrate in a growth hormone assay (antibody G2 coated microtitre plates overcoated with casein)

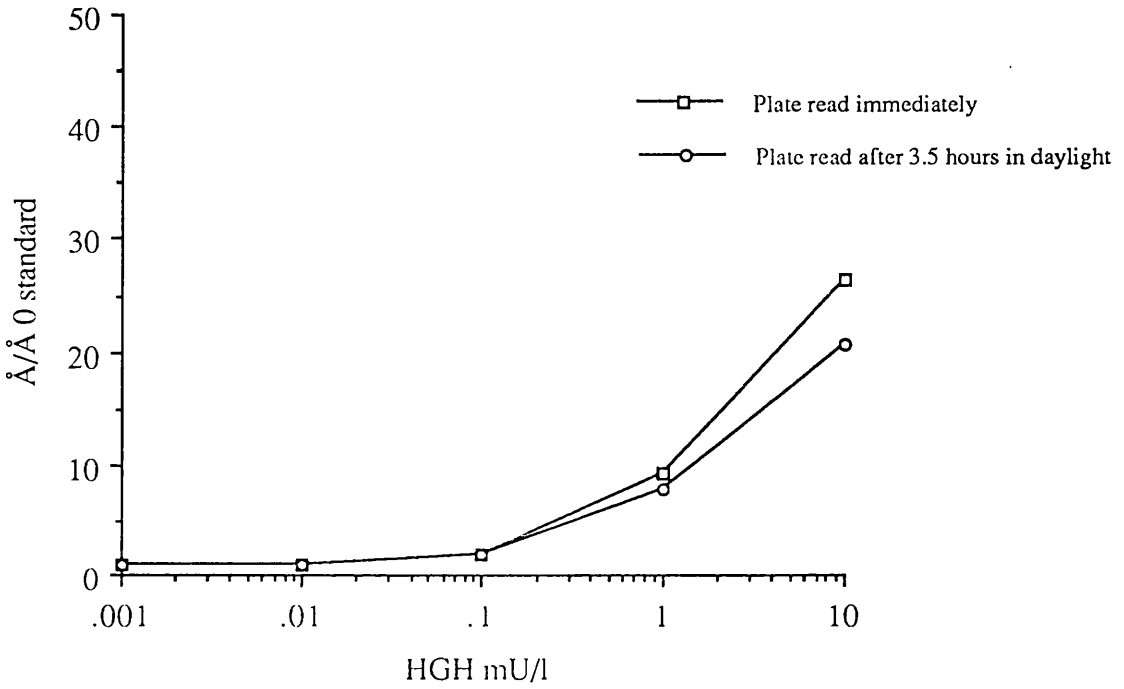
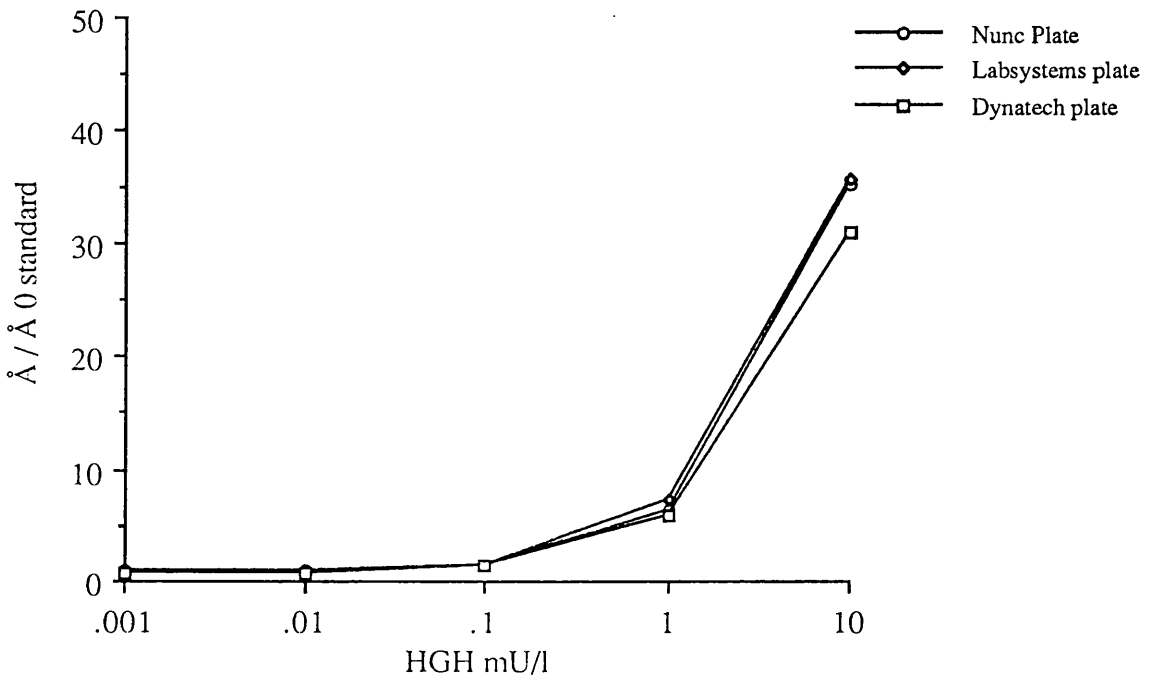


Figure 3.2.16
Comparison of different manufacturers micotitre plates in a growth hormone assay (antibody G2 plates overcoated with casein)



A high binding plate from Labsystems was assessed to determine if there was any enhancement of binding or improvement of coating precision but this was not found to be the case (Figure 3.2.17).

3.2.10 Edge effects

In a number of assays the blank value for the zero standard gave higher readings than the lowest standard and this was thought to be possibly due to edge effects on the plates. In order to investigate this phenomenon, a complete plate of blanks was set up and mean absorbances were calculated for each row and each column to identify if there were differences across the plate and up and down the plate. There appeared on occasion to be a slight effect across the plate but this was not reproducible (Figure 3.2.18).

3.2.11 Fluorescence assay using HPPA substrate

HPPA was used as a fluorescence substrate to investigate if there was a potential gain in sensitivity or in measuring range over TMB.

Peroxidase label was diluted over a range of concentrations to assess the range of the fluorescence plate reader (Figure 3.2.19).

Dialysed and undialysed urine from a normal adult was spiked at a number of levels with growth hormone and assayed to investigate the presence of a urine matrix effect (Section 3.3.3). Two different types of plates were used to ensure that there were no significant variations in coating between different types of plate (Section 3.3.3).

From these experiments it was concluded that HPPA had a much wider working range than TMB, as shown by Tuuminen et. al. (1991), but that the poor background

Figure 3.2.17
Labsystems enhanced binding plate
(antibody G2 microtitre plate overcoated with casein)

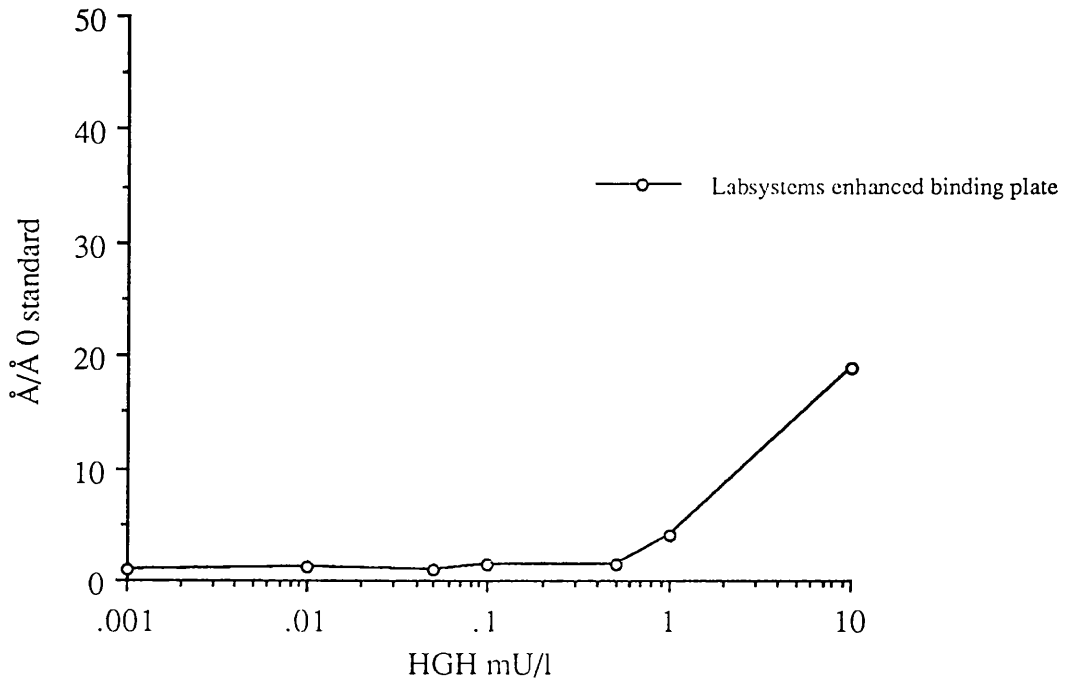


Figure 3.2.18
Investigation of the prescence of edge effects in a microtitre plate Nunc (antibody G2 coated microtitre plate overcoated with casein)

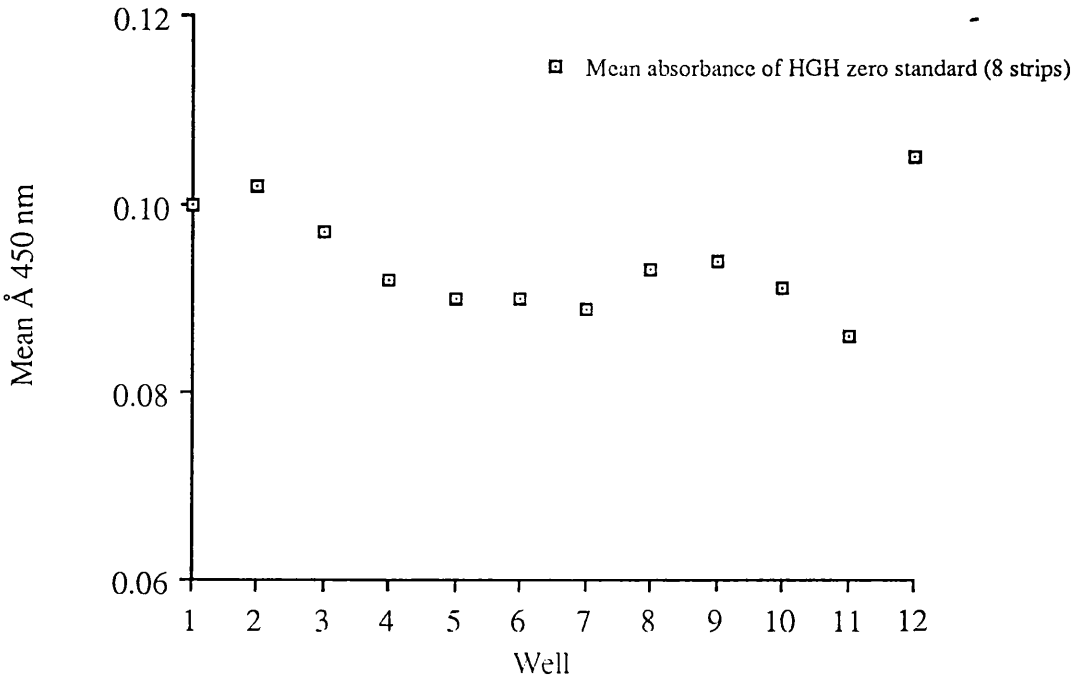
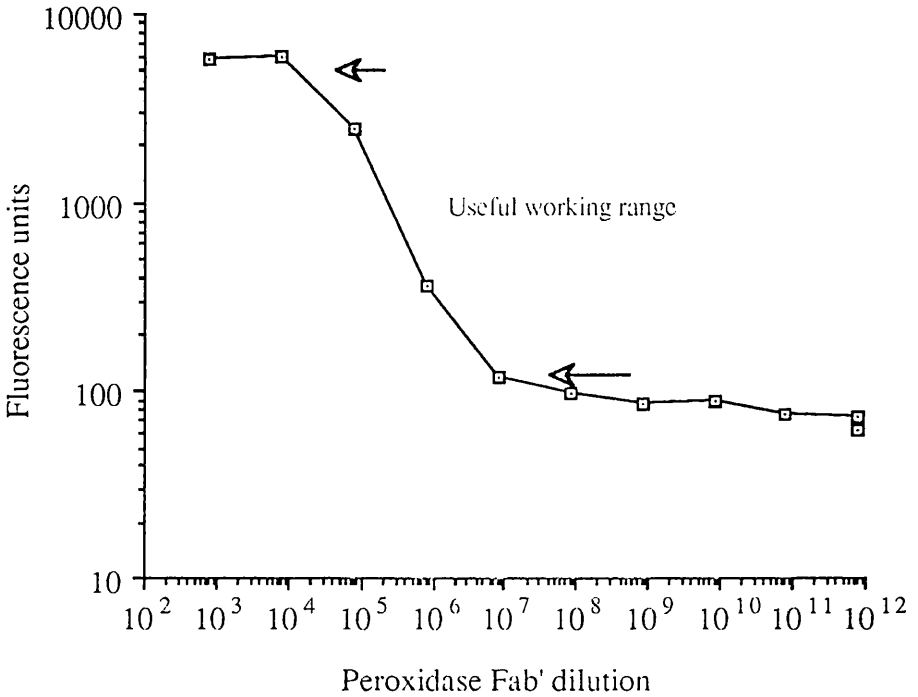


Figure 3.2.19
Fluorescence of diluted Fab'-peroxidase label



readings obtained from the substrate limit the potential sensitivity obtainable. This background problem is due to impurities in the commercially available substrate, which are not easily removed. It may be that plate readers in general whether they are colourimetric or fluorimetric are not as sensitive as the best fluorescence spectrometers, and this may be the price to pay for automation against using labour intensive single cell detectors.

3.2.12 Enhanced luminescence assay

The assay was adapted to use an alternative enhanced luminescence peroxidase substrate which consists of a luminol derivative, a peracid salt and a substituted phenol enhancer. The enhancer increases the amount of light produced and prolongs its emission. (Whitehead et. al. 1983) The standard curve obtained is shown (Figure 3.2.20) and from this initial trial run this substrate appears to provide a better signal to noise ratio and a lower detection limit than that of HPPA or TMB and within the range necessary for a viable urine growth hormone assay. Unfortunately the equipment was not available for an extensive evaluation of the assay.

3.3 APPLICATION OF THE ASSAY TO URINE SPECIMENS

3.3.1 Linearity of the growth hormone assay in the presence of urine

A urine sample from a normal adult was serially diluted in assay buffer (neat x2 x4 x8). These diluted urine samples were spiked with growth hormone standard and then subsequently diluted in assay buffer to provide a range of standards (0.001-10 mU/l). The sets of standards were assayed using antibody G2 coated microtitre plates overcoated with casein and using TMB substrate. The standard curves produced were compared with the equivalent buffer standard curve in order to assess the application of the assay in a urine matrix (Figure 3.3.1). Although both the neat urine and the diluted

Figure 3.2.19
Growth hormone standard curve using a luminescence substrate (antibody G2 coated microtitre plate overcoated with casein)

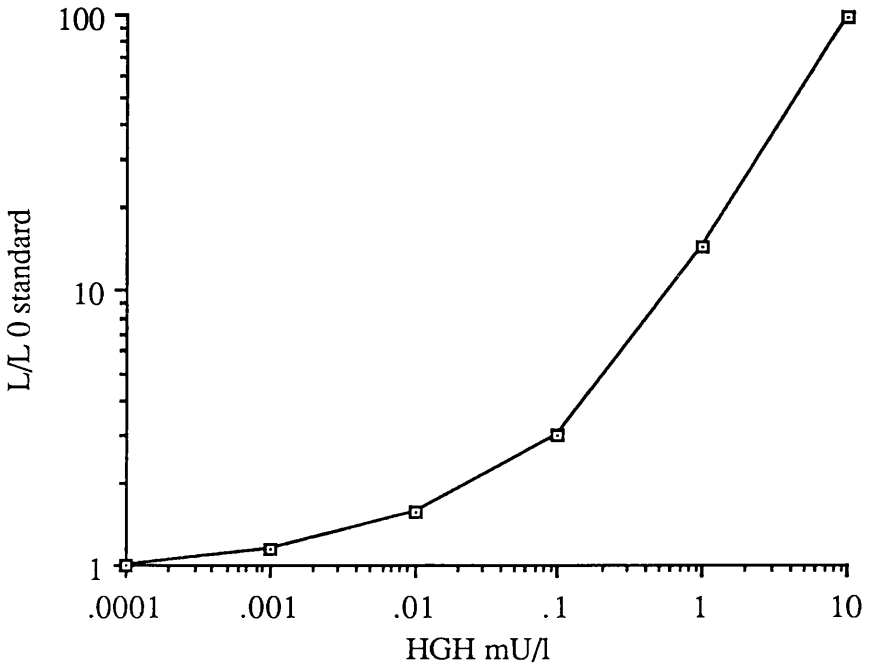
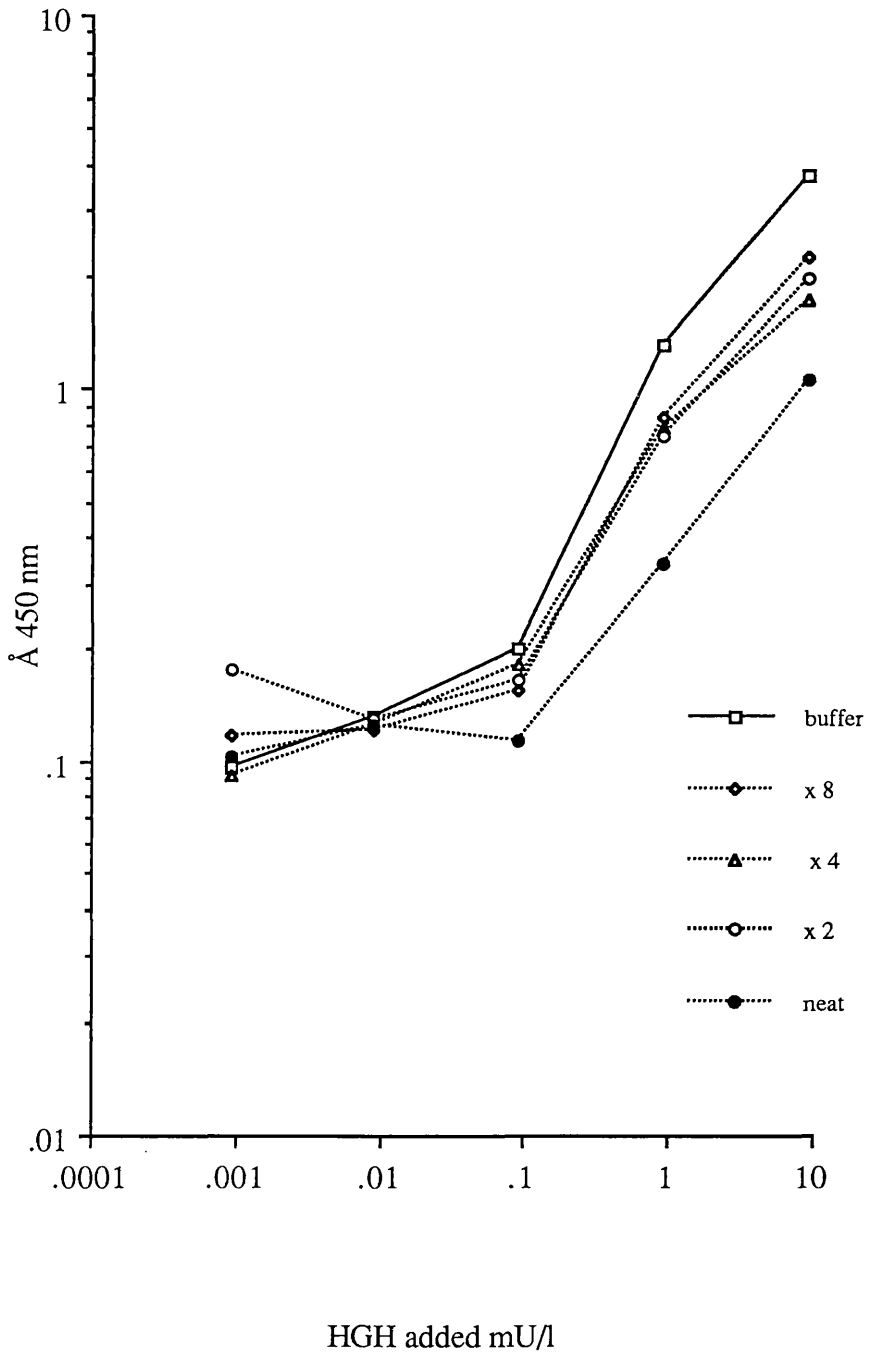


Figure 3.3.1
Diluted urine spiked with growth hormone



urine standards show linearity with the buffer standards, there is a considerable matrix problem which requires further investigation.

3.3.2 Recovery of human growth hormone in urine

A urine sample was serially diluted in assay buffer and growth hormone standard added. The diluted urine was then assayed. The absorbance produced in the assay for each sample relative to a buffer standard is shown (Figure 3.3.2). Clearly there is an interfering substance present in urine which dilutes out in buffer.

3.3.3 The effects of the urine matrix on the assay

An early morning urine sample was divided into two aliquots and one was dialysed overnight into plate assay buffer. Standard curves were prepared by diluting growth hormone UK 9 standard in plate assay buffer, urine, and dialysed urine. The standards were assayed to determine if the matrix effects present in urine could be removed by dialysing the urine into buffer.

Duplicate standard curves were prepared in urine, buffer and dialysed urine and assayed using either TMB (Figure 3.3.3) or HPPA substrate (Figure 3.3.4) These are shown with both linear and with log Y axes (Figure 3.3.5, Figure 3.3.6a). Fluorescence assay standard curves were prepared using Labsystems G2 (Figure 3.3.6a), NUNC G2 (Figure 3.3.6b), and NUNC G3 coated plates (Figure 3.3.6c).

The urine standard curves showed a marked effect on the binding of the standards whereas the dialysed urine showed binding which was intermediate between the urine and the buffer standards which indicated that although dialysis did reduce the matrix effect it did not entirely remove it. The matrix effects may also vary from sample to

Figure 3.3.2
Diluted urine from a normal adult spiked with growth hormone standard

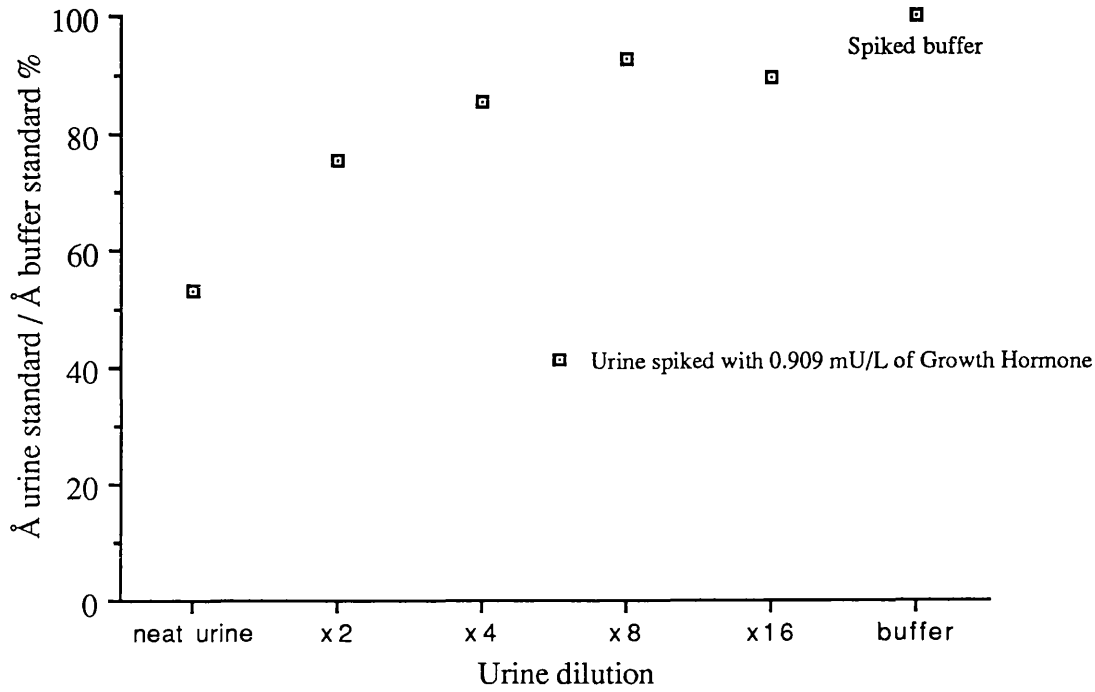


Figure 3.3.3
Growth hormone assay standard curves in buffer, urine and dialysed urine (TMB substrate)

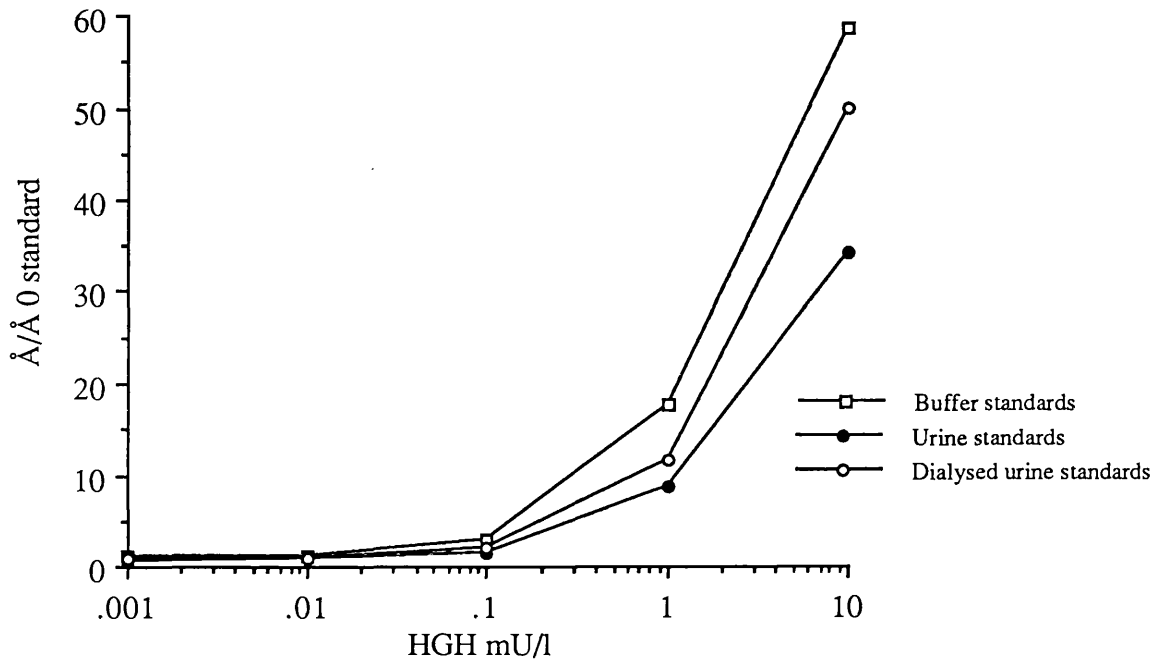


Figure 3.3.4
Growth hormone assay standard curves in buffer, urine and dialysed urine (HPPA fluorescence substrate)

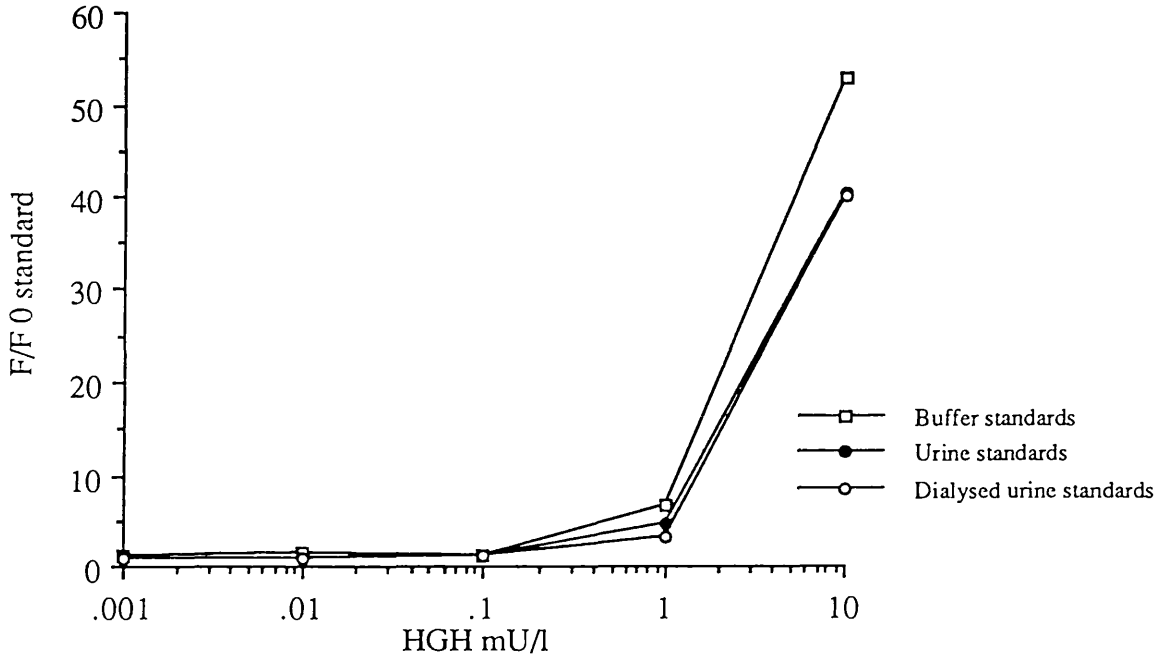


Figure 3.3.5
Growth hormone assay standard curves in buffer, urine and dialysed urine (TMB substrate)

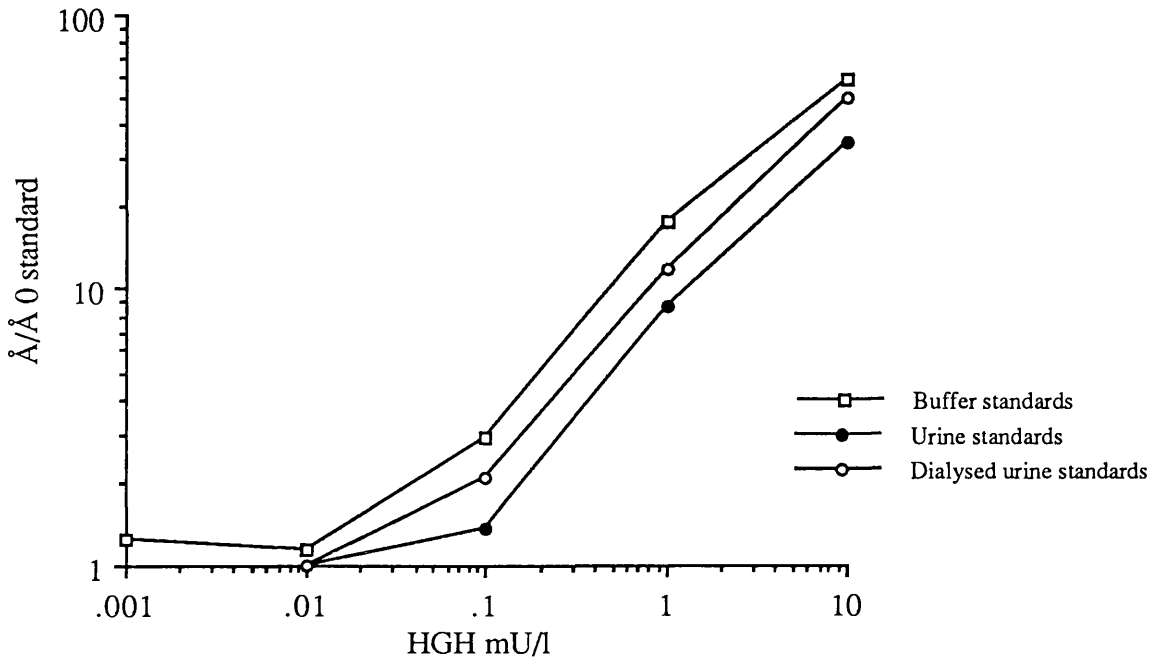


Figure 3.3.6 a
Fluorescence growth hormone assay standards in buffer, urine and dialysed urine (Labsystems G2 plate)

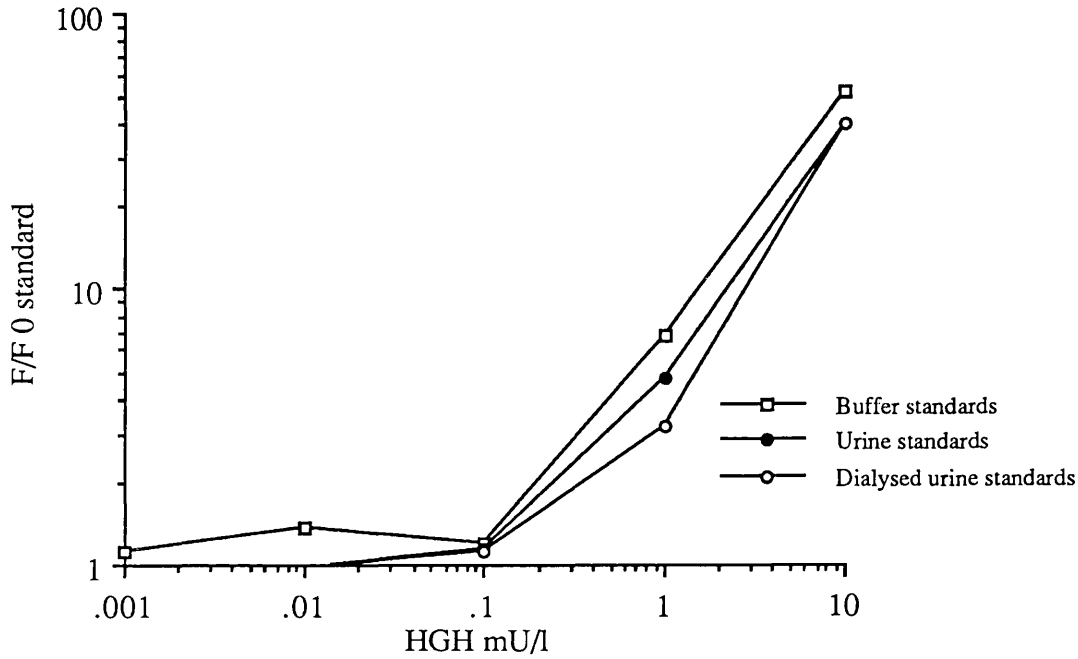


Figure 3.3.6 b
Fluorescence growth hormone assay standards in buffer, urine and dialysed urine (NUNC G2 plate)

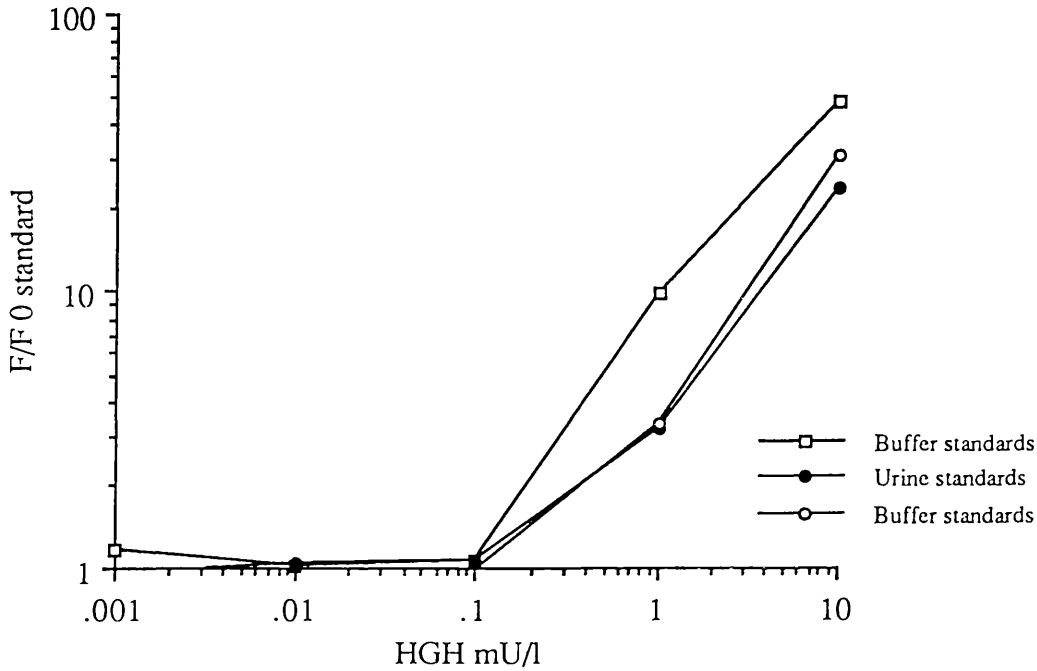
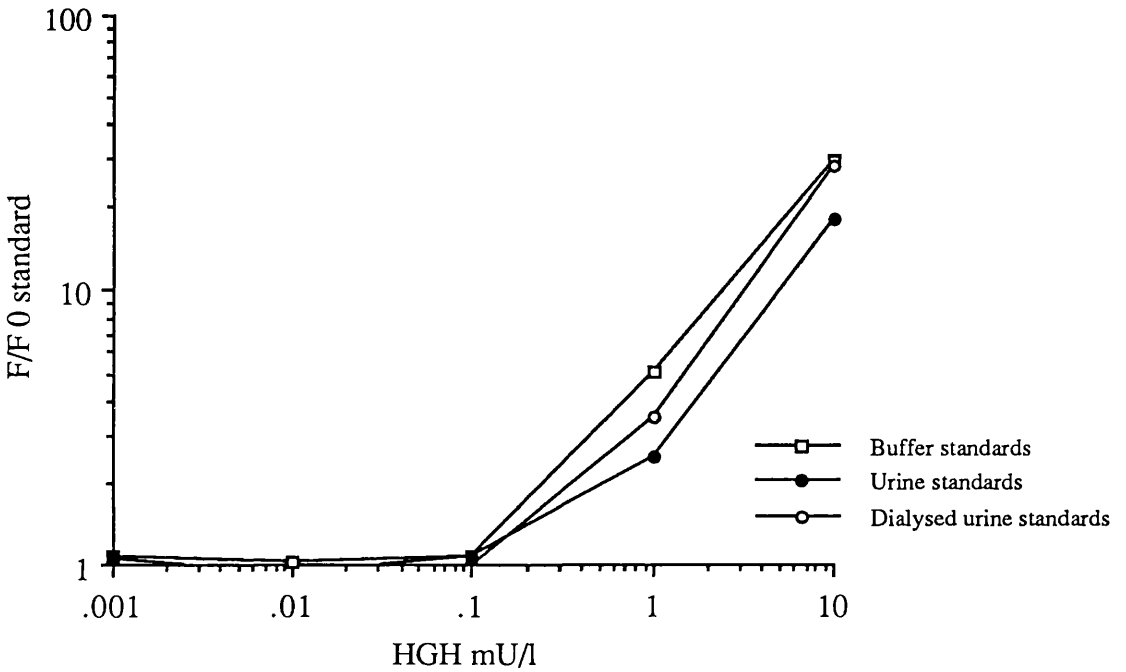


Figure 3.3.6 c
Fluorescence growth hormone assay standards in buffer, urine and dialysed urine (NUNC G3 plate)



sample depending on whether they are 24 hour samples or early morning samples and depending on the concentrating ability of the particular patient's kidneys.

3.3.4 Dilution of urine from an acromegalic subject

A urine sample was obtained from a known untreated acromegalic patient. This sample was split into two aliquots and one was dialysed overnight. A series of dilutions of the dialysed and undialysed urines were produced and the urines were then assayed using HPPA substrate. A buffer standard curve was also prepared for comparison. NUNC G2 (Figure 3.3.7 a), NUNC G3 (Figure 3.3.7 b), and Labsystems G2 coated plates (Figure 3.3.7 c) were used and all showed similar parallelism of the diluted urine with the urine standards but in each case the undialysed urine dilutions showed much lower binding.

3.3.5 Investigation of the possible presence of a high molecular weight interferent

It was thought that the differences in the standard curves obtained when comparing standards diluted in urine with standards diluted in buffer and dialysed urine may be due to a high molecular weight interferent. In order to attempt to investigate this problem the assay was modified and the label and diluted standards were incubated in a separate uncoated plate and then transferred to the coated plates. Hence the labelled antibody was allowed to bind to the growth hormone before the coated antibody, which is the reverse of the conventional assay. The standard curves obtained from this modified assay still showed differences between the buffer, urine and dialysed urine standards as in the conventional assay (Figure 3.3.8). The binding for the transferred assay was much lower than for the conventional assay. This method also lacks a washing step between the reagent additions. For these reasons it was decided not to proceed further with this assay format.

Figure 3.3.7 a
Diluted urine from a patient with untreated Acromegaly (NUNC G2 plate)

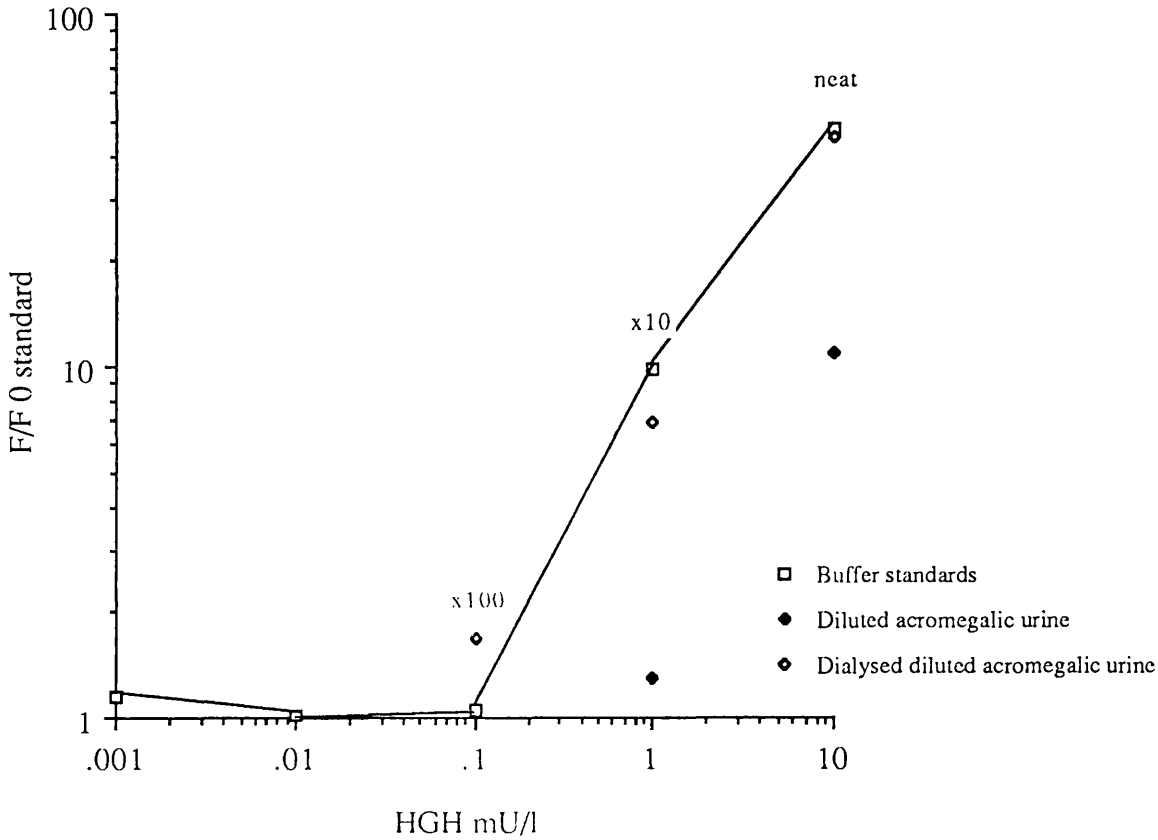


Figure 3.3.7 b
Diluted urine from a patient with untreated Acromegaly (NUNC G3 plate)

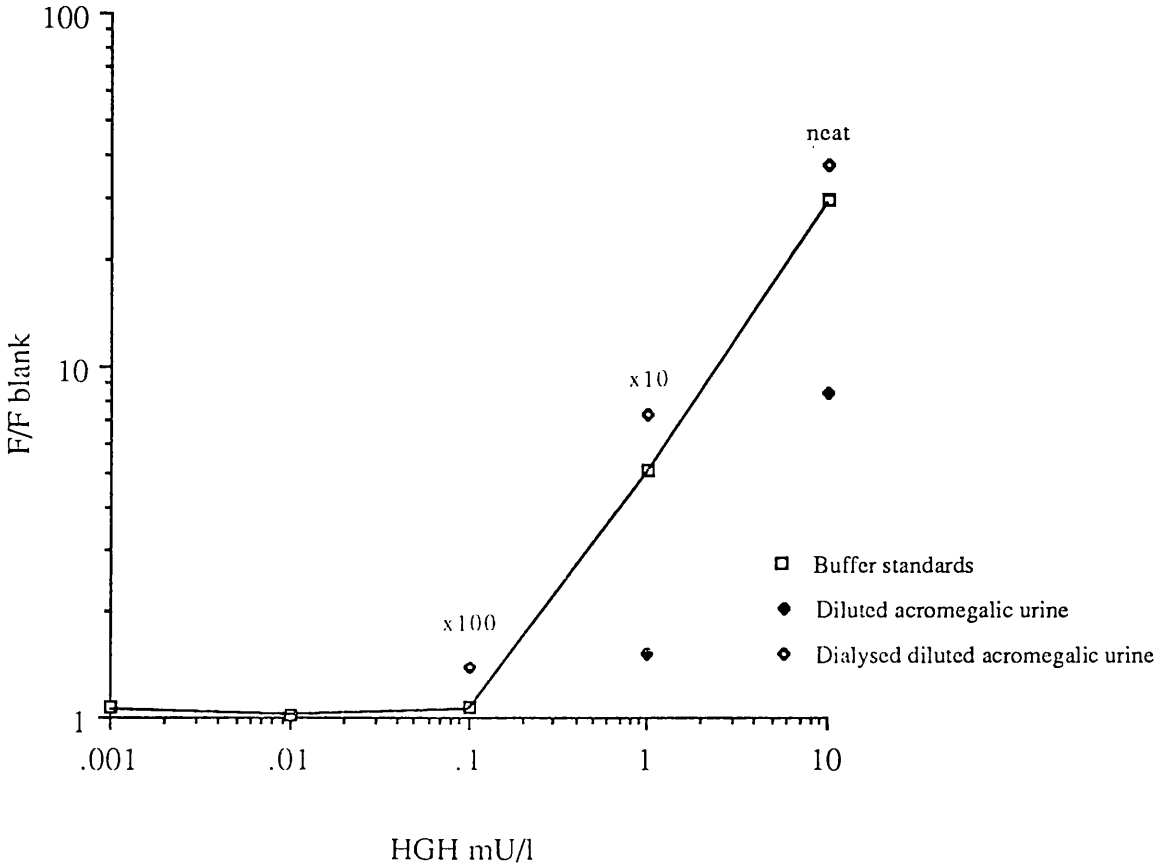


Figure 3.3.7 c
Diluted urine from a patient with untreated Acromegaly (Labsystems G2 plate)

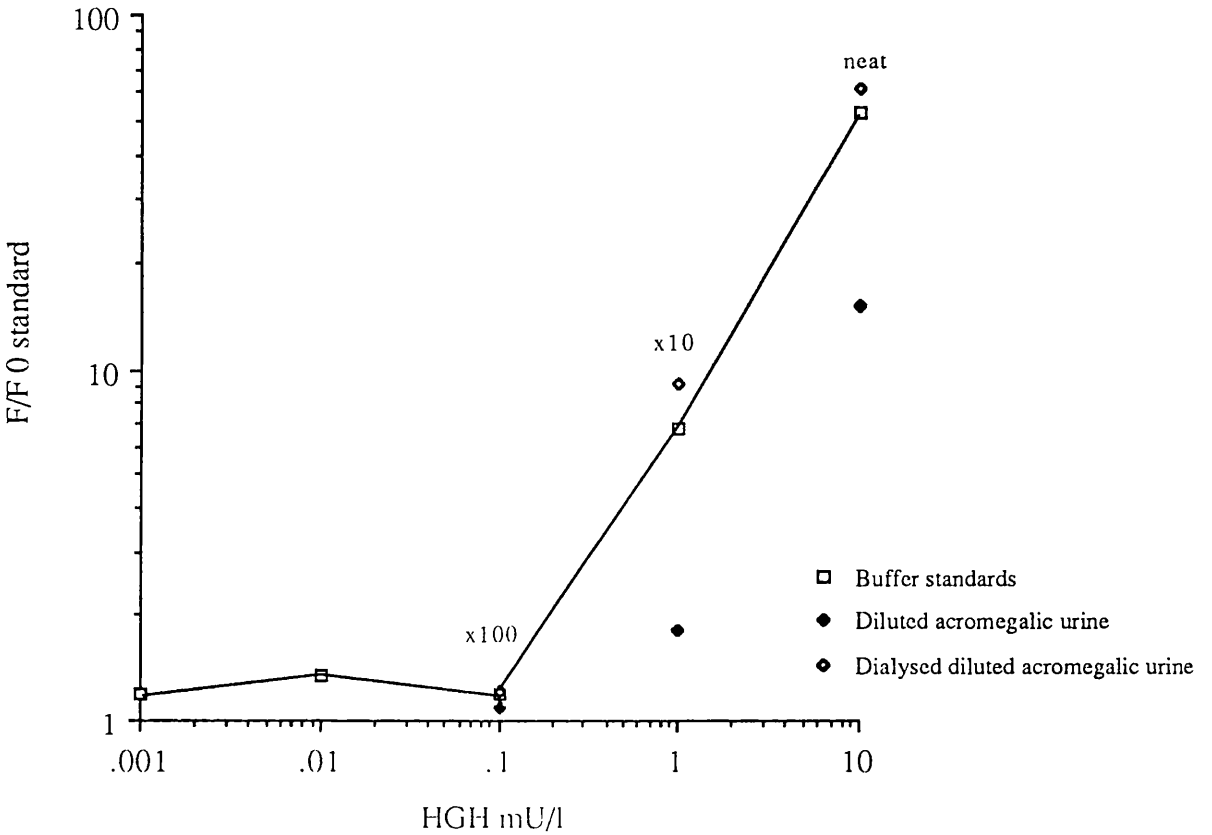
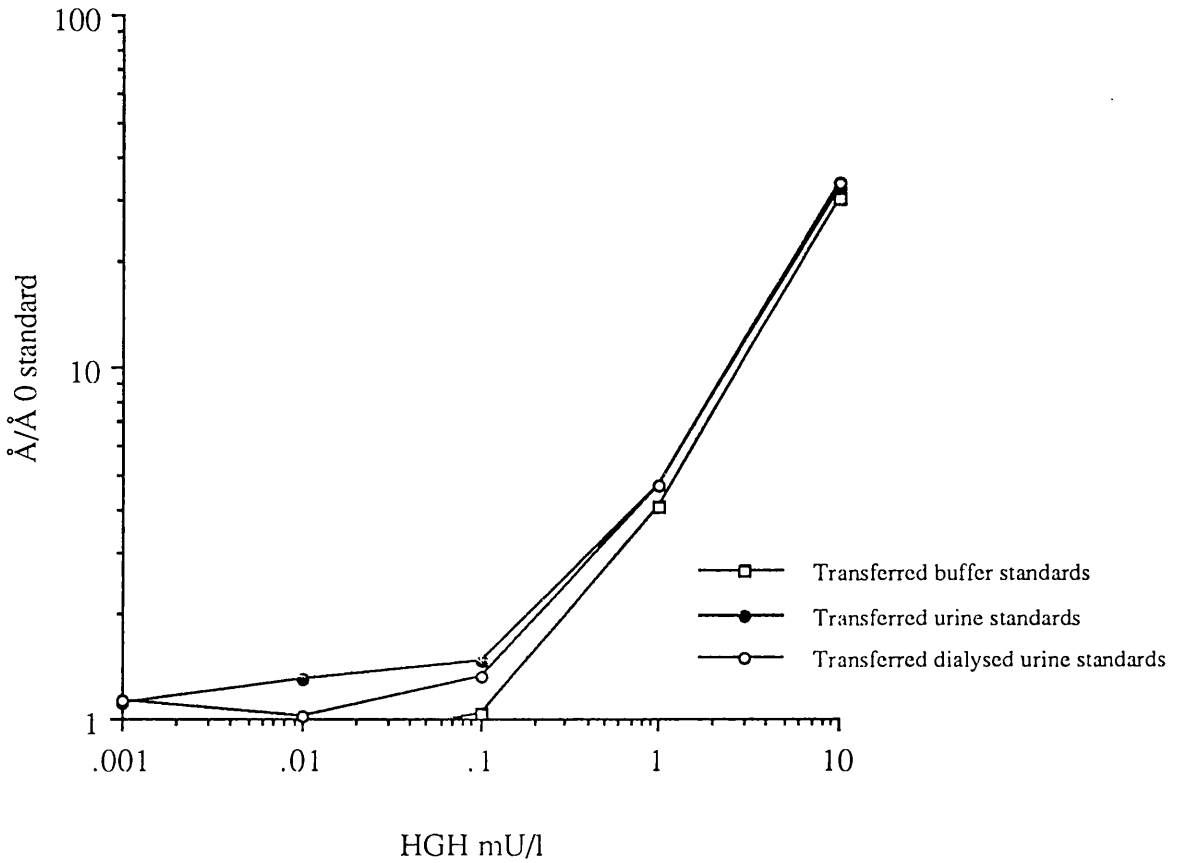


Figure 3.3.8
Growth hormone "transfer" assays in buffer urine and dialysed urine



CHAPTER 4 : DISCUSSION AND CONCLUSIONS

CHAPTER 4 DISCUSSION AND CONCLUSIONS

4.1 Conjugate production

The original aim of this project was to develop a fluorescence enzyme immunoassay for urinary growth hormone similar to the method reported by Hashida et.al. (1987). A large portion of the time was spent attempting to produce an Fab' fragment of sheep polyclonal antiserum using the method reported by Ishikawa et. al. (1988). This was not successful as the $F(ab')_2$ produced could not be further reduced to Fab', perhaps because the method was not fully optimised or for other reasons. For example it was thought that the $F(ab')_2$ may have been resistant to reduction by mercaptoethylamine and therefore mercaptoethanol and thiomersal were examined as alternatives, without success. There was also the possibility that the pepsin digestion left the $F(ab')_2$ in a form which could not be further reduced or that the particular isotype of IgG was simply pepsin resistant. Alternatively there was the possibility that it may not in fact be $F(ab')_2$ which had been produced, but this was unlikely since some initial work to verify the binding of the fragment to growth hormone after pepsin digestion, using radiolabeling methods, showed binding of the $F(ab')_2$ to growth hormone.

Following a successful attempt to produce Fab' by a kit method marketed by Bioprobe International Incorporated, the Ishikawa method was abandoned and a conjugate of Fab' and peroxidase was produced using the maleimide method. The recovery of peroxidase-maleimide conjugate was not entirely satisfactory and some work requires to be done if this method is to be used in the future. Since an adequate supply of peroxidase label was produced to complete this project, and time was limited, optimization was considered unnecessary.

4.2 ELISA development

The original method published by Hashida et. al. (1987) utilised 3 millimetre polystyrene beads as the separation system to which the monoclonal antibody was coated by simple adsorption. Some initial experiments showed no particular advantage of this method over plastic microtitration plates, although in theory the large surface area of the beads may allow coating of more protein than the wells of the plates. The major disadvantage of the beads was that there was no available automated colourimeter or fluorimeter which could adequately detect the colour or fluorescence produced by the enzyme reaction and so it was decided to concentrate efforts on the development of a plate assay but to retain the use of beads as a possible alternative. It is also possible to attach the monoclonal antibody to microparticles for use in a fluorescence or a luminescence system such as the Abbott IMX system but these were outwith the scope of this project. Alternatively an Fab' fragment can be attached covalently to plastic surfaces using maleimide linking reagents which ensures that the fragment is correctly orientated on the surface to allow antigen binding.

Once the peroxidase conjugate was produced the initial assays performed using OPD substrate provided such a small colour change that the project was almost abandoned. However some alternative substrates, namely ABTS and TMB, provided a far greater colour change. This allowed initial optimization of an ELISA method to be carried out and some work investigating problems such as the high blank values, light effects and edge effects was performed. It was later discovered that these were not uncommon problems with plate assays and require considerable time and effort for optimization. Many of the assays performed appeared to have higher binding in the wells at the ends of the strips so that for example the zero standard in the A1 position was often higher than the first standard in the A2 position. The experiments which were carried out in order to confirm this, initially showed a slight edge effect when themean absorbances were calculated for each of the strip positions across eight different strips. This

experiment did not provide the same effect when it was repeated a second time. Edge effects can be due to a combination of three factors. Firstly, there can be uneven distribution of light to the different well positions. This effect was minimised by incubating the plates in the dark during the enzyme reaction following the observation that the surplus TMB substrate solution developed a slight blue colour during the incubation period. As an additional precaution the substrate was made up fresh immediately before use. Secondly, edge effects can be due to variations in the drying of the protein on the plate between steps, and in particular, between washing and addition of substrate, excessive drying leads to loss of enzyme activity. This effect was minimised by addition of substrate as soon as was possible after washing the plates. There is evidence that washing is the most critical step in achieving assay precision and that it is essential that no remnants of the wash solution remain as even tiny amounts of wash solution can lead to significant suppression of colour development. Attempts were made to ensure the complete removal of the wash solution by 'bashing' the plates onto paper towels, but this was less than satisfactory. An alternative method was to use a manual aspirator to remove the remaining liquid. A related problem is that of vacuum control of the aspirator on automated washers, where too low a vacuum pressure causes incomplete drying of the wells while too high a vacuum leads over drying and to loss of enzyme activity. There may also be considerable variability in the aspiration from one nozzle to the next. Thirdly, edge effects can be caused by temperature gradients across the plate which lead to differences in binding and in colour development. Many commercial systems now use specially designed incubators which maintain the temperature across the plates to within very tight limits of around 0.2 °C, with negligible variation from well to well. Unfortunately this equipment was not available at the time.

Another recurring problem was that of outliers which were wells with unexpectedly large colour changes. This may be due to problems with the plate coating or washing or due to contamination during the assay. Great care was required to prevent

contamination from one well to another. One source of possible contamination which was discovered was the dispensing head of the plate washer, which was not adjusted for height correctly, and tracked across the meniscus of the liquid in the wells when returning from the last strip to the first. The plate shaker speed had to be set fast enough to allow proper mixing but prevent splashing. Finding a suitable dispensing device for the ELISA reagent addition was difficult since it had to be precice, accurate, fast enough to prevent excessive variation in incubation times but should not eject the liquid so fast that it squirted the liquid back out of the wells. Several models were used and all were found to have limitations. Outliers can also be caused by variation in the amounts of wash solution added to each well, caused by salt deposits on the dispenser nozzles or due to air bubbles which become trapped in the wash tubing and when dislodged result in addition of insufficient wash solution to some wells. These problems can only be prevented by scrupulous maintenance of the plate washers and careful priming of the wash solution. The wash solution was always primed through several times before use to prevent contamination from the wash solutions of other users which may contain azide, which is reported to reduce peroxidase activity (Ishikawa et. al 1983).

Variation in the coating of wells is a perpetual problem in plate assays and careful maintenance of coating temperatures and drying are essential. It is desirable that the coated protein should form a monolayer on the surface of the well since multilayers are unstable and can interfere with the assay by causing protein-protein interactions. Hence it is necessary to carefully optimise the protein and overcoating concentrations for the assay. Some commercial kit manufacturers dry the plates by centrifugation to ensure consistent drying. In addition commercial companies coat plates in large batches and carry out quality control tests of the coating efficiency on sample plates from each batch. In the method used here the plates were washed in saline between coating steps and stored with moist filter paper in sealed bags. Storage of coated plates would have to be further investigated if the assay were to be used routinely.

Initially lyophilised bovine albumin was used as an over-coating or blocking reagent to reduce non-specific binding to the wells. Following advice that casein or non-fat dried milk solution could provide lower non-specific binding than BSA, some comparisons were carried out (Livesey and Donald 1982). From these comparisons there appeared to be a small improvement in the non-specific binding on using the dried milk blocking reagent and this was adopted in the later assays.

The TMB supplied by sigma did not entirely dissolve in buffer, therefore it was centrifuged briefly before use to remove the remaining undissolved material.

4.3 FEIA development

It was always the intention to develop an assay which used a fluorescence substrate since the reports of Ishikawa claimed that this allowed sufficient sensitivity to measure urinary growth hormone. Unfortunately no equipment was available locally which could adequtely measure the fluorescence of HPPA. It was not until limited use of a fluorescence platereader was arranged by Labsystems UK and Labsystems Finland that the FEIA assay could be investigated. This work showed that the use of HPPA provided a wider working range than TMB but there was no gain in sensitivity due to high background readings from the substrate, probably due to impurities. These conclusions agree with the findings of Tuuminen et. al. (1991). Purification of the substrate is apparently not easily achieved, and the substrate which was recrystallised in house provided no improvement in background signal. (Imagawa et. al. 1983), (Hashida et. al. 1983) In addition there appears to be considerable batch to batch variation in the purity of the substrate. Sigma now supplies HPPA in tablet form which may be of higher purity than the crystalline product available originally and which is more convenient to use. HPPA is available from an alternative Japanese supplier but the cost prohibits its use. There are fluorescence substrates available for other enzymes which in theory may provide better sensitivity than HPPA and luminescence substrates are also available which may also allow lower sensitivity (Zaitsu 1980).

4.4 Enhanced Luminescence Assay

Following the completion of the practical part of this project a luminescence platereader and an enhanced luminescence substrate became available for limited use. Standard curves were set up as previously and these showed a far wider measuring range and lower detection limit (0.001 mU/l) than that obtained using TMB or HPPA substrates. This is very encouraging, but unfortunately there was not any opportunity to optimise the method and the assay simply followed the protocol for the Kodak Diagnostics Amerlite TSH 30 assay from which the substrate was obtained. It is likely that the use of this or a similar substrate could lead to a useful working urine growth hormone assay.

4.5 Urine assay

Several experiments were carried out to examine the matrix effects of urine on the assay. In many of the reported assays urine is pretreated by dialysis into assay buffer to prevent interference from the wide variations in salt concentrations present in the matrix of urine. Ideally urine samples could be added to the wells of the plates and incubated with the coated antibody, but as demonstrated (Figure 3.3.3) there is considerable difference between the binding of the urine standards and buffer standards. Two options were available, the first being a sample dilution in buffer which would help to standardise the pH and reduce the salt concentration, this unfortunately would also reduce the amount of growth hormone present and therefore also reduces the potential sensitivity of the system. Secondly pre-dialysis of the sample was considered and the results showed this to be effective in reducing the effects of the interference although there was still a residual effect on the binding. It is possible that the dialysis was simply incomplete or that there was a high molecular weight interferent present. An attempt was made to investigate the presence of this interferent but this was not particularly successful and requires some further investigation . Although in theory dialysis is tedious to perform there are systems available to improve upon the use of

dialysis tubing when handling large numbers of samples or, alternatively, the use of extraction columns may be worth examination.

4.6 Recent developments

A urine growth hormone method has been published by Turner et. al. (1993) using an immunochemiluminometric assay (ICMA). The assay uses a sheep polyclonal anti growth hormone antibody coated to tubes and a monoclonal antibody labeled with an acridinium ester. The antibodies were produced by the Institute of Biochemisty, Glasgow Royal Infirmary and the Scottish Antibody Production Unit and are the same as those used in this project. This assay is reported to have a detection limit of 1.1 mU/L. The method requires dialysis of urine samples to prevent interference from urea and NaCl which, as their concentrations increased, were shown to lead to a reduction in growth hormone recovery. The clinical studies showed good correlation between serum and urine growth hormone concentrations in healthy volunteers, who were given intravenous growth hormone, and in patients with acromegaly. The study also examined pre-pubertal children of normal and short stature and showed that the short stature children excreted less growth hormone and grew at a slower rate than the normal stature children. Considerable intra-individual variation was found in growth hormone secretion and the authors recommended that sequential urine growth hormone analyses should be performed, and the results interpreted in conjunction with growth velocity measurements. The authors also recommended further examination of the pattern of renal excretion of growth hormone. Alternatives to dialysis, were examined as it was found cumbersome to perform, these included using ethyl or cyano bonded cartridges but the recoveries obtained were around 50% as compared to 85-90% for dialysis. The team also found it was necessary to add albumin to the dialysis buffer to prevent growth hormone adhering to the dialysis tubing.

This method provides a useful comparison since the antibodies used were the same as those used in this project but the labeling technique was different and it is evident that

the use of acridinium esters provides a very sensitive labeling system. The success of this method is encouraging since it shows that the antibodies are capable of providing the required sensitivity provided the labeling is adequate and it may be possible to produce a suitable assay in the future.

4.7 Final conclusions

There are a number of future possibilities which may be explored in order to produce a viable urinary growth hormone assay. The best available pair of matched antibodies, which may be in the form of antibody fragments or genetically altered antibodies, is the essential basis of a sensitive assay and if they are carefully chosen may limit cross reaction from other hormones. A highly sensitive label is required and this may be an enzyme using a luminescence substrate similar to that used by Kodak Diagnostics Limited or alternatively it may be a fluorescence substrate such as Attophos, an alkaline phosphatase substrate which is reported to be highly sensitive. (Attophos Product Information Bulletin 1991) The separation system may be based on microparticles as in the Abbott IMX system, or may be an automated plate processing system such as that marketed by Kodak Diagostics Ltd. where all the reagent addition steps, washing steps and incubation temperatures are carefully controlled within a purpose built analyser. Automation may provide a useful improvement in precision and ensure that the batch to batch assay conditions are identical, essential in an assay such as this which requires the limits of sensitivity to be stretched.

The problem of the interference from the matrix of urine also requires to be addressed in order to identify if NaCl, urea and glucose are responsible, or some other urine component (Moreira-Andres et. al. 1993), (Baumann and Abramson 1983). Dialysis of the urine does not entirely remove this interference therefore it may in part may be due to a large molecule which prevents the binding of growth hormone to the antibody binding sites by steric hindrance or by binding to growth hormone itself alters its ability to bind to the antibody.

It may be that the role of urine growth hormone measurement in the future will be as a non invasive screening method for assessing growth hormone secretion and the replacement dose in growth hormone treatment for growth hormone deficiency but it may be less useful in mild forms of growth hormone insufficiency (Moreira-Andres et. al. 1993).

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