THE PRODUCTION OF MONOCLONAL ANTIBODIES TO HUMAN TSH AND THEIR USE IN THE DESIGN OF IMMUNOMETRIC ASSAYS FOR SERUM AND NEONATAL BLOOD SPOT TSH

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DECLARATION

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

To Mum & Dad

´Not that the story need be long, but it will take a long while to make it short´.

Henry David Thoreau

ABBREVIATIONS

ALT	alanine aminotransferase
ALTM	all laboratory trimmed mean
AFP	Alpha Foeto-Protein
AELIA™	amplified enzyme linked immunoassay
ACE	angiotensin - converting enzyme
B	Bound
BSA	Bovine Serum Albumin
CDI	carbonyldiimidazole
CEA	Carcinembryonic Antigen
cm	centimetre
CHEMELIA	chemical enzyme linked immunoassay
ca	circa
c.v.	coefficient of variation
CH	congenital hypothyroidism
cpm	counts per minute
CK	creatine kinase
cAMP	Cyclic adenosine monophosphate

^{o}C	degrees Celsius
DNA	Deoxyribonucleic Acid
DIT	Diiodotyrosine
DMSO	dimethyl sulphoxide
DELFIA	dissociation- enhanced lanthanide fluoro-immunoassay
DA	dopamine
ELISA	enzyme - linked immunosorbent assay
Eu	europium
EQAS	External Quality Assessment Scheme
FITC	fluorescein isothiocyanate
FSH	Follicle Stimulating Hormone
F	Free
FT_4	Free Thyroxine
FCA	Freund´s complete adjuvant
FIA	Freund´s incomplete adjuvant
GST	glutathione S-transferase
g	gram
GH	Growth Hormone
GHRIH	Growth hormone release - inhibiting hormone
HCG	Human Chorionic Gonadotropin
HAT	Hypoxanthine; Aminopterin; Thymidine
HGPRT´ase	Hypoxanthine - guanine phosphoribosyl-transferase

IFMA	Immunofluorometric Assay
IgG	Immunoglobulin G
IMA	Immunometric Assay
IRMA	Immunoradiometric Assay
IQ	intelligence quotient
IRP	International Reference Preparation
K	Kilo - $X10^3$
l	litre
LH	Luteinising Hormone
u	micro - $X10^{-6}$
m	milli - $X10^{-3}$
mm	millimetre
mol	moles
MIT	Monoiodotyrosine
n	nano - $X10^{-9}$
NPY	neuropeptide Y
HEPES	N - 2 - Hydroxyethylpiperazine - N′ - 2 - ethanesulfonic acid
EPPS	N - [2 - Hydroxyethyl] - piperazine - N′ - 3 - propane - sulfonic acid
NSS	Normal Sheep Serum

PKU	phenylketonuria
p	pico - $X10^{-12}$
PEG	polyethylene glycol
PRL	Prolactin
RIA	Radioimmunoassay
^{125}I	radioisotope of iodine
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SAPU	Scottish Antibody Production Unit
SAFUR	Scottish Automated Follow-up Register
SHBG	sex hormone - binding globulin
SAM	Sheep anti-mouse γ-globulin
S.D.	Standard deviation
SEM	standard error of mean
TSH	Thyroid Stimulating Hormone, Thyrotropin
TRH	thyrotrophin releasing hormone
T_4	Thyroxine, 3, 5, 3′, 5′ - tetraiodothyronine
TBG	thyroxine binding globulin
T_3	3, 5, 3′ - triiodothyronine

U	Unit
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation

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SUMMARY

The potential for the application of antibodies as analytical and preparative reagents was greatly expanded by the development of techniques for the production of monoclonal antibodies (Kohler and Milstein, 1975, 1976). The properties of monoclonal antibodies are particularly suited to application in immunometric assays and indeed overcome all the major problems associated with conventional antisera. Antibodies of only moderate affinity may be used in sensitive immunometric assays with detection limits up to two orders of magnitude greater than can be achieved in competitive immunoassays with the same antibody (Buchegger et al, 1981). The work of this thesis was concerned with the production of monoclonal antibodies to human TSH and their use in the design of immunometric assays for serum and neonatal blood spot TSH.

Chapter 3 deals with the production of a variety of monoclonal antibodies to TSH. These antibodies were characterised in terms of affinity, specificity and isotype. The results indicated that 5H8 and 2G2 may be of extreme practical value in the development of a two-site immunoradiometric assay (IRMA) for TSH. A number of such assays for the measurement of TSH in serum and in neonatal blood spots were then developed using both monoclonal and polyclonal antisera.

Chapter 4 seeks to illustrate the clinical role and suitability of the monoclonal - polyclonal serum TSH assay developed in this project. The assay successfully distinguished all cases of untreated primary hypothyroidism as having an elevated serum TSH and all cases of untreated hyperthyroidism as

having an undetectable serum TSH (<0.2 mU/l). On the basis of this information, together with the performance data described in Chapter 3, the assay was introduced in 1984 as the 'routine' serum TSH assay for the laboratory at Glasgow Royal Infirmary, processing some 20,000 serum specimens per annum.

Regarding the Scottish Screening Programme for congenital hypothyroidism, at the end of 1983 the monoclonal - polyclonal IRMA replaced the two-polyclonal IRMA of Sutherland et al (1981). Cumulative statistics and predictive values of the data obtained from The National Screening Programme with the two In-house IRMA TSH assays are recorded in Chapter 5 and they reveal that the monoclonal - polyclonal IRMA has continued to maintain the excellent clinical data obtained from the Scottish Screening Programme.

Alternative high specific activity labels and the implications of these developments for the serum TSH assay service in Glasgow Royal Infirmary and the Scottish Screening Programme for congenital hypothyroidism are discussed in Chapter 6. It is almost certain that the clinical value of a serum TSH assay will be enhanced by improving the detection limit from 0.2 mU/l to 0.02 mU/l. Such an improvement is not possible with the existing monoclonal and polyclonal antibodies and an ^{125}I-label. The most straightforward way to achieve this improvement is to move to a non-isotopic label that is compatible with the available antibodies.

The work presented in Chapter 4 of this thesis (Sections V and VI) has shown that many patients receiving thyroxine replacement therapy have undetectable serum TSH levels, both in the in-house IRMA and using the Delfia assay. The interpretation

of these results is discussed in Chapter 6. However, the role of sensitive TSH assays in monitoring patients receiving thyroxine replacement has still to be fully evaluated.

CHAPTER 1

INTRODUCTION

I GENERAL INTRODUCTION TO THE SUBJECT

The work presented in this thesis originated at a time when assays for TSH were becoming more topical. A two-site immunoradiometric assay employing two polyclonal antibodies for both serum and blood spot TSH had previously been developed in this laboratory. The prime objective of the studies now described was the production of monoclonal antibodies to human TSH and the use of these antibodies in the design, optimisation and validation of improved immunometric assays for the measurement of TSH in serum and in neonatal blood spots.

II MONOCLONAL ANTIBODIES AS DIAGNOSTIC REAGENTS

a) Introduction

It has always been recognised that immunological methods suffer from several limitations which significantly restrict their applicability and usefulness. Firstly, there are often substantial practical difficulties in purification of antigens from human sources, for use in immunisation, standardisation and the isolation of specific antibodies. Secondly, it is not always simple to generate and continue

producing antibody with appropriate specificity and other properties. All conventional antisera contain complex mixtures of different antibodies of varying specificity and avidity, recognising various aspects of antigenic structure. Further, individual sera from different animals or even at different times in a given animal, differ in the mixture of antibodies they contain and so do not necessarily have the same properties. The heterogeneity of the immune response in vivo results from the fact that antigen indirectly stimulates the clonal proliferation of many different antibody - producing cells (McConnel et al, 1981), each one secreting a single antibody species (one type of heavy chain and one light chain). It is very difficult to resolve the complex (polyclonal) mixture of antibodies into its individual components, and only relatively crude fractionation on the basis of gross differences in avidity or specificity is generally possible.

The potential for the application of antibodies as analytical and preparative reagents was greatly expanded by the development of techniques for the production of monoclonal antibodies (Kohler and Milstein, 1975, 1976). Monoclonal antibodies are the products of individual cloned cell lines which may be grown in vitro, and as such they are homogeneous preparations with defined specificity and affinity.

Over the last decade there has been an explosion of publications describing the production and use of monoclonal antibodies in many areas of pure research and clinical medicine. These have been the subject of several general

reviews (Goding, 1980; McMichael and Bastin, 1980; Edwards, 1981; Sevier et al, 1981; Yelton and Scharff, 1981; Eisenbarth and Jackson, 1982).

b) Production of Monoclonal Antibodies

1) Introduction

In retrospect the principle underlying the successful production of monoclonal antibodies is deceptively simple (Milstein, 1980). Lymphocytes from immunised animals will not themselves grow *in vitro* but by fusion with myeloma cell lines they may be immortalised (Figure 1.1). The hybrid myeloma (hybridoma) cells which result retain the important properties of both cell types involved in the fusion. Thus not only do they grow indefinitely as tumour cells like the myeloma but also continue to produce antibody characteristic of the lymphocyte. The careful choice of fusion partners was the key to this success, these being not only from the same species (originally the mouse) but the same basic cell type as well. As a necessary refinement the myeloma cells were engineered to have an enzyme deficiency so that after cell fusion hybrids but not parental cells survived to grow in a selective medium (see Section b)6). This is important because fusion efficiency is low (of the order of 1 in 10,000 cells) so that initially hybrids are greatly outnumbered by myeloma cells. Hybrid cells may be cloned and individual colonies, each secreting a different homogeneous defined

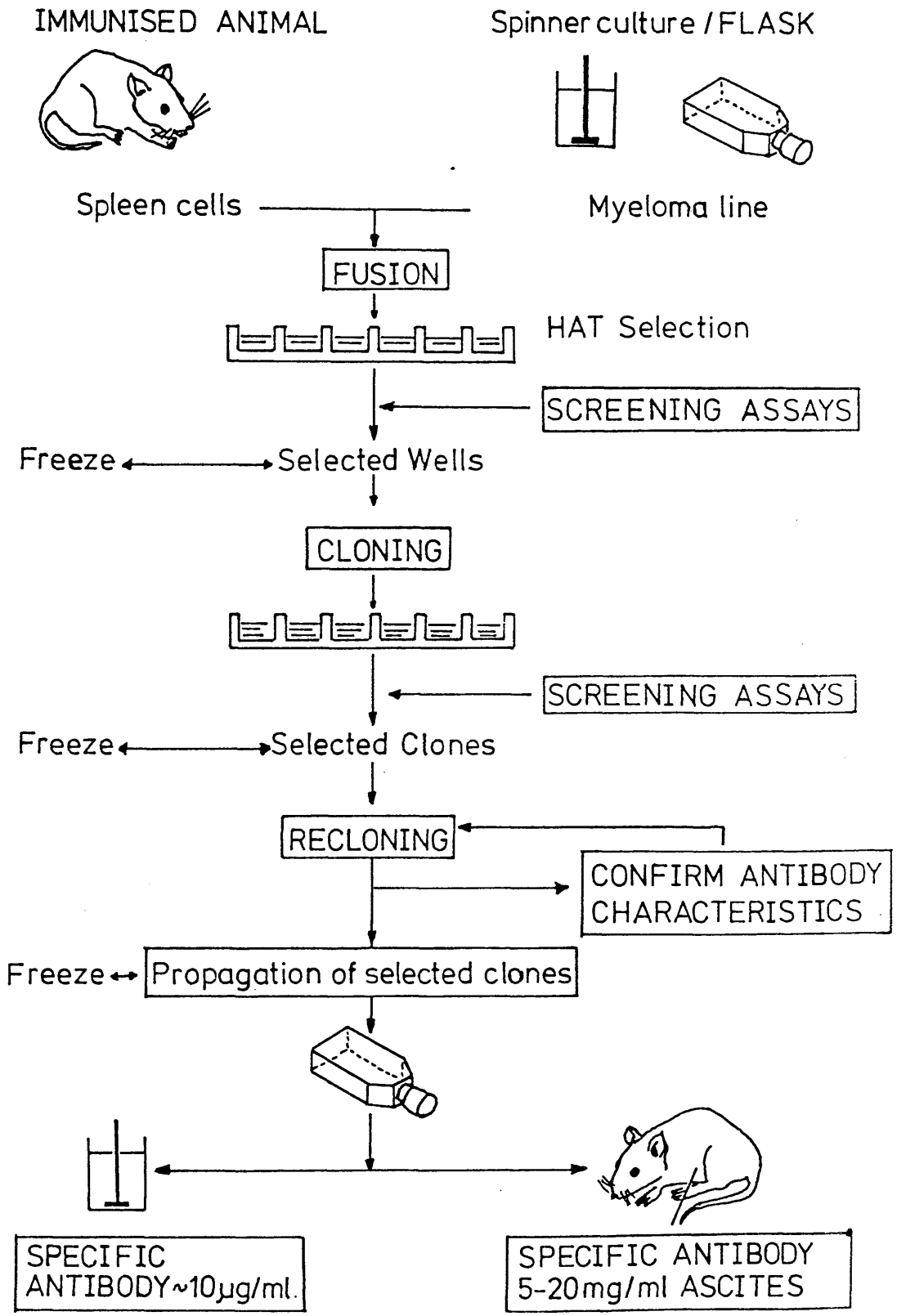


Figure 1.1

Typical schedule for monoclonal antibody production

antibody, grown in tissue culture or as tumours in animals for antibody production. Cells can be maintained indefinitely in culture or stored frozen until required.

It is worth mentioning that the interest which led to the production of hybrid myeloma cells was the structure and organisation of immunoglobulin genes, and the molecular mechanism of antibody diversification (Milstein and Kohler, 1977; Milstein, 1980). Thus, the original work used sheep red blood cells as a model system for which antibodies could be simply produced and analysed. However, the far-reaching implications of the technique for the whole of immunology and biochemistry were immediately obvious, and monoclonal antibodies are now produced routinely in many laboratories, the basic principles and procedures for cell fusion remaining much as first described (Kohler and Milstein, 1975; Galfre et al, 1977).

2) Choice of Cells for Fusion

The choice of myeloma cell line for culture and animal for immunisation are two interdependent variables which must first be considered. Stable antibody-secreting hybrids are generally not produced from fusions between cells of different species due to preferential chromosome loss from one or other of the parent cells. Thus the range of possibilities is limited by the availability of myeloma cell lines. It is a long and difficult process to derive these and is not easily accomplished with most species. The cell

lines of established value were all derived from BALB/c mice or Lou rats (Galfre and Milstein, 1981; Reading, 1982). Some myeloma lines secrete their own immunoglobulin and continue to synthesise this in hybrid cells where the myeloma and lymphocyte heavy and light chains are randomly assembled. As a result the specific antibody containing the correct combination of heavy and light chains may contribute only a small proportion of the immunoglobulin made by such cells. However, variant myeloma lines which synthesise light chains only or no immunoglobulin at all are now available. Providing they undergo efficient fusion to produce stable hybrids, such lines are clearly to be preferred for most purposes because of the simpler nature of the immunoglobulin produced in the hybrids. Several such lines are freely available, while others may only be used under licence for commercial purposes. All these cell lines grow vigorously in standard tissue culture media, either in suspension or more or less loosely attached to surfaces, with a doubling time of the order of 20 h.

Attempts at heterogeneous fusions between standard myeloma lines and lymphocytes of different species have been reported. Reasonable success has been achieved from fusions between rat and mouse cells (Galfre et al, 1977) and stable hybrids between mouse myeloma and human lymphocytes have been described (Schlom et al, 1980; Valente et al, 1982). Attempts to make use of rabbit, hamster and bovine lymphocytes have not been very fruitful, the resulting hybrids generally being unstable and not necessarily

secreting normal immunoglobulins (Yarmush et al, 1981; Sanchez-Madrid et al, 1983).

Therefore, it is necessary to immunise mice or rats in order to carry out cell fusions with the presently available myeloma lines. The most important factor determining the choice of animal is the relative responsiveness to a given antigen, and for the difficult antigens it may be necessary to attempt immunisation of several strains of both mice and rats, including so-called high responders (Goding, 1980; Ivanyi and Davies, 1982). Other things being equal, rats may be preferred because of the apparently greater yield of stable antibody-secreting hybrids (Clark and Milstein, 1981), and potential eventual yield of antibody from tumours grown in vivo (Galfre and Milstein, 1981). It is also an advantage to be able to use myeloma and lymphocytes from the same inbred strain to facilitate the subsequent growth of hybrid cells in vivo in this strain. Inter-strain hybrids can only be grown as tumours in the corresponding F_1 hybrid animals. Inter-species hybrids lead to still more problems and must be grown in vivo in 'nude' mice or rats (Noeman et al, 1982).

Normally the spleen is taken as a source of immunocompetent lymphocytes for fusion when working with rats and mice. Other cells may be used, however, particularly in the preparation of human antibodies.

3) Immunisation Techniques

A good immunisation is the key to a successful fusion experiment. The potential yield of monoclonal antibodies is directly related to the size of immune response in vivo, and the properties of the monoclonal antibodies will on average reflect those detected in the immunised animal.

Mouse and rat immunisation is no different from that of larger animals and preferred protocols reflect the same mixture of prejudice, anecdote and serious study which has governed conventional antiserum production. Thus frequent, repeated injections have been employed by some workers, while others, perhaps more reliably, advise longer intervals, especially before a final boost (Goding, 1980; Galfre and Milstein, 1981). Similarly, quantitites of antigen used have been very variable. Different procedures have rarely been systematically investigated and in any case the optimum conditions will probably differ for different antigens and animals. The initial immunisation is generally given subcutaneously or intraperitoneally using an emulsion of microgram quantities of antigen in Freund's complete adjuvant. One or more boosts with emulsified or soluble material may be given at intervals of a few weeks. The final boost is given intraperitoneally or intravenously in saline so as to maximise the acute stimulation of spleen cells. To increase the chances of obtaining at least one good responder it is worth immunising as many animals including different strains, as the available antigen permits.

Immunogen purity is not in itself important as subsequent screening of antibodies and cell cloning can be used to ensure specificity of the final product. However, if grossly impure preparations are used the dominant antigen may not necessarily be the one of interest. It is usually desirable to substantially purify the material both for immunisation and screening, although antigens of as little as 1-4% purity have occasionally been used sucessfully (Secher and Burke, 1980; Van den Berg et al 1982).

The way in which the antigen is presented for immunisation will influence the characteristics of monoclonal antibodies subsequently obtained just as in the production of conventional antisera. Thus, in the case of haptens, attention must be given to the chemistry of coupling to carrier protein to avoid masking of important chemical groups. Subunits or fragments may be used for immunisation with protein antigens in order to concentrate the immune response and subsequent experimental effort on particular epitopes. However, it is always possible that some monoclonal antibodies to such fragments will not react well with intact native molecules. It has been reported that monoclonal antibody specificity and affinity depends on the immunisation protocol used. Thus the highest affinity and most specific antibodies for progesterone were obtained after prolonged immunisation (Fantl et al, 1982).

4) Immune Response Assessment

The production of monoclonal antibodies is in no way 'magical'. The technique of cell fusion can only immortalise lymphocytes and corresponding antibodies which were present in vivo in the immunised animal. The properties of antibodies made in vivo may change with time and after successive boosts, so that the accumulated serum antibody does not precisely reflect that being made by the most recently stimulated cells, which are selected preferentially in the process of fusion (Kohler and Milstein, 1976; Clark and Milstein, 1981). Apart from this qualification, the chances of obtaining a monoclonal antibody with particular properties will be directly related to the concentration of corresponding antibodies in the serum of the spleen donor. Hence it is necessary to screen immunised animals in order to select for fusion those with the best antibody titre with required properties.

Experience has shown that the yield of positive hybrids is greatest when fusions are performed 3-4 days after boosting, whereas serum antibody concentration does not peak until 7-10 days. Screening must therefore be performed at the optimum time after a penultimate boost, or in the very short time available after a final boost. For this purpose animals may be test bled from the tail and the serum antibody titre determined in a convenient screening assay. Below a certain titre the chances of obtaining good monoclonal antibodies become very small.

If it is important to obtain antibodies with a particular pattern of specificity, it may be worthwhile seeking evidence for such an antibody population in serum (Siddle and Soos, 1981; Fantl et al, 1982). For example, in making antibodies to pituitary hormones such as TSH, there is much variability between animals in the relative incidence of antibodies to α and β subunits, independently of the overall titre. Those animals making antibody predominantly against α subunit invariably yield only similar monoclonal antibodies which therefore react equally with other hormones such as LH and FSH (Siddle and Soos, 1981).

5) Cell Fusion Techniques

It is not clear what the biochemical mechanisms underlying cell fusion are (Knutton and Pasternak, 1979), but technically the process is straightforward and takes only a short time. An important factor in determining success at this stage is that both types of parental cell should be in the best possible condition. It is necessary that myeloma cells are in rapid, exponential growth phase in culture, and lymphocytes stimulated effectively by a final boost of antigen 4 days previously. Myeloma cells and lymphocytes (one mouse or half of a rat spleen) are mixed and spun down into a pellet. Polyethylene glycol, the fusogen, is added carefully and then slowly diluted out. Precise cell numbers, temperature, timing and polyethylene glycol concentration do

not appear to be critical and a number of different protocols have been described and used successfully (Galfre and Milstein, 1981).

The yield of viable hybrid cells is of the order of some hundreds or thousands representing only a small proportion of the parental cells. The fraction of hybrid cells secreting immunoglobulin varies with the myeloma line used for fusion (Clark and Milstein, 1981), and the proportion of these which secrete the required specific antibody is dependent on the success of the immunisation. In general, approximately 1-10% of hybrids may be making specific antibody although much higher figures have been claimed in some cases and with weakly immunised animals lower values are always possible.

Other methods of cell fusion include antigen-directed fusion using polyethylene glycol (Kranz et al, 1980), electric field-induced fusion (Vienken and Zimmermann, 1982) and antigen-directed electric field-induced fusion (Lo et al, 1984).

6) Cell Culture and Cloning

Following fusion a preliminary growth phase is normally preferred before cloning, although attempting to clone cells immediately is possible. Conditions should be made as favourable as possible for hybrid growth with a high foetal calf serum concentration and, if necessary, the addition of feeder cells. It is vitally important to adopt sterile technique throughout as fungal infections can

spread quickly once introduced and destroy a lot of effort and perhaps cell lines which are irreplaceable. During the initial growth period of 10-14 days, hybrid cells multiply, doubling every day or so. The parental lymphocytes do not survive in culture, and the parental myeloma cells are selectively killed by the HAT (hypoxanthine, aminopterin, thymidine) medium technique (Goding, 1980; Galfre and Milstein, 1981). By way of explanation, cells can synthesize DNA in two ways, either by 'de novo' synthesis or via the so-called 'salvage' pathway using exogenous or endogenous sources of preformed bases (Figure 1.2). If myeloma cells are grown in the presence of a purine analogue, for example, 8-azaguanine or 6-thioguanine, the hypoxanthine-guanine phosphoribosyltransferase (HGPRT'ase) enzyme catalyses the incorporation of the purine analogue into DNA where it interferes with normal protein synthesis and so the cells die. The gene coding for the HGPRT'ase enzyme is on the X chromosome and so only a single copy per cell is expressed. Eventually cells will arise that are deficient in the HGPRT'ase gene and therefore do not incorporate the purine analogue. These HGPRT'ase deficient cells are unable to utilise hypoxanthine and so synthesise ribonucleotides only by 'de novo' synthesis. A selective medium containing aminopterin, hypoxanthine and thymidine (HAT medium) was introduced (Littlefield, 1967). Aminopterin is an analogue of folic acid and binds very tenaciously to folic acid reductase thus blocking the co-enzymes required for 'de novo' synthesis of DNA. To grow in this medium a cell must make DNA via the 'salvage' pathway. Thus, if myeloma cells,

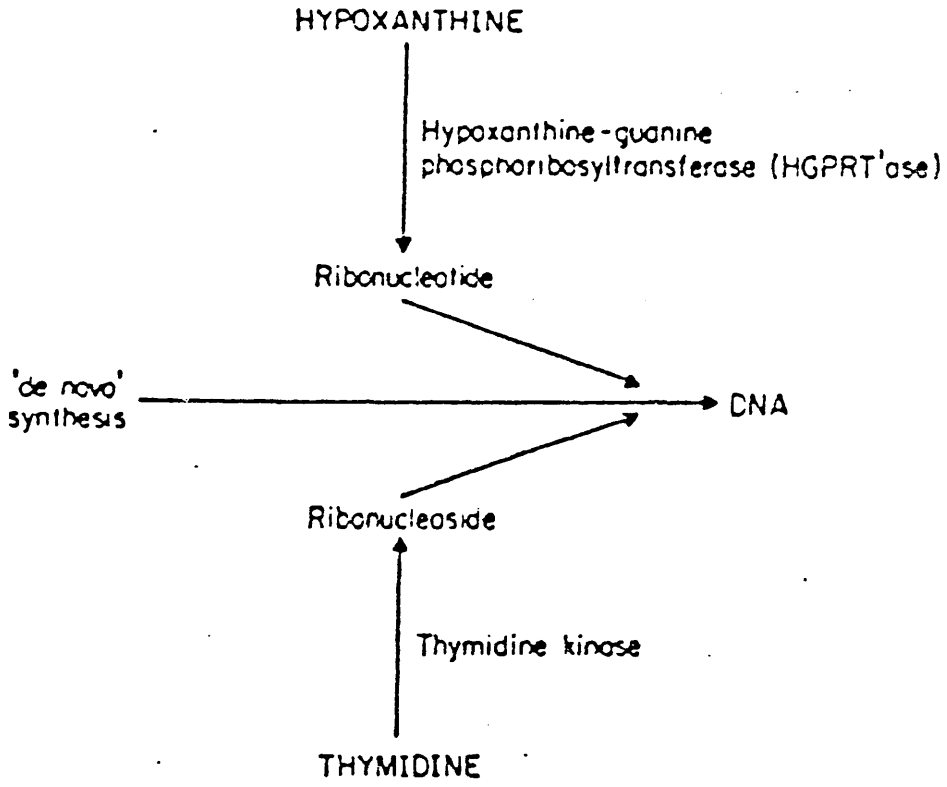


Figure 1.2

Synthesis of DNA by 'de novo' synthesis and via the 'salvage' pathway using exogenous or endogenous sources of preformed bases

deficient in HGPRT´ase, are fused with normal lymphocytes and then placed in HAT medium, only the hybrids between myeloma and normal cells will grow; the myeloma cell provides immortality and the lymphocyte provides the HGPRT´ase enzyme.

Even during early growth it is important to segregate the cell fusion products into a number of different subcultures. This reduces competition between hybrids growing at different rates, facilitates identification of distinct antibody-positive hybrids, and simplifies the subsequent task of cloning. Several different strategies may be employed. Cells may be distributed into about 50 2 ml cultures, each of which will then contain many different hybrids. It is then, initially, a relatively small task to screen for positive cultures, but cloning from these cultures may subsequently demand the screening of very large numbers of clones to identify those arising from a small proportion of positive cells. At the other extreme, cells may be distributed into hundreds or even thousands of 0.2 ml cultures, such that each culture well contains only one or very few different hybrids. Initial screening is then a formidable task but the effort subsequently necessary to isolate positive clones is reduced. However, it has been shown that the probability of hybrid cell survival decreases with post-fusion cell dilution (De Blas et al, 1981), and initial monoclonality may be undermined by the production of non-secreting variants in an originally positive culture.

Cloning should be carried out as soon as possible after positive subcultures have been identified. This may be

achieved by growing hybrids at limiting dilution in micro-titre trays with feeder cells, such that some wells receive only a single hybrid cell which develops into a more or less compact colony after a few days. Alternatively, cells may be grown at low dilution in semi-solid agar as a way of physically separating the clones of cells until they are large enough to be transferred individually to culture wells.

During hybrid growth and cloning it is often found that antibody secretion appears unstable. This may be due to overgrowth by negative cells so that the fraction of positive cells and antibody concentration decreases in successive subcultures. Also, chromosome loss from hybrid cells, especially in the early days after fusion may give rise to non-secreting variants. Failure to obtain positive clones even from subcultures which appear strongly positive may reflect a low proportion of positive cells, or an inherently poor cloning efficiency of some cell lines which will not grow well from low concentrations. Some degree of hybrid instability may persist indefinitely, necessitating periodic recloning to ensure maintenance of overwhelmingly positive cells. Chromosome loss, however, decreases with time, and initially it is normally sufficient to perform two clonings to ensure and confirm that a monoclonal cell line has been obtained.

7) Screening Assays

It is necessary, both in the initial screening of subcultures, and in the subsequent identification of positive clones, to have a good assay for the detection of specific antibody. There are a lot of possible types of screening assay depending on the nature and availability of antigen (Galfre and Milstein, 1981). The chosen assay must be sensitive enough to enable antibody at concentrations of less than 1 ug/ml in supernatant medium from cell cultures to be detected. Also, it must be simple enough to give quick answers on very large numbers of samples. However, it need not be precisely quantitative nor at this stage totally specific.

Most generally applicable are the assays which make use of either insolubilised or labelled antigen (Figure 1.3). Protein antigens may be adsorbed onto a solid phase, often the wells of plastic microtitre trays, and successively incubated with putative monoclonal antibody and then second (anti-immunoglobulin) antibody or protein A, which is radioactively labelled or conjugated to an enzyme which is easily assayable. Relatively large (milligram) amounts of antigen which need not be pure, are required for this method. Alternatively, soluble antigen may be labelled for reaction with monoclonal antibody, which has been previously or is subsequently bound to solidphase linked second antibody or protein A. Very small (microgram) quantities of antigen are required for this method, which preferably should be relatively pure. Either type of assay may pick up false

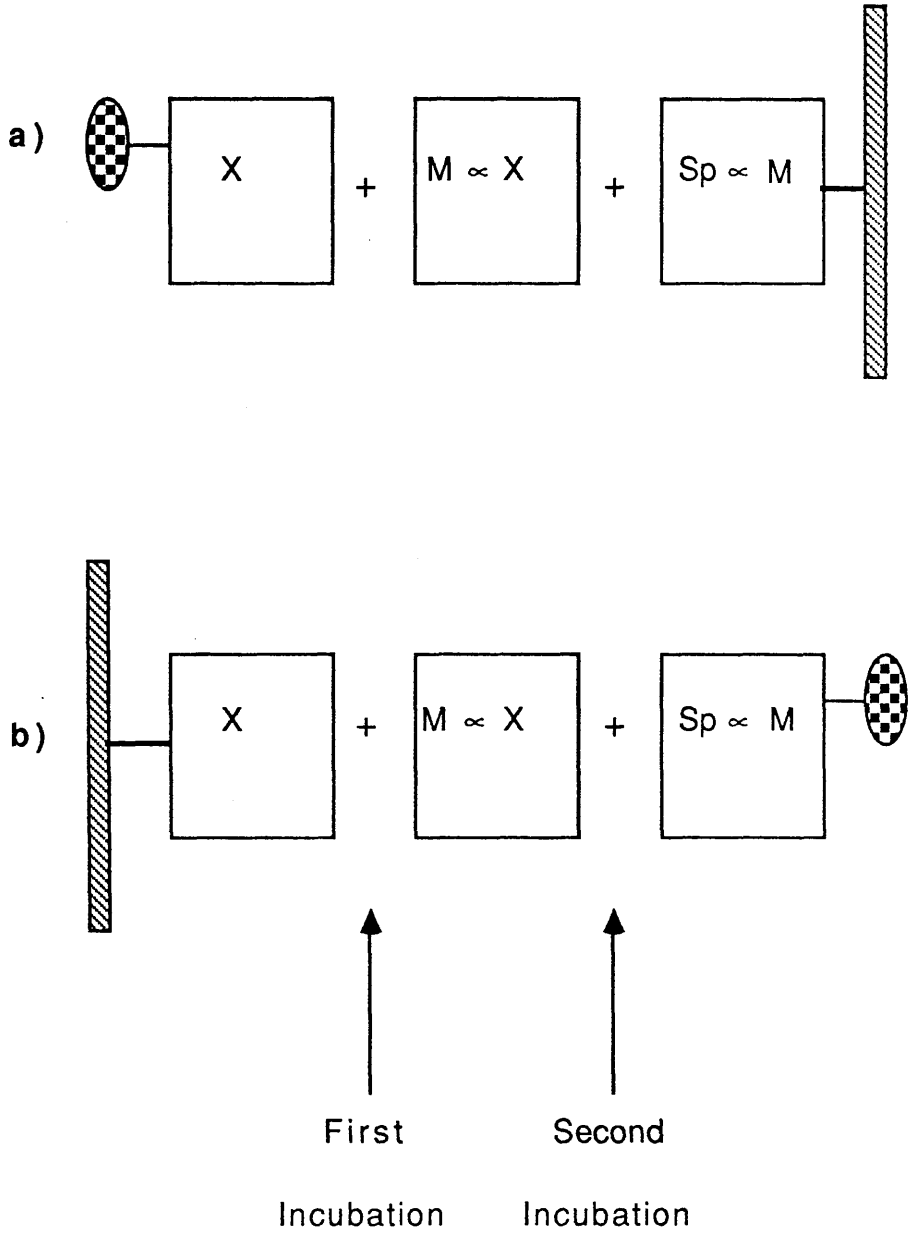


Figure 1.3

Principle of commonly used screening assays for the detection of monoclonal antibodies. Antigen, represented as X, is either suitably labelled (a) or bound to solid phase (b) before reaction with monoclonal antibody M α X. The presence of monoclonal antibody is detected by addition of a second, anti-immunoglobulin antibody Sp α M, which is either coupled to solid phase (a) or labelled (b)

positives due to antibodies directed against impurities if the same impure antigen is used for immunisation and screening. Some additional confirmation of antibody specificity will eventually be required in this case.

Other screening methods with general applicability include haemagglutination using antigen-coated erythrocytes (Coombs, 1981) and complement - dependent lysis with antigens present in or attached to cell surfaces. In some cases, assays may be based on the biological activity of an antigen, in particular enzyme activity, and the ability of antibody to precipitate, bind or inhibit this activity.

Potential cross-reacting or interfering compounds may be included in screening assays so as competitively to inhibit reaction of non-specific antibodies with antigen.

8) Bulk Production and Purification of Antibodies

Cloned cell lines can be grown in quantity for antibody production either *in vitro* or *in vivo*, and the preferred method depends on available facilities, scale of operation and eventual purity required. Before large-scale culture, cells should be adapted to grow in medium supplemented with the lowest possible concentration of foetal calf serum, to ease antibody purification and reduce expense. Litre quantities per day of spent medium containing antibody at 10-100 ug/ml can be produced in spinner cultures. Alternatively, cells may be grown as intraperitoneal (ascites) tumours in animals, depending on the availability

of compatible recipient strains as described previously. Ascitic fluid and serum from tumour bearing animals contains relatively concentrated antibody at 1-10 mg/ml, mice yielding approximately 10 ml of fluid per animal in sequential taps over 2-3 weeks and rats considerably more. Tumour fluid antibody is contaminated by rodent serum proteins including immunoglobulins which may present problems if it is necessary to purify antibodies to homogeneity.

Monoclonal antibodies may be obtained in fairly pure form by simple physicochemical methods such as salt precipitation, ion exchange chromatography and gel filtration (Goding, 1980; Galfre and Milstein, 1981; Bruck et al, 1982; Parham et al, 1982). Affinity chromatography on protein A - Sepharose (Pharmacia) can yield very pure antibody from spent medium but not all immunoglobulin classes bind well to protein A, particularly in the rat, and the capacity is limited by the expense of reagents. Affinity purification on immobilised antigen or anti-immunoglobulin antibody also has a relatively limited capacity and may require potentially damaging conditions for elution of monoclonal antibody.

c) Properties of Monoclonal Antibodies

1) Introduction

The properties of monoclonal antibodies are not intrinsically different from those of normal serum immunoglobulins. Apparent differences result from the fact

that the behaviour of serum antibodies reflects the mean or composite properties of a mixture, whereas each monoclonal antibody has the particular physical and biological properties conferred by its unique molecular structure and single epitope recognition. The properties of an antibody from a cloned cell line should be unchanging. It is possible that an antibody may be susceptible to some form of processing *in vivo* (such as limited proteolysis) which would change the properties of tumour fluid antibody relative to that produced by cells *in vitro*. However, only rarely have differences between ascites and culture fluid antibody been reported (Wang et al, 1982). Monoclonal antibody characterisation is necessary to assign priorities for culture and antibody production, and to assess suitability for a particular application. This requires the determination of concentration, affinity, fine specificity, epitope recognition and isotype, while also looking out for unusual physical properties.

2) Concentration and Avidity

Just as for antiserum, the titre of monoclonal antibodies in culture or tumour fluids is determined by assessing the effect of serial dilution on the performance in any convenient screening assay. The titre (dilution for half-maximal reactivity) is a function of both the concentration and avidity of antibody, and these parameters may both be determined by Scatchard analysis. By studying

the binding of antigen to a limiting amount of antibody as a function of antigen concentration, data for such a plot are obtained. Monoclonal antibodies for monovalent antigens should give linear Scatchard plots indicative of a bimolecular reaction with a single binding affinity. With multivalent antigens the situation is more complex because of the possibilities for formation of ternary and higher order complexes (Jacobsen et al, 1982).

Affinities reported of monoclonal antibodies for monovalent antigens vary over nearly four orders of magnitude (10^7 - 10^{11} M^{-1}). It is often said that monoclonal antibodies are of low affinity, and compared to the best available polyclonal antisera this is frequently correct. The isolation of monoclonal antibodies of relatively low affinity partly reflects the preponderance of such antibodies in vivo in most animals. In monoclonal antibody populations, as for serum antibodies, high affinities are usually in the minority.

3) Fine Specificity

Antibody fine specificity in discriminating between the antigen of interest and related compounds may be investigated in several ways. Direct binding of radioiodinated materials to antibody can be tested. By comparing the potencies of unlabelled compounds to inhibit binding of labelled antigen to antibody, a quantitative estimate of relative affinities

may be obtained. Alternatively, potential cross-reactants may be tested for their ability to prevent binding of antibody to immobilised antigen.

In theory, it is possible that monoclonal antibodies may show reactivity towards molecules completely unrelated to the antigen of interest, because of the chance occurrence of epitopes with similar chemical groupings. Such cross-reaction would not be a serious problem with a polyclonal antiserum for which overall reactivity depends on the recognition of many different epitopes on a molecule by a heterogeneous mixture of antibodies. In practice, such unforeseen cross-reactions have not been found with monoclonal antibodies, indicating that chance homology of epitopes on unrelated molecules occurs rarely if at all.

4) Epitope Definition

It is particularly useful in relation to methods of application requiring compatible pairs or mixtures of antibodies to define the epitopes recognised by different antibodies on a given antigen. Differences in fine specificity of antibodies for structurally related molecules or fragments of antigen may indicate distinct epitopes (Retegui et al, 1982; Lillehoj et al, 1982), though not necessarily sites which are sufficiently separated in space to allow simultaneous binding of the corresponding antibodies (Ivanyi, 1982; Soos and Siddle, 1983). Spatially separated epitopes may be resolved by competition binding studies.

However, the existence of competition between two antibodies in binding to antigen does not prove identity of epitope, but merely proximity considering the large size of immunoglobulin molecules and the potential for steric hindrance over a substantial part of the antigen. Epitope analysis may be performed by testing for competition either between labelled and unlabelled antibodies for binding to immobilised antigen, or between soluble and immobilised antibodies for binding of labelled antigen (Ivanyi, 1982; Soos and Siddle, 1983). The two methods may not give identical results because of additional steric hindrance resulting from immobilisation of antigen. Antibodies to distinct epitopes may also be identified by their ability to complement one another in systems which require multivalent recognition of antigen, such as precipitation or agglutination (Miggiano et al, 1981). Monoclonal antibodies may be encountered for multiple distinct epitopes or a small number of dominant antigenic determinants depending on the size and nature of individual antigens. Moreover, as a result of differences in the predominant antibodies produced in individual animals, the spectrum may vary between fusions.

5) Isotype Determination

The class and subclass of immunoglobulin heavy chain substantially influence the biological and physical properties of monoclonal antibodies, including their ability to fix complement (McConnell et al, 1981) and the conditions

under which monovalent or bivalent antibody fragments may be produced (Parham et al, 1982). The isotype may be determined by several methods, which make use of differences in electrophoretic mobility, reactivity with protein A or recognition by subclass - specific antisera in binding, precipitation or haemagglutination assays. Most mouse monoclonal antibodies are of the IgG_1 subclass, reflecting the major type of immunoglobulin in serum but other classes are encountered not infrequently. IgM antibodies are obtained particularly when fusions are performed after a single immunisation, because of the importance of IgM in the primary immune response.

6) Physical Properties

Conventional antisera differ little in their mean physical properties such as stability on freezing and thawing, storage or iodination, susceptibility to changes in buffer concentration and pH, and ease of salt precipitability. Unusual sensitivity of a subpopulation of serum antibodies to some particular treatment may pass unnoticed against a background of predominantly stable antibody. However, when similarly treated, a sensitive monoclonal antibody may lose activity completely. Thus although most monoclonal antibodies are, like antisera, stable reagents which can be easily handled, atypical behaviour may be encountered. Physical properties which affect ease of adsorption or conjugation of antibodies on to

solid phase, and long-term stability, are by no means trivial considerations for routine diagnostic assays (Davis et al, 1983). Even the choice of buffer may be crucial for the optimal performance of some monoclonal antibodies (Buchegger et al, 1982b). It is difficult and usually unnecessary to investigate systematically the properties of every monoclonal antibody under a wide range of conditions, but as a precaution any new procedure should first be tested on a small scale before committing a large amount of potentially valuable antibody.

d) Application of Monoclonal Antibodies in Diagnostic Assays

In principle, monoclonal antibodies either singly or in combination, can be used in place of conventional antisera in any type of assay. However, in situations where adequate sensitivity, specificity and convenience are already provided by assays employing freely available polyclonal antibodies, there is not much to be gained by the expensive pursuit of monoclonal antibodies which may ultimately prove inferior to existing reagents.

The bulk of assays utilising antibodies may be broadly classified into competitive immunoassays, in which the analyte is the labelled component, or immunometric assays, in which antibody is labelled (Figure 1.4). In either type of assay, radioactive isotopes, enzymes, fluorescent molecules or luminescent compounds may be used as label.

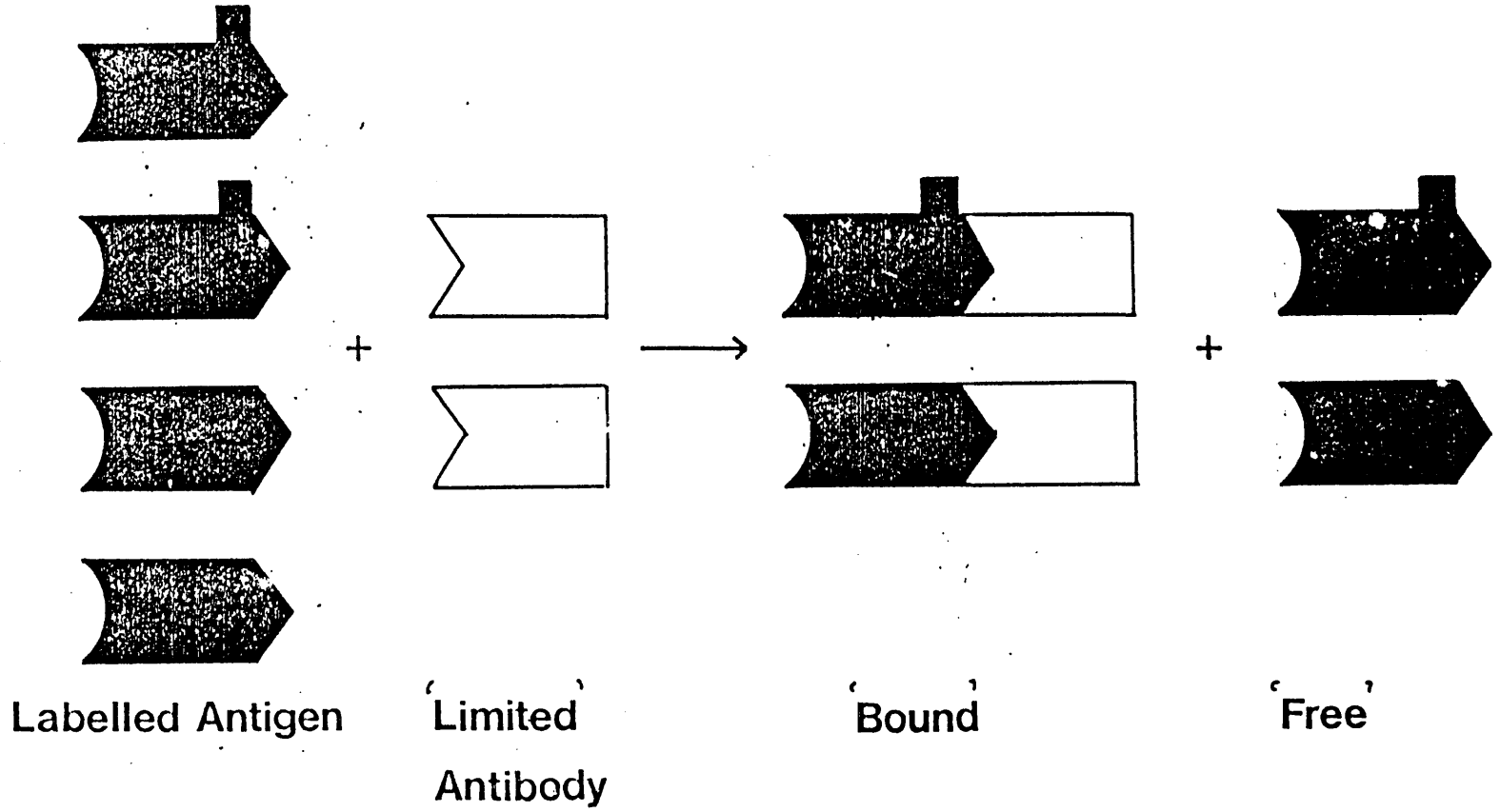


Figure 1.4

(a) Diagrammatic representation of the principles of saturation analysis. A limited quantity of reagent (antibody) is reacted with a relative excess of analyte (antigen) to form bound and free fractions. Monitoring of the bound (or free) component is accomplished by addition of labelled analyte

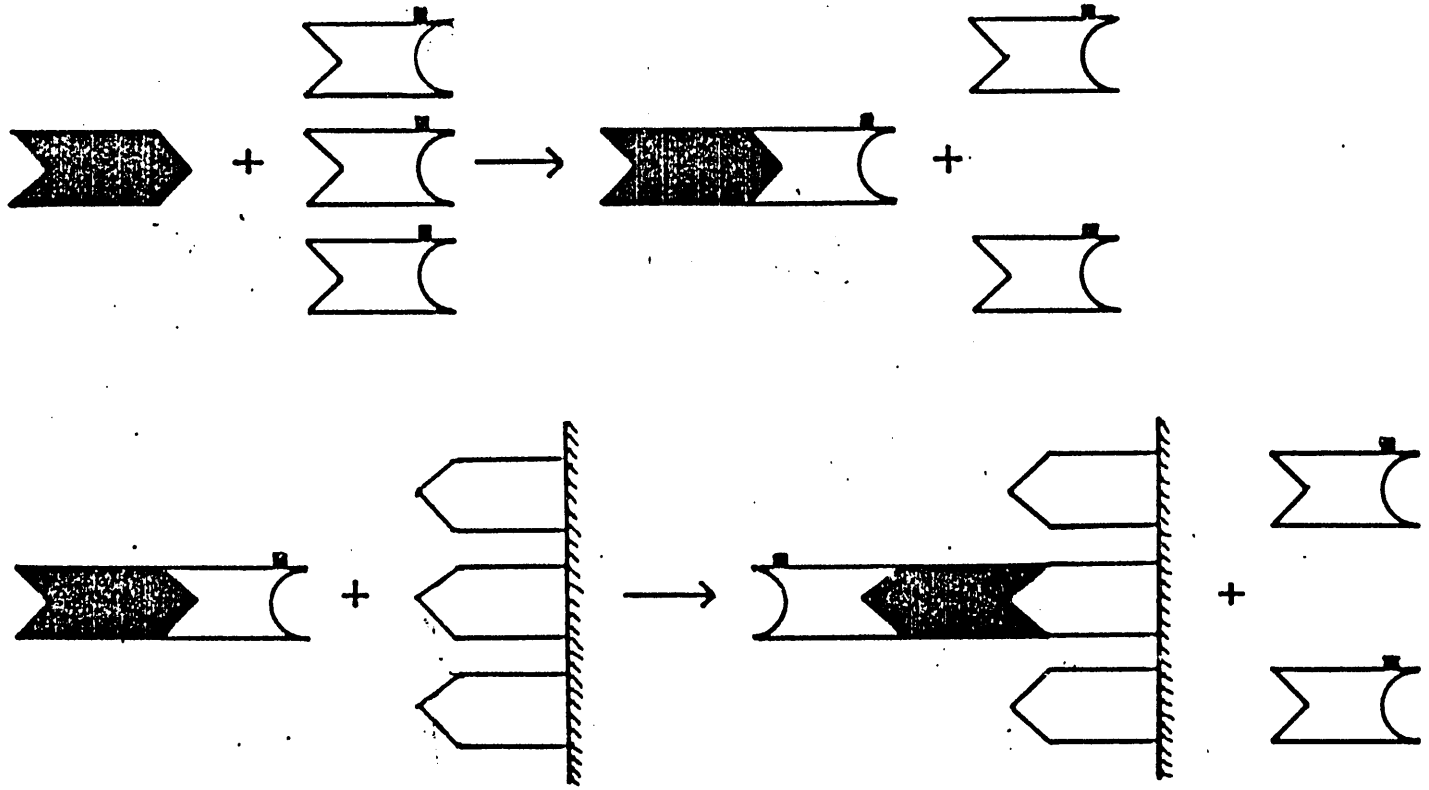


Figure 1.4

(b) Diagrammatic representation of the principle of the two-site IRMA (see text for details)

In competitive immunoassays, standard or unknown antigen competes with labelled antigen for binding to a limiting amount of antibody. A separation step is normally required in order to determine the distribution of label between 'bound' and 'free' fractions. This may be achieved, for instance, by coupling antibody to solid phase, by use of a second (anti-immunoglobulin) antibody, or by some other means of selective precipitation or adsorption of antibody or antigen.

Immunometric assays in principle provide a direct measure of antigen by its combination with labelled antibody present in excess; a separation step is usually required to determine the amount of label bound. Originally this was achieved by addition of excess antigen coupled to solid phase to remove unbound antibody (Miles and Hales, 1968). More commonly, a two-site assay protocol is used (Woodhead et al, 1974) in which antigen is adsorbed on to a solid phase containing a second specific antibody before or after reaction with labelled antibody. This requires that antigen binds simultaneously to two different antibody molecules and places a lower limit on the size of antigen which may be quantitated in this way. Homogeneous assays using labelled antibodies without a separation step have been proposed (Sevier et al, 1981) but not as yet put to practical use.

The theoretical and practical advantages of immunometric assays over competitive immunoassays have now been amply documented (Miles and Hales, 1968; Woodhead et al, 1974; Ekins, 1981; Hunter and Budd, 1981; Hunter et al, 1983). These include improvement in speed, increased sensitivity,

wider working range, and greater specificity for intact, biologically active molecules. Other points in favour of the immunometric assay are the lack of dependence on purification and labelling of antigen, which may alter its immunoreactivity, and the convenience of a separation step based on the two-site principle. Labelled polyclonal serum antibodies have, however, found only relatively restricted application in immunometric assays. Several factors have contributed to this. Firstly, such assays consume large amounts of antibodies, because these are used as excess rather than limiting reagents. Secondly, antibody must be purified from the bulk of serum immunoglobulin, of which it may constitute as little as 1%, for labelling to high specific activity. Purification is achieved by specific adsorption to and elution from immobilised antigen, a process which itself requires substantial amounts of purified antigen (Hales and Woodhead, 1980). The elution of bound antibody, usually at low pH, may damage its reactivity or fail to recover the fraction with highest avidity. A final problem with two-site assays is their requirement for two antibodies which compete minimally for binding sites on the antigen. Such antisera may arise by chance in different animals, although polyclonal antibodies usually at best show substantial overlap in epitope reactivity. Distinct epitope reactivities may sometimes be engineered by immunisation with, or adsorption of sera against, specific fragments of antigen.

It is obvious that the properties of monoclonal antibodies are particularly suited to application in immunometric assays, and indeed overcome all the major problems associated with conventional antisera. Thus, monoclonal antibodies for multiple distinct epitopes on a given antigen may be produced in large quantitites, and easily purified. Antibodies of only moderate affinity may be used in sensitive immunometric assays with detection limits up to two orders of magnitude greater than can be achieved in competitive immunoassays with the same antibody (Buchegger et al, 1981; Weeks et al, 1981; Bosch et al, 1982; Van den Berg et al, 1982; Hunter et al, 1983). In contrast, monoclonal antibodies have few advantages over polyclonal antisera for use in conventional immunoassays. Many monoclonal antibodies of indifferent affinity will produce immunoassays of inferior sensitivity, and only in terms of specificity is there any benefit to be expected.

e) Monoclonal Antibodies in Two-Site Immunometric Assays

1) Introduction

Two-site assays using monoclonal antibodies have been established for several antigens with a variety of different methods for labelling, types of solid phase, and incubation protocols. The particular configuration chosen usually reflects local preferences and expertise, and in any case the best conditions will differ for each antigen and antibody.

There are advantages in having monoclonal antibodies for both solid phase and labelled reagents, although they have also been used in conjunction with polyclonal antisera (Weeks et al, 1981; Brock et al, 1982; Buchegger et al, 1982a; Hunter et al, 1982). In some cases this is simply due to the lack of availability of two suitable monoclonal antibodies. In others, polyclonal antibodies have been deliberately preferred for coupling to solid phase in spite of lower purity, because of better avidity (Hunter et al, 1983).

2) Labelling of Monoclonal Antibodies

Monoclonal antibodies were originally labelled either by radioiodination (Hurrell et al, 1981; Secher, 1981; Weeks et al, 1981; Hunter et al, 1982; Shimuzu et al, 1982) or by conjugation with enzymes (Uotila et al, 1981; Brock et al, 1982; Wada et al, 1982). Monoclonal antibody labelling techniques, and the relative advantages of different labels are much the same as in applications of other labelled proteins (Schall and Tenoso, 1981). Ease of preparation, shelf-life, effect of labelling on antibody activity, sensitivity of detection and instrumentation availability must be considered. Owing to the development of microplate photometers for rapid quantitation of large numbers of samples, and in response to propaganda about the hazards of radioactivity, the popularity of enzyme labels has increased. The sensitivity of enzyme methods may be considerably increased by the introduction of amplification steps in which

the primary product of the antibody - conjugated enzyme is used as a recycled cofactor in a subsequent colour-generating reaction (Self, 1985).

Other forms of labelling with luminescent compounds (Woodhead et al, 1982) or fluorescent molecules (Sioni and Kojola, 1983), are now finding their way into routine use. They are likely to increase in significance as relevant detection systems improve and become more widely available. The potential for much greater sensitivity using such labels, arising from the magnitude of effective signal per labelled antibody molecule, has been demonstrated (Weeks et al, 1983a, 1983b; Petterson et al, 1983).

3) Solid Phase Linked Monoclonal Antibodies

Several different matrices have been used for coupling of antibody to produce solid phase reagent. Antibody may simply be adsorbed on to plastic surfaces such as the wells of microtitre trays (Uotila et al, 1981; Brock et al, 1982). The incubation vessel then provides the solid phase. This greatly facilitates washing procedures and is conveniently linked to spectrophotometric detection of end points by plate-readers. Long-term stability of adsorbed antibody and standardisation on a large scale may, however, give rise to problems. Various particulate materials have also been used for covalent coupling or adsorption of antibodies, including cellulose (Weeks et al, 1981), Sepharose (Hurrell et al, 1981), polystyrene (Buchegger et al, 1982b), glass (Gard et

al, 1983) or various plastics (Gard et al, 1984; Soos et al, 1984). Depending on the nature of the particles, separation and washing may be achieved by sedimentation or filtration techniques. The use of finely - divided cellulose or Sepharose beads has the advantage that these may be conveniently manipulated by automated immunoassay equipment (Bagshawe and Mitchell, 1983). The choice of solid phase will also be influenced by the assay protocol, and whether it is desired to carry out incubations with solid phase and labelled antibodies simultaneously or sequentially.

The available matrices differ in their capacity for coupling of antibody, and in their tendency to bind material non-specifically. The amount of effective antibody bound to solid phase should be optimised in order to give an adequate capacity for antigen with the smallest possible quantity of solid phase, so that non-specific binding of labelled antibody is minimised. The avidity of solid phase antibody is also important in this context. Mixtures of different antibodies of relatively low affinity can be used to produce a high avidity solid phase which binds antigens simultaneously at two sites (Buchegger et al, 1982b).

4) Standard Diluent and Assay Buffers

In principle, standard solutions must be diluted in the same medium as constitutes the samples to be added in the assay. For measurement of human serum samples, it is therefore necessary to have access to analyte-free serum for

preparation of standards, unless it can be shown that there are no non-specific effects of serum, compared to a chosen buffer medium, on the assay reactions. Serum effects are generally less pronounced in immunometric assays than in competitive immunoassays. This is because minor perturbations of the antigen-antibody reaction are not so serious when antibody excess drives the binding reaction to completion. Moreover, it is possible, if necessary, to carry out the critical reaction with labelled antibody after prior adsorption of antigen on to solid phase, and washing to remove any interfering serum components. It may be possible to obtain antigen-free serum under particular physiological or pathological conditions for use as a diluent, when serum effects are a problem. Traces of immunoreactive material may be removed from serum by immunoadsorption using specific immobilised monoclonal antibodies, or sometimes, and less satisfactorily, by non-specific adsorption on to material such as activated charcoal. Alternatively, non-human serum may be employed from a species in which the corresponding antigen is absent or at least does not cross-react with the antibodies used.

The nature and exact pH of assay buffer is normally not a critical parameter. Protein or detergent may be added to assay and washing buffers with the aim of decreasing the non-specific binding of labelled antibody to solid phase (Uotila et al, 1981). Direct binding of labelled antibody to solid phase, independent of antigen, can also arise because of cross-linking by anti-immunoglobulin antibodies present in a small proportion of human sera, and would potentially produce

false high assay values (Buchegger et al, 1982b; Hunter et al, 1983). Prevention of this artefact is possible by the addition of non-immune mouse or other appropriate serum in the assay buffer to saturate any anti-immunoglobulin activity.

5) Incubation Protocols

The reactions of antigen with labelled and solid phase antibodies may be allowed to occur simultaneously or sequentially. The incubation times necessary depend on the kinetics of individual antibody-antigen reactions, and thus on the properties and concentration of each antibody. It may take many hours to reach true equilibrium in two-site assays, but typically incubation times of the order of 1-16 h for each incubation step have been used. However, shorter incubations are possible but there is a progressive loss of sensitivity as the binding achieved decreases.

In principle, because solid phase and labelled antibodies are deliberately chosen not to compete for binding sites, monoclonal antibodies lend themselves particularly to single-step, simultaneous incubation protocols. The great advantage of such a method is its simplicity, but it is not without its problems. Very high levels of antigen may exceed the binding capacity of solid phase within the assay incubation, resulting in a biphasic standard curve, with limited useful working range (Normura et al, 1982; Wada et al, 1982; Davis et al, 1983; Hunter et al, 1983). This is a

practical problem only with analytes such as HCG and AFP which potentially may vary in concentration over several orders of magnitude. A real possibility of ambiguity in interpretation of a given level of binding of labelled antibody then exists. In these circumstances it is impractical to add enough solid phase to guarantee excess under all conditions. To resolve the ambiguity suspect values may be reassayed at one or more dilutions, or a two-step assay protocol may be used so as to avoid it (Nomura et al, 1982; Davis et al, 1983).

In a forward two-step protocol, antigen is first bound to solid phase, which is then washed to remove excess antigen or interfering compounds before reaction with labelled antibody. Difficulties with excess antigen do not arise as the response reaches a plateau at high antigen concentrations, although for precise quantitation it may still be necessary to assay 'high' samples at several dilutions. If polyclonal antiserum is used on solid phase, it is likely that at least some of the antigen will be bound in such a way that the binding site for labelled monoclonal antibody is no longer accessible, with consequent loss of sensitivity.

Potential competition between polyclonal solid phase and monoclonal labelled antibody is avoided in the reverse two-step protocol, in which antigen is reacted with labelled antibody before addition of solid phase.

6) Specificity and Standardisation of Two-Site Assays

The interference from cross-reacting substances in two-site assays depends on whether these are recognised by one or both of the antibodies. If a cross-reactant is bound to some extent by both antibodies, a 'false' positive signal will result and the assay will be more or less non-specific. The magnitude of cross-reaction within the assay may be greater than that expected from the relative affinity with individual antibodies, because of the use of excess reagent conditions (Wada et al, 1982). In the limit, a cross-reactant may render the same assay response as an equivalent concentration of analyte, although binding to one or both antibodies with much lower affinity (Ekins, 1981). No modification of assay design can easily overcome this problem, and the specificity of individual antibodies therefore becomes of paramount importance.

If one antibody is effectively completely specific but the other shows a strong cross-reaction with material other than the analyte of interest, a different problem arises. Cross-reactant then competes with analyte for binding to the non-specific antibody and leads to an artefactually low estimate of analyte concentration in the two-site assay. Such interference has been noted for instance in a TSH assay where the solid phase antibody reacts also with LH and HCG (Wada et al, 1982). This problem can be solved by using the non-specific antibody in such an excess that the binding of cross-reactant becomes unimportant. However, in practice this may be very difficult. As an alternative, an assay

configuration may be chosen so that the sample is first reacted with specific antibody on solid phase in a forward two-step protocol. Potentially cross-reacting material can then be removed by washing before addition of 'non-specific' labelled antibody. The best solution is to avoid the use of cross-reacting antibodies completely. In the case of glycoprotein hormones for instance, antibodies for multiple hormone-specific epitopes can be obtained so that the use of antibodies to the common α-subunit in two-site assays is unnecessary (Gard et al, 1984; Soos et al, 1984).

Standardisation of monoclonal antibody two-site assays, and correlation with existing assays which employ polyclonal antibodies may also present problems if the analyte in standards or samples is potentially heterogeneous (Bangham, 1983). Two-site assays can be designed which are specific in their measurement of intact biologically active polypeptides and do not detect fragments, aggregates or otherwise modified antigen such as might give a response in other assays. In some circumstances this specificity may be an asset but a potential problem in others, depending on whether the heterogeneity is naturally occurring or introduced during processing and storage, and on whether total immunoreactivity or the concentration of specific forms of antigen provides the better diagnostic index. Therefore, it is most important that the results obtained using any new assay with monoclonal antibodies be thoroughly compared with those from assays in previous use. Where differences do arise, in overall quantitation or with particular samples, further investigation must decide which of two conflicting values is

more valid as a physiological measure or diagnostic criterion. It should never be assumed that a monoclonal antibody assay value is unreliable just because it is different, when actually it may be an earlier assay taken as reference which is prone to previously unrecognised problems.

7) Assay Optimisation

Two-site assay optimisation requires consideration mainly of achievable sensitivity and breadth of working range, along with speed and convenience. The factors affecting assay performance include concentration and affinity of antibodies, specific activity of labelled antibody, magnitude of non-specific binding, incubation times and technical reproducibility (Ekins, 1981; Hunter at al, 1982, 1983; Jackson et al, 1983). In practice the optimum conditions chosen are a compromise between the conflicting requirements of sensitivity, working range and speed. The practical value of precision profiles in the objective comparison of different assay protocols has been well documented (Hunter et al, 1982, 1983; Ekins, 1983).

In a two-site assay the detection limit is determined by the smallest amount of specific binding of labelled antibody which can be significantly resolved from non-specific (zero antigen) binding. This will depend on the absolute and relative magnitudes, and the reproducibility, of both specific and non-specific binding. In the limit when antigen

is saturated with labelled antibody, the signal from specific binding is a function only of antigen concentration and the specific activity of labelled antibody. In practice conditions approaching saturation can be achieved with antibodies of high affinity, indicating that the factor then limiting sensitivity is specific activity. This places a limit on the achievable sensitivity with radioactive labels within reasonable counting times whatever the relative level of non-specific binding. Only by the use of other methods of labelling can greater sensitivity be obtained, for instance with luminescent and fluorescent compounds, which offer a higher specific activity, in terms of measurable signal per mole of antibody.

Binding of antibody to antigen, in theory, may always be driven to completion regardless of affinity, by use of a sufficient excess of antibody. The non-specific binding, however, will also rise with increasing concentration of antibody, and if anything the ratio of specific to non-specific binding will decrease. Thus, although immunometric assays are theoretically reagent excess methods, in practice sensitivity may be greatest at limiting antibody concentration. Although low affinity antibodies added in excess can yield assays of reasonable sensitivity, these can never match the performance with antibodies of high affinity which produce a higher level of specific binding at a given antibody concentration.

The theoretical limit, at the upper end of the working range is dictated by the antibody concentration available, and the point at which antigen excess is reached. Thus, in

contrast to sensitivity, breadth of working range is favoured by high concentrations of antibody. The speed of reaction, similarly, may be increased, and the incubation time decreased, by use of high antibody concentrations.

In practice it is clear that the sensitivity of many assays is limited by the magnitude of 'non-specific binding' of labelled antibody, or the measured signal at zero antigen. This is a function of the background on detection systems as well as the true antigen-independent binding of labelled reagent to solid phase or incubation vessel. It is therefore an aspect of assay performance which merits attention at least as much as the concentration and affinity of antibodies to be used, and conditions should always be chosen to give the lowest possible non-specific binding.

III EVOLUTION OF ASSAYS FOR THE MEASUREMENT OF TSH

a) Historical Aspects

Estimation of TSH was originally performed using bioassay techniques. Subsequently, immunoassays and receptor assays have been employed. These assays can be classified as either structurally or functionally specific (Ekins, 1977).

Functional assays assess the effects of the hormone on a specific biological system as a measure of analyte activity. The original TSH bioassays were of this type and are,

historically, the first examples of quantitative TSH assays (Table 1.1). TSH bioassays can be further subdivided into two groups; those relying upon changes in thyroidal histology, and those employing radioisotopic methods to monitor thyroid responsivity. An example of the former is that of D'Angelo and Gordon (1950) in which the stimulatory effect of repeated intraperitoneal injection of test sera into starved tadpoles, on the height of thyroid acinar cells was determined. Histological methods, however, were gradually superseded by the technically simpler radioisotopic techniques. The best known example here is the McKenzie mouse bioassay (1960) in which mice fed a low iodine diet and primed with radioiodine are injected with the sample and the subsequent radioiodine released from the thyroid into the circulation expressed as a percentage increase over control values. Since 1960, the focus has been on the development of *in vitro* bioassay techniques since the use of thyroid slices, cells or plasma membranes, rather than whole animals, was expected to achieve greater sensitivity and reproducibility. Brown and Munro (1967) introduced the main *in vitro* assay competitor to the McKenzie *in vivo* assay. Here, thyroid lobes were removed from mice previously fed on a low iodine diet and injected with radioiodine *in vivo* to label intrathyroidal iodine stores prior to incubation *in vitro* with thyroid stimulating hormone. Radioiodine released *in vitro* was then employed to quantitate the degree of stimulation. The original sensitivity claimed was 100 mU/l but this was improved by Williams and Wolff (1971) to about 30 mU/l by decreasing assay incubation times. A detection

TABLE 1.1

HISTORICAL REVIEW OF TSH ASSAYS

Assay Principle	Reported Sensitivity	Converted to S.I.	Reference
(a) Functionally Specific Assays			
1. Cell Height Change in vivo	40 μU/ml	0.4 U/l	D'Angelo & Gordon (1950)
2. Release of radioiodinated iodothyronines in vivo	25 μU/0.5 ml	50 U/l	McKenzie (1960)
3. . . . . in vitro	0.050–0.100 mU/ml	0.1 U/l	Brown & Munro (1967)
4. . . . . in vitro	0.030 mU/ml	30 mU/l	Williams & Wolff (1971)
5. Radioiodine organification in vitro	0.007 mU/0.4 μl incubation medium/0.1 ml serum	70 mU/l	Kirkham (1962)
6. . . . . in vitro	0.01 mU/ml	10 mU/l	Desbarats-Schonbaum et al (1967)
7. . . . . in vitro	0.005 mU/ml	5 mU/l	Manley et al (1969)
8. Adenylcyclase stimulation in vitro	0.05–0.10 mU/ml	50–100 mU/l	Lissitsky et al (1973)
9. . . . . in vitro	0.010 mU/ml	10 mU/l	Kasagi et al (1982)
10. Lysosomal Dye Formation in vitro	10^{-11} U/ml	0.1 μU/l	Bitensky et al (1963)
(b) Structurally Specific Assays			
11. Radioreceptor Assay	0.125 mU/ml	125 mU/l	Manley et al (1974)
12.	0.010–0.015 mU/ml	10–15 mU/l	Smith & Hall (1974)
13.	0.128 nU/ml	0.128 μU/l	Mehdi & Nussey (1975)
14. Radioimmunoassay	2.5 μg/l	*~ 25 mU/l	Odell et al (1965)
15. " " " " " "	1.5 μg/l	~ 15 mU/l	Utiger (1965)
16. " " " " " "	0.5 μU/ml	0.5 mU/l	Hall et al (1971)

* one international unit = 13.5 mg of international standard

limit of 70 mU/l was achieved by Kirkham (1962) using isolated guinea pig thyroids and radioiodine organification *in vitro* following organification inhibition *in vivo*. Modifications of this technique gave sensitivities down to 5 mU/l (Table 1.1).

In the early 1970's another form of functionally specific assay came into use. This type of assay relied upon the specific interaction of the hormone with its receptor on isolated thyroid cell membranes and the consequent activation of adenyl cyclase. TSH activity being quantitated as cAMP production. A TSH assay with a sensitivity of 370 mU/l was produced by Lissitsky (1973) with porcine thyroid cells or membranes. Sensitivities approaching 10 mU/l have been achieved by Kasagi et al, (1982) with improved techniques based on cAMP production.

A TSH assay which returned to the measurement of the effect of TSH on stimulating colloid endocytosis was described by Bitensky et al (1963). Here, phagolysosomes which form on fusion of colloid droplets and lysosomes exhibit an increased permeability to the compound leucyl-beta-naphthylamide in the presence of TSH. In the lysosome, protease activity releases beta-naphthylamide which can then complex with copper-fast-blue reagent. The intensity of the blue colour detected by integrating scanning microdensitometry, is proportional to TSH added. This system proved to be very sensitive, giving minimum detection limits of 0.001 mU/l.

However, the majority of functionally specific assays are deficient in either sensitivity (McKenzie assay) or sample capacity (cytochemical assay).

Potentially larger sample capacity is offered by structurally specific assays where the number of molecules which have a unique structural component rather than a biological effect are measured. Unlike functionally specific assays, results can, therefore, be expressed in terms of mass or moles. Immunoassays belong to this category since they depend upon the specific reagent (antibody), recognising and interacting with a unique part of the analyte molecule. It has been suggested by Ekins (1977) that structurally specific assays are only valid if both the standard and unknown materials are structurally unique and identical. TSH preparations are, however, a heterogeneous mixture of large molecules and it is likely that different molecular forms (isohormones) and subunits of TSH exist in samples and standards. TSH standard preparations can exhibit different relative potencies in bio- and immuno-assay, reflecting their heterogeneous composition (and differences of assay specificity). To allow comparisons, TSH assays are standardised against an international reference preparation (IRP) currently WHO 80/558 and results are expressed as units of biological activity (mU/l) rather than mass (moles/l).

The two main types of structurally specific assays are based on the reaction of TSH with either a specific antibody in the case of immunoassay or thyroid membrane receptors in the case of radioreceptor assays. The latter depends on the reaction of labelled and sample TSH with the receptor and

following incubation, the quantitation of the bound or free fraction. Using labelled TSH pre-purified with a crude thyroid homogenate, Manley et al, (1974) obtained an assay sensitivity of 125 mU/l. This detection limit was improved to 0.128 mU/l by Mehdi and Nussey (1975) following the preparation of a highly purified receptor by detergent solubilisation of the cell membrane.

Immunoassay is the most widely used structurally specific assay. The earliest radioimmunoassay (RIA) for TSH was published by Odell et al (1965). They achieved an assay sensitivity of approximately 25 mU/l. Various technical refinements including improvements in antisera and tracers have given assays of greater sensitivity, Hall et al, (1971) achieving a sensitivity of 0.5 mU/l. Although TSH RIA's proved to be more reliable than the earlier bioassays, the mean assay coefficient of variation is of the order of 10% in the best systems and the prolonged incubation times required meant that few centres were able to obtain a result within 2-3 days of receiving the sample. In 1971 Addison introduced the concept of the two-site immunoradiometric assay (IRMA). This followed Miles and Hales (1968) pioneering work on the original single-site immunoradiometric assay and provided the potential for improved analytical performance.

b) Relative Merits of Competitive and Immunometric Assays for the Immunoassay of TSH

One of the major drawbacks associated with the use of competitive assays for the measurement of TSH has been their lack of sensitivity, typically of the order of 1 to 4 mU/l (Durham, 1985), resulting in a proportion of normal subjects having undetectable TSH levels. With such systems, generally, around 3 days were required to produce a result due to the prolonged incubation times necessary, and only a limited working range was possible (typically 5-15 mU/l TSH). At low TSH levels between laboratory - agreement was very poor yielding a C.V. of approximately 80% at a TSH level of 2 mU/l compared with a between laboratory C.V. of 15% at 20 mU/l TSH. This was because these methods were prone to non-specific matrix effects at low levels and also due to the fact that early reagents cross-reacted with HCG. Thus the investigation of suppressed, subnormal TSH levels was beyond most routine methods and TSH measurements were, therefore, applied to the detection and monitoring of primary hypothyroidism.

The introduction of the two-site immunoradiometric assay provided the potential for improved analytical performance. Among the advantages offered by such systems were that of greater speed, wider working range (typically 0.5-200 mU/l TSH), lack of serum effects and increased sensitivity. By replacing polyclonal antisera with specific monoclonal antibodies and by employing alternative non-isotopic labels, the sensitivity of TSH assays was further improved.

Detection limits of <0.05 mU/l have been quoted by manufacturers and the sensitivity is such that discrimination between euthyroid and hyperthyroid patients is now possible (Caldwell et al, 1985). Also of clinical significance is the fact that improvements in TSH methodology have reduced the need for TRH tests to clarify borderline results (Durham, 1985).

With these systems a rigorous definition of assay sensitivity became necessary which was applicable to individual batches of reagents such that deterioration and changes in reagents would not compromise assay performance. The assessment of assay sensitivity by precision profile analysis is described in the next section.

c) Immunoassay Design and Evaluation

The first published radioimmunoassay, for insulin, by Berson and Yalow in 1959 was followed by Ekins in 1960 with the presentation of a saturation assay for serum T_4. Since then both groups have differed in their approach to immunoassay design. Assay sensitivity was assessed by Berson and Yalow, in terms of the slope of the dose response curve (Berson et al, 1964). Ekins, (1977; 1976) on the other hand, pointed out that the slope of a dose response curve changes depending on the parameters (% B, B/F, F/B versus concentration plotted arithmetically or logarithmically). If the slope is assessed in the absence of statistical

considerations contradictory results relating to assay precision may be obtained. The statistical approach advocated by Ekins can be applied to both RIA's and IRMA's.

The basis of this technique is to obtain estimates of the error associated with each dose. The errors in the response (R) for a given dose (D) (Figure 1.5) are assumed to be normally distributed around a mean value and this variation can be defined algebraically (Equation 1). The slope and/or the error (R) changes with dose, implying that

$$\text{S.D. of dose estimation } (\Delta D) = \frac{\text{S.D. of response } (\Delta R)}{\text{slope of dose - response curve}} \quad (1)$$

$$= \frac{\Delta R}{dR/dD}$$

the observed ΔD will differ over the concentration range of interest. This changing relationship between ΔD and D is the precision profile and is a fundamental indicator of assay performance. It can be used not only for the direct comparison of assays for the same analyte, but also to assess the effects of minor protocol changes on assay performance. The precision profile is derived as follows. Replicate estimations of a given dose allow the standard deviation of the response (ΔR) to be obtained. A linear response-error-relationship (RER) is derived by plotting the response versus

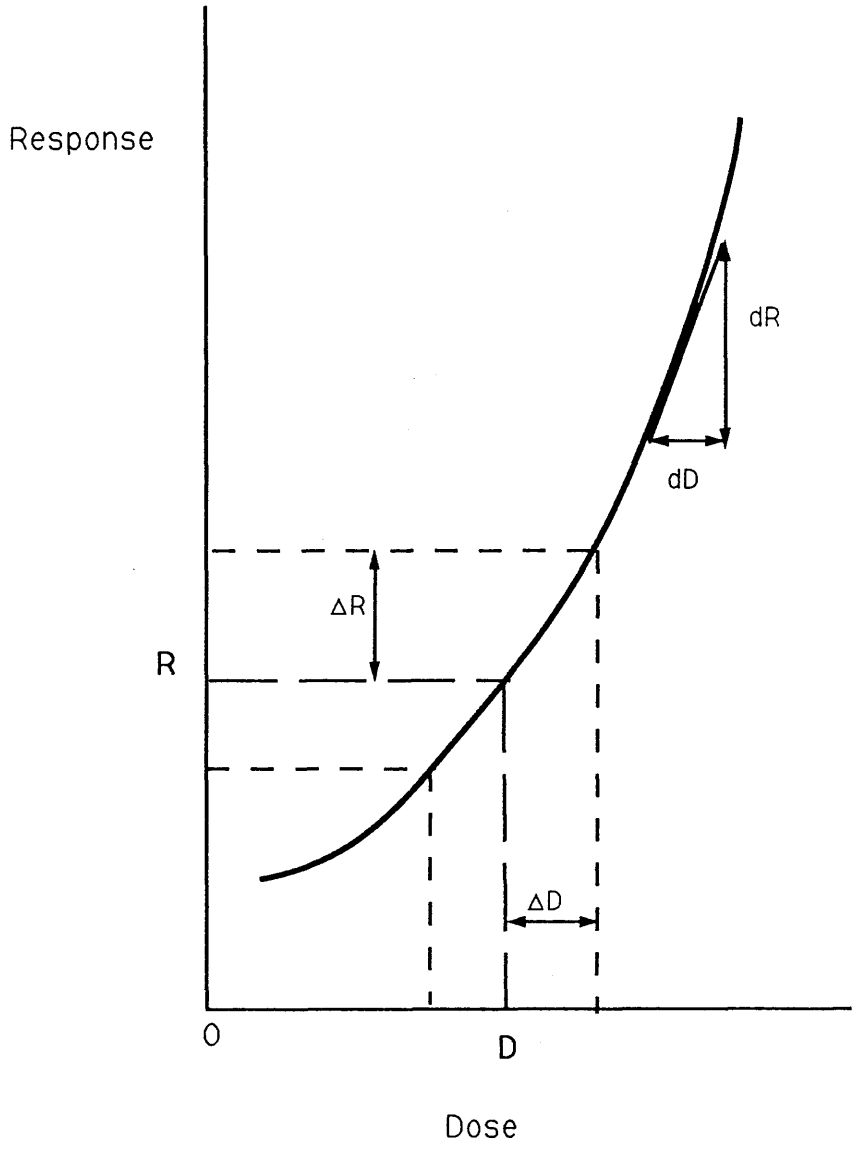


Figure 1.5

Diagrammatic representation of mathematical components used for calculation of assay errors: Dose (D); Response (R); Error in Dose (ΔD); Error in Response (ΔR); Gradient of Curve (dR/dD)

the obtained ΔR, pooled from a number of assays. Therefore, for a given dose, D, the response obtained, R (Figure 1.6a) will have an error in response ΔR derived from the RER (Figure 1.6b). By substituting ΔR into Equation 1 the absolute error in the measurement of dose D can be obtained. A plot of the relative error ($\Delta D/D \times 100\%$: coefficient of variation [C.V.]) obtained for a range of doses gives the heteroscedastic precision profile (Figure 1.6c).

d) The Performance Characteristics of Radioimmunoassay Versus Immunometric Assay for TSH

There have been substantial changes in the techniques for thyroid hormone measurement in recent years, most notably a move to non-isotopic and coated tube methods. It is for TSH that two-site immunometric assays (IMA) have had the most profound impact on laboratory practice and performance. The performance characteristics of radioimmunoassay versus immunometric assay for TSH have been studied by Mackenzie et al (1988). The main conclusions are recorded below.

The first immunometric assay for TSH was the Corning Immophase System which used purified polyclonal antisera, and although it was a good system at the time it was gradually superseded by the newer and ever more numerous two-site monoclonal kits. Between 1983 and 1987 there has been a move away from in-house to kit assays and, within kit assays, a move from RIA to IMA with the result that by the end of 1987 over 80% of laboratories were using IMA assays.

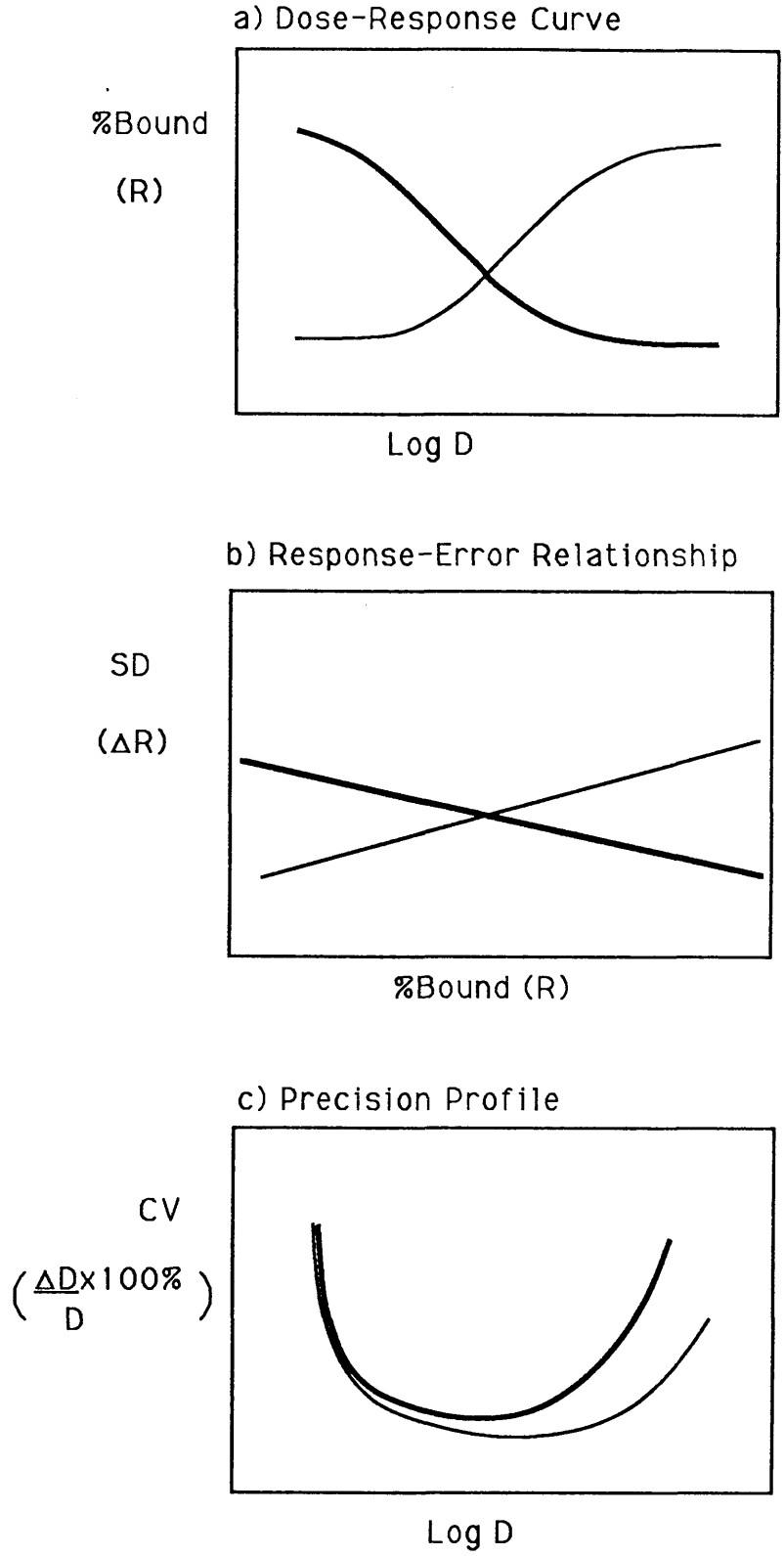


Figure 1.6

Diagrammatic representation of the major plots used for error analysis in saturation analysis (dark line) and excess reagent assays (light line)

Concurrent with the availability of these new kits, there has been an increase in the number of laboratories performing TSH assay. This reflects increased clinical applicability and changes in testing strategy. The "high sensitivity" TSH assays are claimed to be more sensitive, specific and robust than their RIA counterparts, with between-batch CVs typically around 5% at 1 mU/1.

When sera which contained low levels of TSH such as sera from T_3 - suppressed and thyrotoxic subjects were spiked with the 1st IRP, it was shown that there was almost quantitative recovery on all specimens with a concentration above 5 mU/1. At lower TSH levels there was apparent over-recovery due to loss of assay specificity and precision near the detection limit, and the target, the All-Laboratory Trimmed Mean (ALTM) was not reliable below 5 mU/1.

In 1982 the calculation and reporting of cumulative bias (BIAS) and variability of bias (VAR) for individual participants was introduced. Even at concentrations above 5 mU/1 less than 20% of laboratories could achieve a VAR below 10%. This reflected the imprecision of RIA procedures at that time.

After the improvement in 1983, more material with concentrations at the lower end of the range, was distributed by the UK EQAS, to allow laboratories to assess their performance critically. Initially the BIAS and especially the VAR worsened, but as a greater proportion of laboratories adopted IMA procedures overall performance improved.

In 1985 it was shown that the already sizeable group of IMA users obtained quantitative recovery at levels between

1.0 and 5.0 mU/l when T_3 - suppressed sera were spiked with the 2nd IRP.

It is interesting to note that the method groups with a positive bias also tend to over-recover added TSH (2nd IRP) while those with a negative bias under-recover. Since virtually all methods show good parallelism, standardisation procedures seem to account for these differences.

Occasionally users of a single kit method will, for a particular specimen pool, uniformly obtain results which are out of consensus with the other methods. This suggests the presence of interfering factors eg. human anti-mouse antibodies, which affect the monoclonal antibodies of that kit only. This problem is not seen in methods based on polyclonal antibodies.

At subnormal TSH levels, even highly sensitive assays can be affected by the matrix composition. Such an effect of matrix on IMA has been demonstrated by T_3 - suppressed, immunoabsorbed and thyrotoxic sera giving slightly different results with different systems (Swift et al, 1985).

The use of different detection systems, including ^{125}I, chemiluminescence, enzyme endpoint or dissociation-enhanced lanthanide fluorescent procedures, does not appear to be associated with any characteristic pattern of performance. However, there is a tendency for non-isotopic methods to under-recover added TSH. It is difficult to determine whether this is inherent in the detection system or the methodology, or simply reflects the standardisation procedures used. Similarly, some of the newer methodologies appear to be more precise than their isotopic counterparts,

but again this may simply be due to their being used in a dedicated system with little, if any, scope for procedural manipulation.

There has also been a move towards the use of IMA in screening for neonatal hypothyroidism. Major analytical problems, primarily of under-recovery and imprecision have been highlighted by the UK EQAS for Neonatal Blood Spot TSH. Although unsatisfactory, these do not appear to have been translated into serious errors in classification, ie. false negatives.

e) Non-Isotopic Immunoassay Methods for TSH

1) Introduction

At present, the only viable alternatives to a radioisotopic label are those using some form of optical end point. Of the many such methods available those employing enzymes or enzyme cofactors as labels are well established (O'Sullivan et al, 1979), whilst other families of assays rely on the quantitation of fluorescent (Smith et al, 1981) or chemiluminescent (Collins et al, 1983) emitted light.

The theoretical advantage of an optical immunoassay is that a very large signal can be obtained in a short period of time from a label that has a long shelf-life. However, optical immunoassays are very prone to 'blank' problems caused by non-specific factors in biological fluids which can scatter, quench or enhance light emission. As a result, the

signal:noise ratio of many optical immunoassays has been disappointingly low and has limited the potential sensitivity of the system.

2) Enzyme Immunoassay

The main potential advantage of using an enzyme as a label is the amplification of signal effected by the enzyme acting on several substrate molecules. Many enzyme immunoassay techniques have become established, of which 'ELISA' has proved to be useful in the measurement of TSH.

The 'ELISA' (enzyme-linked immunosorbent assay), in its most popular form, uses the principle of two-site immunometric analysis with one antibody coated to the wells of a microtitre plate. Antigen binds to this antibody. The second antibody, bearing the enzyme label, binds to a different epitope on the antigen to complete 'sandwich' formation. The unbound label is removed by aspiration and washing, the colour is developed by substrate addition and quantitated by spectrophotometric scanning of the plate. Several commercial systems of this type are available for TSH measurement including BCL 'ENZYMUN' and 'SOPHEIA' (Diagnostic Products Corporation), both employing solid phase Ab-coated tubes, as well as Hybritech 'TANDEM', a monoclonal IRMA with a 'bead' separation step.

A novel two-site immunometric assay, the Novoclone™ 'AELIA™' (amplified enzyme linked immunoassay, Novo Biolabs), has been described for serum TSH

based on the principle of enzyme amplification (Wheatley et al, 1987). One antibody is labelled with alkaline phosphatase which, at the end of the immunoassay, is used to catalyse a chain of reactions with signal amplification at each stage (Roddis et al, 1985). The sensitivity of the system looks better than that of a good IRMA. The Boots Celltech 'CHEMELIA' (chemical enzyme linked immunoassay) system is another assay adopting a modified enzyme approach. In this coated well assay no 'stop reagent' is used, leading to improvement in assay performance as it removes a step which is prone to error (Reid et al, 1988). The Serono 'SEROZYME' assay can also be placed in this group. In this system one antibody is labelled with alkaline phosphatase, the other with fluorescein isothiocynate (FITC). Separation is achieved by incubation with anti-FITC antibodies coated onto magnetic particles. After washing and incubation with the enzyme substrate, phenolphthalein monophosphate, the reaction is stopped by the addition of 'stop reagent'. Finally, the tubes are read at three wavelengths, 630 nm (to blank), 550 nm and 492 nm.

3) Fluorescence Immunoassay

Fluorimetry is the technique whereby incident light at one wavelength is absorbed by a fluorophore and emitted at a higher wavelength.

In this area of optical immunoassay, the most promising development is time-resolved fluorescence immunoassay as

illustrated by the Pharmacia 'DELFIA' (dissociation-enhanced lanthanide fluoroimmunoassay system). Two approaches have been used to reduce background fluorescence and so increase sensitivity. Firstly, europium chelates have been used as the fluorophore. These have a large Stokes shift (difference in wavelength between incident and emitted light) and a relatively long-lived fluorescence. Secondly, a fluorimeter has been designed which delays the measurement of the emitted light by 400 μs, during which time non-specific background fluorescence will largely have disappeared (Soini and Hemmila, 1979). This combined approach has allowed a two-site immunofluorometric assay for TSH to be developed. The DELFIA serum TSH assay has a signal:noise ratio in excess of 10^3 giving great sensitivity, at least as sensitive as the best IRMA, with a counting time of only 1 s per well.

4) Chemiluminescence Immunoassay

Chemiluminescence is the light emitted as a consequence of the de-excitation of a reactant or a product of a particular chemical reaction. The emission of photons from a chemical reaction is less affected by background interference than is the case for other optical techniques, and the efficient counting of the photons should permit sensitive immunoassays to be developed around chemiluminescent labels. One disadvantage of the technique is that the chemical reaction can only take place once - in other words, an assay cannot be recounted.

The original work in chemiluminescence immunoassay was based on the luminol family of labels but recently aryl acridinium esters have been preferred, and it has been possible to develop the most sensitive assays yet reported for peptide hormones by the use of these esters in two-site immunometric assays (Weeks et al, 1984). Ciba-Corning Diagnostics have adopted this technology in their TSH 'MAGICLITE' assay. The system also uses a solid phase separation method based on magnetic particles.

5) Enhanced Luminescence Immunoassay

A novel non-isotopic immunoassay system has been reported folowing the observation that the horseradish peroxidase catalysed chemiluminescence of a luminol derivative could be enhanced several fold by the addition of D-luciferin, a synthetic component of the firefly bioluminescence system (Whitehead et al, 1983).

Such a system, 'AMERLITE' has recently been launched by Amersham International for the measurement of TSH by two-site immunometric analysis. The assay offers a sensitivity of 0.04 mU/l with a working range of 0.09 to 200 mU/l (Squire and Gimlette, 1987).

IV THE CONTROL OF TSH SECRETION AND ITS ROLE IN NORMAL THYROID PHYSIOLOGY

a) Hypothalamic Control of TSH Secretion

While the dominant hypothalamic control over TSH is stimulatory via thyrotrophin releasing hormone (TRH), thyroid hormones exert a powerful, dose-related negative feedback control over TSH synthesis and release, acting at pituitary and possibly hypothalamic levels (Snyder and Utiger, 1972). As small increases in serum triiodothyronine (T_3) and thyroxine (T_4) levels reduce basal and TRH-stimulated levels, small decreases in T_3 and T_4 levels induced by short-term administration of pharmacological doses of iodide, lead to elevation in basal and TRH-stimulated TSH levels (Saberi and Utiger, 1975).

Recent evidence indicates, however, that the central neurotransmitter, dopamine (DA), has a physiological inhibitory role in the control of TSH secretion in man (Scanlon et al, 1980a); there is also evidence of a similar role for somatostatin (growth hormone release-inhibiting hormone, GHRIH) (Peters et al, 1983). Thus, hypothalamic control over TSH synthesis and release in man is more complex than previously envisaged and has both stimulatory and inhibitory components. Oestrogens and glucocorticoids may each have a role in the modulation of hypothalamic-pituitary-thyroid function and must be considered in the overall picture of TSH regulation (Figure 1.7).

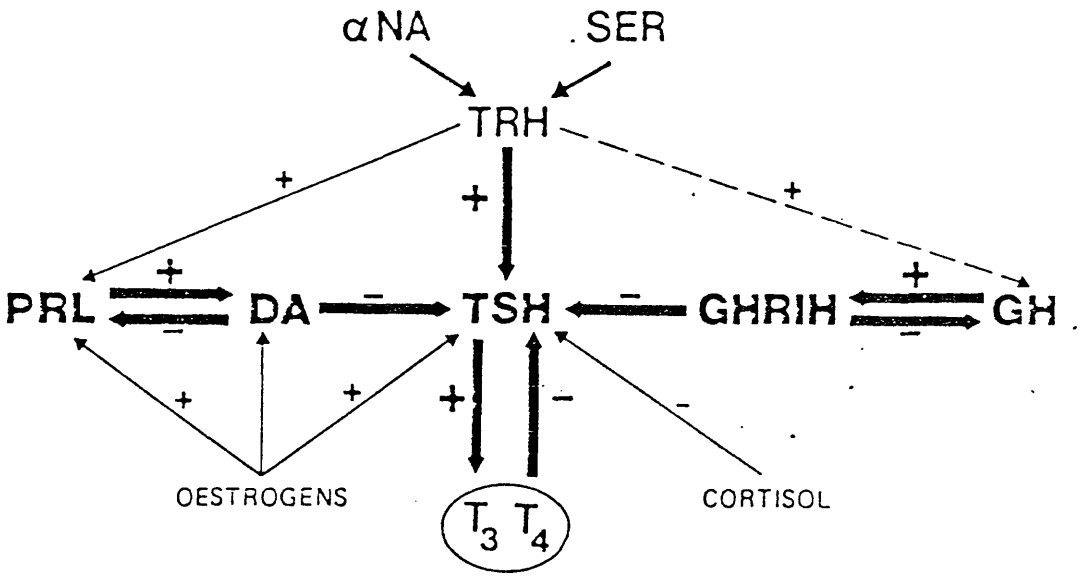


Figure 1.7

Schematic outline of some of the major pathways and interactions which control TSH secretion

b) Circadian Variation in Normal Subjects

There is a clear and well documented circadian variation in basal TSH levels in normal subjects (Weeke, 1973; Weeke and Laurberg, 1976; Chan et al, 1978). TSH levels rise during the evening before the onset of sleep reaching a zenith at about 2300-2400 hours. Thereafter levels decline slowly to a nadir around 1100 hours. The mechanisms underlying this circadian TSH change are unknown. Certainly the rise in TSH levels during the evening before the onset of sleep is not due to a decline in dopaminergic inhibition of TSH release. Recent studies have demonstrated that the dopaminergic inhibition of TSH release is greater at night than during the day (Scanlon et al, 1980b) and DA may be acting to reduce the degree of nocturnal elevation in TSH levels.

c) Neural Regulation of Thyroid Hormone Secretion

During the last few years, evidence has accumulated showing that the thyroid gland is richly innervated not only by adrenergic and cholinergic nerves, but also by peptidergic nerves. The physiological role of these nerves is, however, not clear, but it is evident that TSH is not the only regulator of thyroid hormone secretion (Ahrén, 1986).

As of now, these neural influences are as follows: 1) Adrenergic nerves stimulate basal thyroid hormone secretion

by both α - and β - adrenoceptor mechanisms; 2) Adrenergic nerves inhibit TSH - induced thyroid hormone secretion mediated by norepinephrine and possibly neuropeptide Y (NPY); 3) Cholinergic nerves impair TSH - induced thyroid hormone secretion; and 4) Vasoactive intestinal peptidergic nerves stimulate basal and potentiate TSH-induced thyroid hormone secretion. The exact role of these nerves in thyroid physiology remains to be elucidated.

d) Actions of TSH on the Thyroid

TSH influences many aspects of thyroid structure and function: the size and vascularity of the gland, the height and activity of the follicular epithelium, and the amount of colloid are all controlled by TSH. Every step of the thyroid hormone biosynthetic pathway (Figure 1.8) is stimulated by TSH, as are numerous aspects of cell metabolism - eg. glucose utilisation, oxygen consumption, phospholipid synthesis, and RNA synthesis. These actions begin within a few minutes of administration of TSH and have been attributed to activation of adenyl cyclase after combination with a receptor site on the cell surface. The resulting formation of cyclic AMP leads, via an effect on messenger RNA, to synthesis of proteins concerned in the individual steps of thyroid hormone synthesis - ie. the iodide trap, iodine incorporation into thyroglobulin and thyroid-hormone release into the circulation.

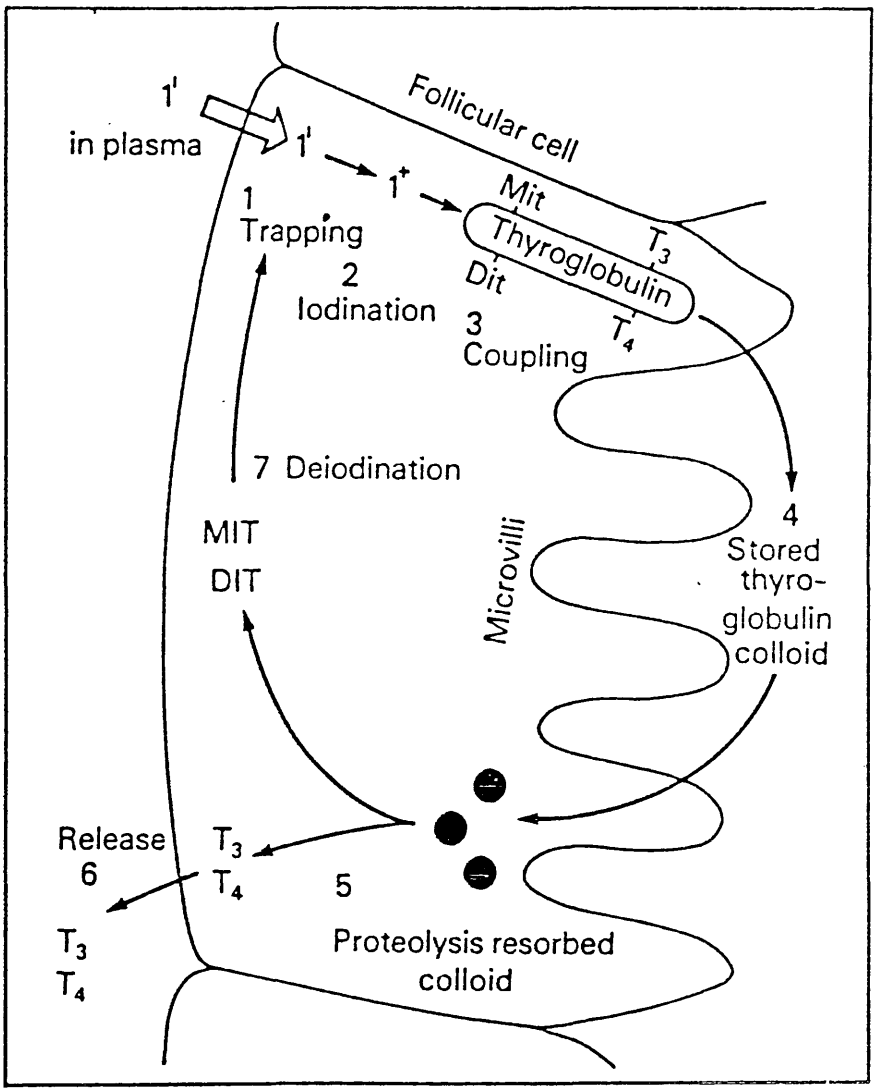


Figure 1.8

Synthesis, storage and secretion of thyroid hormones

V ADAPTATION OF TSH ASSAYS TO SCREENING FOR CONGENITAL HYPOTHYROIDISM

a) Congenital Hypothyroidism

Congenital hypothyroidism (CH) is a term coined to describe a variety of conditions associated with an underactive thyroid gland at the time of birth. If untreated within a few weeks the baby will become mentally deficient (cretinism). The incidence of CH in the UK is now established as 1:3500 births (John, 1987). For some years now screening of all neonates has been advocated to identify this readily treatable condition, although there have been differences between centres in the nature of the specimen used and the hormone measured (See Chapter 5).

b) Specimen Collection

The earliest specimen that can be collected from a neonate is a sample of cord blood and this specimen has been used by some groups for screening for CH (Walfish, 1983). However, the collection of cord blood is not a routine practice and it is, therefore, susceptible to problems of logistics. Furthermore, thyroid physiology changes markedly within the first few hours of birth such that reference ranges for the thyroid related hormones tend to be very wide,

leading to a greater chance of overlap between normal neonates and those affected with CH in the perinatal period (John, 1987). Consequently, the use of cord blood is less favoured than the use of heel prick blood collected onto filter paper cards. As such specimens were already being collected from virtually all neonates in the UK and in the USA for the phenylketonuria screening programme it has proved logistically very simple to use a further aliquot of this specimen for screening for CH. In the USA and in Europe heel prick blood samples are collected between 3 and 7 days after birth, in the UK the specimens are collected between 6 and 10 days, either in hospital or by a visiting midwife. Results of screening programmes are available within 14-30 days of birth - in adequate time to minimise the risk of severe mental retardation. The collection of heel prick blood specimens in the UK is now so well organised that >95% of babies have a suitable sample submitted for screening.

c) Screening Assay

In the first screening programmes the assay of T_4 in filter paper blood spots was adopted and its ability to detect hypothyroid infants was confirmed. However, it was soon realised that using T_4 alone was not sufficient as many of the affected cases, particularly with ectopic thyroid tissue, had normal T_4 values and around 30% of hypothyroid infants could be missed. Conversely, normal pre-term infants were found to have levels of T_4 similar to levels of T_4 in

hypothyroid infants. As well as prematurity, low birth weight, thyroxine binding globulin deficiency and congenital or acute illnesses were all causes of a low concentration of T_4. Recall rates of between 1.5 and 5% have been reported in those programmes using T_4 alone as the screening assay (John, 1987). By taking a second blood spot from the original PKU card and analysing for TSH, this recall rate can be reduced. The number of samples which had to be assayed for TSH varied in the different programmes but was between 3 and 20%. This approach had the advantage of using the cheaper and simpler T_4 assay for all the samples whilst reserving a TSH assay for fewer specimens. Ten years ago, TSH reagents for large screening programmes were difficult to prepare, good antisera were scarce and the methods generally took 2-5 days to produce results. However, this did not deter some European centres from setting up screening programmes utilising a TSH assay as the primary test (Illig et al, 1977). This appears to be the most sensible choice as TSH is the most sensitive indicator of primary hypothyroidism in adults and would probably be so in infants. In cases of infants with ectopic thyroid glands in whom thyroid function may still be compensated, T_4 levels may be normal but TSH is generally raised. In Europe, using TSH alone, the recall rate varied between 0.03 and 0.9%.

In the UK, all screening laboratories rely on TSH as the screening assay, except East Anglia where screening is based on a total T_4 with a supplemental TSH assay on those specimens within the lowest 20% of T_4 values. In those screening centres more experienced in immunoassay, in-house

(generally immunoradiometric) assays were set up (Sutherland et al, 1982; John and Woodhead, 1982), others developed radioimmunoassays (Edwards et al, 1980; Moore and McMillan, 1983; Griffiths et al, 1985) but most used commerical kits. Two-site assays were preferable as screening assays because results could be available within 24 h of receipt of a sample. Other advantages were high sensitivity, ease of automation and, particularly in dealing with large sample numbers, their robust methodology. The Japanese have been very successful at introducing enzyme immunoassays (Naruse and Irie, 1983) for mass screening and future developments should see the gradual replacement of two-site TSH assays based on radioiodine labels with alternative non-isotopic labels.

In routine use the between batch imprecision of the TSH assay for filter paper blood spots is rarely better than 20% but since there is virtually no overlap in TSH values between hypothyroid and normal infants this level of imprecision is adequate. It follows, therefore, that it is not necessary to assay samples in duplicate and many centres use only a single blood spot sample without detriment to their screening programme.

Secondary hypothyroidism is a rare condition which occurs only in one in 100,000 births. Most infants have a deficiency of multiple pituitary hormones, ie. have panhypopituitarism, and will invariably present with other clinical symptoms such as hypoglycaemia, micropenis in the male or central nervous system abnormalities, cranial vault or facial structures. Only in those screening programmes which utilise

T_4 as the initial screening test will secondary cases be detected but, screening programmes are now directed to identifying cases of primary hypothyroidism because of the very much higher incidence.

VI AIMS OF THE WORK DESCRIBED IN THIS THESIS

1. To develop techniques for the production of mouse monoclonal antibodies to human TSH.

2. To establish methods for the characterisation of monoclonal antibodies to human TSH such that selection may be made of those reagents suitable for use in immunometric assays.

3. To develop a variety of two-site immunometric assays for human TSH based on the monoclonal antibodies produced and/or available polyclonal antisera.

4. To optimise one or more two-site immunometric assays for human TSH and to validate their use for the measurement of TSH in serum and in neonatal blood spots.

5. To assess the value of two-site immunometric assays for human TSH in a variety of clinically based situations.

CHAPTER 2

MATERIALS AND METHODS

I PRODUCTION OF MONOCLONAL ANTIBODIES

a) IMMUNISATION

Human TSH (Calbiochem, Iodination grade, potency 5.5 IU/mg), 20 ug in 0.2 ml saline was emulsified (by sonication in an ice bath) with 0.6 ml Freund's complete adjuvant (FCA). Six balb/c mice were given primary immunisations by intraperitoneal injection (26 g ½" hypodermic needle) of 2.5 ug TSH in 0.1 ml FCA followed by a minimum of two "booster" immunisations of identical dose and mode of injection in Freund's incomplete adjuvant (FIA). Time intervals of three weeks were allowed between injections.

Three days prior to fusion a donor mouse was injected intravenously (via the tail vein) with 2.5 ug TSH in 0.1 ml sterile physiological saline.

b) SERUM ANTIBODY EVALUATION

1) BLOOD SAMPLING

Mice were anaesthetised with ether, the ventral tail vein punctured with a sterile 23 g needle and the exuding blood absorbed onto filter paper card to form a discrete spot. All the experimental mice were colour coded to aid identification.

2) BLOOD SPOT ELUTION

The blood spot was allowed to dry and a 6 mm disc punched into a test tube containing 1.0 ml 0.9% NaCl. This approximated to an initial dilution of 1:500, ie. 0.002 ml serum/6 mm disc.

3) ANTIBODY DILUTION CURVES

(i) REAGENTS

(1) Assay diluent:- 0.05 mol/l sodium phosphate buffer containing 0.154 mol/l NaCl, 0.1 % (w/v) BSA and 0.05% (w/v) NaN_3 at pH 7.4.

(2) Iodinated TSH antigen:- stock labelled ^{125}I-TSH was diluted to give 30,000 cpm/0.1 ml (See Section IIa).

(3) Sheep anti-mouse γ globulin immuno-adsorbent:- the stock solution (20 mg/ml) was diluted 1:4 with assay buffer to give a working solution of 1 mg/0.2 ml (See Section IIIc).

(ii) ASSAY

12 mm diameter tubes, Sarstedt 55.484, were used throughout.

Test sera were analysed in duplicate at final dilutions of 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000 as follows. Total count tubes were also prepared.

(1) 0.1 ml test serum at appropriate dilution.

(2) 0.1 ml ^{125}I-TSH (approx. 30,000 cpm).

The tubes were vortexed and incubated overnight at ambient temperature. Sheep anti-mouse γ globulin (1 mg/0.2 ml) was added and the tubes incubated on an orbital shaker at 300 rpm for 60 minutes also at ambient temperature. The incubation tubes were given

three wash, centrifugation, aspiration cycles with 2 ml 0.9% NaCl containing 0.2% Tween 20 and the immunoadsorbent pellets (bound fractions) counted on a gamma counter (Nuclear Enterprises 1600) set on the normalised mode. Binding expressed as percentage of total counts was then plotted against dilution (log scale) for each animal.

4) DISPLACEMENT STUDIES

To give an indication of antibody avidity, displacement was assessed using a modification of the assay protocol outlined above. Two sets of diluted test sera were set up, one with 0.05 ml unlabelled, purified TSH (code no 80/558, dose 25 mU/l), the other with 0.05 ml assay diluent.

The mouse yielding antibody of highest titre and greatest displacement (highest avidity by definition) was selected for hybridisation.

c) PREPARATION OF CELL CULTURE MEDIA

All cell culture media components were purchased from Flow Laboratories (UK) and combined under aseptic conditions as required.

1) MYELOMA CELL MEDIUM

100 ml RPMI - 1640 (Roswell Park Memorial Institute) buffered with 20 mmol/1 HEPES (N-2-Hydroxyethylpiperazine - N' - 2-ethanesulfonic acid).

10 ml heat inactivated foetal bovine serum (approx. 10%).

1 ml 200 mmol/1 - Glutamine (2mM).

2 ml Penicillin/Streptomycin (100 u/ml, 100 ug/ml respectively).

1 ml Fungizone (250 ug/ml) (2.5 ug/ml).

2) HYBRIDISATION MEDIUM (2 X HAT MEDIUM)

100 ml RPMI - 1640 buffered with 20 mmol/1 HEPES.

20 ml foetal bovine serum (approx. 20%).

1 ml 200 mmol/1 - Glutamine (2mM).

2 ml Penicillin/Streptomycin (100 u/ml, 100 ug/ml respectively).

1 ml Fungizone (2.5 ug/ml).

4 ml (50 x concentrate) HAT (Hypoxanthine; Aminopterin; Thymidine).

d) PREPARATION AND MAINTENANCE OF MOUSE MYELOMA CELL LINE FROM FROZEN STOCK

1) PREPARATION

The mouse myeloma cell line X63. Ag8 - 653 (Flow Laboratories) was used in all hybridisations. Aliquots of cells (10 x 10^6 cells/ml) were stored in liquid nitrogen (-196°C). An aliquot was removed, as required, from storage, thawed rapidly by immersion in a 37°C water bath and transferred aseptically to a 20 ml sterile universal bottle. Prewarmed myeloma cell medium (5 ml) was added dropwise slowly with gentle agitation. The universal bottle was then centrifuged (5 min, 800 rpm) and the supernatant discarded. The washing procedure was repeated and then a further 5 ml of medium was added to the washed pellet and the cells counted by haemocytometer. The concentration of cells was then adjusted to approximately 200,000 cells/ml with further medium and transferred to 25 ml flasks, stoppered and incubated at 37°C.

2) MAINTENANCE

The myeloma cells were maintained in logarithmic growth (minimum density 2.5 x 10^5 cells/ml, maximum density 1.5 x 10^6 cells/ml, doubling time approximately 16-24 hours) gradually expanding

the volume before transference to a continuous spinner flask culture (Techne) and further expansion prior to fusion. Cells were counted and viability assessed daily by haemocytometer and trypan blue dye exclusion (Flow Laboratories). When sufficient cells (150 x 10^6) of high viability (>90%) were obtained hybridisation was performed.

e) HYBRIDISATION

1) MYELOMA CELLS

In general 3 fusions were performed simultaneously. The required volume of medium containing 150 x 10^6 myeloma cells was centrifuged at 800 rpm for 5 minutes and the supernatant discarded. The cell pellet was washed once in RPMI, resuspended in 15 ml RPMI and split into 3 x 5 ml aliquots of 50 x 10^6 myeloma cells in sterile universal bottles.

2) SPLEEN LYMPHOCYTES

The spleen from an immunised mouse preselected for the presence of high avidity antibody was removed under sterile conditions, placed in a petri dish (5.5 cm diameter) containing 10 ml RPMI and carefully teased with scalpel blades to yield a cell suspension. The contents of the dish were

then transferred to a sterile universal bottle and the large fragments of tissue allowed to settle. The supernatant cells were removed, centrifuged at 800 rpm for 10 minutes and resuspended in 15 ml RPMI. After lysing the red cells, spleen cells were counted and adjusted to approximately 150 x 10^6 cells. Three aliquots of spleen cells (5 ml), each of 50 x 10^6 cells, were prepared so that each hybridisation would have identical numbers of myeloma and spleen cells.

3) POLYETHYLENE GLYCOL 1500 (PEG)

Polyethylene glycol 1500 (BDH 29575, 500 g fused solid, melting point 46°C) (10 g) was weighed into a 20 ml measuring cylinder and 5-6 ml RPMI - 1640 added. After heating in a 50°C water bath until all the PEG dissolved, the pH was adjusted to pH 7 with 0.1 mol/l NaOH. Additional warmed RPMI - 1640 was added to a total volume of 20 ml (ie. 50% solution). The PEG solution was then sterilised either by membrane filtration (0.22 u) into a sterile universal bottle or by preparing 5 ml aliquots in bijoux vials and autoclaving for 15 minutes (120°C).

4) PREPARATION OF PERITONEAL MACROPHAGES (FEEDER CELLS)

RPMI - 1640 (3 x 5 ml) was prepared in syringes with 19 g needles. Three balb/c mice were killed and dissected to reveal the abdominal skin and 5 ml RPMI injected by entering above the sternum and allowing the needle to lie over the right lobe of the liver. The abdomen was massaged to suspend peritoneal cells and RPMI withdrawn into the syringe very slowly. The three peritoneal washings were pooled into a 20 ml sterile universal bottle, centrifuged for 5 minutes at 800 rpm, resuspended and counted. The cells were then diluted in hybridisation medium to a density of 30,000 cells/ml and distributed to wells and flasks as appropriate.

5) HYBRIDISATION PROTOCOL

The 5 ml aliquots of spleen and myeloma cells were mixed and centrifuged for 10 minutes at 800 rpm. The supernatant was decanted and the universal bottle containing the cell pellet placed in a beaker of water at 37°C. PEG (0.8 ml) was added dropwise over 1 minute and after a further minute 10 ml warm RPMI was added over 5 minutes at a gradually increasing rate of addition. The mixture

of cells was centrifuged for 10 minutes at 800 rpm, washed in RPMI and resuspended in 60 ml prewarmed hybridisation medium (2 x HAT medium).

f) HAT SELECTION

The hybridisation products were plated out, by Transplate 96 (COSTAR), into 5 x 96 well microtitre plates (0.2 ml/well), pre-prepared 24 hours earlier with peritoneal macrophage feeder cells in 2 x HAT medium. All plates were sealed with sellotape and incubated at 37°C for 10-14 days before examination for the presence of growing hybrids and subsequent screening for antibody.

g) SCREENING FOR TSH ANTIBODY

All wells with growing hybrids were screened for antibody by radioimmunoassay using a solid-phase second antibody separation technique.

Approximately 0.2 ml of cell supernatant was removed aseptically from each well of each fusion plate and fresh medium replaced. All plates were resealed with sellotape and incubated at 37°C.

1) REAGENTS

(i) Assay diluent:- 0.05 mol/l sodium phosphate buffer containing 0.154 mol/l NaCl, 0.1% (w/v) BSA and 0.05% (w/v) NaN_3 at pH 7.4

(ii) Iodinated TSH antigen:- stock labelled ^{125}I-TSH was diluted to give 30,000 cpm/0.1 ml.

(iii) Sheep anti-mouse γ globulin immuno-adsorbent:- the stock solution (20 mg/ml) was diluted 1:4 with assay buffer to give a working solution of 1 mg/0.2 ml.

2) ASSAY:

12 mm diameter tubes, Sarstedt 55.484, were used throughout. Total count tubes and assay test samples were prepared in singleton owing to the low volumes of supernatant available.

(i) 0.05 ml cell supernatant.
(ii) 0.1 ml ^{125}I-TSH (approx. 30,000 cpm).

The tubes were vortexed and incubated overnight at ambient temperature. Sheep anti-mouse γ globulin (1 mg/0.2 ml) was added and the tubes incubated on an orbital shaker at 300 rpm for 60 minutes also at ambient temperature. The incubation tubes were

given three wash, centrifugation, aspiration cycles with 2 ml 0.9% NaCl containing 0.2% Tween 20 (v/v) and the immunoadsorbent pellets (bound fractions) counted on a gamma counter (Nuclear Enterprises 1600) set on the normalised mode. Binding expressed as percentage of total counts was then calculated for each well.

In general, depending on the number of positive wells obtained, cell supernatants yielding binding greater than 10% were subjected to a second line test to assess antibody avidity by displacement analysis. Here, cell culture supernatants were diluted 1:2, 1:4, 1:8 etc and analysed in the presence or absence of a displacement dose of standard TSH (code no 80/558, dose 100 mU/l [0.05 ml/tube]). The displacement was calculated (Bo-Bd/Bo x 100% where Bo = binding in the absence of TSH and Bd = binding in the presence of TSH) at each dilution with those wells giving the greatest displacement (highest avidity by definition) selected for cloning.

3) SPECIFICITY STUDIES

To determine antibody specificity, cell culture supernatants were diluted 1:2, 1:4, 1:8 etc and

analysed in the presence or absence of Human Chorionic Gonadotropin (HCG) (dose 25,000 u/l, 0.05 ml/tube).

h) CLONING

Cell lines were cloned by limiting dilution. An initial dilution was made in hybridisation medium to obtain approximately 800 cells/ml. Doubling dilutions were then performed in a 24 x 2 ml well plate to give 8 dilutions in total. Ninety-six well microtitre plates containing 0.1 ml macrophages (approx. 3,000 cells) were used for cloning (1 plate/cell line). 0.05 ml of each dilution was pipetted into a row of 12 wells, the plates sealed with sellotape and incubated at 37°C. Approximately ten days later plates were examined for cell growth and screened for antibody (screening as described in Section Ig). Positive clones grown from few cells were selected for further cloning, each cell line being subjected to at least 2 cloning stages before expansion and ascitic fluid production.

i) EXPANSION OF SELECTED CLONES

Selected clones were transferred from clone plates to 2 ml wells and allowed to expand before seeding in 25 ml and 100 ml flasks. Expansion was continued in further

flasks until sufficient cells were obtained for cell freezing (to conserve the cells line) and ascites production in balb/c mice.

(1) PRISTANE PRIMING OF BALB/C MICE

Pristane (tetramethyl pentadecane, Sigma T7640, 0.5 ml per mouse) was injected intra-peritoneally at least 1 week before the injection of hybrids to form ascitic fluid (single injections of pristane create a favourable environment for the growth of hybridomas).

(2) PREPARATION OF HYBRIDS FOR INJECTION

Sufficient cells for the injection of 10 mice at 10×10^6 cells/mouse were centrifuged, the supernatant removed and the pellet washed once in RPMI. The hybrids were resuspended in a further 5 ml RPMI and injected intra-peritoneally at 0.5 ml/mouse.

(3) PRODUCTION OF ASCITIC FLUID

At time intervals of 10-14 days after innoculation the abdomen of each mouse was punctured with a 19G needle and the ascitic fluid (approximately 5 ml) allowed to drain

into a universal bottle. The ascitic fluid was clarified by centrifugation and stored at -20°C until required. In general this procedure was repeated every 2-3 days throughout the remaining life-span of the animals.

j) DETERMINATION OF CLASS AND SUBCLASS SPECIFICITY OF ANTIBODIES BY THE OUCHTERLONY IMMUNODIFFUSION TECHNIQUE

Mouse monoclonal typing kits (Serotec) were used for antibody class and subclass determination.

1) PRINCIPLE

The concentrations of mouse monoclonal immunoglobulins in growing hybridoma culture supernatants vary between 1-20 mg/l when antibody producing clones are established. SEROTEC sheep anti-mouse immunoglobulins (G, A, M and light chains) sera detect immunoglobulin production but not the individual immunoglobulin specificity.

The test comprises diffusing the six antisera (directed against the mouse classes and subclasses IgG1, IgG2a, IgG2B, IgG3, IgA and IgM) against the neat culture supernatants in the immunodiffusion plates.

2) PREPARATION

The plate was removed from the container and the lid opened for 5-10 minutes at room temperature so that any condensation water could evaporate from the gel surface or the wells.

3) TEST PROCEDURE

Approximately 0.075 ml of the test culture supernatant was pipetted into the large central well of one of the rosettes. 0.01 ml of each of the six antisera, IgG1, IgG2a, IgG2b, IgG3, IgA and IgM were then pipetted, in a documented fashion, into each of the outer 6 wells of the same rosette. The plate was then tightly closed and stored flat at room temperature, for 24-48 hours. After this time the immunoprecipitate relevant to the immunoglobulin specificity was visible. For concentrations of monoclonal immunoglobulin below 1 mg/l more culture supernatant was pipetted into the central well and left for a further 24 hours. For concentrations greater than 20 mg/l, samples were diluted into the test range since protein concentrations greater than the indicated ranges may have prevented the development of precipitates due to antigen excess.

k) PURIFICATION PREPARATION OF IgG FROM MOUSE ASCITIC FLUID

In general ascitic fluid of the same tap number ($\geqslant 3$) were pooled to a volume of 20 ml and adjusted to pH 5 with 0.1 mol/l acetic acid. N-octanoic acid, BDH (1.76 ml/20 ml ascitic fluid) was added dropwise with stirring. After stirring for 30 minutes the material was centrifuged at 1500 g for 20 minutes and the supernatant (IgG) retained. The pellet was washed with 20 ml 0.1 mol/l sodium bicarbonate pH 8, the centrifugation repeated and the supernatants pooled. The IgG was thoroughly dialysed against 0.01 mol/l $NaHCO_3$ pH 8, concentrated by ultrafiltration (Amicon Corporation) and the protein concentration measured (Schachterle, 1973). The material was stored at -20°C until required.

(I) CELL FREEZING

Aliquots (5 ml) of Dimethyl Sulphoxide (DMSO), Sigma D5879 (1 ml = 1.10 g) in glass universal bottles were autoclaved (120°C for 15 minutes) and stored at room temperature. As required a 10% solution of DMSO in ice cold foetal bovine serum was prepared for the cryo-preservation of cells. Cells for freezing were centrifuged, the medium discarded and the pellet washed

once in RPMI. The freezing mixture was added dropwise to the cell pellet with continuous shaking and the cells aliquoted into labelled freezing vials at a density of 10 x 10^6 cells/ml. The vials were sealed in a 1 cm thick polystyrene box and allowed to freeze overnight at -70°C after which they were stored in liquid nitrogen (-196°C) until required.

II) IODINATIONS

a) PREPARATION OF LABELLED ^{125}I-TSH

TSH was iodinated by an enzymatic (lactoperoxidase) solid-phase method, (Karonen et al, 1975).

1) PROCEDURE

Sodium phosphate buffer pH 7.4 (0.5 mol/l, 0.02 ml) was added to 2 ug (0.01 ml) TSH (SFA [3-5] ASF), courtesy Professor W Butt, Birmingham and Midland Hospital for Women, followed by 0.005 ml ^{125}I-Na (0.5 mCi, carrier-free, Amersham IMS 30) and 0.01 ml locally prepared solid-phase lactoperoxidase (0.01 ml stock diluted in 1 ml 0.05 mol/l sodium phosphate buffer pH 7.4, 200 ng). The reaction was started by the addition of 0.01 ml hydrogen peroxide (H_2O_2, approx. 30% w/v, BDH, Analar, 0.01 ml in 250 ml distilled water, 120 ng) and allowed

to proceed for 30 minutes, vortexing every 5 minutes. The reaction was quenched by the addition of 0.02 ml TSH assay diluent (0.05 mol/l sodium phosphate buffer pH 7.4 containing 0.9% [w/v] NaCl, 0.1% [w/v] BSA and 0.05% [w/v] NaN_3).

2) PURIFICATION OF ^{125}I-TSH

The reaction mixture was applied to a 74 x 1 cm column of Ultrogel AcA 54 (LKB 2204-540 UK) stored in 0.05 mol/l sodium phosphate buffer pH 7.4 and primed prior to use with TSH assay diluent. Fractions (100 x 1 ml) were collected overnight on a FRAC-300 fraction collector with P-1 peristaltic pump (Pharmacia) and counted for 10 seconds on a gamma radioisotope counter (Wilj). A profile of counts against fraction number was plotted and the monomer TSH peak identified (see Figure 2.1). Approximately 8 fractions containing monomer ^{125}I-TSH were pooled and a 0.01 ml sample counted for 1 minute on a Nuclear Enterprises 1600 gamma counter to calculate the dilution which would be required to give 30,000 cpm/0.1 ml. Aliquots (0.5 ml) were prepared and stored at -20°C until required (shelf-life was found to be at least 4 weeks).

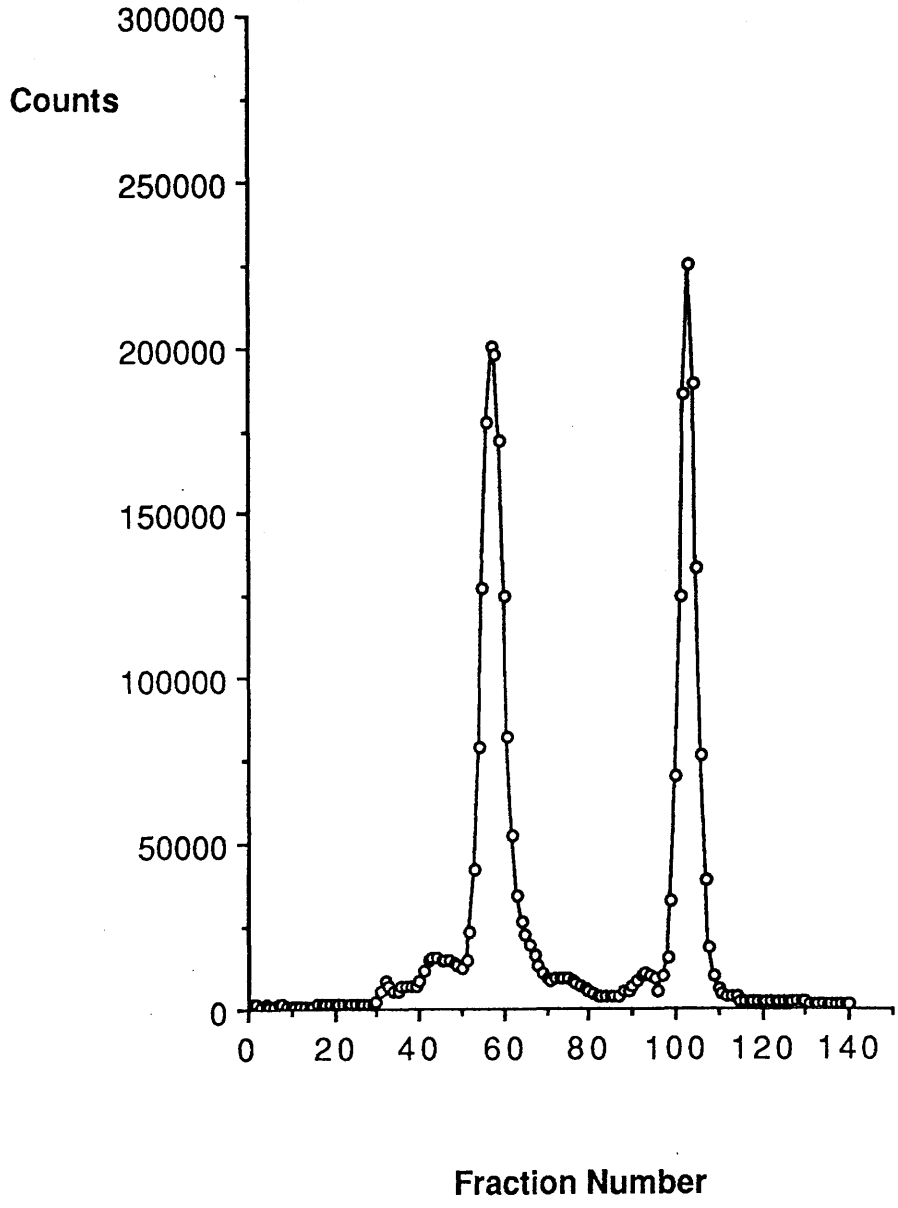


Figure 2.1

Ultrogel AcA 54 chromatography of iodinated TSH

b) PREPARATION OF LABELLED ^{125}I-TSH POLYCLONAL ANTIBODY

1) PREPARATION OF SOLID-PHASE COUPLED TSH FOR IMMUNO-SELECTION

Carbonyldiimidazole (CDI) activated cellulose (100 mg) was rotated end over end overnight in a 12 mm diameter tube (Sarstedt 55.484) with 1 ml of 0.05 mol/l barbitone buffer pH 8 containing 100 ug TSH (Calbiochem, Iodination grade, potency: 5.5 IU/mg). The contents of the tube were then washed into a polypropylene tube (Henley) with 0.5 mol/l sodium bicarbonate pH 8, centrifuged at 2000 g for 20 minutes and the supernatant discarded. This was followed by 3 x 20 minute washes with 0.5 mol/l sodium bicarbonate pH 8 (10 ml) and 2 sodium acetate buffer pH 4 (10 ml) washes of 20 minutes and 60 minutes respectively. The solid-phase coupled TSH was sonicated (3 x 45 seconds) in 5 ml acetate buffer, centrifuged and the supernatant discarded before a further 10 ml wash in the same buffer overnight. The material was then washed 3 times in 10 ml 0.1 mol/l borate buffer pH 8, resuspended in 0.5 ml and stored at 4°C until required.

2) IMMUNOSELECTION OF TSH ANTIBODY

Sheep TSH antiserum (S1779, 0.1 ml, courtesy of Professor R P Ekins, the Middlesex Hospital Medical School, London, UK) was rotated end over end overnight with solid-phase coupled TSH in 0.1 mol/l borate buffer pH 8 (0.5 ml). The material was washed 3 times with 10 ml 0.05 mol/l sodium phosphate buffer pH 7.4 containing 0.9% (w/v) NaCl, once with 10 ml 0.9% (w/v) NaCl containing 0.2% (v/v) Tween 20 and 3 times with 10 ml 0.05 mol/l sodium phosphate buffer pH 7.4. Finally the solid-phase pellet was resuspended in a further 1 ml of 0.05 mol/l sodium phosphate buffer pH 7.4 and stored at 4°C until required.

3) IODINATION AND PURIFICATION OF IMMUNOSELECTED TSH ANTIBODY

Sodium phosphate buffer pH 7.4 (0.5 mol/l, 0.125 ml) was added to 0.25 ml of immunoselected sheep anti-TSH in a 10 ml conical polypropylene tube (Henley), followed by 0.05 ml ^{125}I-Na (Amersham IMS.30) (5 mCi). The reaction was started by pipetting 0.05 ml freshly prepared chloramine T (4 mg/ml in 0.05 mol/l sodium phosphate buffer pH 7.4) into the reaction tube. After stirring for 40 seconds employing a triangular spinbar (Radleys, UK), the reaction was quenched with 5 ml of assay

buffer (0.1 mol/l borate buffer pH 8 containing 0.2% (v/v) Tween 20, 0.5% (v/v) normal sheep serum and 0.05% (w/v) NaN_3). The tube was centrifuged, the supernatant discarded and the solid-phase pellet washed 4 times in 5 ml of 0.001 N HCl pH 3, discarding all supernatants. HCl pH 2 (0.01 N, 1 ml) was added to the pellet, mixed, centrifuged and the supernatant placed in a further 10 ml tube containing 0.5 ml of 0.5 mol/l borate buffer pH 8.0. Sodium bicarbonate pH 8.0 (0.1 mol/l) was then added to the solid-phase pellet, mixed, centrifuged and the supernatant pooled with previously collected material. The pellet was discarded and the retained supernatants in borate buffer applied to a 100 x 1.6 cm C type column containing Sepharose 6B (Pharmacia) primed prior to use with assay buffer (as above). Fractions were collected (200 x 30 drops [about 1 ml]) at a flow rate of approximately 10 ml/hour, on a FRAC-300 fraction collector with P-1 peristaltic pump (Pharmacia). A column profile was plotted (see Figure 2.2), fractions containing anti-TSH were identified, pooled and mixed. The iodinated TSH antibody was then stored in aliquots at -20°C until required. Each aliquot contained sufficient radio-iodinated antibody for 300 tubes at 25,000 cpm initially.

c) PREPARATION OF LABELLED ^{125}I-TSH MONOCLONAL ANTIBODY

TSH monoclonal antibody (5H8) was iodinated by an enzymatic (lactoperoxidase) solid-phase method.

1) PROCEDURE

Sodium phosphate buffer pH 7.4 (0.5 mol/l, 0.01 ml) was added to 20 ug (0.01 ml) 5H8 IgG followed by 0.005 ml ^{125}I-Na (0.5 mCi, carrier free, Amersham IMS 30) and 0.01 ml locally prepared solid-phase lactoperoxidase (0.01 ml stock diluted in 1 ml 0.05 mol/l sodium phosphate buffer pH 7.4, 200 ng). The reaction was started by the addition of 0.01 ml hydrogen peroxide (H_2O_2, approx. 30% w/v, BDH, Analar, 0.01 ml in 250 ml distilled water, 120 ng) and allowed to proceed for 30 minutes, vortexing every 5 minutes. The reaction was then quenched by the addition of 0.02 ml assay diluent (0.1 mol/l EPPS (N-[2-Hydroxyethyl]-piperazine-N'-3-propane-sulphonic acid) buffer pH 8.0 containing 0.2% (v/v) Tween 20, 0.5% (v/v) normal sheep serum and 0.05% (w/v) NaN_3).

2) PURIFICATION OF IODINATED ANTIBODY

The reaction mixture was applied to a 35 x 1.5 cm column of Sepharose 6B (Pharmacia) stored in 0.1 mol/l EPPS buffer pH 8.0 and primed prior to use

with assay diluent (as above). Fractions (100 x 1 ml) were collected overnight on a FRAC-300 fraction collector with P-1 peristaltic pump (Pharmacia) and counted for 10 seconds on a gamma counter (Wilj). A profile of counts against fraction number was plotted and the 5H8 peak identified. Approximately 8 fractions containing ^{125}I-TSH monoclonal antibody were pooled and a 0.01 ml sample counted for 1 minute on a Nuclear Enterprises 1600 gamma counter to calculate the dilution which would be required to give 100,000 cpm/0.2 ml. Aliquots (0.5 ml) were prepared and stored at -20°C until required.

III) PREPARATION OF SOLID-PHASE ANTIBODIES

a) CELLULOSE SOLID-PHASE TSH ANTIBODY

1) IgG FRACTIONATION

The caprylic acid precipitation method of Steinbuch and Audran (1969) was used to prepare an IgG fraction from sheep TSH antiserum.

Sheep TSH antiserum (S-117, 60 ml) was titrated to pH 5.0 with 0.1 mol/l acetic acid and 3.6 ml of a 91% solution of n-octanoic acid (BDH) was added drop-wise. After stirring for 30 minutes the

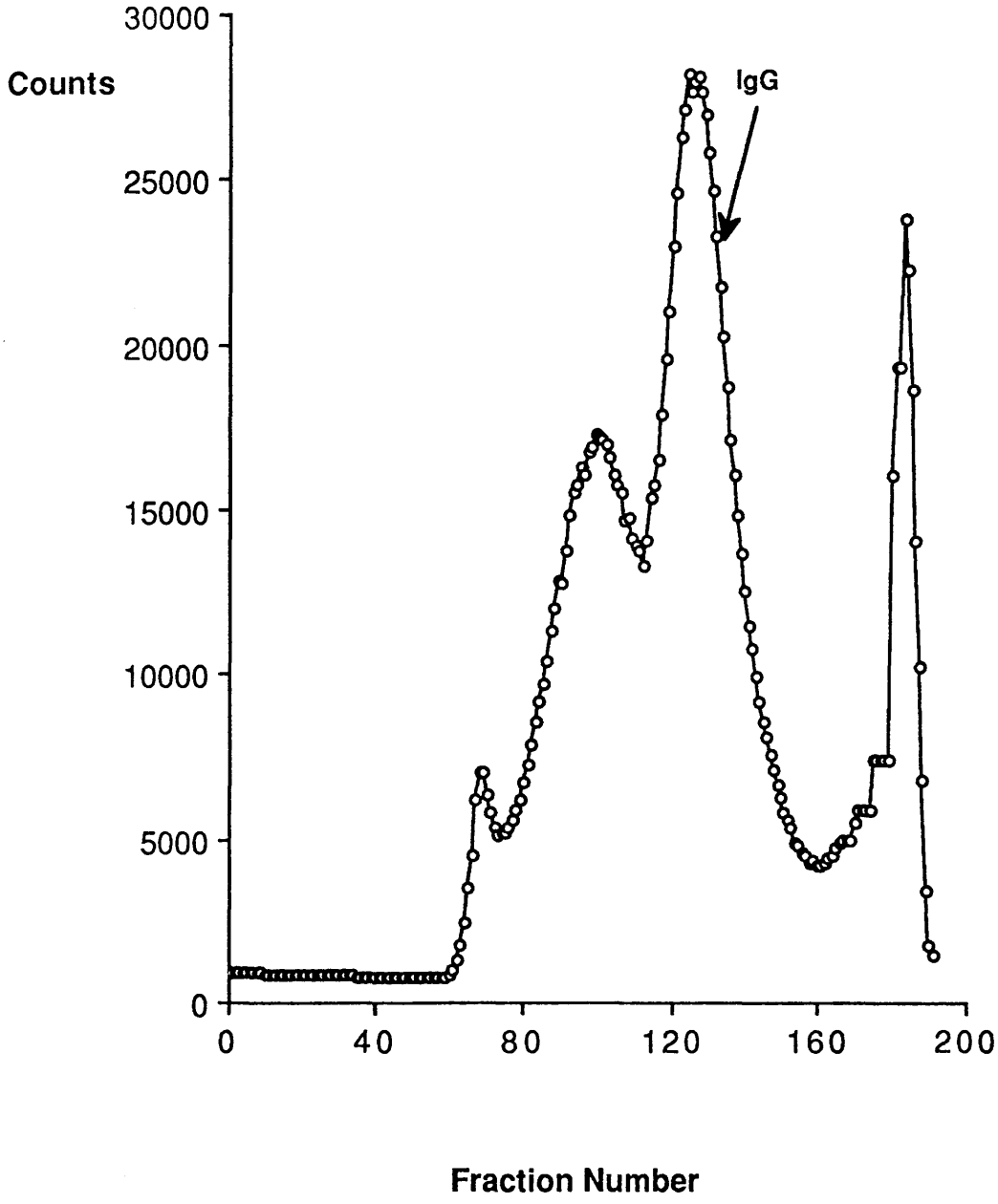


Figure 2.2

Sepharose 6B gel chromatography of immunoselected TSH antibody

material was centrifuged for 45 minutes at 2,000 g, the supernatant recovered and the pellet washed with 30 ml of 0.1 mol/l sodium bicarbonate pH 8. Following re-centrifugation of the washed pellet, the supernatants were pooled and dialysed overnight at ambient temperature against 4 l of 0.01 mol/l sodium bicarbonate, pH 8. The resultant solution had a volume of approximately 80 ml and a protein concentration of approximately 80 mg/ml.

2) CARBONYLDIIMIDAZOLE ACTIVATION OF MICROPARTICULATE CELLULOSE

Carbonyldiimidazole activation was carried out essentially by the method of Chapman and Ratcliffe (1981) in an anhydrous organic solvent with the introduction of imidazolyl carbamate groups into the polymer (Figure 2.3). The remaining imidazole groups are then reacted with peptide amino groups in aqueous alkaline medium to give an N-alkyl-carbamate derivative of the immobilised peptide.

Microparticulate cellulose (Sigmacell Type 20, Sigma, 5 g) was weighed into a 50 ml conical flask fitted with a ground glass stopper, CDI (Sigma) (0.61 g/25 ml acetone, 0.15 mol/l) added and the reaction left to proceed for 60 minutes at 20°C with vigorous stirring. The activated cellulose was recovered by filtration over a glass microfibre

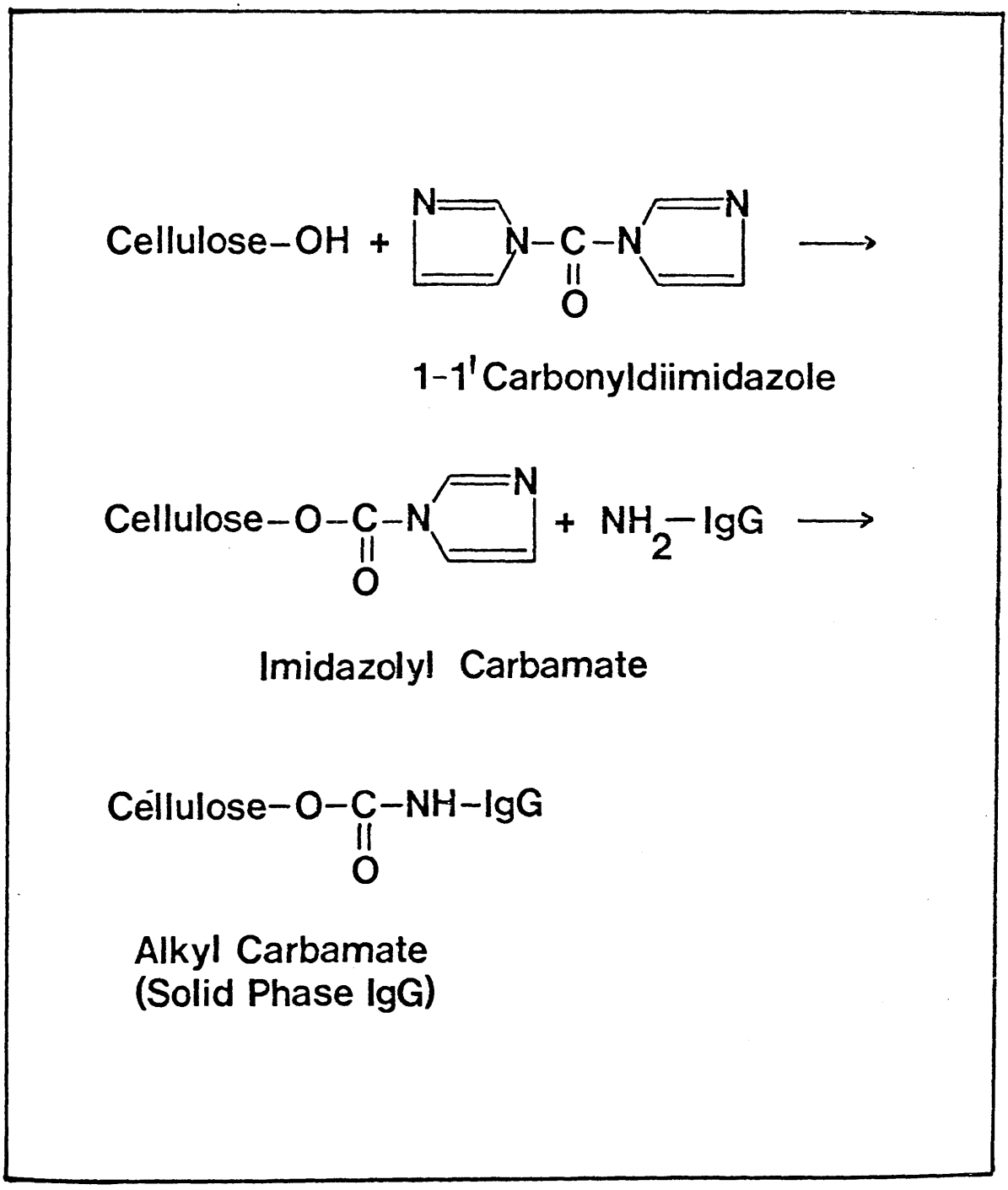


Figure 2.3

Activation of cellulose by the carbonyldiimidazole method

filter (Whatman GF/A, retention efficiency 2 microns) and washed with 3 x 100 ml aliquots of acetone. The activated cellulose was then allowed to dry in air, and stored in a tightly sealed container at -20°C.

Prior to coupling the activated cellulose was allowed to reach ambient temperature. One gram was weighed into a polystyrene tube and mixed with 5 ml of IgG solution (10 mg/ml in 0.05 mol/l barbitone buffer pH 8.0), briefly vortexed to form a mobile slurry, stoppered and rotated end over end for 16-18 hours at ambient temperature. The subsequent washing procedure followed that of Wide (1969). The protein remaining in solution was recovered by centrifugation and the immunoadsorbant washed repeatedly with 50 ml aliquots of the buffers shown below and recovered by centrifugation (10 minutes at 1,500 g) between each wash cycle.

1. 0.5 mol/l sodium bicarbonate buffer pH 8.0, rotate 20 minutes.
2. Repeat.
3. 0.1 mol/l acetate buffer pH 4.0, rotate 60 minutes.

4. 0.1 mol/l acetate buffer pH 4.0, sonicate for 30 seconds, rotate 16-20 hours.
5. 0.9% NaCl, rotate 20 minutes.
6. Repeat.

The immunoadsorbant was then stored in isotonic saline at 4°C until required. Larger preparations were accomplished simply by increasing the volumes of coupling and wash solutions.

b) SEPHAROSE SOLID-PHASE TSH ANTIBODY

1) CARBONYLDIIMIDAZOLE ACTIVATION OF SEPHAROSE

Activation of Sepharose was carried out by the CDI method of Chapman and Ratcliffe (1981), as for microparticulate cellulose.

Calibrated Sepharose CL-4B solution (200 ml) (=100 ml settled gel, Pharmacia) was transferred to a sintered glass funnel (porosity 3, capacity >500 ml) and dehydrated by washing the gel successively with 500 ml aliquots of distilled water, 30%, 50%, 70% and 100% acetone (Analar, BDH). The gel was gently stirred when fresh wash solutions were added and not allowed to dry at any stage. The dehydrated gel slurry was transferred, by careful resuspension in further acetone, to a 200 ml

calibrated conical flask and 4.87 g carbonyldiimidazole (Sigma) (0.15 mol/l) added. The flask was sealed with a ground glass stopper and stirred for 60 minutes before transferring the activated gel back to the sintered glass funnel and rehydrating by washing successively with 500 ml of 100%, 70%, 50%, 30% acetone, distilled water and finally EPPS coupling buffer (Sigma) (0.1 mol/l EPPS, pH 8 containing 0.05% (w/v) NaN_3). Again the gel was not allowed to dry and was resuspended gently between washes. The activated gel was then transferred to a poly-carbonate bottle (calibrated to 200 ml) by washing with coupling buffer and 120 mg TSH IgG was added prior to rotation end over end for 18-24 hours.

2) WASHING PROCEDURE

The subsequent washing procedure followed that of Wide (1969). The protein remaining in solution was recovered by centrifugation and the gel washed repeatedly with 100 ml aliquots of the buffers shown below and recovered by centrifugation (15 minutes at 1,200 g) between each wash cycle as follows:-

1. 0.5 mol/l sodium bicarbonate buffer pH 8.0, rotate 20 minutes.

2. Repeat.
3. 0.1 mol/l acetate buffer pH 4.0, rotate 60 minutes.
4. 0.1 mol/l acetate buffer pH 4.0, rotate 16-20 hours.
5. 0.9% NaCl, rotate 20 minutes.
6. Repeat.

The gel was then stored in isotonic saline at 4°C until required. Larger preparations were accomplished simply by increasing the volumes of coupling and wash solutions.

c) SEPHAROSE SOLID-PHASE SHEEP ANTI-MOUSE GAMMA-GLOBULIN

Solid-phase sheep anti-mouse gamma-globulin (SAM) was prepared in a similar fashion to that of Sepharose solid-phase TSH antibody except that 25 ml of sheep antiserum to mouse gamma-globulin (SAPU, Law Hospital, Carluke) was coupled by rotation end over end for 18-24 hours with the activated gel. The gel was stored at 4°C in 0.9% saline until required.

IV) STANDARDS

a) PREPARATION OF DRIED BLOOD-SPOT TSH STANDARDS

Standard TSH (2nd I.R.P. hTSH, code no 80/558) was dissolved in 50 ml TSH free human serum to give a concentration of 740 mU/l TSH. Aliquots (5 ml) were prepared in polypropylene tubes (Henley) and stored at -20°C. As required, usually at 2 monthly intervals, 5 ml of the stored TSH standard was removed and diluted with 5 ml packed human red blood cells (courtesy of the Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Lanarkshire) previously washed 3 times with 0.9% (w/v) saline to give a stock standard of 375 mU/l TSH in whole blood.

Further dilutions in simulated whole blood (50% packed cells, 50% TSH free serum) gave a series of standard concentations 375, 188, 94, 47, 23, 12, 6, 0 mU/l whole blood. Blood spots (0.025 ml) were prepared on filter paper cards (Schleisser and Schuell grade 2992, old number 903, cellulose) which were then allowed to dry overnight at ambient temperature. Post air drying the spots were sealed in polythene with silica gel dessicant (6-20 mesh, BDH) and stored at -20°C until required.

b) PREPARATION OF TSH DRIED BLOOD-SPOT QUALITY CONTROL POOLS

Standard TSH (80/558) was diluted in TSH free human serum to give TSH concentrations of 185, 92.5 and 46.25 mU/l respectively and aliquots (1 ml) of the calibrated serum were prepared and stored at -20°C until required. Packed cells (1 ml) were added to yield a set of quality controls with values of 92.5, 46.25 and 23.13 mU/l whole blood respectively and blood-spots (0.025 ml) were prepared on filter paper cards. The cards were allowed to dry overnight at ambient temperature prior to sealing in polythene with silica gel desiccant and storage at -20°C until required.

c) PREPARATION OF TSH DRIED BLOOD-SPOT DRIFT QUALITY CONTROL POOL

Packed cells (15 ml) were added to calibrated serum (74 mU/l TSH) to give a drift value of 37 mU/l whole blood. Blood-spots (0.025 ml) were prepared as above.

d) PREPARATION OF TSH HORSE SERUM STANDARDS

The contents of an ampoule of TSH standard (2nd I.R.P. hTSH, code no 80/558) were dissolved in 0.5 ml of 0.05 mol/l sodium phosphate buffer pH 7.4. The solution was

made up volumetrically to 200 ml with TSH free horse serum (Gibco, Paisley, UK) to give a stock standard of 185 mU/l TSH which was then double diluted in further horse serum (100 ml) to obtain standard values of 185, 92.5, 46.25, 23.1, 11.6, 5.8, 2.9, 1.4, 0.72, 0.36 and 0.18 mU/l TSH respectively. Aliquots (0.5 ml) were prepared and stored at -20°C until required.

V) ASSAY PROTOCOLS

a) TWO-SITE IRMA FOR BLOOD-SPOT TSH

The assay protocol for the measurement of TSH from dried blood discs is shown in Figure 2.4. The assay diluent used was 0.1 mol/l borate buffer, pH 8.0 containing 0.5% (v/v) normal sheep serum (SAPU, Law Hospital, Carluke), 0.2% (v/v) Tween 20 (Sigma) and 0.05% (w/v) NaN_3.

b) TWO-SITE IRMA FOR SERUM TSH EMPLOYING 2 POLYCLONAL ANTIBODIES

Sarstedt 55.484 tubes (12 mm diameter) were used throughout and standards, quality controls (Lyphochek, BIO-RAD), samples and total counts were all set up in duplicate. The assay diluent used was 0.1 mol/l borate

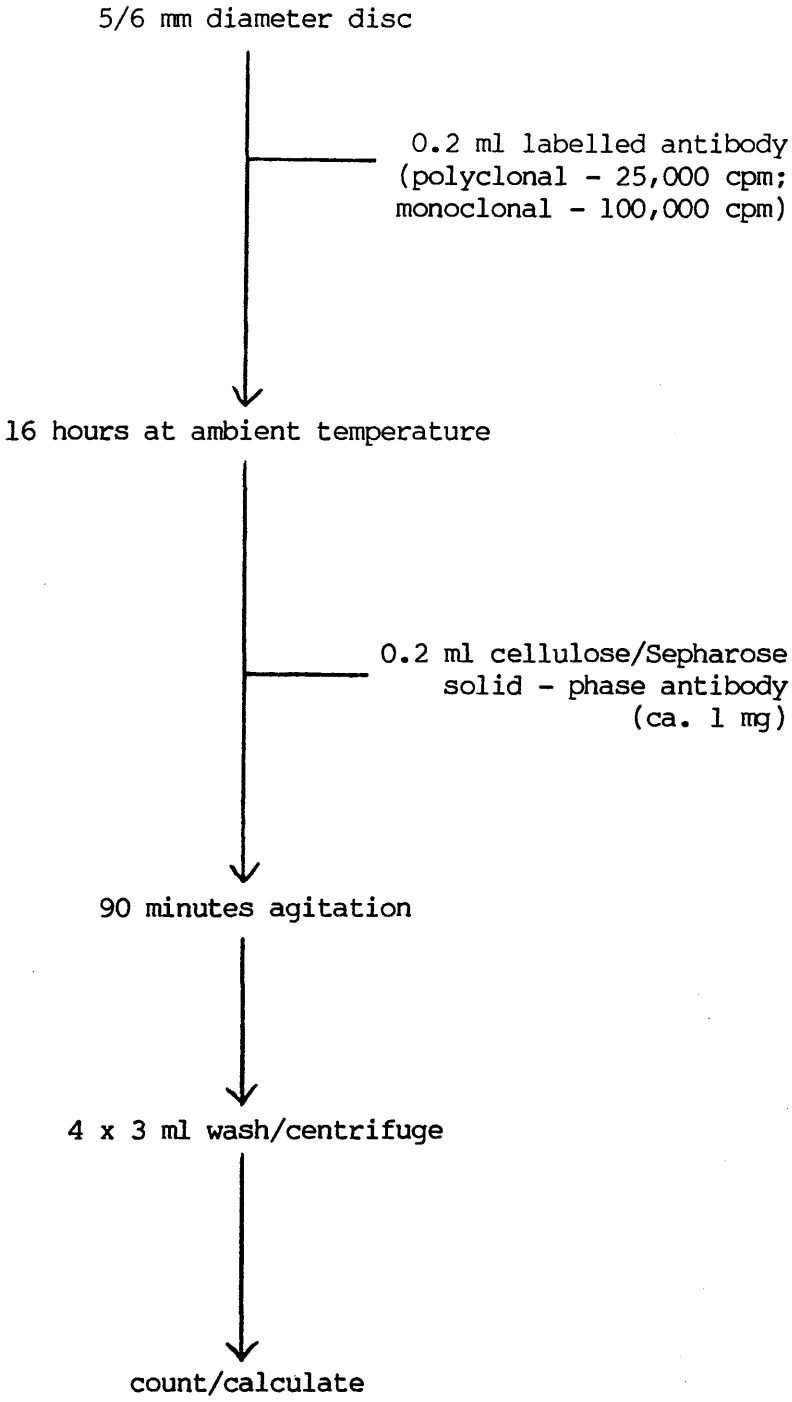


Figure 2.4

Assay protocol for the measurement of TSH from dried blood discs

buffer, pH 8.0 containing 0.5% (v/v) normal sheep serum, 0.2% (v/v) Tween 20 and 0.05% (w/v) NaN_3. The assay protocol is shown in Figure 2.5.

c) TWO-SITE IRMA FOR SERUM TSH EMPLOYING MONOCLONAL AND POLYCLONAL ANTIBODIES

Sarstedt 55.484 tubes (12 mm diameter) were used throughout and standards, quality controls, samples and total counts were all set up in duplicate. The assay diluent used was 0.1 mol/l EPPS buffer, pH 8.0 containing 0.5% (v/v) normal sheep serum, 0.2% (v/v) Tween 20 and 0.05% (w/v) NaN_3. The assay protocol is shown in Figure 2.6. The same protocol was adopted when a monoclonal antibody replaced the solid-phase polyclonal antibody.

d) ALTERNATIVE SEPARATION PROCEDURE - SUCROSE LAYERING TECHNIQUE

To achieve greater assay sensitivity sucrose layering separation was adopted (Wright and Hunter, 1983).

1) PRINCIPLE

Sepharose CL-4B solid-phase is a dense particle and sediments relatively quickly under gravity without

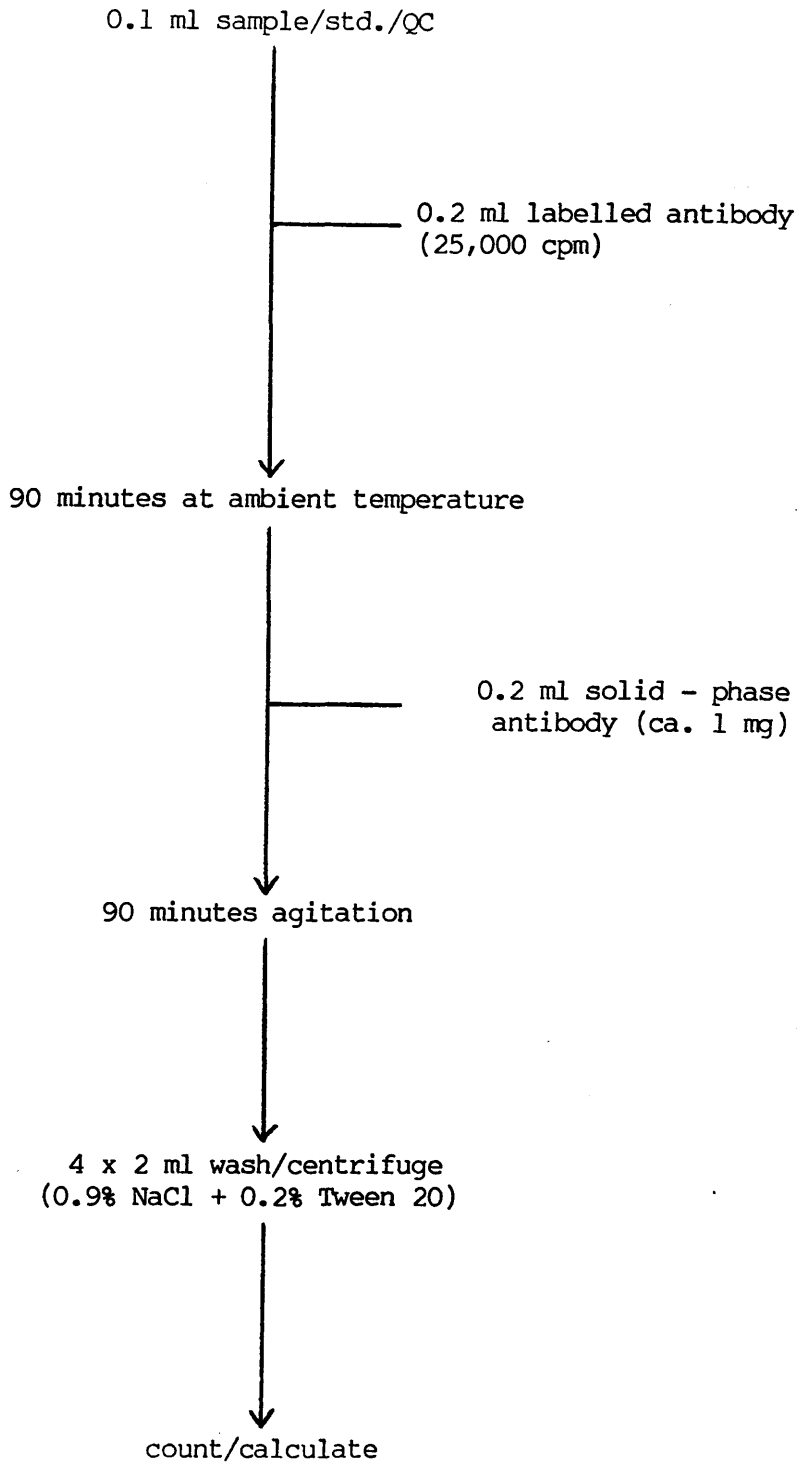


Figure 2.5

Assay protocol for serum TSH employing 2 polyclonal antibodies

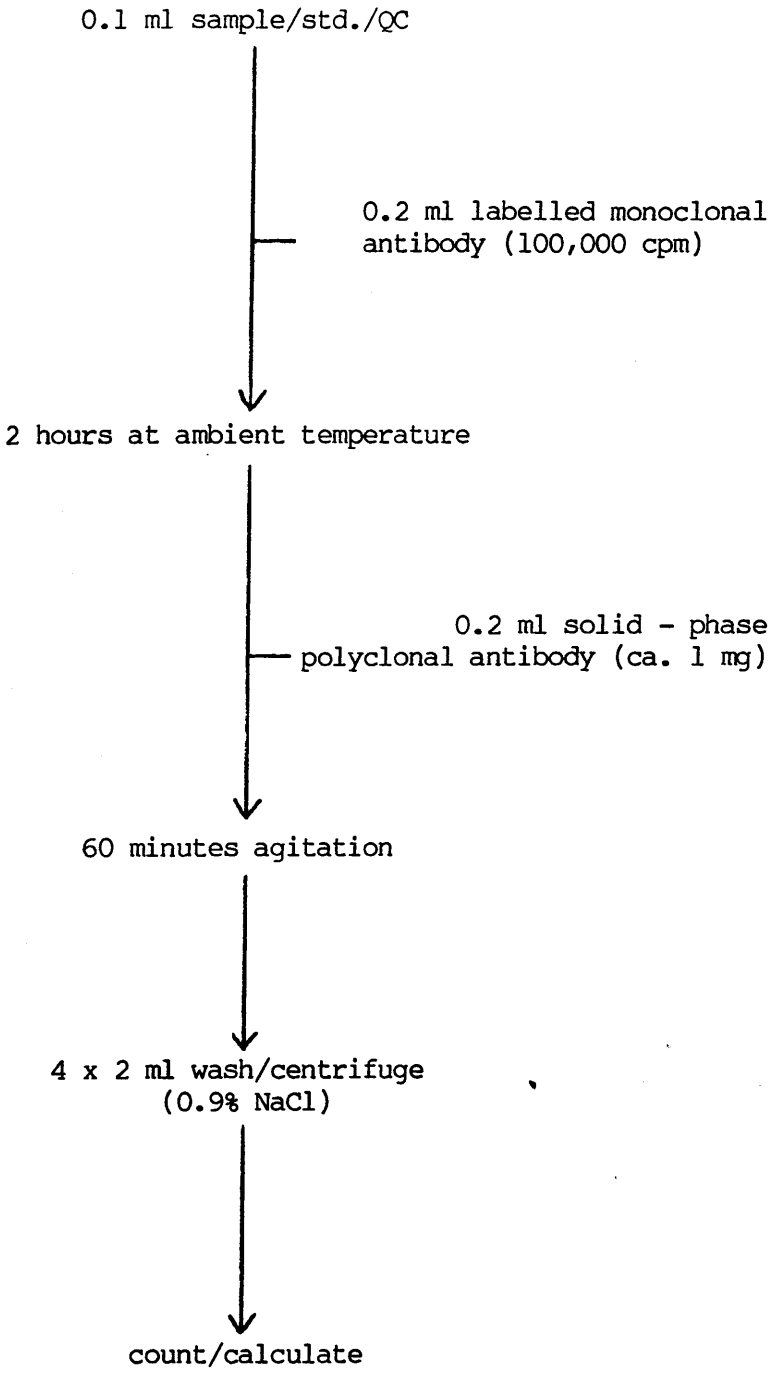


Figure 2.6

Assay protocol for serum TSH employing monoclonal and polyclonal Antibodies

the need for centrifugation. At the end of the agitation incubation (1), wash buffer is added and the solid-phase is allowed to settle for about 5 minutes (2). This step enhances separation efficiency as particles of solid-phase are washed off the sides of the tube. A relatively dense sucrose solution is then layered below the incubate by running it through a hollow probe resting on the bottom of the tube (3). This raises the incubate above the surface layer and lifts the solid-phase (bound fraction) at the interface of the two layers. Over a period of 15 minutes the particulate solid-phase falls through the sucrose, leaving behind the incubate and the remaining labelled antibody (free fraction) (4). The upper layer of incubate and most of the sucrose are now removed by aspiration through a second probe (5). This leaves 0.3-0.5 ml sucrose containing all the solid-phase at the bottom of the tube, ready for counting (6). For optimum sensitivity and precision this separation procedure is repeated. By performing the separation step twice a lower blank can be achieved.

2) REAGENTS

Wash buffer: 0.9% (w/v) NaCl containing 1% (v/v) Tween 20.

Sucrose solution: 10% (w/v) sucrose containing 1% (v/v) Tween 20 and 0.1% (w/v) NaN_3.

3) METHOD

Sarstedt 55.526 tubes (12 mm diameter, volume = 5 ml) were used throughout. The method was as follows:-

1. Add 1 ml wash buffer.
2. Allow solid-phase to settle for about 5 minutes.
3. Add 3 ml sucrose solution.
4. Wait 15 minutes.
5. Aspirate upper layer of incubate and most of sucrose.
6. Repeat steps 1 to 5.

VI) OTHER METHODS

a) PROTEIN ESTIMATION

Protein estimations were carried out with appropriate buffer blanks essentially by the method of Schacterle and Pollack (1973), based on the original work by Lowry et al (1951).

Reagents: Alkaline copper reagent (A); 20 g $Na_2 CO_3$, 4 g NaOH, 200 mg sodium tartrate and 100 mg copper sulphate $5H_2O$ were separately dissolved and sequentially made up to a final volume of 200 ml with distilled water (dH_2O).

Folin-Ciocalteau reagent (B); pre-prepared (BDH) was diluted 1:25 with distilled H_2O prior to use.

Protein standards: A stock solution of 50 mg bovine serum albumin (BSA)/50 ml distilled H_2O was double diluted (x 4) prior to use (Figure 2.7).

Protocol: 0.20 ml standard, buffer blank or test was mixed with 0.20 ml reagent A, vortexed, and allowed to stand at 22°C for 10 minutes. 1.0 ml of reagent B was s added, the solution vortexed, incubated for 5 minutes at 55°C, cooled and absorbance measured (A_{650}).

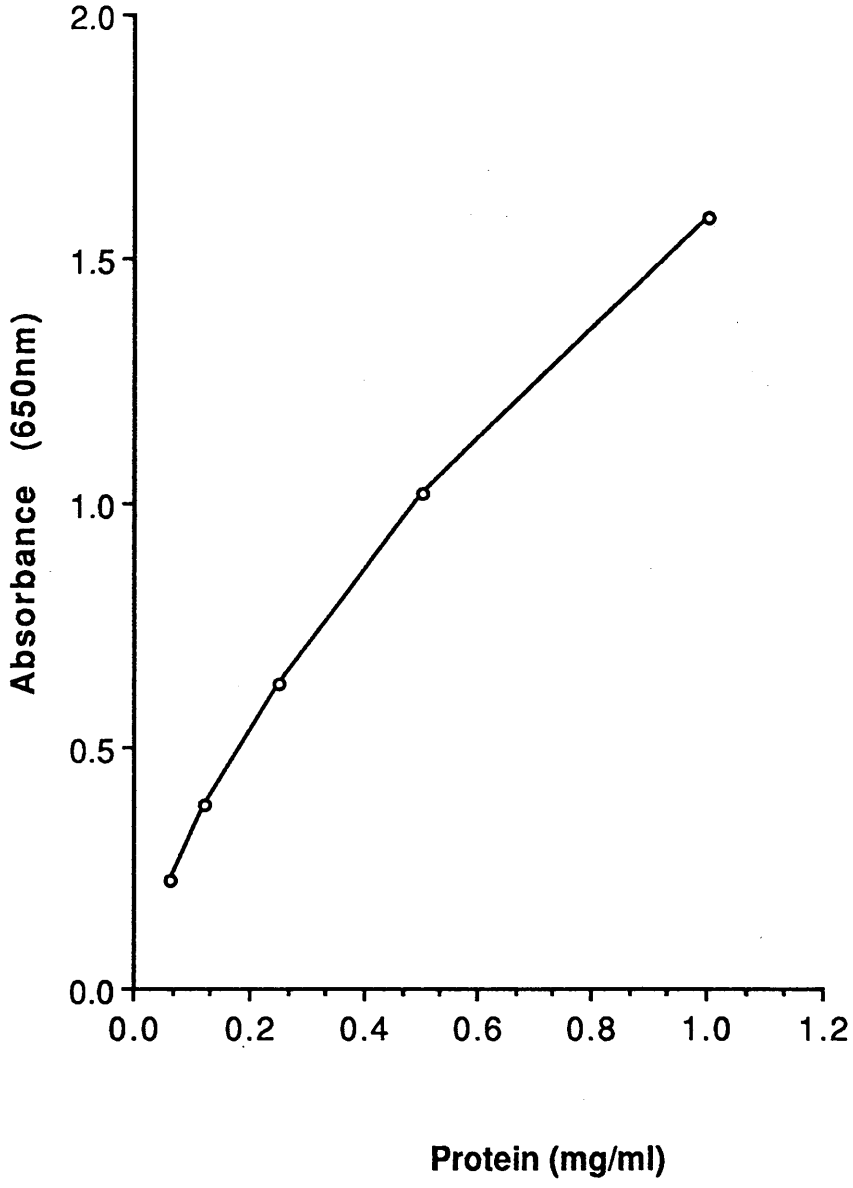


Figure 2.7

Protein standard curve

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES TO HUMAN TSH AND THE DEVELOPMENT OF A VARIETY OF TWO-SITE IMMUNOMETRIC ASSAYS FOR THE MEASUREMENT OF TSH IN SERUM AND IN NEONATAL BLOOD SPOTS

I MOUSE SERUM ANTIBODY EVALUATION

Selection of a single mouse (usually from a batch of 6) for use in a fusion experiment was based on an assessment of antibody titre and avidity. Avidity was assessed by analysis with a dose of 25 mU/l TSH (WHO 68/38). The mouse yielding the best titre and displacement was chosen for fusion. Antibody dilution and displacement curves were set up for each mouse as described in Chapter 2, Section Ib. Figure 3.1 shows typical results from a mouse subsequently selected for fusion. The lower curves represent initial blood samples from the mouse (3 weeks post primary immunisation), whilst the upper curves are identical representations several weeks later following two further booster immunisations. Figure 3.2 shows typical antibody dilution and displacement curves from a mouse rejected due to poor titre and displacement.

Since the chances of obtaining a monoclonal antibody with particular properties is directly related to the concentration of corresponding antibodies in the serum of the

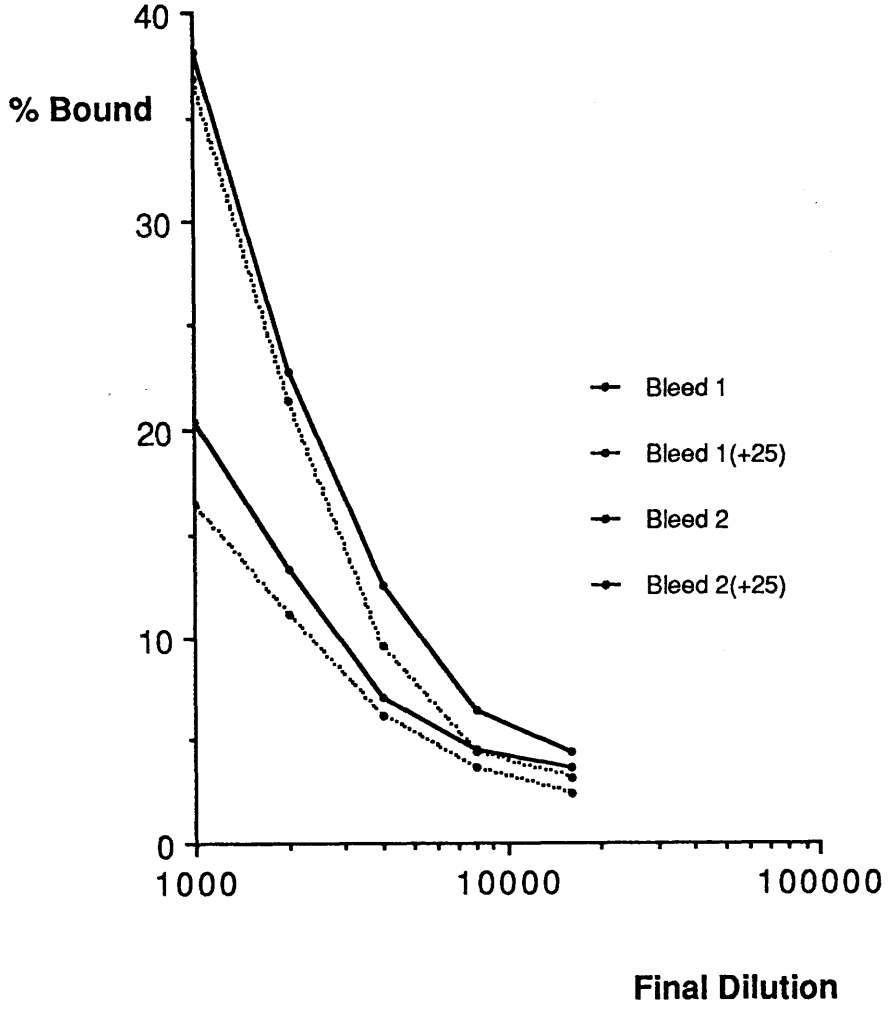


Figure 3.1

Antibody dilution and displacement curves from a mouse subsequently selected for fusion

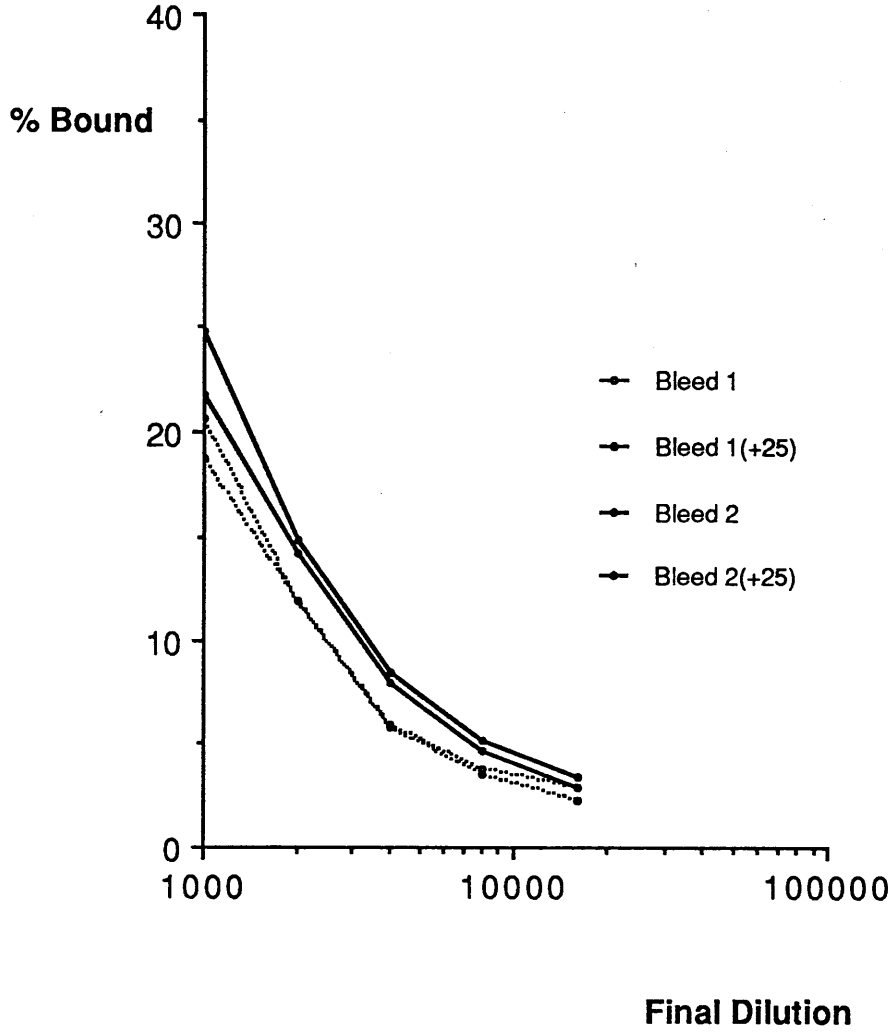


Figure 3.2

Antibody dilution and displacement curves from a mouse rejected from fusion due to poor titre and displacement

spleen donor, during splenectomy blood was removed by cardiac puncture and antibody dilution and displacement curves (10, 25 and 100 mU/l TSH) performed on the serum (Figure 3.3). The spleen from this mouse yielded a large number of antibody secreting hybrids on fusion.

II SELECTION OF ANTIBODIES POST-FUSION

a) OVERALL OUTCOME OF CELL FUSION EXPERIMENTS

A total of 6 monoclonal antibodies to TSH were obtained from 25 fusions. Additional positive hybridomas were identified in initial screening assays. Some of these were not cloned because preliminary tests for binding of hCG showed the antibodies to be non-specific, or because the level of ^{125}I-TSH binding was suggestive of low affinity antibody. There were also a few hybridomas for which attempts at cloning failed and occasionally contamination destroyed potentially useful cell lines.

b) PRODUCTION OF MONOCLONAL ANTIBODY TD.5H8.C5.D8

5H8 was the product of a fusion whose initial screening for binding to ^{125}I-TSH gave 155 hybridomas with binding levels $\geqslant$5% from a total of 1500 wells (10%). Sixty seven hybridomas bound $\geqslant$10% ^{125}I-TSH (5%). These were subsequently reanalysed to determine antibody

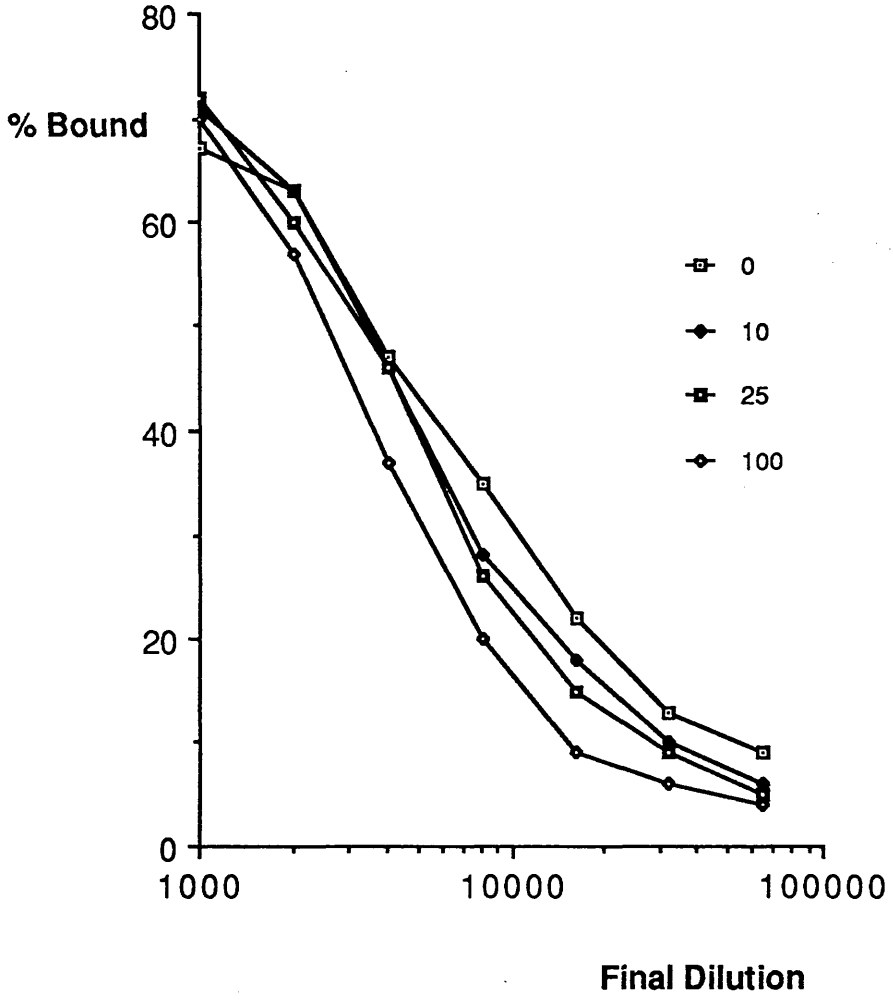


Figure 3.3

Antibody dilution and displacement curves performed on the serum of a spleen donor mouse

avidity by displacement analysis. Table 3.1 shows a typical distribution of positive hybridomas in a 96 well plate screened for TSH antibody. 5H8 (D5/H8) with a binding level of 71.0% is highlighted. Table 3.2 shows the displacement analysis results for those hybridomas eventually selected for cloning, ie. those hybridomas producing the most avid antibodies. The 7 hybridomas were further assessed at 3 dilutions (1:3, 1:9 and 1:27) in the presence of hCG to determine the specificity of the antibodies obtained (Table 3.3). All 7 hybridomas were established in 2 ml wells prior to cloning. However, 5 hybridomas had lost their ability to produce TSH antibody after primary cloning, probably due to overgrowth by non-secreting hybridomas in a single culture containing multiple hybridomas, together with chromosome segregation conspiring against stability of expression (Galfre and Milstein, 1981). Attempts at recloning from 2 ml wells were not attempted since D5/H8 and E3/G1, the remaining hybridomas were considered the most useful. Table 3.4 shows results after initial cloning for the 2 remaining positive hybridomas (D5/H8 and E3/G1). Cells from 3 of the wells of the D5/H8 clone plate and 2 wells of the E3/G1 plate were then subjected to a second cloning stage. Positive plates are shown in Table 3.5 (D5/H8/C5 and E3/G1/B8). From these plates cells from 10 wells were expanded into flasks. Antibody dilution analysis was conducted on all flask supernatants in the presence and absence of hCG (dose 20,000 u/1) and TSH (25 and 100 mU/1). One of the

TABLE 3.1

TYPICAL DISTRIBUTION OF POSITIVE HYBRIDOMAS IN A 96 WELL PLATE SCREENED FOR TSH ANTIBODY

PLATE D5

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.2	1.5	.9	1.2	1.2	1.1	9.3	1.3	1.5	1.9	3.6	1.1
B	1.4	1.4	1.3	1.5	1.4	1.0	2.6	1.6	13.5	1.4	1.1	1.1
C	1.3	1.0	1.1	1.4	1.5	2.3	5.4	1.6	1.1	1.1	2.2	1.0
D	1.1	1.0	1.6	1.3	1.5	.8	1.0	1.5	1.3	1.0	4.6	1.2
E	17.4	5.3	9.4	1.5	1.2	1.3	1.4	1.2	1.4	1.9	1.0	1.2
F	1.0	1.3	5.0	5.2	1.0	1.3	1.4	1.2	1.3	2.8	1.1	1.2
G	15.5	1.1	1.2	1.4	1.7	1.1	1.6	1.7	3.2	1.5	1.2	1.2
H	1.3	2.0	14.1	1.5	1.1	1.4	1.1	71.0	1.8	1.1	.9	2.7

TABLE 3.2

DISPLACEMENT ANALYSIS RESULTS FOR THOSE HYBRIDOMAS EVENTUALLY SELECTED FOR CLONING

CODE	BO	B500	% INHIBITION +
C2/A2	65.22	61.93	5.0
D1/H3	76.24	77.24	-
*D5/H8	71.03	70.77	0.4*
E1/E5	62.76	67.90	-
E1/E6	64.73	64.37	-
E3/G1	43.15	22.87	47.0
F2/D4	64.39	63.52	1.4

$$+ \% \text{ Inhibition} = \frac{BO - B500}{BO} \times 100$$

TABLE 3.3

DETERMINATION OF ANTIBODY SPECIFICITY

DILUTION	BO	BTSH	BhCG
C2/A2			
neat	53.9	54.9	52.1
1:3	47.8	47.6	47.7
1:9	40.1	39.4	36.3
1:27	28.5	28.2	29.8
D1/H3			
neat	63.0	63.8	62.8
1:3	52.8	54.4	54.4
1:9	41.4	42.3	39.1
1:27	24.6	25.0	24.8
D5/H8			
neat	56.1	55.7	55.2
1:3	51.3	48.2	51.4
1:9	44.8	44.6	46.3
1:27	40.3	38.3	39.7
E1/E5			
neat	55.8	56.2	57.2
1:3	44.2	43.7	43.9
1:9	33.9	32.2	34.1
1:27	21.6	21.6	21.2
E1/E6			
neat	53.8	54.3	57.6
1:3	52.0	50.7	51.7
1:9	45.4	43.0	44.2
1:27	36.4	36.6	36.6
E3/G1			
neat	24.9	15.0	2.0
1:3	18.3	5.6	1.1
1:9	9.1	2.7	1.3
1:27	4.8	1.5	1.4
F2/D4			
neat	52.7	54.4	51.4
1:3	38.9	39.2	32.4
1:9	23.3	23.8	17.2
1:27	12.5	12.1	7.0

TABLE 3.4

RESULTS AFTER INITIAL CLONING FOR D5/H8 AND E3/G1

PLATE D5/H8

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			1.3	0.9		10.3	2.8					
			0.6	0.9		2.0	1.8					
			0.8	0.9		1.0	1.1					
			1.3	0.7		1.3	0.8					
C			2.5		63.8		2.4					
			2.5		60.6		2.3					
			1.0		29.4		0.9					
			0.6		6.6		0.6					
D		0.9	2.4			0.7	67.9		1.2	0.5		
		0.8	0.7			0.6	62.8		0.8	0.7		
		0.7	1.0			0.7	38.8		0.6	0.7		
		1.2	0.7			0.9	13.9		0.8	0.6		

TABLE 3.4 CONTINUED:

PLATE E3/G1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.7		0.7			0.7		0.6	0.6	1.0		
	0.5		0.5			0.4		0.6	0.8	0.3		
	0.6		0.6			0.8		1.0	0.8	0.7		
	0.9		0.8			0.5		0.6	0.6	0.4		
B	0.5		0.7			0.6		57.0	0.6	0.6		0.7
	0.5		0.6			0.5		58.3	0.8	0.7		0.5
	0.7		0.9			0.5		28.6	0.6	0.6		0.6
	0.9		0.6			0.6		10.5	0.5	0.6		0.7
C			0.7	0.6	0.6	15.5		0.8	0.5	0.9		0.6
			0.4	0.4	0.6	3.0		0.4	0.5	0.4		0.6
			0.6	0.8	0.5	1.6		0.7	0.6	0.9		0.6
			0.6	0.5	0.5	0.6		0.6	1.0	0.5		0.6
D	1.6	0.7	0.5		0.5	0.9	0.6		0.6	0.6		
	0.6	0.4	0.5		0.7	0.7	0.7		0.6	0.7		
	0.8	0.6	0.8		0.7	0.7	0.7		0.7	0.6		
	0.4	0.8	0.8		0.7	0.8	0.8		0.6	0.6		

TABLE 3.5

RESULTS AFTER SECOND CLONING FOR D5/H8/C5 AND E3/G1/B8

PLATE D5/H8/C5

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B						40.3 37.8						
						19.6 3.6						
C		25.6 7.1		20.7 5.2			20.1 4.2	32.1 17.5				
		3.0 1.3		2.3 1.2			3.4 0.9	7.4 1.7				
D					34.5 24.6		30.8 14.1	44.6 43.9				
					9.5 1.7		7.0 1.1	30.8 12.9				

TABLE 3.5 CONTINUED

PLATE E3/G1/B8

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			16.3	2.0	4.0							
			2.8	1.4	2.4							
			1.8	2.3	1.2							
			1.0	1.3	1.4							
C					11.7	4.0	0.8	1.3	9.1			
					2.1	1.5	1.0	1.3	1.3			
					2.3	1.9	1.0	0.8	2.4			
					0.8	1.0	1.1	0.6	1.4			
D						1.1	1.3					
						2.8	1.2					
						1.1	1.7					
						1.2	1.1					

hybridomas (E3/G1) had lost its ability to produce TSH antibody but 5H8, potentially the most useful cell line was still producing antibody (Figure 3.4). All the results indicated that 5H8 was an antibody directed against the β subunit of TSH which after full characterisation may be of practical value in a two-site IRMA for TSH.

c) PRODUCTION OF MONOCLONAL ANTIBODIES TQ5, TR2 AND TS5

Selection of TQ5, TR2 and TS5 was performed in a similar manner to 5H8. Cells from an immunised mouse spleen weighing 295 mg were fused with myeloma cells (viability 90%) yielding 98 positives (⩾10% binding), ie. 7%. Of these 98 positives, 52 (3%) bound ⩾20% ^{125}I-TSH and were subsequently subjected to displacement analysis (dose 500 mU/l TSH) to determine antibody avidity. The 52 positives were also assessed in the presence of hCG (dose 20,000 u/l) to determine antibody specificity. Results are shown in Table 3.6 for 6 hybridomas which were considered most suitable for cloning. Before second cloning, displacement and specificity analysis was repeated. Results are shown in Table 3.7 for 4 hybridomas selected for further cloning. Prior to expansion 3 of the 4 hybridomas were established in 2 ml wells and reassessed as previously described (Table

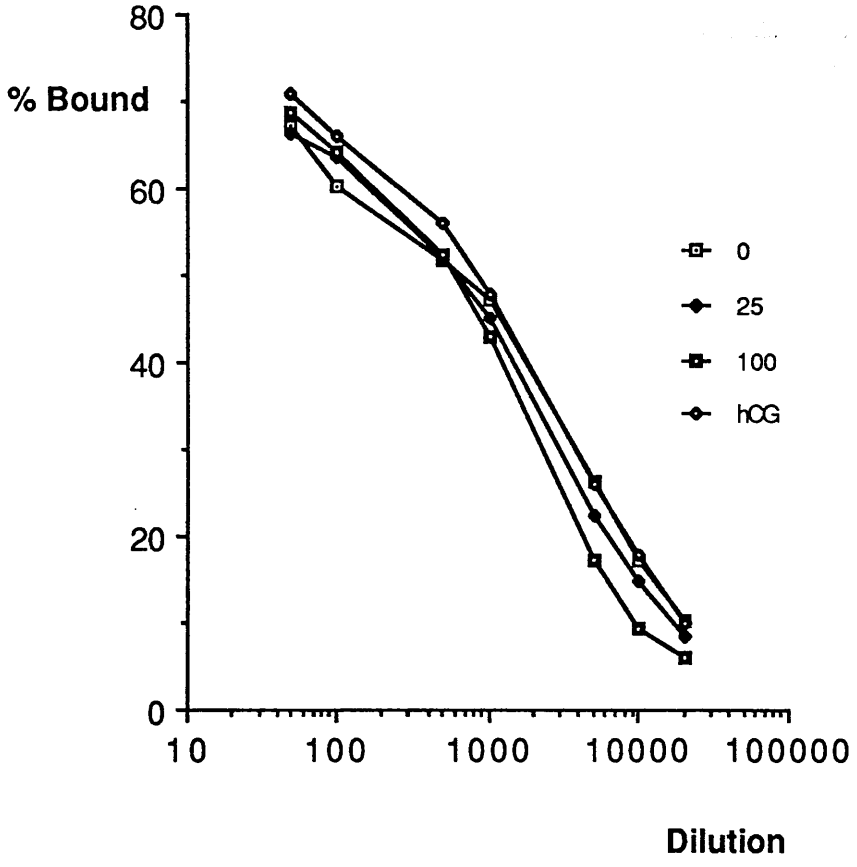


Figure 3.4

Antibody dilution analysis of 5H8 post second cloning and expansion

TABLE 3.6

a) DETERMINATION OF ANTIBODY AVIDITY AND SPECIFICITY PRIOR TO CLONING DILUTION

	DILUTION															
	1:2				1:8				1:32				1:128			
CELL LINE	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I
TQ5/D8	20.0	21.1	18.9	-	12.0	12.1	10.7	-	5.4	5.0	4.5	7.4				
				5.5				10.8				16.7				
TR2/E1	26.2	24.7	24.5	5.7	17.8	15.2	14.6	14.6								
				6.5				18.0								
TS1/F9	34.5	33.0	31.7	4.3	29.2	27.1	26.2	7.2	17.2	17.6	14.8	-				
				8.1				10.3				14.0				
TS4/D5	45.9	46.7	46.1	-	39.4	39.9	37.2	-	29.5	30.0	23.5	-				
				-				5.6				20.3				
TS4/F8	54.7	40.6	40.6	25.8	42.2	32.1	32.2	23.9	17.8	13.7	14.2	23.0	4.3	4.1	3.7	4.7
				25.8				23.7				20.2				
TS5/C7	11.4	7.1	7.4	37.7	18.2	3.5	3.2	80.8								
				35.1				82.4								

b) 5H8 CONTROL

DILUTION	% Bo	% Bd	% BHCG	% INHIBITION
1:2000	52.3	51.2	50.9	
1:4K	53.6	53.7	52.4	
1:8K	51.4	53.8	51.9	
1:16K	49.9	51.5	49.5	
1:32K	49.8	50.9	49.8	
1:64K	49.5	46.4	46.0	7.1
1:128K	43.3	44.6	41.4	4.4
1:256K	39.8	39.4	34.5	13.3
1:512K	42.8	35.9	26.4	38.3
1:1024K	31.2	30.1	15.6	50.0

TABLE 3.7

DETERMINATION OF ANTIBODY AVIDITY AND SPECIFICITY PRIOR TO SECOND CLONING DILUTION

	DILUTION											
	X4				X16				X64			
CELL LINE	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I
TQ5/D8/C11	46.5	45.1	47.9	3.0	26.0	24.2	25.0	6.9	10.0	8.0	8.3	20.0
				-				3.8				17.0
TR2/E1/D11	17.0	13.2	13.8	22.4	2.5	3.1	2.3	-	1.5	1.0	1.4	33.3
				18.8				8.0				6.7
TS4/D5/C10	26.5	25.8	14.3	2.6	11.0	10.4	4.3	5.5	3.6	3.0	2.1	16.7
				46.0				60.9				41.7
TS5/C7/E9	11.1	8.6	9.1	22.5	5.6	4.5	4.8	19.6	2.0	2.5	2.3	-
				18.0				14.3				-

3.8). A full characterisation of the 3 products from this fusion was performed at a later stage, the results of which are presented in Section III.

d) PRODUCTION OF MONOCLONAL ANTIBODIES TX.2G2 AND TW.4F12

Myeloma cells of 99% viability were fused with spleen cells from an immunised mouse (weight of spleen = 0.5 g). In the initial screening assay 18 (1%) positive hybridomas were identified (binding $\geqslant$10% ^{125}I-TSH) (Figure 3.5). The majority (72%) of the positive cell lines were poor binders (10-14.9% ^{125}I-TSH) suggestive of low affinity antibody. Only one hybridoma (2G2) bound greater than 50% of the label. Several hybridomas were cloned once but only 2G2 and 4F12 were selected for further cloning and expansion. Both hybridomas were injected into mice to produce ascitic fluid. The fusion products were fully characterised at a later stage, the results of which are presented in Section III.

III CHARACTERISATION OF MONOCLONAL ANTIBODIES

a) INTRODUCTION

The glycoprotein hormones thyroid stimulating hormone, luteinising hormone, chorionic gonadotrophin and

TABLE 3.8

DETERMINATION OF ANTIBODY AVIDITY AND SPECIFICITY PRIOR TO EXPANSION

DILUTION

	X4				X16				X64			
CELL LINE	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I	% Bo	% Bd	% BHCG	% I
TQ5/ D11	38.4	37.8	38.5	1.6	23.8	22.8	22.0	4.2	12.4	10.9	12.7	12.1
D8/C11				-				7.6				-
E11	45.4	46.5	44.8	-	26.5	29.3	29.7	-	22.7	17.6	17.6	22.5
				1.3				-				22.5
F11	46.6	47.0	43.3	-	29.3	29.0	29.9	1.0	17.5	18.1	20.3	-
				7.1				-				-
TR2/ D10	35.2	34.1	33.0	3.1	15.8	18.3	17.1	-	12.5	9.6	9.2	23.2
E1/D11				6.3				-				26.4
E11	43.9	43.6	42.1	0.7	26.2	27.8	30.3	-	16.5	15.8	14.2	4.2
				4.1				-				13.9
F11	42.1	44.5	42.8	-	28.3	29.8	29.7	-	14.5	16.7	16.4	-
				-				-				-

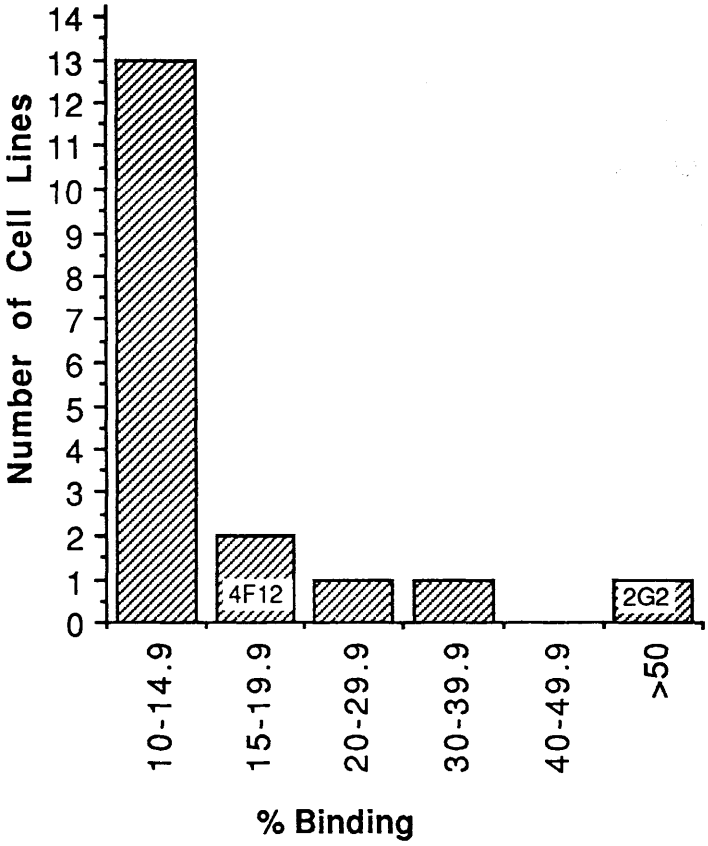


Figure 3.5

Positive hybridomas identified in the intitial screening assay leading to the production of monoclonal antibodies TX.2G2 and TW.4F12

follicle stimulating hormone constitute a family of structurally related polypeptides (Pierce and Parsons, 1981). They share a common alpha-subunit, and have variable degrees of sequence homology in their specific β-subunits. All the hormones have complex carbohydrate groups attached to both subunits.

The similarities in primary sequence and tertiary structure are reflected in the immunological properties of the hormones. Thus antisera raised against one hormone normally cross-react with others of the group (Vaitukaitis et al, 1976). More specific antisera have been obtained by adsorption to remove cross-reacting antibodies, or by immunisation with isolated subunits, peptide fragments or chemically modified preparations rather than intact native molecules (Vaitukaitis et al, 1976; Swaminathan and Braunstein, 1978; Ghai et al, 1980). However, it remains difficult to obtain conventional antibodies of defined specificity for use in sensitive immunoassay. It is to be expected that suitable monoclonal antibodies (Galfre and Milstein, 1981) might be highly specific for a given hormone if directed against a unique structural feature. The ease with which such antibodies may be obtained will depend on the relative immunogenicity of specific and homologous structures in a given hormone.

b) ANTIBODY AFFINITY AND SPECIFICITY

IgG fractions of ascitic fluid were used as a source of antibody. The dilution of each IgG fraction which bound half-maximally 100 pg of ^{125}I-TSH (30,000 cpm) was determined (Figure 3.6) and used in experiments to determine antibody affinity and specificity.

Standard curves were performed in duplicate. Standard TSH (SFA (3-5) ASF standard range 0-10,000 mU/l, 0.1 ml) and antibody (0.1 ml) were incubated overnight with approximately 100 pg of ^{125}I-TSH (0.1 ml). Solid-phase sheep anti-mouse gamma globulin (0.2 ml) was added and the reaction tubes agitated for 60 minutes prior to washing and counting. Figure 3.7 displays standard curves for each of the 6 monoclonal antibodies. The data for binding in the presence of TSH were used to construct Scatchard plots (Figure 3.8) from which the avidity and concentration of the antibody were estimated. Avidities ranged from 1.7×10^7 (4F12) to 6.3×10^9 1/mol (5H8) (Table 3.9).

Antibody specificity, with respect to cross-reaction with LH, FSH and HCG, was assessed by testing for displacement of binding between unlabelled hormones and ^{125}I-TSH. Specificity analysis was performed on 5H8 and 2G2, the antibodies of highest avidity. Standard hormone (0.1 ml), TSH (SFA [3-5] ASF, 0-2,000 ug/l), LH (LH10, 0-1,000 ug/l), FSH (FSHCPDS-30, 0-1,000 ug/l) (Dr

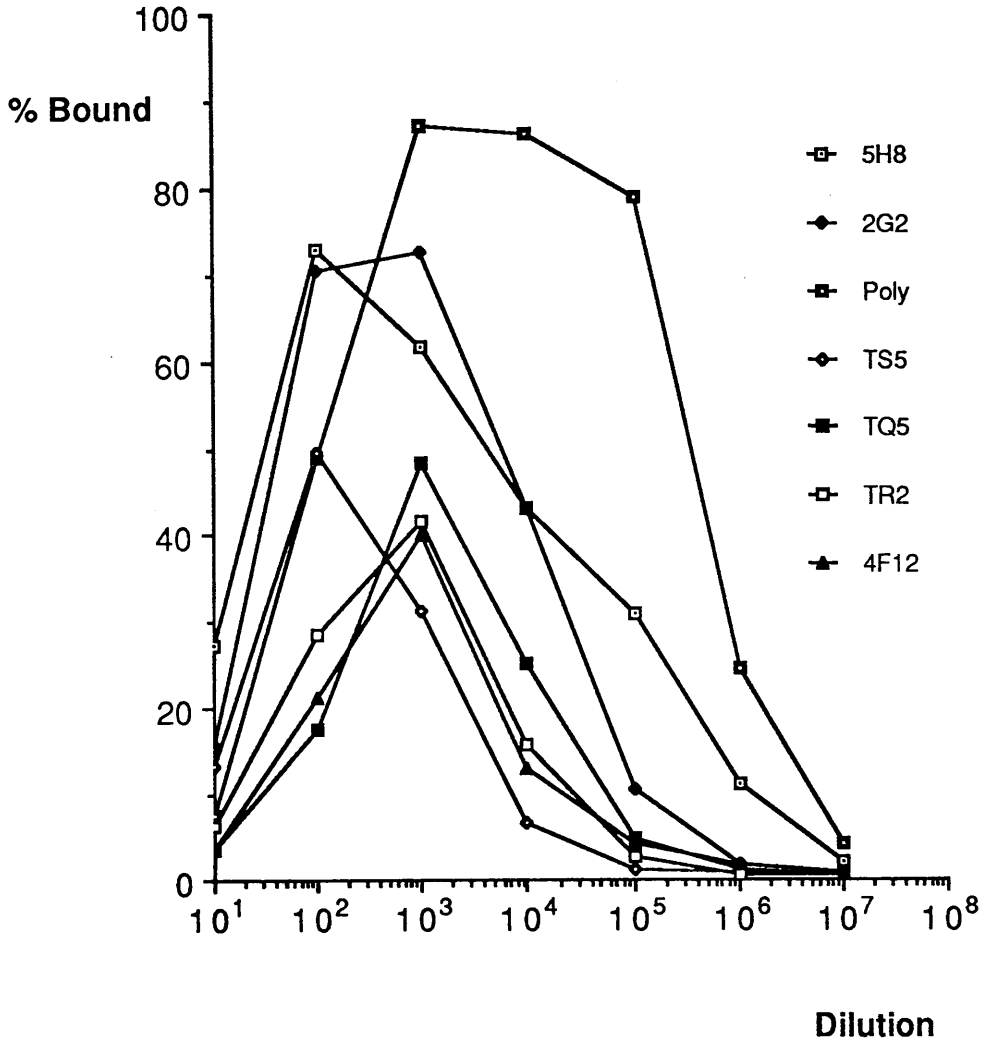


Figure 3.6

Determination of the dilution of each IgG fraction which bound half-maximally 100 pg of ^{125}I-TSH (30,000 cpm)

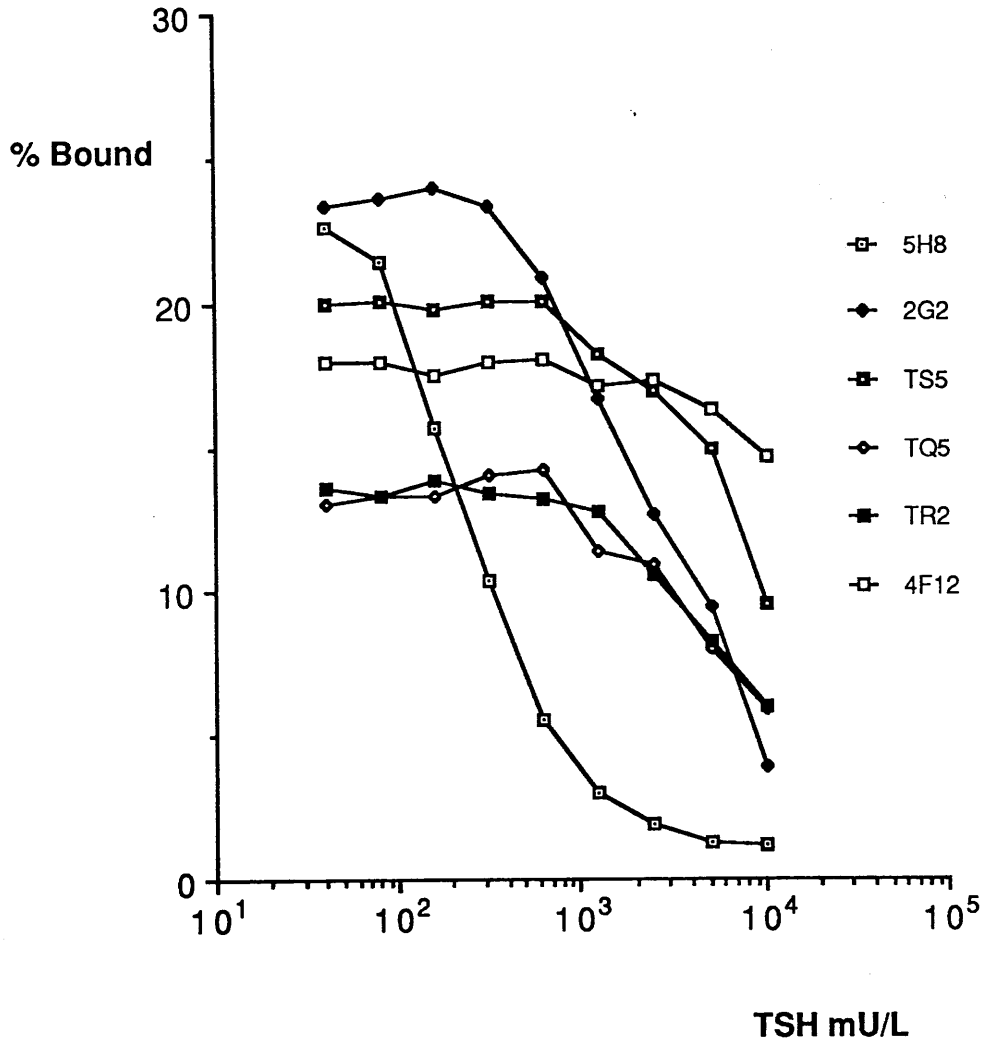


Figure 3.7

Standard curves for each of the 6 monoclonal antibodies

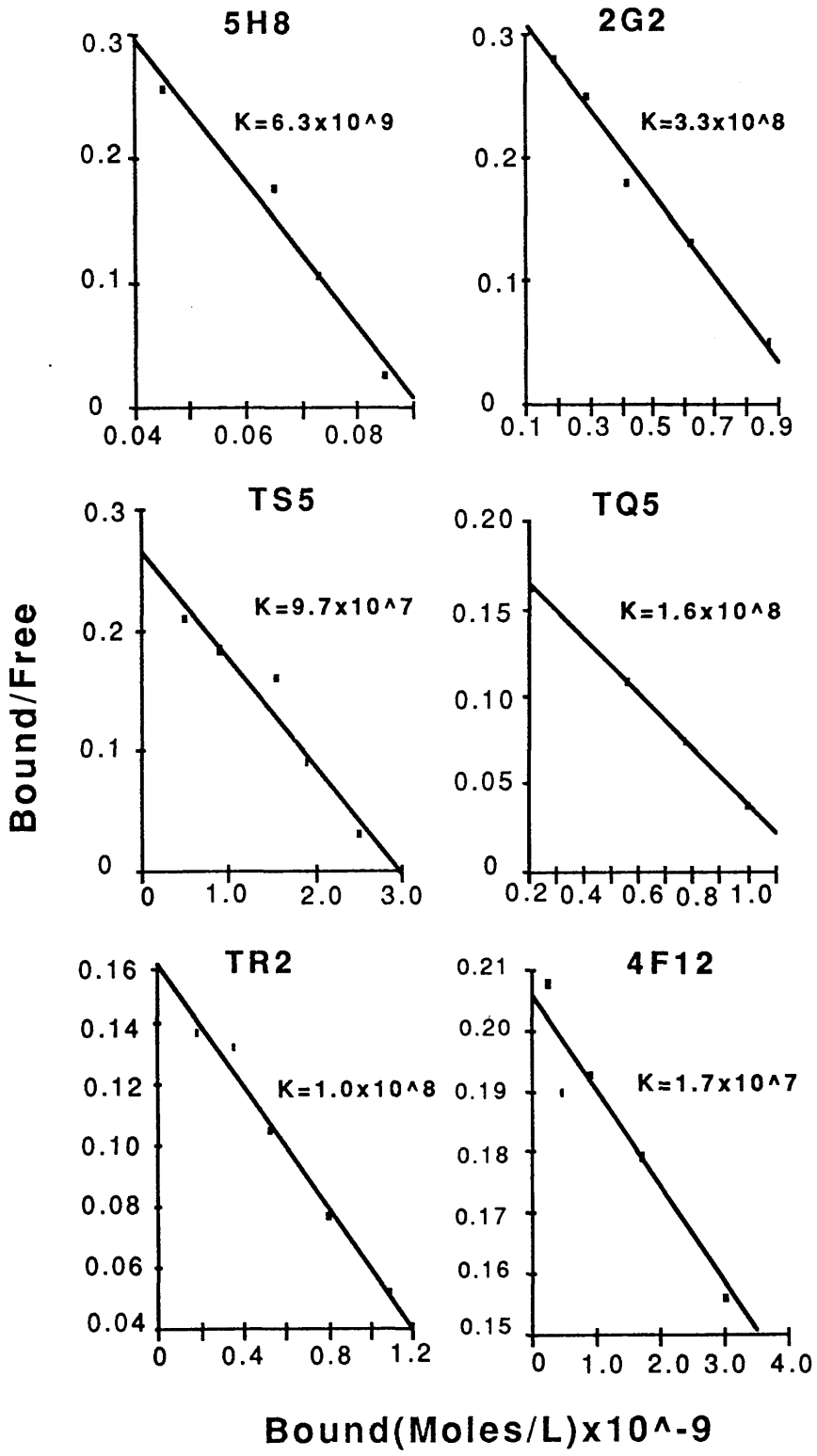


Figure 3.8

Scatchard plots for each of the 6 monoclonal antibodies

TABLE 3.9

ANTIBODY AFFINITY AND CONCENTRATION

ANTIBODY	AFFINITY (litres/mole)	[BINDING SITES] (moles/l)
5H8	6.3×10^{9}	9.60×10^{-11}
2G2	3.3×10^{8}	1.05×10^{-9}
TQ5	1.6×10^{8}	1.24×10^{-9}
TR2	1.0×10^{8}	1.58×10^{-9}
TS5	9.7×10^{7}	2.85×10^{-9}
4F12	1.7×10^{7}	1.21×10^{-8}

S Lynch, Birmingham and Midland Hospital for Women) or HCG (BCL, Lewes, UK, 0-10,000 ug/l) was incubated overnight with 5H8 or 2G2 antibody (0.1 ml) and ^{125}I-TSH (0.1 ml). Solid-phase sheep anti-mouse gamma globulin (0.2 ml) was added and the reaction tubes agitated for 60 minutes prior to washing and counting. The concentration of each hormone producing 50% inhibition of ^{125}I-TSH binding was determined. 5H8 showed no cross reactivity with any of the hormones (Figure 3.9) whereas 2G2 cross-reacted with all 3, LH (97.1%), FSH (82.6%) and HCG (84.9%) (Figure 3.10).

5H8 and 2G2 were then subjected to incubation with preparations of α (N-745-A) and β (AFP-3292B) TSH (National Hormone and Pituitary Programme (NIDDK), University of Maryland School of Medicine, USA) in a similar fashion to that outlined above and % bound versus log concentration (ug/l) plotted (Figure 3.11 and 3.12 respectively). The results indicated that 5H8 was an antibody specific for the β-subunit of TSH whereas 2G2 was specific for the α-subunit. Slight displacement was observed with high concentations of α TSH in the case of 5H8. A similar effect was noted for 2G2 with high concentrations of β TSH. This was attributed to a small degree of contamination with whole TSH (approximately 4% in each case).

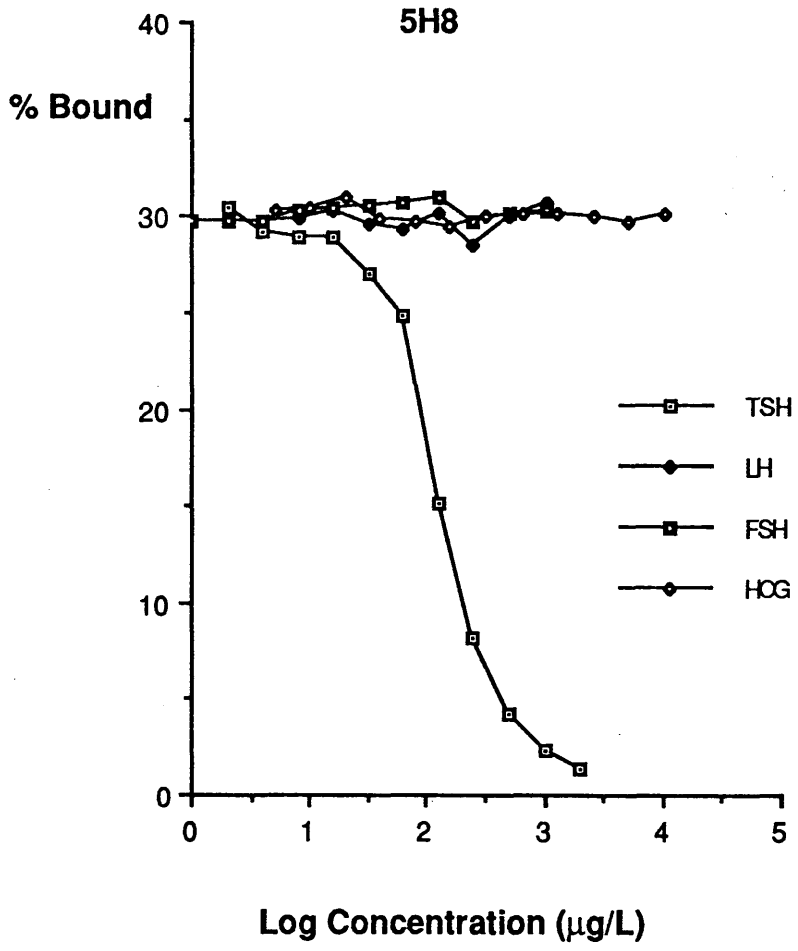


Figure 3.9

Specificity analysis of monoclonal antibody 5H8

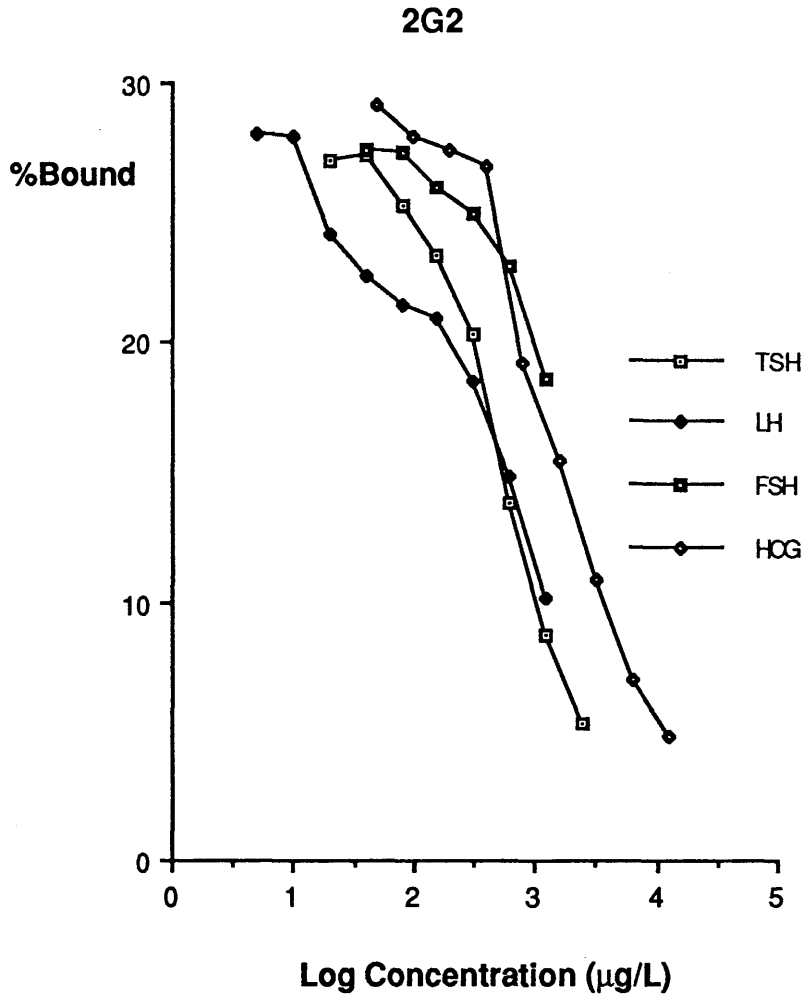


Figure 3.10

Specificity analysis of monoclonal antibody 2G2

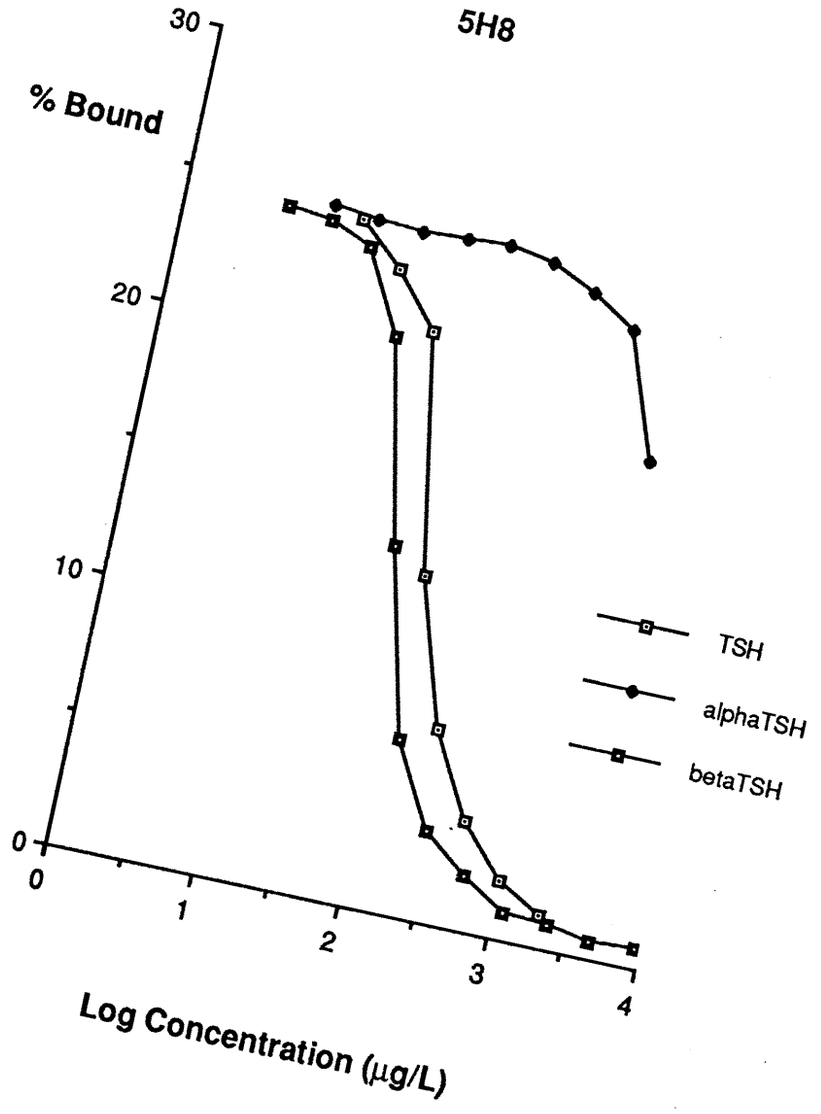


Figure 3.11

Subunit specificity analysis of monoclonal antibody 5H8

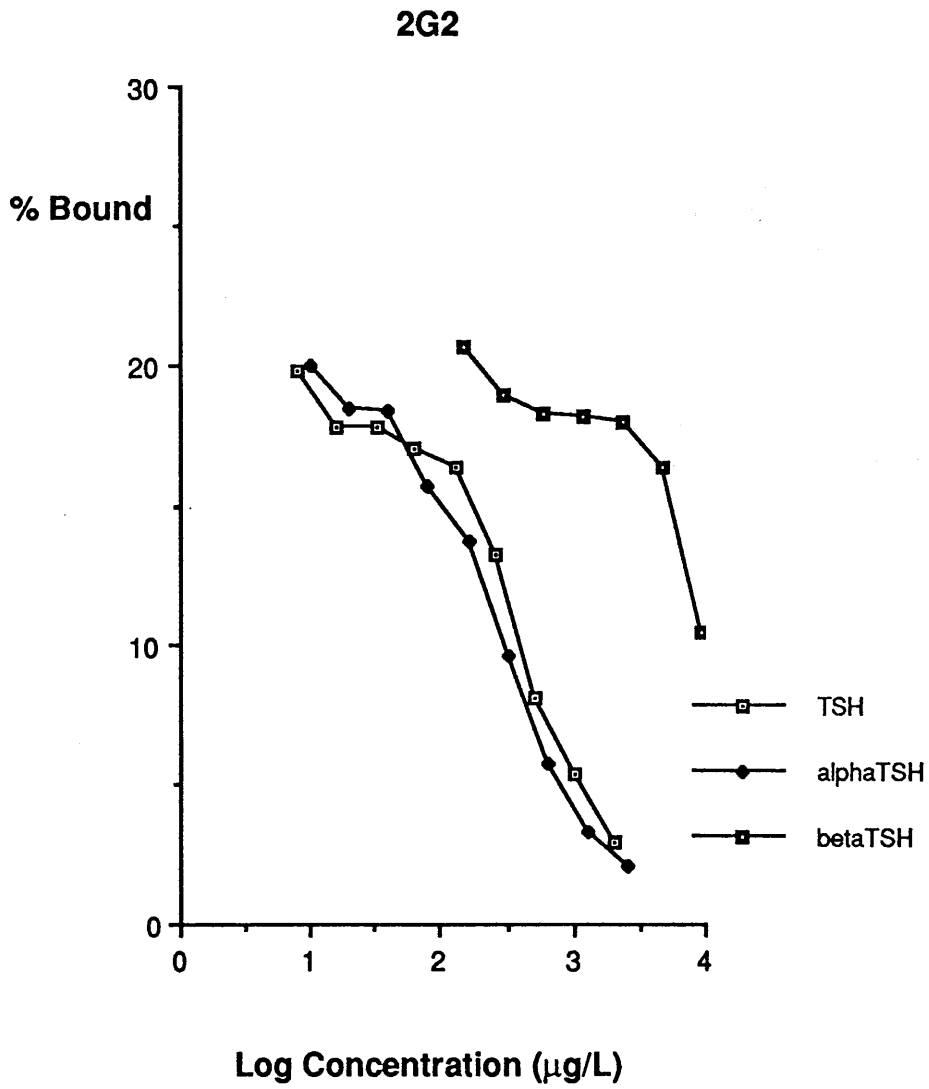


Figure 3.12

Subunit specificity analysis of monoclonal antibody 2G2

The above results indicate that in terms of avidity and specificity 5H8 and 2G2 may be of extreme practical value in the development of a two-site IRMA for TSH.

c) ISOTYPE ANALYSIS

Culture supernatants from suspensions of twice-cloned hybrid cells, grown to maximum density, were used as a source of antibody. The isotype of all 6 antibodies was determined. Results are shown in Table 3.10. It was of interest to note that the two antibodies, 5H8 and 2G2, of highest affinity were both of the IgG_1 subclass.

IV PREPARATION OF ^{125}I-LABELLED TSH MONOCLONAL ANTIBODY

Iodination of monoclonal antibody 5H8 was performed as described in Chapter 2, Section IIIc. 5H8 IgG fractions (20 ug, tap ⩾3) were iodinated with 0.25 and 0.5 mCi carrier-free radioiodine yielding labels with specific activities estimated as 9 and 21 uCi/ug respectively (based on incorporation of radioiodine into the protein) (Figure 3.13). Both labels were incubated at various count rates (100,000, 200,000 and 400,000 cpm/0.2 ml) with buffer ('o' TSH standard) and 30 mU/l TSH and the signal:noise ratios calculated (Table 3.11). All blanks were higher than expected due to the washing procedure not being optimised at

TABLE 3.10

DETERMINATION OF ISOTYPE

ANTIBODY	ISOTYPE
5H8	IgG1
2G2	IgG1
TQ5	IgG2a
TR2	IgG2b
TS5	IgG2b
4F12	IgG2a

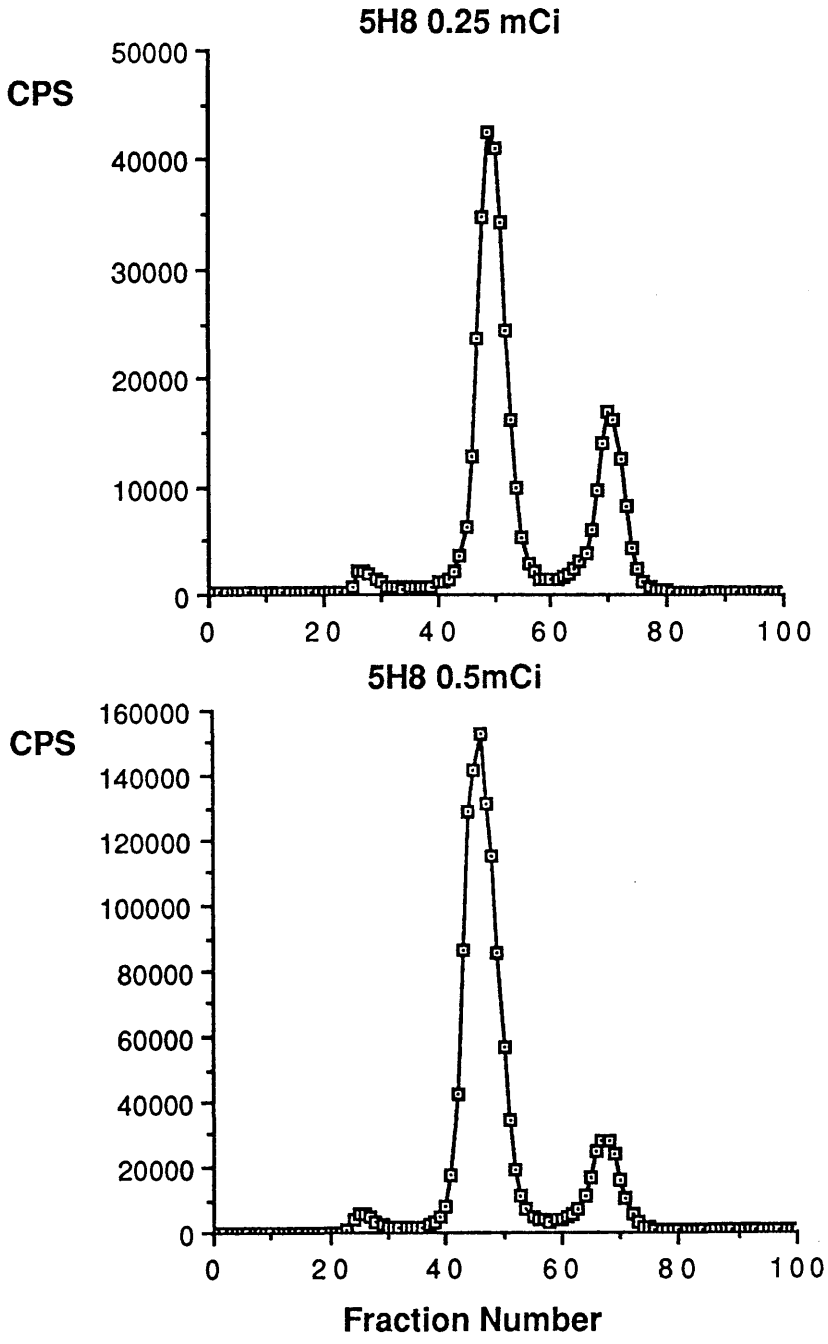


Figure 3.13

Profiles from the iodination of monoclonal antibody 5H8 with 0.25 and 0.5 mCi ^{125}I

TABLE 3.11

EFFECT OF SPECIFIC ACTIVITY AND LABEL MASS ON ASSAY SIGNAL: NOISE RATIO

21 µCi/µg LABEL

COUNTS ADDED	APPROX. MASS OF LABEL	SIGNAL: NOISE RATIO (30/0)
400,000 cpm	16 ng	4.2
200,000 cpm	8 ng	11.0
100,000 cpm	4 ng	20.0

9 µCi/µg LABEL

COUNTS ADDED	APPROX. MASS OF LABEL	SIGNAL: NOISE RATIO (30/0)
400,000 cpm	40 ng	3.1
200,000 cpm	20 ng	8.8
100,000 cpm	10 ng	13.0

this stage but the results strongly suggested that the higher specific activity label at 100,000 cpm/tube (approximately 4 ng) would render the better assay system and was therefore used in future experiments.

V DEVELOPMENT OF A TWO-SITE IRMA FOR SERUM TSH EMPLOYING LABELLED MONOCLONAL ANTIBODIES AND SOLID-PHASE POLYCLONAL ANTISERA

a) INTRODUCTION

In the optimisation of a two-site assay it is necessary to consider chiefly the achievable sensitivity and breadth of working range, together with speed and convenience. The factors which affect assay performance include affinity and concentration of antibodies, specific activity of labelled antibody, magnitude of non-specific binding, incubation times and technical reproducibility (Ekins, 1981; Hunter et al, 1982, 1983; Jackson et al, 1983). In reality, the optimum conditions chosen are a compromise between the conflicting requirements of sensitivity, working range and speed.

b) CELLULOSE OR SEPHAROSE AS SOLID-PHASE MATERIAL?

Buffer standard curves (0-100 mU/l TSH (WHO 80/558) in 0.1 mol/l borate buffer pH 8.0 containing 0.5% normal sheep serum, 0.2% Tween 20 and 0.05% sodium azide) were set up in duplicate using monoclonal antibody 5H8 as the labelled reagent. Standard and label were incubated for 90 minutes at ambient temperature prior to 90 minutes agitation with either cellulose or Sepharose solid-phase polyclonal TSH antibody (S117, Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire). Figure 3.14 displays standard curves obtained adopting this protocol. A lower non-specific binding (0.32%) and a higher specific binding (46%) was achieved with the Sepharose solid-phase in comparison with the cellulose preparation (0.68% and 39% respectively). As a result of this the signal:noise ratio for Sepharose was more than double (146) that for cellulose (58). The results suggested that Sepharose was a better solid-phase matrix for this system than cellulose because of its apparent greater capacity for coupling antibody, and its lesser tendency to bind material non-specifically.

c) INCUBATION PROTOCOL OPTIMISATION

For simplicity, single-step incubation protocol was attempted with a 60 minute agitation incubation with Sepharose solid-phase antibody. However, the binding

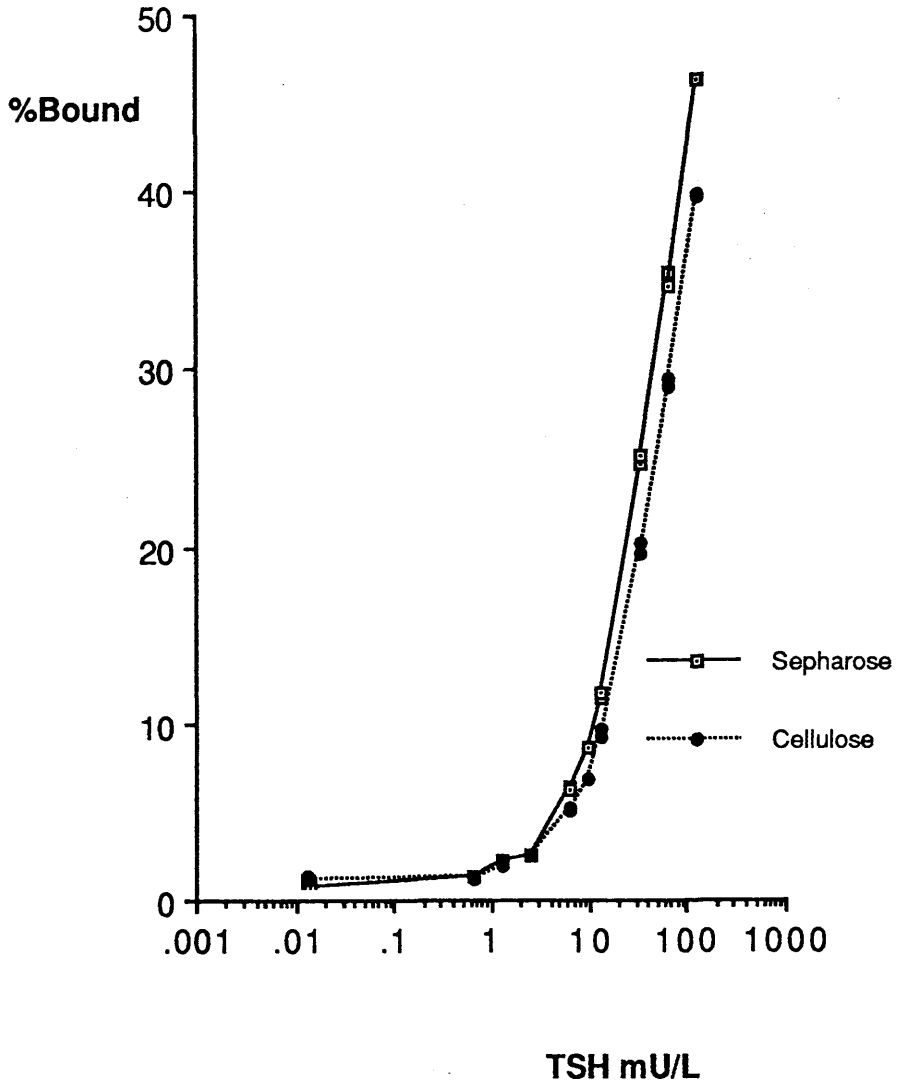


Figure 3.14

Standard curves with cellulose and Sepharose as the solid-phase material

achieved was poor, of the order of 16%, and the non-specific binding high, 0.5%, giving a signal:noise ratio of only 32 and an insensitive assay (Figure 3.15).

Standard curves (0-100 mU/l TSH) were performed with cellulose and Sepharose solid-phase antibodies to optimise the first incubation step (incubation of standard with labelled antibody). Incubation times of 30 minutes, 1 hour, 3 hours and 5 hours were selected with the agitation incubation remaining constant at 90 minutes. Non-specific binding ranged from 0.41% (Sepharose, 1 hour) to 0.90% (cellulose, 5 hours). Specific binding ranged from 28% (cellulose, 30 minutes) to 44% (Sepharose, 5 hours) (Figure 3.16). The optimal signal:noise ratio was achieved with the 1 and 3 hour Sepharose systems and a 2 hour first incubation step was therefore adopted.

However, although the specific binding looked promising for such a system, the non-specific binding was still unusually high for a two-site IRMA employing a monoclonal antibody. A possible explanation for the high blank phenomenon was inadequate washing of the assay, 3 washes being the adopted protocol to date. As a quick, simple experiment, before proceeding to optimise the agitation incubation, the Sepharose (1 hour) curve was further washed, resulting in an

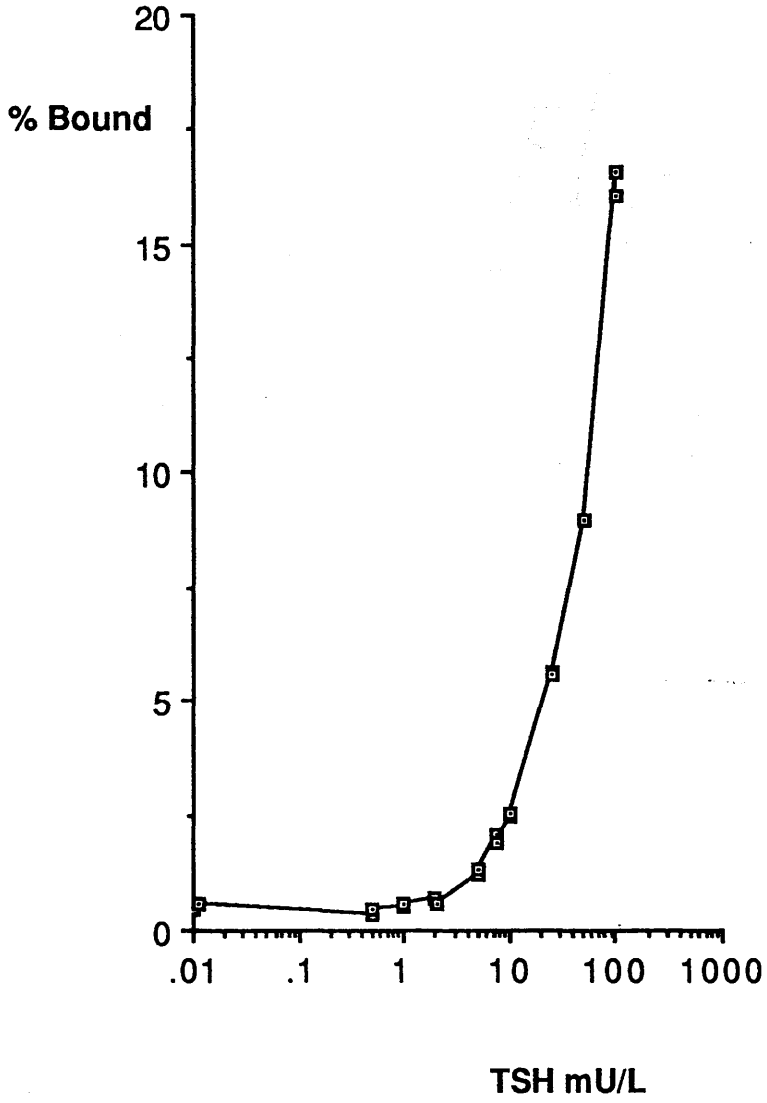


Figure 3.15

Standard curve produced after a single-step incubation protocol was attempted with a 60 minute agitation incubation with Sepharose solid-phase antibody

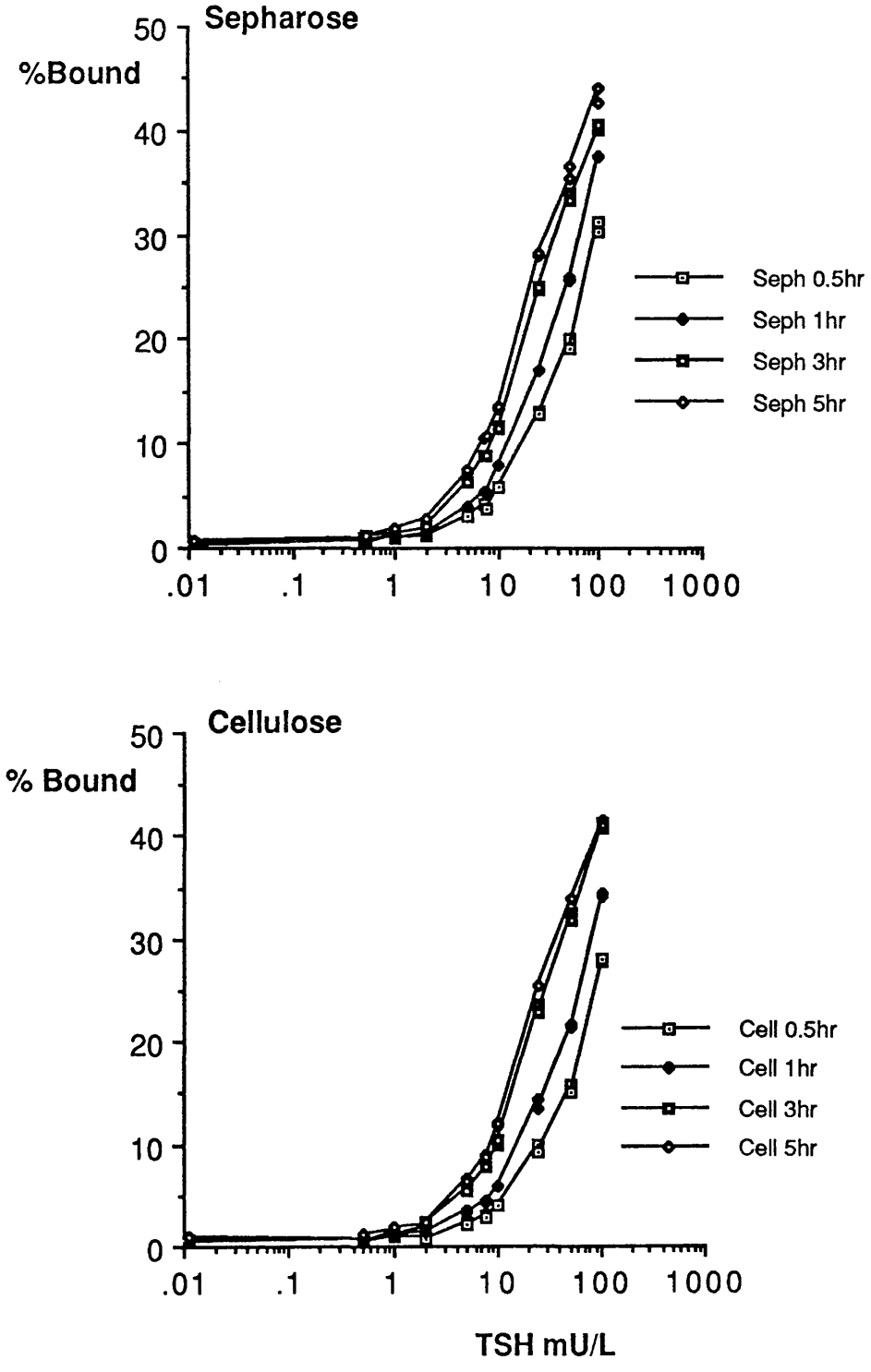


Figure 3.16

Optimisation of first incubation step (incubation of standard with labelled antibody) with cellulose and Sepharose solid-phase antibodies

improvement in the non-specific binding from 0.41% to 0.16% and in signal:noise ratio of 92 to 225 (Figure 3.17).

Standard curves (0-100 mU/l TSH) were performed with both cellulose and Sepharose solid-phase antibodies to optimise the agitation incubation. Incubation times of 30 minutes, 1 hour, 3 hours, 5 hours and 17 hours for both solid-phase matrices were selected, the first incubation remaining constant at 2 hours. Figure 3.18 displays the curves obtained. Non-specific binding ranged from 0.09% (Sepharose, 1 hour) to 0.27% (cellulose, 17 hours). Signal:noise ratios ranged from 101 (cellulose, 17 hours) to 381 (Sepharose, 3 hours). However, there was little difference between the signal:noise ratios for Sepharose, 3 hours (381) and Sepharose , 1 hour (365). As a consequence of this, and to suit the working day of the routine laboratory, an overall incubation protocol of 2 hours first incubation, followed by a 1 hour agitation with Sepharose solid-phase antibody, was adopted.

d) OPTIMISATION OF NUMBER OF WASHING STEPS

A TSH standard curve was performed under optimum incubation conditions. Post agitation the assay tubes were washed 3 to 9 times with 2 ml volumes of isotonic saline containing 0.2% Tween 20, the radioactivity being

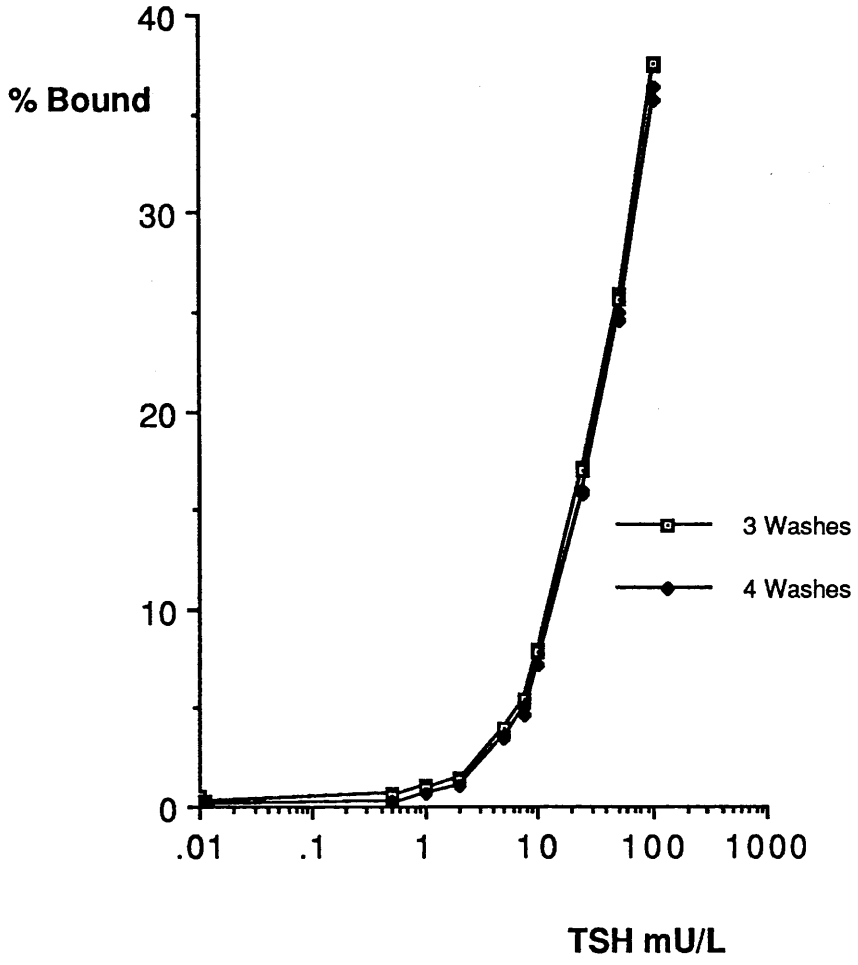


Figure 3.17

Dose-response curves obtained after 3 and 4 washes

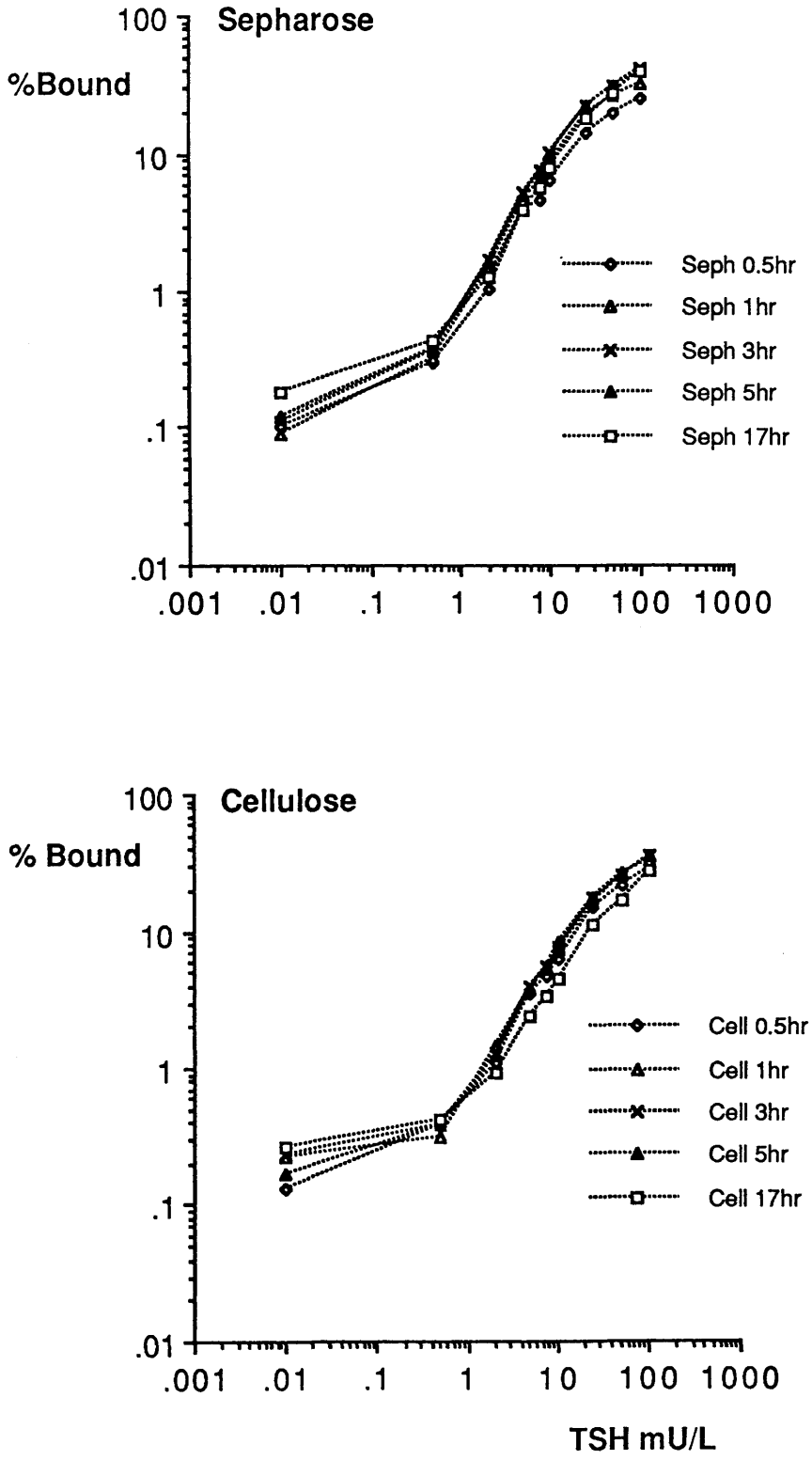


Figure 3.18

Optimisation of the agitation incubation with cellulose and Sepharose solid-phase antibodies

counted after each washing stage. Washing 3 times was found to be totally inadequate, giving a blank of 0.48% (Figure 3.19) and a signal:noise ratio of 71. Four washes improved the system, yielding a blank of 0.13% and a signal:noise ratio of 254. No further improvements were seen after 6 washes (blank 0.04%, signal:noise ratio 733).

e) OPTIMISATION OF WASH VOLUME

Standard curves were washed three and four times with 2 ml and 2.5 ml volumes of isotonic saline containing 0.2% Tween 20. Results are shown in Figure 3.20. As before, 3 washes were found to be inadequate although a slight improvement was observed with greater wash volume. With 4 washes, an increase in wash volume reduced the blank from 0.13% (signal:noise ratio 294) to 0.08% (signal:noise ratio 503) with the potential of producing a very sensitive system. Further increases in wash volume had little effect on the blank level but 3 washes were found to be sufficient when a wash volume of 3 ml was used. A similar level of non-specific binding was noted when isotonic saline without Tween 20 was used as the wash solution. Moreover, increasing Tween 20 concentrations did not appear to affect the blank level.

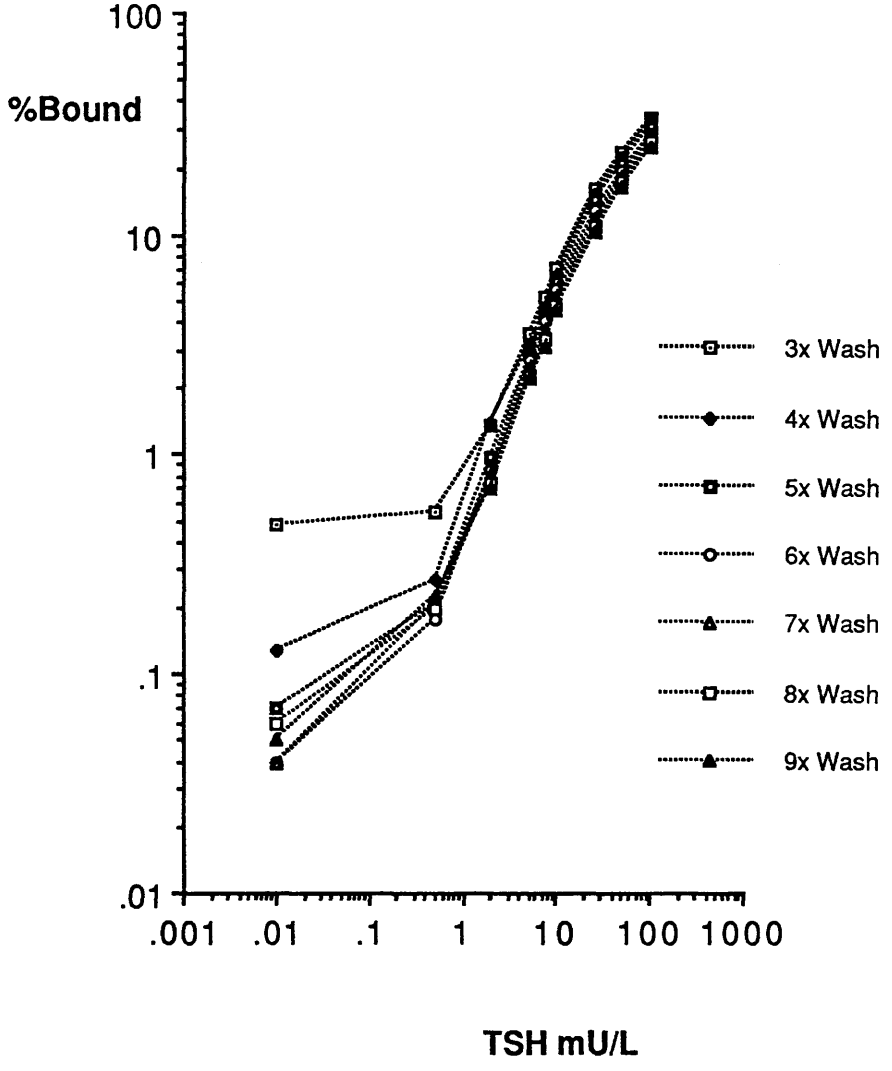


Figure 3.19

Dose-response curves obtained after washing 3 to 9 times with 2 ml volumes of isotonic saline containing 0.2% Tween 20

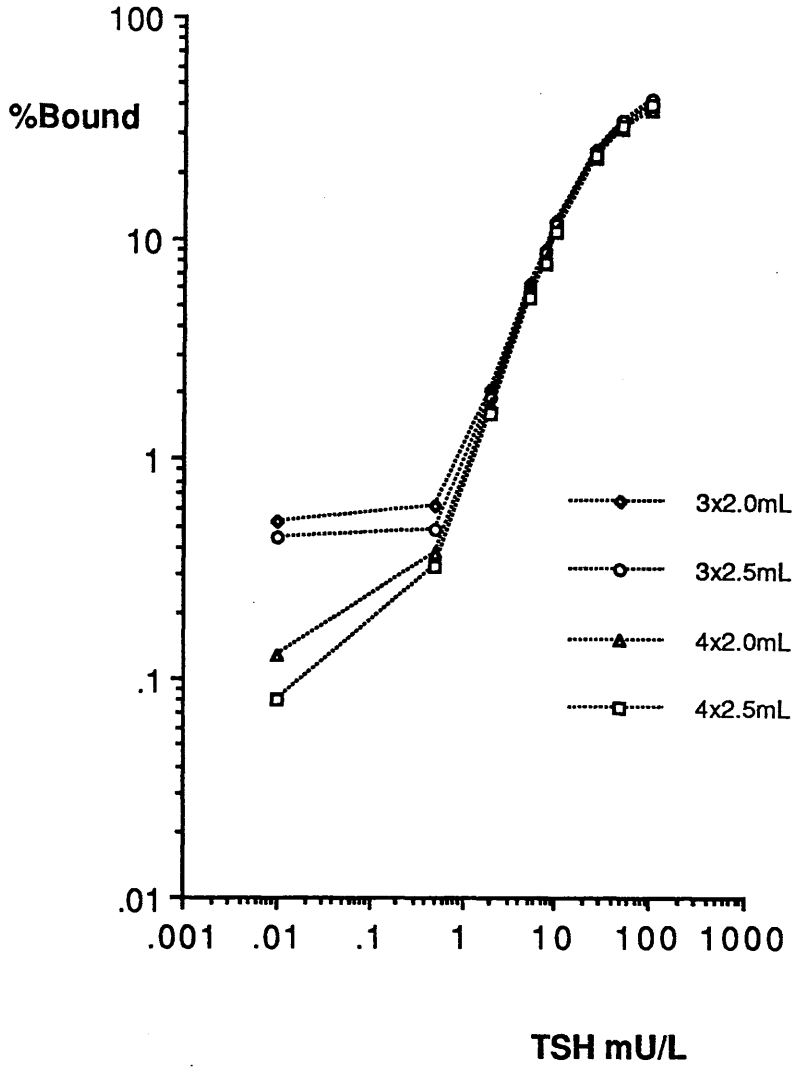


Figure 3.20

Dose-response curves obtained after washing three and four times with 2 ml and 2.5 ml volumes of isotonic saline containing 0.2% Tween 20

f) OPTIMISATION OF BUFFER SYSTEM

Standard curves (0-100 mU/l TSH) were performed in various buffers, 0.05 mol/l phosphate pH 7.4, 0.05 mol/l phosphate pH 7.4 containing 0.9% NaCl, 0.05 mol/l barbitone pH 8.6, 0.1 mol/l EPPS pH 8.0 and 0.1 mol/l borate pH 8.0. All buffers also contained 0.5% normal sheep serum, 0.2% Tween 20 and 0.05% NaN_3. Similar standard curves were obtained for all buffer systems with the exception of phosphate pH 7.4 which gave a high blank (0.44%) and poor specific binding, of the order of 15% (Figure 3.21). On the basis of ease of preparation EPPS, pH 8.0 was chosen as assay diluent.

g) LABEL REPURIFICATION

Aliquots (1 ml) of labelled 5H8 were subjected to repurification by column chromatography (Sepharose 6B) (Figure 3.22) and by end over end rotation for 10 minutes with Amberlite, 10 and 30 days post iodination, in an attempt to increase the shelf-life of the label. Figure 3.23 displays standard curves obtained 10 days post iodination for untreated, Amberlite treated and column repurified 5H8. The results indicated that after 10 days little or no benefit was to be gained by label repurification. However, after 30 days storage of label, specific binding was vastly improved by treatment with Amberlite (10% to 17%) and further

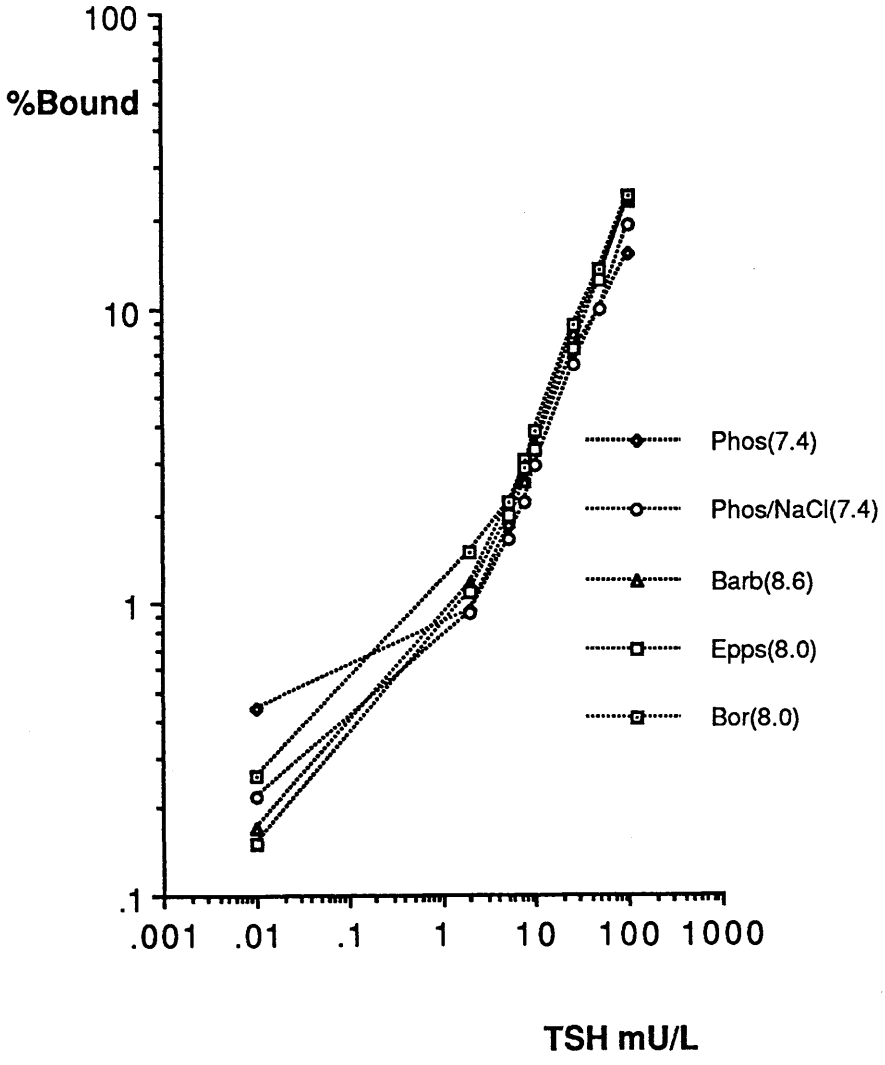


Figure 3.21

Optimisation of buffer system

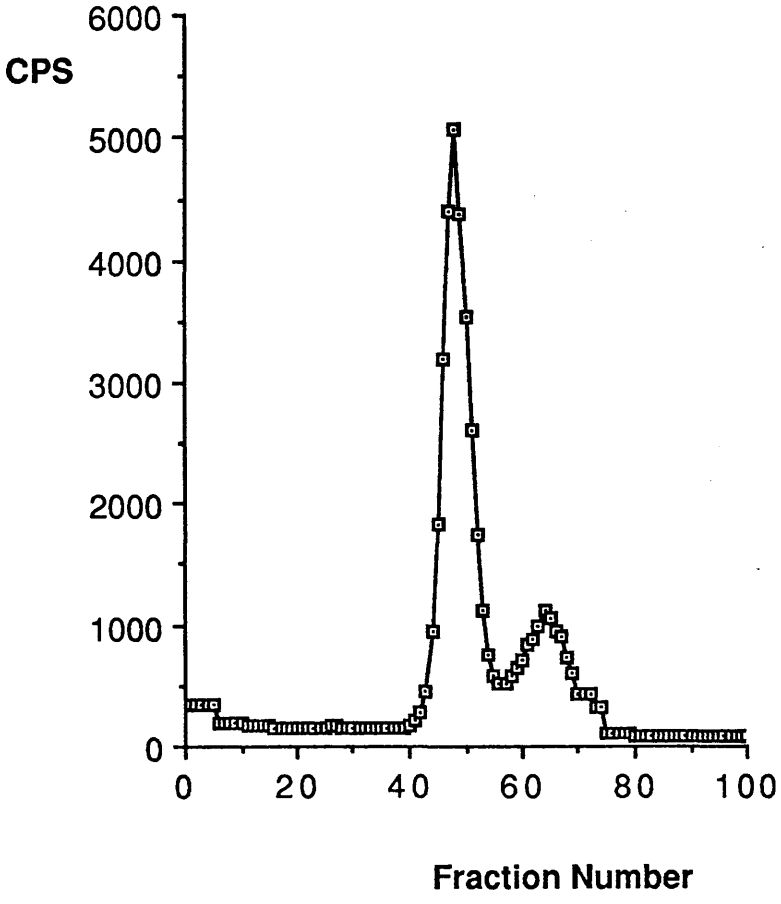


Figure 3.22

Repurification of labelled 5H8 by column chromatography (Sepharose 6B)

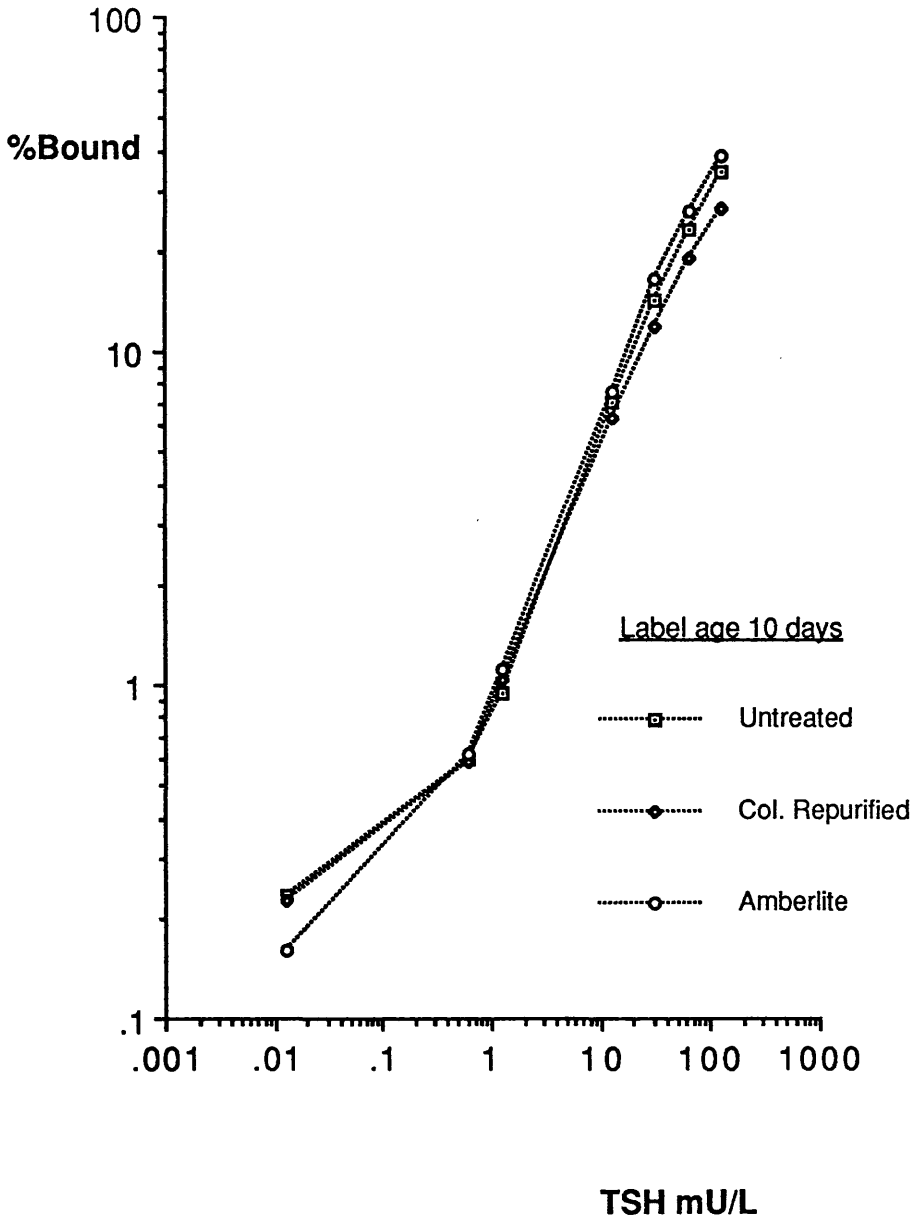


Figure 3.23

Standard curves obtained 10 days post iodination for untreated, Amberlite treated and column repurified 5H8

improved to 25% after column repurification (Figure 3.24). No detrimental effect on non-specific binding with label age was observed.

h) PARALLELISM

Serum samples from six hypothyroid patients were serially diluted in assay buffer (EPPS, pH 8.0), and assayed in duplicate against a buffer standard curve (0-100 mU/l TSH). Results of 2 of such samples are shown in Figure 3.25, indicating that hypothyroid serum samples diluted in parallel to the buffer standard curve.

i) HIGH-DOSE HOOK EFFECT

At very high antigen concentration many immunoradiometric assays show a phenomena which is known as the 'high-dose hook effect'. In this situation, where the labelled antibody can only bind a fraction of the total antigen concentration, a maximum signal is only achieved provided that the antigen concentration does not exceed the capacity of the solid-phase. When the antigen concentration exceeds the capacity of the solid-phase there is a progressive loss of signal. This leads to a falsely low antigen concentration being measured. For this reason an

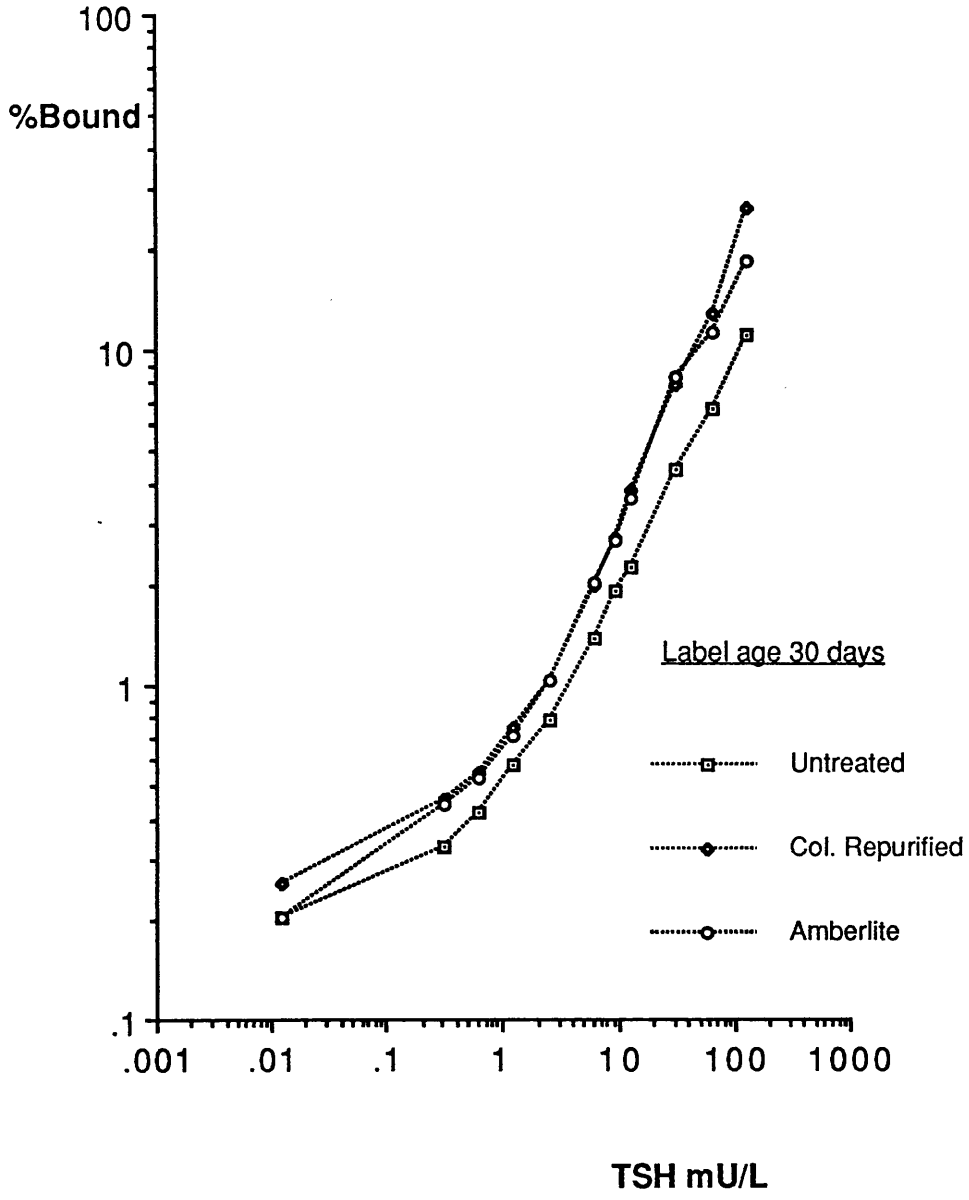


Figure 3.24

Standard curves obtained 30 days post iodination for untreated, Amberlite treated and column repurified 5H8

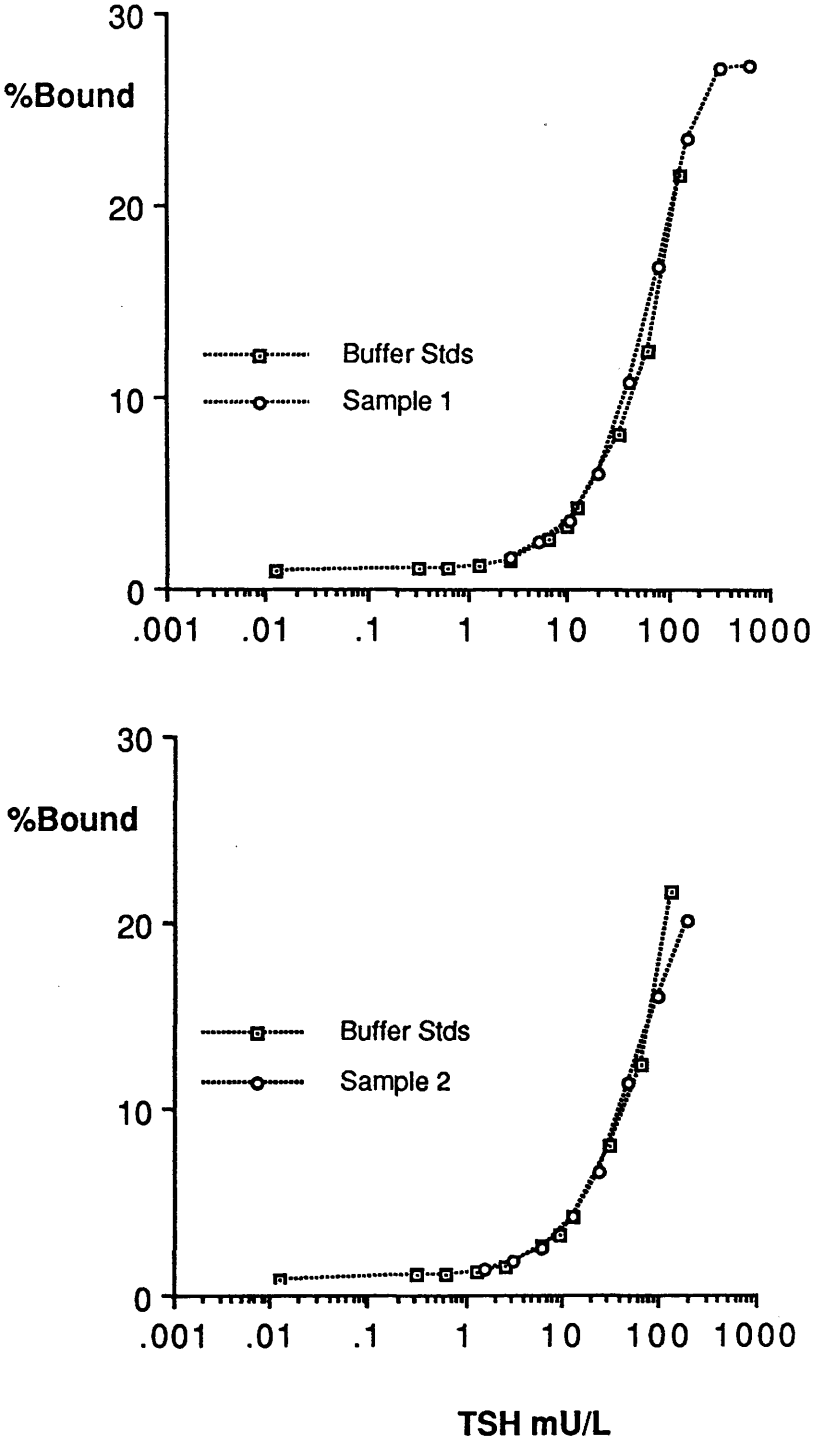


Figure 3.25

Parallelism studies with serum from 2 hypothyroid patients

examination of whether such an effect takes place must be performed before increasing the range of the standard curve. An extended buffer standard curve was performed covering the range 0-500 mU/l TSH. Such a curve is shown in Figure 3.26. At a standard concentration of around 250 mU/l TSH, the curve began to plateau. This was not considered to be a problem since this TSH level was generally much higher than the patients' samples to be measured.

j) RECOVERY OF ADDED I.R.P.TSH (WHO 80/558)

In principle, solutions of standard must be diluted in the same medium as constitutes the samples to be added in the assay. For measurement of human serum samples it is therefore necessary to have access to analyte-free serum for standard preparations unless it can be shown that there are no non-specific serum effects compared to a chosen buffer medium, on the assay reactions.

Traces of immunoreactive material were removed from human serum by immunoadsorption using 100 mg Sepharose solid-phase TSH polyclonal antibody. Serum (100 ml) was rotated end over end overnight with the Sepharose preparation, centrifuged for 30 minutes, and the immunoadsorbed serum removed and tested. Untreated serum gave a binding level of 1.14%, but following immunoadsorption this level was reduced to 0.35%.

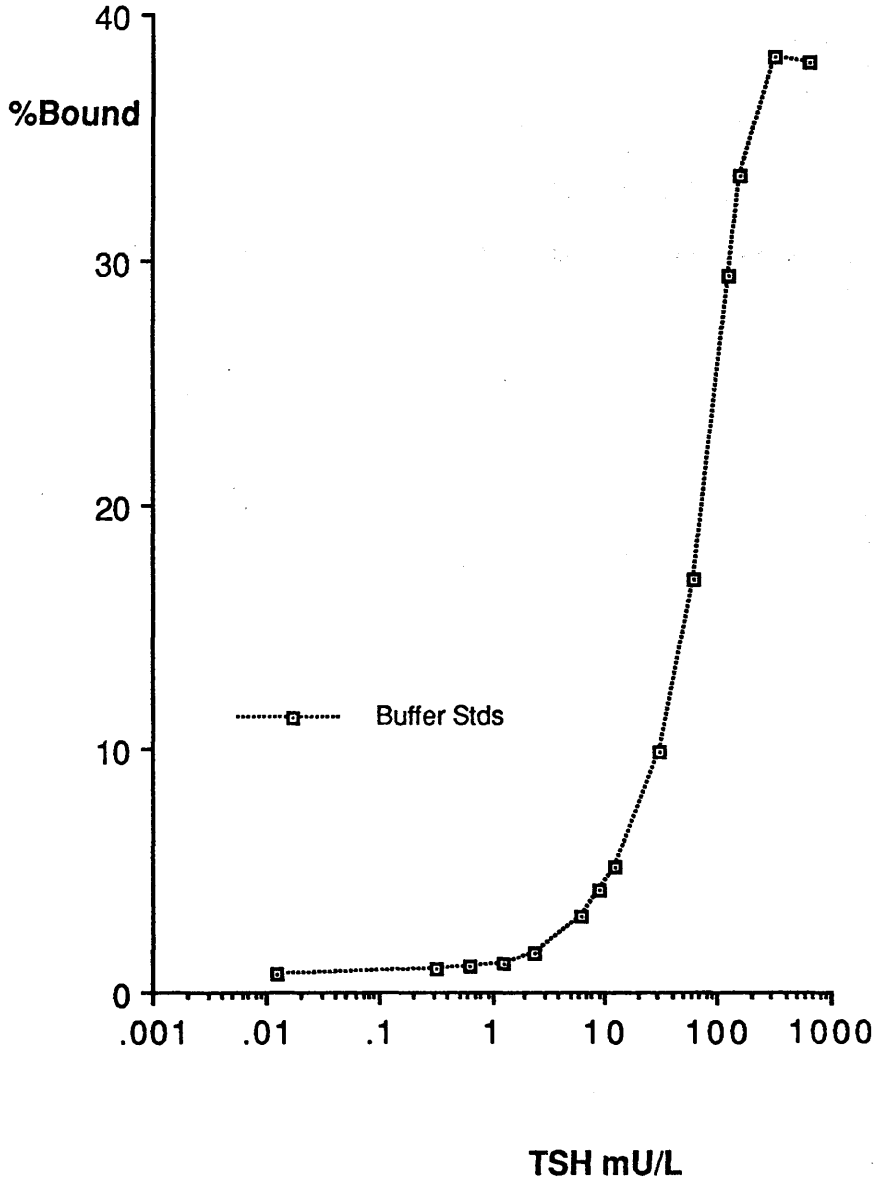


Figure 3.26

High-dose hook effect

To obtain TSH free serum for recovery pools, 5 healthy volunteers were given T_3 and the following day 20 ml of blood was removed. Aliquots of each of the 5 sera were spiked with 1, 10 and 50 mU/l TSH and assayed against buffer and serum standard curves (0-100 mU/l TSH) under optimum conditions (Figure 3.27). Results are displayed in Table 3.12. Better recoveries were obtained with the serum standard curve indicating the need for a serum standard matrix.

Spiked sera (3) at 1, 10 and 50 mU/l TSH were assayed against 3 different serum standard curves (0-200 mU/l TSH), human, horse (Gibco, Paisley, UK) and foetal calf (Flow Laboratories, UK) sera. Standard curves are displayed in Figure 3.28. Results shown in Table 3.13 indicated that foetal calf serum was a poor substitute for human serum as far as recovery of added TSH was concerned. Horse serum, on the other hand, gave comparable recoveries to those obtained previously with human serum and was subsequently selected for the TSH standard matrix because of its availability and cost.

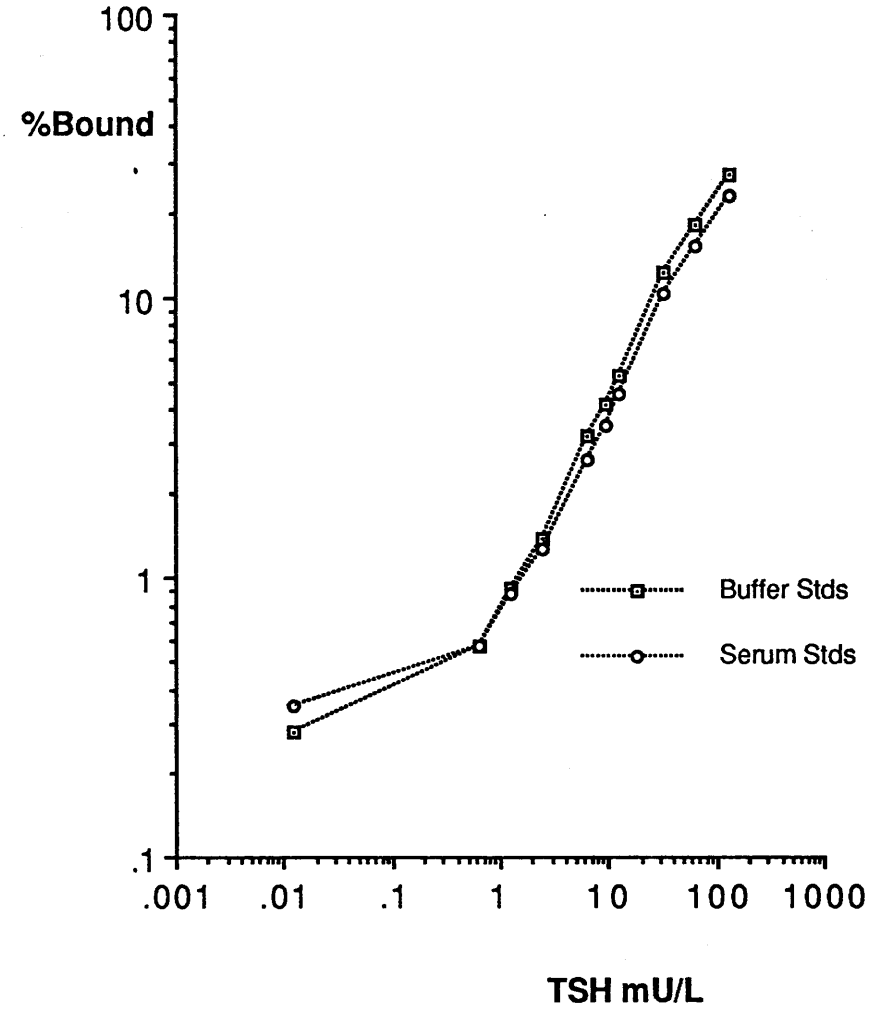


Figure 3.27

Buffer and serum dose-response curves

TABLE 3.12

RECOVERY OF ADDED I.R.P. TSH 80/558 FROM BUFFER AND HUMAN SERUM

SERUM	SERUM STD. CURVE	BUFFER STD. CURVE
A		
1	100%	103%
10	76%	70%
50	89%	70%
B		
1	90%	90%
10	80%	69%
50	95%	76%
C		
1	70%	70%
10	83%	74%
50	96%	81%
D		
1	100%	100%
10	83%	75%
50	96%	79%
E		
1	90%	100%
10	87%	78%
50	94%	78%
	MEAN = 89%	MEAN = 81%

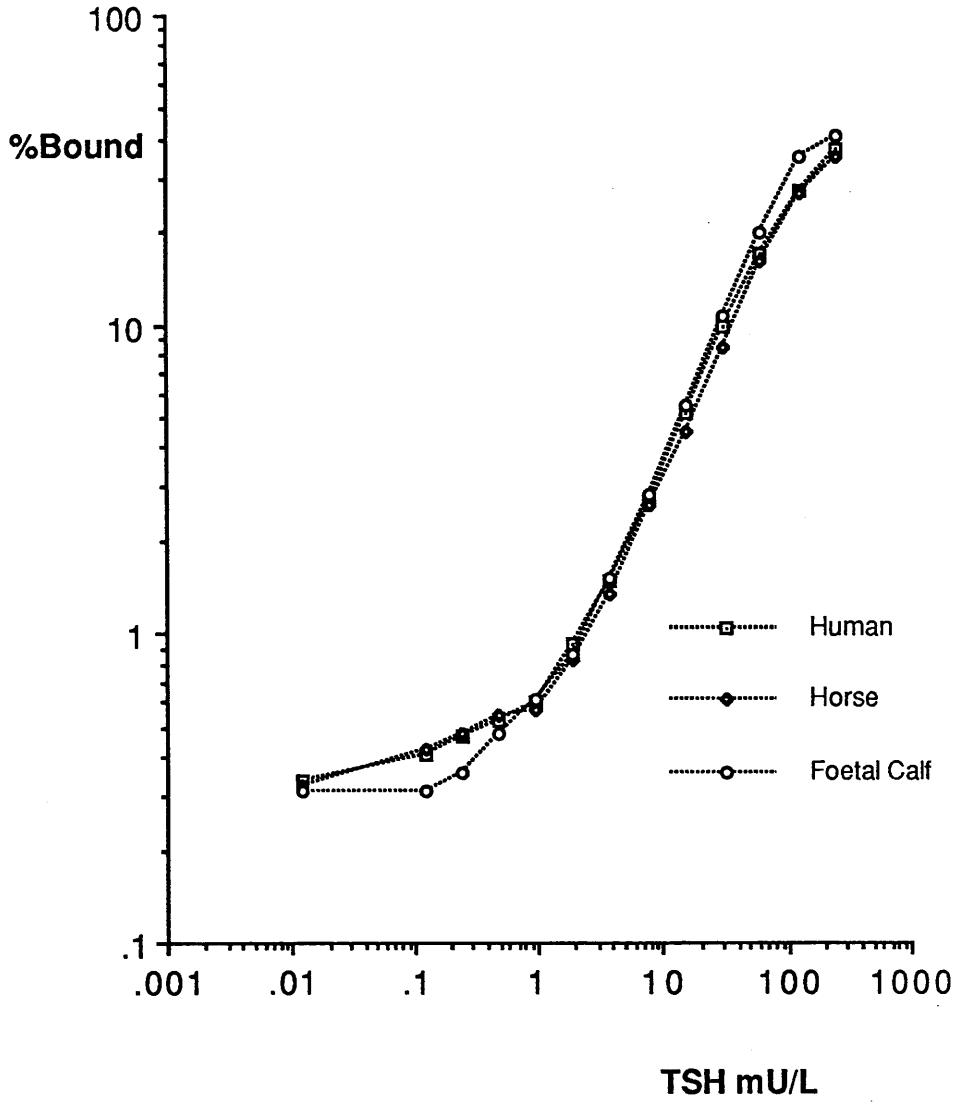


Figure 3.28

Human, horse and foetal calf serum standard curves

TABLE 3.13

RECOVERY OF ADDED I.R.P. TSH 80/558 FROM THREE DIFFERENT SERA

SERUM	HUMAN SERUM	HORSE SERUM	FOETAL CALF SERUM
A			
1	60%	50%	40%
10	82%	92%	73%
50	73%	80%	61%
B			
1	80%	80%	70%
10	92%	104%	86%
50	101%	115%	85%
C			
1	60%	70%	70%
10	90%	108%	85%
50	96%	106%	80%
	MEAN = 82%	MEAN = 89%	MEAN = 72%

k) COMPARISON BETWEEN CENTRIFUGATION AND DENSITY SEDIMENTATION AS A SEPARATION SYSTEM FOR THE SERUM TSH IRMA

Standard curves (0-100 mU/l TSH) were set up in duplicate under optimum conditions. One curve was separated by the usual centrifugation method (3 x 3 ml washes with isotonic saline), the other by density sedimentation (2 x 3 ml washes with 10% sucrose), as described in Chapter 2, Section Vd. Standard curves for both systems are shown in Figure 3.29. No difference in specific binding was noted between systems, but a vast improvement in non-specific binding resulted with the density sedimentation system over the centrifugation system. Precision profile analysis (intra-assay) (Chapter 1, Section IIIc) was performed, giving sensitivities (dose levels at 22% CV) of 0.4 and 0.2 mU/l TSH for centrifugation and density sedimentation respectively (Figure 3.30). With the centrifugation system a working range (<10% CV) of 1.2 to 200 mU/l TSH was achieved whereas the density sedimentation system offered a wider working range of 0.7 to 200 mU/l TSH.

(1) NORMAL RANGE

To establish a normal range for the TSH two-site IRMA, 476 normal human serum samples (male and female) were obtained from blood donors via the Blood Transfusion

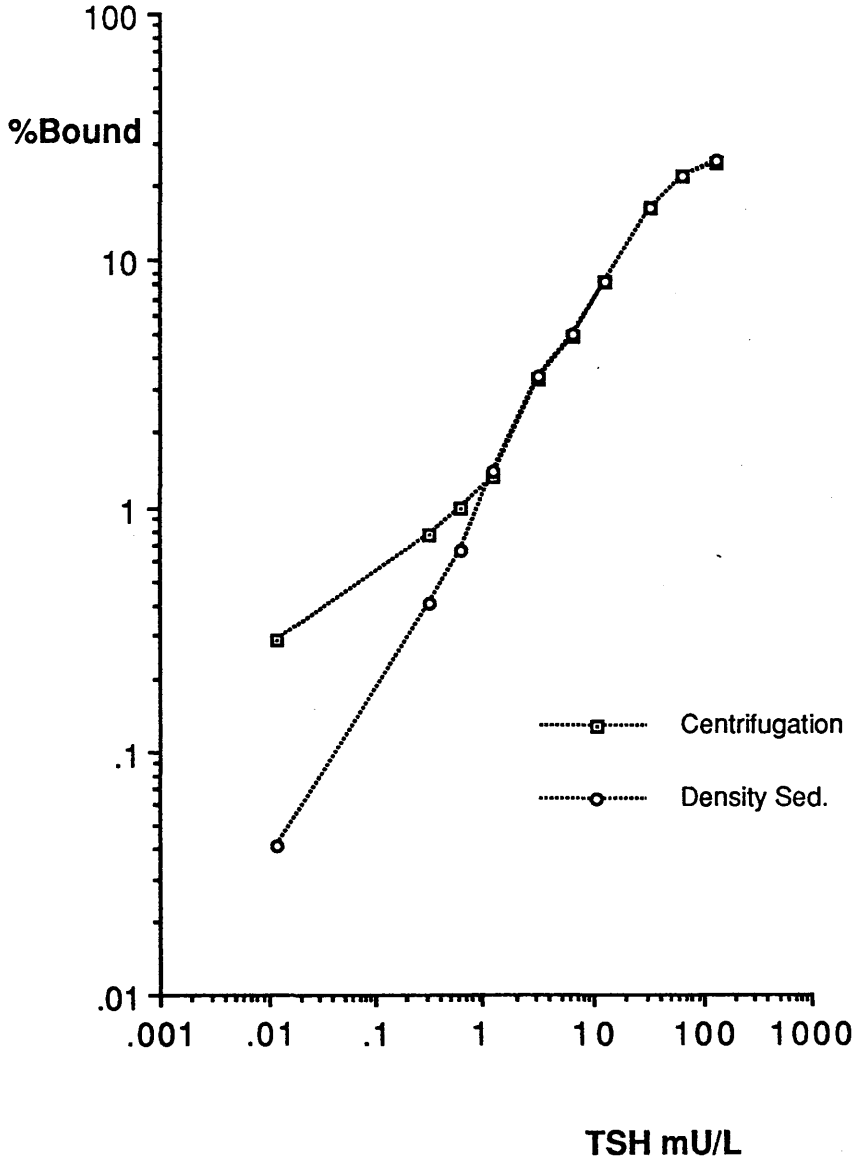


Figure 3.29

Comparison between centrifugation and density sedimentation as a separation system for the serum TSH IRMA

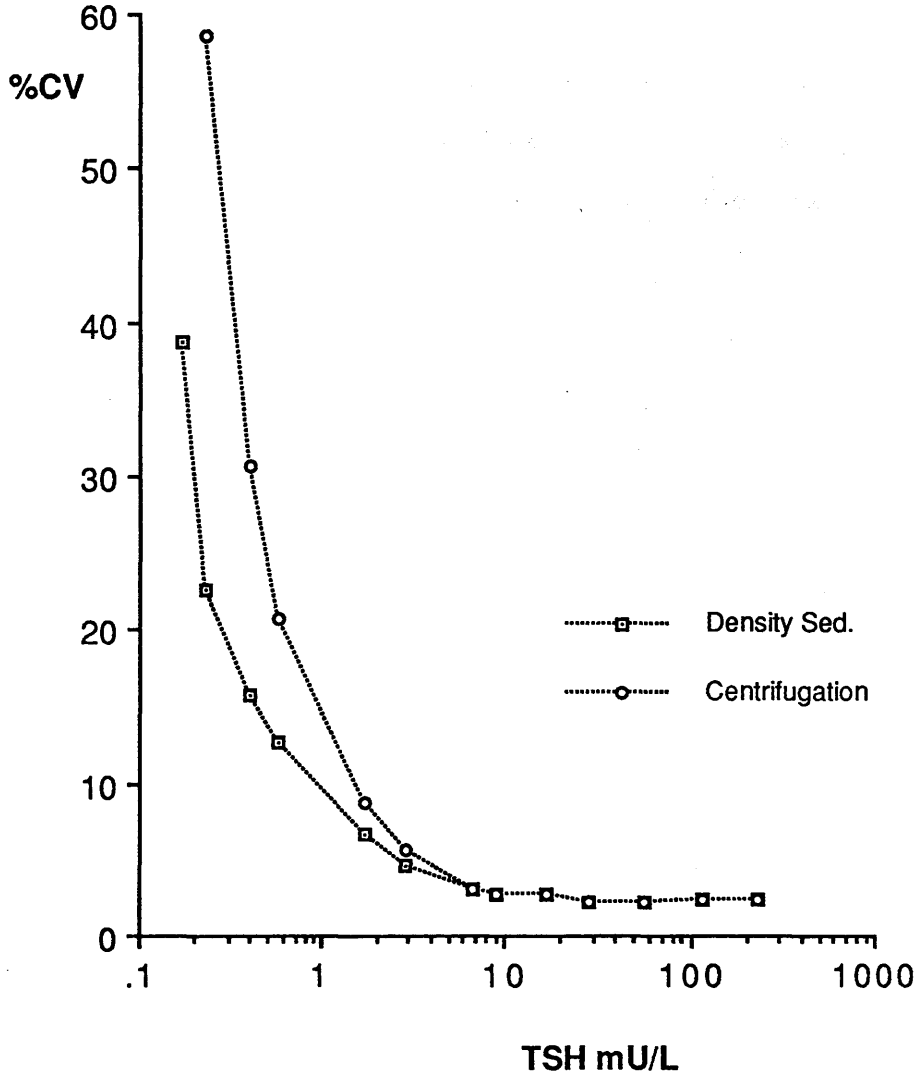


Figure 3.30

Precision profile analysis (intra-assay) for centrifugation and density sedimentation separation systems

Service, Law Hospital, Carluke, Lanarkshire, and each sample assayed in duplicate using the density sedimentation separation system. Figure 3.31 shows a histogram representation of the results produced for the 476 samples. Figures 3.32 and 3.33 display the normal range obtained for males (n=211) and females (n=265) respectively. Both groups produced similar sample means, 1.51 $\pm$ 0.73 for males and 1.46 $\pm$ 0.76 for females. On the basis of this, a normal range (95% of the population) of 0.35 to 3.5 mU/1 TSH was established, as illustrated in Figure 3.34.

VI DEVELOPMENT OF A TWO-SITE IRMA FOR BLOOD-SPOT TSH EMPLOYING LABELLED MONOCLONAL ANTIBODIES AND SOLID-PHASE POLYCLONAL ANTISERA

a) INTRODUCTION

An ideal screening assay for neonatal hypothyroidism requires to be quick and simple to perform, rugged and reliable in routine practice and have adequate sensitivity and specificity. With the above criteria in mind, the blood-spot assay was optimised in a similar manner to that of the serum TSH assay.

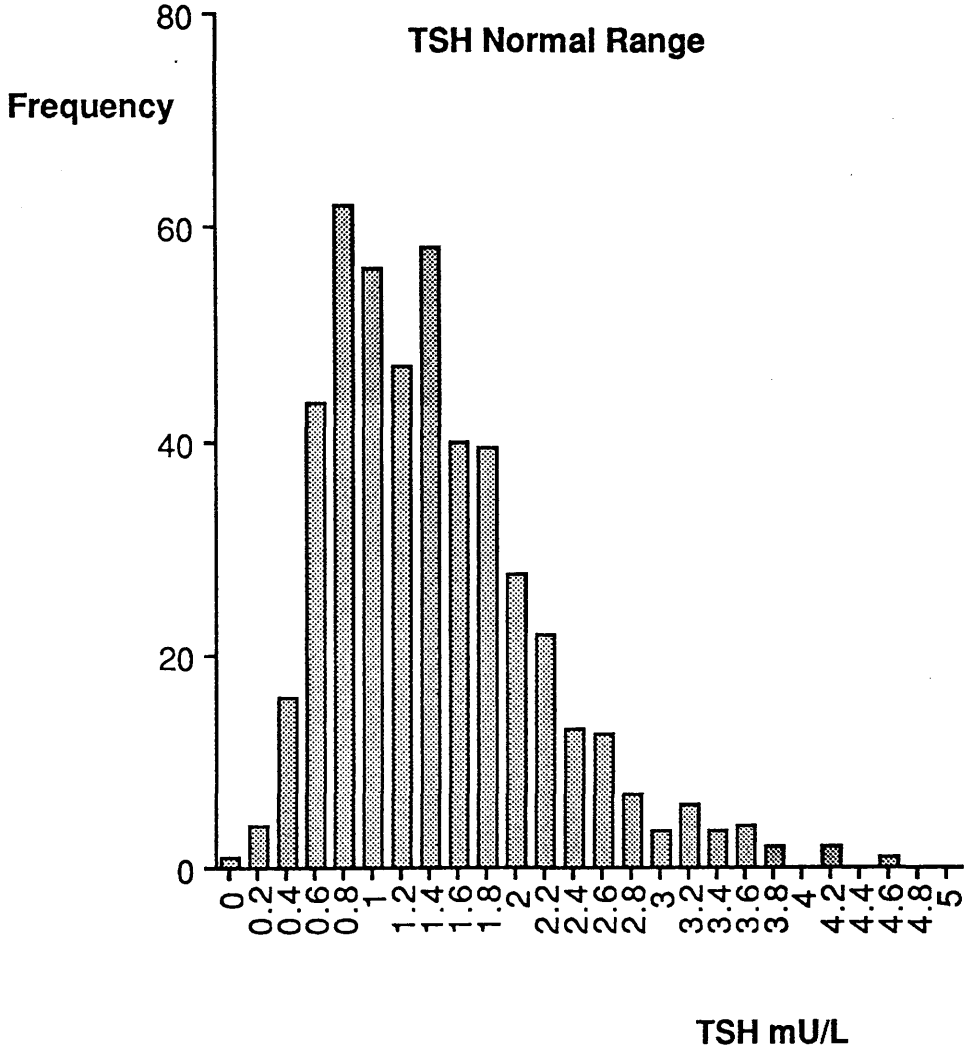


Figure 3.31

Histogram representation of the results produced for the 476 normal human serum samples (male and female) obtained from blood donors

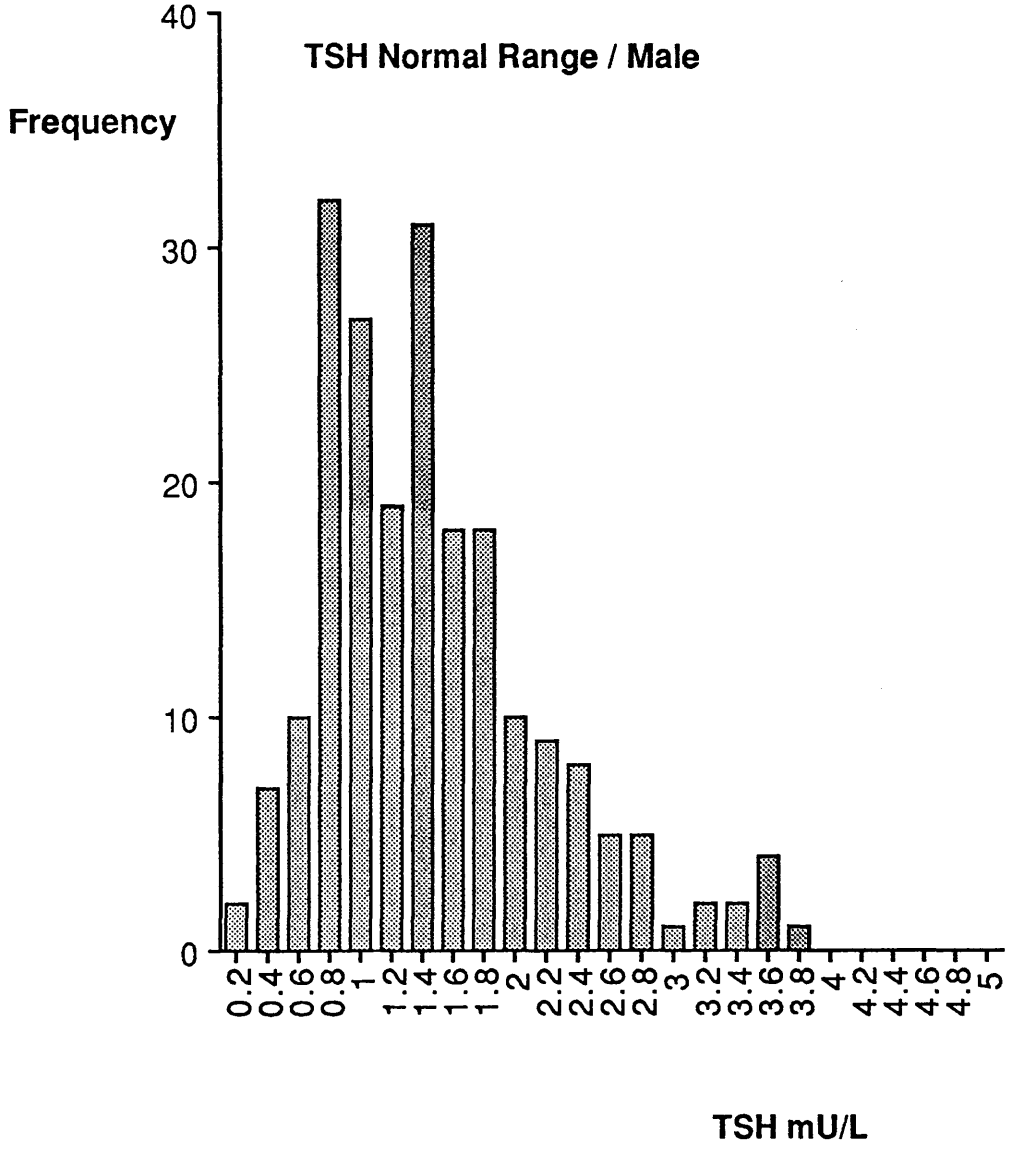


Figure 3.32

Normal range obtained for males (n = 211)

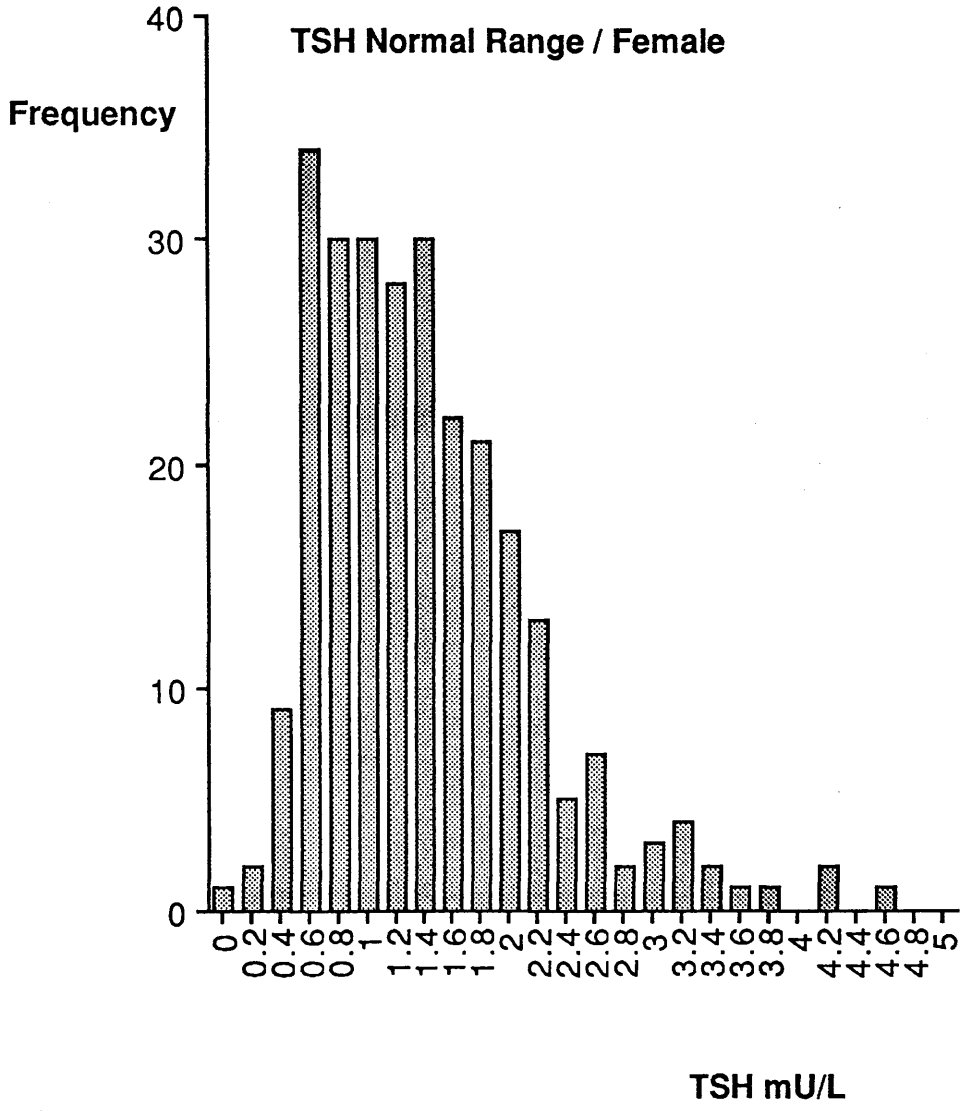


Figure 3.33

Normal range obtained for females (n = 265)

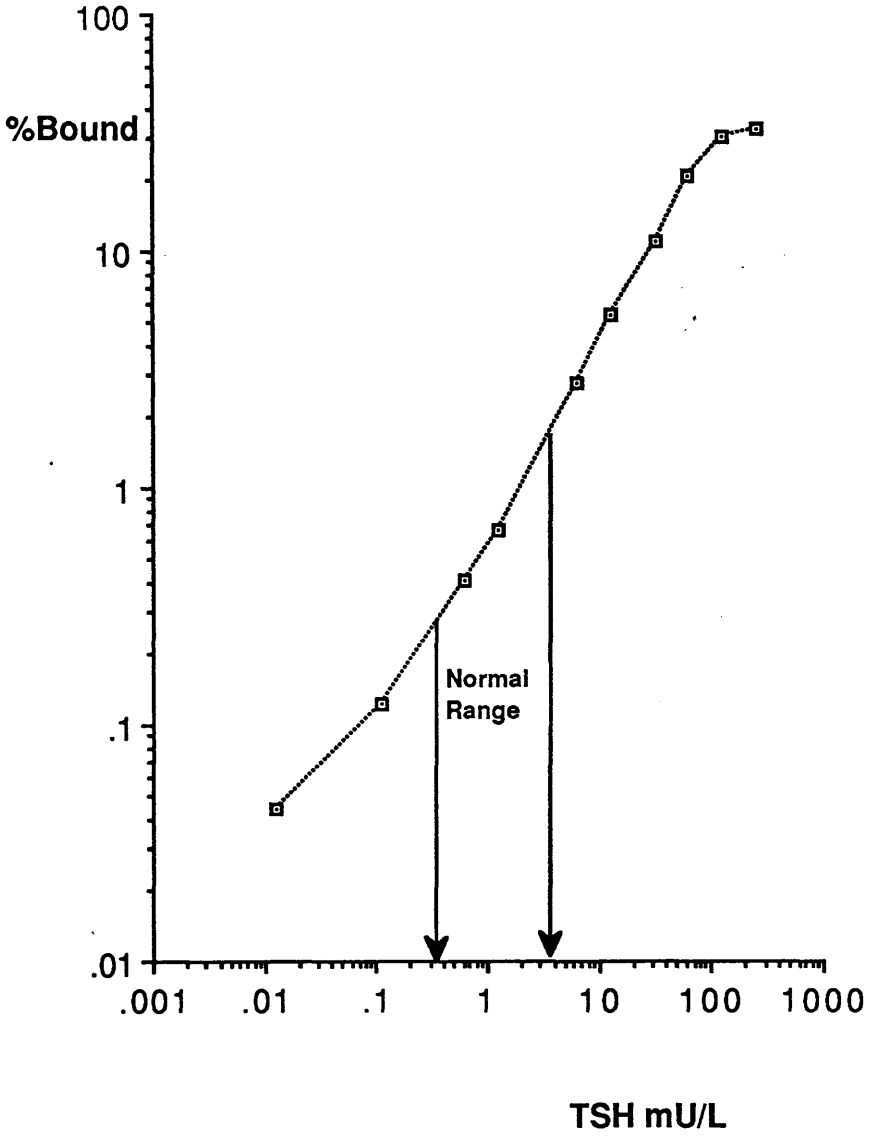


Figure 3.34

TSH dose-response curve displaying the established normal range (95% of the population) of 0.35 to 3.5 mU/l TSH for the serum IRMA

b) CELLULOSE OR SEPHAROSE AS SOLID-PHASE MATERIAL?

Standard TSH (0-210 mU/l), in the form of a 6 mm diameter blood-spot, was incubated overnight with 0.2 ml labelled 5H8 (100,000 cpm = 4 ng) in 0.1 mol/l borate pH 8.0 containing 0.5% normal sheep serum, 0.2% Tween 20 and 0.05% NaN_3, prior to 90 minutes agitation with either cellulose or Sepharose solid-phase polyclonal TSH antibody (S117). The tubes were then washed 4 times with 2 ml volumes of 0.9% NaCl containing 0.2% Tween 20. Similar results were obtained for both systems, as shown in Figure 3.35, each giving a non-specific binding of the order of 0.7% and a signal:noise ratio of 35.

c) WASH SOLUTION

Standard curves (0-300 mU/l TSH) were washed either with isotonic saline or isotonic saline containing 0.2% Tween 20. The results shown in Figure 3.36 indicated that there was no requirement for Tween 20 in the wash solution for the blood-spot assay, as was the case for the serum TSH assay. This was a change brought about by the introduction of the monoclonal antibody as label, since both serum and blood-spot assays employing labelled polyclonal antibodies required Tween 20 in the wash solution.

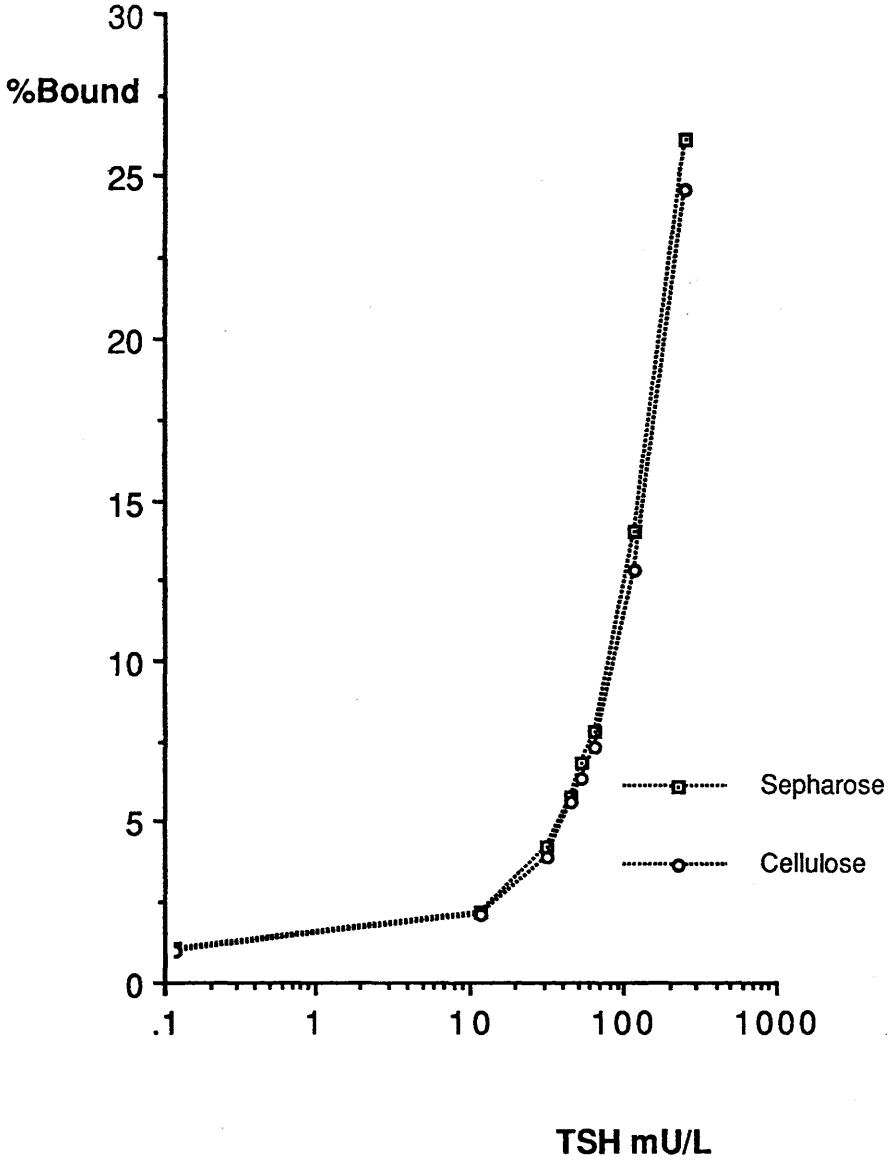


Figure 3.35

Blood-spot standard curves with cellulose and Sepharose as the solid-phase material

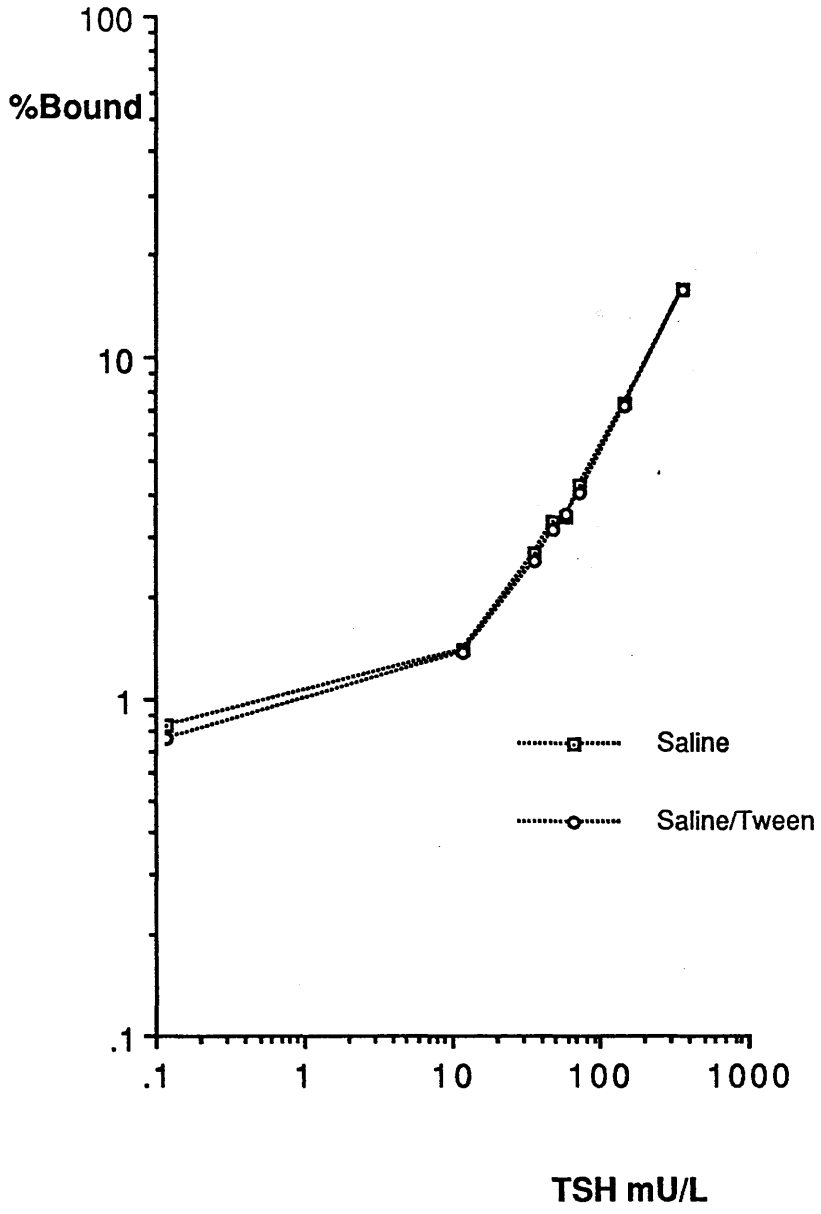


Figure 3.36

Comparison between saline and saline/Tween as the wash solution for the blood-spot TSH IRMA

d) EFFECT OF SAMPLE SIZE

Standard curves (0-300 mU/l TSH) were performed in duplicate, either with a 5 or 6 mm diameter disc. Results are shown in Figure 3.37. A dramatic decrease in specific binding was observed with the 5 mm disc (18% to 11%) and a corresponding decrease in signal:noise ratio, indicating the importance of sample size.

e) RECOVERY OF ADDED I.R.P. TSH (WHO 80/558).

TSH was added to TSH free serum, before mixing with packed red cells, to give recovery pools with values of 25, 50 and 100 mU TSH/l whole blood. These recovery pools yielded mean recoveries of 96%, 103% and 110% respectively when assayed against a blood spot standard curve

VII 2-MONOCLONAL IRMA FOR SERUM TSH

IgG fractions of monoclonal antibodies 2G2, 4F12, TS5, TQ5 and TR2 were obtained by treating 5 ml volumes of ascitic fluid (tap >3) with 0.5 ml caprylic acid (n-Octanoic acid). Each monoclonal antibody IgG fraction (10 mg) was then coupled to Sepharose Cl-4B (20 ml) prior to assay, with 5H8 as label. A standard curve (0-185 mU/l TSH) with polyclonal

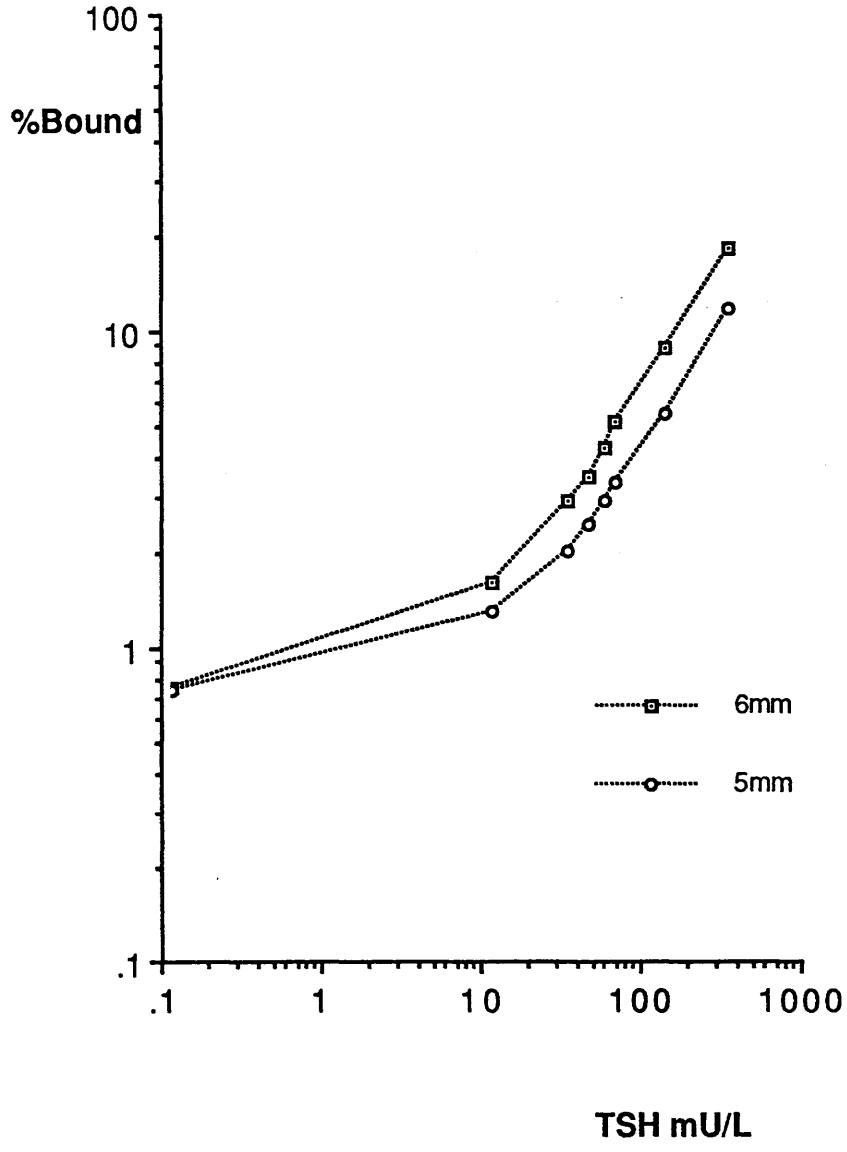


Figure 3.37

Effect of sample size on the blood-spot TSH IRMA

TSH antibody coupled to Sepharose Cl-4B was also set up as a control. Results are shown in Figure 3.38. Three of the antibodies, TS5, TR2 and TQ5, gave very poor signal:noise ratios of 10, 2 and 2 respectively, compared with that of the polyclonal antibody (138). 4F12 yielded a steeper standard curve with a signal:noise ratio of 44, but the best monoclonal antibody system was obtained with 2G2 with a signal:noise ratio of 195, as expected from previous avidity and specificity studies (Section IIIb).

VIII 2-MONOCLONAL IRMA FOR BLOOD-SPOT TSH

The performance of monoclonal antibodies 2G2 and 4F12 was assessed in the blood-spot assay, using 5H8 as label and Sepharose solid-phase polyclonal TSH antibody as a control. Results are shown in Figure 3.39. Similar blanks were obtained for all 3 systems, 0.74%, 0.72% and 0.72% for 2G2, 4F12 and polyclonal antibody respectively. As before, 4F12 produced a poor signal:noise ratio (9), whereas 2G2 gave a signal:noise ratio of 21, which was comparable to that of the polyclonal antibody (22).

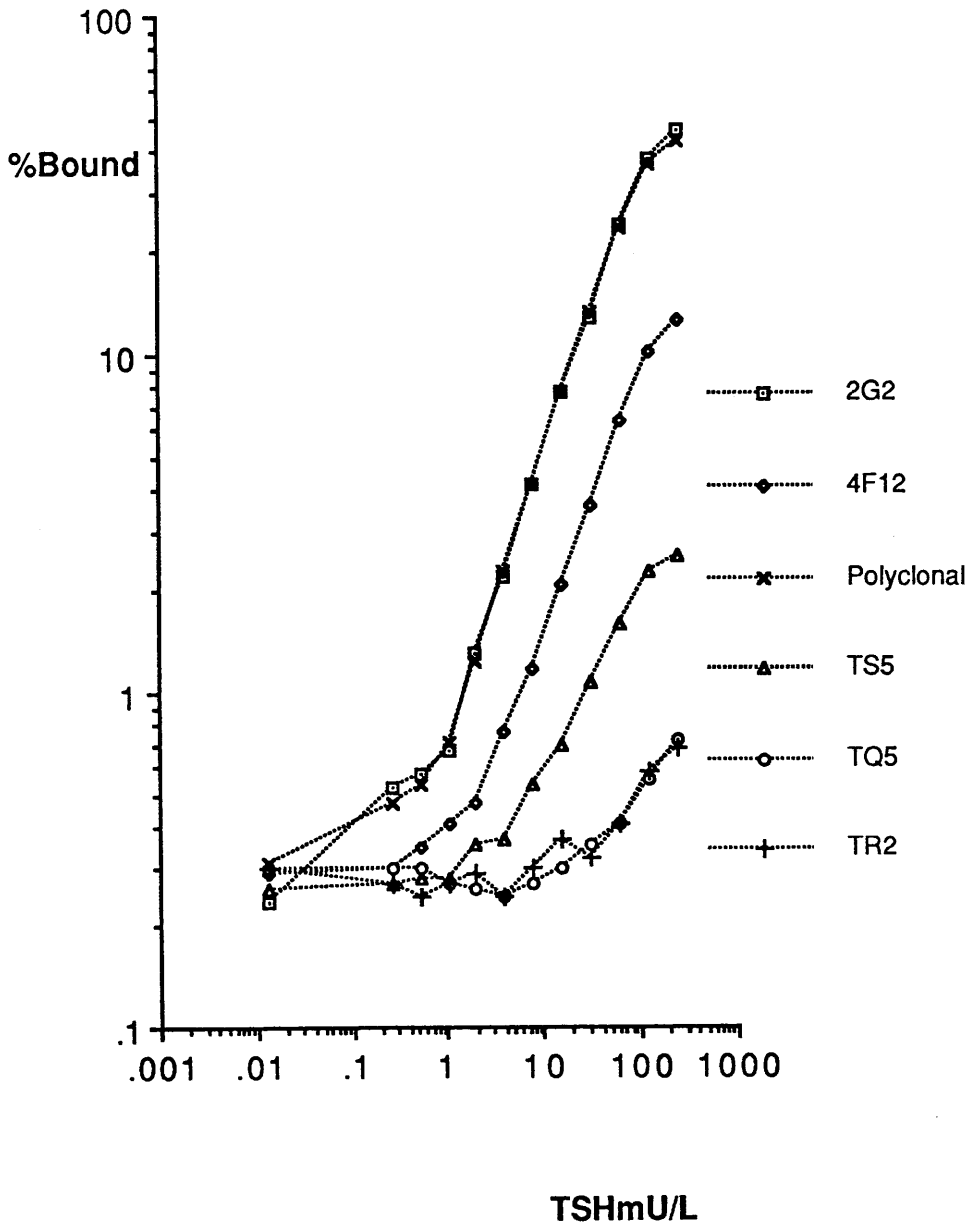


Figure 3.38

Dose-response curves obtained with 5 different monoclonal antibodies coupled to Sepharose Cl-4B solid-phase using 5H8 as label

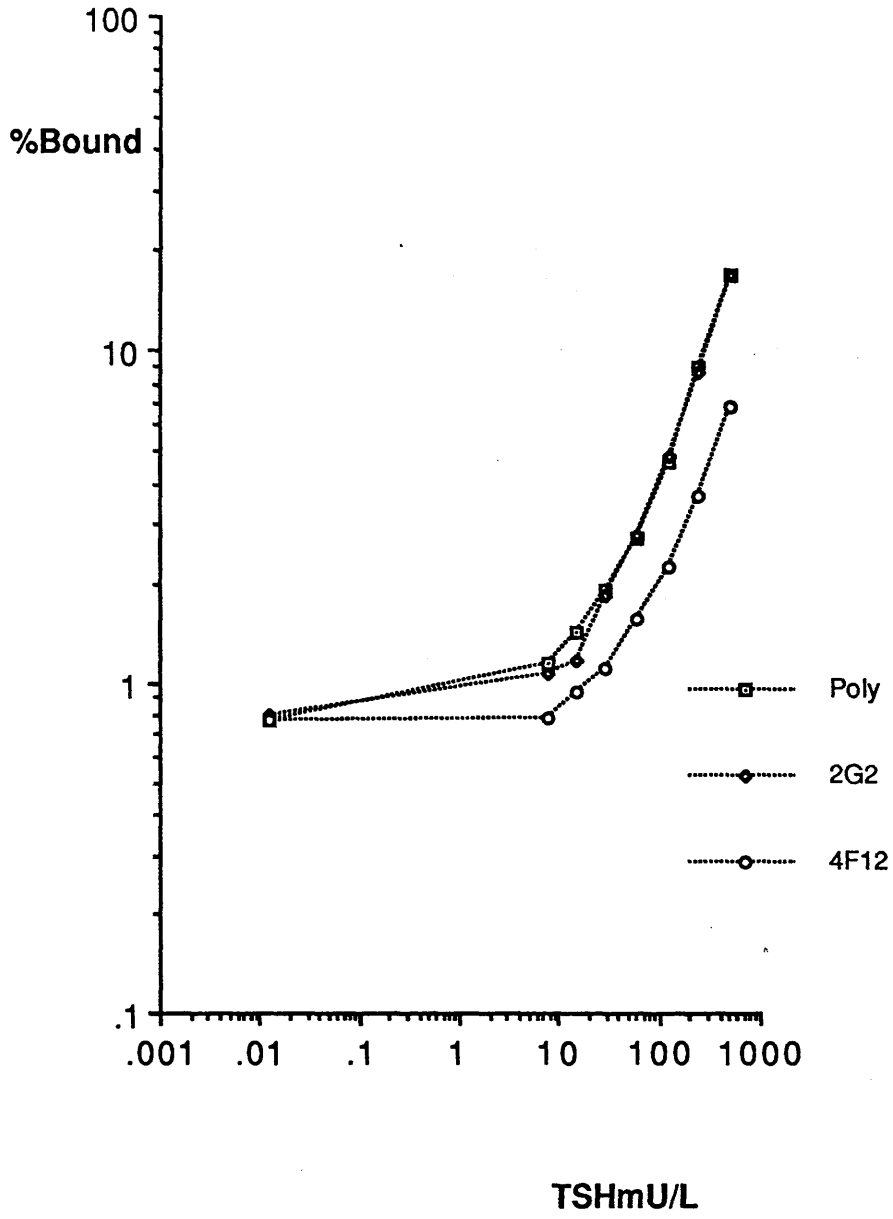


Figure 3.39

Blood-spot standard curves obtained using 5H8 as label with monoclonal antibodies 2G2 and 4F12 as solid-phase reagents

IX COMPARISON OF 3 SYSTEMS FOR THE MEASUREMENT OF TSH IN DRIED BLOOD AS A SCREENING METHOD FOR NEONATAL HYPOTHYROIDISM

a) INTRODUCTION

The screening procedure adopted for the detection of neonatal hypothyroidism, established in Scotland in 1980 (Sutherland et al, 1981) employed a two-site immunoradiometric assay (IRMA) for TSH with sheep polyclonal antisera as solid-phase and radiolabelled reagents (Sutherland et al, 1982). This assay was rapid, but the immunoaffinity purification of antisera prior to radiolabelling was expensive, tedious and time consuming. These disadvantages were overcome by the production of several mouse monoclonal antibodies to TSH. These were carefully characterised (Section III) and the better avidity antibodies, 5H8 and 2G2, gradually incorporated into the screening assay as the monoclonal antibody production programme evolved.

Initially, an IRMA with 5H8, a monoclonal antibody specific to the β-subunit of TSH as radiolabel and an IgG fraction of a polyclonal TSH antiserum (S117), produced by the Scottish Antibody Production Unit, covalently linked to Sepharose Cl-4B solid-phase was devised. Later modification replaced the polyclonal antibody with a second monoclonal antibody, 2G2, of alternative epitope specificity to that used as the radiolabel.

These IRMA variants were compared using precision profiles (Chapter 1, Section IIIc) to establish the best combination to use as a screening method for neonatal hypothyroidism.

b) METHOD

Blood-spots (5 or 6 mm diameter discs) were incubated for 16 hours at ambient temperature with 0.2 ml of labelled antibody. The assay tubes were then agitated for 90 minutes with 0.2 ml of solid-phase antibody (approximately 1 mg) prior to 4 wash/centrifuge cycles with 3 ml of isotonic saline. The tubes were then counted for one minute, the total assay time being less than 24 hours.

(c) RESULTS

Standard curves and precision profiles are illustrated in Figure 3.40 to Figure 3.42 for all IRMA variants using 5 and 6 mm blood-spots. The results obtained are summarised in Table 3.14. The IRMA employing a monoclonal antibody as radiolabel and a solid-phase polyclonal antibody appeared to be the best combination with respect to precision and sensitivity. No further improvements were observed with the introduction of a

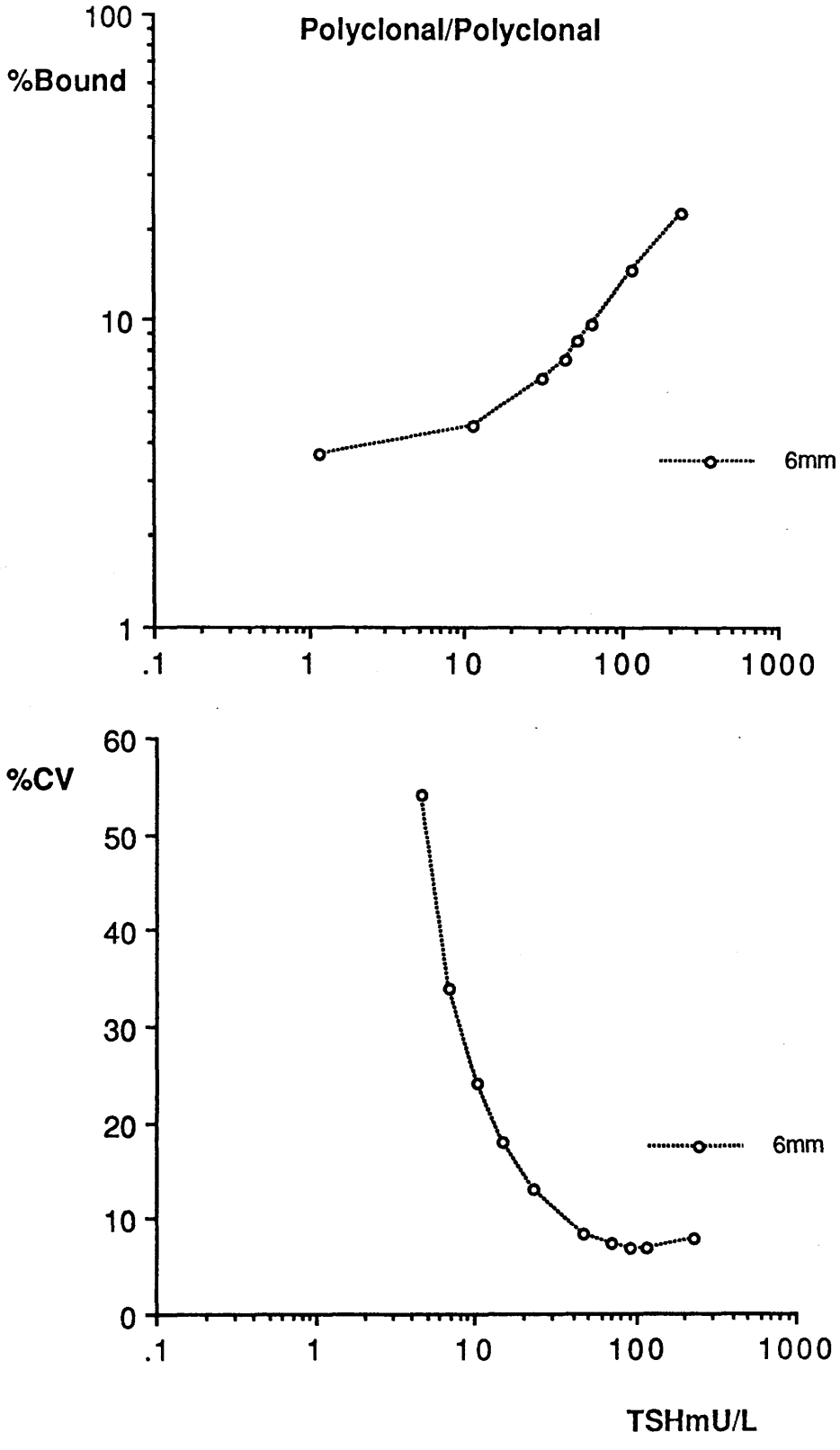


Figure 3.40

Dose-response curve and precision profile for the polyclonal/polyclonal IRMA using a 6 mm blood-spot

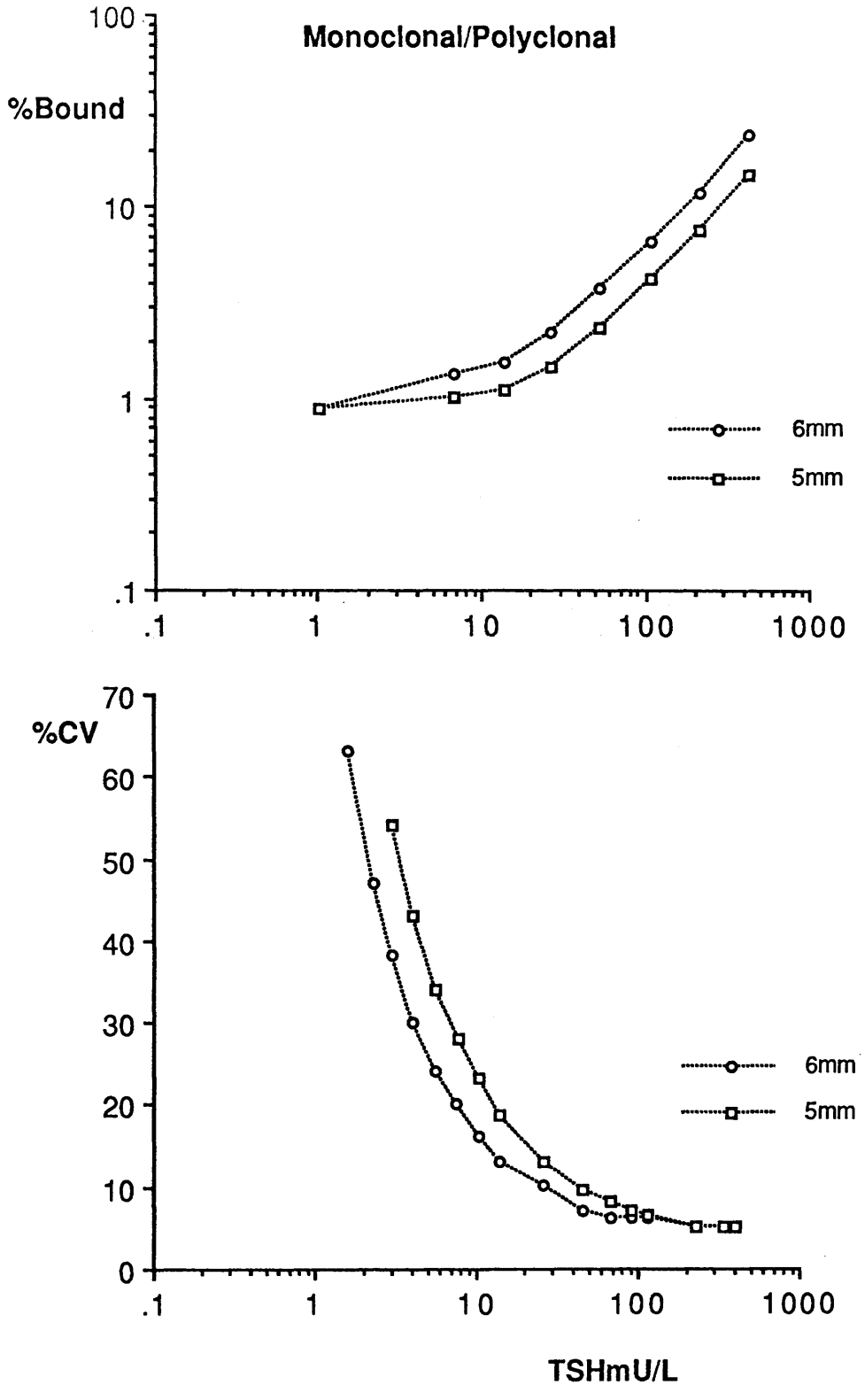


Figure 3.41

Dose-response curves and precision profiles for the monoclonal/ polyclonal IRMA using 5 and 6 mm blood-spots

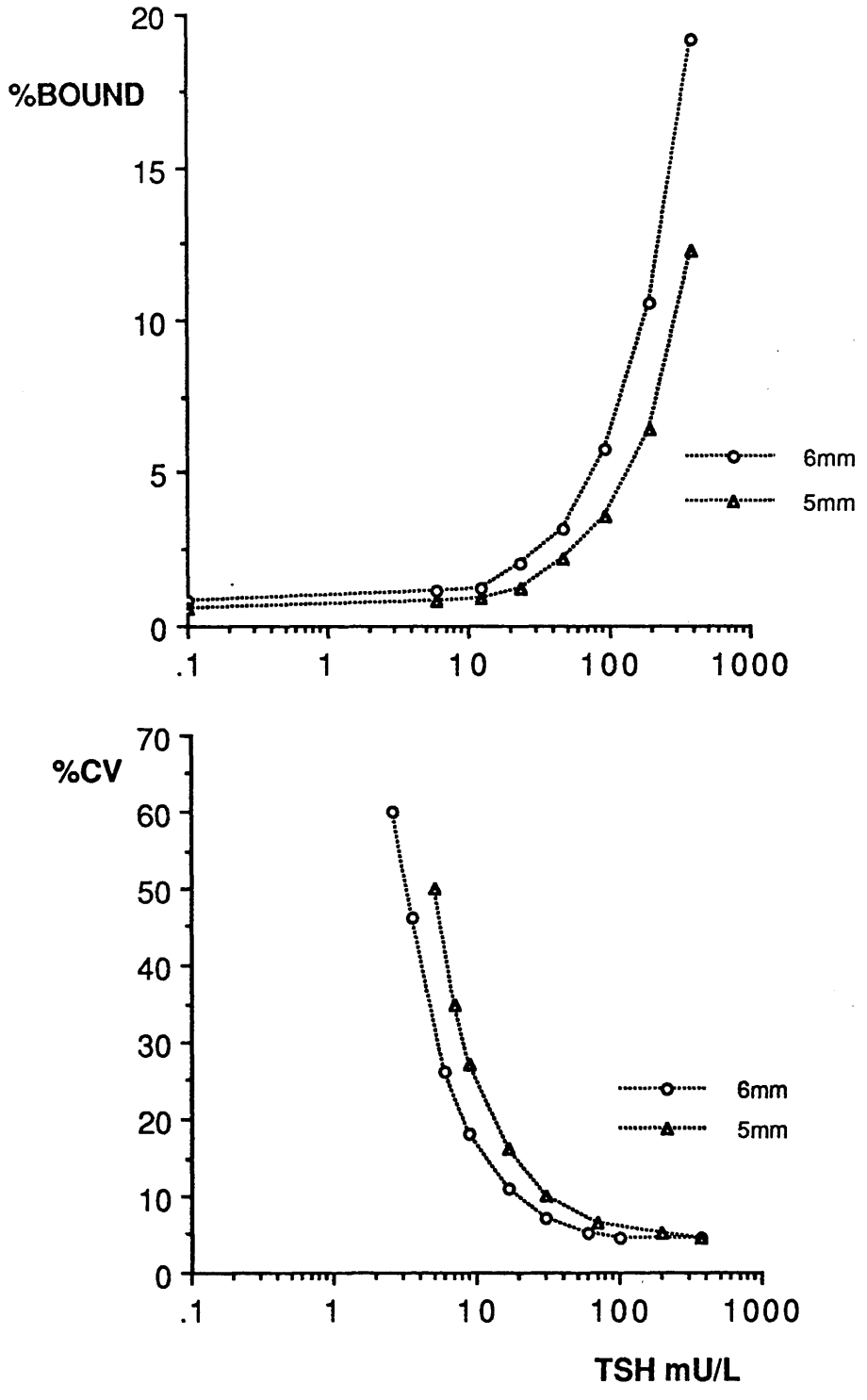


Figure 3.42

Dose-response curves and precision profiles for the monoclonal/ monoclonal IRMA using 5 and 6 mm blood-spots

TABLE 3.14

COMPARISON OF 3 SYSTEMS FOR THE MEASUREMENT OF TSH IN DRIED BLOOD AS A SCREENING METHOD FOR NEONATAL HYPOTHYROIDISM

	2 POLYCLONAL IRMA	MONO./POLY. IRMA	2 MONOCLONAL IRMA
SENSITIVITY (mU/L)	10 (6 mm)	5 (6 mm) 9 (5 mm)	7 (6 mm) 11 (5 mm)
C.V. <10% (mU/L)	27-210 (6 mm)	23-375 (6 mm) 31-375 (5 mm)	23-375 (6 mm) 31-375 (5 mm)
BLANK (%)	3.4 (6 mm)	0.72 (6 mm) 0.69 (5 mm)	0.74 (6 mm) 0.60 (5 mm)
200/0 RATIO	6 (6 mm)	15 (6 mm) 10 (5 mm)	16 (6 mm) 11 (5 mm)

second monoclonal antibody. However, the development of the two-site IRMA for TSH using two monoclonal antibodies directed against different epitopes raises the possibility, currently under exploration of adapting the system to microtitre plate technology.

CHAPTER 4

CLINICAL ASSESSMENT OF THE MONOCLONAL - POLYCLONAL IRMA AS APPLIED TO THE MEASUREMENT OF TSH IN HUMAN SERUM

I INTRODUCTION

The measurement of TSH in serum is now to be regarded as a routine test in most Clinical Biochemistry Laboratories in the UK. The reason for this relates to the key role of TSH in thyroid physiology (Chapter 1, Section IV). An individual with no history or symptoms of thyroid disease will have a detectable TSH within fairly tightly defined limits, the release of TSH will be pulsatile and a diurnal rhythm will exist. As a result T_4 and T_3 are controlled within euthyroid reference ranges.

Thyroid disease is very common, occurring in over 2% of the population, especially in middle aged women (F:M ratio 12:1) (Hall et al, 1980). In adults there are basically three types of thyroid disease:-

(1) goitre - this is a swelling of the neck and can either be diffuse or focal. It is often not accompanied by abnormal thyroid hormone secretion, therefore, the TSH level is normal and for this reason this type of thyroid disease is not discussed further.

(2) hypothyroidism - this is almost always primary in origin, characterised biochemically by a low T_4 and a raised TSH. Occasionally hypothyroidism is autoimmune with the production of blocking auto-antibodies which bind to the TSH receptor but do not mimic TSH action. The clinical features include lack of energy, cold intolerance, dryness of the skin and hair, weight gain, constipation, hoarseness of the voice, typical facial appearance and prolongation of the relaxation phase of the tendon reflexes. All patients with symptomatic hypothyroidism require therapy with T_4.

(3) hyperthyroidism (thyrotoxicosis) - in the UK this is almost always primary in origin, characterised biochemically by a raised T_4 and T_3 and a low TSH. The 3 main presentations in the UK are Graves' Disease, toxic multinodular goitre and single thyroid toxic adenoma. The aetiology of hyperthyroidism is commonly auto-immune with the production of thyroid stimulating auto-antibodies. The clinical features include heat intolerance, weight loss, diarrhoea, irritability and nervousness. Hyperthyroidism can be treated with antithyroid drugs, radioactive iodine or by surgery to remove part of the thyroid gland. From the above it will be clear that serum TSH in association with some index of T_4 (or FT_4) or T_3 (or FT_3) status is vital in the investigation of thyroid disease. Numerically TSH

assays are used most frequently to a) exclude thyroid disease in view of the non-specific symptoms referred to above, b) monitor the treatment of thyroid disease and c) diagnose thyroid disease.

The remainder of this Chapter seeks to illustrate the clinical role and suitability of the monoclonal-polyclonal serum TSH assay developed in this project and described in detail in Chapter 3, Section V.

II DIAGNOSTIC VALUE OF THE MONOCLONAL - POLYCLONAL SERUM TSH ASSAY

a) INTRODUCTION

In order to demonstrate the clinical value of the developed assay, the TSH reference ranges need to be established in unequivocal untreated thyroid disease.

b) PATIENTS AND METHODS

Serum samples from a total of 170 patients attending the Thyroid Clinic at Glasgow Royal Infirmary, collected and stored from the time of the initial diagnosis of their thyroid disease, were studied. Of these, 109 were diagnosed as having primary hypothyroidism on the basis

of their symptoms, a low or low normal serum T_4 result and an elevated serum TSH result in the polyclonal - polyclonal IRMA previously used routinely in this laboratory. The remaining 61 subjects were diagnosed as having hyperthyroidism on the basis of their symptoms and an elevated serum total T_4 and/or serum total T_3 (reference range for T_4 = 55-144 nmol/l and T_3 = 0.9-2.8 nmol/l). Serum TSH was analysed in these serum samples by the developed monoclonal polyclonal assay.

c) RESULTS

The TSH levels obtained are displayed in Figure 4.1. The reference value obtained from 476 blood donors (Chapter 3, Section V.L) is displayed as a shaded area. It is clear that the monoclonal - polyclonal serum TSH assay successfully distinguishes all cases of primary hypothyroidism as having an elevated serum TSH and all cases of hyperthyroidism as having an undetectable serum TSH (<0.2 mU/l - Chapter 3, Section V.K).

d) DISCUSSION

The monoclonal - polyclonal In-house IRMA performs as might be expected from our understanding of thyroid physiology and literature about other TSH assays. On the basis of this information, together with the

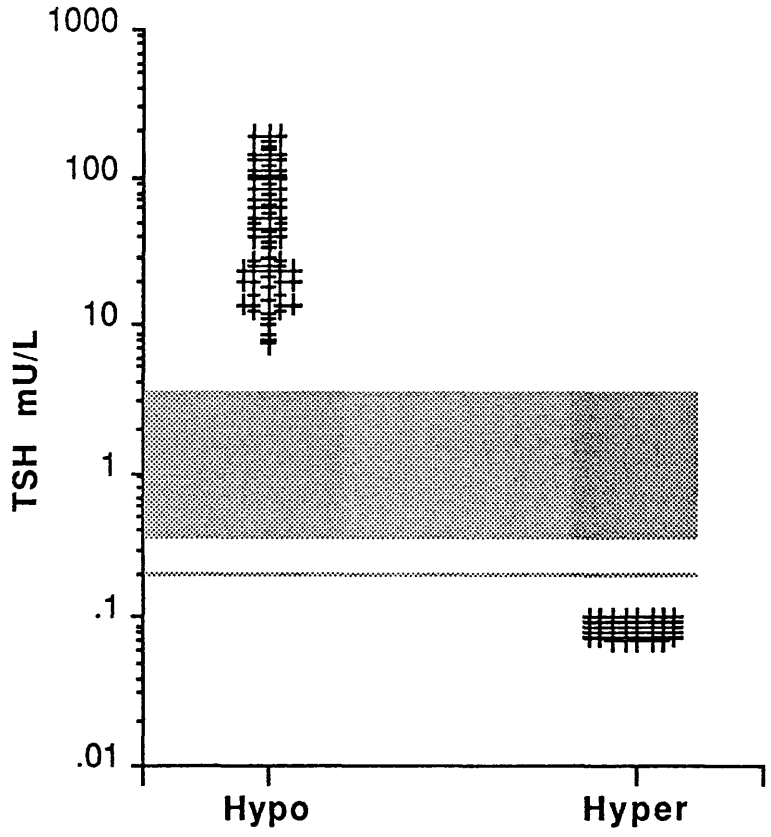


Figure 4.1

TSH reference ranges in unequivocal untreated thyroid disease. The shaded area represents the reference value obtained from 476 blood donors

performance data described in Chapter 3, the assay was introduced in 1984 as the 'routine' serum TSH assay for the laboratory at Glasgow Royal Infirmary, processing some 20,000 serum specimens per annum.

III COMPARISON OF IN-HOUSE IRMA WITH COMMERCIALLY AVAILABLE ALTERNATIVES (1984/85)

a) INTRODUCTION

During the period of development of the In-house TSH IRMA a number of commercial immunometric assay systems for measuring serum TSH became available. It was considered important to compare both the clinical and technical performance of the in-house assay with these systems. Three such systems were chosen for this study and each will be described briefly below. In each case a minimum of 6 kits (600 tubes/wells) were evaluated with a protocol that aimed to assess intra-assay precision and accuracy from a variable number of clinically defined serum samples. Because of the timing of these comparisons it was not possible to measure the TSH from the same serum samples in all four assays.

b) COMMERCIAL TSH METHODS EMPLOYED:

1) TSH IRMA MAIACLONE (SERONO DIAGNOSTICS LTD)

This is a magnetic antibody immunoradiometric method. Standard (0.5, 1.0, 5.0, 10, 30 and 50 uIU/ml) or sample (0.2 ml) was incubated with 0.1 ml ^{125}I Anti-TSH reagent for 2 hours at room temperature. TSH IRMA Separation Reagent (0.2 ml) was added and the tubes agitated prior to incubation for 5 minutes, again at room temperature. The tubes were then placed in the Magnetic Separator for 2 minutes and the supernatant decanted (Figure 4.2). Finally, the tubes were washed in phosphate-saline buffer containing BSA and NaN_3 (0.5 ml) before counting for at least 2 minutes.

2) SUCROSEP^{TM} TSH IRMA (BOOTS-CELLTECH DIAGNOSTICS LTD)

The TSH IRMA that was originally developed at Boots-Celltech was based on the non-centrifugation sucrose separation system (SUCROSEP^{TM}). Standard or sample (0.1 ml) was reacted with ^{125}I-labelled antibody to produce an antibody-antigen complex. Subsequently, a second antibody, coupled to solid-phase, which recognises a second, distinct epitope on the analyte was added. The solid-phase of the

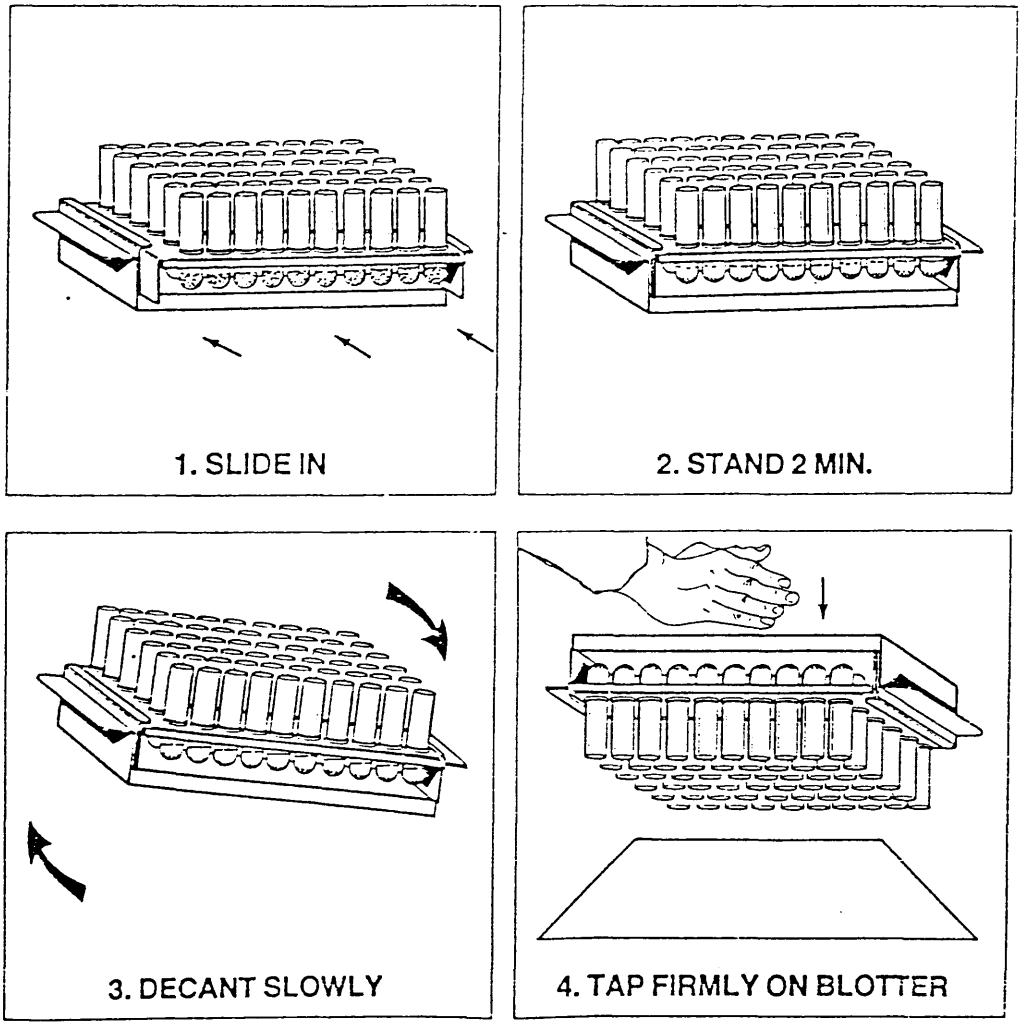


Figure 4.2

TSH IRMA MAIACLONE magnetic separation technique

SUCROSEP™ assay is based on Pharmacia's Sephacryl S300. Separation was brought about by employing the sucrose layering technique developed by Hunter (Wright and Hunter, 1983) (Figure 4.3). Sephacryl S300 solid-phase falls relatively quickly under gravity without the need for centrifuges or rare earth magnets. Agitation was required during the incubation period to maintain the solid-phase in suspension and allow the reaction to proceed (1) At the end of the incubation, wash buffer was added and the solid-phase was allowed to settle for about 5 minutes (2) This step enhances separation efficiency. A relatively dense sucrose solution (10%) was then layered below the incubate by running it through a hollow probe resting on the bottom of the tube (3) This raises the incubate above the sucrose layer and lifts the solid-phase (bound fraction) at the interface of the two layers. Over a period of 15 minutes the particulate solid-phase falls through the sucrose, leaving behind the incubate and the remaining labelled antibody (free fraction) (4) The upper layer of incubate and most of the sucrose were removed by aspiration through a second probe (5) This left 0.3-0.5 ml of sucrose containing all the solid-phase at the bottom of the tube, ready for counting (6) For optimum sensitivity and precision this separation procedure was repeated.

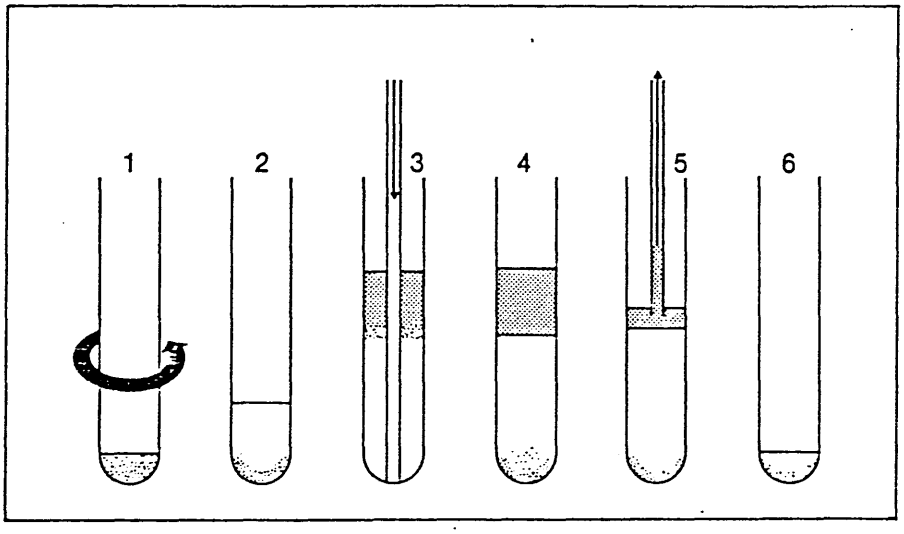
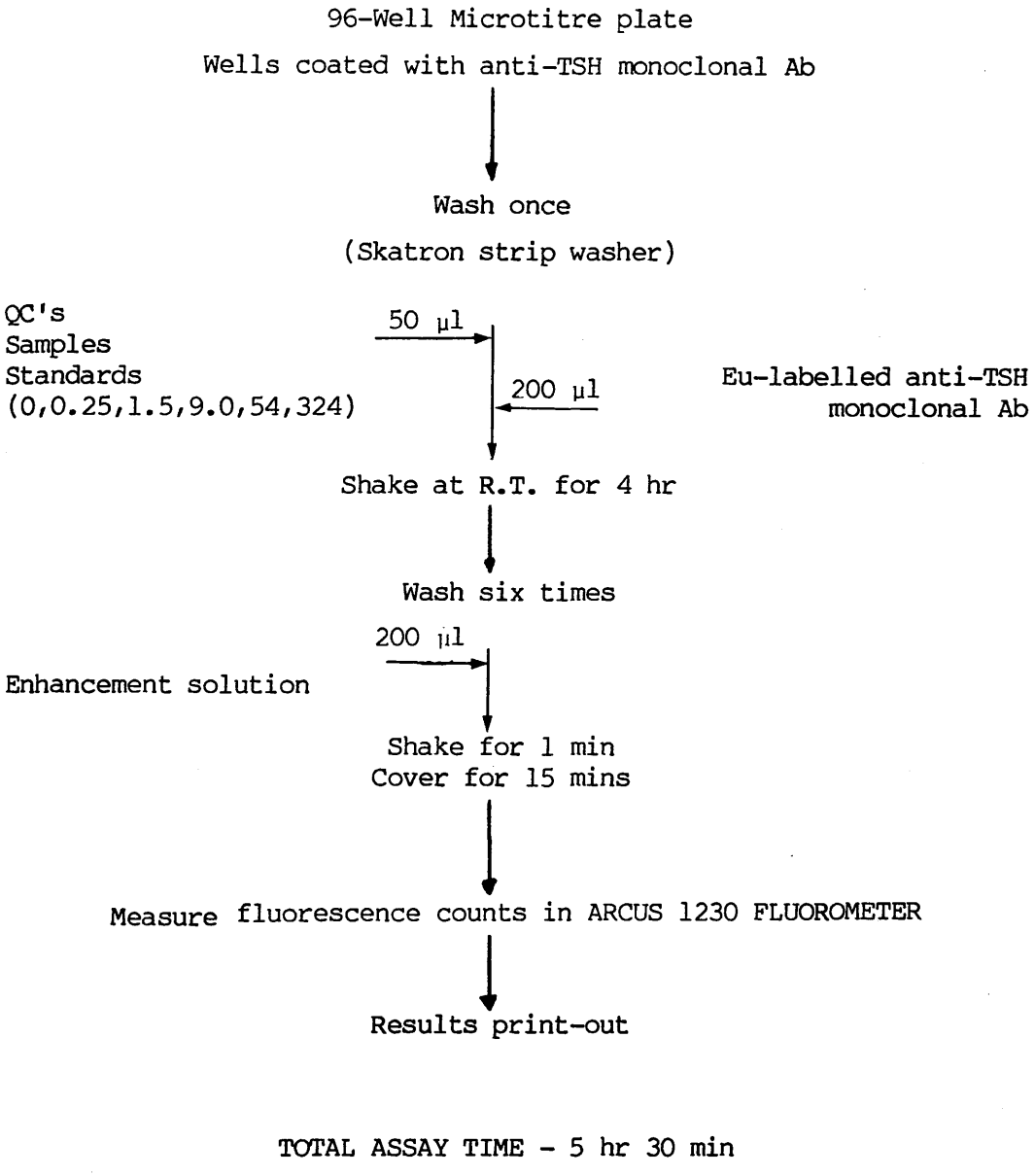


Figure 4.3

The principle of SUCROSEP™ separation (see text for details)

3) DELFIA^{TM} TSH IFMA (PHARMACIA DIAGNOSTICS LTD)

'DELFIA^{TM}' is a solid-phase, two-site immunofluorometric assay (IFMA) which employs two monoclonal antibodies directed against different antigenic determinants on the β subunit of the TSH molecule. One monoclonal antibody is physically adsorbed to a microtitre strip-well in which the assay is performed. The other monoclonal antibody is labelled with a europium (Eu) chelate. Europium is the label of choice because it has a wide excitation wavelength band, a narrow emission wavelength band, a large Stokes shift and a long fluorescence decay time relative to other conventional fluorophores. Before measuring fluorescence, an enhancement solution is added to promote the dissociation of Eu^{3+} cations from the labelled antibody into solution where they form highly fluorescent chelates with components of the enhancement solution. Non-specific background fluorescence is minimised by counting in the time-resolved mode, whereby a delay of 400 us elapses after excitation prior to measurement of the fluorescence. During this delay, virtually all short-lived fluorescence disappears. The whole excitation/counting cycle is completed in 1s. A flow chart for the method appears in Figure 4.4.



TOTAL ASSAY TIME - 5 hr 30 min

Figure 4.4

Flow chart of DELFIA^{TM} TSH assay protocol

c) RESULTS

Typical standard curves and intra-assay precision profiles for the TSH IRMA MAIACLONE, SUCROSEP™ TSH IRMA, and DELFIA™ TSH IFMA are displayed in Figures 4.5, 4.6 and 4.7 respectively. The mean intra-assay imprecision data obtained for each assay system are displayed in Table 4.1. Linear regression analysis was performed on the results obtained from each of these commercial TSH assays with the corresponding results from the In-house polyclonal - monoclonal IRMA and this data is summarised in Table 4.2. The serum TSH results obtained from each assay in defined clinical situations are presented as follows: euthyroid reference value in Table 4.3, reference value for individuals with various non-thyroidal illnesses in Table 4.4, subjects with untreated hypothyroidism in Table 4.5 and subjects with untreated thyrotoxicosis in Table 4.6.

d) DISCUSSION

The objective of this study was to compare the practicability and performance of four high sensitivity TSH assays. Whilst all four assays showed characteristics much improved from traditional radioimmunoassays for TSH, no single high sensitivity TSH assay out-performed its competitors. An assay by assay summary is given below:

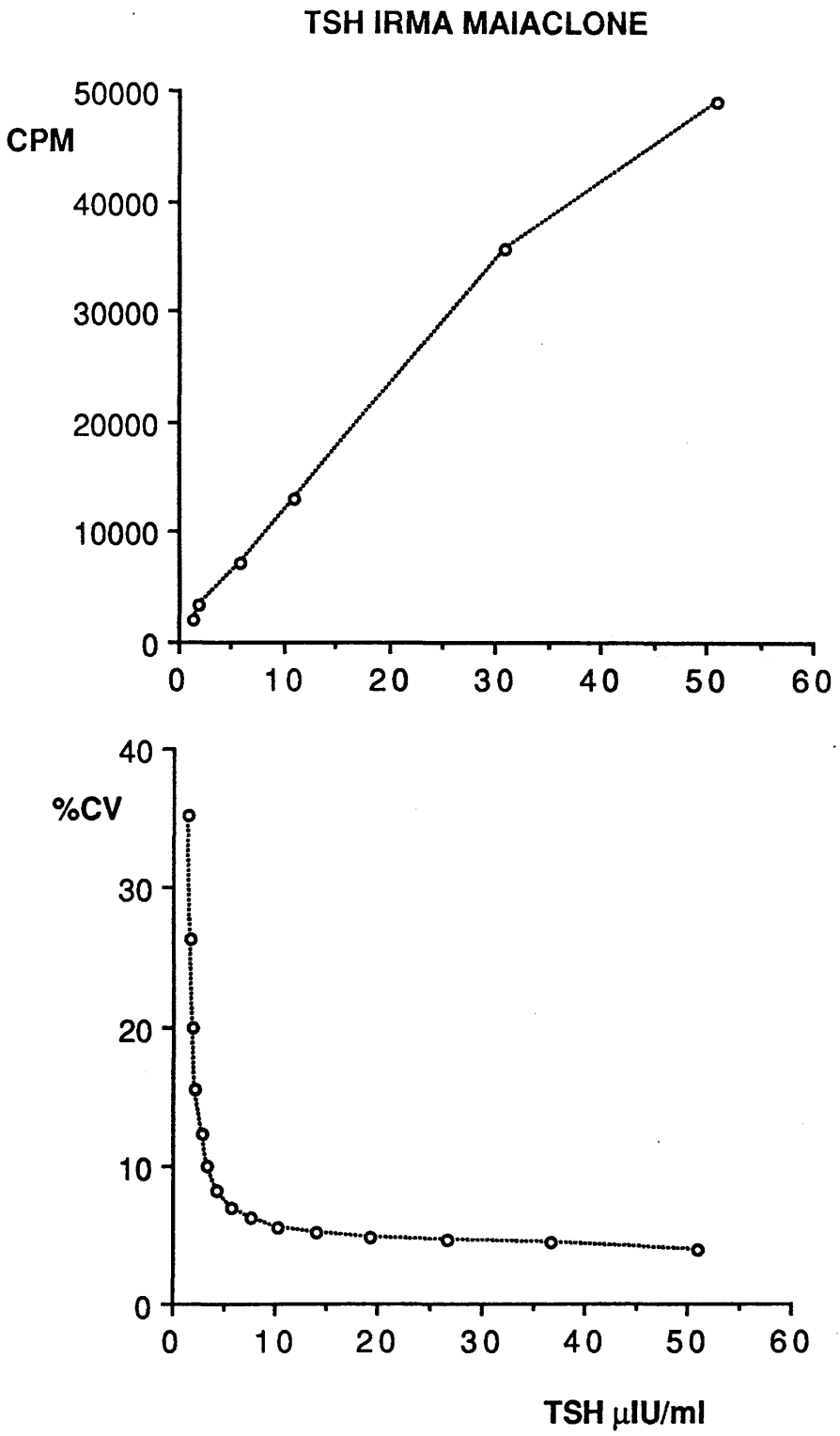


Figure 4.5

Typical standard curve and intra-assay precision profile for the TSH IRMA MAIACLONE

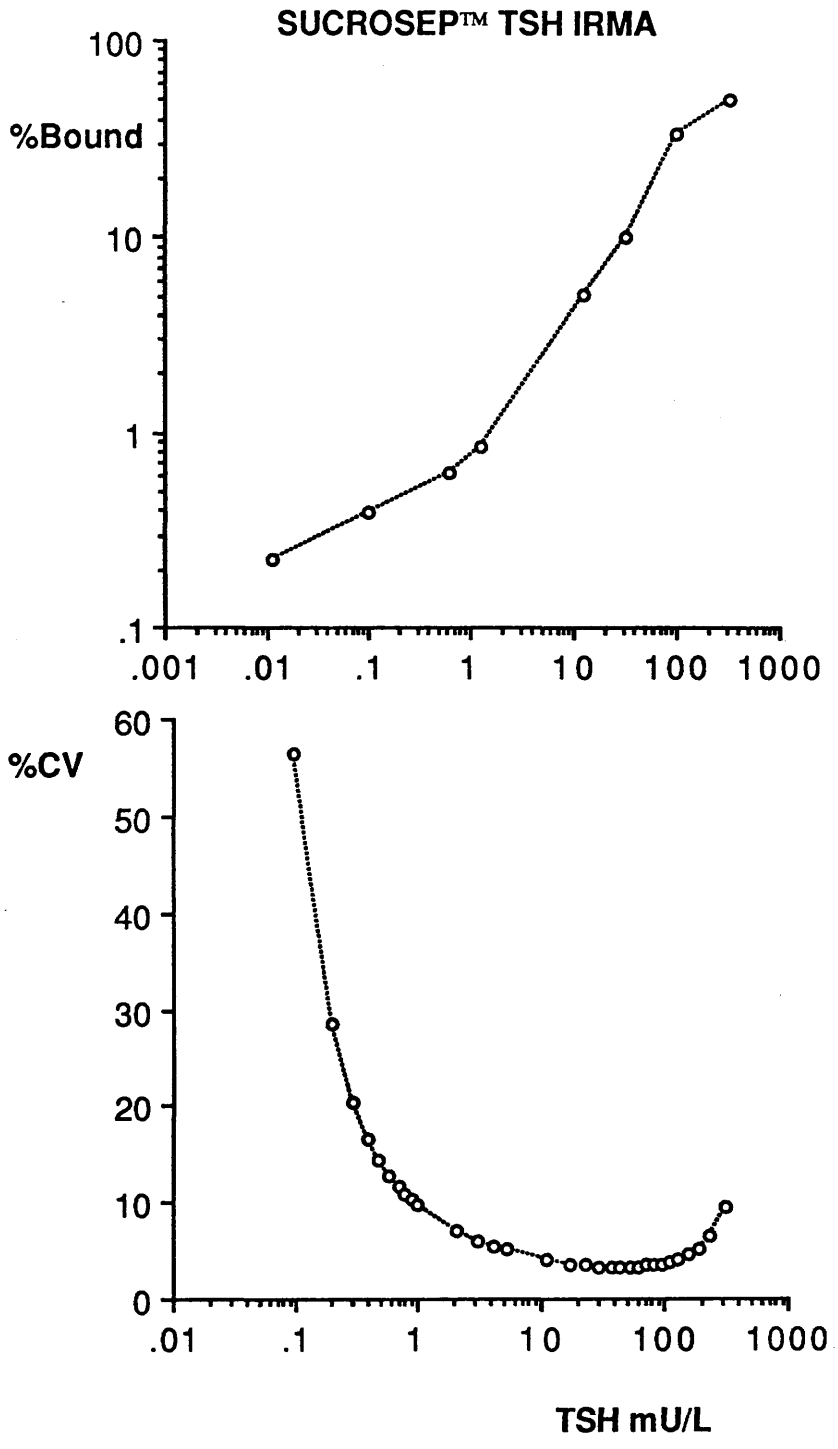


Figure 4.6

Typical standard curve and intra-assay precision profile for the SUCROSEP™ TSH IRMA

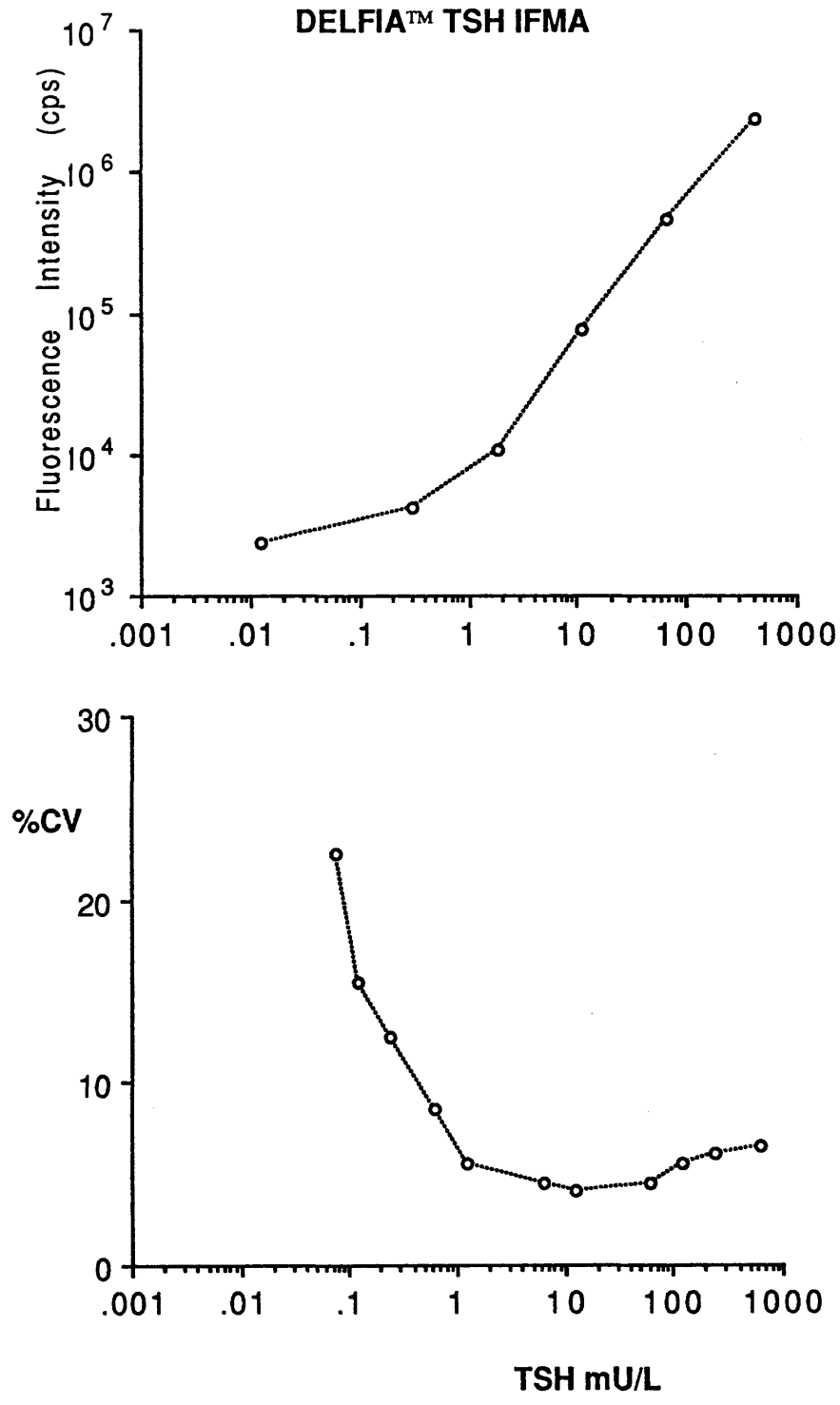


Figure 4.7

Typical standard curve and intra-assay precision for the DELFIA™ TSH IFMA

TABLE 4.1

MEAN INTRA-ASSAY IMPRECISION DATA OBTAINED FOR FOUR DIFFERENT SERUM TSH ASSAYS

Assay	Euthyroid Mean-mU/l (CV)	Hypothyroid Mean-mU/l (CV)
In-house IRMA	1.63 (7.3%)	26.8 (4.7%)
DELFIA™ hTSH	1.20 (6.3%)	82.1 (3.9%)
TSH MAIACLONE	1.34 (4.5%)	17.1 (3.1%)
SUCROSEP™ TSH	0.61 (10.6%)	42.3 (3.2%)

TABLE 4.2

CORRELATIONS WITH IN-HOUSE IRMA

Assay	n	m	c	r
DELFIA™ hTSH	45	1.04	+0.06	0.991
TSH MAIACLONE	45	1.10	+0.52	0.995
SUCROSEP™ TSH	45	1.05	+0.73	0.964

TABLE 4.3

EUTHYROID REFERENCE RANGE FOR SERUM TSH

Assay	Mean (mU/l)	Range (mU/l)	n
In-house IRMA	1.51	0.35 - 3.50	476
DELFIA™ hTSH	1.52	0.47 - 3.84	83
TSH MAIACLONE	2.40	0.5 - 8.5	57
SUCROSEP™ TSH	2.46	0.21 - 8.0	70

TABLE 4.4

SERUM TSH VALUES IN NON-THYROIDAL ILLNESS

Assay	Mean (mU/l)	Range (mU/l)	n
In-house IRMA	1.60	<0.13 - 4.9	21
DELFIA™ hTSH	1.63	0.12 - 4.1	21
TSH MAIACLONE	2.26	0.26 - 6.1	21
SUCROSEP™ TSH	2.21	0.22 - 5.7	21

TABLE 4.5

SERUM TSH VALUES IN UNTREATED PRIMARY HYPOTHYROIDISM

Assay	Range (mU/l)	n	% >Top Std.
In-house IRMA	8.1 - >185	116	1.8
DELFIA™ hTSH	15 - >324	28	3.6
TSH MAIACLONE	8.0 - >50	33	32
SUCROSEP™ TSH	8.4 - >240	58	14

TABLE 4.6

SERUM TSH VALUES IN UNTREATED THYROTOXICOSIS

Assay	2.5 SD Sensitivity (mU/l)	n	n > Sensitivity
In-house IRMA	0.13	43	-
DELFIA™ hTSH	0.022	46	1 (0.05)
TSH MAIACLONE	0.20	21	1 (0.50)
SUCROSEP™ TSH	0.06	41	-

1) IN-HOUSE IRMA

This assay uses a practicable 0.1 ml serum sample to achieve a sensitivity of 0.13 mU/l (+ 2.5 SD) and a working range (<10% CV) of 0.65 - 185 mU/l. The assay requires relatively complex manipulations and all reagents have to be made locally. However, it is very much cheaper to run than the commercial kits (4 p per tube excluding labour). The assay also tends to read approximately 5% lower than the other assays and has poor precision on a 0.30 mU/l recovery pool.

2) TSH MAIACLONE

This IRMA is heavy on serum (0.2 ml) and requires at least one additional standard to extend the working range beyond 50 mU/l. TSH MAIACLONE is the least sensitive of the assays tested (0.20 mU/l [+ 2.5 SD]) but appears to have adequate sensitivity for clinical discrimination. The kit struggles to achieve good precision on a low level recovery pool, but it is simple to use and relatively cheap to run (62 p per tube).

3) SUCROSEP™ TSH

This kit uses a convenient sample volume (0.1 ml) to achieve a very wide working range of 0.60 - 254 mU/l and a sensitivity of 0.06 mU/l (+ 2.5 SD). However, the assay is rather 'clumsy' to perform. SUCROSEP™ TSH is a medium cost assay (70 p per tube) that is capable of excellent clinical discrimination.

4) DELFIA™ hTSH

This kit employs only 0.05 ml of serum to achieve a sensitivity of 0.02 mU/l (+ 2.5 SD) and a working range (<10% CV) of 0.20 - 324 mU/l. It requires at least one additional standard. The kit is easy to use, although thorough washing is vital for optimal performance. At a list price of 100 p per tube, the DELFIA™ hTSH kit is too expensive for many laboratories.

Considering the small numbers of specimens analysed, the intra-assay imprecision data obtained on all TSH assays tested was satisfactory. The correlation coefficient obtained from the comparisons with the In-house IRMA indicated excellent ranking of specimens in all cases. However, the gradient from the linear regression analyses differed for each of the assays. This

fact is borne out by the different euthyroid reference values obtained (Table 4.3) and this formed the basis of the next Study to be described in this Chapter.

As far as the euthyroid reference range was concerned, the TSH MAIACLONE and SUCROSEP^{TM} TSH yielded higher results than the other two methods (Table 4.3). However, discrimination between clinical groups for untreated patients was excellent with all methods (Table 4.5 and 4.6). Samples from patients with non-thyroidal illness (n=21, mean T_4 = 49 nmol/l; mean T_3 = 0.3 nmol/l) were also subjected to analysis by all 4 methods and a separate reference range established (Table 4.4). In all cases this reference range agreed well with the corresponding euthyroid reference range displayed in Table 4.3.

The results from this study were presented at the 1986 Meeting of the UK EQAS participants held in Cardiff.

IV A STUDY OF POTENCY DIFFERENCES IN IMMUNOMETRIC ASSAYS FOR SERUM TSH

a) INTRODUCTION

Whilst comparing the TSH results obtained with the polyclonal-polyclonal and monoclonal-polyclonal In-house assays and the commercially available assays described in the previous section, it became apparent that there was a variable bias between the results obtained from these assays. To investigate this systematically a study was designed.

b) PATIENTS AND METHODS

This study was divided into two sections. Four different serum TSH assays were employed in each section of the study - (i) In-house polyclonal-polyclonal IRMA (ii) In-house monoclonal-polyclonal IRMA (iii) SUCROSEP™ TSH IRMA (iv) DELFIA™ TSH IFMA.

In the first section of the study formal recovery experiments were performed for each of the TSH assays examined using different additions of the 2nd International Reference Preparation for TSH (WHO 80/558).

In the second section of the study ten women, newly diagnosed for primary hypothyroidism were studied prior to any replacement thyroxine and following one month of regular replacement with each of three daily doses of thyroxine (i) 0.05 mg (ii) 0.01 mg (iii) 0.15 mg.

c) RESULTS

1) RECOVERY OF ADDED TSH 80/558

Recovery experiments were performed at three different levels of added TSH for each of the four assays. The data displayed in Table 4.7 were obtained from ten replicates in every experiment. Within the precision limits of the study it can be concluded that all four assays recover added TSH quantitatively.

2) SERUM TSH LEVELS IN UNTREATED AND TREATED HYPOTHYROIDISM

Table 4.8 records the mean serum TSH result (± SEM) obtained from the ten hypothyroid women prior to and following stepwise replacement therapy with T_4. For each assay the untreated mean serum TSH level is taken as 100% and the mean serum TSH level for each replacement dose is expressed as a percentage of this. It is apparent that there are marked

TABLE 4.7

RECOVERY DATA OF TSH 80/558 IN FOUR DIFFERENT SERUM TSH ASSAYS

		TSH Added (mU/l)	Mean Recovery (%)	S.D. (%)	C.V. (%)
I.	2 Polyclonal IRMA	8.3	110	9.7	8.8
		15.8	106	7.7	7.3
		47.7	104	3.6	3.5
II.	Monoclonal/Polyclonal IRMA	1.0	90	4.5	5.0
		10.0	94.7	9.6	10.1
		50.0	95.2	10.0	10.5
III.	'Boots-Celltech' IRMA	0.54	100.5	15.4	15.3
		12.2	95.5	3.8	4.0
		50.0	98.5	6.5	6.6
IV.	Pharmacia DELFIA™ IFMA	0.88	103	4.3	4.2
		4.40	95.4	6.4	6.7
		17.6	96.7	7.6	7.9

TABLE 4.8

SERUM TSH LEVELS IN TEN HYPOTHYROID WOMEN PRIOR TO AND DURING STEPWISE REPLACEMENT THERAPY WITH THYROXINE

Daily Dose T_4 (mg)	Mean Serum T_4 (nmol/l)	Assay I			Assay II			Assay III			Assay IV		
		Mean TSH (mU/l)	SEM	%	Mean TSH (mU/l)	SEM	%	Mean TSH (mU/l)	SEM	%	Mean TSH (mU/l)	SEM	%
Nil	34	31.8	9.0	100	71.4	19	100	95.5	23	100	82.4	21	100
0.05	77	11.5	3.3	36	24.0	6.7	34	33.8	11	35	28.3	8.8	34
0.10	101	2.0	0.8	6	4.7	1.6	7	4.4	2.0	5	3.9	1.7	5
0.15	114	<0.5	-	-	1.0	0.4	1	0.36	0.10	0.4	0.22	0.06	0.3

Assay I - In-house Polyclonal-Polyclonal IRMA

Assay II - In-house Monoclonal-Polyclonal IRMA

Assay III - SUCROSEP™ TSH IRMA

Assay IV - DELFIA™ TSH IFMA

differences between assays in the measured levels of TSH in the untreated hypothyroid subjects. However, the percentage suppression of TSH with each successive dose of T_4 agrees very well between assays.

d) DISCUSSION

The results confirm a systematic difference between measured TSH levels and may help to explain the differences in reference values observed in the previous study. A likely explanation for this phenomenon is that differences exist between the carbohydrate content of I.R.P. TSH and that of circulating TSH. The extent of the observed difference is determined by the antibodies used in each assay. In particular, monoclonal antibodies will only recognise a single epitope on the TSH molecule and the affinity of binding of this epitope may well differ according to the nature of carbohydrate moieties attached to the protein backbone of the TSH molecule. It is quite probable that the carbohydrate content of TSH 80/558 will have been modified during its isolation and purification. In the absence of an independent assessment of the true accuracy of a TSH assay, validation must rely upon the ability to recover 80/558. By this latter criterion all four of the TSH

assays studied were valid but it is not possible to determine which, if any, of them measures the 'true' serum TSH level.

This work was presented at the 1985 British Endocrine Societies' Meeting in Oxford and was the first documented example of such potency differences which are now widely recognised for glycoprotein hormones such as TSH, LH, FSH and tumour markers such as carcinoembryonic antigen (CEA).

V EVALUATION OF THYROID FUNCTION SCREENING TESTS IN THE SCOTTISH AUTOMATED FOLLOW-UP REGISTER

a) INTRODUCTION

The Scottish Automated Follow-up Register (SAFUR) is a computerised follow-up scheme providing a service for over 8,000 patients derived from endocrine clinics in five main hospital centres. All abnormal results are screened by a thyroid specialist after the first contact and before the final decision is made on further investigation and treatment (Hedley et al, 1984; Toft, 1983).

The principal aim of this study was to evaluate the clinical and economic benefits of using new technology thyroid function tests (immunoradiometric assay and immunofluorometric assay), compared to the conventional radioimmunoassays for TSH and T_4.

b) PATIENTS AND METHODS

1) Phase 1: 1135 consecutive specimens were obtained from the SAFUR during 1985/86. The vast majority of these were from patients who had been treated previously by radioiodine, surgery or drugs for thyrotoxicosis. At the time of sampling 711 subjects were receiving maintenance thyroxine. The remaining 424 were receiving no therapy.

Serum total T_4 was measured by conventional RIA in the Department of Chemical Pathology at Aberdeen Royal Infirmary under the direction of Dr Ivan Reid. Serum TSH was measured in Glasgow by two methods - (i) In-house monoclonal-polyclonal IRMA and (ii) DELFIA IFMA.

2) Phase 2: As a result of the data obtained in Phase 1, it was decided to study a smaller number of subjects in depth. Accordingly six categories of subject were defined on the basis of the TSH results obtained with the In-house IRMA:

(i) Thyroxine TSH <0.35 mU/l

(ii) Thyroxine TSH 0.35 - 3.50 mU/l

(iii) Thyroxine TSH >3.50 mU/l

(iv) Non-thyroxine TSH <0.35 mU/l

(v) Non-thyroxine TSH 0.35 - 3.50 mU/l

(vi) Non-thyroxine TSH >3.50 mU/l

The main computer at the University of Glasgow was used to allocate the 1135 specimens from Phase 1 of the study into each of these six categories and to present them in random order within each category. The first 37 specimens from each category (with adequate serum volume >1 ml) were selected for further study. In one category, only 34 specimens were available for further study.

Serum total T_4 and two measurements of TSH were already to hand from Phase 1 of the study. In addition, serum free thyroxine (FT_4) and free triiodothyronine (FT_3) were measured using the appropriate Amerlex-M radioisotopic assay (Amersham International plc).

c) RESULTS

1) Phase 1: The serum T_4 results obtained from the 1135 specimens covered a wide range of values (66-265 nmol/l). The agreement between the TSH results obtained by the two methods was excellent (r=0.991). Of the specimens measured, 484 (43%) gave undetectable results (<0.20 mU/l) in the in-house IRMA whilst 494 (44%) gave undetectable results (<0.05 mU/l) in the DELFIA assay.

2) Phase 2: Results are expressed in terms of the total T_4, FT_4 and FT_3 concentrations measured in each of the specimens in the six categories specified in the Patients and Methods Section. This data are presented in Figures 4.8-4.10 inclusive.

d) DISCUSSION

1) Phase 1: The high percentage of undetectable TSH results obtained in this study (approximately 43%) was unexpected and was not consistent with the opinion being expressed at the time of the study that an undetectable TSH result was indicative of active hyperthyroidism (Toft, 1985; Allen et al, 1985). Clinical assessment of these patients (data not presented) certainly did not support the view

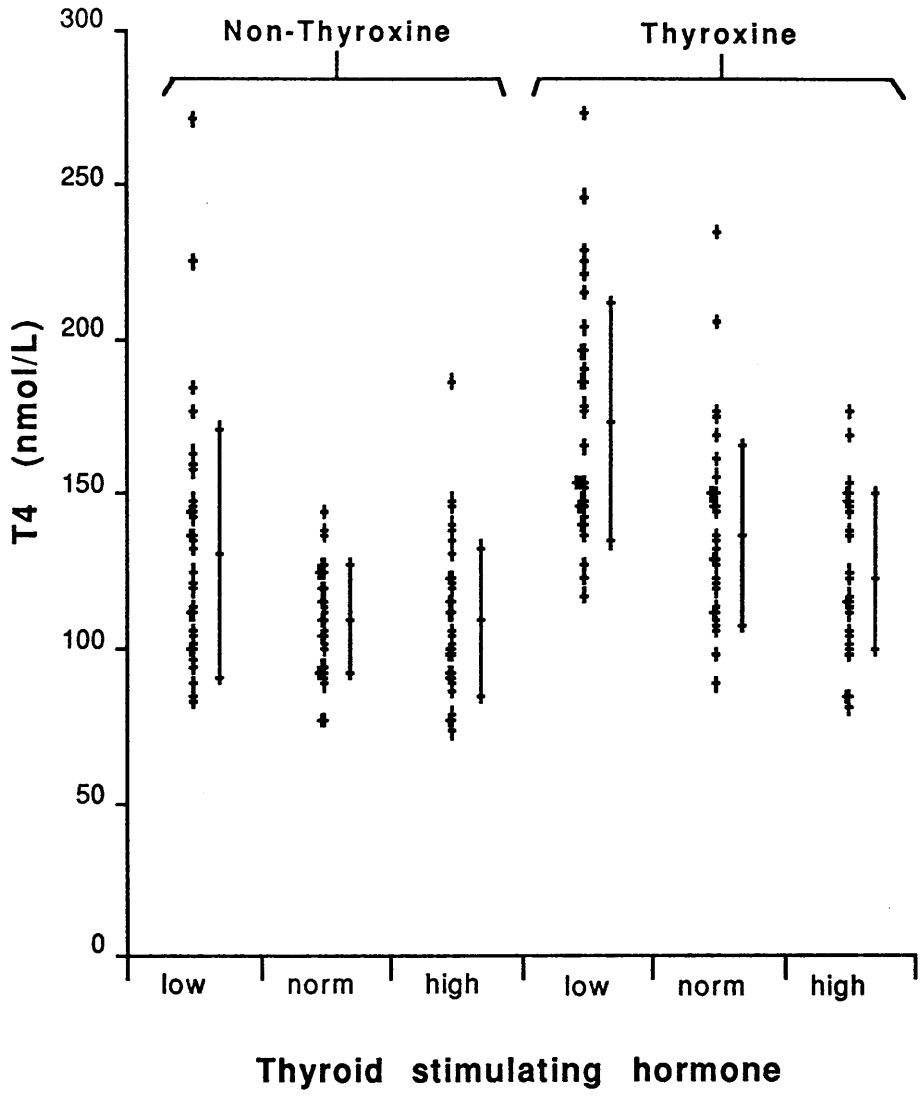


Figure 4.8

T4 concentrations in non-thyroxine and thyroxine treated patients classified by TSH concentration

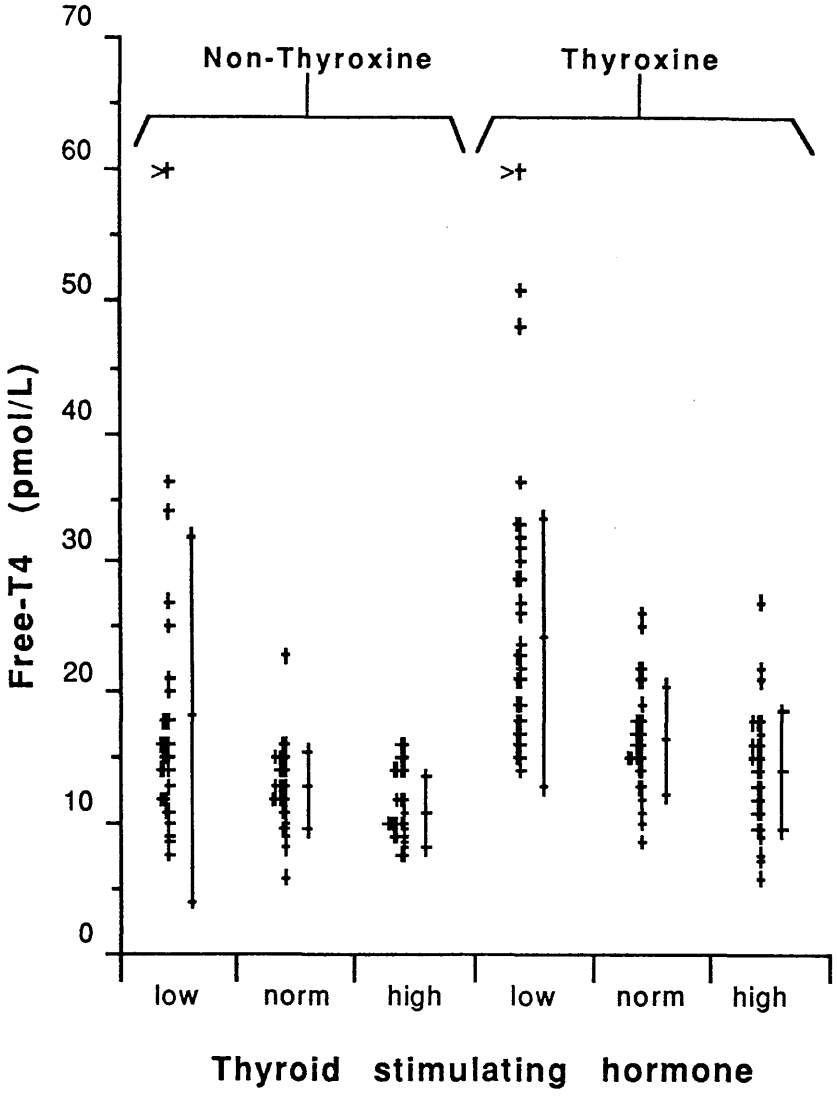


Figure 4.9

FT4 concentrations in non-thyroxine and thyroxine treated patients classified by TSH concentration

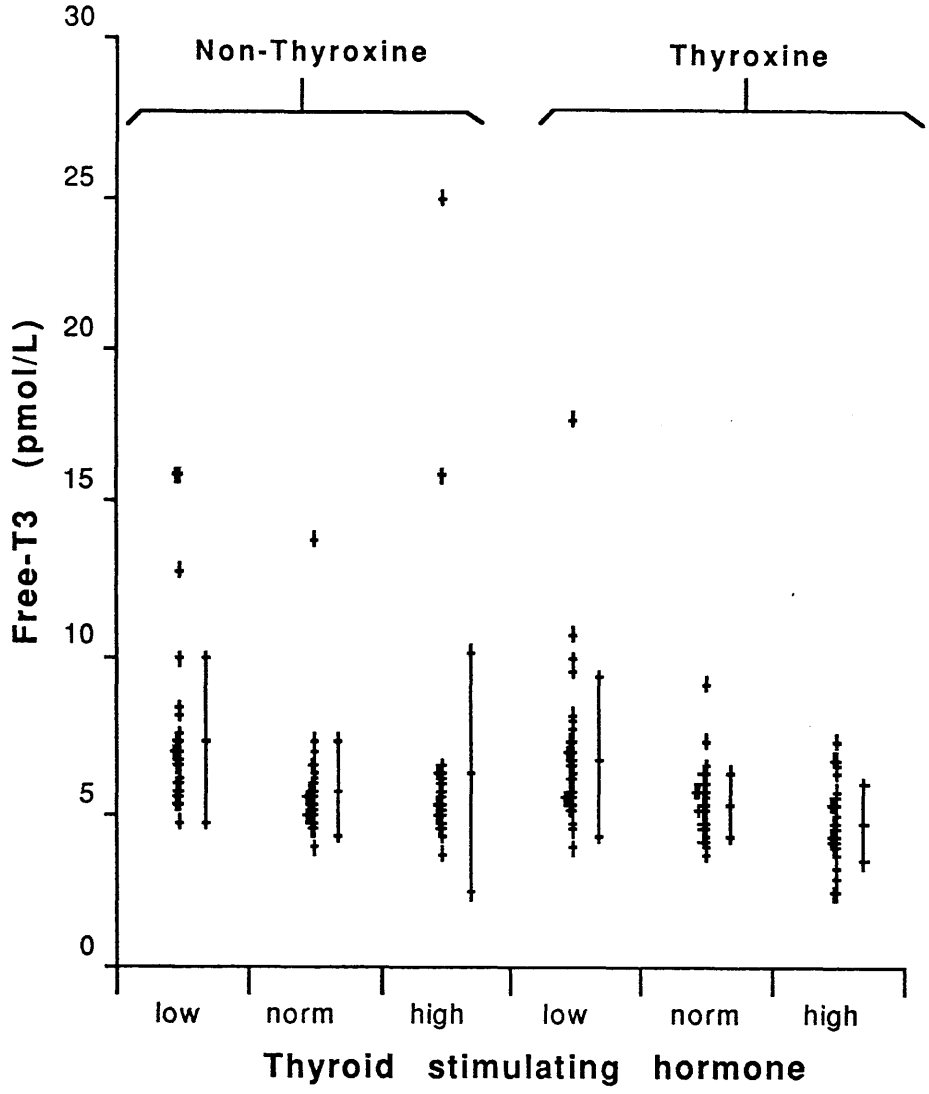


Figure 4.10

FT3 concentrations in non-thyroxine and thyroxine treated patients classified by TSH concentration

that 43% of the subjects had symptoms of hyperthyroidism. Therefore, it was decided to study a sub-population of this group of patients in more detail in order to evaluate the significance of an undetectable TSH result (Phase 2).

Since the completion of this study, several other groups have produced data to show the high percentage of undetectable TSH results in subjects who have been treated for thyroid disease (Pollock and Ratcliffe, 1986). It is now widely accepted that an undetectable TSH result in such patients is not necessarily indicative of active hyperthyroidism.

2) Phase 2: The total T_4 data presented in Figure 4.8 indicate that in all cases the mean T_4 level was higher in the thyroxine treated group than in the corresponding category of non-thyroxine treated patients although these differences did not always achieve statistical significance. By the same token the mean T_4 level for both thyroxine treated and non-thyroxine treated patients was higher in the undetectable TSH groups than in either of the other categories. However, there was considerable overlap between categories.

The FT_4 data presented in Figure 4.9 accord well with the total T_4 data discussed above and the same conclusions apply.

The FT_3 data presented in Figure 4.10 reveal no significant differences between any of the patient categories.

It may be concluded from this study that a sensitive TSH assay offers no obvious clinical and/or economic benefit to SAFUR since the high level of TSH undetectability was not in any way consistent with the clinical status of patients.

The conclusion from Phase 2 of the study is that there is a very poor correlation between serum TSH and any of the indices of T_4 or T_3 measured which calls into question the value of performing thyroid function tests to monitor the progress of subjects who have been treated for thyroid disease. This topic is addressed in more detail in the next study.

The data from this study have been presented in part at the UK EQAS Users Group Meeting in Cardiff in 1986 and to the Scottish Society for Experimental Medicine in Edinburgh in 1987 (Hedley et al, 1987).

VI ARE BIOCHEMICAL TESTS OF THYROID FUNCTION OF ANY VALUE IN MONITORING PATIENTS RECEIVING THYROXINE REPLACEMENT?

a) INTRODUCTION

The development and application of analogue free thyroid hormone assays (Midgeley and Wilkins, 1981) and sensitive thyroid stimulating hormone assays (Wehmann and Nisula, 1984) during the past five years has led to the reappraisal of the best biochemical tests of thyroid function in different disease states. There is considerable controversy over which tests should be used to monitor patients receiving thyroxine replacement for primary hypothyroidism, in whom the detection of over-replacement poses a problem. Different authors have claimed that over-replacement is best detected by measuring the serum concentration of total thyroxine (Jennings et al, 1984), total triiodothyronine (Rendell and Salmon, 1985), free thyroxine (Beckett et al, 1985), or free triiodothyronine (Pearce and Himsworth, 1984). The value of some of these tests has been questioned (Oldfield et al, 1985; Caldwell et al, 1985), while others have recommended measuring both thyroid stimulating hormone, using a sensitive method, and free triiodothyronine (Toft, 1985; Allen et al, 1985). Euthyroid patients receiving thyroxine replacement have higher serum thyroxine and free thyroxine concentrations

than normal euthyroid subjects. This is not so for serum triiodothyronine and free triiodothyronine concentrations (Pearce and Himsworth, 1984; Johansen et al, 1978; Kurtz et al, 1980).

This study was undertaken to establish a reference range for thyroid function tests in patients receiving thyroxine replacement and to try to select the best test or combination of tests to monitor treatment.

This study was conceived at about the same time as the data were beginning to emerge from the SAFUR study discussed previously. However, the present study was a collaboration with a different group of physicians and the data from the SAFUR study in no way influenced the design of this study.

b) PATIENTS AND METHODS

A prospective study was carried out in 148 hypothyroid patients (with primary hypothyroidism or after radioiodine treatment or thyroidectomy) attending a thyroid outpatient clinic. There were 131 female and 17 male patients aged, respectively, 14-89 (mean (SD) 52.9 (15.1)) years and 18-75 (52.6 (13.6)) years. They had been taking thyroxine replacement for more than three months, and none were taking drugs known to interfere with thyroid hormone metabolism, protein binding, or the

assays used. The prescribed daily dose of thyroxine varied from 50-300 ug (mean 124.6 (49.5)). The patients were classified as clinically euthyroid, hyperthyroid, or hypothyroid by the examining physician after a full history and clinical examination and with use of a modified Wayne clinical diagnostic index (Wayne, 1960). The clinical assessments were carried out by three Consultants and a Registrar, who were experienced in thyroid disease. Subsequently blood samples were taken between 0900 and 1130 and the serum stored at -20°C before assay.

Total thyroxine and triiodothyronine concentrations were measured by in-house radioimmunoassay and thyroid stimulating hormone by the in-house monoclonal-polyclonal immunoradiometric assay described in Chapter 3, Section V The detection limit for the assay of thyroid stimulating hormone was 0.1 mU/l (Biggart et al, 1985). Analogue free thyroxine and free triiodothyronine were measured using Amerlex Kits (Amersham International).

c) RESULTS

On clinical examination, supported by the Wayne index, 108 patients were judged to be euthyroid, 22 to be hyperthyroid and 18 to be hypothyroid. Table 4.9 shows the results of biochemical tests in these three groups.

TABLE 4.9

RESULTS OF BIOCHEMICAL THYROID FUNCTION TESTS IN 148 TREATED HYPOTHYROID PATIENTS

	Euthyroid patients (n=108)		Hyperthyroid patients (n=22)		Hypothyroid patients (n=18)	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Total thyroxine (nmol/l)	134 (32)	48-236	175 (59)	94-300	95 (34)	43-156
Free thyroxine (pmol/l)	23 (7)	8-41	37 (20)	15-95	17 (8)	7-39
Total triiodothyronine (nmol/l)	1.9 (0 4)	1.3-3.1	2.1 (0.4)	1.4-3.3	1.7 (0.4)	1.0-2.3
Free triiodothyronine (pmol/l)	5.5 (1.5)	2.1-12.3	6.9 (1.9)	3.6-11.4	4.2 (1.4)	2.1-6.4
Thyroid stimulating hormone* (mU/l)		<0.1-19.7		<0.1-14.4		<0.1-123.5

*Thyroid stimulating hormone was undetectable (<0.1 mU/l) in 34 euthyroid, 9 hyperthyroid, and 3 hypothyroid patients, so the mean was not calculated.

To test whether individual clinicians showed bias in diagnosing hypothyroidism or hyperthyroidism the distribution of the Wayne index scores assigned to patients by each of the four clinicians was examined by Kruskal-Wallis analysis of variance. There was no significant difference in the four median scores (Figure 4.11).

The mean dose of thyroxine received by patients who were hyperthyroid was higher (137 (42) ug) but not significantly different from the dose received by patients who were euthyroid (128 (50) ug). Hypothyroid patients received a significantly lower dose (88 (38) ug) than euthyroid patients ($p<0.01$). There was no significant difference in the mean age of the patients in each clinical group.

Table 4.10 shows the conventional reference ranges and new reference ranges for euthyroid patients receiving thyroxine (95% confidence intervals of the cumulative frequency distribution). Table 4.11 shows the number of patients in each clinical group who were misclassified by the conventional and new reference ranges. Thyroid stimulating hormone was undetectable by the sensitive assay - that is, below 0.1 mU/l - in 34 of the euthyroid patients, nine of the hyperthyroid patients, and three of the hypothyroid patients. Thyroid stimulating hormone concentration was raised in 21

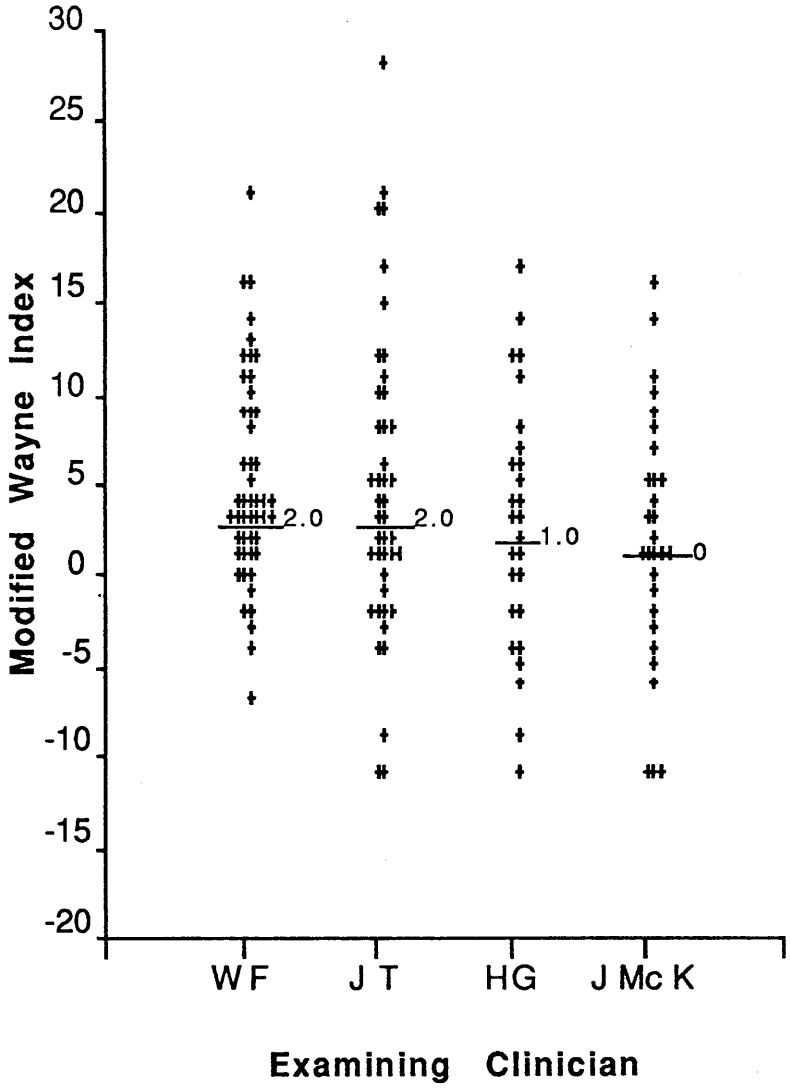


Figure 4.11

Distribution of modified Wayne index scores assigned to patients by the four examining clinicians. Horizontal bars represent median scores

TABLE 4.10

REFERENCE RANGES FOR THYROID FUNCTION TESTS

	Conventional range	Range for patients receiving thyroxine
Total thyroxine (nmol/l)	55–144	90–195
Free thyroxine (pmol/l)	9–25	12–36
Total triiodothyronine (nmol/l)	0.9–2.8	1.3–2.7
Free triiodothyronine (pmol/l)	2.9–8.9	3.0–8.6
Thyroid stimulating hormone (mU/l)	0.35–5.0	<0.1–13.7

TABLE 4.11

NUMBERS OF PATIENTS MISCLASSIFIED BY CONVENTIONAL AND NEW REFERENCE RANGES

(FIGURES ARE NUMBERS OF PATIENTS FALLING BELOW AND ABOVE RANGES)

	Euthyroid patients (n=108)				Hyperthyroid patients (n=22)				Hypothyroid patients (n=18)			
	Conventional Range		New Range		Conventional Range		New Range		Conventional range		New Range	
	Below	Above	Below	Above	Below	Above	Below	Above	Below	Above	Below	Above
Total thyroxine	1	33	3	3	0	14	0	7	4	1	8	0
Free thyroxine	1	34	1	5	0	15	0	9	3	2	8	1
Total triiodothyronine	0	1	3	3	0	2	0	2	0	0	3	0
Free triiodothyronine	1	3	1	4	0	4	0	5	0	4	4	0
Thyroid stimulating hormone	53	21	0	6	13	4	0	1	3	13	0	7

euthyroid patients (range 5.0-19.7 mU/l) and four hyperthyroid patients (range 5.0-11.4 mU/l).

Table 4.12 shows the diagnostic sensitivity, specificity, and false positive rate (Galen and Gambino, 1975) for each test, calculated using the conventional and new reference ranges.

d) DISCUSSION

The major assumption in this study is that the clinical assessment of patients receiving thyroxine replacement by doctors experienced in thyroid disease is correct. This study attempted to establish the role of biochemical measurement of thyroid hormone concentrations in helping this diagnosis.

At present there is no variable that can readily be measured satisfactorily to assess the end organ response to thyroid hormone action in patients receiving thyroxine replacement. The serum concentration of thyroid stimulating hormone is unsatisfactory as the thyrotrophs in the anterior pituitary are more sensitive to changes in the concentration of thyroxine in the circulation than other tissues, which rely more on triiodothyronine (Larsen et al, 1981).

TABLE 4.12

DIAGNOSTIC SENSITIVITY, SPECIFICITY AND FALSE POSITIVE RATE

OF THYROID FUNCTION TESTS

Test	Sensitivity (%)		Specificity (%)		False positive rate (%)	
	Hyperthyroid patients	Hypothyroid patients	Hyperthyroid patients	Hypothyroid patients	Hyperthyroid patients	Hypothyroid patients
			Conventional Reference Range			
Total thyroxine	64	22	69	99	70	20
Free thyroxine	68	17	69	99	69	25
Total triiodothyronine	9	-	99	-	33	-
Free triiodothyronine	18	-	97	-	43	-
Thyroid stimulating hormone	59	72	51	80	80	63
			New Reference Range			
Total thyroxine	32	44	96	96	30	33
Free thyroxine	41	44	95	99	36	12
Total triiodothyronine	9	17	97	96	60	67
Free triiodothyronine	23	22	96	99	44	20
Thyroid stimulating hormone	-	39	-	94	-	50

The data indicate that the reference ranges for serum total thyroxine, analogue free thyroxine, and thyroid stimulating hormone in patients receiving thyroxine replacement are different from conventional reference ranges. It is clear from Table 4.12 however, that serum thyroid hormone and thyroid stimulating hormone concentrations cannot be used with any degree of confidence to classify patients as receiving satisfactory, insufficient, or excessive amounts of thyroxine replacement. There is little difference between the ability of concentrations of total and analogue free thyroxine to detect over-replacement; the poor diagnostic sensitivity and high false positive rate associated with such measurements render them virtually useless in clinical practice. Concentrations of total triiodothyronine, analogue free triiodothyronine and thyroid stimulating hormone are also incapable of satisfactorily indicating over-replacement. The tests perform equally badly in detecting under-replacement.

A question that remains to be answered convincingly is whether it is clinically necessary to measure thyroid hormone concentrations in patients receiving thyroxine replacement. The standard replacement dose in Europe and America was 200-400 ug a day until 1973, when it was halved to 100-200 ug a day on the basis of biochemical measurements of thyroid hormone concentrations (Evered et al, 1973; Stock et al, 1974). No study has shown that this reduction in the standard dose has had any

clinically beneficial effects. Different groups have shown changes in sodium metabolism (Bell et al, 1983), hepatic enzyme activity in serum (Beckett et al, 1985) and systolic ejection time intervals (Jennings et al, 1984) in patients receiving high doses of thyroxine, but such measurements have not been shown to be of any relevance to patient care.

This study indicates that biochemical tests of thyroid function are of little, if any, value clinically in patients receiving thyroxine replacement. Most patients are rendered euthyroid by a daily dose of 100 or 150 ug of thyroxine. Further adjustments to the dose should be made according to the patient's clinical response. In this laboratory 36% of all thyroid function tests are performed to monitor thyroxine replacement. To stop doing these tests in such patients would cause considerable saving in the costs of reagents in laboratories using commerical kits.

These findings emphasise the need for laboratories to make their users aware that the reference ranges for serum thyroxine, free thyroxine, and thyroid stimulating hormone concentrations in patients receiving thyroxine replacement are considerably different from the conventional ranges; they should also point out the limitations of these ranges. This is especially important for General Practitioners and non-specialists, who generally rely on the biochemical findings more than

specialist endocrinologists do in managing these patients. The conclusions in this study also have major implications for schemes such as the Scottish Automated Follow-Up Registry as described in the previous study.

This work was published in the British Medical Journal (Fraser et al, 1986).

VII THYROID DYSFUNCTION AND HYPERCHOLESTEROLAEMIA IN THE GENERAL POPULATION

a) INTRODUCTION

Hypercholesterolaemia, one of the three major risk indices for coronary heart disease, is common in the United Kingdom. Many factors such as diet, genetic predisposition and hormonal imbalance contribute to its high prevalence. It has been shown that in the general population the mean plasma cholesterol is significantly higher in subjects with marginal increases in plasma TSH concentration (Tunbridge et al, 1977). This is more marked in females than males (Tunbridge et al, 1977). The contribution of hypothyroidism to hypercholesterolaemia in the United Kingdom is unknown. This study examines the prevalence of thyroid dysfunction in hypercholesterolaemic subjects in the general population.

b) PATIENTS AND METHODS

A cohort of 2250 males and females (age range 25-59 years) was selected at random from the population of the East-end of Glasgow as part of a coronary risk factor screening programme (Guyer et al, 1985). Plasma cholesterol measurement was made after a 12 hour overnight fast. Blood was collected in plastic EDTA tubes, centrifuged at 1000 g, at room temperature for 10 minutes and plasma separated and stored at 4°C. Plasma TSH measurements were made on all subjects from this study with a fasting plasma cholesterol $\geqslant$8.0 mmol/l and in the first 100 subjects with cholesterol levels $\geqslant$7.0 mmol/l. Non-fasting plasma cholesterol and TSH measurements were also made in a control population of 500 blood donors. This group had an age range from 18-65 years and were seen by the Glasgow and West of Scotland Blood Transfusion Service.

A TRH test was performed, in all, except two, of those subjects with TSH 5-25 mU/l to help assess the significance of borderline TSH results. The TRH test (Ormston et al, 1971) was performed by giving 200 ug of TRH (TRH-Roche, Roche Products Ltd, Welwyn Garden City, AL7 3AY, UK) as a bolus intravenous injection. Blood was collected for TSH measurements immediately before and 20 minutes after the injection.

Cholesterol was determined by the cholesterol CHOD-PAP Enzymatic Method (Boehringer Mannheim GMbh Diagnostics) using a centrifugal analyser (Encore; Baker Instruments). Plasma TSH was measured by the "in-house" monoclonal-polyclonal immunoradiometric assay described in Chapter 3, Section V Serum T_4 and T_3 were measured by "in-house" radioimmunoassays employing antisera obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, ML8 5ER, UK, with ^{125}I-labelled T_4 and T_3 obtained from Amersham International (Amersham International plc, Buckinghamshire, HP7 9LL, UK).

c) RESULTS

The mean + SD cholesterol level in the screened population (5.81 + 1.18 mmol/l) was higher than that in the control group of blood donors (5.53 + 1.25 mmol/l). Ninety individuals in the former group (4% of the population) had plasma cholesterol values >8.0 mmol/l while in 261 the concentrations were between 7.0 - 8.0 mmol/l. Thus, overall plasma cholesterol exceeded 7.0 mmol/l in 15.6% of the population.

The distribution of the TSH levels in the 90 subjects with cholesterol levels >8.0 mmol/l and in the first 100 subjects identified by the screening programme with cholesterol levels >7.0 mmol/l are given in Table 4.13.

TABLE 4.13

THE DISTRIBUTION OF TSH LEVELS IN BOTH HYPERCHOLESTEROLAEMIC GROUPS ARE COMPARED WITH THOSE FOUND IN THE BLOOD DONORS USING X-SQUARED TEST AND YATES CORRECTION FOR SMALL NUMBERS

TSH (mU/l)	Control Group Blood Donors	SCREENED POPULATION Cholesterol ⩾7.0 mmol/l	Cholesterol ⩾8.0 mmol/l
	No of Subjects	No of Subjects	No of Subjects
< 5.0	486	95	78
> 5.0	14	5 (NS)	12 (p<0.001)
>10.0	7	2 (NS)	7 (p<0.001)
>25.0	2	2 (NS)	4 (p<0.01)

Table 4.14 shows the distribution of TSH levels in the 90 subjects with cholesterol values $\geqslant$8.0 mmol/l; and Table 4.15 documents the thyroid function test results of those with abnormal TSH levels. The 10 females in this group of 12 subjects with abnormal TSH levels were all more than 40 years old.

d) DISCUSSION

Hypothyroidism is a readily treatable common cause of hypercholesterolaemia and as such ought not to be missed. This study was set up prospectively to establish the value of screening for hypothyroidism as part of screening programmes for hypercholesterolaemia.

Subjects 1, 2, 3 and 4 were hypothyroid, though they did not consider themselves to have symptoms which necessitated a visit to their General Practitioner. This is not an uncommon feature of hypothyroidism as one blood donor felt fit enough to give blood with TSH levels of 187 mU/l. None of the subjects with borderline TSH levels, in whom a TRH test was performed, were taking medication which might have interfered with the biochemical thyroid function tests. In all cases hypothyroidism was not suspected on the basis of the history or clinical examination, yet their response to TRH was abnormal and consistent with mild primary hypothyroidism.

TABLE 4.14

THE DISTRIBUTION OF TSH LEVELS IN FEMALE AND MALE SUBJECTS WITH CHOLESTEROL LEVELS ≥8.0 mmol/l. THESE HAVE BEEN COMPARED WITH THOSE FOUND IN THE BLOOD DONORS USING X-SQUARED TEST

SCREENED POPULATION WITH CHOLESTEROL ≥8.0 MMOL/L

TSH (mU/l)	Females No of Subjects	Males No of Subjects
< 5.0	38	40
> 5.0	10 ($p<0.001$)	2
>10.0	6 ($p<0.001$)	1
>25.0	3 ($p<0.01$)	1

TABLE 4.15

THYROID FUNCTION TESTS IN THE 12 SUBJECTS WITH FASTING PLASMA CHOLESTEROL ≥8.0 MMOL/L WHO HAD RAISED TSH LEVELS. IN THE SIX SUBJECTS IN WHOM A TRH TEST WAS PERFORMED THE SERUM TSH LEVEL (20 MINUTES POST TRH STIMULATION), IS ALSO GIVEN. SUBJECTS 4* AND 12* WERE MALES

[The laboratory reference range for TSH = 0.35-5.0 mU/l; T_4 = 55-144 nmol/l and T_3 = 0.9-2.8 nmol/l]

	Cholesterol (mmol/l)	TSH (mU/l)	TSH (mU/l) Post TRH	T_4 (nmol/l)	T_3 (nmol/l)
1	8.65	185	-	35	1.3
2	8.75	102	-	23	0.7
3	8.10	100	-	26	1.1
4*	8.3	35	-	45	1.6
5	8.25	16.0	-	67	1.8
6	8.95	14.0	65	87	1.9
7	8.05	13.0	-	71	1.4
8	8.2	11.0	67	63	1.6
9	9.15	9.6	145	96	2.2
10	10.7	7.8	40	45	1.2
11	9.1	7.0	65	97	1.7
12*	8.6	6.4	38	100	1.6

The significance of a borderline elevated TSH level in patients who are clinically euthyroid is uncertain. It has been suggested that "borderline hypothyroidism" is a risk factor in women for coronary artery disease (Dean and Fowler, 1985), although this has not been universally accepted. The finding in this study that this group is selected by looking at hypercholesterolaemia lends weight to the argument that they do indeed have significant thyroid disease which may put them at increased risk of coronary artery disease (Becker, 1985).

The prevalence of hypercholesterolaemia in females rises markedly during the fifth decade of life. In the cohort examined 0.8% of the 25-39 year old females had cholesterol $\geqslant$8.0 mmol/l. This increased to 5.1% in the 40-59 year old age group. No such changes were noted in males. This increase has previously been attributed to menopausal changes. The data from this study indicated that thyroid disease may be an important additional contributory factor in as many as 20% of cases of hypercholesterolaemia in females over 40 years old.

The results of this study confirm that screening for thyroid disease is worthwhile but it must be selective. Screening patients with cholesterol $\geqslant$7.0 mmol/l showed no significant enrichment of hypothyroid patients over the general population. In subjects with cholesterol

$\geqslant$8.0 mmol/l a significant number had unsuspected hypothyroidism and there was an unequivocal increase in the frequency of abnormal thyroid function tests. Whether or not people who appear clinically euthyroid, but have borderline elevations in their TSH, should be treated with thyroxine because they have an increased risk of developing coronary heart disease warrants further investigation.

This study has been published in Clinica Chimica Acta (Series et al, 1988).

CHAPTER 5

SCREENING FOR CONGENITAL HYPOTHYROIDISM IN SCOTLAND

I INTRODUCTION

Almost a century ago a role was recognised for thyroid extract in treating cretinism - the physical and mental manifestation of congenital hypothyroidism. During the past 20 years several well designed studies have shown a clear benefit of thyroxine therapy in treating the mental defects of congenital hypothyroidism but it has become clear that early diagnosis is necessary in order to maximise the chances of the affected infant having a normal intelligence quotient (IQ) later in childhood (Klein et al, 1972; Macfaul & Grant, 1977; Money et al, 1978; Alm et al, 1978; Brock Jacobsen & Brandt, 1981). With this objective in mind screening programmes for congenital hypothyroidism were commenced.

The first pilot screening programmes were set up in Quebec with the measurement of T_4 concentration in filter paper blood spots (Dussault et al, 1975) and in Pittsburgh with the measurement of TSH concentration in cord blood (Klein et al, 1974). Modifications of these programmes were used by other screening centres in North America and Europe. Committees of the American and European Thyroid Associations were formed to deliberate and suggest optimum strategies for screening. In 1979, at the time of the first International meeting in Quebec, much of

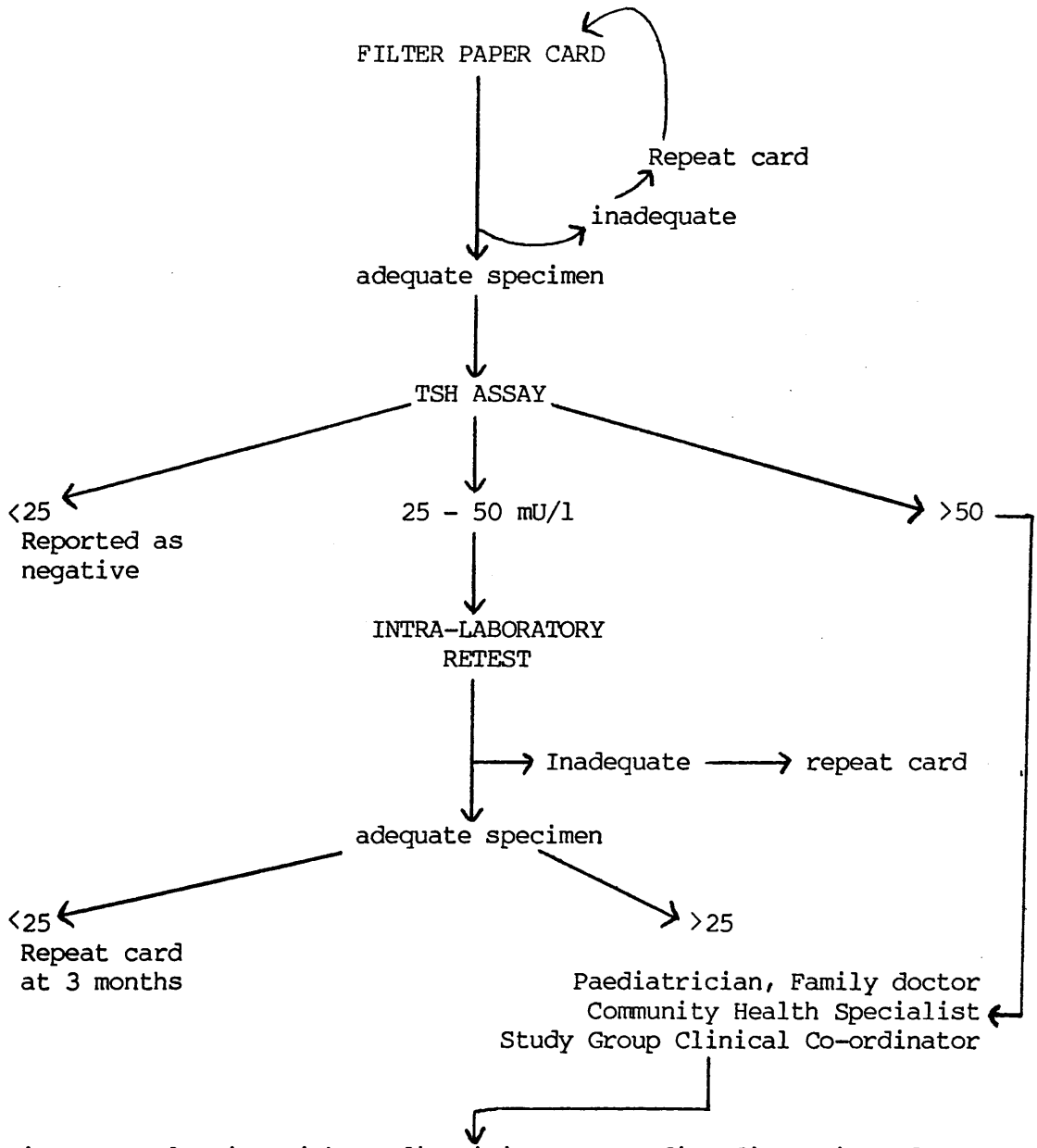
North America, Japan, Australia and Europe had screening programmes in operation (Burrow & Dussault, 1980). Progress was much slower in the United Kingdom and in 1981 health authorities were recommended to start screening. By 1983, all regions had instituted screening programmes mainly using the existing phenylketonuria screening blood spots. Most screening laboratories relied on pre-existing radioimmunoassay expertise and in Scotland, Northern Ireland and Wales, screening has been concentrated in the centres of Glasgow, Belfast and Cardiff respectively. In England there are 23 laboratories involved in screening for congenital hypothyroidism. Some of these, such as in the North Thames area, screen a large population with 90,000 births a year, whereas in other areas, such as Yorkshire where six screening centres exist, some laboratories screen only 3,000 births a year.

A scheme for the external quality assessment of the measurement of TSH in blood spots was established from Manchester and run in conjunction with the UK EQAS for thyroid hormones. This scheme now operates from Birmingham. UK screening centres can participate in this as well as similar schemes operating from France and Germany. A decision was made early on by the Medical Research Council to set up a register, similar to that for phenylketonuria, to record all cases of congenital hyporthyroidism detected by the screening programmes. The data that are collected on these infants should provide evidence of the efficacy of the screening programmes in the UK, and the epidemiological and clinical information will be useful in assessing the factors of importance in the aetiology of thyroid failure.

In normal neonates birth is the trigger for profound changes in the activity of the thyroid gland. Thus, there is a marked TSH surge (plasma levels up to 100 mU/l) within 30 minutes of birth which subsides at 24 hours and returns towards baseline by 3 days of age. The TSH surge causes a rise in serum T_4 levels and a more marked rise in serum T_3 as there is a switch of peripheral T_4 metabolism to T_3 rather than to the metabolically inactive reverse T_3 (Fisher & Klein, 1981). Healthy pre-term infants who are delivered before full maturation of the thyroid - pituitary axis show similar changes with lower TSH and T_4 concentrations, so that at birth T_4 levels are generally lower than full-term infants.

It will be clear from the preceding paragraph that in normal infants aged greater than 3 days the pituitary - thyroid axis has matured to resemble that of the adult so that a heel-prick blood spot taken at 5-10 days of age should reveal relatively low levels of TSH and relatively high levels of T_4, both within reasonably tight reference values. Against this background, it is clearly possible to determine the elevated levels of TSH associated with a deficient thyroid gland as revealed in congenital hypothyroidism although there are some differences as to the action limit for TSH concentration in neonatal blood.

Most screening programmes regard a TSH concentration greater than 20 mU/l (expressed as whole blood) as positive (Mitchell et al, 1978), whereas in other programmes a higher figure of 50 mU/l is taken as a cut-off value. In the Scottish screening programme (Figure 5.1), TSH results less than 25 mU/l are regarded as normal; at between 25 and 50 mU/l a second blood spot is collected for a repeat estimation; and above 50 mU/l, the infant and mother are seen by paediatrician and a full clinical and



First consultation with paediatricians to confirm diagnosis and establish base-line:

1) Clinical Assessment
2) Thyroid Function Tests on venous sample
3) X-ray (for bone maturity)
4) Photograph

↓

1 - 4 reassessed at 3, 6, 12, 24 months

Figure 5.1

Screening protocol used for detection and follow-up of infants with congenital hypothyroidism

biochemical evaluation is carried out. When the clinician is alerted by a positive screening test and clinical evidence of hypothyroidism is also present, treatment can be started. Not all infants who are subsequently confirmed to have congenital hypothyroidism will have clinical signs; the decision to treat these while awaiting a confirmatory serum result may rely on local experience.

In the majority of cases, the diagnosis is in no doubt as the serum results show the classic changes of a low total T_4 and free T_4 with a raised TSH. T_4 treatment in these infants should be started without delay. Problems arise in those infants who display normal concentrations of T_4 with only slightly elevated TSH concentrations. In some of these, if left untreated, the TSH level returns to normal within a few weeks or even months. In others, the TSH starts to rise with falling T_4 and free T_4 concentrations. In the large New England screening programme a diagnosis of hypothyroidism is made when the infant's serum TSH concentration is greater than 40 mU/l (New England Congenital Hypothyroidism Collaborative, 1982). Further serum samples are requested if it is between 20 and 40 mU/l, and a diagnosis of hypothyroidism is only made when the TSH concentration exceeds 40 mU/l or is greater than 20 on three occasions. It was found that only six out of 36 infants with TSH values in the 20 to 40 mU/l range required thyroxine therapy, whereas the remainder eventually displayed normal hormonal concentrations and were clinically well. Clinically and biochemically, these infants pose a difficult problem and a full investigation with a careful questioning of the mother as to her exposure to any antithyroid compounds needs to be made. Serum T_4 concentrations can be decreased in low birth

weight premature babies which emphasises the requirement for normal thyroid function values to be adjusted for gestational age and birth weight (Brock Jacobsen et al, 1977; Oddie et al, 1977; Cuestas, 1978).

Once the diagnosis of congenital hypothyroidism has been confirmed it is normal practice to perform a thyroid scan to determine the aetiology of the defect (De Groot & Reilly, 1981), although other less invasive techniques, such as the measurement of thyroglobulin (Czernichow et al, 1983; Black et al, 1982; Gons et al, 1983) or ultrasound (Dammacco et al, 1985) may also distinguish between the infant with no thyroid gland or ectopic thyroid tissue.

Before the institution of screening for congenital hypothyroidism retrospective studies had revealed the incidence of the condition to be in the range 1/6000 - 1/7000 (Brock Jacobsen & Brandt, 1981; Alm et al, 1978). With screening programmes, the incidence has been remarkably constant in many parts of the world at about one in 4000. This is more common than phenylketonuria, with an incidence of approximately one in 14000. In the UK in the 3 years up to the end of 1984, over 2 million births have been screened, with 607 babies identified as hypothyroid, giving an incidence of one in 3515 (Table 5.1). This higher incidence seen in screening programmes is probably due to their sensitivity in detecting the mildest forms of hypothyroidism in infants who would previously have maintained sub-optimal levels of thyroid hormones. In some of these, further failure of their thyroid gland occurs and they would have presented to a paediatrician at a later age, but it may be that in a proportion they can function more or less normally with reduced thyroid hormone reserve.

TABLE 5.1

NUMBERS OF BABIES SCREENED AND INCIDENCE OF CONGENITAL HYPOTHYROIDISM BETWEEN 1982 AND 1984 IN THE UK

	Numbers of Babies Screened	Positive Cases	Incidence
N Ireland (Belfast)	81 799	26	1:3146
Wales (Cardiff)	105 014	27	1:3889
England	1 749 175	502	1:3484
Scotland (Glasgow)	197 844	52	1:3805
Totals	2 133 832	607	1:3515

Figures for England, N Ireland and Wales are taken from the annual returns to the MRC Register for congenital hypothyroidism.

The reported frequency of various types of defect differs and this might be due to differences in the studied populations or may be due to technical problems in identifying the defect. The most common forms of defect identified by thyroid scanning have been the various dysgeneses of the thyroid which make up 80-90% of all cases (Naruse & Irie, 1983). About one third of these had an ectopic gland, the other two-thirds had a hypoplastic or aplastic gland. Approximately 10% had normal glands and a smaller percentage had large glands suggesting the presence of one of the dyshormonogenesis.

Although the cause of the thyroid gland dysgenesis is unknown, its occurrence in those areas of the world where screening takes place has been very similar. Genetic factors may well play a part as there is a greater chance of thyroid disease in affected families. Congenital hypothyroidism is twice as common in girls as boys. It may be too soon to say if there are real differences in incidence amongst racial groups, but it appears that it may be uncommon in Spanish and Negro infants (Brown et al, 1981) and a high incidence was found amongst Asian infants in Birmingham (Griffiths et al, 1985). It seems that there is an increased incidence of congenital hypothyroidism in Down's Syndrome and in one study the incidence was 1:141, or about 28 times greater than in the general population (Fort et al, 1984). The cause of the thyroid dysfunction was unclear as none had agenesis or ectopia of the thyroid gland. In most cases their biochemical abnormality was mild, but in some there was subsequent progression to hypothyroidism.

The common occurrence of subjects with thyroxine binding globulin (TBG) deficiency has also been revealed by screening with

thyroxine. Approximately one in 9000 infants are affected. It has an X linked dominant inheritance (Fisher et al, 1979) and is an entirely harmless condition. Apart from confirming a low TBG concentration, it does not require any further treatment.

Treatment for congenital hypothyroidism is oral replacement with sodium L-thyroxine, usually at an initial dose of 10 ug/kg per day. If there is any diagnostic difficulty, some advocate that replacement therapy should be started; it is better to reassess early and withdraw treatment at a later date. The dosage can be gradually increased during the early years to the adult dosage of 100-200 ug per day by 12 years of age. The correct dosage can be monitored by the absence of clinical signs or symptoms of hypothyroidism or hyperthyroidism and by the serum concentrations of T_4 and TSH. The majority of infants will show normal levels of T_4 and TSH with replacement but some will have persistently raised serum TSH concentrations despite being clinically euthyroid and having a normal T_4 concentration. This occurs in infants with evidence of intra-uterine hypothyroidism and is thought to be due to impairment of normal feedback control of iodothyronine on TSH secretion (Schultz et al, 1980). Any attempt to suppress TSH concentrations to normal in these infants will induce clinical thyrotoxicosis and may be associated with impaired neurological development and possible development of craniosynostosis (Weichsel, 1978; Penfold & Simpson, 1975).

II THE SCOTTISH SCREENING PROGRAMME FOR CONGENITAL HYPOTHYROIDISM

The screening programme for congenital hypothyroidism in Scotland was commenced at Stobhill Hospital (National Inborn Errors Laboratory) in mid 1979. At first the assay was performed only on blood spots from infants born in West of Central Scotland but by 1980 it had been extended to a National Service. Initially a commercial system was used for measuring the blood spot TSH concentrations but in 1980 the two polyclonal IRMA of Sutherland et al (1981) was introduced with reagents being provided by the Department of Clinical Biochemistry at Glasgow Royal Infirmary. This ran routinely until the end of 1983 when it was replaced by the monoclonal - polyclonal IRMA developed as described in this thesis. In 1981 the UK EQAS for blood spot TSH measurement was introduced and the Scottish Scheme has participated in every distribution made by the EQAS since that date.

III RESULTS OBTAINED FROM THE SCOTTISH SCREENING PROGRAMME FOR CONGENITAL HYPOTHYROIDISM

Between 1979 and the end of 1987 more than 95% of babies born in Scotland were entered in the Scottish Screening Programme. The number of infants screened each year and the number of positive cases of congenital hypothyroidism identified are recorded in Table 5.2. Overall a total of 546,965 infants were screened during this period and 141 cases of congenital

TABLE 5.2

NUMBER OF INFANTS SCREENED AND NUMBER OF POSITIVE CASES OF CONGENITAL HYPOTHYROIDISM BETWEEN 1979 AND 1987 IN SCOTLAND

Year	No of Infants screened	No of +ve cases	Method used
1979	10,036	5	Commercial Kit
1980	68,784	13	2 Poly. IRMA
1981	69,572	17	2 Poly. IRMA
1982	66,864	13	2 Poly. IRMA
1983	65,137	23	2 Poly. IRMA
1984	65,843	16	Mono./Poly. IRMA
1985	67,338	21	Mono./Poly. IRMA
1986	66,187	13	Mono./Poly. IRMA
1987	67,204	20	Mono./Poly. IRMA

hypothyroidism were identified, giving an overall incidence of 1/3879 births. In Table 5.3 are recorded the cumulative statistics and predictive values of the data obtained from The National Screening Programme with the two In-house IRMA TSH assays. For comparative value the corresponding data from the Welsh National Screening Programme has been extracted from the work of John (1987) and also included in Table 5.3. It is apparent from these data that all three assay systems have produced almost identical clinical performance characteristics. The one false negative result from The Scottish Screening Programme was correctly classified by the assay but incorrectly transcribed by the operator.

The analytical performance of the blood spot TSH assay has been judged internally by the inclusion in each assay of three quality control pools. Each month the coefficient of variation is derived from the results obtained on approximately 20 observations of each pool. A summary of these internal quality control data is displayed in Figure 5.2.

The results obtained from The Scottish Screening Programme in the UK EQAS for blood spot TSH measurement are recorded in Figure 5.3. Throughout Figure 5.3 the Scottish result has been compared with the 'target' result from EQAS rather than with the all laboratory trimmed mean (ALTM). Significant differences between the 'target' and ALTM results have been shown in EQAS, reflecting the inherent difficulties of preparing standards for blood spot TSH assays and of recovering TSH quantitatively from the blood spots.

TABLE 5.3

CUMULATIVE STATISTICS AND PREDICTIVE VALUES FOR THE SCOTTISH AND WELSH SCREENING PROGRAMMES

	Scotland+ 1979-83	Scotland* 1984-87	Wales 1981-85
Infants screened	280,393	266,572	160,224
True positive cases (TP)	71	70	34
Other elevated TSH (FP)	94	80	33
True negative cases (TN)	280,228	266,422	160,157
Missed cases (FN)	0	1	2
Incidence	1/3949	1/3808	1/4712
Sensitivity	100%	98.59%	94.44%
Specificity	99.97%	99.97%	99.98%
Efficiency	99.97%	99.97%	99.98%
Predictive value of +ve	43.0%	46.7%	50.7%
Cost per +ve result	£1,100	£1,350	£1,500

+ - commercial system (1979), 2 polyclonal IRMA (1980 - 83)

* - monoclonal - polyclonal IRMA

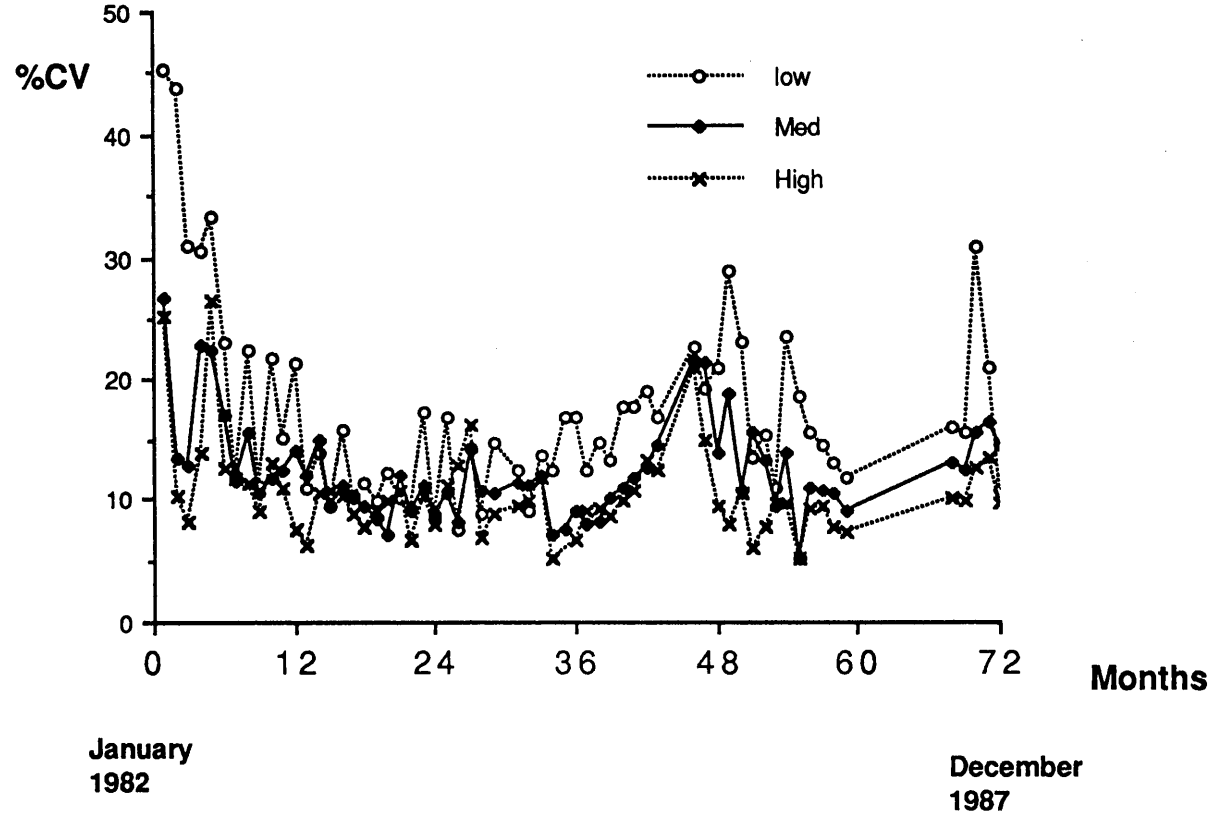


Figure 5.2

Summary of internal quality control data for the blood spot TSH assay

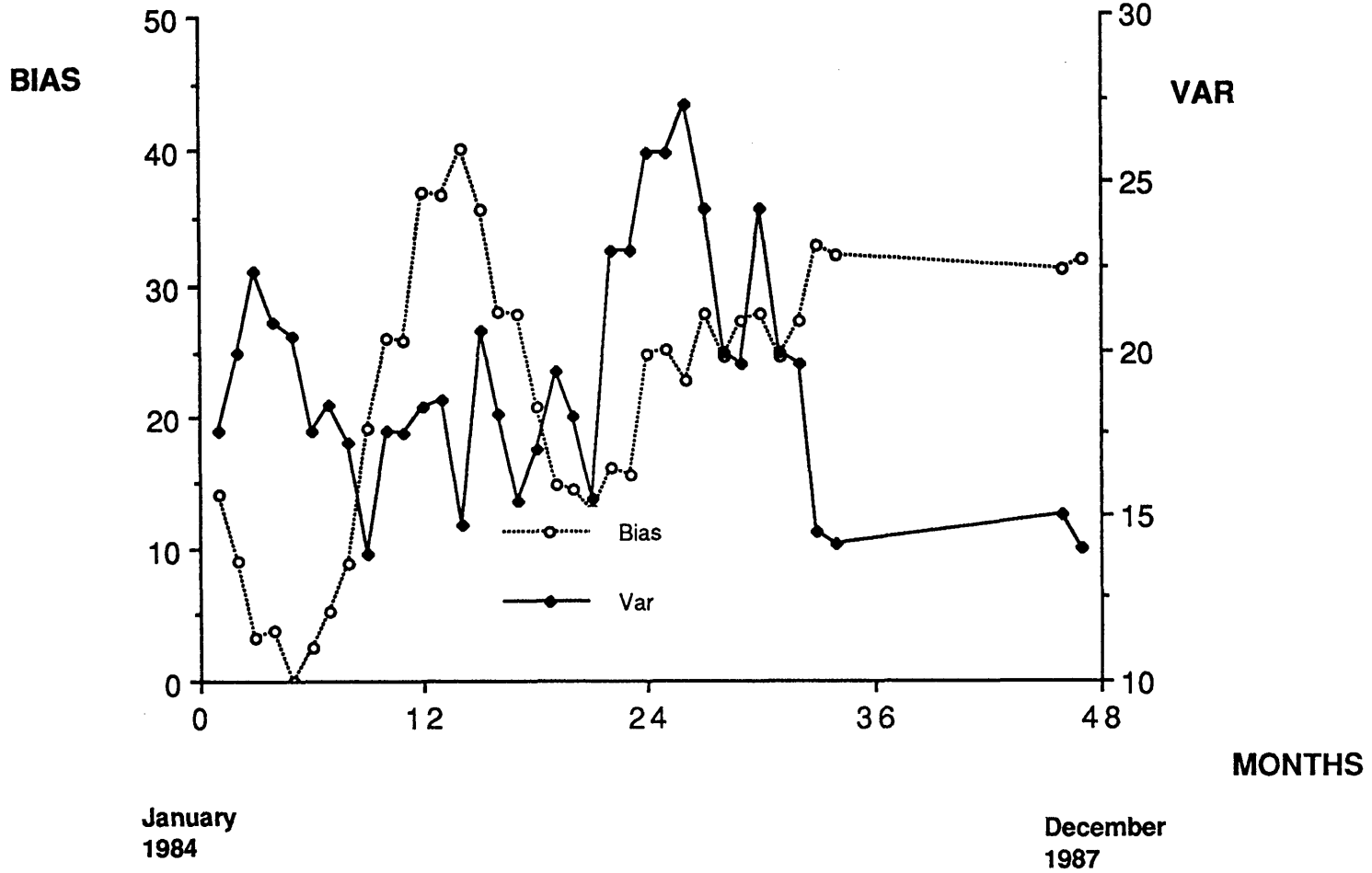


Figure 5.3

Results obtained from The Scottish Screening Programme in the UK EQAS for blood spot TSH measurement

IV DISCUSSION

The analytical challenge of measuring TSH concentrations in a few microlitres of blood eluted from a filter paper disc is a very great one. It is hardly surprising, therefore, that the mean assay imprecision revealed in Figure 5.2 is greater than that encountered for the serum TSH assay based on the same reagents in which some 20 times the volume of serum is taken for assay. By the same token, the EQAS performance data recorded in Figure 5.3 leaves considerable room for improvement, although the assay has performed as well as most of the assays registered in EQAS during the past 7 years.

It is fortunate that blood spot levels of TSH tend to be very elevated in positive cases of congenital hypothyroidism for such discrimination partially offsets the problems of assay imprecision. The clinical performance characteristics displayed in Table 5.3 make satisfying reading for they compare well with the best data published from around the world (John, 1987) and they reveal that the monoclonal - polyclonal IRMA developed with the work described in this thesis has continued to maintain the excellent clinical data obtained from the Scottish Screening Programme.

Future developments on assays for measuring blood spot TSH must seek to reduce the assay imprecision. Such an improvement is likely to be forthcoming with the modification of the very sensitive systems now being used for serum TSH. Such systems, often based on non-isotopic labels of very long shelf-life, should result in an improvement in precision at the critical TSH level of

25 mU/l in the blood spot eluate. This greater assay precision will give confidence to the operator although it is unlikely to make any major difference to the clinical performance characteristics of the National Screening Programme.

The determination of the value of screening programmes for congenital hypothyroidism may be assessed both in terms of the benefit to the mental development of the individual and also as the cost savings to the health care budget.

The infants from the first screening programmes are now approaching life in their teens. Assessment of these patients will show whether the opportunity of early diagnosis and treatment, made possible by screening, provides a favourable outcome in terms of normal development and avoidance of neurological disturbances. The results so far are very encouraging. Treating early avoids the major stigmata of cretinism and in the large New England collaborative study it was shown that the IQ and mental development were the same in patients who were on adequate replacement as in controls (New England Congenital Hypothyroidism Collaborative, 1984). At 2 years of age there was no difference between patients and controls by the Bayley Mental Development Index and at 3, 4 and 5 years the Stanford-Binet IQ scores were the same in each group. No significant correlation with ultimate IQ was found for any clinical or laboratory data except for adequate T_4 replacement.

In the only other report from a large centre the Quebec group demonstrated that their patients had significantly lower scores than did the controls and lower intelligence was found in those with lowest T_4, T_3 and retarded bone age at time of diagnosis (Glorieux et al, 1983). Others have found normal IQ values in

children tested but there is some evidence that there might be minor problems in other neurological areas such as fine motor co-ordination and speech disorders (Naruse and Irie, 1983). It is not known whether this is related to the age on diagnosis, previous deficit of thyroid tissue or adequacy of replacement and most treated infants are too young to assess whether these difficulties will persist or improve.

In the first mass screening programme in Quebec the analysis of the cost of providing a screening service and the savings from the long-term care of patients without screening showed that there was an appreciable cost benefit to screening (Burrow and Dussault, 1980). Using the criteria of cost - benefit analysis, the costs were computed within the framework of a universal health care assurance scheme (a) with an existing PKU screening programme, (b) without a programme and (c) in a free-enterprise system. The costs in the first example would include the cost of reagents, any additional equipment and technical and computer facilities required to provide the hypothyroid screening within an existing PKU screen. In the last option, the costs would include hospital costs, sample collection including recalls, treatment, laboratory space and medical fees. Any savings in costs would come from the elimination of costs needed to look after an undiagnosed case. It was assumed that at least 50% of these cases would end up with an IQ of less than 85, would require some light to moderate care in an institution and spend on average 1 week in hospital before diagnosis or for treatment of complications. Over a 5 year period the benefits´ ratios in each instance are (a) 3.9, (b) 1.8 and (c) 1.2. It was only when screening for hypothyoidism was introduced that their genetics screening programme changed from a net deficit

with PKU and tyrosinaemia screening to one of net economic benefit. This was due to the absence of any effective long-term treatment for tyrosinaemia and the low incidence of PKU of one in 35,000 births. The introduction of hypothyroid screening and the eradication of a treatable form of mental retardation, even without a favourable cost benefit, must be looked on as one of the major advances in preventive medicine.

The previous section of this Chapter has provided strong evidence of the clinical value of a screening programme for Congenital Hypothyroidism both to the individual and to the health care budget. Thus, screening programmes are likely to continue for many years to come, although there are now some interesting developments which may lead to a reassessment of the programmes in the long-term.

Thus whilst most children seem to benefit sufficiently from existing screening programmes, for some, the ability to detect in utero-hypothyroidism and to start treatment before birth may improve their chances of avoiding some of the behavioural disorders. Ultrasonography has been used to detect a goitre in a foetus and measurement of TSH in the amniotic fluid revealed a raised level so that thyroid hormone replacement could be commenced on the first day of life (Kourides et al, 1984). The low levels of TSH in amniotic fluid from third trimester pregnancies of less than 0.15 to 0.55 mU/l could only be measured after prior concentration of the amniotic fluid (Kourides et al, 1982), but these levels in unconcentrated fluid may well be detected by the more sensitive assays for TSH now available. The problem remains of the appropriate choice of patients for

amniocentesis, but mothers at known risk, such as those with familial goitre or those taking antithyroid medication, warrant further study.

The recent detection of thyroid - growth blocking antibodies in the blood of 15 out of 34 mothers who subsequently gave birth to hypothyroid infants could lead to a pre-natal diagnostic test (Van Der Gaag et al, 1985). Using a cloned rat thyroid cell line ($FRTL_5$), Dussault has shown that in cells deprived of TSH for 5 days, after the addition of TSH and maternal immunoglobulin, there was a significantly greater increase in ^{125}I uptake by the thyroid cell membranes, in those mothers of hypothyroid infants compared with mothers of normal infants (Dussault and Bernier, 1985) The mechanism by which these maternal antibodies are responsible for increasing the iodine uptake by the cell membranes remains to be elucidated. This has encouraged him to start screening 25,000 pregnant women to evaluate the false-positive and false-negative rate of this test and its role in predicting a pregnancy at risk of producing a hypothyroid infant. Foetal therapy has already been attempted by injecting T_4 intramuscularly to the foetus, or into the amniotic fluid (Van Merle et al, 1975), or in rats by giving 3,5-dimethyl-3′-isopropyl-l-thyroxine to the mother. This is a thyroid hormone analogue which crosses the placenta, and prevents foetal goitre formation (Comite et al, 1978), so that foetal diagnosis and treatment becomes a real possibility.

CHAPTER 6

GENERAL DISCUSSION

I EVOLUTION OF TSH ASSAYS

a) THE USE OF ^{125}I AS THE LABEL IN IMMUNOMETRIC ASSAYS

Using radioiodine labelled reagents, it is possible to measure concentrations of substances down to 10^7 molecules/ml (approx. 10^{-14} mol/litre) (Ekins, 1987). But aside from the sensitivity which the use of radioactive isotopes confers upon immunoassays, an important attribute to radioactive measurement is its relative invulnerability to environmental interference. Radioisotopic disintegration is not affected by physical or chemical factors, and provided counting conditions are standardised, highly accurate estimates of radioactive content can readily be made using cheap and easily operable equipment.

However, notwithstanding these advantages, considerable pressure has existed for some years to find substitutes for radioisotopes for use in the present context. Amongst the principal reasons for this are the widespread public

perception and fear of health hazards associated with radioactivity (leading to legal constraints on the use of radioisotopes in many countries), the logistic and quality-control problems arising from the limited shelf-life of radiolabelled reagents and the expense of (automatic) radioactive sample measuring equipment. A further disadvantage of radioisotopes stems, paradoxically, from the very invulnerability of radioactive disintegration to environmental influence referred to above, implying that it is impossible to devise simple "homogeneous" assay systems in which labelled reaction products do not require physical separation prior to measurement.

Some of these arguments are of doubtful validity. For example, the amounts of radioactivity involved are exceedingly small, and the health hazards that they present in the normal hospital laboratory negligible, being often outweighed by the potential dangers arising from carcinogens, viruses and other toxic agents which often form part of the laboratory environment. Similarly, the costs attaching to the use of radioactive techniques are generally greatly exaggerated. Counting equipment, the only specialised apparatus normally required, is now cheap and reliable, the cost of the more sophisticated types of sample counter arising primarily from the automatic sample changing and data processing facilities which they provide. Indeed no currently visualised alternative non-isotopic methodology

offering comparable sensitivity and precision to the radioisotopic techniques, and having similar sample handling capacity, has any chance of being significantly cheaper.

In short, the various labels utilised for immunoassay purposes each possess individual attribute, any one (or combination) of which may be of special advantage in particular situations; none is likely to emerge as a "universal" label, applicable in all the varied circumstances in which immunoassays find application.

However, analyte concentrations below 10^7 molecules per millilitre lie below the detection limits of current methods. Though it is sometimes argued that it is unnecessary to develop techniques of sufficient sensitivity to penetrate into this region, specific examples occur, even in endocrinology, in which it would be clinically or otherwise useful to increase the sensitivity of present techniques.

In order to fully exploit the advantages of non-competitive assay designs, it is clearly necessary to identify labels of higher specific activity than that of commonly used radioisotopes thus non-isotopic techniques form the necessary basis on which major improvements in immunoassay sensitivity will inevitably rest.

b) ALTERNATIVE HIGH SPECIFIC ACTIVITY LABELS IN IMMUNOMETRIC ASSAYS

Alternative high specific activity labels have been utilised for immunoassay purposes. The conversion of many molecules of substrate by a single enzyme molecule implies an amplification of the specific activity of enzyme-labelled antibody molecules assuming high sensitivity of detection of the reaction product. Examples of high sensitivity enzyme-labelled antibody techniques that have exploited this phenomenon are the USERIA technique of Harris et al (1979) and the method of Shaleve et al (1980), relying on radioactive and fluorogenic substrates respectively. More recently, somewhat more complex methods of amplification of the enzyme - generated signal have been devised which rely, not on direct observation of the reaction product per se, but on observation of the latter's catalytic effect on a secondary enzyme system, which remains essentially dormant until thus activated (Stanley et al, 1985; Johannsson et al, 1985). Typical systems governed by this principle have been utilised in the development of "ultrasensitive" immunoassays for TSH and other comparable analytes.

Chemiluminescent labels, in spite of quantum efficiencies generally considerably lower than 100%, are also, in principle, capable of yielding higher specific activities than radioactive isotopes, and hence higher immunoassay

sensitivities, enabling, for example, TSH to be measured at concentrations down to 0.01 mU/l or lower (Weeks et al, 1984; Kemp et al, 1984).

Another form of chemiluminescent assay combines the attributes of luminometric measurement with the signal amplification provided by the use of enzyme labels. Originating from the studies of Whitehead et al (1983), assays based on the use of antibodies labelled with horseradish peroxidase and luminescent substrates have recently been launched by Amersham International Enhancement of the luminescent yield of the peroxidase system may be achieved by the use of phenols (Thorpe et al, 1985), resulting in further signal amplification and an increase in assay sensitivity.

Fluorescent labels are potentially capable of yielding very high specific activities since each labelled molecule may be induced to yield many photons in response to exposure to a high energy light input. Fluorescent markers also have a number of other attractive features, including the possibility of repeated, confirmatory, measurements on the same sample, and of observation of their spatial distribution on a solid surface (thus allowing the ready development of multiple immunoassays in the same sample). The major drawbacks associated with conventional fluorescent measurements are the background fluorescence generated by many biological substances, plastics etc and the bleaching effects deriving from continuous exposure of the

fluorophore to high intensity light. These effects conspire to limit the assay sensitivities which are conventionally attainable by such methods. However, these disadvantages may be largely overcome by recourse to pulsed-light, time-resolving fluorescence measurement techniques.

In conclusion, advantages stem, in practice, from the combined use of "non-competitive" immunoassay designs and high specific activity non-isotopic labels. Most of the newer techniques now becoming commercially available reflect this concept. Consequently they are closely comparable in overall performance, such minor differences as exist primarily reflecting differences in the characteristics of the immunological systems on which the assays are based (deriving from the qualities of the antibodies used, etc) rather than in the particular labels used. Therefore, in practice, the decision as to which of the commercially available systems to use is likely to be dictated more by such considerations as cost, the range of kits available, the necessity for, and dependance on, fully automated equipment, ease of servicing, etc, rather than on scientific performance *per se*.

c) IMPLICATIONS OF THESE DEVELOPMENTS FOR THE SERUM TSH ASSAY SERVICE IN GLASGOW ROYAL INFIRMARY AND THE SCOTTISH SCREENING PROGRAMME FOR CONGENITAL HYPOTHYROIDISM

It will be clear from the previous two sections that the use of ^{125}I-based immunometric assays for TSH will diminish in the years ahead and the implications of this trend must be considered in the local environment in order to ensure that the best possible service will be maintained.

There is much still to learn about the physiology of thyroid hormones, especially in subjects treated for thyroid disease (see Section II). However, it is almost certain that the clinical value of a serum TSH assay will be enhanced by improving the detection limit from 0.2 mU/l to 0.02 mU/l. Such an improvement is not possible with the existing monoclonal and polyclonal antibodies and an ^{125}I-label. The most straightforward way to achieve this improvement is to move to a non-isotopic label that is compatible with the available antibodies.

The Delfia serum TSH assay (Pharmacia Diagnostics Ltd) has been shown to have the required additional sensitivity, but it is expensive to run (see Chapter 4, Section IIId). Therefore, for an experimental period of 6 months it was decided in October 1988 to introduce the Delfia TSH assay into Glasgow Royal Infirmary as a back up assay to the in-house IRMA. The Delfia assay will only be performed on those

serum specimens yielding a TSH result of <0.5 mU/l in the in-house IRMA. It is anticipated that approximately 10% of the routine TSH work load will be processed by this back up assay. An assessment of the cost/benefit ratio of the Delfia TSH assay is expected to be made in early 1989.

A more long-term solution to the problem may lie in the local production of europium labelled TSH monoclonal antibody for use in a Delfia type of assay. Collaborative work between other members of the Glasgow Laboratory and Dr T Lovgren of Pharmacia, Finland have established the feasibility of this approach and Pharmacia Diagnostics Ltd have recently agreed to supply Glasgow with the necessary europium chelates and methods to effect local manufacture.

The implications of these developments for the screening programme for congenital hypothyroidism are slightly different. Improved blood spot TSH sensitivity is unlikely to improve substantially the predictive values obtained from the screening programme (Chapter 5, Section III) despite an improvement in imprecision around 25 mU/l. However, the adoption of a non-isotopic label will enable the production of large batches of reagent of long shelf-life and this should greatly simplify the task of reagent provision for the screening programme. Such a change will require to be accommodated within existing budgets and so the use of the Delfia assay itself (or any other fully commercial system) is contraindicated. However, the use of a Delfia type of assay

based on local reagents is likely to be feasible and this will almost certainly represent the next phase of assay development for the screening programme.

II THE INTERPRETATION OF SERUM TSH RESULTS IN SUBJECTS BEING TREATED WITH ORAL THYROXINE

It is now well established that highly sensitive assays for serum TSH can reliably distinguish the undetectable levels found in thyrotoxic patients from normal levels (Seth et al, 1984). However, the role of sensitive TSH assays in monitoring patients receiving thyroxine replacement has still be to be fully evaluated.

The work presented in Chapter 4 of this thesis (Sections V and VI) has shown that many patients receiving T_4 replacement therapy have undetectable serum TSH levels, both in the in-house IRMA and using the Delfia assay. These data have been confirmed by other workers (Semple et al, 1985). Does this mean that all these patients are receiving excessive doses of oral T_4? This question remains to be answered fully but it is generally recognised that the pituitary differs from some other tissues in that T_3 derived from local conversion from T_4 within the cell occupies a greater proportion of T_3 nuclear receptors than T_3 derived from serum (Larsen, 1982). This greater sensitivity of the pituitary to serum T_4 has led to the assumption that it is an unrepresentative target tissue regarding the assessment of overtreatment with T_4.

As alternatives to the more sophisticated measurements of peripheral tissue responses described (Goolden et al, 1971; Jennings et al, 1984; Wilcox and Levin, 1986; Coindre et al, 1986), Gow et al (1987), used simple measurements of constituents in serum known to be altered in patients with overt thyroid disease and related these to TSH secretion. High concentrations of alanine aminotransferase (ALT), liver-specific glutathione S-transferase (GST), sex hormone-binding globulin (SHBG), and angiotensin-converting enzyme (ACE) have all been described in hyperthyroidism (Beckett et al, 1985; Ashkar et al, 1971; Anderson, 1974; Smallridge et al, 1983), whereas concentations of T_4-binding globulin (TBG) and creatinine may be decreased (Ahmed and Smethurst, 1980; Bradley et al, 1974). Elevated concentrations of creatine kinase (CK) are found in hypothyroidism (Doran and Wilkinson, 1975). Changes in the concentrations of these analytes reflect altered entry to (due to changes in synthesis or membrane permeability) or clearance from the blood. These markers are arguably crude and insensitive indicators of thyroid status which cannot serve to assess thyroid status in an individual. However, when many of these markers are measured in groups of patients with overt hyperthyroidism, unequivocal statistically significant abnormalities are found.

Gow et al (1987), demonstrated a relationship between the serum markers used to assess tissue thyroid status and TSH levels measured by a sensitive assay in patients taking T_4. Such a relationship was not found between the serum markers and free T_3 measurements. Some patients had abnormally high ALT, GST, SHBG, and ACE levels at higher T_4 doses. These abnormalities were not as marked as those in patients with untreated overt

hyperthyroidism, but in addition to the complete suppression of TSH secretion, they provided evidence of a generalised tissue over-exposure to thyroid hormones. On the basis of this work, Gow et al (1987) concluded that TSH secretion could be used as a sensitive and representative test of peripheral tissue exposure to thyroid hormones in patients receiving T_4 replacement therapy.

Recently a study has been set up by Carr et al (1988) to examine the results of TRH tests using a sensitive IRMA for TSH, along with FT_3 and FT_4 measurements and clinical observations in a group of treated hypothyroid patients, each of whom was tested on a series of different daily thyroxine dosages. The objective was to collect data at the dosage which resulted in a normal response to TRH for each patient, and at dosages slightly above and below this. The data were used to examine the variation in TRH responsiveness, FT_3 and FT_4, with slight changes in thyroxine dosage, and to test the hypothesis that the basal TSH (IRMA) level in a patient receiving thyroxine replacement is capable of indicating a normal or abnormal response to TRH, and can be useful as a guide to biochemically correct dosage.

The data of Carr et al (1988) indicated that clinical observations were relatively insensitive and failed to detect significant differences between patients receiving thyroxine at various dosages within the narrow range studied. The measurements of FT_3 and FT_4 levels, while showing significant variations over this dosage range for the whole group of patients, were also poor indicators of thyroxine dosage for individual patients.

In contrast, the TRH test showed great sensitivity to small changes in thyroxine dosage; in the majority of cases a change of dosage of only 25 ug was sufficient to modify the TRH response from a normal to a clearly abnormal value.

Furthermore, patients receiving thyroxine in a dosage which resulted in the most normal response to TRH, all appeared clinically euthyroid. It was concluded that the TRH test was able to determine a replacement dosage of thyroxine which equated with the patient being clinically euthyroid, and which correlated well with results of circulating thyroid hormone measurements in individuals and a population of patients, and it was also sensitive to small changes in thyroxine dosage. Basal TSH alone correlated well with TRH response (within the critical range close to normal) and was only slightly less sensitive than the TRH test itself to changes in dosage of thyroxine (Carr et al, 1988).

It has been stated that lack of fine tuning has never been shown to have deleterious consequences (Pearce and Himsworth, 1984), though no long-term study has been conducted to explore this by the deliberate use of replacement regimes based on high and low dosage in relation to a standard such as the TRH test.

In the meantime, the value of routinely adjusting thyroxine dosage according to any test of thyroid function remains controversial.

III CONCLUSIONS AND FUTURE DEVELOPMENTS

The original aims of this thesis have largely been fulfilled. Techniques for the production of mouse monoclonal antibodies to

human TSH have been developed and methods for the characterisation of such antibodies established in order to select those reagents suitable for use in immunometric assays. A variety of two-site immunoradiometric assays for human TSH based on the monoclonal antibodies produced and available polyclonal antisera have been developed and optimised and their use validated for the measurement of TSH in serum and in neonatal blood spots. Finally, the value of the monoclonal - polyclonal immunoradiometric assay for human TSH has been assessed in a variety of clinically based situations.

At the time this work was being carried out, non-isotopic systems for the measurement of TSH were beginning to become commercially available. Such assays offered greater sensitivity than those employing iodine as label. In this laboratory the future should see the replacement of iodine with a non-isotopic label and the subsequent development of assays for both serum and neonatal blood spot TSH.

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