ENZYME IMMUNOASSAY AS AN ANALYTICAL TOOL IN CLINICAL CHEMISTRY.

being a thesis presented by

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in application for the degree of
Master of Science

ABSTRACT.

Immunoassay is a technique used extensively in clinical chemistry. It is a useful technique which in its most popular form of Radioimmunoassay (RIA) uses radioactive labels. As an alternative to RIA Enzyme immunoassay (ELISA) offers the advantages of the antigen-antibody reaction without the drawbacks of using a radio label.

In the work presented, ELISA was investigated as an alternative to RIA for the measurement of TSH in patients suspected of having thyroid disease. The development, performance and assessment of the assay are presented.

To further develop the technique, an assay for estimating albumin in the urine of diabetic patients was developed. Again the performance and assessment are presented. The method was then used to quantitate the albumin excretion in a selected group of non-insulin dependent Type II diabetics

DECLARATION.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United college of St. Salvator and St. Leonard, University of St. Andrews and at the Department of Clinical Chemistry, Victoria Hospital, Kirkcaldy under the supervision of Dr G Kemp and Dr I R F Brown.

Alasdair M Mc Bain.

CERTIFICATE

I hereby certify that Alasdair M McBain has spent seven terms engaged in research work under my direction, that he has fulfilled the conditions of Ordinance No. 51 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Master of Science.

Dr G. D. Kemp.

ACKNOWLEDGEMENTS

I wish to thank Dr Kemp and Dr Brown for their time and attention, always given freely and without question. I also wish to acknowledge the late Dr Andrew P Kenny for stimulating the original idea for this work.

I am indepted to Fife Health Board for their support throughout and to Boots Celltech Ltd for their gift of antiserum. I also wish to thank Dr I W Campbell for allowing me to study his patients

DEDICATION

To my Father and Mother for their support in the early years and with love and affection to my wife Isabelle, and children David, Gavin and Kathryn.

List of Non-Standard Abbreviations.

RIA.....Radioimmunoassay.

TSH.....Thyrotrophin.

T_4.....Thyroxine.

T_3.....Triiodothyronine

LH.....Luteinising Hormone.

FSH.....Follicle Stimulating Hormone.

TRH.....Thyrotrophin Releasing Hormone.

EMIT.....Enzyme Multiplied Immunoassay Technique.

ELISA.....Enzyme Linked Immunosorbent Assay.

HRP.....Horseradish Peroxidase.

IgG.....Immunoglobulin G.

SAPU.....Scottish Antibody Production Unit.

PBS.....Phosphate Buffered Saline.

SD.....Standard Deviation.

CV.....Coefficient of Variation.

AER.....Albumin Excretion Rate.

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SECTION 1

INTRODUCTION

INTRODUCTION.

The use of antigen-antibody reactions as a quantitative technique was first introduced by Yallow & Berson (1959). In that example the extent of reaction was measured by the incorporation of a radioactive label. Known as Radioimmunoassay (R.I.A.) the technique has found widespread application in the field of hormone analysis, offering a degree of specificity and sensitivity unheard of previously.

Antibodies labelled with enzymes were first developed in the 1960s for use in identifying antigens in histological preparations in a purely qualitative fashion. The observation by Engvall & Perlman (1971) that antibodies could be bound to solid phase materials allowed the development of Enzyme Immunoassay as a quantitative technique of some considerable importance in the field of clinical chemistry.

The Clinical Chemistry Department of the Victoria Hospital has used R.I.A. for some time now to assist in the diagnosis and monitoring of thyroid disease in Fife and has found it a useful and reliable technique for routine use. However the cost of using radiolabelled reagents, and their limited shelf life, coupled with the hazards and strict guidelines for the use and disposal of such substances, encouraged the development of non-isotopic methods of immunoassay as alternatives to existing methods of analysis.

Part of the work carried out for this thesis was related to determining the feasibility of using an

enzyme immunoassay as an alternative technique for monitoring thyroid function. This was achieved by developing and assessing an assay for estimating Thyroid Stimulating Hormone (T.S.H. Thyrotrophin) and comparing it with the R.I.A. method in use at the time.

As well as being directly involved in thyroid monitoring the Department is also involved in the monitoring of diabetic patients requiring assessment of their glycaemic control. This is done routinely by measuring their blood glucose and Haemoglobin A_1.

In 1982 Viberti et al reported that the measurement of micro quantities of albumin in the urine could be used to predict clinical nephropathy in patients with insulin-dependent diabetes mellitus. The Diabetic and Clinical Chemistry Departments were very interested in measuring these low quantities of urinary albumin (microalbuminuria) but no commercial kits for urinary albumin were available and so the development of an enzyme immunoassay, using the experience gained from the TSH method, was investigated.

1.1. ASSESSMENT OF THYROID FUNCTION.

The diagnosis of thyroid dysfunction may be possible purely on clinical assessment, but increasingly nowadays the diagnosis of thyroid disease and its subsequent treatment and monitoring requires quantitation of some, if not all, of the hormones involved. Indeed most hospital laboratories can now offer specific measurement of thyroxine (T_4), triiodothyronine (T_3), and TSH on a routine basis, which should enable diagnosis of the majority of thyroid related illnesses.

1.1.1. Chemical Nature of TSH.

Thyrotrophin (TSH) can be isolated from the human anterior pituitary. It is a glycoprotein with a molecular weight of about 28,000 containing about 15% carbohydrate and can be dissociated into two distinct sub-units designated the α and β sub-units (Liao & Pierce, 1970). These two sub-units show marked similarities with the sub-units of human Luteinising Hormone (LH), and human Chorionic Gonadotrophin (HCG) as well as with those of Follicle Stimulating Hormone (FSH). Indeed it has been demonstrated that the α sub-units are identical or nearly so in their primary amino acid structure such that each α sub-unit can combine with a β sub-unit to yield the appropriate activity for the particular β sub-unit (Pierce, 1971). Since the α sub-units are identical it follows that the immunological specificity for each hormone must lie on the β sub-unit (Cornell & Pierce, 1973).

When considering an immunoassay to measure these hormones it is advantageous to use antisera which are as specific for the antigenic material as possible. The present study used antisera which were raised against the β sub-unit of TSH.

1.1.2. Control of Thyroid Hormones.

TSH released from the anterior pituitary controls the output from the thyroid gland of the hormones T_4 and T_3. In turn the TSH output from the pituitary is regulated by Thyrotrophin Releasing Hormone (TRH) from the hypothalamus and by the circulating free T_4 and free T_3 (Zilva & Panall, 1984).

In the healthy individual both thyroid hormones are tightly bound to thyroid hormone binding proteins such that only about 0.015% of total plasma T_4 and 0.5% of total plasma T_3 exist as the free hormones (Hoffenberg & Ramsden, 1983). None the less it is the circulating levels of the free hormones which are metabolically active and are responsible both for the metabolic effects of the hormones and their regulation of the TSH output from the pituitary. This control is normally excercised via a negative feedback mechanism whereby the output of TSH and also possibly TRH is inhibited. It is now considered that free T_3 accounts for much of the total thyroid hormone activity in plasma and hence exerts the most influence on TSH release from the pituitary.

The main uses of the measurement of these hormones in cases of suspected thyroid disease are in helping to

divide patients into euthyroid, hypothyroid and hyperthyroid states as well as monitoring their subsequent treatment (Evered, 1976). The measurement of TSH in addition to T_3 and T_4 is of considerable importance in the diagnosis and monitoring of all of these states.

In the normal euthyroid patient it is now widely accepted that the level of TSH is usually below 7 mu/L (Ismail, 1981) and about 10% of these patients will have levels which lie below the limits of detection of RIA (Tunbridge & Hall, 1976). There are no significant differences between healthy men, women and children greater than 1 year old, and values in the elderly are only marginally raised. Using a highly sensitive technique Patel et al (1972) reported a circadian rythm of TSH reaching its highest level about 3 a.m. and its lowest about 8 p.m. They also reported that levels within a 24 h period could vary by as much as 2.4 mu/L for individual patients.

In cases of clear clinical hypothyroidism the total serum T_4 is generally well below the reference range and subsequently, the TSH is usually markedly elevated (Utiger, 1965 ; Herschmann & Pittman, 1971). Indeed the measurement of plasma TSH is of particular benefit in the early diagnosis of hypothyroidism or in subclinical hypothyroidism where the raised TSH may be the only pointer to disease in the absence of any symptoms attributable to thyroid hormone insufficiency. Patient who have been diagnosed as being hypothyroid

are generally treated with L-thyroxine (Evered 1976), such replacement therapy being considered adequate when the patient's symptoms are relieved and the basal level of TSH is reduced to the normal range.

So in addition to diagnosis and monitoring of the adequacy of the replacement dose, the measurement of TSH may also give an indication of a patient's compliance with their treatment.

It is arguable, however, that correct diagnosis of hypothyroidism requires that both T_4 and TSH levels are measured since not all cases of low serum T_4 are due to hypofunction of the thyroid gland. In cases where hypothyroidism is suspected secondary to pituitary hypofunction, the serum T_4 level may be low, due to lack of stimulation, but the TSH level would be inappropriately low due to the pituitary dysfunction.

In primary hyperthyroidism, or thyrotoxicosis, the serum T_4 and/or T_3 levels are elevated and the TSH levels are below normal. Until very recently most methods available for measuring TSH were too insensitive to detect subnormal levels of TSH. The introduction of an immunoradiometric assay for TSH (Seth et al, 1984) using a monoclonal antibody allowed estimation of the very low levels of TSH expected in thyrotoxicosis. A " high sensitivity " method was not available during this study and the RIA method used routinely could not detect low levels of TSH . Basal estimations using this method were therefore of limited use in the diagnosis

of thyrotoxicosis. However the TSH response to the administration of TRH was of use in such cases. In patients who are thyrotoxic there is usually a failure to respond to TRH due to the suppressive effects of the elevated levels of thyroid hormones. However it should be emphasised that failure to respond to TRH is not in itself diagnostic of primary hyperthyroidism.

The TRH test may also be of value in diagnosing a patient as being euthyroid or hypothyroid, since both these states also have fairly typical responses to TRH stimulation.

In summary, TSH measurement has a valid and important part to play in diagnosing all types of thyroid disorders. Although the above paragraphs outline the possible uses very briefly it is hoped that they do emphasise the importance of measuring TSH in patients suspected of having thyroid disease.

1.2. URINARY ALBUMIN EXCRETION.

The loss of protein through the kidney in normal healthy individuals is usually not greater than approximately 80 mg/day. Levels of protein which are greater than this are normally indicative of disease.

Significant proteinuria may be due to increased permeability such as in nephrotic syndrome or orthostatic proteinuria or may be due to tubular damage such as occurs in pyelonephritis. In cases of glomerular damage, albumin is usually the predominant protein found in the urine. Proteinuria can also occur

more rarely due to a large amount of circulating low-molecular weight protein such as Bence Jones protein.

In diabetes mellitus associated with nephrotic syndrome, the increased glomerular permeability is thought to be secondary to the diabetes and the development of clinical proteinuria (ie. protein >0.5 g/day) has been a recognised consequence of the disease. Some 30% of such diabetic subjects may then go on to develop renal failure (Deckert et al, 1978) but by the time the proteinuria is detected renal damage is too severe to stop the progression (Viberti et al, 1982a).

The measurement of levels of albumin in the urine, prior to the development of overt clinical proteinuria, may allow more intensive therapy to delay the development of glomerular damage and hence delay the clinical nephropathy.

Keen & Chlouverakis (1963) developed an RIA method for measuring such "subclinical" levels of urinary albumin thus allowing the investigation of the effects of stricter control.

Mogensen (1971) observed increased urinary albumin excretion at the time of diagnosis and before treatment in Type I juvenile diabetics. Later Viberti et al (1979) reported that the increased excretion of albumin was due to glomerular damage and that a regime of stricter glycaemic control could reduce the albumin excretion presumably by reducing the glomerular permeability.

Subsequently, the Steno group (1982) confirmed the findings of Viberti and also suggested that the reduction in urinary albumin excretion perhaps signalled an alteration in the course of the development of the renal disease associated with diabetes mellitus. In the same year Viberti et al (1982) confirmed that microalbuminuria could be used as a predictor of the development of clinical nephropathy in insulin dependent diabetics. Subsequent reports by Jarret et al (1984) and Mogensen (1984) suggested that the same is also true for non-insulin dependent, Type II diabetics.

As part of a long term study (1 year's duration) of Type II diabetics , it was decided to assess their urinary albumin excretion before and during treatment with oral hypoglycaemic agents. Consideration of the experience gained in the TSH assay development suggested that a double antibody sandwich type of ELISA could perhaps also be used for albumin.

1.3. ENZYME LINKED IMMUNOSORBENT ASSAY.

The introduction of enzyme immunoassay as an important quantitative technique (Engvall & Perlman, 1971, Van Weemen & Schuurs, 1971) has been followed by a steadily increasing interest, over the last decade or so, in non-isotopic methods of analysis utilising a wide variety of different markers or labels (Oellerich, 1980).

There are basically two variations of enzyme immunoassay which, although based on the same broad principle of using some form of labelled antibody or antigen, differ markedly in the way in which they achieve results. The two types of assay are known as Homogeneous and Heterogeneous enzyme immunoassay.

Homogeneous enzyme immunoassay or Enzyme Multiplied Immunoassay Technique (EMIT) utilises a system which requires no separation of the bound and free enzyme label because the enzymatic activity of the hapten-enzyme conjugate is influenced by the antibody binding (Rubenstein, 1972). This technique is specially suited to the detection of low molecular compounds or haptens.

Heterogeneous enzyme immunoassay also known as Enzyme Linked Immunosorbent Assay (ELISA) differs from EMIT by virtue of the fact that it requires separation of the bound and free enzyme label before estimating the enzymic activity of the bound fraction. This is usually achieved by coupling antibody or antigen to a solid phase material.

Since its introduction ELISA has undergone many investigations in relation to the type of solid phase used. The initial work carried out by Engvall & Perlman (1971) used microcellulose as the solid phase but soon they introduced plastic tubes as their support (Engvall & Perlman, 1972). Initially the plastic solid phase consisted of polystyrene tubes with the antibody or antigen being passively adsorbed onto the surface by means of an incubation at the appropriate pH. More recently however many workers have obtained excellent results with different types and forms of plastic solid phase.

For example, there have been assays described which used polystyrene stirring rods as the solid phase (Felgner, 1977). Activated nylon beads have also produced good results (Hendry & Herrman, 1980). Microtitre plates have found favour because of their ease of handling and small volume requirements. Both polystyrene (Voller et al, 1976) and polyvinyl chloride (MacDonald & Kelly, 1978) forms have been used. Latex (Schall et al, 1978) and sepharose (Joyce et al, 1977) have also been used successfully as solid support.

The information obtained from the above work suggested that microtitre plates would probably be most suited to routine use. Engvall and Ruoslahti (1979) suggested that the binding characteristics of the polyvinyl chloride plates were superior to those of the polystyrene. Therefore polyvinyl microtitre plates

were used as the solid phase throughout the work carried out for this submission.

Incubation times and temperatures have been investigated by several workers, however the conclusions reached by Saunders (1979) and Yolken & Leister (1981) are in agreement. They conclude that, in general, incubation times are longer than required and could be cut if the temperature were increased to 37°C. For this work the incubation times are those which were the shortest and most convenient for routine use without the need to increase the temperature.

In its most basic form there are two types of ELISA assay which can be used. These are a) the Non-Competitive or Sandwich assay and b) the Competitive assay. The general form of each is set out in Figure 1 which indicates the broad approach to each type of assay but there have been many modifications to both types throughout the development of ELISA. Reviews such as those by Wisdom (1976) and Schuurs & Van Weemen (1977) catalogue such modifications.

Initially Van Weemen & Schuurs (1974) had suggested that labelled antibody methods (sandwich type) were not as sensitive or as economical as labelled antigen methods (competitive type). This has not been upheld by more recent work which has in fact suggested that the sandwich assay using labelled antibody is particularily suited to measuring antigens which have a relatively low serum concentration. This is only true of those antigens which have more than one

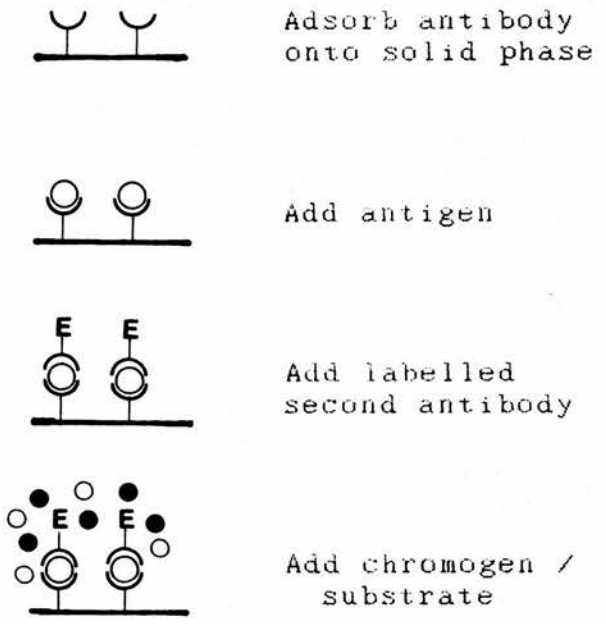

Figure 1a. Sandwich Type enzyme immunoassay

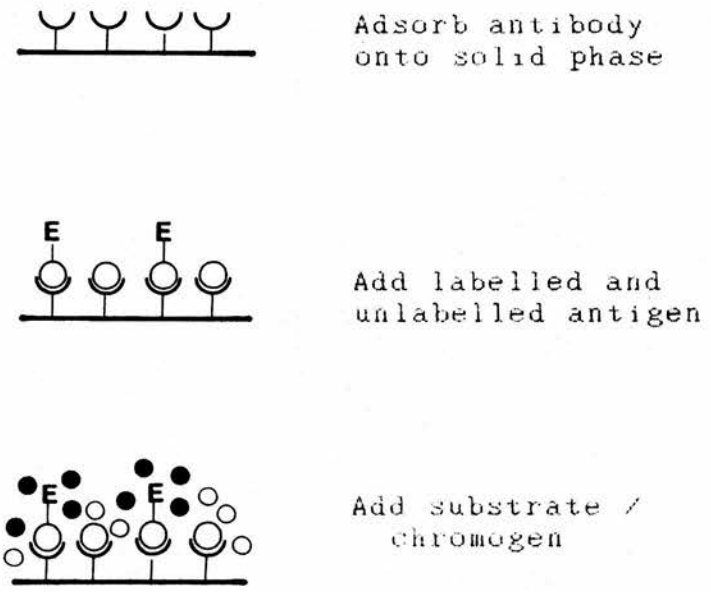

Figure 1b. Competitive type enzyme immunoassay

antigenic determinant or epitope.

When considering TSH as the antigen to be estimated the total number of epitopes is unclear but there is certainly more than one. This indicates its suitability for estimation by a sandwich type assay assuming both antibodies in the sandwich could recognise different epitopes on the TSH. Also, the serum concentration of TSH in healthy individuals is relatively low, the reference range being < 5.7 mu/L (determined by sampling individuals from the local euthyroid population).

In addition, the use of a competitive assay for the measurment of human TSH would have meant using purified human TSH to produce a label (see Figure 1). The cost implications for the required quantities of human hormone made this a very unfavourable proposition. Consequently the work carried out developing the assay to measure TSH used the double antibody sandwich ELISA.

There have been methods published in the past for the measurement of TSH by enzyme-immunoassay techniques but these, in the main, were considered unsuitable for routine application using equipment readily available in the department.

Miyai et al (1976) published one of the first ELISA methods for TSH and used a double antibody competitive technique with alkaline phosphatase as the label. This particular assay had a detection limit of 10 mu/L, well above the normal reference range, and

also took a total of four days to complete. Albert et al (1978) incorporated their TSH assay into a combined thyroid screening protocol using ELISA but again the TSH estimation took at least 40 h to complete. In this case no solid phase was used but the bound enzyme-antibody-TSH complex was precipitated by the addition of a second antibody. Using a fluorophotometric end point, Kato et al (1980) improved on the sensitivity reported earlier but once again the assay took at least two days to complete. Imigawa et al (1982) produced an ELISA method using polystyrene balls as the solid phase and reported a sensitivity down to 0.1 mu/L. The activity of β- D - galatosidase was estimated fluorometrically. All of the above methods were considered unsuitable for routine use in a department which could average about one hundred TSH measurements each week.

Radioimmunoassay was by far the most popular way of measuring TSH and the method used in the department (Section 2.5) had a sensitivity in routine use of 1.5 mu/L (departmental figures) and could be completed within the scope of a normal working day. As part of the aim of this study was to investigate the feasibility of using an ELISA technique as an alternative to the RIA method for measuring TSH, it was considered essential that such a technique be as convenient to operate as the RIA method , as well as being analytically comparable.

The initial work to be carried out in this study

was the investigation relating to TSH. However it was hoped that the experience gained from that work would enable ELISA to be introduced more widely into the laboratory and the possibilities of doing so were continually under review.

In 1984 a report by Mogensen, relating to urinary albumin excretion in diabetics, highlighted an area which could be foreseen as being very useful in the monitoring of the diabetic population in the Region. Whilst an RIA procedure had been described earlier by Woo et al (1978) it was not feasible to use this method in our department. Also there were no commercial kits available, at the time, which could allow us to measure the low quantities of albumin. Thus ELISA was considered as a suitable alternative method to measure urinary albumin.

1.4. CHOICE OF PREPARATIVE METHODS.

The decision to employ a sandwich assay necessitated that an antibody-enzyme conjugate was prepared and used as the label in the reaction sequence. Preparation of antibody-enzyme conjugate is of prime importance in the performance of any assay since the quality of such preparations may be reflected in the final assay performance. Two choices were immediately apparent before a label could be produced. First of all an appropriate enzyme had to be chosen and secondly, but not necessarily unrelated, the method of linking the enzyme to the antibody had to be selected.

1.4.1. Choice of Enzyme.

Since 1971 several enzymes have been used in immunoassays the choice being determined by the following criteria:

a) The enzyme should be readily avaliable in a high degree of purity and at a low cost.

b) It should have a high specific activity at a pH appropriate for the antigen- antibody reaction.

c) It should normally have an assay method that is sensitive, simple,cheap and precise and not be affected by factors present in the biological fluid.

d) It should form a stable antibody conjugate.

e) It should possess reactive groups which allow linking with other molecules and yet retain enzyme activity

The most widely used enzymes have been Alkaline Phosphatase (EC 3.1.3.1.), Horseradish Peroxidase (HRP, EC. 1.11.1.7.), and β-D-Galactosidase (EC.3.2.1.23.) (Voller, Bartlett & Bidwell, 1978). Their wide use does not necessarily imply that they are ideal but only that they have been found to be the most satisfactory. At a meeting organised by the National Institute of Health in Bethesda, USA in 1976 the conclusion reached was that alkaline phosphatase and horseradish peroxidase were about equal in performance but that HRP be preferred because of its availability.

(Schuurs & Van Weemen, 1977). It has also been considered that because alkaline phosphatase is an endogenous enzyme in human blood then perhaps it was better not to use it for a system using human serum samples. A comparison of the costs also indicated that the HRP was considerably cheaper. For these reasons HRP was chosen as the enzyme to be used in the production of the antibody-enzyme conjugates.

1.4.2. Crosslinking Methods

The choice of HRP as the enzyme label offered a further choice in relation to the method of crosslinking the enzyme with the antibody.

The use of glutaraldehyde as the coupling agent for the conjugation of enzymes with proteins was first reported in 1969 by Avrameas. This one-step method produced conjugates which retained a substantial part of their immunological and enzymatic activity. It was therefore used in the production of the enzyme conjugates employed in the first ELISA methods (Engvall & Perlman, 1971 ; Van Weemen & Schuurs, 1971) The protocol was very simple and technically undemanding, involving the reaction of the dialdehyde, glutaraldehyde, with the amino groups of the protein to be conjugated. The Schiff bases so formed underwent further reaction to yield a variety of products. The main disadvantage of this method lay in the fact that the glutaraldehyde reacted at different rates with different proteins which led to non-selective polymerisation of one or other of the reactants

(Kennedy et al, 1976). Subsequently Clyne et al (1973) reported that the yield of useful conjugate from this method was usually in the region of 5%.

An alternative but similar method, the Two - Step Glutaraldehyde method was reported by Avrameas & Ternynck in 1971. This improved on the one step method of earlier but its use was limited to HRP. The method relied upon the inability of HRP to self polymerise after the activation by glutaraldehyde. This was a result either of unfavourable configuration (Avrameas & Ternynck, 1971) or because of the blockage of the majority of its amino groups by the naturally occuring allyl isothiocyanate in horseradish (Nakane et al, 1966). The reaction scheme is set out in Figure 2 and shows the reaction of the N-terminal group of the enzyme with one of the aldehyde groups of the glutaraldehyde (Habeeb & Hiramoto, 1966). After removal of the excess glutaraldehyde, the "activated" enzyme was then conjugated to the antibody, the second aldehyde group reacting with the amino residues of the antibody. The final product consisted of low molecular weight products in low yield (approx 10%) but gave increased sensitivity over those labels produced by the one step method (O'Sullivan & Marks, 1981).

The widespread use of the above method prevailed until in 1974 Nakane & Kawaoi suggested that the carbohydrate moiety of the HRP, not required for enzyme activity, be used for the formation of aldehyde groups by oxidation with sodium periodate. The reaction scheme

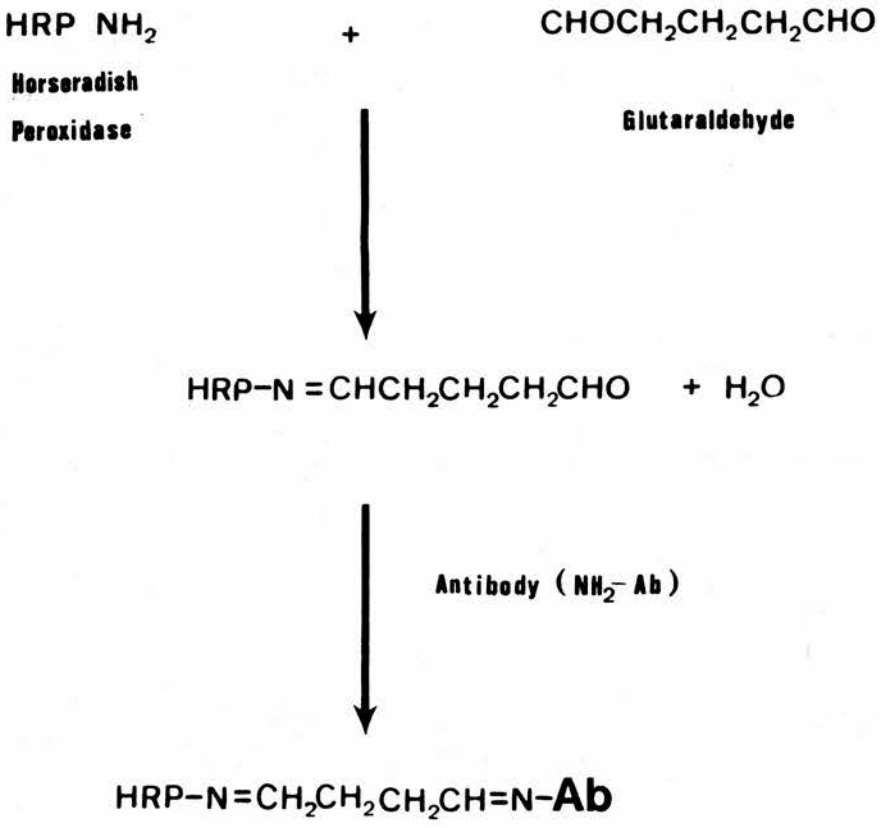

Figure 2: Reaction sequence for the Glutaraldehyde method of conjugation.

for this Periodate Oxidation method is shown in Figure 3. It shows the reaction involved several steps. The first required that the amino groups of the HRP be blocked by reaction with 1-fluoro-2,4-dinitro benzene (FDNB). Once blocking had taken place the carbohydrate residues of the HRP were then oxidised to aldehyde by the periodate. Reaction took place between these aldehyde groups and the free amino groups of the antibody. Finally stabilisation of the product occurred by reduction with borohydride.

Nakane & Kawaoi originally reported high activities of both enzyme and antibody in the conjugate but since then Saunders (1979) has demonstrated that in practice the yields are lower but still higher than those obtained by the glutaraldehyde methods. The method was, however, more complex and more time consuming in its preparation.

It was proposed that during the course of this study both the Periodate oxidation and the Two-Step glutaraldehyde methods be evaluated for their suitability in routine use.

1.5. MONOCLONAL ANTIBODIES.

The production of antibody by immunisation of a suitable laboratory animal is a well established and valuable procedure. Such an immunisation will induce a polyclonal response to the antigen being used and this has a number of important drawbacks. The antiserum produced will consist of a number of different classes of heterogenous immunoglobulins directed towards

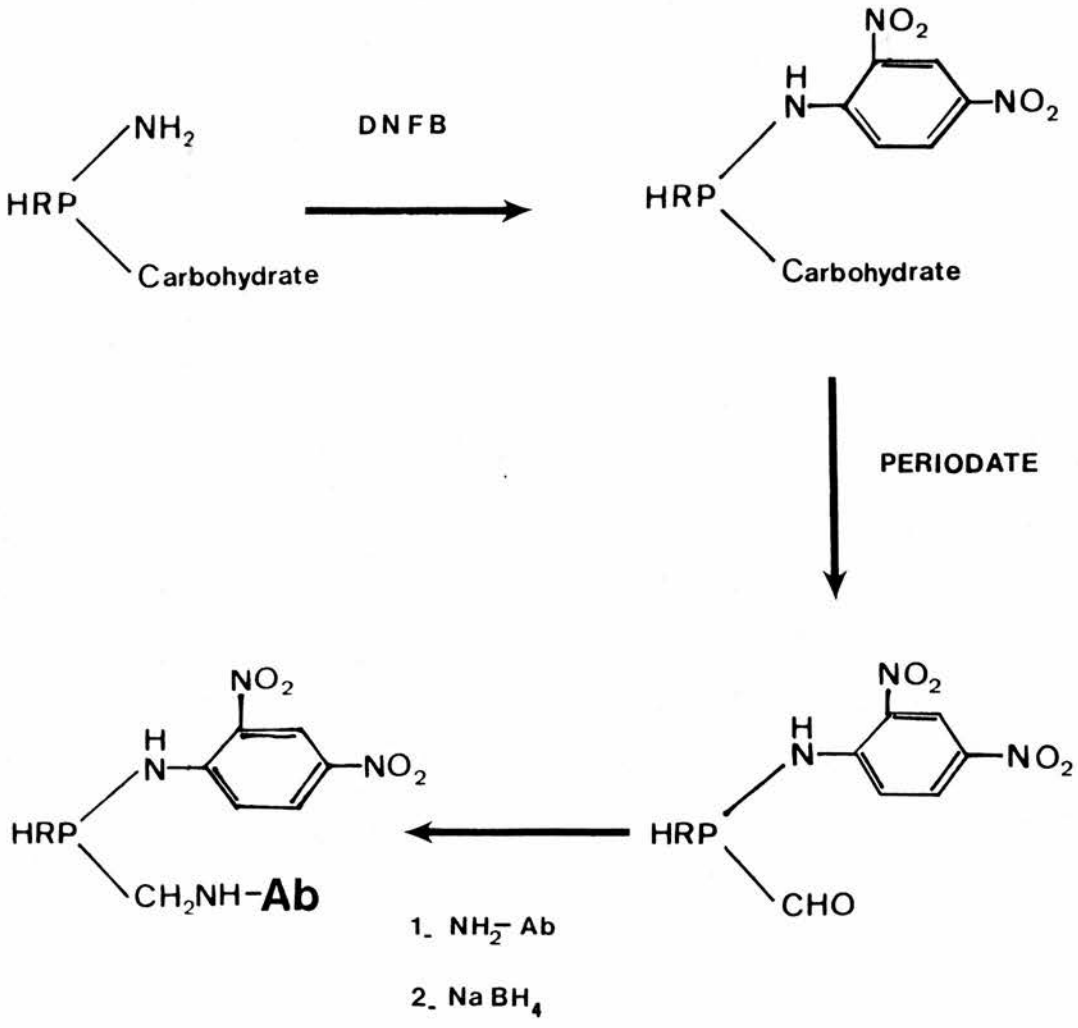

Figure 3. Reaction Scheme for Periodate Oxidation Reaction

different antigenic determinants. This has meant definition of specificity as well as standardisation is very diffuclt for most antisera.

The concept of antibody production was changed dramatically when Kohler & Milstein (1975)synthesized monoclonal antibodies of a single predetermined specificity. To accomplish this they fused a myeloma cell with a mouse spleen cell which had been immunised to respond to a certain antigen. The resultant hybridoma cell was then grown on to continuously secrete antibody of predefined specificity derived from the spleen cell. The cell line may be grown either in culture medium or in the body of a live animal as an ascitic tumour.

The similarities of the glycoprotein hormones such as FSH, LH, HCG and TSH made them ideal models for the production of monoclonal antibodies to their immunologically specific subunits. Ridgeway et al (1982) published details of this technique producing monoclonal antibody to TSH. The monoclonal used in the present work was produced in similar fashion to their method and was specific to the β subunit of human TSH.

The aim of the present study was therefore to investigate the possible use of ELISA to measure TSH and if possible to use the experience gained from that development to set up an ELISA method for urinary albumin.

SECTION 2

MATERIALS AND METHODS

2.1. ESTIMATION OF PROTEIN CONTENT OF ANTISERUM.

The method of Lowry et al (1951) was used to estimate the protein content of each antiserum.

2.1.1. Materials.

Human Normal Immunoglobulin (IgG): A solution of IgG at a concentration of 150 mg/L was obtained from the Blood Transfusion Service, Protein Fractionation Centre, Edinburgh. This solution was diluted with saline (9 g/L sodium chloride) to cover a range of 1-5 mg/mL.

Solution 1- Alkaline Tartrate: 0.2 mol/L sodium carbonate and 2.0 mmol/L sodium potassium tartrate dissolved in 0.1 mol/L sodium hydroxide solution.

Solution 2- Copper Sulphate: 6.0 mmol/L copper sulphate solution.

Solution 3- Alkaline Copper Reagent: 5mL of solution 2 was added to 45 mL of solution 1. This reagent was prepared fresh each day before use.

Solution 4- Folin Ciocalteau's Phenol Reagent obtained from B.D.H. Chemicals, Poole, Dorset, U.K. This solution was diluted 1:2 with deionised water before use.

2.1.2. Method: 0.1 mL of blank (saline), standards or test was added to 5.0 mL of Alkaline Copper reagent, the solutions were mixed and left at room temperature for 15 min. 0.5 mL of Folin Ciocalteau's reagent was then added to each tube with immediate mixing on a vortex mixer and the solutions

were incubated a further 30 min at room temperature. The absorbance was read at 720 nm on a Pye Unicam SP 1800 spectrophotometer.

A straight line standard graph of absorbance versus protein concentration in mg/mL was constructed and the unknown concentration read from the graph.

Using the above procedure the protein content of the untreated polyclonal antibody used in the TSH assay was found to be in the range 32.5 - 35.5 mg/mL and that of the monoclonal antibody to TSH was found to be 10.1 mg/mL.

2.2. ANTISERUM PURIFICATION METHODS.

Two methods of purifying the antiserum were used and compared.

2.2.1. Salt Fractionation Technique. (Kekwick 1940).

2.2.1.1. Materials:

Phosphate buffer: 0.075 mol potassium dihydrogen phosphate plus 0.075 mol disodium hydrogen phosphate made up to 1 Litre with deionised water.

Sodium sulphate (anhydrous): B.D.H. Chemicals Ltd, Poole, Dorset, U.K.

Dialysis tubing: Visking size 1 - 8 / 32" from Medicell International Ltd, London, U.K.

Minicon - B concentrators: Amicon Ltd, Stonehouse, Gloucs, U.K.

Sheep anti- human TSH: Polyclonal antiserum raised in sheep against the β fraction of human TSH. Supplied by the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, U.K

Mouse anti- human TSH: Monoclonal antiserum raised against human β TSH. This preparation was generously donated by Boots Celltech Ltd and was supplied in the form of mouse ascitic fluid.

2.2.1.2. Method:

This method was used to precipitate the IgG selectively.

A volume (1-2 mL) of antiserum was dialysed against 1 Litre of the phosphate buffer for 1 h. After dialysis each volume had the equivalent of 18 mg of sodium sulphate per mL of antiserum added. This mixture was stirred before being centrifuged at 3,000 rpm for 10 min. The supernatant was then discarded and the precipitate redissolved in the phosphate buffer to 40% of its original volume. Again the solution was precipitated with the sodium sulphate (12 mg/mL) and the precipitate redissolved in the phosphate buffer.

To remove excess salt, the solution was exhaustively dialysed against changes of deionised water. Finally the volume of solution was adjusted to produce a solution containing approximately 10 mg/mL of purified IgG. This adjustment meant either the dilution of solutions that were too concentrated by the addition of normal saline or concentrating in a Minicon filter for solutions which were too dilute. Adjustment to this concentration allowed easier handling at the later stages of antibody-enzyme conjugation and application of the antibody to the solid phase. Aliquots of the prepared antibody were stored at -20°C.

2.2.2. Octanoic Acid Technique. (Steinbuch & Audran, 1969). This method precipitated proteins other than IgG, retaining the IgG in the supernatant.

2.2.2.1. Materials.

Acetate buffer (pH 4.0): 0.06 mol/L of sodium acetate in deionised water adjusted to pH 4.0 with glacial acetic acid

Acetate buffer (pH 5.7): 0.015 mol/L of sodium acetate in deionised water adjusted to pH 5.7 with glacial acetic acid. This buffer was made up in 5 Litre volumes.

Octanoic acid: n-Octanoic acid, specially pure, from B.D.H. Chemicals, Dorset, U.K.

Saline: 0.15 mol/L sodium chloride in deionised water.

2.2.2.2. Method.

To 1.0 mL of antiserum was added 2.0 mL of the pH 4.0 acetate buffer and the solution mixed. With vigorous mixing, 0.075 mL of the octanoic acid was added dropwise and the mixture stirred for 30 min. This solution was centrifuged at 3,000rpm and 10°C for a further 30 min. The solution was left overnight at 4°C before being respun. The supernatant was removed and dialysed against 5 x1 Litre changes of the pH 5.7 acetate buffer at 4°C. This was followed by 2 x1 Litre changes of saline . A further centrifugation removed any remaining solid material and finally the volume was adjusted, as before, to give a protein content of about 10 mg/mL.

Again the purified antibody was divided and stored at -20°C.

2.3. ANTIBODY ENZYME CONJUGATION METHODS.

Two methods of preparing antibody-enzyme conjugates were considered (Section 1.4.2.) for use in the project. The Two -Step Glutaraldehyde method of Avrameas & Ternynck (1971) was evaluated and compared with the Periodate Oxidation technique suggested by Nakane and Kawaoi (1974).

2.3.1. Two - Step Glutaraldehyde method.

The chemical basis of this technique was discussed in Section 1.4.2. and is illustrated in Figure 2.

2.3.1.1. Materials:

Peroxidase (E.C.1.11.1.7.): Horseradish Type VI, R.Z.= 3.0, from Sigma Chemical Co, Poole, Dorset, U.K.

Glutaraldehyde: 50% w/w from B.D.H. Chemicals Ltd, Dorset, U.K.

Phosphate buffer: 0.1 mol/L di-Sodium hydrogen phosphate added to 0.1 mol/L potassium dihydrogen phosphate to give a pH = 6.8 .

Carbonate-Bicarbonate buffer: 0.1 mol/L sodium carbonate added to 0.1 mol/L sodium hydrogen carbonate to obtain a pH = 9.6 .

Glycine: 0.2 mol/L in deionised water.

Ammonium sulphate: B.D.H. Chemicals, Dorset U.K.

Purified anti-human TSH antibody: see Section 2.2.1.2.

2.3.1.2. Methods.

The contents of an ampoule of peroxidase (approx 7 mg) were dissolved in 0.2 mL of the phosphate buffer and 5 μL of glutaraldehyde was added. This mixture was allowed to stand at room temperature for 18 h, and was then dialysed against 3 x 1 Litre changes of deionised water. A volume equivalent to 7 mg of purified IgG was added followed by 0.4 mL of the carbonate - bicarbonate buffer and this mixture left for 24 h at 4°C. Glycine was added (0.1 mL) and the mixture left a further 2 h before being dialysed against several changes of saline.

The labelled antibody was then precipitated at 4°C with an equal volume of saturated ammonium sulphate before being dissolved in distilled water (1.5 mL) and dialysed once more against saline.

This solution was then ready for use in evaluating and assessing the method. It was stored at 4°C and dilution was carried out from this solutuion when required.

2.3.2. Periodate Oxidation technique.

The chemical basis of this method was also discussed in Section 1.4.2. and is further illustrated in Figure 3.

2.3.2.1. Materials.

Peroxidase: as described in Section 2.3.1.1.

Sodium carbonate: 0.3 mol/L in deionised water, pH 8.1 .

1-Fluoro-2,4-Dinitrobenzene (FDNB): 0.05 mol/L in absolute ethanol.

Sodium periodate: 0.06 mol/L in deionised water.

Ethanediol: 0.16 mol/L in deionised water.

Carbonate-Bicarbonate buffer: 0.01 mol/L sodium carbonate added to 0.01 mol/L sodium hydrogen carbonate to achieve a pH = 9.5 .

Sodium borohydride : B.D.H. Chemicals, Poole, Dorset, U.K.

Phosphate buffered saline (PBS): supplied in tablet form by Oxoid Ltd, Hampshire , U.K. or made up by dissolving 0.15 mol/L of sodium chloride, 6.0 mmol/L disodium hydrogen phosphate and 1.5 mmol/L potassium dihydrogen phosphate in deionised water, pH 7.3 . The tablets were dissolved, 10/Litre, in deionised water.

2.3.2.2. Method.

FDNB (0.1 mL) was added to a solution containing 5 mg of peroxidase dissolved in 1.0 mL of the sodium bicarbonate. This mixture was gently mixed at room temperature for 1 h. 0.1 mL of sodium periodate was added and again the mixture was gently mixed for 30 min. Ethanediol (0.1 mL) was added and a further incubation of 1 h at room temperature carried out. This solution was then dialysed against 3 x 1 Litre changes of the carbonate-bicarbonate buffer.

After dialysis a volume of the antibody solution containing 7 mg of protein was added to 3.0 mL of the peroxidase-aldehyde solution and mixed gently for 3 h

at room temperature. Finally 5 mg of sodium borohydride was added and the final mixture left overnight at 4°C before further dialysis against PBS.

2.4. STANDARD THYROTROPHIN PREPARATION.

2.4.1. Materials.

Standardisation of the TSH assay was achieved by using the World Health Organisation's 1st International Reference Preparation of Human Thyroid Stimulating Hormone (Pituitary TSH). est 1974. (1st IRP). This standard reference material was obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London. and had an activity defined as : 36.6 mg of preparation was equivalent to one International Unit.

The material was supplied in sealed ampoules each of which contained, by definition, 0.15 units of TSH.

TSH free horse serum: from Sera Labs, Sussex U.K.

Chicken serum: from Flow Laboratories, Irvine, U.K.

Phosphate Buffered saline: PBS as in section 2.3.2.1.

2.4.2. Method.

The contents of one ampoule were dissolved in 0.5 mL of PBS and the solution transferred to 80 mL of carrier serum in a volumetric flask (100 mL). Once made up to mark this stock solution contained 1500 mU/L of TSH.

The stock solution was divided into 2 mL portions

and stored deep frozen at -20°C.

Working standards covering a TSH concentration range of 1.5-75.0 mU/L were made up from the stock standard and carrier serum each time the assay was performed. The use of carrier serum allowed the standard material to be stored and used in a matrix similar to that of the patient sera being tested.

Both carrier sera mentioned above were tested and the results are reported in Section 3.2.2.1.

Standardisation of the Corning Immophase method was also carried out with reference to the 1st IRP and this allowed direct comparison of results obtained from the ELISA and RIA methods.

2.5. RADIOIMMUNOASSAY METHOD FOR TSH.

The radioimmunoasay method employed in the assessment of the ELISA method was that used routinely in the Department of Clinical Chemistry, Victoria Hospital, Kirkcaldy for the measurement of TSH in sera of patients with suspected thyroid disease. It is marketed as a kit by Ciba-Corning Diagnostic Ltd, Halstead, Essex, U.K. under the trade name "Immophase TSH".

In principle this technique was of the two-site immunoradiometric assay or IRMA type of RIA. A schematic representation is shown in Figure 4. The ELISA method developed is directly analogous to this type of RIA.

2.5.1. Materials.

Each kit came prepacked with the following

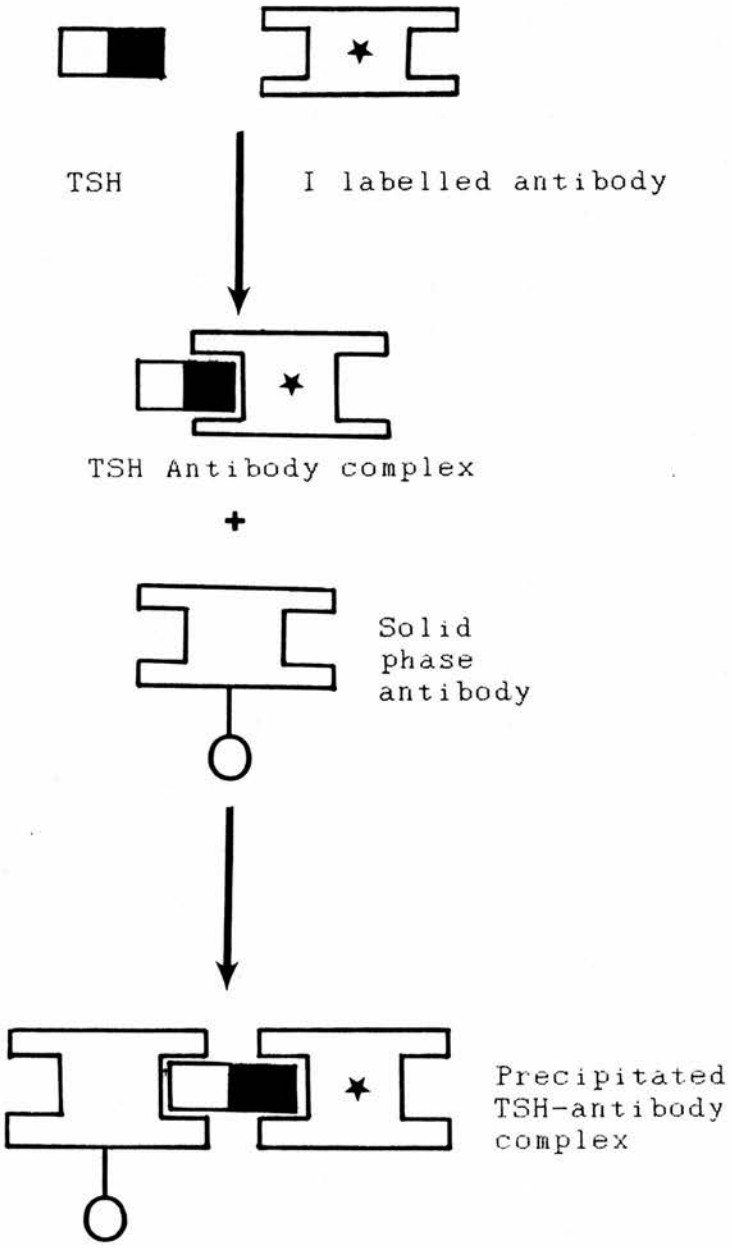

Figure 4: Reaction scheme for Corning TSH method.

reagents.

Solid phase TSH antibody: goat anti-human TSH covalently bound to glass beads in phosphate buffered saline.

Labelled TSH antibody: ^{125}Iodine labelled anti-human TSH in phosphate buffered saline containing bovine albumin, goat serum, rabbit serum and red dye. Each vial contained <5 μCi of radioactivity.

Both the above antisera were polyclonal in nature.

TSH standards: a complete set of standards to cover the range 0-60 mU/L.

2.5.2. Method.

A volume of 50 μL of standard, control or test was added to 50 μL of tracer and mixed. The mixture was allowed to incubate for 3 h at room temperature. Following incubation 0.25 mL of solid phase antibody was added to each tube, mixed and incubated for a further 1 h at room temperature. Finally 1.0 mL of deionised water was added before centrifugation for 10 min. Within 30 min of centrifugation all tubes were decanted and counted on a Beckman 310 gamma counter.

Counts/min of radioactivity were directly related to TSH concentration and all measurements were carried out in duplicate. Results were plotted as the log of the reciprocal of the counts bound against the log of the TSH concentration. This gave a linear relationship as shown in Figure 5. The performance characteristics of this assay are shown in the results and discussion sections.

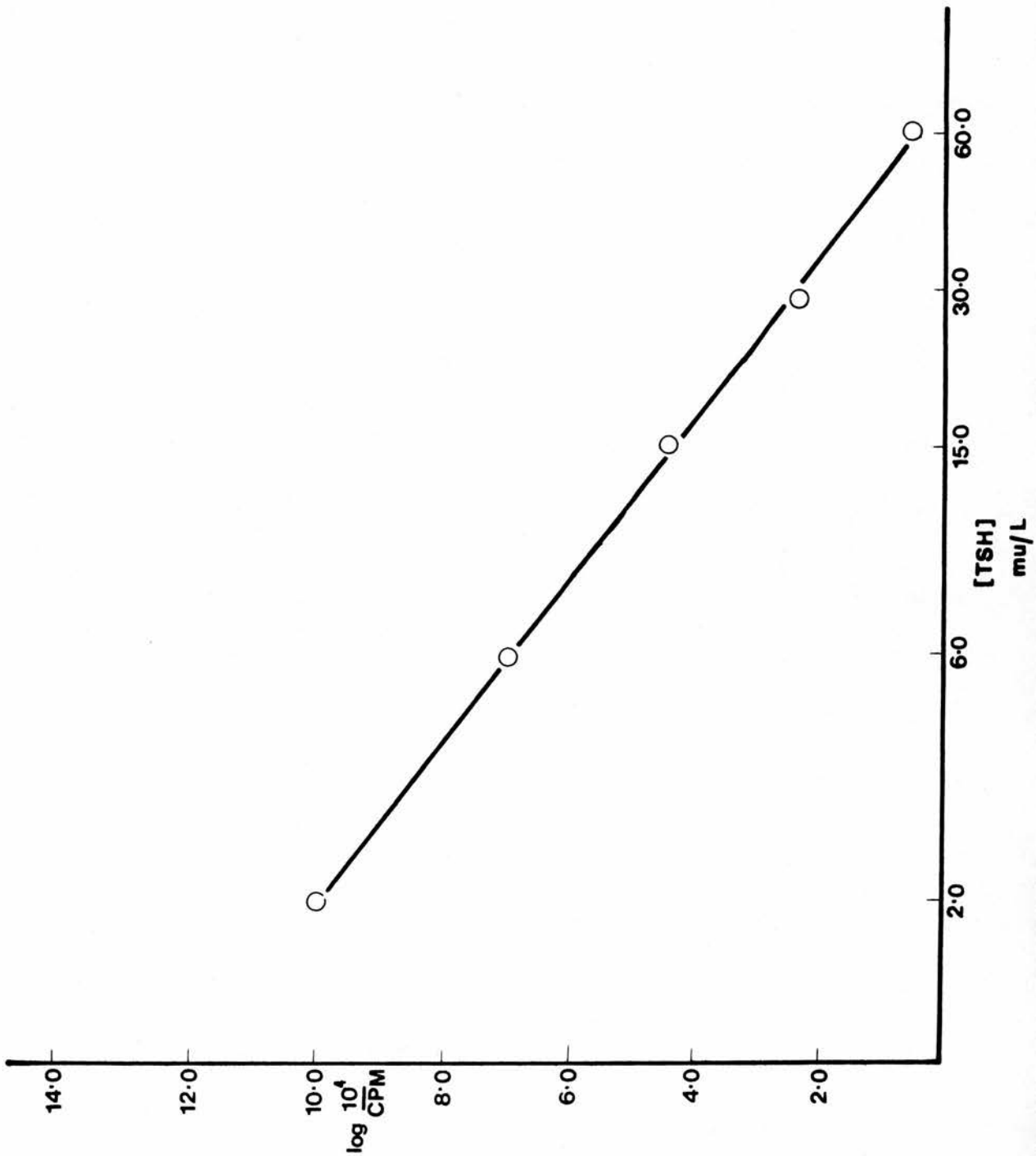

Figure 5. Dose Response Curve for the RIA method for TSH

2.6. ELISA TSH METHOD.

The reagents listed are those used throughout the development of the TSH assay. Modifications to these are reported in the results section as the assay was optimised. The final method protocol is also defined in the results section. (Section 3.2.5.1.)

2.6.1. Materials.

Sheep anti-human TSH: as described in Section 2.2.1.1. containing 50 u/mL HCG to minimise cross reaction as recommended by SAPU.

Mouse anti-human TSH: as described in Section 2.2.1.1.

Coating buffer: 0.015 mol/L sodium carbonate added to 0.035 mol/L sodium hydrogen carbonate to give a pH = 9.6 .

Wash solution: 0.01% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) in phosphate buffered saline, pH 7.4

Chromogen buffer: 5.0 mmol/L of o-phenylenendiamine dissolved in a solution containing 0.125 mol/L disodium hydrogen phosphate and 25 mmol/l of citric acid, pH 6.0 . This solution was stored in 20 mL portions at -20°C. For use, a 20 mL portion was thawed and 10 μL of 30% (w/v) of hydrogen peroxide was added.

Polyvinyl microtitre plates: M29 from Dynatech Laboratories

2.7. STANDARD ALBUMIN PREPARATION.

2.7.1. Materials.

Standardisation of the Albumin assay was achieved by using powdered human albumin obtained from Sigma Chemical Co Ltd, Poole, Dorset, U.K. This product was composed of Cohn fraction V and had a purity of 99% .

Diluent solution: as prepared in Section 2.8.1.

2.7.2. Method.

A stock solution (200 mg/L) was prepared by dissolving 20 mg of the standard albumin in 100 mL of diluent solution. The stock solution was divided into 1 mL portions and stored deep frozen at -20°C . Working standards covering the range 2-1000 µg/L were made up from stock standard and diluent solution each time the assay was performed.

2.8. ELISA ALBUMIN METHOD.

The reagents listed below are those used throughout the Albumin assay development. Any modifications are reported in the results section. Reagents common to TSH and Albumin methods were prepared in the same way.

2.8.1. Materials.

Coating buffer: as in Section 2.6.1.

Wash solution: as in Section 2.6.1.

Chromogen buffer: as in Section 2.6.1.

Diluent solution: 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) in phosphate buffered saline, pH 7.4 .

Rabbit anti-human albumin: Polyclonal antiserum raised in rabbits against human albumin.

Purchased from Dako Ltd, High Wycombe, Bucks, U.K. (1st Antibody).

Goat anti-human albumin: Polyclonal antiserum raised in goat against human albumin. Purchased from Miles Laboratories Ltd, Slough, U.K.

Rabbit anti-goat IgG: Polyclonal antiserum raised in rabbit against goat immunoglobulin G and labelled with horseradish peroxidase and purchased from Miles Laboratories.

As before the microtitre plates used were the M29 polyvinyl plates from Dynatech and all absorbance readings were taken on a Micro-ELISA reader (Vitatron UK, Maidenhead, U.K.) at a wavelength of 490 nm.

All urine samples were diluted, in the first instance , 1:100 with diluent solution. Subsequent dilutions were carried as out necessary.

Section 3

TSH Results Section

3.1. GENERAL ASSAY PROTOCOL FOR TSH.

The general method protocol used in the optimisation and assessment of the assay for TSH was that of a sandwich immunoassay. Figure 6 illustrates the principle.

The double antibody sandwich technique involved passively adsorbing antibody to human TSH onto a solid phase, in this case polyvinyl microtitre plates. This antibody was then allowed to react with the antigen, TSH, in solution so that the antigen was then bound to the solid phase antibody. The sandwich was then completed by attaching a second antibody, labelled with the enzyme, to the bound antigen.

The amount of bound enzyme was dependent on the amount of bound TSH. It follows that the concentration of TSH is directly proportional to the enzyme activity.

The choice of technique, enzyme and solid phase have already been discussed in the the previous sections.

3.2. OPTIMISATION OF THE TSH METHOD.

3.2.1. Purification of antiserum.

Both the Octanoic acid and Salt fractionation techniques described in Section 2.2. were used to purify the antiserum and the resulting antibody preparations were compared. To assess the purified IgG solutions an antibody-enzyme conjugate was prepared for each using the Two-step Glutaraldehyde procedure (Section 2.3.1.). The dose response curves are compared

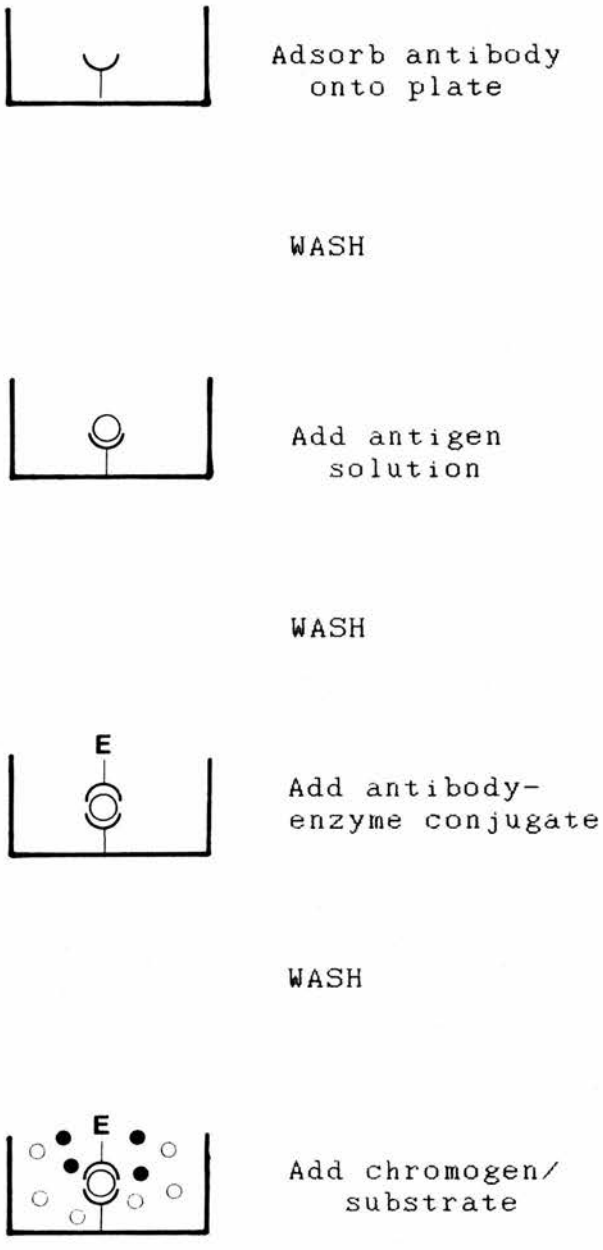

Figure 6. Principle of the double antibody sandwich technique for measuring TSH.

in Figure 7. Saline was used as the diluent for the standard material.

It was noted that the curves produced were similar and parallel. This would tend to indicate that both preparations had similar specificity and contained antibodies of similar immunoglobulin classes. The curves also show that the antiserum produced using salt fractionation had a marginally higher blank value which was reflected throughtout the complete standard curve. Whilst a difference of this magnitude in blank values could be attributed to many other factors, it was felt that, in this case, the difference between them was due to the salt fractionation method producing a slightly less pure product. This conclusion is supported by the fact that the small difference covered the complete standard curve and both curves were produced on the same plate using the same reagents. It is possible, therefore, that the difference in blank values was due to a slight increase in non-specific binding associated with the salt fractionated preparation.

In practice it was difficult to eliminate all the salt from the salt fractionated antiserum by dialysis. Even after exhaustive dialysis undesirable deposits of salt were still in evidence.

In comparing the final product from each, the Octanoic acid method gave a " cleaner " product with which to work. In all subsequent work the Octanoic acid method was used to purify the antiserum.

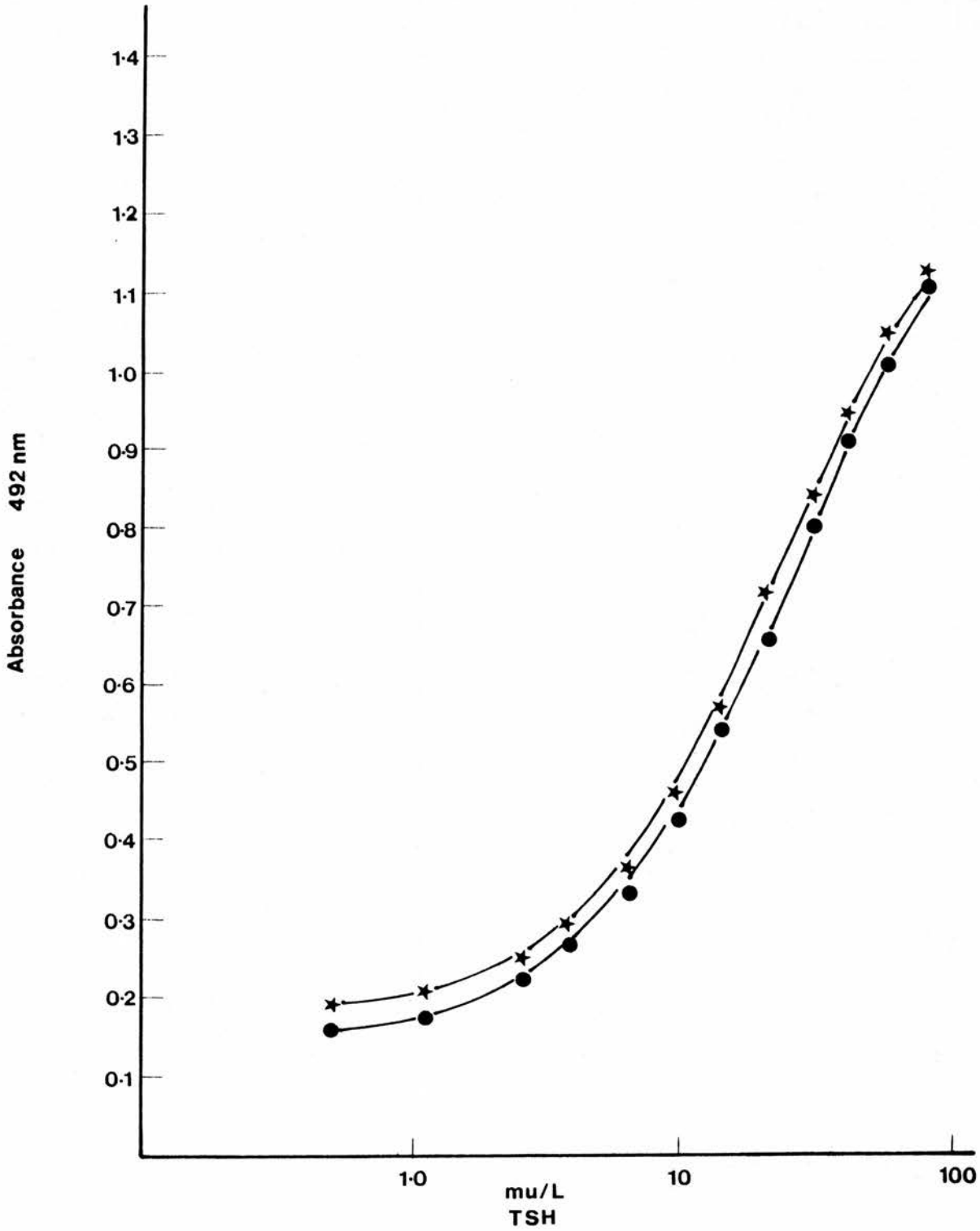

Figure 7. Dose-response curves obtained using antisera purified by different techniques.

✶—✶ salt fractionation. ●—● acid precipitation.

3.2.2. Initial Assessment of Glutaraldehyde Conjugation Method.

Although two methods of conjugation were discussed earlier in Section 1.4.2., in the first instance an antibody-enzyme conjugate was prepared using the two-step glutaraldehyde procedure. Once prepared this antibody-enzyme solution was doubly diluted in chicken serum from 1:250 to 1:8,000 . A dose response curve for each dilution was plotted and assessed using an arbitrarily chosen dilution of 1:1,000 (10 μg/mL) for the 1st antibody. The standards were prepared in chicken serum as described in Section 2.4.2. The curves are shown in Figure 8.

From the data presented in the graph an antibody-enzyme conjugate dilution of 1:1,000 was chosen as being the most suitable. The results using this dilution covered the widest range of absorbance units which had all the points of the curve within the reading range of the spectrophotometer.

In general this dilution produced an acceptable standard curve. One notable point, however, was the absorbance of the zero standard of 0.22 absorbance units. This was considered higher than was acceptable and efforts were made to reduce this value at least to 0.2 units. This would have made it comparable with those results obtained earlier using saline as the standard diluent.

3.2.2.1. Investigation of high blank.

To assess the chicken serum for cross reacting

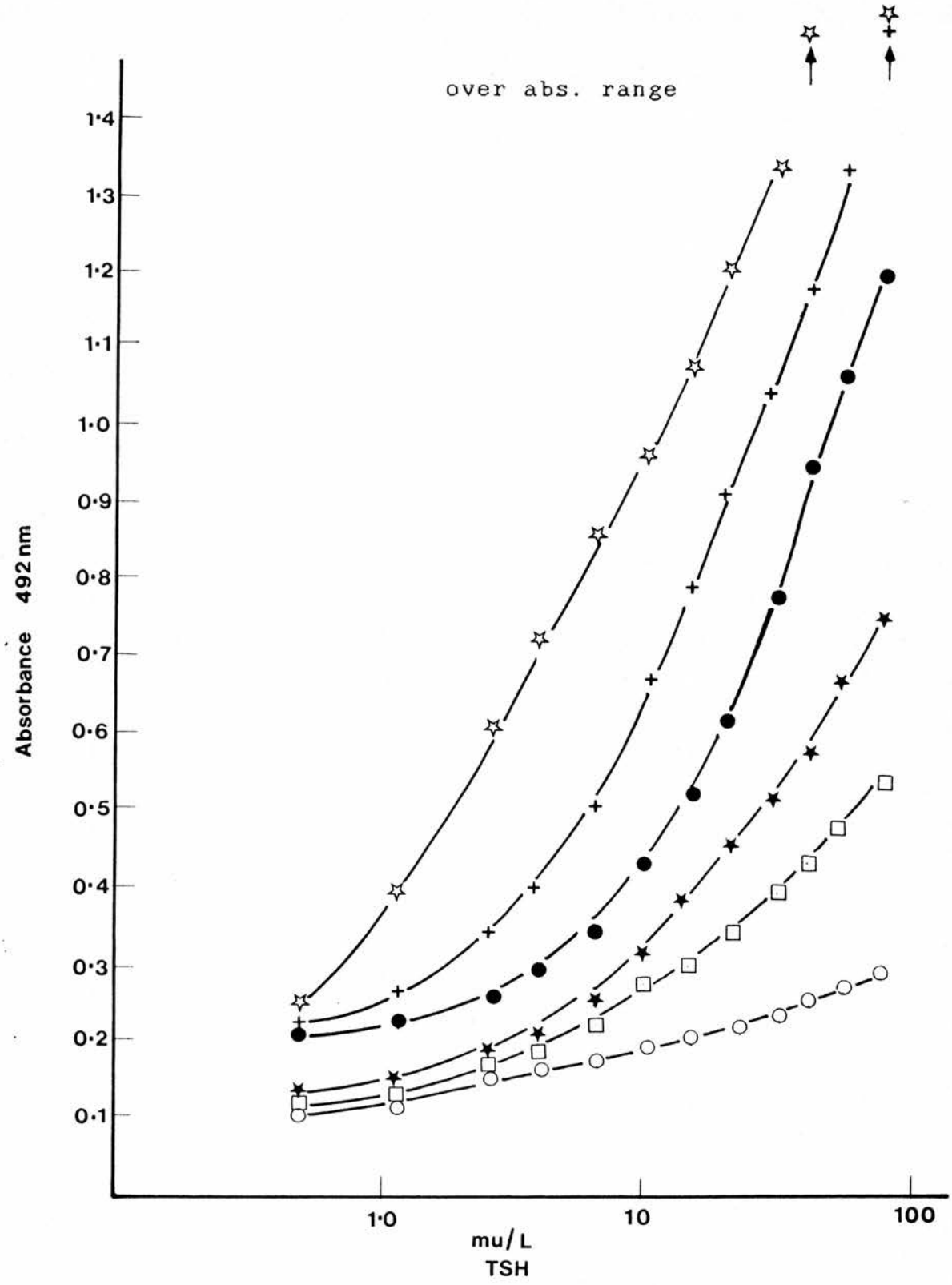

Figure 8. Dose Response curves for doubling dilutions of antibody-enzyme

1:250
1:500
1:1,000
1:2,000
1:4,000
1:8,000

material the following tests were carried out.

Luteinising hormone (L.H.) and Follicle Stimulating Hormone (F.S.H) were measured in the chicken serum and found to be undetectable using an assay set up to measure human gonadotrophins (Hunter & Bennie, 1979).

Human Chorionic Gonadotrophin (H.C.G.) was estimated using an ELISA method for measuring H.C.G. (Mehta & MacDonald, 1982) and again found to be undetectable.

TSH was also estimated using the RIA method to be used in the comparison (Section 3.3.3.) and described in Section 2.5.2 . The level of TSH was found to be below the detection limit of the assay.

Saunders (1979) and Engvall (1980) suggested that increasing the surfactant concentration of the buffers used in the assay could lead to a lesser degree of non-specific binding. In this assay, increasing the surfactant to a maximum of 0.2% of the wash buffer did not affect the blank values mentioned above.

A change of carrier serum from chicken serum to TSH free horse serum was investigated. This led to an improvement in the zero standard readings with no detrimental effect to the rest of the curve. It appeared therefore that the use of the chicken serum was associated with a slight increase in absorbance at all points of the standard curve

Results are presented in Table 1 and the findings are further discussed in Section 5.1..

In all subsequent work the carrier serum used for standard preparation and antibody-enzyme dilution was TSH free horse serum.

Table 1. Absorbance values associated with changes to buffers

Variable Factor	Zero dose Absorbance	Maximum dose Absorbance
0.9% saline	0.12	1.08
Chicken serum	0.22	1.18
Horse serum	0.16	1.15
Chicken serum & Increasing surfactant conc	0.22	1.20

3.2.3. Comparison of preparative methods for antibody-enzyme conjugation.

A comparison of the glutaraldehyde and periodate methods of conjugation was carried out. Protocols for each method have been described in Section 2.3. Comparative dose-response curves are shown in Figure 9.

Bearing in mind that any assessment of comparative methods should take account of their ease of preparation and yield of product, as well as the quality of product, the methods were compared from practical as well as analytical viewpoints. A comparison of the methods is discussed more fully in Section 5.1., but for the purposes of routine use with an acceptable end product, the two-step glutaraldehyde procedure was adopted and used throughout the assay

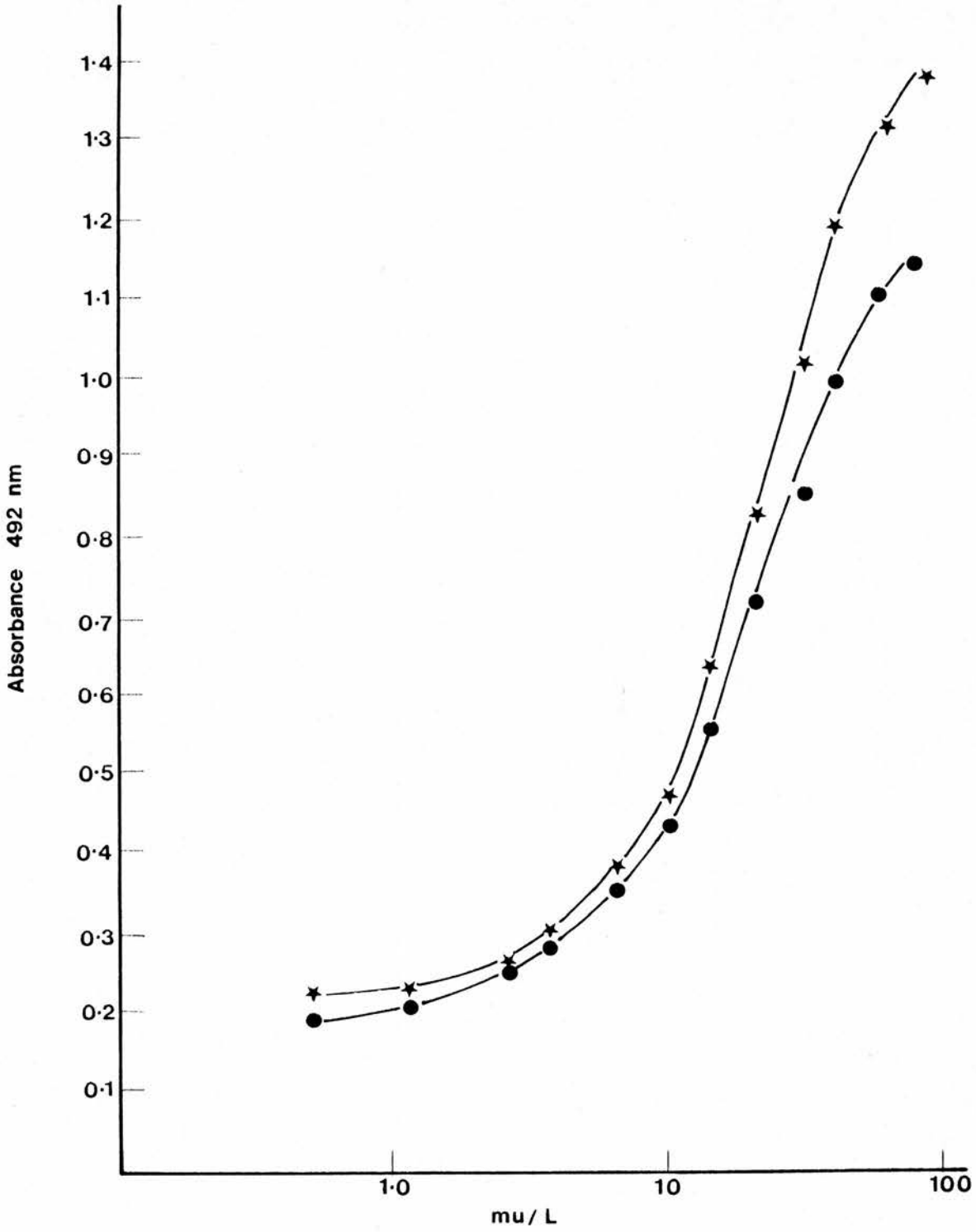

Figure 9. Dose-response curves for periodate oxidation and glutaraldehyde methods of conjugation.

●—● glutaraldehyde method ★—★ periodate method

assessment.

3.2.4. Comparison of monoclonal and polyclonal antibody preparations.

Before use the monoclonal antibody described in Section 2.2.1.1. was purified using the Octanoic acid method as before. The protein content was 10.1 mg/mL.

An antibody-enzyme conjugate was prepared, using the purified antiserum, by the glutaraldehyde method, and assessed for a suitable working dilutionas before.

The dilution of monoclonal antibody-enzyme conjugate which came closest to the dilution of 1:1,000 of the polyclonal conjugate in terms of a comparable standard curve was one of 1:500. A comparison of the four possible combinations of solid phase antibody and labelled conjugates is presented in Figure 10.
The two curves obtained using labelled polyclonal antibody gave an acceptable performance. That which also used polyclonal antibody on the solid phase was of course the system used to set up the assay, the other using monoclonal antibody as the solid phase gave absorbance values which were lower. The lower readings may reflect the higher specificity of the monoclonal antibody. The curve generated by using monoclonal antibody on both sides of the sandwich is no more than expected if the monoclonal is truely monoclonal, recognising only a single epitope on the TSH molecule. The relative differences in the dose response curves are discussed further in Section 5.2.

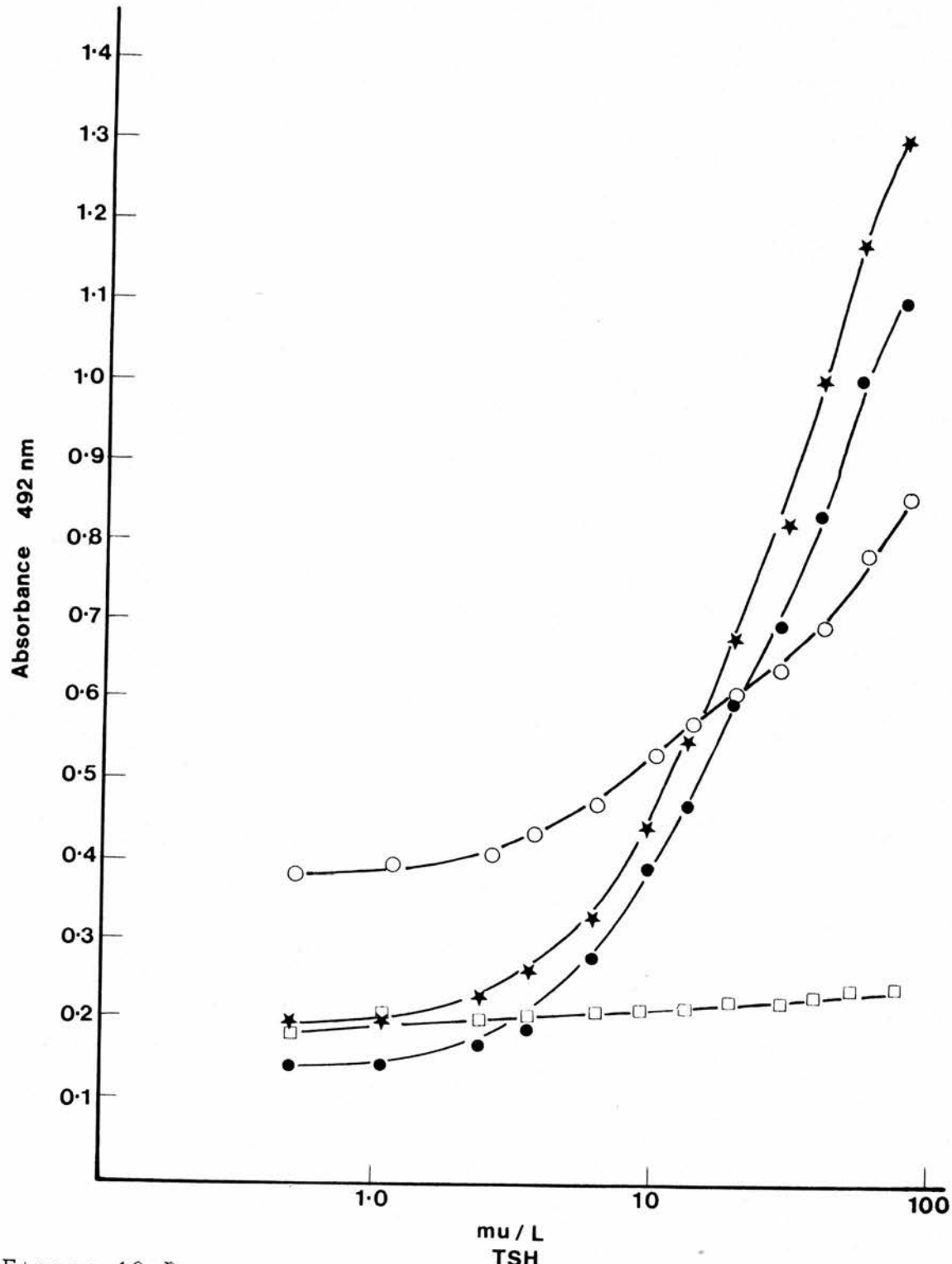

Figure 10. Dose-response curves obtained using different combinations of monoclonal and polyclonal labels.

○—○ solid phase polyclonal/monoclonal label
★—★ solid phase polyclonal/polyclonal label
●—● solid phase monoclonal/polyclonal label
□—□ solid phase monoclonal/monoclonal label

3.2.5. Evaluation of Assay for TSH.

Factors such as incubation times and temperatures as well as different solid phases have been reviewed and discussed in the Introduction. In optimising the current assay, account has been taken of such factors but the final choice has been influenced by the requirement to develop a rapid assay suitable for routine use

3.2.5.1. Final definition of assay.

Definition of the method reagents has already been given in Section 2.6. of the Methods section. However final antibody dilutions and incubation times are set out below in Table 2.

Table 2. Final Assay times and dilutions

Reagent	Dilution	Incubation times
Solid phase antibody (mono)	1:1000	18 h
Test/Standard	undiluted	40 min
Labelled antibody (poly)	1:500	40 min
Chromogen/ Substrate	-	45 min

For convenience all incubations were carried out at room temperature and the incubation times, whilst not critical, were strictly adhered to in order to achieve proper precision. The defined conditions above were used in all measurements during the assessment of the

analytical performance of this assay.

3.3. ASSESSMENT OF THE TSH METHOD.

The perfomance of this ELISA method was assessed. Precision, accuracy, detection limit and correlation with the existing RIA method (Section 2.5.) were the parameters used to judge its performance.

3.3.1. Estimation of Assay Precision.

3.3.1.1. Within batch precision.

To assess within batch precision of the assay, two pools of human serum were collected. The first consisted of serum from patients with normal TSH values, i.e. < 5.7 mU/L, as measured by the Corning RIA method used routinely in the laboratory. These patients had no previous history of thyroid disease. This pool was used as the Low Control (1). The second was made up from the sera of patients who had displayed raised TSH values, i.e. > 20 mU/L, this was used as the High Control (1).

The TSH concentration was measured by setting up 24 duplicates of each on two separate plates, each plate being run through the assay as set out in Section 3.2.5.1.

Using the results obtained, the Standard Deviation (S.D) and Coefficient of Variation (C.V.) were calculated for each control. These are shown in Table 3.

Table 3. Within Batch precision for the Low and High control sera

	n	Mean RIA value (mU/L)	Mean ELISA value (mU/L)	S.D.	C.V. (%)	Comparative RIA C.V. (%)
Low Control (1)	24	3.9	3.3	0.45	13.6	13.7
High Control (1)	24	64.0	50.6	1.44	2.8	4.3

The precision figures are compared with similar figures from the RIA method in Section 5.1. of the discussion. It was also noted that the mean results obtained using the ELISA method were lower than those by the RIA despite the fact that both methods were standardised using the same material. (1st International Reference preparation Section 2.4.2.)

3.3.1.2. Between batch precision.

A further two pools of sera collected, again there was a Low control (2) (TSH = 6.4 mU/L) and a High control (2) (TSH = 46.0 mU/L). Both controls were divided into 1 mL portions and stored frozen at -20°C. Each had TSH measured on 14 consecutive days and the results used to calculate the S.D. and C.V. for between batch analyses. These are shown in Table 4 .

Table 4. Between Batch precision for

Low and High control sera (2).

	n	Mean RIA value mU/L	Mean ELISA value mU/L	S.D.	C.V. %	Comparative RIA C.V. %
Low control(2)	14	6.4	5.7	0.59	10.3	10.9
High control(2)	14	46.0	44.9	3.94	8.8	8.1

The between batch precision was very comparable with that of R.I.A. Overall precision of the assay is discussed more fully in Section 5.1..

3.3.2. TSH assay Detection Limit.

The detection limit of the assay was determined by taking the mean absorbance of the zero standard for 24 readings on one microtitre plate and adding 2 S.D. for those readings. This absorbance value was then interpolated from the standard curve generated on the same plate and a value in mU/L obtained. Using the above procedure the following figures were obtained;

Mean Zero standard absorbance = 0.1722

Standard Deviation = 7.2×10^{-3}

Coefficient of Variation = 4.6%

This gave a detection limit of ;

0.1722 + 0.0140 = 0.1866 Abs. units

when rounded up to 0.19 and read from the graph this gave a value of 1.1 mU/L of TSH.

This value is comparable to similar figures for other R.I.A. methods. (D.H.S.S. Preliminary Report 1985). Fuller discussion is given in Section 5.1.

3.3.3. Accuracy of the TSH Assay.

The accuracy of the method was assessed using two procedures, comparison of results obtained by using another method (R.I.A.), and analytical recovery.

3.3.3.1. Correlation with RIA Method.

Patient samples with TSH values in the range 1.3-60 mU/L were assayed and the results were compared with those obtained by the sandwich RIA used routinely in the laboratory. Using the method of least squares regression, the two methods are related by the equation;

$$Y = 0.86X + 1.69$$

where Y is the ELISA method and X the RIA. The same data gave a regression coefficient (r) = 0.9808.

These results are expressed graphically in Figure 11. It can be clearly seen that whilst the correlation was good the results obtained by the ELISA method were, in general, lower than those obtained by the RIA method

This can be confirmed by the more critical method of analysing the data suggested by Bland and Altman (1986). They suggest that plotting the mean TSH value for the two methods against the difference between the two results gives a superior graphical presentation of the agreement between two methods. Figure 12. presents the agreement between the two TSH methods. Again it shows that agreement is close but also that there is a

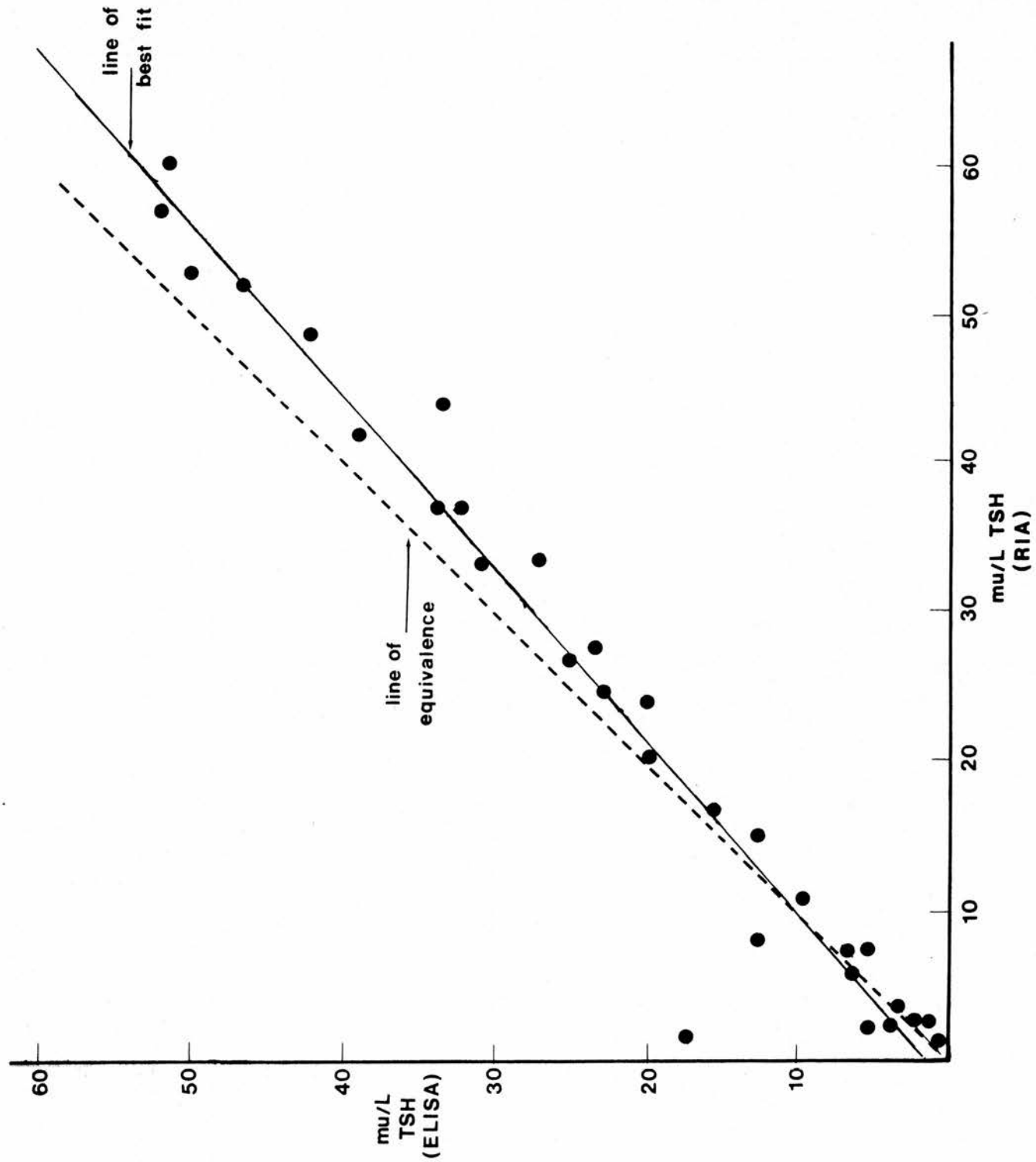

Figure 11. Graph of Correlation Between RIA and ELISA Methods for TSH.

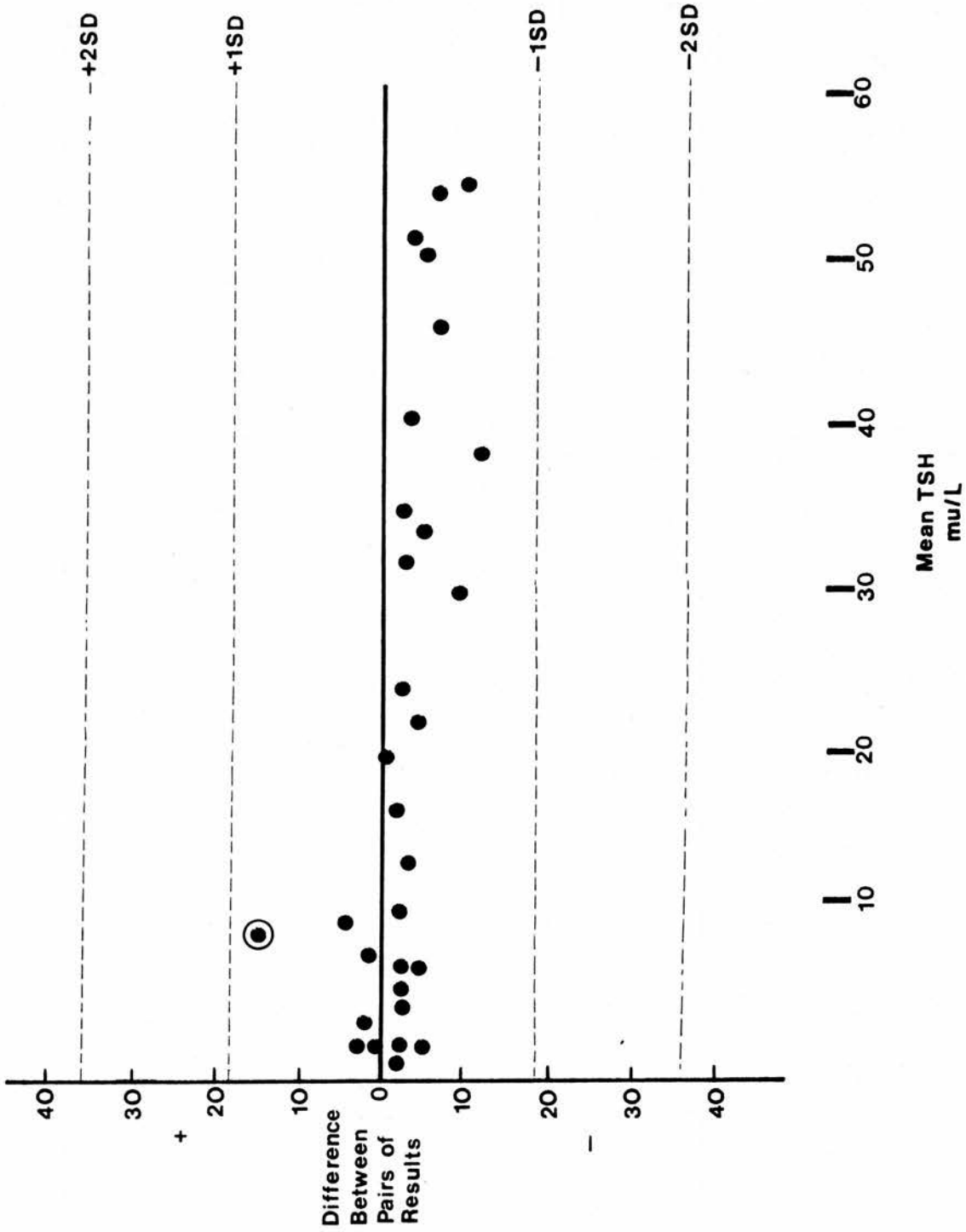

Figure 12. Graph of the Mean TSH Values Against the Difference Between the Results from the Two Methods

bias towards low results by the ELISA method

3.3.3.2. Analytical Recovery.

One other method of assessing accuracy is to determine the recovery of known quantities of TSH. This was carried out in the following way.

Serum was obtained from a thyrotoxic male patient and known amounts of TSH were added to different aliquots of this serum. It was important that the serum used in this experiment be human. The patient's thyrotoxicosis ensured that the TSH level of the serum was undetectable (confirmed by RIA) and that because it was male serum any possible cross reaction with human gonadotrophins (LH,FSH,HCG) would be minimised.

Recovery was assessed at three points on the curve, the details are given in Table 5.

Table 5. Recovery of TSH added to thyrotoxic male serum

Amount of TSH added	Volume of Serum (mL)	Nominal TSH (mU/L)	Measured TSH (mU/L)	Recovery (%)
10 µL of 500 mU/L	1.0	5.0	4.9	98
50 µL of 1500 mU/L	2.0	37.5	36.0	96
50 µL of 1500 Mu/L	1.0	75.0	72.0	96

The results shown in this section indicate that, in general the use of this method to measure TSH in

human samples can at least be equivalent to the current RIA method in use in this department.

SECTION 4

ALBUMIN RESULTS SECTION

4.1. GENERAL ASSAY PROTOCOL FOR ALBUMIN.

The estimation of albumin in urine was carried out using a technique similar to that used in the TSH assay i.e. a sandwich enzyme-immunoassay, which in this case included an additional antibody step. Figure 13 illustrates the principle and also indicates the additional step.

In the work carried out for TSH, the antisera were donated and were not commercially available. There are however numerous suppliers of antisera to many types of immunogens and in some cases the antibody can also be purchased already labelled with enzyme. In order to try out some commercial preparations, the albumin assay was established using antisera purchased from U.K. suppliers. However it was not possible, at the time, to arrange for supplies of an anti-human albumin which was labelled with peroxidase. It was possible to purchase an anti-goat IgG linked to peroxidase and also a goat anti-human albumin. These were used to create an additional sandwich layer thus enabling the bound albumin to be labelled and quantitated.

4.2. OPTIMISATION OF THE METHOD.

4.2.1. Determination of Antibody Dilutions.

4.2.1.1. 1st Antibody Dilution.

To determine the appropriate dilution of 1st antibody, doubling dilutions of the antibody (1:1250-1:20,000) in coating buffer were tested using a complete standard curve and arbitrarily chosen dilutions of 1:2,500 for the 2nd antibody and 1:1,000

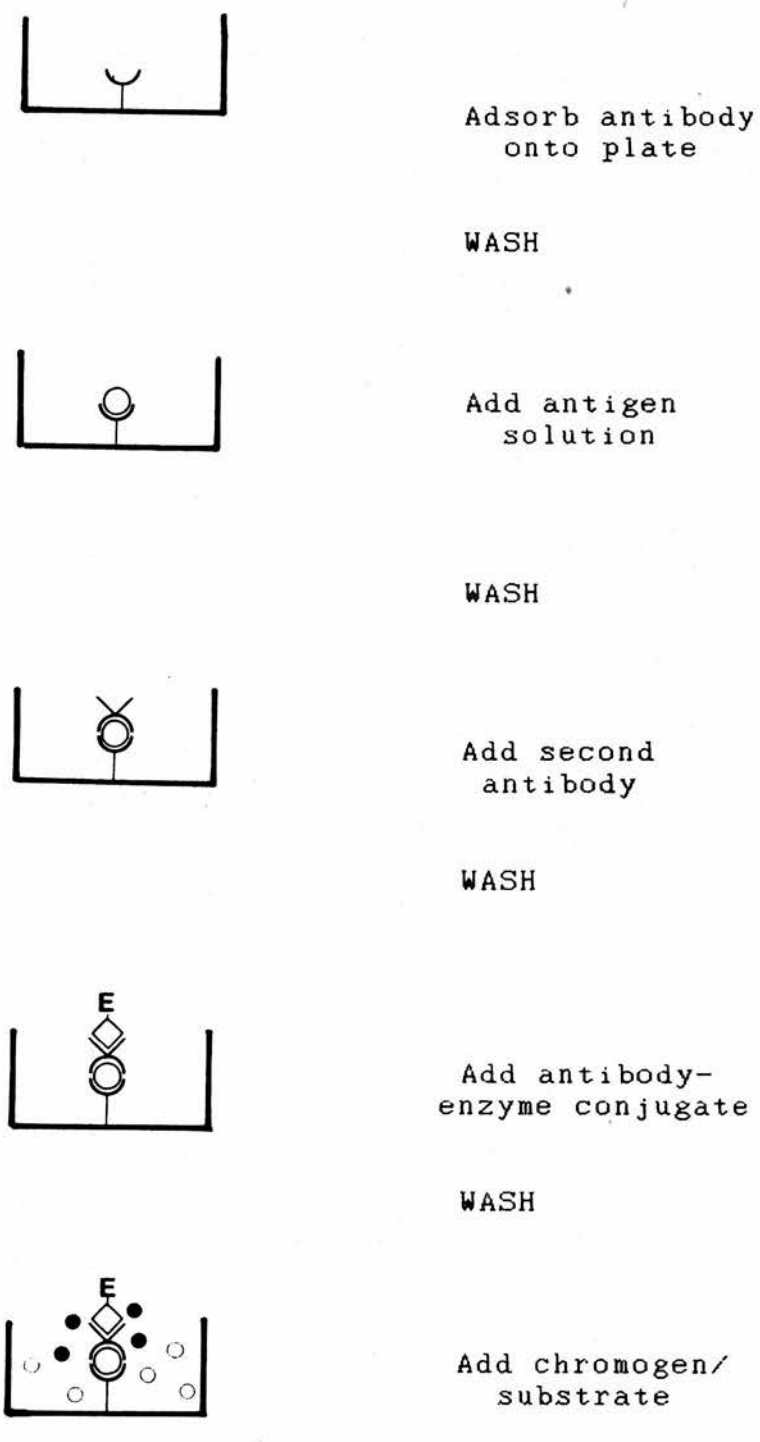

Figure 13. Principle of Double Antibody Sandwich Technique for Measuring Albumin.

for the antibody-enzyme conjugate. These were dilutions based on the TSH assay dilutions.

The absorbances obtained using the above dilutions were all extremely high (>2.0 abs units) and showed no indication of falling at the lower albumin concentrations. This suggested that the antibody-enzyme conjugate was somehow flooding the plate and this could have been due to one of the two following factors:-

1) The dilution of 2nd antibody was too small.

OR

2) The dilution of antibody-enzyme was too small.

To determine which of the above was causing the high absorbance readings, the experiment was repeated using a 2nd antibody dilution of 1:10,000. Similar extremely high absorbance values were observed, indicating that the concentration of the antibody-enzyme conjugate was too high and that the label was being non-specifically bound to the plate.

Once again the initial experiment was repeated with dilutions of 1:10,000 for 2nd antibody and 1:5,000 for antibody-enzyme conjugate. The results are shown in Figure 14 and indicate a suitable dilution of 1st antibody was 1:10,000.

4.2.1.2. Determination of Label Dilution.

Using the above dilutions of 1:10,000 for both 1st and 2nd antibodies, the antibody-enzyme conjugate was diluted from 1:5,000 - 1:20,000. The results are shown in Figure 15.

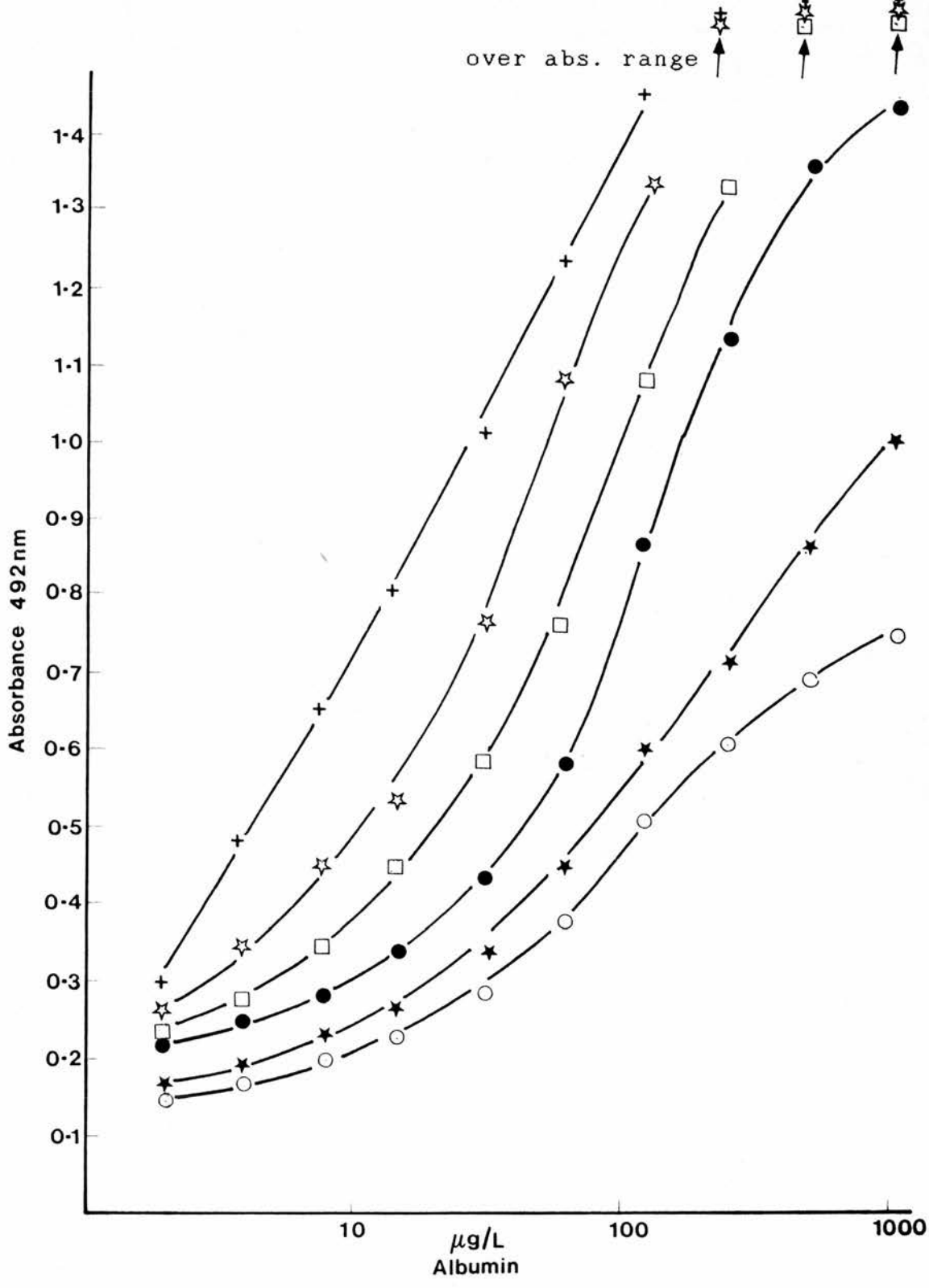

Figure 14: Dose response curves for first antibody dilution in albumin method.

○—○ 1:40,000
□—□ 1:5,000
✶—✶ 1:20,000
☆—☆ 1:2,500
●—● 1:10,000
+—+ 1:1,250

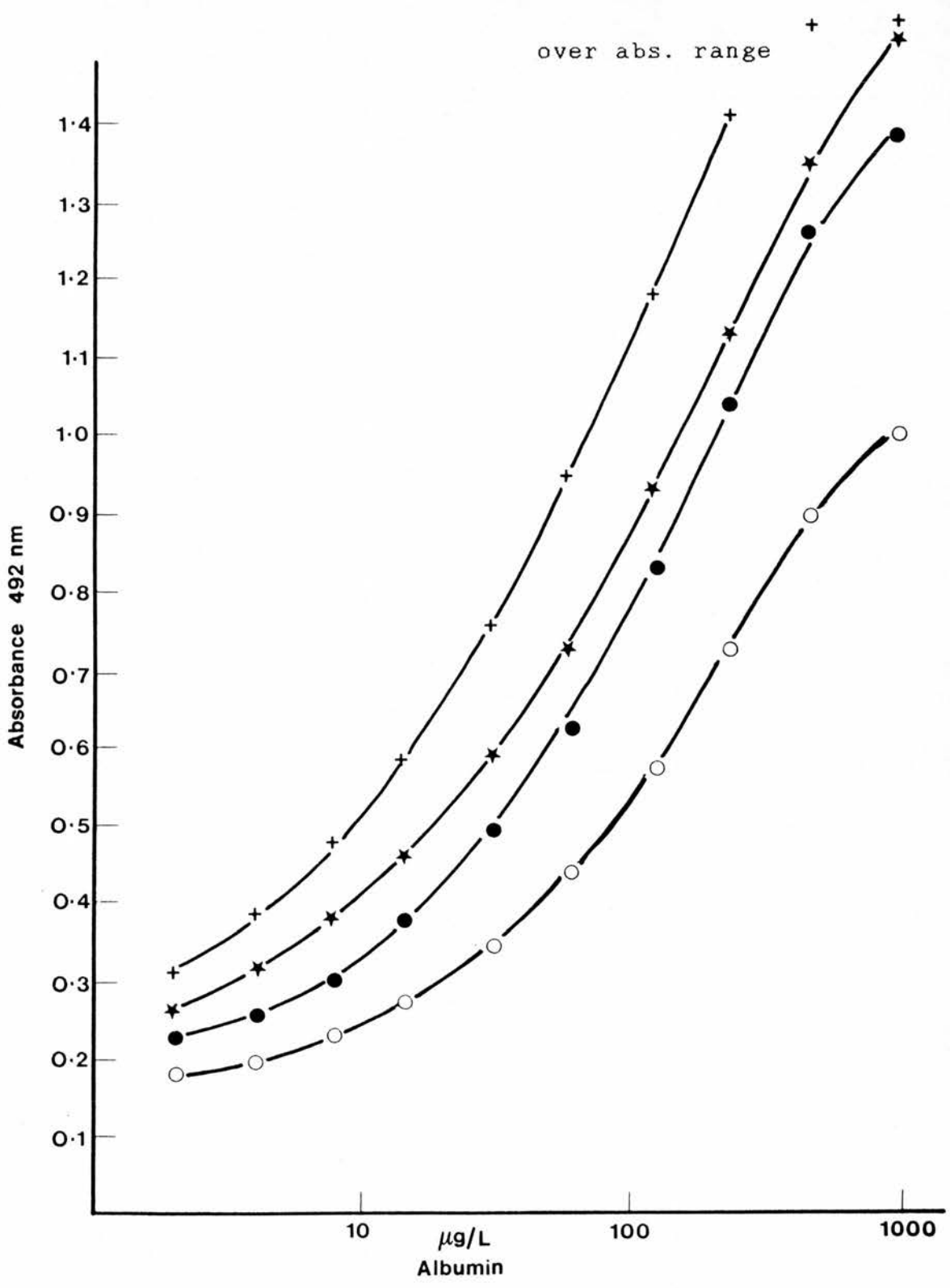

Figure 15. Dose response curves for differing dilutions of antibody-enzyme conjugates

○—○ 20,000 ✶—✶ 10,000

●—● 15,000 +—+ 5,000

Again, as in Section 3.2.2., the curve offering the widest range of absorbance along with the lowest zero standard value was chosen as being the most appropriate dilution of the antibody-enzyme conjugate. In this case a 1:10,000 dilution of conjugate was considered appropriate.

All the curves to this point were generated using incubation times which were similar to those described in section 3.2.5.1.and whilst the dose response curves were acceptable, the zero standard absorbance values for the chosen dilutions were considered to be too high. (> 0.2 abs units)

4.2.1.3. Incubation Time of the Label.

Since this assay was to be used to measure albumin in the urine of diabetic patients, the use of a carrier serum for the antibody-enzyme conjugate was not indicated, neither was the use of a blocking protein such as bovine serum albumin. It was considered to be unwise to use any potentially cross reacting substance in the assay unless it was absolutely necessary. One possible means however of reducing non-specific binding was to shorten the incubation time and, if required, to reduce the dilution of the conjugate. Three dilutions of conjugate were tested. Since an attempt was being made to reduce the incubation time, the highest dilution tried was that already chosen in Section 4.2.1.2. (1:10,000). The other two dilutions were 1:5,000 and 1:2,500. All three were tested at 40 min

and 20 min incubation times. The dose response curves are shown in Figure 16.

It can be seen from the curves that the decrease in antibody-enzyme conjugate dilution, along with the shortening of the incubation time, achieved the desired effect of lowering the zero standard absorbances yet retaining an acceptable absorbance range for the standard curve. The dilution of 1:5,000 incubated for 20 min was chosen for the analytical assessment of the method.

4.2.2. Final Definition of the Albumin Assay.

Definition of the assay reagents has already been given in Section 2.8. but final dilutions are given below in Table 6.

As in the TSH assay, the incubations were carried out at room temperature and the incubation times were again strictly adhered to in order to maintain precision. The defined conditions above were used in the assessment of the performance of this assay and were also used during the measurement of albumin in the urine from the diabetic subjects.

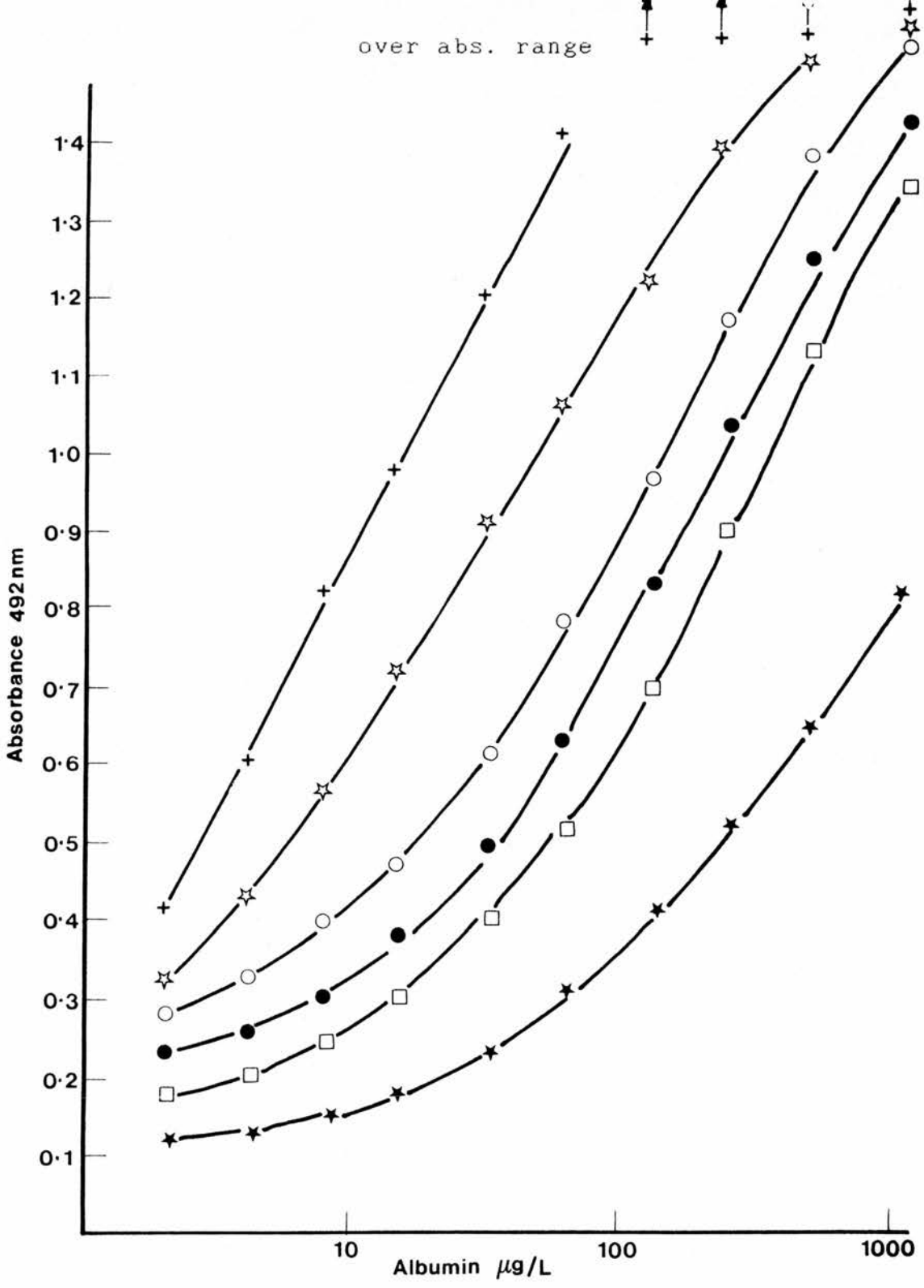

Figure 16 : Dose response curves for antibody-enzyme dilutions at 20 and 40 min incubations.

1:2500	1:5000	1:10000
☆—☆ 20min	□—□ 20min	★—★ 20min
+— + 40min	○—○ 40min	●—● 40min

Table 6. Final Assay Times and Dilutions for Albumin

Assay Step	Dilution	Incubation time
1st antibody	1:10,000	40 min
Test urine	1:100	40 min
2nd antibody	1:10,000	40 min
Antibody-enzyme conjugate	1:5,000	20 min
Chromogen substrate	---	20 min

4.3. ASSESSMENT OF THE ALBUMIN ASSAY.

The performance of the albumin assay was assessed. Precision and accuracy were estimated in a similar fashion to those in the TSH assay. Comparison with an alternative method was not possible, no other method being available at the time, but the ELISA method was used to measure the albumin excretion rates of healthy individuals and also of diabetic subjects attending outpatient clinics at the Victoria Hospital.

4.3.1. Within Batch Precision.

Precision within batch was assessed by estimating the albumin in two separate patient samples of urine. They were taken from diabetic subjects attending regular outpatient clinics at the hospital.

Albumin was estimated on 26 and 22 duplicate dilutions of Urine 1 and Urine 2 respectively, on two separate microtitre plates. Each plate included a standard curve.

As before the S.D. and C.V. of each set of results were calculated and are shown in Table 7.

Table7. Within Batch Precision for Albumin Assay

	n	Mean Value mg/L	S.D. mg/L	C.V. %
Urine 1	26	10.8	0.9	8.6
Urine 2	22	363	19.6	5.4

Whilst comparison of these figures with those of another assay was not possible, the figures shown in the above table do reflect on the general performance of this assay and were considered acceptable for an assay of this sort. (cf.Table 3)

4.3.2. Between Batch Precision.

Two further urine samples, again from diabetic patients were used to assess between batch variability. Each urine was divided into 1 mL portions and stored frozen at -20°C. Each urine was then measured on consecutive assays. (Urine 3 on 12 occasions, Urine 4 on 14 occasions.) Results are presented in Table 8.

Table 8. Between batch Precision for the Albumin Assay

	n	Mean value mg/L	S.D.	C.V. %
Urine 3	12	96.0	7.2	7.5
Urine 4	14	440	36.1	8.2

Once more the above figures reflect a satisfactory performance for an immunoassay technique and both sets of precision figures are discussed further in Section 5.2.

4.3.3. Accuracy of the Albumin Assay.

During the development of this assay no other means of measuring the low levels of albumin was available and for this reason it was necessary to restrict the assessment of method accuracy to the estimation of analytical recovery of added albumin.

4.3.3.1. Assessment of Analytical Recovery.

Recovery was assessed by the measurement of albumin in urine before and after the addition of a solution containing standard albumin. This was carried out at two points on the curve and the results are shown in Table 9.

Table 9. Recovery of Added Albumin

Added Albumin	Urine Volume mL	Nominal Albumin mg/L	Assigned Albumin mg/L	Measured Albumin mg/L	Recovery %
200 µL (2.0g/L)	10	3.0	43.0	44.0	102
100 µL (0.5g/L)	10	0.3	5.3	5.9	111

These recovery figures are discussed in Section 5.2. It was not considered necessary to define a detection limit for this assay since, unlike TSH, levels at the lower end of the dose response curve were

not considered to be of clinical importance. The lower standard, however, was readily distinguished from zero and urines with albumin levels below this value were subsequently reported as < 0.2 mg/L.

4.4. QUANTITATION OF URINE ALBUMIN EXCRETION.

The urinary albumin excretion of healthy and diabetic subjects was estimated using this assay. Urine to be measured was collected over a timed 3h period from fasting individuals and the albumin excretion rate (AER) calculated as μg of albumin/ minute.

4.4.1. Results from Healthy Subjects.

The albumin excretion rates for each of twelve healthy, fasting volunteers were calculated. The range of albumin obtained was 0.5 - 10.4 μg/min, equivalent to a concentration range of 0.4 - 9.5 mg/L. The results are shown in Figure 17. and are discussed in Section 5.2. In this group renal function was assumed to be normal.

4.4.2. Results from Diabetic Subjects.

As part of a larger study, urine was collected from 43 Type II diabetic patients. These were non-insulin dependent diabetics whose clinical assessment indicated unsatisfactory glycaemic control by diet alone and who therefore required to commence treatment with an oral hypoglycaemic agent. Urine was collected upon entry to the study and again after six months of strict glycaemic control.

Figure 18. shows the improvement in the blood glucose and haemoglobin A_1 levels over this period.

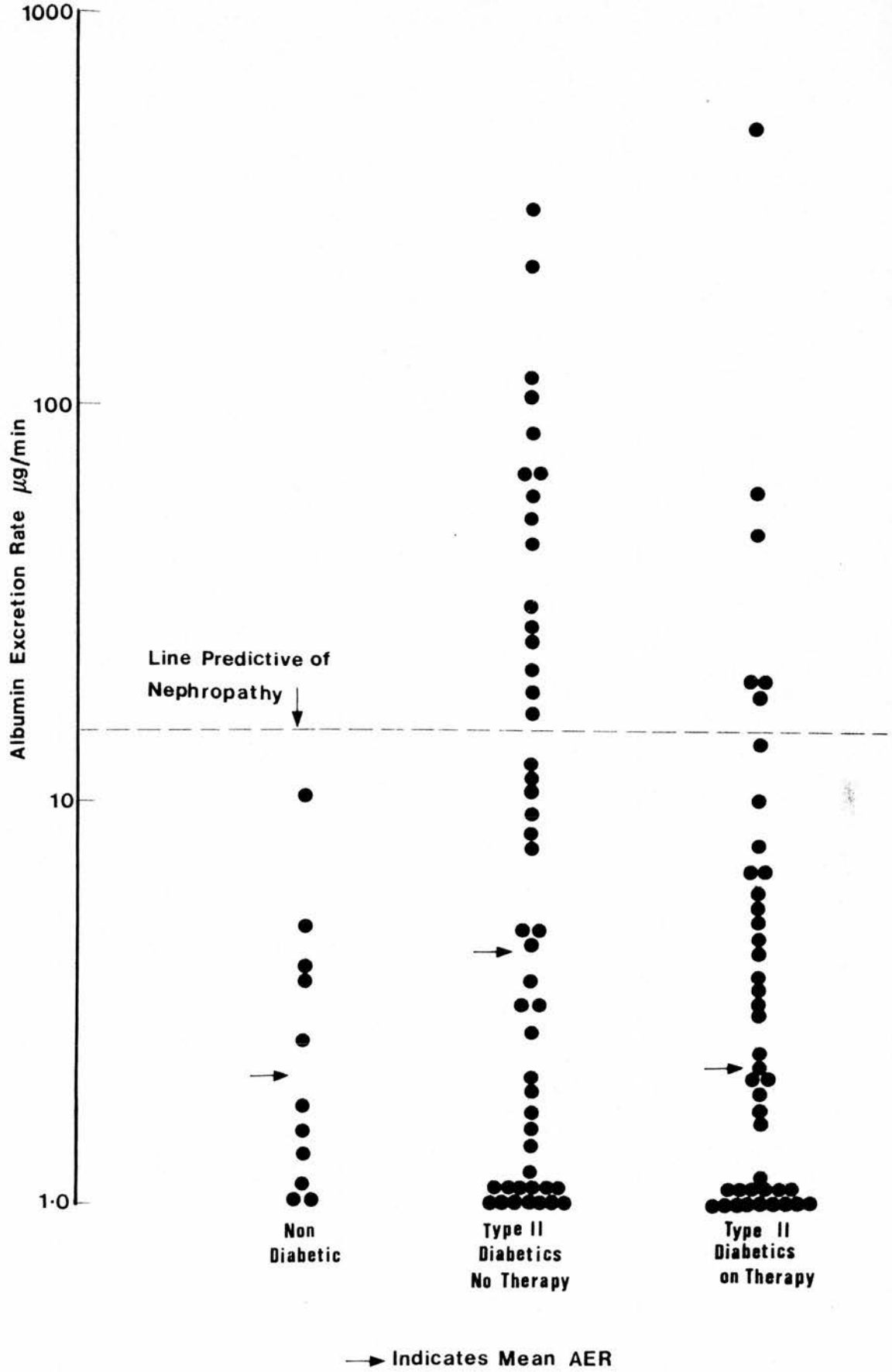

Figure 17 Urinary albumin distribution for non diabetic and diabetic patients before and after therapy.

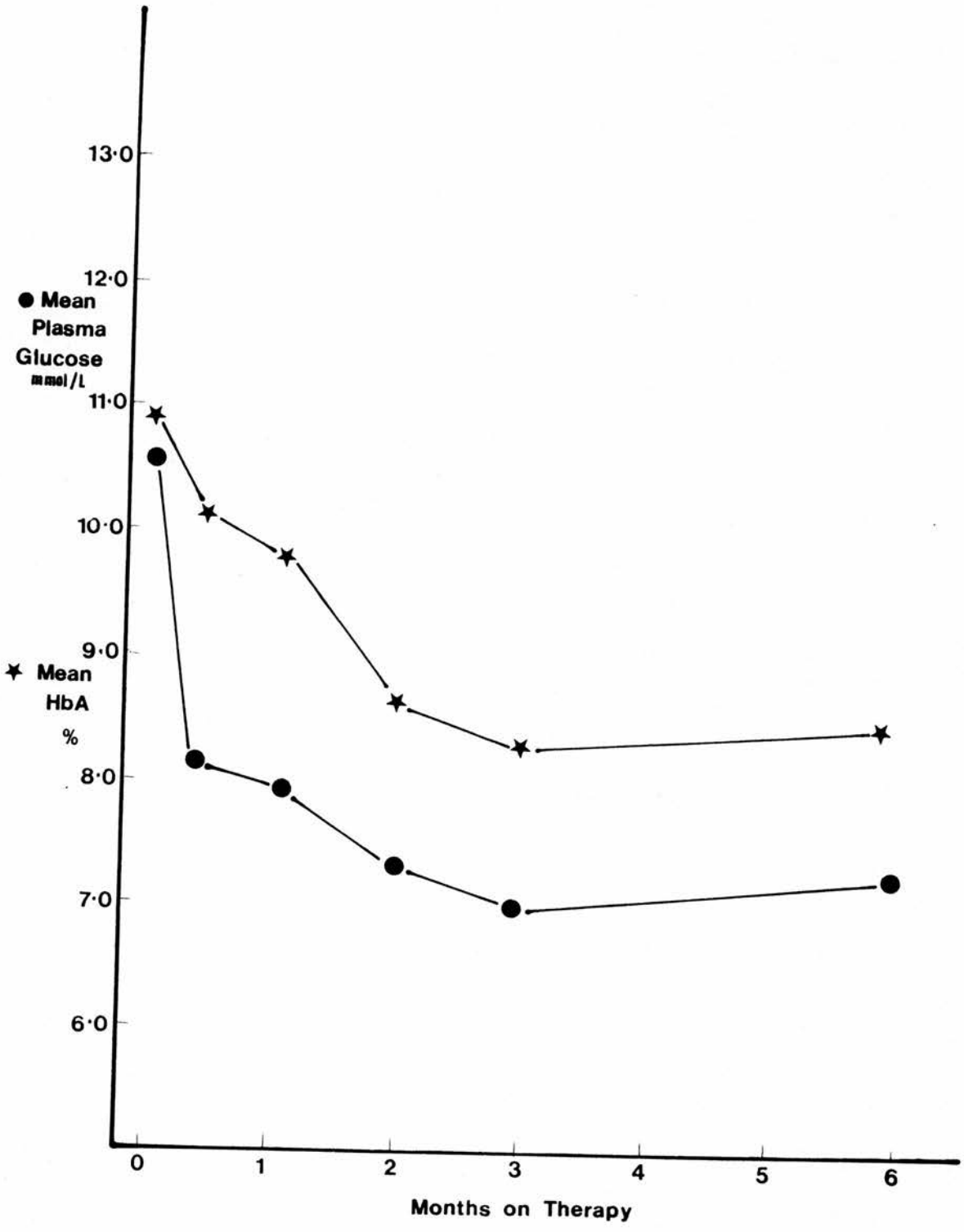

Figure 18. Graph Showing the Improvement in Glycaemic Control for Diabetic Subjects During the Study

Figure 17. shows the results of the albumin excretion rates at the beginning and end of the same period. The range obtained was 0.2 - 315 μg/min, equivalent to a concentration range of 0.2 - 200 mg/L and a mean AER of 4.22 μg/min. After 6 months on an oral hypoglycaemic agent their diabetic control was greatly improved and the mean AER was 2.26 μg/min. This covered a range of 0.2 - 522 μg/min and was equivalent to a concentration of 0.2 - 84 mg/L. The difference in the mean AERs was not statistically significant ($t = 2.07$, $P < 0.05$).

Section 5

Discussion and Conclusions.

5.1. The TSH Assay.

The possibility of using ELISA as an alternative to RIA was identified very soon after the introduction of the technique. In an editorial in the Lancet (1976) it was recognised that not only was ELISA capable of replacing RIA in centralised facilities such as area laboratories, but also that it could possibly undergo further development into the " dip-stick " area of sample testing. Even then it was recognised ELISA had the advantages of immunotechnology but could use it more cheaply than RIA.

Earlier Saunders & Wilder (1974) had referred to the enzyme labelled antibody technique as being useful in " screening for any disease that elicits host immune response to the antigen of the etiologic agent ". Since then the use of immunoassay in general has increased considerably. In particular, RIA has become an analytical tool used in the vast majority of clinical chemistry laboratories. Inherent in this increase was the necessity to develop antiserum for use in the assays and consequently the choice of antisera has widened considerably.

The quality of a particular antiserum is one of the major factors affecting the quality of an individual assay and of course purification of that antiserum is also of considerable importance.

The two techniques tested in this study (Kekwick, 1940. Steinbuch & Audran, 1969) are widely used by routine clinical chemistry laboratories. Although

affinity chromatography may produce purer antiserum (Stites, 1978) the results achieved by the octanoic acid and salt precipitation methods gave sufficient purity for routine use with associated ease of operation.

The dose response curves in Figure 7 show that both techniques produced purified IgG of similar specificity. The curves were parallel and close together . There was in fact little to choose between the methods in terms of product. However the curve produced using the octanoic acid technique did give a curve with a lower absorbance at the zero standard. This small difference was thought to be due to this method producing a slightly more homogenous solution leading to less non-specific binding of the labelled antibody.

It should be noted that both these curves were produced using standard material dissolved in saline. Desirable absorbances of less than 0.2 units were achieved at the zero dose level.

In the assay itself, the use of chicken serum as a carrier serum for the standard material was considered. This was to reduce any diferences between samples and standards arising from possible matrix efects. This practice is used routinely in assays where different matrices of standards and test samples could result in bound antibody expressing a different avidity for the same antigen (Fraser & Peake, 1980). An alternative would have been to dilute the samples in saline, but it

was considered better to use the sera undiluted if possible.

Since the measurement of TSH was to be carried out on human serum samples, it was considered necessary to utilise a carrier serum for standards and conjugate. Of course the best carrier serum would have been TSH free human serum but this was not available in the large quantities required. Chicken serum had been used however in RIA procedures for human alpha fetoprotein (Masseyeff, 1974). It had also been used in some ELISA techniques (Maiolini, 1975. MacDonald & Kelly, 1978) as a carrier serum for the antibody-enzyme conjugates , with a view to blocking non-specific binding of the conjugate to the solid phase.

Chicken serum was used in the present work and the curves produced are shown in Figure 8. They demonstrate the response to doubling dilutions of the antibody-enzyme conjugate and indicate that a dilution of 1:1000 was appropriate.

Comparison of this curve with that obtained for the saline dilutions (Figure 7) show that the curves were similar but that the zero-dose absorbances for the chicken serum were higher and greater than 0.2 units. Since this was true for all dilutions it meant that a small yet undesirable degree of conjugate binding was taking place. This had to be associated with the use of chicken serum. It meant that either the chicken serum contained cross reacting substances or that chicken serum did not block non-specific binding

of antibody-enzyme conjugate.

Cross reactivity was checked by testing the chicken serum for LH, FSH and HCG in assays for the human forms of these proteins. No significant levels were observed. TSH was also tested and showed no detectable levels.

The non-specific binding of antibody-enzyme conjugate was tested by increasing the surfactant concentration of the buffers used in the various stages of the assay (Saunders, 1979. Engvall, 1980). Increasing the surfactant to 0.2% made no significant difference. Saunders also suggested that the wash solution should be at a slightly alkaline pH. The wash buffer used in the assay was pH 7.4.

The above results all pointed to interference in the assay from the use of chicken serum. An alternative carrier serum, TSH free horse serum, was investigated using the protocol as before . The change in absorbance values for the zero and maximum dose concentrations are shown in Table 1. Horse serum allowed the dose response curves to approximate to those produced by the saline.

The comparison of methods for conjugation of the HRP to antibody produced results which were opposed to those reported by Nakane (1974) and O'Sullivan & Marks (1981). They reported that the periodate oxidation method gave a much higher efficiency of conjugation of the enzyme to the antibody and therefore a more efficient product. That being the case it would have

been expected that this method would have produced a much steeper dose response curve than the glutaraldehyde method (Adams & Wisdom, 1971). Figure 9 shows the comparison of the dose response curves. The curves are similar and almost superimposable at the lower end of the curve and only diverge at the higher TSH concentrations. Since the lower section of the curve was where the steeper response was required this offered no obvious advantage over the glutaraldehyde method. Nakane (1974) had reported that the optimum ratio for HRP to immunoglobulin should have been 2.3:1 for general use. He also reported that the quality of conjugate differed from batch to batch of HRP. Saunders (1979) also reported that ratios of HRP to antibody which exceeded 3:1 showed a marked decrease in activity and that the molecular ratio was difficult to control. The curve in Figure 9 was the best achieved from several preparations using the periodate technique. Several preparations showed no activity at all, each with a different batch of HRP. It was concluded that the variability of the method did not make it suitable for routine use.

The glutaraldehyde method by contrast always gave a useful product, presumably because conjugates prepared in this way gave more homogenous products, with conjugate molecules comprising of one molecule HRP and one molecule protein (Avrameas & Ternynck, 1971). Therefore, although the periodate method possessed the potential for a more efficiently produced conjugate,

the glutaraldehyde method demonstrated a more robust and consistent method for routine use. It gave a slightly less steep but none the less acceptable dose response curve.

Figure 10 showed the standard curves produced using the various combinations of polyclonal/ monoclonal antibodies.

The response observed with the monoclonal antibody on both sides of the sandwich indicates that the antibody is truly monoclonal and that all the binding sites recognised by that antibody are being bound by the solid phase antibody. This was also shown by De Groote et al (1983). This curve also indicates that if there is non-specific binding of the label then it is a small effect and remains consistent over the whole curve The slight increase in absorbance is most likely due to the instability of the chromogen (Porstmann et al, 1981)

Both curves using the polyclonal-HRP as the label demonstrate that they were of use in the assay. The system using solid phase polyclonal antibody and the polyclonal label was of course that used in the initial investigation of the assay. Using monoclonal antibody on the solid phase with this label produced a similar curve with a significantly lower set of absorbance values i.e. the monoclonal appeared to be binding less material than the polyclonal. In fact the greater specificity of the monoclonal antibody (Kohler & Milstein, 1975) most likely accounts for

the drop in absorbance.

Similar systems by Brock el al (1982) and Porstmann et al (1983) also reported that the combination of solid phase monoclonal and labelled polyclonal gave improved results over the solid phase polyclonal and monoclonal label. De Groote et al (1983) reported the opposite . The results are of course dependent on the affinity each individual monoclonal has for its particular binding site.

The curve with polyclonal solid phase and monoclonal label gave rather surprising results. The reagents used for this curve were the same as those for the others but the curve was much more flat and had higher absorbance values. No explanation could be found but it may be that the polyclonal antibody was binding a non-specific protein or immunoglobulin with a high affinity for the monoclonal. Scharff et al (1981) point out that whilst monoclonal antibodies are predictable and homogenous this does not exclude cross reaction with non-specific proteins.

Assessment of the precision of this assay was carried out in similar fashion to that suggested by Broughton (1980). Pooled human serum allowed for an evaluation of the within-batch precision at normal and elevated levels of TSH. The coefficients of variation for the low and high values respectively were 13.7% and 2.8% . These compare favourably with the results shown for the RIA method which gave C.V.s of 13.7% and 4.3% respectively for the same samples. Table 3 shows these

results and also shows the concentrations for both the low and high controls. The ELISA method produced results which were lower than those by the RIA.

The between-batch figures for the precision of the two methods (Table 4) are also very similar and therefore acceptable. Again the concentration results obtained from the ELISA method are lower than those for the RIA.

Throughout the precision studies, the standardisation of both methods was achieved using the same reference material. The difference in TSH concentrations must ,therefore, be truly method associated.

The assessment of method accuracy helped in the interpretation of this difference. The regression coefficient between the two methods of course only indicates that there is a linear relationship and in this case the correlation is acceptable.The line of correlation, however,indicates the ELISA method is measuring only 86% of the RIA estimates.The difference of 14% equates well with the average difference of 13% exhibited in the precision studies. The recovery from human serum of known quantities of added TSH, however, indicates that the ELISA method is performing satisfactorily (Swift & Ratcliffe, 1985). A slight under recovery (maximum 4%) was illustrated but the graph constructed by using the criteria laid down by Bland & Altman (1986) shows clearly that the ELISA method has a negative bias when compared with RIA.

One possible explanation of this could be cross reaction in either method. In the ELISA technique cross reaction with the most commonly related substances has been shown to be negligible. The same is also true, according to the manufacturers, of the RIA method. If cross reaction were to be the answer it would have to block binding of the labellled conjugate in ELISA or increase the binding of the radioactive label in the RIA.

Bearing in mind that the ELISA method used a monoclonal antibody it is most likely that the lower values observed merely reflect the higher specificity one expects from monoclonal antibodies (Kohler & Milstein, 1975). Indeed subsequent work on IRMA techniques directly analogous to the ELISA (Seth et al, 1984) confirm that systems using monoclonals in the main will give lower results than polyclonal RIA.

One patient result in the correlation did not fall into this pattern (circled in Figure 12). The RIA method gave a result of 3.2 mu/L a normal euthyroid result. The ELISA method gave a result of 17.4 mu/L. The patient was considered to be clinically euthyroid, had no history of thyroid disease and was a 37 year old male. Repeat analysis confirmed the first set of results. There apppeared to be no explanation for this anomalous result . Subsequent reports (Swift & Ratcliffe, 1985) have shown that a small proportion of the euthyroid population have significant levels of unknown substances which can interfere in those assays

using monoclonal antibodies. This is seen in particular in assays which use two monoclonals. It is possible that in this patient's serum, non-specific binding of such a substance allowed a substantial increase in labelled conjugate to be bound, hence providing a falsely elevated result.

In general the ELISA has been shown to be as reliable as the routinely used RIA method for TSH. The Corning Immophase has consistently preformed well in the UKEQUAS scheme for TSH run by the D.H.S.S. (Swift & Ratcliffe, 1984) and this implies that the ELISA method could be used satisfactorily as a routine assay.

5.2. THE URINARY ALBUMIN ASSAY.

The preparation of antibody-enzyme conjugates has already been shown to be of considerable importance in the development of any new assay. In the light of increased demand, many companies now offer a selection of prepared labelled antibodies. The decision to measure urine albumin concentrations allowed the use of some of these commercial preparations. These were used as supplied, with no further purification.

The use of an additional layer into the albumin assay shown in Figure 13 could have been avoided by preparing a labeled antibody-enzyme conjugate, as in the TSH assay, using the goat anti-albumin and HRP. However, the flexibility of the technique was demonstrated by introducing the additional layer in the sandwich. The goat-anti goat system was chosen to prevent the labelled antibody from cross reacting with

the solid phase antibody which was raised in rabbit.

The initial dilutions used to determine the required dilution of solid phase (1 st) antibody were loosely based on those used in the TSH assay but of course each system required determination of its own optimum dilutions and times. These would not necessarily be the same for different systems or even different antisera in the same system. Each would be dependent on the affinity of the antibody for its antigen, the avidity of the antibody , the concentration of the antigen, etc., and would be unique for a particular assay.

The final dilutions used in the albumin assay were much higher than those for the TSH. This was due to the related factors of urine concentration of albumin and of solid phase binding of antibody. A defined surface area, such as the well of a microtitre plate, has a finite capability to bind protein and in cases such as this it is necessary to dilute the sample before use. Thus the antibody was also required to be diluted to suit. In addition ,the commercial antisera used had a higher protein content (typically > 20 mg/mL) than those used for the TSH assay and therefore required greater dilution. It may be useful to " normalise " the protein concentration of each antiserum particularily to allow a closer continuity from one batch to another.

The consequence of inappropriate dilution was excessive levels of absorbance but by correct

manipulation of the dilutions, suitable dose response curves were easily achieved (Figure 14.).

The initial very high absorbances (Section 4.2.) were thought to be due to non-specific solid phase binding of the antibody enzyme conjugate, probably due to the very high concentration chosen initially. The technique of using a series of dilutions to determine the appropriate dilution to be used has been widely used by other workers (Pledger & Belfield, 1983. MacDonald et al, 1980. Hindawi et al, 1980) and can be carried out particularily easily on microtitre plates, each dilution being accomodated in a separate row of wells on the plate.

The decision not to employ a blocking protein in this assay again indicated the flexibility of the technique. The adjustment of incubation times (Figure 16) demonstrated that for this type of assay the system is easily manipulated to achieve the desired results.

Whilst an alternative method for urine albumin was not available for direct comparison, the precision figures (between and within batch) were considered acceptable at the concentrations to be measured.

Estimation of the accuracy was necessarily restricted to the analytical recovery. The figures show (Table 9) that there is a slight over recovery associated with the assay but once again they are acceptable for an assay of this sort.

Unlike the TSH assay it was not considered

necessary to determine the detection limit. The main use of this assay was to detect abnormal rises in urine albumin concentration. Since the method could readily distinguish the bottom standard from zero and was therefore capable of detecting normal urine albumin concentrations, it was not considered relevant to determine its lower limit of detection.

Comparison of the precision, recovery and detection limit achieved were comparable with those subsequently reported by Watts et al (1986), Silver et al (1986) and Mohammed et al (1984). It confirmed that the method could be used to determine urine albumin. The use of commercial antibodies proved to be satifactory. The experience gained from the TSH assay allowed the assay to be set up quickly and efficiently. The results show that it was suitable for use and it was employed to screen a selected group of diabetic patients.

The accepted form of reporting urine albumin results was to express them as an excretion rate (AER) such as μg/min. Viberti et al (1979) and Jarret et al (1984) concluded that diabetic patients with an AER greater than 15 μg/min were considered to have a greater chance of developing nephropathy. Figure 17 shows the line corresponding to this rate in relation to the AERs for the groups of normals and diabetics before and after treatment. The range of excretion rates for the healthy volunteers (0.5-10.4 μg/min, mean 2.92 μg/min) was similar to that reported by Watts et

al (1986a) and no value was above the line predictive of nephropathy.

For the diabetics at entry to the study , the range of excretion rates was considerably wider a point also noted by Mogensen (1984). The excretion rates for these Type II diabetics were similar to those reported by Jarret et al (1984) and indicated a concentration range of 0.2 - 200 mg/L . A number of patients (16) showed an AER > 15 μg/min. This represented 30% of the diabetics included in the study and this detection rate was similar to that observed by Mogensen (1984) (32.7%).

After six months of stricter control (Figure 17), it can be seen that the number of patients with an AER > 15 μg/min fell to 6 (13.9%). All of those who had an AER > 15 μg/min at this time had also been in this group before treatment commenced. Possibly these patients had progressed to irreversible glomerular damage and would therefore progress more quickly to clinical nephropathy.

The overall mean AERs before and after treatment were 4.22 μg/min and 2.26 μg/min respectively and at the moment it is assumed that a drop in the AER is a reflection of an improvement in renal function. Some workers (Steno group, 1982.) have reported that with conventional therapy they have observed no improvement in insulin dependent patients as far as urine albumin is concerned. Much of the work associating increased AER with early mortality has been of a retrospective

nature and so the usefulness of the results reported above will only be determined by monitoring the patients for some time.

5.3. GENERAL CONCLUSIONS.

The work presented in the preceding chapters has shown that, for the analytes chosen, ELISA can provide an efficient and accurate means of assessing their concentration in biological fluid. The technique proved to be a robust, easy to handle system which gave little problem in the day to day production of results. The reagents, antibodies and labelled conjugates have a long shelf life, are cheap and are easily stored.

The work has shown that it is possible to take an antiserum and construct an assay, including labelled conjugate, to suit the particular requirements of the work being carried out. On the other hand, assays may be set up quickly and efficiently with "ready made" antibodies and labels should this be required.

The assessment of TSH has progressed further since this work was carried out. The new " high sensitivity " methods have changed the strategy for thyroid function testing but whilst radio labelled assays such as the IRMA TSH are now extensively used, it is possible that enzyme amplification or time resolved fluorescence will direct more workers to the non-isotopic methods of analysis.

The discovery and estimation of new proteins and hormones is a continuous process requiring assays which are quick and easy to set up and above all offer

acceptable performance. ELISA is well suited to such situations. The use of urinary albumin estimations is still a relatively new concept and the assay reported here was set up with ease using readily available reagents. Assuming availability of antibodies , then it should be possible to use ELISA to measure analytes such as acute phase proteins, pregnancy associated proteins and tumour markers .

In conclusion , ELISA has been shown to be a useful analytical tool for use in clinical chemistry laboratories. It has been shown to be adaptable, easy to use and cost effective. Perhaps in the near future it will be widely accepted and as such, allow its further development

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