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Local Development of PEG Assisted Double Antibody T_4-Radioimmunoassay

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In order to replace expensive ready-made commercial radioimmunoassay (RIA) kits with high quality local techniques a local *Thyroxine (T_4)* RIA was developed and evaluated. Four basic components of immunoassay system, antibody, radiotracer, standards and precipitating solutions were locally prepared. Twelve rabbits were immunized to produce primary antibodies against T_4 using T_4 conjugated with human serum albumin (HSA) and emulsified with Freund's adjuvant as immunogen. Results showed that 4 out of 12 rabbits responded well to the immunogen and a maximum titre of 1:9700 was achieved with a working dilution from 1:1000 to 1:10000. Radioidination of Triiodothyronine (T_3) to produce radiotracer, ^{125}I-T_4 (and ^{125}I-T_3) by chloramine-T method showed that 31% of radioactivity was bound to T_3 and 50% to T_4 with specific activities of (11.1-14.8 MBq/µg) and (1.85-2.22 MBq/µg) respectively. Second antibody against T_4 was raised in two sheep. Results of optimization of second antibody showed a working dilution 1:60 for T_4-RIA. Precipitating solution to separate antigen antibody complex was prepared by mixing sheep anti-rabbit serum (SARS) with phosphate buffer containing 4% polyethlene glycol (PEG) at working dilution. PEG assisted double antibody T_4-RIA showed more than 7% error in the concentrations below 10 nmol/l with high precision at higher concentrations. The results of quality control samples showed that the values were within expected limits. The values correlated well with the commercial techniques (ICN; Corr. Coeff=0.99) and the values of patient samples determined in commercial as well as in house techniques did not differ significantly ($p>0.5$). The sensitivity of T_4 assay system was 2.5nmol/l. It is, therefore, concluded that in house assay technique has acceptable quality and fits on all standards of good quality.

Key words: Local development, Radioimmunoassay, Separation.

Introduction

Immunoassays are most widely used analytical tools and have been successfully applied to an extensive range of substances, including both large and small molecules, cells, cellular components and viruses. Their applications in estimation of hormones, tumour markers and drugs in body fluids have greatly influenced the diagnostic and therapeutic strategy. Although the historical development has spanned over a century, the immunoassay techniques became very popular in last three decades. Initially immunoassays with radioactive and non-radioactive tracers were developed at almost the same time approximately 41 years ago (Berson and Yalow 1957; Berson and Yalow 1959; Ekins *et al* 1968). The radioimmunoassay techniques became more popular because of good sensitivity, specificity and precision. In the late 80's non-isotopic techniques were also encouraged at many laboratories of the world. There were however sufficient reasons for retaining the use of isotopic techniques (Marks 1985; Turton 1988; Edwards 1994) which still compete well with the non-isotopic methods (Sajid 1999).

An immunoassay is based on interaction between analyte (labeled and unlabelled) and a specific antibody. The resultant antigen antibody complex can be separated from unreacted (or unbound analyte) by a number of separation systems. However, all of them have some disadvantages. For example systems based on differential migration are expensive and not very practicable, whereas those based on adsorption and fractional precipitation are associated with significant misclassification errors (Obell 1980; Chard 1980) which results in inefficiency and lack of ruggedness. The most suitable technique has therefore to be selected after comparison with other techniques.

The labeled analyte is prepared by labeling of the analyte (which may be synthetic or extracted from human tissues) with an isotope. Initially those isotopes were preferred which are most common elements of biological fluids. For example ^{3}H and ^{14}C but mostly they required liquid scintillation counting which is inconvenient and expensive in comparison with the measurement of gamma radiation and needs scintillation well counters. The low energy of beta radiation of ^{3}H also reduces the counting efficiency. The low decay rate of ^{3}H and ^{14}C with long half lives gives them low specific activity and correspondingly low count rates. This reduces their utility for high sensitivity assays. All other isotopes occurring in

biological materials also have suboptimal characteristics for use in radioligand assays. Either the half-life is very short, which makes their use impracticable, or the type of radiation is less suitable e.g., ^{32}P with it's high beta energy. Certain biological compounds contain other elements with radioisotopes that are more suitable for *in vitro* detection; examples are cyanocobalamine labeled with ^{57}Co and thyroid hormones in which natural ^{127}I can be exchanged for ^{125}I or ^{131}I. ^{125}I is more suitable because of low gamma energy (emitted by electron capture; 35 kev) and long half-life (60.2 days). The isotope is usually supplied in the specific activities of 74-92.5 MBq/μmol (555-740 MBq μg) and is attached to the analyte by oxidation of iodide to reactive atomic iodine which replaces the iodine atom of the molecule to be labeled (for example in iodination of T_3 and T_4). ^{125}I can also be used to iodinate those compounds which contain ring structure of tyrosyl residues (similar to that found in T_3 and T_4). Here ^{125}I replaces one or two hydrogen atoms of ring structure. Iodination of TSH with ^{125}I is an example. Almost all proteins contain tyrosyl residues and can therefore be labeled with ^{125}I. The antibodies (immunoglobulins) are also proteins and can be labeled with ^{125}I for the development of immunoradiometric assays (IRMA) (Bolton and Hunter 1973).

The binding reagent or antibody is a very important component of an assay system. The antibodies or immunoglobulins are produced by B-lymphocytes. After administration into an animal it is believed that immunogen binds to the receptors on the surface of lymhocytes. This binding induces the production of immunoglobulins with a surface of the variable portion that can bind this particular antigen. Then the lymhocytes carrying this information start multiplying, and all the new lymphocytes so produced carry with them the ability to produce antibodies against this particular immunogen. The basic structure of all immunoglobulins is similar with a pair of large polypeptide chains (heavy chains, H), that consist of 446 amino acids, each associated with a smaller chain (light chain, L) 214 amino acid long. There are five known classes of immunoglobulins IgG, IgM, IgA, IgD, and IgE. It is IgG which is important in immunoassays. The chains are joined together by disulphide bonds (Fig 1).

Each immunoglobulin molecule has the ability to bind two antigen molecules, one at each of the two binding sites, which

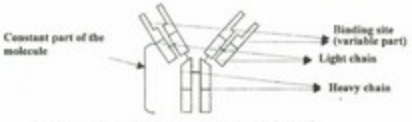

Fig 1. Structure of immunoglobulin (antibody).

are located at the outer open end of each pair of H-L chains. Differences in the chemical composition at these two ends of the molecules (the so called variable portions of the chains) are the structural basis for the differences in the binding characteristics of different antibodies.

The quality of an assay is highly dependent on the properties of the antibody used in an assay system and is assessed by sensitivity, specificity, precision and accuracy. The specificity is predominantly a property depending on the nature of antibody. The sensitivity (minimum quantity detectable) is limited by two factors; the avidity of the receptor (antibody) and the specific activity of the labeled analyte (or radiotracer). The specificity (ability of the assay to measure one specific compound and no other) denotes the uniqueness of binding and the avidity (firmness of the binding or energy in the binding between the ligand and the receptor) denotes the strength of binding. To be able to measure the minute amounts of hormones and other biologically reactive compounds with sufficient sensitivity, avidity has to be very high. Optimal antisera in this respect are sometimes very rare, so the avidity of antibodies is usually the limiting factor for the sensitivity of an RIA. Precision and accuracy are determined mainly by performance of the assays.

In Pakistan, the immunoassay services were introduced in late 70s and the work has been limited to the use of commercial radioimmunoassay kits. Constant dependence on expensive foreign technology is a great lead on country's economy. About 20,00,000 rupees (~35000 US$) are annually required at our institute for the purchase of RIA kits for thyroid related hormones. This work was an attempt to establish local techniques of reagent production to save the foreign exchange. Many other associated benefits like early release of results and increased scientific skills were also expected from this work.

The strategy and production schemes applied for preparation of in house T_4 assay reagents were similar to those applied to the development of T_3 assay and described elsewhere (Sajid 2001). The work was started with the production of antisera against T_4 and proceeded through preparation of assay buffer, radiotracer, standards and optimization of conditions for antibody dilution. Initially 15% PEG precipitation was tried which was then replaced with PEG assisted second antibody precipitation. Standard quality control procedures were applied to confirm the quality of locally developed techniques.

Materials and Methods

Production of T_4-antisera; Preparation of immunogen Chemicals arranged (i). *T_4 (Thyroxine):* SIGMA Chemical Company, USA) (ii). HSA (Human Serum Albumin: SIGMA Chemical Company) (iii). Dimethyl formamide (SIGMA Chemi-

cal Company) (iv). CDI (Morpho CDI:1-Cyclohexyl-3 (2-morpholinoethyl)-carbodiimide metho-p-toulene sulphonate :Aldrich Chemical Company, USA) (v). *Adjuvants:* Two types of adjuvants were used. *Freund's Complete Adjuvant* was used at the time of primary injection. *Freund's Incomplete Adjuvant*. This was used in booster injections. The composition is given later on.

Preparation of T_4 conjugate(immunogen). Small molecules (haptens) like T_3 and T_4 (Molecular weights 650 and 777 respectively) can not induce immunogenic response directly. The approximate limit for immunogenicity is molecular weight 1000 daltons to 10000 daltons. If a hapten is chemically coupled to a large molecule, the combination of the hapten and the large molecule (usually a large protein) acts as a complete antigen or immunogen. In very early studies by Sheehan (1956) and Good friend *et al* (1964) used water soluble carbodiimides as coupling agents for the production of antibodies against T_3 and T_4. These molecules are first conjugated to human serum albumin (HSA) or bovine serum albumin (BSA). Carbodiimide is used to induce peptide bond between T_4 (the carboxyl group) and primary lysine residues (amino group) of HSA or BSA (Herbert 1978; Thorell and Larson 1978). Figure 2 schematically illustrates the reaction. The T_4–HSA conjugate was therefore prepared using method similar to that described by Herbert (1978).

20 mg of T_4 (Thyroxine: SIGMA Chemical Company, USA) and 50 mg of HSA were dissolved in 5 ml of dimethyl

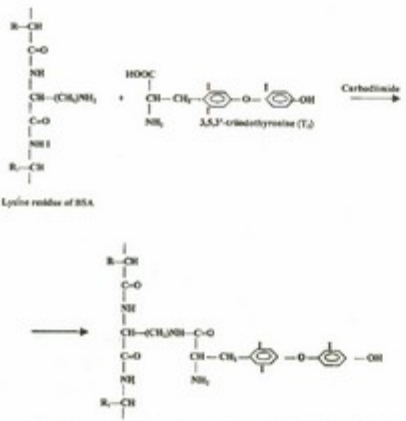

Fig 2. Coupling of T_3 (a dipeptide) to a lysine residue of BSA by means of carbodiimide.

formamide and 25 ml of distilled water respectively. 30 mg of morpho CDI was added to the albumin solution. T_4 solution was then mixed dropwise with the albumin solution with continuous stirring. pH of the mixture was continuously monitored by a pH meter and maintained at 5.5 using either 0.01 M NaOH or 0.01 M HCl. After 10 minutes of incubation at room temperature an additional 10 mg of morpho CDI was added to the incubation mixture, which was kept at room temperature in the dark. The final volume of this mixture was 32 ml having a calculated T_4 level of 804375.8 nmol T_4 /l (625,000 ng/ml or 20mg T_4/32 ml). 5 µl aliquot of this solution was drawn, diluted to 12.5 ml with phosphate buffer (pH7.5, 0.05M, 1%BSA) providing an approximate concentration of *322 nmol T_4 /l (250 ng T_4/ml)* and saved for T_4-RIA to be performed later. The above mixture was then dialyzed against continuously running water in the dark.

Dialysis procedure. A 15 cm long dialysis tube (Dialysis tubing, SIGMA, benzolated, avg. flat width 32 mm, inflated volume approx. 100 ml/ft, suitable for retaining compounds with molecular weight over 2000 daltons, human serum albumin has mol.wt ≅ 68000) was soaked in distilled water for 1 h. One end of the tube was closed with a knot. The other end was closed by inserting a 10 mm diameter rubber stopper through which a cannula was placed. The tubing was tied to the stopper with a string. One end of this string was used to fasten the dialysis bag with a stand to keep it suspended in the beaker containing distilled water. A small weight was also suspended at the lower end of the bag.

The solution to be dialyzed was injected via the cannula into the dialysis bag. The dialysis bag was immersed into 1000 ml of distilled water stirred continuously using a magnetic stirrer. The water was changed after every 6 hrs. The dialysis was carried for 72 hrs. The volume of the dialyzed mixture was made upto 32 ml using distilled water. 5 µl of this mixture was also drawn, diluted to 12.5 ml with phosphate buffer and saved for RIA.

T_4 concentration of the samples drawn before and after dialysis was measured by RIA using a commercial kit (Amersham, UK). The final mixture was aliquoted in 0.5 ml fractions in sterile vials, lyophilized and stored at 4 °C until use.

Preparation of emulsion for injection. T_4-HSA conjugate was emulsified in normal saline and Freund's adjuvant. The adjuvant acts as a non-specific stimulator to the lymphoid system, causing a foreign body reaction with granuloma formation. It also prolongs the adsorption of the immunogen, thereby extending the phase during which the lymphocytes of the host are exposed to the immunogen. Two types of adjuvants were used for immunizations as sug-

gested by Herbert (1978). The procedure of preparation was as under.

One ml of Fruend's adjuvant (complete for primary injection; contains Arlacek A, mannide monooleate, Bayol F (paraffin oil), and heat killed and dried mycobacteria (Myobacterium tuberculosis) and incomplete for booster injections (does not contain heat killed bacteria) and 1 ml of immunogen (Contents of one vial of T_4-conjugate (prepared above) were reconstituted to 2.5 ml using isotonic saline to get immunogen at concentration, 0.3 mg/ml were drawn separately into two 10 CC syringes. Syringes were mounted against each other via the connecting piece (plastic tube). The contents were then pumped back and forth between the syringes until the mixture became thick. (It should be very thick, at least as thick as whipped cream or else the emulsion will not be stable). Since the plunger becomes difficult to press (into the syringe) when emulsion begins to thicken, the plunger was pressed against the surface of a laboratory bench for increased power. After about 5 minutes of pumping, a good emulsion was ready for use. To test the stability of the emulsion a drop of emulsion was allowed to float on lukewarm water and remain there for at least 5 minutes to be certain that oil and water phases do not separate. (Note: An unstable emulsion may give a poor immunogenic stimulus, since the water soluble immunogens are resorbed rapidly. In case of immunogens with high biologic activity, this may even hurt the animals (Thorell and Larson 1978a, 1978b).

One milliliter (~150 µg) of this emulsion was injected to each rabbit. Best responses are generally expected using low doses of immunogen. This also reduces the production of unwanted anti bodies (Munso *et al* 1983).

Animals, route and schedules of injection. Twelve rabbits were selected, weighing from 800g to 1.5 Kg. These were purchased from local market. Four rabbits were male and eight were females. These animals were kept and fed at institute's small animal house during the months from mid January to mid April and randomly labeled as Rabbit-1, Rabbit-2, --- Rabbit-12. Wooden tags were hanged in their necks to maintain the identification. Usual green grass was provided as food. The floor of animal house was filled with natural soil to provide them natural environment. Proper arrangements were made for the ventilation and sunlight. Males were kept separate from females to prevent breeding.

Twenty multiple subcutaneous injections over the back of each rabbit were given. Immunogen emulsified in Freund's complete adjuvant was used for primary injection. In subsequent booster injections Freund's incomplete adjuvant was emulsified with the immunogen. The volume of each injection was around 40 µl. The back of the animal was shaved prior to the injections (Munso and Wallac 1978; Butt *et al* 1983).

A 5ml blood sample was taken 30 days after the primary injection for testing of the antibody titre and at the same time a booster dose (immunogen emulsified in incomplete adjuvant) was given. Further booster doses were given at intervals of 30 days. The animals were finally bled 120 days after primary immunization. The blood was collected from ear vein by rubbing the ear with xylol. The serum was separated and preserved with 1% sodium azide.

Testing of T_4 antisera in rabbits (dilution curve experiments). Antisera from 12 rabbits were collected and tested for anti-T_4 titre by dilution curve experiments. Here 15% cooled polyethylene glycol (PEG) was used to precipitate antigen-antibody complex (Thorell and Larson 1978b; Cheung and Slaunwhite 1976; Desbuquois and Aubach 1971; Chard 1980). Procedural details are given below.

Reagents Prepared. (i). Assay buffer (phosphate buffer); pH:7.5, 0.05M, 1%BSA, sodium azide:0.05%): (a).Disodium hydrogen phosphate 12-hydrate (Na_2HPO_4. $12H_2O$; MW= 359.14), 17.85g, was dissolved in 900 ml distilled water with slight heating. The volume was made up to 1000 ml with distilled water. This was labeled as Solution-I. (b). Sodium di-hydrogen phosphate 2-hydrate (NaH_2PO_4. $2H_2O$; MW=155), 1.95g was dissolved in approximately 200 ml distilled water and was made up to 250 ml. This was labeled as Solution-II.

The pH of solution-I was adjusted to 7.5 by addition of solution II. To it then sodium azide and BSA both in concentration of 0.5gm/litre were added.

(ii). ^{125}I- T_4/ANS solution (≈35000 cpm /50 µl). Diluted the concentrated solution of the tracer using assay buffer to get approximately 35000 counts per 50 ml volume. Added 2 mg per ml ANS (8-aninlono 1- naphthalene sulphonic acid) into this solution.

Thyroxine has very high affinity for plasma proteins especially thyroxine binding globulin (TBG). About 80 % of T_4 circulating in blood is bound to TBG which has a single high affinity binding site for T_4. It is, therefore, necessary to block this binding site to get specific antigen antibody reaction. ANS is therefore used to inhibit this binding of thyroxine to plasma proteins in patient samples. Such binding interferes with antigen antibody complex. Thiomersal (merthiolate, salicylate, tetrachlorothyronine (a halogenated thyroxine derivative), and phenytoin (dilantin) have also been used for this pupose. (Thorell and Larson 1978a, 1978c).

(iii). 15% Polyethylene glycol, MW=6000 (PEG). 15g of PEG was dissolved in 100 ml assay buffer followed by the addition

of 0.1g Triton X100 (Edwards 1983). Polyethylene glycol or PEG is a polymer that soaks up in water and provides a well controlled antibody precipitation system. IgG is precipitated with 15% PEG.

Materials required. (i). Polystyrene assay tubes. (ii). Gamma Counter (Cap-Ria, USA, 16-channel). The counter was coupled with IBM compatible computer for data analysis.

Procedure for testing T_4-antibody titre in rabbits using 15% PEG precipitation: Following dilutions of antiserum were prepared using assay buffer: 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800, 1:25600, 1:512000, 1:1024000, 1:2048000, and 1:4096000. Pipetted out 100 µl of aliquots of these Ab-dilutions into appropriate tubes labelled in duplicate. Duplicate tubes were also prepared for total activity and non-specific binding (no separation of bound from free can be 100% complete and there will always be some residual free antigen concentration associated with the bound fraction. This is non-specific binding which needs to be estimated with considerable degree of accuracy as errors in it's determination have a disproportionate effect on low bound to free ratios in an assay) 100 µl of assay buffer was added in these tubes. Then sequentially following additions were made in each tube. 50 µl of hormone free serum per tube (this is to increase the yield of precipitate). 50 µl of ^{125}I-T_4/ANS solution per tube.

After this all tubes were kept at room temperature (18-20 hrs) and then following day the mixtures were incubated at 4 °C followed by the addition of 0.5 ml of 15% PEG into each tube. After thorough mixing with a vortex mixer, the mixtures were allowed to stand for 15 minutes at 4 °C. Centrifuged the tubes for 30 minutes at 3000 rpm followed by decantation of liquid contents. The precipitates were counted for 90 seconds using a gamma counter and the bound counts were analyzed on the computer.

Preparation of Iodinated Tracers for T_4 (And T_3) RIAs. A variety of methods for iodination of T_3 and T_4 are available in the literature (Butt *et al* 1983). For this study we selected the method described by Edwards *et al* (1985) using Chloramine-T oxidation (Greenwood *et al* 1963) because this method gives tracers of high specific activity. Moreover two tracers (^{125}I-T_3 and ^{125}I-T_4) can be obtained in single iodination experiment followed by purification by gel filtration.

Principle and reaction mechanisms. Radioactive iodine used for labelling is usually supplied in iodide form. The iodide is oxidized to a reactive cation, which attacks aromatic ring of tyrosine. The mechanism of reaction is explained below:-

Radioactive iodine is incorporated into the aromatic ring of tyrosine in two step reaction. In the first step radioiodidide (^{125}I) is oxidized to reactive cation ($H_2O^{125}I^+$) which then attacks on the nucleophilic aromatic ring. The reaction is schematically explained in Fig 3.

The reaction conditions were maintained in such a way that one radioactive iodine atom is incorporated per molecule. The formation of molecules containing more than one radioactive atom may be unstable because of high radiation damage. The final mixture contains a number of species, which needs to be separated. The purification step is therefore essential and is explained in procedures given below.

Methodology of iodination by chloramine-T method. Reagents prepared. (i). Preparation of phosphate buffer (pH 7.5; 0.05M). Procedure of preparation of phosphate buffer was the same as described for preparation of assay buffer except that addition of sodium azide and BSA was excluded.

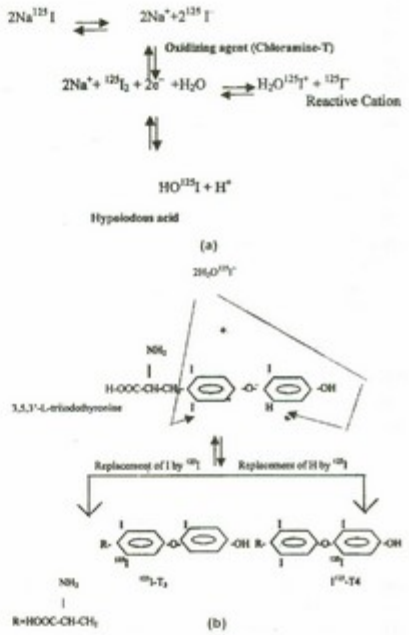

Fig 3. Mechanism of interaction of radioactive iodide with T_3 molecule: (a) oxidation of radioactive iodine by chloramine-T method. (b) Electrophilic attack of reactive cation on aromatic rings.

(ii). *T_3 Solution*, Conc=1 µg/5 µl(0.2mg/ml), pH8.0. 2mg of T_3 was taken in a clean 10 ml graduated beaker or volumetric flask and 4 ml distilled water was added into it. Added 1N NaOH drop wise with stirring until a clear solution was obtained. Diluted the solution to 10 ml with distilled water. Adjusted pH to 8.0-8.5 using 1 M NaH_2PO_4 solution taking care that no turbidity appears.

(iii). *^{125}I-Nal Solution*, Conc=5mCi/50 µl. Provided by the manufacturer (Amerlex International, UK). Centrifuged at 600 rpm for 5 minutes to remove any particles.

(iv). *Chloramine-T solution*, Conc=20 µg/5µl(4 mg/ml) 20mg Chloramine-T was added to 3 ml phosphate buffer pH7.5. Stirred until dissolved. Made up the volume to 5ml.

(v). *Na-Metabisulfite(NaMBS) Solution*, Conc=20 µg/5 µl (4 mg/ml): 80 mg of NaMBS ($Na_2S_2O_5$) was dissolved in 10 ml phosphate buffer (pH7.5) and made up the volume to 20 ml.

(vii). *Potassium Iodide Solution, Conc=10 µg/µl)*. Took 100 mg of KI in 1 ml phosphate buffer (50mM, pH7.5).

(viii). *Bicarbonate buffer*, 50mM, pH9, (MW of $NaHCO_3$=84): 4.2g of $NaHCO_3$ was dissolved in 900 ml of distilled water. pH of the solution was adjusted to 9 with 1M NaOH(40g/l) The volume was made up to 1000 ml with distilled water.

(ix). Preparation of Sephadex G-25 (Superfine) Column. 8 g of sephadex-G25-50(Superfine; Bead Size:20-50µm, (Pharmacia, USA) was taken and swollen up in 25 ml phosphate buffer over 18-24 hrs. Packed in 30 x 1 cm column size.

Equipment and disposables arranged were:-

Single well gamma Counter
Fraction Collector (Frac-300, Pharmacia, Sweden)
Polystyrene tubes
Poly ethylene gloves
Micropippetes (SOCOREX, Germany)

Following reaction procedure was followed:-

Following reagents were added in following quantities into a clean micro test tube in rapid succession.

Phosphate buffer	10 µl
1 mCi ^{125}I-NaI/10 µl	10 µl and vortexed
1 µg T_3/5 µl	5 µl vortexed
20 µg Chl-T/5 µl	5 µl vortex mixed for 20 sec
20 µg NaS_2O_5/ 5 µl	15 µl vortex
1000 µg KI/100 µl	100 µl vortex mix.

Applied the mixtures to a sephadex G-25 column (Column size: 30 x 1cm). Waited for 15-20 minutes to achieve adsorption equilibrium. Eluted the column with 50 mM $NaHCO_3$ (pH9). Collected 2 ml fractions at setting 3 of peristaltic pump (flow rate 14 ml/hr). Counted the fractions using single well gamma counter. Plotted counts of above fractions (Table 1) versus tube number

Table 1
T_4 levels in the dialysis mixture

	T_4 concentration (nmol /l)	Recovery
Calculated value (before dialysis)	322	——
Measured value before dialysis	264±18(SD)	82%
Measured value after dialysis	199.5±17	76%

(radiochromatogram) and identified three peaks (Fig-9). The first was unreacted ^{125}I-peak, the second being ^{125}I-T_3 peak and the last one is ^{125}I-T_4 peak. Pooled the desired peak fractions containing radioactive T_3 and T_4. To each added 0.01 N HCl dropwise until pH was 7.5. Diluted each pool to radioactive concentration of 5-10 µCi/ml, adding phosphate buffer, containing 1% albumin, 2% manitol and 0.1% cystine.

For preparation of working ^{125}I-T_4 solution or tracer, diluted the concentrated solution of the tracer using assay buffer to get approximately 35000 counts per minute per 100 µl volume. Added 2 mg per ml of T_4 inhibitor, ANS into this solution.

Conditions for an ideal tracer. As already stated many methods of iodination are available but chloramine-T (Greenwood *et al* 1963) is the most widely used iodination procedure. The principles and requirements of the iodination reaction have been reviewed (Hunter 1973) and optimum conditions for some steps in the reaction have been investigated. An ideal tracer would be a homogenous preparation of the antigen which has been labeled without loss of affinity for the antibody being used. Methods are available for checking the heterogeneity of the tracer, (Bolton 1977; Bolton and Hunter 1973) which results due to changes in the antigen. The changes in the antigen may result due to (a) the presence of the label (^{125}I), (b) chemical changes resulting from exposure to reagents used in the labelling, such as the oxidant and reducing agent. (c) Handling and exposure to microgram quantities of highly purified protein and unprotected by carriers. (d) radiation damage. Generally unexpected low binding in an assay indicates a damaged (heterogenous) tracer.

Optimization of T_4-antibody quantity for use in T_4 RIA. The most important step in designing a radioimmunoassay is to determine the antibody concentration, which gives an assay system of required sensitivity and precision in the desired clinical range. The experimental set up for this purpose would first require selection of optimum quantity of radiotracer, which is another parameter to adjust the sensitivity of an assay system.

Optimal concentration of radiotracer in our set up. Optimal amount of radiotracer would be that which could ideally saturate all the binding sites of the antibody at optimal dilution. However, practically this is not possible. Efforts were therefore made to establish optimized conditions (Hunter 1983) We have observed that simple arithmetic assessments are often very useful in selecting the optimum concentration of radiotracer. Following was our strategy to prepare workable solution of radiotracer for our assays.

The matter was started from expected or desired clinical which was decided on the basis of normal range reported in literature. In T_4, we were interested in setting up an assay for levels within, above and below the normal range. Following calculations were therefore performed .

Expected normal range (Thorell and Larson 1978c) = 45-120 ng/ml (58-154 nmol/l)

So the desired working range of the assay may be= 10-500 ng/ml (this should normally cover hypo, hyper and euthyroid subjects)

For a sample of volume, 50 µl, 10 ng T_4 /ml will correspond to 0.5 ng T_4 /50 µl (500pg/50µl or 10000 pg/ml).

This means we need to add 500 pg of ^{131}I-T_4 or tracer into each tube in our assay system.

We therefore require a solution of concentration, 0.5 ng ^{125}I-T_4/50 µl.

For a specific activity (see iodination section) of 50 µCi/µg or 60 nCi/ng, this will correspond to 25 nCi ^{125}I-T_4/50 µl. The expected count rate can therefore be calculated as under.

25 nCi = 3.7 x 10 x 25 dps= 55500 dpm

cpm = dpm x efficiency

For a gamma counter of 70% efficiency,

55500 dpm x 0.70 = 38850 cpm.

Thus for our assay system we are interested in, we need to prepare a tracer of concentration, approximately 30000-40000 cpm/ 50 µl to get precise results in the region of desired working limit. This concentration, however, is an estimate and the actual concentration may be different because as already described there are many factors which lead to the reduced binding. These are related to the heterogeneity of the tracer. Rate of radioactive decay must also be taken into account.

Optimal concentration of antibody: Ideally an optimum concentration would be that which is completely saturated by the amount of tracer we have selected and prepared. However, in practical, this can not be achieved because the antigen-antibody reaction is affected by a number of factors (Thorell and Larson 1978c). For example in many antigen-antibody systems primary reaction is followed by secondary reactions. The primary antigen-antibody complexes join together and form aggregates (and these aggregates having large lattices reduce their solubility in water which cause them to precipitate. Other secondary effects of antigen antibody reaction are agglutinations and cell toxic reactions.. In RIA it is assumed that only the primary reversible antigen antibody reaction occurs (except in separation with double antibody) and follows the law of mass action (Here an equilibrium between concentrations of the reactants (antigen and antibody concentrations) and the product (the antigen-antibody complex) is achieved. The optimal concentration of antibody would therefore be that which leads to the equilibrium and gives minimum error in the results. It can also be determined from dilution curve experiment. The procedural details are given below.

Dilution curve experiment. Briefly, following dilutions of antiserum (rabbit-1) were prepared in assay buffer: 1:50; 1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800 and aliquoted in two sets of tubes for determination Bo and Bx. 100 µl of Ab-dilutions were aliquoted into appropriate tubes except NSB tubes where 100 µl of assay buffer was added. Other reagents were 50 µl of ^{125}I-T_4/ANS (≈35000 cpm) into each tube, 50 µl of hormone free serum into each tube of set-1, 50µl of T_4 -standard of conc. 20 nmol/l into each tube of set-2. The mixtures were vortexed and incubated at room temperature overnight. 0.5 ml of 15% PEG was added at the end of incubation. The tubes were finally centrifuged for 30 minutes at 3000 rpm, decanted and counted the precipitate for 90 seconds.

Production of sheep anti-rabbit serum.SARS (2nd antibody) for use in PEG assisted double antibody precipitation: Principle of PEG assisted double antibody precipitation. The non-specific binding is high when we use 15% PEG to separate antigen-antibody complex which is generally associated with significant misclassification errors, variable behaviour of damaged tracer, serum effects and interference in primary antigen-antibody reaction (Obell 1980; Chard 1980). This much non-specific binding could affect the working range of the assay. The precipitation with second antibody could overcome these difficulties. This increases specificity of separation of antibody, and hence the bound fraction, from the free fraction. At the end of the incubation, an anti-IgG antibody is added that is specific to the species in which the primary antibody was raised. The divalent nature of antibodies is responsible for the formation of a lattice consisting of cross-linked antibodies and analyte. More than one molecule of antibody may be bound to each molecule of analyte, at different epitopes, each molecule of primary antibody may bind to two molecules of analyte, and each molecule of second antibody can bind to two molecules of primary antibody. The lattice of cross linked molecules

precipitates and separation is achieved centrifugation and decantation. PEG assisted second antibody precipitation combines the benefits of second antibody and PEG methods: specificity and speed. The PEG which is used at lower concentration of 4%, hastens the precipitation of the cross linked matrix (Wild and Davies 1994).

Second antibody is produced by immunizing a different animal species with immunoglobulin G (IgG) purified from the species in which the first or primary antibody was raised, giving an antiserum capable of precipitating the first antibody. In our case we raised primary antiserum in rabbits. We therefore immunized sheep with rabbit IgG.

Preparation of immunogen.Reagents Prepared/procured. Immunogen. (Rabbit IgG, Sigma, USA). IgG fractions of non-immune animal sera were prepared by the manufacturer by caprylic acid fractionation as described by Steibuch and Audran (1969) followed by DEAE ion-exchange chromatography . Immunogens thus prepared were assessed for homogeneity by immunoclectrophoresis as described by Erlanger *et al* (1959).

Adjuvants.(i) Freund's complete adjuvant: This was used at the time of primary injection. Standard Scottish antibody production (SAPU) protocol (Chapman *et al* 1983) was used i.e., 0.1 mg IgG in 2.5 ml phosphate buffer was emulsified in Freund's complete adjuvant and administered by multisite intramuscular injections. The total volume of emulsion was 5 ml. It was injected at 10 sites.

(ii). Freund's incomplete adjuvant. In booster injections same procedure was used but Freund's complete adjuvant was replaced with Freund's incomplete adjuvant. Booster injections were given at 1 month interval. The injection sites were near the primary injection sites.

Procedure of making the emulsion. Rabbit IgG was dissolved in phosphate buffer to make a concentration of 0.25 mg/ 2.5 ml. One ml of this solution was mixed with Fruend's adjuvant using the procedure described for anti-T_4 (and anti-T_3) antibody production.

Animals, route and schedules of injection. The choice of animals for production of second precipitating antisera is governed by several criteria. First, since the bulk of first antibodies have been reported to be raised in guinea pigs, rabbits, sheep or goats, other species are required for precipitating sera. Second, precipitating antisera are usually of low titre, operating at initial dilution of 1:50, so requiring the provision of large volumes of antisera and consequently the use of large animals. These criteria are usually satisfied by the use of donkeys, sheep or goat. Large and mature animals are therefore recommended (Steibuch and Audran 1969; Erlanger *et al* 1959). In our study we used two sheep, kept at MINAR's animal house. The animals were fed on usual green grass and grains (gram seeds). Two to three litre bleeds were taken within seven days after each boost . Blood was collected from jugular vein by venepuncture after each booster injection. The serum was separated and preserved with 1% sodium azide. Unfortunately one sheep died in the beginning because of disease and study could only be continued in one sheep.

Testing of second antibody in sheep and it's optimization (dilution curve experiments). As discussed in production of primary antibody optimum dilution of the second antibody is also required to set up good quality assays if we are employing second antibody precipitation technique to separate free and bound fractions. The design of dilution curve we followed was similar to that used for dilution experiment for primary antibody. An optimized quantity of non-immune serum from the appropriate first antibody species is normally added to increase the precipitate and simplify the separation.

Reagents prepared. (i) *Assay buffer (phosphate buffer);* pH:7.5, 0.05M, 1%BSA, sodium azide(0.05%) (ii) ^{125}I-T_4/ANS solution (»35000 cpm /50 µl). Diluted the concentrated solution of the tracer using assay buffer to get approximately 35000 counts per 50 ml volume. Added 2 mg per ml ANS into this solution. (iii) 4% Polyethylene Glycol, MW=6000 (PEG). Dissolved 4 grams of PEG in 100 ml assay buffer. (iv). T_4 solution of concentration, 20 ng/ml.

Materials arranged. (1) Pure sheep anti-rabbit serum (SARS). (2) Non-immune rabbit serum (3) Polystyrene tubes (4) Assay buffer (5) Gamma counter.

Procedure. Following dilutions of sheep anti-rabbit were prepared in assay buffer: 1:2 1:4; 1:8; 1:16; 1:32; 1: 64; 1:128; 1:256; 1:512. As previously described two sets of tubes were prepared for setting up optimum dilution experiment. Added 50 µl of ^{125}I-T_4 (»35000 cpm) into each tube. Added 50 µl of assay buffer into each tube of set-1 and then 50µl of T_4-solution of conc.20 ng/ml into each tube of set-2. All of these were vortexed. 50 µl of non-immune rabbit serum was also added into each tube of both the sets.The mixtures were kept at room temperature overnight (18-24h) followed by an additional incubation of 1 hr at 4 °C. 0.5 ml of 4% PEG was finally added to precipitate the complex. The mixtures were finally centrifuge for 30 minutes at 3000 rpm, decanted and counted.

Preparation of working T_4 standards. Standard material was prepared in a way similar to that described for T_3. The working standards were prepared in hormone free serum . A stock standard of concentration 643.5 nmol T_4/l was prepared from a solution of T_4 of concentration, 1287 nmol/l (1mg/ml)

using assay buffer as diluent (Chapman *et al* 1983; Jerne and Perry 1965; Malan *et al* 1983). This stock was further diluted to concentrations 321.5, 160.8, 80.4, 40.2, 20.1, and 10 nmol T_3/l respectively using T_3 free serum. T_3 free serum was also used as zero or control standard.

Quality control sera. Quality control sera of low, medium and high concentration were purchased from Amersham, U.K. The range of expected values as supplied by the manufacturer is given in Table 2. (Chapman *et al* 1983; Jerne and Perry 1965; Malan *et al* 1983).

T_3-Radioimmunoassay using PEG assisted 2nd antibody precipitation: Assay design and protocols. Reagents and Other Items Required: (1) Standard Solutions. Prepared above (2) Anti T_3-antibody at dilution 1:8000 (3) ^{125}I-T_3 solution; conc. ≈34000cpm /50µl-prepared above (4) Anti-rabbit antibody or second antibody—The pure anti-serum was diluted using assay buffer to a dilution of 1:60 (5) 4 % PEG in assay buffer. (6) Polystyrene assay tubes (1cm x 6cm). (7) Well type gamma counter

Assay design. Labelled the assay tubes in following sequence.

Tube No.	Specification
1-2	Total activity tubes (T)
3-4	Non specific binding tubes(NSB)
5-6	0.0 nmol/l Calibrator (C1)
7-8	10 nmol/l Calibrator (C2)
9-10	20.1 nmol/l Calibrator (C3)
11-12	40.2 nmol/l Calibrator (C4)
13-14	80.4 nmol/l Calibrator (C5)
15-16	160.8 nmol/l Calibrator (C6)
17-18	321.5 nmol/l Calibrator (C7)
19-20	QC-1 (Quality control serum of low concen tration)
21-22	QC-2 (Quality control serum of medium con centration)
23-24	QC-3 (Quality control serum of high concen tration)
25-26	Sample-1, placed a total of 30 samples in dupli cate until tubes 83-84
85-86	QC-1
87-88	QC-2
89-90	QC-3
91-92 - 93-94	Sample-31, placed a total of 30 samples in dupli cates until tubes, 139-140
⋮	
139-140.	
141-142	QC-1
143-144	QC-2
145-146	QC-3

Assay Procedure. Added 50 µl of standard, unknown and control sera into appropriate tubes. 50 µl of rabbit anti-T_3 antibody solution was added into all tubes except totals and NSB tubes where 50 µl assay buffer was added. 50 µl of ^{125}I-T_3 (50000cpm; ANS=1mg/ml), 50 µl of normal rabbit serum (NRS) and 50 µl of second antibody were then added into each tube. Incubated the mixtures at room temperature overnight (18-24h). 0.5 ml of 4% PEG at 4 °C was added at the end of incubation. Vortexed and allowed the mixtures to stand for 15 minutes at 4 °C and then centrifuged the contents. Counted the precipitates (bound fraction) for 90 seconds.

Data analysis. The data analysis philosophies of Dudley (1981), Rodbard *et al* (1978) and Ekins (1978-81) were followed. The programs developed by WHO, were used for data analysis of T_3 assay.

Results and discussion

Production of anti-T_3. Conjugation. The T_3 levels measured before and after dialysis are given in Table 1. These show that about 76% of the added T_3 was coupled to the albumin and retained in the reaction mixture. The T_3-albumin conjugate concentration in the final mixture was approximately 1.50 mg/ml. The quantity produced was therefore sufficient for a number of immunizations. The low recovery compared to the expected values may be due to experimental errors and impurities in the commercial preparations.

Antibody titres. The antibody dilution curves of some of the rabbits obtained from sera of different rabbits at 5 weeks intervals are shown in Figs 4-6. The antibody titres observed at 50% tracer binding are given in Table 3. Studies in all rabbits could not be continued because of poor response and mortalities due to disease and unfavourable climate.

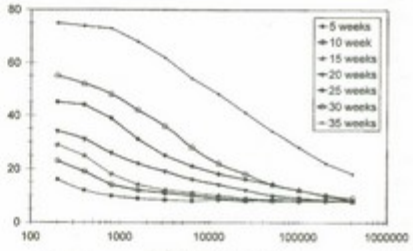

Fig 4. T_3 assay: Rabbit-1. Antibody dilution curves. Blood samples collected at 5 weeks intervals.

Table 2
Typical assay data obtained on a T_4-RIA, applying second antibody precipitation

Tube No	Specification	CPM1	CPM2	Mean	%CV	%B/T
1-2	T	34551	34041	34296	1.1	—
3-4	NSB	589	635	612	5.2	1.8
5-6	C1 (0.00)	21041	20505	20763	1.8	60.5
7-8	C2 (10)	18040	18120	18080	0.3	53.83
9-10	C3 (20.1),	15340	14944	15142	1.8	44.2
11-12	C4 (40.2)	11449	11528	11488	0.5	33.5
13-14	C5 (80.4)	7899	8010	7954	1.0	23.2
15-16	C6(160.8)	4500	4360	4430	2.2	12.9
17-18	C7(321.5),	2650	2580	2515	1.9	7.6
19-20	QC1	14690	14898	14794	1.0	43.1
21-22	QC2	8424	8563	8493	1.1	24.8
23-24	QC3	4723	4680	4702	0.6	13.7
25-26	U-1	2961	2995	2978	0.8	8.68
27-28	U-2	3072	3184	3128	2.5	9.12
29-30	U-3	4911	4345	4628	8.7	13.5
31-32	U-4	9583	9425	9504	1.2	27.7
33-34	U-5	5443	4996	5469	0.7	15.95
35-36	U-6	14876	14379	14628	2.4	42.65
37-38	U-7	6747	7003	6876	2.6	20.05
39-40	U-8	7477	7413	7445	0.6	21.70
41-42	U-9	9711	9824	9767	0.8	28.5
43-44	U-10	3817	4048	3933	4.2	11.47
45-46	U-11	14752	14791	14772	0.2	43.07
47-48	U-12	7229	7615	7423	3.7	21.64
49-50	U-13	6430	6592	6510	1.8	18.99
51-52	U-14	18355	17995	18174	1.4	52.99
53-54	U-15	7029	7073	7051	0.5	20.56
55-56	QC1	14743	14958	14850	1.0	43.3
57-58	QC2	8291	8473	8382	1.5	24.4
59-60	QC3	4726	4694	4710	0.5	13.7
61-62	U-16	9311	9364	9338	0.4	27.3
63-64	U-17	6533	7041	6787	5.3	19.79
65-66	U-18	4751	5043	4897	4.2	14.28
67-68	U-19	3254	3232	3242	0.5	9.46
69-70	U-20	3704	4016	3878	5	11.31
71-72	U-21	7697	7439	7569	2.4	22.07
73-74	U-22	16314	16067	16190	1.1	47.21
75-76	U-23	6926	6704	6815	2.3	19.87
77-78	U-24	8359	8873	8615	4.2	25.12
79-80	U-25	9533	9287	9409	1.8	27.44
81-82	U-26	8334	8520	8894	1.6	24.57
83-84	U-27	9159	9245	9200	0.6	26.83
85-86	U-28	7611	7962	7790	3.2	22.71
87-88	U-29	7686	7425	7554	2.4	22.03
89-90	U-30	6971	7332	7151	3.6	20.85
91-92	QC1	14530	14642	14586	0.5	42.5
93-94	QC2	8150	8050	8100	0.9	23.6
95-96	QC3	4360	4440	4400	1.3	12.8

The results of antibody titre varied in rabbits. Rabbit-1, 1kg, male, actively responded to the immunogen (Fig 4). The response was very steep after fourth boost. A maximum titre of 1: 9700 was observed after 35 weeks.Rabbit-2 responded excellently but could not survive after 2nd booster injection although the titre at that time was 1:8300 (this seemed to be showing the most active response). Rabbits-3 responded positively but could not live long. Rabbit-10 (1.5Kg, female) showed a very good response (titre, 1:7000). Rabbit-12 (1.1Kg female) also responded very well (Fig 5) and the observed titre after 7th boost was 1:9200 Studies on other rabbits could not be continued because some of them died and others did not respond to the immunogen very well. Response in some of the rabbits was very low e.g., rabbit-6 (Fig 6). All this suggests

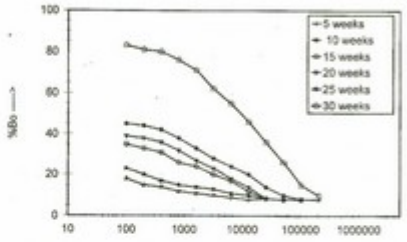

Fig 5. T_4 assay: Rabbit-12. Antibody dilution curves. Blood samples collected at 5 weeks intervals.

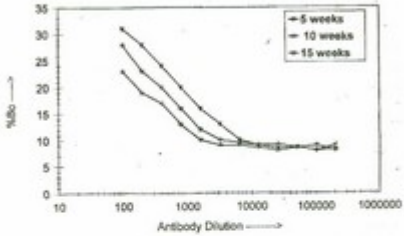

Fig 6. T_4 assay: Rabbit-6. Antibody dilution curves. Blood samples collected at 5 weeks intervals.

Table 3

T_4-Antibody titres in rabbits determined 35 weeks after the primary injection

Rabbit Identification	Weeks after primary injection	Titre*	Remarks
Rabbit-1, male, 1.5 Kg body weight	35	1:9700	Continued to live for two years when it was finally bled. It gave high quantities of antibody, which could be useful for future work.
Rabbit-2, male, 1.2 Kg body weight	10	1:8300	Gave good response but died after 10 weeks.
Rabbit-3, female, 1.0Kg body weight	15	1:2500	This rabbit showed a good response to the immunogen and developed significant quantities of antibodies but died because of sickness.
Rabbit-4 male, 1.0 Kg body weight	15	1:300	Could not survive
Rabbit-5 female, 1.0 Kg body weight	10	low	Could not survive long.
Rabbit-6, female ,1.6 Kg body weight	10	low	Could not survive
Rabbit-7, male ,1.6 Kg body weight	5	low	Responded very low
Rabbit-8. female ,1.6 Kg body weight	15	low	Response very low
Rabbit-9, female 800 g body weight	10	low	Could not survive
Rabbit-10, female 910 g body weight	35	1:7000	Good response
Rabbit-11, female 860 g body weight	5	low	Response poor
Rabbit-12, female 895 g body weight	30	9200	Good response

*Titre is defined as the dilution at 50% binding of tracer with the antibody.

that at least four rabbits had responded very well to the immunization. Serum from all the test bleeds was collected and mixed with sodium azide (to prevent bacterial growth) and stored at −20 °C. Antibody of Rabbit-1 was first used for future experimental work. The very high titres of the antibody suggest that the home made antibody could be sufficient for millions of tests.

Optimization of antibody concentration. The dilution curve obtained by plotting bound counts in two sets of tubes against antibody dilution (rabbit-1) is shown in Fig 7. Bo Curve shows the binding of the tracer to the antibody in the absence of any unlabelled T_4. It can be appreciated that about 50% of the tracer is tagged to the antibody at the point of maximum binding. The Bx curve i.e., the curve obtained in the presence of unlabelled T_4 splits well from the Bo curve in the middle which suggests that the avidity of the antibody is high and a sensitive assay can be developed from this antibody.

An error curve can be constructed from these two curves by plotting the reciprocal of the difference of counts between two curves at various dilutions against the dilution of the primary antibody. (The ratio Bx / (Bo-NSB) can also be plotted against the antibody dilution, as we have done in T_3 assay). The curve thus obtained is shown in Fig 8, which depicts that the antibody will work very well from dilutions 1:1000 to 1:10000. The minimum error point is around 1:8000, which was selected as the optimum antibody dilution. This confirms that there were significant quantities of antibody in our rabbit serum and that many optimum assays could be developed using this cheap locally developed antibody. According to our initial estimates the quantity of serum we have collected could be sufficient for millions of tubes. (i.e., 1 CC serum will be sufficient for about 160000 tubes).

In conclusion the local techniques were successful in producing antibody of high titre which is very sensitive to low concentrations of T_4. This antibody could develop many low cost assays.

Preparation of iodinated tracers for T_4 (and T_3) RIA. The radiochromatogram obtained after purification through sephadex is shown in Fig 9. The analysis of counting data (Table 1) showed that 31% of the total activity appears as ^{125}I-T_3, 50% as ^{125}I- T_4 and 15% as free iodide. 4% of total activity is lost as contaminations. The specific activities of these tracers calculated from counting data of iodination experiments were: T_3= 300-400 μCi/ mg , T_4= 50-60 μCi/ mg.

Production of second antibody. Antibody titre. The dilutions observed in sheep after booster injections are shown in Table 4. The maximum dilution, which we obtained, was 1:60. The sheep did not respond more after 4th booster.

Optimization of second antibody. The dilution curves obtained after analysis of counting data of one of our experiments on optimization of second antibody is shown in Fig 10.

The system seems to be very sensitive to T_4 and there is a very good splitting of the two curves near the maximum binding. If we correlate this splitting (i.e., the reciprocal of the

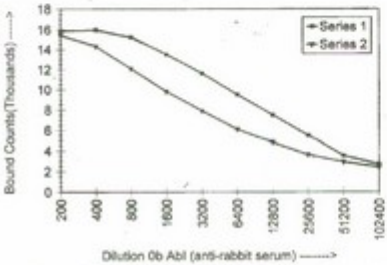

Fig 7. T_4 assay: Optimization of primary T_4 antibody (Bo and Bx against antibody dilution).

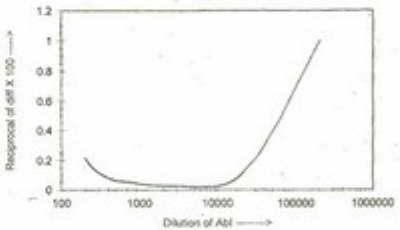

Fig 8. T_4 assay: Testing of anti-rabbit serum for optimum antibody dilution by 15% PEG precipitation.

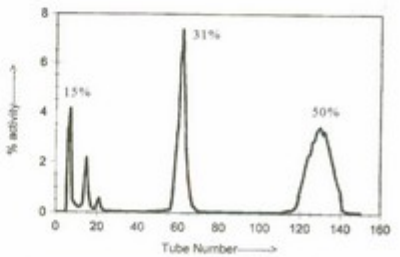

Fig 9. Radiochromatogram of radioiodination products obtained after purification through sephadex column. Radiochromatogram

difference in the count rate of the two curves X100) with the antibody dilution, we get another curve shown in Fig 11. Here the lowest point indicated the dilution where the sensitivity of the system will be highest. This may also be called the minimum error point. As can be read from the curve the dilution of second antibody at this point is 1:60. This is therefore the working dilution of the second antibody.

T_4-Radioimmunoassay using PEG assisted 2nd antibody precipitation . Typical assay data obtained using above protocol is given in Table 2. The assay statistics and curve parameters obtained using WHO data reduction programs are given in Table 5.

The standard curve plotted using the counting data obtained in above protocol is shown in Fig 12. The curve parameters show that the standard data is perfectly fit in logit-log system of coordinates. The non-specific binding is about 2%. This is consistent with findings of others (Edwards 1983; Edward *et al* 1985). The use of second antibody therefore improved the separation in our T_3 and T_4 assays. The response error relationship and precision profiles are shown in Fig 13 and 14 respectively. The curves show more than 7% error in the concentrations below 10 nmol/l. The system is also performing well at higher concentrations and has therefore a very wide working range.

The observed values of quality control sera are given in Table 6. The values are within the expected range. Mean within assay drift is acceptable. The correlation of the values using this in house technique with those obtained using commercial kits is shown in Fig 15. The assay correlates (Correlation Coefficient=0.99) well with the commercial techniques.

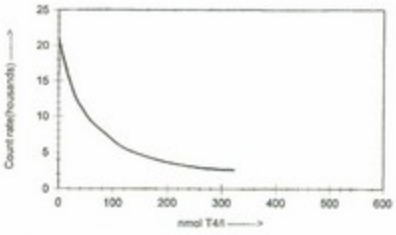

Fig 12. T_4 RIA standard curve (double antibody precipitation).

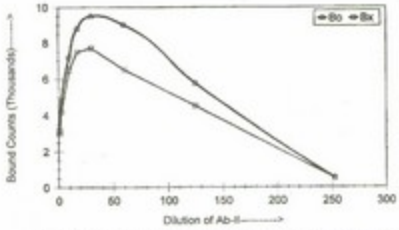

Fig 10. T_3 assay: T_4 optimization of second antibody. Total counts = 28835 NSB=228

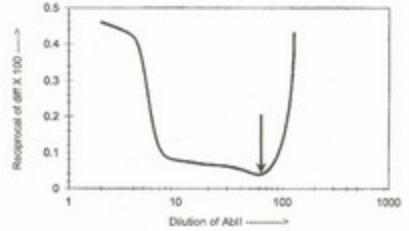

Fig 11. T_4 assay: Optimization of second antibody. The error curve. Total counts = 28835 cpm NSB = 228

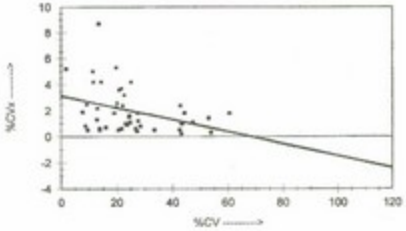

Fig 13. T_4 RIA (double antibody method): Response error relationship.

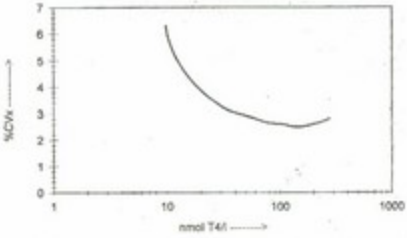

Fig 14. T_4 RIA (double antibody). Imrecision profile.

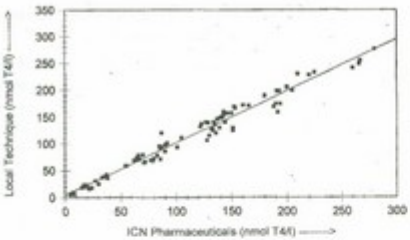

Fig 15. Correlation of local T_4 values (double antibody assay) with commercial kit. Corr. coeff = 0.99, Regression: a = 4.37 b = 0.97.

Table 4
Anti-rabbit antibody titres in sheep determined in first four months after primary injection

Months	First	Second	Third	Fourth
Antibody titre	1:20	1:30	1:40	1:60

Table 5
Double antibody T_4-assay: Overall assay statistics

Assay counting statistics	Minimum %CV=0.2
	Maximum %CV=8.7
	Mean %CV=1.99
	Median %CV=1.5
RER parameters	A=3.15
	B=-0.046
Curve parameters	Logit-Log corr. coefficient= -0.9947
	Logit-log slope = - 1.151
	Logit-log intercept = 4.553
	ED50=53.82
	ED95=4.109

Table 6
Double antibody T_4-RIA. Expected and observed values of quality control sera

Specification	Expected Values (nmol T_4/l)		Observed Values (nmol T_4/l)
	Mean	Range	Mean
QC-1 (Low control)	20.7	16.5-24.9	24.1±0.63
QC-2 (Medium)	68.3	54.6-82	75.3±1.5
QC-3 (High)	177	141-213	162.5±3.2

To summarize, the locally developed PEG assisted double antibody assay system was found to be working very well at all clinical regions (hypo, hyper, normal). The assay precision was excellent and the system seemed to fit well on all Standards of clinical assessment.

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