CHAPTER II

MATERIALS & METHODS

Materials.

A. Instruments:

1. Automatic refrigerated centrifuge, Sorvall, superspeed, RC-2B, USA.
2. Automatic refrigerated centrifuge, Sorvall, RC-3, USA.
3. Philco-Ford refrigerator, no frost, USA.
4. Upright freezer, Rosenlew, Finland.
5. Automatic Gamma Scintillation Counter, Model 1280, LKB Ultragamma, Sweden.
6. Water bath with shaker, controlled temperature at 37±2 C, Gallenkamp, England.
7. Hepaire, laminar flow, Model 5'EC, England.
8. Blood volumeter, Model THC-3, Throcomp Elscint.
9. Analytical balance, Type H-16, E. Mettler, Zürich, Switzerland.
10. pH-meter, Model 7030, Electric Instrument Ltd., Surrey, England.
11. Vortex mixer, Lab Line Instruments, Inc., Melrose Park, Illinois, USA.

12. Digester, 1007 Digester DS 6, Tecator, Sweden.
13. Distilling Unit, 1002 Distilling Unit, Tecator, Sweden.
14. Magnetic Stirrer-plate, Sybron Corporation, Nuova 7, USA.
15. Glass-to-glass homogeniser, Jencons, USA.
16. Burette, diameter 1 x 30 cm, NS 12, Bistabil, W.Germany.
17. Minicolumn with diameter 0.8 x 16 cm, Catalog. No. NEA-050, England.
18. Strong metal sieve (1.5 mm.mesh).
19. Scalpels, forceps, glass pestle and glass plate.
20. 10 x 75 and 10 x 100 mm. glass tubes, Kimble products, USA.
21. Automatic pipettes; 50, 100, 200 µl and 1 ml., Medical Laboratory Automation, Inc., Mount Vernon, N.Y. 10550, USA.
22. Disposable pipette tips for 25-100 µl, 200 µl and 1 ml, Medical Laboratory Automation, Inc., Mount Vernon, N.Y. 10550, USA.
23. Disposable micropipettes; 5, 10 and 20 µl, Yankee, Micropet, USA.
24. Sephadex G-100-40 Ultrafine (Sigma Chemical Company, USA) was soaked in NaCl/Tris/BSA buffer, pH 7.5 overnight before packing the column.
25. 1% BSA in NaCl/Tris/BSA buffer, pH 7.5
26. Human Thyroid tissues were collected from operation room, Department of Surgery and autopsy room, Department of Pathology and Department of Forensic Medicine.

B. Experimental reagents:

1. Highly purified hormone for radioiodination: bTSH (approx. 25 I.U.bTSH/mg), 114 µg/vial, was generously donated by Dr.J.G.Pierce, University College of Los Angeles, School of Medicine, Los Angeles, California, USA., dissolved in 0.228 ml TSH buffer to give a concentration of 5 µg TSH per 10 µl and immediately stored frozen at -20°C.

2. Standard bTSH for standard curve (MRC 53/11): 1.48 I.U. bTSH/tab, was available from the World Health Organization and 4 tabs of standard bTSH were dissolved in 1.15 ml TSH buffer to give a concentration of 128 mIU per 100 µl. This standard solution was aliquoted (100 µl) and stored at -20°C.

3. Phosphate Buffer Saline (PBS): 3.5814 g of disodium hydrogen phosphate 12 hydrate, 8.766 g of sodium chloride (E.Merck, Darmstadt, Germany) and 0.2 g of sodium azide (Fisher Scientific Company, USA.) were dissolved in 800 ml distilled water, adjusted to a pH of 7.8 with diluted hydrochloric acid and then made up to 1 litre with distilled water.

4. TSH buffer: 0.5% BSA in PBS.

5. $Na^{125}I$ for protein iodination (IMS.30), 100 mCi/ml, specific activity of 16.85 mCi/µgI was purchased from Radiochemical Centre, Amersham, England.

6. Iodogen Coated tubes: Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril, Pierce Chemical Co., Rockford, IL. USA.) 1 mg was dissolved in 25 ml Chloroform and 30 µl was aliquoted into iodination tube. They were allowed to dry by blowing with gentle stream

of nitrogen gas (approx. pressure 5 kg/cm^3).

7. Homogenisation Buffer or Tris buffer, 10 mM Tris-HCI, pH 7.5: 0.79 g of Tris (hydroxymethyl, aminomethane hydrochloride, Sigma Chemical Company, USA.) was dissolved in 400 ml of distilled water, adjusted to a pH of 7.5 with diluted sodium hydroxide, made up to 500 ml with distilled water and stored at $4^{\circ}C$.

8. Assay Buffer or NaCl/Tris/BSA Buffer, 40 mM NaCl, 10 mM Tris-HCl, pH 7.5, containing 0.1% W/V bovine serum albumin (BSA): 1.1688 g of sodium chloride (E.Merck, Darmstadt, Germany) and 0.79 g of Tris-HCl (Sigma Chemical Company, USA.) were dissolved in approximate 400 ml distilled water and adjusted to a pH of 7.5 with diluted sodium hydroxide. 500 mg of BSA (Sigma Chemical Company, USA.) was added, made up to 500 ml with distilled water and stored at $4^{\circ}C$

9. 50 mM Phosphate Buffer, pH 7.5 : 3.5490 g of disodium hydrogen phosphate anhydrous (Mallinckrodt Inc., USA.) were dissolved in 400 ml distilled water, and the volume was filled up to 500 ml with distilled water (solution I). 0.975 g of sodium dihydrogen phosphate 2-hydrate (May & Baker Ltd., Dagenham, England) was dissolved in 100 ml distilled water and was filled up to 125 ml with distilled water (solution II). The pH of solution I was adjusted to 7.5 by addition of solution II.

10. Coating Buffer, pH 7.4: 7.9 g of sodium chloride, 5 g BSA and 3.8 g disodium ethylenediaminetetra-acetate (May & Baker Ltd., Dagenham, England) were dissolved in a mixture of 800 ml distilled water and 100 ml of 0.1 M phosphate buffer, pH 7.6. The pH was adjusted to 7.4,

made up to 1 litre with distilled water and stored at 4°C.

11. Elution Buffer, 2M NaCl, 1 g BSA/1 : 5.844 g of sodium chloride were dissolved in 40 ml distilled water and 0.05 g of BSA was added on the surface. The volume was adjusted to 50 ml with distilled water and stored in refrigerator.

12. 50 mM Sodium bicarbonate: 0.042 g of sodium bicarbonate (E. Merck, Darmstadt, Germany) was dissolved in 10 ml 50 mM phosphate buffer, pH 7.5 and prepared freshly before use.

Methods

The methods are divided into 4 parts:

A. Preparation of thyroid membranes: Human thyroid tissue was transfered on ice to laboratory cold room (4°C). Complete membrane preparation was carried out at 4°C, using the method of Kermode et al. (1981)[86]. Fat and connective tissue were removed from the portion of thyroid tissue which was then weighed, chopped finely with cold scalpel and squeezed the cellular tissue through a cold sieve with glass pestle into beaker, adding drops of Tris-HCl buffer at times to keep the tissue cool. The sieved tissue was homogenised in 5 volumes of Tris-HCl buffer by ten up=and=down strokes by means of a tight-fitting all glass to glass homogeniser. The homogenate was centrifuged at 800 g for 5 min at 4°C and recentrifuged the supernatant fractions at 25,000 g for 20 min at 4°C. This pellet was then resuspended in NaCl/Tris/BSA buffer, pH 7.5 to give a membrane concentration of 0.15 g equivalent/ml (defining 1 g equivalent thyroid membrane is that prepared from 1 g weight of

chopped thyroid tissue). Aliquots of thyroid-membrane resuspension (2 ml/tube) were stored at -20°C until assayed.

B. Radioiodination of TSH: Highly purified bTSH was iodinated with $Na^{125}I$ by iodogen method[87,88]. The procedure was performed inside the laminar flow carbinet at room temperature. Thirty to forty µl of normal saline was added to a prealiquoted 5 µg bTSH/10 µl, and mixed gently by bubbling air through the micropipette. This bTSH solution was carefully transfered to the bottom of iodogen coated tube and 0.5 mCi $Na^{125}I$ was added and mixed throughly for 2 min. The iodination reaction was stopped by adding of 200 µl 50 mM sodium bicarbonate and mixed well for another 5-10 min. The reaction products were chromatographed on a 1 x 25 cm column of Sephadex G-100-40 in NaCl/Tris/BSA buffer. Fifty to Sixty fractions of 0.5 ml were collected in tubes containing 1% BSA in NaCl/Tris/BSA buffer, pH 7.5. The ^{125}I-labelled TSH peak fraction was kept frozen at -20°C for up to 4-5 weeks. Specific activity of ^{125}I-TSH and yield of iodination were determined by the following equation

$$\text{Specific activity} = \frac{\text{Radioactivity in } ^{125}\text{I-TSH peak}(\mu\text{Ci})}{\text{Weight of hormone } (\mu\text{g})}$$

$$\text{Iodination percentage} = \frac{\text{Radioactivity in } ^{125}\text{I-TSH peak counts}}{\text{Total radioactivity counts in all fractions}} \times 100$$

C. Receptor Purification of ^{125}I-labelled TSH: Before using in radioreceptor assay, ^{125}I-labelled TSH was subjected to repurification by receptor adsorption. Thyroid membranes (300 mg-equiv.) were precipitated by centrifugation at 25,000 g for 10 min at 4°C and then the membrane pellet was resuspended with 200 ng ^{125}I-labelled TSH in 1 ml NaCl/Tris/BSA buffer, pH 7.5 and incubated for 30 min at 37°C. At the end of incubation, 2 ml cold NaCl/Tris/BSA buffer, pH 7.5 were immediately added and the membranes were precipitated by centrifugation at 25,000 g for 10 min at 4°C. The membrane pellet was rinsed twice by resuspending in 3 ml NaCl/Tris/BSA buffer, pH 7.5 and recentrifuging. The bound TSH in thyroid pellet was eluted by re-incubation with 1 ml 2 MNaCl, 1 g BSA/l for a further 30 min at 37°C, followed by centrifugation at 25,000 g for 10 min at 4°C. The supernatant fraction was chromatographed on 1 x 15 cm column of Sephadex G-100-40 in NaCl/Tris/BSA buffer, pH 7.5. The receptor-purified ^{125}I labelled TSH was eluted in a single peak (see Fig. 8, page 33) and stored at -20°C for use within 3 days.

D. Radioreceptor assay of ^{125}I-TSH: Duplicate incubation mixtures were prepared at 4°C to set standard curve. Frozen stock of standard bTSH (128 mIU/100 µl) was thawed and diluted with NaCl/Tris/BSA buffer, pH 7.5 to give the concentration of 64,32,16,8,4,2,1,0.5,0.25 and 0.125 mIU/ml respectively. Therefore, 50 µl of each concentration of standard bTSH, 100 µl (15 mg-equiv.) thyroid membranes and 100 µl NaCl/Tris/BSA buffer, pH 7.5 were mixed and incubated at 37°C for 10 min. Fifty µl (40-60 pg) of cold ^{125}I-receptor purified bTSH was

immediately added, gently mixed and incubated at 37°C for 2 h. Fifty µl of ^{125}I-receptor purified bTSH in triplicate was measured for total radioactivity. The incubation tubes were allowed to stand in ice-bath for 10 min., 1 ml cold NaCl/Tris/BSA buffer, pH 7.5 was added, gently mixed and centrifuged at 25,000 g for 20 min at 4°C. The supernatant was discarded and the radioactivity in the pellets were measured in an automatic gamma counter.

Membrane-free incubations (control) were run in each assay using 250 µl NaCl/Tris/BSA buffer, pH 7.5 and 50 µl ^{125}I-receptor purified TSH to correct for the non-specific adsorption of ^{125}I-labelled TSH to the incubation tube. Specific binding (S) percentage for each membrane sample was calculated by the following equation;

$$S = \frac{(M-C)}{(T-C)} \times 100$$

where, M = Membrane pellet counts

C = Control pellet counts

T = Total radioactivity counts

Sigmoid curve was obtained by plotting S as a vertical scale against log concentration of unlabelled TSH as a horizontal scale. The bound to free ratio (B/F = S/100-S) for each membrane sample and the amount of TSH bound to the thyroid membranes (S x amount of TSH incubated) were calculated. Therefore, Scatchard analysis[89] was performed by plotting B/F ratio against the concentration of TSH bound in order to analyse the properties of each thyroid membrane sample. The slope of

this plot is related to the association constant (K_a) for the binding and whose intercept (on horizontal scale) is the binding capacity. Moreover, protein concentrations in thyroid membranes were determined by the method of Kjeldahl[90].