CHAPTER II

MATERIALS, REAGENTS AND METHODS

Materials

1.	Bovine serum albumin, crystallized	(Sigma chemical)
2.	Barbituric acid GR	(Merck)
3.	Barbital sod.	(BDH lab reagent)
4.	Complete Freund's adjuvant	(Difco laboratories)
5.	Incomplete Freund's adjuvant	(Difco laboratories)
6.	Hydrochloric acid GR	(Merck)
7.	Sodium hydroxide	(Merck)
8.	Acetic acid	(Merck)
9.	Sodium azide	(Difco laboratories)
10.	Sodium ethylene diamine tetra-acetate	(May and Baker)
11.	Disodium hydrogen phosphate	(May and Baker)
12.	Sodium chloride	(Merck)
13.	Sodium hydrogen phosphate	(Merck)
14.	Sodium bicarbonate	(Merck)
15.	Purified agar	(Difco Laboratories)
16.	Agarose for electrophoresis	(Merck)
17.	Trichloroacetic acid	(Merck)
18.	Ponceau S dye	(Sigma Chemical)
19.	Cellulose acetate strip (50 x 200 m.m.)	(Carl Schleider and Schiill)
20.	Normal rabbit serum (NRS)	
21.	Normal human serum (NHS)	
22.	Thyroid stimulating hormone (TSH)	
23.	Normal human albumin (NHA)	

Materials

24. Human immunoglobulin (HIG)
25. NaI^{125} (Amersham)
26. Sheep anti - rabbit immunoglobulin (Siriraj hospital)
27. Rabbit anti - human immunoglobulin
28. Rabbit anti - human thyroglobulin
29. Sephadex G 200 (Pharmacia)

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Animal Use

Three female white rabbits weigh about 2 kg., 5-7 months of age are used in this study.

Equipments and glassware

1. pH meter (Orient)
2. Magnetic stirrer and Magnetic bar
3. Autogamma Counter (Packard)
4. MSE cool spin
5. Fume hood.
6. Vertex mixer
7. Eppendorf pipette 5, 10, 20, 50, 100, 100-1000 μl
8. Disposable pipette tip
9. Measuring cylinder
10. Deep freeze refrigerator
11. Ultrasonic elcaner
12. Autoclave
13. Beakers
14. Stiring rods
15. Glass funnels
16. Glass column 13 x 300 m.m.
17. Glass slide
18. Glass test tubes 7.5 ml.
19. Electrophoretic apparatus (Schandon Southern, SAE 2525)
20. Hot plate
21. Cork borer
22. Forcep
23. Glass plate

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Reagents

1. Veronal buffer 0.1 M. pH 8.6

5,5-diethyl barbituric acid	3.68	gm.
sodium diethyl barbiturate	20.60	gm.
distilled water qs.	1.00	litre

adjust pH 8.6 with NaOH or HCl

2. Ponceau S 0.2 % w/v in 7.5 % Trichloroacetic acid

Trichloroacetic acid	7.50	gm.
Distilled water qs.	100.00	ml.
Ponceau S	0.20	gm.

3. Normal saline 0.9 %

NaCl	9.00	gm.
Distilled water qs.	1.00	litre

4. $NaHCO_3$ 0.05 M.

$NaHCO_3$	42.0	mg.
Distilled water qs.	10.0	ml.

5. Stock solution of Phosphate buffer 0.5 M. (pH ~ 7.2)

SOLUTION A	Na_2HPO_4 (0.5 M.)	17.74	gm.
	Distilled water qs.	250.0	ml.
SOLUTION B.	$NaH_2PO_4 \cdot 2H_2O$ (0.5 M)	19.50	gm.
	Distilled water qs.	250.0	ml.

Stock solution 0.5 M. Phosphate Buffer (pH ~ 7.2)

Solution A	80	ml.
Solution B	20	ml.

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6. Phosphate buffer 3.5 mM. pH 7.2 (Eluting buffer)

Stock solution 5	7.00	ml.
Distilled water qs.	1.00	litre

adjust pH 7.2 with NaOH or HCl

7. Phosphate buffer 30 mM pH 7.2

Stock solution 5	60.00	ml.
Distilled water qs	1.00	litre

adjust pH 7.2 with HCl or NaOH.

8. Assay buffer

NaCl (0.12 M)	7.02	gm.
EDTA (0.01 M)	3.72	gm.
NaN_3 (0.02 % w/v)	0.20	gm.
BSA (0.25 % w/v)	2.50	gm.
Phosphate buffer 30 mM pH 7.2 qs (7)	1.00	litre

adjust pH 7.2 with HCl or NaOH.

9. Acetic acid 5 %

Glac. Acetic acid	50.00	ml.
Distilled water qs.	1.00	litre

10. Standard thyroglobulin solution

Weigh 1 mg Thyroglobulin and dissolve in assay buffer then dilute to 10,50,100,500,1,000 ng/ml.

11. Rabbit anti-human thyroglobulin solution

Rabbit anti-human thyroglobulin antiserum 100 µl dilute into 1:20,000 times with assay buffer

12. Sheep anti-rabbit immunoglobulin solution (1:4)

Sheep anti-rabbit immunoglobulin antiserum 1 ml. dilutes into 4.0 ml. (1:4) with assay buffer.

13. Normal rabbit serum solution (0.3 %)

Normal rabbit serum 300 µl dilutes into 100 ml. with assay buffer.

Methods

1. Electrophoretic procedure

A pencil line is drawn across the cellulose acetate strip about one-third of the distance from one end after which the strips are dipped in the veronal buffer and blotted lightly between filter paper strips. The wet cellulose acetate strips are then placed in the electrophoresis tank so that the pencil line is nearest the cathode end. It is preferable, though not essential, to pass current through the strips for about 15 minutes to arrow equilibium conditions to be attained. After this time the normal serum and HTg solution are spotted in a straight line along the pencil mark. Application is best carried out using a micro-pipetle or capillary. Approximately 5 µl of undiluted serum is spotted per one site. The course of electrophoresis is best followed by staining the serum prior to application by addition of a few crystals of bromphenol blue. This dye is fairly tightly bound to serum albumin during the course of electrophoresis and does not alter the mobility of albumin or other serum components. Neither does the dye interfere with subsequent staining of the strip. Albumin is the fastest running component of serum so that progress of electrophoresis can be gauge by progress of the blue band. Excess bromophenol blue moves ahead of the albumin as a purplish band and eventually moves off the strip as electrophoresis progresses. (14)

Satisfactory separation of serum proteins is effected by appling a current of about 8-10 mA, 550-510 V, for 80 minutes. At the end of a run the current is switched off and the strip is stained with Ponceau S solution 0.2 % w/v in trichloroacetic acid 7.5 %. Proteins

need not be heat-denatured prior to the application of this stain since the trichloroacetic acid present denatures the proteins directly. Strips are immersed for 15 minutes in a solution of Ponceau S. The strips are then washed in several rinses of 5 % acetic acid until a relatively clear background is obtained. The strip is finally washed in methanol and air-dried. (14)

2. Production of Antibodies

A. Immunization

Emulsified human thyroglobulin 1 ml. (6 mg) in complete Freund's adjuvant 2 ml (ratio 1:3) using vertex mixer in high speed to obtain w/o paste emulsion.

Immunized each 3 rabbits with emulsion dose 2 mg/rabbit Intradermal injection at 30 sites along the vertebral colume.

B. Booster injection

Booster injection were made at 3 weeks intervals.

1. First booster dose.

Emulsified HTg 1 ml (3 mg) in incomplete Freund's adjuvant 2 ml (ratio 1:3) using vertex mixer in high speed to obtain w/o paste emulsion.

Booster dose 1 mg/rabbit.

Intradermal injection at 10 sites along the vertebral column

2. Next booster dose 0.5 mg/rabbit

C. Rabbit serum

1. Normal rabbit serum

Rabbit serum was drawn from the artery of ear before immunization about 5 ml/rabbit

2. Rabbit antiserum

About 5-10 ml. of rabbit antiserum was drawn from the artery of ear every 10 days after injection.

3. Immuno-diffusion Agar (Ouchterlony) method

Dissolve Agarose 0.5 % w/v or purified agar 1.5 % in Veronal buffer, add NaN_3 0.02 % w/v, melt agar on the hot plate until clear solution was obtained. Pipet 3.5 ml and pour over the slide, left it hards. Reservoirs are molded into agar on a flat surface. The wells are filled with appropriate solutions of antigens and antiserum, covered the slide in glass plate to prevent evaporation, and observed periodically for several days. Precipitin bands may become visible in 6-24 hours. (8).

Test for :

A. Can rabbits produce anti-HTg ?

B. Has rabbit anti-HTg only specific to HTg ?

C. How much the dilution titre has rabbit anti-HTg ?

4. Labelling human thyroglobulin by Iodogen method

A. Preparation of Sephadex G. 200 column.

Sephadex G 200 0.5 gm was added into 25 ml Phosphate buffer 3.5 mM. pH 7.2 overnight and packed into glass column 13 x 200 m.m. which give the constant flow rate of 5 drops per 2 minutes. Saturated column with 1.0 % BSA 5 ml until the solution above the sephadex was about 3 m.m. stopped the flow.

B. 0.05 M. $NaHCO_3$ was prepared fleshly.

C. HTg solution

Weigh HTg and dissolve in normal saline or Veronal buffer then dilute to required concentration.

D. Equipments for labelling are already prepared.

1. NaI^{125}
2. Eppendorf pipette and tip
3. Dropping pipette with buble
4. Ice bath
5. Fume hood
6. Iodogen tube
7. reagents.

E. Labelling procedure (15)

1. Put Iodogen tube in ice bath
2. Pipette HTg solution 10 µl (15 µg) into the Iodogen tube
3. Add 5 µl. NaI^{125} (500 µCi)
4. Add 20 ul $NaHCO_3$ immediately, swirl for 8 minutes (or 10, 12, 15 minutes)
5. Add 200 ul $NaHCO_3$ into the tube to stop reaction
6. Transfer the mixture with dropping pipette into the solution on sephadex G.200 column and elute with Phosphate buffer 3.5 mM. + 0.25 % BSA
7. Collecting 1.00 ml fractions about 30-40 tubes.
8. Pipette 50 µl from each fraction tube
9. Count for 1 minute in Autogamma counter.
10. Plot curve cpm VS fraction number
11. Calculate the specific activity of $HTgI^{125}$

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F. Calculation Specific activity

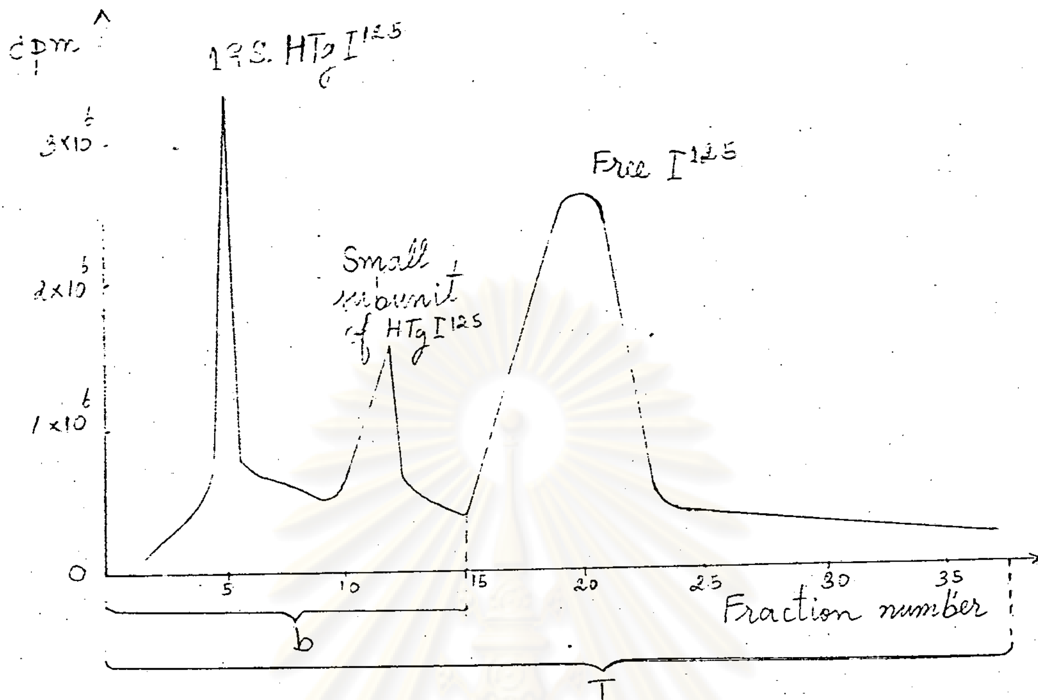

Fig. 4 $HTgI^{125}$ cpm/Fraction number

T = Total count (cpm)

b = total count of I^{125} bound (cpm)

$\therefore$ % Bound = $\frac{b}{T} \times 100$ = B %

Bound 100 % from I^{125} = 500 µCi ($\because$ 1 µl NaI^{125} = 100 µCi)

Bound B from I^{125} = $\frac{500 \times B}{100}$ = 5B µCi

HTg was added to reaction = 15 µg

$\therefore$ Specific activity = $\frac{5B}{15} = \frac{B}{3}$ µCi/µg

5. HTg-RIA procedure

1. Labelled tube NS,Bo, 10,50,100,250,500,1000 ng/ml and unknown sample tubes (duplicate).
2. Pipette unknow serum 100 µl into unknown tube.
3. Pipette standard solution 100 µl into standard tube.
4. Pipette assay buffer 200, 100 µl into NS,Bo tube respectively.
5. Add rabbit-antiserum solution (1:20,000) 100 µl into each tube except NS tube.
6. Mix on the vertex mixer and incubate 37 $^{\circ}$C 4 hrs.
7. Add HTgI^{125} 100 µl (20,000 cpm) into each tube.
8. Mix on the vertex mixer and incubate 37 $^{\circ}$C 18 hrs.
9. Add normal rabbit serum solution 0.3 % v/v 100 µl into each tube.
10. Add sheep anti-rabbit Ig (1:4) 100 µl into each tube.
11. Mix on the vertex and incubate 37 $^{\circ}$C 2.5 hrs.
12. Take into cold room 4 $^{\circ}$C 10 minutes.
13. Add cold assay buffer 500 µl into each tube.
14. Mix on the vertex and centrifuge 2,400 rpm for 30 minutes.
15. Pour supernate and place the tubes in an inversion rack to drain for 10 minutes
16. Count the precipitate for 1 minute in an Autogamma counter.
17. Correct each value for the percentage of HTgI^{125} bound VS. the concentration of standcerd HTg on graphic paper (Standard Curve)
18. Determine sample values directly from the curve.