CHAPTER II

MATERIALS REAGENTS AND METHODS

MATERIALS

1. Albumin, bovine, crystallised (Sigma Chemical)
2. 8 - Anilino - 1 - naphthalene sulphonic acid (ANS) (Eastman Kodak Corp. USA)
3. Barbituric acid G.R. (Merck)
4. Charcoal activated extrapure (Merck)
5. Complete Freund's adjuvant (Difco Laboratoies)
6. 1 - ethyl - 3(3 - dimethyl - aminopropyl) aminopropyl) carbodiimide HCl (Sigma Chemical)
7. Hydrochloric acid G.R. (Merck)
8. Incomplete Freund's adjuvant (Difco Laboratories)
9. Methanol absolute G.R. (Merck)
10. Methyl cellulose
11. Monoiodotyrosine (Sigma Chemical)
12. Nitrogen gas
13. Nomal rabbit serum
14. Normal pool serum
15. Sodium chloride (Merck)
16. Sodium hydroxide (Merck)
17. Sodium barbital (BDH Lab. Reagent)
18. Sulphuric acid (BDH Lab. Reagent)
19. T_4 - stable thyroxine. Sod. salt (Sigma Chemical)
20. T_4^{125} - high specific 1200 uci/ug (Amersham)

21. Triiodothyronine (Sigma Chemical)

22. Triethylamine (Merck)

23. Tetrahydrofuran (BDH Lab. reagent)

REAGENT

1. T_4-free Serum

5 ng T_4I^{125} is added to every 10 ml pooled normal serum and left to equilibrate for 1 hour. 1 g Norit A charcoal is added and mixed at room temperature for 4 hours. The mixture is centrifuge for 20 min at 30,000 xg and the supernatant removed. The serum is finally passed disposable 0.45 u millipore membrane to remove residual traces of charcoal and sterile. The serum is aliquoted and deep frozen for no hormone serum.

2. 0. I N HCl

HCl	8.3 ml
Distilled water q.s. to	1000.0 ml

3. 0. I NaOH

NaOH	4
Distilled water q.s. to	1000.0 ml

4. Barbitone buffer pH 8.6, 0.05 M

Diethyl barbituric acid	1.83 g
Sodium barbitone	10.3 g
20% BSA	0.5 ml
Deionized water q.s. to	1000 ml

5. 8 - Anilino - 1 - naphthalene sulphonic acid 4 mg/ml

ANS	40 mg
Barbitone buffer pH 8.6 q.s. to	10 ml

Prepare freshly before use.

6. Charcoal suspension 10 mg charcoal/100 ul

Methyl cellulose	100 mg
Barbitone buffer q.s. to	20 ml
Charcoal activated extrapure	2 g

7. Standard non - radioactive T_4

7.1 50% Propylene glycol sol^n

7.2 Standard stock T_4 (1)

T_4 - Sod	2.28 mg
50% Propylene glycol	2.0 ml

7.3 Standard stock T_4 (2)

Std. stock T_4 (1)	500 ul
Barbitone buffer q.s. to	50 ml

7.4 Standard stock T_4 (3) (60 ng/ml)

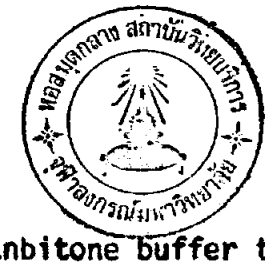
Std. stock (2)	500 ul
Barbitone buffer q.s. to	100 ml

7.5 Diluted standard stock T_4 (3) to 30, 10, 5, 2.5, 0.625 0.156 ng/ml

8. T_4I^{125} Sol^n (T_4 = 15-20 pg/50 ul)

T_4I^{125}	100 ul
Banbital buffer q.s. to	10 ml

Prepare freshly before use and store in dark bottle.



9. Monoidodotyrosine Sol^{n} (MIT)

Weigh 1 mg MIT and dissolve in Banbitone buffer then dilute to 0.1, 1, 10, 100, 1000, 10000, 100000, 1000000 ng/ml.

10. Triiodothyronine Sol^{n} (T_3)

Weigh 1 mg T_3 and dissolve in 0.1N NaOH 1 ml then dilute to 0.1, 1,10, 100, 1000, 10000, 100000, 1000000 ng/ml.

Animal Use

Six rabbits weigh about 2000 gm

Equipments and glassware

1. Kipp apparatus
2. pH Meter (Orient)
3. Magnetic stirror
4. Magnetic bar
5. Autogamma counter (Packard)
6. MSE cool spin
7. Fume hood
8. Dialysis tubing
9. Vortex mixer
10. Eppendrof pipette, 5, 10, 20, 50, 100 ul
11. Disposable pipette tip
12. Repette 5 ml
13. Repette 10 ml
14. Measuring cylinder
15. Watch glass
16. Deep freeze refrigerator

17. Lyophilizer
18. Lead shield bottle
19. Ultrasanic cleaner
20. Autoclave
21. Tissue homoginizer
22. Volumetric flask
23. Stirring rod
24. Beaker 2000 ml
25. Pasteur pipette
26. 10 x 75 mm Plastic test tube
27. Eppendorf centrifuge 3200
28. Disposable millipore 0.45 u

METHOD

I. Prepanation of Antigens

A. Conjugation of $T_4.CH_3.HCl$ to BSA

a) Esterification of thyroxine

1. 100 mg T_4 + 30 ml Methanol (Absolute) + 12 ul T_4I^{125} (1200 uci/ug) count = 812465/.1 min = 2.455 ng
2. Prepare HCl gas using NaCl + H_2SO_4 conc. pour in small amount into a Kipp apparatus and bubbing into (1) for 40 min.
3. Warm the reaction to 37°C the precipitate will form then concentrate the solution by passing N_2-gas, will have supernate and precipitate (ppt.)

Dissolve ppt. in warm methanol at 30-40°C then add distilled water dropwise.

Isolate the ppt. and count = 502,950/.1 min = 1.520 ng

Calculation

T_4I^{125} 2.455 ng form ester — 1.52 ng

100 x 10^6 ng " — $\frac{1.52 \times 100 \times 10^6}{2.455}$

= 61.914 mg

b) Conjugate $T_4.CH_3.HCl$ to BSA

1. Dissolve 50 mg $T_4.CH_3.HCl$ in 0.1N NaOH 2.5 ml
2. Dissolve 100 mg BSA in distilled water
3. Weigh carbidiimide 75 mg dissove in 0.1N NaOH in small amount and add into (1) mix by using magnetic stirror.
4. Add (2) into (3) slowly and mix (pH 9.6) and adjust pH = 9.0 with 0.1N HCl
5. Leave reaction overnight at 4°C
6. Wash dialyze tubing for 3-4 hours in distilled water, washed inside and out by turning tubing inside out. Reverse to correct phase and knot. Check for leaks by filling with distilled water.
7. Dialyzed against 5 litres of distilled water with 3-4 change (3 days) in the dark.
8. Count 1 ml aliquot of each change.

	cpm/ml	Total Volume	Total count/m
1st dialyzate	52	1800	93,600
2nd "	68	1300	88,400
3rd "	87	1000	87,000
4th "	50	1000	50,000
		Total	319,000
		=	0.0964 ng

Calculation

I^{125} $T_4.CH_3.HCl$ conjugate to BSA = 1.520 - 0.0964 ng

= 1.4236 ng

I^{125} $T_4.CH_3.HCl$ 1.52 ng conjugate to BSA 1.4236 ng

$T_4.CH_3.HCl$ 50 mg " $\frac{1.4236 \times 50}{1.52}$

= 46.8289 mg

B. Conjugation of T_4 to BSA

1. Weigh out 100 mg of T_4 dissolve in 50% Tetrahydrofuran (T.h.f) 4 mls.
2. Add 10 ul T_4I^{125} (50 uci/ug) to the solution, exposing to light as little as possible.
3. Add carbodiimide (slightly molar excess) to the T_4 solution. Store in the dark. Measure radioactivity = 471174 count/.1 min = 85.724 ng ..... Sol^nA.

4. Weigh out 200 mg BSA dissolve in 10 ml distilled water. When complete dissolve add 10 ml T.h.f stirring.
5. Add about 50 ul triethylamine ..... Sol^{n}B.
6. Mix sol^{n}A and B. Stir for 5 min in the dark store 24 hours in the dark in beaker covered with watch glass at 4^{o}C.
7. Wash dialyze tubing as A.b.6 (p. 13).
8. Filled solution from 6 into dialyze tubing and dialyzed in the dark against distilled water for 3-4 changes (5-6 litres of water)
9. Count 1 ml aliquot of each change.

	Count per min	Total Vol.	Total count
1st dialyzate	700	1500	1050000
2nd "	460	1500	690000
3rd "	350	1000	350000
4th "	280	1000	280000
			Total = 2370000
			= 43.119 ng

Calculation

$I^{125}T_4$ Conjugate to BSA = 85.724 - 43.119 ng

= 42.605 ng

$I^{125}T_4$ 85.724 ng conjugate to BSA = 42.605 ng

T_4 100 mg = $\frac{42.605 \times 100}{85.724}$

= 49.7002 mg

10. Divide (8) in 1 mg T_4 aliquot into the vial and lyophilize.

II. Production of Antibodies

A. Immunization

Emulsified $T_4.CH_3.HCl$ - BSA (1 mg of T_4) and T_4 - BSA (1 mg of T_4) in complete Freund's adjuvant ratio 1:3 using Thomas Teflon Pestle Tissue Homogenizers to obtain W/O emulsion.

Immminize 3 rabbits with each emulsion dose 300 ug/rabbit. Intradermal injection at 30-50 sites along the vertebral column (3).

B. Booster injection and antibodies estimation

Booster injection were made at 3 weeks intervals. The antibodies were estimate by incubating with T_4I^{125} (50 pg)

Estimation procedure

1. Add 200 ul barbitone buffer into the plastic test tube.
2. Add 100 ul antiserum.
3. Add 100 ul T_4I^{125} (50 pg).
4. Add 500 ul barbitone buffer. Count total count for 1 min then incubate overnight at $4^{o}C$.
5. Add 100 ul charcoal suspension and mix.

6. Centrifuge 2400 rpm for 25 min.
7. Separate the supernate with Pasteur pipette and count the supernate (Bound) for 1 min.
8. Calculate the % Bound.

C. Antibody titration

Antibody titres were estimated by incubating T_4I^{125} (20 pg) with increasing amount of antiserum dilution.

D. T_4-RIA procedure

1. Labelled tube : C.B, B_o 0.625, 2.5, 10, 30, 60 ng/ml
2. Pipette to 10 ul T_4-free serum into the set of standard tubes.
3. Pipette unknown serum 10 ul into unknown tube.
4. Add 100 ul standard into each std. tube and add 100ul barbitone buffer into C.B, B_o and unknown tube.
5. Add 100 ul ANS sol^n (4 mg/ml). Mix and leave for 5-10 min.
6. Add 50 ul T_4I^{125} (20 pg) into each tube and mix.
7. Add 100 ul T_4 antisera into each tube except tube C.B*.
8. Adjust the total volume of each tube to 900 ul.
9. Incubate overnight at 4^oC.
10. Add 100 ul cool charcoal suspersion into each tube.
11. Centrifuge 2400 rpm for 25 minutes.
12. Separate the supernate (Bound) by using Pasteur pipette.
13. Count the supernate for 1 min.