CHAPTER II

MATERIALS AND METHODS

1. Materials.

1.1 Samples for the determination of vitamin B_{12} contents.

1.2 Vitamin B_{12} (Cyanocobalamin) Standard was purchased from Dista Products Limited. Spike.

1.3 ^{57}Co - vitamin B_{12} standard was purchased from The Radioactive Centre, Amersham.

1.4 Norit "A" Charcoal was obtained from Amend Drug and Chemical Co., Inc., New York, N.Y

1.5 National Formulary Intrinsic Factor (NFIF) was obtained from the National Formulary, American Pharmaceutical Association, Washington, D.C

1.6 Haemoglobin was prepared from blood.

1.7 Normal saline solution (0.9 % NaCl) was obtained from Siriraj Hospital.

1.8 Sodium cyanide, Sodium acetate, Ammonium sulphate, Toluene, and Concentrated hydrochloric acid were of analytical reagent grade.

2. Equipments.

2.1 Well-type Scintillation Counter (Nuclear-Chicago Model 181 B, Illinois, U.S.A).

2.2 Internation Portable Refrigerated Centrifuge Model PR-2.

2.3 Spectrophotometer (Bausch and Lomb).

2.4 Beckman pH - meter.

3. Methods of preparation of reagents for the determination of vitamin B_{12} contents.

3.1 Vitamin B_{12} (Cyanocobalamin) Standard B.P

A stock solution of 1 mg per ml of standard cyanocobalamin in distilled water was prepared. One-tenth ml of this solution was made up to 100 ml with distilled water, then 0.1 ml of this solution was diluted to 100 ml with distilled water to provide a working solution of 1000 pg of cyanocobalamin per ml.

3.2 ^{57}Co-vitamin B_{12} solution.

^{57}Co-vitamin B_{12} which has a specific activity of approximately 15 $\mu Ci/\mu g$ was purchased from Amersham, England. It was diluted with distilled water to a final solution of 1000 pg ^{57}Co-vitamin B_{12} per ml, and could be stored at $5^{o}C$ for up to 3 months without lossing the radioactivity.

3.3 National Formulary Intrinsic (NFIF).

A stock solution of 100 mg NFIF in 100 ml of 0.9 % saline solution was prepared. One ml of this stock solution was made up to 100 ml with normal saline solution to provide a working solution of 10 µg NFIF per ml. This solution had a B_{12}-binding capacity of approximately 800 pg per ml.

The stock NFIF solution was stored at-20 °C. The working NFIF solution might have a slight drop in the B_{12} binding capacity with such storage but this did not affect the assay since the crucial point was not the use of exactly 10 µg of NFIF but use of that quantity of NFIF which would bind approximately 60 per cent to 80 per cent of the added ^{57}Co-vitamin B_{12}.

3.4 Haemoglobin - coated charcoal.

This was prepared by mixing equal volumes of a 5 per cent aqueous suspension of Norit "A" pharmaceutical grade charcoal and 0.25 per cent aqueous solution of haemoglobin. This mixture was stored at 5 °C, it could be stored for up to a month without lossing the activity.

Five per cent aqueous suspension of Norit "A" charcoal was prepared by adding a portion of distilled water to 5 gram of charcoal. The solution was shaked well and diluted to 100 ml.

Ten per cent haemoglobin solution was prepared as follows : (Jonxis and Huisman, 1958). Discarded human red cells were thrice washed with 0.9 % saline solution, then hemolyzed with an equal volume of distilled water, followed by addition of one-half volume of toluene. The mixture was shaken vigorously for 5 minutes, then centrifuged for 15 minutes at 3000 rpm. The top two layers, consisting of toluene and cell debris, were discarded; and the bottom layer, consisting of

haemoglobin, was passed through Whatman No.1 filter paper to yield a clear red filtrate. The haemoglobin concentration was adjusted with distilled water to 10 gram per 100 ml.

Dilution of 0.25 per cent aqueous solution of haemoglobin was prepared by 2.5 ml of 10 per cent solution of haemoglobin was diluted to 100 ml with distilled water.

3.5 Acetate buffer (1 % solution)

Ten grams of sodium acetate was dissolved in distilled water and adjusted to pH 4.8 with acetic acid. Then diluted to a final volume of 1 liter.

3.6 Sodium cyanide solution (1 % solution).

One gram of sodium cyanide was dissolved in distilled water and adjusted to a final volume of 100 ml.

3.7 0.25 N. Hydrochloric acid.

About 21 ml of concentrated hydrochloric acid was diluted to 1000 ml with distilled water.

3.8 Ammonium sulphate solution (25 % solution).

Twenty-five grams of ammonium sulphate was dissolved in distilled water and was adjusted to a final volume of 100 ml.

4. Measurement of serum vitamin B_{12} level.

4.1 Standardization of National Formulary Intrinsic Factor concentrate (NFIF).

Five hundred picograms of ^{57}Co-vitamin B_{12} in 0.5 ml of distilled water was added into each of 11 test-tubes containing various amount (1 µg to 10 µg) of intrinsic factor with one ml of normal saline solution. Let the solution stand for 30 minutes at room temperature, and 2 ml of Hb-coated charcoal was added. All test-tubes were placed on a rotex mixer for 10 seconds and centrifuged at 2,800 rpm for 30 minutes. The supernatant was decanted into plastic tube and counted in a well type scintillation counter. The amounts of intrinsic factor were plotted against the binding capacity. The amount of intrinsic factor which had binding capacity of 60 per cent to 80 per cent ^{57}Co-B_{12} was used in the assay.

4.2 Procedure for standardization of ^{57}Co-B_{12}.

Every new lot of ^{57}Co-vitamin B_{12} (1000 pg/ml) prepared for use in the assay was standardized by reversed isotope dilution against the vitamin B_{12} standard (1000 pg/ml). Two solutions of 0.5 ml of ^{57}Co-B_{12} were added into two test-tubes in which one test-tube contains 0.5 ml of cold B_{12}, and the other contains 0.5 ml of normal saline solution. One-half ml of intrinsic factor was added to these tubes and mixed on a Rotex mixer for 10 seconds. Let stand for 30 minutes. Two ml of haemoglobin coated charcoal was added and the contents were mixed again. The tubes were centrifuged at 3,000 rpm for 30 minutes and the supernatant fluid was decanted into counting tubes and counted in a well-type scintillation counter.

The actual quantity of $^{57}Co\text{-}B_{12}$ was calculated for any percentage change in the $^{57}Co\text{-}B_{12}$ binding capacity of the NFIF by using the following equation :

$$pg\ ^{57}Co\text{-}B_{12} = pg\ Cold\ B_{12}\left(\frac{B'}{B - B'}\right)$$

where B = Net cpm of tube containing NFIF and $^{57}Co\text{-}B_{12}$

B' = Net cpm of tube containing NFIF, $^{57}Co\text{-}B_{12}$ and standard cold B_{12}

4.3 Preparation of serum samples :

Blood was obtained from fasting subjects using either acid-washed sterile syringes, or disposable plastic syringes. The blood was allowed to stand for approximately three hours at room temperature after tranfer of blood from syringes to acid-washed screw-top tubes. The clots are 'rimmed' with glass rods or wooden applicators sticks, and centrifuged for five minutes at 3,000 rpm. The supernatant was drawned into the acid-washed or disponsable pipettes and frozen at -20 °C. Sera were assayed in duplicated without any further treatment.

4.4 Assay Procedure.

Vitamin B_{12} contents were assayed by the radioisotope dilution and coated charcoal technique as described by Lau et al., (1965). The principle of radioisotope dilution is applied, using the unknown quantity of nonradioactive B_{12}

released from serum to dilute the specific activity of a known quantity of $^{57}Co\text{-}B_{12}$. A solution of intrinsic factor concentrate (IFC) with a B_{12} binding capacity less than the quantity of added $^{57}Co\text{-}B_{12}$ is used to bind a portion of the mixture of radioactive and nonradioactive B_{12} - i.e, to "biopsy" the pool of B_{12}. The B_{12} not bound to IFC is removed by addition of coated charcoal. This series of events is depicted in figure.

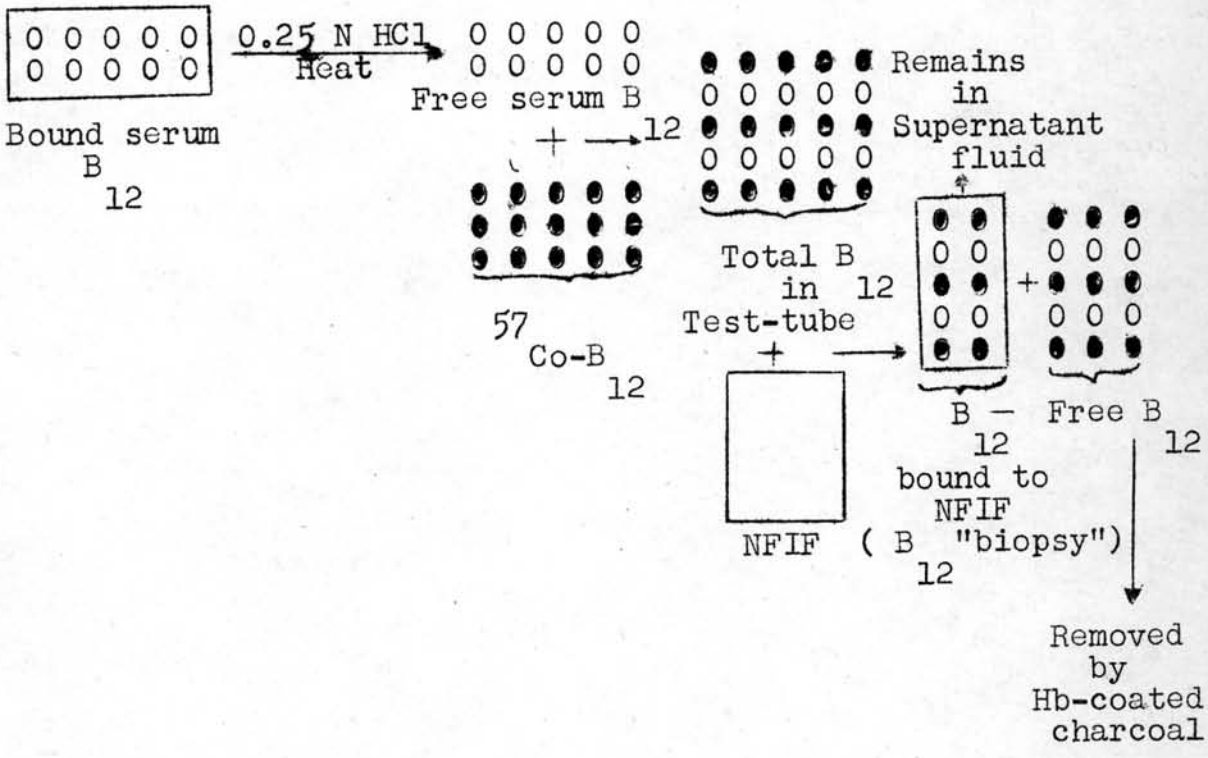

Fig. 1 Schematic representation of serum vitamin B_{12} assay using radioisotope dilution and Hb-coated charcoal (From Lau et al., 1965).

Tests were done in duplicate in 15 ml centrifuge test tubes. One-half ml of unknown serum was added to 1.5 ml of 0.9 % normal saline solution in the tubes for unknown serum and serum supernatant control (see Table 1, p.18). The tubes for NFIF control and NFIF supernatant control contain 2.0 ml normal saline solution. These were run in parallel to correct for volume changes caused by heating. To each tube 0.5 ml of 0.25 N hydrochloric acid was added. The tubes were capped with cotton wool, heated in a boiling water-bath for 15 minutes, and then cooled with tap water. Five tenths ml ^{57}Co-B$_{12}$ was added to each tube and the contents were mixed for 10 seconds on a Rotex mixer. Five tenths ml of NFIF was added to the unknown serum and NFIF control, and equal volume of saline solution was added to the supernatant control. The contents were mixed again and stand for 30 minutes, then 2 ml of Hb-coated charcoal was added and mixed before the tubes were centrifuged at 3,000 rpm for 30 minutes. The supernatant fluid was decanted into counting tubes. The radioactivity in the supernatant fluid was counted in a well-type scintillation counter.

4.5 Calculation of serum B$_{12}$ level.

The radioactivity of the supernatant controls (cpm) were subtracted from those for the unknown and the NFIF control respectively, in order to obtain net counts. The serum B$_{12}$ level was calculated from the following formula :

TABLE 1

SERUM VITAMIN B_{12} ASSAY PROTOCOL

	Sequence of Addition and ml of Reagents to Add :									
	0.9 % Saline	Unknown Serum	0.25 N HCl		1000 pg/ml Co 57-B_{12}	10 µg/ml Std NFIF	0.9 % Saline		Hb-coated Charcoal	
Unknown serum	1.5	0.5	0.5	Mix. Cap tubes with cotton wool. Heat in boiling water bath for 15 min. Cool with tap water.	0.5	0.5	0	Mix for 10 seconds and let stand for 30 minutes	2.0	Mix. Centrifuge at 3000 rpm for 30 min. Count radioactivity in supernatant fluid.
Supernatant control (serum)	1.5	0.5	0.5		0.5	0	0.5		2.0	
NFIF control	2.0	0	0.5		0.5	0.5	0		2.0	
Supernatant control (NFIF)	2.0	0	0.5		0.5	0	0.5		2.0	

Mix for 10 seconds.

$$\text{pg } B_{12} \text{ per ml of serum} = 2 \times \text{pg } {}^{57}\text{Co-B}_{12} \left(\frac{B}{B'} - 1 \right)$$

where B = Net cpm of NFIF control tube.

B' = Net cpm of tube with unknown serum.

4.6 Derivation of formula

Let M = mass of ^{57}Co-B_{12} added and R its radioactivity (cpm)

Let m = mass of ^{57}Co-B_{12} bound by NFIF and B its radioactivity (cpm).

Specific activity of ^{57}Co-B_{12} $\frac{R}{M} = \frac{B}{m}$ (1)

$$R = \frac{B \times M}{m} \quad (2)$$

Let B' = radioactivity (cpm) of ^{57}Co-B_{12} bound by NFIF after dilution of M by a mass of m' cold B_{12}

New specific activity after radiodilution

$$\frac{R}{M + m'} = \frac{B'}{m} \quad (3)$$

substituting for R from equation (2)

$$\frac{B \times M/m}{M + m'} = \frac{B'}{m} \quad (4)$$

$$\frac{B \times M}{m} = \frac{B' (M + m')}{m}$$

$$B \times M = B' (M + m')$$

$$\frac{B}{B'} \times M = M + m'$$

$$m' = \left[\frac{B}{B'} \times M\right] - M$$

$$m' = M\left[\frac{B}{B'} - 1\right] \qquad (5)$$

$$\text{Thus pg } B_{12} = \text{pg } {}^{57}\text{Co-}B_{12}\left[\frac{B}{B'} - 1\right]$$

5. Determination of vitamin B_{12} concentration in fish sauce.

5.1 Preparation of fish sauce dilution.

One ml of fish sauce was diluted to $\frac{1}{5}$ or $\frac{1}{10}$ with normal saline solution.

5.2 Assay procedure.

The assay was made in 0.5 ml of diluted fish sauce by the radioisotope dilution and coated charcoal technique as described above

6. Determination of vitamin B_{12} concentration in soya-bean sauce .

6.1 Preparation of soya-bean sauce solution.

The solution of soya-bean sauce was mixed with 25 % ammonium sulphate solution in equal volume. Then, the solution was centrifuged to obtain an aliquot of clear solution.

6.2 Assay procedure.

The assay was made in 0.5 ml of diluted soya-bean sauce by the method as described above.

7. Determination of vitamin B_{12} concentration in fermented fish.

7.1 Extraction of fermented fish.

One-half ml of sodium cyanide solution and 50 ml of sodium acetate buffer were added to 5 grams of fermented fish. The mixed solution was hydrolyzed in a boiling water-bath for 30 minutes, then the extracted solution was filtered through a No 40 Whatman filter paper into a 100 ml volume metric flask. The residue was extracted again with sodium acetate buffer, and then was filtered. Finally the filtrate was adjusted to a measured volume with sodium acetate buffer. The resulting solution was used to assay vitamin B_{12} concentration.

7.2 Assay procedure.

The determination of vitamin B_{12} contents was made in 0.5 ml of extracted solution by the method described above.