CHAPTER II

MATERIALS AND METHODS

I. Materials

1.1. Vitamin B_{12} contents were determined in meat, poultry and eggs, fishes and sea foods, vegetable, fruits, milk, dairy products and miscelleneous articles purchased from the local markets.

1.2. Vitamin B_{12} (Cyanocobalamin) standard was purchased from British Drug Houses (BDH).

1.3. ^{57}Co-cyanocobalamin, intermediate specific activity ^{57}Co-cyanocobalamin, was purchased from the Radiochemical center, Amersham, England.

1.4. Norit "A" neutral activated charcoal was purchased from Amend Drug and Chemical Co, Inc, New York, N.Y.

1.5. Chicken serum

Chicken blood was obtained from a poultry plant. Serum separated from chicken blood which has been allowed to clot at 37^{o}C. was suitably diluted with saline, and its unsaturated Vitamin B_{12} binding capacity ($UB_{12}BC$) was determined.

1.6. 3.5% PVP (Polyvinylpyrrolidone) was purchased from CHI SHENG chemical works Co. LTD. TAIWAN. Republic of CHINA.

1.7. Sodium acetate, Potassium cyanide, Glutamic acid, Sodiumtetraborate and Glacial acetic acid. All chemicals were Analar grade.

2. Equipments

2.1. Auto-Gamma Scintillation Spectrometer Model 5200 (Packard)

2.2. International portable refrigerated centrifuge Model PR-2 (IEC)

2.3. Spectrophotometer

2.4. Beckman pH-meter

2.5. Metler analytical balance

2.6. Blender

2.7. Low-temperature chest (-20^{o}C)

2.8. Mixer

2.9. Water-bath

2.10. Automatic pipette with plastic tip
(100, 500, 1,000 µl)

3. Method of preparation of reagents

3.1. Vitamin B_{12} (Cyanocobalamin) standard

10.0 mg of crystallized Vitamin B_{12} was accurately weighted out and dissolved in 10 ml volumetric flask with 25% ethyl alcohol. Further dilution were made in order to get 1,000 pg/ml solution. Standard solution was kept in the dark at -20°C.

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

The ^{57}Co-B_{12} solution obtained from Amersham, having a specific activity of 10 microcuries per microgram was diluted with distilled water to 1,000 pg/ml solution. This solution was kept in the dark at 4°C.

3.3. Specific binder-chicken serum

Stock solution 220 millitre of chicken serum was mixed with 0.2 ml of 20% sodium azide and stored at -20°C.

Working solution: The stock solution was freshly diluted to get 1: 250 solution with distilled water. The percentage of radioactivity bound in the absence of added cold Vitamin B_{12} for this dilution compared to the total counts was about 80%.

3.4. PVP Coated Charcoal

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

addition of 7 ml of dialysed PVP solution.

3.5. 0.1 M Borate buffer, pH 8.5

38.137 gm of $Na_2B_4O_4 \cdot 10H_2O$ was dissolved in a 1,000 ml volumetric flask with distilled water. The pH was adjusted to 8.5 with boric acid solution and diluted to the final concentration of 3.8137 gm%.

3.6. Acetate buffer (1% solution)

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

3.7. Potassium cyanide solution (1% solution)

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

3.8. Glutamic acid buffer

1.2 gm of L-glutamic acid was dissolved in 200 ml distilled water. This provided a solution of pH 3.3. One ml of 0.4% KCN solution was immediately added before using.

4. Extraction of samples

4.1. The general extraction was done by the method of Coates et al (1953).

50 ml of 1% sodium acetate buffer solution was added to each 10 gm of sample and 0.5 ml of cyanide solution. The solution was heated on steam-bath for 30 minutes, cooled and diluted if necessary. The extracted solution was filtered through a No.40 Whatman filter paper. The filtrate was used to assay for Vitamin B_{12} concentration.

4.2. Milk and milk products extraction.

1 ml or 1 gm of sample was added into 4 ml glutamic acid buffer (pH 3.3). The solution was boiled in a boiling for 30 minutes, cooled and filtered if necessary. The clear solution was used to assay for Vitamin B_{12} concentration.

5. Measurement of Vitamin B_{12} concentration in some foods.

The contents of Vitamin B_{12} in some Thai foods were assayed by a radioisotopic method using chicken serum as the Vitamin B_{12} binder and PVP coated charcoal to seperate free and bound Vitamin B_{12}. The principle of a radioisotope method depends on measurement of the dilution of added radioactive Vitamin B_{12} by unlabelled Vitamin B_{12} presented in the test sample. The test samples were deproteinized to liberate the Vitamin B_{12} from the binders. Radioactive Vitamin B_{12} (usually as $^{57}CoB_{12}$) and a binder for Vitamin B_{12} were added. Free and bound vitamin were then seperated by charcoal

method. By measurement of the radioactivity in the unbound supernatant, the radioactive Vitamin B_{12} could be calculated and therefore the unlabelled sample Vitamin B_{12} content was estimated.

5.1. Standardization of chicken serum

The unsaturated Vitamin B_{12} binding capacity ($UB_{12}BC$) of chicken serum was determined by a modified method of Lau et al, 1965. The chicken serum was appropriately dilute (1: 250) so that 0.2 ml would bind about 80% of 300 pg $^{57}CoB_{12}$ in this assay. In the presence of equal volumes (0.3 ml) of both cold Vitamin B_{12} (1,000 pg/ml) and hot Vitamin B_{12} ($^{57}CoB_{12}$ 1,000 pg/ml) the binding capacity was 50%.

Determination of chicken serum Vitamin B_{12} binding capacity $UB_{12}BC$.

Duplicate tubes were used throughout the experiment.

a. 0.3 ml of $^{57}CoB_{12}$ solution (1,000 pg/ml) was added to 0.3 ml of borate buffer in a 15 ml glass centrifuge tube.

b. 1.5 ml of distilled water and 0.3 ml of cyanocobalamin standard solution (1,000 pg/ml) were added.

c. Mix twice, various concentration of chicken serum were added to the mixed solution, and incubated for 45 minutes at room temperature.

d. 0.5 ml of PVP coated charcoal was added, mixed and centrifuged at 2,500 rpm for 45 minutes. The supernatant was decanted into a counting tube and was counted in an Auto-Gamma Scintillation Spectrometer (binder supernatant cpm). The amount of chicken serum which had the binding capacity about 80 per cent of $^{57}CoB_{12}$ (300 pg) was used in the assay. On the basis of this determination, a dilution of the binder was made to that its B_{12} binding capacity was reduced to 200 - 300 pg/ml (Newmark et al, 1973). The $UB_{12}BC$ of the undiluted chicken serum used in the present study was diluted to 1: 250.

Calculation of B_{12} binding capacity

The amount of Vitamin B_{12} bound in any tube containing binding protein was calculated as follow:

pg. Vitamin B_{12} bound

$$= \frac{\text{binder supernatnant (cpm) -control supernatant (cpm)}}{\text{Total radioactivity (cpm)}} \times \text{pg}\ ^{57}CoB_{12}\text{added}$$

5.2. Procedure for standardization of $^{57}CoB_{12}$

Every new lot of $^{57}CoB_{12}$ (1,000 pg/ml) prepared for use in the assay was standardized by reverse isotope dilution against the Vitamin B_{12} standard solution (1,000 pg/ml). The sequence of addition and ml of reagents added were shown in Table I.

The actual quantity of $^{57}CoB_{12}$ present could be calculated for any percentage change in the $^{57}CoB_{12}$ binding capacity of the chicken serum by using the following equation:

$$pg\ ^{57}CoB_{12} = pg\ Cold\ B_{12} \quad \frac{B'}{B - B'}$$

where

B = Net cpm of tube containing chicken serum and $^{57}CoB_{12}$

B′ = Net cpm of tube containing chicken serum, $^{57}CoB_{12}$ and standard cold B_{12}

This equation is derived from equation (5) shown under calculation of Vitamin B_{12} concentration.

Assay Procedure

Table I Summarized the assay procedure. All tests were done in duplicate in 15 ml glass centrifuge tube. One half ml of extracted solution was used in the assay.

Calculation of Vitamin B_{12} Concentration

The concentration of Vitamin B_{12} in the extracted solution was calculation from the following formula:

$$\mu g\ B_{12} \text{ in } 100 \text{ gm of sample} = 2 \times pg\ {}^{57}CoB_{12} \times \frac{B}{B'} - 1 \times dil^{n} \times 10^{-4}$$

where

B = Net cpm of chicken serum tube

B′ = Net cpm of tube with unknown sample

Derivation of Formula

Let

M = mass of ${}^{57}CoB_{12}$ added and R is its radioactivity (cpm)

m = mass of ${}^{57}CoB_{12}$ bound by chicken serum and B is its radioactivity (cpm)

Specific Activity of ${}^{57}CoB_{12}$

$$\frac{R}{M} = \frac{B}{m} \tag{1}$$

$$R = \frac{B\ M}{m} \tag{2}$$

Let B = radioactivity (cpm) of ${}^{57}CoB_{12}$ bound by chicken serum after dilution of M by a mass of m′ cold B_{12}

New specific activity after radiodilution

$$\frac{R}{M + m'} = \frac{B'}{m} \tag{3}$$

Substituting for R from equation (2)

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

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

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

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

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

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

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

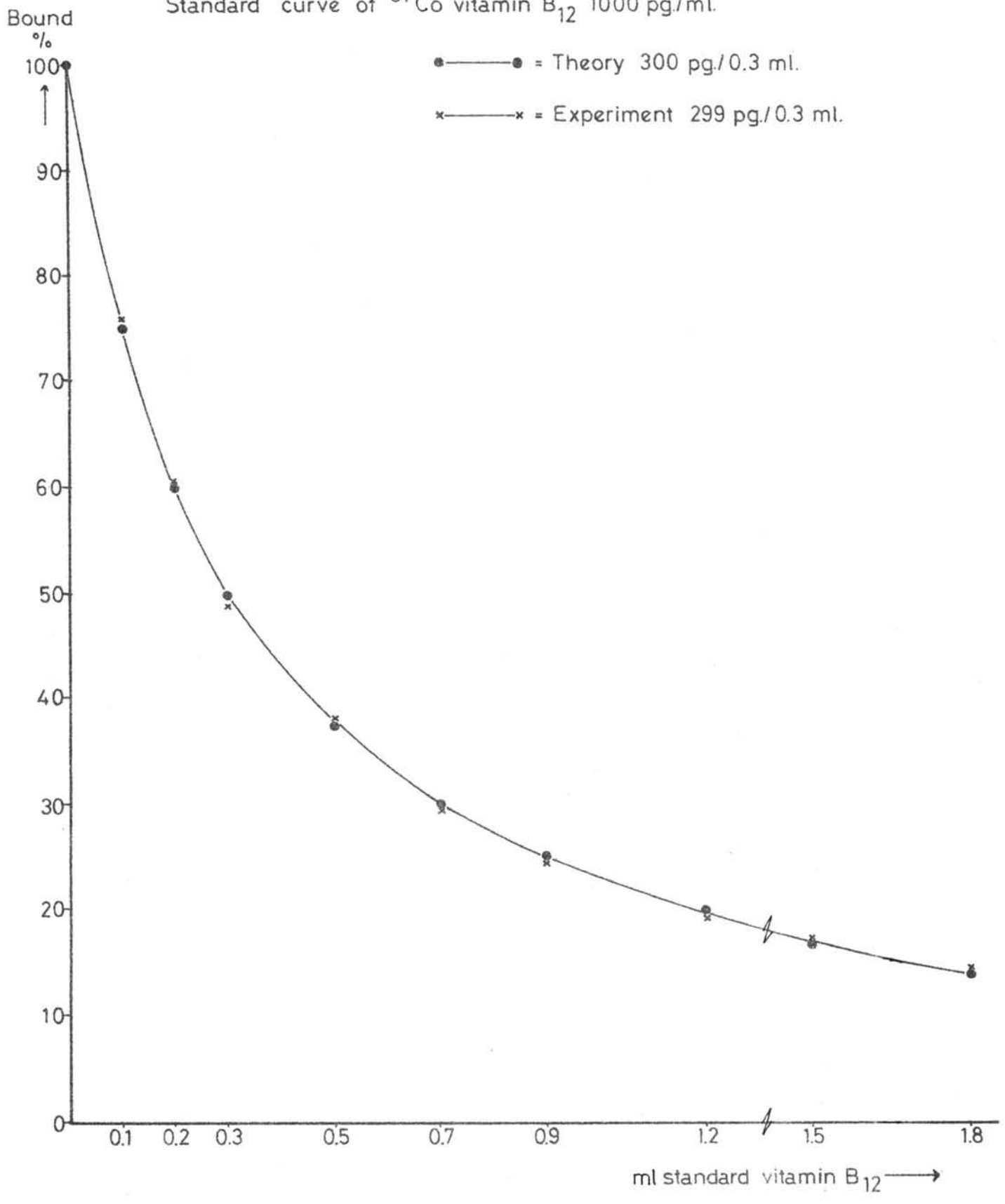
Table I

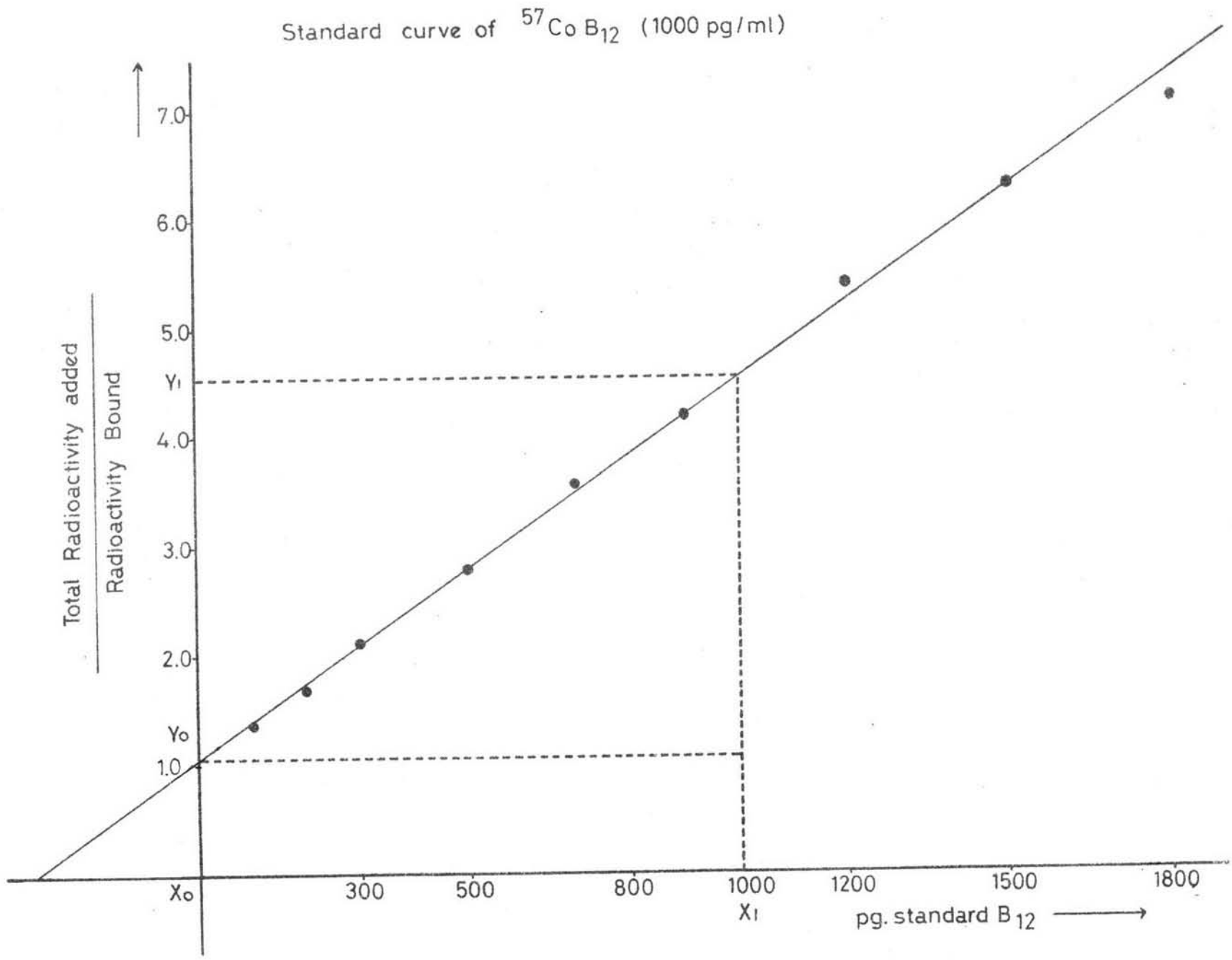
Protocol for the standardization of $^{57}CoB_{12}$ and the assay of Vitamin B_{12}

Tube No.	Sequence and ml. of reagents added											
	Deionized Distilled water	Borate Buffer pH 8.5	Cold B_{12} 1000 pg/ml	57 Co B_{12} 1000 pg/ml		Chicken Serum 1:250		PVP coated charcoal		% Bound	pg $^{57}CoB_{12}$	Total cpm. / BF
1	0	0.3	1.8	0.3	Mix for 10 seconds	0.2	Mix for 10 seconds, Incubate at room temperature for 30 minutes	0.5	Mix, centrifuge at 3000 r.p.m. for 30 minutes Count radioactivity in supernatant	14.28	300	7.1
2	0.3	0.3	1.5	0.3		0.2		0.5		16.67	300	6.26
3	0.6	0.3	1.2	0.3		0.2		0.5		20.0	300	5.37
4	0.9	0.3	0.9	0.3		0.2		0.5		25.0	300	4.19
5	1.1	0.3	0.7	0.3		0.2		0.5		30.0	300	3.55
6	1.3	0.3	0.5	0.3		0.2		0.5		37.5	300	2.81
7	1.5	0.3	0.3	0.3		0.2		0.5		50.0	300	2.16
8	1.6	0.3	0.2	0.3		0.2		0.5		60.0	300	1.74
9	1.7	0.3	0.1	0.3		0.2		0.5		75.0	300	1.37
10	1.8	0.3	-	0.3		0.2		0.5		100.0	300	1.04
11	2.0	0.3	-	0.3		-		0.5		0	300	
Unk Sample	1.3	0.3	Extracted Solu 0.5	0.3		0.2		0.5		Unk.	300	

Unk = Unknown cpm. = Count per minute

pg = picrogram BF = Bound fraction


Standard curve of ^{57}Co vitamin B_{12} 1000 pg./ml.
Bound %
100
90
80
70
60
50
40
30
20
10
0
●——● = Theory 300 pg./0.3 ml.
×——× = Experiment 299 pg./0.3 ml.
0.1
0.2
0.3
0.5
0.7
0.9
1.2
1.5
1.8
ml standard vitamin B_{12} ⟶


Standard curve of $^{57}Co\ B_{12}$ (1000 pg/ml)
Total Radioactivity added / Radioactivity Bound
7.0
6.0
5.0
Y1
4.0
3.0
2.0
Y0
1.0
X0
300
500
800
1000
X1
1200
1500
1800
pg. standard B 12