หอสมุดกลาง จุฬาลงกรณมหาวิทยาลัย

CHAPTER II

MATERIALS AND METHODS.

Materials.

1. Vitamin B_{12} (Cyanocobalamin) Reference Standard (BDH.)
2. ^{57}Co-vitamin B_{12} Standard, specific activity of approximatly 10 µCi./ml., Code No CT 12 for TMSA. (The Radiochemical Center, Amersham, England.)
3. Charcoal. (Norit 'A' neutral pharmaceutical grade decolorizing carbon, Amend Drug and Chemical, Co., Irington, N.J.)
4. Chicken Serum.
5. Polyvinyl-pyrrolidone, PVP, solution. (Chi Sheng Chomical Works Co., Ltd., Taiwan.)
6. Potassium cyanide (E. Merck.)
7. L-glutamic acid (Chromatographically homogenous, BDH.)
8. Sodium tetraborate powder. (M & B)
9. Boric acid (E. Merck)
10. Vitamin B_{12} tablet, 50 and 100 µg. (PS. Chemical)

Instruments.

1. Centrifuge, Internation Portable Refrigerated Centrifuge, Model PR-2. (Internation Equipment, Co., USA.)
2. Electrical Balance (E. Mettler, Type H16.)
3. pH meter (Beckman)

4. Deep Freezer -20 C. (Low Temperature Chest Model CA-230. A, Ohinishi Netsugaku, Co., Ltd., Japan)
5. Mixer (Super-mixer, Labline Instruments, Inc., USA.)
6. Analytical balance (Harvard Trip Balance, Ohaus Scale Corp. Union, N.J.)
7. Micropipetting system (100, 500, and 1000 ul.) with disposible plastic trips (Oxford Sampler Model Q., Oxford Laboratories, Inc.)
8. Packard Autogamma Scintillation Spectrometer (Model 5220)
9. Waterbath (Chicage Surgical and Electrical, CO., Division of Labline, Inc., Illinois.)
10. Magnetic stirrer (Micromagnetic, Labline Instruments, Inc. Illinois.)

Methods for Preparations of Reagents for the Determination of Vitamin B_{12} Content

1. Vitamin B_{12} (Cyanocobalamin) standard solution.

1.1 Stock standard solution (0.1 mg./ml.). Ten milligrams of cyanocobalamin were dissolved in 100 ml. of distilled water. This solution was stored in the freezer -20° C.

1.2 Working solution of cyanocobalamin (1000 pg./ml.) One ml. of stock standard solution (0.1 mg./ml.) 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 solution of 1000 pg./ml. of cyanocobalamin.

2. ^{57}Co-vitamin B_{12} solution. One ml. of ^{57}Co-B_{12} stock solution which has a specific activity of approximately 10 μCi./ml.

or 0.667 µg./ml. was diluted with 666 ml. of distilled water. This solution containing 1000 pg./ml. could be stored at 4°C for up to 3 months without lossing the activity.

3. Sodium borate neutralizing buffer (0.1 M. solution) pH 8.5

Sodium borate buffer was prepared by dissolving 19.068 g. of sodium tetraborate powder in a 500 ml. volumetric flask with 200 ml. of distilled water and the pH was adjusted to 8.5 with M. Boric acid solution. Then made up to a final volume of 500 ml.

4. L-glutamic acid/potassium cyanide extracting buffer, pH 3.3

3.0 grams of L-glutamic acid and 0.01 grams of potassium cyanide were dissolved in 500 ml. of distilled water. This provided a solution of pH 3.3.

L-glutamic acid dissolved more easily in water if the solution is heated. However, it must be ensured that buffer is recooled to room temperature before the addition of potassium cyanide. This solution was prepared immediately before use. To simplify the addition of such a small amount of potassium cyanide, made a 0.4% of potassium cyanide solution and added 0.5 ml. of this to 100 ml. of the L-glutamic acid buffer.

5. Polyvinyl-pyrrolodone-coated charcoal solution. This solution was prepared by adding a portion of distilled water to 10.0 g. of charcoal. The solution was shaked well, then mixed with 14 ml. of the dialysed polyvinyl-pyrrolidone and diluted to 200 ml. with distilled water. This solution can be kept at 4°C not more than two weeks.

6. Chicken serum. Chicken serum, seperated chicken blood which had been allowed to clot in the refrigerator overnight,

was diluted with distilled water. In the presence of equal volume (0.3 ml.) of both cold vitamin B_{12} (1000pg./ml.) and hot vitamin B_{12} (^{57}Co-B_{12} 1000 pg./ml.) the binding capacity was 50%. Its unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) was determined by a modified method of Lau et.al.(1965).

Determination of the vitamin B_{12}-binding capacity of chicken serum ($UB_{12}BC$). Duplicate tubes were used throughout the experiment.

a. 0.3 ml. of ^{57}Co-B_{12} solution (1000pg./ml.) was added to 0.3 ml. of borate buffer in a 10 ml. glass tube.

b. 1.5 ml. of distilled water and 0.3 ml of cyanocobalamin standard solution (1000 pg./ml.) were added.

c. The content were mixed twice and various concentrations of chicken serum were added.

d. The content were mixed again, and incubated at room temperature for 45 minutes.

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e. 0.5 ml. of PVP-coated charcoal was added with continuous stirring of the charcoal suspension using a magnetic stirrer and mixed three times, centrifuged at 2500 rpm. for 45 minutes. The supernatant was decanted into a counting tube and was counted in a Gamma Scintillation Spectrometer (binder supernatant cpm.)

The amounts of chicken serum which had the binding capacity about 80% of 300 pg./ml. of ^{57}Co-B_{12} was used in this assay. On the basis of this determination a dilution of the binder was made up so that its B_{12}-binding capacity was reduced to 200-300 pg./ml. The $UB_{12}BC$ of undiluted chicken serum used in the present studies were diluted to 1:250.

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Calculation of B_{12}-binding capacity. The amount of B_{12} bound in any tube containing binding protein was calculated as follow :

$$\text{pg.}B_{12}\text{ bound} = \frac{\text{binder supernatant cpm. - control supernatant cpm.}}{\text{total cpm.}} \times {}^{57}\text{Co-}B_{12}\text{ added.}$$

Controls. The control should be included in the determination of B_{12}-binding capacity by protein coated charcoal techniques, in order to correct the binder superbatant in the present of $^{57}Co\text{-}B_{12}$ not removed by the charcoal (Gottlieb et. al., 1965). For this purpose supernatant radioactivity (control supernatant cpm.) should be determined after addition of charcoal to tube in which water was added in place of the B_{12}-binding protein. Preliminary experiments showed that the amounts of $^{57}Co\text{-}B_{12}$ not removed by protein-coated charcoal was insignificantly affected, i.e., always less than 2%, by a wide range of pH and ionic strengths (Newmark et. al., 1973).

The total radioactivity. In each experiment, the total activity of $^{57}Co\text{-}B_{12}$ were determined by adding the same amount of $^{57}Co\text{-}B_{12}$ into the water with the equal volume instead of other compounds. The radioactivity (total cpm.) of 0.3 ml. of this solution were then measured.

Methods for preparations of specimens for determination of vitamin B_{12}

1. Cow's milk and its preparations.

1.1 Fresh cow's milk samples were collected from the farm at the Kasetsart University. They were Holstein Fresian and Brown Swiss strains.

1.2 Pasteurized cow's milk samples were prepared by incubating the fresh cow's milk samples at 63°C for 30 minutes(Lampert, 1975)

1.3 Sterilized cow's milk samples were prepared by autoclaving the fresh cow's milk at 121°C (pressure 15 lbs.) for 10 minutes.

2. Powdered milk samples. They were purchased from the market. The samples were diluted with distilled water by using 5.0 grams of powdered milk in 30 ml. of water.

3. Condensed milk and evaporated milk samples. They were purchased from the market. The samples were determined without any dilution.

4. Human milk samples. Milk samples were collected from lactating mothers at the Rajvithi Hospital, Bangkok.

For supplemented group, samples of breast milk were obtained from the mothers giving oral supplement of vitamin B_{12} tablets, 150 and 300 µg. and samples of milk were obtained daily for determination of vitamin B_{12} level.

Subjects in group I took vitamin B_{12} 150 µg. per day for 3 to 5 days.

Subjects in group II took vitamin B_{12} 300 µg. per day for 3 to 5 days.

5. Cheese and butter. The samples were purchased from the market. One half ml. of 0.4% of potassium cyanide solution and 80 ml. of L-glutamic acid buffer were added to 25 grams of sample. The solution was hydrolysed in a waterbath at 100°C for 30 minutes. The extracted solution was adjusted in a volumetric flask of 100 ml., then the extracted solution was filtered through Whatman filter paper. The filtrate was used for assaying vitamin B_{12} concentration.

Assay Method.

Vitamin B_{12} content were assayed by the radioisotope dilution method as described by Lau et. al.(1965). Chicken serum was used as the vitamin B_{12}-binding protein in the present study since chicken serum is known to have a very high unsaturated-B_{12} binding capacity (Green et. al., 1969; Kidroni and Grossiwicz, 1969; Newmark et. al., 1973)

1. General principle. There are three main steps in the technique. Firstly, the unknown vitamin B_{12} is released from its protein complexes by boiling at acid pH. Secondly, the released B_{12} is allowed to compete with a known amount of isotopically labelled B_{12} for binding to a limited amount of B_{12}-binding protein. And lastly, the protein-bound portion is seperated from the remaining free vitamin B_{12} with protein-coated charcoal. Measurement of the amount of the bound ^{57}Co-B_{12} is the determination of the degree of radioisotope dilution, and therefore is the amount of unknown vitamin B_{12} present. In comparing with a reference curve constructed from a series of standard containing known amount of cyanocobalamin, the amount of vitamin B_{12} could be estimated.

2. Procedure for standardization of $^{57}Co\text{-}B_{12}$ Every new lot of $^{57}Co\text{-}B_{12}$ (1000 pg./ml.) prepared for use in the assay was standardised by reversed isotope dilution technique against the vitamin B_{12} standard (1000 pg./ml.). Sequence of addition and ml. of reagents added were shown in table 1.

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

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

where B = net cpm. of the tube containing CS. and $^{57}Co\text{-}B_{12}$

B' = net cpm. of the tube containing CS., $^{57}Co\text{-}B_{12}$ and cold-B_{12}

3. Assay procedure. The assay was summerized in table 1. All tubes in the assay were set up in duplicate. Unknown samples and coated charcoal were dispensed with an automatic sampler (Oxford micro-pipetting system). One tenth ml. of unknown samples, i.e., human milk, evaporated milk, condensed milk, powdered milk and cow's milk was used in the assay, but for extracted cheese and butter one ml. of each sample was used instead of 0.1 ml.

4. Calculation of results. Following the principle of radio-isotope dilution method, the bound radioactivity presented in the super-natant diminished in the presence of increasing amounts of non-radioactive vitamin B_{12}. The standard curve was constructed by plotting the ratio

Table 1. Assay Protocol (Sequence of addition and ml. of reagents to add)

Tube	L-glutamic buffer	Distilled water	Borate buffer	Standard vitamin B_{12} 1000 pg./ml.	^{57}Co-vitamin B_{12} 1000 pg/ml.	Chicken serum in dilution 1:250	PVP - coated charcoal.	bound ^{57}Co-B_{12} / total B_{12} %	Remark.
1	-	1.8	0.3	-	0.3	0.2	0.5	100.0	
2	-	1.5	0.3	0.3	0.3	0.2	0.5	50.0	
3	-	1.3	0.3	0.5	0.3	0.2	0.5	37.5	
4	-	1.0	0.3	0.8	0.3	0.2	0.5	27.3	
5	-	0.8	0.3	1.0	0.3	0.2	0.5	23.1	
6	-	0.6	0.3	1.2	0.3	0.2	0.5	20.0	
7	-	0.3	0.3	1.5	0.3	0.2	0.5	16.7	
8	-	2.0	0.3	-	0.3	-	0.5		
0.1 ml. of unknown samples	0.4	1.3	0.3	-	0.3	0.2	0.5		Unknown samples
1.0 ml. of unknown samples	-	0.8	0.3	-	0.3	0.2	0.5		Standardization of ^{57}CoB_{12}

mixed, capped with cotton wool, heating in waterbath at 100° C for 15 min.

mixed twice

mixed, three times, stand at room temperature for 45 min.

mixed three times, centrifuged at 2500 rpm. for 45 min., decanted supernatant to count in Gamma Scintillation.

of total counts added/counts bound, on the ordinate (y) against the amount of standard vitamin B_{12} concentration added, on the abscissa (x). The standard curve yields a straight line when the system obeys the principle of radioisotope dilution technique(Green et.al., 1974). A typical standard curve was shown in Fig. 2 and 3. The linear line facilitated the calculation of results. From the equation for a straight line, $y = bx + a$, the amount of vitamin B_{12} presented in an unknown (x) may be calculated as :

$$x = 1/b(y - a)$$

where b = the slope

a = y - axis intercept.

y = value of total counts/counts bound for the sample.

Alternatively, the amount of vitamin B_{12} in an unknown sample were calculated from the following equation :

When 1.0 ml. of sample was used in the assay,

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

And when 0.1 ml. of sample was used,

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

where B net cpm. of tube containing CS. and $^{57}Co\text{-}B_{12}$

B' net cpm. of tube containing CS., $^{57}Co\text{-}B_{12}$ and sample.

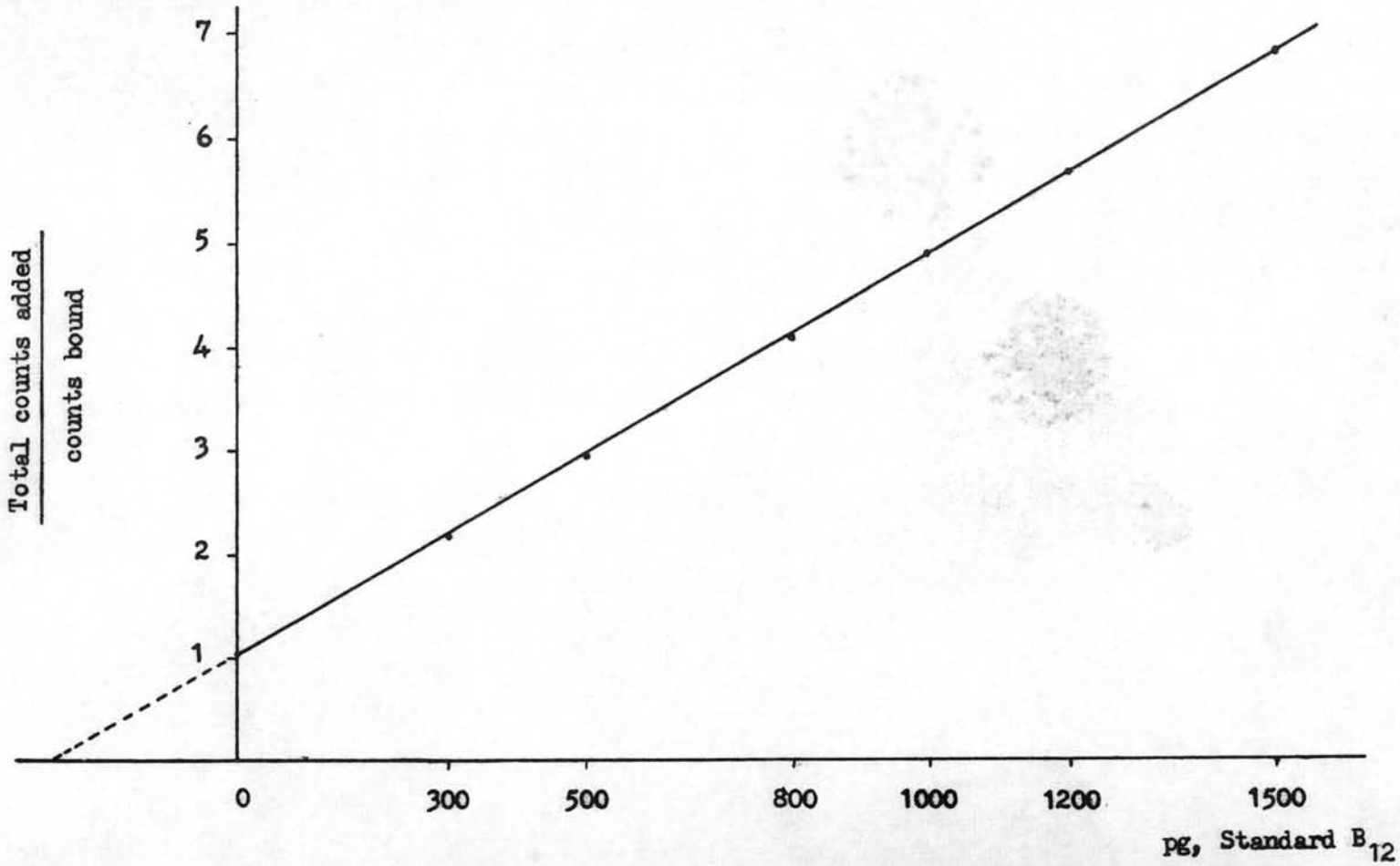

Figure 2 A typical standard curve. Tubes containing 0, 300, 500, 800, 1000, 1200. and 1500 pg standard vitamin B_{12} are shown

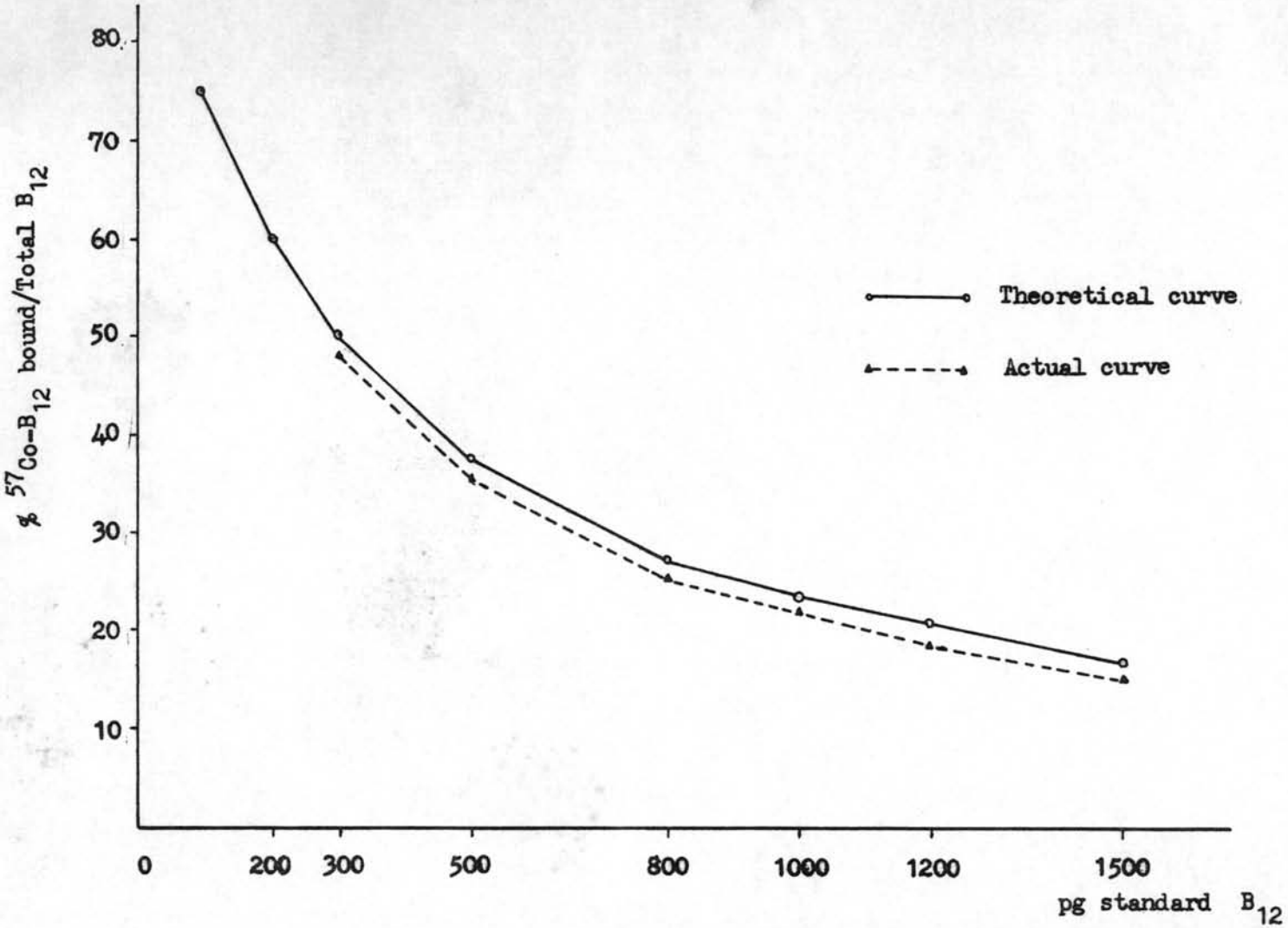

Figure 3 A standard curve. Tubes containing 0, 300, 500, 800, 1000, 1200 and 1500 pg standard vitamin B_{12} are shown