RADIOISOTOPIC DETERMINATION OF SERUM FOLIC ACID, RED CELL FOLATE AND SERUM VITAMIN B_{12} CONCENTRATION OF BLOOD DONORS IN ZARIA

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A Thesis submitted to the Post-graduate School in partial fulfillment of the requirements for the degree of Master of Science, Nuclear Physics.

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DECLARATION

This work, "Radioisotopic Determination of Serum Folic Acid, Red Cell Folate and Serum Vitamin B_{12} Concentration of Blood Donors in Zaria" is the original work of the author. It has never been submitted to any other Institution, Organization or bodies for any award other than to Ahmadu Bello University, Zaria, Nigeria.

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ABSTRACT

A simple model for calculating the concentrations of two important vitamins (folic acid and vitamin B_{12}) in human blood donors using the radioassay technique has been described. An .analysis of this model has shown that there is need to establish optimal working concentrations of reagents different from those recummended by the suppliers of the radioassay kits, depending vn the age of the kit, if high assay sensitivity is to be achieved.

It has also been shown that the radioassay kits of Amersham give very widely spread cocentrations of serum folic acid (SFA)»red cell folate (RCF) and serum vitamin B_{12} (SB_{12}) even with the symptom-free donors. These concentrations showed very poor correlations with those obtained by the microbiological assay using the microorganism L. casei for subjects in Zaria, due prtbably to differences in the response of the two assays.

"The mean SPA, RCF and SB_{12} levels for the symptom-free blood donors in Zaria were found tc be 6.2 + 2.2 μg/l, 318 ± 75ug/l, 383 ± 27 ng/l respectively by the technique. The normal reference ranges for these donors were found to be 1 - 37 (μg/l, 98 - 1035 μg/l and 99 - 1482 ng/l for SFA, RCF and SB_{12} respectively.

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Finally I wish to thank my family for their support and patience during the time of this study.

DEDICATIONS

This piece of work is dedicated to my Father MR. MALLAM GWANZUWANG and my late Mother MRS. TALYIEN MALLAM.

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CHAPTER ONE

1.00 INTRODUCTION AND LITERATURE REVIEW

1.10 AIM

Progress in the knowledge of vitamins, as in other fields of science depends upon the development of effective tools and manipulations for obtaining quantitative data. There has been advancement from the stage at which weeks or months of laborious animal feeding experiments were required before even the approximate amount of a vitamin could be indicated by plus or minus signs, to the point at which the concentration of the better known vitamins can be determined much more accurately by chemical, microbiological or physical procedures within a few days and in some cases, within a few hours. Data secured by these methods have been essential for the isolation, identification and biological evaluation of vitamins, and in studies of their roles in intermediary metabolism and health.

Vitamin B_{12} and folic acid have been areas of special attention mainly because of their relative importance in health. Measurements of these

vitamins has shown that their deficiency is actually responsible for various conditions. In fact, folic acid deficiency has been associated with megaloblastic anaemia in pregnancy, especially twin pregnancy, which is common in most parts of Nigeria (Fleming, 1968a; Ojo, 1965; Osifo, 1970), kwashiorkor (Ebbs, 1966), and sickle cell disease (Watson-Williams, 1965); sprue and liver disease. Vitamin B_{12} deficiency which results from the autoimmune destruction of the parietal cells of the stomach (pernicious anaemia), other gastric conditions, chronic intestinal disease and infestation with D. latum is particularly significant in the pathogenesis of some neurological and psychiatric disorders (Frenkel, 1973; Osuntokun et al, 1969).

Standard methods for the measurement of these vitamins before 1961 were based on the response of microorganisms when fed on these vitamins and are the most conventional procedures in most laboratories today. These methods are, however, time consuming as at least an overnight incubation must be allowed for the microorganism to grow. They are also complex and require the use of standard solutions. The results obtained by these methods can be adversely affected by drugs, especially antibiotics, antimalarials, tranquilizers and anticonvulsants (Chanarin, 1979).

With the advent of the use of radioactive vitamin B_{12} to determine the quantity of the vitamin in unknown solutions (Herbert, 1959) and the independent development of these techniques by Yerlow and Berson and Ekins in 1970, (Ekins, 1971), a number of techniques for the assay of these two vitamins using their radioactively labelled analogues were quickly developed. These radio-assay techniques were reported to be much simpler than the microbiological methods and fast since an assay batch of fifty samples could be completed in a working day especially if automatic radiation counters are available. They were reported to be well reproducible and commercial kits for these radioassays have been developed.

Despite these great improvements, there is still considerable uncertainty caused by differences in vitamin values found by different methods, even by the same methods in different laboratories. The multiplicity of vitamin methodology using radioactively labelled analogues indicates not only the general importance of the subject but also its complexities and difficulties. In fact, the radioassay techniques for the measurement of folic acid and vitamin B_{12} have found little application here in Nigeria. In Zaria particularly, there has been only one report of the measurement of vitamin B_{12} by the radioassay (Fleming et al, 1978). It is therefore the purpose of this work to:

i) Establish the radioassay techniques in Zaria

ii) Apply these techniques and the microbiological assay methods to find serum folate levels (SFA) which gives the current folate status of an individual, red cell folate (RCF) which is a measure of the long standing folate level and the radioassay technique for the measurement of serum vitamin B_{12} (SB_{12}) for 'normal' people. To calculate the correlation between the two assay techniques if any.

iii) To make a comparative study of the levels between symptom free Nigerians, elites and non-elites and caucasians here and abroad in order to identify or locate geographical, social and nutritional differences.

iv) To apply the radioassay techniques to the study of SFA, RCF and SB_{12} levels in various disease conditions encountered in the study area.

v) To extend these techniques to in vivo absorption studies.

However, due to limitation on the availability of materials, only (i) and (ii) could be carried out.

(iii) was partly attempted based on results from the literature while (iv) and (v) needed approval by the appropriate hospital ethics committee. It is worthwhile mentioning that the microbiological method was used mainly for the purpose of comparative studies while the radioassay method was assessed in detail.

1.20 BASIC PHYSIOLOGY OF FOLIC ACID.

Before considering the methods used for the measurement of folic acid and vitamin B_{12}, it is necessary to consider the basic physiology of these vitamins in order to understand how they can be measured. The term 'folic acid' specifically refers to the non-reduced pteroyl-monoglutamic acid, and may be used as a generic term to describe a whole range of water soluble compounds within the vitamin B complex (Malin, 1975). This range, also termed 'folates' include more than a hundred and forty closely related pteroic acid compounds of which only about thirty have been identified (Baugh et al, 1974). The basic structure of folic acid, Fig. 1.1, consist of three portions, namely, pteridine, para-amino-benzoic acid and an l-glutamic acid. It has a molecular weight of about 441 and a melting point of 360°C. It is insoluble in common organic solvents except methanol and phenol.

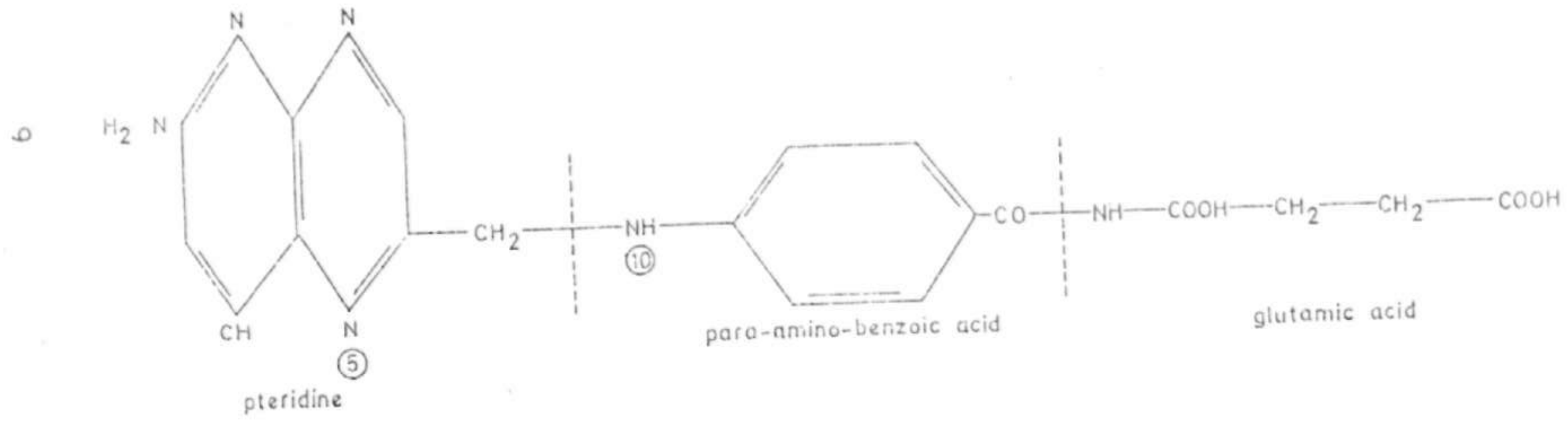


Fig. 1.1 BASIC STRUCTURE OF FOLIC ACID

The solutions are stable in the dark and break up into a pteridine portion and a para-amino-benzoate when exposed to light. It has extinction coefficients of 565, 350, and 195 at 255 nm, 282nm and 365nm in 0.1N sodium hydroxide solution respectively (Chanarin,1979).

Three modifications can be made to the basic structure of folic acid shown in Fig. 1.1. The first modification is conjugation where the monoglutamic acid group is replaced by two or more glutamic acid groups yielding polyglutamates. The second is the reduction of folic acid to physiological forms which are biochemically active. These forms have an extra two or four hydrogen atoms in the pteridine portion thus giving rise to dihydrofolates and tetrahydrofolates. Lastly, an additional one-carbon unit such as methyl (CH_3), formyl (CHO-) or any other one-carbon unit may be added to positions 5 and 10 or held as a bridge between these positions yielding methylfolates or formylfolates. This change is termed condensation.

Folates are present in varying concentrations in food of both animal and plant origin with the highest concentrations found in the liver and yeast. The highest concentrations in raw foods around the study area is in sweet potatoes, 1.94 ng/g dry weight, (Huq, 1981). They are however easily destroyed by oxidation in atmospheric oxygen, heat and ultraviolet

light, yielding pteridine and a para-amino-benzoate. Therefore, the folate content of food declines with storage and drops abruptly with cooking. The reduced polyglutamate forms are the ones found predominantly in nature.

The absorption of folates is limited both by its availability and the ability of the small gut in man to take up folates. Evidence, however, shows that not less than 20%, with an improvement over low doses, of the folate content of food is available for absorption and may go up to 80% or above especially if the folates are in the monoglutamate forms as in bananas and lima beans (Hoffbrand, 1974; Malin, 1975; Chanarin, 1979). Folate requirement for man are influenced by age, state of health, drug use and abuse and dietary intake. Herbert (1977), suggested a minimum of 50 μg as pteroylglutamic acid and up to 300 μg as food folate. Food and Agricultural Organization/World Health Organization (FAO/WHO) recommends as daily intakes:

40 μg for children 0 - 6 months

60 μg for children 7 - 12 months

100 μg for children 1 - 12 years

200 μg for children 13 years and above and

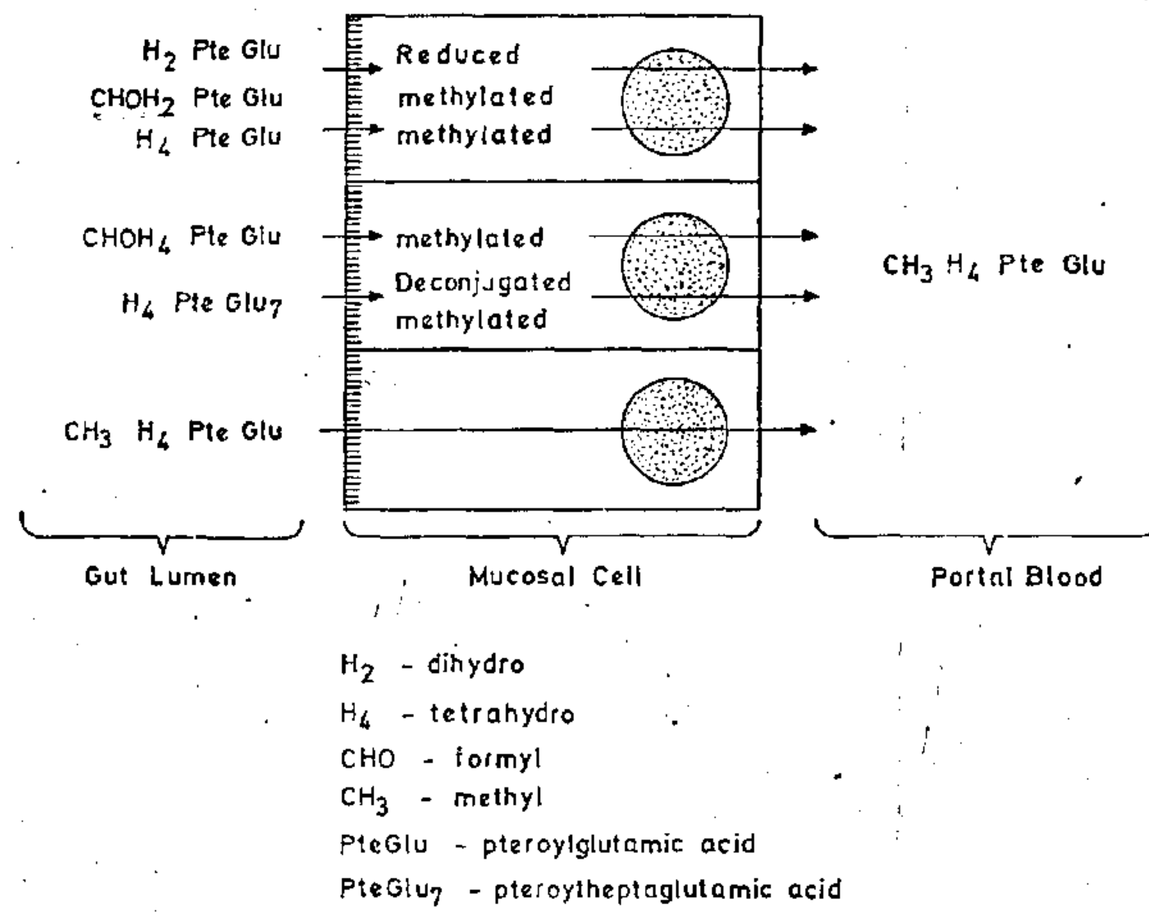
200 - 300μg for pregnant and lactating mothers

(Malin, 1975).

Dietary folates are absorbed by an active process in the jejunum with very little in the ileum and none in the large gut. As they pass through the intestinal cell, the folate molecules are altered so that normally only methyltetrahydrofolate appears in the blood. The polyglutamates have to be deconjugated by '<u>folate conjugase</u>'at a pH optimum of 4.5 to monogluamates and methylated in the intestinal epithelial cell before they enter the portal blood. Non-reduced and partially reduced forms are reduced to tetrahydrofolates by the enzyme' <u>dihydrofolate reductase</u>' and methylated before entering the portal blood, (Fig. 1.2).

After absorption some folates are bound to proteins in plasma for transportation. These protein binders prevent renal excretion of folates and folate uptakes by cells in tissue culture. They also play a role in intra-cellular polyglutamate synthesis. Folates are stored mainly in the liver and kidney and the stores are sufficient to maintain health for only a few months (Herbert, 1962). Folate excretion and loss occurs in the urine, saliva, skin and the bile. Folate in the bile is largely reabsorbed so constituting an enterohepatic circulation.

Folates function as coenzymes in the transfer of single carbon units in a number of important pathways. The carbon units that may be transferred



H_2 - dihydro

H_4 - tetrahydro

CHO - formyl

CH_3 - methyl

PteGlu - pteroylglutamic acid

$PteGlu_7$ - pteroytheptaglutamic acid

Fig. 1.2 BLOCK DIAGRAM OF FOLATE ABSORPTION
(AFTER CHANARIN ET AT, 1976)

include methyl (CH_3-), methylene (CH_2-), methenyl (CH=) and formyl (CHO-). The source of the carbon is generally the three carbon amino-acid serine, which gets transferred to the two-carbon-amino-acid glycine. The most important of these reactions in man include thymidine synthesis by the addition of an extra carbon to de-oxyuridine and the synthesis of purine. Thymidine and purine are required for deoxyribosenucleic acid (DNA) synthesis while purine is require in the synthesis of ribosenucleic acid (RNA) in most dividing cells. Other reactions that have been demonstrated to involve folates in man are the formation of methionine from homocysteine which is important in the metabolism of vitamin B_{12}, and the conversion of histidine to glutamic acid (Hoffbrand, 1974).

Deficiencies of folic acid are responsible for a number of conditions in man already mentioned. The aetiology of these deficiencies include:- Nutritional causes due to food scarcity, prolonged storage, ignorance and overcooking. In Nigeria, nutritional causes have been attributed to seasonal availability and over cooking (Fleming, 1968a). The second cause of folate deficiency is malabsorption due to intercurrent infections such as chronic tuberculosis and tropical sprue. High demands for folates due to physiological factors such as growth and pregnancy especially if accompanied by malaria

or sickle-cell or in twin pregnancy and pathological factors such as tumour growth and haemolysis may also result in folate deficiency. Drug use and abuse especially antimalarials and antibiotics may also interfere with the metabolism of folates and may result in deficiency.

1.30 BASIC PHYSIOLOGY OF VITAMIN B_{12}

Vitamin B_{12} or cobalamin as it is often referred to is a member of a family of closely related compounds called corrinoids. Its basic structure, Fig. 1.3., consist of two portions, namely, a planar corrin ring and a nucleotide portion lying in a plane nearly at right angles to the former. The corrin ring consist of four pyrrole groups linked to a cobalt atom. The nucleotide does not contain purine or pyrimidine as does nucleic acids but instead the base is 5.6 dimethyl-benziminazole with ribose as the sugar. The glycoside linkage is α unlike the β linkage in nucleic acids. It has a molecular weight of about 1355 in the cyanocobalamin form, fairly soluble in water giving an aqueous solution showing absorption maxima at 278, 361 and 550mm and stable at pH 4 to 7.

The β-positions of the corrin nucleus can be substituted by organic ligands such as cyanide, hydroxo, deoxyadenosine and methyl, yielding cyanocobalamin, hydroxo - or aquo - cobalamin

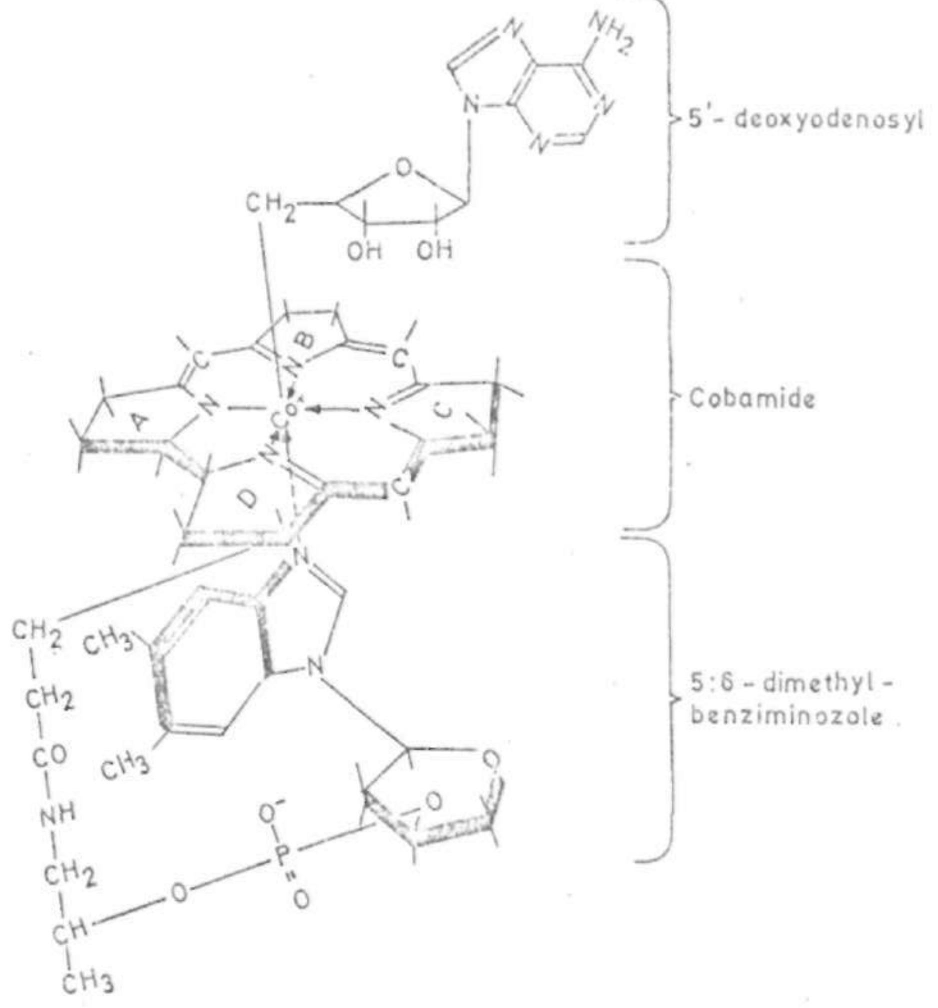


Fig. 1.3 BASIC STRUCTURE OF VITAMIN B_{12}
(AFTER CHANARIN ET AL, 1976).

deoxyadenosylcobalamin and methylcobalamin respectively. Methylcobalamin and deoxyadenosylcobalamin are the predominant forms of vitamin B_{12} found in nature while cyanocobalamin has been the form used most commonly for the study of vitamin B_{12}.

Vitamin B_{12} arises from bacterial synthesis. Animals including man, must therefore obtain the preformed vitamin either by direct absorption from the foregut as in ruminants or from food of animal origin where it is stored mainly in the liver (1 μg/g) and the kidney (0.8 μg/g). It is not required by the plant kingdom and vegetables and fruits are totally devoid of it except by bacterial contamination. Soils and natural water may also contain vitamin B_{12} due to bacterial contamination (Hoffbrand, 1974). Generally, vitamin B_{12} is not destroyed by cooking although under alkaline conditions and in the presence of ascorbic acid, some may be lost at high temperatures (Ellenbogen, 1975).

Seventy percent of vitamin B_{12} taken in with food is absorbed on average. About 1 μg may produce haematological response in a patient with megaloblastic anaemia due to vitamin B_{12} deficiency (Chanarin, 1979). Estimates of total human adult body stores by both microbiological and radioisotope techniques indicate levels ranging from 2 to 11 mg with a mean of 3 - 5mg. To maintain these body stores it has been suggested that daily requirements of vitamin B_{12} for human

adults is about 2 - 5 μg (Chanarin, 1979). The WHO (1970) recommended as daily requirement of food vitamin B_{12} the following figures:

0 -	3 years	0.3 μg
4 -	9 years	1.5 μg
10 -	and above	2.0 μg
	pregnancy	3.0 μg
	lactation	2.5 μg (Mollin et.al, 1976).

The absorption of physiological doses of vitamin B_{12} in the human alimentary tract takes place exclusively in the ileum. The dose absorbed is inversely related to that ingested. When vitamin B_{12} is ingested, it is first released from the food by proteolytic enzymes at an acid pH (Cooper and Castle, 1930,). Upon release, vitamin B_{12} is bound to a macropolysaccharide intrinsic factor (IF) secreted by the parietal cells of the stomach into the gastric juice of animals including man (Castle et al, 1930). It has a molecular weight of about 55000. Presumably, it has two functional sites, a vitamin B_{12} binding site and a receptor site which attaches to a receptor mechanism on the surface of the ileum. The binding sites of IF are protected against enzymatic digestion and heating by the bound vitamin B_{12} during transport to the ileum where IF can then promote the uptake of the bound vitamin. IF also protects the vitamin B_{12} from

uptake by intestinal bacteria.

In the ileum, specific receptors take up the intrinsic factor-vitamin B_{12} (IF-B_{12}) complex. The number of such receptors limit the amount of vitamin B_{12} that is absorbed at any one time. The IF-B_{12} complex is then transported across to the ileal brush borders by a slow energy requiring process where the vitamin is released from the complex into the portal blood. Vitamin B_{12} alone reaches the portal blood after a delay of 3 - 4 hours with maximum blood levels being reached 8 - 12 hours after oral ingestion (Chanarin et al, 1976).

When the amount of ingested vitamin B_{12} is large, however, a passive absorption mechanism becomes operative together with the IF mediated mechanism. This passive mechanism is thought to occur by diffusion (Cooper and Castle, 1930).

After the absorption of physiological amounts of vitamin B_{12}, it enters the blood and is bound to transport proteins, transcobalamin I (TCI) and transcobalamin II (TCII) located in the β- and α-globulin fractions of plasma respectively. Absorbed vitamin B_{12} goes first to TCII which is thought of as the true transport binder and gives up vitamin B_{12} fairly rapidly. Later it appears on TCI whose functions are not well known but it tends to retain the vitamin much longer and is thought of as the

storage form of vitamin B_{12} (Carmel, 1972). TCII is synthesized in the liver while TCI arises principally from the neutrophils and probably to a lesser extent from other tissues. The levels of TCII and TCI have been reported to be higher in Nigerians than in Europeans (Fleming et al, 1978).

Vitamin B_{12} is lost each day from the body mainly through the urine. It has been suggested that this is done at a constant rate proportional to the body stores in the order of about 0.1% daily (Heysell et al, 1966). Absolute losses will therefore become smaller and smaller as the body store is depleted and would help to slow down the process leading to deficiency.

Vitamin B_{12} plays an important role in man for normal blood production and for normal function of nerve tissue. It is needed for normal folic acid coenzyme activity in cells. Vitamin B_{12}, especially as methylcobalamin, is needed for the conversion of homocysteine to methionine (an essential amino acid). This conversion involves the addition of a methyl group. Folic acid participates in this reaction where methyltetrahydrofolate acts as the methyl donor to vitamin B_{12} which is then transferred to homocysteine. Deoxyadenosylcobalamin is concerned with two categories of enzymatic reactions, namely, a number of characteristic rearrangements brought

about by the aid of adenosylcobalamin and the reduction of ribosenucleotide triphosphates to deoxyribose-nucleotide triphosphates which are the building blocks of DNA. This second reaction forms the basis for a microbiological assay of vitamin B_{12} in man and other animals. One characteristic rearrangement, the interconversion between methylmalonyl CoA (methylmalonic acid) and succinyl CoA (Succinic acid) is important in man and has been postulated to be responsible for the pathogenesis of neuropathological lesions associated with vitamin B_{12} deficiency (Beck, 1975).

A deficiency of vitamin B_{12} may disturb any of the aforementioned functions thus leading to ill health. Vitamin B_{12} deficiency, though rare in Nigeria, results most commonly from malabsorption but inadequate intake due to socio-economic and religious reasons (as in vegan and vegetarians) may also cause deficiency. Increased demand as in pregnancy and disturbed metabolism due to antagonist of the vitamin such as drugs are factors that may also lead to its deficiency. Malabsorption may result if the normal sequence of absorption is interrupted in states such as gastric disease (e.g. Addisonian pernicious anaemia, juvenile pernicious anaemia, gastric carcinoma and gastreotomy) and intestinal diseases due to bacterial proliferation after surgery as in the blind loop syndrome. Other intestinal diseases include

Diphyllobothrium latum and *Giardia lamblia* infestation which has been seen in Nigerians but are not important (Wright, 1980), chronic tropical sprue reported by Watson-Williams and Fleming (1966) and ileal resection and by-pass.

Two main effects, megaloblastic anaemia and related phenomena and certain neurological and psychiatric abnormalities are known to occur in human vitamin B_{12} deficiency while elevated serum vitamin B_{12} may be due to some hepatic disease through this has not been confirmed in the case of Nigerians (Fleming *et al*, 1978).

1.40 BASIC PRINCIPLES OF THE RADIOASSAY

As mentioned earlier in section 1.10, folic acid and vitamin B_{12} may be assayed by chemical, microbiological and physical methods. Microbiological methods have found their use in routine assay. They are based on the observation that certain microorganisms require specific vitamin for growth. Using a basal medium complete in all respect except for the vitamin under test, growth responses of the organism are compared quantitatively in standard and unknown solutions. These methods have passed through several modifications and a number of different microorganisms, generally lactic acid bacterial (*Lactobacilli*), have been used (Freed, 1966). In particular, *Lactobacillus casei* (*L. casei*) is the commonly used bacterium for folate assays while

Lactobacillus leichmannii (*L. leichmannii*) and *Euglena gracillis* (*E. gracillis*) are used commonly for vitamin B_{12} microbiological assays.

The radioassays on the other hand utilise the basic mechanism of the absorption and transportation of the vitamins for their assays. The basic principle of the radioassay of folic acid and vitamin B_{12} in any fluid (Fig. 1.4), popularly known as "competitive protein binding radioassay" is a subcategory of "isotope dilution assay". If one wishes to measure compound P. (the vitamin, referred to as the ligand) from the fluid, a radioactively labelled ligand P^* is first added to the biological medium under test. The free ligand may then be extracted from its mileau if necessary. Subsequently, the extracted compound is mixed with a specific binding reagent Q in such relative concentrations that part of P. and P* bound with Q and part remains unbound. The bound and free ligands can then be separated by any of different modalities. The fraction of labelled ligand P* bound to Q will be inversely proportional to the fraction of unlabelled ligand P. This fraction can therefore be compared quantitatively for standard ligand and unknown solution using any dose-response variables.

The basic assumptions underlying the behaviour of the radioassay systems are:-

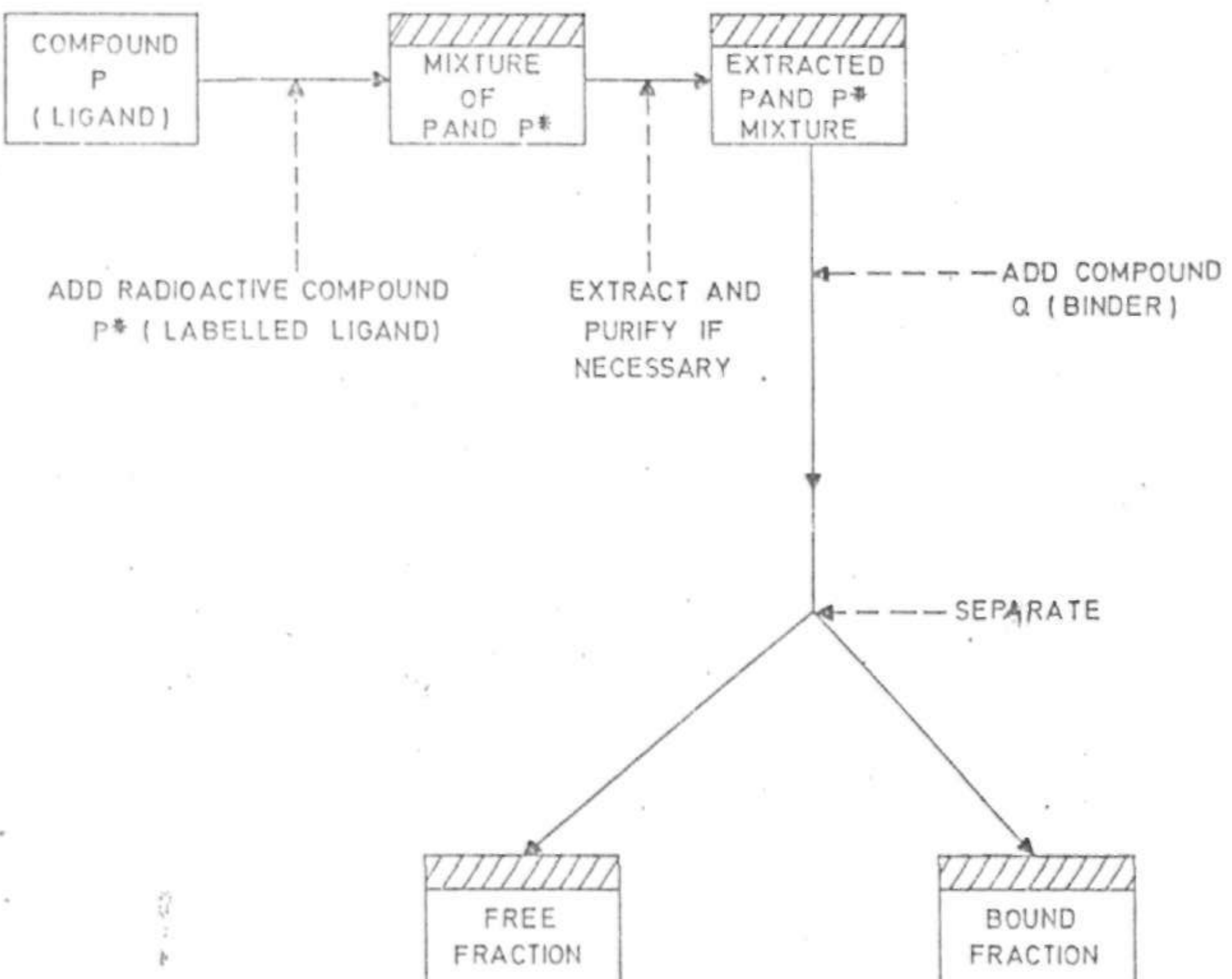


Fig. 1.4 BLOCK DIAGRAM OF THE BASIC PRINCIPLE OF THE COMPETITIVE PROTEIN RADIOASSAY PROCEDURE.

i) The ligand should be present in a homogeneous form, consisting of only one chemical species.

ii) The binding reagent should be present in only one homogeneous chemical form.

iii) Both ligand and binding reagent are univalent i.e there is a one to one molecular reaction.

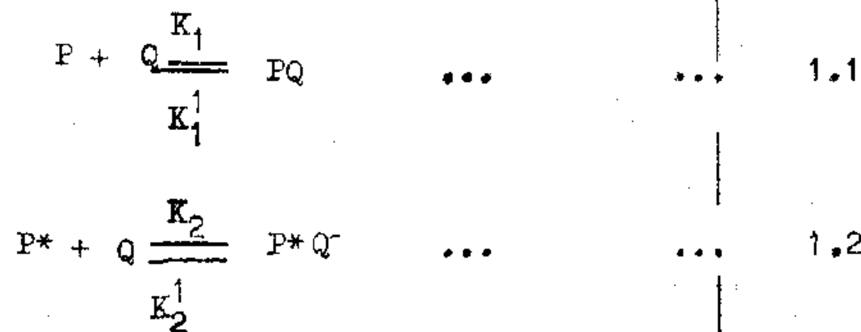
iv) The reaction is according to the first law of mass action.

v) Radioactively labelled and unlabelled ligand have the same physical and chemical properties except for the presence of the label on P*.

vi) The ligand and the binder react until equilibrium is reached.

vii) The bound and free fractions can be separated perfectly without disturbing this equilibrium.

Under these conditions, the radioassay system can be portrayed as:-



$$P + Q \underset{K_1^1}{\overset{K_1}{\rightleftharpoons}} PQ \quad \ldots \quad \ldots \quad 1.1$$

$$P^* + Q \underset{K_2^1}{\overset{K_2}{\rightleftharpoons}} P^*Q \quad \ldots \quad \ldots \quad 1.2$$

Where K_1, K_1^1 and K_2, K_2^1 are the association and

dissociation constants respectively for the first and second reactions.

(1.1) and (1.2) with assumptions (iv) and (v) leads to a Bound to Free ratio (B/F) of

$$B/F = K (q-B) \quad \ldots \quad \ldots \quad 1.3$$

where $K = K_1/K_1^1 = K_2/K_2^1$ is the equilibrium constant, q is the concentration of molecular binding sites on Q and B is the total concentration of the bound ligand (Feldman and Rodbard, 1971).

Equation (1.3) completely characterizes the radioassay dose-response under the assumptions made and provides the basis for using the B/F ratio as the response variable. It may however be re-expressed in terms of other response variables such as bound-to-total (B/T), initial bound to total (BO/T) ratios and the concentration of the labelled ligand-binder complex (P*Q). The last response variable (P*Q) is directly proportional to the total number of counts present in the bound fraction for any given levels of specific activity, reaction volume and counting time (Feldman and Rodbard, 1971).

Taking into account the above mentioned assumptions and reaction kinetics, the concentration of unlabelled ligand, (P), can be expressed as a function of the concentrations of labelled ligand, (P*), binder (Q) the concentration of labelled ligand binder complex (P*Q) and the equilibrium constant K. This yielded the following equation (see appendix 1.1).

$$(P) = (P^*)\left[\frac{(Q)}{(P^*Q)} - 1 - \frac{1}{K((P) - (P^*Q))}\ldots\right] \quad \ldots 1.4$$

If (P^*), (Q) and K are known then 1.4 gives a dose response curve for (P) and (P^*Q) directly. This equation was adopted in the present work.

A number of different modalities for the measurement of SFA and RCF have been describe in the literature employing different binding determinants, radioactively labelled ligand and separation methods. Some of these are summarized in Table 1.1. Most of the earlier methods employed tritiated folates (^{3}H) and iodinated folates (^{125}I) for the radioassay. The procedures are fairly complex and involved the counting of beta radiation. The method of Johnson et al (1977) which is simple and used for the measurement of whole circulating folate using Se^{75} gamma emitter was therefore preferred given by Radiochamical Centre Amersham, England.

Table 1.2 is a summary of the different methods used in the measurement of serum vitamin B_{12} (SB_{12}). Some of the methods had problems of incomplete extraction in the absence of cyanide. In particular the method of Grossowicz et al (1962) did not work well using Nigerian plasma probably due to incomplete extraction from binding proteins and the even more heterogeneous binders in Nigerian plasma (Fleming, personal communication).

TABLE 1.1 SOME FOLATE RADIOASSAY METHODS

AUTHOR	RADIO-ISOTOPE	SEPARATION	BINDER	FRACTION COUNTED	REMARKS
Zalusky and Herbert (1968)	^3H	Whole Milk	Albumin-coated charcoal and entrifugation	Bound fraction in super-natant	Lack sufficient sensitivity to measure endogeneous serum folate β-counting not easy.
Waxman et al (1971)		Powdered milk	Albumin-coated charcoal and centrifugation	"	β-counting problems
Dunn & Foster (1973)	^3H	β=lactoglobolin fraction of milk	Serum-coated charcoal and centrifugation	"	β-counting problems
Kamen & Caston (1974)	^3H	Hog Kidney	Albumin-coated charcoal and centrifugation	"	Not used for whole serum β-counting problems.
Mantzos (1975)	^3H	Pig-plasma	Dextran-coated charcoal and centrifugation		β-counting problems

AUTHOR	RADIO-ISOTOPE	BINDER	SEPARATION	FRACTION COUNTED	REMARKS
Rothenberg et al (1972) Hill and Dawson (1977) Rothenberg and DaCosta (1976)	^{125}I	Purified whole milk	Dextran-coated charcoal and centrifugation	Free fraction on charcoal [illegible]	Iodine very volatile
Johnson et al (1977)	^{75}Se	Porcine Serum	Albumin-coated charcoal and centrifugation	Bound fraction in supernatant	Measures whole circulating folate Employed in present work.

The method of Raven *et al* (1969) which employs the s ame instruments as for the folate radioassay method chosen was preferred especially so that the kits are supplied by the same manufacturers.

TABLE 1.2 SOME SERUM VITAMIN B_{12} RADIOASSAY METHODS

AUTHOR	RADIO-ISOTOPE	BINDER	SEPARATION	FRACTION COUNTED	REMARKS
Barakat & Ekins (1961)	^{57}Co	Pooled Human Serum	Dialysis	Bound Fraction in dialysis bag	Incomplete Serum B_{12} extraction in absence of Cyanide
Grossowicz *et al* (1962)	^{60}Co	Human Serum	Albumin-coated charcoal	Free fraction in charcoal	Inhomogeneous binder in serum
Rothenberg (1961)	^{57}Co	Hog-Intrinsic-Factor	Filtration	Free fraction in supernatant	-
Lau *et al* (1965)	^{57}Co	Hog-Intrinsic-Factor	Albumin-coated charcoal	Bound fraction in supernatant	Incomplete serum B_{12} extraction in absence of cyanide
Frenkel *et al* (1966)	^{60}Co	Human serum	DEAE-Cellulose	Free fraction in charcoal	Inhomogeneous binder in serum

AUTHOR	RADIO-ISOTOPE	BINDER	SEPARATION	FRACTION COUNTED	REMARKS
Raven *et al* (1969)	^{57}Co	Hog-Intrinsic-Factor	Albumin-coated Charcoal	Bound fraction in supernatant	Used in this work (Complete extraction in presence of cyanide)
Wilde & Killander (1971)	^{57}Co	Hog-Intrinsic-Factor	Solid Phase Matrix Sephadex and Cellulose	Bound fraction in supernatant	Binder Stabilised by attaching to solid phase matrix

CHAPTER TWO

2.00 GAMMA - RADIATION: SCINTILLATION COUNTING AND INSTRUMENTATION:

2.10 ELEMENTS OF SCINTILLATION COUNTING

When measuring radioactivity, several different types of measurements can be made. These include factors like the type of radiation, the energy of the radiation, the time of arrival at the detector, the amount of energy deposited in some system per unit time (dose) and the number of particles per unit time emitted by the source (relative or absolute). Relative radioactivity measurements can be used to compare the activity of a series of samples by utilizing identical condition for counting all the samples. The type of radiation to be detected and counted dictates, tp a large extent the type of detecting instrument that may be used, and for a successful radioassay to be carried out, a knowledge of the radioisotopes and the detecting equipment is important. For this reason, some characteristic properties of some of the radioisotopes that have been used in the assay of folic acid and serum vitamin B_{12} are shown in Table 2.1.

In deciding which of these radioisotopes is to be used in a particular radioassay, many different factors, often interrelated and sometimes conflicting, must be considered. The most important of these factors are: the size and number of samples to be assayed, the half-life, nature of emitted radiation and order of activity of the radioactive source, the order of accuracy of the measurement required, the time available for the assay including preparation of sample, and the need to preserve the samples for other test and the relative cost of the equipment needed for the different assays. Gamma radiation sources, being relatively penetrating are not very much absorbed in the sample and usually samples can be assayed directly by these means without any special preparation. This is not true of assay procedures based on beta-radiation measurements which often depend critically upon self-absorption effects. Thus, beta-ray assay procedures are more complex than those based on gamma-rays and usually involve special methods for sample preparation thus increasing the time required for the assay. For these reasons, measurements of folic acid and vitamin B_{12} levels based on selenium-75 (^{75}Se) and Cobalt - 57 (^{57}Co) gamma-ray sources supplied by Amersham International were preferred in this work with a view to developing them to routine work if found suitable.

For the counting of gamma-radiation, gas counters, scintillation counters as well as semiconductor counters have been used. Gas counters are not very sensitive gamma-ray detectors since these radiations are very penetrating and are not easily stopped by the gas detectors. Semiconductor counters, which operate on the same idea as gas counters except that instead of ion-pairs in a gas, hole-electron pairs are produced in the semiconductor detecting materials, have proved to be very efficient for gamma-ray detection. With very high energy resolution and compact sizes, they are very expensive but are especially useful in gamma-ray spectroscopic work. Scintillation detectors on the other hand are usually chosen for gamma-ray assay procedures because of their high intrinsic efficiency for gamma-ray detection and their ability to resolve radiations of different energies (Dubley, 1971).

The detection mechanism of scintillation detectors utilizes the emission of light induced by radiation in luminescent materials called fluors. When a gamma-ray falls on a fluor, it interacts with the material by any of three methods, namely, photoelectric effect, compton scattering and pair production, dissipating all or some of its energy. The photons so produced may cause excitation of the fluor molecules and subsequent de-excitation of these molecules leads to the emission of light flashes

known as scintillation. Pulses of light emitted during the scintillation process may then be detected by a sensitive photocathode of a photomultiplier tube and converted into current pulses with magnitudes proportional to the energy lost in the fluor. These current pulses are then amplified by a factor of $10^5 - 10^8$ in the photomultiplier and then processed further in associated electronic equipment; e.g. amplified by a linear amplifier by a factor $10 - 10^3$, sorted according to amplitude by a pulse-height analyser and counted by a scaler (Fig. 2.1).

Various fluors or scintillators with distinctive characteristics suitable for particular applications have been developed. Solid inorganic fluors are commonly used for gamma-ray or X-ray measurements while liquid organic fluors are used commonly for beta-ray measurements particularly on samples containing radio-isotopes that emit low-energy beta rays. Liquid fluors may also be used for gamma-ray detection in special applications requiring very large detectors. Solid plastic fluors are sometimes used instead of liquids when large gamma ray detectors are required. Table 2.2 gives selected characteristics of the most common inorganic and organic crystals and typical values for liquids and plastic fluors.

Of the inorganic crystal fluors, thallium-activated sodium iodide or NaI (Tl), are amongst the best and commonly used for gamma ray detection

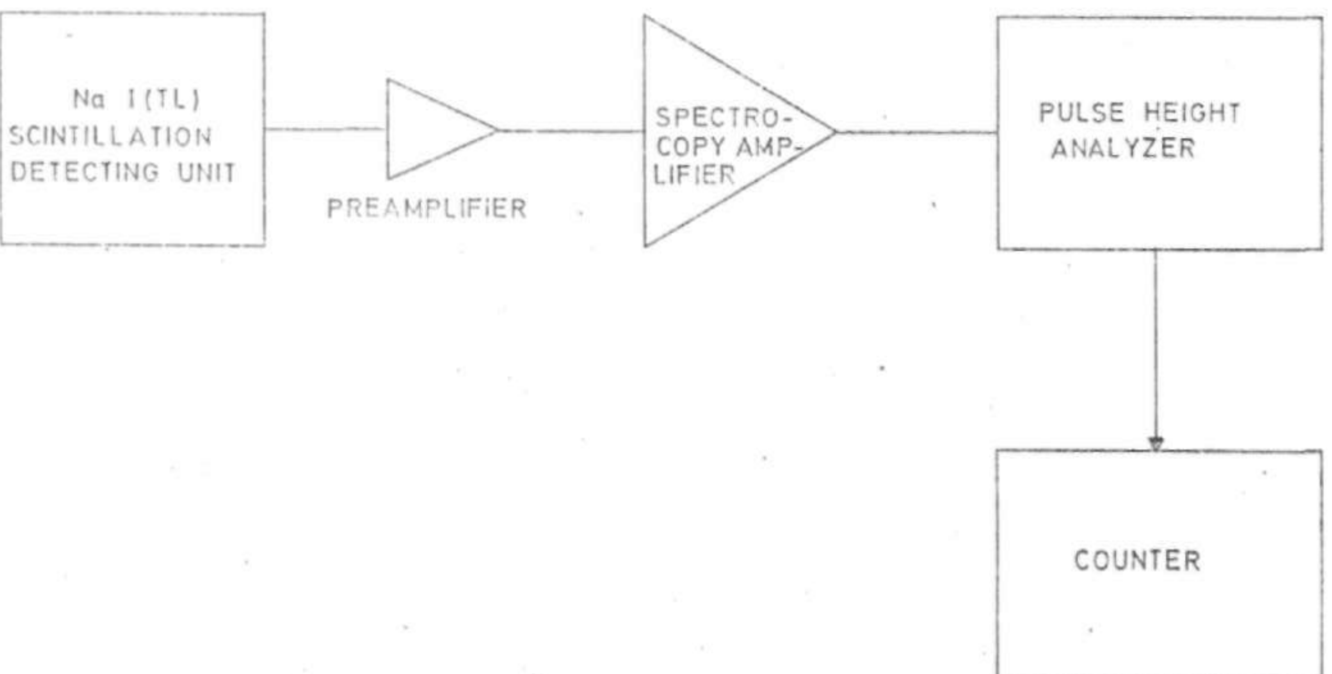


Fig. 2.1 BLOCK DIAGRAM OF SCINTILLATION DETECTION AND COUNTING PROCESS.

TABLE 2.2: CHARACTERISTICS OF VARIOUS SCINTILLATION PHOSPHORS

MATERIAL	WAVELENGTH OF MAXIMUM EMISSION (nm)	DECAY CONSTANT (μs)*	SCINTILLATION CUT OFF WAVELENGTH (nm)	INDEX OF REFRACTION**	DENSITY (kg/m^3)	HYGROSCOPIC	Y-SCINTILLATION CONVERSION EFFICIENCY (%)
NaI(Tl)	410	.23	320	1.85	3.67×10^3	Yes	100
CaF_2(Eu)[1]	435	.94	405	1.47	3.18×10^3	No	50
ScI(Tl)	565	1.0	330	1.80	4.51×10^3	No	45
CaF_2(Eu)	470	.94	450	1.96	3.49×10^3	Yes	35
CsI(Na)	420	.63	300	1.84	4.51×10^3	Yes	80
Liquids	350-450	0.002-0.008	Varies	Varies	0.86×10^3	n/a	20-30
Plastics	350-450	0.002-0.020	Varies	Varies	0.06×10^3	n/a	20-30

* Room Temperature, best single exponential decay constant.

** At emmission maximum.

n/a Not applicable.

1 Primarily used for neutron detection

and counting. Their high density ($3.67 \times 10^3 kg/m^3$) increases the probability that an incident gamma-ray will undergo an interaction with the crystal atom and their high atomic number (Z=53 for Iodine) favours the photoelectric interaction process and thereby the generation of a pulse representing the full energy of the gamma-ray quantum. They have the shortest decay half-life (0.25ns) making them suitable for count rates as high as 10^4 c/sec to be recorded without serious pile-up of pulses provided they can be accepted by the associated electronic equipment. NaI(Tl) crystals convert particle energy to light with the greatest efficiency of any commonly available fluor (approximately 15% energy conversion ratio). They are available in single crystals or polycrystalline forms in a wide variety of sizes and geometries. A typical shape for the crystal is a right circular cylinder with sizes up to 3 inches in diameter and 3 inches high. These 3" x 3" crystals are most widely used in routine gamma-counting and Fig. 2.2 is a diagram of a typical scintillation detector crystal mounted a photomultiplier.

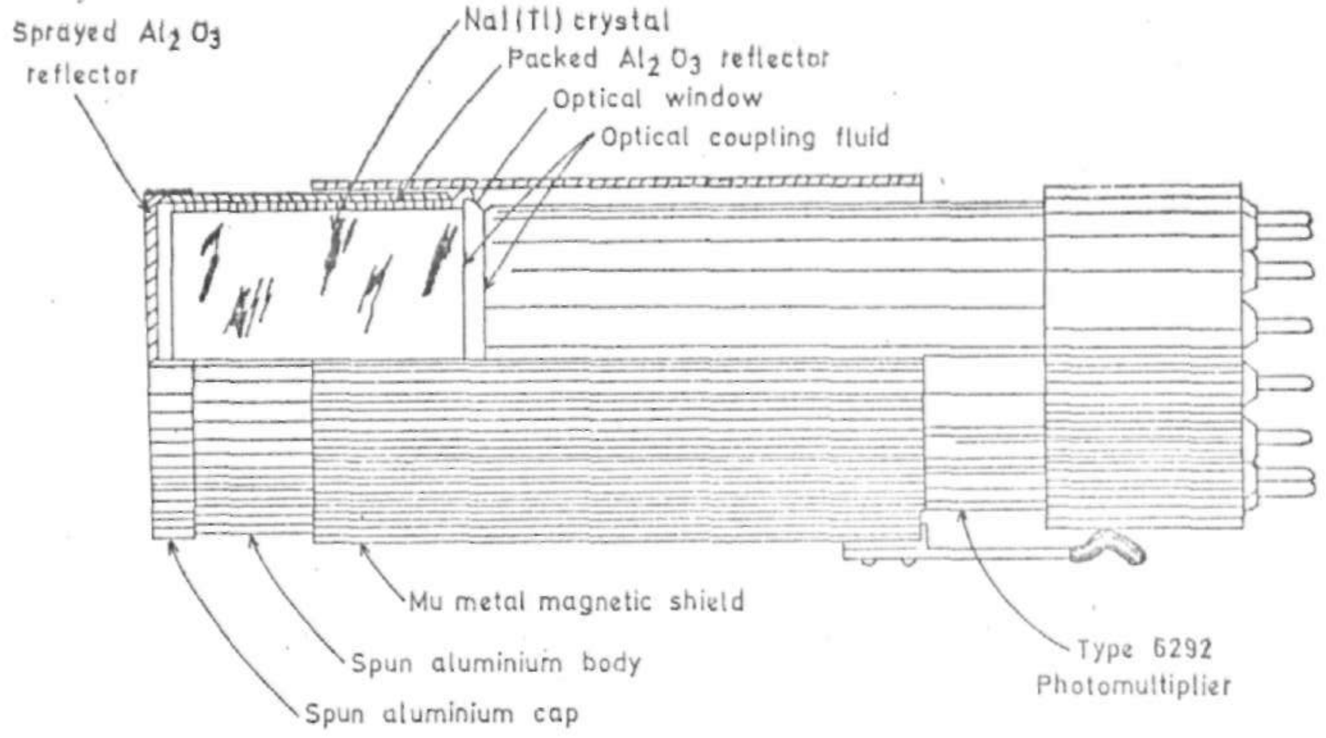


Fig. 2.2 DIAGRAM OF A TYPICAL SCINTILLATION DETECTOR UNIT. A Na I(Tl) CRYSTAL MOUNTED ON A PHOTOMULTIPLIER. (AFTER: BELCHER, 1971)

2.20 GAMMA-RAY DETECTION EFFICIENCY AS DEPENDENT ON CRYSTAL SIZE AND SHAPE

The response of a NaI(Tl) crystal to gamma-rays of various energies depends upon crystal size and shape and upon the location of the radiation source. The intrinsic efficiency, E, of the scintillation counting crystal for the gamma-rays may be defined as:

$$E = \frac{N}{No} \times 100 \quad \ldots \quad \ldots \quad 2.1$$

where No is the total number of gamma-rays per second emitted by the source and N is the number of interactions within the crystal. But if D is the source disintegration rate per unit time and fg is the fraction of this that give rise to gamma-rays, then,

$$No = Df_g \quad \ldots \quad \ldots \quad \ldots \quad 2.2$$

for the simple case of a point source located at the axis of a right circular cylindrical crystal shielded from spurous events.

If E_T is the probability of a gamma-ray striking the detector giving rise to a countable pulse and θ is the solid angle subtended by the detector with the sources, then

$$N = Df_g E_T \theta \quad \ldots \quad \ldots \quad 2.3$$

Substituting 2.2 and 2.3 in 2.1 yields the efficiency of the crystal as:

$$E = E_T \theta \quad \ldots \quad \ldots \quad 2.4$$

The probability of gamma-rays giving rise to countable pulse on striking the detector E_T depends on the energy of the gamma-ray emitted while the solid angle θ depends on the distance from the source to the detector. Plots of the intrinsic efficiency as a function of the gamma-ray energy and source-to-crystal distance show that it increases with decreasing energy and decreasing source to crystal distance for any particular crystal size and shape. An advantage of this therefore is that once the intrinsic efficiency of the crystal has been obtained, another crystal of the same size will have the same intrinsic efficiency for a source at the same distance and direction. The pulse heights may differ between the crystals and another photomultiplier may give a different resolution but the peak areas of the gamma-ray spectra do not depend upon the pulse height or resolution.

2.30 SCINTILLATION COUNTERS FOR SMALL SAMPLES: THE WELL-TYPE SCINTILLATION COUNTER AND ASSOCIATED ELECTRONIC EQUIPMENT

Although detector crystals have high efficiencies for low energy gamma-rays, such gamma-rays are also easily absorbed by the detector housing and sample. At energies below 100keV, detector housing and sample absorption may be well above 10% (Harshaw, 1978). To achieve high counting efficiency especially when the source is contained within a small volume, the

source to crystal distance can be made as small as possible. To this end, 'well-type' crystals have been used (Fig. 2.3). These 'well-type' crystals have geometrical efficiency of nearly unity at the bottom of the well and of 0.5 for an element of sample at the top of the well. A heavy lead shield surrounding the assembly of crystal and photomultiplier tube ensures that the background counting rate due to stray radiation is kept low.

The processing of signals produced by the detector consists of some combination of three basic operations, namely, amplification, shaping and analysis done by associated electronic equipment. Most counting system failure and malfunction occur in the electronic component which together with the geometry, size and quality of the crystal determine the shape of the gamma-ray spectrum and hence the overall counting efficiency of the systems. The basic electronic equipment needed for solid scintillation counting has been shown schematically in Fig. 2.1. The pre-amplifier is located very close to the detector crystal (at times in the detector housing) to minimize distortion of signals from the detector by electrical noise originating in the cable. In the preamplifier, preliminary shaping and amplification of the signal takes place. The amplifier then acts on the signal from the pre-amplifier to further change its size

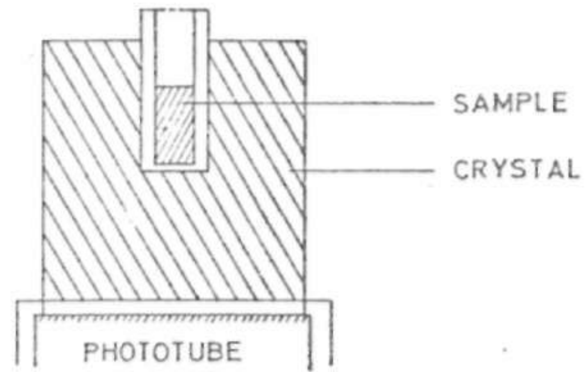


Fig. 2.3 CROSS-SECTION OF A WELL-TYPE NaI(Tl) CRYSTAL SCINTILLATION COUNTER FOR GAMMA-RAY MEASUREMENT ON SMALL SAMPLES.

thus improving the signal-to-cable noise ratio and shape to prevent pulse pile-up. This signal is then passed over to a pulse height selector device (or a pulse height analyser) which produces a standard size pulse each time a signal whose height is greater than some preset height enters the unit. standard output pulse can then be counted on a scaler or used to operate other electronic circuitry as in spectrometers.

2.40 CHARACTERISTICS OF GAMMA-RAY SPECTRA AND DETERMINATION OF OPTIMUM COUNTER SETTING CONDITION

When the number of pulses from a scintillation counting system are plotted against their size or energy, the graph obtained is called the scintillation spectrum. These spectra classify the number of particles or quanta interacting in the scintillator according to their energy deposition. Even though successive gamma-rays from a radioactive source may have the same energy, the corresponding peak in the pulse-height spectrum may have an appreciable width. This arises from statistical fluctuations in the conversion of gamma-ray energy into electrical pulses. The conventional measure of the width of the peak is its full width at half-maximum height. The width of a peak as a percentage of its energy is taken as the energy resolution of the detector. The resolutions helps to seperate peaks and for NaI(Tl) it has

typical values of 10-20%. However, these values may go up with lower gamma-ray energies where the photoelectric interaction process dominates and for the larger crystals within which compton-scattered gamma-rays are more likely to have a subsequent photoelectric interaction and thus be totally absorbed.

For every scintillation counter measurement, use is made only of some portion of the spectrum. High counting rates desired may suggest the use of nearly all the spectrum but the selection of a limited range for example covering the total absorption peak, may give a better overall accuracy by preferential exclusion of the background. This limited range combined with long counting periods gives more accurate count-rates since the relative error in the count-rates reduces with the square-root of the counting period.

To establish the optimum counter settings, preliminary measurements of the pulse-height spectrum, establishing the approximate correspondance between it and the counter settings were made for each of the radioisotopes to be used. The spectra are shown in Appendix 2.5 for ^{75}Se and ^{57}Co for comparison. The resolution of the counter was found to be higher (approximately 50%) than typical values for NaI(TI) crystals due probably to fluctuations in the conversion of the gamma-ray energy to

electrical signals. On the basis of these the counter was set at the peak channels of 85KeV and 98KeV for ^{75}Se and ^{57}Co respectively. A window width of 20% was allowed in both cases.

CHAPTER THREE

3.00 MATERIALS AND METHODS

3.10 SPECIMENS

Blood samples collected by venipuncture from 'normal' adult donors at the Blood Transfusion Service of Ahmadu Bello University Hospital (BTS-ABUH), Zaria, were divided into three fractions of about 2.5 ml and the first fraction allowed to clot and serum separated from it. This serum was stored in bottles containing 5mg of ascorbic acid to prevent auto-oxidation of endogenous folates and labelled for serum folic acid assay. The second fraction was treated in the same manner as the first but stored in bottles without ascorbic acid for serum vitamin B_{12} assay.

The third fraction was added to a specimen bottle containing ethylenediamine tetraacetic acid (EDTA). To release monoglutamates which are the only forms of folates that could be taken up by the microorganism used in the microbiological assay, 0.1ml of blood was added to 1.9ml of freshly prepared ascorbic acid solution (10g/1). This fraction was labelled for whole blood folate assay. These specimens

were stored at $-20^{\circ}C$ until needed and the remaining EDTA treated specimens used for packed cell volume (PCV) readings.

3.20 FOLATE RADIOASSAY:

3.21 Assay Components.

Assay kits for the radioassay of total circulating folate in both serum and whole blood were supplied by Amersham International. Each kit contained a vial each of ^{75}Se - selenofolate (activity, 3 µCi), porcine serum binder and lysine buffer all freeze-dried. Others were five reference standards of N^5-methyltetrahydrofolate in caprine serum, charcoal adsorbent, freeze dried and 50 polypropylene assay tubes. The only additional reagent required for the assay were ascorbic acid and distilled water.

To carry out the assay, other supplementary equipment used include 10ml pipettes, 25ml measuring cylinders, micropipettes and microsyringes capable of dispensing 0.1ml to 1ml accurately. Others were water bath, magnetic stirrer, tube racks, plastic counting tubes, a scintillation counting unit, a centrifuge capable of at least 1000 revolutions per second (rps) and an incubator.

The reagents were reconstituted and used according to the manufacturers instruction.

3.22 Assay Procedure.

Amersham International, the supplying firm recommended the method of Johnson et al, (1977). This procedure was followed in this work. Duplicate aliquots of 0.2ml of each standard or sample (serum or whole blood) were pipetted into polypropylene assay tubes and diluted with 0.4ml of reconstituted lysine buffer to stabilize denatured folate then swirled gently. All tubes were placed in a boiling water bath for five minutes to denature endogenous binding proteins and then cooled at ambient temperatures for 15 minutes. 0.1ml of reconstituted ^{75}Se-selenofolate solution was added to each clear solution followed by 0.1ml of porcine serum protein binder and the contents of the tube mixed properly and incubated at room temperature for 30 minutes. This allowed binding of the folates to the binder to take place.

0.2ml of reconstituted charcoal adsorbent suspension was then added to each tube, mixed thoroughly and centrifuged for 20 minutes at a speed of 200rps. The supernates containing equal volumes of protein-bound folates were decanted into the counting tubes leaving the 'free' folates absorbed on the charcoal. The radioactivity in each bound fraction was then measured by counting in a well-type scintillation counter (J & P Engineering, model Ms 310) set for ^{75}Se as described in chapter Two.

These count rates were corrected for backgroung.

3.23 CALCULATIONS

A preliminary estimation of the mean SFA and whole blood folate (WBF) levels was done by the conventional graphical method. The corrected mean count rates for each of the standard tubes was used to plot a five point dose-response curve of the mean count rate of the duplicate tubes against the concentration of N^5-methyltetrahydrofolate in the standards. The folate levels of each of the unknowns was then interpolated from this curve. The weighted mean of the duplicates were then taken as the mean folate levels. These standard curves are shown in appendice 2.1 and 2.2 respectively. The main method used for calculating the mean SFA and WBF levels was based on equation 1.4. Equation 1.4 was transformed into a parametric equation.

$$Y = P_1 \left[\frac{P_2 P_4}{X} - 1 - \frac{P_4}{P_3(P_1 P_4 - X)} \right] \quad \ldots \quad 3.1$$

where $Y = (P)$

$X = (P^*Q)$ counts per minute (cpm)

$P_1 = (P^*)$

$P_2 = (Q)$

$P_3 = K$

and P_4 = conversion factor from cpm to actual concentrations. To correct for any contamination in

the system and pipetting errors, a counting base level parameter P_5 was introduced so that 3.1 could be written as

$$Y = P_1 \left[\frac{P_2}{2} - 1 + \frac{1}{P_3(Z - P_1)} \right] \quad \ldots \quad 3.2$$

where $Z = \frac{X - P_5}{P_4}$

Initial estimates for the parameter P_1 to P_5 were made on the basis of the activity of the initial labelled ligand and the counting efficiency of the counting unit along with the properties of the ligand and binder. A non-linear least squares program based on the Marquardt (1963) subroutines developed by Verheijen (private communication) was used to fit equation 3.2 to obtain the best estimates for P_1 to P_5. An example of the output of this program is attached in appendix 3.1. With the values of the parameters so obtained for the standards, the mean corrected count rates were then used to calculate the concentrations of SFA and WBF for each sample.

The same procedures for calculation were adopted for both SFA and WBF. The red cell folate RCF levels were then calculated from both SFA and WBF levels by equation 3.3 which corrects for the dilution of the whole blood and for the serum folate concentration in the packed cells of the blood samples.

The equation is

$$RCF = \frac{g \times WBF - SFA\ (1 - PCV)}{PCV} \quad \ldots \quad 3.3$$

where g is the dilution factor which is 20 for the radioassay and PCV is expressed as a ratio. All other symbols have the usual meaning as used earlier.

3.30 FOLATE MICROBIOLOGICAL ASSAY

3.31 Assay Components.

The method adopted for the microbiological assay of folate is based on a technique developed for the turbidometric measurement of the growth of Lactobacillus casei (L. casei) on a medium where the only source of the folate is the standard or unknown sample.

a) Media and Test Organism.

Bacto microassay culture agar (Difco Laboratories), Bacto B_{12} innoculum broth (Difco Laboratories) Folic acid assay medium (BBL) and the test organism, L. casei (NCIB Torry Research Station), stored at 4°C and maintained by subculturing once in three months at the Department of Haemalology ABU were used according to manufacturers instructions.

b) Standard Solutions.

10mg of pteroylglutamic acid (British Drug House), was dissolved in 100ml of 0.1M phosphate buffer at pH7, The stock standard was portioned into 1ml fractions in disposable plastic vials and stored at -20°C. When required for assay a vial was thawed on the day of the assay used once and discarded.

c) Control Serum and Haemolysate.

Plasma from discarded unhaemolyzed stored blood was used to prepare the control serum by adding 500mg of ascorbic acid (Sigma Company) to 100ml of plasma. This was divided into fractions of approximately 1ml and stored at - 20°C until needed, when it may be thawed, used once and discarded.

d) Innoculum.

On the day before the assay, *L. casei* was subcultured from agar to broth and incubated overnight. The overnight growth was re-subcultured on the morning of the assay to broth. Four 30ml screw-capped bottles containing 10ml assay medium and 10ml ascorbic acid solution were autoclaved at the same time with the bulk of the assay medium and ascorbic acid solution After six hours incubation the culture was centrifuged and the supernatant broth discarded. The organisms were then washed thrice in sterile 20ml aliquots of single strength medium and the final resuspension used as the innoculum.

3.32 Assay Procedure

The assay procedure is that of Fleming *et al*, (1971) for the aseptic addition method of Herbert (1966). A fresh solution of medium and 200mg to 100ml aqueous solution of ascorbic acid were prepared and autoclaved at 0.7kg/m^2 (10psi) for 10 minutes on the day of the assay. The solution and all the

glass wares were allowed to cool down before adding the sample to avoid precipitation of the proteins.

A known aliquot of stock standard solution was diluted 1 in 500,000 with sterile ascorbic acid solution. Triplicate standards were then set up in the tubes containing 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0ml of the diluted standard solution and the volume made up to 2ml with ascorbic acid.

The unknown specimens were then pipetted into two sets of duplicate tubes of 0.04ml and 0.02ml respectively. The control serum and haemolysate were set up in a similar way, 2ml of ascorbic acid solution was added to all the tubes using a semi-automatic microlitre dispenser. 2ml of the assay medium was dispensed into all the tubes followed by a drop of innoculum to all except the first triplicate blank (negative blank). A close fitting lid was placed onto the rack and the mixture in all the tubes incubated at 37°C for 48 hours.

3.33 <u>Readings and Calculations.</u>

The tubes were shaken to disperse the <u>L. casei</u> cells before reading turbidity on a spectronic 20 (Bausch & Lomb) at 520nm with the negative blank used to zero the spectronic 20. The average readings of the triplicate standards and the duplicate assay tubes were calculated and the mean of the positive

blank subtracted from all of these.

A dose - response curve relating turbidity reading to the PGA content of the standard tubes was constructed (Appendix 2.4.) The folate content of the assay tubes was then interpolated from the standard curve. If the first serum tube (0.04ml) contained 'a' ng and the second serum tube (0.02ml) contained 'b' then the serum folate was 25a ng/ml and 50b ng/ml respectively. If the haemolysate tubes (whole blood) on the other hand, contained 'x' ng and the second tube contained 'y' ng then the whole blood folate activity is 500x ng/ml and 1000y ng/ml respectively. Calculations for the red cell folate is done using equation 7 of section 3.23 but with the dilution constant g = 1.

3.40 VITAMIN B_{12} RADIOASSAY

Each vitamin B_{12} Radioassay kit from Amersham contained a vial each of cyano (^{57}Co) cobalamin (activity 2.5 µCi), 1ml hog intrinsic factor, six reference standards of cyanocobalamin in buffer solution. 1ml each and half a millilitre vial of one percent weight by volume aqueous potassium cyanide solution. Others are dilute hydrochloric acid, charcoal adsorbent freeze-dried which was reconstituted in the same manner as for folic acid radioassay, and 100 polypropylene assay tubes. The only additional reagent was distilled water and further the same equipment as listed for folate radioassay (section 3.2

were used.

All reagents were supplied ready for use except the charcoal adsorbent. A denaturing reagent was prepared by dispensing the 0.5ml potassium cyanide solution and 1ml cyano (^{57}Co) cobalamin solution into the vial containing dilute hydrochloric acid and mixed thoroughly.

3.42 Procedure.

Duplicate aliquots of 0.2ml of the standards and unknown samples were pipetted into the polypropylene assay tubes arranged and labelled on a metal tube rack. One millilitre of the denaturing reagent was added to each tube, mixed properly and all the tubes placed in a boiling water bath for five minutes then in cold water for another five minutes ensuring in each case that the water level covers the level of the contents in the tubes. 0.2ml aliquots of hog intrinsic factor solutions was added immediately to each tube and mixed thoroughly. All assay tubes were allowed to cool for 30 minutes at room temperature to allow binding of the cobalamin to hog intrinsic factor to take place.

0.2ml aliquots of the charcoal suspension was added into each tube and allowed to stand for five minutes after mixing thoroughly. The tubes were centrifuged for 15 minutes at 1500rps at room temperature and equal volumes of the supernatant

liquid decanted into counting tubes. The radioactivity in each tube was quantified in a well-type scintillation counter set for ^{57}Co counting (Chapter two).

3.43 Calculations.

The calculation of the serum vitamin B_{12} level were done in the same manner as for the serum folic acid except that for the graphical interpolation a six point dose-response curved was used instead of five (Appendix 2.3).

CHAPTER FOUR

4.00 RESULTS, DISCUSSIONS AND CONCLUSIONS

4.10 RESULTS OF THE ASSAYS

Serum folic acid (SFA) assay results by both radioassay and *L. casei* methods for the twenty donors are presented in Figure 4.1. These results were transformed into logarithmic value for statistical analysis. The means and standard deviations calculated for both assays are indicated in fig. 4.1. While the mean SFA levels for the twenty donors was found to be the same by both assay procedures (6.2 μg/l), the distribution of SFA levels about these means was much wider for the radioassay than the *L. casei* assay. Five donors has SFA radioassay levels above the maximum *L. casei* assay level of 13 μg/l while another five had SFA radioassay levels below the *L. casei* assay minimum of 3 μg/l. The sample means and other statistical values estimated for the assays are shown in Table 4.1.

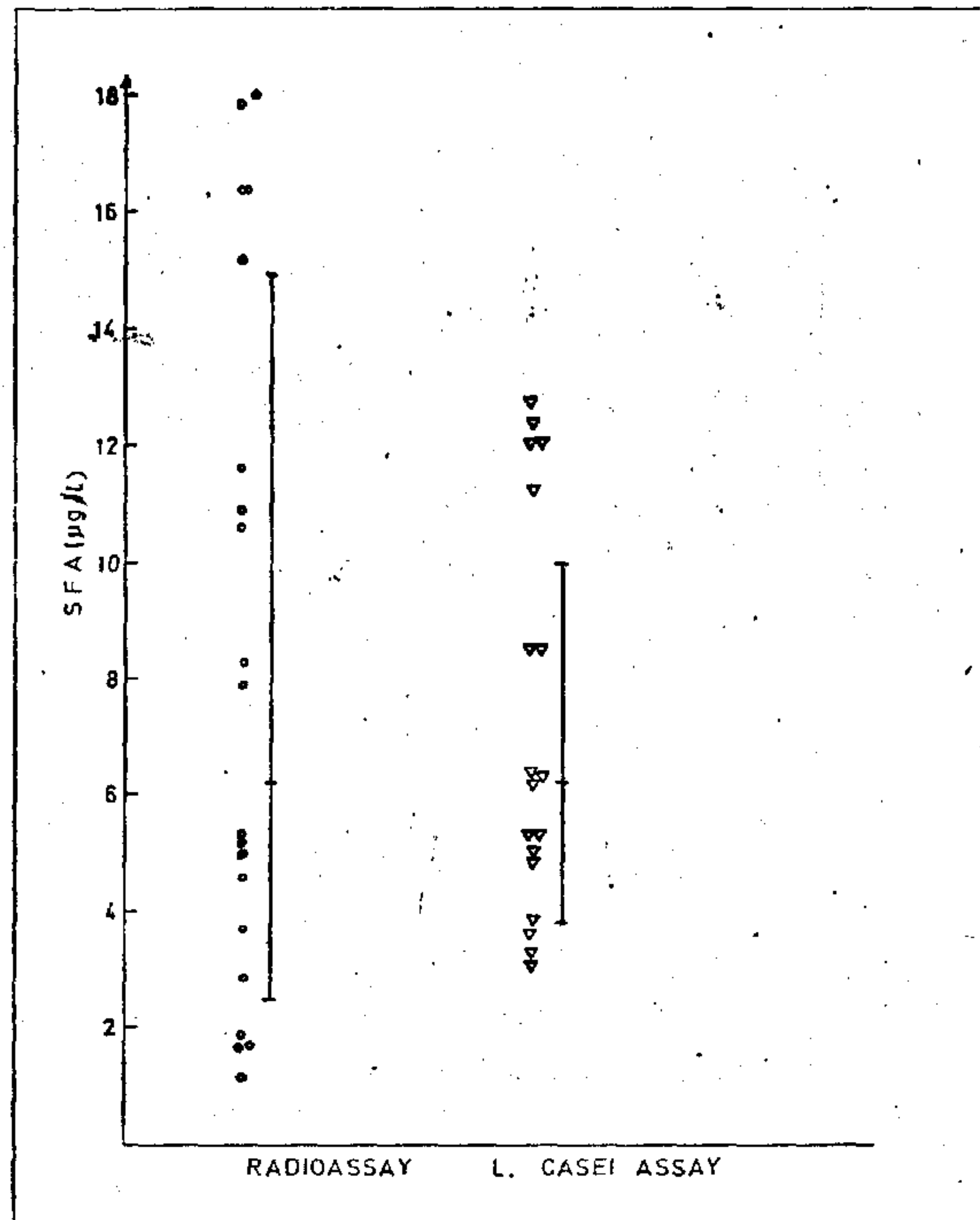


Fig. 4.1 SERUM FOLIC ACID LEVELS (µg/l) FOR 20 DONORS WITH MEAN ± 1SD

TABLE 4.1 RADIOASSAY AND MICROBIOLOGICAL ASSAY FOR SERUM FOLIC ACID, RED FOLATE AND SERUM VITAMIN B_{12} CONCENTRATIONS FOR 20 BLOOD DONORS

VITAMIN	Concentration in µg/l for folate and ng/l for Vitamin B_{12}			
	METHOD	MEAN	RANGE	95% CONFIDENCE LIMITS
SERUM FOLIC ACID (SFA)	Radioassay	6.2	1.2-23.3	1.0-36.9
	L. CASEI	6.2	3.2-12.6	2.3-16.3
RED-CELL FOLATE (RCF)	Radioassay	318.4	123.8-981.5	97.9-1035.1
	L. CASEI	301.0	157.9-370.4	195.4-464.5
SERUM VITAMIN B_{12} (SB_{12})	Radioassay	382.8	92.0-1123.0	98.9-1482.4

Figure 4.2 gives the distribution of the red cell folate, RCF, levels measured by both radioassay and L. casei assay procedures with the mean and standard deviation indicated. The sample mean RCF level by the radioassay procedure (318.4 μg/l) was found to be significantly higher ($p = 0.05$) than that calculated by the L. casei assay (301 μg/l). Again the distribution of RCF radioassay levels is much wider than for the L. casei assay (Table 4.1). Two donors had RCF radioassay levels below 150 μg/l while six had levels above 370 μg/l the minimum and maximum levels found by the L. casei assay respectively.

The serum vitamin B_{12} radioassay results are presented in Figure 4.3. The mean vitamin B_{12} level was found to be 382.8ng/l. One donor had a serum vitamin B_{12}, SB_{12}, level above 1000ng/l while four had levels below 200ng/l the normal range recommended by the manufacturers of the kits. No microbiological assay for serum vitamin B_{12} was performed.

4.20 DISCUSSION OF ASSAY RESULTS

4.21 Sources of Measurement Errors and Factors Affecting the Radioassay Sensitivity.

Since the microbiological assay procedures have been standardized and are in routine use in the study area, only the sources of measurement errors and factors that may affect the radioassay sensitivity will be considered. The radioassay results were calculated using equation 3.2 (Chapter III).

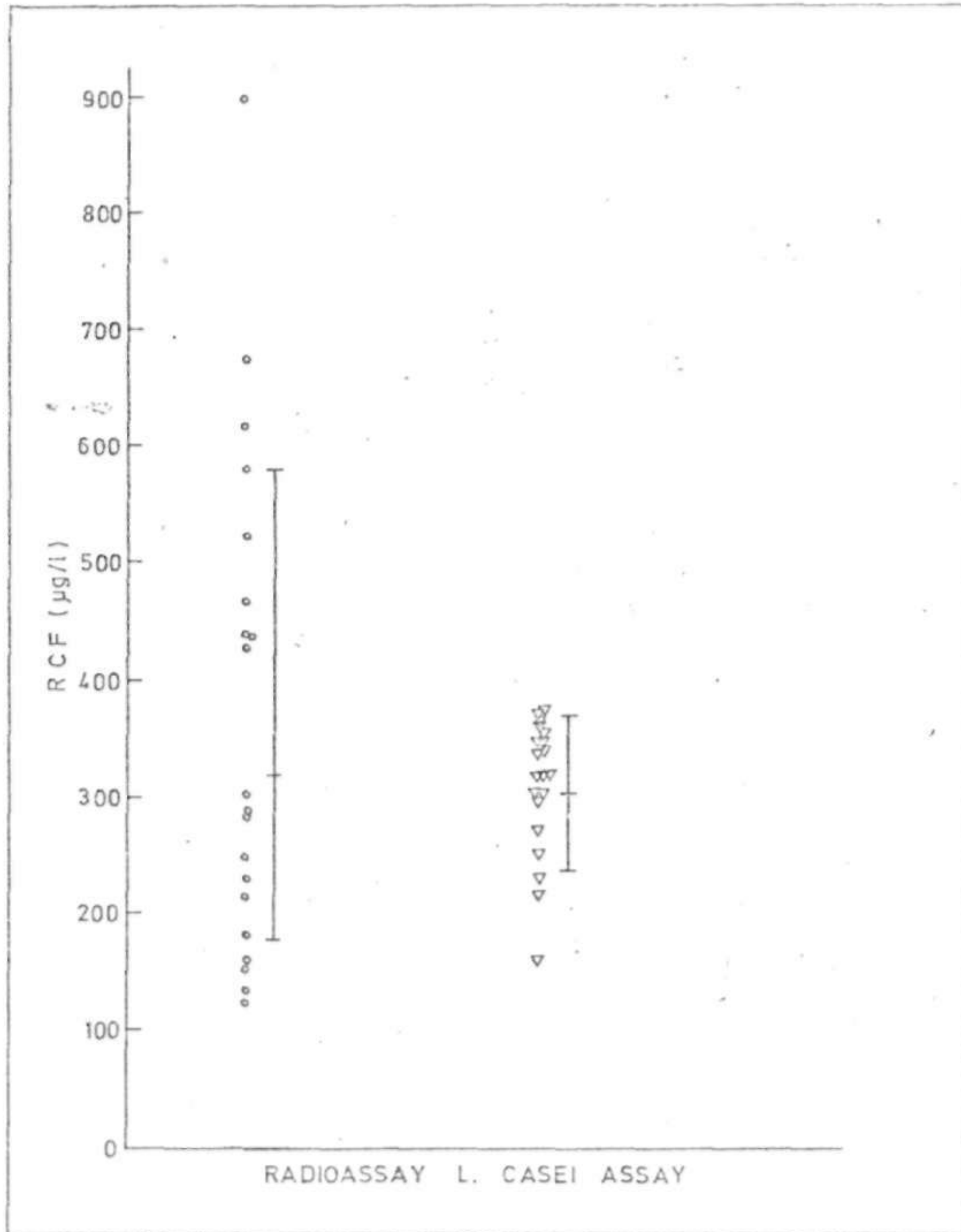


Fig. 4.2 RED CELL FOLATE (µg/l) FOR 20 DONORS WITH MEAN ± 1SD

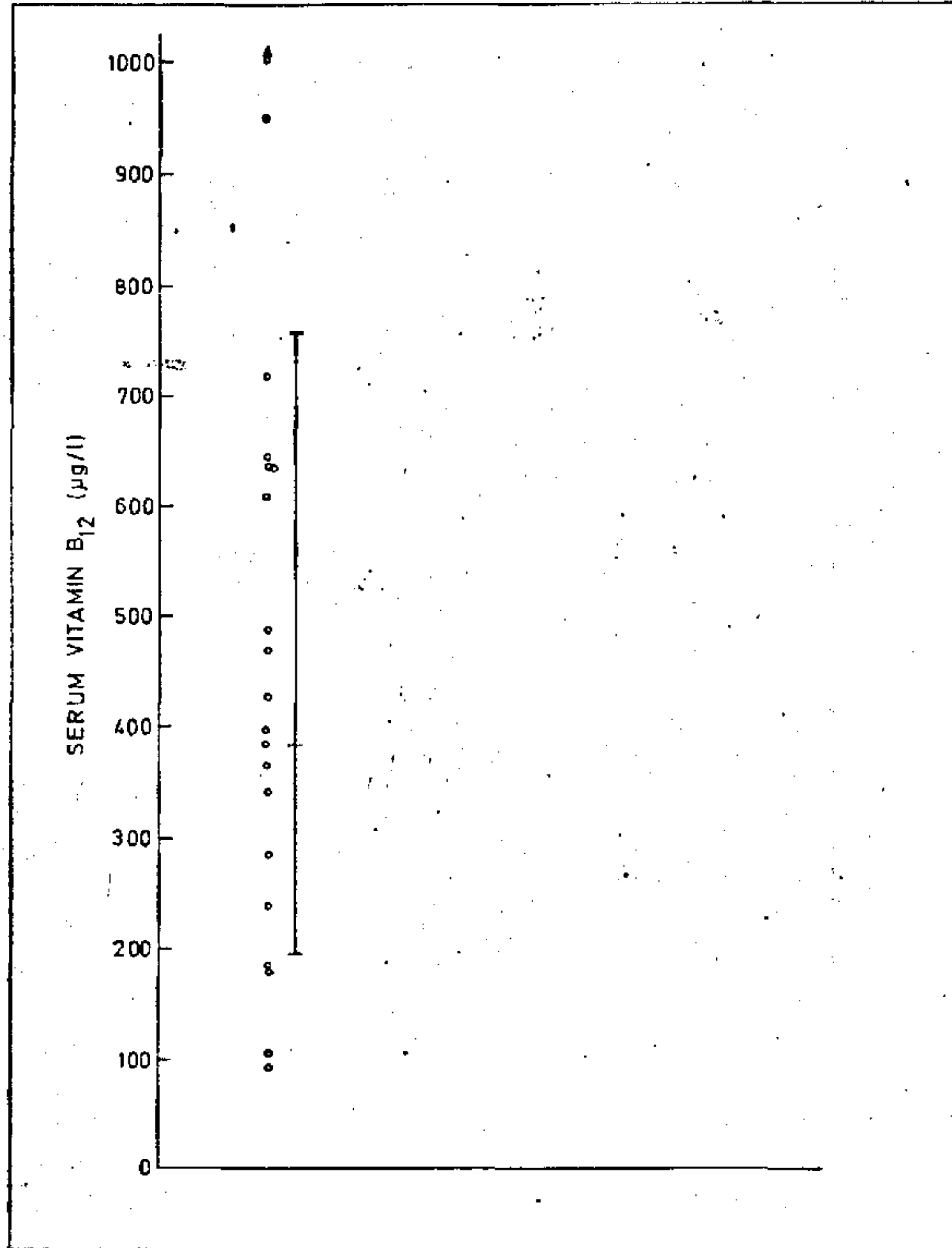


Fig. 4.3 SERUM VITAMIN B_{12} (µg/l) FOR 20 DONORS WITH MEAN ± 1SD

It is therefore expected that any errors in the determination of the parameters in this equation will affect the final sensitivity of the assay and an analysis of this equation should give guidelines on how these errors might be reduced.

Sources of measurement errors in the determination of vitamin levels by this method might arise from random variations in pipetting of the ligands, the binder and buffer solutions. These random variations will therefore lead to an erroneous determination of the starting concentrations of these reagents and hence wrong quantities substituted in equation 3.2. These random variations can be taken care of by use of automicrodispensers in pipetting. Similarly, there may be contamination in the system arising from the pipetting of the reagents and low specificity, especially if kits are old. This leads to cross-reactivity and hence to poor estimation of the vitamins in question. Random variations in the temperature, ionic strength and/or pH may result in gelling of the samples during assay and variability in the equilibrium constant K and hence erroneous determinations. If all the aforementioned sources are removed by the introduction of the counting base level earlier designated as P_5, then the only major source of measurement error left will be in the variation of the count rates resulting from the random nature of radioactive decay and

the counting efficiency of the counting equipment.

Considering equation 3.2, and assuming that the concentration of the labelled ligand (P*Q) in the bound fraction is small compared with the initial concentration of the labelled ligand started with, then equation 3.2 reduces to:

$$y = \frac{q_1}{x - q_3} - q_2 \quad \ldots \quad \ldots \quad 4.1$$

where y = concentration of unlabelled ligand

x = countrate

$q_1 = p_1\ p_2\ p_4$

$q_2 = p_1 + \frac{1}{p_3}$

$q_3 = p_5$ and p_1, p_2, p_3, p_4 and p_5 have meanings as designated in chapter III. By differentiation of the above formula one can derive the error:

$$\left|\frac{dy}{y}\right| = \frac{q_1}{y\ (x - q_3)^2} \times \left|\frac{dx}{x}\right| \quad \ldots \quad \ldots \quad 4.2$$

where $\left|\frac{dy}{y}\right|$ is the relative error in y and $\left|\frac{dx}{x}\right|$ is the relative error in the countrate x. Substituting x yields.

$$\left|\frac{dy}{y}\right| = \frac{(y + q_2)^2}{yq_1} \left[\frac{q_1}{(y + q_2)} + q_3\right] \frac{dx}{x} \quad \ldots \quad 4.3$$

Therefore a plot of the relative error in the concentration as a function of the concentration should vary as equation 4.3. Figures 4.4 to 4.6 are plots of these relative measurement errors as a function of the concentrations for SFA, RCF and

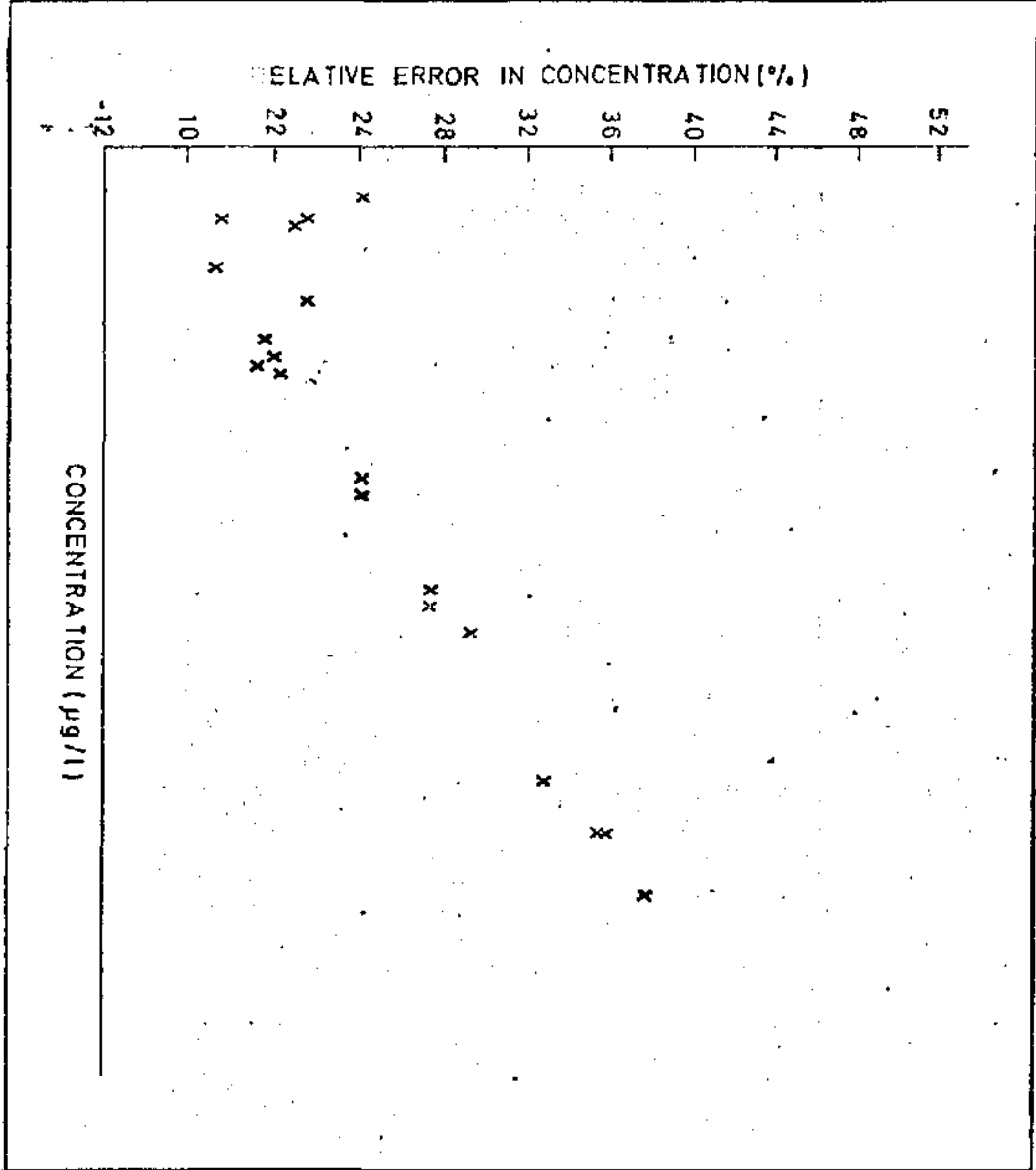


Fig. 4.4 PLOT OF RELATIVE ERROR IN CONCENTRATION (%) AGAINST THE CONCENTRATION FOR SERUM FOLIC ACID RADIOASSAY

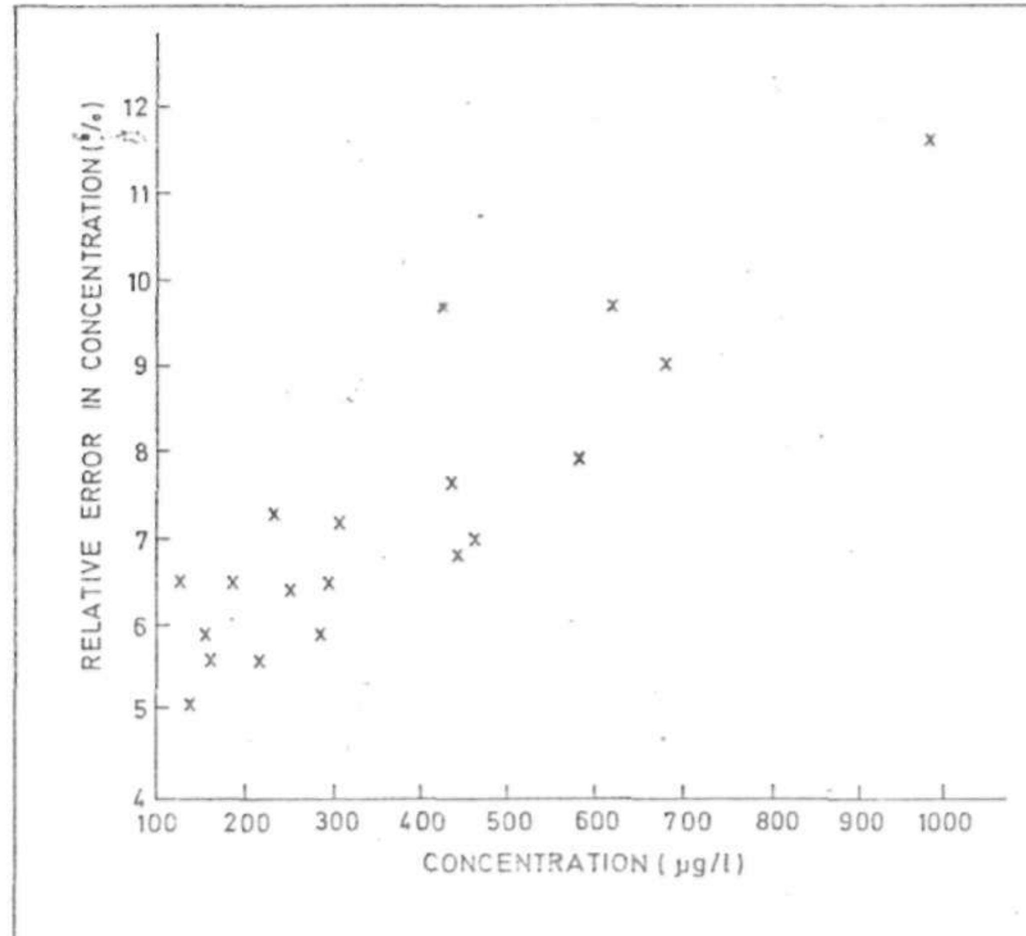


Fig. 4.5 PLOT OF RELATIVE ERROR IN CONCENTRATION (%) AGAINST THE CONCENTRATION FOR RED CELL FOLATE RADIOASSAY

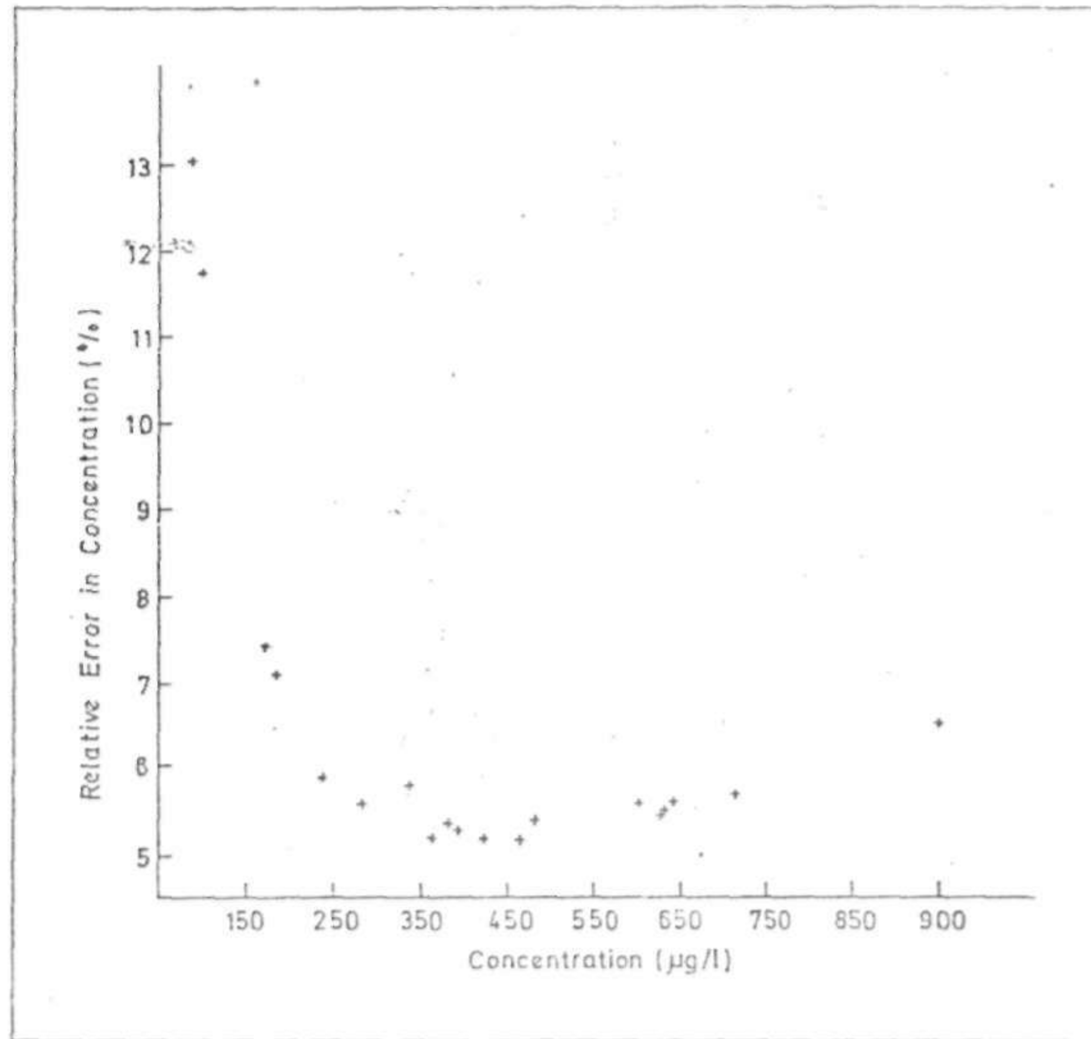


Fig. 4.6 PLOT OF RELATIVE ERROR IN CONCENTRATION (%) AGAINST THE CONCENTRATION FOR SERUM VITAMIN B_{12} RADIOASSAY

SB_{12} respectively. Figure 4.7 show theoretical plots of equation 4.3 for various values of q_3/q_1 assuming that the counting time was long enough to produce a 1% error in the countrate.

Plots in Figure 4.4 to 4.6 are very similar in shape to those of the theoretical plots (Figure 4.7). However, from the theoretical plots, there is a decrease in the relative magnitude of the errors as the ratio of q_3 to q_1 is decreased. This decrease implies that the sensitivity of the assay is improved or that measurement errors are reduced. But the ratio of the parameters

$$\frac{q_3}{q_1} = \frac{p_5}{p_1 p_2 p_4}$$

will decrease if the counting base level p_5 is decreased, i.e., if the pipetting and other random errors are reduced to minimum, or if the countrates are increased relative to the counting base level p_5. Therefore an increase in the concentration of the labelled compound ($(P^*) = p_1$) will improve the sensitivity. The same effect will be achieved if the concentration of the binder ($(Q) = p_2$) is increased as these will lead to higher countrates in the bound fraction. Longer counting times that will reduce the relative error in the countrates especially with old assay kits and poor efficiency counters are desirable.

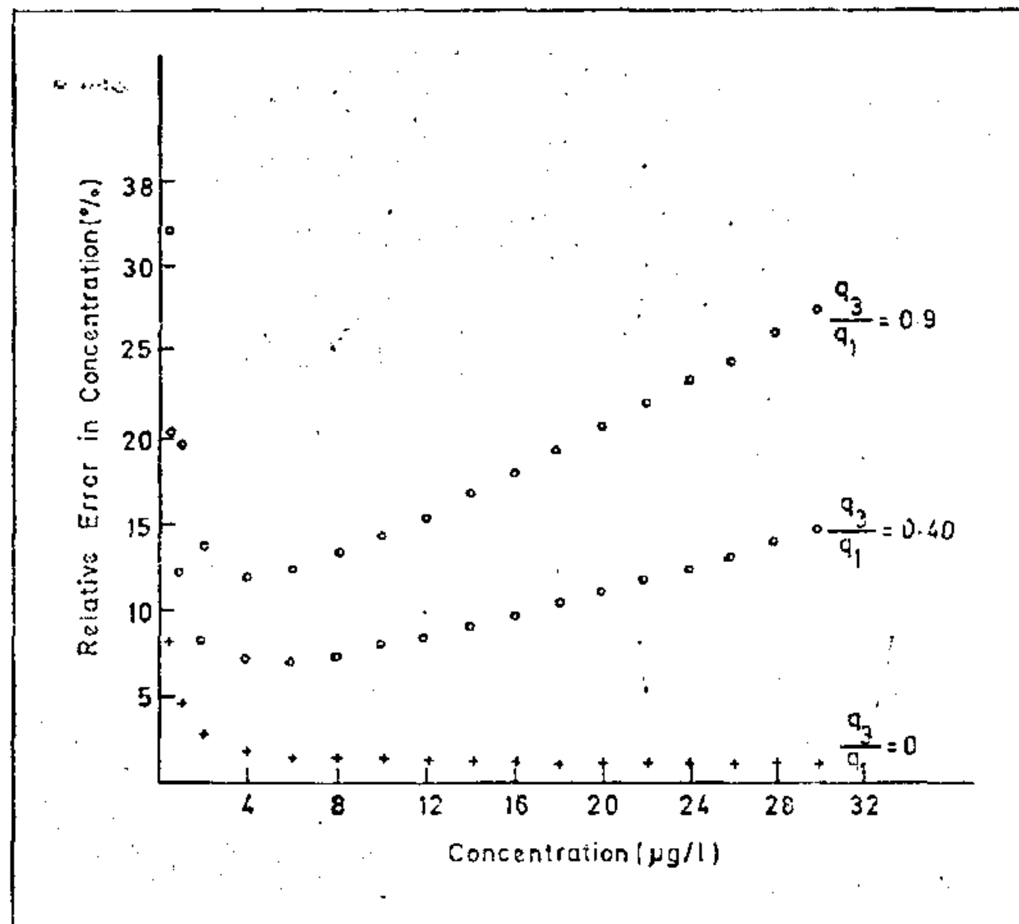


Fig. 4.7 THEORETICAL RELATIVE ERROR PLOTS FOR RADIOASSAY FOR q_3/q_1

4.22 Correlation of the Radioassay and *L. casei* Assay Results for Folic Acid.

Regression analysis for the two methods are summarised in Table 4.2 with the linear correlation scatter plot given in Fig. 4.8. A look at the table reveals that the linear correlation between the radioassay and *L. casei* assay was generally disappointing for the twenty donors taken together (correlation coefficients r = 0.22 for both SFA and RCF with slopes of 0.59 and 0.89 respectively). If the SFA levels and the WBF levels were taken together the correlation coefficient for the donors was not any better (r = 0.33). In fact a similarly poor linear correlation and wide radioassay spread have been observed with the ^{75}Se-selenofolate radioassay kit in Nigerians (Akinsete-personal communication).

However, when the assay results of four donors which showed significant disparity between the radioassay and the *L. casei* assay were excluded, the linear correlation improved significantly for the SFA assays and the SFA/WBF assays taken together (r = 0.76 and 0.68 respectively). This did not however show any significant improvement of the correlation for the red cell folate assays. The lack of improvement of the correlation for the RCF could be due to the fact that in calculating the RCF levels by the radioassay method, a dilution factor

TABLE 4.2 REGRESSION ANALYSIS FOR FOLATE RADIOASSAY AND MICROBIOLOGICAL ASSA

VITAMIN	X-AXIS	Y-AXIS	COEFFICIENT OF CORRELATION (r)	SLOPE(a_1)	INTERCEPT(a_0)	NO. OF SAMPLES
SFA	*L. Casei*	RIDA*	.76	1.25	0.23	16
			.32	0.59	0.32	20
RCF			.38	0.82	0.42	16
			.32	0.89	0.45	20
SFA/ BF			.68	1.20	0.18	32
			.33	0.67	0.29	40

*RIDA = RADIOASSAY

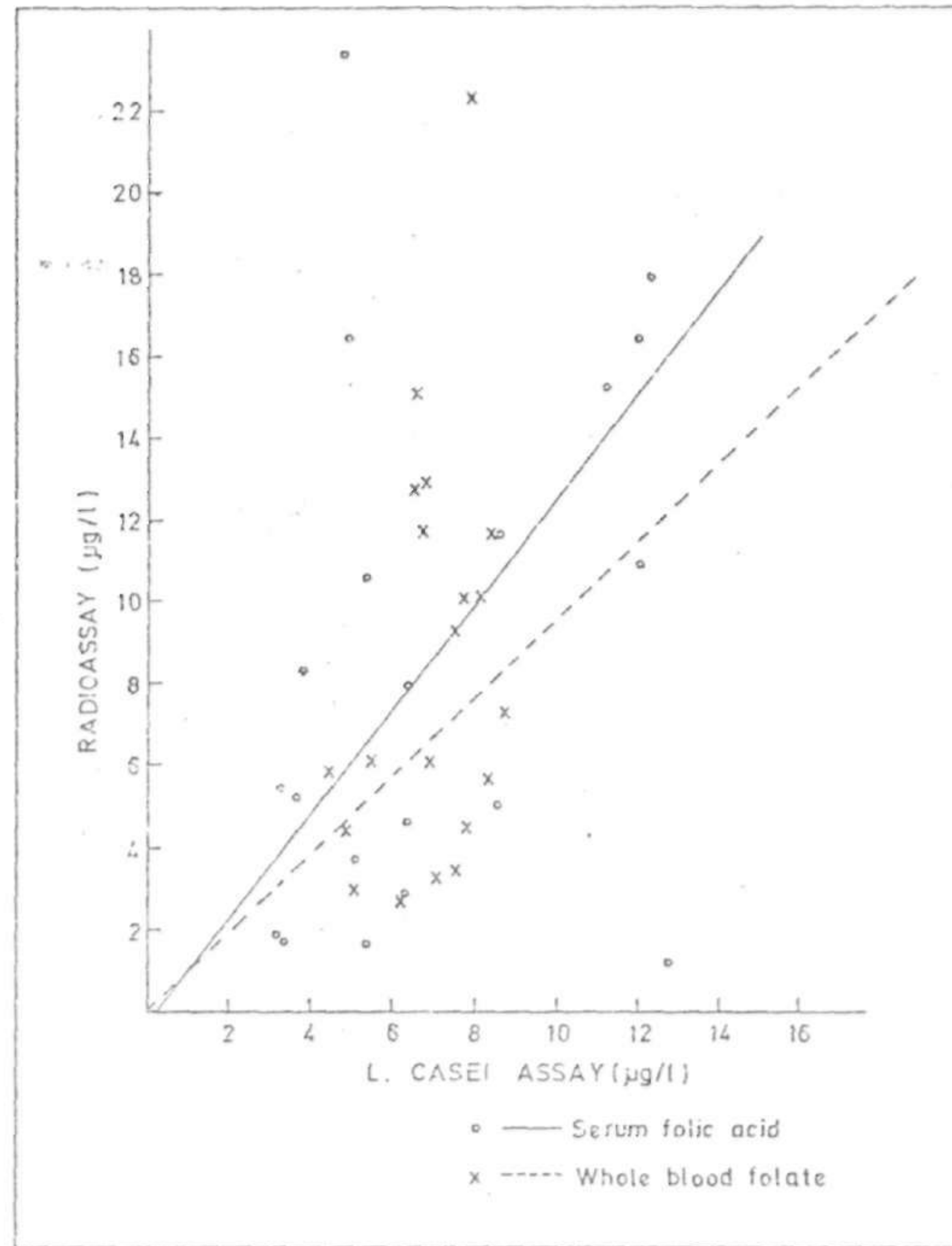


Fig. 4.8 SCATTER DIAGRAM OF FOLATE RADIOASSAY AGAINST L. CASEI ASSAY

significantly larger than unity was used which did not arise in the calculation for the RCF by L. casei.

The linear correlation between the two assays for folic acid for the sixteen donors agrees very well with what was found by Dawson et al (1980), using the Amersham kit (r = 0.68) although it is smaller than what was quoted by Johnson et al (1977) (r = 0.82). Two factors could be responsible for the poor correlation between the two assay procedures. The first factor is that due to measurement errors in the determination of the folate levels. This has been discussed in the previous section. The second is the intrinsic difference that exist in the response of the two assays. The differences may arise from the fact that the radioassay kit was designed to measure total circulating folate while the L. casei method measures only monoglutamates since the microorganism respond only to this form of folates. Any incomplete conversion of folates from other forms to monoglutamates will lead to a falsely low estimation of body folates by the L. casei assay. Similarly low folate levels may be estimated using the L. casei assay if the donors were taking antibiotic and cytotoxic drugs at the time of sampling. This factor was difficult to ascertain since most of the donors were illiterates.

4.30 EVALUATION OF THE RADIOASSAY RESULTS AND DEDUCTIONS FROM THE RESULTS

The results of the radioassays are compared with levels obtained by different methods and different seasons for Nigerian donors in the study area (Huq , 1981), non-Nigerians (Fleming et al, 1971), and with levels published using the ^{75}Se-selenofolate radioassay kit (Dawson et al, 1980) for serum folic acid and red cell folate (Table 4.3). From this table, the serum folic acid, SFA, radioassay levels (range 1.2 - 23. μg/l; mean 6.2 μg/l) had a significantly higher mean ($p = 0.05$) than the result of Huq (1981) (1.5 - 7.9 μg/l; mean 3.3 μg/l): for healthy donors within the study area during the dry season but not significantly different from the mean (5.1 μg/l) reported for Australian female nurses and technicians (Fleming et al, 1971). The differences between the SFA radioassay results and of Huq (1981) can be attributed to three factors namely: the measurement errors (17 - 28% for radioassay) the intrinsic differences in the response of the two methods and seasonal variation in the SFA levels. The first two factors had been discussed in sections 4.21 and 4.22. The higher levels recorded in this study, even by the L. casei, assay method may be due to the fact that the investigation was carried out at the peak of the rains (September) when folate rich foods were available. A significantly higher mean (9.9 μg/l)

TABLE 4.3 COMPARISON OF FOLATE ASSAY CONCENTRATION

ASSAY	SERUM FOLIC ACID				RED CELL FOLATE			
	NUMBER OF SAMPLES	MEAN µg/l	95% CONFIDENCE INTERVAL µg/l	RESULTS (p =0.05)	NUMBER OF SAMPLES	MEAN ug/l	95% CONFIDENCE INTERVAL ug/l	results* (p = 0.05)
RADIO-ASSAY I	20	6.2	1.0-30.9	-	20	318.4	97.9-1035.1	-
HUQ II	50	3.4	1.5-7.9	S	50	249.9	173.3-360.0	S
FLEMING III	95	5.1	2.4-10.7	NS	95	216.0	116.0-400.2	S
DAWSON IV	20	5.3	1.9-13.6	NS	20	a	113.0-820.0	-

a - Not available

S - Significant

NS - Not Significant

* - Results of significant test for differences of the mean with present work.

SOURCES

I = Present radioassay results

II - HUQ (1981)

III - FLEMING et al (1971)

IV - DAWSON et al (1980).

was reported for southern Nigerian donors during the rains (Fleming, 1968a). There was no significant difference between the SFA radioassay levels and those reported by Dawson et al (1980) using a similar kit (1.9 - 13.6μg/l; mean 5.3μg/l).

The red cell folate RCF, levels reported in the present study (range 123.8 - 981.5μg/l· mean 318.4μg/l) follows a similar trend with the SFA results when compared with the report of Huq (1981) (173.3 - 360.0μg/l; mean 249.9μg/l) but significantly higher than what was reported by Fleming et al (1971) (116.8 - 400.2μg/l; mean 216.0μg/l). Although RCF is a measure of long standing folate status it is expected that these long standing stores will fluctuate with decrease or increase in folate intake especially over a period of months. This is reflected by the significant difference between the L. casei assay result and the report of Huq (1981). Dawson et al (1980) did not quote results for the RCF levels but the normal reference range for the ^{75}Se-selenofolate RCF assay is 200 - 800μg/l.

Table 4.3 also show the 95% confidence interval for the mean SFA and RCF levels by the radioassay method. It will be observed that this confidence interval is much wider than the actual ranges recorded in the assay, reflecting a very wide spread in the assay results which may be due to a poor performance by the ^{75}Se-selenofolate kit. This same wide spread

was observed when the kit was used for a bigger sample in Nigeria (Akinseto-personal communication). These 95% confidence interval for the means are normally taken as the reference range for normal populations. Based on this, the normal SFA and RCF levels by the radioassay were found to be 1.0 - 36.9 μg/l and 97.9 - 1035.1 μg/l respectively.

The serum vitamin B_{12} radioassay results were similarly compared with the serum vitamin B_{12} levels reported for Nigerians by a radioassay procedure which used saliva as the binder (Fleming et al, 1978) and the results published for Nigerians using the microbiological assay method employing the micro-organism Lactobacillus leichmanni (L. leichmannii) and the radioassay results published for non-Nigerians (Dawson et al, 1980) in Table 4.4. The differences between the present vitamin B_{12} assay mean and the Fleming et al (1978) radioassay mean for Nigerians was not significant but the difference with the L. leichmannii assay was significant reflecting the differences in response again. The levels were significantly higher than what was reported by Dawson et al. (1980) (mean 279.0 ng/l) reflecting the trend of elevated serum vitamin B_{12} levels observed in Nigerians (Fleming et al, 1978). The normal reference range (95% confidence interval) was found to be 98.9 - 1482.4ng/l.

TABLE 4.4 COMPARISON OF SERUM VITAMIN B_{12} RADIOASSAY VALUES WITH PUBLISHED VALUES:

ASSAY	NO. OF SAMPLES	MEAN ng/1	95% CONFIDENCE INTERVAL OF THE MEAN	RESULTS*
RADIO-ASSAY(I)	20	382.8	98.9-1482.4	-
FLEMING (II)	233	575	545-608.0	S
FLEMING et al(III)	20	502	250.0-1000.0	S
DAWSON et al (IV)	20	279	205-792	S

S = Significant

* = Results refer to result of Test of significance of the difference of the mean of the assay and the mean of the present radioassay values.

SOURCES:

I. Present work. (II) Fleming (1968b)

III. Fleming et al 1978. (IV) Dawson et al 1980.

4.40 RECOMMENDATIONS FOR FURTHER WORK:

This study was not as extensive to give very conclusive results as to the SFA, RCF and SB_{12} levels and normal reference of the limited sample size due to shortage of assay kits and materials. To establish the radioassay procedure firmly a well equipped laboratory with regular supply of assay

kits is recommended. If this is made available, further work can then be carried out by performing assays on subjects who are suspected on the basis of other clinical data to be deficient and comparing the results with those reported for the non-elite symptom-free donors in the study. The particular area of concern should be the folate radioassay since folate deficiency is more common, and the handling and mantainance of the microbiological assays is much more tedious and time consuming, especially when very few samples are handled by the laboratory.

It has been shown that assay sensitivity could be increased with increase in labelled ligand and/or binder concentrations. However, practical work was not carried out to establish the optimum working concentrations. This should be attempted in any further work especially with ageing assay kits where the activities of the labelled ligands may not be appreciably higher than background activities when dealing with small volumes. To cut down the counting base level to a minimum, variations in pipetting and decanting of volumes should be avoided by use of automicrodispensers which were not available in the present work.

From the discussions in section 4.30 it was observed that there appears to be a seasonal variation of the folates. This trend may be confirmed if assays are carried out during different seasons.

This will allow for reference ranges to be fixed for the different seasons in clinical work. Closely related to this is the unresolved question of geographical variation. Formal references ranges could be established for different localities and socio-economic groups in further work.

It is recommended that further work should include the extension of the radioassay method to the study of various disease conditions and in vitro absorption of vitamins. It should also include the use of the assay technique in measuring the folate and vitamin contents of foods.

4.50 CONCLUSIONS:

These assays were performed with a view to establishing firmly the radioassay procedure and using it to assess the folic acid and vitamin B_{12} levels of human sera in the study area. The work however did not quite meet all the desired objectives due to shortage of assay material. However, a simple model for calculating the concentration of the two vitamins in human blood has been described for the radioassay procedure. This model eliminates the problem of manual linear interpolation of concentrations from dose-response curves especially if computer facilities are available. An analysis of this model showed that there is need to establish optimal working concentrations of reagents different from those recommended by the suppliers of the radioassay kits depending on the age of the kit if high assay sensitivities and accuracy is to be achieved. This has not been established as there was shortage of assay materials and has been recommended in any further radioassay work.

It has also been shown that the Amersham ^{75}Se-selenofolate radioassay kit gave very widely scattered concentrations for the symptom-free donors in the study area. The linear correlation of these concentrations with those by the standardized L. casei microbiological assay was dissappointing due probably to the differences in the responses

of the two assays. In fact the ^{75}Se-selenofolate kit had been withdrawn and replaced by a vitamin B_{12}/ folic acid dual assay kit employing ^{125}I as the label at the time of this report. Further radioassay work should be done using this dual assay kit which may be economical

From the results of this study, the mean serum folic acid, SFA, red cell folate, RCF, and serum vitamin B_{12} (SB_{12}) levels by the radioassay technique were found to be 6.2 μg/l, 318.4 μg/l and 383ng/l respectively. The normal reference ranges for the symptom-free donors by the radioassay method were also found to be 1.0-36.9 μg/l, 97.9-1035.1 μg/l and 98.9-1482.4ng/l for SFA, RCF and SB_{12} respectively. These reference ranges are recommended as useful guides for any radioassay procedure in the study area but can only be used in conjunction with other clinical data for diagnosis of folate and vitamin B_{12} status.

APPENDIX 1.1 DERIVATION OF THE EQUATION USED FOR DETERMINATION OF THE CONCENTRATION OF LIGAND IN THE RADIO ASSAY PROCEDURE

For the radioassay procedure described in the text, the following reactions take place:-

$$P + Q \underset{K_1^1}{\overset{K_1}{\rightleftharpoons}} PQ \quad \ldots\ldots\ldots\ldots\ldots \ (1).$$

$$P^* + Q \underset{K_2^1}{\overset{K_2}{\rightleftharpoons}} P^*Q \quad \ldots\ldots\ldots\ldots\ldots \ (2)$$

where the symbols have the usual meanings as given in the text.

At equilibrium, and if the reagents were well mixed, then

$$\frac{P}{P^*} = \frac{P_f}{P^*_f} = \frac{PQ}{P^*Q} \quad \ldots\ldots\ldots \ (3)$$

and

$$P_fQ_fK = PQ \quad \ldots\ldots\ldots\ldots \ (4)$$

$$P_f^*Q_fK = P^*Q \quad \ldots\ldots\ldots\ldots \ (5)$$

where P_f, P_f^* and Q_f are the unbound ligands and binder respectively and K is the equilibrium constant.

From the first law of mass action assumed and the assumption of univalent binding we have

$$PQ + P_f = P \quad \ldots\ldots\ldots\ldots\ldots\ldots \ (6)$$

$$P^*Q + P_f^* = P^* \quad \ldots\ldots\ldots\ldots\ldots\ldots \ (7)$$

$$Q_f + PQ + P^*Q = Q \quad \ldots\ldots\ldots\ldots \ (8)$$

Substituting equations (4) and (5) into equations (6), (7) and (8) yields

$$P_f \ (1 + Q_f K) \ = \ P \quad \dots\dots\dots\dots\dots \quad (9)$$

$$P^* \ (1 + Q_f K) \ = \ P^* \quad \dots\dots\dots\dots\dots \quad (10)$$

$$Q_f \ (1 + P_f K + P_f^* K) \ = \ Q \quad \dots\dots\dots\dots \quad (11)$$

Substituting equation (11) into equation (10) gives

$$P_f^* \ (1 + P_f K + P_f^* K \ + QK) \ = \ P^* \ (1 + P_f K + P_f^* K)-$$

or since from (3) $P_f = P_f^* \ \ P_f^* \frac{P}{P^*}$

$$P_f^{*2} K \ (P + P^*) + P_f^* \ P^*(1 + QK - (P^* + P)K) - P^{*2}$$
$$= 0 \quad \dots\dots\dots \quad (12)$$

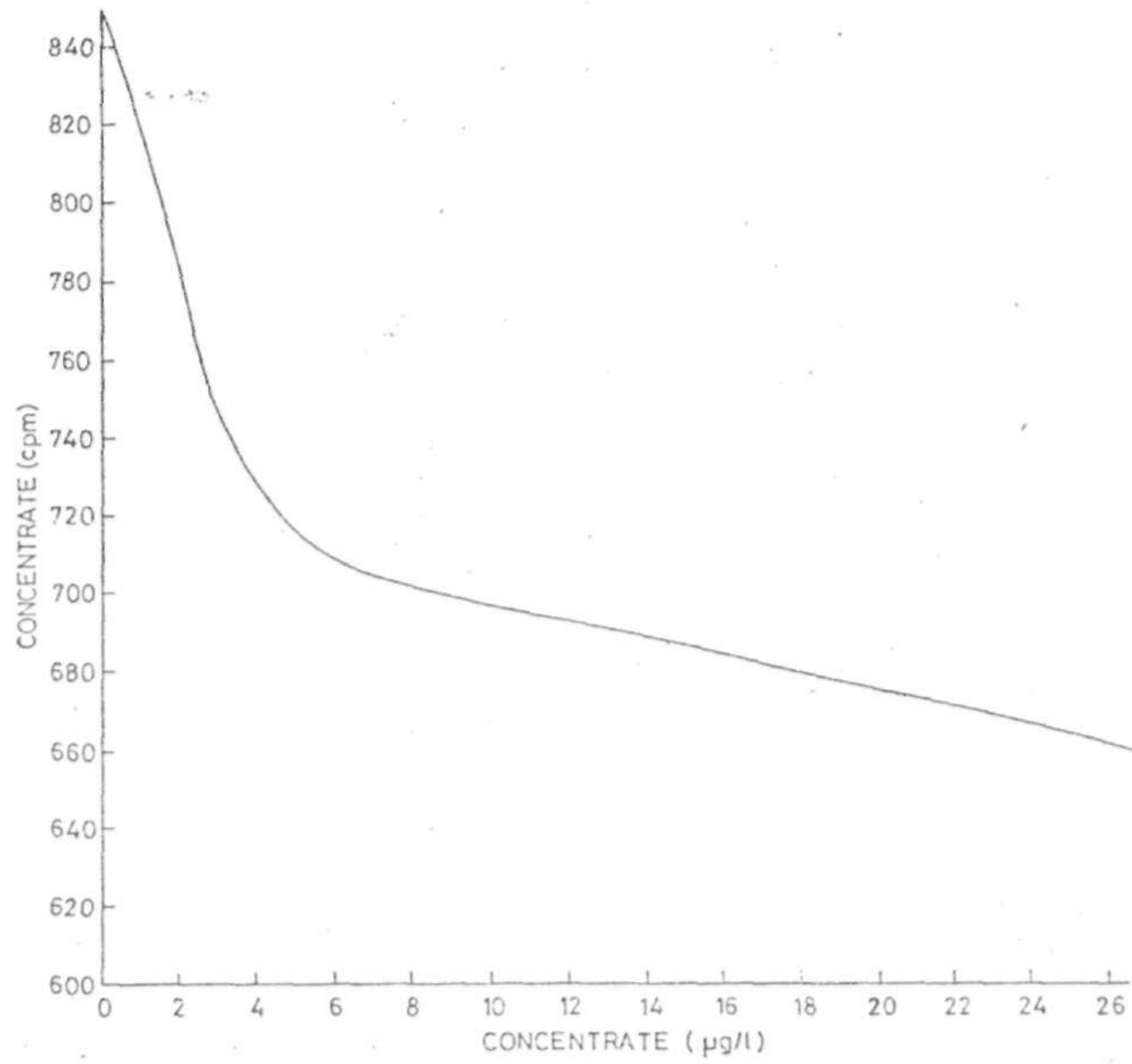
Expressing P as a function of the input variables from (12) gives

$$P \ = \ P^* \left[\frac{Q}{(P^* - P_P^*)} \quad - \quad 1 \ - \frac{1}{KP_f^*} \right] \quad \dots\dots \quad (13)$$

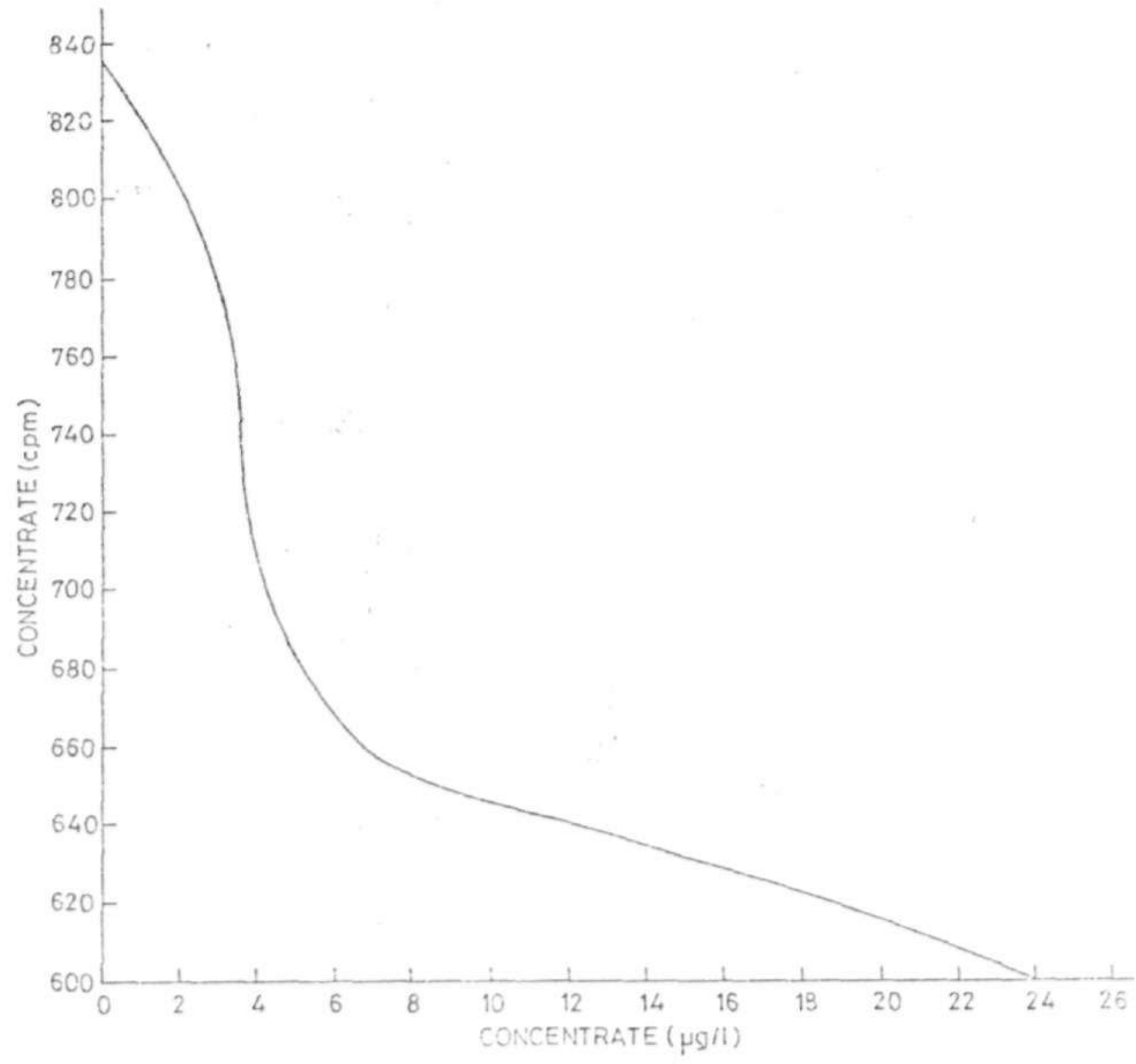
and using equation (7) we can rewrite equation (13) as

$$P \ = \ P^* \left[\frac{Q}{P^* Q} \ - 1 - \ \frac{1}{(P^* Q - P^*)K} \right]$$

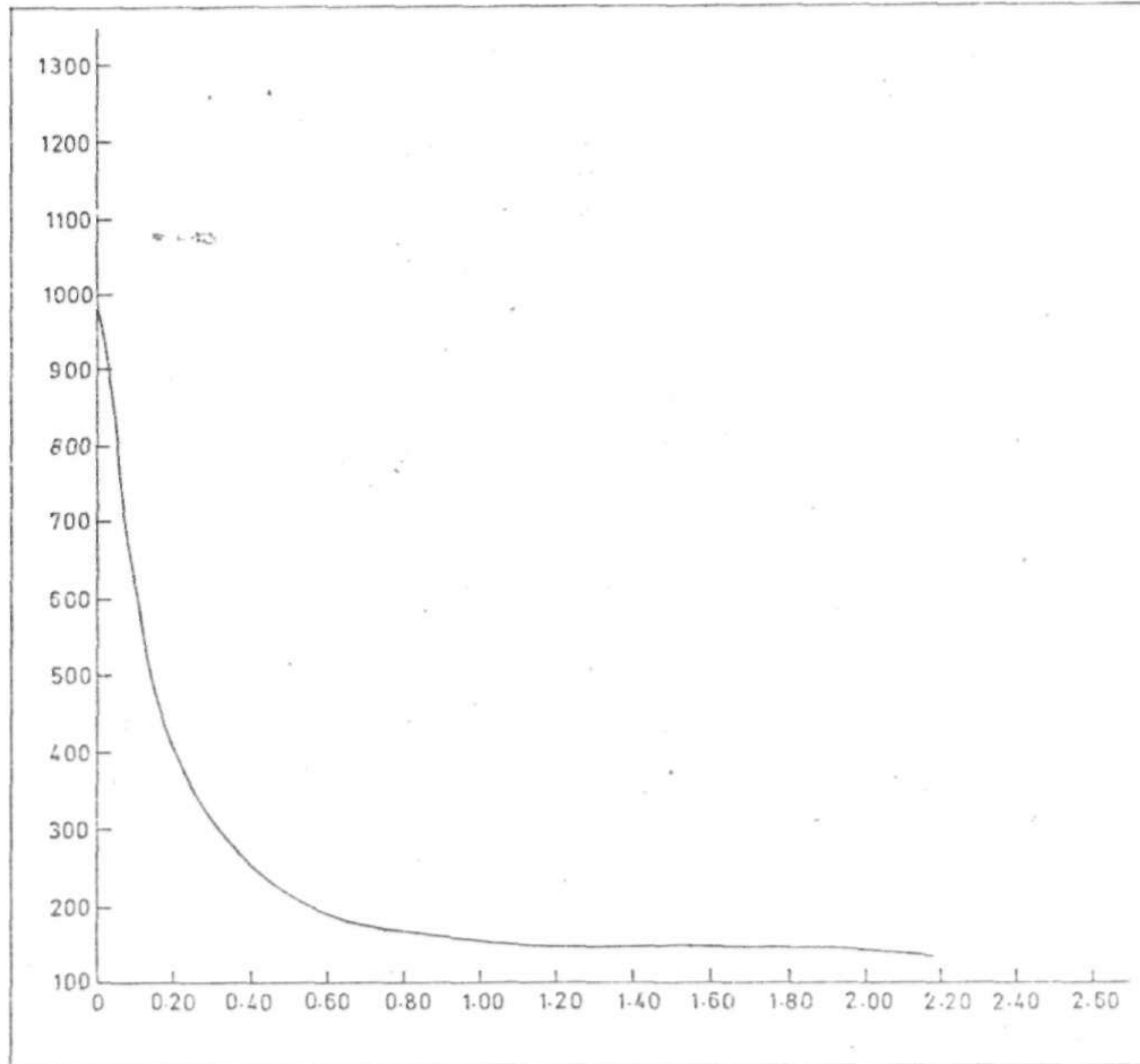
which is equation 1.4 in the text.



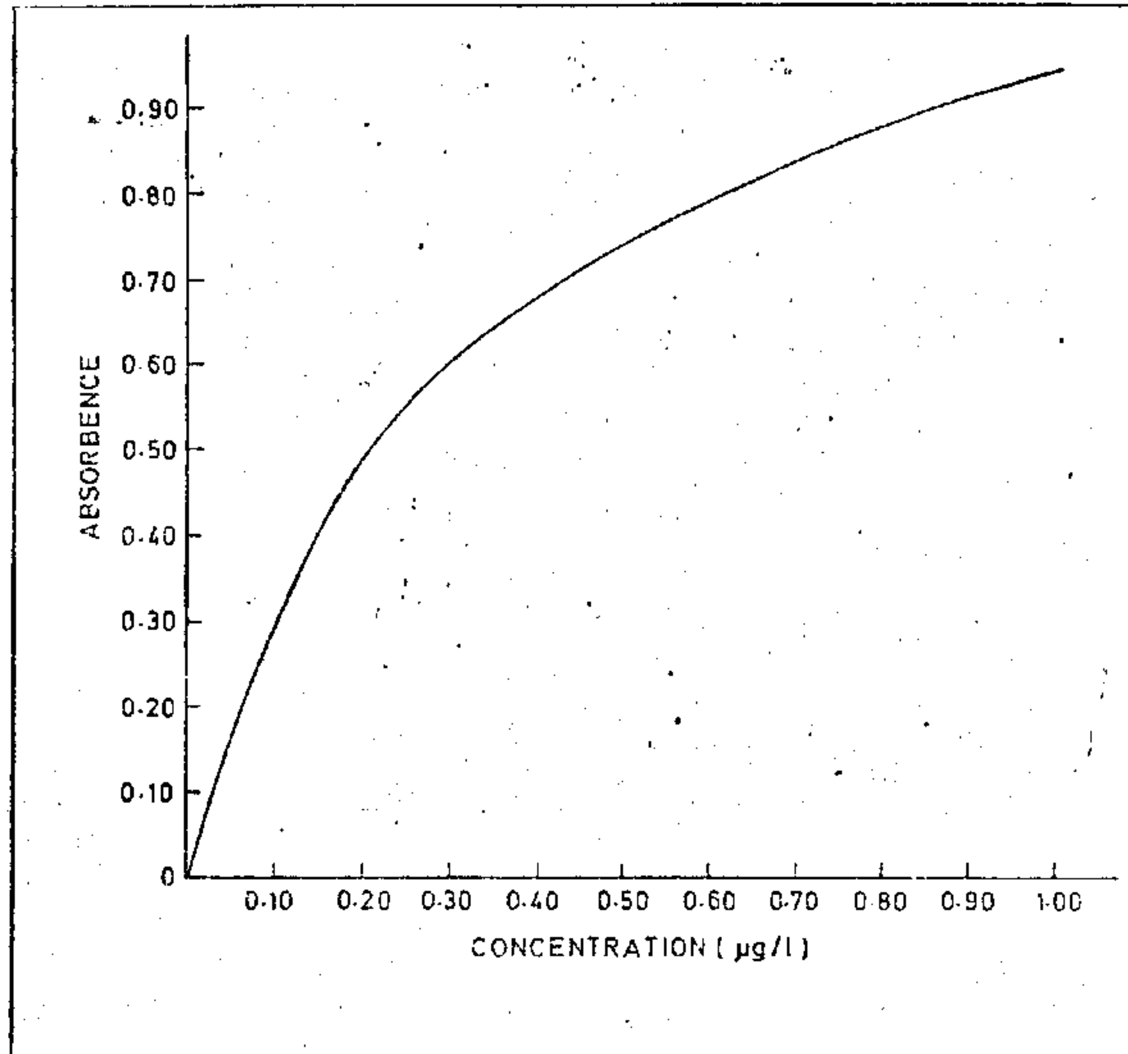
APPENDIX 2.1 STANDARD CURVE FOR SERUM FOLIC ACID RADIO ASSAY.



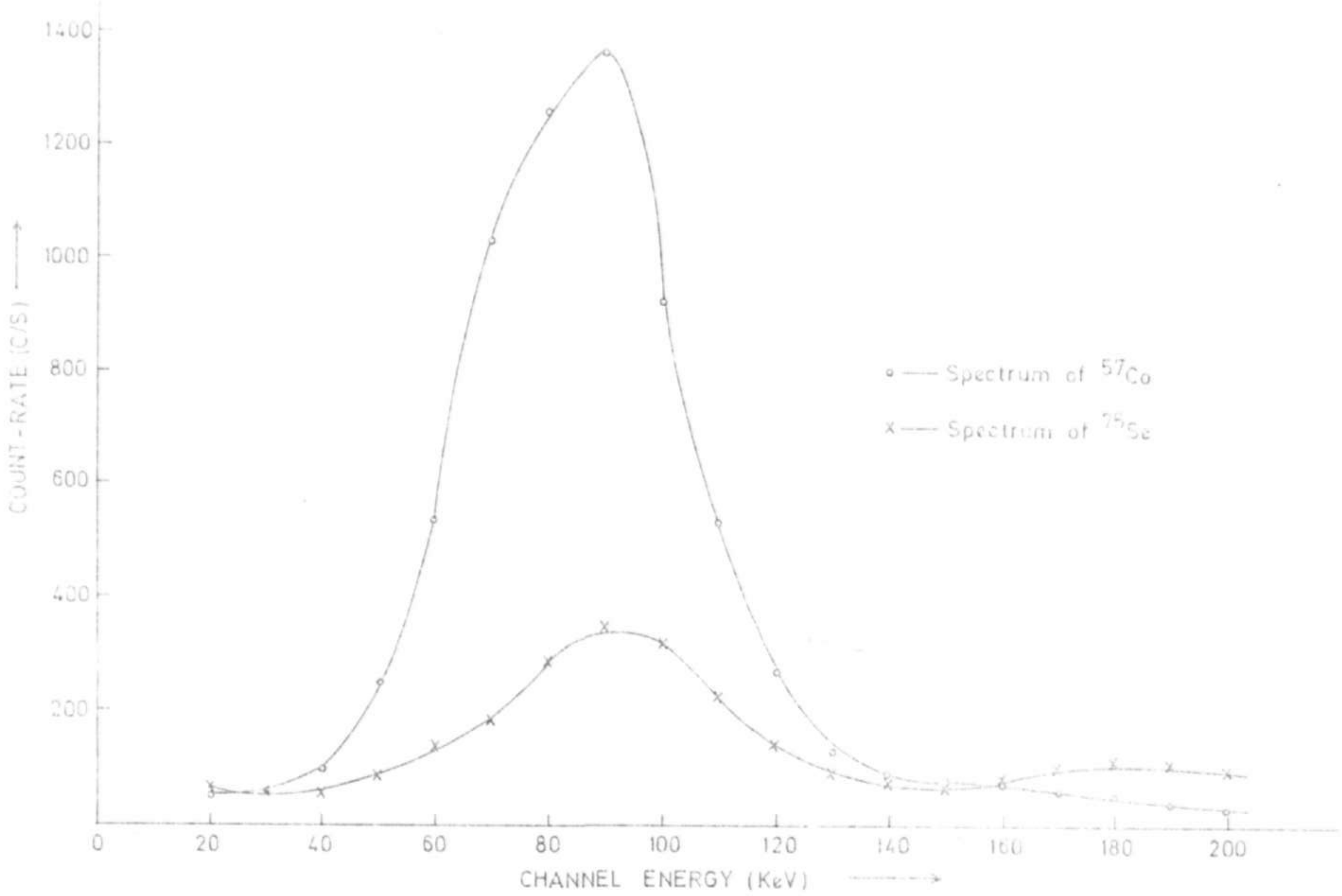
APPENDIX 2·2 STANDARD CURVE FOR RED CELL FOLATE RADIOASSAY.



APPENDIX 2.3 STANDARD CURVE FOR SERUM VITAMIN B_{12} RADIOASSAY USING RAW DATA BACKGROUND CORRECTION.



APPENDIX 2.4 STANDARD DOSE-REPONSE CURVE FOR L. CASEI ASSAY



APPENDIX 2.5 ENERGY SPECTRA OF ^{75}Se AND ^{57}Co USING THE J AND P ENGINEERING MS 310 WELL-TYPE SCINTILLATION COUNTING SYSTEM.

```
[illegible]
ED DATA SET# 1- 6 DAMPING FACTOR# [illegible]
 OF ITERATIONS 14 SUM(D**2) 7.41259
 1 [illegible]  ERR .729494   D .100000E-03
 2 -2.23867     ERR .567575   D .100000E-04
 3 35.7644      ERR 1568.7    D .100000E-04
 4 [illegible]  ERR 7923.17   D [illegible]
 5 125.394      ERR 56.6767   D [illegible]

[illegible] RADIDASSAY S P HALLAM [illegible]

OUTPUT RESULTS/ 1 INDEPENDENT VARIABLE(S)

      X.            Y.          W.          FO.          DIFF.          RED.DIFF.

 1    0           959.000     15.0000     958.966     .337359E-11   .252833E-03
 2  [illegible]   878.500     17.0000     855.7[illegible]  22.7867  [illegible]
 3  [illegible]   414.000     12.0000     46[illegible].636  -46.6[illegible]  -.524276
 4  [illegible]   333.000     [illegible] 278.562     54.4345       .[illegible]93377
 5  [illegible]   221.000     8.50000     205.430     15.5705       .247119
 6  [illegible]   174.000     7.00000     155.155     [illegible]   -.[illegible]3752

[illegible] OF RESULTS.

 X DATA POINT
 * FUNCTION VALUES
 E ARITHMETIC ERROR
 + DATA POINTS NOT INCLUDED IN L.S. FIT.

 SCALE = 6.15000
```

3.1 Sample computer output of non-linear least squares adjustment.

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