THE ESTIMATION AND SIGNIFICANCE OF THE LEVEL OF VITAMIN B₁₂ IN SERUM

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SINCE ROSS (1950) described the estimation of vitamin B₁₂ in human serum, many papers on this subject have appeared. In this paper an attempt will be made to review some of the methods and results which have been described, from the standpoints of diagnosis and significance in relation to the requirements of patients for vitamin B₁₂. Lack of space precludes detailed descriptions of methods.

Methods

Normal human serum contains a few hundred micro-microgrammes ($\mu\mu\text{g.}$) of vitamin B₁₂ per ml. A micro-microgramme is one-millionth of a millionth of a gramme, or 1/1,000,000,000 of a milligramme. No chemical method is capable of measuring such small amounts. Specialized techniques are necessary and, with one exception, all methods employ microbiological assays. Certain micro-organisms require vitamin B₁₂ for growth in defined media, which must contain all nutrients, other than vitamin B₁₂, needed by the organism. Over a certain range, which varies according to the organism used, the concentration of vitamin B₁₂ is proportional to the amount of growth, which can be assessed either turbidimetrically or by measuring the changes in pH of the medium, or by titrating the amount of acid produced in the medium, after growth. The concentration of vitamin B₁₂ in serum can be computed by comparing the growth responses to known amounts of serum, or suitable extracts of serum, with those produced by known quantities of vitamin B₁₂.

The principles underlying microbiological assays are simple, but technically the methods present considerable difficulty. Cleanliness, attention to apparently trifling details of technique and experience are necessary to ensure satisfactory results. These aspects of microbiological assay methods were discussed by Girdwood (1960a).

Organisms

Methods using *Euglena gracilis* var. *bacillaris* (Ross, 1952) and Z strain (Hutner, Bach and Ross, 1956) and *Lactobacillus leichmannii* (Rosenthal and Sarett, 1952; Spray, 1955) are most common,

while *Ochromonas malhamensis* (Mollin and Ross 1955; Baker, Ziffer, Pasher and Sobotka, 1958) and the *Escherichia coli* mutant 113-3 (Grossowicz, Aronovitch and Rachmilewitz, 1954) are also employed. *Euglena* and *Ochromonas* offer advantages in greater sensitivity and specificity, *Euglena* responding to as little as 0.25 $\mu\mu\text{g.}$ vitamin B₁₂ per ml. medium (Hutner and others, 1956). However, they require longer periods of incubation (three to eight days) at special temperatures, and *Euglena* needs intense and uniform illumination.

For general work with human subjects *Lactobacillus leichmannii* appears to be the best organism, giving results in one to two days and making up in simplicity and reliability what it lacks in specificity and sensitivity. Complete media, the use of which for determinations of vitamin B₁₂ in serum was first described by Meynell, Cooke, Cox and Gaddie (1957), are available commercially (Difco Laboratories through Baird and Tatlock (London) Ltd.; Danochemo A/S, Copenhagen, Denmark, through George T. Gurr Ltd., London). The preparation of complicated media is thus eliminated, although in my experience this process is relatively simple, and, having developed a method using media prepared in the laboratory (Spray, 1955), I have not changed since the commercial media were marketed.

The growth of *Lactobacillus leichmannii* is promoted by analogues of vitamin B₁₂ as well as by the vitamin itself, and also by desoxyribosides, such as thymidine. While the latter may complicate studies on the serum of animals (Booth and Spray, 1960), human serum does not appear to contain such interfering compounds (Spray, 1955).

Preparation of Serum for Assay

Most of the vitamin B₁₂ in serum is bound to proteins, in which state it is unavailable to the test organism. The vitamin is released from the proteins by heat, either by adding diluted whole serum to the medium and heating the mixture (Ross, 1952), by heating diluted serum before addition to the medium (Grossowicz and others, 1954) or by previously heating diluted, buffered serum to precipitate the proteins and release the

vitamin B₁₂ simultaneously. Some authors include traces of cyanide at this stage, while others do not (Table 1). The use of cyanide gives higher results with *Lactobacillus leichmannii* (Girdwood, 1960b), and Matthews (1962) has shown that without cyanide vitamin B₁₂ is not completely extracted from the proteins. I found it impossible to recover added vitamin B₁₂ from serum quantitatively in the absence of cyanide (Spray, 1955).

Whether or not to include cyanide is thus an important question, the answer to which depends on the method used. The results with *Euglena*, where diluted whole serum is added to the medium, are not affected by cyanide (Hutner and others 1956); when proteins are precipitated before adding the serum to the medium there seems to be good evidence that cyanide should always be used in order to ensure complete release of vitamin B₁₂ from proteins, whatever test organism is used. If all laboratories employing methods involving preliminary precipitation of proteins would use a standard method of extraction, including cyanide, it seems likely that results from different centres would become comparable one with another. This suggestion is supported by the good agreement between the results of parallel estimations carried out by Dr. D. M. Matthews at the Royal Free Hospital, London, and myself on 13 samples of

serum (Fig. 1). At present, because of the differences in normal ranges (Table 1), results can

FIG. 1.—Graph showing the relationship between the values for vitamin B₁₂ activity obtained in two laboratories on 13 samples of serum. The dotted lines indicate the limits of the normal ranges found in the two laboratories. There is close agreement between the results in the low and normal range, but some discrepancy on the two samples with higher levels. This discrepancy is not sufficient to vitiate the value of the test.

TABLE 1
CONCENTRATIONS OF VITAMIN B₁₂ IN THE SERUM OF CONTROL SUBJECTS AND PATIENTS WITH PERNICIOUS ANÆMIA REPORTED BY DIFFERENT AUTHORS

Authors and Country	Organism	Extraction Procedure	µg. Vitamin B ₁₂ Activity per ml. Serum					
			Controls			Pernicious Anæmia		
			No.	Mean	Range	No.	Mean	Range
Unglaub, Rosenthal and Goldsmith (1954)—U.S.A.	<i>Lactobacillus leichmannii</i>	Heat precipitation of proteins, no cyanide	31	210	70–420	6	40	10–50
Meynell and others (1957)—G.B.	" "	" "	43 90*	281 244	105–672 85–625	20	41	5–50
Girdwood (1960b)—G.B.	" "	" "	267*	281	110–810	241	—	<50–100
Spray and Witts (1958)—G.B.	" "	Heat precipitation of proteins, with cyanide	123	450	150–1,000	92	64	10–100
Matthews (1962)—G.B.	" "	" "	70	480	120–1,150	40	—	<50–100
Nieweg, Faber, de Vries and Kroese (1954)—Holland	" "	Serum diluted, autoclaved with medium	36	528	310–1,050	17	<79	<5–100
Halsted, Carroll and Rubert (1959)—U.S.A.	" "	Not stated	333	470	110–1,260	16	37	16–100
Grossowicz and others (1954)—Israel	<i>Escherichia coli</i> 113-3	Serum diluted, heated with cyanide	30	—	200–1,000	8	79	50–100
Lear, Harris, Castle and Fleming (1954)—U.S.A.	<i>Euglena gracilis</i> var. <i>bacillaris</i>	Serum diluted, heated with medium	20	532	292–856	33	39	0–100
Pitney and Beard (1955)—U.S.A.	" "	" "	?	212	86–460	16	13	0–100
Mollin and Ross (1957)—G.B.	" "	" "	223	356	100–900	320	<38	<10–100
Cooper (1959)—U.S.A.	<i>Euglena gracilis</i> Z strain	" "	32* 27*	499 305	—	22 11	61 44	—
Barakat and Ekins (1961)—G.B.	" "	Saturation analysis	12	626	330–840	3	49	11–100

* Hospital patients used as controls.

usually be referred only to the normal range of the particular laboratory performing the investigation. However, the normal ranges obtained by those authors who use cyanide (Grossowicz and others, 1954; Spray and Witts, 1958; Matthews, 1962) agree closely and results given by the method of Grossowicz and others (1954) are almost identical with those obtained when the proteins are precipitated by heat in the presence of cyanide (Rachmilewitz, Izak, Hochman, Aronovitch and Grossowicz, 1957). These observations provide further support for the suggestion that cyanide should always be used.

Accuracy and Reproducibility

Microbiological assays are subject to inherent errors. Whilst careful technique and attention to detail may minimize such errors, microbiological assays will never approach the levels of accuracy obtainable by chemical methods. It is essential, therefore, to assess the accuracy of the method in each laboratory by measuring the recovery of known amounts of vitamin B₁₂ added to a number of different samples of serum, and the reproducibility of the results by assaying the same sample of serum a number of times. In my hands recoveries of added vitamin B₁₂ from 10 samples of serum varied between 80 and 152%, with means of 108% with 250 µg. vitamin B₁₂ added per ml. serum, and 112% with 500 µg. per ml. added (Spray, 1955). A sample of pooled human serum was assayed 59 times between February 1958 and July 1959. No deterioration in vitamin B₁₂ activity was detected in deep-frozen batches of this sample during this period and the values ranged from 250 to 440 µg. vitamin B₁₂ per ml. (mean 326, standard deviation 45). These results are comparable with data published by others (Rosenthal and Sarett, 1952; Girdwood, 1960b).

Because of the errors of these methods, there is a certain range on either side of the limits of normal in which the results are equivocal when based on only one or two determinations, as is usual. This must be borne in mind when interpreting 'low normal' or 'slightly subnormal' results.

Other Methods

A method using radioactively-labelled vitamin B₁₂ and 'saturation analysis' for the determination of vitamin B₁₂ in plasma was recently described (Barakat and Ekins, 1961). When known amounts of radioactively-labelled vitamin B₁₂ are added to samples of a standard plasma some becomes bound to proteins and some remains free. The ratios of the free and bound fractions, which can be separated by dialysis, are plotted against the amounts of radioactive vitamin B₁₂ added. The resulting calibration curve can then be used to

measure unknown amounts of non-radioactive vitamin B₁₂ extracted from samples of plasma to be tested.

No other reports of the use of this method have yet appeared. It may offer advantages where the facilities and skill for microbiological work are not available, but in terms of the number of manipulations and the apparatus required it appears to be more cumbersome than the microbiological methods. It is reported to give results comparable with the latter (Table 1).

Results

The validity of these methods for diagnosing deficiency of vitamin B₁₂ depends primarily on a comparison of the results from control subjects with those from patients with classical untreated Addisonian pernicious anaemia. A selection of results published by various authors in these two groups of subjects is given in Table 1. This list is not intended to be exhaustive, but to illustrate the considerable differences found in normal ranges according to the test organism and the method of treating serum, and to show that in patients with untreated pernicious anaemia the results are almost always below the normal range.

Low results may be encountered in a number of other conditions, including subacute combined degeneration of the spinal cord due to deficiency of vitamin B₁₂, patients with cerebral manifestations of deficiency of vitamin B₁₂, dietary deficiency of vitamin B₁₂, fish tapeworm anaemia, patients who have had a partial or total gastrectomy, the malabsorption syndrome, intestinal diverticulosis and the blind loop syndrome, pregnancy whether normal or accompanied by megaloblastic anaemia, and megaloblastic anaemia due to treatment with anti-convulsant drugs (Heinrich, 1954; Wokes, Badenoch and Sinclair, 1955; Nyberg and Östling, 1956; Spray and Witts, 1958; Girdwood, 1960b; Smith, 1960). The significance of low values will be discussed in the next section.

Pathologically high values have been reported in various types of leukaemia, in polycythaemia vera and in myelosclerosis and haemochromatosis (Mollin and Ross, 1955; Spray and Witts, 1958). In leukaemia and polycythaemia, with the exception of chronic myeloid leukaemia where the level seems to be consistently high, the result in a particular patient may be normal or raised and little diagnostic information is obtained from the investigation. High results are also found in liver disease, where the level may fall as the patient improves. The test may have some diagnostic application (Rachmilewitz, Stein, Aronovitch and Grossowicz, 1958) in distinguishing hepatocellular from extra-hepatic obstructive jaundice.

Significance of the Level of Vitamin B₁₂ in the Serum in Relation to the Amount of the Vitamin in the Tissues

Mollin and Ross (1953) studied the changes in the morphology of the bone marrow and in the serum vitamin B₁₂ level in patients with pernicious anaemia after injections of vitamin B₁₂. They concluded that the level of vitamin B₁₂ in the serum was maintained at the expense of that in the tissues. This hypothesis was supported by Pitney and Beard (1955). Whilst the diagnostic value of the serum vitamin B₁₂ level in pure vitamin B₁₂ deficiency is fully established, the relationship of the level in serum to the amount of the vitamin in the tissues is clearly important in assessing the value of the estimation in conditions other than pernicious anaemia and closely related diseases.

It is difficult to investigate this problem directly in man. In rats we found that after total gastrectomy the levels of vitamin B₁₂ in both serum and liver decreased, but the concentration in serum became subnormal earlier than that in liver (Booth and Spray, 1960). Evidence for a similar relationship was found in normal rabbits (Simnett and Spray, 1961). One recent observation supports the view that these relationships obtain in man, and other reports provide circumstantial evidence pointing to the same conclusion. Adams (personal communication) measured the vitamin B₁₂ in the liver of a patient who died some time after a total gastrectomy. The level of vitamin B₁₂ in the serum had been subnormal for 10 months before death, but the liver contained 356 µg. vitamin B₁₂ (0.254 µg./g.). These values are slightly below the lowest figures found in 17 control subjects, but are much higher than the levels found in the livers of untreated patients with pernicious anaemia by others (Wolff, Drouet and Karlin-Weissman, 1951; Nelson and Doctor, 1958). It seems, therefore, that as in our rats, the level of vitamin B₁₂ in the liver of this patient decreased more slowly than that in the serum. Sobotka, Baker and Ziffer (1960) found that the concentration of vitamin B₁₂ in the red cells of three normal subjects was as much as four times less than that in the plasma, while three out of four patients with untreated pernicious anaemia had a higher concentration of vitamin B₁₂ in the red cells than in the plasma. As deficiency of vitamin B₁₂ develops, therefore, the concentration in plasma appears to fall before that in red cells. If the level of vitamin B₁₂ in red cells is a measure of that in other tissues, these results support the hypothesis. No further data on levels of vitamin B₁₂ in red cells have appeared.

Some subjects seem to maintain health and normal blood formation in the face of low levels of vitamin B₁₂ in their serum due to impaired

absorption or reduced dietary intake. In the Department a number of subjects with 'latent pernicious anaemia' have been studied at intervals for up to eight years (Callender and Spray, 1962). Several have shown abnormally low levels of vitamin B₁₂ in the serum for several years while they were in good health and showed little or no evidence of abnormalities in their blood. Vegans, a group of strict vegetarians whose diet is completely devoid of animal protein, may be in good health and have a comparatively normal blood picture after many years on the diet, yet their serum may contain abnormally low amounts of vitamin B₁₂ (Wokes and others, 1955). Both types of subject probably possess sufficient vitamin B₁₂ in their tissues for normal haemopoiesis to continue for a long time despite the reduced levels in their serum. The fall in the levels of vitamin B₁₂ in the serum of such subjects probably precedes depletion of the vitamin in their tissues, and may be the first sign of their failure to absorb adequate amounts of vitamin B₁₂.

Low results for the concentration of vitamin B₁₂ in the serum have been reported in conditions where there may be increased demand for the vitamin as a result of stress or deficiency of some other substance required for normal blood formation. Such conditions include normal pregnancy (Heinrich, 1954), iron-deficiency anaemia (Cox, Meynell, Gaddie and Cooke, 1959), idiopathic steatorrhoea with megaloblastic anaemia due to deficiency of folic acid (Mollin and Waters, 1961) and in some patients who absorbed crystalline vitamin B₁₂ normally after partial gastrectomy (Deller, Richards and Witts, 1961). The patients with steatorrhoea also absorbed vitamin B₁₂ normally, and their diet appeared to contain adequate amounts of vitamin B₁₂. The levels of vitamin B₁₂ in the serum increased in the patients with iron-deficiency anaemia after treatment with iron, and in the patients with steatorrhoea after treatment with folic acid. If the tissues must become depleted of vitamin B₁₂ before the levels in serum can fall below normal, all these groups of patients should have shown overt signs of deficiency of vitamin B₁₂. Apart from the megaloblastic anaemia attributable to deficiency of folic acid in the patients with steatorrhoea, no signs of deficiency of vitamin B₁₂ were reported except in two of the patients with partial gastrectomies, in whom the signs were minimal.

Adams (1961) found that after injections of 1000 µg. vitamin B₁₂, patients with pernicious anaemia retained about six times more vitamin B₁₂ than after doses of 50 or 100 µg., yet the concentration of vitamin B₁₂ in their serum remained within the normal range for only a short time longer after the large doses than after the smaller

ones. It was suggested that some of the vitamin B₁₂ retained after the large doses was broken down in the body. This suggestion is unnecessary if the larger amounts retained after injections of 1000 µg. were taken up by the tissues, and the levels of vitamin B₁₂ in the serum were not maintained at the expense of the tissues.

All these observations can be explained if the level of vitamin B₁₂ in the serum becomes subnormal before the supplies of the vitamin in the tissues are exhausted, and in the light of the data presented above it seems reasonable to accept this relationship as the best explanation of the facts. Some caution is therefore necessary in the diagnostic interpretation of low serum vitamin B₁₂ values. Although a low result will indicate deficiency of vitamin B₁₂ in most patients in whom the test is performed for diagnostic purposes, it does not seem to be a legitimate conclusion that the tissues are depleted of vitamin B₁₂ in every patient with a low level. The observation must be assessed in the light of the clinical picture and the results of other laboratory tests, particularly the morphology of the bone marrow and the peripheral blood, the presence or absence of achlorhydria and of gastric mucosal atrophy, and the absorption of radioactively-labelled vitamin B₁₂ and of other substances such as fat and folic acid. It should then be possible to see whether there is some defect, such as deficiency of iron or of folic acid, which might cause a temporary reduction in the level of vitamin B₁₂ in the serum. If evidence for a defect of this sort is found, the concentration of vitamin B₁₂ in the serum may return to normal when medication other than vitamin B₁₂ is given.

Estimation of vitamin B₁₂ in serum may be of paramount importance in patients suspected to have subacute combined degeneration of the spinal cord in whom the blood picture is comparatively normal, and in patients with cerebral manifestations of deficiency of vitamin B₁₂. The test has undoubted value in confirming the diagnosis in untreated pernicious anaemia, although

low results may be obtained in some patients with megaloblastic anaemia due to other causes. However, a normal value in a patient with megaloblastic anaemia suggests that the patient is not suffering from pernicious anaemia. The possibilities of the patient having received vitamin B₁₂ before the blood was taken, and of contamination of the sample from syringes or containers, must of course be considered first. In the absence of any such explanation for an anomalous result, some intestinal lesion, dietary deficiency, or drug treatment must be considered. The results of other tests, such as those mentioned above, should enable a definite diagnosis to be made.

A further point requiring consideration in interpreting values for serum vitamin B₁₂ in individual patients is the standards of normality to which the results are referred. The variation between laboratories has already been discussed. Most normal ranges are based on values from as few as 20 up to 200 to 300 control subjects. Even from these comparatively small groups the lower limit of the normal range may be as much as 10 times less than the upper limit, and it is evident that data are required from much larger groups of control subjects to establish a true basis for comparison. Little is known about the influence of racial and dietary factors on the serum vitamin B₁₂ values in healthy subjects (Matthews, 1962). These factors, together with the inaccuracies of the methods, must all be considered in interpreting results. Nevertheless, determination of the level of vitamin B₁₂ in serum has been, and will continue to be, a valuable tool in the study of the megaloblastic anaemias and the roles of vitamin B₁₂, folic acid and related compounds in blood formation.

I am grateful to Dr. J. F. Adams, Western Infirmary, Glasgow, W.1, for permission to publish details of his patient with total gastrectomy; to Dr. D. M. Matthews, Royal Free Hospital, London, W.C.1, for supplying some of the data included in Table 1; and to Professor L. J. Wiggs for his interest in my work on this subject which has been carried out in his department.

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