THE VITAMIN B₁₂ CONCENTRATIONS OF SERUM AND URINE OF NORMALS AND OF PATIENTS WITH MEGALOBLASTIC ANAEMIAS AND OTHER DISEASES

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(RECEIVED FOR PUBLICATION SEPTEMBER 28, 1951)

Until recently deficiency of vitamin B₁₂ in man could only be recognized by finding the characteristic clinical and haematological signs of the deficiency and by demonstrating that these signs were cured or relieved by treatment with the vitamin. Changes in the vitamin B₁₂ content of the tissues could only be inferred from the responses of patients to treatment.

The recent introduction of highly sensitive methods of microbiological assay has made it possible to measure the actual vitamin B₁₂ content of body fluids. *Euglena gracilis* var. *bacillaris* is the most sensitive organism for microbiological assay (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes, 1949; Ross, in the press), and the purpose of this paper is to report the results of assays with this organism and to demonstrate the value of the assay method in the study of megaloblastic anaemias.

The serum and urinary vitamin B₁₂ concentrations of patients with pernicious anaemia have been found to be much lower than those of both normal subjects and of patients suffering from other diseases. The concentrations found in patients suffering from megaloblastic anaemias other than pernicious anaemia varied. In some the concentrations were as low as those found in pernicious anaemia, but in others the vitamin B₁₂ concentrations were normal.

Materials and Methods

Serum.—Blood was obtained by venepuncture, using a dry syringe, and was allowed to clot in a sterile glass container at 37° C. The serum was removed after one hour at this temperature and kept frozen until assayed. Assays were usually made within three days of collection.

Urine.—Twenty-four hourly collections were made, using toluene as preservative. In most instances each sample was stored at 4° C. immediately after being passed. Assays were usually made within three days of collection.

Method of Assay.—The vitamin B₁₂ concentration in the serum and urine was measured by a modification of the assay method introduced by Hutner *et al.* (1949), in which the green alga *Euglena gracilis* was used as the test organism. Details of the technique will be published in the next issue of this journal. As little as 10 µg. per ml. of vitamin B₁₂ are detectable in serum and urine with this method.

* In receipt of a grant from the Medical Research Council.

Material with vitamin B₁₂-like growth activity for *Euglena gracilis* may occur in serum either free or in combination with some substance, probably protein (Ross, 1950). The combined form is microbiologically inactive and it is necessary to free the vitamin from combination before it can be assayed. Heating dilutions of the test fluids at 100° C. has been used to accomplish this.

The values given below have been expressed as either "uncombined (or free)," "combined," or "total." Uncombined vitamin B₁₂ is the amount detected by assay after heating in a 56° C. water bath for 30 minutes. (Heating at 56° C. does not liberate vitamin B₁₂ from combination but is done to reduce contamination.) Total vitamin B₁₂ is the amount detected after heating in a 100° C. water bath for 15 minutes, and combined vitamin B₁₂ is the difference between the total and the uncombined concentrations.

Blood Counts.—Venous blood collected into ammonium and potassium oxalate mixture was used throughout.

Red Cell Counts.—These were made at a dilution of 1:200, using Bürker or Neubauer counting chambers. A minimum of 2,000 cells were counted for each count.

Haemoglobin.—Haemoglobin was estimated by various modifications of the alkaline-haematin or oxyhaemoglobin methods. Readings were made either with a photoelectric colorimeter or with the M.R.C. grey-wedge photometer.

Packed Cell Volume.—This was measured by Wintrobe's method. The tubes were centrifuged at 3,000 r.p.m. for 30 minutes.

Reticulocyte Counts.—These were made on dry films after staining red cell suspensions with 0.4% cresyl blue for 30 minutes at 37° C. The reticulocytes present in at least 1,000 cells were counted.

Results

The Vitamin B₁₂ Concentration of Serum.—The total and combined serum vitamin B₁₂ concentrations of 65 normal subjects aged between 15 and 40 years are given in Table I. In the same table are included the levels of 63 patients with various conditions other than megaloblastic anaemia, and of 43 patients with pernicious anaemia in mild, moderate, or severe relapse. It can be seen that the differences between the total and the combined serum vitamin B₁₂ concentrations of each group are small, indicating that most of the vitamin in these sera is in the combined form.

Normals and Patients with Conditions other than Megaloblastic Anaemia.—The total serum vitamin B₁₂ concentrations of the normal group ranged from 100 to 720 µµg. per ml., with a mean of 358 µµg. per ml. The combined concentration ranged from 100 to 620 µµg. per ml., with a mean of 329 µµg. per ml. The mean vitamin B₁₂ serum concentration found in patients with conditions other than megaloblastic anaemias was not significantly lower than in the normal subjects. The individual level of a few patients in this group was, however, low (Table II).

Patients with Pernicious Anaemia.—The serum vitamin B₁₂ concentrations of patients suffering from pernicious anaemia in relapse were very much lower than the values found in the other groups and ranged from less than 16 to 105 µµg. per ml. The mean concentration given in Table I for this group of patients is certainly too high, because in 24 instances the concentrations were too low for definite figures to be obtained. These results were not included in those from which the mean was calculated. In 11 of these sera containing only traces of vitamin B₁₂ the amount present was less than 40 µµg. per ml.; in 13 it was less than 20 µµg. per ml.

TABLE I

TOTAL AND COMBINED SERUM VITAMIN B₁₂ CONCENTRATIONS OF NORMALS AND OF PATIENTS WITH PERNICIOUS ANAEMIA IN RELAPSE AND WITH VARIOUS DISEASES OTHER THAN MEGALOBLASTIC ANAEMIA

Group	Age	No. of Subjects	Total Vitamin B ₁₂ Concentration (μg. per ml.)			Combined Vitamin B ₁₂ Concentration (μg. per ml.)		
			Range	Mean	S.E. of Mean	Range	Mean	S.E. of Mean
1. (a) Normal subjects	15-40	65	100-720	358	±16.1	100-620	329	±14.6
(b) Healthy subjects attending hospital with minor complaints	51-70	27	110-620	322	±28.9	110-540	295	±27.7
(c) Healthy subjects attending hospital with minor complaints	71-93	31	60-600	259	±31.6	30-600	234	±29.1
2. Patients with miscellaneous diseases ..	5-76	63	60-680	316	±20.2	40-680	298	±19.9
3. Patients with pernicious anaemia in relapse	28-74	43	<16-105*	43†	±3.3	<16-105*	43†	±3.3

* The range in this group is based on the observations made on 87 sera.

† The mean is based on 63 observations, because the values of 24 sera were too low to be measured. The true mean is therefore less than the recorded figure.

TABLE II

TOTAL AND COMBINED SERUM VITAMIN B₁₂ CONCENTRATIONS OF PATIENTS (GROUP 2 OF TABLE I) WITH DISEASES OTHER THAN MEGALOBLASTIC ANAEMIA

Disease	No. of Patients	No. of Observations	Age	Total Vitamin B ₁₂ Concentration (μg. per ml.)		Combined Vitamin B ₁₂ Concentration (μg. per ml.)	
				Range	Mean	Range	Mean
Haemolytic anaemia ..	6	7	45-74	140-600	354	140-600	315
Polycythaemia vera ..	6	6	39-53	90-420	228	90-420	228
Acute leukaemia ..	6	6	6-35	180-400	336	175-400	306
Chronic leukaemia and generalized reticulo-sarcoma	6	6	33-74	150-400	356	150-400	346
Multiple myeloma ..	2	3	54-64	80-200	120	40-160	97
Iron-deficiency anaemia ..	7	8	32-73	130-680	325	130-680	325
Haemorrhagic diseases ..	3	5	39-54	130-440	234	90-440	226
Aplastic anaemia ..	1	1	18	170	—	170	—
Acute infections ..	4	4	24-35	240-470	365	240-470	352
Miscellaneous conditions‡	22	22	41-73	100-680	360	50-680	340

‡ This group included patients suffering from cardiac failure, various neurological conditions, including alcoholic neuritis, and various types of carcinoma. One patient was suffering from severe scurvy.

In Fig. 1 the vitamin B₁₂ concentrations of the sera of these patients has been plotted against the red cell counts at the time the sera were obtained. In each case the concentration was below the normal range, and tended to be lower in the more anaemic patients. It was our impression that the lowest levels were found in those

FIG. 1.—The serum vitamin B₁₂ concentrations found in 43 patients with pernicious anaemia in relapse are plotted against the patient's red cell count at the time the serum was obtained. The limits of the normal range are indicated by the horizontal unbroken lines; the mean normal serum concentration is indicated by the broken line.

patients in whom the disease was progressing rapidly. Where the blood count was static rather higher levels were found, particularly when patients had red cell counts of 2.5 m. per c.mm. or more.

Almost half the samples of sera were obtained from patients receiving a normal diet; the other samples were taken after the patients had been deliberately placed on a diet of low vitamin B₁₂ content. No significant difference in the serum concentrations was found between these two groups and no spontaneous remissions occurred during the periods of observation.

Subjects of Same Age and Economic Groups as Patients with Pernicious Anaemia.—The normal range given in Table I was based on observations made on healthy doctors, technicians, and nurses. The patients with pernicious anaemia on whom studies were made were older, poorer, and less well cared for, and therefore it seemed desirable to measure the vitamin B₁₂ concentrations of the sera of non-anaemic subjects of similar age and living under comparable conditions. For this purpose sera were obtained from patients who were attending the out-patient department with minor medical or surgical conditions or were in hospital fully convalescent from minor ailments. The results are shown in Table I, Groups 1b and 1c.

TABLE III
INITIAL RED CELL COUNTS, SERUM VITAMIN B₁₂ CONCENTRATIONS, ASSOCIATED CONDITIONS, AND RESPONSE TO TREATMENT OF PATIENTS WITH MEGALOBLASTIC ANAEMIA OTHER THAN PERNICIOUS ANAEMIA

Case No.	Sex	Age	Associated Condition	Vitamin B ₁₂ Concentration of Serum (μg. per ml.)	Therapy	Red Cell Count (m. per c.mm.)		Reticulocyte Response			Bone Marrow Findings		Remarks
						Initially	15 Days after Therapy	Initial %	% at Peak of Response	Peak Day	Initially	4 Days after Therapy	
1	F.	57	Ileo-transverse colostomy 3 yrs. previously	<20	20 μg. B ₁₂ intramuscularly	1.4	2.5	1.0	48	7th	Meg.*	Norm.†	Histamine-fast achlorhydria. Fat absorption: 87% on diet containing 50 g. fat (6-day balance)
2	F.	33	Tabes mesenterica and entero-anastomosis 6 yrs. previously	60 (2 observations)	20 μg. B ₁₂ intramuscularly	2.4	3.2	5.0	22	8th	Meg.	Norm.	Free HCl in gastric juice after histamine: 44% of dried weight of faeces was fat
3	M.	51	Total gastrectomy. 2 yrs. previously. Ileo-ileostomy 18 months previously	20-40 (6 observations)	40 μg. B ₁₂ intramuscularly	1.9	2.8	<0.1	10	6th	Meg.	Norm.	Severe fatty diarrhoea 2 mths. Fat absorption: 30% on diet containing 50 g. fat (6-day balance)
4	F.	56	Total gastrectomy Oesophago-jejunostomy 2 yrs. previously	50 (2 observations)	Spontaneous remission	1.4	—	2.0	27	—	Meg.	Norm.	Spontaneous remission followed enrichment of diet with crude casein. The remission was associated with a rise in the serum B ₁₂ concentration to within the normal range. Fat absorption: 80% on diet containing 50 g. fat (6-day balance)
5	F.	34	Pregnancy	350-370 (3 observations)	40 μg. B ₁₂ intramuscularly	2.3	1.8	1.0	4	7th	Meg.	Meg.	Free HCl in gastric juice 2 yrs. previously when admitted with similar condition. Negligible response to vitamin B ₁₂
				230-250 (2 observations)	60 μg. folic acid intramuscularly	2.2	2.3	5.0	11	5th	Meg.	Meg.	Good subjective response, but negligible haematological response to a very small dose of folic acid
				320-350 (3 observations)	6 mg. folic acid intramuscularly	2.3	3.2	8.0	21	6th	Meg.	Norm.	Good response to single injection of 6 mg. folic acid
6	F.	58	? Idiopathic steatorrhoea	600	120 μg. B ₁₂ intramuscularly 20 mg. folic acid daily	1.4	1.1	0.4	12	9th	Meg.	Meg.	Initial R.B.C. count 0.4 m. per c.mm. Transfused before treatment. Free HCl in gastric juice. Fat absorption: 80% on diet containing 50 g. fat (6-day balance)
						1.1	2.7	4.0	47	6th	Meg.	Norm.	
7	M.	53	Idiopathic steatorrhoea	25	60 μg. B ₁₂ intramuscularly	0.8	—	0.5	—	—	Meg.	—	Free HCl in gastric juice. Diagnosed as suffering from idiopathic steatorrhoea elsewhere on the history and on the findings of a fat balance. Responded to treatment with vitamin B ₁₂

The mean serum level of the patients aged 51–70 years (Table I, Group 1b) was lower than that of the healthy subjects aged 15 to 40 years, and was similar to that of the patients suffering from serious conditions (Table I, Group 2). The apparent difference was, however, not statistically significant. The mean total and combined serum vitamin B₁₂ concentrations of the patients aged between 71 and 93 years was, however, significantly lower (Table I, Group 1c) than that of the normal group ($P < 0.001$). Many of them had disabilities common to their age group, but none was seriously ill or anaemic.

Patients with Megaloblastic other than Pernicious Anaemia.—Seven patients were studied. The initial red cell counts, serum vitamin B₁₂ concentrations, associated conditions, and responses to therapy are shown in Table III. The serum vitamin B₁₂ concentrations of five patients (Cases 1 to 4 and Case 7) were as low as those found in patients with pernicious anaemia; three of these patients (Cases 1 to 3) responded excellently to intramuscular injections of vitamin B₁₂. One patient (Case 4) developed a spontaneous remission before treatment which was associated with an increase of the serum vitamin B₁₂ concentration to within the normal range. One patient (Case 7), treated at another hospital, also responded to treatment with vitamin B₁₂ by injection.

Two patients, one with megaloblastic anaemia of pregnancy and one with a megaloblastic anaemia of doubtful aetiology, possibly idiopathic steatorrhoea, had normal serum vitamin B₁₂ concentrations before treatment. The first patient (Case 5) failed to show a significant red cell or reticulocyte response to an injection of 40 μg . of crystalline vitamin B₁₂, but responded well to a single injection of 6 mg. of folic acid. The other (Case 6) failed to respond to a single injection of 120 μg . vitamin B₁₂, but responded maximally to treatment with folic acid.

Diurnal and Day-to-day Observations in Normals and Patients with Pernicious and Other Anaemias.—Serum was obtained at frequent intervals during 48 hours from two normal subjects. The values found in one subject ranged from 290 to 350 μg . per ml., and in the other from 105 to 130 μg . per ml. (Table IV). Although the diet was not restricted, no relation was noted between the vitamin B₁₂ concentration and the taking of food. In Table IV are also given the results of similar assays carried out over an eight-hour period on four patients with iron-deficiency anaemia, on one patient with aplastic anaemia, and on one patient with pernicious anaemia in relapse. These patients were fasting while the studies were made.

Serum was also taken at frequent intervals from two normal subjects for three weeks. The concentrations found are shown graphically in Fig. 2. The levels in one patient ranged from 100 to 210 μg . per ml., and in the other from 110 to 400 μg . per ml. In Fig. 2 are also shown the vitamin B₁₂ concentrations of the serum of a patient with pernicious anaemia in relapse studied over a similar period. The values found ranged between 16 and 90 μg . per ml. Serial assays were also carried out on the serum of a patient with iron-deficiency anaemia during the intense regeneration produced by oral iron therapy. The vitamin B₁₂ concentration of the serum did not alter significantly during 30 days.

Uncombined Vitamin B₁₂ in Serum.—Uncombined (free) material with growth activity for the *Euglena* similar to vitamin B₁₂ was present in the sera of 54 subjects of the groups included in Table I in concentrations of from 40 to 160 μg . per ml. (mean 81 μg . per ml.). In the sera of all the patients with pernicious anaemia, and

TABLE IV
DIURNAL OBSERVATIONS IN TOTAL SERUM VITAMIN B₁₂ CONCENTRATION OF TWO NORMALS
AND SIX ANAEMIC PATIENTS

Clinical Condition	Vitamin B ₁₂ Serum Concentration ($\mu\text{g. per ml.}$)					
	0	4 hrs.	8 hrs.	12 hrs.	24 hrs.	48 hrs.
Normal	345	290	320	320	305	290
"	120	115	130	120	130	105
Iron-deficiency anaemia	170	230	210	—	—	—
" " "	680	640	720	—	—	—
" " "	150	120	120	—	—	—
" " "	215	225	265	—	—	—
Aplastic anaemia	170	170	255	—	—	—
Pernicious anaemia in relapse	45	45	40	—	—	—

of the remaining 132 subjects included in this Table, uncombined material was present in amounts of less than 40 $\mu\text{g. per ml.}$

Observations made on 15 sera were excluded from Table I because the serum vitamin B₁₂ concentrations were far higher than those found in other sera. These high values, which ranged from 1,000 to more than 4,000 $\mu\text{g. per ml.}$, were due to the presence of large amounts of uncombined material, the combined vitamin B₁₂ concentrations not being raised above the usual levels found in sera of the respective groups. These high free concentrations have been found in the sera of normal subjects and of patients with pernicious anaemia in relapse. Samples of sera taken 24 hours before or after the specimen containing the high free concentration have usually contained no free material. In one normal subject, nevertheless, large amounts were present in four specimens taken during a 12-hour period. In one patient with pernicious anaemia specimens of serum containing no free material were obtained three hours before and three hours after a sample had been taken which contained a very high concentration of this substance.

FIG. 2.—The variation in the total vitamin B₁₂ concentration of the serum of two normal subjects and of one patient with pernicious anaemia in relapse during 25 days. The limits of the normal range are indicated by the horizontal broken lines. Curves A and B represent the changes in the serum concentration of the normal subjects, Curve C the serum concentration of the patient with pernicious anaemia.

Vitamin B₁₂ Concentration of Urine.—The amount excreted in the urine in 24 hours by six normal subjects is given in Table V. The total daily excretion ranged from 0.11 to 0.27 μg .

The daily urinary excretion by eight patients with pernicious anaemia in relapse is given in Table VI. The values found, with one exception, were much lower than those found in the normal subjects. In seven patients less than 0.05 μg . was excreted daily; in a specimen from one patient the excretion was within the normal range.

TABLE V
DAILY EXCRETION OF VITAMIN B₁₂ IN URINE OF SIX NORMALS

Volume of Urine (ml.)	Vitamin B ₁₂ Concentration (μg . per ml.)	Total Vitamin B ₁₂ Excretion (μg . in 24 hours)
1,740*	140	0.24
1,420	80	0.11
1,115	250	0.27
2,055	100	0.21
1,400	94	0.13
1,300*	116	0.16
1,900	85	0.16
1,350	115	0.16
1,570	100	0.16

* Three separate collections were made from the first subject and two collections from the fourth.

TABLE VI
DAILY EXCRETION OF VITAMIN B₁₂ IN URINE OF EIGHT† PATIENTS WITH PERNICIOUS ANAEMIA IN RELAPSE

Volume of Urine (ml.)	Vitamin B ₁₂ Concentration (μg . per ml.)	Total Vitamin B ₁₂ Excretion (μg . in 24 hours)
910	25	0.02
1,360	<40	<0.05
1,135	<20	<0.02
1,385	<20	<0.03
1,846	<20	<0.04
1,740	<16	<0.03
840	32	0.027
1,415	100	0.15

† In seven patients the amount of vitamin B₁₂ excreted was much lower than that excreted by normal subjects. In the last patient the daily excretion was similar to the excretion in normal subjects.

Discussion

It is only very recently that the first results of the microbiological assay of vitamin B₁₂ in body fluids have appeared in the literature. Ross (1950) in a short communication reported his preliminary results using *Euglena gracilis*, and Couch, Olcese, Witten, and Colby (1950), who used *L. leichmannii* as test organism, reported the results of assays made on the whole blood of 10 normal human subjects and of a number of animal species. The latter organism was also used by Chow, Lang, Davis,

Conley, and Ellicott (1950) for the assay of the vitamin B₁₂ content of urine, but was found by them to be insufficiently sensitive to detect the vitamin in the urine of normal subjects. Girdwood (1951) has, however, reported the results of assays with this organism of the vitamin B₁₂ content of the urine of normal subjects and patients suffering from megaloblastic anaemia. Though the values for normal subjects were similar to those reported here he did not demonstrate a clear-cut difference between the urinary excretion of the vitamin by normal subjects and patients with pernicious anaemia.

Euglena gracilis is a more sensitive test organism than the lactobacilli which have been used for vitamin B₁₂ assay, and its use, as now reported, made it possible not only to demonstrate the presence of vitamin B₁₂-like material in serum and urine, but also to measure its range in health and disease. The concentrations of this material in the serum of normal subjects and of patients suffering from conditions other than megaloblastic anaemia were found in this study to be similar. The levels in the serum of two patients (Table III, Cases 5 and 6) suffering from megaloblastic anaemia refractory to treatment with vitamin B₁₂ were not different from those found in normal subjects, but the serum concentrations of patients suffering from pernicious anaemia or other megaloblastic anaemias which responded to treatment with vitamin B₁₂ were below the normal range.

The Specificity of the *Euglena* for Haemopoietically Active Vitamin B₁₂.—The observations summarized in the previous paragraph suggest that the material measured is actually vitamin B₁₂. The much smaller amount of vitamin B₁₂-like material found in the urine of patients with pernicious anaemia in relapse compared with normal subjects also supports this view. Nevertheless it is not certain that vitamin B₁₂ is the only material in serum and urine which is capable of promoting the growth of the *Euglena*. As already mentioned, we excluded the observations made on 15 sera from the results given in Table I. These sera were excluded because their total vitamin B₁₂ equivalent was far higher than that found in the other 186 sera examined. This high total concentration was due to the presence of considerable amounts of uncombined material having a vitamin B₁₂-like activity for the *Euglena*. Five of these sera were from patients with pernicious anaemia in relapse, but in spite of the high concentrations found, which would have been equivalent to as much as 6 μ g. of vitamin B₁₂ circulating in the blood stream, no haematological remission occurred. Yet the same patients subsequently responded well to parenteral or oral administration of the vitamin. Two explanations of this finding seem possible. First, material may be actually present in the blood stream of these patients and, although not haemopoietically active, have growth activity for the *Euglena* similar to vitamin B₁₂. If this is so, it may possibly be of cobalamin nature and enter the blood from either intestine or the tissues. Secondly, material may not be an actual constituent of the patient's blood, but may result from contamination of the blood during its collection and preparation for assay either from needle, syringe, or glassware previously in contact with vitamin B₁₂, or with material with similar activity for the *Euglena*. The method of assay is so sensitive that traces of vitamin B₁₂ which might remain after thorough washing and sterilization of syringes and needles previously used for injecting vitamin B₁₂ could conceivably affect results. Throughout the investigation every effort was made to ensure that the syringes and needles used for administration of vitamin B₁₂ were kept apart from

those used for withdrawal of blood. Nevertheless, it is possible that in certain instances this arrangement may have broken down. The presence of free material was not confined to the 15 sera just referred to. As already mentioned, other sera contained free material in measurable though small amounts. The nature and source of this are also uncertain.

The total concentration of material having a vitamin B₁₂-like activity for the Euglena may not, therefore, in all cases be an accurate measure of the amount of haemopoietically active vitamin B₁₂ present in the patient's blood stream. However, the concentration of *combined* vitamin B₁₂-like material seems to be closely correlated with the nutritional state of the body in respect of vitamin B₁₂. In all patients suffering from megaloblastic anaemias which responded to treatment with vitamin B₁₂, the serum concentrations of the combined material were below the range of the concentration of this material in normal subjects. In no other conditions were such low concentrations found. Further, when vitamin B₁₂ was given parenterally to patients with pernicious anaemia in relapse the serum concentrations of the combined material increased to within the normal range, and, as long as this concentration remained above the lower level of the normal range, haemopoiesis in the bone marrow was normoblastic. When the combined concentration fell below the limits of the normal, megaloblastic haemopoiesis reappeared.

The observation that the combined concentration reflects the body's needs for vitamin B₁₂ is of interest in view of the demonstration by Ternberg and Eakin (1949) that crystalline vitamin B₁₂ is bound by material present in gastric juice. This bound form of vitamin B₁₂, like the combined form of the material in serum, is not available to micro-organisms until freed from combination. Callender and Lajtha (1951) have also shown that, although crystalline vitamin B₁₂ alone had no effect in maturing megaloblasts in tissue culture, the incubated mixture of vitamin B₁₂ and normal gastric juice was effective in this respect. Normal serum, in which, presumably, the vitamin was in the combined form, was also effective. The true significance of the combination of vitamin B₁₂ with components present in serum and gastric juice is still uncertain, as it is known that vitamin B₁₂ combines with great facility with a wide variety of protein-like substances (Meyer, Eppstein, Bethell, and Hall, 1950; Beerstecher and Altgelt, 1951; Bird and Hoevet, 1951). Some of these combinations are not specifically active in patients with pernicious anaemia (Hall, Morgan, and Campbell, 1950).

Serum Vitamin B₁₂ Concentrations and Megaloblastosis of the Bone Marrow.—

The vitamin B₁₂ concentrations found in the patients with pernicious anaemia, all of whom showed recognizable megaloblastic haemopoiesis on marrow biopsy, ranged from less than 16 to 105 $\mu\mu\text{g.}$ per ml. No free material was present in these sera. The upper limit of the serum vitamin B₁₂ concentration at which it is possible to recognize the presence of megaloblastic change in the marrow is probably between 80 and 100 $\mu\mu\text{g.}$ per ml. These levels have been found in patients who showed only minimal signs of anaemia as judged by an examination of the peripheral blood. Serum concentrations of 60 to 100 $\mu\mu\text{g.}$ per ml. have been found in single specimens of serum taken from three patients in the 71 to 93 age group not proved to have been suffering from a megaloblastic anaemia. Similarly in one patient with multiple myeloma and hyperglobulinaemia, total serum vitamin B₁₂ concentrations of 80 and 100 $\mu\mu\text{g.}$ per ml., 40 to 60 $\mu\mu\text{g.}$ of which was combined material, were found on two occasions.

Although the patient was anaemic, haemopoiesis was normoblastic at the time of the first serum assay. Thus, serum concentrations below 80 $\mu\mu\text{g.}$ per ml. may very occasionally not be associated with detectable clinical or haematological signs of deficiency. Whether such low values are due to technical errors in preparing specimens for assay, or to the error of the assay method itself, or to another cause, is uncertain.

Summary

The vitamin B₁₂ concentrations of the serum and urine of normal subjects, of patients with megaloblastic anaemia, and of patients suffering from various other conditions have been assayed, using *Euglena gracilis* as the test organism.

The total concentrations in the sera from normal subjects ranged from 100 to 720 $\mu\mu\text{g.}$ per ml. (mean 358 $\mu\mu\text{g.}$). The combined vitamin B₁₂ concentration ranged from 100 to 620 $\mu\mu\text{g.}$ per ml. (mean 329 $\mu\mu\text{g.}$). Similar values were found in sera from patients suffering from conditions other than megaloblastic anaemia.

The vitamin B₁₂ concentrations of the sera of patients suffering from pernicious anaemia in relapse and of patients with certain other types of megaloblastic anaemia which responded to treatment with vitamin B₁₂ were below the normal range. The amount of vitamin B₁₂ excreted daily in the urine by these patients was much less than the amount excreted by normal subjects. Patients suffering from megaloblastic anaemia which was refractory to treatment with vitamin B₁₂ were found to have normal serum vitamin B₁₂ concentrations.

The material in sera which has a vitamin B₁₂-like activity for the growth of the *Euglena* was present in some sera in both an uncombined and combined form. Uncombined material was only found in 30% of sera, usually in small concentrations. Certain sera, however, contained large amounts of uncombined material. The significance of this uncombined material is discussed, and reasons are given for believing that the concentration of the combined material represents haemopoietically active vitamin B₁₂.

We wish to thank Dr. J. V. Dacie for advice and encouragement during this investigation; the physicians at Hammersmith Hospital for their help and co-operation; Dr. J. M. Chalmers, Dr. G. A. Matthews, and Dr. A. I. Ross for sera from patients under their care; Glaxo Laboratories Ltd. for supplying the vitamin B₁₂ used in this study; Lederle Laboratories Division, Cyanamid Products Ltd., for the folinic acid used; Miss J. Davenport, Mrs. H. M. Stephen, Mr. L. H. Turnbull, and Mr. L. Wallett for technical assistance.

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