Erythrocyte Vitamin B_{12} Activity in Health, Polycythemia, and in Deficiency of Vitamin B_{12} and Folate

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THERE HAS BEEN RELATIVELY LITTLE WORK on the estimation of erythrocyte vitamin B_{12} activity in man, and consequently there are few reports of its activity in disease states. Sobotka et al.[1] reported low levels in four cases of pernicious anemia (P. A.), using *E. gracilis* and *O. malhamensis*. This was confirmed by Biggs et al.,[2] who also reported the levels found in treated cases of polycythemia vera and in various leukemic states using *L. leichmannii.*

Recently, we have developed a method for measuring vitamin B_{12} activity in erythrocytes using *E. gracilis.* This paper reports the levels found in control subjects, in primary and secondary polycythemia, and in vitamin B_{12} and folate deficiency states.

Materials and Methods

Methods

Blood samples were taken for routine hematological estimations using sequestrene as the anticoagulant. Heparinized blood samples were used for plasma vitamin B_{12} and folate determinations, and for erythrocyte vitamin B_{12} estimations.

Routine hematological methods, including red-blood-cell volume measurements, as described by Dacie and Lewis,[3] were used. The method of Anderson[4] for the measurement of serum vitamin B_{12} modified by using Difco medium was used to measure plasma vitamin B_{12}, and the method of Waters et al.[5] for the measurement of serum folate, modified by the use of 10 mg. ascorbate/ml. plasma, and a Difco *L. casei* assay medium, was used for plasma folate assay. In our laboratory, using these methods, similar results for folate and for vitamin B_{12} levels were obtained whether serum or plasma was assayed. The normal range for plasma folate is 2.2–18 ng./ml.,[6] and for serum vitamin B_{12} 150–1000 pg./ml.[4]

Erythrocyte vitamin B_{12} activity was measured using *E. gracilis.* Heparinized blood was centrifuged at 1500 g. for 30 minutes and the plasma removed for vitamin B_{12} and folate

From the University Department of Therapeutics, Royal Infirmary, Edinburgh, Scotland.
First submitted January 2, 1969; accepted for publication July 3, 1969.
Supported by Glaxo Laboratories, Ltd.

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assay. The erythrocytes collected in such a way that contamination with leucocytes was avoided, were washed once with isotonic saline, recentrifuged as before, and 2 ml. of packed cells washed into 2 ml. of acetate buffer (0.4 M and pH 5.3) and 16 ml. of distilled water, giving a 1/10 dilution. In a few cases the initial saline washings were assayed and negligible amounts of vitamin B_{12} were found. The proteins were then precipitated by autoclaving at 10 lbs. pressure for five minutes and removed, the supernatant being used for assay. Biochemical analysis of the supernatant by a modification of the method of Meulemans[7] showed that less than 5–10 mg. protein/100 ml. of filtrate were present and this is insignificant. At this dilution (1/10) the supernatant was found to inhibit the growth of *E. gracilis,* an effect due to the acetate buffer which was eliminated by a final assay dilution of 1/60 (Fig. 1). Subsequent investigation has shown that the in-

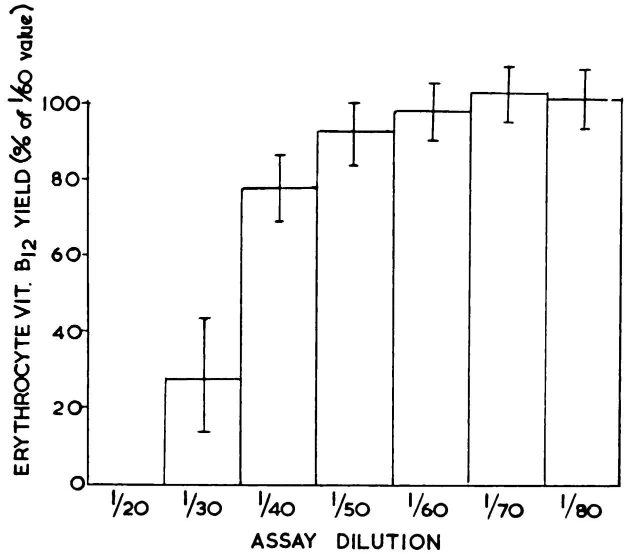

Fig. 1.—Erythrocyte vitamin B_{12} yield at increasing assay dilutions.

hibiting effect of acetate buffer could be eliminated by substituting citrate–phosphate buffer (0.1 M of citrate and 0.2 M of phosphate at pH 5.3) which had no inhibiting effect. Results using this buffer are similar to those obtained at a 1/60 dilution using acetate buffer. Aqueous standard tubes containing 0–12 pg./ml./tube were used, and all samples were assayed in duplicate. Vitamin B_{12} recovery studies were carried out on erythrocytes from three normal persons using the citrate–phosphate buffer. Addition of 100 and 200 pg./ml. of vitamin B_{12} in each case gave a recovery range of 97 per cent–128 per cent. Individual values are given in Table 1.

Table 1.—Recovery of Vitamin B_{12} from Erythrocytes (three normal subjects)

Subject Number	Vitamin B_{12} Recovery (pg./ml.) Basal Level	100 pg./ml. Added	200 pg./ml. Added
1	120	261	411
2	140	233	344
3	135	255	368

Subjects

Measurements were carried out in the following groups:

(1) *Normal Subjects* (29). These were healthy blood donors and laboratory staff with normal peripheral blood counts.

(2) *Deficiency of Vitamin B_{12} or Folate (27).* Twenty-four patients who had been

Table 2.—Folate Deficient Patients (Hematological Investigations and Diagnoses)

No.	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Cyanocobalamin Absorption Schilling Test	Cyanocobalamin Absorption Whole Body Count	Folate Plasma (ng./ml.)	Marrow	Diagnosis
1	24	208	16 per cent	–	0.8	Megaloblastic	Primary Malabsorption
2	21	132	5.7 per cent (+IF*—6 per cent)	–	2.0	Megaloblastic	Primary Malabsorption
				–			
3	93	216	–	–	2.0	Megaloblastic	Epileptic on Phenytoin Treatment
4	87	132	–	59 per cent	2.2	Megaloblastic	Alcoholic
5	87	168	–	31 per cent:59 per cent	1.6	Megaloblastic	Idiopathic Folate Deficiency
6	120	124	–	–	1.6	–	Crohn's Disease
7	132	292	11.1 per cent	–	1.5	Megaloblastic	Rectal Carcinoma
8	195	621	–		1.6	–	Rheumatoid Arthritis

* I.F. = Intrinsic Factor.

Table 3.—Primary Polycythemia Patients (Hematological Investigations)

No.	Sex	Hb (G per cent)	Peripheral Blood R.B.C. (M/cu. mm.)	Peripheral Blood W.B.C. (cu. mm.)	Platelets (1000s/cu. mm.)	Blood Volume R.B.C. (ml./Kg.)	Blood Volume Plasma (ml./Kg.)	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Folate Plasma (ng./ml.)
1	F	19.0	6.5	9000	736	–	–	198	688	5.0
2	F	20.4	7.7	10400	580	63.0	26.4	111	1120	4.8
3	F	18.6	5.7	9800	360	51.8	31.0	120	816	5.5
4	F	17.4	6.5	5700	635	34.7	38.6	171	452	5.0
5	M	17.9	6.0	11300	442	43.5	22.0	198	1088	11.6
6	F	17.2	8.1	13700	320	55.0	37.0	114	544	5.0
7	F	16.2	6.0	18000	550	45.4	31.0	159	836	4.2
8	M	20.0	7.5	8000	250	46.4	25.0	165	1384	3.6
9	M	17.0	6.2	13400	650	42.0	44.4	152	336	4.3
10	M	19.0	8.1	19100	330	63.0	42.0	51	620	7.8
11	F	20.4	9.1	19700	205	68.8	31.7	54	960	4.4
12	M	18.0	7.2	21200	305	60.0	38.0	90	552	3.0
13	F	14.8	5.0	46000	670	35.7	44.0	60	452	2.9
14	F	19.1	7.4	12600	200	48.0	37.0	66	728	4.1

found to have a megaloblastic marrow picture were studied. Eighteen of these cases were then classified as vitamin B_{12} deficient (plasma vitamin B_{12} < 100 pg./ml.), and six cases as folate deficient (plasma folate < 2.4 ng./ml.).

In three cases marrow examination was not carried out. One such patient was vitamin B_{12} deficient as shown by a plasma vitamin B_{12} level of 48 pg./ml. and a plasma folate value of 20.1 ng./ml. In two cases folate deficiency was diagnosed from plasma folate levels of 1.6 and 1.6 ng./ml. with plasma vitamin B_{12} levels of 621 and 124 pg./ml., respectively.

All the patients suffering from vitamin B_{12} deficiency had pernicious anemia. The diagnosis and information regarding B_{12} absorption in the folate deficient group is shown in Table 2.

(3) *Polycythemia* (22). In 17 cases the red blood cell volume was above 35 ml./Kg. in males and above 30 ml./Kg. in females. In five cases volume studies were not considered justifiable. Four were children with congenital heart disease who had all been cyanosed from birth, were still so when blood was collected, and who had PCV values above 55 per cent. One was a pregnant woman with the clinical features of polycythemia vera including splenomegaly, Hb. 19 Gm./100 ml. PCV 60 per cent, WBC 9000/cu. mm., platelets 736,000 cu.mm., leucocyte alkaline phosphatase 163 counts per 100 consecutive neutrophils (control 64 counts), and a marrow picture showing hyperplasia of the erythroid, myeloid, and megakaryocytic series.

These cases were then classified into those in whom no cause for polycythemia could be found (polycythemia vera), and those in whom polycythemia was secondary to a recognized lesion. The latter group included four cases of congenital cyanotic heart disease, two cases of chronic bronchitis and cor pulmonale without clinical signs of cardiac failure, and two cases of erythrocytosis, accompanied in one by a large bronchial carcinoma and in the other by a calcified intracerebral lesion of unknown type. In no case had treatment for the polycythemia been given.

In the statistical analysis of results, the Behrens–Fisher test was used, and all significant differences were at the 5 per cent level.

Results

Polycythemia

Figure 2 shows the values obtained for erythrocyte and plasma vitamin B_{12} activity in control subjects, and in primary and secondary polycythemia. The mean values in controls were, for erythrocytes, 158 pg./ml. of packed cells (S.D. 31 pg.), and for plasma 349 pg./ml. (S.D. 133 pg.); in primary polycythemia, for erythrocytes, 122 pg./ml. of packed cells (S.D. 53 pg.), and for plasma 755 pg./ml. (S.D. 299 pg.); in secondary polycythemia, for erythrocytes, 170 pg./ml. (S.D. 92 pg.), and for plasma, 692 pg./ml. (S. D.$\pm$234 pg.).

The difference between the erythrocyte vitamin B_{12} level in controls and in primary polycythemia was significant, but that between controls and secondary polycythemia was not. The differences between the controls and the two polycythemic groups for plasma vitamin B_{12} was significant.

Table 3 shows hematological data for patients with primary polycythemia. The final five patients are those with abnormally low erythrocyte vitamin B_{12}. These patients do not show any feature which distinguishes them from those with a normal erythrocyte vitamin B_{12} level, and none of them had an excessively low plasma folate.

Figure 3 shows the yield of vitamin B_{12} from erythrocytes as a percentage of the 1/60 dilution value in normals and four cases of polycythemia, three of whom had low red cell vitamin B_{12} levels, at varying assay dilution. No in-

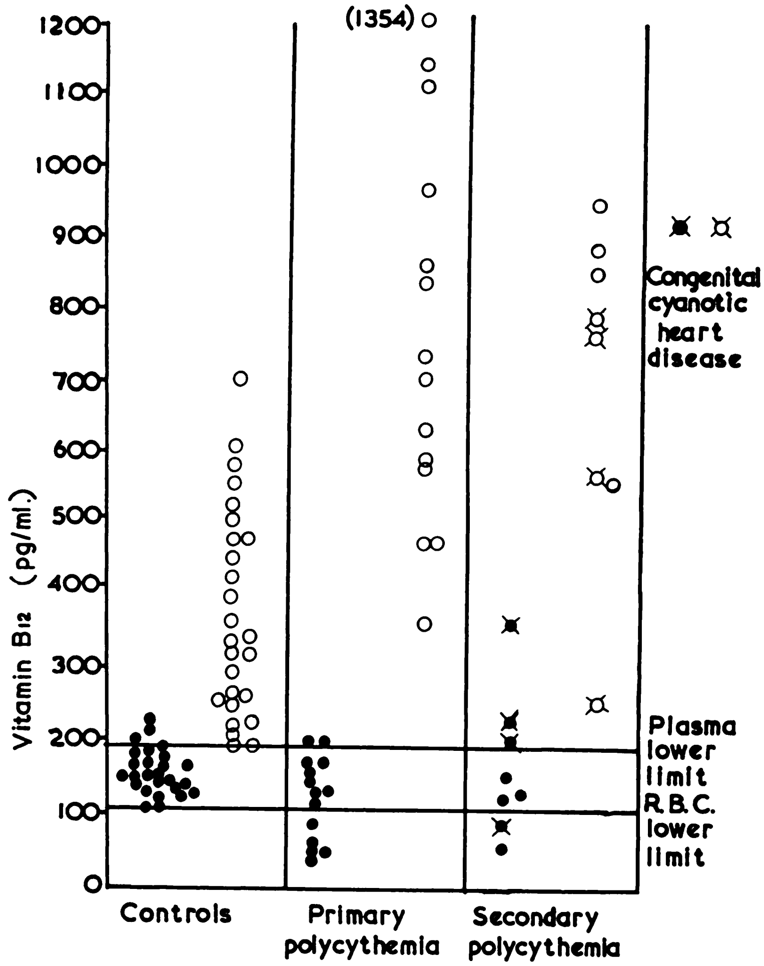

Fig. 2.—Erythrocyte (filled circles) and (plasma open circles) vitamin B_{12} levels in controls and polycythemia.

hibitor peculiar to the patients with primary polycythemia was demonstrated.

Table 4 shows the hematological data and diagnosis in those patients with secondary polycythemia.

B_{12} and Folate Deficiency

Figure 4 shows the values obtained in vitamin B_{12} and folate deficiency as compared to the same controls. The mean values for the vitamin B_{12} deficient patients were, for erythrocytes, 53 pg./ml. of packed cells (S.D. 32 pg.), and for plasma, 32 pg./ml. (S.D. 28 pg.), and for the folic acid deficient group, for erythrocytes, 95 pg./ml. of packed cells (S.D. 57 pg.), and for plasma, 237 pg./ml. (S.D. 165 pg.).

The difference between the erythrocyte vitamin B_{12} level in controls and in vitamin B_{12} deficiency was significant, as was the difference between controls and patients suffering from folate deficiency.

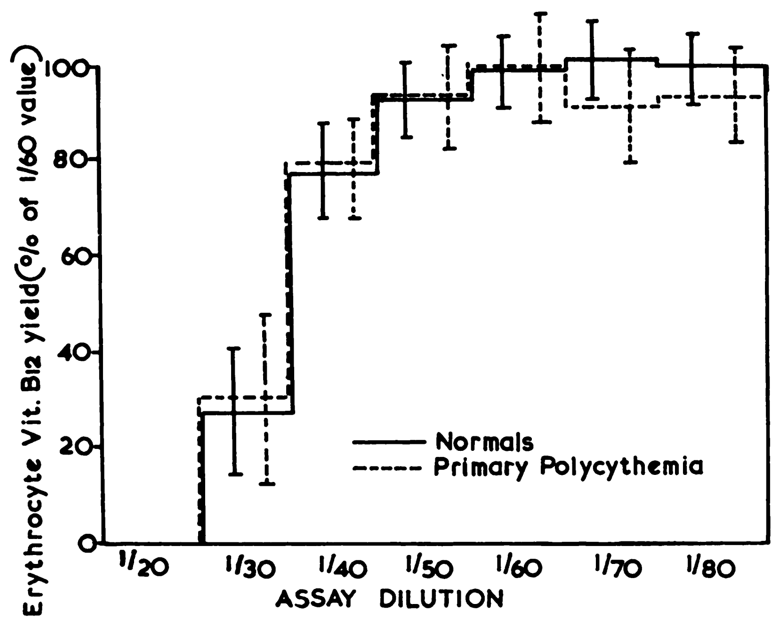

Fig. 3.—Erythrocyte vitamin B_{12} yield at increasing assay dilution in normals and primary polycythemia (four cases).

There was no significant difference between the erythrocyte vitamin B_{12} levels in vitamin B_{12} and folate deficiency. The differences between the controls, and both the vitamin B_{12} and folate deficient groups with regard to plasma vitamin B_{12} were significant, the level in the vitamin B_{12} deficient group being significantly lower than in the folate deficient group.

Discussion

The results obtained in this study for the erythrocyte vitamin B_{12} activity in normal persons agrees with those obtained by other workers.[1,2,8] Regarding patients with primary polycythemia (polycythemia vera), it has already been shown[2] that, in a treated group of patients, the erythrocyte B_{12} level is significantly below normal. This study confirms these findings in untreated patients, but it is noted that only five of the 14 patients had levels below the normal range (lower level 110 pg./ml.). These five patients were not clinically different from others, and in the group as a whole there was no relationship between the erythrocyte vitamin B_{12} level and the hemoglobin, white cell count, platelet count, red cell mass, plasma folate, or plasma vitamin B_{12} level. As shown in Fig. 3, no effective erythrocyte inhibitor could be found in the patients studied. No reason could be found for the reduced level of vitamin B_{12} in the erythrocytes. In untreated secondary polycythemia, the erythrocyte vitamin B_{12} level of the group as a whole was not significantly different from that of the normals, but there were two patients in the group with levels below the normal range, one with congenital cyanotic heart disease (P.C.V. 75 per cent), and one with cor pulmonale due to chronic bronchitis (red cell volume

Table 4.—Secondary Polycythemia Patients (Hematological Investigations and Diagnoses)

No.	Sex	P.C.V. per cent	Blood Volume R.B.C. (ml./Kg.)	Blood Volume Plasma (ml./Kg.)	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Folate Plasma (ng./ml.)	Diagnosis
1	M	57	40.6	37.5	128	880	2.3	Bronchial Carcinoma
2	F	62	47.4	33.0	126	948	6.0	Calcified Cerebral Lesion
3	M	65	68.0	31.0	153	524	4.4	Chronic Bronchitis Cor Pulmonale
4	M	58	61.0	39.0	75	852	2.8	"
5	F	55	–	–	222	520	6.3	Tetralogy of Fallot
6	F	57	–	–	201	784	3.4	"
7	F	70	–	–	360	764	6.2	"
8	M	75	–	–	93	260	4.0	"

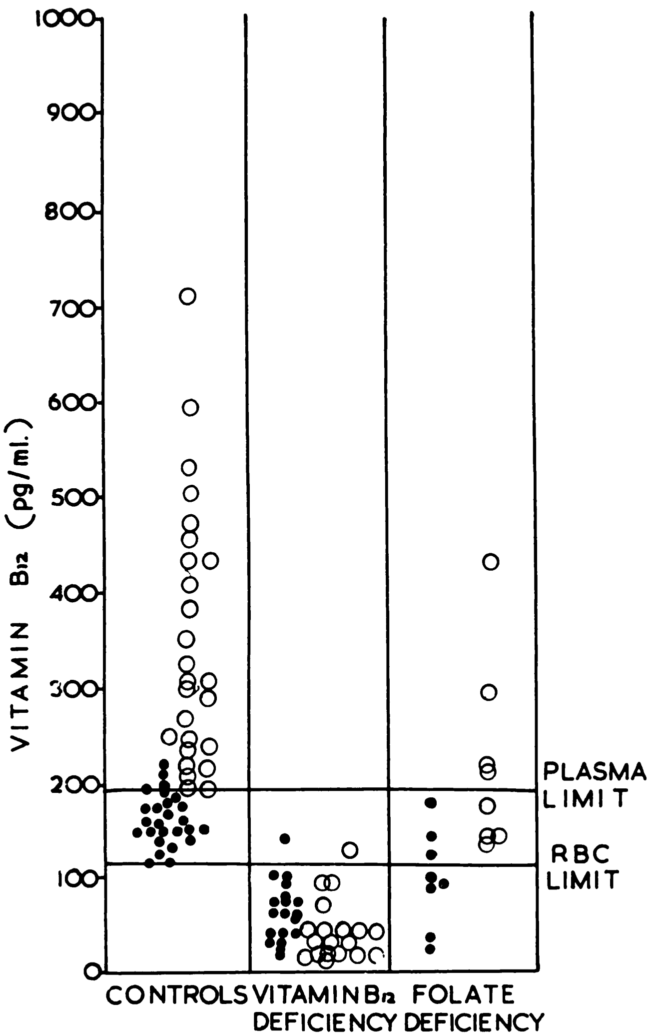

Fig. 4.—Erythrocyte (filled circles) and plasma (open circles) vitamin B_{12} levels in controls, and in vitamin B_{12} and folate deficiency.

61 ml./Kg.). No explanation was found for this, but it presents the possibility that a low erythrocyte vitamin B_{12} level may be related to polycythemic states in general, rather than to the primary form alone. It is known that the plasma vitamin B_{12} may be high in primary polycythemia,[9] and this finding is confirmed here In addition, five of the eight secondary cases had levels above the normal range, though two of these were the patients with isolated erythrocytosis who might in reality be primary cases.

It has already been shown in small numbers of cases that the erythrocyte vitamin B_{12} level is low in pernicious anemia.[1,2,8] This reduction is less than that of the plasma so that the erythrocyte concentration exceeds that of the plasma, and this is the reverse of the normal finding. This was confirmed, but it was found that the level of vitamin B_{12} in erythrocytes is not always greater than in plasma. In five of the 19 patients, the plasma vitamin

B_{12} level exceeded the erythrocyte level, and in one they were the same. Two patients deserve special mention: in one with frank pernicious anemia (Hb 7.8 Gm./100 ml. : marrow megaloblastic) the level of vitamin B_{12} in the plasma was virtually unrecordable at 8 pg./ml., yet the erythrocyte level was normal at 144 pg./ml., while in the other, a patient found to have pernicious anemia during infertility investigations (Hb 12.2 Gm./100 ml.: marrow megaloblastic), the plasma vitamin B_{12} reading was 120 pg./ml. and that of the erythrocyte 67 pg./ml. In both patients the plasma folate level was normal. Clearly the relationship of the plasma to the erythrocyte vitamin B_{12} is not a simple one. There was no relationship between either the erythrocyte or plasma vitamin B_{12}, and the hemoglobin or red cell count in these patients.

We have found no previous reports of erythrocyte vitamin B_{12} levels in folate deficiency. Five of the eight cases studied had levels below the normal range, and in all of these the plasma vitamin B_{12} was normal. In one, a case of primary malabsorption, cyanocobalamin absorption was poor (Schilling test 6 per cent), and in two it was normal. Thus, in folate deficiency, a low erythrocyte vitamin B_{12} level is not related to malabsorption of cyanocobalamin. As has been reported by other workers, the plasma vitamin B_{12} was found to be low in this group, but no relationship between the plasma and erythrocyte vitamin B_{12} was found.

Summary

A method for the estimation of vitamin B_{12} in erythrocytes using *E. gracilis* is described. In 29 normal subjects the mean value was 158 pg./ml. (S.D. 31 pg.) packed cells.

Five of 14 patients with untreated primary polycythemia had very low erythrocyte vitamin B_{12} levels, and in the group as a whole the value was significantly less than normal. In eight patients with secondary polycythemia the erythrocyte vitamin B_{12} did not differ from normal, though two patients had values below the normal range.

In 19 cases of pernicious anemia the mean erythrocyte vitamin B_{12} level was 53 pg./ml. (S.D. 32 pg.) packed cells which was significantly reduced. There was no constant relation between the erythrocyte and plasma levels. In eight cases of folate deficiency the erythrocyte vitamin B_{12} level was significantly reduced, with a mean value of 95 pg./ml. (S.D. 57 pg.) packed cells.

ACKNOWLEDGMENTS

We would like to thank Mr. D. A. Williams of the Department of Statistics, Edinburgh University, for carrying out the statistical analysis on our results, and Mr. J. Baird, A.I.M.L.T., for his technical assistance.

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