Vitamin B₁₂ levels in erythrocytes in normal subjects and in pernicious anaemia

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SYNOPSIS A reliable method is described for the assay of vitamin B₁₂ activity in erythrocytes using *Lactobacillus leichmannii*. The results are shown for age, sex, and blood group in normal subjects, and before, during, and after therapy in vitamin B₁₂ deficiency anaemia.

The normal range is 85-225 pg/ml (mean = 155 ± 35 pg/ml). Raised levels were found in the newborn, and subnormal levels in subjects aged 70 years and over and in males aged 10 to 19 years. There were no differences in erythrocyte vitamin B₁₂ levels between the ABO or Rhesus blood groups.

In pernicious anaemia low levels are found before treatment (mean = 91 pg/ml), followed by a rapid rise during vitamin B₁₂ therapy (mean peak = 482 pg/ml), and falling more slowly towards normal after the initial haematological response.

Most of the work on vitamin B₁₂ levels previously reported in the literature has been performed either on serum or on plasma. Since vitamin B₁₂ is essentially an intracellular substance, however, it is particularly relevant to know something of its concentration within cells as well. Erythrocytes were the obvious choice for this purpose since they are the most easily obtained of all body cells, and because it is known that various changes occur in them in pernicious anaemia (Price-Jones, 1929; Israels, 1939; Scott, 1939).

Levels in normal whole blood (Table I) have been reported by Couch, Olcese, Witten, and Colby (1950), Sobotka, Baker, and Ziffer (1960), and by Rosenthal and Brown (1954). but these are open to ambiguous interpretation. In unwashed normal erythrocytes Kelly and Herbert (1967), using a radioactive assay, obtained a range of 72 to 520 pg/ml, and Biggs, Mason, and Spray (1964), using the lactobacillus, found 110-500 pg/ml.

Biggs *et al* (1964) considered that washing the erythrocytes was unnecessary, despite the fact that one patient in their series, who was suffering from myeloid leukaemia, showed a fall in the vitamin B₁₂ content of the red cells from 745 pg/ml to 440 pg/ml after two washings of the cells in saline.

In normal subjects vitamin B₁₂ levels in erythrocytes are usually lower than those in plasma, as shown in Table I. Thus it is preferable to remove traces of plasma from erythrocytes before assay to ensure the absence of any error from this source. Also, blood from patients receiving vitamin B₁₂ therapy was to be tested in the present survey, and experiments on blood taken within 60 hours after the injection of 1,000 µg cyanocobalamin showed (Table II) that washing the cells reduced their vitamin B₁₂ level by up to 400%, depending on the plasma B₁₂ levels. The reduction in normal subjects who were not receiving vitamin B₁₂ was up to 40 pg/ml, ie, up to 20%. These results confirmed the need to wash the cells before assay.

It will be noticed from Table I that in only two of the reports were the erythrocytes washed before examination (Sobotka *et al*, 1960; Kato, 1958). In both of these communications only a relatively small number of subjects were examined (three and 20 respectively) and it is clearly desirable that to establish the normal range of vitamin B₁₂ concentration in erythrocytes accurately a larger series of investigations is needed.

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Reference	No. of Patients	Source	Vitamin B ₁₂ Level (pg/ml)	
			Range	Mean
Couch <i>et al</i> (1950)	10	Whole blood	600-1,400	800
Sobotka <i>et al</i> (1960)	3	Washed RBCs	140-185	165
		Whole blood	260-425	332
		Plasma	320-620	513
Rosenthal and Brown Jr. (1954)	—	Whole blood		260
Biggs <i>et al</i> (1964)	50	Unwashed RBCs	110-500	213
		Plasma	150-1540	603
Kelly and Herbert (1967)	50	Unwashed RBCs	72-520	209
		Plasma	196-584	392
Kato (1958)	20	Washed RBCs	135-230	195
		Plasma		362
Present work	256	Washed RBCs	80-280	155
		Plasma	140-920	310

Table I Vitamin B₁₂ activity in normal subjects

Subject	Plasma	Vitamin B ₁₂ Level (pg/ml)				
		Number of Erythrocyte Washes				
		Nil	1	2	3	4
<i>Pernicious anaemia</i>						
1	600	380	280	260	240	—
2	2,100	1,800	780	530	500	—
3	2,600	3,100	1,800	960	800	800
4	3,800	2,600	1,200	240	166	166
5	1,200	—	430	270	—	270
<i>Normal</i>						
1	280	220	190	184	—	—
2	540	260	220	220	220	—
3	760	108	112	112	108	—
4	360	160	140	140	—	—
5	292	160	—	144	—	—
6	230	180	—	160	—	—

Table II The effect of washing the erythrocytes on vitamin B₁₂ level in cases of pernicious anaemia on intramuscular Cytamen and in normal subjects

Materials and Methods

The method used was based upon a modification by Biggs *et al* (1964) of Spray's technique (1955) for the estimation of vitamin B₁₂ in serum. After the investigation of various factors involved, including the extraction of B₁₂ from unhaemolysed cells and from cells haemolysed after thawing from -20°C, buffer pH, various concentrations of cyanide, interference by protein, and length of incubation, some alterations were made to the original method. These are included in the following section, together with the reasons for introducing the changes.

METHOD

Ten ml blood was collected, by venepuncture, into heparin. Plasma was separated by centrifuging and the red cells were washed twice in 0.9%

saline and centrifuged. The buffy layer was removed with the supernatant fluid. (Three washes were required if the patient was receiving vitamin B₁₂.) The cells were then stored at -20°C until the assays were performed, usually within four weeks.

Extracts of the erythrocytes were prepared in 1 oz universal containers by adding 2 ml of washed erythrocytes to 2 ml 0.4 M-acetate buffer (pH 5.2), 16 ml deionized water, and a drop of 0.5% (w/v) sodium cyanide and mixing. The samples were then autoclaved at 110° for 30 minutes (Spray, 1955) and centrifuged. From each sample amounts of clear supernatant fluid, each of 5 ml, were placed into each of three 5 in. × ½ in. test tubes. Standards of 0.25, 50, 100, 200, 300, and 400 pg of cyanocobalamin were also prepared. To each was added 5 ml of double-strength assay medium (Difco) and the tubes were capped and placed in a water bath at 60°C for 30 minutes to maintain sterility. After cooling to 37°C, 1 drop of washed suspension of actively growing *Lactobacillus leichmannii* was added to each tube. The tubes were incubated at 37°C for 72 hours and finally titrated with 0.2 N sodium hydroxide using phenolphthalein as indicator.

The medians of the test titrations were read off a graph made from the standards on semi-log paper.

THE TEST ORGANISM (*LACTOBACILLUS LEICHMANNII*)

An important factor in obtaining reliable assay was found to be in the use of an actively growing lactobacillus (Spray, 1955). This was obtained by repeated subculture of the organism until six- to eight-hour growth could be seen to be abundant. A minimum of two subcultures usually needed to achieve this. Adequate washing of the lactobacillus is also required to remove any vitamin B₁₂ present in the culture medium (Spray, 1955) and thereby improve the 'sensitivity' of the method. Thus traces of vitamin B₁₂ inoculated into the assay tubes gave a higher blank reading and masked small amounts of the vitamin present in the sample being assayed. Good results, with very low blank titrations, were obtained by the 'three-tubes transfer' technique of washing. The actively growing culture is centrifuged at 3,000 rpm for five minutes, the supernatant decanted, and the tube drained by inversion on blotting paper. The organisms at the bottom of the tube were removed to a clean tube by adding 1 or 2 drops of assay media directly onto the deposit. The tube was filled with assay media, mixed, and centrifuged. Washing was then repeated twice. The washed organisms were then diluted with assay medium until the suspension matched approximately Brown opacity tube no. 1 (400,000 organisms per ml) and were ready for inoculation.

Age, Sex, and No. of Subjects		Erythrocyte Vitamin B ₁₂ Activity (pg/ml)					
		Mean $\pm$ SD		Range	Em	t	P
<i>At birth</i>							
Males	(3)	172	28	144-200	16	1.06	0.4
Females	(6)	190	41	144-250	17	2.11	0.08
M & F	(9)	184	36	144-250	12	2.3	0.05
<i>10-19 Years</i>							
Males	(6)	109	41	80-182	16	2.7	0.05
Females	(8)	162	25	132-200	8.9	0.78	0.5
M & F	(12)	146	42	80-200	12	0.75	0.5
<i>20-29 Years</i>							
Males	(8)	152	22	100-168	7.7	0.04	0.97
Females	(8)	160	23	124-196	8	0.63	0.6
M & F	(16)	156	22	100-196	5.5	0.18	0.9
<i>30-39 Years</i>							
Males	(7)	141	27	111-170	10	1.4	0.3
Females	(5)	156	10	142-164	4.2	0.24	0.9
M & F	(12)	147	22	111-170	6.3	1.27	0.3
<i>40-49 Years</i>							
Males	(8)	169	42	110-232	15	0.94	0.4
Females	(6)	149	36	114-180	15	0.04	0.97
M & F	(14)	160	36	110-232	9.5	0.5	0.7
<i>50-59 Years</i>							
Males	(5)	163	27	138-200	12	0.7	0.6
Females	(5)	158	21	124-178	9.2	0.3	0.8
M & F	(10)	160	23	124-200	7.1	0.7	0.5
<i>60-69 Years</i>							
Males	(2)	134	42	104-164	30	0.7	0.6
Females	(9)	159	20	120-196	6.7	0.6	0.6
M & F	(11)	154	24	104-196	7.3	0.01	0.99
<i>70 & Over</i>							
Males	(1)	128	—	—	—	—	—
Females	(8)	138	22	112-180	7.8	2.2	0.06
M & F	(9)	137	21	112-180	7	2.57	0.05
<i>All Ages</i>							
Males	(38)	151	35	80-232	5.7	0.7	0.5
Females	(55)	158	27	112-250	3.6	0.83	0.5
M & F	(93)	155	31	80-250	3.2	—	—

Table III Vitamin B₁₂ activity related to age and sex

RECOVERY OF ADDED COBALAMIN FROM ERYTHROCYTES

Assays were made on washed erythrocytes to which vitamin B₁₂ had been added in various concentrations. Recoveries obtained were 100% $\pm$ 6%.

ACCURACY OF THE METHOD

Assays were made on 50 samples of human erythrocytes. Fifty-four per cent of the results agreed within $\pm$ 5% of the initial levels and 70% were within $\pm$ 10%. The standard deviation was $\pm$ 10%.

Results in Normal Subjects

The mean and standard deviation of vitamin B₁₂ activity in erythrocytes in 256 apparently normal subjects was 155 $\pm$ 35 pg/ml. The complete range was 80 to 280 pg/ml but 95.5% of readings obtained from the normal population had levels between 85 and 225 pg/ml.

The results for age and sex in 93 subjects are shown in Table III and the results relating to blood group in Table IV. The age group 0 to 9 years was not examined, apart from nine newborn infants. There was a slightly significant rise above the mean ($P = 0.05$) at birth and slight falls below the mean age 10-19 years in males ($P = 0.05$) and also at age 70 years and over ($P = 0.05$). There were no statistically significant differences between the remaining age groups of 10 to 69 years. There were also no significant differences between the various ABO or Rhesus blood groups.

Furthermore there was no apparent relationship between plasma and erythrocyte vitamin B₁₂ activity in normal subjects (Fig. 1); thus plasma levels could be high with low normal erythrocyte levels. However, greater variability was observed in the plasma levels, ie, 140 to 920 pg/ml, than in the erythrocyte levels, 80 to 280 pg/ml, which suggests that the erythrocyte level is a more critical indication of the cell content of vitamin B₁₂ than is the plasma level; the latter level probably reflects the nature of the subject's diet.

Blood Group	Rh	No. of Subjects	Mean	SD	Range	Em	t	P
O	Positive	41	161	36	96-250	5.6	1.08	0.3
O	Negative	26	159	43	82-260	8.5	0.5	0.7
A	Positive	46	155	36	88-220	5.2	0	1.0
A	Negative	25	166	41	112-280	8.2	1.3	0.3
B	Positive	18	145	35	86-216	8.3	1.2	0.3
AB	Positive	7	151	14	126-160	5.3	0.76	0.95
<i>All ABO groups</i>								
Rh positive		112	150	36	88-250	3.4	1.47	0.2
Rh negative		51	162	36	82-280	5.0	1.4	0.2
O positive and negative		67	160	39	82-260	4.7	1.06	0.3
A positive and negative		71	159	38	88-280	4.5	0.9	0.4
All groups		163	155	37	82-280	2.9	—	—
<i>Normal controls plus blood donors</i>								
		256	155	35	80-280	2.2	—	—

Table IV Vitamin B₁₂ activity in erythrocytes from blood donors

Fig. 1 The relation of the plasma and erythrocyte vitamin B₁₂ levels in normal subjects.

Erythrocyte Vitamin B₁₂ Activity in Pernicious Anaemia

The diagnosis of pernicious anaemia was confirmed in each case by the peripheral blood picture, sternal marrow examination, histamine-

fast achlorhydria, and plasma vitamin B₁₂ level of 102 pg/ml or less. None had clinical evidence of steatorrhoea and no patients with postgastrectomy vitamin B₁₂-deficiency anaemia were included in this series.

The relatively high initial plasma B₁₂ level in the patients with pernicious anaemia is partly due to low blank titrations causing an increased sensitivity of the method for vitamin B₁₂. (High blank titrations may mask small quantities of B₁₂.) It is also partly due to selection of patients with pure B₁₂ deficiency and the exclusion of those with combined folate or iron deficiency. The range of plasma B₁₂ values for the whole series was 20-102 pg/ml (mean = 50 pg/ml).

Progress was assessed by the packed cell volume (PCV) readings performed on the Hawksley microcentrifuge and by haemoglobin estimation (100% = 14.8 g%). All responded typically to vitamin B₁₂ therapy.

The behaviour of the erythrocyte B₁₂ levels is shown in Fig. 2 and Table V, and the main haematological details are presented in Table VI. Patients 1, 2, and 3 received Cytamen, 1,000 µg on alternate days for one week, then 1,000 µg every month. Patients 4 and 5 received 100 µg twice weekly during the period of surveillance.

The vitamin B₁₂ activity in the erythrocytes was low (mean = 87 pg/ml) before therapy. After intramuscular vitamin B₁₂ therapy there was rapid rise which coincided approximately with the reticulocyte response. The peak level observed (mean = 427 pg/ml) during the period of surveillance was approximately five times the initial level. This is a highly significant rise statistically ($P = <0.001$) both in relation to the level before treatment and to the normal mean level of 155 pg/ml.

The vitamin B₁₂ concentration then returned gradually after this initial peak towards normal limits over a period of one to two months. At the end of the haematological and B₁₂ response (approximately 70 days) the level was 188 pg/ml which, although above normal, was not significantly so ($P = 0.06$).

The patterns of change were similar in all the patients, and those observed in case 1 are described in detail as an example. The initial Hb was 4 g%, reticulocyte count 2.2%, and vitamin B₁₂ activity in plasma was 98 pg/ml and in erythrocytes 104 pg/ml. In response to a dosage of cyanocobalamin intramuscularly of 1,000 µg on alternate days, the reticulocyte count rose to a peak of 26.4% on the sixth day. On this same day a maximum level of 520 pg/ml of B₁₂ was observed in the erythrocytes. The reticulocyte count then declined to 10.2% on the 12th day but at this stage the erythrocyte B₁₂ level still remained high (450 pg/ml). The reticulocyte count continued to fall and reached 0.4% by the 29th day, but the erythrocyte B₁₂ level fell more slowly and was still 270 pg/ml on this day, and

the erythrocyte B₁₂ level did not, in fact, return to normal (190 pg/ml) until the 91st day. The haematological response was good and the

Hb rose steadily throughout to reach 9.6 g% on the 29th day and 13.3 g% on the 91st day.

Discussion

In obtaining reliable results in the microbiological assay of vitamin B₁₂ by the use of *Lactobacillus leichmannii* the two most important factors were found to be the use of an actively growing organism and the thorough washing to remove traces of exogenous vitamin B₁₂ from the organism before inoculation. Neglect of these two steps could cause irregularities in the results. Thus, Biggs *et al* (1964) and Kelly and Herbert (1967) have reported vitamin B₁₂ activities in unwashed erythrocytes in which the upper levels in normal subjects were found to be at least twice as high as the upper limits reported in washed red cells by Sobotka *et al* (1960) and Kato (1958) and in those reported in this communication (Table I). The difference is most probably due either to vitamin B₁₂ in the film of plasma of unwashed red cells, which is loosely attached to the red cell membrane, or, possibly, that dialysable vitamin B₁₂ may be present in the cell or its membrane. The increased levels of vitamin B₁₂ activity found in the newborn may be due to the large numbers of immature cells present. The significantly subnormal levels in subjects over the age of 70 years may be related to the fall in plasma levels of the vitamin which is known to occur in the elderly (Chow, Wood, Horonick, and Okuda, 1956; Boger, Wright, Strickland, Gylfe, and Ciminera, 1955).

The subnormal levels in males aged 10 to 19 years may be due to the rapid rate of growth at this age, when the haemopoietic tissues compete with other tissues for vitamin B₁₂, folic acid, and iron. Other factors must be involved, however, since the levels in females of this age were normal.

In pernicious anaemia the concurrence of a rise in vitamin B₁₂ activity in the erythrocytes with the reticulocyte response suggests that a factor may be common to both, ie, the presence of increased quantities of assayable vitamin in immature or developing cells. In this connexion it should be recalled that Schilling (1964) noted

Fig. 2 Erythrocyte vitamin B₁₂ levels in pernicious anaemia during therapy.

Case No.	Erythrocyte B ₁₂ Levels (pg/ml)				
	Initial	Maximum	Day Observed	Final	Day
1	104	520	6	190	70
2	54	800	7	206	69
3	92	1,200	9	156	70
4	76	490	7	170	75
5	98	420	11	204	70
Mean	85	686	8	185	71
P	<0.001	<0.001		0.06	

Table V Erythrocyte vitamin B₁₂ levels in pernicious anaemia before, during, and after therapy with B₁₂

Case No.	Age (yr)	Sex	Days Observed	Dose of CN-B ₁₂ in First Week (μg)	Haemoglobin (g%)		Reticulocyte Response		Plasma B ₁₂ Level (pg/ml)	
					Initial	Final	Maximum (%)	Day No.	Initial	Final
1	72	F	91	3,000	3.9	13.3	26.4	6	98	5,000
2	63	F	69	3,000	5.8	14.8	24.0	6	66	400
3	64	F	96	2,000	5.2	14.0	16.0	9	70	440
4	61	F	114	2,000	7.7	14.0	16.0	7	100	280
5	76	F	94	100	8.9	13.4	—	—	76	376

Table VI Details of responses of five cases of pernicious anaemia during initial two to three months on vitamin B₁₂ therapy

that the uptake of radioactive B_{12} by immature erythrocytes coincided with the reticulocyte count. It is also of interest, however, that the increased non-radioactive vitamin B_{12} activity still persists from some time after the reticulocyte count has returned to normal. This suggests that some cells which show no visible sign of immaturity on routine screening (eg, the presence of reticulins, polychromasia, or stippling) may, nevertheless, still show microbiological signs of incomplete maturity, as demonstrated by persisting raised vitamin B_{12} activity. It is also pertinent to speculate whether this means that the fully mature cell loses some vitamin B_{12} during maturation, or whether the vitamin is gradually incorporated into the protein structure of the erythrocyte in such a way as to make it no longer releasable by simple autoclaving in the presence of cyanide. In this connexion it has been shown by Mooney and Heathcote (1967) that only 3% of the total vitamin B_{12} present in casein is releasable by autoclaving at 10 lb pressure for 30 minutes, and it is reasonable to infer that similar binding may occur during the complete maturation of other proteins, including erythrocytes, as well. This point requires further study.

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