VITAMIN B₁₂ IN PERNICIOUS ANAEMIA: PARENTERAL ADMINISTRATION

BY

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The isolation of a red crystalline anti-pernicious-anaemia factor from liver was achieved almost simultaneously by Rickes, Brink, Koniuszy, Wood, and Folkers (1948) in America, and by Lester Smith and Parker (1948) in Great Britain. American workers were aided greatly by microbiological assays (Shorb, 1948), whereas in Britain attention was concentrated on testing each fraction in patients with pernicious anaemia in relapse. The 84 clinical tests begun in 1946* which guided Lester Smith's purification to the final stage of crystallization are of little medical interest and will not be described.

This report concerns only the pure crystalline material isolated by Lester Smith in Glaxo Laboratories, Greenford, which has proved to be identical with vitamin B₁₂. Chemical and physical properties are reported elsewhere by Lester Smith (1948), and by Fantès, Page, Parker, and Lester Smith (in the press).

Material and Methods

The 53 cases selected had the usual diagnostic features of pernicious anaemia, including megaloblastic marrow and histamine-fast achlorhydria. A few patients had relapsed after stopping treatment, but most had never been treated.

To limit the intake of extraneous haematopoietic factors during the initial test periods, meat, poultry, offal, meat products, yeast products such as "marmite," eggs, fresh fruit, and vitamin preparations were excluded from the diet, which contained white or National flour, processed cereals, sugar, jam or syrup, dried fruit, vegetables, butter and other fats, white fish, cheese, and 1 pint (570 ml.) of milk daily.

Reticulocytes were counted daily by a wet method using brilliant cresyl blue. Haemoglobin was determined by the cyan-haematin method of King and Gilchrist (1947). Red blood cells were counted every five days with N.P.L.-tested pipettes on a Bürker haemocytometer. Blood was diluted 1 in 200 with Hayem's fluid and at least six areas 1 by 0.05 mm. were counted. A test designed to measure the accuracy of counts by taking morning and afternoon specimens in 10 patients with anaemia showed that the standard error was $\pm 6\%$.

For a control period of 7 to 10 days treatment was withheld so that patients showing signs of spontaneous remission could be excluded. A shorter control period was permitted if anaemia increased rapidly. A few cases were rejected because of inter-current illness and one because of hypoplasia of the marrow. Also excluded were results rendered invalid by deterioration of vitamin B₁₂ in very dilute solution (1 μg . per ml.)—a difficulty which has since been overcome.

Vitamin B₁₂ was given intramuscularly as a single dose. Doses were graded logarithmically from 1.25 to 160 μg . An extra group was given 6.5 or 7.5 μg . (mean 7.2) because a level of dosage between 5 and 10 μg . was then believed to be critical (see later).

Patients with red blood cell counts above and below the mean level of 2,000,000 per c.mm. were, so far as possible,

distributed equally in the groups receiving low, medium, and high doses.

Results

There were no unpleasant side effects, and sensitization to vitamin B₁₂ was not encountered. The magnitude of the haematopoietic response was gauged partly from the

TABLE I.—List of All Responses

	Dosage in μg .	Reticulocyte Response (%)	Red Blood Cell Counts (Millions per c.mm.) at Days Indicated						
			0	5	10	15	20	25	30
1	1.25	5.2	1.33	1.46	1.60	1.26			
2	"	Nil	2.23	2.12	2.27	2.36	2.58	2.62	2.54
3	"	Nil	2.61	2.56	2.49	2.45			
4	2.5	11.0	1.17	1.24	1.46	1.32			
5	"	9.2	1.20	1.31	1.59	1.49	1.31		
6	"	12.2	1.68	1.68	2.20	2.25	2.21		
7	"	13.0	1.74	1.98	2.40	2.39	2.43	2.06	
8	"	7.6	1.74	1.69	1.95	1.73	1.68		
9	"	12.6	2.65	2.77	3.07	3.05	3.35	3.62	
10	5.0	P.T.	1.23	1.02	1.85	2.18	1.86		
11	"	12.2	1.49	1.69	2.56	2.62	2.54		
12	"	12.6	1.56	1.58	2.58	1.89			
13	"	P.T.	1.57	1.93	2.30	2.80	3.12	3.36	
14	"	10.2	1.88	1.84	2.93	4.02*	3.87		
15	"	7.6	3.00	3.17	3.76	3.75	3.70		
16	6.5 or 7.5	17.8	1.21	1.52	1.93	2.28	2.28		
17	"	29.2	1.31	1.37	2.77	2.71			
18	"	P.T.	1.33	1.58	2.58	2.52			
19	"	25.0	1.77	1.66	2.98	3.40	3.41		
20	"	19.6	1.77	1.99	2.31	2.59	2.99		
21	"	P.I. (12)	1.89	2.04	2.46	2.26			
22	"	25.8	1.96	1.77	2.79	2.44			
23	"	10.6	2.02	2.26	2.68	3.84	3.42		
24	"	9.2	2.07	2.15	2.63	3.01	2.82		
25	"	P.I. (34)	2.24	2.58	3.47	3.82	4.17		
26	"	6.8	2.24	2.35	3.35	3.04			
27	"	3.8	2.33	2.44	2.71	3.22	3.00		
28	"	P.I. (17)	2.71	2.93	3.69	3.92	3.51		
29	"	P.I. (27)	3.00	3.19	3.60	3.32			
30	10.0	P.T.	1.10	1.57	2.60	2.86	2.80		
31	"	P.T.	1.34	1.46	2.08	2.06			
32	"	30.6	1.40	1.62	2.68	3.16	3.00		
33	"	38.6	1.43	1.80	2.78	2.98	3.05		
34	"	P.I.	1.45	1.83	2.59	2.24			
35	"	P.I. (31)	2.10	2.26	2.76	2.82			
36	"	P.I. (21)	2.26	2.31	2.96	3.08	3.63	3.26	
37	"	P.I. (22)	2.44	2.62	3.60	3.69	3.63		
38	"	P.I.	2.54	2.32	3.80	3.75			
39	"	P.I. (11)	2.54	2.73	3.09	3.12	3.01		
40	"	P.I. (32)	3.00	3.18	3.75	4.12	4.01		
41	"	P.I. (26)	3.04	3.23	4.16	4.13	3.96		
42	"	P.I. (44)	3.05	3.24	4.10	4.37			
43	"	P.I.	3.05	3.23	4.01	4.22	4.56	4.04	
44	20.0	41.8	0.97	1.26	2.26	2.66	2.96	3.17	3.15
45	"	27.6	1.26	1.50	2.28	2.39	2.61		
46	"	> 15	1.46	1.55	2.49	3.26	3.50	3.61	3.45
47	"	34.0	1.62	1.64	2.55	2.65	2.59		
48	"	28.0	1.76	1.91	2.86	3.27	3.53	3.39	
49	"	18.4	2.28	2.45	3.49	3.55	3.87	4.19	4.09
50	"	P.I. (47)	2.59	2.73	3.19	3.59	3.51		
51	"	P.I. (39)	3.01	3.38	3.71	3.76	3.81		
52	40.0	P.T.	0.98	1.45	2.44	2.78		3.53	3.45
53	"	22.8	1.35	1.44	2.83	2.96	2.66		
54	"	P.T.	1.45	1.40	2.53	3.17	3.21	3.33	3.72
								(4.54 at days)	4.9
55	"	38.0	1.77	2.14	3.09	3.28	3.83	3.64	
56	"	P.T.	2.02	2.31	3.18	3.57	3.73	3.90	3.74
57	"	7.2	2.06	2.45	3.09	3.46			4.11
58	"	5.2	2.19	2.38	2.90	3.44	2.86		
59	"	P.I.	2.28	2.53	3.08	3.67	4.32		
60	"	P.I. (53)	2.66	2.90	4.41	4.30			
61	"	3.4	3.03	3.18	4.61	4.45			
62	80.0	P.I. (1)	1.22	1.90	3.03	3.46	3.51	3.57	
63	"	P.I. (5)	1.31	1.99	2.76	3.00	3.17	3.53	3.23
64	"	P.T.	1.32	1.79	3.23	3.66	4.22	4.30	4.22
65	"	26.2	1.59	1.93	2.79	3.66	3.98	4.06	
66	"	P.I. (8)	1.68	2.11	3.04	3.19	4.25	4.10	
67	"	26.4	1.70	1.81	2.82	3.27	3.41	4.27	4.16
68	"	P.I. (7)	2.06	3.41	3.52	3.87	3.33		
69	"	P.I. (6)	2.13	2.31	3.12	4.14	4.11	4.20	
70	"	P.I. (3)	2.45	2.61	3.76	4.16	3.91	4.06	4.52
71	"	16.2	2.66	3.03	3.34	4.17			
72	160.0	19.4	1.27	1.50	2.32	3.42	3.45	3.29	
73	"	21.0	1.71	2.02	3.17	3.72	3.79	3.70	

The highest count is in black figures. In a few instances counts recorded at 20 to 30 days were made a day or so before or after the time indicated. P.T. = previous transfusion. P.I. = previous injection; a number in brackets indicates the previous response; where no number appears the previous injection was of material not referred to in this paper (see under "Increase of Red Blood Cells" below).

* This freak response is excluded from calculations for Figs. 4 and 5 and Tables II and IV.

reticulocyte crisis, but mainly from the rate of increase of red blood cells. A summary of 73 responses in 53 patients is given in Table I. An example is shown in Fig. 1.

*In the early stages of this investigation some additional tests were carried out in Stobhill Hospital, Glasgow, by Dr. J. R. Lauckner, whose co-operation is gratefully acknowledged.

FIG. 1.—Response to repeated injections of Vitamin B₁₂ in a patient with pernicious anaemia.

Reticulocytes

Reticulocytes usually began to rise on the second or third day, reaching a peak on the fifth or sixth day (counting the day of treatment as 0). There was a definite association between high dosage and early reticulocyte peak. With large doses—20 to 160 µg.—the peak usually occurred on the fifth day; with doses below 10 µg. the peak was usually on the sixth or occasionally the seventh day.

Reticulocyte responses of 39 patients, none of whom had had previous transfusion or injection, were compared with an "expected" response based on results to liver extract given intramuscularly (Isaacs and Friedman, 1938). Fig. 2 shows the actual responses at each initial red blood cell count. In Fig. 3 results are expressed as percentages of the expected response. Individuals varied widely in their response to a given dose. In more than 50% (10 out of 17) of the patients who received doses of 10 µg. and upwards the expected response was exceeded, whereas with smaller doses (1.25–7 µg.) this response was seldom attained (four times in 22 patients). A high reticulocytosis was not always the precursor of a satisfactory rise of red blood cells, confirming previous criticisms of the reticulocyte response as

a measure of potency (Murphy, 1933; Ungley, 1936; Della Vida and Dyke, 1942; Fullerton, 1945).

Increase of Red Blood Cells

A better yardstick was the rate of increase of red blood cells. This depended not only on the dose of vitamin B₁₂ but also on the initial count, and these factors varied independently of one another. Thus a formula became necessary to judge whether rates of increase were satisfactory or not.

For its construction we needed more data than were provided by the first responses in the 40 patients who had had no previous treatment. We had to decide whether to include certain additional data—namely, nine responses in patients who had been transfused with a small number of red blood cells, the survival of which could be followed by differential agglutination (Dr. Walker), and 24 responses in patients who had previously received small amounts of vitamin B₁₂ (20 of them in the course of this trial). Such responses were not included unless there had been a satisfactory control period of seven to ten days of stationary or falling counts, as described under "Material and Methods" above. Where doubt existed a marrow puncture was performed and a further injection was given only if the marrow was megaloblastic.

In the nine transfused cases the average increase in the patient's own red blood cells in 15 days was 1,470,000, compared with an "expected" average increase of 1,400,000 calculated by the formula given below.

In the 24 patients who had relapsed after small amounts of vitamin B₁₂ the average increase of red blood cells in 15 days was 1,220,000, whereas the expected average was 1,250,000.

In the 33 cases in these two groups the actual average increase was 1,290,000 compared with an "expected" increase of 1,290,000. Although, as described below, the "expected" increase has been calculated from a group of 63 patients which includes these 33, it is apparent from the above averages that the 30 patients who did not receive any previous treatment could not have differed significantly from the 33 patients who had had previous treatment. It was therefore considered justifiable to include the latter in the trial and to deal with them as normal cases. They are identified in Table I by P.I. (Previous Injection) and P.T. (Previous Transfusion). This neither assumes nor proves that such past treatment never has a serious effect on the rate of increase of red blood cells, but implies that such effects, if present, could be ignored in this series.

In all there were 73 satisfactory tests in 53 patients available for analysis.

Increase of Red Blood Cells in 15 Days

Increase of red blood cells in 15 days was the most useful criterion of response. Della Vida and Dyke (1942) have shown that the average weekly increase over the first two weeks of treatment depends on the initial level and varies inversely with it. Thus patients with fewer red blood cells at the beginning of treatment respond more rapidly than those who have higher counts, and conversely.

For each group of patients receiving similar doses a line was calculated by methods comparable to those of Della Vida and Dyke, and in every case, except for those receiving 2.5 µg., there was a marked relationship between the 15th-day increase and the initial count. But individually

FIG. 2.—Reticulocyte responses (peak) in 39 patients to their first injection of vitamin B₁₂ compared with expected response to liver extract calculated from formula of Isaacs and Friedman (1938).

FIG. 3.—Reticulocyte response expressed as percentage of expected response. Crosses indicate the mean response at each level of dosage. Doses are spaced logarithmically.

none of these relationships could be considered statistically significant, because of the few cases involved, the largest group being 14. It was therefore necessary to calculate a formula making allowances for the differences in dosage and differences in initial blood counts at the same time.

The problem was simplified when it became apparent that over a certain range of dosage (5–160 μ g.) the response varied according to the logarithm of the dose. (In practice this means that doubling the dose produces a constant increase in response.)

Out of 64 cases* receiving 5–160 μ g. 63 were therefore used to calculate the formula by methods of multiple correlation, giving the following results:

$$I_{15} = 1.008 - 0.153 E + 0.245 D - 0.017 E \times D.$$

Where I_{15} = Increase in red blood cells (millions per c.mm.) on the 15th day.

E = Initial red blood cell count (millions per c.mm.) on the day of injection.

D = Dose on a logarithmic scale —e.g. (5 μ g. = 1; 10 μ g. = 2; 20 μ g. = 3; 40 μ g. = 4).

The increases that may be expected according to this formula are given in Table II,

and the lines have been superimposed upon Fig. 4.

This formula is applicable only for doses between 5 and 80 μ g.—or possibly 160 μ g.—and for patients whose initial

TABLE II.—Expected Increase in 15 Days
 $I_{15} = 1.008 - 0.153 E + 0.245 D - 0.017 E \times D$
Standard Deviation of I_{15} = 0.33

Initial Red Blood Cell Count (Millions)	5 μ g.	10 μ g.	20 μ g.	40 μ g.	80 μ g.
1.00	1.08	1.31	1.54	1.77	2.00
1.10	1.07	1.29	1.52	1.75	1.97
1.20	1.05	1.27	1.50	1.73	1.95
1.30	1.03	1.25	1.48	1.70	1.92
1.40	1.01	1.23	1.46	1.68	1.90
1.50	1.00	1.21	1.44	1.66	1.87
1.60	0.98	1.19	1.42	1.64	1.85
1.70	0.96	1.18	1.40	1.61	1.83
1.80	0.94	1.16	1.38	1.59	1.80
1.90	0.93	1.14	1.36	1.57	1.78
2.00	0.91	1.12	1.34	1.55	1.76
2.10	0.89	1.10	1.32	1.53	1.73
2.20	0.88	1.08	1.30	1.51	1.71
2.30	0.86	1.06	1.28	1.48	1.68
2.40	0.84	1.04	1.26	1.46	1.66
2.50	0.82	1.02	1.24	1.44	1.64
2.60	0.81	1.01	1.22	1.42	1.61
2.70	0.79	0.99	1.19	1.39	1.59
2.80	0.77	0.97	1.17	1.37	1.57
2.90	0.75	0.95	1.15	1.35	1.54
3.00	0.74	0.94	1.13	1.32	1.52
3.10	0.72	0.92	1.11	1.31	1.50

E = Initial red blood cell count in millions. I_{15} = Increase in red blood cells on the 15th day. D = Dose on a logarithmic scale (5 = 1; 10 = 2; 20 = 3; 40 = 4; 80 = 5).

The error made in using these tables will be less than the Standard Deviation in about 68% of all individual cases.

*Case 14 was excluded, as the response was most exceptional.

FIG. 4.—Actual increase of red blood cells in 15 days in 73 instances after vitamin B₁₂. The lines are calculated from a formula which takes account of dosage and initial red blood cell count. The dotted line corresponds to the formula of Della Vida and Dyke (1942) for "average" responses to liver extract.

red blood cell count lies between one and three millions per c.mm. Patients vary considerably in their response to treatment, but only 32% of responses will differ from the figures given in Table II by more than $\pm 330,000$. For groups of patients, however, the average increase of a group will be more accurate (e.g., for a group of 10 cases the average increase will usually be forecast by Table II with an accuracy of $\pm 100,000$).

The following is a brief explanation of the meaning of the formula, simplified by omitting the slight correction for the factor $E \times D$. If a patient has an initial red blood cell count of 2,000,000 and is given an injection of 10 $\mu\text{g.}$, the increase in

15 days will be about 1,120,000, making his actual count on the 15th day 3,120,000. For every doubling of the dose in the range 5 to 80 $\mu\text{g.}$ there will be an additional increase of about 220,000, so that the increase in 15 days if the patient had received 20 $\mu\text{g.}$ would be 1,340,000, and if 40 $\mu\text{g.}$, 1,560,000. Similarly, for every million cells above 2,000,000 at the initial count the 15-day increase will be reduced by about 200,000, so that the increase for a patient with an initial count of 3,000,000 receiving 10 $\mu\text{g.}$ is about 920,000. Similarly, for every million cells below 2,000,000 there will be an additional increase of about 200,000, so for a patient with an initial count of 1,000,000 the increase would be about 1,320,000 per c.mm.

The expected response found by Della Vida and Dyke for their "average" group was attained in our series by doses in the region of 10 $\mu\text{g.}$, and only in this limited sense can amounts of this order be regarded as the "minimal effective dose" (Ungley, 1948b).

To check the validity of the formula the expected increases in 15 days were calculated for all 63 patients and compared with the actual increases obtained. The differences between these figures are given in Table III, where it will be noted that in over three-quarters of all the cases the error was less than 400,000. (The standard deviation of this table is $\pm 320,000$, which corresponds with that quoted in Table II.)

Dosage Response Curve Based on Increase of Red Blood Cells in 15 Days

The basic assumption made in calculating the formula was that the mean response to a given dose was proportional

TABLE III.—Differences Between Estimated and Observed Increase of Red Blood Cells in 15 Days

Estimate	Difference in Million R.B.C.	Cases
Too small	0.60-0.79	2
	0.40-0.59	4
	0.20-0.39	12
	0.01-0.19	13
Accurate	0	2
	0.01-0.19	12
	0.20-0.39	11
	0.40-0.59	5
Too large	0.60-0.79	2
		63

to the logarithm of the dose. While this is a justifiable assumption over a given range, it is unlikely to be true over the total range of possible doses.

It was therefore necessary to examine the assumption more closely to see if it was justifiable and at what stage it broke down. Accordingly, the difference between actual and expected increases was calculated for each case; for doses outside the range of Table II the formula was extrapolated. The mean difference for each dose group is given in Table IV and Fig. 5.

TABLE IV.—*The Mean Difference Between Observed and Expected Increase in 15 Days*

Dose in $\mu\text{g.}$	No. of Cases	Mean Initial R.B.C. (Millions per c.mm.)	Mean Difference (Millions per c.mm.)
1.25	3	2.06	-0.52
2.5	6	1.70	-0.40
5.0	5*	1.77	-0.07
7.0	14	1.99	+0.02
10.0	14	2.20	+0.05
20.0	8	1.87	-0.10
40.0	10	1.98	-0.02
80.0	10	1.81	+0.05
160.0	2	1.49	-0.02

*i.e., excluding Case 14.

FIG. 5.—Dose response curve to graded doses of vitamin B₁₂ (spaced logarithmically). Continuous lines = expected response according to formula in text. Difference between observed means and expected increase is shown by vertical lines. Dot = initial count; length of line = deviation from expected response; arrow shows whether deviation was above or below expectation. The relationship between response and log. dose did not hold for doses below 5 $\mu\text{g.}$

From Table IV it is apparent that doses below 5 $\mu\text{g.}$ failed to produce a response proportional to the logarithm of the amount used. But for doses from 5 $\mu\text{g.}$ to 160 $\mu\text{g.}$ there is no significant departure from the logarithmic curve. Consequently, with the data available it may be concluded that between the stated limits the logarithmic is the most satisfactory approximation to the true dose response curve.

Below the stated limits a dose of 2.5 $\mu\text{g.}$ produced fair reticulocytosis but a slow increase of red blood cells which was poorly sustained. The smallest amount given so far

has been 1.25 $\mu\text{g.}$, which produced slight reticulocytosis in one out of three cases with little or no increase in red blood cells in 15 days. Further tests are necessary to complete the curve and examine its behaviour for dosage levels at and above 160 $\mu\text{g.}$

An incidental finding is the considerable variation of the response, which is greater with small than with large doses (see Table VII). Variation in response might be due to a number of causes. Errors due to deterioration of vitamin B₁₂ in solution and inaccuracy in measurement of dosage are believed to be small. Variation in the rate of absorption from the site of injection is probably significant only in so far as it affects the rate of excretion of vitamin B₁₂ in the urine, itself probably an important factor. Individual requirements probably vary with such factors as age, sex, body weight, pyrexia, and metabolic rate. Another possible source of error is variable ingestion, utilization, and reserves of accessory haematopoietic factors, such as folic acid, which are present even in the restricted diet. The smaller variation in response after large doses suggests that they neutralize some of the sources of variability.

Increase at 5, 10, and 20 Days

Although the increase in 15 days was the most useful criterion for judging the potency of vitamin B₁₂, counts were also available for the 5th, 10th, and 20th days.

The increase in 5 days was slight—usually about 15% of the increase at 15 days (Tables V and VI). But precisely

TABLE V.—*Mean Red Blood Cell Counts During First 20 Days of Treatment*

Dose in $\mu\text{g.}$	No. of Cases	Mean Red Blood Cell Count at				Cases Followed 20 Days		
		0 Day	5 Days	10 Days	15 Days	No.	R.B.C. 0 Day	20 Days
1.25	3	2.06	2.05	2.12	2.02	1	2.23	2.58
2.5	6	1.70	1.78	2.11	2.04	5	1.80	2.20
5.0	6	1.79	1.87	2.66	2.88	5	1.83	3.02
7.0	14	1.99	2.13	2.85	3.03	8	2.02	3.02
10.0	14	2.20	2.39	3.21	3.33	9	2.25	3.52
20.0	8	1.87	2.05	2.85	3.14	8	1.87	3.28
40.0	10	1.98	2.22	3.21	3.51	6	1.84	3.44
80.0	10	1.81	2.29	3.14	3.66	9	1.72	3.78
160.0	2	1.49	1.76	2.75	3.57	2	1.49	3.62

TABLE VI.—*Increases as a Percentage of that Reached in 15 Days*

Dose in $\mu\text{g.}$	5 Days	10 Days	15 Days	20 Days
5	7	80	100	109
7	13	83	100	114
10	17	90	100	113
20	14	77	100	112
40	16	80	100	105
80	25	72	100	111
160	13	60	100	103
All	17	81	100	110

the same characteristic variations were discernible, the increase varying with the logarithm of the dose and inversely with the initial count. The greatest rise was between the 5th and 10th days, by which time about 80% of the increase obtained in 15 days had occurred. Again, the same variables operated and had to be sorted out by a formula similar to that given above for the 15th-day counts. As a measure of response the 10th-day counts would have been nearly as useful as the 15th-day counts, but the chance of error was less with the greater increase in 15 days.

The 20th-day counts unfortunately were not complete in all cases, as some 21 patients who had not responded

well to treatment needed further injection before reaching the 20th day and had to be withdrawn from the trial. The remainder are therefore not a representative group, but are biased by the survival of the fittest. In this group the average increase by the 20th day was about 110% of the increase in 15 days.

FIG. 6.—Mean red blood cell counts at fixed times after graded doses of vitamin B₁₂ (spaced logarithmically).

FIG. 7.—Mean response to graded doses of vitamin B₁₂ (from same data as Fig. 6).

Fig. 6 shows the relationship between erythrocyte increase and dosage at various fixed intervals of time. In Fig. 7 the same data are arranged to show the behaviour of the erythrocyte counts over a period of 15 days for various fixed levels of dose. These figures show fairly clearly the relationship between time and dose and emphasize that higher doses produce responses which are not only greater but last longer. Fig. 8 shows the usual form of curve of increase of erythrocytes in the first three weeks after doses of 5–160 μ g. (see Table VI).

FIG. 8.—Proportion of increase of red blood cells which occurred in 5, 10, 15, and 20 days after a single dose of vitamin B₁₂. This form of curve holds true for most cases receiving doses of 5 to 160 μ g. (see Table VI).

Total Dosage to Complete Remission

The amount of vitamin B₁₂ required to raise the red blood cell count above 4,500,000 in 32 cases varied from 15 to 140 μ g. and the time from 15 to 118 days. Individual findings are not recorded, since variations depended partly on the size and spacing of the doses. If red blood cell counts were allowed to fall before subsequent doses were given, then more time and material were required for complete remission. Moreover, remission was attained less economically with a single large dose than with the same amount of material given in divided doses at fortnightly intervals. Daily requirements for vitamin B₁₂ could not be determined accurately from such data.

Other Findings

Changes in the *bone-marrow* from megaloblastic to normoblastic erythropoiesis after liver extract are described by Davidson, Davis, and Innes (1942) and after Lester Smith's non-crystalline red-pigmented material by Lorri-man (1948). Similar changes following crystalline vitamin B₁₂ were observed in the present series by Drs. G. Lorri-man and R. B. Thompson (personal communication).

Initial *white blood cell counts* before treatment in 32 patients ranged from 1,500 to 8,300 (mean 4,300 $\pm$ S.E.260 per c.mm.). When remission was attained (red blood cells 4,500,000 per c.mm. or over) white blood cell counts in these patients ranged from 4,000 to 8,800 (mean 6,200 $\pm$

170 per c.mm.). *Platelet counts*, too, usually rose to normal levels; Fig. 1 shows a typical response. Counts before treatment in 42 patients ranged from 20,000 to 283,000 (mean $105,500 \pm 7,000$). Counts 1 to 12 weeks later (mean 3.5 weeks) ranged from 120,000 to 560,000 (mean $305,500 \pm 16,000$). The difference between means both for white blood cells and for platelets is highly significant.

Within the first few days there was a dramatic increase in appetite and well-being, followed later by a gain in weight. Disturbances such as nausea, vomiting, and diarrhoea were relieved.

Soreness of the tongue usually disappeared within a few days, recurring only if the effects of an injection were allowed to wear off. Three exceptions are mentioned under "Maintenance." Clean smooth tongues became partially or completely repapillated and sometimes furred.

Neurological and certain other findings are discussed below.

Maintenance

Excluding cases with subacute combined degeneration, 21 patients were treated long enough (6 to 15 months) to give some indication of requirements for maintenance. The standard dose was 10 μ g. every two weeks, although in two instances the interval was lengthened to three and four weeks, and in one the dose was increased because of sore tongue. To simplify further discussion the exceptions will be considered first.

Six women showed evidence of iron deficiency after 8 to 15 weeks, with low mean corpuscular haemoglobin concentration (M.C.H.C.); normality was restored by a course of ferrous sulphate.

Three of these women and one man mentioned below had isolated red blood cell counts of just below 4,000,000 per c.mm. in the maintenance period. In one of the women the low count followed an attack of influenza; the man had intermittently a sore tongue and some macrocytosis (mean M.C.V. 96.5 c. μ ; range 90.4 to 101.6 c. μ), indicating that his requirements were not completely met by 10 μ g. a fortnight.

Another patient complained of very slight soreness of the tongue at each attendance. A third had intermittently a red sore tongue, which was not relieved even when doses were stepped up to 80 μ g. Neither of these two patients had abnormal blood findings during the maintenance period.

The mean values mentioned below were derived from the sum of all findings for each patient (including the exceptional cases) after red blood cells first reached 4,500,000 per c.mm.

Mean red blood cell counts ranged from 4,480,000 to 5,120,000 (mean 4,950,000). Mean haemoglobin values ranged from 11.1 to 14.7 g. (mean 13.8 g.) per 100 ml.

Except in the six women who developed iron deficiency, haemoglobin increased at a rate adequate to maintain a normal M.C.H.C. Mean values for M.C.H.C. for all cases ranged from 28.1 to 34.8% (mean 30.9%).

By the time the red blood cell counts had reached 4,500,000 the M.C.V. had usually fallen to normal (76 to 94 c. μ). Mean values during maintenance ranged from 81.1 to 96.5 c. μ (mean 89.6 c. μ). A few patients had isolated values of 96 to 99 c. μ , but only the one man already mentioned had any suggestion of persistent macrocytosis.

Findings in eight patients with subacute combined degeneration who received much larger doses of vitamin B₁₂ (see below) are of interest. Mean red blood cell counts

ranged from 4,850,000 to 5,550,000 per c.mm.; mean haemoglobin levels ranged from 13.8 to 15.4 g. per 100 ml. M.C.H.C. was always within the normal limits; so was M.C.V., apart from one isolated reading of 96.7 c. μ .

Neurological Manifestations

Twenty-one patients in this series have been followed for six to 15 months and others for shorter periods; neurological manifestations improved or remained unchanged and fresh symptoms have not developed.

More direct evidence of the efficacy of vitamin B₁₂ against the neurological manifestations of pernicious anaemia was obtained by treating established cases of subacute combined degeneration (Ungley, 1948a, 1949). The first patient began treatment with Lester Smith's red but not yet crystalline material in December, 1947, changing to crystalline vitamin B₁₂ six months later. Now eight patients have received pure vitamin B₁₂—usually 40 μ g. a week—for a minimum of 10 months. Using a quantitative method of assessing neurological status, significant improvement was demonstrated in each case. Comparison with 44 patients treated before the war showed that vitamin B₁₂ was as effective as liver extract—crude or refined—in the treatment of subacute combined degeneration (Ungley, 1949).

Routine dosage of vitamin B₁₂ must cover wide individual variations in requirement and maintain reserves. Uncomplicated cases without neurological involvement may be given 40 to 80 μ g. initially, followed by 20 μ g. weekly for three months and 30 μ g. every three weeks thereafter. Patients with subacute combined degeneration should receive weekly at least 40 μ g. for six months and 20 μ g. thereafter. Some will need more: at the least sign of relapse—lingual, haematological, or neurological—or if infection develops, dosage should be temporarily doubled or trebled.

Our findings amplify earlier reports on the effect of vitamin B₁₂ given parenterally in pernicious anaemia (West, 1948; Ungley, 1948a, 1948b, 1949; Hall and Campbell, 1948; Spies, Stone, Kartus, and Aramburu, 1948; Berk, Denny-Brown, Finland, and Castle, 1948; Bethell, Meyers, and Neligh, 1948; Spies, Suarez, Garcia Lopez, Milanes, Stone, Lopez Toca, Aramburu, and Kartus, 1949; West and Reisner, 1949).

Subsequent papers will describe the effects of vitamin B₁₂ administered via the alimentary tract; its effect in non-Addisonian megaloblastic anaemias; its effect in certain cases of megaloblastic anaemia showing rapid elimination of transfused red blood cells; and the effect in pernicious anaemia of factors related to vitamin B₁₂, including thymidine and Lester Smith's slow-moving red component.

Summary

Seventy-three responses to single doses of vitamin B₁₂ injected intramuscularly were observed in 53 patients with pernicious anaemia in relapse.

Doses were graded logarithmically from 1.25 to 160 μ g. Conclusions are based on the means of a number of responses at each dose level. Reticulocyte responses were compared with the "expected" response to liver extract according to Isaacs and Friedman (1938), but a better yardstick was the increase of red blood cells in 15 days.

While doses of 1.25 μ g. had little or no effect, 2.5 μ g. produced a definite but small response. Responses to 5 and 7 μ g. were on average somewhat below, and to 10 μ g. just up to expectation, both in respect to reticulocytes (Isaacs and Friedman, 1938) and to increase of red blood cells in 15 days (Della Vida and Dyke, 1942, formula for "average" group).

Only in this limited sense can 10 μ g. be regarded as a minimal effective dose. Larger doses produced still greater increase of red blood cells in 15 days, the mean response being roughly proportional to the logarithm of the dose, at least in the range 5 to 80, or possibly even 160 μ g. From a formula devised to express this relationship the expected response to any dose within the range was calculated. Most of the responses actually obtained did not differ from the expected response by more than 330,000 per c.mm.

Further tests at high and low levels of dosage are needed to complete the dose response curve, which will probably be sigmoid in form, rising gradually, then steeply, from the threshold dose, and flattening out to a horizontal level when supramaximal doses are reached.

Maintenance doses of 10 μ g. of vitamin B₁₂ every two weeks were satisfactory in 18 out of 21 patients followed for six to 15 months. The exceptions were three patients whose sore tongue was not completely relieved, one having macrocytosis in addition. Six women developed iron deficiency at eight to 15 weeks, relieved by ferrous sulphate.

Fresh neurological symptoms did not develop. Direct evidence of an effect in subacute combined degeneration is published elsewhere (Ungley, 1949).

I am indebted to medical, nursing, technical, and clerical members of the staff for their co-operation, and to general practitioners who referred untreated cases of megaloblastic anaemia: further progress in this field depends on their continued help. I am also indebted to Dr. E. Lester Smith and his colleagues in the Research Division of Glaxo Laboratories Ltd., Greenford, who prepared and supplied all the crystalline vitamin B₁₂ used in these investigations.

APPENDIX

It was necessary to calculate from the data in Table I formulae which would give the number of red blood cells to be expected in a given patient after a given dose.

Regression lines were calculated between increase in red blood cells at 15 days (I_{15}) and the initial count (E) for each dose. These were as follows (Table VII):

Dose (μ g.)	No. of Cases	Regression Line I_{15} in millions	Correlation	Std. Dev. of I_{15} in millions
2.5	6	$I_{15} = 0.162 + 0.101 E$	+0.23	0.45
5.0	5	$I_{15} = 1.039 - 0.091 E$	-0.18	0.50
7.2	14	$I_{15} = 1.487 - 0.226 E$	-0.25	0.45
10.0	14	$I_{15} = 1.432 - 0.130 E$	-0.26	0.34
20.0	8	$I_{15} = 1.935 - 0.339 E$	-0.57	0.35
40.0	10	$I_{15} = 1.824 - 0.149 E$	-0.55	0.14
80.0	10	$I_{15} = 2.362 - 0.285 E$	-0.47	0.27

These lines indicate a consistent relationship between low initial red blood cells and high response at 15 days for all doses between 5 and 80 μ g. There is, moreover, a suggestion that the dosage effect is on a logarithmic scale.

A fresh regression line was calculated for doses between 5 and 80 μ g. for three variables I_{15} , E, and D, where D was the dose on a logarithmic scale—i.e., 5 μ g.=1; 10 μ g.=2; 20 μ g.=3; 40 μ g.=4; 80 μ g.=5. (On this scale the intermediate dosage of 7.2 μ g. corresponded to 1.5.) This gave formula 2:

$$I_{15} = 1.168 - 0.199 E + 0.193 D$$

Number of cases = 61

Corrected correlation between I_{15} and D = +0.60

" " " " I_{15} and E = -0.31

Standard deviation of I_{15} = 0.33 millions

This formula, used for preparatory work on dosage response, compels the dosage lines to run parallel, whereas perhaps they should converge. To test this hypothesis another formula was calculated in which a fourth factor was introduced, $C = D \times E$. If there was a tendency for the lines to converge this would be apparent in the value of C. At this stage further data were available for two cases receiving 160 μ g., and as these appeared

to fall accurately on the extrapolation of formula 2 they were included in the calculation of formula 3, which was as follows:

$$I_{15} = 1.008 - 0.153 E + 0.245 D - 0.017 D \times E$$

Number of cases = 63

Standard deviation of I_{15} = 0.33 millions.

This is the formula used in the text.

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The drawing up of a series of measures designed to strengthen health administrations in underdeveloped countries in the fight against tuberculosis is one of the main results achieved by the Expert Committee on Tuberculosis of the World Health Organization, which recently concluded a five-day session in Copenhagen. The importance of training health personnel in underdeveloped areas was stressed repeatedly by the experts. They pointed out that the World Health Organization could be of great assistance in setting up centres to train local personnel in all phases of tuberculosis control work and to serve as demonstration centres for medical and auxiliary personnel of the area. The committee further suggested that WHO might give useful information on certain types of hospitals which could be set up in a relatively rapid and economical way. Health education was recognized by the committee as an essential tool in tuberculosis control. It was urged that WHO should encourage national and international voluntary organizations to inform the public on all aspects of the tuberculosis programme carried out in a particular country with the purpose of securing active support for it. The value of tuberculin-testing and of B.C.G. vaccination as the only practical way known so far of producing specific resistance against tuberculosis was reaffirmed by the committee. Further, it was emphasized that B.C.G. immunization could be fully effective only if used as a part of a general programme in addition to the application of other available preventive measures. Mass vaccination with B.C.G. was specially recommended for countries with high tuberculosis infection and mortality rates. In connexion with the B.C.G. programme the committee heard a report on the work and plans of the research office which has been set up by WHO in Copenhagen to evaluate the results of tuberculin-testing and B.C.G. vaccination. The results to be expected from this WHO research group will be of great value to future planning of anti-tuberculosis campaigns on a world-wide basis.