VITAMIN B₁₂ CONCENTRATIONS OF SERUM AND URINE IN THE FIRST 72 HOURS AFTER INTRAMUSCULAR INJECTIONS OF THE VITAMIN

BY

D. L. MOLLIN AND G. I. M. ROSS*

From the Department of Pathology, Postgraduate Medical School of London

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The vitamin B₁₂ concentrations of serum and urine can be assayed microbiologically with *Euglena gracilis* as test organism (Ross, 1950, 1952; Mollin and Ross, 1952). The mean vitamin B₁₂ concentration of the serum of normal subjects was found to be 350 $\mu\text{g.}/\text{ml.}$ The lower limit of the range in normal subjects was 100 $\mu\text{g.}/\text{ml.}$ The concentrations in the serum of patients with untreated pernicious anaemia were below the normal range; the mean concentration was less than 40 $\mu\text{g.}/\text{ml.}$ In serum the vitamin was found to be combined with some material, probably protein, which rendered it microbiologically inactive until it was freed from combination by heating at 100° C.

In this paper are reported the changes in the vitamin B₁₂ concentrations of the serum and urine of patients with untreated pernicious anaemia during the first 72 hours after intramuscular injection of the vitamin. In addition, the amounts of vitamin B₁₂ excreted by these patients following injections are compared with the amounts excreted by normal subjects given similar injections of the vitamin.

Materials and Methods

Observations were made on 31 patients with pernicious anaemia in relapse. Twenty-seven of them were in hospital receiving a diet from which meat was excluded and in which fish, butter, cheese, and eggs were restricted. The normal subjects studied were either healthy young doctors or patients in hospital fully convalescent from minor disorders.

Solutions of crystalline vitamin B₁₂ were given by intramuscular injection. Patients usually received the vitamin B₁₂-deficient diet for five to seven days before treatment.

Method of Assay.—Details of the assay technique used have been published elsewhere (Ross, 1952). The method employs *Euglena gracilis* as test organism and is based upon that introduced by Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949).

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In order to obtain good comparison between the values in sera taken serially from the same patient, specimens collected at short intervals have been assayed in one assay batch, and such assays have been repeated at least once. In this way slight differences in values due to variations in the growth of the test organism from one assay batch to another have been minimized. It has also been found advisable to assay batches of sera or urine at the same dilutions as far as possible, for such fluids may give greater calculated values in relatively strong concentrations than in more dilute solutions. This is presumably due to the presence of supplementary growth factors, the effect of which is removed by high dilution.

Other Technical Methods.—These have been described elsewhere (Mollin and Ross, 1952).

Concentrations of Vitamin B₁₂ in Serum after Injections

Effect of Single Injections.—Six patients with pernicious anaemia in relapse were given intramuscular injections of 40, 80, or 160 $\mu\text{g.}$ of vitamin B₁₂. The total and combined vitamin B₁₂ concentrations of the serum of these patients were assayed at frequent short intervals after injection. A typical experiment is illustrated in Fig. 1 (see also Table I, Case 1a).

The changes which occurred in the serum vitamin B₁₂ concentrations of the other five patients were similar and are also shown in Table I (Cases 2a, 3a, 4, 5, and 6). Normal combined concentrations were usually present by the first hour after injections and did not increase significantly during the following 24 hours.

Effect of Larger Doses.—Serum vitamin B₁₂ concentrations in the first 24 hours after an injection were higher and remained raised longer after the larger doses (Table I). This was also noted when patients were given a second, larger intramuscular dose of vitamin B₁₂ after a first injection of 40 $\mu\text{g.}$, the interval between injections being

FIG. 1.—Vitamin B₁₂ concentrations in the serum and urine of a patient with pernicious anaemia after an intramuscular injection of 40 μ g. B₁₂. The difference between the total and combined serum concentrations represents the amount of uncombined vitamin B₁₂ present.

long enough for the serum vitamin B₁₂ concentration to have fallen to its low pre-treatment level. In Table I (Case 3a and b) and in Fig. 2 are shown the different responses of a patient to successive intramuscular injections of 40 and 160 μ g. Similar changes followed successive injections of 40 and 80 μ g. (Table I, Case 1a and b).

Effect of Repeated Doses.—Three injections of 40 μ g. of vitamin B₁₂ were given to another patient at 48-hour intervals. The changes in the total and combined vitamin B₁₂ concentrations of the serum were similar after each of these injections, with highest concentrations after one hour and with all the vitamin in combined form after 24 hours (Table I, Case 2a, b, and c, and Fig. 3).

This patient was then given injections of 40 μ g. of vitamin B₁₂ daily for 33 days (total dose 1,440 μ g.). A further 40 μ g. was given 48 hours after the last injection. The serum vitamin B₁₂ concentration immediately before this last injection was much higher than before the numerous daily

injections. This was mainly due to the presence of a large amount of uncombined vitamin. Following the last dose of 40 μ g. the increase in the total concentration of the vitamin was not greater than after the very first injection. This increase was due to a rise in the level of uncombined vitamin, the combined level not being significantly increased. This suggests that the repeated doses had saturated the available vitamin B₁₂-combining capacity of the serum (Table I, Case 2e, and Fig. 3).

Persistence of Uncombined Vitamin. B₁₂.—The total and combined vitamin B₁₂ concentrations in the serum of 30 patients with pernicious anaemia in relapse were assayed 24, 48, and 72 hours after injection. All the vitamin in the serum of 14 patients who received injections of 20 or 40 μ g. of vitamin B₁₂ was in the combined form 24 hours after injection (mean concentration 210 μ g./ml.). Sixteen patients received injections of 80, 160, or 320 μ g. of vitamin B₁₂; the vitamin was all combined within 24 hours in the serum of 10 patients (mean concentration 355 μ g./ml.); in six patients uncombined vitamin B₁₂ was also present (mean combined concentration 680 μ g./ml.). In the serum of only one

patient was uncombined vitamin found 48 hours after the injection. It appeared, therefore, that 24 to 48 hours after large injections more vitamin B₁₂ may be present in the circulation than can be bound by the serum. Where this was so the combined concentration was maximal, usually being from 300 to 1,000 μ g./ml.

The actual combined concentration of the vitamin, following daily injections great enough to saturate the binding capacity of the serum and leave uncombined vitamin in circulation, varied considerably from patient to patient, and was usually higher than after single injections. Two patients who were given frequent large saturating doses of vitamin B₁₂ had high serum total vitamin B₁₂ concentrations with both combined and uncombined material present. The mean combined B₁₂ concentrations of the sera of these two patients during saturation were 320 μ g./ml. (five observations) and 760 μ g./ml. (seven observations) respectively. But, once a

FIG. 2.—Total vitamin B₁₂ concentrations in the serum and urine of a patient with pernicious anaemia after injections of 40 µg. and 160 µg. (Tables I and II, Cases 3a and b). The excretion after 40 µg. is represented by the hatched area and after 160 µg. by the total enclosed area.

high combined concentration in the serum of a particular patient had been produced by repeated injections, further large doses did not alter this concentration significantly.

Urinary Excretion of Vitamin B₁₂ after Injections

Pattern of Excretion in First 24 Hours.—The urinary excretion of vitamin B₁₂ by seven patients with pernicious anaemia following intramuscular injections of 40, 80, 160, or 320 µg. of vitamin B₁₂, and by one patient with leukaemia after an injection of 1,000 µg., was measured at frequent intervals during the first 24 hours. The results of these assays are shown in Table II.

The greatest part of the excretion of the vitamin occurred during the first four hours after the injections, and more than 96% of the total excretion occurred in the first 12 hours. Assay of individual specimens collected in the first four-hour period showed that the greater part

of the excretion occurred in the first one to two hours. The duration and the total amount of the excretion, however, varied in proportion to the size of the dose (Table II). In some patients, given injections of 320 µg. or more of vitamin B₁₂, the urinary excretion of the vitamin was raised above the level of the excretion in normal untreated subjects for at least 48 hours after the injection (Table II, Cases 9 and 11).

Relation of Vitamin B₁₂ Concentrations in Serum and Urine during First 24 Hours after Injection.—The rate of excretion of vitamin B₁₂ in the urine was greatest while the concentration of uncombined vitamin in the serum was highest (Tables I and II and Figs. 1, 2, and 3). When the uncombined vitamin was present in all specimens of serum collected during the first 24 hours after injection urinary excretion of vitamin B₁₂ was more prolonged (Tables I and II, Case 2e).

Total Excretion of Vitamin B₁₂ after Injections.

The amount of vitamin B₁₂ excreted by 19 patients in the first 24 to 48* hours after injections was measured. Sixteen injections were given to patients in initial relapse; 11 injections were given to patients who had been previously treated but whose bone marrow had reverted to megaloblastic haemopoiesis and/or whose serum B₁₂ concentration had again fallen below the normal range. The results are given in Table III.

The average total and percentage excretion of each dose increased as the dose was increased, but there were wide variations in the amount excreted by individual patients given the same dose. The amount excreted was not related to the severity of the anaemia at the time of the injections.

Comparison of Excretion of Vitamin B₁₂ by Normals and by Patients with Pernicious Anaemia.

—Twenty normal subjects were given intra-

* In most instances, except with the largest doses, excretion was complete within 24 hours.

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TABLE I

SERUM VITAMIN B₁₂ CONCENTRATIONS OF PATIENTS WITH PERNICIOUS ANAEMIA AFTER INTRAMUSCULAR INJECTIONS OF VITAMIN B₁₂ OR LIVER EXTRACT

Case No.	R.B.C. (m./c.mm.)	Dose (i.m.) μ g. B ₁₂	Total and Combined Vitamin B ₁₂ Concentrations (μ g./ml.)													
			0 hr.		1 hr.		3 hr.		6 hr.		8 hr.		24 hr.		48 hr.	
			(i)*	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
1‡(a)	1.5	40	50	50	700	300	500	150	280	230	230	230	210	210	200	200
(b)	3.1	80	90	90	2,400	320	—	—	1,320	720	960	610	910	810	200	200
2 (a)	1.9	40	20	20	1,700	800	1,000	200†	440	290	860	640	400	400	220	220
(b)	1.8	40	220	220	1,800	800	1,350	470†	610	370	600	440	400	400	150	150
(c)	2.0	40	150	150	1,500	950	880	415	880	620	640	560	100	100	—	—
(e)	3.5	40	1,120	800	2,400	860	2,080	800	1,920	1,440	—	—	1,120	720	1,000	880
3‡(a)	1.3	40	16	16	350	45	290	—†	215	110	120	60	110	110	80	80
(b)	1.6	160	20	20	3,360	1,280	2,240	440	800	425	1,360	1,270	250	250	200	200
4	3.2	40	40	40	1,120	620	940	100	1,000	450	250	250	200	200	240	240
5	1.3	80	55	40	900	100	850	250	—	—	415	380	200	200	250	250
6	1.5	160	50	50	4,600	2,200	—	—	1,280	240	—	—	760	230	495	135
10‡(a)	1.8	40 liver extract	50	50	2,200	600	1,240	320	—	—	—	—	240	240	115	115
(b)	2.7	40 liver extract	70	70	1,480	160	1,320	320	520	140	460	295	245	155	—	—

* (i) Total vitamin B₁₂ concentrations. (ii) Combined vitamin B₁₂ concentrations.

† Samples taken two hours after the injection.

‡ Initial injections were given to patients in relapse; subsequent injections were given to Cases 1, 3, and 10 when the serum B₁₂ concentrations had fallen to subnormal levels.

TABLE II

URINARY EXCRETION OF VITAMIN B₁₂ AFTER INTRAMUSCULAR INJECTIONS

Case No.	Intramuscular Dose (μ g.)	Excretion of Vitamin B ₁₂ (μ g.)						
		Pre-injection (0-24 hr.)	Post-injection					
			0-4 hr.	4-8 hr.	8-12 hr.	12-24 hr.	Total in 24 hr.	24-48 hr.
1 (a)	40	0.04	1.6	0.43	0.03	0.07	2.1	0.56
(b)	80	0.02	36	22	0.12	0.10	58	—
2* (a)	40	< 0.04	1.1	1.9	0.22	0.18	3.4	< 0.1
(b)	40	< 0.01	3.4	—	0.26	< 0.08	3.7	< 0.2
(c)	40	—	3.0	—	0.3	0.06	3.4	—
(d)	40	—	—	3.6	—	0.62	4.2	—
(e)	40	—	3.8	11	0.56	0.35	16	1.1
(f)	40	1.4	—	—	14	—	14	—
3 (a)	40	< 0.02	5.8	0.31	0.08	0.08	6.3	0.05
(b)	160	< 0.02	70	38	11	1.1	120	0.11
4	40	< 0.05	5.4	0.02	0.02	0.02	5.5	< 0.05
7	40	< 0.03	3.1	0.06	0.01	0.006	3.2	—
8	80	0.02	24	1.8	0.07	0.21	26	0.14
9	320	—	156	40	—	7.8	204	7.9
11†	1,000	0.21	458	95	1.9	8.9	564	6.6

* Case 2 received the first three injections at 48-hour intervals and a fourth injection 24 hours later. Thirty-two daily injections of 40 μ g. were then given, and 48 hours after the last of this series a further 40 μ g. was injected (Case 2e); the final injection of 40 μ g. was given 72 hours later (Case 2f).

† Patient was suffering from leukaemia. All other subjects had pernicious anaemia.

muscular injections of either 40, 80, 160, or 1,000 μ g. of vitamin B₁₂. The amounts excreted are shown in Table IV. There was no significant difference between the proportion of each dose excreted by normal subjects and by patients with pernicious anaemia in relapse.

Excretion of the Vitamin after Repeated Injections.—The amount of vitamin B₁₂ excreted by two patients with pernicious anaemia given injections of 40 μ g. was studied before and after saturating doses of vitamin B₁₂ in order to determine whether a larger proportion of the dose would be

FIG. 3.—Total vitamin B₁₂ concentrations of the serum and urinary B₁₂ excretion of a patient with pernicious anaemia after repeated intramuscular injections of B₁₂ (Tables I and II, Cases 2a, b, c, and e).

TABLE III

TOTAL AND PERCENTAGE EXCRETION OF INTRAMUSCULAR DOSES OF VITAMIN B₁₂ BY PATIENTS WITH PERNICIOUS ANAEMIA

No. of Observations	Initial R.B.C. Range	Intra-muscular Dose (μg.)	Total Amount of B ₁₂ Excreted after Injection (μg.)		Percentage Excretion of Injected Dose	
			Range	Mean	Range	Mean
6	1.3-3.2	40	0.3-6.3	3.5	0.75-16	9
9	1.1-3.7	80	4.2-58	22	5-73	27.5
8	1.2-3.7	160	22-121	66	14-75	41.5
3	2.6-4.2	320	49-343	196	15-107	62
1	3.2	1,000	588	588	58.8	58.8

excreted following saturation. The details of the treatment given and amounts of the vitamin excreted by the first patients are shown in Table II (Case 2) and in Fig. 3. The average excretion in the first 24 hours after the first four injections was 3.7 μg. (Case 2a, b, c, and d), and was 16 μg. and 14 μg. after the two injections following the period of saturating doses (total given 1,440 μg.) (Case 2e

and f). More B₁₂ was also excreted during the second 24 hours after the injections when the serum vitamin B₁₂ concentrations were high and uncombined vitamin was present in all the samples. In a similar experiment carried out on the second patient (Table II, Case 3), the increase in the amount of excretion was less definite.

Repeated large injections of vitamin B₁₂ were given to a patient with leukaemia whose serum B₁₂ level was normal. After the first injection of 1,000 μg., 560 μg. was excreted in the first 24 hours and 6.6 μg. in the 24- to 48-hour period. Injections of 1,000 μg. were then given daily for seven days. The average amount of vitamin B₁₂ excreted in the first 24 hours after each dose was 510 μg. in the 24- to 48-hour period after the last of these saturating doses 88 μg. was excreted. A further 1,000 μg. injection was given the next day; in the 24- to 48-hour period after this injection 553 μg. was excreted.

TABLE IV

EXCRETION OF INTRAMUSCULAR VITAMIN B₁₂ BY NORMALS WITH COMPARISON OF EXCRETION BY PATIENTS WITH PERNICIOUS ANAEMIA

No. of Subjects	Intra-muscular Dose B ₁₂ (μg.)	Amount of Vitamin B ₁₂ Excreted (μg.)		Percentage Excretion of Injected Dose	
		Normal Subjects		Normal Subjects (Mean)	Patients with Pernicious Anaemia (Mean)
		Range	Mean		
5	40	1.5-3.2	2.1	5.3	9
5	80	2-31	22	27.5	27.5
5	160	35-41	54	33.5	41.5
5	1,000	385-670	553	55.3	58.8

Serum and Urine Concentrations after Injections of Liver Extract

One patient with pernicious anaemia was given two injections of liver extract at an interval of 17 days. Each dose contained the equivalent of 40 μg. of vitamin B₁₂. The concentrations of vitamin B₁₂ in the serum in the first 24 hours after these injections are shown in Table I (Case 10a and b

After the first injection 4.2 μ g. was excreted ; after the second 2.5 μ g. was excreted.

Less detailed studies were made on several other patients after injections of liver extracts. In all cases the changes in the serum and urine vitamin B₁₂ concentrations were comparable with those found after equivalent injections of crystalline vitamin B₁₂. Girdwood (1951) using *L. leichmannii* also found in one experiment that a similar amount of vitamin B₁₂ was excreted in the urine after equivalent injections of crystalline B₁₂ and of liver extract.

Discussion

Conley, Krevans, Chow, Barrows, and Lang (1951) and Chow (1951) have used *Lactobacillus leichmannii* to follow the changes in the B₁₂ concentration of the plasma of patients with pernicious anaemia given injections of vitamin B₁₂. However, with this organism they were only able to detect concentrations of the vitamin in the plasma for a few hours after large intravenous injections. The results in this paper show that changes in the vitamin B₁₂ concentrations of serum and urine after small intramuscular injections of vitamin B₁₂ can be readily followed by assay with *Euglena gracilis*. It seems probable that with this method any parenteral dose of vitamin B₁₂ sufficient to produce a definite reticulocyte response in a patient with pernicious anaemia will cause a detectable change in the serum concentration of the vitamin at least immediately after the injection.

Although changes in serum concentrations of the vitamin can be detected after large doses of B₁₂, such as 3,000 μ g. given by mouth, the reticulocyte response is thought likely to be more sensitive for detecting the slower absorption of small amounts of vitamin B₁₂ from the gastro-intestinal tract.

Serum Vitamin B₁₂ Concentrations after Intramuscular Injections.—The concentrations of vitamin B₁₂ in serum were highest within the first hour after intramuscular injections of from 40 to 1,000 μ g. of vitamin B₁₂. At this time amounts greater than could be bound by the serum were present, so that a proportion of the circulating vitamin was uncombined. The concentrations after the larger doses were greater than after the smaller doses, and uncombined vitamin B₁₂ in the serum was usually present for a longer time after the larger doses (Table I). The serum B₁₂ concentration remained within the normal range for at least 72 hours, except in one patient (Table I, Case 3a). The duration of normal levels after injections will be reported in a later paper. The changes in the

vitamin B₁₂ concentrations of serum after injections of refined liver extract were similar to those which followed injections of crystalline vitamin B₁₂. This is not surprising in view of the fact that the vitamin is usually in the uncombined form in these extracts. The total concentrations reached no doubt vary with the rate of absorption of the dose, the rate of excretion in the urine, and the rate of removal of the vitamin from the circulation by the tissues, including the bone marrow.

Normal subjects with a plasma volume of 3 litres and an average serum B₁₂ concentration of 350 μ g./ml. have approximately a total of 1 μ g. of B₁₂ in circulation in the plasma ; all of it is in the bound form. Patients with pernicious anaemia in relapse, on the other hand, may have concentrations less than 40 μ g./ml. and therefore a total of less than 0.1 μ g. in the plasma. Within one hour after an intramuscular injection of 40 μ g. given to a patient with pernicious anaemia the total in the plasma rises to about 3 μ g. and falls within 24 hours to approximately 0.75 μ g. In view of the fact that as much as 35 μ g. out of a 40 μ g. injection is not excreted, it appears that the greater part of the dose is stored in the body outside the plasma and that the plasma concentration in patients with pernicious anaemia receiving no further treatment is maintained by drawing upon this store, and possibly also by the absorption of very small amounts of vitamin B₁₂ from the intestine.

Specificity.—It has been suggested by Hendlin (1949) that the *Euglena* assay is not specific for vitamin B₁₂ in view of the fact that the organism may respond to material with no B₁₂ activity in "animal tests." No pure substance, however, was known to replace B₁₂ for the *Euglena* until a red crystalline substance, Factor A, distinct from the vitamin B₁₂ group, was isolated from the rumen contents and faeces of calves by Ford, Kon, and Porter (1951). This can replace vitamin B₁₂ for the growth of *Euglena gracilis* ; it has also been shown by Dr. J. N. M. Chalmers to be haemopoietically active in pernicious anaemia (Coates, Ford, Harrison, Kon, and Porter, 1952). However, because of the close relationship between the changes in serum levels and the amounts of crystalline B₁₂ injected, we feel reasonably certain that the results we have reported in this paper represent assays of vitamin B₁₂. Nevertheless, occasional samples of serum and also of urine from both normal subjects and patients with pernicious anaemia have been found to contain large amounts of some substance, not considered to be vitamin B₁₂ or to be haemopoietically active, but

which has a marked growth-promoting effect for *Euglena gracilis*. This material has been found in approximately 6% of all sera tested and has been found also in some samples of serum and urine kindly supplied by Dr. C. C. Ungley. The characteristics and possible significance of this material have been discussed elsewhere (Mollin and Ross, 1952), and for reasons given in that paper the results of the assays of these few sera have been excluded from the values reported in this paper. Because of this finding it may be necessary to assay more than one pre-treatment sample of serum, preferably taken on successive days, or more than one 24-hour collection of urine, in order to establish B₁₂ deficiency. The presence of this material in occasional specimens of urine may account for the rather high values of vitamin B₁₂ sometimes found in the urine of patients with pernicious anaemia in relapse by *Euglena* assay (Mollin and Ross, 1952) and possibly also by *L. leichmannii* assay (Girdwood, 1951).

Combined and Uncombined Vitamin B₁₂.—Sera collected within half an hour of giving intramuscular injections of B₁₂ to patients with pernicious anaemia contained the vitamin in combined form in concentrations as high as in normal subjects. In pernicious anaemia, therefore, there is no deficiency in the serum of material able to bind the vitamin. The combination of B₁₂ in the serum of patients with pernicious anaemia resisted heating to the same extent as the combination of B₁₂ in the serum of normal subjects (Ross, 1952). Further tests have shown that the serum of patients with untreated pernicious anaemia bound the vitamin when this was added to it *in vitro*. The binding capacity of sera of normal subjects and of patients with pernicious anaemia was, however, limited in that combined concentrations of only 200 to 600 µg./ml. were attained—combined concentrations similar to those normally found *in vivo*. Excess vitamin remained uncombined even after incubation in the serum at 4° C., 22° C., or 37° C. for periods up to 24 hours. There was no change in the proportion of combined and uncombined vitamin in sera assayed at intervals over a period of several months.

Daily saturating injections of up to 1,000 µg. of vitamin B₁₂ did not greatly raise the extent to which the serum of either normal subjects or of patients with pernicious anaemia could combine with vitamin B₁₂. This suggests that *in vivo* the maximal level of combined vitamin is determined by the binding capacity of the serum and that the binding capacity is not increased by repeated injections.

Significance of Combined Vitamin B₁₂.—The significance of the binding of vitamin B₁₂ in serum is uncertain. Our observation that the excretion of B₁₂ after intramuscular injections is greatest when the concentration of uncombined vitamin in the serum is highest (Fig. 1) suggests that combination helps to retain the vitamin in the blood stream. The binding may be necessary for transport of the vitamin from stores or intestine to tissues which require it.

Ternberg and Eakin (1949) showed that vitamin B₁₂ was bound by some substance present in normal gastric juice in such a way that it became non-dialysable and microbiologically inactive. Ross (1950) demonstrated that vitamin B₁₂ was bound by material in serum. Further tests have shown that the vitamin in serum is non-dialysable when combined, but dialysable when uncombined. Callender and Lajtha (1951) claimed that either normal serum, or a mixture of normal gastric juice and vitamin B₁₂ added to pernicious anaemia serum, "ripened" megaloblasts in bone marrow culture, whereas vitamin B₁₂ alone added to pernicious anaemia serum did not ripen these cells. They suggested that the ineffectiveness of B₁₂ in pernicious anaemia serum was due to the absence of "intrinsic factor" in the serum of patients with pernicious anaemia. Our observations, however, indicate that vitamin B₁₂ is in fact bound by pernicious anaemia serum apparently in the same way as it is bound by normal serum. It appears, therefore, that this "ripening" effect of serum is not directly related to ability to bind B₁₂. Callender and Lajtha (1951) also claimed that heating serum at 56° C. for two hours greatly reduced its ripening effect. This degree of heating, however, is insufficient to liberate B₁₂ from the bound form in serum. Spray (1952) suggested that the intrinsic factor and the binding substance in gastric juice might also be different. He reported that heating a mixture of neutral normal gastric juice and vitamin B₁₂ at 95° C. for 20 minutes destroyed the intrinsic factor but only liberated a small amount of vitamin B₁₂ from combination.

Urinary Excretion of Vitamin B₁₂.—In general the results of our urine assays using the *Euglena* are similar to those which have been obtained by workers using *Bact. coli* or *L. leichmannii* assays (Chow, Lang, Davis, Conley, and Ellicott, 1950; Chesterman, Cuthbertson, and Pegler, 1951; Conley *et al.*, 1951; Girdwood, 1951; Lang Harte, Conley, and Chow, 1952; Sokoloff, Sanne-man, and Beard, 1952). In particular, the excretion of an increased percentage of the larger

doses, the rapid excretion in the first few hours, and the greater excretion of a given dose after saturation—first shown by Chesterman *et al.* (1951)—have been confirmed. Our observations suggest that the amount of the vitamin excreted in the urine after a given dose is increased when daily injections of B₁₂ have previously been given so that B₁₂ is present in the serum in an uncombined form throughout the period between injections. We found no significant difference in the amounts of B₁₂ excreted in urine by normal subjects and by patients with pernicious anaemia after injected doses of 40 to 1,000 µg. The excretion after injections of less than 40 µg., however, was not studied.

Summary

Vitamin B₁₂ concentrations in serum and urine have been assayed using *Euglena gracilis* as test organism before and after intramuscular injections of the vitamin. The B₁₂ concentrations in the sera of patients with pernicious anaemia in relapse rose after injections to within or above the range of concentrations found in normal subjects and reached highest levels within the first hour. They remained within the normal range for more than 72 hours, except in one patient.

The urinary excretion of the vitamin was greatest during the first few hours after injections at the time when relatively large amounts of uncombined vitamin were present in the serum. The B₁₂ combining capacity of serum appeared to be limited in both *in vivo* and *in vitro* tests.

The percentage of the injected dose excreted increased with the size of the dose, rising from approximately 7.5% of 40 µg. injections to 60% of 1,000 µg. injections. Normal subjects and

patients with pernicious anaemia excreted similar amounts of the vitamin after injections.

Injections of liver extracts of known B₁₂ content caused changes in B₁₂ concentrations in serum and urine similar to those which followed injections of the equivalent amounts of crystalline vitamin B₁₂.

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REFERENCES

- Callender, S. T., and Lajtha, L. G. (1951). *Blood*, **6**, 1234.
 Chesterman, D. C., Cuthbertson, W. F. J., and Pegler, H. F. (1951). *Biochem. J.*, **48**, li.
 Chow, B. F. (1951). *J. Nutr.*, **43**, 323.
 ———, Lang, C. A., Davis, R., Conley, C. L., and Ellicott, C. E. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 156.
 Coates, M. E., Ford, J. E., Harrison, G. F., Kon, S. K., and Porter, J. W. G. (1952). *Biochem. J.*, **51**, vi.
 Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., and Lang, C. A. (1951). *J. Lab. clin. Med.*, **38**, 84.
 Ford, J. E., Kon, S. K., and Porter, J. W. G. (1951). *Biochem. J.*, **50**, ix.
 Girdwood, R. H. (1951). *Edinb. med. J.*, **58**, 309.
 Hendlin, D. (1949). Unpublished observations quoted by Zucker, T. F., and Zucker, L. M. (1950). *Vitam. and Horm.*, **8**, 22.
 Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 118.
 Lang, C., Harte, R. A., Conley, C. L., and Chow, B. F. (1952). *J. Nutr.*, **46**, 215.
 Mollin, D. L., and Ross, G. I. M. (1952). *Journal of Clinical Pathology*, **5**, 129.
 Ross, G. I. M. (1950). *Nature, Lond.*, **166**, 270.
 ——— (1952). *Journal of Clinical Pathology*, **5**, 250.
 Sokoloff, M. F., Sanneman, E. H., and Beard, M. F. (1952). *Blood*, **7**, 243.
 Spray, G. H. (1952). *Biochem. J.*, **50**, 587.
 Ternberg, J. L., and Eakin, R. E. (1949). *J. Amer. chem. Soc.*, **71**, 3858.