The urinary excretion of assayable vitamin B₁₂ and radioactivity after parenteral ⁵⁸Co B₁₂ in man

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SYNOPSIS Evidence is presented that after injection of radioactive vitamin B₁₂ in man, there is a close correlation between the amount of radioactivity excreted and the amount of assayable vitamin B₁₂ excreted, and thus that the amount of radioactivity excreted is a true measure of the vitamin B₁₂ excreted. The possible reasons for this occurrence are discussed and it is suggested that in the body vitamin B₁₂ does not exist as such but as an analogue or active derivative.

It is generally assumed that, in man, vitamin B₁₂ (cyanocobalamin) is absorbed from the intestinal tract and functions in the body as an active substance without undergoing any chemical change. This view is based largely on the results of recovery experiments in which known amounts of vitamin B₁₂ are added to tissues; the increase in microbiological activity, as measured by assay methods considered to be specific for vitamin B₁₂, is proportional to the amount of added vitamin B₁₂ and hence it seems likely that the original microbiological activity of the tissue is also due to vitamin B₁₂.

If a patient who had no vitamin B₁₂ in his body and was not excreting any radioactive substance in his urine were given an injection of radioactive vitamin B₁₂ it would therefore be a reasonable assumption that the amount of radioactivity then found in the urine would be a true indication of the amount excreted via the kidney. For example, if this hypothetical patient were given 1,000 µg. radioactive vitamin B₁₂ intramuscularly and 20% of the radioactivity was found in the urine then that 20% would represent 200 µg. of vitamin B₁₂. The normal subject, however, has between 1,500 and 5,000 µg. of non-radioactive vitamin B₁₂ in his body and the simple process outlined above cannot be assumed to occur because the possibility must be considered that the injected radioactive B₁₂ might equilibrate or mix with the non-radioactive body stores. Under these circumstances the amount of radioactivity appearing in the urine after an injection of radioactive vitamin B₁₂ would not be a true indication of the amount excreted but would represent only a fraction of that excreted. For example, if a patient with 3,000 µg.

non-radioactive vitamin B₁₂ in his body were given 1,000 µg. radioactive vitamin B₁₂ intramuscularly and 20% of the radioactivity was excreted in the urine then that 20% would represent 20% of (1,000 + a proportion of 3,000) microgrammes vitamin B₁₂ depending on the completeness of equilibration.

Preliminary investigations showed that the normal subject excreted about 70% of the radioactivity of an injection of 1,000 µg. radioactive B₁₂: it was obvious that complete equilibration could not have taken place otherwise this would have represented about 2,800 µg. if the body stores were 3,000 µg. It was clearly important to determine whether any significant degree of equilibration did in fact occur and this could be detected by injecting radioactive vitamin B₁₂ and comparing the amount excreted in the urine as measured by the radioactivity present and by microbiological assay. If there was agreement with the two methods this would indicate that equilibration had not taken place whereas if equilibration did occur the amount of assayable vitamin B₁₂ in the urine would be greatly in excess of the amount indicated by the radioactivity present. For example, if a subject were given 1,000 µg. radioactive vitamin B₁₂ and 50% of the radioactivity were excreted and a total of 500 µg. of vitamin B₁₂ was found by assay then this would be evidence against equilibration of significant degree: if, however, 50% of the radioactivity were excreted and 750 µg. of vitamin B₁₂ were found by assay this would suggest that the 1,000 µg. of radioactive vitamin B₁₂ had equilibrated with 500 µg. of the body stores of non-radioactive vitamin B₁₂.

Such an investigation supposes that the assay is accurate and fails to take into account the possibility of a constant error which would cause misleading

results. This error can be detected by duplicating the experiment in patients who have no body stores of vitamin B₁₂. For example, if a normal subject were given 1,000 µg. radioactive vitamin B₁₂ intramuscularly and 50% of the radioactivity were excreted in the urine and 600 µg of vitamin B₁₂ was found by assay this would suggest either that there had been some equilibration or that the assay result was too high due to a constant error. If the same results were obtained in a patient with no body stores of vitamin B₁₂ then it would be clear evidence that the assay was giving a high result. Repeated injections of radioactive vitamin B₁₂ can be given to such control patients without prejudicing the results. If equilibration did occur then the injected radioactive vitamin B₁₂ would equilibrate with that remaining in the body from the previous injection. The use of control subjects would not only serve to reveal a constant error in the assay if such were present but would also reveal the degree of any equilibration which took place.

If it could be shown that equilibration of injected radioactive vitamin B₁₂ and non-radioactive body stores of vitamin B₁₂ did not occur then this finding would be of both practical and academic importance. Such a conclusion is drawn from the studies reported here.

PLAN OF THE EXPERIMENT

The object of the experiments was to compare the urinary excretion of radioactivity and of total assayable vitamin B₁₂ after parenteral injection of ⁵⁸Co vitamin B₁₂ in normal subjects and control subjects who were suffering from vitamin B₁₂ deficiency. To cover the usual range of doses used in clinical practice three different doses of ⁵⁸Co vitamin B₁₂ were used.

PATIENTS, MATERIALS, AND METHODS

The control patients were seven in-patients suffering from severe vitamin B₁₂ deficiency. Five were diagnosed as having Addisonian pernicious anaemia on the basis of a macrocytic anaemia, megaloblastic bone marrow, histamine-fast achlorhydria, low serum vitamin B₁₂ level, and a reticulocyte response and rise in peripheral blood values after treatment with parenteral ⁵⁸Co vitamin B₁₂ only; after the study reported here the absorption of oral ⁵⁸Co vitamin B₁₂ was studied in these patients with and without intrinsic factor by the method of Schilling (1953) and the results supported the diagnosis. One patient was diagnosed as having adult coeliac disease on the basis of a macrocytic anaemia, megaloblastic bone marrow, free gastric acid, excess fat in the faeces, a low serum vitamin B₁₂ level, and reticulocyte response and rise in peripheral blood values after treatment with ⁵⁸Co vitamin B₁₂ only; Schilling tests on this patient showed malabsorption of vitamin B₁₂ unaffected by the addition of intrinsic factor

One patient came under observation with a macrocytic anaemia, megaloblastic bone marrow, and a low serum vitamin B₁₂ level four years after total gastrectomy. He also responded to parenteral ⁵⁸Co vitamin B₁₂ and Schilling tests showed that he failed to absorb it unless it was given with intrinsic factor. The control patients were treated by ⁵⁸Co vitamin B₁₂ intramuscularly daily.

The normal patients were in-patients with a variety of diseases in which depletion of body vitamin B₁₂ stores is not a known occurrence. All had normal serum vitamin B₁₂ levels. These patients were given a single intramuscular injection of ⁵⁸Co vitamin B₁₂.

The nature and objects of the experiment were explained to the patients, all of whom cooperated willingly.

⁵⁸Co vitamin B₁₂ was obtained from the Radiochemical Centre, Amersham, and diluted to a concentration of 0.5 µg., specific activity 0.5 µc., in 5 ml. normal saline, dispensed in dark glass rubber-capped bottles, autoclaved and stored at +4°C when not in use. The vitamin B₁₂ content was checked by microbiological assay every week the batch was in use. Ampoules of ⁵⁸Co vitamin B₁₂ for injection were prepared in the three dose ranges by adding 2 ml. of the ⁵⁸Co vitamin B₁₂ solution to 1,140 and 540 µg. non-radioactive vitamin B₁₂ and 3 ml. of the ⁵⁸Co vitamin B₁₂ solution to 54 µg. non-radioactive vitamin B₁₂. The same tuberculin syringe was used for the preparation of these solutions and the preparation of the standard for isotope counting. Intramuscular injections were made by standard methods.

Urine was collected directly into acid-washed dark glass bottles. Pretreatment 24-hour collections were made on all patients. The total volume was noted and aliquots taken for isotope counting and microbiological assay. Samples for assay were diluted as required with vitamin B₁₂-free water and stored at -20°C. till used: assays were performed as soon as possible.

ISOTOPIC METHODS The radioactivity in 450 ml. aliquots of urine contained in plastic bottles was measured by the end-on method in a suitably modified Ecko scintillation counter type N 550 with thallium-activated sodium iodide crystal, 3.81 cm. diameter and 2.54 cm. deep, shielded by 3 in. of lead, and an Ecko automatic scaler type N 530A. A standard containing 0.2 µc. ⁵⁸Co vitamin B₁₂ and 2,000 µg. vitamin B₁₂ as a carrier in 450 ml. water was used. All samples were counted until 10,000 counts had been recorded in the 1,140 µg. and 540 µg. series, or until 5,000 counts had been recorded in the 54 µg. series.

MICROBIOLOGICAL ASSAYS were conducted by a modification of the methods of Ross (1952) and Hutner, Bach, and Ross (1956) using *Euglena gracilis* 3 strain as the test organism. Samples were assayed in at least two different assay batches.

STATISTICAL METHODS The results were analysed by standard statistical methods.

RESULTS

No radioactivity and only a negligible amount of assayable vitamin B₁₂ was found in the pretreatment

collections in normal and in control patients. The results of the microbiological assays expressed as total assayable vitamin B₁₂ excreted in microgrammes and the amount of radioactivity excreted expressed as a percentage of the dose together with the regression line and 95% confidence limits for the control patients are plotted in Figs. 1, 2, and 3. The essential statistical results are set out in Table I.

It will be observed that the mean results for excretion in the control and normal patients do not differ greatly. This aspect will be discussed in a further communication.

DISCUSSION

A clear implication of the results is that, for practical purposes, parenteral radioactive vitamin B₁₂ in the doses used, does not equilibrate with the body stores of non-radioactive vitamin B₁₂, at least in the 24 hours after injection. In the 54 µg. series it is permissible for the regression lines and standard deviation

FIG. 1. Correlation of assayable vitamin B₁₂ and radioactivity excreted after injections of 54 µg. ⁵⁸Co vitamin B₁₂ in normal and control subjects.

FIG. 2. Correlation of assayable vitamin B₁₂ and radioactivity excreted after injections of 540 µg. ⁵⁸Co vitamin B₁₂ in normal and control subjects.

FIG. 3. Correlation of assayable vitamin B_{12} and radioactivity excreted after injections of 1,140 $\mu\text{g. } ^{58}\text{Co}$ vitamin B_{12} in normal and control subjects.

TABLE I

ESSENTIAL STATISTICAL RESULTS OBTAINED IN THE INVESTIGATION

Series ($\mu\text{g.}$)	Patients	No. of Readings	Mean Values		Coefficient of Correlation (r)	Regression of Isotope (x) on Assay (y)	S.E. of Estimate (%)
			Assayable B_{12} ($\mu\text{g.}$)	Radioactivity % of Dose			
54	Controls	30	6.83	17.06	0.9740	$x = 1.8809y + 4.2131$	2.256
54	Normals	18	5.828	14.12	0.9546	$x = 1.9374y + 2.8259$	2.078
54	Controls	33	392.36	71.27	0.8220	$x = 0.12731y + 21.314$	3.70
540	Normals	14	373.00	64.72	0.9482	$x = 0.15675y + 6.2541$	1.88
1,140	Controls	30	825.97	66.63	0.8517	$x = 0.05937y + 17.595$	6.911
1,140	Normals	15	827.88	69.97	0.8270	$x = 0.056961y + 22.807$	5.079

tion curves to be extended to meet the abscissa and ordinate. The lower SD curve for the control group (Fig. 1) lies on the point abscissa O, ordinate O, while for the normal group the lower SD curve meets the ordinate at the point abscissa O ordinate -1.88, the regression line for the controls meets the ordinate at the point abscissa O ordinate 2.83 and the upper SD curve for the controls meets the ordinate at the point abscissa O ordinate 7.54. These findings are good evidence that the lack of equilibration occurs in the entire dose range up to 1,140 µg.

It is difficult to explain the phenomenon on the basis of the concept that vitamin B₁₂ is present as such in man. An obvious explanation would be that the injected radioactive vitamin B₁₂ is excreted so rapidly that there is insufficient time for equilibration to occur; detailed observations on the rate of excretion of injected ⁵⁸Co vitamin B₁₂ suggest that this explanation is unlikely to be wholly satisfactory.

The explanation may be related to the fact that the preponderant mass of vitamin B₁₂ in the body is tissue bound. The process of binding involves at least physical changes, since the assayable vitamin B₁₂ in liver is microbiologically active but is not dialysable, and it is pertinent to consider whether the process may not also involve chemical changes in the structure of the vitamin B₁₂ molecule, *i.e.*, whether vitamin B₁₂ absorbed from dietary sources is converted to an analogue in the process of binding to body tissues. If this were the case one would not expect the injected radioactive vitamin B₁₂ to equilibrate with the analogue until it also has been converted by the process of binding, by which time it would be fixed to the tissues and no longer free to be excreted. The proportion which is excreted would be that which the tissues have been unable to bind in the time available.

Recently biochemical evidence has been produced which suggests that a very considerable proportion of the cobalamins in rabbit liver are present not as vitamin B₁₂ but as coenzymes which may be the metabolically active form of vitamin B₁₂. There is also evidence that these coenzymes can be converted to vitamin B₁₂ by procedures commonly used in

microbiological assays of tissues such as exposure to heat, light, or to cyanide ions (Weissbach, Toohey, and Barker, 1959). This suggests that in microbiological assays the substance which is actually measured is vitamin B₁₂ derived from body stores of the microbiologically active form. In addition, one of these coenzymes, 5, 6-dimethylbenzimidazolylcobamide coenzyme, has been shown to be as metabolically active in patients with pernicious anaemia as vitamin B₁₂ at the same dosage level (Wasserman, Estren, Brody, and Herbert, 1960).

It is also clear from the results that when man is given such injections of radioactive vitamin B₁₂ the radioactivity found in the urine in the subsequent 24 hours is a true indication of the amount of assayable vitamin B₁₂ excreted and that this occurs regardless of its body stores. This finding is of considerable practical importance for it means that the isotope method can be used with confidence instead of the microbiological assay in determining the total urinary loss of vitamin B₁₂ after an injection of radioactive vitamin B₁₂, at least for the doses used. The isotope method, which makes use of a very small dose of radioactivity, has obvious advantages over the assay method; as a technical method it is inherently more accurate and reliable results may be obtained quickly and with little expenditure of time or labour. The practical application of this method will be dealt with in subsequent papers.

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