

# Results with radioisotopic assay of serum B<sub>12</sub> using serum binding agent

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**SYNOPSIS** Results obtained with a radioisotope method for assay of serum vitamin B<sub>12</sub> (Ekins and Sgherzi, 1965) are described. This method, which uses serum as binding agent, is simple, easy to set up, and suitable for routine diagnostic use. It gives results extremely similar to those obtained with standard microbiological assays.

In recent years, several methods have been described for the radioisotopic assay of serum vitamin B<sub>12</sub>, some using intrinsic factor as binding agent (Rothenberg, 1961, 1963; Lau, Gottlieb, Wasserman, and Herbert, 1965; Raven, Walker, and Barkhan, 1966) and others using serum (Barakat and Ekins, 1961, 1963; Grossowicz, Sulitzeanu, and Merzbach, 1962; Ekins and Sgherzi, 1965; Frenkel, Keller, and McCall, 1966). In principle, such methods have overwhelming advantages over microbiological assays, but some of them have proved troublesome to set up, to have unexpected technical snags, and to give erratic and low results (*e.g.*, Hall, 1966a; Brafield, 1966; Raven and Barkhan, 1966). We have found the method of Ekins and Sgherzi remarkably simple and easy to set up, and it gives results closely similar to those obtained by standard microbiological methods. This paper gives details of the technique used, reports the results obtained, and compares them with those given by a standard microbiological method.

## PRINCIPLE

A series of cyanocobalamin standards is prepared, and a small, constant amount of labelled cyanocobalamin added to each, followed by a constant amount of binding agent (normal human serum). The amount of binding agent used is such that in the weakest standards most of the cyanocobalamin will be bound, and in the strongest, most of the cyanocobalamin will be free. After incubation to allow B<sub>12</sub> binding, bound and free cyanocobalamin are separated by adding coated charcoal, which adsorbs the free. Free and bound radioactivity are then determined. The ratio free counts/bound counts is a function of the total cyanocobalamin present, and a standard curve may be constructed. In estimating serum B<sub>12</sub>, the several

forms of B<sub>12</sub> in serum are extracted and converted to cyanocobalamin by heating with a cyanide-containing buffer. An aliquot of the extract is treated in the same way as the standards, and its cyanocobalamin concentration determined by reference to the standard curve. Ekins refers to assays of this general type as 'saturation analysis'.

## REAGENTS

1 <sup>57</sup>CO-CYANOCOBALAMIN<sup>1</sup> (specific activity about 100 mC./mg.) Store deep-frozen.

*Working solution* Prepare a solution containing 1,000 μg./ml. in acetate-cyanide buffer. Store deep-frozen. Make freshly at monthly intervals.

## 2 STANDARD CYANOCOBALAMIN SOLUTIONS

*Stock standard* Prepare a stock standard containing 1 μg./ml. by dilution of standard cyanocobalamin solution (Glaxo) with acetate-cyanide buffer. Store at 4°. Prepare freshly every eight weeks.

*Working standard* (1,000 μg./ml.) Make up 1 ml. of stock standard to 1,000 ml. with water. Prepare freshly before each assay.

3 BINDING AGENT Take normal human serum, divide into 1 ml. aliquots and store deep-frozen. On the day of the assay, mix 1 ml. of serum with 0.3 ml. of KCN solution (40 mg. KCN in 100 ml. water). Do not use serum after more than three months' storage.

4 ACETATE-CYANIDE BUFFER<sup>2</sup> NaOH, 12 g., 45 ml. glacial acetic acid and 40 mg. KCN, made up to 1 litre and pH adjusted to 4.5.

5 SALINE BUFFER One vol. 0.85% NaCl + 6 vol. acetate-cyanide buffer.

<sup>1</sup>Radiochemical Centre., Amersham; or Philips-Duphar, Holland - through M.E.L. Equipment Co., Crawley.

<sup>2</sup>The presence of albumin prevents rapid sedimentation of the charcoal, making it easier to dispense. 'Coating' with albumin is not necessary for the function of the charcoal in the assay.

**6 COATED CHARCOAL SUSPENSION** To 3.75 ml. acetate-cyanide buffer add 1.25 ml. 20% bovine albumin (Stayne Laboratories) and 250 mg. activated charcoal (B.D.H. acid-washed decolorising) and mix well. Make freshly before each assay.

#### SPECIAL APPARATUS

Repette syringe (2 ml.) for addition of charcoal suspension (Jencons Ltd.) Two Hamilton ultramicro syringes (50  $\mu$ l.) for  $^{57}\text{Co-B}_{12}$  and binding agent (V. A. Howe & Co.). Rotary mixer (Matburn Ltd). Whirlmixer (Fisons Scientific Apparatus). Glass incubation tubes, 50  $\times$  10 mm. (Scientific Supplies Co.). Plastic stoppers for incubation tubes (Aimer Products Ltd.). Centrifuge buckets adapted for incubation tubes.

#### PROCEDURE

Standards and serum extracts should be put up in duplicate.

**PREPARATION OF STANDARDS** Prepare in 10 ml. plastic disposable tubes, 1-7. Place working standard (2 ml.) in tube 1, add saline buffer (8 ml.) and mix. Place saline buffer (3 ml.) in tubes 2-7; transfer 3 ml. from tube 1 to tube 2 and mix; continue to tube 6, *i.e.*, doubling dilutions. The resulting standards contain 200, 100, 50, 25, 12.5, and 6.25  $\mu\text{g.}$  cyanocobalamin per ml. Tube 7 is the blank.

**EXTRACTION OF SERUM** To serum (1 ml.) in a centrifuge tube add acetate-cyanide buffer (6 ml.). Cap with aluminium foil. Heat in boiling bath 20 minutes. Cool, and filter off precipitate (Whatman no. 41).

**ANALYSIS** Place  $^{57}\text{Co-B}_{12}$  working solution (20  $\mu$ l.) in a 50  $\times$  10 mm. glass tube, add standard (1 ml.) or serum extract (1 ml.) and binding agent (25  $\mu$ l.) Mix thoroughly. Stand one hour at room temperature, followed by one hour at 4°C. Add coated charcoal suspension (100  $\mu$ l.). Mix by rotation 30 min. at 4°C. Centrifuge charcoal down. Remove all supernatant from charcoal deposit with a fine-tipped Pasteur pipette and place in a separate tube. Count both supernatant (= bound cyanocobalamin) and charcoal (= free cyanocobalamin) in a well-type gamma scintillation counter. Calculate ratios of free counts/bound counts for all samples, and construct standard curve. The  $\text{B}_{12}$  concentration in the unknowns can then be read. Serum  $\text{B}_{12}$  concentration = concentration in serum extract  $\times$  7.

#### RESULTS

A representative standard curve is shown in Figure 1. The form of the standard curve varies slightly according to the serum used as binding agent. The sensitivity of the assay may be increased greatly by reducing the amount of binding agent (the slope of the linear part of the standard curve being inversely

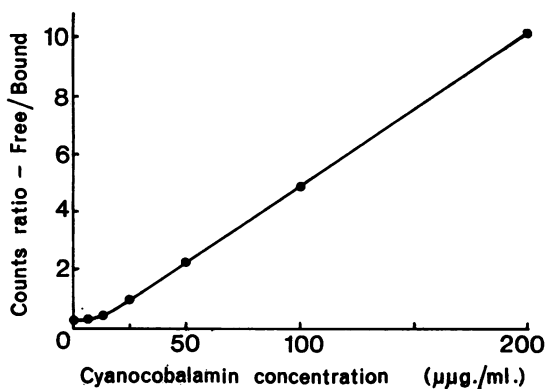


FIG. 1. A standard curve.

related to the quantity of binding agent used), though increased sensitivity is achieved at the expense of some loss of accuracy in measuring higher  $\text{B}_{12}$  levels. The amount of binding agent used in the present technique, which is half that used by Ekins and Sgherzi, is chosen as particularly suitable for diagnostic use. It is such that serum  $\text{B}_{12}$  concentrations in the critical region around 150 to 200  $\mu\text{g.}$  per ml. fall on a virtually linear part of the standard curve, and give a free/bound ratio not far removed from unity (at which the most accurate estimates of the ratio are obtained).

Serum  $\text{B}_{12}$  concentrations measured in 100 healthy people (laboratory staff and students, 58 men and 42 women, age range 19-66, mean age 32) ranged from 180 to 935  $\mu\text{g./ml.}$ , mean 482  $\mu\text{g./ml.}$ , S.D. 171, S.E. 17. (The 98% range was 184 to 919  $\mu\text{g./ml.}$ ). In 20 cases of untreated pernicious anaemia, the values ranged from 10 to 115  $\mu\text{g./ml.}$ , mean 58, S.D. 28, S.E. 6.3 (Fig. 2). In 53 sera from healthy people, hospital patients, and cases of pernicious anaemia, duplicate estimations were performed by the present method, and by microbiological assay with *L. leichmannii*. The majority of the microbiological assays were kindly carried out by Dr. G. H. Spray, of the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, using a method previously described (Spray, 1955). The results (Fig. 3) correspond well. There was no significant difference between the mean value obtained in these sera by the isotope assay (311, S.E. 36) and that by *L. leichmannii* (320, S.E. 37). All values found to be subnormal by one method were subnormal by the other.

#### DISCUSSION

The normal results obtained with this assay (mean

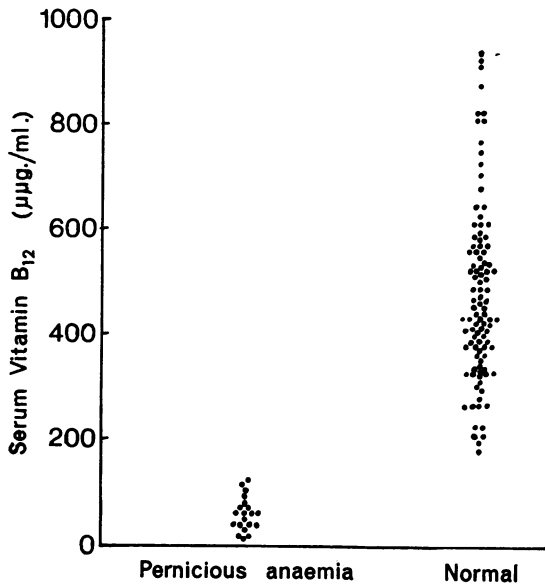


FIG. 2. Results obtained in normal subjects and cases of untreated pernicious anaemia.

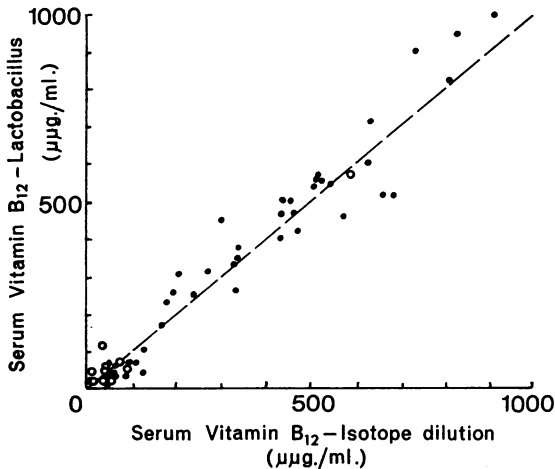


FIG. 3. Comparison of results by the isotope assay and by the *L. leichmannii* assay. The broken line represents the relationship that would be obtained if isotope and microbiological results were identical.

- comparison with Dr. Spray's laboratory.
- comparison with assays performed in this and other laboratories.

482 µµg./ml., range 180 to 935) are extremely similar to those given by standard microbiological methods. Using *L. leichmannii*, Spray and Witts (1958) reported a mean of 450 (range 150 to 1,000). Matthews (1962) reported 480 (120 to 1,150), and using *E.*

*gracilis*, z strain, Anderson (1964) reported 472 (165 to 925). In a more recent and larger group of control subjects, Spray (personal communication, 1966) found a mean of 421 µµg./ml, range 140 to 1200. Direct comparison of the results of the present assay with those of the *L. leichmannii* estimation is also satisfactory. The normal range obtained in the present work is slightly narrower than that reported for some microbiological assays; this may reflect a reduction in experimental error. The normal results also appear to compare well with those of the isotope assay of Frenkel *et al.* (1966): range 165 to 800, no mean given.

It is likely that the good correspondence with accepted microbiological methods is contributed to by the use of cyanide in the extraction procedure which converts all forms of B<sub>12</sub> believed to be present in blood (methylcobalamin, coenzyme B<sub>12</sub>, and hydroxocobalamin, Lindstrand and Ståhlberg, 1963; Lindstrand, 1964; Lindstrand, Wilson, and Matthews, 1966) to cyanocobalamin. It has repeatedly been shown that when cyanide is omitted in extraction of B<sub>12</sub> from serum, recovery of the vitamin is both inconsistent and low (*e.g.*, Spray, 1955; Girdwood, 1960), probably owing to adsorption of some of the B<sub>12</sub> to the precipitate of serum proteins (Matthews, 1962); and in 'saturation analysis' it is essential that not only should all the B<sub>12</sub> be extracted from the serum, as in any satisfactory method, but that it should all be converted to that form (cyanocobalamin) which is used as standard. This point has recently been stressed by Hall (1966b).

The theoretical basis of the assay is critically discussed by Ekins and Sgherzi (1965) who stress that it appears to contain certain minor sources of inaccuracy which have not yet been eliminated. They point out that assays of successive dilutions of serum extract do not give precisely the expected result, and that assays performed at high dilutions tend to give a slight overestimate of serum B<sub>12</sub> concentration. In spite of these reservations, it is clear that when a standard technique is used, the assay gives very satisfactory results, and its simplicity makes it particularly suitable for routine use. The advantages of this type of assay are very great. They include elimination of the uncertainties inseparable from microbiological techniques, specificity, very high sensitivity if required, speed (the whole assay can be carried out in less than a day), cheapness (the cost of materials is less than that of a microbiological assay using commercial media) and, since the number of tubes used is small and washing of glassware is largely eliminated, considerable saving of labour. In addition, such assays are unlikely to be affected by antibiotics or by other drugs or their metabolites. We are in full agreement with Ekins

and Sgherzi that the present method is sufficiently accurate for diagnostic purposes, and is superior to microbiological assays.

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## REFERENCES

- Anderson, B. B. (1964). *J. clin. Path.*, **17**, 14.  
 Barakat, R. M., and Ekins, R. P. (1961). *Lancet*, **2**, 25.  
 —, — (1963). *Blood*, **21**, 70.  
 Brafield, A. (1966). *Lancet*, **2**, 966.  
 Ekins, R. P., and Sgherzi, A. M. (1965). In *Radiochemical Methods of Analysis*, vol. 2, p. 239. International Atomic Energy Agency, Vienna.  
 Frenkel, E. P., Keller, S., and McCall, M. S. (1966). *J. Lab. clin. Med.*, **68**, 510.  
 Girdwood, R. H. (1960). *Scot. med. J.*, **5**, 10.  
 Grossowicz, N., Sulitzeanu, D., and Merzbach, D. (1962). *Proc. Soc. exp. Biol., (N.Y.)*, **109**, 604.  
 Hall, C. A. (1966a). *Lancet*, **2**, 186.  
 — (1966b). *Ibid.*, **2**, 862.  
 Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965). *Blood*, **26**, 202.  
 Lindstrand, K. (1964). *Nature (Lond.)*, **204**, 188.  
 —, and Ståhlberg, K. G. (1963). *Acta med. scand.*, **174**, 665.  
 —, Wilson, J., and Matthews, D. M. (1966). *Brit. med. J.*, **2**, 988.  
 Matthews, D. M. (1962). *Clin. Sci.*, **22**, 101.  
 Raven, J. L., and Barkhan, P. (1966). *Lancet*, **2**, 1078.  
 —, Walker, P. L., and Barkhan, P. (1966). *J. clin. Path.*, **19**, 610.  
 Rothenberg, S. P. (1961). *Proc. Soc. exp. Biol., (N.Y.)*, **108**, 45.  
 —, (1963). *J. clin. Invest.*, **42**, 1391.  
 Spray, G. H. (1955). *Clin. Sci.*, **14**, 661.  
 —, and Witts, L. J. (1958). *Brit. med. J.*, **1**, 295.