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Improved method for measuring vitamin B_{12} in serum using intrinsic factor, $^{57}CoB_{12}$, and coated charcoal

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SYNOPSIS An improved and simplified method is described for the measurement of vitamin B_{12} in serum using intrinsic factor, $^{57}CoB_{12}$, and coated charcoal. The extraction of serum in the presence of cyanide and the incorporation of B_{12}-deficient serum into the intrinsic factor control has increased the accuracy of the method for both sera and crystalline B_{12} solutions. There are interesting differences between the results obtained for some sera by the isotope and *L. leichmannii* methods and the reasons for these differences are discussed.

A method for the measurement of vitamin B_{12} (B_{12}) in serum by radioisotope dilution using intrinsic factor, $^{57}CoB_{12}$, and coated charcoal was described be Lau, Gottlieb, Wasserman, and Herbert (1965). With some modifications this method appeared to be satisfactory for the routine assay of B_{12} in serum (Raven, Walker, and Barkhan, 1966) but further experience with the method in the assay of many hundreds of sera has brought to light several discrepancies. It was found (1) that for occasional sera, irrespective of their B_{12} values, the isotope method gave falsely low values and in the case of some B_{12}-deficient sera even negative values; (2) sera with B_{12} levels greater than 500 to 600 pg/ml gave lower values in the isotope method than in the microbiological (*L. leichmannii*) method; (3) falsely low values were obtained when crystalline B_{12} solutions were assayed.

We studied the reasons for these anomalous results and found that there were two factors affecting the accuracy of the method. The first was that the use of cyanide in the extraction process resulted in higher values for those sera with B_{12} levels over 500 pg/ml (Raven, Walker, and Barkhan, 1967; Raven, Robson, Walker, and Barkhan, 1968) and the second that serum increased the binding of B_{12} by intrinsic factor (Rothenberg, 1961; Raven *et al*, 1968). By the incorporation of B_{12}-deficient serum into the intrinsic factor control it was possible to eliminate the problem of falsely low values found previously in the assay of crystalline solutions and of some sera (Raven *et al*, 1968).

As a result of these observations the radioisotope method has been improved and simplified and in this paper we describe in detail the modified method that has evolved. In addition we present the results of B_{12} assays carried out with this method in comparison with those obtained by microbiological assay (*L. leichmannii*) for a large number of normal, B_{12}-deficient, folate-deficient, and postgastrectomy sera.

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MICROBIOLOGICAL ASSAY

The organism was *Lactobacillus leichmannii* (NCB 8117) obtained from the Torry Research Station, Aberdeen, Scotland, and the method was that of Rosenthal and Sarett (1952), modified by Spray (1955), and by the use of Difco brand of B_{12} inoculum broth USP.

RADIOISOTOPE ASSAY

^{57}Co-vitamin B_{12} ($^{57}CoB_{12}$) was obtained from the Radiochemical Centre, Amersham, Bucks. Preparations with different specific activities ranging from 10 to 180 $\mu c/\mu g$ were tested and all were found to be satisfactory. Those of low specific activity (10 to 15 $\mu c/\mu g$) were used in a concentration of 1,000 pg/ml, while those of high specific activity (70 to 180 $\mu c/\mu g$) were used in a concentration of 250 pg/ml. The increased cost of the high specific activity preparations is outweighed by their advantages in providing higher count rates in the samples and an increase in sensitivity of the method at low serum B_{12} levels; the high specific activity $^{57}CoB_{12}$ is recommended for routine assays. Each batch of $^{57}CoB_{12}$ is diluted to the required concentration according to the Amersham values and the B_{12} concentration of the diluted solution checked by a microbiological method. When the isotope method is working satisfactorily it may be used for checking new batches of $^{57}CoB_{12}$ solutions and there is no need to rely on a microbiological method, *eg*, if the same volume of intrinsic factor solution binds 80% of a 250 pg/ml $^{57}CoB_{12}$ solution and 70% of a new $^{57}CoB_{12}$ solution, then the B_{12} concentration of the new solution is

$\frac{80}{70} \times 250$, *ie*, 286 pg/ml. Standardization by reverse isotope dilution (Lau *et al*, 1965) has not proved satisfactory and is no longer used by us. It tended to overestimate the concentration of $^{57}CoB_{12}$ solutions.

The diluted $^{57}CoB_{12}$ is kept in 20 ml aliquots at −20°C.

COATED CHARCOAL Ten grams of acid-washed activated charcoal powder (British Drug Houses), and 6·7 ml of 30% bovine albumin (Armour) are made up to 400 ml with deionized water. This may be kept for several days at 4°C but is usually prepared freshly before each assay. The BDH charcoal is not suitable for coating with haemoglobin (Lau *et al*, 1965; Herbert, Gottlieb, and Lau, 1966) or with dextran.

INTRINSIC FACTOR A hog intrinsic factor (IF) concentrate (48743/131) made by Lederle Laboratories has been used for all the assays. A stock solution of 100 mg/100 ml is prepared and after filtration through Whatman no. 1 filter paper is stored in 1·5 ml aliquots in plastic tubes at −20°C. Under these conditions of storage, the IF solution is satisfactory for assay purposes for up to six months. The working IF solution is prepared according to the concentration of the $^{57}CoB_{12}$ being used and the correct concentration is that which will bind approximately but not more than 80% of the $^{57}CoB_{12}$ present; for $^{57}CoB_{12}$ solutions containing 250 pg/ml the correct dilution of the stock IF solution prepared from our Lederle IF is approximately 1 in 900 ml. When the B_{12} binding capacity of the stock IF solution falls slightly during storage, the concentration of the working IF solution may be increased appropriately.

The Lederle preparation is no longer being marketed but Armour preparations are available commercially and batch numbers KF1751, LH0254, and LM2251 were found to be quite satisfactory for this method. They are, however, less potent than the Lederle IF and the stock IF solution needs to be more concentrated (100 mg/25 ml).

CYANIDE SOLUTION Of 0·1% (w/v) NaCN solution, 100 ml is diluted up to 500 ml with deionized water.

SERA Sera were obtained from normal subjects and hospital patients and were stored at −20°C. Most sera were assayed within two weeks of collection. For testing the reproducibility of the method some sera were stored for periods of up to 18 months.

CYANOCOBALAMIN STANDARD A solution of 1,000 pg/ml was prepared from Cytamen (Glaxo) brand of cyanocobalamin B.P. The concentration was checked a number of times and in different dilutions by microbiological assay.

PREPARATION OF TEST SERUM Into a 20-ml screwcapped glass Universal container place 1 ml serum, 2 ml N/4 HCl, and 7 ml cyanide solution. Then autoclave, with the screw cap in position, for 15 minutes at 115°C (a domestic pressure cooker with a 10 lb weight is used). After cooling, add 2 ml $^{57}CoB_{12}$ and then place 3 ml of the mixture into each of three 10 ml polystyrene tubes (Stayne Laboratories Ltd.), the first tube (tube 1) containing 1·5 ml water and the second and third tubes (tubes 2 and 3) each containing 1·5 ml working IF solution. With the tubes in this sequence it is possible to use an automatic dispenser (Warner-Chilcott) without rinsing between each serum. After mixing allow the tubes to stand for 30 minutes, and then to each add 2 ml coated charcoal. Mix, centrifuge the tubes horizontally at 2,000 rpm for 20 minutes, and decant 3 or 5 ml from each into counting tubes.

PREPARATION OF INTRINSIC FACTOR CONTROL An IF control with a serum content approximating that of the test serum mixture but depleted of free B_{12} is prepared as follows:

Into a Universal container place 2 ml acid, 7 ml cyanide solution, and 1 ml of any serum which has a B_{12} level of less than 300 pg/ml and has not been stored for more than one month. Then autoclave the mixture as before and allow to cool. Add 2 ml coated charcoal and centrifuge at 2,000 rpm for 20 minutes. After centrifugation decant 10 ml of the supernatant into another Universal container.

The IF control is prepared in duplicate and it has been found easier to avoid carry-over of charcoal by preparing three containers as described above and decanting enough for the duplicate controls. The IF control is then treated in exactly the same way as the test serum, *ie*, adding $^{57}CoB_{12}$, transfer to polystyrene tubes, etc.

PREPARATION OF RADIOACTIVE CONTROL This control is prepared similarly to the test serum using the same automatic syringes and dispensers, except that all the reagents apart from the $^{57}CoB_{12}$ are replaced by their same volume of water.

COUNTING Each sample is counted in a well type scintillation counter for 100 seconds.

CALCULATION OF THE SERUM B_{12} CONCENTRATION Serum

$$B_{12}\ (\text{pg/ml}) = C \times \left(\frac{IF}{S} - 1\right) \times 2$$

where C = B_{12} concentration in pg/ml of the $^{57}CoB_{12}$
S = net counts in the test serum
= average of tubes 2 and 3 − tube 1
IF = net counts in IF control
= average of tubes 2 and 3 − tube 1

SIMPLIFIED METHOD

For the rapid screening of sera it is possible to modify the above method by the use of a common serum background, so that all the reaction steps are carried out in one container, and the number of counting tubes per serum sample is two instead of three. The use of a common serum background tends to produce falsely low serum B_{12} values for some old sera and grossly haemolysed sera, and for occasional freshly collected sera.

PREPARATION OF TEST SERUM Into a glass Universal container, place 1 ml serum, 1 ml N/4 HCl, and 3 ml cyanide solution. Autoclave at 115°C for 15 minutes,

allow to cool, and add 1 ml $^{57}CoB_{12}$ (250 pg/ml, specific activity 70 to 180 $\mu c/\mu g$). Mix and then add 2 ml of IF solution (1 ml stock IF solution diluted 1 in 600 to 650). Mix and stand for 30 minutes. Add 1 ml coated charcoal, mix and centrifuge, and then decant 3 ml supernatant into each of two counting tubes.

PREPARATION OF INTRINSIC FACTOR CONTROL Prepare charcoal-treated serum extract (artificially B_{12}-depleted serum) as described above and place 5 ml in a Universal container. Then treat as described for the test serum, *ie*, add $^{57}CoB_{12}$, mix, add 2 ml IF solution, etc.

BACKGROUND CONTROLS Two are required, one for the sera and one for the IF control. They are prepared in exactly the same way as the test serum and the IF control except that the 2 ml IF solution is replaced by 2 ml water. Small volumes of the test sera are pooled and 1 ml of this pool is used for the serum background.

CALCULATIONS Serum B_{12} (pg/ml) $= C \times \left(\frac{IF}{S} - 1\right)$

where C = concentration in pg/ml of the $^{57}CoB_{12}$
IF = counts in IF control − counts in IF background control
S = counts in test serum − counts in serum background control.

USE OF A DILUTION CURVE

Although all the serum results given in this paper were obtained by means of an intrinsic factor control and the equation shown on page 206, it is possible to use a dilution curve. We have shown previously (Raven *et al*, 1968) that the results obtained by the two different methods of calculation are almost identical.

For the estimation of B_{12} in serum, a serum with a known B_{12} content (between 900 and 1,100 pg/ml and determined accurately by repeated microbiological and radioisotope assay) is doubly diluted (up to 1:32) to give B_{12} concentrations of approximately 1,000, 500, 250, 125, 60, and 30 pg/ml. The dilutions are then treated in exactly the same way as for test sera. For the calculations, a graph is constructed on semi-logarithmic paper with the net counts for each of the dilutions plotted on the logarithmic scale and the B_{12} values on the linear scale.

ASSAY OF CRYSTALLINE CYANOCOBALAMIN AND HYDROXOCOBALAMIN SOLUTIONS

For assay of these solutions some modifications of the method are required. When a large number of solutions is to be assayed it is convenient to use a dilution curve made from doubling dilutions of a 1,000 pg/ml cyanocobalamin solution, *ie*, 1,000, 500, 250, 125, 60, and 30 pg/ml. The standards and the test solutions are prepared in the same way as sera except that they are not autoclaved. The net counts of the standard solutions are plotted against their respective B_{12} concentrations on semi-logarithmic paper and a curve constructed (counts on the log scale).

When only one or two solutions are to be assayed it is more convenient to use an intrinsic factor control and to calculate the B_{12} values of the solutions from the same equation as above.

Into a Universal container, place 5 ml cyanide solution, 2 ml N/4 HCl, 1 ml of the crystalline B_{12} solution being tested, and 2 ml of a 1 in 20 dilution in water of B_{12}-deficient (less than 100 pg/ml) serum. This mixture is then treated in the same way as for sera, *ie*, autoclaving, cooling, addition of $^{57}CoB_{12}$, transfer to polystyrene tubes, etc. An appropriate IF control is prepared similarly except that the 1 ml test crystalline B_{12} solution is replaced by 1 ml water.

RESULTS

COMPARISON OF RADIOISOTOPE AND *L. leichmannii* RESULTS Five hundred and fifty sera obtained from normal subjects and a variety of unselected hospital patients were assayed by both the *L. leichmannii* and the $^{57}CoB_{12}$ methods. The results (Figure 1) show a fairly good correlation between the two methods, although the isotope method tended to give higher values for those sera which gave values of between 250 and 600 pg/ml by the *L. leichmannii* method.

NORMAL RANGE FOR THE RADIOISOTOPE METHOD Sera were obtained from 723 haematologically normal subjects. The B_{12} values obtained ranged from 175 to 1,400 pg/ml with a mean of 455 pg/ml (Figure 2).

B_{12}-DEFICIENT, FOLATE-DEFICIENT, AND POSTGASTRECTOMY PATIENTS Sera obtained from 92 patients with haematological and clinical evidence of B_{12} deficiency were assayed by both the *L. leichmannii*

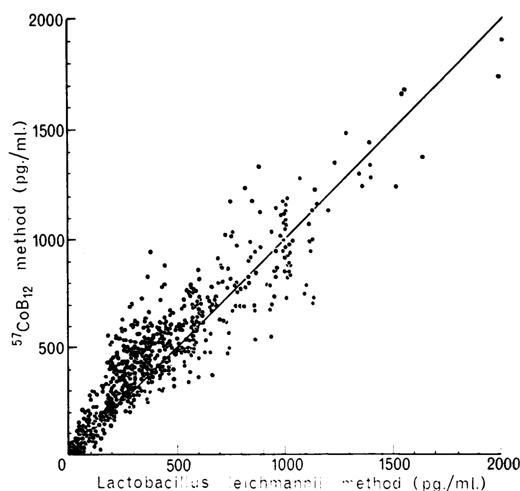

FIG. 1. *Comparison of the B_{12} values obtained for 550 sera by the* L. leichmannii *method and the $^{57}CoB_{12}$ method.*

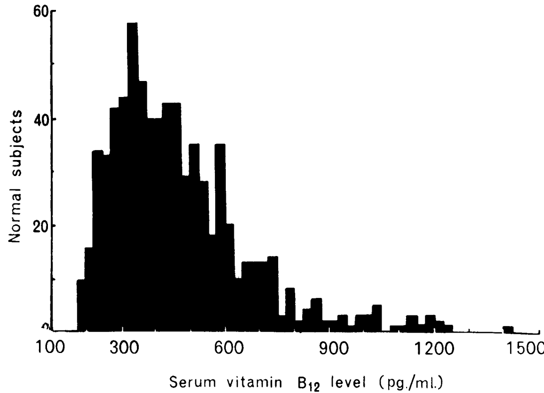

FIG. 2. *Distribution of the serum B_{12} values obtained for 753 normal subjects by the $^{57}CoB_{12}$ method. Mean serum vitamin B_{12} level is 455 pg/ml.*

and the $^{57}CoB_{12}$ methods. These patients consisted of 71 with pernicious anaemia, three with intestinal malabsorption, 17 with postgastrectomy anaemia, and one who was a vegan. Values of less than 200 pg/ml were obtained for all the sera (Fig. 3), but the isotope values tended to be higher than the microbiological values.

Sera obtained from 89 patients with clinical and haematological evidence of folate deficiency were assayed by both the *L. leichmannii* and $^{57}CoB_{12}$ methods. The causes of the folate deficiency were varied, and included malnutrition 52, malabsorption 17, drugs 6, pregnancy 4, intermittent dialysis 3, haemolytic anaemia 2, others 5. Patients with folate deficiency following gastric surgery have been excluded from this group and are considered separately. The results are shown in Figure 4. Values of less than 200 pg/ml were obtained by both methods for 20 sera and a further 18 gave values of less than 200 pg/ml by the *L. leichmannii* method but within the normal range by the $^{57}CoB_{12}$ method. The causes of the folate deficiency in this group were malabsorption 6, malnutrition 5, drugs 3, pregnancy 2, haemolytic anaemia 2.

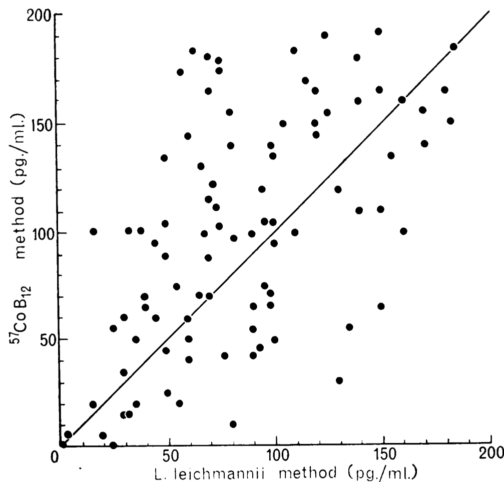

FIG. 3. *Comparison of the B_{12} values obtained by the* L. leichmannii *method and $^{57}CoB_{12}$ method for 92 sera from patients with B_{12} deficiency.*

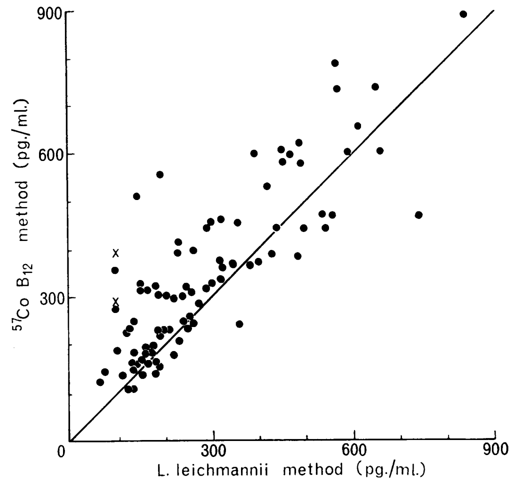

FIG. 4. *Comparison of the B_{12} values obtained by the* L. leichmannii *method and the $^{57}CoB_{12}$ method for 89 sera from patients with folate deficiency.* (× = *heroin/barbiturate addicts*).

Sera obtained from 96 patients who had had gastrectomies (93 partial, 3 total) were assayed by both the *L. leichmannii* and the $^{57}CoB_{12}$ methods. Fourteen of the patients had clinical and haematological evidence of B_{12} deficiency and 11 evidence of folate deficiency. The results are shown in Figure 5

TABLE I

SUMMARY OF THE VITAMIN B_{12} VALUES OBTAINED BY $^{57}CoB_{12}$ AND *L. leichmannii* METHODS FOR SERA FROM 96 POSTGASTRECTOMY PATIENTS

Patients	Serum B_{12} (pg/ml)	Number of Sera: $^{57}CoB_{12}$ Method	Number of Sera: L. leichmannii Method
With evidence of B_{12} deficiency	< 200	14	14
With evidence of folate deficiency	< 200	2	6
	> 200	9	5
Without evidence of B_{12} or folate deficiency	< 200	6	30
	> 200	65	41

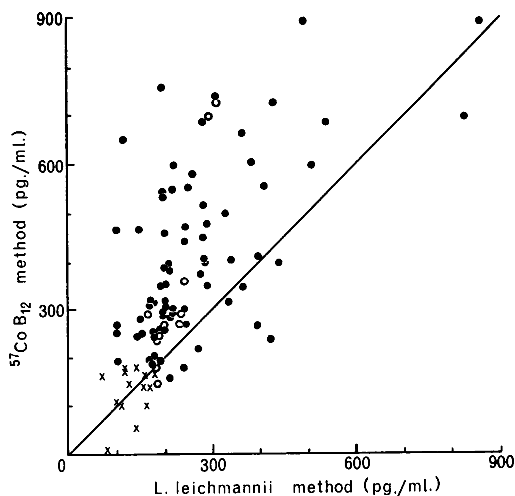

FIG. 5. *Comparison of the B_{12} values was obtained by the* L. leichmannii *method and the $^{57}CoB_{12}$ method for 96 sera from postgastrectomy patients.* × = *with evidence of B_{12} deficiency;* ○ = *with evidence of folate deficiency;* ● = *without evidence of B_{12} or folate deficiency.*

and Table I. All the 14 B_{12}-deficient patients had values of less than 200 pg/ml by both methods. Of the remaining 71 patients without evidence of B_{12} or folate deficiency, serum B_{12} levels of less than 200 pg/ml were found in 30 by the *L. leichmannii* method but in only six by the $^{57}CoB_{12}$ method.

REPRODUCIBILITY OF THE RADIOISOTOPE METHOD

SERA A number of sera were assayed repeatedly both in the same batch and over a period of 18 months. The results (Table II) show a satisfactory reproducibility.

Doubling dilutions of a single serum were assayed on five different occasions by the $^{57}CoB_{12}$ method. The expected values for this serum were obtained by repeated microbiological assay. Table III shows that the values obtained by the isotope method were reasonably close to the expected values.

TABLE III

RESULTS OBTAINED ON FIVE DIFFERENT OCCASIONS BY THE $^{57}CoB_{12}$ METHOD FOR DOUBLING DILUTIONS OF THE SAME SERUM

Serum Dilution	*Expected B_{12} Values (pg/ml)*	*Observed B_{12} Values (pg/ml)*				
		1	*2*	*3*	*4*	*5*
Undiluted	960	1,020	820	980	940	1,050
1:2	480	515	470	470	495	510
1:4	240	250	270	250	245	250
1:8	120	120	125	115	110	135
1:16	60	35	50	30	50	50
1:32	30	15	60	0	15	15

Ninety-eight sera were assayed in duplicate in the same batch by the $^{57}CoB_{12}$ method. The results obtained are plotted against each other in Figure 6 and show close agreement.

CYANOCOBALAMIN SOLUTIONS Satisfactory agreement with the expected value was obtained when three cyanocobalamin solutions were assayed on a number of different occasions over a period of 18 months (Table IV).

TABLE IV

REPRODUCIBILITY OF $^{57}CoB_{12}$ METHOD OVER 18 MONTHS IN ASSAY OF THREE CYANOCOBALAMIN SOLUTIONS

Expected B_{12} Values (pg/ml)	*Observed B_{12} Values (pg/ml)*
1,000	915, 1,080, 96C, 940, 820, 1,160, 1,060, 1,070
500	485, 485, 520, 480, 530, 480
200	190, 175, 200, 190, 140, 185

TABLE II

REPRODUCIBILITY OF THE $^{57}CoB_{12}$ METHOD FOR SERA BOTH IN A SINGLE BATCH AND OVER A PERIOD OF 18 MONTHS

Times of Assay	*Serum No.*	*No. of Assays*	*Serum B_{12} (pg/ml)*	*Mean*
Over 18 months	484	5	15, 10, 0, 65, 25	25
	982	12	0, 20, 60, 50, 0, 0, 30, 0, 20, 45, 50, 40	25
	949	15	0, 55, 45, 20, 100, 10, 90, 50, 20, 10, 40, 25, 35, 20, 60	40
	851	6	25, 30, 100, 55, 55, 55	55
	593	7	95, 115, 105, 65, 80, 65, 95	90
	633	4	230, 250, 215, 265	240
	1,002	5	255, 275, 260, 260, 260	260
	1,001	14	570, 450, 490, 510, 500, 395, 525, 490, 520, 515, 530, 430, 450, 440	490
	932	5	930, 970, 890, 880, 880	910
Single batch	476	5	0, 0, 0, 10, 10	5
	856	6	200, 205, 220, 195, 210, 225	210
	1,001	6	430, 480, 420, 460, 480, 460	455
	1,216	31	700, 660, 670, 700, 620, 740, 720, 670, 720, 690, 640, 690, 650, 730, 780, 685, 680, 760, 730, 740, 740, 680, 740, 690, 720, 680, 760, 780, 760, 840, 820	720

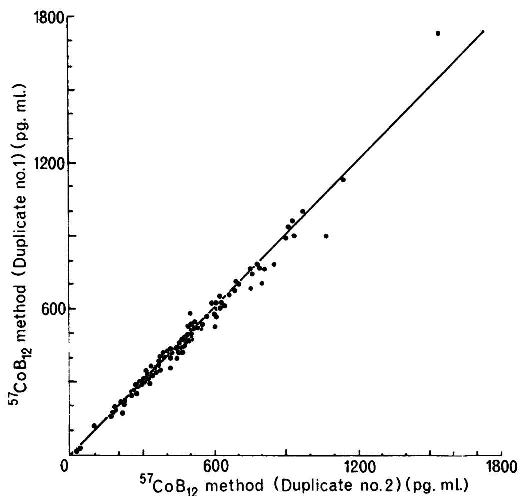

FIG. 6. *Comparison of the B_{12} values obtained for 98 sera assayed in duplicate by the $^{57}CoB_{12}$ method.*

DISCUSSION

The modified method described in this paper has been in use for about 18 months and has been found to be a distinct improvement on the methods described earlier (Lau *et al*, 1965; Raven *et al*, 1966). The results have correlated reasonably well with those obtained by microbiological assay using *L. leichmannii*, and the addition of B_{12}-deficient serum to the intrinsic factor control and of cyanide to the test sera has improved the accuracy of the method for both sera and crystalline B_{12} solutions.

We have continued to use intrinsic factor as the B_{12} binder rather than serum, as described by other workers (Barakat and Ekins, 1961, 1963; Grossowicz, Sulitzeanu, and Merzbach, 1962; Frenkel, Keller, and McCall, 1966; Matthews, Gunasegaram, and Linnell, 1967; Rothenberg, 1968) because it allows simplification of the radioisotope method in that deproteination and filtration are not required, the time of binding between IF and B_{12} is shorter than that between serum and B_{12} (Matthews *et al*, 1967), and the calculation of the results is simpler. The problem of instability of IF solutions reported by Rothenberg (1968) has not arisen in our work because our working IF solutions have been more concentrated, and furthermore the use of serum in the IF control gives greater uniformity of binding.

Less than 2% of normal sera gave B_{12} values between 175 and 200 pg/ml and the lower limit of normal by the method described is taken at 200 pg/ml. Sera from patients with pernicious anaemia have occasionally given B_{12} values as high as 190 pg/ml but so far all sera from patients with clinical and haematological evidence of B_{12} deficiency, whatever the cause, have given values of less than 200 pg/ml.

It is well known that patients with folate deficiency may also have subnormal serum B_{12} levels and that when the folate deficiency is corrected, the B_{12} values return to normal. We have found that when the *L. leichmannii* assay gave either a low normal or subnormal B_{12} value in patients with folate deficiency, the isotope method gave a similarly low value, but there have been some exceptions with the isotope method giving normal values and the *L. leichmannii* method subnormal values.

The serum B_{12} levels in postgastrectomy patients are of special interest. Serum B_{12} values of less than 200 pg/ml were found in all patients who had clinical and haematological evidence of B_{12} deficiency. However, many of the sera from patients without clinical and haematological evidence of B_{12} deficiency gave normal values with the isotope method but subnormal values with the *L. leichmannii* assay, thus producing a poor correlation between the two methods for this group of sera.

Now that the isotope method is working satisfactorily, it is necessary to find an explanation for any discrepancies between the results obtained by the $^{57}CoB_{12}$ and microbiological methods. As mentioned previously, both normal and B_{12}-deficient sera tend to give higher results by the isotope method. The discrepancies have been particularly marked with some folate-deficient and many postgastrectomy sera, with the microbiological assay giving low results and the isotope method normal values. It seems unlikely that these differences are due to inaccuracy on the part of the isotope method, because it is as efficient in detecting B_{12}-deficient sera as the *L. leichmannii* method and its reproducibility is equal to, or superior to, that of the microbiological assay. The two methods cannot be detecting different forms of B_{12}, because in each method the extraction process is carried out in the presence of cyanide, thus converting all forms of B_{12} in the serum to cyanocobalamin. In addition we have been unable to find any evidence of an *L. leichmannii* inhibitor in those sera giving different results by the two methods, except of course in sera from patients receiving antibiotics.

It seems very likely that the discrepancies are due to the different extraction processes used by the two methods to separate B_{12} from its binding proteins. The isotope method employs autoclaving (115°C for 15 minutes) in the presence of N/4 HCl while the microbiological method uses deproteination at *p*H 5-5·6. Rothenberg (1968), using a $^{57}CoB_{12}$ method with serum as the B_{12} binder, has reported that for

pernicious anaemia sera the extraction process described by Lau *et al* (1965) tended to give higher B_{12} values than those obtained when the extraction was carried out by deproteination at *p*H 5·6; he made no comment about other sera. Since he had previously found that extraction by deproteination gave recovery values of approximately 95% for cyanocobalamin added to serum (Rothenberg, 1963), he considered that it was unlikely that inefficient extraction could explain the lower B_{12} values obtained by his extraction process and suggested that the heating of serum at a low *p*H might release some substance which interfered with the binding between $^{57}CoB_{12}$ and serum. However, he assumed that recovery of cyanocobalamin added to serum can be equated with recovery of endogenous B_{12} from serum and this assumption remains unsupported.

Preliminary studies in which we have used the isotope method to assay the protein precipitate formed during the *L. leichmannii* extraction process suggest that for many sera appreciable amounts of B_{12} are left behind in this precipitate. We have noticed that when the two methods have given similar values for sera, little B_{12} can be recovered but when the two methods have given markedly different results for a serum the difference between the two results often equals the amount of B_{12} detected in the protein precipitate.

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