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Assay of Serum Vitamin B_{12} Concentration Using Co^{57}-B_{12} and Intrinsic Factor. (26840)

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Athough many proteins are capable of binding Vit. B_{12}, the greatest avidity for the vitamin is exhibited by intrinsic factor (I.F.). Barlow and Frederick(1) reported that purified intrinsic factor concentrates reveal several distinct protein components on paper electrophoresis but the most intense binding of Co^{60}-labeled Vit. B_{12} was observed in the region with the least amount of stainable protein material.

Bunge and coworkers(2) have clearly shown that Co^{60}-B_{12} (B_{12}*) and unlabeled Vit. B_{12} are biologically identical and compete for the binding sites of normal human gastric juice.

In agreement with observations of other investigators(1) initial studies in this laboratory using paper electrophoresis demonstrated that in mixtures of I.F. and B_{12}*, where there was insufficient I.F. to bind all the B_{12}*, 2 peaks of radioactivity could be demonstrated; the free B_{12}* remaining close to the origin and the B_{12}*-I.F. complex moving toward the anode. If concentrations of I.F. and B_{12}* were kept constant while concentration of unlabeled Vit. B_{12} was varied, the ratio of bound B_{12}* to free B_{12}* (B/F) decreased progressively with increase in concentration of unlabeled B_{12} in consequence of the competitive inhibition of binding of the labeled vitamin.

These results are analogous to the observations of Berson, Yalow *et al.*(3) on binding of insulin-I^{131} to insulin binding antibodies, in studies employing paper electrophoresis and chromatography. The observed inverse relationship between the ratio of bound to free I^{131}-insulin (B/F) and total concentration of insulin present was exploited in development of an immunoassay for insulin in plasma(4).

In the present communication the application of these principles to assay of Vit. B_{12} in plasma is described.

Methods. Since the specific activity of commercially available labeled B_{12} is at present too low to yield adequate counting rates in small amounts of serum extracts containing tracer quantities of B_{12}*, electrophoretic separation of free and bound B_{12}* proved inexpedient. Therefore, attempts were made to separate bound and free Vit. B_{12}* by precipitation of I.F.-B_{12}* complexes from larger quantities of serum extract than could be conveniently analyzed by paper electrophoresis. Several protein precipitants (TCA, phosphotungstic acid, etc.) produced co-precipitation of free Vit. B_{12} and could not be used for the present purpose. The Somogyi, barium hydroxide-zinc sulfate method(5) proved satisfactory.

Because of its higher available specific activity, Co^{57}-B_{12} (5 $\mu c/\mu g$)† was used instead of the Co^{60}-labeled vitamin.

Preparation of plasma extracts. Most or all of the Vit. B_{12} in serum is present as a heat-labile Vit. B_{12}-protein complex. When serum is acidified and heated the proteins are precipitated and Vit. B_{12} is liberated. Ac-

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† Purchased from Merck, Sharp & Dohme, Rahway, N. J.

cordingly, Vit. B_{12} extracts were prepared by diluting one ml of the test serum with 2 ml of distilled water and 1 ml of 0.1 N hydrochloric acid. After agitation at 100°C for 30 minutes, the precipitated proteins were separated by centrifugation and one ml of the supernatant (representing 0.25 ml of the original serum) was assayed for Vit. B_{12} content. Recovery by this extraction procedure of 100 $\mu\mu$g of Co^{57}-B_{12} added to serum was 85-88%. Recovery of added unlabeled Vit. B_{12} is reported below.

Preparation of standard solutions. Since the binding of Vit. B_{12} to intrinsic factor may be influenced by such factors as pH, and concentrations of various electrolytes notably calcium(6), it is essential that a "standard curve" be obtained under conditions which simulate as closely as possible those obtained in the presence of unknown samples. Since these conditions have not yet been completely determined, standard solutions, containing known amounts of Vit. B_{12} have been prepared using an extract of serum from a Vit. B_{12} deficient patient. Fig. 1 illustrates the different curves obtained with the same amount of Co^{57}-B_{12} and I.F. added to unlabeled B_{12} in distilled water and B_{12} deficient serum extract.

The serum extract employed for preparation of the standard solution used in the present study (serum C) contained the lowest concentration of Vit. B_{12} yet encountered as evidenced by the least inhibition of binding of Co^{57}-B_{12}. Intrinsic factor‡ was progressively diluted in this extract until a quantity was reached (0.00035 mg) in which 65%-75% of 100 $\mu\mu$g Co^{57}-B_{12} was bound under conditions to be described. A series of 1 ml mixtures was then prepared in which concentration of unlabeled crystalline Vit B_{12} ranged from 0-250 $\mu\mu$g/ml but in which concentrations of Co^{57}-B_{12} (100 $\mu\mu$g/ml) and I.F. (added last in all cases) were maintained constant. After one hour incubation at room temperature with gentle agitation, 1.5 mg of human serum albumin was added to all tubes in negligible volume (6 μl) to serve as carrier protein in the precipitation. This was followed by addition of 0.5 ml of 5% $ZnSo_4$. $Ba(OH)_2$ was then added dropwise with agitation after each drop until the endpoint of $ZnSo_4$ precipitation was reached. Amount of $Ba(OH)_2$ used was previously determined by titration with phenolphthalein indicator(5). The filtrate, representing free Vit. B_{12}, was assayed for radioactivity in a well type scintillation counter, using a gamma ray spectrometer with a sensitivity of 1.6×10^6 CPM/μc Co^{57}-B_{12}. Each sample was counted for a minimum of 10,000 counts.

‡ Hog Intrinsic Factor concentration kindly supplied by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N. Y.

In the absence of I.F. and serum albumin no Co^{57}-B_{12} was precipitated. In the absence of I.F. but with serum albumin present at the concentrations noted considerably less than 5% Co^{57}-B_{12} was precipitated.

For determination of Vit. B_{12} in unknown samples, test serum was prepared as described, and 1.0 ml of the extract was substituted for the unlabeled Vit. B_{12} used in the standard solution.

Vit. B_{12} concentrations were determined in the sera of 8 normal subjects, 8 patients with megaloblastic anemia, 1 patient with acute monocytic leukemia and 1 patient with chronic myelogenous leukemia.

Results. The ratio of bound Co^{57}-B_{12} to free Co^{57}-B_{12} (B/F) is plotted as a function of unlabeled Vit. B_{12} concentration added to serum extract C to yield a standard curve (Fig. 2). Serum C was then assayed against the standard curve and proved to fall out on the zero point. Vit. B_{12} concentrations in extracts from unknown serum samples were determined from the B/F ratios obtained with these extracts by reference to the standard curve, as indicated in Fig. 2. Results are given in Table 1. Samples A through H were from patients with megaloblastic anemias, presumed to be deficient in B_{12}. Range of B_{12} levels is zero to 72 $\mu\mu$g per ml of serum. Samples I through P are normal sera in which Vit. B_{12} concentrations ranged from 104 to 328 $\mu\mu$g per ml.

Recovery of crystalline Vit. B_{12} added to serum before extraction is summarized in Table II. Correction for 15% loss in extraction has been made to permit estimate of the accuracy of the assay procedure. Range of

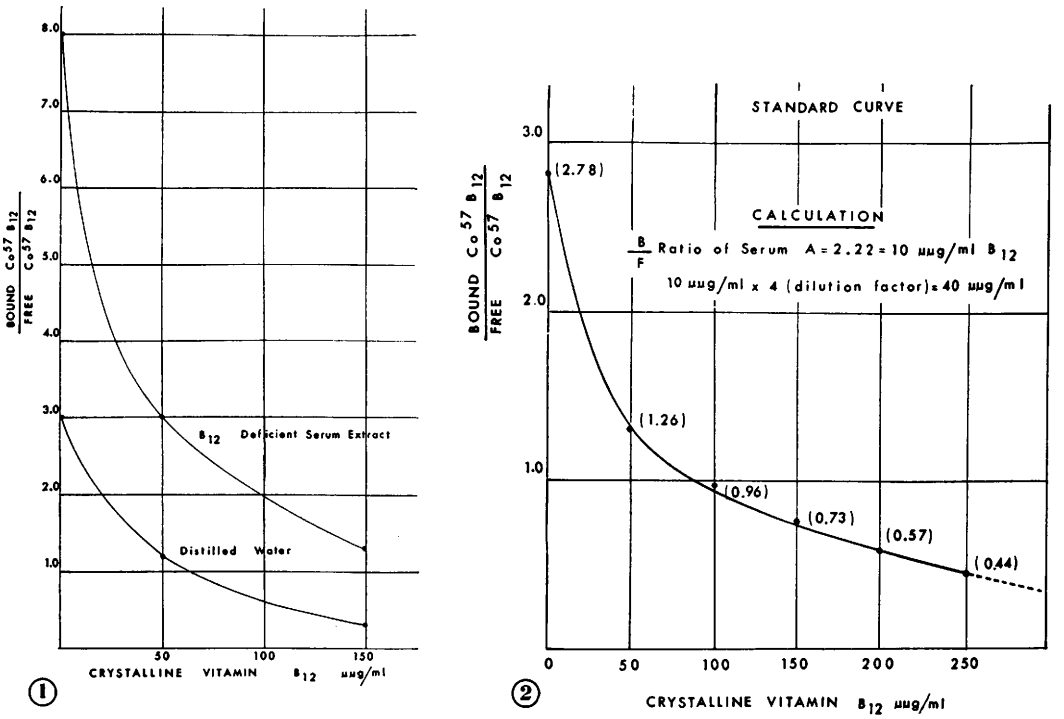

FIG. 1. Different curves obtained using the same amount of I.F. and $Co^{57}B_{12}$ in distilled water and B_{12} deficient serum extract.

FIG. 2. Standard curve obtained by addition of crystalline Vit. B_{12} to extract of serum "C."

recovery was from 83 to 112%.

Since it is known that leukemic patients have a high serum B_{12} concentration, 2 such sera were assayed. Both had levels well over the normal range. The serum of the patient with acute monocytic leukemia showed a B/F value at the end of the curve, representing 1000 $\mu\mu$g per ml. The value for the serum of the patient with chronic myelogenous leukemia fell off the curve, yielding, by extrapolation, an estimate of 1500 to 2000 $\mu\mu$g per ml.

Discussion. The method described depends on an accurately assayed commercial preparation of crystalline Vit. B_{12}, since it is used in preparation of the standard curve. Variations in the preparations of I.F. or the presence of non-specific binders should not affect this method because the quantity used is constant for both standard solutions and unknown samples.

The values obtained by this method, as presently described, do not include the endo-

TABLE I. Serum Vitamin B_{12} Concentration in Patients with Megaloblastic Anemia, Leukemia, and Normal Controls.

Serum	Clinical condition	% free Co^{57}-B_{12}	Co^{57}-B_{12}, Bound / Free	Vit. B_{12} conc., $\mu\mu$g/ml
A	Megaloblastic anemia	31.0	2.22	40
B	"	28.8	2.47	20
C*	"	25.7	2.90	0
D	"	31.0	2.22	40
E	"	31.0	2.22	40
F	"	33.6	1.98	68
G	"	32.6	2.06	56
H	"	34.0	1.94	72
I	Normal	43.6	1.29	200
J	"	37.0	1.70	104
K	"	45.3	1.20	224
L	"	48.4	1.06	304
M	"	45.2	1.20	224
N	"	42.2	1.37	176
O	"	49.6	1.02	328
P	"	46.4	1.15	248
AL	Acute leukemia	69.5	.44	1000
CL	Chronic "	81.4	.23	1500-2000

* This serum extract used as standard.

TABLE II. Total Recovery of Crystalline Vitamin B_{12} Added to Serum before Extraction. All values in $\mu\mu$g/ml.

Serum (Table I)	Endogenous Vit. B_{12} level	B_{12} added to serum before extraction	Expected additional B_{12} in extract*	Vit. B_{12} level assayed	% recovery of expected total
E	40	100	85	104	83
F	64	150	127	192	100
G	56	200	170	188	83
J	104	100	85	236	112
K	224	150	127	312	89
L	304	200	170	504	106
M	224	300	255	408	85

* Correction for 15% loss secondary to extraction procedure.

genous B_{12} level of the serum extract used in preparation of the standard curve. However, this error is minimized by choosing a serum with the lowest B_{12} level (least inhibition of binding of 100 $\mu\mu$g Co^{57}-B_{12} by I.F.).

Serum Vit. B_{12} concentrations in normal controls and in patients with pernicious anemia and leukemia reported above agree, in general, with levels obtained employing microbiological assay procedures(7,8). A more precise and critical comparison can be made only when many more sera are assayed simultaneously by both methods.

Summary. Based on some observed properties of the B_{12}-I.F. complex, an assay procedure is described for determining serum Vit. B_{12} levels using isotopically labeled Vit. B_{12} and intrinsic factor. 83%-112% of crystalline Vit. B_{12} added to serum was recovered in the assay. Concentrations of Vit. B_{12} in normal, B_{12} deficient, and leukemic sera were in the ranges, 104-328 $\mu\mu$g/ml, 0-72 $\mu\mu$g/ml and over 1000 $\mu\mu$g/ml respectively.

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Antigen Elimination from the Site of Arthus Phenomenon in the Guinea Pig* (26841)

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Almost 60 years have passed since Arthus (1), using horse serum injected into rabbits, described the phenomenon that bears his name, but its effect on the removal of antigen from the injection site has not been clearly delineated. The gross physical manifestations of this reaction are usually apparent 60 minutes after injection of the antigen, and thus one might anticipate an effect on antigen removal during the first few hours after in-

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