Vitamin B_{12} Binding Capacity of Serum in B_{12} Deficiency.* (28908)

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Vitamin B_{12} in serum is bound to the seromucoid fraction of the serum proteins(1,2). By microbiologic assay of electrophoretic fractions of serum most of the vitamin has been found in the α_1 globulin(3,4). If serum is incubated with radioactive or non-radioactive cyanocobalamin and dialyzed, the added vitamin is detected predominantly in the β-globulin(4,5). In serum electrophoregrams, obtained at pH 4.5, the "native" vitamin has been found in the anodically migrating acidic seromucoids, while added vitamin remains at point of origin with the other proteins(2). These findings have led to the conclusion that the "specific" B_{12}-binding protein in normal serum is saturated and added vitamin is bound to other proteins(2,5). One could, therefore, assume that only in the presence of lower than normal B_{12} serum levels some of the "specific" binding protein is unsaturated.

The results of the present study suggest that normally only approximately one-third of the "specific" binding protein is saturated and that this binding protein has a diminished serum concentration in megaloblastic anemia due to B_{12} deficiency.

Methods and materials. B_{12} serum levels were determined according to the U. S. Pharmacopeia XV *Lactobacillus leichmannii* method modified by Mendelsohn and Watkin(6). All samples were autoclaved prior to microbiologic assay and no attempt was made to determine the amount of B_{12} available to *L. leichmannii* without autoclaving. This amount, usually designated "free" B_{12}, depends on the technic used for B_{12} assay(7,8), it is approximately 25% of the total in the *L. leichmannii* assay(8) and is not identical with dialyzable B_{12} which we could not detect in normal serum.

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B_{12} binding capacity was measured by incubating for 4 hours in the dark at 4°C 2 ml-serum-aliquots with increasing amounts of Co^{57}-B_{12} to result in concentrations of 1.2 to 9 mμg/ml in all samples. In addition, from 4 normals and all B_{12} deficient patients serum samples with lower concentrations were prepared (0.025-0.3 mμg/ml; in case of the lowest concentration 6 ml serum samples were used). Following determination of the radioactivity of each tube the sera were transferred to Visking No. 20 tubing and dialyzed at 4°C against 500 ml of 0.15 M NaCl for 48 hours with one change after 24 hours. This method of equilibrium dialysis has been found to yield similar results as exhaustive dialysis in running tap water. After dialysis the outer surface of the tubing was rinsed and the content transferred to counting tubes. The radioactivity of the empty bags was determined separately and the counts added to those of the dialyzed serum. The samples were counted in a well-counter equipped with an automatic sample changer. The counting error was less than one per cent. The counts were corrected for background and for coincidence loss.

Electrophoresis (e.p.) was performed in agar gel, pH 8.8, 0.03 M sodium barbital buffer, ionagar No. 2 (Consolidated Laboratories, Inc.)(9). The heated gel (approximately 60-70 ml) was poured on large glass plates 8 × 25 cm. The dialyzed serum samples (approximately 2 ml) were mixed with 0.5 ml of gel and introduced into grooves (20 × 0.4 cm) which had been cut into the hardened gel. In a separate small slit, at the same distance from the electrodes a small amount of serum stained with amido-Schwarz was applied as marker to assure reproducibility of the electrophoretic distances of the protein fractions. The duration of e.p. was usually 90 minutes and the applied potential was 13-15 volts/cm. After e.p. the gel was

divided into 0.5 cm segments, the radioactivity of each segment was counted and microbiological assay for B_{12} performed on eluates of the segments obtained by freezing and thawing. The recovery of radioactivity after e.p. of the dialyzed samples was 90-96%

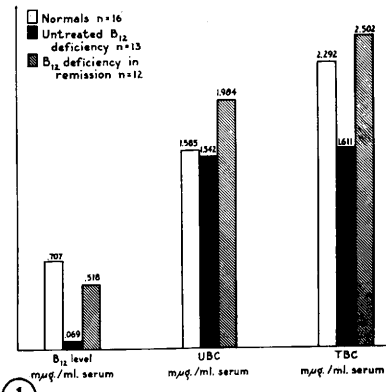
For *radioautography* the agar gel plates after e.p. were dried under infrared light, placed on photographic film (Eastman Royal X-Pan) with the dried agar surface in contact with the film and exposed for 9-27 days.

The *seromucoid fraction* was prepared from 10 ml-aliquots of the dialyzed sera according to the technic of Winzler(10). The perchlorate soluble fraction was precipitated with phosphotungstic acid(10) and radioactivity determined in this precipitate and the perchlorate insoluble material. The recovery was nearly 100%. For e.p. of the seromucoid fraction the supernatant after perchlorate precipitation was dialyzed for 72 hours against large volumes of distilled water which was under continuous agitation and was changed 5-6 times daily. The dialysate was lyophilized and redissolved in 2 ml of distilled water and electrophoresed in agar gel at pH 8.8 (sodium barbital 0.03 M). Measurement of radioactivity in the segments and radioautography were performed as described above. These experiments were also performed with the isolated α and β fractions which after their isolation were incubated with Co^{57}-B_{12}, dialyzed and reelectrophoresed.

The sera were obtained from 16 normal adults, 13 patients with B_{12} deficiency (12 with pernicious anemia (p.a.), one after ileal resection), and 12 patients with p.a. in remission. No special consideration was given to age and sex.

Two normals and 2 patients with p.a. in relapse were given 100 μg of cyanocobalamin intramuscularly daily for 4 days and the binding capacity determined prior to each dose and 24 hours after the last one.

Results. The mean B_{12} serum level ($\pm$ S.D.) of the 16 normals (Fig. 1) was 0.707 $\pm$ 0.190 mμg/ml. It was 0.069 $\pm$ 0.050 mμg/ml in patients with B_{12} deficiency and 0.518 $\pm$ 0.255 mμg/ml in patients in remission. The B_{12} level was determined in



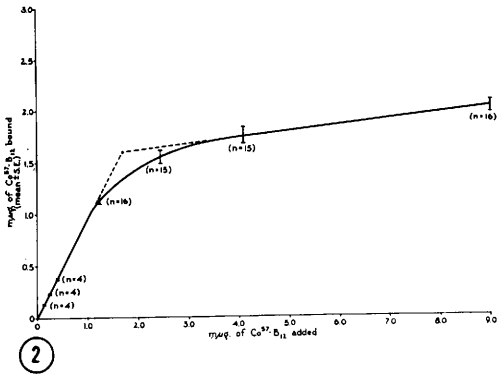


FIG. 1. Serum B_{12} levels, unsaturated and total binding capacity in normals and patients with B_{12} deficient megaloblastic anemia in relapse and complete remission.

FIG. 2. Co^{57}-B_{12} binding capacity of normal serum.

several of these samples following dialysis, but no dialyzable B_{12} was detected. Thus the entire amount of native vitamin was considered to be protein bound.

Following incubation of sera with increasing amounts of Co^{57}-B_{12} all sera were found to bind additional vitamin (Fig. 1,2). This finding is in complete or partial agreement with the results of several authors(1,5,11,12, 13). The plotted values formed a biphasic curve suggesting 2 or more binding substances (Fig. 2). The initial steep slope was considered to be caused by a "primary" binder of high affinity for the vitamin and the shallow slope by "secondary binders." The intersection of the 2 extended slopes was defined as "unsaturated binding capacity" (UBC). The mean UBC for 16 normals was

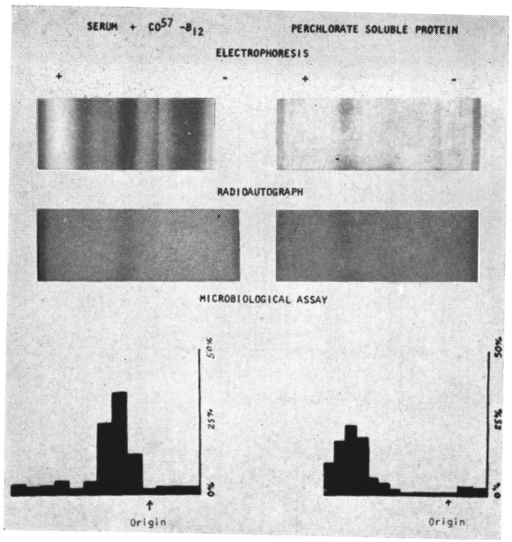


FIG. 3. Serum electrophoresis in agar gel, radioautographs and segmental microbiological assay for B_{12} before and after perchlorate precipitation.

1.585 ± 0.335 mμg/ml; it was 1.542 ± 0.478 mμg/ml in 13 patients with B_{12} deficiency, and 1.984 ± 0.321 mμg/ml in 12 patients with B_{12} deficiency in complete remission.

The total binding capacity (TBC) was defined as the sum of the B_{12} level and the UBC. This definition appeared to be justified since in original serum no dialyzable vitamin was detected. The TBC (Fig. 1) was lower in patients with megaloblastic anemia due to B_{12} deficiency than in normals, but was normal in patients in remission.

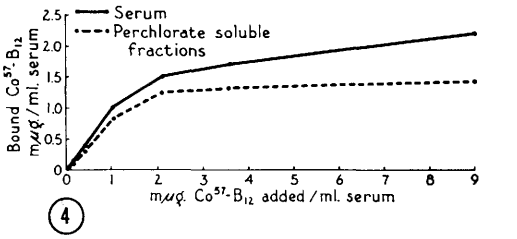
Radioautographs showed the bulk of the added Co^{57}-B_{12} to be in the β-globulin fraction (Fig. 3). Radioactivity was detectable in other fractions only when the content of dialyzed serum was around 2.5 mμg Co^{57}-B_{12} per ml. The β-globulin binding was also noted in serum from all B_{12} deficient patients. Even after incubation with such a small amount as 0.075 mμg of Co^{57}-B_{12} per ml of B_{12} deficient serum no radioactivity was detected in the α-globulin. When, however, the same sera were subjected to perchlorate precipitation and the supernatant electrophoresed, the Co^{57}-B_{12} binding protein migrated as α_1-globulin in all samples, whether a very small amount or an amount commensurate with the UBC had been incubated with the serum (Fig. 3). This shift from the β to the α_1 fraction was also noted in the microbiological assay of segments of the electrophoregram (Fig. 3). When the seromucoid was prepared from the isolated β-globulin fraction which had been incubated with Co^{57}-B_{12} and electrophoresed, most of the radioactivity was found in the α_1 fraction.

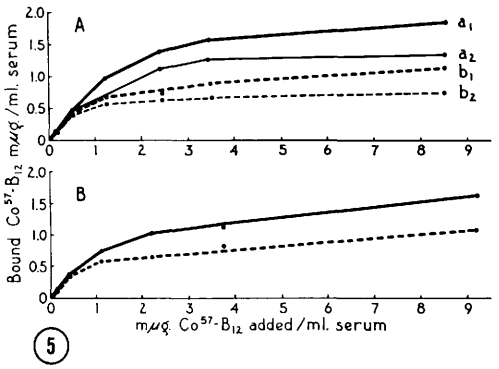
In the sera from 3 normals the seromucoid bound radioactivity was determined at each level of added Co^{57}-B_{12} (Fig. 4). Up to the point of the UBC more than 80% of the protein bound vitamin was found in the seromucoid. At higher levels this proportion diminished and there was only a slight increase of the absolute values beyond this point. This phenomenon was also observed in serum from one patient with p.a. in relapse and in early remission.

The binding of B_{12} *in vivo* was investigated before and after intramuscular injection of 400 μg cyanocobalamin in 4 equally divided daily doses, into 2 normals (Fig. 5) and 2 patients with p.a. in relapse (Fig. 6). In either case a diminution of the UBC was noted. In the patients with p.a. practically no UBC was left as evident from the absent initial steep slope of the binding curve. The increase in B_{12} serum levels had led to an apparently complete saturation of the primary binding protein in the patients with p.a. and only partial saturation in the normal. The latter was also noted in the perchlorate soluble fraction (Fig. 5).

Discussion. Several studies have shown that serum B_{12} is bound to the seromucoid fraction(1,2) and migrates with α_1-globulin (1-5). Since added vitamin is predominantly found in the β-globulin fraction, it might be assumed that in normal serum the α_1-globulin binder is completely saturated and that the binding of added vitamin occurs in other protein fractions. While our findings also indicate that binding occurs in other than perchlorate soluble proteins at high levels of added vitamin, they strongly suggest that the seromucoid fractions contain a binding protein which is normally not completely saturated. Since the seromucoid level in normal serum is approximately 600 μg/ml(14) and the B_{12}-TBC is approximately 2.5 mμg/ml

it is likely that only a very small component of the seromucoid fraction, probably less than 1 μg/ml participates in the binding of B_{12}. It is possible that this fraction represents a specific B_{12}-binder. The findings of Weinstein *et al*(1) suggest that this component is not orosomucoid.





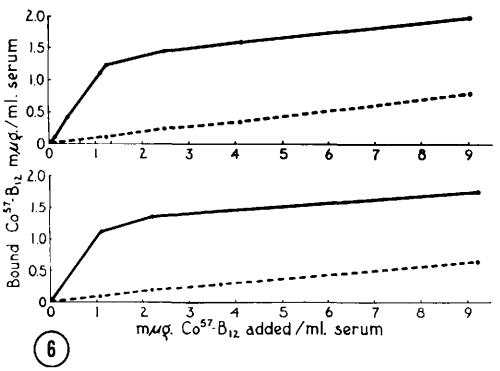


FIG. 4. Co^{57}-B_{12} binding capacity of serum and of supernate after perchlorate precipitation.

FIG. 5. A. a_1: Co^{57}-B_{12} binding capacity of normal serum (B_{12} level: 0.475 mμg/ml) and b_1: 24 hr after 4 daily doses of 100 μg of B_{12} intramuscularly (B_{12} level 1.750 mμg/ml). a_2: binding curve of supernate of serum a_1 after perchlorate precipitation b_2: same of serum b_1. B. Same as A in another normal. Perchlorate precipitation was not performed. B_{12} serum level 0.530 mμg/ml before B_{12} administration and 1.26 mμg/ml afterward.

FIG. 6. Same as fig. 5 in 2 patients with addisonian anemia in relapse. B_{12} serum levels before 4-day treatment were 0.112 mμg/ml and 0.068 mμg /ml, after treatment 1.89 mμg/ml and 2.25 mμg/ml.

The change in e.p. mobility of Co^{57}-B_{12}-binding seromucoid from β to a_1 after precipitation of the perchlorate-insoluble protein is not fully explained. The question must be raised if this shuttling of the Co^{57}-B_{12}-seromucoid *in vitro* reflects a physiological mechanism which causes absorbed or injected vitamin to be bound first to β-globulin and later to a_1-globulin, where most of the native vitamin is detected. This change in the e.p. mobility of the binding protein could be caused by the seromucoid-B_{12} complex attaching itself during the *in vitro* incubation to the β-globulin. To test this possibility we isolated the seromucoid-Co^{57}-B_{12} complex with a mobility and incubated it with serum. No change in electrophoretic mobility of the seromucoid-B_{12} occurred. This problem requires further study, but it is of interest that Hall(15) recently demonstrated sequential changes in the pattern of plasma binding of Co^{57}-B_{12} after its intravenous injection.

The UBC in serum of patients with B_{12} deficiency is not significantly different from the normal, but because of the initial low B_{12} level the TBC is diminished. The fact that in p.a. apparently less binding protein is available is also illustrated by the marked diminution of UBC following injection of the vitamin, in contrast to the normal, in whom the injection of an equal amount of the vitamin still leaves UBC available (Fig. 5,6). On the other hand, a tracer dose of B_{12}, injected intravenously, disappears from the plasma of a B_{12} deficient individual at a slower rate than from normal plasma(15,16,17). This could suggest a stronger than normal binding capacity in B_{12} deficient plasma, but another explanation must be considered: the tissues in B_{12} deficiency might have diminished binding capacity for the vitamin, and, therefore, be incapable of receiving the vitamin from the plasma with the expected increased avidity. One could postulate that vitamin B_{12} stimulates the synthesis of its binding protein in the tissues as well as in the plasma.

Summary. 1. Human serum has been found to have a limited binding capacity for Vit. B_{12}. The B_{12} binding protein, a seromucoid

fraction, appears to be normally only approximately 30-40% saturated. In Vit. B_{12} deficiency the unsaturated binding capacity is normal but total binding capacity is diminished. 2. It is postulated that the slower than normal disappearance of Co^{57}-B_{12} from the plasma of B_{12} deficient individuals is caused by diminished binding capacity in the tissues.

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Effects of Hydrobiotites upon Strontium-89 and Cesium-137 Retention By Ruminant Animals.* (28909)

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The literature is well documented with reports concerned with the movement and physiological behavior of radio-strontium and cesium(1-4), and extensive data are available on various dietary and therapeutic measures for limiting absorption and deposition in monogastric animals(5-7). However, there is little information on complexing agents suitable for use with ruminant animals. In that more than half of our added body burden of radioactivity originates from milk and milk products, and another one-fourth is derived from meat and animal products(8-9), there is a potential need for a practical procedure for safeguarding this portion of our food-chain against chronic ingestion of low levels of radioactive materials. Several reports(3,10-12) have indicated various degrees of success with commercial vermiculite for influencing excretion routes, and for decreasing skeletal retention of Sr-89 and of Cs-137. Initial studies in this laboratory(13) have revealed interesting possibilities for special processed vermiculite ores for selective sorption of these cations in the rumen for subsequent excretion, unabsorbed.

This study was initiated, therefore, to investigate the binding qualities for Sr-89 and Cs-137 of a refined vermiculite, verxite, a nearly pure exfoliated hydrobiotite, *in vivo* and *in vitro*, in an effort to prevent gastro-intestinal absorption of these cations in the ruminant animal.

Methods and materials. The *in vitro* studies consisted of incubating tracer levels of Sr-89

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