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Research Article

ELECTROPHORETIC STUDIES OF THE VITAMIN B_{12}-BINDING PROTEIN OF NORMAL AND CHRONIC MYELOGENOUS LEUKEMIA SERUM *

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The serum in chronic myelogenous leukemia (CML) has an increased concentration of vitamin B_{12} (1, 2) and an increased capacity to bind added vitamin B_{12} either *in vivo* (3–5) or *in vitro* (2, 6, 7). The vitamin B_{12} of normal and CML serum moves with the α-globulins when serum is electrophoresed at pH 8.6 (1, 8, 9). The seromucoid, the fraction of serum containing glycoproteins which comprises about 1.5 per cent of the serum proteins (10), moves with the α-globulins when electrophoresed at pH 8.6 (11). Furthermore, it has been found that the seromucoid fraction isolated from CML serum is capable of binding amounts of added cobalt^{60}-labeled vitamin B_{12} (B_{12}*) sufficient to account for the increased binding capacity of the whole serum (12). The possibility that the binding protein of both normal and CML serum for native vitamin B_{12} (B_{12}BP) is in the seromucoid fraction was investigated.

METHODS

Venous blood was collected in the fasting state from normal subjects and from patients with CML in hematological and clinical relapse. About one to two hours after clotting, the serum was separated and either used immediately or stored at −20°C. The seromucoid fraction of serum was prepared by the method of Winzler, Devor, Mehl and Smyth (10) as follows: one volume of serum was added to one volume of 0.4 M sulfosalicylic acid, thoroughly mixed for five minutes, and then centrifuged. The supernatant containing the seromucoid was separated and neutralized with 1 N NaOH. The neutralized seromucoid and the original serum from which it had been prepared were dialyzed against large volumes of 0.15 M NaCl for 72 hours at 4 to 6°C. and its vitamin B_{12} concentration then determined by *Euglena gracilis* assay (13). When used for electrophoresis, the seromucoid was dialyzed for 72 hours against large volumes of distilled water, lyophilized, and the dried protein then dissolved in the appropriate buffer. Seromucoid or serum containing bound (nondialyzable) B_{12}* [1] was prepared by adding an amount of B_{12}* in excess of the binding capacity (4 mμg. B_{12}* per ml. of normal serum or its equivalent seromucoid and 12 mμg. per ml. of CML serum or its equivalent seromucoid). The seromucoid was then dialyzed against large volumes of distilled water for 72 hours at 4 to 6° C. and lyophilized, while the serum was dialyzed against 0.15 M NaCl.

Lyophilized Cohn plasma fractions (I through VI),[2] prepared by Method VI (14), were dissolved in sufficient 0.15 M $NaHCO_3$ to bring them to their equivalent plasma volumes, dialyzed for 48 hours against 0.15 M NaCl, and the vitamin B_{12} concentration determined as was the vitamin B_{12} concentration of the original plasma.

Zone electrophoretic separation of serum or lyophilized seromucoid was performed using starch blocks as the supporting medium. The general technique described by Kunkel (15) was used except that the electrophoresis was carried out at pH 4.5 which permitted separation of the B_{12}BP from the bulk of serum proteins. The starch blocks were prepared from slurries of starch [3] that had been previously washed once with distilled water and twice with an acetate-sodium chloride buffer pH 4.5, ionic strength 0.10. The buffer consisted of 0.04 M sodium acetate and 0.06 M NaCl with concentrated HCl added to bring the pH to 4.5. The following samples were electrophoresed: *a*) a mixture of 1.0 ml. of serum and 0.5 ml. of acetate buffer; *b*) serum containing bound (nondialyzable) B_{12}* in a volume of 1.5 to 2 ml.; *c*) lyophilized seromucoid dissolved in a solution of 1 ml. of 0.15 M NaCl and 0.5 ml. acetate buffer; and *d*) lyophilized seromucoid containing bound B_{12}* dissolved as in *c*. Samples were applied to a 4 to 5 mm. transverse slit at the center of the block. In some experiments, serum containing bound B_{12}* was run in parallel on the same starch block as the native serum. Electrophoresis was carried out for 16 hours at 5 to 8° C. using a current flow of 36 ma. for a 31 cm. long, 10 cm. wide, 1.5 cm. thick starch block. At the termination of electrophoresis a maximum

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[1] Kindly supplied by Dr. N. S. Ritter, Merck and Co., Rahway, N. J.

[2] Kindly prepared by the Plasma Protein Foundation, Boston, Mass.

[3] Fisher hydrolyzed potato starch, Fisher Scientific Co., New York, N. Y.

TABLE I

The native vitamin B_{12} concentration of the seromucoid of normal and chronic myelogenons leukemic (CML) sera

Subject		Serum B_{12} concentration	Seromucoid B_{12} concentration	Seromucoid fraction
		μμg./ml.	*μμg./ml.*	%
Normal				
1	S.W.	525	435	83
2	L.S.	125	122	98
3	A.N.	732	451	62
4	M.B.	732	539	74
5	T.C.	681	544	80
6	S.J.	332	208	63
Mean		521	383	77
Range		125–732	123–544	62–98
CML				
1	H.W.	6,096	5,976	98
2	A.M.	15,121	15,101	100
3	C.M.	9,220	9,010	98

pH change of 0.2 pH unit was found at either end of the gel. After drying at room temperature (0.5 to one hour) the gel was cut into transverse segments so that the origin was included in one segment (O segment). Other segments were designated as anodal (A) or cathodal (C) and identified by the number of segments from the O segment. Sections of the block at the cathodal and anodal ends well outside the range of migration of the proteins were removed to serve as blanks. Each segment was suspended in 8 ml. of distilled water, the contents periodically mixed for 1.5 hours, and then centrifuged. The supernatant was removed and centrifuged again. The vitamin B_{12} concentration of these eluates was then determined by *E. gracilis* assay. The per cent recovery of vitamin B_{12} from the starch block in 19 electrophoretic analyses of serum or seromucoid was equal to an average of 93 per cent ± 14, range, 75 to 115 per cent.

The protein concentration of starch eluates was determined by Kunkel and Tiselius' modification (16) of the Folin-Ciocalteu procedure (17). Protein concentration was plotted in terms of optical density with necessary corrections made for the volume of eluate analyzed. Sialic acid was determined by the method of Werner and Odin (18) using 2 ml. of the eluates. Because of the negligible reading of the blank with Baker's iso-amyl alcohol, further purification of the iso-amyl alcohol was not necessary.

In preliminary experiments, it was found that albumin and the bulk of the globulins remained at the origin or migrated cathodally when serum was electrophoresed at pH 4.5. Acidic α_1-glycoprotein,[4] isolated from Cohn Fraction VI (19), was dissolved in a solution composed of 1 ml. 0.15 M NaCl and 0.5 ml. acetate buffer, electrophoresed on a starch block (pH 4.5) and the protein and sialic acid concentration of 1.0 cm. segments of the block determined.

After electrophoresis, the starch segments containing nondialyzable B_{12}* were placed in test tubes and the radioactivity determined in a plastic scintillation well counter (20) having a background of 385 to 400 counts per minute. Samples with higher counting rates, such as *a*) the origin and first four cathodal segments of normal sera, *b*) anodal segments of the seromucoid and *c*) the anodal and cathodal segments of CML sera, were counted long enough to give a 10 to 15 per cent counting error. The anodal segments of the normal sera contained small amounts of radioactivity (as little as five to 10 counts per minute over background) and were counted for three to four 10 minute periods with 10 minute counts of background alternating between each sample count. The radioactivity of the B_{12}* solution added to serum was counted in the same well counter using similar geometry, and the concentration of vitamin B_{12} in this solution was also determined. From the *a*) specific activity of the added B_{12}* and *b*) radioactivity found in each starch segment, the amount of B_{12}* (*μμg.*) per segment was estimated. The total vitamin B_{12} bound by the proteins in any starch segment was calculated by summing of the native vitamin B_{12} (determined by *E. gracilis* assay) and of the nondialyzable B_{12}*.

RESULTS

Vitamin B_{12} concentration of the seromucoid

The seromucoid contained an average of 77 per cent, range 62 to 98 per cent, of the vitamin B_{12} of whole normal serum (Table I). Despite a great increase in serum vitamin B_{12} concentration, 98 to 100 per cent of the vitamin was recovered from the seromucoid in three patients with CML.

Vitamin B_{12} concentration of Cohn plasma fractions

Vitamin B_{12} was found in all six Cohn plasma fractions. Fraction V contained the highest percentage of the plasma vitamin (27 per cent) while Fraction VI had only 14 per cent.

Vitamin B_{12} concentration of fractions of the seromucoid separated by electrophoresis

The distribution of native vitamin B_{12}, protein and sialic acid in the seromucoid, obtained from normal serum following starch gel electrophoresis at pH 4.5, is shown in Figure 1. The protein and sialic acid were concentrated in segments anodal to the origin with peak levels found in the A-3 and A-4 segments. A similar distribution of protein and sialic acid was found when a crystalline preparation of the acidic α_1-glycoprotein was electro-

[4] Kindly supplied by Dr. Karl Schmid, Massachusetts General Hospital, Boston, Mass.

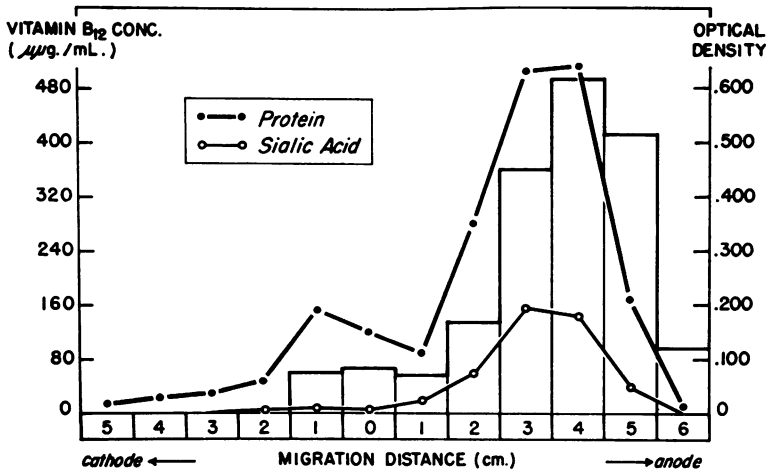

FIG. 1. NATIVE VITAMIN B_{12} CONCENTRATION OF FRACTIONS OF NORMAL SEROMUCOID SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT PH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density is plotted on the right ordinate with protein the solid dots ●——●, and sialic acid the open dots ○——○.

phoresed at pH 4.5. The vitamin B_{12} was also found anodally with peak concentrations at A-3 to A-5 segments. The B_{12}* bound to the normal seromucoid had a distribution similar to the native vitamin, with peak concentrations at A-2 to A-4 segments (Figure 2).

The distribution of protein, sialic acid and vitamin B_{12} in the CML seromucoid was similar to that of the normal, with peak concentrations found at A-3 to A-4 segments (Figure 3). Furthermore, the distribution of added B_{12}* bound by CML seromucoid was similar to that of the native vitamin, with peak concentrations located at A-2 to A-4 segments (Figure 4).

Vitamin B_{12} concentration of fractions of serum separated by electrophoresis

Electrophoresis of a normal serum at pH 4.5 (Figure 5) is representative of the findings in a group of eight such sera. In sharp contrast to the seromucoid, there was a negligible amount of protein in A-2 to A-6 segments with the peak pro-

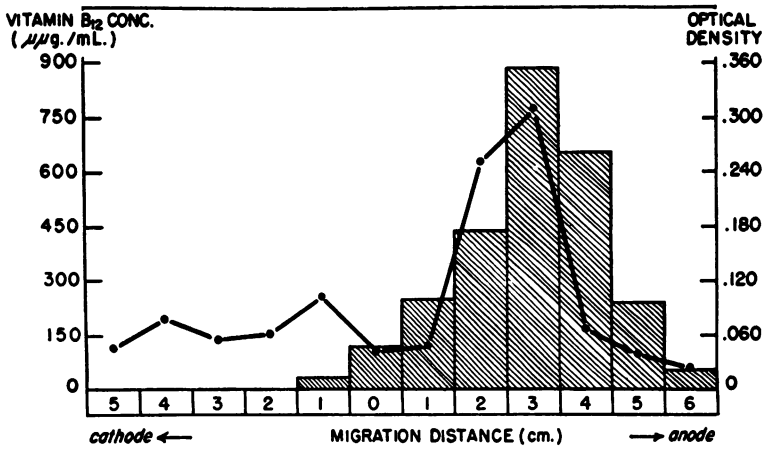

FIG. 2. DISTRIBUTION OF BOUND (NONDIALYZABLE) B_{12}* AMONG FRACTIONS OF NORMAL SEROMUCOID SEPARATED BY STARCH GEL ELECTROPHORESIS AT PH 4.5

The concentration of B_{12}* is plotted on the left ordinate and is represented by the ruled boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

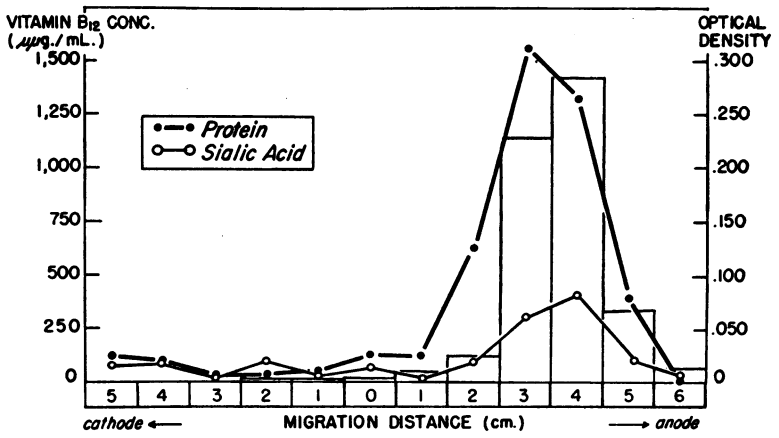

FIG. 3. NATIVE VITAMIN B_{12} CONCENTRATION OF FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIA SEROMUCOID SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT pH 4.5

Vitamin B_{12} concentration plotted on the left ordinate is represented by the open boxes. Optical density is plotted on the right ordinate with protein the solid dots ●——● and sialic acid the open dots ○——○.

tein concentration at the O segment (A-1 or C-1 in some sera). However, virtually all of the native vitamin B_{12} was recovered anodally (83 to 92 per cent found in segments A-2 to A-6), with peak concentrations in A-3 to A-5 segments. The distribution of the native vitamin B_{12} and of the nondialyzable B_{12}* bound to the same serum is shown in Figure 6 and is representative of the findings in a group of seven such sera. The native vitamin had the same anodal distribution as previously described. Only a small amount of B_{12}* was found anodally, segments A-2 to A-6 containing an average of 80 μμg. per ml. ± 17, range, 28 to 183 μμg. per ml. The proteins with the greatest binding ability for the added B_{12}* were found at the origin and C-1 to C-3 segments. The binding of B_{12}* by A-2 to A-6 segments was equal to an average of 6 per cent ± 4, range, 3 to 15 per cent of the total bound radiovitamin. The saturation of the B_{12}BP (located at A-2 to A-6 segments) as estimated from the ratio of the native bound vitamin to the total vitamin that could be

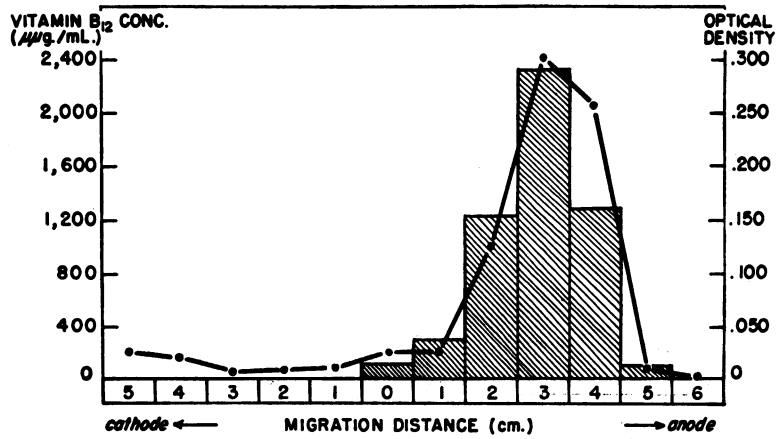

FIG. 4. DISTRIBUTION OF BOUND (NONDIALYZABLE) B_{12}* AMONG FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIA SEROMUCOID SEPARATED BY STARCH GEL ELECTROPHORESIS AT pH 4.5

The concentration of B_{12}* plotted on the left ordinate is represented by the ruled boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

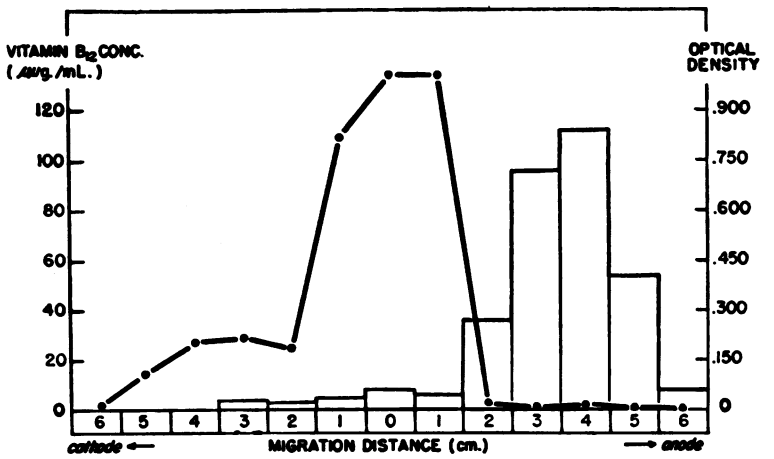

FIG. 5. NATIVE VITAMIN B_{12} CONCENTRATION OF PROTEIN FRACTIONS OF NORMAL SERUM SEPARATED BY STARCH GEL ELECTROPHORESIS AT pH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

bound and was equal to an average of 78 per cent ± 13, range, 57 to 94 per cent.

The distribution of vitamin B_{12} in a CML serum (Figure 7) is representative of a group of four such sera. As in the normal, the protein concentration of the A-2 to A-6 segments was negligible, while the native vitamin B_{12} was virtually all found anodally with peak concentrations at A-3 to A-5 segments. The distribution of the native vitamin and of the nondialyzable B_{12}* (Figure 8) is representative of a group of three such sera. The native vitamin had the same anodal

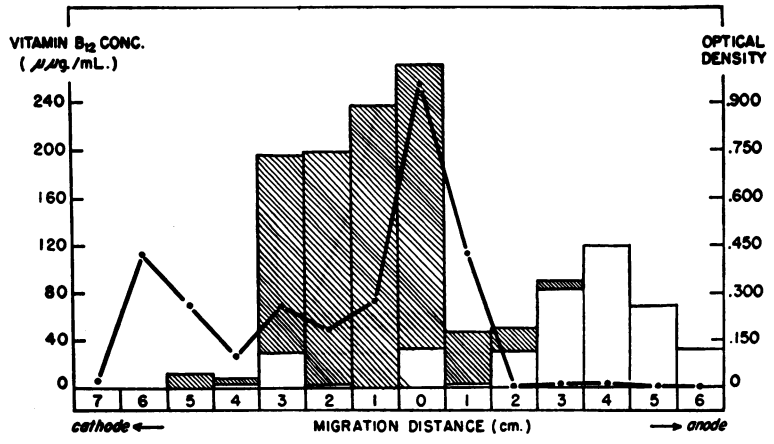

FIG. 6. DISTRIBUTION OF NATIVE VITAMIN B_{12} AND OF BOUND (NONDIALYZABLE) B_{12}* AMONG THE PROTEINS OF NORMAL SERUM SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT pH 4.5

Normal serum and an aliquot of the same serum containing bound (nondialyzable) B_{12}* were electrophoresed on the same starch block. The native vitamin shown by the open boxes was measured by *Euglena gracilis* assay while the bound radiovitamin, shown by the ruled boxes, was measured by counting the radioactivity in each segment. The solid dots ●——● refer to protein concentration.

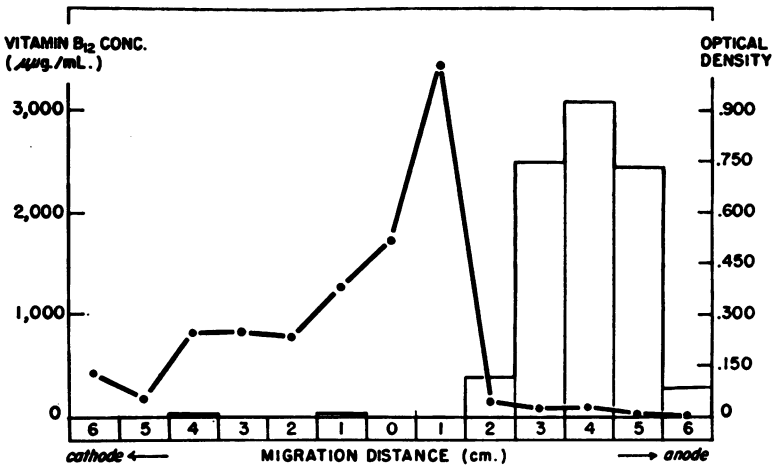

FIG. 7. NATIVE VITAMIN B_{12} CONCENTRATION OF PROTEIN FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIC SERUM SEPARATED BY STARCH GEL ELECTROPHORESIS AT pH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

distribution as described above. However, in contrast to the normal, a large amount of the added B_{12}* (2,200 to 3,973 μμg. per ml.) was bound by the A-2 to A-6 segments. The binding of the radiovitamin by these anodal segments was equal to 48 to 78 per cent of the total serum binding of B_{12}*. The per cent saturation of the B_{12}BP located at A-2 to A-6 segments ranged from 35 to 75 per cent, as calculated by the method outlined above.

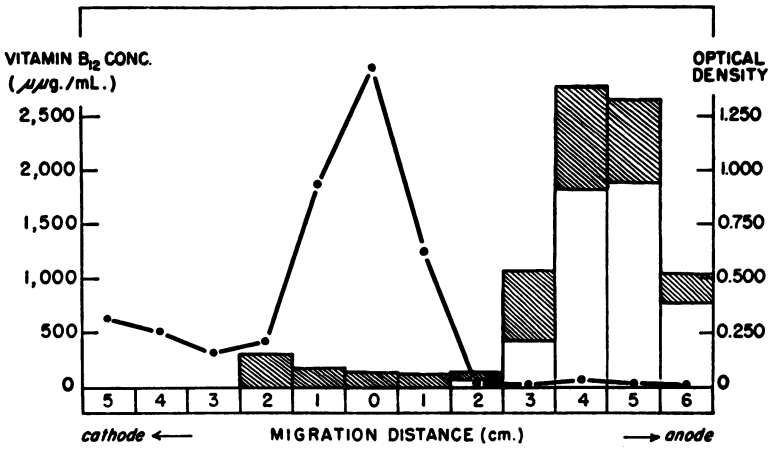

FIG. 8. DISTRIBUTION OF NATIVE VITAMIN B_{12} AND OF BOUND (NONDIALYZABLE) B_{12}* AMONG THE PROTEINS OF CHRONIC MYELOGENOUS LEUKEMIC (CML) SERUM SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT pH 4.5

CML serum and an aliquot of the same serum containing bound (nondialyzable) B_{12}* were electrophoresed on the same starch block. The native vitamin shown by the open boxes was measured by *Euglena gracilis* assay while the bound radiovitamin shown by the ruled boxes was measured by counting the radioactivity in each segment. Note the change in scale of vitamin B_{12} concentration plotted on the left ordinate from that of Figure 6. The solid dots ●——● refer to protein concentration.

DISCUSSION

A large fraction of the native vitamin B_{12} of normal serum was found in the seromucoid. Cohn Fraction VI, thought to be similar to the seromucoid in composition (19), contained little of the vitamin. In Method VI of Cohn and associates, the major protein fractions of plasma are isolated by a series of precipitations which depend on changes in pH, ionic strength and ethanol concentration (14). Some coprecipitation of the seromucoid routinely occurs when it is prepared from serum by precipitation of the serum proteins with sulfosalicylic or perchloric acid (10), and similarly it may be presumed that coprecipitation of the B_{12}BP with each Cohn plasma fraction may account for the absence of selective concentration of the vitamin in Fraction VI.

Since the great bulk of serum proteins have isoelectric points of 4.5 or higher, they either remained at the origin or migrated cathodally at this pH. However, due to the more acidic isoelectric point of the B_{12}BP, it migrated anodally with the other proteins of the seromucoid. The native vitamin B_{12} in serum or seromucoid preparations was usually distributed over a relatively wide anodal area of the starch block, with peak concentrations found in a 2 to 3 cm. width. Trailing of the protein and/or diffusion of the B_{12}BP during the electrophoretic run may account for the wide distribution of the vitamin and does not necessarily indicate the presence of more than one binding protein. The anodal distribution of vitamin B_{12} was simliar to, but not identical with, that of the main protein and sialic acid peaks of the seromucoid or its chief constituent, the acidic α_1-glycoprotein (19, 21). Despite their somewhat similar mobility, the B_{12}BP is probably not the acidic α_1-glycoprotein, since *1*) the B_{12}BP was not selectively precipitated with Cohn Fraction VI, whereas the acidic α_1-glycoprotein has been found only in this fraction (19); and *2*) Fahey, McCoy and Goulian fractionated serum on a diethylaminoethyl cellulose ion-exchange column and found that the B_{12}BP was eluted off before the acidic α_1-glycoprotein (22). A small amount of the native vitamin B_{12} of normal serum (10 per cent) was found scattered among cathodally moving proteins. It is impossible to know whether this represents technical error in the measuring of small amounts of the vitamin or another B_{12}BP.

The B_{12}BP of CML serum had properties similar to the normal B_{12}BP since *1*) it was found in the seromucoid, and *2*) had anodal mobility when electrophoresed on starch gel at pH 4.5. Mendelsohn, Watkin, Horbett and Fahey found that both the normal and CML B_{12}BP behaved similarly when serum was chromatographed on a diethylaminoethyl cellulose ion-exchange column (23). These chemical similarities between the normal and the CML B_{12}BP strongly suggest that the increased concentrations of vitamin B_{12} uniformly found in CML serum in relapse is due to an increase in concentration of the normal B_{12}BP rather than to an abnormal protein. Despite the tremendous increases in concentration of the B_{12}BP in CML sera (amounting to as much as 100-fold in one patient we have studied), only slight increases in the concentration of the total seromucoid fraction have been reported in this disease (24). However, the concentration of B_{12}BP in normal serum is probably minute as compared to that of the total seromucoid. Thus, the B_{12}BP of a typical normal serum, when completely saturated, can bind approximately 0.65 mμg. per ml. If the molecular weight of the B_{12}BP is similar to that of the orosomucoid, *i.e.*, 44,000 (25), and if one molecule of the B_{12}BP can bind one molecule of vitamin B_{12} (molecular weight, 1,343), the concentration of normal B_{12}BP would be equal to 2.20 μg. per 100 ml. serum. Even a 100-fold increase in concentration of B_{12}BP, to 0.22 mg. per 100 ml., would represent only an increase of 0.2 per cent over the normal concentration of the seromucoid, an increase well within the error of the chemical determination. It would appear that the elevations in seromucoid concentration found in CML are contributed by constituents of the seromucoid other than the B_{12}BP.

When additional B_{12}* was added to normal serum, the bound (nondialyzable) radiovitamin was almost all associated with proteins found at the origin or cathodal segments, whereas a very small amount of the added B_{12}* was bound by the anodally migrating B_{12}BP. This suggests that at the levels of vitamin B_{12} found in normal serum, the B_{12}BP approaches saturation and, therefore, the addition of further vitamin results

in the binding of the vitamin to nonacidic proteins. In CML serum on the other hand, there appears to be a commensurate increase of the $B_{12}BP$ in association with the increased level of vitamin B_{12}, so that although the relative saturation of the former remains approximately normal, the absolute binding capacity for added vitamin B_{12} is greatly increased.

The $B_{12}BP$ has chemical properties similar to those of the acidic α_1-glycoprotein and the α_2-glycoprotein isolated from Cohn Fraction VI (19, 26). Thus, they are all relatively heat resistant, are all soluble in sulfosalicylic or perchloric acid, are all precipitated by phosphotungstate in 2 N HCl and all have acidic isoelectric points as compared with other serum proteins (11, 12, 19, 21, 26, 27). The acidic α_1-glycoprotein (19) and the α_2-glycoprotein (26) are mucoid glycoproteins, *i.e.*, proteins containing significant amounts of firmly bound hexosamine (28). This would suggest that the $B_{12}BP$ may be a mucoid glycoprotein, although direct confirmation of such a structure must await its isolation in pure form. It is of interest that intrinsic factor (29, 30) and erythropoietin (31) are probably also mucoid glycoproteins. The available evidence suggests that the $B_{12}BP$ is not, however, identical with either of these substances. Thus, intrinsic factor has a differing electrophoretic mobility at pH 8.6 (32) and its binding sites for vitamin B_{12} are relatively heat labile (33). Recently, we have found a vitamin B_{12}-binding substance in CML urine which resembles that found in CML serum (34). This urinary material, when given orally to patients with pernicious anemia in amounts sufficient to bind an oral dose of B_{12}*, had no intrinsic factor activity. Also, increased erythropoietin levels have been reported in such diseases as chronic lymphatic leukemia (35), carcinoma of the cervix (35), secondary polycythemia (36) and hypoplastic anemias (37), whereas vitamin B_{12} levels are not particularly increased in these conditions.

It seems reasonable to suppose that the $B_{12}BP$ functions as a transport protein for vitamin B_{12}. The seromucoid fraction, representing a readily accessible, easily prepared, and easily concentrated source of the $B_{12}BP$, should aid in experiments designed to elucidate the role played by this protein in vitamin B_{12} metabolism.

SUMMARY

1. A method for the separation of the vitamin B_{12}-binding protein ($B_{12}BP$) of normal and chronic myelogenous leukemia serum by electrophoresis on starch gel at pH 4.5 has been described.

2. The $B_{12}BP$ of normal serum has been identified as a constituent of the seromucoid fraction of serum. It is a protein with an electrophoretic mobility at pH 4.5 similar to that of the acidic α_1-glycoprotein, the most acidic protein found in serum. However, it was not selectively precipitated with Cohn Fraction VI.

3. The $B_{12}BP$ of chronic myelogenous leukemia serum had properties similar to the normal $B_{12}BP$, suggesting that the great increase in vitamin B_{12} levels found in chronic myelogenous leukemia sera results from an increased concentration of the normal $B_{12}BP$ rather than from an abnormal protein.

4. Only a very small fraction of the cobalt^{60}-labeled vitamin B_{12} bound by normal serum as determined by dialysis was bound to the $B_{12}BP$. In contrast to the normal, the largest fraction of the cobalt^{60}-labeled vitamin B_{12} bound by chronic myelogenous leukemia serum was bound by the $B_{12}BP$.

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