Identification of the Vitamin B₁₂-Binding Protein in the Serum of Normals and of Patients with Chronic Myelocytic Leukemia

By ROBERT S. MENDELSOHN, DONALD M. WATKIN, ANN P. HORBETT AND JOHN L. FAHEY

RECENT STUDIES have demonstrated that virtually all the vitamin B₁₂ in normal serum is bound to serum proteins.¹ In chronic myelocytic leukemia the serum B₁₂ concentration may be increased from 2 to 30 times normal levels.²⁻⁴ At these increased levels, all or almost all of the B₁₂ is protein-bound. However, normal serum can bind completely only an amount of B₁₂ equal to two or three times the normal concentration.¹,³,⁵ Thus the increased serum B₁₂ concentration of chronic myelocytic leukemia can be attributed either to an increase in the normal B₁₂-binding protein or to the presence in the serum of components normally absent.

These studies were undertaken to characterize the B₁₂-binding protein of normal serum and of serum in chronic myelocytic leukemia. The present studies were conducted entirely with serum containing B₁₂ bound in vivo to the serum proteins. In vitro B₁₂ addition was not employed in order to avoid any artifacts attributable to overloading the normal B₁₂-binding system or to contaminating materials.

The recent introduction by Peterson and Sober of chromatographic procedures for protein fractionation has made possible new approaches to the separation and characterization of serum proteins.⁶⁻⁷ Normal and pathologic sera have been studied by Fahey, McCoy and Goulian,⁸ employing a modified anion-exchange cellulose chromatographic procedure. This chromatographic procedure and electrophoretic procedures, employed individually and in sequence, have been used in this study to characterize the B₁₂-binding protein in the serum of normal subjects and of patients with chronic myelocytic leukemia.

Microbiologic assay was used to identify the B₁₂. The results suggest that an alpha-1 globulin binds B₁₂ in normal serum and that an increased concentration of this protein in the serum of patients with chronic myelocytic leukemia accounts for the elevated serum B₁₂ levels in this disease.

METHODS

A modification described in detail elsewhere⁹ of the U.S.P. method using Lactobacillus leichmannii, A.T.C.C. #7830, was utilized in measuring the B₁₂ concentrations in all sera and serum fractions. The mean and standard error of B₁₂ levels determined by this method in 31 normal subjects was 0.533± 0.030 in μg./ml. This mean level in normal subjects is similar to that reported from a study in which Euglena gracilis was the test organism. Two major advantages of the L. leichmannii over the E. gracilis method are the 20-hour incuba-
IDENTIFICATION OF THE VITAMIN B₁₂-BINDING PROTEIN

...tion time and the more complete recovery values obtained with the former. Although somewhat less specific than *E. gracilis* in that *L. Leichmannii* will respond to desoxyribose if purine bases are also present in the media, *L. leichmannii* require for growth 1000 times as much desoxyribose as B₁₂. This factor is therefore largely eliminated from serum by the dilution involved in the method. The convenience, accuracy and rapidity of this method compared to the *E. gracilis* assay suggest it as the method of choice in investigations of the type here presented.

Paper electrophoretic analyses and separations were carried out on 0.008-0.050 ml. of serum by standard procedures. Each serum was run in duplicate, one strip was stained with bromphenol blue, and the other was cut into 0.5 cm. sections which were then added to the assay media and the B₁₂ content measured.

Electrophoresis of 1.0-1.25 ml. serum samples was done on polyvinyl blocks by a modification of the method of Müller-Eberhard and Konkel. The blocks were divided into 0.5 or 1.0 cm. segments which were then eluted by displacement filtration. Aliquots of the eluate were assayed microbiologically for B₁₂. Protein determinations were carried out by standard biuret technics or, in very dilute solutions, by measurement of optical density at 2840 angstroms utilizing the barbiturate buffer as a blank. Each block segment containing B₁₂ was also characterized by analytical paper electrophoresis after concentration.

Serum protein chromatography was carried out by a technic described in detail elsewhere. After dialysis to equilibrium with the starting buffer, approximately 20. ml. of serum was introduced onto a column of diethylaminoethyl-cellulose and eluted with sodium phosphate buffer of gradually increasing molarity and hydrogen ion concentration. After the distribution of protein in the effluent was determined (optical density at 2800 angstroms) aliquots from the individual 4 or 8 ml. effluent fractions were combined into 22 pools for localization of B₁₂ activity. Aliquots from the effluent tubes containing B₁₂ activity were then combined into smaller pools for more precise delineation of B₁₂ distribution.

RESULTS

The results of these electrophoretic and chromatographic studies are presented graphically in figures 1-4. The serum levels and the recovery of B₁₂ after serum fractionation are recorded in table 1.

The distribution of B₁₂ after block electrophoresis of the sera of three normal subjects and two patients with chronic myelocytic leukemia is shown in figure 1. The highest concentration of B₁₂ in serum from both normal and leukemic subjects appears in the alpha-1 globulins. When electrophoresis was performed on the polyvinyl blocks, B₁₂ was sometimes detected in the gamma, beta, alpha 2, albumin or pre-albumin regions. The reason for these constant minor-sites of B₁₂ recovery is not known.

The distribution of B₁₂ in proteins separated by paper electrophoresis of sera of two patients with chronic myelocytic leukemia is illustrated in figure 2. In each instance the greatest B₁₂ concentration is in the alpha-1 region. The small amount of B₁₂ present in normal serum precluded B₁₂ detection in fractions obtainable by paper electrophoresis of such serum.

The distribution of B₁₂ in proteins separated by column chromatography from the serum of two normal subjects and two patients with chronic myelocytic leukemia is shown in figure 3. The protein-bound B₁₂ was eluted in the same region, just prior to the site of ceruloplasmin elution, with both

*Diethylaminoethyl-cellulose was generously made available by Drs. E. A. Peterson and H. A. Sober of the Laboratory of Biochemistry, National Cancer Institute.*
the normal and pathologic seras. There was no evidence of a qualitative
difference in B12 distribution between the two types of sera. As would be
expected there was a marked quantitative difference in B12 obtained at the
elution site. This B12 was not dialyzable nor ultrafiltrable and is presumed
to be protein-bound.

The chromatogram effluent in the region of high B12 activity contains sev-
eral protein components. Therefore the proteins in this region of three of
the chromatograms were concentrated by ultrafiltration and then further
separated by polyvinyl block or paper electrophoresis. The results of micro-
biologic B12 assay, illustrated in figure 4, demonstrate that the B12 in this
region of both normal and chronic myelocytic leukemia serum chromatograms
is bound to alpha-1 globulins.

Fig. 1.—The distribution of B12 in proteins separated by block electrophoresis from the
sera of 3 normal subjects and 2 patients with chronic myelocytic leukemia. Note that the
B12 concentration scale for serum from leukemic patients is 10 times the scale for normal
serum. B12 concentration is indicated by the shaded blocks and protein distribution by
the area bounded by the solid line.
IDENTIFICATION OF THE VITAMIN B₁₂-BINDING PROTEIN

Fig. 2.—The distribution of B₁₂ in proteins separated by paper electrophoresis from the sera of 2 patients with chronic myelocytic leukemia. Protein distribution (solid line) is presented on an arbitrary scale determined by scanning the bromphenol-blue-dyed paper strips with an automatic recording densitometer. B₁₂ distribution is indicated by the shaded areas.

DISCUSSION

No qualitative difference could be demonstrated between the B₁₂-binding proteins of normal serum and of serum in chronic myelocytic leukemia, although the serum B₁₂ levels in the chronic myelocytic leukemia sera were 10–20 times greater than normal. Both electrophoretic and chromatographic technics of protein separation and characterization, employed separately and in sequence, indicated only one type of protein carrying in-vivo-bound B₁₂.

The B₁₂-binding protein was characterized electrophoretically as an alpha-1 globulin, consistent with previous reports. The spread among fractions obtained here by block electrophoresis was not evident on paper electrophoresis (cf. J.S., fig. 1 vs. fig. 2). Furthermore, dividing the paper electrophoretic strip into 0.5 cm. sections focuses on the primarily alpha-1 localization of the B₁₂-binding protein. The uniform curve of B₁₂ distribution, extending into the alpha-2 and albumin regions on occasion, would account for the B₁₂ distribution in these regions observed by others who divided strips into sections corresponding to the gross electrophoretic components.
The distribution of B₁₂ in proteins separated by column chromatography from the sera of 2 normal subjects and 2 patients with chronic myelocytic leukemia. Note that the B₁₂ concentration scale is 20 times that used for the sera of normals in the case of J. S. and 5 times normal in the case of R. L. B₁₂ concentration is indicated by the shaded blocks and protein distribution (D₂₅₀) by the area bounded by the solid line. In the R. L. chromatogram, the protein-bound hexose distribution of the latter portion of the chromatogram is represented by the dashed line. The location of ceruloplasmin is indicated by a small c above the appropriate peak near the end of the chromatogram.

The pattern of B₁₂-binding protein distribution on anion-exchange cellulose chromatography is also consistent with a single protein entity. This protein is characteristically eluted after the major albumin peak and also after a peak containing considerable amounts of alpha-2 globulin, but before the peaks of alpha-1 glycoprotein and ceruloplasmin elution. When the distribution of protein-bound carbohydrate (hexose) was determined in the chromatogram of a chronic myelocytic leukemia serum (fig. 3), it was evident that the B₁₂-binding protein was not associated with the major glycoprotein components of this region.

Not only was the same chromatographic distribution of protein-bound B₁₂ found in the serum of normal and chronic myelocytic leukemia subjects, but further examination of the B₁₂-containing chromatogram region demonstrated the association of B₁₂ with the alpha-1 component. This is consistent with the findings on electrophoretic examination of whole serum and suggests that the increased serum B₁₂-binding capacity in chronic myelocytic leukemia is due to an increased amount of the normal B₁₂-binding protein.

Although the results of the present work are interpreted as indicating that the high B₁₂ level in the serum of patients with chronic myelocytic leukemia is associated primarily with an increase in the level of the normal B₁₂-binding
IDENTIFICATION OF THE VITAMIN B₁₂-BINDING PROTEIN

**Fig. 4.** The distribution of B₁₂ among proteins of the chromatogram regions containing high levels of B₁₂. Paper electrophoresis was performed on the concentrated effluent from region 1340 to 1390 ml. of the J. S. chromatogram and from region 1250 to 1360 ml. of the R. L. chromatogram. Protein distribution on these strips is presented as in figure 2. Block electrophoresis was performed on concentrated effluent from region 1270 to 1380 ml. of the H. A. chromatogram, and the protein distribution (D₂₅₄) presented as a dotted solid line. B₁₂ distribution after electrophoresis is indicated in each instance by the shaded area.

protein, it is possible that the increased serum B₁₂-binding capacity might be due to an increase in the number of B₁₂-binding sites on the normal B₁₂-binding protein. However, there is no specific evidence to support such a postulate. This question arises because the B₁₂-binding protein is measured indirectly, by means of the associated B₅₂ activity. Direct measurement of the B₁₂-binding protein is not yet feasible. The amount of B₁₂-binding protein in normal serum is probably very small. Assuming that 1 molecule of B₁₂ (molecular weight = 1347) is bound to 1 molecule of binding protein (molecular weight about 150,000) and that the normal level of 0.51 milli-micrograms of B₁₂ represents 50 per cent of the total binding capacity, the total amount of B₁₂-binding protein present in 1 ml. of normal serum would be 0.11 micrograms. This represents 0.00016 per cent of the total serum protein and about 0.003 per cent of the alpha-1 component.

The site of origin of the B₁₂-binding protein is unknown. The possibility that the products of leukemic cell destruction contaminate the sera sufficiently to account for the elevated B₁₂ levels has not been ruled out. Neither has an hepatic source been established. Although serum B₁₂ levels may be
elevated in association with extensive hepatic disease\textsuperscript{11,13} the pathologic process causing the $B_{12}$ elevation in severe liver disease apparently differs from that operating in chronic myelocytic leukemia. Stevenson, in a preliminary report, observed\textsuperscript{14} that the plasma clearance of intravenously administered radioactive vitamin $B_{12}$ in severe liver disease was normal and did not resemble the prolonged clearance seen in chronic myelocytic leukemia.\textsuperscript{15} Also, the hepatic pathology seen in chronic myelocytic leukemia does not resemble that seen in the liver diseases associated with serum $B_{12}$ elevation.

The data presented here are compatible with the view that the serum $B_{12}$ level in chronic myelocytic leukemia is elevated due to abnormal metabolism of the $B_{12}$-binding protein. At the present time it is uncertain whether this is directly related to the myelocytic disorder or is a separate manifestation of the basic disease process.

**Summary**

Vitamin $B_{12}$-binding proteins in the serum of normal subjects and of patients with chronic myelocytic leukemia have been compared. The in-vivo-bound $B_{12}$ was utilized to identify the binding protein. Column protein chromatography and block and paper electrophoresis were employed individually and in combination to characterize the $B_{12}$-binding protein.

$B_{12}$ was found to be bound primarily to an alpha-1 globulin in both normal individuals and in patients with chronic myelocytic leukemia. No qualitative difference was found in these proteins.

The increased amounts of $B_{12}$-binding protein in the serum of patients with chronic myelocytic leukemia would seem to be attributable to abnormal metabolism of the same protein that binds $B_{12}$ in normal serum.

**Summario in Interlingua**

Esseva comparate le proteinas que liga vitamina $B_{12}$ in le sero de subjectos normal e de patientes con chronic leucemia myelocytic. Le vitamina $B_{12}$ que habeva essite ligate in vivo esseva utilitate pro identificar le proteina effectuante le ligation. Chromatographia proteinic a columnas e electrophorese a bloco e a papiro esseva emplante individualmente e in combination pro characterisar le proteina que liga vitamina $B_{12}$.

Esseva trovate que vitamina $B_{12}$ es ligate primarimente a un globulina alpha-1, tanto in individuos normal como etiam in patientes con chronic leucemia myelocytic. Nulle differentia qualitative esseva trovate inter le proteinas in le duo casos.

Le quantitate de proteina ligante vitamina $B_{12}$ esseva plus alte in le sero de patientes con chronic leucemia myelocytic. Isto es probablemente atribuibile a un anormalitate metabolic del mesme proteina que etiam liga vitamina $B_{12}$ in sero normal.

**References**

IDENTIFICATION OF THE VITAMIN B₁₂-BINDING PROTEIN

Identification of the Vitamin B₁₂-Binding Protein in the Serum of Normals and of Patients with Chronic Myelocytic Leukemia

ROBERT S. MENDELSON, DONALD M. WATKIN, ANN P. HORBETT and JOHN L. FAHEY