THE PLASMA VITAMIN B_{12} BINDING SUBSTANCE: I. ITS DETECTION IN THE SEROMUCOID FRACTION OF PLASMA FROM NORMAL SUBJECTS AND PATIENTS WITH CHRONIC MYELOCYTIC LEUKEMIA *

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It is well known that the striking increases in serum vitamin B_{12} concentrations seen in patients with chronic myelocytic leukemia (CML) are associated with an increased plasma binding capacity for B_{12}. The latter has been demonstrated *in vitro* (1–4) using microbiologic assay or dialysis. *In vivo* studies in patients with CML have demonstrated a delay in the plasma disappearance of either an intravenously (5, 6) or orally (7) administered dose of $Co^{58}B_{12}$ and would appear to confirm the *in vitro* findings.

The association of a high serum B_{12} with a high plasma binding capacity has stimulated investigation into the nature of the plasma binding substance. Previous attempts to characterize the B_{12} binding substance have been primarily concerned with defining its electrophoretic mobility, and thus far actual isolation and chemical characterization have not been achieved (1, 8–10). The fact that B_{12} apparently exists in a macromolecular complex at several stages of its metabolism, including gastrointestinal absorption (11), plasma transport (1) and liver storage (12), led us to consider whether or not a similar type of substance binds B_{12} in each case. Since intrinsic factor, which plays a key role in the gastrointestinal absorption of B_{12}, is thought to be a glycoprotein (13), we undertook the present studies to determine whether or not the B_{12} binding substance of plasma is also a glycoprotein.

Co^{58} vitamin B_{12} was used to label the serum B_{12} binding protein, either *in vitro* or *in vivo*. Glycoproteins were isolated according to modifications of the methods of Winzler, Weimer, and co-workers (14, 15). The results obtained with plasma from patients with CML were compared to those obtained with plasmas from subjects having normal serum B_{12} concentrations. The latter were either normal bank blood donors or patients with malignant disease and normal serum B_{12} concentrations, and in the text are referred to as "control" subjects.

METHODS

Microbiologic assay. A modification described in detail elsewhere (16) of the U. S. P. method using *Lactobacillus leichmannii,* American Type Culture Collection No. 7830, was utilized in measuring the B_{12} concentrations of sera and serum fractions. The mean and standard error of B_{12} levels determined by this method in 31 normal subjects was 0.533 ± 0.030 mμg. per ml.[1]

Radioactivity assay. The $Co^{58}B_{12}$ had an original specific activity of 2.38 μc. per μg.[2] One mμg. of this material gave approximately 1,900 cpm above a background of 180 cpm. All counting was done in a well-type scintillation counter. Samples were counted for a period of time sufficient to give a counting error of less than 3 per cent.

Biologic activity of the labeled vitamin was confirmed by *L. leichmannii* assay and found to be 93 per cent of that given by the manufacturer. Suitable dilutions were made with the buffer described below.

Perchloric-phosphotungstic acid precipitation. The *in vitro* studies were done as follows. To 3 ml. aliquots of plasmas in dialysis bags was added 1 ml. of solutions containing sufficient $Co^{58}B_{12}$ to provide concentrations ranging from 0.1 to 100 mμg. of $Co^{58}B_{12}$ per ml. of plasma. The bags were tied, and the contents mixed by inversion and incubated at room temperature for one hour. Each bag was placed in a 125 ml. Erlenmeyer flask and 100 ml. of buffer was added.[3] Dialysis was performed for a total

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[1] The abbreviation mμg. as used in this paper means 10^{-9} gram.

[2] Purchased from Merck and Co., Inc., Rahway, N. J.

[3] The buffer used was 0.15 M sodium phosphate adjusted to pH 7.3 and diluted with nine parts of 0.85 per cent sodium chloride.

of 72 hours at 4° C. with replacements of the buffer at 24 and 48 hours. The contents of each dialysis bag was transferred to a 4 ml. counting vial and counted against a standard. Each vial was then emptied into a 50 ml. beaker to which was added 18 ml. of 0.85 per cent saline. The saline facilitated rinsing of the vial and served to dilute out the glycoproteins, thus minimizing loss by coprecipitation with the perchloric acid precipitable proteins. Ten ml. of 1.8 M perchloric acid was slowly added to each beaker with mixing and five minutes later the contents filtered through Whatman No. 12 folded filter paper. The dried filter paper with its precipitate was folded and transferred to a 4 ml. counting vial. The clear perchloric acid filtrate was collected in a 50 ml. centrifuge tube, 5.6 ml. of 5 per cent phosphotungstic acid in 2 N HCl was added and the contents were mixed by inversion. Five minutes later this mixture was centrifuged at 3,000 rpm for 30 minutes. The clear supernatant was decanted and a 4 ml. aliquot assayed for radioactivity. The phosphotungstic acid precipitate was redissolved in 4 ml. of 1 N NaOH and also assayed for radioactivity.

Calculations were made as follows:

A. per cent bound to whole plasma =

$$\frac{\text{cpm whole plasma after dialysis}}{\text{cpm added}} \times 100;$$

B. per cent bound to a protein fraction =

$$\frac{\text{cpm of that fraction}}{\text{cpm whole plasma after dialysis}} \times 100;$$

C. mμg. bound to a protein fraction =

$$\frac{(\text{m}\mu\text{g. } Co^{58}B_{12} \text{ originally added})\ (A)}{10{,}000}\ (B);$$

D. per cent recovery =

$$\frac{\text{sum of cpm from each protein fraction}}{\text{cpm whole plasma after dialysis}} \times 100.$$

"MP-1" isolation. Plasma was fractionated according to a modification of the method of Weimer, Mehl and Winzler (15). In the *in vitro* studies, 1 ml. of a $Co^{58}B_{12}$ solution containing 20 mμg. per ml. was added to 20 ml. of plasma (1 mμg. $Co^{58}B_{12}$ per ml. plasma) and the mixture incubated at 4° C. for one hour. In the *in vivo* studies, endogenous labeling of plasma with radioactive $Co^{58}B_{12}$ was carried out as described in the section on *in vivo labeling.*

To one volume of plasma, previously labeled with $Co^{58}B_{12}$ in either of the above manners, 1.2 volumes of saturated $(NH_4)_2SO_4$ were slowly added with mixing. After this mixture had stood at 4° C. for 16 hours, a precipitate ("Ppt. A") was separated by centrifugation for 30 minutes at 2,000 rpm. The supernatant was decanted, reduced to pH 4.75 with 1 N HCl and allowed to stand for 16 hours at 4° C. A precipitate ("Ppt. B") was separated by centrifugation as before and the supernatant was reduced to pH 3.7 with 1 N HCl. After this supernatant had stood for another 16 hours at 4° C., a precipitate ("Ppt. C") was again separated by centrifugation. The supernatant was saturated with $(NH_4)_2SO_4$ and the "MP-1" fraction thereby precipitated. This precipitation was facilitated by transferring the pH 3.7 supernatant to a dialysis sac which was then suspended in a beaker containing saturated $(NH_4)_2SO_4$ with an excess of the solid salt. After the sac had remained suspended in solution for 72 hours at 4° C., a precipitate ("MP-1") formed in the sac and subsequently was separated by centrifugation. Precipitates A and B, which contained the bulk of the added radioactivity, were redissolved in water and treated with perchloric and phosphotungstic acid in a manner similar to that described above. The resulting precipitates were assayed for radioactivity. Calculations were performed as described above.

In vivo labeling. After overnight fasts, patients were given 0.57 μg. of $Co^{58}B_{12}$ by mouth and fed one hour later. Blood samples were withdrawn into heparinized syringes at three hour intervals up to 12 hours, then at 24 and 36 hours. After assaying the radioactivity in 4 ml. of whole plasma from each sample, 10 to 20 ml. of plasma from the sample having the highest cpm was fractionated according to the previously described methods. The cpm in each fraction were expressed as the per cent of cpm present in whole plasma.

RESULTS

A. Perchloric-phosphotungstic acid method

1. In vitro addition of $Co^{58}B_{12}$. The results obtained by perchloric-phosphotungstic acid precipitation of plasmas (to which 0.120 mμg. $Co^{58}B_{12}$ per ml. had been added) obtained from five subjects with normal serum B_{12} concentrations and five patients with CML and increased B_{12} concentrations are listed in Table I. The phosphotungstic acid precipitates contained a mean of 78 per cent of added $Co^{58}B_{12}$ for the control group and a mean of 77 per cent for the CML group. Perchloric acid precipitates contained a mean of 17 per cent and 11 per cent for the two groups, respectively. Total recovery ranged from 80 to 113 per cent of the added material. The dialysates contained no radioactivity indicating practically complete binding at this concentration.

2. Binding of $Co^{58}Cl_2$. In order to exclude the possibility that radioactivity present in each fraction represented free cobalt, the binding characteristics of $Co^{58}Cl_2$ were studied.[4]

When $Co^{58}Cl_2$ equivalent to the amount of Co^{58} present in 1 mμg. of $Co^{58}B_{12}$ was added to plasma, and a procedure otherwise identical to that used

[4] $Co^{58}Cl_2$ obtained from Oak Ridge National Laboratory was carrier-free and had an original concentration of 1.26 mc. per ml.

TABLE I

In vitro addition of 0.600 mμg. $Co^{58}B_{12}$ per 5 ml. of plasma (0.120 mμg. per ml.) followed by perchloric-phosphotungstic acid precipitation

Subject	Diagnosis	Serum B_{12}	Per cent of added $Co^{58}B_{12}$		Recovery
			In perchloric acid Ppt.	In phosphotungstic acid Ppt.	
		mμg./ml.			
I. Normal B_{12}					
A	Normal	0.461	16	79	95
B	Normal	0.265	13	71	84
J. Q.	Partial resection of ileum	0.299	18	95	113
O. C.	Prostatic carcinoma	0.353	17	80	97
D. S.	Embryonal rhabdomyosarcoma	0.497	20	66	86
Mean			17	78	95
II. High B_{12}					
L. R.	Chronic myelocytic leukemia	1.200	6	83	89
M. B.	Chronic myelocytic leukemia	2.681	7	78	85
O. F.	Chronic myelocytic leukemia	7.825	8	72	80
O. F.	Chronic myelocytic leukemia	7.983	13	73	86
J. S.	Chronic myelocytic leukemia	13.475	23	81	104
Mean			11	77	89

for $Co^{58}B_{12}$ was followed, the recoveries of added material were as follows:

	Per cent of cpm added
Whole plasma (after dialysis)	22.2
Phosphotungstic acid precipitate	1.89
Perchloric acid precipitate	8.84

thus demonstrating that with equal amounts of Co^{58}, at the concentrations indicated, that which is added as $Co^{58}Cl_2$ is not as effectively bound to whole plasma as is B_{12} (22 vs. 95 per cent), and that in contrast to B_{12}, most of the Co^{58} is found in the perchloric acid precipitate. The fact that the sum of the material recovered in the two precipitates does not equal that which was present in the dialyzed plasma may indicate that perchloric acid had freed some of the bound Co^{58} in the case of $Co^{58}Cl_2$.

3. Precipitation in the presence of an excess of nonradioactive B_{12}. Because of the possibility that the appearance of B_{12} in the phosphotungstic acid precipitate of a perchloric acid filtrate represented an artifact introduced by the action of perchloric acid on the original binding protein, by causing release of the bound vitamin, radioactive B_{12} was allowed to bind with whole plasma, and the subsequent perchloric and phosphotungstic acid precipitations were carried out in the presence of an excess of nonradioactive B_{12}.

Two 3 ml. aliquots of plasma were combined with 0.1 mμg. of $Co^{58}B_{12}$ per ml. plasma. Both were processed in the same manner (see Methods), but for the fact that following dialysis and prior to the addition of perchloric acid, 100 mμg. of nonradioactive B_{12} per ml. of plasma was added to Sample "A." Recovery of radioactivity in the phosphotungstic acid precipitate was not influenced by the presence of an excess of nonradioactive B_{12}.

	$Co^{58}B_{12}$ mμg./ml. plasma	Nonradioactive B_{12} mμg./ml. plasma	Recovery of $Co^{58}B_{12}$: Perchloric acid Ppt. %	Recovery of $Co^{58}B_{12}$: Phosphotungstic acid Ppt. %
A	0.1	100	16.7	74.4
B	0.1	0	12.0	71.4

4. In vitro addition of increasing amounts of $Co^{58}B_{12}$ to normal and CML plasma. The preceding studies suggest that most of the $Co^{58}B_{12}$ added to plasma at a concentration of 0.120 mμg. per ml. can be recovered in the phosphotungstic acid precipitate. These experiments compare the results obtained with the addition of increasing amounts of B_{12} to plasmas of five normal bank blood donors to that obtained on the plasma from eight patients with CML (Table II and Figures 1 and 2).

a. Bound to whole plasma (Table II). For all subjects studied, virtually all of the added $Co^{58}B_{12}$

TABLE II

In vitro addition of $Co^{58}B_{12}$; per cent bound to whole plasma and absolute amount in perchloric and phosphotungstic acid precipitates *

Subject	Serum B_{12}	WBC	Per cent bound to whole plasma: $Co^{58}B_{12}$ added (*mμg./ml.* plasma) 0.1	1.0	10.0	100.0	mμg. bound to protein fractions: $Co^{58}B_{12}$ added (*mμg./ml.* plasma) 0.1 Perch.	0.1 Phosph.	1.0 Perch.	1.0 Phosph.	10.0 Perch.	10.0 Phosph.	100.0 Perch.	100.0 Phosph.
	mμg./ml.													
Normals														
A	0.461		95	93	18	5	0.012	0.079	0.110	0.745	0.370	1.26	3.18	2.54
B	0.510		92	99	18	4	0.019	0.073	0.186	0.724	0.448	1.29	1.81	1.83
C	0.320			97	16	4			0.157	0.725	0.368	1.16	1.51	2.02
D	0.400			93		5			0.123	0.766			1.82	3.30
E	0.380			95	12				0.183	0.699	0.299	0.83		
Mean				95	16	5			0.110	0.732	0.288	1.135	2.08	2.42
CML														
D. L.	9.725	13,600		100	89	24			0.070	0.840	0.690	6.18	2.96	18.60
J. L.	8.700	5,300		92	50	8			0.097	0.764	0.640	4.06	1.91	5.03
O. F.	7.937	100,000		97	98	15			0.074	0.877	1.086	8.56	2.64	11.62
L. E.	7.800	20,000			86	12					0.907	7.33	2.09	9.66
J. S.	7.300	14,600			44	7					0.565	3.56	1.55	4.95
R. F.	2.033	52,000		95	22	4			0.090	0.770	0.420	1.67	1.37	2.32
M. B.	2.000	81,600			18	4					0.111	0.69	1.05	2.37
P. P.	1.875	7,200		88	10	3			0.160	0.720	0.240	0.73	1.01	1.42

* Per cent bound = cpm after dialysis/cpm added; perch. = perchloric acid precipitate; phosph. = phosphotungstic acid precipitate (seromucoid).

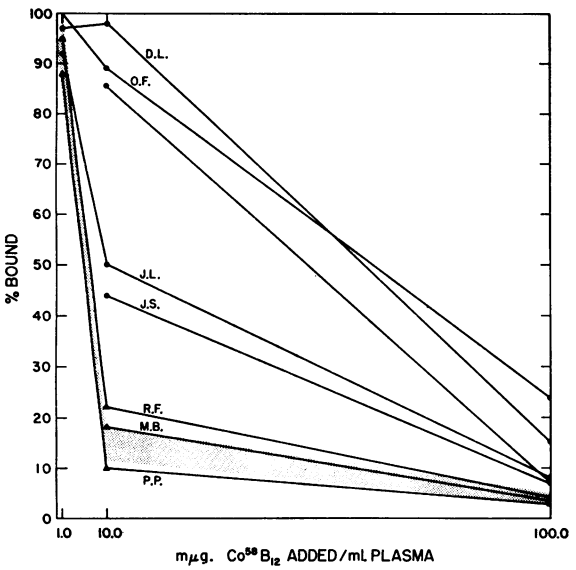

FIG. 1. PER CENT OF $Co^{58}B_{12}$ BOUND TO WHOLE PLASMA FOLLOWING *IN VITRO* ADDITION OF 1.0, 10.0 AND 100 MμG. PER ML. OF PLASMA AND DIALYSIS

The shaded area indicates the range in subjects with a normal serum B_{12}. Closed circles represent CML subjects with a serum B_{12} greater than 2.1 mμg. per ml.; closed triangles represent CML subjects with a serum B_{12} less than 2.1 mμg. per ml.

was nondialyzable (83 to 100 per cent) when added at a concentration of 0.1 or 1 mμg. per ml. plasma; with the addition of larger amounts, the per cent bound fell off sharply in the normal B_{12} concentration group, but remained high in those CML patients with a high serum B_{12}. At a concentration of 10 mμg. of added $Co^{58}B_{12}$ per ml. plasma, five normal subjects bound a mean of 16 per cent (range = 12 to 18) of the added material, whereas the eight patients with CML and a high B_{12} bound a mean of 47 per cent (range = 10 to 89). When 100 mμg. of $Co^{58}B_{12}$ was added per ml. of plasma, normals bound a mean of 4.5 per cent (range = 4 to 5) and CML patients bound 9.3 per cent (range = 3 to 24). Within the CML group those subjects with a higher B_{12} concentration bound a greater per cent of the added material than did those with a less striking elevation. Three subjects, R. F., M. B. and P. P., had a pattern similar to that of the normals. They had serum B_{12} concentrations in the 2.0 mμg. per ml. range. The other five CML subjects with increased binding had serum B_{12} concentrations in the 7.0 mμg. per ml. range.

b. Bound to phosphotungstic acid precipitate (Table II). The increased binding capacity for added $Co^{58}B_{12}$ by plasma from patients with CML was found to be associated with an increased recovery of radioactive material in the phosphotungstic acid precipitate. In the five subjects with CML whose plasma bound a greater than normal amount of $Co^{58}B_{12}$, the absolute amount of $Co^{58}B_{12}$ found in the phosphotungstic acid precipitate when 10 mμg. of $Co^{58}B_{12}$ per ml. of plasma was added, had a mean of 5.94 mμg. per ml. plasma, whereas normals at the same concentration of added $Co^{58}B_{12}$ had a mean of 1.14 mμg. per ml. in their phosphotungstic acid precipitate. At a concentration of 100 mμg. of $Co^{58}B_{12}$ per ml. of plasma, the five CML subjects had a mean of 10.01 mμg. per ml. in their phosphotungstic acid precipitate, whereas the normals had a mean of only 2.42 mμg. per ml. The three previously mentioned CML subjects in partial remission had a normal amount of $Co^{58}B_{12}$ bound in their phosphotungstic acid precipitate.

Inspection of Figure 2 demonstrates that in

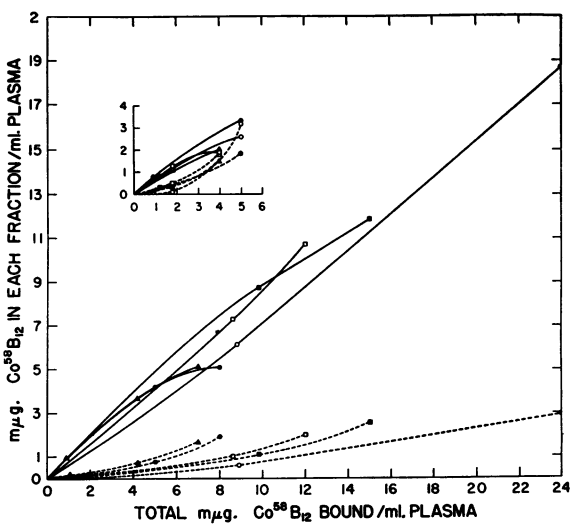

FIG. 2. THE ABSOLUTE AMOUNT OF $Co^{58}B_{12}$ BOUND TO PHOSPHOTUNGSTIC ACID PRECIPITATE, I.E., SEROMUCOID (SOLID LINE CURVES) AND PERCHLORIC ACID PRECIPITATE (INTERRUPTED LINE CURVES)

The main graph is on sera from five subjects with CML. The insert (same scale) is on five sera with a normal B_{12} concentration. "Total mμg. $Co^{58}B_{12}$ bound" is that present following dialysis. The three points on each subject's curve correspond to concentrations of 1, 10 and 100 mμg. per ml. plasma of added $Co^{58}B_{12}$.

TABLE III

*The per cent of $Co^{58}B_{12}$ recovered in various protein fractions, using the procedure for isolation of "MP–1"**

		MP–1 fractionation					Distribution of $Co^{58}B_{12}$ in subfractions of A and B					
		Distribution of $Co^{58}B_{12}$ in $(NH_4SO_4)_2$ fractions					Ppt. A			Ppt. B		
	Serum B_{12}	Ppt. A	Ppt. B	Ppt. C	MP–1	Total recovery	Perch.	Phosph.	Total recovery	Perch.	Phosph.	Total recovery
	mμg./ml.	%	%	%	%	%	%	%	%	%	%	%
Normals												
A	0.710	70.70	3.88	0.36	5.72	80.66	13.66	89.20	102.90	5.99	87.47	93.46
B	0.580	71.88	4.15	0.34	0.36	76.73	10.96	100.81	111.77	10.90	86.10	97.00
C	0.603	74.89	5.34	1.07	8.18	89.48	9.83	87.67	97.50	5.36	80.96	86.32
D	0.440	64.11	5.18	0.25	8.39	77.93	9.61	94.72	104.33	2.72	55.16	57.88
E	0.640	65.03	4.59	4.96	4.84	79.42	23.11	60.29	83.40	4.20	89.44	93.64
F	0.950	56.01	4.30	1.51	4.62	66.44	31.14	87.55	118.69	8.00	83.00	91.00
G	0.328	69.84	5.91	1.82	10.61	88.18	29.55	68.91	98.46	4.52	62.73	67.25
H	0.425	75.48	3.37	1.52	6.72	87.09	23.19	52.96	76.15	6.37	92.58	98.95
Mean		68.49	4.59	1.48	6.18	80.74	18.88	80.26	99.15	6.01	79.68	85.68
CML												
D. L.	17.150	26.28	23.55	2.21	18.90	70.94	8.04	83.15	91.19	4.58	76.96	81.54
J. L.	8.700	30.04	19.16	17.58	5.01	71.79	6.47	66.37	72.84	6.14	83.95	90.09
J. S.	7.300	51.03	15.72	1.52	9.28	77.65	8.21	78.46	86.67	6.97	73.58	80.55
R. F.	1.975	56.85	17.70	1.18	11.22	85.77	9.62	70.02	79.64	5.66	83.43	89.09
M. B.	1.550	65.89	9.24	0.57	9.56	85.26	15.29	75.07	90.36	5.17	63.22	68.39
P. P.	1.187	67.86	9.80	0.78	7.89	86.33	14.63	83.56	98.19	9.57	73.17	82.74
Mean		49.65	15.86	3.97	10.31	79.64	10.38	76.11	86.49	6.35	75.72	82.09

* $Co^{58}B_{12}$ added *in vitro* (1 mμg. per ml.); ppts. A, B, C and MP–1, see methods; perch. = perchloric acid precipitate; phosph. = phosphotungstic acid precipitate.

plasma from subjects with a normal serum B_{12} concentration, when more than 2 mμg. of $Co^{58}B_{12}$ is bound per ml. of whole plasma, the per cent of total bound vitamin recovered in the phosphotungstic acid precipitate falls off, whereas that in the perchloric acid precipitate rises. In contrast, in none of the eight subjects with CML did the per cent of bound B_{12} present in the phosphotungstic acid precipitate decrease until bound B_{12} exceeded 4 mμg. per ml. of plasma, and in two, the per cent did not fall off until bound B_{12} exceeded 8 mμg. per ml. of plasma.

5. In vivo labeling. Plasma from two subjects, previously labeled *in vivo,* was fractionated according to the perchloric-phosphotungstic acid method. The results were as follows:

D. S. (embryonal rhabdomyosarcoma)

	Serum B_{12} = 0.478 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (10 ml.)	100	
Perchloric acid Ppt.	0	0
Phosphotungstic acid Ppt.	73	73

J. L. (CML)

	Serum B_{12} = 8.225 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	520	
Perchloric acid Ppt.	0	0
Phosphotungstic acid Ppt.	303	58.3

B. MP-1 fractionation

The fact that most of the radioactive B_{12} bound to plasma either *in vitro* or *in vivo* could be recovered in the phosphotungstic acid precipitate of a perchloric acid filtrate in both normals and CML's indicated that the major plasma B_{12} binding substance was present in the "seromucoid fraction" of plasma. It was, therefore, of interest to determine whether or not the substance was identical with the "MP-1" fraction of seromucoid obtained by Weimer, Mehl and Winzler (15).

Plasma from eight normal bank blood donors and plasma from six patients with CML were labeled *in vitro* with $Co^{58}B_{12}$ (1 mμg. per ml. plasma). The plasmas were fractionated according to a modification of the MP-1 isolation procedure of Winzler and the per cent recovery of added $Co^{58}B_{12}$ in each fraction was determined. The results are listed in Table III and are summarized as follows:

	No. of subjects	Ppt. A	Ppt. B	Ppt. C	MP-1	Total recovery
Normals	8	68.49	4.59	1.48	6.18	80.74
CML's	6	49.65	15.86	3.97	10.31	79.64

They indicate that MP-1 is not the major B_{12} binding substance in either normals or CML's. Most of the radioactivity was found in Ppts. A and B. Subsequent treatment of each of these with perchloric acid and phosphotungstic acid resulted in good recovery of radioactivity (82 to 99 per cent) in the phosphotungstic acid precipitate thus further demonstrating the "seromucoid" nature of the binding substance (Table III).

Using the same techniques, fractionation of plasma labeled *in vivo* was carried out in two subjects:

D. S. (embryonal rhabdomyosarcoma)

	Serum B_{12} = 0.478 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	200	
Ppt. A	120	60
Ppt. B	0	0
Ppt. C	0	0
MP-1	6	3
	Total	63

J. L. (CML)

	Serum B_{12} = 8.225 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	510	
Ppt. A	56	10.9
Ppt. B	156	30.6
Ppt. C	0	0
MP-1	197	38.6
	Total	80.1

DISCUSSION

It has been previously demonstrated that the phosphotungstic acid precipitate of a perchloric acid filtrate of plasma represents approximately 1.5 per cent of the total plasma protein and is rich in glycoproteins (14). This fraction is referred to as seromucoid (14).

Seromucoid is a heterogeneous material. Its major component was isolated in an electrophoretically homogeneous state by a series of ammonium sulfate precipitations by Weimer, Mehl and Winzler (15) and has been designated oroso-

mucoid or "MP-1." A second component has been demonstrated by electrophoresis and designated "M-2" (17). Experiments in progress suggest the presence of several further components (18).

The data presented in the present study suggest that the plasma B_{12} binding substance in both subjects with normal serum B_{12} concentrations and in CML patients with high serum B_{12} concentrations appears in the seromucoid fraction of plasma. That radioactivity represented the presence of B_{12} and not simply Co^{58} was demonstrated by the poor recovery of radioactivity in the seromucoid fraction when plasma was labeled with $Co^{58}Cl_2$. Because of the possibility that perchloric acid had stripped the $Co^{58}B_{12}$ from its original binding protein thus allowing it to bind secondarily to a glycoprotein, the perchloric acid precipitation was performed in the presence of a high concentration of nonradioactive B_{12}. This did not impair recovery of radioactivity in the phosphotungstic acid precipitate. The results of *in vivo* labeling further support the concept that the native substance is in the seromucoid fraction.

The addition of increasing amounts of $Co^{58}B_{12}$ brought out differences between normals and patients with CML when concentrations greater than 1 mμg. per ml. of plasma were reached. Thus, at 10 mμg. per ml. control plasma bound 16 per cent of added $Co^{58}B_{12}$, whereas plasma from CML patients with a high serum B_{12} bound a mean of 47 per cent. Studies by others (3, 4), also using dialysis methods, have demonstrated a comparable increase in the binding capacity for added B_{12} ("unsaturated binding capacity") in plasma from CML patients, though variations in the technique used probably account for differences in absolute values obtained by these authors.

With control plasma, when a concentration greater than approximately 2 mμg. of bound $Co^{58}B_{12}$ per ml. of plasma was reached, the per cent bound by the seromucoid fraction diminished whereas the per cent bound by the perchloric acid precipitate increased. This suggests relative saturation of the normal B_{12} binding substance at about the 2 mμg. per ml. level and subsequent secondary binding of additional B_{12} by perchloric acid precipitable protein. In two CML patients with high serum B_{12} concentrations, however, even at 10 mμg. of bound $Co^{58}B_{12}$ per ml. plasma, there was no decrease in the per cent bound to the seromucoid fraction.

More specific identification of the B_{12} binding material was attempted by using the scheme of Weimer, Mehl and Winzler (15) for the isolation of MP-1. These studies indicate that in sera from subjects with normal B_{12} concentrations the MP-1 fraction is not the major B_{12} binding substance. Instead, the bulk of radioactivity was found in Ppt. A (globulins) and could be separated from it by precipitation with perchloric and phosphotungstic acid. Plasma from CML patients, especially those with a high serum B_{12}, displayed a variable pattern with less activity noted in Ppt. A and more in Ppts. B, C and MP-1. The significance of this difference remains to be determined.

Though the present study clearly demonstrates that the plasma B_{12} binding substance appears in the seromucoid fraction of plasma, purification and chemical characterization of the material are required before one can say with certainty that it is a glycoprotein. Similar procedures are necessary to resolve the question of whether the increase in B_{12} binding substance seen in CML represents an excess of the normally occurring material or the presence of a chemically similar but abnormal material having B_{12} binding capacity. Studies on the further purification of the B_{12} binding protein by anion-exchange cellulose column chromatography (19) of the seromucoid fraction are now in progress (18).

The data presented appear to be in accord with the previously known facts concerning the plasma B_{12} binding substance. During electrophoresis at pH 8.6 in veronal, Pitney, Beard and Van Loon (1) found most of the endogenous bound B_{12} in the α-globulin fraction of serum as did Ostrowski, Skaryzynski and Zak (9) and Heinrich and Erdmann-Oehlecker (8). In a previous report from this laboratory (10), these findings were confirmed using paper or block electrophoresis followed by microbiologic assay of the endogenous B_{12} present in each fraction. It is known that under the same conditions of electrophoresis the bulk of the seromucoid fraction of plasma has a similar mobility (17). It is of interest that in all of these electrophoretic studies smaller amounts

of B_{12} were noted in the albumin and β-globulin fractions. It is not yet clear whether these fractions bind some B_{12}, since the spreading may be due to imperfections of methods.

The divergent results of Miller and Sullivan (4), who found that following the addition of 15 mμg. of $Co^{60}B_{12}$ per ml. of serum and paper electrophoresis at pH 8.6 in veronal the greatest radioactivity appeared in the β-globulin fraction in normals and in the α_1-fraction of sera from CML patients, may reflect the effects of *in vitro* "overloading" in the normal sera as a result of the unphysiologic concentration of added radioactive B_{12}.

Mendelsohn, Watkin, Horbett, and Fahey (10) have previously reported that when whole serum was fractionated by anion-exchange cellulose (DEAE) column chromatography a single B_{12} peak was obtained. The latter had the mobility of α_1-globulin whether obtained from normal or CML sera. On their chromatogram the B_{12}-containing fraction did occur in an area of relatively high protein-bound carbohydrate (hexose) concentration. It did not correspond with the site of elution of orosomucoid (MP-1) thus confirming the present finding that MP-1 is not the major B_{12} binding substance.

Recently, Miller and Sullivan have reported (20) that normal serum mucoprotein remaining after sulfosalicylic acid precipitation of proteins retained 13 per cent of total serum B_{12} binding capacity and CML mucoproteins so prepared retained 59 per cent. It is possible that the mucoprotein techniques used may impair the subsequent binding properties of the material. The present procedure of allowing B_{12} to bind to whole plasma and then fractionating appears to give better recovery of the B_{12} protein complex. The poorer recovery obtained by Miller and Sullivan may also be due to the use of sulfosalicylic rather than perchloric acid.

The fact that Gregory and Holdsworth (21) have described a B_{12} binding protein in sow's milk which has the characteristics of a glycoprotein and that a glycoprotein substance has been isolated from urine (22) which has strong B_{12} binding activity lends further support to the originally proposed hypothesis that various normally occurring B_{12} binding proteins are chemically similar. The subjects of the exact interrelationship between these substances as well as their physiologic and biochemical functions bear further investigation.

If, as data from the present studies imply, the elevated serum B_{12} levels seen in CML are associated with an increase of a specific B_{12} binding glycoprotein present in the seromucoid fraction, the finding is of interest in terms of the relation between neoplastic disease and glycoprotein metabolism. Total seromucoid as well as MP-1 and MP-2 are known to be elevated in the sera of human subjects with neoplastic disease (23) and also in certain tumor bearing animals (24). Total seromucoid is also known to rise in a variety of diseases associated with traumatic, inflammatory or degenerative tissue changes. These findings have been well reviewed by Winzler (23), Greenspan (25), and Moschides, Stefanini, Magalini and Kistner (26). In addition, however, there is evidence that the carbohydrate to protein ratio of seromucoid may vary in different diseases (27, 28) and also that the relationship between carbohydrate components themselves may vary (29). It has been previously suggested (26) that, since seromucoid is a heterogeneous material, these changes could result from the alteration in a specific component of this fraction. The present data suggest that such is the case in plasma of subjects with CML, in which the B_{12} binding protein is increased. The source of this material as well as the cause for its increase in this disease is at the present time unknown.

SUMMARY

The fact that intrinsic factor is thought to be a glycoprotein led us to investigate the B_{12} content of the seromucoid fraction of plasma from normal subjects and patients with chronic myelocytic leukemia (CML). When 0.1 to 1.0 mμg. of $Co^{58}B_{12}$ per ml. was added to plasma from six normal subjects and eight patients with CML and free B_{12} was removed by dialysis, 77 per cent and 83 per cent, respectively, of the bound radioactivity for the two groups was found in the seromucoid fraction. When 10 to 100 mμg. of $Co^{58}B_{12}$ per ml. was added to plasma from patients with CML, an association between the increased binding capacity of this plasma and the increased recovery of radioactivity in the seromucoid fraction

was demonstrated. The failure to recover substantial radioactivity from the "MP-1" (orosomucoid) fraction following its isolation by ammonium sulfate precipitation from the labeled plasma of normal subjects and patients with CML indicates that orosomucoid is not the major plasma B_{12} binding protein. The fact that the B_{12} binding protein is present in the seromucoid fraction of plasma suggests that it is a glycoprotein. In order to establish this, further purification and chemical analysis of the protein are required. These studies are now in progress.

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