JCI The Journal of Clinical Investigation

# THE *IN VITRO* BINDING OF COBALT$^{60}$ LABELED VITAMIN $B_1$ BY NORMAL AND LEUKEMIC SERA

Aaron Miller

*J Clin Invest.* 1958;37(4):556-566. https://doi.org/10.1172/JCI103638.

Research Article



# THE *IN VITRO* BINDING OF COBALT$^{60}$ LABELED VITAMIN $B_{12}$ BY NORMAL AND LEUKEMIC SERA

By AARON MILLER with the technical assistance of JOHN F. SULLIVAN

*(From the Radioisotope and Medical Services, Boston Veterans Administration Hospital, and The Department of Medicine, Boston University School of Medicine, Boston, Mass.)*

(Submitted for publication August 17, 1957; accepted December 26, 1957)

In chronic myelogenous leukemia both the serum concentration of vitamin $B_{12}$ and the capacity of the serum to bind added vitamin $B_{12}$ are markedly increased (1–3), suggesting an abnormality of the serum proteins in this disease. *In vitro* measurements of vitamin $B_{12}$ binding capacity employing a microbiological assay method have not demonstrated a qualitative difference between normal and leukemic sera in respect to the binding of added vitamin $B_{12}$ by the various serum protein fractions (1, 4). In the present study an equilibrium dialysis technique using cobalt$^{60}$ labeled vitamin $B_{12}$ has been employed to measure the binding capacity of normal and leukemic sera for added vitamin $B_{12}$ and to identify the protein fractions which bind the added vitamin $B_{12}$.

## METHODS

### *A. Experimental plan*

Venous blood samples were obtained from patients in the fasting state. After two to three hours at room temperature the serum was separated under sterile conditions and stored at $-20°$ C. until tested. The serum concentration of vitamin $B_{12}$, the capacity of the serum to bind added cobalt$^{60}$ labeled vitamin $B_{12}$ and the distribution of the added vitamin $B_{12}$ among the serum proteins were determined. Studies were carried out on 13 patients with chronic myelogenous leukemia, 8 patients with other types of leukemias, 8 patients with nonmalignant granulocytic leukocytoses, 2 patients with polycythemia vera and 22 control subjects, the latter group being composed of healthy laboratory personnel or ambulatory hospitalized patients suffering from functional disorders.

### *B. Analytical procedures*

*Serum vitamin $B_{12}$ concentration.* "Total" vitamin $B_{12}$ concentration of the serum was determined by the modification of Lear, Harris, Castle, and Fleming (5) of the *Euglena gracilis* method of Ross (6). "Free" vitamin $B_{12}$, measured on occasional serum samples from normal subjects and patients with chronic myelogenous leukemia, constituted 15 per cent or less of the total serum vitamin $B_{12}$.

*Serum vitamin binding capacity.* Following the addition of a known excess quantity of radioactive vitamin $B_{12}$ to serum, the degree of binding of the added vitamin $B_{12}$ could be determined by a modification of the equilibrium dialysis method as described by Klotz, Walker, and Pivan (7). To duplicate or triplicate 2 ml. samples of serum was added 2 ml. of a solution containing 0.40 m$\mu$g. of Co$^{60}$ vitamin $B_{12}$ and 5 ml. of 1/15 M phosphate buffer (pH 7.3). This mixture was placed in a Visking[1] cellulose dialysis sac at 22° C. A protein-free blank prepared in triplicate with phosphate buffer in place of serum was run simultaneously. Each dialysis bag was placed into 3 by 17 cm. test tubes and the amount of radioactivity in each sample determined by means of a plastic scintillation well counter. After one hour incubation at 22° C., the bags were dialyzed against 250 ml. of 1/15 M phosphate buffer, pH 7.3. Initially, the dialysis was carried on for 24 hours at 22° C. with constant agitation but subsequently this was performed for 48 hours at 4° C. without agitation. Equilibrium was attained in both situations, and similar values for vitamin $B_{12}$ binding were obtained under both experimental conditions. At the conclusion of dialysis each bag was rinsed externally in running water for one-half to one minute after which its radioactivity was determined in the plastic scintillation well counter. The bags were then slit in two, emptied of their contents, washed for one-half to one minute in running water and recounted for the measurement of the residual radioactivity bound to the bag. The unsaturated vitamin $B_{12}$ binding capacity of the serum (UBBC) was determined from the following calculations:

*1.* Fraction of added $Co^{60}B_{12}$ bound per ml.

$$= \frac{(a)\ Co^{60}B_{12} \text{ in bag postdialysis (cpm)} - Co^{60}B_{12} \text{ bound to bag (cpm)}}{Co^{60}B_{12} \text{ added predialysis (cpm)} \times \text{ml. serum} - \text{mean } Co^{60}B_{12} \text{ in blank calculated as in } (a)}$$

*2.* UBBC, m$\mu$g./ml. = $Co^{60}B_{12}$ added predialysis (m$\mu$g.) $\times$ calculation *1.*

[1] Secured from Visking Corporation, Chicago, Illinois.

No correction for Donnan's equilibrium was needed as vitamin $B_{12}$ is essentially un-ionized (8). The total vitamin $B_{12}$ binding capacity of the serum (TBBC) was obtained from the sum of the serum vitamin $B_{12}$ concentration and the UBBC.

*Technical considerations.* Two ml. of a 1:50 dilution of $Co^{60}$ vitamin $B_{12}$ of specific activity 0.97 $\mu$c./1.00 $\mu$g. were used in each determination of the UBBC unless otherwise stated. This was equivalent to 22 to 23,000 cpm under the counting conditions used. Radioactivity measurements were made in a large plastic scintillation well counter (well, 3.7 cm. in diameter and 10 cm. in depth) with other physical characteristics as previously described (9). A $Co^{60}$ standard was counted each morning to correct for daily variations in the counter. The geometry of counting in pre- and postdialysis samples was constant. Samples were counted sufficiently long to give a counting error of less than 2 per cent except for the counting of empty dialysis bags where less than 5 to 7 per cent counting error was obtained.

The concentration of the stock $Co^{60}$ vitamin $B_{12}$ used for the measurement of the UBBC was determined by the Euglena gracilis assay. Due to the known photolability of the cyano group of the vitamin $B_{12}$ molecule, aliquots of the stock $Co^{60}B_{12}$ solution were exposed to a fluorescent light source at 4° C. for a 12 day period. The binding of the $Co^{60}$ vitamin $B_{12}$ solution exposed to light was 80 per cent greater than a similar aliquot of the vitamin $B_{12}$ solution protected from light. For this reason, the $Co^{60}$ vitamin $B_{12}$ was shielded from any source of light during all phases of the binding measurement. Serum, diluted 1 to 4 with 1/15 M phosphate buffer, pH 7.3, had a similar binding capacity as that of undiluted serum. Increasing or decreasing the amount of $Co^{60}$ vitamin $B_{12}$ over a fourfold range, slightly increased, or did not affect, the amount of vitamin $B_{12}$ bound in both the normal and leukemic sera (Table I).

Experiments designed to evaluate the effect of temperature on vitamin $B_{12}$ binding indicated that similar amounts of $Co^{60}$ vitamin $B_{12}$ were bound by serum at 4° C. and 22° C. No difference in binding was noted between aliquots of serum dialyzed at 4° C. and 22° C. The binding of radioactive material to the dialysis bag was influenced by the dialysis temperature however, 4 to 8 per cent of the $Co^{60}$ vitamin $B_{12}$ becoming bound at 22° C. as compared to 1 to 2 per cent at 4° C. The firmness of this bond is reflected by the fact that only 10 to 20 per cent of the bag radioactivity disappeared after 48 hours of dialysis. Dialysis against distilled water, phosphate buffer, pH 7 to 8, dextran solution, barbital buffer (pH 8.6) and saline had no effect on binding. Multiple successive freezing and thawing of serum did not affect binding capacity. The UBBC of serum stored at −20° C. remained stable over a six month period. In a patient with chronic myelogenous leukemia, no difference in vitamin $B_{12}$ binding was found between serum collected, as described previously, and heparinized plasma separated from the cellular elements at 4° C. immediately after venipuncture. Serum with increased vitamin $B_{12}$ binding showed better agreement between duplicate samples than those with normal binding. The vitamin $B_{12}$ binding values showed a maximum difference in duplicate samples of 15 per cent in serum with binding greater than 4.00 m$\mu$g. per ml., 20 per cent with binding from 1.50 to 4.00 m$\mu$g. per ml., and 35 per cent in samples with binding less than 1.50 m$\mu$g. per ml. If sufficient serum was available, vitamin $B_{12}$ binding was remeasured when the upper limits of precision were approached. The variation in the serum vitamin $B_{12}$ concentration, the UBBC, and the TBBC of a normal subject over a two week interval of time is shown in Table II.

TABLE I

*The relationship between the quantity of added vitamin $B_{12}$ and the unsaturated vitamin $B_{12}$ binding capacity of serum aliquots*

| | Amount of vit. $B_{12}$ added per ml. serum m$\mu$g. | Unsaturated vit. $B_{12}$ binding capacity m$\mu$g./ml. |
|---|---|---|
| Normal serum A | 5 | 1.62 |
| | 10 | 1.86 |
| | 20 | 2.08 |
| Normal serum B | 4 | 1.33 |
| | 20 | 1.56 |
| Myelogenous leukemic serum | 10 | 7.20 |
| | 50 | 7.30 |

TABLE II

*Vitamin $B_{12}$ content and binding capacity of serum and the distribution of serum bound vitamin $B_{12}$ as determined at different times in a normal subject*

| Subject | Serum $B_{12}$ conc. m$\mu$g./ml. | UBBC* m$\mu$g./ml. | TBBC† m$\mu$g./ml. | Fraction of vitamin $B_{12}$ bound to serum | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Albumin % | α-1 globulin % | α-2 globulin % | β-globulin % | γ-globulin % |
| A. M. | | | | | | | | |
| 4/1/56 | 0.35 | 1.96 | 2.31 | 14 | 3 | 28 | 42 | 13 |
| 4/8/56 | 0.34 | 2.20 | 2.54 | 14 | 6 | 26 | 37 | 17 |
| 4/15/56 | 0.33 | 2.25 | 2.58 | 18 | 0 | 31 | 33 | 18 |

* UBBC refers to unsaturated vitamin $B_{12}$ binding capacity.
† TBBC refers to total vitamin $B_{12}$ binding capacity.

TABLE III

*The vitamin $B_{12}$ content and binding capacity of serum in 22 normal subjects as compared to 10 untreated cases of chronic myelogenous leukemia*

| | Serum vit. $B_{12}$ concentration mμg./ml. | | UBBC* mμg./ml. | | TBBC† mμg./ml. | |
|---|---|---|---|---|---|---|
| | Range | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. |
| Normal | 0.27–1.27 | 0.64 ± 0.10 | 1.00–2.40 | 1.51 ± 0.56 | 1.32–2.81 | 2.23 ± 0.43 |
| Chronic myelogenous leukemia | 4.20–14.30 | 8.09 ± 4.55 | 4.75–14.25 | 8.87 ± 4.12 | 10.45–26.05 | 16.86 ± 7.78 |

* UBBC refers to unsaturated vitamin $B_{12}$ binding capacity.
† TBBC refers to total vitamin $B_{12}$ binding capacity.

*Electrophoresis.* One ml. of serum and 15 mμg. of $Co^{60}$ vitamin $B_{12}$ (in 0.5 ml.) were allowed to bind for one hour at 22° C. and then dialyzed at 4° C. for 48 hours against frequent changes of phosphate buffer, pH 7.3. Then, 0.12 ml. aliquots of the serum containing bound $Co^{60}$ vitamin $B_{12}$ were electrophoresed for 18 hours on 4 by 47 cm. Schleicher and Schuell paper No. 2043B, using veronal buffer pH 8.6 (ionic strength, 0.08) and a voltage of 110 to 114. At least four individual strips equivalent to 0.48 ml. of the serum-$Co^{60}$ vitamin $B_{12}$ mixture were run on each patient. Strips were then stained by a ninhydrin spray method. No loss of radioactivity occurred after staining the strips. This was in contrast to the bromphenol blue or amido black methods of staining where considerable losses of radioactivity were encountered. Under the conditions used, the albumin fraction usually moved 9 to 12 cm. from the origin while the gamma globulin moved slightly towards the anode (0.05 to 1.5 cm.). "Free" $Co^{60}$ vitamin $B_{12}$ (vitamin in saline solution) remained at the origin or moved slightly towards the anode (0.5 to 1 cm.). In serum which had not been dialyzed containing large amounts of unbound $Co^{60}$ vitamin $B_{12}$, the free radiovitamin had a mobility similar to that in saline solution, occupying an area just adjacent to or overlapping that occupied by the gamma globulins. Each stained protein fraction was then sepa-

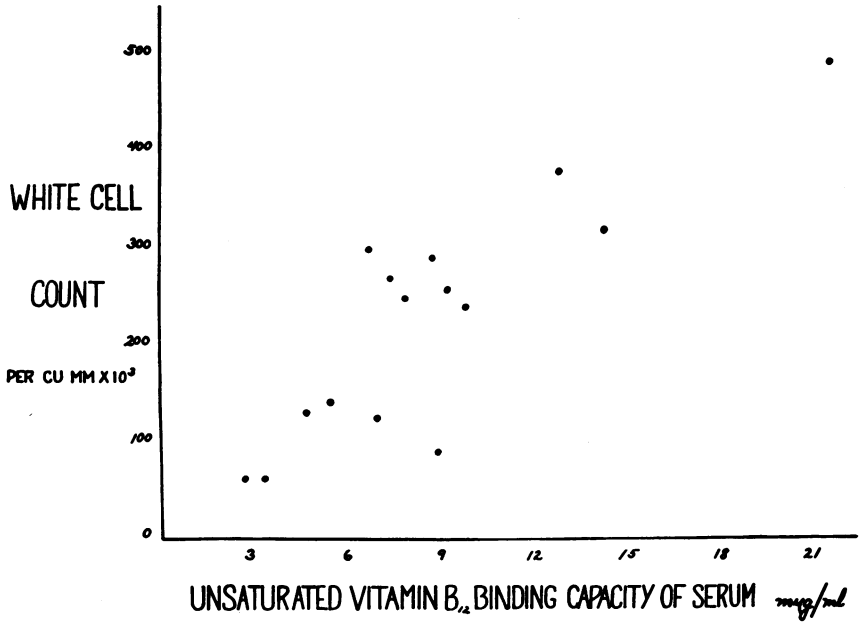


FIG. 1. CORRELATION OF THE WHITE CELL COUNT WITH THE UNSATURATED VITAMIN $B_{12}$ BINDING CAPACITY IN 10 PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN RELAPSE

The above points represent the white cell count and the unsaturated vitamin $B_{12}$ binding capacity of a patient with chronic myelogenous leukemia in relapse. Some patients, studied at different times during one or more relapses, are represented by more than one point. The coefficient of correlation between the above two measurements was 0.88.

TABLE IV

*Tabular summary of vitamin $B_{12}$ data in treated and untreated cases of chronic myelogenous leukemia*

| Patient | | WBC per cu. mm. $\times 10^2$ | Serum $B_{12}$ conc. *mμg./ml.* | UBBC *mμg./ml.* | TBBC *mμg./ml.* | Therapy |
|---|---|---|---|---|---|---|
| F. C. | 10/4/56 | 3,814 | 14.30 | 12.75 | 27.05 | X-ray to spleen |
| | 10/29/56 | 280 | 11.53 | 1.70 | 13.23 | |
| | 12/7/56 | 623 | 3.30 | 2.75 | 6.05 | |
| | 4/5/57 | 5,005 | 6.50 | 20.00 | 26.50 | X-ray to spleen |
| | 4/15/57 | 1,506 | 5.50 | 14.40 | 19.90 | |
| | 4/20/57 | 749 | 6.20 | 8.99 | 15.19 | |
| T. M. | 6/13/56 | 2,109 | 7.83 | 9.22 | 17.05 | P-32 |
| | 6/28/56 | 1,942 | 7.65 | | | |
| | 8/10/56 | 2,470 | 7.91 | 8.72 | 16.63 | X-ray to spleen |
| | 8/23/56 | 391 | 7.90 | 3.79 | 11.69 | |
| | 9/6/56 | 234 | 7.68 | 4.36 | 12.04 | |
| | 10/4/56 | 107 | 4.20 | 1.24 | 5.44 | |
| | 11/15/56 | 129 | 2.45 | 0.90 | 3.35 | |
| J. C. | 10/4/56 | 2,000 | 4.35 | 7.80 | 12.15 | X-ray to spleen |
| | 10/17/56 | 324 | 4.05 | 1.53 | 5.58 | |
| | 11/13/56 | 286 | 1.76 | 1.37 | 3.13 | |
| | 12/13/56 | 625 | 3.45 | 3.40 | 6.85 | |
| | 1/17/57 | 1,397 | 5.56 | 5.50 | 11.06 | |
| | 2/21/57 | 2,254 | | 6.54 | | |
| S. M. | 10/23/56 | 900 | 7.94 | 8.96 | 16.90 | Myleran® |
| | 12/13/56 | 145 | 3.80 | 2.90 | 6.70 | |
| T. E. | 9/11/56 | 766 | 2.25 | 5.00 | 7.25 | 5 weeks after incomplete response to X-ray, Myleran® begun |
| | 11/15/56 | 59 | 2.19 | 2.18 | 4.37 | |
| G. L. | | 185 | 0.94 | 1.40 | 2.34 | 1 yr. following treatment with Myleran® |
| S. D. | | 100 | 1.70 | 0.90 | 2.60 | 6 months following X-ray to spleen |
| S. B. | | 1,201 | 4.20 | 7.00 | 11.20 | |
| A. R. | | 1,311 | 5.70 | 4.75 | 10.45 | |
| R. L. | | 2,200 | 4.65 | 7.45 | 12.10 | |
| T. W. | | 3,000 | 14.00 | 6.70 | 20.70 | |
| T. K. | | 3,212 | 8.90 | 14.25 | 23.15 | |
| V. P. | | 1,920 | 9.00 | 9.85 | 18.85 | |

rated by cutting out each individual protein fraction of the electrophoretic strip. Each of the combined stained protein fractions was then counted in a sodium iodide well counter. As low counting rates were usually found, each fraction was counted for 10 minutes, with the background for each sample determined by 10 minute counting preceding and following the sample count. The chief binding protein fractions in normal sera (beta- and alpha-2 globulins) counted from three-fourths to one and one-half times background, whereas the other fractions counted 10 to 40 per cent above background. In sera with high UBBC's the chief binding protein counted from two to four times background. The binding in each protein fraction was expressed as (*a*) per cent $Co^{60}$ vitamin $B_{12}$ bound calculated by dividing the radioactivity found in each protein fraction by the total radioactivity in all fractions times 100, and as the (*b*) mμg. of $Co^{60}$ vitamin $B_{12}$ bound per ml. calculated from the UBBC and calculation (*a*). The variation in vitamin $B_{12}$ binding by the serum protein fractions of a normal subject measured over a two week interval of time is shown in Table II.

## RESULTS

### *Serum vitamin $B_{12}$ concentration and binding capacity*

*Normals.* In 22 normal subjects the mean serum vitamin $B_{12}$ concentration was equal to 0.64 mμg. per ml. (range, 0.27 to 1.27 mμg per ml.), the mean UBBC equal to 1.51 mμg per ml. (range, 1.00 to 2.40 mμg. per ml.), and the mean TBBC equal to 2.23 mμg. per ml. (range, 1.32 to 2.81 mμg. per ml.) (Table III).

*Chronic myelogenous leukemia.* The average serum $B_{12}$ concentration, UBBC, and TBBC were greatly increased over normal in 10 patients with active chronic myelogenous leukemia, the first by a factor of 12, the second by a factor of 6, and the third by a factor of 7 (Table III). An excellent correlation was found between the white blood cell count and the UBBC in patients in relapse, the

coefficient of correlation equal to 0.88 (Figure 1). A poorer correlation was found between the white blood cell count and the serum $B_{12}$ concentration (a coefficient of correlation of 0.58). With successful treatment of the disease (X-ray or Myleran®), the UBBC fell rapidly towards normal, paralleling the decline in white blood cell count, this phenomena being noted as soon as five days after the onset of therapy. Restoration of normal UBBC levels occurred within two weeks after the onset of treatment in one patient (Patient J. C., Table IV). By contrast, the concentration of vitamin $B_{12}$ in the serum did not begin to decline for as long as two to three weeks after the onset of treatment. Even in complete remission serum $B_{12}$ levels remained elevated with the sole exception of Patient G. L. (Table IV). With relapse, the serum vitamin $B_{12}$ level and the UBBC rose together.

*Other leukemias.* The serum vitamin $B_{12}$ content, the UBBC, and TBBC were normal in three patients with chronic lymphocytic leukemia except for a slight increase in TBBC in one of the three (Table V). Two patients with chronic monocytic leukemia had elevated serum $B_{12}$ levels, UBBC's, and TBBC's approaching or equalling that found in chronic myelogenous leukemia. In contrast to chronic myelogenous leukemia, a poor correlation existed between the white blood cell count and the UBBC. After a partial response to X-ray treatment, the serum $B_{12}$ level fell to normal whereas the UBBC and TBBC were elevated (Patient T. D.). One of three patients with acute leukemia had a moderate increase in UBBC and TBBC associated with a normal serum $B_{12}$ level. This patient had a dramatic clinical response to cortisone associated with a decrease in the UBBC and TBBC to normal.

*Leukocytosis* Eight patients with neutrophilic leukocytoses due to varying causes with or with-

TABLE V

*Tabular summary of vitamin $B_{12}$ data in cases other than chronic myelogenous leukemia*

| Diagnosis and clinical comments | WBC per cu. mm. $\times 10^2$ | Serum $B_{12}$ conc. mμg./ml. | UBBC mμg./ml. | TBBC mμg./ml. |
|---|---|---|---|---|
| **Chronic lymphocytic leukemia** | | | | |
| T. B. | 4,000 | 1.06 | 1.65 | 2.71 |
| G. M. | 610 | 0.64 | 1.12 | 1.76 |
| M. G. | 3,866 | 1.27 | 1.97 | 3.24 |
| **Chronic monocytic leukemia** | | | | |
| T. D. 9/19/56 | 486 | 6.00 | 9.00 | 15.00 |
| 11/15/56 (Partial response to X-ray treatment) | 129 | 0.50 | 5.50 | 6.00 |
| J. M. | 32 | 1.90 | 5.20 | 7.10 |
| **Acute leukemia** | | | | |
| T. C. | 154 | 0.23 | 1.74 | 1.97 |
| B. M. | 1,862 | 0.73 | 1.26 | 1.99 |
| S. C. 4/20/57 | 250 | 0.26 | 3.97 | 4.23 |
| 4/29/57 (Good response to cortisone) | 41 | 0.20 | 1.96 | 2.16 |
| **Leukocytoses** | | | | |
| C. C. Pulmonary tuberculosis and bacterial pneumonia | 352 | 0.98 | 1.55 | 2.53 |
| T. D. Polycythemia vera with leukemoid reaction | 580 | 0.85 | 6.30 | 7.15 |
| J. F. Epidermoid carcinoma of throat with metastases | 322 | 3.73 | 5.60 | 9.33 |
| J. E. Septicemia | 484 | 0.42 | 3.86 | 4.28 |
| T. C. Pulmonary tuberculosis with leukemoid reaction | 300 | 0.40 | 2.79 | 3.19 |
| J. A. Carcinoma of lung with secondary infection | 300 | 0.50 | 2.70 | 3.20 |
| S. H. Polycythemia vera, postoperative after lobectomy for carcinoma of lung | 534 | 0.25 | 2.94 | 3.19 |
| T. A. ? Myeloid metaplasia | 692 | 0.74 | 6.15 | 6.89 |
| **Polycythemia vera** | | | | |
| S. M. | 100 | 0.57 | 2.40 | 2.97 |
| T. S. | 144 | 1.96 | 1.48 | 3.44 |
| **Miscellaneous** | | | | |
| J. R. Chronic pancytopenia of ? etiology | 28 | 0.27 | 2.75 | 3.02 |

TABLE VI

*The binding of $Co^{60}$ labeled vitamin $B_{12}$ by the protein fractions of serum from normal subjects*

| Subject | Albumin | | α-1 globulin | | α-2 globulin | | β-globulin | | γ-globulin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. |
| T. M. | 11 | 0.18 | 7 | 0.11 | 29 | 0.47 | 32 | 0.52 | 21 | 0.34 |
| F. M. | 5 | 0.06 | 8 | 0.10 | 32 | 0.40 | 38 | 0.48 | 17 | 0.21 |
| J. A. | 6 | 0.11 | 7 | 0.12 | 23 | 0.41 | 48 | 0.85 | 16 | 0.28 |
| H. L. | 7 | 0.09 | 6 | 0.08 | 18 | 0.26 | 49 | 0.61 | 20 | 0.25 |
| J. C. | 4 | 0.08 | 1 | 0.02 | 16 | 0.31 | 66 | 1.29 | 13 | 0.25 |
| A. C. | 8 | 0.09 | 3 | 0.03 | 29 | 0.31 | 43 | 0.46 | 17 | 0.18 |
| A. M. | 18 | 0.35 | 8 | 0.16 | 29 | 0.57 | 37 | 0.73 | 8 | 0.16 |
| F. M. | 8 | 0.18 | 0 | 0.00 | 31 | 0.68 | 44 | 0.97 | 17 | 0.37 |
| S. M. | 4 | 0.10 | 4 | 0.10 | 47 | 1.14 | 32 | 0.78 | 13 | 0.31 |
| F. M. | 13 | 0.21 | 0 | 0.00 | 33 | 0.54 | 40 | 0.65 | 14 | 0.23 |
| T. D. | 11 | 0.26 | 2 | 0.05 | 34 | 0.82 | 56 | 1.34 | 17 | 0.41 |
| T. G. | 11 | | 5 | | 19 | | 41 | | 24 | |
| M. S. | 3 | | 4 | | 17 | | 57 | | 19 | |
| S. F. | 6 | | 2 | | 32 | | 45 | | 15 | |
| M. L. | 7 | | 4 | | 31 | | 48 | | 10 | |
| T. M. | 8 | | 5 | | 38 | | 33 | | 16 | |
| P. R. | 16 | | 6 | | 16 | | 41 | | 21 | |
| T. B. | 12 | | 6 | | 24 | | 45 | | 13 | |
| Range | 3–18 | 0.06–0.35 | 0–9 | 0–0.12 | 16–47 | 0.26–1.14 | 32–66 | 0.46–1.34 | 10–24 | 0.16–0.41 |
| Mean ± standard deviation | 9±4 | 0.16±0.09 | 4±3 | 0.07±0.06 | 28±7 | 0.59±0.19 | 44±10 | 0.79±0.38 | 16±4 | 0.27±0.09 |

out leukemoid reactions were studied (Table V). The commonest finding was a normal serum vitamin $B_{12}$ concentration and an elevated UBBC and TBBC (six of eight patients). One patient (J. F.) had an elevated serum vitamin $B_{12}$ concentration, UBBC, and TBBC approximating those found in chronic myelogenous leukemia while the last patient had a normal serum $B_{12}$ concentration, UBBC, and TBBC.

*Other diseases.* One patient with polycythemia vera had a normal serum vitamin $B_{12}$ level and UBBC but a slightly increased TBBC while another patient with this disease had an elevation of vitamin $B_{12}$ content and TBBC. A patient with chronic pancytopenia of unknown etiology had a normal serum vitamin $B_{12}$ level with a slight increase in UBBC and TBBC.

### *Electrophoresis of serum*

*Normals.* In 16 of 18 normal subjects the beta globulins bound the greatest per cent of the added $Co^{60}$ vitamin $B_{12}$, average of 44 per cent, range, 32 to 66 per cent (Table VI). The alpha-2 globulins were also an important binding fraction containing an average of 28 per cent of the added $Co^{60}$ vitamin $B_{12}$, with a range of 16 to 47 per cent, and were the chief binding proteins in the two other subjects. Smaller amounts of radioactivity were bound by the gamma globulin and albumin fractions, whereas binding by the alpha-1 globulins was negligible (average, 4 per cent; range, 0 to 9 per cent). The amount of vitamin $B_{12}$ bound per protein fraction expressed as mμg. per ml. is also shown in Table VI.

*Chronic myelogenous leukemia.* In contrast to normal subjects the alpha-1 or alpha-2 globulins were the main binding protein fractions of the serum for the added $Co^{60}$ vitamin $B_{12}$ (Table VII). Thus, the alpha-1 globulins contained an average of 38 per cent of the added $Co^{60}$ vitamin $B_{12}$, approximately nine times that of normal sera, and was the main binding protein in 5 of the 10 patients. The alpha-2 globulins bound an average of 34 per cent of the added $Co^{60}$ vitamin $B_{12}$, approximately 20 per cent more than that of normal serum, and was the main binding protein in four patients. When expressed in mμg. per ml. the increase in $Co^{60}$ vitamin $B_{12}$ binding by the alpha-1 globulins was approximately 47 times greater than that of normal serum as compared to the sixfold increase over normal by the alpha-2 globulins. The percentage of added $Co^{60}$ vitamin

TABLE VII

*The binding of cobalt$^{60}$ labeled vitamin $B_{12}$ by the protein fractions of serum from patients with chronic myelogenous leukemia in relapse*

| Patient | Albumin | | α-1 globulin | | α-2 globulin | | β-globulin | | γ-globulin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. |
| T. M. | 3 | 0.28 | 56 | 5.16 | 16 | 1.48 | 10 | 0.92 | 15 | 1.38 |
| S. B. | 7 | 0.49 | 25 | 1.75 | 24 | 1.68 | 31 | 2.17 | 13 | 0.91 |
| A. R. | 9 | 0.43 | 24 | 1.14 | 34 | 1.62 | 19 | 0.90 | 14 | 0.66 |
| F. C. | 3 | 0.38 | 56 | 7.14 | 29 | 3.70 | 10 | 1.28 | 2 | 0.26 |
| S. M. | 4 | 0.36 | 52 | 4.66 | 23 | 2.06 | 13 | 1.17 | 8 | 0.72 |
| J. C. | 4 | 0.31 | 30 | 2.34 | 33 | 2.57 | 23 | 1.79 | 10 | 0.78 |
| R. L. | 1 | 0.07 | 21 | 1.57 | 55 | 4.10 | 18 | 1.34 | 5 | 0.37 |
| T. W. | 0 | 0.00 | 42 | 2.81 | 42 | 2.81 | 10 | 0.67 | 6 | 0.40 |
| T. K. | 1 | 0.14 | 36 | 5.13 | 52 | 7.41 | 7 | 1.00 | 4 | 0.57 |
| V. P. | 4 | 0.39 | 39 | 3.84 | 33 | 3.25 | 20 | 1.97 | 4 | 0.39 |
| Range | 0–9 | 0.00–0.49 | 21–56 | 1.14–7.14 | 16–55 | 1.62–7.41 | 7–31 | 0.67–2.17 | 4–14 | 0.26–1.38 |
| Mean ± standard deviation | 4±2 | 0.29±0.18 | 38±13 | 3.55±2.29 | 34±12 | 3.07±2.04 | 16±7 | 1.32±0.66 | 8±5 | 0.64±0.32 |

$B_{12}$ bound by the beta globulins was decreased to about one-third that found for normal serum while slighter decreases were also found for the gamma globulin and albumin fractions. Despite the decrease in the per cent of added $Co^{60}$ vitamin $B_{12}$ bound by these fractions, modest elevations in the mμg. of vitamin $B_{12}$ bound per ml. of serum were found although these values overlapped those found in normal serum. With successful treatment, the serum protein binding pattern reverted towards that found in normal subjects and, with complete remission, a normal pattern of binding was found (Table VIII).

*Other leukemias.* The binding of $Co^{60}$ vitamin $B_{12}$ by the serum proteins of patients with chronic lymphocytic leukemia was similar to that of normal serum (Table IX). Elevated binding by both the alpha-1 and alpha-2 globulins was found in one patient with chronic monocytic leukemia, whereas in two other patients with chronic mono-

TABLE VIII

*The effect of treatment on the binding of cobalt$^{60}$ labeled vitamin $B_{12}$ by the protein fractions of serum from patients with chronic myelogenous leukemia*

| Patient | Albumin | | α-1 globulin | | α-2 globulin | | β-globulin | | γ-globulin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. |
| J. C. | | | | | | | | | | |
| 10/4/56 Rx | 4 | 0.31 | 30 | 2.34 | 33 | 2.57 | 23 | 1.79 | 10 | 0.78 |
| 10/17/56 (After Rx) | 2 | 0.03 | 8 | 0.12 | 28 | 0.42 | 39 | 0.60 | 23 | 0.35 |
| 1/17/57 (Relapse) | 4 | 0.22 | 11 | 0.61 | 42 | 2.31 | 27 | 1.49 | 16 | 0.88 |
| 2/21/57 (Relapse) | 2 | 0.13 | 22 | 1.35 | 42 | 2.75 | 22 | 1.35 | 12 | 0.79 |
| F. C. | | | | | | | | | | |
| 10/4/56 Rx | 3 | 0.38 | 56 | 7.14 | 29 | 3.70 | 10 | 1.28 | 2 | 0.26 |
| 10/29/56 (After Rx) | 11 | 0.19 | 27 | 0.46 | 22 | 0.37 | 26 | 0.44 | 14 | 0.24 |
| 12/7/56 (Relapse) | 2 | 0.06 | 20 | 0.55 | 55 | 1.51 | 18 | 0.50 | 5 | 0.14 |
| 4/5/57 Rx | 2 | 0.40 | 35 | 7.00 | 52 | 10.40 | 8 | 1.60 | 3 | 0.60 |
| 4/15/57 Rx | 2 | 0.29 | 40 | 5.76 | 47 | 6.77 | 7 | 1.01 | 4 | 0.58 |
| 4/20/57 | 7 | 0.63 | 25 | 2.25 | 51 | 4.59 | 12 | 1.08 | 5 | 0.45 |
| G. L. Remission | 6 | 0.08 | 7 | 0.10 | 46 | 0.64 | 34 | 0.48 | 7 | 0.10 |

TABLE IX

*The binding of cobalt$^{60}$ labeled vitamin $B_{12}$ by the protein fractions of serum from patients with varying types of leukemia and leukocytoses*

| Diagnosis | Albumin | | α-1 globulin | | α-2 globulin | | β-globulin | | γ-globulin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. | Vit. $B_{12}$ bound % | Vit. $B_{12}$ bound mμg./ml. |
| Chronic lymphatic leukemia | | | | | | | | | | |
| T. B. | 8 | 0.13 | 3 | 0.05 | 30 | 0.50 | 47 | 0.78 | 12 | 0.19 |
| G. M. | 7 | 0.14 | 2 | 0.04 | 31 | 0.61 | 45 | 0.89 | 15 | 0.30 |
| Chronic monocytic leukemia | | | | | | | | | | |
| J. M. | 4 | 0.21 | 3 | 0.16 | 40 | 2.08 | 41 | 2.13 | 12 | 0.62 |
| T. D. 11/15/56 (After Rx) | 1 | 0.06 | 18 | 0.99 | 54 | 2.97 | 22 | 1.21 | 5 | 0.28 |
| Acute leukemia | | | | | | | | | | |
| T. C. | 8 | 0.14 | 6 | 0.11 | 20 | 0.35 | 35 | 0.61 | 31 | 0.54 |
| S. C. 4/20/57 | 6 | 0.24 | 3 | 0.12 | 39 | 1.55 | 39 | 1.55 | 13 | 0.52 |
| 4/29/57 (After Rx) | 11 | 0.22 | 3 | 0.06 | 30 | 0.59 | 49 | 0.96 | 7 | 0.14 |
| Leukocytoses | | | | | | | | | | |
| J. F. | 0 | 0.00 | 25 | 1.40 | 58 | 3.25 | 14 | 0.78 | 3 | 0.17 |
| J. A. | 7 | 0.19 | 5 | 0.14 | 18 | 0.49 | 45 | 1.22 | 25 | 0.68 |
| S. H. | 7 | 0.20 | 6 | 0.18 | 18 | 0.53 | 53 | 1.56 | 16 | 0.47 |

cytic leukemia and acute leukemia, increased binding by the alpha-2 globulins was found (Table IX).

*Leukocytoses.* Patient J. F., with the increase in serum vitamin $B_{12}$ concentration, UBBC, and TBBC, also demonstrated increased binding of $Co^{60}$ vitamin $B_{12}$ by the alpha-1 and alpha-2 globulins (Table IX). Two other patients with slight increases in the UBBC and TBBC had normal patterns of $Co^{60}$ vitamin $B_{12}$ binding by the serum proteins.

## DISCUSSION

Amounts of vitamin $B_{12}$ bound by the sera of normal subjects as determined in this study differ greatly from those previously obtained using microbiological assay procedures (3, 4, 5). Such differences in results are not surprising in view of the different methods employed. The binding of vitamin $B_{12}$ is defined physicochemically in a dialysis method, *i.e.,* by the ability of the free vitamin to traverse a cellulose membrane. This experimental system is not subject to the variables inherent in microbiological assay procedures.

A relatively constant amount of vitamin $B_{12}$ was bound by normal and leukemic sera when increasing concentrations of $Co^{60}$ vitamin $B_{12}$ were allowed to bind with normal and leukemic sera before dialysis. A similar limit in the vitamin $B_{12}$ binding has been reported using a dialysis system (10). Recently, others have reported different results using a dialysis method differing slightly from that used in this study (11). They found no limitation in the amount of vitamin $B_{12}$ bound by normal serum when the concentration of $Co^{60}$ vitamin $B_{12}$ was progressively increased. No reason for these differences in results is apparent at present.

In normal sera, using microbiological methods, the alpha globulins have been found to be the chief binding proteins for added vitamin $B_{12}$ (4). However, the beta globulins bound the greatest amount of added $Co^{60}$ vitamin $B_{12}$ in the present study. The alpha-2 globulins were next in vitamin $B_{12}$ binding ability but only negligible amounts were bound by the alpha-1 globulins. In previous investigations, excess vitamin $B_{12}$ was added to serum, the serum proteins fractionated by paper electrophoresis, and the vitamin $B_{12}$ content of the isolated protein fractions then determined. The considerable vitamin $B_{12}$ found in the beta globulin fraction was ascribed to "free" vitamin $B_{12}$,

*i.e.*, that available to *Euglena gracilis* without preliminary heating of the serum. However, we have found that a large fraction of the $Co^{60}$ vitamin $B_{12}$ "bound" by normal serum as determined by dialysis is measured as "free" by the *Euglena gracilis* assay (12). The vitamin $B_{12}$ in human liver homogenates has been found to be "free" as measured by *Euglena gracilis* even though the vitamin $B_{12}$ was associated with a protein which had a mobility similar to the beta globulins on electrophoresis (13). Actually, on electrophoresis "free" vitamin $B_{12}$ (the vitamin in saline solution) moves with the endosmotic flow and not as a beta globulin. It seems apparent that the differences in our findings and those previously reported are due to the different methods employed for the determination of vitamin $B_{12}$ binding. The interpretation of the *Euglena gracilis* method of determining serum vitamin $B_{12}$ binding is difficult if the vitamin $B_{12}$ moving with a protein on electrophoresis can be measured as "free."

It is of interest that the vitamin $B_{12}$ content of normal serum is primarily bound to the alpha-1 and alpha-2 globulins (4), whereas added vitamin $B_{12}$ was principally bound by the beta and alpha-2 globulins. The interpretation of these findings is not certain. It may be that the alpha-1 globulins are already saturated with vitamin $B_{12}$ accounting for their inability to bind additional vitamin $B_{12}$, whereas the alpha-2 globulins are only partially saturated and still capable of binding added vitamin $B_{12}$. The ready binding of added vitamin $B_{12}$ by the beta globulins could be due to an *in vitro* avidity of the protein for the vitamin having no significance *in vivo*. However, *in vivo* binding of vitamin $B_{12}$ by the beta globulins cannot be ruled out. Small amounts of the serum vitamin $B_{12}$ content were found bound to the beta globulins in some normal sera (4). A protein in human liver homogenates resembling a beta globulin on electrophoresis is also capable of binding vitamin $B_{12}$ (13). The small amount of vitamin $B_{12}$ bound to the beta globulins *in vivo* could result if its rate of serum turnover was rapid as compared to the vitamin bound to the alpha globulins.

The vitamin $B_{12}$ concentration, UBBC, and TBBC were all greatly elevated in chronic myelogenous leukemia as has been noted by other workers (1–3). The increase in UBBC was a labile abnormality decreasing rapidly to normal with the onset of treatment in contrast to the gradual decline in serum vitamin $B_{12}$ levels following treatment. Of the other leukemias studied, only the patients with chronic monocytic leukemia had elevations in vitamin $B_{12}$ concentration, UBBC, and TBBC approaching that found in chronic myelogenous leukemia. Elevated vitamin $B_{12}$ levels have also been reported in monocytic leukemia (3, 14), although no increase in binding capacity was found using the *Euglena gracilis* binding method (3). Similarities between the monocytic and myelogenous leukemic processes are suggested by these findings. Patients with granulocytic leukocytoses of varying etiologies usually had normal serum vitamin $B_{12}$ levels with increased UBBC's and TBBC's although the increase in UBBC and TBBC was usually not in the range found in chronic myelogenous leukemia. Thus, serum vitamin $B_{12}$ levels were more helpful in distinguishing these patients from those with chronic myelogenous leukemia. However, this was not uniformly so, and Mollin and Ross have reported increased serum vitamin $B_{12}$ levels in two patients with chronic leukocytoses (2).

In contrast to the findings in normal sera, the alpha-1 and alpha-2 globulins were the proteins responsible for tremendous increase in the UBBC of chronic myelogenous leukemic sera. The increase in binding by the alpha-1 globulins was most striking as negligible amounts of vitamin $B_{12}$ were bound by this protein in normal serum. Similar increased binding of added vitamin $B_{12}$ by the alpha-1 and alpha-2 globulins of myelogenous leukemic sera had been found, using microbiological assay techniques (1). However, the difference between normal and myelogenous leukemic sera was not described by the above workers since they found the alpha-1 and alpha-2 globulins of normal serum to be the chief binding proteins for added vitamin $B_{12}$. The vitamin $B_{12}$ content of myelogenous leukemic serum is also bound to the alpha globulins (1). It is of interest that, following treatment, the alpha globulins containing the vitamin $B_{12}$ content of the serum were cleared slowly from the circulation, whereas the alpha globulins responsible for the increased binding of added vitamin $B_{12}$ disappeared rapidly from the serum. Patients with an increased UBBC due to diseases other than chronic myelogenous leukemia always had increased binding of vitamin

$B_{12}$ by the alpha-2 globulins but only occasionally had elevated alpha-1 globulin binding. This would suggest that the increase in alpha-1 globulin binding of vitamin $B_{12}$ is the more specific change in chronic myelogenous leukemia. The percentage $Co^{60}$ vitamin $B_{12}$ bound by the other three protein fractions of myelogenous leukemic sera was decreased, whereas the $Co^{60}$ vitamin $B_{12}$ bound in mμg. per ml. was slightly increased as compared with the increase in alpha-1 or alpha-2 globulin binding.

The cause of the increased concentration of vitamin $B_{12}$ in chronic myelogenous leukemia serum is unknown. The rise in serum vitamin $B_{12}$ concentration to levels greater than the TBBC of normal serum and the invariable increase in UBBC both suggest an etiological relationship between the increased binding capacity and the hypervitaminemia. If vitamin $B_{12}$ is bound more firmly than normal by the binding substances in myelogenous leukemic sera, a decreased serum turnover of the vitamin with a resultant rise in its serum concentration would ensue. Rates of serum turnover of bound vitamin $B_{12}$ have been determined in chronic myelogenous leukemia following the intravenous administration of $Co^{60}$ vitamin $B_{12}$ (15). However, the estimation of turnover rates for normal subjects was not possible due to the small amounts of bound $Co^{60}$ vitamin $B_{12}$ remaining in the serum after 24 hours. The absence of hypervitaminemia despite the increase in UBBC in diseases other than chronic myelogenous leukemia is somewhat against such a hypothesis. Hypervitaminemia could also result from an excess amount of vitamin $B_{12}$ entering the serum without any change in the serum turnover rate of the vitamin. Myelogenous leukemic leukocytes dying intravascularly could be the source of the added vitamin (2, 16). Against such a hypothesis is the absence of an increased concentration of vitamin $B_{12}$ in myelogenous leukemic leukocytes (2, 16). If the survival time of chronic myelogenous leukemic leukocytes was shorter than that found in the other leukemias, an increased amount of vitamin $B_{12}$ could also be presented to the serum. However, studies of white cell survival have demonstrated that the leukocytes in chronic myelogenous leukemia have a longer survival time than those of the acute leukemias (17).

The chemical nature of the vitamin $B_{12}$ binding substances in myelogenous leukemic sera moving electrophoretically with the alpha globulins is also obscure. Whether these substances are proteins found normally in serum, but increased in chronic myelogenous leukemia, proteins of the serum that have been altered by the disease process, or abnormal tissue proteins foreign to normal serum, cannot be answered by this study.

## SUMMARY

1. An equilibrium dialysis method for the determination of the vitamin $B_{12}$ binding capacity of serum (UBBC) has been described. The serum concentration of vitamin $B_{12}$, the UBBC, and the total vitamin $B_{12}$ binding capacity (TBBC) of sera from normal subjects and patients with chronic myelogenous leukemia, other leukemias and leukocytoses have been determined.

2. In normal subjects, the average serum vitamin $B_{12}$ concentration was equal to 0.64 mμg. per ml., the UBBC to 1.51 mμg. per ml., and the TBBC to 2.23 mμg. per ml.

3. Patients with chronic myelogenous leukemia had an average serum vitamin $B_{12}$ concentration of 8.09 mμg. per ml., a UBBC of 8.87 mμg. per ml., and a TBBC of 16.86 mμg. per ml. Following treatment, the UBBC fell rapidly to normal, while the decline in serum vitamin $B_{12}$ concentration was a delayed and more gradual one.

4. Two patients with chronic monocytic leukemia, and one patient with leukocytosis had an increase in serum vitamin $B_{12}$ concentration and UBBC. Six of eight patients with leukocytosis and one of three patients with acute leukemia had normal serum vitamin $B_{12}$ levels but elevated UBBC's. Patients with chronic lymphocytic leukemia had a concentration of vitamin $B_{12}$ and UBBC within the normal range.

5. The beta globulins bound an average of 44 per cent of the added $Co^{60}$ vitamin $B_{12}$ (average, 0.79 mμg. per ml.) and was the chief binding protein in 16 of 18 normal subjects. The alpha-2 globulin was next in vitamin $B_{12}$ binding ability (an average of 28 per cent of the added $Co^{60}$ vitamin $B_{12}$ and 0.59 mμg. per ml.). The alpha-1 globulins bound the least $Co^{60}$ vitamin $B_{12}$ of any of the five protein fractions.

6. The alpha-1 globulins were the chief binding protein in 5 of 10 patients with chronic myeloge-

nous leukemia, binding an average of 38 per cent of the added $Co^{60}$ vitamin $B_{12}$ (3.55 mμg. per ml.). The alpha-2 globulins were the chief binding protein in 4 of the 10 patients, binding an average of 34 per cent of the added $Co^{60}$ vitamin $B_{12}$ (3.07 mμg. per ml.). With treatment of the disease, the pattern of vitamin $B_{12}$ binding by the serum proteins returned towards normal.

7. An increase in the binding of $Co^{60}$ vitamin $B_{12}$ by the alpha-1 and alpha-2 globulins was noted in one patient with chronic monocytic leukemia, and one other patient with leukocytosis. Four other patients with elevated UBBC's of varying degree, due to diseases other than chronic myelogenous leukemia, had an increase in vitamin $B_{12}$ binding by the alpha-2 globulins but no increase in alpha-1 globulin binding.

8. In addition to an increased serum concentration of, and binding capacity for, vitamin $B_{12}$ in chronic myelogenous leukemia, there is an abnormal distribution of added vitamin $B_{12}$ among the serum protein fractions in this disease.

ACKNOWLEDGMENTS

We are indebted to Merck and Company who kindly supplied the cobalt$^{60}$ labeled vitamin $B_{12}$ used in this study.

## REFERENCES

1. Beard, M. F., Pitney, W. R., and Sanneman, E. H. Serum concentrations of vitamin $B_{12}$ in patients suffering from leukemia. Blood 1954, **9**, 789.
2. Mollin, D. L., and Ross, G. I. M. Serum vitamin $B_{12}$ concentrations in leukaemia and in some other haematological conditions. Brit. J. Haemat. 1955, **1**, 155.
3. Raccuglia, G., and Sacks, M. S. Vitamin $B_{12}$ binding capacity of normal and leukemic sera. J. Lab. clin. Med. 1957, **50**, 69.
4. Pitney, W. R., Beard, M. F., and Van Loon, E. J. Observations on the bound form of vitamin $B_{12}$ in human serum. J. biol. Chem. 1954, **207**, 143.
5. Lear, A. A., Harris, J. W., Castle, W. B., and Fleming, E. M. The serum vitamin $B_{12}$ concentration in pernicious anemia. J. Lab clin. Med. 1954, **44**, 715.
6. Ross, G. I. M. Vitamin $B_{12}$ assay in body fluids using Euglena gracilis. J. clin. Path. 1952, **5**, 250.
7. Klotz, I. M., Walker, F. M., and Pivan, R. B. The binding of organic ions by proteins. J. Amer. chem. Soc. 1946, **68**, 1486.
8. Smith, E. L., Ball, S., and Ireland, D. M. $B_{12}$ vitamins (cobalamins). 2. Neutral, basic and acidic cobalamins. Biochem. J. 1952, **52**, 395.
9. Hine, G. J., and Miller, A. Large plastic well makes efficient gamma counter. Nucleonics Oct., 1956, **14**, 78.
10. Rosenthal, H. L., and Sarett, H. P. The determination of vitamin $B_{12}$ activity in human serum. J. biol. Chem. 1952, **199**, 433.
11. Bertcher, R. W., and Meyer, L. M. $Co^{60}$ vitamin $B_{12}$ binding capacity of normal human serum. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 169.
12. Miller, A. Unpublished observations.
13. Pitney, W. R., Beard, M. F., and Van Loon, E. J. The vitamin $B_{12}$ content of electrophoretic fractions of liver homogenates. J. biol. Chem. 1955, **212**, 117.
14. Beard, M. F., Pitney, W. R., Sanneman, E. H., Sakol, M. J., and Moorhead, H. H. Serum concentrations of vitamin $B_{12}$ in acute leukemia. Ann. intern. Med. 1954, **41**, 323.
15. Miller, A., Corbus, H. F., and Sullivan, J. F. The plasma disappearance, excretion and tissue distribution of cobalt$^{60}$ labelled vitamin $B_{12}$ in normal subjects and patients with chronic myelogenous leukemia. J. clin. Invest. 1957, **36**, 18.
16. Thomas, J. W., and Anderson, B. B. Vitamin $B_{12}$ content of normal and leukaemic leukocytes. Brit. J. Haemat. 1956, **2**, 41.
17. Osgood, E. E., Tivey, H., Davison, K. B., Seaman, A. J., and Li, J. G. The relative rates of formation of new leukocytes in patients with acute and chronic leukemias. Cancer (Philad.) 1952, **5**, 331.