Vitamin B\(_{12}\) Transport in Blood. I. Congenital Deficiency of Transcobalamin II

By Elisabeth Gimpert, Majo Jakob, and Walter H. Hitzig

Some characteristics of vitamin B\(_{12}\) binding and transport in the serum of an infant with congenital hereditary transcobalamin II (TC II) deficiency were studied using the following parameters and methods: vitamin B\(_{12}\) level and binding capacity; electrophoretic mobility in polyacrylamide gel electrophoresis; various immunodiffusion and absorption experiments, using a specific anti-TC II antiseraum and the patient’s serum as antigen. The results of these studies point to a deficient synthesis of TC II. Parenteral administration of high doses of vitamin B\(_{12}\) was followed by rapid and complete clinical remission and the appearance of vitamin B\(_{12}\) binder in the \(\alpha_2\) region which is similar to “fetal binder.” Thus, very high concentrations of vitamin B\(_{12}\), either carrier free or bound to this \(\alpha_2\) binder, were able to correct the disturbed physiology of TC II deficiency, presumably by normalization of DNA-thymine synthesis.

Under physiologic conditions, vitamin B\(_{12}\) in human blood is bound to specific plasma proteins. The main carrier proteins are transcobalamin I and II (TC I and TC II), but several others have been described—fetal binder, leukemia binder, polycythemia binder.\(^1\) TC I and TC II are proteins with electrophoretic mobilities of \(\alpha_1\) globulin and \(\beta\)-globulin, respectively, and plasma concentrations of 60 \(\mu\)g/liter for TC I and 25 \(\mu\)g/liter for TC II.\(^4\) The transcobalmins can be characterized and distinguished from each other by measuring the total vitamin B\(_{12}\) binding capacity of plasma, by the distribution of this capacity in chromatographic and electrophoretic fractions, and by immunologic methods using specific antisera. Clinical studies of congenital defects or molecular anomalies may yield new data concerning their physiologic functions.

We investigated an infant with congenital TC II deficiency. A few weeks after birth the child presented with severe life-threatening signs: pancytopenia due to bone marrow insufficiency, severe malabsorption due to atrophy of the intestinal mucosa, and marked hypogammaglobulinemia with absence of specific antibody production after antigenic stimulation. A rapid and complete return to normal of all these disturbances followed the parenteral administration of high doses of vitamin B\(_{12}\) (e.g., neutrophils rose from 280 to 12,000 within 6 days, thrombocytes from \(30 \times 10^6\) to \(1060 \times 10^6\) within 11 days), and remission was maintained by weekly intramuscular injections of the vitamin. It would seem that the clinical manifestations of congenital absence of TC II can be overcome by pharmacologic doses of vitamin B\(_{12}\). Family studies in our patient suggested that the deficiency was inherited as a recessive autosomal trait, similar to the families reported by Hakami et al.\(^8\) and Scott et al.\(^9\). The clinical

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The studies described below were done in an attempt to understand better the cause of TC II deficiency and the compensatory mechanisms taking over the physiologic function of TC II.

MATERIALS AND METHODS

Blood was obtained from the patient (B.A.) before and at varying intervals (2 wk and 3 mo) after parenteral vitamin B₁₂ therapy, from eight hematologically normal relatives of the patient (B.A.), and from ten normal volunteers (laboratory and hospital personnel) as controls. The serum was immediately separated and stored at −20°C until used.

Anti-TC II antiserum was generously provided by Dr. Ch. A. Hall (Albany), who had produced it in rabbits, using his own highly purified TC II preparation as antigen.³⁴

Commercial preparations of cobalamin labeled with ³⁷Co (obtained from Philips AG, Petten, Holland, through Galenica, Berne, Switzerland) with a specific activity of approximately 250 mCi/mg B₁₂ were diluted to a final concentration of 1 ng B₁₂/ml.

Routine methods were used for the hematologic work-up of the patient, his relatives, and controls.

Serum vitamin B₁₂ level was measured by radioisotopic assay using hemoglobin-coated charcoal.¹² Serum unsaturated vitamin B₁₂ binding capacity (BC) was determined by the method described by Gottlieb et al. with some of our own modifications: the serum samples were treated with 1 g/liter sodium cyanide in acetate buffer 0.125 M, pH 4.6, to stabilize free B₁₂ liberated by heating the TC II/B₁₂ complex to 100°C for 20 min. Carrier serum instead of intrinsic factor (0.5 ml of AB serum pool) was added, mixed for 60 min, and then treated with hemoglobin-coated charcoal to remove unbound vitamin B₁₂. Radioactivity in the supernatant was counted in a Packard gamma-counter Model 3002. In our own preliminary experiments the results obtained by use of the AB serum pool as a carrier were more reproducible than those obtained by use of intrinsic factor.

Polyacrylamide gel electrophoresis (PAGE) was performed with 20 μl of serum labeled with 1 μCi/ml ⁵⁷Co-cobalamin in a discontinuous TRIS-glycine buffer system with a gel concentration of 70% Albumin stained with bromphenol blue and unlabeled vitamin B₁₂ were used as markers for electrophoretic mobility and endosmosis, respectively. After 105 min separation time at 5 mA per tube, the migration distances were 60–70 mm. The whole length of the gel was then cut into 2-mm slides with a razor blade, and counted.

Immune reactions were studied using Ouchterlony’s two-dimensional immunodiffusion,¹⁵ immunoelectrophoresis,¹⁶ and simple and “tandem” crossed immunoelectrophoresis.¹⁷,¹⁸ For absorption studies, equal amounts (usually 0.5 ml) of anti-TC II antiserum and serum were mixed, incubated for 20 min at 37°C, and then kept at 4°C overnight. The mixture was dialyzed against barbital buffer (pH 8.6, ionic strength 0.05) for 3 days, and the absorbed sample was subjected to PAGE, immunoelectrophoresis, and/or other immunodiffusion procedures.

RESULTS

Serum Level of Vitamin B₁₂ and Serum Unsaturated Binding Capacity (Fig. 1)

Patient B.A. had a normal serum level of vitamin B₁₂ but virtually no unsaturated binding capacity (BC). Free binding capacity became available (more than 90%) 4 wk after the start of therapy and was the same in blood taken 1 hr after B₁₂ injection and immediately before the next dose, i.e., 7 days later. On the other hand, serum vitamin B₁₂ levels were very high (>2000 pg/ml) 1 hr after injection, and dropped to normal within 1 wk (Fig. 1). The serum levels of these two parameters in the patient’s relatives could be divided into two groups, one equal to normal values and the other intermediate between that of the normal controls and the patient’s.
 Immune Reactions

In different immunodiffusion systems, the anti-TC II antiserum provided by Dr. Hall produced three lines of specific precipitate upon reaction with normal serum. In Ouchterlony plates, normal serum after absorption with pure anti-albumin and pure anti-IgG gave one single line. The patient’s serum tested in a similar way formed no precipitate whatsoever (Fig. 2).

Immunoelectrophoresis (ImEP) of normal serum developed with anti-TC II antiserum showed one out of three lines of specific precipitate similar to IgG.
in mobility, position, and shape. The second line was a small precipitate in the albumin region and the third in the β-region. This third line was not produced when the patient’s serum was used as antigen (Fig. 3). During vitamin B₁₂ therapy, the β-precipitate was likewise absent, thus excluding the possibility that therapy had induced TC II synthesis (Fig. 3B).

These specific precipitates were identified by tandem crossed immunoelectrophoresis (Fig. 4), using an intermediate gel containing anti-TC II and reference gels containing anti-IgG and anti-albumin, respectively. Reactions of identity between the precipitates of patient and control serum were observed for IgG and for albumin, thus proving the presence of both corresponding antigens.

In a crossed immunoelectrophoresis (Fig. 5), normal serum and patient’s serum were run against anti-TC II antiserum in the second (perpendicular) direction. The antiserum concentration was adjusted so as to remove the IgG and albumin precipitates, but retain the TC II-anti-TC II precipitate. The optimum concentration was found to be 10% (v/v). Two peaks of slightly different mobilities were found with normal serum but none with the patient’s serum.

**Polyacrylamide Gel Electrophoresis**

Electrophoretic separation of vitamin B₁₂ carrier proteins was performed after the addition of ⁵⁷Co-labeled cobalamin to the serum samples. In Fig. 6 normal serum and the patient’s serum are compared: one peak with an electrophoretic mobility of 3.6 identifies TC I (in our own experiments, albumin does not bind ⁵⁷Co-labeled cobalamin). A second peak with the electrophoretic mobility of β-globulin (1.7) is present with control sera but absent in the serum of patient B.A. This β-peak was of intermediate height in the presumed heterozygous family members. It looks exactly like Fig. 7B, which, however, results from an artificial mixture of normal and patients serum 1:1. Planimetry of the area under the two peaks and calculation of the quotient (α-region)/(β-region) gave in both parents and three other relatives numerical values intermediate between normal controls and the patient’s (Table 1).
Fig. 4. Tandem crossed intermediate gel electrophoresis: the samples to be compared (patient’s serum, PS; normal serum, NS) were placed in two wells at a distance of 8 mm in an "empty" agarose gel and separated electrophoretically (anode to the right). Then intermediate gel slabs containing anti TC II (1) were applied longitudinally on both sides, and reference gels containing anti-IgG (2 on top), and antialbumin (3 on the bottom). Subsequently, the second electrophoretic run, performed in the perpendicular direction (anode at the bottom) developed the specific precipitates of IgG in 2 ("iceberg" in the top of the figure) and of albumin (bottom) showing identity with corresponding lines in the intermediate anti-TC II antiserum. The great difference in the height of the IgG-peaks in 2 is due to the hypogammaglobulinemia of the patient. The albumin precipitate in the intermediate gel 3 is pushed far outside to the interphase due to antigen excess.

Fig. 5. Crossed immunoelectrophoresis with normal serum. The first electrophoretic run was done in "empty" agarose in horizontal direction (bottom, anode to the right, 4 V/cm, 3 hr). Subsequently the second-dimension gel containing anti TC II was poured (top of figure), and the second electrophoresis in perpendicular direction (anode at bottom) was run overnight. Two specific precipitates of β-mobility are developed. No precipitates were formed with patient’s serum.
Fig. 6. Polyacrylamide gel electrophoresis (see text). Anode to the left. Color markers indicate place of application of sample including endosmosis (vitamin B₁₂) and migration of albumin stained with bromphenol blue. Radioactivity of added ⁵⁷Co-cobalamin was counted in 2-mm-wide fractions. (A) Control serum. (B) Patient before therapy. (C) Patient under therapy. The broken lines indicate the TC II activity in the control serum.

Fig. 7. Polyacrylamide gel electrophoresis absorption studies. Same arrangement as Fig. 6. (A) Control. (B) Patient and control serum mixed in equal amounts. (C) Control serum absorbed with antisemum anti-TC II. (D) Patient's serum absorbed with anti-TC II and subsequently mixed with equal amounts of control serum.
Table 1. Planimetric Quantitation of Vitamin B₁₂ Binders

<table>
<thead>
<tr>
<th>Subject With Symbol in Family Tree</th>
<th>TC₈, 100</th>
<th>TC₁, 100</th>
<th>(TCII-α-TCII), 100</th>
<th>Remarks</th>
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<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>range (N = 10)</td>
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<td></td>
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<tr>
<td>Patient B.A. IV 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before therapy</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under therapy</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>New binder, not TC II</td>
</tr>
<tr>
<td>Relatatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father III 13</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother III 18</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aunt III 11</td>
<td>20</td>
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</tr>
<tr>
<td>Uncle III 15</td>
<td>32</td>
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<td></td>
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<tr>
<td>Uncle III 16</td>
<td>26</td>
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<tr>
<td>Grandfather II 8</td>
<td>31</td>
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<tr>
<td>Uncle III 14</td>
<td>42</td>
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<td>Mixtures and absorption studies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Patient + control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>27</td>
<td></td>
<td></td>
<td>Like heterozygous</td>
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<tr>
<td>Patient + α-TC II</td>
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<tr>
<td>1:1</td>
<td>0.01</td>
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<tr>
<td>Control + α-TC II</td>
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<td></td>
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</tr>
<tr>
<td>1:1</td>
<td>1</td>
<td>47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient + α-TC II + control 1:1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20</td>
<td>Free α-TC II</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>available</td>
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The following experiments were performed to further characterize the missing β-binder in patient B.A.'s serum (Fig. 7): PAGE of a mixture of equal parts (1:1) of normal and patient's serum showed a β-peak of intermediate size similar to the group of heterozygous relatives (Fig. 7B, Table 1). Incubation of the mixture overnight gave a similar result, thus suggesting the absence of an inhibitor of TC II in the patient's serum.

If normal serum was first absorbed with anti-TC II, the β-peak disappeared, and a new smaller peak with a faster electrophoretic mobility (α-2) was seen (Fig. 7C). The TC I peak remained unchanged, indicating the absence of anti-TC I in the antiserum.

If the patient's serum was similarly absorbed with anti-TC II and then mixed with equal amounts of normal serum, the same changes occurred, i.e., disappearance of the β-peak and appearance of a smaller α-2 peak (Fig. 7D). This indicates that even after absorption with the patient's serum, the anti-TC II activity was still available to react with the TC II present in the normal serum.

After treating B.A. with high intramuscular doses of vitamin B₁₂, his serum separated by PAGE revealed a new peak moving more rapidly than TC II, i.e., in the α-2 region with an electrophoretic mobility of 2.2 (Fig. 6, Table 2). This new material did not react with anti-TC II neither in the Ouchterlony test nor in immunoelectrophoresis (Figs. 2 and 3).
Table 2. Electrophoretic Mobility of Vitamin B₁₂ Binders in PAGE

<table>
<thead>
<tr>
<th>Subject With Symbol in Family Tree</th>
<th>TC I</th>
<th>TC II</th>
<th>Other Binders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (N = 10) range</td>
<td>3.3–3.95</td>
<td>1.16–1.91</td>
<td></td>
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<tr>
<td>Patient BA. IV 5</td>
<td>3.60</td>
<td>Absent</td>
<td>2.1/2.16 fetal?</td>
</tr>
<tr>
<td>Before therapy</td>
<td>3.2/3.37</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>Under therapy before/after inj.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relatives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father III 13</td>
<td>3.16</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Mother III 18</td>
<td>3.54</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>Aunt III 11</td>
<td>3.40</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>Uncle III 15</td>
<td>3.39</td>
<td>1.79</td>
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</tr>
<tr>
<td>Uncle III 16</td>
<td>3.38</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>Grandfather II 8</td>
<td>3.96</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Uncle III 14</td>
<td>3.42</td>
<td>1.88</td>
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<td>Mixtures and absorption studies</td>
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<tr>
<td>Patient + control 1:1</td>
<td>3.77</td>
<td>1.78</td>
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<tr>
<td>Patient + α-TC II 1:1</td>
<td>3.60</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Control + α-TC II 1:1</td>
<td>3.43</td>
<td>2.89 (α-TC II-TC II)</td>
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<tr>
<td>(Patient + α TC II) + control 1:1</td>
<td>3.70</td>
<td>1.69</td>
<td>2.93 (α-TC II-TC II)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The transport of vitamin B₁₂ from the gut lumen to its intracellular sites of action is a complex phenomenon. The study of congenital disorders has greatly helped in its understanding, and presently at least four such disturbances can be located (besides exogenous vitamin B₁₂ deficiency):

1. Pernicious anemia due to absence of intrinsic factor synthesis manifests itself, at the earliest, after the age of 1 yr because a normal fetal liver has stored amounts of vitamin B₁₂ sufficient for this length of time (with the exception of the rare children born to mothers suffering themselves of vitamin B₁₂ deficiency during pregnancy). It has all the characteristic features of pernicious anemia as in later life.²⁰

2. In the Imerslund-Gräsbeck syndrome,¹⁹,²⁰ the transport of the intrinsic factor-vitamin B₁₂ complex through the ileal cell is impaired. This involves at least three phases,²¹ the presence of specific receptors in the ileal cell, of Ca²⁺ ions, and of a releasing enzyme which liberates B₁₂ from intrinsic factor.²² Again, the first signs of the deficiency are recognized after the end of the second year of life. Proteinuria is regularly associated with this disorder.

3. Impaired uptake by the blood stream.⁵,²¹,²³-²⁸ After having crossed the epithelial cell of the gut, vitamin B₁₂ is usually picked up by TC II which carries it to every cell in the body. The uptake of the vitamin by the cell membrane is apparently also mediated by TC II. These two important functions would
seem to account for the severe and early clinical manifestations of congenital TC II deficiency (patients of Hakami et al., Scott et al., and our own patient).

4. Deficiency of TC I was described in two brothers aged 47 and 46. They were hematologically normal, and the deficiency was an accidental finding made during the course of vitamin B₁₂ studies in the older brother after gastrectomy. The younger brother suffered from multiple sclerosis.

It is not surprising then that the clinical manifestations of congenital disturbances in vitamin B₁₂ transport are quite heterogeneous. They range from no apparent disturbance in TC I deficiency to predominantly hematologic abnormalities in pernicious anemia and Imerslund-Gräbeck syndrome, to the severe, life-threatening, and rapidly progressing disease associated with TC II deficiency.

Because the family history of our patient suggested a hereditary basis (two older brothers, nr IV-3 and IV-4 in the pedigree, died under identical signs) the following etiologic mechanisms were considered:

1. Deficient synthesis. At no time with any of the various methods used, could a protein similar to TC II in the patient's serum be demonstrated (Figs. 2–4, 6, and 7). One-half of the normal activity of TC II was found in both parents and in three paternal relatives, while two other relatives had normal binding activity and normal electrophoretic distribution (Tables 1 and 2). Specific anti-TC II antiserum was not exhausted after mixing with the patient's serum (Fig. 7). This is the most sensitive indication of the absence of the corresponding antigen TC II in patient BA.

2. In vitro, the presence of an inhibitor impairing TC II activity was excluded by the mixing experiment (Fig. 7) when the expected amount of activity (corresponding to a simple diluting effect) was found. One would expect that an inhibitor, had it been present, would inactivate considerably greater volumes of normal serum. The other possibility of an anti-TC II autoantibody could be excluded because prolonged incubation of the mixture equally had no effect.

3. Hypercatabolism of TC II in the patient would as well affect normal TC II, destroying an increasing amount during incubation in the above mixing experiments (Fig. 7).

4. The presence of an antigenically intact TC II molecule with deficient function was excluded by immunoelectrophoresis, the Ouchterlony test, and the failure to absorb anti-TC II antiserum with the patient's serum, thus excluding the presence of a cross-reacting antigen (Fig. 7).

All the evidence is in favor of severely impaired TC II synthesis in the patient.

**Effects of Vitamin B₁₂ Therapy**

The efficacy of high doses of vitamin B₁₂ suggests an auxiliary mechanism, substituting for the lacking TC II activity. Actually, after treatment a vitamin B₁₂ binder was found (Fig. 6), which is clearly distinguishable from TC I and TC II by its intermediate electrophoretic mobility and its very high binding capacity (Fig. 1). It shows no immunologic cross-reaction with anti-TC II. These three characteristics suggest the previously described "fetal binder" or similar proteins which have been designated as "third binder" or TC III by several workers.
Complete regression of all clinical signs proves that vitamin B\textsubscript{12} at very high concentrations can penetrate into the cell. This might be a simple diffusion effect,	extsuperscript{4} but the simultaneous appearance of a binder with \(\alpha\)-2 mobility leads us to consider also a possible carrier function of this substance. Other explanations would be that this \(\alpha\)-2 binder could be a by-product of cell metabolism, excluding leukocytes which release a "granulocyte-" or "leukemia-binder" resembling TC \textsubscript{I},	extsuperscript{20} or according to some of its characteristics it could be one of the vaguely defined R binders.	extsuperscript{19} Finally, the fact that its electrophoretic mobility is equal to the "fetal binder" suggests a hypothetical reactivation of a fetal protein synthesis.	extsuperscript{30}

A valid study object is the bone marrow: recent studies\textsuperscript{40,43} in megaloblastic anemias have proven that in cobalamin deficiency inadequate DNA-thymine synthesis results from decreased conversion of \(^\text{\textsuperscript{14}}\)N-methyltetrahydrofolic acid to tetrahydrofolic acid. This conversion is catalyzed by an enzyme itself dependent on a cobalamin coenzyme, which is presumed to be methyl-B\textsubscript{12}, the main substance carried by TC II. We have not yet been able to perform such studies with the serum of our patient, but our clinical data\textsuperscript{10} prove that not only the bone marrow, but also other rapidly replicating cells, such as the epithelium of the intestinal mucosa and the antibody-forming lymphoid cells, need a similar supply of vitamin B\textsubscript{12}.

ACKNOWLEDGMENT

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Vitamin B12 transport in blood. I. Congenital deficiency of transcobalamin II

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