Cyanocobalamin, Ascorbic Acid and Pteroylglutamates in Normal and Megaloblastic Bone Marrow

By E. V. Cox, D. M. Matthews, M. J. Meynell, W. T. Cooke and R. Gaddie

With the isolation of vitamin B₁₂,"¹² the synthesis of folic acid² and demonstration of the therapeutic activity of these substances, it has been accepted that megaloblastic anemia is a deficiency disorder as a direct result of reduction in body content of vitamin B₁₂ or pteroylglutamates or both. This view is supported by the occurrence of reduced serum levels of vitamin B₁₂ in patients responding to vitamin B₁₂ therapy and evidence suggesting folic acid deficiency in those with megaloblastic anemia of pregnancy and some patients with megaloblastic anemias who have normal serum levels of vitamin B₁₂."¹²

This hypothesis, however, is not entirely satisfactory. There are many reports of patients with vitamin B₁₂ deficiency and normoblastic erythropoiesis."¹³-²³ In Addisonian pernicious anemia, an erythropoietic inhibitor as an additional factor has already been suggested.²⁴ In order to explain the varying clinical patterns in Addisonian anemia with and without neurologic features, Mollin and Ross²⁷-²⁸ suggest differences in the amount of available folic acid and its related compounds. Spray and Witts,"²⁹ Girdwood,"³⁰ Cox et al."³¹ and Chanarin et al."³² have produced evidence of a disordered folic acid metabolism in Addisonian anemia. Thompson and Ungley"³² were unable to explain the megaloblastic anemia of pregnancy and the puerperium solely on a deficiency basis. Indeed, Ungley"³³ and Girdwood"³¹,³⁶ considered that in some instances this condition might be ascribed to a mal-utilization of the folic acid complex rather than to a reduction in the amount of folic-acid-like substances in the body. Since a megaloblastic anemia responding to ascorbic acid therapy has been described,"³⁴,³⁵ it would seem that the absence of this vitamin plays some role in the production of this type of dyserythropoiesis. This is in contradiction to the findings in monkeys"³⁶ and man"³⁷ in which the megaloblastosis of scurvy was considered to be due to an associated folic acid deficiency. Since low plasma concentrations of ascorbic acid"³⁸,³⁹ and evidence of a disturbed metabolism of ascorbic acid are found in pernicious anemia,"⁴⁰-⁴² the possibility exists that the manifestations of vitamin B₁₂ deficiency may be influenced by the concentration of ascorbic acid.

The purpose of this investigation was to ascertain to what extent specific deficiencies play a part in the etiology of megaloblastic anemia. It was assumed that a deficiency should be demonstrable in the tissue in which the abnormality existed. Therefore, the serum level of vitamin B₁₂, the Streptococcus faecalis activity of heparinized blood (folic acid activity—F.A.A.) and the plasma level of ascorbic acid in 12 normal subjects and 32 patients...
with hematologic disorders were determined simultaneously with estimations of these substances in the nucleated cells of bone marrow aspirates.

METHODS AND MATERIAL

Methods

Marrow samples were obtained by needle puncture of the sternum. In a few of the pregnant women they were taken from the iliac crest. One to 2 ml. of marrow was rapidly withdrawn and a small portion used to make films. The remainder was placed in a heparinized, siliconed tube, shaken and transferred to 2 or more Wintrobe tubes. These were centrifuged at 3000 rpm for 30 minutes. The volumes of the separated components were noted. Plasma and any fatty material floating on top of the plasma column were discarded. The layer of marrow cells was removed with a Pasteur pipette to a small beaker. Six, 8 or 10 ml. of 0.85 per cent saline (depending upon the marrow cell volume) were added and an even suspension made by repeatedly drawing up and ejecting cells and saline with a 10 ml. graduated pipette. Three ml. of the cell suspension was removed for a cell count and estimation of vitamin B\textsubscript{12} and F.A.A. To the volume remaining, an equal quantity of 10 per cent trichloracetlc acid was added with shaking. After standing for 5 minutes, the specimen was centrifuged and an estimation for ascorbic acid content performed on the supernatant.

Differential counts on the marrow films included at least 400 cells. Megaloblast counts signified the number of megaloblasts together with those erythroblasts which were so immature that they could not be differentiated into megaloblasts or normoblasts expressed as a percentage. Erythroblast counts included all nucleated red cell precursors. Cell counts on the marrow cell suspension were performed in suitable dilution in an improved Neubauer chamber. The number of red cells in the suspension rarely exceeded that of the nucleated cells, and after the preliminary experiments only the latter were counted.

All glassware used before and during the preparation of the cell suspension was siliconed, with the exception of the Wintrobe tubes.

Venous blood was taken for a full blood count and the estimation of serum vitamin B\textsubscript{12}, F.A.A. and plasma ascorbic acid. When the estimations of F.A.A. and vitamin B\textsubscript{12} were not carried out immediately, the suspensions were stored at —10 C. for up to 7 days; that for ascorbic acid was performed immediately.

Vitamin B\textsubscript{12} was estimated in the serum and marrow suspension with Lactobacillus leichmanii by the method of Meynell, Cooke, Cox and Gaddie,\textsuperscript{9} the normal range being between 105 to 450 \(\mu\)g. per ml. Ascorbic acid was determined in the plasma by the method of Roe and Kuether\textsuperscript{46} with the modification that incubation with 2,4-dinitrophenyl-hydrazine was shortened to 2 hours at 60 C. without significant alteration in the recovery estimations.

F.A.A. in the bone marrow suspensions and heparinized blood was assayed with Streptococcus faecalis by a modification of the method of Teply and Elvehjem.\textsuperscript{45} To 2.5 ml. heparinized blood, 2.5 ml. of 1 per cent acetate buffer (pH 4.6) and 20 ml. distilled water were added, the final dilution of the blood being 1 in 10. The tubes were placed in a water bath at 100 C. for 30 minutes, cooled and centrifuged. The reaction of the supernatant was adjusted to pH 5.9 with N. NaoH. To one or 5 ml. of supernatant, 5 ml. of folic acid double strength media ("DIFCO") were added. If 1 ml. was taken, 4 ml. of distilled water was added, so that in either case, the final volume was 10 ml. A standard of 8 tubes containing amounts of folic acid varying from 0.001 to 0.008 \(\mu\)g. per 10 ml. was set up with each batch of assays. All tubes were sterilized at 121 C. for 3 minutes and after cooling were inoculated with a standard culture of Streptococcus faecalis N.T.C.T. 8123. After incubation for 20 hours at 37 C., these were read turbidimetrically against the standard curve.

Levels below 2.0 \(\mu\)g. per ml. cannot be estimated. The mean F.A.A. obtained when one specimen of blood was assayed on 10 separate occasions was 3.92, S.D. 0.17, range 3.8 to 4.2 \(\mu\)g. per ml. The mean of 5 separate blood specimens taken from one patient
within 1 hour was 3.76 S.E. 0.10 range 3.6 to 3.8 mµg. per ml. The mean diurnal variation judged from assaying blood from 8 subjects, taken at 10 a.m., 2 p.m. and 5 p.m. was 0.13 S.D. 0.16, range, -0.4 to +0.4 mµg. per ml. The mean F.A.A. in the blood of 35 medical students and staff was 5.55 S.D. 3.4, range 2.0 to 22.0 mµg. per ml: only one subject had a blood F.A.A. above 10 mµg. In two normal subjects who had weekly assays for 10 weeks, the mean F.A.A. in blood was 7.3 and 7.6, S.D. = 0.5 and 0.45, ranges 6.8 to 8.0 and 7.0 to 8.4 mµg., respectively.

Clinical Material

Control observations were obtained from 10 normal subjects and two hospital patients with no detectable disease, aged 20 to 70 years. Thirty-two patients of comparable age with hematologic disorders formed the abnormal group: 10 patients with pernicious anemia (table 1) had a macrocytic anemia, megaloblastic erythropoiesis, histamine-fast achlorhydria, normal radiologic appearance of the stomach and small bowel, normal fecal fat excretion and remitted completely with therapy with vitamin B₁₂. Two patients had adult celiac disease, normal serum levels of vitamin B₁₂ and a megaloblastic anemia. Two patients had lesions of the small intestine (regional enteritis and enterococci), low serum levels of vitamin B₁₂ and a megaloblastic anemia responding to vitamin B₁₂ therapy. Six patients with megaloblastic anemia of pregnancy (table 2) developed the condition within two months before or three weeks after delivery and remitted completely after a short course of folic acid therapy. Two pregnant patients with a nonmegaloblastic anemia were also studied. Eleven patients had iron deficiency and hypochromic anemia (table 3); in three of these, the anemia was due to alimentary blood loss, in two to poor diets, in two to adult celiac disease; in two the anemia followed gastrectomy, in one, regional enteritis; and in one the cause was not discovered. All had low serum iron levels. Five of them had abnormally low serum concentrations of vitamin B₁₂ and another two had low levels falling below normal within two months of the initial observations. Erythropoiesis in these seven patients was normoblastic, though two showed occasional giant metamyelocytes and one of these had macronormoblasts in addition.

One patient had scurvy and an iron-deficient hypochromic anemia, normoblastic hyperplasia of the marrow and a plasma ascorbic acid of less than 0.02 mg. per 100 ml.

RESULTS

Serum Vitamin B₁₂, Plasma Ascorbic Acid and Blood F.A.A.

Control subjects.—Serum vitamin B₁₂ levels had a mean level of 240 mµg./ml., S.E. 30.9, range 105 to 450. The mean level of plasma ascorbic acid was 0.74 mg./100 ml., S.E. 0.097, range 0.28 to 1.17. The heparinized blood had a mean F.A.A. of 6.8 mµg./ml., S.E. 1.27, range 3.6 to 16.0 mµg./ml.

Pernicious anemia (table 1).—The serum vitamin B₁₂ concentrations were 100 mµg./ml. or less in every case. Plasma ascorbic acid was reduced, mean level 0.47 mg./100 ml. S.E. 0.079. The mean level of F.A.A., 3.1 mµg./ml. S.E. 0.85, though falling within the range of the levels in our normal group, was significantly different from the normal mean \( t = 7.07, n = 18, p = 0.001 \), i.e., the group as a whole fell consistently in the lowest range of normal. There was no correlation between these results and the hemoglobin level, the packed cell volume or the number of megaloblasts per 100 nucleated marrow cells.

Megaloblastic anemia of pregnancy (table 2).—Four of the six patients had reduced serum levels of vitamin B₁₂. All six had F.A.A. in blood in the lower range of normal. There was no correlation between the concentrations of vitamin B₁₂, F.A.A. or plasma ascorbic acid with either the degree of anemia or of marrow megaloblastosis.
Table 1.—Patients With Pernicious Anemia

<table>
<thead>
<tr>
<th>Case no.</th>
<th>RBC (X 10^6 cu.mm)</th>
<th>Hb. (Gm.%)</th>
<th>Serum B12 (µg./ml.)</th>
<th>Blood F.A.A. (µg./ml.)</th>
<th>Plasma ascorbic acid (µg./%)</th>
<th>Megalo-blasts (%)</th>
<th>Erythro-blasts (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.42</td>
<td>39% = 5.8</td>
<td>16.5</td>
<td>80</td>
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<td>0.26</td>
<td>9.75</td>
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<td>2</td>
<td>1.30</td>
<td>37% = 5.5</td>
<td>17</td>
<td>40</td>
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<td>0.31</td>
<td>21.5</td>
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<tr>
<td>3</td>
<td>1.48</td>
<td>34% = 5.0</td>
<td>15</td>
<td>100</td>
<td>3.4</td>
<td>0.85</td>
<td>18.25</td>
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<tr>
<td>4</td>
<td>1.46</td>
<td>36% = 5.3</td>
<td>17</td>
<td>80</td>
<td>2.8</td>
<td>0.39</td>
<td>39.25</td>
</tr>
<tr>
<td>5</td>
<td>3.69</td>
<td>87% = 12.9</td>
<td>39.5</td>
<td>100</td>
<td>2.0</td>
<td>0.36</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>1.90</td>
<td>57% = 8.5</td>
<td>23</td>
<td>25</td>
<td>2.4</td>
<td>0.45</td>
<td>12.5</td>
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<tr>
<td>7</td>
<td>1.23</td>
<td>35% = 5.3</td>
<td>16.5</td>
<td>95</td>
<td>4.0</td>
<td>0.12</td>
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<tr>
<td>8</td>
<td>1.97</td>
<td>54% = 8.0</td>
<td>23.0</td>
<td>60</td>
<td>2.0</td>
<td>0.76</td>
<td>9.75</td>
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<tr>
<td>9</td>
<td>1.16</td>
<td>35% = 5.2</td>
<td>15</td>
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<td>19.5</td>
<td>40</td>
<td>9.0</td>
<td>0.41</td>
<td>29.75</td>
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Table 2.—Patients With Megaloblastic Anemia of Pregnancy

<table>
<thead>
<tr>
<th>Case no.</th>
<th>RBC (X 10^6 cu.mm)</th>
<th>Hb. (Gm.%)</th>
<th>Serum B12 (µg./ml.)</th>
<th>Blood F.A.A. (µg./ml.)</th>
<th>Plasma ascorbic acid (µg./%)</th>
<th>Megalo-blasts (%)</th>
<th>Erythro-blasts (%)</th>
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<td>11</td>
<td>2.01</td>
<td>7.7</td>
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<td>—</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>2.2</td>
<td>5.5</td>
<td>21.5</td>
<td>90</td>
<td>1.8</td>
<td>0.3</td>
<td>20</td>
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<tr>
<td>16</td>
<td>1.23</td>
<td>5.3</td>
<td>17.5</td>
<td>365</td>
<td>2.6</td>
<td>0.54</td>
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Hypochromic anemia (table 3).—Five of the 11 patients had low serum levels of vitamin B₁₂ and no megaloblasts in the bone marrow, while two developed low serum levels within two months of the initial observations. The mean F.A.A. of whole blood was normal, 6.0 µg./ml., S.E. 1.45, but the plasma ascorbic acid (mean = 0.53 mg./ml., S.E. 0.11, range 0.28 to 1.56) was significantly reduced (t = 4.37, n = 19 and p = <0.001). Again no correlation existed between these findings and the anemia or marrow counts.

Marrow Findings

Volume.—In normal subjects, the mean volume of marrow cells was 73 cu. mm. per ml. of aspirate, volumes comparable to those recorded by others. The volume was slightly increased in iron deficiency anemia (mean 83 cu.mm., range 43 to 270 S.E. 24.4) and greatly increased in megaloblastic anemia (mean 197 cu.mm., range 42 to 490 S.E. 29.5; figs. 1 and 2).

Vitamin B₁₂, F.A.A. and ascorbic acid content of marrow cells (table 4).—The results of the estimations of vitamin B₁₂, F.A.A. and ascorbic acid have been expressed in two ways: relative to the unit volume of packed marrow cells and to the cell count expressed per million cells.

Pernicious anemia. Patients with Addisonian anemia showed a significant reduction in the vitamin B₁₂ content of the marrow whether expressed in relation to volume of packed cells (t = 5.2, n = 20, p = <0.001) or their number, i.e., per million (t = 3.78, n = 18, p = <0.01). The concentrations of F.A.A. and ascorbic acid were markedly reduced by unit volume of packed
Table 3.—Patients With Iron Deficiency Anemia

<table>
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<th>RBC (× 10⁶ cu.mm)</th>
<th>Hb. (Gm.%)</th>
<th>P.C.V.</th>
<th>Serum B₁₂ (µg./ml.)</th>
<th>Blood F.A.A. (µg./ml.)</th>
<th>Plasma ascorbic acid (mg.%)</th>
<th>Erythroblasts (%)</th>
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<td>2.46</td>
<td>4.1</td>
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<td>18</td>
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<td>34.0</td>
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<td>1.56</td>
<td>31</td>
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<td>21</td>
<td>2.9</td>
<td>8.0</td>
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<td>10.7</td>
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<td>5.2</td>
<td>0.28</td>
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<td>33</td>
<td>62</td>
<td>2.2</td>
<td>0.41</td>
<td>34</td>
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</table>

*Serum B₁₂ levels fell to below normal limits in those cases within 2 months.

Fig. 1.—B₁₂, folic acid activity and ascorbic acid content of bone marrow expressed per unit volume of packed marrow cells.

Norm. = normal subjects.
P.A. = Addisonian pernicious anemia.
M.A. = non-Addisonian megaloblastic anemia.
M.A.P. = megaloblastic anemia of pregnancy.
Fe. Def. = iron deficiency normoblastic anemia.

marrow cells (F.A.A. \( t = 7.548, n = 20, p < 0.001 \); ascorbic acid \( t = 5.370, n = 20, p < 0.001 \)). The concentrations of these substances per million cells did not differ significantly from the normal. In other words, though the concentrations of F.A.A. and ascorbic acid were reduced in the cells, the amounts of each per million cells were unaltered.

MEGALOBLASTIC ANEMIA OF PREGNANCY. Vitamin B₁₂ was reduced per ml. of packed marrow (\( t = 3.302, n = 16, p < 0.01 \)) and also per million cells, though the reduction was not statistically significant when expressed per million cells. Concentration of ascorbic acid per unit volume was reduced significantly (\( t = 3.626, n = 16, p < 0.01 \)), and there was a significant reduction in F.A.A. when expressed per million cells (\( t = 2.169, n = 14, p < 0.05 \)).
HYPOCHROMIC ANEMIA. Those patients with persistently normal serum levels of vitamin \(B_12\) had concentrations of vitamin \(B_12\) and F.A.A. within the normal range; concentrations of ascorbic acid were raised both per unit volume and per million cells. The five patients with low serum levels of vitamin \(B_12\) showed significant reductions in marrow vitamin \(B_12\), both per unit volume (\(t = 2.718, n = 15, p < 0.02\)) and per million cells (\(t = 2.831, n = 13, p < 0.02\)). There was no significant difference between this group and those having Addisonian anemia. The concentrations of F.A.A. and ascorbic acid in this group were within the normal range.

SCURVY. In the one patient investigated, ascorbic acid concentration was low, 1.9 mg. ascorbic acid per 100 ml. packed cells and 0.018 \(\mu\)g per million cells. The concentrations of vitamin \(B_12\) and F.A.A. were within normal limits.

OTHER ANEMIAS. In the two patients with adult celiac disease with megaloblastic anemia and normal serum levels of vitamin \(B_12\), the concentration of vitamin \(B_12\) was in the lower range of normal when expressed as micromicrograms per milliliter but completely normal when expressed per million cells. The concentrations of both F.A.A. and ascorbic acid were greatly reduced, expressed in either way.

In two patients with anatomic defects of the small bowel, low serum levels of vitamin \(B_12\) and megaloblastic anemias, the concentrations of vitamin \(B_12\) and ascorbic acid were low (figs. 1 and 2). In one, the concentration of F.A.A. was normal, but in the other the concentration was low, comparable to that found in patients with Addisonian anemia.

**Correlation Between Findings in Peripheral Blood and Marrow Cells**

In the two patients with hypochromic anemia in whom the serum levels of vitamin \(B_12\) fell to subnormal levels within two months of the observations, the levels of \(B_12\) in the marrow were low. Among the remaining patients 18 of the 21 patients with serum levels of vitamin \(B_12\) below 105 \(\mu\)g./ml. had con-
centrations in the marrow aspirates of 8000 μg./ml. or less and 19 had less than 21 μg. million cells. On the other hand, only 4 of 18 patients with normal serum levels of vitamin B12 had concentrations in the marrow below 8000 μg. per ml. and less than 21 μg. per million cells. There was a direct relationship between the serum levels and marrow content of vitamin B12 when expressed per unit volume ($r = +0.4807, d.f. = 35, p = <0.01$) but not when expressed per million cells ($r = +0.3046, d.f. = 35, p = >0.05$). The concentrations of F.A.A. in the blood and marrow showed no correlation (per ml. $r = +0.1758, d.f. = 32, p >0.10$; per million cell $r = +0.0562, d.f. = 31, p = >0.10$). A significant correlation was found between the plasma ascorbic acid and the concentration per milliliter in the marrow ($r = +0.3961, d.f. = 35, p = <0.02$) and per million cells ($r = 0.3933, d.f. = 33, p = <0.02$).

**Discussion**

Mollin and Ross\(^2\) demonstrated a correlation between the serum levels of vitamin B12 and the appearance of megaloblasts in the marrow of patients with Addisonian anemia who were allowed to relapse. However, not all patients with reduced serum levels of vitamin B12 have megaloblastic changes in the marrow, as is shown in the five patients with iron deficiency anemia and low serum levels of vitamin B12. Although there are some exceptions in the patients with megaloblastic anemia of pregnancy and in the two patients with hypochromic anemia who subsequently developed low serum concentration, in general, patients with low serum concentrations have low marrow concentrations, irrespective of the cytology.

In seeking an explanation, certain points must be considered. The assay method with *Lactobacillus leichmanii* is not specific, and other substances such as desoxyribosides may also be included in the measurement. The amount by which this may cause error has been estimated by Wolff, Royer and Karlin\(^5\) to be 5 to 15 per cent. Davidson, Leslie and White\(^3\) have shown that the megaloblasts have a high content of desoxyribosides; this could mask any difference between the content of vitamin B12 of megaloblastic and normoblastic marrows associated with low serum levels. Such an explanation is unlikely, since the reduction of concentration of vitamin B12 in both serum and marrow, whether expressed per unit volume or per million cells, is of the same order as that seen in the patients with Addisonian anemia.

*Streptococcus faecalis* activity (F.A.A.) in the given microbiologic assay conditions reflects the activity of the pteroylglutamate complex in terms of the known amounts of folic acid. This measure of the pteroylglutamate complex leaves much to be desired, since different members of this complex have different degrees of microbiologic activity. In other words, qualitative changes within the complex may not be detected though a reduction of F.A.A. might be indicative of a reduced concentration of a part of the whole of the complex. Thus, the apparently lower concentration of F.A.A. per milliliter in the marrow of Addisonian anemia suggests an associated pteroylglutamate deficiency which could be of importance in the development of megaloblastic erythropoiesis. There is, however, no significant reduction from normal of F.A.A. content per
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million cells so that the reduction per milliliter could be the result of the larger cell size and the increased amount of active bone marrow in the body as a whole—the result but not the cause of megaloblastic changes. Similarly, the significantly lower concentration per unit volume of ascorbic acid in the marrow of Addisonian anemia patients could be the result of the increase in amount of active marrow. The disordered metabolism of both folic acid and ascorbic acid in Addisonian anemia also seems to be associated with rather than the cause of the disordered erythropoiesis. That there is no significant deficiency of either ascorbic acid or folic acid and its related compounds is indicated by the rarity with which these substances are required in the therapy of Addisonian anemia.

The incidence of low serum levels of vitamin B12 in pregnancy is significant, 20 per cent according to Heinrich and in a group of 100 anemic patients as high as 70 per cent. In the megaloblastic anemias of pregnancy, therapy with vitamin B12 was used initially without success, although Holly later obtained a response to vitamin B12 and ascorbic acid when administered together. In 1952, Nieweg reported one patient and has since presented other examples. Further patients with megaloblastic anemia of pregnancy responding to vitamin B12 therapy have been reported by others. Forshaw has shown that large doses of vitamin B12 are more likely to be effective. Nieweg and his associates pointed out the dual nature of this disorder, some patients having low serum levels of vitamin B12 and normal levels of F.A.A., others, normal serum levels with low values for F.A.A. and responding only to folic acid therapy.

In four of the six patients with megaloblastic anemia of pregnancy now presented, the serum concentrations of vitamin B12 were low. One of these patients had completely normal marrow concentrations while the two with normal serum levels of vitamin B12 had low concentrations in the marrow. Two patients who proved to have no dyshematopoiesis had normal marrow levels (8500 and 10,300 µg./ml and 437 and 620 µg./million cells, respectively), even though one had a serum level of 95 µg./ml. In only one of the patients with megaloblastic anemia of pregnancy were the concentrations of F.A.A. and ascorbic acid within normal range: this was the patient with the low serum level and normal marrow concentration of vitamin B12. It is possible that the megaloblastosis in this patient was due to resistance to folic acid or vitamin B12 during pregnancy, as suggested by Badenoch et al. or the result of interference with folic acid utilization by unknown factors. Though interference by an as yet unidentified factor cannot be ruled out, some of our results suggest a combined deficiency of vitamin B12, folic acid and ascorbic acid. Since patients with a dual deficiency of vitamin B12 and folic acid show an incomplete or no response to vitamin B12 and require folic acid to achieve remission, the infrequent response to vitamin B12 in megaloblastic anemia of pregnancy could be due to the associated folic acid deficiency. High dosage of vitamin B12 may be successful therapeutically because the small amounts of folic acid present are enabled to be used more effectively. Thus the correction of two deficiencies, i.e., those of vitamin B12 and ascorbic acid, might produce a response in the presence of reduced levels
of folic acid and provide the explanation for the good results seen by giving vitamin B₁₂ and ascorbic acid together.⁵⁴,⁵⁹ However, though our results indicate that megaloblastic anemia of pregnancy can occur without a detectable deficiency in the marrow and that others have multiple deficiencies, it is more than probable that cases do exist with either a simple deficiency of folic acid or vitamin B₁₂, as was suggested by Nieweg et al.⁶³

Finally, in all the megaloblastic anemias there was a significant reduction of ascorbic acid in the bone marrow. It has been shown that vitamin B₁₂ has a "sparing" effect on the plasma concentrations and utilization of ascorbic acid.⁴² However, the low concentrations of ascorbic acid were not confined to megaloblastic anemias due to deficiency of vitamin B₁₂. Indeed, one of the patients with adult celiac disease, megaloblastic anemia and normal marrow and serum levels of vitamin B₁₂ had a concentration of ascorbic acid in the marrow as low as that found in the patient with scurvy. The significance of these observations is obscure.

**Summary**

The B₁₂ activity as estimated by *Lactobacillus leichmannii*, the folic-acid-like activity by *Streptococcus faecalis* (F.A.A.) and the ascorbic acid concentration have been determined in the blood and buffy coat of bone marrow of normal subjects, 10 patients with pernicious anemia in relapse, a group of patients with non-Addisonian megaloblastic anemia and some patients with iron deficiency.

A correlation between the serum B₁₂ and the plasma ascorbic acid and their respective levels in bone marrow was observed. The marrow and serum B₁₂ levels in pernicious anemia were abnormally low, but they did not differ from a group of 5 patients with hypochromic normoblastic anemia who had both low serum and marrow levels. The concentration of F.A.A. in the marrow of patients with pernicious anemia was reduced, but it was felt that this was more likely a manifestation of the megaloblastic anemia rather than a causative factor.

One of six patients with megaloblastic anemia of pregnancy had no detectable deficiency, while the other five had reduced B₁₂, folic acid and ascorbic acid concentrations. The possible therapeutic implications are discussed.

There was a significant reduction in the bone marrow concentration of ascorbic acid in all patients with megaloblastic anemia.

**Summario in Interlingua**

Le activitate de B₁₂ (estimate secundo *Lactobacillus leichmannii*), le activitate de acido folic o su equivalente (secundo *Streptococcus faecalis*), e le concentration de acido ascorbic esseva determinate in le sanguine e le coagulo blanc de medulla ossee ab subjectos normal, ab 10 patientes con anemia perniciose in recidiva, ab un gruppo de patientes con anemia megaloblastic non-addisonian, e ab certe patientes con carentia de ferro.

Esseva trovate un correlation inter le valores pro B₁₂ del sero e acido ascorbic del plasma e le correspondent valores in le medulla ossee. Le nivellos medullari e seral pro B₁₂ esseva anormalmente basse in anemia
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perniciose, sed illos non differeva ab le nivellos trovate in un gruppo de 5 patientes con anemia normoblastic hypochronic qui etiam havaeva basse valores de B12 in lor sero e lor medulla. Le concentration del activitate equivalente a acido folic in le medulla de patientes con anemia perniciose esseva reduce, sed le conclusion pareva justificate que isto esseva plus probablemente un manifestation del anemia megaloblastic que un factor in su causation.

Un de sex patientes con anemia megaloblastic de pregnantia havaeva nulle detegibile carentia, durante que le altere cinque havaeva reducecentra- tiones de B12, acido folic, e acido ascorbic. Le possibile signification therapeutic de iste observationes es discutite.

Esseva notate un reduction significative del concentration medullari de acido ascorbic in omne le patientes con anemia megaloblastic.

REFERENCES
18. Victor, M., and Lear, A. A.: Subacute combined degeneration of the spinal...
46. Roe, J. A., and Kuether, C. A.: The determination of ascorbic acid in whole blood and urine through the
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2.4 dinitrophenylhydrazine derivative of dehydroascorbic acid. J.Biol.Chem. 147:399, 1943.


Cyanocobalamin, Ascorbic Acid and Pteroylglutamates in Normal and Megaloblastic Bone Marrow

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