Experimental Megaloblastic Anemia in Young Guinea Pigs

By Rolv K. Slungaard and George M. Higgins

Zuelzer and Ogden,\(^1\) in 1946, described anemia in infants that was associated with the development of megaloblasts in the bone marrow. They proposed the name “megaloblastic anemia of infancy” for this condition. A further consideration of this disease entity appeared in 1953.\(^2\)

Relatively few studies have reported the production of megaloblastosis in animals. This may be due to the fact that most animals synthesize ascorbic acid, the lack of which is presumably of importance in the development of megaloblastosis. Thus, the monkey, which depends on exogenous sources of ascorbic acid, has been the animal more extensively studied.

Wills and Bilimoria,\(^3\) in 1932, produced megaloblastic anemia in monkeys by dietary means, but the most comprehensive study on experimental megaloblastosis was reported by a group of investigators at the University of Minnesota in a later series of papers.\(^4\)-\(^11\) Using milk diets, they explored the role of ascorbic acid in the induction of a megaloblastic anemia that was indistinguishable from the megaloblastic anemia of infancy. They evaluated the effects of ascorbic acid, pteroylglutamic acid (PGA), folic acid and vitamin B\(_{12}\) in the treatment of this anemia.

Guinea pigs have not been extensively used, although this animal, like the monkey, depends for its requirements of ascorbic acid on exogenous sources and thus should prove a satisfactory test animal for such studies. Antimetabolites and sulfonamides have been given to guinea pigs in an attempt to inhibit the function or prevent the synthesis of essential vitamins.\(^12\)-\(^15\) In these experiments certain changes resembling some of those seen in megaloblastic anemia of infancy were produced. These changes may be due to some specific action on hematopoiesis or to a toxic effect on the cells themselves.

The present report is the result of an attempt to study certain etiologic factors in megaloblastic anemia of infancy by the use of animals other than the monkey, using purified diets exclusively. Since histories of inadequate diets almost always are obtained in infants who have this disorder, it would appear logical to attempt the production of megaloblastic anemia in animals by means of known dietary deficiencies. Our experiments, therefore, were designed to observe the results of a single dietary deficiency and then to induce multiple deficiencies of essential hematopoietic metabolites. Vitamin B\(_{12}\), PGA and ascorbic acid have all been implicated in the clinical histories, and all of these metabolites have been used clinically in the treatment of the disorder. A study of the effects of these deficiencies, either singly or in combination, appeared to be a reasonable approach to the problem.

From the Mayo Clinic and the Mayo Foundation, Rochester, Minnesota.

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EXPERIMENTAL MEGALOBLASTIC ANEMIA

MATERIALS AND METHODS

Guinea pigs were chosen as test animals because, as already indicated, they rely on a dietary source of ascorbic acid and because they are inexpensive and easy to maintain. Disadvantages in their use are: (1) they are herbivorous and the results obtained are not directly applicable to omnivorous human beings; (2) they are susceptible to infections, which obviously could introduce an unknown factor; (3) they succumb rather readily to dietary deficiencies and (4) samples of blood are not easily obtained in very young animals.

In order to produce a distinctly defined dietary deficiency, it was necessary to use a purified diet in which all components were known. Such a diet for guinea pigs has been recently described by Reid and Briggs; it is composed of casein, sucrose, dextrose, cornstarch, corn oil, cellophane spangles, a salt mixture and vitamins. Test diets used in the present experiments differed only in the restriction of PGA, vitamin B$_2$ or ascorbic acid or of combinations thereof.

Young lactating animals were removed from their mothers and given these diets at the age of 4 to 7 days. Diets deficient in one of these three essential nutrients were given to each of three groups of eight animals. Diets deficient in two of the three essential nutrients were given to each of two groups of eight animals and to one of seven, while diets depleted of all three substances were given to a group of four animals. One group of eight animals was fed the complete, purified fortified diet and served as a control group. The adequacy of this purified diet was tested by a comparison of the growth of these animals with the growth of eight animals which were fed a regular commerical stock diet and kept under environmental conditions identical to those of the test groups.

The animals were weighed twice each week and their daily intake of food was recorded. In each experiment using diets deficient in ascorbic acid, either alone or in combination with other nutrients, two series of animals were used; in series I ascorbic acid was withheld from the beginning of the experiment, and in series II the ascorbic acid component of the diet, initially adequate, was decreased gradually and then completely eliminated after 4 weeks.

Biopsy of bone marrow was performed at regular intervals, usually at 30, 60 and 90 days. Bone marrow was obtained, however, whenever the clinical condition of the animal appeared critical. After appropriate staining of the marrow, 500 cells were counted and a complete myelogram was constructed. In the identification of megaloblasts and giant metamyelocytes, the criteria laid down by Jones and by Downey were used. Myeloid-erythroid (M-E) ratios were calculated in each instance.

Samples of blood were obtained by cardiac puncture at the same time that the specimens of bone marrow were obtained. Values for hemoglobin, the cell volume per cent (hematocrit) and the mean corpuscular hemoglobin concentration (MCHC) were determined. Erythrocyte and reticulocyte counts were made and the mean corpuscular volume (MCV) was calculated. Smears of peripheral blood were studied for hypochromasia, macrocytosis, anisocytosis and polychromatophilia. In certain instances bacteriologic studies of the stools were made. Necropsy of most animals was done. Stained sections were prepared from several organs, including the adrenal glands.

RESULTS

Detailed nutritional data are to be published elsewhere, but the essential manifestations of the various deficiencies will be set forth in connection with the hematologic data. Animals fed the complete purified diet and maintained under identical conditions attained weights comparable to weights of animals fed the commercial stock ration. Thus, it was evident that such a purified diet was entirely adequate for guinea pigs and that deviations encountered in the groups of test animals must, therefore, be due to the specific deficiencies we had instituted.

* We are grateful to Dr. Pease, of the Section of Clinical Pathology of the Mayo Clinic, for assistance in the morphologic interpretation of our preparations.
TABLE 1.—Comparison of Average Differential Counts (Per Cent) of Bone-Marrow Cells in Two Groups of Eight Guinea Pigs Each, One Fed Commercial Stock Diet (CS), the Other Fed Purified Complete Diet (PC)

<table>
<thead>
<tr>
<th>Days on Diet</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>CS</td>
<td>PC</td>
<td>CS</td>
</tr>
<tr>
<td>Pronormoblasts</td>
<td>1.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Basophilic normoblasts</td>
<td>8.6</td>
<td>1.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Polychromatic normoblasts</td>
<td>10.2</td>
<td>27.4</td>
<td>13.4</td>
</tr>
<tr>
<td>Orthochromatic normoblasts</td>
<td>0.2</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>9.5</td>
<td>3.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>33.6</td>
<td>29.7</td>
<td>32.8</td>
</tr>
<tr>
<td>Polymorphonuclear granulocytes</td>
<td>13.3</td>
<td>4.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>21.4</td>
<td>32.0</td>
<td>29.1</td>
</tr>
<tr>
<td>Reticuloendothelial cells</td>
<td>0.6</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Blast cells</td>
<td>1.3</td>
<td>0.3</td>
<td>2.1</td>
</tr>
<tr>
<td>M-E ratio</td>
<td>3.78:1</td>
<td>1.27:1</td>
<td>2.94:1</td>
</tr>
</tbody>
</table>

TABLE 2.—Comparison of Average Values in Peripheral Blood in Two Groups of Eight Guinea Pigs Each, One Fed Commercial Stock Diet (CS), the Other Fed Purified Complete Diet (PC)

<table>
<thead>
<tr>
<th>Days on Diet</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>CS</td>
<td>PC</td>
<td>CS</td>
</tr>
<tr>
<td>Hemoglobin, Gm. per 100 cc.</td>
<td>13.8</td>
<td>13.5</td>
<td>15.0</td>
</tr>
<tr>
<td>±0.45</td>
<td>±0.43</td>
<td>±0.41</td>
<td>±0.39</td>
</tr>
<tr>
<td>Hematocrit, cell volume per cent</td>
<td>36.5</td>
<td>33.9</td>
<td>39.3</td>
</tr>
<tr>
<td>±1.82</td>
<td>±1.04</td>
<td>±1.50</td>
<td>±2.31</td>
</tr>
<tr>
<td>Erythrocytes, millions per cubic millimeter</td>
<td>5.13</td>
<td>5.38</td>
<td>5.77</td>
</tr>
<tr>
<td>±0.39</td>
<td>±0.14</td>
<td>±0.27</td>
<td>±0.28</td>
</tr>
<tr>
<td>MCV, cubic microns</td>
<td>71.9</td>
<td>63.3</td>
<td>68.4</td>
</tr>
<tr>
<td>±3.09</td>
<td>±1.07</td>
<td>±2.32</td>
<td>±1.42</td>
</tr>
<tr>
<td>Reticulocytes, per cent</td>
<td>0.7</td>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Normal hematologic values for the strain of pigs we were using were first assembled. The values given in the literature differ considerably, due essentially to the fact that authors failed to take cognizance of the age factor. Therefore, data obtained from samples of the cardiac blood and of bone marrow were assembled from eight animals which were provided the commercial stock diet at 4 to 7 days of age. Samples of cardiac blood and of bone marrow were obtained when the animals were 30, 60 and 90 days of age. During this period the MCHC decreased from 39.9 Gm. per 100 cc. of packed cells, recorded on the thirtieth day, to 34.4 Gm. on the ninetieth day of age. The mean of data obtained from three animals on the 114th day was 31.6 per cent. Data assembled from the blood and bone marrow of eight animals of the same age which were fed the purified diet did not differ significantly from those obtained from animals fed the stock ration (tables 1 and 2). Therefore, this purified diet was considered...
### Table 3.—Effect of Vitamin Deficiencies on Bone Marrow in 51 Guinea Pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin deficiency in diet</td>
<td>C</td>
<td>PGA</td>
<td>B₁₂</td>
<td>C, PGA</td>
<td>C, B₁₂</td>
<td>PGA, B₁₂</td>
<td>C, PGA, B₁₂</td>
</tr>
<tr>
<td>Animals in each group</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Megaloblastosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Giant metamyelocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>With megaloblastosis</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Days before megaloblastosis appeared</td>
<td>—</td>
<td>26.5</td>
<td>30</td>
<td>I* 17</td>
<td>—</td>
<td>35</td>
<td>23</td>
</tr>
<tr>
<td>Spontaneous remissions</td>
<td>—</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>—</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>M:E ratio† &gt; 7:1, with megaloblastosis</td>
<td>0</td>
<td>0</td>
<td>1(+1/-3)</td>
<td>1(+1/-2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M:E ratio† &lt; 0.5:1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1(-2/+1)</td>
<td>6‡</td>
<td>1(-2/+1)</td>
<td>6§</td>
<td>1(-3/+2)</td>
<td>2‖</td>
<td>2(0/+3)</td>
</tr>
<tr>
<td>With megaloblastosis</td>
<td>2(-1/+2)</td>
<td>3(-1/+2)</td>
<td>3(-1/+2)</td>
<td>1(-1/+2)</td>
<td>2(0/+3)</td>
<td>2(0/+3)</td>
<td></td>
</tr>
</tbody>
</table>

* Roman numerals indicate series I and II.
† Numbers in parentheses indicate myelopoietic (before slant) and erythropoietic activity (after slant). Both are graded from +3 to −3; zero denotes normal activity.
‡ Activity was −1/+3 in four animals without megaloblastosis.
§ Activity was −1/+3 in three animals without megaloblastosis.
‖ Activity was −1/+2 in one animal without megaloblastosis.
adequate for normal hematopoiesis in the guinea pig, as well as for growth and development.

Normal hematologic values accepted for the guinea pigs used in this study and fed the purified adequate diet were as follows: hemoglobin, more than 12 Gm. per 100 cc. of blood; hematocrit, 30 to 45 per cent; erythrocytes, 5,000,000 to 7,000,000 per cubic millimeter; mean corpuscular volume (MCV), 60 to 80 cubic microns; reticulocytes, less than 1 per cent and myeloid-erythroid (M-E) ratio, 0.5:1 to 7:1.

The hematologic data obtained from 51 animals included in the seven test groups fed the deficient diets are summarized in the accompanying tables; table 3 includes the data obtained from bone-marrow biopsies and table 4 the findings in the peripheral blood.

Deficiency of Ascorbic Acid.—When ascorbic acid (vitamin C) was withheld from the diet (group 1), megaloblastosis or giant metamyelocytes did not appear in any animal. Anemia developed in four animals, two of series I (vitamin withheld from the start of the experiment) and two of series II (vitamin withdrawn after the experiment was under way). The MCV was increased in two animals and decreased in five. Pronounced reticulocytosis was present in all eight instances.

The clinical condition was characterized by loss of weight, loss of appetite, onset of diarrhea and death within a relatively short time (26 to 75 days). At necropsy, hemorrhage into the internal organs was commonly found. The adrenal cortices were usually hypertrophic and only sparse amounts of lipid substance were present.

Deficiency of Pteroylglutamic Acid.—Of the animals fed the diet deficient in PGA (group 2), four of the eight presented evidence of megaloblastosis of an intermediate type during the first 30 days of the experiment (table 3). Two of these animals died shortly thereafter, as did two that did not show this feature. On the sixtieth day one of the two remaining animals with megaloblastosis showed evidence of a spontaneous remission; at this time the other animal still had megaloblasts of intermediate type, whereas by the eightieth day megaloblasts of the classic type had developed. This animal likewise had a spontaneous remission later. Giant metamyelocytes were seen in the bone marrow in three of the four animals which had megaloblastosis; in the first animal which displayed a spontaneous remission, giant metamyelocytes were found after the megaloblasts had disappeared. The M-E ratios were abnormally low in six animals due to a mild depression of myelopoiesis with a simultaneous increase in erythropoietic activity. The latter was less pronounced in the two animals in which megaloblastosis was present.

Anemia of relatively mild degree developed in four animals (table 4). It appeared in 23 to 190 days and was associated with megaloblastosis in two animals. The MCV was decreased in three animals, none of which showed megaloblastosis. Reticulocytosis was present in three animals, none of which showed megaloblastosis.

The animals in this group had diarrhea and loss of weight, and their intake of food was low during the first 30 days, when megaloblasts first appeared in the
| Table 4.—Effect of Vitamin Deficiencies on Peripheral Blood in 51 Guinea Pigs |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|
|                                | 1     | 2     | 3     | 4     | 5     | 6     | 7     |
| Vitamin deficiency in diet     | C     | PGA   | B₁₂   | C, PGA | C, B₁₂ | PGA, B₁₂ | C, PGA, B₁₂ |
| Animals in each group          | 8     | 8     | 8     | 8     | 7     | 8     | 4     |
| Animals with < 12 Gm. of hemoglobin per 100 cc. | I 2(10.7) | I 1(10.5) | II 2(10.9) +2? | II 1(9.4) | II 3(8.9) | 4(10.3) | 4(11.0) |
| Days on diet before anemia appeared | I 22s 93.7; range = 23 to 190 | I 22 | I 34 | I 20 | 64.4; range = 41 to 100 | 19.5 |
| Anemia with megaloblastosis     |       |       |       |       |       |       |       |
| Total                           | 0     | 1 + 1?| 0     | 5 + 2?| 0     | 3 + 2?| 2     |
| Classic type                    |       | 1 + 1?|       | 1?    |       | 2 + 1?| 2     |
| Intermediate type               |       | 5 + 1?|       | 1?    |       | 1 + 1?|       |
| MCV > 80 cubic microns         |       |       |       |       |       |       |       |
| Total                           | 2     | 0     | 2     | 1     | 2     | 2     | 0     |
| With megaloblastosis            | 0     | 0     | 0     | 0     | 0     | 2     | 0     |
| MCV < 60 cubic microns         |       |       |       |       |       |       |       |
| Total                           | 5     | 3     | 3     | 4     | 4     | 2     | 4     |
| With megaloblastosis            | 0     | 0     | 0     | 0     | 0     | 2     | 0     |
| Reticulocytosis (1%+)           |       |       |       |       |       |       |       |
| Total                           | 8     | 3     | 2     | 5     | 3     | 1     | 0     |
| With megaloblastosis            | 0     | 0     | 0     | 1     | 0     | 1     | 0     |

* Roman numerals indicate series I and II.  
† Numbers in parentheses in this horizontal column indicate average value for hemoglobin.  
‡ Question mark indicates animals which were questionably anemic.  
§ One animal changed from an MCV of 60 to an MCV of 80.
bone marrow. Four animals dying during this stage showed atrophic adrenals that were almost completely devoid of cortical lipid material (fig. 1).

The surviving four animals of this group appeared to adjust to the deficiency and no clinical abnormalities were observed, although one of them continued to show megaloblastosis. Daily weight gains decreased and one animal in apparently good condition died on the 110th day. Necropsy revealed bilateral adrenal hemorrhage as the only pathologic finding. The adrenal cortex was hypertrophied and contained normal amounts of lipid material (fig. 2); this was also the finding in the other animals of this group surviving for more than 90 days. Platelets were not counted routinely in this study, but smears of the peripheral blood showed that they were obviously reduced in most instances when a diet deficient in PGA was fed.

Deficiency of Vitamin B12.—The diet deficient in vitamin B12 (group 3) produced megaloblasts of the intermediate type in one animal by the thirtieth day (fig. 3). Giant metamyelocytes were identified at the same time, but they also were observed in one animal which did not reveal megaloblasts. Both changes disappeared spontaneously. The M-E ratio was increased in one instance due to greatly decreased erythropoiesis; the ratio was decreased in another due to depression of myelopoiesis. This was not associated with megaloblastosis.

Anemia developed in three animals after an average of 115 days; it was not

![Fig. 1. a. Adrenal gland from a guinea pig fed stock ration. b. Adrenal gland from a guinea pig fed a diet deficient in PGA. There is atrophy of the adrenal cortex in the PGA-deficient animal and very little lipid material is present (both Sudan IV; ×35).](image-url)
associated with megaloblastosis. Deviations from the normal in MCV and reticulocyte counts were observed in three instances. Clinically the animals were apparently well, although gain in weight and their life span were definitely decreased. Necropsy usually showed no significant abnormalities. The adrenal glands of these animals displayed cortical hypertrophy with normal content of lipides.

**Combined Deficiency of Ascorbic Acid and Pteroylglutamic Acid.** The highest incidence of megaloblastosis in any one group (seven of eight animals) occurred when the diet was deficient in both ascorbic acid and PGA (group 4). The megaloblasts in one animal were of the classic type, whereas in six they were of the intermediate type. Giant metamyelocytes developed with the megaloblastosis in all but one animal, but they also appeared in the one animal of this group which did not show megaloblastosis. The time required for the development of megaloblastosis was closely related to the degree of ascorbic acid deficiency; it was 17 days on the average in series I and 36 days in series II. Spontaneous remission occurred in one animal, which revealed only a few megaloblasts of the intermediate type at any time. The M-E ratios were decreased in three animals with megaloblastosis. This decrease again was due to an increased erythropoietic activity associated with a slight depression in myeloid activity. An even more

![Fig. 2](image-url)

**Fig. 2.** Adrenal gland from a guinea pig fed a diet deficient in PGA. The animal was doing well after an initial period of diarrhea when it died suddenly on the 110th day. Moderate hypertrophy of the adrenal cortex was present, with normal amounts of lipid material. Small zones of hemorrhage are scattered throughout the cortex (Sudan IV; ×35).
pronounced increase in erythropoietic activity was encountered in three animals at a time when megaloblastosis was not present.

Anemia was demonstrated in six animals, developing in about 34 days in animals of both series I and II. Cardiac puncture was not attempted in the remaining two animals in view of the risk involved. However, smears of peripheral blood from ear veins revealed pronounced hypochromasia and they have been listed as “questionably anemic.” The MCV was normal in all instances when megaloblasts were present. At other times an abnormally high or low MCV was observed. Reticulocytosis was observed in five animals but only once in association with a megaloblastic marrow.

The clinical picture of animals comprising this group included loss of weight, loss of appetite, and bloody diarrhea. Death resulted after relatively short periods (27 to 39 days in series I and 38 to 55 days in series II). Hemorrhage into internal organs was found at necropsy in most animals. The adrenal cortex was hypertrophic and contained sparse amounts of lipid material. Adrenal hemorrhage was encountered in several animals.

Combined Deficiency of Ascorbic Acid and Vitamin B₁₂.—The results in the animals receiving diets deficient in both vitamin B₁₂ and ascorbic acid (group 5) did not differ essentially from those obtained when the diet was deficient in ascorbic acid alone (group 1).

Combined Deficiency of Pteroylglylutamic Acid and Vitamin B₁₂.—Removal of both vitamin B₁₂ and PGA (group 6) from the diet resulted in changes only slightly different from those encountered in deficiency of PGA alone (group 2). Megaloblastosis developed in five of eight animals; it was classic in type in two. Giant metamyelocytes were seen in five animals, four of which showed megaloblastosis. Spontaneous remission was noted in three instances and pronounced depression of myeloid activity was present in one. Anemia of mild degree was
seen in all instances of megaloblastosis, and the MCV was increased in two of them. The clinical picture, nutritional data and findings at necropsy closely resembled those obtained in deficiency of PGA.

Combined Deficiency of Ascorbic Acid, Pteroylglutamic Acid and Vitamin B₁₂.—The four animals receiving the diet deficient in PGA, vitamin B₁₂ and ascorbic acid died in 15 to 25 days (group 7). Megaloblastosis of the classic type developed within an average of 23 days in two animals. Giant metamyelocytes were seen in one animal with and one without megaloblasts in the bone marrow. Megaloblastosis was accompanied by extremely low M-E ratios due to greatly increased erythropoietic activity. Anemia developed in all four animals and the MCV was low in all. Reticulocytosis was not present. The clinical course, nutritional data and findings at necropsy were similar to those seen when the diet was deficient in ascorbic acid alone.

Concerning morphologic changes in the peripheral blood, a deficiency in ascorbic acid alone produced a microcytic, hypochromic picture (fig. 4a). The MCHC was 33.8 per cent as compared to a normal value for that age group of 37.5 per cent. Since internal hemorrhage developed in these animals, the decreased MCHC may have been due to bleeding. Deficiency in PGA alone resulted in some instances in a dimorphic picture of microcytosis and macrocytosis.

Fig. 4.—a. Peripheral blood from a guinea pig fed a diet deficient in ascorbic acid for 22 days. Microcytosis and hypochromasia are present. b. Peripheral blood from a guinea pig fed a diet deficient in ascorbic acid, PGA and vitamin B₁₂ for 22 days. Both macrocytosis and microcytosis are present (both Wright's stain; X950).
This was more prevalent when a deficiency in PGA was combined with a deficiency in vitamin B₁₂ and was most obvious when a deficiency in both PGA and ascorbic acid was imposed (fig. 4b). Characteristic morphologic changes were not seen in connection with a deficiency in vitamin B₁₂ alone.

**Results of Treatment**

In most instances pronounced megaloblastosis developed in animals which were moribund, whereas megaloblastosis of less severe degree usually reversed itself spontaneously. Treatment, however, was instituted for one animal of group 6, fed the diet deficient in both PGA and vitamin B₁₂. Megaloblasts of the classic type first had appeared in this animal on the twenty-second day but it was not anemic. Spontaneous remission had taken place when examination was done on the fortieth day; this was only temporary, for megaloblasts again were present on the 100th day and were again of the classic type (fig. 5a). The animal was anemic at that time. The value for hemoglobin was 9.8 Gm. per 100 cc. of blood and erythrocytes numbered 4,650,000 per cu. mm.; the hematocrit was 29.5 per cent, the MCHC was 33.2 per cent and the MCV was 63.4 cubic microns.

![Fig. 5.](image)

*Fig. 5. a. Bone marrow from a guinea pig fed a diet deficient in PGA and vitamin B₁₂ for 100 days. Three basophilic megaloblasts, a giant metamyelocyte and a normal metamyelocyte are seen. b. Bone marrow from a guinea pig fed a diet deficient in PGA and ascorbic acid and receiving treatment with ascorbic acid for 6 days prior to aspiration of the bone marrow. One promegaloblast and several polychromatik megaloblasts can be seen; note “halo” around the nucleus of the promegaloblast (both Wright’s stain; ×1,200).*
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The reticulocyte count was 0.6 per cent. Anisocytosis, hypochromasia and macrocytosis were present. The clinical condition, however, was apparently good.

Intraperitoneal injection of vitamin B12 in daily amounts of 1.5 micrograms was started. The reticulocytes were counted every other day but a response did not occur. The animal lost appetite and weight; it died after 16 days of treatment. Samples of bone marrow and of blood were taken just before death. The bone marrow did not contain megaloblasts. Only a few myeloid cells were present but erythropoietic activity was normal and the cells were shifted to the left. Hemoglobin measured 7.9 Gm., the hematocrit was 23.5 per cent and the MCHC was 33.6 per cent; erythrocytes numbered 3,700,000 and the MCV was 63.5 cubic microns. The reticulocyte count was 1.0 per cent. Pronounced anisocytosis was present.

For more data on possible recovery, treatment of two more animals was instituted. One animal (A) had been fed the diet deficient in PGA for 3 weeks. Ascorbic acid was then removed from the diet and 2 weeks later megaloblastosis of the intermediate type was present. Treatment initially consisted of the intraperitoneal injection of 50 mg. of ascorbic acid daily. A reticulocytic response was not observed. The animal lost weight and its dietary intake further decreased. Biopsy of bone marrow done after 6 days of such treatment revealed large numbers of megaloblasts of the classic type (fig. 5b). PGA in doses of 10 mg. per day also given intraperitoneally was then added to the ascorbic acid. A reticulocytic response was noted on the third day and on the fifth day reticulocytes had increased to 10.4 per cent. The appetite increased and gain in weight was recorded. Study of bone marrow after 6 days of such combined treatment failed to reveal any megaloblasts but giant metamyelocytes still were present. Cardiac sampling

Fig. 6.—Bone marrow from a guinea pig fed a diet deficient in PGA and ascorbic acid prior to treatment with PGA. Several polychromatric megaloblasts and a giant metamyelocyte are seen (Wright's stain; X1,200).
was not done during the period of treatment because of the risk involved but at the end of the period samples of heart blood were entirely normal.

A second animal (B) in the same category was studied. This animal was born to a mother fed a diet deficient in both PGA and ascorbic acid. The animal was allowed to remain with the mother for 4 weeks and was then isolated and provided the same deficient diet. On the 56th day of age, classic megaloblasts were found in the bone marrow (fig. 6). Myelopoietic activity was greatly depressed, whereas erythropoietic activity was correspondingly increased. Hemoglobin measured 11.6 Gm., the hematocrit was 34.0 per cent and the MCHC was 34.1 per cent; erythrocytes numbered 4,400,000 and the MCV was 77.3 cubic microns. Reticulocytes numbered 0.4 per cent. Macrocytosis was found in the peripheral blood.

Intraperitoneal injection of PGA in daily doses of 10 mg. was then started. Reticulocytosis of 3.1 per cent was noted the next day and the number had increased to 10.9 per cent on the sixth day of treatment. At this time the bone marrow did not contain megaloblasts or giant metamyelocytes; both myelopoiesis and erythropoiesis were normally active. However, the condition of the peripheral blood had not improved; the hemoglobin had decreased to 9.4 Gm., the hematocrit was 30.0 per cent, the MCHC was 31.3 per cent; erythrocytes numbered 3,850,000 and the MCV was 77.9 cubic microns. Pronounced polychromatophilia was noted in the peripheral blood. The animal was killed after 6 days of treatment. Necropsy showed adrenal cortical hypertrophy of moderate degree. No signs of hemorrhages were present. Two siblings kept on the same dietary regimen but not given PGA died at this time; necropsy revealed extensive hemorrhage in the internal organs in both.

**Comment**

Megaloblastic anemia of infancy is often classified as a macrocytic anemia. In the present study, macrocytosis, as evidenced by an MCV greater than 80 cubic microns, was encountered in association with megaloblastosis in only two animals, both of which had consumed a diet deficient in both PGA and vitamin B₁₂. On the other hand, microcytosis associated with megaloblastosis also was found in two animals, both of which received a diet deficient not only in PGA and vitamin B₁₂ but in ascorbic acid as well. A dimorphic picture consisting of both macrocytosis and microcytosis was most frequently associated with megaloblastosis so that normal values for the MCV were usually encountered. Therefore, the presence or absence of megaloblastosis cannot be evaluated by an appraisal of the peripheral blood alone. However, it is noteworthy that macrocytosis was observed most often in animals eating the diet deficient in vitamin B₁₂.

The production of this dimorphic picture of cellular size in the peripheral blood may be explained in two ways. 1. Microcytosis is characteristic of a deficiency in ascorbic acid, a condition usually associated with pronounced reticuloctytosis. This increased regeneration thus results in the appearance of a number of regenerative macrocytes in the blood stream. While the predominant feature is microcytosis and hypochromasia, yet macrocytes usually appear together with
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the microcytes. 2. However, this dimorphic picture also was observed without signs of increased regeneration. It was found not only in animals fed a diet deficient in PGA alone but when both PGA and vitamin B₁₂ were withheld. The dimorphic picture in the peripheral blood in these instances may be explained by the concept of Zuelzer and Rutzky,² who maintained that deficiencies in certain hematopoietic substances affect both large, primitive normoblasts and smaller, more mature ones. The former will change into large megaloblasts and mature to become megalocytes, whereas the latter will be transformed into small megaloblasts and become small erythrocytes.

Spontaneous remission often occurred when mild degrees of megaloblastosis had developed. For example, one animal eating the diet deficient in PGA changed from a megaloblastic to a normoblastic erythropoiesis twice during the 90-day period. We were using young and rapidly growing animals, so that growth in itself may bear a relationship to these spontaneous remissions. Weight charts showed for example that megaloblastosis often occurred in animals which had large daily gains in weight (7 to 10 Gm.), whereas smaller daily gains (up to 4 Gm.) were recorded for animals in which normoblastic erythropoiesis was the rule. In view of the effect of a deficiency in PGA on growth, it appears possible that a relative deficiency of this metabolite may become obvious due to increased demands during periods of rapid growth.

Spontaneous remissions also may be explained on the basis of a change in the bacterial flora in the intestinal tract. It is known that certain bacteria produce PGA and vitamin B₁₂ and that others depend on these substances for their normal growth. Spontaneous remissions may be due to an increase of the bacteria producing such hematopoietic substances or to a decrease of organisms consuming these substances.

In order to evaluate these factors, bacteriologic investigation of feces was done on certain deficient animals (table 5). Only one animal had diarrhea at the time

<table>
<thead>
<tr>
<th>Vitamin Deficiency</th>
<th>Findings on Stool Culture</th>
<th>Erythropoiesis in Bone Marrow*</th>
<th>Clinical Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>E. coli, fairly Numerous; Aerobacter; Str. faecalis</td>
<td>Nbl</td>
<td>Good; no diarrhea</td>
</tr>
<tr>
<td>PGA</td>
<td>E. coli, few; Str. faecalis; micrococci</td>
<td>I Mbl</td>
<td>Good; no diarrhea</td>
</tr>
<tr>
<td>PGA</td>
<td>E. coli, numerous; Proteus</td>
<td>Nbl</td>
<td>Severe diarrhea</td>
</tr>
<tr>
<td>PGA, B₁₂</td>
<td>E. coli, several; diphtheroids</td>
<td>Mbl</td>
<td>Good; no diarrhea</td>
</tr>
<tr>
<td>PGA, B₁₂</td>
<td>E. coli, numerous; diphtheroids</td>
<td>I Mbl</td>
<td>Good; no diarrhea</td>
</tr>
<tr>
<td>PGA, B₁₂</td>
<td>E. coli, few; diphtheroids</td>
<td>Mbl 4 days after culture</td>
<td>Diarrhea developed 6 days after culture</td>
</tr>
<tr>
<td>PGA, B₁₂</td>
<td>E. coli, few; diphtheroids</td>
<td>I Mbl</td>
<td>Good; no diarrhea</td>
</tr>
</tbody>
</table>

* Nbl = normoblastic; I Mbl = intermediate megaloblastic; Mbl = megaloblastic.
Escherichia coli was demonstrated, and diarrhea began in one 6 days after E. coli was found in the stools. In most instances certainly, any correlation between the type of intestinal flora and the morphologic appearance of bone marrow was not recognized. E. coli normally is not present in the stools of guinea pigs and it is presumed that its presence in these animals may be of some importance. E. coli usually produces PGA but certain mutant strains are said to require PGA for their growth. The presence of diphtheroids in addition to E. coli in the stools of four animals showing megaloblastosis is of interest. These organisms were not found in the feces of the normal animals in this study. Little is known, however, about their role in the metabolism of either PGA or vitamin B₁₂, so that their presence is difficult to evaluate.

The occurrence of diarrhea and its relationship to megaloblastosis are noted in table 6. In group 3 (deficiency in vitamin B₁₂), diarrhea did not develop in any animal. In group 1 (deficiency in ascorbic acid), four of the eight animals had diarrhea. It was usually severe, often bloody and occurred as a terminal event 4 to 5 days prior to death. All eight animals in group 2 (PGA-deficient diet) had diarrhea after 2 to 3 weeks of the diet, but megaloblastosis occurred in but four, two of which died. One of the survivors continued to show megaloblastosis and one had a spontaneous remission after the diarrhea ceased. Of the four animals which did not have megaloblastosis, two died while having diarrhea and two lived for a long period after the diarrhea ceased but megaloblastosis did not appear.

In group 4 (deficiency in ascorbic acid and PGA), diarrhea and megaloblastosis were terminal events in two animals in series II. In a third one, megaloblastosis developed before the diarrhea appeared. In group 4, series II, diarrhea and megaloblastosis were coincident in two animals, whereas megaloblastosis appeared in a third but spontaneous remission took place before diarrhea developed. Thus, in this group of seven animals, four exhibited the simultaneous appearance of diarrhea and megaloblastosis.

In group 5, which was fed the diet deficient in ascorbic acid and vitamin B₁₂, five animals had diarrhea but megaloblastosis did not develop in any of them.

In group 6 (deficiency in PGA and vitamin B₁₂), only one animal had megaloblastosis, which was accompanied 2 days later by diarrhea. E. coli was recovered from the stools of this animal.

In group 7 (deficiency in all three factors), diarrhea accompanied by megaloblastosis appeared in one animal.
Thus, moderate or severe diarrhea developed in each group except group 3, which was fed the diet deficient only in vitamin B\textsubscript{12}, and megaloblastosis accompanied the diarrhea in certain animals of each of the other groups except group 5, which was fed the diet deficient in ascorbic acid and vitamin B\textsubscript{12}. Therefore, diarrhea appeared to be a contributing factor to the onset of megaloblastosis.

Diarrhea may alter the intestinal flora and it may interfere with the absorption of hematopoietic substances. The importance of intestinal factors in the development of megaloblastosis is further indicated by the association of megaloblastic anemia with sprue, celiac disease and intestinal strictures and fistulas, and also by the reports of successful treatment of some of these anemias by the peroral administration of antibiotics.\textsuperscript{24, 25} Infections also are known to play a role in the development of megaloblastic anemia; according to May and associates,\textsuperscript{4} infections increase the demand for PGA. In our study the animals were housed individually in clean separate cages and signs of infection were never identified at any necropsy. Therefore, it is our opinion that the diarrhea encountered was noninfectious and that infections did not play any role in the development of megaloblastosis unless one considers the presence of E. coli in the stools of some animals as an infection.

The failure of subsequent use of vitamin B\textsubscript{12} to allow survival of the single animal which had eaten the diet deficient in both PGA and vitamin B\textsubscript{12} may be due to the fact that the latter is effective only at higher metabolic levels in the synthesis of nucleic acid. Intraperitoneal injection of vitamin B\textsubscript{12} did induce the reversal of erythropoiesis from a megaloblastic type to a normoblastic one, which may be explained by assuming that some PGA still persisted in the bodily stores when treatment was instituted, and that the vitamin B\textsubscript{12} provided a rapid utilization of such PGA as was available. Should the bone marrow have a special affinity for these nutrient substances, the synthesis of nucleic acid may have proceeded normally in this organ, while at the same time a rapid disruption of these processes in other organ systems may have occurred, leading to death of the animal.

In the case of animal A of the recovery series (fed a diet deficient in ascorbic acid and PGA), treatment with ascorbic acid alone did not prevent the development of more severe megaloblastosis. When PGA was given, reticulocytosis ensued and rapid improvement both hematologically and clinically took place. This indicates clearly that a deficiency of PGA was the more important of these two factors in the development and maintenance of megaloblastic erythropoiesis in this animal.

The rapid development of reticulocytosis and the reversal from megaloblastic to normoblastic erythropoiesis in animal B on receiving PGA point to the same conclusion. However, the anemia became more severe and the MCHC decreased from 38 to 31 per cent. Internal hemorrhage had not occurred, so that the increasing anemia was not due to loss of blood.

Deficiency of both PGA and vitamin B\textsubscript{12} as a cause of megaloblastic anemia apparently is well established. Pernicious anemia is usually considered to be the result of a deficiency in vitamin B\textsubscript{12}, and the megaloblastic anemia of pregnancy is the result of a deficiency in PGA. The other megaloblastic anemias are grouped in between and presumably result from combined deficiencies of these metabo-
bites. According to our data on guinea pigs, megaloblastic anemia of infancy may be due chiefly to a deficiency in PGA but it may develop as a result of a deficiency in vitamin B₁₂. Of eight animals fed a diet deficient in vitamin B₁₂ (group 3), only one revealed intermediate megaloblasts in its bone marrow and one showed giant metamyelocytes. However, it must be recalled that the guinea pig is an herbivorous animal and that man is omnivorous; thus, the two may depend on entirely different sources for their supply of vitamins. Of some importance may be the fact that man normally harbors E. coli in his intestinal tract and this organism may produce vitamin B₁₂. E. coli was not found in cultures of the feces of normal animals in the present group and they were fed a synthetic diet completely devoid of vitamin B₁₂. This may account for the rare occurrence of megaloblastosis in animals lacking vitamin B₁₂ in their diet.

A deficiency in ascorbic acid plays some unknown role, for it certainly appears to enhance the development of megaloblastosis in the absence of PGA. May and his coworkers¹⁸ were of the opinion that such a deficiency imposed an increased demand on the organism for folic acid. Other workers²⁶,²⁷ have suggested that ascorbic acid may play some role in the conversion of PGA to folic acid, which is thought to be the active substance in bone marrow.

We did not encounter any constant relationship between the clinical condition of the animal and the development of megaloblastosis. The severe clinical picture usually seen in infants, however, was closely paralleled in our animals which were fed diets deficient in both ascorbic acid and PGA, as well as in some of the animals fed the diet deficient in PGA alone during the early stages. On the other hand, megaloblastosis developed in animals which were apparently in good clinical condition. Thus, clinical findings by themselves are not a reliable indicator of megaloblastosis. The severe signs and symptoms encountered in megaloblastic anemia of infancy probably are due to a number of deficiencies rather than to such specific deficiencies of PGA or of vitamin B₁₂. One of the most important of such associated deficiencies doubtlessly is that of ascorbic acid.

Finally, reference is made to the animal fed the diet deficient in PGA which died on the 110th day while it was apparently in good clinical condition. Extensive bilateral adrenal hemorrhage was the only abnormal finding at necropsy. Smears of peripheral blood were not obtained in this instance but, as mentioned previously, other animals in this group had decreased numbers of platelets in the peripheral blood. This indicates that other serious complications accompany a deficiency in PGA. The “antimegaloblastic” treatment advocated by Zuelzer and his co-workers¹,² when a deficiency in PGA is suspected (diarrhea, infections) certainly appears to be more than justified.

**SUMMARY AND CONCLUSIONS**

Megaloblastosis has been produced in young guinea pigs by feeding them purified diets deficient in pteroylglutamic acid (PGA) or vitamin B₁₂ or both. The withholding of ascorbic acid from the diet did not produce megaloblastosis but it greatly enhanced its development when PGA was also withheld. Spontaneous remission took place in several instances.

The possible role of growth of the animal and changes in the bacterial flora of the gastrointestinal tract has been discussed. Bacteriologic studies appeared to
indicate a possible interrelationship between the occurrence of Escherichia coli in the stools of guinea pigs consuming deficient diets and the development of megaloblastosis. Diarrhea and infections are considered important in the pathogenesis of megaloblastic anemia but infections were not observed in animals of the present study. However, diarrhea was a prominent feature and appeared to be a factor associated with megaloblastosis.

Anemia was usually present in association with megaloblastosis; however, megaloblastosis of a mild degree appearing in some of the animals fed the diets deficient in PGA or vitamin B₁₂ was not accompanied by anemia. When ascorbic acid was withheld in addition, then anemia always developed.

A dimorphic picture of macrocytosis and microcytosis was found most commonly in the peripheral blood of animals with megaloblastosis. This has been explained on the basis of regenerative macrocytosis or as a disturbance of the maturation of erythrocytes in such deficiencies.

Data obtained from peripheral blood are not a reliable indicator of megaloblastosis, nor is the clinical condition of the animal indicative of the pathologic state.

One animal which had been fed a diet deficient in vitamin B₁₂ and PGA and which showed megaloblasts in its bone marrow was given injections of vitamin B₁₂. The megaloblastosis was corrected but the animal died on the sixteenth day of treatment.

Two animals fed diets deficient in PGA and ascorbic acid were subsequently given injections of PGA and ascorbic acid. The results demonstrated that administration of ascorbic acid alone did not interrupt the development of severe megaloblastosis in the continuing absence of PGA. When PGA was given, reticulocytosis ensued and there was a reversal of megaloblastic erythropoiesis.

**SUMMARIO E CONCLUSIONES IN INTERLINGUA**

Megaloblastosis esseva producite in juvemre porcos de India per mantener los super purificate dietas deficiemte in acido pteroylglutamic o in vitamina B₁₂ o in ambes. Le suppression de acido ascorbic in le dieta non produceva megaloblastosis sed promoteva su disveloppamento quando acido pteroylglutamic esseva etiam supprimite. In plure casos, remissiones spontanea occurreva.

Es discutite le possibile rolo del crescentia del animal e alteratioures in le flora bacterial del tubo gastrointestinal. Studios bacteriologic pareva indicar un relation possibile inter le occurrentia de *Escherichia coli* in le febes de porcos de India a dietas deficiente e le disveloppamento de megaloblastosis. Diarrhea e infectiones es considerate como important in le pathogenese de anemia megaloblastic, sed in le animales del presente studio nulle infectiones esseva observate. Del altere latere, diarrhea esseva un constatatious prominente e pareva esser un factor associate con megaloblastosis.

Anemia esseva usualmente presente in association con megaloblastosis. Sed megaloblastosis de leve grado, occurrence in alcunes del animales mantenite super dietas deficiente in acido pteroylglutamic o vitamina B₁₂, non esseva accompaniante de anemia. Si in plus etiam le acido ascorbic esseva supprimite, anemia se disveloppava sin exception.

Un configuration dimorphe de macrocytosis e microcytosis esseva notate le
plus comummente in le sanguine peripheric de animales con megaloblastosis. 
Isto es explicate super le base de macrocytosis regenerative o como un disturba-

tion del processos de maturation del erythrocytos in casos de tal deficientias. 
Datos obtenite ab le sanguine peripheric non es de valor conclusive como indi-
catores de megaloblastosis, e etiam le condition clinic del animal non indica su 

stato pathologic.

Un del animales que habeva recipite dietas deficiente in vitamina B₁₂ e acido 

pteroylglutamic e que mostrava megaloblastosis in le medulla ossee espeva 

tractate con injectiones de vitamina B₁₂. Le megaloblastosis eseva corrigite,

sed le animal moriva le dece-sextie die del tractamento.

Duo animales mantenite super dietas deficiente in acido pteroylglutamic e 

acido ascorbic reciepeva subsequentemente injectiones de ille substantias. Le 

resultatos indicava que acido ascorbic sol non interrumpe le disveloppamento 
de serve megaloblastosis si acido pteroylglutamic continua esser absente. Quando 

acido pteroylglutamic eseva administrate, reticulocytosis se disvelopoppava e un 

reversion del erythropoiiese megaloblastic sequave.

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EXPERIMENTAL MEGALOBLASTIC ANEMIA

Experimental Megaloblastic Anemia in Young Guinea Pigs

ROLV K. SLUNGAARD and GEORGE M. HIGGINS