Experimental Production of Nutritional Macrocytic Anemia in Swine

V. Hematologic Manifestations of a Combined Deficiency of Vitamin B₁₂ and Pteroylglutamic Acid

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In previous reports from this laboratory, the hematologic manifestations of experimentally induced pteroylglutamic acid deficiency and of vitamin B₁₂ deficiency have been described.

The deficiency of pteroylglutamic acid was produced in a total of 56 swine by feeding a purified diet containing casein and supplemented with seven B vitamins, succinylsulfathiazole and a folic acid antagonist. The hematologic manifestations observed were severe macrocytic anemia, leukopenia with proportionately greater reduction in polymorphonuclear cells than in mononuclear cells, slight thrombocytopenia and hyperplasia of the bone marrow. The bone marrow contained many large immature nucleated red cells which, by virtue of their size and immaturity, resembled the megaloblasts seen in the marrow of patients with pernicious anemia. However, these cells differed from the megaloblasts of pernicious anemia in that their nuclear chromatin was not as delicate as that seen in “true” megaloblasts. These cells are perhaps more correctly referred to as “macronormoblasts” rather than “megaloblasts.”

All of the hematologic manifestations of a deficiency of pteroylglutamic acid were alleviated rapidly and completely by the administration of this vitamin or by the administration of any one of its three known conjugates. Purified liver extract and vitamin B₁₂ were found to possess little or no hematopoietic activity under these conditions. Indeed, all of the hematologic manifestations of a deficiency of pteroylglutamic acid appeared in animals given 15 U.S.P. units of liver extract every fifteen days from the beginning of the experiment.

The authors are indebted to Dr. A. Gibson, Merck and Company, Inc., Rahway, N. J., for the supplies of thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate, inositol, choline chloride and crystalline vitamin B₁₂; to Dr. E. L. Sevringhaus, Hoffmann LaRoche, Inc., Nutley, N. J., for biotin and vitamins A, D, E and K; to Dr. H. J. Prebluda, U. S. Industrial Chemicals, Inc., New York, N. Y., for the liberal supplies of DL-methionine; to Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., for the pteroylglutamic acid and the crude methyl folic acid antagonist; and to Dr. W. P. Boger, Sharp and Dohme, Inc., Philadelphia, Pa., for the generous supplies of succinylsulfathiazole.

Miss Helen Ashenbrucker, Mr. George Trappett and Mr. Ocie Hadley gave valuable technical assistance.

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In another series of experiments a total of 70 pigs were fed succinylsulfathiazole and a purified diet containing soybean alpha protein in place of casein in an effort to produce a deficiency of vitamin B₁₂. The age of the animals when started on the experimental diet ranged from 2 to 28 days. Methionine, iodinated casein, desiccated thyroid and pteroylglutamic acid were added to the diet of certain animals and omitted from the diet of the other pigs. Gastrectomy was performed in 9 of the animals.

Growth of the animals on the above soybean protein diets without added vitamin B₁₂ was retarded as compared with the growth of animals on the same diet supplemented with this vitamin. The administration of vitamin B₁₂ to the deficient animals resulted in rapid growth.

Of the 39 animals not receiving B₁₂ and surviving for a sufficiently long period of time for adequate evaluation, 13 failed to develop anemia, 16 developed a mild anemia, and only in 10 did a moderately severe anemia appear. The anemia was normocytic and in 24 pigs was accompanied by a moderately severe neutropenia. Differential cell counts on the sternal marrow were normal except for a slight increase in the proportion of normoblasts. In spite of the fact that definite and sometimes marked reticulocyte increases followed the administration of vitamin B₁₂, the anemia and leukopenia were neither consistently nor completely corrected.

The failure to observe macrocytic anemia with a megaloblastic bone marrow in the vitamin B₁₂ deficient swine was surprising since patients with megaloblastic anemia (pernicious anemia) respond so dramatically to small doses of this vitamin. Two explanations for this failure were offered: (1) the deficiency was not sufficiently severe to produce such a change in the hemopoietic system, or (2) the presence of pteroylglutamic acid in the diet as a contaminant in the soybean protein prevented the development of megaloblastic anemia even in the absence of vitamin B₁₂. The latter possibility seemed not unlikely since patients with pernicious anemia respond hematologically to the administration of this vitamin in the absence of vitamin B₁₂.

In order to put the second possibility to experimental test, animals were fed the “vitamin B₁₂ deficient” vegetable protein diet but a folic acid antagonist was added. It is the purpose of the present report to describe the hematologic changes which developed in these animals. Data are also presented concerning the hematologic response following the administration of each vitamin alone and of both vitamins given simultaneously.

A preliminary report of this work has been published.

**Experimental Procedure**

Twenty weanling Chester-White pigs, 21 to 28 days of age and derived from three different litters, were used for these experiments. All the animals were housed in individual cages and were handled by methods previously described.

All of the animals received the following basal diet in amounts of 30.7 Gm. (152 calories) per Kg. of body weight per pig per day: soybean alpha protein* 31.2 per cent, sucrose 37.0 per cent, lard 26.6 per cent, salt mix No. 3 5.2 per cent. Succinylsulfathiazole in an amount

* Eighty-six and six tenths per cent protein. Purchased from Glidden Company, Soya Products Division, 5165 W. Moffat Street, Chicago 39, Ill.
of 2.0 per cent and crude methyl folic acid antagonist in an amount of 0.4 per cent were added to the basal diet. In addition, all animals received 3,000 units of vitamin A, 600 units of vitamin D, 1 mg. of vitamin E and 1 mg. of vitamin K per Kg. of body weight per week by mouth. The vitamins were supplied in crystalline form by placing them in capsules and administering them orally three times a week. The quantities given were as follows (mg. per Kg. of body weight per day): thiamin hydrochloride 0.25, riboflavin 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20, calcium pantothenate 0.50, para-aminobenzoic acid 0.10, inositol 0.20 and biotin 0.10. A methionine supplement and choline chloride were added to the basal diet in the amounts indicated in table 1.

Because the soybean alpha protein contains about 1 µg. of pteroylglutamic acid activity per Gm. as determined by microbiologic assay with Lactobacillus casei, the amount of folic acid antagonist fed to these animals was increased to twice that which was given in our previous experiments3,5 in which a purified casein diet was used.

**Table 1**—Litter Data and Supplementary Dietary Details

<table>
<thead>
<tr>
<th>Litter No.</th>
<th>Pig Numbers</th>
<th>Days of Age</th>
<th>Choline mg/Kg/day</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11-93 – 11-96</td>
<td>28</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>12-17 – 12-24</td>
<td>21</td>
<td>3</td>
<td>None*</td>
</tr>
<tr>
<td>III</td>
<td>12-45 – 12-52</td>
<td>24</td>
<td>30</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

* On the seventy-fifth day of the experiment the animals 12-22, 12-23 and 12-24 were given 10 Gm. of DL-methionine per day for sixty days. Thereafter, methionine was included in the diet in amounts of 0.3 per cent.

**Table 2**—Morphologic Data Prior to and During the Deficiency (20 Animals)

<table>
<thead>
<tr>
<th>Group</th>
<th>Days on Exp.</th>
<th>RBC 10⁶ cu. mm.</th>
<th>Hgb. Gm.</th>
<th>V.R.C. 10⁶</th>
<th>MCV cu.µ</th>
<th>MCH %</th>
<th>MCHC %</th>
<th>Retics</th>
<th>PMN 10⁶ cu. mm.</th>
<th>MN 10⁶ cu. mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to deficiency</td>
<td>0</td>
<td>8.25</td>
<td>14.4</td>
<td>44</td>
<td>33</td>
<td>2.2</td>
<td>15.3</td>
<td>4.1</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.491 ±0.87</td>
<td>±2.1 ±3.1</td>
<td>±1.2</td>
<td>±1.4</td>
<td>±0.88</td>
<td>±3.27</td>
<td>±1.36</td>
<td>±2.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>91</td>
<td>4.19</td>
<td>9.1</td>
<td>64</td>
<td>22</td>
<td>1.7</td>
<td>8.5</td>
<td>1.4</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.868 ±4.50</td>
<td>±2.2 ±1.3</td>
<td>±0.85</td>
<td>±2.38</td>
<td>±1.22</td>
<td>±2.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures represent mean ± S.D. V.P.R.C., volume of packed red cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PMN, polymorphonuclear cells; MNC, mononuclear cells.

Hematologic studies (red blood cell count, hemoglobin, volume of packed red cells, red cell indexes, reticuloocyte, total leukocyte and differential leukocyte counts) were performed weekly on all animals throughout the entire experiment. The cellular composition of the sternal marrow was studied by the technic outlined previously.6

**RESULTS**

The morphologic data on the blood are summarized for all 20 pigs in Table 2. In every instance anemia developed. The anemia ranged in severity from volumes of packed red cells of 16 to 35 ml./100 ml. In two-thirds of the animals the volume of packed red cells was less than 30 ml./100 ml. The anemia was definitely although not markedly macrocytic. The mean corpuscular volume prior to the deficiency ranged from 46 to 61 cu.µ, whereas after the deficiency had developed, in only 4 animals was the mean corpuscular volume less than 61 cu.µ. In general,
the longer the duration of the deficiency the greater was the degree of macrocytosis. This correlation was noted previously.²

The anemia was normochromic, there being no reduction in the mean corpuscular hemoglobin concentration. No significant reticulocytosis accompanied the anemia. There was leukopenia, which, as in the deficiency of pteroylglutamic acid alone,¹ ² was due to a proportionately greater reduction in neutrophils than in mononuclear cells.

On examination of the peripheral blood smears a marked degree of anisocytosis with many macrocytes was observed. Poikilocytosis was not pronounced. An increase above the normal in Howell-Jolly bodies, nucleated red cells and polychromatophilia was present. The neutrophils, although reduced in numbers, were not morphologically remarkable except for an occasional multi-segmented one.

Differential cell counts performed on smears obtained by needle aspiration of the sternum during life and stained with Wright’s stain revealed a marked increase in the proportion of nucleated red cells. Many of these cells were normoblasts, morphologically indistinguishable from the cells in normal pig marrow. However, many were larger than normal and showed a considerable degree of immaturity (“macronormoblasts”). In addition, there were also cells present in small numbers which were larger and more immature and which presented a fine nuclear chromatin structure. These cells included basophilic, polychromatolytic acidophilic forms. Karyorrhexis and multiple Howell-Jolly bodies were present. In general, the neutrophils were unaffected although a few multi-segmented neutrophils and giant metamyelocytes of the pernicious anemia type were observed.*

Examination of microscopic sections† of the livers of the animals receiving the high choline diet (30 mg./Kg./day) and the methionine supplement revealed an entirely normal histologic pattern. This was true regardless of whether or not pteroylglutamic acid, vitamin B₁₂, pteroylglutamic acid together with vitamin B₁₂ or neither vitamin had been given. The livers of the animals (11-94, 11-95, 11-96) fed the low choline diet (3 mg./Kg./day) without methionine supple-

* Dr. O. P. Jones of the University of Buffalo and Dr. Dorothy Sundberg of the University of Minnesota kindly examined two of the bone marrow preparations. Dr. Jones’s opinion was as follows: “Macrocytes and their precursors which are not normal members of the erythroblastic series have been produced. Although the marrow is predominantly normoblastic and macronormoblastic, there are quite a few cells that can be classified as atypical megaloblasts. For the most part the neutrophils seem to be unaffected but a few giant metamyelocytes and band forms are present. The atypical megaloblasts referred to are like those described in human cases of partial and temporary deficiencies of anti-pernicious anemia principle.”

Dr. Sundberg stated: “I believe that the developing erythrocytes in this preparation show the morphologic features characteristic of megaloblasts. These cells are probably not morphologically identical with either the megaloblasts of man or of monkey, but I am willing to accept them as megaloblasts. Any features which seem unusual, I think, can be attributed to species differences. There are very occasional neutrophils with five or six reasonably delicate nuclear lobes and a few promyelocytes and metamyelocytes which seem about double the normal size and which have reniform nuclei. These cells are the only ones which might be taken as evidence of some concomitant alteration in the cells of the neutrophilic series.”

† We are indebted to Dr. F. D. Gunn, Professor of Pathology, University of Utah, for these examinations.
NUTRITIONAL MACROCYTIC ANEMIA IN SWINE. V

Mention revealed a rather marked, diffuse fatty metamorphosis such as was described in our previous report. All 3 of these animals had been treated with both pteroylglutamic acid and vitamin B₁₂ prior to the termination of the experiment. The livers of 6 of the 8 animals receiving the low choline diet supplemented from the seventy-fifth day of the experiment with methionine showed a moderate degree of fatty infiltration, necrosis of the liver cells, or both necrosis and fatty infiltration. The livers of the remaining 2 animals in this group were entirely normal.

Ataxia or other manifestation of neurologic disease were not observed. The icterus index of all the animals remained within normal limits. The pigs appeared somewhat unsteady and hair growth was poor, although large areas of alopecia were not present. The stools were at all times dark in color and semi-solid.

**Table 3—Hematologic Response to Vitamin B₁₂**

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Before Therapy</th>
<th>After Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCV cu. μ</td>
<td>V.P.R.C.</td>
</tr>
<tr>
<td></td>
<td>m. /100 ml.</td>
<td>Remics</td>
</tr>
<tr>
<td>11-94</td>
<td>63</td>
<td>27</td>
</tr>
<tr>
<td>11-95</td>
<td>58</td>
<td>26</td>
</tr>
<tr>
<td>12-24</td>
<td>58</td>
<td>16</td>
</tr>
<tr>
<td>12-47</td>
<td>69</td>
<td>21</td>
</tr>
<tr>
<td>12-48</td>
<td>67</td>
<td>24</td>
</tr>
</tbody>
</table>

Mean: 63 23 2.0 | 13.0 7 30 — 79

Five μg were given i.m. daily for ten days. In pigs 12-24, 12-47 and 12-48 this was followed by 50 μg. i.m. weekly.

For symbols see table 2.

**Response to Vitamin B₁₂**

Five animals were treated with vitamin B₁₂ after they had become deficient. The results are summarized in table 3. Following this therapy there was a moderate increase in reticulocytes which reached a maximum six to ten days after the institution of therapy. The reticulocytosis was followed by an increase in the volume of packed red cells in 4 of the 5 animals. In no animal, however, was the anemia completely relieved. No significant increase in the total leukocyte count was observed following treatment.

Two days following vitamin B₁₂ therapy, as judged by smears obtained by needle aspiration of the sternum, the cellularity of the marrow seemed to be greater than before. Differential counts revealed that approximately 90 per cent of the cells were of the erythroid series. Two weeks later this hypercellular response had subsided and thereafter the myeloid:erythroid ratio remained constant at approximately 0.5:1. The atypical megaloblasts tended to disappear although an occasional one persisted. The erythroblasts were predominantly of the macronormoblastic type and the bone marrow morphology was not significantly different from that previously described in swine deficient in pteroylglutamic acid.¹ ²
Three of the animals were given repeated injections of vitamin B₁₂ at weekly intervals for sixteen to twenty-one weeks. In these animals the volume of packed red cells increased to approximately 30 ml./100 ml. and remained at this level for the entire period of vitamin B₁₂ therapy. The course of one such animal is shown in detail in figure 1. The subsequent administration of pteroylglutamic acid resulted in a return of both the peripheral blood and the bone marrow to normal.

The growth stimulating effect of vitamin B₁₂ was striking when the growth of treated animals was compared with that of animals given no therapy (fig. 2). Of considerable interest is the observation that after vitamin B₁₂ therapy the degree of macrocytosis increased significantly and persisted to a degree greater than that observed in untreated animals (table 4). A similar increase in the degree of macrocytosis was observed previously following liver extract or B₁₂ therapy in animals fed a casein diet deficient in pteroylglutamic acid.¹ ²

Response to Pteroylglutamic Acid

Five animals were treated with pteroylglutamic acid. In 3 of these the initial course of therapy was followed by weekly injections of pteroylglutamic acid for a period of sixteen weeks. In the remaining 2 (12-20 and 12-21) only four weekly injections were given. The results are summarized in table 4.

In all the animals except one the administration of pteroylglutamic acid was followed by a significant reticuloctysis. The reticuloctysis peak appeared some-

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**Fig. 1.**—Development of anemia in a pig fed a diet deficient in vitamin B₁₂ and in pteroylglutamic acid and the response to vitamin B₁₂. The animal was given an initial course of 50 μg. of vitamin B₁₂ intramuscularly daily for ten days. This was followed by a reticuloctysis of 25 per cent and a temporary increase in the V.P.R.C. from 10 to 28 ml./100 ml. After seventeen days vitamin B₁₂ was again given and a second response occurred. On 50 μg. of vitamin B₁₂ weekly the V.P.R.C. reached a plateau at 25 to 30 ml./100 ml. In spite of the vitamin B₁₂ therapy the macrocytosis persisted. The administration of pteroylglutamic acid (PGA) resulted in alleviation of the anemia and the macrocytosis.

Methionine supplementation was begun on the seventy-fifth day and continued throughout the remainder of the experiment.

M.C.V., mean corpuscular volume; V.P.R.C., volume of packed red cells; retics., reticulocytes; W.B.C., total leukocyte count; P.M.N., polymorphonuclear cells.

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what earlier than after vitamin B₁₂ therapy and was followed by a substantial rise in the volume of packed red cells to nearly normal values within twenty to thirty days. With the disappearance of anemia the mean corpuscular volume

![Graph showing growth curves for 8 pigs.](image)

**Fig. 2.—Growth curves for 8 pigs.** Each line represents the mean growth of 2 pigs. All 8 pigs were from litter number III. On the thirteenth week 2 pigs (12-47 and 12-48) were treated with vitamin B₁₂ (10 μg. i.m. daily for ten days and then 50 μg. i.m. weekly), 2 pigs (12-49 and 12-50) were treated with pteroylglutamic acid (5 mg. i.m. daily for 10 days and then 7 mg. i.m. weekly), and 2 pigs (12-51 and 12-52) were given both vitamins simultaneously in the above amounts. Two animals (12-45 and 12-46) were not treated and served as littermate controls. Growth is expressed in weight/initial weight in order to make the starting points of the curve exactly comparable.

**Table 4—Hematologic Response to Pteroylglutamic Acid**

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Before Therapy</th>
<th>After Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCV cu. μ</td>
<td>V.P.R.C. ml./100 ml.</td>
</tr>
<tr>
<td>12-20</td>
<td>60</td>
<td>29</td>
</tr>
<tr>
<td>12-21</td>
<td>52</td>
<td>26</td>
</tr>
<tr>
<td>12-23</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>12-49</td>
<td>63</td>
<td>24</td>
</tr>
<tr>
<td>12-50</td>
<td>61</td>
<td>25</td>
</tr>
</tbody>
</table>

Mean ............ 60 27 1.6 21.0 4 39 58

Five mg. were given i.m. daily for ten days. In pigs 12-23, 12-49 and 12-50 this was followed by 7 mg. i.m. weekly.

For symbols see table 2.

decreased to within the normal range. The total leukocyte counts tended to increase transiently after therapy and then decreased to the previous low values.

In the 3 animals observed for sixteen weeks after the initial rise in the volume
of packed red cells there was a slow progressive fall to values of approximately 35 ml./100 ml. (table 4). The course of one such animal is shown in detail in figure 3. The subsequent administration of vitamin B₁₂ was followed by a second reticulocytosis and a return of the blood and bone marrow to normal.

After the administration of pteroylglutamic acid the bone marrow reverted to normal except for a slight increase in the proportion of normoblasts to myeloid cells as compared with normal marrow. Macronormoblasts and megaloblasts disappeared entirely.

As shown in figure 2 the administration of pteroylglutamic acid resulted in stimulation of growth approximately equal in degree to that caused by vitamin B₁₂.

![Graph](image)

**Fig. 3.**—Development of macrocytic anemia in a pig with the double dietary deficiency and the response to pteroylglutamic acid (PGA). Following the administration of PGA the reticulocytes increased to 36 per cent, the V.P.R.C. increased from 28 to 42 ml./100 ml. and the M.C.V. declined. On continued PGA therapy anemia again developed and was then relieved by the administration of vitamin B₁₂.

Methionine supplementation was begun on the seventy-fifth day and continued throughout the remainder of the experiment.

For symbols see figure 1.

**Response to Vitamin B₁₂ and Pteroylglutamic Acid Given Simultaneously**

Three pigs were treated with both vitamins in optimal amounts simultaneously (table 5). This combined therapy resulted in a pronounced reticulocytosis which was greater in degree than had been observed following the administration of either vitamin alone, complete relief of the anemia, a decrease in the mean corpuscular volume to within the normal range, an increase in the total leukocyte and differential cell count to normal, and a return of the bone marrow morphology and differential count to normal. After sixteen to twenty-two weeks of maintenance therapy the blood and bone marrow remained normal. The course of one such animal is presented in detail in figure 4.

The combined therapy resulted in considerably greater growth than that which followed the administration of either vitamin alone (fig. 2).
**Table 5—Hematologic Response to Both PGA and Vitamin B₁₂**

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>V.R.</th>
<th>Mcv.</th>
<th>Retics</th>
<th>V.P.R.C.</th>
<th>Mcv.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu.µ</td>
<td>ml./100 ml.</td>
<td>%</td>
<td>ml./100 ml.</td>
<td>cu.µ</td>
</tr>
<tr>
<td>12-22</td>
<td>72</td>
<td>21</td>
<td>3.2</td>
<td>42.0</td>
<td>5</td>
</tr>
<tr>
<td>12-51</td>
<td>66</td>
<td>21</td>
<td>0.8</td>
<td>22.0</td>
<td>3</td>
</tr>
<tr>
<td>12-52</td>
<td>69</td>
<td>26</td>
<td>0.4</td>
<td>26.0</td>
<td>4</td>
</tr>
</tbody>
</table>

Mean: 69 23 1.5 30.0 4 45 57

Five mg. of PGA and 50 µg. of vitamin B₁₂ were given daily (i.m.) for ten days followed by 7 mg. of PGA and 50 µg. of vitamin B₁₂ weekly (i.m.).

For symbols see table 2.

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**Fig. 4.—Development of macrocytic anemia in a pig with the double dietary deficiency and the response to the simultaneous administration of both vitamin B₁₂ and PGA. Methionine supplementation was begun on the seventy-fifth day and continued throughout the remainder of the experiment.

For symbols see figure 1.

**Table 6—Summary of the Results of Prolonged Therapy**

<table>
<thead>
<tr>
<th>Therapy</th>
<th>No. of Pigs</th>
<th>Maximum Retics %</th>
<th>V.P.R.C. ml./100 ml.</th>
<th>Mcv. cu.µ</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>4.0</td>
<td>25</td>
<td>60</td>
<td>Macronormoblastic</td>
</tr>
<tr>
<td>B₁₂</td>
<td>5</td>
<td>13.0</td>
<td>30</td>
<td>70</td>
<td>Macronormoblastic</td>
</tr>
<tr>
<td>PGA</td>
<td>3</td>
<td>21.0</td>
<td>35</td>
<td>50</td>
<td>Normoblastic</td>
</tr>
<tr>
<td>B₁₂ + PGA</td>
<td>3</td>
<td>30.0</td>
<td>45</td>
<td>57</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**Discussion**

A comparison of the hematologic changes associated with a deficiency in swine of pteroylglutamic acid, of vitamin B₁₂ and of both these vitamins is presented in table 7. The combined deficiency differs from pteroylglutamic acid deficiency in several respects. These are: (1) a few cells are found in the bone marrow which
more nearly approach in their morphology the megaloblasts of pernicious anemia than those seen in pteroylglutamic acid deficiency alone; (2) the anemia responds partially to the administration of vitamin $B_{12}$ therapy; (3) although the anemia responds initially to the administration of pteroylglutamic acid, after several months a partial hematologic relapse occurs, in spite of continued therapy; and (4) the blood and bone marrow are converted to normal only after the administration of both vitamins.

The experimental disorder produced in swine by a dual deficiency of vitamin $B_{12}$ and pteroylglutamic acid resembles in different degrees the various conditions now usually classed under the title of "megaloblastic macrocytic anemias." We postulated earlier that these anemias represent in various degrees combinations of deficiencies of both of these vitamins. It was suggested that refractory

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Anemia</th>
<th>Bone Marrow Morphology</th>
<th>Hemopoietic Response to</th>
</tr>
</thead>
</table>
| PGA $B_{12}$ | Macrocytic | Macronormoblastic | ++++ 0 +++
| PGA $B_{12}$ | Normocytic | Normoblastic | 0 + +
| PGA $B_{12}$ | Macrocytic | Macronormoblastic | +++ + ++
| PGA $B_{12}$ | Few megaloblasts | | ++ + ++

TABLE 8—Comparison of Nutritional Macrocytic Anemia in Swine with Pernicious Anemia in Human Subjects

<table>
<thead>
<tr>
<th>Similarities</th>
<th>Dissimilarities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrocytic anemia</td>
<td>Bone marrow not markedly megaloblastic</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>No neurologic disease</td>
</tr>
<tr>
<td>Slight thrombocytopenia</td>
<td>No evidences of hemolysis</td>
</tr>
<tr>
<td>Hyperplastic bone marrow</td>
<td>No pronounced morphologic alterations in the leukocytes</td>
</tr>
<tr>
<td>Hyperferremia</td>
<td>Normal plasma copper level</td>
</tr>
<tr>
<td>Response to $B_{12}$</td>
<td>Incomplete response to $B_{12}$</td>
</tr>
<tr>
<td>Low normal free erythrocyte protoporphyrin</td>
<td></td>
</tr>
</tbody>
</table>

megaloblastic anemia and achreastic anemia represent deficiencies of pteroylglutamic acid alone and that a counterpart of these human disorders is pteroylglutamic acid deficiency in swine. It was proposed that megaloblastic anemia of infancy, megaloblastic anemia of pregnancy, nutritional macrocytic anemia, tropical and nontropical, and sprue, represent dual deficiencies of pteroylglutamic acid and vitamin $B_{12}$, in different cases more of one and less of the other; and that pernicious anemia is the result chiefly of a deficiency of vitamin $B_{12}$. It might be suggested now that the dual deficiency in swine described in the present study represents the experimental counterpart of human nutritional macrocytic anemia. For reasons which will be presented shortly, it lacks important features of human pernicious anemia.

Similarities between the dual deficiency in swine and pernicious anemia are
listed in table 8. In addition it may be pointed out that, as in the experimental disorder, hemopoietic responses to both pteroylglutamic acid and to B₁₂ have been observed in pernicious anemia. Another point of similarity is that, in patients with pernicious anemia maintained on pteroylglutamic acid for two or three years, a hematologic relapse may occur which can be relieved by the administration of liver extract. Presumably the effect of liver extract would also be produced by the administration of B₁₂.

A distinguishing feature of pernicious anemia is the development of neurologic changes. These have appeared in some cases in spite of the administration of pteroylglutamic acid. They have not been observed so far in pernicious anemia subjects maintained on adequate amounts of B₁₂. Furthermore, the administration of this vitamin has brought relief not only of the hematologic manifestations but, to some extent, those referable to the nervous system. Thus, pernicious anemia would seem to be the ultimate consequence of a deficiency of vitamin B₁₂ although it is clear that pteroylglutamic acid is in some way related.

Neither in the experimentally induced vitamin B₁₂ deficiency reported earlier nor in the dual deficiency described here have all the features of pernicious anemia been reproduced. The explanation of this contradiction may be that, although a deficiency of vitamin B₁₂ severe enough to impair growth in pigs has been produced, the deficiency may not have been severe enough to interfere with hemopoiesis to the extent often encountered in pernicious anemia or to impair the function of the nervous system. Although animals fed a highly purified casein diet containing at most only traces of vitamin B₁₂, excrete 30 to 40 μg. of this vitamin daily in the feces, it is not likely that bacterial synthesis of vitamin B₁₂ in the gastro-intestinal tract and absorption therefrom would counteract to a serious degree the effects of a dietary deficiency of vitamin B₁₂ for it has been shown that patients with pernicious anemia in relapse excrete large amounts of this vitamin in the feces. It is much more probable that the gastric “intrinsic factor,” which presumably was unaltered in our experimental animals, served to secure for them out of a diet deficient in but not completely lacking vitamin B₁₂, enough of this essential substance to prevent the development of a degree of deficiency which would result in the development of all of the features of pernicious anemia. If this is true, then it is unlikely that a severe deficiency can be produced experimentally without removing the source of intrinsic factor. It is possible that a sufficiently severe deficiency of B₁₂ might be produced with the aid of a metabolic antagonist of that vitamin. It may be pointed out that our earlier attempts to produce a deficiency of pteroylglutamic acid in the pig by dietary deficiency alone failed to bring about a severe or persistent anemia. This was only accomplished when a folic acid antagonist was used as well. Thus far, surgical attempts to deprive animals of “intrinsic factor” have been unsuccessful.

That species differences between man and experimental animals make it difficult to produce pernicious anemia experimentally is a possibility which has been suggested from time to time. One obvious difference between man and the pig is that the latter is able to synthesize its own ascorbic acid while man is not. May and his co-workers have shown that, in the monkey, an animal which like man depends on the diet for ascorbic acid, in the presence of dietary ascorbic acid a deficiency of pteroylglutamic acid is not characterized by macrocytic
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anemia or megaloblastic bone marrow. Only when such animals are deficient in both ascorbic acid and pteroylglutamic acid, does macrocytic anemia accompanied by megaloblastic anemia appear. It is uncertain, at this stage of our knowledge, whether or not the difference between man and the pig in respect to ascorbic acid is of any significance in regard to attempts to reproduce experimentally the various aspects of the megaloblastic macrocytic anemias.

A third hypothesis which might explain the failure of the swine disorder to more closely simulate pernicious anemia is that, in addition to vitamin B₁₂, pteroylglutamic acid and the alimentary “intrinsic factor,” some additional factor may be involved in the pathogenesis of pernicious anemia. This could be a hitherto undiscovered metabolic essential. Again, that a toxic, hemolytic agent is concerned in the pathogenesis of pernicious anemia has been postulated from time to time. This concept has been revived once more as the result of experiments in rats with “blind” intestinal segments in which macrocytic anemia has been observed. The significance of these observations in relation to our experimental studies in swine is now being explored. Recently, Lajtha, as well as Thompson, have reported observations which they interpret as indicating that in the serum of patients with untreated pernicious anemia there is a factor which inhibits the ripening of megaloblasts to normoblasts in bone marrow cultures. If this is true, then, until a method is devised for increasing the amount of this “inhibitor” in the serum of swine, it is unlikely that the entire pernicious anemia syndrome will be reproduced experimentally in animals.

It should also be noted that our experimental goal in the pig depends in part on the assumption that porcine megaloblasts should be identical morphologically with the megaloblasts of human subjects. This assumption is based partially on the observation of Ehrlich that the primitive erythroblast of the early human embryo is identical with the pernicious anemia megaloblast. It depends also on our own study of the primitive erythroblast of pig embryos which we have found to be more nearly identical to pernicious anemia megaloblasts than are the cells observed in the marrows of our deficient swine. Therefore, at least until all other explanations for the differences between the pig and the human anemia have been excluded, we will continue to demand that the marrow of the pig with experimentally produced “pernicious anemia” should be morphologically identical to the marrow of patients with pernicious anemia in relapse. Likewise one might hope that with the production of a sufficiently severe deficiency of vitamin B₁₂, changes in the nervous system might develop and also that, by some means, manifestations of increased pigment excretion might be induced.

**Summary**

A total of 20 swine were fed a diet adequate in all known respects except that soybean protein was substituted for casein, succinylsulfathiazole and a folic acid antagonist were added, and vitamin B₁₂ and pteroylglutamic acid were withheld from the vitamin supplement.

The animals developed macrocytic anemia, leukopenia and neutropenia, accompanied by erythroid hyperplasia of the bone marrow. The erythroblasts consisted mainly of immature macronormoblasts but a few atypical megaloblasts were also observed.
The anemia responded rapidly and completely to the administration of both vitamin B₁₂ and pteroylglutamic acid. The administration of pteroylglutamic acid alone resulted in an immediate return of the blood and bone marrow to within normal limits but after several months there was a partial hematologic relapse in spite of continued therapy with this vitamin. The administration of vitamin B₁₂ alone resulted in only partial remission of the anemia and the bone marrow remained macronormoblastic although the megaloblasts tended to disappear.

Growth of the animals was stimulated by the administration of either vitamin but the administration of both vitamins simultaneously resulted in the greatest rate of growth.

No manifestations of neurologic disturbances or of increased pigment excretion were observed in the deficient swine.

REFERENCES

Experimental Production of Nutritional Macrocytic Anemia in Swine: V. Hematologic Manifestations of a Combined Deficiency of Vitamin B12 and Pteroylglutamic Acid

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