Hematologic Manifestations of Vitamin B₁₂ Deficiency in Swine*


As reported previously from this laboratory, there are several striking similarities between the morphologic and chemical changes in the blood of pteroylglutamic acid-deficient pigs and of patients with pernicious anemia. In both, the anemia is macrocytic and is accompanied by leukopenia and slight thrombocytopenia. In both conditions the plasma copper is essentially normal and the plasma iron level is increased. In pernicious anemia the amount of free protoporphyrin in the erythrocytes tends to be either normal or somewhat below normal. Likewise, in the experimental deficiency in swine, free erythrocyte protoporphyrin is lower than is found in the normal animal. Both conditions are characterized by similar morphologic changes in the nucleated red cells of the marrow and, finally, the hematologic manifestations of both conditions are alleviated by the administration of pteroylglutamic acid.

There are, however, a number of differences between the experimental macrocytic anemia and pernicious anemia. Although the nucleated red blood cells in the marrow of the experimental animals in some ways resemble the megaloblast series of cells seen in the marrow of patients with pernicious anemia in relapse, they are not in all respects identical with them. Furthermore, neurologic disturbances, glossitis, signs usually interpreted as evidence of increased blood...
destruction and certain morphologic alterations in the leukocytes characteristi-
cally seen in pernicious anemia have not been observed in the pteroylglutamic
acid-deficient pigs. Again, purified liver extract, vitamin B\textsubscript{12} and thymine have
been shown to be effective in the treatment of the hematologic manifestations of
pernicious anemia but these substances are relatively ineffective in relieving the
anemia in swine. Thus, as pointed out previously\cite{2,4} the experimental porcine
anemia corresponds more closely to certain pteroylglutamic acid-responsive, liver-
refractory megaloblastic anemias described in human subjects (“achrestic ane-
mia,” “refractory megaloblastic anemia,” macrocytic anemia of pregnancy and
megaloblastic anemia of infancy) than to pernicious anemia.

Because of the dissimilarities between this porcine anemia and pernicious
anemia it was thought to be important to observe the hematologic effects of a
deficiency of vitamin B\textsubscript{12} in swine. Although the diets used in the above experi-
ments contained at most only a trace of vitamin B\textsubscript{12} as determined either by
microbiologic assay\cite{*} or by assay in human subjects with pernicious anemia,\cite{2}
the protein used was from an animal source. Since a deficiency of vitamin B\textsubscript{12}
has been produced in mice\cite{7,8}, rats,\cite{9-12} chicks\cite{13-16} and pigs\cite{17-20} by feeding a diet
in which the protein was derived from a vegetable source, experiments have
been carried out in swine in which casein was replaced by soybean protein. A
preliminary report of this work has appeared\cite{21}.

It has been observed by many investigators that the administration of desic-
ccated thyroid or of iodinated casein to rats markedly increases the requirement
for vitamin B\textsubscript{12}\cite{7,9,10,12,15,22}. Furthermore, it has been shown in several different
ways that methionine has a sparing action on vitamin B\textsubscript{12}\cite{23-26}. For these reasons,
desiccated thyroid and iodinated casein were given certain animals while others
were fed diets low in methionine. Diets were also included in our study which
were not supplemented with pteroylglutamic acid since it seemed possible that
the presence of this vitamin in large amounts might protect the animals from
the development of anemia. Attempts to produce a combined deficiency of
vitamin B\textsubscript{12} and pteroylglutamic acid by the use of a folic acid antagonist in
addition to the “B\textsubscript{12} deficient” vegetable protein diet will be described in a
later report.

In human subjects, the so-called “intrinsic factor” of normal gastric juice
appears to be concerned with the absorption of vitamin B\textsubscript{12}\cite{29}. With the hope
that vitamin B\textsubscript{12} deficiency might be accentuated by gastrectomy, this operation
was performed in one group of animals which were also fed the “B\textsubscript{12} deficient,”
low-methionine, vegetable protein diet without pteroylglutamic acid supple-
mentation. Again, because May and his co-workers\cite{30} were able to show that
ascorbic acid prevents the development of megaloblastic anemia in monkeys
deficient in pteroylglutamic acid, d-glucosaccharic acid, an ascorbic acid an-
tagont,\cite{31,32} was administered to one gastrectomized pig.

On the other hand, in an attempt to relieve the deficiency by means other
than the administration of vitamin B\textsubscript{12}, several animals were treated with
aureomycin since this antibiotic has been shown to contain “animal protein
factor” activity in the pig\cite{33,34}.

\* One and one-tenth milligamma per Gm. of whole diet.
EXPERIMENTS

For these studies a total of 70 Chester-White pigs were used. All animals were housed in individual cages and were handled by the methods previously described. The general outline of the experiments is given in table 1.

Experiment I

Forty-three baby pigs, 2 to 7 days of age, were fed the alpha protein* (isolated soybean protein), "synthetic milk" diet described by Neumann, Kriider and Johnson except that sucrose was substituted for glucose, our swine salt mixture No. 3 was used in place of theirs, and methionine was not added except in group C (table 1). The "milk" was homogenized in a Waring blender, warmed to 37°C and fed ad libitum at 9 a.m., 12 noon, 5 p.m. and 9 p.m., until the pigs were 12 days of age, at which time the number of feedings per day was reduced to three and the "milk" was fed cold. No difficulty was encountered in getting 2 to 7 day-old baby pigs to consume such a diet from feed troughs. When the pigs were approximately one month of age they were fed the following basal diet in amounts of 30.7 Gm. (152 calories) per Kg. of body weight per pig per day: alpha protein 30.0 per cent, sucrose 37.4 per cent, lard 26.6 per cent, salt mixture No. 3 6.0 per cent.

Sulfasuxidine formed 2.0 per cent of the basal diet of all animals. 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. Crystalline choline chloride was added to the basal diet daily in amounts of 10 mg. per Kg. of body weight. The other 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, biotin 0.10, pteroylglutamic acid 0.02.

* Eighty-six and six-tenths per cent protein. Purchased from Glidden Company, Soya Products Division, 5165 W. Moffat Street, Chicago 39, Ill.

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The animals were divided into 3 groups and received additional supplements as noted in table 1.

These 2 to 7 day old pigs developed severe diarrhea after the first several days on the "synthetic milk" diet and 22 of them died before they were 30 days of age.

Experiment II

Twenty-seven pigs, 21 to 28 days of age were fed the following basal diet in amounts of 33.7 Gm. (152 calories) per Kg. of body weight per pig per day: alpha protein 31.2 per cent, sucrose 37.0 per cent, lard 26.6 per cent, salt mixture No. 3² 5.2 per cent.

Sulfasuxidine, vitamins A, D, E and K were given all the animals as in Experiment I. Crystalline choline chloride was added to the basal diet daily in amounts of 3 mg. per Kg. of body weight. The other "B" vitamins were supplied in crystalline form as in Experiment I except that no para-aminobenzoic acid or pteroylglutamic acid was included.

The animals were divided into 5 groups as outlined in table 1.

Throughout the entire time of study all the animals had diarrhea and passed dark brown, liquid stools. Nevertheless, all animals in groups A, B, C and E survived for at least fifteen weeks.

When gastrectomy was performed, this was carried out under ether anesthesia. Approximately 90 per cent of the stomach was removed and the small remaining esophageal portion was sutured to the duodenum by an end to side anastomosis leaving a short 2 to 3 cm. blind pouch of duodenum. Spleenectomy was performed in order to facilitate the gastrectomy. Only 4 animals (11-75, 11-77, 11-90, 11-92) survived the operative procedure. Two died from operative errors and 3 died several weeks after gastrectomy as a result of herniation of the abdominal contents into the thoracic cavity.

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

RESULTS

Experiment I

Growth: The growth of the animals in both groups A and B without methionine supplementation was exceedingly poor (fig. 1). After the addition of methionine to the diet, growth of the animals in both groups was greatly accelerated. However, the animals receiving vitamin B₁₂ (group A) grew considerably better than those on the same diet without vitamin B₁₂ (group B). The average daily weight gain for the two groups for the period between the eighth and twenty-second weeks was as follows: group A, 269 Gm.; group B, 169 Gm.

Four animals (11-39, 11-40, 11-54, 11-58) in group B were treated with crystalline vitamin B₁₂ intramuscularly. Beginning in the twenty-second week pig 11-54 was given 30 µg. the first day and 10 µg. daily for the next nine days. Beginning in the twenty-sixth week pig 11-58 was given 30 µg. of vitamin B₁₂ daily for ten days. In both animals this therapy was followed by a marked acceleration in growth (fig. 1). The growth response of a third animal (11-40) to 10 µg. of vitamin B₁₂ daily for 10 days and 10 µg. per week thereafter is shown in figure 2. The fourth animal (11-39) died two weeks after therapy was instituted.

The animals in group C (basal diet plus desiccated thyroid) grew poorly in spite of the fact that methionine was included in the diet. The average daily weight gain in grams for the first twelve weeks was only 72. Six animals (11-29, 11-31, 11-33, 11-34, 11-36, 11-37) in this group were treated with vitamin B₁₂. Each of these animals was given a total of 130 µg. of the vitamin intramuscularly.
over a period of six to ten weeks. In each instance a marked stimulation in growth occurred (fig. 2). One (11–29) of two litter mates, each weighing approximately 9 Kg., was treated with vitamin B₁₂. Ten weeks later the animal receiving vitamin B₁₂ (11–29) weighed 30 Kg. and the untreated animal (11–31) weighed 13.3 Kg. The untreated animal (11–31) was given vitamin B₁₂. During the six weeks immediately prior to B₁₂ therapy this animal had gained 2.9 Kg. In the same period immediately following therapy the weight gain was 9.5 Kg. (fig. 2)

**Blood and bone marrow:** The hematologic changes in the peripheral blood for all groups of animals in Experiment I are summarized in table 2. No significant alterations occurred in the peripheral blood of the animals in group A. In groups B and C anemia was variable in occurrence and degree. In table 3 the incidence and severity of anemia in these two groups are tabulated. In 6 animals no anemia was present; in 8 the anemia was only slight; and in 3 it was moderately severe. When present, the anemia was normocytic except

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*This material consisted of the dried, coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.*

**Graph:**

*Fig. 1.—Experiment I. Growth curves for groups A (basal diet plus vitamin B₁₂) and B (basal diet only). The growth response to vitamin B₁₂ is also shown for two animals in group B given this vitamin after they had become deficient.*
in 2 pigs (11-36, 11-54). In these 2, the anemia was slightly macrocytic. However, in each pig reticulocytosis was present.

In the 4 pigs of group A ("control") the total leukocyte count varied between 10,000 and 14,000 per cu. mm, while the absolute neutrophil count varied between 3,000 and 5,000 per cu. mm. Although the mean total leukocyte count was not significantly altered from the normal in any of the groups in which vitamin B₁₂ deficiency was produced, in 4 pigs in each of groups B and C the total leukocyte count was below 10,000 per cu. mm. Again, in 3 pigs in group B and in 8 pigs of group C the absolute neutrophil count was less than 3,000 per cu. mm. Thus,
in individual animals some neutropenia was observed. Morphologic alterations of the leukocytes such as nuclear multi-segmentation were not encountered.

Differential counts on the sternal marrows of the anemic animals revealed a slight increase in the proportion of erythroid cells. In no instance were true megaloblasts seen or even cells resembling megaloblasts such as those observed in the bone marrow of pteroylglutamic acid-deficient swine.

**Histopathology:** With one exception, 6 animals in group B (11-39, 11-41, 11-56, 11-64, 11-62, 11-66) and 4 animals in group C (11-30, 11-32, 11-35, 11-38) were autopsied prior to receiving any therapy. Pig 11-39 received 5 μg. of vitamin B₁₂ two weeks prior to death. In addition, pig 11-63 of group A received vitamin B₁₂ from the beginning of the experiment and served as a control. Sections were made from sternal, costal and femoral marrows, liver, spleen, kidney, stomach, small intestine, large intestine, tongue, lung, skeletal muscle and cardiac muscle.

Sections of the liver from all of the animals examined except the one “control” (11-63) showed some degree of degenerative change or accumulation of fat, with or without necrosis of the parenchyma. Diffuse fatty metamorphosis in

<table>
<thead>
<tr>
<th>V.P.R.C. (ml/100 ml)</th>
<th>Number of Pigs</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>40+</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>35-39</td>
<td>8</td>
<td>47</td>
</tr>
<tr>
<td>30-34</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>25-29</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

V.P.R.C. refers to volume of packed red cells.

an important degree occurred in 3 (11-30, 11-32, 11-56). In pig 11-35 vacuolation of the hepatic cords was limited to the central and mid-zones in association with granular degeneration and atrophy of the cord cells. In pig 11-64 zones of vacuolated cells surrounded necrotic lobular centers.

Necrosis, graded as 1 plus to 3 plus, involved the livers of 5 pigs (11-38, 11-39, 11-41, 11-62, 11-64) and apparently arose as a centrilobular degeneration, followed by necrosis, disintegration and liquefaction of cord cells, which spread peripherally to involve entire lobules and groups of lobules. Most of the necrotic lesions appeared to be of recent origin. Leukocytic infiltrate was scanty and consisted predominantly of polymorphonuclear neutrophils. In a few foci monocytes and lymphocytes predominated and in many necrotic lobules no leukocytic infiltrate was evident. The liver of the animal in group A receiving vitamin B₁₂ (11-63) was essentially normal. These changes are illustrated in figure 3.

The sections of myocardium of 2 of the pigs (11-62, 11-63) showed simple atrophy. In 4 of the pigs (11-35, 11-38, 11-39, 11-64) there were numerous areas of focal degeneration in the myocardium. Extensive areas of degeneration and
Fig. 3.—Showing the pathologic changes in the liver in vitamin B12 deficient swine compared with a “control.” (A) Pig II-63 (Group A, Experiment I). This pig received crystalline vitamin B12, 10 μg. intramuscularly twice weekly from the beginning of the experiment. The liver was essentially normal. (B) Pig II-62 (Group B, Experiment I). A litter mate of pig II-63 receiving the same diet except that no vitamin B12 was given. Neither of the animals received methionine. There is marked necrosis of the liver cells and minimal zonal fatty degeneration. (C) Pig II-82 (Group A, Experiment II). This pig received methionine and 900 μg. of vitamin B12 twenty-eight days prior to the time of sacrifice. There is marked fatty infiltration and necrosis of the liver cells. (D) Pig II-79 (Group C, Experiment II). This pig received the same diet as pig II-82 except that no pteroylglutamic acid or methionine was added to the diet. Note the marked fatty infiltration with minimal necrosis of the liver cells.
necrosis were found in sections of the left ventricular myocardium of one pig (11-56). These were infiltrated more or less densely by lymphocytes and monocytes (focal interstitial myocarditis). The myocardium was essentially normal in sections from 4 animals (11–30, 11–32, 11–41, 11–66). Focal hyaline degeneration and a moderate degree of atrophy were found in the skeletal muscle of all of the pigs examined.

The bone marrow sections revealed essentially normal cellularity for pigs of the corresponding age in all except 3. The marrows of pigs 11–35, 11–62 and 11–63 were considered to be hypoplastic. Serous atrophy of marrow fat was evident in numbers 11–62, 11–63 and 11–66.

No consistent abnormalities were found in the sections of spleen, kidney, stomach, tongue, intestine or lung.


The administration of vitamin B₁₂ was followed in three instances by a reticulocytosis greater than 20 per cent, in four instances by a moderate reticulocytosis (10 to 20 per cent) and in two instances by a slight reticulocytosis (5 to 10 per cent). In one animal no significant reticulocytosis was observed. It is noteworthy that marked reticulocytosis developed in 2 pigs (11–37, 11–39) even in the absence of anemia.

The degree of red cell regeneration following vitamin B₁₂ therapy was variable. In 7 of the pigs no significant or sustained red cell regeneration developed. In 3 pigs (11–31, 11–54, 11–58) a significant degree of red cell regeneration took place. The course of one of these animals (11–54) is presented in detail in figure 4. This pig was the most severely anemic animal in the two deficient groups and represents the most definite hematologic response to vitamin B₁₂ which was observed. In 5 pigs, as exemplified by pig 11–37 (fig. 5), a transient decrease in the volume of packed red cells occurred following the administration of vitamin B₁₂. Such decreases took place whether or not anemia was present at the time of therapy, and regardless of the degree of reticulocytosis which followed therapy.

Experiment II

Growth: The growth curve for each of the 5 groups of animals, all of which received the B₁₂ deficient diet, is presented in figure 6. The average weight gain in grams per day for each group is given in table 5.

The addition of methionine to the diet enhanced growth considerably as can be seen by comparison of the growth curves of groups A and B. The omission of pteroylglutamic acid from the diet resulted in poor growth (group C) as compared with that of animals receiving pteroylglutamic acid (group B). This was rather surprising since the alpha protein used contained approximately 1 µg. pteroylglutamic acid activity per gram as determined by microbiological assay with Lactobacillus casei. This suggests that under the conditions of these experiments the requirement for pteroylglutamic acid was increased.
HEMATOLOGIC MANIFESTATIONS OF VITAMIN B\textsubscript{12} DEFICIENCY

Table 4.—Experiment I: Results of Therapy with Vitamin B\textsubscript{12}

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Total Dose B\textsubscript{12} (mcg)</th>
<th>Duration of Treatment (days)</th>
<th>V.P.R.C ml/100 ml.</th>
<th>Retic. %</th>
<th>After Therapy</th>
<th>V.P.R.C. ml/100 ml.</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-29</td>
<td>5</td>
<td>1</td>
<td>33</td>
<td>0.4</td>
<td>11</td>
<td>33</td>
<td>23</td>
</tr>
<tr>
<td>11-31</td>
<td>100</td>
<td>10</td>
<td>35</td>
<td>1.0</td>
<td>2</td>
<td>46</td>
<td>21</td>
</tr>
<tr>
<td>11-33</td>
<td>155</td>
<td>56</td>
<td>35</td>
<td>4.8</td>
<td>6</td>
<td>39</td>
<td>56</td>
</tr>
<tr>
<td>11-34</td>
<td>130</td>
<td>56</td>
<td>50</td>
<td>2.4</td>
<td>4</td>
<td>46</td>
<td>56</td>
</tr>
<tr>
<td>11-36</td>
<td>100</td>
<td>10</td>
<td>35</td>
<td>1.2</td>
<td>5</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>11-37</td>
<td>130</td>
<td>10</td>
<td>45</td>
<td>2.8</td>
<td>11</td>
<td>45</td>
<td>59</td>
</tr>
<tr>
<td>11-39</td>
<td>5</td>
<td>1</td>
<td>43</td>
<td>1.4</td>
<td>26.6</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>11-40</td>
<td>100</td>
<td>10</td>
<td>38</td>
<td>4.8</td>
<td>13.8</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>11-54</td>
<td>120</td>
<td>10</td>
<td>28</td>
<td>5.0</td>
<td>33.3</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>11-58</td>
<td>300</td>
<td>10</td>
<td>36</td>
<td>1.8</td>
<td>12.6</td>
<td>12</td>
<td>27</td>
</tr>
</tbody>
</table>

Mean...................... 38.2 2.6 16.1 40.4

V.P.R.C. refers to volume of packed red cells.

V. P. R. C. refers to volume of packed red cells.

Fig. 4.—Experiment I. Pig 11-54, Group B ("B\textsubscript{12} deficient"). Hematologic response to the intramuscular administration of vitamin B\textsubscript{12}.

M.C.V., mean corpuscular volume; V.P.R.C., volume of packed red cells; retic., reticulocytes; W.B.C., white blood cells; P.M.N., polymorphonuclear cells including metamyelocytes, neutrophils, eosinophils, and basophils.

The gastrectomized animals (group D) received neither methionine nor pteroylglutamic acid and growth in this group was the poorest of all of the groups.
The addition of iodinated casein to the diet markedly suppressed growth as can be seen by comparing the growth curve of group E with that of group B (fig. 6).

Two animals (11–80, 11–83) in group A were treated by giving 50 µg. of vitamin B₁₂ intramuscularly daily for fifteen days. The remaining 2 animals of this group (11–81, 11–82) were given recrystallized aureomycin, 4 mg. orally per Kg. of body weight per day. The effects on growth are presented in figure 7. Growth was enhanced by vitamin B₁₂ but not by aureomycin. The 2 pigs (11–80, 11–83) treated with vitamin B₁₂ were then given aureomycin without appreciable effect on growth. The 2 pigs (11–81, 11–82) given aureomycin initially were later injected with a total of 600 µg. of vitamin B₁₂ over a fifteen day period, the antibiotic being continued. This resulted in resumption of growth.

Two animals in group B died before the effects of therapy on growth could be evaluated. Of the 2 remaining animals, one (11–85) was treated initially with 10 Gm. of methionine daily and the other (11–84) was given 50 µg. of vitamin B₁₂ intramuscularly daily for fifteen days (fig. 8). Neither form of therapy had an appreciable effect on growth. Both animals were then given 4 mg. of aureomycin per Kg. of body weight per day. The pig (11–84) which had

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**Figure 5.**—Experiment I. Pig 11-37, Group C ("B₁₂ deficient plus desiccated thyroid"). Hematologic response to the intramuscular injection of vitamin B₁₂ and to oral liver residue. Note the marked reticulocytosis which appeared in the absence of anemia, the slight decrease in the volume of packed red cells immediately after vitamin B₁₂ administration was begun and the delayed rise in volume of packed red cells. The neutropenia was unaffected by vitamin B₁₂. Liver residue had no significant effect.

For symbols see figure 4.
previously received vitamin B₁₂ continued to grow at the same rate as before whereas the animal which had not been given this vitamin practically ceased to grow. The latter animal (11-85) was then given 50 µg. of vitamin B₁₂ daily for twelve days when growth was resumed (fig. 8).

The animals in groups C, D and E either died before the effects of therapy on growth could be adequately evaluated or were not treated.

**Blood and bone marrow:** The peripheral blood changes for each of the 5 groups of animals are summarized in table 5.

The average volume of packed red cells for the 4 animals in group A was 38 ml./100 ml. with a range of 32 to 42 ml./100 ml. The mean volume of packed red cells of animals of the same age receiving the same diet but supplemented with vitamin B₁₂ (Experiment I, group A) was 44 ml./100 ml. with a range from

### Table 5.—Experiment II: Summary of the Hematologic Data

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Pigs</th>
<th>Days on Experiment</th>
<th>Average Wt. Gain Gm./day</th>
<th>R.B.C. X 10⁶/µl</th>
<th>Hgb. Gm./100 Gm.</th>
<th>V.P.R.C. X 10³ cu.mm/100 Gm.</th>
<th>M.C.V. cu.mm</th>
<th>M.C.H. mg</th>
<th>M.C.H.C. %</th>
<th>Retic. %</th>
<th>W.B.C. X 10³/µl</th>
<th>P.M.N. %</th>
<th>M.N.C. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>144 (134-148)</td>
<td>6.83 (6.6-7.9)</td>
<td>13.1 (11.5-14.2)</td>
<td>37.9 (32-42)</td>
<td>56 (53-58)</td>
<td>20 (14-20)</td>
<td>35 (31-36)</td>
<td>1.2 (0.6-2.0)</td>
<td>13.4 (11.1-14.2)</td>
<td>13.0 (12-14)</td>
<td>2.4 (1.8-3.0)</td>
<td>7.1 (6.0-8.0)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>130 (113-148)</td>
<td>5.25 (4.0-6.0)</td>
<td>10.0 (9.1-11.1)</td>
<td>29.3 (27.3-31.1)</td>
<td>57 (52-61)</td>
<td>19 (18-21)</td>
<td>31 (30-33)</td>
<td>1.3 (1.1-1.5)</td>
<td>10.0 (8.8-11)</td>
<td>10.2 (9.1-12)</td>
<td>3.1 (2.3-3.9)</td>
<td>7.1 (6.0-8.0)</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>121 (78-161)</td>
<td>6.03 (5.7-6.5)</td>
<td>12.5 (12-13)</td>
<td>36.4 (33.3-38)</td>
<td>61 (57-60)</td>
<td>21 (19-23)</td>
<td>31 (30-33)</td>
<td>4.7 (3.7-5.2)</td>
<td>11.2 (9.1-12)</td>
<td>11.5 (10-13)</td>
<td>3.5 (2.5-4.5)</td>
<td>7.1 (6.0-8.0)</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>104 (83-121)</td>
<td>7.09 (5.7-8.2)</td>
<td>11.9 (10-14)</td>
<td>36.1 (30-42)</td>
<td>51 (44-55)</td>
<td>17 (15-18)</td>
<td>33 (32-34)</td>
<td>1.4 (0.6-1.8)</td>
<td>10.6 (9.1-12)</td>
<td>10.2 (9.1-12)</td>
<td>3.8 (3.1-4.5)</td>
<td>7.1 (6.0-8.0)</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>107 (78-97)</td>
<td>9.35 (8.0-10.6)</td>
<td>16.0 (13.7-17.1)</td>
<td>45.1 (40-50)</td>
<td>48 (46-50)</td>
<td>17 (16-18)</td>
<td>35 (31-36)</td>
<td>3.2 (2.4-3.6)</td>
<td>11.7 (9.8-12)</td>
<td>11.7 (10-13)</td>
<td>3.0 (2.1-3.6)</td>
<td>7.1 (6.0-8.0)</td>
</tr>
</tbody>
</table>

Group A, basal diet plus PGA and methionine; Group B, basal diet plus PGA; Group C, basal diet only; Group D, basal diet only plus gastrectomy; Group E, basal diet plus indented casein.

V.P.R.C., volume of packed red cells; M.C.V., mean corpuscular volume; M.C.H., mean corpuscular hemoglobin; M.C.H.C., mean corpuscular hemoglobin concentration; W.B.C., white blood cells; P.M.N., polymorphonuclear cells; M.N.C., mononuclear cells.

42 to 48 ml./100 ml. Thus the omission of vitamin B₁₂ resulted in a mild anemia. This anemia was normocytic and normochromic.

The omission of the methionine supplement from the vitamin B₁₂ deficient diet (group B) resulted in definite anemia (average volume of packed red cells, 29 ml./100 ml.) of moderate severity as compared with animals on the same diet plus additional methionine (group A). Again the red cell indexes were within normal limits.

The omission of both pteroylglutamic acid and methionine (group C) was associated with less anemia than in group B. This may be explained by the fact that the growth of the animals was considerably less than in group B and consequently their total blood volumes were considerably less. Again the red cell indexes were within the normal limits.

The gastrectomized animals receiving no methionine or pteroylglutamic acid supplement (group D) developed only a mild anemia. The animals receiving
iodinated casein and pteroylglutamic acid (group E) failed to develop anemia. Growth of the animals in both groups D and E was exceedingly poor.

A significant degree of leukopenia was not present in any of the animals in the above groups. Definite neutropenia (neutrophils below 3,000 per cu. mm.) was present in all animals in groups A and B, in 3 of 5 animals in group C, in none of the animals in group D, and in two of the 5 animals in group E. Mor-

![Graph showing growth curves for different groups.](image)

**Fig. 6**—Experiment II. Growth curves. For explanation see text and table 1.

phologic changes in the neutrophils such as nuclear multisegmentation were not observed.

The sternal marrow differential counts in all groups revealed a slight increase in the proportion of erythroid cells but no cells resembling megaloblasts were observed.

**Histopathology:** Autopsies were performed on 1 pig in group A (11–82), 1 pig in group B (11–86), 5 pigs in group C (11–81, 11–73, 11–79, 11–88, 11–89),
HEMATOLOGIC MANIFESTATIONS OF VITAMIN B12 DEFICIENCY

4 pigs in group D (11-75, 11-77, 11-90, 11-92) and 3 pigs in group E (11-99, 12-00, 12-01). Histologic examinations were performed as in Experiment I.

Either fatty infiltration, necrosis or both were observed in the livers of all animals. Fatty infiltration was the predominant change in 7 pigs. Necrosis without fatty metamorphosis was observed in 2 pigs, and both necrosis and fatty infiltration were present in 5 pigs.

Four pigs (11-77, 11-90, 11-92, 11-82) had been treated with vitamin B12 fifteen, eight, thirty-two and twenty-eight days respectively prior to death.

**Fig. 7.—**Experiment II. Group A. Growth response in B12 deficient pigs to the administration of vitamin B12 and aureomycin. Two pigs (11-80, 11-83) were treated initially with vitamin B12 followed by aureomycin. The other 2 pigs (11-81, 11-82) were treated first with aureomycin and later with vitamin B12. Note that the administration of aureomycin to the vitamin B12 deficient pigs resulted in cessation of growth, whereas the administration of the antibiotic had no appreciable effect after vitamin B12 had been given. The administration of vitamin B12 to the pigs receiving aureomycin stimulated growth.

Two animals (11-82, 11-86) had previously received aureomycin. Marked necrosis of the liver cells was present in all of these except pig 11-92. In this animal central and mid-zone degeneration were moderate and necrosis was minimal.

Areas of focal degeneration in the myocardium were observed in only 3 of the 14 pigs. Slight atrophy or focal hyaline degeneration of the skeletal muscles was observed in the sections from 12 of the animals. In only 1 (11-79) were hyaline degeneration and necrosis considered to be moderately advanced, involving nearly one-half of the fibers represented in the samples, and in this animal the
striated muscle of the tongue also showed focal hyaline degeneration of moderate degree. Samples from 5 pigs (11-82, 11-88, 11-89, 11-90, 11-92) showed slight interstitial edema of skeletal muscle. The degree of cellularity of the bone marrow was essentially normal in all except pig 11-82 in which there was a disproportionately large number of normoblasts in a diffusely hyperplastic marrow. Slight serous atrophy of marrow fat was evident in pig 11-90.

![Experiment II](image)

**Fig. 8.**—Experiment II. Group B. Growth response in B₁₂ deficient pigs to methionine, aureomycin and vitamin B₁₂. The administration of aureomycin to a vitamin B₁₂ deficient pig caused a diminution in the growth rate which was reversed by vitamin B₁₂ (pig 11-85). The administration of aureomycin after vitamin B₁₂ had been given had no effect on growth.

No consistent histologic alteration was found in the thyroid glands of those pigs which were treated with desiccated thyroid substance or with iodinated casein as compared with those of the other pigs in the group.

The other organs and tissues which were examined microscopically showed no consistent abnormalities. No significant incidental infectious disease was discovered in any of the animals of this series.

**Hematologic response to therapy:** All 4 animals (11-80, 11-81, 11-82, 11-83) in group A and 2 animals (11-84, 11-85) in group B were treated. Pigs 11-80, 11-83 and 11-84 were treated initially with vitamin B₁₂ and then with aureomycin. Pigs 11-81 and 11-82 were treated first with aureomycin and then with
HEMATOLOGIC MANIFESTATIONS OF VITAMIN B₁₂ DEFICIENCY

TABLE 6.—Experiment II: Hematologic Response to Vitamin B₁₂ and Aureomycin in Groups A (Basal Diet Plus PGA and Methionine) and B (Basal Diet Plus PGA only)

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>V.P.R.C. Prior to Therapy ml. 100 ml.</th>
<th>After Vitamin B₁₂</th>
<th>After Aureomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retic. Peak ml.</td>
<td>V.P.R.C. ml. 100 ml.</td>
<td>Retic. Peak ml.</td>
</tr>
<tr>
<td>11-80</td>
<td>32</td>
<td>5.4</td>
<td>46</td>
</tr>
<tr>
<td>11-83</td>
<td>41</td>
<td>7.0</td>
<td>47</td>
</tr>
<tr>
<td>11-84</td>
<td>28</td>
<td>13.8</td>
<td>34</td>
</tr>
<tr>
<td>Mean</td>
<td>34</td>
<td>42</td>
<td>42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>After Aureomycin</th>
<th>After Vitamin B₁₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-81</td>
<td>42</td>
<td>0.6</td>
</tr>
<tr>
<td>11-82</td>
<td>37</td>
<td>0.8</td>
</tr>
<tr>
<td>11-85*</td>
<td>36</td>
<td>1.8</td>
</tr>
<tr>
<td>Mean</td>
<td>38</td>
<td>30</td>
</tr>
</tbody>
</table>

* Previously given methionine. V.P.R.C. refers to volume of packed red cells.

Fig. 9.—Experiment II. Hematologic response to aureomycin and vitamin B₁₂ in two pigs. The administration of aureomycin to the vitamin B₁₂ deficient pig (11-82) did not significantly influence the developing anemia, which later responded to vitamin B₁₂. The administration of aureomycin after vitamin B₁₂ had been given had no striking immediate effect on the blood (pig 11-84).

For symbols see figure 4.
vitamin B₁₂. Pig 11–85 was treated first with methionine and then with aureomycin and finally with vitamin B₁₂. The amounts of these agents and the dosage schedules are given in the preceding section on growth. The hematologic responses are summarized in table 6.

The animals treated initially with vitamin B₁₂ all developed reticulocytosis and showed red cell regeneration. The administration of aureomycin after vitamin B₁₂ had no further effect on the blood. The animals treated initially with aureomycin failed to develop reticulocytosis and, in fact, this therapy was followed by a decrease in the volume of packed red cells. The administration of vitamin B₁₂ to these pigs while still receiving aureomycin resulted in reticulocytosis and alleviation of the anemia.

One pig (11–85) was treated initially with 10 Gm. of methionine daily for ten days. This therapy had no appreciable effect on the blood. Neutropenia when present was generally not relieved by either vitamin B₁₂ or aureomycin therapy.

In figure 9 the effects of aureomycin and vitamin B₁₂ on the volume of packed red cells are shown in detail in 1 animal (11–82) treated first with aureomycin and then vitamin B₁₂, and in 1 animal (11–84) treated first with vitamin B₁₂ and then with aureomycin.

All 4 of the pigs (11–75, 11–77, 11–90, 11–92) in group D were treated with
vitamin B₁₂ but all died shortly after therapy was begun. Prior to B₁₂ therapy 1 pig (11–90) was given an ascorbic acid “antagonist,” d-glucoscorbic acid¹⁻⁵ in an amount of 5 per cent of the basal diet for twelve days. The results are presented in figure 10. This compound had no demonstrable hematologic effects on the blood or bone marrow. The “tyrosyl” excretion⁴ in the urine was unaltered and the whole blood and plasma “ascorbic acid” levels, as measured by the method of Roe and Kuether⁶ rose appreciably. No detrimental effects on the well being of the pig were noted. Indeed there was no evidence that d-glucoscorbic acid acted as an effective anti-ascorbic acid agent in the pig in this dosage.

**Discussion**

Judging by the impaired growth of pigs receiving the soybean protein diet and the growth response when they were given crystalline vitamin B₁₂, it may be concluded that a deficiency of this vitamin had been produced in our experimental animals. The hematologic alterations which accompanied the deficiency, however, were of an extremely mild order. Anemia, when present, was rarely severe nor was it macrocytic or accompanied by clinical evidences of neurologic disease. Morphologic alterations in the neutrophils and in the nucleated red
cells of the marrow, similar to those which occur in pernicious anemia were not present.

There were few signs of hematologic disturbance whether or not the pigs were started on the experimental diets during the first or the fourth week of life and whether or not a methionine supplement, iodinated casein, desiccated thyroid, or pteroylglutamic acid was added to the diet, or gastrectomy performed. Furthermore, when a significant degree of anemia or neutropenia was present, these alterations were neither consistently or completely corrected by the administration of vitamin B₁₂. This was true even though definite reticulocyte increases frequently took place following vitamin B₁₂ therapy. In fact, little or no correlation was observed between the degree of anemia prior to therapy and the maximal reticulocyte increase after therapy (fig. 11); nor was there any correlation between the reticulocyte maximum and the degree of red cell regeneration following therapy.

As pointed out already, the omission of methionine as well as vitamin B₁₂ was associated with the development of a more severe grade of anemia than occurred when only vitamin B₁₂ was lacking. The question may be considered whether or not the existence of the double deficiency influenced the response to therapy with vitamin B₁₂. Comparing the responses in pigs 11–82 and 11–84 (fig. 9), the former of which had received methionine, it would appear that a less satisfactory response occurred when a double deficiency existed. As a whole the most definite changes following administration of vitamin B₁₂ were observed in pigs which were deficient in B₁₂ but not deficient in methionine (11–80, 11–81, 11–82, 11–83).

Although a deficiency of vitamin B₁₂, at least as determined by growth response to the crystalline vitamin, has been produced by a number of different workers in mice, rats, chicks and pigs, few references to hematologic alterations have been made. When hemoglobin or leukocyte values have been given, in many instances the changes from the normal do not appear to have been striking. In the chick, Nichol et al. observed no difference in the hemoglobin values of deficient birds and those receiving vitamin B₁₂. In the rat, Emerson found that the red and white cell counts in both the deficient group and in the groups receiving vitamin B₁₂ fell within normal limits. Zucker and Zucker observed definite leukopenia in their rats at the “time of the crisis.” Borson et al. however, found not only severe leukopenia and neutropenia, but also some anemia in weanling rats raised from vitamin B₁₂ deficient mothers. No evidence of a hemorrhagic diathesis or of thrombocytopenia was encountered. Nucleated red cells were observed in the peripheral blood but all of these were of the normoblastic type and were easily recognized as such. The bone marrows of the animals were hyperplastic with a high degree of erythroblastic as well as myeloid activity. Death of the animals appeared to be correlated with the severity of the leukopenia and neutropenia. The administration of vitamin B₁₂ but not of pteroylglutamic acid restored the blood picture to normal and initiated a sustained increase in weight.

In the pig, Cunha et al. observed slightly subnormal hemoglobin values when “animal protein factor” was not included in the diet. However, vitamin B₁₂ concentrates alone did not influence the hemoglobin level. Heinle, Welch
and Shorr reported that vitamin B₁₂ deficient swine receiving pteroylglutamic acid develop a severe normocytic anemia which responds to vitamin B₁₂ therapy. Megaloblasts were not observed in the marrow. Unfortunately, none of the details of these experiments have been published.

Neumann, Johnson and Thiersch have reported that vitamin B₁₂ deficiency in the pig is associated with a mild normocytic anemia, neutrophilia and no alteration in the morphology of the nucleated red cells in the marrow. An increase in hemoglobin from approximately 9 to 11 Gm. per cent followed the administration of either vitamin B₁₂ or purified liver extract. However, these investigators made no comment concerning a similar increase in hemoglobin which occurred spontaneously in the untreated group.

The same group of investigators, in a later paper reported that in young pigs fed a soybean protein, vitamin B₁₂ deficient diet and given a folic acid antagonist, "The blood and bone marrow symptoms of this combined vitamin B₁₂-folic acid deficiency were cured by either crystalline vitamin B₁₂ or by folic acid therapy." The only evidence which they offered in support of this statement is that, immediately following removal of the folic acid antagonist from the diet and the simultaneous administration of vitamin B₁₂, the reticulocytes rose to a mean value of 10 per cent as compared with 3 per cent for the animals not given B₁₂. Since "other blood data showed no significant differences" one wonders about the importance of these differences in reticulocyte averages.

Our own experience with the pig would suggest that such findings must be interpreted with caution as reticulocyte counts in the pig fluctuate greatly. In a second experiment with baby pigs fed a similar diet but of lower protein content, together with sulfathalidine but not supplemented with the folic acid antagonist, a "marked" reticulocytosis following vitamin B₁₂ injections and "a second marked" reticulocyte response to folic acid administration, consisted of an increase in reticulocytes from 2 to 7 per cent. Since there were "no other differences in the blood picture between the basal and the treated groups," we seriously doubt the validity of conclusions based on such meager evidence. In this report it is not stated whether or not neurologic abnormalities were observed although in the earlier, preliminary paper the authors indicated that 2 pigs developed a marked ataxia.

In view of the striking hematopoietic response following the administration of vitamin B₁₂ in pernicious anemia, the failure to produce a megaloblastic macrocytic anemia in swine in which vitamin B₁₂ deficiency was produced is surprising. There are at least three reasonable explanations for this failure. The first is that the deficiency, though severe enough to impair growth, was not sufficiently pronounced to produce hematologic or neurologic alterations corresponding to those seen in pernicious anemia in human subjects. That this explanation might be, at least in part, correct is suggested by the observations, already described, of Borson et al. in weanling rats raised from vitamin B₁₂ deficient mothers. Certainly this possibility cannot be eliminated until pigs born of vitamin B₁₂ depleted mothers have been reared on a deficient diet.

The second possibility is that in the pig a deficiency of only vitamin B₁₂ does not result in changes similar to those of pernicious anemia in man. There is considerable evidence that both vitamin B₁₂ and pteroylglutamic acid are con-
cerned in hemopoiesis and that they are interrelated in their action. Furthermore, it has been repeatedly demonstrated that the administration of pteroylglutamic acid to patients with pernicious anemia completely alleviates the anemia in the vast majority of patients. Possibly in the pig the presence of pteroylglutamic acid prevents the development of blood changes which are characteristic of pernicious anemia. Perhaps, even when no pteroylglutamic acid is included in the diet, enough is available to an animal to prevent the development of macrocytic anemia.

A third possibility involves the relationship of ascorbic acid to pteroylglutamic acid, and thereby possibly to vitamin B₁₂ metabolism. As mentioned earlier, it has been shown that, in the monkey at least, megaloblastic anemia does not develop in the presence of ascorbic acid. The pig is capable of synthesizing its own ascorbic acid.

Jukes et al. as well as Carpenter have demonstrated that the administration of recrystallized aureomycin to pigs receiving a diet containing vegetable protein markedly enhances growth. Similar results have been obtained by Stokstad and Jukes in chicks. Lichtman et al. have shown that the administration of aureomycin to patients with pernicious anemia produces a hematologic remission. In the experiments reported here, the administration of aureomycin to vitamin B₁₂ deficient pigs resulted in marked impairment of growth and may have even accentuated the anemia. When aureomycin was administered to such animals after vitamin B₁₂ had been given, no effect on either the blood or growth was observed. One wonders whether, in our experiments aureomycin accentuated the vitamin B₁₂ deficiency, possibly by diminishing the synthesis of this vitamin in the intestinal tract. In seeking an explanation for the difference between our own observations and those cited above, it must be pointed out that Jukes et al. and Carpenter used considerably younger pigs, the diets were not identical as regards the type of proteins used, the fat content or the degree of deficiency in vitamin B₁₂ and finally, the amount of aureomycin used in our experiments was larger than that employed by them.

In previous experiments in swine fed a diet similar to the one used in the studies reported here, but differing in that purified casein was fed rather than soybean alpha protein, we were unable to demonstrate a growth response to vitamin B₁₂. One wonders, therefore, why a soybean protein diet predisposes to a deficiency of vitamin B₁₂. That the vitamin B₁₂ content of the alpha protein of soybean is lower than that of purified casein is not indicated by assay. The alpha protein diet was found to contain 2.06 milligram of vitamin B₁₂ per Gm., as determined by microbiologic assay with Lactobacillus lactis Dornier (No. 8000), while the purified casein diet contained only 1.1 milligram per Gm.* Another explanation might be that certain amino acids may have a "sparring action" on vitamin B₁₂ and that the concentration of these amino acids is low in the soybean protein. It has been demonstrated that methionine has a "sparring action" on vitamin B₁₂ and that vitamin B₁₂ is intimately concerned with methionine synthesis. Our experiments confirm the observation that soybean protein is partially deficient in methionine. This may be the

* We are indebted to Mr. J. F. Roland, Head of the Nutrition Section, Armour and Company, Chicago, Ill., for these determinations.
reason why soybean protein predisposes to a deficiency of vitamin B\textsubscript{12} but it is also possible that certain amino acids which require vitamin B\textsubscript{12} for their utilization are present in excess in alpha protein. Still another explanation is that in animals fed the soybean protein diet the intestinal flora is altered in such a way that less vitamin B\textsubscript{12} is available to the animal from this source.

Several reports have appeared in the literature recently which indicate that vitamin B\textsubscript{12} has a choline-sparing action,\textsuperscript{23, 24} a marked lipotropic effect when injected into rats receiving a high fat diet\textsuperscript{48} and that it has a protective effect against experimental hepatic injury.\textsuperscript{30, 31} In the experiments reported here the livers of all 25 animals autopsied, with the exception of the 1 animal receiving vitamin B\textsubscript{12} from the beginning of the experiment, showed marked fatty infiltration or necrosis, or both. These changes occurred in the presence of a diet containing adequate amounts of protein and vitamin E and were present regardless of whether or not a methionine supplement was given. It should be pointed out, however, that the fat content of the diet was relatively high and that the amount of choline added to the diet may not have been adequate under these conditions. Four animals were treated with vitamin B\textsubscript{12} eight to thirty-two days prior to death. This did not seem to influence the pathologic changes in the liver.

**SUMMARY**

In an effort to produce a deficiency of vitamin B\textsubscript{12} a total of 70 pigs were fed a purified diet containing soybean alpha protein in place of casein. One group of animals was started on the diet at 2 to 7 days of age. A second group began at 21 to 28 days of age. Methionine, iodoated casein, desiccated thyroid and pteroylglutamic acid were added to the diet of certain animals and omitted from the diet of other pigs. In addition, 9 pigs were gastrectomized. Forty-three of the animals survived for a sufficiently long period of time for adequate evaluation of the results of the experiment.

Severe liver damage was observed in 24 of the 25 animals autopsied. The only animal not showing liver damage received vitamin B\textsubscript{12} from the beginning of the experiment. Necrosis of the liver cells, fatty infiltration, or both, occurred in the presence of a high fat diet containing apparently adequate amounts of protein, choline, vitamin E and methionine. These pathologic changes were apparently prevented but not reversed by the administration of vitamin B\textsubscript{12}.

Growth of the animals on the above diets without added vitamin B\textsubscript{12} was retarded as compared with the growth of animals on the same diet supplemented with this vitamin. The administration of vitamin B\textsubscript{12} to the deficient animals resulted in rapid growth.

Of the 39 animals not receiving vitamin B\textsubscript{12}, 13 failed to develop anemia, 16 developed a mild anemia and in 10 a moderately severe anemia was present. When present, 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. These hematologic alterations were neither consistently or completely corrected by the administration of vitamin B\textsubscript{12} in spite of the fact that definite and sometimes marked reticulocyte increases followed.
When methionine deficiency was associated with vitamin B12 deficiency, anemia appeared to be more severe.

The administration of aureomycin, an "animal protein factor," did not stimulate growth and failed to induce a hemopoietic response.

There was no macrocytic anemia, the bone marrow was not megaloblastic, and neurologic disturbances or morphologic alterations in the neutrophils were not observed.

These results are in contrast to those obtained in pigs with an experimentally produced deficiency of pteroylglutamic acid. Such animals develop macrocytic anemia, leukopenia and a macronormoblastic type of bone marrow.

It is not possible to give with any assurance the reason why megaloblastic anemia was not produced in the "B12-deficient" animals. This may have been due to the fact that (1) the deficiency was not sufficiently severe to result in such a change in the hemopoietic system; or (2) because pteroylglutamic acid prevents the development of megaloblastic anemia even in the absence of vitamin B12.

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Hematologic Manifestations of Vitamin B$_{12}$ Deficiency in Swine

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