EXPERIMENTAL PRODUCTION OF A NUTRITIONAL MACROCYTIC ANEMIA IN SWINE

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THE PURPOSE of this paper is to describe the experimental production of a macrocytic anemia in swine made deficient in pteroylglutamic acid. This subject is of interest because of the morphologic resemblance of the experimental anemia to pernicious and related macrocytic anemias, and because it is known that the hematologic manifestations of these anemias respond to pteroylglutamic acid. A preliminary report of this work has appeared.

REVIEW OF THE LITERATURE

Since the contributions of Minot and Murphy in 1927 and of Castle in 1929 concerning the etiology of pernicious anemia, many attempts have been made to produce macrocytic anemia in animals by a variety of different approaches. However, the entire pernicious anemia syndrome has not yet been produced in the experimental animal.

Several investigators have reported the production of macrocytic anemia in swine. Miller and Rhoads as early as 1935 fed swine a canine black-tongue producing diet and observed a symptom complex which included anemia, lesions of the oral mucous membranes, gastric "achlorhydria," diarrhea and motor weakness of the extremities. The disorder was thought to be associated with a loss of the anti-pernicious anemia activity of the gastric secretion and liver. Remissions of the anemia and amelioration of symptoms were induced by the administration of liver extract. Contrary to their claim, however, the anemia was not actually macrocytic. The average mean corpuscular volume in the animals with "macroyctic" anemia was reported to be 59.5 c. The mean corpuscular volume in normal swine is about 56 ± 5.16 c. Furthermore, intermittent periods of achlorhydria may be observed in normal swine.

Smith, Reiser and Harrell observed a macrocytic anemia in weanling pigs on a prolonged partial deficiency of the vitamin B complex but spontaneous cure of the anemia ensued while the pigs remained on the same diet and without treatment. The diet used was a modification of the Goldberger black-tongue producing diet.
and was almost identical to that used by Miller and Rhoads. The mean corpuscular volumes reached 74 to 97 cu. The inclusion of 10 per cent brewer's yeast in the diet protected the animals. McGowan and Sinclair found that young pigs kept on a ration of corn, fish meal and "draft" became ill with anemia and jaundice and liver damage were observed. The anemia was described as macrocytic and the femoral marrow was red and cellular. Reticulocyte increases occurred following the administration of raw liver, the anemia disappeared and the bone marrow became normal. Lawrason and Cronkite observed a macrocytic anemia and achlorhydria in two pigs exposed to atom bomb ionizing radiation at Bikini. In one animal the bone marrow was hyperplastic and "megaloblastic-like." The relation of these instances of anemia in experimental animals to nutritional deficiency is not clear.

Welch, Heinle and colleagues have reported the production of macrocytic anemia with megaloblastic hyperplasia of the bone marrow in pigs maintained on a highly purified diet essentially free of extrinsic factor and to which sulfasuxidine and a folic acid antagonist were added. One of the animals responded to the administration of crude sodium caseinate together with a 95 per cent ethanol extract of crude casein and normal human gastric juice. This animal later relapsed and was treated successfully with a single injection of 15 units of liver extract. A second animal responded rapidly to four daily intramuscular injections of 10 mg. of pteroylglutamic acid. A third pig became critically ill and was successfully treated with a combination of purified liver extract, pteroylglutamic acid and niacinamide. These investigators concluded that purified liver extract is effective in correcting the anemia initially but found that relapses developing after liver-induced remissions were refractory to liver extract. Extract prepared from the liver of a pig raised on the purified casein diet and given pteroylglutamic acid was hemopoietically inactive when assayed in a patient with pernicious anemia. An extract from the liver of the same animal after a month on the same diet, except that crude casein containing "extrinsic factor" replaced the purified casein, was active when so assayed. An extract prepared from normal pig liver was even more active. It was concluded that pteroylglutamic acid can elicit a complete hematopoietic response in pigs on a purified diet poor in extrinsic factor. Our own studies in swine have been alluded to already and will be discussed more fully later.

In monkeys, Wills reported the production of a severe macrocytic anemia with a megaloblastic bone marrow. The animals were given a diet similar to that in common use among the poorer class of Mohammedans in Bombay, where nutritional macrocytic anemia is common. It consisted of polished rice, margarine, salt, iron, white bread, cod liver oil, and either tomatoes or carrots. The anemia responded rapidly following the administration of either marmite or Campolon but purified anti-pernicious liver extracts were ineffective. Bartonella infections were not present.

The earlier work in dogs fed a modified Goldberger black-tongue producing diet was inconclusive. Several of the dogs had been splenectomized and it was later demonstrated that the anemia was complicated by Bartonella infection and that such an infection was capable of itself producing a severe anemia. Later a rela-
relationship between nicotinic acid deficiency and the development of macrocytic anemia was suggested by the work of Handler and Featherston. Dogs fed three different types of diet deficient in nicotinic acid developed severe macrocytic anemia. A sharp reticulocyte response and subsequent elevation of the red cell count and hemoglobin followed the administration of nicotinic acid but not when purified liver extract was given. More recently Ruegamer, Brickson, Torbet and Elvehjem observed that when young growing dogs were fed a niacin-deficient purified ration containing 1 per cent sulfasuxidine, weight loss and signs of black-tongue developed. Small doses of niacin were only partially effective in combating the loss of weight. Pteroylglutamic acid helped to produce a more consistent response to niacin but had no apparent effect on the macrocytic anemia which appeared and became progressively more severe. Diarrhea was present and in the most deficient animals a pronounced, flaccid type of paralysis was observed. As little as 0.05 ml. of purified liver extract (20 "units"/ml) was effective in bringing about a complete remission of the anemia. Bone marrow studies were not mentioned. This work would seem to be of considerable importance if it can be confirmed since it represents the only liver extract responsive, macrocytic anemia associated with neurological symptoms to be reported to date in experimental animals. The administration of choline or acetylcholine has been claimed by Davis to produce a macrocytic anemia in dogs but this has not been confirmed.

Wills produced macrocytic anemia in rats but found that the anemia was due to Bartonella infection. Watson, Cameron and Witts have reported that the formation of a blind intestinal loop in rats leads to the development of a macrocytic anemia which responds to purified liver extract. The bone marrow contained increased numbers of proerythroblasts and basophilic erythroblasts. A striking difference between this anemia and that seen in nutritional macrocytic anemia and in pernicious anemia is the fact that marked reticulocytosis (10 to 40 per cent) was present in the rats prior to therapy.

It has been reported that in some instances a deficiency of copper in sheep and cattle is associated with a macrocytic anemia but detailed morphologic studies have not been done and this work needs confirmation.

Pteroylglutamic acid deficiency in the rat is associated with severe normocytic anemia, granulocytopenia, lymphopenia and thrombocytopenia. However, such a deficiency in the chick has been described as leading to the development of macrocytic anemia and the mean corpuscular volume has been reported to be increased from the normal of 137 cμ to 161. The anemia is accompanied by severe leukopenia, due mainly to lymphopenia, the absolute numbers of neutrophils being maintained. The thrombocytes also diminish in number. In none of these species has the anemia responded to purified liver extract therapy. The entire stomach of various species of animals including the dog, cat, rat, pig and monkey has been removed by a number of investigators. This approach has not resulted in the production of macrocytic anemia in any species. Petri and his group in a large series of experiments extending over a period of nearly twenty years have failed to produce macrocytic anemia in either the dog or the pig. They
have observed, however, that total gastrectomy in swine leads to the complete disappearance of the anti-pernicious anemia substance in the liver and that nicotinic acid therapy subsequent to the resection can prevent this loss.\(^{33}\)

**Experimental Procedure**

Including the four animals described in the preliminary report,\(^1\) a total of 32 weanling Chester-White pigs, 21 to 28 days of age were used in this study. The animals were housed in individual cages and were fed the purified diet from the day they were received which was also the day of weaning.

Two types of basal diet were fed, a 10 per cent and a 16 per cent protein diet, the compositions of which were as follows:

<table>
<thead>
<tr>
<th></th>
<th>per cent</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>26.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>57.7</td>
<td>73.8</td>
</tr>
<tr>
<td>Lard</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Salt mixture(^3)</td>
<td>5.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Two types of casein were used in different animals, Sheffield's New Process (crude) casein and Sheffield's alcohol-extracted (purified) casein. The latter was prepared from Sheffield's 'high nitrogen casein'\(^4\) by presoaking for 18 hours with cold 98 per cent methanol at pH 6, followed by a continuous 24 hour extraction with hot methanol. The extracted casein was then tray dried to remove the residual methanol. It has been demonstrated previously in this laboratory by assay in patients with pernicious anemia in relapse that the Sheffield New Process (crude) casein contains significant amounts of 'extrinsic factor' activity.\(^1\) The Sheffield alcohol-extracted (purified) casein has been assayed in a similar manner for extrinsic factor activity. The procedure of assay was as follows. The patient was hospitalized and during the assay periods liver, meat, meat products, milk and poultry were excluded from the diet. Bread, cereals, sugar, fats, vegetables and fruits were permitted in the amounts desired. Daily for ten days, 50 grams of the casein to be assayed were incubated at 37°C for two hours with 10 to 2.00 ml. of normal human gastric juice at pH 1.5 to 3.5. The incubation mixture was then strained through cheese cloth; the filtrate was neutralized to pH 5.0 and administered immediately to the patient. The results are shown in figure 1. The purified casein, in the quantity given, contained insignificant amounts of extrinsic factor activity, whereas the same quantity of crude casein apparently carried an amount adequate to give a significant reticulocytosis and rise in volume of packed red cells. Following this response, the administration of one U.S.P. unit of purified liver extract daily for ten days did not produce a further reticulocytosis.

Depending upon the type and amount of casein in the basal diet, the animals were divided into four groups as follows:

- **Group A**—Crude casein, 10 per cent.
- **Group B**—Crude casein, 16 per cent.
- **Group C**—Purified casein, 10 per cent.
- **Group D**—Purified casein, 10 per cent plus 15 U.S.P. units of purified liver extract (\(^*\)1039, Parke, Davis, 15 U.S.P. units per ml.), administered intramuscularly every 15 days from the beginning of the experiment.

The basal diet was fed in amounts of 36.5 grams (152 calories) per kilogram of body weight per pig per day. Sulfasuxidine was added to the basal diet of all animals in amounts of 0.0 per cent. All animals were given a crude methyl-folic acid antagonist prepared\(^4\) by allowing 2,4,5-triamino-6-hydroxyprimidine and p-amino benzoil-L (+)-glutamic acid to react with 2, 3-dibromobutyraldehyde.\(^36\) This antagonist was administered either in capsules (0.06 Gm. per kilogram of body weight) or added to the diet (0.1 per cent). Natola (Parke, Davis, 55,000 units of vitamin A, 11,000 units of vitamin D per gram), 0.056 gram per kilogram of body weight per week supplemented the basal diet. Vitamins

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\(^*\) Prepared by heating freshly separated "fat-free" milk to 110-115°F and precipitating the casein with dilute muriatic acid at pH 4.5-4.6. The casein is then repeatedly washed to reduce the free acid and mineral constituents to a minimum and is then dried continuously.

\(^†\) By Dr. M. E. Hultquist and Dr. J. M. Smith, Jr., of the Calco Chemical Division, American Cyanamid Company, Pearl River, N. Y.
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 kilogram of body weight per day):

- Thiamin hydrochloride: 0.25 mg.
- Pyridoxine hydrochloride: 0.20 mg.
- Riboflavin: 0.12 mg.
- Calcium pantothenate: 0.50 mg.
- Nicotinic acid: 0.01 mg.
- Choline chloride: 0.01 mg.

In addition, all animals received crystalline biotin, 50 μg per kilogram of body weight per week intramuscularly.

Hematologic studies (red blood cell count, hemoglobin, volume of packed red cells, reticulocyte count, total leukocyte count, differential leukocyte count and platelet count) were performed weekly on each animal throughout the entire experiment.

The cellular composition of the bone marrow was studied in each animal at the onset of the experiment and at the time of development of the deficiency, as well as before and after each therapeutic test. Specimens of bone marrow were obtained by aspiration of the sternal marrow with standard 16 gauge sternal puncture needles. A small amount of marrow fluid, usually less than 0.3 ml., was withdrawn into a clean dry syringe and then cover-glass preparations were drawn and stained with Wright’s stain. Differential cell counts were made on 500 to 1,500 cells. Because of the large amount of material the differential counts will not be presented in detail in this publication. Photomicrographs of several types of cells as well as detailed differential counts were presented in the preliminary report.

The total 24 hour urinary excretion of total hydroxyphenyl compounds (tyrosine, p-hydroxyphenyllactic and p-hydroxyphenylpyruvic acids), expressed as the tyrosine equivalent and referred to as “tyrosyl,” was determined by the Folin and Ciocalteu method as modified by Medes. The urine, made weakly acid with acetic acid, was shaken with fuller’s earth to remove coloring matter and impurities capable of reacting with mercuric sulfate in subsequent procedures. The intensity of the final color was measured in the Evelyn photoelectric colorimeter, using filter a.m. Commercial tyrosine, three times recrystallized served as a standard. Allantoin was determined in the urine by the method of Young and Conway, using the Evelyn photoelectric colorimeter and filter a.m. Recrystallized potassium allantoate served as a standard. Urinary uric acid was determined by a modification of the Kern and Stransky method.

RESULTS

General: The animals deficient in pteroylglutamic acid presented an untidy appearance with thin, lusterless hair. Their growth was poor, although it was no more impaired than that of the low-protein controls not given the antagonist. On the 26 per cent casein diet growth was good but, by comparison with growth...
Table 1.—Summary of the Data on the Anemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Pigs</th>
<th>Days on Experiment</th>
<th>Prior to Deficiency</th>
<th>During Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R.B.C. Mill./c.mm.</td>
<td>Hgb. Gm. %</td>
</tr>
<tr>
<td>A......</td>
<td>12</td>
<td>110</td>
<td>±16.9</td>
<td>7.76</td>
</tr>
<tr>
<td>B......</td>
<td>4</td>
<td>160</td>
<td>±37.2</td>
<td>7.12</td>
</tr>
<tr>
<td>C......</td>
<td>11</td>
<td>69</td>
<td>±11.4</td>
<td>8.19</td>
</tr>
<tr>
<td>D......</td>
<td>5</td>
<td>76</td>
<td>±5.8</td>
<td>7.79</td>
</tr>
</tbody>
</table>

Group A, Crude casein, 10 per cent; Group B, Crude casein, 16 per cent; Group C, Purified casein, 10 per cent; Group D, Purified casein, 10 per cent plus liver extract, (15 units every 15 days).

VPRC, volume of packed red cells.
MCV, mean corpuscular volume.
MCH, mean corpuscular hemoglobin.
MCHC, mean corpuscular hemoglobin concentration.
curves of animals fed the basal diet plus 3 to 6 grams of yeast per kilogram of body weight, there was a significant impairment of growth. At 120 days of age the four deficient animals given a 26 per cent casein diet (group B) weighed on the average 24.1 kilograms and at 180 days 46.1 kilograms. The weight of animals given yeast and no antagonist was 25 kilograms at 120 days of age and 67 kilograms at 180 days.

In addition, the deficient animals became listless, weak and ate poorly. Moderately severe diarrhea was present and the stools were somewhat orange-yellow in color, due presumably to the presence of antagonist. No oral lesions were observed. Spontaneous partial remissions of the pancytopenia occurred from time to time in the course of the experiment (figures 6 and 7). These remissions were usually associated with a slight reticulocytosis of 6 to 8 per cent. It is unlikely that the remissions were due to contamination of the diet since only one of two pigs kept in separate pens but eating out of a common trough might have a spontaneous response. A more plausible explanation would be that a favorable change in the synthesis of pteroylglutamic acid by the intestinal flora took place at times. Once the deficiency had become fully established, however, no further spontaneous remissions were observed.

It should be noted that the four pigs (10-53, 10-54, 10-56 and 10-64) described in the preliminary report were depleted of pteroylglutamic acid for 80 to 120 days before the crude methyl folic acid antagonist was administered. These four pigs are included in group A. The remainder of the animals described here were given the antagonist from the beginning of the experiment.

**Red Blood Cells:** All four groups of animals developed severe macrocytic anemia (table 1), the only difference between the groups being in the rapidity of development and degree of macrocystosis. The administration of 26 per cent crude casein (group B) rather than 10 per cent crude casein (group A) delayed the onset of anemia by an average of 40 days. Anemia developed most rapidly in the animals receiving 10 per cent purified casein (group C), severe anemia being present in about 69 days as compared with the 160 days required in the animals fed 26 per cent crude casein (group B). As can be seen by inspection of the values for group D (table 1), the administration from the beginning of the experiment of 15 U.S.P. units of liver extract every 15 days did not prevent nor delay the appearance of anemia. The animals in group D were given a diet identical with that of group C.

The anemia in all four groups of animals was macrocytic. The mean corpuscular hemoglobin concentration was normal. However, the macrocystosis in group B was marked, whereas in group C it was only slight. The factors which seemed to determine the degree of macrocystosis are illustrated in figure 2. From this and from inspection of table 1 it can be seen that the degree of macrocystosis increased as time went on. An additional factor appeared to be the amount of protein in the diet since the group receiving 26 per cent casein (group B) developed a greater degree of macrocystosis in the same period of time than did the group receiving 10 per cent casein (group A). In the former group mean corpuscular volumes as great as 104 cu were observed.

The first change noted in the red cells was a marked anisocytosis. About an equal
number of macrocytes and microcytes were present. As the deficiency progressed
the proportion of macrocytes increased. Poikilocytosis was not prominent at any
time. An increase above the normal in the number of Howell-Jolly bodies, nucleated
red blood cells and polychromatophilic cells generally took place. The anemia was
associated with a slight reticulocytosis of 2 to 3 per cent (table 1).

![Graph showing degree of macrocytosis.]

**Fig. 1.**—Degree of macrocytosis in animals fed the crude, 16 per cent casein diet (Group B, solid line) compared with that in animals fed a crude, 10 per cent casein diet (Group A, broken line). The data represent the means of values in all 4 pigs of Group B and 6 pigs studied for a corresponding length of time in Group A.

**Table 2.**—Summary of the Data on Leukocytes and Platelets

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Pigs</th>
<th>Days on Experiment</th>
<th>Prior to Deficiency</th>
<th>During Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>WBC X 1000/µl</td>
<td>PMN X 1000/µl</td>
</tr>
<tr>
<td>A</td>
<td>12</td>
<td>120</td>
<td>17.5 ± 3.43</td>
<td>5.6 ± 1.37</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>160</td>
<td>17.0 ± 7.77</td>
<td>5.8 ± 4.96</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>69</td>
<td>17.0 ± 6.6</td>
<td>6.3 ± 3.81</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>76</td>
<td>17.1 ± 3.82</td>
<td>6.7 ± 3.07</td>
</tr>
</tbody>
</table>

Group A, Crude casein, 10 per cent; Group B, Crude casein, 16 per cent; Group C, Purified casein, 10 per cent; Group D, Purified casein, 10 per cent plus liver extract, 15 units every 15 days.

PMN, polymorphonuclear cells including neutrophils, eosinophils and basophils.

MNC, mononuclear cells including lymphocytes and monocytes.

**Leukocytes** (table 2): Marked leukopenia accompanied the anemia, the absolute number of leukocytes being reduced to 50 to 58 per cent of their original values. The leukopenia resulted from a reduction in both polymorphonuclear and mononuclear cells but there was a proportionately greater reduction in polymorpho-
nuclear cells (82 per cent) than in mononuclear cells (44 per cent). All four groups of animals developed these changes to the same degree but the changes occurred much more rapidly on the purified than on the crude casein. Giant metamyelocytes and multinucleated neutrophils such as those seen in the blood smears of patients with pernicious anemia were not observed in the blood of the pigs.

Platelets (table 2): Slight thrombocytopenia developed. The values for individual pigs varied considerably but in general there was a slight reduction at the time of maximal anemia. No differences were noted between the four groups. Spontaneous rises occurred occasionally (figures 6 and 7).

*Sternal Marrow:* Differential cell counts on marrow obtained from the deficient animals revealed rather striking alterations from the normal. These are only summarized in table 3 since detailed differential counts were presented in the preliminary report.¹ No significant differences were noted between the four groups of animals. For this reason all the deficient animals are included in one group in the table.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Non-Deficient Pigs</th>
<th>Deficient Pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblasts</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Promyelocytes and Myelocytes</td>
<td>9.2</td>
<td>12.1</td>
</tr>
<tr>
<td>Metamyelocytes and PMN Neutrophils</td>
<td>49.3</td>
<td>22.2</td>
</tr>
<tr>
<td>Normoblasts</td>
<td>32.3</td>
<td>35.2</td>
</tr>
<tr>
<td>&quot;Megaloblasts&quot;</td>
<td>0.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Leukocyte:Erythroid Ratio</td>
<td>2.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values represent means.

There was a marked reduction in polymorphonuclear neutrophils and metamyelocytes and a slight increase in the proportion of myelocytes, promyelocytes and myeloblasts in the marrow of the deficient animals. The leukocyte-erythroid ratio was decreased. In addition, extremely immature nucleated red cells were present which differed from the basophilic normoblasts seen in the marrow of normal pigs and in those fed diets low in protein or deficient in iron. These cells were large, measuring 12 to 15 microns in diameter, and their nuclear chromatin was delicate and meshlike. In some cells the chromatin showed a tendency to clump; in others the chromatin appeared finely granular and more homogeneous. A delicate nuclear membrane separated the relatively large nucleus from the homogeneous basophilic cytoplasm. The more immature of these cells contained two or three distinct nucleoli. Later stages were present, including the orthochromatic stage. This entire series of cells constituted about 15 per cent (3 to 40) of all the bone marrow cells. Photomicrographs were included in the previous report.¹

These cells resembled closely the megaloblasts seen in the marrow of patients with pernicious anemia, the only distinct difference being that the nuclear chromatin was not as fine and meshlike as that seen in megaloblasts in man. Whether
or not the cells described are pig megaloblasts is a matter for conjecture but for purposes of discussion in this paper these cells will be referred to as megaloblasts in order to distinguish them from the immature red cells (normoblasts) seen in other types of anemia in swine. It is noteworthy that these megaloblasts were present in the bone marrow in the same proportion in group D as in group A, B and C, in spite of the administration of liver extract from the beginning of the experiment.

"Tyrosyl," Allantoin and Uric Acid Excretion: The data on the urinary excretion of "tyrosyl," allantoin and uric acid, both before and one month after pteroylglutamic acid therapy, are summarized in table 4. These represent the means of three consecutive daily determinations in each of 5 animals.

The term "tyrosyl" is used to refer collectively to the hydroxyphenyl compounds (tyrosine, p-hydroxyphenyllactic acid and p-hydroxyphenylpyruvic acid) as determined by the method of Folin and Ciocalteau.36 No significant change was noted in the excretion of "tyrosyl." Determinations were done daily on 2 animals for thirty days following therapy. No consistent increase or decrease was noted either within the first day or two, or later. The amount excreted per day by a single animal varied from day to day as much as 575 mg.

The 24 hour urinary excretion of allantoin and uric acid was not significantly different before therapy as compared with the excretion one month later. However, immediately following therapy, in association with the reticulocytosis, there was a marked increase in the excretion of allantoin as shown in figure 3. A similar increase in uric acid excretion did not occur.

Pathologic Studies*: Autopsy material including sternal, rib and femoral bone marrow, liver, spleen, stomach, kidney, lung, skeletal muscle and cardiac muscle, was obtained from 7 untreated animals in which a deficiency of pteroylglutamic acid had been produced. The bone marrows showed a striking cellular hyperplasia. Megakaryocytes were present in approximately normal numbers except in the marrow of pig 10-88. This animal had a marked thrombocytopenia (72,000 per cu.mm.) just prior to death, and the number of megakaryocytes was reduced.

Microscopic examination of the liver, spleen, kidneys, lungs and cardiac muscle failed to reveal any significant abnormalities although small areas of interstitial

* We are indebted to Dr. F. D. Gunn, Professor of Pathology, University of Utah, for these studies.
hemorrhage were seen in sections of the lungs of two animals. An increase in hemosiderin was not observed in any of the organs studied. Areas of atrophy, hyalinization and segmental necrosis were present in the sections of skeletal muscle. Similar changes have been observed in the muscles of animals fed a diet low in protein.

Response to Various Therapeutic Agents: The increases in reticulocytes and volume of packed red cells following therapy with pteroylglutamic acid compounds (23 animals) are summarized in table 5. Representative examples are illustrated in detail in figures 4, 5, 6, 7 and 8. In every instance except two, a reticulocytosis of...

![Graph](image)

Fig. 3.—Urinary excretion of uric acid and allantoin (pig 10-73, Group B) following a single intramuscular injection of 10 mg of pteroylglutamic acid. Note the marked increase in the excretion of allantoin during the period of reticulocytosis. The excretion of uric acid remained relatively constant.

11 per cent or greater followed therapy with a pteroylglutamate. In half of the animals treated there was a reticulocytosis of 21 to 42 per cent. The reticulocyte curve rose sharply with a peak on either the third or fourth day following therapy, as illustrated in figure 4, in all but two instances. In these the peak was reached on the second and fifth days, respectively. Simultaneously with or just following the reticulocytosis there occurred a rapid rise in the volume of packed red cells and a return to normal or near normal values within two to three weeks. Frequently the increase was as great as 10 to 15 ml./100 ml. in the first week following therapy (figure 4). In all instances except two the increase was greater than 10 ml./100 ml. and in one-half of the animals the rise was greater than 15 ml./100 ml. The mean corpuscular volume returned to normal more slowly and frequently macrocytosis persisted in the absence of anemia.

An initial leukocyte increase, generally greater than 5000 per cu.mm., invariably followed this type of therapy (table 8). After several weeks the leukocytes then decreased somewhat but in 10 of the 23 animals so treated the values for total leukocytes were sustained within the normal range. A thrombocytosis also oc-
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curred in almost every instance but due to the great variability in the number of platelets the results are difficult to interpret.

In the bone marrow pteroylglutamic acid therapy was associated with a reversion to normal. There was a decrease in the megaloblast-like cells, an increase in the myeloid-erythroid ratio and a "shift to the right" in the myeloid series. Frequently large masses of platelets, covering several low-power fields, were present in the bone marrow preparations.

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Compound</th>
<th>Dose (mg. I.M.)</th>
<th>Before Therapy</th>
<th>After Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PGA</td>
<td>V.P.R.C. ml., 100 ml.</td>
<td>Retics %</td>
</tr>
<tr>
<td>10-53</td>
<td>PGA</td>
<td>100</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>10-54</td>
<td>PGA</td>
<td>2.0</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>10-56</td>
<td>PGA</td>
<td>2.0</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>10-64</td>
<td>PGA</td>
<td>2.0</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>10-72</td>
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<td>2.0</td>
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</tr>
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<td>PHGA</td>
<td>10</td>
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<td>2</td>
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PGA, pteroylglutamic acid.
PDGA, pteroyldiglutamic acid.
PTGA, pteroyltriglutamic acid.
PHGA, pteroylheptaglutamic acid.
V.P.R.C., volume of packed red cells.

Pteroyldiglutamic acid (figure 4), pteroyldiglutamic acid (figure 8) and pteroyl-
heptaglutamic acid were as effective as pteroylglutamic acid (table 5) in restoring
the blood and bone marrow to normal. The one animal (10-97) treated with the
diglutamate, one (10-98) of the four animals treated with the triglutamate and
both animals (10-85, 10-96) treated with the heptaglutamate were fed the purified
casein diet and so presumably had received little "extrinsic factor."

The effects of therapy with various commercial liver extracts and with vitamin
Fig. 4.—Pig 10-97 (Group C, purified casein, 10 per cent). Note the marked reticulocytosis and increase in volume of packed red cells (Ht), leukocytes (WBC), and polymorphonuclear cells (PMN) following the intramuscular administration of 50 mg. of pteroyldiglutamic acid.

MCV refers to mean corpuscular volume in cc; Ht. refers to volume of packed red cells, ml/100 ml; retic refers to reticulocytes, per cent; WBC refers to total leukocyte count, thousands per c.mm.; PMN refers to polymorphonuclear cells, thousands per c.mm.; Plat. refers to platelets, times 100,000 per c.mm.

Fig. 5.—Pig 10-72 (Group B, crude casein, 26 per cent). Note the reticulocytosis and gradual increase in the volume of packed red cells (Ht.) following the intramuscular administration of 2 ml. (No. 1039, 30 U.S.P. units) of purified liver extract. This represents the greatest response to liver extract observed. Compare with figures 7 and 8. After becoming anemic again this animal failed to respond to a second injection of liver extract (No. 1039, 30 U.S.P. units) although it responded promptly to 20 mg of pteroylglutamic acid, intramuscularly. It is noteworthy that this animal was receiving 26 per cent crude casein and consequently had available liberal amounts of extrinsic factor. For symbols see figure 4.
B₁₂ are presented in table 6. Representative examples are illustrated in figures 5, 6, 7, 8. A significant reticulocytosis of more than 10 per cent occurred in 33 per cent
Liver extracts given intramuscularly in a single injection. V.P.R.C. refers to volume of packed red cells.

Liver extract 1039, Parke, Davis and Co.; 1124, Armour and Co., 20 mg. solids per ml; 1067, Armour and Co.; 1063, Armour and Co., containing in vitro factors of Hays (43); 1066, Armour and Co., one ml. derived from 46 grams of fresh liver.

animals given the purified casein diet than in the animals fed crude casein. There was no correlation between the number of units of liver extract given and the degree of response. The greatest reticulocytosis observed following liver extract therapy...
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was 24 per cent (pig 10-72, figure 5). After becoming anemic a second time, this animal then failed to respond to a second injection of liver extract but responded promptly to 30 mg. of pteroylglutamic acid. Pig 10-92 (figure 6) was given the purified casein diet and 25 U.S.P. units of liver extract every two weeks from the beginning of the experiment. When severe anemia developed, 10 ml. of liver extract (150 units/ml.) was administered in a single injection. In spite of the previous administration of liver extract, reticulocytosis appeared which reached a peak of 21 per cent on the 12th day following therapy. However, the reticulocyte

Table 7.—Results of Therapy With Various Other Substances

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Therapy</th>
<th>Before Therapy</th>
<th>After Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compound</td>
<td>Route</td>
<td>Dose</td>
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<tr>
<td>10-76</td>
<td>Thymine</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
<tr>
<td>10-77</td>
<td>Thymine</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
<tr>
<td>10-81</td>
<td>Thymine</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
<tr>
<td>10-81</td>
<td>Thymine</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
<tr>
<td>10-77</td>
<td>Uracil</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
<tr>
<td>10-77</td>
<td>Adenine</td>
<td>Oral</td>
<td>5 Gm.</td>
</tr>
<tr>
<td>10-80</td>
<td>Xanthopterin</td>
<td>I.M.</td>
<td>20 mg.</td>
</tr>
<tr>
<td>10-80</td>
<td>Xanthopterin</td>
<td>Oral</td>
<td>25 mg.</td>
</tr>
<tr>
<td>10-78</td>
<td>Tyrosine</td>
<td>Oral</td>
<td>10 Gm.</td>
</tr>
</tbody>
</table>

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

Table 8.—The Initial Rise in the Leukocytes Following Pteroylglutamic Acid Therapy (23 pigs) as Compared With Liver Extract Therapy (17 pigs)

<table>
<thead>
<tr>
<th>Initial Rise in WBC X 1000/c.mm</th>
<th>Pteroylglutamic Acid Compounds No. Pigs</th>
<th>Liver Extracts No. Pigs</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1–5</td>
<td>4</td>
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<td>5–10</td>
<td>12</td>
<td>2</td>
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<tr>
<td>10–15</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

curve was irregular and was not accompanied by a rise in volume of packed red cells.

A significant increase in the volume of packed red cells following liver therapy was observed in only 5 pigs (10-54, 10-56, 10-72, 10-77, 10-80) and in these the rise was 8, 12, 14, 13 and 8 ml./100 ml. respectively. By comparison, pteroylglutamic acid therapy was followed by a rise in the volume of packed red cells greater than 10 ml./100 ml. in 91 per cent of the trials. The rise was prompt as well as marked. In general, the mean corpuscular volume was little affected by liver extract therapy (figures 6, 7, 8).

An initial increase in leukocytes following liver therapy occurred in 12 out of 15 trials but it was not as marked as the rise following pteroylglutamic acid therapy (table 8). In 10 instances the leukocyte level was sustained within the normal
range. Thrombocyte increases occurred irregularly but in general the increases were not sustained.

The changes in the bone marrow which followed liver therapy were of the same type and degree as the changes noted in the peripheral blood. In most instances, as the number of leukocytes rose following the injection of liver, there was an increase in metamyelocytes and neutrophils and an increase toward normal in the leukocyte-erythroid ratio. In the bone marrow of those animals in which the anemia responded partially to liver therapy there was a reduction in the relative numbers of megaloblast-like cells but in no instance did they disappear entirely. If no response to liver was observed, no changes were noted in the bone marrow.

Crystalline vitamin B₁₂ was administered to one pig (table 6). The reticulocytes rose from 1 to 6 per cent and the volume of packed red cells increased from 20 to 31 in 2.9 days.

The results of therapy with various other substances are presented in table 7. Uracil, adenine and tyrosine were inactive in the doses given. Xanthopterin in a single injection of 20 mg. (1 mg. kilogram of body weight) produced, in a single animal, a slight reticulocytosis of 9 per cent and a small unsustained rise in volume of packed red cells. This was then followed by 25 mg. (1.9 mg. kilogram) of xanthopterin daily by mouth for five days without a further response. A response to thymine with a reticulocytosis of 21 per cent was observed in one animal (10-81). Although the volume of packed red cells, leukocytes and platelets increased, the increase was not maintained and a second course of thymine was ineffective. In two other pigs (10-76, 10-77) no response followed the administration of thymine.

**DISCUSSION**

Macrocytic anemia, leukopenia due to a proportionately greater reduction in polymorphonuclear than in mononuclear cells, slight thrombocytopenia, and a bone marrow picture showing erythroid hyperplasia and immature red cells resembling the megaloblasts of pernicious anemia, developed in swine fed low or high levels of crude or purified casein and given sulfasuxidine and a pteroylglutamic acid antagonist. A clear cut and sharp hemopoietic response was observed in such animals whenever pteroylglutamic acid was given. The administration of liver extract, however, was associated with only a slight effect. Thus, in 10 out of 15 trials, no or only slight activity was observed. In 5 trials a significant reticulocytosis occurred but the reticulocyte peak was delayed and the curve was flat as compared with that produced by pteroylglutamic acid. Although an increase in volume of packed red cells followed liver extract therapy in 5 pigs, this increase was delayed, submaximal and unsustained.

These blood and bone marrow changes developed in the experimental animals not only when crude casein containing “extrinsic factor” was fed, but even in pigs injected every 15 days with liver extract containing 15 units of anti-pernicious anemia principle. Thus it seems clear that a hematologic syndrome with morphologic characteristics similar to those of pernicious anemia can be produced experimentally in the pig in the presence of the anti-pernicious anemia liver factor. This condition would appear to be similar to the pteroylglutamic acid-responsive, liver-
refractory megaloblastic anemias described in human subjects; namely, tropical
macrocytic anemia,\textsuperscript{44, 45, 46} macrocytic anemia of pregnancy,\textsuperscript{47} achrestic anemia,\textsuperscript{48} and refractory megaloblastic anemia.\textsuperscript{49}

It is not likely that the response to liver extract, such as it was, can be attributed
to the pteroylglutamic acid content of the extracts. More than 50 \mu g of pteroyl-
glutamic acid has been found to be required by the pig in order to elicit a significant
response (table 6). The extracts used were found by assay with \textit{Lactobacillus casei}
in our laboratory as well as by others\textsuperscript{39} to contain only about 1 to 5 \mu g of the vita-
min per ml. and the greatest response to liver extract (pig 10-71) occurred following
the injection of only 2 ml. Furthermore, there was no correlation between the
number of ml. of extract given and the degree of response. Again, extracts contain-
ing no anti-pernicious anemia activity (nos. 1063 and 1066) but possessing the same
quantities of pteroylglutamic acid were ineffective. The less highly purified extract
(no. 1067, 4 units/ml.) containing about 5 \mu g of pteroylglutamic acid per ml. was
no more effective in the pig than the more highly refined extract (no. 1124, 70
units/ml.) which contained only about 1 \mu g per ml.

Since the response following the administration of crystalline vitamin B_{12}* was
of the same type as that following purified liver extract, it is reasonable to assume
that the effectiveness of the liver extracts was due to their vitamin B_{12} content (or
to a chemically related substance) rather than to a third factor and that in those
pigs responding to liver extract there existed a partial deficiency of the liver
factor in addition to the pteroylglutamic acid deficiency. Consistent with this is
the observation that pig 10-72 (fig. 5) failed to respond to a second injection of liver
extract after becoming anemic a second time. Presumably the deficiency had been
satisfied by the injection of liver extract and a second response was therefore not
obtainable.

Animals receiving crude casein containing considerable extrinsic factor activity
responded as well to liver extract as did those receiving purified casein. It must be
concluded, therefore, that a partial deficiency of liver principle developed in spite
of the availability of extrinsic factor. On the other hand, pigs fed a diet similar in
all respects to the low protein diet used in these experiments, with the exception
that a pteroylglutamic acid antagonist was not included, did not respond at all to
liver extract.\textsuperscript{51} This suggests that in pteroylglutamic acid deficiency the require-
ment for a factor in liver extract (vitamin B_{12}) is increased. One may speculate
whether pteroylglutamic acid plays a role in the release, absorption or synthesis
of vitamin B_{12} in the intestinal tract.

The hypothesis has been presented\textsuperscript{42} that pteroylglutamic acid functions in some
way in the synthesis of thymine and that B_{12} serves as a coenzyme which is con-
cerned with the conversion of thymine to thymidine. According to this hypothesis,
the curative effects of pteroylglutamic acid in pernicious anemia depend upon
increased thymine synthesis, which, by mass action, leads to the formation of
thymidine. The effectiveness of large amounts of thymine in pernicious anemia is
explained on a similar mass action hypothesis. If this view is correct, then pigs

* This finding has been confirmed in five additional pigs.
deficient in pteroylglutamic acid should respond to large doses of thymine. Such was not the case.

The pteroylglutamic acid deficient swine, receiving either crude or purified casein, responded rapidly and maximally to each of the three pteroylglutamic acid conjugates tested. This indicates that in these animals there was adequate utilization of the conjugates. If the animals were actually in a state of liver factor depletion, as suggested by assays of the livers of similar animals, then it is difficult to accept the hypothesis that the liver factor is necessary for the proper utilization of the naturally occurring conjugates. However, it must be admitted that it is much easier to find flaws in current hypotheses concerning the role of pteroylglutamic acid and the anti-pernicious anemia factor in metabolism than it is to offer an explanation which is wholly satisfactory.

Swendseid, Wandruff and Bethell have found that the urinary excretion of total phenols and hydroxyphenyl acids is increased in patients with pernicious anemia in relapse and that within twenty-four hours following therapy with liver extract there is a marked reduction in the phenolic fraction containing the hydroxyphenolic acids. It has also been claimed that liver suspensions from pteroylglutamic acid deficient rats are better able to oxidize tyrosine after the addition of pteroylglutamic acid than in the absence of this substance and that either pteroylglutamic acid or anti-pernicious anemia liver extracts are capable of reducing the increased keto acid and tyrosyl excretion in scorbutic guinea pigs. The results of the tyrosyl excretion studies presented here fail to indicate the presence of a defect in tyrosine metabolism in pigs.

The markedly increased excretion of allantoin in the urine during the period of reticulocytosis following therapy with pteroylglutamic acid is similar to the increase in uric acid excretion which occurs in patients with pernicious anemia following therapy with liver. However, since a similar increased excretion takes place during the regenerative phase following hemorrhage, it is likely that this merely represents increased hemopoietic activity and a rapid turnover of nucleic acids in the bone marrow.

Summary

1. A deficiency of pteroylglutamic acid has been produced in 32 swine fed a purified diet containing casein and supplemented with seven B vitamins, sulfasuxidine and a folic acid antagonist. The casein was fed at two levels, 10 and 2.6 per cent. Two types of casein were used: a crude preparation possessing significant intrinsic factor activity and a purified casein with little activity.

2. The hematologic manifestations observed were (a) severe macrocytic anemia, (b) leukopenia, due to a proportionately greater reduction in polymorphonuclear than in mononuclear cells, (c) slight thrombocytopenia, and (d) hyperplastic bone marrow with an increase in immature nucleated red cells which resemble the megaloblasts seen in the bone marrow of patients with pernicious anemia.

3. The feeding of a 2.6 per cent rather than a 10 per cent crude casein diet did not prevent but did delay the onset of the blood changes. Anemia developed most rapidly in the animals receiving 10 per cent purified casein.
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4. The group receiving 26 per cent casein developed a greater degree of macrocytosis in the same period of time than did the group receiving 10 per cent casein. In all groups the degree of macrocytosis increased as the duration of the anemia increased.

5. The hematologic manifestations were not delayed nor was their development prevented by the intramuscular administration of 15 U.S.P. units of liver extract every 15 days.

6. The blood and bone marrow returned rapidly to normal following the administration of pteroylglutamic acid, pteroyldiglutamic acid, pteroyltriglutamic and pteroylheptaglutamic acid. Thymine and xanthopterin had little or no activity. Tyrosine, adenine and uracil were inactive.

7. Purified liver extracts and crystalline vitamin B₁₂ were found to possess some hemopoietic activity in several animals but the activity was considerably less than that of the pteroylglutamic acid compounds.

8. The urinary excretion of "tyrosyl" (hydroxphenyl compounds) was not abnormal in the pteroylglutamic acid deficient pigs and was not altered by either pteroylglutamic acid or liver extract therapy.

9. The urinary excretion of allantoin and uric acid did not differ significantly from the normal. Immediately following therapy with pteroylglutamic acid, however, in association with the reticulocytosis and lasting for the same period, there was a marked increase in the excretion of allantoin.

10. The results suggest that both pteroylglutamic acid and a factor in liver extract similar to or identical with vitamin B₁₂ are required for normal hemopoiesis in the pig.

ACKNOWLEDGEMENTS

The crude methylfolic acid antagonist, xanthopterin, and the pteroylglutamic acid compounds, with the exception of pteroylheptaglutamic acid, were kindly furnished by the Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. T. H. Jukes and Dr. S. M. Hardy.

Sulfasuxidine was generously furnished by Sharp & Dohme, Inc., Philadelphia, Pa., through the courtesy of Dr. W. A. Feirer.

Pteroylheptaglutamic acid and Natola were supplied by Parke, Davis & Company, Detroit, Mich., through the courtesy of Dr. A. E. Sharp and Dr. J. J. Piffner.

Biotin was obtained from Hoffmann-LaRoche, Inc., Nutley, N. J., through the courtesy of Dr. E. L. Sevringhaus.

The vitamins, with the exception of pteroylglutamic acid and biotin and including vitamin B₁₂ were kindly furnished by Merck and Company, Inc., Rahway, N. J., through the courtesy of Dr. A. Gibson and the late Dr. D. F. Robertson.

Experimental liver extracts (No. 1114, 1063, 1066 and 1067) were generously furnished by Armour and Company, Chicago, Illinois through the courtesy of Dr. E. E. Hays.

We are indebted to Mrs. Darlene Kehl, Mr. George Trappett, and Mr. Ocie Hadley for technical assistance.

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EXPERIMENTAL PRODUCTION OF A NUTRITIONAL MACROCYTIC ANEMIA IN SWINE

G. E. CARTWRIGHT, BETTY TATTING, HELEN ASHENBRUCKER and M. M. WINTROBE