blood samples were taken from patients chosen at random. In Fig. 1 the temperatures at which each clotting time was determined is plotted against the corresponding clotting time, and points determined with the same sample of blood are connected by broken lines. The solid line represents the estimated average slope of these broken lines. Its position in the figure is based on the average normal value of 5 min. 28 sec. previously determined at about 26°C. At this temperature, 4 to 6 3/4 min. is considered the normal range. It is obvious from the curve that winter-summer variations of room temperature, which may range from 19°C to 37°C in temperate climates, can result in considerable changes in the normal range of clotting time.

It is recommended that a curve such as shown in Fig. 1 be used to evaluate clotting time results whenever this determination is performed at room temperature.

Summary. The effect of room temperature on the clotting time of whole blood determined by the rotating tube method is described. The clotting time may be significantly prolonged in cool environments, and shortened in warm weather. A curve is described to correct for this factor.

16672

Pteroylglutamic Acid Deficiency in Mice: Hematologic and Histologic Findings.*

DAVID R. WEIR, ROBERT W. HEINLE, AND ARNOLD D. WELCH.

From the Departments of Medicine and Pharmacology, School of Medicine, Western Reserve University, and the University Hospitals of Cleveland.

The production of a deficiency of pteroylglutamic acid (PGA) can be accomplished in various mammalian species by feeding a purified diet containing the known essential vitamins, excluding PGA, to which has been added either succinylsulfathiazole, a PGA antagonist, or both. In the deficient state the animals have shown varying degrees of reduction of all circulating elements in the blood stream. Reports of bone marrow findings have been conflicting and incomplete and have not been reported previously for mice.

Procedure. Deficiency may be established in weanling mice more quickly than in adults. Weanling animals of a Swiss strain with an average weight of about 20 g were placed in wire mesh cages and were allowed distilled water ad libitum. They were fed unlimited quantities of a PGA free diet of the following percentage composition:

Glucose ("Cerelose")	59.1
Casein ("Labco" vitamin free)	20.0
Cellulose ("Cellu-flour" or "Ruffex")	5.0
Salts USP (Smaco)	3.8
Accessory salts*	0.2
Choline chloride	0.2
Inositol	0.1
Thiamine hydrochloride	0.0005
Riboflavin	0.001
Pyridoxine hydrochloride	0.0005
Niacinamide	0.005
Calcium pantothenate	0.0055
Ascorbic acid	0.01
2-methyl-1, 4-napthoquinine	0.001
Biotin	0.00001
Hydrogenated vegetable oils ("Primex")	8.0
Corn oil containing vit. A, D, and E†	2.0

* This research was made possible by a grant from the Price McKinney Memorial Fund and is part of a study aided by a grant from the United States Public Health Service.

* Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; $FeSO_4$, 10; $MnSO_4$, 1; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 1; NaI, 0.3; NaF, 0.1; $CoCl_2 \cdot 6H_2O$, 0.01.

† Two grams of the corn oil preparation contained vitamin A, about 4500 units; vitamin D, about 560 units; and mixed tocopherols, about 2.7 mg.

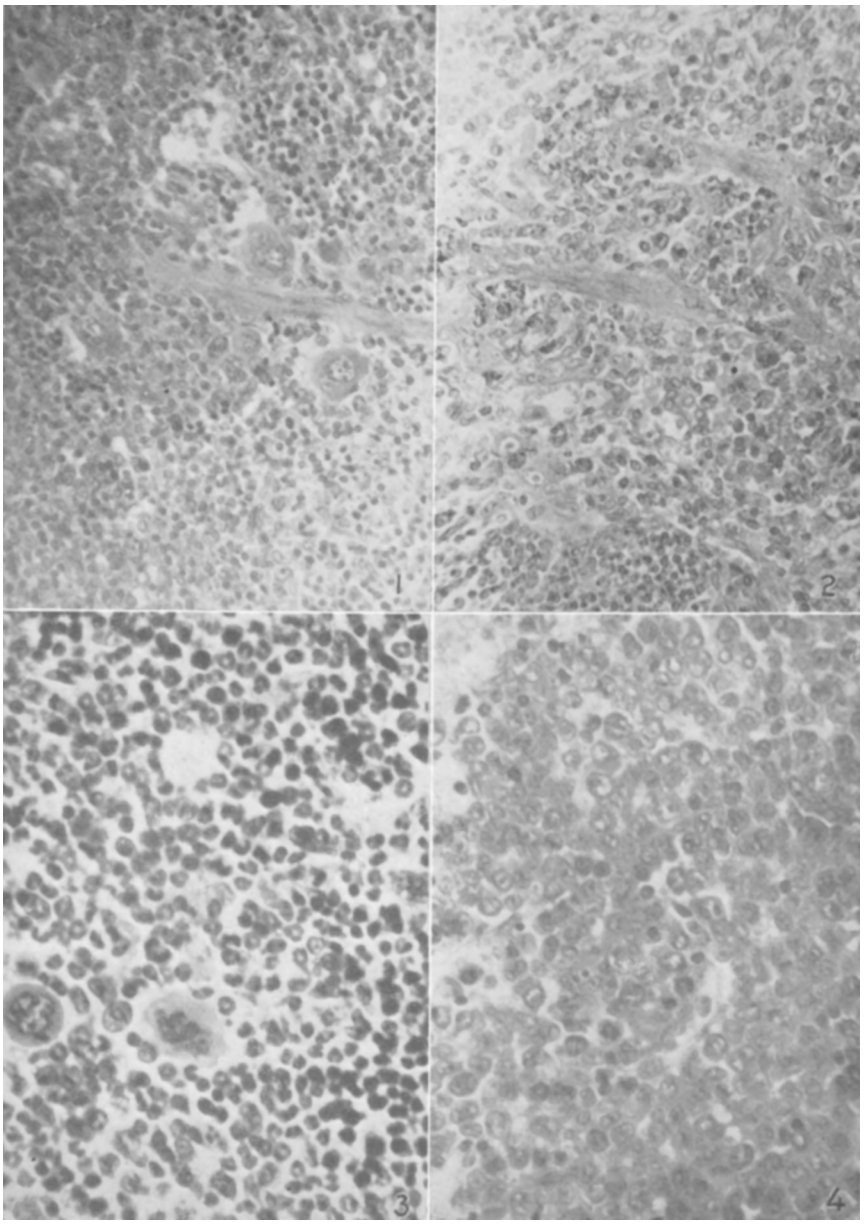

PLATE 1. Normal Spleen. ×246. Note the degree of cellularity and the numerous nucleated red blood cells and megakaryocytes. Actually this spleen is from a mouse that had been deficient and was cured by the administration of PGA.

PLATE 2. Deficient Spleen. ×246. The cellularity is much reduced. Nucleated red blood cells and megakaryocytes have practically disappeared. Extensive deposits of hemosiderin are present but these cannot be appreciated in the absence of color-reproduction. The coarsely granular appearance of some of the cells is due to hemosiderin.

PLATE 3. Normal Marrow. ×410. Note the presence of numerous nucleated red blood cells, polymorphonuclear leucocytes, and megakaryocytes. The absence of fat is normal in the mouse.

PLATE 4. Deficient Marrow. ×410. Large immature cells with nucleoli and mitoses predominate. Almost no polymorphonuclear leucocytes or nucleated red cells are seen and megakaryocytes have disappeared.

Five-tenths percent of a crude antagonist of PGA (Lederle N-67A)[1†] was added to the diet. In a preliminary experiment succinylsulfathiazole[‡] also was added but it was found that the majority of animals died before the desired evidences of deficiency had developed. Therefore the sulfonamide was withheld for the first month and then added at a level of 1.0% in the diet. With this modification only a very small percentage of the animals died in the preliminary stages of the experiment. On this regimen, deficiency was established in all animals within 50 to 60 days. A number then were killed for histologic studies. The remainder of the group was divided into 3 sub-groups and given varying amounts of PGA in the form of solutions of 'Folvite' (Lederle). Daily subcutaneous injections of 20, 2, and 0.2 μg were used. Care was taken to protect the solutions from exposure to sources of ultra-violet radiation. At weekly intervals the animals were weighed and white blood cell counts were done. Normally leucocyte counts of mice are not less than 6000 per cmm. When levels below 4000 were reached, it was considered that PGA deficiency was established. Red blood cell counts were then done; and, during the period of treatment with PGA, daily reticulocyte counts also were performed.

Hematologic Findings. After 30 to 40 days of the above regimen the white blood cell counts begin to fall to low normal levels. By 50 days many counts are below the minimal normal level of 6000, and by 60 days practically all the counts are below 4000 with some as low as 1000. Both lymphocytes and granulocytes participate in this leucopenia. There is no consistent change in the differential count; the normal lymphocyte-to-granulocyte ratio is preserved. Red blood cell counts done at the time when the leucocytes had fallen to levels of less than 6000 per cc showed that a moderate to marked anemia had developed. From a normal count of about 10 million erythrocytes per cc the count was usually reduced to 4 to 6 million with some counts as low as 2 million per cc.

The entire blood picture may be restored rapidly to normal by the parenteral administration of PGA while the animals are still on the dietary regimen with the added antagonist and succinylsulfathiazole. A maximal response was obtained by giving daily subcutaneous injections of 20 μg of a solution of 'Folvite' (Lederle). There is a reticulocyte rise in 2 days which usually reaches a maximum of about 30% in 7 days. The erythrocyte counts become normal within 15 to 20 days. Also there is a very rapid regeneration of leucocytes, normal counts being obtained within 3 to 5 days. More remarkable is the fact that abnormally high white counts may occur. In one animal the initial leucocyte count of 2000 per cc rose to 57,000 per cc 3 days after PGA was started, and to 85,000 per cc 3 days later; after another 3 days it was below the normal maximum of 25,000 per cc.

During the first month prior to the administration of succinylsulfathiazole there is a moderate gain in weight. On the addition of this drug to the purified diet the mice soon cease to gain in weight and many animals show losses of a few grams. When PGA is given, weight gain is resumed at a rapid rate.

[1] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

† Generously supplied by the Lederle Laboratories Division of the American Cyanamid Co.

‡ Kindly furnished by the Medical Research Division of Sharp and Dohme, Inc.

Also the ruffled fur characteristic of the deficient state disappears.

Histologic Findings. See Plates 1-4. The spleen of normal mice is an active hematopoietic organ. Many megakaryocytes are present and numerous foci of nucleated red blood cells are scattered diffusely through the pulp. There are a few polymorphonuclear leucocytes and occasionally immature cells. Mitoses are rarely seen. In PGA deficiency the hematopoietic activity of the spleen practically ceases. Megakaryocytes and nucleated red cells may entirely disappear. Polymorphonuclear leucocytes are decreased in number. The pulp becomes sparsely cellular and shows large amounts of hemosiderin, both in the free form and phagocytized by macrophages. As in pernicious anemia, the hemosiderin probably represents iron released by the normal breakdown of erythrocytes. In PGA deficiency it is not utilized because of the decreased formation of erythrocytes. The splenic follicles did not show any consistent change from the normal. Treatment with PGA restores the histology of the spleen to normal, although some of the hemosiderin may persist. In a few animals, in the deficient state, there was a moderate increase in the number of immature cells in the splenic pulp in addition to the findings described above. This was never as prominent in the spleen as in the bone marrow.

The marrow of normal mice contains practically no fat. The cellular elements consist of nucleated red blood cells, megakaryocytes, polymorphonuclear leucocytes, a few immature cells, a few lymphocytes, and rare mitoses. In PGA deficiency a very marked change occurs. The marrow becomes solidly packed with large immature cells containing nucleoli and showing numerous mitoses. Only a few identifiable nucleated red cells remain. Polymorphonuclear leucocytes and megakaryocytes may disappear entirely. The picture is one of maturation arrest with overproduction of immature cells. Positive identification of these immature cells has not been made. They have not been identified as either myeloblasts or megaloblasts; it seems likely they fall into the category of primitive blastic cells. After treatment with PGA the marrow cannot be distinguished from that found in normal mice.

In a few of the animals small areas of necrosis were present in the liver. Both in this experiment and in preliminary experiments these areas of necrosis had been found in animals which had died before the development of the PGA deficiency as evidenced by leucopenia and anemia.

Discussion. There is general agreement that PGA deficiency produces a reduction of all circulating elements in the blood streams of various animals and that this reduction may be corrected by the administration of PGA. The appearance of immature elements in the peripheral blood has not been described. Reports on the histologic findings have been variable. Bethell, Swenseid, and Rosenman[2] using rats found that the nucleated cell count of the marrow fell slightly below normal. The majority of the marrow cells were undifferentiated primitive forms, with relative increase in myeloblasts and early erythroblasts. Myelocytes, late erythroblasts and megakaryocytes practically disappeared. The situation could be corrected by extracts of liver or yeast containing folic acid. Gross, Axelrod and Bosse[3] using rats, stated that the marrow findings varied from almost complete aplasia to intense hyperplasia and concluded that the pathologic changes included progressive hypoplasia of the bone marrow. Endicott, Daft and Ott,[4] using rats, described a decrease of all elements of the marrow, lymphoid exhaustion of the spleen, and absence of splenic hematopoiesis. They observed a pancytopenia of the marrow without maturation arrest or hyperplasia of immature elements. Doan,[5] working with monkeys, described hypoplasia of the marrow with result-

2 Bethell, F. H., Swenseid, M. E., and Rosenman, R. H., *J. Clin. Invest.*, 1944, **23**, 926.

3 Gross, P., Axelrod, A. E., and Bosse, M. D., *Am. J. Med. Sci.*, 1944, **208**, 642.

4 Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

5 Doan, C. A., *Am. J. Med. Sci.*, 1946, **212**, 257.

ing peripheral panhematopenia; detailed studies of the marrow were not included in the report. Heinle, Welch, George, Epstein, and Pritchard,[6] using swine, found megaloblastic hyperplasia of the marrow. Franklin, Stokstad, Belt, and Jukes,[1] using rats, observed an increase of nucleated red blood cells in the marrow, an increased number of blast cells, and a marked reduction of the polymorphonuclear leucocytes. They stated that the maturation of cells of the erythroid series and the production of mature granulocytes were seriously impaired. They did not stress hyperplasia with maturation arrest. Cartwright, Fay, Tatting, and Wintrobe,[7] using swine, found a reduction of polymorphonuclear leucocytes and metamyelocytes, a slight increase in earlier forms of the myeloid series, and a significant decrease in the leucocyte-erythrocyte ratio. They observed immature and nucleated red cells which they considered to be similar in many ways to those seen in the marrow of patients with pernicious anemia in relapse.

The exact role of PGA in the formation and maturation of blood cells has not been elucidated. Certainly it participates in an important way. If it were an essential growth factor necessary for the formation of the most primitive blood cells we would expect pancytopenia of bone marrow, rather than maturation arrest and hyperplasia of immature elements. It may be inferred, then, that PGA does not participate in the production of the most primitive blood elements, but rather that it must be present to allow maturation beyond the most primitive stages. In pernicious anemia in relapse, and in some cases of agranulocytosis, a similar although an apparently selective maturation arrest with hyperplasia of immature elements is seen. The production of primitive elements is not abolished and may even be in excess. It has been postulated that in red cell formation the role of the anti-pernicious anemia factor of liver is to act in conjunction with PGA or its conjugates to bring about the maturation of red blood cells beyond the megaloblastic stage. This would fit into our hypothesis that PGA may not be necessary for the formation of the most primitive blood elements, but that it is concerned with reactions essential to maturation such as loss of nuclear material.

Summary. 1. A deficiency of pteroylglutamic acid in mice produces a reduction of all cellular elements of the circulating blood.

2. In the deficient state, the bone marrow of mice shows maturation arrest with hyperplasia of immature elements and marked reduction of more adult forms.

3. It is suggested that pteroylglutamic acid may not be necessary for the formation of the most immature blood elements, but must be present to allow maturation after these primitive elements are formed.

[6] Heinle, R. W., Welch, A. D., George, W. L., Epstein, M., and Pritchard, J. A., *Proc. Central Soc. Clin. Research*, 1947, **20**, 7.

[7] Cartwright, G. E., Fay, J., Tatting, B., and Wintrobe, M. W., *J. Lab. and Clin. Med.*, 1948, **33**, 397.