in particular Lt. Commander John J. Halbert, MC, USNR, Capt. Bailey, VC, AUS, and Capt. Catlin, CAC, AUS, and of Lt. (JG) Williams, MC, USNR, and other members of Naval Medical Research Unit Number One, Berkeley. The facilities of the Virus Laboratory in Berkeley were generously made available by Dr. M. D. Eaton, Director, Virus Laboratory, California State Department of Public Health.

15960
Chemical Antagonism of Pteroylglutamic Acid in a Pig; Hematopoietic Effect of Extrinsic and Intrinsic Factors.*

ARNOLD D. WELCH, ROBERT W. HEINLE, GEORGE SHARPE, WALTER L. GEORGE, AND MARTIN EPSTEIN.

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

It has been established that pteroylglutamic (folic) acid (PGA) is concerned with the formation of various cells of the blood, particularly erythrocytes and granulocytes. The vitamin has been studied in several animal species, including chicken, turkey, rat, dog, and monkey, as well as in man.

In man, with various types of macrocytic anemia, including that seen in pernicious anemia and in sprue, a striking hematologic response is usually obtained when PGA is administered.[1,2,3,4] This response cannot be differentiated clearly from that characteristically produced by refined liver extracts,† or from that caused by the administration, in pernicious anemia in relapse, of normal gastric juice (containing intrinsic factor) together with a heat-stable substance (extrinsic factor) found in beef skeletal muscle, casein, and other natural materials.[5] The roles of the non-PGA antipernicious anemia (APA) factor of liver extracts and of the extrinsic factor have been most difficult to study, because the effects produced in man have not been demonstrable in animals, even in those with anemia or leucopenia induced by a deficiency of PGA.[6,7,8]

Only swine have offered promise for studies of this character. The liver of this species is rich in the APA factor and is widely employed in the manufacture of liver extracts used in the treatment of macrocytic anemias. It was shown by Miller and Rhoads[9] that, under certain dietary conditions, swine develop an anemia that responds to injections of liver extract. Most striking, however, was the report of Cartwright, Wintrobe and Hum-

* This investigation was supported by a grant-in-aid from the United States Public Health Service. The chemical antagonist of PGA and a large proportion of the vitamins used in the purified diets were supplied by the Lederle Laboratories Division, American Cyanamid Company. The succinylsulfathiazole was generously supplied by the Medical Research Division, Sharp and Dohme, Inc. Crude sodium caseinate was donated by the Borden Company.

† Refined liver extracts contain insignificant amounts of determinable PGA, often less than 0.001 mg per cc.

1 Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *South. Med. J.*, 1945, **38**, 707.

2 Darby, W. J., and Jones, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 259.

3 Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. Clin. Med.*, 1945, **30**, 1056.

4 Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V., *J. Biol. Chem.*, 1946, **164**, 787.

5 Castle, W. B., *Harvey Lectures*, 1934-35, **30**, 37.

6 Day, P. L., Langston, W. C., Darby, W. J., Wahlin, J. G., and Sims, V., *J. Exp. Med.*, 1940, **72**, 463.

7 O'Dell, B. L., and Hogan, A. B., *J. Biol. Chem.*, 1943, **149**, 323.

8 Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 112.

9 Miller, D. K., and Rhoads, C. P., *J. Clin. Invest.*, 1935, **14**, 153.

10 Cartwright, G. E., Wintrobe, M. M., and Humphreys, I., *J. Lab. Clin. Med.*, 1946, **31**, 423.

phreys[10] describing the effect in a weanling pig of a diet containing "Labco" vitamin-free casein (26.1%), sucrose, lard (11%), salts, succinylsulfathiazole (2%), choline chloride, inositol, thiamine, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate, and *p*-aminobenzoic acid. This dietary regimen produced a severe depression of growth and marked hematologic as well as other changes. The volume of packed red blood cells was reduced to 21 cc from 50 cc per 100 cc of blood; the anemia was characterized as normocytic. After 120 days on the diet, intramuscular administration of biotin (1 mg per day) for 17 days caused no apparent improvement and 1 cc of purified liver extract was then administered by the intramuscular route daily for 10 days. A definite reticulocytosis (peak, 9.4% on tenth day) occurred and the hemoglobin concentration and the volume of packed red cells promptly increased. The growth response was striking, so that 50 days after the liver therapy the weight had doubled (from about 29 kg).

By analogy with results obtained in other species, it may be postulated that this pig developed a deficiency of PGA. In the rat, a comparable dietary regimen produces a deficiency that responds promptly to combined therapy with biotin and PGA.[11,12,13] In addition to deficiencies of these factors, a deficiency of the extrinsic factor also may have developed, since it has been shown by Castle *et al.*[14] that "Labco" vitamin-free casein contains little or none of this substance, as evidenced by tests in human patients. The possibility that deficiencies of PGA and of extrinsic factor can be corrected by the administration of materials found in refined extracts of liver, essentially free from PGA,[14] has important implications and is deserving of further study. Although the production of such a syndrome in swine offers a number of disadvantages, particularly that of expense, a program of research directed toward the isolation and study of antianemic factors would be facilitated greatly by the availability of an animal species in which a suitable deficiency could be produced. Accordingly, swine were placed on a diet closely resembling that of Cartwright *et al.*[10]

Experimental. Six Berkshire pigs 9 weeks of age were employed; the weights varied from 3.9 to 9.6 kg. One animal, (No. 1 ♀, 3.9 kg) was fed a commercial diet designed for the feeding of swine; the other animals were given a purified diet of the following composition: casein ("Labco" vitamin-free), 20 g; glucose ("Cerelose"), 48.7 g; hydrogenated vegetable oils ("Primex"), 18 g; corn oil, containing vitamins A, D, and E,‡ 2 g; salt mixture (U.S.P. No. 2), 3.8 g; accessory salts,§ 0.2 g; cellulose ("Cellu-flour"), 5 g; succinylsulfathiazole, 2 g; choline chloride, 0.2 g; inositol, 0.1 g; thiamine hydrochloride, 0.5 mg; riboflavin, 1 mg; pyridoxine hydrochloride, 0.5 mg; nicotinamide, 5 mg; calcium pantothenate, 5.5 mg; ascorbic acid, 10 mg; 2-methyl-1,4-naphthoquinone, 1 mg; biotin, 0.02 mg. In addition, two animals (No. 2♂, 8.5 kg, and No. 3♀, 6.9 kg) were given a daily oral supplement of synthetic PGA, 0.02 and 0.2 mg per kg, respectively. Pigs No. 4♂ (8.5 kg), No. 5♀ (9.6 kg), and No. 6♀ (7.3 kg) were given no PGA. After 16 days on this dietary regimen, the diet of pig No. 6 was supplemented with a chemical antagonist of PGA. This antagonist was used in the crude form, as described in the accompanying paper by Franklin, Stokstad and Jukes,[15] and was fed at a level of 0.1%¶ of the diet.**

11 Black, S., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 308.

12 Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.

13 Welch, A. D., and Wright, L. D., *J. Nutr.*, 1943, **25**, 555.

14 Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. J., and Ham, T. H., *Science*, 1944, **100**, 81.

‡ 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.

§ 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.

15 Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 368.

¶ The crude antagonist was prepared by Drs. M. E. Hultquist and J. M. Smith, Jr., Calco Chemical Company, by reacting 2,4,5-triamino-6-hydroxy-

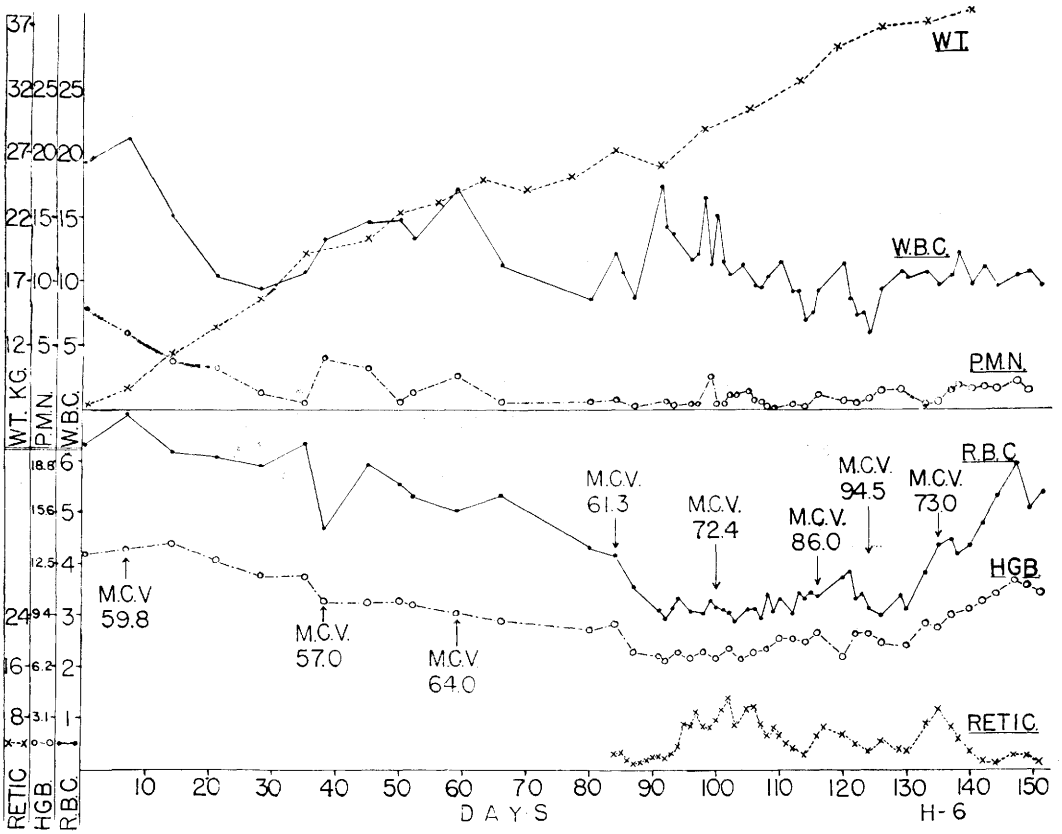

FIG. 1.

Hematologic and weight changes in a pig fed a purified diet containing 2% succinylsulfathiazole. After 16 days, the diet was supplemented with an antagonist of pteroylglutamic acid; this supplementation was continued throughout the experiment. On the 83rd day, and for a total of 10 days, normal human gastric juice was administered daily, together with an alcoholic extract of crude casein. For 14 days only, beginning on the 83rd day, the diet contained crude sodium caseinate, in place of vitamin-free casein ("Labco").

W.B.C. = White blood cells $\times 10^3$.
P.M.N. = Polymorphonuclear leucocytes $\times 10^3$.
HBG = Hemoglobin in g/100 cc.
R.B.C. = Red blood cells $\times 10^6$.
RETIC = Reticulocytes in %.
M.C.V. = Mean corpuscular volume in cubic microns.

Results. In contrast to the striking result obtained by Cartwright *et al.*,[10] no evidence of any deficiency in growth or in hematopoiesis was observed in those swine given the highly purified diet containing succinylsulfathiazole, except in the case of the one animal also given the chemical antagonist. The reason for this difference in the findings of the two laboratories cannot be stated. Of possible significance were the omission of *p*-amino-

pyrimidine and *p*-amino-benzoyl-l(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described by Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667. The percentage of crude antagonist in subsequent lots of diet was varied in proportion to the potency of the antagonist, as determined by Dr. E. L. R. Stokstad by microbiological assay using *S. faecalis R.*

** We are much indebted to these workers for supplying the antagonist and for the frequent discussions of their and our studies.

benzoic acid and the inclusion of biotin in our diet.

In the pig given the crude antagonist of PGA the growth rate gradually decreased and a state of severe anemia developed with the characteristics indicated in Fig. 1. A patchy alopecia appeared, but the hair-loss was not extensive. Profuse diarrhea was noted, the animal became listless and evidenced an unwillingness to stand, although no signs of neuromuscular degenerative changes were found. The appetite, which previously had been excellent, quite rapidly diminished to such a degree that the caloric intake became critically low (minimal daily caloric intake, about 18 calories per kg), and the continued survival of the animal became doubtful.

Therapy. At this time the vitamin-free casein of the diet was withdrawn and replaced by crude sodium caseinate in equivalent amount. Because of the low food intake, the pig was given in addition, by daily gastric intubation, an extract of crude casein in an amount equivalent to about 100 g of casein, together with from 80 to 150 cc of fresh neutralized human gastric juice. Since alcohol-extracted casein has been shown by Castle *et al.*[14] to be essentially free of extrinsic factor, we employed an extract of casein prepared by exhaustive treatment of crude casein with ethanol (95%) at 140°C;†† the extract was then concentrated and extracted with petroleum ether to remove fats and contaminating oils. The sodium caseinate substituted for the purified casein, and an alcoholic extract of crude casein entirely comparable to that used in the pig, contained extrinsic factor, as demonstrated by tests in patients with pernicious anemia in relapse. None of the materials administered contained appreciable amounts of microbiologically determinable PGA.‡‡

After a total of 10 days of supplementation by gastric intubation and 14 days of feeding sodium caseinate in the diet, the pig was returned to the highly purified diet. As a result of the supplementation, improvement in appetite and vigor were rapid and unmistakable. Within 10 days after supplementation was begun the appetite improved and the animal was stronger and much more alert. The stools, previously very diarrheic, assumed a semi-solid consistency, although they were still of a greenish-black color. Within another week the animal appeared to be normal with respect to appetite, alertness and strength. On the eleventh day after supplementation was begun, the reticulocyte count began to increase; 3 days later the level was 9% and a peak of 11% was attained after an additional 5 days. Other hematologic data are shown in Fig. 1.

It is to be noted that the improvement in appetite, growth and hematopoiesis, initiated by the supplementation, has continued unabated to the present date, approximately 10 weeks from the cessation of therapy, despite the continued administration of a purified diet, succinylsulfathiazole, and the antagonist of folic acid.

Discussion. As has been mentioned previously, no adequate explanation can be offered to account for the failure to obtain results comparable to that of Cartwright, Wintrobe and Humphreys,[10] when the purified diet free from the antagonist of PGA was used. Possibly the inclusion of biotin in our diet may account for the different result; for example, a facilitation of the synthesis of PGA may have occurred. In any case, the inclusion of the chemical antagonist of PGA in the same diet severely depressed growth and the formation of erythrocytes and leucocytes.

It cannot now be stated that the response of the pig to the administration of a crude source of extrinsic factor, together with normal human gastric juice, was due to the combined effect of the two materials. A preliminary trial of the casein extract alone was prevented by the severity of the syndrome that developed. It is unlikely that gastric juice would

†† The alcoholic extract of crude casein was generously supplied by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡‡ The samples of gastric juice administered were not analyzed for PGA; however, no sample so far tested has contained more than 2 μg of PGA/100 cc. Microbiologic assay of the sodium caseinate, after tryptic digestion and treatment with hog kidney conjugase, indicated a PGA content of 1.6 μg/g; the alcoholic extract of casein supplied not more than 0.1 μg of PGA daily.

have been efficacious by itself, since the studies of Castle and his associates have shown adequately that intrinsic factor alone produces no significant hematopoietic effect in human patients with pernicious anemia in relapse. Whether the pig had developed a deficiency of intrinsic factor, of course, cannot be stated, although a gastric analysis after stimulation with histamine, about 3 weeks prior to the initiation of therapy, indicated a high total acidity with absence of free hydrochloric acid. Studies designed to answer some of these questions are now in progress.

Summary. Interference with the metabolism of pteroylglutamic acid in the pig, through the use of a crude chemical antagonist, interrupts growth and significantly inhibits the formation of erythrocytes and of granulocytes. This interference is removed, despite continued feeding of the antagonist, by administration of a crude source of extrinsic factor (essentially free of PGA), together with normal human gastric juice. This finding affords an experimental animal with which to study the mechanism of action of antianemic substances and their functional relation to folic acid; also, a suitable bioassay tool is offered for guiding the isolation of antianemic factors of unknown chemical composition.

The administration of a purified diet similar to that successfully used by Cartwright *et al.*[10] failed to produce a failure in growth and in hematopoiesis in swine. It is suggested that this failure may possibly be attributable to the presence of biotin in the diet employed in this study.

15961

Acceleration of Pteroylglutamic Acid Deficiency in Mice and Chicks by a Chemical Antagonist.

A. L. FRANKLIN, E. L. R. STOKSTAD, AND T. H. JUKES.

From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

In another communication[1] the effect on rats of a synthetic preparation "antagonistic" to pteroylglutamic acid was described. The present article describes the results obtained with mice and with chicks which received the same preparation. An acceleration of pteroylglutamic acid deficiency was observed, and the effect was prevented by adding pteroylglutamic acid to the diet at levels sufficient to overcome the action of the antagonistic preparation.

Experimental. Rockland female mice, 5 to 6 weeks old, were kept in wire-floored cages. Five animals were used per group. The following basal diet was fed (diet 1) glucose (Cerelose), 72 g; washed casein (Labco), 20 g; salt mixture,[2] 4 g; corn oil (Mazola) *plus* vitamins A, D and E, 3 g; succinylsulfathiazole, 1 g; choline chloride, 0.1 g; inositol, 0.1 g; niacinamide, 5 mg; calcium pantothenate, 5 mg; thiamine HCl, 1 mg; riboflavin, 1 mg; pyridoxine HCl, 1 mg; *p*-aminobenzoic acid, 1 mg; 1-acetoxy-2-methyl-4-naphthyl sodium phosphate, 0.5 mg; biotin, 0.02 mg. Three grams of the corn oil preparation contained vitamin A (acetate), 1500 U.S.P. units; vitamin D (Delsterol), 200 A.O.A.C. chick units; mixed tocopherols, 34 mg.

The antagonist was prepared by condensing 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoyl-*l*(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described elsewhere.[3] The preparation was carried out by Dr. M. E. Hultquist and Dr. J. M. Smith, Jr.* The reaction product was used without purification. A similar product, using *p*-aminobenzoyl-*d*(—)-glutamic acid has been stated to have "displacing" activity for pteroylglutamic acid in the growth

1 Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, in press.

2 Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.