Biological Activity of a Derivative of the Citrovorum Factor.* (19141)

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In 1948 Sauberlich and Baumann(1) described a factor (CF) present in liver which is necessary for the growth of *Leuconostoc citrovorum* 8081. The synthesis of a substance, "leucovorin"(2,3), "folinic acid SF" (4,5), 5-formyl-5,6,7,8-tetrahydro pteroylglutamic acid, with the biological properties of CF has recently been described. Leucovorin, unlike synthetic pteroylglutamic acid (PGA), has been shown to be active in reversing the growth inhibiting effects of Aminopterin (4-amino PGA) on *L. citrovorum*(6-8), mice(6), rats(9), chick embryos(10), and mouse leukemia(11). Studies in patients with Addisonian pernicious anemia indicate that leucovorin is active when given parenterally in amounts of 0.75 to 3 mg daily for 10 days (12-16). In infants with megaloblastic anemia this factor has been found to be active in amounts as small as 75 μg per day when given parenterally(17). Following the administration of PGA to man and rats, CF is excreted in increased amounts in the urine(18). Weight for weight CF appears to be more effective than PGA in converting megaloblasts to normoblasts in bone marrow cultures(19). These data, in addition to the observations that the conversion of PGA to CF by rat liver slices is inhibited by the administration of Aminopterin and that CF is capable of preventing this inhibition(9,20), have led to the conclusion that in the body limited amounts of PGA may be converted to CF. It has also been suggested that ascorbic acid and vit. B_{12} are concerned in this conversion of PGA to the biologically more active CF(20,21).

When CF is allowed to stand at room temperature in dilute acid it loses its activity for *L. citrovorum* but its activity for *S. faecalis* is retained(2,6). Because of the possibility that the hydrochloric-acid-treated leucovorin may be similar to the product which occurs when naturally-occurring CF is exposed to the normal action of gastric juice, it was thought to be of interest to study the bio-

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TABLE I. Comparative Microbiological Activity of Citrovorum Factor, Acid-Treated Leucovorin and Pteroylglutamic Acid.

Compound tested	*Leuconostoc citrovorum* Microbiological activity expressed as %	*Streptococcus faecalis* Microbiological activity expressed as %
Pteroylglutamic acid	0.001	100
Acid-treated citrovorum factor	2.0	53
Citrovorum factor	100	50

TABLE II. Effect of Citrovorum Factor (CF), Acid-Treated Citrovorum Factor (ATCF) and Pteroylglutamic Acid (PGA) on Toxicity of 4-Aminopteroyl Glutamic Acid for Mice. Supplement injected 3x weekly.

Group No.	4-NH_2 PGA	CF	ATCF	PGA	1 day	4 days	5 days
1281	—	—	—	—	20.2 (10)	22.2 (10)	23.1 (10)
82	10 γ	—	—	—	20.5 (10)	17.9 (10)	16.8 (8)
83	10 γ	10 γ	—	—	20.7 (10)	21.3 (10)	21.6 (10)
84	10 γ	—	30 γ	—	20.6 (10)	20.5 (10)	19.9 (10)
85	10 γ	—	100 γ	—	20.6 (10)	21.8 (10)	22.2 (10)
87	10 γ	—	—	100 γ	20.4 (10)	19.0 (10)	17.5 (10)

	8 days	10 days	12 days	15 days
1281	24.7 (10)	25.2 (10)	25.8 (10)	26.8 (10)
82	—	—	—	—
83	22.0 (10)	21.8 (10)	21.1 (10)	20.7 (9)
84	19.5 (6)	19.0 (4)	22.5 (2)	25.0 (1)
85	23.6 (10)	23.6 (10)	23.2 (10)	24.5 (9)
87	16.3 (3)	18.5 (2)	17.0 (2)	16.0 (1)

logical activity of this product (ATL) in bacteria and mice and in patients with pernicious anemia in relapse.

Experiments. Leucovorin, 5-formyl-5,6,7,8-tetrahydro-pteroylglutamic acid(2,3) was synthesized by Drs. B. Roth, M. E. Hultquist, and J. M. Smith, Jr., Calco Chemical Division, American Cyanamid Company. A solution for clinical use was prepared by dissolving the calcium salt in water and adjusting the concentration to 3 mg per ml calculated as the anhydrous free acid. 0.15 mg of which corresponds to 1,000,000 "units"(22). The solution was placed in 1 ml ampules and sterilized by autoclaving. "Acid treated leucovorin" (ATL) was prepared by allowing leucovorin solution (4 mg per ml) to stand at pH 2 for 24 hours at 25°C. The solution was then neutralized, brought to a concentration equivalent to 3 mg per ml of original leucovorin and sterilized by autoclaving. *Leucovorin citrovorum* 8081 and *Streptococcus faecalis* 8043 were used in this study. The assay technic with *L. citrovorum* was that described by Sauberlich(17) with leucovorin as a standard. The microbiological assay procedure with *S. faecalis* followed that of Flynn *et al.*(23) with synthetic pteroylglutamic acid (PGA) as a standard. In the experiments with mice the procedure of Franklin *et al.*(24) was used. Carworth Farms CF-1 strain white mice weighing about 20 g were placed on a purified diet without PGA and containing 1% succinylsulfathiazole. Three patients with Addisonian pernicious anemia in relapse were studied. The patients were hospitalized and during the assay periods, liver, meat, meat products, milk and poultry were excluded from the diet. Bread, cereals, sugar, fat, vegetables, and fruits were permitted in the amounts desired.

Results. The response of *L. citrovorum* and *S. faecalis* to pteroylglutamic acid, leucovorin

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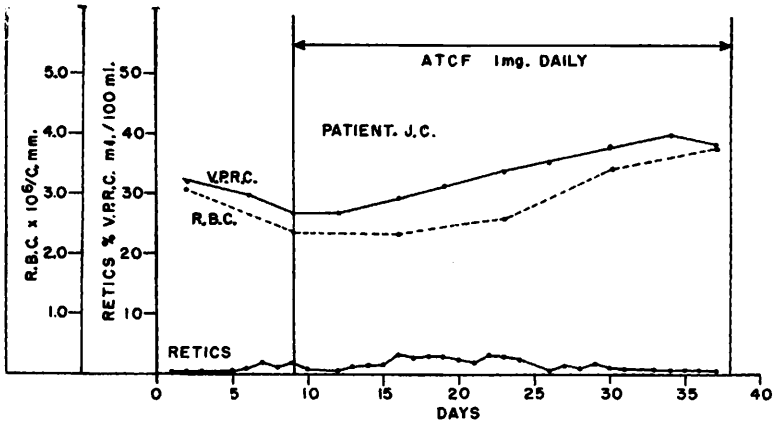

FIG. 1. Response of a patient (J. C.) with pernicious anemia in relapse to the intramuscular administration of 1 mg of acid-treated leucovorin (ACTF) daily for 29 days.

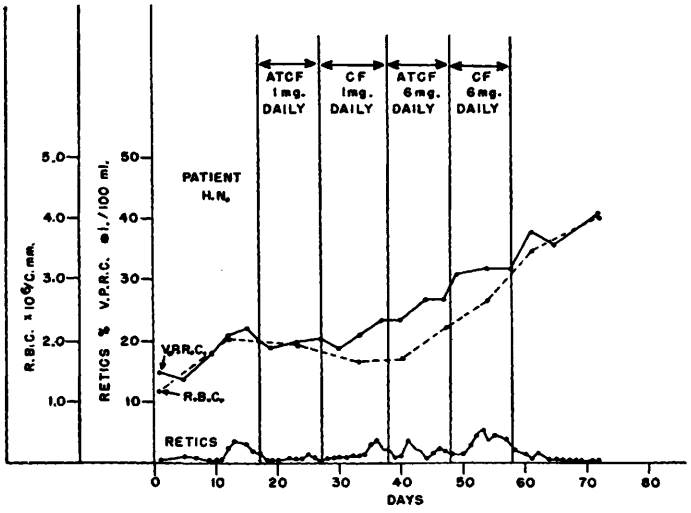

FIG. 2. Response of a patient (H. N.) with pernicious anemia in relapse to the intramuscular administration of acid-treated leucovorin (ATCF), and leucovorin (CF).

and acid-treated leucovorin (ATL) are shown in Table I. About 100,000 times more PGA was required than leucovorin for equivalent growth of *L. citrovorum*. As reported elsewhere(25) PGA was about twice as active as leucovorin for *S. faecalis*. Exposure of leucovorin solutions to pH 2 at 25°C resulted in inactivation of leucovorin activity, while the *S. faecalis* activity was unaltered.

The ability of leucovorin, ATL, and PGA to reverse the toxic effects of 4-amino-pteroylglutamic acid (Aminopterin) in mice is summarized in Table II. Reversal of the lethal effect of 10 μg of Aminopterin (90% survival) was obtained with 10 μg of CF. A somewhat larger amount of leucovorin was noted previously to be required to produce a gain in weight equal to that of a control group not receiving Aminopterin(2). PGA was almost completely ineffective in reversing Aminopterin toxicity in mice. Under the conditions of this experiment ATL was about one-tenth as active as leucovorin in reversing the effect of the antagonist.

The hematologic responses of the 3 patients with pernicious anemia in relapse to various doses of intramuscularly administered ATL and leucovorin are shown in Fig. 1-3. The

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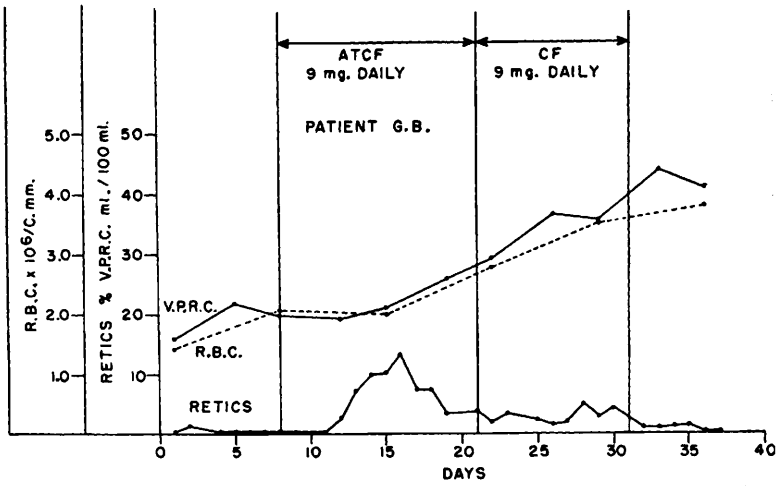

FIG. 3. Response of a patient (G. B.) with pernicious anemia in relapse to the intramuscular administration of acid-treated leucovorin (ATCF), and leucovorin (CF).

administration of 1 mg of ATL daily for 29 days was associated with an increase in the volume of packed red cells (VPRC) from 27 to 40 ml/100 ml and a rise in the red cell count (RBC) from 2.37 million per cmm to 3.80 in this period of time, although a significant reticulocyte response was not observed (Fig. 1). The administration of 1 mg ATL to a second patient (Fig. 2) for 10 days was without apparent effect on the RBC, VPRC or reticulocytes. However, the subsequent administration of 1 mg of leucovorin daily for 10 days was associated with a slight increase in reticulocytes to 3.8% and a significant rise in the VPRC. Six mg of ATL was then given daily for 10 days and the RBC and VPRC continued to increase. When 6 mg of leucovorin was given daily for 10 days a reticulocyte rise to a peak of 5.6% took place and the increase in RBC and VPRC continued. The third patient (C.B.), (Fig. 3) was given 9 mg of ATL daily for 13 days. This resulted in a rise in reticulocytes to 13.4% and a significant increase in RBC and VPRC. The administration of 9 mg of leucovorin daily for 10 days following this was associated with a very slight secondary reticulocytosis of 5%.

Discussion. In confirmation of earlier studies it is apparent that, although *S. faecalis* can utilize either PGA, CF or ATL for its requirement for folic acid, *L. citrovorum* can utilize only CF. The studies with mice suggest that in the presence of Aminopterin the mouse is able to convert the acid-treated product to CF to a limited extent. The results in patients with pernicious anemia in relapse indicate that the acid-treated product is less active than leucovorin. This was apparent at levels of 1, 6 and 9 mg. Evidence published to date(12-16) as well as the observations reported here, indicate that amounts of leucovorin greater than 1.0 mg are required to produce a satisfactory hemopoietic response in cases of pernicious anemia. Davidson and Girdwood(15) failed to obtain maximal reticulocyte responses after the administration of a single injection of 12 mg. Meyer *et al.*(13) obtained the expected reticulocyte maximum in only one of 4 patients given 3 mg intramuscularly each day for 10 days. Ellison *et al.*(14) found that 4 patients treated with 0.75 to 1.5 mg of leucovorin daily developed maximal red cell rises at the end of 3 weeks, although the reticulocyte responses were only about one-half that expected.

It seems, therefore, that the therapeutic doses of leucovorin and PGA are of the same order since the oral administration of PGA in amounts of 1.0 to 2.5 mg per day results in satisfactory although sub-optimal responses in patients with pernicious anemia(25). This would seem to indicate that the conversion of PGA to CF is not greatly disturbed in patients with pernicious anemia. Such a finding some-

what contradicts the hypothesis that vit. B_{12} is concerned in the conversion of PGA to CF (21) for, if such were true, CF should be considerably more active than PGA on a weight for weight basis.

Summary. Exposure of citrovorum factor (leucovorin) solutions to pH 2 for 24 hours at 25°C resulted in inactivation of *L. citrovorum* activity but produced no alteration in the activity for *S. faecalis*. The acid-treated leucovorin was about one-tenth as active as leucovorin in reversing Aminopterin toxicity in mice. When administered to patients with pernicious anemia in relapse the acid-treated leucovorin was less active than leucovorin.

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Effect of Hypoxemia on Edema Formation in Perfused Isolated Rat Hind Limb.* (19142)

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The effect of hypoxemia on capillary function has been the object of numerous investigations since Landis(1) noted that complete anoxemia caused a fourfold increase in capillary filtration rate in the frog mesentery. Maurer(2,3) used the rate of flow and protein content of the cervical lymph in dogs to indicate a change in permeability of the capillaries during respiratory hypoxemia. In all cases lymph flow increased steadily with progressing hypoxemia to as low as 7 volumes % oxygen content. However, this change cannot be attributed to hypoxemia alone when considering that many other factors affect the overall flow of lymph. Warren and Drinker(4) studied the lung lymph flow in the same manner using a level of hypoxemia as low at 4.4 vol. %, and found that lung lymph flow consistently increased. Further indirect evidence that graded hypoxemia causes graded increases in capillary permeability was reported by Pochin (5), who occluded the blood vessels at the base of the rabbit's ear for 2 to 24 hours and noted an increase in the thickness of the ear in proportion to the time of occlusion. Hopps and Lewis(6) found that anoxemia did not facilitate the passage of antibody globulin nor of T-1824 through the vascular epithelium of the guinea pig. McMichael and Morris(7) as well as Henry *et al.*(8) utilized the procedure of venous congestion of the upper extremities of human beings. The former authors found no increase in swelling of the arm at 9.5 vol. % oxygen content and the latter no change in calculated loss of protein from the blood of the congested arm at 60 to 70% oxygen saturation of the blood. However, at 15 to 20% saturation or 4 to 6 vol. % oxygen content a significant loss of protein occurs(9). Stead and Warren(10) noted that in patients with chronic emphysema in whom the blood was 50 to 60% saturated, no signs of protein "leakage" were present. Perfusion technics in the frog were used by Danielli(11) and Saslow(12) with different interpretations

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