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Activity of Citrovorum Factor for the Chick.* (19794)

G. M. BRIGGS, M. R. SPIVEY, J. C. KERESZTESY, AND M. SILVERMAN.
From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Bethesda, Md.

Sauberlich and Baumann(1) reported the existence of a substance which was closely related to folic acid and which was necessary for the growth of *Leuconostoc citrovorum.* The isolation of citrovorum factor (CF) in crystalline form from horse liver was reported by Keresztesy and Silverman(2). This substance was found to be approximately twice as active for *L. citrovorum* as the synthetic compound, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (leucovorin(3), folinic acid-SF (4)). The biological activity of the natural crystalline product in folic acid deficient animals has not been reported thus far. Various reports have indicated that synthetic compounds with CF activity prepared from folic acid are at least partially active in folic acid deficient chicks. Hill and Briggs(5) reported that a synthetic form of folinic acid which differed from folinic acid-SF† was as effective as pteroylglutamic acid (PGA) when given in the diet. Broquist *et al.*(6) recently found that leucovorin was 50% as active as PGA when injected. Orally administered leucovorin, however, had considerably lower activity. The authors suggested that leucovorin might be a mixture of diastereoisomers in order to account for its having only one-half as much activity as PGA when injected. Recently, Sauberlich(7) compared the activity for the chick of PGA with a concentrate of CF and concluded that they had the same activity when incorporated in the diet.

Because of the differences in the reported activities of the various products and because of the recent availability of crystalline natural CF, it became of interest to determine the comparative efficacy of crystalline CF, folinic acid-SF, leucovorin, and PGA for the chick.

Experimental. New Hampshire female chicks from a commercial hatchery were equilibrated according to weight in groups of 6 and placed on experiment when one day old. They were raised in electrically heated brooders with wire screen floors. Feed and water were supplied *ad libitum* and the experiments were terminated at the end of 4 weeks. The basal diet, which contained no added folic acid, consisted of the following in g/kg: Cerelose (glucose) 615, vitamin-free casein 200, gelatin 80, DL-methionine 3, corn oil 40, Chick Salts A 60,‡ and choline chloride 2.

* The sample of leucovorin was supplied by Lederle Laboratories, Pearl River, N. Y., and the sample of folinic acid-SF by Dr. William Shive, University of Texas, and Eli Lilly and Co., Indianapolis.

† A personal communication from Dr. William Shive informs us that the folinic acid used by Hill and Briggs(5) was a purified amorphous synthetic sample which differed from folinic acid-SF, and did not contain any free or conjugated PGA.

‡ Sixty g of Chick salts A contributed the following in g/kg of diet: $CaCO_3$ 15, K_2HPO_4 9, Na_2HPO_4 7.3, $Ca_3(PO_4)_2$ 14, NaCl 8.8, $MgSO_4 \cdot 7H_2O$ 5, ferric citrate (16.7% Fe) 0.4, $MnSO_4 \cdot 4H_2O$ 0.42, KI 0.04, $ZnCO_3$ 0.02 and $CuSO_4 \cdot 5H_2O$ 0.02. All salts were U.S.P. grade or purer.

TABLE I. Effect of Pteroylglutamic Acid and CF Active Compounds on Chick Growth.

Group	Supplement/kg basal diet, mg	Avg wt in g at 4 wk, S.E.,* and No. of chicks† Series 6	Series 9	Series 16 (with aureomycin)‡	Series 24 (with aureomycin)‡
1	0	205 ± 9.5(12)	254 ± 21 (6)	148 ± 18.4 (5)	148 ± 25.2 (5)
	Pteroylglutamic acid				
2	.1	334 ± 25.8 (6)	323 ± 29 (5)	222 ± 21.8 (6)	262 ± 18.7 (6)
3	.2	331 ± 13.6 (6)	380 ± 23.6 (6)	—	—
4	.4	376 ± 22.3 (6)	—	—	—
5	3	371 ± 38.4(12)	411 ± 15.3 (6)	368 ± 15 (6)	345 ± 18.2 (6)
	Leucovorin				
6	.1	—	—	136 ± 9 (6)	—
7	.2	231 ± 22.6 (6)	260 ± 24.6 (6)	—	—
8	.4	262 ± 27.3 (6)	—	—	—
9	3	—	428 ± 32.8 (6)	—	—
	Citrovorum factor				
10	.1	224 ± 23.4 (5)	—	150 ± 14.3 (6)	157 ± 14.3 (6)
11	.2	248 ± 5.5 (5)	—	—	—
	Folinic acid-SF				
12	.25	238 ± 26.3 (6)	—	—	—
13	.5	232 ± 35 (6)	—	—	—
14	3.2	—	341 ± 26.1 (6)	—	—
	Pteroylglutamic acid inj.				
15	.1§	—	284 ± 12.1 (6)	264 ± 12.6 (5)	248 ± 8.2 (6)
	Leucovorin inj.				
16	.1§	—	—	190 ± 16.5 (6)	—
17	.2§	—	339 ± 13.6 (6)	—	—
	Citrovorum factor inj.				
18	.1§	—	373 ± 22.8 (6)	251 ± 15.3 (6)	232 ± 18.2 (6)

* S.E. determined by method of Mantel(8).

† No. of chicks remaining at end of experiment given in parentheses (6 chicks started per group). Groups 1 and 5 in Series 6 are averages of 2 groups.

‡ Each group in Series 16 and 24 was given 50 mg of aureomycin/kg of diet.

§ Injections made 3 times per week. All levels given were in proportion to amount of folic acid consumed by Group 2 during preceding period (see text).

The following vitamins were used (in mg/kg): thiamine hydrochloride 8, riboflavin 8, Ca pantothenate 20, nicotinic acid 100, pyridoxine hydrochloride 8, D-biotin 0.3, vit. B_{12} 0.02, vit. A acetate 3, vit. D_3 0.02, alpha-tocopherol acetate 10, and 2-methyl-1,4-naphthoquinone 1. The B vitamins were added to the diet by the use of dry premixes with Cerelose as the carrier. The fat soluble vitamins were added from stock solutions with corn oil as the carrier. Aureomycin was added to each diet in series 16 and 24 at a level of 50 mg/kg in order to enhance the severity of the deficiency (unpublished data). In series 6 and 9 the barium salt of the natural CF was used, whereas the calcium salt was used in series 16 and 24. In all instances the calcium salt of leucovorin and the free form of folinic acid-SF were used. All of these compounds as well as PGA were made up in slightly alkaline aqueous solution for subcutaneous injection and kept under refrigeration. The amounts administered are given in the table as the free forms. The amount of each compound injected corresponded to the amount of PGA consumed between injection periods by the group fed 0.1 mg PGA/kg of diet. Injections were given subcutaneously 3 times weekly.

Results. The average 4 week weights of the various groups from 4 different series are given in Table I. The addition of PGA to the basal diet resulted in typical growth responses. The feeding of CF, leucovorin, or folinic acid-SF at low levels resulted in substantially less growth than corresponding levels of PGA. Relatively high amounts of leucovorin and folinic acid-SF, however, caused good growth. Large amounts of CF were not used because of lack of sufficient amount of this compound.

CF and leucovorin were considerably more

active when injected than when fed. Natural CF when injected appeared to be as active as PGA and somewhat more active than leucovorin (see series 9 and 16). A study of the significance of these differences by Fisher's "t" test (Snedecor) showed that these differences between injected CF and leucovorin were significant in series 16.

In general, the quality of feathers obtained by feeding or injecting the various CF active compounds could be correlated directly with the growth response.

Discussion. From the results obtained it appears that when crystalline CF is injected it is as active as PGA in the stimulation of the growth of chicks. Leucovorin is not as active as CF when injected. Broquist *et al.*(6) have previously reported that injected leucovorin was approximately half as active as PGA. This finding is substantiated by your data.

The low activity of CF, leucovorin, and folinic acid-SF when given in the diet may be due to possible modification of these compounds under the acid conditions which exist in the gizzard of the chick. Under such conditions it is known that alteration of the CF molecule may occur(9-11), whereby it might become inactive or unavailable to the chick. Further studies are necessary to explain the reported discrepancies in activity of the various compounds. Our results do not preclude the possibility that CF active compounds may be found in the future with chick activity when fed. The reports of Hill and Briggs(5) and Sauberlich(7) suggest that such a possibility exists.

It has been reported by Frost and Dann (12) that PGA was approximately twice as active when given by injection to chicks as when given orally. Our evidence indicates that the route of administration did not affect the activity of PGA although the method and time of injection may account for differences in this report.

Summary. 1. Crystalline citrovorum factor obtained from horse liver was approximately as active when injected as pteroylglutamic acid in counteracting folic acid deficiency in the chick. Under the same conditions citrovorum factor appeared to be somewhat more active than leucovorin. 2. Citrovorum factor, leucovorin, and folinic acid-SF when given in the diet were substantially less active than pteroylglutamic acid.

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