Synthesis of Citrovorum Factor From Folic Acid By Liver Slices; Augmentation By Ascorbic Acid.* (17806)

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An important role of ascorbic acid in the development of megaloblastic anemia has been indicated by various clinical observations, and these have been reviewed by Vilter (1) and by May, *et al.*(2). Studies of experimental deficiencies in the human infant(2), monkey(3), guinea pig(4), rat(5), and chick (6) have suggested that ascorbic acid is involved in the metabolism of pteroylglutamic acid (PGA). The studies described in this paper indicate that ascorbic acid may participate in the metabolic alteration of PGA to form a compound or compounds with remarkable activity for *Leuconostoc citrovorum* 8081. The citrovorum factor(s)(CF), first described by Sauberlich and Baumann(7), which is required for the growth of this organism, is considered to be closely related to PGA(8-12). In this report, further evidence for the relationship between the two factors is afforded by the observations that (i) the liver of PGA-deficient rats contains a greatly reduced amount of CF as well as of PGA, (ii) the enzymatic formation of CF by rat liver slices is greatly increased by the addition of PGA, and (iii) the yield of CF, under these conditions, is augmented significantly by the addition of ascorbic acid.

Experimental. Weanling albino rats (Sprague-Dawley-Holtzman strain) were depleted of PGA on a purified diet containing, in g per 100 g: "vitamin-free" casein (Labco) 18, cystine 0.2, glucose (Cerelose) 67.5, cellulose (Celluflour) 2, salts IV(13) 4, succinylsulfathiazole (Sulfasuxidine) 2, hydrogenated vegetable oil (Primex) 4, fortified corn oil (supplying 2200 I.U. vitamin A, 300 I.U. vitamin D, and 2.5 mg of α-tocopherol acetate) 2, choline chloride 0.1, and a mixture of B-vitamins 0.2 (supplying thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, each 0.5 mg, nicotinic acid and calcium pantothenate, each 2.0 mg, inositol 10 mg, and menadione 0.1 mg). After about 5 weeks on this diet, individual rats weighing from 80 to 100 g were selected for the tissue studies when the acute state of PGA-deficiency was indicated by rapid loss of body weight and severe diarrhea. The normal rats (av. wt. 200 g) were raised on a complete stock diet. The rats were stunned, decapitated, bled and the liver was removed. Liver slices, prepared with the Stadie-Riggs micro-

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1. Vilter, R. W., in Lejwa, A., *Symposia on Nutrition of the Robert Gould Research Foundation*, Cincinnati, Ohio, 1947, v1, 179.

2. May, C. D., Nelson, E. N., Salmon, R. J., Lowe, C. U., Lienke, R. I., and Sundberg, R. D., *Bull. Univ. Minn. Hosp., and Minn. Med. Found.*, 1950, v21, 208.

3. May, C. D., Nelson, E. N., and Salmon, R. J., *Proc. Central Soc. Clin. Res., J. Lab. Clin. Med.*, 1949, v34, 1724.

4. Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.*, 1948, v172, 851.

5. Rodney, G., Swendseid, M. E., and Swanson, A. L., *J. Biol. Chem.*, 1947, v168, 395.

6. Dietrich, L. S., Nichol, C. A., Monson, W. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v181, 915.

7. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

8. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1949, v181, 871.

9. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

10. Anker, R. M., Boehne, J. W., and Welch, A. D., *Fed. Proc.*, 1950, v9, 351.

11. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

12. Bond, T. J., Bardos, T. J., Sibley, M., and Shive, W., *J. Am. Chem. Soc.*, 1949, v71, 3852.

13. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

TABLE I.
Synthesis of Citrovorum Factor by Rat Liver Slices.

Tissue	Units of citrovorum factor per g of liver, fresh wt. Additions to vessels containing liver slices* None	Ascorbic acid, 10 mg	PGA, 100 μg	PGA, 100 μg, + ascorbic acid, 10 mg
Normal rat liver				
Exp. 4 (1)†	2880	—	5530	7500
" 5 (1)	2220	4160	3850	5810
" 5 (1)	1710	4375	3660	10930
PGA-deficient rat liver				
Exp. 1 (3)	147	215	3020	6340
" 3 (5)	73	85	2500	4460
" 4 (4)	36	—	4200	8700
PGA-deficient rat liver				
Exp. 2 (3)	88	190	3460	6600 (5 mg)‡ 8000 (10 mg)‡ 6500 (30 mg)‡

* Liver slices (1 g) were suspended in air-equilibrated Krebs-Ringer phosphate (5 ml). All vessels were incubated at 37° for 2 hrs in a constant temperature water bath with shaking.

† Each vessel contained slices from the number of animals indicated by the figure enclosed within parentheses.

‡ The figures expressed parenthetically indicate the amount of ascorbic acid added in these cases (PGA, 100 μg).

tome(14), were placed in consecutive beakers (20 ml) in regular sequence. The beakers, which contained chilled Krebs-Ringer phosphate (pH 7.4) and, where indicated, PGA, were weighed to the nearest 0.01 g before and after addition of the slices (approximately 1 g). In some cases freshly neutralized ascorbic acid was added immediately before the vessels were agitated for 2 hours in a covered, constant temperature (37°) water bath. The final volume of fluid in each beaker was 5 ml. After heating at 100° for 5 minutes the contents of each vessel were ground in a glass homogenizer. The mixture was transferred to a glass-stoppered test tube, adjusted to pH 6.5, diluted to 25 ml, steamed at 100° for 10 minutes, thoroughly mixed, and filtered. The clear filtrates were assayed microbiologically with *Leuconostoc citrovorum* 8081, using the basal medium described by Sauberlich and Baumann(7). Under the conditions of this assay, added PGA was so greatly diluted as to exert no determinable growth-promoting action on the microorganisms.† Turbidimetric readings were made after about 20 hours at 37° using the Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

Results. The results, presented in Table I, are expressed in terms of units of CF activity.‡

The liver slices of normal rats were found to contain, after incubation for 2 hours, about 2000 units of CF activity per g, wet weight. This amount was doubled when incubation was carried out in the presence of PGA (100 μg) or in the presence of ascorbic acid

14. Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, 1944, v154, 687.

† As has been shown elsewhere (Nichol, C. A., and Welch, A. D., *Fed. Proc.*, 1950, v9, 367) the response of *Leuconostoc citrovorum* to the factor(s) derived from PGA is potentiated by thymidine and other naturally occurring substances. However, so great were the dilutions of the liver tissue employed in the determinations of the high CF levels, that the findings recorded were not significantly influenced by these factors.

‡ Units of activity employed in this study cannot be compared directly with those used by other workers in this field where both titrimetric and turbidimetric assays have been used and reference standards have differed. In this laboratory a liver fraction is used as a constant reference standard; one unit is defined as that amount needed, per 10 ml assay tube, to produce half-maximal turbidity in 20 hours at 37°.

(10 mg), while a combination of PGA and ascorbic acid increased the level of CF 3- to 5-fold. Although considerable variation was noted in the amount of CF in individual rats, the differences between treatments showed a very consistent trend in the tissue replicates.

The use of PGA-deficient rats offered a real advantage in that only a small amount of CF was present or was produced during the 2-hour incubation of liver slices from these animals. Under these conditions ascorbic acid (10 mg) exerted little effect on the yield of CF, while the addition of PGA (100 μg) produced nearly as much CF as was found in the normal liver tissue after incubation with PGA; similarly, incubation with both PGA and ascorbic acid gave values for CF which were approximately double those obtained with PGA alone. The effect of ascorbic does not appear to be specific since it can be produced also by glucoascorbic acid. Extensive studies of other compounds with a high reducing potential have not yet been made, but glutathione under the conditions tested appears to be much less active. In contrast to slices, homogenates of normal or PGA-deficient rat liver form very little CF from added PGA.

Discussion. The results presented indicate clearly that enzyme systems are present in rat liver that are able to convert PGA to a substance highly active for *Leuconostoc citrovorum*. The favorable effect of ascorbic acid and glucoascorbic acid on this synthesis might suggest that CF is readily oxidized; however, concentrates of CF are remarkably stable, except in an acid medium, and do not appear to be attacked readily by atmospheric oxygen. It is possible that in the series of reactions involved in the conversion of PGA to CF, reduction may occur. Presumably, CF is more closely related in structure to a prosthetic group of which PGA serves as a precursor.

Although the microbiological potency of pure CF is not known, studies of concentrates (10) suggest that under the conditions of these experiments only a small fraction of the added PGA is converted to CF. Despite the fact that normal rat liver usually contains less than 10 μg of PGA per g, as determined with *Lactobacillus casei*(15), addition of ascorbic acid produced slightly more CF from materials present in this tissue than did the addition of 100 μg of PGA in the absence of ascorbate. Apparently the substances present in liver that augment the growth of *L. casei* are more readily converted to a substance or substances active for *Leuconostoc citrovorum* than is synthetic PGA.

The physiological significance of the augmentation by ascorbic acid of the synthesis of CF from PGA, is indicated also by studies now in progress in rats and in human subjects. Preliminary findings show that the urinary excretion of CF, which is augmented strikingly by the administration of PGA, is further increased from 2- to 4-fold by doses of ascorbic acid ranging from 10 to 20 times that of PGA.

The fact that ascorbic acid increases the formation of CF from PGA suggests the possibility that a severe deficiency of vitamin C might depress the metabolic alteration of PGA to CF. In keeping with this possibility is the demonstrated relationship of scurvy to the appearance of megaloblastic anemia in human infants and monkeys, a condition relieved by treatment with PGA (May, *et al.*)(2,3). Also, in another species unable to synthesize ascorbic acid, the guinea pig, a deficiency of vitamin C leads to an abnormality in the metabolism of tyrosine, a condition also relieved by relatively large doses of PGA(4). It is reasonable to postulate that the conversion of PGA to CF is limited by a severe deficiency of ascorbic acid, so that effective amounts of the essential derivative are no longer obtained from PGA unless the intake of folic acid is materially increased.

Summary. Rat liver slices, normal or folic acid-deficient, are shown to form in the presence of pteroylglutamic acid a factor required for the growth of *Leuconostoc citrovorum* 8081. Ascorbic acid augments significantly the yield of the factor. The rela-

15. Wright, L. D., Skeggs, H. R., and Welch, A. D., *Arch. Biochem.*, 1945, v6, 15.

tionship of ascorbic acid to the metabolic activity of folic acid is discussed.

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Procaine and Autonomic Innervation. (17807)

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Hunt(1) reported that procaine may counteract the influence of acetylcholine on blood pressure and heart rate. Hazard, Corteggiani and Cheymol(2) showed that intravenous injection of procaine paralyzes the cardiac vagus innervation and suppresses the bradycardia by acetylcholine(3), physostigmine(4) and nicotine(5), while the hypotensive effects of acetylcholine are maintained. The hypertensive effect of acetylcholine in atropinized animals may be changed to a hypotensive by procaine. Harvey(6) and Dutta(7) stated that procaine may block conduction in the first cervical sympathetic ganglion. Laqua (8) showed that procaine protects the guinea pig against physostigmine intoxication. Conway *et al.*(9) reported that physostigmine and di-isopropylfluorophosphate(DFP) protect mice against procaine intoxication, while neostigmine and carbamic acid, N,N-dimethyl- 4 -dimethylamino-3-isopropylphenyl ester, methiodide (DIP) lower the LD50 of procaine.

Experiments have been performed on 24 dogs anaesthetized with chloralosane to investigate the mechanism of action of procaine on autonomic innervation.

The observations may be summarized as follows: 1. Small doses of procaine (10-40 mg/kg) given intravenously to 11 atropinized dogs decrease acetylcholine (60-240 γ/kg) hyperpnea and change the hypertensive effect to an arterial hypotension, while the vasopressor reflexes of carotid sinus origin are unaltered.

2. High doses of procaine in 15 normal dogs (40-300 mg/kg) curarize first intercostal respiratory muscles, then the diaphragm and finally the skeletal muscles, and suppress bradycardia induced by vagus stimulation or by nicotine. Small doses of acetylcholine (30-60 γ/kg) provoke arterial hypotension, without bradycardia. Higher doses of acetylcholine still induce bradycardia, which is in turn suppressed by higher doses of procaine. Vasopressor reflexes of carotid sinus origin or induced by central vagus nerve stimulation are first depressed and then blocked by high doses of procaine. Vasomotor synaptic stimu-

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1. Hunt, R., *Am. J. Physiol.*, 1918, v45, 250.

2. Hazard, R., Corteggiani, R., and Cheymol, J., *C. R. Soc. Biol.*, 1946, v140, 83.

3. Hazard, R., and Cheymol, J., *C. R. Soc. Biol.*, 1943, v137, 280.

4. Hazard, R., *Actualités pharmacologiques, Masson et Cie*, 1949.

5. Hazard, R., *C. R., Soc. Biol.*, 1942, v136, 459.

6. Harvey, A. M., *Bull. Johns Hopkins Univ.*, v65, 223.

7. Dutta, N. K., *Brit. J. Pharmacol.*, 1946, v4, 197.

8. Laqua, *Thesis Breslau*, 1920, cit. by Schiereller, *Arch. exp. Path.*, 1925, v108, 78.

9. Conway, A. C., Ting, K. S., and Coon, J. M., *J. Pharm. Exp. Ther.*, 1949, v96, 472.