On the Metabolic Alteration of Pteroylglutamic Acid.*† (20298)

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The enzymatic formation of a derivative of folic acid (FA) which promotes the rapid growth of the microorganism, *Leuconostoc citrovorum* (ATCC 8081), was demonstrated by incubating slices of rat liver with pteroylglutamic acid (PGA) in an air-equilibrated medium(2). Under similar conditions, however, homogenates of rat liver failed to form more than trace amounts of citrovorum factor (CF) from pteroylglutamic acid(3). The effectiveness of ascorbic acid in augmenting the amount of CF formed by the slices suggested that anaerobic conditions might favor the reaction. Data presented here show that satisfactory yields of CF were obtained when homogenates of liver were incubated in the presence of PGA under an atmosphere of nitrogen. Also, different tissues have been compared with respect to their capacity to form CF from PGA *in vitro* and the hepatic concentration of CF was determined with respect to time following the parenteral administration of PGA to rats previously depleted of FA.

Experimental. Weanling albino rats were depleted of FA by feeding a purified casein-cerelose diet containing succinylsulfathiazole which was described previously(2). Rhode Island Red chicks of mixed sex were fed a similar FA-deficient diet supplemented with gelatin (10%). The normal rats were maintained on a complete stock diet. The chicks and rats were used for experiments *in vitro* when signs of FA-deficiency were apparent. Like FA, *CF must be released* from the conjugated forms in which it occurs naturally before it is active for the assay organism, but liver is an excellent source of such hydrolytic enzymes(4). In the studies of the concentration of CF in the liver of rats which were injected intraperitoneally with PGA, satisfactory release of CF was obtained by autolysis of homogenates of fresh liver under toluene at 37°. The homogenates (1:4) were prepared in 0.1 M phosphate buffer of pH 6.5. Studies on the rate of release of CF showed that although autolysis for 12 hours was insufficient, maximal values were obtained after 18 hours. In this study, 10 cc aliquots of homogenates prepared from the individual livers were autolyzed for 20 hours, then steamed for 10 minutes, diluted to 25 cc and filtered. The preparation and incubation of the tissue slices was described previously(2). Homogenates were prepared in a glass homogenizing-tube with 0.1 M phosphate buffer, pH 7.0, so that 5.0 cc of homogenate contained 1 g of fresh tissue. After incubation, the tissues were steamed for 10 minutes. The filtrate of each sample was diluted appropriately for assay using *Leuconostoc citrovorum* in the medium described by Sauberlich(5). Following incubation of the 10 cc assay tubes for 18 hours at 37°, turbidity readings were made using a Klett-Summerson photoelectric colorimeter with a No. 66 filter. The barium salt of synthetic citrovorum factor was used as a reference standard; the concentration was expressed in terms of the free acid. Since synthetic CF (leucovorin or folinic acid-SF) as the free acid, was found to be 50% as active as PGA when

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† As used by Welch and Nichol(1) the term pteroylglutamic acid (PGA) has been restricted to the synthetic compound, and citrovorum factor (CF) to the substance or substances active in promoting the rapid growth of *Leuconostoc citrovorum* 8081, while the term folic acid (FA) has been considered as a generic term applicable to a group of substances, including PGA and CF, which stimulate the growth of *Lactobacillus casei* and *Streptococcus faecalis*.

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TABLE I. Comparison of the Capacity of Various Tissues to Form Citrovorum Factor.

Tissue*	Yield of CF after incubation: Pteroylglutamic acid None	Pteroylglutamic acid 100 μg/vessel	% conversion of PGA to CF
	mμg CF/g of tissue, wet wt		
Liver	273	22400	22.13
Kidney	175	534	.36
Pancreas	25	172	.15
Spleen	8	102	.09
Small intestine	7	75	.07
Brain	4	22	.02
Lung	2	15	.01
Ventricle	2	7	<.01
Pectoral muscle	<1	4	<.01
Blood	<1	<1	0
Bone marrow	3	121	.12

* The tissues, obtained from FA-depleted chicks, were suspended in 5 cc of Krebs-Ringer-phosphate which contained sodium ascorbate (10 mg). The vessels, containing approximately 1 g of tissue slices (blood sample—3 cc/vessel), were incubated at 37° for 1 hr under an atmosphere of nitrogen.

assayed for *Lactobacillus casei* and *Streptococcus faecalis* activity, it was assumed that only 50% of the synthetic vitamin was active for the organism, *Leuconostoc citrovorum;* this assumption has been discussed elsewhere (1). The amounts of CF referred to throughout these studies are calculated on the basis of the naturally-occurring material, which is regarded as exactly twice as active as the synthetic material presently available. For comparison of these with other studies in which the results were related directly to a synthetic reference standard, the amounts of CF reported in such studies must be halved.

Results. The data presented in Table I indicate that the conversion of PGA to CF occurs mainly in the liver. This comparison was consistent with the results of two similar experiments using tissue from chicks. A small amount of CF was formed by all tissues tested (with the exception of blood). Although the assay organism responds slowly when PGA is added to the medium in very large amounts (5 to 10 μg per assay tube), the low value obtained for the sample of blood incubated in the presence of 100 μg of PGA demonstrates that under the conditions of the assay this material was inactive. However, as an additional control, PGA was added to filtrates of the control samples in amounts corresponding to that present in the samples which were incubated with PGA. The control values were not increased by this treatment. Although the amount of CF formed by bone marrow was small, it should be noted that the increase relative to the control value was greater than in any other tissue, with the exception of liver.

Since the concentration of CF in the livers of FA-depleted rats was approximately one-tenth of that which occurred in the livers of normal rats, the increase in the hepatic concentration of CF following administration of folic acid could be clearly demonstrated (Table II). Within one hour following the intraperitoneal injection of PGA, large amounts of CF were present in the livers of the previously depleted rats. Thereafter the concentration of CF decreased, and, after 24 hours, the amount of the vitamin retained in the tissue corresponded closely to that observed in the livers of normal rats.

A comparison of the effect of aerobic and anaerobic conditions on the formation of CF was carried out by incubating slices or homogenates of liver under an atmosphere of nitrogen and, simultaneously, a duplicate series of tissue replicates under oxygen. The results of representative experiments are presented in Table III. The hepatic tissue used in all these experiments was obtained from FA-depleted rats or chicks, a circumstance which minimized the amount of CF released from conjugates of the vitamin during the incuba-

TABLE II. Rate of Appearance of Citrovorum Factor in Hepatic Tissue of Rats Following Intraperitoneal Administration of Pteroylglutamic Acid (PGA).

Time (hr) following I.P. dose of PGA (10 mg/kg)	No. of rats	Citrovorum factor content of liver, μg/g of tissue, wet wt: Avg	Range
Folic acid-deficient rats			
Control (untreated)	6	.13	.07– .22
1	4	3.84	2.23–5.12
2	7	2.77	1.53–3.95
4	3	1.99	1.76–2.19
24	6	1.57	.81–2.65
Normal rats			
Control (untreated)	5	1.27	.92–1.47
24	5	2.23	2.14–2.50

TABLE III. Formation of Citrovorum Factor by Liver Preparations Incubated Aerobically and Anaerobically with Pteroylglutamic Acid (PGA).

Additions/vessel* PGA, μg	Ascorbic acid, mg	Yield (μg) of CF/g tissue, wet wt, following incubation under atmosphere of Oxygen	Nitrogen
	Rat liver slices		
0	0	.007	.027
0	10	.032	.055
100	0	.220	1.26
100	5	.920	2.64
100	10	1.51	2.83
100	20	2.10	2.36
	Rat liver homogenate		
0	0	.005	.287
0	20	.015	.514
100	0	.062	2.56
100	10	.253	3.23
100	20	.780	2.51
	Chick liver slices		
0	0	.351	.496
0	10	.592	.596
100	0	3.34	8.80
100	5	11.0	15.2
100	10	12.9	12.9
100	20	15.6	12.7
	Chick liver homogenate		
0	0	.082	.318
0	20	.398	.367
100	0	.470	8.63
100	10	5.73	17.2
100	20	9.52	18.1

* Each vessel contained approximately 1 g of liver slices suspended in 5 cc of Krebs-Ringer-phosphate which contained the additions indicated *or* 4.5 cc of a 1:4 homogenate of liver in neutral phosphate buffer (0.1 M) and 0.5 cc of buffer or the additions indicated. The vessels were incubated at 37° for 1 hr.

tion. Consistently higher yields of CF were obtained under anaerobic conditions. When rat liver slices were incubated in the presence of 100 μg of PGA under an atmosphere of oxygen, the yield of CF was much smaller than that obtained in the duplicate incubated under nitrogen. The addition of sodium ascorbate increased the yield of CF in each case, but 5 mg of ascorbate was more effective under anaerobic conditions than 20 mg under an atmosphere of oxygen. In the experiment using homogenized rat liver, poor yields of CF were obtained in the aerobic incubation, even in the presence of ascorbate, but under anaerobic conditions the formation of CF occurred readily without the addition of ascorbate. In the experiments using chick liver, larger yields of CF were obtained, and a similar favorable effect of anaerobic conditions and the presence of ascorbate was observed. Homogenates of chick liver were capable of forming CF when incubated under oxygen in the presence of ascorbate.

Discussion. The conversion of PGA to CF appears to occur mainly in the liver. The capacity of other tissues to form CF seems to be quite limited. However, it is of interest that bone marrow is able to form an appreciable amount of CF, relative to the control value for this tissue. In cultures of bone marrow obtained from untreated patients with pernicious anemia, Thompson(6) observed that maturation of the megaloblasts was induced by PGA (10 to 50 μg per cc) and Callender and Lajtha(7) found that CF was more effective than PGA in causing the disappearance of megaloblasts in similar cultures. Consequently the ability of bone marrow to form even a small amount of CF may be of real importance with relation to the function of CF in this tissue.

Goldin *et al.*(8) demonstrated that doses of FA administered one hour prior to Aminopterin protected mice against otherwise lethal doses of the antagonist and that this protection did not occur when PGA and the analogue were given at the same time. Data presented here show that PGA was converted rapidly to CF in the liver of intact rats and saturation of the tissue with CF, a substance which can compete effectively with Aminopterin, occurred within one hour following administration of PGA. Although a large dose of PGA (10 mg per kg) was administered, only about 1 μg of CF per g of tissue was retained in the liver at the end of a 24-hour period.

Both anaerobic conditions and the presence of ascorbate facilitated the formation of CF. Studies now in progress indicate that ascorbic acid inhibits the enzymatic destruction of CF during the aerobic incubations. However, since ascorbate increased the yield of CF in the anaerobic medium, it may also participate directly in the formation of CF. The finding that the enzymatic formation of CF proceeds well in cell-free preparations of liver makes possible a detailed study of the properties of the system and the mechanism of the reaction.

Summary. The liver appears to be the main site of the conversion of pteroglyglutamic acid (PGA) to citrovorum factor (CF) in the body. Other tissues had very limited capacity to form CF, with the exception of bone marrow which, relative to the small amount present in this tissue, formed an appreciable quantity of the factor. The hepatic tissue of rats which had been depleted of folic acid and CF was flooded with CF within one hour following the intraperitoneal injection of PGA. Homogenates of rat or chick liver, when incubated with PGA under an atmosphere of nitrogen, were capable of forming CF. Little CF was found when similar preparations were incubated under oxygen. The yield of CF was consistently increased by the presence of ascorbate in the incubation mixture.

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Hemagglutination with Certain Arthropod-Borne Viruses.* (20299)

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Specific hemagglutination (HA) with neurotropic viruses was first described by Hallauer(1) for the Columbia SK and Columbia MM viruses, by Lahelle and Horsfall(2) for the GD VII virus, and by Olitsky and Yager (3) not only for the Columbia SK and Columbia MM, but also for the Mengo and encephalomyocarditis viruses. Other reports have followed, largely confirming these findings; but departures from them were recorded by Sabin and Buescher(4) and Sabin(5), who included several other encephalitis viruses. The former(4) reported success with Japanese B encephalitis, West Nile disease, and Russian Far Eastern encephalitis viruses; the latter(5) reported success not only with these agents, but in addition with St. Louis encephalitis and Western equine encephalitis viruses.

Recently Sweet, Chanock, and Sabin(6) showed the presence of hemagglutinins associated with 2 immunologically distinct types of dengue virus, Hawaiian and New Guinea C. The method of preparation of viral extracts, the pH range needed for positive reactions, and the conditions under which serum inhibition of HA took place were found to be rigid and exacting and varied from virus to virus. MacDonald(7), in a recent step forward, readily prepared specific HA extracts from the brains of newborn mice infected with Murray Valley encephalitis virus. Prior studies in this laboratory have shown the superiority of the brains of newborn mice as source material for the preparation of complement fixation antigens. It was therefore

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