Metabolic Interrelationship between Folic Acid, Vitamin B_{12}, and the Citrovorum Factor.* (20530)

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Present evidence indicates the presence in avian liver of 2 enzyme systems which are responsible for an increase in microbiologically determinable citrovorum factor (CF). A report by Hill and Scott(1) suggests that ascorbic acid increases the CF content of chick liver homogenate by activating a CF conjugase present in chick liver. Nichol(2) reported that formation of CF from pteroylglutamic acid (PGA) in chick liver homogenate at an optimal pH of 6.2 proceeded well under anaerobic conditions. Recently, Broquist *et al.*(3) reported 39% to 54% conversion of added PGA to CF by resting cells of a strain of *S. faecalis* resistant to 4-amino-10 methyl pteroylglutamic acid(4). Such high enzymatic formation of CF was obtained only when the resting cells were incubated with PGA and a supply of "formate" in presence of a reducing environment.

The metabolism of "formate" is influenced by dietary PGA according to reports by several groups(5-8). Reports have appeared to indicate a participation of vit. B_{12} in the conversion of PGA to CF(9-11) but the evidence does not seem conclusive. It is the purpose of this report to determine the effect of supplementing the chick diet with varying levels of vit. B_{12} and PGA on the *in vitro* synthesis of CF from added PGA by the liver homogenates.

Experimental. Straight run (New Hampshire males X Single Comb White Leghorn female) crossbred chicks were used for all the studies. Birds were housed in electrically heated batteries with raised screen floors. The chicks were wingbanded, weighed and randomized into 12 groups of 12 birds each at one day of age. Weights were recorded at weekly intervals. The basal diet used in the present studies was the same as reported by Couch *et al.*(12) with the exception that the diet contained 69.5% Cerelose, 22.5% soybean protein (Drackett 220), 2.5% soybean oil, 6 mg/kg α-tocopherol, 10,000 I.U. vit. A/kg, and 2,000 I.C.U. vit. D_3/kg. PGA and vit. B_{12} were not added to the basal diet since the latter was supplemented with 3 different levels of PGA (2, 100, 400 mg/kg) and 2 levels of vit. B_{12} (30, 500 γ/kg).

At the end of the 4-week period, 3 chicks from each group were sacrificed, the livers were removed and immediately chilled with ice for 2 minutes; 20% homogenates were then prepared in 0.08 M sodium potassium phosphate buffer pH 6.3, using the Potter-Elvehjem glass homogenizer. Equal amounts of the 3 homogenates were mixed and representative homogenates thus obtained from the various groups of birds were immediately tested for PGA to CF conversion studies as follows: 5 ml aliquots of the various homogenates were pipetted in duplicates into 50 ml Erlenmeyer flasks containing 5 ml of the above mentioned phosphate buffer and also aliquots of solutions containing substances to be described. The final volume in each flask was made to 11 ml with water and the solutions were layered with toluene. Then the flasks were tightly rubber-stoppered and nitrogen was introduced by the use of hypodermic needles thrust through the rubber stoppers (13). After flushing with nitrogen, the needles were carefully withdrawn and the flasks were incubated on a reciprocating shaker at 37°C for 2 hours. The rubber stoppers were removed and replaced by the usual

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TABLE I. Conversion of PGA to CF by Liver Homogenates from Chicks Fed a Low Vit. B_{12}-PGA Diet Supplemented with Varying Levels of Vit. B_{12} and PGA.

Group No.	Supplements to basal diet: PGA, mg/kg	Supplements to basal diet: Vit. B_{12}, γ/kg	Vit. B_{12}, mγ/g liver	γ PGA added/flask*: 0	50	100	250
				γ CF/g liver homogenate			
1	0	0	18	1.3	13.6	25.8	45.5
2	0	30	129	1.4	17.1	30.0	55.4
3	0	500	364	3.2	27.5	43.4	64.9
4	2	0	12	3.6	17.3	25.2	61.0
5	2	30	165	3.7	23.4	45.8	55.0
6	2	500	340	12.8	36.7	66.1	75.6
7	100	0	46	2.7	20.0	30.2	62.5
8	100	30	286	9.6	33.4	51.0	71.3
9	100	500	611	8.5	37.4	72.5	121.0
10	400	0	18	4.2	25.8	39.5	67.4
11	400	30	256	10.7	43.6	71.8	108.8
12	400	500	406	12.6	31.9	44.6	54.5

* Flasks components: 5 ml of 20% liver homogenate in .08 M sodium-potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of PGA solution, made to a total volume of 11 ml with H_2O. Flasks were incubated for 2 hr under N_2.

cotton plugs and all the samples were autoclaved for 5 minutes at 120°C. After cooling, the samples were homogenized in a Waring Blendor, neutralized, made up to volume, filtered, and the filtrate was assayed. The CF content was determined using the single strength basal medium VI of Steele *et al.*(14) with acid hydrolyzed casein to supply part of the amino acids. *Leuconostoc citrovorum* ATCC 8081† was used as the test organism and Leucovorin (Lederle) was employed as the standard. Since the latter has been reported to be half as active as the CF isolated from horse liver(15), the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF. The cultures were incubated for 72 hours at 37°C and growth was determined by titrimetric procedure.

Liver samples of 6 chicks from each group were pooled and the vit. B_{12} determinations were run in triplicates. The method of Yacowitz *et al.*(16) modified by addition of 50 γ KCN per g of liver homogenate was used for liberating vit. B_{12}(17). Determinations of vit. B_{12} were made on the treated liver samples using the titrimetric method of Skeggs *et al.* (18) with *Lactobacillus leichmannii* 4797 as the test organism and the crystalline vit. B_{12} as the standard.

Results. It is apparent from the results of Table I that supplementing of chick diet either with increasing amounts of vit. B_{12} or PGA increased the conversion of added PGA to CF by the liver homogenate. This is largely true of results from chicks of Groups 1 to 11 while on the other hand liver homogenates from chicks receiving highest levels of vit. B_{12} and PGA (Group 12) gave a lowered PGA to CF conversion. Also homogenates of liver from chicks receiving 400 mg PGA/kg of diet (Group 10) gave an increase in PGA to CF conversion which was comparable to supplementation of the diet with 500 γ vit. B_{12}/kg (Group 3). This would suggest that the 2 vitamins may influence the converting mechanism by separate pathways.

It was of interest to determine if the CF synthesized by chick liver homogenate from added PGA was identical with the synthetic CF (Leucovorin). This was accomplished by analyzing the filtrate prepared for CF assays, using bioautographic procedures described by Doctor and Couch(19). Paper strips were developed using wet symmetrical collidine and also using a solvent system composed of 50%

† A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study on the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier by Pederson (*Bact. Rev.*, 1949, v13, 225).

TABLE II. Influence of Adding Vit. B_{12} and Formate on Conversion of PGA to CF by Liver Homogenates from Chicks Fed a Low Vit. B_{12}-PGA Diet Supplemented with Varying Levels of Vit. B_{12} and PGA.

Group No.	Supplements to basal diet: PGA, mg/kg	Supplements to basal diet: Vit. B_{12}, γ/kg	Substances added/flask*: None	PGA, 50 γ	Formate, 10 mg + vit. B_{12}, 10 γ	Formate, 10 mg + vit. B_{12}, 10 γ + PGA, 50 γ
			γ CF/g liver homogenate			
1	0	0	1.3	13.6	1.1	17.1
2	0	30	1.4	17.1	1.3	20.3
3	0	500	3.2	27.5	3.8	30.3
4	2	0	3.6	17.3	3.4	23.6
7	100	0	2.7	20.0	2.9	17.8
10	400	0	4.2	25.8	4.5	28.0

* Flask components: 5 ml of 20% liver homogenate in .08 M sodium potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of solutions containing substances described to make a total volume of 11 ml. Flasks were incubated for 2 hr under N_2.

ethanol, 15% *n*-butanol, 10% ammonia, and 25% water(19). Results of both these studies indicated that the compound synthesized from PGA by chick liver homogenate moved on paper strips at the same rate as did synthetic CF (Leucovorin).

Supplementing the chick diet with increasing amounts of vit. B_{12} increased by many fold the vitamin content in the liver (Table I). Supplementing with 100 mg PGA/kg also resulted in an increased storage of vit. B_{12} in the liver. Supplementation of the basal diet with 400 mg PGA/kg produced a slight decrease in the vit. B_{12} content of the livers as compared to values obtained when 100 mg PGA/kg were fed. The soybean protein used in the basal ration supplied 50 γ PGA/kg of diet and 15 γ CF/kg. Average weights of the birds at 4 weeks of age ranged from 293-355 g. No apparent differences were obtained which could be correlated with dietary changes.

In order to investigate whether feeding a diet low in vit. B_{12} resulted in a low storage of "formate" in the liver and to determine if vit. B_{12} is directly concerned in some way with the converting mechanism, studies were conducted to determine the influence of *in vitro* addition of vit. B_{12} and "formate" on the PGA to CF conversion. Results of this study presented in Table II show only a slight stimulation of PGA to CF conversion by *in vitro* addition of 10 γ vit. B_{12} together with 10 mg sodium formate. The data may be interpreted to suggest that vit. B_{12} may have to be converted into a type of coenzyme system concerned with the synthesis of CF from added PGA but the failure of added formate in giving an appreciable increase in the conversion is even more intriguing(3,20).

Discussion. Investigations by Drysdale *et al.*(6) where PGA deficiency decreased the incorporation of formate into C-2 and C-8 of the liver adenine and guanine and that of Skipper *et al.*(7), in which the incorporation of formate into total nucleoprotein of mice is decreased by antagonist-induced PGA deficiency suggest a probable CF-catalyzed utilization of formate in the synthesis of purines(21). Recently, Buchanan and Schulman(20) have reported a more direct influence of CF in a preferential incorporation of radioactive formate into C-2 of inosinic acid. It is apparent that under the presently described experimental conditions an increased conversion of PGA to CF is obtained in liver homogenates from chicks receiving increasing levels of PGA or vit. B_{12}. In order to ascertain if this increase in conversion was largely due to a general increase in metabolic activity of the liver, studies on the endogenous oxygen uptake were conducted. It was observed that supplementation with vit. B_{12} or PGA did not appreciably affect the endogenous oxygen uptake.

Since vit. B_{12} is not effective in reversing the effects of certain PGA analogues which are known to block the transformation of PGA to CF, it is conceivable that dietary vit. B_{12} and PGA may in some way prevent

the inactivation of CF synthesized by the liver homogenate and thereby cause an apparent increase in CF production. The evidence does not, however, rule out the possibility that a specific effect of vit. B_{12} and PGA is involved.

Summary. Supplementing a low vit. B_{12}-PGA chick diet with either increasing levels of vit. B_{12} (30 γ and 500 γ/kg) or increasing levels of PGA (2, 100, and 400 mg/kg) resulted in an increased capacity by the liver homogenates to convert added PGA to CF. The results further suggest that the 2 vitamins may influence the converting enzyme system through different mechanisms. *In vitro* addition of formate and vit. B_{12} to the liver homogenate gave only a slight stimulation of PGA to CF conversion by the liver homogenate.

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Effect of Aureomycin upon Growth and Maturation of *Lebistes reticulatus*. (20531)

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Recent work in nutrition has indicated that addition of aureomycin to the diet increased the rate of growth of newborn or immature animals(1-7). Jukes *et al.* produced acceleration of growth in pigs using aureomycin supplemental feeding(1). These results have been confirmed and extended to other animals as the chick(2-7). Growth-enhancing effect of aureomycin in premature children has also been described(8). Recently it has also been shown that aureomycin supplemental feeding enhanced the growth of rats made diabetic with alloxan(9).

The above work demonstrating the anabolic effect of the broad spectrum antibiotic in mammals and birds and in experimentally induced diabetes made it of interest to study this propensity of the drug in lower vertebrates. To this end the experimental report herein is concerned with the observations on