METABOLISM OF PTEROYLGLUTAMIC ACID AND THE CITROVORUM FACTOR IN PATIENTS WITH SCURVY [1, 2]

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The citrovorum factor (CF) is closely related chemically and metabolically to pteroylglutamic acid (PGA) (1–5). CF replaces PGA in the nutrition of certain microorganisms (3, 6) and in some species of animals (6, 7); and also reverses aminopterin activity (3, 7–11). A relationship of CF to PGA is further indicated by the increased urinary excretion of CF which occurs when PGA is fed (12–16). The hematopoietic effect of CF given to patients with pernicious anemia (17–20), or with other nutritional anemias (21, 22), or to monkeys with megaloblastic anemia induced by dietary means (23) also attests to the metabolic activity of this factor.

The synthesis of CF from PGA by rat liver slices is augmented by ascorbic acid (24). This indicates that ascorbic acid may play a role in the conversion of PGA to CF. In order to investigate the role of ascorbic acid in the metabolic relationship of PGA to CF in man, the urinary excretion of CF was determined following PGA administered orally to two patients with scurvy and to a non-scorbutic subject before, during and after ascorbic acid therapy. The results of these studies are presented and indicate that one role of ascorbic acid in man is to facilitate the conversion of PGA to CF.

PATIENT MATERIAL AND METHODS

Two men with scurvy and one non-scorbutic male comprise the subjects of this study. The two scorbutics, T. C. age 66, and F. O'D. age 65, were bachelors who lived alone in single rooms, and gave histories of ingesting inadequate quantities of food and of prolonged dietary ascorbic acid deficiency. Clinical evidences of scurvy included multiple perifollicular hemorrhages most prominent on the legs and forearms, ecchymoses especially of the posterior thighs, and hyperkeratotic folliculosis with crinkled, brittle hairs, most apparent on the abdominal wall and thighs. Neither patient showed hypertrophic or bleeding gums. Ascorbic acid was not present in the buffy coat of peripheral venous blood samples obtained from each of these patients. Neither patient had significant anemia. The hemoglobin concentrations were 13.7 gm. and 12.8 gm. per 100 ml., and the red blood cell counts 4.19 million and 4.20 million per cu. mm., respectively. Other blood studies including white blood cell counts, differential count, platelet count, prothrombin time, bleeding time, and coagulation time were within normal limits. A tourniquet test done on a forearm of each of these patients was markedly positive. All of the abnormalities noted were promptly and completely reversed following the administration of ascorbic acid.

The non-scorbutic subject, J. O'C. age 49, was a chronic alcoholic and an epileptic. He had been in the hospital for two months during which time an adequate diet was provided prior to the initiation of the studies described here. At this time physical examination and the usual routine laboratory studies, including tests of liver function, revealed no abnormalities. The peripheral venous buffy coat ascorbic acid content was 44 mg. per cent.

The patients with scurvy were examined shortly after hospital entry and immediately transferred to the Thorndike Metabolic Ward. They did not receive food or medication prior to the institution of the dietary regimen and the studies described. During the entire periods of study these patients were kept at rest in bed and were offered diets consisting of boiled milk and rice, crackers, sugar, coffee, and water. These items of food supplied minimal, if any, quantities of ascorbic acid. The non-scorbutic subject was ambulatory and was maintained on a constant well-balanced dietary regimen pro-

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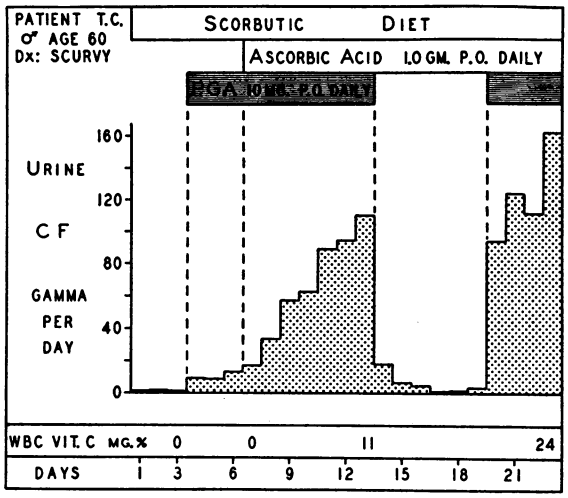

FIG. 1. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A PATIENT WITH SCURVY BEFORE AND AFTER ASCORBIC ACID THERAPY

viding 75 gm. of protein and 2,800 calories daily during this study.

The three subjects were observed during initial control periods of three days. Each was then given 10 mg. of PGA [4] orally daily in two equal doses at 9 a.m. and 5 p.m. for from three to six days. During a third period of study of each patient, PGA was continued in the dosage stated and in addition, 1 gm. of ascorbic acid was administered daily for from six to nine days. The ascorbic acid was given orally in the cases of T. C. and J. O'C., and intramuscularly as sodium ascorbate in the case of F. O'D. in four equal doses at 9 a.m., 1 p.m., 5 p.m., and 9 p.m. PGA was then discontinued, but the basal diets and ascorbic acid continued for from six to nine days. In the case of the scorbutic patients, PGA was administered orally on a second occasion according to the same dosage schedule previously employed while ascorbic acid therapy and the basal diets were continued unchanged. Thus, the patients served as their own controls, since observations on the urinary excretion of CF following PGA taken orally were made before and after the alleviation of the scorbutic state by adequate therapy with ascorbic acid.

A final period of study in F. O'D. consisted of the oral supplementation of his diet with 10 mg. of PGA daily for six days without supplemental ascorbic acid. The final period of study in J. O'C. consisted of six days on the constant diet and was therefore identical to the initial study period in this patient.

During these studies the urine specimens were collected and immediately placed in a refrigerator. After completion of each 24-hour urine collection the daily urine volumes were measured, and aliquots stored in a deep freeze at — 20° C. for subsequent microbiologic assay for PGA and CF activity. The microbiologic assays for CF were done according to the method of Sauberlich (1, 12) using a synthetic crystalline material with *L. citrovorum* activity (leucovorin) as the reference standard, and for PGA activity by the growth response of *Lactobacillus casei* (25). Buffy coat ascorbic acid concentrations were measured by the method of Butler and Cushman (26).

[4] The pteroylglutamic acid (folvite) and the crystalline citrovorum factor (leucovorin) used in these studies was generously provided by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

RESULTS

The results of these studies are presented in Figures 1, 2, and 3. T. C., Figure 1, a patient with scurvy, excreted about 1.2 gamma of urinary CF daily during three initial days of study. When 10 mg. of PGA were administered orally daily for three days, urinary CF excretion ranged from 9.1 to 13.3 gamma daily. One gram of ascorbic acid was then given orally daily for seven days, in addition to PGA. Urinary CF excretion was 16.9 gamma on the first day of ascorbic acid therapy and gradually increased to 110.4 gamma by the seventh day. PGA was then omitted but ascorbic acid continued. Urinary CF returned within four days to initial levels. After six days PGA was given again in addition to ascorbic acid. On this occasion on the first day of PGA administration urinary CF was 93.9 gamma, and averaged 132.6 gamma daily during the three additional days of the final study period.

The second patient with scurvy, F. O'D., Figure 2, excreted about 0.4 gamma of urinary CF

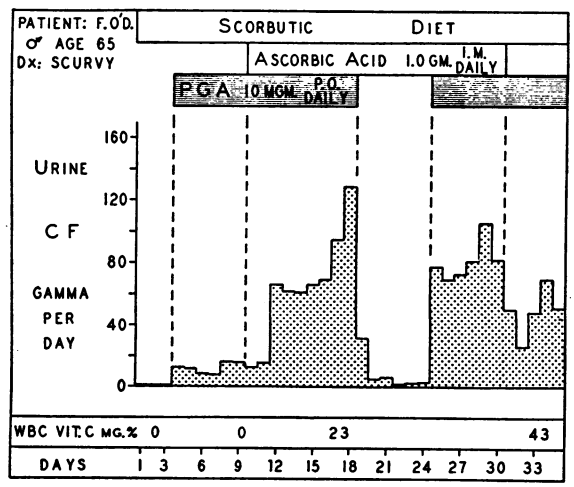

FIG. 2. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A PATIENT WITH SCURVY BEFORE AND AFTER ASCORBIC ACID THERAPY

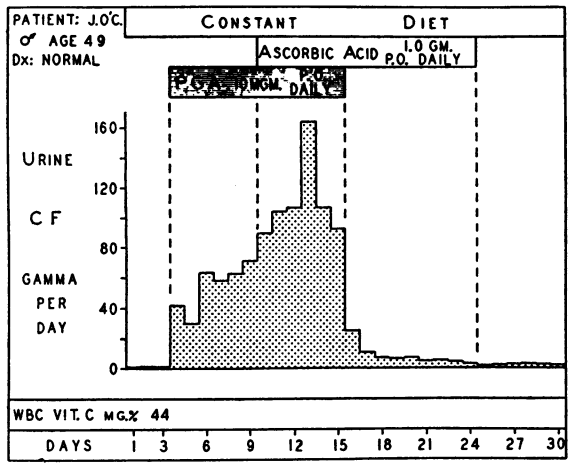

FIG. 3. URINARY EXCRETION OF CF FOLLOWING ORAL PGA IN A NON-SCORBUTIC MALE BEFORE AND AFTER ASCORBIC ACID THERAPY

during the first three days of study. Urinary CF ranged from 7.9 to 16.1 gamma daily when 10 mg. PGA were given orally daily for six days. During the next six days of study, 1.0 gm. of ascorbic acid was given intramuscularly daily to this patient in addition to PGA. Urinary CF was 12.6 gamma on the first day of ascorbic acid therapy, 15.2 gamma on the second day, and ranged from 61.8 to 129.3 gamma daily for four additional days. Ascorbic acid was then continued, but PGA omitted for six days. Urinary CF returned promptly to initial levels. For the next six days, PGA was again given in addition to ascorbic acid. The readministration of PGA was accompanied on the first day of this medication by the urinary excretion of 77.9 gamma of CF, 69.7 gamma on the second day, and 72.5 to 106.0 gamma daily for four additional days of this study period. Finally, ascorbic acid was discontinued, but PGA continued for five days during which time urinary CF excretion averaged 49.3 gamma daily.

J. O'C., Figure 3, a non-scorbutic control patient, excreted approximately 0.9 gamma of urinary CF daily during the first three days of study. When 10 mg. of PGA were given orally, urinary CF excretion rose promptly to 41.8 gamma on the first day and averaged 54.3 gamma daily during the six days of PGA administration. One gram of ascorbic acid was then given orally daily for six days in addition to the PGA. Urinary CF excretion was immediately further increased, and averaged 116.1 gamma daily for this period. Then for nine days, ascorbic acid was continued alone. The urinary CF excretion gradually decreased to 3.0 gamma daily. The urinary CF excretion during a final period of study which was comparable to the initial study period was again very low, ranging from 1.9 to 2.9 gamma daily.

DISCUSSION

A small amount of CF was excreted in the urine by the two patients with scurvy before PGA administration and was slightly increased by the oral administration of PGA before ascorbic acid therapy. However, maximal urinary CF excretion occurred in these patients only when both PGA and ascorbic acid were given. The relatively small increases in urinary CF excretion which were observed in the scorbutic patients when PGA alone was given during the scorbutic state or during the first several days of combined ascorbic acid and PGA therapy, which has also been noted by others (16), is in marked contrast to the immediate and large increases in urinary CF excretion which occurred in these same patients given PGA following adequate therapy with ascorbic acid. A prompt and great increase in urinary CF occurs when PGA is given orally to normal subjects and this increase is further augmented by the administration of ascorbic acid (14, 16). Similar results were obtained in the present study of a non-scorbutic subject during six days of PGA administered orally alone, and then six days of combined PGA and ascorbic acid. Thus the findings in the patients with scurvy given PGA are also markedly different from those observed in the non-scorbutic individual. However, following adequate therapy with ascorbic acid, these patients responded to PGA administration in a manner similar to that observed in the non-scorbutic individual. The failure to observe high urinary CF values in the three subjects studied during those periods of dietary supplementation with ascorbic acid alone indicates that the maximal increases in urine CF excretion observed when both PGA and ascorbic acid were given cannot be attributed to an effect of ascorbic acid upon the constituents of the diets provided.

Large amounts of PGA are known to stimulate the growth of *L. citrovorum* (1). Since the CF

values varied independently from the constant intakes of PGA, it is unlikely that the urinary excretion of PGA in large amounts following its oral administration accounts for the observed alterations in urinary CF excretion. This is obvious from comparing the urine CF values obtained in the patients with scurvy before and during ascorbic acid therapy. Likewise, the urine CF values were not related directly to ascorbic acid administration. However, in order further to validate the microbiologic assay of the urines studied for CF activity content, all urine specimens were assayed for PGA and ascorbic acid content. Although the urines obtained during periods of PGA administration contained very high concentrations of PGA, in no case was this of sufficient magnitude, taking into account the dilution factors used in the assays for CF, to allow for growth stimulation of *L. citrovorum*. Likewise, ascorbic acid alone or in combination with PGA in the concentrations encountered in the urines studied failed to replace CF for the growth of *L. citrovorum*.

The urinary CF excretion pattern observed in patient T. C., Figure 1, might suggest that initial saturation with PGA may be necessary before maximal urinary CF excretion occurs. The urinary PGA excretion data do not, however, indicate an initial retention of PGA. In fact, the quantities of PGA excreted were comparable before and after ascorbic acid therapy. Illustrative data obtained in one of the patients studied are presented in Table I. The failure to observe a gradually increasing CF excretion in patient F. O'D., Figure 2, during six days of PGA administration before ascorbic acid therapy also makes it unlikely that the gradually increasing urinary CF excretion noted in patient T. C., Figure 1, would have occurred even if ascorbic acid had not been given.

The results of the PGA assays on the urines also demonstrate that the failure of maximal urinary CF excretion to occur in the scorbutic state is not related to a failure of absorption of the orally administered PGA in the absence of ascorbic acid since in both patients with scurvy, large amounts of urinary PGA were excreted daily before ascorbic acid therapy (Table I).

The relatively small but significant increase in urinary CF excretion noted in the patients with scurvy given PGA orally is not readily explained.

TABLE I

Urinary PGA and CF excretion following oral PGA before and during ascorbic acid therapy in a patient with scurvy (T. C.)

Day of study	Oral medication		Urinary excretion	
	PGA	Ascorbic acid	PGA	CF
	mg.	*gm.*	γ	γ
1			8.0	1.1
2			4.4	1.3
3			6.9	1.1
4	10.0		5,400	9.1
5	10.0		5,500	8.9
6	10.0		7,200	13.3
7	10.0	1.0	8,400	16.9
8	10.0	1.0	8,500	33.4
9	10.0	1.0	6,800	57.6
10	10.0	1.0	5,600	62.4
11	10.0	1.0	6,600	89.1
12	10.0	1.0	6,800	94.5
13	10.0	1.0	5,900	110.4
14		1.0	190	18.1
15		1.0	15.7	6.2
16		1.0	10.2	4.6
17		1.0	2.7	0.9
18		1.0	3.7	1.2
19		1.0	8.4	3.6
20	10.0	1.0	6,600	93.9
21	10.0	1.0	6,100	123.9
22	10.0	1.0	8,000	111.4
23	10.0	1.0	8,100	162.5

This may be related to the large doses of PGA used. Other possibilities are that although ascorbic acid was not found in the buffy coats of the patients studied, they were not totally deficient in ascorbic acid, or perhaps that reducing substances other than ascorbic acid may contribute to the biosynthesis of CF from PGA. In this regard, glucoascorbic acid as well as ascorbic acid markedly increased the urinary excretion of CF in rats given PGA, while cysteine and glutathione were without effect (16).

Urinary CF did not increase promptly in the patients with scurvy after ascorbic acid was given. Several days of ascorbic acid therapy were required before outputs of CF comparable to those observed in normal individuals occurred. This delay in CF excretion may be related to the quantity of ascorbic acid required to restore adequate tissue concentrations of this substance, or it may indicate tissue storage of CF occurring before excretion of this material commences.

The results of these studies indicate that PGA is closely related metabolically to CF in adult man, in confirmation of similar findings by others (12–16). In addition, the data presented indicate that

ascorbic acid greatly facilitates the conversion of PGA to CF, and suggest that one of the biochemical functions of ascorbic acid in man is to take part in the biosynthesis of CF from PGA.

SUMMARY AND CONCLUSIONS

The urinary excretion of CF was determined microbiologically following PGA administered orally to two men with scurvy and to one non-scorbutic adult before, during and after ascorbic acid therapy.

A small amount of CF was excreted by the patients with scurvy and by the non-scorbutic subject during the initial control period. Urinary CF was increased slightly when PGA was administered orally to the patients during the scorbutic state. However, maximal CF excretion, comparable to that observed in the non-scorbutic individual, occurred in the patients with scurvy only after adequate therapy with ascorbic acid.

The data indicate that CF is metabolically related to PGA, and that one role of ascorbic acid in man is to provide for the conversion of PGA to CF.

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