THE EFFECT OF ASCORBIC ACID ON THE ENZYMATIC FORMATION OF THE CITROVORUM FACTOR*

BY CHARLES A. NICHOL†

(From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio)

(Received for publication, March 30, 1953)

May and coworkers (1-3) demonstrated that the production of a scorbutic state in monkeys led to the development of a megaloblastic anemia, a condition which responded promptly to treatment with pteroylglutamic acid (PGA) or to very much smaller amounts of synthetic citrovorum factor (leucovorin or folinic acid-SF). It has been shown that the administration of ascorbic acid to normal human subjects ingesting PGA caused a 3-fold increase in the amount of citrovorum factor (CF) excreted in the urine (4-6). Also, scorbutic patients were shown to excrete little or no CF in the urine when given similar test doses of PGA, even though absorption of the substance was apparently normal (6); the excretion of significant amounts of CF occurred only after prolonged treatment with ascorbic acid (6, 7). Nichol and Welch (8) reported that the formation of CF by slices of rat liver incubated in a medium containing PGA was augmented by the presence of ascorbic acid. Finding conditions suitable for the activity of this system in cell-free preparations of liver (9) has made possible a more detailed study of the effect of ascorbic acid on the enzymatic formation from PGA of compounds which, unlike PGA, promote the rapid growth of Leuconostoc citrovorum.

EXPERIMENTAL

Preparation of Tissue—Rhode Island red chicks, obtained from a commercial hatchery, were fed a purified diet which contained casein, gelatin,

* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

† Scholar in Cancer Research of the American Cancer Society. Present address, Department of Pharmacology, Yale University School of Medicine, New Haven 11, Connecticut.

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 folie 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.
glucose, succinylsulfathiazole (2 per cent), and adequate micronutrients, with the exception of folic acid. When signs of folic acid deficiency were apparent, the birds were decapitated and the livers were removed and placed in ice-cold isotonic KCl. Homogenates were prepared in a glass homogenizing tube with the addition of cold phosphate buffer (0.1 M, pH 6.2) so that 6 ml. contained 1 gm. of tissue, wet weight. In each experiment the several reaction vessels contained aliquots of the same homogenate.

Incubation Procedure—Small beakers (20 ml.) were held in a rack which oscillated in a 37° water bath and was equipped with two hoods in order that duplicate vessels might be equilibrated separately with gases, the one with 100 per cent nitrogen and the other with 100 per cent oxygen. The volume of the incubation mixture in each experiment was 4.0 ml.; this consisted of 3.0 ml. of a 1:5 homogenate of liver, 0.5 ml. of a solution of pteroylglutamic acid (440 γ per ml.), and 0.5 ml. of buffer or a freshly prepared solution of sodium ascorbate in buffer (added immediately before the incubation). In some experiments, ascorbate was compared with other reducing agents similarly prepared.

Microbiological Assay—Immediately following incubation, the samples were heated at 15 pounds pressure for 20 minutes, transferred to calibrated glass-stoppered tubes, diluted to 25 ml. with distilled water, shaken thoroughly, and filtered. The addition of ascorbate to aliquots of various samples at the end of the incubation and immediately before heating did not alter the yield of CF. The clear filtrates were assayed microbiologically with L. citrovorum (ATCC No. 8081), with the medium described by Sauberlich (10). After incubation for 18 hours at 37°, the turbidity of the 10 ml. assay tubes was measured in a Klett-Summerson photoelectric colorimeter with filter No. 66. A solution of the barium salt of synthetic CF was used as a reference standard; the activity was expressed in terms of the free acid. Assay of standard solutions of synthetic CF (leucovorin) for microbial activity (Lactobacillus casei; S. faecalis), in comparison with PGA, indicated that on a weight basis only 50 per cent of synthetic CF was active. Since all CF activity should be measured by such assays, it was assumed that only 50 per cent of the material present in the synthetic reference standard was active for L. citrovorum; the significance of this situation has been discussed elsewhere (11). Accordingly, in these studies the results of assays for CF are expressed in terms of the naturally occurring material which was regarded as being exactly twice as active for the assay organism as was the synthetic CF employed. In other studies, in which the amounts of CF reported are based directly on standards of similar

* Leucovorin and pteroylglutamic acid were kindly supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.
synthetic material, it will be necessary, for comparison with these results, to halve the former values. The yields of CF are expressed in micrograms per gm. of liver, wet weight.

Results

The rate of formation of CF under aerobic and anaerobic conditions was studied both in the presence and in the absence of added ascorbate (Fig. 1). Duplicate series were incubated at the same time under atmospheres of oxygen and nitrogen. In the vessels incubated under oxygen without addition of ascorbate, negligible yields of CF were observed, with the exception of the first sample which was incubated for 15 minutes. In the comparable series containing ascorbate (20 mg. per 4 ml.), marked synthesis of CF occurred, which led to a maximal yield within 2 hours. The differences due to the addition of ascorbate were not as marked in the incubation under nitrogen because a considerable yield of CF was obtained in the control series. However, the presence of ascorbate did increase the yield, and by the end of 3 hours more than 25 per cent of the added folic acid was converted to CF. In both series with incubation under nitrogen, a progressive increase in the formation of CF continued throughout the 3 hour period.

Fig. 1. A comparison of the effect of aerobic and anaerobic conditions on the rate of formation of CF from PGA by a chick liver homogenate. See the text for the details.

Fig. 2. The effect of ascorbate on the formation of CF from PGA by a chick liver homogenate. See the text for the details.
The effect of smaller amounts of ascorbate (2.5, 5.0, and 10.0 mg. per 4 ml.) was observed in a similar experiment in which the replicate series were incubated under oxygen (Fig. 2). In the control series without added ascorbate, a small initial production of CF was observed after 15 and 30 minutes, but after 1 hour or longer negligible amounts of CF could be detected. The addition of 2.5 mg. of ascorbate (per 4 ml.) resulted in rapid formation of CF; 5.0 mg. caused a nearly maximal effect since the addition of twice this amount of ascorbate caused but little further increase in yield. The largest yield in each of these series was obtained after incubation for approximately 1 hour; with longer periods, progressively smaller amounts of CF were recovered.

The stability of natural CF, when incubated with homogenates of chick liver under aerobic conditions, was then studied. Natural CF was formed enzymatically by incubating PGA with a homogenate of chick liver under nitrogen; at the end of 1 hour the atmosphere of nitrogen was replaced by oxygen, sodium ascorbate (10 mg. per 4 ml.) was added quickly to one series, and buffer was added to the control vessels. The incubation was continued and the amount of CF was observed at intervals for a period of 2 hours (Fig. 3). CF disappeared rapidly in the homogenate of liver following exposure to oxygen, whereas the presence of ascorbate not only stabilized the CF which had been formed during the anaerobic incubation but...
permitted continued formation of CF under aerobic conditions. In a similar comparison, synthetic CF (50 \gamma) was incubated with a homogenate of chick liver under oxygen; 95 per cent of the CF activity was recovered at the end of 1 hour when 10 mg. of sodium ascorbate per vessel were added at the beginning of the incubation, but in the absence of ascorbate only 50 per cent of the activity was recovered.

The effect of analogues of ascorbic acid and other reducing agents on the formation of CF is shown in Fig. 4. While isoascorbate duplicated the effect of ascorbate, cysteine (at twice the molar concentration) was without effect. Under comparable conditions glucoascorbate also duplicated the effect of ascorbate, while glutathione had no effect; similar results with a liver slice system have been reported previously (8).

When the addition of ascorbate was delayed for 1 hour, at which time little CF could be detected, the synthesis of CF was initiated promptly and the rate of formation of CF during the next 30 minutes was apparently greater than that observed when ascorbate was added at the beginning of the incubation (Fig. 4).

DISCUSSION

The chemical synthesis of CF (leucovorin and folinic acid-SF) involves the addition of 4 atoms of hydrogen to the pteridine ring of pteroylglutamic acid and the replacement of one of these hydrogens by a formyl group; the product has been identified as 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (12, 13). The enzymatic formation of CF apparently involves this over-all reaction. In the chemical synthesis of CF, Shive et al. (14) have reported that the yield was increased when large amounts of ascorbic acid were present during the catalytic hydrogenation of formylfolic acid and the heating of the product; a similar result was obtained by maintaining an inert atmosphere during the procedure (15). However, in the studies described herein the effect of ascorbate on the formation of CF could not be attributed to its presence during the heat treatment of the samples following the incubation, nor did it have any effect when incubated with heat-inactivated tissue. Analogues of ascorbate (glucoascorbate and isoascorbate), which have negligible antiscorbutic potency, duplicated the action of ascorbate in augmenting the yield of CF. Since cysteine and glutathione were ineffective in this respect, a certain structural specificity would appear to facilitate the participation of compounds related to ascorbic acid in the enzymatic reaction. A similar effect was observed in experiments with intact animals; these showed that the administration of ascorbate or glucoascorbate increased the urinary excretion of CF by rats receiving intraperitoneal injections of PGA, while cysteine and glutathione were ineffective (6).

The amount of CF formed (110 \gamma per gm. of liver, wet weight) by the
homogenates incubated under optimal conditions was about 20 times the amount of CF which is found in the liver of normal chicks and more than 100 times the amount of CF which could be released from the folic acid-depleted tissue used in these experiments. Under different experimental conditions, Hill and Scott (16) obtained no formation of CF when PGA was incubated with dilute homogenates of chick liver in the presence of ascorbic acid. These results do not support their conclusion that "the primary rôle of ascorbic acid in increasing the CF content of chick liver homogenates is due to activation of the enzyme which liberates CF from a bound form present in the liver." The ascorbic acid occurring naturally in the tissue would be adequate for the formation of CF for a considerable period since under anaerobic conditions it would be protected from destructive oxidation. In the homogenates incubated under oxygen, the small peak observed after 30 minutes in the series without added ascorbate (Fig. 2) may represent the duration of action of the ascorbate occurring in the tissue. The duration of action of the added ascorbate was approximately 1 hour. Under aerobic conditions in the absence of ascorbate, either CF is not formed or that which is formed is destroyed rapidly. The occurrence of some metabolic alteration of PGA during the aerobic incubation is indicated by the more rapid formation of CF following the delayed addition of ascorbate (Fig. 4). The accumulation of a derivative of PGA, which in the presence of added ascorbate can be rapidly converted to CF, is suggested by these findings.

The observation that both natural and synthetic CF are susceptible to enzymatic destruction under aerobic conditions draws attention to the stability of this vitamin in metabolic studies. The experiments by Hill and Scott (16) on the enzymatic release of CF from conjugates require revaluation in terms of the action of ascorbic acid in preventing the destruction of CF. However, the rôle of ascorbic acid in the enzymatic formation of CF is not simply in preventing destruction of the product of the reaction since its presence increases the formation of CF under either aerobic or anaerobic conditions. The postulate that ascorbic acid is required for the metabolic formation of CF is supported also by the observations of May et al. (1-3) that the development of a megaloblastic anemia in experimental monkeys (which responded to treatment with folic acid or CF) was dependent upon the use of a scorbutigenic diet and by the observations of Welch et al. (6) that scorbutic patients (in marked contrast to normal individuals) were unable to form CF from test doses of PGA.

**SUMMARY**

Homogenates of chick liver which were incubated anaerobically in the presence of ascorbate were capable of converting up to 25 per cent of
added pteroylglutamic acid to *citrovorum* factor. Ascorbic acid not only suppressed the enzymatic destruction of natural and synthetic CF which occurred under aerobic conditions, but also facilitated the active formation of CF. Glucoascorbate and isoascorbate duplicated the action of ascorbate in promoting the formation of CF, whereas cysteine and glutathione were ineffective in this respect.

It is a pleasure to acknowledge the interest and advice of Dr. Arnold D. Welch in this investigation.

**BIBLIOGRAPHY**

THE EFFECT OF ASCORBIC ACID ON THE ENZYMATIC FORMATION OF THE CITROVORUM FACTOR
Charles A. Nichol

_J. Biol. Chem._ 1953, 204:469-475.

Access the most updated version of this article at [http://www.jbc.org/content/204/1/469.citation](http://www.jbc.org/content/204/1/469.citation)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/204/1/469.citation.full.html#ref-list-1](http://www.jbc.org/content/204/1/469.citation.full.html#ref-list-1)