A POLYGLUTAMATE FORM OF CITROVORUM FACTOR SYNTHESIZED BY *BACILLUS SUBTILIS*

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The discovery of the citrovorum factor (CF) in yeast and in liver (Sauberlich and Baumann, 1948) was accompanied by the observation that a portion of the material existed in a bound form, from which it was liberated by incubation with an enzyme system ("conjugase") found in hog kidney (Bird *et al.*, 1946). Bound forms of CF were found by Iwai *et al.* (1954) in higher plants, and these also contained an enzyme system capable of releasing free CF. By analogy with the bound form of pteroylglutamic acid obtained from yeast (Pfiffner *et al.*, 1946), that of CF has been considered to be a γ-linked heptaglutamate. Using a special bioautographic technique, Doctor and Couch (1953) studied the chromatographic behavior of the bound form of CF: the agar medium was inoculated with *Leuconostoc citrovorum* (now termed *Pediococcus cerevisiae*), together with a conjugase preparation from chick pancreas; in this manner, the presence of a bound form of CF (presumably a heptaglutamate) in commercial extracts of mammalian liver, in yeast and in autoclaved chicken liver, was confirmed.

In contrast to the heptaglutamate form of CF, those derivatives of CF which contain only one or two additional γ-linked units of glutamic acid are active for *L. citrovorum*; this was shown by Shive *et al.* (1950) who synthesized the diglutamate and triglutamate forms, as well as CF (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid). The chromatographic behavior and enzymatic hydrolysis of the diglutamate and triglutamate forms of CF have been studied by Bolinder *et al.* (1953).

Using bioautographic techniques, Winsten and Eigen (1950) demonstrated that a liver preparation contained, in addition to CF, two other materials active for *L. citrovorum*. That these were polyglutamate forms of CF was suggested by their disappearance after incubation with the rat stomach conjugase system while the amount of free CF increased. Not only animal tissues and yeast, but also seaweeds (Bánhidi and Ericson, 1953), lichen (Sjöström and Ericson, 1953), and marine algae (Ericson *et al.*, 1953) contain, in addition to free CF, unidentified compounds with activity for *L. citrovorum*; comparison of these with the synthetic triglutamate and diglutamate forms of CF suggested that in lichen a triglutamate and in marine algae a diglutamate form of CF were present (Ericson, 1953).

Simultaneously with our studies, referred to in an earlier note (Hakala and Welch, 1955), the observation that a triglutamate form of CF can be derived from *Streptococcus faecalis* was made independently in this laboratory by Zakrzewski and Nichol (1955). Also, several complex pteridine polyglutamates, some with activity for *L. citrovorum*, have been detected in *Clostridium cylindrosporum* by Wright (1955).

In this paper, evidence that *Bacillus subtilis* contains a γ-linked triglutamate form of CF (or a labile precursor of it) is presented. An enzymatic preparation of a similar compound from "Teropterin" (synthetic triglutamate form of pteroylglutamic acid) and comparisons of it with the substance present in *B. subtilis* are described.

MATERIALS AND METHODS

Pteroyl-L-glutamic acid (PGA), "diopterin" (γ-linked pteroyl-L-glutamyl-L-glutamic acid, PG_2A), "teropterin" (γ-linked pteroyl-L-glutamyl-L-glutamyl-L-glutamic acid, PG_3A), and synthetic CF ("leucovorin"), i. e., 5-formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, were obtained from the American Cyanamid Company, through the courtesy of T. H. Jukes (Lederle Laboratories Division) or J. M. Smith, Jr. (Calco Division).

The stock culture of *B. subtilis* was carried on potato agar slants, and the cell mass used as a source of the growth factor was grown in a liquid salts-glucose medium (pH 7.3) containing, per L, the following ingredients: glucose, 10 g; KH_2PO_4, 2 g; $(NH_4)H_2PO_4$, 3.5 g; NaCl, 4 g;

$MgSO_4$, 0.1 g; $CaCl_2$, 0.1 g; and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.1 g. Incubation for 3 to 4 days at 35 C gave, per L, 600 mg of cells (dry weight, about 150 mg); the cells were removed by centrifugation and washed twice with water. The supernatant medium and the cells were studied separately.

The growth factor for *L. citrovorum* was extracted from the cells of *B. subtilis* by three methods: (1) extract A was prepared by suspending the washed cells in phosphate buffer of pH 7.0 (0.05 M) and incubating for 4 hr at 37 C; (2) extract B was made by heating the cell suspension described above for 10 min at 120 C; and (3) extract C was prepared by sonic oscillation of the cell suspension described above using a magnetostriction oscillator (Model R-22-3, 50 watts, 9 k.c.) of the Raytheon Manufacturing Company. In each case, cell debris was separated by centrifugation.

Strips of Whatman No. 1 filter paper, 1 in wide, were used for descending chromatography with phosphate buffer (pH 7.0, 0.1 M) as the solvent. For bioautographic analysis these strips were divided lengthwise into two or three narrower strips and placed on solid media inoculated with either *Streptococcus faecalis* strain ATCC 8043, *Lactobacillus casei* strain ATCC 7469 or *Leuconostoc citrovorum* (*Pediococcus cerevisiae*) strain ATCC 8081. The solid media used were those of the AOAC, for strains 8043 and 7469 (Lepper, 1950), and that of the USP XIV, for strain 8081, except that each was supplemented with agar, 1.5 per cent. The same media, without agar, were used for "tube assays" and bacterial growth was followed by measuring turbidity in the Klett-Summerson photoelectric colorimeter (filter 66). Since the growth curves obtained with the conjugated forms of CF differed from those seen with free CF, the relative activities of the compounds were expressed in terms of the amounts required to produce half-maximal growth of the organisms. The reference standard, synthetic CF (leucovorin), a racemic mixture, was employed in amounts double those of the natural material, a circumstance taken into account in the calculations.

Partial purification of the factor formed from synthetic PG_3A by a cell-free extract of an A-methopterin-resistant strain of *S. faecalis* (*S. faecalis*/A) was attained by paper chromatography coupled with adsorption on and elution from charcoal (Darco G-60). Initially, the substance formed was separated on large sheets of filter paper (Whatman No. 3), using phosphate buffer (pH 7, 0.1 M) as the solvent; when dry, the sheets were divided horizontally into strips (1 cm wide). Each strip was eluted with water and an aliquot was rechromatographed on a fresh paper strip for bioautographic analysis in order to determine the position and the apparent degree of homogeneity of the growth factor present. The fractions which yielded only one zone of growth with the desired R_f value (0.90) were combined, the pH was adjusted to 4.5 with HCl, and charcoal (Darco G-60) was added. After agitation for about 5 min, the supernatant was removed by filtration; of the original activity for *L. citrovorum* only 2.2 per cent was not adsorbed. The charcoal was eluted for 5 min with a mixture containing equal amounts of ethanol (95 per cent) and ammonia water (7 per cent NH_3); 96.3 per cent of the original activity for *L. citrovorum* was recovered in the eluate. The entire procedure was repeated with the nearly salt-free eluate. Since the polyglutamates of CF were easily hydrolyzed by ammoniacal alcohol (see Results) the last step of the purification involved paper chromatography at pH 7.

RESULTS

Bioautograms obtained with extract A of *B. subtilis* (table 1) revealed a single zone (R_f 0.90), with activity for both *L. citrovorum* and *L. casei*, but without activity for *S. faecalis*; a material with the same properties was present in extracts B and C and also in the supernatant medium S. In addition, medium S contained other substances with activity for both *L. citrovorum* and *S. faecalis*; these appeared at R_f 0.71 (agreeing with that of free CF) and at R_f 0.83 (considered to be the form of CF containing two residues of glutamic acid). From extracts B and C only one zone of growth, R_f 0.90, for *L. citrovorum* was obtained. For *L. casei*, some samples gave, in addition, a zone of growth at R_f 0.72; this zone, which supported the growth of neither *S. faecalis* nor *L. citrovorum*, is believed to represent the triglutamate form of pteroylglutamic acid (PG_3A). Of the three test organisms used, only *L. casei* could be inhibited by the antibiotic (Salle and Jann, 1945) released by *B. subtilis* into the medium, but this sub-

TABLE 1

Microbial assays of Bacillus subtilis extracts before and after digestion with chicken pancreas conjugase

Bacillus subtilis Extract and the Test Organism	Original Extract			Extract After Conjugase Digestion		
	R_f value	Corresponding compound	Equivalent of dry cells required for ½ maximal growth	R_f value	Corresponding compound	Equivalent of dry cells required for ½ maximal growth
						μg
Extract A:						
Streptococcus faecalis	—		6.1 mg	0.82	Diglutamate form of CF*	
Lactobacillus casei	0.89	Triglutamate form of CF*		0.8	Diglutamate form of CF	
Leuconostoc citrovorum	0.90	Triglutamate form of CF	820 μg	0.81	Diglutamate form of CF	
Extract B:						
Streptococcus faecalis	—		3.1 mg	0.81	Diglutamate form of CF	69
Leuconostoc citrovorum	0.90	Triglutamate form of CF	91 μg	0.83	Diglutamate form of CF	73
Extract C:						
Streptococcus faecalis	—		7.0 mg	0.82	Diglutamate form of CF	154
Leuconostoc citrovorum	0.90	Triglutamate form of CF	546 μg	0.81	Diglutamate form of CF	377

* CF = Citrovorum factor.

stance caused no interference under our experimental conditions.

Enzymatically prepared triglutamate form of CF. It is of interest to compare the factor found in *B. subtilis* with the compound formed enzymatically from the synthetic triglutamate form of PGA (teropterin). Bioautographic examination of this material (table 2) demonstrated contamination with the diglutamate form and with PGA. Incubation of teropterin with resting cells of *S. faecalis* showed that these cells were able to transform free PGA to CF, as had been reported (Nichol, 1954), but that substances corresponding to the di- or tri-glutamate forms of CF were not found in the incubation mixture. Therefore, an incubation with a *cell-free* sonic extract of *S. faecalis* cells was carried out under the conditions described by Nichol (1954) for the conversion of PGA to CF. Thus, to the extract of 2.5 g (wet weight) of the bacteria in 50 ml, was added sodium formate (100 mg), sodium ascorbate (85 mg), glucose (180 mg), adenosine triphosphate (38 mg), diphosphopyridine nucleotide (66 mg) and Teropterin (5 mg); the mixture was incubated at 37 C for 3 hr. Table 2 indicates that CF and its triglutamate form were derived from teropterin in this system (bioautograms did not reveal the presence of the diglutamate form of CF). Incubations were made simultaneously with "folvite" (PGA) and diopterin (PG_2A), but no compound with the chromatographic and microbiologic characteristics of the factor from *B. subtilis* was found in these incubation mixtures.

From 5 mg of teropterin a total activity for *L. citrovorum* equivalent to 256 μg of CF was obtained. The mixture was purified by repeating three times the entire procedure described under Methods. The final product, the activity of which was equal to 80 μg of CF, was recovered and assayed microbially (table 3). These assays indicate that our preparation of the triglutamate form of CF has very little activity for *S. faecalis.*

Effect of hydrogen ion concentration. The total activity for *L. citrovorum* released from the cells of *B. subtilis* into extract A was least when the extraction was done in the pH range 6 to 8, but with moderate increases in acidity (pH 5) or

TABLE 2

Bioautograms of "teropterin" before and after incubation with cell-free extract of Streptococcus faecalis/A (Nichol, 1954)

Organism	"Teropterin"		"Teropterin" Incubation Mixture	
	R_f value	Corresponds to compound	R_f value	Corresponds to compound
Streptococcus faecalis strain 8043	0.31 0.54	PGA PG_2A	0.71	CF*
Lactobacillus casei strain 7469	0.33 0.55 0.71	PGA PG_2A PG_3A	0.71 0.89	CF Triglutamate form of CF
Leuconostoc citrovorum strain 8081	—		0.71 0.89	CF Triglutamate form of CF

* CF = Citrovorum factor.

TABLE 3

Microbial assays of the partially purified triglutamate form of citrovorum factor (isolated from "teropterin" incubation mixture) before and after digestion with chicken pancreas conjugase

Organism	Partially Purified Triglutamate Form of Citrovorum Factor		After Conjugase Digestion	
	R_f value	Relative amounts required for ½ maximal growth	R_f value	Relative amounts required for ½ maximal growth
Streptococcus faecalis strain 8043	—	35	0.83	0.74
Lactobacillus casei strain 7469	0.89	1.0	0.81	1.0
Leuconostoc citrovorum strain 8081	0.90	1.0	0.82	0.74

alkalinity (pH 9) the amount of activity obtained was augmented. However, after extraction at pH 10.2 two zones of growth for *L. citrovorum* appeared in the bioautograms; these occurred at R_f 0.90 and 0.83. The appearance of the zone at R_f 0.83 is attributable to partial hydrolysis of the triglutamate with the formation of the diglutamate form of CF; extraction at pH levels below 3 led to a rapid disappearance of all activity for *L. citrovorum*. When extract A was heated to 100 C in the presence of 0.5 N HCl the activity for *L. citrovorum* was completely destroyed within 15 min and a mixture of compounds with activity for *L. casei* was formed. At pH 12 and 100 C, new zones of growth for *L. citrovorum* and *L. casei* appeared in the extracts within 15 min; these corresponded not only to the triglutamate and diglutamate forms, but also to compounds of unknown composition.

Evaporation on the water bath of an eluate (ethanol-ammonia-water) derived from a charcoal adsorbate of the enzymatically treated PG_3A, which on the basis of bioautograms contained a single compound (triglutamate form of CF), caused a stepwise hydrolysis of the glutamic acid units, so that after evaporation the presence of three CF-like compounds was revealed. These appeared to be CF (R_f 0.71) and the diglutamate form (R_f 0.82), each with activity for *L. casei*, *S. faecalis*, and *L. citrovorum*, as well as the parent triglutamate (R_f 0.90), with activity for *L. casei* and *L. citrovorum*, but *not* for *S. faecalis*. Thus, exposure of the triglutamate form of CF (derived from PG_3A) to alkali resulted mainly in hydrolysis of the γ-glutamyl peptide bonds, while acid, as with free CF (Cosulich *et al.*, 1952), destroyed all activity for *L. citrovorum*; the effects on the polyglutamate obtained from *B. subtilis* were indistinguishable.

Digestion with conjugases. Incubation at pH 7 and 37 C for 18 hr with a lyophilized preparation of chicken pancreas conjugase[1] converted the material in extracts of *B. subtilis*, as well as the enzymatically prepared and partially purified triglutamate form of CF, to a compound with R_f 0.82, apparently the diglutamate form of CF. This finding is in agreement with that of Dabrowska *et al.* (1949), who demonstrated that the triglutamate form of PGA (PG_3A), when incubated with the highly purified conjugase of chicken pancreas, yields the diglutamate form, PG_2A, rather than PGA itself. Incubation of our two substrates with chick pancreas conjugase (the enzyme preparation contained essentially

[1] The conjugase preparation of chicken pancreas was kindly supplied to us several years ago by Doctor M. Laskowski of Marquette University, and had been stored at the temperature of a deep freezer.

no microbial activity) increased the activity of each for *S. faecalis* by about 50-fold and for *L. citrovorum* by only 25 to 45 per cent (tables 1 and 3); the initial high activity of each for *L. casei* was unchanged. These results with crude extracts of *B. subtilis*, and with the material prepared enzymatically from PG_3A, demonstrate the negligible activity of the triglutamate form of CF for *S. faecalis*, as compared with the high activity of the diglutamate form. When incubated at pH 4.5 and 37 C for 18 hr with a crude preparation of hog kidney conjugase (Bird *et al.*, 1946; Hill and Scott, 1952), an enzyme known to release free PGA from its conjugates (Pfiffner *et al.*, 1946), both the original triglutamate and the diglutamate form of CF were hydrolyzed to CF (R_f 0.71). An enzyme resembling hog kidney conjugase in its action apparently was present in extract C of *B. subtilis* since an unheated sample accumulated CF during storage, while the amount of the triglutamate form diminished.

Effect of the triglutamate form of CF on the inhibition by "aminopterin" of L. citrovorum. Based on CF units, the triglutamate form of CF present in extract B of *B. subtilis* was as effective as free CF in preventing inhibition of the growth of *L. citrovorum by aminopterin.*

DISCUSSION

The presence in *B. subtilis* of an oligo-glutamate form of CF containing at least three glutamic acid residues was suspected because cell extracts prepared in different ways supported the growth of *L. citrovorum* and *L. casei*, but had relatively little activity for *S. faecalis*. It is possible that the *L. casei* activity found in *B. subtilis* cultures by Burkholder *et al.* (1945) was caused by the same factor, although at that time it was considered to be vitamin B_c or its conjugate. Our hypothesis was based on the fact that the γ-linked triglutamate form of PGA, originally termed "fermentation folic acid," had been found to possess high activity for *L. casei*, but only about one-fiftieth that of PGA for *S. faecalis* (Hutchings *et al.*, 1946; Suarez *et al.*, 1946). On the other hand, Ericson (1953) regarded both diglutamate and triglutamate forms of CF as active for *S. faecalis*, whereas Wright (1955) found no activity for *S. faecalis* in any of the polyglutamyl pteridine compounds found in *Clostridium cylindrosporum.*

Evidence for the identity of the oligo-glutamate form of CF in *B. subtilis* with the triglutamate form was provided by the enzymatic formation of a compound with the same characteristics from the triglutamate form of PGA. Both compounds, although not isolated in pure form, had the same microbial and chromatographic properties and their behavior toward acid, alkali, and enzymatic digestion was identical. Although *S. faecalis* cells are very inefficient in their use of the triglutamate form of either CF or PGA for growth, and the intact cells fail to convert PG_3A to the corresponding form of CF, an enzyme system capable of performing this conversion is present within the cells. Thus, these intact cells have a selective capacity for utilizing PGA (and presumably the diglutamate form), but not the triglutamate forms; according to Hutchings *et al.* (1947) PGA and PG_2A are equally active, on a molar basis, in promoting the growth of *S. faecalis*. Yet the triglutamate form of CF is normally present within these cells (Zakrzewski and Nichol, 1955). Under the conditions tested, cell-free extracts of *S. faecalis* did not lengthen the peptide chain of monoglutamate or diglutamate forms of PGA.

Enzyme systems capable of splitting the γ-linkage of the polyglutamate forms seem to be quite common; they occur to a varying extent in the tissues of many species: avian, mammalian, and in higher plants (Bird *et al.*, 1945, 1946; Laskowski *et al.*, 1945; Iwai *et al.*, 1954); their presence in bacteria is indicated by these studies of cell-free extracts of *B. subtilis*. In general it appears that not only CF but its oligo-glutamate and polyglutamate forms also are widely distributed, being found not only in animal tissues, higher and lower plants, but also in bacteria. Evidence for the presence of a higher conjugate of CF in *B. subtilis* was not found; neither the bioautographic technique using chicken pancreas preparation in the medium (Doctor and Couch, 1953) nor prolonged digestion with chicken pancreas conjugase caused an increase in activity for *L. casei*. That this treatment caused a slight (25 to 45 per cent) increase in activity for *L. citrovorum* is interpreted possibly to indicate more efficient use of the diglutamate form of CF by *L. citrovorum.*

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SUMMARY

Evidence is presented to indicate that *Bacillus subtilis* synthesizes a form of citrovorum factor (CF) which contains three units of glutamic acid per molecule. The CF derivative prepared enzymatically from synthetic γ-linked pteroyl-L-glutamyl-L-glutamic acid with the aid of cell-free extracts of *Streptococcus faecalis* strain A and the CF derivative obtained from *B. subtilis* had identical microbial and chromatographic properties; in addition, their patterns of degradation with acid, alkali, and enzymes did not differ.

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