OCCURRENCE AND PROPERTIES OF A CONJUGATED FORM OF LEUCONOSTOC CITROVORUM FACTOR*

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Sauberlich and Baumann discovered the *Leuconostoc citrovorum* factor (CF) in 1948 (1). Since that time several workers (2–9) have investigated the biological properties of CF and determined its relationship to pteroylglutamic acid (PGA) and its enzymatic release from natural materials. The CF content of Reticulogen is increased 5 per cent on incubation with hog kidney enzyme (1). A 2- to 3-fold increase in CF resulted when samples of beef liver and beef round were incubated with pancreatin for 48 hours (10). A 12-fold increase in the CF content of a brewers' yeast has been obtained on incubation with hog kidney enzyme (11). Chick liver contains a CF-liberating enzyme which is activated by ascorbic acid, according to reports of Hill and Scott (12, 13). Winsten and Eigen (14) used bioautographic techniques and were able to separate four factors which are active for *L. citrovorum* 8081.

The present report is concerned with the separation and occurrence of a conjugated form of CF in yeast extract (Difco), liver fraction L (Wilson), and fresh autoclaved chick liver.

EXPERIMENTAL

Preparation of Hog Kidney and Chick Pancreas Enzymes—Fresh hog kidney was homogenized with 3 parts of distilled water, centrifuged, and the centrifugate filtered through Super-Cel (15). The clarified extract was stored in the frozen state. The chick pancreas enzyme was purified by the method of Laskowski *et al.* (16, 17). 100 gm. of fresh chicken pancreas were homogenized in a Waring blendor with 2 volumes of 0.1 M phosphate buffer, pH 7. After autolysis for 24 hours at 37° under toluene, the autolysate was centrifuged and the middle brownish layer separated from the settled residue and upper fat layer. This brownish liquid was treated with an equal volume of 0.1 M tricalcium phosphate suspension. The adsorp-

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tion complex was separated from the supernatant by centrifugation and discarded. The centrifugate was chilled to 5°, mixed with an equal amount of ice-cold absolute ethanol, and maintained at 5° for about an hour, centrifuged, and the supernatant solution discarded. The precipitate was suspended in 100 ml. of 0.1 M phosphate buffer, pH 7, and thoroughly mixed. After centrifugation, the supernatant solution was collected and stored frozen in tubes. The chick pancreas enzyme prepared in this manner did not contain any measurable CF activity while the hog kidney enzyme contained activity equivalent to 5 to 10 mμgm. of CF ($C_{20}H_{23}N_7O_7$) per ml.

Paper Strip Chromatography with Wet Symmetrical Collidine—Descending chromatography of Winsten and Eigen (14, 18) was used to separate growth factors for *L. citrovorum* 8081 found in yeast extract (Difco), liver fraction L (Wilson), liver concentrate paste (Wilson, 1:20), and liver extract (Wilson). 10 to 15 μl. of the materials to be tested were spotted individually on a $\frac{1}{2} \times 16$ inch paper strip (Eaton-Dikeman No. 613). Wet symmetrical collidine was run over the strips for 40 hours. The strips were air-dried and cut lengthwise into two $\frac{1}{4} \times 16$ inch strips. One of these was bioautographed on nutrient agar containing purified chick pancreas enzyme and seeded with *L. citrovorum* 8081. The remaining strip was bioautographed on nutrient agar seeded with *L. citrovorum* 8081, but not containing chick pancreas enzyme. The nutrient agar was made up by using the single strength basal Medium VI of Steele *et al.* (19) with acid-hydrolyzed casein as the source of part of the amino acids and with 2 per cent Bacto-agar added.

Release of CF by Chick Pancreas and Hog Kidney Enzymes—CF was released from four samples of yeast, yeast extract, and liver preparations, and from autoclaved chick liver by digestion with chick pancreas and hog kidney enzymes, according to the procedure of Sreenivasan *et al.* (20). The CF content was determined by using the single strength basal Medium VI of Steele *et al.* (19) with acid-hydrolyzed casein to supply a part of the amino acids. *L. citrovorum* 8081 was used as the test organism and the crystalline calcium salt of CF (Leucovorin, Lederle) was employed as the standard. All the data are expressed as the anhydrous-free acid ($C_{20}H_{23}N_7O_7$). The cultures were incubated for 16 hours at 37°. Growth was measured by turbidity.

Aliquots of a 10 per cent solution of yeast extract were treated as follows: (*a*) no treatment, (*b*) digestion with chick pancreas enzyme (20), (*c*) digestion with hog kidney enzyme (20), (*d*) digestion with chick pancreas enzyme, followed by digestion with hog kidney enzyme (20). Enzyme hydrolysates were concentrated *in vacuo* to a volume equal to the original 10 per cent extract (100 mg. of yeast extract per ml.). 15 μl. of samples

a, *b*, *c*, and *d* and 10 μl. of Leucovorin solution (250 mμgm. per ml.) were chromatographed according to the descending technique (14) with wet collidine as the developing agent. Paper strips were dried, cut lengthwise, and bioautographed as described above.

Paper Strip Chromatography with Water-Miscible Solvent System—The conjugated form of CF was separated by using a water-miscible solvent system composed of 50 per cent ethanol, 15 per cent *n*-butanol, 10 per cent ammonia, and 25 per cent water. This system was developed after several water-immiscible systems had failed to move the conjugated form of CF. These included the butanol-acetic acid system of Patridge (21). A chick liver preparation was made in the following manner: 50 gm. of fresh chilled liver were homogenized in a Waring blendor with 5 volumes of water. The homogenate was autoclaved for 15 minutes at 15 pounds pressure, cooled, and filtered. The filtrate was concentrated *in vacuo* to 25 ml. 15 μl. of a 10 per cent solution of yeast extract, a 10 per cent solution of liver fraction L, and of a chick liver solution prepared as described above were each spotted on a $\frac{1}{2} \times 13$ inch paper strip (Eaton-Dikeman No. 613) and the strips developed by ascending technique for 48 hours. After air drying, the strips were cut lengthwise into two $\frac{1}{4}$ inch strips. One of these was bioautographed on nutrient agar containing purified chick pancreas enzyme and seeded with *L. citrovorum* 8081. The remaining strip was bioautographed on nutrient agar seeded with *L. citrovorum* 8081 but not containing chick pancreas enzyme.

Methods for Studying Properties of Growth Factors Active for L. citrovorum 8081—10 per cent solutions of yeast extract and of liver fraction L were used as the starting materials in all of the experiments described below.

Yeast extract and liver fraction L were each stored in 0.5 N H_2SO_4 and 0.5 N NaOH at room temperature for 24 hours and were also autoclaved in the same strength of acid and base for 15 minutes at 15 pounds pressure. Each of these solutions was neutralized to pH 6.8. Growth factors for *L. citrovorum* 8081 were separated by means of ascending paper strip chromatography with the water-miscible solvent system as the developing agent. The presence of the CF conjugate was detected by bioautographing half of each strip (cut lengthwise) on nutrient agar containing purified chick pancreas enzyme and seeded with *L. citrovorum* 8081.

Yeast extract and liver fraction L were each extracted sixteen times with equal volumes of *n*-butanol at pH 3 and 9. The *n*-butanol extracts and aqueous extracts were each combined and concentrated *in vacuo* to a volume corresponding to 100 mg. per ml. of the starting material. The distribution of the growth factors was determined as described above.

Solutions of yeast extract and liver fraction L were treated with Norit A at pH 3. The charcoal adsorbate was removed by filtration and was

eluted twice with hot 5 per cent ammonia in 50 per cent ethanol. The filtrate and the combined eluates were each concentrated *in vacuo* to a volume equivalent to 100 mg. per ml. of the starting material and the distribution of the growth factors was determined as described above.

Solutions of yeast extract and liver fraction L in 0.08 M phosphate buffer at pH 5 were each dialyzed against the same buffer. The distribution of the growth factors in the dialysate and the protein fraction was studied as described above.

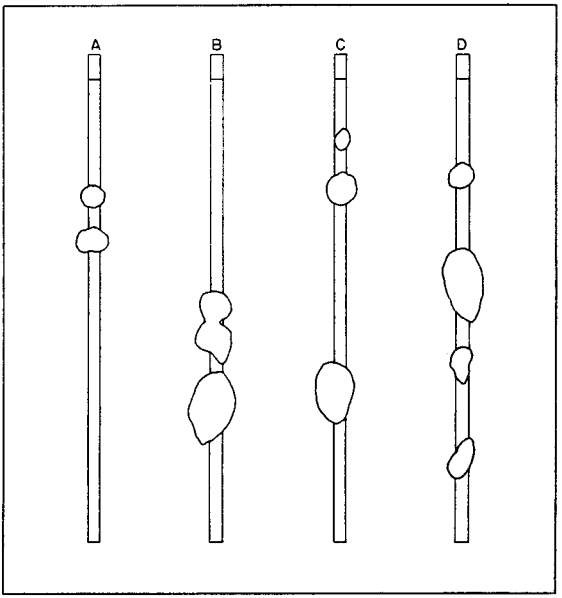

FIG. 1. Tracings of bioautographs. Test organism, *L. citrovorum* 8081. Strip A, 15 μl. of 10 per cent solution of yeast extract (Difco); Strip B, 15 μl. of 10 per cent solution of liver fraction L (Wilson); Strip C, 10 μl. of 10 per cent solution of liver concentrate paste (Wilson, 1:20); and Strip D, 10 μl. of liver injection 2, U. S. P., crude (Wilson). The strips were bioautographed on a plate containing 300 ml. of nutrient agar.

RESULTS AND DISCUSSION

It is apparent from Figs. 1 and 2 that a growth factor for *L. citrovorum* 8081 has been liberated from Strips A and B (yeast extract and liver fraction L respectively) by incubation of the chromatographed strips on nutrient agar containing purified chick pancreas enzyme. The compound from which this growth factor is liberated will be hereby designated as a conjugate of CF. It is not present in liver paste or liver extract, as shown by the absence of it on Strips C and D (Fig. 2), which were also incubated

on the nutrient agar containing purified chick pancreas enzyme. Other growth factors illustrated in Figs. 1 and 2 are active for *L. citrovorum* 8081, regardless of the presence of chick pancreas enzyme in nutrient agar.

CF occurs in bound forms which are released by treatment with purified chick pancreas enzyme or with hog kidney enzyme (Table I). CF activity of four yeast samples, yeast extract, liver fraction L, liver paste, and fresh autoclaved chick liver, was increased in all instances by incubation with

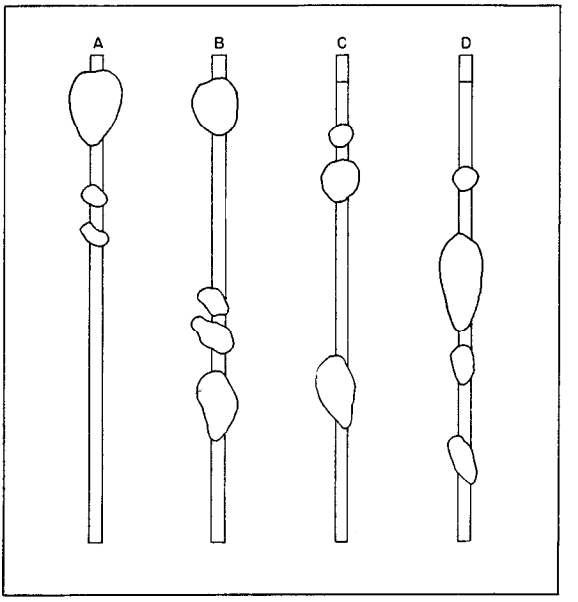

FIG. 2. Tracings of bioautographs. Test organism, *L. citrovorum* 8081. Strips A, B, C, and D represent the same samples as in Fig. 1, but were bioautographed on a plate containing 300 ml. of nutrient agar mixed with 10 ml. of chick pancreas enzyme (see the text for the method of treatment).

purified chick pancreas enzyme or with hog kidney enzyme. The hog kidney enzyme was somewhat more effective in releasing CF from samples used in this study than was chick pancreas enzyme. These findings are in accord with earlier reports.

In Fig. 3 are shown the tracings of bioautographs of yeast extract before (Strip A) and after various enzyme treatments (Strips B, C, and D). Strip E represents Leucovorin (Lederle). These strips were bioautographed on nutrient agar containing purified chick pancreas enzyme. The other half of these strips, when bioautographed, gave the same picture as Fig. 3, except for the absence of the stationary conjugated CF spot present on Strip A. Incubation of yeast extract with chick pancreas enzyme con-

verted the three growth factors shown on Strip A into a single compound (Fig. 3, Strip B). A different growth factor appeared on incubation of yeast extract with hog kidney enzyme (Strip C). Hog kidney enzyme can convert the compound liberated by incubation of yeast extract with chick pancreas enzyme into the growth factor which appears on direct incubation of yeast extract with hog kidney enzyme (Fig. 3, Strip D). The compound which results on incubation of yeast extract with hog kidney enzyme, or with chick pancreas enzyme followed by hog kidney enzyme, moves on paper strips at the same relative rate as the crystalline Leucovorin (Lederle). From the data of the present investigations it appears that

TABLE I

Release of CF from Yeasts and Liver Samples by Chick Pancreas and Hog Kidney Enzymes

Sample*	No enzyme treatment†	With chick pancreas enzyme	With hog kidney enzyme
	γ per gm.	*γ per gm.*	*γ per gm.*
Torulopsis utilis (sulfite waste liquor)	1.0	5.4	7.6
Anheuser-Busch, primary grown	0.3	20.6	25.6
Fleischmann, active dry	0.5	9.4	11.9
Red Star, active dry	0.2	11.0	12.5
Yeast extract (Difco)	0.7	39.0	59.7
Liver fraction L (Wilson)	6.1	8.1	15.0
Liver concentrate paste (Wilson, 1:20)	4.7	6.2	9.3
Chick liver	0.0	0.3	1.8

* The values of CF are based on dry weight of the material.

† The hot water extract of the samples was assayed directly. In case of chick liver, the amount of CF in this column was 25 mμgm. per gm.

CF, like PGA, occurs in natural materials in various forms having a different number of glutamic acid moieties joined by γ linkages to the same basic unit. If such is the case, the differences between the action of chick pancreas enzyme and hog kidney enzyme noted in Fig. 3 might be explained on the basis of earlier observations (22, 23) in which chick pancreas conjugase was designated as γ-glutamic acid carboxypeptidase and hog kidney conjugase as simply a carboxypeptidase.

The stationary conjugated form of CF (Figs. 2 and 3) was moved by developing the strips with a water-miscible solvent system described in this paper. A growth factor active for *L. citrovorum* 8081 is liberated from this compound on incubation of chromatographed Strips A (yeast extract), C (liver fraction L), and E (chick liver) in Fig. 4 on nutrient agar containing purified chick pancreas enzyme. This growth factor did not appear when the remaining halves of these strips were incubated on nutrient agar not

containing the purified chick pancreas enzyme (Fig. 4, Strips B, D, and F). The conjugated form of CF present in yeast extract, liver fraction L, and chick liver moved on the paper strips at the same relative rate and therefore may be identical or else very closely related. These data offer additional evidence for the existence of a CF conjugate since a solvent system

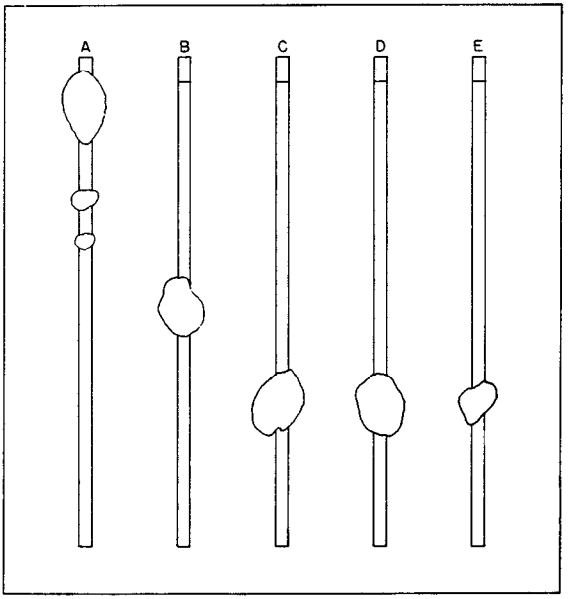

FIG. 3. Tracings of bioautographs. Test organism, *L. citrovorum* 8081. Strip A, 15 μl. of a 10 per cent solution of yeast extract (Difco); Strip B, the same concentration as Strip A, but the yeast extract was pretreated with chick pancreas enzyme; Strip C, the same as Strip A after incubation with hog kidney enzyme; Strip D, the same as Strip A after incubation with chick pancreas enzyme followed by kidney enzyme; Strip E, 10 μl. of solution containing 2.5 mμgm. of CF standard (Leucovorin, Lederle). Strips were bioautographed on a plate containing 300 ml. of nutrient agar mixed with 10 ml. of chick pancreas enzyme.

was developed which moved this compound obtained from three different crude sources at the same relative rate.

The CF conjugate present in yeast extract and in liver fraction L exhibits similar properties with regard to stability to heat, acid, and alkali, solubility in *n*-butanol at acid or alkaline pH, adsorption on Norit A, and behavior on dialysis. CF conjugate present in yeast extract or liver fraction L was found to be stable to autoclaving for 15 minutes at 15 pounds pressure in 0.5 N NaOH, but was destroyed by a similar treatment in 0.5 N H_2SO_4. Storage at room temperature for 24 hours in 0.5 N NaOH or 0.5

N H_2SO_4 gave the same results. Acid lability of the CF conjugate indicates that the CF part of the conjugate may be converted to a PGA-like compound by such a treatment. The CF conjugate was found to be insoluble in *n*-butanol at pH 3 or at pH 9, while the free forms of CF migrated to *n*-butanol layer at pH 3 but not at pH 9. The insolubility of CF conjugate in water-immiscible organic solvents suggests a probable peptide structure. The CF conjugate and the free forms of CF are adsorbed on Norit A at

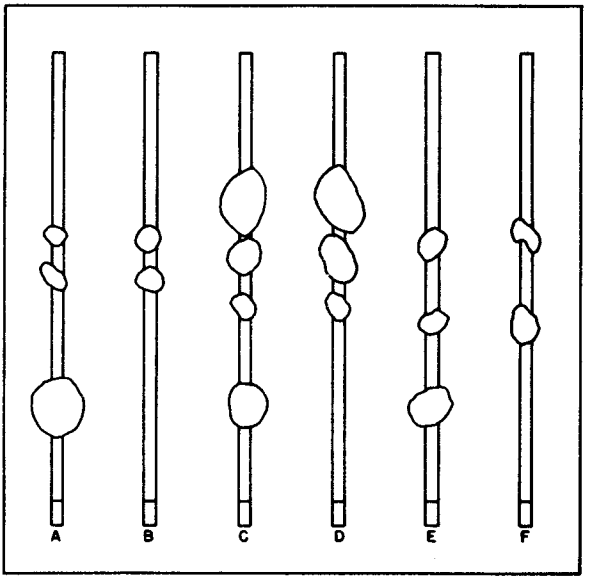

FIG. 4. Tracings of bioautographs. Test organism, *L. citrovorum* 8081. Strips A, C, and E were bioautographed on a plate containing 300 ml. of nutrient agar mixed with 10 ml. of chick pancreas enzyme, while Strips B, D, and F were incubated on a plate containing 300 ml. of nutrient agar alone. Strips A and B, 15 μl. of a 10 per cent solution of yeast extract (Difco); Strips C and D, 15 μl. of a 10 per cent solution of liver fraction L (Wilson); Strips E and F, 15 μl. of the chick liver preparation.

pH 3, as shown by the absence of these factors in the unadsorbed fraction. When bioautographed, the eluate was found to contain the CF conjugate and the free forms of CF. The charcoal adsorption and elution properties may be accounted for by the presence of a basic amino group on the CF part of the structure (9). Although CF conjugate dialyzes considerably more slowly than the free forms of CF, when dialyzed over longer periods it was completely dialyzable.

It is interesting to note that yeast extract, liver fraction L, and chick liver all three contain the conjugated form of CF, indicating a possible common rôle played by CF in plant and animal cells.

SUMMARY

Occurrence of a closely related or identical form of CF conjugate has been noted in yeast extract, liver fraction L, and chick liver. There is a large increase in the CF content of yeasts and liver samples tested upon treatment with purified chick pancreas enzyme or hog kidney enzyme. Bioautographic analysis of the enzyme-digested yeast extract reveals striking differences between the action of chick pancreas enzyme and hog kidney enzyme. Preliminary observations on properties of the CF conjugate indicate a probable similarity to PGA conjugate.

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