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Occurrence of *Crithidia* Factors and Folic Acid in Various Bacteria

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Crithidia factors and folic acid were found to be widely distributed in culture fluids and in cells of 27 species of bacteria, when cultured under aerobic conditions into the stationary phase. Most bacteria excreted more *Crithidia* factors and folic acid than they retained in their cells. One *Crithidia* factor produced by *Serratia indica* and one produced by *Bacillus cereus* differed from biopterin in their chromatographic behavior. The factor excreted by *S. indica* appeared to be a 2-amino-4-hydroxy-6-substituted pteridine on the basis of $KMnO_4$ oxidation and ultraviolet absorption spectra. One of the folate compounds excreted by this organism was shown to be identical to 5,10-methylidynetetrahydrofolic acid by bioautography.

2-Amino-4-hydroxy-6-substituted pteridines, such as biopterin, are termed "*Crithidia* factors." These factors promote the growth of the trypanosomid flagellate *C. fasciculata* when grown on a chemically defined medium limiting in folate (5). *Crithidia* factors have been found in human urine (11), royal jelly (2), *Drosophila* eyes (3), frog skin (4), yeast, and liver (8). During a survey of natural materials in this laboratory, the factors were found to be widely distributed in vegetables, beans, cereals, livers (bovine and chicken), dry yeast, and royal jelly. The factor in royal jelly and in livers was identical with biopterin, but that in vegetables was different in chromatographic behavior. Although Wacker et al. (15) found three *Crithidia* factors in cell-free extracts of spinach, grass, and several microorganisms which differed from biopterin in chromatographic behavior, the details of their structures have not been proposed. The present investigation deals with the distribution of *Crithidia* factors and folic acid in the culture fluids and cells of 27 species of bacteria. It also describes some properties of the factors produced by *Serratia indica* and *Bacillus cereus*.

MATERIALS AND METHODS

Organisms and media. All bacteria listed in Table 1 were obtained from the IFO culture collection (Institute for Fermentation, Osaka, Japan). The strain of *C. fasciculata* ATCC 12857 was kindly supplied by S. H. Hutner, Haskins Laboratories, New York, N.Y. Stock cultures of the 27 species were cultivated on agar slants prepared as follows. Sliced potatoes (20 g) and 3 g of commercial bakers' yeast were boiled for 30 min with 100 ml of water, and squeezed through a cotton cloth. To this filtrate, 0.5 g of commercial beef extract, 1.5 g of glycerol, 0.5 g of glucose, 2 g of agar, and 1 g of T. G. C. medium (Daigo-Eiyo Co., Osaka; containing 0.5 g of L-cystine, 2.5 g of NaCl, 5.5 g of glucose, 5 g of yeast extract, 15 g of peptone, 0.5 g of sodium thioglycolate, 1 ml of 0.1% resazurin, and 0.75 g of agar per 30 g of powder) were added and heated to dissolve completely. After the medium had cooled, the *p*H was adjusted to 7.4 with 10% NaOH, and it was autoclaved for 10 min. The final *p*H of the medium was 7.0 to 7.1. Bacteria were cultured in Massen's medium enriched with Casamino Acids (containing 40 g of sucrose, 10 g of asparagine, 2 g of $Na_2HPO_4 \cdot 12H_2O$, 0.7 g of DL-malic acid, 2.5 g of Na_2CO_3, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCO_3$, and 5 g of Casamino Acids, per liter). These cultures were supplemented, when necessary, with the following vitamin solution: 1 μg each of thiamine-HCl, riboflavine, calcium pantothenate, nicotinic acid, and *p*-aminobenzoic acid, 0.005 μg of biotin, 2 μg of pyridoxal-HCl, 0.2 ng of cyanocobalamin, and 1 mg of ascorbic acid per ml. Since Casamino Acids (Difco, vitamin-free) was found to be contaminated with *Crithidia* factor, a filtrate after treatment with 2% Norit A (w/v; American Norit Co.) at *p*H 3.5 was used as the source of amino acids in the experiments. The solution of treated Casamino Acids and vitamins, previously sterilized by autoclaving, was added aseptically to filter-sterilized Massen's medium. To investigate the properties of the factor, *S. indica* was cultured on a synthetic medium containing 4% sucrose, 1% $(NH_4)_2SO_4$, 0.2% $Na_2HPO_4 \cdot 12H_2O$, 0.25% Na_2CO_3, 0.04% $MgSO_4 \cdot 7H_2O$, and 0.001% $CaCO_3$. In this medium, this organism reached full growth and produced the factor as well as it did in Massen's medium enriched with 0.5% Casamino Acids. *B. cereus* was cultured on Massen's medium enriched with Casamino Acids as described above. The stock culture of *C. fasciculata* contained 0.5% polypeptone, 0.5% yeast extract, 1.5% sucrose, 0.01%

liver fraction "L" (Nutritional Biochemicals Corp., Cleveland, Ohio), and 2.5 mg of hemin per 100 ml (Sigma Chemical Co.; reference 5). For *C. fasciculata*, the medium of Guttman et al. (5), was used with the following modification: Hycase SF was replaced by 1% Casamino Acids, 0.01% L-phenylalanine, and 0.01% L-tyrosine, and Tween 80 was added to the medium at a final concentration of 0.3%. With this modification, the medium was quite transparent, even after heat sterilization, and incubation time was shortened to 3 days. *Streptococcus faecalis* R ATCC 8043, used in tetrazolium bioautography to determine the form of folic acid, and *Lactobacillus casei* ATCC 7469, used in the folic acid assay, were grown in a uniform medium described previously (7).

Culture conditions. Bacteria were cultured aerobically on a rotary shaker at 28 to 29 C in the dark, almost to the stationary phase (in most cases, for 4 to 5 days, but for *Pseudomonas* sp. and *Serratia* sp., for 2 to 3 days).

Assay for the Crithidia factor and folic acid in cultured microorganisms. Cell extracts were prepared as follows. The cells were harvested by centrifugation and dried at 70 to 80 C for 4 to 5 hr. Dried cells were ruptured with sea sand, and then were suspended in 0.1 M acetate buffer (*p*H 4.5, containing 0.05% ascorbic acid). This suspension was boiled for 15 min and then filtered. The filtrate was assayed for the *Crithidia* factor by use of *C. fasciculata* and for folic acid by use of *L. casei*. In the microbiological assay for the *Crithidia* factor and folic acid, Coleman colorimeter cuvettes (19 by 105 mm, round) were used for the cultures. A 2-day-old culture of *C. fasciculata* was inoculated and incubated in a slanted position at 25 C for 3 days in the dark. Culture conditions for the folic acid assay were described previously (7). Culture fluids were microbiologically assayed immediately after appropriate dilutions. Growth of *C. fasciculata* and *L. casei* was monitored by measuring their turbidities at 675 nm with a Coleman Universal spectrophotometer.

Paper chromatography of culture fluids for S. indica and B. cereus. After removal of cells by centrifugation, the factor in the culture fluids (500 ml) of *S. indica* or *B. cereus* was adsorbed on Norit A (2.5 g), and eluted with ethyl alcohol-5% NH_4OH (1:1, v/v). The eluate was evaporated to dryness in vacuo below 40 C. The residue was spotted on Whatman no. 1 chromatography paper, and was chromatographed in the ascending direction in *n*-propanol-1% NH_4OH (2:1, v/v), and in *n*-butyl alcohol-acetic acid-water (4:1:1, v/v). To assay activity of the factor on the paper chromatograms, strips were cut at 5-mm intervals along the chromatograms. Each segment was added directly to the assay medium before sterilization.

Purification and characterization of the factor in the culture fluid of S. indica. The Norit eluate described above was purified chromatographically by use of Toyo no. 2 filter paper (Toyo-Roshi Co., Tokyo, Japan), and by successive development with the two solvent systems described above. The blue fluorescent area on the final chromatogram, which had *Crithidia* factor activity, was cut out and eluted with water. This eluate was concentrated in vacuo. The concentrate was submitted to oxidation with alkaline $KMnO_4$ (13), and its absorption spectrum was measured with a Hitachi model 139 spectrophotometer (Hitachi Co., Tokyo, Japan).

Tetrazolium bioautography. The methods for bioautography and characterization of folic acid with *S. faecalis* have been described (6). Authentic 5,10-methylidynetetrahydrofolic acid, synthesized by hydrogenation of 10-formylfolic acid, was kindly supplied from the laboratory of Eisai Co., Tokyo, Japan.

Purification of biopterin. L-*erythro*-2-Amino-4-hydroxy-6-dihydroxy-propyl pteridine (biopterin, 60% purity) was purchased from K & K Laboratories (Jamaica, N.Y.). This preparation was further purified chromatographically by use of a ChroMax column (58 by 360 mm, 0.5 kg/cm²; developer, *n*-propanol-1% NH_4OH, 2:1, v/v; LKB-Produkter AB, Stockholm, Sweden; reference 9) and a phosphocellulose column (3 by 33 cm; developer, water; Brown Co.; reference 14). This purified biopterin was used as the standard in the assay for the *Crithidia* factor (12).

RESULTS

Distribution of Crithidia factor in various bacteria. The *Crithidia* factor is widely distributed in culture fluids and cells of various bacteria (Table 1). Of these, *B. cereus*, *Escherichia coli*, *Pseudomonas aureofaciens*, *S. indica*, and *S. marcescens* excreted more of the factor into the medium (biopterin equivalent: 50 to 120 ng/ml) than did the other cultures (0.1 to 10 ng/ml). On the other hand, cell extracts of *Aerobacter aerogenes*, *E. coli*, and *P. aureofaciens* contained more of the factor (30 to 70 ng/mg of dry cells) than did extracts of the other bacteria (1 to 20 ng/mg). Most organisms, except *B. roseus* and *Micrococcus ureae*, excreted more of the factor into the medium than they retained in their cells. *S. indica*, particularly, excreted 90 times more factor than was retained in its cells.

Distribution of folic acid in various bacteria. Table 1 shows that *B. cereus*, *P. aeruginosa*, *P. aureofaciens*, and *Aeromonas hydrophila* excreted more folate into the medium (50 to 160 ng/ml) than did the other organisms (1 to 20 ng/ml). Cell extracts of *P. convexa*, *P. aureofaciens*, *P. putrefaciens*, *S. indica*, and *Flavobacterium aquatile* contained more folate (40 to 120 ng/mg) than did extracts of other bacteria (1 to 10 ng/mg). Most organisms, except *F. aquatile*, excreted more folate into the media than they retained in their cells. *A. hydrophila*, *B. cereus*, *Gluconobacter melanogenus*, and *S. marcescens* excreted 100-fold more folate and *P. aeruginosa* excreted 1,000-fold more folate than was retained.

Comparison of the Crithidia factor produced by S. indica and B. cereus with biopterin. As shown

TABLE 1. *Amounts of Crithidia factor and folic acid produced by various bacteria*

Bacterium[a]	Culture fluids		Cells			
	Crithidia factor[b]	Folic acid[c]	*Crithidia* factor[b]		Folic acid[c]	
			Amt/mg (dry wt)	Amt/ml[d]	Amt/mg (dry wt)	Amt/ml[d]
	ng/ml	*ng/ml*	*ng*	*ng*	*ng*	*ng*
Acetobacter xylinum[e] IFO 3144	1.6	—	10.6	5.3	0.8	0.4
Achromobacter liquidum IFO 3084	1.2	3.3	11.9	0.18	18.1	0.27
Aerobacter aerogenes IFO 3317	1.2	2.5	53.8	1.6	2.5	0.07
Aeromonas hydrophila IFO 3820	12.8	47.4	7.2	2.2	1.9	0.6
Bacillus alvei[e] IFO 3343	8.2	4.3	1.4	2.9	—	—
B. cereus IFO 3131	92.5	162.4	22.9	30.7	0.9	1.2
B. coagulans[e] IFO 3886	8.2	7.8	5.2	10.4	—	—
B. roseus IFO 3041	0.1	0.6	4.5	0.9	0.8	0.16
B. sphaericus IFO 3525	2.5	0.9	2.7	0.18	0.3	0.02
Brevibacterium acetylicum IFO 12146	7.2	1.2	0.9	0.06	0.5	0.03
Corynebacterium equi IFO 3730	1.7	37.2	3.6	3.6	16.3	16.3
Erwinia carotovora IFO 3057	5.6	26.4	2.3	0.37	1.0	0.16
Escherichia coli IFO 3806	55.0	14.6	72.0	1.7	—	—
Flavobacterium aquatile IFO 3772	2.0	0.6	3.5	0.7	38.1	7.6
Gluconobacter melanogenus[e] IFO 3293	1.1	32.0	1.7	0.5	0.8	0.2
G. suboxydans[e] IFO 3130	0.5	14.3	1.8	0.3	—	—
Micrococcus flavus[e] IFO 3242	17.0	13.8	2.9	9.0	0.8	2.4
M. luteus IFO 3763	0.1	0.4	12.0	0.24	10.0	0.2
M. lysodeikticus IFO 3333	0.8	5.5	1.9	0.95	10.4	5.2
M. ureae IFO 3767	0.1	36.2	4.4	2.2	9.8	4.9
Pseudomonas aeruginosa IFO 3898	24.0	136.0	—	—	1.1	0.07
P. aureofaciens IFO 3521	47.0	57.7	29.8	7.4	44.5	11.0
P. convexa IFO 3773	9.2	14.7	10.5	1.4	115.0	15.4
P. fluorescens IFO 3081	10.2	23.6	12.8	4.3	13.0	4.4
P. putrefaciens IFO 3908	9.6	21.9	6.1	0.4	50.0	3.4
Serratia indica IFO 3759	120.5	17.4	12.0	1.4	55.8	6.8
S. marcescens IFO 3046	47.8	22.8	16.4	1.3	4.3	0.3

[a] Cultured in Massen's medium enriched with 0.5% Casamino Acids.
[b] Biopterin equivalent.
[c] Assayed with *L. casei*.
[d] Amount of *Crithidia* factor or folic acid in cells obtained from each 1 ml of the culture fluid.
[e] Cultured in Massen's medium enriched with 0.5% Casamino Acids and vitamins as described in the text.

in Table 1, *S. indica* and *B. cereus* excreted much more of the factor than did other bacteria. It was of interest to investigate whether or not the *Crithidia* factor produced by *S. indica* or *B. cereus* was identical with biopterin. The *Crithidia* factor in the culture was concentrated by Norit treatment and submitted to chromatography and histography. The major blue fluorescent spot of the concentrate of the culture fluid of *S. indica* had strong *Crithidia* factor activity, with R_F values different from those of biopterin (Fig. 1). The concentrate of the culture fluid of *B. cereus* contained two strong blue fluorescent spots having *Crithidia* factor activity (Fig. 2B): one, showing the major *Crithidia* factor activity, was different in R_F value from biopterin; the other, showing minor *Crithidia* factor activity, was similar to that of biopterin, in that it was not separated from biopterin by solvent A (Fig. 2A).

Some properties of the Crithidia factor and of folate produced by S. indica. The ultraviolet absorption of the alkaline $KMnO_4$ oxidation product of the factor was identical with that of authentic 6-carboxypterin, synthesized by the method of Waller et al. (16): λ_{max}, 261 and 363 nm in 0.1 N sodium hydroxide. R_F values of the two materials were indistinguishable in the following systems: *n*-propanol-1% NH_4OH (2:1), R_F 0.15; *n*-butyl alcohol-acetic acid-water (4:1:1), R_F 0.18; isopropanol-5% boric acid (4:1), R_F 0.09; and 4% sodium citrate, R_F 0.38. The ultraviolet absorption spectra of the factor at various *p*H values were similar to those of the 6-alkyl pterins, i.e., biopterin and neopterin: λ_{max}, 255 and 363 nm in

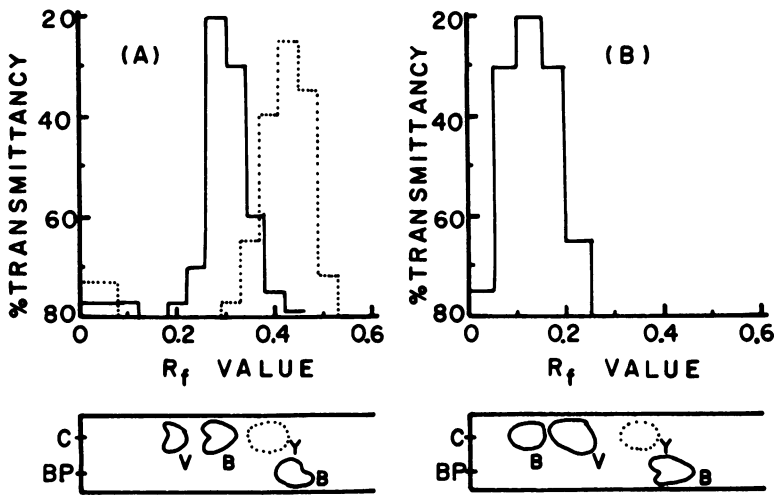

FIG. 1. *Histograms (upper) and paper chromatograms (lower) of the culture fluid (C) of S. indica and a synthetic biopterin (BP). The following solvents were used: (A), n-propanol-1% NH_4OH (2:1); (B), n-butyl alcohol-acetic acid-water (4:1:1). Solid line and dotted line in histogram A indicate the Crithidia factor activity of the culture fluid and biopterin, respectively. V, B, and Y on the paper chromatograms reveal violet, blue, and yellow fluorescent color demonstrated under an ultraviolet lamp.*

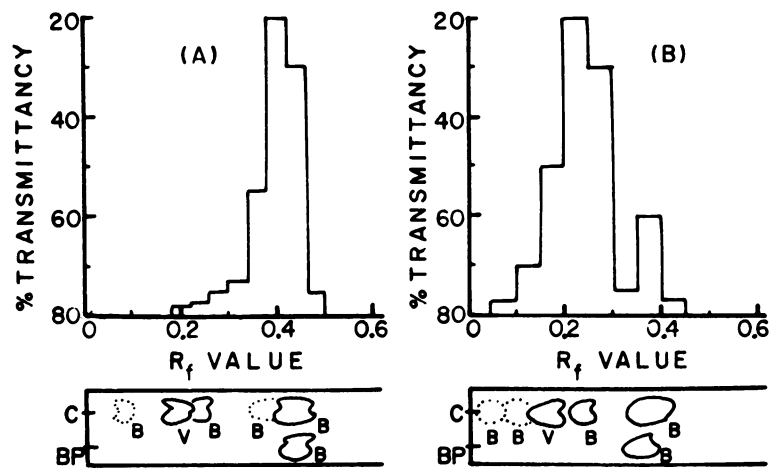

FIG. 2. *Histograms (upper) and paper chromatograms (lower) of the culture fluid (C) of B. cereus. Conditions and abbreviations were identical with those in Fig. 1.*

0.1 N sodium hydroxide, 249 and 323 nm in 0.1 N hydrochloric acid, and 234 (shoulder), 273, and 345 nm in water. However, R_F values of the *Crithidia* factor, biopterin, and neopterin differed from one another: *n*-propanol-1% NH_4OH (2:1), 0.24, 0.47, and 0.31; *n*-butyl alcohol-acetic acid-water (4:1:1), 0.12, 0.44, and 0.20, respectively. One of the folate compounds excreted by *S. indica* was identical with 5,10-methylidyne-tetrahydrofolic acid as shown by tetrazolium bioautography with *S. faecalis* R (6).

DISCUSSION

Crithidia factors in some higher plants and microorganisms have been shown to differ from biopterin (15). The present paper indicates that the *Crithidia* factor(s) was produced by many bacteria, and that *S. indica* and *B. cereus* produced more of the *Crithidia* factor than did other bacteria (Table 1). Comparison of Fig. 1 and 2 suggested that the major components of the *Crithidia* factors produced by *S. indica* and *B.*

cereus are different, and that both differ from biopterin. The minor component of the *Crithidia* factor produced by *B. cereus*, migrating at the same rate as biopterin (Fig. 2B), is probably different from biopterin itself, because it has weak *Crithidia* factor activity in spite of the strong fluorescence intensity on the paper chromatogram.

Biopterin and L-neopterin (6-L-*erythro*-trihydroxypropyl pterin) have strong growth-promoting activities for *C. fasciculata* (12), and this organism responds weakly to some other 6-substituted pterins, i.e., pterin (2-amino-4-hydroxypteridine), 6-methyl-, 6-hydroxymethyl-, 6-formyl-pterins, etc. (1, 10). In view of its growth-promoting activity for *C. fasciculata* and its similarity to biopterin or neopterin in ultraviolet absorption spectra at different *p*H values, it is suggested that the true form of the *Crithidia* factor produced by *S. indica* may be a stereoisomer of biopterin or neopterin, or 6-substituted pterin. Further work on the chemical structure is in progress.

Addendum. The *Crithidia* factor produced by *S. indica* was subsequently isolated in pale-yellow spiny crystals, and was identified as 2-amino-4-hydroxy-6-(L-*threo*-1′,2′,3′-trihydroxypropyl)-pteridine, i.e., L-*threo*-neopterin. The details will be published elsewhere.

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