JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4608–4612
0021-9193/88/104608-05$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 10

Analysis and Characterization of the Folates in the Nonmethanogenic Archaebacteria

ROBERT H. WHITE

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 19 January 1988/Accepted 20 July 1988

A detailed analysis of the folate coenzymes in the nonmethanogenic archaebacteria has been performed. By using the *Lactobacillus casei* microbiological assay for folates, the levels of folates in *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* were found to be 3.7 and 8.3 ng/g (dry weight) of cells, respectively, compared with 88,000 and 28,000 ng/g (dry weight) of cells in *Halobacterium halobium* and *Halobacterium* strain GN-1, respectively. The levels of folates found in the *Sulfolobus* spp. were ~100 times less than those found in the typical eubacterium, whereas the levels in the halobacteria were ~10 times higher. The folate in *Sulfolobus solfataricus* was shown to consist of only 5-formyltetrahydropteroylglutamate, and the folate in *Halobacterium* strain GN-1 was shown to consist of only pteroyldiglutamate. The low folate levels in the *Sulfolobus* spp. are the same as those found in the methanogenic bacteria, suggesting that another C_1 carrier may function in these cells.

Folate and its polyglutamates are well-established coenzymes involved in C_1 metabolism in both eubacteria and eucaryotes (1), and until recently, they were the only recognized C_1 carriers known to function in nature. However, during work on the nature of the coenzymes involved in the reduction of carbon dioxide to methane in the methanogenic archaebacteria, a new pterin-containing coenzyme, methanopterin, was discovered and chemically characterized (22, 23). On the basis of its structural similarity to folic acid and on the basis of biochemical evidence that it can serve as a single carbon carrier at the oxidation level of methenyl (6, 24), methylene (7, 14), and methyl (8–10), there is little doubt that methanopterin functions in a manner analogous to that of folate in these cells. In addition, existing biosynthetic evidence indicates that methanopterin is the first example of a structurally modified folate (25–27). It also appears that folates are absent or at very low levels in these bacteria (5, 14, 17). The occurrence of this unique pterin-containing coenzyme in the methanogenic archaebacteria suggests that other structurally modified folates may be present in the other two groups of archaebacteria, i.e., the extremely thermophilic bacteria and the extremely halophilic bacteria. To address this possibility, the levels and the structures of compounds possessing folate bioactivity in these organisms have been investigated.

MATERIALS AND METHODS

Chemicals and supplies. Nitrobenzoyl chloride was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Folinic acid (5-formyltetrahydrofolic acid), folic acid, α-L-glutamyl-L-glutamic acid, and γ-L-glutamyl-L-glutamic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Pteroyldi-γ-L-glutamic acid [$Pte(Glu)_2$] was obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. Vitamin assay Casamino Acids were obtained from Difco Laboratories, Detroit, Mich. Silica gel 60 F-254 thin-layer chromatographic plates were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Methanopterin was obtained from *Methanobacterium formicicum* and purified by anion-exchange chromatography as described by Leigh and Wolfe (18).

Growth of organisms. *Sulfolobus solfataricus* isolate 98-3, supplied by Tom Langworthy of the University of South Dakota, Vermillion, and *Sulfolobus acidocaldarius* (ATCC 33909) were grown for 3 to 4 days at 72 to 74°C in a medium consisting of 1.3 g of $(NH_4)_2SO_4$, 280 mg of KH_2PO_4, 250 mg of $MgSO_4 \cdot 7H_2O$, 75 mg of $CaCl_2 \cdot H_2O$, and either 1 g of tryptone, 1 g of vitamin-free Casamino Acids or 0.5 g of vitamin-free Casamino Acids, and 0.5 g of glycine or 0.5 g of serine per liter of tap water. This medium is similar to that of Brock et al. (2) except for the deletion of trace metals. Stock cultures of *Sulfolobus* spp. were maintained in 5 ml of the medium in screw-top tubes and transferred weekly after 3 days of growth at 72 to 74°C. *Halobacterium halobium* R_1 and *Halobacterium* strain GN-1, supplied by Barbara J. Javor of the Scripps Institution of Oceanography, La Jolla, Calif., were grown for 3 to 4 days at 39°C on the complex medium described by Javor (16).

To determine the effect of the growth medium on the cellular content of folate, cells were grown in 2.8-liter Fernbach flasks containing 1.5 liters of medium, aerated by shaking at 150 rpm. The large quantity of cells needed for the isolation and characterization of folates was grown in 20-liter plastic carboys, each containing 18 liters of medium. The carboy cultures were mixed and aerated by the addition of sterile air at a rate of 0.3 liters per min through three glass frits mounted at the bottom of the bottle. The carboys, containing the media, were fitted with a thermometer, autoclaved for 1.5 h at 121°C, and cooled to 72°C before inoculation. A 10% inoculum of cells, grown in the Fernbach flasks, was used to inoculate the carboys. Sterile techniques were used at all times. The *Sulfolobus* cells were isolated from the medium first by concentration by membrane filtration with a Millipore Pellicon Cassette System with a 0.5-μm-pore-size filter and then by centrifugation (10,000 × *g*). The *Halobacterium* cells had to be isolated by centrifugation because of cell lysis during membrane filtration.

Extraction of the bacteria. *Sulfolobus* cells were extracted at 100°C for 10 min with 5 ml of 0.1 M mercaptoethanol per

g (wet weight) of cell pellet. After centrifugation to remove the insoluble material, the supernatant was assayed for folate and fractionated by the DEAE-Sephadex column as described below. Large-scale extraction of up to 60 g (wet weight) of *Sulfolobus* cells was conducted with boiling methanol-water (9:1 [vol/vol]) in a Soxhlet extractor.

The halobacteria (100 to 200 g) were extracted with 5 ml of water per g (wet weight) of cells for 30 min at 100°C with vigorous stirring. (The stirring was required to break up the thick mass generated during lysis of the cells.) After cooling, the red suspension was adjusted to pH 3.0 from its initial pH of 6 to 7 by the addition of 10 mg of citric acid per g (wet weight) of original cell pellet and 6 M HCl. The precipitate was removed by centrifugation (30,000 × *g*) for 30 min, and the resulting clear, red solution was stirred for 30 min with 1 g of norite per 100 ml of extract. The norite was separated from the solution by centrifugation and washed five times with water (100 ml) and two times with 50% ethanol (100 ml). The folate activity was eluted from the norite by heating with 50% ethanol containing 5% ammonia at 70°C for 1 h followed by filtration through a Celite bed to give a clear, greenish-yellow solution which was dried in vacuo.

Chromatography of cell extracts. Cell extracts were applied to a DEAE-Sephadex column (1.5 by 26.5 cm) equilibrated in 0.04 M phosphate buffer at pH 5.8. The folates were eluted from the column with a linear gradient generated by mixing 200 ml of the 0.04 M phosphate buffer with 200 ml of the 0.04 M phosphate buffer containing 2 M NaCl. Fractions (5 ml) were collected and assayed for UV absorbance, conductivity, and folate activity.

Folic acid assay. Folic acid was assayed microbiologically by using *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469 by the procedure supplied by ATCC for the use of this culture. Folic acid was used to generate the standard curve for the assay, and the average values of appropriately diluted duplicates of each sample were used to determine the levels of folate.

Zinc-hydrochloric acid cleavage of the folates and the purification and identification of the cleavage products. On the basis of bioactivity, fractions containing folate were pooled, concentrated 10-fold by evaporation, and reductively cleaved by one of the procedures outlined by Foo et al. (11). The folate derivatives extracted from the *Sulfolobus* spp. were cleaved by procedure 1, which involves the cyclization of any 5- or 10-formyl-H_4Pte(Glu)$_x$ to 5,10-methylenyl-H_4Pte(Glu)$_x$ under acidic conditions in the presence of mercaptoethanol, followed by reduction of the resulting 5,10-methylenyl-H_4Pte(Glu)$_x$ with $NaBH_4$ to 5-methyl-H_4Pte(Glu)$_x$. The 5-methyl-H_4Pte(Glu)$_x$ was then oxidatively cleaved to the *p*-aminobenzoyl-(Glu)$_x$ [*p*-AB-(Glu)$_x$] by the acid-base treatment used in procedure 1. The reaction mixture was adjusted to pH 5.8 and centrifuged to remove the ZnO precipitate; the *p*-AB-(Glu)$_x$ released by the cleavage reaction was identified as described below.

The folate derivatives isolated from the halobacteria by carbon absorption were cleaved by procedure 2, which involves the Zn-HCl cleavage step but omits the $NaBH_4$, $HgCl_2$, and base treatment steps. After the reaction mixture was adjusted to pH 5.8 and centrifuged to remove the ZnO precipitate, the sample was applied to a Bio-Gel P-2 column (3 by 35 cm) and eluted with water (5 ml per fraction). The *p*-AB-(Glu)$_2$ eluted at fraction 17 (void volume of the column), the salts eluted at fraction 37, and the 6-methylpterin eluted at fraction 50. The position of elution of the *p*-AB-(Glu)$_2$ was determined by fluorescence intensity after a portion of each fraction was reacted with fluorescamine (12), and the position of elution of 6-methylpterin was determined by its fluorescence. Fractions containing the *p*-AB-(Glu)$_2$ were adjusted to pH 3.0, absorbed to a Dowex 50 column (5 by 10 mm), washed with water, eluted with 3 M ammonia, and purified by preparative thin-layer chromatography. The position of migration of the described compound was determined by spraying the plate with Ehrlich reagent (13). The *p*-AB-(Glu)$_2$ isolated from the halobacteria and converted into the trimethyl ester by reaction for 2 h with 3 M HCl in methanol was identified by mass spectrometry (M^+, *m/z* 437, 2.0% base peak; $M^+ - 32$, *m/z* 405, 9.0% base peak, and base peak at *m/z* 120).

The *p*-AB-Glu that resulted from the cleavage of the 5-formylfolate-containing fractions, which were isolated from the *Sulfolobus* spp., was identified in two ways. First, the cleaved material was fractionated on the DEAE column described above, the fractions were reacted with fluorescamine, and the position of elution of the *p*-AB-Glu was established by fluorescence. In the second method, the cleavage reaction mixture was adjusted to pH 5.8, centrifuged to remove the precipitated ZnO, and reacted with fluorescamine. The resulting fluorescamine adduct was isolated by absorption on a C_{18} Sep-Pack cartridge (Waters Associates, Melford, Mass.), followed by elution with methanol. The elution of the fluorescamine adduct from the C_{18} cartridge was easily monitored by using a fluorescent light. Known samples of *p*-AB, *p*-AB-Glu, and *p*-AB-(Glu)$_2$ were prepared in a similar manner. After thin-layer chromatography, these samples had R_fs of 0.47, 0.42, and 0.08 with the solvent system acetonitrile-water-pyridine (90:10:10 [vol/vol/vol]), respectively, and 0.73, 0.64, and 0.30 with the solvent system acetonitrile-formic acid (88%) (90:10 [vol/vol]), respectively. The fluorescamine adducts were visualized on the plates as green spots by exposing the dried plates to UV light. The spots were not stable over time, fading after several hours. (Since the halobacteria had much more folate bioactivity than the *Sulfolobus* spp., the cleavage products were easily identified on the thin-layer plates by UV absorbance for the *p*-AB-Glu and by both fluorescence and UV absorbance for the 6-methylpterin.) In addition, the *p*-AB-Glu could be identified on the plate after spraying with the Ehrlich spray. By using the solvent system acetonitrile-formic acid (88%) (95:5 [vol/vol]), *p*-AB-Glu, *p*-AB-Gln, *p*-AB-Glu-α-Glu, and *p*-AB-Glu-γ-Glu had R_fs of 0.49, 0.31, 0.16, and 0.12, respectively. Known pterin samples of 6,7-dimethylpterin, 6-methylpterin, 7-methylpterin, and pterin, assayed by thin-layer chromatography by using the solvent system acetonitrile-water-formic acid (88%) (80:20:10 [vol/vol/vol]), had R_fs of 0.54, 0.51, 0.47, and 0.43, respectively. The pterins were visualized as fluorescent spots when the thin-layer plates were exposed to UV light.

Fluorescamine analysis of *p*-substituted anilines. To a 10 μM solution of the *p*-substituted aniline in 0.04 M phosphate buffer at pH 5.8 was added 15 μl of a 15-mg/ml solution of fluorescamine in acetone. After 30 min at room temperature, the fluorescence excitation and emission spectrum of the fluorescent adducts were obtained by using a Perkin-Elmer 650-40 fluorescence spectrometer.

Synthesis and characterization of *p*-aminobenzoyl-containing peptides. A 50-μmol portion of the appropriate peptide was dissolved in 0.4 ml of 0.5 M NaOH, and 0.4 ml of ethanol was added. *p*-Nitrobenzoyl chloride (10 mg) was added in one portion with stirring at room temperature, and after dissolution occurred (~10 min), a second 10-mg portion of *p*-nitrobenzoyl chloride was added. After an additional 1.5 h of stirring, 0.05 ml of 1 M NaOH, 0.8 ml of 50% ethanol,

TABLE 1. Folic acid levels in bacteria

Organism	Medium used[a]	Folic acid content (ng/g [dry wt] of cells)[b]
Sulfolobus solfataricus	Tryptone	3.9
	Casamino Acids + Gly	8.9
	Casamino Acids + Ser	8.3
Sulfolobus acidocaldarius	Tryptone	5.7
	Casamino Acids + Gly	8.9
	Casamino Acids + Ser	8.3
Halobacterium halobium R_1	Peptone	28,000
Halobacterium strain GN-1	Peptone	88,000
	Peptone[c]	25,000 (cells)
		51,000 (medium)
Escherichia coli	Glucose + Casamino Acids	1,100[d]
Bacillus subtilis	Glucose + Casamino Acids	1,100[d]
Pseudomonas fluorescens	Glucose + Casamino Acids	3,600[d]

[a] All cells were grown in shake flasks unless otherwise indicated.
[b] Values are the average of two or more bioassays of the folic acid in the samples, with folic acid as the standard.
[c] Cells were grown in a 20-liter carboy and separated from the medium as described in the text. Both the cell pellet and the resulting medium were then assayed for folic acid.
[d] Values are those of Leigh (17).

and 10 mg of PtO_2 were added and the mixture was stirred under a hydrogen atmosphere at atmospheric pressure for 40 min. After removal of the Pt by filtration, the resulting *p*-aminobenzoyl peptides were purified by preparative thin-layer chromatography. The peptides were easily identified on the thin-layer plates by their UV absorbance or by their reaction with Ehrlich reagent (13). Peptides were eluted from the thin-layer plates with water, absorbed onto Dowex 50W-8X H^+, eluted with 3 M ammonia, and reacted for 3 h at room temperature with 2 M HCl in methanol for the formation of the methyl esters. Mass spectral analysis of the resulting methyl esters by direct insertion at 70 eV gave clear molecular ions for all of the peptide derivatives tested.

RESULTS AND DISCUSSION

Halobacteria and *Sulfolobus* spp. are generally grown on a complex medium composed of folate-containing constituents, e.g., yeast extract. Thus, any folate found in cells grown on this medium could simply be derived from the medium and not be produced by the cells. To alleviate this potential problem, the cells used in these experiments were grown on a folate-free medium. Finding a suitable medium was not a problem since both organisms are known to grow on a wide variety of carbon sources (2, 16). Vitamin-free Casamino Acids were chosen for the initial carbon source. Growth rates and yield of cells with this carbon source were about the same as with a medium containing yeast extract, indicating that the cells are able to generate not only their own folates, but also all of the other coenzymes required for their metabolism.

Assay of the folate levels in two *Halobacterium* strains and two *Sulfolobus* species by using the *L. casei* assay (Table 1) showed a 1,000-fold difference in the folate levels of the halobacterial strains and the *Sulfolobus* spp. The folate levels in the *Sulfolobus* spp. were approximately 100-fold less than those found in eubacteria such as *Escherichia coli* and *Bacillus subtilis* (17). The folate levels in the halobacteria were approximately 10-fold greater than those in these eubacteria. The level of folate activity, 3.9 ng/g (dry weight) of cells for *S. solfataricus* and 5.7 ng/g (dry weight) of cells for *S. acidocaldarius*, was found to increase slightly in both bacteria when they were grown on a medium containing glycine and serine (Table 1). This increase could possibly be explained by an increased requirement for folate, since both serine hydroxymethyltransferase and the glycine cleavage enzyme require folate as a coenzyme (20). The levels of folate activity in the two strains of halobacteria tested were quite different, with strain GN-1 containing about three times more activity than *H. halobium*. The levels in strain GN-1 also depended on the method used to grow the cells. In addition, a large amount of activity was found in the growth medium after the GN-1 cells were isolated (Table 1).

Chromatography of extracts of *S. solfataricus*, extracted in the presence of 0.1 M mercaptoethanol, showed one major peak of bioactivity eluting at the same position as in a known sample of 5-formyl-H_4-PteGlu (Fig. 1). The elution position of the bioactivity eliminated consideration of folate derivatives (indicated in the figure) as well as the folyl polyglutamates, which elute at a higher salt concentration (3, 19). Because of the small amount of material present, the compound could only be detected by bioassay; thus, the structure had to be confirmed indirectly by performing known chemistry on the sample and measuring changes in the chromatographic behavior of the products. Acidification of the active fractions in the presence of 0.1 M mercaptoethanol led to the formation of 5,10-methenyl-H_4-PteGlu, which was reduced to 5-methyl-H_4-PteGlu by reduction with $NaBH_4$. Rechromatography of the treated sample on the DEAE column showed the biological activity to now elute at the position of 5-methyl-H_4-PteGlu.

Subjecting the material in the original bioactive fractions to the entire reductive cleavage procedure (procedure 1 [11]) resulted in the release of *p*-AB-Glu as one of the fragments. The structure of the *p*-AB-Glu was confirmed by the formation, isolation, and characterization of its fluorescamine adduct. The fluorescence excitation and emission spectra of this adduct (maximal excitation, 400 nm; maximal emission, 499 nm) were the same as those observed for the fluorescamine adduct of authentic *p*-AB-Glu. From the fluorescence data shown in Table 2, it is clear that the adduct arises from a *p*-substituted aniline containing an unsaturated group at the *para* position (Table 2). This is in contrast to *p*-substituted anilines with saturated carbon at the *para* position, such as the cleavage product derived from methanopterin, which consistently have longer wavelength excitation and emission spectra. The isolated fluorescent adduct also

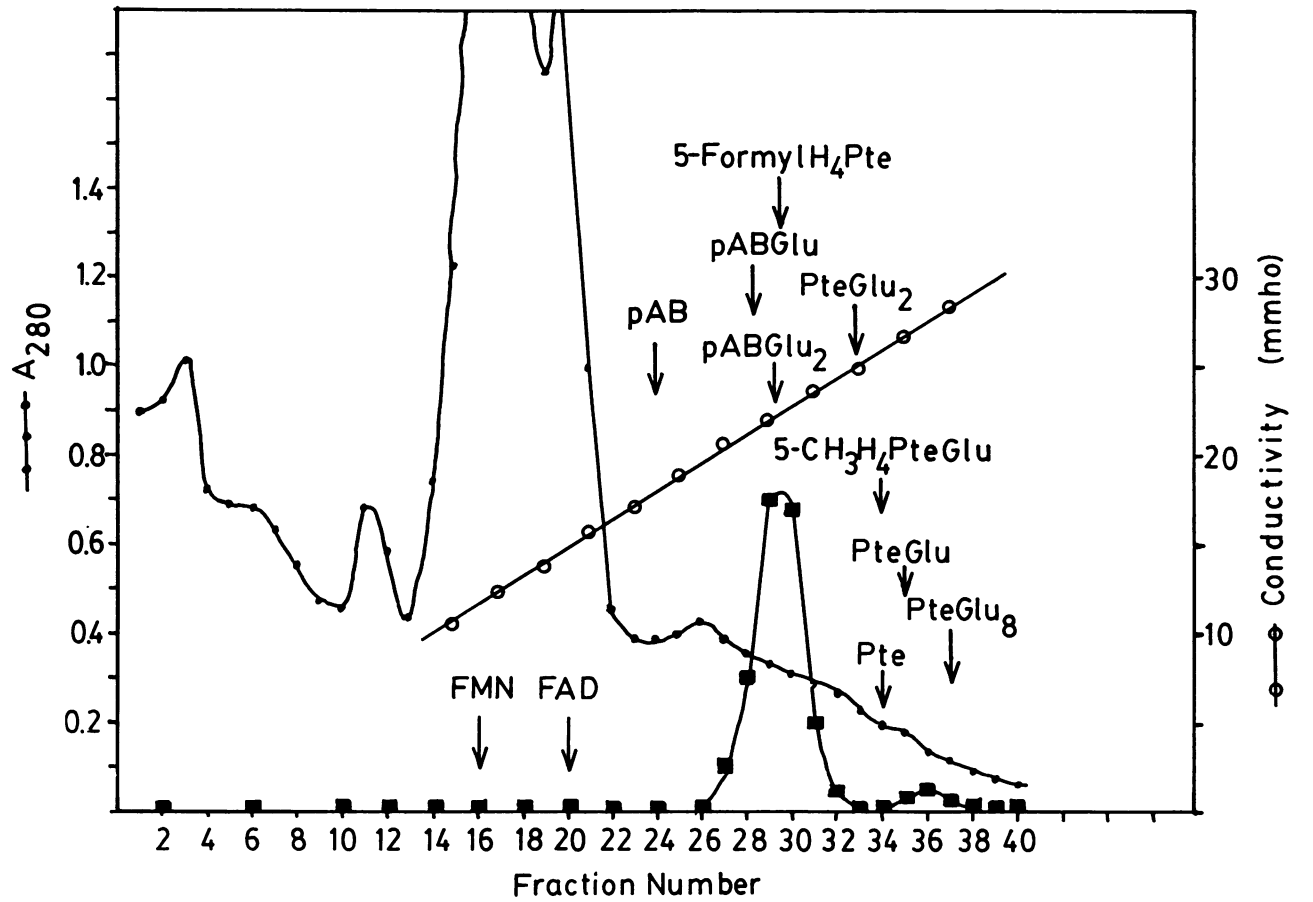

FIG. 1. Separation of an extract of *S. solfataricus* on a DEAE-Sephadex column.

had the same R_f as the *p*-AB-Glu adduct of fluorescamine in both the acetonitrile-water-pyridine and acetonitrile-formic acid solvent systems. On the basis of this information, and on the basis of the fact that the bioactive fractions gave a positive assay in the very specific bioassay for folate, it is concluded that the predominant material responsible for the positive folate assay in *S. solfataricus* is 5-formyl-H_4-PteGlu.

An identical analysis of the folate activity in the halobacteria was not carried out because the large amount of salt present in the halobacterial cell pellets made ion-exchange chromatography of the extract difficult and because the cells had such high levels of folate activity. Thus, the activity of the extracts was absorbed onto carbon and eluted with ammonia to separate the fclate-active compound from the salt. This procedure, similar to that used in the first isolation and identification of folic acid in 1948 (15, 21), resulted in the recovery of over 95% of the activity present in the original cell extract. Chromatography of the carbon-eluted material on the DEAE-Sephadex column, followed by measurement of the bioactivity of the fractions, gave a very broad peak of activity which eluted from about fractions 9 to 35. To determine whether this broad peak was composed of more than one compound, fractions 19 and 29, representing widely spaced fractions with relatively high activity, were combined and rechromatographed on the same column under identical conditions. Analysis of the resulting fractions gave a single peak of bioactivity eluting at fraction 24, indicating that the peak was composed of only one compound. [The position of elution was somewhat earlier than with $Pte(Glu)_2$ because of the salt present in the fractions from the first column.] Zinc-hydrochloric acid cleavage of this peak and analysis of the resulting arylamine fragments on the same DEAE-Sephadex column showed one peak eluting at fraction 29. The UV spectrum of this peak showed an absorption maximum at 273 nm, the same as for the *p*-aminobenzoyl peptides. The fluorescamine adduct also gave the same fluorescence excitation and emission spectra as that observed for the fluorescamine adduct *p*-AB-$(Glu)_2$ (Table 2).

TABLE 2. Fluorescence data of fluorescamine adducts of *p*-substituted anilines

Compound	Maximal excitation (nm)	Maximal emission (nm)
p-AB	400	499
p-AB-Glu	404	502
p-Aminobenzenesulfonic acid	398	498
p-Aminobenzenesulfonamide	399	494
p-Aminobenzyl alcohol	403	514
p-Aminophenylacetic acid	407	519

Separation of the Zn-HCl cleavage products on a Bio-Gel P-2 column allowed the separation of the *p*-AB-$(Glu)_2$ and pterin cleavage products from the salt. A comparison of the mass spectrum of the trimethyl ester of the isolated *p*-AB-$(Glu)_2$ with that of the alpha- and gamma-linked *p*-AB-$(Glu)_2$ showed that only the gamma-linked compound was the same as the unknown. The identity of the isolated compound as

gamma linked was also confirmed by thin-layer chromatography of the methyl esters with the acetonitrile-formic acid solvent system, which was able to resolve these two isomers.

The pterin cleavage product from this peak was identified as 6-methylpterin by thin-layer chromatography with the solvent system acetonitrile-water-formic acid, which is able to separate 6-methylpterin, 7-methylpterin, 6,7-methylpterin, and pterin. Thus, the halobacterial strain GN-1 contains gamma-linked Pte$(Glu)_2$ as its principal folate.

In conclusion, it is clear that both the halobacteria and the *Sulfolobus* spp. contain folates but that the concentrations in the cells vary greatly, with 1,000 times more folate in the halobacteria than in the *Sulfolobus* spp. In addition, the chain lengths found in these bacteria, PteGlu in the *Sulfolobus* spp. and Pte$(Glu)_2$ in the halobacteria, are quite atypical of those generally found in other bacteria which contain mixtures of polyglutamates from Glu_3 to Glu_7 (4).

The very small amounts of activity found in the *Sulfolobus* spp. is in the range of activities of folate found by Leigh (17) in the methanogenic bacteria. The low folate activity in the methanogenic bacteria can be explained by methanopterin functioning as the C_1 carrier in these cells in place of folate. Methanopterin, but not folate, has been shown to function in the enzymes methenyl-tetrahydromethanopterin cyclohydrolase (6) and serine hydroxymethyltransferase (14), and it is presumed that the rest of C_1 metabolism in these cells which is normally carried out by folates, i.e., the biosynthesis of purines and histidine, is carried out with methanopterin. Attempts, however, to find methanopterin in the fractions of extracts of *Sulfolobus* spp., separated by DEAE-Sephadex chromatography, has led to negative results (R. H. White, unpublished results), indicating that a new C_1 carrier may be functioning in these cells. Also, methanogenic bacteria and *Sulfolobus* spp. have been found to be resistant to the sulfa drugs sulfanilamide, sulfadiazine, and sulfathiazole, which inhibit folate biosynthesis, whereas the halobacteria, which I have shown here to contain a folate, are sensitive to these sulfa drugs (R. H. White, unpublished results).

ACKNOWLEDGMENTS

I thank Kim Harich for running the mass spectra, Jia Y. Juan for conducting the folic acid assays, and Linda D. White for editing the manuscript.

This work was funded by National Science Foundation grant DMB-8718216.

LITERATURE CITED

1. **Blakley, R. L., and S. J. Benkovic (ed.).** 1984. Folates and pterins, vol. 1. John Wiley & Sons, Inc., New York.
2. **Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss.** 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Mikrobiol. **84**:54–68.
3. **Brown, J. P., F. Dobbs, G. E. Davidson, and J. M. Scott.** 1974. Microbial synthesis of folate polyglutamates from labelled precursors. J. Gen. Microbiol. **84**:163–172.
4. **Cossins, E. A.** 1984. Folates in biological materials, p. 1–59. *In* R. L. Blakley and S. J. Benkovic (ed.), Folates and pterins, vol. 1. John Wiley & Sons, Inc., New York.
5. **Donnelly, M. I., J. C. Escalante-Semerena, K. L. Rinehart, Jr., and R. S. Wolfe.** 1985. Methenyl-tetrahydromethanopterin cyclohydrolase in cell extracts of *Methanobacterium*. Arch. Biochem. Biophys. **242**:430–439.
6. **Donnelly, M. I., and R. S. Wolfe.** 1986. The role of formylmethanofuran:tetrahydromethanopterin formyltransferase in methanogenesis from carbon dioxide. J. Biol. Chem. **261**:16653–16659.
7. **Escalante-Semerena, J. C., J. A. Leigh, K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Formaldehyde activation factor, tetrahydromethanopterin, a coenzyme of methanogenesis. Proc. Natl. Acad. Sci. USA **81**:1976–1980.
8. **Escalante-Semerena, J. C., J. A. Leigh, and R. S. Wolfe.** 1984. New insights into the biochemistry of methanogenesis from H_2 and CO_2, p. 191–198. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. American Society for Microbiology, Washington, D.C.
9. **Escalante-Semerena, J. C., K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Tetrahydromethanopterin, a carbon carrier in methanogenesis. J. Biol. Chem. **259**:9447–9455.
10. **Escalante-Semerena, J. C., and R. S. Wolfe.** 1985. Tetrahydromethanopterin-dependent methanogenesis from nonphysiological C_1 donors in *Methanobacterium thermoautotrophicum*. J. Bacteriol. **161**:696–701.
11. **Foo, S. K., D. J. Cichowicz, and B. Shane.** 1980. Cleavage of naturally occurring folates to unsubstituted *p*-aminobenzoyl-poly-γ-glutamates. Anal. Biochem. **107**:109–115.
12. **Furness, R. A. H., and P. C. Loewen.** 1981. Detection of *p*-aminobenzoylpoly(γ-glutamates) using fluorescamine. Anal. Biochem. **117**:126–135.
13. **Heacock, R. A., and M. E. Mahon.** 1965. The colour reactions of the hydroxyskatoles. J. Chromatogr. **17**:338–348.
14. **Hoyt, J. C., A. Oren, J. C. Escalante-Semerena, and R. S. Wolfe.** 1986. Tetrahydromethanopterin-dependent serine transhydroxymethylase from *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **145**:153–158.
15. **Hutchings, B. L., E. L. R. Stokstad, N. Bohonos, N. H. Sloane, and Y. SubbaRow.** 1948. The isolation of the fermentation *Lactobacillus casei* factor. J. Am. Chem. Soc. **70**:1–3.
16. **Javor, B. J.** 1984. Growth potential of halophilic bacteria isolated from solar salt environments: carbon sources and salt requirements. Appl. Environ. Microbiol. **48**:352–360.
17. **Leigh, J. A.** 1983. Levels of water-soluble vitamins in methanogenic and nonmethanogenic bacteria. Appl. Environ. Microbiol. **45**:800–803.
18. **Leigh, J. A., and R. S. Wolfe.** 1983. Carbon dioxide reduction factor and methanopterin, two coenzymes required for CO_2 reduction to methane by extracts of *Methanobacterium*. J. Biol. Chem. **258**:7536–7540.
19. **Parker, D. J., T.-F. Wu, and H. G. Wood.** 1971. Total synthesis of acetate from CO_2: methyltetrahydrofolate, an intermediate, and a procedure for the separation of the folates. J. Bacteriol. **108**:770–776.
20. **Schirch, L.** 1984. Folates in serine and glycine metabolism, p. 399–431. *In* R. L. Blakley and S. J. Benkovic (ed.), Folates and pterins, vol. 1. John Wiley & Sons, Inc., New York.
21. **Stokstad, E. L. R., B. L. Hutchings, and Y. SubbaRow.** 1948. The isolation of the *Lactobacillus casei* factor from liver. J. Am. Chem. Soc. **70**:3–5.
22. **Van Beelen, P., J. F. A. Labro, J. T. Keltjens, W. J. Geerts, G. D. Vogels, W. H. Laarhoven, W. Guijt, and C. A. G. Haasnoot.** 1984. Derivatives of methanopterin, a coenzyme involved in methanogenesis. Eur. J. Biochem. **139**:359–365.
23. **Van Beelen, P., A. P. M. Stassen, J. W. G. Bosch, G. D. Vogels, W. Guijt, and C. A. G. Haasnoot.** 1984. Elucidation of the structure of methanopterin, a coenzyme from *Methanobacterium thermoautotrophicum*, using two-dimensional nuclear-magnetic-resonance techniques. Eur. J. Biochem. **138**:563–571.
24. **Van Beelen, P., J. W. Van Neck, R. M. de Cock, G. D. Vogels, W. Guijt, and C. A. G. Haasnoot.** 1984. 5,10-Methyenyl-5,6,7,8-tetrahydromethanopterin, a one-carbon carrier in the process of methanogenesis. Biochemistry **23**:4448–4454.
25. **White, R. H.** 1985. Biosynthesis of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane by methanogenic bacteria. Arch. Microbiol. **143**:1–5.
26. **White, R. H.** 1986. Biosynthesis of the 7-methylated pterin of methanopterin. J. Bacteriol. **165**:215–218.
27. **White, R. H.** 1986. Stereochemistry of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane portion of methanopterin. J. Am. Chem. Soc. **108**:6434–6435.