JOURNAL OF BACTERIOLOGY, Nov. 1975, p. 884-892

Vol. 124, No. 2
Printed in U.S.A.

# Polypeptide Nature of Growth Requirement in Yeast Extract for *Thermoplasma acidophilum*

PAUL F. SMITH, THOMAS A. LANGWORTHY,* AND MARIE R. SMITH

*Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota 57069*

Received for publication 7 July 1975

The active component(s) in yeast extract required by *Thermoplasma acidophilum* for growth is polypeptide in nature. A fraction from yeast extract was isolated and partially characterized as one or more peptides of molecular weight about 1,000 containing 8 to 10 amino acids. Although it was composed largely of basic and dicarboxylic amino acids, only one amino group per molecule was free. The polypeptide(s) appeared to bind avidly to cations. No other organic compounds except glucose were needed by *Thermoplasma*. Among several hundred known compounds tested, only glutathione plus $Fe^{2+}$ or $Fe^{3+}$, clostridial ferredoxin, and spinach ferredoxin elicited any growth response.

The nutritional requirements of members of the class *Mollicutes* are complex. Chemically defined media have been devised for only a very limited number of species (17). In no instance are any of these media considered more than barely adequate. The recent discovery of the obligately acidophilic thermophilic mycoplasma *Thermoplasma acidophilum* (7) offered an organism with the least complex nutritional demands among the *Mycoplasmatales*. This organism will multiply readily in a medium composed of inorganic salts and glucose when supplemented with 0.1% yeast extract. The apparent simplicity of this culture medium together with the curiosity associated with a wall-less organism demanding a harsh environment gave impetus to a study of the nutritional requirements of *Thermoplasma*. This paper presents the results of a study to define the component(s) supplied by the yeast extract necessary for the growth of *T. acidophilum*.

## MATERIALS AND METHODS

The strain of *T. acidophilum* used for this study was isolate 122-1B2 (ATCC 25905). It was maintained by serial transfer in a medium containing 0.02% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.025% $CaCl_2 \cdot 2H_2O$, 0.3% $KH_2PO_4$, and 0.1% yeast extract (Difco). The pH was adjusted to 2 with 10 N $H_2SO_4$. After autoclaving, glucose was added to a final concentration of 1%. Normally a 5% inoculum was added and incubation was carried out with shaking at 59 C (18). Nutritional experiments used the basal salts medium supplemented with glucose but devoid of yeast extract. Chemical compounds and various fractions of yeast extract were assayed by the typical dose response method in concentrations varying from 0.001 to 1.0 mg/ml. Contamination with other microorganisms was not a factor. Only three other known organisms grow under such harsh conditions: *Sulfolobus acidocaldarius* (5), *Bacillus acidocaldarius* (6), and an iron-oxidizing organism (4) similar to *Sulfolobus*. Even these are unable to grow in the medium used for *Thermoplasma* because of their sensitivity to the high phosphate concentration. *Thermoplasma* easily tolerates a phosphate concentration of 1%. Assays were performed in 25-ml volumes contained in 125-ml Erlenmeyer flasks. In those instances where insufficient material was available for assay in 25-ml volumes, 5-ml volumes in tubes were incubated statically. However, under these cultural conditions growth achieved only about one-third of the usual maximum. Volume changes were avoided by maintaining a humid incubator. Controls consisted of unsupplemented inoculated medium, which served as the blank for optical density (OD) measurements, and medium containing 0.2, 0.6, and 1.0 mg of yeast extract per ml. Early in the study it was found that more reproducible results could be obtained by supplementation of the basal salts medium with known mixtures of amino acids, vitamins, purines, and pyrimidines. Even with these supplements no growth occurred in control flasks. Routinely each 100 ml of basal salts medium was supplemented with 1.0 ml of minimal essential medium (Eagle)-amino acids (50×) without glutamine, 1.0 ml of minimal essential medium (Eagle)-vitamin solution (100×), 6.0 mg of glutamic acid, 1.0 mg each of adenine, guanine, cytosine, and uracil, and 0.5 mg of thymine. The addition of these supplements eliminated the possibility of these compounds being the sought-after factor(s) during fractionation of yeast extract. Growth was assayed by measurement of OD at 540 nm using a Bausch & Lomb Spectronic 20 spectrophotometer.

A wide variety of separation techniques was used in the development of a fractionation procedure to isolate the active component(s) from yeast extract. The fractionation procedure developed is as follows.

One hundred grams of yeast extract (Difco) was dissolved with heating in deionized water to give a final volume of 200 ml. Absolute ethanol was added with stirring to give a final ethanol concentration of 70%. The precipitate formed was removed by centrifugation at 8,000 rpm for 20 min at O C in a Sorvall RC-2B centrifuge. Additional absolute ethanol was added with stirring to 570 ml of the supernatant fluid from the first precipitation giving a final ethanol concentration of 95%. The precipitate was collected by centrifugation, washed twice with absolute ethanol, and dried in vacuo at 60 C. The yield of the 70 to 95% ethanol precipitate (fraction I) was 12.7 g. A 20% (wt/vol) aqueous solution of fraction I was heated at 70 C with an equal volume of 90% phenol (21). After cooling and centrifugation to separate the solvent layers, the upper aqueous phase was discarded. The phenol phase was re-extracted at 70 C with an equal volume of water four additional times. A small volume of water (10%) was added to the phenol phase, which was extracted four times with a volume of diethyl ether twice the volume of the phenol phase. The residual aqueous phase from the ether extraction was made 95% with ethanol, and the resulting precipitate was collected by centrifugation and freeze dried. A yield of 0.95 g (fraction II) was obtained from 12.7 g of fraction I. A solution of fraction II (20 mg/ml) in 0.1 M sodium phosphate buffer, pH 8.0, was incubated with a few milligrams of trypsin overnight at 37 C. A drop of toluene was added to prevent bacterial contamination. Samples (10 mg) of trypsin-treated fraction II were loaded onto a column (1 by 45 cm) of Corning controlled-pore-size glass of pore diameter 7.5 nm and particle size 120/200 mesh. The exclusion limit for globular proteins on this column is approximately 28,000. The column was eluted with water, 2-ml fractions being collected. All of the growth-promoting activity appeared in the 30- to 34-ml fraction representing an apparent molecular weight of 1,000. The yield from 32 mg of fraction II was 19.7 mg (fraction III).

Protein was determined by the method of Lowry et al. (14); nucleic acids by absorption at 260 nm; phosphorus by the method of Ames (1); neutral sugars by the phenol-sulfuric acid method (2); total iron by the colorimetric assay with bathophenanthroline (9); sulfur by the chloranilate assay (8); and free amino groups by the ninhydrin method (16). Spectrophotometric measurements were made on Beckman DU, Beckman IR5A, and Beckman DK2A instruments. Enzymatic hydrolyses were carried out under conditions optimal for each enzyme. Acid and alkaline hydrolyses were performed under nitrogen in Teflon-lined screw-cap tubes. Fractions used for amino acid analyses were hydrolyzed in 6 N HCl. After removal of the acid in vacuo, the hydrolysates were analyzed on a Beckman amino acid analyzer, model 116, by the method of Spackman et al. (19). Descending paper chromatography used Whatman no. 1 paper and the following solvents: *n*-propanol/ammonia/water, 6:3:1 (vol/vol); *n*-butanol/acetic acid/water, 4:1:5 (vol/vol); and water-saturated phenol.

All known chemical compounds tested were derived from commercial sources except rhodotorulic acid, a gift from J. B. Neilands, University of California, Berkeley, and a synthetic pentapeptide ($NH_2$-serine-cys-val-ser-cys-COOH), a gift from H. Tanaka, Kyoto University, Kyoto, Japan.

## RESULTS

The complexity of yeast extraction prompted an initial search for a simpler crude biological material as a source of the factor(s) supplied by yeast extract. Certain peptones possessed some activity, but yeast extract appeared as the richest source (Table I). The results were the same both in the presence and absence of the amino acid, vitamin, and purine and pyrimidine supplements. Subsequently all media for assay of growth responses contained these supplements. Likewise no growth occurred upon the use of several defined media designed for cultivation of tissue cells and thermophilic bacteria. Acid-hydrolyzed casein was inactive whereas enzymatic digests of casein retained some minimal activity (Table 1).

Yeast extract was chosen for fractionation, in spite of its complexity, because this material represented the richest source of the growth factor(s). Several lots of yeast extract were examined for growth-promoting activity. Figure 1 shows an example of the variability with regard to both growth promotion and growth inhibition. Most, if not all, of the inhibitory activity appeared to be retained within dialysis casing

TABLE 1. *Sources of growth factor for Thermoplasma*

| Materials[a] | Growth response ($OD_{540}$) |
|---|---|
| Proteose peptone no. 3[b] | 0.120 |
| Peptone[b] | 0.080 |
| Tryptose[b] | 0.140 |
| Tryptone[b] | 0.085 |
| Phytone[c] | 0.076 |
| Protone[b] | 0 |
| Casitone[b] | 0.135 |
| Neopeptone[b] | 0.180 |
| Polypeptone[c] | 0.155 |
| Thiotone[c] | 0.065 |
| N-Z amine, type A[d] (pancreatic digest of casein) | 0.090 |
| N-Z amine, type E[d] (tryptic digest of casein) | 0.110 |
| Hycase[d] (acid-hydrolyzed casein) | 0 |
| Soy peptone[d] | 0.055 |
| Yeast extract[b] | 0.455 |

[a] Final concentration for each was 1 mg/ml; incubation time was 48 h.

[b] Difco Laboratories, Detroit, Mich.

[c] BBL, Division of BioQuest, Cockeysville, Md.

[d] Sheffield Chemical Co., Norwich, N.Y.

whereas most but not all of the growth-promoting activity passed to the outside (Fig. 2). These results suggested that the active component(s) was either a portion of or tightly bound to some biopolymer.

Initial experiments were performed to determine what class of chemical compound in yeast extract possessed the growth-promoting activity. For this purpose yeast extract was subjected to a variety of treatments (Table 2). Ashing completely destroyed the activity. The growth requirement was nonlipoidal since no activity was apparent in diethyl ether, acetone, chloroform-methanol (2:1, vol/vol), or methylal-methanol (4:1, vol/vol) extracts nor was the activity of the residues reduced. Methanol extraction resulted in almost equal distribution of activity in both extract and residue. About 50% of the activity could be removed by adsorption with activated charcoal (Norit A). However, this activity could not be eluted in significant amount by use of acid, alkali, various buffers of differing ionic strength, or organic solvents. It could be shown that the growth factor(s) was adsorbed by demonstrating growth of the organism when the Norit A, preadsorbed with yeast extract, was placed in the basal medium within a dialysis casing. As will be described later, apparent irreversible adsorption also occurred with ion exhange resins, rendering them useless for fractionation. Ammonium sulfate and trichloroacetic acid precipitates of yeast extract were inactive. The inactivity of the trichloroacetic acid precipitate may have been due to residual acid not removed by ether extraction. Treatment with N NaOH at 100 C for 18 h caused a 40

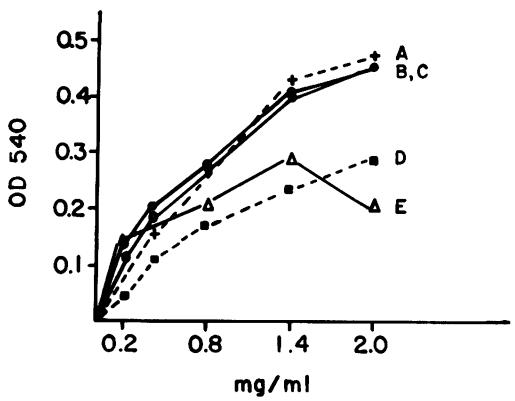


FIG. 1. *Growth response of Thermoplasma to different lots of yeast extract.*

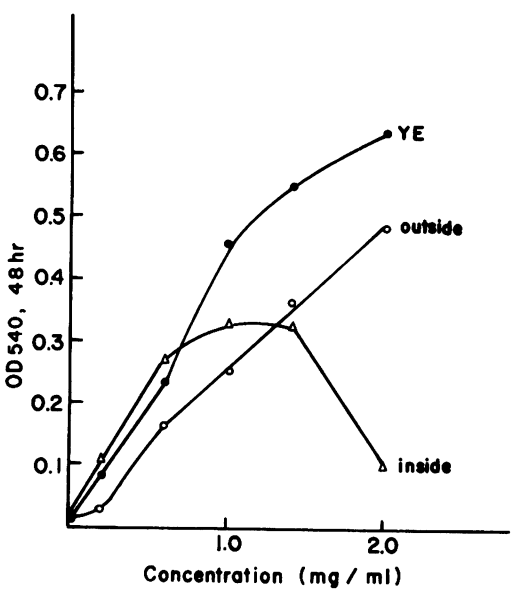


FIG. 2. *Growth response of Thermoplasma to the dialysate and the dialyzed portion of yeast extract.*

TABLE 2. *Effect of various treatments of yeast extract on growth response by Thermoplasma*

| Treatment[a] | Growth response ($OD_{540}$) |
|---|---|
| None | 0.475 |
| Ashing | 0 |
| Methanol extract | 0.125 |
| Methanol residue | 0.175 |
| Norit adsorption (supernatant fluid) | 0.175 |
| Ether extract | 0 |
| Ether residue | 0.545 |
| Chloroform-methanol (2:1, vol/vol) extract | 0 |
| Chloroform-methanol residue | 0.514 |
| Acetone precipitate | |
| 0–50% | 0.585 |
| 50–70% | 0.605 |
| 70–90% | 0.390 |
| Supernatant fluid from 90% acetone | 0.045 |
| Trichloroacetic acid precipitate (10%) | 0 |
| Ammonium sulfate precipitate (saturated) | 0.030 |
| Pronase | 0.383 |
| Pepsin | 0.373 |
| Papain | 0.398 |
| Chymotrypsin A | 0.420 |
| Trypsin | 0.398 |
| N NaOH, 100 C, 24 h | 0.255 |
| 0.1 N HCl, 37 C, 18 h | 0.520 |
| 0.1 N HCl, 100 C, 1h | 0.515 |
| 0.1 N HCl, 100 C, 18 h | 0.345 |
| N HCl, 37 C, 18 h | 0.580 |
| N HCl, 100 C, 10 min | 0.590 |
| N HCl, 100 C, 2 h | 0.515 |
| N HCl, 100 C, 18 h | 0.135 |

[a] Final concentration for each was 1 mg/ml. Ash was equivalent to 1 mg of original yeast extract.

to 75% reduction in activity. Mild acid hydrolysis increased activity, whereas strong acid hydrolysis destroyed all growth-promoting activity. Proteolytic enzymes appeared to produce some diminution of the activity of unfractionated yeast extract. These results tended to suggest that the growth-promoting activity was proteinaceous in character.

During the course of fractionation efforts several possible leads presented themselves. These results led us to assay the growth response to a wide variety of chemical compounds. These included carbohydrates, peptides, nucleosides, nucleotides, lipids, tricarboxylic acid cycle intermediates, amino sugars, D-amino acids, polyamines, polyphosphates, nucleic acids, proteins, and inorganic sulfur compounds. With three exceptions these efforts were fruitless. An aged lot of glutathione, clostridial ferredoxin (type I), and spinach ferredoxin (type III) (13) (Sigma Chemical Co., St. Louis, Mo.) elicited weak growth responses (Table 3). The addition of $Fe^{2+}$ or $Fe^{3+}$ to the glutathione increased the activity slightly. A fresh lot of glutathione was without activity, but the addition of $Fe^{2+}$ or $Fe^{3+}$ generated a slight growth response. Both lots of glutathione were indistinguishable from each other by paper chromatography and infrared, ultraviolet (UV), and visible spectra. Since glutathione decomposes by release of glutamic acid as pyrrolidone-5-carboxylic acid and by the cyclization of the cysteinylglycine to a piperazine-like compound capable of cation chelation (12), attempts were made to determine whether either of these breakdown products was active. None of these nor oxidized glutathione exhibited any activity either in the presence or absence of $Fe^{2+}$ or $Fe^{3+}$. The stimulatory activity of $Fe^{2+}$ and $Fe^{3+}$ prompted an examination of the possible growth-promoting activity of a wide variety of iron-binding compounds (15). These included the hydroxamate-type siderochromes, the phenolic acids, hemes, ferredoxin models, ferritin, citrate, polydicarboxylic amino acids, and inorganic iron salts. None of these exhibited any activity.

TABLE 3. *Growth response of Thermoplasma to glutathione and ferredoxins*

| Compound[a] | Growth response ($OD_{540}$) |
|---|---|
| Yeast extract | 0.550 |
| Glutathione, reduced, old lot | 0.067 |
| +$FeCl_2$ | 0.073 |
| +$FeCl_3$ | 0.059 |
| +CuCl or $CuCl_2$ | 0 |
| Glutathione, oxidized, old lot | 0.070 |
| Glutathione, reduced, new lot | 0.010 |
| +$FeCl_2$ | 0.029 |
| +$FeCl_3$ | 0.029 |
| +CuCl or $CuCl_2$ | 0 |
| Glutathione, oxidized, new lot | 0.005 |
| Spinach ferredoxin | 0.040 |
| Clostridial ferredoxin | 0.042 |

[a] Final concentration of yeast extract and glutathione, 1 mg/ml; of ferredoxins, 0.1 mg/ml; of inorganic salts, 10 mg/ml.

Preliminary experiments indicated that the growth-promoting activity in yeast extract could be concentrated by precipitation with polar organic solvents. Although methanol, ethanol, and acetone could achieve this end, ethanol was selected for the initial fractionation step. Figure 3 shows the specific activity of precipitates formed by increasing the concentration of ethanol. Some activity precipitated with 33% ethanol concentration only from complete yeast extract. None appeared in this fraction from the external dialysis fluid. Increasing activity appeared in precipitates formed in the 50 to 67%, 67 to 80%, and 80 to 96% ethanol concentrations. No additional increase in specific activity could be achieved by narrowing the ranges of ethanol concentrations between 60 and 95%. Little or no activity remained in the supernatant fluid of the 95% ethanol concentration. The ratio of $OD_{260/280}$ decreased as growth-promoting activity increased. Nevertheless a considerable amount of $OD_{260}$-absorbing material remained in the active fractions. Subsequent fractionation used the precipitate formed with ethanol concentrations between 70 and 95% (fraction I). Most of the activity precipitated at acetone concentrations between 50 and 90%. Prior treatment of yeast extract in N HCl at 100 C for 10 min resulted in more activity being precipitated at ethanol concentrations of 50 to 70% and

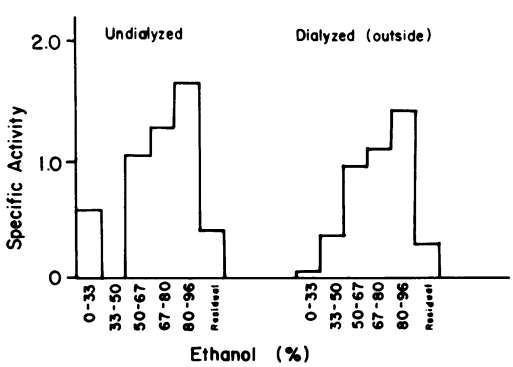


FIG. 3. *Specific activities of various ethanol precipitates of undialyzed and dialyzed yeast extract. Specific activity is defined in Table 7.*

more diffusion of total activity in precipitates over the 50 to 95% ethanol concentrations. No gain in specific activity was obtained by varying the pH or ionic strength of the yeast extract before ethanol treatment. Since fraction I still represented a rather crude preparation, additional treatments were carried out with the purpose of further defining the class of chemical compound responsible for growth promotion (Table 4). Similarly to unfractionated yeast extract, fraction I possessed some susceptibility to proteolytic enzymes. Phosphomonoesterases and phosphodiesterases had no effect, as was the case also with carbohydrate-splitting enzymes. The effects of acid and alkaline hydrolysis were identical to those effects on unfractionated yeast extract. Treatment with sodium borohydride and esterification had no effect, although the stability of the ester derivatives is uncertain under the hot acid conditions required for growth of *Thermoplasma*. *N*-acetylation markedly reduced activity. Treatment with 1-fluoro-2,4-dinitrobenzene completely abolished growth-promoting activity. All residual reagent was removed by ether extraction. Paper chromatography of this derivative yielded three spots, none of which was able to elicit a growth response. Oxidation with performic acid and with sodium periodate completely abolished activity. However, with these two reagents neutralized reagent controls inhibited growth when yeast extract served as source of the growth factor(s). Formate ions and halogen ions, in particular iodide and bromide, also were found to be greatly inhibitory. Treatment of fraction I with 0.17 M ethylenediaminetetraacetic acid rendered it inactive for supporting growth. The treated fraction was repeatedly washed through an ultrafilter. The chelating agent completely inhibited growth at a level of $10^{-3}$ M in the crude growth medium. Presumably the ethylenediaminetetraacetic acid was bound and could not be removed by ultrafiltration.

TABLE 4. *Effect of various treatments of fraction I (ethanol precipitate) from yeast extract on growth response by Thermoplasma*

| Treatment[a] | Growth response ($OD_{540}$) |
|---|---|
| None | 0.342 |
| Pronase | 0.276 |
| Pepsin | 0.292 |
| Papain | 0.228 |
| Chymotrypsin A | 0.255 |
| Trypsin | 0.223 |
| Phosphomonoesterase | 0.331 |
| Phosphodiesterase | 0.332 |
| N NaOH, 100 C, 24 h | 0.062 |
| N HCl, 100 C, 6 h | 0.294 |
| Sodium borohydride reduction | 0.340 |
| *N*-acetylation | 0.071 |
| Esterification | 0.340 |
| Periodate oxidation | 0 |
| Performic acid oxidation | 0 |
| 1-Fluoro-2,4-dinitrobenzene derivative | 0 |

[a] Final concentration of each component was 1 mg/ml.

Ultrafiltration of fraction I through controlled-pore-size filters resulted in all of the growth-promoting activity passing through the PM10 filter (Amicon Corp., Lexington, Mass.) and being totally retained by the UM2 filter. This behavior suggested a molecule ranging somewhere between 1,000 and 10,000 molecular weight. All of these results suggested some compound(s) of proteinaceous nature. However, the heavy contamination with $OD_{260}$-absorbing material that could account for all of the phosphorus in fraction I necessitated further attempts at purification.

By using the anion resin Dowex-1-formate or Dowex-1-$OH^-$, all of the growth-promoting activity and the bulk of the fraction loaded onto the columns eluted as one peak with water. It was concluded that the component(s) sought was not bound by anion exchange resins. On the other hand, the strongly acidic cation exchange resin Dowex-50-$H^+$ irreversibly bound almost all of the activity. Only a small amount of the original activity could be eluted with 2 N HCl. Incorporation of the resin from the top of the column into the basal culture medium permitted growth to occur. The resin alone had no inhibitory or stimulatory effects. Repeated attempts gave comparable results. Use of weakly acidic cation exchange resins failed to resolve the growth-promoting activity from the $OD_{260}$-absorbing material. All of the activity and most of the mass eluted with water from carboxymethyl cellulose columns. Similar results were obtained with Bio-Rex 70 carboxylic resin (Bio-Rad Laboratories, Richmond, Calif.), using 0.002 N HCl as eluting solvent. No significant increase in specific activity was achieved. Therefore, ion exchange chromatography was abandoned as a separation method.

Passage of fraction I through a Sephadex G-100 column using water as eluant resulted in the appearance of one peak well beyond the void volume. As with the other pore sizes of Sephadex, the growth-promoting activity was skewed away from the protein peak as determined by the Lowry method (14). Passage of fraction I through a column of Sephadex G-25

resulted in the appearance of at least three peaks of growth-promoting activity and two protein peaks (Fig. 4A), some appearing in the void volume. The fraction comprising tubes 19-22 from the Sephadex G-25 column could be further subdivided by passage through a column of Sephadex G-15 (Fig. 4B). These results suggested that the growth-promoting activity was associated with molecules of varying size but still within the range of 1,000 to 10,000 molecular weight. Pretreatment of fraction I with N HCl at 100 C for 2 h followed by passage through a column of Sephadex G-15, in an attempt to reduce the active components to a common size, resulted in the appearance of three active peaks, none of which eluted in the void volume. In all of these gel filtration studies insufficient material was accumulated to determine specific activity with accuracy. Unfortunately, all of the fractions collected from Sephadex columns retained considerable $OD_{260}$-absorbing material as well as protein; hence no separation of a single chemical class was achieved.

Several fractions from Sephadex chromatography were subjected to paper chromatography. The best resolution was obtained by using *n*-propanol/ammonia/water, 6:3:1 (vol/vol). All of the growth-promoting activity migrated at about $R_f$ 0.3. Although several ninhydrin-positive spots were apparent, the region exhibiting growth activity was only weakly ninhydrin positive and lay very near the only UV-absorbing spot. Successful isolation of the growth-promoting activity from the UV-absorbing material was achieved. However, only minute quantities were recoverable by this method. The isolated material exhibited no significant absorption in the UV and visible spectra: the infrared spectrum was very suggestive of secondary amines. Total acid hydrolysis yielded only amino acids (Table 5). The large preponderance of arginine, ammonia, glutamic acid, and aspartic acid suggested a histone- or protamine-like molecule.

TABLE 5. *Amino acid composition of various fractions of yeast extract supporting growth of Thermoplasma*

| Amino acid[a] | Fraction II (mol/100 mol) | Fraction III (mol/100 mol) | Paper chromatography[b] (mol/100 mol) |
|---|---|---|---|
| Lysine | 16.33 | 4.54 | 2.47 |
| Histidine | 4.50 | 1.18 | 0.95 |
| Ammonia | 16.63 | 15.97 | 17.06 |
| Arginine | 8.11 | 2.92 | 39.68 |
| Aspartic acid | 7.87 | 15.40 | 6.74 |
| Threonine | 3.97 | 4.43 | 2.36 |
| Serine | 4.76 | 5.36 | 4.02 |
| Glutamic acid | 5.50 | 11.79 | 7.01 |
| Proline | 4.05 | 6.21 | 3.48 |
| Glycine | 9.53 | 9.02 | 5.83 |
| Alanine | 4.05 | 4.73 | 3.53 |
| Valine | 3.76 | 4.34 | 3.09 |
| Methionine | 0.54 | 0.70 | |
| Isoleucine | 3.37 | 3.75 | 1.64 |
| Leucine | 3.22 | 4.59 | 2.16 |
| Tyrosine | 1.86 | 2.37 | |
| Phenylalanine | 1.96 | 2.74 | |

[a] No half-cystine was detected.

[b] Fraction purified by descending paper chromatography in *n*-propanol/ammonia/water, 6:3:1 (vol/vol).

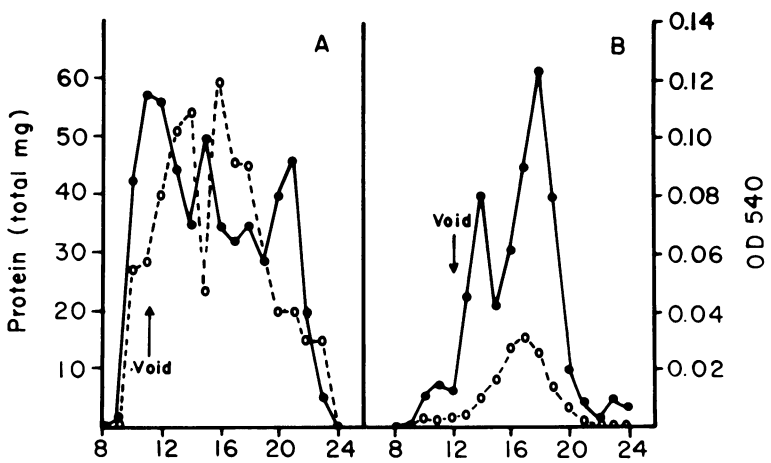


FIG. 4. *Separation of growth-promoting activity in fraction I by gel filtration. (A) Fraction I on Sephadex G-25; (B) fractions 19-22 from (A) on Sephadex G-15. Symbols: ○, Protein; ●, growth response. Columns were 2.5 by 50 cm; flow rate, 1 ml/min; fraction volume, 12 ml; eluant, water. One gram of fraction I was loaded onto the G-25 column. Fractions were assayed in 5-ml cultures.*

However, a variety of known histones and protamines as well as their partial acid hydrolysates proved inactive in support of growth. Likewise increasing the concentrations of amino acids, individually and in combination, up to 100 times the amount in the basal medium was without effect.

The inefficient and cumbersome nature of paper chromatography for isolation of sufficient quantities of the growth factor led to further attempts at fractionation. Our results at this point indicated that the active material was peptide in nature. Therefore an attempt was made to remove the presumed nucleic acid contaminants. Treatment of fraction I with a variety of nucleases followed by gel permeation chromatography (in this instance controlled-pore-size glass beads) and gel permeation chromatography using 2 M NaCl to effect breaking of ionic bonding were unsuccessful in separating nucleic acid from protein. Finally, successful separation of the growth-promoting activity from $OD_{260}$-absorbing material was achieved by use of hot 45% aqueous phenol extraction. Essentially all of the activity was recovered from the phenol phase (fraction II) (Table 6). All of the dry weight could be accounted for as Lowry-reactive protein. It was devoid of phosphorus, sugars, and amino sugars. The visible spectrum exhibited no absorption, the UV spectrum exhibited a slight peak at 275 nm, and the infrared spectrum exhibited a pattern typical of secondary amines. Paper chromatography in *n*-propanol/ammonia/water revealed a weakly ninhydrin reactive spot at $R_f$ of about 0.3. Only this area of the paper supported growth.

Passage of fraction II through a column of controlled-pore-size glass (nominal pore size of 7.5 nm) using water as eluant resulted in the appearance of one peak calculated to have a molecular weight of 1,000 (Fig. 5). However, solution of fraction II in 3 M KCl, although yielding only one peak, resulted in its appearance almost at the pore volume of the column. In addition to the growth activity, inorganic salt also eluted at this volume. It would appear that the growth factor was binding salt or cation, a conclusion corroborated by its irreversible binding to cation exchange resins. Treatment of fraction II with 2 or 6 N HCl at 100 C for varying time periods resulted in loss of growth-promoting activity but no change in elution characteristics on the controlled-pore-size glass. Paper chromatography of fractions from these columns showed a disappearance of the active component and an increase in quantity and number of strongly ninhydrin-reactive spots indicative of hydrolysis. Treatment with Pronase (Protease type II, Sigma Chemical Co., St. Louis, Mo.) gave the same results as acid treatment whereas treatment with trypsin, chymotrypsin, or pepsin resulted in increased activity with the same elution characteristics as the untreated fraction II dissolved in KCl. Trypsin-treated fraction II after passage through the

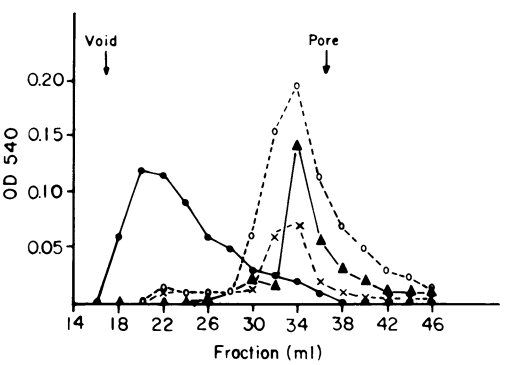


FIG. 5. *Filtration of fraction II through controlled-pore-size glass beads of pore size 7.5 nm. Column (1 by 45 cm) was loaded with 10 mg and eluted with water. Two-milliliter fractions were collected. Symbols: ●, Untreated fraction II; ○, trypsin treated; △, fraction II in 3 M KCl; ×, fraction II hydrolyzed for 60 min in 6 N HCl at 100 C and then neutralized with 6 N KOH. Fractions were assayed in 5-ml cultures.*

TABLE 6. *Fractionation of yeast extract for growth-promoting activity for Thermoplasma*

| Fraction | Yield (g) | Sp act[a] | Recovery (%) |
|---|---|---|---|
| Yeast extract | 100 | 1.00 | 100 |
| Ethanol precipitate (70–95%) (fraction I) | 12.7 | 1.51 | 19.2 |
| Phenol phase from hot 45% phenol extraction of fraction I (fraction II) | 0.95 | 2.71 | 2.6 |
| Trypsin-treated fraction II | | 4.50 | |
| Eluate from controlled pore-size-glass beads of trypsin-treated fraction II (fraction III) | 0.14 | 21.47 | 3.0 |

[a] Specific activity = $OD_{540}$ per milligram per milliliter. Calculations were made by determining growth responses to varying concentrations and then striking an average. Specific activity of yeast extract was arbitrarily set as 1.00.

controlled-pore-size glass resulted in a preparation (fraction III) devoid of a minor ninhydrin-positive component contaminating fraction II. Fraction III was devoid of $OD_{260}$-absorbing material, phosphorus, sugars, and amino sugars. Amino acids could account for its entire mass. Ninhydrin assays revealed the presence of about one free amino group per molecule of molecular weight 1,000. Table 5 presents the amino acid analysis of various fractions. Noticeably absent or present in very low amounts were the S-containing amino acids. Fraction II possessed an abundance of basic amino acids and dicarboxylic acids probably existing as their amides. Treatment with trypsin significantly lowered the lysine, histidine, and arginine content (fraction III) with a concomitant increase in the amide forms of aspartic and glutamic acids. The fraction obtained by paper chromatography was composed mainly of arginine, asparagine, and glutamine. These results tend to suggest the necessity for amino groups, either free or bound, in a polymeric form. Either a particular sequence of amino acids or their linkage or both appear necessary since polyasparagine, polylysine, and polyarginine of molecular weights approximating 5,000 are ineffective replacements for the polypeptide(s) isolated from yeast extract.

A summary of the fractionation of the growth-promoting activity from yeast extract is shown in Table 6. The final fraction consisted only of Lowry-reactive material of presumed molecular weight of about 1,000 with a specific activity 22-fold that of yeast extract. It was composed solely of amino acids and consisted of one or more oligopeptides containing no more than 8 to 10 amino acids. Its avid binding characteristics led us to assess the growth-promoting activity of cyclic peptide antibiotics that can serve as membrane ionophores. None of these examined (gramicidin, bacitracin, and polymyxin) exhibited any growth-promoting activity. The idea also occurred that basic amino acids may be too highly charged at the low pH of growth; hence permeation of such molecules may be impeded. However, use of *N*-acetyl basic amino acids, singly or in mixtures, failed to replace the requirement for fraction III.

The isolated active polypeptide(s) could completely replace the requirement for yeast extract. The amino acid, vitamin, and purine/pyrimidine supplements were found to be unnecessary (Table 7).

TABLE 7. *Growth response of Thermoplasma to fraction III[a] from yeast extract in presence and absence of various supplements*

| Addition | Growth response ($OD_{540}$) |
|---|---|
| None | 0.230 |
| Vitamin mixture[b] | 0.225 |
| Purine-pyrimidine mixture[b] | 0.160 |
| Vitamines + purines-pyrimidines | 0.160 |
| Amino acids + vitamins + purine-pyrimidine mixture | 0.225 |

[a] Final concentration was 0.4 mg/ml.
[b] See text for description.

## DISCUSSION

The nutritional requirements of *Thermoplasma* appear to be simple. Other than inorganic ions, there is a need for a carbon and energy source, supplied by glucose, and one or more oligopeptides. It is not clear whether even glucose is required since the organisms will continue to multiply for several transfers in the absence of glucose. The oligopeptide requirements is absolute. In this respect *Thermoplasma* differs from *Sulfolobus* (5, 7), which can grow autotrophically or heterotrophically in the presence of small amounts of yeast extract. This distinction may be a reflection of the nature of the outer envelopes of the two organisms. *Sulfolobus*, although devoid of peptidoglycan, does possess an ultrastructural element overlying the cytoplasmic membrane (20), whereas *Thermoplasma* is bounded only by a cytoplasmic membrane. The relatively large amount of oligopeptide required and its apparent avid cation-binding property suggest that the oligopeptide is not a nutritional requirement but rather bears some relationship to the high $H^+$ concentration of the growth environment of *Thermoplasma*. Any speculation on the function of the oligopeptides would be meaningless until the structures of active molecules are defined. Obviously there are specific structural requirements since many proteins or partial hydrolysates of these proteins cannot supply the needs of the organism. In particular, the behavior of the oligopeptide from yeast extract on ion exchange resins and the amino acid composition mimic the histones and protamines (11), yet neither of these basic proteins nor their partial acid hydrolysates exhibit any growth-promoting activity. On the other hand, weak activity is exhibited by both clostridial and spinach ferredoxins, signifying some similar structural segments. The minimal activity of glutathione is suspected to be the result of some degradative product (12), perhaps a larger polymer than the original tripeptide, since aging or some other treatment is necessary for manifestation of the activity. The known characteris-

tics of the oligopeptide requirement would tend to suggest a role as ion scavenger for some trace metal requirement, protection of the organism at its surface from the high $H^+$ concentration, involvement in ion transport, or as a supply of essential amino acids in a form permeable in the very acid environment in which the organism occurs. Peptide requirements of other microorganisms appear to result from a need of an appropriate permeable or exogenously non-metabolizable form of an amino acid (10). This explanation in the case of *Thermoplasma* does not seem appropriate since high concentrations of amino acids cannot override its oligopeptide requirement as is the case with other peptide-requiring microorganisms. Definition of the function of the oligopeptide requirement must await a sorting of the obvious mixture of peptides in the most purified fraction and delineation of their structures.

Some relationship is indicated between the nutritional requirements of *Thermoplasma* and it ecological incidence. *Thermoplasma* has been found to occur naturally only in the vicinity of burning coal refuse piles (3). None have been found in acid hot springs, the source of other obligately acidophilic thermophilic microorganisms, all of which appear capable of autotrophic growth. Obviously the coal refuse piles are rich in carbon and in organic material derived from decaying plants and animals. Activated carbon adsorbs and concentrates the necessary factors for growth found in yeast extract. These factors are tightly bound to the activated charcoal but are released in sufficient amount in the hot acid environment to allow growth to occur. Therefore, in the natural environment carbon probably is required to concentrate and localize the oligopeptides demanded by *Thermoplasma*, thereby restricting its natural habitat to hot acid carboniferous areas.

## ACKNOWLEDGMENTS

This study was supported in part by Public Health Service research grant 5R01AI04410-13 from the National Institute of Allergy and Infectious Diseases.

We are indebted to R. J. Peanasky for the amino acid analyses.

## LITERATURE CITED

1. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases, p. 115–118. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
2. Ashwell, G. 1966. New colorimetric methods of sugar analysis, p. 85–95. *In* E. F. Neufeld and V. Ginsberg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
3. Belly, R. T., B. B. Bohlool, and T. D. Brock. 1973. The genus *Thermoplasma*. Ann. N.Y. Acad. Sci. 225:94–107.
4. Brierley, C. L., and J. A. Brierley. 1973. A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. Can. J. Microbiol. 19:183–188.
5. 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.
6. Darland, G., and T. D. Brock. 1971. *Bacillus acidocaldarius* sp. nov., an acidophilic thermophilic spore-forming bacterium. J. Gen. Microbiol. 67:9–15.
7. Darland, G., T. Brock, W. Samaonoff, and S. F. Conti. 1970. A thermophilic, acidophilic *Myoplasma* isolated from a coal refuse pile. Science 170:1416–1418.
8. Dittmer, J. C., and M. A. Wells. 1969. Quantitative and qualitative analysis of lipids and lipid components, p. 482–530. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol. 14. Academic Press Inc., New York.
9. Doeg, K. A., and D. M. Ziegler. 1962. Simplified methods for the estimation of iron in mitochondria and submitochondrial fractions. Arch. Biochem. Biophys. 97:37–40.
10. Guirard, B. M., and E. E. Snell. 1962. Nutritional requirements of microorganisms, p. 33–93. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 4. Academic Press Inc., New York.
11. Hnilica, L. S. 1972. The structure and biological functions of histones. CRC Press, Cleveland, Ohio.
12. Isherwood, F. A. 1959. Chemistry and biochemistry of glutathione, p. 3–16. *In* E. M. Crook (ed.), Glutathione, Biochemical Society Symposium no. 17. Cambridge University Press, Cambridge.
13. Lovenberg, W. 1974. Ferredoxin and rubredoxin, p. 161–185. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press Inc., New York.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
15. Neilands, J. B. 1974. Iron and its role in microbial physiology, p. 3–34. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press Inc., New York.
16. Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67:10–15.
17. Smith, P. F. 1971. The biology of mycoplasmas. Academic Press Inc., New York.
18. Smith, P. F., T. A. Langworthy, W. R. Mayberry, and A. E. Hougland. 1973. Characterization of the membranes of *Thermoplasma acidophilum*. J. Bacteriol. 116:1019–1028.
19. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino-acids. Anal. Chem. 30:1190–1206.
20. Weiss, R. L. 1974. Subunit cell wall of *Sulfolobus acidocaldarius*. J. Bacteriol. 118:275–284.
21. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraction von Bakterien mit Phenol/Wasser. Z. Naturforsch. 7b:148–155.