Growth of Puccinia graminis f. sp. tritici on a Defined Medium

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ABSTRACT

Sustained axenic growth of *Puccinia graminis* f. sp. *tritici*, race 126-ANZ-6,7, was obtained on a defined medium consisting of 1% agar, 3% glucose, Czapek's minerals, Burkholder and Nickell's trace elements, and a mixture of 16 amino acids in the proportions found in purified casein hydrolysate. Mycelial growth from uredospores, measured at 28 days, on this medium was equal to that on media containing casein hydrolysates and superior to that on Evans' peptone. We developed the defined medium initially screening 25 commercial peptones and then comparing representative products which did and did not support growth. Only six peptones were effective when incorporated directly into a basal medium; however, six more products supported growth after additional acid hydrolysis. Analyses showed distinct amino acid patterns (high levels of glutamate in nine out of 10 effective hydrolysates and high levels of glycine in noneffective ones) but no unique levels of trace elements. Since purified casein, hydrolyzed 24 hr, supported growth as well as the best commercial products, its amino acid composition was mimicked to prepare the artificial medium. When amino acids were supplied in the proportion found in Bactopeptone or when trace elements were omitted, the defined medium did not support growth.

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Three species and several physiological races of *Puccinia* have now been cultured independently of their hosts (5, 9, 14, 18; I. Wahl, *unpublished data*). This method of axenic culturing of rust fungi provides a new research tool for the investigation of rust metabolism and host-parasite relationships.

However, the necessity for use of nondefined media prepared from natural products for successful growth of the rust fungi in culture has prevented critical work on the relationship between growth and pathogenicity. If rust fungi can be grown satisfactorily on defined media, it should be feasible to study three longstanding questions involving host-parasite interactions: How is nutrition of the rust pathogen related to (i) its virulence; (ii) the ability of uredospores to infect intact leaves; and (iii) resistance and susceptibility of the host?

Investigators have shown that successful growth in culture depends on the type of peptone used in the medium (4, 12, 19). However, no explanation exists as to why one peptone is superior to another, since the nutritional requirements for rust growth are not understood. Evans' peptone plus yeast extract (19), Evans' peptone alone (3, 6), and casein hydrolysates (4) have all been reported as satisfactory organic nitrogen sources. No synthetic medium has been tried which supports growth as well as do hydrolysates of natural products (9).

The purpose of the present study was to develop a chemically defined medium for the growth of one race of *Puccinia graminis tritici* by screening a range of commercial peptones and related natural products, analyzing their components, and then preparing an artificial medium similar to the composition of the peptone or product which most effectively promotes growth in culture.

MATERIALS AND METHODS.—*Maintenance of uredospores.*—*Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., race 126-ANZ-6,7 (obtained from American Type Culture Collection, Rockville, Md.), was maintained on *Triticum aestivum* L. 'Little Club'. All uredospores stored for more than 7 days were lyophilized by the method described by Sharp & Smith (13) and kept at 4 C. Prior to inoculation, the lyophilized uredospores were hydrated in a water-saturated atmosphere for 24 hr at 20 C and then floated on glass distilled water at a density of 3 mg/cm^2 for 12-18 hr at 4 C. The uredospores were collected on a sintered glass filter, dried for 45 min by moving air, mixed with 10 volumes of talc, and dusted on wheat seedlings at the two-leaf stage (8 days). The plants were atomized with water, covered with premoistened plastic bags, and placed in an environmental growth chamber (20 C and 1,500 ft-c at plant height) at the start of an 8-hr night followed by a 16-hr day. The plants were left under these lighting and temperature conditions for 36 hr, after which the plastic bags were removed. Flecking first appeared 6 days after inoculation, and uredospores were collected on the 9th day and at 3-day intervals thereafter with a cyclone spore collector.

Aseptic uredospore production.—Inoculated wheat leaves were harvested when flecking first appeared. The leaves were surface sterilized for 4 min in a fresh saturated solution of calcium hypochlorite containing 0.001% Tween 20 (polyoxyethylene sorbitan monolaurate). After surface sterilization, all manipulations with leaves and spores were carried out aseptically with sterile glassware and solutions. The individual leaves were washed in water and placed in 18-mm screw cap test tubes containing 0.5 ml of 2.0% sucrose. The test tubes were incubated in an environ-

mental growth chamber under the same temperature and lighting used to produce nonaseptic spores. At 4 days, when uredospore production was at its maximum, the leaves were removed from the test tubes and placed in pairs in 9-cm petri dishes which were held for 24 hr in desiccators containing activated silica gel. The uredospores were then mechanically removed from the leaf surface and used immediately.

Basal medium and bioassay.—The basal bioassay medium contained 3.0% glucose, 1.0% Difco Certified Bacto-agar, and Czapek's mineral solution (7) with 14.4 mg/liter ferric EDTA instead of ferrous sulfate. The final assay medium in all cases was adjusted to pH 6.4 with HCl and autoclaved at 121 C for 20 min.

All bioassays were performed by seeding aseptic uredospores on 9 cm plastic petri dishes containing the bioassay medium. The majority of the spores were distributed in clumps ranging in size from 50 to 400 spores. The seeded culture plates were incubated in the dark at 13-15 C in plastic moisture chambers containing a half-saturated copper sulfate solution to retard the growth of bacteria and fungi on the inside of the container. Cultures were examined periodically from inoculation up to 56 days for type and quantity of growth. During the initial experiments, growth on basal medium plus Evans' peptone (Evans Medical Ltd., Liverpool, Speke, England) was used as a standard to compare products being tested; subsequently, Acidicase (BBL, Division of Bioquest, Cockeysville, Md.) was substituted for Evans' peptone as the standard. The plates were numerically rated from 0-4 for the amount of growth present at 28 days as follows: 0 = inhibition of spore germination; 1 = germination but no growth; 2 = slight growth; 3 = amount of growth observed on Evans' peptone; and 4 = amount of growth observed on Acidicase (full descriptions of growth are given under RESULTS).

Contamination tests.—To determine whether the rust mycelial growth obtained in culture was free of other organisms, cultures were checked for contamination. The medium and mycelium from eight petri dishes containing 28-day cultures were aseptically cut into five 1- X 3-cm blocks, and each block was placed in a test tube containing bacterial nutrient broth to test for the presence of bacteria. Tubes were incubated for 2 weeks at 25 C. The same test was repeated using potato-dextrose agar slants to test for the presence of contaminating fungi. In both tests, no growth occurred by the end of 2 weeks.

Purification and hydrolysis of casein.—Eight hundred g of nonfat dried milk were dissolved in 13 liters of glass distilled water at 4 C, and the casein was precipitated at its isoelectric point (pH 4.6) by the slow addition of HCl. The supernatant liquid was siphoned off, the precipitate was washed 3 times with water, and the casein was redissolved by adjusting the pH to 7.6 with NaOH. The process of precipitating, washing, and resuspending was repeated 3 times. The final precipitate was collected by filtration, freeze-dried, and ground to a powder in a blender.

The casein was hydrolyzed by dissolving 10 g in 150 ml of 6 N HCl and refluxing at 100 C for 6, 12, 24, or 48 hr. The HCl solution was removed under partial pressure in a rotary evaporator with a dry ice trap for rapid evaporation. The residue, a thick brown syrup, was resuspended in water, clarified by shaking 10 min with 5 g activated charcoal, and filtered. The filtrate was adjusted to pH 7.0 with NaOH and allowed to stand overnight at 4 C, and the flocculent precipitate which appeared was removed by filtration and discarded. After the final filtrate was freeze-dried, the resulting hydrolysate was weighed and stored.

Analyses.—For gel filtration chromatography, 1-g samples of Acidicase and Evans' peptone were each dissolved in 10 ml water and layered on top of separate Sephadex G-25 columns (2.5 X 145 cm; flow rate, 1 ml/min). Ten-ml fractions were collected and assayed for ninhydrin-positive materials (11).

Total nitrogen was determined by the Kjeldahl procedure (8). For elemental analysis, 1-g samples were ashed and assayed by emission spectroscopy at the Georgia Soil Testing and Plant Analysis Laboratory. Amino acid analyses were done on a Beckman/Spinco Model 120-B analyzer by the Department of Biochemistry, University of Georgia.

RESULTS.—*Description of growth.*—At 28 days, cultures on different media showed marked distinctions in growth and differentiation. The following patterns of growth were observed for each of the five ratings listed under MATERIALS AND METHODS.

Plates rated 0 showed ca. 10% germination. Most of the germ tubes present had ruptured tips and limited branching. Plates rated 1 (Fig. 1) showed normal germination with a limited number of cross walls and some branching. There was no evidence of osmotic damage. In plates rated 2 (Fig. 2, 3), a number of cross walls occurred in profusely branching germ tubes. From 8 to 28 days, a limited amount of mycelium was produced. The hyphae were 3-6 μm in diam, about one-half the width of the germ tubes. By 28 days, the production of a brown, water-soluble pigment which diffused into the medium indicated that the mycelium was senescing. None of the plates rated 0-2 showed any further development after 28 days; no uredospore or teliospore production was observed. Plates were discarded after 56 days.

In plates rated 3, maximum germination occurred within 4 days, with profuse branching of the germ tubes followed by a period of no apparent growth for 1-4 days. From the 8th to the 21st days, the branched germ tubes produced abundant mycelium which was hyaline and thin-walled, with numerous septa. At 28 days, the colony had developed a dense, cream-colored, cottony mycelium which was both embedded and aerial. Subsequently, a buff, round, flat, fleshy stroma 2-10 mm in diam was formed. By the 5th week, small amounts of the staling pigment, which had appeared in type 2 plates at 28 days, were present. During the senescent period (5-8 weeks after inoculation), the stroma produced a sheathlike coating of noncellular material. The production of uredospores (15-19 μm X 32-38 μm) was first evident at 6 weeks. In addition, bulbous structures 45-120 μm in diam and haustorialike structures previously described by Williams (17) appeared. Teliospores

were found at 8 weeks. The stroma began to take on a brownish-black, water-soaked appearance with an almost woody texture.

The pattern of development for plates rated 4 (Fig. 4, 5) was similar to those rated 3 except that growth was superior. The mycelium at 28 days was

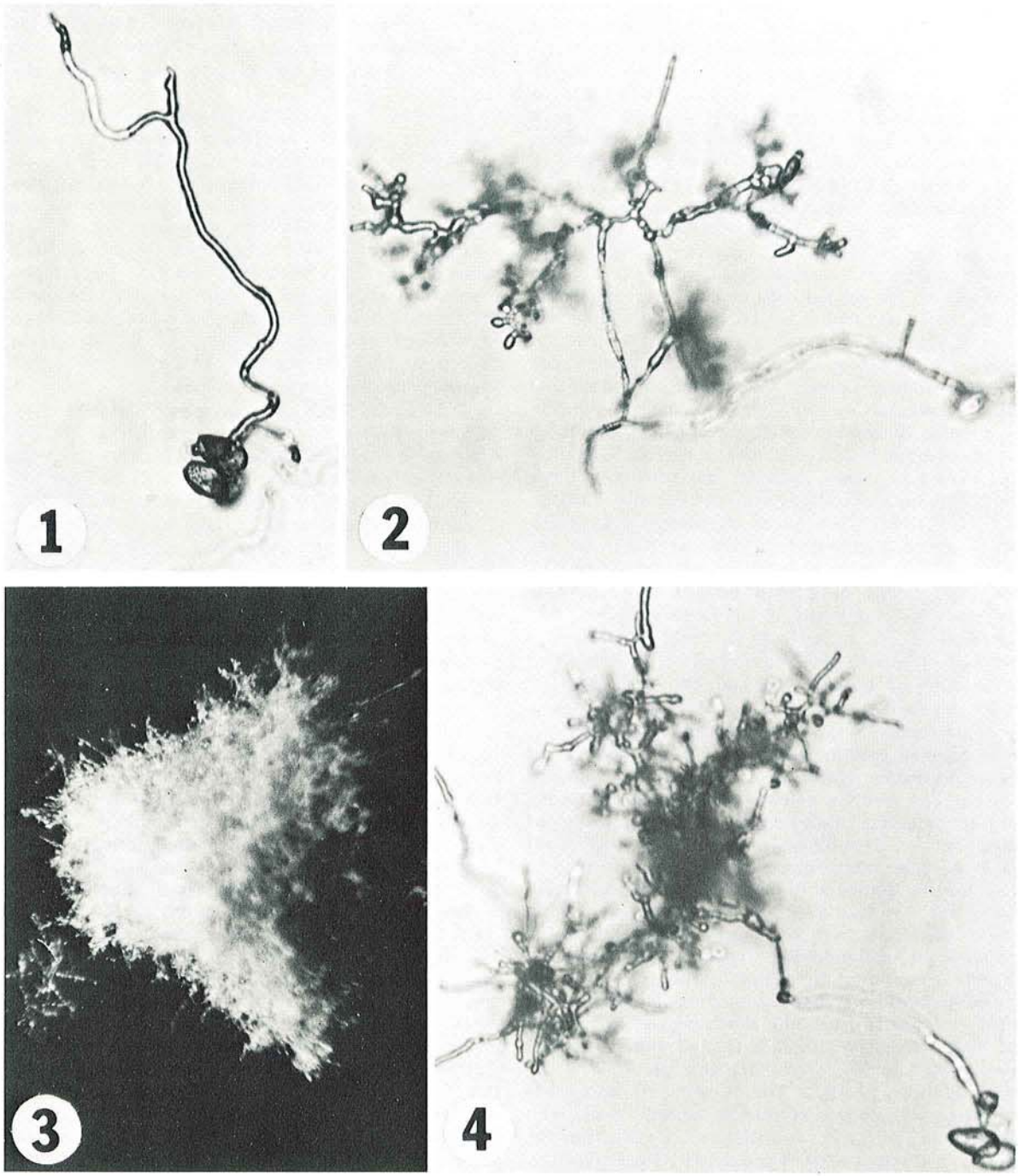

Fig. 1-4. 1) Germ tube from single uredospore of *Puccinia graminis tritici* on type 1 plate (basal medium) at 28 days (× 275). **2)** Branched germ tube from single uredospore on type 2 plate (defined casein hydrolysate medium minus trace elements with ashed Acidicase) at 28 days (× 240). **3)** Growth produced by group of spores on type 2 plate (defined casein hydrolysate medium minus trace elements with ashed Acidicase) at 28 days (× 90). **4)** Highly branched germ tube from single uredospore on type 4 plate (defined casein hydrolysate medium) at 28 days (× 240).

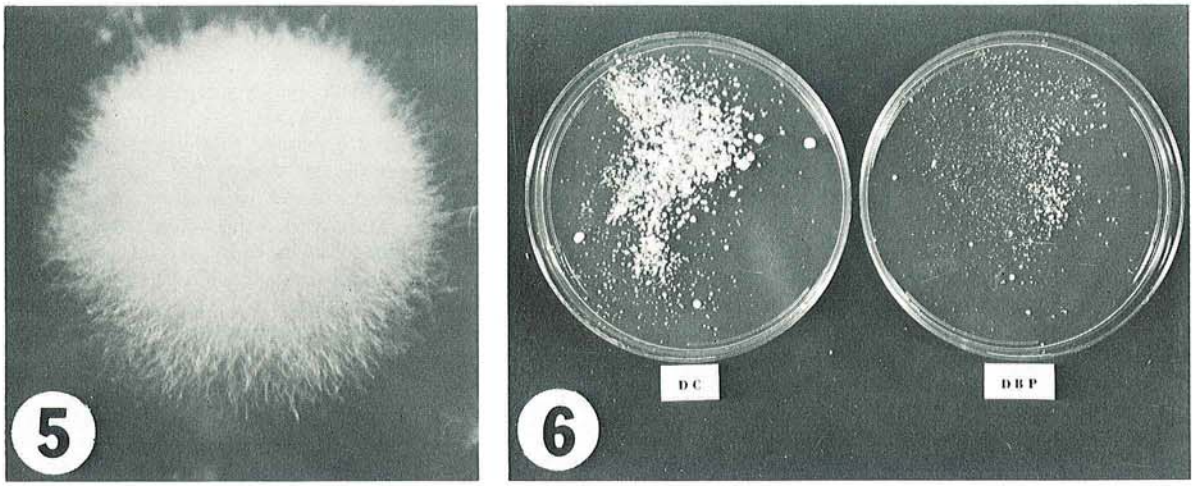

Fig. 5-6. **5)** Dense aerial mycelium of colony of *Puccinia graminis tritici* on type 4 plate (defined casein hydrolysate medium) at 28 days (× 43). **6)** Comparison of type 4 growth on defined casein hydrolysate medium (DC) and type 2 growth on defined Bacto-peptone medium (DBP), both at 28 days.

TABLE 1. Description of commercial peptones and related products and their ability to support growth of *Puccinia graminis tritici* before and after hydrolysis

Product[a]	Description	Growth rating[b]	
		Before hydrolysis	After hydrolysis
Acidicase[c]	Acid hydrolysate of casein	4	4
Acidicase (low salt)[c]	Desalted form of Acidicase	3	3
Bacto-peptone[d]	Hydrolysate of animal tissue	0	0
Biosate[c]	Plant and animal protein hydrolysate, yeast autolysate, and pancreatic casein digest	0	3
Casamino acids (vitamin free)[d]	Acid hydrolysate of casein to its amino acids	4	4
Casein hydrolysate (acid)[e]	Acid hydrolysate of casein	4	
Casein hydrolysate (enzymatic)[e]	Enzymatic hydrolysate of casein	4	
Casitone[d]	Pancreatic digest of casein	1	3
Evans' peptone[f]	Acid and enzymatic digest of whale muscle	3	3
Gelysate[c]	Pancreatic digest of gelatin	1	1
Lactalysate[c]	Pancreatic digest of lactalbumin	2	3
Milk-protein hydrolysate[c]	Pancreatic digest of milk proteins	1	3
Myosate[c]	Pancreatic digest of heart muscle	1	3
Neopeptone[d]	Enzymatic digest of protein	0	
Phytone[c]	Papaic digest of soya meal	0	
Potato-dextrose[d]	Potato extract and glucose	0	
Proteose peptone[d]	Protein digest	1	
Proteose peptone No. 2[d]	Modified Proteose peptone	1	
Proteose peptone No. 3[d]	Modified Proteose peptone No. 2	1	
Soytone[d]	Enzymatic digest of soya meal	0	
Thiotone[c]	Peptic digest of animal tissue	1	
Trypticase[c]	Pancreatic digest of casein	0	3
Tryptone[d]	Pancreatic digest of casein	0	
Tryptose[d]	Industrial secret	0	
Yeast extract[d]	Yeast autolysate	2	

[a] Added to basal bioassay medium at concentrations of 0.1% and 0.6% (w/v); each assay replicated 20 times.

[b] 0 = inhibition of spore germination; 1 = germination but no growth; 2 = slight growth; 3 = amount of growth observed on Evans' peptone; 4 = amount of growth observed on Acidicase.

[c] BBL, Division of Bioquest, Cockeysville, Maryland.

[d] Difco Laboratories, Detroit, Michigan.

[e] Nutritional Biochemical Corporation, Cleveland, Ohio.

[f] Evans Medical Ltd., Liverpool, Speke, England.

more aerial and cottony, and was almost white rather than cream. The mycelial mat was 5-15 mm in diam. The resulting stroma was lighter in color, more aerial, and less dense and fleshy. Production of uredospores, as well as senescence, occurred 2 weeks later than in type 3 colonies. During the senescent period (7-9 weeks), some teliospores, large quantities of the staling pigment, and the noncellular sheath were produced.

Stromata rated 3 and 4 were maintained for 3 months by a transfer to fresh medium every 6 weeks. By this time, all stromata appeared brownish-black and water-soaked, and had numerous teliospores on the surface. The interior of each stroma was made up of thick-walled cells resembling sclerenchyma tissue. A few thin-walled peripheral mycelial cells which contained cytoplasm (shown by trypan-blue staining) were still evident. A few plates were maintained for as long as 18 months, but no further changes were observed.

Screening of commercial peptones.—In order to select for analysis peptones which most effectively promoted growth in culture, 25 commercial products (Table 1) were added individually at a concentration of 0.1% (w/v) to the basal bioassay medium. Five products supported growth equal to or superior to Evans' peptone: Acidicase, Acidicase (low salt), Casamino acids, Casein hydrolysate (acid), and Casein hydrolysate (enzymatic) (Table 1).

Effective peptones were further tested to determine their optimal concentration in culture media. Acidicase, Casamino acids, Casein hydrolysate (acid), Casein hydrolysate (enzymatic), and Evans' peptone were tested at 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, and 1.2% (w/v) in the basal bioassay medium. Concentrations above 1.2% were not used because of the difficulty in keeping more than 1.2% Evans' peptone in solution. The results showed that 0.6% peptone gave the best growth; therefore, subsequent bioassays were performed at this concentration. The initial peptone screening of 25 products was repeated using a concentration of 0.6%, and the results were the same relative to the standard as with the lower concentration of 0.1%.

After these assays, Acidicase was selected to replace Evans' peptone as the standard for growth since it consistently gave a higher growth rating at equal concentrations and since its composition was simpler.

The ability of completely hydrolyzed casein to support growth better than a partially hydrolyzed muscle preparation (Evans' peptone) indicated that free amino acids, and not peptides, are important for growth. Therefore, it seemed logical that some peptones might be more effective if they were completely hydrolyzed to their individual amino acids. Twelve commercial peptones, selected as representative preparations which did and did not support growth, were acid-hydrolyzed for an additional 24 hr, then clarified and neutralized as described for casein. The hydrolysates were incorporated into the basal bioassay medium at a concentration of 0.6% and rated for stimulation of growth. Of the products tested, only four supported growth rated as 3 or 4 prior to additional hydrolysis. However, after hydrolysis, six additional products (Biosate, Casitone, Lactalysate, Milk-protein hydrolysate, Myosate, and Trypticase) supported growth (Table 1). Hydrolysis had no effect on Bacto-peptone and Gelysate.

Hydrolysis of casein.—Since the details of preparation of most commercial media are not generally known, it appeared necessary to prepare an hydrolysate of a natural product in order to be sure that no materials were added during commercial processing that might affect rust growth. Casein was the obvious choice for hydrolysis, since it is easy to purify and several commercial casein preparations were equally good in supporting growth. Cow's milk casein was separated from other proteins, hydrolyzed, clarified, neutralized, and incorporated into the basal medium at a concentration of 0.6%. Six, 12, 24, and 48-hr hydrolysates all produced growth equal to the Acidicase standard [rating, 4]. Nonhydrolyzed casein supported no growth [rating, 1].

Analyses.—If the amino acids in certain protein hydrolysates are responsible for growth, fractionation of peptones should give fractions that do and do not support growth. This was tested by subjecting Acidicase and Evans' peptone to gel filtration on Sephadex G-25. Fractions were collected, assayed by ninhydrin, and pooled into groups which were freeze-dried and incorporated into the basal medium at a concentration of 0.6%. The ninhydrin-positive fractions from both Acidicase and Evans' peptone promoted growth in culture equal to that of the nonfractionated controls [rating, 4 for Acidicase and 3 for Evans' peptone]. Ninhydrin-negative fractions did not support growth [rating, 1].

Additional evidence that amino acids are the major organic constituents of growth-promoting hydrolysates was obtained from other assays. When Acidicase was desalted by extraction with HCl in acetone (15) and chromatographed two-dimensionally on silicic acid thin-layer plates, 17 ninhydrin-positive spots were present. No additional spots were produced after a charring with H_2SO_4. Acidicase also gave a negative test for carbohydrate when assayed by the anthrone method (10).

Analysis of the 24- and 48-hr casein hydrolysates, dried over phosphorous pentoxide, yielded 9.4-9.8% total nitrogen and 38-39% ash. The moisture content of freeze-dried 24- and 48-hr casein hydrolysates, determined by drying over phosphorous pentoxide to constant weight, was 7.1-9.2%. By comparison, the moisture content of Acidicase was 9.2%. By using the accepted value of 15.8% nitrogen in casein (16), the calculated percentage of amino acids varied from 59 to 62%. These values compare favorably with the 64% amino acids determined by automated amino acid analysis.

The results of elemental analysis of 12 commercial peptones and a 48-hr hydrolysate of purified casein are shown in Table 2. These peptones are the same ones that were bioassayed after additional hydrolysis (Table 1); however, they were analyzed for inorganic elements before the extra hydrolysis. A comparison of the levels of both major and trace elements re-

TABLE 2. Elemental composition of 13 peptones and related products

Product	Minerals												
	%				μg/g								
	P	K	Ca	Mg	Mn	Fe	Cu	Zn	Al	Mo	Sr	Ba	Na
Acidicase	0.42	0.03	0.03	0.00[a]	17	36	4	12	24	1.6	18	0[b]	78,750
Acidicase (low salt)	0.21	0.07	0.07	0.00[a]	13	45	5	5	10	1.8	3	1	4,600
Biosate	0.89	0.98	0.13	0.07	6	15	5	5	48	2.0	6	0[b]	21,750
Casamino acids	1.05	0.33	0.01	0.00[a]	3	19	4	5	>0	1.4	9	0[b]	45,000
Casitone	0.63	0.17	0.07	0.03	4	22	22	5	35	1.9	3	1	23,750
Evans' peptone	0.78	1.21	0.06	0.10	6	51	5	38	4	2.3	3	1	7,750
Lactalysate	0.32	0.54	0.74	0.03	6	68	9	12	55	2.8	3	1	4,500
Milk-protein hydrolysate	0.61	0.16	0.34	0.02	4	18	5	5	94	1.7	9	1	42,250
Myosate	0.49	0.78	0.02	0.07	3	27	6	8	28	2.1	3	1	12,500
Trypticase	0.70	0.10	0.18	0.00[a]	6	16	6	7	78	2.1	5	1	44,250
48-hr hydrolysate of casein	0.42	0.18	0.09	0.00[a]	44	84	30	11	128	2.3	20	11	NA[c]
Bacto-peptone	0.22	0.16	0.01	0.01	4	22	5	7	35	1.9	2	0[b]	13,250
Gelysate	0.10	0.07	0.04	0.02	3	28	13	9	10	2.3	2	0[b]	9,250

[a] Less than 0.01%.
[b] Less than 1 ppm.
[c] NA = information not available.

vealed no consistent pattern between the products that supported growth before hydrolysis (both Acidicases, Casamino acids, Evans' peptone), those that supported growth after hydrolysis but not before (Biosate, Casitone, Lactalysate, Milk-protein hydrolysate, Myosate, Trypticase), and those that did not support growth even after hydrolysis (Bacto-peptone, Gelysate) (Table 2). Extreme differences often existed between levels in different products that promoted growth: 0.03% K in Acidicase versus 1.21% in Evans' peptone; 5 μg/g Zn in Casamino acids versus 38 μg/g in Evans' peptone; 18 μg/g Sr in Acidicase versus 3 μg/g in Acidicase (low salt). The high amounts of Na were due to the salts formed during neutralization of acid in the preparation of the products.

Amino acid analyses were made on the 24-hr and 48-hr hydrolysates of purified casein and on 10 commercial peptones subjected to additional hydrolysis. Since Acidicase and Casamino acids were found to contain few, if any, peptides, they were analyzed directly as the commercial preparations. The other eight peptones were analyzed after the additional hydrolysis. The results shown in Table 3 indicate distinct differences in the amino acid composition of the effective [products CH_1, CH_2, 1, 4, 5, 8, 9, 12, 13, 22] and noneffective [products 3, 10] peptones. Nine of the 10 products which promoted growth had glutamic acid in greatest concentrations (17-30%). These products had low concentrations of glycine, phenylalanine, and tyrosine; tryptophan was completely absent. Aspartic acid, leucine, lysine, and proline were present in moderately high concentrations. Evans' peptone was somewhat different in that it contained only 10% glutamic acid, and had lysine as the major constituent. The composition of the two noneffective products (Bacto-peptone and Gelysate) was almost identical. The concentration of glycine was nearly twice that of any other amino acid present. Amino acids in moderately high concentrations were alanine, arginine, aspartic acid, glutamic acid, lysine, and proline. The presence of tryptophan indicates that large amounts of this amino acid must have been present prior to the additional 24-hr hydrolysis, since it is usually destroyed by acid hydrolysis.

Composition of defined medium.—The amino acid composition of the 24-hr hydrolysate of casein ('CH_1', Table 3) was used as the basis for formulation of a defined medium. Since a dried preparation of this casein hydrolysate contained 62% amino acids, a bioassay medium made with 0.6% casein hydrolysate contained 0.372% amino acids. Thus, a synthetic medium was prepared with 3.72 g/liter of a mixture of individual amino acids (Sigma Chemical Co., St. Louis, Mo.) in the same proportion as those in the casein hydrolysate.

Since the hydrolysates of natural products contained trace elements not supplied in the basal medium, and since the proportions of these elements did not appear critical for growth (Table 2), a standard trace element solution was used with the amino acid mixture.

The final synthetic casein hydrolysate medium contained 10 g/liter agar; 30 g/liter glucose; Czapek's mineral solution (7) with 14.4 mg/liter ferric EDTA instead of ferrous sulfate; 2 ml/liter Burkholder & Nickell's trace element solution (2) minus ferric tartrate; and the following amino acids in mg/liter: alanine, 114; arginine, 159; aspartic acid, 281; glutamic acid, 863; glycine, 71; histidine, 122; isoleucine, 201; leucine, 292; lysine, 287; methionine, 110; phenylalanine, 125; proline, 376; serine, 207; threonine, 163; tyrosine, 110; valine, 242.

The results from four different experiments with a total of 140 replicates showed that growth on the

TABLE 3. Amino acid composition of 12 peptones and related products

Amino acid	Product[a]											
	CH_1	CH_2	1	4	5	8	9	12	13	22	3	10
Ala	3.0[b]	2.9	4.0	4.9	3.3	3.4	5.5	3.8	9.3	3.2	9.2	8.6
Arg	4.9	3.3	3.4	4.4	4.0	4.0	13.2	3.3	6.8	2.8	12.8	10.0
Asp	7.6	6.7	12.0	8.4	7.2	7.7	7.1	9.0	10.1	7.2	6.9	6.9
Cys-[c]	0.0	0.0	tr[d]	tr	0.0	1.5	0.0	0.0	0.0	tr	0.0	0.0
Cys	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glu	23.2	31.5	30.3	21.6	22.6	25.4	10.3	22.7	16.9	22.6	10.9	11.7
Gly	1.9	1.7	2.3	2.9	1.9	2.0	3.6	1.9	7.0	1.5	23.5	27.6
His	3.3	2.4	2.7	3.4	3.0	1.9	12.1	3.4	2.8	2.7	1.4	1.7
Ile	5.4	4.6	2.4	5.4	4.9	5.5	3.5	5.3	5.0	5.8	2.1	1.6
Leu	7.8	8.6	4.2	11.1	8.2	10.0	6.7	11.7	9.0	10.1	4.1	3.2
Lys	7.7	6.6	8.5	8.3	0.1	8.6	22.3	10.9	7.7	9.1	7.2	5.2
Met	3.0	2.3	2.8	2.5	3.0	3.4	1.9	2.4	2.8	3.1	1.2	1.1
Phe	3.4	2.9	2.8	2.0	4.5	2.4	1.1	1.6	1.6	2.0	0.4	tr
Pro	10.1	9.7	9.6	8.0	11.4	7.6	2.7	9.1	5.7	13.3	9.6	12.9
Ser	5.6	4.8	4.9	5.4	5.8	4.5	2.8	4.2	4.4	5.1	3.4	3.8
Thr	4.4	3.8	4.2	4.3	4.5	4.0	3.5	3.8	4.8	4.2	2.3	1.8
Try	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.7
Tyr	3.0	2.6	1.3	0.6	tr	0.8	0.6	0.4	0.5	0.6	tr	0.0
Val	6.5	5.8	4.0	6.8	6.4	7.2	3.3	6.8	5.6	6.6	3.3	2.1

[a] In the subheading below CH_1 is the 24-hr acid hydrolysate of casein and CH_2 is the 48-hr acid hydrolysate of casein; all other numbers refer to the corresponding commercial products in Table 1.
[b] Percentage of total amino acids.
[c] Half cystine.
[d] Trace.

defined casein hydrolysate medium was consistently type 4 (Fig. 4, 5, 6; Table 4). In each experiment, growth appeared identical on defined medium, Acidicase, and casein hydrolysate.

Other synthetic media.—When trace elements were omitted from the defined casein hydrolysate medium, growth was type 2 (Table 4). If ashed Acidicase (added to the medium in an amount equivalent to the inorganic elements supplied by 0.6% Acidicase) was substituted for the trace elements, growth was also type 2 (Fig. 2, 3).

A synthetic Bacto-peptone medium, prepared with a mixture of amino acids in the same proportion as those found in Bacto-peptone (Table 3), did not support growth (Fig. 6; Table 4).

In order to determine if the tryptophan present in Bacto-peptone was inhibiting growth, we prepared defined media with and without tryptophan at 63 mg/liter (the level present in a Bacto-peptone medium). Growth on synthetic Bacto-peptone medium was not increased when tryptophan was omitted; conversely, growth on synthetic casein hydrolysate was not inhibited when tryptophan was added (Table 4).

DISCUSSION.—One of the major problems in understanding the nutrition of race 126-ANZ-6,7 of *Puccinia graminis tritici* has been the necessity of including undefined natural products in the growth media. Initially, Williams et al. (18) added yeast extract to a basal medium containing carbohydrate and major elements; later they found that growth was improved when the yeast extract was supplemented with Evans' peptone (19). Bushnell (3) found yeast extract unsatisfactory, but Evans' peptone without the yeast extract gave good results. Others have reported that growth on Evans' peptone was not improved by the addition of yeast extract (6, 20). Casein hydrolysates were shown as effective as Evans' peptone, and mixtures of casein hydrolysates and Evans' peptone were even better (4). Other peptones, such as Difco Bacto-peptone, have repeatedly failed to support growth (20; W. R. Bushnell, *personal com-*

TABLE 4. Growth of *Puccinia graminis tritici* on different defined media

Synthetic media	Growth rating[a]
Synthetic casein hydrolysate	
Complete[b]	4
Plus tryptophan	4
Minus trace elements	2
Synthetic Bacto-peptone	
Complete[c]	2
Minus tryptophan	2

[a] 2 = slight growth; 4 = amount of growth observed in Acidicase.
[b] Basal bioassay medium, amino acids in the same proportion as those in a casein hydrolysate, and Burkholder & Nickell's trace elements.
[c] Basal bioassay medium, amino acids in the same proportion as those in Bacto-peptone, and Burkholder & Nickell's trace elements.

munication). Some of these variations may be due to variations in commercial products used for media. The cation composition of yeast extract and peptones, for example, apparently varies not only among producers but also among batches from the same producer (1). However, the fact that some peptones are consistently effective in culture media, whereas others are not, indicates that growth of the rust fungus is controlled to some extent by the composition of the organic nitrogen source in the medium.

Attempts to grow race 126-ANZ-6,7 on defined media have only been partially successful. Kuhl et al. (9) reported some growth on a mixture of 20 amino acids, on the sulfur group of amino acids, and on aspartic acid plus some of the sulfur amino acids. However, growth on these combinations was no more than half that on a yeast extract, Evans' peptone control.

When the present work was started, several classes of compounds appeared to be candidates for supporting growth in culture since commercial peptones are essentially complex mixtures of peptides, amino acids, inorganic elements, and vitamins. However, screening experiments showed that peptides were not the critical factor because the rust fungus grew on Casamino acids (Table 1), which is composed of totally hydrolyzed casein. The results of gel filtration chromatography of Acidicase and Evans' peptone showed that the fraction associated with the ninhydrin-positive peak promoted growth. Thin-layer chromatography of Acidicase revealed the presence of 17 ninhydrin-positive spots which could be small peptides or amino acids. There was no evidence of contaminating substances. This increased the probability that combinations of amino acids were important in determining growth. A noncommercial preparation of purified casein was subjected to a time-course hydrolysis to determine whether prolonged hydrolysis would destroy its growth-promoting ability. The 48-hr hydrolysate still gave type 4 growth. After hydrolysis for 48 hr, the critical components would probably be in the inorganic element or amino acid portion of the hydrolysate.

Since Acidicase, Casamino acids, and casein hydrolysates (both acid and enzymatic) supported growth, the question arose as to why Casitone and Trypticase, which were also casein products, failed. Since several products, including these, were effective after 24 hr of additional hydrolysis, some of the commercial preparations prepared by enzymatic digestion probably contained peptides which did not promote growth. Thus, the rust organism apparently cannot utilize amino acids which are tied up in peptides or intact protein for sustained growth. Also, the free amino acid balance in a product is altered after additional hydrolysis.

Amino acid analyses revealed a pattern in the distribution of amino acids in the products which did and did not promote growth. The nearly identical amino acid composition of Bacto-peptone and Gelysate (Table 3), two products which did not support growth (Table 1), showed high concentrations of glycine and the presence of tryptophan. The products which did promote growth were high in glutamic acid, much lower in glycine, and lacking tryptophan. If the fungus requires a specific combination of amino acids, then it should have been possible to duplicate the complex mixture of amino acids in one of the effective peptones and obtain growth.

However, supplying a synthetic mixture of amino acids was effective only if trace elements were added. Type 2 growth occurred when the synthetic casein hydrolysate medium contained amino acids without trace elements. Since elemental analysis did not show any particular distribution of elements which would account for growth, Burkholder & Nickell's trace elements (2) minus ferric tartrate (ferric ion supplied in Czapek's minerals) was selected because it resembled the composition in some peptones. Together, Czapek's and Burkholder & Nickell's solutions furnished all elements shown in the peptone analyses except Ca, Al, Sr, and Ba. Strangely, ashed Acidicase did not substitute for Burkholder & Nickell's elements. Apparently, something in the ash was inhibitory to growth.

The failure of the synthetic Bacto-peptone medium to support growth (Fig. 6; Table 4) indicates that the different amino acid compositions in casein hydrolysate and Bacto-peptone are critical for growth of the rust fungus. The only qualitative difference in the amino acids of the two products is in tryptophan, which is absent in casein hydrolysate and present in Bacto-peptone as 1.8% of the total amino acids. To test a possible role of tryptophan, synthetic Bacto-peptone and synthetic casein hydrolysate, each with and without tryptophan, were bioassayed. The presence or absence of tryptophan did not affect the respective types of growth on each medium (Table 4), showing that tryptophan is not inhibitory.

The fact that the 16 amino acids found in casein hydrolysate do not promote growth when combined in different proportions in the synthetic Bacto-peptone minus tryptophan, together with our previous observations of growth on different peptones, supports the hypothesis that the balance of certain amino acids is a determining factor in obtaining in vitro growth of race 126-ANZ-6,7 of wheat stem rust.

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