Nutritional Requirements for the Growth in Pure Culture of the Myxomycete *Physarum rigidum* and Related Species

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SUMMARY

Physarum rigidum grew and sporulated in pure culture at pH 4.2 on a partially defined medium containing: mineral salts, glucose, yeast extract, haematin, casein hydrolysate. The inoculum equiv. 0.22 mg. protein/25 ml. grew to equiv. 21.5 mg. protein/25 ml. in about 12 days. *Physarum flavicomum* varieties 1 and 2 also grew and sporulated on this medium. The yeast extract and haematin were essential for growth; the casein hydrolysate was not. Omission of glucose resulted in a 50% decrease in growth yield. Ethanol, galactose, glycerol, lactose, mannitol, mannose, potato starch, raffinose or sorbitol could replace glucose, but the growth yield was decreased. Fructose, inulin, sucrose and the pentoses and carboxylic acids tested did not support growth.

INTRODUCTION

Although there are over 450 known species of myxomycetes, the plasmodia of less than 10% of them have been grown in the laboratory in crude culture. Of these, only a few species have been grown on media which did not contain living or killed micro-organisms as a food source. According to Alexopoulos (1963) these include only *Licea flexuosa*, *Fuligo cinerea* and *Physarum polycephalum*. Ross (1964) reported the growth of *Physarella oblonga* and *Physarum flavicomum* on a partially defined medium but observed a gradual decline in the growth rate of the latter (Ross & Sunshine, 1965). Our previous paper (Henney & Henney, 1968) gave a genetic and morphological characterization of *P. rigidum* and *P. flavicomum* varieties 1 and 2. The present paper gives details for growth of these organisms in pure culture on a partially defined medium. They have been serially transferred on solid and liquid medium for more than 1 year with no alteration in characteristics or growth rate. This is the first report of the laboratory cultivation of *P. rigidum* in pure or crude culture. The composition of the medium developed is qualitatively and quantitatively different from those previously reported for other myxomycetes but is similar to that reported for *P. polycephalum* (Daniel & Rusch, 1961). Some of the nutritional requirements for *P. flavicomum* reported by Ross (1964) and Ross & Sunshine (1965) were not substantiated.

METHODS

Cultures. Axenic clones (myxamoebae) of the organisms studied were established by the methods previously described (Henney & Henney, 1968). Axenic plasmodia were produced by mixing two compatible clones together on half-strength corn meal agar

with the addition of formalin-killed *Aerobacter aerogenes* (or *Escherichia coli*). The cultures were incubated for several weeks at 22° after which time small plasmodia began to appear. Small amounts of sterile, long-cooking, ground oats were sprinkled onto the plasmodia. Incubation was continued until the plasmodia had reached a large size. Small pieces of the plasmodia were cut out, placed in the same bacteriological test media used for testing purity of the myxamoebae and incubated at 25° and 37° for 1 month. When bacterial growth developed in any of the test media, the plasmodial cultures were discarded and the entire procedure repeated. The axenic plasmodia thus developed could be maintained in pure culture for long periods of time. However, after several months of cultivation on oats, they accumulated large amounts of 'slime' and died unless transferred for a few times on the killed bacteria medium. The organisms also utilized oats overlaid with agar.

Plasmodia were cut from these plates, using a surgical blade sterilized in a flame, and transferred to sterile partially defined test media in glass Petri dishes. The cultures were subcultured to fresh media every 7 days. All transfers were made in a culture room with an air-filtration system and positive pressure. The Petri dishes were incubated at 22° and kept in the dark except during periods of examination.

Plasmodium was adapted to the liquid medium by transferring a block of agar carrying plasmodium to a sterile 500 ml. Erlenmeyer flask containing 50 ml. liquid medium. The flask was incubated inclined so that the liquid did not touch the plasmodium while it crawled onto the glass. The liquid was then allowed to touch the plasmodium until the latter floated, and the flask was aerated on a New Brunswick Scientific Co. (New Brunswick, New Jersey, U.S.A.) Model G 25 Gyrotory shaker at 170 rev./min. and room temperature (22° to 25°). The glass window of the shaker was covered to exclude light, and the pure cultures were exposed to light only during periods of examination. The plasmodium was soon converted into numerous microplasmodia which enabled replicate samples to be removed for inocula and analyses. Size of microplasmodial inoculum was measured as equiv. mg. protein/ml.

Stationary liquid cultures did not degenerate. The microplasmodial inoculum would form branching filaments, anastomose and crawl along the flask bottom while some floated to the top and adhered to the glass at the air/liquid interface. The plasmodia also grew well in 3 l. batches in a New Brunswick Fermentor Model FS 305 at about 1 l. air/min. at 200 rev./min. and room temperature.

Glassware. All glassware was cleaned with detergent and rinsed with tap water and glass-distilled water. Pipettes were cleaned in sulphuric+chromic acid cleaning solution and rinsed as above.

Water. Laboratory-distilled water (about 25 p.p.m. NaCl) was redistilled in a Corning Glass Works (Corning, New York, U.S.A.) Distillation Unit Model AG-3; the final product had less than 1 p.p.m. NaCl. This glass-distilled water was used throughout the work.

Reagents and substrates. All reagents were of Analytical Reagent grade. The substrates glucose, inulin, lactose, D-mannitol, raffinose, sorbitol and D-xylose were Difco (Detroit, Michigan, U.S.A.) products. Baker (Phillipsburg, New Jersey, U.S.A.) potassium acetate and purified potato starch, Eastman (Rochester, New York, U.S.A.) L-malic acid, Matheson (Norwood, Ohio, U.S.A.) fumaric acid, glycerol, sodium pyruvate and sucrose, and Calbiochem (Los Angeles, California, U.S.A.) ascorbic acid were used. L-Arabinose C.P. and D-galactose C.P. were obtained from Pfanstiehl

Laboratories (Waukegan, Illinois, U.S.A.), and sodium citrate U.S.P. from Allied Chemical (New York, N.Y., U.S.A.). Malonic acid, D-mannose and sodium succinate were from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.), and β -D-fructose, inositol, DL-lactate, and D-ribose from Sigma (St Louis, Missouri, U.S.A.).

Media. The composition of the medium finally developed is given in Table 1. Experiments at different pH values were made by changing the proportions of citric acid to potassium phosphate while maintaining the same total molarity. Growth was also determined at various concentrations of the basal salt mixture.

Table 1. *Medium for growth of Physarum rigidum*

Complete medium	
Basal salts mixture solution (pH 4.0)*	100 ml.
Trace elements solution†	0.1 ml.
Glucose (Difco)	5.0 g.
Yeast extract (Difco)	5.0 g.
N-Z Case (Sheffield)	5.0 g.
Agar (Difco)	25.0 g.
Glass-distilled water	to 1000 ml.

Autoclave (121°, 15 min.) and cool to about 50°; add 1 ml. sterile 0.25% (w/v) haematin (haemin (Eastman, Rochester, New York, U.S.A.) in 1% NaOH; autoclaved); pour completed medium into sterile Petri dishes; final pH is about 4.2. Omit agar for liquid cultures.

* Basal salts mixture solution (pH 4.0). Add successively to about 700 ml. glass-distilled water (final volume to 1000 ml.) with stirring (g.): citric acid, 29.78; K_2HPO_4 , 33.10; NaCl, 2.50; NH_4NO_3 anhydrous, 10.00; $MgSO_4 \cdot 7H_2O$, 1.00; $CaCl_2 \cdot 2H_2O$, 0.50.

† Trace elements solution. Dissolve successively in 95 ml. glass-distilled water with stirring (g.): citric acid, 5.00; $ZnSO_4 \cdot 7H_2O$, 5.00; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1.00; $CuSO_4 \cdot 5H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.05; H_3BO_3 anhydrous, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.05; $Co(Cl)_2 \cdot 6H_2O$, 0.05.

Difco Tryptone, Proteose-peptone, or Casamino acids and Sheffield (Norwich, New York, U.S.A.) N-Z Amine type A could be substituted for the Sheffield N-Z Case with no effect on growth yield. However, the first two protein hydrolysates led to abundant slime production on agar media. N-Z Case is a tryptic digest of casein in powder form and N-Z Amine is a pancreatic casein hydrolysate.

For liquid cultures, 50 ml. of media were used in 500 ml. Erlenmeyer flasks or 25 ml. in 250 ml. flasks; increased quantities of media resulted in decreased growth yields. A few drops of microplasmodial suspension (measured as equiv. mg. protein/ml.) were used as inoculum.

Protein analyses. Growth of the plasmodia was measured as an increase in protein content (mg./ml. culture) determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin (Armour, Kankakee, Illinois, U.S.A.) as a standard. Samples of the culture medium (2 ml.) or the inoculum samples were centrifuged at about 6000 g for 5 min. The yellow pigment was removed by incubating the pellet with 2 ml. acetone + 2 ml. 10% (w/v) trichloroacetic acid solution for a few hours at room temperature, or overnight at 4°. The suspension was centrifuged at 12,000 g for 5 min., and the pellet dissolved in 0.4 N-NaOH by boiling for about 5 min. Samples were then analysed for protein content.

RESULTS

In general, the plasmodia did not grow well in plastic Petri dishes. This was especially true of *Physarum rigidum*, but *P. flavicomum* variety 1 grew better than the others under these conditions. This inhibition was quite significant and seemed to be due to

the lower humidity in the plastic dishes (because of less tightly fitting lids) rather than to the presence of a metabolic inhibitor.

Plate 1 shows cultures of *Physarum rigidum*, *P. flavicomum* variety 1 and *P. flavicomum* variety 2 on complete medium. There were distinct stable differences in morphology and colour among the plasmodia when grown under identical environmental conditions. The plasmodium of *P. rigidum* was yellow with a greenish tinge, very thin and spread out with an almost smooth edge. *P. flavicomum* variety 1 was almost orange, much thicker and grew in long finger-like projections. *P. flavicomum* variety 2 was lemon yellow, grew in a thin sheet but without the smooth edge or the long finger-like projections of the other two. Pure cultures of these organisms fused with themselves but not with each other. This extends the reports on crude cultures of plasmodia of different species which did not fuse (Alexopoulos, 1963) and different geographical isolates of the same species which did not fuse (Gray, 1945) to pure cultures as well. Carlile & Dee (1967) have recently reported similar results on fusion with pure cultures of *P. polycephalum*.

The organisms often sporulated after 10 days of incubation on the solid medium. *Physarum flavicomum* variety 1, in particular, formed fruiting bodies in at least 80 % of the cultures. These spores, and those of *P. rigidum*, germinated yielding viable myxamoebae which increased in numbers and fused to form plasmodia in the presence of formalin-killed bacteria. Therefore the complete life-cycles of both *P. rigidum* and *P. flavicomum* have been completed in pure culture. *P. flavicomum* variety 1 also readily sporulated on solid medium in plastic Petri dishes.

The optimum temperature range for plasmodial growth of both species was between 22° to 25°; plasmodia did not survive above 30° or below 10°.

The only essential nutritional requirements for *Physarum rigidum* were the basal salts mixture, a carbohydrate, the yeast extract, and haematin (Table 1). Omission of the trace elements solution, the protein hydrolysate (N-Z Case) and of ammonium nitrate from the basal salts mixture had no effect on the final growth yield. The initial growth rate, however, was slower when a protein hydrolysate was omitted (Fig. 1). According to Difco Laboratories, Inc., Bacto Yeast Extract contains 13 amino acids. The complete medium is presented here (Table 1) since it may have application for other myxomycetes with more stringent growth requirements. The essential requirements for *P. rigidum* were also essential for *P. flavicomum*.

The optimum concentration of yeast extract was 0.5 % (w/v), haematin 2.5 µg./ml. (Fig. 1, 2) and carbohydrate 0.5 % (w/v). The concentration of essential nutrients was critical; in many instances a twofold increase in the optimum concentration resulted in death of the plasmodia. These concentration optima were not determined for *Physarum flavicomum*.

Physarum rigidum grew well at pH 3.0 to 4.2; 4.2 was usually selected since at the higher value haematin was more soluble and the agar solidified better. The pH value of the medium always increased during growth, regardless of the carbon source.

Although the basal salt mixture at the standard concentration of 0.77 % (w/v) or $\frac{1}{2}$ concentration were equally effective, the higher concentration was selected because of its greater buffering capacity. A twofold increase or a tenfold dilution of the standard salt mixture resulted in about a 50 % decrease in the maximum growth yield. *Physarum flavicomum* had the same optima of pH value and salt mixture concentration.

A typical growth curve for *Physarum rigidum* is shown in Fig. 3. After the maximum

plasmodial protein content was attained, there was a rapid decrease in this fraction. *P. flavicomum* variety 1 plasmodia developed a maximum protein content about 2.5 times greater than *P. rigidum*.

Older cultures accumulated abundant amounts of viscous materials when grown in shake culture with certain carbohydrates, especially in the presence of protein hydrolysates. Galactose, glucose, mannose and most carbohydrates containing them were most active in slime production. The plasmodia were separated from the growth media by centrifugation. The slime, collected by winding it on a glass rod from ethanol-layered supernatant fluid, was soluble in boiling water and gave no colour with iodine; its composition and production are being investigated. Presumably the casein hydrolysate was carbohydrate-sparing and enhanced polysaccharide production.

Fig. 1. Yeast extract (●) and casein hydrolysate (N-Z Case) (○) requirements of *Physarum rigidum*. Shake cultures were analysed for protein after 8 days incubation in various concentrations of nutrients. The inocula contained 0.10 mg. protein/25 ml.

Fig. 2. Effect of variable concentrations of haematin on growth of *Physarum rigidum*. The shake cultures were analysed for protein at intervals for a total of 14 days. The maximum protein/25 ml. of medium attained during this time period is plotted. The inocula contained 0.13 mg. protein/25 ml.

Fig. 3. Growth of *Physarum rigidum* in shake culture on the complete medium. Two-ml. samples were removed and analysed for protein. The inoculum contained 0.22 mg. protein/25 ml.

Some of the carbon sources tested with *Physarum rigidum* are given in Table 2. All aldohexoses and alcohols tested, lactose, potato starch and raffinose gave good growth. Those substances which did not give good growth (at 0.5 %, w/v) included: arabinose, fructose and its polymer inulin, inositol, ribose, sucrose, and xylose. Acetate, ascorbate, citrate, fumarate, DL-lactate, L-malate, malonate, pyruvate and succinate were all toxic at a concentration of 0.5 % (w/v).

Table 2. *The effect of carbon sources on growth of Physarum rigidum*

Compound	Growth yield* (max. mg. protein/ 25 ml.)	Ratio of growth to growth with glucose	Compound	Growth yield* (max. mg. protein/ 25 ml.)	Ratio of growth to growth with glucose
Glucose	22.5 ± 1.1	1.00	Sorbitol	17.2 ± 1.3	0.76
Lactose	20.6 ± 2.4	0.92	Starch (potato)	16.8 ± 1.0	0.75
D-Mannitol	20.1 ± 0.1	0.89	Glycerol	15.0 ± 0.0	0.67
Ethanol	19.9 ± 0.4	0.89	D-Fructose	12.6 ± 1.4	0.56
D-Mannose	19.8 ± 1.7	0.88	Sucrose	12.0 ± 0.8	0.53
Raffinose	19.0 ± 1.1	0.84	D-Ribose	12.0 ± 0.1	0.53
D-Galactose	18.6 ± 1.5	0.83	None	11.8 ± 1.3	0.52

* Mean value ± average deviation from the mean based on four experiments.

DISCUSSION

The growth requirements of *Physarum rigidum* differ from those of *P. polycephalum* as reported by Daniel & Rusch (1961). We found no absolute requirement for Tryptone and the optimum concentration of yeast extract was 3.3 times greater than for *P. polycephalum*. However, the optimum haematin concentration of 2.5 µg./ml. was the same for both organisms on a partially defined medium (Daniel, Kelley & Rusch, 1962). The growth rate and pH optimum were lower in the case of *P. rigidum* and important differences existed in the utilization of carbon sources. Daniel & Baldwin (1964) reported that *P. polycephalum* utilized fructose well, ethanol slowly, but that potato starch, D-galactose and glycerol supported little or no growth. Our results with these substrates and *P. rigidum* were just the reverse. Fructose and its polymer inulin were not utilized and the other compounds were utilized rather well.

The observations of an increase in pH value and formation of viscous polymers during growth of *Physarum rigidum* suggests that the organism might tend to polymerize available carbohydrates rather than actively catabolize them. The ready use of the sugar alcohols could provide an available source of biochemical energy in the form of reduced pyridine nucleotides.

The medium reported here for *Physarum flavicomum* is much simpler than, and quite different from, that indicated by Ross (1964) and Ross & Sunshine (1965). Most importantly, we found no requirement for corn meal agar, or for quinic or gallic acids either for growth or sporulation of this organism as reported by those authors. There were also significant differences in optima of pH value and salt concentration. It should be noted that we used the same variety of *P. flavicomum* as used by them (variety 1), but a different isolate. The present work was done with the PHILIPPINE II isolate while they worked with the AFRICA isolate. These two isolates formed a compatible allelic series (Henney & Henney, 1968).

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1.0 cm. wire

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EXPLANATION OF PLATE

Pure cultures on complete medium. (a) *Physarum rigidum*, *P. flavicomum* variety 1, and *P. flavicomum* variety 2; (b) *P. rigidum* and *P. flavicomum* variety 2; (c) *P. rigidum* and *P. flavicomum* variety 1; (d) *P. flavicomum* variety 2 and *P. flavicomum* variety 1. The length of the wire is 1.0 cm.