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A defined minimal medium for axenic strains of *Dictyostelium discoideum*

(slime mold/auxotroph selection)

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ABSTRACT **A defined, minimal medium for the cellular slime mold *Dictyostelium discoideum* is reported.**

Dictyostelium discoideum is the species of cellular slime mold most frequently used to study problems of development. The unicellular amoebae of this organism can be made to aggregate and form a rudimentary tissue containing two major cell types. The shift from growing amoebae to aggregating social amoebae is brought about by starvation. Many enzyme activities increase during aggregation and later development (1) and these may reflect changes in gene activity. It is our intention to approach problems of gene regulation in *Dictyostelium* with genetic and biochemical techniques. Until now the genetic approach has been limited to parasexual methods (2, 3) and suffers from a dearth of selectable chromosomal markers. A defined medium would solve this problem by letting us isolate auxotrophs.

Slime mold amoebae usually grow by degrading ingested bacteria. In 1967, Sussman and Sussman (4) isolated a strain that could grow in a broth made of Proteose Peptone, yeast extract, glucose, phosphate, liver extract, and fetal calf serum. Since then, the liver extract and fetal calf serum have been removed (5, 6) and other axenic strains have been isolated (6, 7). Two recessive mutations on the second and third linkage groups distinguish axenic strains from the wild type and allow them to grow axenically (8). Marin (9) was able to grow the AX3 strain in a medium lacking Proteose Peptone and containing a reduced amount of yeast extract. Growth in this medium was dependent on 11 amino acids, as shown by single omission experiments. Watts and Guest (10), using auxotrophic strains of *Escherichia coli*, were able to specify five vitamins required by *D. discoideum*.

Our strategy for creating a totally defined medium was to assemble all of the compounds known to occur in Proteose Peptone and yeast extract (11, 12) and to supplement them with nutrients known to be essential for the growth of other unicellular eukaryotes. The axenic strain AX3 grew in this basal medium immediately. All unnecessary compounds have now been removed and the conditions under which the cells grow best have been defined.

MATERIALS AND METHODS

Strains. The axenic strain AX3 was used in this study. Amoebae growing in HL/5 medium were harvested by centrifugation during logarithmic phase, washed twice with phosphate buffer (17 mM Na_2HPO_4/KH_2PO_4, pH 6.0), and resuspended in the same buffer at a density of 2×10^6/ml. Five milliliters of medium in 50-ml erlenmeyer flasks were inoculated with the cell suspension to a density of 4×10^4 amoebae per ml. The cultures were incubated at 21–22° on a gyratory shaker (120 rpm; ¾-inch stroke). Cells were counted with a hemacytometer. The cells were tested for their ability to aggregate and form fruiting bodies as described by Sussman (13).

Composition of the Media. The HL/5 medium contained (per liter): glucose, 10 g; yeast extract (BBL), 5 g; Thiotone (BBL), 5 g; Proteose Peptone (Difco), 5 g; KH_2PO_4, 0.34 g; $Na_2HPO_4 \cdot 7H_2O$, 0.67 g; and dihydrostreptomycin sulfate, 50 mg. The final pH was 6.5–6.7 and the medium was sterilized by autoclaving for 20 min at 121°. The defined medium described in the final column of Table 1 was constructed from concentrated stock solutions of amino acids, glucose, vitamins, salts, trace metals, and buffer. Stock solutions were stored frozen. The complete medium was autoclaved for 20 min at 121° and can be stored in the cold for several months.

Materials. All the amino acids, "Good" buffers, ascorbic acid, cyanocobalamin, folic acid, lipoic acid, riboflavin, thiamine·HCl, adenosine, uridine, sugars (except glucose), and dihydrostreptomycin sulfate were obtained from Sigma. Special agar (Noble) was obtained from Difco. Biotin, *i*-inositol (*meso*), nicotinic acid, calcium pantothenate, and pyridoxine·HCl were supplied by Gibco; vitamin D_3 and vitamin E (acetate) were bought from Nutritional Biochemical Co. Choline chloride and *p*-aminobenzoic acid were purchased from Eastman. All other chemicals were reagent grade. Single distilled water supplied by the Biological Laboratories was used in all preparations.

RESULTS

The compositions of HL/5 and the initial basal medium are shown in Table 1 along with two simplifications resulting in the final minimal medium (FM). Comparison of columns M1 and M2 shows that 16 compounds were eliminated during the initial simplification of the basal medium. The omission of sodium acetate and the decrease in NaCl improved the growth rate, while omission of the other compounds, mostly vitamins, had no effect on growth.

Amino Acids. The amino acids listed in column M2 were removed one at a time. Arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine were absolutely essential for growth, as reported by Marin (9). Omitting alanine, aspartic acid, glutamine, serine, and tyrosine had no significant effect on growth rate or yield; but eliminating cysteine and cystine or proline and hydroxyproline decreased both growth rate and yield, resulting in a maximum density of about 3×10^6/ml. Asparagine and glutamic acid improved growth (Table 2). This stimulation could not be achieved by replacing asparagine and glutamic acid with equimolar concentrations of aspartic acid and glutamine or with glucose and ammonium chloride. (See *Note Added in Proof.*)

Decreasing or increasing the concentrations of all essential amino acids by 50% slowed growth and gave lower yields. The optimal concentration of each essential amino acid has not been separately determined except for phenylalanine and proline.

Vitamins. Requirements for biotin, folic acid, lipoic acid, riboflavin, and thiamine were demonstrated by single omission

Table 1. Composition of growth media for *Dictyostelium discoideum*

Component	HL/5*	M1†	M2	M3	FM
Glucose (mM)	>56	56	56	56	56
Amino acids (mM)					
L-Arginine‡	4.4	3.3	3.3	3.3	3.3
L-Asparagine	—	2.3	2.3	2.3	2.3
L-Cysteine	—	0.83	0.83	1.7	1.7
Glycine	15.6	12.0	12.0	12.0	12.0
L-Glutamic acid	9.7	3.4	3.4	3.4	3.4
L-Histidine‡	1.6	1.4	1.4	1.4	1.4
L-Isoleucine	4.3	4.6	4.6	4.6	4.6
L-Leucine	7.2	6.9	6.9	6.9	6.9
L-Lysine‡	5.9	4.9	4.9	4.9	4.9
L-Methionine	1.9	2.0	2.0	2.0	2.0
L-Phenylalanine	3.1	3.0	3.0	6.0	3.0
L-Proline	> 6.3	3.5	3.5	7.0	7.0
L-Threonine	4.3	4.2	4.2	4.2	4.2
L-Tryptophan	0.52	1.0	1.0	1.0	1.0
L-Valine	5.9	6.0	6.0	6.0	6.0
L-Alanine	—	2.25	2.25	—	—
L-Aspartic acid	6.9	2.3	2.3	—	—
L-Cystine	0.58	0.42	0.42	—	—
L-Glutamine	—	3.4	3.4	—	—
L-Hydroxyproline	—	3.1	3.1	—	—
L-Serine	—	1.9	1.9	—	—
L-Tyrosine	2.5	2.2	2.2	—	—
Vitamins (mg/liter)					
Biotin	0.023	0.022	0.022	0.022	0.020
Cyanocobalamin	0.005	0.005	0.005	0.005	0.005
Folic acid	0.12	0.11	0.11	0.11	0.20
Lipoic acid	—	0.4	0.4	0.4	0.4
Riboflavin	0.4	0.45	0.45	0.45	0.5
Thiamine·HCl	0.53	0.6	0.6	0.6	0.6
L-Ascorbic acid	—	2.0	2.0	2.0	—
Calcium pantothenate	0.59	0.75	0.75	0.75	—
Pyridoxine·HCl	0.18	0.125	0.25	0.25	—
Choline chloride	30	4	—	—	—
Nicotinic acid	3.7	4	—	—	—
p-Aminobenzoic acid	0.13	0.13	—	—	—
Putrescine·2HCl	—	5	5	—	—
Salts (mM)					
K_2HPO_4	5§	3.4	2	2	5
NaH_2PO_4	—	6.6	3	3	—
NaOH	7	7	8.2	3.3	2.0
NaCl	6	10	—	—	—
$NaHCO_3$	—	1.0	1.0	1.0	0.2
NH_4 formate	—	1.0	1.0	1.0	—
NH_4Cl	—	—	—	—	1.0
$CaCl_2$	0.31	0.2	0.2	0.2	0.02
$FeCl_3$	0.20	—	0.1	0.1	0.10
$MgCl_2$	0.49	0.35	0.4	0.4	0.4
Trace elements (μM)¶					
Na_2EDTA	—	130	130	13	13
H_3BO_3	—	18	18	1.8	1.8
$CoCl_2$	—	7	7	0.7	0.7
$CuSO_4$	6.4	6	6	0.6	0.6
$(NH_4)_6Mo_7O_{24}$	—	0.8	0.8	0.08	0.08
$MnCl_2$	1.4	26	26	2.6	2.6
$ZnSO_4$	13	80	80	8	8
Dihydrostreptomycin sulfate (mg/liter)	50	200	50	50	50
pH (before autoclaving)	6.6	6.4	6.4	6.4	6.5

* The composition of HL/5 is calculated from data found in refs. 11 and 12 and data sheets supplied by Difco Laboratories.

† Medium M1 also contained (per liter): 0.3 mmol of adenosine, 0.33 mmol of uridine, 2.5 mmol of sodium acetate, 0.2 mmol of $(NH_4)_2SO_4$, 0.22 mmol of $FeSO_4$, 0.125 mg of pyridoxal·HCl, 0.125 mg of pyridoxamine, 0.5 mg of hematin, 2.0 mg of *i*-inositol, 0.5 mg of Tween 80, 0.06 mg of tocopherol-acetate, 0.05 mg of cholecalciferol, 2.5 mg of indoleacetic acid, and 1.0 mg of stigmasterol.

‡ Added to media M1-FM as L-arginine·HCl, L-histidine·HCl·H_2O, and L-lysine·HCl.

§ HL/5 contained in addition 3.4 mM phosphorus.

¶ The known composition of HL/5 included the following trace elements: 0.04 μM As, 0.6 μM Pb, and 170 μM SiO_2.

Table 2. Effect of asparagine and glutamic acid on growth rates

Medium*	Doubling time (hr)
— Asparagine — glutamic acid	16
+ Asparagine	14
+ Glutamic acid	15
+ Asparagine + glutamic acid	13

* Medium composition as in column M3 (Table 1) with asparagine and glutamic acid omitted.

experiments. In a medium lacking biotin the cells ceased growth after about three generations. In media without folic acid, riboflavin, or thiamine·HCl, growth ceased after one to two doublings. The absence of lipoic acid allowed growth, but the final density did not exceed 3×10^6/ml. The requirement for cyanocobalamin did not appear until the cells had grown for about 10 generations in a medium lacking this vitamin. Even washed spores will germinate and divide for six generations in the absence of cyanocobalamin. However, a strain of AX3 obtained from M. Brenner did not show any requirement for cyanocobalamin, even after 40 generations. This is in agreement with the results of Watts and Guest (10), who could not demonstrate a requirement for cyanocobalamin using the axenic strain AX2 (6), while they were able to show the requirement for the other five vitamins. The other vitamins and putrescine were declared nonessential after at least 30 generations of normal growth in media lacking them. When the concentrations of the six essential vitamins were halved or doubled, only the decrease of folic acid slowed growth and therefore the folic acid concentration was raised from 0.11 to 0.20 mg/liter.

Carbohydrate. The glucose concentration was optimized with medium M3. The best concentration of glucose was close to 1% (wt/vol). Without glucose there was hardly any growth. When cells were harvested from a logarithmic phase culture, washed free of medium in phosphate buffer, and inoculated at a density of 8×10^5/ml in a medium lacking glucose, the final density of this culture did not exceed 2.5×10^6/ml. D-Mannose, fructose, maltose, and trehalose supported growth at concentrations of 1% (wt/vol), and 1% glycerol supported slight growth. D- and L-arabinose, D- and L-fucose, D-galactose, lactose, mannitol, L-rhamnose, D-ribose, sorbitol, sucrose, and D-xylose could not replace glucose.

Buffer. The buffer in the medium is the only source of phosphate. Maximum growth rates occurred at phosphate concentrations of 1–12 mM and maximum yields were reached at about 4 mM. Without the phosphate buffer there was no growth. Since dramatic pH changes occur during growth (Fig. 1), the effect of several "Good" buffers on the growth of the amoebae was tested in medium M3 (Table 1). The following buffers, all with pK_a in the 6–7 range, were adjusted to pH 6.5 with NaOH or HCl. The numbers in parentheses indicate the highest concentration (mM) at which growth rate and yield were unaffected: *N*-(2-acetamidol)-2-aminoethanesulfonic acid (Aces) (10), *N*-(2-acetamidol)iminodioacetic acid (Ada) (2), [bis-(2-hydroxyethyl)imino]-tris-[(hydroxymethyl)methane] (Bis-Tris) (5), 1,3-bis[tris(hydroxymethyl)amino]propane (Bis-Tris-propane) (2), 2-(*N*-morpholino)ethanesulfonic acid (Mes) (5), and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) Pipes (5).

Salts. The various salt concentrations were optimized in medium M3. Concentrations of 0–2 mM sodium or potassium bicarbonate permitted optimal growth, while 5 mM bicarbonate increased the doubling time by 1–2 hr. The doubling

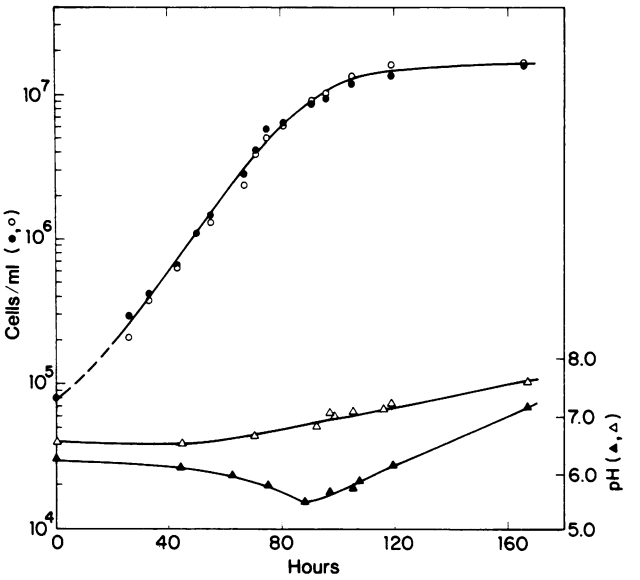

FIG. 1. Growth curves and pH changes of AX3 in 100 ml of HL/5 (○, △) or 100 ml of FM (●, ▲) in 500-ml erlenmeyer flasks. The cultures were incubated at 21–22° on a gyratory shaker (150 rpm; 1-inch stroke).

time also increased 1–2 hr in the absence of ammonium chloride or ammonium formate. The addition of 20 mM potassium chloride or 10 mM sodium chloride permitted near optimal growth, but 50 mM potassium chloride or 20 mM sodium chloride decreased the growth rate considerably, and the addition of 50 mM sodium chloride did not permit any growth. When the Na/K ratio is 20 or higher, growth is suboptimal. No clear-cut requirement for calcium chloride was demonstrated, and the concentration was decreased to 20 μM. In the absence of $MgCl_2$ or $FeCl_3$ little or no growth occurred. Maximum yields were obtained at 0.1 mM $FeCl_3$ and 0.4 mM $MgCl_2$. The 10-fold decrease in the concentrations of trace elements (compare columns M2 and M3, Table 1) has not affected growth after 30–40 generations. After the cells were subcultured in a medium lacking trace elements, the final cell densities reached were only 3×10^6/ml. Full growth could be restored by the addition of 10 μM $ZnCl_2$ with final cell densities of 1.9×10^7/ml; when the $ZnCl_2$ concentration was raised to 100 μM, the final densities were 3.6×10^7/ml. An additional mixture of trace elements, including As, Al, F, I, Pb, Sn, Se, and Cr, had no effect.

Growth Conditions. The growth rates achieved in the minimal medium are shown in Fig. 1 and Table 3. The growth of the amoebae in HL/5 and minimal medium (FM) are indistinguishable when 100-ml volumes are used in half-liter flasks. Fig. 1 also shows that the pH of the minimal medium creeps up as the cells reach stationary phase after an initial sharp drop at the end of exponential growth. In small flasks the minimal medium is better than the HL/5; in 125-ml flasks with 25 ml of medium, the HL/5 supports faster growth. Filter-sterilized minimal medium supports growth as well as autoclaved medium.

The growth of cells in minimal medium was affected by several other conditions. The optimal pH in an autoclaved medium is about 6.2. Because autoclaving lowers the pH (Fig. 2), the medium was made up to pH 6.5. Cells grown in the defined medium (autoclaved or filter-sterilized) developed normally when harvested from cultures with densities of 2×10^6 to 1.2×10^7/ml. Prolonged exposure to light destroyed the medium; addition of fresh vitamins did not restore it, so light

Table 3. Growth of *Dictyostelium discoideum* in HL/5 and FM

Cells and growth conditions*	Initial density (cells/ml)	HL/5		FM	
		Doubling time (hr)	Final density (cells/ml)	Doubling time (hr)	Final density (cells/ml)
AX3					
5 ml in 50-ml flasks†	8×10^4	14–18§	4×10^6–8×10^6§	13	2.2×10^7
25 ml in 125-ml flasks‡	4.5×10^4	11.5	1.5×10^7	13	2.2×10^7
100 ml in 500-ml flasks‡	8×10^4	12	1.7×10^7	12	1.6×10^7
AX3 from M. Brenner					
100 ml in 500 ml flasks‡	7×10^4	9.5	—	16.5	—

* Cultures were incubated on a gyratory shaker at 21–22°.
† 120 rpm; ¾-inch stroke.
‡ 150 rpm; 1-inch stroke.
§ With 5 ml of HL/5 in 50-ml flasks there are large variations from flask to flask.

should be avoided during long incubations. The growth rates of the cells in minimal medium were increased as much as 30% by including 0.05–0.10% (wt/vol) Noble agar. Because we are not sure of the purity of the agar, we have not included it in the medium reported here.

Whereas cultures in HL/5 started growing from densities as low as 5×10^2 cells per ml, cultures in the minimal medium did not usually grow when started at densities of less than 2×10^3 cells per ml. When cultures were incubated in microtiter plates (Linbro, IS-MRC-96) in 0.1-ml volumes fewer cells were required for growth. With the mean number of cells per well equal to 10, in minimal medium, all wells incubated in the dark at 21° without shaking produced visible colonies in 2–3 weeks. With the mean number of cells per well equal to 1, less than 8% of the wells produced colonies. The same experiment done with HL/5 produced colonies in 50% of the wells.

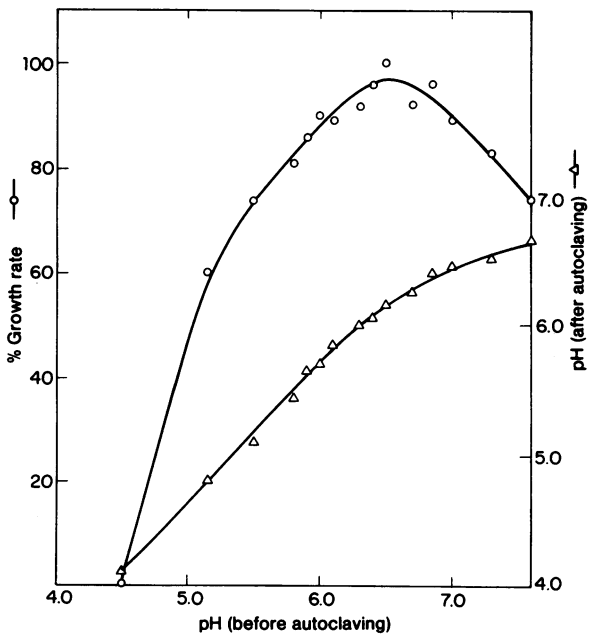

FIG. 2. Amoebae of *D. discoideum* (strain AX3) were inoculated at a density of 5×10^4 cells per ml in 5 ml of medium of varying pH in 50-ml erlenmeyer flasks and incubated at 21–22° on a gyratory shaker (120 rpm; ¾-inch stroke). The media were prepared as in column FM (Table 1), except that KH_2PO_4 replaced K_2HPO_4, and the final pH was adjusted with 1 M KOH. Autoclaving caused a significant change of the pH (△). The percent growth rate (○) = 100 × (minimum doubling time/doubling time).

The cloning efficiency of AX3 in minimal medium in microtiter plates could be increased to 50% by providing them with a feeder layer of 1000 NC-4 cells per well. NC-4, the nonaxenic parent strain of AX3, cannot grow in HL/5 or minimal medium.

We have tried other axenic strains in the minimal medium, but not all grow as well as the strain maintained as AX3 in this laboratory, for which the medium was optimized. A version of AX3 obtained from M. Brenner grew well in medium M2, but grew poorly in the final minimal medium (Table 3). The new V12 axenic strains developed by Williams (7) grow in the minimal medium (Williams, personal communication). A temperature-sensitive, cycloheximide-resistant derivative of AX2, obtained from M. B. Coukell, grew in HL/5 and minimal medium with a doubling time of 16–18 hr.

DISCUSSION

The medium described here is both minimal and defined. The cost is about 1.5 times that of HL/5. Once stock solutions have been made, little further effort is required. It is important to realize that not all strains of AX3 grow equally well in this minimal medium, and it is likely that the strains maintained in various laboratories, though called AX3, are not identical.

Our version of AX3 does not require any nutrient not listed in the last column of Table 1. Like most organisms, *D. discoideum* synthesizes all of its nucleic acid precursors, and therefore a defect in any of a number of steps will make the cells dependent on exogenous precursors. Seven of the amino acids are not required. Among the vitamins, the cells can do without pantothenate, pyridoxine, nicotinic acid, choline, and inositol. All steroids, fatty acids, and lipids must be synthesized *de novo*. We have begun to select a series of vitamin, amino acid, and nucleic acid auxotrophs, which should increase the number of selectable genetic markers many fold. Such mutants will make genetic analysis by parasexual and sexual methods much easier. Sexual genetic analysis in *D. discoideum* is now at an early stage of development, but can be done (Warren and Kessin, unpublished data).

This medium is useful for several other purposes. It makes radioisotope labeling more efficient. Preliminary efforts to synchronize the division cycles of the cells by omission of the essential amino acid tryptophan indicate that the cells divided synchronously after tryptophan was restored to the medium.

Note Added in Proof. When asparagine and glutamic acid were omitted from medium FM (Table 1) the final yields were low (final cell densities less than 5×10^6/ml). The absence of cysteine allowed

good growth. Omission of cysteine and proline together allowed very little or no growth.

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