NUTRITIONAL REQUIREMENTS FOR VEGETATIVE GROWTH OF *MYXOCOCCUS XANTHUS*

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ABSTRACT

DWORKIN, MARTIN (Indiana University Medical Center, Indianapolis, Ind.). Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J. Bacteriol. **84**:250–257. 1962.—This investigation was part of a program to clarify the environmental regulation of fruiting-body formation in a fruiting myxobacterium. By use of a dispersed-growing strain of *Myxococcus xanthus*, the nutritional requirements for vegetative growth have been defined. Exponential growth will take place on a medium containing 17 amino acids and salts. The generation time is 8 to 10 hr. Various optima have been established. There are no requirements for exogenous vitamins, and, with the exception of glycogen, a variety of organic materials do not significantly stimulate growth.

It is unrealistic to hope that one can critically study the relationship between an organism and its environment without controlling the nutritional milieu. In general, physiological studies of microorganisms should be carried out, whenever possible, in chemically defined media. Specifically, when investigating the effect of the environment on microbial morphogenesis, this must be a *sine qua non*. Moreover, an understanding of the nutritional requirements of an organism is apt to provide insight into a number of facets of the nature of that organism. To a large extent, the ecological role of a microbe will be related to its nutritional capacity, and an examination of its metabolic apparatus may be broadly guided by a knowledge of its nutritional needs. However, the most compelling reason for our interest in the nutrition of a fruiting myxobacterium is the oft-described relationship between the nutritional milieu of a microbe and its morphogenetic development. The processes of sporulation and germination in *Bacillus* have been shown to be nutritionally dependent (Hardwick and Foster, 1952; Hills, 1950). The formation of perithecia in *Melanospora* can be regulated by the nature and concentration of various constituents of the medium (Asthana and Hawker, 1936), and fruiting-body formation by *Myxococcus* (Oetker, 1953) and *Chondromyces* (Kühlwein, 1950) has been shown to depend upon the nature of the medium. The clarification of this influence is likely to lead to an understanding of the nature of the interaction between the environment and the regulatory mechanisms controlling morphogenesis.

Although a number of investigators have examined the stimulatory or inhibitory effect of various nutrilites (Norén, 1955; Oxford, 1947) and the suitability of various complex media (Oetker, 1953), there has been no clear definition of the nutritional requirements of any of the fruiting myxobacteria. To a large extent, investigations in this area have been restricted by the lack of a good quantitative method for measuring myxobacterial growth. Growth occurred either as a ring around the culture tube, in which case the thickness of the ring was measured (Norén, 1955), or as colonies on solid media, in which case colony diameter was the parameter of growth (Oetker, 1953). The use of dispersed-growing variants of *Myxococcus* by Loebeck and Klein (1956) and by Holt (1960) paved the way for an analytical approach to the problem.

By use of such dispersed-growing variants, this investigation has been concerned with defining the nutritional requirements for the vegetative growth of *Myxococcus xanthus*.

MATERIALS AND METHODS

Organisms. Unless otherwise indicated, the data to be described were obtained with a variant of *M. xanthus* referred to as VC. This organism was isolated by John Holt while at Purdue University. Under appropriate conditions, VC grows in a dispersed state and, as such, is suited for turbidi-

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metric measurement of growth. After about 100 serial transfers in complex liquid medium, this culture lost the ability to form either microcysts or fruiting bodies, while retaining its characteristic, gliding motility. Holt (1960) also observed this phenomenon. The defined medium, as finally developed, was also tested for its ability to support the growth of two other strains of *M. xanthus*, designated FB and MC. Strain FB, obtained from the stock culture collection of the bacteriology department of the University of California in Berkeley, will undergo the complete developmental cycle, forming microcysts and fruiting bodies. Strain MC was derived from FB, and will form microcysts but not mature fruiting bodies. Both of these strains will grow in a dispersed state.

Growth conditions. Dispersed growth of vegetative cells was obtained by inoculating 40 ml of CT medium in a 250-ml Erlenmeyer flask and incubating at 30 C (±1 C) with shaking (about 75 to 100 rotations per minute). CT medium contains Casitone (Difco), 2%; $MgSO_4$, 0.1%; K_2HPO_4-KH_2PO_4, 0.01 M (pH 7.2); and distilled water. (Casitone is an enzymatic hydrolyzate of casein.) To avoid precipitation, the $MgSO_4$ was added aseptically after the other components were autoclaved. Growth on CT agar is vegetative, with no microcysts or fruiting bodies formed. For microcyst or fruiting-body formation, a medium prepared by autoclaving a suspension of *Escherichia coli* in distilled water (about 5×10^8 cells/ml) and agar (2%) for 1.5 hr was used. This medium is referred to as CA. Cultures were maintained by daily transfer in CT liquid. With each transfer, the culture was streaked on a CT plate and, in the case of MC and FB, also on a CA plate, to maintain the ability to form microcysts and fruiting bodies.

For growth measurements, cultures were grown in 250-ml Erlenmeyer flasks with Klett tubes attached. Growth was measured with a Klett-Summerson colorimeter using the green (no. 57) filter. For nutritional experiments, the inoculum was prepared by centrifuging cells out of an 18- to 24-hr-old culture. The cells were resuspended in distilled water, and the experimental flasks were inoculated to about 10^8 cells/ml.

Chemicals. All amino acids and peptides were obtained from the Cyclo Chemical Corp., Los Angeles, Calif., as the L isomers unless otherwise stated.

RESULTS

Growth on complex media. M. xanthus will grow well on a medium containing enzymatic digests of protein. Its growth on CT medium is illustrated in Fig. 1. The generation time is about 3.5 hr. Under these conditions, the stationary phase is followed by rapid autolysis of the cells. The maximal Klett reading usually obtained is about 500, representing a cell concentration of about 2×10^9 organisms/ml. We have not been able to increase significantly either the rate of growth or the maximal yield of cells, and have therefore adopted the growth pattern on CT as the standard of measurement. The principal parameter used for evaluating growth was the generation time during exponential multiplication.

A number of protein hydrolyzates were compared to Casitone. Although there was no striking difference among the various enzymatic digests of protein, the acid hydrolyzates were significantly inferior (Table 1). Since acid hydrolysis of protein will destroy tryptophan and cysteine, the effect of adding these amino acids (0.1 mg/ml) back to the acid hydrolyzates (Casamino Acids and Acidicase) was tested. This did not improve the suitability of the acid hydrolyzates. The media containing the acid hydrolyzate contained a significant amount of sodium chloride (0.38%). To eliminate the possibility that growth was in-

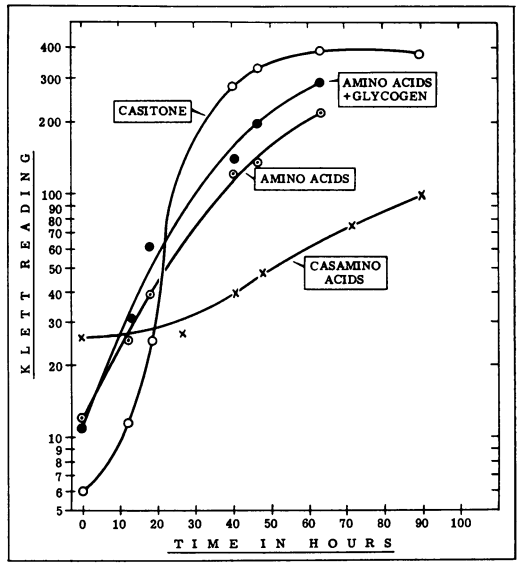

FIG. 1. *Growth of Myxococcus xanthus VC on various media.*

hibited by the presence of sodium chloride, an equivalent amount of sodium chloride was added to the CT medium. There was no inhibition of growth. As an additional control, a 10% solution of Casamino Acids was electrically desalted and then tested. There was no change in its ability to support growth.

Growth-factor requirements. Although Norén (1955) indicated that growth of *M. virescens* was stimulated by the presence of a variety of vitamins, Oxford (1947) found that a vitamin mixture had no effect on the growth of the same organism. We were unable to demonstrate any stimulatory effect of vitamins on the growth of *M. xanthus*.

In an attempt to detect a requirement for steroids or fat-soluble vitamins, 2 g of finely powdered Casitone were placed in a Soxhlet apparatus and extracted for 48 hr with diethyl ether. There was no alteration in the ability of the Casitone to support growth. This would seem to indicate the absence of any fat-soluble vitamin or steroid requirements. The ability of vitamin-free Casitone (Difco) to support growth was tested. This material is prepared from casein which is sufficiently free of vitamins to serve in assay media for nicotinic acid, thiamine, pantothenic acid, biotin, vitamin B_{12}, and folic acid (Difco Laboratories, 1953). Growth was equal to that obtained with regular Casitone. After the defined medium had been developed, the addition of a vitamin mixture (riboflavine, 0.5 μg/ml; *p*-aminobenzoic acid, 1.0 μg/ml; pyridoxal-PO_4, 2 μg/ml; thiamine·HCl, 0.1 μg/ml; calcium pantothenate, 0.4 μg/ml; nicotinamide, 0.4 μg/ml; biotin, 0.01 μg/ml; folic acid, 0.05 μg/ml; B_{12}, 0.005 μg/ml) had no stimulatory affect on growth.

TABLE 1. *Growth on various protein hydrolyzates*

Hydrolyzate (1%)	Generation time	Highest Klett reading
	hr	
Enzymatic hydrolyzates		
Casitone (Difco)	4	337
Trypticase (Difco)	5	419
Tryptone (Difco)	6	467
Milk protein hydrolyzate (BBL)	6	381
Phytone (BBL)	7	361
Lactalysate (BBL)	9	398
Peptone (Difco)	10	234
Acid hydrolyzates		
Casamino Acids (Difco Certified)	28	100*
Acidicase (BBL)	44	75*

* Growth was still taking place.

TABLE 2. *Effect of addition of single amino acids to a 0.5% Casamino Acids-salts medium*

Amino acid (0.5 mg/ml)	Amino acid (0.5 mg/ml)
Stimulatory	Histidine
Tyrosine	β-Alanine
Asparagine	Canavanine
Leucine	Carnosine
Isoleucine	Putrescine
Proline	Cystathionine
Phenylalanine	Homocystine
Inhibitory	*Indifferent*
Glutamine	Cystine
Tryptophan	Djenkolic acid
Methionine	Serine
Alanine	γ-Aminobutyric acid
Glycine	Cysteine·HCl
Valine	Glutamic acid
α-Aminobutyric acid	Arginine
α-Aminoisobutyric acid	Betaine
β-Aminobutyric acid	Cadaverine
DL-Threonine	Sarcosine
Aspartic acid	Cysteic acid
Lysine	Glycocyamine

Amino acid requirements. The superiority of the enzymatic hydrolyzate over the acid hydrolyzate implied that peptides were a better source of amino acids than were the free amino acids themselves. In view of the extreme difficulty in either duplicating the complex mixture of peptides in Casitone or in isolating, identifying, and supplying the required peptides, an alternate approach was sought. This approach was suggested by the work of Kihara, Ikawa, and Snell (1961), who showed that the stimulation by peptides of the growth of *Streptococcus faecalis* reflects not a requirement for the peptide *per se* but an overcoming of an amino acid imbalance or antagonism. This imbalance presumably inhibits the entry of the required amino acid into the cell. This view has proven fruitful in clarifying the role of peptides in bacterial nutrition. Accordingly, our approach was to attempt to duplicate the Casamino Acids mixture with known amino acids and then to vary the levels of the component amino acids. However, all of our initial attempts to obtain any vegetative growth with mixtures of amino acids failed.

TABLE 3. *Effect of individual amino acids as determined by elimination from the defined medium*

Amino acid	Optimal concn*
	mg/ml
Required	
Leucine	1.00
Isoleucine	0.50
Proline	0.50
Methionine	0.05–0.50
Alanine	0.05–0.50
Valine	0.10–0.50
Glycine	0.05–0.25
Djenkolic acid†	0.10–0.25
Stimulatory	
Tyrosine	0.60
Asparagine	0.50
Arginine	0.10
Histidine	0.05–0.25
Lysine	0.25–0.75
Phenylalanine	0.015–0.15
Threonine	0.10
Serine	0.10–0.50
Tryptophan	0.05–0.50

* Those amino acids for which a single concentration is given demonstrated no well-defined optimal range.

† In the absence of cystine.

Norén (1955) was able to demonstrate effects of single amino acids on the growth of *M. virescens* when the amino acids were added to a basal medium containing low levels of Casamino Acids. We utilized this approach by adding single amino acids at a level of 0.5 mg/ml to a limiting amount of Casamino Acids (0.5%). Under these conditions, distinct patterns of stimulation and inhibition of growth were noted and it was possible to determine the approximate level above which any particular amino acid was toxic. Table 2 lists the amino acids tested and the effect of these amino acids on the growth of vegetative cells in the presence of 0.5% Casamino Acids.

On the basis of these results, a synthetic medium was then devised containing the stimulatory and indifferent amino acids at levels of 0.5 mg/ml and the inhibitory amino acids at levels of 50 μg/ml (at which concentration they did not inhibit growth on 0.5% Casamino Acids). This mixture supported growth of VC. The rate of growth, however, was extremely low. Eliminating individual amino acids from this synthetic medium permitted us to determine which amino acids were essential or stimulatory and which were indifferent. Table 3 lists those amino acids whose omission from the mixture reduced growth. Eight amino acids were required and nine were stimulatory. When all nine stimulatory amino acids were eliminated, however, no growth took place.

Without consideration of the possible concentration interactions, concentration optima were determined by varing the concentration of single amino acids. The optimal concentration for a number of amino acids was quite narrow. Glycine, for example, was absolutely required for growth. The optimal concentration was 50 μg/ml, and concentrations greater than 250 μg/ml completely inhibited growth. Table 3 lists for some amino acids the range of concentrations within which optimal growth occurred. For the other amino acids, the optimal concentration either varied from experiment to experiment or could not be clearly defined.

On the basis of these experiments, we were able to construct a synthetic medium consisting of salts and 17 amino acids. The composition of this medium is described in Table 4. Growth on this medium is illustrated in Fig. 1.

Occasionally, a batch of medium would not sup-

TABLE 4. *Composition of defined medium*

Component	Concn
	mg/ml
Tyrosine	0.6
Asparagine	0.5
Leucine	1.0
Isoleucine	0.5
Proline	0.5
Arginine	0.1
Histidine	0.05
Glycine	0.05
Lysine	0.25
Methionine	0.05
Phenylalanine*	0.15
Tryptophan*	0.05
Serine	0.1
Threonine	0.1
Valine	0.1
Djenkolic acid	0.1
Alanine*	0.05
Glycogen	3.0
$MgSO_4$	1.0
K_2HPO_4-KH_2PO_4 (pH 7.6)	0.01 M

* Used at 1.0 mg/ml for FB and MC.

TABLE 5. *Effect on growth of the addition of various organic materials to the amino acids-salts medium*

Addition (0.1%)*	Addition (0.1%)
Marked stimulation	*Inhibition*
Glycogen (shellfish)	Mannose
Glycerol	Galacturonic acid
Slight stimulation	Sodium citrate
Fructose	Fumaric acid
Glucose	Lauryl alcohol
Arabinose	Capric acid
Soluble starch	Adenine sulfate†
Glucuronic acid	Guanine
Glucosaminic acid	*Indifferent*
Ammonium malate	Maltose
Succinic acid	Sorbose
Uracil	Cellobiose
Xanthine	Sucrose
Yeast nucleic acid	Trehalose
	Raffinose
	Xylose
	Sodium acetate
	L-Tartaric acid

* All carbohydrates, sugar acids, and glycerol were autoclaved separately and added aseptically.
† Added at 0.01%.

port any growth. The only known appropriate variable was the occasional use of DL amino acids rather than the L isomers. The inhibitory effect of the D isomer was investigated. Each amino acid required in the medium was supplied singly as the DL mixture at twice the usual concentration of the L isomer, and the effect on growth was determined. DL-Leucine completely inhibited growth, while DL-isoleucine caused a partial inhibition. The effect of adding twice the normal level of L-leucine or L-isoleucine was tested. In neither case did this result in equivalent inhibition.

Optimal concentration of inorganic components. The optimal concentration of $MgSO_4$ in the synthetic medium was determined and was found to lie between 0.1 and 0.2%. No growth took place in the absence of $MgSO_4$, and the growth rate was decreased at 0.3 and 0.03%. The optimal level of phosphate was 0.01 M. Growth was inhibited at 0.03 and 0.003 M.

Two mixtures of trace elements [(i) Metals "44" (Cohen-Bazire, Sistrom, and Stanier, 1957); and (ii) $MgSO_4$, 500 μg/ml; $MnSO_4$, 5 μg/ml; $ZnSO_4$, 5 μg/ml; $FeSO_4$, 10 μg/ml; $CaCO_3$, 2 μg/ml] were tested with the synthetic medium and were found to exert no marked stimulatory effect on growth. The trace-element mixtures did, however, stimulate the production of a bright-red extracellular pigment. This phenomenon was encountered on numerous occasions and could not be unequivocally related to the rate or amount of growth. Adding an ashed residue of Casitone to the synthetic medium had no stimulatory effect on growth.

Optimal pH. There was no sharp pH optimum for vegetative growth. In the defined medium, growth was inhibited at pH 6.8 and below, and was maximal between pH 7.2 and 8.2. It must be kept in mind that the inhibitory effect of phosphate precluded its use at concentrations higher than 0.01 M. Its buffering capacity was therefore quickly exceeded and the pH experiment measured only the effect of the initial pH.

Effect of organic materials. The effect on growth of a wide variety of organic compounds was tested (Table 5). These materials were added to the amino acid-salts mixture. Although many of the compounds tested showed a slight stimulatory effect (e.g., starch, uracil, succinic acid), glycogen was the most effective, reducing the generation time from 11 to 8 hr. This is consistent with the observation of Norén (1955), who reported that *M. virescens* can utilize glycogen and starch. It seemed at first unlikely that this was a nutritional effect due to the glycogen, since commercially available glycogen was found to be frequently contaminated with nitrogenous materials and since the optimal effect was at a rather high concentration (0.3%). Also, neither glucose nor maltose showed any marked stimulatory effect. However, glycogen was precipitated three times from hot water by 95% ethyl alcohol and lost none of its growth-stimulating activity, despite a considerable reduction in ninhydrin-positive material.

In another experiment, the fate of glycogen in a growing culture was followed by a quantitative anthrone test (Carroll, Longley, and Roe, 1956) for carbohydrates and a quantitative iodine test (Van der Vies, 1954) for polysaccharide (Fig. 2). Determinations were made on the cells and on the culture filtrate. The level of iodine- and anthrone-reactive material in or on the cells remained low and constant. In the filtrate, however, during the early phase of growth there was a sharp decrease in iodine-reactive material (polysaccharide). There was no corresponding decrease in anthrone-reacting material, indicating the presence of non-polymerized carbohydrates in the supernatant

culture fluid. After about 1 day of growth, the level of iodine-reacting material started rising, terminating in a level of polysaccharide considerably higher than that found in a culture without glycogen.

Effect of dipeptides. At this point it was necessary to consider the basis for the more rapid growth on the Casitone medium (generation time of 3.5 hr) as compared to the synthetic medium (generation time of 8 hr). Since vitamins and trace elements had been effectively ruled out, the enhanced growth was probably due to the superiority of the peptide as a source of amino acids. Accordingly, single dipeptides were added (0.25 mg/ml) to the complete amino acid medium; 36 dipeptides, containing all the required and stimulatory amino acids except djenkolic acid, were tested. A variety of peptides stimulated growth but in no case was the rate of growth equal to that obtained on CT. When all of the stimulatory peptides were added to the synthetic medium, growth was enhanced (generation time of 6.5 hr) but was still considerably inferior to that obtained on CT. The stimulatory dipeptides were: methionine amide·HCl, alanyl-isoleucine, prolyl-glycine, prolyl-leucine, valyl-valine, α-glutamyl-tyrosine, tyrosyl-glycine, prolyl-tyrosine, phenylalanyl-leucine, alanyl-phenylalanine, isoleucyl-alanine, and isoleucyl-leucine.

After combining all of the above optima, vegetative growth was obtained which, while inferior to growth on CT, was superior to that on Casamino Acids. A comparison of growth on the various media is illustrated in Fig. 1. The generation time obtained on CT was 3.5 hr; on Casamino Acids, 28 hr; on the amino acid medium, 11 hr; and on the amino acids-glycogen medium, 8 hr.

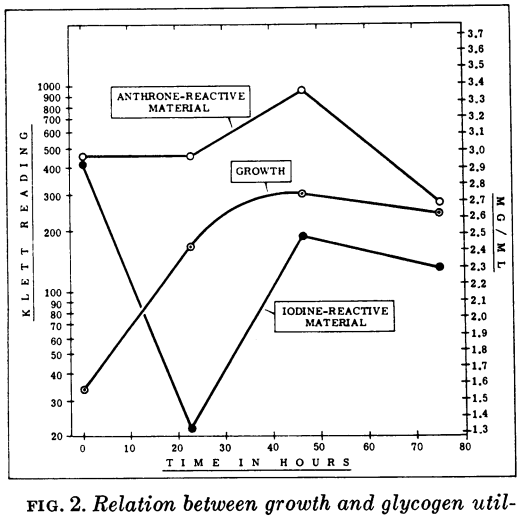

FIG. 2. *Relation between growth and glycogen utilization in Myxococcus xanthus VC.*

Growth of MC and FB. The amino acids-salts medium was tested for its ability to support vegetative growth of the MC and FB strains. Neither was able to grow under these conditions. When the levels of tryptophan, phenylalanine, and alanine were raised to 1 mg/ml, growth took place with a generation time of 12.5 hr for both MC and FB. This was reduced to 10 hr in the presence of 0.3% glycogen. The terminal growth obtained was considerably higher than that obtained with the VC variant.

Growth on solid medium. Ultimately, it is our hope to study fruiting-body formation under conditions of defined nutrition. It was necessary, therefore, to be sure that vegetative growth of FB would take place on solid media. When the defined medium was solidified with 1% agar (Oxoid) and inoculated with FB or VC, typical vegetative, colonial growth took place. No microcysts or fruiting bodies were formed. As in the liquid medium, growth was enhanced by the presence of 0.3% glycogen.

Serial transfer in defined medium. It is important to demonstrate that growth in the defined medium is not dependent on either the carry-over of complex material with the inoculum or on the intracellular accumulation of some essential nutrient present only in the complex medium. Accordingly, serial passage in the defined medium (Table 4) was attempted and was successful. There was no change in the growth patterns of VC or FB after five transfers on the defined medium, with or without glycogen.

DISCUSSION

The principal purpose of this investigation has been accomplished, namely, the formulation of a defined medium for vegetative growth of *M. xanthus*. Many questions, however, remain unanswered. Unlike a simple glucose-salts medium, it is impossible at this point to decide what is serving as the energy source, nitrogen source, and carbon source. Are some amino acids being oxidized while others are incorporated, or do many serve a dual function? The clarification of the roles of the individual components of the medium will have to await a more detailed metabolic

study. The fact that some amino acids are stimulatory rather than absolutely required may reflect either a rate-limiting synthesis of the particular amino acid or the presence of the amino acid as a component of the slime of the inoculum. The latter factor may be responsible for the difficulty encountered in establishing optimal concentrations for some of the amino acids in the medium.

The nature of the medium poses some questions. Magnesium sulfate is required at an unusually high concentration (0.1%), yet this was clearly shown to be the optimal level. Leucine, and in the case of substrains FB and MC, tryptophan, phenylalanine, and alanine are required at levels of 1 mg/ml. At lower levels, either growth does not take place at all or the rate is considerably lower. These relatively high levels may be required to overcome permeability difficulties or to overcome the antagonistic effects among some amino acids. This is consistent with the observation that peptides serve the organism much more effectively than the corresponding amino acids. It is interesting that, despite the superiority of CT over the amino acid medium, adding dipeptides to the amino acids does not significantly enhance growth. Either the dipeptide is not sufficiently large or the relative position of the amino acid in the dipeptide is critical.

The superiority of djenkolic acid as a sulfur source is unusual. It was found to be superior to cysteine, cystine, homocystine, and lanthionine. Although djenkolic acid is not a component of bacterial protein, it has been shown to serve satisfactorily as a sulfur source for *Streptococcus bovis* (Prescott, 1961). The cell surface of *M. xanthus* is sensitive to cysteine and to sulhydryl compounds in general, forming spheroplasts in their presence (Dworkin and Voelz, 1962). The relative unsuitability of cystine may be a reflection of its low solubility. The optimal concentration of djenkolic acid is about twice the highest concentration of cystine obtainable under the conditions of culture.

The role of glycogen is unclear. Its function is not solely a physical one, since it disappears from the medium. The appearance in the medium of anthone-positive material which does not react with iodine implies that the glycogen is hydrolyzed extracellularly. Since the growth of the cells is not appreciably stimulated by glucose, maltose, or by sugars in general, it is unlikely that the function of the glycogen is to provide an external source of sugar. The ΔF of phosphorolysis of glycogen is +1.5 kcal, so it is unlikely that the process furnishes energy to the cell. In the presence of glycogen, the final level of iodine-reactive material in the culture filtrate is considerably higher than that found in the absence of glycogen. Since this is preceded by a sharp decrease in iodine-reactive material, it is likely that this final level represents polysaccharides synthesized by the organism. Holt (1960) has shown that the slime of *M. xanthus* contains about 20% polysaccharide. The role of the slime in vegetative growth as well as in the formation of microcysts and fruiting bodies will undoubtedly prove to be an important one.

The ability to cultivate a fruiting myxobacterium under defined nutritional conditions places the future goals of this investigation within reach. Preliminary experiments indicate that proper manipulation of the constituents of the defined solid medium can control fruiting-body formation. We are now in a position to examine the specific relation between fruiting and the nutritional milieu in the hope of clarifying the regulatory mechanisms which control fruiting-body formation.

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