NATURE OF THE GROWTH FACTOR FOR THE COLORLESS ALGA PROTOTHECA ZOPFII

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The detailed studies of Barker (1) on the metabolism of *Prototheca zopfii* demonstrated that development of this organism did not take place in media containing a suitable carbon source and inorganic or amino nitrogen only. Yeast autolysate or some other source of complex organic material was found requisite for development. The quantity of yeast extract required for growth, however, was very small. In media containing low concentrations of this material development of the organism could be greatly increased by the addition of an ammonium salt, such as ammonium chloride. Although this substance could not serve as a substitute for yeast autolysate, quantitative studies showed that a large portion of the nitrogenous cell material could be synthesized from the ammonia nitrogen. The maximum cell yield of *Prototheca* was shown to be a complicated function of the amounts of yeast autolysate and ammonia nitrogen available. From these experiments Barker concluded: "Yeast autolysate or some other complex material is indispensable for the development of this alga."

It seemed logical to investigate the possibility, indicated in the findings of Barker, that yeast autolysate contributed one or more essential growth factors. A detailed study of this phase was attractive because the identification of such factors would make it possible to investigate their function in the metabolism of this organism.

Material and Methods

Organism.—The strain of *Prototheca zopfii* used was No. 7322, one of the several maintained in the pure culture collection at the Hopkins Marine Station. It was selected from the group on the basis of its rapid development in a simple liquid medium.

Medium.—Cultures of the organism were maintained on yeast agar containing 2 per cent glucose.

For studies on the growth factor requirements of the organism, a basal liquid medium of the following composition proved satisfactory:

	per cent
Glass-distilled H_2O	
NH_4CL	0.10
$MgSO_4$	0.02
KH_2-Na_2HPO_4 (pH 7.0)	0.20
Glycerol	0.50

For growth factor studies it is imperative that all tests for substances, functioning as nutrilites for a particular organism, be carried out in a basal medium which is as simple as possible and yet contains *all* other elements necessary for growth of the organism. The composition of the glycerol mineral medium fulfills these requirements closely enough, since in the absence of yeast extract or of the active components of this material practically no growth occurs, whereas the addition of these materials gives rise to a profuse development.

Glycerol was chosen as a carbon source in preference to glucose because glycerol is not acted upon by *Prototheca* under anaerobic conditions. While glucose is aerobically converted into cell material and carbon dioxide, anaerobically it is quantitatively fermented into lactic acid (1). Therefore, the use of glycerol has the great advantage over glucose in that the acidity of the medium resulting from the growth of the organism on glycerol will be solely that arising from fermentation of stored carbohydrate products. This obviates the necessity of the addition of large quantities of calcium carbonate to the culture medium as a buffer. Glycerol has the added advantage as a substrate for growth factor tests in that it does not undergo decomposition or polymerization when sterilized in the presence of phosphates as does glucose. The products resulting from heat sterilization of sugar have been found to influence the growth of microorganisms, exerting a toxic effect on some and acting as growth-promoting substances for others (Stanier (2), and Fulmer, Williams, and Werkman (3)). Finally, glycerol can much more easily be obtained free from minute amounts of organic impurities, which might serve as growth factors, than can carbohydrates.

To determine if autoclaving resulted in a serious destruction of growth factors, duplicate series of varying concentrations of yeast autolysate were prepared using sterile autolysate and heat-sterilized medium base. One series was autoclaved at 15 pounds pressure for 20 minutes and both series were inoculated with equal amounts of a dilute *Prototheca* suspension. Cell yield determinations for each series indicated conclusively that under the conditions employed in the tests the growth promoting substances are quite heat-stable and that sterilization may be safely accomplished by autoclaving.

Glassware used for all growth factor experiments was scrupulously cleaned with cleaning solution and at least four final rinsings with distilled water.

Culture Methods.—Although *Prototheca* is capable of carrying on an anaerobic metabolism it is totally unable to develop under strictly anaerobic conditions (1). In tests for the activity of growth factors it is essential that the conditions of aeration approach the optimum in order that the growth of the organism will be a function of the amounts of the growth substances present rather than being restricted by a limited oxygen supply. Quantitative experiments were therefore carried out with cultures in shallow layers in rotating bottles. Unless otherwise stated these cultures were incubated at 30° C. for a period of 7 days.

Although satisfactory growth curves were obtained with cultures growing in liquid media dispensed in 50 ml. volumes in Florence flasks of 125 ml. capacity, such curves

indicated reduced oxygen tension to be a limiting factor in the growth of *Prototheca* in flasks. This was especially true at the higher concentrations of growth-promoting substances. To obtain more nearly optimum aerobic conditions under which to grow *Prototheca*, a method of culture was devised which would allow the surface of the liquid medium to be very large as compared with its volume. This was accomplished by using 25 ml. volumes of medium in 150 ml. narrow mouth glass bottles having an inside diameter of approximately 5 cm. When the bottles were placed on their sides the greatest depth of the liquid was about 1 cm. To further insure uniform aerobic conditions the bottles were placed in a rolling machine and continuously rolled at the rate of about 18 R.P.M.

In order to compare the growth of *Prototheca* in 25 ml. amounts of medium in bottles with that in 50 ml. amounts in flasks, graduated series of concentrations of yeast autolysates were set up in each type of culture vessel and incubated for a period of 10 days. Cell yield measurements for each series are recorded in Fig. 1. These

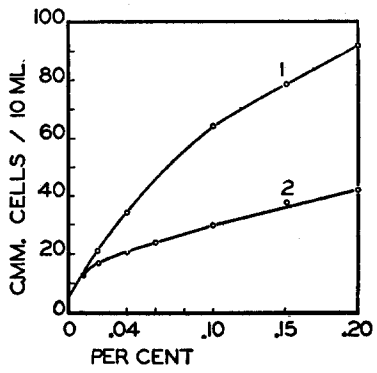

FIG. 1. Comparison of the growth of *Prototheca* in bottles (1), and in flasks (2), in media containing varying concentrations of yeast autolysate.

clearly demonstrate that growth of *Prototheca* in flasks under semi-aerobic conditions is definitely inferior to that produced under aeration such as is obtained in shallow layers continuously rolled. Further experiments showed that with the higher yeast extract concentrations double the amount of growth could be obtained in bottles in 1 week as that obtainable in flasks during a 2 week period of incubation.

Quantitative Determination of Cell Yield.—Determination of cell yield was accomplished by centrifuging aliquot portions of the cultures. Ordinarily, 10 ml. were centrifuged in Hopkins vaccine tubes for 10 minutes at 2700 R.P.M. The results so obtained are quite reproducible and of an accuracy sufficient to show a linear relationship between cell volume and the amount of growth factor in the lower concentrations of this material.

Fractionation of Yeast Autolysate and Activity of the Fractions

Since Barker had shown yeast autolysate to contain substances essential for the growth of *Prototheca* this material was selected as a source from which to

attempt the isolation of the active constituents. Preliminary extraction tests using ether, chloroform, and 95 per cent ethanol as solvents were carried out on aliquot samples of yeast autolysate adjusted to pH 2.5, 7.0, and 9.0. The autolysate used in these tests was prepared by letting pressed yeast autolyze in the presence of chloroform according to the method of Willstätter (4). Additional extractions using ether and 95 per cent ethanol as solvents were carried out in a Soxhlet extraction apparatus on a dehydrated yeast extract powder (Difco standardized). Appropriate amounts of the soluble and insoluble fractions obtained for each pH value for each solvent were mixed with the basal medium to provide a wide series of media for each fraction. These were inoculated with equal amounts of a *Prototheca* suspension. Cell volume determinations made on aliquot samples from each culture showed that the growth-promoting substances for *Prototheca* are not extracted by ether or chloroform from acid, neutral, or alkaline solutions, as in each case the total activity remained in the insoluble fraction. Cell yield values obtained in the series of media containing the various ethanol fractions showed that the nutrilites for the alga are alcohol-soluble. In addition, growth of the organism in the fractions obtained in the alcoholic extraction of yeast autolysate which had been adjusted to pH 9.0 indicated that the growth-promoting substance had undergone some destruction.

Comparison of Active Material with Known Growth Factors.—The solubility characteristics of the active substance in the various solvents tested, strongly suggested the possibility of its association with the group of B vitamins known at the time these investigations were undertaken. More specifically, its sensitivity to alkali indicated a striking similarity to the properties possessed by vitamin B_1 (thiamin). Consequently, a number of experiments were set up with the mineral glycerol medium, to which a graded series of concentrations of three members of the B group (thiamin, riboflavin, and nicotinic acid) had been added. Growth determinations showed that only those media which contained vitamin B_1 permitted development of *Prototheca* and that the further addition of riboflavin and nicotinic acid did not result in any greater cell yield than was obtained with thiamin alone. The maximum growth in the presence of thiamin alone approximated closely that previously observed in the presence of yeast extract. Comparative experiments using the basal medium enriched with yeast autolysate and with thiamin in different concentrations corroborated this. Additional experiments, in which combinations of low concentrations of yeast autolysate and thiamin were used, showed the activity of the two materials to be additive. Serial subcultures in the glycerol medium with added vitamin B_1 have shown *Prototheca* capable of the same level of growth for many transfers.

These tests consequently established the chemical nature of the factor which is responsible for the activity of yeast autolysate in the growth of *Prototheca*. It can be asserted that the only nutrilite required by this alga is thiamin. Its

activity is very high; concentrations as low as 3×10^{-11} M permit a detectable growth of the alga. The response to increasing concentrations of thiamin is shown in Table I and part of the data has been plotted in Fig. 2. This figure shows that the relationship between thiamin concentration and cell yield is virtually linear to 1×10^{-7} M thiamin. At this concentration growth is practically as heavy as the maximum obtainable with larger amounts of the vitamin. Considerably higher concentrations of vitamin B_1 seem to show a tendency to

TABLE I

Growth of Prototheca in Various Concentrations of Thiamin

Molar concentration of thiamin	Cell yield	Molar concentration of thiamin	Cell yield
	mm.³ per 10 ml.		*mm.³ per 10 ml.*
1×10^{-10}	7	5×10^{-8}	56
3×10^{-10}	9	8×10^{-8}	80
1×10^{-9}	12	1×10^{-7}	96
3×10^{-9}	15	3×10^{-7}	110
1×10^{-8}	20	1×10^{-6}	105
2×10^{-8}	32	1×10^{-5}	100
3×10^{-8}	40	3×10^{-5}	97

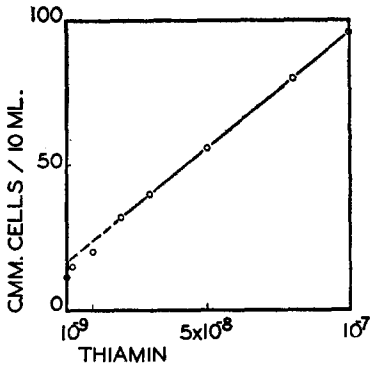

FIG. 2. Relationship between molar concentration of thiamin and cell yield.

exert a slight inhibition. Although the accuracy of determination of cell yield at this cell concentration is insufficient to stress this depressant effect, it may be stated that such results were consistently obtained and that Schopfer (5) has made similar observations.

Thiamin and Its Components As Growth Factors

Thiamin Requirements of Plants and Animals.—The general need for thiamin —as a vitamin for animals, as a necessary growth factor for many microorganisms, and as a hormone (6) for plant cells—shows that it plays an important rôle in the growth of the most diverse types of cells.

The elucidation of the chemical configuration of thiamin and its simple quantitative cleavage into the thiazole and the pyrimidine portions has made possible detailed studies concerning the replaceability of thiamin by its major constituents and by laboratory-synthesized analogues of these constituents. This approach has revealed that both the higher and lower animals require the complete thiamin molecule. Even the protozoa not obviously derivable from algae (ciliates, trypanosomes) behave in this manner. On the other hand many plant tissues and plant-like microorganisms are less exacting and are capable of fulfilling their thiamin requirements from a mixture of the two components of the thiamin molecule as effectively as from the whole molecule. Some of the microorganisms are even able to satisfy their growth requirements from the thiazole or the pyrimidine component supplied singly. In this connection it is interesting to note that certain of the fungi considered as typical plant parasites, resemble the animals in that they require the entire thiamin molecule for growth.

The microorganisms can thus be divided into five groups according to their relationship to thiamin and to its components:

(*a*) Organisms requiring the whole thiamin molecule: The Lwoffs (7 to 10) have shown the ciliate *Glaucoma piriformis* and three species of the flagellate *Strigomonas* to resemble the animals in their vitamin B_1 requirements; Robbins (11) reports ten fungi of the genus *Phytophthora* to likewise require the complete thiamin molecule.

(*b*) Organisms needing thiamin or both of its components in equimolar concentrations: Knight (12) showed *Staphylococcus aureus* to need thiamin or its components for growth, neither part alone functioning in this capacity. Similar findings were made for the molds *Phycomyces blakesleeanus* and *Phycomyces nitens* by Schopfer and Jung (13) and by Robbins and Kavanagh (14) and by Sinclair (15). Schopfer (5) found the fungus *Pilaira moreaui* to require both components also. Lwoff and Dusi (16 to 18) broadened the known list of organisms capable of utilizing mixtures of both components with their findings for the flagellates *Polytoma caeca* and *Chilomonas paramecium*. Robbins and Kavanagh (19) included the yeast *Torula laurentii*, and the basidiomycetes *Ustilago violacea* and *U. scabiosae* were added by Schopfer and Blumer (20).

(*c*) Types of organisms requiring only the pyrimidine component: In the long list of fungi whose growth factor requirements were investigated by Schopfer (21) we find five species of *Rhodotorula* and one of *Dematium* representing the yeasts, and the zygomycetes *Absidia ramosa*, *Parasitella simplex*, and *Pilaira anomala* which are capable of satisfying their growth factor requirements from the pyrimidine component alone. Robbins and Kavanagh (22) found two representatives of *Pythium* and one of *Phytophthora* to include in this type.

(*d*) The fourth group of organisms, those capable of utilizing the thiazole portion alone, includes *Mucor ramanianus* (Müller and Schopfer (23)), and the protozoa *Polytoma caudatum* (Lwoff and Dusi (17)), and *Polytoma ocellatum* (Lwoff and Dusi (24)). It may be pointed out that these two species of protozoa are related to the Chlamydomonas group of green algae and thus can be expected to possess thiamin requirements characteristic of the plant-like organisms.

(*e*) The fifth type comprises a large group of bacteria, yeasts, molds, and algae which are capable of normal development in the absence of any external source of thiamin or its components.

Identification of the Growth Factors for Prototheca zopfii

Although thiamin was found to satisfy the growth factor requirements of *Prototheca*, further tests were made to determine whether this organism needs the whole vitamin molecule, a mixture of the thiazole and pyrimidine components, or one of the components alone as the active principle.

TABLE II

Cell Yield in Mm.[3] *per 10 Ml. of Prototheca Cultures Grown in the Presence of Different Concentrations of Thiamin and of Various Pyrimidine Preparations*

Molar concentration	Thiamin	Pyrimidine						
		3	3 B	4	5	5 W	6	7 M
5×10^{-6}	104	8	9	8	7	7	4	10
1×10^{-6}	94	8	9	8	7	8	4	7
5×10^{-7}	96	8	9	8	7	8	4	8
1×10^{-7}	94	8	7	8	7	7	4	8
5×10^{-8}	86	8	7	8	7	9	4	7
1×10^{-8}	32	8		8	7	9	4	7
5×10^{-9}	17	8	7	9	7	8	4	7
1×10^{-9}	6	6	7	8	7	8	4	5
Control	2							

3	= 2-methyl-4-amino-5-aminomethyl pyrimidine	I. G. Farbenindustrie
3 B	= 2-methyl-4-amino-5-aminomethyl pyrimidine	Buchman (supplied by Dr. James Bonner)
4	= 2-methyl-4-amino-5-hydroxymethyl pyrimidine	I. G. Farbenindustrie
5	= 2-methyl-4-amino-5-chloromethyl pyrimidine	I. G. Farbenindustrie
5 W	= 2-methyl-4-amino-5-chloromethyl pyrimidine	Winthrop
6	= 2-methyl-4-amino-5-aminomethyl pyrimidine	I. G. Farbenindustrie
7 M	= 2-methyl-4-amino-5-ethoxy pyrimidine	Merck

Growth of Prototheca on the Thiazole and on the Pyrimidine Component.—In experiments conducted to determine the ability of the alga to grow on thiazole or pyrimidine alone, media containing various concentrations of five thiazole preparations[1] and seven pyrimidine analogues[1] were made up in glycerol mineral medium and sterilization was accomplished by autoclaving.

Cell yield measurements in media containing pyrimidine alone showed about equal but very slight development for all samples, with one exception, and in no case did an increase in concentration over a 5,000-fold range cause an appreciable increase in cell yield. Data for these growth tests are presented in Table II.

[1] These preparations were kindly supplied by the I. G. Farbenindustrie, Winthrop Chemical Company, Merck and Company, and Dr. James Bonner and Dr. E. R. Buchman of the California Institute of Technology.

The results obtained with four different samples of 4-methyl-5-hydroxyethyl thiazole and one sample of the benzoic acid ester of the same compound are recorded in Table III. They show that, while the benzoic acid ester was without any activity whatever, the effects of the four "natural" thiazole compounds on the growth of *Prototheca* were far from comparable. Cell yields ranged from an amount equal to that obtained in the controls to very nearly the maximum volume obtainable from growth on the whole vitamin. Since the thiazoles used in these tests were different preparations of the same compound, the purity of those permitting heavy growth was not above suspicion. Additional growth

TABLE III

Cell Yield in Mm.³ per 10 Ml. of Prototheca Cultures Grown in the Presence of Different Concentrations of Thiamin and of Various Thiazole Preparations

Molar concentration	Thiamin	Thiazole				
		1	1 W	1 M	1 B	2
5×10^{-6}	104	70	86	84	21	2
1×10^{-6}	94	24	42	90	10	2
5×10^{-7}	96	18	28	84	6	2
1×10^{-7}	94	9	12	51	2	2
5×10^{-8}	86	7	8	33	3	2
1×10^{-8}	32	4	3	12	3	2
5×10^{-9}	17	4	5	2	3	2
1×10^{-9}	6	3	2	2	2	2
Control	2					

1	= 4-methyl-5-hydroxyethyl thiazole	I. G. Farbenindustrie
1 W	= 4-methyl-5-hydroxyethyl thiazole	Winthrop
1 M	= 4-methyl-5-hydroxyethyl thiazole	Merck
1 B	= 4-methyl-5-hydroxyethyl thiazole	Buchman (supplied by Dr. James Bonner)
2	= 4-methyl-5-hydroxyethyl thiazole benzoic acid ester	I. G. Farbenindustrie

tests showed three of the thiazoles to be seriously contaminated to different extents with pyrimidines or substances capable of acting in that capacity. Contaminating substances in the fourth thiazole were of sufficiently low concentration to be detectable only when used in excessively high concentrations; concentrations up to 10^{-5} M failed to provide a satisfactory source of growth factors for *Prototheca*.

Growth of Prototheca on Mixtures of the Natural Thiazole and Pyrimidine Components.—Experiments were carried out to determine the ability of *Prototheca* to grow in a series of media containing graded concentrations of mixtures of the purest thiazole and one of the natural pyrimidine preparations. The results of one experiment are summarized in Table IV. They show unequivocally that *Prototheca zopfii* is able to utilize a combination of the two com-

ponents in place of the whole thiamin molecule and that the components are required in equimolar proportions. The cell yield is determined by the component of the thiamin molecule that is present in the smaller amount.

The data obtained in these experiments give rise to some further comments. Both in the absence and in the presence of very low concentrations of thiazole the growth of *Prototheca* appears to be due to some extent to the amount of pyrimidine present. This suggests the presence of a limited supply of thiazole in either the medium, the inoculum, or as a contaminant of the pyrimidine. The presence of thiazole as an impurity of the pyrimidine should have resulted in increased growth in the excessively high concentrations used to test this component alone and, therefore, can be ruled out.

TABLE IV

Cell Yield in Mm.³ per 10 Ml. of Prototheca Cultures Grown in the Presence of Different Concentrations of Mixtures of the Purest Thiazole and Pyrimidine

Molar concentration		Pyrimidine 3B								
		0	1×10^{-10}	3×10^{-10}	1×10^{-9}	3×10^{-9}	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}
Thiazole 1 B	3×10^{-7}	2	3	5	12	22	42	92	92	94
	1×10^{-7}	2	3	5	12	22	42	100	100	96
	3×10^{-8}	2	3	5	12	21	44	92	92	94
	1×10^{-8}	2	3	5	12	21	41	54	54	48
	3×10^{-9}	2	3	5	10	17	22	22	22	22
	1×10^{-9}	2	3	4	10	14	15	14	14	15
	3×10^{-10}	2	3	4	10	12	11	11	12	12
	1×10^{-10}	2	3	4	8	11	12	11	11	11
	0	2	3	4	8	9	10	9	11	10

The occurrence in nature of thiazole unaccompanied by an equivalent quantity of pyrimidine is apparently so rare that the chemicals used in the basal medium would seem most unlikely as a source of thiazole contamination. If thiazole were introduced with the inoculum it can be inferred that the cells used for this purpose must have contained an excess of thiazole. Special experiments, however, to test the storage of thiazole by *Prototheca* gave negative results.

The higher concentrations of the mixtures allow a total cell yield closely approximating the maximum growth obtainable in cultures growing in media containing the complete vitamin or yeast autolysate. A number of experiments have shown that maximum cell yield is obtained at a concentration approximately 3×10^{-8} M for each component of the thiamin molecule. It is interesting to note that this is about one-tenth of the concentration necessary when the whole molecule is used. This observation is in line with that of Schopfer who found *Phycomyces blakesleeanus* to require approximately twice as much thia-

min as a mixture of the two components in order to give comparable maximum yields.

Since the alga cannot grow unless supplied with both pyrimidine and thiazole, *Prototheca zopfii* represents another member of the previously mentioned group (*b*).

SUMMARY

Barker's study on the nutritive requirements of *Prototheca zopfii* indicated that this colorless alga fails to grow in the absence of small amounts of yeast extract. A study of the growth factor requirements of *Prototheca* has shown that the active constituent of yeast extract necessary for the growth of this organism is thiamin (vitamin B_1). Thiamin can fully replace the complex yeast material and allows, in the basal medium used, a maximum cell yield in concentrations of $1\text{–}3 \times 10^{-7}$ M.

Thiamin as such, however, is not essential for the growth of *Prototheca zopfii*. The alga can develop equally well if supplied with both the thiazole and pyrimidine constituents of this vitamin. These appear to be needed in equimolar proportions. Maximum cell yield is obtained with 3×10^{-8} M concentrations of the two components.

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