Mutual relations of protozoans and symbiotic Algae in *Paramecium bursaria*

III. Nutritive requirements of the *Chlorella* clone occurring as an endosymbiont of *Paramecium bursaria*

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INTRODUCTION

The clone *Chlorella* occurs always as an inner partner in symbiosis with the protozoan *Paramecium bursaria*. The algae *Chlorella* find appropriate growth and developmental conditions in the host cell, and are transferred from generation to generation during division of the host cell. A close symbiosis and its durability are the causes of great difficulty encountered in separating the animal from the algae (Pringsheim 1928; Siegel 1960; Pado 1965). Thus the question arises whether — owing to a longlasting endosymbiotic coexistence in absolutely different conditions in relation to the free-living algae — the nutritive requirements of the endosymbiotic algae will not be quite different.

The general tendency to a nutritive compounds exchange, which takes place in many symbiotic organisms, has been long known. According to Buchner (1953) such investigators as: Penard, Graber, Doflein found a trophic interaction of algae and the animal organism in the symbiosis of *Paramecium bursaria*. More recent investigations, on the other hand, (Pringsheim 1925, 1928; Parker 1926; Loefer 1936 a, b) show that the algae leave a considerable part of the assimilates at the disposal of the protozoan. This among other things allows the protozoan to survive for months in light and sterile conditions in an environment of sterile water, in the same environmental conditions, however, the protozoans deprived of algae die much sooner.

Recent investigations by Pado (1967) on photosynthesis in *Paramecium bursaria* show in a quantitative way the important role of the photosynthetic process in the metabolism of the whole symbiotic system.

As regards the results obtained, it seems a necessity to stress the possible conveniences resulting for the algae from symbiosis with an animal organism. Algae enclosed within a protozoan cell find good protection, water supply, mineral salts and large amounts of CO₂. The concomitant establishment of a positive phototaxis in *Paramecium bursaria* (Wichterman 1948) indicates that exposure to light is also more advantageous for symbiotic than for free-living algae. Besides certain advantages concerning the microenvironment in which the algae live, it cannot

be excluded that the animal partner supplies the endosymbionts with some chemical compounds necessary or favourable for their growth. Advanced morphological changes of endosymbiotic algae in relation to the corresponding free-living clones have been established in numerous cases (Keeble and Gamble 1905, 1907, 1910; Younge 1936; Younge and Nicholls 1940).

Vitamin and hormonal connections established in lichens (Quispel 1943; Tobler 1944; Zehnder 1949) may suggest that besides morphological changes some biochemical changes making the algae dependent on the metabolism of the host occur in the algae-animal metabolism.

The latter aspect of the symbiosis physiology has not been well recognized as yet. A precise species specification of the organisms entering into symbiosis (Goetsch 1924; Pringsheim 1928; Siegel 1960) seems to indicate a correlation of biochemical nature between the symbiotic partners.

The present investigations were aimed at finding an answer to the question whether the clone of the symbiotic zoochlorella occurring in *Paramecium bursaria* is identical with the free-living representatives of the *Chlorella* genus, or if it is distinguished by special biochemical properties.

MATERIAL AND METHOD

A pure culture of *Chlorella* isolated from protozoans cultured under light of high intensity (2700 lux) was used for investigations on the nutritive requirements of algae. Sterilization of the protozoans was carried out according to Wichterman's (1953) method modified by Siegel (1960), without applying antibiotics. The procedure of purification of the infusoria from bacteria consisted in 6–7 successive washings with sterile nutrient solution in sterile conditions. Transfer of the protozoans for the successive washing was controlled under a stereoscopic microscope and carried out according to the previously given method (Pado 1965). Control of sterility was carried out by bacterial inoculation of samples taken from each washing. Inoculations were made on Petri dishes:

1. with Loefer (1936 c) nutrient solution solidified with 2 % agar and
2. on nutrient solution prepared from dry agar medium (Suche Podłoże Agarowe Zwykłe produced by Wytwórnia Surowic i Szczepionek, Warszawa) — 220 g per 1 l. of water. As regards growth values, this nutrient solution corresponds fully to the ordinary agar medium (MPB — meat — peptone broth) prepared from fresh meat. The pH of the medium 4.0, 7.0, 10.0 was adjusted by means of 15 % HCl or 10 % NaOH. Nutrient solution was poured into Petri dishes and sterilized by autoclaving under 2 atm. for 30 min.

Sterilized protozoans were transferred to test tubes with 8 ml of Loefer's nutrient solution each (1936 a) of the following composition: proteose peptone 5.00 g, glucose 5.00 g, $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$ 0.14 g, NaCl 0.10 g, KH_2PO_4 0.10 g, FeCl_3 traces, distilled H_2O up to 1000 ml. pH of the nutrient solution 6.5–6.7 was adjusted by means of 1N NaOH. Protozoans (5–8) were placed in each sample.

Fig. 1. The shaking machine used for the cultures of algae.

A series which within 72 hours following sterilization did not show any infection of the nutrient in the test tubes was considered sterile and used for further investigations.

Test tubes were placed in a thermostat illuminated with 1700 lux at 27°C. The presence of protozoans was controlled every day under a stereoscopic microscope. After a period of 25—30 days free algae originating from break up of the infusoria were noticed in the test tubes, where they formed green deposits on the bottom. In order to create better developmental conditions for the freed algae, the content of the test tubes was stirred several times a day by shaking or by placing the test tubes in a shaking machine in a light thermostat.

About 1.5 month or even later following inoculation, the amount of algae was sufficient to be used as an inoculum for further investigations. Before this, however, the cultures were subjected once again to a sterility test according to the previously described method of inoculation on agar medium. The sterility test was negative and did not show the presence of any bacteria in the cultures. Green agglomerations of *Chlorella* algae grew in the dishes with Loefer's nutrient solution, provided the inoculum concentration was high enough. A sterile culture of *Chlorella* algae was used for the main investigations.

Knop's nutrient (1884) of the following composition was the basic mineral nutrient solution: $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$ 0.80 g, KNO_3 0.20 g, KH_2PO_4 0.20 g, MgSO_4 0.20 g, FeCl_3 -traces, distilled water up to 1000 ml. pH = 6.5 was adjusted by means of 1N NaOH. The nutrient solution was modified by means of glucose, yeast extract or vitamins of group B in the way described in the following chapter. Glucose was a p.a. preparation. The yeast extract was prepared by pouring 1000 ml. distilled water over 10 dkg yeast. After boiling the solution was put aside for 12 hours in

a high glass cylinder for the suspension to settle down. The poured off yeast water was still subjected to decantation and centrifugation.

Vitamins B_{complex}, B₁, B₆, B₁₂, were obtained from pharmaceutical preparations (ampoules) admitted for medical treatment by the Ministry of Health. Thiazole-L, (Light England) was used 2-Methyl-4-amino-5-methyl-ethoxy-pyrimidine was available from the Biochemical Institute, Polish Academy of Sciences, Warsaw.*

Table 1

Growth of the endosymbiotic *Chlorella* — strain on the Knop mineral nutrition with addition of vitamins

Time of culture in days since inoculation	Cell number per 1 ml				
	Knop	1% glucose	yeast extract	0.025% vitam. B _{complex}	0.01% vitamin B ₁ (thiamine)
0	5.185×10^6	5.185×10^6	5.185×10^6	5.185×10^6	5.185×10^6
4	5.333×10^6	10.419×10^6	16.098×10^6	20.000×10^6	32.641×10^6
8	5.037×10^6	7.703×10^6	33.777×10^6	39.111×10^6	54.419×10^6
12	5.086×10^6	6.617×10^6	39.654×10^6	39.703×10^6	68.395×10^6

Nutrient solution was poured out into 50-ml flasks with a flat bottom, 20 ml into each, and after sterilization by autoclaving, inoculated with a sterile suspension of algae by means of specially scaled pipettes. The amount of algae applied for the inoculum was equal in every series. The amount of algae cells, — both in the inoculum and in various growth phases of the culture, was determined by the use of Burk's chambers; the technique applied was similar to that used for counting red blood cells. Two samples from each flask were counted at a time.

Flasks with nutrient solution inoculated with a known amount of algae were placed on a specially constructed shaking machine with frequency of about 120 shakes/min and 30 mm amplitude, driven by an electric motor. This shaking machine (fig. 1) was placed in a light thermostat with constant temperature 27°C. Light intensity on the culture level was 2300 lux. Shaking given to the culture proved to be very advantageous, it accelerated the growth of the culture and prevented the algae from falling down and sticking to one another.

The increment of algae on individual nutrient solutions was calculated on every 4th day following inoculation. Two flasks — subsequently eliminated from further experiments — were used each time for determination of the suspension density.

* The author wishes to express his gratitude to doc. dr K. L. Wierzchowski for making this preparation available to him.

Before determination of the amount of algae cells the loss of the volume due to evaporation was completed up to the starting value (20 ml). Control counts were carried out 4, 8, 12 and sometimes 18 days after inoculation.

Micrographs of endosymbionts cultured for 14 days on a mineral nutrient solution according to Knop with 0.01 % vitamin B₁ concentration and of algae immediately after isolation from *Paramecium bursaria* were used for the quantitative evaluation of morphological changes in algae. The pictures obtained were projected and the cell diameter measured on the pictures; the longer dimension of every cell being measured.

RESULTS

The first phase of experiments aimed at checking if the obtained clone of endosymbionts is able to live and reproduce in light on a nutrient solution containing only mineral compounds. Knop's nutrient solution proved to be quite insufficient

Fig. 2 Growth curves of endosymbiotic *Chlorella*—strain cultured in various media

1 — Mineral medium + vitamin B₁ 0.01%; 2 — mineral medium + vitamin B complex 0.025%; 3 — mineral medium + yeast extract; 4 — mineral medium + vitamin B₁₂ 0.01%; 5 — mineral medium + glucose 1%; 6 — mineral medium + vitamin B₆ 0.01%; 7 — mineral medium

x axis — time of growth in days; y axis — number of cells in 1 ml

as nutritive medium. No growth was recorded either after 14 days or even a much longer time (fig. 2).

Addition of glucose in the amount of 1 % also remained without any influence (fig. 2).

A distinct reproduction of cells, on the other hand, was observed on nutrient solution with yeast extract. Although the qualitative and quantitative composition of the yeast water is not precisely known it is certain, however, that it contains, great amounts of nitrogen compounds and vitamins of group B.

Table 2

Growth of the endosymbiotic *Chlorella* — strain on the Knop mineral nutrition with addition of vitamins

Time of culture in days since inoculation	Cell number per 1 ml			
	0.02% thiamine	0.03% thiamine	0.01% vit. B _{complex}	0.025% vit. B _{complex}
0	5.185×10^6	5.185×10^6	5.185×10^6	5.185×10^6
4	36.938×10^6	18.962×10^6	29.728×10^6	20.000×10^6
8	75.061×10^6	35.851×10^6	45.975×10^6	39.111×10^6
12	75.604×10^6	37.432×10^6	54.024×10^6	39.703×10^6

In order to make sure if the above mentioned growth can be associated with the presence of vitamins of group B in the yeast extract, vitamin B_{complex} (0.025 % concentration) was added to Knop's nutrient solution in further investigations.

In these conditions growth proceeded actively.

Fig. 3 Growth curves of endosymbiotic *Chlorella* — strain cultured in various media:

1 — Mineral medium + vitamin B₁ 0.02%; 2 — Mineral medium + vitamin B₁ 0.01%; 3 — Mineral medium + vitamin B_{complex} 0.01%; 4 — Mineral medium + vitamin B_{complex} 0.025%; 5 — Mineral medium + vitamin B₁ 0.03%

x axis — time of growth in days; y axis — number of cells in 1 ml

The favourable influence of vitamin B_{complex} prompted us to examine the influence of individual vitamins of group B. The following vitamins were applied: B₁, B₆, B₁₂.

Vitamin B₁ proved to be most favourable. In the concentration applied (0.01 %) it gave best growth results of all the nutrient solutions applied (fig. 2). Addition of other vitamins of group B, on the other hand, did either gave no results or the division of cells was so scarce (fig. 2) that it could be due to a certain contamination

with vitamin B₁. This would be evidence that vitamin B₁ is a necessary factor for development of endosymbiotic algae *Chlorella* in light conditions.

The influence of concentration of vitamin B₁ and vitamin B_{complex} was the subject of further investigations. In both cases the concentration of vitamins influences growth to a certain extent. A double dose of vitamin B₁ causes a much stronger growth of algae. The dose 0.02 % of vitamin B₁ in the nutrient solution proved to be the optimum concentration for the clone examined. Further increase of concentration of vitamin B₁ to 0.03 and 0.04 % acts in an inhibitory way on the growth of the culture, the higher the concentration the greater being the inhibition (table 2 fig. 3).

Vitamin B_{complex}, on the other hand, influences the growth of the culture optimally in a concentration 0.01 % (table 2 fig. 3).

As it results from the above given data, the endosymbiotic *Chlorella* clone is a defective form in relation to the free-living *Chlorella* species. While the latter are complete autotrophs, the symbiotic zoochlorella is an auxanotroph with ability to photosynthesis and growth on a mineral nutrient solution only in the presence of vitamin B₁.

Table 3

Growth of the endosymbiotic *Chlorella* — strain on the Knop mineral nutrition with addition of vitamins

Time of culture in days since inoculation	Cell number per 1 ml				
	0.01% thiazole	0.005% thiazole +0.005% pyrimidine	0.01% pyrimidine	0.02% thiamine	0.01% thiazole second transfer
0	5.185×10^6	5.185×10^6	5.185×10^6	5.185×10^6	5.185×10^6
2	5.333×10^6	5.432×10^6	5.283×10^6	22.222×10^6	14.765×10^6
4	5.382×10^6	5.283×10^6	5.135×10^6	37.086×10^6	24.470×10^6
6	33.185×10^6	20.000×10^6	5.333×10^6	56.888×10^6	37.086×10^6
8	38.716×10^6	28.797×10^6	5.185×10^6	71.703×10^6	45.234×10^6
10	40.493×10^6	28.197×10^6	5.135×10^6	74.123×10^6	47.456×10^6
12	39.111×10^6	28.098×10^6	5.086×10^6	72.888×10^6	50.024×10^6
14	40.197×10^6	28.493×10^6	5.135×10^6	—	52.790×10^6

In view of the results obtained, the problem of the kind of disturbances in the mechanism of vitamin B₁ synthesis in algae seemed to be interesting and worth examination. The question in point was whether the endosymbiotic *Chlorella* must

Fig. 4 Growth curves of endosymbiotic *Chlorella* — strain cultured in various media:

1 — Mineral medium + vitamin B₁ 0.02%; 2 — Mineral medium + thiazole 0.01%; 3 — Mineral medium + thiazole 0.005% + pyrimidine 0.005%; 4 — Pyrimidine 0.01%

x axis — time of growth in days; y axis — number of cells in 1 ml

Fig. 5 Growth of endosymbiotic *Chlorella* — strain cultured in mineral medium (Knop) with:

a — T+P thiazole + pyrimidine; b — T thiazole; c — P pyrimidine; d — B₁ vitamin

be given the above growth factor in its final form or whether it preserves the ability of an at least partial synthesis of vitamin B₁.

The series of experiments, which were to give an answer to the above question, were carried out on nutrient solutions of the following composition:

1. Knop+0.01 % thiazole
2. Knop+0.01 % pyrimidine (2-methyl-4-amino-5-methyl-ethoxypyrimidin)
3. Knop+0.005 % thiazole and 0.005 % pyrimidine
4. Knop+vitamin B₁ 0.02 % — control.

The highest growth rate was recorded when vitamin B₁ was used in the mineral nutrient solution; the growth of the culture, however, proceeded as well on nutrient

solution with both compounds of vitamin B₁ (thiazole+pyrimidine) as in the case when the nutrient solution contained thiazole only. The above given data are plotted in table 3 figs. 4, 5.

It results from these data that the examined *Chlorella* clone need not necessarily obtain ready vitamin B₁. The cells of algae are able to combine both elements of vitamin B₁, provided they have been introduced together into the nutrient solution. They also show an ability — and this is most noteworthy — to synthesize pyrimidine within their own competence — and to build it on the thiazole supplied.

Thiazole, thus, is the factor which is indispensable for the growth and development of algae cultures on a mineral nutrient solution.

The course of growth of algae cultures on thiazole and thiazole+pyrimidine requires a separate discussion. In the initial period, 4–6 days after inoculation of

Fig. 7a The cells of endosymbiotic *Chlorella* immediately after isolation from the protozoan
 Fig. 7b The cells of the endosymbionte *Chlorella* — strain cultured on Knop medium with vitamin B₁
 (magnifications of both pictures the same)

the nutrient solution containing thiazole, the culture did not show any growth; the amount of algae remains at that time almost at the same level (fig. 4). Then the growth processes becomes intensively activated. Owing to this a distinct change in the colour of the nutrient solution — from colourless to green — can be observed. Within the next 3—4 days the amount of cells stabilizes at a constant level. The presented (fig. 4) course of growth of the culture may suggest that the initial inhibition of growth and division processes is caused by lack of certain enzymes, which are formed later (adaptation enzymes).

In order to check up this supposition nutrient solution with thiazole was inoculated with an inoculum taken from 14–16 day—old cultures on mineral nutrient solution with thiazole, i.e. with algae taken from the period when the culture was at its maximum growth. If the previous supposition concerning the existence of adaptation enzymes is right, organisms possessing previously formed “adaptation enzymes” are, thus transferred by inoculation of the algae from such a culture on to a fresh nutrient solution with thiazole. The presence of these enzymes should be manifested by growth beginning already from the first days after inoculation.

The results obtained indicate the existence of such enzymes as the course of growth is now very similar to that obtained for the control, i.e. mineral Knop nutrient solution +0.02 % vitamin B₁. Division of algae takes place from the very first days following inoculation as shown in table 3 and fig. 6.

During the multiple countings of cells while examining the growth of cultures, some differences in morphology of the algae cells and specially in their dimensions were observed. It was found that cells of the endosymbionts are immediately after isolation from the host cell, three times the size of those cultured for a few days on mineral nutrient solution with addition of vitamin B₁ (figs 7a and 7b). The mean dimensions of cells immediately after isolation from *Paramecium bursaria* and of those from cultures grown for 14 days on mineral nutrient solution according to Knop with vitamin B₁ equal $11.9 \pm 0.196 \mu$ and $3.3 \pm 0.237 \mu$, respectively.

Further differences concern the cell structure. In the population of algae isolated immediately from the protozoan *Paramecium bursaria* a high percentage of cells possess a chloroplast already divided into 2 or 4 chloroplasts, a characteristic feature of the stage preceeding division (stage L₃ or L₄ according to the symbolics by Tamiya and others 1962). Whereas, in the population of cells cultured on mineral nutrient solution with vitamin B₁ the percentage of such cells is considerably lower.

The supposition may be advanced that life conditions created for the algae by the symbiotic protozoan prolong the developmental phase L₃—L₄; in cultures of isolated algae, on the other hand, this phase is shortened in favour of D—L.

DISCUSSION

The mineral nutrient solution applied in the above given experiments according to Knop proves to be quite unsatisfactory for the growth and development of algae in light conditions.

It would seem that the life of algae in symbiosis handicapped them in their ability of biosynthesis, as revealed distinctly after isolation from the host organism.

What concerns nutrient requirements of symbiotic algae these have been little recognized as yet, zoochlorellae were the object of numerous investigations, but only Mc Laughlin and Zahl (1957) deal with nutrient requirements of zooxantella. Haffner (1925) maintains that the considerably varied nutrient requirements of endosymbiotic algae should be ascribed to the diversity of clones. Beijerinck (1890) maintains that glucose and peptone are of vital importance for endosymbiotic *Chlorellae*.

Recent investigations on zoochlorella isolated from *Paramecium bursaria* (Loefer 1936; Ehret 1953; Siegel 1960) show that there exists a possibility of maintaining on aseptic cultures to nutrient solution containing 5 % of mineral salts with addition of glucose and peptone (Loefer's nutrient solution). Its composition suggests a heterotrophic character of the symbionts. On the other hand, it can be affirmed — on the grounds of a series of investigations with special attention paid to photosynthesis of the complex *Paramecium bursaria* — that the green symbionts assimilate at a high rate making in many cases the whole symbiotic system photoautotrophic (Pado 1967).

An analogous situation is encountered in lichens where some not definitely known growth factors are often required by the symbiotic algae for full photosynthesis. Neither is it known yet whether these growth factors are taken up by the myxomycete directly from the medium, or if they are able to produce them themselves and transfer them subsequently to the algae.

Addition of yeast extract to the nutrient solution resulted in a vigorous growth of endosymbiotic algae cultures. This result suggests that vitamins from group B, specially abundant in yeast seem to be responsible for the growth of the symbionts. Vitamin B_{complex} applied in the nutrient solution with a positive result corroborated this hypothesis.

The action of vitamin B₁ in the metabolism of a living organism has been already recognized to a great extent. The derivative of vitamin B₁ — pyrophosphate thiamin appears as a coenzyme of decarboxylase and aldehyde transferase. Thiamine plays here an essential part in the oxydative decarboxylation of pyruvic acid and α -ketoglutaric acid.

The presence of vitamin B₁ is indispensable for the cell metabolism. In contrast to animals, the majority of plants are able to synthesize this vitamin. There are plants, however, which have lost this ability. As Schopfer (1934) established *Phycomyces blakesleanus* belongs to this group. Fries (1948) found a partial or total sensitivity to thiamine deficiency.

The loss of ability of vitamin B₁ synthesis varies in individual organisms. Lilly and Barnett (1959) divide micro-organisms into 4 groups with reference to their sensitivity to vitamin B₁:

a) organisms which require an intact molecule of vitamin B₁ (thiamine) have no ability to synthesize this compound even if they are supplied with both components, thiazole and pyrimidine.

b) organisms capable of synthesizing complete vitamin B₁ from the supplied mixture of thiazole and pyrimidine.

c) organisms capable of synthesizing thiazole and combining it with pyrimidine supplied from outside.

d) organisms capable of synthesizing pyrimidine and combining it with the thiazole supplied.

It should be stressed that complete vitamin B₁ (thiamine) is the only substance active and indispensable in metabolism. Leonian and Lilly (1940) showed that

the presence of one substance as well as of both does not cause any growth until thiamine is formed from them.

On the grounds of the present data (fig. 4 and 5) the endosymbiotic *Chlorella* clone should be classified to organisms of the (d) group.

The curve of growth of the endosymbiont culture after addition of the two components separately — thiamine or thiazole only (fig. 4) — deserves special attention. Control cultured on complete vitamin B₁ manifest a gradual and regular growth from the first days following inoculation. In experimental cultures, on the other hand, (T) and (T+P), an almost complete inhibition of division processes was observed in the initial period of 4–6 days. The vigorous growth which started after the lapse of 4–6 days can be most probably associated with production of certain adaptation enzymes. The result obtained from cultures where algae cultured on a mineral nutrient solution with thiazole were used as inoculum corroborate this presumption. A transfer of such algae on to a fresh nutrient solution gives an immediate growth effect, as in this case, presumably, the cells of the inoculum already possess the adaptation enzymes (fig. 6).

The endosymbiotic clone *Chlorella* shows after isolation from *Paramecium bursaria* cells ability to photosynthetic production of organic materials only in the presence of vitamin B₁. Since photosynthetic production has been revealed in the whole symbiotic complex (Pado 1967), it may be assumed that in normal symbiosis vitamin B₁ is supplied to the algae by the host organism.

A one-sided or mutual dependence of the symbiotic partners based upon exchange of growth factors has already been reported many times. These data chiefly refer to lichens. Tobler (1944) proved that *Xanthoria parietina* requires vitamin B₁ or at least its component — thiazole for germination of exospores; the optimal effect was obtained at a concentration of 1:500 000. Leonian (1936) established that four algae: *Chlorella viscosa*, *Coccomyxa simplex*, *Oocystis negelii* and *Scenedesmus flavescens* are species which — besides carbohydrates — supply *Phytophthora cactorum*, their symbiont, with a growth factor — thiamine.

In the lichen *Placodium saxicola* the myxomycete component shows requirement for vitamin B₁ or its components on which its development depends. Zehnder (1949) proved that *Placodiomyces saxicolae* takes vitamin B₁ from its green endosymbiont *Cystococcus*.

The quantitative requirement for vitamin B₁ varies in individual species. For *Placodiomyces saxicolae* Zehnder (1949) gives as optimal 0.004 γ /ml, Muller (1941) for *Mucor ramanianus* 0.08 γ /ml stressing, however, the fact that the optimum of vitamin B₁ activity increase with the duration of the experiment, and this is connected with varying requirements depending on the developmental stage of the given organism.

In the present investigations vitamin B₁ in 0.02 % concentration proved to be the optimum dose for *Chlorella* with *Paramecium bursaria*. There must exist some mechanisms regulating the number of endosymbionts in a symbiotic complex, which would permit to keep a certain balance and prevent one symbiont from complete predominance over the other. These mechanisms, however, are still little known.

Intracellular digestion of the algae surplus, observed by Young (1944) for *Tridacna* can be regarded as the simplest form. Many suggestions have been made to explain the drop in division rate of algae by a more active uptake of organic compounds from them (Pasher 1929 a b; Geitler 1938). The mechanism of changes in activity of assimilates uptake by the heterotrophic symbiont has not been elucidated. The established fact of a certain dependence of symbionts in the line of vitamin exchange shows a new possibility of elucidating the mechanisms of symbiotic balance.

It may be supposed that when a protozoan culture remains in darkness, the control of the protozoan over the algae runs along the line of limiting the amount of vitamin B₁; this causes a decrease of the division rate or, what is less probable, it may influence the division rate, decreasing it by an increase in the amount of vitamin B₁ over the optimal dose.

The changes established in the size of cells of algae cultured on vitamin B₁ in comparison with the cell size immediately after isolation from the protozoan may suggest that vitamin B₁ plays a certain, so far not precised part in the development of algae, which is morphologically manifested. Tamiya, Morimura and Yokota (1962) examined the influence of many substances upon the developmental cycle of *Chlorella elipsoides*. Some of the examined substances acted in a very specific way. Thus, 8-azaguanine and 8-azaxanthine caused an excessive growth of *Chlorella* cells, inhibiting the division ability at the same time. Other substances as e.g. phenyl-lactic acid or 5-bromouracil in certain concentrations did not influence the growth of algae cells in any way, but the number of divisions (normally n-4) increased greatly what in turn resulted in a smaller than normal size of the daughter cells. It cannot be excluded that the differences recorded in the size of cells of *Chlorella* occurring as a symbionts and cultured on vitamin B₁ may be caused by a specific influence of this compound disturbing the normal rythm of growth and division. It may also be that in this case vitamin B₁ influences the endosymbionts in a similar way by an increase of the number of divisions (*n*), it may also be that the cells acquire sooner the division ability before reaching their appropriate size.

SUMMARY

The aim of the present paper was to determine the nutrient requirements of the endosymbiotic *Chlorella* clone which occurs as an inner symbiont of the protozoan *Paramecium bursaria*.

The data obtained proved that the isolated clone of zoochlorella is a vitamin-deficient mutant which requires a supply of vitamin B₁ from outside for normal development and activity of the photosynthetic apparatus. On the basis of the above given results, some conclusions are drawn concerning the mechanism of regulation of the algae number in a protozoan cell, possibly by regulating the amount of the thiamine supplied by the protozoan.

The concentration of vitamin B₁ influencing the development of the culture of the endosymbiotic *Chlorella* has been calculated and determined at about 0.02%.

It has also been established that vitamin B₁ which is a necessary factor inducing growth need not be supplied to the algae in ready form, it suffices when thiazole — one of its components — is introduced into the nutrient solution.

Morphological differences mainly in the size of the cells of the endosymbionts have also been revealed. *Chlorella* cells cultured for a time on a mineral nutrient solution with vitamin B₁ are almost 1/4 the size of the ones isolated from the protozoan *Paramecium bursaria*.

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Wzajemna współzależność pierwotniaków i symbiotycznych glonów u Paramecium bursaria

III. Wymogi odżywcze szczepu *Chlorella* występującego jako endosymbiont *Paramecium bursaria*

Streszczenie

W powyższej pracy starano się określić wymagania pokarmowe endosymbiotycznej *Chlorella*, który to szczep występuje jako wewnętrzny symbiont z pierwotniakiem *Paramecium bursaria*.

W wyniku uzyskanych danych stwierdzono, że wyizolowany szczep zoochlorelli jest mutantem witaminowo-deficytowym, który dla swego normalnego rozwoju oraz normalnego funkcjonowania aparatu fotosyntetycznego wymaga dostarczenia z zewnątrz witaminy B₁. W związku z powyższym wysunięto też pewne wnioski odnośnie mechanizmów regulacji liczby glonów w komórce pierwotniaka, widząc tę możliwość w regulacji ilości dostarczanej przez pierwotniaka tiaminy.

Obliczono, jaka ilość witaminy B₁ ma optymalny wpływ na rozwój kultury endosymbiotycznej *Chlorella* określając ją na około 0,02%.

Wykazano też, że witamina B₁ będąca tym koniecznym czynnikiem wyzwalającym wzrost, nie musi być podawana w gotowej formie, lecz wystarczy dostarczenie do pożywki jednego jej składnika, jakim jest tiazol.

Stwierdzono również różnice w morfologii, głównie wielkości komórek endosymbiontów. Komórki *Chlorella* hodowane przez pewien czas na pożywce mineralnej z witaminą B₁ są prawie czterokrotnie mniejsze od wyizolowanych bezpośrednio z pierwotniaka *Paramecium bursaria*.