

## On the mechanism of biosynthesis reorientation in *Chlorella* under the influence of factors limiting cellular division

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With 5 figures and 1 table in the text

### Abstract

We have studied the effect of cycloheximide (CHI) on the reorientation of biosyntheses which takes place in *Chlorella* under nitrogen starvation.

In a nitrogen free medium and under conditions favouring high rates of photosynthesis the strain *Chlorella pyrenoidosa* 82 accumulates large quantities of lipids while another strain, *Chlorella* sp.K, accumulates starch. The predominant synthesis of lipids does not take place in *Chlorella pyrenoidosa* 82 if CHI is added to the culture at the moment of its transfer to the N-free medium. CHI has no effect on the response to nitrogen deficiency of the strain *Chlorella* sp.K and, in fact, the behaviour of the lipid accumulating strain in presence of CHI resembles that of the starch accumulating strain.

We conclude that enzymes for the predominant lipid synthesis are of adaptive nature and that their formation in the cell is induced by the transfer to the nitrogen free medium. The time course of CHI inhibition shows that the synthesis of these enzymes is completed within 1.5 hour following this transfer.

Key words: *Chlorella*, nitrogen starvation, lipid synthesis, starch synthesis, cycloheximide, adaptive enzymes, proteosynthesis inhibition.

P D C \* : SS 12; BN 02, 03; PY 04, 052, 101, 111; BC 04, 05, 06, 103; EC 14; CH 05; GC 03, 04, 30; ME 08, 13, 15; UC 01.

### Introduction

It is known that nitrogen starvation (SPOEHR & MILNER 1949, KLJAČKO-GURVIČ & SEMENENKO 1965, KLJAČKO-GURVIČ 1966, ŽUKOVA & KLJAČKO-GURVIČ 1969, GITELZON et al. 1966), supraoptimal temperature (SEMENENKO et al. 1969, SEMENENKO & ZVEREVA 1972), solutions with high ionic strength

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(SEMENENKO et al. 1972) and inhibition by mitomycin C (SEMENENKO & RUDOVA 1972) cause uncoupling of synthetic and reproduction processes in the cells of *Chlorella*. This is accompanied by essential changes in the synthetic activity of the cell, which becomes usually diverted to a single predominant pathway producing a storage material such as lipid or starch.

The particular direction of the diverted syntheses under uncoupled conditions does not depend on the nature of the factor that produced the disturbance. It is rather determined by the genotype of the algal strain and we have shown that among the strains of *Chlorella* forms accumulating lipids and other ones, synthesising predominantly carbohydrates, can be found (KLJAČKO-GURVIČ 1964, KLJAČKO-GURVIČ & SEMENENKO 1965, SEMENENKO & ZVEREVA 1972).

To understand the regulatory mechanism underlying the reorientation of biosyntheses in *Chlorella* at uncoupling of their cellular functions it is essential to know whether this transition requires the synthesis of corresponding enzymes and, if so, what protein synthesizing systems are involved. We have attempted in the present paper to answer some of these questions by the method of inhibitor analysis widely used in the study of adaptive enzyme induction (cf. HAVKIN 1969, KRETOVIČ 1972).

The analysis performed earlier in our laboratory revealed that if lipids accumulate in *Chlorella pyrenoidosa* 82<sup>1</sup>, in response to nitrogen starvation and to other factors inducing predominant lipid synthesis, they form globules localized in the cytoplasm (KLJAČKO-GURVIČ et al. 1968, VLADIMIROVA et al. 1968). On the other hand, if starch is accumulated under uncoupled conditions it is, as can be expected on general grounds, localized in the chloroplast. That this is actually so in the strain *Chlorella* sp. K.<sup>1</sup> was proved in our laboratory (SEMENENKO et al. 1969).

On the basis of these facts it could be anticipated that the enzymic systems responsible for synthesis of reserve lipids were localized in the cytoplasm and that the application of cycloheximide (CHI), the specific inhibitor of protein synthesis on the cytoplasmic 80S ribosomes (ŠEMIJAČEK et al. 1961, ROODYN & WILKIE 1968, SPIRIN & GAVRILOVA 1971, SMLIE et al. 1967) should suppress their formation. That CHI can be successfully used in algae, including

<sup>1</sup> The authors prefer to use in this paper the designation of the two algal strains which they had used in many communications before. The strains are also kept under these labels in the culture collection of algal strains in Dr. SEMENENKO's laboratory. The unambiguous designation (and correct taxonomic position) of the strains is as follows: *Chlorella pyrenoidosa* 82 ≡ *Chlorella vulgaris* BEIJ. var. *vulgaris* strain PRINGSHEIM/Praha - Ac. 82 (it has been obtained from the Algarum, muscorum, hepaticarumque in culturis collectio, Praha, through courtesy of Academician S. PRÁT - personal communication by the authors). *Chlorella* sp. K ≡ *Chlorella vulgaris* BEIJ. var. *vulgaris* strain KOSSIKOV 1961/132-2 (see ŠETLÍK et al. Arch. Hydrobiol./Suppl. 49, Algolog. Studies 14: 70-104, 1975.) - The Editor.

*Chlorella*, for selective inhibition of 80S ribosome activity without affecting the syntheses on 70S ribosomes has been proved by numerous authors (MORRIS 1966, SMILLIE et al. 1967, SMILLIE & SCOTT 1969, EVANS 1971, TOMOVA et al. 1969, KRETOVIČ et al. 1970, EVSTIGNEVA et al. 1971).

### Material and methods

The experiments were carried out with the strains "*Chlorella pyrenoidosa* 82" and "*Chlorella* sp. K"<sup>2</sup>. We have shown earlier that under influence of various factors affecting cellular division the former strain starts to produce predominantly lipids while the latter switches over to carbohydrate synthesis (KLJAČKO-GURVIČ & SEMENENKO 1965; ŽUKOVA et al. 1969). In this paper we describe experiments in which the reorientation of biosyntheses was triggered by nitrogen starvation.

The algae were grown in special small-volume all-glass vessels with plate parallel walls of the algal space (SEMENENKO & AFANASEVA 1972) in a batch culture regime under continuous illumination ( $4.3 \times 10^4$  erg cm<sup>-2</sup> sec<sup>-1</sup>) by fluorescent tubes from one side. Tamiya's nutrient solution was used (TAMIYA 1963) aerated with air containing 1.7% (v/v) of CO<sub>2</sub>. Optimum temperature was used for the growth of each strain i. e. 26° C for *Chlorella pyrenoidosa* 82 and 36° C for *Chlorella* sp. K.

The growth of the population was measured by counting cell numbers in the haematocrit chamber and by determining the amount of dry weight per unit volume of the culture. Total nitrogen was measured by the KJELDAHL method in lyophilized samples taken from the culture during the experiments. Protein content was estimated by multiplying the total nitrogen figures by the coefficient 6.25. Carbohydrates were assessed in the fresh suspension by the phenol-sulfuric acid reagent (DUBOIS et al. 1956). The quantity of lipids was calculated as the difference between the sum of protein + carbohydrate and the amount of dry weight. We have confirmed by preliminary experiments that the results obtained by this difference method correspond well with direct lipid determinations by infrared spectrophotometry at the wavenumber 1750 cm<sup>-1</sup> where the typical band of lipid carbonyl bonds is located (KLJAČKO-GURVIČ & MARTEMJANOVA 1974).

In performing the experiments we have precultured the algae to the density of 2 to 3.10<sup>8</sup> cells ml<sup>-1</sup>. The suspension was then centrifuged, washed with water and resuspended in the nitrogenfree medium either with or without CHI. Both variants were further exposed to the same light and temperature conditions as before. All operations were carried out under sterile conditions.

<sup>2</sup> See footnote on p. 186.

Table 1. Cycloheximide effect on biosynthesis reorientation during nitrogen starvation in 2 strains of *Chlorella*. (Results of analyses after 48 hours of nitrogen starvation.)

Variants	Dry weight mg/ml	Increment of d. wt.	Protein		Carbohydrates		Lipids	
			mg/ml	Increment mg/ml	% of d. wt. increment	mg/ml	Increment mg/ml	% of d. wt. increment
<i>Chlorella pyrenoidosa</i> 82								
Initial	1.91		0.93		0.33		0.64	
(-N) control	3.88	1.97	0.73	-0.20	1.70	1.37	1.44	0.80
(-N) + cycloheximide 3 µg	3.27	1.36	0.76	-0.17	1.70	1.37	0.80	0.16
(-N) + cycloheximide 10 µg	2.47	0.56	0.89	-0.04	1.17	0.84	0.40	-0.24
<i>Chlorella</i> sp. K								
Initial	1.36		0.67		0.46		0.22	
(-N) + control	4.06	2.70	0.58	-0.09	1.96	1.50	1.51	1.29
(-N) + cycloheximide 3 µg	4.14	2.78	0.51	-0.16	2.31	1.85	1.31	1.09
(-N) + cycloheximide 10 µg	2.82	1.46	0.46	-0.21	1.85	1.39	0.50	0.28

In preliminary experiments we have established the efficient concentration of CHI. We have tested a range of concentrations between 3 and 30  $\mu\text{g ml}^{-1}$ . We have found that the lowest concentration (3  $\mu\text{g ml}^{-1}$ ) was already fully efficient in suppressing proteosynthesis on 80S ribosomes in *Chlorella* cells. Higher concentrations from 10  $\mu\text{g ml}^{-1}$  onwards caused various side effects which were particularly pronounced at the highest level of the antibiotic. We have also observed that a single application of the dose 3  $\mu\text{g ml}^{-1}$  retained its inhibitory activity for 36 to 48 hours. Thereafter the manifestations of CHI activity faded, due to either detoxication or degradation of the antibiotic. Thus, in long term experiments, we have used 5  $\mu\text{g ml}^{-1}$  of CHI as the basic dose and we have added this quantity repeatedly every 48 hours.

## Results

### Comparison of CHI effects on the biosynthesis during nitrogen starvation of the "lipid" and "carbohydrate" strain of *Chlorella*

As can be seen from Table 1 and Figs. 1 and 2 in the presence of CHI the shift to nitrogen free medium does not provoke the predominant lipid synthesis in *Chlorella pyrenoidosa* 82.

A cycloheximide concentration of 3  $\mu\text{g ml}^{-1}$  in the culture is sufficient to produce full effect. The comparison of the CHI treated and untreated variant under these conditions shows that in both the amount of protein per unit volume of culture slightly decreased. The net gain in dry weight of the CHI containing subculture was numerically nearly identical to the increase of

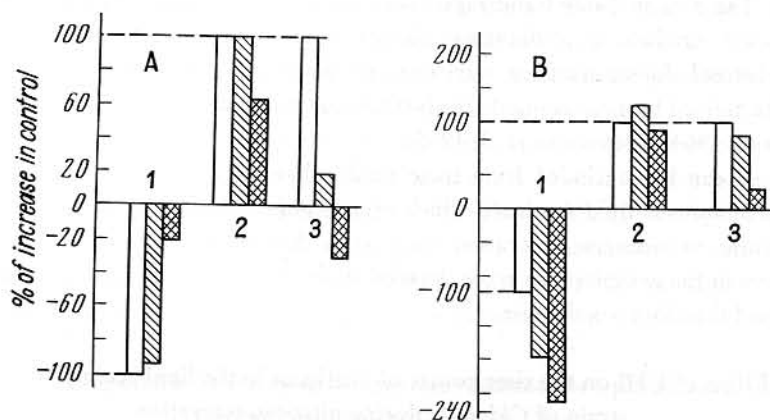


Fig. 1. Variation in the three main cellular components under nitrogen starvation in absence and in presence of CHI. The changes after 48 hours in N-free medium are expressed for the CHI treated variants in per cent of the change in the control without CHI. A - *Chlorella pyrenoidosa* 82. B - *Chlorella* sp. K. Groups of columns - 1 - proteins; 2 - carbohydrates; 3 - lipids. White columns - control (without CHI); hatched columns - 3  $\mu\text{g ml}^{-1}$ ; cross-hatched columns - 10  $\mu\text{g ml}^{-1}$  CHI.

weight in the carbohydrate fraction while the quantity of protein disappeared was compensated for by lipid synthesis. The latter was consequently, of a rather limited extent (approx. 10 % of the total net change in dry weight).

With the higher CHI concentration ( $10 \mu\text{g ml}^{-1}$ ) similar trends are observed except that a net decrease in lipids was recorded. This may be, perhaps, due to some secondary effect of the antibiotic.

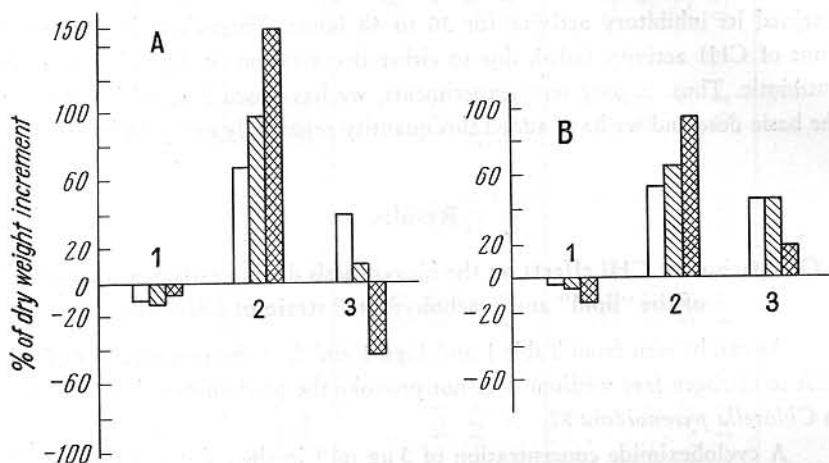


Fig. 2. Variation in the three main cellular components under nitrogen starvation in absence and in presence of CHI. The changes after 48 hours in N-free medium are expressed as percentage of the dry weight increment in each variant. Meaning of other symbols as in Fig. 1.

The data in Table 1 and Figs. 1 and 2 show further that  $3 \mu\text{g ml}^{-1}$  of CHI did not produce any dramatic change in the distribution of substances synthesized during nitrogen starvation in the strain *Chlorella* sp. K which is characterized by predominant starch synthesis under these conditions (KLJAČKO-GURVIČ 1964, SEMENENKO et al. 1969).

It can be concluded from these results that the diversion of metabolism to predominant lipid synthesis, which occurs with *Chlorella pyrenoidosa* 82 in response to nitrogen starvation requires a functional protein synthesizing system in the cytoplasm. A more detailed study of this interesting model system seemed therefore worthwhile.

#### Effect of CHI on the time course of syntheses in the lipid accumulating strain of *Chlorella* during nitrogen starvation

In the above experiments the algae were kept in the nitrogen free medium for a relatively short period of time (48 hours), during which the typical reorientation of biosyntheses did not run to completion. Moreover the composition of the cells has been analyzed only at the end of the experiment.

To learn more about the nature of the interference of CHI with the lipid metabolism during nitrogen starvation it was important to follow the time course of the synthetic processes in the control and in the CHI treated variant. Fig. 3 A shows that in the course of a long-lasting nitrogen starvation a steady and regular increase in the relative content of lipids is observed, while the percentage of carbohydrates rises substantially during the first 48-hour interval, but decreases slowly afterwards.

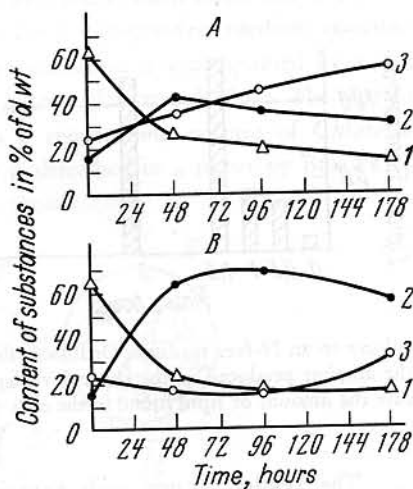


Fig. 3. Time courses of synthetic processes in *Chlorella pyrenoidosa* 82 under nitrogen starvation. Abscissa: time from the transfer of the culture to the N-free medium. Ordinate: content of three main cellular components in per cent of dry weight. A - control variant, without CHI. B -  $5 \mu\text{g ml}^{-1}$  CHI added simultaneously with transfer to N-free medium and repeatedly in 48 hour intervals. Curve 1 - proteins; 2 carbohydrates; 3 - lipids.

The curves in Fig. 3 B indicate what happens if  $5 \mu\text{g ml}^{-1}$  of CHI are added to the culture at the moment of its transfer to the nitrogen free medium. There is no increase in the percentage of lipids in the dry weight during the first five days of the experiment and the content of carbohydrates rises to a much higher level than in the control (Fig. 3 A) before it starts to sink slowly.

The results of this experiment show that when protein synthesis is suppressed by CHI the cells of the "lipid" strain of *Chlorella* behave in the same way as the cells of the "carbohydrate" strain. This makes us assume that starch synthesis under nitrogen deficiency conditions is mediated by constitutive enzymes while the diverted lipid metabolism is an adaptive property that requires induction and synthesis of the respective enzymes.

#### The time course of synthesis of enzymes required for lipid accumulation during nitrogen starvation in *Chlorella*

If the enzymes required for the predominant lipid synthesis are of adaptive nature and the shift of the culture to the nitrogen-free medium provides the factor inducing their synthesis, then this synthesis should be running for a limited time only after the exclusion of nitrogen.

To prove that this is actually so we have added CHI into a series of subcultures at various times from the transfer of the culture to the nitrogen free medium. CHI was added into the culture after 30 minutes, 1 hour, 1.5, 2, 4, 6 and 12 hours from the start of the nitrogen starvation.  $5 \mu\text{g ml}^{-1}$  of CHI was added to each sample and they were grown further under optimum conditions for photosynthesis. To compensate for the degradation of CHI with time a dose giving final concentration of  $5 \mu\text{g ml}^{-1}$  was added to each variant 48 hours after the first addition of the inhibitor.

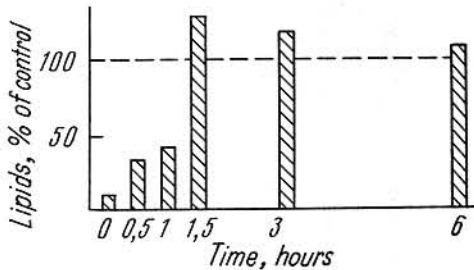


Fig. 4. The efficiency of CHI inhibition in relation to the time of its application. Abscissa: time of CHI application after the transfer of the culture to an N-free medium. Ordinate: the amount of lipids synthesized in per cent of the amount produced in the control variant, not treated with CHI. The columns indicate the amount of lipid found in the cells 48 hours after the transfer to N-free medium.

The results of one such experiment are graphically demonstrated in Fig. 4. A complete abolition of the diverted lipid synthesizing capacity is observed only if CHI is added simultaneously with the shift to the N-free medium. If applied 1.5 hour following this shift and at all longer intervals CHI does not interfere with the appearance of full capacity for lipid synthesis, equivalent to that observed in the control. CHI additions to the culture after 30 minutes and 1 hour in the nitrogen free medium results in a partial development of the lipid synthesizing capacity.

### Discussion

We think that the results presented in this paper can be interpreted in one way only. The transfer of *Chlorella pyrenoidosa* 82 into conditions unfavourable for balanced growth, e. g. to a N-free medium, triggers synthesis of adaptive enzymes which are responsible for the predominant synthesis of lipids during the unfavourable treatment. The enzyme synthesis is completed within 1.5 hour and cycloheximide applied after this period has no effect on the reorientation of synthetic processes elicited by the unfavourable conditions (nitrogen starvation).

It is interesting that the time required for induced enzyme synthesis in this case (1 to 1.5 hour) is comparable to that necessary for synthesis of another adaptive enzyme in *Chlorella*, i. e. NADP-dehydrogenase, as described by TOMOVA et al. (1969) and KRETOVIČ (1972).

Since the synthesis of the enzymes is accomplished on a nitrogen-free medium it follows that it occurs on the expense of inorganic and organic reserves in the cells and that also redistribution and reutilization of the nitrogen already bound in cellular structures takes place. Such behaviour is known e. g. from studies on yeast cells incubated on a nitrogen-free medium containing galactose; synthesis of the induced galactosidase is accompanied by a decrease in activity of enzymes fermenting glucose (KRETOVIČ 1967). The change of activity of a number of enzymes in a synchronous culture of *Chlorella pyrenoidosa* shifted to a N-free medium is described in a paper by BERGER & PIRSON (1967).

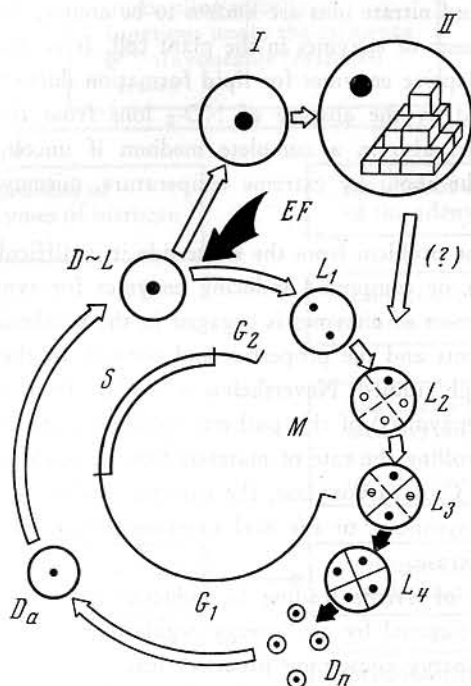


Fig. 5. Schematic diagram showing where, in the cell cycle of *Chlorella*, the reorientation of biosynthesis and the development of a "specialized" cell starts under the influence of an unfavourable (extreme) factor (EF).  $D_n$  to  $L_4$  - stages of the *Chlorella* life cycle according to TAMAYA (1963).  $G_1$ ,  $S$ ,  $G_2$ ,  $M$  - phases of the eucaryotic cell cycle. I - period of enzyme induction; II - predominant synthesis of reserve substances.

Thus at uncoupling of cellular functions the cells of *Chlorella* acquire new qualities. The chronologically determined sequence of events characteriz-

ing the normal cell cycle is disturbed and the cell is transformed into a peculiar specialized cell with a prolonged cell cycle and a new set of enzymes opening specific pathways of biosynthesis (see Fig. 5).

This transition is of particular interest in the wider context of self-regulation of cellular processes. The cell placed into certain conditions has "to decide" not only what enzyme it has to synthesize in this situation (this mechanism can be imagined according to JACOB & MONOD (1961) but also at the expense of what protein new enzymes should be synthesized and how the selective splitting of the unnecessary enzymes should be realized. These problems call for system analysis and take us to the field of molecular physiology (GREEN & GOLDBERGER 1967, BONNER 1965, KURSANOV 1972, KOROTAEV 1973).

In looking for a possible mechanism that could be operative in our case the following facts have to be considered:

Ammonium and nitrate ions are known to be among the most important regulators of syntheses of enzymes in the plant cell. It is clear, however, that the synthesis of adaptive enzymes for lipid formation during nitrogen starvation is not induced by the absence of  $\text{NO}_3^-$  ions from the medium. Lipid accumulation occurs also in a complete medium if uncoupling of cellular functions is brought about by extreme temperature, mitomycin C inhibition etc.

Looking at the problem from the other side it is difficult to identify the intracellular factor or compound inducing enzymes for synthesis of reserve lipids. A great number of enzymes is engaged in the synthesis of triglycerides and their components and the properties and controls of these enzymes have not yet been enough studied. Nevertheless it is clear from the present study that one or more enzyme(s) of this pathway become(s) induced and it is most probably one controlling the rate of material flow through the pathway. This may be the acetyl CoA carboxylase, the enzyme catalyzing the rate limiting step in fatty acid synthesis, or the acyl carrying protein (ACP), or the acyl and malonyl transferase.

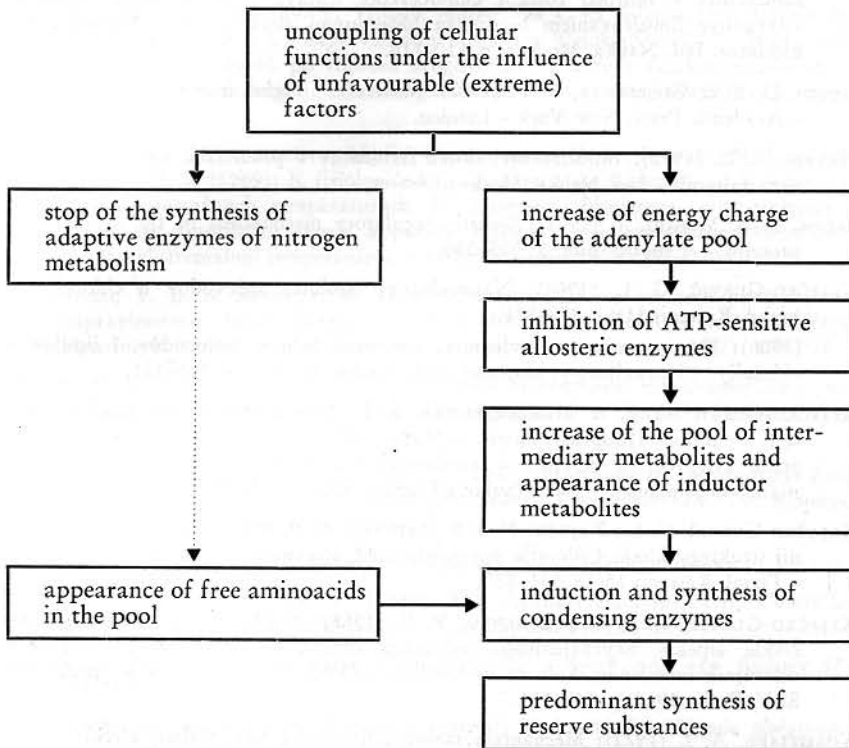
The sequence of events leading to induction of the lipid synthesizing enzymes may be triggered by an "energy regulation" mechanism. When cell division (and all energy consuming processes leading to it) is blocked, while the photosynthetic apparatus is fully functional, the "energy charge" of the adenylate system becomes high. This condition may trigger both starch and lipid synthesis.

In the first case we may imagine that high ATP concentration brings about allosteric inhibition of phosphofructokinase thus causing accumulation of fructose-6-phosphate. The latter, on its turn, may be transformed into starch through glucose-1-phosphate ADPG particularly if hexokinase activity is suppressed. The stimulating effect of ATP on hexoso-di-phosphatase may result

in a still higher accumulation of fructoso-6-phosphate. This are perhaps the reactions occurring during nitrogen-starvation in those *Chlorella* strains which accumulate starch.

In the lipid strains, high level of ATP may inhibit citrate-syntase which catalyses the entrance of acetyl-CoA into the KREBS cycle. The reduced consumption of acetyl-CoA for respiratory and anaplerotic functions of the KREBS cycle may lead to its better availability for fatty acid synthesis if the corresponding enzymes are available.

Thus the control of biosynthesis in the photosynthesising cells with uncoupled cellular functions involves a complex chain of interrelated events that can be represented by the following diagram:



The influence of external factors resulting eventually in the predominant synthesis of different compounds is thus realised through a chain of many reactions and thus plays a role of an indirect trigger. Consequently, the mechanism corresponds to the pattern suggested by WADDINGTON (1966).

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