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ADENINE NUCLEOTIDE LEVELS AND PHOTOPIGMENT SYNTHESIS IN A GROWING PHOTOSYNTHETIC BACTERIUM

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SUMMARY

Cell contents of AMP, ADP, ATP and bacteriochlorophyll were measured during exponential photoanaerobic growth of *Rhodopseudomonas spheroides* (Strain Y. de Klerk) under conditions which evoked an initial lag in synthesis of bacteriochlorophyll. During early stages of exponential phase, oscillations occurred in adenine nucleotide levels. In particular, a sharp drop in ATP level preceded onset of bacteriochlorophyll synthesis. An inverse relation was obtained between ATP cellular concentration and rate of bacteriochlorophyll synthesis. Results were consistent with the hypothesis that ATP levels alone, and not the energy charge parameter, were decisive in control of regulation mechanisms for photopigment synthesis.

INTRODUCTION

It is well known[1] that while, under anaerobic conditions in the light, growth of the non-sulfur purple bacteria *Rps. spheroides* is immediate after inoculation, there can be conditions under which there is no chlorophyll synthesis for more than one generation. The duration of this lag depends upon the light intensity. Light energy is converted to chemical energy, in the form of ATP, which is the ultimate energy source for biosynthesis in all types of cells. It is then tempting to suggest[2,3] that changes in the amount of ATP within the cells may constitute one of the means whereby control of bacteriochlorophyll synthesis is established.

The inhibition of bacteriochlorophyll synthesis caused by introduction of O_2 into cultures of facultative heterotrophes has been studied in detail by COHEN-BAZIRE *et al.*[1] and LASCELLES[4]. As shown by experiments made under diverse conditions[5-7] of culture and growth, the endogenous ATP level varies very rapidly with O_2 tension or light and can undergo large oscillations in the presence of O_2 and substrate.

In an effort to test the hypothesis of a correlation between ATP levels and bacteriochlorophyll synthesis, we have assayed adenine nucleotide levels during the early phase of growth of a *Rps. spheroides* strain. The "energy charge" parameter of ATKINSON AND WALTON[8] and ATKINSON AND FALL[9] defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP), which strongly affects the activity of enzymes which utilize ATP, has also been determined because it has been suggested that the kinetics of bacteriochlorophyll synthesis in response to sudden changes in light intensity or O_2

tension involve a direct effect on enzymes concerned in the synthesis of bacteriochlorophyll rather than repression of the formation of these enzymes[10].

MATERIALS AND METHODS

Cell culture

Rps. spheroides (Strain Y. de Klerk) was grown in 2-l bottles filled with the modified Hutner medium described by DE KLERK *et al.*[11], under illumination by tungsten lamps and continuous N_2 bubbling, at 30°. The illumination was strong enough to insure a maximum growth rate, with a mass doubling time of 105 min. Inoculation was made with 24-h cultures, diluted to obtain an initial cell density of about 0.04–0.06 $A_{1200\,nm}$. A typical growth curve is exhibited in Fig. 1. Growth was measured by turbidimetry (one A unit corresponded to $4 \cdot 10^9$ cells/ml). Samples were drawn every 10, 30 or 60 min under sterile conditons with an automatic syringe.

Assays

Bacteriochlorophyll was determined in the intact cells by measurements of $A_{850\,nm}$, with the method of SHIBATA[12]. The relationship between concentration and absorbance was linear (as confirmed by determinations made after methanol extraction[1]). For the standardized conditions used in this work: $\varepsilon_M(850\,nm) = 1 \cdot 10^5$. Variations in intensity of light or O_2 tension were avoided, as the ratio of the two bacteriochlorophyll peaks at 870 and 850 nm in *Rps. spheroides* varied appreciably when these conditions were changed.

For determinations of ATP, ADP and AMP, the bacterial suspension was rapidly injected into an ice-cold centrifuge tube and cells harvested by spinning 10 min at 3000 × g in a refrigerated centrifuge. The freshly harvested cells containing not more than 20 nmoles of ATP were extracted with 0.5 ml of 0.3 M H_2SO_4, 30 min at room temperature[13]. We did not use $HClO_4$ extraction which caused a 30 % quenching of luminescence in the luciferin–luciferase assay[14] and displaced the peak of maximum emission[15], or trichloroacetic acid extraction which caused a 10 % loss[15]. The extracts were stable at least 6 h at 0° in 0.3 M H_2SO_4. They were neutralized and measured immediately by the luciferin–luciferase procedure adapted from that of PRADET[16]. We used lyophilized Worthington firefly extract which dit not contain adenine nucleotides, pyruvate kinase or myokinase. On the same sample ATP + ADP was measured as ATP after 6 min incubation at 34° with pyruvate kinase, and phosphoenolpyruvate[16], and ATP + ADP + AMP as ATP with the same assay procedure after 30 min incubation at 34° with myokinase[16]. ADP and AMP were determined by difference.

Routine checks with internal standards showed that there was no degradation of ATP or ADP during heating at 34° and no excitation of luminescence which arose from the bacterial extract itself.

The ATP content was assayed by the initial flash of light obtained when the luciferin–luciferase mixture was injected into the cuvette containing the neutralized bacterial extract, the intensity being recorded simultaneously as the amplified signal from an XP 1003 photomultiplier tube (S 20) (La Radiotechnique) of high sensitivity (60 A/lumen). Under the conditions of our work (lyophilized extract, high Mg^{2+} concentration) the decay in luminescence was quite slow and the initial peak remained constant for at least 7 sec. Thus, we could monitor the photocurrent of the photo-

multiplier tube amplified on a 150 Keithley microammeter (the time response of which was 2 sec) with a Moseley recorder (time response $\leqslant$ 0.5 sec) (see refs. 17 and 18). With this device we were able to measure as little as $1 \cdot 10^{-12}$ mole of ATP.

RESULTS AND DISCUSSION

Variation of ATP levels during inoculation

RAMIREZ AND SMITH[5] observed a 4–5-fold increase in the ATP levels on anaerobic illumination of intact cells of *Rhodospirillum rubrum*. As can be seen in Table I, we observed similar phenomena. Dilution carried out by rapid inoculation did not change bacteriochlorophyll content, but there was an approx. 3-fold increase in ATP levels. This latter augmentation exhibited the following characteristics:

(1) It was extremely sensitive to light. RAMIREZ AND SMITH[5] remarked that exposure of their cell cultures to even dim room light resulted in considerable ATP formation. In our experiments if inoculation was performed in dim room light we observed the 3-fold augmentation. If one observed strict precautions to exclude light, there was no change in ATP levels (Table I).

(2) It depended only on illumination. We can see in Table I that the same phe-

TABLE I

ATP, ADP, AMP LEVELS, CELL MASS AND BACTERIOCHLOROPHYLL CONTENT OF 24-H ANAEROBIC LIGHT-GROWN CELLS MEASURED BEFORE AND AFTER THEIR INOCULATION UNDER DIFFERENT CONDITIONS

ATP, ADP, AMP, Σ and bacteriochlorophyll expressed as nmoles/$A_{1200\ nm}$. Σ is the sum: ATP + ADP + AMP.

	$A_{1200\ nm}$	*nmoles*/$A_{1200\ nm}$				
		Bacterio-chlorophyll	*ATP*	*ADP*	*AMP*	Σ
Dilution in room light and in fresh medium, pH 6.8:						
Inoculum	1.2	24	0.55	1.15	1.9	3.6
After dilution	0.045	22	1.78	0.2	0.2	2.2
Dilution in the dark and in fresh medium, pH 6.8:						
Inoculum, Expt. 1	1.42	18	1	1.3	1.1	3.4
Expt. 2	1.44	19.5	1.4	1.2	0.9	3.5
After dilution, Expt. 1	0.13	18	1.1	1.9	0.65	3.6
Expt. 2	0.16	19.5	1.3	1.6	0.9	3.8
Dilution in room light and phosphate buffer, pH 7.4, 0.01 M:						
Inoculum	1.2	24	0.7	1.2	2	3.9
After dilution	0.12	21	2.1	0.17	0.04	2.3
Dilution in room light and in the supernatant medium of the 24-h culture, pH 7.8:						
Inoculum	1.08	19	0.95	1.45	1.25	3.6
After dilution	0.1	18	2.6	0.75	0.7	4

nomenon was observed when inoculation was made either in buffer, or in the supernatant medium of a 24-h-old culture, at the same pH as in the original inoculum.

(3) It persisted for several minutes when O_2 was introduced after illumination (see ref. 5).

Thus, it appeared justified to conclude that the levels of ATP, ADP, AMP, attained in cells illuminated anaerobically during growth remained constant during the time involved in sampling and extraction, using precautions described above.

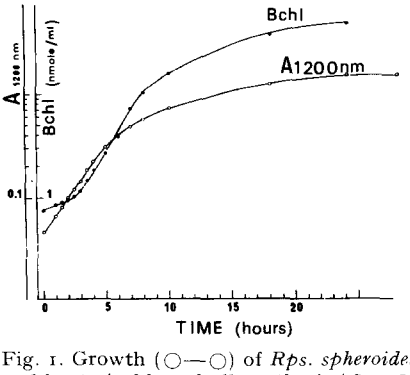
ATP and bacteriochlorophyll synthesis during growth

The data presented in this paper are results of ten different growth experiments.

Fig. 1 shows that under the conditions used there is a lag period in bacteriochlorophyll synthesis for about 3 h, although growth is immediate. In all experiments the bacteriochlorophyll synthesis starts when the $A_{1200\ nm}$ is around 0.1. The lag period in bacteriochlorophyll synthesis is best seen in Fig. 2, which shows the cellular level of bacteriochlorophyll along with that of ATP. Plotting nmole/$A_{1200\ nm}$, as a function of cell mass, we note that the bacteriochlorophyll cell content diminishes at first, then remains constant for a short period, after which bacteriochlorophyll synthesis starts, bacteriochlorophyll content increasing to reach the value attained in the cells originally used for inoculation.

In the corresponding development of ATP levels, one can distinguish two periods: (1) An early phase which shows oscillations. (2) Intermediate and stationary phase where ATP reaches a rather constant level which is the level in the original inoculum. It appears that bacteriochlorophyll synthesis starts when the ATP level, after some oscillations, reaches a certain low level. If this level is not low enough, bacteriochlorophyll synthesis will not start. To establish this correlation, we studied the variation of these two parameters during the first 3 h of growth, taking samples every 10 min (Fig. 3).

In Fig. 4 results of Figs. 2 and 3 are plotted in another manner to show bacteriochlorophyll or ATP concentrations, rather than cell levels, as a function of exponen-



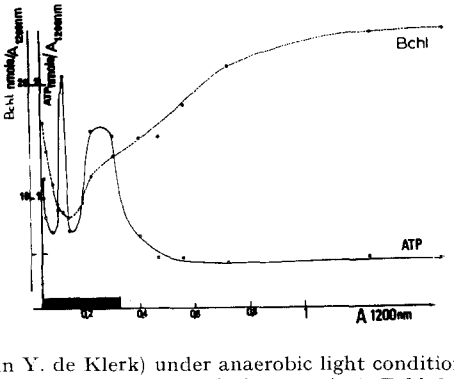


Fig. 1. Growth (○—○) of *Rps. spheroides* (Strain Y. de Klerk) under anaerobic light conditions, and bacteriochlorophyll synthesis (●—●) (expressed in nmoles per ml of suspension). Bchl, bacteriochlorophyll.

Fig. 2. Development of ATP (□—□) and bacteriochlorophyll (●---●) cellular levels as a function of cell mass during growth. The black zone represents the exponential phase of growth. Measurements are made every 30 min.

tial growth. Exponential growth ($A_{1200\ nm}$) is represented on the abscissa on a logarithmic scale. In this plot the slopes of the bacteriochlorophyll curves are the different rates of bacteriochlorophyll synthesis. During the lag phase this rate is zero or essentially zero. While the exact moment of onset of bacteriochlorophyll synthesis is not easily decided, synthesis, once begun, reaches a rapid rate abruptly. To each such ini-

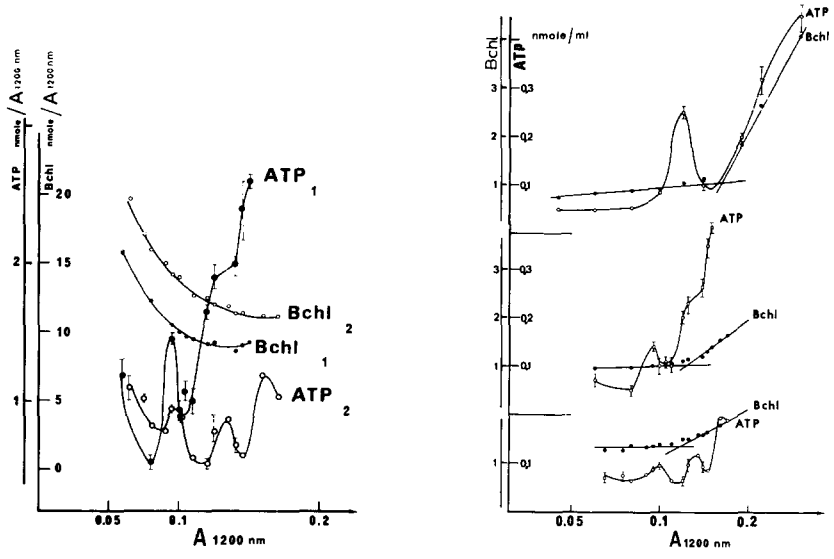


Fig. 3. Development of ATP and bacteriochlorophyll synthesis as a function of cell mass during exponential growth, expressed as nmoles/$A_{1200\ nm}$, for two different runs (Expt. 1, ●—●; Expt. 2, ○—○). Measurements made every 10 min. Bars represent experimental ATP deviation of duplicates or triplicates.

Fig. 4. Kinetics of bacteriochlorophyll (●—●) and ATP (○—○) synthesis. The abscissa represents cell mass on a log scale, the ordinate ATP and bacteriochlorophyll concentration, per ml of suspension. The upper curve is the same as Fig. 2, the two others as Fig. 3. Bars represent ATP experimental deviations.

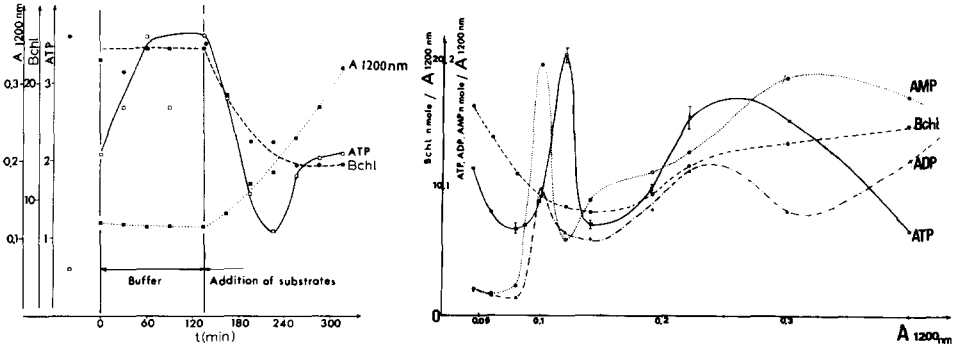


Fig. 5. Effect of the addition of substrates on ATP (□—□) and bacteriochlorophyll (●—●) levels and growth (■ ··· ■), on an anaerobic light culture of *Rps. spheroides* inoculated in phosphate buffer (0.01 M, pH 7.4). ATP and bacteriochlorophyll expressed as nmoles per $A_{1200\ nm}$.

Fig. 6. Bacteriochlorophyll (●---●), ATP (□—□), ADP (▲-·-▲), AMP (○ ··· ○) levels as a function of cell mass during exponential growth (same experiment as Fig. 2).

tial increase in bacteriochlorophyll synthesis rate, there seems to correspond a decrease in ATP. Eventually, bacteriochlorophyll reaches a level adequate to support photophosphorylation rates needed to satisfy ATP needs of cell metabolism.

Fig. 5 exhibits results of another type of experiment which shows the same phenomenon. When growth is halted by inoculation in phosphate buffer (0.01 M, pH 7.4) instead of growth medium, bacteriochlorophyll level remains constant, while the ATP level continues to increase to a very high value, due to phosphorylation without growth and ATP utilization. As soon as the substrates are added, growth starts and the bacteriochlorophyll level decreases. Meanwhile ATP level decreases to a low level whereupon bacteriochlorophyll synthesis commences. It appears that in this special case where the initial ATP level is very high, there are no oscillations.

ADP, AMP and energy charge variations during growth

During exponential growth, adenine nucleotide levels exhibit oscillations (Fig. 6). Invariably an increase in AMP is followed in time by an increase in ATP, when simultaneously AMP decreases; the variations in ADP are much more damped and show a rather continous increase. Synthesis of the three nucleotides exhibit a lag, but shorter than that of bacteriochlorophyll. For clarity only ATP levels have been shown in Fig. 4. AMP synthesis is similar, shifted in time, preceding ATP, and ADP increases slowly during the same time period. Recalling the work of ATKINSON and co-workers[8,9] and that of PRADET[16], this is the manner in which relative proportions of the individual adenine nucleotides should be expected to vary as functions of the energy charge, defined as half the average number of anhydride-bound phosphate groups per adenosine moiety: $1/2\ \varepsilon/\Sigma = (\mathrm{ATP} + 0.5\ \mathrm{ADP})/(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP})$, provided there is equilibration between the three nucleotides monitored by adenylate kinase (EC 2.7.4.3) which catalyses the following reaction: ATP + AMP $\rightleftarrows$ 2 ADP. If this is not the case, the concept of energy charge cannot be applied.

Table II shows values calculated for the apparent equilibrium constant of

TABLE II

VALUES OF THE APPARENT EQUILIBRIUM CONSTANT OF ADENYLATE KINASE EXPRESSED AS $K = [\mathrm{ATP}]\,[\mathrm{AMP}]/[\mathrm{ADP}]^2$, IN TWO DIFFERENT EXPERIMENTS

Fig. 3, Expt. 1		*Fig. 6*	
t (*min*)	*K*	*t* (*min*)	*K*
0	10	0	5.8
60	8	60	5
90	2	90	9
100	0.7	120	1.8
110	0.3	150	3.1
120	0.8	180	1
130	1.4	210	1.7
140	0.9	240	1.4
150	0.9	300	3.5
160	2.0	360	0.7
170	2.7		
180	2.7		
190	5.6	24 h	0.6

adenylate kinase $K = [ATP][AMP]/[ADP]^2$, using our data on adenine nucleotide levels during growth. One can see that this quantity is not constant and that values are very different from those found in various tissues, which have been reported to average between 0.7 and 1.0 (see ref. 19).

Only in stationary phase does K appear to approach a constant value consistent with reported averages. It is therefore not surprising that neither $1/2\ \varepsilon/\Sigma$, ATP/ADP, nor ATP/AMP show a better correlation with bacteriochlorophyll synthesis than does ATP itself (Fig. 7). It is obvious that all these derived parameters reflect merely variations in ATP.

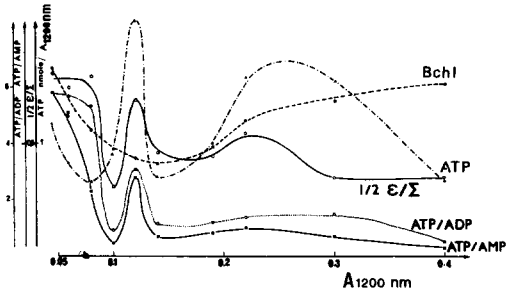


Fig. 7. ATP (△–·–△) and bacteriochlorophyll (●---●) levels, and ATP/ADP (□ · · · □) and ATP/AMP (■—■) ratios and energy charge $1/2\ \varepsilon/\Sigma$ (see text) (○—○) as function of cell mass during exponential growth (same experiment as in Fig. 2).

Finally, we include results of an experiment in which effects of exogenous ATP on bacterochlorophyll synthesis were determined. While growth appeared unaffected, bacteriochlorophyll synthesis was inhibited as expected if endogenous levels of ATP were maintained high, either by direct entry of ATP or by an indirect effect such as a stimulation of endogenous ATP synthesis.

CONCLUSIONS

A clear-cut inverse correlation is seen to exist between bacteriochlorophyll synthesis and ATP levels in this strain of *Rps. spheroides*. It is possible that this correlation is general among the photosynthetic bacteria and reflects some basic characteristic of porphyrin biosynthesis.

The well-known findings of COHEN-BAZIRE *et al.*[1] can be rationalized easily by variations in ATP levels such as seen in our researches. Thus, the lag in bacteriochlorophyll synthesis they observed after transition from dim to bright light could have been the result of a rapid increase in ATP levels. The reverse results they found in transition from bright to dim light could be ascribed to rapid decreases in ATP levels. It will be desirable to test these conclusions by extension of their transition experiments to include assays of adenine nucleotide levels.

Our results are also consistent with those of SCHÖN[20], who has demonstrated that in growing cultures of *R. rubrum* a rate of bacteriochlorophyll synthesis higher than the growth rate is associated with a low ATP/AMP ratio.

Very interesting work is that of GAJDOS AND GAJDOS-TORÖK[21] which has led them to conclude that the intracellular concentration of ATP may play an important

role in the quantitative regulation of the biosynthesis of porphyrins. Even though their experiments have been done under rather drastic conditions and with much different test systems, their conclusions are relevant in that they appear to demonstrate:

(1) That ATP enters the living cell. This had been reported previously by SANTARIUS AND HEBER[22], and our results (Fig. 8) are consistent with these findings; that is, if ATP is added in the growth medium, and the experiment conducted as usual, the synthesis of bacteriochlorophyll is drastically inhibited, the growth rate being unaltered. (It is not certain that ATP in fact entered the cells in the experiments we conducted, as no direct determination of such entry was made. Thus, it is possible ATP was metabolically dissociated at the membrane and bacteriochlorophyll synthesis inhibited by indirect means as a consequence of entry of metabolic fragments. Experiments to establish whether or not exogenous ATP raises the endogenous level of ATP are in prospect.)

(2) There is an inverse correlation between ATP concentration in the medium and prophyrin synthesis.

(3) ATP variations precede porphyrin modification.

These conclusions are identical to ours, if one substitutes bacteriochlorophyll for porphyrin. It is of interest that pathological conditions[21] as well as physiological conditions lead to the same conclusions.

The mechanism of action of ATP on porphyrin biosynthesis is not yet elucidated. The results of GAJDOS AND GAJDOS-TORÖK[21] provide a basis for the belief that it is located in the very early steps of synthesis preceding condensation of glycine and succinate to δ-aminolevulinic acid. Many factors have been considered in the regulation of the porphyrin biosynthesis, the impairment of which lead to excess porphyrin formation as, for instance, feed-back inhibition of the δ-aminolevulinic acid synthetase system. As suggested by GOODWIN[23], certain cellular control processes based on negative feed-back control loops induce oscillations of the cellular content of metabolic intermediates. Oscillations of nucleotide cellular levels, such as we notice in the early

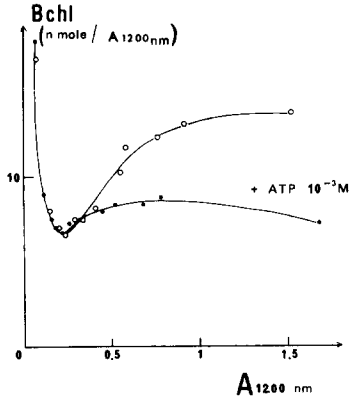


Fig. 8. Effect of exogenous ATP (1 mM) on bacteriochlorophyll synthesis during growth under usual conditions (see text). Growth curves were unaltered by presence of exogenous ATP. Bacteriochlorophyll synthesis in normal growth medium (○—○) and in normal medium *plus* ATP (1 mM) (●—●).

stage of growth, after a short lag preceding the start of bacteriochlorophyll synthesis have also been reported, under other conditions[20,22].

In any case, it appears clear that control of bacteriochlorophyll synthesis by the energy-charge parameter does not appear to operate during exponential growth in this bacterial strain. ATP levels alone appear to be decisive.

Extension of these experiments to include study of correlations under a variety of transition conditions other than the one of dilution used in the present research is projected with the objective of locating the site of action of ATP as a control factor in the biosynthetic enzyme chain which provides the basis for elaboration of the bacterial photosynthetic membrane.

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