Kinetics of Synthesis and Utilization of Adenosine Triphosphate by Intact Cells of *Rhodospirillum rubrum**

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ABSTRACT: The kinetics of formation then disappearance of adenosine triphosphate in intact adenosine triphosphate starved cells of *Rhodospirillum rubrum* were followed after initiation and cessation of illumination or oxygenation or a combination of these. Samples of bacteria were collected from a flow apparatus under various conditions into $HClO_4$ and the adenosine triphosphate level of the supernatant samples was measured by the luciferin–luciferase method. Adenosine triphosphate synthesis on illumination of anaerobic bacteria showed an initial rate of 0.33 nmole sec^{-1} per mg dry weight that was independent of temperature between 4 and 37°. The maximal rate following oxygenation at 22° was around 0.17 nmole sec^{-1} per mg dry weight and this varied with temperature.

The initial rate on illumination in the presence of O_2 was similar to that seen on illumination of anaerobic cells. The level of adenosine triphosphate attained on illumination was higher than that reached on oxygenation, but illumination following oxygenation brought the level to the same as that attained on anaerobic illumination. The data support the postulate of two separate and probably independent electron transport phosphorylating systems.

The facultative photoheterotrophes, *Rhodospirillum rubrum*, have been observed to synthesize ATP rapidly when an anaerobic suspension of the bacteria with a low ATP content is illuminated or oxygenated (Ramirez and Smith, 1968). Under the experimental conditions used the synthesis of ATP was so rapid that details of the kinetics could not be obtained with the simple methodology employed. However the data clearly showed that the steady-state level of ATP attained during 20–60 sec of illumination was greater than the steady state reached after addition of oxygen. The rate of utilization of ATP following exhaustion of oxygen in the dark was found to be quite low (Ramirez and Smith, 1968).

In an effort to gain a deeper understanding of the reactions involved in ATP synthesis and utilization in *R. rubrum*, a method was devised for more rapid mixing and sampling so that both the early and the later kinetics of the changes in ATP level of the intact bacteria could be followed after initiation or cessation of illumination or oxygenation or a combination of these. The data show that the initial rate of ATP synthesis following illumination is high and independent of temperature between 4 and 37°. The rate following oxygenation is always lower and varies with temperature in the same manner as a usual enzymatic reaction. Under the conditions of the experiments reported here, the utilization of ATP was slow after the exhaustion of oxygen in the dark, but this increased markedly after a brief period of illumination. The data are best explained in terms of two electron transport chains coupled to phosphorylating systems which appear to be localized in different compartments within the cells.

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Experimental Section

Culture and Preparation of Bacteria. R. rubrum, Van Niel strain 1, were grown in bottles filled with the modified Hutner medium described by Cohen-Bazire *et al.* (1957) under illumination by photoflood lamps in a bath at 30°. The bacteria were harvested by centrifugation when the absorbance reached about 0.6 in a Coleman Junior spectrophotometer at 680 mμ in a test tube of 13-mm o.d. A thick suspension was made in 20 mM phosphate buffer (pH 6.8) to give a calculated absorbance at 680 mμ of 6. A suspension of the bacteria with an absorbance of 1 at 680 mμ contained around 2.1 mg dry weight and 35 μmoles of bacteriochlorophyll per ml.

The freshly harvested bacteria were either used immediately in the experiments, or after storage in concentrated suspension at 4° for no longer than 48 hr. Before beginning each experiment, the concentrated suspension of cells was incubated anaerobically in the dark in the presence of substrate (25 mM malate or 17 mM β-hydroxybutyrate) for 30–120 min at 30°. This was done to reduce the level of ATP so that accurate measurements of the kinetics of ATP synthesis could be made. This incubation to attain a low level of ATP changes the properties of the bacteria somewhat; these changes will be documented in a subsequent paper. It must be kept in mind that all of the experiments reported in this paper were made with bacterial suspensions that had been "aged" to achieve a low ATP content.

Apparatus for Rapid Mixing and Sampling of Bacteria. Figure 1 is a schematic diagram of the device used for rapidly mixing the bacterial suspension with buffer saturated with air, oxygen, or nitrogen, then rapidly removing the mixed sample. Syringes A and B, mounted on a plastic frame, contained 0.45 ml of a bacterial suspension having an absorbance of 6 at 680 mμ and the same volume of buffer saturated with the appropriate gas, respectively. The contents of the two syringes were injected through the jets of Hamilton three-way valves into syringe C, from which the contents could be collected after

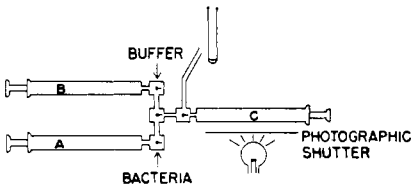

FIGURE 1: Schematic diagram of the mixing and sampling apparatus.

measured time intervals into a test tube containing 0.2 ml of 35% $HClO_4$. While in syringe C, the aerobic or anaerobic bacterial sample could be illuminated by a 500-W projector lamp placed behind a photographic shutter; this light intensity gave maximal rates of ATP synthesis. Syringe B was filled from a reservoir bottle of buffer and syringe A from a 20–50-ml syringe containing anaerobic bacterial suspension strirred with a magnetic stirrer. The apparatus, bacteria, and reagents were kept in a constant-temperature room at the desired temperature before beginning each experiment. The minimal mixing plus sampling time obtainable with the apparatus was 1.9 sec.

After injection of the bacteria into the $HClO_4$, the sample was mixed rapidly by agitation on a Vortex shaker, then stored in an ice bath until an experiment was completed. The $HClO_4$ in each suspension was then neutralized by the addition of 0.3 ml of a mixture composed of 22.5 ml of saturated KOH, 17.5 ml of 1 M KCl, and 60 ml of 2 M Tris buffer, adjusted to bring the pH of the neutralized mixture to 7. The neutralized tubes were centrifuged for 15 min at 3000*g* and the supernatant fluids were decanted. The pH of each supernatant was checked with a miniature pH electrode or with pH indicator paper, and the ATP content of each was assayed with the luciferin–luciferase method described in the following paragraph.

Measurement of ATP Content. A luciferin–luciferase mixture was obtained by extracting 100 mg of desiccated firefly tails (Sigma Chemical Co.) with 10 ml of 0.1 M phosphate buffer (pH 6.8) by grinding in an agate mortar, then incubating at room temperature for 30 min. The mixture was centrifuged at 3000*g* and the decanted supernatant fluid was allowed to stand for 1 hr in the cold, recentrifuged, and the sedimented material was discarded.

The ATP content of the neutralized $HClO_4$ extracts from the bacteria was assayed by recording the signal from the initial flash of light when 0.20 ml of the aerated luciferin–luciferase mixture was injected into quartz cuvets containing 50 μl of the bacterial extract plus 1.8 ml of the reaction mixture described by Bergmeyer (1963) except that the concentration of Tris buffer was doubled. The intensity of the initial light flash was recorded as the amplified signal from a 1P21 photomultiplier tube. There was excellent proportionality between the ATP content of a sample and the signal from the phototube, as seen in Figure 2. It was possible to measure 10^{-13} mole of ATP in the 2.05-ml reaction mixture described above; the samples usually contained around 10^{-11} mole of ATP.

Measurement of Oxygen Content of the Bacterial Suspensions. When measuring the change in ATP content of a bacterial suspension following exhaustion of oxygen in the dark, the oxygen content was monitored by means of a Clark-type oxygen electrode in the air-free chamber described previously (Ramirez and Smith, 1968). Samples could be removed from the chamber for ATP assay at invervals of 10 or more sec after the exhaustion of oxygen; this proved to be rapid enough for the rates of change observed.

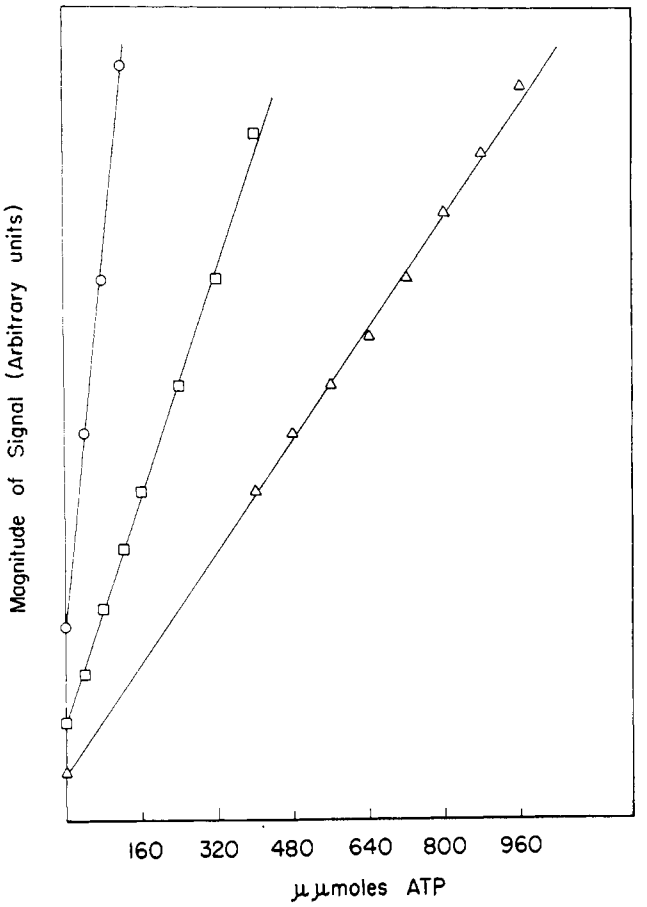

FIGURE 2: Plot showing proportionality between the ATP added to the reaction mixture and the intensity of the signal from the initial flash of light obtained on injection of the luciferin–luciferase mixture into cuvets containing the same concentrations of $HClO_4$ and neutralizing mixture as in the bacterial extracts. The three plots were derived using three different extents of amplification. Each point represents the average of three determinations.

Results

Figure 3 plots the changes in ATP level following illumination or addition of oxygen to a suspension of bacteria which had been incubated anaerobically in the dark with substrate at room temperature for at least 30 min (see Experimental Section); the experiment was carried out at 22°. The data of the lower continuous curve were obtained by mixing anaerobic bacteria in syringe A with oxygen-saturated buffer in syringe B into syringe C of the mixing and sampling apparatus, then injecting the contents of syringe C at measured times after mixing into $HClO_4$ for measurement of the ATP content. The values for the upper curve were obtained by mixing anaerobic bacteria with buffer saturated with nitrogen into syringe C, then collecting the contents of syringe C into $HClO_4$ after different periods of illumination. The dashed curve shows the results obtained when the bacteria were first mixed with oxygenated buffer and then illuminated. The insert plots the observations on ATP synthesis during more prolonged illumina-

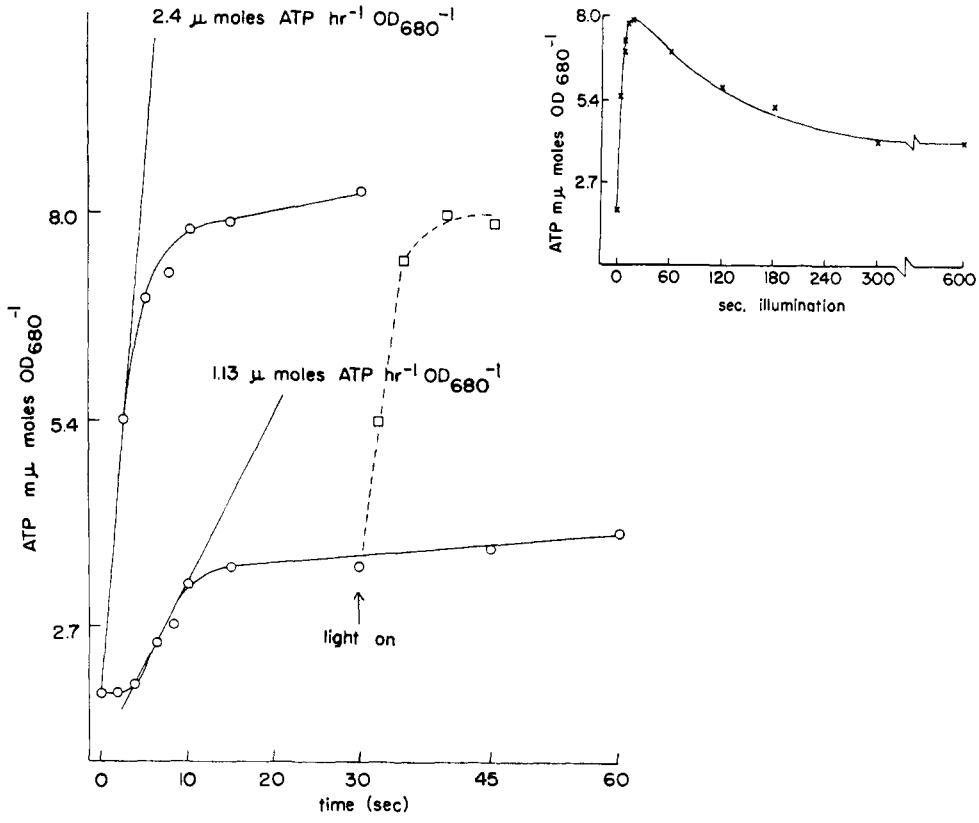

FIGURE 3: ATP formation in *R. rubrum* on oxygenation or illumination or both at 22°. The bacterial suspension having an absorbance of 6 at 680 mμ and having been "aged" for 30 min (see text) was diluted with an equal volume of buffer saturated with oxygen or nitrogen into syringe C of the mixing and sampling apparatus. The lower continuous curve plots the ATP content after addition of oxygen and the upper curve the content following addition of buffer saturated with nitrogen, then illumination. The maximal rates of ATP formation were calculated from the slopes traced on the two plots. The dashed curve plots the changes of ATP content on illumination of a previously oxygenated sample. The insert shows the changes in ATP content during more prolonged illumination on a different time scale.

tion on a slower time scale. The data show that rapid ATP synthesis ensues immediately on illumination and that at this temperature a maximal ATP content is reached after about 30–40 sec, then the ATP level decreases until a final lower steady state is reached after about 300 sec of illumination. The kinetics following oxygen addition are somewhat different. There is a short lag period before ATP synthesis is seen and the maximal rate attained is only about one-half that seen on illumination, as calculated from the slopes of the lines traced on Figure 3 (see Table I). Also the maximal level of ATP reached during illumination was higher than the level attained on addition of oxygen in the dark. As seen in the dashed curve, the initial rate and the final level of ATP attained when a previously oxygenated suspension was illuminated were similar to those observed on illumination in the absence of oxygen. The steady-state concentration reached after the overshoot was usually the same or slightly less than that reached on oxygenation alone.

When the experiments charted in Figure 3 were repeated at 4°, the high level of ATP attained initially on illumination remained at this level for 300 sec and decreased only slightly during several additional minutes of illumination. On oxygenation at this temperature the level of ATP reached was again less than that seen on illumination, as at 22°, and this level sometimes increased slowly after the initial rapid synthesis. At 4° the rate of synthesis following oxygen addition was considerably less than that at 22°, but the initial rates observed on illumination were similar at the two temperatures. At 4° the initial rapid rate was followed by a lower rate; the data from a typical experiment at 4° are plotted in Figure 4. Averages of the initial rates of ATP synthesis and the maximal levels reached in a number of experiments at 4 and 22° are summarized in Table I. In a few experiments at 37° the initial rate

TABLE I: Rate of ATP Formation on Oxygenation or Illumination of Whole Cells of *R. rubrum* and ATP Steady States Reached.

	ATP Level (nmoles ATP OD_{680}^{-1})			Rate of ATP Formation (μmoles of ATP) hr^{-1} OD_{680}^{-1})	
	Illumination				
Temp (°C)	Maximum	Steady State after 10 min	Oxygenation	Illumination	Oxygenation
22	7.5	3.8	4.0	2.40	1.13
4	7.5	5.3	4.0	1.90	0.37

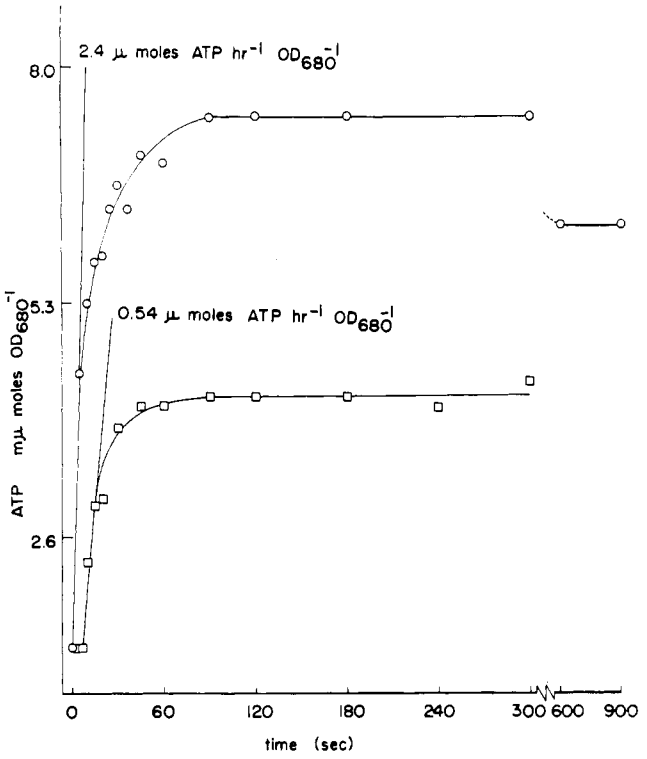

FIGURE 4: ATP formation on oxygenation (□—□) or illumination (○—○) of an anaerobic suspension of *R. rubrum* at 4°. The procedure was the same as that described for Figure 3, including incubation at 30° to reduce the ATP level.

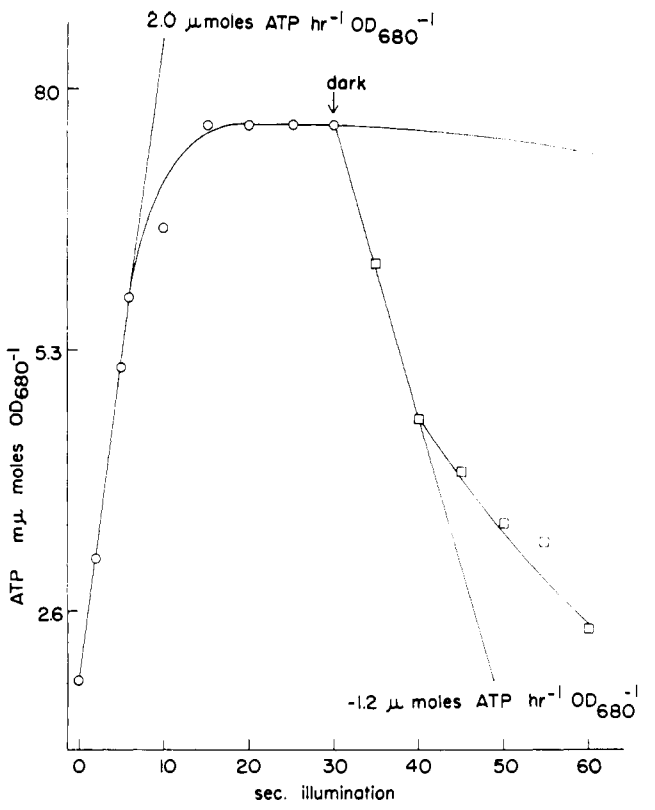

FIGURE 5: ATP formation during illumination of *R. rubrum* for 30 sec, then the utilization of ATP on turning off the light. Experiment performed at 22°; the experimental details were as in Figure 3.

of light-induced ATP formation showed no significant increase over that at 22°. The steady-state level of ATP attained on prolonged illumination was lower as the temperature was increased, but the level reached after oxygenation was similar at 4 and 22°.

Figure 5 illustrates the decrease in the ATP level after the termination of a 30-sec period of illumination at 22°. Similar observations on rates of ATP utilization after two intervals of illumination at 4 and 22° are summarized in Table II (averages of three experiments). The rates are very low at 4°. At 22° the rate of utilization of ATP increases as the period of illumination is increased; after 15, 30, 60, and 300 sec of illumination the ratios of the rates were about 2:3:4:8. The rate of utilization of ATP after the exhaustion of oxygen in the dark was immeasurably low both at 4 and 22° in these bacteria which had been "aged" to reduce the ATP level before beginning the experiments.

TABLE II: Rates of ATP Utilization in Whole Cells of *R. rubrum*.

	Rate of Utilization (μmoles of ATP hr^{-1} OD_{680}^{-1})	
	Illumination	
Temp (°C)	After 30 sec	After 300 sec
22	1.15	1.80
4	0	0.15

Discussion

The bacteria employed in all of the experiments in this paper were "aged" by anaerobic incubation in the dark in the presence of substrate in order to reduce the cellular ATP to a low level. After "aging" in this manner, the properties of the bacteria differ in a number of ways from those of the freshly harvested ones, which have a high level of ATP. In contrast with the latter, the "aged" cells show a low rate of respiration initially, but this increases with time after addition of oxygen or is immediately increased by a brief period of illumination. The rate of utilization of ATP after exhaustion of oxygen in the dark is less in the "aged" cells than in the freshly harvested ones and the increase in ATP level on illumination of dark oxygenated cells is greater in the "aged" bacteria. These reversible changes which result on reduction of the ATP level will be documented in a subsequent publication; the changes in properties do not affect the conclusions suggested by the data reported in this paper.

The data presented can best be explained by the postulate (Smith and Baltscheffsky, 1959; Ramirez and Smith, 1968) of two separate electron transport phosphorylating systems. One reacts only on illumination and will be referred to in subsequent discussions as the L system; the other (referred to as the [L or O_2] system) is the dark respiratory chain system which

can also react with an oxidant produced on illumination. The two systems appear to be localized in different compartments within the cells. The final level of ATP reached on oxygenation of the bacteria in the dark is the same at 4 and 22°, although the rate of utilization of ATP is essentially zero at both temperatures, and the level reached is lower than the maximal level attained on illumination. Thus all of the cellular adenine nucleotide available to react on illumination does not participate in the dark oxygen-linked reaction. Illumination of the oxygenated bacteria brings the ATP level to that attained rapidly on illumination in the absence of oxygen, pointing to the involvement of the oxygen-linked chain plus an additional one in the ATP synthesis during illumination. The decrease in ATP after the overshoot on illumination at 22° brings the level about down to that attained following oxygenation, giving further evidence of two compartments. Finally, the obvious combination of rapid and slower kinetics of ATP synthesis on illumination at 4° would be in agreement with the postulate that one of the ATP-synthesizing systems is that coupled to the [L or O_2] chain, the rate of which is decreased by lowering the temperature.

The postulate of two separate electron transport phosphorylating systems in *R. rubrum* is supported by evidence from other types of experimentation (Ramirez and Smith, 1968; Hind and Olson, 1968). Gibson and Morita (1967) have suggested that their observation that only part of the adenine nucleotides of *Chromatium* is involved in the rapid reactions following illumination and cessation of illumination also may be an indication of compartmentalization. The nature of the possible compartmentation within the cells of *R. rubrum* is not known, but the presence of numerous internal membranes which may form a continuum throughout the cells is well documented (Cohen-Bazire and Kunisawa, 1963; Holt and Marr, 1965). The extent of these membranes, which appear to be the source of the chromatophore fraction of broken-cell extracts, varies with the growth conditions and time of growth (Cohen-Bazire and Kunisawa, 1963; Hickman and Frenkel, 1959).

As mentioned above, the rate of decrease in the ATP level in these "aged" bacteria in the dark after exhaustion of oxygen is very low, even at 22°. The rate of dark decay then increases after even short periods of illumination, an increase as much as fourfold being observed between 15 and 300 sec of illumination. Something is formed during illumination which leads to the maximal rate of ATP utilization. Similar observations were made with *Chromatium* by Gibson and Morita (1967). The reactions which are participating in the utilization of ATP are not known; synthesis of protein or other synthetic reactions may be involved. The reduction of pyridine nucleotide on illumination follows a time course similar to that observed here for ATP synthesis (Amesz, 1963) and the suggestion has been made that this occurs by reversed electron transport, utilizing ATP or a high-energy presursor of ATP (Chance and Nishimura, 1960; Bose and Gest, 1962). Whatever its explanation, the increase in the rate of utilization of ATP with time of illumination can explain the kinetics of the change in ATP level during illumination at 22°, since during the initial phase of synthesis the rate of utilization is low and this increases with time to produce the "overshoot" and the drop to the lower steady-state level. The initial high steady-state level is maintained at 4°, because of the low rate of utilization at this temperature. The steady-state level also remains constant following oxygenation in the dark because the rate of utilization is low at both 4° and at 22°. There is no evidence for reactions rapidly utilizing ATP, such as the reduction of pyridine nucleotide by reversed electron transport, associated with the [L or O_2] pathway.

The initial rate of ATP synthesis on illumination was essentially the same at 4, 22, and 37°, indicating that the rate-limiting step in the reactions of the L systems is not an enzymatic reaction with a temperature coefficient like those characteristic of usual enzymatic reactions. This observation differs from those of Nishimura with *R. rubrum* chromatophores, where he found that the rate of photophosphorylation (presumably the L reaction) at 26° was about double that at 15° (Nishimura, 1962). However, Nishimura made measurements of pH change rather than direct measurements of ATP formation, and there may have been some change in the system during the preparation of the chromatophores. In contrast with the light-induced ATP synthesis by the L system, the rate-limiting step in the ATP synthesis by the [L or O_2] system (following oxygenation in the dark or the slower synthesis in the light at 4°) has a temperature coefficient like that of usual enzymatic reactions, and is thus different from the one in the L system.

The maximal ATP content of *R. rubrum*, attained on illumination at 4°, was 4 nmoles/mg dry weight, as compared with average values of 12–14 and around 14–16 nmoles per mg dry weight found in *Chromatium* (Gibson and Morita, 1967) and in the nonphotosynthetic *E. coli* (Cole *et al.*, 1967), respectively. The half-time for light-induced ATP synthesis in *R. rubrum* of around 2 sec and the initial rate of 0.33 nmole/mg dry weight sec^{-1} in *R. rubrum* are similar to the values of 2 sec and 0.4 nmoles per mg dry weight sec^{-1} observed with *Chromatium* (Gibson and Morita, 1967). The highest rate of photophosphorylation reported for the isolated chromatophore fraction of *R. rubrum* (Baltscheffsky, 1961) is not far from that of light-induced phosphorylation in the intact cells, when both are based on bacteriochlorophyll content. Apparently the membrane fragments composing the chromatophore fraction can be obtained in a state where the phosphorylating enzymes are properly exposed for rapid reaction with added ADP and inorganic phosphate, in contrast with observations made with the dark oxygen-linked system in cell-free extracts (Ramirez and Smith, 1968; Geller, 1962). The rate of ATP synthesis in the dark on introduction of oxygen was 0.17 nmole sec^{-1} per mg dry weight at 22°. This rate is comparable with rates seen with nonphotosynthetic bacteria (Cole *et al.*, 1967; P. B. Scholes and F. Welsch, 1968, unpublished data) and with the rate of phosphorylation of exogenous ADP by mammalian mitochondria (Eisenhardt and Rosenthal, 1968). There is no lag seen in ATP synthesis on illumination of *R. rubrum*, but there is an apparent lag of about 4 sec following addition of O_2 at either 4 or 22°. The reason for the lag is not known.

Calculations based on the literature value of 1 mole of ATP required for the synthesis of 10.5 g dry weight of bacteria (Bauchop and Elsden, 1960; Senez, 1962) and the measured generation time of 4 hr at 30° indicate that even the rapid rate of ATP synthesis during illumination is not enough to support the bacterial growth. In fact, the rate of ATP synthesis is only about $^1/_{20}$ of the calculated required rate, the same as the discrepancy in similar calculations of Gibson and Morita (1967) with *Chromatium*. One possible source of the discrepancy in *R. rubrum* is that the maximal rate of ATP synthesis in freshly

harvested bacteria incubated to reduce the ATP level less extensively appears to be somewhat greater (as much as twofold) than that found in the "aged" bacteria used in these experiments.

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Studies on the Biosynthesis of Lincomycin. IV. The Origin of Methyl Groups*

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ABSTRACT: The bioorgin of the methyl groups of lincomycin has been studied. Using radioactive techniques and mass spectroscopy it was determined that in addition to the SCH_3 and NCH_3 groups of this antibiotic, the CCH_3 group present in the amino acid moiety is derived from C_1 donors. The results obtained present a unique example of biological methylation reactions on nitrogen, sulfur, and carbon occurring in the same biological system.

Lincomycin (Ia), as has been reported (Mason *et al.*, 1962; Herr and Bergy, 1962; Hoeksema *et al.*, 1964), is an antibiotic produced by an actinomycete designated *Streptomyces lincolnensis* var. *lincolnensis*. Soon after the discovery of lincomycin it was observed (Argoudelis *et al.*, 1965) that fermentations of *S. lincolnensis* produce 4′-depropyl-4′-ethyllincomycin (Ib) (U-21,699) in addition to lincomycin. Vapor-phase chromatography indicated that the composition of the mixture of antibiotics produced by *S. lincolnensis* is *ca.* 95% lincomycin and 5% 4′-depropyl-4′-ethyllincomycin. One of the characteristic structural features of lincomycin and 4′-depropyl-4′-ethyllincomycin is the presence of NCH_3, SCH_3, and two CCH_3 groups. As part of our work on the biosynthesis of the antibiotics produced by *S. lincolnensis* we have examined the bioorigin of the methyl groups present in the lincomycin molecule. The present paper describes the results of this study.

Experimental Section

Counting Procedures. Radioactivity was determined with an automatic Packard Tri-Carb liquid scintillation spectrometer, Model 3000 (Packard Instrument Co., Inc.).

Procedures described by E. Rapkin (Packard Technical Bulletin, No. 6, March 1963) were generally used. Specifically, samples for counting were prepared by mixing 0.5-ml aliquots of the aqueous solution of substances to be counted with 15 ml of a scintillator consisting of 200 ml of xylene, 600 ml of dioxane, 600 ml of methyl Cellosolve, 14 g of 2,5-diphenyloxazole, 700 mg of bis[2-(5-phenyloxazolyl)-1-benzene], and 112 g of naphthalene. Xylene and methyl Cellosolve were purified by procedures outlined in Weissberger (1956). Naphthalene was recrystallized from absolute ethanol. Sample glass vials were supplied by Packard Instrument Co., Inc.

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