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SYNTHESIS OF ADENOSINE TRIPHOSPHATE IN INTACT CELLS OF *RHODOSPIRILLUM RUBRUM* AND *RHODOPSEUDOMONAS SPHEROIDES* ON OXYGENATION OR ILLUMINATION

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SUMMARY

1. The ATP content of intact cells of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* was measured under different conditions using the sensitive luciferin-luciferase method.

2. The rate of decrease in ATP in the intact bacteria in the dark in the absence of O_2 is low; ATP formation following illumination or oxygenation is extremely rapid and the initial rates are similar with the two procedures.

3. P:O ratios measured for ATP formation coupled to oxidation of β-hydroxybutyrate by O_2 are as high as 2.8. Maximal rates of light-induced ATP synthesis at 10° were around 70–75 μmoles ATP/h per mg bacteriochlorophyll.

4. The ATP content of the intact cells reaches a higher level during illumination than after oxygenation, and illumination of aerobic cells increases the level to that obtained on illumination of anaerobic bacteria.

5. ATP formation linked to dark respiration of intact cells of *R. rubrum* is inhibited by methylphenazonium methosulfate; light-induced ATP synthesis is inhibited by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and both processes are inhibited by carbonylcyanide trifluoromethoxyphenylhydrazone.

6. Respiration of the bacteria could be completely inhibited on illumination; after cessation of illumination the respiration rate was increased. Both the inhibition of respiration and the subsequent stimulation are blocked by substances which inhibit light-induced formation of ATP.

7. The data tend to favor the existence of two separate electron-transport chains in these photosynthetic bacteria.

INTRODUCTION

Rhodospirillum rubrum and *Rhodopseudomonas spheroides* can obtain energy for growth from oxidation–reduction reactions leading to the reduction of oxygen or in-

Abbreviations: PMS, methylphenazonium methosulfate; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; FCCP, carbonylcyanide trifluoromethoxyphenylhydrazone.

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volving substances produced on illumination. As expected, cell-free extracts of these bacteria can catalyze the phosphorylation of ADP to ATP coupled to the oxidation of substrates in the dark by oxygen or during illumination. Some experimental evidence led to the suggestion that two separate electron-transport systems are involved, one activated only during illumination and the other capable of both supporting dark respiration and of transferring electrons to an oxidant produced on illumination[1,2]. Alternate schemes[3–5] have proposed that one complex cycle of electron-transport pigments mediates both the dark reactions with oxygen and the light-induced reactions, with most of the pigments used as a common pathway.

Although some physical separation of the dark respiratory chain system from one catalyzing light-induced phosphorylation of ADP can be achieved by differential centrifugation of broken cell extracts of *R. rubrum* (L. SMITH AND M. BALTSCHEFFSKY, unpublished observations), the data from such experiments are neither clear-cut nor easily reproducible. The maximal rates of respiration or of phosphorylation coupled to dark respiration in cell-free extracts are often low compared to the rates of light-induced phosphorylation[1,6]. As a different approach to the problem, we initiated experiments measuring the formation of ATP in intact cells of *R. rubrum* and *R. spheroides*, using the sensitive luciferin-luciferase[7] method to measure the ATP content. The method has been applied successfully to such measurements in whole cells of non-photosynthetic bacteria[8]. The data show that ATP is formed very rapidly on either illumination or oxygenation of anaerobic cells of these bacteria. However, the content of ATP attained in aerobic cells in the dark is less than that in illuminated cells. The additional formation of ATP on illumination of aerobic cells is slower than the initial rate of ATP formation on illumination or oxygenation of anaerobic cells. The respiratory chain-linked phosphorylation and the light-induced phosphorylation can be selectively inhibited, and studies with selective inhibitors show the two processes to have similar rates. The well-known inhibition of respiration by illumination[9] is prevented by substances that inhibit the light-induced formation of ATP. Consideration of all of the various data appears to favor the two separate electron-transport chains described above.

METHODS

Culture of bacteria

Rhodospirillum rubrum (VAN NIEL Strain 1) and *Rhodopseudomonas spheroides* (VAN NIEL Strain 2.4.1) were grown for 48 h under illumination in a bath at 29–30° in 500-ml bottles filled with the modified Hutner medium described by COHEN-BAZIRE, SISTROM AND STANIER[10]. A blue-green mutant of *R. rubrum* obtained through the kindness of Dr. JACK W. NEWTON was grown similarly, except for the addition of a small quantity of yeast extract to the medium (around 0.2 mg per ml). The bacteria were harvested by centrifugation, then resuspended in either fresh growth medium or in 0.01 M phosphate buffer, pH 7.0. For some experiments the cells were washed several times with the buffer before the final suspension was made. The suspensions used for measurements of respiration and of ATP content contained around 0.1 mg of bacteriochlorophyll per ml, as measured by the method of COHEN-BAZIRE, SISTROM AND STANIER[10].

Measurement of respiration

Rates of O_2 uptake were measured with a CLARK oxygen electrode[11] supported in a plastic collar which could be screwed into a plastic chamber of 3.5-ml capacity. A tuberculin syringe was joined to the chamber with a micrometer attached to the barrel, so that measured amounts of fluid could be rapidly added to or removed from the chamber through a small metal tube fitted with thin plastic tubing. Any air bubbles in the chamber could also be removed through the small tube. The oxygen electrode was connected by means of a banana plug to a battery set at 0.6 V and the electrode current amplified and recorded with a Servariter (Texas Instrument Model PWD). A second plastic chamber surrounding the first provided a means for circulating water from a constant temperature bath around the suspension of bacteria. The contents of the chamber were stirred by a magnetic stirrer.

Measurement of ATP formation and utilization

For assay of ATP content, 0.1-ml samples of the bacterial suspensions were removed rapidly from the chamber described above into small centrifuge tubes containing 0.04 ml of cold 17.5 % $HClO_4$ and mixed by agitation on a Vortex shaker. Samples thus treated were kept in an ice bath until an experiment was completed, then the $HClO_4$ was neutralized by the addition to each tube of 0.04 ml of a mixture containing 17.5 ml of saturated KOH, 22.5 ml of 1 M KCl and 60 ml of 0.1 M Tris buffer. This mixture had been shown to bring the pH of an equal volume of the $HClO_4$ to between 7.0 and 7.2. In contrast to the observations of STRANGE, WADE AND DARK, with *Aerobacter aerogenes*[8], we found that the same amount of ATP was extracted from the photosynthetic bacteria at room temperature and at ice bath temperature. After neutralization the tubes were centrifuged for 5 min at 10000 $\times g$ and the ATP content of the supernatant fluids measured using the luciferin-luciferase mixture in desiccated firefly tails (kindly supplied by Dr. W. D. MCELROY) ground in a mortar with cold 0.1 M phosphate buffer, pH 7.0 (ref. 12, p. 572). 50-μl aliquots of the supernatant fluids were added rapidly to 1.50 ml of a mixture containing $KHPO_4$, NaCl, $MgCl_2$ and Tris buffer (ref. 12, p. 565, Method d) *plus* 0.21 ml of the firefly extract and 0.01 ml of 0.1 M AMP in a cuvette with a 1-cm light path. Rapid mixing was obtained by adding the liquid on the end of a glass rod made nearly to fit the inside of the cuvette. In a completely dark room, the rod was inserted rapidly into the cuvette through a hole just above it in a compartment adjacent to the photomultiplier tube of a Farrand spectrofluorometer. The signal from the initial flash of light was recorded on a Brown recorder. A calibration curve was made for each experiment by adding different amounts of ATP (Sigma Chemical Co.) to a sample of supernatant fluid from the bacteria or to a solution containing the same concentrations of $HClO_4$ and neutralizing fluid. The recordings from the initial flashes showed a linear relationship to the ATP added.

Oxygenation and illumination of the bacterial suspensions

A suspension of bacteria in growth medium or in 0.01 M phosphate buffer, pH 7.0, containing substrate (usually 0.017 M β-hydroxybutyrate) was aerated at 30° in the dark and two aliquots added to $HClO_4$ while the suspension was still aerobic. The suspension was then put into the air-free chamber containing the oxygen electrode with water circulating in the outer compartment at 30°. Samples were taken at

intervals of 15 to 20 min after the suspension became anaerobic until the ATP content had decreased substantially (usually after 1 h or more). Water at 10° was circulated around the chamber, then either: (a) The chamber was illuminated by a microscope lamp 1 to 2 inches from the wall of the chamber and samples were rapidly collected. The light intensity was about 20000 lux at the wall of the chamber. (b) A known volume of buffer saturated with oxygen at 10° was rapidly added to the stirred contents of the chamber through the small tube by means of the syringe and micrometer. The subsequent O_2 uptake was recorded by the oxygen electrode, while samples were removed every 10 sec into $HClO_4$.

Inhibitors to be tested were added to the bacterial suspension several minutes before the beginning of the experiment. Concentrations and sources of the inhibitors used are given in the legends to the figures and table.

RESULTS

The initial observations showed that the decrease in ATP content of the bacterial suspensions was very slow during anaerobic incubation in the dark and that the formation of ATP following illumination or oxygenation was extremely rapid. At 10° there was less than 10% decrease in the ATP content of a washed suspension of *R. spheroides* during anaerobic incubation for 1 h in the dark with succinate as substrate; the decrease was even less when the bacteria were suspended in fresh growth medium with malate as substrate. The ATP content decreased most rapidly on incubating at 30° with β-hydroxybutyrate, but even here at least an hour was required to reach a fairly low level (Fig. 1). The formation of ATP on oxygenation or illumination was so rapid at 30° (Fig. 1), that the final procedure adopted for studying the kinetics of ATP synthesis was that of incubation of the bacteria in the dark at 30° to reduce the content of ATP, then changing the temperature to 10° and collecting

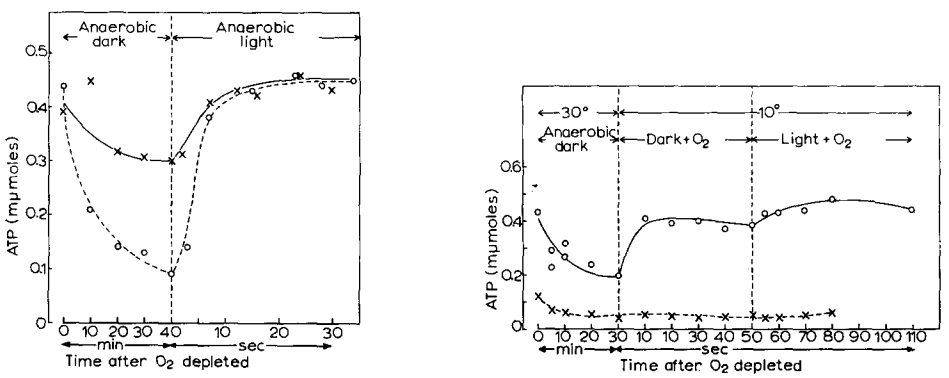

Fig. 1. Decrease in ATP content of the blue-green mutant of *R. rubrum* on incubation in the dark in the absence of O_2 and formation of ATP on illumination at 10° and 30°. The bacteria were washed in 0.01 M phosphate buffer, pH 7.0, and suspended in the same buffer containing 0.017 M β-hydroxybutyrate. The ordinate represents mμmoles ATP in 0.03 ml bacteria. O---O, 30°; ×—×, 10°.

Fig. 2. Formation of ATP on oxygenation then illumination of anaerobic cells of *R. rubrum* in the presence, ×---×, and absence, O—O, of FCCP. The bacteria were suspended in 0.01 M phosphate buffer, pH 7.0, containing 0.0125 M β-hydroxybutyrate. The FCCP was added several minutes before the bacteria were aerated. The ordinate represents the ATP in 0.03 ml bacteria.

samples as rapidly as possible after addition of oxygen or illumination. After a bit of practice (and the combined efforts of three people during collection of the samples), reproducible data were obtained, once certain difficulties were appreciated. Great care had to be exerted to collect the samples into the $HClO_4$ without introduction of oxygen or exposure to light. Exposure of the cells to even rather dim room light resulted in considerable ATP formation. Thus the chamber and all but the tip end of the exit tube were covered with black cloth or tape and samples were delivered rapidly into the centrifuge tubes just above the $HClO_4$ solution and mixed with a Vortex mixer. Some of the zero time readings (taken before the bacteria were placed in the plastic chamber) are probably too high because of exposure to room light.

The data of Figs. 2 and 3 show that the rates of ATP formation are high (and similar) following oxygenation or illumination; even at 10° the initial rates were difficult to measure with the set-up used. The interesting observation is that more ATP is formed on illumination than on oxygenation. Illumination of oxygenated cells after the ATP content had reached a plateau resulted in additional ATP formation, but the same final level of ATP was rapidly reached on illumination in the absence of oxygen; then the addition of oxygen yielded no further production of ATP. The two-step synthesis of ATP on addition of first oxygen, then light to anaerobic bacteria was reproduced in numerous experiments. The rate of formation of ATP on illumination of oxygenated cells was always lower than the initial rate seen on oxygenation or illumination of the anaerobic cells. The kinetics of ATP formation on illumination of anaerobic cells may have rapid, then slower phases, but the data are not good enough to be sure of this. Fig. 4 shows that the initial rate of the light-induced formation of ATP in *R. rubrum* is rapid in the presence of methylphenazonium methosulfate (PMS), which inhibits the dark respiratory chain-linked phosphorylation, and that the final level of ATP is not as great as on illumination in the absence of PMS.

The kinetics of ATP formation were the same in the wild-type and the blue-green mutant of *R. rubrum* and in *R. spheroides*.

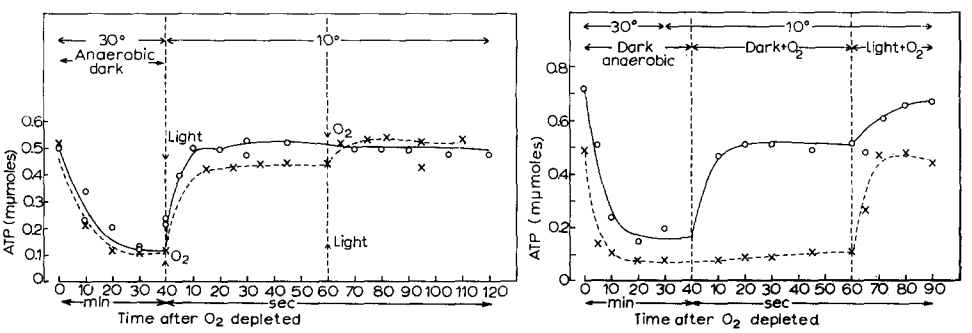

Fig. 3. Effect of oxygenation, then illumination, ×---×, or of the reverse procedure, O—O, on the blue-green mutant of *R. rubrum* suspended in 0.01 M phosphate buffer, pH 7.0, containing 0.017 M β-hydroxybutyrate. Ordinate represents ATP in 0.03-ml samples of the bacteria.

Fig. 4. Formation of ATP following oxygenation, then illumination of an anaerobic suspension of *R. rubrum* in 0.01 M phosphate buffer containing 0.017 M β-hydroxybutyrate, in the absence, O—O, and presence, ×---×, of $5 \cdot 10^{-4}$ M PMS. The ordinate represents the ATP content of 0.03-ml samples of the bacteria. The PMS was added several minutes before the beginning of the experiment.

In the experiment of Fig. 4 the P:O ratio (the initial rate of ATP formation divided by 1/2 the corresponding rate of dark O_2 uptake) equals 2.8 in the absence of PMS. This same ratio was obtained with several cultures of *R. rubrum* oxidizing β-hydroxybutyrate. Lower values (around 2) were found in a few other experiments.

The initial rates of light-induced ATP formation, expressed in terms of the bacteriochlorophyll content, were somewhat variable. In three experiments 70–75 μmoles of ATP were formed per h per mg bacteriochlorophyll at 10°; in three other experiments the rates were lower: 17–52 μmoles per h per mg bacteriochlorophyll.

The rate of light-induced formation of ATP in both *R. rubrum* and *R. spheroides* was decreased in the presence of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) at concentrations around 5–10 μM (Fig. 5). PMS ($5 \cdot 10^{-4}$ M) completely inhibited the phosphorylation linked to respiration in *R. rubrum*, but not light-induced phosphorylation. Surprisingly, PMS completely inhibited light-induced phosphorylation in *R. spheroides*, even at lower concentrations. In the presence of 5.6 μ*M* carbonylcyanide trifluoromethoxyphenylhydrazone (FCCP), there was no ATP formation in *R. rubrum* following either oxygenation or illumination (Fig. 2).

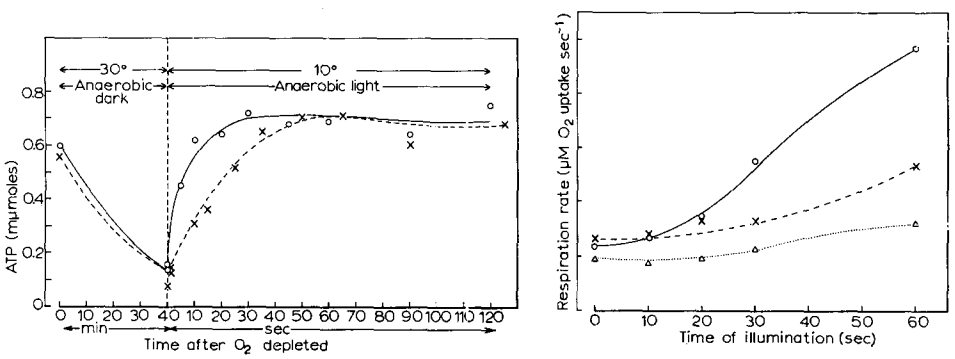

Fig. 5. Effect of HQNO on ATP formation during illumination of *R. spheroides*. The bacteria were suspended in 0.01 M phosphate buffer containing 0.017 M β-hydroxybutyrate. O—O, no HQNO; ×---×, 4 μM HQNO added several minutes before the beginning of the experiment. The ordinate represents ATP content of 0.017-ml samples of bacteria.

Fig. 6. Effect of HQNO on stimulation of respiration of *R. rubrum* after illumination. Bacteria suspended in 0.01 M phosphate buffer, pH 7.0, respiring with endogenous substrate, were illuminated successively in the plastic chamber described in METHODS for the periods indicated. O—O, no HQNO; ×---×, 2.5 μM HQNO; △....△, 12.5 μM HQNO.

The respiration of *R. rubrum* was inhibited by illumination, as previously observed[9]; in our experiments complete inhibition was obtained. It was also found that the respiration rate increased progressively after brief periods of illumination (Table I and Fig. 6) with either endogenous substrate or with added β-hydroxybutyrate. Both the inhibition of respiration by illumination and the increase of the respiratory rate after the inhibited period were removed by substances shown to inhibit the light-induced phosphorylation. Fig. 6 illustrates the inhibitory effect of HQNO on the stimulation of respiration after illumination of *R. rubrum*. The respiration of *R. rubrum* in the dark was not inhibited by 2.5 to 12.5 μM HQNO, but after several minutes exposure to even the lowest concentration, the inhibitory effect of illumination on the respiration was decreased. The same was true with 10 μM FCCP.

TABLE I

EFFECT OF LIGHT ON THE RESPIRATION OF *R. rubrum*

Expt. No.		*Respiration rate* ($\mu M\ O_2\ sec^{-1}$)
1	Endogenous substrate, dark	0.098
	Light on, 1 min	0
	Light off	0.249 for 1 min, then began to decrease
	Light on, 1 min	0
	Light off	0.405 for 30 sec, then decreased
	Light on, 1 min	nearly 0
	Light off	0.492 for 1 min, then decreased
2	Endogenous substrate, dark	0.110
	β-Hydroxybutyrate added	0.174
	Light	0 for 40 sec, then began to increase
	Light off	0.484

DISCUSSION

The rate of decrease of the ATP content in *R. rubrum* or *R. spheroides* in the dark in the absence of O_2 is low, whether the cells are suspended in buffer or in growth medium; with malate or succinate in the medium a low ATP content may be attained only after incubation for several hours. The more rapid decrease in ATP content in the presence of β-hydroxybutyrate probably results from its reaction with ATP leading to polymer formation[14]. There is no fermentative formation of ATP in the presence of FCCP, which inhibits ATP synthesis following illumination or oxygenation. However, the limitation of growth under anaerobic conditions in the dark does not seem to be due to a lack of ATP, unless there is a problem of localization within the cells.

In contrast to the slow decrease in ATP content, the formation of ATP in both species is very rapid when anaerobic suspensions with a low ATP content are illuminated in the presence of all substrates tested. At 10° the rate of light-induced phosphorylation was as high as 75 μmoles ATP per h per mg bacteriochlorophyll; phosphorylation on oxygenation showed similar rates. Light-induced ATP formation by cell-free extracts or chromatophore fractions of *R. rubrum* can show considerably higher rates under optimal conditions at 25 or 30° (refs. 15, 16). Since the initial rates of ATP formation in the intact cells were too high to measure accurately at temperatures exceeding 10°, it is not known whether the lower rates with the bacteria result from a lack of "optimal conditions".

The P:O ratios obtained for the dark respiratory chain-linked phosphorylation are comparable to those found with mammalian mitochondria oxidizing DPN-linked substrates. Thus the low values obtained with broken cell extracts of *R. rubrum*[6,17] do not reflect a dark oxidative phosphorylation system of lower efficiency in these bacteria, but are evidence of disruption of the system on breakage of the cells.

More ATP is produced on illumination of anaerobic cells of these photosynthetic bacteria than on oxygenation, and illumination of dark aerobic cells brings the ATP content to the level observed on illumination in the absence of O_2. The initial rates of ATP formation following illumination or oxygenation are both very rapid,

but the rate of ATP formation on illumination of oxygenated cells is lower. Two possible explanations for these observations could be offered: (a) there are two electron-transport-phosphorylating systems with different localization within the cells, or (b) there are either separate or one complex system fed from two sources and also a competing ATP splitting reaction, and the light-induced formation of ATP is more rapid. The competing reaction would then limit the ATP level attained on oxygenation. It would have to be an ATPase with a high K_m for ATP, like the one observed in chromatophores of *R. rubrum* by NISHIMURA[16] to fit with the slow decrease of ATP observed in the dark in anaerobiosis. Since our data indicate similar initial rates of ATP formation and similar levels of ATP reached on oxygenation as on illumination in the presence of PMS (which inhibits the respiratory chain-linked phosphorylation), they agree better with the postulate of two independent pathways.

The evidence obtained with inhibitors with cell-free extracts[1,6] and with the intact cells argues against the participation of the identical electron-transport chain in the dark respiratory chain-linked phosphorylation and that only induced on illumination. Concentrations of HQNO which completely inhibit the light-induced ATP formation in extracts of *R. rubrum* which have no respiratory chain-linked phosphorylation[1] partially inhibit light-induced ATP formation in intact cells. This substance has been shown to block oxidation of cytochrome *b* and reduction of cytochrome *c* in other systems[18,19] and also does this in intact cells of *R. rubrum* on illumination, but not on oxygenation[1] except at much higher concentrations[20]. PMS, which appears to be able to form a by-pass of some members of an electron-transport chain, eliminates some ATP-forming step(s) in the dark respiratory chain system of *R. rubrum*, but not in that responding exclusively to light. In general, the observations made with inhibitors in intact cells and in cell-free extracts or chromatophore fractions of these bacteria[1,6,20–22] are in agreement. With the intact cells several minutes may be required after the addition of the inhibitors for maximal effects to be manifest.

There is other evidence against the postulate that the same electron-transport chain functions in the exclusively light-induced reactions and in the dark respiratory chain. Photosynthetic bacteria synthesize more than one *c*-type cytochrome, and some of these appear to be specifically involved in electron transport stimulated by light. In fact, in *Chromatium* the *c* cytochromes may be involved in different pathways of photometabolism[23]. Some cytochrome *c* remains reduced in the dark in aerobic cells of *R. rubrum* and *R. spheroides* while the cytochrome *b* is nearly all oxidized[24,25], and illumination of *R. rubrum* results in oxidation of cytochrome *c* which is not oxidized on oxygenation[2]. Dark-grown cells of *R. rubrum* have a cytochrome *c* on an electron-transport chain oxidized by oxygen[2] and also some which remains reduced in the presence of oxygen[26], the latter presumably part of a non-functional chain which lacks the photochemical apparatus. The two ATP-forming systems show different stabilities to rupture of the cells, the respiratory chain-linked one being more unstable. The data presented here do not give evidence for or against the suggestion of VERNON AND ASH[27] that there is some interrelationship of the ATP-forming systems at the level of dehydrogenases, with bifurcation of the electron-transport sequences. Interaction at this level might also be explained by changes in the concentrations of oxidized and reduced coenzymes or of the different adenine nucleotides (see below).

The kinetics of ATP formation and utilization were similar with *R. spheroides*, *R. rubrum* and the mutant of *R. rubrum*. The mutant appears to be unchanged from

the wild type as far as the phosphorylating systems are concerned. A surprising difference between *R. rubrum* and *R. spheroides* is the inhibition of light-induced ATP formation in the latter but not the former by the concentration of PMS employed. This does not appear to be a permeability phenomenon, since PMS can stimulate light-induced phosphorylation in cell-free extracts of *R. rubrum*[17], but inhibits it in extracts of *R. spheroides* (M. E. NAVA AND J. RAMÍREZ, unpublished observations). HQNO inhibits light-induced phosphorylation in intact cells or cell-free extracts of both species (ref. 1 and M. E. NAVA AND J. RAMÍREZ, unpublished observations).

Both the respiratory chain-linked and the light-induced phosphorylating systems are bound to insoluble membrane fragments in broken-cell extracts of *R. rubrum*. Although only the cytoplasmic membrane and internal membranes which appear to arise from invaginations of it are seen in electron micrographs of thin sections of the bacteria[28–30], broken cell extracts of *R. rubrum* and *R. spheroides* yield membrane fractions with different compositions and enzymatic activities[31,32]. The system of *R. rubrum* catalyzing dark respiratory-chain linked phosphorylation has been reported to have a different composition from that catalyzing light-induced phosphorylation[6]. The relevance of these findings to the localization of the activities within the cells is not yet clear.

As demonstrated previously[9], the respiration of *R. rubrum* was inhibited by illumination; in our experiments complete inhibition could be obtained. There was a stimulation of respiration following the cessation of illumination, as observed by FORK AND GOEDHEER[13]. The inhibitory effect of light on respiration has been attributed to competition between O_2 and the photooxidant for electrons furnished by a common electron-transport pathway[3,4]. An alternate explanation, suggested by VERNON[5] is that the inhibition results from increased formation of ATP and thus decrease of phosphate acceptor available to the coupled systems; thus the competition is for phosphate acceptor, rather than for electrons. This postulate is supported by our observation that the inhibition by illumination is removed by substances which inhibit the light-induced formation of ATP. Also our experiments showed the rates of ATP formation on oxygenation and illumination (and thus presumably the rates of electron transport) to be similar, arguing against a more rapid rate of electron transport to the photooxidant. FORK AND GOEDHEER[13] also observed decreased inhibition of respiration by illumination of PMS-treated *R. rubrum*, which could be explained by the decreased ATP formation in the presence of this substance. KATOH[33] observed some inhibition of the respiration of a suspension of chromatophores from *Rhodopseudomonas palustris* by illumination, even in the absence of added ADP. In fact, the respiration was inhibited by ADP. He feels that his observations rule out the competition for phosphate acceptor as an explanation for the inhibition. He did not ascertain to what extent oxidation and phosphorylation were obligately coupled in the extracts with which he worked.

The stimulation of respiration following a period of illumination was eliminated in the presence of HQNO in our experiments and of PMS in the experiments of FORK AND GOEDHEER[13]. The delay of several minutes before the full effect of the HQNO was seen may be due to a slow permeation into the cells. The explanation of the increase in respiration rate after a period of illumination is uncertain. The demonstrated increase in reduced pyridine nucleotide on illumination[34] could be an explanation; the suggestion has been made that this is an energy-requiring reaction[35,36].

However, AMESZ[37] found that the reduction of pyridine nucleotide was only partially inhibited by concentrations of HQNO higher than those which inhibited the increase of the respiration rate of our experiments.

The data reported here were gathered using very simple methodology. More rapid collection of samples for ATP analysis combined with a judicious combination of inhibitors and substrates should yield answers to some additional questions. These experiments are in progress.

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REFERENCES

1 L. SMITH AND M. BALTSCHEFFSKY, *J. Biol. Chem.*, 234 (1959) 1575.
2 L. SMITH AND J. RAMÍREZ, *Arch. Biochem. Biophys.*, 79 (1959) 233.
3 M. NISHIMURA AND B. CHANCE, *Biochim. Biophys. Acta*, 66 (1963) 1.
4 T. HORIO AND M. D. KAMEN, *Biochemistry*, 1 (1962) 1141.
5 L. P. VERNON, *Ann. Rev. Plant Physiol.*, 15 (1964) 73.
6 D. M. GELLER, *J. Biol. Chem.*, 237 (1962) 2947.
7 B. L. STREHLER AND W. D. MCELROY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 871.
8 R. E. STRANGE, H. E. WADE AND F. A. DARK, *Nature*, 199 (1963) 55.
9 C. B. VAN NIEL, *Advan. Enzymol.*, 1 (1941) 263.
10 G. COHEN-BAZIRE, W. R. SISTROM AND R. Y. STANIER, *J. Cell. Comp. Physiol.*, 49 (1957) 25.
11 L. C. CLARK, *Trans. Am. Soc. Artificial Internal Organs*, 2 (1956) 41.
12 H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, pp. 559–573.
13 D. C. FORK AND J. C. GOEDHEER, *Biochim. Biophys. Acta*, 79 (1964) 249.
14 J. M. MERRICK AND M. DOUDOROFF, *Nature*, 189 (1961) 890.
15 M. BALTSCHEFFSKY, *Acta Chem. Scand.*, 15 (1961) 215.
16 M. NISHIMURA, *Biochim. Biophys. Acta*, 64 (1962) 345.
17 D. M. GELLER AND F. LIPMANN, *J. Biol. Chem.*, 235 (1960) 2478.
18 J. W. LIGHTBOWN, *J. Gen. Microbiol.*, 11 (1954) 477.
19 J. W. LIGHTBOWN AND F. W. JACKSON, *Biochem. J.*, 63 (1956) 130.
20 M. NISHIMURA, *Biochim. Biophys. Acta*, 66 (1963) 17.
21 D. M. GELLER, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 161.
22 H. BALTSCHEFFSKY AND M. BALTSCHEFFSKY, *Acta Chem. Scand.*, 14 (1960) 257.
23 W. J. VREDENBERG AND L. N. M. DUYSENS, *Biochim. Biophys. Acta*, 79 (1964) 456.
24 B. CHANCE AND L. SMITH, *Nature*, 175 (1955) 803.
25 M. NISHIMURA AND B. CHANCE, *Studies on Microalgae and Photosynthetic Bacteria*, Japan. Soc. Plant Physiol., Univ. of Tokyo Press, 1963, p. 636.
26 S. TANIGUCHI AND M. D. KAMEN, *Biochim. Biophys. Acta*, 96 (1965) 395.
27 L. P. VERNON AND O. K. ASH, *J. Biol. Chem.*, 235 (1960) 2721.
28 E. S. BOATMAN, *J. Cell Biol.*, 20 (1964) 297.
29 S. C. HOLT AND A. G. MARR, *J. Bacteriol.*, 89 (1965) 1402.
30 G. COHEN-BAZIRE AND R. KUNISAWA, *J. Cell Biol.*, 16 (1963) 401.
31 G. COHEN-BAZIRE AND R. KUNISAWA, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1543.
32 R. LANGRIDGE, P. D. BARRON AND W. R. SISTROM, *Nature*, 204 (1964) 97.
33 S. KATOH, *J. Biochem. Tokyo*, 49 (1961) 126.
34 L. N. M. DUYSENS AND G. SWEEP, *Biochim. Biophys. Acta*, 25 (1957) 13.
35 B. CHANCE AND M. NISHIMURA, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 19.
36 H. GEST AND S. K. BOSE, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 121.
37 J. AMESZ, *Biochim. Biophys. Acta*, 66 (1963) 22.