Reactions of *Rhodospirillum rubrum* Extract with Cytochrome c and Cytochrome c₂*

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(Received for publication, November 18, 1958)

Observations from a number of different experimental approaches have implicated cytochrome pigments in oxidation-reduction reactions resulting from the illumination of photosynthetic tissues (1–6). Possible relationships of these cytochromes to those of the respiratory chain pigments which react with oxygen in the dark have been considered.

Vernon (7) isolated a soluble cytochrome in good yield from the photosynthetic bacterium *Rhodospirillum rubrum*. This pigment, which has been designated cytochrome c₁ (8), has absorption spectrum properties nearly identical with those of mammalian cytochrome c, but the oxidation-reduction potential resembles that of the cytochrome f found in the chloroplasts of green plant tissues. Vernon and Kamen (3, 4) studied the oxidation of mammalian cytochrome c and the bacterial cytochrome c₂ by sonic extracts of a number of photosynthetic bacteria in the light and in the dark and concluded that all contained both a dark cytochrome c oxidase and a cytochrome c photooxidase and that the relative amounts of the two enzymes could be correlated with the relative aerobicity of the organisms.

The present paper describes some properties of cell-free extracts of *Rhodospirillum rubrum* and further studies on the reactions of these extracts with mammalian cytochrome c and bacterial cytochrome c₂. The dark oxidation of either cytochrome was found to be very slow, and the photooxidation of the cytochrome c does not appear to be an enzymatic reaction. These two reactions cannot play significant roles in the metabolism of the bacteria.

**EXPERIMENTAL**

*Culture of Bacteria and Preparation of Cell-free Extracts—Rhodospirillum rubrum*, van Niel strain S1, were obtained from Dr. M. Kamen and grown under illumination in the medium described by Gest et al. (9). To prepare the broken-cell suspension, the bacteria were washed once with 0.02 M phosphate buffer, pH 7.4, then ground with powdered glass in a mortar, after the procedure of Tissières (10). The deep purple extract was used in those experiments where “cell-free extract” is indicated. A “washed particulate fraction” was obtained from the extract by collecting the material sedimented by centrifugation at 22,000 × g for 40 minutes, then washing twice with buffer by recentrifugation. For a few experiments cell-free extracts were prepared by treatment of a cell suspension for 5 minutes in a sonic oscillator.1 Dry weight measurements were made on samples of washed cells or dialyzed extracts dried to constant weight at 110°C.

*Preparation of Cytochromes c and c₂—Mammalian cytochrome c, kindly furnished by Dr. E. C. Slater, was prepared from horse heart by the method of Keilin and Hartree (11) and further purified by adsorption on an ion exchange resin, as described by Margoliash (12). Cytochrome c₂ from *R. rubrum* was purified by the method of Vernon and Kamen (13), omitting the electrophoresis on filter paper. It was also prepared by another method, some parts of which were suggested by Dr. S. R. Elsden;2 this method avoids heating the bacteria with trichloroacetic acid. The bacteria were washed twice with weak phosphate buffer, then “sloppy dried” in a vacuum desicator. The dry powder was extracted with 20 volumes, then with 10 volumes of cold 2 per cent sodium borate, removing the precipitate each time by low-speed centrifugation. Trichloroacetic acid was added to the cold extract to give a final concentration of 0.145 M, then the procedure of Vernon and Kamen was followed, all steps being carried out in the cold. The cytochrome c₂ prepared by the two methods appeared to have similar absorption spectra and reactions with the *R. rubrum* extract.

Cytochrome c and c₂ were reduced by treatment with hydrogen and platinum black. The cytochrome in 0.01 M phosphate buffer at pH 7.0 was gassed for 10 minutes with nitrogen, which had been freed from oxygen by exposure to heated copper wire. Then the mixture was treated with hydrogen for about 1 hour, then with nitrogen again for 10 minutes. The platinum black was removed by centrifugation.

*Manometric Experiments—*The respiratory of whole cells or extracts diluted with buffer was measured in Barcroft differential manometers at 30°C with KOH in the center well to absorb any evolved CO₂. The effect of illumination on oxygen uptake was tested in a bath with a glass side; strong light was reflected to the bottom of the Barcroft vessel by a mirror placed in the bath.

*Spectrophotometric Measurements—*The oxidation of reduced cytochrome c or cytochrome c₂ was followed by observing in a Uvispek or a Beckman DU spectrophotometer the change in optical density at 550 mp after the addition of the bacterial extract to the reduced cytochrome. An equal concentration of *R. rubrum* extract was added to the reduced cytochrome. An equal concentration of *R. rubrum* extract was added to the reduced cytochrome.
Rubrum extract. The mixture contained 0.1 ml. of extract
made with the apparatus designed by Keilin and Hartree (14).
The stimulating light from a tungsten lamp was filtered through
two thicknesses of Wratten 88A filter, which removes light of
wave length shorter than 720 μm. Tests with light-scattering
suspensions, such as milk or colorless bacteria, showed that the
phototube was unaffected by the filtered stimulating light.

RESULTS

Respiratory Activity of Cell-free Extracts of R. rubrum—The
rubrum extracts were added to the buffer in the blank cuvette.
Most of the observations on the effects of illumination were
made with the apparatus designed by Keilin and Hartree (14).
The stimulating light from a tungsten lamp was filtered through
two thicknesses of Wratten 88A filter, which removes light of
wave length shorter than 720 μm. Tests with light-scattering
suspensions, such as milk or colorless bacteria, showed that the
phototube was unaffected by the filtered stimulating light.

The QO₂ of the extract oxidizing succinate
was as high as 9.3, compared with 15.1 for whole cells and 18.4
for the washed particulate suspension with this substrate.
The washed particles showed no oxygen uptake in the absence of
added substrate and oxidized only succinate rapidly. The addi-
tion of cytochrome c to the extracts or washed particles did
not affect the rate of oxygen uptake.

The QO₂ of the extracts made with powdered glass when oxida-
ting succinate was sometimes only one-half as great as that of
the whole cells, and the QO₂ of the extracts prepared in the sonic
vibrator was usually only about one-third that of the intact cells.

As found by van Niel (15), the respiration of the intact cells
was always inhibited by light, but in our experiments, the effect
was highly variable in extent (from 38 to 100 per cent inhibition).
In contrast, an inhibition of respiration of the cell-free extracts
was always inhibited by light, but in our experiments, the effect
was highly variable in extent (from 38 to 100 per cent inhibition).

Oxidation of Reduced Cytochrome c and Cytochrome c₂ by R.
rubrum Extracts—1. Dark reactions: The data of Fig. 1 show
the optical density changes at 550 μm after the addition of 0.2
ml. of a strong extract of R. rubrum to reduced cytochrome c
(15 μM) in the dark. As found by Vernon and Kamen (3, 4),
there is a slow cyanide-sensitive oxidation (decrease in optical
density at 550 μm) in the dark. Since this reaction is very slow
compared to the oxidation of cytochrome c by comparable con-
centrations of extracts known to contain cytochrome c oxidase,
an attempt was made to assess the importance of this reaction
as a part of the dark respiratory chain of the organisms. This
was done by comparing the rate of oxidation of the cytochrome
c with the rate of oxygen uptake of the same extract oxidizing
succinate. It was assumed in the calculations that 1 mole of
oxygen is equivalent to 4 moles of cytochrome c. The data of
Table I show that in a typical experiment the rate of oxygen
uptake calculated to correspond to the observed rate of oxidation
of cytochrome c (calculated from rate constant X concentration
of extracts known to contain cytochrome c oxidase, then expressed
as μliters of O₂) is very slow compared with the observed respiration rate of the extract
oxidizing succinate. The calculated rate of O₂ uptake at 15 μM
mammalian cytochrome c was always less than 10 per cent of
the observed rate and was even slower when the bacterial cyto-
chrome c₂ was oxidized.

When cyanide was added to inhibit the dark oxidation, the
rubrum extracts could reduce oxidized cytochrome c slowly;
subsequent addition of succinate increased the rate of reduction
(see Fig. 1). With the washed particles, no reduction of cyto-
ochrome c was observed until substrate was added. Thus, the
slow rates of cytochrome c oxidation, which were measured with-
out added substrate, cannot be explained on the basis of opposing
back reactions.

2. Light-induced reactions: Fig. 2 shows the increase in the
rate of oxidation of cytochrome c when the mixture of reduced cytochrome c and R. rubrum extract is illuminated with infrared
light. It can be seen that illumination resulted in a more rapid
decrease in optical density at 550 μm after the slow rate
of dark oxidation either stopped, or there was a slow reduction
of cytochrome c (increase in optical density). The optical den-
sity changes during continuous illumination are plotted in Fig.
3. Here the steady state value reached does not represent
complete oxidation of the cytochrome c, as shown by the fur-
ther oxidation of cytochrome c by ferricyanide. The same mix-
ture of oxidized and reduced cytochrome c is obtained on il-
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TABLE I
Comparison of respiration rate of R. rubrum extract with rate of
oxidation of cytochrome c.

<table>
<thead>
<tr>
<th>Observed O₂ uptake</th>
<th>Calculated O₂ uptake (corresponding to oxidation of cytochrome c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μl. per hour</td>
<td>μl. per hour</td>
</tr>
<tr>
<td>47.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

In both measurements the same R. rubrum extract was used in
0.03 M phosphate buffer, pH 7.4. In the respiration measurement,
succinate was added as substrate to a final concentration of 0.03
M. The concentration of cytochrome c was 15 μM.

Entirely similar results were obtained on illumination whether
the R. rubrum extract was prepared in a sonic vibrator or with
powdered glass.

In a number of experiments it was observed that the rate of
reduction of cytochrome c in the dark in the presence of cyanide

FIG. 1. Oxidation and reduction of cytochrome c in the dark by
rubrum extract. The mixture contained 0.1 ml. of extract
(about 3.5 mg. dry weight) in 0.05 M phosphate buffer, pH 7.4,
containing 15 μM reduced mammalian cytochrome c.
FIG. 2. Oxidation of cytochrome c by R. rubrum extract during intermittent illumination. The reaction mixture contained 0.1 ml. of R. rubrum extract in 0.05 M phosphate buffer, pH 7.4, containing 15 µM reduced mammalian cytochrome c. Illumination was as described in text.

FIG. 3. Oxidation of cytochrome c by R. rubrum extract during continuous illumination. Same extract as in Fig. 2.

and succinate was increased after a period of illumination. It was also observed that the decrease in optical density at 550 µm after illumination of the extract plus cytochrome c was greater than that which would have been expected from the oxidation of cytochrome c alone. In other words, there was a decrease in absorption of something in the R. rubrum extract at this wave length.

When the R. rubrum extract is heated at 70° for 30 minutes, the suspension precipitates in small particles; the particles do not settle when suspended in 1.5 M sucrose. When this suspension of heated particles is added to reduced cytochrome c, no dark oxidation is observed, but the cytochrome c is still oxidized on illumination.

In all of the experiments described above, the reaction mixture was aerobic. As observed by Vernon and Kamen (3), no oxidation of cytochrome c was observed under anaerobic conditions, either in the dark or the light.

DISCUSSION

The data reported show that extracts of R. rubrum which are capable of active respiration do not have significant cytochrome c oxidase or cytochrome c2 oxidase activities. The lack of an active cytochrome c oxidase agrees with the observation that intact cells of these bacteria have no cytochrome c3 (2). It has also been shown (16) that broken-cell extracts of a number of other kinds of bacteria do not oxidize mammalian cytochrome c, except at a very low rate. Spectrophotometric observations of intact cells of R. rubrum showed that the cytochrome c2 remains largely reduced in the presence of air (2). Thus, although Vernon and Kamen concluded that R. rubrum possesses a dark cytochrome c oxidase (3, 4), quantitative measurements show that the slow rate of reaction could have no physiological significance and that the cytochrome c2 is not a part of the bacterial respiratory system. Further evidence against a function for a cytochrome c2 oxidase is given by the observation that after a period of illumination, the slow oxidation in the dark of cytochrome c3 (or cytochrome c) stops, although illumination produces no inhibition of the respiratory rate.

Although the rate of oxidation of reduced cytochrome c during illumination is greater than the oxidation in the dark, it is not an extremely rapid reaction. Illumination results in a steady-state level of cytochrome c which is still partly reduced. It would appear that this steady-state mixture of oxidized and reduced cytochrome c is the result of reaction with oxidized and reduced substances formed on illumination. The oxidation of reduced cytochrome c or c3 on illumination does not appear to be an enzymatic reaction, since it is not inactivated by heating the extract at 70° for 30 minutes, when the proteins of the extract have precipitated as fine particles. The heated precipitate re-suspended in strong sucrose retains its activity. Vernon and Kamen (3) reported that the "photooxidase" activity of R. rubrum extract was lost on boiling; but heating will coagulate the mixture, and they do not report an attempt to examine the re-suspended precipitate. As reported by Vernon and Kamen (3) also, the light-induced oxidation takes place only in the presence of air. Since the bacteria do not evolve oxygen on illumination (15) and grow well anaerobically in the light, a photo-oxidase requiring oxygen would be of little importance to the bacterial metabolism. The data in the following paper show that the endogenous cytochrome c2 of R. rubrum extracts is not oxidized on illumination except when there is concurrent phosphorylation.

An enzyme system from digitonin-treated spinach chloroplasts has been described (17) which will oxidize mammalian cytochrome c on illumination. Unlike the bacterial extracts, the chloroplast system was found to be heat-labile, but in both systems there is a requirement for oxygen.

An enzyme system from digitonin-treated spinach chloroplasts has been described (17) which will oxidize mammalian cytochrome c on illumination. Unlike the bacterial extracts, the chloroplast system was found to be heat-labile, but in both systems there is a requirement for oxygen.

On preparation of cell-free extracts of R. rubrum, the inhibitory effect of illumination on the respiration is lost. An explanation for this is suggested in the following paper.

Two observations suggest that oxidizing or reducing substances can accumulate in solution after illumination of R. rubrum extracts or cells. (a) The rate of reduction of soluble cytochrome c by extracts containing succinate in the dark is increased after a period of illumination. (b) We also confirmed the observation of Vernon and Kamen (3) that illumination of a mixture of re-
duced cytochrome c and intact cells of *R. rubrum* results in an oxidation of the cytochrome. Since it is unlikely that the cytochrome penetrates the cells, it must either react with oxidizing substances at the cell surface or such substances released into the medium.

**SUMMARY**

1. Cell-free extracts and washed particle suspensions of *Rhodospirillum rubrum* are described which can respire in the presence of added substrates.

2. The extracts of *R. rubrum* can oxidize mammalian cytochrome *c* or the bacterial cytochrome *c* at a low rate in the dark. The rate of oxidation of either cytochrome is very low compared to the rate of oxygen uptake of the extracts oxidizing succinate. This and other data indicate that cytochrome *c* is not a part of the bacterial respiratory chain.

3. The rate of oxidation of cytochrome *c* (or *c*2) by *R. rubrum* extract is increased by illumination with infrared light, but this more rapid oxidation is not inactivated when the extract is heated at 70° for 30 minutes, even though the proteins of the extract are precipitated. Thus the oxidation of cytochrome *c* on illumination does not appear to be an enzymatic reaction. The reaction, which requires oxygen, is also not important in the metabolism of these bacteria.

**Acknowledgment**—The author would like to express her deep appreciation to Professor D. Keilin for his continuing interest and helpful suggestions, and to the Molteno Institute for hospitality.

**REFERENCES**

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