SOME HAEM PROTEIN-LINKED PYRIDINE NUCLEOTIDE OXIDATION SYSTEMS IN *RHODOSPIRILLUM RUBRUM**

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SUMMARY

Ammonium sulfate fractionation of a crude citrate extract obtained from lyophilized cells of *R. rubrum*, a facultative photoheterotrophe, yields a soluble enzyme preparation which contains "bound" bacterial cytochrome *c* ("cytochrome c_2") and variant heme protein ("RHP"). The following light-insensitive reactions, enzymically-catalyzed by this preparation, have been noted: (a) TPNH oxidase (b) DPNH oxidase (c) TPNH-cytochrome c_2 reductase (d) DPNH-cytochrome c_2 reductase (e) TPNH-RHP reductase (f) DPNH-RHP reductase (g) TPNH-2,6-DCPI reductase and (h) DPNH-2,6-DCPI reductase. The reaction between DPNH and 2,6-DCPI indicates 10-fold purification of the enzyme with respect to the initial crude extract. Using this preparation, some characteristics of these catalyzed reactions have been determined.

The evidence obtained from studies of heat inhibition and variations with pH suggests that reactions (g) and (h) are catalyzed by different enzymes, the latter being much more reactive. The rates of oxidase reactions (a) and (b) are very small, being 1/300 and 1/50 respectively of those found for DPNH and TPNH using 2,6-DCPI as H-acceptor.

The bound RHP is rapidly oxidized, whereas cytochrome c_2 is only partially oxidized when the enzyme preparation is aerated. When DPNH is added in air, only cytochrome c_2 is reduced. Under anaerobic conditions, both endogenous heme proteins are reduced to the maximum extent, but at different rates. These preliminary studies are in accord with an oxidation scheme in which dehydrogenation of the substrate DPNH proceeds via RHP to both cytochrome c_2 and oxygen as hydrogen acceptors.

INTRODUCTION

The participation of haem proteins in photosynthesis is well authenticated[1,2]. Studies relevant to an understanding of mechanisms involved have been made, particularly a number of investigations on the metabolism of purple photosynthetic bacteria,

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; 2,6-DCPI, 2,6-dichlorophenol-indophenol; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide.

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such as the facultative photoheterotrophe, *Rhodospirillum rubrum*, and the photoanaerobe, *Chromatium*. These have included exploratory tests for the occurrence in cell-free extracts of light-sensitive and light-insensitive oxidase activities associated with the bacterial haem proteins[3–5], adaptive synthesis of haem proteins during illumination of cell suspensions[6], simultaneous light-stimulated oxido-reductions[7,8], photophosphorylation[9–13], and *in vivo* studies of light-induced shifts in the oxidation states of cellular haem proteins[14–16]. However, data bearing on partial reactions at the enzyme level are meager, and the enzymic basis for the action of haem proteins remains obscure.

In *Rhodospirillum rubrum*, most of the haem proteins present in the photoactive portions of the cell can be accounted for as a mixture of two compounds[4]. One of these is a *c*-type cytochrome ("cytochrome c_2") and the other is a variant haem protein, called "RHP". Both of these proteins have been isolated and procedures established for their preparation in relatively large amounts with high purity[17,18] and even in crystalline form[18]. The availability of these haem protein preparations together with advances in technology of enzyme isolation procedures makes feasible a re-investigation and extension of earlier work which was done using very crude preparations of pyridine nucleotide-oxidases[3].

In this paper we will present results of orientation experiments on partially purified enzyme systems which confirm previous suggestions[3], based on the early work with the cruder preparations, that DPNH- and TPNH-linked-RHP reductases exist which are coupled to reduction of cytochrome c_2.

MATERIALS AND METHODS

R. rubrum was grown anaerobically in 20-l bottles under conditions of continuous illumination at room temperature (approx. 30°) in the synthetic medium described by COHEN-BAZIRE, SISTROM AND STANIER[19]. After incubation for 3–4 days, the cells were harvested using a Sharples centrifuge, washed with water, lyophilized and stored at −5°.

Crystalline cytochrome *c* was prepared according to the method of NOZAKI *et al.*[20], using as starting material a horse heart muscle cytochrome *c* sample commercially available from Sigma Chemical Company, St. Louis, Mo.

To obtain a crude enzyme preparation, the lyophilized powder of light-grown cells was mixed in a Waring blendor with 0.1 *M* sodium citrate in the proportion 10 ml per gram of cells. The mixture was adjusted to pH 7 with 20 % aqueous KOH, then extracted with continuous stirring in a cold room (4–5°) for one day. To the extract, solid ammonium sulfate was added to 40 % saturation, followed by centrifugation in the cold at 18,000 rev./min for 2 h in a Servall automatic superspeed centrifuge. To the supernatant, solid ammonium sulfate was further added up to 65 % saturation. The resulting precipitate was collected by centrifugation and resuspended in 10 volumes 45 %-saturated aqueous ammonium sulfate. The suspension was centrifuged at 40,000 rev./min for 2 h in a Spinco preparative centrifuge. The resulting supernatant was supplemented with saturated aqueous ammonium sulfate to 65 % saturation. After centrifuging, the precipitate was dissolved in distilled water, then dialyzed against distilled water in a cold room for two days with several changes of outside water. The dialyzed solution (I) was lyophilized (II).

Approximately 50 mg of (II) was obtained from 10 g of the lyophilized cells used as the starting material. The lyophilized powder was stored at —5°. Just before use, distilled water was added to the lyophilized powder to make a solution containing approximately 50 mg/ml. The mixture was centrifuged at 40,000 rev./min for 2 h using a Spinco preparative centrifuge. A large amount of colorless material was precipitated by this centrifugation, and was easily removed by this procedure. The resulting clear, reddish brown supernatant was used as an enzyme preparation (III). The purification steps are summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION OF COENZYME – 2,6-DCPI REDUCTASE FROM *R. rubrum*

Purification steps	*Volume (ml)*	*Total extinction**		*Protein (mg)*	*Total enzyme units ($-\Delta E_{600\ m\mu}^{DPNH}$/min)*	*Specific activity (Enzyme units/mg protein)*
		$E_{275\ m\mu}$	$E_{256-259\ m\mu}$			
Lyophilized cells	—	—	—	50,000**	—	—
Extract	454	97,000	160,000	4,090	139	0.035
45–65% saturated ammonium sulfate (I) fraction	54	1,260	1,820	221	94	0.42
Lyophilized enzyme (II)	—	—	—	241**	87	0.36
Enzyme preparation (III)	5.4	945	1,260	151	70	0.46

* Absorption maximum at 256–259 mμ.
** Dry weight.

Absorbancies were measured at approx. 22°, using a Cary ratio-recording spectrophotometer, model 14, furnished with a potentiometer providing two absorbancy scales-0.0–1.0 and 1.0–2.0. Anaerobic spectrophotometric measurements were made with a Thunberg type cuvette. In a typical experiment, the cuvette (1-cm optical path) was first evacuated, then flushed with helium gas. This procedure was repeated until no more bubbling occurred despite vigorous shaking of the cuvette during final evacuation.

Percentage saturation of ammonium sulfate was calculated according to the table given by GREEN AND HUGHES[21] when solid salt was used to make a solution of appropriate concentration. When saturated aqueous ammonium sulfate was added, an amount of salt solution saturated at room temperature (approx. 22°) was added as calculated from simple dilution. All salting-out procedures were carried out at pH 6.5–7.0.

Protein concentration was determined by a modified biuret reaction[22] in which the influence of salt was counteracted by the use of the same concentration of the salt solution in controls.

To measure coenzyme-2,6-DCPI reductase activity, the following reaction mixture was used: an appropriate amount of enzyme preparation (see Fig. 1); 0.5 ml of 0.1 *M* buffer tris(hydroxymethyl)aminomethane-HCl, "Tris", pH 8.0, used in all assays unless indicated otherwise; 0.1 ml of 10^{-3} *M* aqueous 2,6-DCPI; 0.1 ml of 0.006 *M* reduced coenzyme (TPNH or DPNH); and water to make the total volume 1.2 ml. The reaction was started by rapid addition of the reduced coenzyme solution, and decrease in absorbancy at 600 mμ was followed at 22° in the air. Automatic

recording was started precisely 10 sec after the beginning of the reaction. Readings were made for the following 30 sec.

The enzyme activity, $-\Delta E^{DPNH}_{340\,m\mu}$/min, was expressed simply as three times the experimental readings from 20 sec to 40 sec after the beginning of reaction. One enzyme unit was defined as the amount of enzyme giving a value of 1.0 in $-\Delta E^{DPNH}_{600\,m\mu}$/min. Specific activity of the enzyme was defined as units/mg of protein.

To measure reduced coenzyme oxidase activity, the same experimental conditions as for the reduced coenzyme–2,6-DCPI reductase activity were used, except that water was added instead of the dye. The oxidation of reduced coenzyme, $-\Delta E^{DPNH}_{340\,m\mu}$/min, was assayed by change in absorbancy at 340 mμ.

The oxidation of the ascorbate-reduced horse-heart cytochrome *c* was measured according to the method of KAMEN AND VERNON[3], except that the enzyme preparation was used instead of the sonic extract.

RESULTS

Oxidation of reduced coenzymes by Preparation (III)

Both DPNH and TPNH could be oxidized by 2,6-DCPI in the presence of (III). The rate of oxidation of DPNH was an order of magnitude higher than that of TPNH, as shown in Fig. 1. The relative rates varied somewhat in different batches of (III). In the range of the enzyme concentrations used, the activities showed linear dependence with amount of the enzyme preparation used. All of the following experiments on reduced coenzyme–dye reductase activity were confined to this range of enzyme concentration.

If 2,6-DCPI was omitted from the reaction mixture, oxidation of both coenzymes could still proceed. The rates for DPNH and TPNH oxidation were roughly one-three

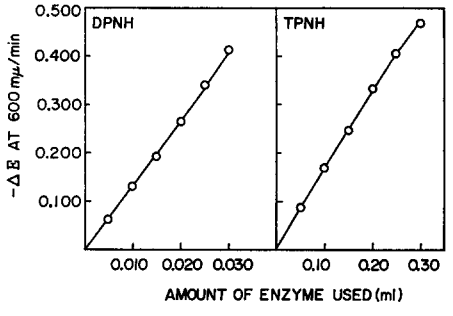


Fig. 1. Reduced coenzyme – 2,6-DCPI reductase activities of enzyme preparation III.

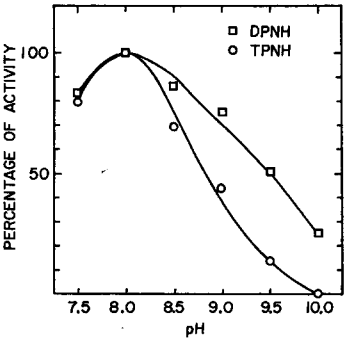


Fig. 2. Influence of pH on the reduced coenzyme – 2,6-DCPI reductase activity of enzyme preparation III. Tris buffer (0.1 *M*) was used for the pH range from 7.5 to 9.0, and glycine–NaOH buffer (0.1 *M*) for the higher pH range.

hundredth and one-fiftieth that in the presence of the dye. The activities of the enzyme preparation for the reduced coenzyme–dye reductase and the reduced coenzyme oxidase were insensitive to irradiation with light of wave lengths effective in bacterial photosynthesis.

The optimal pH of the reduced coenzyme–2,6-DCPI reductase activity was found to be 8 for both DPNH and TPNH, as shown in Fig. 2.

The marked difference in behavior of the two curves in the alkaline pH-range shown in Fig. 2, could indicate that two different enzymes were involved. This conclusion was consistent with the fact that the ratio of the two reductase activities was altered during the purification steps given in Table I.

Effect of heating on oxidation of reduced coenzymes by (III)

When (III) was heated at 50° and at pH 8 (0.08 *M* Tris buffer), the reduced coenzyme–2,6-DCPI reductase activity was first markedly stimulated, then gradually depressed, as shown in Fig. 3. After 20 min of heating, the enzyme preparation oxidized

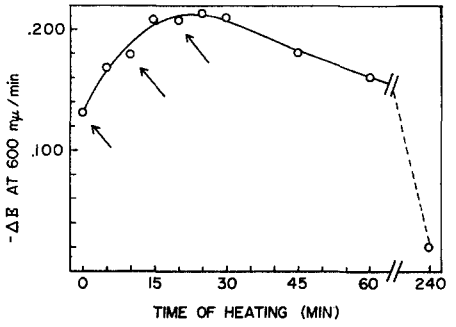


Fig. 3. Effect of heating at 50° on DPNH – 2,6-DCPI reductase activity of enzyme preparation III. One volume of III was mixed with five volumes of 0.1 *M* Tris buffer (pH 8.0), then the mixture was heated at 50°. From the mixture, aliquots were pipetted at times indicated and immediately chilled in a slurry of ice–water. The reduced coenzyme – 2,6-DCPI reductase activity was not changed when the preparation was maintained in the chilled state for one day. Hence DPNH – 2,6-DCPI reductase activity of stored aliquots could be assayed at the same time after conclusion of the 4-h heating experiment. Arrows refer to aliquots withdrawn for assay of DPNH-cytochrome c_2 reductase (see Fig. 7).

neither DPNH nor TPNH in the absence of the dye. However, this amount of heating gave maximal activation of the reduced coenzyme–dye reductase activity (see Fig. 3). No visible turbidity appeared even after 1 h of heating. The stimulation of dye reductase activity by heating varied in different preparations, increases in ratio of activity of heated to untreated preparations ranging from 50 to 90 %.

Spectrophotometric analyses

To investigate further the mutual relationship of RHP and cytochrome c_2 during the reduced coenzyme oxidations, purification was halted at a point where the enzyme system still contained endogenous RHP and cytochrome c_2 in such amounts that oxidation and reduction of these haem proteins could be spectrophotometrically assayed readily. The neutral pH indicated in the preparative procedure (Table I) was dictated by the need to avoid excessive denaturation of the oxidase system which was found to become inactivated rapidly at pH < 7. This enzyme preparation (III) contained less cytochrome c_2 than RHP. Thus, it was calculated that $4.6 \cdot 10^{-4}$ *M* RHP and $3.5 \cdot 10^{-5}$ *M* cytochrome c_2 were present, based on preliminary values of molecular extinction of RHP obtained by BARTSCH AND KAMEN[17] together with a value for the molecular extinction of the reduced cytochrome c_2, at 550 mμ, of $28 \cdot 10^6$. This result followed from the fact that RHP was more readily precipitated than cytochrome c_2, at the concentrations of ammonium sulfate used (Table II).

Before the lyophilization of the enzyme preparation (See METHODS), more than 80 % of the cytochrome c_2 present in the enzyme preparation existed in its reduced form. The lyophilization changed approximately 60–70 % of the cytochrome c_2 into its oxidized form. On the other hand, the RHP present in the enzyme preparation was always found in its oxidized form at the first step in which RHP could be spectrophotometrically detected.

When DPNH was added to (III) in air, the endogenous cytochrome c_2 was rapidly reduced, while the endogenous RHP remained in its oxidized form. The more DPNH was added to the enzyme preparation, the more reduced the cytochrome c_2 became.

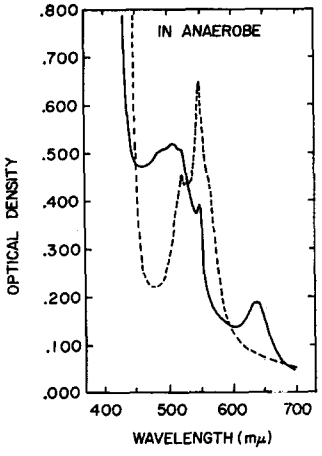


Fig. 4. Anaerobic reduction by DPNH of the RHP and cytochrome c_2 present in enzyme preparation III. A Thunberg type cuvette contained the following components: 0.30 ml of the enzyme preparation, 1.00 ml of Tris buffer (0.1 *M*, pH 8.0), 0.20 ml of 0.006 *M* DPNH and water to make the total volume 3.00 ml. DPNH was added from the side arm of the cuvette after the evacuation of the cuvette. ———, absorption spectrum of the enzyme preparation before the addition of DPNH. The reading was carried out from 700 mμ to the lower wavelengths, time required being 10 sec for the spectrophotometer to scan 10 mμ. ------, absorption spectrum of the enzyme preparation reduced by DPNH. The scanning was begun 5 min after the mixing of DPNH from the side arm. No corrections were made for concentration changes caused by addition of DPNH and evacuation of the cuvette.

If DPNH was added to (III) under anaerobic conditions, both the RHP and cytochrome c_2 were reduced to the same extent (see Fig. 4). This degree of reduction was equal to that obtained by addition of sodium dithionite in excess. The reduction of the cytochrome c_2 was much faster under anaerobic conditions, in spite of the negligible auto-oxidizability exhibited by pure cytochrome c_2. Since the normal redox-potentials of RHP and cytochrome c_2 have been reported to be approximately 0.0 and 0.3 V, respectively[4,16], this fact indicated that (III) contained at least one enzyme which could catalyze the reduction of RHP itself, and that the reduction of RHP could not have been effected to any appreciable extent *via* reduced cytochrome c_2.

Kinetics of the reduction of the RHP and cytochrome c_2 present are shown in Figs. 5-A and 5-B. It was assumed that the decrement of O.D. at 640 mμ resulted wholly from disappearance by reduction of the absorption peak at this wavelength of the oxidized RHP (Curve A in Fig. 5-A). Both RHP and cytochrome c_2 contributed

to the absorption peak at 550 mμ. Therefore, the increment of O.D. at 550 mμ was assumed to result from the reduction of both endogenous RHP and cytochrome c_2 (Curve A in Fig. 5-B). The comparison in rate between Curves A in Fig. 5-A and Fig. 5-B indicated that the cytochrome c_2 was much more rapidly reduced by DPNH than the RHP.

During this work, cytochrome c_2 and RHP were prepared in their crystalline forms from LG cells of *R. rubrum*[18]. If pure cytochrome c_2 was added to (III) under anaerobic conditions, the reduction of the RHP by DPNH showed a longer latent period than in the case of the enzyme preparation without such addition, as shown

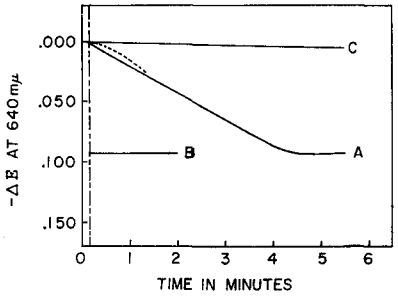


Fig. 5-A. Anaerobic reduction by DPNH of the RHP present in the enzyme preparation. Experimental conditions were the same as for Fig. 4, except that the reaction was followed at a fixed wavelength, 640 mμ. Curve A, with the enzyme preparation; Curve B, sodium dithionite added at the end of experiment traced in Curve A; Curve C, with the enzyme preparation previously heated at 50° for 10 min (see Fig. 3). For explanation of dotted line see text.

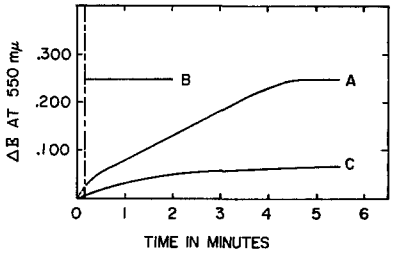


Fig. 5-B. Anaerobic reduction by DPNH of the RHP and cytochrome c_2 present in the enzyme preparation. Experimental conditions were the same as for Fig. 5-A, except that the reaction was followed at a fixed wavelength, 550 mμ. Curve A, with the enzyme preparation; Curve B, sodium dithionite added at the end of experiment traced in Curve A; Curve C, with the enzyme preparation previously heated at 50° for 10 min (see Fig. 3).

by the dotted line in Fig. 5-A. If air was admitted into the cuvette containing the enzyme preparation reduced by DPNH, the RHP was rapidly oxidized. Gentle shaking of the cuvette sufficed to completely oxidize the RHP. On the contrary, only 5–10 % of the cytochrome c_2 was oxidized despite vigorous shaking of the cuvette. It was also noted that reduced horse heart cytochrome *c* could be slowly but steadily oxidized by (III) in air.

When (III) was heated at 50° for 10 min, the endogenous RHP was reduced hardly at all by DPNH under anaerobic conditions, while cytochrome c_2 present was slowly reduced, as shown in Fig. 6. The kinetics of the reductions of both hemoproteins are shown in Curves C of Fig. 5-A and Fig. 5-B. If the initial velocities in increment of absorbance at 550 mμ were compared for (III) and for the heated enzyme preparation, both having been supplemented by addition of the crystalline oxidized cytochrome c_2, then it was found that reduction of the cytochrome c_2 by DPNH was greatly diminished in rate by the heating, as shown in Fig. 7. The same general result was observed using TPNH. Heating of (III) at 50° for 20 min inactivated the aerobic oxidation of reduced coenzymes in the absence of 2,6-DCPI and the anaerobic reduction of the RHP by the reduced coenzymes.

Further purification of coenzyme–2,6-DCPI reducing enzyme

The enzyme could be further purified by repeated fractionation with saturated

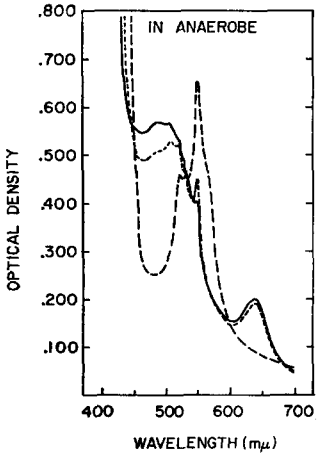


Fig. 6. Anaerobic reduction by DPNH of the cytochrome c_2 and RHP present in the enzyme preparation previously heated at 50° for 10 min. Experimental conditions were the same as for Fig. 4, except that the heated enzyme preparation was used. ———, before the addition of DPNH *in vacuo* from the side arm of the cuvette; - - - - -, after the addition of DPNH (the scanning was started 8 min after the addition of DPNH); – – – – –, sodium dithionite added in excess after termination of scanning.

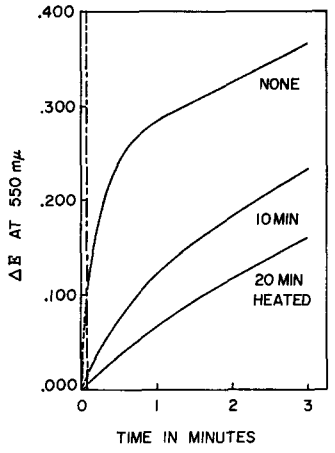


Fig. 7. Anaerobic reduction by DPNH of the cytochrome c_2 present in the enzyme preparation which was externally supplemented with cytochrome c_2. Experimental conditions were the same as for Fig. 5-B, except that $1.30 \cdot 10^{-2}$ μmoles of crystalline cytochrome c_2 in its oxidized form was externally added to the reaction mixture. Curve None, with the enzyme preparation without heating; Curve 10 min, with the enzyme preparation heated at 50° for 10 min; Curve 20 min, with the enzyme preparation heated at 50° for 20 min (see Fig. 3). It was assumed that the initial rates resulted from the reduction of cytochrome c_2 but not RHP (refer text and see the dotted line in Fig. 5 A).

aqueous ammonium sulfate, as shown in Table II. The 55–60 % saturated ammonium sulfate fraction showed the strongest reduced coenzyme–2,6-DCPI reductase activity of the four fractions obtained. This fraction contained the least amounts of RHP and cytochrome c_2. The specific enzyme activity was enriched a hundred-fold compared to that of the first extract (see Table I).

The aerobic DPNH-oxidizing activity was lost from all fractions after the ammonium sulfate fraction. However, a mixture of the 55–60 % and 78–100 % saturated ammonium sulfate fractions showed aerobic DPNH-oxidizing activity approximately 0.2 as much as the original enzyme solution, indicating the possibility that the oxidation might occur *via* endogenous RHP contained in the 78–100 % fraction. Critical experiments on this problem will be repeated when much more active preparations are available.

DISCUSSION

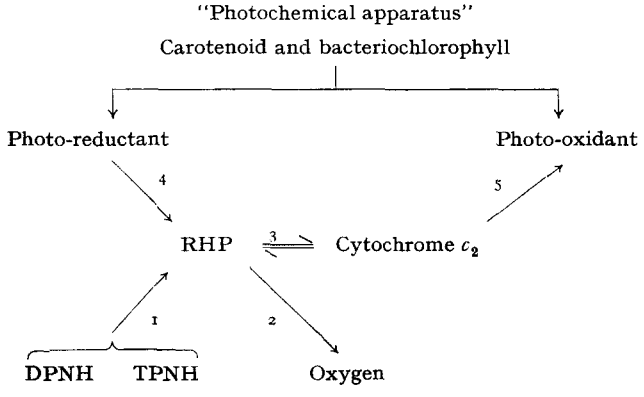
The experiments presented in this report extend previous observations relating to the light-insensitive haem protein mediated pyridine nucleotide oxidases[3]. It is seen that the diaphorase (dye reductase) activity can be differentiated from the oxidase activity solely by heat treatment. Thus, short-term heating at 50° abolishes oxidase activity whereas the diaphorase action is markedly stimulated. Further, the ability of the pyridine nucleotide oxidases to use either RHP or cytochrome c_2 as terminal

acceptors in place of oxygen is greatly diminished by the same heat treatment which abolishes the oxidase activity. The total oxidase, or haem protein reductase, activity is smaller by two orders of magnitude than the diaphorase activity so that the possibility seems very remote, although not excluded, that inactivation of oxidase causes activation of diaphorase in a strictly reciprocal relation.

The kinetics of the oxidation–reduction sequence induced in endogenous cytochrome c_2 and RHP where DPNH is added either aerobically or anaerobically, appear to support strongly the assumption that RHP is the primary H-acceptor of the haem protein reductase and that cytochrome c_2 reduction proceeds via RHP. A consequence of this assumption is construction of the *R. rubrum* pyridine nucleotide oxidase system as comprising an RHP-reductase with the rapidly auto-oxidizable RHP acting as terminal oxidase when air is present. Thus, the same scheme formulated previously from studies of crude sonicates[3] appears to hold for the partially purified soluble electron transfer system described herein.

The fact that cytochrome c_2 is very sluggishly oxidized but very rapidly reduced even under aerobic conditions confirms the observations of SMITH on crude cell-free extracts of *R. rubrum* in which it was observed that succinate oxidation proceeded much more rapidly than oxidation of cytochrome c_2 (see ref. 5). As a working hypothesis, the role of cytochrome c_2 can be taken to be that of mediating oxidation of the electron transport chain by the photo-oxidant produced during photosynthesis.

The reaction scheme which is essentially a restatement of the one discussed by KAMEN AND VERNON[3] can be presented diagrammatically as follows:



In the scheme, reaction 1 is catalyzed by reduced coenzyme–RHP reductase. Under aerobic conditions, the resulting RHP in its reduced form can be oxidized rapidly by oxygen (reaction 2). Under anaerobic conditions, the RHP reduced by the reduced coenzymes can reduce cytochrome c_2 much more rapidly than under aerobic conditions, probably non-enzymically, as in reaction 3. Cytochrome c_2 in its reduced form, if added in excess to this system, could partially reduce RHP which exists wholly in its oxidized form under aerobic conditions because of its great auto-oxiizability (reverse reaction 3). The reductant and oxidant produced by the photochemical apparatus of light-grown cells of *R. rubrum* in the light can be assumed to reduce and oxidize RHP and cytochrome c_2 by reactions 4 and 5.

Thus, RHP is accorded a key position in electron transport shunting reduction

TABLE II

FURTHER AMMONIUM SULFATE FRACTIONATION OF COENZYME – 2,6-DCPI REDUCTASE

Fractions (% saturation with Ammonium sulfate)	*Total extinction*				*Total enzyme activities*	
	E (mμ)*	$E^{ox.}_{640\ m\mu}$	$E^{red.}_{550\ m\mu}$ *RHP + cytochrome* c_2	$E^{red.**}_{550\ m\mu}$ *Cytochrome* c_2	*DPNH 2–2,6-reductase activity (units)*	*DPNH oxidase activity* $(-\Delta E^{DPNH}_{340\ m\mu}/min)$
Original enzyme solution	$1{,}074_{(256)}$	—	—	—	64.4	0.220
55–60	$32_{(270)}$	0.040	0.176	0.016	17.1***	(±)
60–78	$208_{(259)}$	0.752	2.500	0.134	2.24	(±)
78–100	$154_{(259)}$	1.554	5.952	0.306	0.68	(—)
Supernatant§	$420_{(256)}$	0.480	6.112	4.770	0.00	(—)

* These values show extinctions at u.v. wavelengths at which each fraction shows absorption maximum.

** These values were calculated from the following equation:

$$E^{red.}_{550\ m\mu\ (\text{cytochrome } c_2)} = E^{red.}_{550\ m\mu\ (\text{RHP + cytochrome } c_2)} - E^{ox.}_{640\ m\mu\ (\text{RHP})} \times 3.63$$

*** This fraction contained 4.6 mg of protein, specific enzyme activity being 3.72.

§ The enzyme solution saturated with ammonium sulfate was centrifuged in the cold at 18,000 rev./min for 30 min with the use of a Servall automatic superspeed centrifuge. Resulting supernatant had turbidity.

equivalents (electrons, or H-atoms) either through cytochrome c_2 to the photooxidant, or directly to oxygen. In this way, an enzymic basis is provided for the remarkable competitive interaction between light and dark metabolism in facultative photoheterotrophes—a phenomenon noted long ago by NAKAMURA[23] and others[24]. Alternative explanations based on inactivation of light-sensitive components have been proposed, the most recent being given by VERNON[25] in connection with his observations on light suppression of DPNH-oxidase activity in *R. rubrum* chromatophores.

The present results suffer from the limitations imposed by the relatively weak activities obtained despite the mild character of the procedures employed. It will be necessary to continue efforts to obtain preparations of greater activity before resuming systematic studies to test further the hypothesis presented regarding the role of haem proteins in the metabolism of the photoheterotrophes. Such efforts will involve use of a variety of procedures based on more care in avoidance of extremes in pH and temperature, as well as more attention to removal of salts and traces of heavy metals.

ACKNOWLEDGEMENTS

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