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SEPARATION OF RESPIRATORY REACTIONS IN *RHODOSPIRILLUM RUBRUM*: INHIBITION STUDIES WITH 2-HYDROXYDIPHENYL

J. OELZE and M. D. KAMEN*

Lehrstuhl für Mikrobiologie der Universität, Biologie II, 78 Freiburg, Schänzlestr. 9 (G.F.R.)

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SUMMARY

1. Respiration of chemotrophically and phototrophically grown *Rhodospirillum rubrum* is inhibited by 2-hydroxydiphenyl.

2. Membrane-bound NADH oxidase and NADH: cytochrome *c* reductase are inhibited also. The inhibitor constant for both reactions (K_i) is 0.075 ± 0.012 mM. NADH dehydrogenase is not inhibited significantly.

3. The inhibition of succinate:cytochrome *c* reductase is associated for chemotrophic membranes with $K_i = 0.22 \pm 0.03$ mM and for phototrophic membranes with $K_i = 0.49 \pm 0.09$ mM. Succinate dehydrogenase is not affected by 2-hydroxydiphenyl.

4. Cytochrome oxidase is inhibited only slightly.

5. While NADH-dependent reactions in both phototrophic and chemotrophic membranes are inhibited maximally more than 95 %, succinate-dependent reactions can be inhibited more than 95 % only in chemotrophic membranes. In phototrophic membranes the maximum inhibition of succinate-dependent reactions is about 70 %.

6. The type of inhibition in both cases 2 and 3 is non-competitive.

7. While the reduction of *b*-type cytochrome is inhibited by 2-hydroxydiphenyl, the degree of ubiquinone reduction is not influenced. The data suggest that the site of inhibition is localized between ubiquinone and cytochrome *b*.

8. Implications of these data for the respiratory electron transport system in *R. rubrum* are discussed.

INTRODUCTION

Electron transport from succinate, or NADH, to oxygen in chemotrophically grown *Rhodospirillum rubrum* has been claimed to proceed via specific dehydrogenases through a common pool of ubiquinone(s) and thence through a single cyto-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

* Present address: Department of Chemistry, University of Southern California, Los Angeles, Calif. 90007, U.S.A.

chrome-mediated chain [1, 2]. However, some evidence indicates that in certain *Rhodospirillaceae*, a degree of separation in pathways exists [3–5]. As reported herein, it is feasible to detect significant diversity in pathways by means of the bacteriostatic and fungistatic agent, 2-hydroxydiphenyl. This compound, like other diphenyl derivatives (e.g. diphenylamine), is an effective inhibitor of carotenoid biosynthesis [6, 7]. It also exerts marked effects on processes in *R. rubrum*, other than pigment synthesis. Thus, normal development of photosynthetically active intracytoplasmic membranes is hindered [8]. Further, cells fail to grow aerobically in the presence of 2-hydroxydiphenyl at concentrations which ailow anaerobic photosynthetic growth of the same cell suspension (unpublished observation). One may surmise from reports of respiratory inhibition by 2-hydroxydiphenyl and diphenylamine in mycobacteria and plant mitochondria [9, 10] that 2-hydroxydiphenyl can affect respiration in *R.rubrum*. Consistent with this expectation are the results presented in this paper which show not only that both NADH and succinate oxidations are inhibited, but (more significantly) that there are quantitatively different effects. These are seen by monitoring redox levels of cytochrome components, as well as by determination of inhibitor constants (K_i). Taken together with observations on redox states of the ubiquinone components, it appears that the site of inhibition is localized for both substrates between the ubiquinone pool and the *b*-type cytochromes.

MATERIALS AND METHODS

Cultivation of bacteria and membrane isolation

R. rubrum, strain S1, was grown chemotrophically (fully aerated in the dark) and phototrophically (anaerobically in the light) as described previously [11]. Bacteria were harvested from the late phase of exponential growth by centrifugation. After washing, the cells were suspended in 0.05 M phosphate buffer (pH 7.6) and homogenized twice with a cooled French pressure cell. All the following steps were performed at 4 °C. Particles in the 15 900 $\times g$ (20 min) supernatant were sedimented at 144 000 $\times g$ (60 min). The resultant crude membrane fraction was washed once in phosphate buffer and used either immediately for the determination of ubiquinone redox states or stored at −80 °C.

Enzyme assays

2-Hydroxydiphenyl and membranes were added as indicated under Results. All reactions were run at 30 °C.

NADH and succinate oxidase activities were determined polarographically with a Model 53 Biological Oxygen Monitor System (Yellow Springs Instrument Co., Yellow Springs, Ohio). Reaction mixtures contained 0.05 M phosphate buffer (pH 7.6). Reactions were initiated by addition of substrate (1 mM NADH or 4 mM sodium succinate). The following enzyme activities were determined spectrophotometrically. NADH oxidase was measured at 340 nm by the oxidation of 0.15 mM NADH [12]. Variations in the NADH molarities used are indicated in the Results. NADH dehydrogenase (EC 1.6.99.3) and succinate: 2,6-dichlorophenolindophenol reductase (EC 1.3.99.1) activities were measured by assay of 2,6-dichlorophenol (DCIP) reduction at 578 nm. The reaction mixtures contained 0.05 M phosphate buffer (pH 7.6), 2 mM KCN, 0.06 mM DCIP, 0.15 mM NADH or 6 mM sodium succinate. The

same assay system was used for succinate dehydrogenase (EC 1.3.99.1) except for the addition of 0.4 mM phenazine methosulfate, and 4 mM sodium azide instead of KCN. Activities for DCIP reduction were calculated on the basis of the extinction coefficient of 20.6 $mM^{-1} \cdot cm^{-1}$ at 578 nm. Assay mixtures for both NADH : cytochrome *c* reductase (EC 1.6.99.3) and succinate : cytochrome *c* reductase (EC 1.3.99.1) contained 0.05 M phosphate buffer (pH 7.6), 4 mM sodium azide, 0.05 mM horse heart cytochrome *c*, and, unless stated otherwise, either 0.15 mM NADH or 6 mM sodium succinate. Cytochrome reduction was measured with an Eppendorf photometer (filter Hg 546 nm). Cytochrome oxidase activity (EC 1.9.3.1) was determined by assay at 550 nm [13]. Horse heart ferrocytochrome *c* was obtained by reduction with ascorbate. Oxidized and excess ascorbate were removed by chromatography on Sephadex G-25. The final reaction mixture contained 0.025 mM ferrocytochrome *c*. Specific activities were calculated by using the extinction coefficients of 21 $mM^{-1} \cdot cm^{-1}$ (filter Hg 546 nm) and 19 $mM^{-1} \cdot cm^{-1}$ at 550 nm.

Determination of the ubiquinone redox state

Membrane-bound quinones were reduced in 0.05 M phosphate buffer (pH 7.6) by 1 mM NADH or 4 mM sodium succinate. The assays were performed in Thunberg cuvettes which were evacuated and flushed with nitrogen three times. Then the cuvettes were incubated for 15 min at 30 °C. Quinones were extracted rapidly [14]. To remove 2-hydroxydiphenyl, extracts were spotted under a stream of N_2 on silica gel plates (F254, Woelm, Eschwege, Germany) which were developed for 10 min with a mixture of methanol and water (9 : 1, v/v) under N_2. From the resultant chromatogram the quinones were removed manually by scraping, and then extracted with ethanol. The redox state was determined spectroscopically by measuring the reduced and oxidized forms [14]. The quinone fraction consisted of ubiquinone and highly autoxidizable rhodoquinone. Rhodoquinone contributed 17 % of the total absorption of oxidized quinones at 275 nm. To calculate ubiquinone redox levels total oxidized and reduced quinones were corrected by this value. Separation of ubiquinone and rhodoquinone was achieved by thin-layer chromatography [15].

Cytochrome difference spectra

Reduced minus-oxidized difference spectra of cytochromes in situ were obtained under aerobic conditions with a Cary 14 R spectrophotometer at 20 °C. Substrate concentrations were 3 mM NADH and 12 mM sodium succinate, respectively. Measurements in 0.05 mM phosphate buffer (pH 7.6) were done 3 min after the addition of NADH and 10 min after the addition of sodium succinate.

Protein determinations

Protein determinations were performed according to Lowry et al. [16].

Chemicals

Horse heart cytochrome *c*, phenazine methosulphate, and DCIP were obtained from Serva Entwicklungslabor, Heidelberg, Germany. NADH was from Boehringer, Mannheim, Germany. 2-Hydroxydiphenyl was from Fluka AG, Buchs SG, Switzerland; a highly purified sample was a generous gift from Dr B. Maudinas, Université de Nancy, France.

RESULTS

Endogenous respiration of whole cells

The distinct inhibition of chemotrophic growth observed previously (unpublished), suggests that the respiratory chain of *R. rubrum* is affected by 2-hydroxydiphenyl. The results of Table I show that there is indeed an inhibition of oxygen uptake by chemotrophically, as well as by phototrophically, grown cultures. The degree of inhibition is the same for both cultures. The values noted were obtained within a few seconds after the addition of inhibitor.

TABLE I

OXYGEN UPTAKE BY CELLS OF *R. RUBRUM*

Cells were suspended in air-saturated culture medium at a concentration of 580 μg protein per ml. Oxygen uptake was measured for 5 min without 2-hydroxydiphenyl. Then, inhibitor was added as indicated and oxygen uptake followed for 6 min.

2-Hydroxydiphenyl (mM)	% Oxygen uptake by	
	Chemotrophic cells	Phototrophic cells
0	100	100
0.059	67	70
0.29	15	13

Localization of the inhibitory site

The various sections of the respiratory electron transport chain of isolated membranes are inhibited to a different extent. Table II summarizes the effects of 0.29 mM 2-hydroxydiphenyl on reactions, as determined by optical assays. NADH oxidase and NADH : cytochrome *c* reductase are affected equally and are the most sensitive. This is true for both chemotrophic and phototrophic membranes. Succinate : cyto-

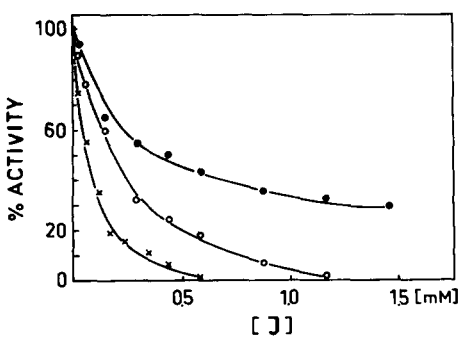

Fig. 1. Relative activities of respiratory chain reactions in the presence of different concentrations of 2-hydroxydiphenyl (J). × — ×, NADH oxidase (determined polarographically and spectroscopically) and NADH : cytochrome *c* reductase of membranes isolated from chemotrophic and phototrophic cells of *R. rubrum*. Succinate oxidase and succinate : cytochrome *c* reductase from chemotrophic (○ — ○) and phototrophic (● — ●) cells.

TABLE II

INHIBITION OF RESPIRATORY ACTIVITIES IN MEMBRANES OF *R. RUBRUM*

Membranes (about 30 μg protein per ml assay) of chemotrophically and phototrophically grown cells were incubated in the presence of 0.29 mM 2-hydroxydiphenyl. The data are presented as percent inhibition.

Source of membranes	NADH			Succinate			Cytochrome *c* oxidase
	Oxidase	DCIP dehydrogenase	Cytochrome *c* reductase	DCIP reductase	Cytochrome *c* reductase	Phenazine methosulphate/ DCIP dehydrogenase	
Phototrophic cells	88	<5	89	40	43	<5	10
Chemotrophic cells	90	<5	88	61	60	<5	10

chrome *c* reductase as well as succinate : DCIP reductase on the other hand are inhibited differently depending on the origins of membranes tested. With phototrophic membranes the inhibition is less than with chemotrophic membranes. Dehydrogenases either for NADH or succinate are not inhibited significantly. Only very slight inhibition can be observed for the terminal cytochrome oxidase.

TABLE III

OXIDATION-REDUCTION LEVELS OF UBIQUINONE IN MEMBRANES OF *R. RUBRUM*

Membranes of chemotrophic cells (3 mg/ml) were incubated anaerobically at 30 °C with either NADH (1 mM) or succinate (4 mM). The concentration of 2-hydroxydiphenyl was added as indicated. After 15 min the reactions were stopped. Correspondingly oxygen uptake was measured under aerobic conditions.

Substrate	2-Hydroxy-diphenyl (mM)	% Reduction of total ubiquinone	% Oxygen uptake
NADH	—	20	100
NADH	0.59	26	9
Succinate	—	15	100
Succinate	0.59	15	25

The degree of inhibition by various concentrations of 2-hydroxydiphenyl was measured for NADH oxidase and cytochrome *c* reductase, as well as for succinate oxidase and cytochrome *c* reductase (Fig. 1). While the percentages of inhibition fit one curve for both of the NADH-dependent reactions, regardless of the nature of the membranes, the inhibition of succinate-dependent reactions was greater with chemotrophic membranes than with phototrophic membranes. NADH-dependent reactions were inhibited more than 95 % by 0.59 mM 2-hydroxydiphenyl. Succinate-dependent

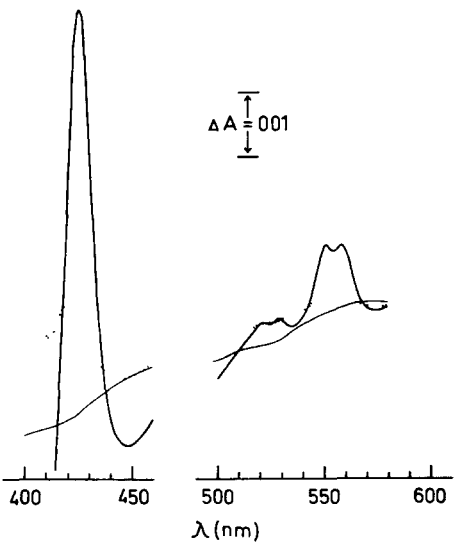

Fig. 2. Difference spectra of membranes from chemotrophically grown *R. rubrum*. NADH reduced-minus-oxidized (—); NADH reduced-minus-oxidized plus 0.59 mM 2-hydroxydiphenyl (· · ·). Measurements 3 min after NADH (3 mM) addition. Protein, 1.5 mg per ml.

reactions with chemotrophic membranes were inhibited more than 95 % by 1.18 mM 2-hydroxydiphenyl. The maximum inhibitory effect obtained with phototrophic membranes was 70 % at higher concentrations. The data presented in Table III indicate that the reduction of ubiquinone is not inhibited by 2-hydroxydiphenyl. With both NADH and succinate the same degree of reduction of ubiquinone can be achieved in the presence and absence of inhibitor. Respiration on the other hand is significantly diminished for both substrates when measured as oxygen uptake. The relatively high proportions of oxygen uptake in the presence of inhibitor result from the high membrane protein concentration of 3 mg per ml which is needed to obtain reasonably high amounts of quinone for measurement.

The ability to reduce cytochromes by either NADH (Fig. 2) or succinate (Fig. 3) has been tested by tracing reduced-minus-oxidized difference spectra. Thus it is demonstrated that neither succinate nor NADH-dependent cytochrome reductions are normal when membranes are incubated in the presence of 2-hydroxydiphenyl. The maximum at 560 nm for the *b*-type cytochrome is greatly decreased and the maximum at 551 nm for the *c*-type cytochrome is only slightly influenced by the inhibitor. The inhibitory effects also can be observed in the Soret region where an absorbance decrease and a slight shift of the absorbance maximum to shorter wavelengths is noted.

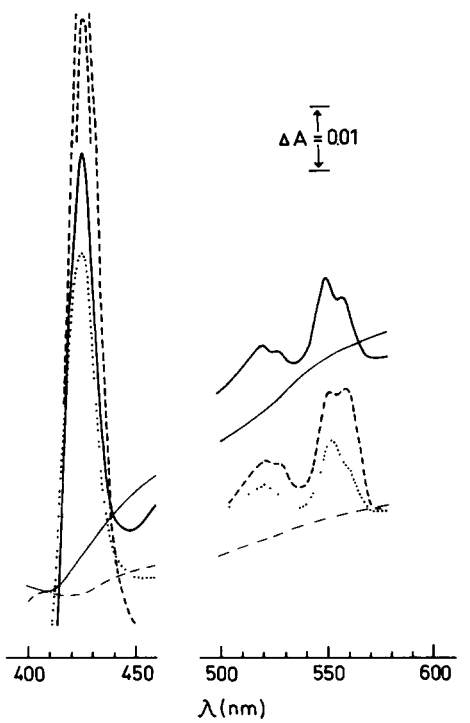

Fig. 3. Difference spectra of membranes from chemotrophically grown *R. rubrum*. Succinate reduced-minus-oxidized (—); succinate reduced-minus-oxidized plus 0.59 mM 2-hydroxydiphenyl (· · ·); dithionite reduced-minus-oxidized (- - -). Membrane protein: 1.5 mg per ml. Measurements 10 min after succinate (12 mM) addition.

Determination of inhibitor constants and of the inhibition type

The different rates of 2-hydroxydiphenyl inhibition upon various respiratory chain activities imply different inhibitor constants for NADH- and succinate-dependent reactions. The inhibitor constants and the type of inhibition were determined simultaneously according to Dixon [17]. As shown by Figs 4–6, the inhibition was of the non-competitive type for both substrates. The linearity between the inhibitor concentration and the reciprocal velocity ($1/v$) indicated a one-to-one relation between inhibitor and independent site of inhibition. The inhibitor constants were computed from the regression lines of nine independent Dixon plots. NADH oxidase and NADH cytochrome *c* reductase exhibit an identical inhibitor constant $K_i = 0.075 \pm 0.012$ mM for membranes of chemotrophic and phototrophic cells. In the case of succinate : cytochrome *c* reductase a constant $K_i = 0.22 \pm 0.03$ mM is obtained for chemotrophic membranes and $K_i = 0.49 \pm 0.09$ mM for phototrophic membranes. Since

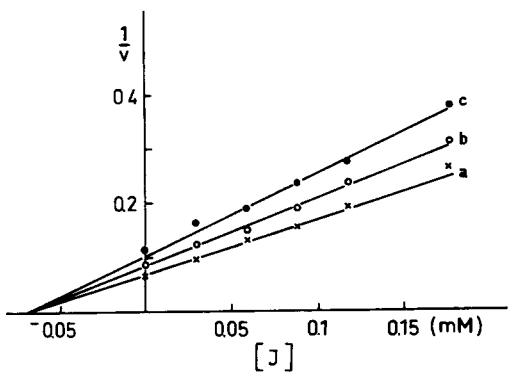

Fig. 4. Dixon plot of NADH : cytochrome *c* reductase activities in the presence of 2-hydroxydiphenyl (J). The assay contained 25 μg membrane protein per ml. Membranes were isolated from phototrophically grown cells. NADH concentrations: a, 0.06 mM; b, 0.015 mM; c, 0.0075 mM. *V*, velocity (nmol NADH oxidized per ml per 10 min).

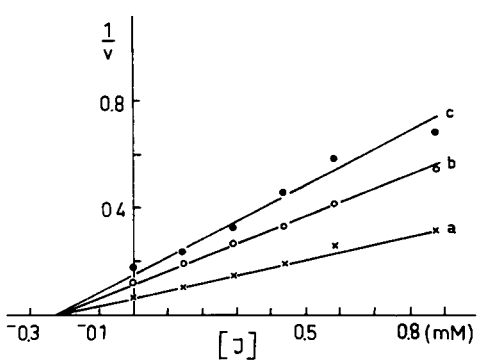

Fig. 5. Dixon plot of succinate : cytochrome *c* reductase activities in the presence of 2-hydroxydiphenyl (J). 20 μg protein of membranes isolated from chemotrophic cells were used per ml of assay. Succinate concentrations: a, 6 mM; b, 0.6 mM; c, 0.3 mM. *V*, velocity (nmol horse heart cytochrome *c* per ml per 10 min).

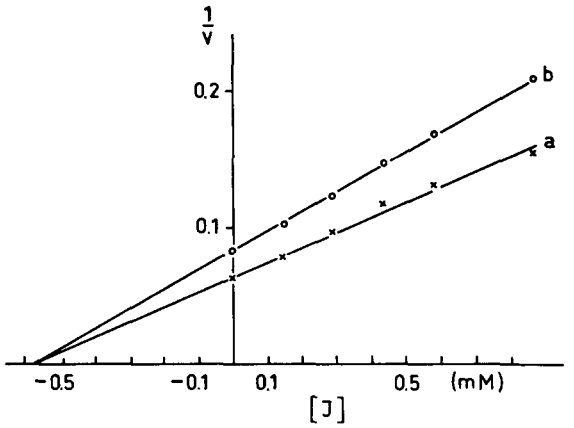

Fig. 6. Dixon plot of succinate : cytochrome *c* reductase activities in the presence of 2-hydroxydiphenyl (J). 30 μg protein of membranes isolated from phototrophically grown cells was used per ml of assay. Succinate concentrations: a, 6 mM; b, 0.6 mM.

in phototrophic membranes the highest degree of inhibition observable is 70 % (Fig. 1), half-maximum inhibition of the affectable activity (65 % of the total activity) would result at about the same concentration of 2-hydroxydiphenyl as in chemotrophic membranes.

DISCUSSION

Respiration of whole cells is inhibited by 2-hydroxydiphenyl to an identical extent regardless of the history of the cultures examined (Table I). The same is true for NADH-dependent reactions measured with isolated membranes of chemotrophically and phototrophically grown *R. rubrum* (Table II). However, succinate-dependent reactions exhibit considerable differences in the degree of inhibition when a comparison is made between chemotrophic and phototrophic membranes (Table II). These findings indicate that extrapolation from the isolated membrane systems to those operative in oxygen uptake in whole cells is better based on the properties of the NADH-dependent, rather than succinate-dependent, reactions. There is the additional suggestion that NADH oxidation and succinate oxidation do not share the same site of inhibition a rationalization consistent with the different inhibitor constants obtained for NADH and succinate, respectively. The existence of two inhibitor constants for succinate : cytochrome *c* reductase can be understood on various bases. For example, there may be hindered accessibility and less reactivity in the site of phototrophic membranes. Also, one may suppose an increased amount of essential constituents at the site of inhibition as well as the presence of an independent pathway not present in chemotrophic membranes. In this latter connection previous resutls [2] on substrate oxidation in membranes derived from phototrophic *R. rubrum* have been interpreted as arising from different pathways. Therefore the presence of an additional less-sensitive succinate oxidase pathway in phototrophic cells is a reasonable interpretation of the data. This also is supported by the observation only for phototrophic membranes that a considerable proportion (about 30 %) of succinate : cytochrome *c*

reductase and succinate oxidase activities remain uninhibited even at highest attainable concentrations of 2-hydroxydiphenyl (Fig. 1). Obviously such considerations are not relevant for the case of NADH-dependent pathways because no change in degrees of inhibition is noted in comparing chemotrophic and phototrophic systems.

The rate-limiting reaction of the respiratory chain was first proposed by Keister and Minton [18] at the level of the initial dehydrogenases. Later, however, they indicated that terminal cytochrome oxidase(s) might be rate-limiting [2]. Our data show that the inhibitor constants for NADH oxidase and NADH-cytochrome *c* reductase are identical while cytochrome oxidase is scarcely inhibited. The results show the same inhibitor concentration necessary for 50 % inhibition of both succinate oxidase (Fig. 1) and succinate : cytochrome *c* reductase (Figs 1 and 5). Thus it can be concluded that the above statements for NADH-dependent reactions are valid for succinate-dependent reactions also. Therefore the terminal oxidase cannot be the rate-limiting reaction of the respiratory chain.

The redox reactions of cytochromes are affected by the presence of inhibitor (Figs 2 and 3) whereas the redox reactions of ubiquinones are not (Table III), a result which is difficult to reconcile with any suggestion other than that the size of inhibition is localized between ubiquinone and the cytochromes. Usually, cytochrome c_2 is not included into the respiratory chain of *R. rubrum* [1, 2, 19], hence the *b*-type cytochrome only needs further discussion. While a recent proposal for the respiratory chain places cytochrome *b* prior to ubiquinone [19], the results presented here are in agreement with previous schemes which indicate that cytochrome *b* reduction is mediated by ubiquinones [1, 2]. Among the different possibilities for the action of 2-hydroxydiphenyl between ubiquinone and cytochrome *b*, it seems plausible that ubiquinone is the actual site of inhibition. The following arguments may be presented in elaboration of this suggestion. Different inhibitor constants for NADH and succinate infer that no electrons are exchanged before the site of inhibition. The quinones involved in the respiratory electron transport are postulated to exist in separate pools, as has been proposed previously for substrate oxidation in mitochondria and *Rhodopseudomonas palustris* [4, 20]. These pools can be reduced in the presence of 2-hydroxydiphenyl. If it is assumed additionally that, in the presence of inhibitor, the reduced quinone pool involved cannot be reoxidized (neither by cytochromes, nor by the adjacent quinone pool), then the existence of different inhibitor constants for NADH and succinate is a necessary consequence. As might be expected from these considerations, experiments in progress (unpublished) concerning the action of 2-hydroxydiphenyl on light-dependent electron-transport reactions have revealed that the light-driven NADH reduction by succinate is also strongly inhibited. This reaction is mediated by the two dehydrogenases and by ubiquinone, the latter being the only possible sensitive constituent.

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