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ENERGY TRANSDUCTION IN PHOTOSYNTHETIC BACTERIA

VI. RESPIRATORY SITES OF ENERGY CONSERVATION IN MEMBRANES FROM DARK-GROWN CELLS OF *RHODOPSEUDOMONAS CAPSULATA*

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SUMMARY

Membranes prepared from *Rhodopseudomonas capsulata* grown heterotrophically in the dark perform phosphorylation linked to oxidation of NADH and succinate, with $P/2e^-$ ratios of about 0.5 and 0.15, respectively. The localization of the sites of energy conservation was investigated by observing the respiration-induced quenching of the fluorescence of atebrine.

Energization of the membrane can be demonstrated when NADH is oxidized by O_2, ferricyanide or Q_1, when succinate is oxidized by O_2 or by oxidized diaminodurene, and during the oxidation of reduced diaminodurene.

Antimycin A completely inhibits energization between succinate and O_2 or succinate and diaminodurene; however, it only inhibits partially NADH or succinate oxidases and energization between NADH and O_2. KCN inhibits NADH oxidase in a biphasic way: the first level of inhibition is observed at concentrations which block the oxidation of exogenous cytochrome *c* or of diaminodurene and energization between succinate or ascorbate–diaminodurene and O_2. The second level corresponds to the inhibition of the antimycin-insensitive oxidase.

The results are interpreted as evidence of the presence in these bacteria of a respiratory chain branching after the dehydrogenase system, one arm of the chain being sensitive to antimycin A and low concentrations of KCN and capable of energy conservation, the other being represented by a completely uncoupled system.

INTRODUCTION

The respiratory system of facultative photosynthetic bacteria has received relatively little attention in comparison with the large amount of research dealing with their photosynthetic apparatus. Nevertheless aerobic metabolism can be no less important than photometabolism since these organisms are capable of high rates of growth in the dark in the presence of oxygen[1].

Our present limited knowledge on the composition and function of the respir-

atory chain of non-sulfur purple bacteria is concerned primarily with the nature of the electron transport components and their interaction with substrates[2–6]. Very little information is available so far on the localization of energy-conserving sites in the respiratory electron chain of *Athiorodaceae*. It has been demonstrated that the inhibition of cyclic phosphorylation by antimycin A in *Rhodosporillum rubrum* photosynthetic membranes can be overcome by low concentrations of phenazine methosulfate; on this basis it has been proposed that a site of energy conservation is located between an antimycin-sensitive component and reaction center bacteriochlorophyll[7]. However, this evidence could not be reproduced in *Rps. capsulata*[8]. Evidence for a second site in cyclic photophosphorylation, again in *Rh. rubrum*, has been obtained fom the demonstration of cross-over effects and from the demonstration that ATP or PP_i induce reverse electron flow reactions between a *b*-type and a *c*-type cytochrome[9]. In addition, a third site was postulated by Keister *et al.* [10] between NADH and fumarate, in order to rationalize the mechanism of energy-dependent reduction of NAD^+ by succinate. All these observations were further supported in the work by Isaev *et al.*[11], who were able to demonstrate energy conservation coupled to oxidation of NADH by oxygen or of succinate by ferricyanide or upon illumination in the presence of antimycin A and ascorbate–*N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Membranes prepared from facultative photosynthetic bacteria, grown aerobically, perform oxidative phosphorylation utilizing NADH or succinate as substrates[12,13]; however the low $P/2e^-$ ratios usually obtained in these preparations are a technical hindrance in elucidating further the sequence of redox reactions of the electron transport carriers and the associated energy-converting steps. Recently this problem has been approached in photosynthetic membranes of *Rps. rubrum* by an indirect method, namely measuring the distribution of permanent anoins as a probe of membrane energization[11]. In this study we have attempted to localize the energy coupling sites of respiration in membranes from *Rps. capsulata*, grown aerobically, by means of respiration-induced quenching of atebrine fluorescence. This phenomenon, which has been shown to be related to energization of phosphorylating membranes[14,15], has been studied by us in parallel with measurements of phosphate esterification in the presence of suitable electron flow inhibitors and electron donors and acceptors.

MATERIALS AND METHODS

Photosynthetic cultures of *Rps. capsulata*, strain St. Louis (American Type Culture Collection 23 782) were grown in the medium described by Ormerod *et al.*[16], transferred to a small flask (25 ml) containing the same medium and grown for many generations in the dark with continuous mechanical shaking. A small inoculum of these aerobic cultures was then transferred to Fernbach bottles and strongly aerated by bubbling air through the cultures and shaking with a mechanical rotatory device. After 20 h the bacteria, which appeared to be slightly pinkish, were harvested, washed once with 0.1 M glycylglycine (pH 7.2) containing 8 mM $MgCl_2$ and broken in a French pressure cell operating at 16000 lb/inch^2. The cell extract was freed of the largest debris and unbroken cells by centrifugation for 20 min at $40000 \times g$ and the membrane fraction was sedimented at $150000 \times g$ for 40 min. The membrane

fragments were suspended in the same buffer and kept in ice. All the experiments were performed within a few hours of preparation of the membranes. The degree of coupling of the system was found to be considerably decreased after 8–15 h of storage. Oxidation of NADH and reduction of ferricyanide or cytochrome *c* were measured spectrophotometrically at 340 nm, 420 nm and 550 nm, respectively, using a Cary Model 15 double beam spectrophotometer. The rates of oxidation of succinate, ascorbate–diaminodurene and ascorbate–cytochrome *c* were measured polarographycally using a Yellow Springs O_2 electrode (Model 5400). The routine reaction mixture for all these assays contained (in a volume of 2 ml for the spectrophotometric measurements and 6.8 ml for the polarographic): glycylglycine (pH 7.2), 100 mM; $MgCl_2$, 10 mM; EDTA, 1 mM; bovine serum albumin, 1 mg/ml; and particles (0.1 to 0.2 mg protein/ml). The various reactions were started by addition of NADH, 0.15 mM; sodium succinate, 20 mM; sodium ascorbate, 3 mM; oxidized mammalian cytochrome *c*, 0.012 mM; or diaminodurene, 0.12 mM. The amounts of other electron donors, electron acceptors and inhibitors are indicated for each experiment (see Results).

When necessary, anaerobic conditions were achieved by flushing the reaction mixture containing all the reagents except particles and bovine serum albumin, with N_2 for 10 min; these last two components were added from the side arm of the Thunberg cuvette after the flushing was completed. Other additions were made with microliter syringes through a rubber septum.

Phosphorylation was assayed by measuring the incorporation of $^{32}P_i$ into glucose 6-phosphate as previously described[17]. For routine experiments, when NADH or succinate were the substrates used, the procedure followed was the one described in ref. 18; otherwise experimental conditions are described under Results.

Measurements of fluorescence were made at a 90° with a filter fluorimeter[19].

Proteins were assayed by the method of Lowry *et al.*[20].

RESULTS

NADH and succinate oxidases and associated phosphorylations

Membranes prepared from *Rps. capsulata*, grown heterotrophically under the conditions described above, synthetize ATP coupled to the oxidation of NADH and succinate. Although the oxidase rates can vary considerabley in different preparations, the $P/2e^-$ ratios measured in membranes from cells harvested in the early stationary phase of growth fall between 0.4 and 0.5 for NADH and between 0.15 and 0.2 for succinate. As previously reported[18], phosphorylation linked to both substrates is completely inhibited by uncouplers and oligomycin; NADH-dependent activities are also sensitive to rotenone.

The effect of KCN on NADH and succinate oxidase activities and coupled phosphorylations is shown in Fig. 1. The rate of NADH oxidation shows a clear biphasic dependency upon KCN concentration, suggesting the presence of two different sites of action of the inhibitor. The concentrations of KCN at which the two phases are 50% inhibited are approximately 5.10^{-6} and 6.10^{-4} M, respectively. By contrast, the inhibition of succinate oxidation appears to be monophasic with an apparent K_i of approximately $5 \cdot 10^{-5}$ M.

Phosphorylation rates show similar patterns of inhibition: the NADH-

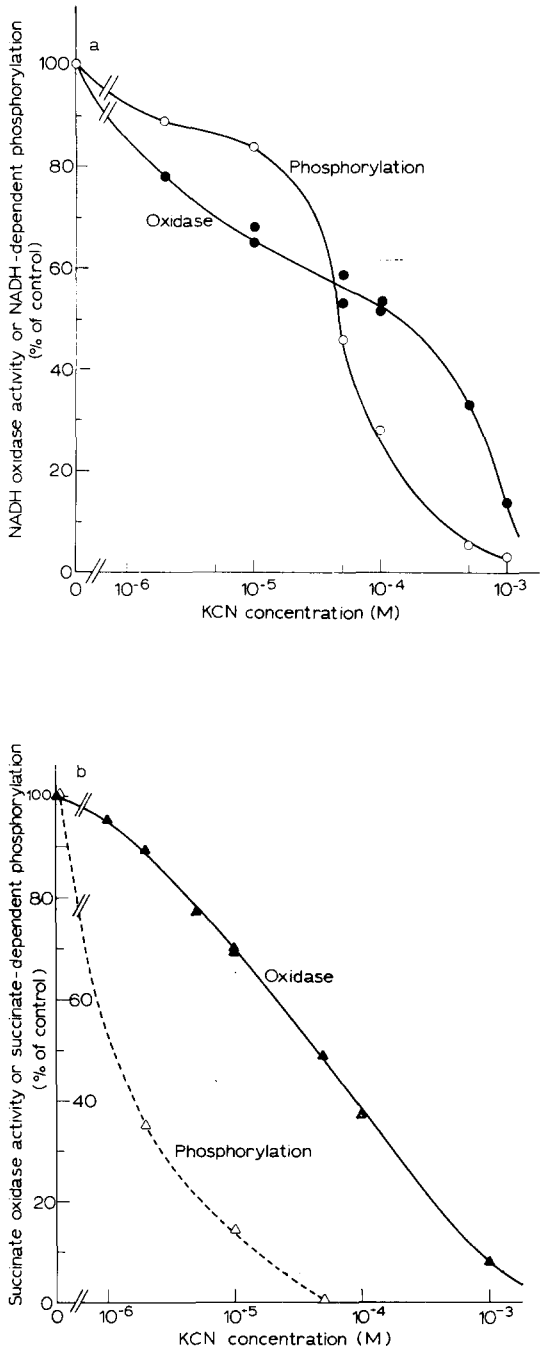

Fig. 1. Profile of NADH (a) or succinate (b) oxidases and related phosphorylation activities as a function of KCN concentration. NADH and succinate oxidases, in the absence of inhibitor, were 13.9 and 4.5 μmoles/h per mg protein, respectively; under similar conditions NADH- or succinate-dependent oxidative phosphorylation were 6.4 and 1.3 μmoles/h per mg protein.

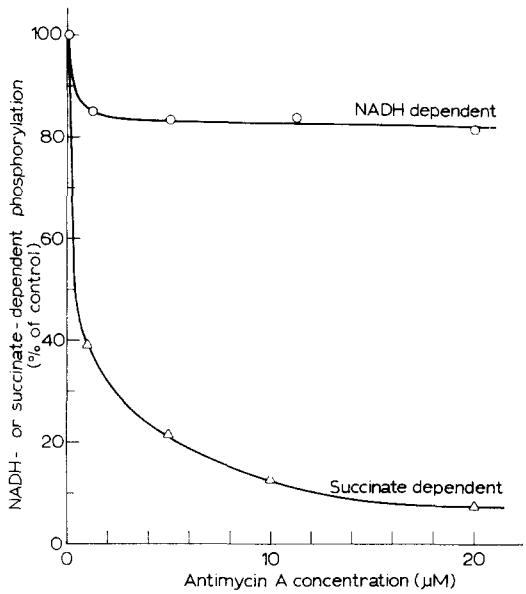

Fig. 2. Effect of antimycin A on NADH or succinate-linked oxidative phosphorylation. Control activities were 7.6 μmoles/h per mg protein for NADH-dependent phosphorylation and 1.4 μmoles/h per mg protein for succinate-dependent phosphorylation.

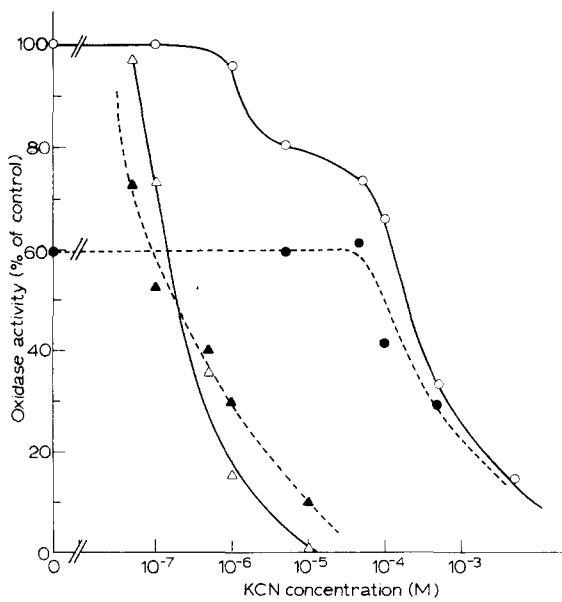

Fig. 3. Inhibition by KCN of NADH (○—○), ascorbate–cytochrome *c* (▲- - -▲), and ascorbate–diaminodurene (△—△) oxidases as a function of inhibitor concentration. Control activities were: 36 μmoles/h per mg protein for NADH oxidase; 8.7 μmoles/h per mg protein and 24 μmoles/h per mg protein for ascorbate–cytochrome *c* and ascorbate–diaminodurene oxidases, respectively. The curve indicated as ●—● refers to the rate of NADH oxidase in presence of antimycin A (20 μM).

dependent activity is rather insensitive to KCN at concentrations below 10^{-5} M, whereas the succinate dependent one is 70% inhibited at this concentration.

Oxidative phosphorylation shows similar difference in sensitivity to antimycin A (Fig. 2): in the presence of the inhibitor the maximum inhibition of NADH-dependent phosphorylation is only 18% of the control (ocasionally inhibition reaches values of 30%), while at the same concentration phosphorylation coupled to succinate oxidation is nearly completely inhibited. In agreement with the results previously reported with respiratory membranes from *Rsp. rubrum*[2] and *Rps. capsulata*, strain Kb1 (ref. 4), both NADH and succinate oxidases are only inhibited maximally between 50 and 60% by antimycin A.

These observations together with the results described above may be interpreted as evidence fo two pathways of oxygen consumption. Indeed it can be shown (Fig. 3) that the residual NADH oxidase activity measured in the presence of antimycin A is completely insensitive to KCN concentrations lower than $5 \cdot 10^{-5}$ M. However KCN was extremely effective in inhibiting the oxidation of substrates with more positive potentials such as sodium ascorbate *plus* mammalian cytochrome *c* or sodium ascorbate *plus* diaminodurene: these oxidases were 50% inhibited at 10^{-7} M and $5 \cdot 10^{-7}$ M, respectively.

Oxidation of NADH and succinate by different electron acceptors

The data presented in Table I show the rates of NADH and succinate oxidations by O_2 or, in anaerobiosis, in the presence of different exogenous electron acceptors and their sensitivity to antimycin A and rotenone. The results confirm and extend the data previously published by Klemme *et al.*[4]., with membrane preparations

TABLE I

EFFECT OF ANTIMYCIN A AND ROTENONE ON VARIOUS REACTIONS CATALYZED BY RESPIRATORY MEMBRANES FROM *RPS. CAPSULATA*

The measurements were performed as described under Materials and Methods. The concentrations of the various electron acceptors used were; ferricyanide, 2.5 mM; equine cytochrome *c*, 0.027 mM; Q_1, 0.05 mM; sodium fumarate, 3 mM. The activities are expressed as μmoles of substrate oxidized/h per mg of protein

Electron donor	*Electron acceptor*	*Activity (μmoles/h per mg)*	*Plus antimycin A (10 μM)*		*Plus rotenone (8 μM)*	
			Activity (μmoles/h per mg)	%	*Activity (μmoles/h per mg)*	%
NADH	O_2	13.9	6.9	50	0.4	2.9
NADH*	Ferricyanide	109	114	104	75	68.5
NADH**	Cytochrome *c*	5.9	0.8	13.6	0.3	5.1
NADH*	O_1	14.3	15.5	110	2.9	20.3
NADH*	Fumarate	0.08	Not inhibited		Inhibited	
Succinate	O_2	4.5	2.6	58	–	—
Succinate*	Ferricyanide	10.8	3.2	30	—	—
Succinate**	Cytochrome *c*	1.7	0.25	14.7	—	—

* Activities measured in anaerobiosis.

** Activities measured in the presence of $4 \cdot 10^{-3}$ M KCN.

from *Rps. capsulata*, strain Kb1. All NADH-dependent reactions are sensitive to rotenone though to different degrees; NADH–ferricyanide and NADH–Q_1 reductases are inhibited by 31 and 80%, respectively. Antimycin A is generally less effective but exerts a more selective action: this inhibitor does not affect the oxidation of NADH by "Site I" electron acceptors, like Q_1 and fumarate, but drastically inhibits the reduction of exogenous cytochrome *c*.

Succinate oxidation both by ferricyanide and cytochrome *c* is strongly inhibited by antimycin A (70 and 85%, respectively) but an antimycin A-insensitive pathway is responsible for about 50% of the overall oxidation rate present with both NADH and succinate oxidases.

Membrane energization by partial reactions of respiration

The study of the relationship between different partial reactions of the respiratory chain (Table I) and energization of the membranes was accomplished using atebrine as a probe for the high energy state. It has indeed been demonstrated that the fluorescence of this dye is largely quenched in the presence of energized vesicular phosphorylating systems[14,15]. More specifically it has been suggested that the quenching is related to an active uptake of this diamine into the inner compartment of the vesicle, following acidification of this phase. For this reason the quenching of certain fluorescent amines (with a pK higher than 10) has been proposed as a tool for a direct measurement of the transmembrane pH difference[21].

Recent experiments have demonstrated that in a liposome system[22] this model holds quantitatively for the monoamine 9-aminoacridine (pK=10), but only qualitatively for the diamine atebrine (pK_1=7.9; pK_2=10.5; see ref. 21), but we have chosen atebrine as a probe in this study because of its faster response and

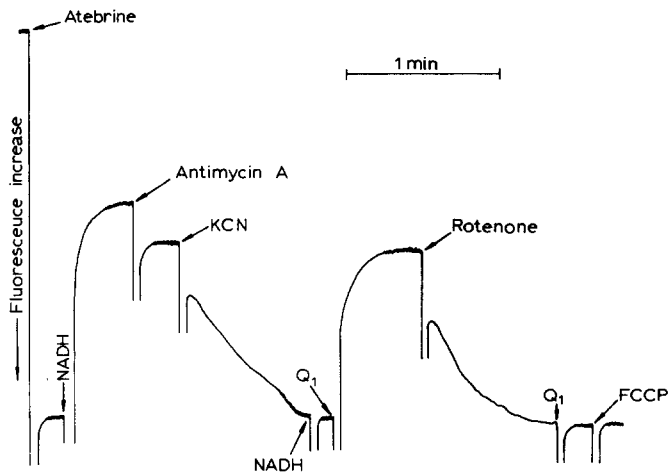

Fig. 4. Quenching of the fluorescence of atebrine induced by oxidation of NADH by oxygen or by an artificial electron acceptor (Q_1). The assay contained, in a final volume of 2.5 ml: glycylglycine (pH 8.0), 100 μmoles; $MgCl_2$, 12.5 μmoles; KCl, 250 μmoles; bovine serum albumin, 1.25 mg; EDTA, 2.5 μmoles; valinomycin, 4 μg; atebrine, 10 nmoles and respiratory membranes corresponding to 1 mg of protein. The following additions were made: NADH, 0.5 μmole; KCN, 1 μmole; Q_1, 0.1 μmole; rotenone, 20 nmoles; antimycin A, 25 nmoles; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 25 nmoles.

higher sensitivity to small pH differences. However all the results presented below can also be obtained using 9-aminoacridine. Valinomycin and KCl, which have been shown to enhance the rate and extent of atebrine fluorescence quenching, were used throughout these experiments.

The effect of different inhibitors and electron acceptors on the quenching of atebrine fluorescence induced by the oxidation of NADH is shown in Figs 4 and 5. Addition of NADH to an aerobic suspension of respiratory membranes of *Rps. capsulata* (Fig. 4) causes a decrease in fluorescence which is only slightly reversed by antimycin A; $4 \cdot 10^{-4}$ M KCN completely abolishes the quenching. If at this point an artificial electron acceptor such as Q_1 is added, a decrease in fluorescence is observed which is completely sensitive to rotenone.

The experiments illustrated in Fig. 5 indicate that in the presence of KCN the oxidation of NADH by ferricyanide is also able to induce a considerable quenching of the fluorescence of the dye. The energization is inhibited by rotenone, but is insensitive to antimycin A. Since we have shown that the oxidation of NADH by Q_1 or ferricyanide is not completely sensitive to rotenone, it must be concluded that these dehydrogenase reactions proceed through multiple pathways which are not all equivalent in energy conservation.

From this first set of results it appears clear that the oxidation of NADH by

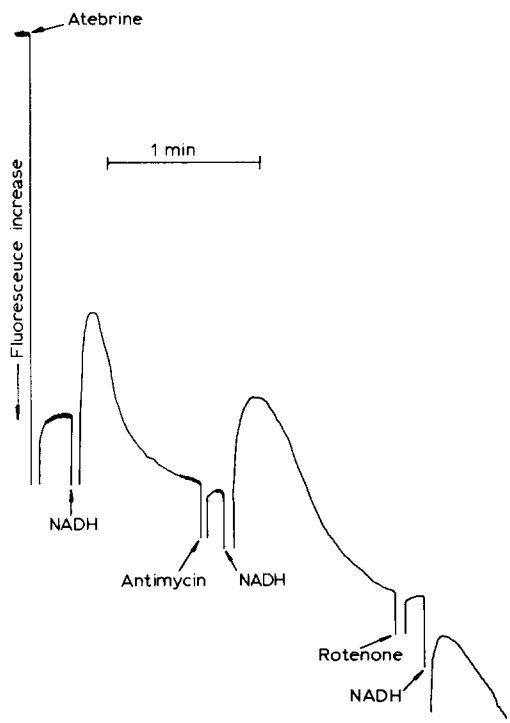

Fig. 5. Membrane energizatior dependent on oxidation of NADH by ferricyanide. Conditions as in Fig. 4, except that the assay contained 5 μmoles of potassium ferricyanide and 1 μmole of KCN before the addition of atebrine. The shifting of the baseline is due to the reduction of ferricyanide and the resulting decrease in absorbance of the excitation light.

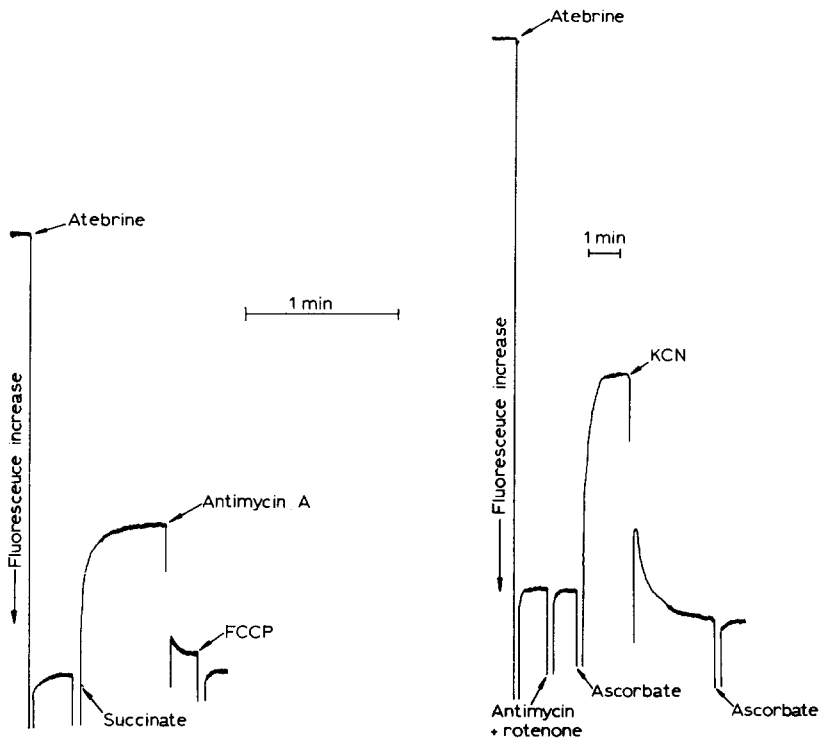

Fig. 6. Quenching of atebrine fluorescence revealing energization of the membrane linked to oxidation of succinate. Conditions as in Fig. 4; sodium succinate added, 20 μmoles.

Fig. 7. Quenching of atebrine fluorescence revealing energization of the membrane depending on oxidation of ascorbate–diaminodurene. Conditions as in Fig. 4 except that diaminodurene (0.1 μmole) was already present in the assay mixture before the addition of atebrine. The amount of sodium ascorbate added was 2.5 μmoles.

O_2 or, in the presence of KCN, by Q_1 or ferricyanide leads to energization of the membrane and that this state is easily revealed by the use of atebrine.

The effect of the addition of succinate on the fluorescence of atebrine is shown in Fig. 6. The quenching induced by addition of succinate to an aerobic suspension of membranes is smaller than that obtained in the presence of NADH and is completely reversed by antimycin A. These results agree with those obtained by measuring directly the rates of NADH- or succinate-dependent oxidative phosphorylation in the presence or absence of this electron transport inhibitor.

Using the same technique we also studied the response to other electron donors of a more positive oxidation reduction potential. In the presence of antimycin A and rotenone the ascorbate–diaminodurene couple induces a significant decrease in fluorescence (Fig. 7); the reversal of the quenching by KCN indicates a locus of membrane energization between this electron donor system and oxygen.

Though the effects of antimycin A on the energization linked to oxidation of succinate or ascorbate–diaminodurene clearly differentiate between the site of entry of electrons from these two-electron donors into the respiratory chain, it is possible

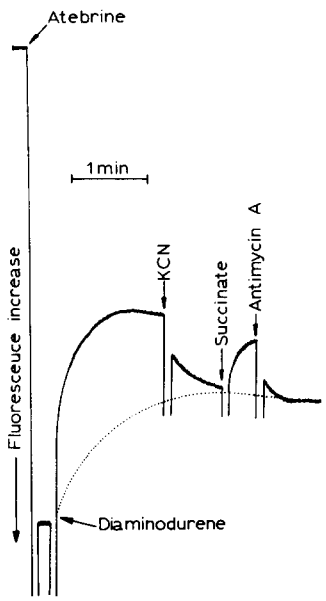

Fig. 8. Energization of the membrane depending on oxidation of reduced diaminodurene by oxygen and reduction of oxidized diaminodurene by succinate. The assay contained respiratory particles corresponding to 1.5 mg protein. The following additions were made: diaminodurene, 1 μmole; KCN, 1 μmole; succinate, 5 μmoles; antimycin A, 20 nmoles. The displacement of the baseline is due to the increase in absorbance of the excitation light following oxidation of reduced diaminodurene; this phenomenon is resolved from the true quenching of fluorescence due to energization of the membrane, if the latter is eliminated by the addition of 20 nmoles of gramicidin (dotted line).

in principle, that in both cases the effect observed is related to the same energy converting site. It is unlikely that the effects of ascorbate–diaminodurene are due to a direct interaction of ascorbate with the electron transport chain because substrate amounts of diaminodurene ($E'_0 = +0.245$ V) alone are capable of inducing a measurable energization, which is sensitive to KCN (Fig. 8). Moreover the presence of two different coupling sites between succinate and O_2 was confirmed by the following experiment: A substrate amount of reduced diaminodurene was extensively oxidized by respiratory membranes (in an energy yielding process) and the reaction was blocked by the addition of KCN (4.10^{-4} M), which at this concentration completely inhibits the coupled oxidation of succinate (Fig. 1b). At this stage, addition of succinate was able to induce a second energization cycle which was now completely inhibited by antimycin A. Thus diaminodurene can act both as an electron donor in the reduced form and an electron acceptor from succinate in the oxidated state and these two reactions are both coupled to energization.

Oxidative phosphorylation by segments of the respiratory chain

We have been able to show that partial oxidation reactions of the respiratory chain which can energize the membrane are also able to drive ATP synthesis (Table II). Incorporation of $^{32}P_i$ into ATP could be demonstrated when an anaerobic suspension of membranes was supplemented with NADH and Q_1; the reaction was inhibited by oligomycin and uncouplers.

TABLE II

TYPICAL OXIDASE AND PHOSPHORYLATION ACTIVITIES OF RESPIRATORY MEMBRANES FROM *RPS. CAPSULATA*

The experimental conditions used for measuring NADH- and succinate-dependent activities were described under Materials and Methods. The measurement of NADH–Q_1 reductase and linked phosphorylation were performed in anaerobic Thunberg cuvettes as follows: a reaction mixture containing all the components, except NADH, carrier-free $^{32}P_i$ and Q_1 was made anaerobic by flushing with nitrogen. In order to avoid any incorporation of $^{32}P_i$ due to oxidation of NADH by traces of oxygen still present in the reaction mixture, NADH was added first to the measuring cuvette, followed by addition of carrier-free $^{32}P_i$. The reaction was started by adding Q_1 (0.1 mM) and the oxidation of NADH was monitored spectrophotometrically; 0.1 ml of cold 25% trichloroacetic acid was added to both reference and measuring cuvettes after all Q_1 was reduced. The values presented for ascorbate–diaminodurene and ascorbate–cytochrome *c* oxidases and associated phosphorylations were obtained by measuring both activities directly in the oxygen electrode chamber. All components, including diaminodurene or cytochrome *c*, were already present before the reaction was started by the addition of ascorbate; a few seconds after starting the reaction, carrier-free $^{32}P_i$ was added and the reaction was allowed to reach the complete exhaustion of oxygen. A 2-ml sample was then added to precooled centrifuge tubes containing 0.2 ml of 25% trichloroacetic acid. All measurements were run in duplicate; control experiments in the presence of oligomycin or in the absence of the substrate were also performed. Special care was also used for the extraction of the inorganic phosphate: after addition of carrier glucose 6-phosphate (0.3 mM) the phosphomolybdate complex was extracted twice with isobutanol–benzene (1:1 by vol.) saturated with water followed by two other extractions, one with isobutanol and one with ethyl ether.

Electron donor	*Electron acceptor*	*Oxidase rate (μmoles/h per mg)*	*Substrate oxidized (nmoles)*	*ATP formed (nmoles)*	*P/2e⁻*
NADH	O_2	26.7	467	228	0.46
Succinate	O_2	9.4	363	55	0.15
Diaminodurene–ascorbate	O_2	20.9	2680*	157	0.06
NADH	Q_1	22.3	225	53	0.23
Cytochrome *c*–ascorbate	O_2	6.45	3360*	154	0.05

* Measured as natoms of oxygen consumed.

Similarly, phosphorylation coupled to oxidation of ascorbate–diaminodurene could be obtained and the incorporation was again inhibited by oligomycin and uncouplers, but was completely insensitive to antimicyin A and rotenone. Synthesis of ATP, which was insensitive to antimycin A, was also obtained by oxidation of ascorbate *plus* mammalian cytochrome *c* (compare ref. 4, Table V). This reaction represents another and more direct demonstration of energy conservation linked to cytochrome oxidase. Energy conservation at this site could not be confirmed using atebrine because the Soret band of cytochrome *c* overlapped the atebrine absorption spectrum too heavily.

The experimental $P/2e^-$ ratios obtained with the different oxidative phosphorylating systems are summarized in Table II; the numerical values indicated are typical of those consistently obtained with NADH or succinate dependent phosphorylations, but are merely indicative for the other systems due to the low $P/2e^-$ ratios obtained. It should be noted however that the experimental $P/2e^-$ values associated with the oxidation of ascorbate–diaminodurene are consistently lower

than that measured with succinate in spite of the higher rates of oxidation of the ascorbate–diaminodurene couple.

DISCUSSION

All the results described above have been obtained in membrane preparations from *Rps. capsulata*, strain St. Louis, grown heterotrophically in the dark under the conditions described; at the present we do not know if these properties hold also for photosynthetically grown cells since no data are available. Therefore, the following discussion will be primarily concerned with the properties of the respiratory system in cells grown aerobically and will not attemtp to consider the interrelationship between the photosynthetic and respiratory electron transport chain.

Before discussing the sites of energy conservation it is necessary to comment briefly about the effect of antimycin A and KCN on the different oxidase activities.

Antimycin A inhibits succinate or NADH oxidases by only about 50%. It almost completely blocks cytochrome *c* reduction by either NADH or succinate in the presence of $4 \cdot 10^{-3}$ M KCN. The inhibition of the NADH oxidase by KCN is biphasic in the concentration range between 10^{-6} and $5 \cdot 10^{-3}$ M. The first phase of the KCN inhibition is saturated at concentrations comparable to those which completely inhibit oxidation of ascorbate–cytochrome *c* or ascorbate–diaminodurene. The second phase corresponds to the inhibition of that portion of the NADH oxidase activity which is insensitive to antimycin A.

Succinate oxidation is also inhibited by KCN concentrations ranging from 10^{-6} and $5 \cdot 10^{-3}$ M but no biphasicity is clearly observable. Finally the presence of a slow but definite rate of reduction of fumarate by NADH confirms that the two dehydrogenase systems must interact through some common component.

The simplest way of explaining these results is to assume that NADH and succinate oxidation proceed to oxygen through a branched pathway, with the branch point situated on the substrate side of the antimycin A block. According to this model, cytochrome *c* only donates or accepts electrons after the site of antimycin A inhibition on the pathway which is sensitive to low concentrations of KCN. The other arm of the branched chain, assumed to be that which is blocked by high concentrations of cyanide, may be merely the result of an autooxidizable component of the respiratory chain.

On the basis of this scheme the energization data are readily interpreted. Energization linked to NADH oxidation, which is also largely present when antimycin A or low concentrations of KCN are added, must be located at least partially before the antimycin A-sensitive site. The demonstration of energization associated with NADH oxidation by Q_1, or ferricyanide which is insensitive to KCN or antimycin A, can be considered as direct evidence for this site.

On the other hand the greater sensitivity to antimycin A and cyanide of succinate-linked energization indicates that all the energy-conserving steps coupled to the oxidation of this substrate must involve electron flow through a pathway which contains the sites of action of these two inhibitors. The exact number of these sites is difficult to assess. The experimentally determined $P/2e^-$ ratios for succinate oxidation are always higher than those observed for the oxidation of ascorbate–diaminodurene or ascorbate–cytochrome *c*. An estimation of the number

of coupling sites on this basis cannot be very accurate, since the experimental $P/2e^-$ ratios can depend on the rate of oxidation[23]. It should be noted, however, that the rate of succinate oxidation is always considerably lower than that of cytochrome *c* or diaminodurene oxidases. It appears therefore that there is more than one coupling site linked to oxidation of succinate.

Oxidation of the couples ascorbate–diaminodurene or ascorbate–cytochrome *c* induces an energization of the membrane which is insensitive to antimycin A in contrast to the energization dependent upon succinate oxidation; this energization was also confirmed by direct measurements of $^{32}P_i$ incorporation both using ascorbate–diaminodurene or ascorbate–cytochrome *c* as electron donors. In addition we have shown that it is possible to obtain an energy-dependent quenching of atebrine fluorescence by adding substrate amounts of reduced diaminodurene, which, once oxidized, can act as an electron acceptor from succinate in an energy-yielding reaction. These two reactions which are sensitive to KCN and antimycin A, respectively, possibly represent a direct demonstration of the presence of two coupling sites.

The present study has shown that the measurement of atebrine fluorescence during energization can be used to localize energy coupling sites in preparations of phosphorylating membranes characterized by low $P/2e^-$ ratios. In addition this study demonstrates that, if the proposed model for the mechanism of the quenching is correct[21], a good correlation can be demonstrated between proton translocation and phosphorylation in every site of energy conservation of aerobic bacterial membranes, as required by the chemioosmotic coupling hypothesis.

In summary, the picture that can be drawn from these observations bears considerable similarity to that of the electron transport chain of mammalian mitochondria, with in addition a very active non-phosphorylating pathway insensitive to antimycin A and sensitive to high concentrations of KCN. The presence of branched chains has been proposed also for other respiratory systems[24–26].

Our conclusions partially disagree with the proposal by Klemme and Schegel[4] that ATP formation is linked only to succinate oxidation. It has to be noted however that in their studies a different strain of *Rps. capsulata* (Kb1) was used, along with a more disruptive procedure for breaking the cells, *i.e.* sonication.

It is not possible to suggest a physiological role for the non-phosphorylating pathway at present. The antimycin A-insensitive oxygen consumption could be an expression of a second, non-phosphorylating terminal oxidase or, as suggested above, due to the autooxidizability of some electron carrier. In either case, the presence of this pathway would explain the low $P/2e^-$ ratios routinely observed in these preparations. In this connection it should be recalled that in intact photosynthetic cells of *Rsp. rubrum*, respiration can be totally inhibited by light and the inhibition be reversed by uncouplers[27]. Though the nature of the endogenous substrate of respiration is unknown, these data may be interpreted as showing that "*in vivo*" only a phosphorylating respiratory chain exists.

After this manuscript was completed we became aware (H. Gest, personal communication) that a number of respiratory mutants of *Rps. capsulata* have been isolated by Marrs and Gest[28]. The scheme we have proposed above agrees remarkable well with the characteristics of growth and the properties of respiration observed in these mutants. However, it was observed that mutants with a lesion on the final

cytochrome *c* oxidase are still capable of aerobic growth on a medium containing malate. This observation suggests that an alternative pathway of respiration, possibly identical with the antimycin A-insensitive pathway described in this paper is operative also "*in vivo*" at least under the extreme conditions existing in these mutants.

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REFERENCES

1 Horio, T. and Kamen, M. D. (1970) *Ann. Rev. Microbiol.* 24, 409
2 Taniguchi, S. and Kamen, M. D. (1965) *Biochim. Biophys. Acta* 96, 395–428
3 Thore, A., Keister, D. L. and San Pietro, A. (1969) *Arch. Mikrobiol.* 67, 378–396
4 Klemme, J.-H. and Schlegel, H. G. (1969) *Arch. Mikrobiol.* 68, 326–354
5 Whale, F. R. and Jones, O. T. G. (1970) *Biochim. Biophys. Acta* 223, 146–157
6 Sasaki, T., Motokawa, Y. and Kikuchi, G. (1970) *Biochim. Biophys. Acta* 197, 284–291
7 Baltscheffsky, H. and Ardwidsson, B. (1962) *Biochim. Biophys. Acta* 65, 425–428
8 Klemme, J.-H. and Schlegel, H. G. (1968) *Arch. Mikrobiol.* 63, 154–169
9 Baltscheffsky, M. (1969) *Arch. Biochem. Biophys.* 121, 46–53
10 Keister, D. L., Yike, N. J. (1967) *Arch. Biochem. Biophys.* 121, 415–422
11 Isaev, A. A., Liberman, E. A., Samilov, V. D., Skulachev, V. P. and Tsofina, T. M. (1970) *Biochim. Biophys. Acta* 216, 22–29
12 Geller, D. M. (1962) *J. Biol. Chem.* 237, 2947–2954
13 Yamashita, J., Yoshimura, S., Matuo, Y. and Horio T. (1967) *Biochim. Biophys. Acta* 143, 154–172
14 Kraayenhof, R. (1970) *FEBS Lett.* 6, 161–165
15 Azzi, A., Fabbro, A., Santato, M. and Gherardini, P. L. (1971) *Eur. J. Biochem.* 21, 404–410
16 Ormerod, J. G., Ormerod, K. S. and Gest, H. (1961) *Arch. Biochem. Biophys.* 94, 449–463
17 Baccarini Melandri, A., Gest, H. and San Pietro, A. (1970) *J. Biol. Chem.* 245, 1224–1226
18 Melandri, B. A., Baccarini Melandri, A., San Pietro, A. and Gest, H. (1971) *Science* 174, 514–516
19 Melandri, B. A., Baccarini Melandri, A., Crofts, A. R. and Cogdell, R. J. (1972) *FEBS Lett.* 24, 141–145
20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
21 Schuldiner, S., Rottemberg, H. and Avron, M. (1972) *Eur. J. Biochem.* 25, 64–70
22 Deamer, W. D., Prince, R. C. and Crofts, A. R. (1972) *Biochim. Biophys. Acta* 274, 323–335
23 Tsou, C. A. and van Dam, K. (1969) *Biochim. Biophys. Acta* 172, 174–176
24 Jones, C. W. and Redfearn, E. R. (1967) *Biochim. Biophys. Acta* 143, 340–353
25 Bendall, D. S. and Bonner, W. D. (1971) *Plant Physiol.* 47, 236–245
26 Lambowitz, A. M., Smith, E. W. and Slayman, C. W. (1972) *J. Biol. Chem.* 247, 4850–4858
27 Oelze J. (1972) in *Proc. 2nd Int. Congr. on Photosynthesis Research, Stresa, 1971* (Forti, G., Avron, M. and Melandri, B. A., eds), Vol. I, pp. 649–654, Dr W. Junk N. V. Publishers, The Hague
28 Marrs, B. and Gest, H. (1973) *J. Bacteriol.* 114, 1045–1051