BBA 46027

ENERGY COUPLING IN *HYDROGENOMONAS EUTROPHA*

M. ISHAQUE AND M. I. H. ALEEM

Thomas Hunt Morgan School of Biological Sciences, Department of Microbiology, University of Kentucky, Lexington, Ky. (U.S.A.)

(Received June 29th, 1970)

SUMMARY

1. Cell-free extracts from autotrophically grown *Hydrogenomonas eutropha* catalyzed oxidative phosphorylation yielding P/O ratios of approx. 2.0 with H_2 or NADH, 1.0 with succinate and 0.6 with ascorbate as the electron donors.

2. The phosphorylation coupled to the oxidation of H_2 or NADH was inhibited by the flavoprotein inhibitors such as rotenone, amytal and atabrine; these inhibitors had little effect on the phosphorylation coupled to succinate oxidation.

3. With H_2, NADH and succinate as electron donors, a marked inhibition of phosphorylation was caused by antimycin A or 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). The phosphorylation coupled to ascorbate oxidation, however, was not affected by either the flavoprotein inhibitors or by antimycin A and HQNO. Although cyanide or azide had little or no effect on the oxidation of ascorbate, the coupled phosphorylation was strongly inhibited.

4. The oxidative phosphorylation with H_2, NADH, succinate and ascorbate as electron donors was inhibited by various uncouplers such as 2,4-dinitrophenol, 2,6-dibromophenol, pentachlorophenol, *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) and dicumarol without any appreciable inhibition of the O_2 consumption.

5. The cell-free extracts also catalyzed a succinate- or ascorbate-linked ATP-dependent reversal of electron transfer from ferrocytochrome *c* to NAD^+ and this process was inhibited by the flavoprotein inhibitors, antimycin A or HQNO and 2,3-dimercaptopropanol. The reverse electron flow was markedly sensitive to oligomycin as well as to various uncouplers of oxidative phosphorylation.

INTRODUCTION

The process of oxidative phosphorylation has been demonstrated in chemosynthetic bacteria belonging to the genera Nitrobacter[1-4] and Thiobacillus[5-7]. However, little information is available concerning the mechanism of electron transport and coupled energy conversion processes in the facultative autotroph, Hydrogenomonas which uniquely catalizes the oxidation of H_2 by O_2, a process commonly known as the "Oxyhydrogen or Knallgas reaction". Under autotrophic growth conditions the oxidation of four H_2 molecules results in the assimilation of one molecule of CO_2 involving

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

an efficient operation of the Calvin–Benson cycle[8–10]. The mechanism of H_2 oxidation appears to involve a hydrogenase and the normal components of the electron transport chain[11–16]. However, BONGERS[14] reported recently that the phosphorylation coupled to the oxidation of H_2 was not mediated by NAD^+ but involved the electron transport chain between H_2 and cytochrome *b* only which was oxidized by a "unique" cytochrome *b* oxidase in cell-free extracts from autotrophically grown Hydrogenomonas H-20. Such a conclusion is not in harmony, however, with the energetics of the observed CO_2 assimilation driven by H_2 oxidation. Studies were undertaken therefore, to reinvestigate the status of oxidative phosphorylation in *H. eutropha*. The experimental results show that all the conventional sites of energy conservation are operative in this organism and the cell-free preparations exhibit a high level of phosphorylation coupled to the oxidation of H_2, NADH, succinate and ascorbate. Evidence is presented that the phosphorylation coupled to H_2 oxidation is also mediated by NAD^+.

MATERIALS AND METHODS

Culture of bacteria and preparation of cell-free extracts

H. eutropha was grown in an inorganic medium as described by REPASKE[9]. A gas mixture containing 70 % H_2, 20 % air and 10 % CO_2 was circulated through 16 l of medium during the incubation period. After 24 h of growth at 30° the cells were harvested in a DeLaval continuous flow centrifuge at 5° and washed twice with Tris–HCl (pH 8.0). For the preparation of cell-free extracts, 20 g (wet weight) of cells were brought into suspension by mixing with 20 ml of 0.05 M Tris–HCl (pH 8.0) containing 0.3 M sucrose, 1.0 mM $MgCl_2$, 0.5 mM EDTA (disodium salt) and 0.5 mM GSH. The cells were then disrupted by passing twice through an AMINCO French Pressure Cell at 18000 lb/inch^2. The cell debris were removed by centrifugation at 10000 × *g* for 30 min in a Sorvall RC-2 refrigerated centrifuge and the supernatant fraction was used as the enzyme source.

Experimental procedures

Oxidation of NADH, succinate, and ascorbate was measured polarographically at 30°. The reaction mixture in a 3.0-ml volume contained: 270 μmoles of Tris–HCl (pH 8.0) ,9 μmoles of $MgCl_2$, 30 μmoles of KF, 9 μmoles of P_i, 4.5 μmoles of ADP and cell-free extracts containing 4–6 mg of protein as indicated in the tables. H_2 oxidation was measured in a similar medium by standard manometric methods at 30°. The gas phase used was 50 % H_2 and 50 % air. Phosphorylation was measured in parallel experiments using the same reaction mixtures. Samples for ATP measurements were taken 3 min after substrate addition and deproteinized according to the procedure of GIBSON AND MORITA[17]. The amount of ATP formed was determined by the luciferin–luciferase assay as described by STREHLER[18]. The same reaction mixture without substrate was used in control experiments and the results have been corrected for the small endogenous O_2 uptake or ATP values. The P/O ratios reported in this paper were determined after 3 min of incubation at 30°.

The difference absorption spectra were obtained in a Cary Model 14 recording spectrophotometer and protein was determined by the biuret method of GORNALL *et al.*[19].

RESULTS AND DISCUSSION

Phosphorylation coupled to the oxidation of various substrates

The data in Table I show that the oxidation of H_2, NADH, succinate and ascorbate was efficiently coupled with phosphate esterification. It may also be seen that contrary to the findings of BONGERS[14], there was not any marked difference in the rates of oxidation and coupled phosphorylation with either H_2 or NADH as the electron donor, and that both H_2 and NADH oxidations yielded equally good P/O ratios of 1.6 and 1.8, respectively. However, with succinate the P/O ratios ranged between 0.9 and 1.2 while the ascorbate oxidation yielded P/O ratios of 0.6 .The experimental data thus suggest that all of the three conventional sites of energy conservation were functional in the electron transport chain of autotrophically grown *H. eutropha*.

Participation of NAD^+ in H_2 oxidation and coupled phosphorylation

The data presented in Table II indicate that addition of NAD^+ to the cell-free preparations caused a small stimulation in the rate of H_2 oxidation as well as the coupled ATP synthesis. When also NADH trap (*e.g* pyruvate–lactate dehydrogenase) was added, both the oxidation of H_2 and coupled phosphorylation were markedly decreased. Thus the NADH trap seems to be effective with endogenous NADH and the

TABLE I

PHOSPHORYLATION COUPLED TO THE OXIDATION OF VARIOUS SUBSTRATES IN *H. eutropha*

The experimental conditions were similar as described in MATERIALS AND METHODS. The substrates used were 10 μmoles of NADH, 20 μmoles of succinate and 20 μmoles of ascorbate; the enzyme preparation contained 4.5 mg protein. Gas phase for H_2 oxidation was 50% hydrogen *plus* 50% air. The reaction was terminated after 3 min.

Substrate	*O_2 consumed (natoms)*	*ATP formed (nmoles)*	*P/O*
H_2	115	185	1.60
NADH	110	198	1.80
Succinate	99	90	0.91
Ascorbate	81	51	0.63

TABLE II

EFFECT OF NADH-TRAPPING SYSTEM ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF H_2 AND NADH

Experimental conditions were similar to those described in Table I, except that 0.5 mM NAD^+ and 2.0 mM NADH were used where indicated. NADH-trap contained 1.0 mM pyruvate and 40 μg of lactate dehydrogenase, (Sigma Chemical Co., Type 1).

Treatment	*O_2 consumed (natoms)*	*ATP formed (nmoles)*	*P/O*
H_2 (control)	175	254	1.45
H_2 + NAD^+	185	296	1.60
H_2 + NAD^+ + NADH-trap	62	34	0.55
NADH	180	310	1.72
NADH + NADH-trap	0	0	0.00

pyridine nucleotide appears to be a member of the *H. eutropha* respiratory chain involved in the oxidation of H_2 and coupled energy generation.

Effect of uncouplers and respiratory chain inhibitors

Since the phosphorylation associated with the oxidation of H_2, NADH, succinate or ascorbate was markedly inhibited by low concentrations of 2,4-dinitrophenol, 2,6-dibromophenol, pentachlorophenol, CCCP and dicumarol without any appreciable inhibition of the O_2 consumption (Table III), it is clear that the phosphate is esterified mainly by the process of electron transport-linked oxidative phosphorylation involving all of the conventional coupling sites. This conclusion is further supported by the observed inhibition of oxidative phosphorylation by the flavin inhibitors, HQNO or antimycin A, and cyanide or azide. The data in Table IV show that low concentrations of the flavoprotein inhibitors such as rotenone, amytal and atabrine caused about 80 % inhibition of the phosphorylation coupled to the oxidation of either H_2 or NADH without significantly inhibiting the rate of O_2 consumption. A similar effect was observed with HQNO and antimycin A. It would thus appear that in the flavoprotein or HQNO-blocked electron transport chain, the transport of electrons from H_2 or NADH to O_2 is mediated by a nonphosphorylating pathway which is probably catalyzed by the NADH–cytochrome *c* oxidoreductase and the observed P/O ratios of 0.3 might be due to the participation of the terminal segment of the respiratory chain involving the action of ferrocytochrome *c*–O_2 oxidoreductase.

It is of interest to note that the phosphorylation coupled to the oxidation of

TABLE III

EFFECT OF UNCOUPLERS ON OXIDATIVE PHOSPHORYLATION IN *H. eutropha*

The experimental conditions were similar to those described in Table I, except that various uncouplers were added as indicated and the enzyme preparation contained 6.0 mg of protein.

Substrate	*Uncoupler*	*Concn. (mM)*	*O_2 consumed (natoms)*	*ATP formed (nmoles)*	*P/O*
H_2	None	—	170	270	1.60
	2,4-Dinitrophenol	0.05	163	0	0.00
	2,6-Dibromophenol	0.05	165	30	0.18
	CCCP	0.01	171	73	0.43
	Dicumarol	1.00	156	0	0.00
NADH	None	—	163	316	1.93
	2,4-Dinitrophenol	0.01	144	57	0.40
	2,6-Dibromophenol	0.05	144	0	0.00
	Pentachlorophenol	0.05	153	0	0.00
	CCCP	0.04	158	5	0.03
	Dicumarol	1.00	158	0	0.00
Succinate	None	—	168	201	1.20
	2,4-Dinitrophenol	0.01	193	15	0.08
	2,6-Dibromophenol	0.05	166	15	0.09
	Pentachlorophenol	0.01	193	9	0.06
	CCCP	0.01	170	42	0.24
	Dicumarol	1.00	168	24	0.14
Ascorbate	None	—	138	80	0.58
	2,4-Dinitrophenol	0.01	138	0	0.00
	2,6-Dibromophenol	0.05	132	25	0.19
	Pentachlorophenol	0.01	124	9	0.07
	CCCP	0.01	138	28	0.20

TABLE IV

EFFECT OF RESPIRATORY CHAIN INHIBITORS ON OXIDATIVE PHOSPHORYLATION IN *H. eutropha*

Experimental conditions were the same as described in Table I, except that various inhibitors were employed as indicated. The reaction mixture contained 6 mg of enzyme protein. R represents the relative respiration rate with various substrates. Actual R values for the oxidation of H_2, NADH, succinate and ascorbate were 170, 158, 138 and 130 natoms O_2, and 255, 258, 165 and 80 nmoles of ATP were formed coupled to the oxidation of H_2, NADH, succinate and ascorbate, respectively, without any inhibitor.

Inhibitor	*Concn.*	*Substrates*							
		H_2		*NADH*		*Succinate*		*Ascorbate*	
		R	*P/O*	*R*	*P/O*	*R*	*P/O*	*R*	*P/O*
None	—	100	1.50	100	1.67	100	1.20	100	0.61
Rotenone	10 μM	95	0.32	80	0.00	80	1.14	99	0.56
Amytal	10 μM	97	0.21	97	0.49	82	1.16	96	0.62
Atabrine	10 μM	93	0.27	100	0.24	90	0.92	98	0.64
HQNO	2.5 μg	94	0.37	100	0.28	87	0.15	97	0.58
Antimycin A	5.0 μg	88	0.30	91	0.32	62	0.10	98	0.56
Cyanide	0.1 mM	96	0.92	—	—	—	—	95	0.23
Cyanide	1.0 mM	86	0.15	—	—	—	—	83	0.07
Azide	0.1 mM	99	0.81	—	—	—	—	97	0.20
Azide	1.0 mM	88	0.19	—	—	—	—	87	0.09

succinate or ascorbate was not affected by rotenone, amytal or atabrine. Although HQNO and antimycin A caused about 90 % inhibition of the phosphorylation linked with the oxidation of NADH, H_2 and succinate, ATP formation coupled to the ascorbate oxidation remained unaffected. These observations are in complete accord with the behavior of the electron transport components observed in the presence of added rotenone and antimycin A or HQNO when NADH or succinate served as the electron donors (Figs. 1 and 2). Thus an addition of 5 mM NADH to the phosphorylating enzyme preparations caused the reduction of flavoproteins and cytochromes of *b*- and *c*-type. However, NADH failed to reduce cytochromes in the presence of added rotenone. Although NADH oxidation occurred in the presence of rotenone, this process did not result in phosphate esterification (Table IV). It was observed that unlike NADH, H_2 catalyzed the reduction of cytochromes of *b*- and *c*-type with partial reduction of the flavoprotein(s) in the presence of added rotenone which did not block the oxidation of H_2 but the coupled phosphorylation was inhibited to an extent of 70 %. In the presence of antimycin A or HQNO H_2 reduced cytochrome *b* predominantly; cytochrome *c* was also reduced but to a lesser extent but the phosphorylation was markedly inhibited. However, it is likely that in the antimycin A-blocked system the reduction of cytochrome *c* was catalyzed by the NADH–cytochrome *c* oxidoreductase due to the reduction of endogenous NAD^+ by H_2. Since little or no inhibition of the O_2 consumption is observed with NADH or H_2 in the rotenone- or antimycin A-blocked system, it may be inferred that the flavoprotein(s) as wel as cytochrome *b* are oxidized directly by O_2 and, therefore, the inhibition by rotenone of the phosphorylation coupled to H_2 or NADH oxidation, as well as the inhibition by antimycin A of the phosphate esterification can be readily explained on the basis of these observations.

The cytochromes were also reduced when the cell-free preparations were treated

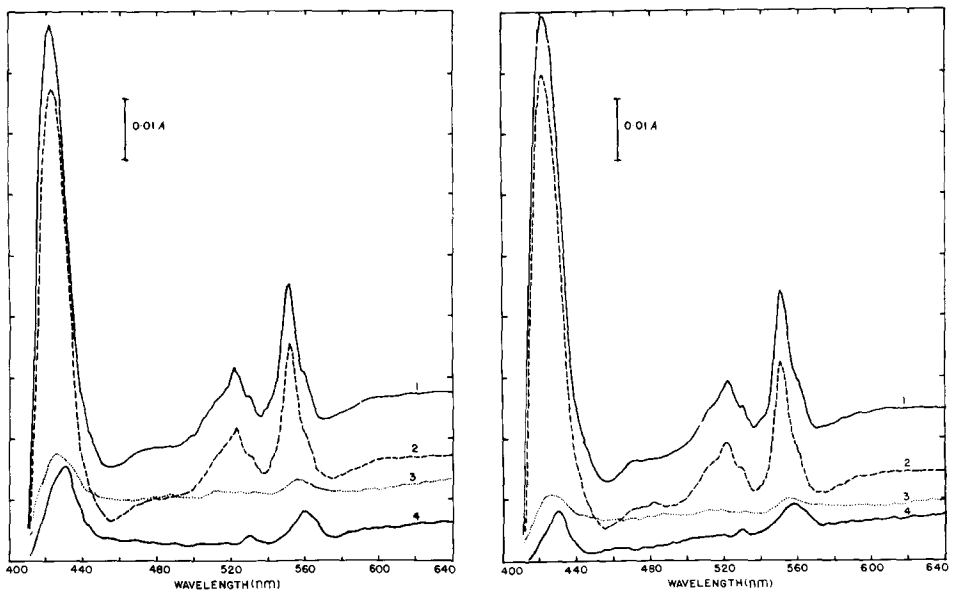


Fig. 1. Effect of rotenone, thenoyltrifluoroacetone and antimycin A upon the reduction of cytochrome system by NADH. Reaction mixture in a final volume of 2.0 ml contained cell-free extracts containing 2.0 mg protein, 96 μmoles of Tris–HCl (pH 8.0) and 10 μmoles of NADH. Trace 1 indicates the difference absorption spectra obtained 10 min after the addition of NADH to the test cuvette. Trace 2 represents the difference spectra in the presence of 0.1 mM thenoyltrifluoroacetone in both cuvettes and NADH in the test cuvette. Trace 3 indicates the difference spectra in the presence of 0.2 mM rotenone in both cuvettes and NADH in the test cuvette. Trace 4 represents the difference spectra in the presence of 2.5 μg antimycin A in both cuvettes and NADH in the test cuvette.

Fig. 2. Effect of rotenone, theoyltrifluoroacetate and antimycin A upon the reduction of cytochrome system by succinate. Experimental conditions were similar as described in Fig. 1 except that 40 μmoles of succinate were added to the test cuvette. Trace 1 represents the steady-state difference absorption spectra obtained 10 min after the addition of succinate. Traces 2, 3 and 4 represent the absorption spectra obtained in the presence of 0.2 mM rotenone, 0.1 mM thenoyltrifluoroacetone and 2.5 μg of antimycin A, respectively.

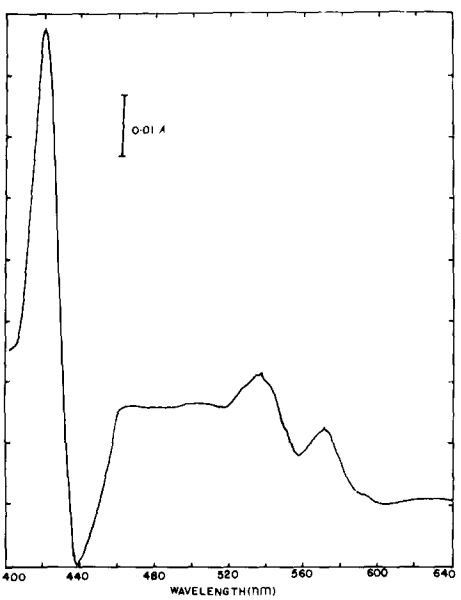
with succinate. In the presence of antimycin A or HQNO, however, there was no initial reduction of cytochrome *c*; cytochrome *b* was reduced but it was subsequently oxidized (probably by O_2). Succinate dehydrogenase was completely inhibited in the presence of 0.1 mM thenoyltrifluoroacetone as evidenced by the failure of succinate to reduce either cytochrome *b* or *c*. Rotenone, however, did not inhibit succinic dehydrogenase as the cytochrome systems were reduced by succinate in the presence of rotenone. The NADH dehydrogenase on the other hand was unaffected by added thenoyltrifluoroacetone.

The oxidation of H_2 as well as that of ascorbate was virtually unaffected by 0.1 mM cyanide or azide but the coupled phosphorylations were inhibited by about 50–60 %. Although 1.0 mM cyanide or azide caused only about 15 % inhibition of H_2 and ascorbate oxidation, over 90 % inhibition of the concomitant phosphorylation occurred under these conditions. It would thus appear that in the terminally blocked electron transport chain with H_2 as the electron donor, the electrons are accepted by O_2 directly from the flavoprotein and/or cytochrome *b* level. However, with ascorbate as the electron donor, the ferrocytochrome *c* may be oxidized through the mediation of

cytochrome *o* when the cytochrome oxidase components (a^+a_3) are blocked by cyanide or azide. If this happens to be the case then it would appear that cytochrome *o* is not involved in the process of energy conservation in the terminal segment of the respiratory chain of *H. eutropha*. We have observed that cytochrome *o* was indeed present in cell-free preparations (Fig. 3). The dithionite-reduced *plus* CO *minus* reduced difference absorption spectra showed the α, β, and γ peaks at 570–573, 540 and 421 nm, respectively.

Energy-linked reversal of the respiratory chain in H. eutropha

Of particular interest is the ability of the phosphorylating enzyme preparations from *H. eutropha* to catalyze an ATP-dependent reversal of electron transfer from succinate or ascorbate to NAD^+. The energy-linked reversibility of the reactions of oxidative phosphorylation has been well established in mammalian systems[20–31] as well as in chemosynthetic bacteria[32–36] and study of the partial reactions can be helpful towards elucidation of the loci of energy-coupling sites in the electron transport chain. We have observed that under optimal conditions the rate of succinate-linked ATP-



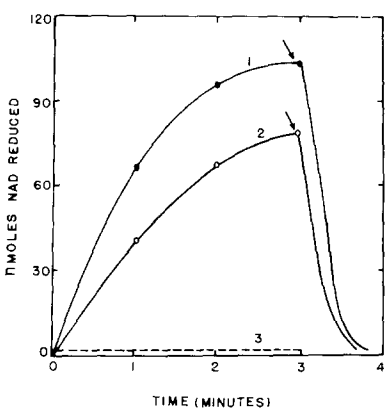


Fig. 3. Reduced *plus* CO *minus* reduced absorption spectra of *H. eutropha* cell-free extracts. Reaction mixture in a total volume of 2.0 ml contained cell-free extract containing 20 mg protein and 100 μmoles of Tris–HCl (pH 8.0). Both the sample and the reference cuvettes were treated with 0.3 mM dithionite and a stream of O_2-free CO was bubbled into the sample cuvette for about 2 min.

Fig. 4. ATP-dependent NAD^+ reduction by succinate or ascorbate in *H. eutropha*. The reaction was carried out in Thunberg-type cuvettes of 1 cm light path. Reaction mixture in a total volume of 3.0 ml contained 220 μmoles of Tris–HCl (pH 8.0), 10 μmoles of $MgCl_2$ and 3 μmoles of NAD^+. The treatment cuvette in addition, was supplied with 3 μmoles of ATP. The side arm of the cuvettes contained enzyme containing 10 mg of protein and 7 mM succinate or ascorbate. The cuvettes were evacuated and the reaction was started by tipping in the contents of the side arm. The rate of absorption change was recorded at 340 nm. Traces 1 and 2 represent the rate of ATP-dependent NAD^+ reduction by succinate and ascorbate, respectively. The arrows indicate the disappearance of absorption under anaerobic conditions upon the addition of NADH-trapping system consisting of 1 mM pyruvate and 40 μg of lactate dehydrogenase (Type 1, Sigma Chemical Co.).

dependent reduction of NAD^+ in *H. eutropha* (calculated from the linear part of the reaction) was 7–8 nmoles/min per mg of enzyme protein. In order to confirm that the increase in absorption at 340 nm was due to the NADH formed, pyruvate–lactate dehydrogenase trap was added at the end of the experiment which caused an immediate disappearance of the absorption (Fig. 4).

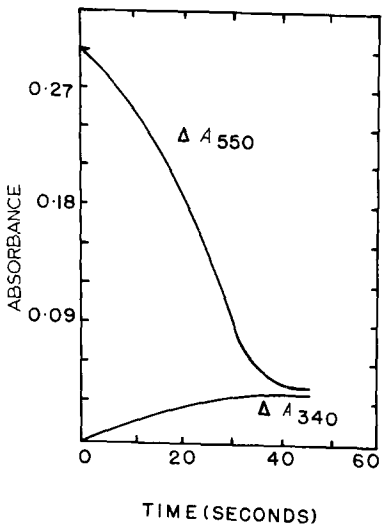


Fig. 5. ATP-driven oxidation of mammalian ferrocytochrome *c* coupled to the reduction of NAD^+. Experimental conditions were the same as given in Fig. 4, except that 50 μM mammalian ferrocytochrome *c* and NAD^+ were placed in the side arm during evacuation. No succinate or ascorbate was present in the reaction mixture. The absorbance changes at 550 and 340 nm were recorded simultaneously with a dual split-beam spectrophotometer equipped with a dual-pen recorder.

TABLE V

EFFECT OF INHIBITORS ON THE ATP-DEPENDENT NAD^+ REDUCTION BY SUCCINATE AND ASCORBATE IN *H. eutropha*

Experimental conditions were the same as described in Fig. 4, except various inhibitors and uncouplers were used as indicated. In the absence of inhibitors the succinate- and ascorbate-linked ATP-dependent rate of NAD^+ reduction was 60 and 40 nmoles/min, respectively.

Inhibitor	*Concn.*	*% inhibition of NAD^+ reduction with*	
		Succinate	*Ascorbate*
Thenoyltrifluoroacetone	0.10 mM	100	100
Rotenone	0.10 mM	65	67
Atabrine	0.10 mM	65	50
Amytal	1.00 mM	57	44
2,4-Dinitrophenol	0.50 mM	100	100
2,4-Dibromophenol	0.50 mM	70	50
Pentachlorophenol	0.50 mM	100	100
CCCP	0.50 mM	100	100
Dicumarol	1.00 mM	86	50
2,3-Dimercaptopropanol	2.00 mM	60	100
Oligomycin	1.2 μg/mg protein	100	82
Antimycin A	1.2 μg/mg protein	82	100
HQNO	1.2 μg/mg protein	100	70

When mammalian cytochrome *c* reduced with H_2 and palladium asbestos was employed as the electron donor, the enzyme preparations catalyzed an ATP-driven oxidation of ferrocytochrome *c* and concomitant reduction of the pyridine nucleotide under anaerobic conditions. The reaction was complete in about 40 sec during which period approx. 45 nmoles of cytochrome *c* were oxidized per 20 nmoles of NAD^+ reduced and yielded a stoichiometry of 2:1 (Fig. 5).

Effect of inhibitors on the ATP-dependent NAD^+ reduction in H. eutropha

Table V shows that the ATP-dependent reduction of NAD^+ by either succinate or ascorbate was sensitive to the flavoprotein inhibitors. Thus 0.1 mM rotenone or atabrine and 1 mM amytal caused about 60% inhibition of energy-dependent reduction of the pyridine nucleotide by succinate or ascorbate. Rotenone, atabrine and amytal at the concentrations used did not, however, inhibit the oxidation of either succinate or ascorbate to any significant extent (see Table IV). Inhibitors which block electron transfer between cytochrome *b* and cytochrome *c* (*e.g.* antimycin A or HQNO) when used at a concentration of 1.2 μg/mg protein effectively caused an 80–100% inhibition of NAD^+ reduction by succinate or ascorbate. The possibility cannot be excluded, however, that the inhibitory effect of antimycin A or HQNO at such concentrations was due to an uncoupling action. The succinate or ascorbate-linked energy-dependent NAD^+ reduction was also markedly inihbited by 2,3-dimercaptopropanol, an antagonist of quinones and vitamin K analogues. The energy transfer from ATP to drive the reverse electron flow process was strongly blocked by oligomycin when used at a concentration of 1.2 μg/mg enzyme protein. Likewise, the process was highly sensitive to low concentrations of uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol, 2,6-dibromophenol, pentachlorophenol and dicumarol. Since the electron transport and coupled phosphorylations as well as the energy-dependent reversal of electron transfer show remarkably similar inhibition patterns with respect to rotenone, antimycin A or HQNO, and various uncoupling agents, it seems likely that the same electron transport chain is involved in the forward and back reactions.

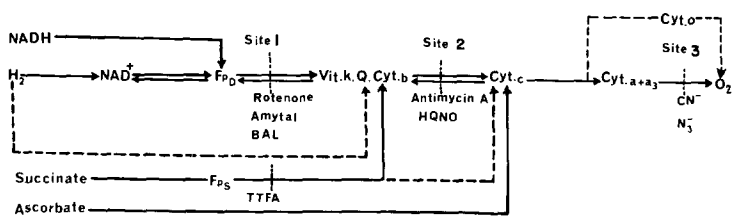


Fig. 6. Proposed pathways of electrons and energy transfer reactions in autotrophically grown *H. eutropha*. The electron transport from NADH to O_2 is mediated by the complete electron transport chain. About 60–70% of H_2 oxidation is mediated by NAD^+, flavoproteins and the cytochrome system and there appears to be a NAD^+ bypass of electron transfer to cytochrome *b* which can be oxidized directly by O_2 in an antimycin A-blocked system. In a cyanide- or azide-blocked system, the electron donors are oxidized by the mediation of cytochrome *o* and this pathway of electron transport is not coupled to phosphorylation. Bulk of the succinate oxidation is mediated by cytochrome *b* although succinate–cytochrome *c* oxidoreductase activity is also present. The ATP-dependent reversal of electron transfer is effected from the level of cytochromes *c* and *b*. Both the oxidative phosphorylation as well as its reversal is markedly sensitive to the inhibitors of coupling sites 1 and 2 and to the uncouplers of oxidative phosphorylation. The high level of oxidative phosphorylation coupled to the oxidation of H_2, NADH, succinate and ascorbate provides the evidence that all of the three phosphorylation sites are functional in *H. eutropha*. Abbreviations: BAL, 2,3-dimercaptopropanol; TTFA, thenoyltrifluoroacetone.

On the basis of our experimental data the over-all electron transfer scheme in *H. eutropha* is presented in Fig. 6.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant, GB 14661, from the National Science Foundation, by University of Kentucky Research Foundation, and by U.S. Public Health Service Biochemical Research Support (Grant No. 5S05-FR07114-02). The authors are grateful to Dr. Roy Repaske of the National Institute of Health for providing us a pure culture of *Hydrogenomonas eutropha*. We are also grateful to Professor Lucille Smith for helpful discussions and for providing us with the ATP-measuring apparatus.

REFERENCES

1 M. I. H. ALEEM AND A. NASON, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 763.
2 I. FISCHER, AND H. LOUDELOUT, *Biochim. Biophys. Acta*, 110 (1965) 259.
3 L. KIESOW, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 981.
4 M. I. H. ALEEM, *Biochim. Biophys. Acta*, 162 (1968) 338.
5 A. J. ROSS, R. L. SCHOENHOFF AND M. I. H. ALEEM, *Biochem. Biophys. Res. Commun.*, 32 (1968) 301.
6 E. A. DAVIS AND E. J. JOHNSON, *Can. J. Microbiol.*, 13 (1967) 873.
7 J. S. COLE AND M. I. H. ALEEM, *Biochem. Biophys. Res. Commun.*, 38 (1970) 736.
8 A. SCHATZ, *J. Gen. Microbiol.*, 6 (1952) 329.
9 R. REPASKE, *J. Biol. Chem.*, 237 (1962) 1351.
10 L. PACKER, AND W. VISHNIAC, *Biochim. Biophys. Acta*, 17 (1955) 153.
11 L. PACKER, *Arch. Biochem. Biophys.*, 78 (1958) 54.
12 R. L. LESTER, AND F. L. CRANE, *J. Biol. Chem.*, 234 (1959) 2139.
13 D. E. ATKINSON, *J. Biol. Chem.*, 218 (1956) 557.
14 L. BONGERS, *J. Bacteriol.*, 93 (1967) 1615.
15 F. H. BERGMANN, J. C. TOWNE AND R. H. BURRIS, *J. Biol. Chem.*, 230 (1958) 13.
16 B. A. MC FADDEN AND C. L. TU, *Biochem. Biophys. Res. Commun.*, 19 (1965) 728.
17 J. GIBSON AND S. MORITA, *J. Bacteriol.*, 93 (1976) 1544.
18 B. M. STREHLER, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1965, p. 563.
19 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
20 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1544.
21 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1569.
22 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1555.
23 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1562.
24 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1577.
25 M. KLINGENBERG AND P. SCHOLLMEYER, *Proc. 5th Intern. Congr. Biochem., Moscow*, Pergamon Press, Macmillan Co., New York, Vol. 5, 1963, p. 46.
26 M. KLINGENBERG AND P. SCHOLLMEYER, *Biochem. Biophys. Res. Commun.*, 4 (1961) 323.
27 H. LOW, I. VALLIN AND B. ALM, in B. CHANCE, *Energy-Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 5.
28 E. C. SLATER AND J. M. TAGER, in B. CHANCE, *Energy Linked Functions of Mitochondria*, Academic Press, New York, N.Y. 1963, p. 97.
29 L. PACKER, *Biochim. Biophys. Acta*, 74 (1963) 127.
30 L. ERNSTER, *Proc. 5th Intern. Congr. Biochem., Moscow*, Pergamon Press, Macmillan Co., New York, Vol. 5, 1963 p. 115.
31 L. ERNSTER AND C. P. LEE, *Ann. Rev. Biochem.*, 33 (1964) 729.
32 M. I. H. ALEEM, H. LEES AND D. J. D. NICHOLAS, *Nature*, 200 (1963) 759.
33 M. I. H. ALEEM, *J. Bacteriol.*, 91 (1966) 729.
34 M. I. H. ALEEM, *Biochim. Biophys. Acta*, 113 (1966) 216.
35 M. I. H. ALEEM, *Biochim. Biophys. Acta*, 128 (1966) 1.
36 D. L. SEWELL AND M. I. H. ALEEM, *Biochim. Biophys. Acta*, 172 (1969) 467.