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Phosphorylation in Hydrogen Bacteria

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The electron-transport system of cell-free extracts obtained from *Hydrogenomonas* H-20 has been studied with particular reference to phosphorylation associated with the oxyhydrogen reaction. Cell-free preparations of this organism exhibit oxidative phosphorylation with hydrogen and succinate as electron donors. This activity could be uncoupled with a number of agents. Ratios of phosphorylative activity to oxidative activity observed varied from 0.2 to 0.7. Factors affecting the efficiency of phosphorylation were examined. Inhibitor and spectrophotometric studies indicated that phosphorylation with hydrogen as electron donor occurs exclusively at a site in an abbreviated electron transport chain between H_2 and cytochrome *b*. The possible occurrence of a cytochrome *b* oxidase and the requirement for a quinone are discussed, as well as the correlation between the abbreviated pathway and the energy generation by the cell. Evidence is presented which indicates that nicotinamide adenine dinucleotide does not participate in the hydrogen oxidation path which is coupled to adenosine triphosphate formation.

Molecular hydrogen is utilized by hydrogen bacteria for reductive and energy-yielding purposes. In this process, which is mediated by hydrogenase, two reactants required for CO_2 assimilation are generated: reduced nicotinamide adenine dinucleotide ($NADH_2$) and adenosine triphosphate (ATP).

The generation of $NADH_2$ with molecular hydrogen as electron donor has been demonstrated in hydrogenomonads (*Hydrogenomonas eutropha* and *H. ruhlandii*; 20, 24) and other hydrogenase-containing bacteria (e.g., *Clostridium pasteurianum* and *C. kluyveri*; 22). Repaske (24) demonstrated a requirement for flavin mononucleotide as a cofactor in the reduction of nicotinamide adenine dinucleotides (NAD) by extracts of *H. eutropha*. No such requirement was found for *H. ruhlandii*.

Little is known concerning the enzymatic oxidation of $NADH_2$ by hydrogenomonads. Repaske and Lizotte (25) reported two pathways in crude cell-free extracts of *H. eutropha*: a menadione-dependent reductase specific for $NADH_2$ and a menadione-independent cytochrome *c* reductase. Since extraction with cyclohexane did not alter these activities, participation of quinones as electron acceptors in the oxidation of $NADH_2$ seemed unlikely.

On the other hand, quinones are found in many microorganisms, including *H. eutropha* (17). Kasket and Brodie characterized two quinones in *Escherichia coli*. They observed that coenzyme Q_{10} (CoQ_{10}) mediated electron transport from succinate to cytochrome *c* and oxygen, and they found that naphthoquinone was involved in $NADH_2$ cytochrome *c* reductase (15). Evidence has implicated vitamin K_1 in the oxidation of a variety of substrates in extracts of *Mycobacterium phlei* (3, 4). Evidence will be presented which indicates that a quinone may also be involved in the oxidation of hydrogen in cell-free preparations of hydrogenomonads.

Phosphorylation linked to the oxidation of a variety of organic substrates has been described with particulate and crude extracts isolated from heterotrophic bacteria. The ratios of phosphorylative activity to oxidative activity (P/O) observed with preparations of different organisms varied, but generally, depending on the electron donor used, values in the order of 0.5 to 2.0 were observed (2). Also, with inorganic substrates, ATP generation has been reported (1, 7, 21, 26), but comparatively little work has, so far, been done with preparations of chemoautotrophs. We have obtained crude cell extracts of an autotrophically grown hydrogenomonad which are capable of oxidizing hydrogen, and this oxidation is coupled to phosphorylation. This paper will describe observations concerning the efficiency of ATP formation coupled to the oxidation of hydrogen and of other substrates in cell-free extracts of *Hydrogenomonas* H-20.

MATERIALS AND METHODS

Culture procedure. Hydrogenomonas H-20 (kindly supplied by H. G. Schlegel, Institut für Mikrobiologie

der Universität, Göttingen, Germany) was grown autotrophically in a liquid culture containing: 0.02 M potassium phosphate (*p*H 6.5), 16 × 10^{-3} M urea, 5 × 10^{-4} M $MgSO_4$, and 2 × 10^{-5} M $Fe(NH_2)_2(SO_4)_2$. No trace elements were added, since contaminating salts in the major constituents apparently sufficed. The cultures (120 ml of suspension per 500-ml flask) were incubated in a rotary shaker at 33 C with a gas phase containing 70% H_2, 20% O_2, and 10% CO_2, according to a method described by Repaske (23). To enhance gas diffusion, the 500-ml flasks were provided with indentations.

Extract preparation. After 16 hr of incubation, the cells obtained from a 1% inoculum were collected by centrifugation, washed twice, and resuspended (approximately 10 mg, dry weight/ml) in 0.1 M tris(hydroxymethyl)aminomethane (Tris) chloride (*p*H 7.0); they were subsequently disrupted by ultrasonic treatment (Branson, 20 kc/sec) for 1.5 to 2 min. The 14,000 × *g* supernatant fluid, designated as crude extract, was used for the experiments described.

Phosphate esterification. Phosphorylation with a number of substrates was measured in reaction mixtures containing 3 to 4 mg of extract protein per ml and, in micromoles per milliliter: $MgCl_2$, 10; inorganic phosphate, 3.0; adenosine diphosphate (ADP), 1.5; glucose, 20; NaF, 10; and 0.2 mg of yeast hexokinase. The same reaction mixture without substrate was used as a control in all experiments. The amount of esterified phosphate was determined after 10 to 15 min of incubation at 30 C as the difference in inorganic phosphate between sample and control, according to Fiske and SubbaRow (8) and, in some experiments, as the formation of esterified phosphate by the method of Hagihara and Lardy (9). Good agreement between both methods was obtained.

Addition of ATP to the reaction mixtures did not significantly affect the rate of disappearance of inorganic phosphate in the presence of hydrogen as electron donor, or cause an increase in inorganic phosphate by incubation under air. NaF, however, was routinely added to the reaction mixtures to inhibit possible adenosine triphosphatase or phosphatase activity.

Oxygen consumption. Oxygen uptake was determined in parallel experiments by use of a Teflon membrane-covered gold-silver Clark-type electrode.

Lactate determination. The formation of lactate was measured according to Warburg et al. (29). With this method, the concentration of lactate in the reaction mixture is determined as a reduction of NAD in the presence of lactate dehydrogenase (E.C. 1.1.1.28) at *p*H 9.6.

RESULTS

P/O ratios. In Table 1, the phosphorylative and oxidative activity observed in a number of individual cell-free preparations is recorded. Under conditions of experimentation, a linear rate of phosphorylation was observed during 10 to 15 min of incubation under hydrogen and oxygen. All experiments reported here were restricted to this time of incubation. The disappearance of inorganic phosphate and the consumption of oxygen observed under these conditions gave P/O ratios on the order of 0.5.

All cofactors, generally believed to be required for phosphate esterification, proved to be essential (Table 2). Other phosphate acceptors in place of ADP stimulated phosphate esterification. Adenosine monophosphate, guanosine diphosphate, and inosine diphosphate were equally as effective as ADP, whereas cytosine diphosphate (CDP) was somewhat less active.

The disappearance of inorganic phosphate from the medium implies the generation of ATP, which in turn is converted to glucose-6-phosphate in the presence of hexokinase (E.C. 2.7.1.1) and glucose. Evidence for the formation of glucose-6-phosphate was the observed reduction of added nicotinamide adenine dinucleotide phosphate

TABLE 1. *Phosphate esterification coupled to hydrogen oxidation*[a]

Prepn	Δ μmoles of inorganic phosphate/hr	Δ μatoms of O_2/hr	P/O
1	4.35	10.0	.44
2	4.68	10.4	.45
3	3.64	8.1	.45
4	4.36	8.2	.53
5	6.48	8.4	.76
6	5.76	10.5	.55

[a] The standard reaction mixtures (see Materials and Methods) contained 3 to 5 mg of protein per ml. Gas phase, 70% H_2 and 30% O_2 ; temperature, 30 C.

TABLE 2. *Cofactor requirement for phosphate esterification*[a]

Components of mixture	Δ μmoles of inorganic phosphate/hr
Complete mixture	4.7
Plus 2 μmoles of ATP	4.6
Plus 2.5 μmoles of NAD	4.8
Minus $MgCl_2$	3.4
Minus ATP trap	1.7
Minus NaF	4.5
Minus ADP	0.5
Minus ADP + AMP	5.5
Minus ADP + GDP	4.0
Minus ADP + CDP	3.1
Minus ADP + IDP	4.3

[a] For complete reaction mixture, see Materials and Methods. Where indicated, ADP was replaced by an equivalent amount of phosphate acceptor. The protein content was 4.2 mg/ml. The reactions were carried out at 30 C.

(NADP) in the presence of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49). NADP reduction was obtained in reaction mixtures which were preincubated with H_2 and O_2. The amount of NADP formed was in agreement with the observed disappearance of inorganic phosphate.

Table 3 shows the phosphorylation observed in association with the oxidation of various substrates. The rate of phosphorylation with succinate as a substrate was four- to fivefold lower than the rates observed with hydrogen as substrate. The P/O ratios with succinate as a substrate were relatively low, and varied between 0.40 and 0.20. If hydrogen and succinate were provided simultaneously, the rate of phosphorylation was approximately equal to the sum of the rate observed with hydrogen and with succinate. Also, an equivalent increase in O_2 uptake was observed (see Table 3). These observations indicate that two different phosphorylating sites are involved. The site of phosphorylation with hydrogen as a substrate is probably located at a lower potential than the sites operating in the presence of succinate. If one presumes that succinate enters the electron-transport chain at the cytochrome *b* level, the bulk of phosphorylation with hydrogen as substrate must occur between the H_2 and the cytochrome *b*.

The effects of a number of uncoupling agents tested in this system are recorded in Table 4. Carbonyl cyanide *m*-chlorophenyl hydrozone (CCCP), in a concentration of 10^{-6}, inhibited phosphate esterification to half the control value, whereas even 10^{-4} M CCCP had little effect on the rate of oxygen consumption. The effectiveness

TABLE 3. *Effect of various substrates on phosphorylation*[a]

Substrate	Δ μmoles of inorganic phosphate/hr	Δ μatoms of O_2/hr	P/O
H_2	6.47	12.4	.52
Succinate	1.23	6.8	.18
H_2 + succinate	7.51	16.9	.44
β-Hydroxybutyrate	0.38	1.1	.35
β-Hydroxybutyrate[b]	0.52	4.9	.11
$NADH_2$	0.11	5.4	.02

[a] Gas phase: 70% H_2 + 30% O_2 or air. Concentrations per milliter of substrates in standard reaction mixture: 10 μmoles of succinate; 6.4 μmoles of $NADH_2$; 10 μmoles of β-hydroxybutyrate; protein concentration was 4.5 mg/ml and the temperature was 30 C.

[b] In this instance, 0.2 μmole of NAD per ml was added to the reaction mixture.

TABLE 4. *Effect of uncouplers on oxidative and phosphorylative activity*[a]

Uncoupler	Δ μmoles of inorganic phosphate	Δ μatoms of O_2	P/O
Control	3.87	7.10	0.55
CCCP, 7×10^{-7} M	2.40	7.30	0.33
CCCP, 7×10^{-6} M	1.17	6.55	0.08
CCCP, 7×10^{-5} M	0	5.20	0
Control	3.96	7.95	0.50
DNP, 10^{-5} M	3.18	7.80	0.41
DNP, 10^{-4} M	2.46	7.25	0.34
DNP, 10^{-3} M	1.05	7.05	0.15
Control	5.52	9.6	0.58
Dicumarol, 10^{-4} M	2.82	8.9	0.32
Dicumarol, 10^{-3} M	0	9.3	0

[a] Reaction mixture contained 4 mg of protein per ml. Gas phase: 70% H_2 + 30% O_2; temperature, 30 C.

of CCCP as uncoupling agent thus seems somewhat less than observed with mammalian mitochondrial systems (10, 11).

The results (Table 4) obtained with dinitrophenol (DNP) classified *Hydrogenomonas* in the group of organisms which are weakly sensitive to this uncoupler (half inhibition at 3×10^{-4} M) compared with ~10-fold greater sensitivity observed in DNP-sensitive organisms such as *M. phlei* (3, 4). The oxyhydrogen reaction was only slightly affected by the DNP concentrations employed. Dicumarol uncoupled phosphorylation similarly to DNP. Oxidative activity was not affected by Dicumarol. The data reported indicate that the phosphorylation observed occurs above substrate level.

Oxidation at the cytochrome b level. The results reported above suggested an oxidation by O_2 at the level of cytochrome *b*. This could be further demonstrated by spectrophotometric observations. As illustrated in Fig. 1, the distinct cytochrome *b* absorption (Fig. 1, curve 1, maxima at 562 and 528 mμ), observed upon incubating cell-free preparations with hydrogen, disappeared when air was introduced in the presence of cyanide, whereas cytochrome *c* (Fig. 1, curve 2, maxima at 552 and 520 mμ) remained reduced. In the presence of cyanide, cytochrome *c* was reduced even in the presence of air, so that upon flushing with hydrogen the only change was the reduction of cytochrome *b* (Fig. 1, curve 3). Replacement of hydrogen by air yielded reoxidation of cytochrome *b* (Fig. 1, curve 4). The observations illustrated in Fig. 1 indicate that, under certain conditions, cytochrome *b* can react

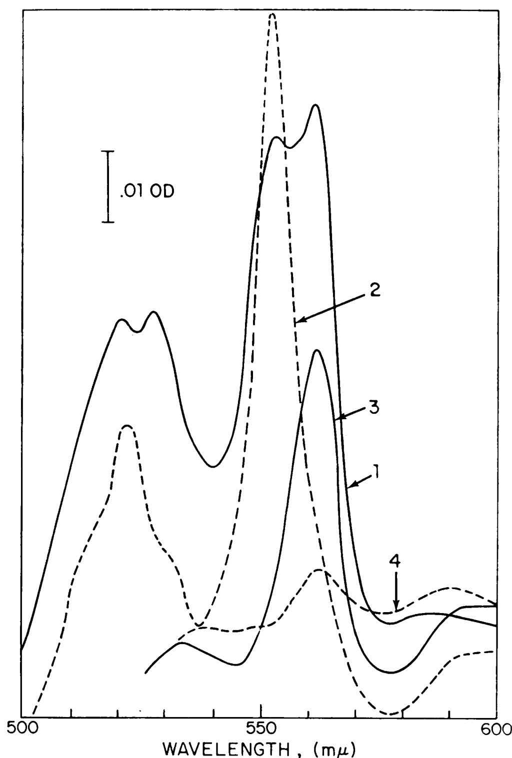

FIG. 1. *Oxidation of reduced cytochrome b by oxygen. Sample (Thunberg) cuvette containing 2 ml of extract (10 mg of protein per ml) was gassed with hydrogen. Reference cuvette contained 2 ml of extract in air. After incubation for 5 min at room temperature, spectrum 1 was obtained. Subsequently, cyanide was tipped from the side arm (final concentration, 10^{-4} M), the hydrogen in the sample cuvette was replaced by air, and spectrum 2 was obtained. Spectra 3 and 4 were obtained with 10^{-4} M CN^- in both reference and sample cuvette. Spectrum 3 was measured after flushing the sample with hydrogen and the reference with air. Spectrum 4 was obtained subsequently after flushing the sample cuvette with air. Measurements were made at room temperature in a Cary model 15 spectrophotometer.*

directly with oxygen. It is not clear whether this reaction between oxygen and cytochrome *b* occurs to the same extent in the absence of CN^-. However, from the relatively low P/O values observed with succinate (see Table 3), and from the inability of antimycin A to significantly inhibit oxidative activity with this substrate (see Table 6), one would assume that also in the absence of CN^- some auto-oxidation at the cytochrome *b* level occurs.

Effects of inhibitors. Table 5 describes the effects of various inhibitors upon phosphorylative and oxidative activity with hydrogen as substrate. The P/O ratios observed in the presence of cyanide, azide, and carbon monoxide are about equal to the values found in the absence of these inhibitors. The decrease in the rate of oxidation and of phosphorylation can be attributed to the partial inactivation of hydrogenase due to these inhibitors. This finding, reported earlier by Hyndman, Burris, and Wilson (12) was confirmed. Also, antimycin A proved to be an ineffective inhibitor of phosphorylation accompanied by hydrogen oxidation. The highest antimycin A concentration tested (40 μg/ml) gave an inhibition of only 50%. On the other hand, 2N-heptyl-4-hydroxyquinoline N-oxide (HQNO) proved an effective inhibitor of phosphorylation with hydrogen as electron donor. The effects of HQNO and antimycin A on phosphorylation with hydrogen and with succinate as substrate provided strikingly different results. Phosphorylation coupled to the oxyhydrogen reaction was severely inhibited by HQNO concentrations on the order of 1 μg/ml, whereas similar concentrations had little effect on the phosphorylative activity coupled to succinate oxidation. However, in the latter case, antimycin A proved extremely effective (Table 6). (No inhibition of hydrogenase activity, measured as methylene blue reduction, was observed with HQNO concentrations of 8 μg/ml.

TABLE 5. *Effect of respiratory chain inhibitors on oxidative and phosphorylative activity with hydrogen as substrate*[a]

Inhibitor	Percentage of control oxidative activity	Percentage of control phosphorylative activity	P/O
Control[b]			.46
CN^-, 3 × 10^{-4} M	38	37	.48
N_3^-, 10^{-4} M	100	98	.45
CO, 20%	93	100	.49
Antimycin A, 2.5 μg/ml	97	95	.45
Antimycin A, 40 μg/ml	97	62	.30
HQNO, 0.7 μg/ml	46	31	.46
HQNO, 1.4 μg/ml	35	15	.19
Rotenone, 5 × 10^{-4} M	98	87	.41
Amytal, 10^{-3} M	98	75	.37

[a] Incubation under 70% H_2 + 30% O_2 of complete reaction mixtures (see Materials and Methods). Protein concentration, 4 mg/ml; incubation temperature, 30 C. Incubation time was 10 min.

[b] Values in the control with no inhibitors were as follows: Δ μmoles of O_2/hr, 9.2; Δ μmoles of inorganic phosphate/hr, 4.26.

TABLE 6. *Effect of respiratory chain inhibitors on oxidative and phosphorylative activity with succinate*[a]

Inhibitor	Percentage of control oxidative activity	Percentage of control phosphorylative activity	P/O
Control[b]			0.33
Antimycin A, 2.5 μg/ml	84	47	0.19
Antimycin A, 5 μg/ml	69	0	0
HQNO, 0.7 μg/ml	91	98	0.36
HQNO, 1.4 μg/ml	80	89	0.37
HQNO, 2.8 μg/ml	70	81	0.39

[a] Incubation under air of complete reaction mixtures (see Materials and Methods). Protein concentrations: 4 mg/ml; incubation temperature, 30 C; succinate concentration, 10 μmoles/ml; incubation time, 20 min.

[b] Values in the control with no inhibitors were as follows: Δ μmoles of O_2/hr, 4.65; Δ μmoles of inorganic phosphate/hr, 1.56.

As will be discussed later, the reduction of NAD by H_2 was not inhibited by HQNO.)

The results indicate that HQNO acts at a lower potential than antimycin A, and, if one assumes the succinate-fumarate couple to be at a potential of cytochrome *b*, the site of action of HQNO must be located at a lower potential than cytochrome *b*. A similar inhibitory action of HQNO was also observed with *E. coli* by Lightbown and Jackson (18). The results imply that, with H_2 as substrate, oxidation occurs of an electron carrier located before the antimycin A-sensitive site and either at, or after, the HQNO-sensitive site. If the reactive site of HQNO is close to cytochrome *b*, oxidation at such a potential can be surmised.

Both amytal and rotenone, potent inhibitors of phosphorylation in mitochondrial systems (5, 6, 16, 27) proved ineffective with respect to oxidative and phosphorylative activity in the hydrogenomonad system. A similar observation concerning electron transport was reported by Jacobs and Wolin (13, 14).

NAD participation. Observations by Repaske (24) have shown that the presence of trace amounts of oxygen significantly decreases the reduction by hydrogen of added NAD. If, however, oxygen was provided in the presence of hydrogen under otherwise similar conditions, a rate of hydrogen oxidation was observed commensurate with the rate of NAD reduction in the absence of oxygen. In addition, Repaske and Lizotte (25) observed a relatively low rate of $NADH_2$ oxidation in unsubstituted extracts of *H. eutropha*. These findings make the participation of NAD in the electron-transport sequence from hydrogen to oxygen questionable. To investigate this aspect in more detail, experiments were conducted in the presence of a $NADH_2$ trapping system and a NAD analogue. It was assumed that the lactate dehydrogenase (E.C. 1.1.1.28) trap would quantatively oxidize $NADH_2$, thus preventing electron flow through the chain and consequently inhibiting phosphorylation. That the trapping system was functioning was evident from the formation of lactate in the reaction mixture. As shown in Table 7, neither the oxidative nor the phosphorylative activity was affected by NAD, the trapping system, or the NAD analogue. These observations support the assumption that an effective electron flow from hydrogen to oxygen exists, which, to a large extent, can bypass NAD, and that NAD is not acting as a cofactor in the phosphorylation associated with the oxyhydrogen reaction.

Role of quinones. The occurrence of CoQ_8 in *H. eutropha* was reported by Lester and Crane (17), but Repaske and Lizotte (25) were unable to demonstrate a Q_8 mediated oxidation of $NADH_2$. Inasmuch as quinones have been shown to be essential cofactors in the oxidative metabolism, e.g., *Escherichia coli* (15), and since it was found that Dicumarol uncoupled phosphorylation associated with hydrogen oxidation (see Table 4), its participation in electron transport seemed likely. To test this assumption, the effect of cyclohexane extraction on phosphorylative and oxidative activity was tested. Lyophilized cells were treated with cyclohexane and, subsequently, the crude extract was prepared as usual. (The ultraviolet spectrum of the concentrated supernatant fluid exhibited the distinct absorption characteristic of a quinone.) The rate of phos-

TABLE 7. *Effect of $NADH_2$-trapping system and 3-acetyl-pyridine-NAD analogue on phosphorylative activity*

Treatment	Δ μmoles of inorganic phosphate	Δ μatoms of O_2	P/O
Control	6.4	10.2	.64
+ $NADH_2$ trap	6.2	9.9	.63
Control (+5 × 10^{-5} M NAD)	6.5	8.5	.76
+ $NADH_2$ trap	6.3	8.7	.72
Control	6.6	9.2	.72
+ NAD analogue (5 × 10^{-4} M)	7.0	10.4	.67

[a] Reaction mixture contained 5 mg of protein, 10 μmoles of pyruvate, and 20 μg of lactic dehydrogenase (Sigma Chemical Co.) per ml. Gas phase: 70% H_2 + 30% O_2; temperature, 30 C.

phorylation and of hydrogen oxidation was measured with these extracts, and the results were compared with preparations of lyophilized cells which were not extracted by cyclohexane. The results shown in Table 8 indicate that cyclohexane extraction completely eliminates phosphorylation and, also, that the oxidative activity is strongly diminished.

The decline in oxidative activity due to extraction with cyclohexane varied, but a residual activity of approximately 25% was observed in all cell-free preparations and in whole cells. A residual oxidative activity of the same order of magnitude was consistently observed in cell-free preparations which were not extracted, but were inhibited by HQNO (see Table 5). These observations suggested the existence of a pathway which is not mediated by an extractable cofactor and is not inhibited by HQNO. To investigate this assumption, the effect of HQNO was tested on cell-free preparations which were extracted by cyclohexane. As recorded in Table 8, the residual oxidative activity was found to be relatively insensitive to HQNO. Thus, the residual flow observed with preparations not treated with cyclohexane but inhibited by HQNO, and the residual flow observed with cyclohexane-treated preparations may well occur via a similar pathway.

To characterize this pathway further, attempts were made to determine whether these extracts were still able to reduce NAD in the presence and the absence of HQNO. It was found that the rates of NAD reduction by cyclohexane-extracted preparations were of the same order of magnitude as the rates observed with preparations not treated with the solvent, and that the reduction of NAD was insensitive to HQNO (see Table 8). These observations may be taken as presumptive evidence that the residual electron flow which is observed after cyclohexane extraction, or in the presence of HQNO, occurs via a pathway in which NAD functions as a cofactor. This pathway may be identical to $NADH_2$ cytochrome *c* reductase characterized by Repaske and Lizotte (25) in cyclohexane-extracted preparations of *H. eutropha*.

DISCUSSION

The data presented demonstrate that cell-free extracts of hydrogen bacteria contain enzyme systems required for phosphorylation coupled to hydrogen oxidation. The formation of ATP was observed to be limited to the segment of the

TABLE 8. *Effect of cyclohexane extraction on electron transport and phosphorylation*[a]

Treatment	Gas phase	Δ μmoles of inorganic phosphate/hr	Δ μatoms of O_2 / hr	P/O	Δ μmole $NADH_2$/hr
Control[b]	70% H_2 + 30% O_2	1.72	8.64	0.20	—
Extracted	70% H_2 + 30% O_2	0	2.48	0	—
Control	70% H_2 + 30% O_2		3.30		
+ 5 μg of HQNO	70% H_2 + 30% O_2		1.56		
	100% H_2				1.94
+ 5 μg of HQNO	100% H_2				2.10
Extracted	70% H_2 + 30% H_2		1.76		
+ 5 μg of HQNO	70% H_2 + 30% H_2		1.45		
	100% H_2				0.81
+ 5 μg of HQNO	100% H_2				0.89
Whole cells[c]					
Control	70% H_2 + 30% O_2		12.9		
+ 5 μg of HQNO	70% H_2 + 30% O_2		4.14		
Extracted	70% H_2 + 30% O_2		3.77		
+ 5 μg of HQNO	70% H_2 + 30% O_2		3.77		

[a] Lyophilized cells were extracted with cyclohexane for 30 min and then centrifuged; the remaining cyclohexane was removed from the pellet by vacuum. Subsequently, extracted and nonextracted pellets were resuspended in Tris chloride buffer, and the cell-free extracts were prepared in the usual manner.

[b] Reaction mixture contained approximately 4 mg of protein; all rates were calculated per milligram of protein.

[c] Protein content estimated at 70% of dry weight.

respiratory chain between hydrogen and cytochrome *b*. The evidence supporting this view was obtained from inhibition studies with cyanide, azide, and carbon monoxide, as well as with antimycin A. The fact that uncoupling agents (DNP, CCCP, and Dicumarol) abolish phosphorylation, although they are ineffective with respect to oxidative activity, indicates that phosphorylation is limited to oxidative reactions.

A low sensitivity to DNP (3×10^{-4} M gave approximately 50% inhibition) was observed. Similar observations were made by Rose and Ochoa (26) with the phosphorylation system of *Azotobacter*. They observed, moreover, that DNP as uncoupler was particularly ineffective in the presence of hydrogen as electron donor, and they suggested that insensitivity could be related to the fact that the phosphorylation system of *Azotobacter* is already uncoupled. This phenomenon could also account for the relatively low sensitivity to DNP of the hydrogenomonad system. However, the relatively low effectiveness of DNP as uncoupler in the presence of hydrogen as electron donor may also be the result of its reduction to 2-amino-4-nitrophenol, as Naik and Nicholas (19) observed with *Azotobacter*.

Of interest are the results which have been presented concerning the electron-transport inhibitor HQNO, particularly in respect to the inability of antimycin A to substitute for HQNO. The results presented show that HQNO inhibited phosphorylative and oxidative activity with hydrogen as electron donor. Similar concentrations caused no inhibition of hydrogenase activity or of phosphorylation linked with succinate oxidation. The HQNO effect must thus be linked with an electron carrier between hydrogenase and cytochrome *b*, and prevent the reduction of cytochrome *b*. Although the precise interaction of HQNO with the electron-transport chain has evaded detection, its inability to inhibit the reduction of NAD would lead one to assume that the site of action is at a potential which is relatively close to cytochrome *b*.

The nature of the mechanism involved in the oxidation of cytochrome *b* is not understood. Clearly, a rapid decomposition of H_2O_2, presumably formed upon the reaction of cytochrome *b* with oxygen, would occur in this catalase-positive organism. However, preliminary results have indicated that, also in the presence of catalase inhibitors (CN^-, N_3^-, and CO), no appreciable accumulation of H_2O_2 is found. Similarly, reaction mixtures equilibrated with an atmosphere of hydrogen decomposed added H_2O_2 too slowly to accommodate the relatively high rate of hydrogen oxidation observed with these extracts. It therefore seems questionable that the disposal of peroxide, if formed, occurs via a peroxidative oxidation of hydrogen, a mechanism demonstrated in *Acetobactor peroxidans* (28) and *Vibrio succinogenes* (13, 14).

Relatively little is known about the electron-transport sequence at the reducing site of the chain. Since we have been unable to demonstrate an inhibiting effect on phosphorylation and hydrogen oxidation of the presence in the reaction mixtures of NAD analogue or of an NAD-trapping system, it would appear that NAD plays no part in the electron-transport sequence of the oxyhydrogen reaction. This assumption is further supported by the fact that $NADH_2$ oxidation proceeds at a relatively slow rate. The observations of Repaske (24, 25), that the reduction of NAD by hydrogen is strongly inhibited by trace amounts of oxygen and that its oxidation requires the addition of menadione (K_3) support the view that NAD is not participating in the sequence of reactions leading to ATP formation by the system described in this paper.

At the present time, little can be said concerning the inability of amytal and rotenone to substantially inhibit electron transport and ATP formation in the hydrogenomonad system. Similar observations concerning NAD participation and amytal inhibition were made by Jacobs and Wolin (14) with the vibrio system. The fact that NAD plays no role in either system may be related to the fact that these inhibitors are ineffective. Inhibition by these inhibitors is observed with intact mitochondria (5) and submitochondrial particles (27). Electron transport of the hydrogenomonad and the vibrio system apparently proceeds through an amytal-insensitive pathway in which NAD does not participate and cytochrome *b* functions as a terminal oxidase.

A tentative electron-transport scheme which in part accounts for the above findings is illustrated in Fig. 2. In this scheme, "X" represents a primary acceptor which is not yet identified. A quinone is included in the "abbreviated" pathway. Although the exact function of quinones in the electron transport of hydrogenomonads has yet to be defined, observations presented in this paper concerning the effects of cyclohexane treatment indicate their possible involvement in oxidative phosphorylation and electron transport of the hydrogenomonad system.

Experiments described in this paper support the conclusion that the abbreviated chain ($H_2 \rightarrow$ cytochrome *b* $\rightarrow O_2$) is a predominant pathway. It is not yet known whether this reaction between

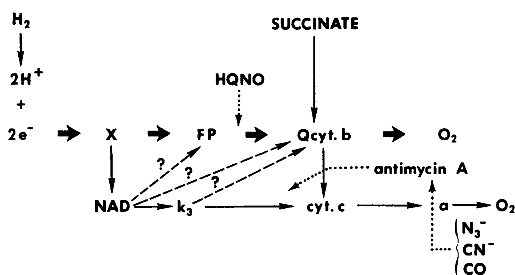

FIG. 2. *Tentative electron-transport scheme (see text).*

cytochrome *b* and oxygen is a preparative artifact, a culturally induced phenomenon, or an alternate pathway employed for the sake of simple expediency. It may well be that the operation of the inefficient abbreviated pathway can provide the cell with energy much more rapidly than energy can be obtained via the common respiratory chain. Studies concerning the growth yield, to be reported in a forthcoming paper, suggest this as a reasonable hypothesis.

The scheme presented here must be regarded as a tentative one, because several facts, particularly with respect to electron transfer from $NADH_2$ to oxygen, have not been experimentally established. On the other hand, it may well be that the primary function of pyridine nucleotide, reduced by "X," is to provide reducing equivalents for the reduction of CO_2, rather than to provide the cell with energy via oxidative phosphorylation. This formulation could, in principle, account for several phenomena discussed here.

ACKNOWLEDGMENTS

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