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Phosphorylation and the Reduced Nicotinamide Adenine Dinucleotide Oxidase Reaction in *Streptococcus agalactiae*

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Cell-free extracts from aerobically grown *Streptococcus agalactiae* cells possess a reduced nicotinamide adenine dinucleotide (NADH) oxidase which is linked to oxygen. It is inhibited by cyanide, although cytochromes evidently are not involved. Adenosine triphosphate (ATP) formation occurs during the reaction, but 66 to 75% of the total ATP is formed nonoxidatively. The remaining 25 to 35% of the ATP formation is related to the oxidation of NADH. The formation of ATP in the oxidative reaction can be prevented by excluding oxygen or adding cyanide to prevent NADH oxidation. It can also be prevented by adding methylene blue or pyruvate, which bypasses electron transport to oxygen, but does not interfere with NADH oxidation. Potential sources of ATP, such as glycolysis, the pyruvate oxidase reaction, or the oxidative pentose cycle, are not present, and the high nonoxidative ATP formation is ascribed to the adenylate kinase reaction. The reaction requires adenosine diphosphate (ADP) as a phosphate acceptor. NADH oxidation is independent of ADP. Antimycin A, amytal, and 2,4-dinitrophenol decreased, but did not prevent, oxidative formation of ATP. P:O ratios ranged from 0.15 to 0.25. All of the oxidative activity was in the soluble portion of the cell-free extracts.

Gallin and VanDemark (6) have reported evidence for oxidative phosphorylation during reduced nicotinamide adenine dinucleotide (NADH) oxidation with cell-free extracts of *Streptococcus faecalis* 10Cl. Smalley et al. (15) determined molar growth yields of *S. faecalis* 10Cl grown aerobically and obtained evidence to substantiate the occurrence of oxidative phosphorylation. No other reports of oxidative phosphorylation linked to the respiration of streptococci, or other bacteria not containing cytochromes have been made. The respiration of cell suspensions and the NADH oxidation by cell-free extracts of *S. agalactiae* 50 are both inhibited by cyanide. No cytochromes have been found. Under aerobic conditions, cell suspensions metabolized up to 50% of the glucose used by the oxidative route, yielding acetate, acetylmethylcarbinol, and carbon dioxide (10). The subject of this report is a measurement of adenosine triphosphate (ATP) formation during NADH oxidation by cell-free extracts prepared from aerobically grown cells of a culture of *S. agalactiae*.

MATERIALS AND METHODS

The culture of *S. agalactiae* 50 was from the collection at the National Animal Disease Laboratory (10). Cell-free extracts of *S. agalactiae* 50 from cells grown aerobically were prepared, and NADH oxidase activity was assayed as described earlier (10). The extracts contained 2 to 4 mg of protein per ml as determined by the method of Stadtman et al. (16). The extracts were usually assayed for activity on the day they were prepared; if stored for several days, they were held at −60 C. After thawing, they were kept in an ice bath during use.

Measurement of phosphorylation. Phosphorylative activity was usually assayed by the spectrophotometric procedure of Pinchot (13), though the manometric method (2) also was used.

Reactions leading to ATP formation in the cell extracts were as follows:

$NADH_2$ oxidase reaction

$$NADH + H^+ + ADP + P_i \xrightarrow{\frac{1}{2}O_2} NAD^+ + ATP + H_2O$$

Adenylate kinase (endogenous) reaction

$$2\ ADP \underset{\text{(EC 2.7.4.3)}}{\rightleftharpoons} AMP + ATP$$

Spectrophotometric measurement of ATP was done with hexokinase, glucose-6-phosphate dehydrogenase, Mg^{++}, glucose, and nicotinamide adenine dinucleotide phosphate (NADP) as reagents. The reactions were as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow[(\text{EC } 2.7.1.1)]{\text{hexokinase, } Mg^{++}} \text{glucose-6-phosphate} + \text{ADP} + H^+$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow[(\text{EC } 1.1.1.49)]{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconic acid} + \text{NADPH} + H^+$$

Determination of ATP, NADH, inorganic phosphate, and lactic acid. When the manometric method was used for the measurement of phosphorylation, the reaction vessels were removed from the manometers and immediately placed in an ice bath. The flask contents were quickly transferred into small tubes and placed in a boiling-water bath for 10 min to stop enzymatic activity. After centrifugation, ATP was determined in a sample of the supernatant liquid by the method of Kornberg (9). NADH was determined spectrophotometrically from the extinction of 6.22×10^3 liters $\times$ mole$^{-1} \times$ cm^{-1} (8). Inorganic phosphate was determined by the Fiske and SubbaRow method (17).

After the spectrophotometric measurement of phosphorylation, lactic acid in the reaction mixture was determined by the lactic dehydrogenase procedure (1). A 1-ml amount of 10% trichloroacetic acid was added to the contents of the absorption cell when the reaction was terminated, the volume made to 5 ml, and the mixture was centrifuged. A 1-ml portion of the supernatant fluid was neutralized to *p*H 7, treated with 0.5 ml of 20% $CuSO_4$ and 0.5 g of $Ca(OH)_2$, and made to 5 ml. The mixture was centrifuged and samples of the supernatant fluid were assayed for lactic acid.

Phosphorus-32 experiments. ^{32}P was obtained as $Na_3{}^{32}PO_4$ from Nuclear Corp., Skokie, Ill. The reaction mixture, in a 2-ml volume, contained 200 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.4, 0.2 μmole of NADH, 5 or 10 μmoles of inorganic phosphate, and $Na_3{}^{32}PO_4$ to give 15,000 to 30,000 counts per min per μmole of inorganic phosphorus. After a reaction period of 4 to 10 min, during which the oxidation of NADH was followed by measuring the decrease in absorbancy at 340 nm in a Beckman DU-2 spectrophotometer, 0.2 ml of 50% trichloroacetic acid was added, and the precipitated protein was separated by centrifugation. The supernatant fluid (1.8 ml) was treated by the procedure of Nielsen and Lehninger (11) to remove the inorganic phosphate. A portion of the aqueous phase was dried on planchets under a heat lamp and counted in a thin-window gas-flow counter (Nuclear-Chicago, model 186A). (I am grateful to Milton Allison for assistance in carrying out the experiments with ^{32}P.) A control with heat-inactivated cell-free extract was used in all experiments.

An alternative method of Crane and Lipmann (4), in which the nucleotides were absorbed on charcoal, was also used. The Norit A was dried on planchets under a heat lamp and counted in a thin-window gas-flow counter.

Anaerobic reactions. All of the reactants except the cell-free extract were flushed in an anaerobic absorption cell for 6 min with N_2 freed from O_2 by passage over heated copper. The N_2 inlet was by needle through a rubber ampoule closure. The exit was closed, the N_2 inlet needle was removed, and the reaction was initiated by injecting cell-free extract through the rubber closure. Care was taken that the syringe was completely filled with extract to eliminate all oxygen except that dissolved in the 0.1 ml of extract injected.

Adenylate kinase activity of crude cell-free extracts. A 0.1-ml amount of an active cell-free extract (2.5 mg of protein per ml) was incubated for 10 min at room temperature with 1 μmole of adenosine diphosphate (ADP) and 300 μmoles of Tris buffer, *p*H 7.4, in a volume of 3 ml. The reaction mixture was then placed in a boiling-water bath to destroy enzyme activity and was centrifuged; a sample of the supernatant fluid was used to measure ATP by the procedure of Kornberg (9). A control consisted of 0.1 ml of the cell-free extract and 0.9 ml of distilled water. This mixture was heated in a boiling-water bath for 60 min to inactivate adenylate kinase and then cooled, after which 1 μmole of ADP and 300 μmoles of Tris buffer were added in a final volume of 3 ml. The ADP preparation was free from ATP activity.

Adenosine triphosphatase. Adenosine triphosphatase activity of the cell-free extracts was measured by incubating a 1-ml reaction mixture containing 100 μmoles of Tris buffer, *p*H 7.4, 1 μmole of ATP, 10 μmoles of $MgSO_4$, and 0.10 ml of cell-free extract for 15 min at room temperature. A 1-ml amount of 10% trichloroacetic acid was added, and samples of the clear supernatant fluid were analyzed for inorganic phosphate.

Sources of chemicals. NADH, NADP, guanosine-5-diphosphate (GDP), cytidine-5-diphosphate (CDP), ATP, hexokinase, and glucose-6-phosphate dehydrogenase were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Adenosine monophosphate (AMP) was purchased from Sigma Chemical Co., St. Louis, Mo. ADP and antimycin A were purchased from Nutritional Biochemicals, Corp., Cleveland, Ohio, and sodium amytal was a gift from Eli Lilly & Co., Indianapolis, Ind.

RESULTS

Phosphorylative activity of cell-free extracts. Cell-free extracts of *S. agalactiae* 50 form ATP during NADH oxidation. However, 65 to 75% of the total ATP formed during the assay of phosphorylative activity is formed in the absence of NADH oxidation. Table 1 shows typical results obtained on measurement of phosphorylative activity with six different cell-free extracts.

The total reduced NADP (NADPH) minus the NADPH formed in the nonoxidative reaction is that linked to NADH oxidation, and is the value from which the P:O ratio was calculated. It is also referred to as the "net" ATP formed. Each mole of NADPH formed was presumably equivalent to a mole of ATP. Under aerobic conditions, there was always ATP formed in excess of the nonoxidative values. Assays of phosphorylative activity made manometrically, in the absence of the glucose-hexokinase trap for ATP, gave P:O ratios of the same magnitude (Table 2).

NADH oxidation was depressed by anaerobic conditions or by cyanide. When cyanide was present, NADH oxidation was reduced about 40 to 50%, and there was no ATP formed in excess of that formed in the nonoxidative reaction. Under anaerobic conditions, NADH oxidation was depressed as much as 80%, and the "total" NADPH values were much less than that measured in the nonoxidative reaction. A negative value would appear in column 4 of Table 2 if the "nonoxidative" NADPH was subtracted from the unusually low "total" NADPH value. There was no net increase in ATP under anaerobic conditions or with cyanide present (Table 3).

The need for ADP as a phosphate acceptor is shown in Table 4, where AMP, GDP, and CDP were substituted for ADP. No ATP was formed in the absence of ADP, though NADH oxidation was the same regardless of which nucleotide was added as a potential phosphate acceptor. The respiration proceeding through the NADH oxidase system in *S. agalactiae* does not appear to be tightly coupled with phosphorylative activity, since substrate oxidation was not dependent on the presence of ADP.

TABLE 1. *Phosphorylation and NADH oxidation in cell-free extracts of S. agalactiae*[a]

Expt	Amt (μmoles) per mg of extract protein				P:O ratio
	NADH oxidized	Total NADPH	Nonoxidative NADPH	NADPH from NADH oxidation	
1	0.414	.300	.202 (68)[b]	.098	.24
2	0.705	.540	.353 (66)	.187	.27
3	0.830	.795	.540 (68)	.225	.27
4	1.100	.770	.580 (75)	.190	.17
5	0.492	.350	.264 (76)	.086	.18
6	0.565	.402	.295 (74)	.107	.19

[a] Reactants: 300 μmoles of Tris buffer, *p*H 7.4; 100 μmoles of glucose; 15 μmoles of $MgCl_2$; 0.3 μmole of NADH; 0.3 μmole of NADP; 0.5 μmole of ADP; hexokinase, 1.5 units (5); glucose-6-phosphate dehydrogenase, 0.4 unit (12), 0.15 mg of crystallized bovine albumin; cell-free extract, 0.2 to 0.4 mg of protein. Volume, 3.0 ml. Reaction initiated by addition of NADH. NADH omitted in measuring nonoxidative NADPH formation. Hexokinase, glucose-6-phosphate dehydrogenase, NADP, and ADP omitted in measuring NADH oxidation.

[b] Percentage of total NADPH formed nonoxidatively.

TABLE 2. *Manometric measurement of phosphorylation during NADH oxidation by S. agalactiae 50*[a]

Expt	NADH (μmoles) oxidized	ATP (μmoles) formed	O_2 (μmoles) consumed	O_2 (μatoms)	P:O ratio
1	No NADH	1.15	0.31		
	2.86	1.33	1.10	1.58	.114
2	No NADH	1.08	0.13		
	2.86	1.42	1.45	2.64	.129
3	No NADH	1.50	0		
	3.00	1.80	1.20	2.40	.125

[a] Reactants: 5 μmoles of K_2HPO_4; 4 μmoles of NADH; 0.4 μmole of ADP; cell-free extract equivalent to 1 to 1.25 mg of protein. Volume, 2.0 ml.

Several compounds known to inhibit oxidative phosphorylation in mammalian mitochondrial systems were tested for their effect on the phosphorylative activity of *S. agalactiae* (Table 5). In each, the cell-free extract and all components of the reaction mixture (except NADH) were incubated with the inhibitor for 5 min before initiation of the reaction with NADH. Antimycin A, amytal, and 2,4-dinitrophenol reduced, but did not abolish, "net" ATP formation. Cyanide caused complete inhibition of "net" ATP synthesis and reduced NADH oxidation. Methylene blue and pyruvate both abolish "net" ATP synthesis without affecting NADH oxidation.

Pyruvate added to the reaction mixture in the absence of NADH caused no increase in the endogenous ATP formation, which suggested that a pyruvate oxidase reaction could not be a source of endogenous ATP (Table 6).

Lactic acid analyses were made after termination of the NADH oxidase reaction in the complete reaction mixture, which included the glucose-hexokinase ATP trap, and there was no increase in lactic acid over that found in the controls run with inactivated extracts. This finding appeared to rule out glycolysis as a source of the endogenous ATP. These data are shown in Table 6.

The possibility existed of NAD acting as an electron acceptor when glucose was present to yield pyruvate and NADH. The NADH would then provide a substrate for ATP formation with-

TABLE 3. *Phosphorylation and NADH oxidation in cell-free extracts of S. agalactiae: effect of cyanide and anaerobic conditions*[a]

Conditions	Amt (μmoles) per mg of extract protein				P:O ratio
	NADH oxidized	Total NADPH	Nonoxidative NADPH	NADPH from NADH oxidation	
Aerobic	.830	.795	.540 (68)[b]	.255	.32
Anaerobic					
(1)	.310	.030	.460		.00
(2)	.178	.094	.363		.00
No cyanide	.820	.645	.477 (74)[b]	.168	.21
Cyanide, 0.001 M	.520	.477	.477 (100)		.00
No cyanide	.860	.557	.456 (82)	.101	.12
Cyanide 0.001 M	.437	.475	.485 (100)		.00

[a] Reactants: solution volume and concentration of reactants same as in Table 1. Reactants incubated with cyanide for 5 min before starting reaction by addition of NADH.
[b] Percentage of total NADPH formed nonoxidatively.

TABLE 4. *Phosphorylation and NADH oxidation in cell-free extracts of S. agalactiae: phosphate acceptors*[a]

Conditions	Amt (μmoles) per mg of extract protein				P:O ratio
	NADH oxidized	Total NADPH	Nonoxidative NADPH	NADPH from NADH oxidation	
Complete	.650	.527	.415 (79)[b]	.112	.18
− ADP	.650	.00	.00 (—)	.00	.00
− ADP, + AMP	.650	.00	.00 (—)	.00	.00
− ADP, + GDP	.650	.00	.00 (—)	.00	.00
− ADP, + CDP	.650	.00	.00 (—)	.00	.00

[a] Reactants: 0.5 μmole of AMP, ADP, GDP, or CDP per 3.0 ml. All other reactants the same as in Table 1.
[b] Percentage of total NADPH formed nonoxidatively.

out the formation of lactic acid. However, NAD (or NADP) was not reduced by cell-free extracts of *S. agalactiae* when they were incubated in the presence of glucose. The reaction was carried out in an atmosphere of N_2 to prevent subsequent oxidation of the NADH by the oxidase activity on the extract. Substrate level phosphorylation appears not to be a source of net ATP.

When 1 μmole of ADP was incubated with crude cell-free extract for 10 min, 0.34 μmole of ATP activity was found. This was equal to 0.137 μmole of ATP per mg of protein in the cell-free extract, and it confirms the presence of adenylate kinase activity in the absence of the components of the ATP trapping system.

The adenylate kinase from *S. agalactiae* resembled rabbit muscle myokinase (3). It was not inactivated at 100 C for 10 min, and at equilibrium ADP was 69% converted to AMP and ATP.

No adenosine triphosphatase activity was found in the cell-free extracts.

The reaction was conducted in the absence of glucose, hexokinase, NADP, and glucose-6-phosphate dehydrogenase and in the presence of ^{32}P-*ortho*-phosphate to determine whether phosphorus was esterified during NADH oxidation. In a number of experiments when P:O ratios were calculated from the labeled inorganic phosphate esterified and NADH oxidized, the values ranged from 0.01 to 0.80 (Table 7). In the heat- or acid-inactivated cell-free extract controls, there was always some radioactivity that was not removed by successive washing of the Norit A or by extraction of the phosphomolybdic acid complex with *n*-butyl alcohol-benzene. The omission of ADP resulted in negligible incorporation of radioactivity in the organic fraction. The radioactivity in the organic fraction when NADH was omitted was subtracted from the radioactivity

TABLE 5. *Phosphorylation and NADH oxidation in cell-free extracts of S. agalactiae: inhibitors of NADH oxidation and phosphorylation*[a]

Inhibitor	Concn (M)	Amt (μmoles) per mg of extract protein				P:O ratio
		NADH oxidized	Total NADPH	Nonoxidative NADPH	NADPH from NADH oxidation	
None	—	0.820	.645	.477 (74)[b]	.168	.21
Amytal	0.25×10^{-2}	0.770	.605	.520 (86)	.085	.12
Antimycin A	0.80×10^{-5}	0.813	.530	.448 (84)	.082	.10
Cyanide	1.0×10^{-3}	0.520	.477	.477 (100)	.00	.00
None	—	0.850	.815	.575 (71)	.240	.28
2,4-DNP[c]	0.80×10^{-5}	0.900	.695	.583 (84)	.112	.12
None	—	1.00	.690	.540 (78)	.150	.15
Pyruvate	0.33×10^{-3}	0.98	.630	.655 (100)	.00	.00
Methylene blue	0.80×10^{-5}	1.02	.523	.490 (94)	.033	.032

[a] Reactants: except for inhibitors, same as in Table 1; volume, 3.0 ml; inhibitors incubated for 5 min with reactants before initiating the reaction with NADH.
[b] Percentage of total NADPH formed nonoxidatively.
[c] 2,4-Dinitrophenol.

measured in the complete reaction mixture in calculating the P:O ratio.

When the cell-free extract was centrifuged at 100,000 × *g* for 1 hr, a reddish-brown pellet of particulate material was sedimented. The particulate fraction contained less than half the total NADH oxidase activity of the cell-free extract and formed no ATP above that in the nonoxidative reaction. When the supernatant and particulate fractions were recombined, the NADH oxidase and phosphorylative activity were the same as that of the supernatant fluid. The NADH oxidase and phosphorylative activity was all in the soluble portion of the cell-free extract (Table 8).

TABLE 6. *Absence of glucolysis and pyruvate oxidase during measurement of phosphorylative activity*

Expt	Lactic acid (μmoles) in total reaction[a]				NADPH (μmoles) formed in total reaction[b]	
	Complete	Minus NADP	Minus NADH	Plus inactivated enzyme[c]	No NADH	No NADH + 1 μmole of pyruvate
1	0.90	0.90	0.80	0.90	0.113	0.117
2	0.41	0.49	0.47	0.50	0.151	0.151
3	0.80	0.78	0.78	0.82	0.168	0.153

[a] Reactants same as in Table 1, except for omissions. Volume, 3.0 ml.
[b] Reactants same as in Table 1, except for omission of NADH and addition of pyruvate.
[c] Enzyme inactivated with trichloroacetic acid.

DISCUSSION

The NADH oxidase of *S. agalactiae* does not appear to have an electron transport chain which is efficient in synthesizing ATP. Though ATP formation was dependent on the presence of ADP, the oxidation of NADH was independent of ADP. About two-thirds to three-fourths of the total ATP formed was formed nonoxidatively. The remaining 25 to 30% of the ATP formed appeared to be related to NADH oxidation. This "net" ATP or oxidative ATP formation could be prevented by excluding oxygen from the reaction mixture, by using cyanide as an NADH oxidation inhibitor or bypassing electron flow to oxygen with methylene blue or pyruvate. This evidence suggests that a small but definite portion of the ATP synthesis by *S. agalactiae* is regulated by NADH oxidation with oxygen as the electron acceptor, and may be referred to as oxidative phosphorylation. Endogenous ATP formation was rapid and was not dependent on oxygen.

The reason for the low "total" NADPH values in the anaerobic experiments (Table 3) is not known. One would expect these values to equal the "nonoxidative" NADPH values if the adenylate kinase activity was the same in the complete reaction system as it was when measured separately. The "total" NADPH values in anaerobic measurements were always much lower than the nonoxidative NADPH. NADH oxidation was

TABLE 7. *^{32}P Incorporation into nucleotide during NADH oxidation by cell-free extracts of S. agalactiae 50*

Reactants[a]	NADH oxidized (μmoles)	Counts per min incorporated per mg of protein	Corrected for inactivated extract	^{32}P incorporated (μmoles)	P:O
Complete[b]	0.57	7,400	3,370	.263	.27
− NADH	—	5,480	1,350	.105	
− ADP	0.57	5,500	1,370	.107	
Control	0	4,130			
Complete	0.77	42,816	5,536	.430	.12
− NADH	—	41,800	4,520	.341	
− ADP	0.77	34,164	0		
Control	0	37,280			
Complete	0.50	57,232	6,627	.418	.83
− NADH	—	50,681	76		
− ADP	0.50	45,451	0		
Control	0	50,605			
Complete	0.46	563	270	.0197	.013
− NADH	—	480	187	.0137	
− ADP	0.46	355	62	.004	
Control	0	293			

[a] Reactants: 200 μmoles of Tris buffer, *p*H 7.4; 0.2 μmole of NADH; 0.5 μmole of ADP; 5 or 10 μmoles of inorganic phosphate; 9 μmoles of $MgCl_2$; ^{32}P as Na_3PO_4 to give 15,000 to 30,000 counts per min per μmole of inorganic phosphate. Volume, 2.0 ml.

[b] Procedure of Crane and Lipmann (4); others, procedure of Nielsen and Lehninger (11).

inhibited by as much as 80% when oxygen was excluded. Cyanide did not prevent NADH oxidation as completely as exclusion of oxygen, and the "total" NADPH value was equal to that of the "nonoxidative" NADPH formed. A net increase in ATP is dependent on respiration linked to oxygen.

The formation of ATP from ADP by cell-free extracts without the presence of the glucose-hexokinase trapping system suggested that the endogenous ATP formation is due to adenylate kinase activity. This view is further supported by evidence for the lack of a pentose oxidative cycle in whole cells and of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity in cell-free extracts (10), and also by evidence for a lack of glucolysis or pyruvate oxidase activity in the cell-free extracts. The cell-free extract was unable to catalyze the reduction of NAD or NADP with glucose as the substrate.

2,4-Dinitrophenol, the classical uncoupling agent for oxidative phosphorylation in the more highly organized systems of mammalian mitochondria and of some bacteria, decreased but did not abolish the oxidatively formed ATP. This fact and the lack of dependence of NADH oxidation on the presence of a phosphate acceptor indicate that *S. agalactiae* possesses an uncoupled or loosely coupled phosphorylative system. Antimycin A and amytal also decreased but did not abolish oxidatively linked phosphorylation. Evidence that flavine reduction is the first step in electron transport during NADH oxidation (10) would suggest the presence in the electron transport chain of at least one phosphorylation site. The P:O ratios measured were all considerably less than one, which suggests that this single electron transfer step in cell extracts is not tightly coupled to ATP synthesis. It is of interest that, when cell-free extracts were tested for ability to split inorganic phosphate from ATP, no adenosine triphosphatase activity was found. Coupling factor F_1 isolated from beef heart mitochondria possessed adenosine triphosphatase activity (14, 18). Low P:O ratios seem to be a characteristic of soluble bacterial oxidative systems (7). The oxidative system of *S. agalactiae* remains in the supernatant fluid when cell-free extracts are centrifuged at 100,000 × *g*.

TABLE 8. *Phosphorylation and NADH oxidation in cell-free extracts of S. agalactiae: fractionation of extract by centrifugation at 100,000 × g, 1 hr, 4 C*[a]

Prepn	Amt (μmoles) per mg of extract protein				P:O ratio
	NADH oxidized	Total NADPH	Nonoxidative NADPH	NADPH from NADH oxidation	
Cell-free extract	1.11	.780	.557 (71)[b]	.223	.20
Supernatant fluid (S), 100,000 × g, 1 hr	0.750	.492	.376 (63)	.116	.16
Particulate (P)	0.390	.420	.465 (100)	.00	.00
Combined (S + P)	0.750	.415	.308 (74)	.107	.14

[a] Reactants: same as in Table 1.

[b] Percentage of total NADPH formed nonoxidatively.

Data suggesting oxidative phosphorylation during NADH oxidation were reported for *S. faecalis* (6, 15). Though *S. faecalis* and *S. agalactiae* are both homofermentative lactic acid bacteria in the absence of oxygen, they respire slowly and their ability to respire is enhanced when the cells are grown aerobically. The phosphorylative activity associated with NADH oxidation in these two streptococci has some important differences. The P:O ratios for the NADH oxidase reaction in the two organisms are similar. In contrast to *S. agalactiae*, oxidative phosphorylation in *S. faecalis* was reported to be completely inhibited by 2,4-dinitrophenol and antimycin A, and to be insensitive to cyanide (6). ATP formation in *S. agalactiae*, linked to NADH oxidation, is cyanide sensitive, and is not completely inhibited by 2,4-dinitrophenol or antimycin A; the NADH oxidation is independent of the phosphate acceptor, ADP. *S. agalactiae* has an active adenylate kinase, whereas *S. faecalis* was not reported to have adenylate kinase activity (6).

The P:O ratios were similar when measured manometrically, spectrophotometrically, or by ^{32}P incorporation. The P:O ratios measured by ^{32}P incorporation represented greater extremes, but, because of the low incorporation and high values obtained in the controls, the values are not as reliable as the manometric or spectrophotometric assays. Nevertheless, the indications are that there is ATP synthesis which is linked to electron transport during NADH oxidation. It is not an efficient energy-conserving system and, because of this fact, its value in the energy economy of the organism is perhaps limited. About 66% of the glucose degraded with aerobically grown cell suspensions is converted to lactic acid, and the remainder is converted to acetate, carbon dioxide, and acetylmethylcarbinol (10). From 100 moles of glucose, there would be an estimated 132 moles of lactic acid and 132 moles of ATP formed. From the 33 moles of glucose not fermented, because of the NADH oxidase reaction, there would be 66 moles of pyruvate and 66 moles of NADH to be oxidized. No evidence for a pyruvate oxidase reaction has been found in the cell-free extracts. If there were no other ATP-yielding reactions (with a measured P:O ratio of 0.25), the net gain is 16 moles of ATP above that of substrate phosphorylation resulting from NADH oxidation. Presumably, a flavine nucleotide reduction is the first step in the electron transport chain of the NADH oxidase preparation. A comparison of cellular yields per mole of glucose consumed with *S. agalactiae* under aerobic and anaerobic growth conditions may allow a further evaluation of the respiratory pathway in terms of energy available to support cell synthesis. It will be of interest to note whether P:O ratios determined by the in vivo and in vitro procedures are similar.

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LITERATURE CITED

1. Bergmeyer, H. V. 1963. Methods of enzymatic analysis, p. 266–270 (translation from original German title "Methoden der Enzymatischen Analyse"). Academic Press Inc., New York.
2. Brodie, A. F. 1963. Oxidative phosphorylation systems: microbial, p. 284–294. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 6. Academic Press Inc., New York.
3. Colowick, S. P. 1951. Adenylate kinase (myokinase), p. 148–150. *In* J. B. Summer and K. Myrbäck (ed.), The enzymes, vol. 2, P + I. Academic Press Inc., New York.
4. Crane, R. K., and F. Lipmann. 1953. The effect of arsenate on aerobic phosphorylation. J. Biol. Chem. **201**:235–243.
5. Darrow, R. A., and S. P. Colowick. 1962. Hexokinase from baker's yeast, p. 226–235. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
6. Gallin, J. I., and P. J. VanDemark. 1964. Evidence for oxidative phosphorylation in *Streptococcus faecalis*. Biochem. Biophys. Res. Commun. **17**:630–635.
7. Gelman, N. S., M. A. Lukayanova, and D. N. Astrovski. 1967. Respiration and phosphorylation of bacteria. Plenum Press, New York. Translation from Russian, Ed. G. P. Pinchot.
8. Horecker, B. L., and A. Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. **175**:385–390.
9. Kornberg, A. 1950. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. J. Biol. Chem. **182**:779–793.
10. Mickelson, M. N. 1967. Aerobic metabolism of *Streptococcus agalactiae*. J. Bacteriol. **94**:184–191.
11. Nielsen, S. O., and A. L. Lehninger. 1955. Phosphorylation coupled to the oxidation of ferrocytochrome C. J. Biol. Chem. **215**:555–570.
12. Noltmann, E. A., C. J. Gubler, and S. A. Kuby. 1961. Glucose-6-phosphate dehydrogenase (Zwischenferment). J. Biol. Chem. **236**:1225–1230.
13. Pinchot, G. B. 1957. A rapid method for measuring phosphorylation coupled to oxidation of reduced diphosphopyridine nucleotide. J. Biol. Chem. **229**:11–23.
14. Racker, E. 1965. Mechanisms in bioenergetics. Academic Press Inc., New York.
15. Smalley, A. J., P. Jahrling, and P. J. Van Demark. 1968. Molar growth yields as evidence for oxidative phosphorylation in *Streptococcus faecalis* 10C1. J. Bacteriol. **96**:1595–1600.
16. Stadtman, E. R., G. D. Novelli, and F. Lipmann. 1951. Coenzyme A function in acetyl transfer by the phosphotransacetylase system. J. Biol. Chem. **191**:365–376.
17. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric techniques, 3rd ed., Burgess Publishing Co., Minneapolis.
18. Vallejos, R. J., S. G. VandenBergh, and E. C. Slater. 1968. On coupling factors and oxidative phosphorylation. Biochim. Biophys. Acta **153**:509–520.