RESPIRATORY PATHWAYS IN THE *MYCOPLASMA*

I. LACTATE OXIDATION BY *MYCOPLASMA GALLISEPTICUM*

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ABSTRACT

SMITH, S. L. (Cornell University, Ithaca, N.Y.), P. J. VAN DEMARK, AND J. FABRICANT. Respiratory pathways in the *Mycoplasma*. I. Lactate oxidation by *Mycoplasma gallisepticum*. J. Bacteriol. **86**:893–897. 1963.—Resting cells of *Mycoplasma gallisepticum* 293 required the addition of nicotinamide adenine dinucleotide, thiamine pyrophosphate, and flavine mononucleotide for the maximal rate of sodium lactate oxidation. Inhibitor studies, as well as spectrophotometric and chemical assays, indicate that the pathway of electron transport to oxygen during lactate oxidation does not involve heme catalysts, and is mediated by flavin-linked enzyme systems. The presence of reduced nicotinamide adenine dinucleotide-specific lactic dehydrogenase, menadione reductase, ferricyanide reductase, and reduced nicotinamide adenine dinucleotide oxidase activities was detected in cell-free extracts. No cytochrome *c* reductase or reduced nicotinamide adenine dinucleotide peroxidase activity was detected in these extracts.

It has been shown by various workers (Rodwell and Rodwell, 1954; Tourtellotte, 1960; Neimark and Pickett, 1960) that glycolysis represents the major metabolic pathway in the fermentative *Mycoplasma* with lactic acid being the major end product under anaerobic conditions. However, with the exception of these studies, little is known concerning the respiratory pathways and energy-yielding mechanisms of the fermentative pleuropneumonia-like organisms (PPLO). Since lactate is a substrate readily used by most strains of *Mycoplasma*, a study of its oxidation by a number of strains of PPLO has been undertaken in our laboratory. The present paper is a report of the studies of lactate oxidation by *M. gallisepticum* (Edward and Kanarek, 1960) 293.

MATERIALS AND METHODS

Culture. M. gallisepticum 293 originally was isolated in 1956 from a field case of chronic respiratory disease in poultry (Calnek and Levine, 1957). Its serological characteristics were described by Fabricant (1960). Since isolation, the organism has been maintained on laboratory media in continuous subculture for over 250 transfers. For this study, the organism was grown from a 1% inoculum in a broth medium consisting of 2.5% beef heart infusion, 10% horse serum, 1% yeast hydrolysate, 0.05% thallium acetate, and 1 million units per liter of penicillin on a reciprocal shaker at 37 C. The cells were harvested after 48 hr by centrifugation at 12,000 × *g* for 20 min and washed twice in distilled water. Resting-cell suspensions were prepared by resuspending the cells in approximately $\frac{1}{20}$ of the original growth volume of distilled water.

Cell-free extracts were prepared by sonic oscillation of a 10% suspension of cells in a 0.5% glutathione solution in a 10-kc oscillator for 10 min. The cell debris was removed by centrifugation at 20,000 × *g* for 1 hr.

Manometric procedures. The oxidation of sodium lactate was followed by conventional manometric methods with air as the gas phase at 37 C; each Warburg vessel contained 100 μmoles of phosphate buffer (pH 7.0), 50 μmoles of sodium lactate, and approximately 3 mg of cells, with a total volume of 3.0 ml per flask. The center well of each vessel contained .15 ml of 40% KOH to absorb carbon dioxide.

Spectrophotometric and chemical assays. Nicotinamide adenine dinucleotide (NAD)-linked enzymes were assayed spectrophotometrically by measuring the change in absorbancy at 340 mμ with a Beckman model DU spectrophotometer at 27 C. The reduction of ferricyanide was determined at 400 mμ and cytochrome *c* at 550 mμ.

Catalase activity was determined by measuring

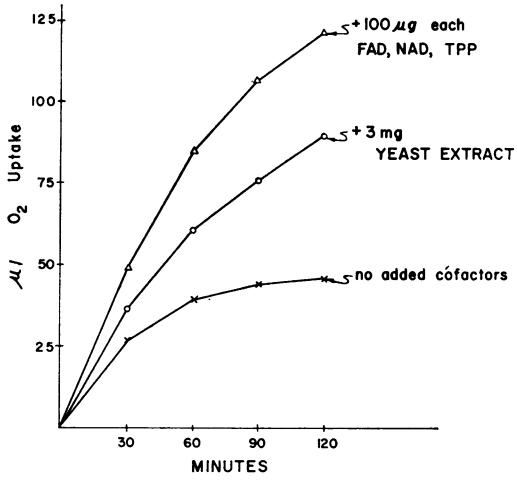

FIG. 1. *Oxidation of sodium lactate by resting cells of Mycoplasma gallisepticum. Each Warburg flask contained 100 μmoles of phosphate buffer (pH 7.5), 3.2 mg (dry weight) of cells, 60 μmoles of sodium lactate and, where indicated, 100 μg each of FAD, NAD, and TPP or 3 mg of yeast extract, with a total fluid volume of 3.0 ml. The endogenous respiration was subtracted from the data shown.*

TABLE 1. *NAD requirement for lactate oxidation*

Addition*	Qo_2 (dry wt)
None	31.5
NAD (10^{-4} M)	48.5
NADP (10^{-4} M)	33.4
Niacin (10^{-4} M)	30.4

* Each flask also contained 10^{-4} M FAD and TPP.

the breakdown of hydrogen peroxide when incubated with cell suspensions at 25 C; the iodometric method of Herbert (1955) was used to determine hydrogen peroxide disappearance.

The flavine level of this organism was determined on an extract prepared by heating a cell suspension in acid solution at 120 C for 15 min, followed by rapid cooling and centrifugation at 12,000 × *g* for 30 min. The flavine level of the extract was determined by the difference in absorbancy at 450 mμ, between the extract in the oxidized state in air, and after reduction with hydrosulfide under anaerobic conditions with an anaerobic cuvette.

The benzidene tests for heme-containing compounds were made on extracts prepared according to the procedure of Morrison and Stotz (1957). Spectrophotometric studies at 25 C, with sodium hydrosulfite to reduce any heme components, were used to determine the possible presence of cytochromes. The protein level of cell extracts was determined by the trichloroacetic acid method of Stadtman, Novelli, and Lipmann (1951).

RESULTS

Lactate oxidation by resting cells. Resting-cell suspensions of *M. gallisepticum* 293 were found to oxidize sodium lactate slowly. However, as shown in Fig. 1, this oxidation was markedly stimulated by the addition of yeast extract. The addition of a combination of NAD, thiamine pyrophosphate (TPP), and flavine adenine dinucleotide (FAD), could replace yeast extract in the stimulation of this oxidation.

The initial rapid rate of O_2 uptake with no added cofactors may be due to low residual levels of the required cofactors, which are dissipated during the early stages of the oxidation, within the cells.

Elimination of any of these three cofactors resulted in a marked decrease in respiratory activity.

This requirement of NAD, TPP, and FAD for resting-cell respiration by *M. gallisepticum* appears analogous to the requirement of NAD and coenzyme A for the respiratory activity of *Rickettsia*, as reported by Paretsky and co-workers (1958). However, lactate oxidation by this strain of *M. gallisepticum* was not stimulated by the addition of coenzyme A, α-lipoic acid, or magnesium ions. In view of the sensitivity of this oxidation to 10^{-3} M sodium arsenite and the previous report of Tourtellotte (1960) of the requirement for coenzyme A and lipoic acid in the pyruvate metabolism of *M. gallisepticum* 5969, the lack of stimulation by these cofactors does not mean that these cofactors are not involved in the present oxidation.

As shown in Table 1, the requirement for NAD was not replaced by the addition of an equivalent level of nicotinamide adenine dinucleotide phosphate (NADP) or nicotinic acid. Flavine mononucleotide (FMN) but not riboflavine would replace the stimulation by FAD (Table 2). As also shown in Table 2, the stimulation of this oxidation by FMN was reversed by the addition of 10^{-3} M Atabrine (quinocrine hydrochloride; Winthrop Chemical Co., New York, N.Y.), as would be expected with a flavine-mediated enzyme

system. It was further found that thiamine would replace TPP in stimulating lactate oxidation.

The effect of various inhibitors on lactate oxidation is illustrated in Table 3. In contrast to lactate oxidation by *Escherichia coli*, the aerobic dissimilation of this substrate by *M. gallisepticum* was insensitive to 2×10^{-3} M sodium azide. The use of a gas phase of 90% carbon monoxide–10% oxygen in the dark, as compared with a control gas phase of 90% nitrogen–10% oxygen also failed to inhibit the *Mycoplasma* oxidation.

M. gallisepticum possessed no catalase activity as measured by the breakdown of hydrogen peroxide by resting cells. This lack of catalase activity is similar to the observations of Weibull and Hammarberg (1962), who found negligible catalase activity in several other species of *Mycoplasma*. Attempts to demonstrate heme-containing compounds in cell extracts (see Materials and Methods) by the benzidine test were negative, indicating the absence of these compounds. Spectrophotometric studies failed to reveal absorption spectra characteristic of the heme or porphyrin components of a cytochrome-containing system either in the visible range or in the Soret band regions.

As shown in Table 4, the rate of oxidation of lactate increased with increasing oxygen concentration, with a maximal rate of oxidation occurring in a 100% oxygen atmosphere. Such an increase in oxidation rate with increased oxygen tension would seem to indicate a low affinity of the terminal respiratory sites involved in lactate oxidation for atmospheric oxygen, which is characteristic of a flavine-terminated respiratory system. The total flavine level was assayed spectrophotometrically by determining the difference in absorbancy at 450 mμ of an extract in the oxidized state in air and after reduction with hydrosulfite under anaerobic conditions. The flavine level, as determined by this method, averaged 2.1×10^{-10} moles per mg of cell dry weight. This flavine level would seem relatively high, analogous to that found in certain lactic acid bacteria.

Investigations with sonic extracts. As shown in Fig. 2, cell-free extracts contained a NAD-specific lactic dehydrogenase. Although not shown on this figure, the dehydrogenase was specific for *l*(+)-lactic acid. This data is similar to that previously reported by Tourtellotte (1960) with *M. gallisepticum* 5969.

These sonic extracts also oxidized reduced NAD (NADH) in the presence of menadione and ferricyanide (Fig. 3) as electron acceptors, indicating the presence of a NADH-menadione reductase and a NADH-ferricyanide reductase. The high endogenous activity is due to the presence of a NADH oxidase. This NADH oxidase activity varied with differing enzyme preparations but was always considerably less than the level of menadione and ferricyanide reductase activity. During the oxidation of NADH with oxygen as the electron acceptor, near stoichiometric levels of hydrogen peroxide accumulated. During the course of these studies on NADH oxidation, it was observed that intact cells of this strain would oxidize this reduced pyridine nucleotide. This reaction, as followed manometrically, is illustrated in Fig. 4. As shown in this figure, in contrast to the oxidation of NADH by

TABLE 2. *Flavine requirement for lactate oxidation*

Addition*	Q_{O_2} (dry wt)
No flavine	27.6
FAD (10^{-4} M)	48.5
FMN (10^{-4} M)	57.0
Riboflavine (10^{-4} M)	31.8
FMN and 10^{-3} M Atabrine	29.4

* Each flask also contained 10^{-4} M NAD and TTP.

TABLE 3. *Effect of inhibitors on the oxidation of sodium lactate*

Inhibitor	*Mycoplasma gallisepticum*		*Escherichia coli*	
	Q_{O_2}	Per cent inhibition	Q_{O_2}	Per cent inhibition
None	14.1	—	42	—
10^{-3} M NaCN	15.7	—	0	100
10^{-3} M NaN_3	9.2	33	19.5	54
90% N_2–10% O_2 atmosphere	8.4	—	38.5	—
90% CO–10% O_2 atmosphere	9.5	—	21.2	55

TABLE 4. *Effect of oxygen tension on lactate oxidation*

Oxygen level	Q_{O_2} (dry wt)
%	
21 (air)	15.6
50	27.0
75	32.9
100	36.7

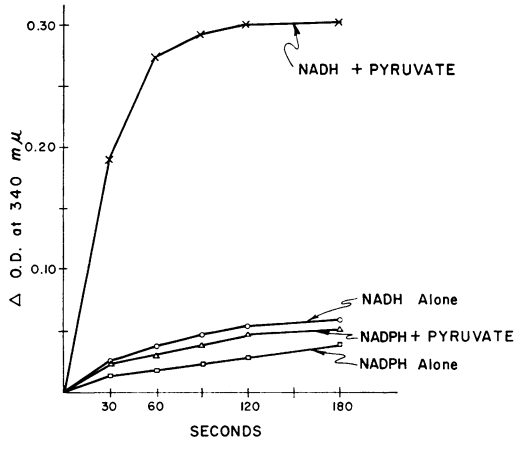

FIG. 2. *Lactic dehydrogenase activity of sonic extracts of Mycoplasma gallisepticum. Each cuvette contained 100 μmoles of phosphate buffer (pH 7.5), 0.35 mg of enzyme protein, and, where indicated, 0.33 μmoles of NADH or NADPH, 20 μmoles of sodium pyruvate, and water to a final volume of 3.0 ml.*

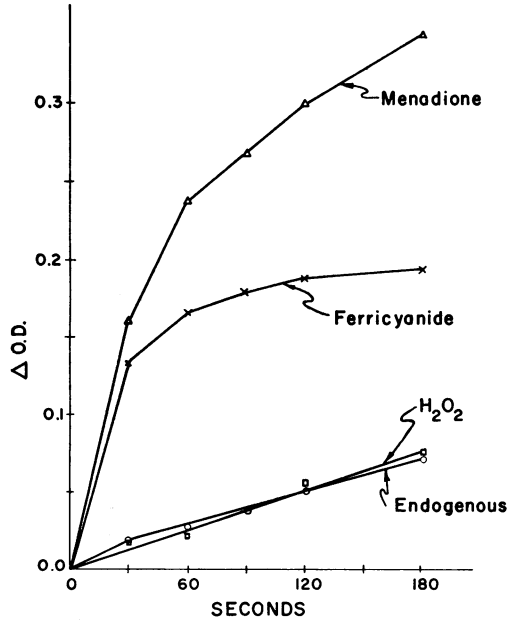

FIG. 3. *NADH oxidation by sonic extracts of Mycoplasma with menadione, ferricyanide, and H_2O_2 as acceptors. Ferricyanide reduction was measured by the change in optical density at 440 mμ and the other reactions at 340 mμ. Each cuvette contained 100 μmoles of phosphate buffer (pH 7.5), 0.3 μmoles of NADH, 0.35 mg of enzyme protein, and, where indicated, 0.2 μmoles of menadione, ferricyanide, or H_2O_2, and water to a final volume of 3.0 ml.*

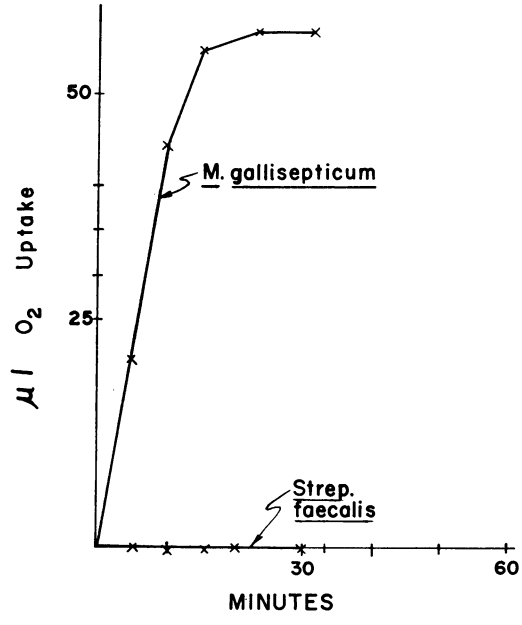

FIG. 4. *NADH oxidation by resting cells of Mycoplasma gallisepticum. Each Warburg flask contained 100 μmoles of phosphate buffer (pH 7.5), 2.0 mg of NADH, and, as indicated, 2.5 mg of M. gallisepticum cells or 1.8 mg of Streptococcus faecalis cells, with a total fluid volume of 3.0 ml.*

intact *Mycoplasma* cells, cells of *Streptococcus faecalis* failed to oxidize this reduced nucleotide. This difference in ability to oxidize NADH would seem to depend upon the presence or absence of a cell wall, since protoplasts of this *Streptococcus* readily oxidized NADH (P. J. VanDemark, *unpublished data*).

As also shown in Fig. 3, the rate of NADH oxidation in the presence of hydrogen peroxide was no greater than the endogenous activity, indicating the lack of NADH peroxidase activity in these preparations. No cytochrome *c* reductase activity, as measured by an increase in absorption at 550 mμ due to the reduction of cytochrome *c*, was detected in these extracts.

DISCUSSION

The required addition of NAD, thiamine, and FMN or FAD for a maximal rate of lactate oxidation by *M. gallisepticum* is an indication not only that these cofactors are functioning in this oxidation but that these cells require these cofactors nutritionally preformed or that their synthesis is slow and thus rate-limiting in lactate

oxidation. This cofactor requirement is also an indication of a difference in permeability between PPLO and normal bacteria. It would appear that, in the preparation of resting-cell suspensions of PPLO, these cofactors are leached from the cells during washing and, as a result, an external source of these cofactors must be provided to restore maximal activity. Also related to this difference in permeability is the ability of intact cells of this PPLO strain to oxidize NADH. Since the studies of Smith (1962) showed that the spheroplasts of *Bacillus subtilis* are capable of NADH oxidation, the greater permeability of PPLO may well correlate with their lack of a cell wall. However, one cannot so simply explain the relative insensitivity of PPLO to osmotic changes in contrast to the osmotic sensitivity of the spheroplasts of bacteria.

The present study of the respiratory pathways of *M. gallisepticum* would indicate that the PPLO are similar in nature to the *Lactobacteriaceae*, as they lack the heme-containing respiratory systems and the terminal respiration is flavine-mediated. The homolactic nature of the fermentation of PPLO is another example of this resemblance to the lactic acid bacteria.

ACKNOWLEDGMENT

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