Regulation of Lactate Production in *Streptococcus bovis*:
A Spiraling Effect That Contributes to Rumen Acidosis

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ABSTRACT

When *Streptococcus bovis* was grown in batch culture with 6 g/L glucose at pH 6.7, maximum specific growth rate was 1.47 h$^{-1}$, and lactate was the primary fermentation product. In continuous culture at pH 6.7 and growth rate equal to .10 h$^{-1}$, little lactate was formed, and formate, acetate, and ethanol accounted for most of the product. When extracellular pH decreased to 4.7, intracellular pH declined to 5.4, and organisms switched back to lactate production. Intracellular concentration of fructose 1,6-diphosphate of batch culture cells was greater than 12 mM, a concentration that allowed maximal lactate dehydrogenase activity. When *Streptococcus bovis* was grown in continuous culture at pH 6.7, intracellular fructose-1,6-diphosphate declined to .4 mM, a concentration which gave little lactate dehydrogenase activity at pH 6.5 or greater. Decreasing pH of continuous culture to 4.7 increased intracellular fructose-1,6-diphosphate concentration to .8 mM. This concentration was still limiting if lactate dehydrogenase was assayed at pH 6.5, but nearly maximal activity was obtained when enzyme was assayed at pH 5.5. The small increase in fructose-1,6-diphosphate and decreased requirement of lactate dehydrogenase for fructose-1,6-diphosphate under acidic assay conditions, accounted for increased lactate production during low pH (4.7) continuous culture. These and other aspects of lactate regulation by *Streptococcus bovis* are discussed as factors leading to rumen acidosis. This pattern of regulation also helps to explain why rumen acidosis is difficult to reverse.

INTRODUCTION

The rumen is well buffered by bicarbonate, phosphate, proteins, forage cell walls, and volatile fatty acids, and in ruminants fed forage, ruminal pH is generally near neutrality. However, if large quantities of starch are added to the diet, ruminal pH decreases to 5.0 or less. At ruminal pH less than 5.5, animals often suffer from indigestion, and feed intake decreases. In more extreme cases, rumen ulceration, parakeratosis, liver abscess, and even death can result (24).

Early work by Hungate et al. (8) demonstrated that rumen acidosis was associated with an initial overgrowth of *Streptococcus bovis* and the inability of lactate-utilizing bacteria to ferment all the lactate that was produced. *Streptococcus bovis* produces lactate at high growth rates, but it changes to an acetate, formate, and ethanol fermentation at slow growth rates if pH is greater than 6.0 (19). At pH less than 5.0, *S. bovis* produces lactate even at slow growth rates (5, 20, 21).

Regulation of lactate production by *S. bovis* leads to a spiraling effect of ever-increasing lactate production and lower pH. In animals fed forage, *S. bovis* grows slowly because substrate is limiting, and acetate, formate, and ethanol are its primary fermentation products. If animals are fed larger amounts of starch, *S. bovis* grows faster and increases lactate production. Lactate, a strong acid, decreases rumen pH. As pH decreases, the growth rate of *S. bovis* declines (23, 28), but the organism does not switch back to an acetate, formate, and ethanol fermentation. Instead, it continues to produce lactate, reduces rumen pH even further, and creates a niche for lactobacilli that also make lactate. The following experiments...
studied the regulation of lactate production by *S. bovis*.

**MATERIALS AND METHODS**

**Cell Growth**

The JB1 strain of *Streptococcus bovis* was used (18). Recent work has indicated that this strain is characteristic of the species (22). The media contained (per liter) 292 mg K$_2$HPO$_4$, 292 mg KH$_2$PO$_4$, 480 mg (NH$_4$)$_2$SO$_4$, 480 mg NaCl, 100 mg MgSO$_4$·7H$_2$O, 64 mg CaCl$_2$·2H$_2$O, 4 g Na$_2$CO$_3$, .6 g cysteine hydrochloride, 1.0 g Trypticase (BBL Microbiology systems, Cockeysville, MD), .5 g yeast extract, and glucose (amounts given in Table 1). The media were prepared under anaerobic conditions as described by Hungate (7). Incubations were at 39°C in batch (500 ml vessel) or continuous cultures (New Brunswick model C30 chemostat, 360 culture vessel). In some cases, pH of the media was decreased by adding concentrated HCl. When pH declined to 4.7 there was little buffering capacity, so 10 mM propionate was added. At least a 99% turnover of medium was allowed to pass through the continuous culture vessel between samplings. Optical density readings indicated the culture was at steady state.

**Analyses**

Cells for assay of lactate dehydrogenase (LDH) were removed from the culture vessel (360 ml) and centrifuged immediately (30,000 × g, 0°C, 5 min). Supernatant sample was frozen (−15°C) and the cell pellet was resuspended in 67 mM phosphate buffer (pH 6.7, 0°C) and recentrifuged. The resulting pellet was resuspended in 2 ml of 80 mM phosphate buffer (pH 7.0, 0°C) and sonicated for 30 min (Branson model 200 sonicator, 30% duty cycle, maximum power output for microtip, 0°C). Unbroken cells were removed by centrifugation (30,000 × g, 15 min, 0°C) and the crude extract was frozen (−15°C). Lactate dehydrogenase was assayed by a method similar to (3). The cell extract was diluted with 80 mM phosphate buffer, and the complete assay mixture (3 ml) contained 80 mM histidine buffer of different pH, 4 mM pyruvate, .3 mM nicotinamide adenine dinucleotide, reduced (NADH), 1.2 mM fructose-1,6-diphosphate.
(FDP), 1.5 mM phosphate buffer, 20 mM MgCl₂·6H₂O, and approximately 1 to 2 μg protein/ml assay mixture. The absorbance was followed at 340 nm (Gilford Model 260 spectrophotometer, cuvettes of 1 cm light path, room temperature). Nicotinamide adenine dinucleotide, reduced oxidase was estimated from the rate of NADH oxidation in the absence of pyruvate, and this small value was subtracted from the total rate of NADH oxidation.

Cells for FDP determinations were diluted by one-fourth with crushed ice and mixed vigorously in an ice bath (0°C). When the ice inside the vessel melted, the cell suspension was centrifuged (30,000 × g, 5 min, 0°C). Supernatant was frozen (−15°C) and the pellet was washed in 67 mM phosphate buffer and recentrifuged. Resulting pellet was treated with 10% perchloric acid and sonicated for 30 min to break the cells (see preceding paragraph). Cell debris was removed by centrifugation (30,000 × g, 15 min, 0°C), and supernatant was treated with a 1.25 times excess of K₂CO₃ to remove perchlorate as insoluble potassium salt. Fructose-1,6-diphosphate was assayed by the enzymatic procedure of Racker (16).

Fermentation products in cell-free medium were assayed by high-pressure liquid chromatography (Beckman model 334 liquid chromatograph, model 156 refractive index detector, model 421 CRT data controller, CR1A integrator), 50-μl loop, at 50°C using a Bio-Rad HPX-87H organic acid column. Glucose utilization was measured with an enzymatic procedure employing hexokinase and glucose-6-phosphate dehydrogenase (2). Cells washed with distilled water were dried on aluminum pans (105°C) to estimate cellular dry matter. Optical density was determined at 600 nm (Gilford 260 spectrophotometer, cuvettes with 1 cm light path). Previous work indicated that S. bovis is very resistant to cell breakage and is not sensitive to osmotic shock (22).

Intracellular pH

Intracellular pH was measured in cells grown in batch cultures by an acid distribution method originally described by Riebeling et al (16). This method is based on the assumption that undissociated forms of weak acids diffuse through the lipophilic cell membrane freely so that internal and external concentrations equilibrate. Distribution of the ionized form of the acid then becomes a function of gradient between internal and external pH.

As the culture grew and extracellular pH declined, samples were removed from the 500-ml incubation vessel (39°C) with a 2-ml hypodermic syringe. Replicate samples were injected into separate tubes (13 × 100 mm, 39°C filled with CO₂, capped with butyl rubber stoppers) that contained [7-14C] benzoate (1.00 μCi, 10 μCi/μmol), [1,2-14C] polyethylene glycol (1.00 μCi, .1 μCi/mg), or ¹H₂O (1.00 mCi, .25 μCi/mg). All three isotopes were obtained from Dupont, New England Nuclear, Boston, MA. After 5 min of incubation with radioactive isotopes, two .9-ml samples of culture were rapidly centrifuged through silicon oil (Dexter Hysol 550, Hysol Co., Olean, NY, Fisher model 235A micro centrifuge, 1.5 ml polypropylene tubes, 13,750 × g, 5 min, 22 C). Fifty-microliter samples of supernatant were removed for scintillation counting (10 ml Aquasol-2, Dupont, New England Nuclear, Boston, MA). Centrifuge tubes and contents were frozen (−15°C). When supernatant was solid, the bottom of the centrifuge tubes (containing the cell pellets) was removed with a pair of dog nail clippers. Pellets were allowed to dissolve in scintillation fluid for 6 h before counting.

Intracellular space was calculated from the difference in specific activity of ¹H₂O and [1,2-14C] polyethylene glycol. The [7-14C] benzoate counts in the pellet were corrected for a small amount of extracellular fluid contamination. Intracellular pH was calculated from specific activity of [7-14C] benzoate in the supernatant, and the counts of [7-14C] benzoate in the pellet using the Henderson-Hesselbach equation. Benzoate was used as a pH indicator because it readily diffuses through lipids and because it is not produced by S. bovis.

Statistics

All incubations and analyses were performed in triplicate, and individual values were highly reproducible. Standard errors of the mean were always less than 5%.
RESULTS AND DISCUSSION

Lactate Production

When *S. boris* JB1 was grown in batch culture with 6 g/L glucose at pH 6.7, maximum specific growth rate (μ) was 1.47 h⁻¹ and lactate was the primary fermentation product (Table 1). In chemostats with pH 6.7 and μ equal to .10 h⁻¹, little lactate was formed and formate, acetate, and ethanol accounted for most of the product. When extracellular pH of chemostat grown cells (μ = .10 h⁻¹) was decreased to 4.7, the organism switched back to a lactate fermentation. These results agree with earlier reports (20, 21) and indicate that lactate production was influenced by both growth rate and extracellular pH.

Effects of pH on Lactate Dehydrogenase

When specific activity of LDH was measured at pH 7.0, there was an inverse, rather than direct, relationship between the amount of lactate produced and LDH specific activity (Table 1). Further work, however, indicated that pH influenced LDH activity (Figure 1). Little activity was observed at alkaline pH. Similar results were reported by Wolin (32). He indicated that the LDH of *S. boris* was rapidly inactivated by exposure to pH greater than 7.5. As pH was decreased from 7.0 to 5.5 in these experiments there was an increase in activity. This increase was most dramatic with extracts from cells grown at pH 6.7 (Figure 1). Cells grown at pH 4.7 had less activity, but the optimum was also at pH 5.5. A similar effect of pH on LDH activity was reported for *Lactobacillus casei* (3).

Intracellular pH

For many years, it was assumed that intracellular pH of bacteria was maintained at a neutral or slightly alkaline pH. More recent work indicates that intracellular pH can decrease significantly when bacteria are grown in acidic environments (6, 10, 12, 14, 15, 17). When *S. boris* was grown in a batch culture containing 52 mM glucose, growth rate was rapid (1.42 h⁻¹) and the accumulation of lactate closely paralleled the increase in optical density (Figure 2a). The increase in lactate was associated with a modest decline in pH during the first 2 h and a more abrupt decrease between the 2 and 3 h. By 3 h, extracellular pH had declined to 4.7 and growth ceased even though glucose remained in the medium. Between 3 and 4 h, there was little change in glucose, lactate, or extracellular pH. When intracellular pH was estimated by acid distribution, intracellular pH remained more alkaline than extracellular pH (pH 4.5 to 7.0), but there was a definite decline in intracellular pH as extracellular pH decreased (Figure 2b). At a extracellular pH of 4.7, intracellular pH was approximately 5.4. This intracellular pH nearly coincided with the pH optimum of the LDH (Figure 1) and helps to explain the large amount of lactate in low pH chemostats (Table 1).

Effects of Fructose-1, 6-Diphosphate on Lactate Dehydrogenase

Wolin (32) reported that streptococcal LDH had a requirement for FDP, and we were unable to detect significant activity in the absence of FDP. This requirement for FDP was, however, dependent on the pH at which the enzyme was assayed (Figure 3). At pH 6.5, greater than 1.6
mM FDP was required for maximum activity, and very little activity was observed with less than .4 mM FDP. When the enzyme was assayed at pH 5.5, greater than 80% of the maximum activity was observed at .4 mM FDP.

We measured intracellular FDP concentrations to see if variations in FDP could be responsible for changes in lactate production (Table 1). At a growth rate of 1.47 h⁻¹ in batch culture (pH 6.7), FDP concentration was 42.8 nmol/mg dry weight. A similar FDP was reported by Yamada and Carlson (33). Assuming that bacteria are approximately 30% dry matter (9), the intracellular concentration of FDP would have been 13.0 mM. This concentration was more than enough for maximum LDH activity and lactate production (Figure 3).

In chemostat culture (pH 6.7 and growth rate of .10 h⁻¹), FDP concentration declined to 1.3 nmol/mg dry weight or .4 mM (Table 1). This concentration of FDP gave near maximum activity if LDH was assayed at pH 5.5, but little activity was seen when the enzyme was assayed at pH 6.5 (Figure 3). Assuming that intracellular pH was going to be greater than 6.5 under these growth conditions (Figure 2b), most of the decrease in lactate production could be explained by a decline in intracellular FDP.

Figure 2. a. The decline in extracellular pH during growth in batch culture, (●), optical density; ○, extracellular pH (pHₑ; Δ, glucose; and △, lactate). b. The relationship between extracellular pH (pHₑ) and intracellular pH (pHᵢ).

Figure 3. Effect of fructose-1,6-diphosphate (FDP) concentration on the relative activity of lactate dehydrogenase (% of maximum activity). Extracts were obtained from cells in batch culture (μ = 1.47 h⁻¹) at pH 6.7 (●, ●), in a chemostat (μ = .10 h⁻¹) at pH 6.7 (Δ, ●), and in a chemostat at pH 4.7 (●, ●). Results are when the enzyme was assayed at pH 5.5 (○, Δ, ●) and at pH 6.5 (●, Δ, ●).
When *S. boris* was grown in chemostats ($\mu = 0.1 \text{ h}^{-1}$) at pH 4.7, FDP concentration was 2.7 nmol/mg dry weight or .8 mM (Table 1). This concentration was still limiting when LDH was assayed at pH 6.5, but nearly maximum activity was observed at pH 5.5 (Figure 3). A small increase in FDP and decrease in the requirement of LDH for FDP at pH less than 6.5 would increase lactate production (See Table 1).

**Effects of Phosphate and Magnesium on Lactate Dehydrogenase**

Wolin (32) reported that phosphate and magnesium were required for LDH activity and we also examined the effect of these ions. If crude extracts were dialyzed or diluted manyfold with histidine buffer (pH 6.5, 0°C), LDH activity decreased markedly. As much as 80 mM phosphate was required to stabilize the enzyme. Actual enzyme catalysis, however, was inhibited by high concentrations of phosphate. If the enzyme was assayed at pH 6.5 there was an increase in activity up to 15 mM phosphate and a decrease thereafter (Figure 4). At pH 5.5, phosphate concentrations greater than 1.5 mM caused a decrease in activity. Effects of magnesium were also dependent on pH. Magnesium was not required for maximal activity at pH 5.5, but greater than 8 mM magnesium was required when the enzyme was assayed at pH 6.5.

**Effects of Pyruvate and Ratio of Nicotinamide Adenine Dinucleotide, Reduced, to Nicotinamide Adenine Dinucleotide**

When the reciprocal of pyruvate concentration was plotted against the reciprocal of enzyme velocity, the Lineweaver-Burk transformation was not linear (Figure 5a). These results indicate that the enzyme did not follow typical enzyme kinetics. At low substrate concentrations, the velocity was lower than a Michaelis-Menten relationship would predict. Hill plots gave slopes $> 1$ (Figure 5b). A slope $>1$ indicates that the enzyme has more than one site that binds substrate. Results were similar with *Escherichia coli* (27) and *Selenomonas ruminantium* (30).

We also assayed the LDH with different ratios of NADH to nicotinamide adenine dinucleotide (NAD) (Figure 6). As the log of the NADH to NAD ratio increased, there was a small but linear increase in activity. Experiments by Wimpenny and Firth (31) indicated that log (NADH/NAD) of facultative bacteria ranged from $-0.12$ to $+0.56$. Based on these results, changes in the intracellular ratio of NADH to NAD were unlikely to have a significant effect on lactate production in *S. boris*.

We did not examine the LDH of *S. boris* by electrophoresis because enzyme kinetics indicated that regulation by isoenzymes (25) was probably not important. There was some change in specific activity but the optimal pH (Figure 1), responses to FDP (Figure 3), requirements for phosphate (Figure 4), Michaelis constants (Figure 5a), and effects of NADH/NAD on the LDH were remarkably similar for all three growth conditions (Figure 6).

**Pyrurate Formate Lyase**

Streptococci convert pyruvate to acetyl-coenzyme A and formate with the enzyme pyruvate formate lyase (PFL) (1, 11, 13, 26, 29), and Japanese workers (1) showed that this enzyme was important in the regulation of pyruvate metabolism. We attempted to assay the enzyme under the strictly anaerobic con-
Figure 5. a. Lineweaver-Burk plot of $1/S$ ($1$/mM pyruvate) vs. $1/V$ ($1$/µmol nicotinamide adenine dinucleotide, reduced/mg protein/min X 10) when the enzyme was assayed at pH 5.5 (●) and pH 6.5 (▲). b. Hill plot when the enzyme was assayed at pH 5.5 (●) and pH 6.5 (▲). pH 5.5 plot had a slope of 1.54 and an $r^2$ of .90. pH 6.5 plot had a slope of 1.76 and an $r^2$ of .92. Results were similar when the enzyme was obtained from cells grown in chemostats (pH 4.7 or 6.7).

Figure 6. Effect of log nicotinamide adenine dinucleotide, reduced/nicotinamide adenine dinucleotide (NADH/NAD) on the relative activity of lactate dehydrogenase (% of maximum activity). Extracts were obtained from cells in batch culture ($\mu = 1.47$ h$^{-1}$) at pH 6.7 (●), in a chemostat ($\mu = .10$ h$^{-1}$) at pH 6.7 (●), and in a chemostat at pH 4.7 (●). Results are when the enzyme was assayed at pH 5.5 (●, ▲, ○) and at pH 6.5 (●, ▲, ●).

Conclusions

Lactate production by *S. boris* is regulated by a variety of physiological factors and probable sites of regulation are outlined in Figure 7.
When ruminants are fed forages, starch is scarce, and the growth rate of *S. bovis* is restricted by the availability of a suitable energy source. At slow growth rates, intracellular FDP concentrations are low, LDH is not activated, and there is little lactate production. In the absence of lactate, the rumen fluid remains well buffered, intracellular pH remains near neutral or slightly alkaline, and pyruvate formate lyase favors the production of formate and acetate.

When large amounts of starch are added to the diet, growth of *S. bovis* is no longer restricted by energy source and grows faster than other species of rumen bacteria. As growth rate increases, FDP and possibly pyruvate concentrations increase within the cell and these intermediates activate the LDH. Triose phosphate concentrations may also increase, which would act to inhibit the pyruvate formate lyase. The net effect of these changes is a switch from acetate and formate production to lactate production.

Lactate is a ten times stronger acid than the volatile fatty acids, and accumulation of lactate eventually exceeds the buffering capacity of rumen fluid. As rumen pH declines, the growth rate of *S. bovis* decreases, and the fermentation could theoretically switch back to acetate and formate production, thereby alleviating acidosis. The low extracellular pH, however, induces other changes. When extracellular pH decreases, intracellular pH also declines. The decline in intracellular pH, in turn, decreases the requirement of LDH for FDP and increases the maximum velocity of the LDH. Pyruvate formate lyase is inhibited by low pH, and the metabolism spirals toward ever greater lactate production.
production and lower pH. The results obtained in these experiments support practical observations that once ruminants succumb to acidosis the condition is difficult to reverse (24).

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REFERENCES


