Control of Lactate Production by *Selenomonas ruminantium*: Homotropic Activation of Lactate Dehydrogenase by Pyruvate

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Selenomonas ruminantium produced one mole of D(–)-lactate per mole of glucose used at all dilution rates in ammonia-limited continuous culture. In contrast, lactate production varied according to the dilution rate when glucose was the limiting nutrient. At dilution rates of less than 0.2 h^{-1} , acetate and propionate were the main fermentation products and lactate production was low. At dilution rates above 0.2 h^{-1} , the pattern changed to one of high lactate production similar to that under ammonia limitation. Experiments with cell-free extracts of *S. ruminantium* showed that D(–)-lactate dehydrogenase had sigmoidal kinetics consistent with homotropic activation of the enzyme by its substrate, pyruvate. This feature allows *S. ruminantium* to amplify the effects of relatively small changes in the intracellular concentration of pyruvate to cause much larger changes in the rate of production of lactate. Some confirmation that this mechanism of control occurs under physiological conditions was obtained in glucose-limited culture, in which the sigmoidal increase in lactate production was accompanied by a linear increase in pyruvate excretion as the dilution rate increased.

INTRODUCTION

The pattern of fermentation products of *Selenomonas ruminantium* growing on glucose in continuous culture changes according to the dilution rate (D). As D increases, lactate tends to replace acetate and propionate in the extracellular medium (Hobson, 1965*a*; Scheifinger, Latham & Wolin, 1975), until, as in strain GA192 of Scheifinger *et al.* (1975), lactate may become the sole fermentation product.

Regulation of the lactate dehydrogenase (LDH) of several micro-organisms, including the rumen bacteria *Streptococcus bovis* (Wolin, 1964) and *Butyrivibrio fibrisolvens* (van Gylswyk, 1977), is achieved by fructose 1,6-bisphosphate being an obligatory activator of the enzyme, so that cells can respond to an accumulation of glycolytic intermediates by activation of LDH and removal of pyruvate by conversion to lactate, thereby regenerating NAD^+ . A similar type of control was suspected for *S. ruminantium* by Scheifinger *et al.* (1975) on the basis of observed differences in fermentation products at different dilution rates, but no activation of LDH by fructose 1,6-bisphosphate was found.

In view of the probable importance of lactate in interspecies cross-feeding in the rumen, and the role of *S. ruminantium* in this activity (Wolin, 1975), the regulation of lactate production of *S. ruminantium* was re-examined in carbon- and nitrogen-limited continuous cultures. Control was again found to be exerted at LDH, but in this bacterium the enzyme was subject to homotropic activation by its substrate, pyruvate, rather than to activation by fructose 1,6-bisphosphate.

METHODS

Organism. *Selenomonas ruminantium* WPL 151/1 was isolated in this Institute from the rumen of a barley-fed sheep by S. O. Mann. This strain was an obligate anaerobe and did not use DL-lactate as a growth substrate. It was maintained by weekly subculture in the liquid form of medium 1 of Kurihara *et al.* (1968).

Continuous culture. The growth medium contained (per litre final volume): minerals solutions (a) and (b) (Hobson, 1969), 150 ml each; trace metals (Clark & Holms, 1976), 10 ml; 0.075 % (w/v) resazurin, 4 ml; vitamins solution, a 10-fold concentration of the vitamin content of the complete medium of Scott & Dehority (1965), 100 ml; glucose, 20 mmol; ammonium chloride, 10 mmol in glucose-limited culture or 1.2 mmol in ammonia-limited culture; reducing solution, 100 ml. Reducing solution contained (in boiled, CO₂-gassed distilled water): 4 % (w/v) NaHCO₃; 0.25 % (w/v) Na₂S; 0.25 % (w/v) Na₂S₂O₄. It was prepared immediately before use and was sterilized by filtration. Minerals solutions and trace metals were prepared in batches, autoclaved at 121 °C for 15 min and stored at 4 °C until required. Vitamins solution was sterilized by filtration and stored at 4 °C. Other solutions were prepared fresh.

All constituents of the medium apart from the reducing solution were mixed with boiled, glass-distilled water and autoclaved in 5 l batches (109 °C, 45 min). The reducing solution was then added and the reservoir was gassed by the method of Hobson (1965b).

Continuous culture apparatus was based on the system developed by Hobson (1965b) and Hobson & Summers (1967), and modified so that peristaltic pumps delivered growth medium (MHRE 22; Watson-Marlow, Falmouth, Cornwall) and 1 M-NaOH for pH control (Minipump; Schuco Scientific, London). Samples were removed from the culture by a sampling hood similar to that described by Baker (1968) except that anaerobic conditions were maintained. The culture volume was 100 or 300 ml, depending on the dilution rate required.

Samples were taken from the culture after a minimum flow of 5 culture volumes; these were immediately chilled on ice, and a portion of each was centrifuged (3000 g, 4 °C, 15 min). Culture supernatants were stored at -20 °C and pellets at -60 °C. No attempt was made to exclude air from samples.

Analyses. Glucose in the inflowing medium and in the supernatant fluid from centrifuged culture samples was determined in an automatic analyser by the glucose oxidase method (Morley, Dawson & Marks, 1968). Lactate was measured by the microdiffusion method of Conway (1957), in which lactate is non-stereospecifically oxidized to acetaldehyde by ceric sulphate. L(+)-Lactate was assayed using L(+)-lactate dehydrogenase (Boehringer). Acetate and propionate were determined directly on acidified culture supernatants by the gas chromatography method of Fell *et al.* (1968) with peak areas estimated using a Pye Unicam DP80 digital integrator. A standard solution of 30 mM-acetate and 10 mM-propionate was used for calibration. The lactate dehydrogenase assay of Kubowitz & Ott (1943) was adapted for the measurement of pyruvate: samples were incubated in a 1 ml assay mixture containing 42 µmol KH₂PO₄, 0.2 mg NADH, and 10 µl lactate dehydrogenase (5 mg ml⁻¹) at pH 7.4, and pyruvate was estimated from the ΔA_{340} . Succinate was assayed by the method of Clark & Porteous (1964). The protein content of cell-free extracts was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Cell density was calculated from the turbidity at 650 nm of diluted samples from cultures, measured using a Pye Unicam SP600 spectrophotometer. The standard curve of turbidity against dry weight was linear up to an absorbance of 1.0, and samples were diluted to an absorbance of less than 0.7 for estimation of cell density. Washed cell suspensions were dried at 105 °C overnight for calibration of the dry weight/turbidity relationship.

Preparation of cell-free extracts. Pellets from continuous culture samples were resuspended in cold 30 mM-KH₂PO₄ buffer pH 7.0 and disrupted using a Soniprobe (Dawe Instruments, London) at a current of 5 A for 5 min, with alternate 30 s on/off periods. The sample was kept on ice throughout. Whole cells were removed by centrifugation (11 600 g, 4 °C, 1 h) and the supernatant liquid was either used immediately or stored at -60 °C.

Lactate dehydrogenase. LDH activity was assayed in cell-free extracts by measurement of the rate of NADH oxidation at 340 nm, using a Pye Unicam SP1800 spectrophotometer. The assay mixture, incubated at 25 °C, contained (in 3 ml): 60 µmol KH₂PO₄ buffer pH 7.0; 0.75 µmol NADH; 50 µmol sodium pyruvate; 0.5 ml cell-free extract. The reaction was started by addition of NADH. NADH oxidase was estimated from the rate of NADH oxidation in the absence of pyruvate, and this value was subtracted from the total initial rate of NADH oxidation to give LDH activity.

NAD-independent LDH activity was assayed using phenazine methosulphate and 2,6-dichlorophenol-indophenol (Gregolin & Singer, 1963).

Chemicals. Pyridoxin, HCl, thiamin, HCl, NADH, NADPH, NAD⁺, DL-lactic acid, fructose 1,6-bisphosphate, AMP, bovine serum albumin, ATP, phenazine methosulphate, 2,6-dichlorophenolindophenol, Trizma base and imidazole were obtained from Sigma, L(+)-lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate and ADP from Boehringer, and sodium dithionite from May & Baker. All other chemicals were obtained from BDH.

Fig. 1

Fig. 2

Fig. 1. Fermentation products in glucose-limited continuous culture: ○, lactate; ●, acetate; □, propionate; ■, pyruvate.

Fig. 2. Fermentation products in ammonia-limited continuous culture: symbols as in Fig. 1.

RESULTS

Production of fermentation acids in continuous culture

Selenomonas ruminantium produced acetate, propionate and lactate at all dilution rates in both glucose- and ammonia-limited continuous cultures. However, the rate of production of each acid varied according to the limiting substrate and the dilution rate.

Under glucose limitation (Fig. 1), production of lactate was low at low dilution rates, and rose slowly up to $D = 0.2 \text{ h}^{-1}$. Thereafter lactate production increased rapidly, eventually reaching 1.0 mole of lactate per mole of glucose consumed at $D = 0.41 \text{ h}^{-1}$. The maximum specific growth rate of *S. ruminantium* is 0.44 h^{-1} in this medium. Production of acetate and propionate fell with increasing dilution rate (Fig. 1). Succinate was a minor product often detected in the medium, but its production was variable, and in any case was always less than 4% of the glucose carbon fermented. Production of pyruvate, another minor fermentation product, increased linearly with dilution rate (Fig. 1). No butyrate was found in any sample. Carbon balances were constructed, making the assumption that carbon constituted 50% of the dry weight of bacteria. At all values of D except 0.20 and 0.21 h^{-1} , cell material, acetate (+ CO_2), propionate, lactate, succinate ($-\text{CO}_2$) and pyruvate accounted for $102.0 \pm 4.5\%$ of the glucose carbon consumed. At $D = 0.20, 0.21 \text{ h}^{-1}$, recovery of carbon in the above products fell to 84.1 and 77.4% respectively.

Under ammonia limitation (Fig. 2), lactate production was high throughout. The production of pyruvate was also generally higher than under glucose limitation; however, under ammonia limitation, pyruvate production fell as D increased, until at values of D near the maximum specific growth rate, its yield [about $23 \text{ mmol (mol glucose)}^{-1}$] was similar in both types of culture, as was lactate production. The formation of acetate and propionate in ammonia-limited cultures was independent of dilution rate above $D = 0.19 \text{ h}^{-1}$, but at lower D the production of these acids, particularly of propionate, did change with

Table 1. *Lactate dehydrogenase content and rate of lactate production of S. ruminantium*

Samples were removed from continuous cultures, harvested, sonicated and assayed for LDH as described in Methods. Specific activity of LDH in cell-free extracts is expressed in $\mu\text{mol NADH oxidized (mg protein)}^{-1} \text{ h}^{-1}$. The production of lactate is expressed both as concentration and as the specific rate of production by the culture, in $\mu\text{mol lactate (mg dry wt)}^{-1} \text{ h}^{-1}$.

Limiting nutrient	D (h^{-1})	Specific activity of LDH	Lactate concn (mM)	Rate of lactate production
Glucose	0.15	28	1.3	0.3
Glucose	0.18	11	1.5	0.9
Glucose	0.21	19	4.3	0.9
Glucose	0.29	15	13.5	6.7
Glucose	0.34	19	15.6	7.3
Ammonia	0.05	4	9.3	1.7
Ammonia	0.13	4	6.9	7.1
Ammonia	0.22	7	6.8	26.5
Ammonia	0.32	12	5.3	21.0

Table 2. *Effects of some potential regulators of lactate dehydrogenase activity*

The overflow from an ammonia-limited culture ($D = 0.39 \text{ h}^{-1}$) was collected at 4°C and harvested by centrifugation (11 600 g , 4°C , 15 min). A cell-free extract was prepared and LDH was assayed as described in Methods. Activities are expressed as percentages of the rate of NADH oxidation in the absence of added potential effectors. The standard error of the mean of four replicate assays was always less than $\pm 7\%$ of the full LDH activity.

Addition	Concn (mM)	LDH activity (%)
None	—	100
Fructose 1,6-bisphosphate	0.5	100
Glucose 6-phosphate	1.0	98
NAD^+	1.0	84
AMP	1.0	111
ADP	1.0	75
ATP	0.4	72
ATP	1.0	46
ATP	2.0	49
Fumarate	5.0	97
Succinate	5.0	99

changing D . An unusually high recovery of carbon was associated with ammonia-limited cultures. For $D \geq 0.19 \text{ h}^{-1}$ the recovery was $112.1 \pm 3.8\%$, and at $D < 0.19 \text{ h}^{-1}$ the apparent recovery of carbon was even greater (e.g. 158% at $D = 0.13 \text{ h}^{-1}$). Again, succinate was a variable minor fermentation product in most samples.

While lactate was measured routinely by the non-stereospecific method of Conway (1957), several samples from both types of culture at several dilution rates were analysed by the stereospecific enzymic method. No L(+)-lactate was found in the supernatant fluid from any culture sample.

Properties of lactate dehydrogenase of S. ruminantium

NAD-linked LDH activity was measured in the supernatant fraction of centrifuged, sonicated samples from continuous cultures. The LDH of glucose- and ammonia-limited *S. ruminantium* at different dilution rates varied (Table 1), but not in a way which accounted for the observed differences in the rates of lactate production in continuous culture.

Enzyme activity was stable to freezing, both of whole bacteria and of sonicated samples, and on storage at -60°C for several weeks. It was also completely stable in sonicated samples at room temperature for at least 6 h. NADP could not replace NAD as cofactor for the enzyme, and the product was D(—)-lactate. No activity was associated with the particulate

Fig. 3. Influence of pyruvate concentration on lactate dehydrogenase activity. The overflow from an ammonia-limited continuous culture was collected at 4 °C and harvested by centrifugation (11 600 g, 4 °C, 15 min). A cell-free extract was prepared and LDH was assayed as described in Methods. Each assay mixture contained 138 μ g protein. Results are expressed in terms of the initial rate of NADH oxidation [v ; μ mol NADH oxidized (mg protein) $^{-1}$ h $^{-1}$] and pyruvate concentration (s ; mM). (a) Lineweaver-Burk double reciprocal plot. (b) Hill plot, using $V_{\max}$ determined from plot (a). (c) Double reciprocal plot: $1/v$ against $1/s^2$.

fraction. NAD-independent LDH activity in whole sonicated samples was less than 2% of the NAD-linked activity. Fructose 1,6-bisphosphate did not affect NAD-independent LDH.

Several potentially regulatory compounds were tested for their effects on LDH activity (Table 2). Of these, ATP and, to a lesser extent, ADP and NAD $^{+}$ caused inhibition, while fructose 1,6-bisphosphate, glucose 6-phosphate, AMP, fumarate and succinate had no significant effect. Fructose 1,6-bisphosphate also did not activate LDH in assays in which 20 mM-Tris pH 7.6 replaced phosphate as the buffer in which bacteria were disrupted and in which LDH was assayed.

Enzyme kinetics

LDH activity was measured over a range of pyruvate concentrations, and the results were graphed in several ways. A Lineweaver-Burk plot of the data was clearly non-linear (Fig. 3a), and a Hill plot (Fig. 3b) confirmed that the disproportionately low activity of LDH at low pyruvate concentrations was a consequence of a cooperative effect of the substrate on enzyme activity. For the experiment illustrated by Fig. 3, the Hill coefficient was 1.6 and the substrate concentration at $\frac{1}{2}V_{\max}$ [i.e. where $v/(V_{\max} - v) = 1$] was 5.4 mM. A double reciprocal plot of $1/v$ against $1/s^2$ (Fig. 3c) gave a graph that was linear except at the highest concentrations of pyruvate.

The kinetic experiment was repeated using cell-free extracts from other glucose- and ammonia-limited cultures at different growth rates, with very similar results. Hill coefficients were similar, and again the $1/v$ against $1/s^2$ graph appeared linear at all but the highest values of s .

DISCUSSION

Hobson (1965*a*) and Scheifinger *et al.* (1975) observed that the fermentation pattern of *S. ruminantium* changed according to the dilution rate in glucose-limited continuous culture, such that acetate and propionate production fell as D increased and lactate became an increasingly predominant product. Their results did not, however, show that the increase was in two phases and that a transition from low lactate production to high lactate production took place at around $D = 0.2 \text{ h}^{-1}$. Ammonia-limited continuous culture, where carbon and energy were presumably in excess at all D values and lactate production was always high, complemented glucose-limited culture in support of the suggestion by Scheifinger *et al.* (1975) that the activity of LDH might be regulated by levels of intracellular pools of metabolic intermediates, such that accumulation of metabolites would activate LDH and trigger conversion of pyruvate to lactate. The possibility that LDH activity was controlled by regulation of the enzyme's synthesis was eliminated since no correlation was found between the LDH content of cultures and their lactate-producing activity.

Lactate dehydrogenases of a wide range of life forms are subject to metabolic regulation (Everse & Kaplan, 1973), and those of several micro-organisms require fructose 1,6-bisphosphate for activation (Wolin, 1964; de Vries *et al.*, 1970; Jonas, Anders & Jago, 1972; Holland & Pritchard, 1975; Eisenberg, Elchisak & Rudd, 1976; van Gylswyk, 1977; Crow & Pritchard, 1977). However, as also found by Scheifinger *et al.* (1975), the LDH of *S. ruminantium* showed no response to added fructose 1,6-bisphosphate. Of some other possible effectors, only ATP significantly influenced enzyme activity, 2 mM-ATP causing more than 50% inhibition. It seems unlikely that inhibition of LDH by ATP could be primarily responsible for the transition in rates of lactate production discussed above, particularly since the ATP pool of *S. ruminantium* does not change markedly with growth rate (Hobson & Summers, 1972). It is more likely that ATP is a secondary regulator of LDH, as it is for the fructose 1,6-bisphosphate-activated enzyme in *Rothia dentocariosa* (Eisenberg *et al.*, 1976) and the pyruvate-activated enzyme of *Butyrivibacterium rettgeri* (Wittenberger, 1968), so that LDH is partially inhibited by high energy levels and activated by high levels of intracellular pools of glycolytic intermediates.

The kinetic experiments with cell-free extracts clearly showed that the LDH of *S. ruminantium* did not display classical Michaelis-Menten kinetics in response to changes in pyruvate concentration. Enzyme activity increased with increasing substrate concentration more rapidly than could be accounted for by simple saturation of a single binding site on the LDH molecule. Analysis of these data by a Hill plot indicated that LDH possessed two binding sites for pyruvate, such that binding of pyruvate to the molecule was positively cooperative and resulted in activation of the enzyme. It was therefore concluded that LDH of *S. ruminantium* is an allosteric enzyme subject to homotropic activation by its substrate, pyruvate.

The D(-)-lactate dehydrogenases of *Butyrivibacterium rettgeri* (Wittenberger & Fulco, 1967; Wittenberger, 1968) and *Escherichia coli* B (Tarmy & Kaplan, 1968) have kinetic properties strikingly similar to those of the LDH of *S. ruminantium* which was also found to be a D(-)-lactate dehydrogenase (EC 1.1.1.28). The enzymes of all three bacteria were allosterically activated by pyruvate, were inhibited by ATP, and were linked specifically to NAD^+ as cofactor. Indeed the *E. coli* enzyme gave a Hill plot similar to that for *S. ruminantium* with a gradient of 1.7 (cf. 1.6 for *S. ruminantium*) and a pyruvate concentration at $\frac{1}{2}V_{\text{max}}$ of 7.2 mM (cf. 5.4 mM) at pH 7.5. The *B. rettgeri* enzyme had a slightly higher affinity for pyruvate (Wittenberger & Fulco, 1967), but was otherwise similar.

Tarmy & Kaplan (1968) pointed out that a consequence of the regulatory characteristics of *E. coli* LDH was that the reduction of pyruvate to lactate would follow virtually second order reaction kinetics at low concentrations of pyruvate. The graph of $1/v$ against $1/s^2$ showed that this was also true for the LDH of *S. ruminantium*.

Pyruvate occupies a central position in the metabolism of *S. ruminantium*, at the junction

between glycolysis and the biosynthetic reactions of fatty acid and amino acid synthesis, the energy-yielding pathways of acetate and propionate production, and lactate production. So that the biosynthetic reactions may continue under all growth conditions, it is essential that LDH should never cause entire depletion of the pyruvate pool. This is achieved by LDH being inactive or only partially active at low pyruvate concentrations, and only becoming fully active in response to high levels of pyruvate. A similar interpretation of the physiological consequences of this type of kinetic control was made by Tarmy & Kaplan (1968).

Some confirmation of this reasoning was the relationship found between lactate and pyruvate production in continuous culture. Leakage of pyruvate into the medium, which was presumably a function of intracellular pyruvate concentration, increased linearly with increasing growth rate, yet lactate production was sigmoidal in relation to growth rate. At low D , the pyruvate concentration was low, LDH was largely inactive and the proportion of glucose fermented to lactate was minimal. As the pyruvate concentration passed a critical level at $D = 0.2 \text{ h}^{-1}$, LDH was activated and lactate became the major product. Under ammonia limitation, with the carbon source in excess, the pyruvate pool was always high, and the molar ratio of lactate produced/glucose fermented was approximately 1.0 at all dilution rates.

The recovery of carbon in known products from glucose-limited cultures was complete, except in the region of transition from mainly volatile acid production to mainly lactate production. A similar 'loss' of carbon in glucose-limited cultures of two other strains of *S. ruminantium* can be calculated from the results of Scheifinger *et al.* (1975), the loss again occurring only at the transition phase in the production of fermentation acids. The fate of this carbon is not known. The cause of the high carbon recovery in ammonia-limited cultures, apparently much greater than 100%, is also unknown, but is not unprecedented in the study of rumen bacteria (e.g. Latham & Legakis, 1976). As the only sources of carbon other than glucose were HCO_3^- in the reducing solution and the CO_2 gas phase, it must be concluded that considerable CO_2 fixation may occur in ammonia-limited *S. ruminantium*, particularly at low dilution rates where the ATP pool and pools of other metabolic intermediates are likely to be high. CO_2 is essential for optimal growth of *S. ruminantium* in batch culture (Dehority, 1971), and it may be that CO_2 uptake is, under some circumstances, quantitatively more significant than its virtually catalytic role in the propionate fermentation pathway (Hobson & Summers, 1972).

It should be noted that some strains of *S. ruminantium* form DL-lactate or only the L(+)-isomer (de Vries, van Wijck-Kapteyn & Oosterhuis, 1974; Scheifinger *et al.*, 1975), and so this mechanism of metabolic control may not be applicable to other strains.

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