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Effect of Amino Acids on the Heat Production and Growth Efficiency of *Streptococcus bovis*: Balance of Anabolic and Catabolic Rates

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***Streptococcus bovis* JB1 grew nearly twice as fast (0.9 versus 1.6 h^{-1}) and had a 40% greater growth yield (18 versus 12.5 mg of protein per mmol of glucose) when an ammonia-based medium was supplemented with amino acids, but the glucose consumption rate (88 μmol mg of protein^{-1} h^{-1}) and specific rate of heat production (2.1 mW/mg of protein) were unaffected. Amino acid availability had little effect on the catabolic rate, but the specific heat decreased 40% (8.8 to 5.2 J/mg of protein). These growth rate-dependent changes in metabolic efficiency were fivefold greater than the maintenance energy. Chloramphenicol (100 mg/l), an inhibitor of protein synthesis, caused a gradual decrease in anabolic (growth) rate, but there was little change in the rate of glucose consumption and the specific heat increased. When growth was inhibited by iodoacetate, the catabolic and anabolic rates both declined and there was no increase in specific heat. On the basis of these results, the benefit of amino acid supplementation was largely explained by the balance of anabolic and catabolic rates. When amino acids were available, the anabolic and catabolic rates were more closely matched and less energy was spilled as heat.**

For ruminant animals, microbial protein is a major source of amino acids and the efficiency of microbial protein synthesis can have a major impact on meat and milk production (10, 14). Most rumen bacteria can utilize ammonia as a nitrogen source, but the growth of many species is stimulated by amino acids (1). Pure cultures of rumen bacteria grew as much as 40% more efficiently when they were supplemented with protein hydrolysate (7), and the flow of microbial protein from the rumen was 30% greater when a urea-based diet was supplemented with casein (9).

The effect of amino acids on rumen microbial growth efficiency remains uncertain (8). Stouthamer's calculations (23) indicated that amino acid availability should have little impact on bacterial growth efficiency, as the cost of transport would offset any saving in biosynthesis. Polymerization reactions require far more energy than monomer synthesis.

Estimates of microbial growth efficiency have often been confounded by differences in growth rate and maintenance energy, variations in cell composition, and lack of accurate determination of ATP production. In most instances, there has been no indication of the amount of the energy source which was used as a carbon source. Despite some attempts to account for these variations (3, 12, 13, 15, 16, 24, 25), few studies directly assessed the natural inefficiency of all biological processes, i.e., heat production. Microcalorimetry can provide a reliable and continuous estimate of bacterial heat production (17, 18, 21).

The diversity and complexity of the rumen microbial ecosystem complicates the study of rumen microbiology (10) and makes the choice of a model organism difficult. *Streptococcus bovis* can be a predominant organism when the diet is rich in cereal grain (10), and it exhibits the following useful attributes: (i) a simple and well-defined fermentation scheme, (ii) a rapid growth rate, (iii) some resistance to oxygen, (iv) a lack of storage polysaccharide, (v) simple nutritional requirements, and (vi) an ability to use either ammonia or amino acids as a nitrogen source (18, 26).

Previous work showed that the growth yields of *S. bovis* were increased by addition of an amino acid source (7). The experiments described here indicated that this stimulation was due to a better balance of catabolic and anabolic rates, which decreased the loss of metabolic energy as heat.

MATERIALS AND METHODS

Cell growth. *S. bovis* JB1 was grown anaerobically at 39°C in 350-ml fermentation vessels in a basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 600 mg of cysteine hydrochloride, 3 g of glucose, microminerals, and vitamins (7), and the medium was supplemented with Casamino Acids (Difco, Detroit, Mich.) as indicated. During growth in continuous culture (145-ml culture vessel), at least 98% of the medium turned over between samplings. The specific growth rate of exponentially growing cells was estimated from the increase in optical density (1-cm-diameter cuvettes, 600 nm), and preliminary work indicated that the ratio of protein to optical density (160 μg of protein ml^{-1} optical density $unit^{-1}$) was not influenced by the specific growth rate or iodoacetate.

Heat production. Bacterial heat production was measured with an LKB 2277 bioactivity monitor equipped with semiconducting Peltier elements as thermopiles and gold flow cells. The instrument was calibrated and stabilized with an internal electric heat source. The flow cell was sterilized with 95% ethanol and 1 M HCl. Bacteria and medium were pumped through the flow cell at a rate of 40 ml/h (effective volume, 0.678 ml). The flow cell temperature was set at 39.00°C, and total transit time from the culture vessel through the flow cell and back to the culture vessel was approximately 4 min. Heat production (microwatts per milliliter) of exponentially growing cultures was directly porportional to optical density (intercepts at the origin with correlation coefficients of greater than 0.95).

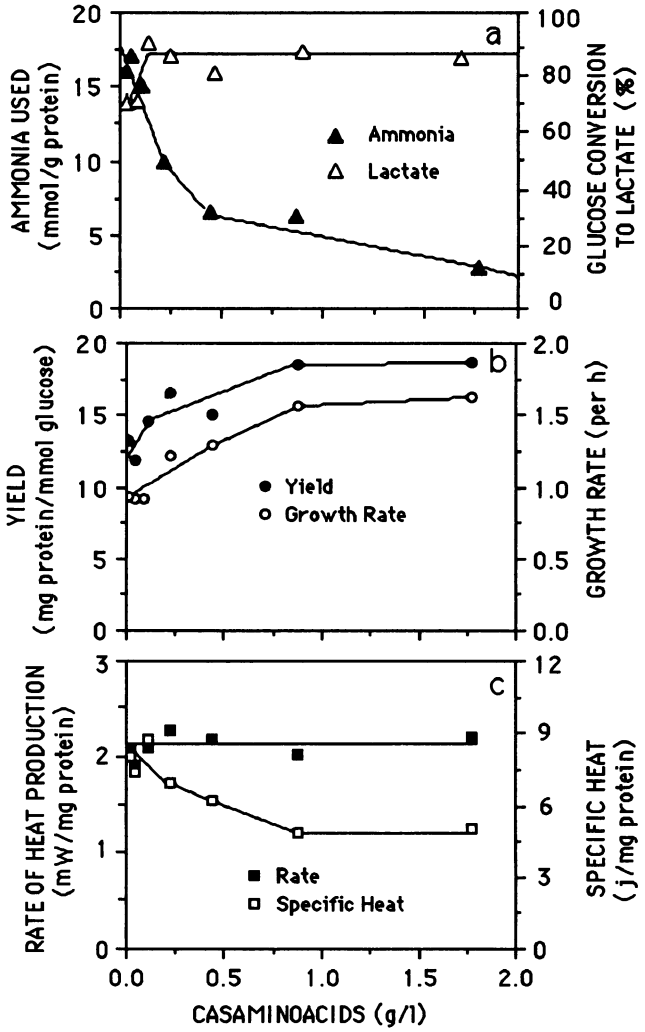

FIG. 1. Effect of Casamino Acids on ammonia utilization and lactate production (a), yield and specific growth rate (b), and rate of heat production and specific heat of protein synthesis (c) of *S. bovis*.

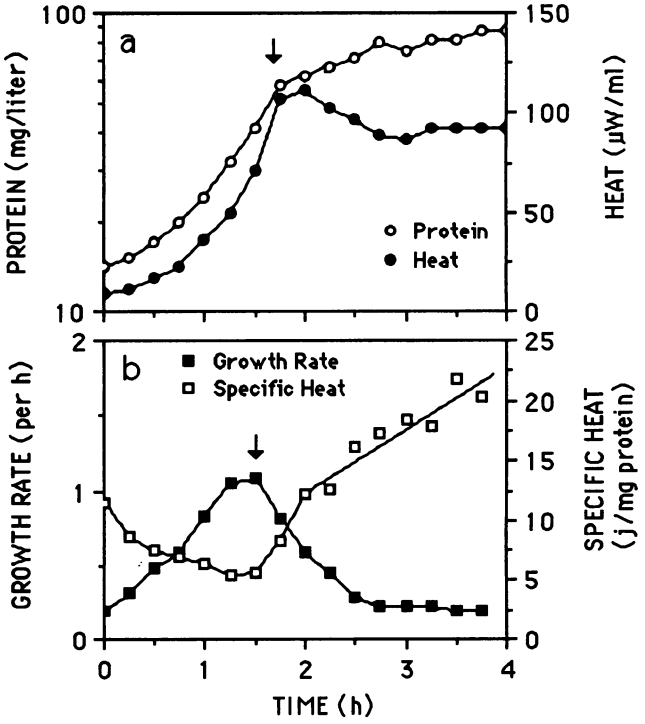

FIG. 2. Effect of chloramphenicol (10 μg/ml at 1.5 h [arrow]) on the growth and heat production of *S. bovis* in batch culture with 1.8 g of Casamino Acids per liter (a). The relationship between the specific growth rate and specific heat of protein synthesis is shown in panel b. The inoculum was grown in a medium lacking Casamino Acids.

Other analyses. Glucose was quantified by an enzymatic method using hexokinase and glucose 6-phosphate dehydrogenase (4). Protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 15 min) was assayed by the method of Lowry et al. (11). Cell carbohydrate was assayed by the anthrone reaction (2). Lactate, ethanol, and fermentation acids were determined by high-pressure liquid chromatography with a Beckman 334 liquid chromatograph equipped with a 156 refractive index detector and a Bio-Rad HPX-87H organic acid column. The sample size was 20 μl, the eluant was 0.0065 M H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50°C.

RESULTS

Effects of amino acids. When batch cultures of *S. bovis* JB1 were supplemented with Casamino Acids, ammonia utilization declined and the specific growth rate increased (0.9 versus 1.6 h^{-1}; Fig. 1). Amino acid supplementation caused a 40% increase in growth yield, but the specific rate of bacterial heat production was unaffected. Lactate was the only fermentation product detected, and there was little change in recovery of glucose as lactate. *S. bovis* did not accumulate large amounts of anthrone-reactive material, and the ratio of carbohydrate to protein (0.27 ± 0.04 mg/mg) was not affected by the supply of amino acids.

Effects of chloramphenicol and iodoacetate. When cultures lacking amino acids were inoculated into medium containing 1.8 g of amino acids per liter, the specific growth rate was initially low and during this time the specific heat (specific rate of heat production divided by specific growth rate) was 12 J/mg of protein (Fig. 2). As the rate of protein synthesis increased, specific heat declined. When chloramphenicol was added to the culture, the optical density continued to increase, but there was a gradual decrease in the rate of protein synthesis (1.0 to 0.2 h^{-1}). Because the rate of protein synthesis declined at a faster rate than heat production, specific heat increased dramatically.

Inocula supplemented with 1.8 g of amino acids per liter did not lag, and the cells soon had a specific growth rate of 1.6 h^{-1} (Fig. 3). Iodoacetate, an inhibitor of glycolysis, also caused a decrease in specific growth rate when it was added in a stepwise fashion, but in this case the specific heat did not increase significantly. The titration of iodoacetate was, however, not always predictable. When another dose of iodoacetate (20 μM) was added at 2 h, glucose consumption and heat production were completely eliminated and the specific heat declined to 0 (data not shown).

Maintenance energy. When *S. bovis* was grown in glucose-limited chemostats, cultures that received 1.8 g of amino acids per liter had theoretical maximum growth yields nearly

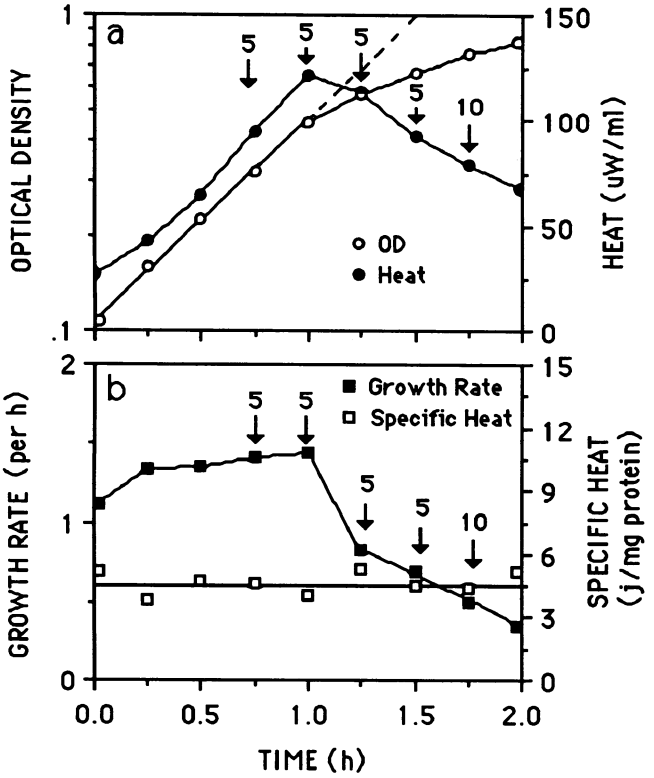

FIG. 3. Effect of iodoacetate (5 or 10 μM at the arrows) on the growth and heat production of *S. bovis* in batch culture (a). The relationship between the specific growth rate and specific heat of protein synthesis is shown in panel b. The inoculum was grown in a medium containing 1.8 g of Casamino Acids per liter.

twice as high as those of cultures having only ammonia (Fig. 4). However, amino acids had little effect on the maintenance energy coefficient, i.e., the slope of the plot (8.5 versus 7 μmol of glucose per mg of protein per h for 1.8 and

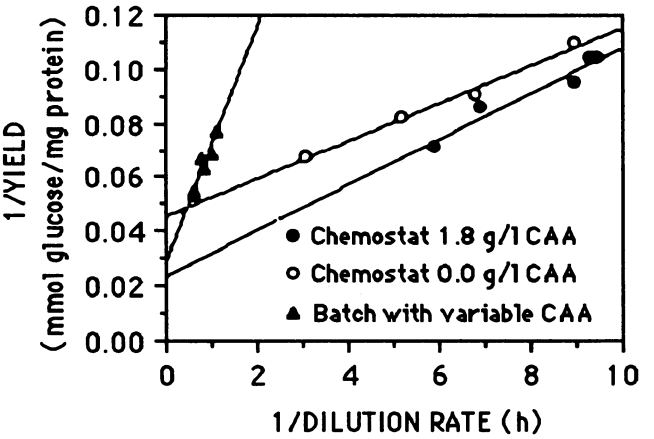

FIG. 4. Double-reciprocal plot of 1/yield versus 1/dilution rate (16). The theoretical maximum growth yields (1/intercept to the ordinate) were 37.7 and 21.9 mg of protein per mmol of glucose, and the maintenance energies (slope) were 8.5 and 7.0 μmol of glucose mg of protein^{-1} h^{-1} for 1.8 (●) and 0.0 (○) g of Casamino Acids (CAA) per liter, respectively. The relationship between 1/yield and 1/specific growth rate for batch cultures that received various amounts of Casamino Acids is also shown (▲).

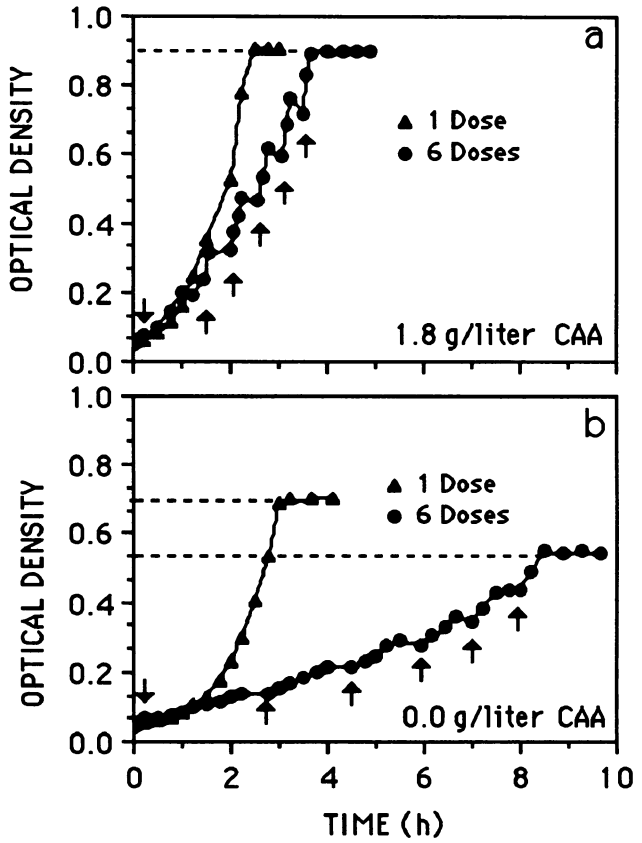

FIG. 5. Effect of discontinuous glucose addition on growth of *S. bovis* when either 1.8 (a) or 0.0 (b) g of Casamino Acids per liter was available in batch culture. The cultures received either a single dose of 10 mM glucose at time zero or six doses of glucose (1.67 mM at each of the arrows).

0.0 g of amino acids per liter, respectively). In continuous culture, acetate, formate, and ethanol (1:2:1) were the end products of glucose fermentation.

Discontinuous growth. *S. bovis* cultures that received 1.8 g of amino acids per liter and a single dose of 10 mM glucose grew without a lag, and the specific growth rate was 1.6 h^{-1} (Fig. 5). When the same amount of glucose was provided in six doses and the consecutive doses were not added until the culture had stopped growing for 15 min, *S. bovis* resumed its maximum specific growth rate and achieved the same final optical density (0.90). Cultures that received ammonia (no amino acids) and a single dose of 10 mM glucose had a long lag period, but they eventually grew at 1.0 h^{-1} and the final optical density was 0.75. When the cultures had ammonia as the only nitrogen source and glucose was given in six pulse doses, the specific growth rate was never greater than 0.45 h^{-1} and the final optical density was 25% lower (0.75 versus 0.53).

DISCUSSION

When amino acids were added to the *S. bovis* cultures, the efficiency of growth (yield) was 40% greater (Fig. 1b) and this difference could not be explained by a change in fermentation end products (Fig. 1a) or an accumulation in cell polysaccharide. Cultures supplemented with amino acids grew faster than those which had only ammonia as a

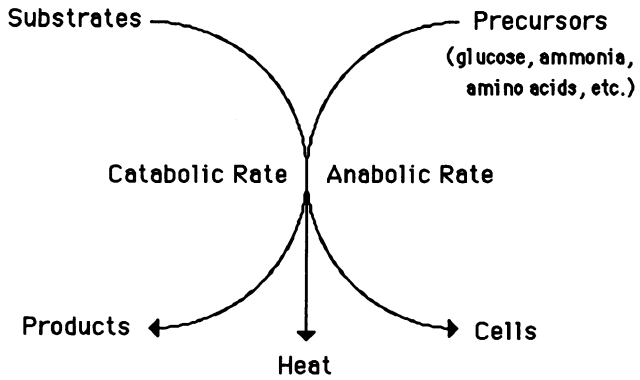

FIG. 6. Coupling of catabolism and anabolism.

nitrogen source (Fig. 1b), but maintenance energy could not explain the 40% difference in yield. The amino acid-dependent change in the glucose consumption rate of the batch cultures was fivefold greater than the maintenance rate, and maintenance never accounted for more than 15% of the total glucose consumption rate (Fig. 4).

Continuous cultures of *Klebsiella aerogenes* that were limited by ammonia, potassium, or sulfur consumed glucose faster than those which were limited by glucose, and the investigators explained this increase in glucose consumption by a variable or growth rate-dependent maintenance coefficient (12, 16). The use of a variable maintenance coefficient, however, introduces another question: why should cells which are energy limited require less maintenance energy than cells which are not energy limited?

The amino acid-dependent increase in glucose consumption for *S. bovis* is more easily explained by an imbalance of anabolic and catabolic rates and the dissipation of excess energy as heat (Fig. 6). When the cells were deprived of amino acids, the anabolic (growth) rate decreased (Fig. 1b) but the rate of heat production, the catabolic rate, was unaffected (Fig. 1c). When the rate of catabolism exceeded the rate of anabolism, the specific heat increased. The idea that the cells were responding to the balance of anabolic and catabolic rates was supported by the effects of chloramphenicol and iodoacetate. When the cells were treated with chloramphenicol, a protein synthesis or anabolic inhibitor, the rate of protein synthesis decreased. Once again, there was little change in the catabolic rate and the specific heat increased (Fig. 2b). Iodoacetate, a glycolytic or catabolic inhibitor, also caused a decrease in specific growth rate, but in this case the catabolic and anabolic rates both decreased and there was little increase in specific heat (Fig. 3b).

In *Escherichia coli*, ammonia limitation can create an energy-consuming futile cycle of ammonia through the cell membrane (6). Such a mechanism, however, cannot explain the amino acid-dependent changes in glucose consumption that were observed with *S. bovis*. *S. bovis* always had excess ammonia and glucose (Fig. 1). Previous experiments showed that nitrogen-limited and chloramphenicol-treated *S. bovis* cultures dissipated heat by an *N,N'*-dicyclohexylcarbodiimide-sensitive mechanism (21). On the basis of these earlier results, it appeared that growth-independent heat was being created by a futile cycle of protons through the cell membrane and the activity of the membrane-bound proton ATPase (18).

Lag is a common feature of the bacterial growth cycle, but the cause of the lag period has been a curiosity. Are the cells unable to take up an energy source immediately? Are the cells unable to initiate protein synthesis? Is a large portion of the cells unable to grow? With *S. bovis*, the lag period could not be explained by the inability of the cells to transport and metabolize glucose. The rate of glucose metabolism was faster than the specific growth rate, and the additional energy was dissipated as heat (Fig. 2). Brooks and Meers (5) noted that the growth efficiency of a methanol-utilizing pseudomonad decreased dramatically when the energy source was provided discontinuously (continual lags in growth) although the interval between feedings was short. With *S. bovis*, a discontinuous glucose feeding cycle only decreased the yield, but only if ammonia was the nitrogen source. When amino acids were available, *S. bovis* grew without a lag and little energy was spilled (Fig. 5).

Several questions arise: (i) are energy-spilling reactions a novel characteristic of rapidly growing organisms which exploit energy-rich environments, (ii) are energy-spilling reactions a common feature of bacteria, and (iii) is there any advantage in spilling energy? The experiments of Schonheit et al. (22) showed that *Methanobacterium thermoautotrophicum* was able to "uncouple growth from methane formation when both H_2 and CO_2 concentration is high," even though methanogens are normally characterized as slowly growing and energy-starved bacteria. Mixed rumen bacteria grew 60% less efficiently when limited by nitrogen (20), but recent work has indicated that not all rumen bacteria spill energy. When *Bacteroides ruminicola* B_14 was grown in nitrogen-limited medium, little energy was spilled but there was a dramatic (10,000-fold) reduction in viable cells (19). *S. bovis* JB1, a bacterium capable of spilling energy, did not show a decrease in viability when nitrogen was limiting and glucose was in excess (data not shown). The latter comparison indicates that energy spilling is an advantageous strategy for cell survival.

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