Metabolism of Exogenous Arginine and Glucose by Starved *Streptococcus lactis* in Relation to Survival

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SUMMARY

Arginine or glucose accelerated death of *Streptococcus lactis* starved in phosphate buffer. Both substrates were metabolized at linear but different rates and the rate of ATP production from glucose metabolism was about 7.5 times that for arginine metabolism. Mg^{2+} reduced glucose-accelerated death and abolished arginine-accelerated death to prolong survival. When glucose was fed continuously to organisms starved in phosphate buffer containing Mg^{2+} , survival was related to the rate of glucose addition and hence to the rate of metabolism. By feeding low concentrations of glucose, the viability of most of the population was extended from about 1 to 4 days. The maintenance energy requirement appeared to be in the region of 0.045 to 0.09 mg. glucose/mg. dry wt bacteria/hr. The glycolytic activity of starved organisms declined at varying rates depending on the starvation environment but could not be correlated directly with loss of viability. Exogenous arginine substantially reduced the lethal effect of adverse pH values.

INTRODUCTION

Streptococcus lactis ferments carbohydrate mainly by means of the Embden-Meyerhof pathway, and in an earlier investigation (Thomas & Batt, 1969*a*) the lactic acid produced was found to account for approximately 93 % of the glucose fermented. Apart from carbohydrate, arginine is the only substrate reported from which *S. lactis* can obtain energy. Catabolism of arginine by *S. lactis* produced ornithine, carbamyl phosphate and ammonia and the enzymes catalysing these reactions were characterized by Korzenovsky & Werkman (1953, 1954). When *S. lactis* was starved in the presence of a fermentable carbohydrate, accelerated death occurred (Thomas & Batt, 1968). Addition of Mg^{2+} reduced this effect while incubation of starved organisms with arginine plus Mg^{2+} gave extended survival indicating that the nature of the energy source was critical for survival.

Postgate & Hunter (1962) reported that glycerol-limited *Aerobacter aerogenes* died more rapidly when washed and starved in phosphate buffer containing glycerol than in phosphate buffer alone. This phenomenon was termed 'substrate-accelerated death' and has since been investigated intensively for *A. aerogenes* (see review by Postgate, 1967). Strange & Dark (1965) reported that the death rate of *A. aerogenes* increased with the rate of substrate metabolism while exogenous Mg^{2+} decreased the rate of

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degradation of intracellular RNA without significantly affecting substrate metabolism. The mechanism of substrate-accelerated death is not fully understood. However, Strange & Dark (1965) attributed glycerol-accelerated death, at least in part, to the formation of an unidentified toxic product of glycerol metabolism. They suggested that added Mg^{2+} prevented the accumulation or formation of this product. During glucose-accelerated death of *A. aerogenes* the bacterial ATP pool increased significantly whereas in the presence of glucose plus Mg^{2+} there was little change (see Discussion, Strange & Hunter, 1967). In this connexion, Postgate & Hunter (1964) showed that glycerol-accelerated death of *A. aerogenes* was largely abolished by uncoupling agents. Any proposed mechanism should be consistent with the fact that while exogenous glucose accelerates death the death rate may decrease in parallel with increasing intracellular polyglucose levels (Strange, Dark & Ness, 1961).

In contrast to the effect of carbon substrates on starving organisms discussed above however, there are several recent reports of decreased death rates resulting from the intermittent addition of small amounts of glucose to *Escherichia coli* starved in buffer containing Mg^{2+} (Clifton, 1966; McGrew & Mallette, 1962, 1965; Mallette, 1963). However, the evaluation of some of these results is difficult since the experimental procedure did not preclude regrowth (see Dawes & Ribbons, 1964; Postgate, 1967).

The present investigation was undertaken in an attempt to define the role of exogenous, energy-yielding substrates in relation to bacterial survival.

METHODS

General procedures. The organism (*Streptococcus lactis* ML₃), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969*b*). With experiments which involved the continuous feeding of glucose, washed organisms suspended in phosphate buffer were placed in a triple-necked quickfit flask (1400 ml. capacity) held in an incubator at 30°. The culture was agitated by a magnetic stirrer and phosphate buffer containing glucose was added at a constant flow rate using a D.C.L. Micropump (F. A. Hughes and Co., England) attached to a reservoir flask fitted with a constant head device.

Analytical methods. The products of arginine metabolism were estimated using the basic column of a Beckman model 120 C automatic amino acid analyser. The glycolytic activity of starved organisms was determined by the method of Thomas & Batt (1969*a*).

RESULTS

The effect of energy sources on the survival of starved *Streptococcus lactis* is shown in Fig. 1. Both arginine and glucose accelerated the death rate. Mg^{2+} reduced glucose-accelerated death while arginine + Mg^{2+} gave extended survival times.

Arginine was metabolized at a linear rate of 2.35 μ mole/mg. dry wt bacteria/hr (Fig. 2). This rate theoretically corresponds to 2.35 μ mole ATP/mg. dry wt bacteria/hr (based on the stoichiometric reaction:

The glycolytic activity of *Streptococcus lactis* ML₃ was 0.29 μ mole lactate/mg. dry wt bacteria/min. (Thomas & Batt, 1969*a*) and, since anaerobic fermentation of

glucose produces 1 mole of ATP/mole lactate, this glycolytic activity represents the formation of $17.7 \mu\text{mole ATP/mg. dry wt bacteria/hr.}$ Therefore the theoretical rate of ATP generation by *S. lactis* is about 7.5 times greater with 10 mM-glucose than with 10 mM-arginine. Arginine metabolism yielded the theoretical amount of ornithine and slightly less than the theoretical amount of ammonia (Fig. 2), confirming the findings of Korzenovsky & Werkman (1954). In earlier survival experiments (Thomas & Batt, 1968) washed suspensions with $20 \mu\text{g. dry wt bacteria/ml.}$ were incubated with 10 mM-arginine. Assuming the above linear rate, these organisms would require more than 200 hr for complete arginine metabolism, so that arginine was probably not limiting in these experiments.

Fig. 1

Fig. 2

Fig. 1. Effect of glucose and arginine on survival of *Streptococcus lactis*. Washed organisms, harvested from the end of the growth phase in routine medium, were resuspended at $20 \mu\text{g. dry wt/ml.}$ in $0.075 \text{ M-phosphate buffer (pH } 7.0, 10 \mu\text{M-EDTA)}$ containing: $\circ$, no addition; $\bullet$, 1 mM-MgSO_4 ; $\square$, 10 mM-glucose ; $\blacksquare$, $10 \text{ mM-glucose} + 1 \text{ mM-MgSO}_4$; $\triangle$, 10 mM-arginine ; $\blacktriangle$, $10 \text{ mM-arginine} + 1 \text{ mM-MgSO}_4$. Cultures were incubated at 30° and viability was determined by slide-culture.

Fig. 2. Arginine metabolism by *Streptococcus lactis*. Bacteria were harvested from the routine medium at the end of the growth phase, washed twice and resuspended in $0.075 \text{ M-phosphate buffer (pH } 7.0; \text{ containing } 10 \mu\text{M-EDTA, } 1 \text{ mM-MgSO}_4 \text{ and } 10 \text{ mM-L-arginine)}$. The organisms were incubated at 30° at $4.9 \text{ mg. dry wt/ml.}$ At intervals, samples were withdrawn, immediately cooled to -10° , centrifuged and the supernatants filtered. Supernatant samples were frozen (-20°) and later analysed for arginine ($\circ$), ornithine ($\square$) and ammonia ($\triangle$), using the basic column of an amino acid analyser (see Methods).

Certain published findings with other bacteria have suggested that the extended survival of starved *Streptococcus lactis* with added arginine, as compared with glucose, may have been due to a lower rate of substrate metabolism (see Discussion). In order to test this possibility, experimental conditions were established so that glucose could be fed continuously to starved organisms. Buffered glucose solutions were added to cultures at constant flow rates with different glucose concentrations being used in each experiment. Some typical results of viability measurements on the starved organisms are shown in Fig. 3. Starved *S. lactis* is capable of fermenting glucose at a maximum rate of about $8.8 \mu\text{mole/mg. dry wt bacteria/hr}$ (Thomas & Batt, 1969a).

When glucose was fed continuously to starved organisms in excess of this rate the death rate increased as expected (Fig. 3). By adding $1.5 \mu\text{mole glucose/mg. dry wt bacteria/hr}$, a level which would be expected to produce ATP at about the same rate as added arginine (see above), the survival curve resembled that of organisms starved in the presence of arginine + Mg^{2+} (Fig. 1). Feeding lower levels of glucose further enhanced survival until an optimum level was reached at 0.25 to $0.5 \mu\text{mole glucose/mg. dry wt bacteria/hr}$ (Fig. 3). After 120 hr starvation at a feed rate of $0.25 \mu\text{mole glucose/mg. dry wt bacteria/hr}$ (viability 40 %), organisms were harvested for examination by electron microscopy (Thomas & Batt, 1969*d*).

Fig. 3. Effect of feeding starved *Streptococcus lactis* continuously with different concentrations of glucose. For each experiment, washed organisms harvested from 35 ml. routine medium were resuspended at 30° in 500 ml. 0.075 M -phosphate buffer (pH 7.0) containing 1 mM - MgSO_4 and $10 \mu\text{M}$ -EDTA. The initial bacterial mass was $45\text{--}50 \mu\text{g. dry wt/ml}$. Phosphate buffer, containing different glucose concentrations in each experiment, was added continuously (13 additions/min.) to the stirred culture at a flow rate of 7.0 ml./hr . Samples (0.2 ml.) were removed periodically for determination of viability, pH and turbidity. Glucose feed rates ($\mu\text{mole glucose/mg. dry wt bacteria/hr}$) were: ○, 15.0; ●, 1.5; □, 0.5; ■, 0.25; △, 0.05; ▲, 0.00.

Starved cultures were progressively diluted but since survival was independent of bacterial density in the presence of added Mg^{2+} (Thomas & Batt, 1968), this should not have influenced the present results. The pH drop was always less than 0.15 units. At low feed rates the turbidity of starved cultures, after correction for dilution, showed a slow decline while at higher feed rates the decline was more rapid. At low glucose feed rates, viable organisms after 50 to 100 hr starvation had short division lag times similar to those at the onset of starvation. By contrast, rapid glucose metabolism gave rise to long division lags (Thomas & Batt, 1968) and more rapid RNA breakdown (Thomas & Batt, 1969*b*).

Organisms which were starved under conditions giving the maximum range of

death rate were examined for comparative glycolytic activities. No general correlation between glycolytic activity and survival was found (Table 1). Incubation of starved organisms with glucose caused a more rapid loss of glycolytic activity (Table 1). With the bacterial concentration used, 10 mM-glucose was metabolized in about 5 hr and 10 mM-arginine in about 20 hr. The survival curve for the high glucose feed rate flattened off unexpectedly (Fig. 3), possibly as a result of decreased glycolytic activity. Similarly the survival curves at low feed rates flattened off at low viabilities but in these experiments the lower glucose feed rates may have been capable of supporting only part of the total population.

Table 1. *Glycolytic activity of starved Streptococcus lactis*

Organisms were washed and resuspended at 30° in 100 ml. 0.075 M-phosphate buffer (pH 7.0, 10 μ M-EDTA) containing the additions given in the first column. The cell mass was 0.21 mg. dry wt/ml. At intervals, samples (10 ml.) were removed and centrifuged, the organisms were washed and resuspended in phosphate buffer (2 ml.) and the glycolytic activity determined as described in Methods. Glycolytic activity is given in terms of μ moles lactate/mg. dry wt bacteria/min., % viabilities are given in parentheses.

Additions to above buffer	Time (hr)							
	0.5	1.5	3	5	8	10	22	27
	Glycolytic activity							
None	0.274 (99)	0.247	0.230	0.194 (96)	0.175 (57)	0.174 (15)	0.155 (1)	0.133
+ MgSO ₄ (1 mM)	0.279 (99)	0.264	0.234	0.206 (99)	0.185 (96)	0.164 (98)	0.158 (72)	0.155 (36)
+ MgSO ₄ (1 mM)+L-arginine (10 mM)	0.285 (99)	0.269	0.262	0.218	0.189	0.193 (97)	0.148 (98)	0.144 (91)
+ MgSO ₄ (1 mM)+L-arginine (10 mM) + casamino acids (0.5 %)	0.286 (99)	0.272	0.241	0.231	0.178	0.170 (98)	0.157 (95)	0.140 (87)
+ Glucose (10 mM)	0.269 (99)	0.243 (72)	0.181 (19)	0.162 (11)	0.137 (1)	0.118	0.052	0.045
+ MgSO ₄ (1 mM)+glucose (10 mM)	0.277 (99)	0.260	0.205	0.171 (99)	0.121 (84)	0.091 (61)	0.016 (23)	0.011 (4)

Starved organisms were previously shown to be very sensitive to pH changes, the optimum pH for survival being near 7.0 (Thomas & Batt, 1968). Since Dawes & Ribbons (1962, 1964) have suggested that an energy source is required for intracellular pH control in starved bacteria it seemed appropriate to re-examine the pH effect on *Streptococcus lactis* in the presence of an added energy source. As addition of either carbohydrate or arginine alone produced accelerated death of starved *S. lactis* (Fig. 1) it was decided to re-examine the pH effect with added Mg²⁺, which abolished arginine-accelerated death (Fig. 1), in addition to incubations with arginine + Mg²⁺. Addition of Mg²⁺ decreased the death rate at all pH values (Fig. 4b), while arginine + Mg²⁺ produced a further marked decrease in the death rates (Fig. 4c). The initial pH values in Fig. 4 (a), (b) did not change significantly over the starvation period. After 48 hr starvation with 10 mM-arginine (Fig. 4c), initial pH values had risen by only about 0.2 pH units. This rise was presumably due to ammonia production.

Fig. 4. Effect of pH on survival of *Streptococcus lactis* ML₈. Buffers were prepared from 0.1 M-Na₂HPO₄ and 0.05 M-citric acid containing 10 μ M-EDTA. Curve (a). Bacteria were harvested from 20 ml. culture at the end of the growth phase, washed and resuspended in 5 ml. phosphate-citrate buffer (pH 7.0), 0.5 ml. of this suspension was added to 10 ml. buffer at 30° to give 190 μ g./ml. and final pH values indicated. Curve (b). Suspensions prepared as for (a) at similar pH values; each suspension contained 1 mM-Mg²⁺. Curve (c). Suspensions prepared as for (a) but at 80 μ g. dry wt bacteria/ml. The buffer contained 10 mM-arginine + 1 mM-MgSO₄ and pH values were adjusted to those above by addition of HCl. Results were graphed and a summary is presented. Before viability determination samples were diluted in phosphate-citrate buffer containing 1 mM-Mg²⁺ (pH 7.0).

DISCUSSION

The addition of either glucose or arginine to *Streptococcus lactis* starved in phosphate buffer resulted in accelerated death while the presence of Mg²⁺ either reduced or eliminated this effect. The only metabolic product likely to be common to both glucose and arginine metabolism is ATP. Both arginine and glucose were metabolized at constant rates by starved organisms and the rate of ATP generation was calculated to be about 7.5 times greater with 10 mM-glucose than with 10 mM-arginine, in agreement with the results of Forrest (1965) for starved *Streptococcus faecalis*. Assuming 93 % conversion, a glycolytic activity of 0.29 μ mole lactate/mg. dry wt bacteria/min. corresponds to fermentation of about 1.7 mg. glucose/mg. dry wt bacteria/hr. Even allowing for energy yield differences from glucose metabolism, this rate was much greater than

the maintenance energy requirement of approximately 0.07–0.09 mg. glucose/mg. dry wt bacteria/hr for aerobic metabolism of *Escherichia coli*, *Aerobacter aerogenes* and *Aerobacter cloacae* (see Pirt, 1965). Assuming a similar maintenance energy requirement for *S. lactis*, then clearly there is no economy of metabolic energy for maintenance of starved *S. lactis* and the linear rate of glucose fermentation (Thomas & Batt, 1969a) indicates that the metabolism of starved organisms continues at rapid rates. From data obtained for the growth of *S. lactis* (Thomas, 1968) it can be calculated that growing organisms should produce approximately 1.2 μ moles lactate/mg. dry wt bacteria/min. while glycolytic activity measurements (Thomas & Batt, 1969a), showed that non-growing organisms produced 0.29 μ mole lactate/mg. dry wt bacteria/min. A simple explanation for the observed linear rates of glycolysis and arginine fermentation in starved *S. lactis* would be that these processes were not subject to feedback control and hence ATP could be generated in amounts which were far in excess of requirements. A similar conclusion was drawn by Forrest & Walker (1965a) using *S. faecalis*.

It seemed possible that the extended survival of starved *Streptococcus lactis* organisms with added arginine, compared with glucose, was due to the lower rate of ATP production since McGrew & Mallette (1962) reported an increase in the survival times of starved *Escherichia coli* on the intermittent addition of very small amounts of glucose. In contrast, most other workers have used relatively high carbohydrate/cell mass ratios and have not observed extended survival. Glycerol- and succinate-accelerated death decreased on reducing the substrate concentration and was undetectable at low concentrations (Postgate & Hunter, 1964). It could be informative to re-examine the effect of low glycerol concentrations on survival in the presence of added Mg^{2+} . Strange & Dark (1965) have observed that increased rates of glycerol oxidation produced increased death rates adding to a considerable amount of evidence which suggested that the rate of substrate metabolism, and the rate of ATP production, may be critical for survival. In the present investigation experiments involving the continuous addition of glucose to starved *S. lactis* indicated that survival was profoundly affected by the rate of glucose metabolism. An optimum feed rate of 0.25–0.5 μ mole glucose/mg. dry wt bacteria/hr extended survival from about 1 to 4 days. This corresponds to a maintenance energy requirement for starved *S. lactis* of approximately 0.045–0.09 mg. glucose/mg. dry wt bacteria/hr which is of the same order as that reported for aerobic bacteria (see Pirt, 1965). Although glucose fermentation by *S. lactis* yields less energy than in aerobic organisms, Marr, Nilson & Clark (1963) calculated that for *E. coli* approximately half the maintenance energy requirement may be used for resynthesis so that the actual maintenance requirement for *S. lactis* may be lower than that for *E. coli* since resynthesis of protein and RNA by starved *S. lactis* occurs to a much more limited extent (Thomas & Batt, 1969c).

In the absence of Mg^{2+} , the death rate of *S. lactis* when harvested from the lactose-limited growth medium and starved in phosphate buffer was accelerated to similar degrees by the addition of either glucose, galactose, fructose or lactose (Thomas & Batt, 1968), all of which are considered to be metabolized via the glycolytic pathway. By contrast, the death rate of glycerol-limited *Aerobacter aerogenes* was not accelerated by added glucose or ribose (Postgate & Hunter, 1964). Although these substrates were shown to be metabolized, there was no indication that the rate of their metabolism was similar to that in organisms grown with glucose or ribose limitations where these

substrates accelerated the death rate of starved organisms. Since the activity of constitutive enzymes may vary according to growth conditions (Pardee, 1961), it seems possible that substrates not included in the growth medium may be metabolized at slower rates by starved organisms. However, the strain of *A. aerogenes* used by Strange & Dark (1965) showed carbon-accelerated death with carbon sources besides the one which had been used as the growth-limiting substrate.

Several reports have indicated that rapid metabolism of energy-yielding substrates by starved bacteria is accompanied by an increased rate of polymer hydrolysis (e.g. Strange & Dark, 1965; Thomas & Batt, 1969*b*) while other investigations have demonstrated that the breakdown of protein and nucleic acid in starved bacteria is suppressed by agents inhibiting energy-yielding reactions (see Halvorson, 1962). Slow substrate metabolism may supply the maintenance energy requirement without deleterious effects.

The metabolism of exogenous substrates does not always appear to be coupled to the energy requirements of starved organisms and it seems possible that the rapid depletion of reserve polymers in some bacteria may present a similar situation with respect to endogenous substrates. Burleigh & Dawes (1967) have suggested that the rapid metabolism of polyglucose may cause substrate-accelerated death. Reserve polymers may exert a sparing action on essential cell constituents but they seem to enhance survival only if they are broken down at comparatively slow rates (e.g. see Strange *et al.* 1961; Sierra & Gibbons, 1962; Sobek, Charba & Foust, 1966; Zevenhuizen, 1966). However, Strange & Hunter (1967) also pointed out that the enhanced survival of nitrogen-limited *Aerobacter aerogenes* may be due to the relatively high Mg^{3+} content rather than the glycogen reserves.

In a number of starvation environments, the glycolytic activity of *Streptococcus lactis* fell steadily from the onset of starvation and was not correlated with viability. Other workers have, however, found a correlation between the activity of the catabolic enzymes and survival in support of an early hypothesis associating bacterial death with enzyme inactivation (Rahn & Schroeder, 1941). For example, Postgate & Hunter (1962) found that the glycerol dehydrogenase and glycerol oxidase activities of starved *Aerobacter aerogenes* declined in parallel with viability, while the endogenous respiration rate of the glycerol-limited organisms was not directly related and, in fact, declined more rapidly. Similarly, Burleigh & Dawes (1967) reported a correlation between the survival of aerobically starved *Sarcina lutea* and their ability to oxidize exogenous glutamate and glucose. In the present study the glycolytic activity of *S. lactis* was not maintained with exogenous carbohydrates or amino acids including arginine, in contrast to the findings of Walker & Forrest (1964) and Forrest & Walker (1965*b*) with *Streptococcus faecalis*. Indeed, added glucose produced a more rapid decline in glycolytic activity.

Although it has been proposed that energy is required for intracellular pH control in starved bacteria (Dawes & Ribbons, 1962, 1964), this has not been experimentally demonstrated. *Streptococcus lactis* had a sharp pH optimum for survival which is perhaps consistent with the absence of an endogenous energy source. In this connexion, the addition of arginine was shown to produce a marked decrease in death rates at all pH values. At acid pH values this effect could have been due to a neutralizing action of the NH_3 produced from arginine metabolism. However, arginine also prolonged survival at alkaline pH and it seemed likely that the energy produced from

arginine metabolism was directly responsible for reducing the lethal effect of adverse pH values.

The growth of lactic acid bacteria in milk normally ceases when the lactic acid formed produces inhibitory acid conditions. Although growth ceases, the organisms remain viable and continue to ferment the large excess of lactose present but at a much slower rate. It is possible that the energy produced by the continuing fermentation could be an important factor for the survival of these organisms in sour milk.

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