Survival of *Streptococcus lactis* in Starvation Conditions

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SUMMARY

The survival characteristics of washed *Streptococcus lactis* ML3 organisms, suspended in phosphate buffer, have been examined at the growth temperature. Mg^{2+} markedly prolonged survival. High bacterial concentrations extended survival times, probably because Mg^{2+} was excreted by the bacteria. Surviving organisms in some conditions showed prolonged division lags, especially in the absence of Mg^{2+} . Salt concentration had little effect on survival within wide limits; the optimum pH value for survival was near 7.0. Survival times increased considerably at lower temperatures. Agitation and aeration tended to decrease survival. Death was not influenced by the phase of growth at which cells were harvested from a lactose-limiting medium. Casamino acids increased survival markedly in the presence of Mg^{2+} ; arginine was almost as effective as the complete mixture of amino acids while other individual amino acids tested gave only slight increases in survival times. Fermentable carbohydrates accelerated death of starving organisms irrespective of the growth phase from which they were harvested and of the limiting nutrient; the accelerated death was reduced by addition of Mg^{2+} .

INTRODUCTION

Apart from the extensive findings of Postgate, Strange, and collaborators on the survival characteristics of *Aerobacter aerogenes* (Postgate & Hunter, 1962, 1963*a*, 1964; Strange, Dark & Ness, 1961; Strange & Dark, 1965; Strange & Hunter, 1966), the literature contains few precise measurements of the survival of bacteria held at growth temperatures in buffered suspensions. Postgate & Hunter (1962, 1963*a*) stressed the pitfalls in interpreting bacterial survival measurements and the ambiguities which may arise from toxic elements in suspending solutions, cryptic growth and growth on impurities.

Postgate & Hunter (1962, 1963*a, b*, 1964) observed increased survival times in the presence of Mg^{2+} and Ca^{2+} and the phenomenon of 'substrate-accelerated death' which occurred when starved *Aerobacter aerogenes* were exposed to the growth-limiting substrate. Exceptions were the response of sulphate- and magnesium-limited cells to the addition of sulphate and magnesium ions respectively. However, Strange & Dark (1965) repeated these experiments and concluded that this phenomenon was less general than was claimed by Postgate & Hunter (1964) and was in fact restricted to the lethal effect of carbon energy sources metabolized by *A. aerogenes* in the absence of added Mg^{2+} . Strange & Hunter (1966) later withdrew this conclusion, having succeeded in observing nitrogen-accelerated death. Death of *A. aerogenes* in starved sus-

pensions did not involve alteration or degradation of the osmotic barrier (Postgate & Hunter, 1962). Harrison (1960) first observed the extension of survival times at high cell concentrations and claimed there was an optimum density for survival above which the death rate increased. Postgate & Hunter (1962, 1963a) confirmed these findings in general but did not observe the reversed death rate at high bacterial concentrations. Extended survival and maintenance of respiratory activity of *Escherichia coli* resulting from the metabolism of a carbon energy source in the presence of Mg^{2+} was observed by Clifton (1966). McGrew & Mallette (1962, 1965) maintained *E. coli* at constant viability with a very low threshold concentration of glucose, but Clifton (1966) pointed out that their experimental conditions did not preclude regrowth.

The industrial importance of lactic streptococci has led to several studies on their survival, mainly in milk cultures at low temperatures (Gibson, Landerkin & Morse, 1965, 1966; Lamprech & Foster, 1963; Cowell, Koburger & Weese, 1966). No detailed measurements have been reported on the survival of lactic streptococci, or closely related bacteria, at growth temperatures under starvation conditions. The present paper describes a study of factors which affect the survival of *Streptococcus lactis* under starvation conditions.

METHODS

Organism. *Streptococcus lactis* ML3 was obtained from the cheese starter collection of the New Zealand Dairy Research Institute. The master culture was maintained in skim-milk media as described by Whitehead, Briggs, Garvie & Newland (1956) for *Streptococcus cremoris* HP (D.R.I.).

Medium. The culture medium, developed for this work from known nutritional requirements of *Streptococcus lactis*, contained (g./l.): lactose monohydrate, 5.0; sodium acetate, 1.0; glycerol, 1.0; Casamino acids (Difco), 5.0; peptone (Difco), 0.5; L-asparagine, 0.1; DL-tryptophan, 0.05; L-arginine, 0.1; adenine, 0.005; guanine, 0.001; uracil, 0.005; xanthine, 0.005; pyridoxine hydrochloride, 0.001; pyridoxal hydrochloride, 0.0002; nicotinic acid, 0.001; calcium pantothenate, 0.001; biotin, 0.0001; riboflavin, 0.0001; thiamine hydrochloride, 0.0001; folic acid, 0.0001; *p*-aminobenzoic acid, 0.0002; $NaHCO_3$, 4.2; Na_2HPO_4 , 4.25; KH_2PO_4 , 2.7; $MgSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot 4H_2O$, 0.001; disodium ethylenediaminetetraacetate (EDTA), 0.004. The lactose, $NaHCO_3$, and vitamin components were sterilized separately using a Seitz filter, which had been prewashed with 200 ml. de-ionized water, and added aseptically to the other components which had been autoclaved at 115° for 20 min. The medium had a final pH of 7.3. This medium is not completely defined because peptone is a component; in subsequent descriptions it will be referred to as the routine medium.

Growth conditions and resuspension procedures. All growth cultures and washed suspensions were incubated at 30° in static conditions unless otherwise stated. The routine medium was inoculated from the skim-milk culture and, after 20 daily transfers of 1 % inocula in this medium, a number of small tubes of routine medium were inoculated. These cultures were frozen rapidly (solid CO_2 + ether) towards the end of the growth phase and stored at -75° to -80°. When required, cultures were removed, thawed in a 30° water bath (thawed cultures had 50-80 % viable organisms after 5 months storage), and used to inoculate 10 ml. fresh routine medium. Organisms were prepared for survival studies as follows. After 10-20 daily transfers (70-140

generations), growth phase organisms were inoculated into 25 ml. medium and growth was followed turbidimetrically. At the end of the growth phase a sample (usually 6 ml.) was removed and the organisms were centrifuged down (30,000*g* for 1 min.). The packed organisms were rinsed thoroughly and then dispersed in sterile phosphate buffer (see below), centrifuged (30,000*g* for 1 min.), rinsed and finally suspended in 5 ml. sterile phosphate buffer (pH 7.0). All washings and resuspension buffers were at 30°. Manipulations were done aseptically and were completed in about 15 min., thus minimizing changes in temperature and chemical environment. These procedures left insignificant traces of growth medium (equivalent to a 10^{-7} dilution) and repeated centrifugation had no measurable effect on the survival curves of the organisms.

The phosphate buffer used was 0.075M- $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$, (pH 7.0) containing 10^{-5} M-EDTA, unless otherwise stated. No measurable change in pH value occurred with suspensions of equiv. 10 mg. dry wt organisms/ml. Small volumes (0.2 ml.) of the washed suspension were used to inoculate 10 ml. phosphate buffer (+ substrates, etc., where specified) in metal-capped test-tubes which were incubated in a water bath at 30°. Suspensions prepared in this manner contained equiv. $20 \pm 2 \mu\text{g}$. dry wt organisms/ml. (equivalent to about 6×10^7 chains/ml. or about 1.6×10^8 cocci/ml.) and had an initial viability of about 99 %. Usually samples were removed at intervals and placed directly on agar slides for viability measurements. In experiments with dense populations some dilution was necessary before doing viability determinations. Where the effect of a toxic metal or growth inhibitor was being examined the organisms were centrifuged down and washed in phosphate buffer before the viability determination.

Growth measurement and cell-counting procedures. Growth was followed by taking samples at intervals from the culture and measuring the turbidity at 600 m μ with a Beckman DB Spectrophotometer. Dilutions were made in distilled water where necessary, equivalent dilutions in phosphate buffer gave the same turbidities. Bacterial dry weights were calculated from the turbidities of bacterial suspensions after calibration with suspensions of known dry wt/ml. Bacterial dry weights were obtained by taking known volumes of suspensions in distilled water and drying to constant weight at 100°. All cell masses are recorded as mass units dry wt bacteria/ml. Total counts were made in a Petroff-Hausser chamber with a phase-contrast microscope (magnification $\times 310$). The 4 large squares at each corner of the grid were counted for each sample (400–500 chains each). Counts are of individual chains separated by at least 2 coccal diameters unless otherwise specified. The number of cocci/chain was determined by counting the individual cocci in a total of 250 chains for each sample (magnification $\times 700$).

Viability measurement. The percentage of viable organisms in resuspended systems was determined by the slide-culture method of Postgate, Crumpton & Hunter (1961). The only modification to the method found necessary was the use of stainless-steel annuli instead of brass annuli, to avoid copper toxicity. The agar medium used had the following composition (g./100 ml.); lactose monohydrate, 2.0; Casamino acids (Difco), 1.0; peptone (Difco), 0.8; yeast extract (Difco), 0.1; agar (Davis), 1.0; Na_2HPO_4 , 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; final pH 7.0. The agar was filtered (Whatman no. 15) before autoclaving and centrifuged hot before slide preparation to remove debris. Slides were incubated at 30° for times sufficient to allow 4–5 generations which provided adequate differentiation between live and dead organisms. *Streptococcus*

lactis ML3 occurred mainly as diplococci, but chains with up to 6 cocci were observed. Slides were counted in green light using a phase-contrast microscope with an eyepiece grid (magnification $\times 310$). As a routine, 8 typical fields with 40–60 objects/field were counted on duplicate slides; on slide cultures of organisms with long division lags, 10 fields were counted with 20–35 objects/field to minimize errors from overgrowth of dead organisms. Conventional colony counts were made by the standard pour-plate method (Cruickshank, 1965) with phosphate buffer diluent and the agar medium described above.

Analytical methods. Magnesium and copper were measured in cell-free supernatant fluids and buffer solutions respectively, by using a Techtron AA3 atomic absorption spectrophotometer (Techtron Pty. Ltd., Australia) under standard operating conditions. Standard magnesium solutions and samples contained in phosphate buffer were diluted 1/10 with de-ionized water to appropriate concentrations. Phosphorus interference was suppressed by using lanthanum. Cellular magnesium content was determined by the method of Webb (1966). Perchloric acid was added to copper standards and samples to a final concentration of 10% (v/v). Ammonium pyrrolidine dithiocarbamate (1%) and methyl isobutylketone were added (Allan, 1961) and the copper determined in the organic phase after shaking. Lactose was measured by the anthrone method of Richards (1959). Lactic acid was determined by the method of Barker & Summerson (1941).

Materials. Reagents of analytical grade were used when available. All water was distilled and then de-ionized by passage through a mixed bed ion-exchange resin (Permutit 'Biodeminrolit').

RESULTS

Growth of organism. The mean generation time (doubling of turbidity) of *Streptococcus lactis* ML3 during the logarithmic growth phase in batch culture was 46 ± 4 min. Analyses showed that lactose was the growth-limiting substrate. The pH changed from 7.3 to 6.3 ± 0.1 at the end of growth; the yield was 0.98 ± 0.03 mg. dry wt organism/ml., corresponding to about 8×10^9 cocci/ml.

Numbers of organisms in resuspended systems. It was important in the present investigation to determine whether the total number of cocci in a given suspension remained constant on prolonged incubation. For this reason total counts/ml. were made as well as slide counts by the method of Postgate *et al.* (1961), which determines only the ratio of viable/total organisms. Four washed suspensions (equiv. 50 μ g. dry wt organisms/ml.) in phosphate buffer were sampled over a 28 hr period. For total chain counts, quadruplicate samples from one suspension and single samples from the other suspensions were counted. The viable chain counts given in Table 1 are the means of plate counts from samples of all flasks diluted in duplicate and plated in duplicate (i.e. 16 plates). The number of cocci/chain and the % viability figures from slide culture are means of duplicate counts from each suspension (see Methods). The results in Table 1 show that *Streptococcus lactis* ML3 organisms had a tendency to clump in phosphate buffer on prolonged storage. This was the reason for the decrease in total chain counts but there was no significant decrease in total numbers of cocci for at least 28 hr. The 20% decrease in turbidity was probably a result of endogenous metabolism and leakage of cellular material into the suspending buffer. There appeared to be a constant systematic error in the % viability results obtained by

normal methods, similar to that noted by Norris & Powell (1961), Postgate *et al.* (1961), and Strange *et al.* (1961). These low figures were probably the result of errors in the total count determination, since the survival curves were very similar when plate count viabilities were expressed as percentages of the initial plate count (taken as 99 %).

Table 1. *Constancy of total cell numbers and comparison of survival measurements on Streptococcus lactis ML3 in washed suspensions at 30°*

Organisms were harvested at end of growth phase, washed twice, and resuspended in 0.075 M-phosphate buffer (pH 7.0, containing 10^{-5} M-EDTA) at equiv. 50 µg. dry wt organism/ml. with gentle agitation provided by a magnetic stirrer.

Suspension incubation time (hr)...	1	3	5	7	10	24	28	S.D.*
(1) Total chain count ($\times 10^{-8}$ /ml.)	1.52	1.47	1.36	1.45	1.42	1.12	1.10	± 0.07 (7)
(2) Viable chain count ($\times 10^{-8}$ /ml.)	1.19	1.16	1.10	0.52	0.22	0.03	—	± 0.08 (16)
(3) % viability ((2)/(1) $\times 100$)	78	79	81	36	15	3	—	—
(4) % viability (slide count)	99.5	98	96.5	65	28	6	—	—†
(5) Cocci/chain or clump	—	2.64	—	2.71	—	3.56	3.45	± 0.05 (8)
(6) Total coccal count ((1). (5) $\times 10^{-8}$ /ml.)	—	3.88	—	3.93	—	3.99	3.79	± 0.22
(7) Turbidity	0.173	0.165	0.157	0.149	0.143	0.140	0.139	—

* Standard deviation (no. of measurements/sample). † See Fig. 1.

The slide culture technique of Postgate *et al.* (1961) was evaluated for *Streptococcus lactis* ML3 in the following way. Six washed suspensions were prepared in phosphate buffer (see Methods). Duplicate samples were removed from each suspension at times designed to give measurements at high, medium and low viabilities. Slide preparation and counting was performed as described in Methods. An analysis of variance was carried out on the % viability figures for each sampling time. The standard deviation from the mean was computed together with the 95 % confidence interval for two slides prepared from one suspension (Fig. 1). At high viabilities (94–99 %), variance between tubes was negligible and the accuracy of the method was high, but variance increased with moribund populations which had variable cell division times. Variance was largely a function of division lag time. The survival curve of *S. lactis* ML3 starved in buffered suspension was essentially sigmoid in form.

Survival in resuspended systems

Survival measurements on all resuspended systems were repeated at least three times. Survival curves showed small fluctuations with different batches of organisms but trends between systems were always the same.

Effect of ethylenediaminetetra-acetate (EDTA). Early experiments on survival in phosphate buffer produced irregular results and rapid death rates, these were decreased on addition of 5×10^{-6} M-EDTA (Fig. 2). Lower concentrations had only a slight effect while high concentrations of EDTA (10^{-2} M) accelerated death, possibly as a result of its capacity to destabilize cell walls (Gray & Wilkinson, 1965; Asbell & Eagon, 1966) and ribosomes (Wade, 1961). A toxic metal impurity was thus probably present in the buffer (Postgate & Hunter, 1962). Extraction of phosphate buffers with organic metal complexing reagents (see Methods) revealed an average of 0.06 p.p.m. copper (about 10^{-6} M-copper). When trace amounts of Cu^{2+} (10^{-6} M) were added to suspensions

containing 5×10^{-6} M-EDTA the survival curve was similar to that for buffer without EDTA (Fig. 3a).

Fig. 1

Fig. 2

Fig. 1. Variance in viability determinations on washed suspensions of *Streptococcus lactis* for 95% confidence limits. Duplicate counts on six similar populations were determined using the slide culture technique. For details see text.

Fig. 2. Effect of EDTA on survival of *Streptococcus lactis*. *Streptococcus lactis* harvested at the end of the growth phase was washed and resuspended in 0.075 M-phosphate buffer at 20 μ g./ml. and incubated at 30°, pH 7.00 ± 0.05 in static culture. Tubes contained the following concentrations of EDTA: $\blacktriangle$, 10^{-2} M; $\circ$, 10^{-5} M; $\bullet$, 5×10^{-6} M; $\triangle$, 10^{-6} M; $\square$, no EDTA. Viabilities were determined by slide culture.

Effect of divalent metal ions. The effect of divalent metal ions was studied with suspensions containing sufficient EDTA to remove the toxic effect of contaminant copper. Hg^{2+} (10^{-6} M) caused complete death within 5 min. while the toxicity of other ions tested decreased in the order $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+} > \text{CO}^{2+} > \text{Zn}^{2+} > \text{Pb}^{2+}$ (Fig. 3a). From literature reports (Perrin, 1964) this would also appear to be the approximate order of decreasing stability of the metal-EDTA complexes so that it is unlikely that any of these ions displaced the contaminant copper from its complex. Ca^{2+} , Mn^{2+} , and Sn^{2+} (10^{-5} M and 10^{-4} M) were without effect.

Addition of Mg^{2+} produced greatly increased survival times (Fig. 3b), maximum survival being afforded with about 10^{-4} M- Mg^{2+} . Mg^{2+} also prolonged survival of organisms in phosphate buffer without EDTA. When incubated with $\text{Mg}^{2+} + \text{Cu}^{2+}$ the toxic effect of Cu^{2+} was decreased (Fig. 3b). These observations are similar to those made by MacLeod & Snell (1950) for *Lactobacillus arabinosus* and by Abelson & Aldous (1950) for *Escherichia coli*. The experiments described show two effects of Mg^{2+} : (i) a protective effect decreasing Cu^{2+} toxicity, (ii) a stabilizing effect (see Discussion).

Fig. 3. Effect of divalent metal ions on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2. Tubes contained 5×10^{-6} M-EDTA in 0.075 M-phosphate buffer. Part (a): ○, no addition; ▲, +Pb(NO₃)₂; □, +ZnSO₄; ●, +CoCl₂; △, +FeSO₄; ■, +NiCl₂; ×, +CuSO₄; all salts 10^{-5} M. Part (b): ■, no addition; remaining tubes contained MgSO₄ at the following concentrations: □, 10^{-3} M; ○, 10^{-4} M; △, 10^{-5} M; ●, 10^{-4} M + CuSO₄ (10^{-5} M).

Effect of bacterial concentration. Very dense suspensions (equiv. 7.8 mg. dry wt organism/ml. or about 6×10^{10} cocci/ml.) survived best, with decreasing survival times at lower bacterial concentrations (Fig. 4a). Measurements of Mg²⁺ excretion by the organisms (see Methods) showed that at high bacterial concentrations Mg²⁺ leakage from the organisms was sufficient to produce stabilizing concentrations (Fig. 4a). Analyses of whole bacteria immediately after washing in buffer indicated 0.41 % (w/w) magnesium. The concentration (7×10^{-4} M) of Mg²⁺ in the supernatant fluid with equiv. 7.8 mg. dry wt organisms/ml. represented a loss of 0.22 % (w/w) magnesium from the organisms (i.e. 54 % of the cellular magnesium). From these results it seemed likely that Mg²⁺ excretion by the organisms was the cause of extended survival at high bacterial concentrations. Additional support for this conclusion was obtained in a similar experiment where the addition of 10^{-4} M-Mg²⁺ gave almost identical survival curves at each bacterial concentration (Fig. 4b). High bacterial concentrations also prolonged survival in phosphate buffer without EDTA, presumably because the copper toxicity was decreased by the excreted Mg²⁺ in a similar manner to that described in the preceding section.

Effect of growth phase and media composition. The survival of organisms taken from different growth phases in the lactose-limiting medium were compared. Organisms from the mid-logarithmic phase, the end of the growth phase and from 1 and 2 hr after growth had ceased showed no measurable differences in the survival curves. This is in

Fig. 4. Effect of cell concentration on survival of *Streptococcus lactis*. Cells were harvested from 500 ml. of culture at the end of the growth phase, washed and resuspended in 60 ml. $0.075 M$ -phosphate buffer with $10^{-5} M$ -EDTA. Part (a) At intervals viability determinations were made, after sample dilution where necessary, and 5 ml. samples withdrawn from each suspension. The samples were centrifuged and the cell-free supernatants carefully removed and filtered (sintered glass, porosity 5) into tubes. These samples were frozen and later analysed for magnesium (see Methods). Viabilities of washed suspensions (50 ml.) containing 7.8 mg. dry wt bacteria/ml. ($\bigcirc$ — $\bigcirc$), 0.77 mg./ml. ($\square$ — $\square$), 0.078 mg./ml. ($\times$ — $\times$) and 0.019 mg./ml. ($\triangle$ — $\triangle$) are shown. Supernatant Mg^{2+} concentrations at the same cell densities were $\bullet$ — $\bullet$, $\blacksquare$ — $\blacksquare$, $\times$ — $\times$, and $\blacktriangle$ — $\blacktriangle$ respectively. Part (b). Cell suspensions were prepared as in part (a), containing: $\bigcirc$, 8.1 mg./ml.; $\square$, 0.80 mg./ml.; $\triangle$, 0.079 mg./ml. Each suspension contained $10^{-4} M$ - Mg^{2+} .

agreement with the observations of Postgate & Hunter (1962), who stressed the importance of the nutritional status of the population and suggested that for a genetically uniform population the growth phase has only a small effect on the survival time during starvation. Attempts to find a satisfactory non-carbohydrate-limiting medium were unsuccessful. When the lactose concentration was increased to 1% growth ceased, presumably because of the inhibitory pH value produced (about pH 4.7); analyses indicated the presence of 0.08% lactose at the end of growth. These organisms, when washed and resuspended, showed a slightly increased death rate as compared with organisms from the routine medium. When the amino acid concentration was decreased from 0.5% to 0.05% the growth rate was substantially decreased (doubling time 2–2.5 hr as compared with about 46 min. for routine medium), and resuspended organisms from this medium also showed higher death rates than normal (50% decrease in viability in 4.5–5 hr).

Effect of salt concentration. In de-ionized water containing $10^{-5} M$ -EDTA the viability of resuspended organisms decreased to 50% 3.6 hr. In $Na_2HPO_4 + KH_2PO_4$ buffer (pH 7.0) containing $10^{-5} M$ -EDTA, survival times decreased slightly with increasing buffer concentration. Organisms in phosphate buffer at 0.075 M, 0.150 M and 0.300 M decreased to 50% viability in 7.2 hr, 6.1 hr, and 6.0 hr, respectively. Ringer solution, with and without $10^{-5} M$ -EDTA, gave 50% viabilities after 6.7 hr and 3.8 hr, respectively. All times were ± 0.3 hr for replicate samples.

Effect of pH value. In phosphate + citrate buffer at 30° *Streptococcus lactis* ML3 had a sharp optimum for survival near pH 7.0 (Fig. 5). Survival times decreased sharply on decreasing the pH value and 50 % decrease in viability occurred in 0.5 hr at pH 4.0. It was therefore of interest to re-examine the observations of Harvey (1965), who concluded that *S. lactis* ML3 organisms held in broth culture at pH 4.2 were damaged but maintained complete viability for 5 hr. Organisms were grown and placed in a growth medium at pH 4.2 as described by Harvey (1965). Measurements showed that while the turbidity increased by 20 % in 3 hr there was no increase in viable or total

Fig. 5. Effect of pH on survival of *Streptococcus lactis*. Buffers were prepared from 0.1 M- Na_2HPO_4 and 0.05 M-citric acid containing 10^{-5} M-EDTA. Cocci 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 $\mu\text{g.}/\text{ml.}$ and final pH's (± 0.05) of: Δ , 7.93; $\circ$, 6.98; $\square$, 6.55; $\bullet$, 6.00; $\blacktriangle$, 4.90; $\blacksquare$, 4.03. Before viability determination a 1/10 dilution was made in phosphate-citrate buffer, pH 7.0.

bacterial numbers, contrary to the interpretation of Harvey (1965), and 97–99 % of the organisms survived for 6 hr. This indicated a very marked protective effect of nutrients at low pH values. Organisms harvested from the same broth medium (pH 6.5) and starved in phosphate buffer gave similar survival times to washed organisms from the routine medium starved in phosphate buffer.

Effect of temperature. The death rate decreased markedly on lowering the incubation temperature of washed suspensions from 45° to 3° (Fig. 6a, b). Note the time scale for Fig. 6b is ten times that for Fig. 6a. In contrast, Postgate & Hunter (1962) observed decreased death rates on lowering the incubation temperature from 50° to 20°, but found accelerated death again below 20°.

Effect of atmosphere and agitation. Gentle agitation produced by a magnetic stirrer had no measurable effect on survival curves as compared with those of static suspensions, but vigorous agitation (magnetic stirrer) generally gave more rapid death

(50% decrease in viability in 2–3 hr). Both air and O_2 -free N_2 increased the death rate when gently bubbled through washed suspensions (50% decrease in viability in 4.5–5 hr for air, and in 3–3.5 hr for N_2). These observations are difficult to explain but it is possible that agitation washed out soluble intracellular components. Vigorous agitation did not produce significant chain breakage. The results imply that special E_h values were required for survival, as would befit a micro-aerophilic organism.

Fig. 6. Effect of temperature on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2, the washed cocci being inoculated into phosphate buffer equilibrated to the storage incubation temperature. Part (a): □, 45°; △, 37°; ○, 30°; part (b): ○, 30°; ■, 22°; ▲, 16°; ●, 3°. Temperatures are $\pm 1^\circ$, slide cultures were incubated 30°.

Effect of metabolic inhibitors. The addition of 0.01 mM-iodoacetate decreased survival times sharply (Table 2). Methylene blue at concentrations above 0.02 mM showed an immediate bactericidal effect, while at 0.001 mM the death rate increased slightly. The addition of 2.7 mM sodium arsenate and 2.4 mM-sodium fluoride also produced a slight increase in death rate. Sodium azide, 2,4-dinitrophenol, potassium cyanide and sodium malonate did not significantly influence the survival curves, although all experiments were made under conditions of low O_2 -tension. Before slides were prepared for viability determination the inhibitors were removed by washing (see Methods), this procedure had no noticeable effect on the viability of control populations.

Effect of substrates

Carbohydrates. Lactose, glucose, galactose and fructose (10^{-2} M; Fig. 7a) produced markedly increased death rates in washed suspensions (50% decrease in viability in 1–1.5 hr) irrespective of the growth phase of the organisms and limiting nutrient (50% decrease in viability in 0.5–0.7 hr for organisms from amino acid limited medium). The suspension remained at pH 7.0 and analyses for lactic acid indicated that fermentation of all carbohydrates took place. Sodium lactate (10^{-2} M) had no effect on

Fig. 7. Effect of nutrients on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2 in phosphate buffer containing 10^{-5} M-EDTA. Part (a): $\circ$, no addition; $\blacktriangle$, +lactose; $\bullet$, +glucose; $\square$, +galactose; $\triangle$, +lactose + Mg^{2+} (10^{-3} M). All carbohydrate supplements were 10^{-2} M. Part (b): $\blacktriangle$, + Mg^{2+} ; $\circ$, +Casamino acids (Difco) (0.5%) + Mg^{2+} ; $\square$, +arginine (10^{-2} M) + Mg^{2+} ; $\blacksquare$, +alanine, aspartic acid, glutamic acid (each 10^{-2} M) + Mg^{2+} ; $\bullet$, +glycerol (10^{-2} M) + Mg^{2+} ; all Mg^{2+} supplements 10^{-3} M; $\triangle$, growth medium minus lactose, vitamins and $NaHCO_3$.

Fig. 8. Division lag times for *Streptococcus lactis* from various resuspended systems. Suspensions were prepared as for Fig. 2 in phosphate buffer containing 10^{-5} M-EDTA. Viabilities for the suspensions: +0.5% Casamino acids (Difco) + 10^{-3} M- Mg^{2+} ($\circ$ — $\circ$), + 10^{-3} M- Mg^{2+} ($\square$ — $\square$), no addition ($\triangle$ — $\triangle$), + 10^{-2} M-lactose ($\times$ — $\times$). Division lags of cells in the same systems are indicated. $\bullet$ — $\bullet$, $\blacksquare$ — $\blacksquare$, $\blacktriangle$ — $\blacktriangle$, $\times$ — $\times$.

survival times. Accelerated death was markedly reduced in all cases on addition of 10^{-3} M- Mg^{2+} , giving survival times similar to control systems without carbohydrate or Mg^{2+} (Fig. 7a). Addition of 10^{-2} M-ribose had no effect. Analysis for lactic acid showed *Streptococcus lactis* ML3 did not ferment exogenous ribose to lactate in washed suspensions.

Table 2. *Effect of metabolic inhibitors on survival of Streptococcus lactis in buffered suspensions at 30°*

Organisms were harvested at end of growth phase, washed twice, and resuspended in 0.075 M-phosphate buffer at equiv. 20 μ g. dry wt. organism/ml. Buffer (pH 7.0, except where indicated) contained 10^{-5} M-EDTA and inhibitor as below.

Inhibitor	Concentration (mM)	Death rate*
None (pH 7.0)	—	7.2
None (pH 6.5)	—	6.0
2,4-Dinitrophenol	1.10	6.8
	0.11	6.6
Iodoacetic acid†	0.11	1.1
	0.01	1.9
Methylene blue	0.010	2.9
	0.005	5.0
	0.001	6.7
Potassium cyanide	1.54	7.0
	0.77	6.8
Sodium arsenate	2.70	6.1
	0.54	6.8
Sodium azide	1.54	6.8
	0.15	7.5
Sodium fluoride	2.38	6.9
	0.24	7.3
Sodium malonate†	6.75	6.3
	0.67	6.0

* Time (hr) for 50% decrease in viability (± 0.3 hr).

† Buffer adjusted to pH 6.5.

Amino acids and other growth media components. Organisms resuspended in routine medium without lactose, vitamins and $NaHCO_3$, survived for a longer period than organisms resuspended with Mg^{2+} only (Fig. 7b). In the former system no detectable change in total numbers occurred up to 48 hr in 2 suspensions containing equiv. 20 μ g. dry wt organism/ml., one suspension contained penicillin G (100 units/ml.) to prevent cell growth and provide a control. Casamino acids + Mg^{2+} gave equivalent survival as in routine medium without lactose, vitamins and $NaHCO_3$, while arginine + Mg^{2+} was almost as effective (Fig. 7b). Alanine, aspartic acid and glutamic acid, which were found to comprise most of the free amino acid pool in these organisms, produced a marginal increase in survival time when incubated together with washed organisms + Mg^{2+} . Glycerol + Mg^{2+} also produced a marginal increase in survival time while 10^{-2} M-sodium acetate + 10^{-3} M- Mg^{2+} was ineffective (Fig. 7b). Washed organisms incubated with the vitamins, purines and pyrimidines supplied at the routine medium concentrations were without effect in the presence of 10^{-3} M- Mg^{2+} .

Growth characteristics of survivors

In some resuspended systems surviving organisms had considerably longer division lags than did exponentially growing organisms. These lags were measured (Postgate & Hunter, 1964) in four resuspended systems giving the maximum scatter in survival times. Division lags tended to increase just before the onset of bacterial death, the increase being particularly sharp in systems without Mg^{2+} (Fig. 8). In almost all experiments where organisms were incubated in the absence of Mg^{2+} , surviving organisms in slide cultures showed a large proportion of morphologically aberrant colony forms once the organisms started to die; instead of the typical circular colonies many organisms produced filamentous colonies and these organisms always showed longer division lags.

DISCUSSION

The slide culture method of Postgate *et al.* (1961), is well suited to survival measurement of short-chain lactic streptococci such as *Streptococcus lactis* ML3 and the present results show many similar trends to those found for *Aerobacter aerogenes* by Postgate, Strange and collaborators. Subsidiary stresses were minimized by washing and resuspending cells in a constant chemical environment at a constant temperature, and by incubating suspensions near the optimum survival pH.

Addition of Mg^{2+} considerably extended survival times of starving cells of *Streptococcus lactis*, in agreement with the observations of Postgate & Hunter (1962) for *Aerobacter aerogenes*. Apart from its potential action in reducing the toxic effect of certain metal cations (MacLeod & Snell, 1950; Abelson & Aldous, 1950), Mg^{2+} , together with Na^+ and K^+ , has been established as an important stabilizer of ribonucleoprotein in bacterial cells (Bowen, Dagley & Sykes, 1959; Tempest, Dicks & Hunter, 1966). In experiments not reported here, addition of Mg^{2+} to washed *Streptococcus lactis* ML3 organisms in phosphate buffer markedly suppressed the excretion of pentose and soluble u.v.-absorbing substances. It is probable that maintenance of the integrity of cell ribonucleoprotein is important for cell survival of *S. lactis*.

Streptococcus lactis organisms in dense suspensions (equiv. 7.8 mg. dry wt organisms/ml.) lost 54% of their cellular magnesium, producing a stabilizing Mg^{2+} concentration (7×10^{-4} M) in the suspending buffer. With equiv. 0.77 mg. dry wt organisms/ml. a stabilizing concentration of Mg^{2+} was reached (10^{-4} M) but death was more rapid than at the higher cell density. It is possible that a considerable amount of irreparable damage was done to the organisms before this concentration was reached. Results suggest that Mg^{2+} excretion by starving organisms was a major factor in prolonging survival of dense populations and further support for this conclusion was obtained when the addition of 10^{-4} M- Mg^{2+} produced almost identical survival times at all population densities. This result contrasts with the observation of Postgate & Hunter (1963a) who showed a population effect in the presence of optimal Mg^{2+} concentration. Cryptic growth (Harrison, 1960; Postgate & Hunter, 1962) in dense populations is unlikely for organisms with such exacting nutritional requirements as *S. lactis*. In contrast to the present results Postgate & Hunter (1964) could not detect magnesium by chemical methods in concentrates of cell-free supernatants in which 20 μ g. dry wt organisms/ml. populations of *Aerobacter aerogenes* had died. Harrison (1960) first observed the effect of bacterial concentration on survival and reported a

'reversal' of this phenomenon at high concentrations although possible errors are not discussed. If death of *A. aerogenes* at high concentrations is due to anoxia as Harrison suggested, then it is not unexpected to find that *S. lactis*, a facultative anaerobe, did not show a reversed death rate at high bacterial concentrations. Postgate & Hunter (1962, 1963*a*) confirmed Harrison's findings in general but did not observe the 'reversed' population density effect. This discrepancy is explained if anoxia was the cause of the increased death rate at high concentrations of organisms since Harrison (1960) did not aerate the washed suspensions, in contrast to the experimental procedure of Postgate & Hunter (1962).

All the fermentable carbohydrates tested produced accelerated death of washed cells of *Streptococcus lactis* irrespective of the growth phase or growth-limiting nutrient. Accelerated death was markedly reduced on addition of Mg^{2+} , and was not due to a toxic effect of the lactate produced. These results contrast with the observations of McGrew & Mallette (1962, 1965) and Clifton (1966), who reported extended survival of *Escherichia coli* without growth in the presence of glucose and Mg^{2+} . Postgate & Hunter (1963*b*, 1964) observed that addition of certain growth-limiting nutrients to starving suspensions of certain Gram-negative bacteria increased the death rate. This applied to nitrogen-, phosphorus-, and carbon-limited populations and it was suggested that this 'substrate-accelerated death' was a fairly general phenomenon for Gram-negative bacteria. Glycerol-accelerated death was slower, the more dense the population and addition of Mg^{2+} prevented glycerol-accelerated death. This suggests that Mg^{2+} excretion by *Aerobacter aerogenes* might extend the survival of these bacteria in dense populations, but Postgate & Hunter stated that because of interference it was not possible to perform satisfactory magnesium analyses on cell-free buffers in which dense populations had died.

Arginine, which *Streptococcus lactis* converts to ornithine with the production of ATP (Korzenovsky & Werkman, 1953, 1954), produced a remarkable increase in survival time in the presence of Mg^{2+} , indicating that the nature of the energy source was critical for survival. Walker & Forrest (1964) showed maintenance of glycolytic activity of starved *S. faecalis* with exogenous arginine and showed that restoration of glycolytic activity in aged organisms was possible on incubation with Casamino acids and an energy source. The extended survival of *S. lactis* ML3, without growth, in routine growth medium without lactose, vitamins and bicarbonate, was attributable to its Mg^{2+} and amino acid content. Casamino acids presumably prolonged survival of *S. lactis* by providing a limited energy source for cell maintenance and supplying amino acids for cell turnover minimizing breakdown of essential components, and giving the observed short division lags of survivors. It would appear that the division lags for *S. lactis* in various resuspended systems are a function of the extent of cell damage which eventually leads to death.

The insignificant death rate observed in the initial incubation period of *Streptococcus lactis* ML3 in most resuspended systems indicates that intrinsic differences of resistance between individual bacteria are slight. The duration of this period of almost complete viability compared with the phase of rapid death varied widely, depending on the resuspension system and the biological history of the population. The death rate then begins to increase and generally continues at a rapid rate. The actual cause or causes of death of *S. lactis* in those non-toxic buffers which provide maximum stability are probably complex and diverse.

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REFERENCES

- ABELSON, P. H. & ALDOUS, E. (1950). Ion antagonisms in microorganisms: interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc and manganese. *J. Bact.* **60**, 401.
- ALLAN, J. E. (1961). The determination of copper by atomic absorption spectrophotometry. *Spectrochim. Acta* **17**, 459.
- ASBELL, M. A. & EAGON, R. G. (1966). Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas aeruginosa*. *J. Bact.* **92**, 380.
- BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535.
- BOWEN, T. J., DAGLEY, S. & SYKES, J. (1959). A ribonucleo-protein component of *Escherichia coli*. *Biochem. J.* **72**, 419.
- CLIFTON, C. E. (1966). Ageing of *Escherichia coli*. *J. Bact.* **92**, 905.
- COWELL, G. R., KOBURGER, J. A. & WEESE, S. J. (1966). Storage of lactic streptococci. 1. Effect of pH on survival and endogenous metabolism in phosphate buffer. *J. Dairy Sci.* **49**, 1.
- CRUICKSHANK, R. (1965). In *Medical Microbiology*, 11th ed., p. 869. London: E. and S. Livingstone Ltd.
- GIBSON, C. A., LANDERKIN, G. B. & MORSE, P. M. (1965). Survival of strains of lactic streptococci during frozen storage. *J. Dairy Res.* **32**, 151.
- GIBSON, C. A., LANDERKIN, G. B. & MORSE, P. M. (1966). Effects of additives on the survival of lactic streptococci in frozen storage. *Appl. Microbiol.* **14**, 665.
- GRAY, G. W. & WILKINSON, S. G. (1965). The effect of ethylenediaminetetra-acetic acid on the cell walls of some Gram-negative bacteria. *J. gen. Microbiol.* **39**, 385.
- HARRISON, A. P. JUN. (1960). The response of *Bacterium lactis aerogenes* when held at growth temperature in the absence of nutriment: an analysis of survival curves. *Proc. R. Soc. B* **152**, 418.
- HARVEY, R. J. (1965). Damage to *Streptococcus lactis* resulting from growth at low pH. *J. Bact.* **90**, 1330.
- KORZENOVSKY, M. & WERKMAN, C. H. (1953). Conversion of citrulline to ornithine by cell free extracts of *Streptococcus lactis*. *Archs Biochem. Biophys.* **46**, 174.
- KORZENOVSKY, M. & WERKMAN, C. H. (1954). Stoichiometry of the citrulline phosphorylase reaction of *Streptococcus lactis*. *Biochem. J.* **53**, 343.
- LAMPRECH, E. D. & FOSTER, E. M. (1963). The survival of starter organisms in concentrated suspensions. *J. appl. Bact.* **26**, 359.
- MACLEOD, R. A. & SNELL, E. E. (1950). The relation of ion antagonism to the inorganic nutrition of lactic acid bacteria. *J. Bact.* **59**, 783.
- MCGREW, S. B. & MALLETTE, M. F. (1962). Energy of maintenance in *Escherichia coli*. *J. Bact.* **83**, 844.
- MCGREW, S. B. & MALLETTE, M. F. (1965). Maintenance of *Escherichia coli* and the assimilation of glucose. *Nature, Lond.* **208**, 1096.
- NORRIS, K. P. & POWELL, E. O. (1961). Improvements in determining total counts of bacteria. *Jl R. microsc. Soc.* **80**, 107.
- PERRIN, D. D. (1964). In *Organic Complexing Reagents*, p. 100. New York: Interscience.
- POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.
- POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.
- POSTGATE, J. R. & HUNTER, J. R. (1963*a*). The survival of starved bacteria. *J. appl. Bact.* **26**, 295.
- POSTGATE, J. R. & HUNTER, J. R. (1963*b*). Acceleration of bacterial death by growth substrates. *Nature, Lond.* **198**, 273.
- POSTGATE, J. R. & HUNTER, J. R. (1964). Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. *J. gen. Microbiol.* **34**, 459.
- RICHARDS, E. L. (1959). The reaction of lactose with anthrone and its application to the estimation of lactose in casein and other dairy products. *J. Dairy Res.* **26**, 53.

- STRANGE, R. E. & DARK, F. A. (1965). 'Substrate-accelerated death' of *Aerobacter aerogenes*. *J. gen. Microbiol.* **39**, 215.
- STRANGE, R. E. & HUNTER, J. R. (1966). 'Substrate-accelerated death' of nitrogen-limited bacteria. *J. gen. Microbiol.* **44**, 255.
- STRANGE, R. E., DARK, F. A. & NESS, A. G. (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. *J. gen. Microbiol.* **25**, 61.
- TEMPEST, D. W., DICKS, J. W. & HUNTER, J. R. (1966). The interrelationship between potassium, magnesium and phosphorus in potassium-limited chemostat cultures of *Aerobacter aerogenes*. *J. gen. Microbiol.* **45**, 135.
- WADE, H. E. (1961). The autodegradation of ribonucleoprotein in *Escherichia coli*. *Biochem. J.* **78**, 457.
- WALKER, D. J. & FORREST, W. W. (1964). Anaerobic endogenous metabolism in *Streptococcus faecalis*. *J. Bact.* **87**, 256.
- WEBB, M. (1966). The utilization of magnesium by certain Gram-positive and Gram-negative bacteria. *J. gen. Microbiol.* **43**, 401.
- WHITEHEAD, H. R., BRIGGS, C. A. E., GARVIE, E. I. & NEWLAND, L. G. M. (1956). The influence of cultural conditions on the characteristics of *Streptococcus cremoris* strain HP. *J. Dairy Res.* **23**, 315.