

## Degradation of Cell Constituents by Starved *Streptococcus lactis* in Relation to Survival

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### SUMMARY

*Streptococcus lactis* organisms were grown in lactose-limited batch culture and transferred, after washing, to phosphate buffer at the growth temperature. Soluble protein was released from viable organisms into the suspending buffer and the intracellular free amino acid pool declined steadily with the components appearing in the suspending buffer; a net increase in the total amount of free amino acid indicated some protein hydrolysis. RNA was hydrolysed, resulting in the release of u.v.-absorbing bases and ribose from the organisms. Conditions which promoted rapid RNA breakdown also produced rapid death rates and long cell division lags in surviving organisms. After 28 hr starvation in buffer containing  $Mg^{2+}$ , the bacterial dry wt decreased by 26%; loss of RNA, protein and free amino acids accounted for 10.3%, 7.3% and 2.7% of the total bacterial mass loss. The products of polymer hydrolysis appeared to be released in an undegraded form into the external buffer and there was no appreciable formation of lactate, ammonia or volatile fatty acids, possibly indicating the absence of any important endogenous energy sources. There was no appreciable degradation of DNA or carbohydrate but phospholipid was broken down on prolonged starvation. No polyglucose or poly- $\beta$ -hydroxybutyrate was detected in the organisms.

### INTRODUCTION

The progressive loss of cell constituents which generally occurs when bacteria are starved in buffer at the growth temperature is normally a result of an imbalance in the total anabolic and catabolic reactions. It may also result from other processes such as the leakage or secretion of cell components and intracellular pools. Limited resynthesis or turnover of cell polymers may occur but the net metabolism is catabolic and will eventually result in the death of the organism. The over-all metabolic activities of starved bacteria are normally referred to as their 'endogenous' metabolism which, according to Powell (1967), is effectively a measure of the rate at which bacteria break down their own mass. This field has been extensively investigated with aerobic organisms and published work has been reviewed in a symposium (Lamanna, 1963) and by Dawes & Ribbons (1962, 1964). The functions of endogenous metabolism, as envisaged by Dawes & Ribbons (1962), include the provision of energy for turnover of protein and nucleic acids, osmotic regulation, pH control and the supply of suitable

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substrates for the resynthesis of essential bacterial constituents. However, the control and economy of these energy-requiring processes are ill-defined (Strange, 1967) and, while starved bacteria may exhibit high initial  $O_2$ -uptakes, the  $O_2$ -uptake may fall to a negligible level though significant decline in viability may not occur for a considerable period (Burleigh & Dawes, 1967), which suggests that the amount of energy required for survival may be very small.

Components reported to be degraded in starved organisms include carbohydrate, RNA, protein, free amino acids, peptides, lipids and certain specialized 'reserve' materials. The rates and orders of substrate degradation and the general pattern of endogenous metabolism vary (1) in different organisms, (2) with the growth conditions and hence the chemical and physiological state of the organism and (3) with the physico-chemical conditions of the starvation environment (Strange, 1967). Profound changes in the composition of starved bacteria without concurrent viability loss are well documented. Up to 25 % of the total cell protein was catabolized before a significant decrease in the viability of *Aerobacter aerogenes* occurred (Strange, Dark & Ness, 1961) and it appeared that RNA was to some extent expendable, since up to 50 % of the ribosomal RNA of some organisms was metabolized without death taking place. Within a single species, organisms grown at rapid rates contain much more RNA (Schaechter, Maaløe & Kjeldgaard, 1958) and die more slowly when starved than organisms grown at slow rates (Postgate & Hunter, 1962). Although many conditions which delay RNA degradation, such as the presence of  $Mg^{2+}$  (Strange & Hunter, 1967) or  $D_2O$  (Lovett, 1964), also delay death, no absolute correlation between RNA degradation and death rate has been established (see Postgate, 1967). Burleigh & Dawes (1967) were unable to correlate loss in viability of starved *Sarcina lutea* with the degradation of any single cellular constituent.

An important function of research laboratories serving the dairy industry is the maintenance of lactic acid cultures with high acid producing activities for use as cheese 'starters'. An understanding of the factors affecting the survival and activity of these organisms could therefore be valuable. Only a small number of reports on the metabolism of starved lactic acid bacteria have been published and no correlations between viability and endogenous metabolism have been reported. Most of the reported studies on the metabolism of viable, starved bacteria have involved Gram-negative organisms which can accumulate 'reserves' such as glycogen or poly- $\beta$ -hydroxybutyrate. Since lactic streptococci have no reported 'reserves' and very limited catabolic activities, it was considered that extension of endogenous metabolism studies to *Streptococcus lactis* could be informative. The report that exogenous  $Mg^{2+}$  suppressed RNA degradation but had no effect on the survival of starved *Sarcina lutea* (Burleigh & Dawes, 1967), suggested that some of the important survival characteristics of this Gram-positive organism may be markedly different from those for Gram-negative organisms. Similar findings were made by Dawes with two other Gram-positive organisms (see Discussion, Strange & Hunter, 1967). Further investigations with Gram-positive organisms have been indicated and might more clearly define the general parameters for bacterial survival.

Studies on the metabolism of viable, starved organisms are likely to be significant only if factors affecting survival are investigated concurrently. The results of such an investigation with *Streptococcus lactis* have been previously reported (Thomas & Batt, 1968).

## METHODS

*Organism and culture methods.* *Streptococcus lactis* ML<sub>3</sub> was maintained and sub-cultured using methods and media described previously (Thomas & Batt, 1968).

*Preparation of starved suspensions.* Organisms were harvested at the end of the growth phase by centrifuging (30,000 g for 1 min.) and the deposited bacteria were then rinsed thoroughly, dispersed in phosphate buffer (see below), centrifuged (30,000 g for 1 min.), rinsed and finally resuspended in buffer. The temperature was maintained at about 30° and the procedures, which were performed with aseptic precautions, were completed in 15 to 20 min., thus minimizing changes in temperature and the chemical environment. The phosphate buffer used was 0.075 M-Na<sub>2</sub>HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub> (pH 7.0) containing 10<sup>-5</sup> M-EDTA (plus substrates, etc., where specified).

Bacterial mass and viability (by slide culture) were determined by methods described previously (Thomas & Batt, 1968). Where some settling occurred after prolonged starvation, the organisms were gently agitated by means of a magnetic stirrer. Correction was made for evaporation loss by adding appropriate amounts of sterile water. Samples of supernatant buffer solutions were obtained for chemical analyses by centrifugation and filtration through a membrane filter (0.45 μ; Millipore Filter Corp., Bedford, U.S.A.). When analyses on the bacteria were required, the packed organisms were washed in phosphate buffer and normally resuspended in deionized water. Bacterial mass determinations were carried out at the sampling time, while suspension samples and cell-free supernatant solutions were stored at -20° when not analysed immediately.

*Analytical procedures.* Colorimetric and spectrophotometric measurements in the visible and ultraviolet (u.v.) region were made with either a Beckman DB or a Zeiss PMQII spectrophotometer using 1 cm. glass or silica cells. Polyglucose and poly-β-hydroxybutyrate were assayed by the methods of Strange *et al.* (1961) and Williamson & Wilkinson (1958) respectively.

Protein was determined by the biuret method (Stickland, 1951). Suspensions of *Streptococcus lactis* required heating at 100° for 20 min. in 0.75 N-NaOH for maximum colour development. Dried bovine serum albumin (Sigma, A grade), containing 13.6% N, was used as the standard. The alternative method of Lowry, Rosebrough, Farr & Randall (1951) gave similar results to the biuret method with samples of both supernatants and alkali-hydrolysed cell suspensions. Total cell N and supernatant N were determined by the micro-Kjeldahl method described by Humphries (1956) using a Se/K<sub>2</sub>SO<sub>4</sub> catalyst and methyl red-methylene blue indicator.

Amino acids in bacterial extracts and supernatant samples were measured colorimetrically by the ninhydrin method of Yemm & Cocking (1955). Glycine standards were used and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> standards were included to correct for NH<sub>3</sub>, which was determined separately by the micro-diffusion method of Conway (1947). Buffer samples were adjusted to pH 5.0 before amino acid analysis by addition of HCl or dilution in citrate buffer (pH 5.0). Quantitative measurement of individual amino acids was obtained using a Beckman model 120C automatic amino acid analyser with Beckman custom spherical ion exchange resin. The short column for basic amino acids contained resin type PA 35 to a height of 8 cm. The elution buffer pH was 5.28 and the flow rate 68 ml./hr. The long column for acidic and neutral amino acids contained resin type PA 28 to a height of 58 cm. Buffer, pH 3.28, was used as eluant, being

replaced after 90 min. by a second buffer at pH 4.25. The ninhydrin flow rate was 34 ml./hr and analyses took place over a 4 hr period. Peaks were identified from standard elution times and integrated by the height-width method. The total cellular amino acid composition was determined on organisms harvested at the end of the growth phase and washed twice in distilled water. Washed organisms were freeze-dried, hydrolysed (6N-HCl for 24 hr at 110° in a vacuum sealed tube) and the hydrolysate analysed. The minimum time in boiling water required for complete release of the amino acid pool of *Streptococcus lactis* was  $12 \pm 3$  min. For routine extracts, bacteria were washed and resuspended in deionized water and the suspensions heated for 20 min. at 100° in stoppered tubes. Bacterial debris was removed by centrifugation and the clear extract was pipetted from the tube. A second extraction yielded only a further 1 to 3 % of ninhydrin-positive material and was therefore not carried out routinely.

Bacterial RNA was determined by the method described by Munro & Fleck (1966). Perchloric acid-washed bacteria (10 min. at 0° in 0.5N-HClO<sub>4</sub>) were subjected to alkaline hydrolysis in 0.5N-KOH at 37° for 60 min.; this was shown to give a complete extraction of ribonucleotides. (For convenience, samples and standards were often stored at -20° in alkaline suspension before hydrolysis; storage for up to 24 hr at -20° did not affect analyses.) The hydrolysate was chilled to 0° and ice-cold HClO<sub>4</sub> was added to a final concentration of 0.5N. After 10 min. at 0° the insoluble fraction was sedimented and washed twice in ice-cold HClO<sub>4</sub> (0.5N). Centrifugation was carried out in a Sorvall refrigerated centrifuge (30,900 g for 1 min.). The alkali extract and washings were combined and made up to 25 ml. Samples were filtered through a sintered glass filter (porosity 5/3) before extinction measurement at 260 m $\mu$ . Soluble yeast RNA (Sigma, type III) was used as a standard and was treated in the same way as samples. The standard RNA contained 7.85 % phosphorus and was assumed to be 83 % RNA. Bacterial DNA was extracted and estimated by the methods of Burton (1956). Deoxyribose was used as the standard. Before measuring the extinction of supernatant samples at 257 m $\mu$ , the solutions were deproteinized with 5 % trichloroacetic acid, and the appropriate blanks were included.

Free and purine-bound ribose was estimated in cell-free supernatant or alkali-soluble nucleotide extracts using the orcinol method (Mejbaum, 1939). Total cellular hexose was estimated as glucose by the anthrone procedure of Trevelyan & Harrison (1952) or by the reducing sugar method of Nelson (1944). Lactic acid was measured in deproteinized supernatant samples by the method of Barker & Summerson (1941). Volatile fatty acids were separated by exhaustive steam distillation in a Markham still, after adjusting the supernatant and standard samples to pH 1.0 with H<sub>2</sub>SO<sub>4</sub> and distillates were titrated with 0.01N-NaOH in a stream of N<sub>2</sub>, using phenolphthalein as the indicator.

Lipids were extracted directly from washed organisms by the methanol-chloroform procedure of Vorbeck & Marinetti (1965*a*). A second extraction recovered a further 6 to 9 % of the total lipid, and acid hydrolysis (2.5N-HCl, 16 hr at 100°) of the residue yielded a further 3 to 4 % of the total lipid. Two methanol-chloroform extractions were normally carried out. Extracts were washed with aqueous CaCl<sub>2</sub> (Folch, Lees & Stanley, 1957) to remove non-lipid material and the solvents were removed using a rotary evaporator at 40°. The tared flasks were finally dried to constant weight in a vacuum desiccator over P<sub>2</sub>O<sub>5</sub> and the lipids were estimated gravimetrically. Silicic

acid column chromatography was used to separate lipids into neutral and polar fraction (Hirsch & Ahrens, 1958). Lipid (200 mg.) was applied to the top of the column (250 × 17 mm.) in the minimum amount of chloroform-methanol (4:1) and the neutral lipid was completely eluted with ethanol-free chloroform containing 1% methanol (500 ml.). Polar lipid was then eluted completely using chloroform-methanol (2:1, v/v, 300 ml.). Column separations were monitored routinely by both TLC and phosphorus analyses and the fractions obtained were dried and weighed as described previously.

Glycerides were hydrolysed by refluxing with aqueous 5N-KOH:CH<sub>3</sub>OH (1:1, v/v) for 5 hr; non-saponifiable material was removed by ether extraction. After acidification, free fatty acids were extracted into diethyl ether, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and esterified with diazomethane (Schlenk & Gellerman, 1960). Analysis of the methyl esters was carried out on an Aerograph 600 gas chromatograph (Wilkins Instrument & Research, U.S.A.) using a hydrogen flame ionization detector. Apiezon L on 80-100 mesh celite (10%, w/w) at 196° and polyethylene glycol adipate at 180° were used as stationary phases and the columns were prepared as described by James (1960). The detector and columns were checked periodically with fatty acid standards and the procedures recommended by Horning *et al.* (1964). Peaks were identified by comparison with retention data of standards or with published values; the % composition of the fatty acids was estimated by the height-width method (Horning *et al.* 1964).

Thin-layer chromatography was carried out on glass plates with a layer of silicic acid (silica gel G, Merck) following the procedures of Mangold (1961). Chromatograms, which had been developed in either hexane:diethyl ether:acetic acid (70:30:1, v/v/v) or di-isobutyl ketone:acetic acid:water (80:50:8, v/v/v), were dried before spraying with ninhydrin and the spots giving a positive reaction were marked. Phosphorus-containing components were detected by using the molybdenum spray reagent of Dittmer & Lester (1964). The plate was finally sprayed with 10% H<sub>2</sub>SO<sub>4</sub> and charred at 100° to show all components. Phosphorus was estimated by the method of Burton & Petersen (1960).

*Materials.* Solid reagents for analytical procedures were recrystallized and solvents redistilled if analytical reagent grades were not available. All water was distilled and then deionized by passage through a mixed bed ion-exchange resin (Permutit 'Biodeminrolit').

## RESULTS

*'Reserve' polymers.* Organisms (1.2 g. dry wt) were harvested from the end of the growth phase in the routine medium and extracted for polyglucose and poly-β-hydroxybutyrate (PHB). Analysis of the extracts revealed no trace of either polymer. However, the routine medium was lactose-limiting, so that optimum conditions may not have existed for polymer accumulation. When organisms were harvested at the end of the growth phase and resuspended in the routine medium minus the casamino acids and peptone components, no detectable synthesis of either polyglucose or PHB occurred. Conditions similar to these had been found to be optimal for intracellular polyglucose synthesis in *Streptococcus salivarius* (Hamilton, 1968). Therefore it seemed unlikely that *Streptococcus lactis* was capable of synthesizing these polymers which had been reported as present in many bacterial species. Electron micrographs of thin sections showed no evidence of polyphosphate granules (Thomas & Batt, 1969).

*Changes in bacterial protein and total N.* Little, if any, net protein breakdown occurred in starved suspensions (Fig. 1). Protein was estimated by the biuret method, which measures peptide bonds and by the Folin-Ciocalteu procedure which measures tyrosine (see Methods). Only traces of free tyrosine were released from starved organisms (Table 2). Protein accounted for 48 % of the initial bacterial dry wt and starvation for the 28 hr period resulted in a 26 % bacterial mass loss (Fig. 1).

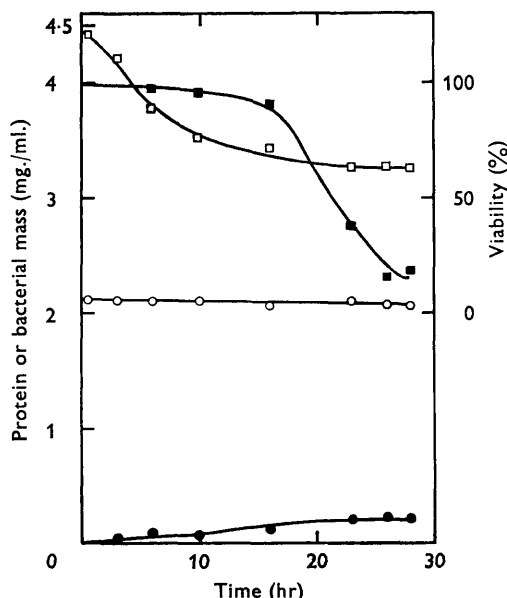


Fig. 1. Changes in protein in starved *Streptococcus lactis*. Bacteria were harvested from the end of the growth phase, washed and resuspended at 30° in phosphate buffer (0.075 M, pH 7.0 containing 1 mM-MgSO<sub>4</sub> + 10 μM-EDTA). At the times indicated, samples of the suspension were removed and a portion immediately deep-frozen together with supernatant samples which were obtained after centrifugation and filtration. Protein analyses were performed on whole suspensions (○) and supernatant samples (●). At each sample time, bacterial density (□) was determined by both turbidity and dry wt measurements and viability (■) was estimated by the slide-culture procedure.

Table 1. Release of protein from starved *Streptococcus lactis*

Suspensions were prepared as for Fig. 1 at initially 4.5 mg. dry wt/ml. but with organisms resuspended in (1) 0.075 M-phosphate buffer + 1 mM-MgSO<sub>4</sub> + 10 μM-EDTA and (2) as for (1) + casamino acids (0.5 %, Difco) + arginine (0.1 %). Supernatant protein was precipitated with 5 % TCA, washed and estimated by the method of Lowry *et al.* (1951); results are expressed as mean mg./ml. of four determinations.

Starvation period (hr)	Suspension (1)		Suspension (2)	
	Viability (%)	Supernatant protein	Viability (%)	Supernatant protein
2.5	99	0.03	—	0.02
6	98	0.06	99	0.06
18.5	93	0.17	99	0.17
23	71	0.20	97	0.21
42	0	0.29	48	0.28

Soluble protein (0.22 mg./ml.), amounting to 10% of the total bacterial protein, was released from starved organisms into the external medium (Fig. 1). The total N in this culture was constant at  $0.49 \pm 0.018$  mg./ml. and the amount of N released into the suspending buffer after 28 hr starvation ( $0.091 \pm 0.016$  mg./ml.) suggested that a considerable amount of non-protein N was involved (protein release accounted for approx. 0.02 mg. N/ml.). The initial turbidity of the whole suspension was 23.1 and the turbidity of supernatant samples was always less than 0.005. The rate of protein release appeared to decrease at about the same time as the death rate increased.

Table 2. Release of amino acids from starved *Streptococcus lactis* organisms

Bacteria were harvested, washed twice and resuspended at 15 mg. dry wt/ml. in buffer (Fig. 1). The intracellular amino acid pool was extracted with deionized water at zero time (see Methods). After 28 hr starvation at 30° the organisms in the buffer suspension were washed and extracted in deionized water. The supernatant buffer was deproteinized by addition of TCA (final concentration 5%). Samples (0.5 ml.) of the extracts, supernatant and hydrolysed bacteria were analysed with an amino acid analyser (see Methods). Results are expressed as  $\mu\text{g. amino acid or NH}_3/\text{mg. dry wt bacteria at 0 hr}$ .

Amino acid	Intracellular pool		Super-natant (28 hr)	Total pool (28 hr)	Hydrolysed bacteria (0 hr)
	0 hr	28 hr			
Lysine	0.95	0.38	8.9	9.3	48.5
Histidine	0.24	0.03	0.71	0.74	8.0
Arginine	—	—	—	—	18.6
Aspartic acid	4.25	0.15	0.70	0.85	67.1
Threonine	1.32	0.10	2.24	2.34	24.9
Serine	0.20	0.03	0.32	0.35	22.5
Glutamic acid	16.3	3.44	11.6	15.0	88.2
Proline	—	0.11	1.27	1.38	19.0
Glycine	1.05	0.22	5.2	5.5	28.8
Alanine	7.3	1.24	15.1	16.4	56.6
Cystine	0.19	—	0.21	0.21	—
Valine	0.33	0.05	1.37	1.42	23.2
Methionine	0.05	—	0.25	0.25	9.7
Isoleucine	0.09	0.03	0.81	0.84	18.1
Leucine	0.35	0.05	1.40	1.45	32.3
Tyrosine	0.17	—	0.57	0.57	13.3
Phenylalanine	0.35	—	0.71	0.71	16.4
Ammonia	0.17	0.25	2.18	2.43	11.5
Total amino acid	33.14*	5.83	51.36†	57.31	495.2‡

\* Plus six unidentified peaks which comprised 3.5% total pool.

† Plus four unidentified peaks which comprised < 1% total pool.

‡ Plus one peak which comprised 3% total pool (probably hydroxylysine).

However, the rate and amount of protein release was not influenced by the presence of exogenous amino acids although these reduced the death rate (Table 1).

*Release of amino acids and ammonia.* The intracellular amino acid pool of *Streptococcus lactis* accounted for more than 3% of the dry wt of freshly suspended organisms and this pool was rapidly depleted on starvation (Fig. 2). Ninhydrin-reactive material appeared concurrently in the external medium and there was a net increase in the total free amino acids on starvation.

The 73% net increase in the total free amino acids after 28 hr starvation (Table 2) suggested that, in addition to leakage of intracellular amino acids, some protein degradation occurred. This increase in amino acid concentrations would correspond

to a 5% breakdown of the total bacterial protein. Colorimetric methods of protein estimation were presumably too insensitive to estimate these small changes accurately (see Fig. 1). No low molecular weight peptides were observed on the amino acid traces but it is possible that if they were present they may have been removed when the samples were deproteinized. The total amount of amino acids obtained from hydrolysed organisms (Table 2) was consistent with earlier analyses for bacterial protein.

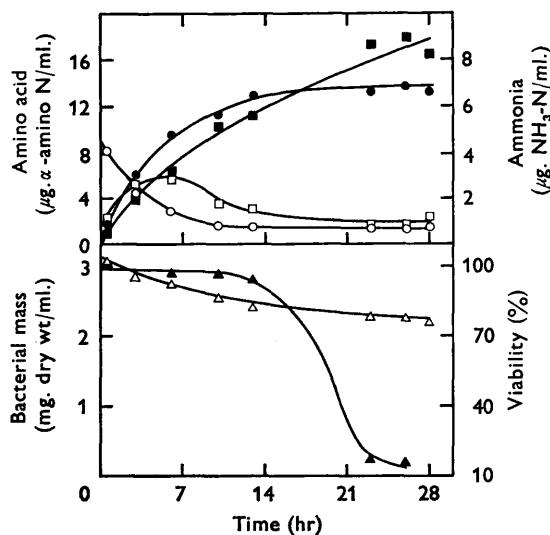


Fig. 2. Release of ninhydrin-reactive material and ammonia from starved *Streptococcus lactis* organisms. Bacteria were harvested, resuspended and sampled at intervals, as described for Fig. 1. Bacterial extracts were prepared and all samples were deep-frozen and later analysed (see Methods). Intracellular free amino acid and ammonia are shown as  $\circ$ ,  $\square$ ; supernatant amino acid and ammonia are shown as  $\bullet$ ,  $\blacksquare$ . Bacterial mass ( $\triangle$ ) and viabilities ( $\blacktriangle$ ) were determined as described in Methods.

Glutamic acid, alanine and aspartic acid made up 49.2%, 22.0% and 12.8% respectively of the initial amino acid pool. The total amount of free aspartic acid was substantially reduced on starvation while the glutamic acid level showed a slight decrease (Table 2). There have been no reports of the catabolism of these amino acids by *Streptococcus lactis*. The total amount of free lysine increased considerably on starvation while other amino acids increased by varying amounts. The levels of amino acids in the intracellular pool reflected the total amino acid composition of the organisms (Table 2). Neither free arginine nor ornithine were detected in any samples and only a small amount of  $\text{NH}_3$  was produced (Table 2). These results suggested that starved *Streptococcus lactis* was unlikely to obtain substantial energy from the amino acid pool.

**Changes in nucleic acids.** A substantial amount of material, with an absorption maximum of 257  $\text{m}\mu$ , was released from starved organisms (Fig. 3). This could be correlated with nucleic acid breakdown and the release of ultraviolet (u.v.)-absorbing purine and pyrimidine fragments.

In the absence of added  $\text{Mg}^{2+}$ , bacterial RNA was broken down at a rapid rate



from the onset of starvation, the degradation products being released into the suspending buffer. Organisms initially contained 20.8 % RNA, which was reduced after 28 hr starvation to 5.5 % RNA (calculated on the initial bacterial dry wt). With added  $Mg^{2+}$ , the death rate was reduced and RNA was broken down only after a considerable lag, the initial RNA level of 20.7 % dry wt being reduced to 10.4 % after 28 hr (Fig. 3).

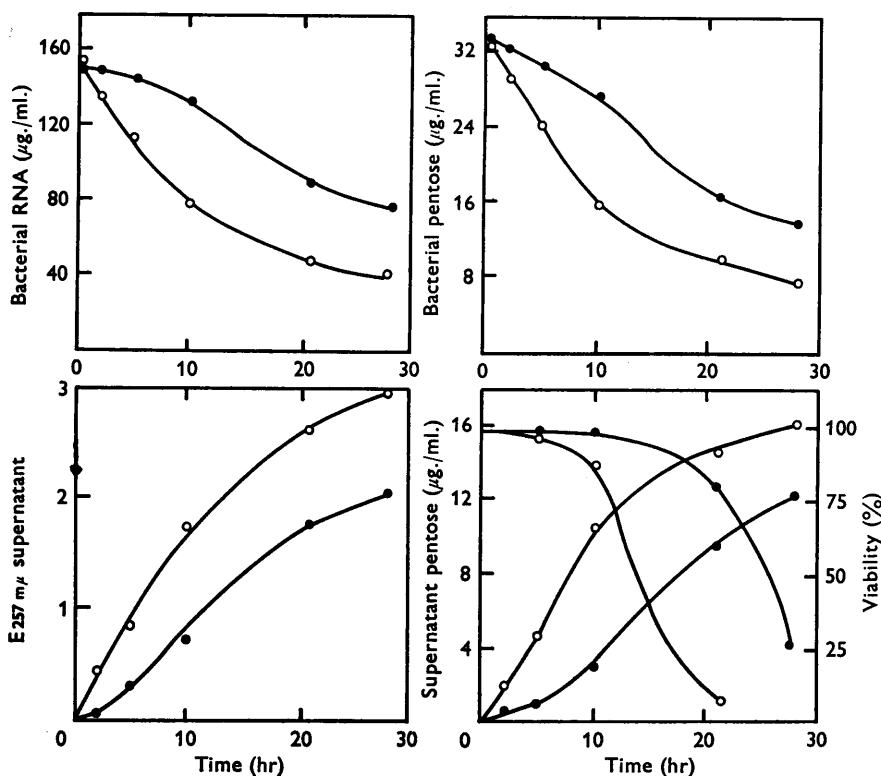


Fig. 3. Breakdown of RNA in starved *Streptococcus lactis* organisms. Bacteria were harvested from routine growth medium at the end of the log-phase, washed once and resuspended at 30° in 0.075 M-phosphate buffer (pH 7.0, + 10  $\mu$ M-EDTA) containing: ○, no addition; ●, 1 mM-MgSO<sub>4</sub>. Bacterial masses were initially 0.74 and 0.73 mg. dry wt/ml. respectively. Samples were removed at the times indicated and immediately centrifuged. Supernatant and cellular extracts were prepared as described in Methods, frozen and later analysed.

Without added  $Mg^{2+}$ , most organisms were still viable when 50 % of the cellular RNA had been lost, although with added  $Mg^{2+}$  most organisms were non-viable at this point (Fig. 3).

In both suspensions loss of cellular orcinol-reactive material was similar to RNA loss but was not balanced by a corresponding increase in supernatant orcinol-reactive material (Fig. 3), suggesting that some ribose may have been catabolized. However, lactic acid was not produced from exogenous ribose (Thomas & Batt, 1968), and since the orcinol reaction does not estimate pyrimidine bound ribose it may not be possible to balance ribose concentrations. In later experiments measurements of the release of u.v.-absorbing material into the suspending buffer were used as a measure of RNA

breakdown after samples had been treated with TCA to remove u.v.-absorbing protein (see Methods).

The rates of RNA breakdown and loss of viability were measured in the presence of substrates which produced the maximum range of death rate and cell division lag time (Fig. 4). Glucose-accelerated death was accompanied by extremely rapid RNA breakdown. Organisms starved in phosphate buffer also showed rapid rates of death and RNA breakdown. Addition of  $Mg^{2+}$  suppressed RNA degradation and the death rate but the presence of exogenous amino acids had only a slight effect on RNA breakdown although they markedly reduced the death rate.

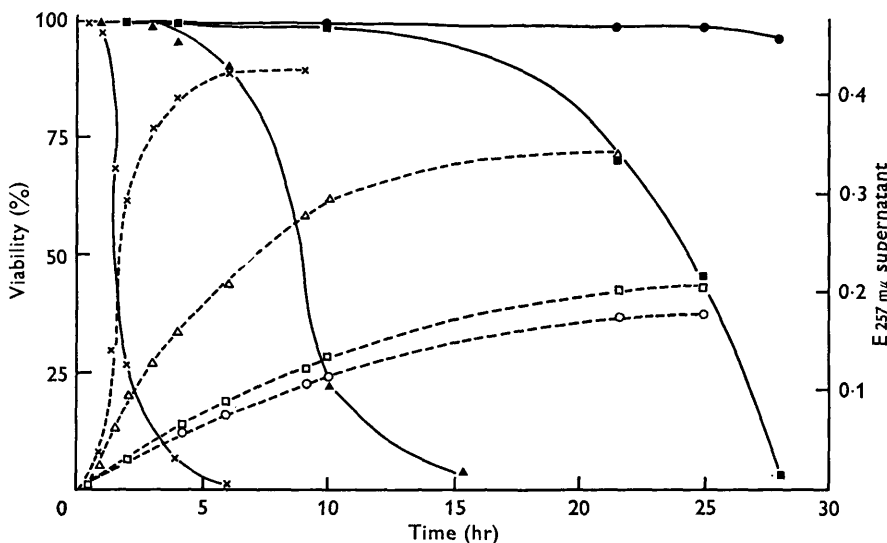


Fig. 4. RNA breakdown and death rate of starved *Streptococcus lactis* organisms. Washed organisms were resuspended at 0.09 mg. dry wt./ml. in phosphate buffer containing 10  $\mu$ M-EDTA. Supernatant  $E_{257m\mu}$  values for the suspensions: ○, +0.5% casamino acids (Difco) +0.1% arginine +1 mM- $Mg^{2+}$ ; □, +1 mM- $Mg^{2+}$ ; △, no addition; ×, +10 mM-glucose; are indicated by dashed lines. Viability curves of organisms in the same systems have solid lines.

No detectable change in bacterial DNA occurred in two experiments where organisms were starved for 16 hr at 0.24 mg. dry wt./ml. in phosphate buffer. The total DNA content of the suspensions remained at  $8.84 \pm 0.17 \mu$ g./ml. (DNA accounted for 3.7% of the bacterial dry wt).

*Changes in carbohydrates.* Analyses for cellular anthrone-positive material and reducing sugars indicated that there was little, if any, carbohydrate breakdown in starved organisms (Table 3). Carbohydrate fermentation would be expected to produce predominantly lactic acid. The small amount of lactate produced (Table 3) suggested that about 2% of the total cellular carbohydrate may have been fermented. Most of the hexose in *Streptococcus lactis* is likely to have a structural role. Other investigators working with other organisms (Strange *et al.* 1961; Postgate & Hunter, 1962; Ribbons & Dawes, 1963; Dawes & Ribbons, 1965; Burleigh & Dawes, 1967) have shown that structural carbohydrate is only slightly degraded, if at all, in starved bacteria.

*Changes in lipids.* *Streptococcus lactis* ML<sub>3</sub>, grown in the routine medium, contained

3.8 % lipid on a dry wt basis (Table 4). A net loss of 10 % of this lipid occurred during a 55 hr starvation period. During this time the polar lipid fraction decreased from 85 % to 70 % of the total lipid and the neutral lipid fraction increased from 12 % to 30 % of the total lipid (Table 4). Spot intensities on H<sub>2</sub>SO<sub>4</sub>-charred thin-layer chromatograms indicated a marked increase in the free fatty acid component of the neutral

Table 3. Cellular carbohydrate in starved *Streptococcus lactis* organisms

Bacteria from the end of the growth phase were washed and resuspended in 0.075 M-phosphate buffer (pH 7.0 + 10  $\mu$ M-EDTA) at 30° and 1.0 mg. dry wt bacteria/ml. Supernatant samples were removed and frozen at intervals. Anthrone-reactive carbohydrate was determined directly on washed organisms. Reducing sugar was measured in washed organisms after storage at -20° in 2.5 N-H<sub>2</sub>SO<sub>4</sub> before hydrolysis. Carbohydrate is expressed as the mean  $\mu$ g. glucose equivalents/ml. of four determinations.

Starvation period (hr)	Viability (%)	Cellular carbohydrate		Supernatant	
		Anthrone + ve material	Reducing sugar	Anthrone + ve material	Lactate ( $\mu$ g./ml.)
0.2	98	77	107	0.2	0.6
1.2	97	77	102	0.6	0.7
2	95	74	105	0.5	0.9
3	95	75	109	0.7	1.2
4	96	74	106	1.0	1.0
5	93	72	106	0.9	1.6
10	68	73	103	0.9	1.8
22	12	74	102	0.8	1.9

Table 4. Changes in lipid components of starved *Streptococcus lactis*

Bacteria were grown at 30° in 3  $\times$  10 l. flasks containing 10 l. routine medium, agitation being provided by a magnetic stirrer. Growth was followed turbidimetrically, and at the times indicated from the end of growth bacteria were harvested using a Sorvall continuous flow centrifuge. With a flow rate of 6 l./hr at 31,000 g, turbidity measurements on the supernatant indicated recovery of > 99 % bacterial dry wt. The packed bacteria were washed twice in distilled water and the lipids extracted, washed and analysed as described in Methods.

Time from end of growth (hr)	0	24	55
Bacterial yield* (g. dry wt)	6.72	5.73	4.97
Total lipid (g.)*	0.256	0.244	0.228
(% bacterial dry wt)	(3.81)	(4.25)	(4.59)
Neutral lipid (g.)*	0.032	0.036	0.070
(% total lipid)	(12.5)	(14.6)	(30.6)
Polar lipid (g.)*	0.218	0.201	0.159
(% total lipid)	(85.2)	(82.5)	(69.7)
Lipid recovery (%)	97.7	97.1	100.3
Polar lipid P (%)	2.37	2.28	1.86
Polar lipid N (%)	0.72	0.67	0.95
Viability (%)	99	96	32

\* Mean of two or more batches.

lipid on starvation. Hydrolysis of polar lipid may explain these changes. The polar lipid phosphorus and nitrogen values observed (Table 4) were similar to those reported by Ikawa (1963) for a range of lactic acid bacteria.

Analysis of the fatty acids in the neutral and polar lipid fractions from *Streptococcus lactis* revealed a composition very similar to that previously reported for this organism

by MacLeod, Jensen, Gander & Sampugna (1962). Starvation brought about changes in the fatty acid composition of the two fractions (Table 5). The relative amount of hexadecanoic acid decreased in the neutral lipid fraction while tetradecanoic acid increased. The cyclopropane acid, lactobacillic acid, increased in both neutral and polar lipid fractions with a corresponding decrease of its immediate precursor, *cis*-vaccenic acid. Knivett & Cullen (1967) reported that *Escherichia coli*, in the post-

Table 5. *Changes in fatty acids in starved Streptococcus lactis.*

The neutral and polar lipid fractions of the lipid extracts in Table 4 were hydrolysed, the fatty acids isolated, esterified and finally identified and quantitatively measured by gas-liquid chromatography (see Methods). Results are the means of four analyses and are expressed as % of the total fatty acid ester in each sample.

Fatty acid (probable identity)	Neutral lipid			Polar lipid		
	0 hr	24 hr	55 hr	0 hr	24 hr	55 hr
<sup>c</sup> 12:0	1.1	1.3	0.9	0.1	0.0	0.2
<sup>c</sup> 14:1	0.5	0.6	0.8	0.4	1.1	1.3
<sup>c</sup> 14:0	8.7	10.3	13.9	13.7	17.4	14.9
<sup>c</sup> 16:1	3.9	4.1	4.2	4.1	3.7	4.2
<sup>c</sup> 16:0	34.7	26.8	25.9	20.8	20.7	21.4
<sup>c</sup> 18:1	24.6	26.8	21.0	30.2	20.3	18.1
<sup>b</sup> 18:0	2.7	2.4	1.4	0.1	1.1	0.8
<sup>c</sup> 19:0 <sup>v</sup>	24.8	27.6	31.7	30.8	35.7	39.2

<sup>c</sup>18:1 represents *cis*-vaccenic acid.

<sup>c</sup>19:0<sup>v</sup> represents lactobacillic acid.

Table 6. *Summary of changes in components of Streptococcus lactis organisms starved at 30° in 0.075 M-phosphate buffer (pH 7.0, 10 μM-EDTA, 1 mM-MgSO<sub>4</sub>)*

(1) Depletion of polymers and amino acid pool (results are expressed as % initial bacterial dry wt)

Starvation period (hr)	Protein*	Amino acid† pool	RNA‡	DNA	Carbo- hydrate§	Lipid	Total	Bacterial dry wt*
0	48	3.3	20.7	3.7	7.7	3.8	87.2	100
28	40.7¶	0.6	10.4	3.7	7.2	3.6	66.2	74
Loss	7.3	2.7	10.3	0.0	0.5	0.2	21.0	26

(2) Formation of products (results of analyses on the suspending buffer expressed as % initial bacterial dry wt)

Starvation period (hr)	Lactate§	NH <sub>3</sub> †	Volatile fatty acid (as acetic acid)	Amino acid†
28	0.21	0.22	0.36	5.73

\* Fig. 1.

† Table 2.

|| Table 4 (24 hr).

¶ Estimated from protein released\* and increase in total free amino acids†.

‡ Fig. 3.

§ Table 3 (carbohydrate = anthrone+ve).

exponential growth phase, showed increases in cyclopropane fatty acids with decreases in the corresponding monoenoic acids and similar changes have been reported with many bacterial species after growth has stopped (see Kates, 1964).

A small amount of volatile fatty acid ( $0.6 \mu\text{eq./mg. dry wt bacteria/24 hr}$ ) was released into the suspending buffer ( $0.075\text{M-phosphate} + 1 \text{ mM-MgSO}_4$ ) by starved *Streptococcus lactis* organisms. The source of this material has not been defined.

A summary of the chemical changes which occurred when *Streptococcus lactis* was starved at the growth temperature is presented in Table 6.

#### DISCUSSION

*Streptococcus lactis* did not accumulate detectable amounts of polyglucose or poly- $\beta$ -hydroxybutyrate in conditions which might be expected to be favourable for their synthesis. No reports have appeared in the literature indicating the presence of these or other storage polymers in lactic streptococci. Only one report has been published on the metabolism of *S. lactis* starved at growth temperatures and this claimed that (a) 'the organism had a substantial endogenous respiration' and (b) 'endogenous lactate or succinate was oxidized after a lag' (Spendlove, Weiser & Harper, 1957). These results were not confirmed by Thomas (1968) and it seems unlikely that lactate or succinate could function as endogenous substrates for an organism which is a homolactic fermenter, possessing no terminal respiratory system.

Whether bacteria possess polymeric storage reserves or not, the constitutive material of starved organisms is ultimately degraded and death ensues. Most of the decrease in cell mass of starved *Streptococcus lactis* organisms could be accounted for by RNA and protein degradation. The rates of RNA breakdown and death were reduced by the addition of  $\text{Mg}^{2+}$ , in contrast to the findings of Burleigh & Dawes (1967) with *Sarcina lutea*. However, these authors examined the effect of added  $\text{Mg}^{2+}$  with suspensions of high bacterial density ( $8.8 \text{ mg. dry wt/ml.}$ ) and it is possible that if  $\text{Mg}^{2+}$  was liberated by the organisms as their RNA was degraded, the effect of added  $\text{Mg}^{2+}$  on survival may have been masked (see Thomas & Batt, 1968) since RNA appeared to be to some extent expendable. The protective effect of  $\text{Mg}^{2+}$  on Gram-negative organisms under conditions of stress has been demonstrated with bacterial concentrations of  $20 \mu\text{g. dry wt/ml.}$  or less (see Postgate & Hunter, 1962, 1964; Strange & Postgate, 1964; Strange & Dark, 1965). These stresses showed population density effects so that the response of *S. lutea* to added  $\text{Mg}^{2+}$  may not be basically different from that defined for Gram-negative organisms. *S. lactis* has both a high RNA content, typical of an organism grown at a rapid rate, and a high Mg content, which is consistent with the probable interdependence of these constituents (Tempest & Strange, 1966). The molar ratio of RNA/Mg was approximately 50, which is of the same order as that observed for *Aerobacter aerogenes* (Tempest & Strange, 1966).

Conditions which accelerated RNA breakdown—such as buffer systems which either contained glucose alone or did not contain  $\text{Mg}^{2+}$ —also produced increased death rates. However, in the presence of  $\text{Mg}^{2+}$ , arginine only slightly suppressed RNA degradation although it extended survival. Hence no general correlation exists between RNA degradation and loss of viability. Although considerable RNA may be degraded without affecting viability, in agreement with results for many other starved bacteria (see Burleigh & Dawes, 1967), it seems likely that a degree of ribosome stability is important for survival of *Streptococcus lactis*. Conditions which accelerated RNA breakdown in other bacteria were generally more lethal (e.g. see Strange & Shon, 1964; Strange & Dark, 1965) and Postgate (1967) has concluded that RNA degrada-

tion is a critical process in the survival of *Aerobacter aerogenes*. However, no absolute correlation between the rates of death and RNA breakdown could be demonstrated.

No evidence was obtained for the catabolism of ribose or bases from degraded RNA, in contrast to findings with most other starved organisms (see Dawes & Ribbons, 1964). It has been suggested that RNA breakdown may continue in viable bacteria as long as mechanisms for RNA resynthesis from precursors remain intact (Burleigh & Dawes, 1967). The present results show that conditions producing maximum rates of RNA degradation and death also produce increased cell division lag times of surviving organisms (Thomas & Batt, 1968). These lags are probably directly influenced by the amount of polymer degradation which has taken place, particularly that of RNA, since the RNA content of bacteria increases with the growth rate (Tempest & Strange, 1966) while the rate of protein synthesis per ribosome particle is constant in growing bacteria (Tempest, Herbert & Phipps, 1967). Most of the protein lost from starved organisms appeared in the external medium as biuret-positive material although the net increase in total free amino acids indicated that some protein had been hydrolysed. Protein hydrolysis occurs in many starved bacteria with subsequent metabolism of the released amino acids (see Dawes & Ribbons, 1964). Although the over-all level of free aspartate (and to a lesser extent glutamate) was reduced, no evidence was obtained for appreciable catabolism of components of the free amino acid pool in *Streptococcus lactis*.

The loss of RNA and protein from starved *Streptococcus lactis* appeared to involve only hydrolytic reactions with the release of undegraded products into the external medium. Viability was not immediately affected and there was no evidence for appreciable energy-yielding catabolism of endogenous substrates. This was consistent with the formation of only trace amounts of the normal end products of fermentation, namely lactate,  $\text{NH}_3$  and volatile fatty acids. The cellular anthrone-reacting material and reducing sugar of *S. lactis* (which probably occurs mainly in structural polymers), together with cellular DNA, were not appreciably degraded in starvation conditions. These results are in agreement with literature reports for other starved organisms. However, Postgate (1967) and Burleigh & Dawes (1967) pointed out that chemical analyses would not detect structural changes in DNA that could cause loss of viability.

Polar lipid and in particular phospholipid, constitutes the main lipid fraction in most lactic acid bacteria (Kates, 1964). In *Streptococcus faecalis*, 94% of the total lipid was found in the membrane fraction (Vorbeck & Marinetti, 1965*b*). In the present investigation, a substantial amount of phospholipid breakdown occurred on prolonged starvation of *Streptococcus lactis*. Since these compounds are known to have important structural and physiological roles in bacterial membranes (Brown, 1964; Salton, 1967) any breakdown of phospholipids may be expected to impair permeability barriers. The possible significance of these observations in the survival of starved *S. lactis* is discussed elsewhere (Thomas & Batt, 1969).

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