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Studies on the Endogenous Metabolism and Senescence of Starved *Sarcina lutea*

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1. When washed suspensions of *Sarcina lutea* are starved aerobically in phosphate buffer at the growth temperature of 37°, the rate of endogenous oxygen consumption decreases to very low values after 10hr., although many of the cells survive for 40hr. If starvation is prolonged further, the bacteria die at a rate of approximately 1·5% of the initial viable population per hour. 2. Oxidation of intracellular free amino acids accounts for most of the observed endogenous oxygen uptake but RNA is also utilized and a portion of the component bases and pentose is degraded and presumably oxidized. Ammonia appears in the supernatant and some pentose and ultraviolet-absorbing nucleotide are released from the cells. DNA, protein and polysaccharide are not measurably degraded. 3. Survival can be correlated with the ability of aerobically starved bacteria to oxidize exogenous L-glutamate and glucose. When starved under nitrogen for 40hr. cells continue to oxidize their endogenous reserves at undiminished rates when transferred to aerobic conditions; on prolonging anaerobic starvation the rate of oxidation declines during the period of most rapid loss of viability. 4. In the presence of Mg^{2+}, RNA degradation during aerobic starvation is almost completely suppressed without affecting the period for which the bacteria survive. 5. Cells grown in peptone supplemented with glucose accumulate reserves of polysaccharide which are metabolized in aerobic starvation, together with free amino acids. Ammonia is evolved and RNA is degraded to a greater extent than in peptone-grown suspensions. Bacteria rich in polysaccharide survive less well than those which are deficient in the polymer; the reason for this phenomenon has yet to be established. 6. In peptone medium, endogenous oxygen uptake and the concentration of intracellular free amino acids decline as growth progresses and they continue to decrease when the organism is held in stationary phase. Under the conditions used, the endogenous Q_{O_2} and free amino acid pool of cells grown in peptone with 2% (w/v) glucose did not decline so markedly and the bacteria contained large amounts of polysaccharide at all stages of growth.

When cells are totally deprived of exogenous substrates, metabolism, if it continues, is predominantly catabolic. Bacterial constituents known to be degraded during starvation include free amino acids and protein, RNA, polysaccharide and lipid. In all cases so far investigated, starved bacteria eventually die, though it has not yet been possible to attribute death to the degradation or inactivation of any single molecular species. Dawes & Ribbons (1962, 1964) have reviewed what is known of the survival and so-called endogenous metabolism of bacteria held in nutrient-free environments.

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Of current interest is whether endogenous metabolism is wholly catabolic or whether available precursors and energy are liberated which will support some degree of macromolecular resynthesis; cell components whose loss is particularly likely to result in death might then be selectively re-formed from dispensable material. This and other potential survival mechanisms are discussed by Dawes & Ribbons (1962, 1964) and the possible protective roles of specialized reserves, such as polysaccharide, poly-β-hydroxybutyrate and polyphosphate, have been reviewed by Wilkinson (1959).

Studies on Gram-positive cocci form a minor proportion of published work on bacterial starvation. Of immediate relevance are the observations of Dawes & Holms (1958*a*) that, after 5hr. starvation,

the rate of endogenous respiration of *Sarcina lutea* had declined from an initial Q_{O_2} (approximately 15) to a value of less than unity. In the same period, about half of the cellular free amino acid pool was oxidized and small amounts of amino acid appeared in the supernatant. Binnie, Dawes & Holms (1960) also found that washed suspensions of *S. lutea* assimilate glucose to form a non-reducing polysaccharide of low molecular weight and that the polymer is degraded in starvation.

The object of the present studies has been to starve *S. lutea* for longer periods of time and to compare the behaviour of cells which are respectively rich and deficient in polysaccharide reserves. The effect of starvation on viability has also been investigated. Preliminary accounts of the work have already been published (Burleigh, Dawes & Ribbons, 1963; Burleigh & Dawes, 1965).

EXPERIMENTAL

Organism. The strain of *S. lutea* used throughout this work was originally provided by Professor E. F. Gale.

Growth and maintenance. The complex medium used was that of Dawes & Holms (1958*b*), having the following composition (g./l.): KH_2PO_4, 6·0; Bacto-Peptone (Difco), 5·0; NaCl, 0·6. The pH was adjusted to 7·1 with 5N-NaOH and, after autoclaving, sterile $MgSO_4,7H_2O$ (10%, w/v) was added to a final concentration of 0·04% (w/v). When glucose was to be included to give final concentration 2% (w/v), it was sterilized as a separate solution and the volume of the main body of fluid was correspondingly reduced.

Cells for starvation studies were grown with forced aeration usually in 10l. round, flat-bottomed flasks fitted with sintered-glass spargers and containing 6l. of medium. The incubation temperature was 37° and 45ml. of a stationary-phase population was used as inoculum. Peptone-grown cells were harvested either after 24hr. when the rate of multiplication was declining or 2–3hr. after the onset of stationary phase, which was finally attained after 32hr. Growth under these conditions was extremely reproducible. Bacteria from glucose–peptone medium were harvested after either 24 or 36hr. while the turbidity of the culture was still increasing. When smaller quantities of bacteria were required, they were grown in a 1l. flask containing 500ml. of medium which was aerated via a capillary aerator.

Cells were maintained at 2° on the appropriate medium, peptone or glucose–peptone, solidified with 2% agar. The organism was subcultured at intervals of up to 1 month.

Preparation of bacterial suspensions for starvation. Large volumes of culture were harvested with an air turbine Sharples no. 1A Laboratory super-centrifuge operated at 50000*g*. The deposited bacteria were cooled in ice as soon as centrifugation was completed. Smaller quantities of cells were harvested at 5000*g* in an MSE Major refrigerated centrifuge at 0°.

Harvested bacteria were washed in ice-cold potassium phosphate buffer (0·067M-KH_2PO_4) at the growth pH 7·1 followed by ice-cold distilled water and were then usually suspended to a density approx. 10mg. dry wt./ml. in the starvation medium, which was phosphate buffer (0·067M-KH_2PO_4; pH 6·8). The phosphate buffers were adjusted to the appropriate pH with 5N-NaOH. Cells were starved at a slightly acid pH so that NH_3 liberated during starvation would be retained in solution.

For studies involving prolonged starvation it is essential to exclude contaminating bacteria when preparing washed suspensions. In early experiments, it was noted that although freshly harvested populations of *S. lutea* might appear homogeneous, contaminating organisms sometimes appeared after suspensions had been starved for 30hr. The contaminants had presumably multiplied by utilizing soluble products of endogenous metabolism released by *S. lutea* and quantitative estimation of such products was consequently seriously hindered. Endogenous Q_{O_2} values were observed to increase when contaminants appeared. To overcome this problem, cultures were always inoculated and suspensions prepared and washed under a hood whose interior had been sterilized with ultraviolet light. Distilled water and buffer solutions used to wash and suspend the bacteria, as well as the Sharples bowl, starvation flasks, centrifuge tubes and caps, were all sterilized and cultures were aspirated directly into the Sharples centrifuge without exposure to the atmosphere.

The organism was starved aerobically by three procedures which have yielded similar results. The method of Dawes & Holms (1958*a*) was initially employed and consisted of passing sterile water-saturated air at a rate of approx. 1l./min./g. total dry wt. of bacteria through a suspension of cells in a Quickfit and Quartz (Q/Q) flask of either 500ml. or 1l. capacity. A second method was to starve cells in a shaken and stoppered conical flask (Q/Q) containing approximately twice as much oxygen as was required for endogenous respiration. This procedure had the advantage that possible changes in cell density, due to condensation or evaporation of water from the suspension, were eliminated and the results of analyses could be related to a single estimate of cell density made at zero time. Cells have also been starved both aerobically and under N_2 in Warburg flasks of approx. 25ml. capacity.

Chemicals. Chemicals of analytical grade (Hopkin and Williams Ltd., Chadwell Heath, Essex; British Drug Houses Ltd., Poole, Dorset) were used wherever possible, otherwise, those of the best available purity from the same suppliers. Orcinol was recrystallized.

Analytical procedures

Bacterial density. Bacterial densities were determined by drying known volumes of suspension in distilled water or phosphate buffer to constant weight at 105°; appropriate correction was made for the amount of solids present in the buffer. Estimates were also made by the turbidimetric procedure of Holms (1957); samples were suitably diluted in distilled water to densities 0·1–0·6mg. dry wt./ml. and mixed with an equal volume of a solution containing formalin (1·6%) and the detergent, Comprox A (2%, v/v) (British Petroleum Ltd., London), which dispersed cell clumps and resulted in more reproducible extinctions. The latter were measured with either a Hilger Spekker absorptiometer and Chance filters OB2 and H508 or with a Unicam SP. 600 spectrophotometer at 570mμ. No decrease in turbidity was noted when cells were allowed to stand in the detergent for 1hr. Extinctions were related to bacterial dry weight by appropriate calibration curves constructed

for cells harvested from peptone and from glucose–peptone media; turbidities of peptone-grown suspensions were approx. 15% higher than those of glucose–peptone suspensions containing an equal weight of bacteria per ml.

Measurement of bacterial viability. Viability was measured by plate counts and by the slide-culture technique of Postgate, Crumpton & Hunter (1961). If either method is applied to *S. lutea*, a potential source of gross inaccuracy is the presence of cell clusters; these occur even in suspensions which have been starved for prolonged periods and they may include as many as several hundred individual bacterial cells. *S. lutea* is known readily to survive exposure to ultrasonic waves (Stumpf, Green & Smith, 1946; Fuchtbauer & Theismann, 1949) and we found that suitable treatment with an ultrasonic probe disaggregated bacterial clumps into groups of one, two and four individual cells. After disaggregation, the highest percentage of tetrads recorded was 15 but more often the value was 6–7% of the total bacterial objects. Pairs of cells were in excess of singles in the ratio approx. 2:1. Consequently, assuming a random distribution of viable cells among these objects, the viable count arrived at by the slide-culture technique employed would be a maximum value. For the distributions given, and with 50% viability of individual cells, we estimate that our technique could give a maximum value of about 70% viability; for 25% viability it could be as high as approx. 31%. As a routine, approx. 6ml. of a suspension containing 0·5–1·0mg. bacterial dry wt./ml. of distilled water was placed in a thin-walled glass vial, surrounded by ice and treated with an MSE ultrasonic probe for two periods of 1min., separated by a cooling interval of 1min. The probe was operated at 18–20kcyc./sec. and was used at submaximal current output (0·5–1·0A), the degree of turbulence in the suspension being adjusted to a point short of maximal, as gauged by ear and eye.

Plate counts were made by serially diluting suspensions treated ultrasonically in sterile phosphate buffer (0·067M) to give a final bacterial concentration approx. 2×10^3–3×10^3 viable units/ml. Portions (0·1ml.) of the diluted suspensions were spread on six replicate plates of Oxoid Nutrient Agar and were incubated at 37° for 48hr. Standard deviations were determined. Colony counts were related to the dry weight of bacteria per unit volume of suspension in the starvation flask.

Both Difco and Oxoid Nutrient Agar were used to prepare slide cultures and gave results which were in good agreement. However, Difco medium was preferred as it contained, on average, less than one dead coccus per field of view, as compared with three or four in the Oxoid product. As Oxoid agar did not readily pass through bacterial filters, results were corrected for its content of dead bacteria; Difco medium was always filtered before use. With the Difco product, counts were performed on suspensions diluted to contain about 40 bacterial objects per field of view, as recommended by Postgate *et al.* (1961). Cells were inoculated on to Oxoid agar as a somewhat denser suspension (70–80 bacterial objects per field) to reduce the relative proportion of extraneous bacteria initially present in the medium. Significant overcrowding of microcolonies did not result. Microcolonies and dead bacterial objects were counted under phase-contrast illumination with a 4mm. dry objective and viabilities were expressed as the percentage of viable units in the population. Dead cells appeared dull and unrefractile under the microscope and they frequently occurred in pairs which were counted as one dead unit. Cultures of dying populations also contained a small proportion of tetrads which were not readily classified as viable or dead on the basis of refractility. These may have been the product of one or two cell divisions or, alternatively, were packets of dead cells which had not been dispersed by ultrasonic agitation. Such objects were arbitrarily classified as viable. They contributed 2–6% to the percentage viabilities of populations which gave rise to few or no microcolonies; their contribution was less at higher viabilities and was negligible at values of 85% and over. Slide cultures of freshly harvested cells were incubated for 8–9hr. at 37° but starved cells required progressively longer incubation periods of up to 12hr. with the Oxoid medium or 14hr. with the Difco medium. When cultures containing few microcolonies were incubated for up to 24hr., undivided cells did not replicate themselves.

Similar survival curves were obtained when determined simultaneously by slide culture and by plate counts (Fig. 2) but, for our purposes, slide culture was more suitable since the greater scatter of plate counts tended to mask the relatively slow loss of viability of peptone-grown suspensions during the first 40hr. of starvation. When viability fell below 75%, slide cultures were less accurate.

The brief period of ultrasonic treatment used to disperse cell suspensions appeared to have little adverse effect on viability. Suspensions of freshly harvested, peptone-grown bacteria were agitated for periods of 2, 5 and 10min. and were respectively 97·6, 96·5 and 95·2% viable, as measured by slide culture. Peptone-grown cells which had been starved for 44hr. and had started to die, were treated ultrasonically for periods of 2, 3 and 5min. and were found to be respectively 81, 83 and 82% viable. There was thus no progressive loss in viability which would have indicated that starved cells are less resistant to ultrasonic stress or that they are adversely affected by suspension in cold, distilled water during the ultrasonic treatment. Undispersed aggregates of cells starved for 90hr., and which were moribund after ultrasonic treatment, did not visibly increase in size when incubated on slide-culture medium for 24hr. Cell pellets were also found to be 94% viable after being stored on ice for 3 days, and in one experiment cells were stored as a pellet on ice overnight before starvation; their subsequent survival was not significantly affected.

Amino acids and ammonia. Free amino acid pools were obtained by immersing bacterial suspensions for 10min. in a boiling-water bath and removing cell debris by centrifugation. The ninhydrin method of Yemm & Cocking (1955) was used to determine the total amount of amino acid in the pools and in cell-free supernatants. Glycine standards were employed. Standards of $(NH_4)_2SO_4$ were also set up to correct for the presence of NH_3 which was estimated separately by nesslerization (Paul, 1958).

Carbohydrate. Total cell hexose was determined as glucose by the anthrone procedure of Trevelyan & Harrison (1952) as adapted for *S. lutea* by Binnie *et al.* (1960). Reducing sugar was determined by the method of Nelson (1944) and free and purine-bound ribose in cell suspensions and cell-free supernatants was estimated by the orcinol method with ribose standards (Mejbaum, 1939). Free glucose gave extinctions with the orcinol reagent which were approx. 2% of those obtained with ribose; intra-

cellular anthrone-reactive glucose was assumed to react to the same extent and results of orcinol determinations were corrected for polysaccharide interference after reference to an appropriate calibration curve. Ribose reacts with the anthrone reagent but under the conditions used it does not give significant interfering colour.

Nucleic acids. Bacterial RNA was extracted as ribonucleotide by the method of Fleck & Munro (1962). Cells were incubated with 0·3 N-KOH for 60 min. at 37° and then chilled in ice, when ice-cold perchloric acid was added to final concentration 0·5 N. After centrifuging, pellets were washed twice with ice-cold perchloric acid (0·5 N); washings were combined with the alkali extract and made to 25 ml. Virtually no more nucleotide was extracted by further treatment with alkali but hydrolysis of alkali-extracted residues with N-perchloric acid at 70° for 20 min. (Paul, 1956) yielded a further quantity of nucleotide equivalent to about 2% of the cell dry weight and this was taken as a measure of DNA. Nucleotide which was soluble in cold acid was obtained by twice extracting cells with ice-cold 0·7 N-perchloric acid for 10 min.; the combined extracts were made to 15 ml.

All centrifuging was carried out at 0° with the superspeed attachment of an MSE Major refrigerated centrifuge for 1 min. at 14000 rev./min. (27400*g*). Extinctions and u.v. spectra were determined with a Cary, Beckmann DB or Unicam SP.800 recording spectrophotometer and were related to standards of hydrolysed yeast RNA and DNA.

Protein. Cell protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after suspensions (1 mg. dry wt./ml.) had been boiled in 2·5 N-NaOH for 3 min. The biuret procedure of Stickland (1951) was also employed; in this case, cell suspensions (2·5 mg./ml.) were boiled in alkali for 8 min. Bovine plasma albumin, fraction V (Armour Pharmaceutical Co., Eastbourne, Sussex) was used as a standard.

Manometric methods

Conventional manometric methods were used and the uptake of O_2 of cell suspensions was measured at 37° in an atmosphere of air with a shaking rate of 100 oscillations/min. Results are expressed as Q_{O_2} values (μl. of O_2 consumed/hr./mg. of bacterial dry wt.). Flask contents were: 1 ml. of cell suspension (5–15 mg./ml.), 1·8 ml. of phosphate buffer (0·067 M, pH 7·1) and, in the centre well, 0·2 ml. of 20% (w/v) KOH. Substrates were dissolved in 0·5 ml. of buffer, whose volume in the main compartment was reduced accordingly.

Paper chromatography

Two-dimensional paper chromatography was performed on cell-free supernatants with Whatman no. 1 paper. The supernatants were concentrated eightfold by evaporating to dryness under vacuum and dissolving the residue in 0·5 ml. of distilled water. Buffer salts did not interfere on the chromatograms. Solvents were (i) descending: propan-2-ol–HCl (sp.gr. 1·19)–water (4:1:1, by vol.); (ii) ascending: butan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (17:1:2, by vol.). Ultraviolet-absorbing spots were eluted in 0·1 N-HCl and were identified by their absorption spectra and R_F values.

RESULTS

Cells grown in peptone medium

Effect of phase of growth on endogenous metabolism. Measurements of endogenous respiration on cells harvested at different stages of the growth cycle revealed a progressive and marked decline in the rate of oxygen consumption as growth progressed. From a value of approx. 30 in mid-exponential growth (after 21 hr.), the endogenous Q_{O_2} fell to 15·9 by the onset of the stationary phase (34 hr.) and thereafter continued to decrease. After 60 hr., Q_{O_2} was 6·3. Endogenous respiration could be correlated with the intracellular content of free amino acids (Fig. 1). In the early stages of growth, cells also contained about 20% more anthrone-reactive carbohydrate, 8% more protein and about 30% more orcinol-reactive pentose and ribonucleotide than those in stationary phase (Fig. 1). The pH of the growth medium did not change.

In a separate experiment, cells freshly harvested after 32·0, 49·5 and 54·0 hr. of growth were found, by slide culture, to be respectively 95, 97 and 94% viable.

Aerobic starvation of washed suspensions of S. lutea. As observed by Dawes & Holms (1958*a*), the endogenous respiration of *S. lutea* declined rapidly during the first 5 hr. of starvation. By 24 hr., Q_{O_2} values had fallen to approximately unity and oxygen continued to be consumed at rates which were barely measurable when starvation was extended up to 72 hr. (Fig. 2 and Table 4). The rate at which starved cells respired was not a measure of their ability to grow and multiply, which was virtually unimpaired after 24 hr.; by 40 hr., between 5 and 10% of the population were dead and the bacteria then died more rapidly at a rate of approx. 1·5% of the initial viable population/hr.

The survival curves of cells starved at densities of 10·2 and 1·0 mg. dry wt./ml. were not markedly different, though onset of death appeared to occur slightly earlier in the more dilute suspension (Table 1). Estimates of viability made by slide culture and by plate counts also agreed well (Fig. 2). The bacteria used in this experiment were harvested 7–8 hr. after reaching stationary phase and had a relatively low Q_{O_2} 11·5. The rate of death during the phase of most active decline was less than usually observed and the population was approx. 50% viable after 75 hr., which compares with values of 10–25% obtained in other experiments when cells were harvested either just before or 2 hr. after the onset of the stationary phase.

Free amino acid pool and ammonia. Chemical analyses confirmed the findings of Dawes & Holms (1958*a*) that free amino acids are collectively the

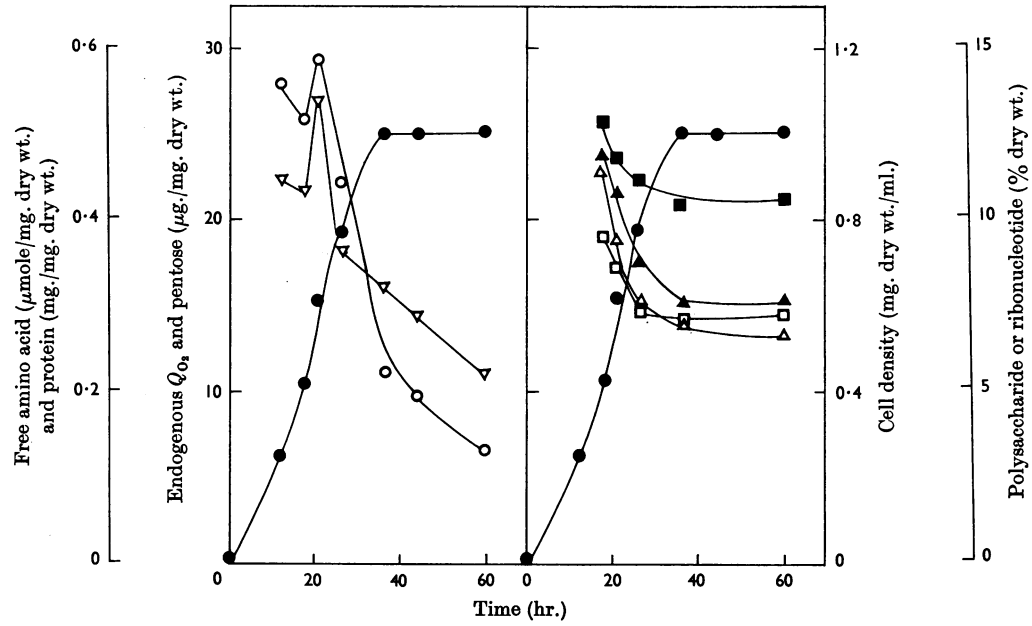

Fig. 1. Effect of growth phase on endogenous Q_{O_2} and amounts of cellular constituents of *Sarcina lutea* in peptone medium. The organism was grown in 6l. of medium with forced aeration (5l. of air/min.). At intervals, cells were harvested, washed in phosphate buffer (0·067M-KH_2PO_4; pH 7·1) followed by distilled water and re-suspended to a suitable volume in phosphate buffer (0·067M; pH 7·1) for chemical analysis and for measurements of endogenous Q_{O_2}. Results were related to the dry wt. of bacteria/ml. of suspension; cell density was determined by turbidity. For clarity of presentation two plots are shown with the growth curve on each to facilitate comparison. Endogenous Q_{O_2}, ○; cell density, ●; polysaccharide, □; protein, ■; pentose, △; ribonucleotide, ▲; free amino acid pool, ▽.

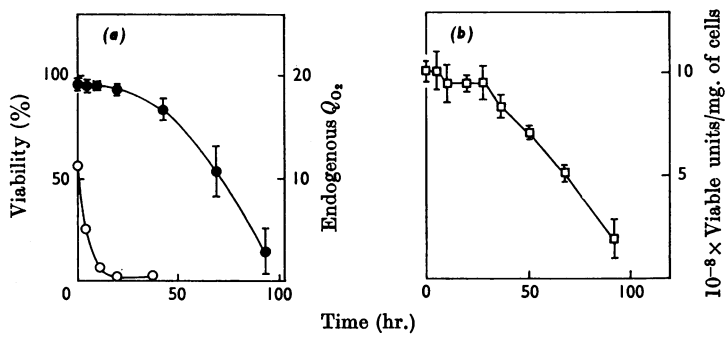

Fig. 2. Starvation of peptone-grown *Sarcina lutea* with forced aeration; effect on endogenous Q_{O_2} and viability as determined by (*a*) slide culture and (*b*) plate counts. The organism was harvested after 40hr.; washed cells were suspended to a density 10·8mg./ml. in 500ml. of phosphate buffer (0·067M-KH_2PO_4; pH 6·8) and aerated at 37° with 5l. of air/min. Endogenous Q_{O_2}, ○; slide-culture viability, ●; plate-count viability, □. Vertical bars represent standard deviations.

major endogenous substrates in the early stages of starvation and that a decrease in the intracellular free amino acid pool can be correlated with the decline in endogenous respiration. Degradation of amino acid was virtually complete after starvation for 10hr. Small amounts of amino acids appeared in the supernatant and these were previously shown by chromatography to consist mainly of

Table 1. *Effect of cell density during starvation on the viability of peptone-grown* Sarcina lutea

The organism was harvested after 36hr. of growth in 500ml. of medium; washed cells were suspended to densities 10·2 and 1·0mg./ml. in 10ml. of phosphate buffer (0·067 M-KH_2PO_4; pH 6·8) and shaken at 37° in stoppered conical flasks. The shaking rate was 110 oscillations/min. Samples were taken periodically for slide-culture determinations on Difco Nutrient Agar.

Period of starvation (hr.)	Viability (%)	
	10mg./ml.	1mg./ml.
0	98·1	98·1
5·5	98·4	98·0
24·0	97·7	95·4
30·5	94·3	92·3
50·5	85·0	70·7
73·0	24·4	15·9

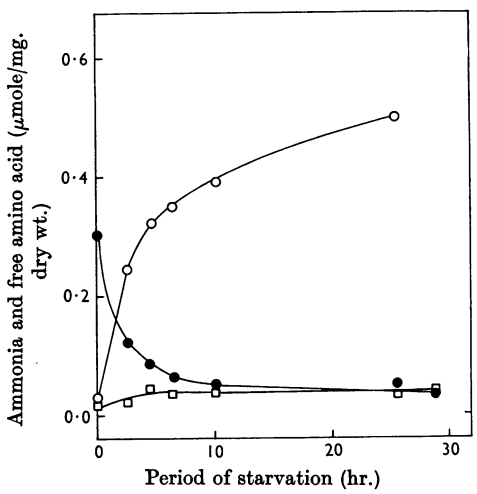

Fig. 3. Release of ammonia and behaviour of the free amino acid pool during starvation of peptone-grown *Sarcina lutea*. The organism was harvested from peptone medium after 24hr., washed cells were suspended to a density 11·6mg./ml. in 300ml. of phosphate buffer (0·067 M-KH_2PO_4; pH 6·8) and aerated at 37° with 3·5l. of air/min. Ammonia in supernatant, ○; intracellular free amino acid, ●; amino acid in supernatant, □.

aspartate, valine and glycine (Dawes & Holms, 1958*a*). Ammonia was released from the cells during starvation (Fig. 3) but the pH of the supernatant did not change and the intracellular concentration of ammonia remained constant at a low value of approx. 0·02 μmole/mg. dry bacterial wt.

Nucleic acid degradation. Since ammonia was liberated in amounts which exceeded those theoretically expected from the deamination of α-amino

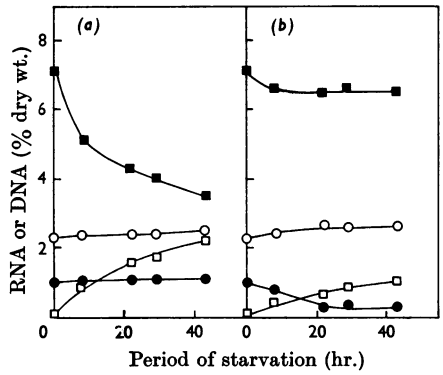

Fig. 4. Comparison of nucleic acid metabolism during starvation of peptone-grown *Sarcina lutea* in the presence and absence of Mg^{2+}. The organism was harvested after 24hr. of growth in 6l. of medium; washed cells were suspended to a density 8·8mg./ml. of (*a*) phosphate buffer (0·067 M-KH_2PO_4; pH 6·8) and (*b*) phosphate buffer (0·067 M-KH_2PO_4; pH 6·8) plus $MgSO_4$ (0·01 M). Suspensions were shaken at 37° in 1l. conical flasks at approx. 250 oscillations/min. RNA, ■; DNA, ○; intracellular nucleotide, ●; nucleotide in supernatant, □.

acids, another nitrogenous substrate was sought. This proved to be RNA, which declined from 7·1% to 3·4% of the cell dry weight during 43hr. of starvation (Fig. 4). Material showing an absorption maximum at 255mμ accumulated in the supernatant and, after 43hr., amounted to 2·2% of the cell dry wt. DNA and the acid-soluble pool remained constant at respectively 2·4% and 1·0% of the dry weight. There was a net loss of ultraviolet-absorbing nucleotide from the system; the degraded material was presumably oxidized and corresponded to 1·4% of the cell dry weight when related to RNA standards. Starvation resulted in cellular orcinol-reactive pentose being reduced from 1·5% to 1·0% of the dry weight in 43hr. and, when expressed as RNA, the corresponding values were 8·4% and 5·9%. The amounts of pentose (as ribose) which were respectively degraded and released from the cells were 0·35% and 0·15% of the dry weight.

When a portion of the same bacterial suspension was starved in phosphate buffer (0·067 M-potassium dihydrogen phosphate; pH 6·8) containing 0·01 M-magnesium sulphate, catabolism of RNA was almost completely suppressed and although a small proportion of the RNA appeared to be degraded in the first 8hr., no subsequent loss was observed. The amount of ultraviolet-absorbing material which appeared in the supernatant was only half of that released by cells starved in Mg^{2+}-free buffer and consisted of components of the acid-soluble pool which had been released from the cells during

Table 2. *Comparison of viability and endogenous Q_{O_2} of* Sarcina lutea *harvested from peptone and starved in the presence and absence of* 0·01 M-*magnesium sulphate*

The organism was harvested after 24hr. of growth in 6l. of medium; washed cells were suspended to a density 8·8mg./ml. in 100ml. of (a) phosphate buffer (0·067 M-KH_2PO_4; pH6·8) and (b) phosphate buffer (0·067 M-KH_2PO_4; pH6·8) plus $MgSO_4$ (0·01 M). The suspensions were shaken at 37° in 1l. conical flasks at approx. 250 oscillations/min.

Period of starvation (hr.)	Mg^{2+} present		Mg^{2+} absent	
	Endogenous Q_{O_2}	Viability (%)	Endogenous Q_{O_2}	Viability (%)
0·0	21·1	96·8	21·1	96·8
8·0	1·5	94·5	1·6	97·9
22·0	1·3	94·8	0·7	
29·0	0·7	95·7		
34·0				89·5
43·0	0·3	84·9		85·6
55·0		55·9		61·5
71·0		24·7		12·6

Table 3. *Effect of starvation on the protein, ribonucleotide and anthrone-reactive carbohydrate of peptone-grown* Sarcina lutea

The suspension of Expt. 1 was prepared, starved and sampled as described in the legend to Fig. 2. In Expt. 2, protein and ribonucleotide were estimated on samples (5ml.) periodically withdrawn from a suspension of cells (40ml.; 12·7mg./ml. of phosphate buffer, pH6·8), which were harvested from 500ml. of medium after 34hr. and starved at 37° in a 500ml. conical flask shaken at 400 oscillations/min. In this experiment, starved bacteria were not separated from their supernatant and results relate to whole-cell suspensions. Ribonucleotide includes acid-soluble nucleotide.

Period of starvation (hr.)	Cellular constituents (% of dry wt.)			
	Expt. 1		Expt. 2	
	Biuret-reactive protein	Total ribonucleotide	Carbohydrate	pH of supernatant
0·0	40·2	9·7	7·0	6·8
4·5			7·8	6·8
10·0	40·5	9·0		
24·0	41·8	8·7		
28·5			7·9	6·7
34·0	40·3	8·6		
42·0			7·7	6·8
49·5	39·8	7·7		
51·0			7·8	6·8
57·0	40·5	7·4		
68·0			8·1	6·8
75·0	39·5	6·9		
92·0			7·7	6·8

starvation. DNA was again stable and, if anything, increased slightly. The survival curves of cells starved in the presence and absence of Mg^{2+} did not significantly differ and patterns of endogenous respiration were also similar (Table 2).

Paper chromatography revealed that the ultra-violet-absorbing material released by starved cells was of low molecular weight. Four components were resolved of which the most intense had the absorption characteristics of guanosine and reacted strongly with orcinol. The remaining spots reacted with this reagent to different extents and probably were adenosine, uridine and cytidine, mixed with unknown amounts of the corresponding free bases. The latter are not adequately resolved from their nucleosides in this system and absorption spectra did not permit clear differentiation to be made. The presence of such free bases would account for the observed discrepancy between RNA contents calculated from u.v. measurements and pentose

analysis. Chromatograms revealed only trace amounts of materials in positions corresponding to phosphorylated derivatives.

Protein and carbohydrate. Significant degradation of cell protein during starvation could not be detected by the two methods used. Anthrone-reactive polysaccharide was also stable (Table 3).

Oxidation of substrates. Though the initial decrease in endogenous Q_{O_2} appeared to be due to the depletion of amino acid substrates, it was of interest to determine the effect of starvation on the cells' ability to oxidize amino acids. Glutamate was selected as it was quantitatively the major endogenous substrate in the early stages of starvation.

In one experiment, cells were starved at a density 5·2 mg. dry wt./ml. in Warburg flasks of 25 ml. capacity. At intervals, flasks were removed from the bath, alkali was added to the centre well and, after re-equilibration, glutamate was added to a final concentration of 0·018 M. Oxygen uptake was measured for 40 min. and the viability of the bacteria was then estimated by slide culture (Table 4).

It was obvious that the capacity of the cells to oxidize glutamate remained high for up to 46 hr. and, indeed, correlated well with cell survival (Table 4). The Q_{O_2} values for glutamate oxidation, corrected for endogenous Q_{O_2}, fell from approx. 80 to 70 after 46 hr. and then declined more sharply in the next 26 hr. to a value of 18. After 46 hr., viability decreased from 96·5% to 88% and, thereafter, at a faster rate so that 92% of the population was dead after 72 hr.

In a further experiment, cells were starved in a 500 ml. conical flask and duplicate samples (0·5 ml.) were periodically transferred to Warburg flasks for measurements of oxygen uptake in the presence of L-glutamate. In this case, the quantity of substrate was reduced to 5 μmoles and the final cell density in the flasks was 1·3 mg./ml. The rate of glutamate oxidation declined from Q_{O_2} approx. 60 to one of 20 in the first 16 hr. of starvation; it then fell more slowly to a value of 9·6 after 70 hr. (Table 5). Glucose and succinate were also tested as substrates; the rate at which succinate was oxidized declined sharply in the initial stages of starvation but Q_{O_2} values of cells metabolizing glucose remained high for 42 hr. and then diminished in parallel with viability (Table 5). Also shown in Table 5 is the effect of anaerobic starvation on the organism's ability to oxidize its endogenous reserves when the gas phase is changed to air. The Q_{O_2} again remained high for 42 hr. and only fell when the cells died. Indeed, up to 22 hr. Q_{O_2} values increased from 25 to 37, a general observation confirmed in three such anaerobic starvation experiments. Under these conditions, the bacteria appeared to survive slightly less well than when starved in air. Decreased survival was more marked in another experiment when only 45% of an anaerobically starved suspension was viable after 45 hr.; in this case, a portion of the suspension was not starved in air for comparison.

Cells grown in peptone–glucose medium

Effect of phase of growth on endogenous metabolism. Added glucose (2%, w/v) did not markedly increase the rate of growth of cultures (mean generation time 6 hr.) although growth continued well after cells in unsupplemented peptone medium had reached stationary phase. In the experiment of Fig. 5 the endogenous respiration of freshly harvested cells declined relatively slowly as growth progressed; after 13 hr., the endogenous Q_{O_2} was 22·0 and it ultimately fell to a value of 15·0 after 87 hr. The carbohydrate content remained high at

Table 4. *Effect of starvation on ability of peptone-grown* Sarcina lutea *to oxidize* L-*glutamate*

Cells were harvested from 500 ml. of medium after 24 hr. of growth. Washed suspensions (2·3 ml.) were starved in phosphate buffer (0·067 M-KH_2PO_4; pH 6·8) at a cell density 5·2 mg./ml. in Warburg flasks of 25 ml. capacity. Taps and side-arms were kept open. At intervals 20% (w/v) KOH was added to the centre well and 0·5 ml. of glutamate was tipped into the main compartment to give final glutamate concentration 0·018 M. After measurement of oxygen uptake for 60–75 min. a portion of the flask contents was removed for slide-culture viability determinations on Difco agar. Two starvation flasks were used for periodically determining endogenous respiration. Alkali was removed from the centre well between Q_{O_2} determinations. Q_{O_2} values are based on maximum linear rates.

Time (hr.)	Q_{O_2} (glutamate)	Q_{O_2} (endogenous)	Viability (%)	
			Difco agar	Oxoid agar
0·0	80·2	21·5	96·5	95·0
8·0	80·2	2·2	96·0	95·7
20·0	74·5	0·6	92·5	88·5
46·0	69·0	0·3	88·0	93·2
72·0	18·1	0·7	7·5	8·0

Table 5. *Effect of aerobic starvation on viability and oxidation of glucose, succinate and* L-*glutamate by peptone-grown* Sarcina lutea *and of anaerobic starvation on viability and oxidation of endogenous reserves*

The organism was harvested after 24hr. growth in 1l. of peptone medium; washed cells were suspended to a density 7·5mg./ml. in phosphate buffer (0·067M-KH_2PO_4; pH6·8). A portion (25ml.) of the suspension was shaken at 250 oscillations/min. in a 500ml. stoppered conical flask and further portions (2ml.) were starved under nitrogen in Warburg flasks (25ml. capacity) shaken at 100 oscillations/min. At intervals samples (0·5ml.) of aerobically starved suspensions were removed for slide-culture determinations of viability and duplicate portions (0·5ml.) were used for Q_{O_2} estimations in the presence of exogenous substrates. Warburg flasks contained 1·8ml. of phosphate buffer (0·067M-KH_2PO_4; pH6·8) and 0·5ml. of glucose (10μmoles), succinate (10μmoles) or L-glutamate (5μmoles) plus, in the centre well, 0·2ml. of 20% (w/v) KOH. The effect of aerobic starvation on endogenous respiration was determined by starving cells in two Warburg flasks which contained 1ml. of suspension (7·5mg./ml.) and 1·8ml. of buffer. Alkali was periodically added to the centre wells and the rate of oxygen consumption measured. The endogenous respiration of anaerobically starved cells (2ml. of suspension/vessel) was similarly determined after changing the gas phase of duplicate flasks to air and adding 0·8ml. of buffer. Oxygen uptake was measured for 40–80min. and a sample (0·5ml.) of suspension was then removed for estimation of viability by slide culture. Difco medium was used. Q_{O_2} values are based on maximum linear rates.

Period of starvation (hr.)	Respiration rate (Q_{O_2})					Viability (%)	
	Endogenous		Aerobically starved cells				
			Glucose	Glutamate (endogenous subtracted)	Succinate		
	Air	N_2				Air	N_2
0·0	24·7	24·7	26·3	59·9	73·2	95·5	
14·0		31·6					94·6
16·5	4·2		28·5	20·0	9·4	95·9	
22·0	1·3	36·8					92·5
42·0	0·9	28·3	29·4	16·7	4·9	78·0	67·0
68·0		3·0					3·0
70·0	0·9		4·8	9·6	4·1	13·0	
86·0	0·5	1·8					

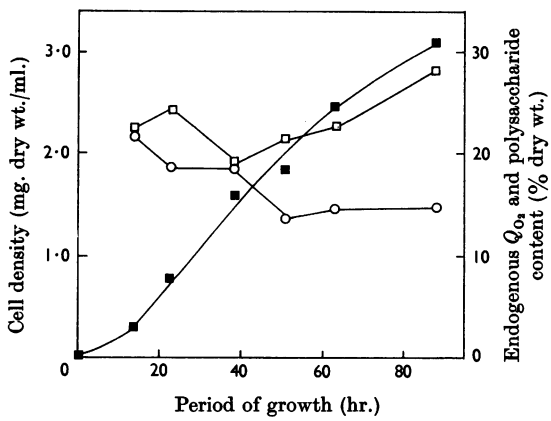

Fig. 5. Effect on endogenous Q_{O_2} and intracellular polysaccharide during growth of *Sarcina lutea* on peptone+2% glucose. The organism was grown in 6l. of medium with forced aeration. At intervals, cells were harvested, washed in phosphate buffer (0·067M; pH7·1) followed by distilled water and re-suspended to a suitable volume in phosphate buffer (0·067M; pH7·1) for chemical analysis and for measurements of endogenous Q_{O_2}. Results were related to the dry wt. of bacteria/ml. of suspension; cell density was determined by turbidity. Endogenous Q_{O_2}, ○; bacterial density, ■; carbohydrate content, □.

values 22–28% of the dry weight and over the same period the free amino acid pool decreased from 0·21 to 0·17μmole/mg. dry wt.

The cell density in peptone–glucose medium was about 3mg. bacterial dry wt./ml. after 80hr.; that of stationary-phase peptone cultures was approx. 1mg./ml.

Starvation of washed suspensions. S. lutea harvested from peptone medium contains anthrone-reactive carbohydrate equivalent to 8–10% of the bacterial dry weight. When 2% (w/v) glucose is added to the medium, the total amount of bacterial carbohydrate is approximately doubled. This excess of polysaccharide is degraded in starvation and, by 22hr., only the basal quantity characteristic of peptone-grown cells remained (Table 7). Keto acids were not detected as accumulated products of polysaccharide degradation. Boiling water extracted virtually all of the utilizable polysaccharide from the cells but removed very little free reducing sugar (Table 6). During starvation, polysaccharide, free amino acids and RNA were degraded simultaneously, ammonia and ultraviolet-absorbing material were released from the cells and the pH of the supernatant remained essentially unchanged (Table 7). The presence of the reserve

Table 6. *Comparison of the extent of utilization of carbohydrate fractions of* Sarcina lutea *grown on glucose–peptone medium*

The organism was harvested after 24hr., washed cells were made to 250ml. in a round-bottomed flask (500ml. capacity; Quickfit and Quartz) and aerated with 2·5l. of water-saturated air/min. Cell density was 11·0mg./ml.

Aeration period (min.)	Total carbohydrate (% dry wt.)	Carbohydrate released by boiling water (% dry wt.)	Carbohydrate extracted from remainder by 75% ethanol (% dry wt.)	Residual carbohydrate (% dry wt.)	Total free reducing sugar (μg./mg. dry wt.)
0	15·8	7·5	0·6	7·8	4·1
45	13·9	5·7	0·6	7·8	3·3
90	13·4	5·2	0·6	7·7	3·0
180	11·5	3·2	0·4	7·9	2·4
270	11·1	1·5	0·5	9·1	0·7

Table 7. *Starvation of* Sarcina lutea *grown on peptone with* 2% *glucose: effect of starvation on cellular constituents, endogenous* Q_{O_2}, *viability and* pH *of supernatant of peptone–glucose-grown* Sarcina lutea

The organism was harvested after 24hr.; washed cells were suspended to a density 14·2mg./ml. in 70ml. of phosphate buffer (0·067M-KH_2PO_4; pH6·8) and starved at 37° in a 1l. conical flask shaken at approx. 200 oscillations/min.

Period of starvation (hr.)	Viability (%)	Endogenous Q_{O_2} (μl. of O_2/hr./mg.)	Polysaccharide (% dry wt.)	RNA (% dry wt.)	Free amino acid (μmole/mg.)	NH_3 in supernatant (μmoles/mg.)	pH of supernatant
0·0	95·1	28·6	17·6	7·20	0·49	0·01	6·92
11·8		6·8	11·7	4·85	0·11	0·58	6·95
22·3	36·5	2·5	9·1	3·36	0·10	0·83	6·95
31·0		1·2	8·8	2·06	0·08	0·76	6·97
49·0		0·9	8·4			1·12	7·10
50·0	8·0						

polysaccharide did not materially affect the rate at which RNA was initially degraded but, beyond 10hr., bacteria from glucose–peptone medium metabolized a disproportionately greater amount of their RNA, which, after 31hr., represented less than 2% of the cell dry weight (Fig. 6).

The endogenous Q_{O_2} of cells from glucose–peptone medium declined in the initial stages of starvation (Fig. 7) but respiration continued at a measurable rate for longer periods than in suspensions devoid of oxidizable polysaccharide; after 5hr., the Q_{O_2} of glucose–peptone cells was approx. 7. Values for peptone-grown suspensions harvested in stationary phase and starved for the same period varied between 1 and 2. Of the latter cells, relatively few died in the first 40hr. of starvation; however, bacteria rich in polysaccharide started to die immediately they were starved and at rates which varied between 2% and 9% of the population/hr. (Fig. 7). The rate of decline was most rapid in suspensions with access to a relatively limited supply of oxygen but the survival of peptone-grown suspensions under these conditions was unaffected (Table 8).

DISCUSSION

Quantitative survival curves are available for relatively few species of bacteria, but it appears that if these organisms are held in environments containing negligible quantities of nutrient, their capacity to grow and multiply is ultimately and invariably lost and they may be considered to have died. Starving cells are subject to a variety of stresses, some of which may be physicochemical in nature. A major question is the relationship between survival and a cell's endogenous metabolism, if this continues during starvation. Such metabolism is essentially catabolic and it must ultimately be detrimental to viability. One specific effect might be that the tensile strength and selective permeability of a bacterium's outer layers are reduced to a point where lysis takes place in the starvation medium. This does not occur in *Aerobacter aerogenes* (Postgate *et al.* 1961) and indeed, the osmotic barrier continues to function in cells which fail to replicate themselves (Postgate & Hunter, 1962). We have found that death of starved *S. lutea* occurs as a failure of morphologically

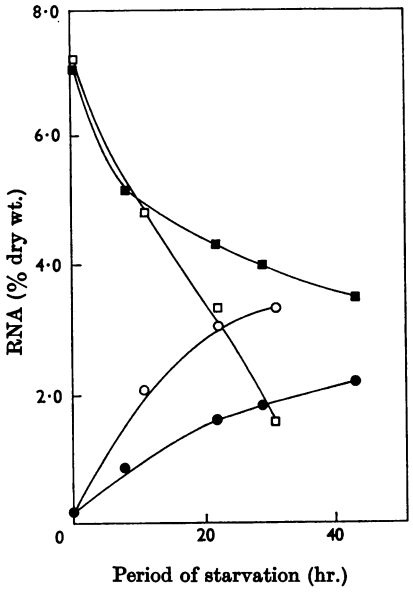

Fig. 6. Comparison of RNA degradation and nucleotide release by *Sarcina lutea* grown on (a) peptone and (b) peptone–glucose media. (a) Cells from peptone medium were harvested after 24hr. of growth in 6l. of medium; washed bacteria were suspended to a density 8·8mg./ml. in 100ml. of phosphate buffer (0·067M-KH_2PO_4; pH6·8) and shaken at 37° in a conical flask (1l.) at approx. 250 oscillations/min. Intracellular RNA, ■; nucleotide in supernatant, ●. (b) Cells from peptone+2% glucose were harvested after 24hr. of growth in 6l. of medium; washed bacteria were suspended to a density 14·2mg./ml. in 70ml. of phosphate buffer (0·067M-KH_2PO_4; pH6·8) and starved at 37° in a conical flask (1l.) shaken at approx. 200 oscillations/min. Intracellular RNA, □; nucleotide in supernatant, ○.

recognizable cells to multiply and that no visible signs of lysis can be detected microscopically even when starved cells have been treated ultrasonically.

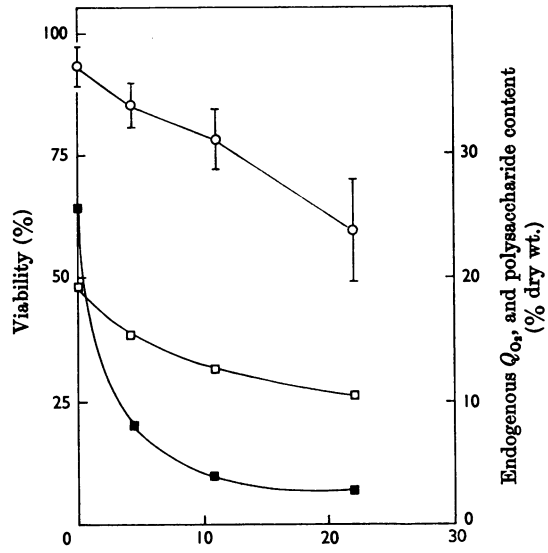

Fig. 7. Effect of starvation on viability, endogenous Q_{O_2} and cell carbohydrate of *Sarcina lutea* grown on peptone +2% glucose. The organism was harvested after 36hr.; washed cells were suspended to a density 11·0mg./ml. in 80ml. of phosphate buffer (0·067M-KH_2PO_4; pH7·1) and starved at 37° in a conical flask (1l.) shaken at approx. 300 oscillations/min. Endogenous Q_{O_2}, ■; carbohydrate content, □; viability, ○.

Table 8. *Comparison of effect of starvation on viabilities of peptone- and peptone–glucose-grown* Sarcina lutea

Expt. 1. Cells harvested after 34hr. from 500ml. of peptone medium, and from a similar quantity of peptone +2% (w/v) glucose, were made to 40ml. in phosphate buffer (0·067M-KH_2PO_4; pH6·8) and starved at respective densities 9·8 and 10·8mg./ml. in stoppered 500ml. conical flasks, sealed with Parafilm. Expt. 2. Cells grown and harvested as under (1) were made to 10ml. in phosphate buffer (0·067M-KH_2PO_4; pH6·8) and starved at respective densities 12·1 and 17·3mg./ml. in 50ml. conical flasks. Viabilities were estimated in Difco agar. In parentheses are recorded μl. of O_2/mg. bacterial dry wt. initially present in the starvation flask.

Expt. 1			Expt. 2		
	Viability (%)			Viability (%)	
Period of starvation (hr.)	Peptone (233·0)	Glucose–peptone (210·0)	Period of starvation (hr.)	Peptone (66·0)	Glucose–peptone (47·0)
0	97·4	92·9	0	95·5	98·0
8·0	96·3	62·3	9·0	97·3	18·1
23·0	94·4	38·8	26·0	92·9	11·7
29·0	93·7	20·7			

We have not been able to correlate the survival of suspensions of peptone-grown *S. lutea* with the degradation or release from the cell of any single species of constituent. Protein, polysaccharide and DNA are stable throughout starvation and the only endogenous substrates which have been detected are intracellular free amino acids, of which glutamate predominates, and RNA. Species which degrade protein include *Escherichia coli* (Dawes & Ribbons, 1965), *A. aerogenes* (Strange, Dark & Ness, 1961), and *Corynebacteria* and *Staphylococcus epidermidis* (R. E. Strange, personal communication). Oxidation of free amino acids is largely responsible for the initial high rate of endogenous respiration but loss of these components, which is virtually complete after 10hr., does not immediately affect viability and most of the cells survive for a further 30hr. The period for which cells remain viable is neither proportional to their rate of endogenous respiration when freshly harvested nor to their initial internal concentration of free amino acids which therefore appear dispensable; indeed, some evidence suggests that the phase of greatest decline in viability is most marked in bacteria with a relatively high initial rate of respiration.

The RNA which is degraded is also dispensable. Approximately 25% of cellular RNA is metabolized in the first 24hr., when the number of dead bacteria does not increase significantly. This behaviour is consistent with that of other starved micro-organisms including *E. coli* (Dawes & Ribbons, 1965), *A. aerogenes* (Strange *et al.* 1961), *Lactobacillus casei* (Holden, 1958) and *Euglena gracilis* (Blum & Buetow, 1963), which in conditions of partial or total starvation degrade a considerable proportion of their RNA without a corresponding loss in viability. Experiments on *Salmonella typhimurium* (Schaechter, Maaløe & Kjelgaard, 1958) and on *Bacillus megaterium* and *A. aerogenes* (Herbert, 1961) have shown that the amount of RNA per cell increases with rate of growth and multiplication; thus freshly harvested bacteria may frequently contain RNA in excess of the amount required to initiate a minimal, finite rate of growth on the recovery medium used to estimate viability. During starvation, the amount of RNA per cell may even decrease to less than this threshold value but this need not be lethal if mechanisms for resynthesizing the polymer from precursors in the recovery medium remain intact. Apart from the necessary enzymes, such mechanisms presumably include a portion of the cells' RNA which acts as a primer for polynucleotide synthesis, whence catabolism of RNA in isolation from other constituents will only be lethal when RNA molecules with a priming function are degraded.

This does not appear to be the cause of death in starved *S. lutea*. Magnesium sulphate suppresses RNA degradation in cell suspensions but does not prolong survival; this lack of protection differs from that recorded for some Gram-negative bacteria under conditions of stress (see e.g. Postgate & Hunter, 1964; Strange & Dark, 1965; Strange & Shon, 1964). Magnesium ions presumably enter the bacteria and prevent the initial disaggregation of ribosomes which precedes RNA catabolism and are known to have a similar effect in *E. coli* (see Mandelstam, 1963) and *Pseudomonas aeruginosa* (Gronlund & Campbell, 1965). Although isolated 70s ribosomes of *S. lutea* are degraded when dialysed in the absence of Mg^{2+}, addition of magnesium acetate (0·01 M) suppresses this process for 40hr. (Brown & Rosenberg, 1962). We have noted that a similar ribosome component is degraded in starving cell suspensions which were reconstituted from freeze-dried material, but these studies have not been extended to freshly harvested bacteria.

Any mechanism of death must be consistent with the fact that cells starved beyond 10hr. consume amounts of oxygen which are insufficient to oxidize more than very small quantities of cellular constituents. Although their rate of respiration is barely detectable, most of the bacteria survive for 40hr.; thereafter, the population declines more rapidly and after 75hr. few viable cells remain.

Between 20 and 45hr. approx. 15 μl. of oxygen is consumed/mg. bacterial dry wt.; about 2 μl. is required to oxidize the small quantities of pentose and ultraviolet-absorbing bases which are degraded in this period and which amount to respectively 1·5 and 0·4 μg./mg. cell dry wt. In the unlikely event of the remaining oxygen being used solely for the oxidation of amino acids derived from protein breakdown, these would be equivalent to about 1% of the dry weight, assuming, after Dawes & Holms (1958*a*), that 1 μmole of amino acid requires 3·5 μmoles for its complete oxidation and that the average molecular weight of the amino acids is 100. It was calculated that, in the first 22hr. of starvation, catabolism of pentose and bases derived from RNA breakdown accounted for less than 2% of the observed oxygen uptake. Measurements of respiration are thus compatible with the failure to detect degradation of macromolecular constituents other than RNA and with the unaltered respiration pattern in the presence of Mg^{2+} ions. The possibility that more than an insignificant degree of macromolecular re-synthesis occurs may also be excluded since the organism is a strict aerobe and has no fermentative abilities which would provide the necessary energy. Glucose is not metabolized anaerobically (Dawes & Holms, 1958*b*) and our studies of anaerobic starvation have shown that, when cells which have been starved under nitrogen for up to 45hr. are transferred to

aerobic conditions, the rate of endogenous respiration remains high so that the bacteria do not apparently ferment the relevant substrates, of which free amino acids presumably predominate. Beyond 40hr., and in the period when viability was declining most rapidly, cells starved under nitrogen also lost their capacity to consume oxygen under aerobic conditions; there was a similar decrease in the ability of aerobically starved suspensions to oxidize exogenous glucose and high concentrations of glutamate, a major component of the internal free amino acid pool.

Leaching of coenzymes such as NAD and NADP from the cell does not appear to be responsible for the diminished rates of oxidation since most nucleotide is released in the early stages of starvation and does not appear to include these compounds. Relatively unspecific inactivation of enzymes appears the most likely explanation and may be a major cause of death in starving *S. lutea*. A decline in the ability of aging haemoglobin to bind oxygen and chromium is one known case of the biological activity of a protein being lost solely as a result of changes in tertiary structure (Walter, 1963). Disaggregation of ribosomes and mutations involving relatively minor changes in the base substituents of DNA are other potentially lethal events which need not result from the catabolism of macromolecules to monomeric sub-units. From the behaviour of cells starved in the presence and absence of Mg^{2+} ions, loss of ribosomes is unlikely to be the critical event preceding death and, indeed, attempts to specify a single, sensitive component may be fruitless. Death is conceivably the result of non-specific changes of conformation in individual macromolecules, enzymes for example, enhanced by weakening of the forces which bind such subcellular entities into the final, organized metabolic unit. Since detectable catabolism was confined to dispensable constituents, lethal stresses may have been physicochemical resultants of the composition, ionic strength and osmotic pressure of the environment; however, though we did not detect degradation of protein, polysaccharide and, in the short term, lipid, cleavage of a few covalent bonds, not necessarily enzymically, within such molecules cannot be excluded and may have contributed to the ultimate weakening of cell structure and organization. Our results on the oxidation of exogenous glutamate suggest that permeability to substrates in the recovery medium is another potentially lethal factor and it may be that internal products of endogenous metabolism are also slightly toxic.

Survival curves of starved peptone-grown *S. lutea* are nearly rectangular in form. This is typical of populations becoming senescent (see Comfort, 1964). In *S. lutea*, what we postulate as causes of death essentially include the chance, non-enzymic disruptions of chemical bonding which Strehler (1962) conceives to be the mechanism of senescence in mammalian cells. Strehler considers that these so-called stochastic reactions are activated by random, local accumulations of thermal energy; as products form, heat is evolved which initiates further deleterious reactions.

We have not been concerned with calorimetry but such measurements have been applied to anaerobically starved *Streptococcus faecalis* whose behaviour bears some analogy to peptone-grown *S. lutea* starved both aerobically and anaerobically (Forrest & Walker, 1963; Walker & Forrest, 1964). Viability of *S. faecalis* was not determined. Ability to ferment glucose was taken as a measure of the integrity of cellular organization and displayed a time-curve which was roughly rectangular, activity declining after being stable for about 10hr. The starving bacteria released some amino acids and generated heat in an amount which exceeded that expected from cleavage of peptide bonds. However, these processes virtually ceased after 5hr. They were not observed when glycolysis was declining and if enzymes were being inactivated by processes involving a large heat output per molecule, including proteolysis and release of soluble end products, the number of molecules involved must have been very few or zero. More likely, a larger number of enzymes were being inactivated by changes in configuration associated with little increment in enthalpy and entropy.

We are aware that senescence in higher organisms involves adverse and probably interacting changes in many cell types and in the extracellular matrix. Nevertheless, starved bacteria may have a wider significance as models of auto-degradative, senescent processes and their effect on cell function, even though the index of function is an unspecialized capacity for replication. We feel that this possibility at least merits further exploration. To this end, Gram-positive cocci are probably better experimental material than Gram-negative rods since they are frequently more resistant to the immediate physical stresses involved in the preparation of washed suspensions.

Survival curves of starved bacteria are frequently complicated by growth and multiplication of a portion of the population at the expense of liberated endogenous metabolites and products of cell lysis. This seems unlikely in our populations of *S. lutea* since lysis was not observed microscopically; starved cells even resisted ultrasonic waves and from the organism's nutritional requirements the limited spectrum of amino acids released in the early stages of starvation is unlikely to support growth. The low respiration rate of starving suspensions and the lack of fermentative energy-

yielding mechanisms are consistent with this view. If significant re-growth occurs aerobically, a starved obligate aerobe should survive less well under nitrogen. Our results eliminate re-growth in the first 24hr., but they are not fully conclusive beyond this point.

Polysaccharide and poly-β-hydroxybutyrate frequently accumulate in bacteria which are grown in media containing excess of carbon. Of current interest is whether the presence of such polymers favours the survival of starving bacteria by, for example, contributing carbon and energy for the re-synthesis of cell components which are required for growth and multiplication in a renewed favourable environment (see Wilkinson, 1959; Dawes & Ribbons, 1962, 1964). *A. aerogenes* survives longer when cells are initially rich in glycogen (Strange *et al.* 1961) and poly-β-hydroxybutyrate similarly appears to protect starving populations of *Micrococcus holodenitrificans* (Sierra & Gibbons, 1962).

We have found that the presence of utilizable polysaccharide adversely affects the survival of starved *S. lutea* and that the reserve material differs from the common conception of bacterial storage products in several ways. It is extracted from the cells in boiling water and in 75% ethanol and, from chromatographic evidence, it was judged to be of low molecular weight by Binnie *et al.* (1960). It is unlikely to contain more than 10 glucose sub-units and may perhaps be more accurately regarded as an oligosaccharide. Unlike the case of glycogen in *E. coli* (Dawes & Ribbons, 1965) and in *A. aerogenes* (Strange *et al.* 1961), metabolism of the polymer does not suppress release of ammonia from starving cells.

We do not as yet know how the polymer exerts its lethal effect. Cells appear to consume less oxygen than is theoretically necessary for the complete oxidation of the amounts of carbohydrate and amino acid which disappear, and viability declines more rapidly when the supply of available oxygen is reduced. However, keto acids do not accumulate during starvation and the pH of the suspension does not decrease; these observations are consistent with the report of Dawes & Holms (1958*b*) that arsenite must be added before these compounds can be detected in suspensions oxidizing exogenous glucose. Relatively greater amounts of RNA are degraded by starving suspensions which are rich in polyglucose but it is not clear whether this is a cause or effect of death. Ribosome behaviour in such cells, and the effect of added Mg^{2+} ions, may well prove interesting. The rapid demise of polyglucose-rich *S. lutea* might conceivably be a particular case of substrate-accelerated death (Postgate & Hunter, 1963, 1964; Strange & Dark, 1965) in which an intracellular substrate, derived from the polyglucose, rather than an exogenous substrate, is the inimical agent, although glucose was not growth-limiting in these cultures.

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