The Survival of Starved Bacteria

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SUMMARY

Samples of populations of *Aerobacter aerogenes* grown in continuous culture on a limited supply of glycerol died linearly with time without significant cryptic growth when aerated in buffered physiological saline at their growth pH and temperature. Death was uninfluenced by atmospheres varying from pure O₂ to 2 % O₂ in N₂ but was accelerated under N₂ in media of various E_h values. Death was accelerated in environments of higher or lower tonicity, in unbuffered media, at pH values above 7, temperature above 40°, by strong illumination. Within limits, lower pH values or temperatures prolonged survival. Death was not immediately accompanied by breakdown of the osmotic barrier. Populations more dense than equiv. 20 µg. dry wt./ml. survived longer, sparser ones died more rapidly. The death rates of the populations studied were not influenced by 30 % i-erythritol or by certain high molecular weight materials. Several metabolic inhibitors were tested; most of them accelerated death or had no effect; three protected transiently. Mg²⁺, Ca²⁺ or Fe³⁺ ions delayed death. A variety of trace elements, inorganic ions, growth factors, or an amino acid mixture, had no significant action; glycerol or intermediate compounds accelerated death. Dying populations showed rapid initial breakdown of intracellular RNA with release of phosphate and base fragments into the medium; most of the ribose was metabolized. Intracellular protein was degraded after a lag; intracellular polysaccharide and DNA were scarcely degraded at all. Endocellular reserves declined much more rapidly than did viability; the Q_o with glycerol and 'glycerol dehydrogenase' activity declined in parallel with viability. Populations permitted to grow at different rates died more rapidly the slower the growth rate; steady states were obtained by slow continuous culture in which constant proportions of the organisms were dead.

For a given growth rate the nature of the limiting nutrient influenced the form of the survival curve: carbon, phosphorus, sulphur or oxygen limitations gave almost linear curves, N-limitation gave a sigmoid curve and Mg-limitation a concave form. In some instances the growth rate influenced the form of the survival curve and the death rate; Mg-limited organisms apparently died faster the faster they grew. The survival of stationary phase populations derived from batch cultures depended markedly on which chemical component of the environment limited growth; 'logarithmic phase' and 'stationary phase' C-limited organisms differed insignificantly in death rates. Partially synchronized populations from batch cultures died marginally more rapidly when harvested just after division ceased.

INTRODUCTION

The survival of bacteria stored in aqueous suspension and subject to no overt stress has been studied by many workers, mostly at the beginning of this century. Shearer (1917) reported that physiological saline was more toxic than distilled water or 1.5 % NaCl to meningococci; Ca or K antagonized this toxicity. Cohen (1922) cited earlier studies and showed that 'Bacterium coli' and 'Bacterium typhosum' in distilled water or dilute buffer died most rapidly at elevated temperatures and high pH values. The two species had different pH optima for survival; in distilled water their death was erratic and 'coincided with apparently insignificant pH variations'. Winslow & Falk (1923*a*) observed that 'B. coli' survived longer in physiological saline than in distilled water and that the pH optima for survival were different in the two media; Winslow & Falk (1923*b*) studied the effect of calcium and sodium ions on the viability of such populations. Winslow & Donoff (1928) and Winslow & Haywood (1931) were concerned with survival in nutrient media in which certain cations prevented growth. Morrison, El Bagoury & Fletcher (1956) observed that certain concentrations of chloramphenicol delayed the death of *Escherichia coli* stored in broth. Cook & Wills (1958) showed that washed buffered populations of *E. coli* maintained high viabilities at room temperature, compared with unwashed or unbuffered aqueous suspension; survival times were influenced by the growth conditions of their populations. Ryan (1959) recorded exponential death of histidineless *E. coli* in spent medium, though during this period the organisms had an appreciable mutation rate. Harrison (1960) reported that below a certain limit dense populations of *Aerobacter aerogenes* taken from logarithmic phase cultures survived longer than sparse populations in a non-nutrient buffer; centrifuged suspensions in which organisms had died contained materials that prolonged the lives of fresh populations. Strange, Dark & Ness (1961) published a study of the survival of stationary phase populations of the same strain of *A. aerogenes* in non-nutrient buffer. They showed that the composition of the growth medium, the phase of growth and the period during which the organisms had been in the stationary phase influenced the survival characteristics of their populations. Death of the populations was preceded and accompanied by degradation of polymeric cell constituents (protein, ribonucleic acid, polysaccharide) and excretion of fragments of these polymers. The mean contents of these polymers within the organisms were influenced by the composition of the medium from which the populations were harvested; so were the rates at which dying populations degraded these polymers, the order in which they were degraded, and the death rates. Strange (1961) reported that these reactions decreased the ability of the organisms to form adaptive enzymes without affecting their viability or ATP content. Ryan (1959) showed that glucose accelerated the death of his histidineless mutants; Strange *et al.* (1961) observed that addition of glucose to moribund populations accelerated their death rate. Harrison (1960) in contrast, reported that traces of glucose prolonged survival.

Studies on survival at ordinary temperatures in non-nutrient aqueous solutions are complicated by three factors. (1) Buffers prepared from highly purified reagents contain impurities which permit limited growth of bacteria. Garvie (1955) showed that this phenomenon could account for apparent 're-activation' of killed bacteria;

Strange *et al.* (1961) encountered a similar effect during their studies. (2) As soon as a portion of the population dies, nutrients may be released into the medium enabling the survivors to multiply. This phenomenon is well known in the ageing of bacterial cultures (e.g. see *Topley & Wilson's Principles* 1955) and was apparent in the work of Winslow & Falk (1923*a*, see Table 1). Ryan (1955) termed it 'cryptic growth' and concluded that it did not occur to any significant extent in non-multiplying populations of histidineless *Escherichia coli* (Ryan, 1959). Strange *et al.* (1961) called it 'regrowth' and showed that it could be prevented by dialysing the suspension or renewing the suspending fluid after filtration. Harrison (1960) invoked the phenomenon (termed 'cannibalism') to account for the increased survival time of dense bacterial suspensions as compared with more dilute ones, but he included within the term maintenance of viability (without growth) at the expense of materials released by dead organisms. (3) The third factor was briefly alluded to by Harrison (1960) but dismissed by him: laboratory glassware, reagents and distilled water may contain materials actively toxic to bacteria.

The aim of the work reported here has been to study the influence of environmental and historical factors on the survival of bacteria in starvation conditions, taking into account these complications as far as practicable. Misleading results due to cryptic growth were avoided by: (a) choosing conditions which provided a rapidly dying population capable of supporting only limited cryptic growth; (b) concentrating on the death of the first 90 % of that population. Multiplication of the stored populations at the expense of nutrient impurities in the storage media was rendered unimportant by: (a) choosing a simple medium (essentially physiological saline with a minimal concentration of a non-nutrient buffer); (b) using initial cell populations sufficiently dense that such residual growth as did occur would contribute negligibly to the apparent viability of the population. Toxicity due to impurities was avoided by: (a) demonstrating that one such toxic agent was metallic in nature and that its action could be eliminated by adding traces of a chelating agent; (b) choosing to work with as dense populations as the earlier considerations permitted so that the proportionate effect of traces of toxic material should be as small as possible.

Variations in survival characteristics due to the phase of growth and cultural conditions were reduced to a minimum by studying steadily growing populations from a continuous culture apparatus in which the growth rate was limited by the supply of energy source. These populations resembled those harvested just at the end of the logarithmic phase of growth of a batch culture. The culture was permitted to grow for many generations at a constant growth rate: variations consequent on transfer from batch culture to continuous culture might thus be expected to have taken place before the work recorded here was done; the mean physiological state of the populations studied should thus have been reasonably constant. Evidence that changes of this character did take place is presented later.

METHODS

Organism. *Aerobacter aerogenes* (NCTC 418) from Professor Sir Cyril Hinshelwood's laboratory was maintained in a continuous culture apparatus designed by Dr D. Herbert of this laboratory. This strain of *A. aerogenes* was incorrectly

designated No. 417 by Postgate, Crumpton & Hunter (1961) owing to a confusion of laboratory records. It was used by Strange *et al.* (1961) and Postgate & Hunter (1961) and is probably that used by Harrison (1960, 1961). The culture vessel contained 100 ml. culture and an air space of roughly equal volume. A defined medium of the following composition was pumped in at an appropriate rate: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (5 mM), $(\text{NH}_4)_2\text{HPO}_4$ (45 mM), K_2SO_4 (10 mM), glycerol (2 g./l.); trace element mixture to give MgO (1.25 mM), CaCO_3 (0.1 mM), FeCl_3 (0.1 mM), ZnO (25 μM), MnCl_2 (25 μM), CuCl_2 (5 μM), CoCl_2 (5 μM), H_3BO_3 (5 μM), Na_2MoO_4 (10 μM), HCl (2.9 mM), sodium ethylenediaminetetra-acetate (EDTA; 1.59 mM); NaOH (about 5 mM) to pH 7.8 when prepared; the pH value fell to 7.4 after autoclaving in batches of 10 l. for 30 min. at 121°. The temperature during growth was maintained at 40° by a thermostatically controlled 150 watt infra-red lamp; the usual dilution rate (proportion of culture volume replaced/hr.) used was $0.25 \pm 0.01/\text{hr}^{-1}$; in these conditions the culture was at pH 7.0 ± 0.1 and the bacterial population equivalent to about 1 mg. dry wt./ml.; bacterial numbers were 2.3 to 2.5×10^9 organisms/ml. as determined by the method of Norris & Powell (1961). Aeration was provided by flowing air at 10 l. hr. over a vortex produced by stirring the culture magnetically; other work here had shown that in these conditions aeration was adequate and that the bacterial population of the culture depended on the glycerol concentration of the influent medium. All data reported here refer to the strain between 4 and 24 months in continuous culture in these conditions. Brief interruptions occurred during the period owing to holidays or trivial failures of the culture device; a reserve of organisms frozen in 10 % (v/v) glycerol and stored at -20° (Postgate & Hunter, 1961), renewed monthly so that the viability did not fall below 90 %, was kept to reinoculate the continuous culture in case of interruption. After such episodes cultivation was continued until the survival curve of the population was normal before sample populations were used for further experiment.

Before the work reported here the strain underwent spontaneous alteration of at least three characters:

(i) The mean length of the organism increased from about 2.5 to about 5 μ , and the scatter of individual lengths became wider than that of the parent culture. On transfer to tryptic meat broth the population regained its original size.

(iii) The minimum mean generation time of the population on transfer to batch culture in the glycerol medium was 55 ± 3 min. as compared with 44 ± 2 min. for the original stock.

(iii) The organisms developed a long lag when plated on tryptic meat agar; in certain circumstances up to 70 % of the organisms did not divide. The original stock maintained on this medium grew rapidly.

The colonial form was uniform; periodic checks on the fermentation properties of the strain showed that it retained the diagnostic reactions characteristic of *Aerobacter aerogenes*. Tests on its antigenicity showed that it remained immunologically similar to the parent stock.

Storage. Bacteria were prepared for survival studies as follows. A sample (usually 5 ml.) was removed from the continuous culture vessel and the organisms washed twice in NaCl (0.85 g./l.) by centrifugation, re-suspended in distilled water to avoid carry-over of salt when special environments were tested, and 1 ml. added to 50 ml. saline-tris buffer (see below) in nominally 8 in. $\times$ 1.5 in. hard glass

tubes plugged with cotton wool. These were incubated in a water bath at 40° and gently aerated with 25 to 50 ml. damp air/min. through a Pasteur pipette pushed through the cotton-wool plug. For protracted survival curves (e.g. Fig. 1), when condensate might leach nutrients from the cotton wool, a rubber seal ('Suba-seal') replaced the cotton-wool plug. The evaporation rate from such suspensions was 1.5–2 ml. in 24 hr.; in experiments lasting longer than this appropriate volumes of sterile distilled water were added to the suspensions. Occasionally 25 or 30 ml. volumes of suspension in 8 in. $\times$ 1½ in. tubes were studied. The saline-tris buffer consisted of 9 vol. 0.137 M-NaCl + 1 vol. 0.048 M 2-amino-2-hydroxymethylpropane 1:3 diol ('tris') + EDTA to 0.316 mM. This amount of buffer maintained the pH value unchanged during aeration for 24 hr. at 40°. Populations stored in this manner contained about 4×10^7 organisms/ml. (equiv. about 20 µg. dry wt. bacteria/ml.; some loss of organisms occurred during washing) having an initial viability of 95–99 %. Samples were taken at intervals to determine viability; where the effect of a growth inhibitor was being examined, populations were centrifuged and re-suspended in saline before the viability determination. This procedure had no influence on the viability of the population though there was a further loss of bacteria during centrifugation. In an experiment on this topic a population of 7.17×10^7 viable organism/ml. had only 6.27×10^7 /ml. after one centrifugation; 3.3×10^6 organisms/ml. were accounted for in the supernatant fluid.

Filtration. During the work reported in the next section it became clear that Millipore filter disks (Oxoid Ltd.) contained materials that could act as energy sources for growth and which in certain circumstances could accelerate the death of starved populations. Such filters were therefore set up, autoclaved and then washed with 400 ml. distilled water followed by 50 ml. saline-tris.

Measurement of viability: The percentage viable organisms in stored populations was measured by slide culture (Postgate *et al.* 1961). The medium was that prescribed above supplemented with Difco yeast extract (1 g./l.); Difco casamino-acids (1 g./l.); Douglas's digest broth (10 %, v/v, Medical Research Council, 1931), Difco agar-agar (15 g./l.). It was filtered hot through a Millipore filter. In experiments with small population densities conventional plate counts on a similar medium were used.

Chemical analyses. Protein was determined by the biuret reaction (Stickland, 1951), boiling the alkaline suspension; bovine serum albumin, fraction 5 (Armour Laboratories) was used as standard. Free amino acids were determined by treatment of equiv. about 20 mg. dry wt. bacteria with acetic acid (100 g./l.; Mattick, Cheeseman, Berridge & Bottazzi, 1956), neutralization of the supernatant fluid with 2N-NaOH and analysis for primary amino groups with β -naphthaquinonesulphonate (Frame, Russell & Wilhelmi, 1943); Difco 'Casamino-acids' solutions were used as standard amino acid mixtures. Polysaccharide was determined by a modification of the anthrone reaction devised by Mr P. J. Phipps (this laboratory; personal communication) with glucose as standard. Ribonucleic acid was measured by the orcinol reaction (Morse & Carter, 1949) with ribonucleic acid (Boehringer and Son) as standard. Deoxyribonucleic acid was measured by the diphenylamine reaction with calf thymus nucleic acid (Gulland, Jordan & Threlfall, 1947) as standard. Polymeric cell constituents were measured on whole organisms without preliminary treatment and thus include diffusible derivatives of the materials

sought. Phosphate was determined by a version of the phosphomolybdate reaction (King, 1946). Colorimetric measurements were made with a Spekker absorptiometer (A. Hilger Ltd., London) or a Unicam spectrophotometer (Cambridge Instrument Co. Ltd., Cambridge). Fluorimetric measurements were made with the Locarte fluorimeter using exciting light of 340–380 m μ and recording wavelengths longer than 470 m μ (maker's filters LF2 and LF6 respectively). Bacterial dry weights were deduced from the optical densities of bacterial suspensions measured in the Spekker with a filter transmitting maximally at 540 m μ , and calibrated with suspensions of known dry wt./ml. from our continuous culture. Determinations of enzyme activity were performed with conventional Warburg manometers at 40°; 'glycerol dehydrogenase' activity was determined by the procedure of Fahmy & Walsh (1952) with the modification that the suspensions were incubated in Thunberg tubes under nitrogen at 40° for 20 min. Fahmy & Walsh used tubes open to air; in our tests incubation under N₂ was found to be necessary with enzyme preparations from the bacteria, and was therefore considered preferable for testing moribund populations.

Materials. Reagents of analytical grade were used when available. Water was distilled and then de-ionized by passage through a mixed bed ion-exchange resin.

RESULTS

Standard survival curves. Populations (equiv. 20 μ g. dry wt. *Aerobacter aerogenes*/ml.) harvested from steady growth at a dilution rate of 0.25 hr.⁻¹ and allowed to die in aerated buffer at 40° and pH 7 showed a linear survival curve in which a constant % of the initial population died per hr. over the first 7–10 hr. A statistically analysed curve with such populations was quoted by Postgate *et al.* (1961); this showed a mean death rate of about 8%/hr. with empirical 95% confidence limits of $\pm 9\%$ for the technique of viability determination used. Most of the survival curves obtained during this work resembled this; the death rate, over the period when the first 70% of the population died (about 5 hr.), was usually between 6% and 12%/hr., though extremes as low as 4.6%/hr. and as high as 17%/hr. were encountered. The reasons for these fluctuations are not known. The survival curve of the population showed considerable reproducibility from day to day for several weeks; sometimes it changed over 2 or 3 days; during the 20 months in which the culture was studied the population showed a trend towards faster death rates. Occasionally survival curves of a slightly sigmoid character were observed, in which an initially descending portion was followed by a steeper linear decline. In all cases the curve flattened off, when after 7–10 hr., the viability had fallen below 30%; by 24 hr. the viable population had fallen below 2%. Because of the occasional variability of the survival curves all experiments reported here included as control a population dying in aerated buffer.

Cryptic growth. This phenomenon leads to an increase in the total count of a population during its death. However, the systematic and operational errors in making a conventional total count are such that small percentage increases in bacterial numbers might be missed (Norris & Powell, 1961). It may be detected by comparing survival curves with and without a non-bactericidal growth inhibitor such as penicillin (see Ryan, 1959); if these are identical it follows that cryptic

growth has not occurred in the absence of bacteriostatic agent. Ordinary cultural experiments showed that penicillin G (100 units/ml.) inhibited growth of equiv. 20 $\mu\text{g./ml.}$ inocula in the glycerol medium for 17 hr. and that chloramphenicol (100 $\mu\text{g./ml.}$) inhibited multiplication for 2 days. The latter observation was confirmed by slide culture on agar containing chloramphenicol; none of 600 organisms watched divided over 24 hr. Table 2 records experiments showing that cryptic growth did not occur in the conditions we used routinely: the survival curves over the first few hours with and without chloromycetin or penicillin were identical.

On prolonged incubation cryptic growth was readily detected (Fig. 1). The maximum cryptic growth of which our populations were capable was determined by experiments of which the following is typical. A suspension of about twice the customary population density (6.75×10^7 viable bacteria/ml.) was left to die at 40° with aeration for 24 hr. in the presence of 100 u. penicillin G/ml. to prevent cryptic growth. By then the viability was 1.3 %. The suspension was filtered through a Millipore membrane and the filtrate assayed with a population from continuous culture which has been made resistant to penicillin by two batch subcultures (about 11 generations) with 200 and 100 u./ml. successively. For assay, 5 vol. filtrate were diluted with 4 vol. Millipore-filtered saline-tris buffer plus 1 vol. culture medium made up without glycerol so as to bring substrates leached from dying organisms to concentrations comparable with those to be expected in the suspensions we routinely studied; the portion of basal medium supplied an excess of mineral nutrients. The diluted filtrate was inoculated with 10^4 penicillin-resistant bacteria/ml. Growth was followed by plate counts and compared with that in a control buffer supplemented with basal medium. The filtrate supported a maximum excess growth of 7.2×10^5 organisms/ml. over the control; these grew as a result of the death of about 3.3×10^7 organisms/ml. Hence the maximum cryptic growth of which our populations were capable was one new organism at the expense of about forty-seven dead ones.

Growth on impurities. Direct assays of the extent of growth permitted by impurities in our reagents and on our glassware were made by inoculating 30 ml. lots of buffer with about 4×10^3 organisms from the continuous culture. Saline-tris buffer supported from 2×10^2 to 8×10^4 viable organisms/ml. as the maximum viable population. Addition of 0.1 vol. of the basal medium (without glycerol) to supply mineral nutrients augmented growth to $2.8\text{--}3.5 \times 10^5$ organisms/ml. Garvie (1955) and Strange *et al.* (1961) obtained values of about 10^6 organisms/ml. for residual growth on impurities in phosphate buffers.

Manipulation of organisms. The populations studied were subject to centrifugation and changes of temperature and chemical environment during preparation for storage. Organisms washed once, twice or three times in saline gave essentially similar survival curves of slope 7.8 %/hr. and initial viabilities of 90–95 %. A repetition of Gossling's (1958) experiments using buffers prepared according to his prescriptions in the manner represented schematically below showed negligible effects of chemical environment on viability.

A temperature decrease to $18\text{--}20^\circ$ was unavoidable and this 'cold shock' (Hegarty & Weeks, 1940) may have caused the 1–8 % of dead organisms usually found after preparation for survival curves. A decrease to 4° did not alter the viability; a decrease to 0° obtained by squirting the suspension on to ice caused a small but

significant kill. Viabilities in an experiment on this topic were 99 % at room temperature, 98 % at 4° and 94.5 % at 0°; freezing in solid CO₂ reduced the viability to below 1 % (see Postgate & Hunter, 1961).

		Viability (%)
Culture	→M/15 phosphate	95.3
	→Ringer	92.7
	→M/15 phosphate	95.5
	→Ringer	26.7
Centrifugations: 1st		2nd

Prior to storage the bacteria spent 3–5 min. in distilled water. This treatment affected the subsequent death rate though it did not measurably influence the initial viability of the population. In a typical experiment organisms suspended in distilled water before storage in buffer died at 10.1 %/hr. compared with 5 %/hr. for those suspended in saline-tris buffer. Exposure to M-NaCl before storage led to a survival curve similar to that obtained after exposure to distilled water.

Need for buffer. The viabilities of populations suspended in unbuffered physiological saline declined to 15 % in 2 hr. and 5 % in 3 hr. Death was accompanied by a change from pH 6.0 to 4.9. In distilled de-ionized water the viability fell to 13 % after 2 hr.

Need for ethylenediaminetetra-acetate. Early in this work the slopes of the survival curves were erratic; they showed an abrupt change on one occasion when a new batch of saline-tris buffer was used; in this batch the organisms all died within 2 hr. and the death rate returned to about 8 %/hr. when EDTA was added. This suggested the buffer might contain a toxic metal. Extraction of 200 ml. of buffer with 8-hydroxyquinoline in CHCl₃ gave a chloroform solution which by spark spectroscopy showed an unusual amount of copper (Dr L. C. Thomas, personal communication). Laboratory-distilled and de-ionized water contained about 2×10^{-7} M-Cu²⁺; its effect was neutralized by EDTA. The routine practice was adopted of adding to the buffer 0.316 mM-EDTA (three times the concentration necessary to neutralize the most toxic batches encountered).

Effect of recovery medium. The medium used for slide culture contained nitrogenous supplements (casein hydrolysate, yeast extract, tryptic meat broth) to reduce the scatter of division lags among the organisms counted. A medium without these supplements gave less accurate viabilities due to overgrowth of late starters by organisms of short lag (Postgate *et al.* 1961). The rich medium gave greater survivals with dying populations: tests with conventional plate counts on the rich medium and the minimal medium gave comparable counts on the two media when the populations tested were more than about 50 % viable; below this value the minimal medium gave consistently lower counts. For example, a 40 % viable population gave a count of 5.8×10^6 organisms/ml. on the rich medium and 3.7×10^6 ml. on minimal medium; a 27 % viable population gave counts of 5.2×10^6 /ml. and 2.9×10^6 /ml. respectively. Replica plating from rich to minimal agar showed that these differences were not due to the appearance of nutritional mutants in the population counted, and we conclude that more of the population died during the lag phase on minimal agar than died during this period on rich agar.

Effect of light. To determine survival curves the bacterial suspensions were stored in a thermostat illuminated by indirect daylight. The illumination at the surface of the water ranged from 4 to 14 ft.-c. according to the weather; appropriate tests showed that this degree of illumination had no killing effect on our populations. For example, a population died at 9.2 %/hr. in our ordinary tubes and in tubes screened by wrapping in aluminium foil. By intensifying the illumination killing by visible light was observed: a population held at 40° in a transparent thermostat was all dead in 4 hr. when illuminated with 100 ft.c. from a 75 W. tungsten filament bulb at pH 6.9 ± 0.05 ; the survival curve showed a sigmoid form. The control, screened with aluminium foil, died linearly at 9.8 %/hr. and was about 60 % viable after 4 hr.

Fig. 1

Fig. 2

Fig. 1. Survival curve of *Aerobacter aerogenes* showing cryptic growth. *A. aerogenes* harvested from continuous culture on glycerol at $D = 0.25 \text{ hr}^{-1}$ was washed twice by centrifugation, resuspended in distilled water, added to 'saline-tris' buffer at 20 µg. dry wt. organisms/ml. and allowed to die at 40° at pH 6.95 ± 0.05 under forced aeration. Points up to 24 hr. by slide culture; including plate counts at 0, 7 and 24 hr.; subsequent points by plate counts alone.

Fig. 2. Effect of population density on death of *Aerobacter aerogenes*. *A. aerogenes* suspensions prepared as for Fig. 1; added to saline-tris buffer and allowed to die at 40° and pH 7.1 ± 0.1 with forced aeration. Tube ○ contained 25 µg. dry wt. organisms/ml.; ● : 2.5 µg./ml.; ○ : 0.25 µg./ml.; ● : 0.025 µg./ml.; ○ : 0.0025 µg./ml. In the first of these the viabilities were determined by slide culture, in the rest by plate counts assuming all the initial viabilities were similar.

Effect of population density on survival curve. Harrison (1960) showed that between populations of 10^5 and 10^8 organisms/ml. the death rate of *Aerobacter aerogenes* was slower the denser the population; at 10^9 organisms/ml. the survival curve became steeper again. Our experiments confirmed Harrison's: denser populations survived longer than sparser ones (Fig. 2); the most dense population we have tested contained about 4×10^8 organisms/ml., probably yet too sparse to show accelerated death.

Effect of temperature. The effect of temperature on the death rate is illustrated in Fig. 3. Death was slower at 30° and 20°; faster at 10, 45 and 50°. A small contribution to the shallower death curves at 20° and 30° may have been due to cryptic

growth. The organisms did not grow at 45° or above; at 60° more than 99 % of the organisms were dead by the time (about 3 min.) the environment had warmed up to this temperature.

The slide cultures prepared from populations dying at 10° and 20° showed many morphologically aberrant forms once death had started. Small bacteria sometimes became relatively large refractile spheres which burst, leaving convoluted forms or 'ghosts'. Slides watched for 7 hr. at 37° showed that most of these bodies did not form micro-colonies; they were recorded as dead.

Effect of deep freezing. Freezing the population in diethylene glycol solution (100 g./l.), which caused no change in viability, accelerated the death of the organisms after thawing. This experiment was described by Postgate & Hunter (1961) and illustrated in their Fig. 1.

Fig. 8. Effect of temperature on death of *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 20 µg. dry wt./ml. and allowed to die at pH 7.0 to 7.1 with forced aeration. Part (a) tubes ●: 40°; ○: 30°; ●: 20°; ●: 10±1°; part (b) ○: 45°, ●: 50°.

Effect of osmotic environment. The concentration of the buffered sodium chloride was that usually described as physiological, though such an environment is in fact hypotonic to the bacterial cytoplasm. The effects of adding materials that would alter this condition were studied.

(i) *Sodium chloride.* Organisms made sensitive to osmotic shock by Nakamura's (1923) procedure (incubation with lysozyme followed by exposure to pH 10) lysed in distilled water and in buffered saline; lysis was much decreased in buffer containing 1.15 M-NaCl. However, the death rate of the ordinary bacteria in buffer containing 1.15 M-NaCl (41.0 %/hr.) was greater than that in the control (5.2 %/hr.). An environment of marine salinity (0.05 M-NaCl buffered with tris) caused accelerated death (60 % died/hr. compared with 17.5 %/hr. in a control); sea water filtered and buffered at pH 7 with the usual concentration of tris was less toxic and the death rate was like that in the control buffer.

(ii) *Polyethyleneglycol or dextran.* High molecular weight substances protect organisms in freeze drying because their osmotic effect occurs at the cell wall (see Record & Taylor, 1960). Dextran (Glaxo Laboratories Ltd., mean m.w. 125,000) 50 g./l. and polyethyleneglycol (mean m.w. 10,000), 50 g./l., provided by Dr B. R.

Record, were tested in saline-tris buffer. Both slightly accelerated death and altered the shape of the survival curve; their effects were small and were not studied further.

(iii) *i-Erythritol*. This compound like glycerol penetrates the osmotic barriers of bacteria freely (Mitchell & Moyle, 1956) including our strain (Postgate & Hunter, 1961). Cultural tests showed that the organisms did not use it for growth. Survival curves in buffer supplemented with 300 g. *i*-erythritol/l., which may be expected to have diluted the cytoplasmic water considerably, were virtually indistinguishable (death rate: 5.2 %/hr.) from controls without this supplement (5.3 %/hr.).

Effect of pH value. Figure 4 illustrates that a pH value below 7.0, at which they had grown, was optimal for survival.

Effect of atmosphere. Removal of CO₂ from the influent air with NaOH did not significantly influence the death rate (12.5 %/hr. without CO₂, 12.4 %/hr. with). Replicate suspensions aerated with O₂ + N₂ mixtures ranging from pure O₂ to 2 % (v/v) O₂ died at similar rates. Replacement of air by commercial 'oxygen free' nitrogen (British Oxygen Company Ltd., O₂ below 5 p.p.m.) gave erratic death rates but usually accelerated death (e.g. 5 %/hr. in control; 21.5 %/hr. with N₂). The increased death rate was usually associated with a decline in pH value; e.g. in N₂ buffered suspensions fell from pH 7.0 to about 6.0 in 5 to 7 hr., reaching about pH 5.3 by 24 hr.; in air the pH value scarcely changed (7.0 to 6.9) over 24 hr. On one occasion the usual pH change did not occur in N₂ but death was nevertheless rapid.

Effect of reducing agents. The death rate in an atmosphere of N₂ was influenced by poisoning the E_h value of the environment with chemicals known to interact with biological systems. With sodium ascorbate (2×10^{-2} M) the death rate was 10.8 %/hr., compared with 21.5 %/hr. with sodium thioglycollate (2×10^{-2} M) and 21.5 %/hr. with N₂ in an unpoised buffer. The control suspension in air died at 5 %/hr. Acid was formed in all the instances in which the environment was anaerobic.

Effects of nutrients

Effect of trace elements. The culture medium used contained trace elements held in solution by a slight molar excess of EDTA. We are not certain that all these supplements were necessary for growth. The trace element mixture, when added to saline-tris buffer in the proportion used in preparing the growth medium, had a preservative effect (death rate 5.7 %/hr. compared with control 13 %/hr.). This phenomenon was analysed by testing the component elements singly at the concentrations used in the culture medium (Table 1). Ca, Mg, or to a slight extent Fe, protected the organisms; all the other elements were without effect when EDTA was present at 316 μ M. The protective effect of calcium was complex: the death rate increased in a parabolic fashion for about 5 hr., then settled to a linear rate equal to that of the control. A test with a 'non-toxic' batch of buffer (see 'need for EDTA') without EDTA showed that Co, Mn, Cu and Zn were then toxic.

Effect of nitrogen. Addition of NH₄Cl (10 g./l.) did not significantly alter the death rate (control: 5.4 %/hr., + NH₄Cl 5.6 %/hr.). Vitamin-free casein hydrolysate 20 g./l. supported growth when the experiment was extended for 24 hr. Over the first 2 hr., however, the survival curve paralleled that of the control, suggesting that amino acids exerted no protective effect.

Effect of potassium and phosphate. Addition of KCl (m/150) accelerated death slightly (control: 6.4 %/hr., +KCl: 10 %/hr.) NaH_2PO_4 (1.25 mM) added to the buffer had no significant effect (control: 5 %/hr., + NaH_2PO_4 : 6 %/hr.).

Death in the growth medium. Populations survived longer when incubated in the spent culture medium from which organisms had been removed (death rate 2.8 %/hr.). This was not due to protective materials excreted by the organisms because the same death rate was obtained in the basal medium prepared without a carbon source. The whole of the protective effect of spent or basal medium was attributable to its trace element content: the death rate in the basal medium (5.3 %/hr.) was somewhat greater than that in saline-tris buffer supplemented with Ca, Mg and Fe (3.7 %/hr.); 10.4 % of a control in ordinary saline-tris buffer died/hr.

Table 1. *Effect of trace elements on death of starved Aerobacter aerogenes*

Survival curves at the growth pH value (7) and temperature (40°) in saline-tris buffer with the supplements below were obtained as described in Methods.

Supplement	Concentration (μM)	Death rate (%/hr.)
FeCl_3	100	12.4
ZnSO_4	25	14
MnSO_4	25	14.7
CuSO_4	5	16
CoSO_4	5	14
H_3BO_3	5	15
NaMoO_4	10	14.8
CaCl_2	100	c. 5.0*
MgCl_2	1250	5.6
All†	—	5.4
None	—	15

* Curve with Ca non-linear; see text.

† Mixture contains Zn, Mn, Cu and Co as chlorides.

Effect of vitamins. Several compounds were tested at concentrations recorded in the literature as having growth factor effects for appropriately exigent organisms. None significantly influenced the death rate of our populations over the first 7 hr. The substances were (conc./ml.): adenine (10 μg .), adenosine (4 μg .), adenylic acid (4 μg .), *p*-aminobenzoic acid (0.1 μg .), biotin (1 m μg .), cyanocobalamin (1 m μg .), glutamine (2 μg .), haemin (c. 1 μg .), *m*-inositol (10 μg .), nicotinamide (0.1 μg .), sodium pantothenate (1 μg .), phthiocol (1 μg .), pteroylglutamic acid (1 m μg .), pyridoxal (2 μg .), pyridoxamine (2 μg .), pyridoxine (2 μg .), riboflavine (0.1 μg .), ribose (2 μg .), thiamine HCl (0.1 μg .), Tween-80 (10 μg .), uracil (10 μg .).

Effect of carbon source. Glycerol (10⁻²M) accelerated death. This phenomenon was not due to toxic materials in the analytical grade glycerol used, since intermediates such as sodium pyruvate, oxaloacetate, α -ketoglutarate, succinate, citrate or DL-malate also accelerated death. Selected data illustrating this phenomenon are given in Fig. 5.

In this context we should record an experimental hazard. In certain experiments it was necessary to filter batches of saline-tris buffer through a Millipore membrane and the survival curves in filtered buffer were steeper (6.7 %/hr.) than the control (4.7 %/hr.). By extracting Millipore membranes with substrate-free growth medium

and testing the extracts microbiologically we showed that this phenomenon was not due to toxic materials in the filter but to carbon substrates which permitted growth in the basal medium and accelerated death in the buffer. Adequate washing of the membranes removed these materials.

Effect of metabolic inhibitors. Death in aqueous suspension is preceded by considerable endogenous metabolism (Strange *et al.* 1961) and it is reasonable to suppose that metabolic inhibitors, provided they are not bactericidal, might influence survival directly. Several known metabolic inhibitors were tested by incorporating them into the routine saline-tris buffer and comparing the death rates of populations in their presence with those in controls. Samples of the dying populations were

Fig. 4

Fig. 5

Fig. 4. Effect of pH value on death rate of *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 20 $\mu\text{g./ml.}$ and allowed to die at 40° under forced aeration at various pH values. ○: 6.1; ●, 6.5; ●, 7.0; ●, 7.45; ●, 7.9; all pH values ± 0.05 .

Fig. 5. Effect of carbon sources on death of *Aerobacter aerogenes*. Suspension of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 20 $\mu\text{g. dry wt./ml.}$ and allowed to die at 40° and pH 7.0 ± 0.1 under forced aeration. ○: controls; ●: plus glycerol; ●: plus sodium pyruvate; ●: plus sodium citrate (all supplements 10^{-3}M.).

centrifuged and re-suspended in saline to remove the inhibitor before determining the viability by slide culture; as mentioned in 'Methods' this procedure had no effect on the viability of the control populations. The compounds were tested at concentrations judged from the literature to be likely to have a marked effect provided that the material reached its site of action. Compounds which showed an immediate bactericidal effect were examined by slide culture to find the maximum concentration that exerted no such effect (see Postgate *et al.* 1961, Table 3) and were tested at one half that concentration. The results are given in Table 2. Yttrium or lanthanum nitrates showed modest preservative effects; controls showed that these were not due to the nitrate ion. With lanthanum these effects were observed in four experiments; lanthanum at $5 \times 10^{-5}\text{M}$ had no effect; $2 \times 10^{-4}\text{M}$ -lanthanum behaved like

the concentration quoted in Table 2; 10^{-3} M-lanthanum had bactericidal effect leaving only 25 % viable organisms at the start of the survival curve. The death rate with pyridine sulphonate (10^{-4} M) was not influenced by adding its metabolite analogue nicotinic acid to 10^{-6} M.

State of the osmotic barrier. Stored suspensions of some micro-organisms liberate purines into the external medium (Holden, 1958; Higuchi & Uemura, 1959).

Table 2. *Effect of metabolic inhibitors on the death rates of starved Aerobacter aerogenes*

Death rates measured at growth temperature (40°) and pH value (7) in a saline-tris buffer; for details see text.

Inhibitor	Concentration (μ g./ml.)	Death rates	
		Control	Test
4-Aminopteroylglutamic acid	50	7.1	7.4
Aureomycin	20*	6.6	6.8
Aza-adenine	100	5.4	5.3
Aza-guanine	100	8.5	10.5
Aza-uracil	100	5.0	5.0
Aza-xanthine	100	8.5	8.4
Chloramphenicol	100	5.6	4.8
<i>p</i> -Fluorophenylalanine	100	7.1	7.7
<i>iso</i> Nicotinic acid hydrazide	100	7.1	7.0
Penicillin G	100*	5.0	1.5
Pyrithiamine	200	5.4	5.1
Sodium azide	100	6.6	8.0
Sodium fluoracetate	100	7.2	7.2
Sodium fluoride	78	7.2	8.4
Sulfathiazole	100	6.6	6.6
Trypan blue	100	7.0	7.2
Anilinonaphthalene-8-sulphonic acid	15†	9.6	9.8
Beryllium nitrate (cryst.)	100	8.0	42
Methylene blue	5‡	9.0	30
Proflavine	20‡	9.0	31
Sodium arsenate	640	9.5	13
Sodium iodoacetate	208	7.2	28
Sodium malonate§	1270	7.2	48
Sodium pyridine-3-sulphonate	16‡	11.0	21.5
Lanthanum nitrate (cryst.)	43	11	9.5
Yttrium nitrate (cryst.)	38	10.5	8.4

Little or effect

Death rate accelerated

Death rate reduced

* Units/ml.

† 50 $m\mu$ mole/ml.; see fig. 9.

‡ Concentrations double those quoted showed an immediate bactericidal effect on a portion of the population.

§ Dead organisms mainly spherical.

Strange *et al.* (1961) showed that these bases were products of endocellular metabolic processes which preceded death. Since breakdown of the osmotic barrier of cells also releases such materials from the cytoplasm it was of interest to discover the state of the osmotic barriers in moribund suspensions.

Mager, Kuczynski, Schatzberg & Avi-dor (1956) showed that, at low solute concentrations, the optical density of living bacterial suspensions depended on the tonicity

of the suspending medium. With *Pasteurella tularensis* the optical density (OD) in 0.4M-MgCl₂ was twice that in distilled water. They interpreted this phenomenon as due to a change in refractive index of the cells consequent upon an adjustment of the water content of the internal environment to some sort of equilibrium with the exterior; it did not occur with heat-killed or disinfectant-killed organisms, they termed this phenomenon the 'optical effect'. It provides a test of whether the osmotic barrier is functioning. With the culture of *Aerobacter aerogenes* used in this work, live organisms showed about 25 % greater OD in 0.4M-NaCl compared with suspensions in distilled water (Fig. 6); equimolar Na₂SO₄, MgCl₂ or glucose showed smaller increments. Heat-killed organisms (10 min., 60°) showed a slight negative effect (OD in 0.4M-NaCl about 90 % of that in water); mixtures of heat-killed + live bacteria showed an optical effect in proportion to the % live organisms over the range +25 % to -10 % of OD in water.

Fig. 6

Fig. 7

Fig. 6. The optical effect with logarithmic phase *Aerobacter aerogenes*. A strong washed suspension of *A. aerogenes* from continuous culture was added to NaCl solutions to give 0.12 mg. dry wt. organism/ml. and the optical densities were measured at 540 m μ . with a 1 cm. light path. Points taken at 2, 10 and 30 min. after preparation of suspensions.

Fig. 7. Permeability and viability of moribund *Aerobacter aerogenes*. *Part a.* Suspension of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 150 μ g. dry wt. organisms/ml. and allowed to die at 40° and pH 7.10 $\pm$ 0.05 with forced aeration. Samples centrifuged and re-suspended in distilled H₂O and 0.4M NaCl for optical effect; diluted in saline for viability. Optical effect expressed as % of range between wholly live cells (+35 %) and cells killed by heating 5 min. at 60° (-10 %). ○: viability; ●: optical effect. *Part b.* Suspension of *A. aerogenes* 20 μ g./ml., pH 7.05 $\pm$ 0.05, otherwise similar to Part a. Viability determined as usual. Permeability to anilino-naphthalene-8-sulphonic acid determined fluorimetrically after addition of dye to 50 m μ mole/ml. Expressed as % of range between untreated suspension and one killed by exposure to 60° for 5 min. ○: viability; ●: fluorescence.

The optical effect was examined with suspensions dying in buffer as usual, except that a ninefold greater concentration of organisms was used (initial OD = 0.22-0.25 in water in 1 cm. cuvette); the extent of the optical effect as % of maximum was compared with the viability. The results of one such experiment are shown in Fig. 7a. Though the figures for the optical effect show a fair scatter, there is no doubt that the optical effect persisted near its maximum extent when

nearly 80 % of the organisms had died. This curve includes a point observed after 24 hr., when over 98 % of the population was dead and the viability was outside the range of the slide-culture technique; nevertheless, over 50 % of the organisms showed the optical effect. It seems that the osmotic barrier was intact after 'death'. Independent support of this finding was provided by the demonstration of an unchanged 'amino acid pool' (see below) and by experiments with anilino-naphthalene-8-sulphonic acid (similar to the toluidine dye used by Newton, 1954) which forms a fluorescent complex with these bacteria only when the osmotic barrier is destroyed. This dye was non-toxic at the concentration used (Table 2); Fig. 7*b* illustrates an experiment showing that organisms in moribund suspensions became permeable to the dye more slowly than the rate at which they died.

Mager (1959) showed that certain aliphatic diamines (notably spermine) delayed osmotic breakdown of sensitive bacteria and of spheroplasts. Since breakdown of the osmotic barrier did not normally accompany death of the organisms studied here one may predict that spermine ought not to influence the death curve. This was so: a population died at 6.6 %/hr. alone, 7.2 %/hr. with 10^{-4} M-spermine.

Biochemical changes accompanying death. Strange *et al.* (1961) showed that *Aerobacter aerogenes* harvested from the stationary phase of growth in a defined medium based on mannitol degraded endocellular ribonucleic acid before death; the catabolism of protein and polysaccharide was less extensive. Our populations, which differed in being harvested while steadily growing, behaved similarly in conditions resembling those used by Strange *et al.* Suspensions of equiv. 10 mg. dry wt. bacteria/ml. aerated in saline-tris at 40° and pH 7.0 ± 0.2 for 17 hr., during which period 98 % of the population died, lost about 50 % of their pentose and 15 % of their polysaccharide. About twice as much inorganic phosphate and about three times as much material which absorbed at about 260 m μ appeared in the medium as was released by heat-killing or treating with a quaternary detergent. About 20 % of the pentose lost by the organisms was detectable in the medium after death. Hence the phosphate and 260 m μ materials were generated by a metabolism which involved pentose fermentation and were not formed by osmotic leakage such as that observed by Mager (1959) from *Neisseria perflava* and *Pasteurella tularensis* in media of low tonicity. The materials which absorbed about 260 m μ had a complex absorption spectrum: the supernatant fluid showed a main maximum at 254 m μ with subsidiary peaks at 247.5, 260 and 265 m μ . Chromatography by Mr H. E. Wade indicated hypoxanthine, uracil, guanine and an unidentified anionic material. Cytosine, cytidine, uracil and inosine were absent.

This population was some 500-times those used for our routine survival studies. Measurements on the rates of breakdown of bacterial constituents were therefore made with shorter term tests on populations concentrated by centrifugation for analysis. Typical figures for the initial composition of the organisms used are given in Table 4. RNA breakdown started immediately and was most rapid in the early stages of death of the population (Fig. 8*a*); in contrast, protein catabolism (Fig. 8*b*) was not detectable during the first few hours, though it ultimately took place. Figure 8*b* includes data in which the 'amino acid pool' was estimated during death; it remained unchanged for several hours. The polysaccharide content showed an initial decrease of about 20 %, then remained constant. Curves for DNA metabolism during death were not obtained because pilot experiments had shown virtu-

ally no change: in two experiments the viabilities of 30 $\mu\text{g./ml.}$ suspensions fell from 96–97 % to 10 and 29 %; the DNA contents of the organisms declined during this period by 12.5 and 3.5 % respectively.

Enzymic changes during death. A few experiments were performed on the state of the respiratory system of our population during death. Figure 9*a* shows that the endogenous Q_{O_2} declined rapidly during the first hour of storage and settled to a low level during the main part of the survival curve. High population densities were necessary for the measurement of the Q_{O_2} ; for practical reasons a much more concentrated suspension was used than would ordinarily have been compatible with the customary death rate. By coincidence the slope of the survival curve was much as usual, possibly because the high population density was in the range where the death rate accelerated with increasing density (Harrison, 1960). The upwards inflexion of the Q_{O_2} value towards the end of the curve may be attributable to cryptic growth.

Fig. 8. Polymer content and viability of moribund *Aerobacter aerogenes*. **Part a.** Suspensions of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 36 $\mu\text{g. dry wt./ml.}$ and allowed to die at 40° and pH 7.2 $\pm$ 0.05 with forced aeration. 10 ml. samples removed at intervals for viability determination and for pentose after centrifugation. Curves incorporate data from three experiments. $\circ$: Viability; $\bullet$: pentose content. **Part b.** Suspensions prepared as for Fig. 1; added to saline-tris buffer at 45 $\mu\text{g./ml.}$ and allowed to die at 40° and pH 7.0 $\pm$ 0.5 with forced aeration. 75 ml. portions concentrated by centrifugation for protein analyses. Data also included for 'free amino-nitrogen' contents of a similar suspension dying at 50 $\mu\text{g./ml.}$ and pH 7.2. Curves incorporate data from three experiments. $\circ$: viability; $\bullet$: protein content; $\bullet$: amino-N content. **Part c.** Suspension prepared as for Fig. 1; added to saline-tris buffer at 60 $\mu\text{g. dry wt./ml.}$ and allowed to die at 40° and pH 7.0 $\pm$ 0.05 with forced aeration. 50 ml. samples removed for duplicate polysaccharide determinations after concentration by centrifugation. $\circ$: viability; $\bullet$: polysaccharide content.

Populations of the culture of *Aerobacter aerogenes* used in this work metabolized glycerol incompletely in the Warburg manometer, taking up about 30 % of the theoretical amount of O_2 for complete oxidation of the substrate before the rate of oxygen uptake declined to about the endogenous value. No increase in the polysaccharide content of the organisms was found at the point at which the Q_{O_2} declined; the fate of the substrate was not studied further. Initial Q_{O_2} values with glycerol are compared with viability of a stored suspension in Fig. 9*b*. The decline

in Q_{O_2} with the growth substrate paralleled the decline in viability. Figure 10 records the dehydrogenase activities of moribund populations with glycerol as hydrogen donor. The 'glycerol dehydrogenase' activity declined roughly in parallel with the viability throughout the experiment; hence the decline in Q_{O_2} with glycerol observed earlier can be attributed at least partly to the enzyme system involved in the initial attack of glycerol. Our strain is known to have not a simple dehydrogenase but a glycerol kinase and a glycerophosphate dehydrogenase (Mr D. Tempest, personal communication).

Fig. 9. Respiration of moribund *Aerobacter aerogenes*. Part a. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer in a Warburg manometer at 4.5 mg. dry wt./ml. and allowed to die at 40° and pH 6.95 $\pm$ 0.05 with shaking and with the apparatus open to air. Periodically the suspension was sampled for viability determination and immediately afterwards the endogenous Q_{O_2} was determined. ○: viability; ●: endogenous Q_{O_2} . Part b. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 100 μ g./ml. and allowed to die at 40° and pH 6.95 $\pm$ 0.05 with forced aeration. Periodically 20 ml. samples were centrifuged, the organisms transferred to Warburg manometer containing a saline phosphate buffer, and the Q_{O_2} with glycerol determined. ○: viability; ●: Q_{O_2} with glycerol.

Survival curves of organisms grown in other conditions

Effect of growth rate on death rate. After over 18 months in continuous culture samples of our population were transferred to a second continuous culture apparatus in which the dilution rate was altered, causing corresponding changes in the imposed doubling time. Figure 11a illustrates the survival curves obtained after the populations had spent at least seven generations at a new dilution rate; it appears that the faster the population grew the slower it died. This generalization has been confirmed twice with carbon-limited populations of this organism: once in experiments not reported here in which survival curves were obtained in phosphate buffer, once in the series of experiments involving unusually slow growth rates (see below) illustrated in Fig. 11b.

The populations obtained at the slowest growth rates contained a proportion of organisms dead at the start, about 15 and 30 % at dilution rates of 0.12 and 0.06 hr⁻¹ respectively (Table 4). This phenomenon occurred in several instances of slow

Fig. 10. 'Glycerol dehydrogenase' activity of moribund *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at $25 \mu\text{g./ml.}$ and allowed to die at 40° and $\text{pH } 6.95 \pm 0.05$ with forced aeration. At intervals viabilities were determined and 40 ml. samples were centrifuged, the organisms re-suspended in a saline phosphate buffer and the dehydrogenase activity towards glycerol determined quantitatively with tetrazolium (see Methods; two experiments recorded). $\circ$: viability; $\bullet$: 'glycerol dehydrogenase' activity.

Fig. 11. Effect of growth rate upon death of *Aerobacter aerogenes*. *Part a.* *A. aerogenes* was grown in continuous culture (glycerol limiting growth) at different doubling times and the survival curves of $20 \mu\text{g./ml.}$ populations in saline-tris buffer at $\text{pH } 7.0 \pm 0.05$ and 40° obtained (see text). The survival curves are corrected for organisms dead at the start of the experiments: the doubling times (t_d) refer to the viable proportion of the population in the continuous culture. The customary dilution rate was 0.25 hr.^{-1} . $\bullet$: $t_d = 57 \text{ min.}$; $\circ$: $1 \text{ hr., } 18 \text{ min.}$; $\circ$: $2 \text{ hr. } 42 \text{ min.}$; $\bullet$: 5 hr. ; $\bullet$: $10 \text{ hr. } 6 \text{ min.}$ (see also table 4). *Part b.* A similar experiment using a strain that had not undergone the alterations described in Methods. $\circ$: $t_d = 2 \text{ hr. } 55 \text{ min.}$; $\bullet$: $5 \text{ hr. } 56 \text{ min.}$; $\bullet$: $11 \text{ hr. } 48 \text{ min.}$; $\bullet$: 21 hr. ; $\otimes$: $2 \text{ hr. } 53 \text{ min.}$ after protracted growth at low dilution rates and viabilities (see table 8).

continuous culture (see Tables 3, 4); the survival curves in Fig. 11 are corrected for the organisms initially dead and refer only to the initially viable proportion of the populations tested. Similarly, the doubling times of the populations given in Tables 3 and 4 are corrected to take the dead organisms into account, since when a proportion of a steady state population is dead the doubling time of the multiplying organisms is no longer $\log 2$ /dilution rate.

Effect of cultural history. We recorded in Methods how our population of *Aerobacter aerogenes* underwent changes before the work reported in this paper was carried out. An experiment with Dr D. Herbert and Mr D. Tempest provided us with the opportunity to repeat and extend the experiment described in the previous paragraph with the unaltered parent strain of *A. aerogenes*. A continuous culture

Table 3. *Viability of populations of Aerobacter aerogenes in slow continuous culture*

The parent stock (see Methods) of *A. aerogenes* was grown in continuous culture at 37° with glycerol limiting growth (see text) and at intervals the dilution rate (proportion of the culture vessel replaced per hour) was halved. Some data from this experiment are included in fig. 11*b*.

Dilution rate (hr. ⁻¹)	Period (days)	Mean viability (%)	No. of determinations	Mean doubling time of viable organisms (hr.)
0.232	3	96.6	4	2.92
0.112	2	94.2	2	5.92
0.055	3	91.3	3	11.8
0.0295	5	86.1	8	21
0.0145	9	76.3	7	38.9
0.0074	24	57.5	19	61.2
0.0038	35	37.8	21	82.0
back to 0.226	4	92.6	4	2.89

was set up differing essentially in that the volume was 2 l., the glycerol concentration (still limiting) was 10 g./l. and the temperature was 37°. The viabilities of the populations at different dilution rates were determined, and in each new steady state duplicate survival curves of populations (equiv. 20 µg. dry wt./ml. at 40° and pH 7.00 ± 0.05) were obtained using a single batch of saline-tris buffer throughout. Some of the data obtained are collected in Table 3 and Fig. 11*b*. Though the death rate was inversely related to the growth rate, in all instances the forms of the survival curves were different from those obtained with our customary population; instead of being arithmetically linear they gave an almost linear plot of probit against time. The steady-state viabilities for a given dilution rate of this population (Table 3) were higher, where they overlapped, than those obtained with our customary population (Table 4) grown in carbon-limiting conditions. Towards the end of the experiment, when the steady-state viability had been low for several divisions, the dilution rate was abruptly restored to the starting value of 0.25 hr.⁻¹. The steady-state viability rose rapidly to over 90 %, yet in spite of the powerful natural selection in favour of longevity that might be expected to have operated during the months of slow continuous culture, the survival curves then obtained did not differ significantly from those observed at the outset (Fig. 11*b*).

Effect of enriching the growth medium. The medium was enriched with yeast

extract and casein hydrolysate; glycerol was still the main component supporting growth. Since these supplements increased the yield (wt. organisms/wt. substrate) the glycerol concentration was lowered to maintain the population in the continuous culture at about equiv. 1 mg. dry wt. organism/ml. The composition of the growth medium was that described in Methods but with 10 g. glycerol and 0.6 g. each Difco casamino acids and Difco yeast extract/l. Organisms grown in these conditions died somewhat faster (18 %/hr.) than the parent culture (16 %/hr.) after a similar number of divisions from the time of inoculation.

Effect of changing limiting nutrient. All the experiments so far recorded refer to populations derived from a continuous culture in which the concentration of glycerol controlled the population density. To study the effect of limitation by other

Table 4. *Influence of growth conditions on various properties of Aerobacter aerogenes populations in continuous culture*

A. aerogenes was grown in continuous culture at different rates and with various nutrients limiting growth. For details of procedures see text; analyses refer to % (w/w) washed, freeze-dried organisms; their accuracy was limited by relatively small amounts of material being available.

(i) Properties of cultures

Limiting nutrient	Dilution rate (hr. ⁻¹)	Population density (mg. dry wt. organisms/ml. culture)	Yield*	Mean viability (%)	t _d † (hr.)
C (21.7 mM glycerol)	0.72	0.92	1.17	98	0.97
	0.5	1.22	1.55	98	1.38
	0.25	1.08	1.38	97	2.75
	0.12	0.84	1.07	85	5.08
	0.06	0.7	0.89	70	8.82
O ₂	0.25	0.49	—	c. 100	2.76
N (5 mM NH ₄ Cl)	0.44	0.52	7.44	96	1.55
	0.24	0.52	7.44	96	2.77
	0.12	0.54	7.7	95	5.69
P (0.4 mM NaH ₂ PO ₄)	0.45	0.64	51.5	97	1.53
	0.27	0.74	60	96	2.54
	0.12	0.88	71	98	5.77
S (0.31 mM Na ₂ SO ₄)	0.41	0.20	200	97	1.68
	0.26	0.24	240	92.5	2.5
	0.12	0.22	220	90	5.35
Mg (20 μM MgCl ₂)	0.43	0.38	391	96	1.58
	0.25	0.57	587	c. 86	2.48
	0.11	0.72	741	62	4.37

(ii) Compositions of organisms

		Protein (%)	RNA (%)	DNA (%)	Polysaccharide (%)
C	0.25	68.6 ± 3.5	12 ± 0.6	3.50 ± 0.03	2.34 ± 0.06
N	0.24	61.3 ± 3.0	14.5 ± 0.7	3.90 ± 0.04	3.80 ± 0.10
P	0.27	77.5 ± 4.5	13.9 ± 0.8	4.43 ± 0.04	2.88 ± 0.07
S	0.26	57.6 ± 2.9	13.7 ± 0.7	3.53 ± 0.03	3.63 ± 0.10
Mg	0.25	74 ± 3.8	17 ± 0.9	4.17 ± 0.04	3.33 ± 0.09

* mg. dry wt organisms/mg. element.

† t_d: mean doubling time of the viable organisms in the culture.

nutrients, portions of our population were transferred to the second continuous culture apparatus and allowed to grow with different components of the medium limiting the population density. The ordinary carbon-limiting conditions gave a population density equiv. 1–1.1 mg. dry wt. organism/ml. Oxygen limitation was obtained by altering the air flow to 0.6 l./hr. and simultaneously decreasing the stirring rate; the population density declined to about half (Table 4). Nitrogen limitation was obtained by using K_2HPO_4 in place of $(NH_4)_2HPO_4$ in the recipe quoted in Methods and adding NH_4Cl to 5 mM. Magnesium limitation was obtained by altering the magnesium content to 20 μM ; sulphur limitation by using KCl in place of K_2SO_4 and adding Na_2SO_4 to 0.81 mM. Phosphorus limitation was obtained by using $NaHCO_3 + NH_4HCO_3$ in place of the phosphates quoted in the recipe and adding NaH_2PO_4 to 0.4 mM; the medium was sterilized by filtration under positive pressure; the culture was buffered to $pH\ 7.0 \pm 0.1$ by incorporating 30 % (v/v) of CO_2 in the gas phase. The organisms were allowed to grow at three dilution rates approximating to 0.12, 0.25 and 0.45 hr^{-1} . Data are collected in Table 4. With C-limitation the yield increased with dilution rate until it passed a maximum where 'wash-out' began; with N- and S-limitation the yield was little influenced by flow rate over the range tested; with P-limitation the yield decreased somewhat at faster flow rates; with Mg-limitation the yield decreased markedly with increasing flow rate (cf. Herbert, 1958). The steady-state viabilities obtained with the S-limited culture probably signify a trend in the direction of the partially dead continuous cultures obtained with very slowly growing C-limited populations. The P- and N-limited culture did not show this phenomenon in the range tested; in other experiments we have observed it with N-limited organisms grown very slowly. The Mg-limited organisms showed the phenomenon to a marked extent.

Strong illumination with visible light accelerated the death of our stored suspensions (see above). The continuous cultures were illuminated with diffuse daylight in day-time and were subject to intermittent illumination by the infra-red heaters which, depending on the type of bulb used, sometimes included considerable amounts of white light. Killing by visible radiation might therefore have contributed to the low steady-state viabilities found in these experiments. A fresh continuous culture of Mg-limited organisms was set up from our customary population and run with strong visible illumination (about 1500 ft.-c.) at a dilution rate of $0.1 \pm 0.01\ hr^{-1}$. When the viability had settled at a steady value for a few days the infra-red heater was changed for one with a red filter (about 80 ft.-c.) for a few days; finally, the culture vessel was wrapped in blackened foil and the temperature maintained at 40° by a water jacket. Throughout this experiment the steady-state viability remained at $75 \pm 5\ %$; we conclude that killing by visible light contributed negligibly to the steady-state viabilities obtained during this work.

The survival curves obtained with these populations showed marked differences. In Fig. 12 survival curves at about 0.25 hr^{-1} are collected; each curve is typical of at least four examples for a given nutrient limitation. In all instances the organisms had spent over 100 generations in their new growth condition because the experiments with Mg-limitation gave some suggestion that the form of the survival curve changed over the first forty or so generations. P- and O-limitation, like the ordinary C-limitation, gave virtually linear survival curves; those of O-limited bacteria were the least steep. S-limitation gave a nearly linear curve, particularly

when corrected for the 10 % of bacteria dead at its start. N-limitation gave a sigmoid curve: a period of slow death was followed by one of rapid decline. The N-limiting medium had a twofold excess of glycerol over that required to account for the population density at $D = 0.25 \text{ hr.}^{-1}$ (Table 4); increasing this excess to fourfold did not significantly alter the survival curves obtained. Mg-limitation gave concave survival curves. All the survival curves flattened after 80–90 % of the population had died; a Mg + Ca mixture (concentrations as in Table 1) prolonged survival of N-, P-, S- and Mg-limited organisms; O-limited organisms were not tested.

The influence of growth rate on death rate in the various conditions of nutrient limitation tested are illustrated in Figs. 13 and 14, which are comparable with

Fig. 12. Effect of nutritional state on death of *Aerobacter aerogenes*. *A. aerogenes* was grown in continuous culture at a dilution rate of 0.25 hr.^{-1} with different components of the medium limiting the growth of the organisms. Survival curves of $20 \mu\text{g./ml.}$ populations in saline-tris buffer aerated at 40° and $\text{pH } 7.00 \pm 0.05$ were obtained (see text). Part (a): ●: limited by S; ○: by N; ◐: by Mg. Part (b): ◐: by O; ●: by P; ○: by C.

Fig. 11a. The generalization that the faster the organisms had grown the slower they died applied to N-, P- and S-limited bacteria, though at the slower growth rates the differences were small compared with those obtained with C-limitation. With N- and P-limitation the survival pattern depended on the length of an initially flatter portion of a sigmoid survival curve; the slopes of the steeper portion did not change markedly among the three growth rates tested. The curves for P-limited organisms illustrate a case in which the flatter portion became undetectably small and the survival curve shifted from sigmoid to linear. The survival of Mg-limited organisms was complicated by the low steady-state viabilities of the populations tested. The actual curves obtained are shown in Fig. 14a, the corrected curves in 14b. The generalization previously observed was reversed in these conditions; the faster these organisms had grown the faster they died (see Discussion). The form of the survival curve shifted with increasing growth rate from sigmoid through linear to concave.

Comparison with stationary phase populations. A well aerated batch culture was inoculated from the continuously growing population and survival curves taken

(a) between the 8th and 9th generation of logarithmic growth, and (b) 100 min. after the population had become stationary due to exhaustion of glycerol. Contrary to expectation, the survival curves were closely similar (Fig. 15).

Some experiments were undertaken to determine the effect of different nutrient limitations on the survival patterns of stationary phase bacteria. Inocula were taken from the continuous culture and allowed to grow as batch cultures (usually under forced aeration) in conditions in which the C, N, Mg or O content of the environment limited growth. After the populations had been at their maximum optical densities for 4–5 hr. the death rates of washed samples were determined in

Fig. 13. Effect of growth rate on death rates of *Aerobacter aerogenes* in different nutritional states. *A. aerogenes* was grown in continuous culture at the dilution rates indicated with different components of the medium limiting the growth of the organisms. The survival curves of 20 $\mu\text{g./ml.}$ populations in saline-tris buffer at $\text{pH } 7.00 \pm 0.05$ and 40° were obtained (see text). Curves are typical of at least four tests in each condition. Part (a) N-limitation at dilution rates of ●: 0.44, ○: 0.24 and ◐: 0.16 hr.^{-1} . Part (b) S-limitation at dilution rates of ●: 0.41, ○: 0.30 and ◐: 0.12 hr.^{-1} . Part (c) P-limitation at dilution rates of ●: 0.42, ○: 0.27 and ◐: 0.12 hr.^{-1} .

the usual way. The experiments described in this and the next paragraph used organisms grown at 37° ; the temperature of the control continuous culture was 37° for their duration. The C-limited population died more rapidly than did a N-limited population, but the survival curve in the latter condition remained linear. Mg-limitation led to an L-shaped survival curve. Oxygen limitation, obtained by growing the organisms in a sealed flask containing a magnetic stirrer, provided the most long-lived population.

Death of synchronized populations. A modified culture apparatus was constructed to provide a continuous supply of synchronously dividing organisms. Its construction (to be described) was based on a principle used by Campbell (1957) to induce partial synchrony in yeasts by subjecting them to repeated short periods of starvation between generations. Figure 16 indicates that this treatment induced partial synchrony with our strain of *Aerobacter aerogenes*. Populations taken from four stages in the cycle showed small differences in their death rates; death was most rapid towards the end of division (70 min.) and at the beginning of the new cycle (10 min.).

Fig. 14

Fig. 15

Fig. 14. Effect of growth rate on death rate of magnesium limited *Aerobacter aerogenes*. *A. aerogenes* was grown in continuous culture at the dilution rates indicated with 20 μM - MgCl_2 limiting growth. The survival curves of 20 $\mu\text{g./ml.}$ population in saline-tris buffer at pH 7.00 ± 0.5 and 40° were obtained (see text). Curves are typical of at least four tests in each condition. Part (a) actual curves at dilution rates of $\bullet$: 0.43, $\circ$: 0.25 and $\bullet$: 0.13 hr.⁻¹. Part (b) same curves corrected for organisms dead initially. Doubling times of viable organisms corresponding to the dilution rates quoted are $\bullet$: 0.16 hr., $\circ$: 2.45 hr. and $\bullet$: 3.8 hr.

Fig. 15. Effect of phase of growth on death of *Aerobacter aerogenes*. *A. aerogenes* was inoculated into a well aerated batch culture and after 8 to 9 generations of 'free' logarithmic growth survival curves of a 20 $\mu\text{g./ml.}$ population in saline-tris buffer at pH 7.00 ± 0.05 and 40° were obtained and compared with similar survival curves obtained later when the same population had completed logarithmic growth owing to exhaustion of glycerol and had been stationary for 100 min. For procedure see text. $\circ$: logarithmic phase organism; $\bullet$: stationary phase organisms.

Fig. 16. Death of synchronized populations of *Aerobacter aerogenes*. *A. aerogenes* was grown at 37° in an apparatus that imposed synchrony on the population as evidenced by part (a); $\circ$: optical density; $\bullet$: plate count (all samples 98 to 100% viable). Survival curves of 20 $\mu\text{g./ml.}$ populations in saline-tris buffer at pH 7.00 ± 0.05 and 37° were obtained with samples taken at intervals through the synchronous division cycle and are quoted in part (b); A: $\bullet$ 10th min.; B: $\bullet$ 30 min.; C: $\bullet$ 50th min.; D: $\circ$ 70th min.

DISCUSSION

Biological integrity of 'dead' micro-organisms. We have used the term 'dead' to describe bacteria that failed to multiply in the arbitrary favourable environment provided by our slide-culture medium. This is a legitimate usage, since microbiologists are usually concerned with the ability of the organisms to initiate a fresh population rather than its survival as an individual. Our organisms retained their osmotic barriers after death and may thus have been in some sense 'alive'; Razumovskaya & Osipova (1958) noticed *Acetobacter melanogenum* organisms which were impermeable to the vital stain acridine orange yet which did not form colonies in plate counts. It follows that the assumption commonly made that dead organisms are permeable to the medium and live organisms are not is not universally true. Since this assumption is the basis of several procedures for the rapid assay of viability (immersion refractometry, vital staining, optical effect, leakage of purine bases), results obtained with these procedures should be interpreted cautiously.

Analysis of stresses. Studies on microbial survival necessarily involve applying some intentional stress (drying, chilling, disinfection, etc.) to the population. Subsidiary stresses may be applied unintentionally. We studied starvation at their growth pH value and temperature as the stress likely (a) to cause least interference with the physiological organization of the organisms and (b) to entail a minimum of subsidiary stresses. Yet our procedure for submitting organisms to starvation proved to engender three subsidiary stresses. The overt stress was limitation of the supply of energy source during growth; subsidiary stresses were: (i) trace element deficiency during death (demonstrated by the protective effect of calcium, magnesium or iron), (ii) a stress due to the pH value of growth not being optimal for survival, (iii) a stress (presumably osmotic) caused by brief exposure to distilled water before storage which committed our populations to a faster death rate than they would otherwise have shown.

Cryptic growth. About fifty of our organisms needed to die to support division of one. Since we have concerned ourselves primarily with the survival of the first 80 % or so of our populations we can be confident that the individual organisms at the beginning and end of the survival curves were the same. This consideration only eliminates cryptic growth as a factor in the survival of population of our C-limited organisms in our routine conditions: equiv. 20 μ g dry wt. organisms/ml. saline-tris buffer at 40°. Cryptic growth may have influenced the survival patterns of organisms studied at other temperatures, in other media or even at higher population densities. It undoubtedly influenced the survival pattern of the organisms when the survival curve was examined over several orders of magnitude (Fig. 1).

Toxicity of buffer. Though we identified copper and neutralized it with EDTA we were unable rigidly to exclude the possibility that a subsidiary stress due to another toxic material in the buffer influenced the survival curves. Three considerations make this possibility unlikely. First, cryptic growth in fact occurred provided one waited long enough; secondly, one would need to attribute rather special properties to the postulated toxic material to account for the variety of survival patterns which were obtained depending on the nutritional status of the population being tested; thirdly, the survival patterns obtained in our routine conditions were unchanged when a saline-phosphate buffer was used instead of saline-tris.

Physical conditions affecting survival. Illumination, though a potential hazard, did not influence our findings. Temperatures below the 40° imposed for growth prolonged survival, but there was a limit below which further cooling accelerated death. This finding is relevant to the custom of storing viable bacteria at refrigerator temperatures: it is possible that at room temperatures survival would be prolonged. The population effect, the dependence of death rate on the concentration of organisms, is a curious phenomenon earlier observed by Harrison (1960) which our experiments entirely confirm. We can add nothing to Harrison's discussion of it beyond the demonstration already mentioned that cryptic growth plays no part in it.

Chemical conditions affecting survival. A pH value lower than 7 favoured survival of our populations, consistent with earlier reports (Cohen, 1922; Winslow & Falk, 1923*a*; Strange *et al.* 1961). Anaerobic conditions accelerated death despite the change to more favourable pH values that usually occurred in these conditions. Inorganic components of the growth medium such as phosphate, potassium, ammonium, sulphate, etc., had no influence on survival though some of these ions have been implicated in the salt balance of microbes. Only certain trace cations, Mg, Ca and to some extent Fe, were protective. Mr R. Strange (personal communication) has observed protection of stationary phase *Aerobacter aerogenes* starved in phosphate buffer by Mg or Ca. The vitamins were unequivocally inactive, but the conclusion that amino acids were inactive must be regarded as tentative since the tests were complicated by growth.

Substrate-accelerated death. Glycerol, the energy source for growth, accelerated death. This observation is consistent with statements in the publications of Ryan (1959) and Strange *et al.* (1961). The phenomenon was not due to toxic impurities in the glycerol since known intermediates in glycerol metabolism also accelerated death; it is being studied further.

Biochemical changes during death. Our populations metabolized their endocellular polymers, thus resembling the organisms studied by Strange *et al.* (1961). Endocellular RNA was metabolized first, the ribose largely oxidized, adenine and cytosine (but not guanine) de-aminated and excreted, inorganic phosphate excreted. Ribose oxidation might well account for the rapid drop in endogenous Q_{O_2} that preceded death. Protein was metabolized later, at such a rate that the 'amino-acid pool' of the bacteria remained unchanged for several hours. Polysaccharide, of which the organisms contained little, was catabolized slightly at the start of the survival curve and then not at all; DNA was scarcely metabolized. Bacteria grown in conditions other than C-limitation may have shown different catabolic patterns (cf. Strange *et al.* 1961), but it seems that with our organisms RNA was the most expendable 'reserve' material.

Rahn & Schroeder (1941), from a study of the catalase and succinic dehydrogenase activities of *Bacillus cereus* near the threshold of thermal death, concluded that enzyme decay did not accompany death. In our experiments 'glycerol dehydrogenase' and 'glycerol oxidase' activities declined in parallel with viability. This parallelism could imply that the relevant enzymic activities remained unchanged until the organism died, when they became negligibly small. Or it may be fortuitous. In either case both this phenomenon and substrate-accelerated death (see above) have an obvious practical relevance to the state of bacteria which are shaken with substrates + buffer for long periods in Warburg manometers.

Effect of metabolic inhibitors. Despite the metabolism that accompanies death none of the metabolic inhibitors tested prolonged survival to any great extent. The protective effect such substances have on aerosols (Webb, 1959*a, b*) must be connected with supplementary stresses involved in aerial suspensions and recovery therefrom. Our failure to observe protection by chloramphenicol contrasts with the report of Morrison, El Bagoury & Fletcher (1956), but Professor R. B. Morrison has informed us that their phenomenon, which occurred in meat-extract broths, was due to inhibition by chloramphenicol of a decarboxylase system which caused the pH value to change to toxic values; our storage conditions did not provide substrates for such an enzyme. Lanthanum and yttrium showed slight protective effects which may have been connected with their anti-phosphatase activity (Clayton, 1959); alternatively, they may have had a sparing action on the organisms' reserves of active ions such as Mg or Ca.

Shapes of survival curves. Despite doubts dating back many years (e.g. Buchanan & Fulmer, 1928), survival curves of dying bacteria are widely expected to be exponential in form. The majority of the C-limited populations of *Aerobacter aerogenes* gave linear survival curves, though after about 80 % had died the curve flattened and could be held to approach an exponential form. For a given growth rate the shape of the survival curve depended on the nutritional status of the population: C-, O-, or S-limitation gave near linear curves, N-limitation gave sigmoid curves and only Mg-limitation gave concave curves approximating to the exponential from the outset.

Effect of biological history. Our strain had undergone the variations reported in Methods. The experiment recorded in Fig. 11*b* involved growing the original laboratory stock which had not changed; the survival curves obtained were smoothly sigmoid and gave linear plots of probit against time. We conclude that the biological history of the population influenced its survival characters, and expectation of this result was one of our reasons for choosing to study organisms that had spent many generations in steady continuous culture. However, our attempt deliberately to select for long-lived organisms was not successful; in contrast Harrison (1961) has briefly noted the isolation of 'starvation resistant' mutants.

Relation of death rate to growth rate. The faster C-limited organisms grew, the slower they died. *Aerobacter aerogenes* growing rapidly with glycerol contains more RNA than slowly growing organisms (Herbert, 1958; his Fig. 11), so this observation is consistent with the suggestion that the longevity of these bacteria was in part determined by their RNA content.

The generalization that the faster the bacteria grew the slower they died applied also to N-, P-, and S-limited populations. Harrison (1961) briefly mentioned a converse relation which (personal communication) applied to N-limited organisms. The only instance of a converse relation occurred with our Mg-limited populations and in this instance the position was complicated by proportions of dead organisms present at the start of the survival curve determination. It could be argued that the most mortal individuals were dead before their survival characters could be examined experimentally, and that the generalization was true in principle even with Mg-limited organisms.

Continuous culture of moribund populations. By decreasing the growth rate until the death rate made an appreciable contribution to the population dynamics, steady

states in which constant proportions of the population were dead were obtained with C-, S- or Mg-limited organisms; Mr D. Tempest has obtained comparable steady states with N-limited *Aerobacter aerogenes*. It seems that these bacteria were obliged either to multiply or die; we were unable to provide them with just sufficient nutrient to maintain themselves indefinitely without dividing. These observations have two consequences of importance. First, a further proportion of the organisms from a moribund population would die during the lag phase of slide culture or plating; hence the viabilities recorded by the techniques were those of the population at the end of its lag phase, not at the time of sampling. This fact probably accounts for the lower viabilities obtained when largely dead populations were plated on a minimal medium compared with those found with the customary 'rich' medium. Secondly, if it is generally true that slow continuous culture of bacteria leads to steady states in which substantial proportions of the populations are dead, then practical installations based on analogous principles (e.g. activated sludge plants; anaerobic digestion of sewage) and natural systems of a similar character (e.g. rumina of animals, sulphur springs) may well be largely populated by dead microbes.

Effect of growth phase. Populations harvested from the stationary phase of growth are often regarded as less fragile than those from the exponential phase (see review by Winslow & Walker, 1939) since they are more resistant to cold shock (Sherman & Albus, 1923; Hegarty & Weeks, 1940; Meynell, 1958; Gorrill & McNeil, 1960), heat shock (Ellicker & Frazier, 1938; Lemcke & White, 1959), decompression (Fraser 1951), desiccation (Lemcke, 1959), freeze-drying (Fry & Greaves, 1951) and other stresses. Our experiments with partly synchronized organisms, which died slightly more rapidly when harvested just before or after division, led us to expect that a proportion of the logarithmic phase organisms from batch cultures of our bacteria would be hypersensitive to death by starvation. In fact this was not so; we could detect no significant differences between the survival patterns of logarithmic phase and early stationary phase populations from batch cultures of our strain. This observation is not necessarily in conflict with the general fragility of other logarithmic phase bacteria, because the examples mentioned above involved different stresses from starvation. But there is a discrepancy between our finding and the report of Strange *et al.* (1961) that stationary phase *Aerobacter aerogenes* from batch culture died considerably less rapidly of starvation in phosphate buffer than did logarithmic phase organisms. Though there are differences in experimental detail between our studies and those of Strange *et al.* (1961), we are inclined to attribute difference in behaviour to differences in the biological histories of the sub-strains studied. Our organisms had spent many months in continuous culture before testing as batch cultures and may have remained physiologically homogeneous on transfer to batch culture compared with organisms subject to repeated batch culture. We suggest that, provided inheritable differences among the individuals in the inoculum are reduced to a minimum, the phase of growth of a population has only a small effect on its susceptibility to death by starvation. Generally speaking, the nutritional status of the population is the most important factor.

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