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Accelerated Death of *Aerobacter aerogenes* Starved in the Presence of Growth-Limiting Substrates

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SUMMARY

Substrate-accelerated death (Postgate & Hunter, 1963*a*) was observed with glycerol-, glucose-, ribose-, ammonium- or phosphate-limited populations of *Aerobacter aerogenes* grown in defined media and starved in non-nutrient buffer; sulphate- or magnesium-limited organisms did not show this. Glucose or pyruvate accelerated death of starved populations obtained from a complex medium. Lactate-accelerated death of *Escherichia coli* and glucose-accelerated death of *Serratia marcescens* were also observed with populations of appropriate nutritional status. Glycerol-accelerated death of glycerol-limited *A. aerogenes* occurred with organisms from batch or continuous cultures grown at various pH values; it showed a population effect and was particularly pronounced in 0·15 M-NaCl buffered with phosphate. Continued presence of glycerol was necessary and the glycerol was metabolized. Survivors showed prolonged division lags. Tricarboxylic acid cycle intermediates, but not glucose or ribose, also accelerated death. Glycerol-accelerated death was not delayed by malonate, fluoride or fluoracetate; iodo-acetate delayed its onset but did not affect its rate; 'uncoupling' agents antagonized it though they were themselves toxic. Glycerol-accelerated death was not accompanied by accelerated breakdown of the osmotic barrier, nor by leakage of materials associated with cold shock nor by acquiring sensitivity to cold shock. No catabolism of DNA or protein accompanied it; polysaccharide was synthesized; no change in the rate of degradation of RNA was observed. Coloured substances, pyridine nucleotides, white-fluorescent material and material which absorbed at 220–230 mμ were released during glycerol-accelerated death. Magnesium ions prevented glycerol-accelerated death.

INTRODUCTION

Postgate & Hunter (1963*a*) showed that substrates which had limited the growth of populations of certain Gram-negative bacteria could accelerate their death when they were subsequently starved in non-nutrient buffer. The phenomenon was termed 'substrate-accelerated death' and was shown with nitrogen-, phosphate- and carbon-limited bacterial populations. Sulphate-limited populations differed in that the carbon source, not sulphate, accelerated death. Magnesium-limited populations did not show substrate-accelerated death. The present paper describes substrate-accelerated death in fuller detail and presents data which exclude certain possible interpretations of glycerol-accelerated death of glycerol-limited *Aerobacter aerogenes*.

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METHODS

Organisms and culture. The variant strain of *Aerobacter aerogenes* NCTC 418, described by Postgate & Hunter (1962), was maintained in the same continuous culture apparatus and was in its third and fourth year of growth at a dilution rate of 0·25 hr^{-1}, temperature 40° and pH value 7·0 ± 0·1. Growth was limited to a yield equivalent to 1–1·1 mg. dry wt. organism/ml. (about $2{\cdot}3 \times 10^9$ bacteria/ml.) by the glycerol concentration (2 g./l.) in the defined medium previously described. (Yields of organism and population densities will be expressed in 'mg./ml.', implying 'equiv. dry wt. bacteria/ml.'.) For growth in other nutritional conditions a second chemostat was inoculated from the first 'master' culture and with the modified media and conditions described by Postgate & Hunter (1962; in that publication the sulphate-limiting medium was incorrectly described [see addendum p. 473). For glucose or ribose limitation the regular medium was used with 2 g./l. of these compounds in place of glycerol; the yields were: 0·95–1 mg./ml. from glucose, and 0·85–0·9 mg./ml. from ribose. In a few experiments organisms other than *A. aerogenes* NCTC 418 were used.

Viability was determined by slide culture (Postgate, Crumpton & Hunter, 1961), except where mentioned, on the glycerol medium supplemented with casein hydrolysate, yeast extract and Douglas's meat digest broth (*Medical Research Council*, 1931); with glucose- or ribose-limited organisms the appropriate compound replaced glycerol. Incubation periods of 4–5½ hr were used for slide culture of populations dying in the presence of substrate because the survivors of substrate-accelerated death showed long division lags (see below).

Starvation. For reasons given in the text two main procedures for starving organisms were used during this work. The 'saline tris procedure' was that described by Postgate & Hunter (1962): the organisms were washed twice in saline, suspended in distilled water and diluted to 20 µg./ml. for starvation at 40° in aerated 0·15 M-NaCl buffered to pH 7·0 ± 0·1 with tris and containing a trace of ethylenediaminetetra-acetate. The 'saline phosphate procedure' avoided exposure to distilled water: organisms were washed only once and the saline was buffered to pH 6·3 (optimal for survival of the strain) or to pH 7·3 with 10 % (v/v) of M/15-sodium potassium phosphate buffer.

Optical and analytical procedures. These were mostly described previously (Postgate & Hunter, 1962). Glycerol was estimated by oxidation with periodate followed by colorimetric determination of formaldehyde with chromotropic acid (Neish, 1950). Magnesium was determined by the Titan Yellow procedure of Garner (1946), scaled down 10-fold and omitting treatment with trichloroacetic acid. Whole bacteria were wet-ashed by repeated evaporation with HNO_3 in the presence of H_2SO_4 before analysis for Mg.

RESULTS

Circumstances in which substrate-accelerated death occurred

Aerobacter aerogenes. When organisms were harvested from continuous culture in the glycerol medium with glycerol limiting growth, and were then starved in saline tris buffer, they showed accelerated death with 10 mM-glycerol; the pH value remained unchanged throughout. Other major components of the growth medium

(sulphate, phosphate, potassium, ammonium) had no influence on the death rate but certain trace elements (Mg^{2+}, Ca^{2+}; and to some extent Fe^{3+}) were protective (Postgate & Hunter, 1962). Further experiments, with the saline tris procedure, showed that glucose (10 mM) or ribose (10 mM) did not accelerate death, though dye-reduction tests indicated that both these substrates were metabolized by the organisms (Table 1). A continuous culture limited by ribose was therefore set up. The population did not show significant ribose-accelerated death when subjected to

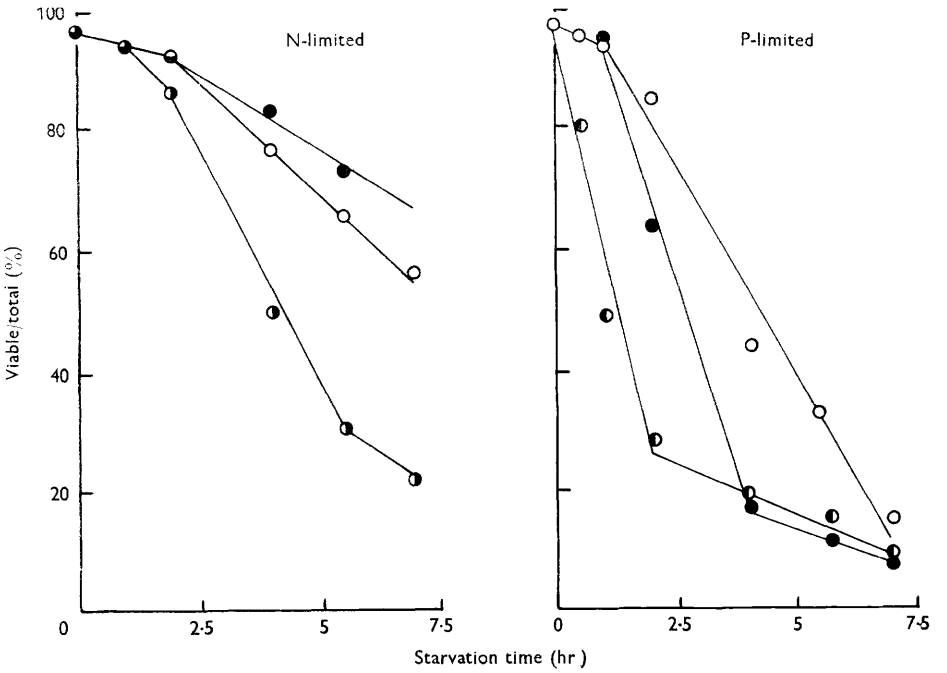

Fig. 1. Substrate-accelerated death of nitrogen- and phosphate-limited *Aerobacter aerogenes*. Organisms harvested from continuous culture, washed and starved at about 20 μg./ml. in aerated saline tris buffer (pH 7·0) at 40°. Viabilities were determined by slide culture (see text). ○, control population; ●, + 10 mM-glycerol; ◑, +45 mM-NH_4Cl; ◐, +10 mM-KH_2PO_4.

Table 1. *Reduction of methylene blue by glycerol-limited Aerobacter aerogenes*

Aerobacter aerogenes organisms from continuous culture were aerated for 15 min. at 40° to remove residual glycerol and 2 ml. portions (equiv. 2 mg. dry wt. organisms) added to 4 ml. portions of saline tris buffer (pH 7·0), containing 10 mM substrates, in Thunberg tubes. After evacuation and equilibration for 6 min. at 40°, 0·5 ml. methylene blue was added (final concn. 0·67 mM) and the decolorization times noted. Means of duplicates are quoted.

Substrate	Decolorization time (min.)
Glycerol	1·16
Glucose	1·83
Ribose	4·85
None	30

the saline tris procedure but a pronounced effect occurred with the saline phosphate procedure (e.g. 8·7 % died/hr in saline phosphate (pH 7·3); 83 % were dead in 0·5 hr with buffer + 10 mM-ribose). Glycerol also accelerated death (93 % dead in 0·5 hr with 10 mM glycerol). A glucose-limited continuous culture gave a population that showed slight glucose-accelerated death with the saline tris procedure but a pronounced effect with saline phosphate (death rate: 17 %/hr at pH 7·3; 94 %/hr with 10 mM-glucose). Continuous cultures in which nutrients other than the carbon-*cum*-energy source limited growth were set up to observe whether glycerol or the limiting

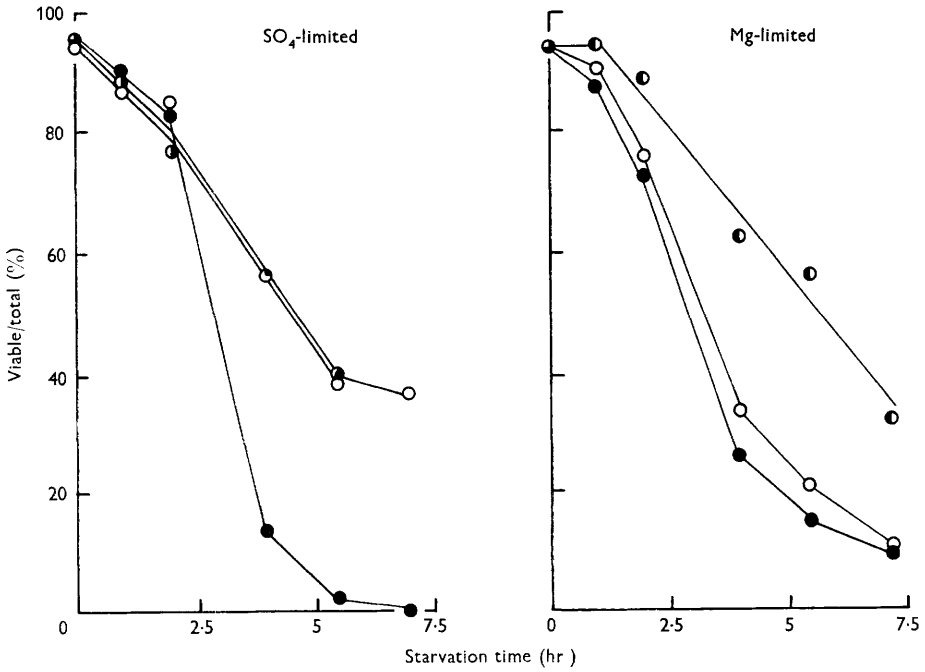

Fig. 2. Effect of substrates on death of sulphate- and magnesium-limited *Aerobacter aerogenes*. Organisms tested as for Fig. 1. ○, control population; ●, +10 mM-glycerol; ◑, +10 mM-Na_2SO_4; ◐, +1·25 mM-$MgCl_2$.

nutrient accelerated death. With the saline tris procedure N-limited organisms showed NH_4-accelerated death, but glycerol delayed death; P-limited organisms showed PO_4-accelerated death and glycerol also accelerated death, though to a lesser extent (Fig. 1). S-limited organisms did not show accelerated death with sulphate but glycerol was active (Fig. 2). Mg-limited organisms showed delayed death with $MgCl_2$ and sometimes with glycerol (Fig. 2). A population of *Aerobacter aerogenes* NCTC 418 was grown in Douglas's meat digest broth and we confirmed the observation of Strange, Dark & Ness (1961) that glucose (10 mM) accelerated its death in saline phosphate at pH 6·5. Sodium pyruvate (10 mM) was also active (Fig. 3); data illustrating the acceleration of death of mannitol-limited stationary phase *A. aerogenes* by mannitol or glucose were quoted by Postgate & Hunter (1963*a*).

Other bacteria. Fig. 4 illustrates accelerated death of lactate-limited *Escherichia coli* (Jepp) by lactate or pyruvate, and of glucose-limited *Serratia marcescens* (M 148) by glucose, both from batch cultures. In general, substrate-accelerated death was less pronounced with stationary phase cultures, and the appearance of the slide cultures of the dying populations suggested to us that cryptic growth (see Postgate & Hunter, 1962) was masking the phenomenon. Cryptic growth was obvious as an increase in optical density of the suspension 'starved' with substrate in unsuccessful attempts to demonstrate substrate-accelerated death with mannitol-limited *Bacillus subtilis*, acetate-limited *Pseudomonas ovalis* and glucose-limited *Candida utilis*.

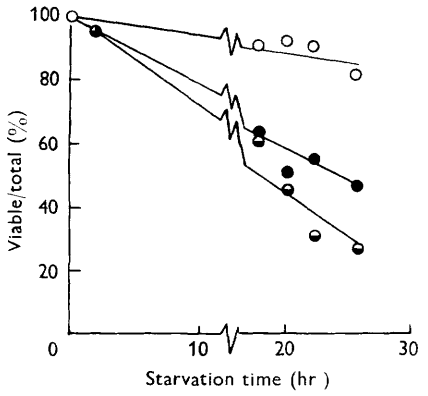

Fig. 3

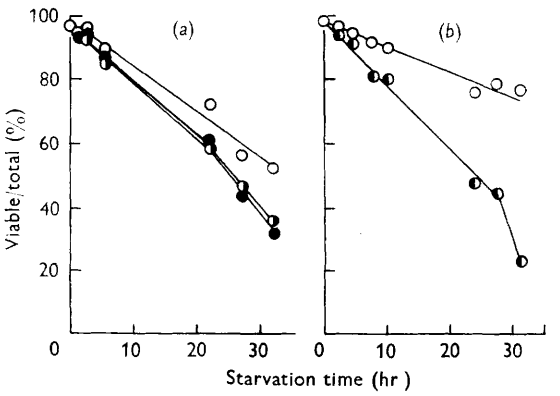

Fig. 4

Fig. 3. Effect of substrates on death of stationary phase *Aerobacter aerogenes* from a complex medium. Organisms grown at 37° in Douglas's meat digest broth were washed twice and starved (at about 20 μg./ml.) in aerated saline phosphate buffer (pH 6·5) at 37°. Viabilities by slide culture on Douglas's meat digest agar. ○, control; ◒, + 10 mM-glucose; ●, + 10 mM-sodium pyruvate.

Fig. 4. Substrate-accelerated death of *Escherichia coli* and *Serratia marcescens*. *E. coli* strain Jepp, and *S. marcescens* M148 were grown at 37° in lactate + salts and glucose + salts defined media, respectively, in shaken flasks containing sufficient air to allow complete oxidation of all of the carbon substrate provided. When in the stationary phase, the organisms were washed and starved at about 20 μg./ml. in aerated saline phosphate buffer (pH 6·5) at 37°. Viabilities were determined by slide culture on the medium mentioned in the text with sodium lactate or glucose in place of glycerol as appropriate. (*a*) *E. coli*; (*b*) *S. marcescens*. ○, control population; ◑, + 10 mM-sodium lactate; ●, + 10 mM-sodium pyruvate; ◐, + 10 mM-glucose.

Glycerol-accelerated death of Aerobacter aerogenes

Effect of washing and storage procedure. Postgate & Hunter (1962) found that a brief exposure to distilled water experienced during preparation for starvation by the saline tris procedure committed the organisms to a faster death rate than would otherwise have occurred. Omission of exposure to distilled water did not abolish glycerol-accelerated death in saline tris buffer. Postgate & Hunter (1962) also reported that the growth pH value of 7·0 was inferior to pH 6–6·5 for survival during starvation. Since tris does not buffer appreciably at pH values below about 7·0 saline phosphate was adopted for general use unless comparison with earlier work with saline tris was required. At comparable pH values, glycerol-accelerated death was more pronounced in saline phosphate buffer than in saline tris buffer: at pH 7·3,

70 ± 10 % of the populations were dead in 1 hr in saline phosphate buffer + 10 mM-glycerol compared with 50 ± 10 % dead/hr in saline tris buffer + glycerol. Control populations without glycerol died at 12–15 %/hr in either buffer.

Effect of washing procedure. Starvation by the saline phosphate procedure at pH 7·3 or 6·3 allowed pronounced glycerol-accelerated death, despite the absence of a pre-exposure to distilled water. When organisms were pre-exposed to distilled water, as for the saline tris procedure, their rates of glycerol-accelerated death were unchanged in saline phosphate buffers, though pre-exposure to water accelerated death in these buffers if glycerol were absent. Organisms washed exclusively in distilled water showed a small but reproducible decrease in their rate of glycerol-accelerated death compared with organisms washed in saline. Of a 97 % viable population starved with 10 mM-glycerol, 53 % of those washed in distilled water were viable after 30 min. compared with 26 % of those washed in saline; by 60 min. both populations had fallen below 2 % viable.

Growth characteristics of survivors. Casual observation suggested that survivors of death accelerated by ammonium, phosphate, glycerol etc. had long lag periods compared with survivors of starvation in plain buffer. The growth characteristics of survivors of glycerol-accelerated death were measured as follows. Populations in saline phosphate buffer (pH 6·4) were starved for 0·5 or 1 hr with and without glycerol, centrifuged, and re-suspended in liquid medium (that used for slide culture but without agar) warmed to 40°. At this point the viability of the population was determined by slide culture, its optical density (OD) measured, and the culture allowed to grow while aerated through a Pasteur pipette. Viabilities were measured during the lag phase and optical densities during growth. A semi-logarithmic plot of the OD increase was extrapolated to an OD equivalent to that of the viable proportion of the original inoculum (e.g. a population of initial OD of 0·03 and viability 66 % was regarded as having a 'viable OD' of 0·02) and the intercept was taken as a measure of the lag. The lags observed were: 150 min. for survivors of a population which had died to 66 % viable during 30 min., 240 min. for one that had died to 10 % viable over 1 hr. A control population starved without glycerol for 1 hr decreased from 99 to 94 % viable and had a lag period of 36 min. No further death of survivors of glycerol-accelerated death during the lag phase was detected by slide culture, and populations derived from the survivors had mean generation times similar to that of the control population (51–54 min.). In these respects the survivors of substrate-accelerated death differed markedly from the survivors of freezing and thawing (Postgate & Hunter, 1963*c*).

Comparison with plate counts. Slide culture can give false values for the viability if the scatter of individual lags is wide, due to overgrowth of dead organisms by colonies from individuals of short lag (Postgate *et al.* 1961). Though longer incubation times were adopted for the study of glycerol-accelerated death (see Methods), some comparisons with plate counts were made. Figure 5 illustrates that the agreement between slide and plate cultures was adequate; the increased lag of survivors of substrate-accelerated death was reflected in a longer incubation period required for the plates to reach a constant count.

Effect of recovery medium. The presence of glycerol in the recovery medium was not necessary for the expression of glycerol-accelerated death. The organism grew equally well on slide cultures on medium prepared according to the usual recipe but

without glycerol. The survival curves of populations suffering glycerol-accelerated death were indistinguishable from those obtained with the glycerol medium. For these experiments the samples of the dying population were centrifuged and re-suspended in saline before slide cultivation to avoid carry-over of glycerol from the starvation buffer.

Effect of pH value of growth. Continuous cultures of *Aerobacter aerogenes* were grown at pH 6·5, 5·5 and 4·9. Populations from these cultures all showed pronounced glycerol-accelerated death when tested at pH 6·5 by the saline phosphate procedure.

Effect of glycerol concentration. Glycerol-accelerated death of 20 μg./ml. populations occurred to similar extents at glycerol concentrations between 10 mM and 100 μM in saline phosphate (pH 7·3); the phenomenon was less pronounced at 50 μM and undetectable at 10 μM (contrast succinate; see below). Typical values after 1 hr of starvation were: 23 % viable with 10 mM-glycerol, 29 % with 100 μM, 41 % with 50 μM; the control without glycerol was 82 % viable, like the population with 10 μM-glycerol.

Effect of population density. Below a certain maximum, death by starvation is slower the denser the bacterial population (Harrison, 1960; Postgate & Hunter, 1963*b*). Glycerol-accelerated death showed a comparable phenomenon (Table 2).

Table 2. *Effect of population density during glycerol-accelerated death of Aerobacter aerogenes*

Washed organisms were starved in aerated saline phosphate buffer (pH 7·3) at 40° and various population densities, with between 10 mM and 50 mM-glycerol. Initial viabilities: 98 %.

Population density (equiv. mg. dry wt./ml.)	Time: 1 hr Viability (%)	Time: 2 hr Viability (%)
2·0	92	69
0·3	46	3
0·02	18	1

Effect of pre-treatment with glycerol. Aerobacter aerogenes organisms (250 mg./ml.) were aerated for 10 min. at 40° in saline phosphate (pH 6·5) with and without 30 mM glycerol, and then were washed, drained and tested for survival in saline phosphate (pH 6·5) as usual. None died during the pre-treatment, and the death rates were similar when the organisms were subsequently starved in the plain buffer. A control suspension not pre-exposed to glycerol showed glycerol-accelerated death. Hence a brief pre-treatment of the organism with substrate did not commit them to a faster death rate than they would otherwise have shown.

Effect of metabolic intermediates. Glucose or ribose did not accelerate death (see above). Postgate & Hunter (1962) had shown, with the saline tris procedure, that the sodium salts of pyruvate, oxaloacetate, α-ketoglutarate, malate, succinate, or citrate accelerated death when used in place of glycerol. These compounds were retested at 10 mM with the saline phosphate procedure at pH 6·3; all accelerated death, though malate had only a small effect. Succinate appeared more effective than glycerol: like glycerol, it showed a maximum effect at 100 μM or above, but gave a perceptible effect at 10 μM; it had no effect at 1 μM. Pyruvate was active over

a concentration range similar to that of glycerol; citrate was only effective at 1 mM and above.

Effect of metabolic inhibitors. Postgate & Hunter (1962) listed a variety of inhibitors tested for an effect on death of *Aerobacter aerogenes* starved in plain saline tris buffer. Certain of those likely to be involved in terminal oxidative metabolism were re-tested for an effect on death accelerated by 10 mM-glycerol, with the saline tris procedure. Sodium fluoracetate (10 mM) or sodium fluoride (1 mM) had no effect on the accelerated death rate. Sodium malonate (2 mM) further accelerated death with glycerol or succinate; at 400 μM it had no effect. Similar results were obtained when the saline phosphate procedure was used. Sodium iodoacetate (1 mM) hastened glycerol-accelerated death of organisms prepared by the

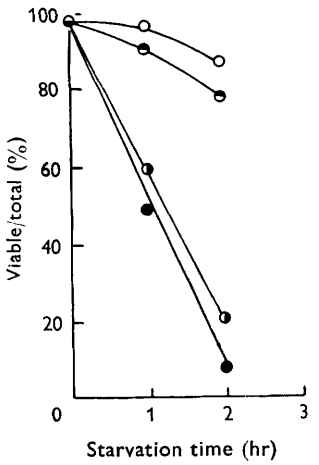

Fig. 5

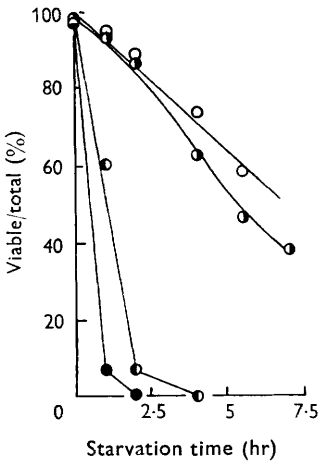

Fig. 6

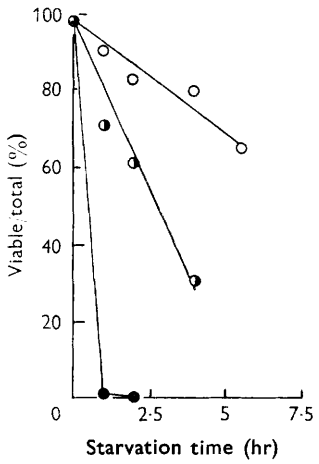

Fig. 7

Fig. 5. Demonstration of glycerol-accelerated death by plate and slide culture. *Aerobacter aerogenes* from a continuous culture was washed and starved at 20 μg./ml. in aerated saline phosphate buffer (pH 6·5) at 40°. Viability by slide culture or by plate count after micropipette dilution (Postgate & Hunter, 1963*c*) in 0·15 M-NaCl; viabilities from plate counts are plotted as % initial value, taken as equal to the initial viability obtained by slide culture. ○, slide culture; ◓, plate count on control population; ●, slide culture; ◑, plate count on population in presence of 10 mM-glycerol.

Fig. 6. Effect of 2,4-dinitrophenol on glycerol-accelerated death of *Aerobacter aerogenes*. *A. aerogenes* prepared as for Fig. 5 and starved in presence of: ●, 10 mM-glycerol; ◐, 1 mM-2,4-dinitrophenol; ◑, 10 mM-glycerol + 1 mM-2,4-dinitrophenol; ○, control population. Samples (1 ml.) were centrifuged and re-suspended in 0·15 M-NaCl before estimating viability by slide culture.

Fig. 7. Glycerol utilization during glycerol-accelerated death of *Aerobacter aerogenes*. *A. aerogenes* prepared as for Fig. 5. ○, viability of control population; ●, + 1 mM glycerol; ◑, proportion of glycerol remaining in centrifuged samples.

saline tris procedure, but with the saline phosphate procedure it delayed the onset of glycerol-accelerated death for up to 1 hr; death subsequently occurred at a rate similar to that of the population without inhibitor. α-Keto-γ-valerolactone-γ-carboxylic acid (KVC) inhibits α-ketoglutarate oxidation (Montgomery & Leyden-Webb, 1954); it was tested at 10 mM with glycerol, α-ketoglutarate or succinate (10 mM) using the saline phosphate procedure and it delayed substrate-accelerated

death with the last two compounds. With glycerol, however, no significant protection by KVC against substrate-accelerated death was observed. These observations suggested a specific protective effect by KVC, but spontaneous hydrolysis of KVC at about pH 7 was found by observing its behaviour alone in saline tris buffer: the pH value dropped from pH 7 to pH 6 in 2 hr, and to pH 5 in 7 hr. Little pH change occurred in the experiments already mentioned, but it is obvious that the agent was not stable in the test conditions. The inhibitors mentioned so far were not unequivocally protective, but two compounds showed clear positive effects with the saline phosphate procedure. Figure 6 shows protection by the uncoupling agent 2,4-dinitrophenol. Though toxic by itself at 1 mM, this substance virtually prevented glycerol-accelerated death at that concentration. A similar effect occurred with azide: it slowed the rate of glycerol-accelerated death but did not prevent it entirely. Azide was tested between 1·5 and 10 mM and showed maximum protection at 5 mM: after 4 hr at pH 6·4, when the populations with azide or glycerol alone were less than 3 % viable, that with azide + glycerol was 26 % viable; controls, with neither substance, were 55 % viable. In all these experiments, azide was more toxic in plain buffer (death rate 30 %/hr with 1·5 mM-azide, and 12 %/hr without azide, in saline phosphate buffer at pH 6·4) than recorded by Postgate & Hunter (1962: 10 %/hr with azide, and 8 %/hr. without azide). Azide was retested with the saline tris procedure and showed an intermediate toxic effect: 20 %/hr with azide, 13 %/hr without.

Utilization of substrate. Glycerol utilization during glycerol-accelerated death was implied by the experiments already described and by later ones (e.g. those on polysaccharide synthesis discussed below). Disappearance of glycerol during glycerol-accelerated death was followed by analysis of centrifuged samples (Fig. 7) and indicated that glycerol utilization continued after all the population was empirically 'dead'. The respiratory quotient of starved organisms declines in parallel with the viability during starvation (Postgate & Hunter, 1962) and the effect of presence of substrate during conventional Warburg respirometry was therefore examined. In a typical experiment, eight Warburg vessels were set up with conventional quantities of organism and glycerol; two without glycerol served as blanks. At intervals, pairs of vessels were removed for viability determination by slide culture. Figure 8 illustrates the most impressive of these experiments; in others glycerol-accelerated death was less pronounced during the 80-min. period but was nevertheless clear. Its extent presumably depended on whether the organisms utilized all the substrate before it 'killed' them.

Effect of magnesium. Magnesium delays death from starvation in saline tris buffer or saline phosphate buffer (Postgate & Hunter, 1962). At 1·25 mM (its concentration in the growth medium) Mg abolished glycerol-accelerated death in both buffers (Fig. 9). Other major components of the growth medium (K_2SO_4, 10 mM; $(NH_4)_2HPO_4$, 4·5 mM) as a mixture had no effect. Experiments were made to determine the minimum effective magnesium concentration: in saline phosphate (pH 6·3), $MgCl_2$ between 6·25 and 12·5 μM brought the death rate of a 20 μg./ml. suspension with 10 mM glycerol back to that of a comparable suspension without glycerol and magnesium; 25 μM-$MgCl_2$ lowered the death rate to that with magnesium but no substrate. $FeCl_3$ (1·6 mM) or $CaCl_2$ (0·1 mM), which had some protective action on organisms starved in plain buffer, did not influence glycerol-accelerated

death at pH 6·4 in saline phosphate buffer. $MnCl_2$ (25 μM), which had no effect on organisms starved in plain buffer, antagonized glycerol-accelerated death to a slight extent. For example, all of an initially 99 % viable population were dead after 1 hr in saline phosphate buffer + 10 mM glycerol (pH 6·4), whereas in a similar solution with $MnCl_2$ they were 27 % viable. The control suspension was 96 % viable.

Polymer catabolism. Death of *Aerobacter aerogenes* by starvation is preceded by the metabolism of certain polymeric cell constituents (Strange *et al.* 1961). Polymer catabolism with and without glycerol was studied, using the saline phosphate procedure and the assay methods cited by Postgate & Hunter (1962). No significant change in the DNA and protein contents of 240 and 300 μg./ml. suspensions,

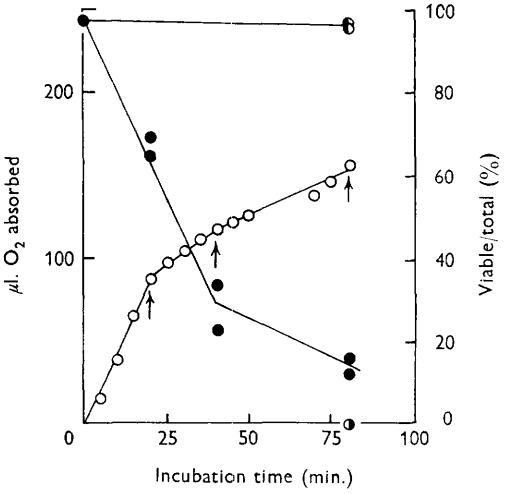

Fig. 8

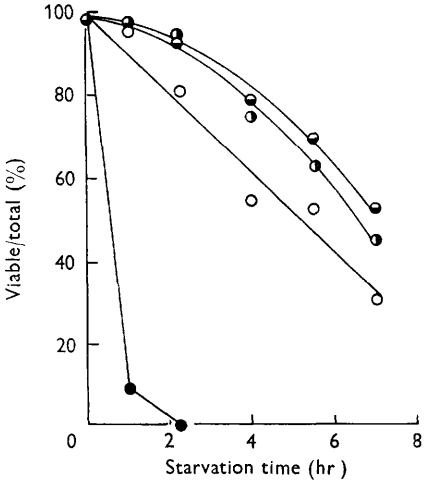

Fig. 9

Fig. 8. Substrate-accelerated death during Warburg respirometry. Warburg vessels containing equiv. 2 mg. dry wt. organism in 2 ml. saline phosphate buffer (pH 7·3); 0·1 ml. KOH (10 M) in centre well; vessels were equilibrated at 40° and, after tipping 3·26 μmole glycerol in 0·2 ml. water as substrate, pairs of vessels were dismantled during the reaction (times indicated by arrows) for viability determination on their contents by slide culture after dilution in 0·15 M-NaCl. ○, mean O_2 uptake values for pairs of vessels with glycerol; ◑, blank pair without glycerol; ●, viabilities of populations in individual flasks with glycerol; ◐, viabilities of blank pair.

Fig. 9. Abolition of glycerol-accelerated death of *Aerobacter aerogenes* by magnesium ions. *A. aerogenes* prepared as for Fig. 5 and starved in saline phosphate buffer (pH 6·3). Viabilities by slide culture. ○, control population; ●, +10 mM-glycerol; ◒, +1·25 mM-$MgCl_2$; ◑, + glycerol + $MgCl_2$.

respectively, occurred at pH 7·3 over 2 hr, although the viabilities fell to 2 and 4 % with 10 mM glycerol. Without glycerol, the viabilities were unchanged at 96–98 %. In three experiments the polysaccharide contents of 750 μg./ml. suspensions (pH 7·3) increased 2·1- to 3-fold in 2–4 hr, while the viabilities fell from 99 % to between 60 and 80 % and the control populations without glycerol remained wholly viable. RNA catabolism, indicated by the Bial reaction, was slower with 100 μg./ml. populations at 7·3 and pH 6·4 than recorded by Postgate & Hunter (1962). This may be related to the longer survival of populations treated by the saline phosphate procedure compared with those prepared by the saline tris procedure. At neither

pH value was the rate of RNA breakdown altered when death was accelerated with 25 mM glycerol, though after 4 hr these populations were 85 % to 98 % dead and the viabilities of control populations without glycerol had scarcely changed from the starting value of 97 %. Both showed a 10–15 % decrease in RNA content over this period.

Excretion of materials. Micro-organisms starved in buffer excrete into the medium materials which absorb radiation at about 260 mμ (see Strange *et al.* 1961; Postgate & Hunter, 1962). Differences in the amounts of such materials were not detected during the death of a 60 μg./ml. population dying in saline phosphate buffer, with or without 30 mM-glycerol, because a strong absorption at about 230–240 mμ appeared which obscured the 260 mμ peak. This absorption reached an apparent maximum in 2 hr, during which time the viability of the population decreased from 99 to 2 %; the control population decreased from 98 to 86 % viable. Twenty-fold concentrates of saline phosphate buffer (pH 6·4) in which 20 μg./ml. populations had died during incubation for 7 hr, with or without 10 mM-glycerol, had different appearances. Those from the populations whose death had been accelerated by glycerol were brownish-yellow in colour and had a pronounced white fluorescence in light of 365 mμ wavelength; the controls were colourless and showed little fluorescence. The cyanide reaction (Ciotti & Kaplan, 1957) indicated that, after glycerol-accelerated death, pyridine nucleotides (equivalent to about 5 μM in the original buffer) were present which were absent from the controls. Since loss of these materials may have been a factor in glycerol-accelerated death, mixtures of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (10 and 33 μM) were tested with the saline phosphate procedure. These compounds did not influence glycerol-accelerated death. Attempts were made to measure the magnesium contents of such concentrates. Analyses of whole bacteria prepared as for the saline phosphate procedure indicated 0·19 % (w/w) Mg; hence our conventional 20 μg./ml. suspensions corresponded to 1·6 μM magnesium. In our hands the Titan Yellow procedure would just have detected the Mg in 20-fold concentrates of such a solution, but analyses of concentrates of buffers after bacterial death with or without glycerol showed no detectable magnesium. $MgCl_2$ added to such concentrates was recovered quantitatively; hence other materials in the concentrates did not interfere with the analysis. Concentrated buffers in which denser (200 μg./ml.) populations had died did interfere, however; they delayed the appearance of the red colour and lowered the blank reading, as well as diminishing the recovery of added $MgCl_2$. Buffers from populations subjected to glycerol-accelerated death for 6·5 hr interfered to a lesser extent than controls from populations without glycerol. Evaporation of such concentrates with H_2SO_4 followed by NaOH, to remove the volatile acids and NH_3 which interfere with the Titan Yellow reaction (Feigl, 1954), did not prevent this interference. Both types of concentrate interfered with the 'magneson' reaction (Anon. 1949).

State of the osmotic barrier. Postgate & Hunter (1962) used the optical effect of Mager, Kuczynski, Schatsberg & Avi-dor (1956), and permeability to a dyestuff that fluoresced in contact with protein, to show that the osmotic barriers of *Aerobacter aerogenes* organisms starved by the saline tris procedure remained functional after death. Comparable experiments with the saline phosphate procedure showed that glycerol-accelerated death at pH 7·3 did not involve accelerated breakdown of the

osmotic barrier. Some experiments with the saline tris procedure, and measurement of the permeability to anilino-naphthalene sulphonic acid fluorimetrically, confirmed that glycerol- or succinate-accelerated death did not involve accelerated breakdown of the osmotic barrier in these conditions.

State of the 'permeability control mechanism'. 'Cold shock' is characterized by release of undegraded ATP and amino acids into the environment and has been interpreted in terms of inhibition of a permeability control mechanism by abrupt chilling (Meynell, 1958; Strange & Dark, 1962). Since cold shock only occurs among organisms from exponentially growing cultures, it was conceivable that exposure of starving organisms to their limiting substrate might induce a state physiologically analogous to susceptibility to cold shock. Leakage of products comparable to those which appear during cold shock, as well as susceptibility to cold shock, were therefore sought during glycerol-accelerated death. Suspensions of *Aerobacter aerogenes* (2 mg./ml.) dying at pH 6·5 in saline phosphate, with and without 50 mM-glycerol, were sampled after 1 and 2 hr (glycerol-accelerated populations fell to 66 % viable; controls unchanged at 97 %), filtered rapidly into an ice-cold receiver through a membrane filter (Oxoid), the filtrates frozen in liquid nitrogen and stored at $-20°$ for examination next day. ATP was sought by the firefly luminescence technique; none was detected. Cold shock would have released readily detectable amounts of ATP from a susceptible population of this density (Strange & Dark, 1962). Chromatographic examination of the filtrates indicated no enhancement of the amino acid excretion in the presence of glycerol. Our populations of *A. aerogenes* do not ordinarily show cold shock (Postgate & Hunter, 1961). No signs of susceptibility to cold shock (increased death after chilling for 30 min. at 4°) appeared during the death of an 80 μg./ml. suspension accelerated by 40 mM-glycerol in saline phosphate (pH 7·3), nor during starvation of a control population without glycerol. The test population decreased from 98 to 17 % viable during the 60 min. starvation period; the control did not die at all. Strange & Dark (1962) showed that certain concentrations of spermine had a slight protective effect against cold shock. Spermine at 10 μM did not influence glycerol-accelerated death of a 20 μg./ml. population at pH 6·3 in saline phosphate buffer; at 100 μM spermine accelerated death.

DISCUSSION

Ubiquity of substrate-accelerated death. Substrate-accelerated death appears to be a fairly general phenomenon associated with the survival of starved *Aerobacter aerogenes*. It can be observed in organisms harvested from complex media, in which the 'limiting' nutrient is not known and may change as the culture grows. It occurs with other Gram-negative bacteria. Substrate-accelerated death differs in principle from the 'suicidal' behaviour of certain mutants (e.g. inositol-less *Neurospora crassa*, Strauss, 1958; thymine-less *Escherichia coli*, Barner & Cohen, 1956), because 'suicidal' behaviour is provoked by withdrawal of the required nutrient in conditions otherwise favourable to growth, whereas our phenomenon is elicited by providing a previously needed substrate in conditions which, nevertheless, remain unfavourable for growth. Substrate-accelerated death of *A. aerogenes* occurred whether the limiting substrate was the nitrogen, phosphorus or carbon-*cum*-energy source; it was not observed when the sulphur source limited growth, though in this instance

the carbon source continued to accelerate death. It seems possible that the sulphur source limited growth indirectly by influencing the rate of some step in carbon utilization. Magnesium-limited organisms did not show substrate-accelerated death, but this is reasonable since Mg ions protect ordinary starved populations, irrespective of nutritional status (Postgate & Hunter, 1962). Magnesium prevented substrate-accelerated death brought about by glycerol.

The fact that the nutritional status of the population determines which substrate, if any, will accelerate death presumably explains certain conflicting reports in the literature cited by Postgate & Hunter (1963*a*), who pointed out the relevance of substrate-accelerated death to the design of physiological experiments which make use of live 'resting' bacteria; e.g. enzyme induction, respirometry and the replacement of 'maintenance energy' by small additions of substrate. Figure 8 illustrates an extreme case of substrate-accelerated death during Warburg respirometry, and though the existence of such a phenomenon need not necessarily invalidate the conclusions obtained from the respirometric experiments, it seems likely from our data that part of the 'uncoupling' effect of compounds such as azide and 2,4-dinitrophenol in respirometry might be connected with the preservation of viability.

Action of magnesium. The antagonism of glycerol-accelerated death by magnesium showed some differences from the protection these ions ordinarily afford against starvation. Calcium ions had no action in place of Mg ions and Fe^{3+} ions were not protective. Manganese ions, which had no protective effect in ordinary starvation (this observation was re-checked with the saline phosphate procedure), may have antagonized glycerol-accelerated death by sparing the organisms' reserves of magnesium ions. Strange & Shon (1964) showed that organisms washed in NaCl solutions lost more magnesium than those washed in distilled water and became more sensitive to a mild heat stress. A comparable retention of stored magnesium might account for the lower sensitivity to glycerol-accelerated death of organisms washed in distilled water compared with those washed in saline.

Mechanism of substrate-accelerated death. The data presented here do not allow a complete account of substrate-accelerated death and do not establish whether the inorganic substrates, ammonium or phosphate, accelerate the death of susceptible populations by a mechanism similar to that of carbon-*cum*-energy sources such as glycerol, glucose or lactate. For the case of glycerol-accelerated death, the excretion of coloured and fluorescent material, together with the existence of a population effect, implicate a loss, from dying organisms, of materials which, once they reach a threshold concentration, prolong the lives of the surviving neighbours (see discussion of population effects by Harrison, 1960, and Postgate & Hunter, 1963*b*). Magnesium ions may be among the relevant materials excreted, but if so their concentration was below the range of analytical procedures available to us. The release of such materials must be precipitated by the presence of glycerol or some active substitute for it; their release required the continued presence of glycerol in the starvation environment but not in the medium used to assess viability; it did not involve accelerated breakdown of the osmotic barrier, nor did changed permeability characters appear that resembled those which render logarithmic-phase organisms susceptible to cold shock. Accelerated breakdown of the endocellular polymers, DNA, RNA and protein, did not occur and polysaccharide was laid down during glycerol-accelerated death.

The long lags shown by survivors of substrate-accelerated death, which occurred with inorganic and with organic substrates, suggests that reclamation of the lost material after transfer to the recovery medium was a slow process, or that the active substrate influenced the organisms' metabolism in such a way that their behaviour then resembled that of bacteria subject to repression of enzyme synthesis. Recovery from repression is known to involve long lags (Bourgeois, Wiame & Lelouchier-Dagnelie, 1960) and several instances are known in which a substrate may repress synthesis of its own enzyme. For example, β-galactosidase synthesis by partly adapted *Escherichia coli* may be repressed by lactose (Mandelstam, 1961) and in this instance not only are intermediates in the metabolism of lactose also repressive but repression is antagonized by 2,4-dinitrophenol. Hypotheses based on analogy with the repression of enzyme synthesis would be consistent with the fact that glycerol continues to be utilized by operationally 'dead' organisms (Fig. 7), despite the finding of Postgate & Hunter (1962) that metabolism of glycerol declined in parallel with viability during death in a plain buffer, because such hypotheses would envisage repression during turnover of some factor essential for subsequent multiplication. The existing complement of catabolic enzymes would be primarily unaffected by the active substrate. However, the chemical disparity of the agents that induce substrate-accelerated death, together with the variety of intermediates that can replace glycerol, and the capriciousness with which utilizable substrates which are not intermediate metabolites may or may not accelerate death, all make it clear that simple repression of an enzyme sequence involved in the utilization of the substrate provides an inadequate theoretical approach. The active substrate must trigger-off some more central process in the regulatory mechanism of the organism, presumably one concerned with the issue or transfer of genetic information for constitutive biosynthetic reactions.

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REFERENCES

ANON. (1949). *The B.D.H. Book of Organic Reagents.* Poole: The British Drug Houses Ltd.

BARNER, H. D. & COHEN, S. C. (1956). The relation of growth to the lethal damage induced by ultra-violet irradiation in *Escherichia coli*. *J. Bact.* **71**, 149.

BOURGEOIS, S., WIAME, J. M. & LELOUCHIER-DAGNELIE, H. (1960). Étude du 'rétrocontrole' de synthèses d'enzymes par des acides aminés au cours de la croissance de *Proteus morganii*. *Biochim. biophys. Acta*, **38**, 136.

CIOTTI, M. M. & KAPLAN, N. O. (1957). Procedures for determination of pyridine nucleotides. In *Methods in Enzymology*. Ed. by S. P. Colowick & N. O. Kaplan. Vol. **3**, p. 890. New York: Academic Press.

FEIGL, F. (1954). *Spot Tests.* Vol. **1**. London: Elsevier.

GARNER, R. J. (1946). Colorimetric determination of magnesium in plasma or serum by means of Titan Yellow. *Biochem. J.* **40**, 828.

HARRISON, A. P., JUN. (1960). The response of *Bacterium lactis aerogenes* when held at growth temperature in the absence of nutriment: an analysis of survival curves. *Proc. Roy. Soc.* B, **152**, 418.

MAGER, J., KUCZYNSKI, M., SCHATZBERG, G. & AVI-DOR, Y. (1956). Turbidity changes in bacterial suspensions in relation to osmotic pressure. *J. gen. Microbiol.* **14**, 69.

MANDELSTAM, J. (1961). Induction and repression of β-galactosidase in non-growing *Escherichia coli*. *Biochem. J.* **79**, 489.

Medical Research Council (1931). *A System of Bacteriology in Relation to Medicine.* London: H.M.S.O.

MEYNELL, G. G. (1958). The effect of sudden chilling on *Escherichia coli*. *J. gen. Microbiol.* **19**, 380.

MONTGOMERY, C. M. & LEYDEN-WEBB, J. (1954). Detection of a new inhibitor of the tricarboxylic acid cycle. *Science*, **120**, 843.

NEISH, A. C. (1950). *Analytical Methods for Bacterial Fermentations. Nat. Res. Council, Canada*, Rep. 46–8–3, Saskatoon.

POSTGATE, J. R. & HUNTER, J. R. (1961). On the survival of frozen bacteria. *J. gen. Microbiol.* **26**, 367.

POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.

POSTGATE, J. R. & HUNTER, J. R. (1963*a*). Acceleration of bacterial death by growth substrates. *Nature, Lond.* **198**, 273.

POSTGATE, J. R. & HUNTER, J. R. (1963*b*). The survival of starved bacteria. *J. appl. Bact.* **26**, 295.

POSTGATE, J. R. & HUNTER, J. R. (1963*c*). Metabolic injury in frozen bacteria. *J. appl. Bact.* **26**, 405.

POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.

STRANGE, R. E. & DARK, F. A. (1962). Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. *J. gen. Microbiol.* **29**, 719.

STRANGE, R. E. & SHON, M. (1964). Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension. *J. gen. Microbiol.* **34**, 99.

STRANGE, R. E., DARK, F. A. & NESS, A. G. (1961). The survival of stationary phase cells of *Aerobacter aerogenes* stored in aqueous suspension. *J. gen. Microbiol.* **25**, 61.

STRAUSS, B. S. (1958). Cell death and 'unbalanced growth' in Neurospora. *J. gen. Microbiol.* **18**, 658.

ADDENDUM

An earlier paper by Postgate & Hunter (1962) contained several errors. We publish the following table of corrections and apologize to anyone who may have been misled.

Reference	*Correction*
Page 236, line 13	*For* $\pm 0{\cdot}01/\mathrm{hr}^{-1}$ *read* $\pm 0{\cdot}01\ \mathrm{hr}^{-1}$
Page 237, line 22	*For* Millipore *read* membrane
Page 240, sketch	*For* 26·7 *read* 96·7
Page 245, last line	*For* $\times 10^{+5}$M *read* $\times 10^{-5}$M
Page 246, table 2	*Insert* no *after* or *in last column*
Page 246, table 2	*For* Fig. 9 *read* Fig. 7 (in note marked †)
Page 248, line 36	*For* uracil *read* uridine
Page 253, table 4 Page 254, line 10	*For* 0·31 mM-Na_2SO_4 *read* 0·031 mM-Na_2SO_4
Page 255, Fig. 12	*Label ordinate* viable/total, %
Page 256, Fig. 13*a*	*Third point in D* = 0·44 *curve should have symbol* ◒
Figs. 2–5, 11, 13–16	*For* % viable total *read* viable/total, %