On Substrate-accelerated Death in *Klebsiella aerogenes*

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SUMMARY

Glycerol-accelerated death of starved, glycerol-limited populations of *Klebsiella aerogenes* occurred if the medium used to assess viability contained glycerol, but not if it contained pyruvate or glucose. Pyruvate-accelerated death of glycerol-limited populations occurred with all three types of recovery medium. Lactose-limited populations showed lactose-accelerated death, with comparable substrate and recovery medium specificity, without change in β -galactosidase content. Inducers of the *lac* operon, whether gratuitous or not, also accelerated death of lactose-limited populations. Cyclic AMP (0.08 mM) or its butyryl derivative (0.02 mM), but not a variety of other nucleotides, prevented substrate-accelerated death but did not abolish the prolonged lags characteristic of survivors of this stress; these findings further support the view that substrate-accelerated death is related to intracellular repression-derepression processes.

INTRODUCTION

Substrate-accelerated death is the phenomenon whereby that substrate which had limited the growth of populations of certain Gram-negative bacteria could accelerate death when the organisms were subsequently starved in non-nutrient buffer (Postgate & Hunter, 1964). It occurred with carbon-, ammonium- or phosphate-limited populations of *Klebsiella aerogenes* (syn. *Aerobacter aerogenes*) but not those limited by magnesium ions or sulphate. Strange & Dark (1965) could not confirm substrate-accelerated death with ammonium- or phosphate-limited populations. Phosphate-accelerated death may have been a technical artifact, but with ammonium-accelerated death the discrepancy was due to one group working with a variant of the strain used by the other: the substrain used by Postgate & Hunter, which differed from the parent in colony form, biochemical reactions, $\mu_{\max}$, size and ability to synthesize polysaccharide, showed ammonium-accelerated death in the hands of Strange & Hunter (1966).

During starvation of *Klebsiella aerogenes* in a saline phosphate buffer, Strange, Dark & Ness (1961) and Postgate & Hunter (1962) observed that the decrease in viability was accompanied by breakdown of polymers and decrease in dry weight. The precise polymers degraded depended on the nutritional status; glycogen or protein was utilized if plentiful, DNA was not used. RNA was always utilized, though less rapidly if glycogen was plentiful, bases being excreted and ribose assimilated. Protein was degraded without changes in the contents of pool amino acids. The osmotic barrier remained intact. Strange, Dark & Wade (1963) reported that, at 37°, the level of ATP increased initially and then declined. Postgate & Hunter (1964) reported that the catabolic patterns were unchanged during glycerol-accelerated death except that polysaccharide was synthesized and nicotinamide nucleotides were excreted into the medium. They noted that the survivors of substrate-accelerated death

exhibited long division lags. Magnesium ions protected populations from glycerol-accelerated death but did not influence the excretion of u.v.-absorbing material; glycerol-accelerated death did not involve enhanced excretion of Mg^{+2} . Sodium azide or 2,4-di-nitrophenol also protected against glycerol-accelerated death though both compounds were toxic alone.

Strange & Dark (1965) proposed that a toxic product of glycerol metabolism could be responsible for glycerol-accelerated death although 2-oxoglutarate, a major product, did not accelerate death. Strange (1967) mentioned that *Klebsiella aerogenes* starved in buffer with glucose showed an increased ATP pool; Mg^{2+} prevented ATP accumulation.

In this paper we report further features of substrate-accelerated death including a role of the recovery medium, its alleviation by cyclic AMP and a study of substrate- and inducer-accelerated death in lactose-limited bacteria. A preliminary account of the effect of cyclic AMP and of recovery media has been given (Calcott & Postgate, 1971).

METHODS

Klebsiella aerogenes (NCTC 418) was grown aerobically at 37° in chemostats (Baker, 1968) of effective volume 220 ml at a dilution rate of $0.23 \pm 0.01 \text{ h}^{-1}$. The population density was limited by the concentration of carbon source in the basal medium of Postgate & Hunter (1962) to 3.4×10^9 (glycerol 2 g/l) or 2.2×10^9 (lactose 2 g/l) organisms/ml, equivalent to 0.92 or 0.60 mg dry wt organisms/ml respectively. The maximum time for which we used continuous culture was three months, a similar period to that employed by Strange & Hunter (1966) and not the four years of Postgate & Hunter (1962). Hence our populations probably corresponded closer to the parent strain than to Postgate & Hunter's substrain.

Culture samples were washed twice in saline phosphate buffer (0.13 M-NaCl+0.02 M-sodium phosphate, pH 7.4) and starved with aeration at 37° in 40 ml buffer (with or without supplements) containing approximately 10^8 organisms/ml as described by Postgate & Hunter (1962).

Viabilities of the starving population were determined by slide culture (Postgate, Crumpton & Hunter, 1961). The recovery media used were modifications of their slide culture medium: the basal continuous culture medium, without carbon source, was supplemented with yeast extract (Oxoid) 0.1 % (w/v); Casamino acids (Oxoid) 0.1 % (w/v), nutrient broth (Oxoid) 10 % (v/v); Bactoagar (Difco) 1.5 % (w/v); and the desired carbon source at 2 % (w/v). The validity of slide culture was checked with conventional viable counts on plates of medium identical to that used for slide culture; the populations were diluted in saline phosphate with micropipettes (Postgate, 1969).

β -Galactosidase was assayed by the method of Strange (1961) except that the bacteria were disrupted with toluene, not benzene. The time courses were carried out at about 25° (room temperature), at 420 nm using a Pye-Unicam SP 1800 spectrophotometer.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin, Fraction V (Sigma Chemical Co., London, S.W. 6) as standard. Magnesium was estimated on a Southern Analytical Atomic Absorption Spectrometer. All chemicals used were of analytical reagent grade.

RESULTS

Specificity of substrate-accelerated death

Death curves, with or without substrate, were linear over the first 80 % of the population as recorded by Postgate & Hunter (1962), but our populations died more slowly. Only the

substrate which had limited growth of the population, or metabolites immediately derived from it, accelerated death. With the glycerol-limited population, glycerol or sodium pyruvate, but not glucose, accelerated death, and with the lactose-limited population, lactose, glucose, pyruvate, but not glycerol, accelerated death (typical data are included in Table 1).

Effect of magnesium

As Postgate & Hunter (1964) reported, Mg^{2+} protected populations from substrate-accelerated death. With our populations, 80 μM - $MgCl_2$ protected completely from glycerol-accelerated death.

Cryptic growth

Normally 10^8 organisms/ml were used, a population density which may be used for slide culture directly. However, at this population density, cryptic growth (Ryan, 1959) obscured the true death-rate (in contrast to the behaviour of Postgate & Hunter's (1964) substrain), but was prevented by addition of benzyl penicillin at 100 international units (i.u.)/ml, a bacteriostatic concentration. The antibiotic did not affect substrate-accelerated death rates because, at lower population densities (5×10^6 or 10^7 organisms/ml), where cryptic growth was very much less, 100 i.u. benzyl penicillin/ml did not alter the glycerol-accelerated death-rate; nevertheless, for comparisons, critical experiments were repeated in the absence of penicillin using initial death-rates, before cryptic growth became significant.

Effect of recovery medium

Sodium pyruvate, glucose and glycerol were assessed as accelerators of death of glycerol-limited populations using recovery media identical except for the major carbon-energy source. Similarly, sodium pyruvate, glycerol, glucose and lactose were tested with lactose-limited populations using four complementary recovery media. Acceleration of death

Table 1. *Specificity of substrate-accelerated death of Klebsiella aerogenes and effect of recovery medium*

Samples from glycerol- or lactose-limited populations were washed twice, suspended at 10^8 organisms/ml in saline phosphate buffer, pH 7.4 (100 i.u. penicillin/ml was present) and starved at 37° under forced aeration with and without 1 mM substrates. Viabilities were determined at intervals by slide culture, linear death rate plotted and their slopes recorded in % dead/h.

Supplements to starvation environment	Major carbon-energy source in recovery medium			
	Lactose	Glycerol	Pyruvate	Glucose
	Death-rate (% dead/h)			
Glycerol-limited population				
Nil	—	5	10	10
Glycerol	—	28	9	9
Sodium pyruvate	—	27	30	24
Glucose	—	8	6	10
Lactose-limited population				
Nil	3	4	5	5
Lactose	20	5	5.5	6
Glycerol	3	5	6	4
Sodium pyruvate	16	15	20	23
Glucose	25	6	6.5	27

depended on the recovery medium (Table 1): glycerol- or lactose-accelerated death was observed only on glycerol or lactose media respectively; pyruvate-accelerated death, which occurred with both glycerol and lactose-limited populations, was expressed on all recovery media; glucose, a catabolite of lactose, only accelerated death of lactose-limited populations but did so on both lactose and glucose media.

Protection by 3'-5' cyclic adenosine monophosphate (cAMP)

2,4-Dinitrophenol prevented de-adaptation of maltose utilization by glucose in yeast (the 'glucose effect') (Spiegelman & Reiner, 1947) and also protected *Klebsiella aerogenes* from glycerol-accelerated death (Postgate & Hunter, 1964). Because catabolite repression in *Escherichia coli* is alleviated by 3'-5' cAMP (see DeCrombrugghe, Perlman, Varmus & Pastan, 1969) its effect on substrate-accelerated death was tested. It protected glycerol-limited populations from glycerol- or pyruvate-accelerated death on all relevant recovery media at 0.1 mM. The following adenosine nucleotides (0.1 mM) did not prevent glycerol-accelerated death: 2'-AMP, 3'-AMP, 5'-AMP, ADP, ATP and adenosine; nor did the cyclic nucleotides, cCMP, cGMP, cTMP, cIMP, cUMP, 2'-3'-cAMP. Protection by cAMP was

Table 2. *Mean division lags of survivors of glycerol-accelerated death of Klebsiella aerogenes*

Samples from glycerol-limited populations were washed twice, suspended at 10^8 organisms/ml in saline phosphate buffer, pH 7.4, and starved under forced aeration at 37° with or without supplements for 4 h. The organisms were harvested by centrifugation and inoculated into a liquid glycerol medium similar to that used for slide culture but without agar. A logarithmic growth curve was plotted and mean lags obtained by back extrapolation (Postgate & Hunter, 1964).

Supplement to starvation environment	Lag (min)	Viability (%)
None	20	85
cAMP (0.1 mM)	25	84.7
Glycerol (1 mM)	60	43
Glycerol + cAMP	55	82

Table 3. *Acceleration of death of starved, lactose-limited Klebsiella aerogenes by lactose and its analogues*

Samples of lactose- or glycerol-limited populations were washed twice, suspended at 10^8 organisms/ml in saline phosphate buffer, pH 7.4 (100 i.u. penicillin/ml was present) and starved with or without supplements (1 mM analogues) under forced aeration at 37°. Viabilities were determined at intervals by slide culture, linear death rates plotted and their slopes recorded in % dead/h.

Recovery medium ...	Lactose-limited		Glycerol-limited	
	Lactose	Glycerol	Lactose	Glycerol
Supplement to starvation environment	Death-rate (% dead/h)			
None	4	4.7	5.5	5.4
Lactose	20	4.0	5.7	6.4
Lactitol	28.3	4.5	4.4	2.4
Lactulose	30.2	4.6	4.5	3.0
Isopropyl-thio- β -D-galactoside	23	6.3	4.7	5.4
Methyl-thio- β -D-galactoside	18.6	5.6	4.7	5.4
Methyl- β -D-galactoside	5.2	4.8	—	—
Raffinose	4.3	4.5	—	—
Lactobionic acid	4.6	6.1	—	—
Phenyl-thio- β -D-galactoside	> 50	> 50	19.0	23.2

—, Not tested.

not due to Mg^{2+} being introduced as an impurity in the cAMP because, though specimens contained traces of Mg^{2+} (as did the buffer), even the most contaminated batches did not contain more than $8 \mu M$ - Mg^{2+} , well below the minimum protective level. cAMP protection was not affected by the presence of penicillin as populations could be protected fully in its absence.

Titration of cAMP and an analogue, 6-*N*-2'-*O* dibutyryl cAMP, which is more active in certain systems (Cehovic, Marcus, Gabbai & Posternak, 1970), gave 0.08 mM and 0.02 mM, respectively, as the lowest concentrations which fully protected our usual populations against glycerol-accelerated death. cAMP (0.1 mM) also protected the lactose-limited populations from lactose-accelerated death.

Lag periods of survivors of glycerol-accelerated death

Survivors of glycerol-accelerated death have extended lags when compared with survivors in saline phosphate buffer (Postgate & Hunter, 1964). cAMP, although protecting a population from glycerol-accelerated death, did not diminish this characteristic lag (Table 2).

These findings were confirmed by a different method. The average number of divisions undergone by microcolonies of survivors on slide cultures during a fixed incubation period is an indication of lag before division started. The mean number of divisions undergone by approximately 300 survivors of 5 h of glycerol-accelerated death, incubated 4 h on a slide culture, was 1.3 compared with 2.6 for the controls without glycerol. cAMP did not alter these values, although it protected the population from glycerol-accelerated death (90 % viable compared with 40 % for unprotected populations). Penicillin was absent from the starvation environment in this test.

Analogues of accelerators of death

Lactose analogues which act as inducers of β -galactosidase synthesis accelerated death of lactose-limited populations. They showed population and recovery medium specificity: they did not accelerate death of glycerol-limited organisms and were only effective with lactose-limited populations if a lactose recovery medium, not a glycerol medium, was used (Table 3). The death-rates in the presence of active analogues were not altered by removal of the inducers by centrifuging before slide culture, nor were they altered by omission of penicillin from the starvation environment. As with true lactose-accelerated death, death induced by lactose analogues was alleviated by 0.1 mM-cAMP or 1 mM- Mg^{2+} . Phenyl-thio- β -D-galactopyranoside, though a lactose analogue, was anomalous in that it accelerated death of either glycerol- or lactose-limited populations on glycerol or lactose recovery media. Magnesium ions or cAMP did not alleviate acceleration of death by this analogue and we conclude that it was acting as a non-specific bacteriocide.

β -Galactosidase content

The levels of β -galactosidase in lactose-limited populations starved with lactose might be relevant to the specificity of lactose-accelerated death (see Discussion). The β -galactosidase content of a population after starving for 3 h with 1 mM-lactose was unaltered from its initial value of 0.5 μ mole *o*-nitrophenol released/mg bacterial protein/min during which time the viability fell to 30 %. A corresponding population starved in saline-phosphate buffer retained similar activity while viability fell to 85 %.

DISCUSSION

Several situations are known in which the response, in terms of viability, of a microbial population to a stress occurs on the recovery medium. With u.v.-irradiated populations, the apparent death rate is higher if rich recovery media are used instead of minimal media (see Howard-Flanders, Simson & Theriot, 1964). Conversely, 'metabolic injury' in freeze-damaged bacteria (see Postgate & Hunter (1963) for references) occurs because apparent viabilities of frozen and thawed populations are greater when determined on rich media than on poorer media. Substrate-accelerated death falls into this class of response, in that the apparent viability depends on the composition of the recovery medium. Table 1 suggests that a subtle medium specificity occurs. If the growth-limiting nutrient (glycerol or lactose) provides the overt stress, death only occurs in media containing that nutrient; if a metabolite (pyruvate) common to several substrates provides the stress, death occurs on media containing any of the substrates. It followed that the overt stress, that of exposing the susceptible population to the growth-limiting substrate, does not actually kill the population. In some ways the stressed populations behave like mutants defective in an enzyme on the catabolic pathway of the death-accelerating substrate, but this analogy is not satisfactory. Only complete loss of β -galactosidase could account, in these terms, for the different specificities of glucose and lactose recovery media recorded in Table 1 for lactose-limited populations exposed to lactose. No evidence for any decline in the content of this enzyme during exposure to lactose was obtained.

Possible mechanisms of substrate-accelerated death have been discussed by Postgate & Hunter (1964), Strange & Dark (1965), Strange & Hunter (1966), Elsdén (1967) and Postgate (1967). We shall not review the whole question but shall add the relevance of our findings to it. Our work with lactose analogues shows that utilization of the substrate, or a metabolite derived from it, is not essential for true acceleration of death, since the inducers isopropyl-thio- β -D-galactoside and methyl-thio- β -D-galactoside both accelerate death of lactose-limited populations but are not utilized. Ability to induce the *lac* operon, however, appears to be essential, because lactobionic acid and raffinose, both non-inducing analogues of lactose, do not accelerate death (Table 4). These observations rule out, at least for the

Table 4. *Certain properties of lactose and its analogues relevant to substrate-accelerated death*

Data compiled from Table 3, Jacob & Monod (1961) and Dr W. H. Holms (personal communication).

Substance added to starvation environment	Ability to accelerate death of lactose-limited bacteria	Ability to induce <i>lac</i> -operon	Ability to enter organism	Ability to be hydrolysed
Lactose	+	+	+	+
Lactitol	+	+	+	+
Lactulose	+	+	+	+
Isopropyl-thio-galactoside	+	+	+	—
Methyl-thio-galactoside	+	+	+	—
Methyl-galactoside	—	—	+	+
Raffinose	—	—	+	—
Lactobionic acid	—	—	?	—
Phenyl-thio-galactoside	*	—	+	—

* Non-specific bactericide, see text.

case of lactose-accelerated death, the idea of Strange & Dark (1965) that a toxic product of substrate utilization was responsible for the accelerated death.

Long lag periods shown by survivors of substrate-accelerated death perhaps indicate that the active substrate influences their metabolism in such a way that they resemble bacteria subject to repression of enzyme synthesis (Postgate & Hunter, 1964). Recovery from repression is known to involve long lags (Bourgeois, Wiame & Lelouchier-Dagnelie, 1960). The analogy is strengthened because cAMP, like 2,4-dinitrophenol, can alleviate substrate-accelerated death and also catabolite repression. The fact that only inducers of the *lac* operon are able to mimic lactose-accelerated death of lactose-limited populations further supports this idea. However, the long lags themselves cannot be the sole reason for substrate-accelerated death since cAMP has no effect on these. Our findings do not yet suggest a coherent account of substrate-accelerated death at the molecular level, but they strengthen the idea that populations suffering from this phenomenon are undergoing acute repression.

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