‘Substrate-Accelerated Death’ of Nitrogen-Limited Bacteria

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SUMMARY

‘Substrate-accelerated death’ (Postgate & Hunter, 1963*a*; 1964) occurred when a nitrogen-limited variant of *Aerobacter aerogenes* NCTC 418 (Postgate & Hunter, 1962) was starved at growth temperature (37° or 40°) in aerated saline buffers containing ammonium ion; it was not observed when the parent strain of *A. aerogenes* or *Escherichia coli* (MRE 162) was grown and starved under similar conditions. Sulphate ion increased the lethal effect of ammonium ion on the variant and magnesium did not abolish either the effect of ammonium or ammonium + sulphate ions. The *A. aerogenes* variant differed from the parent strain in morphology, colonial appearance on nutrient agar, biochemical and immunological reactions and ability to synthesize polysaccharide. In ammonium-limited medium at 37° or 40° at a dilution rate near 0.25 hr⁻¹ the variant contained 3–5% and the parent strain 12–16% of dry weight as polysaccharide; in spent medium or phosphate buffer at 37° with added glycerol the rate of polysaccharide synthesis by the variant was about 25% that of the parent strain. When grown in nitrogen-deficient medium with excess glycerol in batch culture, populations of the variant containing 25% polysaccharide were obtained; the survival of the polysaccharide-rich variant was not affected by ammonium ion.

INTRODUCTION

Strange & Dark (1965) failed to observe ‘ammonium-accelerated death’ of *Aerobacter aerogenes* as reported by Postgate & Hunter (1963*a, b*, 1964). The respective tests differed in that Postgate & Hunter used a variant of *A. aerogenes* grown and starved at 40°, whereas Strange & Dark used the parent strain grown and starved at 37°. Re-investigation has confirmed these findings and shown them to be true whether the organisms were grown and starved at 37° or 40°. Evidently the apparent discrepancy was not due to differences in technique or interpretation of results but to a difference in the organisms themselves. During continuous growth at 40° under nitrogen-limiting conditions at a dilution rate of 0.25 hr⁻¹ the variant contained much less polysaccharide than the parent strain did when growing under similar conditions but at 37°, and this difference was offered by Strange & Dark (1965) as a possible explanation for the divergent results. The present paper records a comparison of various properties of the variant and parent strains of *A. aerogenes*, including their survival in saline buffers with and without ammonium ion, and polysaccharide synthesis under various conditions. Some data obtained with strains of *Escherichia coli* are included.

METHODS

Organisms. *Aerobacter aerogenes*, NCTC 418 and a variant strain of this organism (Postgate & Hunter, 1962); *Escherichia coli*, strains MRE 162 (laboratory strain) and NCTC 8164.

Media and cultural conditions. Organisms were grown at 37° and 40° in chemostats (designed by Dr D. Herbert) with culture vessels containing 100 or 250 ml. medium (Postgate & Hunter, 1962; Strange & Dark, 1965). Defined nitrogen-limiting and carbon-limiting media described by Postgate & Hunter (1962) contained glycerol (2–10 g./l.) as the carbon energy source. Chemostats were inoculated with fully grown shaken batch cultures of the organisms grown in these media. The dilution rate was usually maintained near 0.25 hr⁻¹.

Viability determinations. These were made with slide cultures (Postgate, Crumpton & Hunter, 1961) or viable counts (Strange, Dark & Ness, 1961). The enriched agar medium used for both methods was described by Postgate *et al.* (1961) and contained 0.2% (w/v) glycerol or glucose.

Starvation. Bacteria were separated from the culture and washed twice in the appropriate saline buffer by centrifugation. The bacterial pellet was resuspended (at a concentration of 1–2 × 10⁹ bacteria/ml.) in saline buffer. Volumes of washed bacterial suspension (0.2–0.6 ml.) were added to saline buffer with and without additions (final vol. 10–30 ml.) usually in test tubes (18 × 3 cm.); the test suspensions were aerated with sterile washed air through Pasteur pipettes. When experiments lasted more than 6 hr, suspensions were usually aerated in Dreschel type gas wash-bottles from which effluent air escaped through a water condenser fitted with a filter, or were agitated in Erlenmeyer flasks on a reciprocating shaker.

Saline buffers. Saline tris (Postgate & Hunter, 1962) contained 135 mM-NaCl, 4.8 mM tris buffer and 0.82 mM-EDTA (pH 7.0–7.2); saline phosphate 1 (Postgate & Hunter, 1962) contained 135 mM-NaCl and 6.7 mM sodium potassium phosphate buffer (pH 6.5); saline phosphate 2 (Strange & Dark, 1965) contained 108 mM-NaCl and 20 mM-potassium phosphate buffer (pH 6.5). 'Specpure' saline phosphate (pH 6.7–6.8) contained highly purified NaCl (108 mM), KH₂PO₄ (20 mM) and K₂CO₃ (5.4 mM) (Johnson, Matthey & Co., Ltd., Hatton Garden, E.C. 1) dissolved in glass-distilled water, and the solution was sterilized and stored in polypropylene bottles.

Analytical methods. Bacterial dry weights and total carbohydrate were determined as previously described (Strange & Dark, 1965).

RESULTS

Comparison of some properties of the variant and parent strains of Aerobacter aerogenes

The variant of *Aerobacter aerogenes* appeared and completely replaced the parent strain during continuous cultivation in glycerol-limiting defined medium at 37° and 40° in a chemostat (Postgate & Hunter, 1962). The ability of the variant to replace the parent strain in chemostats at dilution rates of 0.25–0.5 hr⁻¹ was confirmed by mixing in a third chemostat samples from separate chemostat cultures of the two organisms and determining the relative proportion of each organism after various periods of growth (Fig. 1). At dilution rates above 0.5 hr⁻¹, the parent strain out-

grew the variant. As found by Postgate & Hunter (1962), the growth rate of the variant was lower than that of the parent strain during unrestricted growth in defined medium in batch cultures (Fig. 2).

Fig. 1

Fig. 2

Fig. 1. Growth of glycerol-limited parent and variant strains of *Aerobacter aerogenes* in mixed culture in chemostats at various dilution rates. Samples of cultures of the organisms growing separately in chemostats at the given dilution rate were mixed in a third chemostat and the proportion of each organism was determined by differential colony counts after intervals of growth at 37°. Proportion of variant organisms at dilution rates of 0.25 (○, ×), 0.3 (●), 0.5 (△) and 0.62 (□) hr⁻¹. One mixed culture (×) was seeded with 0.1% variant.

Fig. 2. Comparison of the growth rates of parent and variant strains of *Aerobacter aerogenes* in defined medium at 37° with glycerol as the carbon energy source. Medium was inoculated with samples of glycerol-limited cultures (dilution rate, 0.5 hr⁻¹) and aerated at 37°. Turbidities of variant (○) and parent strain (●) cultures were measured in a spectrophotometer at 540 mμ.

Chemostats were inoculated with batch cultures of the variant grown from single colonies on solid medium, but after several days growth, samples of chemostat cultures produced three colonial forms on nutrient agar, all of which differed from parent strain colonies. The relative proportion of the different variant colonies varied from day to day but one type always predominated. The average length of variant organisms was greater than that of parent strain organisms (Postgate & Hunter, 1962). During the present work the parent strain did not alter in colonial appearance during continuous culture in either N-limiting or C-limiting media at 37° or 40° for 3 months.

The biochemical reactions of the variant in peptone water containing various carbohydrates differed from those of a typical *Aerobacter aerogenes*; in general, no gas or much less gas was produced and no acid was apparent with dextrin or starch. The biochemical reactions of pure cultures of the three colonial types of the variant differed slightly from each other.

Table 1. *Effect of ammonium ion on the survival of N-limited bacteria in saline buffers*

Bacteria growing in N-limiting medium (dilution rate $0.23-0.25 \text{ hr}^{-1}$) were washed and starved (3.5×10^7 bacteria/mL) at 37° or 40° in aerated saline buffers with and without NH_4Cl . Viabilities were determined at intervals by slide culture (s.c.) or viable counts (v.c.). 'SP 1', 'SP 2' and 'ST' refer to saline phosphate and saline tris buffers (see Methods).

Organism	Growth temp.	Diluent	pH	Starvation temp.	NH_4Cl	Time (hr)				Viability assay
						0	6	24	48	
<i>A. aerogenes</i> NCTC 418	37°	SP 1	6.5	37°	{ Nil 22 mM }	99	99	98	96	s.c.
						99	99	95	94	
	37°	SP 2	6.5	37°	{ Nil 22 mM }	99	98	97	95	
						99	99	93	89	
	37°	ST	7.0	37°	{ Nil 22 mM }	99	98	94	85	
						99	99	93	82	
	40°	SP 1	6.5	37°	{ Nil 15 mM }	99	96	76	—	
						98	93	80	—	
	40°	SP 1	6.5	40°	{ Nil 15 mM }	98	95	59	—	
						98	92	58	—	
<i>A. aerogenes</i> variant	40°	SP 2	6.5	40°	{ Nil 25 mM }	100	85	76	34	v.c.
						100	88	75	33	
	37°	SP 1	6.5	40°	{ Nil 25 mM }	97	93	44	16	
						99	26	8	1	
	37°	SP 2	6.5	37°	{ Nil 25 mM }	98	93	35	6	
						98	62	11	6	
	40°	SP 1	6.5	40°	{ Nil 25 mM }	98	73	—	—	
						99	39	—	—	
	37°	SP 1	6.5	40°	{ Nil 15 mM }	98	97	96	96	
						98	94	94	95	
<i>E. coli</i> MRE 162	37°	SP 2	6.5	37°	{ Nil 25 mM }	99	99	99	95	s.c.
						97	99	97	97	

The pattern of precipitation lines produced by the variant in agar-gel double diffusion plates with antiserum produced in rabbits against the parent strain indicated that at least two antigens were absent from the variant or were present in much lower concentration.

Effect of ammonium ion on the survival of N-limited bacteria

Samples of bacteria from chemostat cultures of N-limited *Escherichia coli* MRE 162 and the parent and variant strains of *Aerobacter aerogenes* growing at 37° and 40° were washed and starved at 37° and 40° in various saline buffers (see Methods) with and without NH₄Cl (Table 1). The death rate of the variant, which was greater than that of the other organisms in buffer alone, increased in the presence of NH₄Cl.

Table 2. *Effect of magnesium and/or sulphate ions on 'ammonium-accelerated death' of the parent and variant strains of Aerobacter aerogenes*

Bacteria grown in N-limiting medium (dilution rate 0.23–0.25 hr⁻¹) were washed and starved (at 3×10^7 bacteria/ml.) at 40° in aerated saline phosphate 1 with and without additions. Viabilities of suspensions were determined at intervals by slide culture.

Organism	Addition to diluent	Time (hr)				
		0	2	4	7.5	24
		Viability (%)				
Parent strain	Nil	99	98	97	97	98
	15 mM-NH ₄ Cl	98	98	99	97	97
	15 mM-NH ₄ Cl + mM-Na ₂ SO ₄ }	98	99	98	99	97
	15 mM-NH ₄ Cl + mM Na ₂ SO ₄ + mM MgCl ₂ }	98	98	98	98	96
Variant strain	Nil	98	98	95	85	—
	15 mM-NH ₄ Cl	97	97	84	58	—
	mM-MgCl ₂	98	98	94	89	—
	mM-Na ₂ SO ₄	98	98	94	90	—
	15 mM-NH ₄ Cl + mM-MgCl ₂ }	98	97	94	68	—
	15 mM-NH ₄ Cl + mM-Na ₂ SO ₄ }	98	92	50	27	—
	15 mM-NH ₄ Cl + mM-MgCl ₂ + mM-Na ₂ SO ₄ }	98	97	76	39	—

In contrast, the death rates of the parent strain *A. aerogenes* and of *E. coli* were unaffected by NH₄Cl. The effect of NH₄Cl on the variant increased with concentration up to 50 mM in 'Specpure' saline phosphate (Fig. 3). At higher temperatures (43–47°) the death rate of the parent strain was decreased by NH₄Cl (Fig. 4). Sulphate ion (mM) increased the effect of NH₄Cl on the variant (but not on the parent strain) starved at 37° or 40° (Table 2); magnesium ion (mM) slightly decreased but did not abolish either the effect of NH₄Cl or of NH₄Cl + sulphate ion on the starved variant (Table 2). Apparently continuous growth in N-limiting medium in a chemostat was necessary before the variant strain became susceptible to 'ammonium-accelerated death'. NH₄Cl had no effect on the survival of starved

stationary phase variant organisms grown in N-deficient medium in batch cultures (see below) and protected starved glycerol-limited variant organisms grown at a dilution rate of 0.25 hr^{-1} .

Fig. 3

Fig. 4

Fig. 5

Fig. 3. Effect of increasing NH_4Cl on the death rate of N-limited variant of *Aerobacter aerogenes* in 'Specpure' saline phosphate at 40° . Bacteria growing continuously at 40° (dilution rate, 0.25 hr^{-1}) were washed and resuspended at 4.4×10^7 bacteria/ml. in aerated saline phosphate with and without NH_4Cl at 40° . Viabilities (viable counts) at intervals of suspensions in buffer alone (○) and with 10 mM (●), 25 mM (◐), 50 and 100 mM NH_4Cl (◑).

Fig. 4. Effect of NH_4Cl on the death rate of N-limited parent strain of *Aerobacter aerogenes* in saline phosphate at 43 – 47° . Bacteria growing at 40° in a chemostat (dilution rate, 0.23 hr^{-1}) were washed and starved (at 3×10^7 bacteria/ml.) in aerated saline phosphate 2 with and without NH_4Cl (10 mM) at 43° (○, ●), 45° (△, ▲) and 47° (◻, ◼); open symbols, buffer; closed symbols, buffer + NH_4Cl . Viabilities were determined at intervals by slide culture.

Fig. 5. Polysaccharide synthesis by washed suspensions of parent and variant strains of *Aerobacter aerogenes* in the presence of glycerol. Bacteria growing in N-limiting medium in chemostats (dilution rate, 0.25 hr^{-1}) were washed and resuspended (equiv. $580 \mu\text{g}$. dry weight bacteria/ml.) in aerated 0.067 M -sodium potassium phosphate buffer (pH 7.0) containing mM- MgSO_4 and 0.5% (w/v) glycerol at 37° . Samples were taken at intervals from suspensions of variant (○) and parent strain (●) for carbohydrate determination. Rates of polysaccharide synthesis in spent medium + glycerol were similar to those shown.

Polysaccharide synthesis by bacteria grown under N-limiting conditions in chemostats and in N-deficient medium in batch cultures

The previously reported polysaccharide content values of the variant and parent strains of *Aerobacter aerogenes* when growing in continuous culture at 40° and 37° , respectively, under N-limiting conditions (Postgate & Hunter, 1962; Strange & Dark, 1965) were confirmed: at a dilution rate near 0.25 hr^{-1} , the variant contained $< 5\%$ polysaccharide and the parent strain contained 12–16% polysaccharide. Increasing the glycerol concentration in the medium from 0.2 to 1.0% (w/v) or decreasing the growth temperature to 37° had little effect on the polysaccharide content of the variant and did not abolish its 'ammonium-accelerated death'; increasing the growth temperature of the parent strain to 40° did not decrease the polysaccharide content nor make the organisms susceptible to ammonium ion during starvation. A comparison of the rates of polysaccharide synthesis by these organisms

in spent medium and in phosphate buffer (0.067 M) + mM-MgSO₄ containing glycerol was therefore made (Fig. 5). In 2 to 3 hr at 37° the polysaccharide synthesized by the variant was only 20–25 % of that produced by the parent strain. However, despite the slow rate of polysaccharide synthesis by the variant during and after continuous growth in N-limiting medium, in N-deficient medium containing 1 % (w/v) glycerol in shaken flask cultures at 37° for 20 hr, variant organisms containing 25 % or more polysaccharide were produced. The polysaccharide-rich variants of *A. aerogenes* survived much better in saline phosphate than did the continuously growing N-limited variants and its survival was unaffected by NH₄Cl during 96 hr at 37° (Table 3). Neither the parent strain *A. aerogenes* nor *Escherichia coli* NCTC 8164 grown in N-deficient medium in batch cultures, suffered 'ammonium-accelerated death'.

Table 3. *Effect of NH₄Cl on stationary phase variants of Aerobacter aerogenes grown in N-deficient medium (1 % w/v, glycerol) in batch culture*

Washed bacteria (4×10^7 /ml.) were starved in saline phosphate 2 with and without NH₄Cl in shaken flasks at 37°. Viabilities were determined at intervals by slide culture. Initially the bacteria contained 25 % total carbohydrate as glucose.

NH ₄ Cl	Time (hr)			
	0	18	23	96
	Viability (%)			
Nil	100	90	88	45
25 mM	100	94	90	44

DISCUSSION

The apparent disagreement between Postgate & Hunter (1964) and Strange & Dark (1965) about 'ammonium-accelerated death' of *Aerobacter aerogenes* was due to differences in the properties of the parent strain and the variant derived from it during continuous culture. The sensitivity of the N-limited variant to ammonium ion during starvation may be associated with a low content of polysaccharide reserve material as compared with that in the parent strain *A. aerogenes* and in other bacteria grown under N-limiting conditions (Holme & Palmstierna, 1956; Holme, 1957; Wilkinson, 1959). Starved polysaccharide-rich variant organisms, grown in N-deficient batch culture, were insensitive to ammonium ion. However, lack of polysaccharide does not explain the lethal effect of ammonium ion on N-limited variant organisms. Glycerol-limited variants grown at a similar dilution rate also contained little carbohydrate but did not suffer 'ammonium-accelerated death'. Apparently nitrogen limitation does affect the physiology of the organisms so that they may become sensitive to ammonium ion in the absence of a carbon source; perhaps when polysaccharide reserves are present, these supply a source of carbon and energy which allows balanced metabolism to occur, like that during normal growth. If this be true then 'ammonium-accelerated death' will not be observed with members of the Enterobacteriaceae which lay down polysaccharide reserves under conditions of nitrogen limitation.

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