Variation in Content and Distribution of Magnesium, and its Influence on Survival, in *Aerobacter aerogenes* Grown in a Chemostat

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(Received 6 January 1966)

SUMMARY

The magnesium and RNA contents of *Aerobacter aerogenes*, growth-limited by Mg^{2+} , K^+ , NH_4^+ or carbon source, in defined media at 35° increased with growth rate. The results support the view that the amounts of these constituents are functions of the growth rate and are inter-dependent. Up to 26 % of the total Mg^{2+} of bacteria freshly harvested from cultures containing excess magnesium was loosely bound to the bacterial surface; this adsorbed Mg^{2+} was removed by washing with 0.85 % (w/v) NaCl but was unaffected by distilled water. Mg^{2+} -limited bacteria had no surface-adsorbed magnesium. Surface-adsorbed Mg^{2+} stimulated polysaccharide synthesis, and affected the response of bacteria in saline buffer to stresses including starvation, heat-accelerated and substrate-accelerated death, and cold shock.

INTRODUCTION

When the growth rate of *Aerobacter aerogenes* cultures in a chemostat was limited by the supply of Mg^{2+} , the bacterial concentration varied with dilution rate (Tempest, Hunter & Sykes, 1965) which, assuming quantitative uptake of Mg^{2+} from the medium, indicated a variation in cellular Mg^{2+} content with growth rate. This assumption has now been confirmed by direct determination of the distribution of Mg^{2+} in Mg^{2+} -limited cultures by the Titan Yellow method of Gardner (1946) which, when used as described below, gave accurate and reliable results. Furthermore, it was found that the Mg^{2+} content of bacteria growth-limited by substances other than Mg^{2+} , increased with the growth rate. The Mg^{2+} associated with bacteria growth-limited by substances other than Mg^{2+} included some Mg^{2+} that was loosely bound; this 'adsorbed Mg^{2+} ' could be removed by washing with salt solution (Strange & Shon, 1964). Adsorbed Mg^{2+} affected the response of the bacteria to subsequent stresses. The present paper records data which indicate a relationship between the intracellular Mg^{2+} and RNA contents of *A. aerogenes* grown under a variety of growth-limiting conditions and presents evidence which shows that surface-adsorbed magnesium is advantageous for the survival of the organisms in unfavourable conditions.

METHODS

Organism and cultural conditions. *Aerobacter aerogenes* (NCTC 418) was grown in 0.5 l. Porton-type chemostats (Herbert, Phipps & Tempest, 1965) in defined media containing growth-limiting concentrations of Mg^{2+} , glycerol (Tempest *et al.* 1965)

or K^+ . The K^+ -limited medium contained: Na_2HPO_4 , $5.0 \times 10^{-3} M$; $(NH_4)_2H_2PO_4$, $4.5 \times 10^{-2} M$; $(NH_4)_2SO_4$, $2.5 \times 10^{-2} M$; citric acid, $1.0 \times 10^{-3} M$; $MgCl_2$, $1.25 \times 10^{-3} M$; $CaCl_2$ and $FeCl_3$, each $1.0 \times 10^{-4} M$; trace amounts of Mn^{2+} , Cu^{2+} and $NaMoO_4$. Glycerol was added to a final concentration of 30 mg./ml., and K_2SO_4 to $5.0 \times 10^{-4} M$. In addition, ammonium-limited and carbon-limited cultures of *A. aerogenes* were grown in defined media in a smaller chemostat (designed by Dr D. Herbert) as described by Postgate & Hunter (1962), but with a culture volume of 0.25 l. In each case, the dilution rate was progressively increased from 0.1 to 0.8 vol./hr and samples were taken (see below) at several intermediate 'steady state' growth-rate values. Exponential phase *A. aerogenes* organisms were harvested from shaken batch cultures of the organisms in a defined medium containing mannitol (Strange & Dark, 1962).

Survival studies. Washed suspensions of bacteria were subjected to heat stress at 48° as described by Strange & Shon (1964), to carbon substrate accelerated death (Postgate & Hunter, 1963; 1964) as described by Strange & Dark (1965), and to chilling at 0° as described by Strange & Dark (1962). Viabilities were determined with the slide culture method of Postgate, Crumpton & Hunter (1961), with an enriched agar medium similar to theirs except that glucose (0.2%, w/v) replaced glycerol. Saline phosphate contained 0.108 M-NaCl and 0.02 M-potassium phosphate buffer (pH 6.5).

Analytical methods. Magnesium in bacteria and culture filtrates was determined by the Titan Yellow colorimetric method (Gardner, 1946). Mg^{2+} was extracted from bacteria as follows. Duplicate samples of culture (10 ml. containing equiv. 1–10 mg. dry wt bacteria/ml.) were centrifuged at 3000g for 10 min.; the packed cell pellets were suspended in a suitable diluent (see Results) and re-sedimented by centrifugation. The washed pellets were resuspended in ice-cold water (2.5 ml.) and cold 2 N- $HClO_4$ (2.5 ml.) added; after standing at 4° for 15 min., the suspensions were centrifuged and the clear extracts decanted into 10 ml. graduated test tubes. The pellets were re-extracted with cold N- $HClO_4$ (4 ml.) as before and the combined extracts diluted to 10 ml. with N- $HClO_4$. Total removal of Mg^{2+} from the organisms was confirmed by wet ashing the pellets followed by acid extraction and analysis for Mg^{2+} . The Mg^{2+} -content of $HClO_4$ -extracts and culture filtrates was determined as follows. Reaction mixtures containing (final vol., 2.5 ml.) 1 m-mole $HClO_4$, 0.4 mg. gum ghatti, 0.15 mg. Titan Yellow and 2–12 μg . Mg^{2+} were prepared; colour was developed by adding 1 ml. 3 N-NaOH and measured after exactly 5 min. with a Bausch & Lomb 'Spectronic 20' spectrophotometer at 540 m μ . The amount of $HClO_4$ present in the reaction mixtures was important because colour formation decreased as its concentration increased. Under the conditions given, colour formed was directly proportional to Mg^{2+} concentrations between 2 and 12 μg . The presence of interfering substances in acid extracts was assessed by recovery of standard amounts of added Mg^{2+} from extracts. The only substance found seriously to interfere in the assay and which was present in significant concentration in some extracts was Polyglycol P-2000, a polypropylene glycol added to cultures to suppress foaming. This substance was quantitatively removed from extracts by two extractions with an equal volume of 'Analar' grade light petroleum (60–80° fraction). Bacterial dry weights, carbohydrate and RNA-contents were determined as previously described (Tempest *et al.* 1965).

RESULTS

Mg²⁺-content of Aerobacter aerogenes as a function of growth rate and limiting substrate

Previous work (Strange & Shon, 1964) showed that *Aerobacter aerogenes* adsorbed Mg^{2+} from solutions of magnesium salts but this adsorption progressively decreased in the presence of increasing concentrations of Na^+ or K^+ . It therefore seemed likely that bacteria separated from cultures containing excess Mg^{2+} would contain some Mg^{2+} adsorbed to their surface. This possibility was examined by washing samples of bacteria sedimented from cultures, with distilled water and parallel samples with 0.85 % (w/v) NaCl, before acid extraction for Mg^{2+} assay. The results (Table 1) show that, with the exception of Mg^{2+} -limited organisms, the Mg^{2+} contents of water-washed bacteria were significantly higher than those of saline-washed bacteria.

Table 1. *Magnesium content of Mg^{2+} -, glycerol-, K^+ - and NH_4^+ -limited Aerobacter aerogenes grown at various dilution rates, and the effect of the washing procedure on adsorbed magnesium*

Samples of bacteria, separated from chemostat cultures, were washed with distilled water or 0.85 % (w/v) NaCl and extracted with $N-HClO_4$ for Mg^{2+} assay (see Methods). For each growth limitation, figures in column (a) refer to Mg^{2+} content of water-washed bacteria, and in column (b) to Mg^{2+} content of saline-washed bacteria. All values are expressed as g. Mg^{2+} /100 g. dried organisms.

Dilution rate (hr ⁻¹)	Mg^{2+} -limited			Glycerol-limited			K^+ -limited			NH_4^+ -limited		
	(a)	(b)	(a/b)	(a)	(b)	(a/b)	(a)	(b)	(a/b)	(a)	(b)	(a/b)
0.1	0.101	0.101	1.00	0.151	0.130	1.16	0.148	0.124	1.19	0.192	0.175	1.10
	0.118	—	—	—	—	—	0.150	0.137	1.09	0.220	0.191	1.15
0.2	0.168	0.166	1.01	0.210	0.179	1.17	0.178	0.162	1.10	0.227	0.202	1.12
	0.156	—	—	—	—	—	0.182	0.156	1.17	0.264	0.233	1.13
0.4	—	—	—	—	—	—	0.183	0.153	1.20	—	—	—
	0.214	0.216	0.99	0.234	0.198	1.18	0.221	0.186	1.19	0.316	0.258	1.22
0.5	0.220	—	—	0.244	0.208	1.17	0.223	0.186	1.19	—	—	—
	—	—	—	—	—	—	0.224	0.187	1.19	—	—	—
0.6	—	—	—	—	—	—	—	—	—	0.324	0.295	1.10
	0.238	0.248	0.96	0.274	0.231	1.19	0.256	0.216	1.19	0.310	0.270	1.15
0.8	0.246	0.249	0.99	—	—	—	0.240	0.208	1.16	—	—	—
	—	—	—	—	—	—	0.266	0.211	1.26	—	—	—
0.8	0.264	0.264	1.00	0.353	0.292	1.21	0.302	—	—	—	—	—
	0.284	—	—	0.319	0.300	1.06	—	—	—	—	—	—

Irrespective of the washing procedure, however, the concentration of Mg^{2+} in the organisms increased with the growth rate. The results also show that N-limited bacteria grown at low dilution rates contained significantly more magnesium than did bacteria growth-limited by other substances at equivalent dilution rates. It seems probable that the higher concentration of Mg^{2+} in slow growing N-limited bacteria was associated with the presence of a high concentration of bacterial glycogen. Determination of the RNA and Mg^{2+} contents of bacteria growth-limited by Mg^{2+} , K^+ and glycerol showed that the ratio RNA: Mg^{2+} varied only slightly with increasing dilution rate in the range 0.1–0.8 hr⁻¹ (Table 2). The RNA content of Mg^{2+} -limited organisms growing at a dilution rate of 0.2 hr⁻¹ decreased with

temperature (Tempest & Hunter, 1965) and, assuming complete uptake of Mg^{2+} from the environment, the Mg^{2+} content increased from 0.135 % (of bacterial dry weight) at 40° to 0.199 % at 25°; the corresponding increase in RNA content was from 9.4 to 14.9 %.

Table 2. *Cellular RNA:Mg²⁺ ratios in continuous cultures of Aerobacter aerogenes*

Samples of bacteria separated from chemostat cultures, were washed with 0.85 % (w/v) NaCl and extracted with ice-cold 0.5 N-HClO₄ and then with 0.5 N-HClO₄ at 80°. The RNA content of the 80° extract was determined by the orcinol method (see Methods). Similar samples of bacteria were assayed for Mg^{2+} .

Dilution rate (hr ⁻¹)	Mg ²⁺ -limited organisms	Glycerol- limited organisms g. RNA/g. Mg ²⁺	K ⁺ -limited organisms
0.1	76	62	70
0.2	66	52	72
0.4	71	61	79
0.6	67	60	85
0.8	63	55	82

Effect of adsorbed Mg²⁺ on the physiology and survival of Aerobacter aerogenes

It seems probable that the Mg^{2+} which can be removed from glycerol-, K⁺- or N-limited *Aerobacter aerogenes* by suspending the organisms in 0.85 % (w/v) NaCl solution is adsorbed to the surface layer of the organisms. The evidence supporting this assumption is that Mg^{2+} taken up by bacteria suspended in $MgSO_4$ solution is quantitatively removed by resuspension of the separated bacteria in 0.85 % (w/v) NaCl solution (Strange & Shon, 1964). The question arises whether this adsorbed Mg^{2+} serves a functional role in the organisms; various pieces of evidence suggest that it may well do so. For example (1) polysaccharide synthesis by washed suspensions of glycerol-limited *A. aerogenes* in the presence of glycerol was higher with bacteria washed with distilled water than with bacteria washed with saline (Fig. 1). (2) The death rate of water-washed mannitol-limited *A. aerogenes* in saline buffer (pH 6.5) at 40°, with or without mannitol (40 mM) was lower than that of saline buffer-washed bacteria (Fig. 2); the effect of the washing procedure on substrate-accelerated death (Postgate & Hunter, 1964) was very marked. (3) As shown previously (Strange & Shon, 1964), the washing procedure may affect the thermal resistance of bacteria; this was confirmed by heating water-washed and saline phosphate-washed mannitol-limited *A. aerogenes* in saline phosphate (pH 6.5) at 48°: the death rate of water-washed bacteria was lower than that of phosphate-washed bacteria (Fig. 3). To confirm that the lower heat resistance of saline phosphate-washed bacteria was due to loss of adsorbed Mg^{2+} during washing, bacteria were washed successively with saline phosphate (to desorb Mg^{2+}), mM- $MgSO_4$ and finally with distilled water (to remove excess $MgSO_4$). The survival characteristics of these bacteria were similar to those of water-washed bacteria (Fig. 3). Also, the presence of mM- $MgSO_4$ in saline phosphate during heating of saline phosphate-washed bacteria decreased the death rate (Fig. 3). (4) Sudden chilling caused loss of viability of suspensions of exponential phase *A. aerogenes* (Strange & Dark, 1962; Strange & Postgate, 1964) and it was found that the washing procedure affected

the susceptibility of the organisms to cold shock. Water-washed organisms were less susceptible to chilling in saline buffer (pH 6.5) than organisms washed with solutions of NaCl or sodium phosphate buffer (Fig. 4).

Fig. 1

Fig. 2

Fig. 1. Carbohydrate synthesis in washed suspensions of glycerol-limited *Aerobacter aerogenes*. Organisms from a glycerol-limited culture (37° , dilution rate = 0.4 hr^{-1}) were separated from the culture by centrifugation ($3000g$, 5 min.) and washed either twice in water ($\circ$), twice in 0.85% (w/v) NaCl ($\bullet$) or once each, successively, in saline, 0.01 M-MgCl_2 , water (Δ). Washed organisms were resuspended at a concentration equiv. 1 mg. dry wt. bacteria/ml. in $0.067 \text{ M-phosphate}$ (pH 6.5) + 0.05 M-glycerol and incubated at 37° , with aeration. Samples were analysed for carbohydrate as indicated in *Methods*.

Fig. 2. Survival of water-washed and saline phosphate-washed *Aerobacter aerogenes* at 40° in aerated saline phosphate (pH 6.5), with or without mannitol. Continuously grown mannitol-limited bacteria (dilution rate, 0.4 hr^{-1}) were washed twice with distilled water or saline phosphate and resuspended (about 4×10^8 bacteria/ml.) in aerated saline phosphate with and without mannitol (40 mM). Viabilities (slide culture) of suspensions of water-washed ($\circ$, $\bullet$) and saline phosphate-washed (Δ , $\blacktriangle$) bacteria. Open symbols, buffer; closed symbols, buffer plus mannitol.

DISCUSSION

The present results confirm previous reports that bacterial RNA (Herbert, 1961, Neidhardt, 1963) and magnesium (Tempest *et al.* 1965) increase with the growth rate of bacteria. The fact that the ratio of RNA:magnesium in *Aerobacter aerogenes* varied only slightly with dilution rate suggests that a large proportion of the magnesium was associated with the RNA. This view is strengthened by the finding (Tempest & Hunter, 1965) that when the RNA content of magnesium-limited bacteria growing at a fixed dilution rate was varied by altering the incubation temperature, a corresponding change in the magnesium content of the bacteria apparently occurred. Most of the cellular RNA is present in ribosomes which are

known to have magnesium as an integral part of their structure (Edelman, Ts'o & Vinograd, 1960; Rogers, 1964). However, proof that the magnesium requirement of bacteria increases with the growth rate mainly for the purpose of stabilizing an increasing number of ribosomes would require more precise data than those obtained here about the distribution of Mg^{2+} in bacteria. The present results show that in bacteria separated from environments containing an excess of magnesium, a proportion of their magnesium is loosely bound and removed by washing with saline, whereas magnesium-limited bacteria had no loosely bound magnesium associated

Fig. 3

Fig. 4

Fig. 3. Survival of water-washed and saline phosphate-washed *Aerobacter aerogenes* at 48° in aerated saline phosphate (pH 6.5). Continuously grown mannitol-limited bacteria (dilution rate, 0.25 hr⁻¹) were washed: (a) three times with distilled water (○); (b) once with saline phosphate and twice with water (●, ▲); (c) successively with saline phosphate, 1 mM-MgSO₄ and water (△). The washed organisms were resuspended in water and samples were diluted 1/100 (final bacterial concentration about 3×10^8 /ml.) in aerated saline phosphate, with (▲) and without 1 mM-MgSO₄, at 48°. Viabilities were determined by slide culture.

Fig. 4. Effect of washing procedure on the susceptibility of exponential phase *Aerobacter aerogenes* to chilling at 0°. Bacteria growing exponentially in shaken flasks of defined medium at 37° were separated and washed with distilled water (○), 0.15 M-NaCl (●) or 0.075 M-sodium phosphate buffer (pH 6.5) (△). Samples of the washed organisms suspended in the washing liquid were diluted 1/40 (final concentration about 3×10^7 bacteria/ml.) in saline phosphate (pH 6.5) at 0°. Viabilities were determined by slide culture.

with them. While adsorbed magnesium is evidently not necessary for growth, it did affect both the ability of the organisms to synthesize polysaccharide in the presence of glycerol and their resistance to various stresses. Therefore this adsorbed magnesium may have an important functional role in bacteria and this should be considered when preparing washed bacterial suspensions for studies of metabolic activity or survival.

We wish to thank our colleague Dr J. W. Dicks for some magnesium determinations and Mr T. H. Dunham for his skilled technical assistance.

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