The Stability of *Mycoplasma mycoides*

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SUMMARY

The morphology of *Mycoplasma mycoides* was well preserved after washing and suspension in buffered 0.4 M-sucrose solutions, but the survival of viable particles was no better, and the loss of ultraviolet (u.v.)-absorbing substances and the decrease of turbidity was no less than in hypotonic solution (0.01 M-tris HCl or 0.01 M- $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$). The addition of Mg^{2+} , Ca^{2+} , spermidine or spermine (increasing order of activity) decreased the decrease of turbidity and loss of u.v.-absorbing substances. Ca^{2+} and Mg^{2+} , but not spermine, increased the degree of survival of viable particles. Ethylenediaminetetra-acetate (EDTA; 0.01 M) increased the loss of u.v.-absorbing substances, and decreased the turbidity and degree of survival. Ca^{2+} , Mg^{2+} and spermine annulled the effects of EDTA on loss of u.v.-absorbing material and on the turbidity, but only Ca^{2+} prevented the lethal effect of EDTA. Filaments disappeared and cell volume increased when the organisms were transferred from hypertonic to hypotonic solutions; the shape changes were reversible.

INTRODUCTION

Smith & Sasaki (1958) showed that the viability of some *Mycoplasma* strains was much less affected by the osmotic pressure of the suspending medium than had been supposed. Butler & Knight (1960) confirmed this observation and showed also that metal-chelating agents (diethyldithiocarbamate, ethylenediaminetetra-acetate, 8-hydroxyquinoline), manganese dioxide and the use of de-ionized water of high quality increased the degree of survival in suspensions in dilute buffer solutions. In contrast, Ca^{2+} ions have a marked stabilizing effect on bacterial spheroplast suspensions in hypotonic solutions (Tabor, 1962) and spermine, spermidine, streptomycin and polylysine were very effective stabilizers, while monovalent cations, Mg^{2+} and the diamines 1,4-diaminobutane and 1,5-diaminopentane were relatively ineffective.

Razin & Argaman (1963) compared the susceptibility of mycoplasmas, bacterial L-forms, spheroplasts and protoplasts to lysis by osmotic shock, by alternate freezing and thawing and by surface-active compounds. The mycoplasmas and bacterial L-forms were more resistant to osmotic shock than were the bacterial protoplasts and spheroplasts but, like the protoplasts, the mycoplasmas were very sensitive to lysis by surface-active substances. *Mycoplasma* strains differed in their sensitivity to lysis by osmotic pressure changes, strains of *Mycoplasma mycoides* being among the more resistant ones (Razin, 1963). Razin (1964) showed that the *Mycoplasma* strains he examined resisted osmotic shock at 0°, but underwent rapid lysis at 37°. Divalent and polyvalent cations, in concentrations as low as 10^{-5} M, protected *Mycoplasma laidlawii* from osmotic lysis.

METHODS

Organisms. The strain v5 of *Mycoplasma mycoides* and the strain N of *M. capri* were used. The latter strain has been found not to form detectable amounts of polysaccharide (Plackett, Buttery & Cottew, 1963). The organisms were grown in supplemented BVF-OS medium (Plackett *et al.* 1963) and harvested when the turbidity of the cultures had increased to between one quarter and three quarters of the maximum expected value. Within this turbidity range, the organisms were highly filamentous and the ratio of colony count of viable particles to turbidity was approximately constant.

Reagents. The salts used were of AR quality; the water was glass distilled, but no special precautions were taken to prevent metal ion contamination. Spermine (grade B) and spermidine (grade A) were obtained from the California Foundation for Biochemical Research, and 1,4-diaminobutane from E. Light and Co. (Colnbrook, Bucks., England). Sucrose (M solution) was freed from cations by passage through Dowex 50 (H⁺) column. The solutions containing ethylenediaminetetraacetate and divalent metal ions were adjusted to pH 7.4 with NaOH.

Procedure. Ten-ml. volumes of culture were chilled, and centrifuged at 15,000 rev./min. for 20 min. (Spinco, model L, rotor 40). The supernatant fluids were poured off and the walls of the tubes wiped dry with filter paper. The deposits of organisms were then washed twice by resuspension in 10-ml. volumes of the test solutions. The entire process, which occupied about 3.5 hr, was performed in the cold.

Counts of viable particles determinations were made by a plate colony count method after the suspensions had been kept about 1 hr at 0–2°, and again in most experiments after a further period of 1–3 days at 2°. The results are expressed as the % of the colony count of the original culture. Turbidity measurements were made at 650 m μ (Beckman Model DU, 1 cm. light path) on samples of the suspensions (warmed to room temperature) at the end of the storage period, and are expressed as % of the turbidity of the original culture. Leakage of u.v.-absorbing material was measured at 250 m μ in both of the supernatant fluids after washing, and also in the supernatant fluid obtained after centrifuging a sample of the suspension after storage. The sum of these measurements, corrected where necessary for the extinction of the washing fluid, are expressed as % of the extinction of an alkali lysate. For this, the unwashed pellet from 10 ml. culture was incubated at 37° with 1 ml. N-NaOH for 1 hr and the lysate diluted to 50 ml. for measurement.

The volumes of packed cell pellets were measured after centrifugation in graduated capillary-tipped tubes at 1850g for 18 hr at 2°. Polyvinylpyrrolidone (1%, w/v, in the suspending fluid) was used as an indicator for measuring the intercellular volume (Wetherell & Pollack, 1962).

Electron microscopy. Nine volumes of a solution of the same composition as that of the suspending fluid but containing in addition either 4% (w/v) formaldehyde or 5% (w/v) glutaraldehyde were added to 1 vol. of suspension of organisms. Phosphate buffer was used in the suspending fluid to avoid pH changes. After standing overnight at 2°, the organisms were washed and suspended in water. Droplets were air-dried on electron microscope grids before metal-shadowing. No differences in morphology between material fixed in formaldehyde or glutaraldehyde were

detected, but the impression was formed from dark field examination in a light microscope that both fixatives resulted in some shrinkage, and in an increase in the number of beaded filaments.

RESULTS

Effects on turbidity, loss of ultraviolet-absorbing material and survival

*Mg*²⁺, *Ca*²⁺ and *EDTA*. There was a decrease in turbidity, a loss of u.v.-absorbing material from the organisms and a decrease in the colony count after washing the organisms in dilute tris or phosphate buffers (0.01 M, pH 7.4). The magnitude of

Table 1. *The effect of Mg*²⁺ and *Ca*²⁺ on the stability of *Mycoplasma mycoides*

Expt.	Divalent cation salts* (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡	Viable count (%) after stated period at 0–2°	
				1 hr	70 hr
1	None	50	44	13	0.3
	MgSO ₄ (0.001)	78	28	15	0.2
	MgSO ₄ (0.01)	84	24	60	21
	MgSO ₄ (0.1)	95	25	57	41
2	None	60	35	49	8
	CaCl ₂ (0.001)	70	27	57	2.5
	CaCl ₂ (0.01)	87	20	44	27
	CaCl ₂ (0.1)	88	23	45	19

* Salts added to 0.01 M tris HCl (pH 7.4). † Percentage of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

Table 2. *The effect of Mg*²⁺ and *Ca*²⁺ and *EDTA* on the stability of *Mycoplasma mycoides*

Expt.	Additions* (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡	Viable count (%) after stated period at 0–2°	
				1 hr	70 hr
1	None	73	37	56	21
	EDTA (0.001)	71	50	56	23
	EDTA (0.01)	60	55	2.0	0.01
	MgSO ₄ (0.01)	89	29	65	59
	EDTA (0.001); MgSO ₄ (0.001)	89	29	48	45
	EDTA (0.01); MgSO ₄ (0.02)	82	32	8	0.8
2	EDTA (0.01)	35	81	1.0	0.2
	MgSO ₄ (0.01)	83	27	48	22
	EDTA (0.01); MgSO ₄ (0.02)	80	33	22	2.1
	CaCl ₂ (0.01)	97	24	47	32
	EDTA (0.01); CaCl ₂ (0.02)	83	28	28	22
	MgSO ₄ (0.01); CaCl ₂ (0.01)	98	22	48	34
	EDTA (0.01); MgSO ₄ (0.01)	83	28	31	24
	CaCl ₂ (0.02)				

* Additions to 0.01 M tris HCl (pH 7.4). † Percentage of turbidity of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

these changes was about the same for either buffer. Tris buffer was used for most of the experiments to be described. The effect of pH value was not investigated. The addition of MgSO₄ or CaCl₂ in concentrations of 0.01 M or higher decreased these

changes (Table 1), whereas ethylenediaminetetra-acetic acid (EDTA) at 0.01 M increased them, its effect on the loss of viability being greater after storage (Table 2). At one-tenth this concentration, EDTA had no effect on survival, but increased the leakage of u.v.-absorbing material. Mg^{2+} (0.01 g. ions/l.) prevented almost completely the decrease of turbidity, and the loss of u.v.-absorbing material which

Table 3. *The effect of polyamines on the stability of Mycoplasma mycoides*

Polyamine* (M concn.)	Turbidity (%)†	Extinction at 260 m μ (%)‡
None	71	21.4
Spermine (0.0001)	80	18.7
Spermine (0.0001)	86	13.5
Spermine (0.001)	89	11.7
Spermine (0.005)	95	11.7
Spermidine (0.0001)	78	17.0
Spermidine (0.001)	87	13.4
Spermidine (0.005)	97	13.3
1,4-Diaminobutane (0.005)	83	19.5

* Added to (0.01 M tris HCl (pH 7.4). † Percentage of turbidity of culture. ‡ Sum of 260 m μ extinction of washings as % of alkali lysate.

Table 4. *The effect of Mg^{2+} , Ca^{2+} and spermine on the stability of Mycoplasma mycoides*

Expt.	Cation additions (M concn.)	Turbidity (%)†	Extinction at 260 m μ (%)‡	Viable count (%) after stated period at 0–2°		
				2 hr	24 hr	48 hr
1	None	59	35	21	11	—
	MgSO ₄ (0.01)	79	19	39	43	—
	CaCl ₂ (0.01)	87	17	21	25	—
	Spermine (0.001)	82	14	20	3	—
2	None	68	32	42	19	11
	MgSO ₄ (0.01)	90	20	75	81	70
	CaCl ₂ (0.01)	92	16	41	47	41
	Spermine (0.001)	96	15	50	10	4
3	None	76	30	41	—	12
	MgSO ₄ (0.01)	88	24	60	—	28
	Spermine (0.001)	95	18	49	—	6
	MgSO ₄ (0.01)†	104	17	38	—	32
	Spermine (0.001)					

* Added to 0.01 M tris HCl (pH 7.4). † Percentage of turbidity of culture. ‡ Sum of 260 m μ extinction of washings as % of alkali lysate.

occurred in the presence of 0.01 M-EDTA alone, but only partly prevented the effect of EDTA (0.01 M) on survival (Table 2). Ca^{2+} (0.01 g ions/l.) almost completely prevented the lethal effect of EDTA (0.01 M) as well as preventing effects on turbidity and on loss of u.v.-absorbing material (Table 2).

Polyamines. Spermine (0.0001 M) had about the same effect as 0.01 M-MgSO₄ or 0.01 M-CaCl₂ on turbidity and the loss of u.v.-absorbing material. Spermidine (0.001 M) had about the same effect as 0.0001 M-spermine; 1,4-diaminobutane had little effect at 0.005 M (Table 3). Spermine, in contrast to MgSO₄ and CaCl₂, did not

increase the survival of viable particles during storage, although the organisms survived in its presence when MgSO_4 was also added (Table 4). Spermine (0.001 M) was as effective as Ca^{2+} (0.01 g. ions/l.) in preventing the effects of 0.01 M-EDTA on the loss of u.v.-absorbing material and on turbidity (Table 5).

Table 5. *The effect of Ca^{2+} , EDTA and spermine on the stability of *Mycoplasma mycoides**

Tris = 0.01 M-tris HCl (pH 7.4); P = 0.01 M- $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ (pH 7.4); Suc = 0.4 M-sucrose.

Composition (M concn.)	Turbidity (%)*	Extinction at 260 m μ (%)†
Tris + CaCl_2 (0.01)	93	20
Tris + EDTA (0.01)	55	43
Tris + EDTA (0.01) + CaCl_2 (0.02)	80	23
Tris + EDTA (0.01) + spermine (0.001)	76	23
P + EDTA (0.01)	49	43
P + EDTA (0.01) + spermine (0.001)	75	24
P + Suc + EDTA (0.01)	34	51
P + Suc + EDTA (0.01) + spermine (0.001)	73	21

* Percentage of turbidity of culture. † Sum of 260 m μ extinction of washings as % of alkali lysate.

Table 6. *The effect of sucrose, NaCl and KCl on the stability of *Mycoplasma mycoides**

Added solute (M concn.)*	Total solute concn. (molal)	Turbidity†	Extinction at 260 m μ (%)‡	Viable count (%) after 1 hr at 0°
None	0.03	78	22	45
Sucrose (0.25)	0.30	67	30	32
Sucrose (0.4)	0.50	61	25	36
NaCl (0.15)	0.30	65	35	42
NaCl (0.25)	0.50	65	35	30
KCl (0.15)	0.30	70	34	58
KCl (0.25)	0.50	61	33	30

* Added to 0.01 M-tris HCl + 0.01 M- MgSO_4 . † Percentage of turbidity of culture. ‡ Sum of 260 m μ extinction of washings as % of alkali lysate.

Tonicity. In the absence of stabilizing cations, the decrease in turbidity and the loss of u.v.-absorbing material were greater when the organisms were washed and suspended in solutions containing sucrose (0.4 M) than they were in hypotonic solutions. In the presence of stabilizing cations the addition of sucrose made little difference. The effects of adding sucrose (0.25 M, 0.4 M) and of NaCl and KCl (0.15 M and 0.25 M) to tris + MgSO_4 (0.01 M) solution are shown in Table 6. The decrease in turbidity and the loss of u.v.-absorbing material were slightly greater, and survival about the same, in the presence of these solutes as they were in the suspension in hypotonic solution.

Effects on morphology

Observations were made by dark field microscopy and by electron microscopy of formaldehyde-fixed or glutaraldehyde-fixed suspensions of *Mycoplasma mycoides*. The cultures examined contained a high proportion of filamentous organisms, but

other forms were also present. The filaments were preserved after washing and fixing in solutions having a total solute concentration of 0.3 or 0.5 molal (dilute-buffer + sucrose or NaCl) followed by washing in water. There were no filaments in suspensions of organisms washed and fixed in hypotonic (0.03 molal) solutions. Electron micrographs of these suspensions showed electron-dense almost spherical forms, flattened forms and other material. The addition of stabilizing cations or of EDTA had no marked effects on the morphology of the organisms when suspended either in sucrose (0.5 molal) or in hypotonic (0.03 molal) solutions.

Table 7. *Effect of tonicity of suspending medium on the packed cell volume of Mycoplasma strains v5 and N*

Organisms were washed and suspended in 0.5 molal solution. 1 vol. suspension was added to 10 vol. solution of molality 0.03 or 0.5, and the suspensions were then centrifuged in graduated capillary-tipped tubes at 1850 g for 18 hr at 2°. The cell volumes recorded are the pellet volumes from which the intercellular volumes (as determined by the amount of PVP in the pellets) were subtracted.

Strain	Age of culture (hr)	Molal solute concentration*	Cell vol. (μ l.)
v5	40	0.07	41
		0.50	23
v5	18	0.07	18
		0.50	8.5
N	18	0.07	9.6
		0.50	5.4

* Composition of solution: 0.01 M-tris HCl (pH 7.4). + 0.01 M-MgSO₄ + 1 % w/v polyvinylpyrrolidone (PVP) + sucrose 0.02 M (total solute concentration = 0.07 molal), or 0.4 M (total solute concentration = 0.50 molal).

The effects of the tonicity of the suspending fluid on morphology, and the reversibility of these changes, are illustrated in Pl. 1, figs. 1-3; figs. 1a and 1b show the organisms after they had been washed and resuspended in 0.01 M-phosphate + 0.01 M MgSO₄ + 0.001 M-spermine + 0.4 M-sucrose solution (hypertonic solution); figs. 2a and 2b show their appearance after they had been washed in the hypertonic solution and then suspended in a solution of the same composition but lacking sucrose (hypotonic solution); figs. 3a and 3b show their appearance after they had been suspended successively in hypertonic, hypotonic and again hypertonic solutions. The flattened forms seen in Pl. 1, figs. 2a and 2b, may have been spherical in suspension and have collapsed during drying on the grids. Dark field examination of the unfixed suspension in a light microscope showed only what appeared to be spherical forms of various diameters with a maximum of about 1 μ . The rapidity of the shape changes was evident from the complete absence of streaming birefringence in the suspension in hypotonic solution and its immediate reappearance when the organisms were resuspended in hypertonic solutions. Measurements of cell volumes by using polyvinylpyrrolidone to measure the volume of the intercellular space in the cell pellets are shown in Table 7. Organisms were harvested from an exponential phase culture of *Mycoplasma mycoides* strain v5 which contained numerous filaments, from a stationary phase culture of v5 strain which did not contain filaments, and from an exponential phase culture of the *M. capri* strain N which consisted

mostly of short rod-like forms. The cell volumes were, in each case, about twice as great when the organisms were suspended in hypotonic solution as when in hypertonic solution.

DISCUSSION

These observations support those of others (Smith & Sasaki, 1958; Butler & Knight, 1960; Razin, 1963) that *Mycoplasma* organisms survive well in hypotonic solutions. They differ from those reported by Butler & Knight (1960) who found that, within an optimal concentration range, metal chelating agents, including EDTA, increased survival. In the experiments reported here, EDTA decreased survival and turbidity and increased the loss of u.v.-absorbing material, whereas Ca^{2+} and Mg^{2+} had a stabilizing effect. These conflicting results may be due to differences in concentrations of toxic metal ion impurities. The stabilizing effects of divalent cations and of polyamines for *Mycoplasma mycoides* are similar to those described by Tabor (1962) for bacterial protoplasts and spheroplasts and by Razin (1964) for *M. laidlawii*. Razin found that any of a series of divalent and trivalent cations as well as spermine and spermidine at 10^{-5} M and even lower concentrations, prevented lysis during incubation of suspensions of *M. laidlawii* at 37° . Spermine at 10^{-5} M also prevented death in a hypotonic medium. In the experiments reported here, spermine did not prevent death of *M. mycoides*. The observed interactions between Ca^{2+} , Mg^{2+} , spermine and EDTA may be the result of a competition between EDTA and the cell membrane for Ca^{2+} and Mg^{2+} on the one hand, and between Ca^{2+} , Mg^{2+} and spermine for common binding sites on the membrane on the other.

Mycoplasma mycoides responds to changes in the tonicity of the suspending solution by changes in shape and volume. The volume changes for individual undamaged organisms may be larger than those measured for the whole suspensions, because many of the organisms may have been damaged and their property of responding to osmotic pressure changes destroyed during the manipulations. In the case of the highly filamentous organisms of the v5 strain harvested from the 18 hr culture, the volume changes may have been considerably larger than the measurements shown in Table 7 for another reason. *M. mycoides* synthesizes a large amount of polysaccharide which is believed to form an extracellular capsular or slime layer closely surrounding the organisms in young cultures and later shed into the medium (Plackett *et al.* 1963). The suspension of organisms harvested from the younger culture would therefore contain a much larger amount of this extracellular material. The ratio of the volume to the number of turbidity units in the samples centrifuged was about four-fold greater for the suspension from the 18 hr culture than it was for the suspension from the 40 hr culture. The filaments have a very large ratio of surface area to volume. If a filament 0.1μ in diameter and 7μ in length and bounded by a membrane $85 \text{ \AA}$ in thickness were transformed to a sphere of the same surface area, the volumes within the external and internal surfaces of the membrane would increase by factors of 5.5 and 8, respectively; the diameter of the sphere would be 0.8μ . This is about the diameter of the larger forms observed by dark field examination in a light microscope in unfixed suspensions in hypotonic solution. The plastic filamentous mycoplasmas therefore have the potential to increase their volume several-fold by dilution of their contents (that is to decrease their internal osmotic pressure) without an increase in surface area. This may help to explain their

relative tolerance to osmotic pressure changes compared with bacterial protoplasts, whose volume, within a narrower tonicity range, is inversely related to the osmotic pressure of the medium (Weibull, 1955) but which undergo lysis in dilute solutions. Razin (1963) found that the sensitivity of certain *Mycoplasma* strains to osmotic lysis decreased with the age of the culture. The older cultures would contain few filaments. Changes in size, shape, lipid composition of membrane, and internal osmotic pressure are doubtless important also in determining stability. All of these may change with age of culture.

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EXPLANATION OF PLATE

Figs. 1-3 illustrate the effect of the tonicity of the suspending medium on the shape of *Mycoplasma mycoides* organisms. Figs. 1*a*, *b* are of organisms washed and suspended in a solution of 0.01 M-phosphate (pH 7.4) + 0.01 M-MgSO₄ + 0.001 M-spermine + 0.4 M-sucrose (hypertonic solution). Figs. 2*a*, *b* are of organisms washed in hypertonic solution and then suspended in a solution of the same composition but lacking sucrose (hypotonic solution). Figs. 3*a*, *b* are of organisms suspended successively in hypertonic, hypotonic and then hypertonic solutions. The organisms for figs. 1-3 were then fixed for 18 hr at 0° in solutions of the same composition but containing 5% (w/v) glutaraldehyde, and washed and suspended in water. Gold-palladium shadowed. Figs. 1*a*, 2*a* and 3*a*, ×1500; figs. 1*b*, 2*b* and 3*b*, ×8600.