Factors Influencing Osmotic Fragility of Mycoplasma

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SUMMARY

Osmotic lysis of *Mycoplasma* organisms was found to depend on the temperature of incubation. The organisms were practically resistant to osmotic shock at 0°, but lysed rapidly at 37°. Their sensitivity to lysis also depended on the pH value of the suspending medium, being lowest at pH values near neutrality. Divalent and polyvalent cations, in concentrations as low as 10^{-5} M, protected mycoplasmas from osmotic lysis. The parasitic *Mycoplasma* strains showed various degrees of osmotic fragility, *Mycoplasma gallisepticum* being the least fragile. All the parasitic strains tested were, however, more resistant to osmotic lysis than the saprophytic *Mycoplasma laidlawii*. The possibility that osmotic lysis of mycoplasmas involves autolytic processes was tested and is discussed.

INTRODUCTION

Mycoplasma organisms are limited by a thin membrane resembling the plasma membrane of bacteria, and are completely devoid of cell walls (Razin, 1963*a*). In spite of the absence of cell wall, mycoplasmas were found to be much more resistant to osmotic lysis than bacterial protoplasts and spheroplasts (Razin & Argaman, 1963). Further work showed that various *Mycoplasma* strains differed in osmotic fragility and that the saprophytic *Mycoplasma laidlawii* strains had a high degree of osmotic fragility when harvested at the logarithmic phase of growth (Razin, 1963*b*). To elucidate the mechanism of osmotic lysis of mycoplasmas, a study of the factors which influence osmotic fragility was undertaken.

METHODS

Organisms. *Mycoplasma mycoides* var. *mycoides* (PG 1), *M. mycoides* var. *capri* (PG 3), *M. agalactiae* (PG 2), *M. neurolyticum* (PG 28) and *M. gallisepticum* (PG 31) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). A strain of *M. gallisepticum* named (R) was provided by Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovoth, Israel). Strain s 6 of *M. gallisepticum* was obtained from Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *Mycoplasma laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity. This strain was serologically related to *M. laidlawii* strain A.

Suspensions of organisms. The organisms were grown in a modified Edward medium (Razin, 1963*b*) containing 1 % (v/v) Difco PPLO serum fraction. Growth was done in 500 ml. volumes distributed in 1 l. Roux flasks. The flasks were incubated

in inclined positions to improve aeration. The organisms were harvested while the optical density of cultures was still increasing, usually after incubation for 24–48 hr at 37°. The organisms were sedimented by centrifugation at 16,000 *g* for 5 min., washed twice in 0.25 M-NaCl solution and resuspended in the same solution.

Preparation of protoplasts. Protoplasts of *Micrococcus lysodeikticus* were prepared by lysozyme treatment, as described previously (Razin & Argaman, 1963) and suspended in a M-NaCl solution.

Examination of lysis. Test tubes contained 4 ml. of serial twofold dilutions of a 0.25 M-NaCl solution for testing the lysis of mycoplasmas, and of a M-NaCl solution for testing the lysis of protoplasts. The tubes were placed in a 37° water bath and each received 0.1 ml. of organism suspension. The number of viable particles in Mycoplasma suspensions was determined after 30 min. incubation according to Butler & Knight (1960). Simultaneously, the extinction of suspensions at 500 *mμ* was determined in a Unicam SP 500 spectrophotometer. The suspensions were then centrifuged at 20,000 *g* for 10 min., and the extinction of the clear supernatant fluids measured at 260 *mμ*. For testing the effect of temperature on osmotic lysis, extinction measurements were done in a Unicam SP 500 spectrophotometer equipped with a 'constant-temperature cell housing'.

Breakdown of cell membranes. Cell membranes of *Mycoplasma laidlawii* were prepared by osmotic shock of the organisms. Saline-washed packed organisms (1–2 g. wet weight) were suspended in de-ionized water (about 300 ml.), incubated at room temperature for 15 min. and then centrifuged at 16,000 *g* for 5 min. in the cold. The sediment, consisting mainly of unbroken organisms, was discarded and the supernatant fluid centrifuged at 34,000 *g* for 40 min. at 4°. Electron microscopy showed that this sediment consisted of membranes; no unbroken organisms were observed. The sedimented membranes were resuspended in ice-cold 0.01 M-tris buffer (pH 8.0) containing 0.01 M-NaCl. Proteolytic activity in membrane suspensions was tested by incubating 4 ml. samples of suspension at 37° for various periods of time. Extinction at 500 *mμ* of suspensions was measured at the end of the incubation period, and the membranes were sedimented by centrifugation at 34,000 *g* for 40 min. in the cold. The supernatant fluids were separated, concentrated by evaporation to 0.5 ml. volumes and their amino acid content analysed quantitatively by paper chromatography (Giri, Radhakrishnan & Vaidyanathan, 1952; Razin & Cohen, 1963). The coloured spots which appeared on the chromatogram after spraying with ninhydrin were cut off and eluted with 75 % (v/v) ethanol in water containing 0.005 % (w/v) CuSO₄.5H₂O. The colour intensity was determined spectrophotometrically at 540 *mμ*.

RESULTS

Effect of temperature on osmotic lysis

Lysis of *Mycoplasma laidlawii* by osmotic shock was temperature-dependent, and was not instantaneous; the time required for the completion of lysis depended on the incubation temperature (Fig. 1). Results obtained by the viable count technique confirmed those obtained by extinction measurements. The mycoplasmas were only slightly damaged by osmotic shock at 0° (Table 1). The dependence of osmotic lysis on temperature and incubation time was found also with *M. mycoides* var. *capri* and *M. neurolyticum*. The standard technique subsequently adopted for

testing osmotic lysis of mycoplasmas accordingly included incubation of suspensions in the hyptonic solutions at 37° for 30 min. Protoplasts of *Micrococcus lysodeikticus*, tested for comparison, lysed almost instantly in hypotonic NaCl solutions, the lytic process being even more pronounced at 0° than 18° (Fig. 2).

Resistance of heated *Mycoplasma* organisms to osmotic lysis

The degree of osmotic fragility decreased proportionally to the decrease in the number of viable organisms on heating *Mycoplasma* suspensions (Fig. 3). Heat-killed mycoplasmas were thus shown to be resistant to osmotic lysis while protoplasts of *Micrococcus lysodeikticus* heated to the same temperatures remained sensitive to osmotic lysis.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Osmotic lysis of *Mycoplasma laidlawii* at various temperatures of incubation. Test tubes containing 4 ml. de-ionized water were incubated at the temperatures indicated in figure. Each test tube received 0.1 ml. of a washed suspension of organisms in 0.25 M-NaCl and the extinction was measured at 1 min. intervals.

Fig. 2. Osmotic lysis of *Micrococcus lysodeikticus* protoplasts at various temperatures of incubation. Test tubes containing 4 ml. of various NaCl solutions (concentration of NaCl indicated in figure) received 0.1 ml. of suspension of washed protoplasts in M-NaCl. ---, Suspensions incubated at 0°; —, suspensions incubated at 18°.

Fig. 3. Osmotic lysis of *Mycoplasma laidlawii* organisms heated at various temperatures. Washed suspensions in 0.25 M-NaCl were heated for 10 min. at the temperature indicated in figure. Number of viable particles/ml. of unheated suspension, 1.1×10^{11} ; suspension heated at 45°, 1.6×10^{10} ; suspension heated at 55°, 5.0×10^6 ; suspension heated at 65°, 2.5×10^4 . The heated suspensions were tested for osmotic fragility at 37°, as described in Fig. 1.

Effect of pH value on osmotic lysis

Sensitivity of *Mycoplasma laidlawii* to osmotic lysis varied with the pH value of the suspending medium. Sensitivity was lowest at values between pH 7.0 and 8.0 (Fig. 4). The mycoplasmas were somewhat more sensitive to lysis in dilute tris buffer than in phosphate buffer of the same pH value. The higher sensitivity of the mycoplasmas to osmotic lysis at acid or alkaline pH values was found also with *M. mycoides* var. *capri*.

Table 1. *Effect of temperature on viability of Mycoplasma laidlawii in various NaCl solutions*

Test tubes containing 4 ml. of NaCl solution or de-ionized water received 0.1 ml. of a washed suspension of organisms in 0.25 M-NaCl (final concentration of NaCl indicated in the table). Viable counts were made after 30 min. of incubation.

NaCl concentration (M)	Temperature of incubation		
	0°	18°	37°
	No. of viable organisms/ml. suspension		
0.250	4.4×10^9	4.5×10^9	4.5×10^9
0.037	3.6×10^9	2.1×10^9	7.0×10^8
0.006	8.8×10^8	1.1×10^7	8.4×10^5

Table 2. *Protection by cations of Mycoplasma laidlawii and protoplasts of Micrococcus lysodeikticus from osmotic lysis*

Test tubes containing 4 ml. of neutralized solutions of the salts in de-ionized water received 0.1 ml. washed Mycoplasma suspension in 0.25 M-NaCl or washed protoplasts in 1.0 M-NaCl. Extinction of suspensions measured after 30 min. incubation at 37°.

Organism and Salt	Concentration of salt (M)					
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
	Extinction of suspension at 500 mμ					
<i>Mycoplasma laidlawii</i>						
Spermine.4HCl	—	0.42	0.38	0.37	0.37	0.08
Spermidine phosphate	—	0.41	0.40	0.35	0.20	0.08
UO ₂ (NO ₃) ₂ .6H ₂ O	—	0.42	0.39	0.38	0.33	0.23
AlCl ₃ .6H ₂ O	—	0.37	0.36	0.36	0.36	0.30
Fe ₂ (SO ₄) ₃	—	0.45	0.44	0.40	0.39	0.30
FeSO ₄ .7H ₂ O	—	0.40	0.39	0.36	0.34	0.20
MgCl ₂ .6H ₂ O	0.43	0.40	0.40	0.36	0.33	0.20
MnCl ₂ .4H ₂ O	0.42	0.37	0.37	0.37	0.36	0.31
CaCl ₂	0.47	0.39	0.35	0.34	0.18	0.09
BaCl ₂ .2H ₂ O	0.44	0.41	0.42	0.42	0.34	0.22
CuCl ₂ .2H ₂ O	0.45	0.39	0.37	0.37	0.36	0.17
ZnCl ₂	—	0.44	0.40	0.40	0.39	0.30
CdCl ₂ .2½H ₂ O	0.41	0.39	0.39	0.38	0.35	0.30
Hg(NO ₃) ₂	—	0.42	0.38	0.38	0.31	0.13
NaCl	0.43	0.09	0.08	0.08	0.08	0.08
KCl	0.48	0.10	0.10	0.09	0.09	0.09
LiCl	0.39	0.08	0.08	0.08	0.07	0.07
NH ₄ Cl	0.39	0.10	0.08	0.08	0.08	0.08
<i>Protoplasts of M. lysodeikticus</i>						
Spermine.4 HCl	—	0.42	0.20	0.16	0.02	0.02
MgCl ₂ .6H ₂ O	0.27	0.08	0.08	0.07	0.02	0.02
MnCl ₂ .4H ₂ O	0.35	0.18	0.07	0.06	0.02	0.02
NaCl	0.04	0.02	0.02	0.02	0.02	0.02

Protective effect of cations

Trivalent and divalent cations were very effective in protecting *Mycoplasma laidlawii* against osmotic lysis; monovalent cations were completely ineffective. A long series of trivalent and divalent cations as well as spermine and spermidine inhibited osmotic lysis at 10⁻⁵ M or even lower concentrations (Table 2). Spermine prevented the death of *M. laidlawii* organisms in a highly hypotonic medium (Fig. 5). The polyamines and divalent cations afforded some protection against osmotic

lysis of *Micrococcus lysodeikticus* protoplasts, but at much higher concentrations than required to protect mycoplasmas (Table 2).

Osmotic lysis of parasitic *Mycoplasma* strains

In earlier studies parasitic *Mycoplasma* strains were found to be relatively resistant to osmotic lysis (Razin, 1963*b*). The finding that osmotic lysis of *Mycoplasma laidlawii* was temperature-dependent urged us to re-examine the osmotic fragility of the parasitic strains, by using 37° as the incubation temperature. In addition to measuring the extinction of suspensions viable counts were made. By these

Fig. 4

Fig. 4. Osmotic lysis of *Mycoplasma laidlawii* at various pH values. Lysis was examined in NaCl solutions (concentrations indicated in figure) prepared in 0.01 M-phosphate buffer, or in 0.01 M-tris buffer. Extinction measurements (—) and viable counts (---) were made after incubation at 37° for 30 min.

Fig. 5

Fig. 5. Protection by spermine against lysis and death of *Mycoplasma laidlawii* suspended in 0.006 M-NaCl. Viable counts (○) and turbidity measurements (●) were made after incubation at 37° for 30 min.

techniques the parasitic strains were found to lyse and die in hypotonic solutions (Fig. 6). However, the degree of lysis of the parasitic strains rarely reached that of the saprophytic *M. laidlawii* strains. The various parasitic strains differed in osmotic fragility. Thus, all three *M. gallisepticum* strains tested were usually more resistant to osmotic lysis than the other strains of parasitic *Mycoplasma*. The parasitic strains frequently showed variations in osmotic fragility. Apparently one of the factors responsible for these fluctuations is the age of the culture. This had already been demonstrated for *M. laidlawii* (Razin, 1963*b*) and was found to be true also for *M. mycoides* var. *capri*, which was much more sensitive to osmotic lysis when harvested at the logarithmic phase of growth (Fig. 7). On testing osmotic fragility of the parasitic strains, it was noted that the number of viable *M. neurolyticum* organisms significantly decreased during washing and incubation in 0.25 M-NaCl. The toxic effect of NaCl was less pronounced at lower temperatures. *Mycoplasma laidlawii*,

M. mycoides var. *mycoides* and *M. gallisepticum* were not significantly affected by 0.25 M-NaCl. Washing of *M. neurolyticum* in 0.4 M-sucrose + 0.01 M-phosphate buffer (pH 7.0) according to Rodwell & Abbot (1961) was found to be preferable to washing in 0.25 M-NaCl.

Fig. 6. Susceptibility of various *Mycoplasma* strains to osmotic lysis. The extent of lysis in the various NaCl solutions was measured after 30 min. incubation at 37°. Extinction readings at 500 mμ of suspensions (●); extinction readings at 260 mμ of the supernatant fluids of the corresponding suspensions (×); number of viable particles/ml. suspension (○).

Breakdown of Mycoplasma laidlawii membranes

Isolated membranes of *Mycoplasma laidlawii* were examined for disintegration by following the methods devised by Brown (1962). The optical extinction of membrane suspensions decreased markedly during the first 5 min. of incubation at 37°, followed

by an increase in the amount of free amino acids in the supernatant fluids of the suspensions (Fig. 8). However, electron microscopy revealed no gross morphological changes in the membranes, even after incubation at 37° for 1 hr.

Fig. 7

Fig. 8

Fig. 7. Death of *Mycoplasma mycoides* var. *capri* in hypotonic NaCl solutions. Organisms grown in Edward medium were harvested after 22 hr (○), 38 hr (×) or 66 hr (●) incubation at 37°. The washed organisms were suspended in the various NaCl solutions and viable counts made after 30 min. incubation at 37°.

Fig. 8. Proteolysis in suspensions of *Mycoplasma laidlawii* membranes. The membranes were incubated at 37° in 0.01 M-NaCl containing 0.01 M-tris buffer (pH 8.0). Extinction of suspensions (●) and the amount of free amino acids in the supernatant fluids of centrifuged suspensions (○) were determined after various incubation times as described under Methods.

DISCUSSION

The dependence of the osmotic lysis of *Mycoplasma* organisms on temperature may explain the observations of Butler & Knight (1960) that washed *Mycoplasma bovis genitalium* organisms rapidly died in 0.01 M-phosphate buffer at room temperature (about 20°), but survived well in the same hypotonic buffer at 2–4°. Several of the parasitic *Mycoplasma* strains previously found to be relatively resistant to osmotic lysis at room temperature (Razin, 1963*b*) were lysed at 37°. However, even then strains of parasitic *Mycoplasma* in general showed a higher resistance to osmotic shock than strains of saprophytic *Mycoplasma*. Most resistant to osmotic lysis were *M. gallisepticum* strains. This observation might be of some interest in view of the opinion that *M. gallisepticum* should not be regarded as *Mycoplasma* proper (Klieneberger-Nobel, 1963). However, electron microscopy (Ruys & Van Iterson, 1961) and chemical analysis of organisms (Morowitz *et al.* 1962; Razin, Argaman & Avigan, 1963) did not show any fundamental difference between *M. gallisepticum* and other strains of *Mycoplasma*. The lower osmotic fragility shown by several strains of parasitic *Mycoplasma* may in part be due to their being harvested at the decline phase of growth. Like saprophytic organisms (Razin, 1963*b*), parasitic

Mycoplasma organisms were also found to be most sensitive to osmotic shock when harvested in the logarithmic phase of growth. Harvesting of the more exacting Mycoplasma strains at this phase was not always possible, because of their poor and sluggish growth.

Inhibition of osmotic lysis of Mycoplasma fits in well with the known protective effect of di- and multi-valent cations on osmotically fragile organisms and organelles (Tabor, Tabor & Rosenthal, 1961). The ability of very low spermine concentrations to protect Mycoplasma organisms from lysis and death in highly hypotonic media at 37° may be of use in nutritional and metabolic studies, where viability and metabolic activity of the organisms can thus be kept under the above-mentioned conditions.

The temperature dependence of the osmotic lysis of Mycoplasma, and the resistance of heated mycoplasmas to lysis, suggested that the lytic process may involve autolytic enzyme activity. Autolysis might be triggered by initial damage to the cell membrane by osmotic shock. The studies of Brown and his collaborators on the lysis of marine and extremely halophilic bacteria provide certain hints in favour of this hypothesis. The envelope of the extremely halophile *Halobacterium halobium* resembles that of Mycoplasma in being composed of a lipoprotein unit membrane devoid of the mucopeptide polymer (Brown & Shorey, 1963; Brown, 1963). The envelope of a marine pseudomonad was composed of two lipoprotein membranes (Brown, Drummond & North, 1962). The membranes of the pseudomonad, when isolated by osmotic lysis of the organisms, underwent intense autolysis of a proteolytic nature (Brown, 1961). The autolytic process, like the osmotic lysis of Mycoplasma, depended on temperature, pH value and ionic strength of the medium, and was inhibited by di- and multi-valent cations (Brown, 1962). Our results indicate that disintegration and proteolysis of *Mycoplasma laidlawii* membranes might take place on incubation at 37°. However, gross disintegration like that described for membranes of the marine pseudomonad (Brown *et al.* 1962) could not be seen in the electron microscope. Autolytic processes therefore probably play only a secondary role in the clearing of Mycoplasma suspensions after the initial osmotic shock. The studies of Edebo (1961*a-c*) on sonic and osmotic lysis of bacteria may provide another approach to explain the effects of incubation temperature, heat, pH value and polyvalent cations on osmotic lysis. According to Edebo sonic or osmotic lysis of bacteria is a two-step process; the first step involving initial structural damage to the cell envelope, and the second, the dispersion of protoplasm in the surrounding medium. All the factors which influence sonic or osmotic lysis are known to affect the transformation of the protoplasmic gel into a sol, and its dispersion in the medium.

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