Lysis Induced by Sodium Ion and Its Relation to Lytic Enzyme Systems in *Clostridium saccharoperbutylacetonicum*

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SUMMARY

Growing *Clostridium saccharoperbutylacetonicum* was lysed by sodium ion concentrations above 0.1 M (maximum effect at 0.3 M). The rate of lysis depended on the age of the culture. The most rapid lysis occurred in organisms from logarithmically growing cultures which were incubated for 3 to 5 h; organisms from stationary phase cultures were completely resistant to Na⁺-induced lysis. Sodium ion-induced lysis was related to autolysis of the organisms and was greatly affected by pH and temperature. The optimum pH was about 6.0, and the optimum temperature 35 °C. Various chemical inhibitors, including known enzyme inhibitors (such as Cu²⁺ and *p*-chloromercuribenzoate) and fixative agents (such as formalin and glutaraldehyde), inhibited the lysis, while organisms whose growth had been inhibited by antibiotics such as tetracycline, were also resistant to Na⁺-induced lysis. The lysate produced by Na⁺-treatment itself had lytic activity on isolated walls, thought to be due to its content of autolysin.

About 30 to 60 min after mitomycin C treatment (which induced the production of the phage tail-like bacteriocin clostocin O), the organisms were temporarily resistant to Na⁺-induced lysis. However the organisms reverted to the state of high sensitivity to Na⁺ when clostocin O-associated endolysin was produced. We suggest that Na⁺-induced lysis is due to the action of wall lytic enzymes such as autolysin and clostocin O-endolysin.

INTRODUCTION

In a previous paper (Ogata & Hongo, 1973), we showed that growing organisms of *Clostridium* species were lysed by univalent cations such as Na⁺, K⁺, Rb⁺, Cs⁺, Li⁺, and NH₄⁺. Of various pathogenic and non-pathogenic *Clostridium* species, strains of *C. saccharoperbutylacetonicum* were the most sensitive to univalent cations. The lytic action of Na⁺ was displayed at concentrations greater than 0.1 M, this ion having maximum effect at 0.3 M. Bivalent cations such as Ca²⁺, Mg²⁺, Ba²⁺, Co²⁺ and Ni²⁺ at above 0.005 M inhibited this lysis, whereas chelating agents such as EDTA and sodium citrate at concentrations above 0.01 M enhanced it. Therefore, the lysis seemed to depend on antagonism between added univalent cations and bivalent cations present in the medium and on the bacterial surface. However, from our findings it appeared that the lysis might not only be due to such ion antagonism, but to action of some lytic enzymes such as autolysin.

Very similar observations have been made in studies of the sensitivity of *Bacillus subtilis* to saline: when harvested at the logarithmic phase of growth they lost their colony-forming ability in 0.15 M-NaCl (Iizima & Ikeda, 1969). The saline-sensitive organisms were generally competent (i.e. able to take up DNA and produce transformants). It is well known (Young & Spizizen, 1963; Young, 1966; Stewart & Marmur, 1970) that there is a remarkable

correlation between an organism's autolytic propensity and its ability to undergo transformation. Saline sensitivity was also displayed by *Escherichia coli* (Sato, Suzuki, Izaki & Takahashi, 1971; Sato, Izaki & Takahashi, 1972), so that our findings should be compared with reports of other instances of saline sensitivity.

METHODS

Organism. The strain used was NI-4 (ATCC13564) of *Clostridium saccharoperbutylacetonicum* (Hongo & Murata, 1965) previously found to be particularly sensitive to Na⁺-induced lysis.

Medium and cultural conditions. Growth was at 30 °C under lowered atmospheric pressure (5 to 10 mm Hg) in TYA broth (Hongo & Murata, 1965) which contained (g/l distilled water): glucose, 40; Bacto-tryptone (Difco), 6; yeast extract (Daigo Eiyo Kagaku Co.) 2; ammonium acetate, 3; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.4 (approx. 0.001 M-Mg²⁺); FeSO₄·7H₂O, 0.01; at pH 6.5. To obtain a young exponentially growing culture, fresh medium was inoculated with sufficient organisms to produce an initial extinction (*E*) of 0.1 at 660 nm, and unless otherwise mentioned the culture was incubated until its *E*₆₆₀ became 0.30 (10⁸ organisms/ml).

Turbidity measurements. Extinction (*E*) of the culture or bacterial suspension was measured at 660 nm with a photoelectric colorimeter (model 7A, Tokyo Kodon Co. or type-101, Hitachi Co.), as previously described (Ogata, Mihara, Ikeda & Hongo, 1972).

Induction of bacterial lysis by sodium ions. In general, the organisms were grown anaerobically at 30 °C to an *E*₆₆₀ of 0.30. NaCl was added to this logarithmically growing culture to a final concentration of 0.3 M, incubation was continued at 30 °C, and readings of *E*₆₆₀ were made at 5 min intervals.

Induction of bacterial autolysis. Autolysis was induced as described by Kawata & Takumi (1970), and Higgins, Pooley & Shockman (1970). The organisms taken at various times were harvested by centrifugation and washed once with cold distilled water. The washed organisms were suspended in 0.01 M-phosphate buffer (pH 6.0) and allowed to autolyse at 30 °C, *E*₆₆₀ being measured at 5 min intervals.

Antibiotic treatment. Cultures grown anaerobically at 30 °C to an *E*₆₆₀ of 0.25, were treated for 20 min at 30 °C with (μg/ml): chlortetracycline (TC, Takeda Co.), 100; or chloramphenicol (CM, Sankyo Co.), 250; or mitomycin C (MC, Kyowa Hakko Co.), 4. Excess antibiotic was removed by centrifuging for 5 min at 9000 g. The harvested organisms were resuspended in prewarmed TYA broth to an *E*₆₆₀ of 0.25, and the culture incubated in the usual manner with readings of *E*₆₆₀ being made at 30 min intervals. A sample of antibiotic-treated culture was withdrawn at a suitable time and exposed to 0.3 M-NaCl, changes in its *E*₆₆₀ being monitored for 30 to 60 min.

Estimation of the rate of lysis. The rate of lysis was calculated from the equation: rate = $1000 \times (E_0 - E_t)/t$ where *E*₀ is the initial *E*₆₆₀ of the culture or suspension, and *E*_{*t*} is the terminal *E*₆₆₀ after lysis for *t* min.

Preparation of walls and formalin-treated organisms. Walls of strain NI-4 were prepared as described by Kawata & Takumi (1971).

Formalin-treated organisms were prepared by exposing harvested logarithmic phase organisms for 30 min at room temperature, to 0.067 M-phosphate buffer (pH 6.0) containing 5 % (v/v) formalin. The organisms were washed twice with cold phosphate buffer (without formalin), and suspended in similar buffer of pH 5.5.

Assay of lytic activity in the lysate induced by Na⁺-treatment. Lytic activity was assayed

Fig. 1

Fig. 2

Fig. 1. Inhibition of sodium ion-induced lysis of clostridia by various chemical compounds. O, No inhibitor; ●, glutaraldehyde (0.1 %, v/v), OsO₄ (0.1 %, w/v), CuSO₄ (10⁻³ M); ◐, CuSO₄ (5 × 10⁻⁴ M); ◑, CuSO₄ (10⁻⁴ M); Δ, formalin (0.5 %, v/v); ▲, formalin (0.1 %, v/v); ▽, PCMB (10⁻³ M); ▼, PCMB (5 × 10⁻⁴ M); ▲, fradiomycin (500 μg/ml), polymyxin B (500 μg/ml); ▲, FR (100 μg/ml) and PB (100 μg/ml); Δ, uranium nitrate (10⁻³ M). Initial *E*₆₆₀ of each culture was 0.30.

Fig. 2. Lysis rates of sodium ion-induced lysis and autolysis of various aged clostridia. O, Growth curve; ▲, lysis rate of Na⁺-induced lysis; Δ, lysis rate of autolysis.

by the decrease in turbidity of suspensions of walls or of formalin-treated organisms. Na⁺-induced lysate (2.5 ml) and 2.5 ml substrate were suspended in 0.067 M-phosphate buffer (pH 5.5) to a initial *E*₆₆₀ of 0.30; pH 5.5 phosphate buffer was used, since the pH of the lysate was 5.5. A control tube contained lysate boiled for 5 min.

Assay of bacteriocin-associated endolysin. Samples were withdrawn at 30 min intervals from a mitomycin C-treated culture. A portion of each sample was immediately centrifuged at 9000 *g* for 10 min, and the supernatant was assayed for extracellular endolysin activity. Another portion was exposed to 0.3 M-NaCl at 30 °C until lysis was completed, when the lysate was centrifuged at 60 000 *g* for 60 min to remove bacteriocin, and the supernatant was assayed for total (extracellular and intracellular) endolysin activity. To distinguish between endolysin and autolysin, formalin-treated bacteria were used as the substrate in this experiment since endolysin but not autolysin acted on this substrate.

RESULTS

Inhibition of sodium ion-induced lysis

Effect of chemical agents. In preliminary work, it was found that Na⁺-induced lysis was inhibited by small concentrations (10⁻³ to 10⁻⁴ M) of heavy metal cations such as Cu²⁺, Ag²⁺ and Pb²⁺. Other known inhibitors of lysis (Ralston, Lieberman, Baer & Krueger, 1957; Ralston, Baer, Lieberman & Krueger 1961; Doughty & Mann, 1967; Goepfert & Naylor, 1967; Higgins *et al.* 1970) were therefore tested as follows. The compounds were added to the growing cultures between 0 and 5 min before treatment with NaCl. After addition of NaCl, the *E*₆₆₀ was followed for a further 30 min at 30 °C (Fig. 1). Fixatives such as glutaraldehyde, formalin, osmium tetroxide (OsO₄), and uranium nitrate were potent inhibitors of the Na⁺-induced lysis. These fixatives protect the bacteria from auto-

Fig. 3

Fig. 4

Fig. 3. Resistance to sodium ion-induced lysis of tetracycline-treated clostridia. $\circ$ — $\circ$, Growth curve of normal organisms; $\bullet$ — $\bullet$, growth curve of TC-treated organisms; $\circ$ — $\circ$, lysis curve of normal organisms; $\bullet$ — $\bullet$, lysis curve of TC-treated organisms. TC: 100 μ g/ml. Excess TC was removed by centrifugation. NaCl (0.3 M) was added at the various times indicated by the arrows, and incubation was continued for 60 min at 30 °C.

Fig. 4. Resistance to sodium ion-induced lysis of chloramphenicol-treated clostridia. $\circ$ — $\circ$, growth curve of normal organisms; $\blacktriangle$ — $\blacktriangle$, growth curve of CM-treated organisms; $\circ$ — $\circ$, lysis curve of normal organisms; $\blacktriangle$ — $\blacktriangle$, lysis curve of CM-treated organisms. CM: 250 μ g/ml. The other conditions were the same as for Fig. 3.

lysis (Higgins *et al.* 1970). Sodium ion-induced lysis was also inhibited by *p*-chloromercuribenzoate (PCMB), fradiomycin (FR, neomycin, Takeda Co.) and polymyxin B (PB, Pfizer Taito Co.).

These results indicate that some enzymic reaction participates in Na^+ -induced lysis, and that free sulphydryl groups are involved.

Effect of pH. The culture was adjusted to the desired pH with 1 N-NaOH (or 1 N- NH_4OH) and 1 N-HCl before treatment with NaCl. After the addition of NaCl, the E_{660} was followed for 30 min at 30 °C. Lysis was clearly observed at pH 5.0 to 7.0, being most rapid at pH 5.7 to 6.0.

Effect of temperature. The culture was prewarmed or precooled to temperatures between 0 and 50 °C before treatment with NaCl. After the addition of NaCl, the E_{660} was followed for 30 min at each temperature. The rate of lysis increased with temperature to a maximum at 35 °C. No lysis took place below 5 °C or above 40 °C.

Effect of growth phase on sodium ion-induced lysis and autolysis

Portions of a culture were withdrawn at intervals to measure the organisms' sensitivity to Na^+ -induced lysis and their tendency to autolyse (Fig. 2). Actively growing organisms from young cultures were highly sensitive to Na^+ -induced lysis and autolysis. Both sensitivities decreased in organisms taken from older cultures. The rate of Na^+ -induced lysis was minimal at the stationary phase of growth, and that of autolysis was minimal at the late logarithmic phase.

These results suggest that active growth and a propensity for autolysis could be essential for Na^+ -induced lysis.

Fig. 5. Wall lytic activity in the lysate induced by sodium ion treatment. ○, Walls+lysate; ●, walls + heated lysate; × formalin-treated organisms+lysate. Incubation was at 30 °C. Initial E_{660} of each suspension was 0.30.

Resistance to sodium ion-induced lysis of tetracycline- and chloramphenicol-treated organisms

Treatment with antibiotics such as chloramphenicol (CM) results in rapid decrease in the rate of autolysis (Shockman, 1965; Higgins *et al.* 1970; Pooley & Shockman, 1970; Stewart & Marmur, 1970). Organisms were therefore treated with chlortetracycline (TC) or CM and both treatments resulted in inhibition of growth and a decrease in the rate of Na^+ -induced lysis (Figs. 3 and 4). It was also evident that TC and CM were not chemical inhibitors such as FR and PB; TC had a more pronounced effect on both Na^+ -induced lysis and growth than had CM. The TC-treated organisms completely stopped growing about 60 min after exposure to the antibiotic, and developed their insensitivity to Na^+ -induced lysis after some 120 min. With CM-treated organisms, slow Na^+ -induced lysis was demonstrable at all times.

Thus, Na^+ -induced lysis was inhibited by antibiotics known to impair autolysis.

Wall lytic activity in the lysate obtained by sodium ion treatment

As shown in Fig. 5, Na^+ -induced lysate had some lytic activity on isolated walls, but not on formalin-treated organisms. To determine whether or not this lytic activity was enzymic in origin, the Na^+ -induced lysate was boiled for 5 min. The heat-treated preparation did not digest isolated walls (Fig. 5).

This suggests the presence of a lytic enzyme (possibly autolysin) in Na^+ -induced lysate.

Sodium ion-induced lysis of organisms treated with mitomycin C

Another aspect of Na^+ -induced lysis was examined using mitomycin C (MC)-treated organisms in which the production of phage tail-like bacteriocin was induced (clostocin O; Ogata *et al.* 1972).

When a logarithmically growing culture ($E_{660} = 0.25$) was exposed to 4 µg MC/ml, the E_{660} of the culture increased as shown in Fig. 7. Normal lysis (MC-induced lysis) began 180 min after pre-cultivation (Fig. 7), and finished some 5 h or more later (Ogata *et al.* 1972). By treatment with NaCl, Na^+ -induced lysis was provoked at various times. The lysis curve and lysis rate displayed by organisms harvested at different incubation times are shown in Figs. 6 and 7, respectively. MC was evidently not acting as a chemical inhibitor.

Fig. 6

Fig. 7

Fig. 6. Sodium ion-induced lysis of mitomycin C-treated clostridia. O, No MC. The following indicate 0.3 M-NaCl-added times (see O—O line in Fig. 7) with 4 μ g MC/ml: ▲, 0 min; ●, 30 min; △, 60 min; ▼, 120 min; ▽, 180 min. The initial E_{660} was not identical for each culture (E_{660} : 0.25 to 0.42, see O—O line in Fig. 7). The experiment was performed as described in Fig. 3.

Fig. 7. Lysis rate of sodium ion-induced lysis of mitomycin C-treated clostridia and production of clostocin O-endolysin at various times after mitomycin C treatment. O—O, Growth curve of MC-treated organisms; ▲—▲, lysis rate of Na^+ -induced lysis; ●—●, extracellular activity of endolysin; ●—●, intracellular activity of endolysin. Maximum activity of endolysin was 12 units.

During pre-cultivation, MC-treated organisms became relatively resistant to Na^+ -induced lysis, maximum resistance being displayed some 30 min after pre-cultivation. However, the organisms returned to their state of high sensitivity after about 60 min. Their rate of lysis was greater than that of normal organisms, and the 5 min lag period usually observed in the case of normal organisms was absent. The lysis rate increased still further as time proceeded, reaching its maximum at 150 to 180 min, which was also the time at which MC-induced lysis was initiated. A possible explanation of this enhanced sensitivity to lysis is offered below.

Production of bacteriocin-associated endolysin during the development of mitomycin C-treated organisms

Production of phage or of phage tail-like bacteriocin, and the release of these particles, are accompanied by the lysis of the producer organisms (Ralston *et al.* 1957; 1961; Koch & Dreyer, 1958; Kageyama, Ikeda & Egami, 1964; Doughty & Mann, 1967; Goepfert & Naylor, 1967; Bradley, 1969). Such lysis is caused by the breakdown of wall by phage- or bacteriocin-associated endolysin.

Fig. 7 shows extracellular and intracellular development of bacteriocin-associated endolysin (clostocin O-endolysin). No extracellular activity of this endolysin could be detected until 90 min after pre-cultivation, and little active endolysin was present in the organisms at 60 min. The content of endolysin increased with the age of the culture, a portion being released from the bacteria. Maximum activity was observed at the time of MC-induced lysis, and the rate of Na^+ -induced lysis accelerated according to the increased content of active endolysin.

These results indicate that the high sensitivity of MC-induced organisms to Na^+ -induced lysis is likely to be due to the biosynthesis of endolysin.

DISCUSSION

Sodium ion-induced lysis is a specific phenomenon manifested by *Clostridium* species (Ogata & Hongo, 1973). It seemed to be due to ion antagonism between Na^+ added and bivalent cations in the medium and on the bacterial surface, or to some physical action of Na^+ . However, there were indications that the lysis was enzymically caused, e.g. it was inhibited by chemicals known to be inhibitors of enzymes and of autolysis. The cultural conditions which favoured lysis were those in which there was marked synthesis of autolysin. Furthermore, the lysate produced by Na^+ -treatment had lytic activity against isolated walls. Since no lytic enzymes other than autolysin are known to exist in normal growing organisms, our findings suggest that the lysis induced by Na^+ -treatment is catalysed by autolysin. Autolysin may play some part in the normal growth of bacteria (Shockman, Thompson & Conover, 1967; Anderson, Matsushashi, Haskin & Strominger, 1965; Shockman, 1965; Young, 1966; Strominger, Izaki, Matsushashi & Tipper, 1967); action of autolysin on peptidoglycan can cause local weakening of the rigid wall and so facilitate insertion of newly synthesized wall material.

Treatment of rapidly growing organisms with antibiotics, including CM, MC, actinomycin D and cellocidin, results in thickening of their walls (Shockman, 1965; Higgins *et al.* 1970), rapid loss of their ability to autolyse, and a gradual decrease in their content of the active form of autolysin (Shockman, 1965; Higgins *et al.* 1970; Pooley & Shockman, 1970; Stewart & Marmur, 1970). At present, we have no precise knowledge of the condition of antibiotic-treated organisms, but it is clear that TC- or CM-treatment results in a rapid decrease in the rate of Na^+ -induced lysis. Antibiotic-treated organisms developed their insensitivity to Na^+ -induced lysis when growth completely stopped. The continuing sensitivity of CM-treated organisms could be due to the continuing synthesis of autolysin and wall, since CM still permitted growth. These results indicate that Na^+ -induced lysis is inhibited by those antibiotics known to inhibit autolysis. It may also be said that the resistance to Na^+ -induced lysis of antibiotic-treated organisms would depend on the decrease in their content of autolysin and the increase in thickness of their walls.

Temporary resistance to Na^+ -induced lysis of MC-treated organisms could be explained on the same basis as the resistance of TC- or CM-treated organisms. The very high sensitivity of MC-treated organisms would be due to the production of clostocin O-endolysin. This lysin was clearly different from the lysin of normal organisms (autolysin) in its substrate specificity, for it was active against formalin- and chloroform-treated organisms (Sekiguch & Cohen, 1964; Salser, Gesteland & Bolle, 1967) as well as on isolated walls, whilst autolysin was only active on isolated walls. There was much more endolysin than autolysin in the lysate (S. Ogata, Y. Tahara, A. Umeda, S. Yoshino & M. Hongo, unpublished). The endolysin when present in small concentrations in MC-treated organisms at early times may cause expansion of the wall, but when present in greater concentrations, as at later times, it may cause breakdown of the wall. FR and PB were specific inhibitors of both lysins (Ogata *et al.* unpublished), whose enzymic properties will be reported in greater detail in a future paper.

From these observations, it may be concluded that the rapid lysis of strain NI-4 by the addition of Na^+ is due to the action of wall lytic enzymes such as autolysin and bacteriocin-associated endolysin. However, the role of Na^+ remains obscure and is being further investigated in our laboratory.

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