PROPIONATE INDUCED LYSIS OF PROTOPLASTS OF *BACILLUS MEGATERIUM*[1]

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Previous studies (Storck and Wachsman, 1957; Weibull, Beckman, and Bergstrom, 1959; Wachsman, Fukuhara, and Nisman, 1960) on enzyme localization were performed with cell fractions, obtained by lysing protoplasts in media of low osmotic pressure. In an attempt to determine the effect of the tonicity of the lysing medium on the distribution of enzyme activities between the membrane containing and supernatant fractions, a study of methods for protoplast lysis in 0.25 M sucrose was undertaken. This concentration of sucrose is usually sufficient to stabilize the protoplasts of *Bacillus megaterium* (Weibull, 1953). A possible method for lysing protoplasts in a normally isotonic medium was suggested by the recent studies on energy dependent permeation mechanisms in bacteria (Rickenberg et al., 1956; Cohen and Monod, 1957). The lysozyme protoplast of *Escherichia coli* contains a galactoside-permease (Rickenberg, 1957; Sistrom, 1958) which may catalyze the intracellular accumulation of galactosides to a level several hundred-fold greater than that of the extracellular concentration.

The present work shows that propionate, or any one of a variety of other low molecular weight compounds, can induce a rapid lysis of *B. megaterium* protoplasts in 0.25 M sucrose. The resulting lysate contains intact cytoplasmic membranes that appear almost free of contamination with other microscopically detectable cell components. The lytic phenomenon is energy dependent and can best be explained in terms of an osmotic shock, resulting from the active transport and intracellular accumulation of the low molecular weight compound.

MATERIALS AND METHODS

B. megaterium strain KM was grown with vigorous aeration at 30 C on a medium containing 1.0 per cent glucose, 0.5 per cent L-asparagine, 0.5 per cent NH_4Cl, 0.3 per cent K_2HPO_4, 0.1 per cent KH_2PO_4, 0.1 per cent Na_2SO_4, 0.01 per cent $MgSO_4 \cdot 7H_2O$, and 3 $\times$ 10^{-4} M ferric citrate (final pH, 7.0). Cells were harvested by centrifugation at the end of the phase of exponential growth and were washed once with 0.04 M potassium phosphate buffer pH 7.0 containing 2 $\times$ 10^{-3} M $MgSO_4$ (medium B).

Protoplasts were prepared by resuspending cells in medium B containing 0.25 M sucrose and 0.04 per cent lysozyme (final concentration of approximately 9 mg bacterial dry weight per ml). Protoplast formation was complete after 15 min at 30 C. In experiments where washed protoplasts were used, the majority of the contaminating lysozyme and cell wall degradation products were removed by centrifuging the suspension for 5 min at 13,000 $\times$ *g*. The protoplasts were then resuspended in medium B containing 0.25 M sucrose. Both washed and unwashed protoplast suspensions were incubated in an ice bath for periods of anywhere from 30 min to 400 min, prior to use.

For lytic experiments, aliquots of the cooled protoplast suspension were added to colorimeter tubes (13 $\times$ 145 mm) in the cold. The reaction mixtures were adjusted to contain 1.2 mg of protoplast dry weight per ml in medium B, and 0.25 M sucrose in a final volume of 4.0 ml. With the exception of the control tubes, potassium propionate and other lytic agents were usually added at a final concentration of 75 μ moles per ml. The tubes were then sealed with rubber stoppers and were incubated in a horizontal position on a Dubnoff reciprocal shaker at 30 C. Optical densities were determined at zero time and periodically during the incubation, with the Klett-Summerson colorimeter using filter no. 62.

Observations on swelling, contraction, or lysis of protoplasts were made with the Bausch and Lomb phase contrast microscope.

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Growth rates were determined on cultures shaken at 30 C, using 250 ml flasks with attached colorimeter tubes. Experiments on dye reduction were carried out as previously reported (Storck and Wachsman, 1957). The conventional Warburg apparatus was used in manometric experiments.

All compounds used were commercial samples of analytical grade. Gramicidin and sodium propionate were obtained from the Nutritional Biochemicals Corp. and versene from the Hach Chemical Co. Crystalline lysozyme was obtained from Armour and Co., and chloramphenicol from Parke, Davis and Co.

RESULTS

Propionate-induced lysis of protoplasts. Preliminary experiments showed that propionate lysis of protoplasts occurs at 30 C, but not at 4 C, and is dependent upon an adequate supply of oxygen. Therefore, all experiments were performed with gentle shaking at 30 C. In addition, observations with the phase contrast microscope showed that the liberated cytoplasmic membranes were almost free of contamination with other cell components; membranes isolated as a result of protoplast lysis in dilute phosphate buffer contain many more dense granules.

It has been fairly well established that as protoplasts swell, they scatter less light (Mitchell and Moyle, 1956; Sistrom, 1958; Gilby and Few, 1959). This can be detected as a corresponding decrease in optical density. Conversely, protoplast contraction results in an increase in optical density. A similar dependence of optical density on volume has been found for intact bacteria (Mitchell and Moyle, 1956; Kuczynski-Halmann, Avi-Dor, and Mager, 1958), rat liver mitochondria (Tedeschi and Harris, 1958), and erythrocytes (Wilbur and Collier, 1943).

An example of the optical density changes accompanying the propionate lysis of protoplasts is shown in figure 1. Since microscopic examinations showed that protoplast lysis does not occur above 175 Klett Units, it is apparent that lysis is preceded by a rapid swelling of protoplasts, followed by a contraction. This contraction is not observed when propionate induced lysis is extremely rapid (figures 2 and 3). In addition, the rate of lysis is dependent

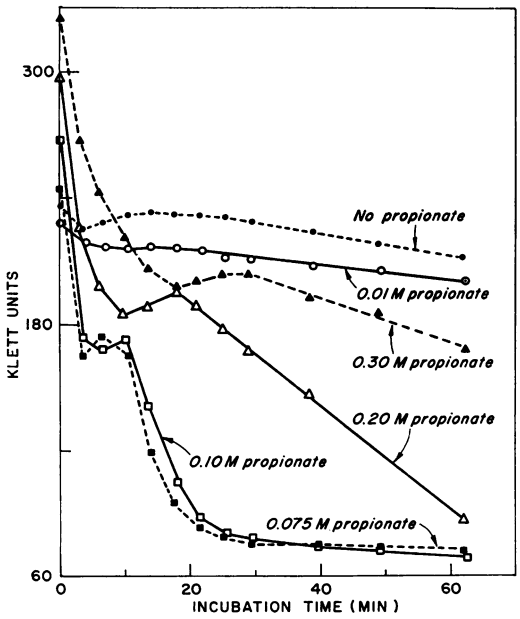

Figure 1. Effect of propionate concentration on protoplast lysis in 0.25 M sucrose. Propionate was added as the potassium salt at zero time. The protoplasts were unwashed and were aged in an ice bath for 30 min prior to the experiment.

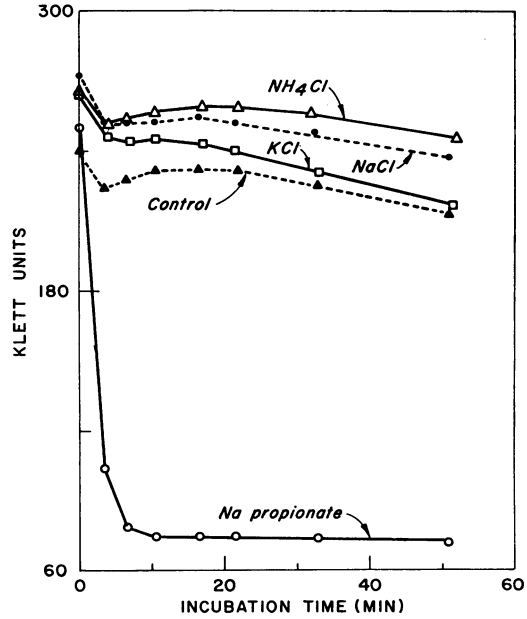

Figure 2. Effect of Na-propionate and inorganic salts on protoplast stability in 0.25 M sucrose. The protoplasts were unwashed and were aged in an ice bath for 130 min. All salts were added at zero time at a final concentration of 0.075 M.

upon the propionate concentration. The most rapid rate occurs in the presence of 0.075 M to 0.10 M potassium propionate, while both 0.30 M and 0.01 M propionate induce little or no lysis.

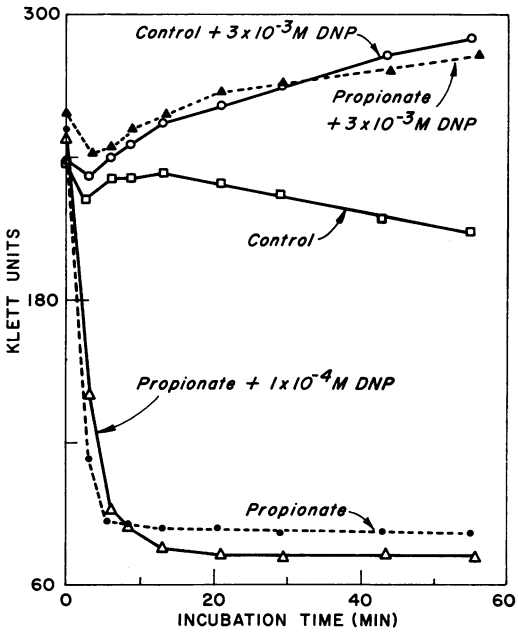

Figure 3. Effect of 2,4-dinitrophenol (DNP) on propionate induced protoplast lysis in 0.25 M sucrose. The protoplasts were unwashed and were aged in an ice bath for 230 min. Propionate was added as the potassium salt (0.075 M).

The control protoplast suspension is stable, showing only minor changes in optical density.

It is also apparent from figure 1 that the zero time readings increase with increasing concentrations of propionate. As determined microscopically, this is due to a contraction of protoplasts. However, even though the optical density at zero time may vary with the tonicity of the medium, the same final optical density is reached upon complete lysis. With 0.075 M propionate, the optical density at complete lysis is approximately 30 per cent of the zero time reading.

Since the potassium salt of propionic acid was used in the experiment of figure 1, it was decided to determine the effect of cations on the lytic phenomenon. As is shown in figure 2, the sodium salt of propionic acid also induces protoplast lysis. Furthermore, neither sodium, potassium, nor ammonium chloride were effective lytic agents. Therefore, lysis may be attributed to the presence of the propionate anion.

A plot of the logarithm of the optical density as a function of time yields at least a two-component curve. It is therefore difficult to determine the rate of lysis in any simple way. As an estimate of the rate, it was decided to consider the time necessary for lysis of 50 per cent of the protoplasts. Based upon microscopic observations, this corresponds to a reading of 120 Klett Units.

As is shown in figure 3, 3×10^{-3} M 2,4-

TABLE 1

*Effect of metabolic inhibitors on propionate lysis of protoplasts in 0.25 M sucrose**

Expt No.	Inhibitor	Concentration	Time for Turbidity Drop to 120 Klett Units	
			− Inhibitor	+ Inhibitor
			min	*min*
43	2,4-dinitrophenol	1×10^{-3} M	2.5	No lysis
44	Potassium cyanide	1×10^{-3} M	2.0	4.5
	Gramicidin†	1.6×10^{-4} M	2.0	15.0
	Arsenite	1×10^{-3} M	2.0	2.0
	Azide	1×10^{-3} M	2.0	5.8
	Chloramphenicol	200 μg per ml	2.0	2.5
45	Ethanol	4.0 per cent	3.5	25.0

* Summary of several different experiments in which unwashed protoplasts were incubated with 0.075 M potassium propionate under the conditions described in Materials and Methods. The metabolic inhibitors were added at zero time at the final concentrations listed above. In all cases the controls showed no lysis.

† Assuming a mol wt of 3,000.

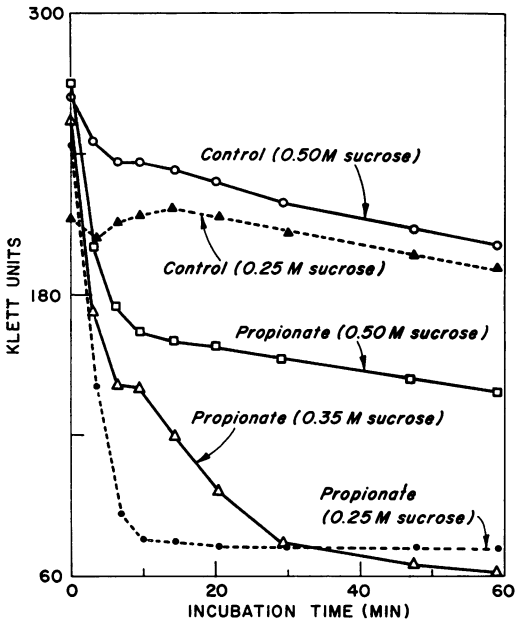

Figure 4. Effect of sucrose concentration on propionate lysis of protoplasts. The protoplasts were unwashed and were aged in an ice bath for 115 min. Propionate was added as the potassium salt (0.075 M).

dinitrophenol completely blocks propionate induced lysis. In most experiments, 1×10^{-3} M 2,4-dinitrophenol was sufficient to prevent lysis. The presence of inhibitory levels of 2,4-dinotrophenol causes the formation of highly dense and contracted protoplasts. The results of several experiments on the effect of other metabolic inhibitors on lysis are summarized in table 1. It is apparent that at the concentrations employed, 2,4-dinitrophenol is the most effective inhibitor. Both 4.0 per cent ethanol and 1.6×10^{-4} M gramicidin are good inhibitors, while chloramphenicol has only a slight effect. This is true even when protoplasts are pre-incubated with chloramphenicol. Since as little as 5 μg of chloramphenicol per ml strongly inhibits the growth of this strain of *B. megaterium* (J. T. Wachsman, *unpublished data*), the findings suggest that protein synthesis is not required for propionate lysis.

The apparent energy requirement for propionate lysis is consistent with a mechanism involving the active transport of propionate across the permeability barrier of the protoplast. The results may be interpreted as indicating

TABLE 2

*Compounds inducing protoplast lysis in 0.25 M sucrose**

Compound Tested	Time for Turbidity Drop to 120 Klett Units	
	+ Test compound (in absence of K-propionate)	− Test compound (in presence of K-propionate)
	min	*min*
Glucose	15.0	2.3
K-acetate	2.9	3.1
K-butyrate	3.6	3.1
K-*n*-valerate	19.0	4.0
Na-L-glutamate	7.6	2.4
L-Asparagine	7.8	3.8
L-Aspartate	26.5	4.3
K-pyruvate	10.8	2.4
K-L-malate	18.3	4.8
K-fumarate	43.0	4.3
K-malonate	11.3	7.5
Na_3-DL-isocitrate	5.5	5.3
K_3-Citrate	2.8	2.4
Versene	3.5	5.0

* Summary of several different experiments in which washed protoplasts were incubated with different compounds under the conditions described in Materials and Methods. All compounds were tested at a final concentration of 75 μmoles per ml in the presence of 2.5×10^{-4} M glucose as an energy source. All results are presented in comparison to the results on lysis in the presence of K-propionate, obtained in the corresponding experiment. The results represent an average of at least 2 experiments. In all cases, the controls showed no lysis. (All compounds neutralized to pH 7.0.)

that the protoplast has the ability to concentrate propionate to a level where the intracellular concentration exceeds that of the extracellular concentration. As a result of the increased internal osmotic pressure, the protoplast takes up water, swells, and eventually bursts. This interpretation is consistent with the finding of an optimal propionate concentration for lysis (figure 1). However, it should be possible to inhibit lysis in the presence of an optimal propionate concentration (0.075 M), by increasing the external osmotic pressure of the medium. The results of such an experiment are shown in

figure 4. Of the sucrose concentrations used, 0.25 M sucrose permits the most rapid rate of lysis. The rate of lysis is decreased in the presence of 0.35 M sucrose and even further by 0.50 M sucrose. On the basis of microscopic observations, less than 20 per cent lysis occurred in the presence of 0.50 M sucrose; the remaining protoplasts were highly contracted.

Effect of other compounds on protoplast stability. A search for other lytic agents showed that 0.075 M glucose induces lysis. Since the lytic phenomenon is energy dependent, it was decided to test for the activity of other compounds in the presence of 2.5×10^{-4} M glucose; this concentration is insufficient to cause lysis. The results of several experiments are summarized in table 2, and an example of the types of optical density changes observed is shown in figure 5. It is apparent that a variety of different organic molecules can induce lysis: sugars, fatty acids, amino acids, etc. Of the compounds tested, only acetate, butyrate, citrate, isocitrate, and versene

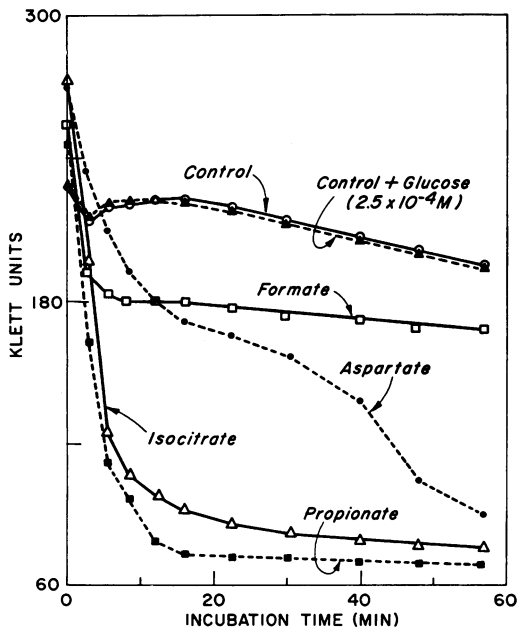

Figure 5. Effect of several compounds on protoplast stability in 0.25 M sucrose. Washed protoplasts were incubated in an ice bath for 190 min. The following compounds were added at zero time at a final concentration of 0.075 M: K-propionate, Na_3-DL-isocitrate, NH_4-formate, or L-aspartate. In addition, all reaction vessels, except one of the controls, contained 2.5×10^{-4} M glucose.

TABLE 3

*Influence of 2,4-dinitrophenol on substrate induced protoplast lysis in 0.25 M sucrose**

Compound Tested	Time for Turbidity Drop to 120 Klett Units	
	− Dinitrophenol	+ Dinitrophenol
	min	*min*
Glucose	12.0	No lysis
K-acetate	3.0	No lysis
K-propionate	4.0	No lysis
K-butyrate	4.5	No lysis
K-*n*-valerate	17.5	40% lysis†
Na-L-glutamate	9.5	No lysis
L-Asparagine	3.5	No lysis
L-Aspartate	9.0	No lysis
K-pyruvate	11.5	35.5
K-L-malate	7.0	26.0
K-fumarate	15.0	30% lysis†
K-malonate	10.5	39.5
Na_3-DL-isocitrate	4.5	4.5
K_3-Citrate	2.5	2.5
Versene	2.5	11.0

* Summary of several different experiments in which washed protoplasts were incubated with a variety of substrates under the conditions described in Materials and Methods. All substrates were tested at a final concentration of 75 μmoles per ml in the presence of 2.5×10^{-4} M glucose, as an energy source. Duplicate reaction vessels containing 2×10^{-3} M 2,4-dinitrophenol were run simultaneously. All the compounds used were neutralized to pH 7.0. In all cases, the controls showed no lysis.

† Based on microscopic observations, since turbidity did not drop to 120 Klett units during the period of incubation.

are as effective as, or better than propionate as lytic agents. The following compounds failed to induce protoplast lysis under the conditions employed in table 2: lactose, L-sorbose, *i* erythritol, *n*-butanol, glycerol, 2,3-butylene glycol, acetoin, ethanol, L-leucine, L-methionine, L-tryptophan, and NH_4-formate.

Since propionate induced lysis is completely blocked by 2,4-dinotrophenol, it was decided to determine the effect of this inhibitor on the lytic ability of other compounds. The results of

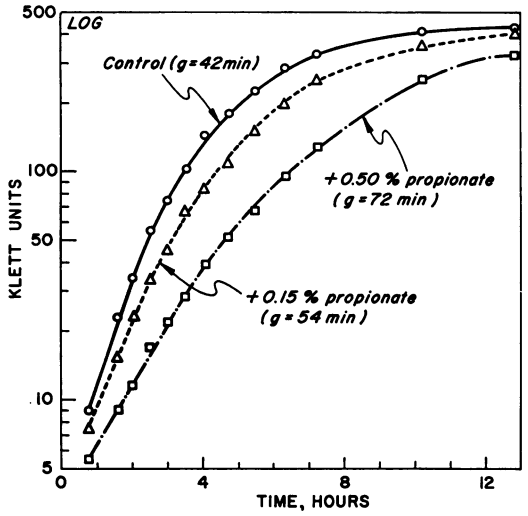

Figure 6. Inhibitory effect of propionate on growth of strain KM. Growth flasks containing the glucose-asparagine medium listed in Methods, were inoculated with an exponentially growing culture. Different amounts of Na-propionate were added and the flasks were incubated with shaking at 30 C. The growth flasks contained attached colorimeter tubes. Growth was measured in the Klett-Summerson colorimeter, using filter no. 64.

several different experiments are summarized in table 3. It is apparent that the action of 2×10^{-3} M 2,4-dinotrophenol varies from a complete inhibition of lysis to no effect, depending upon the lytic agent.

Growth and metabolism in the presence of propionate. The effect of sodium propionate on the growth of *B. megaterium* in the glucose-asparagine medium described above is shown in figure 6. It is apparent that propionate inhibits growth and that the degree of inhibition is a function of the propionate concentration. The generation time varies from 42 min in the absence of propionate to 72 min in the presence of 0.5 per cent propionate.

Manometric experiments were performed with cells grown in the presence and the absence of sodium propionate. Under conditions where there was a rapid oxygen uptake in the presence of either glucose or L-asparagine, no oxygen uptake above the endogenous level was found with sodium propionate. In addition, propionate has no effect on oxygen uptake in the presence of glucose.

Cells grown in the absence of propionate were converted to protoplasts, and were lysed in dilute phosphate buffer. The resulting total lysate was then assayed for the ability to catalyze the reduction for 2,6-dichlorophenol indophenol in the presence of propionate. Under conditions in which the dye was rapidly reduced in the presence of glucose, pyruvate, or α-ketoglutarate, no reduction above that of the endogenous level could be shown with potassium propionate.

DISCUSSION

The results may be explained in terms of the following mechanism for propionate induced lysis. Propionate is actively transported across the permeability barrier of the protoplast and is there concentrated to a level sufficient to render the intracellular medium hypertonic with respect to the extracellular medium. The protoplast then imbibes water, swells, and eventually bursts. This interpretation is consistent with the requirement for energy and for an optimal propionate and sucrose concentration. In addition, this explanation accounts for the observed differences between propionate lysis and detergent lysis of protoplasts. In contrast to the action of propionate, lysis of *B. megaterium* protoplasts by sodium dodecylsulfate occurs at 4 C and it is not inhibited by 2,4-dinitrophenol (Storck and Wachsman, *unpublished data*). Furthermore, detergent action results in a fragmentation of membranes (Gilby and Few, 1957; Wachsman et al., 1960).

The results show that the optical density of a protoplast suspension can vary by as much as 30 per cent, as a result of swelling or contraction of protoplasts. This is in agreement with the report that the protoplast volume of this strain of *B. megaterium* varies inversely with the external osmotic pressure (Weibull, 1955). Lysozyme protoplasts of *E. coli* have also been found to respond osmotically. The optical density of a protoplast suspension, prepared from cells grown in the presence of β-galactosides, can decrease as much as 30 per cent upon exposure to 0.01 M lactose (Sistrom, 1958). The author concluded that the bulk of the intracellular galactoside was in the form of a free solute.

The compounds inducing protoplast lysis may be divided into three groups on the basis of the effect of 2×10^{-3} M 2,4-dinitrophenol on the lytic phenomenon. Lysis by acetate, propionate, butyrate, glucose, L-glutamate, L-asparagine, or

L-aspartate is completely inhibited by this concentration of 2,4-dinitrophenol. Lysis by citrate or isocitrate is not effected by 2,4-dinitrophenol while lysis by *n*-valerate, pyruvate, L-malate, fumarate, malonate, or versene is only partially inhibited by 2,4-dinitrophenol. Either there are several different mechanisms for protoplast lysis, or the systems involved in active transport differ with respect to their ability to utilize a limited energy supply.

Versene has been found necessary for the lysis of some gram-negative bacteria in the presence of lysozyme (Repaske, 1958). In addition, versene has been reported to induce protoplast lysis in *B. megaterium* (Vennes and Gerhardt, 1956). Although the latter investigators make no mention of an energy requirement for lysis, it is significant that the protoplasts were lysed at 37 C.

SUMMARY

Propionate, or any one of a variety of other compounds of low molecular weight, can induce a rapid lysis of protoplasts of *Bacillus megaterium* in 0.25 M sucrose. The cytoplasmic membranes are liberated in an intact state, and appear to be almost free of contamination with other microscopically detectable cell components. Propionate lysis occurs at 30 C, but not at 4 C; it is completely inhibited by 2×10^{-3} M 2,4-dinitrophenol. The results are consistent with the active transport of propionate across the permeability barrier of the protoplast and the concentration of propionate to a level sufficient to render the extracellular medium hypotonic with respect to the protoplast. The protoplast then imbibes water, swells, and eventually bursts. Propionate inhibits the growth of *B. megaterium*, but no propionate oxidation could be detected.

Like propionate, the lysis induced by acetate, butyrate, glucose, L-glutamate, L-asparagine, or L-aspartate is completely inhibited by 2×10^{-3} M 2,4-dinitrophenol.

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