FACTORS AFFECTING THE LYTIC ACTIVITY OF LYSOZYME[1]

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Since the initial discovery of lysozyme by Fleming (1922), numerous attempts have been made to describe the properties of this enzyme. The absence of a reliable method for the determination of enzymatic activity, however, has contributed to the incompleteness and to the inconsistency of the facts found in the literature. The availability of crystalline, egg white lysozyme has made it possible to study quantitatively some of the factors which affect its lytic activity. The assay method reported by Smolelis and Hartsell (1949) has been found to be accurate and highly adaptable to the study of this enzyme. This report concerns its application to the effect of pH, salts, ions, temperature, and manner of preparation of the cell suspension, on the activity of lysozyme.

MATERIALS AND METHODS

Preparation of the cell suspension. To expedite the testing of lytic activity by lysozyme and to reduce the possibility of daily variations associated with the use of live cells of *Micrococcus lysodeikticus*, as noted by Meyer and Hahnel (1946), an effort was made to use killed cells. Lysozyme is capable of causing lysis of both live and dead cells of this species. Boasson (1938) used phenolized cells; therefore, this method was tried first. This technique provided an extremely variable cell preparation especially with pure lysozyme and the spectrophotometric method of assay. After storage of these cells at 4 C reproducible results in replicate tests were not obtained.

Desiccation of the phenol-killed cells with acetone did not provide a satisfactory cell preparation. Such cells gave variable results and were difficult to resuspend properly. It was assumed that acetone influenced the sensitivity of *M. lysodeikticus* by reason of its fat solvent action on certain lipoidal layers near the cell wall, thus changing the physiochemical factors essential to lysozyme action or to cell solubility. Lyophilization of a distilled water suspension of untreated cells provided a satisfactory preparation which could be easily rehydrated, easily stored at 4 C, and would give reproducible results, even though autolytic enzymes might still be active when such cells were employed. *M. lysodeikticus*, strain ATCC 4698, was used in these studies.

Table 1 presents the effect on the sensitivity to lysis obtained when other agents were used to kill *M. lysodeikticus*.

[1] These data constitute, in part, a thesis submitted to the Graduate School of Purdue University in partial fulfillment of the requirements for the Doctor of Philosophy degree. Grateful appreciation is expressed to the Purdue Research Foundation for a research grant while completing these studies.

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An examination of the data shows that treatment of cells by any method prior to exposure to lysozyme invariably caused a decreased lytic response. This is contrary to the findings of Feiner *et al.* (1946). These authors reported that little or no change in cell sensitivity to lysis was observed after the organism was subjected to repeated washings with saline, lyophilization, precipitation with ice cold acetone, one per cent phenol, or exposure to ultraviolet light.

Our data are in agreement with Epstein and Chain (1940) who state that, "Even *M. lysodeikticus* will not be lysed, or incompletely so, if its proteins are denatured by heat, organic solvents (alcohol, acetone, etc.) or iodine."

TABLE 1

*A comparison of the bacteriolytic responses of killed cells**

LYSOZYME DILUTION	AVERAGE PER CENT TRANSMISSION									
	Cell preparations									
	Live cells	Live QF	UVL	Live L	AD	PKL	PKA	RL	P	EL
1:200,000	91.7	88.5	80.2	85.0	75.5	73.0	64.7	61.0	59.7	53.2
1:400,000	86.2	79.7	70.0	76.0	66.9	61.7	61.9	58.6	57.7	52.5
1:800,000	73.5	55.5	53.9	58.7	48.6	46.0	51.0	49.5	49.7	44.7
1:1,600,000	53.0	38.7	40.5	45.2	37.2	35.0	39.0	39.5	49.7	37.0
1:3,200,000	37.5	29.5	32.0	32.7	30.7	30.0	32.2	33.1	33.0	27.4
1:6,400,000	29.0	26.0	26.5	30.0	28.5	26.2	28.9	29.6	29.0	28.0
Control	23.6	23.0	22.9	25.0	25.0	26.0	26.0	26.0	24.2	28.6

* In phosphate buffer pH 6.2; 20 minutes' incubation at room temperature (25 C).

LEGEND:

Live QF—live cells quick frozen
UVL—ultraviolet-killed, lyophilized cells
Live L—live cells lyophilized
AD—live cells acetone dried
PKL—phenol-killed cells lyophilized
PKA—phenol-killed cells acetone dried
RL—Rocall-killed cells lyophilized
P—phenol-killed cells
EL—Emulsept-killed cells lyophilized

On the basis of our data, ultraviolet light was selected as the killing agent, followed by lyophilization and storage of the cells at 4 C until used. It was assumed that the ultraviolet light rendered autolytic enzymes inactive, to a considerable extent, thus minimizing their influence on lysis in any test situation. Presumably this improved the possibilities for a cell suspension to give reproducible results and to more accurately assay the lytic activity of lysozyme alone.

Effect of temperature on the activity of lysozyme. While developing the assay procedure referred to previously, it was necessary to find the temperature at which lysis would take place so that when the results were plotted a geometric relationship existed between the concentration of lysozyme and the extent of lysis. Table 2 presents the data from a typical experiment with purified lysozyme and killed cells of *M. lysodeikticus*, tested in the manner described by Smolelis and Hartsell (1948).

The readings for the experiments done at room temperature gave the desired

linear relationship between the turbidity and the lysozyme concentration. A sufficiently wide range was obtained so that readings for an unknown lysozyme extract could be interpolated thereon. Therefore, this was the temperature selected for the standard assay of lysozyme. An increase in the incubation temperature would have necessitated a higher series of lysozyme dilutions to obtain the required graphic relationship, thus magnifying any errors when preparing dilutions of the enzyme.

As with other enzymatic reactions, lysozyme was much more active when the temperature was elevated. At 37 C and at 52 C greater lysis was noted than at room temperature (25 C). These observations are consistent with those of Hartsell (1948) and Wilson (1950).

TABLE 2

*Effect of temperature on lysis**

LYSOZYME DILUTION	TEMPERATURE		
	R. T. 25 C	37 C	52 C
	Average per cent transmission		
1:200,000	85.0	89.7	90.5
1:400,000	77.3	86.0	86.5
1:800,000	62.0	75.3	81.7
1:1,600,000	46.6	53.7	73.2
1:3,200,000	36.7	41.5	58.7
1:6,400,000	29.3	33.1	43.5
Control Cell suspension	23.0	25.5	26.0

* In phosphate buffer pH 6.2, after 20 minutes' incubation.

Fleming (1922) found that bacteriolysis by lysozyme was a function of the temperature of incubation and that this was related to the heat stability of the enzyme. Inactivation of the lysozyme contained in egg white, sputum, saliva, or tissue extracts took place at 75 C after a 30 minute exposure. Bordet (1928) showed that lysozyme in a 2 per cent solution of acetic acid would withstand 100 C for 45 minutes; however, neutral preparations of the enzyme lost all activity under these conditions. Sandow (1926) observed that heat stability of the enzyme varied with the pH of the diluent. Unpurified egg white was inactivated in 15 minutes at 65 C at pH 8.0, although heating at pH 5.0 for over 60 minutes did not result in any loss of activity. Epstein in a communication to Thompson (1940) indicated that highly purified lysozyme preparations are less stable to heat than impure ones. The following experiments were done to assist in answering the question—how far can the temperature be increased before inactivation of lysozyme is noted?

Lysozyme was exposed to heat by first preparing 25 ml aliquots of a 1:100,000 dilution of lysozyme in phosphate buffer, pH 6.2, in 50 ml volumetric flasks, then heating these in a water bath or autoclave. The temperatures used for the

exposure of the lysozyme were 80, 100, and 121 C; the times were those indicated in the figures. After heating, the flasks were removed and cooled in an ice bath. Additional phosphate buffer was added to make up 50 ml of a 1:200,000 lysozyme dilution. From this concentration twofold dilutions in phosphate

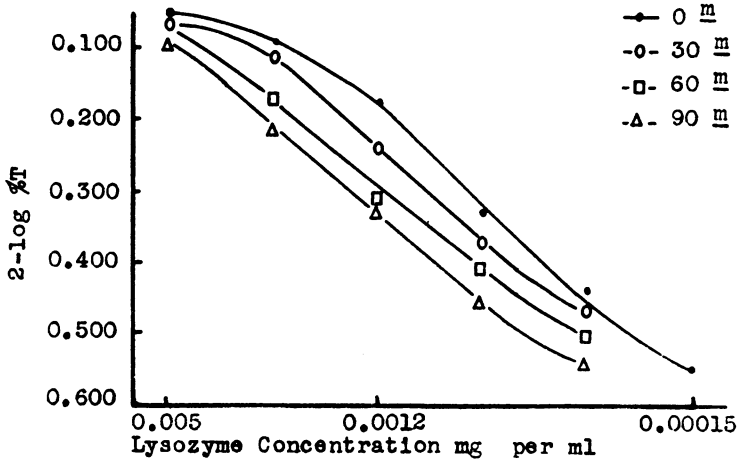

Figure 1. Effect of exposure of lysozyme to 80 C.

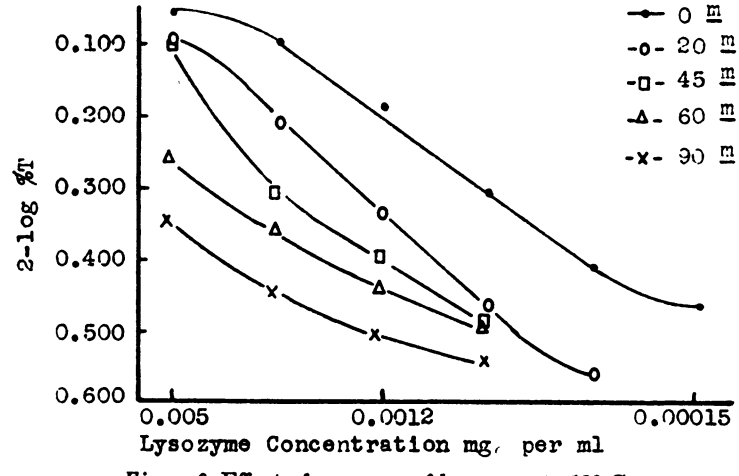

Figure 2. Effect of exposure of lysozyme to 100 C.

buffer were made and individually assayed in the manner described by Smolelis and Hartsell (1948). Figures 1, 2, 3, and 4 show the effect of heat on the lytic activity of lysozyme.

It is evident that the heat stability of lysozyme is an important characteristic of this enzyme. Even in relatively low concentrations in the presence of phosphate

buffer the enzyme loses only 5 per cent of its activity when heated at 80 C for 30 minutes and approximately 25 per cent of its activity after 20 minutes at

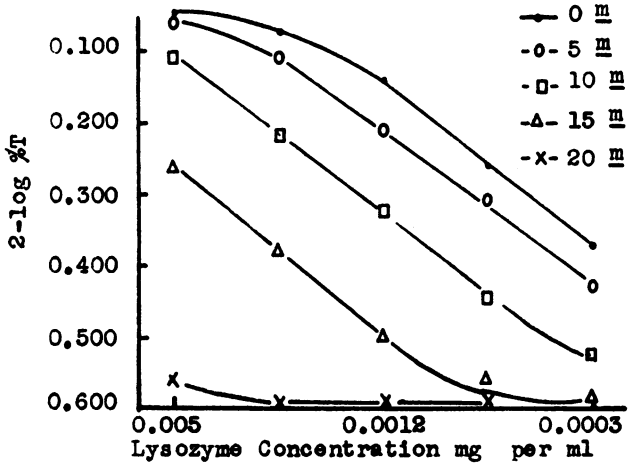

Figure 3. Effect of exposure of lysozyme to 121 C.

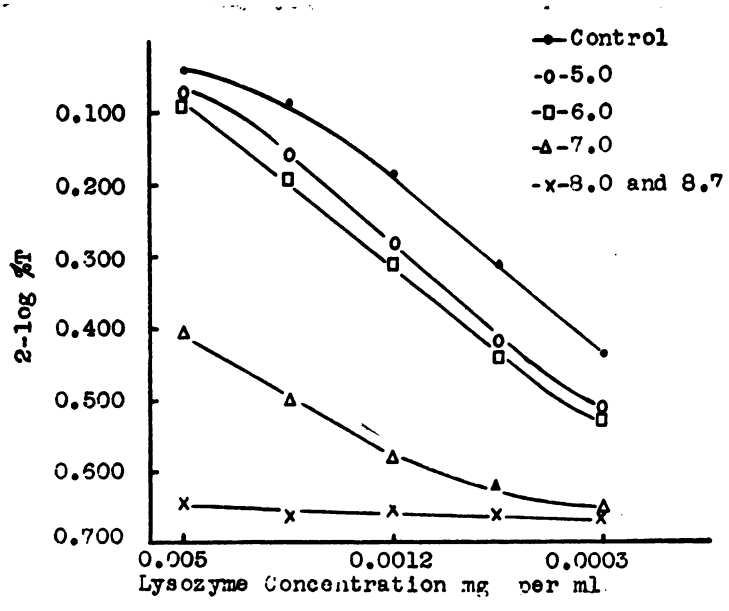

Figure 4. Effect of heating lysozyme at various pH values.

100 C. Lysozyme when dissolved in neutral or acid buffer was quite heat stable, figure 4, when exposed for 30 minutes at 100 C. The greatest loss of activity was observed at pH values higher than 7.0; however, contrary to previous investi-

gators, unheated lysozyme was active in alkaline reactions pH 8.7 and 9.0, created with veronal buffer.[3]

Effect of reaction on the activity of lysozyme. Optimum pH values for visible lysis of *M. lysodeikticus* are reported in the literature as pH 6.2 (Boasson, 1938), pH 6.05 to 6.35 (Thompson, 1941), pH 6.5 (Wilson, 1950), and pH 5.3 (Meyer and Hahnel, 1946). The experimental conditions were quite varied in these investigations, and since differences in the effects of pH on lysozyme activity could now be measured quantitatively, this factor was studied again.

M. lysodeikticus cells and crystalline lysozyme dilutions were made up in Sorenson's phosphate, Michalis' veronal, and potassium phthalate buffers at various pH values. In comparing bacteriolysis in Sorenson's buffer with that in other buffers at the same pH value, differences in the lytic response were observed. Later work dealing with the effect of salts on lysis showed that these

TABLE 3

Effect of pH on lysis

LYSOZYME DILUTION	pH 4.6*	pH 5.2*	pH 6.2†	pH 6.6†	pH 7.0‡	pH 8.7‡
	Per cent transmission after incubation					
1:200,000	34.0	59.5	77.5	78.3	77.2	45.5
1:400,000	32.2	50.0	61.5	60.1	60.7	32.7
1:800,000	29.7	45.5	46.7	45.7	44.4	27.5
1:1,600,000	27.0	32.0	35.7	34.7	32.7	25.5
1:3,200,000	24.7	28.7	31.0	29.5	27.7	25.0

* Phthalate buffer.
† Sorenson's phosphate buffer.
‡ Veronal buffer.

minor variations were due to the chemical nature of the buffers, particularly the cation. Mixtures of cells and buffer without lysozyme served as controls to indicate autolysis, if any.

After the cell, buffer, and lysozyme mixtures were incubated for 20 minutes at 25 C, the turbidities were determined in the usual manner. The results are presented in table 3.

The optimum hydrogen ion concentration at which maximum lysis occurred was shown to be pH 6.6, with a rapid decrease in lytic response at pH values above 7.0 or below 6.2. Quite different conclusions regarding the activity of lysozyme were drawn by Epstein and Chain (1940). They observed that the enzyme would liberate *n*-acetylhexoseamine from the purified substrate at pH values from 2 to 9. A strict comparison is not possible since initial adsorption of substrate on the cell, followed by the solubility of the cellular substances, is quite different from hydrolysis of a purified substrate. If lysis is considered to be a three-step process, i.e., initiation of lysis by lysozyme (adsorption and depolymerization), activity of autolytic enzymes within the cell structure, and

[3] Data are available in the thesis of A. S. on file in the library of Purdue University.

finally solution of cellular residues, then the optimum pH for visible lysis is a mean value at which all three processes will take place; a pH of 6.6 is that value for *M. lysodeikticus*.

Influence of salts and ions on the activity of lysozyme. If crystalline lysozyme is mixed with a suspension of *M. lysodeikticus* cells in triple distilled water, no visible lysis can be observed. The use of normal saline as a diluent will result in cell lysis by lysozyme. Fleming and Allison (1922) found that 0.5 per cent sodium chloride increased bacteriolysis and that higher concentrations caused an inhibition. Boasson (1938) reported that high concentrations of electrolytes caused inhibition. In addition, he found that the valence of the ions in the diluent could be correlated with the extent of inhibition in that the higher the valence the greater the inhibition. Feiner *et al.* (1946) attempted to increase cell susceptibility to lysozyme by the addition of various substances, e.g., ferrous sulfate, sodium arsenate, tyrothricin. Not many of the substances tested showed any effect; the first chemical inhibited lysis while the second increased the titer fourfold.

In the work reported here an attempt was made to clarify and to extend some of the existing information on the influence of various salts and ions on lysozyme activity.

Molar solutions of different salts were prepared in triple distilled water. From these stock concentrations, a number of dilutions were made. To each salt solution enough lysozyme was added to give a final dilution of 1:200,000 of the enzyme. Five ml quantities of the salt-lysozyme preparations were mixed with equal amounts of ultraviolet-killed *M. lysodeikticus* suspension which had been rehydrated in triple distilled water. Control mixtures containing only cells and the different salt solutions were prepared to measure the amount of salt-induced lysis. After 20 minutes at 25 C, the turbidities of the different mixtures were determined with a Coleman spectrophotometer. The results were expressed as the differences in light transmission between the control and the lysozyme containing preparation. The difference in density was considered to be due exclusively to the action of lysozyme. Salt-induced lysis, when noted in the controls, was found to be negligible.

The salts chosen for study were potassium chloride, bromide, chlorate, iodide, phosphate, nitrate, and sulfate; sodium chloride, carbonate, nitrate, and sulfate; magnesium chloride, nitrate, and sulfate; calcium nitrate and chloride; potassium and sodium acetate; sodium and ammonium citrate; sodium lactate; bile salts; and urea. Figure 5 shows the three general patterns of lytic response when different molar salt concentrations were used. The sodium, potassium, and ammonium salts all exerted a similar influence (curve A). The concentrations at peak response, or optimal salt concentrations, were between 0.05 and 0.12 molar. The use of bivalent calcium or magnesium salts resulted in considerably less lysis and a range of maximum lysis at a lower molar salt concentration (curve B). These results corroborate the observations of Boasson (1938) relative to the higher the valence of the salt the less the evidence of lysis. The curve showing the least amount of lysis (curve C) and a disproportionate optimum

salt concentration was typical for those salts whose hydrogen ion concentration in solution was below 4.5. Bacteriolysis in the presence of the organic compounds sodium acetate, sodium and ammonium citrate, and sodium lactate was very similar to that obtained with inorganic potassium and sodium salts.

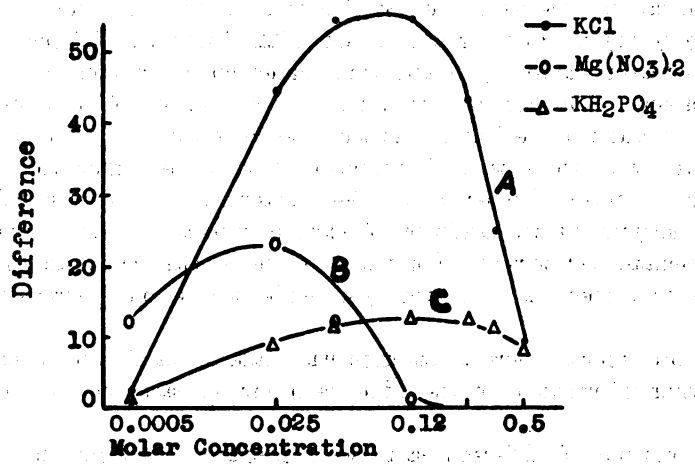

Figure 5. Effect of salts on lysis of cells by lysozyme.

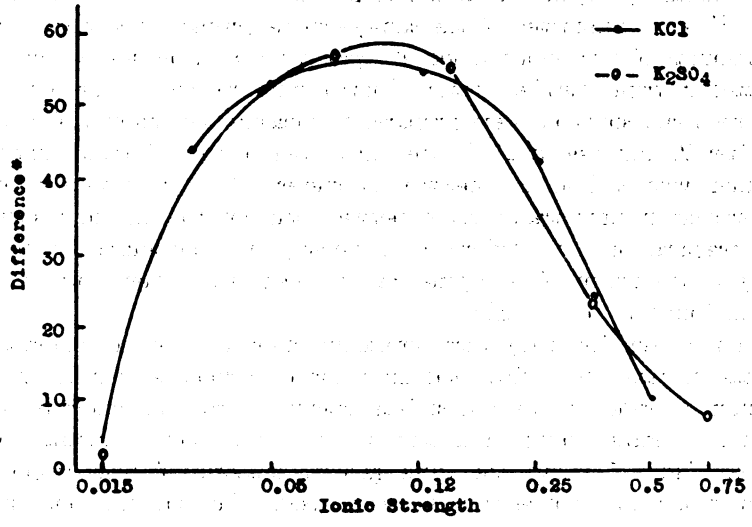

Figure 6. Effect of ionic strength of potassium salts on the lysis of cells by lysozyme.

From these results it is evident that the controlling factor for the action of lysozyme in these salts was the cation. When magnesium or calcium salts were used, for example, the amount of lysis never approached the magnitude obtained

with potassium or sodium salts. This was true irrespective of the anion present. It is interesting to note that when the amount of lysis is plotted against ionic concentration, rather than the molarity of the solutions, a new relationship can be observed.

Figure 6 shows that on the basis of ionic strength there is no difference in the influence of potassium chloride or potassium sulfate on lysis.

Extensive studies were not made on the effect of mixtures of salts on the lysis of *M. lysodeikticus* by lysozyme; however, bile salts (commercial preparation) suppressed lytic activity while Locke's solution or Ringer's solution enhanced lytic action by 10 per cent compared to that obtained in Sorenson's phosphate buffer pH 6.2.

These experiments indicate the controlling influence of the cation in relation to visible lysis. They also reflect a common pattern of action for certain groups of salts and an optimum range of influence for salts on the lytic process. With a 1:200,000 dilution of lysozyme the optimum range for lysis in the presence of the different salts was between molar concentrations of 0.025 and 0.12. Altering the lysozyme concentrations did not change the results. When 0.125 M solutions of sodium chloride, potassium chloride, or ammonium nitrate were used to prepare a series of lysozyme dilutions, the response was found to be constant.

Since no bacteriolysis can be observed when *M. lysodeikticus* is suspended in triple distilled water plus lysozyme, it is evident that the salts are essential to the lytic process. Little is known about the specific effects of ions on the cell membrane. There is the possibility of (1) alteration of membrane permeability which may be necessary before lysozyme can act, (2) reaction of the salt with polar groups on the cell surface, or (3) conditioning the solubility of cellular substances. The sodium ion is known to have low ionic mobility at the surfaces of living plant cells, whereas the potassium ion has a high mobility and is, therefore, more permeable to the nonaqueous layers of the cell wall. Since these cations were essentially the same in their influence on the lysis of killed *M. lysodeikticus* cells, it would seem that the effect of the cation is largely to condition the last phase of bacteriolytic solution of cellular residue rather than upon the first phase where ionic mobility would condition the adsorption of lysozyme on the bacterial cell. Presumably the autolytic enzymes of the ultraviolet-killed cells would not contribute to the lytic process, so the possibility of the cation having a coenzyme relationship in endocellular autolysis or to the exocellular lysozyme, as used in these experiments, seems very remote.

Salts of widely divergent cationic or anionic valences but of the same pH when in solution permitted essentially similar patterns of lytic action on ultraviolet-killed cells.

SUMMARY

A number of the factors affecting the activity of lysozyme have been studied. Ultraviolet-killed lyophilized cells of *Micrococcus lysodeikticus*, after rehydration, provide a suitable cellular suspension for quantitatively measuring the effects of physiochemical factors on bacteriolysis by lysozyme. The heat stability of pure

lysozyme has been quantitatively assessed. At alkaline pH values egg white lysozyme was inactivated though it was observed to be active near neutrality. In phosphate buffer, pH 6.2, highly purified lysozyme can be exposed to 80 C for 30 minutes with only a 5 per cent loss of activity. It will withstand 100 C for 20 minutes with only a 25 per cent loss in lytic capacity.

The optimum pH for visible lysis was found to be 6.6; however, lysis of *M. lysodeikticus* was observed at both alkaline and acid pH values. This observation is at a variance with the statement frequently made in the literature that lysozyme is not active in the alkaline range.

Optimal salt concentrations for maximal lysis were found to depend upon the ionic strength of the cation and upon its valence. In order of increasing efficiency in bacteriolysis of *M. lysodeikticus* the cations can be arranged as follows: calcium, magnesium, ammonium, potassium, and sodium. Although it was possible to determine the effect of the various ions upon lytic response, the function of these ions in the lytic process is believed to be largely as conditioning agents in the solubility of cellular residues in the final phase of lysis.

REFERENCES

BOASSON, E. H. 1938 On the bacteriolysis by lysozyme. J. Immunol., **34**, 281–293.

BORDET, M. 1928 Essais d'extraction du lysozyme. Compt. rend. Soc. Biol., **99**, 1254–1256.

EPSTEIN, L. A., AND CHAIN, E. 1940 Some observations on the preparation and properties of the substrate of lysozyme. Brit. J. Exptl. Path., **21**, 339–355.

FEINER, E. R., MEYER, K., AND STEINBERG, A. 1946 Bacterial lysis by lysozyme. J. Bact., **52**, 375–384.

FLEMING, A. 1922 On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. (London), B, **93**, 306–317.

FLEMING, A., AND ALLISON, J. 1922 A bacteriolytic substance (lysozyme) found in secretions and tissues. Brit. J. Exptl. Path., **3**, 252–260.

HARTSELL, S. E. 1948 The lysozyme activity of rehydrated, spray-dried whole-egg powder. Food Research, **13**, 136–142.

MEYER, K., AND HAHNEL, E. 1946 The estimation of lysozyme by a viscosimetric method. J. Biol. Chem., **163**, 723–732.

SANDOW, A. 1926 The antibacterial activity of egg white. Proc. Soc. Exptl. Biol. Med., **24**, 172–174.

SMOLELIS, A. N., AND HARTSELL, S. E. 1948 The determination of lysozyme. J. Bact., **58**, 731–736.

THOMPSON, R. 1940 Lysozyme and its relation to antibacterial properties of various tissues and secretions. Arch. Path., **30**, 1096–1134.

THOMPSON, R. 1941 Lysozyme and the antibacterial properties of tears. Arch. Ophthal., **25**, 491–509.

WILSON, A. T. 1950 Urinary lysozyme. I. Identification and measurement. J. Pediat., **36**, 39–44.