A STUDY OF THE EFFECT OF LYSOZYME UPON MICROCOCCUS LYSODEIKTICUS

by

FRANCIS L. PENISTON

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INTRODUCTION

Many problems concerning the metabolism of the bacterial cell evolve from the permeability of the outer coat, or coats, of the cell. A more complete insight into the factors that are responsible for variations in the permeability of different cells might aid in obtaining a better understanding of the metabolic processes of a cell. The factors associated with the permeability may, to a considerable extent, control the uptake of food, in its various forms, by the individual cell.

Very few quantitative studies have been conducted upon the outer structures of a cell. The tool, lysozyme, used in this problem to alter these outer structures of the cell has been used by Utter et al. (16) to lyse bacterial cells and free the enzyme systems of the cell in an active form. These liberated systems were further utilized in a series of studies upon cell respiration and metabolism.

Dubos (3, p. 99) believed the action of lysozyme upon a cell resulted in an increased permeability. As a result of this action the cells disintegrated and the cell material diffused into the medium.

Sevage and Miller (13) noticed that sensitized E. typhosa cells underwent lysis when subjected to the action of complement. Immediately after lysis occurred and increase in oxygen uptake was noted. This work showed the possibility of an alteration of the outer cell components and perhaps the alteration of the

permeability of the cell.

This problem was created to determine the action of the enzyme lysozyme upon the respiration of bacterial cells and to determine, if possible, any changes in the cell permeability, measured by a changing oxygen uptake, as a result of the action of the enzyme lysozyme upon the cell wall.

REVIEW OF LITERATURE

A comprehensive search of the literature concerned with the activity of the enzyme lysozyme and research carried out on the organism Micrococcus lysodeiktieus revealed a dearth of information in connection with this problem. Therefore, this following discussion is limited to the properties of lysozyme and the inclusion of work connected with the action of lysozyme upon various susceptible organisms.

The enzyme lysozyme was first isolated by Flemming in 1920. Lysozyme has been found to be very widespread in nature. A few of the sources are white of eggs, secretions of the mucous membranes of the body, buttermilk, and in certain molds, algae and bacteria. The most commonly used source for the production of lysozyme is the white of eggs. Abraham and Robinson (1) were the first workers to produce the enzyme in a crystalline form. An improved schedule for the crystallization of the enzyme has been presented by Fevold and Alderton (5).

Goldsworthey and Florey (7) stated that Flemming found 75

per cent of the organisms isolated from the air to be susceptible to lysozyme activity. Goldsworthey and Florey (7) found this activity to be elicited in three ways: (a) killing without lysis, (b) killing accompanied by lysis, and (c) inhibition of growth. Meyer (9, p. 278) found the resulting lysis to be definitely tied up with the pH of the medium. His observations indicate that lysis occurs only at an alkaline to slightly acid pH value. The optimum activity of the enzyme occurs at pH 4.5, but at this value little to no lysis occurs.

Feiner et al. (4) observed the susceptibility of both living and dead organisms to lytic activity of lysozyme. However, it was noted that precipitation with 95 per cent alcohol or acid acetone followed by autoclaving in alkaline, acid, or formalin solutions rendered the organism highly resistant to complete lysis by the enzyme.

Meyer et al. (11) concluded that lysozyme reacts only in the reduced state. It was found that lysozyme could be inactivated by the presence of sulfahydryl groups, alkali, peroxide, iodine and cupric oxide. It can, however, be reactivated by hydrogen sulfide, hydrogen cyanide and similar compounds.

The substrate attacked by lysozyme is a mucopolysaccharide in high polymer form. Meyer and Hahnel (10) observed the liberation of an acetyl-hexosamine as a result of lysozyme activity. These workers believed the lytic action of lysozyme on susceptible cells cannot be explained on any physical basis such as the lowering of surface tension. The properties of the enzyme did

not show any protease, kinase, amylase, lipase, or phosphatase activity during the course of their experiments.

Roberts (12) was able to demonstrate antibodies against lysozyme and showed the loss of activity by the enzyme upon its contact with immune serum. Flemming and Allison (6) worked with immunological methods to show the differences in lysozyme which had been crystallized from different sources.

As a result of its sensitivity, the organism most widely used in studies on lysozyme is Micrococcus lysodeikticus. This organism was isolated from the air by Flemming. Feiner et al. (4) determined the cultural requirements of this organism. Casein hydrolysate and adenine were found to be indispensible for good growth of the organism. Growth may further be enhanced by the use of a Lactobacillus casei growth factor. Hewitt (8) first demonstrated the expected catalase activity of this particular organism. Results of his work showed that the most luxurious growth resulted from aeration of broth cultures.

In working with lysozyme Boasson (2) observed a swelling in the final stages of lysis of M. lyso. His techniques may be presented as microscopic observations with a staining schedule utilizing a carbol fuchsin, serum, and eosin stain. As a result of this work it was concluded that the cell wall was not destroyed but that the observed swelling of the cell was a resulting mechanical flattening of the cell after the removal of the polysaccharide substrate from the cell wall.

Welshimer and Robinow (17) carried out a similar project

using the enzyme and Bacillus megatherium. By microscopic observation the cells appeared to be autolysed and swollen. By using a special "agar streak plate" method the cells appeared to be drawn out into long thread-like structures. The loss of some structural unit from the cell wall was indicated by this technique.

Dubos (3, p. 195), in his review of the susceptibility of microorganisms to the action of lysozyme, notes that only the saprophytic species were susceptible to lysis, while most of the pathogens were completely unaffected. It was his belief, however, that a number of organisms exist which are potentially pathogenic but are not able to exert their activities due to their susceptibility to this particular enzyme. Therefore they are unable to establish themselves in the potential host. He further states that certain strains of staphylococcus are more virulent as a result of in vitro contact with lysozyme.

Utter and others (16) found the enzyme lysozyme to be very useful in experiments on the oxidation of various substrated by the endoenzymes of M. lysodeikticus. The techniques used were closely in line with those used in this study and are presented more in detail to show a comparable study with, however, a different end point in mind. In the procedure followed by Utter cells of M. lysodeikticus were washed, dried in vacuo, ground in mortars, and stored at low temperatures. The cells were then lysed with lysozyme as needed. This preparation was then used in respiration experiments using various substrates. The

respirometer flasks were set up as follows: 40 mg (dry weight) of cells lysed for 40 minutes, 0.06 M phosphate buffer at pH 6.2, 0.03 M substrate (sodium salts in the case of acids) and water up to a total of 2.0 ml in the flask proper. Also 0.3 ml of 20 per cent KOH was placed in the center well. It was found that the addition of NaCl to the flasks in varying dilutions had a considerable effect upon the oxygen uptake by the lysed cells. Even an increased oxygen uptake stimulated by the addition of the NaCl and the massive numbers of cells used in this series of experiments failed to exceed the value of cubic milliliters of oxygen uptake as observed in the work by this author. The authors were not certain as to the reason, or reasons, for the increased oxygen uptake due to the addition of NaCl. They reasoned that it might be due to an aggregating action of the precipitate, formed upon the addition of the NaCl, thus furnishing a surface for the previously dispersed enzymes to come into contact with each other, with the substrate, or with co-enzymes. The fact that the NaCl might inhibit the action of the lysozyme was also considered.

EXPERIMENTAL

General Methods and Techniques

The organism, Micrococcus lysodeikticus, used throughout this series of experiments was obtained from stock cultures

provided by Dr. J. O. Harris of Kansas State College. These cultures were easily maintained on nutrient agar slants incubated at 30 degrees C.

This organism required 48-96 hours incubation to provide sufficient growth in quantity when grown upon large agar surfaces. The method finally used for producing a mass of cells was aeration of a liquid medium. Compressed air was washed and passed through cotton filters before reaching the growth medium.

All preliminary work was carried out with cells grown in nutrient broth diluted 1:2 to reduce the amount of foaming occurring during active aeration over a period of 24 hours. Fresh agar slants were transferred daily for three or four days prior to innoculation of the nutrient broth. The incubation temperature during aeration was 30 degrees C. The broth was innoculated by suspending the growth on an agar slant in nutrient broth and then pipetting this suspension into the growth medium.

The cells were harvested from the nutrient broth by the use of a Sharples Super Centrifuge. These cells were then washed three times with Ashby's salt solution to remove all substrate from the surface of the cells. After the final washing the cells were resuspended in Ashby's salts. The composition of this salt solution is as follows:

KH_2PO_4	1.8 g
K_2HPO_4	0.7 g
NaCl	0.2 g
$MgSO_4$	0.2 g
$CaCl_2$ $5H_2O$	0.02 g
$CaCO_3$	0.02 g
$FeCl_3$ (soln.)	4 drops
Molybdenum soln.	1.0 ml

$MnSO_4$.....	0.4 g
Molybdic acid.....	0.4 g
KI.....	0.4 g
Water.....	400 ml
Water.....	1.0 liter

The pH of this solution was approximately 7.0.

The stock suspensions of cells were kept at low temperatures and proved to be quite stable. They showed no tendency towards autolysis and during the course of experiments the respiratory activity showed little loss. No attempt was made to correlate any of the results of the preliminary experiments since the number of cells varied with each suspension.

For the final experimental results it was desirable to have a standard viable suspension of cell to check results on a quantitative and comparative basis. This was accomplished by the lyophilization of a heavy suspension of cells obtained in the following manner. Freshly transferred cells of Micro. lysodeikticus were used to innoculate several bottle of diluted nutrient broth and aerated for 24 hours. The cultures were checked for purity and harvested in a Sharples Super Centrifuge. The cells were then washed and resuspended in Ashby's salts solution at pH 7.0; 5 ml amounts of the suspension were then pipetted into small complement type vials of an approximate volume of 25 ml. These vials were plugged with rubber stoppers containing a piece of open glass tubing. The cultures were then quickly shell frozen, packed in dry ice, and stored in a deep freezer for a one-hour "aging" period. The vials were subsequently connected to a vacuum pump via the glass tube, packed

in dry ice and evacuated overnight. Upon termination of the evacuation the vials were removed and the tip of the tubing was sealed off with a flame. These lyophilized cultures were easily resuspended by injecting 15 ml of Ashby's solution into the vials with a syringe. In all cases these resuspended cells were used within two days. However, some samples stored under low temperatures showed good respiratory activity for over a one-week period and showed no autolysis over a four-week period. These lyophilized cultures were not plated out to check the viability of the cells since they were not harvested under aseptic conditions and the only condition demanded was a uniform rate of respiration.

A direct cell count using a Petroff-Hausser bacterial counting chamber showed a cell count of 26.5 to 28.5 billion cells/ml of the lyophilized cultures after resuspension.

Turbidity measurements gave a value of 67 using a 1/100 dilution of the resuspended cells in standardized tubes. The equipment used was a Coleman Universal Spectrophotometer with a PC-4 filter and a setting of 500 Mu.

The respiration apparatus used in this study was the Warburg type micro-respirometer. The techniques used were basically the same as are presented by Umbreit et al. (16).

Throughout the course of the experimental work a stock solution, or suspension, of lysozyme was used in various amounts and dilutions. This stock was made up with 50 mg of Armour and Company crystalline lysozyme in 100 ml of M/100 KH_2PO_4 at pH 4.5.

This suspension was kept refrigerated during the entire period of experimentation and no loss of activity was noted. The amount of lysozyme used in the various experiments has been designated by volume used or by concentration of lysozyme expressed in micrograms per ml. The concentration of the stock solution was 500 micrograms/ml of phosphate buffer at pH 4.0. All dilutions of this stock solution were made with phosphate buffer at pH 7.0 as the diluting liquid.

Various Substrates Utilized by M. lysodeikticus

The preliminary studies upon the respiration of M. lysodeikticus were made using different organic compounds as the substrate. This preliminary work was used only as a screening process to determine which compounds might serve as a source of energy that would enable the organism to carry on active respiration.

For these experiments freshly harvested cells were used in most instances. The numbers of cells used in the preliminary experiments were not standardized. Results were based upon the various controls used during each respiratory test. The cells were stored at low temperatures during the series of studies.

The Warburg equipment was used with the water bath set at 30 degrees C. Duplicate flasks were used in all cases. One flask was used as a barometric control and contained a total liquid volume of 3.4 ml. One pair of flasks was used to measure

the endogenous respiration of the cells. By the endogenous respiration the oxygen uptake of the cells without a substrate is indicated. In these controls 0.7 ml of washed cells were placed in the side arm of the flasks, 0.2 ml KOH and a roll of filter paper were placed in the center well, and 2.5 ml of M/20 phosphate buffer at pH 7.0 was introduced into the flask proper. In the flasks with the substrate, or carbon compounds to be checked, 0.7 ml of cells was pipetted into the side arm, 0.2 ml 15 per cent KOH and a roll of filter paper were placed in the center well, and 0.5 ml of substrate and 2 ml of phosphate buffer were measured into the flask proper. The flasks were attached to respirometers with the system opened to the atmosphere. After allowing the system 15 minutes to come to equilibrium the fluid levels were adjusted and the system closed off to the air. The cells from the side arm were then mixed with the contents of the flask proper. Readings were made every 15 minutes for a two hour period.

During this course of preliminary work many compounds were used in concentrations ranging from M/5 up to M/250. In all cases 0.5 ml of the various substrates at different concentrations was used. Values from this preliminary work showed the desirability of using concentrations in the range of M/20 with concentrations of M/100 for comparison.

The amounts of oxygen uptake recorded during the two hour respiration period were used as criteria for determining which substrates tested should be used. Concentrations of sodium

acetate and ethyl alcohol at a value of M/20 showed the highest amounts of oxygen uptake as measured in cu ml. The sodium acetate showed the most consistent results and was chosen as a standard with which to compare the other substrates. Table 1 shows the results of these initial studies upon the various substrates in comparison to the sodium acetate substrate.

The Effect of pH Upon Lysozyme Activity

Lysozyme shows its greatest activity at a pH value of about 4.5 according to Meyer (9, p. 278). However, lysis decreases with a drop in the pH value. Lysis was indicated in this experiment by a decrease in the turbidity of the cell suspensions.

To check the possible effect of this factor upon the turbidity measurements made at the end of each respiration, the following experiment was carried out: The Coleman Spectrophotometer was used with a PC-4 filter and a setting 500 Mu. All tubes used were obtained from a standardized set. To five tubes containing 3.0 ml of phosphate buffer at pH values of 4.5, 5.0, 5.5, 6.0, and 6.5, respectively, were added 1.0 ml of a 1/10 dilution of the stock lysozyme, 1.0 ml of M/20 acetate, and 1.4ml of the lyophilized cells. A control tube contained 4.0 ml of phosphate buffer at pH 7.0, 1.0 ml of M/20 acetate, and 1.0 ml of a 1/10 dilution of the lysozyme. In the five experimental tubes the pH values were adjusted after adding the cells. The enzyme suspension was added last and upon its addition a reading was

Table 1. The comparison of various substrates to sodium acetate as indicated by oxygen uptake.

Compound	Comparison to Na acetate
Ethyl alcohol	Very good
Na lactate	Fair
Na succinate	Good
Na tartrate	Very poor
Na fumerate	"
Na valerate	"
Xylose	"
Sorbitol	"
Dulcitol	"
Na citrate	"
Mannitol	"
Na gluconate	"
Levulose	"

Table 2. Value in micrograms of lysozyme of various dilutions of stock lysozyme suspension.

Dilutions of stock	Value-micrograms/0.5 ml dilution
1/100	2.50
1/125	2.00
1/150	1.66
1/200	1.00

made on the spectrophotometer corresponding to zero time.

Readings were made at five minute intervals with a total time of 30 minutes recorded.

Results, as are shown in Fig. 1, indicate that most of the lysis occurred within the first 15 minutes. In the case of the tube at pH 4.5 the lysis all occurred within the first 5 minutes.

The Measurement of Turbidities at Varying Lysozyme Concentrations

Some insight into the amount of lysis that could be expected from varying dilutions of lysozyme was necessary to provide an approximate dilution that would result in some lysis and yet not result in the destruction of all cells during the period of respiration.

The results of the previous experiment suggested the use of a buffer diluting solution at a pH of about 6.5. Five tubes from a matched set were used in this experiment. Into four standard tubes were pipetted 3.0 ml of phosphate buffer, 4.0 ml of freshly harvested and washed cells of M. lysodeikticus, and 1.0 ml of M/20 acetate. To one each of these tubes was added 1.0 ml of stock lysozyme diluted 10, 50, 100, and 150 times respectively. The enzyme was added last and an immediate reading corresponding to zero time was recorded. A control tube was set up with the cells replaced by an equivalent volume of buffer. The results of the experiment may be noted in Fig. 2.

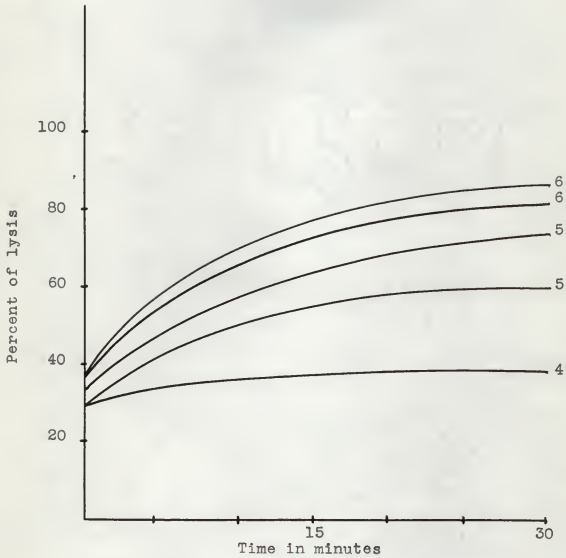

Fig. 1. Lysis at varying pH values.

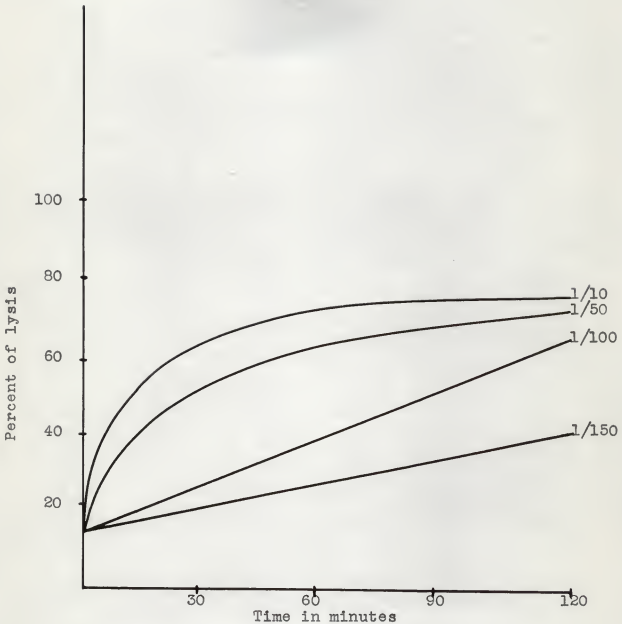

Fig. 2. Lysis at varying lysozyme concentrations.

Cell Respiration with Lysozyme and M/20 Acetate

In studying the effects of lysozyme upon cell respiration it was necessary to run two controls. The first of these was a double flask control on the Warburg equipment to measure the endogenous respiration of the lyophilized cells. These flasks were set up in the same manner as previous experiments indicate and all conditions were similar. Since results proved to be so consistent for the measure of the endogenous respiration this control was not run during every respiration but was checked on different suspensions of the cells at periodic intervals.

The second control used was set up approximately in the same manner as the endogenous controls. In this control, however, 0.5 ml of M/20 sodium acetate was added to the flask proper and the buffer was reduced by 0.5 ml.

The third set of flasks contained 0.7 ml of cells in the side arm. The flask proper contained 1.5 ml buffer, 0.5 ml of M/20 acetate, and 0.5 ml of lysozyme. A roll of filter paper and 0.2 ml of alkali were placed in the center well. It was necessary to use various concentrations of the lysozyme stock solution to find the optimum amount to use. The concentrations used ranged from 50 micrograms to a mathematical value of 1.66 micrograms/ml. The optimum values ranged between 5 and 2.5 micrograms/ml. The most consistent results were obtained using 0.5 ml of lysozyme stock solution containing about 1.66 micrograms. The concentration in micrograms was determined by evalu-

ating dilutions of the stock lysozyme. Table 2 shows the corresponding dilutions and value in micrograms of lysozyme per 0.5 ml of suspension.

By graphing the oxygen uptake at the 15 minute intervals it was possible to compare the amount of oxygen of the lysed cultures with that of the endogenous and the respiration using a M/20 acetate substrate. Fig. 3 shows a typical representation of the type of curves produced during this series of experiments.

The information presented in Table 3 is a summary of the work carried out using M/20 acetate as a substrate. The data presented in this table show the amount of lysozyme per ½ml of diluted stock, the amount of lysis as indicated by a reduction in cells, and the percentage of stimulation, or inhibition, of the oxygen uptake of lysed cultures in comparison to the non-lysed controls. The lysis indicated was determined by measuring the turbidity of a 1/10 dilution of representative flasks at the termination of the respiration. The Coleman equipment was used as mentioned previously.

By inspection of Fig. 3 and Table 3 it can be noted readily that those flasks containing a proper dilution of the lysozyme, cells, and substrate show a definite increase in the amount of oxygen uptake.

The amount of lysis indicated in Table 3 was determined by constructing a standard curve using the cell count of 27.5 billion cells/ml in conjunction with spectrophotometer readings of various dilutions of the stock culture of lyophilized cells.

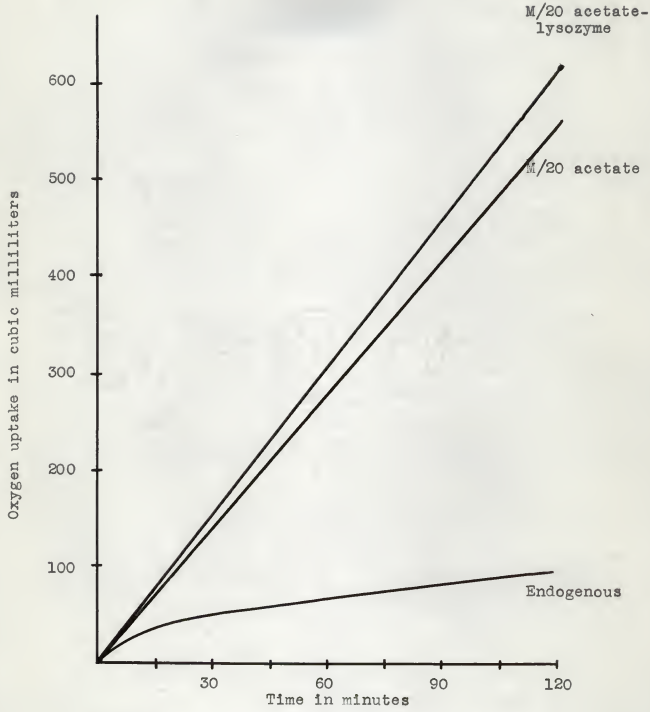

Fig. 3. Oxygen uptake using M/20 acetate and lysozyme dilution 1/100.

Table 3. Percentage of lysis and stimulation of oxygen uptake of cells using M/20 acetate in the presence of varying lysozyme concentrations.

Micrograms of lysozyme ml/2	Percentage lysis	Percent of stimulation
3.33	17.3	16.0
3.33	20.6	14.1
3.33	16.1	9.5
3.33	14.5	9.6
2.50	8.7	10.8
3.33	33.4	8.4
2.50	13.4	10.5
3.33	9.6	11.6
3.33	23.8	3.5
2.50	31.2	8.0

Table 4. Percentage of lysis and stimulation of oxygen uptake of cells using M/100 acetate in the presence of varying lysozyme concentrations.

Micrograms of lysozyme ml/2	Percent of lysis	Percent of stimulation
2.50	43.1	6.4
2.00	27.6	6.5
1.66	27.6	7.7
2.50	21.4	12.5
2.50	18.1	5.1
2.50	24.3	10.2
2.50	24.3	9.9

Reductions in the number of cells could then be found by applying the turbidity readings, taken at the termination of each respiration, to the standard curve. The number of cells lysed is presented on a percentage basis. The percent of stimulation of the oxygen uptake presented in Table 3 is the percent of the total uptake and included the endogenous respiration. Due to its constant value and its questionable position in cell respiration the amount of oxygen uptake due to the endogenous respiration was included and the percentage value was determined with total oxygen uptake.

Cell Respiration with Lysozyme and M/100 Acetate

It was considered desirable to compare the effect of lysozyme upon cell suspensions using a M/20 concentration of acetate with the effect of lysozyme using a M/100 acetate substrate.

This series of respiration studies was set up in an identical manner as those using the M/20 concentration of the substrate. Conditions were duplicated on the Warburg apparatus and turbidities of the individual flasks were determined at the termination of each experiment.

Results of these respiration studies are shown in Table 4. Fig. 1 shows a representative evaluation of the oxygen uptake of M. lysodeikticus, with and without lysozyme, on a M/100 acetate substrate. The endogenous respiration is shown for

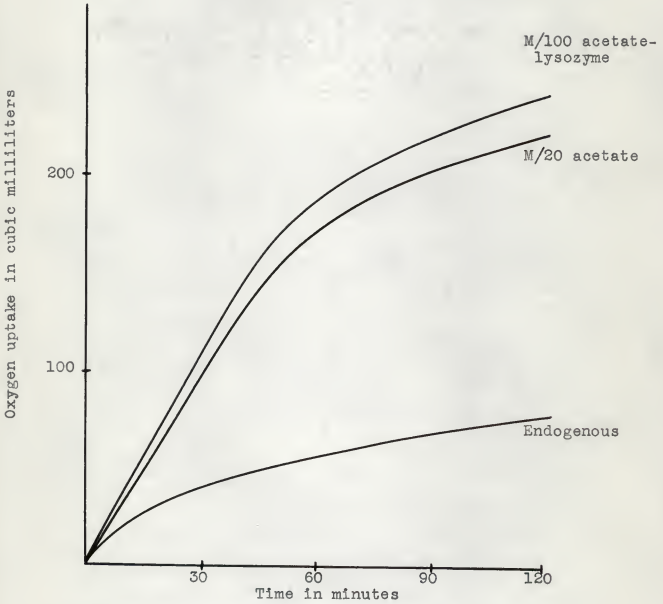

Fig. 4. Oxygen uptake using M/100 acetate and lysozyme diluted 1/100.

comparison.

A constant and rather uniform increase in the oxygen uptake of the lysed cultures may be noted in comparison to those not subjected to the action of lysozyme. This increase, however, was not of the magnitude of that noted when a more concentrated substrate was used. Inspection of the curves shown in Fig. 2 shows the increase as occurring mostly during the second hour of the respiration experiment. The percentage was figured on total oxygen uptake and included the endogenous values.

The Effect of Lysozyme Using a M/20 and M/100 Ethyl Alcohol Substrate

The only other substrate tested that showed good respiration values was ethyl alcohol. Since the molecules of this compound are smaller than those of the acetate, similar respiration studies were made using it as a substrate in the same concentration as the acetate.

Respiration measurements were carried out in the same way as presented for those using acetate as the substrate. Studies were made on both M/20 and M/100 concentrations of the ethyl alcohol. Turbidities of the contents of representative flasks were measured at the end of each respiration.

The results of the experiments upon M/20 ethyl alcohol are shown in Fig. 5 and Table 5. The results using M/100 ethyl alcohol as a substrate are indicated in Fig. 6 and Table 6.

The heavier concentration, M/20, of the ethyl alcohol as a

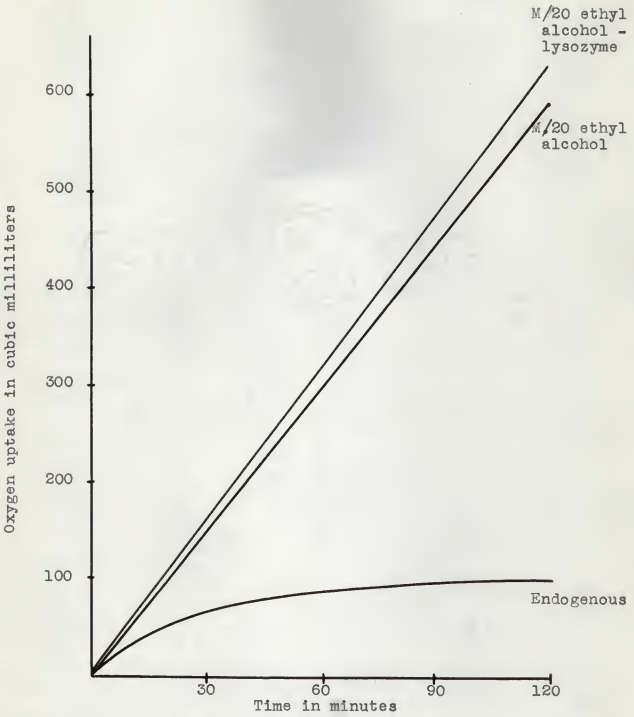

Fig. 5. Oxygen uptake using M/20 ethyl alcohol and lysozyme diluted 1/125.

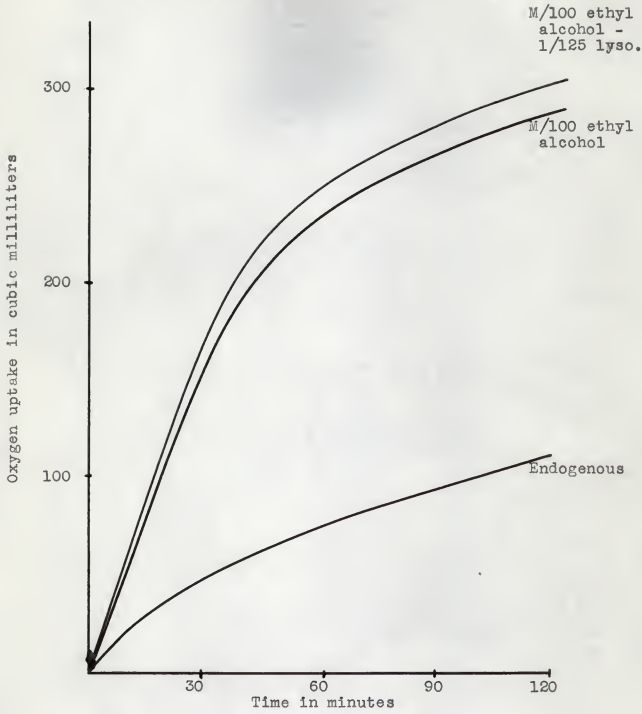

Fig. 6. Oxygen uptake using M/100 ethyl alcohol and lysozyme dilution 1/125.

Table 5. Percentage of lysis and stimulation of oxygen uptake of cells using M/20 ethyl alcohol in the presence of an optimum lysozyme concentration.

Micrograms of lysozyme ml/2	Percent of lysis	Percent of stimulation
2.0	20.0	2.5
2.0	20.9	5.3
2.0	9.2	6.2
2.0	11.4	6.2

Table 6. Percentage of lysis and stimulation of oxygen uptake of cells using M/100 ethyl alcohol in the presence of two lysozyme concentrations.

Micrograms of lysozyme ml/2	Percent of lysis	Percent of stimulation
2.5	11.9	3.5
2.5	11.4	4.0
2.0	11.4	2.0
2.0	11.5	4.0

substrate shows a rate of oxygen uptake comparable to that recorded for M/20 acetate. A smaller percentage increase is noted for lysed suspensions of M. lysodeikticus upon the ethyl alcohol than upon the acetate substrate. In most instances the increase approaches a value approximately half that of acetate. The values for the M/100 concentrations are of the same magnitude.

The Effect of Lysozyme Upon Endogenous Respiration

Meyer and Hahnel (10) stated that the depolymerization of the mucopolysaccharide from the cell by lysozyme resulted in the release of a reducing sugar. This sugar was designated as an acetyl-hexosamine. The possibility that the presence of this sugar might result in the stimulation of oxygen uptake formed the basis for this experiment.

One pair of flasks was used to measure the endogenous respiration as in the experiments concerned with respirations using various substrates. In two other flasks 0.7 ml of cells were pipetted into the side arm and 0.5 ml of lysozyme solution containing 1 2/3 micrograms of lysozyme and 2.0 ml of buffer were placed in the flask proper. A roll of alkali impregnated filter paper was placed in the center well. The third pair of flasks contained 1 1/4 micrograms of lysozyme. All other conditions for the respiration were as previously mentioned. Turbidity measurements were carried out in the same manner as the preceding

ones.

No increase in oxygen uptake as compared to the endogenous uptake was noted using either of the two above mentioned concentrations of the enzyme. A definite lysis had occurred, as was evidenced by the turbidity measurements at the termination of the respiration, and hence acetyl-hexosamine was considered as present. This experiment precluded the possibility that the non-lysed cells might possibly be stimulated to greater oxygen uptake by the presence of any material removed from the cell wall of the lysed cells.

Determinations Using Heated Lysozyme

This series of studies was carried out to determine the effect of inactivated lysozyme upon M. lysodeikticus. This inactivation was brought about by steaming a 1/50 dilution of the stock suspension of lysozyme for 30 minutes. A more concentrated suspension was used to insure a surplus of the enzyme so that any alteration in the normal respiration of the cells could be definitely noted. Since a suspension of this concentration brought about quick and extensive lysis, the effectiveness of the inactivation could be easily determined at the start of the respiration by comparison of the oxygen uptake with that of the controls.

The results of this work showed no change upon the respiration of the cells in the presence of the steamed enzyme. A

substrate of M/20 acetate was used during this investigation.

Controls were run using no lysozyme and lysozyme (untreated) diluted 1/125. All methods used correspond to those previously described in detail. Turbidities were measured at the termination of the experiments and no lysis was noted in the cells in contact with the treated enzyme suspension.

DISCUSSION

The results of this work showed conclusively that a proper concentration of lysozyme and cells resulted in a stimulation of oxygen uptake in the presence of a readily usable substrate. Figures represented in the various tables show that this increase in oxygen uptake was accompanied by lysis of the cells in every case. In no instance was stimulation noted in the absence of a measurable lysis. The preliminary work concerning lysozyme concentrations showed that a heavy concentration of lysozyme resulted in excessive lysis and a resulting cessation of respiration.

Several possible explanations of the stimulation of respiratory activity may be listed as follows: (a) The stimulation resulted from the presence of the acetyl-hexosamine hydrolyzed from the cell walls of the cells. This explanation was largely discredited as a result of the work using cells subjected to lysozyme activity in the absence of a substrate. A quantitative study was conducted on the supernatant liquid of a lysed culture

to determine the presence and amount of reducing sugar present. In all cases lysis was accompanied by the appearance of a reducing sugar. It was then assumed that this sugar was present in the flasks during respiration studies on cells subjected to lysis in the absence of a usable substrate. No consistent increase in the respiration of these cells was noted during the series of experiments on this phase of the problem.

(b) The stimulation may have resulted from an action similar to that noted in the use of very low concentrations of heavy metals and other bactericides. This possibility seemed untenable as a result of a direct comparison of the percentage increase of oxygen uptake using a concentrated and a diluted form of various substrates. It was noted that the average increase of oxygen uptake using a M/20 concentration of acetate was 10.4 per cent. The increase using a M/100 concentration of acetate was 8.2 per cent. These results indicated that the increase was, in part, due to the amount of substrate present rather than an "oligodynamic" action of the lysozyme.

(c) The stimulation may have been due to the use of the enzyme solution as a substrate. This was however discredited by the use of a heavy enzyme concentration inactivated by heat. No stimulation of oxygen uptake was noted during the series of respirations using these heated preparations. The very dilute concentration of the enzyme used also helped to nullify the possibility of using the enzyme as a substrate to increase the oxygen uptake of the cells.

(d) A fourth possible cause of stimulation might have been the alteration of the permeability of the cell wall. The removal of some structural component of the cell wall would introduce the possibility that the substrate could be more readily available and thus result in an increased oxygen uptake by the cell. The removal of some structural unit was consistently indicated by lysis of the cells during the respiration studies. This theory of the alteration of the permeability of the cells is in close agreement with concepts advanced by such workers as Savage and Miller (13), Boasson (2), and Welshimer and Robinow (17).

CONCLUSION

The oxygen uptake of lyophilized cells of Micrococcus lysodeikticus was measured, in the presence of various substrates, by the use of a Warburg microrespirometer. Those substrates giving the best results were acetate and ethyl alcohol. Lactate and succinate gave fair to good results as measured by oxygen uptake of the cells. Larger molecules such as tartrate, xylose, levulose, and others proved to be very poor substrates.

The pH was very important in the respiration studies with lysozyme. Results of these experiments indicated the best pH level to range from 6.5 to 7.0.

The concentration of the lysozyme was important. Concentrations providing 1.66 to 3.33 micrograms of lysozyme per

1/2 ml gave the most consistent results.

The use of an optimum concentration of lysozyme and cells with a dilute and concentrated substrate of acetate and ethyl alcohol showed an increased oxygen uptake by M. lysodeikticus.

The use of inactivated lysozyme resulted in no increase of oxygen uptake.

The presence of lysozyme had no effect upon endogenous respiration.

An alteration of the cell permeability was indicated by an increased oxygen uptake accompanied by a partial lysis of the cells.

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