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The mechanism of the activity of lysozyme on bacteria

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Thesis

THE MECHANISM OF THE ACTIVITY OF LYSOZYME ON BACTERIA

by

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(B.A., Syracuse University, 1950)

Submitted in partial fulfilment of the
requirements for the degree of
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INTRODUCTION

The object of this thesis is to demonstrate and discuss the kinetics of the bacteriolytic action of egg white lysozyme. This is to elucidate further the enzymic nature and the mechanism of lysozyme action. The activity of the enzyme is measured by a turbidimetric method. This method determines the decrease in light absorption of a bacterial suspension which is being lyzed by lysozyme.

REVIEW OF LYSOZYME

INTRODUCTION: In 1922, Fleming (20) found that suspensions of certain air cocci (Micrococcus lysodeikticus) were rapidly and completely dissolved by a bacteriolytic agent present in nasal mucus, tears, egg white, and other biological fluids. Fleming called this agent "lysozyme", and this term has come into general use. Since then, several biological fluids known to dissolve the micrococci, but which differ in their properties, have been loosely called lysozyme. Unless otherwise indicated, all data in this paper are for egg white lysozyme.

PURIFICATION: Partial purification of lysozyme was carried out by Wolff (71), but it was first prepared in crystalline form from egg white by Abraham and Robinson (1). In 1945, Alderton et al. (3) were able to isolate lysozyme in a more purified crystalline form and in much better yields (85 - 90%) than ever before. The lysozyme was prepared from egg white by adsorbing it on bentonite, eluting active proteins with phosphate buffer (pH 7 to 8) and 5% aqueous pyridine, and then eluting the active material with pyridine-sulfuric acid solution at pH 5.0. The eluate is dialyzed and dried in the frozen state. An exact procedure taken from Alderton's method is given in Biochemical Preperations (12).

Crystalline lysozyme can be most readily prepared by direct crystallization from egg white (4). This is done by adding 5% of solid sodium chloride to egg white, adjusting the pH to 9.5 with sodium hydroxide, seeding with a small amount of

crystalline lysozyme and allowing the mixture to stand at 4°C. for 48 hours. Lysozyme is precipitated in crystalline form in 70 - 80% yield. The products can be recrystallized by dissolving in dilute acetic acid and adding 5% sodium bicarbonate. It can also be further purified by rechromatography (66). Chromatographical experiments (66) have demonstrated that by the direct crystallization procedure it is possible to prepare a sample of lysozyme which is over 95% homogeneous chromatographically.

CHEMICAL COMPOSITION: Table II on page four gives a comparison of the amino acid composition of lysozyme as reported by 2 different investigation teams (26,47).

Table I

Nitrogen Distribution Between the Different Groups of Amino Acids (26)

	amino acid N expressed in % of total N
Basic groups	33.3
Acidic groups	8.4
Neutral aromatic groups	8.9
Neutral non-aromatic groups	37.9
Amide and misc. Nitrogen	10.0
Total Nitrogen	98.5

Table II

The Amino Acid Composition of Lysozyme

Reference Constituent	(26) % of lysozyme	(47) molecule	(26) number of residues per molecule	(47)
Sulphur	2.53	2.53	12	11.8
Phosphorus	0.0	0.0	0	0.0
Glycine	5.6	5.7	11	11.3
Alanine	6.1	5.8	10	9.7
Serine	7.2	6.7	10	9.51
Cysteine	0.0	0.0	0	0.0
Cystine	8.0	6.8	5	4.96
Threonine	5.3	5.5	7	6.89
Methionine	2.3	2.06	2	2.06
Valine	6.2	4.8	8	6.11
Leucine	7.0	6.9	8	7.84
Isoleucine	6.2	5.2	7	5.91
Proline	1.3	1.4	2	1.81
Hydroxyproline	0.0	0.0	0	0.00
Phenylalanine	2.3	3.12	2	2.82
Tyrosine	3.8	3.58	3	2.94
Tryptophan	8.3	10.6	6	7.73
Aspartic acid	11.8	18.2	13	20.4
Glutamic Acid	3.3	4.32	4	4.36
Lysine	6.0	5.7	6	5.81
Histidine	1.05	1.04	1	1.00
Arginine	14.8	12.7	13	10.9
Amide N	1.86	1.71	19	18.1
Total N	98.5	98.5	195	198

The formula of isoelectric egg white lysozyme appears to be Gly_{11} Ala_{10} Ser_{10} $[(Cy.S)_2]_5$ Met_2 Thr_7 Val_8 Leu_8 $Ileu_7$ Pro_2 Phe_2 Tyr_3 Try_6 Asp_{13} Glu_4 Lys_6 His_1 Arg_{13} $(NH_2)_{16}$ based on a molecular weight of 14,700 (26).

The amino acid analysis of lysozyme has shown that cysteine is absent, while cystine is present in the molecule. Thus, there are only disulfide bonds present and no -SH groups. These disulfide bonds have been known to supply the cross links between peptide chains of proteins. If cleavage of the disulfide bonds between 2 similar polypeptide chains occurred, there would be a halving of the molecular weight (25). In experiments (25) conducted to prove this, the molecular weight of the reduced and alkylated lysozyme remained the same as the original protein. This suggests that lysozyme consists of a single peptide chain internally crosslinked by disulfide bonds.

CHEMICAL PROPERTIES: Fleming in his original paper (20) reported that lysozyme was soluble in water and physiological saline solution. It was precipitated by chloroform, ether, acetone, alcohol, and toluene without destroying it. According to Meyer et al. (49) lysozyme is basic in nature, being soluble only in acidic aqueous media and insoluble in pure organic solvents and alkaline media. Roberts (60) reported that lysozyme is very soluble in dilute acedic acid. Its behavior upon filtration through Berkefeld filters is as follows: in alkaline media, no lysozyme passes through; in media acidified with acetic acid, only part of it passes; in dilute mineral acid, it is entirely

Table III

Molecular Weight of Lysozyme

Method	Molecular wt.	Reference
ultracentrifuge	18,000	(60) Roberts and Wells, Quart. J. Exptl. Physiol., 27, 89 (1937).
diffusion	13,000	(56) Pasynskii and Plaskew, Compt. rend. acad. sci. U.R.S.S., 48, 579 (1945).
osmotic pressure & ultracentrifuge	17,500	(3) Alderton et al, J. Biol. Chem., 157, 43 (1945). (J.L. Oncley).
sedimentation & diffusion	14,700	(3) Alderton et al., J. Biol. Chem., 157, 43 (1945). (J.L. Oncley)
x-ray diffraction	13,900	(55) Palmer et al., J. Am. Chem. Soc., 70, 906 (1948).
amino acid analysis	14,900	(47) Lewis et al., J. Biol. Chem., 186, 23 (1950).
amino acid analysis	14,700	(27) Fromageot and de Garilhe, Biochem. et Biophys. Acta, 4, 509 (1950).
osmotic pressure	16,600	(25) Frænkel-Conrat et al., J. Am. Chem. Soc., 73, 625 (1951).
light scattering	14,800	(35) Halwer et al., J. Am. Chem. Soc., 73, 2786 (1951).

filterable (49). Saturation with ammonium sulfate precipitates lysozyme but 50% saturation causes only an opalescence (60).

The biuret, glyoxylic acid, Folin-Ciocalteu phenol and nitroprusside reactions are positive (49) and the Molisch test is negative. Bromine in glacial acetic acid is readily decolorized. Roberts (60) reported positive reactions with the xanthoproteic and Millon tests and that lysozyme was precipitated by trichloracetic, sulphosalicylic, flavianic, picric, styphnic, picrolonic and Reinecke acids. Meyer et al. (49) contradicted Roberts by stating that lysozyme was not precipitated by trichloracetic or sulfosalicylic acids and only incompletely by tungstic acid. Perchloric acid precipitated the enzyme. Like most other proteins it is removed from solution by colloidal iron (60). Some salts of heavy metals (as gold or silver) precipitated lysozyme with simultaneous inactivation. Meyer et al. (49) consider purified lysozyme to be a basic polypeptide that is very stable to heat and acids but is inactivated by alkali, peroxide and iodoacetic acid. The basicity of the protein seems to be due to the relatively small quantity of dicarboxylic acids (23). In neutral and especially in alkaline solutions lysozyme is rapidly inactivated by heat (11,61) although it is stable in acid and its stability increases down to pH 4 (18). Alderton (72) found lysozyme to be rather stable in solutions over the pH range of 3-11 at 23°.

Precipitation and inactivation are also obtained by sozoiodolic acid and when lysozyme solutions are iodized with iodine in

potassium iodide (49). Cuprous oxides decrease the activity of lysozyme. The inactivation by iodine or cuprous oxide is partially reversible. Oxidized lysozyme can be reactivated by reducing agents such as sulfite or hydrogen sulfide. Hydrogen cyanide only partially reactivated iodine-inactivated preparations. According to Kertesz and Caselli (42), the biological activity of oxidized lysozyme is approximately 50% of the original activity of reduced lysozyme.

No significant loss of activity takes place on digestion of lysozyme with any of the following enzymes: bacterial proteinase at pH 7.4, mold proteinase at pH 7.4, trypsin at pH 7.4 and papain activated with cysteine at pH 6.0 (3). With suitable substrates no protease, kinase, amylase, lipase, or phosphatase activity is demonstrated by lysozyme (50).

Lysozyme forms insoluble complexes with sodium thymus nucleate and sodium yeast nucleate, but not with adenylic acid (43). Among the proteolytic enzymes, pepsin precipitates lysozyme but trypsin and chymotrypsin do not. Similarly, the plasma proteins, bovine albumin and γ-globulin do not form insoluble complexes with lysozyme. From the effect of pH on the precipitation phenomena, particularly with sodium dodecyl sulfate, Klotz and Walker (43) concluded that electrostatic forces contribute strongly to the stability of the complexes. They also believe that the failure to detect any significant complexing between lysozyme and methyl orange anions as an additional contribution to the importance of van der Waals interactions to the binding

energy of the complexes.

Lysozyme is antigenic and will give specific precipitin reactions (36,60). Lysozymes from varying sources do not give cross reactions, i.e. - are specific, although they possess the common property of lysing M. lysodeikticus (65). Smolens (65) and Wetter (84) confirmed the fact that crystalline lysozyme is antigenic, combines with specific rabbit antisera to a high titer and that antilysozyme rabbit serum inhibits the activity of lysozyme on the test bacteria - M. lysodeikticus.

INACTIVATION AND ACTIVATION EFFECTS: These effects are determined by the lytic activity of lysozyme against a test organism, usually M. Lysodeikticus. Lysozyme is inactivated by various chemical reactions which effect its amino, carboxyl, amide, guanidyl or hydroxyl groups (24). All of the groups seem to be essential for lysozyme activity. Chemical attack on the indole group is less harmful which suggests that the indole group is not essential for lysozyme activity. Reagents such as Ac_2O, AcCl, phenylisocyanate which usually inactivate lysozyme, do not react with lysozyme under anhydrous conditions, as indicated by unchanged solubility and bacteriolytic activity (72). The action of excess iodine led to some loss of activity which was partially regenerated in the early stages by reducing agents. The phenol groups and the disulfide bonds were very unreactive to chemical attack. Fraenkel-Conrat (24) believes that since this stable protein is so readily inactivated by a variety of chemical reagents, it is the chemical changes, rather than the conditions of

reaction, which are responsible for the observed losses of activity. He concludes that numerous types of reactive groups are involved in the "active site" of lysozyme since its lytic activity seems to be very sensitive to most chemical agents.

Smith and Stocker (63) determined that the optimal inactivating efficiency of crystalline lysozyme by straight chain aliphatic compounds occurs in the range C_{12} - C_{16} where it increases with the length of the carbon chain and with concentration. Alkyl sulfates, fatty acids, sodium salts of fatty acids and aliphatic long chain alcohols have been found to inhibit lysozyme if their carbon chain contains more than 12 carbons and the inhibiting effect increases with the solubility of the aliphatic compound. Inactivation of lysozyme by alkyl sulfates could not be reversed by dialysis or precipitation with calcium or barium ions. This inhibition is due probably to changes in the protein molecule itself.

Moss and Martin (52) found lysozyme to be inhibited by sodium alkyl sulfates, sodium decyl benzene solfonate, N-pyridinium chloride, a synthetic zeolite, activated carbon, and by Bentonite (a hydrated aluminum silicate). A combination of Bentonite, a synthetic zeolite and polyamine resin displayed a synergistic action as lysozyme inhibitors.

Andersen (5) did some early work on the inhibitory factors of lysozyme. An oligodynamic action of silver on

lysozyme could not be observed, but copper destroys it in 8 days. Alkalies, acids, and a series of disinfectants inhibit it. Neutralization of the acid restores some of the action; mono- and poly-saccharides have a very small inhibitive action; urea and hexamethylenetetramine have none.

Glassman and Molnar (30) interacted crystalline lysozyme with an anionic, a cationic, and 2 nonionic surface-active agents. Quantitative precipitation of lysozyme by the ionic surface-active agents was obtained. Neither of the nonionic surface-active agents tested caused precipitation or altered the enzymatic activity of lysozyme.

No inactivation resulted from the treatment of lysozyme (59) overnight at 22° C. with 0.1 M alloxan or 2 M hydroxylamine, while 0.1 M sodium hydroxide resulted in about 50% inactivation. A 40% inactivation of lysozyme resulted from its incubation with riboflavin in strong light. In contrast to Meyer's paper (51), Proctor et al. (59) reported that 0.5 M NaCl gave about 50% inhibition and 1.0 M NaCl gave 90% inhibition.

Boasson states that high concentrations of electrolytes (8% NaCl) inhibit the primary reaction, adsorption of lysozyme (10). In this respect the valency of the ions is predominant; the higher the valency of the ions, the stronger the inhibition. This suggests the importance of electrostatic forces in this and similar reactions. Recently Smolelis (81) confirmed this

inhibition by salts.

Ferrous sulfate exerts an inhibition which may be counteracted by sodium pyrophosphate (19). Nihoul (78) reported that ribose nucleic acid, gum arabic and heparin act as lysozyme inhibitors. Lawrence and Klingel (46) found that azosulfonamides suppressed the lytic action of lysozyme in dilutions up to 1:10,000. Heparin and analogous polysulfonates inhibit the action of lysozyme in physiological saline and phosphate buffer solutions of various pH values (8). Sulfanilamide in concentrations up to 1% and sulfathiozole up to 0.1% do not interfere with the activity of lysozyme (53). Fiaccavento (74) found an enhancement, in vitro, of the antibacterial power of sulfonamides by lysozyme. Denaturation and inactivation takes place on passing lysozyme through a filter-candle (60). According to Kertesz and Teti (41), lysozyme that is oxidized by the system polyphenol oxidase-o-dehydroxyphenol(so-called tyrosinase) is completely inactivated, although this inactivation is reversible upon reducing the oxidized lysozyme. The addition of casein to lysozyme increases its lytic action (54).

PHYSICOCHEMICAL PROPERTIES: Ready dialysability of lysozyme was one of the first evidences of its low molecular weight. Dialysis is quite rapid through cellophane and within a few hours, lysozyme activity is the same on both sides of the membrane (60). By diffusing lysozyme through collodion mem-

branes, Gildemeister (29) assigned it a molecular size of less than 30 millimicrons.

Lysozyme is easily denatured in an air-water interface and probably at a water-chloroform interface (60). A highly purified lysozyme preparation produced no appreciable lowering of surface tension in water or saline (50).

Gratia and Goreczky (32) using a modified Henri-Huguenard ultracentrifuge showed that 85,000 r.p.m. lysozyme is more stable than the bactericidal substances (a- and B- lysin) in rabbit serum but shows no concentration in any of the layers.

From the sedimentation rates measured (3) at lysozyme concentration of 1.5 to 0.5% in 0.15 M sodium chloride, values at $S_{20,w}$ were found to be about 1.9 Svedberg units. The average diffusion constant, $D_{20,w}$ is 11.2 X 10^{-7} at 1% lysozyme concentration.

Crystallization of lysozyme (3) has been effected at the isoelectric region (pH 10.8), at pH 7.0 and in acid solutions (pH 3.5 to 5.0). The crystal form appears to vary, depending on the pH of crystallization and the acid used in dissolving the protein. Lysozyme forms one type of crystal below pH 7.0 and another type above pH 7.0 (4). In appearance, the crystals formed at any pH above 7.0 seem identical. They differ chemically, however, in that those formed in the isoelectric region (pH 10.5 -11) are relatively insoluble in water, while those formed below pH 9.0 are increasingly soluble with decreas-

ing pH. lysozyme has been crystallized as the chloride, bromide, iodide, nitrate and carbonate. These crystals are needle-like in form. Attempts (4) have been made to crystallize other salts of lysozyme such as the acetate, sulfate and tartrate, but without success.

Palmer et al. (55) report 2 crystalline modifications of lysozyme chloride; with mother liquor at pH 3.5 - 6.0, tetragonal bipyramidal crystals of the first order appear and from pH 7 to 11, needle-shaped orthorhombic crystals appear. Only the tetragonal crystals have good diffraction patterns. Tetragonal symmetry has been confirmed (55) by taking several sets of 5° oscillation photographs 90° apart around the "C" axis. In all cases these pairs of photographs appear to be identical.

The corrected density (55) at 27° C. of air-dried tetragonal lysozyme chloride is 1.305±0.033 gms./cc. The optical properties of lysozyme chloride are as follows (39): average refractive index, M=1.554, the optical character is uniaxial (+) and the birefringence is 0.003.

Anderson (6) reports the isoelectric point of lysozyme to be about pH 11.0 at 0.01 ionic strength of sodium glycinate and an isoelectric point of pH 11.2 at 0.05 ionic strength of sodium glycinate. Pasynskii and Kastorskaya (57) found the isoelectric point of lysozyme to be at pH 6 by the Tiselius method and that the slope of the mobility vs. the pH

curve in the region of the isoelectric point is 2.5×10^{-5}. They think that the difference between their isoelectric point of 6 and Alderton's (3) 10.5 is explained by the difference in the constitution of the buffers used. Alderton et al. (3) concluded from the electrophoretic study of crystalline lysozyme that lysozyme and the globulin component egg white designated G_1 by Longsworth et al. (48) as being identical.

Electrometric titration (2) of lysozyme showed that this is a basic protein with an acid binding capacity not far from 23 groups per molecule. At pH 11.5 the base-binding capacity in water, at 25° C. corresponded to about 15 groups per molecule.

SPECTROSCOPY: An oxidized lysozyme solution is red and shows an absorption maximum in the visible spectrum at 480 m μ (42). The lysozyme is oxidized by tyrosinase.

The ultraviolet absorption peak of oxidized lysozyme is at 279 mμ (42). The ultraviolet spectrum (28) of lysozyme in acid as well as alkaline solutions corresponds to that of a mixture of six mols of tryptophan, 3 mols of tyrosine and 5 mols. of cystine. The 2 or 3 mols. of phenylalanine in the molecule have practically no influence on the spectrum. It is possible to calculate from the variations of the ultraviolet spectrum of lysozyme at λ = 288-292 m μ as a function of pH, the value of pK = 10.8 for the -OH group of the tyrosine in lysozyme, differing only slightly from that of free

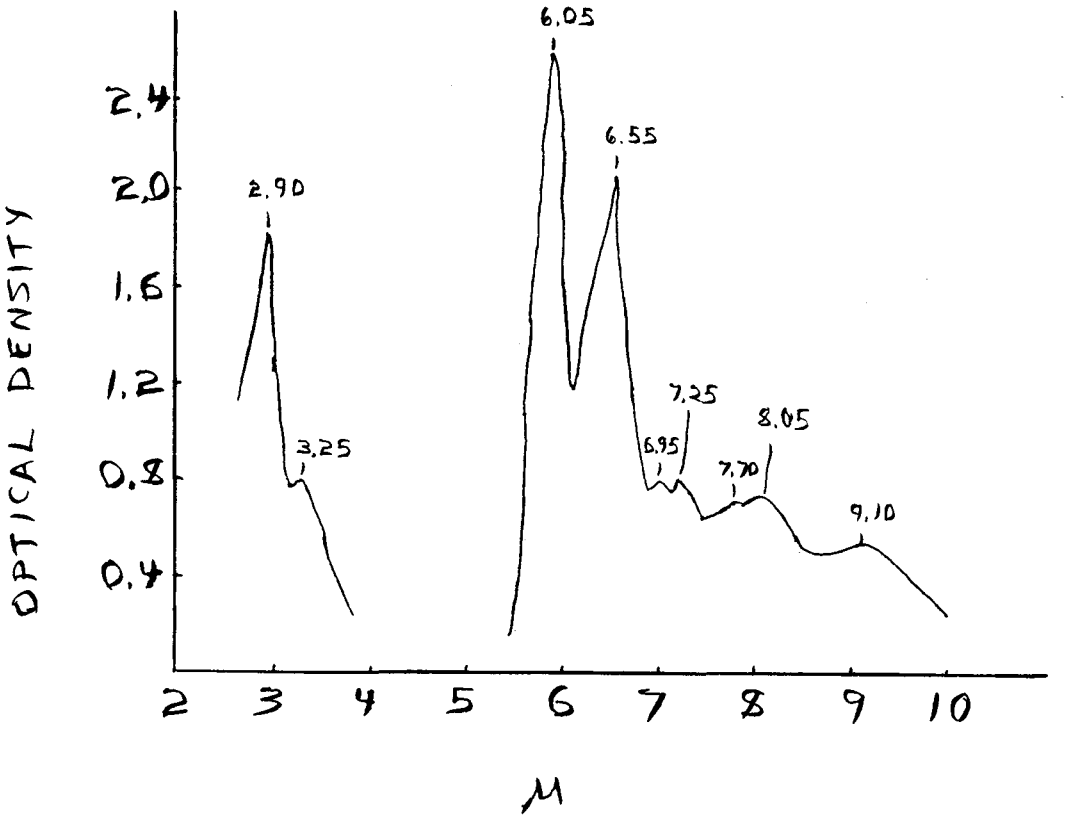
tyrosine which has a pK of 10.1. The variations of the absorption spectrum of lysozyme as a function of pH are reversible, provided the oxidation of the protein in an alkaline medium is avoided.

The infrared spectrum (44) of lysozyme is in the 3 μ region and the primary peak is at 2.90 μ. Since absorption band below 3 μ seems to be characteristic of -OH groups, even when hydrogen bonded, this peak seems to indicate a high content of hydroxyamino acids. This indication is confirmed by the amino acid analyses of lysozyme (26, 47) which reports about 16% hydroxyamino acids. The absorption band at 3.25 μ is probably due to the NH and aromatic C-H vibrations of aromatic amino acids; at 6.05 μ, due to the C=O group; at 6.55 μ, due to the N-H group; at 6.95 μ, due to the C-H vibrations of a methyl group. The band at 9.10 μ would fit the presence of an aliphatic hydroxyamino acid.

Lysozyme formed complexes with sodium dodecyl sulfate which were very suitable for infrared analysis (44). In the spectrum of the complexes there are small differences in the frequencies in the 3 and 6 μ regions which would tend to indicate that the dodecyl sulfate interacts in a rather general fashion with the protein molecule as well as at certain specific foci. The peak at 9.1 μ in lysozyme is replaced by one at 9.5 μ in the complex. If the latter is due to aliphatic hydroxyamino acids, the shift would indicate that the dodecyl sulfate anion interacts strongly with the -OH group.

GRAPH I

INFRARED ABSORPTION SPECTRUM OF LYSOZYME (44)



(WAVE LENGTH, μ)

MECHANISM OF LYSOZYME ACTION:

METHODS OF DETERMINATION: Since the visible process of lysis is the rapid clearing of cloudy or opaque suspensions of susceptible bacteria, Fleming (20) worked out a method for estimating lysozyme concentrations. He used serial dilutions to determine the lowest concentration which would dissolve a known suspension of his micrococci during a given period of incubation. Welshimer and Robimow (70) observed lysis microscopically, whereas more recent workers (7,40) used the electron microscope. Boasson (10) studied lysozyme action by measuring the light absorption of a lysing suspension with a Moll extinctiometer. Later, Meyer and Hahnel (51) measured the decrease of viscosity and correlated it with the concentration of lysozyme used. Smolelis and Hartsell (64) used a Coleman spectrophotometer at a wave length of 540 m μ to measure the decrease of turbidity of bacterial suspension as a result of the action of lysozyme. Recently several men (73,76,79) have reported various modifications in the determination of lysozyme.

FATE OF LYSOZYME DURING LYSIS OF THE ORGANISMS: Fleming (20) showed that when lysozyme is added to a susceptible bacterial suspension, it is rapidly fixed onto the susceptible cells. This adsorption has been generally confirmed (10). Fleming and Allison (22) found further that in the process of lysis there is no neutralization of the lysozyme but that

it may be recovered completely from the dissolved mixture and used over again.

PHYSIOCHEMICAL NATURE OF LYSOZYME ACTION: Dorfman (14), using Abrahams modification of the micro-electrophoretic chamber of Northrop, observed that as soon as lysozyme is added to the bacterial suspension, there occurred a sudden rise in the negative electro-kinetic potential. This persists for 5 to 10 minutes and then falls to the initial value which depends on the pH of the medium. The change in the potential of the bacterial surface accompanies lysis, and is not due to the absorption of lysozyme or to the effect of impurities or substances derived from lysed cells. Lysozyme inactivated by boiling had no appreciable effect on the potential (15). The zeta potential effect of lysozyme is represented by a sigmoid curve similar to that obtained by penicillin (17), but this investigator earlier stated (16) that the zeta potential effect of the bacteriostatic agents did not change with time, while that of the bacteriolytic agents was reversible. These differences in time of the zeta potential effect were tentatively attributed to the more profound metabolic and structural changes of the bacterial cell as caused by the lytic agents, although the primary rise in the potential was identical in both cases (16).

Dorfman (15) concluded that the early stages of bacteriolysis is localized in the cell surface and is accompanied by

a transient change in the electrical charge of the cell surface.

Pasynskii and Kastorskaya (57) assumed that the maximum lytic activity and the maximum absorption of lysozyme by sensitive bacteria as corresponding to the isoelectric point of lysozyme or is at least closely related to the isoelectric point which they believe is at pH 6.0.

Physico-chemical analysis by Boasson (9) shows that lysis takes place in 2 stages: (a) the action of lysozyme on the cell, the velocity of which is chiefly determined by the amount of lysozyme absorbed, and (b) the diffusion of the cell contents into the solution, dependent on the solubility of these substances in that solution. The size of the decomposition particles is proportional to the rate of lysis (13).

Bacteriolysis by lysozyme on both dead or living bacteria behaves as a unimolecular reaction according to several investigators (13, 62, 69). Under the conditions that Holyoke and Johnson (38) employed, they found the rate of action of lysozyme on M. lysodeikticus, measured photometrically, as being nearly proprotional to the enzyme concentration and being nearly inversely proportional to the initial concentration of bacterial cells. With a given amount of lysozyme (1.5 μ gm/ml) the density of cell suspension decreases exponentially with time, until it is reduced to between 20% or 40% of that at the start, followed by a decreasing rate of

action.

CHEMICAL NATURE OF LYSOZYME ACTION: Hallauer (34) was the first to suggest that lysozyme acts on the mucoid fraction in the bacteria. Meyer and co-workers (50) found that the action of lysozyme upon the bacteria, sarcinae, brings about progressive increase in non-protein nitrogen, inorganic phosphorus, and reducing substances. The increase in nitrogen and phosphorus is due to the release of materials from the bacterial cell. The lytic action liberates reducing sugars from the mucoids of polysaccharides of the susceptible sarcinae. The action is apparently on one of the sugar linkages, but the nature of the linkage is unknown. This action is quite specific since a number of glucoproteins and polysaccharides are not susceptible (50).

Pirie (58), Epstein and Chain (18) confirmed and extended the findings of Meyer et al. (50) They prepared from the cells of M. lysodeikticus a high molecular polysaccharide which was hydrolyzed by purified lysozyme into an N- acetylaminohexose and a ketohexose. This was accomplished over a large range of pH (pH 2 to 9). This confirmed that lysozyme is an enzyme belonging to the class of carbohydrases. Also, this polysaccharide has been found in all lysozyme-sensitive bacteria investigated.

Later Meyer and Hahnel (51) found that the mucoid substrate existed firmly bound within the bacterial wall as an

insoluble, highly polymerized, mucopolysaccharide fraction which was depolymerized by lysozyme. With larger concentrations of lysozyme, glucosidic linkages are hydrolyzed and reducing sugar and acetylhexosamine appear.

Hawthorne (37) found that the products formed in the lysis of M. lysodeikticus were several ninhydrin reacting substances which were probably derived from the cell contents. After removal of these substances, no free reducing sugars were present, but on acid hydrolysis, glucose and mannose appeared. Other substances in the hydrolysate were identified as glucosanine and glucuronic acid.

In a review of lysozyme, Meyer (33) summed up the mechanism of lysozyme action as consisting of a depolymerization of a mucopolysaccharide, leading to water imbibition by the organism and to the disorganization of the microbial cell. The cessation of oxygen uptake and the appearance in the supernatant solution of non-protein nitrogen, inorganic phosphorus and reducing substances is due to the breakdown of protein and organic phosphates by bacterial autolytic enzymes, which are apparently responsible for some part of the visible clearing of the bacterial suspension. Gorini (75) found that lysis of M. lysodeikticus by lysozyme is accompanied by proteolysis of the cellular proteins and that the proteinases are only active when Ca is present. Webb (68) adds that heat inactivation of the autolytic enzyme systems may be of

greater significance in explaining the resistance of heat-killed cells to complete lysis by lysozyme, than the fact that cellular proteins are rendered insoluble by heating. Welshimer and Robinow (70) reported that although the action of lysozyme is influenced by the presence of autolytic enzymes, lysozyme can function independently of the autolytic system. The response of the organism to lysozyme differed with heat and formaldehyde inactivation of the autolytic enzymes.

Kern and co-workers (40) report a difference in the action of lysozyme on Staphylococcus aureus and on Micrococcus lysodeikticus. The action of lysozyme on S. aureus is accompanied by a gradual decrease in density at the periphery of the cell. No evidence was found of a rupture of the cell membrane with a spilling of the cellular constituents. Their electron microscope observations of the lysis of M. lysodeikticus showed that lysozyme first causes an enlarging or swelling of the cell, followed by a rupture of the cell wall with a dispersal of the cell contents. With the exception of 2 cell bodies, believed to be nuclei, the cell contents dissolved in the medium. Finally the cell membrane is also destroyed, leaving only the cell bodies.

Lysis of bacteria by lysozyme is proceeded by the conversion of Gram positive cells to Gram negative forms (33). Webb (68) found that when this occured, small amounts of re-

ducing sugar (which came from the carbohydrate material located at the surface of the cells) were liberated. The alteration in Gram staining reaction seems to be due to the accompanying removal of ribonucleic acid from the cell surface. The outer layer of bacterial cells probably consists of ribonucleic acid and carbohydrate in chemical combination.

FACTORS INFLUENCING THE ACTION OF LYSOZYME:

TEMPERATURE: Fleming (20) found that the rate of lysis of bacterial suspensions by lysozyme was directly related to temperature, increasing to a maximum at about 60° C. Others (5,67) have found the maximum action to take place at 55° C. The point at which increase in temperature ceases to increase the rate of action depends on the temperature at which destruction of the enzyme begins, which, in turn, depends on the hydrogen ion concentration (pH) of the material used (67).

HYDROGEN ION CONCENTRATION (pH): Fleming (20) found that the action of lysozyme was influenced by the concentration of hydrogen ions in the medium. He determined that the maximum lysis occured at about the neutral point. Boasson (10) obtained the greatest lysis at pH 6.2 and the finding of Thompson's group (67) confirmed this. It has been definitely established that the optimum pH as determined by the clearing of bacterial suspensions is not actually the most favorable pH for the action of the enzyme, but a composite optimum depending on several factors (10,67). At some point not far

above pH 7 an increase in the lability of lysozyme enters and the destruction of lysozyme may occur at the temperature of incubation. At lower pH the action of lysozyme increases, but below pH 6.2, no visible lysis occurs; after neutralization, however, lysis is immediate. Hallauer (34) was the first one to interpret this phenomenon correctly, viz., the action of lysozyme is not inhibited, but visible lysis cannot occur because the constituents of the cell are insoluble in dilute acid. Boasson (10) showed that the apparent inhibition of lysis by distilled water was due to the same phenomenon and the addition of a small amount of alkali would dissolve the cell contents.

Recently smolelis (81) confirmed Boasson's work. No lysis was observed in triple distilled water but the addition of different salts caused varying degrees of lysis. The optimum salt concentration for maximum lysis was correlated with the chemical nature and pH of the salt. The use of univalent, rather than polyvalent salts, resulted in the highest level of lytic activity. Inhibition of lysis was almost complete at a 0.5 M concentration of most salts. This statement is in complete agreement with Boasson's idea, as stated on page 10. Lobstein (76) reports that very low as well as high chloride concentration in the buffer solution alter lysozyme activity considerably. He thus modified the phosphate buffer (pH 6.2) by the addition of .071 N NaCl, the salt concentration which

gave optimum lytic activity.

Meyer et al. (50), Epstein and Chain (18), both found that the action of lysozyme on the mucopolysaccharide substrate increased with increasing acidity down to about pH 3.5, indicating that the optimum pH obtained by lytic titrations is nowhere in the region of the true optimum pH for the action of lysozyme. The pH curve determined by the viscosimetric method gave the optimum pH for lysozyme activity as pH 5.3 (51). Epstein and Chain (18) further state that lysozyme is active over a wide range from pH 2 to 9. Lysis of M. lysodeikticus occurs, however only on a much more restricted range, no lysis being noticeable below pH 5.

Under increased hydrostatic pressures of 4000 and 8000 pounds/ square inch, the rate of lytic action is faster than at normal pressure at various pH's between 5.65 and 8.20 at 35 degrees C. (38).

BACTERIA: A number of workers (21,31,70) reported that heat-killed cocci were definitely less susceptible to the lytic action of lysozyme than living organisms because of the previously mentioned (p.28) phenomenon that the autolytic enzyme systems of the heat-killed bacteria are inactivated by the heat.

Kopeloff et al. (45) found that the susceptibility of M. lysodeikticus to lysozyme could be somewhat increased by growing them on beef extract agar instead of casein digest agar. They also reported that neither the age of the culture (5 mos.) nor the incubation period of the bacteria (48 hours) within the

limits indicated have any significant influence on the lytic action. Hallauer (34) produced bacteria resistant to lysozyme action by growing susceptible organisms in medium containing sublethal concentrations of lysozyme.

Andersen (5) observed that there are no morphological differences between susceptible and non-susceptible bacteria. Webb (68) believes that varying sensitivities of bacteria to lysozyme, probably are due to different rates of hydrolysis according to carbohydrates specific to the organisms.

Lysis of sensitive bacteria occurs only under conditions when all the substances on which the cell structure depends are in a water-soluble state after the action of lysozyme (18). The state of the bacterial proteins is very important in this connection. M. lysodeikticus will not be lysed or will be only incompletely lysed if its proteins are denatured by heat, organic solvents (alcohol, acetone, etc.) or iodine.

Feiner et al (19) have made qualitative statements which are difficult to interpret semi-quantitatively from our own experiences. They mention:

> "That modifications in the composition of the bacterial medium (including changes in pH and the addition of various peptones, sugars, amino acids, etc.), in the temperature of incubation, and in the period of incubation prior to testing, though not always optimum for growth, do not always affect sensitivity of living organisms to a significant degree to lysozyme. Altered sensitivity could be induced by certain methods of pretreatment of the organisms. Little or no effect resulted from repeated washings with saline, lyophilization, precipitation with ice-cold acetone, treatment with 1% phenol, dialyzation against 0.1 N HCl or exposure to

ultraviolet light. Precipitation with either 95% ethyl alcohol or acid acetone, or autoclaving in alkaline solution, rendered the organisms highly resistant to the action of lysozyme. By contrast, organisms autoclaved in acid solution or formalinized, were almost completely lysed with high dilutions of lysozyme, although it was impossible to obtain complete clearing even with the strongest concentration used. In the actual performance of the test, numerous attempts (19) were made to enhance the sensitivity by the addition of various agents. The majority of the substances had no effect. Of all the substances tested, only sodium arsenite was found to have a distinct and consistent effect."

Recently, Lobstein (77) reported that lyophilized M. Lysodeikticus stored for different periods of time, grew less susceptible to lysozyme as time proceeds and as storage temperature increases. Cells stored at temperatures from -5 to 3° apparently remain unchanged. They also found that cells exposed to ultra-violet light are far superior to acetone, formaldehyde or phenol-treated cells as far as storage and lysozyme susceptibility is concerned.

PROCEDURE

Reagents:

Substrates: Micrococcus lysodeikticus, strain 4698, obtained from the American Type Culture Collection. Sarcine lutea, obtained from Dr. G. Young, Dept. of Bacteriology, Boston University.

Buffer: M/15 phosphate buffer, pH 6.2

(KH_2PO_4 / Na_2HPO_4)

Enzyme: Crystalline lysozyme, purchased from the

Delta Chemical Company, was diluted with the pH 6.2 phosphate buffer.

Instruments: Junior Coleman spectrophotometer and Coleman cuvettes.

Method: The organisms were grown in Difco beef heart infusion broth at 37°C. for 2 to 3 days or until growth was dense enough to warrant harvesting. The organisms were treated with 1% phenol for 1 to 2 hours (including time of centrifugation), then separated by centrifugation and washed 3 or 4 times with phosphate buffer. The concentrate was resuspended in a few ml. of buffer and placed in the refrigerator (4°C.). Portions of the concentrate were diluted up to the desired absorbances when needed. The spectrophotometer was set at zero absorbance with a blank containing phosphate buffer, using a wavelength of 540 mμ. The reaction mixture consisted of 1 ml. of substrate of known absorbance, to which 1 ml. of lysozyme solution of known concentration was added. Both solutions were preheated to a desired temperature before the experiment was started. The cuvettes were taken out of the controlled temperature bath at desired times for readings of absorbance. Readings were made at 1, 3 or 5 minute intervals until the reaction had slowed down noticeably.

The turbidimetric determinations were carried out

essentially according to the method of Boasson (10) and Smolelis and Hartsell (64).

DATA

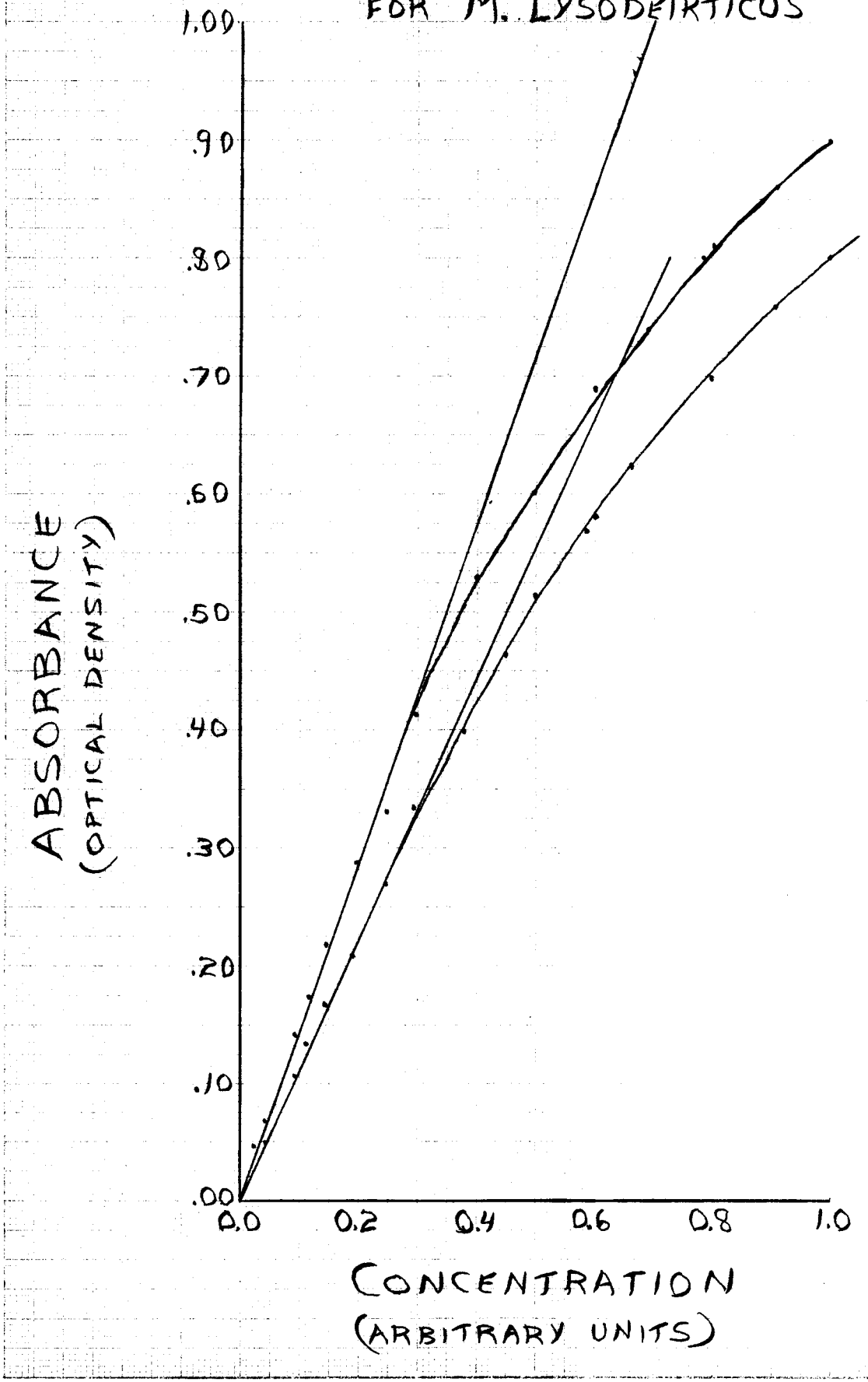
In order to use the turbidimetric method for determining the enzymatic activity of lysozyme, the relationship between bacterial concentration and optical density had to be resolved. According to Graphs II and III bacterial concentration is directly proportional to optical density at low concentrations, but after an optical density of about 0.30 there is an increasing deviation as is shown by the decrease in the slope of the curve. The calibration curves (Graphs II and III) were used to correct the optical density readings. The highest enzyme concentration used exhibited the same light transmission as water and thus had no effect upon the kinetics of reaction except to initially dilute the bacterial suspension in half. If the enzyme solution showed a lower % transmission, than the kinetics would become complicated, due to the adsorption of lysozyme onto the bacterial cell (20).

When the decrease in bacterial light absorption is plotted against time, a characteristic curve is obtained. Graph IV illustrates this. A straight line is obtained (Graph V) when log optical density is plotted against time, thus the kinetics follows a monomolecular equation.

In the graphs of log optical density vs. time good constancy was obtained in the early part of the plot, excluding

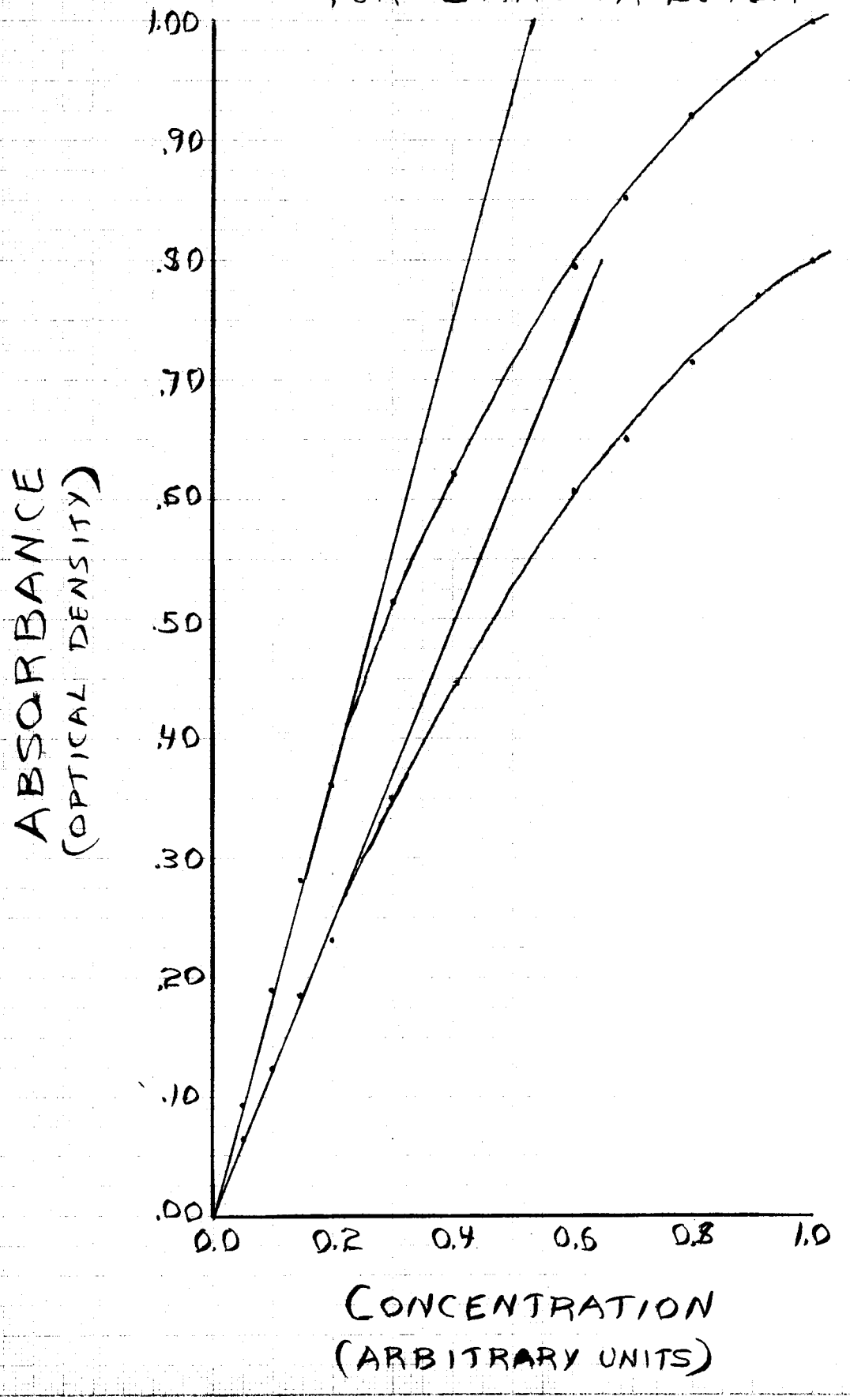
GRAPH II

CALIBRATION CURVE FOR M. LYSODEIKTICUS



GRAPH III

CALIBRATION CURVE FOR SARCINA LUTEA



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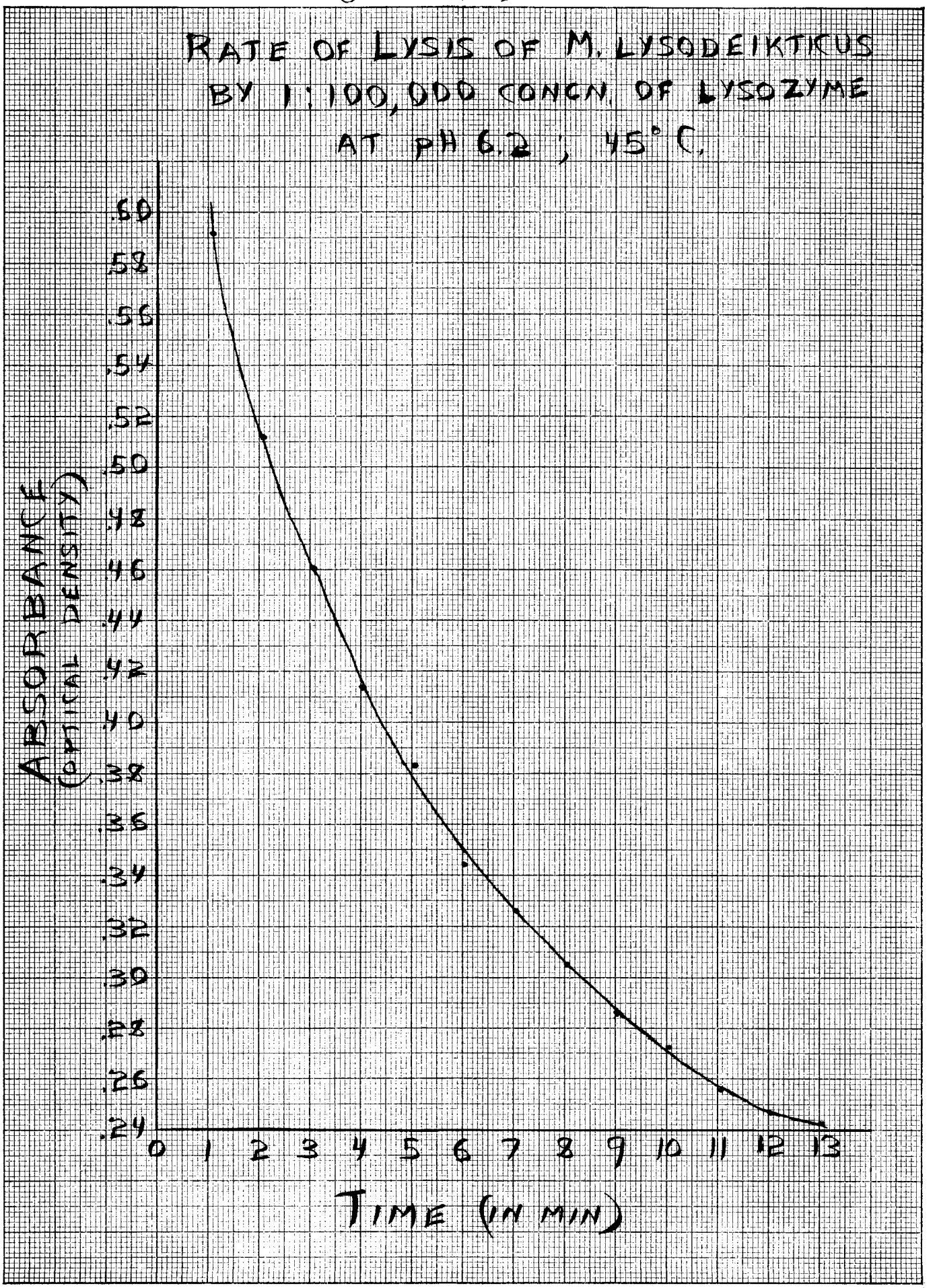
GRAPH IV



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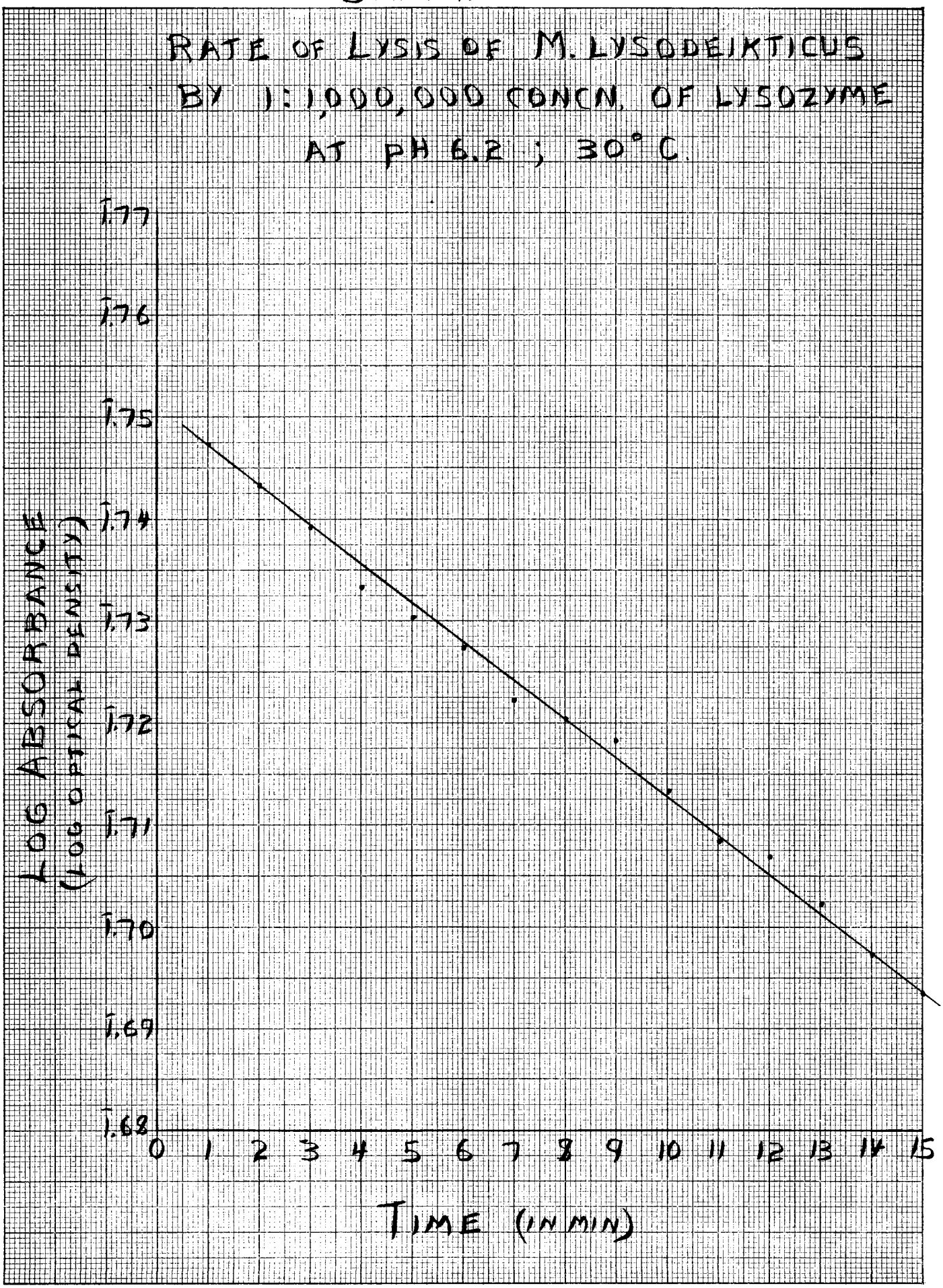
GRAPH V



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the first few points which probably are zero order or some fractional order. The sloping off in the later points was believed due to the inhibition of lysozyme by the end products and the visible insolubility and viscosity of the end products in the phosphate buffer.

The rate constants were not taken from the graphs but were computed in such a manner as to eliminate the human prejudice factor as much as possible. As is illustrated in Table IV, two types of rate constants (k=(-2.3)(-slope)) were calculated. K_1 is the slope from the first minute to any given minute, while K_2 is the slope from minute to minute. Since the rate of reaction slows down due probably to inhibition of end products and, in this experiment, the end products also decrease the light transmission, the first K_1's are assumed to be more accurate and thus the early K_1's are heavily weighed. K_2 helps to point out if and where any break occurs in the rate constants. So by examining both K's one could pick the most accurate group of K_1's and average them for the rate constant of the given kinetic run. The data in the typical kinetic run in table IV is better than the average data collected as is shown by the constancy of K_2 over the entire experiment.

In order to improve the kinetics of the experiments and to determine its variables, a few experiments were run to show qualitatively how the rate of reaction can be affected. The

turbidimetric method was used for determining the activity of lysozyme. Bacteria gave lower rates of reaction when kept in solution for at least 2-3 days before being incubated with lysozyme. It seems as if the more susceptible organisms deteriorated (autolysis?) upon standing (77). As a control M. lysodeikticus was incubated in buffer for 1 hour at 45°, and there was a 4% decrease in concentration. This is believed to be due to autolysis which increases with increased temperatures. The rate of reaction was slower after autolysis of the organism had occurred as compared to non-autolyzed bacteria under the same conditions. The autolyzed bacterial suspension was centrifuged, washed and resuspended so as to wash out the end products of autolysis, but this had no perceptible effect upon the rate of reaction. The rate constant increased from 5.6×10^{-3} MIN^{-1} to 7.7×10^{-3} MIN^{-1} when an emulsifying agent was used as compared to a control reaction without the emulsifying agent. This could be explained via the facilitation of the adsorption of lysozyme onto the surface of the bacteria, prior to the lytic reaction on the bacterial membrane.

Neither M. lysodeikticus nor S. lutea were lyzed by lysozyme after pretreatment of the organisms with the 1% phenol for approximately 18 hours. Pretreatment with 1% phenol for 1 hour seemed to have no effect upon the sensitivity of the organisms to lysozyme (38) but it is doubtful that all the bacteria were killed by 1% phenol after such a short treat-

ment. The phenol, by being in contact with the organisms for a long period of time, probably altered the surface of the bacteria to such an extent as to make them resistant to lysozyme.

Conditions whereby bacteria might change in their sensitivity to lysozyme were eliminated as much as possible. The review of the literature has pointed out some conditions, such as heat action, alcohol or acetone action, storage over long periods of time or at high temperatures, autoclaving and high salt concentrations.

Higher concentrations of lysozyme were used in the experiments on Sarcina Lutea than for M. lysodeikticus since most strains of Sarcinae are less susceptible to lysozyme than M. lysodeikticus (33). The concentrations of lysozyme were chosen so that lysis was rapid and yet slow enough for accurate readings to be made on the photometer.

Lysozyme was inactivated at 65° and no lysis was observed after cooling down from 65°. The optimal temperature for a 2 minute reaction time was about 55° and for a 10 minute reaction time was about 50°. The experiments indicated that the enzyme was being heat-inactivated at 50° but that the process was becoming quite rapid at 55°. Usually the enzyme is incubated for 5 - 10 minutes so the enzyme solution will reach a given temperature equilibrium before being added to the bacterial solution.

TYPICAL KINETIC RUN

BACTERIA: M. Lysodeikticus

LYSOZYME CONCENTRATION: 1:1,000,000

pH: 6.2

TEMPERATURE: 30°C.

INITIAL CONCN. OF BACTERIA: 0.80 (opt. density)

DATA:

TIME	OPTICAL DENSITY	CORR. OPTICAL DENSITY	LOG OPTICAL DENSITY	LOG $\frac{a_1}{a-x}$	K_1 (SLOPE)	LOG $\frac{a-x_1}{a-x_2}$	K_2 (SLOPE)
1	.508	.558	$\bar{1}$.747	----	----	----	----
2	.504	.553	$\bar{1}$.743	.004	.0040	.004	.004
3	.500	.548	$\bar{1}$.739	.008	.0040	.004	.004
4	.495	.541	$\bar{1}$.733	.014	.0047	.006	.006
5	.492	.537	$\bar{1}$.730	.017	.0043	.003	.003
6	.489	.533	$\bar{1}$.727	.020	.0040	.003	.003
7	.485	.527	$\bar{1}$.722	.025	.0042	.005	.005
8	.482	.525	$\bar{1}$.720	.027	.0039	.002	.002
9	.480	.522	$\bar{1}$.718	.029	.0036	.002	.002
10	.476	.516	$\bar{1}$.713	.034	.0038	.005	.005
11	.472	.511	$\bar{1}$.708	.039	.0039	.005	.005
12	.470	.509	$\bar{1}$.707	.040	.0036	.001	.001
13	.466	.504	$\bar{1}$.702	.045	.0038	.005	.005
14	.462	.498	$\bar{1}$.697	.050	.0038	.005	.005
15	.458	.493	$\bar{1}$.693	.054	.0039	.004	.004

TABLE IV

DATA:

Average K_1 (For The Kinetic Run)= 3.96×10^{-3}

Rate Constant= $.00396 \times 2.303 = 9.10 \times 10^{-3}$ MIN^{-1}

Standard Deviation= $\pm 0.29 \times 10^{-3}$

Standard Error= $\pm .078 \times 10^{-3}$

Error= 20%

LEGEND:

a is the initial bacterial concentration.

x is the decrease in concentration.

a_1 is the concentration at the end of the first minute.

Optical density (a-x) is the observed reading from the Coleman photometer.

Corrected optical density was obtained by using the calibration curve. ~~on page 26.~~

Log optical density Log(a-x) is the logarithm of the corrected optical density.

Log $\frac{a_1}{a-x}$ is calculated by subtracting each successive log optical density (Log a-x) from the log optical density (Log a_1) given at the 1 minute mark which is used as the zero time. $\bar{1}.743 - \bar{1}.739 = 0.008$

K's (slope) were calculated from the equation for the monomolecular reaction: $k = \frac{2.303}{t} \text{Log} \frac{a}{a-x}$.

K_1 was calculated from the formula: $\frac{\text{Log} \frac{a_1}{a-x}}{t_1 - t_x} = K_1$

$$\text{Log } \frac{a_1}{a-x} = 0.008 \qquad t_1 - t_x = 2$$

$$K_1 = \frac{0.008}{2} = 0.004$$

(Rate Constant) $k_1 = (-2.303)\ (-K_1)$.

$\text{Log } \frac{a-x_1}{a-x_2}$ is calculated by subtracting each successive log optical density from the previous log optical density.

$\bar{1}.743 - \bar{1}.739 = 0.004$

K_2 (Slope) is calculated from the following formula: $\text{Log } \frac{a-x_m}{a-x_{m/1}} = K_2$

$$t_x - t_{x/1}$$

$$\text{Log } \frac{a-x_m}{a-x_{m/1}} = 0.004 \qquad t_x - t_{x/1} = 1$$

$$K_2 = \frac{0.004}{1} = 0.004$$

(Rate Constant) $k_2 = (-2.303)\ (-K_2)$

DATA:

RATE CONSTANTS

M. Lysodeikticus pH 6.2

Enzyme Concn.	1 : 100,000		1 : 300,000		1: 1,000,000	
Temp	K_1	Log k_1	K_1	Log k_1	K_1	Log k_1
20°	-.0125	$\bar{2}$.462	--	-----	--	-----
25°	-.0150	$\bar{2}$.544	-.0057	$\bar{2}$.121	-.0029	$\bar{3}$.826
30°	-.0170	$\bar{2}$.591	-.0063	$\bar{2}$.158	-.0040	$\bar{3}$.959
35°	-.0220	$\bar{2}$.699	-.0089	$\bar{2}$.309	-.0046	$\bar{2}$.025
40°	-.0280	$\bar{2}$.806	-.0120	$\bar{2}$.441	-.0059	$\bar{2}$.117
45°	-.0340	$\bar{1}$.004	-.0170	$\bar{2}$.591	-.0070	$\bar{2}$.204
50°	-.0590	$\bar{1}$.134	-.0190	$\bar{2}$.634	-.0095	$\bar{2}$.342
55°	-.0708	$\bar{1}$.212	---		-.0110	$\bar{2}$.398

K_1 = Slope

Log k_1 = Log (K_1 x 2.303) = Log (Rate Constant)

TABLE V

DATA:

RATE CONSTANTS

SARCINA LUTEA pH 6.2

ENZYME CONCN.	1 : 10,000		1 : 100,000	
TEMP.	K_1	Log k_1	K_1	Log k_1
25°	-.0146	$\bar{2}$.531	-.0071	$\bar{2}$.210
30°	-.0333	$\bar{2}$.881	-.0114	$\bar{2}$.415
35°	-.0430	$\bar{2}$.997	-.0134	$\bar{2}$.491
40°	-.0420	$\bar{2}$.982	-.0227	$\bar{2}$.716
45°	-.0730	$\bar{1}$.230	-.0320	$\bar{2}$.869
50°	-.0590	$\bar{1}$.130	-.0320	$\bar{2}$.869

TABLE VI

RESULTS

The main group of experiments were conducted to determine the effect of temperature upon the rate of reaction as measured by the rate constant. The only variable in the experiment was the change in temperature. Table VII illustrates the results of such experiments by showing that for every 10^{o} increase in temperature the reaction rate is nearly doubled. The average temperature coefficient (Q_{10}) was 1.75. The Q_{10} values for different enzyme concentrations are similar and the values for the two different species of bacteria compare quite favorably.

The energy of activation (μ) was calculated via the Arrhenius equation from the rate constants and compared by changing the enzyme concentration and/or changing the species of bacteria for each group of experiments. Values from 8,000 to 12,400 were obtained, with the average value being 10,200 cal $\pm$ 1,600. Since μ is characteristic of a chemical reaction, the similarity of μ values seem to indicate that the same enzymatic reaction is occurring at all the given enzyme concentrations and in both bacteria. In the plot of log k vs. the reciprocal of the temperature (Graph VI) a straight line is obtained, thus demonstrating the validity of using the Arrhenius equation.

The rate constants were further utilized in evaluating the relationship between rate constants and enzyme concentra-

tion. Theoretically, if the rate constants follow a first order reaction then the enzyme concentration is directly proportional to the rate constant. Table IX shows that nearly a direct relationship holds at the stronger enzyme concentration but deviates at the weaker enzyme concentration. Following Van Slyke's hypothesis (83) that the complete time required for the decomposition of a substrate molecule consists of 2 intervals--time for combination (adsorption) and time for decomposition, the above deviations at the lower enzyme concentration can be explained. Relatively speaking, there would be more molecules of substrate per molecule of enzyme at the lower enzyme concentration, thus making the time of combination shorter, i.e. more efficient in its enzymatic action, and this would show up in larger rate constants.

RESULTS:

TEMPERATURE COEFFICIENT (Q_{10})*

	M. LYSODEIKTICUS			S. LUTEA	
ENZYME CONCN. TEMP°C	1:100,000	1:300,000	1:1,000,000	1:10,000	1:100,000
20-30°	1.36	-----	-----	-----	-----
25-35°	1.47	1.56	1.59	2.94	1.90
30-40°	1.65	1.91	1.44	1.26	1.99
35-45°	2.00	1.91	1.51	1.70	2.38
40-50°	2.10	1.58	1.67	1.41	1.41
45-55°	1.61	-----	1.58	-----	-----
AVG.	1.70	1.74	1.56	1.83	1.92

(At Constant pH of 6.2)

$$*Q_{10} = \frac{\text{Rate Constant } (k_1) \text{ at Temp}_{n+10^\circ}}{\text{Rate Constant } (k_1) \text{ at Temp}_{n}}$$

Average Q_{10} = 1.75

St'd Deviation = $\pm$0.14

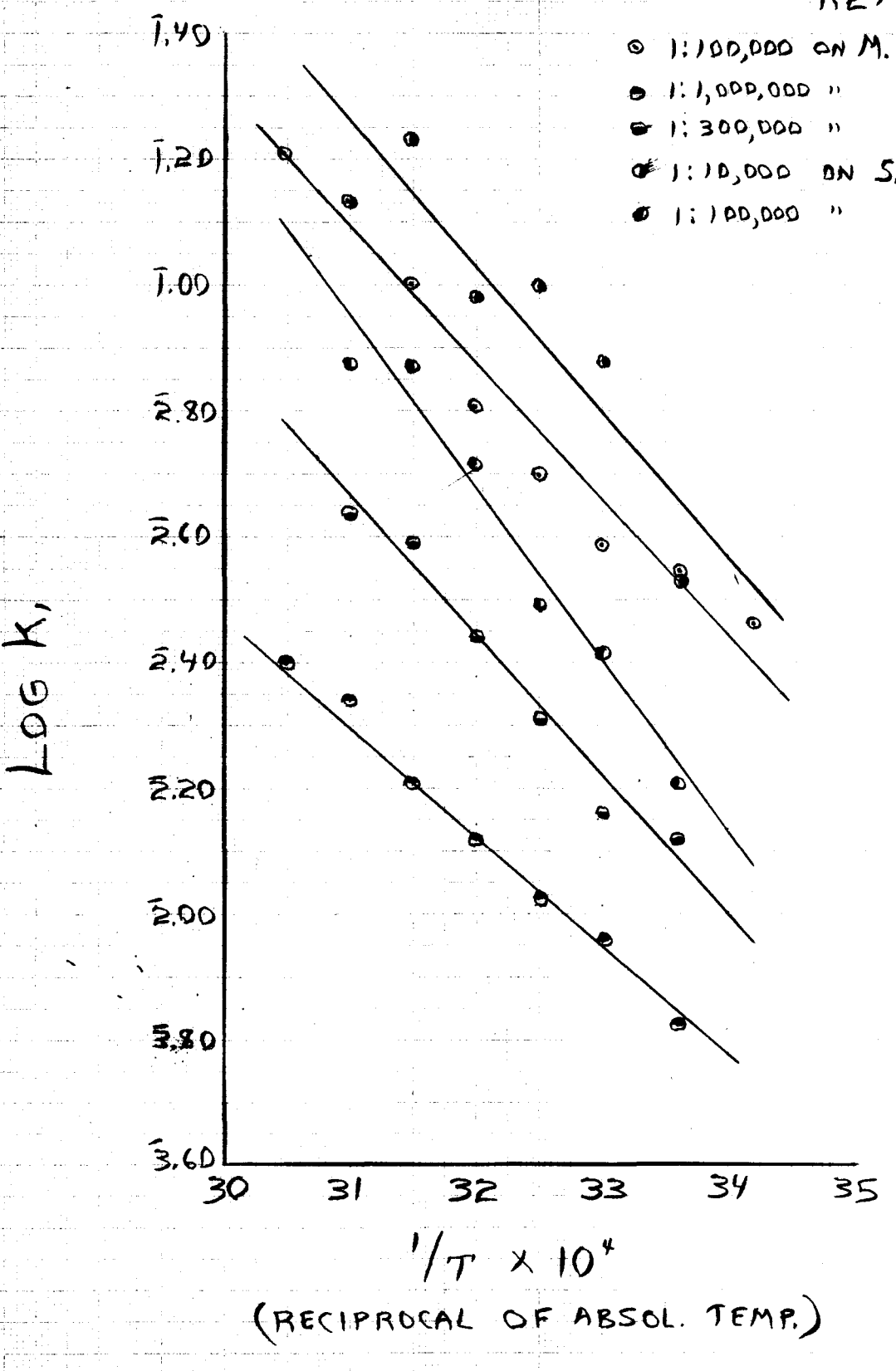
St'd Error = $\pm$0.061

Error = 2.9%

TABLE VII

GRAPH VI

VARIATION OF REACTION RATE CONSTANT WITH TEMPERATURE



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RESULTS:

ENERGY OF ACTIVATION (μ)

CONDITIONS	SLOPE*	μ in (Cal.)
M.Lysodeikticus 1:100,000 pH 6.2	-2,160	≠9,893
M.Lysodeikticus 1:300,000 pH 6.2	-2,208	≠10,113
M.Lysodeikticus 1:1,000,000 pH6.2	-1,733	≠7,937
Sarcina Lutea 1:10,000 pH 6.2	-2,294	≠10,507
Sarcina Lutea 1:100,000 pH 6.2	-2,713	≠12,426

TABLE VIII

*Calculation via the method of least squares

using equation $\text{Log}_{10} k = \left(\frac{-\mu}{2.303R}\right)\frac{1}{T} \neq C$

or slope $= \frac{-\mu}{2.303R} = \frac{-\mu}{4.58}$

Average μ = ≠10175 Cal.

St'd deviation = ≠1602

St'd error = ≠ 715

Error = 7.0%

HEAT OF ACTIVATION

$$\frac{d \ln k}{dT} = \frac{1}{T} + \frac{\Delta H^*}{RT^2} = \frac{\Delta H^* + RT}{RT^2} = \frac{\mu}{RT^2}$$

$$\therefore \mu = \Delta H^* + RT$$

$$\Delta H^*_{298} = \mu - RT = 10175 - 593 = 9582 \text{ CAL.}$$

RESULTS:

RELATIONSHIP OF RATE CONSTANT
($K_1 10^{-5}$) with Enzyme Concentration

M. Lysodeikticus at pH 6.2

Enzyme Concn. Temp. °C	1:100,000 (A)	1:300,000 (B)	Ratio B/A	1:1,000,000 (C)	Ratio C/A	Ratio C/B
25°	150	174	1.2/1	290	1.9/1	1.7/1
30°	170	189	1.1/1	396	2.3/1	2.1/1
35°	220	267	1.2/1	460	2.1/1	1.7/1
40°	280	360	1.3/1	570	2.0/1	1.6/1
45°	440	514	1.2/1	696	1.6/1	1.4/1
50°	590	570	1.0/1	950	1.6/1	1.7/1
AVG.	---	---	1.2/1	---	1.9/1	1.7/1

(Theoretically, the values at constant temp should be equal)

An equivalent factor was used in order to correlate the rate constants at different enzyme concentrations

EQUIVALENT FACTOR: 1:1,000,000 = 1
1:300,000 = 3.3
1:100,000 = 10

$$\frac{K}{\text{EQ. Factor}} = \frac{.0150}{10} = .00150 = 150 \times 10^{-5}$$

TABLE IX

CONCLUSION AND ANALYSIS

In the light of Sizer's paper (80) several interpretations can be made. Our average value of Q_{10} (1.75) value falls within the normal range, but there is not a consistent decrease in Q_{10} with rise in temperature which is often considered characteristic of enzyme reactions. An explanation of this can be inferred from the fact that lysozyme is more stable to heat than most enzymes (49). Our data shows that the reactions seem to follow the Arrhenius equation over a temperature range 20-55°C.

The results in this paper substantiate the theory (80) that "the activation energy of an enzyme system should be independent of different environmental changes, unless those factors alter the catalytic surface of the enzyme." The activation energy was found to be essentially uneffected by (a) changes in temperature (20 to 55°), (b) changes in enzyme concentration, (c) changes in substrate species and (d) changes in pH 5.2-7.2 (unreported experiment).

Sizer (80) also states "that in many instances the same activation energy characterizes the action of an enzyme on different substrates" and also is "presumptive evidence that the same enzyme is acting as a catalyst in the reactions." The similarity of our activation energies adds more evidence to Sizer's observation and substantiates Meyers' (50) and Smolelis" (82) statement that the enzymatic reaction between

lysozyme and the mucopolysaccharide component of the bacterial membrane is quite specific.

"The μ values are not scattered at random but grouped around certain modes, the most important of these being μ=16,000, μ=11,000 and μ=8,000" (80). The average value of 10,200 would fall in very easily with the 11,000 mode.

The data presented in this paper establishes the earlier idea (13,62,69) that bacteriolysis by lysozyme behaves as a unimolecular reaction.

The data also agrees with the physico-chemical analysis of lysozyme action which was reported by Boasson (9). The experiments did not determine if lysis takes place in 2 steps but it did confirm that if there are 2 steps, the velocity of the lysis is determined by the amount of lysozyme adsorbed. Adsorption of lysozyme onto the bacterial wall, rather than the diffusion of the cell contents into the solution, would be the rate-determining step. This is shown by (1) an increase in rate constants as the enzyme concentration decreased and (2) an increase in rate when an emulsifying agent was used.

ABSTRACT

The object of this problem was to demonstrate and discuss the kinetics of the bacteriolytic action of egg white lysozyme. This was done to elucidate further the enzymic nature and the mechanism of lysozyme action.

The activity of the enzyme was measured by a turbidi-

metric method.

Crystalline lysozyme was diluted to various concentrations with phosphate buffer (pH 6.2) and incubated at various temperatures with bacterial substrates such as Micrococcus lysodeikticus and Sarcina lutea. The decrease in optical density was followed on a Coleman spectrophotometer, using a wave length of 540.

The data indicates that the reaction follows the monomolecular equation as others have earlier cited in the literature. The average Q_{10} value was 1.75±0.14. The energy of activation (μ) was found to be 10,200±1600 cal for experiments conducted with lysozyme at three different concentrations and on both substrates. The similarity of "μ" values indicates that the lyzing action of lysozyme is characteristic of the enzyme on both substrates. It appears that the lysozyme is splitting the same type of linkage in the mucopolysaccharide component of the bacterial cell membrane. Thus this enzymatic reaction is quite specific.

It was also shown that the velocity of the lytic action by lysozyme is determined by the amount of lysozyme adsorbed, i.e.- adsorption is the rate-determining step.

BIBLIOGRAPHY

1. Abraham, E.P., and Robinson, R., Nature 140, 24(1937). Crystallization of lysozyme.

2. Abraham, E.P., Biochem. J., 33, 622-30 (1939). Properties of egg white lysozyme.

3. Alderton, G.; Ward, W.H., and Fevold, H.L., J. Biol. Chem., 157, 43-58 (1945). Isolation of lysozyme from egg white.

4. Alderton, G., and Fevold, H.L., J. Biol. Chem., 164, 1-5(1946). Direct crystallization of lysozyme from egg white.

5. Andersen, O., Z. Immunitats., 70, 90-103 (1931). Untersuchungen uber das lysozym aus dem M. lysodeikticus.

6. Anderson, E.A., and Alberty, R.A., J. Phys. & Colloid Chem., 52, 1345-64 (1948). Electrophoretic behavior of proteins.

7. Babudieri, B., and Bietti, G.B., Arch. Ophthalmal., 36, 449-454 (1945). Electron microscope observations.

8. Bergamine, L., and Ferrari, W., Boll. ist. sieroterap. milan, 27, 89-92 (1948). (Chem. Abstr., 43, 3855f (1949) .

9. Boasson, E.H., Acta Brevia Neerland. Physiol., Pharmacol., Microbiol., 6, 59-63 (1936) (in English). Lysozyme action.

10. Boasson, E.H., J. Immunol., 34, 281-93 (1938). Bacteriolysis by lysozyme.

11. Bordet, M., Compt. rend. soc de biol., 99, 1254 (1928). Essais d'extraction du lysozyme.

12. Carter, H.E., "Biochemical Preparations," John Wiley & Sons, New York, 1949, pp. 67-71. Preparation of lysozyme.

13. Dorfman, V.A., and Karakash, E.I., Byull. Eksptl. Biol. Med., 14, 112-14 (1942). Kinetika lizotsimnogo lizisa.

14. Dorfman, V.A., Nature 153, 169-70 (1944). Physicochemical nature of bacteriolysis.

15. Dorfman, V.A., and Moldavskaya, E.A., Am. Rev. Soviet Med., 2, 271 (1945). Physicochemical nature of bacteriolysis.

16. Dorfman, V.A., and Moldavskaya, E.A., Am. Rev. Soviet Med., 3, 500-10 (1946). Physicochemical nature of antibiotic action.

17. Dorfman, V.A., and Shcherbacheva, D.P., Doklady Akad. Nauk. S.S.S.R., 59, 969-72 (1948). (Chem. Abstr. 42, 6403g (1948)).

18. Epstein, L.A., and Chain, E., Brit. J. Exptl. Path., 21, 339-54 (1940). Preparation and properties of the substrate of lysozyme.

19. Feiner, R.R.; Meyer, K., and Steinberg, A., J. Bact., 52,

375-84 (1946). Bacterial lysis by lysozyme.

20. Fleming, A., Proc. Roy. Soc., London, B, 93, 306 (1922). A bacteriolytic element found in tissues and secretions.

21. Fleming, A., and Allison, V.D., Brit. J. Exptl. Path., 3, 252-60 (1922) Lysozyme.

22. Fleming, A., and Allison, V.D., Proc. Roy. Soc., London, s.B, 94, 142 (1923). Observations on lysozyme.

23. Fraenkel-Conrat, H.L., and Olcott, H.S., J. Biol. Chem., 161, 259-68 (1945). Esterification of proteins.

24. Fraenkel-Conrat, H., Arch. Biochem., 27, 109-24 (1950). Essential groups of lysozyme.

25. Fraenkel-Conrat, H.,: Mohammad, A.; Ducay, E.D., and Mecham, D.K., J. Am. Chem. Soc., 73, 625-27 (1951). Molecular weight of lysozyme.

26. Fromageot, C., Cold Spring Harbor Symposia Quant. Biol., 14, 49-54 (1949). Analysis of amino acids in lysozyme.

27. Fromageot, C., and de Garilhe, M.P., Biochim. et Biophys. Acta, 4, 509-17 (1950) (in French). La composition du lysozyme en acides amines.

28. Fromageot, C., and Schnek, G., Biochim. et Biophys. Acta, 6, 113-22 (1950) (in French). Spectre ultra-violet du lysozyme.

29. Gildemeister, E., Zentralbl. f. Bakt., 136, 408 (1936). Untersuchungen uber das lysozyme.

30. Glassman, H.N., and Molnar, D.M., Arch. Biochem. & Biophys. 32, 170 (1951). Inhibition of lysozyme by surface-active agents.

31. Goldsworthy, N.E., and Florey, H., Brit. J. Exptl. Path., 11, 192 (1930). Some properties of mucus.

32. Gratia, A., and Goreczky, L., Z. Immunitats., 93, 18-26 (1938). Das Ultrazentrifugieren der Immunkorper und der normalen. bakteriziden Stoffe.

33. Green, D.E., "Currents in Biochemical Research, Interscience Publishers, Inc., New York, 1946, pp. 277-283. A review of lysozyme (By K. Meyer).

34. Hallauer, C., Zentralbl. f. Bakt., 114, 519 (1929). Ueber das lysozym.

35. Halwer, M.; Nutting, G.C., and Brice, B.A., J. Am. Chem. Soc., 73, 2786-90 (1951). Molecular weight of lysozyme by light scattering.

36. Hartsell, S.E., Proc. Indiana Acad. Sci., 57, 44-53 (1948). A review of lysozyme.

37. Hawthorne, J.R., Biochim et Biophys. Acta, 6, 94-6 (1950) (in English). Products of lysozyme action.

38. Holyoke, E.D., and Johnson, H.F., Arch. Biochem. Biophys., 31, 41(1951). Influence of pressure and pH on rate of lysis.

39. Jones, F.T., J. Am. Chem. Soc., 68, 854-7 (1946). Properties of lysozyme chloride.

40. Kern, R.A.; Kingkade, M.J.; Kern, S.F., and Behrens, O.K., J. Bact., 61, 171-78 (1951). Characterization of lysozyme action.

41. Kertesz, D., and Teti, M., Compt. rend. Soc. biol., 144, 923-4 (1950). Inactivation reversible du lysozyme par le systeme tyrosinase.

42. Kertesz, D., and Caselli, P., Bull. soc. chim. biol., 32, 583-6(1950). Sur le spectre d'absorption du lysozyme oxyde.

43. Klotz, I.M., and Walker, F.M., Arch. Biochem. 18, 319-25(1948). Complexes of lysozyme.

44. Klotz, I.M.; Griswold, P., and Gruen, D.M., J. Am. Chem. Soc. 71, 1615-20(1949). Infrared spectra of lysozyme.

45. Kopeloff, N.; Harris, M.M. and McGinn, B., Am. J. Med. Sci., 184, 632-37(1932). Lysozyme in saliva.

46. Lawrence, C.A., and Klingel, H., Proc. Soc. Exptl. Biol. Med., 52, 129-30(1943). Effect of Azosulfonamides on lysozyme.

47. Lewis, J.C.; Snell, N.S.; Hirschmann, D.J., and Fraenkel-Conrat, H., J. Biol. Chem., 186, 23-35 (1950). Amino acid composition of egg proteins.

48. Longsworth, L.G.; Cannan, R.K., and MacInnes, D.A., J.

Am. Chem. Soc., 62, 2580 (1940). Electrophoretic study of egg white proteins.

49. Meyer, K.; Thompson, R; Palmer, J.W., and Khorazo, D., J. Biol. Chem., 113, 303-9 (1936). The purification and properties of lysozyme.

50. Meyer, K.; Palmer, J.W.; Thompson, R., and Khorazo, D., J. Biol. Chem. 113, 479-86 (1936). The mechanism of lysozyme action.

51. Meyer, K., and Hahnel, E., J. Biol. Chem., 163, 723-32 (1946). Estimation of lysozyme by a viscosimetric method.

52. Moss, J.N., and Martin, G.J., Am. J. Digest. Dis., 15, 412-14(1948). Inhibition of lysozyme.

53. Neter, E., Proc. Soc. Exptl. Biol. Med., 48, 106-9 (1941) Action of sulfamido compounds on lysozyme.

54. Paikina, S.Sh., Chem. Zentr., 1, 499 (1942). Uber de stimulierende wirkung des caseins auf lysozym.

55. Palmer, K.J.; Ballantyne, M., and Galvin, J.A., J. Am. Chem. Soc., 70, 906-8(1948). Molecular weight of lysozyme by the x-ray diffraction method.

56. Pasynskii, A.G., and Plaskeev, V., Compt. rend. acad. sci. U.R.S.S., 48, 579-81(1945) (in English). Diffusion and molecular weight of lysozyme.

57. Pasynskii, A.G., and Kastorskaya, T., Compt. rend. acad. sci. U.R.S.S., 49, 504-7(1945). L'electrophorese du

lysozyme par la methode du Tiselius.

58. Pirie, A., Brit. J. Exptl. Path., 21, 125 (1940). Effect of lysozyme on the union between a phage and bacteria.

59. Proctor, C.M.; Dickman, S.R., and Smith, E.L., Fed. Proc., 8, 239 (1949). Studies on lysozyme.

60. Roberts, E.A.H. and Wells, A.Q., Quart. J. Exptl. Physiol., 27, 89-98 (1937). Preparation and properties of lysozyme.

61. Sandow, A., Proc. Soc. Exptl. Biol. Med., 24, 172 (1926). Antibacterial activity of egg white.

62. Shabordin, D.A., Arch. sci. biol. (U.S.S.R.), 60, 83-6 (1940). (Chem. Abstr., 35, 5918 (1941)).

63. Smith, G.N., and Stocker, C., Arch. Biochem., 21, 383-94 (1949). Inhibition of lysozyme.

64. Smolelis, A.M., and Hartsell, S.E., J. Bact., 58, 731-6 (1949). Determination of lysozyme.

65. Smolens, J., and Charney, J., J. Bact., 54, 101-7 (1947). Antigenicity of lysozyme.

66. Tallan, H.H., and Stein, W.H., J. Am. Chem. Soc., 73, 2976-7 (1951). Studies on lysozyme.

67. Thompson, R., Arch. Path., 30, 1096 (1940). A review of lysozyme.

68. Webb. M., J. Gen. Microbiol., 2, 260-74 (1948). Action of lysozyme on heat-killed, gram-positive micro-

organisms.

69. Welsch, M., Compt. rend. soc. biol. 128, 795-801 (1938) Mise au point d'une technique nephelometrique pour l'etude de la mycolyse.

70. Welshimer, H.J. and Robinow, C.F., J. Bact., 57, 489-99 (1949). Lysozyme and autolytic enzymes.

71. Wolff, L.K., Ztschr. f. Immunitatsforsch. u. exper. Therap. 50, 88 (1927). (Untersuchungen uber das lysozym.

72. Alderton, G. and Fevold, H.L., Federation Proc., 5, 119 (1946). Some properties of lysozyme.

73. Erdos, J. and Hoyo, L., Enzymologia 14, 51-3 (1950) (in German). (Chem. Abstr., 45, 7189b (1951)).

74. Fiaccavento, W. and Natale, D., Arch. sci. med., 91, 378-80 (1951). (Chem Abstr.,45, 9116g (1951)).

75. Gorini, L. and Crevier, M., Biochim. et Biophys. 7, 291-4 (1951) (in French). "Le comportement de la proteinase endocellulaire de Micrococcus lysodeikticus au cours de la lyse de cet organisme par le lysozyme."

76. Lobstein, O.E. and Fogelson, S.J., Am. J. Dig. Dis., 18, 298 (1951). The effect of chloride concentration in the determination of lysozyme activity.

77. Lobstein, O.E.; Jopes, H.S. and Fogelson, S.J., Quart. Bull. Northwestern Univ. Med. Sch., 26, 19-21 (1952). The effect of time and temperature on the susceptibili-

ty of ly ophilized Micrococcus lysodeikticus to lysozyme.

78. Nihoul, E.; Massart, L. and van Heel, G., Arch. Intern. pharmacodyamie 88, 123-5 (1951). (Chem. Abstr., 46, 1613 e (1952)).

79. Pringle, B.H.; DePaulis, D.C. and Pemrick, T.D., Am. J. Clin. Path., 21, 1039-44 (1951). Estimation of lysozyme in certain biologic materials.

80. Sizer, I.W., Advances in Enzymol., 3, 35-62 (1943). Effect of temperature on enzyme kinetics.

81. Smolelis, A.N. and Hartsell, S.E., Bact. Proc., 1950, 106 (1950). The influence of salts on lysozyme activity.

82. Smolelis, A.N. and Hartsell, S.E., Proc. Soc. Exp. Biol. Med., 76, 455-6 (1951). Occurrence of lysozyme in bird egg albumens.

83. Van Slyke, D.D., Advances in Enzymol., 2, 33 (1942). The kinetics of hydrolytic enzymes.

84. Wetter, L.R. and Deutsch, H.F., J. Biol. Chem., 192, 237 (1951). Immunochemical and physical studies on lysozyme.