

The Newer Knowledge of Lysozyme and Bacteria*

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In recent years considerable interest has been aroused in the general subject of microbic associations. The literature has been searched by many investigators to see if the organism of their particular interest has been involved in any special events such as synergism, commensalism, symbiosis, metabiosis, or antibiosis. As the search progressed, the investigator found that the latter phenomenon—antibiosis—was one of the first microbic associations to be studied. And in the literature on the subject, the antibiotic substance, lysozyme, has a distinctive position. Before Fleming recorded his observations about *Penicillium notatum* and staphylococci, he had studied the antibiotic activity of egg white extensively and described (1) the effects on bacteria of the substance he called “lysozyme”. Thompson (2, 3) has reviewed the early literature on lysozyme so the present account concerns the more recent information on the subject.

What is Lysozyme?

Lysozyme is a basic polypeptid of low molecular weight that acts as an enzyme and hydrolyses certain mucopolysaccharides. When this hydrolysis occurs within or on bacteria in the presence of this substance, lysis results,—hence the name “lysozyme”.

The Occurrence of Lysozyme

Lysozyme is apparently widely distributed in nature. In the tissues and secretions of man, it has been found in tears, nasal mucus, saliva, inflammatory exudate, cartilage, spleen, kidney, liver, lungs and lymph glands. Cartilage is particularly high in its lysozyme content. Lysozyme apparently does not occur in normal urine, cerebro-spinal fluid, sweat, aqueous humor and lens tissue. The tissues of rabbits and guinea pigs have less lysozyme than man; dog tissue is mid-way between these two animals in lysozyme content. The occurrence of lysozyme in the tissues of man is apparently influenced at a given time by the status of his health. Colds appear to reduce the amount of lysozyme in the nasal secretions. Stimulation of tear glands by pilocarpine and hypersecretion of the glands reduces the lysozyme titer. The influence of an avitaminosis on the lysozyme content of the tissue is debatable; at least very little reliable information is at hand to clarify this point.

Lysozyme has recently been isolated by Meyer, Hahnel and Steinberg (4) from *Fiscus latex*. *Fiscus* lysozyme is somewhat different chemically and immunologically from egg-white lysozyme and has a much lower potency as determined by lytic activity on bacteria. Cabbage,

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turnip, cauliflower, *Euphorbia*, and even bacteria, themselves, are sources of lysozyme. Castaneda (5) observed lysozyme from donkey milk and noted its activity as being much less than that from egg-white. The wide occurrence of lysozyme, or lysozymes, in nature is apparent.

The principal source of lysozyme is egg-white from which it has been recently isolated in a very highly purified form by Alderton, Ward and Fevold (6). Alderton and Fevold (7) reported the preparation of the chloride, bromide, iodide, nitrate and carbonate of lysozyme in crystalline form. Good yields of the enzyme were obtained by direct crystallizations from homogenized, acidified egg-white to which 5% salt had been added. One of their co-workers, Jones (8) has reported the optical and crystallographic properties of lysozyme chloride in great detail. Crystals of this substance are colorless, tetragonal, tubular by-pyramids of the first order, usually showing a short pyramid of the second order. The shape of the crystal is a rhombic dodecahedron, giving uniaxial (+) inference figures and the face angle is 144° .

The chemical and physical properties of lysozyme

It is appropriate that we review the chemical and physical properties of this substance before considering the mechanism of lysis wherein bacteria are involved.

As stated previously, lysozyme is a basic protein or polypeptid of low molecular weight—reported as 13,000 by Pasynskii and Plaskew (9), 17,500 by Alderton and Fevold (6) and as about 25,000 by Meyer, Thompson, Palmer and Kharazo (10). The methods for determining the molecular weight appears to account for these differences. The molecular size of lysozyme is less than 30 millimicrons as determined by the osmotic pressure and ultracentrifuge methods. Lysozyme is soluble in acidified aqueous mediums and insoluble in alkaline mediums. Pure preparations are less stable but stability increases down to pH 4. The isoelectric point of lysozyme is given as pH 10.5 to 11.00 by Alderton, Ward and Fevold (6), however, Pasynskii and Kastarskaya (11) report the isoelectric point as pH 6. The wide difference is explained by the latter authors as due to the buffers used.

Lysozyme is filterable in acid solutions, is inactivated rapidly by heat in neutral or alkaline solutions, however, partially purified solutions retain their original activity after being kept at 100°C . for thirty minutes at pH 4, (Linz, 12). The lytic activity of lysozyme in rehydrated whole eggs is evident when diluted in phosphate buffer at a pH of 6.2 and heated for 90 minutes at 85°C . (Hartsell, 13). Lysozyme is very active at temperatures of 50°C ., however, satisfactory measurements of the progress of lysis can be made at 37.5°C .

Lysozyme is insoluble in pure organic solvents but is soluble in dilute alcohol and water. According to Linz (12) it is precipitated but not inactivated by chloroform, ether, acetone, alcohol and toluene. It is inactivated by oxidizing agents and the salts of the heavy metals. Since the inactivation can be reversed by H_2S or HCN , Meyer, Thompson, Palmer and Kharazo (10) determined that one active group in the lysozyme molecule is the sulfhydryl radical. Ermoleva (14) determined

the chemical composition of lysozyme as 0.64% sulphur, 0.25% phosphorus, 6.4% hydrogen, 48.65% oxygen and 15.0% nitrogen. The amino acids—tryptophan, tyrosine, cystine, and arginine have been detected in purified preparations of lysozyme. Fraenkel-Conrat and Olcott (15) have esterified lysozyme by alcohols of low molecular weight.

Several factors are known to influence lysozyme activity in the presence of susceptible bacterial cells. Bile and high concentrations of sodium chloride slow down its action on *sarcina*, whereas gastric juice and low concentrations of sulfonamides (1%) do not inactivate it, according to Cabezas and Rubina (16). Feiner, Meyer and Steinberg (17) observed that ferrous sulfate apparently inhibited the sensitivity to lysozyme, and sodium pyrophosphate counteracted this inhibition; sodium arsenate consistently increased the titer four-fold. Sulfathiazole-diazo-symmetrical-carbamide-2R acid completely suppressed lytic action of lysozyme on *M. lysodeikticus*, (Lawrence and Klingel (18)). Either ferrous or ferric iron will overcome egg white inhibition of this organism (Schade and Caroline, (19)). Casein stimulates the action of lysozyme according to Paikina (20), who reasoned that some component other than glycine was responsible for the effect noted, since this amino acid is not present in casein.

The properties of lysozyme as an enzyme have been observed. Meyer, Palmer, Thompson and Kharazo (10) found that lysozyme did not have protease, kinase, amylase, lipase or phosphatase activity. Schultze (21) observed that lysozyme from fresh egg white was free of proteinase activity. However, Meyer and Hahnel (22) regard it as similar to another mucolytic enzyme, hyaluronidase, because, in both systems the chloride ion greatly increases the activity of the enzyme, both affect the substrate by depolymerase activity and both can be reversibly oxidized.

Lysozyme is antigenic and will give specific precipitin reactions. However, lysozyme from human saliva, from cat saliva and from *Fiscus globatra* do not give cross-reactions though they possess the common property of causing lysis in sensitive bacteria. Smolens and Charney (23) confirmed the antigenicity of crystalline lysozyme and observed that antilysozyme rabbit serum inhibits the activity of lysozyme on *M. lysodeikticus*.

The Action of Lysozyme

The lysis of bacterial cells would appear to be a process that could be followed in an orderly manner and that reproducible results could be obtained. However, the difficulties of studying lytic phenomena with bacteria as the test organism are well known. In the early studies, qualitative expression of lysis as it progressed, or as it reached the end point (complete clearing of a given suspension), was sufficient for most experimental purposes. Recently, greater precision was desired in measuring the process. Meyer and Hahnel (22) determined lysozyme viscosometrically with greater accuracy than by the use of bacteriological methods. They found that bacteria vary greatly in their sensitivity to lysozyme according to the media upon which they are grown,

the strain and the method of killing the cells before testing for lysis. Quoting these authors—"The explanation for this variation apparently lies in changes in the autolytic enzymes contained within the bacterial cell, which contribute to a varying degree to the visible clearing of bacterial suspensions." Thus we see that bacteriolysis by lysozyme is a complex phenomenon.

The method of Boasson (24) has been used extensively. In this procedure the progress of lysis is followed with the aid of a Moll-extinctionmeter. As a heavy suspension of the organism is lysed corresponding changes in the reading on the galvanometer are registered. Thompson (25) measured the lysis of sensitive cells photoelectrically. The instrument he used was not indicated in the reference. Fleming (1) measured its action by noting the clearing of suspensions and the destruction of organisms in plate cultures. Thompson (25) also used the latter procedure.

Klemparskaya (26) noted that bacteria may be grouped according to whether they are lysed or whether they are flocculated. He found that the flocculation phenomenon was conditioned by the pH and the concentration of the lysozyme. He believed that the difference in behavior of various organisms was due to their different colloidal structures. Lysozyme has been observed to inhibit the growth of bacterial cells without causing lysis, however no units for measuring this inhibition have been proposed.

Living bacterial cells of susceptible species are more sensitive to lysozyme than dead cells. However, Boasson (24) believes that dead cells are preferable in experiments with lysozyme. These cells killed by acetone, alcohol or heat are somewhat less sensitive than those killed by phenol or cold, neutral acetone. The essential features of the substrate,—that is, the bacterial cell—is that it must contain an acetylated aminopolysaccharide if lysozyme is to cause lysis.

From the work of many investigators it is possible to recognize three phases of the mechanism of lysis of bacteria: 1. adsorption of lysozyme onto the cell surface containing the specific substrate upon which the enzyme acts, 2. depolymerization of the substrate and 3. visible alteration of the cell—so-called "lysis".

The entire process has been extensively studied by Dorfman and his co-workers (27, 28, 29, 30). By using Abramson's modification of the micro-electrophoretic chamber of Northrop they observed that in the adsorption phase there occurred a rise in the electrokinetic potential at the cell surface in the case of sensitive cells (*M. lysodeikticus*). The effect occurs immediately and persists for a short time, after which the potential returns to normal. The reverse of the process is noted in strains, such as *B. subtilis*, that are insensitive to lysozyme. Substances obtained from lysed bacterial cells or impurities in the lysozyme did not influence the kinetics of the reaction. It is interesting to note that Dorfman and his co-workers suggested that electrokinetic potentials are an efficient way of measuring the effects of other antibiotics such as penicillin, bacteriophage and tyrothricin. These substances apparently affect the cell in such a way that electrically charged particles are

dissociated. From the work of other investigators adsorption of lysozyme is inhibited by increasing concentrations of salt. Shabordin (31) observed that bacteriolysis of dead and live cells by lysozyme behaves as an unimolecular reaction. The reaction can be reversed by extraction in acid alcohol.

In the second phase, lysozyme acts specifically on the acetylated aminopolysaccharide in sensitive cells changing it to N-acetylaminohexose and ketose. The aminohexose is present in an insoluble form. Lysozyme is capable of attacking organisms or substrate when either is combined with antibody. Several environmental factors, such as temperature, pH, etc. are known to influence this phase of the reaction. As depolymerization proceeds, reducing sugars appear in solution, oxygen uptake ceases, non-protein nitrogen and inorganic phosphorus appear in solution and the gram reaction of *M. lysodeikticus* becomes gram negative.

The change in the gram reaction is an interesting observation. A brief summary of the bacteria which are sensitive to lysozyme reveals that the gram negatives are usually not affected, whereas the gram positives, especially saprophytic cocci, are lysed. In our studies on the microflora of rehydrated, spray-dried whole egg powder we found sensitive and insensitive strains of micrococci (gram positive) and flavobacteria (gram negative). Chemical studies on the identification of the substrate in these cells have not been made. These findings raise a question about the validity of lysis as an indicator of the presence of the substrate in the bacterial cell.

The third phase of bacteriolysis by lysozyme is closely related to the second phase, since the end point of the process—visible lysis—is accomplished after a succession of effects upon the cell has occurred. As depolymerization progresses there occurs a swelling of the cell as observed under the microscope and an increased permeability of the cell membrane (Babudiere and Bietti, 32). These effects result in the liberation of the contents of the cell. Boasson (24) and Babudiere and Bietti (32) found that the cell membrane was intact after lysis and that the swelling of the membrane is not due to a mechanical artifact. As has been previously indicated autolytic enzymes are believed to aid in the process of destruction. In the residue of what was once intact cells, particles of various sizes appear. Dorfmen (28) measured these particles and considered them proportional to the rate of lysis. Particles from lysed, heat-treated cells had greater size than those from living bacteria or phenolized cells. As lysozyme acts upon the cell it is not used up, however, inhibition of lysozyme by the products of lysis has been noted (Thompson, 2).

Fleming (1) observed that the rate of lysis is directly related to temperature; the rate increasing to a maximum at about 60°C. Anderson (33) and Thompson (2) found that maximum lysis occurred at 55°C. In recent work with lysozyme from rehydrated, spray-dried, whole eggs the author (13) conducted the experiments at 52°C, observing that lysis of sensitive cells was very rapid at that temperature. Other effects of temperature on lysozyme were noted by incubating the cell-lysozyme

suspensions at 85°C for varying periods of time. The rapidity of lytic activity was directly proportional to the time of exposure. As recorded previously, lysozyme from this source was still active on *M. lysodeikticus* after 90 minutes of exposure at 85°C.

The hydrogen ion concentration of the suspending medium in which lysozyme is active influences the process of cytolysis. Fleming (1) found that maximum lysis occurred at a pH of 7.0. However, Boasson (24) observed lysozyme to be most active at pH 6.2. Epstein and Chain (34) exposed lysozyme-cell mixtures at a pH of 4.5 in phosphate buffer and then added a small amount of normal NaOH to check the fact that the cells of *M. lysodeikticus* had been affected. There appears to be little agreement among investigators regarding the correct pH or the optimal suspending medium in which to determine the effect of lysozyme on a culture. It is probable that the cell protein of each strain of micro-organism would have a pH at which its solubility was greatest, which may or may not be the pH at which lysozyme acts best. Meyer, Thompson, Palmer and Kharazo (10) and Epstein and Chains (34) have observed this correlation.

Another factor that influences the lysis of bacterial cells by lysozyme is the medium upon which the culture is grown. Kopeloff, Harris and McGinn (35) found the susceptibility of micrococci to increase if the culture was grown on beef extract agar instead of digest agar. Meyer and Hahnel (22) also found that the sensitivity of *M. lysodeikticus* varied with the medium in which the culture was grown. Since strains of the coccus varied in their sensitivity to lysis, they preferred the viscometric method to the bacteriological method for measuring lytic activity.

Still another factor influences lysozyme activity—that of concentration. Obviously, the rate of lysis would be dependent upon the amount of lysozyme acting upon sensitive cells, however, the sequence of events when an excess of the substance is used under optimal conditions has not been recorded. Thompson and Kharazo (36) found that pathogenic bacteria will be attacked if the concentration is great enough.

Lysozyme and Viruses

Having reviewed what lysozyme is, and how it acts, I would like to direct your attention to the viruses and bacteria, other than *M. lysodeikticus*, upon which lysozyme has been tested.

The only report of the action of lysozyme (undiluted egg white) on viruses to come to the attention of the author is that of Scheidegger (37). No inactivation of vaccine virus, fixed rabies virus, herpes virus, the eastern strain of equine encephalomyelitis virus, influenza virus (human strain W. S. and swine strain Shope) or the virus of fowl plague could be detected.

Lysozyme and Bacteria

Lysozyme is most active on certain saprophytic cocci. *M. lysodeikticus* is the most sensitive of the bacteria. Thompson (3) recorded a summary of the *pathogenic* bacteria previously used in studies on lysozyme and then stated as had Fleming (1), that many strains of *saprophytic* bac-

teria, yeasts and two molds had been found to be sensitive to lysozyme. No complete record of the saprophytic genera used was given. In the author's report (13), referred to previously, the insensitivity of bacilli, *Achromobacter*, *Aerobacter*, *Bacterium* and certain sarcina to lysis of lysozyme is noted. The variable sensitivity of strains of micrococci and *Flavobacterium* are also recorded. *Escherichia coli* like other gram negative bacteria, is not sensitive to lysozyme under the usual conditions for testing bacteriolysis.

However, gram negative bacteria that are frequently pathogenic may be killed by lysozyme. *Vibrio cholerae* has been killed by 25% egg-white; *Eberthella typhosa* by 50% egg-white. *Klebsiella*, brucella, streptococci and most strains of staphylococci are sensitive to lysozyme. Neisseria and mycobacteria are reported as sensitive to partially purified lysozyme. No anaerobes have yet been subjected to lysozyme under experimental conditions; so wrote Thompson in 1940. This statement still holds true. The several factors that condition the process of lysis must be recalled when interpreting the sensitivity of strains, as recorded in the literature and when projecting work for the future. Thompson (2) wrote—"Provisionally it seems justifiable to include lytic actions of lysozyme-containing materials on various saphrophytes as evidence of lysozyme action". But bacteria are subject to variation in their reaction to lysozyme so a negative test for lysis requires further explanation. Either of three possibilities are apparent: (1) the substrate is not within the cell therefore lysozyme could not act upon it, or (2) the substrate was there but no lysis was possible because conditions for the solubility of the cell substances were not optimal, or (3) the substrate was there, the conditions for the solubility of cells substance were optimal but the permeability of the cell wall could not be altered to allow the cell substance to pass from it. In the latter situation inhibition of growth would result, but no lysis would be observed. Only further experimentation will prove the validity of this reasoning. Furthermore, the natural tendency of microorganisms to vary confuses the explanations of bacteriolysis by lysozyme.

Resistance to lysozyme can be cultivated in bacteria. Fleming (1) and many other investigators have observed this phenomenon. Lysozyme resistant cells are not easily phagocyted, which suggests that the lysozyme, —or should we say lysozymes—in the host may serve to cause a pathogenic strain to change if it would continue to invade the host. A positive correlation exists between lysozyme resistance and certain common indicators of virulence in staphylococci, i.e. pigmentation and coagulase production. However, too little information is available to appraise the significance of microbic variation in the process of pathogenesis wherein lysozyme is active.

Reference should now be made to substances closely related to lysozyme—the so-called "avidin-biotin" complex. Lawrence (38) believed that lysozyme was biotin-saturated avidin. The work of Gyorgy and Rose (39); Eakin, Snell and Williams (40); Wooley and Longworth (41) and Alderton, Lewis and Fevold (42) clearly separates avidin, biotin and lysozyme.

The Biological Significance of Lysozyme

Considering the investigations on the occurrence of lysozyme, the mechanism of its action and the effect it has upon the bacterial cell, we should expect certain broad generalizations regarding its biological significance..

The effect of lysozyme on the bacterial cell, as has been indicated by Dubos (43), offers another method whereby the chemical composition of the cell can be discerned. Since lysozyme acts on a specific substrate, lysis of the cell by lysozyme can be construed as evidence of the acetlyated aminopolysaccharide in that cell. Insensitive cells would not possess this substrate if the reasoning, and subsequent experimentation, are correct. The prospect of grouping bacteria into two categories—the *lysozyme-sensitive* (show visible lysis) and the *lysozyme-insensitive* (show no visible lysis)—would appear to have taxonomic significance. However, there is need for caution in using such a grouping until more detailed information about purified lysozyme acting on a wide variety of genera are available. Bacilli, and many gram negative bacteria, are insensitive to lysozyme whereas many cocci, including the pathogenic types, are sensitive.

It remains to be seen whether lysozyme could have more than a limiting effect upon bacteria *in vivo*—perhaps causing a slight variation of the pathogen, making it less susceptible to lysis. Thompson (2), first, considered this possibility when he wrote—"A first step in the evolution from susceptible saprophyte to parasite would certainly require the development of a mechanism of resistance to this enzyme, whether or not this were the only requirement for such a step". Recent investigations do not detract from, or add to, this view.

The significance of lysozyme in the tissues of man cannot be properly appraised. Meyer and Hahnel (22) do not consider lysozyme to have a metabolic function in the host since the wide occurrence of the substrate of lysozyme in extracts of organs has not been shown. Since lysozyme occurs in some tissues in great abundance, and to a lesser extent in other tissues, the prospect of increasing the lysozyme content of all tissues artificially offers an interesting field of investigation. The possible additive effects of lysozyme and other tissue components to prevent the invasion of pathogenic microorganisms offers a fertile field of investigation. In disease, an increase in the lysozyme in the area of the affected tissue appears to have some beneficial effect. Ponomareva (44) found that lysozyme in contact with suppurative surfaces hastened the healing process and promoted granulation without modifying the tissue. A second effect noted in the application of lysozyme was the destruction of many pyogenic bacteria, however, he observed (45) that, if lysozyme was used over a long period of time, lysozyme-fast or resistant strains developed.

Finally, the function of lysozyme in the resistance mechanism of the shell egg has received little attention from investigators. This is an unusual observation since egg-white has served as a ready source of lysozyme for so many years.

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