BACTERIAL LYSIS BY LYSOZYME[1]

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The earlier literature on lysozyme sensitivity of bacteria has been extensively reviewed by Thompson (1940). This paper includes a more complete description of the widely used test organism, *Micrococcus lysodeikticus* (Fleming), and further studies on factors influencing bacterial lysis by lysozyme with particular reference to the immunological behavior of the lysozyme substrate.

DESCRIPTION OF THE ORGANISM

Observations were made on cultures obtained from three different sources: one (C) maintained for many years in this laboratory, another (4698) received from the American Type Culture Collection, and a third (F) received recently from Dr. Fleming. To our knowledge all derive from the original organism isolated by Fleming (1922). For purposes of convenience each will be designated as a separate "strain." All three, with a few minor variations, exhibited the same cultural characteristics and could not be distinguished on the basis of the usual laboratory tests. Immunologically, however, there was a significant difference between the C and 4698 strains on the one hand, and the F on the other.

The organism, a gram-positive micrococcus, grows well on ordinary media within a range of pH 7.0 to 7.6 and at an optimum temperature of 37 C. The colonies in undifferentiated cultures are characteristically yellow, convex, opaque, smooth, and glistening. The F strain when received produced a definitely lighter pigment than the other two, and this difference is still apparent after repeated transfer. In broth all cultures usually remain clear, with a sediment which becomes more and more viscid on aging. Better growth is obtained in shallow layers or with aeration. In agar shakes growth occurs on or very near the surface. Reactions in the usual biochemical tests are generally negative. Gelatin is not liquefied. Nitrates are not reduced. Litmus milk becomes slightly alkaline with no other changes. Glucose, lactose, maltose, saccharose, mannitol, and salicin are not fermented. Indole and H_2S are not produced. Starch is not hydrolyzed.

The 4698 strain was used in experiments to develop a synthetic medium which would support the growth of *M. lysodeikticus*. A basal medium containing vitamin-free acid hydrolyzate of casein (General Biochemicals), 0.5 per cent; cystine, 0.01 per cent; tryptophane, 0.01 per cent; salts A and B (according to Snell and Wright, 1941); and adenine, 5 ppm, resulted in visible but poor growth. None of the following substances when added to the basal medium proved to have a stimulatory effect: thiamine, niacin, pyridoxine, pantothenic acid, riboflavin,

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para-aminobenzoic acid, biotin,[2] choline, uracil, pimelic acid, or strepogenin concentrate.[3] The addition of *Lactobacillus casei* factor,[4] 2.5 ppm, however, definitely enhanced growth, and the further addition of 0.5 per cent glucose before autoclaving gave somewhat better results. A typical experiment is shown in table 1. At least 11 serial transfers were possible in this medium. In preparation, the pH of the basal medium was adjusted to 7.4 to 7.6, the accessory substances added, and 5.0-ml amounts were dispensed in 25-ml Erlenmeyer flasks and autoclaved for 15 minutes at 15 pounds. The pH after sterilization was 7.2 to 7.4. The inoculum was 1 drop (0.05 ml by pipette) of a faintly turbid saline suspension of a thrice-washed 18- to 24-hour broth-grown culture. The flasks were incubated for 4 days at 37 C, and the amount of growth was determined turbidimetrically with a spectrophotometer. At the end of the incubation

TABLE 1

Growth of Micrococcus lysodeikticus (4698) in a synthetic medium

	AVERAGE OF DUPLICATE READINGS AFTER 4 DAYS' INCUBATION AT 37 C
Basal	76
Basal plus glucose	76
Basal plus *L. casei* factor	57
Basal plus *L. casei* factor plus glucose	47
Blank	100

The percentage transmission was read in a Coleman universal spectrophotometer using the uninoculated medium as a standard transparency of 100. A wave length of 580 millimicrons was used.

period the pH of the medium was virtually unchanged in the presence of the growing organisms. Since the medium was slightly turbid to begin with, 0.5 ml of 0.1 N HCl was added to each flask to clear the medium before readings were made.

This medium also supported the growth of the C and F strains, but it is not known whether the requirements of all three strains are identical. The maximum growth obtained after 4 days' incubation in the medium described above was distinctly poorer than a 48-hour growth in ordinary broth. Whether other accessory substances would further stimulate growth in the presence of the *L. casei* factor remains to be determined. It should be noted that the *L. casei* factor used in these experiments was a "fermentation product material." In two experiments in which a synthetic *L. casei* factor, "folvite," was used, no

[2] Biotin was obtained from Merck and Company through the courtesy of Dr. D. F. Robertson.

[3] Strepogenin concentrate was obtained from Dr. D. W. Woolley of the Rockefeller Institute.

[4] The *L. casei* factor used, a "fermentation product material," and also a synthetic preparation, "folvite," were obtained from Lederle Laboratories through the courtesy of Dr. E. L. R. Stokstad.

stimulation was observed. An optimum synthetic medium for *M. lysodeikticus* is still to be defined.

Variation in colonial morphology has been observed. On buffered neopeptone (Difco) beef infusion agar two types were usually seen: one smooth and opaque, of butyrous consistency and easily suspended; the other much more viscid and more translucent, often adherent to the medium. In some cultures the major difference was in the degree of viscidity and the ease of emulsification of the colonies, rather than in the degree of opacity. The organisms from translucent viscid colonies stained very irregularly with many large, pale, ill-defined gram-negative forms present, in contrast to the regular, evenly stained masses of cocci usually seen. This type of dissociation was more clearly manifest in the C and 4698 strains. Similar colonies were observed with the F strain but were less stable.

TABLE 2

Agglutination tests with Micrococcus lysodeikticus

ANTISERUM	STRAIN	SERUM DILUTIONS						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-C	C	++++	++++	+++	+++	+	+	−
	4698	++++	++++	+++	+++	+	+	−
	F	++	−	−	−	−	−	−
Anti-4698	C	+++	+++	+++	+++	+	−	−
	4698	++++	++++	++++	+++	+	−	−
	F	−	−	−	−	−	−	−
Anti-F	C	++	++	++	++	+	−	−
	4698	++	++	++	++	+	−	−
	F	++++	++++	++++	+++	+	+	−

Stable chromogenic mutants from the C and 4698 strains were not infrequently encountered. We have isolated white, pink, and apricot-colored strains. Although extensive studies on the dissociative behavior of these mutants have not been made, it is probable that pigment production varies independently of other characteristics of colonial morphology, as has been described for other organisms (Dubos, 1945).

Although the three strains of *M. lysodeikticus* studied could not be differentiated on the basis of the observations made above, they could be distinguished on the basis of serological tests. Rabbits were immunized with dissociated cultures grown on neopeptone infusion agar to maintain maximum differentiation between smooth and viscid variants. The sera were obtained after 14 intravenous injections of once-washed saline suspensions of living organisms. Agglutination tests were performed in the usual way with organisms grown on either neopeptone or Difco tryptose heart infusion agar. The use of the latter was preferable since on the buffered neopeptone agar the organisms were very difficult to wash off and showed a tendency to autolyze in suspension. This was particu-

larly true of the very viscid variants. Within any one strain no immunological differences could be detected between the smooth and viscid variants by agglutination tests. The C and 4698 strains were always immunologically indistinguishable, but antisera to these strains failed to cross-agglutinate with the F strain beyond a titer of 1:10 or 1:20. Anti-F sera, on the other hand, cross-agglutinated with strains C and 4698 at much higher serum dilutions (table 2). The results of agglutinin absorption tests further emphasized the difference between the C and 4698 strains, and the F strain. It was found that the F strain removed agglutinins for all strains not only from its homologous serum but to a marked degree from anti-C and anti-4698 sera as well, whereas the C and 4698 strains were not capable of removing F agglutinins from F serum. These findings indicate that the American strains, although derived from the same original source, are now distinct from the Fleming strain. Although antisera were not prepared against the chromogenic mutants, suspensions of the latter reacted serologically in the same way as the yellow parent cultures from which they were derived.

SENSITIVITY TO LYSOZYME

Sensitivity to lysozyme was determined by a method previously briefly described (Meyer, 1944). An acid pH is optimum for lysozyme activity; visible lysis of susceptible organisms occurs at an alkaline pH. The organisms from 24- to 48-hour agar slants were suspended in M/15 KH_2PO_4 (pH 4.5) to match the density of a no. 10 $BaSO_4$ standard. To 0.5-ml amounts of serial 2-fold dilutions of pure egg-white lysozyme, in the same buffer, 0.5 ml of suspension were added. The tubes were incubated for 1 hour at 37 C, and, after the addition of 2 drops of N NaOH, were read for clearing of the suspensions. Lysozyme sensitivity was expressed as the highest dilution at which complete lysis of the organism occurred. Within the limits of error of the method, no difference in sensitivity could be detected among the three strains of *M. lysodeikticus* and their variants in repeated tests. As a rule, cultures when tested under the same conditions showed complete lysis at lysozyme dilutions of 1:320,000 to 1:640,000.

Although this method is convenient for the determination of relative sensitivity to lysozyme among different organisms, it is inadequately sensitive for titration and standardization of lysozyme activity. In some instances, lysozyme titers have remained constant over long periods of time in repeated tests with living cultures; in others, unexplainable gross variations in sensitivity occurred from time to time. The usual dilution end point of lysozyme for *M. lysodeikticus* has been between 1:320,000 and 1:640,000, but occasionally the titer has dropped to 1:80,000 or has risen as high as 1:1,280,000 with the same preparation of lysozyme, even when the organism was maintained on the same medium under the same conditions. It may be mentioned here that modifications in the composition of the 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.

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 per cent phenol, dialyzation against 0.1 N HCl, or exposure to ultraviolet radiation. Precipitation with either 95 per cent 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 the enzyme, although it was impossible to obtain complete clearing even with the strongest concentrations used.

In the actual performance of the test numerous attempts were made to enhance the sensitivity by the addition of various reagents. The majority of substances tested had no effect. Ferrous sulfate apparently inhibited the reaction, although the test was difficult to read because of the formation of precipitated ferric hydroxide. Sodium pyrophosphate counteracted this inhibition. Tyrothricin, tryptophane, and the supernatant washings of *M. lysodeikticus* agar slant cultures occasionally seemed to increase the titer, but the results were extremely variable. Of all substances tested, only sodium arsenite was found to have a distinct and consistent effect. With a final concentration of M/200 sodium arsenite, the titer was 4 times higher than that of controls without arsenite.

Isolation from *M. lysodeikticus* of the high polymer form of the lysozyme substrate has recently been accomplished. This mucopolysaccharide substrate is rapidly depolymerized by lysozyme, as indicated by viscosity tests. The method of isolation and characterization of the substrate and the technique of the test are described in detail elsewhere (Meyer and Hahnel, 1946). Optimum conditions for the antibacterial action of lysozyme parallel in general the conditions for its depolymerizing action. The latter test, however, is specific, accurate, and rapid, and is not encumbered by the uncontrollable biological variations which seem to be inherent in tests with the intact organism. A comparison between the bacteriological and chemical tests, not only with regard to egg-white lysozyme but to lysozymes of plant origin as well, is likewise described elsewhere (Meyer, Hahnel, and Steinberg, 1946). For the accurate determination and standardization of lysozyme activity the bacteriological test has been supplanted by a chemical test which is constant and reproducible.

An interesting observation was made with regard to the behavior of an adapted strain of *M. lysodeikticus* which became resistant to egg-white lysozyme. This strain required 16 times as much egg-white lysozyme for complete lysis as the parent C strain from which it was derived and became completely refractory to ficus lysozyme (Meyer, Hahnel, and Steinberg, 1946). Only one-tenth the amount of carbohydrate substrate yielded by the parent strain could be extracted from the adapted strain. Yet the nature of this substrate was apparently unchanged since it was hydrolyzed to the same degree by both egg-white and ficus lysozyme. The complete resistance to ficus lysozyme of the intact adapted organisms could not be explained by the ability of the latter to destroy this enzyme. Supernatants from ficus lysozyme incubated with the adapted strain

revealed no apparent destruction of the enzyme when tested against the susceptible parent strain. Adapted organisms killed by treatment with phenolized saline were also completely refractory. The most probable explanation for the complete resistance of these organisms to ficus lysozyme would seem to be that the polarity of the substrate complex as it exists on the bacterial surface is changed so that it can still combine with the strongly electro-positive egg-white lysozyme but not with the more electro-negative ficus lysozyme.

IMMUNOLOGICAL BEHAVIOR OF THE SUBSTRATE

The lysozyme substrate used in these experiments was obtained from the C strain of *M. lysodeikticus* by fractionated extraction with 0.5 N NaOH and fractionation with alcohol after the removal of proteins and other impurities. This substance is a high polymer mucopolysaccharide fraction which in its present crude state of isolation contains about 5 per cent nitrogen and about 30 per cent hexosamine. It is rapidly depolymerized by lysozyme and hydrolyzed, with the liberation of reducing sugar, half of which can be accounted for as acetylglucosamine. For immunization and precipitin tests the mucopolysaccharide was dissolved in saline. It proved to be antigenic, although weakly so, when tested in a single rabbit. After subcutaneous, intraperitoneal, and intravenous injections of a total of 56.0 mg, the serum obtained gave a very weak precipitin reaction with the substrate (1.0 ml precipitated with 5 to 10 μg of substrate after standing for several days in the icebox) and a very low agglutinin titer against the homologous organism. By contrast, 3 of 4 rabbits[5] immunized with living organisms produced much stronger precipitating antibodies against the substrate, in addition to good agglutinin titers, after 14 intravenous injections; and after a further series of 16 injections, 0.5 ml of all these sera precipitated almost immediately with 10 to 100 μg of substrate.

It was found that a saline solution of substrate in a concentration of 1.0 mg per ml still gave a precipitin reaction after standing in the icebox for 3 weeks. Apparently any changes in viscosity that may have occurred did not affect the combining properties of the substrate. This was borne out by the following experiment in which depolymerized substrate was used. The substrate was prepared for use as in a viscosity test (Meyer and Hahnel, 1946), and, following the addition of lysozyme, samples were withdrawn every 10 minutes over a period of 1 hour. Each sample was diluted in saline to contain 50 μg of substrate per 0.5 ml and added to 0.5 ml of a 1:10 dilution of a strong antiserum. The relative viscosity was 3.6 initially and fell to 1.6 in an hour. All the samples tested as depolymerization progressed showed immediate turbidity and the same amount of precipitate as far as could be judged visually by this kind of a test. The degree of hydrolysis as determined by an increase in reducing sugar was negligible under the conditions of this experiment.

Attempts were made to determine the relationship of the substrate-precipitating antibodies to the agglutinating antibodies. Precipitin tests were set up

[5] Two of these were anti-C and 2 were anti-4698; no differences in their behavior against C substrate were noted.

in which varying amounts of substrate antigen were added to constant amounts of antiserum. With the antibacterial sera used in these experiments, turbidity, followed by precipitation, was immediately apparent. The tubes were placed in the icebox for 48 hours and the amount of precipitate formed noted by visual inspection. After centrifugation, the supernatants were withdrawn from each tube and divided into two portions, one of which was tested for excess antibody by the addition of substrate antigen, the other for excess antigen by the addition of antibody. The results of a typical experiment, as given in the left half of

TABLE 3

Addition of increasing amounts of substrate to 1.0 ml anti-M. lysodeikticus serum no. 367 diluted 1:10

TUBE*	SUBSTRATE ADDED	REACTION†	TESTS ON SUPERNATANTS		EFFECT OF ADDITION OF SUBSTRATE‖ TO SUPERNATANTS FROM TREATED PRECIPITATES			
					Lysozyme-treated		Controls	
	μg		Excess antigen‡	Excess antibody§	Immediate	24 hours	Immediate	24 hours
1	150	+	++++	+				
2	100	+	++++	+	t	++++	−	−
3	80	+	++++	+	t	+++	−	−
4	60	++	++++	+	t	+++	−	v sl t
5	50	+++	++++	+	t	+++	−	t
6	40	+++	++	+	t	+++	−	sl t
7	30	++++	+	+	t	+	−	sl t
8	20	++++	t	+	t	+	−	sl t
9	10	++++	t	++	t	+	−	sl t
10	8	++++	−	++++				
11	5	+++	−	++++				
12	3	+++	−	++++				
13	1	+	−	++++				

* Set up in duplicate; precipitates used for action of lysozyme on precipitated substrate.

† Read after 48 hours' incubation in the cold: to ++++ indicates relative amount of precipitate; t, turbid; v sl t, very slightly turbid.

‡ Tested by addition of 0.5 ml 1:10 dilution of serum.

§ Tested by addition of 2.5 μg substrate to tubes 1 through 7, 5.0 μg to tube 8, 10 μg to tubes 9 and 10, and 100 μg to 11, 12, and 13.

‖ 20 μg substrate added.

table 3, show that there was a considerable range in which both antigen and antibody were present in the same supernatant. Such behavior may be due to the presence of several antigens in the substrate or to some alteration in the process of isolation (Kendall, 1937). When similar supernatants were tested for the presence of agglutinating antibodies against the organism, the titer remained virtually undiminished as far as could be determined by the usual crude agglutination tests. Absorption of antibacterial serum by the organism, on the other hand, removed both precipitating and agglutinating antibodies. Lysozyme substrate thus appears to be one of the antigens of *M. lysodeikticus*, presumably forming part of

the cell surface. The antibody which precipitates the isolated substrate seems to be one of the agglutinins produced by injection of the whole organism.

With regard to the immunological specificity of the substrate little can be said until more data are available. Substrates were prepared from the F strain of *M. lysodeikticus*, and from two other lysozyme-sensitive organisms, *Sarcina lutea* and *Staphylococcus muscae*.[6] Two rabbits had been immunized with the F strain and two with *S. lutea*, using saline suspensions of living organisms. All of these sera had only very weak precipitating antibodies against their homologous substrates, and against the C substrate as well. The anti-C and anti-4698 sera precipitated the F and *S. lutea* substrates to the same degree as the C substrate when observations were made using 0.5-ml amounts of serum and 10 and 50 μg of substrate. The *S. muscae* substrate was not precipitated by any of these sera. These observations warrant the conclusion that the *M. lysodeikticus* and *S. lutea* substrates are closely related immunologically. Until specific sera with good titers can be prepared against all the individual substrates, it will be difficult to determine their exact antigenic relationships. Elucidation of the relationship of these substances to one another and to other antigens in the bacterial cell may require the application of quantitative immunochemical methods, using more highly purified substrates.

Experiments were set up to determine whether whole organisms and the substrate in combination with antibody were still susceptible to the action of lysozyme. The initial observations were made previously on agglutinated organisms. In these experiments, the technique for determining sensitivity was the same as described above, except that saline was used both for suspending the organisms and for dissolving the enzyme. At this pH (6.8 to 7.0) gradual lysis could be observed without the addition of NaOH, although the latter was added before final readings were made. It was found that a suspension of agglutinated *M. lysodeikticus* showed the same sensitivity to lysozyme as a control suspension in normal rabbit serum, i.e., complete lysis at 1:640,000 dilution of lysozyme. In a further test, a constant amount of the enzyme was added to a series of tubes containing organisms agglutinated by serial 2-fold dilutions of antiserum. For this purpose an agglutination test was first set up in the usual way with a serum dilution range of 1:10 to 1:640. After incubation at 37 C for 1 hour and then in the icebox overnight, 10 μg of lysozyme were added to each tube, including an unagglutinated control suspension, and after 1 hour at 37 C the tubes were read for lysis. Lysis of agglutinated cocci always occurred, even in the presence of the highest concentrations of serum, i.e., 1:10, in which the supernatants still showed the presence of excess agglutinating antibodies. This was true for all strains of *M. lysodeikticus* and for *S. lutea* as well.

The ability of lysozyme to attack a precipitated substrate was then studied in the following way: The precipitates formed in the experiment outlined in table 3 were allowed to stand in the cold for 48 hours and then centrifuged in the cold.

[6] The *S. lutea* used in our experiments is as sensitive to lysozyme as *M. lysodeikticus*, lysis occurring at 1:320,000 to 1:640,000 dilutions of lysozyme. *S. muscae* is much less sensitive, lysis occurring at 1:10,000 to 1:20,000 dilutions.

The supernatants were decanted and drained off, and the precipitates resuspended in chilled saline and again centrifuged in the cold. The supernatants were again discarded, the precipitates suspended in 1.0 ml M/15 Sorensen PO_4 buffer, pH 5.0, and 100 μg of lysozyme added in 0.1 ml of the same buffer. For each series of tubes a similar control series was set up without the addition of enzyme. The treated precipitates and controls were incubated for 20 to 24 hours.[7] At the end of this time an unmistakable difference in the appearance between the treated and untreated precipitates was noted. In the controls the precipitates remained as opaque white pellets, whereas in the treated series they appeared as translucent vacuolated material closely adherent to the bottom of the tube and markedly diminished in size. It could be shown that some antibody had been released as a result of lysozyme action.[8] The supernatants were withdrawn from all tubes and the pH adjusted to 7.2. Twenty μg of substrate were added and the immediate reaction was noted. In the supernatants from treated precipitates the tubes became turbid immediately and precipitation was observed on the following day. The control series by comparison remained clear or became very slightly turbid. The findings are summarized in the right half of table 3. The evidence indicates that lysozyme is still capable of attacking organisms or substrate when either is combined with antibody.

The assumption that the substrate occurs as a surface antigen in the bacterial cell and may be covered by antibody during agglutination seems reasonable in view of the fact that the intact organism removes antisubstrate as well as agglutinating antibody. If this assumption is valid, the fact that agglutinated bacteria are still susceptible to lysozyme lysis suggests that with the intact organism, as with the isolated substrate, the enzyme is capable of penetrating the antigen-antibody complex to attack the substrate. It has been demonstrated (Delbrück, 1945) that high concentrations of antibacterial antibody will inhibit the adsorption of bacteriophage onto the bacterial cell. This difference in behavior between a virus and an enzyme may possibly be explained by the difference in size between the two agents.

SUMMARY

Cultures of *Micrococcus lysodeikticus* obtained from three different sources were identical in their cultural characteristics and could be grown in a semi-synthetic medium. On the basis of immunological tests, two of these strains were found to be identical, but the third differed significantly from the other two.

All cultures of the organism and of its variants and chromogenic mutants showed the same sensitivity to egg-white lysozyme when tested under the same

[7] Under these conditions hydrolysis of the substrate can be expected to occur, since it is customary to add 200 μg of lysozyme to 5.0 mg of substrate and test for hydrolysis at the end of 2 hours.

[8] This is not to imply that the precipitates in the control series would not dissociate spontaneously to some extent under the described experimental conditions. Also it should be remembered that some lysozyme is probably still present in the supernatant from the treated precipitates. For this reason the immediate reaction was noted before the enzyme could conceivably hydrolyze the newly added substrate.

conditions. An adapted strain which showed a marked decrease in sensitivity to egg-white lysozyme became completely refractory to ficus lysozyme.

The high polymer mucopolysaccharide lysozyme substrate isolated from *M. lysodeikticus* proved to be weakly antigenic when tested in a single rabbit, giving rise to substrate-precipitating antibodies as well as agglutinins. It was precipitated by antibacterial serum and is one of the antigens of the organism. The substrates of *M. lysodeikticus* and *Sarcina lutea* are closely related immunologically. Depolymerized substrate is still capable of combining with antibody.

Lysozyme is capable of attacking organism or substrate when either is combined with antibody.

REFERENCES

DELBRÜCK, M. 1945 Effects of specific antisera in the growth of bacterial viruses (bacteriophage). J. Bact., **50**, 137–150.

DUBOS, R. 1945 The bacterial cell. Harvard University Press, Cambridge, Mass.

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

KENDALL, F. E. 1937 Studies on serum proteins. J. Clin. Investigation, **16**, 921–931.

MEYER, K. 1944 The relationship of lysozyme to avidin. Science, **99**, 391–392.

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

MEYER, K., HAHNEL, E., AND STEINBERG, A. 1946 Lysozyme of plant origin. J. Biol. Chem., **163**, 733–740.

SNELL, E. E., AND WRIGHT, L. D. 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., **139**, 675–686.

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