THE DETERMINATION OF LYSOZYME[1]

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Since the discovery of lysozyme by Fleming (1922) several authors have proposed different methods for determining the activity of various preparations containing this enzyme. Most of these methods were based upon the clearing of dense suspensions of a susceptible organism without concern for accurate quantitative results. The isolation of highly purified crystalline lysozyme by Alderton and Fevold (1946) suggested the possibility of a method for assaying lysozyme. The procedure described here provides for the rapid and accurate microbiological assay of materials that show lytic activity considered to be due to lysozyme.

Fleming (1922), in his original work, observed the lytic activity of lysozyme either as clear zones on agar plates seeded with *Micrococcus lysodeikticus* or as a clearing of a suspension of the same organism. Sandow (1926) used serial dilutions of egg white in meat infusion broth inoculated with various species of organisms. After incubation these mixtures were observed for growth, as evidenced by the turbidity of the tubes. This procedure was applied to a study of the different organisms affected by lysozyme, the dilution of egg white capable of producing inhibition or sterilization being noted.

Goldsworthy and Florey (1930) devised a scheme of assay which consisted of washing an 18-hour culture of *M. lysodeikticus* with saline and adjusting the opacity of that suspension to that of Brown's barium sulfate standard no. 4. Lysozyme was serially diluted so that each succeeding dilution contained only half as much of the enzyme as the one previous. Then 0.5 ml of each dilution was mixed with an equal quantity of cell suspension. The mixtures were allowed to incubate at 38 C for 1 hour. A unit was defined as the least amount of lysozyme necessary to produce complete lysis.

Rosenthal and Lieberman (1931) in determining the lysozyme content of infant stools mixed a susceptible sarcina with stool extracts. Visual observations of the mixture were made under the microscope. A disappearance of the sarcina cells indicated lysozyme activity.

Boasson (1938) developed a technique involving the use of optical measurements of turbidity. A phenol-killed suspension of the test organism was mixed with various dilutions of lysozyme. The amount and rate of clearing was carefully measured in a Moll extinctometer and correlated with the concentration of lysozyme. Since the activity for the known concentration could be observed accurately, it was possible to compare the extent of activity of an unknown and in this manner to determine the amount of lysozyme present.

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The method herein reported is somewhat similar to the Boasson technique and to the one employed by Goldsworthy and Florey (1930).

Meyer and Hahnel (1946) developed a viscosimetric method for measuring the mucolytic activity of lysozyme. A mucopolysaccharide was used as a substrate for the enzyme. The test was based on the depolymerization of this material, which thereby effected a change in the viscosity of the substrate-enzyme mixture. The change in viscosity could be measured and correlated with the lysozyme concentration. The preparation of this mucopolysaccharide fraction is a very involved and laborious procedure, which does not lend itself to a rapid method of assay.

Meyer, Hahnel, and Steinberg (1946), in reporting on lysozyme of plant origin, used the viscosimetric method to measure mucolytic activity and an optical method to measure the bacteriolytic activity. The optical method was not very different from those used by other investigators. *M. lysodeikticus* cells were suspended in a M/15 phosphate solution and mixed with lysozyme dilutions. After 1 hour of incubation at 37 C, 2 drops of normal sodium hydroxide were added and the clearing was read visually. A unit was expressed as the highest dilution giving complete visible clearing under the conditions specified.

Hartsell (1948) used a suspension of *M. lysodeikticus* in phosphate buffer, pH 6.2, mixed with lysozyme contained in rehydrated, spray-dried, whole egg powder. An incubation temperature of 52 C was used, and clearing of the suspension was observed visually. The lysozyme concentration in a sample was expressed as the reciprocal of the highest dilution causing clearing.

None of the tests described above appear to be capable of providing accurate results with a minimum expenditure of time. The desire for an accurate and rapid method of assay prompted this study.

METHOD

The method for lysozyme assay described here is based on a comparison of light transmissions of crystalline lysozyme dilutions with the values for the substance being tested, after the addition of susceptible cells and incubation. A purified chloride salt of lysozyme[2] is used as the standard. This material was chosen because of its availability and constancy of activity.

M. lysodeikticus Fleming, ATCC 4698, is subcultured on yeast water, veal infusion agar[3] with 0.2 per cent glucose every 24 hours for 3 days. After the final subculturing a suspension of the organism is made in phosphate buffer, pH 6.2, and a large number of Roux bottles containing the same medium are inoculated. A heavy suspension as inoculum with a minimum amount of residual moisture on the surface of the agar will give the highest yield of cells. After 18 hours at 37 C the cells are harvested in phosphate buffer. The suspension is then exposed to ultraviolet light in the following manner: A "mediquartz"

[2] The crystalline lysozyme was supplied by the Western Regional Research Laboratory, U.S.D.A., Albany, California, and The Armour Laboratories, Chicago 9, Illinois.

[3] Ten per cent veal infusion, 10 per cent yeast water, 0.5 per cent salt, 0.5 per cent peptone, and 2 per cent agar.

germicidal lamp is placed about 4 inches above an inclined piece of Venetian glass. The cell suspension is allowed to flow slowly down the rough side of the glass. The procedure is repeated. In this manner the cells are twice exposed to approximately 2,700 microwatts per cm^2,[4] which provides a cell suspension with but few living cells. The organisms are then collected, shell-frozen, and dried in a vacuum. In this way it is possible to obtain approximately 0.15 g of dried cells per 100 ml of medium. The cells are stored at 4 C until needed.

The cell suspension is prepared from the dried cells in Sørenson's phosphate buffer, pH 6.2, prior to the assay. Rehydration is easily accomplished since lyophilized cells can be readily resuspended. The turbidity of this suspension is adjusted to show 10 per cent light transmission in a Coleman spectrophotometer equipped with a PC-4 filter, at a wave length of 540 mμ, and to show 100 per cent transmission with a distilled water blank.

From a 1:10,000 stock solution of crystalline lysozyme, dilutions are prepared in Sørenson's phosphate buffer, pH 6.2. Twofold dilutions, starting with 1:200,000 and progressing to 1:6,400,000, are made, thus giving a range of 0.0007 to 0.025 mg of lysozyme per 5 ml of dilution used. Prior to the test the unknown should be checked for its activity to determine what dilutions are needed to give the same level of activity as the crystalline control.

Before the assay is begun a sufficient number of test tubes are matched so that all tubes will show the same light transmission value with distilled water as a reference. At measured intervals a 5-ml quantity of the lysozyme dilution is mixed with 5 ml of the cell suspension. The same procedure is used for the dilutions of the material being tested. All mixtures are made in duplicate. After 20 minutes' incubation at room temperature, the light transmissions for the various mixtures are recorded and the concentration of the unknown is determined. The results of a typical test are shown in table 1.

With the transmission values for the crystalline lysozyme mixtures, a standard curve is prepared by plotting the transmission against concentration. A log scale is used on the abscissa of the standard curve. The transmission values for the unknown dilutions are located on the ordinate and projected to the standard curve. By projection to the abscissa from these points, the concentration of lysozyme in each dilution is determined. Multiplication by the dilution factor results in the concentration of lysozyme per ml of undiluted extract. Figure 1 represents the curve for the values in table 1.

Among the criteria of adequacy of a test of this nature are the accuracy and reliability of the results. The effectiveness of this test was established in a series of assays using materials that showed lytic activity and were capable of maintaining this power when stored. The first preparation tested was powdered egg albumin, which upon rehydration in phosphate buffer showed high lytic activity. The results of the assay of this material are given in table 2. It is to be noted that the titers are consistent and that the error does not exceed 7 per cent,[5] which is not considered excessive for this type of assay. Some of the

[4] All wave lengths are less than 3,400 A.

[5] Computed from the *A.S.T.M. Manual on Presentation of Data*, Supplement A., A.S.T.M., July, 1947, the data indicate 99 per cent certainty that the error does not exceed 7 per cent.

differences in the values may be attributed to the difficulty of uniform rehydration of the albumin.

TABLE 1

Assay results for rat kidney extract

DILUTION	AVG % T	2-LOG % T	LYSOZYME MG PER ML	
			Diluted* extract	Undiluted† extract
Rat kidney extract				
1:10	68.7	0.1627	0.0021	0.021
1:20	56	0.2518	0.0013	0.026
1:40	41	0.387	0.0006	0.024
1:60	34	0.469	0.0004	0.024
Crystalline control				
1:200,000	82.3	0.0848		
1:400,000	73.7	0.1322		
1:800,000	55	0.2596		
1:1,600,000	40.3	0.395		
1:3,200,000	31.3	0.505		
1:6,400,000	27	0.569		

* Value obtained from figure 1.

† Value obtained by multiplying the amount obtained from figure 1 by the dilution factor.

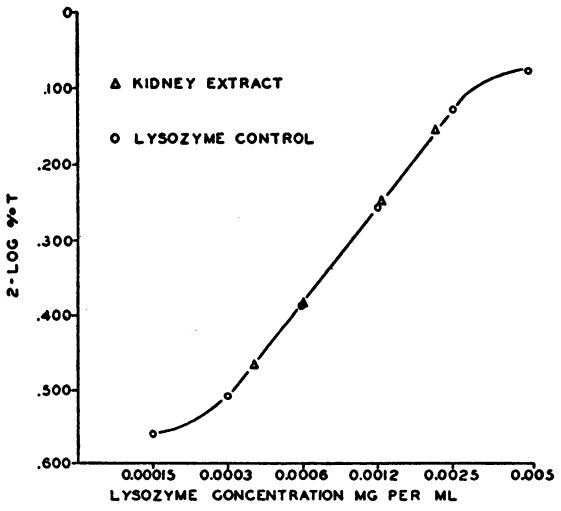

Figure 1. The result of a typical assay with rat kidney extract.

Other preparations with a lower lysozyme concentration were tested by the same method. A rat kidney extract and a hen's egg albumin extract[6] provided

[6] Alderton, Ward, and Fevold (1945) bentonite pyridine extraction, as modified by H. Feldmann, Purdue University; personal communication.

further evidence for the reliability of the test. These results are given in tables 3 and 4. The values are not absolute titers because the extracts were not prepared for quantitative determinations. The rat kidney and the albumin extract both show the same reliability of titers during storage in the icebox for a period of 4 weeks.

TABLE 2

Lysozyme titers of dried egg albumin rehydrated in phosphate buffer, pH 6.2

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per g		*mg per g*
1	20.3	9	22.1
2	19.2	10	20.2
3	19.8	11	19.1
4	20.1	12	16.6
5	20.4	13	18.8
6	21.1	14	16.0
7	21.1	15	17.7
8	20.9	16	21.9

TABLE 3

Lysozyme titers of hen's egg albumin extract

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per ml		*mg per ml*
1	0.80	5	0.85
2	0.79	6	0.87
3	0.89	7	0.91
4	0.91	8	0.91

TABLE 4

Lysozyme titers of rat kidney extract

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per ml		*mg per ml*
1	0.023	5	0.026
2	0.024	6	0.023
3	0.025	7	0.025
4	0.022		

To further demonstrate the practicability of this test several students conducted the assay using the same source of unknown material. The results were similar and the differences were within the limits of experimental error.

SUMMARY

The method of assay for lysozyme described has proved to be sufficiently accurate to recommend its use in testing various materials for lytic activity.

It has been successfully applied to the examination of several preparations containing lysozyme (dried egg albumin, fresh hen's egg albumin, and extracts from animal tissue). The method is rapid and thus makes possible the testing of large numbers of materials in a relatively short time.

A method has been described for the preparation of a large number of *Micrococcus lysodeikticus* cells for use in the turbidimetric assay of lysozyme. It is possible to store these cells at icebox temperatures without any appreciable reduction in their sensitivity when used in assaying lysozyme. Consistent titers for lysozyme activity were obtained in replicate tests.

The method has been shown to be adaptable for both high and low concentrations of the enzyme from natural materials, and gives reproducible results in each instance.

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