

A QUANTITATIVE STUDY OF THE PHOSPHATASE ACTIVITY OF *MICROCOCCLUS PYOGENES*

ERVIN H. BARNES¹ AND JAMES F. MORRIS

Medical Investigation Branch, U. S. Army Hospital, Dugway Proving Ground, Dugway, Utah

Received for publication July 27, 1956

The ability of *Micrococcus pyogenes* var. *aureus* to produce certain toxins and enzymes has been correlated with the causation of disease in animals and human beings. Various laboratory procedures have been devised to determine the activity of these substances as an index of potential pathogenicity. It is known that nonpathogenic strains may exhibit such activity but to a lesser degree. Therefore, a quantitative estimation of the activity of a toxin or enzyme is desirable in determining presumptive pathogenicity.

The most commonly determined substances include coagulase, α -hemolysin, and phosphatase. Of these, phosphatase has received the least attention in regard to quantitative study. The purpose of this paper is to present a quantitative study of the phosphatase activity of a strain of *M. pyogenes* var. *aureus* and to propose a simple rapid technique for measuring the activity of the enzyme.

MATERIALS AND METHODS

The strain of *Micrococcus pyogenes* var. *aureus* used was isolated from a furuncle and was characterized by the production of yellow pigment, coagulase, α -hemolysin (determined by the method of Gillespie and Simpson, 1948), and acid phosphatase. The cells were cultured on nutrient agar (Difco) plates inoculated with 22-hr nutrient broth cultures and incubated at 37 C. After 22 hr the cells were harvested by washing the plates with Sorensen's citric acid-sodium hydroxide buffer, pH 5.6. The suspension was then adjusted to 0.625 optical density with a Coleman Junior spectrophotometer at a wave length of 600 m μ and adjusted to zero with a blank control of buffer, using 10- by 75-mm cuvettes. When any one variable was studied, other than cell concentration, all tubes were prepared with aliquots from the same suspension to insure as nearly

equal a concentration of enzyme per tube as possible.

The substrate used was *p*-nitrophenyl phosphate disodium (Sigma Chemical Company, 1955). Two-tenths ml substrate at 5 g per L concentration were deposited in tubes, stoppered, and frozen until used. When the tests were set up, 0.8 ml of the cell suspension was added to each tube, thus diluting the substrate to 1 g per L and the cell suspension to 0.60 optical density. Buffer, rather than buffered cell suspension, added to the substrate, constituted the control.

The tubes were incubated in a water bath at 37 C. After 30 min, 1 ml of NaOH at pH 12 was added to each tube to stop the reaction and to develop the color of the *p*-nitrophenol liberated. The tubes were then centrifuged at 500 G for 15 min to precipitate the cells. One ml of supernatant was drawn off each tube with a pipette and deposited in a 10- by 75-ml cuvette. The spectrophotometer, set at a wave length of 400 m μ and adjusted to zero with a blank control (incubated buffer and substrate), was used for determination of optical densities. Optical densities of the alkaline solutions were determined, after which 1 drop of concentrated HCl was added to each tube to remove the color of the *p*-nitrophenol. The spectrophotometer was adjusted to zero with the acidified control and the optical densities of the solutions when acidic were determined.

The optical density of alkaline solutions can be attributed to *p*-nitrophenol and extraneous sources, such as residual cells in suspension. The optical density of the acidic solutions is solely of extraneous origin, as *p*-nitrophenol is colorless under acidic conditions. An accurate measurement of the intrinsic optical density of the *p*-nitrophenol is therefore obtained by subtracting the latter value from the former. The total amount of *p*-nitrophenol does not originate entirely from enzymatic hydrolysis, but also from spontaneous hydrolysis and from its presence in the initial substrate. This source of error is cor-

¹Present address: Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

rected when the spectrophotometer is adjusted to zero with the incubated, blank control of buffer and substrate.

p-Nitrophenol, expressed in mmole, was then determined by extrapolation from a standard curve, constructed by determining the optical density of various concentrations of *p*-nitrophenol at pH 12. Because the solutions were diluted 50 per cent when the sodium hydroxide was added, the extrapolated values were corrected by multiplying by a factor of 2. In some instances, the hydrolysis was of such magnitude that the solutions were diluted further for more accurate extrapolation, and the values corrected accordingly. In these instances the control tubes were diluted in a like manner.

RESULTS AND DISCUSSION

Substrate concentration. The activity of the enzyme in relation to the concentration of the substrate was analyzed according to the method of Lineweaver and Burk (1934). The linear regression of $S \div V$ (ratio of substrate concentration to velocity) as a function of S (substrate concentration) was determined (figure 1A). The resultant equation was used to compute the velocity as a curvilinear function of substrate concentration (figure 1B). The substrate concentration that would give the theoretical maximum velocity was calculated to be 255 mmole; 7.6 mmole gave 97.5 per cent of the theoretical maximum activity, and 3.8 mmole or 1 g per L gave 95.9 per cent. All other studies were conducted with 1 g per L substrate concentration for reasons of economy. The Michaelis constant was calculated and found to be 0.0002 M. The data of Martland and Robison (1927) indicate that this constant for bone phosphatase is less than 0.003 M.

The velocity of spontaneous hydrolysis of the substrate was found to be a linear function of the logarithm of the substrate concentration.

Incubation time. The enzymatic hydrolysis of the substrate was found to be a linear function of time for approximately the first 2 hr, thereafter the velocity decreased slowly (figure 2). This departure from linearity may be a reflection of the concentration of the substrate, as the velocity is linear only if the enzyme is saturated. These studies were conducted with 1 g per L substrate, which was found to be theoretically less than saturation (refer to section on substrate concentration). As a matter of consideration, it should

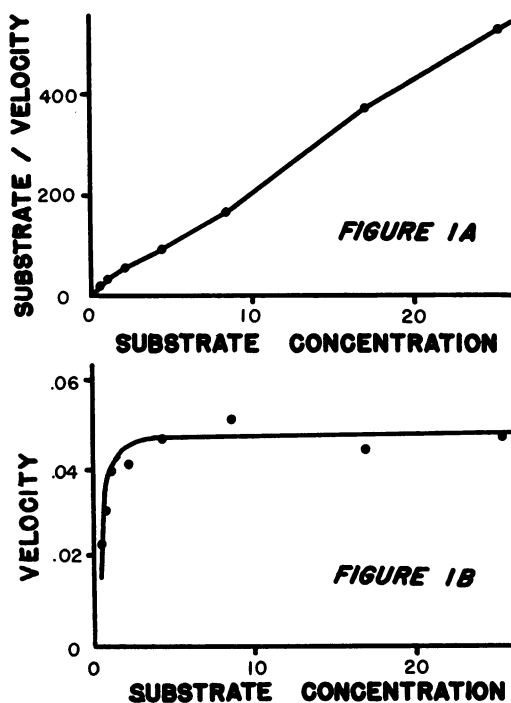


Figure 1. The effect of substrate concentration, expressed in mmole, on the enzymatic velocity as measured by mmole of *p*-nitrophenol per 30 min. The linear regression of $S \div V$ as a function of S . ($S \div V = 3.715 + 20.804 S$). B. The velocity as a curvilinear function of substrate concentration, calculated from the above equation.

be pointed out that determinations beyond 1 hr were made from 1:19 dilutions, and corrected accordingly. Clearly, this increases the limits of error by a like factor. Determinations under 1 hr were made from 1:3 to 1:7 dilutions, and are therefore more accurate than those beyond 1 hr.

The spontaneous hydrolysis of the substrate was found to be a linear function of time, producing 0.96 per cent hydrolysis after 4½ hr incubation.

The enzyme as a variable. *p*-Nitrophenyl phosphate disodium is a phosphomonoester, and on this basis the enzyme could be classified a phosphomonoesterase. The variability of the enzyme was investigated by studying the effect of age and concentration of cells.

Available methods for determining numbers of viable cells involve large errors; therefore cell concentration was considered in a relative manner. Concentrated, buffered cells were used to make a 50 per cent dilution series. The most

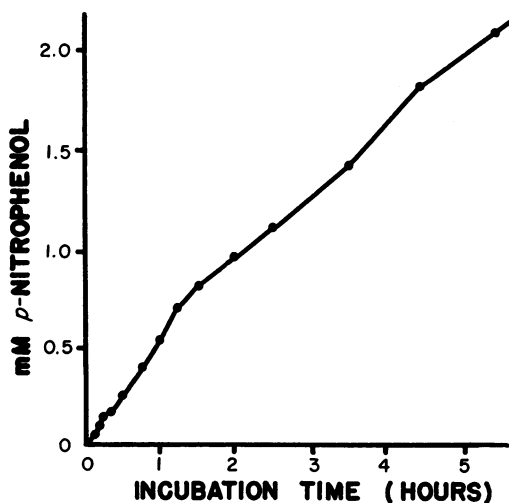


Figure 2. The effect of incubation time on enzyme activity.

dilute suspension, 1:256 the concentration of the original suspension, was considered to be X cell concentration, and the original suspension was then considered to be 256 X . The intermediate dilutions were considered in a like manner.

In order to provide an estimate of the number of cells per ml, a suspension of 0.60 optical density (67 X in relation to the relative cell concentration) was diluted to 10^{-8} . Three replicate pour plates containing 1 ml each, yielded 45, 44, and 45 colonies, the average to two significant digits being 45. The concentration of this suspension then was 45×10^8 cells per ml, or more precisely, clumps of cells per ml. This figure was used to compute the plate count scale in figure 3.

The hydrolysis in relation to the relative cell concentration was found to be a linear function (figure 3). This can be interpreted as reflecting the enzyme concentration as this is the expected relationship.

With a substrate of 1 g per L and incubation time of 30 min, approximately 1×10^8 cells are minimal for detectable activity. A convenient concentration to use with routine determinations would be 50×10^8 cells per ml, a suspension which gives an optical density of 0.50. (This corroborates the suggested 1×10^{10} cells per ml recommended by White and Pickett, 1953.) This concentration is recommended for two reasons:

(1) The magnitude of hydrolysis is such that random error from variation in substrate concen-

tration, cell concentration, incubation time, and dilutions is a small per cent. The coefficient of variation for seven replicate tubes containing 45×10^8 cells per ml was 2.7 per cent, and for tubes containing 14×10^8 cells per ml it was approximately 18 per cent.

(2) One agar slant tube of 22-hr growth is usually sufficient to provide enough cells for 1 ml of this concentration.

"Tween 20" (polyoxyethylene sorbitan monolaurate) was used in concentrations of 0.1 to 20.0 per cent in an attempt to produce uniformity in the suspensions and to delay precipitation of cells during the incubation period. No significant differences were found among seven replicates of the control and various concentrations of tween 20 in magnitude of hydrolysis as measured by an analysis of variance. A comparison of the coefficients of variation for these replicates revealed no differences in variation of practical importance, as there was no relationship between the coefficient of variation and concentration of tween 20.

Age of the culture has a marked effect upon the magnitude of hydrolysis. A 48-hr culture exhibited 86.0 per cent the activity of a 24-hr culture, and a 144-hr culture only 20.8 per cent. The hydrolytic activity appears to be a sigmoid function of culture-age (figure 4) and a reflection

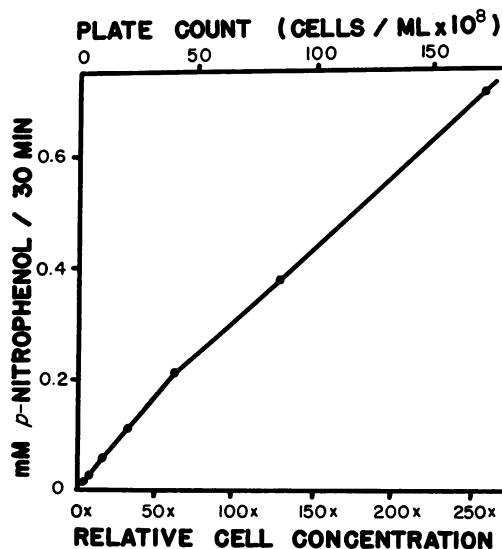


Figure 3. The relationship of cell concentration to enzymatic velocity. The curve was plotted according to the relative cell concentration. The plate count was calculated from dilution pour plates of one cell suspension.

of the physiological age of the cells. Maximum activity is obtained with young cultures.

pH of substrate medium. The pH dependence of enzymes is usually a critical function. This was found to be the case with bacterial phosphatase. A change of 1.4 units of pH on the acid side decreased activity 73.6 per cent and on the basic side by 46.1 per cent. One optimum was exhibited at pH 5.6. Boivin and Mesrobian (1953) found two pH optima, at pH 6 and pH 10, for staphylococci undergoing autolysis induced by toluene and chloroform.

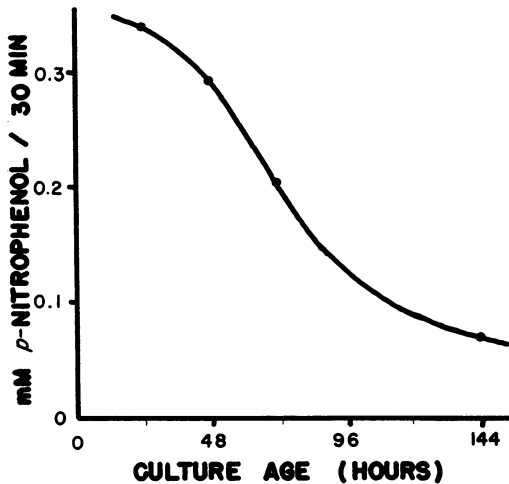


Figure 4. The effect of culture-age on enzyme activity.

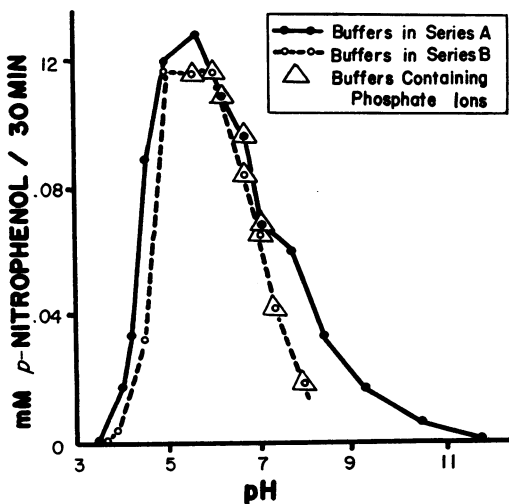


Figure 5. The pH dependence of the enzyme system. Note the inhibition with those buffers containing phosphate ions. (Refer to table 1 for the buffers in series A and B.)

TABLE 1

Buffers used in series A and B for determination of the pH-dependence of the enzyme system

Series A		
pH Range		Buffer
3.2-3.6	KH Phthalate	+HCl
4.0-5.6	KH Phthalate	+NaOH
6.2-7.0	KH ₂ PO ₄	+NaOH
7.7-11.7	Boric acid + KCl	+NaOH
Series B		
pH Range		Buffer
3.7-4.9	Acetic acid	+Na Acetate
5.6-8.0	Na ₂ HPO ₄	+KH ₂ PO ₄

Because no one buffer can be used over a large pH range, different buffers must be employed. Thus, the enzyme is exposed to different ions in different pH ranges. It is well established that certain ions, which might be constituent parts of buffers, inhibit or activate enzyme systems, and hence skew the pH curve. Accordingly, two series of buffers were used (table 1). An unfortunate choice with the phosphate buffers was made as phosphate ions are reported to inhibit acid phosphatase (Roche, 1950). Our results seem to corroborate this report (figure 5). In each pH range where one buffer series contained phosphate ions and the other did not, the former exhibited less activity; and where both series contained phosphate ions, there was little difference. It would appear then, that the pH curve from approximately 5.5 to 7.0 is skewed towards the acid side, since both series contained phosphate ions in this range. Although the phosphate ions appear to inhibit the hydrolysis, it is nonetheless apparent that there was only one optimum and that the pH dependence is a critical function.

ACKNOWLEDGMENT

The authors wish to thank Dr. Stanley Marcus of the Department of Bacteriology, University of Utah for his constructive criticism of the manuscript.

SUMMARY

The phosphomonoesterase activity of a strain of *Micrococcus pyogenes* var. *aureus* was studied in relation to concentration of substrate, incuba-

tion time, concentration of cells, pH dependence, and age of culture. The method used was an adaptation of the technique used for serum phosphatase using *p*-nitrophenyl phosphate disodium.

The method could be utilized for clinical laboratory study, particularly to compare the coagulase, hemolysin, and phosphatase activities of *M. pyogenes*.

REFERENCES

- BOIVIN, A. AND MESROBEANU, L. 1933 Contribution 'a L'etude de la composition chimique des Bacteries. Les Substances phosphorees au cours de L'autolyse bacterienne. Compt. rend. soc. biol., **112**, 611-613.
- GILLESPIE, W. A. AND SIMPSON, P. M. 1948 Pathogenic staphylococci: Detection of A-lysin production on rabbit- and sheep-blood agar plates. Brit. Med. J., **2**, 902.
- LINWEAVER, H. AND BURK, D. 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., **56**, 658-666.
- MARTLAND, M. AND ROBISON, R. 1927 The possible significance of hexosephosphoric esters in ossification. Part VII The bone phosphatase. Biochem. J. (London), **21**, 665-674.
- ROCHE, J. 1950 The Phosphatases, *The enzymes*, Sumner, J. B., and Myrbäck, K., editors. Vol. I, Part 1. Academic Press, Inc., New York, N. Y.
- SIGMA CHEMICAL Co. 1955 Determination of serum acid and alkaline phosphatase and pasteurization of milk products using Sigma 104 phosphatase substrate. Tech. Bull. No. 104.
- WHITE, M. L. AND PICKETT, M. J. 1953 A rapid test for *Micrococcus pyogenes* var. *aureus* for detection of potentially pathogenic strains. Am. J. Clin. Pathol., **23**, 1181-1183.