The pH Value at the Surface of *Bacillus subtilis*

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SUMMARY

The reaction between penicillin and the penicillinase of *Bacillus subtilis* strain 749c, in the cell-bound and the free states, was studied over the range pH 3.9–9.0. The results suggested that the bound penicillinase behaved as though it were reacting in an environment of significantly lower pH value than that of the bulk phase. This effect was not as great as that calculated from electrokinetic data for *B. subtilis* surfaces. Various ways of interpreting the data are discussed.

INTRODUCTION

Following the work of Peters (1931), Danielli (1937) suggested that owing to charge effects, the pH value at a cell surface might be significantly lower than the pH value of the surrounding environment. McLaren & Babcock (1959) pointed out that enzyme reactions at surfaces might yield information about the surface pH value. In the present communication experiments are described in which the cell bound penicillinase of *Bacillus subtilis* strain 749c was used as such a 'molecular pH-meter' for the surface of this organism, by studying its reaction with penicillin. This particular organism was chosen because previous work had been done on the location of its bound penicillinase (Kushner & Pollock, 1961), and a simple iodometric method was available for penicillinase assay (Perret, 1954).

METHODS

Organism. This was *Bacillus subtilis* strain 749c, a penicillinase-constitutive mutant isolated from *B. subtilis* 749 wild strain (Division of Bacterial Physiology, National Institute for Medical Research, London). Although the penicillinase is largely bound to the bacteria during the log phase of growth, it may be obtained free in the supernatant fluid of older cultures; the release of penicillinase was described by Pollock (1961). The organisms were used in suspensions containing the equivalent of 0.45–0.55 mg. dry weight organism/ml., as determined by measurements of opacity in a Hilger and Watts Spectrophotometer and the use of a concentration curve.

Reagents. Buffer solutions were 0.2M-citric acid + sodium hydroxide (pH 3.9–6.5) and 0.2M-potassium dihydrogen phosphate + sodium hydroxide (pH 6.0–8.5).

Benzylpenicillin (crystalline sodium salt, Glaxo, containing 1670 units/mg.) was dissolved (0.25 g.) in 100 ml. 0.2M-phosphate buffer (pH 6.5) and kept in ice. The reagents and method used for the iodometric assay of penicillin were as described by Perret (1954).

The assay of penicillin was carried out in 60 ml. stoppered reagent bottles which were shaken gently in a water bath maintained at 30°. At zero time 1 ml. penicillin solution was added to 4 ml. prewarmed buffer; 5 min. after this either 1 ml. of enzyme solution or 1 ml. of a suspension of washed *Bacillus subtilis* was added. After incubation for 30 min. the residual penicillin was assayed. Twenty-one determinations were made with cell-bound enzyme, and 40 with cell-free enzyme. The penicillinase activity is expressed as percentage maximal activity in each run of experiments.

Measurements of electrophoretic mobility of Bacillus subtilis strain 749c. Organisms in the log phase were washed by centrifugation in various buffers and resuspended in similar solutions. The measurements were carried out in the microelectrophoresis apparatus described by Bangham, Flemans, Heard & Seaman (1958). The electrophoretic mobilities of ten intact bacteria which were not actively motile were measured in each buffer.

RESULTS

The electrophoretic mobilities of Bacillus subtilis strain 749c organisms

The organisms were suspended in 0.2M-citrate buffers at 30° and the results are shown in Fig. 1. From these mobilities it is possible to calculate (see later) the

Fig. 1

Fig. 2

Fig. 1. Electrophoretic mobilities and calculated ζ -potentials of kaolin particles with adsorbed penicillin (○—○), kaolin particles with adsorbed penicillinase (●—●) and *B. subtilis* (○—○) in citrate buffers of pH values indicated.

Fig. 2. Percentage penicillinase activity at different bulk-phase pH, as observed in the 'free' state (●—●) when bound to the surface of *B. subtilis* (Δ—Δ) and as calculated at the surface of *B. subtilis* (× --- ×).

position of the curve for enzyme activity at the bacterial surface from the curve for enzyme activity in the bulk phase. Surface is here used to mean the zone or region in which the pH value is calculated from the ζ -potential (see later). Three curves are therefore plotted in Fig. 2: measured activity in the bulk-phase, measured activity at the bacterial surface, activity at the bacterial surface as calculated from the bulk-phase electrokinetic data.

Discussion of enzyme activity results

The relationships were studied in two parts, first, where activity increased with increasing pH value, and secondly where activity decreased with further increase in pH value. There was a significant departure from linearity when points for which enzyme activity was less than 15 % were included. Omission of these points produced relations in which there was no significant departure from linearity and regression coefficients adequately summarized the rate of change of activity with pH value; these regression coefficients are given in Table 1.

For the part of the curve where enzyme activity and pH value increased together, the difference between the regression coefficients was not significant at the 5 % level ($10\% > P > 5\%$). However, the curves only coincided at the 100 % activity level, which is interpreted as meaning either that their slopes were different, or that the lines were parallel until a high degree of activity was reached, at which point they curved over to coincide. If parallelism be assumed, an analysis of covariance shows the lines to be separated by a highly significant amount ($P < 0.001$). In either case, therefore, it is concluded that the lines are different. As the pH value increased further, activity decreased from 100 % more rapidly for free enzyme than for cell-bound enzyme, the difference between regression coefficients being significant at the 5 % level.

Table 1. *Regression coefficients of percentage activity on pH*

	Cell-bound enzyme	Free enzyme	Calculated reaction at bacterial surface
Activity increasing with increasing pH			
pH range	5.3–6.5	4.8–6.5	5.2–7.1
Activity range	17.0–79.5	16.4–93.7	16.4–93.7
Regression coefficient $\pm$ s.e.	55.0 $\pm$ 4.1	45.6 $\pm$ 2.4	42.6 $\pm$ 2.2
Comparison with cell-bound*	—	†	‡
Activity decreasing with increasing pH			
pH range	7.0–8.5	7.0–8.5	7.5–9.0
Activity range	98.0–82.0	96.6–63.0	96.6–63.0
Regression coefficient $\pm$ s.e.	–10.7 $\pm$ 4.9	–23.4 $\pm$ 3.0	$\times$ 23.5 $\pm$ 3.0
Comparison with cell-bound*	—	‡	‡

* Significance of difference between regression coefficients.

Significance levels: † not significant at conventional 5 % level but $0.05 < P < 0.10$; ‡ $0.01 < P < 0.05$.

For small pH values, the enzyme activities were similar but the cell-bound enzyme increased to 100 % activity faster, the difference between the regression coefficients being significant at the 5 % level. As the pH value increased further, the activity of cell-bound enzyme decreased from 100 % more slowly than did the calculated (surface) pH value; this difference between the regression coefficients was again significant at the 5 % level. The differences in enzymic activity at different pH values between free and cell-bound enzymes are therefore not explicable only in terms of calculated surface pH value.

Measurements of bulk-phase pH values were made at the beginning and end of the

electrophoretic mobility experiments to determine whether changes in bulk pH values could have contributed to the differences observed in the previous mobility experiments. It was found that, with free and cell-bound enzymes, over the range pH 3.9 to 8.0, the maximal difference between initial and final pH value was 0.1 unit. The differences in the curves shown in Fig. 2, therefore, cannot be attributed to differences in bulk-phase pH values.

To determine whether during the penicillinase assay all of the bound penicillinase was released from the *Bacillus subtilis* organisms into the suspending fluid, assays were done in buffers at pH 3.9, 6.75 and 8.0. Two lots of bacteria were used. (i) Bacteria were kept without penicillin in buffer at 30° for 30 min., then penicillin was added, and their penicillinase activity was compared after 30 min. with that of group (ii). (ii) Bacteria were incubated at 30° for 30 min. with penicillin, then centrifuged, resuspended, more penicillin added and the suspension incubated for a further 30 min. At the end of the respective incubation times the suspensions were assayed for residual penicillin. The enzyme activity remaining on the batch (ii) organisms expressed as percentage of the activity of the batch (i) organisms was 100 % at pH 3.9, 65.5 % at pH 6.75 and 86 % at pH 8.0. The losses due to handling being taken into consideration, it would appear that nearly all of the bound penicillinase remained bound during its interaction with penicillin in the experiments so far described.

Experiments were done, in another laboratory with a fresh sample of enzyme, and with a 0.13M-buffer instead of 0.2M as described earlier. Some experiments were done in the presence of kaolin. The pH optima for free enzymic activity was about 1.0 pH unit lower than in the original experiments. A point to be examined was whether a difference could be observed as between free enzyme and that acting at a kaolin surface, and whether this difference corresponded with the difference calculated from measurements of the electrophoretic mobility of the kaolin particles. These experiments were made as a check on the calculations of surface pH value from measurements of electrophoretic mobility, and also to determine whether the penicillinase + penicillin system could be used to measure surface pH.

Experiments with kaolin

The activity of penicillinase was determined in the presence and absence of kaolin particles. It was hoped that the enzyme adsorbed to kaolin particles would simulate 'cell-bound' enzyme. The assay of free penicillinase was carried out as before except that 2 ml. distilled water were added to each 4 ml. of buffer before warming. The assay of kaolin-bound penicillinase was carried out by adding 2 ml. of a 50 % (v/v) suspension of kaolin in distilled water to each 4 ml. of buffer. (The volume of kaolin powder was measured in a measuring cylinder.) After warming, penicillin and later penicillinase were added as previously described. In all, 20 assays were made, each with its own control. Citric acid + sodium hydroxide buffers were used throughout the range pH 3.9–7.0. It was previously found that the presence of kaolin had no detectable effect on the titration of iodine solutions by sodium thio-sulphate as in the Perret method.

In another series of experiments penicillinase was added to citrate buffer and suspended kaolin and incubated at 30° for 5 min. The mixtures were then centrifuged at about 3000g for 5 min. after which each deposit was resuspended in 4 ml.

of 0.2M-citrate buffer + 2 ml. distilled water, and about 1 ml. kaolin powder was added to each supernatant fluid sample. Penicillin solution was added after 5 min. incubation, and an assay of residual penicillin made after a further 30 min. The enzymic activity was associated with the centrifuged deposit of kaolin and no enzymic activity was observed in the supernatant fluid, indicating that the enzyme was bound to the kaolin particles.

Two ml. of the 50% (v/v) suspension of kaolin was incubated with 4 ml. citrate buffer (pH 3.9–6.95) and 1 ml. of penicillin or penicillinase solution for 5 min. at 30°. Samples of these suspensions were then introduced into the electrophoresis apparatus, and the electrophoretic mobilities of the kaolin particles measured; the results are shown in Fig. 1.

The results of experiments with kaolin

The measured activities of the penicillinase in the presence ('bound') and absence ('free') of kaolin are shown in Fig. 3 together with the 'expected' kaolin curve calculated from the 'free' curve and the electrokinetic data. Without statistical analysis it can be seen that the activities of penicillinase are different over the pH range studied in the presence and absence of kaolin, and that the calculated curve for activity in the presence of kaolin fits the observed curve.

Fig. 3. Percentage penicillinase activity at different bulk-phase pH values, as observed in the 'free' state (○—○) when taking place in the presence of kaolin (△—△) and as calculated in the region of kaolin surfaces (× --- ×).

DISCUSSION

A discussion of the underlying theory relevant to the concept of 'surface pH value' was given in a review by McLaren & Babcock (1959). Hartley & Roe (1940) proposed that as the local concentration of ions in the 'surface region' of a particle is

determined by its electrokinetic potential (ζ), the 'surface' hydrogen-ion concentration will be

$$[\text{H}_{\text{Surface}}^+] = [\text{H}_{\text{Bulk}}^+] e^{-\epsilon\zeta/kT}, \quad (1)$$

where ϵ is the electronic charge, k is the Boltzmann constant and T is the absolute temperature. The dissociation constant at the surface (K_S) is given by (Hartley & Roe, 1940).

$$K_S = K e^{-\epsilon\zeta/kT} = K e^{-F\zeta/RT}, \quad (2)$$

where K is bulk thermodynamic dissociation constant, F is the faraday and R the gas constant.

At 30° equation (2) may be rewritten as

$$\text{pH}_S = \text{pH}_B + \zeta/57. \quad (3)$$

(Note that ζ has a negative value.)

Equation (3) may be transformed into the useful, experimentally determinable form from the Helmholtz-Smoluchowski equation which relates ζ -potential to electrophoretic mobility (V).

$$V = \zeta D/4\pi\eta \quad (4)$$

(see Abramson, Moyer & Gorin, 1942), i.e.

$$\zeta = \frac{4\pi\eta}{D} V, \quad (5)$$

where V is mobility towards the cathode in microns/sec/V./cm. of potential gradient, D is the dielectric constant and η the viscosity of the medium at the plane of electrophoretic shear.

Under the conditions of electrophoresis in the experiments described, and substituting for ζ in equation (3) as indicated in equation (5)

$$\text{pH}_S = \text{pH}_B + 0.219 V. \quad (6)$$

The calculated activity curves of penicillinase at surfaces in Figs. 2 and 3 were obtained by extrapolating the percentage activities of the enzyme in the free state to the pH calculated from equation (6).

The points to be discussed are concerned with the results of the experiments in which the enzyme activity curve obtained experimentally for enzyme bound to the surface of *Bacillus subtilis* differed in position from the curve obtained experimentally for free enzyme and from the calculated activity curve for enzyme located at the bacterial surface. The results with kaolin show that given a pH/activity curve for free penicillinase and the electrophoretic mobility of kaolin particles, it is possible to calculate the pH/activity relationship for penicillinase acting at a kaolin surface. These results also imply that from the aspect of enzyme activity, penicillinase acts in the same way in the bulk-phase as at the kaolin surface, since no allowance is made in the various equations for changes in the enzyme. The results of the experiments with kaolin lend additional support to the idea of using enzymes as molecular pH meters to determine the pH value at surfaces (McLaren & Babcock, 1959), although it must be emphasized that the values obtained are those for the sites at which enzyme activity occurs, and not for the 'surface' as a whole. From the data given, under the described experimental conditions, the pH value in the region of the relevant parts of the kaolin surfaces was about 0.85 pH unit less than in the bulk phase.

There is of course the possibility that kaolin-bound penicillinase is not a good model for penicillinase naturally bound at the surface of *Bacillus subtilis*; in the two situations the type of binding may be different, and this might modify the penicillinase and produce a change in the pH/activity relationships. Indeed in *B. cereus* there are two distinct moieties of penicillinase, that liberated and that bound to the cytoplasmic membrane, which apparently differ only in their tertiary configuration yet have strikingly different immunological and enzymic properties and different

Fig. 4. Schematic representations of the hypothetical distribution of charged groups and bound enzymes (E) at parts of the cell surface. A. Uniform distribution of charged groups, electrophoretically indistinguishable from B, where there is a predominantly zonal distribution of groups resulting in C, zones of lowest pH value and separated by regions of pH values intermediate between 'lowest' pH value and bulk-phase pH value. D, distribution of enzymes relative to zones of lowest pH value and regions of intermediate pH values fitting the data from the experiments shown in Fig. 2. E, possible intrazonal distribution of enzymes to account for activity of surface enzymes in an environment of higher than optimal pH value.

pH/activity relationships. According to Garber & Citri (1962) the distinct properties of the cell-bound (γ) penicillinase are closely related to its binding to solid surfaces, including the cell envelope. Whatever the effect of binding, the pH/activity curve for penicillinase in the presence of kaolin is 'calculable', whereas the curve for cell-bound enzyme is not.

McLaren (1960) pointed out that Hartley & Roe's equation for surface pH value gives values at the electrophoretic plane of shear. This plane may be spatially different from the plane at which enzyme/substrate interactions occur.

Apart from these considerations the cause of the discrepancy between the observed and calculated pH/activity relationships of cell-bound penicillinase may be that the bound penicillinase is not superficially located at the surface of *Bacillus subtilis*, in the sense that it is deep to the plane of electrophoretic shear, the plane to which the various calculations apply. 'Deep' in the present discussion means inaccessible to the electrical double-layer of ions which follows the surface contours. If an enzyme be imagined as having its active centre in a 'pit' having a diameter less than that of a hydrated counter-ion, then this centre would not directly respond to surface pH value. If bound penicillinase were thus located, it is difficult to understand how it could react with penicillin at all while in the bound state. However, Kushner & Pollock (1961) showed that the cell-bound penicillinase is not free within the cytoplasm, since rupture of the cell envelope does not release it in a soluble state; up to 98 % of the bound enzyme can be released from the apparently intact bacillus by trypsin, and up to 40 % may be neutralized by specific antiserum.

Another possibility is that under the experimental conditions some of the bound penicillinase had been released and was acting in the free state at a greater pH value than the bound enzyme. Although the possibility that small quantities of enzyme act in this way cannot be ruled out, it seems unlikely that this was occurring to a significant degree, partly because the bacilli were used after washing, when only bound enzyme was present initially, and Kushner & Pollock (1961) have shown this to be firmly bound for short periods of time. Other experiments showed that in spite of handling losses an average of about 84 % of the original penicillinase could be demonstrated as bound to bacilli during 30 min. following a preliminary 30 min. reaction with penicillin. During one 30 min. reaction with penicillin and with no intermediate washing it seems likely that much more than 84 % of the bound enzyme would remain bound.

The last possibility to account for the differences between the observed and calculated pH/activity relationships of the bound-penicillinase is extremely speculative, but may be considered partly because it lends support to similar speculation about the mammalian cell surface (Weiss, 1962). It is well known that the electrophoretic mobility of particles V , is related to their charge density σ by

$$V = \frac{\sigma}{\eta\kappa},$$

where $1/\kappa$ is the thickness of the Debye-Hückel electrical double-layer, which is very much less than the radius of curvature of the particle. Measurements of electrophoretic mobility only give an index of charge 'density' and not of charge 'distribution'. If the charged groups of *Bacillus subtilis* were all located at one point on its surface, then the organism's motion through fluid in the electrophoresis chamber would be complex. If, however, the ionogenic groups on the bacterial surface were arranged in zones or clusters having higher charge density than the spaces between them (Fig. 4B) this would be indistinguishable electrophoretically from a surface having uniformly distributed groups (Fig. 4A). It would be expected that the pH values in the region of the high charge-density zones would be lower than that in the intermediate spaces (Fig. 4C). The pH value in the region of the intermediate spaces would be lower than that in the bulk phase, owing to ion-induced dipole formation in the intermediate space leading in turn to increased local cation (including H^+

ion) concentration. If the bound penicillinase were located in the intermediate spaces as shown in Fig. 4D, then it would be expected that activity/pH curves would show it to be reacting in a region of smaller pH value than the bulk-phase, but of greater pH value than that calculated on a basis of equal surface-charge distribution. In fact, these expectations are realized, as shown in Fig. 2. If there is any truth in this speculation it may be that other enzymes exist at the cell surface located in the postulated zones of high charge density (Fig. 4F). When examined by the type of experiments described here it might be expected that bound-enzymes, in such a location, would behave as though they were at a smaller pH value than that calculated from measurements of electrophoretic mobility. With mammalian cells such an 'intrazonal' localization might perhaps explain how enzymes with very small pH optima such as the lysosomal acid hydrolases (de Duve, 1959) can function at cell surfaces at 'physiological' bulk-phase pH values.

The techniques described offer the possibility not only of the measurement of pH in regions of the cell surface, but also for the localization of enzymes in relation to other structures.

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