The Nature of the Initial Reaction of Oxygen with Two Bacterial Cytochrome Oxidases

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In a previous paper the respiration rates of bacteria and cell-free bacterial preparations as functions of oxygen concentration were described (Longmuir, 1954). The similarity of these functions to the Michaelis function was shown, and thus the reasonableness of calling the concentration of oxygen at which a bacterium or its cell-free preparation respire at half its maximum rate the Michaelis constant (K_m). During the course of this work a paper by Dixon (1953) was published in which the relation of the pH of a solution of an enzyme to the logarithm to base ten of the reciprocal of its Michaelis constant (pK_m) was described. Briefly this relationship is a straight line of slope numerically equal to the change of charge of the enzyme or its substrate when the enzyme-substrate complex is dissociated. If 'substration', as this complex-formation has been called, is associated with a shift of the pK of an ionizing group on the enzyme or substrate, the curve will have a wave. On producing the three straight portions of the curve intersections will be found at the old and new values of the pK .

It seemed that it might be worth while to examine the respiring bacterial preparations in this way. If the logarithm of the reciprocal of the K_m as determined polarographically were to be plotted against the pH, it might be possible to come to some conclusion about the nature of the 'substration' reaction. To obtain a reasonably large part of the curve it would be necessary to determine the K_m over a wide range of pH values; it has been repeatedly stated that it is not possible to determine oxygen polarographically at pH values below 6, as the hydrogen wave overlaps the oxygen wave. However, it would obviously be of great value to be able to determine oxygen over a wide range of pH values, and for the work suggested above it would be essential. Investigation of polarograms at low pH values showed, in fact, that the initial deductions may be incorrect and that the limiting factor is the sensitivity of the biological system to pH change and not the polarographic system.

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It was decided to study the respiratory systems of *Aerobacter aerogenes* and *Bacillus megatherium*, as these two systems had been studied before and, in addition, were thought to have different cytochrome oxidases.

MATERIALS AND METHODS

Materials. These were as previously described (Longmuir, 1954), except that the phosphate buffer was not always brought to pH 7.3, but to the required pH. A.R. H_3PO_4 was boiled with water to hydrolyse any pyrophosphoric acid present, according to the method of Slater (1952), and then a solution of KOH (Polaritan, Hopkins and Williams Ltd.) was added until the required pH was obtained. It is not claimed that such a system can be called a buffer at all pH values: there are regions of the titration curve where there is quite a large change of pH with a small addition of acid or alkali. In order to buffer throughout the entire range of pH over which measurements are made it would really be necessary to use a number of different buffer systems. This was considered undesirable, as it was not known what specific effect different buffers might have on the K_m . If the H_3PO_4 -KOH system is to be used satisfactorily it must be shown that its weak buffering power is adequate to maintain the pH constant for the period of determination, and that the variation in the concentration of the potassium ion does not affect the K_m . It was easy to show that variation of the potassium concentration over a range wider than that to be used subsequently was without effect on the K_m within the limits of experimental error. The problem of the buffering power was more difficult. It was found that when the organisms were ground with the phosphate buffer the pH of the resultant preparation was markedly different from that of the original solution. Two methods were used to deal with this. Either the pH was restored to the original value by the addition of H_3PO_4 or KOH, or the new pH was chosen as the one to be employed, and the other buffer solutions were made up to this pH. In practice the latter method was used for the intermediate pH values and the former for the more extreme values. It was found that during the period between the initial and final calibrations there was no change in the pH of the preparation within the limits of the pH meter used (Electronic Instruments Ltd., Model 30). Thus the requirement of a constant pH during the measurement of K_m was met. If, however, the preparation was allowed to stand for an hour or two in an un-aerated state then the pH became slightly acid, presumably because of fermentation.

It was desirable to obtain points over as wide a pH range as possible, but there are obvious limits. Between the two

limits of pH at which the pK_m was determined (Figs. 3, 4) the respiration rate increased about four- to five-fold on going from the acid to the alkaline side. Beyond these values at either extreme the respiration rate dropped sharply and the pK_m values measured there have not been included in the curves as their validity is doubtful. Even before these regions were reached there was some precipitation of protein and the inactivation of the respiratory system may have been due to this carrying down active matter rather than to a direct inactivation by hydrogen or hydroxyl ions.

Method. The apparatus was the same as previously described.

Theoretical basis of the method. When the rate of the electrode reaction is of the same order of magnitude as the rate of diffusion of the depolarizer to the electrode the Ilkovic equation ceases to be valid. While there is no agreement as to the exact nature of the reactions which O_2 undergoes at the surface of a gold electrode it does seem to be generally agreed that at low pH hydrogen ions enter into the reaction (Kolthoff & Jordan, 1952; Hacobian, 1953) if not directly, at least by their reciprocal relationship with hydroxyl ions. Thus an alteration in the hydrogen-ion concentration will affect the rate of reduction of oxygen at the electrode. Smutek (1953) has shown that progressive alterations in one rate factor in a reduction involving consecutive reactions (such as is known to occur in the electrolysis of O_2) will alter the shape of the polarogram in the manner shown in Fig. 1. Experimentally, it has been shown that reduction of the pH produces a similar change (Fig. 2). This does not prove that the two changes are identical, but it does suggest that what was thought to be an early appearance of the hydrogen wave may be due to oxygen being reduced at the electrode surface more slowly than it diffuses to it. Polarograms determined in the absence of oxygen showed that the region *YZ* is not part of the hydrogen wave.

Since the straight part of the curve *YZ* is about equal in height to the curved region *XY* it might seem that the first half of the curve is due to the reduction of oxygen to hydrogen peroxide and the second to the reduction to water. However, it can be shown that no hydrogen peroxide is formed at any part of the curve and that the value of n (the number of electrons involved in the reaction) is approximately 4 throughout. This was determined by the first method described by Longmuir (1954). The equation relating the current flowing to the concentration of oxygen in solution must therefore be of the form $i = 4ADF(C_0 - C_e)/\delta$, where i is the current flowing; A is the area of the electrode; D is the diffusion coefficient of oxygen through the solution; F is Faraday's constant; δ is the thickness of the diffusion layer; C_0 is the concentration of O_2 in the bulk of the liquid; C_e is the concentration of O_2 at the surface of the electrode.

So long as C_e is a constant fraction of C_0 , and thus $i = kC_0$, we have a reasonably precise method of measuring the concentration of O_2 in solution. However, polarograms obtained with different concentrations of O_2 do not show the same ratio of *XY* to *YZ*. Therefore it is necessary to employ a point on the curve where $C_e = 0$. Determination of the diffusion current as a function of oxygen concentration at different points between *Y* and *Z* confirms that it is only when one approaches *Z* that a straight line is obtained. The calibration procedure used was that used by Longmuir (1954). It may be difficult to locate *Z*. In practice it has

always lain between -0.75 and -0.80 v. Below -0.75 v the calibration curve is not quite straight and above -0.80 v the residual current may be inconveniently high. Even if point *Y* is selected the deviation from a strictly linear relationship would introduce an error of less than 10% in the measurement of O_2 concentration.

When a cell-free preparation is introduced into the polarographic cell O_2 is used up and the diffusion current falls in the manner previously described; the current becomes constant when no more O_2 remains in solution. The value of K_m is then obtained by measuring the distance above this horizontal line at which the slope of the curve is half the maximum value. Sometimes the current

Fig. 1. Theoretical polarograms of the reduction of a substance which takes place by consecutive reactions. The change in the shape of the first curve on the left is brought about by a stepwise change in one of the rate factors.

Fig. 2. Polarograms of oxygen in acid solution. Curve 1 is that obtained initially and curve 2 after 'ageing'. The similarity of these curves to those in Fig. 1 and the significance of regions *XY* and *YZ* are discussed in the text. *Z* is the point normally selected for amperometric determinations. Beyond this point the hydrogen wave begins. If a lower potential is used the diffusion current is no longer linearly related to the concentration of oxygen.

does not become constant but continues to fall slowly. There are a number of explanations of this: (1) zero drift of the instrument; (2) the gradual removal by electrolysis of some other electroreducible substance such as metal ions; (3) the presence in the preparation of a system with a very high affinity for O_2 , i.e. with a very low K_m .

If the preparation is replaced by a different one this drift is usually abolished, so that it cannot be a function of the instrument. In practice we have never found any drift in the instrument we use. If when a little more O_2 is introduced into the cell and the curve is repeated it is found that the whole curve is moved downwards, then the explanation is probably the second. This occurred on one occasion and on investigation it was found that the deionizing column had become exhausted. All solutions made with this batch of water and the results obtained with them were discarded. If, however, the second curve has the same position as the first, as has been found on all but the one occasion, then the final explanation is probably correct. If the experiment is allowed to go on long enough the curve eventually becomes horizontal. This gives further confirmation that the last explanation is the correct one. Examination of such a curve shows that the preparation contains two O_2 -absorbing components, namely, the main one whose K_m is the one normally measured, and a second one with an activity of less than 1% that of the first and a K_m of about 10^{-9} M. The error introduced into the measurement of the K_m of the first component by the presence of the second is less than the experimental error already present.

Spectroscopic examinations were made of the intact cells of *Aero. aerogenes* and *B. megatherium* by means of a Zeiss microspectroscope, a Hartridge reversion spectroscope and a hand model. The bands in the cell-free preparation were indistinct and difficult to see, so precise measurements were made with intact cells. There are reasons, which are discussed below, indicating that the cell-free component contains all the pigments seen in the intact cells, although perhaps not in quite the same proportions.

RESULTS

Cytochrome oxidase of Aero. aerogenes. In Fig. 3 is a plot of the logarithm to base 10 of the reciprocal of the Michaelis constant (pK_m) of a cell-free pre-

paration from *Aero. aerogenes* against pH. It will be seen that a straight line passes within two standard deviations of all but two points. It is possible that the line may begin to curve at either end, and many attempts were made to extend the curve but they always resulted in partial inactivation of the preparations.

Fig. 3. Effect of pH on pK_m of a cell-free preparation of *Aero. aerogenes*. The circles indicate the mean values and the lines extend to two standard deviations either way. The most probable line through these points is a straight line of zero slope.

Fig. 4. Effect of pH on pK_m of a cell-free preparation of *B. megatherium*. The circles indicate the mean values and the lines extend to three standard deviations either way. A possible line through these points has been drawn. The horizontal portions have been extended to meet the sloped line at pH 6.2 and 6.8, the possible values of the pK of a group in the enzyme after and before 'subtraction' respectively. The points at which the extensions of the straight portions meet the sloped line are about 0.3 pK_m distant from the line theoretically required.

Table 1. *Absorption spectra of suspensions of Aerobacter aerogenes and Bacillus megatherium*

Thick washed suspensions of these organisms, grown overnight at 37° in tryptic meat broth shake-bottles, were examined with a Zeiss microspectroscope and a Hartridge reversion spectroscope; the positions of the cytochrome bands were measured with the latter. Both organisms showed 'bacterial' types of cytochrome spectra, the 'classical' a , b and c bands of the mammalian and yeast spectra being replaced by bands usually ascribed to cytochromes a_2 , a_1 and b_1 (Smith, 1954). The centres of the a_2 and b_1 bands were at significantly different wavelengths in the two organisms, and only *Aero. aerogenes* showed a cytochrome a_2 band which was unusually strong. It was from these suspensions that the cell-free preparations were made.

Bands	...	...	...	a_2	a_1	b_1	d
<i>Aero. aerogenes</i>							
Wavelength (m μ)				630	593	561	525-530
Intensity				Very strong	Weak	Strong	Diffuse
<i>B. megatherium</i>							
Wavelength (m μ)				—	597	558	525-530
Intensity				Absent	Rather weak	Strong	Diffuse

Cytochrome oxidase of B. megatherium. In Fig. 4 is a plot obtained from a *B. megatherium* preparation. It was possible to extend the measurements a little further each way, but attempts to substantiate a possible wave in the middle only raised doubts whether there is more than one wave, or whether the whole relationship is one straight line. However, it is not possible to draw a straight line without leaving two or more points more than three standard deviations outside it.

Spectroscopic observations. In Table 1 are the results of the spectroscopic examination of a thick suspension of the organisms. It has been shown that such suspensions of living cells are anaerobic except for a very thin layer at the surface. These bands are therefore of the reduced cytochromes.

DISCUSSION

The system examined is obviously a complex one containing all the enzymes necessary to oxidize glucose. It might contain a system in which glucose is not oxidized through a cytochrome oxidase. However, Tissières (1952) showed that the respiration of cell-free bacterial preparations is cyanide-sensitive and the K_m values of the flavoprotein enzymes are much higher than those of these preparations, suggesting that it is a cytochrome oxidase with which the oxygen combines.

Keilin & Hartree (1953) have shown that in the mammalian cytochrome-cytochrome oxidase system some of the cytochrome oxidase may not be accessible to oxygen. Similarly, Tissières (1951) has shown that *Aero. aerogenes* contains very much more cytochrome oxidase than is necessary for maximum respiration. Thus it is not in general possible to study the activity of the whole of the cytochrome oxidase in a preparation, and presumably this applies to the systems described here with the additional factor that some other enzyme involved in the reduction of the oxidase may be limiting.

The effect of pH on the maximum activity of the systems studied is relatively small, the change of pH from the most acid to the most alkaline increasing the rate about fourfold. This may be an effect on the maximum activity of the oxidase or of a limiting factor; if it is the latter the proportion of cytochrome oxidase studied increases with the pH. If the K_m is independent of the pH this increased amount of oxidase is probably all of the same nature, but, if it alters, the change may be due to a change in the nature of the oxidase involved in the reaction.

The results obtained from a study of extracts of *Aero. aerogenes* are shown in Fig. 3. The interpretation of this relationship, using Dixon's rules (Dixon, 1953), is that the oxidase of this organism

reacts with oxygen without the combining group undergoing any change of charge. The combining group is believed to be iron, and is thought to undergo reversible oxidation to the ferric state on reaction with oxygen. If this is so the ferric iron is not ionized in the range of pH values studied. Alternatively, the iron may not be oxidized in the initial reaction with oxygen.

The results obtained with extracts of *B. megatherium* (Fig. 4) are not so easy to interpret. If the relationship is a straight line then Dixon's rules do not apply. If it consists of two straight lines joined by a wave, as shown, then the interpretation is that oxygen reacts with the respiratory system of *B. megatherium* without any change of its charge, but the pK of one ionizable group in the molecule not directly concerned in the reaction is changed from 6.8 to 6.2. Alternatively, it might be interpreted as the effect produced by a new cytochrome oxidase coming into play as a result of the change in pH.

Whatever interpretation is put on the curve in Fig. 4, it is clear that there is a difference in the cytochrome oxidases in *B. megatherium* and *Aero. aerogenes*. Tissières (1951, 1952) has shown that cytochrome a_2 is the cytochrome oxidase of *Aero. aerogenes*, and although he could not find its band in cell-free preparations, Moss (1954) believes it is present in sufficient amount to account for all the respiration. It will be seen from Table 1 that the cytochrome a_2 band is absent from *B. megatherium* so some other substance must react with oxygen in this organism.

SUMMARY

1. The polarographic determination of oxygen in acid solutions by means of the rotating gold electrode is described.
2. By means of this technique the effect of pH on the K_m for oxygen of cell-free preparations of two organisms has been investigated.
3. These results have been treated by the method described by Dixon (1953).
4. The conclusions to be drawn from this treatment are that the two organisms contain different cytochrome oxidases, and both may react with oxygen without initially undergoing oxidation.

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Ficin-catalysed Reactions: the Affinity of Ficin for some Arginine Derivatives

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We have recently obtained detailed information about the kinetics and mechanism of the trypsin-catalysed hydrolysis of α -benzoyl-L-arginine ethyl ester and amide to α -benzoyl-L-arginine (Gutfreund, 1955*a*; Bernhard, 1955*a, b*). It appeared likely that further insight into the mechanism of enzyme-catalysed hydrolysis reactions could be gained from a comparison of the reactions of two enzymes which are specific for the same substrates. During the course of their pioneer work on the specificity of peptidases Irwing, Fruton & Bergmann (1941) have shown that ficin, an enzyme isolated from fig-tree latex, has the same specificity as trypsin and catalyses the hydrolysis of benzoyl-L-arginine amide. The conditions required for optimum activity of ficin were so different from those of trypsin that one could conclude that the two enzymes catalyse the same reactions via different mechanisms.

Results obtained from varied studies of ficin-catalysed reactions will allow us to map out the steps involved in the formation of the enzyme-substrate complex and its decomposition to enzyme and products. In the present paper we describe the effect of pH on the catalytic activity and on the affinity of ficin for benzoyl-L-arginine ethyl ester, benzoyl-L-arginine amide and benzoyl-L-arginine.

EXPERIMENTAL

Substrates

Benzoyl-L-arginine (BA), its amide (BAA) and its ethyl ester (BAEE) were prepared by the method of Bergmann, Fruton & Pollok (1939).

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The concentration of a nearly saturated stock solution of the zwitterion BA was determined as follows. A sample of BA was dissolved in water and titrated to pH 7.0 with 0.1N-NaOH. The mixture was allowed to stand at 5° for 2 days and then filtered; the filtrate was brought back to pH 2.0 by the addition of N-HCl and the solution was titrated potentiometrically with 0.1N-NaOH. The BA in this solution was found to be 0.02M and its pK_a was 3.40 ± 0.05 at 25°.

Ficin

Crude ficin (100 g.) (L. Light and Co. Ltd., Colnbrook, Bucks) is suspended in 1 l. of 0.01N-HCl and stirred occasionally for 24 hr. The suspension is then dialysed against running tap water for 24 hr. and filtered. To 100 ml. of the filtrate is added 30 g. of $(NH_4)_2SO_4$ and the mixture is left to stand at +4° for 2 hr. and then filtered. The precipitate is washed with a solution containing 24.5 g. of $(NH_4)_2SO_4$ /100 ml., and redissolved in 400 ml. of distilled water, dialysed against distilled water until salt-free and finally dialysed against a mixture of 0.01N-HCl and 0.1M-NaCl. This enzyme stock solution was found to be of constant activity when stored at 4° for 2 months.

A 1% solution of the enzyme in 0.1M sodium phosphate buffer, pH 6.7, was examined in the ultracentrifuge; this was kindly done for us by Mr Per Bro of Yale University. The protein sedimented as one homogeneous boundary with a sedimentation constant S_{20} of 2.56×10^{-13} , corrected to sedimentation in water at 20°. Preliminary osmotic pressure measurements of ficin solutions in 0.1M sodium phosphate, pH 5.05, indicate a mol.wt. of approx. 26 000. On the basis of this mol.wt. the enzymic activity/mole of protein was calculated for