THE RÔLE OF PHOSPHATE IN BIOLOGICAL OXIDATIONS

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Although it has been demonstrated repeatedly that phosphates exercise a catalytic effect in biological oxidations, no one has successfully identified the particular ionic species to which phosphate catalysis is due. In consequence of observations on the stimulus to carbon dioxide production exerted by phosphate solutions on Elodea canadensis, Lyon (1) concluded that the PO₄⁻ ion was the active form. He succeeded in demonstrating that the results could conveniently be expressed by the hyperbolic equation (CO₂-a) (pPO₄⁻-b) = K. Furthermore, reference to the literature seemed to indicate that for lipase in phosphate buffers the following similar relationship was obtained:

\[(\text{Activity of enzyme}) (p\text{PO}_4^-)^a = K\]

where \(p\text{PO}_4^- = -\log (\text{PO}_4^-)\). This direct proportionality between enzyme activity and \(\text{PO}_4^-\) ion concentration would seemingly be an acceptable proof of the catalytic activity of this ionic species.

It happens, however, that other considerations, which the author fully appreciated, render this proof somewhat less convincing. In the first place it is necessary to bear in mind that in almost all of the experiments cited, the \(\text{PO}_4^-\) ion concentration was changed, not by additions of phosphate at constant pH, but by alteration of the hydrogen ion concentration. In consequence effects attributed to the \(\text{PO}_4^-\) ion are inextricably associated with the powerful action of the hydroxyl ion.

The single exception to this method of altering the \(\text{PO}_4^-\) ion concentration was presented as a citation from experiments by Platt and Dawson on pancreatic lipase (2). Here the authors increased the \(\text{PO}_4^-\) ion concentration by additions of phosphate at constant pH, a method which eliminates any effects due to changing hydrogen ion activity. Unfortunately, however, one is not enabled by this means
to increase only the $\text{PO}_4^{3-}$ ion concentration. Over the range of acidity usually investigated a change in the concentration of any one of the phosphate ions would necessarily be associated with proportional increases in the remainder. This is apparent from the following equations

$$
[H^+] = \frac{K_1}{[H_3PO_4]} = \frac{K_4}{[H_2PO_4^-]} = \frac{K_5}{[HPO_4^{2-}]} = \frac{K_s}{[P_0_4^{3-}]}
$$

In consequence hyperbolic relationships corresponding to

$$(\text{Activity of enzyme}) (\text{pPO}_4^{3-}) = K \tag{1}$$

can be obtained with any one of the other ionic species under consideration. For example, it may be shown by derivation from Equation 1 that

$$(\text{Activity of enzyme}) (\text{pK'} + \text{pHPO}_4^{2-}) = K \tag{2}$$

where $K' = \frac{K_s}{[H^+]}$ = a constant

$$K_s = \frac{[H^+] [\text{PO}_4^{3-}]}{[\text{HPO}_4^{2-}]}$$

and $\text{pHPO}_4^{2-} = \log [\text{HPO}_4^{2-}]$

It is therefore apparent that if the data conform to Equation 1, they agree also with Equation 2. We are, in consequence, unable to resort to these equations to identify the particular ionic species to which the catalytic effect of phosphate is due.

Furthermore, we are reminded by Dawson and Platt (3) that the hyperbolic relationship just described is true of pancreatic lipase only under certain limited conditions. Serious deviations are observed in fluids more alkaline than pH 7.2.

To determine the mechanism of phosphate catalysis fairly simple systems are clearly needed. The data of respiration experiments are singularly difficult to analyze because of the unknown nature and concentration of the substrate, the participation of other catalysts (enzymes), and uncertainty about the diffusion of phosphate ions into the cell. We have endeavored to investigate the problem by having
recourse to quite a simple reaction,—the oxidation of glyceric aldehyde by phenolindophenol or other suitable dyestuff. The experiments were conducted at constant hydrogen ion activity in the absence of enzymes and under conditions which permitted precise electrometric observations of the rate of oxidation.

As is indicated in the following paragraphs, we agree with Lyon in attributing phosphate catalysis to the $\text{PO}_4^{3-}$ ion, even though we have been but partly convinced by the evidence presented by him.

**EXPERIMENTAL**

In the first group of experiments the oxidation of glyceric aldehyde (0.0044 M) at different hydrogen ion activities was studied. Methylene blue, 1-naphthol-2 sulfonate indophenol, and phenolindophenol, all 0.00018 M, were used as the oxidizing agents. The hydrogen ion activity was maintained by the use of phthalate, borate, carbonate, and phenylalanine buffers, in each case the buffer concentration being 0.1 M. In those tests in which phosphate was added, it was used in a concentration of 0.05 M.

Controls were also run which were identical with the experimentals except that the substrate, glyceric aldehyde, was omitted.

The experiments were conducted in small soft glass bulbs of about 5 cc. capacity. Each bulb after being blown was drawn off from the parent tube in such a way as to produce a long, slender, capillary outlet. While still hot, the open end was placed in the appropriate buffer and allowed to fill partly on cooling. The bulbs were then boiled to displace all the air with steam. While the contents were still boiling the bulbs were inverted and the buffer expelled. The open ends were promptly plunged into the buffered, glyceric aldehyde—dye solutions. The tiny air bubble which remained in each bulb on cooling was expelled by warming. The tubes were immediately sealed, the bulbs suspended in a bath at $31 \pm 0.02^\circ$, and the time noted. Completion of oxidation was determined by noting the time required for decoloration of the contents. For experiments which have to run many hours, we have found these bulbs superior to vacuum tubes.

The results are presented in Fig. 1. Wherever the pH is expressed to three decimal places the quinhydrone electrode and type K potentiometer were used. In a few instances where the amount of solution
available was small, the pH was determined colorimetrically and expressed to the first decimal place.

The results obtained with methylene blue and phenolindophenol demonstrate that phosphate exercises no catalytic effect at pH 4.77. This conclusion has to be qualified by mention of the aberrant behavior
of naphthol sulfonate indophenol in which catalysis was observed. It is supported, however, by the early work of Löb and colleagues (4, 5) and Witzemann (6) in which the oxidation of glucose by hydrogen peroxide was catalyzed but slightly by acid phosphate solutions. Undissociated phosphoric acid and the \( \text{H}_2\text{PO}_4^- \) ion are therefore eliminated from consideration.

At pH 7.8 to 7.9, in all buffers, phosphate clearly catalyzed the oxidation. The most pronounced effects were observed in borate, in which phosphate additions reduced, in striking fashion, the time of oxidation. As an incidental finding attention should be drawn to the very obvious inverse relationship between the time of oxidation in the borate controls and the oxidation-reduction potential of the dye. This relationship, though not unexpected, was not observed in the phenylalanine controls. We recognize the possibility that phenylalanine may itself have suffered oxidation, and in the continuation of this research we propose to inquire more fully into this question.

In the next group of experiments an exact quantitative study of the phosphate effect was made. Phenolindophenol, in an initial concentration of 0.00037 N, was used as the oxidizing agent in borate buffers (0.1 M) at pH 7.83 to 7.92. The total amount of phenolindophenol initially present was \( 10 \times 10^{-4} \) mols or \( 20 \times 10^{-6} \) equivalents. The oxidation was followed electrometrically by potential measurements on the system \( \text{Pt} / \text{Oxidized dye} / \text{KCl sat.} / \text{Reduced dye} / \text{Sat. KCl} / \text{Hg} / \text{HgCl}_2 \). In every experiment duplicate readings were made by the use of two electrodes of bright platinum. A titration cell almost identical with that described by Clark (7) was used. In it was placed a solution of the dye in the desired buffer. The solution of glyceric aldehyde was placed in an aeration vessel and both solutions were thoroughly deaerated by washing with oxygen-free nitrogen.

1 Nitrogen was freed from oxygen by passing it over tightly rolled copper gauze in a combustion furnace at a temperature of 650 to 700°. Never more than half the length of copper was allowed to become oxidized. It was then reduced with hydrogen. The gas before entering the cell was passed through a wash bottle of distilled water and a spray trap, both in the thermostat. All joints were gas tight. Rubber tubing, which commonly permits oxygen leakage, was not used. Mercury seals, deKhotinsky cement seals, and fused glass joints were used throughout.
Several readings were then made on the buffered dye solution in the cell. 5 cc. of deaerated glyceric aldehyde solution (containing $2.45 \times 10^{-5}$ mols) were added and further readings made at intervals

Fig. 2. A. Borate, 0.1 M; phosphate, 0.000 M; pH 7.833  
B. Borate, 0.1 M; phosphate, 0.002 M; pH 7.921  
C. Borate, 0.1 M; phosphate, 0.005 M; pH 7.913  
D. Borate, 0.1 M; phosphate, 0.010 M; pH 7.900  
E. Borate, 0.1 M; phosphate, 0.020 M; pH 7.889  
F. Borate, 0.1 M; phosphate, 0.050 M; pH 7.833
of 15 minutes or so for about 4 hours. From these readings the equivalents of dye reduced were calculated.

The results are plotted in Fig. 2, Curves A to F. When the equivalents of dye reduced in a given time are plotted against phosphate concentration (Fig. 2, Curves G and H), it is to be observed that the relationship is a linear one.

This observation now permits us to differentiate between the HPO$_4$\textsuperscript{2-} ion and the PO$_4$\textsuperscript{3-} ion in an attempt to identify the ionic species possessed of catalytic activity. At pH 7.8 to 7.9 most of the phosphate is present as HPO$_4$\textsuperscript{2-} but a small amount exists as PO$_4$\textsuperscript{3-}. The concentration of the latter may be calculated from the equation:

$$\frac{[H^+] [PO_4^{3-}]}{K_1 K_2 K_3} + \frac{[H^+] [PO_4^{2-}]}{K_2 K_3} + \frac{[H^+] [PO_4^{-}]}{K_3} + [PO_4^{3-}] = C$$

where $K_1$, $K_2$, $K_3$ are the dissociation constants of phosphoric acid and $C$ the total molar concentration of H$_3$PO$_4$ and the three ionic species. The concentration of HPO$_4$\textsuperscript{2-} is practically equal to total phosphate. The initial concentration of glyceric aldehyde was 0.000089 M. Considering now phosphate solutions of 0.02 M it follows that the ratios

$$\frac{[HPO_4^{-}]}{\text{glyceric aldehyde}} \quad \text{and} \quad \frac{[PO_4^{3-}]}{\text{glyceric aldehyde}}$$

possess the values 225 and 0.055 respectively. The former is already so large that it is difficult to understand by reference to any acceptable

2 The equation given by Clark (8) is

$$E_{\text{calomel}} = E_a - 0.03006 \log \frac{S_2}{S_3} + 0.03006 \log [K_2 + K_3 H^+] + (H^+)^2$$

$$+ 0.03006 \log [H^+] - 0.03006 \log [K_2 + (H^+)]$$

$$E_a = +0.6494 \quad K_2 = 3.6 \times 10^{-10}$$

$$K_3 = 2.3 \times 10^{-11}$$

These constants were assumed to be the same for 31° as for 30°. The numerical factor 0.03006 was changed to 0.03015. The potential of the calomel cell at 31° was +0.2432. After calculating the potential $E_{a'}$, at the given pH, of the system containing equal amounts of reduced and oxidized dye the determination of the amount of reduction was taken from a curve in which per cent reduction was plotted against $(E_a - E_{a'})$, where $E_{a'}$ observed = $E_a + E_{\text{calomel cell}}$.

3 In calculation of PO$_4^{3-}$, the tables by Holt, La Mer, and Chown (9) were employed.
theory of catalysis why further additions of $\text{HPO}_4^{2-}$ should continue
to give a proportional increase in oxidation. On the contrary, the
$\text{PO}_4^{3-}$ ion concentration relative to that of the substrate is so small
that it is readily understandable why a two or three fold increase in
$\text{PO}_4^{3-}$ concentration should provoke a similar increase in substrate
oxidation if to the $\text{PO}_4^{3-}$ ion be attributed the catalytic activity of the
system. We conclude therefore that these results are best interpreted
by regarding the $\text{PO}_4^{3-}$ ion as the catalyst. This conclusion is based
on the premise that the disodium and trisodium phosphates are so
completely dissociated that the possibility of molecular salt catalysis
can be ignored. Professor V. K. La Mer suggests that the linear
relationship between phosphate concentration (at constant pH) and
the rate of oxidation is very likely an example of general basic catalysis
by $\text{PO}_4^{3-}$ ion in the sense used by Brönsted (10-13). We propose to
investigate the subject from this point of view.

SUMMARY

1. The effect of phosphate on the oxidation of glyceric aldehyde
by methylene blue, 1-naphthol 2-sulfonate indophenol, and phenol-
indophenol has been studied.

2. At pH 4.77 in a phthalate-buffered medium phosphate does not
catalyze the reaction.

3. At pH 7.9 in solutions buffered with borate, carbonate, or phenyl-
alanine marked catalysis by phosphate is observed. The effect is
most pronounced in borate.

4. Phosphate catalysis, within the limits studied, is strictly a linear
function of the phosphate concentration.

5. The high concentration of $\text{HPO}_4^{2-}$ and the low concentration of
$\text{PO}_4^{3-}$ relative to that of the substrate virtually demand the conclusion
that the $\text{PO}_4^{3-}$ ion is the active catalytic species.

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