# The Rate of Enzymic Hydrolysis of Phosphoric Esters

## 3. CARBOXY-SUBSTITUTED PHENYL PHOSPHATES

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(*Received* 17 *February* 1950)

Delory & King (1943) published a comparative study of the hydrolysis by faecal alkaline phosphatase of a series of phosphoric esters and established a correlation between the pK values of their second phosphate dissociations, the optimum pH for the enzymic hydrolysis and the Michaelis constants of the esters. They showed that the more acid this phosphate dissociation, the more alkaline was the pH optimum, the greater was the amount of hydrolysis and the lower the Michaelis constant. They found their results consistent with the Martland & Robison (1927) theory of enzyme-substrate combination and with Kay's (1932) interpretation of the pH optima of alkaline phosphatase for different substrates as a balance between increase in the rate of hydrolysis and increase in the rate of enzyme inactivation with increasing alkalinity.

Two more esters, the *o*- and *p*-carboxyphenyl phosphates have been added to this series. For their synthesis the carboxyl groups of salicylic and *p*-hydroxybenzoic acid had to be protected by esterification before phosphorylation of the phenolic hydroxyl group by phosphorus oxychloride. Even then the usual conditions employed for this reaction (King & Nicholson, 1939) were too drastic, and the reaction mixture had to be kept at less than 10° throughout the phosphorylation. The crystalline esters so obtained had a much lower barium and higher phosphorus content than was expected; and when potentiometric titration showed only one titrable hydrogen per atom of phosphorus, and that with a pK of about 1, it was realized that the product was a pyrophosphoric ester and not an orthophosphate. Only later was it discovered that the conditions we had been forced to adopt for the phosphorylation were almost exactly those described by Neuberg & Wagner (1926) for the synthesis of diphenyl pyrophosphate.

Mild alkaline hydrolysis of these pyrophosphates split the pyrophosphate link, and set free the carboxyl group. The carboxyphenyl phosphates were isolated as barium salts; and titration now showed the three dissociations expected, which were ascribed in order of decreasing acidity to the primary phosphate, the carboxyl group and the secondary phosphate dissociations (Table 1).

Table 1. pK *values of carboxyphenyl phosphoric esters*

| | |
|---|---|
| *o*-Carboxyphenyl phosphate | $pK_1$, 0·95; $pK_2$, 3·50; $pK_3$, 6·11 |
| *p*-Carboxyphenyl phosphate | $pK_1$, 1·14; $pK_2$, 3·90; $pK_3$, 6·40 |
| Di-*o*-carbomethoxyphenyl pyrophosphate | $pK_{1 \text{ and } 2}$, 1·05 |
| Di-*p*-carbethoxyphenyl pyrophosphate | $pK_{1 \text{ and } 2}$, 1·10 |

The pH optima for hydrolysis by faecal alkaline phosphatase and the Michaelis constants for these esters were determined, and the results are included in Table 2, which also contains the corresponding data for some of the esters investigated by Delory & King (1943).

## EXPERIMENTAL AND RESULTS

### *Preparation of esters*

*Pyrophosphoric esters*

The procedure of King & Nicholson (1939) was followed, except that the reaction mixture was cooled in an ice bath throughout the addition of $POCl_3$ to the pyridine solution of

Table 2. *Rates of enzymic hydrolysis of phosphoric esters*

| Phosphoric ester | Secondary phosphate pK | Optimum pH | Rate of hydrolysis* | Michaelis constant ($K_m$) |
|---|---|---|---|---|
| *p*-Bromophenyl | 5·44 | 9·96 | 3·4 | 0·0003 |
| Phenyl | 5·73 | 9·73 | 2·4 | 0·0006 |
| *o*-Carboxyphenyl | 6·11 | 9·10 | 1·6 | 0·0010 |
| *β*-Glycero | 6·34 | 8·82 | 1·0 | 0·0012 |
| *p*-Carboxyphenyl | 6·40 | 8·00 | 0·4 | 0·0030 |
| Ethyl | 6·45 | 8·08 | 0·3 | 0·0025 |

* Compared with *β*-glycerophosphate taken as 1; substrate concentrations 0·01 M.

the carboxyl ester, and instead of refluxing for 10 min. it was allowed to stand 24 hr. in the ice chest, and was again cooled during the stage of precipitation with $Ba(OH)_2$. The Ba salts were recrystallized from hot aqueous ethanol, the *di-o-carbomethoxyphenyl pyrophosphate* forming fine needle-like crystals, and the *di-p-carbethoxyphenyl pyrophosphate* glancing platelets.

*Analyses. Di-o-carbomethoxyphenyl pyrophosphate.* (Found: C. 32·0; H, 2·9; P, 10·4; Ba, 24·0. $C_{16}H_{14}O_{11}P_2Ba$ requires: C, 33·0; H, 2·4; P, 10·3; Ba, 23·5%.) *Di-p-carbethoxyphenyl pyrophosphate.* (Found: C, 34·8; H, 2·9; P, 10·3; Ba, 22·6. $C_{18}H_{18}O_{11}P_2Ba$ requires: C, 35·4; H, 3·0; P, 10·1; Ba, 22·5%.)

*Orthophosphoric esters*

After dissolving in water and removal of Ba by the calculated amount of $H_2SO_4$, hydrolysis of the pyrophosphate esters was accomplished by the addition of five equivalents of NaOH. After 18 hr. at 37° only two equivalents of NaOH remained (titration with HCl to phenolphthalein end point). No inorganic phosphate was liberated by this hydrolysis. The carboxyphenyl phosphates were isolated by the addition of $BaCl_2$, either as the $Ba_{1\cdot5}$ salts from alkaline solution or as the half Ba salts from acid (pH <3) solution.

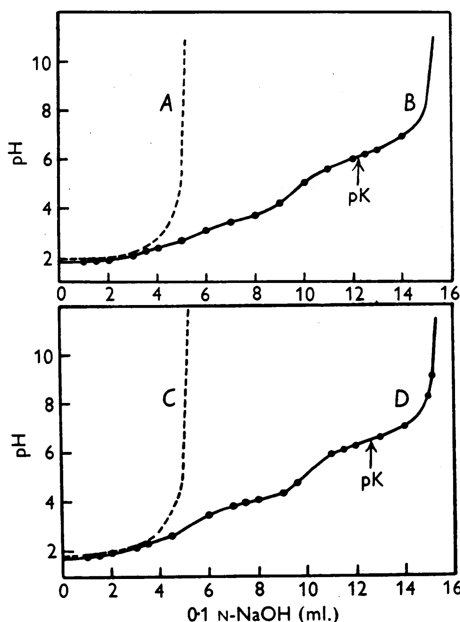


Fig. 1. Dissociation curves for *A*, *o*-carbomethoxyphenyl pyrophosphate; *B*, *o*-carboxyphenyl phosphate (pK 6·11); *C*, *p*-carbethoxyphenyl pyrophosphate; and *D*, *p*-carboxyphenyl phosphate (pK 6·40).

*Analyses.* For the isomeric $Ba_{1\cdot5}$ salts of the carboxyphenyl phosphates. (Found for *barium o-carboxyphenyl phosphate*: P, 7·7; Ba, 49·0; and for *barium p-carboxyphenyl phosphate*: P, 7·6; Ba, 47·8. $C_7H_4O_6PBa_{1\cdot5}$ requires: P, 7·4; Ba, 48·7%.)

*Titration curves.* For the pH values a Muirhead pH meter with a dipping glass electrode was used. The meter was set with standard phthalate (pH 4·00) and borate (pH 9·00) buffers. The Ba pyrophosphate ester (0·25 mmol.) or the Ba orthophosphate ester (0·5 mmol.) was dissolved in a carefully measured volume of water, and treated with the exact amount of $H_2SO_4$ to precipitate the Ba. The solution of the acid was then titrated with $CO_2$-free 0·100 N-NaOH (temp. 18–20°). Three values for each dissociation constant were calculated using the equation $Ka = \frac{[H^+] \times (B + [H^+])}{C - (B + [H^+])}$ for $pK_1$ and $pK_2$, and the equation $pK = pH - \log B/(C - B)$ for $pK_3$ (when $H^+$ is negligible compared with $B$ and $C$ (see Van

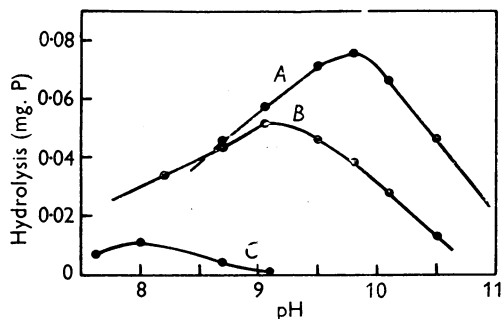


Fig. 2. Optimum pH for hydrolysis by phosphatase of *A*, phenyl phosphate (pH 9·7); *B*, *o*-carboxyphenyl phosphate (pH 9·1); and *C*, *p*-carboxyphenyl phosphate (pH 8·0).

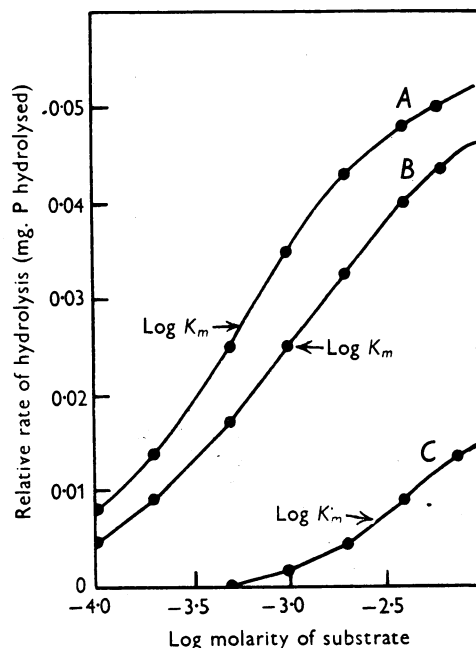


Fig. 3. Curves showing Michaelis effect of phosphoric esters and faecal phosphatase at the optimum pH for each ester. The continuous lines are drawn from the equation $\log \frac{1}{x} = \log \frac{1}{K_m} + \log\left(\frac{V}{v} - 1\right)$, the values of the constant $K_m$ being chosen for the best fit for the determined values. *A*, phenyl phosphate ($K_m$ 0·0006); *B*, *o*-carboxyphenyl phosphate ($K_m$ 0·0010); *C*, *p*-carboxyphenyl phosphate ($K_m$ 0·0030).

Slyke, 1922)). $B$ represents the molar concentration of added NaOH and $C$ the molar concentration of ester in the titration mixture at any given time. (A $pK_1$ and $pK_2$ for the pyrophosphoric esters could not be distinguished by this method.) The graphs for the titration are given in Fig. 1, and the pK values in Table 1.

*Enzyme hydrolysis.* A range of carbonate-veronal buffers covering pH 7·5–10·7, and a potent faecal phosphatase were prepared as described by Delory & King (1943), and their procedure for the determination of the pH optima and Michaelis constants was followed exactly. These results are shown graphically in Figs. 2 and 3.

## DISCUSSION

It was expected, when these two substituted phenyl phosphates were made, that they would show a greater rate of hydrolysis than unsubstituted phenyl phosphate, due to the strong positive inductive, acid-strengthening effect of the carboxyl group. At the pH values used for enzymic hydrolysis, however, the group which is influencing the secondary phosphate dissociation is the negatively charged ionized carboxyl group ($—COO^-$); and the two negative charges on the dissociated primary phosphate and carboxyl groups have outweighed the positive inductive action which we had expected.

These results, however, agree with those of Delory & King (1943) in showing an increase in the amount of hydrolysis and a shift of the pH optimum to a more alkaline reaction with increasing acidity of the substrate.


## SUMMARY

1. *o*-Carbomethoxyphenyl pyrophosphate and *p*-carbethoxyphenyl pyrophosphate have been prepared; they are converted by mild hydrolysis to the orthophosphoric esters *o*-carboxy- and *p*-carboxyphenyl phosphate.

2. These esters are not such strong acids (pK *o*-, 6·11; *p*-, 6·40) as unsubstituted phenyl phosphate (pK 5·73).

3. The optimum pH values for enzymic hydrolysis by faecal phosphatase (*o*-, 9·10; *p*-, 8·00) are not as alkaline as that of phenyl phosphate (9·73). The rates of enzymic hydrolysis are also less.

4. The Michaelis constants ($K_m$ for *o*-, 0·0010; *p*-, 0·0030) are greater than that of phenyl phosphate ($K_m$ 0·0006).



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# The Fermentable Form of Fructose


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(*Received* 15 *December* 1949)

Hopkins (1931) found that when a rapid fermentation of fructose by brewer's yeast is arrested the residual sugar exhibits mutarotation in the positive sense. From this and other observations it was concluded that one form of fructose was specifically fermented or at least preferred, that this form possessed a specific rotation less negative than that of fructose at equilibrium, and was fructofuranose. It was not possible to furnish clear experimental proof since neither $\alpha$-fructopyranose nor any form of fructofuranose could be isolated in the crystalline form. However, a preparation of fructose exhibiting substantially less negative specific rotation than that of the solution at equilibrium can be prepared by slowly fusing normal fructose and cooling suddenly. The rates of fermentation of such preparations have been compared with those of fructose at equilibrium, and of freshly dissolved normal fructose.

## MATERIALS AND METHODS

*Substrates.* D-Fructose recrystallized from ethanol (Harding, 1922), was heated slowly with stirring in a nickel crucible immersed in a glycerine bath until the temperatures stated in Table 1 were attained. The crucible was then plunged into solid $CO_2$. It is important to minimize access of moisture to the sugar throughout.

*Fermentation.* Brewer's top fermentation yeast was used. Rates of fermentation were measured as by Hopkins & Roberts (1935*a*). In the fermentation vessel the brewer's yeast in suspension was brought to the desired temperature and the sugar, in solid form or in solution at equilibrium, was