SUMMARY

1. The inhibition of true and pseudo cholinesterase by a series of organo-phosphorus inhibitors has been determined.

2. Based on the effect of changes of acyl groups in choline esters on their hydrolysis by true and pseudo cholinesterase an explanation of the different

sensitivities of the two enzymes for organo-phosphorus inhibitors has been suggested.

My thanks are due to Mr B. Topley and Dr H. Coates (Albright and Wilson, Ltd.), to Dr G. S. Hartley (Pest Control, Ltd.) and to Dr A. H. Ford-Moore (Chemical Defence Experimental Station) for the generous gifts of the inhibitors used in this paper, and also to Miss J. E. Cremer for valuable technical assistance.

REFERENCES

- Adams, D. H. (1949). *Biochim. biophys. Acta*, **3**, 1.
 Adams, D. H. & Thompson, R. H. S. (1948). *Biochem. J.* **42**, 170.
 Adams, D. H. & Whittaker, V. P. (1949). *Biochim. biophys. Acta*, **3**, 358.
 Aldridge, W. N. (1953a). *Biochem. J.* **53**, 117.
 Aldridge, W. N. (1953b). *Biochem. J.* **53**, 110.
 Aldridge, W. N. & Davison, A. N. (1952a). *Biochem. J.* **51**, 62.
 Aldridge, W. N. & Davison, A. N. (1952b). *Biochem. J.* **52**, 663.
 Augustinsson, K. B. (1951). *Acta chem. scand.* **5**, 712.
 Austin, L. & Berry, W. K. (1952). Personal communication.
 Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950). *J. biol. Chem.* **186**, 69.
 Bournsnel, J. C. & Webb, E. C. (1949). *Nature, Lond.*, **164**, 875.
 Earl, C. J. & Thompson, R. H. S. (1952). *Brit. J. Pharmacol.* **7**, 261.
 Ellis, S. (1947). *J. Pharmacol.* **91**, 370.
 Gliok, D. (1941). *J. biol. Chem.* **137**, 357.
 Hartley, B. S. & Kilby, B. A. (1952). *Biochem. J.* **50**, 672.
 Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.
 Jansen, E. F., Nutting, M. D. & Balls, A. K. (1949). *J. biol. Chem.* **179**, 201.
 Jansen, E. F., Nutting, M. D., Jang, R. & Balls, A. K. (1950). *J. biol. Chem.* **185**, 209.
 Levine, M. G. & Suran, A. A. (1950). *Nature, Lond.*, **166**, 698.
 Mazur, A. (1946). *J. biol. Chem.* **164**, 271.
 Mazur, A. & Bodansky, O. (1946). *J. biol. Chem.* **163**, 261.
 Mendel, B. & Hawkins, R. D. (1947). *Biochem. J.* **41**, xxii.
 Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
 Metcalf, R. L. & March, R. B. (1950). *J. econ. Ent.* **48**, 670.
 Michel, H. O. & Krop, S. (1951). *J. biol. Chem.* **190**, 119.
 Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.
 Nachmansohn, D. & Wilson, I. B. (1951). *Advanc. Enzymol.* **12**, 259.
 Ord, M. G. & Thompson, R. H. S. (1950). *Biochem. J.* **46**, 346.
 Remick, A. E. (1949). *Electronic Interpretations of Organic Chemistry*. London: Chapman and Hall.
 Sturge, L. M. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 518.
 Van Slyke, D. D. (1942). *Advanc. Enzymol.* **2**, 33.
 Whittaker, V. P. (1951). *Physiol. Rev.* **31**, 312.

Studies on Fumarase

2. THE EFFECTS OF INORGANIC ANIONS ON FUMARASE ACTIVITY

By V. MASSEY

Sir William Dunn Institute of Biochemistry, University of Cambridge

(Received 8 March 1952)

It was shown quite early in the history of this enzyme that the activity of fumarase is affected by the addition of inorganic salts. Mann & Woolf (1930), working with whole washed cells of *Escherichia coli*, found that sulphates inhibited slightly the hydration of fumaric acid to L-malic acid; while phosphates and citrates strongly activated this reaction. Similarly, the activating effect of phosphates on the extracted enzyme from animal tissues has been shown by various workers (Quastel, 1931; Clutterbuck, 1928; Davydova, 1947). In this paper is reported the effect of various anions on the activity of crystalline fumarase.

METHODS

The enzyme used was obtained from pigs' hearts by the method already described (Massey, 1951, 1952) and was recrystallized twice before use. The enzyme activity was determined by the spectrophotometric method of Racker (1950), which depends on the formation or disappearance of the double bond of fumaric acid. The fumarate or L-malate concentrations used in these experiments were generally 0.0167M-fumarate or 0.0835M-L-malate. These concentrations are over five times the highest Michaelis constant observed with either substrate. The rate of the reaction is linear at first and then falls off as the substrate concentration decreases and the product is formed (Fig. 1). The values

reported here are the initial velocities, when the reaction rate is linear, and may be expressed as change in fumarate concentration per unit time or as change in optical density per unit time. As there is a direct correlation between the fumarate concentration and the optical density (Massey, 1953), most of the values reported here are, for the sake of convenience, expressed as changes in optical density. Unless otherwise stated, all the results described here were obtained at room temperature.

RESULTS

The effect of pH on the activity of well dialysed fumarase. Contrary to previous claims (Laki, 1941), fumarase when pure is not denatured by exhaustive dialysis against glass-distilled water. When impure,

Fig. 1. The course of hydration of fumarate to L-malate by a small amount of fumarase ($\lambda=2950 \text{ \AA}$).

however, it does precipitate out in an inactive form on dialysis, possibly owing to absorption on a contaminating protein which is insoluble in distilled water. The pH-activity curves of well dialysed solutions of pure enzyme, in the presence of L-malic or fumaric acids neutralized to the desired pH, are shown in Fig. 2. Below pH 7, fumarate behaves as a reasonably good buffer; L-malate is an efficient buffer up to about pH 8, so that the enzyme activity can be determined at constant pH without the addition of any anions other than the substrates. The pH values of the reaction mixtures were checked before the addition of enzyme and during the course of the reaction. Under the conditions used, with small concentrations of enzyme the pH never varied more than 0.1 pH unit during the period of reaction, and in most cases much less than this.

Fumarase in the absence of inorganic anions has quite different pH-activity curves with its two substrates (Fig. 2). With fumarate as substrate, the curve is rather flat with a poorly defined optimum around pH 5.6. However, with L-malate, a symmetrical pH curve with a well defined optimum at pH 7.2 is obtained.

The effect of phosphate. Fig. 2 also shows the effect of 0.06M-phosphate on the pH-activity curves of fumarase with both fumarate and L-malate as substrates. With both substrates the pH optimum is shifted toward the alkaline side, and there is considerable activation over most of the pH range studied.

The effects of phosphate on the hydration reaction are very similar to those found with whole bacteria by Mann & Woolf (1930). The shape of the pH curve in the presence of phosphate, and the pH optimum, are the same as they obtained. The pH

Fig. 2. The effect of pH on fumarase activity in the presence and absence of phosphate. A, 0.0167M-fumarate in the absence of phosphate; B, 0.0167M-fumarate in the presence of 0.06M-phosphate; C, 0.0835M-L-malate in the absence of phosphate; D, 0.0835M-L-malate in the presence of 0.06M-phosphate.

optimum (5.6) of crystallized fumarase in the absence of inorganic salts is considerably lower than the value (6.4) obtained by Mann & Woolf (1930) with washed bacteria. This difference may well be due to the retention of some phosphate by the bacteria even after thorough washing.

Table 1 shows the effect of phosphate concentration on the extent of activation, using 0.0835M-L-malate as substrate at pH 7.4. Maximum activation is quickly reached and is maintained over a considerable range of phosphate concentration. At high phosphate concentrations, however, the activity again falls off. Such effects were found at all pH values investigated.

That the activating effects of phosphate are not due to changes in the affinity of the enzyme for the substrate are shown in Fig. 3 for fumarate as substrate at pH 6.35, and 23°. The initial velocities over a range of fumarate concentration from $m/600$ to $m/120$ are plotted according to the method of Lineweaver & Burk (1934). It is evident that under these conditions, while the enzymic activity is greatly increased by phosphate, the Michaelis constant is unaffected.

Table 1. *The effect of concentration of phosphate on activity of fumarase at pH 7.4*

(The activity values represent the initial increase in optical density over a period of 3 min. when 0.0835M-L-malate is acted on by a constant quantity of salt-free fumarase.)

Concentration of phosphate (M)	Activity
0	37
0.00033	44
0.00066	47
0.00133	49
0.0020	50
0.0033	53
0.0066	55
0.0167	56
0.0835	56
0.167	51
0.333	40

Again, the pH-activity curves in the presence of phosphate are not greatly influenced by the ionic strength. The results shown in Fig. 2 are with 0.06M-phosphate solutions in which the ionic strength varies from 0.06 to 0.18 over the range of pH studied. If, however, the ionic strength of the phosphate is maintained constant at 0.06, the pH-activity curve remains unaltered.

The effect of other anions. Several other anions affect the activity of well dialysed solutions of fumarase. The effects of chloride, sulphate, selenate, arsenite, citrate, borate, phosphate and arsenate are shown in Fig. 4. By comparison it should be noted that with washed bacteria Mann & Woolf (1930) found sulphate slightly inhibitory, and citrate an activator even more efficient than phosphate. These differences may be due to different properties of bacterial and animal fumarases, or more probably are connected with permeability factors. Divalent ions activate to a lesser extent than trivalent ions, and the greater the extent of activation, the further toward the alkaline the pH optimum is shifted. Chloride ions, which act as inhibitor over most of the pH curve, while also shifting the pH optimum considerably toward the alkaline, appear to have little effect on the alkaline branch of the pH-activity curve, but have a very large effect on the acidic

branch. Bromide, iodide and thiocyanate ions behave in the same way as chloride. The inhibition with these monovalent ions occurs also in the presence of phosphate; Fig. 5 shows their effect

Fig. 3. Determination of the Michaelis constant of fumarase with fumarate as substrate at pH 6.35 and 23°. A, in the absence of phosphate; B, in the presence of 0.06M-phosphate.

Fig. 4. The effect of some anions on the dehydration of L-malate by fumarase. Concentration of L-malate, 0.0835M. A, in the absence of added anions; B, 0.03M-sodium selenate or 0.025M-sodium sulphate; C, 0.01M-sodium arsenite; D, 0.01M-sodium citrate; E, 0.2M-sodium borate; F, 0.06M-sodium and potassium phosphate; G, 0.05M-sodium arsenate; H, 0.1M-sodium chloride.

under similar conditions, but in the presence of 0.06M-phosphate. That the inhibition, like the activation with phosphate, has no effect on the Michaelis constant is shown in Fig. 6.

That the activation produced by phosphate, arsenate, etc., may be due to the removal of a contaminating inhibitory metal is unlikely for several reasons. First, all the reactions reported here have

salt-free reaction at pH's 6.3 or 7.9. Similarly, diethyldithiocarbamate in saturated aqueous solution at these pH values has no effect.

DISCUSSION

The results shown here might be explained in terms of the theory of Michaelis & Davidssohn (1911) and Michaelis & Pechstein (1914) that the dissociation of two particular groups, acidic and basic, determines the activity of an enzyme, and that the pH-activity curve is determined by the dissociation constants of these groups.

According to this theory, the optimum activity is at that pH value where the dissociation of the two groups responsible for activity is maximal. It is obvious that the experimental results which support this theory do not really enable us to argue that only one acidic and one basic group are concerned in the activation; theoretically, any number of groups which had pK values close to each other would produce the same results.

Applied to fumarase, it is seen that, with both fumarate and L-malate, it is the alkaline branch of the pH-activity curves which is predominantly altered by the addition of phosphate. This suggests that the basic dissociation constant(s) of the enzyme is affected by the phosphate, rather than the acidic.

Fig. 2 also shows that, in the absence or in the presence of phosphate, the alkaline branches of the activity curves for both fumarate and L-malate are close together, while the acid branches are widely separated. This suggests that the two substrates may also affect the dissociation constants responsible for activation, except that now the effect is on the acidic dissociation constant(s) rather than on the basic. There is evidence (Massey, unpublished) that the substrates and other carboxylic acids are capable of secondary combinations with fumarase at sites other than the active centre(s), and that this type of combination affects the dissociation constants of groups at the active centre.

Effects similar to those of phosphate are shown with all the activating ions investigated. All of the anions found to activate thus appear to alter the basic dissociation constant(s), while having little effect on the acidic dissociation constant(s).

With the inhibiting monovalent anions, the effect seems to be of the opposite kind; here the acidic dissociation constant(s) seem to be affected much more than the basic dissociation constant(s).

It is rather difficult to visualize, however, how the interaction of protein and anion can lead to a shifting of the basic dissociation constant toward the alkaline region, as occurs with the activating ions. Binding of an anion should lead to the suppression of the ionization of a basic group, with a consequent

Fig. 5. The effect of some monovalent anions on fumarase activity. A, L-malate (0.0835 M) in the presence of 0.06 M-phosphate; B, plus 0.1 M-potassium chloride; C, plus 0.1 M-potassium bromide; D, plus 0.1 M-potassium iodide Δ or 0.1 M-ammonium thiocyanate, $\square$.

Fig. 6. Determination of the Michaelis constant of fumarase with fumarate as substrate at pH 6.35, and 23°. A, in the presence of 0.06 M-phosphate; B, in the presence of 0.06 M-phosphate plus 0.01 M-ammonium thiocyanate.

been obtained in solutions employing glass-distilled water in which the concentration of heavy metals is extremely minute. Secondly, 0.033 M-versene (ethylenediaminetetraacetic acid), which is an extremely strong metal-chelating agent, has no effect on the

shift of the basic dissociation constant toward acid pH values. It is also difficult to explain why monovalent ions such as chloride seem to have no effect on the basic dissociation constant, but do affect the acidic dissociation constant; the shift again being toward the alkaline.

Presumably the effects described cannot be due to a direct influence of the anions on the active centre. This has been shown directly for phosphate and thiocyanate at pH 6.35; at this pH neither anion has any effect on the Michaelis constant.

Such shifts may be due to combination of the anions with basic groups adjacent to the acidic and basic groups responsible for activation. The combination of an anion with a basic group adjacent to the activating basic group might be expected to cause an increased ionization of the activating basic group, i.e. the dissociation constant of this group would be shifted toward the alkaline and activation would result over the alkaline range of the pH-activity curve.

If, on the other hand, the anion combined with a basic group adjacent to the acidic group causing activation, then the dissociation of this acidic group would be made more difficult, and the dissociation constant would be shifted toward the alkaline, with a consequent inhibition, especially over the acid branch of the pH-activity curve.

However, while this interpretation fits the experimental data, it gives little clue as to why some anions should combine preferentially with basic groups adjacent to the activating acidic group, and

why others combine preferentially with basic groups adjacent to the activating basic group.

This preferential adsorption at certain sites in the enzyme may be due to differences in size or charge of the anions; all the inhibiting anions are rather small and are monovalent, while the activating anions are much larger and are at least divalent.

However, some experiments with fumarase have shown that some of the changes in activity obtained on the addition of anions can be correlated with gross changes of molecular arrangement of the enzyme. Thus, changes induced in the dissociation constants of the protein, if they do occur, are probably not the only factors effecting the changes in activity.

SUMMARY

1. The effect of pH on fumarase activity in the absence of inorganic anions is described.

2. Sulphate (and selenate), arsenite, citrate, borate, phosphate and arsenate, in increasing order of effectiveness, activate salt-free fumarase and alter the pH of optimal activity.

3. Chloride, bromide, thiocyanate and iodide, in increasing order of effectiveness, inhibit the action of fumarase, either in the presence or absence of activating anions. The extent of the inhibition is markedly dependent on the pH.

I am indebted to Dr M. Dixon, F.R.S., for his advice and encouragement, and to Dr G. Weber and Mr D. Lawrence for many helpful discussions. The work was carried out with a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

REFERENCES

- Clutterbuck, P. W. (1928). *Biochem. J.* **22**, 1193.
Davydova, S. J. (1947). *Biochemistry, Leningr.*, **12**, 135.
Laki, K. (1941). *Enzymologia*, **9**, 141.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Mann, P. J. G. & Woolf, B. (1930). *Biochem. J.* **24**, 427.
Massey, V. (1951). *Nature, Lond.*, **167**, 769.
Massey, V. (1952). *Biochem. J.* **51**, 490.
Massey, V. (1953). *Biochem. J.* **53**, 72.
Michaelis, L. & Davidsohn, H. (1911). *Biochem. Z.* **35**, 386.
Michaelis, L. & Pechstein, H. (1914). *Biochem. Z.* **59**, 77.
Quastel, J. H. (1931). *Biochem. J.* **25**, 888.
Racker, E. (1950). *Biochem. biophys. Acta*, **4**, 211.