LI. THE ACTION OF SALTS ON FUMARASE. I.

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Fumarase is the name given by Batelli and Stern [1911] to the enzyme in animal tissues capable of converting fumaric acid into malic acid. It was shown by Einbeck [1919] that the reaction reached equilibrium when three-quarters of the fumaric acid had been changed, and by Dakin [1922] that the malic acid so formed was entirely in the laevo-form. Clutterbuck [1927, 1928] followed polarimetrically the production of *l*-malic acid from fumaric acid under the influence of the muscle and liver enzyme, and found that the reaction followed a linear course until the equilibrium state was almost reached. He also showed that the velocity of the action was influenced by salts, phosphates at p_H 7·2 producing a marked activation, and chlorides, bromides, fluorides and nitrates an inhibition.

Quastel and Whetham [1924] reported that fumarase was present in very small amount in *B. coli communis*. It was shown by Woolf [1929] that the fumarase activity, in the experiments of these workers, was masked by a side reaction, which could be inhibited by 2 or 4 % propyl alcohol; in presence of this inhibitor *B. coli communis* displayed great fumarase activity. Woolf reported that with the bacterial enzyme reaction curves were obtained similar to those given by Clutterbuck for the animal preparations, the velocity of conversion of fumaric acid remaining constant for the greater part of the reaction. He also showed that phosphates had an accelerating effect on the enzyme at p_H 7·4.

Although there are many known cases of enzyme activity being accelerated or retarded by salts, there are hardly any enzymes for which such effects have been studied in detail. Such an investigation has been carried out by Myrbäck [1926] on salivary amylase. As is well known, sodium chloride has a marked accelerating action on this enzyme. Myrbäck studied the effect of this salt, as well as the action of bromides, iodides, nitrates and chlorates, all of which activate the enzyme. He showed that at any given p_H increasing concentrations of the salt gave first of all an increasing activation, until a maximum effect was obtained which did not change with increase of salt. The curve relating salt concentration to increase in enzyme velocity seemed to be a rectangular hyperbola, or "Michaelis curve," suggesting that the salt,

or one of its ions, combined with the enzyme, the amylase-salt complex being more active than the free amylase. The presence of the salt did not markedly change the affinity of the enzyme for its substrate, so that the salt combined with the enzyme at some group other than that concerned in binding the substrate. On studying the effect of p_H on the activity of the free enzyme and of the various enzyme-salt compounds, he found that the latter were active over a wider p_H range than the salt-free preparation. The p_H-activity curves were related together like the dissociation-residue curves of an ampholyte, in which the p_K of the $H^{\cdot}$ and the OH' ions was shifted to various extents by the added salts. He concluded that the enzyme was an ampholyte, which was catalytically active only in its undissociated or isoelectric form. Combination with a salt altered the degree of dissociation of the enzyme, and hence the proportion of the enzyme in the active form at any given p_H.

It seemed desirable to study the effects of salts on fumarase, and the present paper gives the results of the first part of this investigation. The salts used were the phosphate, citrate and sulphate of sodium. The sodium was always present in large concentration, being used to neutralise the substrate as well as the added acid, and its influence (if any) on the reaction velocity may be taken as constant in all the experiments described. The effects observed can therefore be attributed to the added anions. The phenomena fit in exactly with the idea that the enzyme is an ampholyte acting only in the isoelectric form, and that it combines with anions, the fumarase-anion complex being an ampholyte with different p_K values, as suggested by Myrbäck for salivary amylase.

Experimental.

The enzyme preparation used throughout this work was a suspension of *B. coli communis*. The organism was grown on the surface of tryptic broth agar in Petri dishes for 2 days. The growth was taken up in distilled water and thoroughly washed by centrifuging several times. It was then again suspended in water, aerated for a few hours and shaken mechanically to ensure an even suspension. This stock preparation of the "resting" organism was stored in the refrigerator. It was generally used within a fortnight of preparation, though it retained its activity unimpaired for several weeks at least. Immediately before use the organism was again washed several times with distilled water to remove any trace of salts produced by autolysis, then made up to a suitable strength with distilled water and shaken in the machine. In this way an enzyme preparation could be obtained free from all salts or other soluble materials. The actual experiments rarely lasted more than 18 hours, and during this period there was no significant enzyme destruction or bacterial disintegration, since the reaction velocity remained constant even for a much longer period, and the enzyme remained associated with the cells, the supernatant fluid after centrifuging being entirely inactive. The effects observed, therefore, may be regarded as given by the enzyme *in vivo*.

The long initial linear portion of the curve, when the equilibrium is approached from the fumaric acid side, made it possible to take the initial velocity as proportional to the malic acid produced in a given time, even when the reaction had progressed a considerable distance. It was thus possible to obtain large polarimetric readings, and thereby to obtain a very accurate measurement of the reaction velocity. We have always obtained this initial linearity in the experiments reported below. The enzyme was therefore always working at full saturation with its substrate, so that the effects could not be due to a change in the affinity of the fumarase for fumaric acid.

For each experiment a series of reaction mixtures was made up, in which one factor was continuously varied. These series were of two kinds—those in which the effect of an increasing concentration of a salt was studied at a constant p_H, and those in which the salt concentration was kept constant, but the p_H was varied. In all cases the initial concentration of fumaric acid was $M/10$, the inhibitor being 4 % propyl alcohol. The reaction mixtures were placed in stoppered flasks and incubated at 37°. At suitable intervals 10 cc. samples were taken for the polarimetric estimation of malic acid as the molybdate compound by the method of Auerbach and Kruger [1923]. The procedure described by Woolf [1929] was followed, save that, as 10 cc. samples were taken, the addition of 5 cc. of water was not required. Also, since *l*-malic acid was the only optically active compound present, there was no need to take the control reading in trichloroacetic acid.

For a series at a constant p_H, the various constituents were made up to suitable concentrations separately, and brought to the required p_H with NaOH. The p_H of the bacterial suspension was also adjusted. The required quantities were then mixed and made up to volume with distilled water, and the p_H of the mixture was checked. For a series with varying p_H, two solutions were made up, one more acid and the other more alkaline than the extremes of the p_H range being investigated. Thus, when it was desired to study the p_H-activity curve of the enzyme in presence of $M/5$ phosphate between p_H 4·5 and 9·5, two solutions were made up, each $M/4$ in phosphate and $M/8$ in fumarate, but one acid to p_H 4·5 and the other alkaline to p_H 9·5. The solutions were mixed in various proportions to give the required intermediate p_H values. 20 cc. of each mixture was taken, and to it were added 1 cc. of propyl alcohol, 2 cc. of bacterial suspension, and 2 cc. of distilled water, making the final volume 25 cc. and the concentrations of phosphate and fumarate $M/5$ and $M/10$ respectively. The p_H of the final reaction mixture was then accurately measured initially and at the time of taking each sample. When the p_H was below 8, it was measured electrometrically by the quinhydrone method. Unfortunately the hydrogen electrode is useless in solutions of fumaric acid, so that the more alkaline p_H values had to be estimated by means of indicators. Since many of the reaction mixtures were necessarily unbuffered, the p_H often changed appreciably during the course of the

incubation, and the average of the final and initial readings was assumed to be the p_H during the experiment. But when the difference between the initial and final p_H exceeded 0·1, the experiment was rejected, except in the alkaline citrate mixtures, as noted below.

Results.

The effect of p_H on the activity of the enzyme in the absence of added salts is shown by the full line in Fig. 1. The various p_H values were obtained by neutralising the $M/10$ fumaric acid to different extents with NaOH. It is of course impossible to determine the activity of the enzyme in a completely salt-free medium, since the substrate is itself a salt. It will be seen that a symmetrical curve is obtained, covering the range between p_H 5 and 7·7, with an optimum at about 6·4.

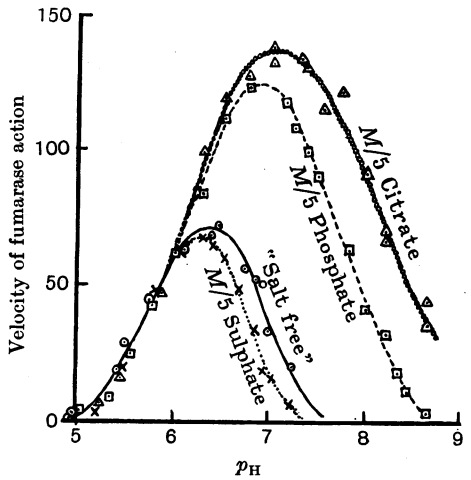

Fig. 1.

Between p_H 6 and 8·8, the addition of phosphates increases the reaction velocity. At any given p_H in this range, as the phosphate concentration is increased the activation is also increased, until a maximum effect is obtained which is the same for all higher phosphate concentrations. The results of a series at p_H 7·4 are shown in Fig. 2. It will be seen that the curve is approximately a rectangular hyperbola, suggesting that the salt combines with the enzyme, to give a more active enzyme-phosphate complex. The apparent Michaelis constant of the phosphate (*i.e.* the concentration at which half the maximum effect is obtained) at this p_H is about 0·0076 M, and the affinity (the reciprocal of the Michaelis constant) about 132. In order to relate the various curves to one another, the maximum velocity in presence of phosphate at p_H 7·4 for each enzyme preparation was taken as 100. In each series of reaction mixtures, a control was included containing the same amount of bacterial suspension in presence of $M/10$ fumaric acid and $M/5$ phosphate at

p_H 7·4, and the velocities in all the other reaction mixtures were expressed as percentages of the velocity in this control. In this way the results of all the series were made quantitatively comparable.

That an ion of the phosphate combines with the enzyme is made to appear more likely by the results at other p_H values. At 6·6 the phosphate-free enzyme itself has a considerable activity, the velocity being slightly under 50. The maximum activity induced by phosphate is about 100. The effect of varying phosphate concentration at this p_H is shown in Fig. 3. The curve is of the same type as that obtained at p_H 7·4, but the Michaelis constant is now

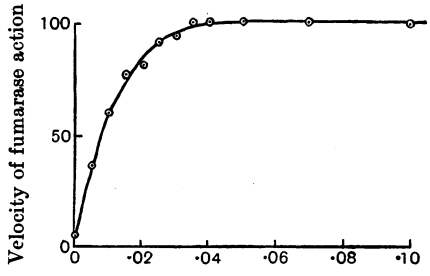

Fig. 2. Effect of phosphate at p_H 7·4.

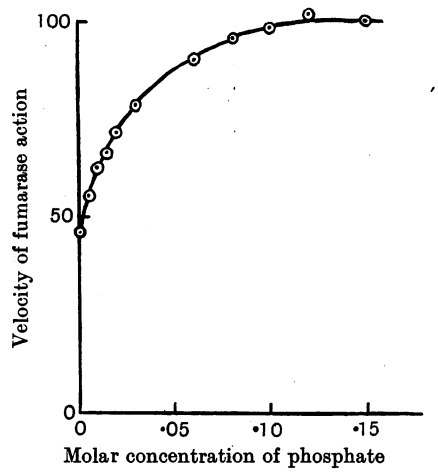

Fig. 3. Effect of phosphate at p_H 6·6.

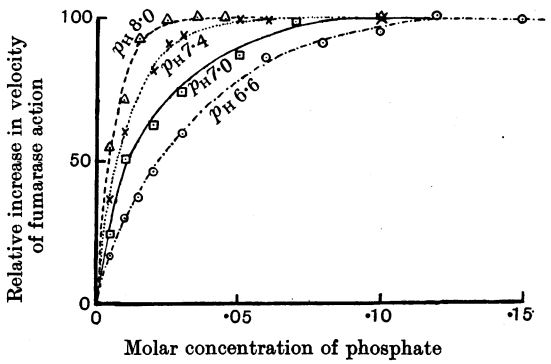

Fig. 4.

0·026 *M* and the affinity only about 38. Similar curves were obtained at p_H 7·0 and 8·0. The maximum velocity at p_H 7 is about 125 and at 8 about 45. The four curves are shown in Fig. 4 reduced to the same scale, the relative increase in velocity being plotted against phosphate concentration. The figure brings out clearly the regular rise of the affinity with p_H. In Fig. 5 is shown

the affinity plotted against p_H. The curve shown is the dissociation curve for the reaction $H_2PO_4' \rightleftharpoons HPO_4'' + H^*$, on to which the affinity at p_H 7·0 has been fitted. It will be seen that the other three points fall very well on the curve. The affinity at any p_H thus seems to be proportional to the concentration of HPO_4'' ions in the solution, making it seem extremely probable that the activating effect is due to an actual combination of the enzyme with this ion.

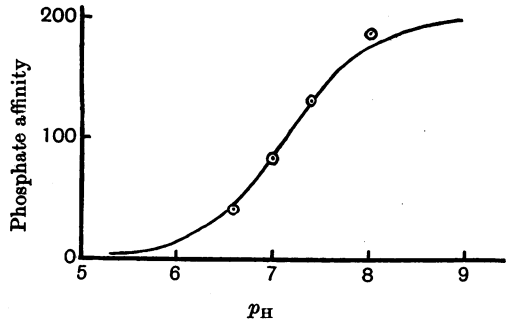

Fig. 5.

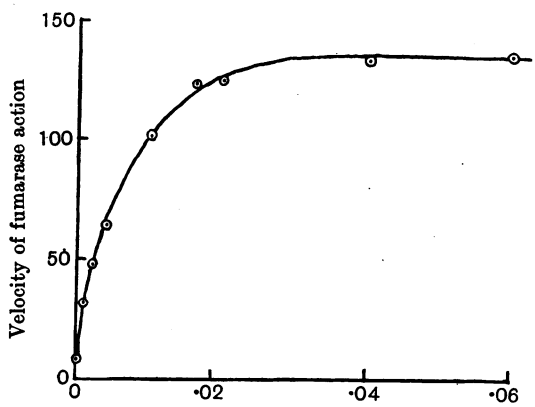

Fig. 6. Effect of citrate at p_H 7·4.

When the phosphate concentration is sufficient to give the maximum activation at any p_H, the enzyme may be regarded as saturated or completely combined with the phosphate ion. A phosphate concentration of $M/5$ is sufficient to effect this over the whole of the significant p_H range. A p_H-activity curve was therefore obtained in presence of $M/5$ phosphate, and is shown in Fig. 1. It will be seen that it is a symmetrical curve, which coincides with the "salt-free" curve between p_H 5 and 6, but then rises above it to an optimum at about p_H 6·9, after which it falls, roughly parallel to the alkaline limb of the "salt-free" curve to reach the horizontal axis at about p_H 8·8.

Citrate behaves similarly to phosphate, but gives a greater activation. Fig. 6 shows the effect of varying concentrations of citrate on the reaction

velocity at p_H 7·4. It will be seen that the maximum velocity at this p_H is about 135, as against 100 for phosphate. The effect of p_H on the citrate affinity is the opposite of that on the phosphate, the affinity rising as the p_H is decreased. The results so far obtained suggest that it is the doubly ionised citrate which is effective, but this is not yet fully worked out. $M/5$ citrate gives maximum activation at all p_H values, and a p_H-activity curve in presence of this concentration is shown in Fig. 1. The curve is similar to that obtained with phosphate, but lies a little outside it over the alkaline range, the fumarase-citrate optimum being at about p_H 7·1. Since neither fumarates nor citrates are buffers over the alkaline range of this curve, the p_H of the reaction mixtures often changed greatly. Consequently a wider latitude than 0·1 p_H had to be allowed for this part of the curve, and the individual points are therefore rather scattered, although the curve as given is probably not far from correct.

Sulphates have a retarding effect over the alkaline part of the "salt-free" curve. $M/5$ sulphate gives the maximum effect, and the fumarase-sulphate p_H-activity curve is also shown in Fig. 1, the optimum being at about p_H 6·2.

The four p_H curves shown in Fig. 1 are related to one another like the dissociation residue curves of an ampholyte, whose acid p_K remains constant but whose alkaline p_K is shifted to different extents by the combination of the various anions. The results are completely explained by the hypothesis that the enzyme is an ampholyte which is active only in its isoelectric form. The enzyme can combine with various bivalent anions, the enzyme-anion complex having an altered dissociation constant for the $H^{\cdot}$ ion. Phosphates and citrates repress the ionisation, thus increasing the proportion of the enzyme molecules in the active form at any p_H in a given range, without affecting the rate at which an active enzyme molecule catalyses the hydration of the substrate. Sulphates encourage the ionisation, thus decreasing the number of active enzyme units, and so retarding the reaction velocity. Thus the effects of salts on fumarase are precisely similar to those found by Myrbäck for salivary amylase.

Preliminary experiments have been made with several other salts, and also on the combined effects of two or more salts. The power of affecting the velocity of fumarase action seems to be possessed by very many salts, and it is proposed to report the results of further studies in another communication.

Summary.

1. "Resting" *B. coli communis*, washed free of all salts and suspended in distilled water, in presence of 2 or 4 % propyl alcohol will convert fumaric acid into its equilibrium mixture with *l*-malic acid at a linear rate for the greater part of the reaction. In the absence of added salts, the range of activity is between p_H 5 and 7·7, the p_H-activity curve being symmetrical, with an optimum at about 6·4.

2. Phosphates accelerate between p_H 6 and 8·8. If at any given p_H the increase in velocity is plotted against phosphate concentration, a rectangular hyperbola ("Michaelis curve") is obtained, suggesting that the phosphate combines with the enzyme non-competitively with the substrate. The "affinity" of the enzyme for phosphate at any given p_H is proportional to the HPO_4'' ion concentration. In presence of 0·2 *M* phosphate, which gives the maximum effect at every p_H, a symmetrical p_H-activity curve is obtained, with an optimum at 6·9.

3. Citrates behave similarly, giving a greater activation at every p_H, and an optimum at 7·1.

4. Sulphates depress the activity, giving an optimum at p_H 6·2.

5. The four p_H curves are related to one another like the dissociation residue curves of an ampholyte, in which the p_K of the OH′ ion is constant, but the p_K of the H· ion is shifted to different extents by the added anions. It seems, therefore, that the enzyme is only active when in the isoelectric state, the salt effect being due to an alteration in the proportion of the enzyme molecules in this active state at any given p_H.

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