SALT EFFECTS ON FUMARASE

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INTRODUCTION:

Inorganic ions exhibit both activating and inhibiting effects on the activity of the enzyme fumarase, whcih catalyses the malic acid-fumaric acid conversion in the Krebs cycle. Fumarase is activated by the presence of the polyvalent anions sulfate, arsenite, citrate, borate, phosphate, and arsenate (in order of increasing effectiveness), with the pH optimum shifted to the alkaline. Phosphate ion has also been observed to have an inhibiting effect.[1] The monovalent anions fluoride, chloride, bromide, thiocyanate, and iodide (in order of increasing effectiveness) inhibit the activity of fumarase, with the pH optimum shifted to the acidic. These effects had been explained by two theories. Massey[2] proposed an adsorption of the monovalent ions on the acidic groups and the polyvalent ions on the basic groups at the active site; Warren[3] proposed a general change in the enzyme's configuration due to ionic strength effects.

Neither of these theories, however, fully explains the phenomena observed by Schmidt[4] in his studies of the effects of bromide and phosphate anions on the kinetics of the fumarase reaction. Non-linear dependence of the maximum velocity and Michaelis constant was observed when the concentration of one anion was held fixed and that of the other varied. Phosphate appeared to increase the Michaelis constant, while bromide appeared to decrease both the Michaelis constant and the maximum velocity. The $(\text{initial velocity})^{-1}$ exhibited sigmoid curves when a strongly activating ion (phosphate) was varied in the presence of a strongly inhibiting ion (bromide). As a function of either component alone, however, the $(\text{initial velocity})^{-1}$ remained a linear function of concentration, thus eliminating the possibility of

allosteric effects.

Schmidt partially explained this data by treating phosphate as a classical competitive inhibitor, bromide as a classical non-competitive inhibitor, and the curves obtained as the interaction of these. However, this did not explain the sigmoid curves obtained when the initial velocity was plotted as a function of one component at a fixed concentration of the other. This research was directed towards a further systemization and explanation of these unexplained phenomena.

EXPERIMENTAL:

All initial velocity measurements were made on a Cary 14 recording spectrophotometer at a wavelength of 250 millimicrons, with a thermostated cell holder set at 27.0 degrees Centigrade. All glassware used was cleaned by washing with concentrated nitric acid, rinsing eight times with distilled water, and rinsing twice with doubly distilled water. All solutions were prepared using doubly distilled water. Solutions were pHed by dissolving the solute in less than the required amount of solvent, adding dropwise solutions of nearly saturated and 0.2M NaOH until a pH meter indicated the correct value had been reached, and then diluting the solution to volume.

The stock solutions prepared were: 1) a 250 ml solution of 1.35M $NaH_2PO_4 \cdot H_2O$ buffered to pH 7.3. 2) a set of eight dilutions from this original phosphate solution with the following ratios of solution to water: eight to one, seven to two, two to one, five to four, four to five, one to two, two to seven, and one to eight. 3) a 250 ml solution of 0.86M NaBr, pHed to 7.3. 4) a 50 ml solution of 0.077M l-malic acid (identical to that purified by Schmidt and originating from CalBioChem), pHed to 7.3. 5) a solution of 0.30 mg/ml fumarase (Sigma Chemical, rated activity 370) in 0.01M $NaH_2PO_4 \cdot H_2O$ at pH 7.3.

The phosphate solutions seemed to hold their pH well over the range of dilution used. The melting point of the l-malic acid was 103.5-104 degrees Centigrade. The fumarase was removed from its shipping solution of ammonium sulfate by a five hour dialysis at five degrees Centigrade against 0.01M sodium dihydrogen phosphate, changing the external solution once after three hours. The concentration of fumarase was determined from the absorption reading of the solution at 280 millimicrons (0.53 O.D. equals 1 mg/ml). The fumarase stock solution was kept under refrigeration and renewed every two weeks.

A typical set of initial velocity measurements was carried out in the following manner: ten test tubes (25 ml) and ten cuvettes (4 ml) were cleaned and allowed to dry. Into each test tube was pipetted 0.70 ml of the stock NaBr solution and 1.00 ml of the stock l-malic acid solution. Then 1.00 ml of each of the nine phosphate solutions was pipetted into a correspondingly labeled test tube, and 1.00 ml of water into the tenth. The test tubes were then equilibrated for twenty minutes by immersion into a 27.0 degrees Centigrade water bath adjoining the Cary. During this time, 0.50 ml of the enzyme solution was diluted to 5.00 ml with 0.01M $NaH_2PO_4 \cdot H_2O$ at pH 7.3 and equilibrated in the bath. The contents of each test tube were in turn transferred to a cuvette, 0.30 ml of the fumarase solution added, and the cuvette inverted twice to mix the contents and run in the Cary for about five minutes. The reciprocal slopes of the resultant lines were plotted vs phosphate concentration to obtain a curve of reciprocal initial velocity vs concentration. The final concentrations for this set of runs was 0.2M NaBr, 0.02M l-malic acid, 0.01 mg/ml fumarase, and phosphate concentrations varying from 0.001M to 0.501M in intervals of 0.05M.

Each final mixture retained its pH of 7.3 throughout the run, except the 0.001M mixture, which became more acidic during the run

and is useless except as a test run solution. All other solutions yielded slopes reproducible to three percent. The enzyme solution was found to have constant activity over the fifty minutes it took to complete the measurements. No change in results was noted if the enzyme solution and l-malic acid were interchanged in order of addition (i.e. if the enzyme was allowed to equilibrate with the salt solution for twenty minutes and then the l-malic acid added to initiate the reaction). Greater accuracy could have been obtained by preparing larger volumes for each run, but three percent was thought sufficient for the purposes of this investigation.

In the initial stages of the investigation, an attempt was made to reproduce Alberty's[5] determination of the equilibrium constant of the malic-fumaric conversion as a function of phosphate concentration at pH 7.3. Despite the use of very rigid experimental conditions (doubly distilled water, immediate use of simultaneously prepared solutions, equilibration of the enzyme with the solution before the addition of l-malic acid), the results obtained were irreproducible with time. This was hypothesized to be possibly due to some sort of bacterial growth in the phosphate stock solutions, although this seems unlikely in view of the short time periods involved. No such irreproducibility was found for the initial velocity measurements, but it is possible that over long periods of time the phosphate stock solutions will deteriorate. Care should be taken to ensure that this does not interfere with the experimental results.

RESULTS:

In contradiction of Schmidt's experimental results, a linear dependence of reciprocal initial velocity on phosphate concentration for a fixed bromide concentration was found. Over the large set of runs made, a least-squares plot approximation gives an excellent straight line with low variance. The experimental conditions for

these runs differed from Schmidt's in that Schmidt used only distilled water from the tap, centrifuged the enzyme to remove the shipping solution rather than dialyzing it, mixed his phosphate solutions independently rather than by dilution, and used only the buffered phosphate solutions to pH the entire mixture.

In attempts to duplicate Schmidt's sigmoid curves, each of the more rigorous conditions employed in this investigation was relaxed in turn until Schmidt's experimental conditions were duplicated in every possible detail. No change was observed even when enzyme from the same company Schmidt had used (CalBioChem) was left in the shipping solution and used, or when distilled water from the tap was used to prepare the solutions (a dead mouse had recently been found floating in the distilled water tank). In fact, no experimental procedure yielding reproducible results could be found which yielded non-linear reciprocal velocity curves.

DISCUSSION:

Although the experimental results obtained in this investigation disagree with Schmidt's experimental results in that particular area, they agree completely with the rest of Schmidt's results and with the general theory of salt interaction Schmidt proposed. If phosphate is treated as a classical competitive inhibitor and bromide as a classical non-competitive inhibitor, all of Schmidt's other plots and the linear dependence of reciprocal initial velocity on phosphate concentration in the presence of bromide can be explained. For a thorough exposition of this theory of anion inhibition, pages 95 through 103 of Schmidt's thesis should be consulted. It should be noted that this new theory of Schmidts offers facets (which are confirmed by his experimental findings) that neither the Massey theory nor the Warren theory can explain.

Since Schmidt obtained extensive sets of sigmoid curves, it is surprising that his results could not be duplicated. Use of identical experimental techniques removed that as a source of change. Either the identical chmeicals or chemicals from the same company were used. Perhaps an effector molecule capable of causing allosteric behavior (such as glycerol phosphate for hemoglobin) was present in one of his basic components, and caused the sigmoidicity of his plots. Unfortunately, no such contaminant could be found, and if such existed, it must have been through an accidental chance that cannot be duplicated.

A second possibility for the existence of these sigmoid curves is that the error in each point might have been high enough so that the plot was, in fact, linear. This cannot be disregarded in that the pHing Schmidt did was so perfunctory and the lines from which he obtained the slopes so short and slight in slope. It is quite possible that a more careful experimental determination would have revealed a straight line.

FOOTNOTES:

[1]R.A. Alberty, V. Massey, C. Frieden, and A.R. Fuhlbrigge, JACS 76, 2485 (1954).

[2]V. Massey, Biochem J. 53, 67(1953).

[3]J.S. Warren, L. Stowring, M.F. Morales, J. Bio. Chem. 241 (2), 309 (1966).

[4]D.E. Schmidt, Part II "Salt Effects on Fumarase" of Doctoral Thesis submitted to Caltech (March, 1967).

[5]Bock and Alberty, JACS 75, 1921 (1953).

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