A STUDY OF THE INFLUENCE OF pH
ON THE ENZYMATIC DECARBOXYLATION
OF OXALACETIC ACID

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A STUDY OF THE INFLUENCE OF pH ON THE ENZYMATIC DECARBOXYLATION OF OXALACETIC ACID

By

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INTRODUCTION

The study of the influence of variations in pH on the rate of an enzyme catalyzed reaction and the determination of the optimum pH for activity are fundamental steps in the characterization of an enzyme.

Such data are available for most of the enzymes which have, so far, been investigated in detail. However, with the exception of some of the proteolytic enzymes, (1), little has been discovered concerning the mechanisms by which the pH of the medium influences enzymatic activity. Theoretically, such influences might be classified as follows:

(i) as an effect of changes in the pH of the medium on the ionization of the substrate, and

(ii) as the effect of changes in pH of the medium on the properties of the apo-enzyme (protein), the co-enzyme or both.

The amphoteric character of proteins would lead one to expect that any change in pH would change the nature of all enzymes to some extent. On the other hand, the non-ionizable, comparatively non-polar character of a few biological substrates, such as the sugars, would not suggest any influence of pH on the nature of the substrate as an important factor in the variation of enzyme activity. If however, the whole group of naturally occurring substrates is examined, it can be seen that non-ionizable substrates constitute the exception rather than the rule. The ionizable character of most of these compounds would lead one to expect that changes in the acidity of the medium could affect both the nature of the substrate and of the enzyme. Of course, it always remains to be

(1)
seen whether effects of changes in the ionic nature of the substrate or
effects on enzyme and co-enzymes are the more important factors in deter-
mining the rate of the reactions at different pH.

Among the important biological enzyme-substrate systems, the indi-
vidual reactions of the tricarboxylic acid cycle, are characterized by
their ionizable substrates and the rather limited range of pH in which
enzyme activity may be demonstrated. Oxalacetic acid, a key substance
of this metabolic cycle, may undergo enzymatic decarboxylation to pyruvic
acid and carbon dioxide, in a reaction which is markedly influenced by
changes in the pH of the medium. In addition, this is a system which is
relatively easy to study, because the reaction may be followed by measur-
ing the liberation of carbon dioxide.

It was therefore considered of interest to study the effect of varia-
tions in pH on the rate of enzymatic decarboxylation of oxalacetic acid
to ascertain (1): the relationship between pH and the shape of the pH —
activity curve, and

(ii), to utilize this data to gain an insight into the mech-
anism of the decarboxylation of the substrate.
HISTORICAL INTRODUCTION

In 1934, Northrop and Kunitz (2), in studies on the isolation and characterization of crystalline trypsin and pepsin, were able to explain the existence of regions of optimal hydrogen ion concentration for the hydrolysis of different protein-substrates.

To explain the acid pH optimum shown by pepsin, it was assumed that this enzyme was only effective when acting on the positively charged protein-substrate. As evidence for this, it was shown that the titration curve of different substrate-proteins, over a given pH range, coincided entirely with the curve obtained for amount of peptic hydrolysis of the same substrate.

In a similar manner, the pH optimum of approximately eight, shown by trypsin, acting on several proteins, could be explained. For this, it was assumed that this enzyme only acts on the negatively charged form of the substrate, and that the enzymatic activity is in proportion to the amount of this form present at a given pH. However, the pronounced decrease in activity shown by this enzyme above pH eight, seemed to be due to an action of base on trypsin itself, since the enzyme could be shown to exist in an active and inactive state, which were in an equilibrium, which was shifted to the inactive form at pH above eight. In this case then, the combination of two factors, each conditioned by pH, but opposing one another in their effects on the rate of the reaction, explained the typical, bell-shaped character of the curve of trypsic activity vs. pH.

(3)
In 1926, Willstaetter, Grassmann and Ambros (3), with similar experimental evidence, indicated that the close relationship between the optimal pH for the action of papain, and the iso-electric point of its protein substrate could be used to postulate that the enzyme was only effective when acting on a protein substrate with no overall charge.

Moelwyn-Hughes (4), on basis of a series of comparisons between optimal enzyme activities and their iso-electric points observed that all enzymes are most effective catalysts within a range of two pH units from their iso-electric points. This would confirm the old postulate of Michaelis (5), that enzymes are only active when in the net-uncharged state.

While an appreciable amount of research has been done concerning the mechanism of the influence of pH on the activity of the proteolytic enzymes, little is known concerning the influence of H—ions on other enzyme systems.

Chance (6) working with peroxidase and catalase systems has shown that in the system hydrogen peroxide—catalase, with formic acid acting as the electron donor, catalase shows a specific requirement for the undissociated form of formic acid. Working at low pH ranges, to prevent influences of enzyme denaturation on the rate of the reaction, Chance was able to show that the rate was practically constant with increasing pH. However, he observed that from the pH where formic acid begins to dissociate to an appreciable extent, onwards, a decrease in enzymatic activity set in, which could be shown to parallel the titration curve of formic acid. Therefore it was postulated that formic acid would only function in this reaction while in the undissociated state, and that the amount of reaction produced was proportional to the availability of this form.

It is interesting to note here that changes in pH within a given range
had no effect on the rate of the enzyme catalyzed reaction, although there must have been, undoubtedly, a change in the charge on the protein.

The enzymatic decarboxylation of oxalacetic acid was first demonstrated in 1941, by Krampitz and Werkman (7), who showed that lysed cells of Micrococcus lysodeikticus promoted the change of this acid to pyruvic acid and carbon dioxide. The enzyme responsible for the reaction, has since then been partly purified, and was shown to have two relatively sharp pH optima at pH 5.2 and pH 7.0 (8).

Similar enzymatic activity was found in pigeon liver extracts, which were shown to actively catalyze the decarboxylation of oxalacetic acid in the presence of Mn²⁺ (9). The enzyme responsible for this reaction has been shown to have its optimum activity at pH 4.5 (10).

Oxalacetic carboxylase has also been isolated, in a highly purified form from Azotobacter vinelandii (11). A broad pH optimum, lying around pH seven has been demonstrated for this preparation. Mn²⁺ or Co²⁺ were found to be required for activity, in this case.

Vennesland and co-workers have obtained extracts having oxalacetic acid carboxylase from a variety of higher plants; in most cases Mn²⁺ or Co²⁺ have been found to be needed for the activity of the preparations.

The preparation obtained from parsley roots (12), has been investigated in more detail, and is believed to have a pH optimum around five. The rate of the enzymatic reaction, which apparently follows first order kinetics at low substrate concentrations, can be accelerated by a variety of divalent cations, as shown by Speck (13).

The decarboxylating activity of certain crystalline seed globulins
has first been reported by Vennesland and Felsher (14). It was observed by these authors, that the enzymatic activity of a preparation obtained from squash seeds, could not be enhanced by the addition of $\text{Mn}^{++}$, in contrary to the results reported for most of the enzymes obtained from the other sources listed. Byerrum, Brown and Ball have shown that a similar preparation, contained 0.12 milligrams of manganese per 100 grams of protein and that this metal was not lost by a repeated washing and dialysis procedure (15). It was also shown by the authors, that the activity of the enzyme obtained from squash seeds could be influenced by the ionic strength and the basic character of the buffer used in the reactions.

Steinberger and Wepsheimer (1b) have studied the $\text{Mn}$-ion catalyzed decarboxylation of dimethyl oxalacetic acid in an effort to produce a model system which could explain the enzymatic decarboxylation of oxalacetic acid. According to the results presented by the authors, the diion of dimethyl oxalacetic acid participates in the reaction by forming a chelate complex with the metal ion. In a previous work (17), the same authors had demonstrated that the spontaneous decarboxylation of the monoethyl-ester of dimethyl oxalacetic acid cannot be accelerated by the addition of metal ions. It is postulated by the authors that since the ester cannot form a complex with added metal ions, no catalytic effect can be expected from them in this case.
EXPERIMENTAL

I - The Influence of Ionic Strength on the Shape of the pH vs. Activity Curve of Oxalacetic Acid Carboxylase.

In those cases where determinations of the optimum pH for oxalacetic acid carboxylase activity have been reported (10, 11, 12) the experiments had been conducted in buffers of equal molarity. Apparently however, no precautions had been taken to avoid the variation of the ionic strength of the buffers, at different pH. In view of the results of Byerrum, Brown, Ball (15), in which it was demonstrated that increasing ionic strength of the system decreases enzymatic activity, it was considered of interest to determine whether the shape of the pH vs. activity curve, obtained using acetate buffers could be a result of the variation in acetate ion concentration, in the different, equimolar buffers.

In the following experiments, the velocities of decarboxylation of a series of enzymatic reactions, conducted in buffers of equal ionic strength, but different pH, were evaluated. The resulting pH vs. activity curves were compared with corresponding results obtained using a series of buffers of equal total molarity. The rates of the enzymatic reactions were determined in a Warburg apparatus, using a substrate of oxalacetic acid, prepared by the method of Schneider (13), and purified by recrystallizations from a warm, acetone-benzene mixture. The enzyme preparation used, was the crystalline globulin isolated from the seeds of Cucurbita pepo, according to the method of Vicken et al. (19). A similar preparation
had first been shown to have oxalacetic acid decarboxylating activity by Vennesland and Felsher (14). Activities were determined by measuring the increase in pressure resulting from the carbon dioxide evolved after given intervals of time. As incubation vessels, one side-arm Warburg flasks, of 15 milliliter capacity were employed. The enzyme was dissolved or suspended in a buffer of desired pH, and a volume containing 3.1 milligrams of protein, was pipetted into the Warburg flask. One tenth milliliter of oxalacetic acid containing one milligram of this substrate, was added to the side arm. Since fairly diluted buffers were employed in all experiments, it was considered advisable to neutralize the solution of the substrate by adding to it enough sodium hydroxide to bring the pH of the solution to approximately four. The oxalacetic acid was dissolved immediately before use and the Warburg flasks were filled and incubated at the same time, since the spontaneous decarboxylation of a solution of this acid was shown to be fast enough to promote a significant decrease in its concentration, over a period of ten minutes. An additional quantity of buffer, was added to give a final volume of three milliliters in the flask. After a ten minute equilibration period at 30° (±1) C, zero-time readings were taken, and the reaction started by tipping the flask to mix enzyme and substrate. Readings were taken at desired time intervals. Except for pauses necessary for the readings, the flasks were constantly shaken at the rate of four oscillations per second. To account for the spontaneous decarboxylation of oxalacetic acid during the enzymatic reaction, control runs, using an enzyme solution which had been heated for 2 1/2 minutes in a boiling water-bath were made at each pH. Experiments
Figure 1. Activity of Oxalacetic acid Carboxylase in acetate buffers of varying pH and constant ionic strength.

Ordinate is activity expressed as the first order rate constant, K/min (based on 5 min. readings) 1 ml. of enzyme (3.12 mg.). 0.1 ml. (1 mg.) oxalacetic acid, 3/4 neutralized with sodium hydroxide. Final volume in flask 3 ml. Final ionic strength of buffer: ● 0.006; ○ 0.0096; ◯ 0.012; ● heat inactivated enzyme (in 0.012 buffer).
Figure 2. Activity of Oxalacetic Acid Carboxylase in acetate buffers of varying pH, varying ionic strength and constant molarity.

Ordinate is activity expressed as the first order rate constant, K/min. (based on 5 min. readings)

1 ml. of enzyme (3.12 mg.). 0.1 ml. (1 mg.) oxalacetic acid, 3/4 neutralized with sodium hydroxide. Final volume in flask 3 ml. Final molarity of buffer, 0.06 ○ active enzyme; ● heat inactivated enzyme.
were always run in duplicate or triplicate. The results of three series of experiments, using acetate buffers of ionic strength of 0.012, 0.0096 and 0.006 respectively, are shown in Figure 1., where velocity, expressed as the first order rate constant, is plotted against pH. Figure 2 shows the results of a series of experiments, covering the same pH range, in which, however, the total molarity of the buffer was maintained constant. By comparing the two sets of data, it can be seen that, over the same range of pH, the shape of the pH vs. activity curve remains essentially unchanged regardless of whether the reactions are run in buffers of constant molarity or of constant ionic strength. It can therefore be concluded that the varying amount of acetate ion present in the equimolar buffers of different pH is not the factor responsible for the bell-shaped character of the pH vs. activity curve. The differences in the height of the curves in Figure 1, would however, indicate that ionic strength definitely has an effect on the rate of the enzyme catalysed reaction.

It should be observed that, although in these experiments, the ionic strength of the buffering system was maintained the same at every pH, changes in the total ionic strength of the medium, according to pH, could not be entirely accounted for. Such changes would be principally produced by two factors: (i) the influence of pH on the formation of the dissociation products of oxalacetic acid, and (ii) the effect of pH on the charge and ion adsorption power of the protein. The first factor, e.g., the variation in the amounts of the mono and divalent anions of oxalacetic acid present in different solutions of pH between 3.7 and 4.7, could be evaluated by the method discussed later (p.16). The effect of this
Figure 3. Variation of the order of the enzymatic reaction with time.

1 ml. of enzyme (3.12 mg.); 0.1 ml. (1 mg.) oxalacetic acid, (O.A.A.) 3/4 neutralized with sodium hydroxide; final volume in flask 3 ml. Final ionic strength of acetate buffer, 0.0096. ◇ pH 3.50; ◆ pH 3.75; ○ pH 4.00; ● pH 4.25.
factor on variations in ionic strength, was shown to be relatively small when compared with the ionic strength of the buffers used, and could be disregarded in this case. On the other hand, it is apparent, that an evaluation of the ionic strength contribution of the protein would involve serious difficulties, and therefore, such a determination was not attempted, in this work.

First order rate constants were used in these experiments, since they had been shown to be a suitable means of expressing the rates of the enzymatic reactions (12, 15). It will be noted however, that the values reported here are always those calculated for the early stages of the reaction e.g. 300 seconds. This seemed necessary, since as plots of the log of remaining substrate against time (Figure 3) indicate, most reactions show a deviation from their first order character after certain intervals of time. This effect can probably be explained by the decrease in overall ionic strength and progressive exhaustion of the buffer, as a consequence of the decarboxylation of the substrate.

II — The Influence of pH on the Solubility of the Enzyme.

Vennesland (12) had observed that the active crystalline globulin fraction of squash seeds became inactive and insoluble in acetate buffers of pH above five.

Later, Byerrum, Brown and Ball (15), were able to show that this enzyme was in suspension in acetate buffers of pH above 4.5, and in solution at lower pH's. At the time however, no attempts had been made to correlate enzyme solubility and oxalacetic acid carboxylase activity.
Figure 4. Solubility of Oxalacetic Acid Carboxylase at different pH

Line A: Protein solubility vs. pH. Ordinate is solubility, expressed as percentage of the amount of protein dissolved at pH 4.13. 53.4 mg. of protein in 50 ml. of acetate buffer of ionic strength 0.02; room temperature; dissolved protein determined in supernatant after centrifugation.

Line B: Enzyme activity vs. pH. Ordinate is activity, expressed as percentage of the first order rate constant, K/min(at pH 4.30, obtained from Figure 1. 1 ml. of enzyme (3.12 mg.); 0.1 ml. (1 mg.) oxalacetic acid, 3/4 neutralized with sodium hydroxide; final volume in flask 3 ml. acetate buffer ionic strength 0.012.

Line C: Protein solubility vs. pH. Acetate buffer of ionic strength 0.1. Other conditions are identical with those for Line A.
Therefore, in this work a measurement of protein solubility in acetate buffers of different pH, was made. For this, weighted amounts of enzyme were dissolved in equal volumes of acetate buffers, of pH’s between 4.10 and 5.00. After standing for thirty minutes at room temperature, the mixtures were centrifuged and the supernatant analyzed for dissolved protein, according to the semi-micro Kjeldahl method outlined by Clark (20). The ammonia produced upon alkalization of the digested mixture, was distilled into ten milliliters of a 4 percent boric acid solution and titrated with 0.02 normal hydrochloric acid, using a methyl red - methylene blue indicator. The total nitrogen content of the globulin was determined by the same method, and found to be 19.1 percent, when calculated for a protein sample which had been dried for 12 hours at 110°C. This value is fairly close to the value of 18.5 percent reported by Vickery et al. (19).

From the weight of the ammonia calculated from the titration data, the protein contents of each solution were ascertained. It was shown that in a buffer of ionic strength 0.02 and pH of 4.13, 94.0 percent of the protein had gone into solution. Curve A of Figure 4 shows the amounts dissolved at the different pH’s, expressed as percent of the weight of protein dissolved at pH 4.13. Curve B on this figure shows the relative rates of the enzymatic reactions, at different pH, obtained from data of Figure 1. In this plot, the first order rate constant at pH 4.30 was considered to be 100 percent enzyme activity, and all other rates were calculated as a percentage of this activity. By comparing the two curves it can be seen that for pH above 4.30, the variation in enzymatic activity is proportional to the decrease in protein solubility over the same pH range. Curve C of Figure 4, represents protein solubility, measured in a
series of buffers of 0.1 ionic strength, under otherwise identical conditions as those used for curve A. The results indicate that the enzyme becomes progressively more insoluble, even at a lower pH, as the ionic strength of the buffer is increased. This relationship might explain the slight shift in the peaks of the curves of Figure 1. However, no definite proportionality is postulated here, and further work would seem indicated to settle this question.

III - The Influence of pH on the Acid–base Binding Power of the Protein and the Relation of this Factor to Enzyme Activity.

As a subsequent step in the investigation of how changes in pH affected the different components of the system, the titration curve of the protein was determined by potentiometric titration. One gram of squash seed globulin was dissolved in 100 milliliters of 0.02 normal hydrochloric acid, and the mixture diluted to 200 milliliters with carbon dioxide-free, distilled water. The solution was titrated with alkali, by adding measured amounts of 0.05 normal, carbon dioxide-free sodium hydroxide to the solution, using constant stirring. The pH was ascertained after each addition, using a Beckmann pH-meter. A five-minute interval was made between the addition of the alkali and the pH reading; this time was considered sufficient to reach the equilibrium point, since no subsequent changes in the pH of the solution took place up to twenty minutes. The results plotted as milliequivalents of $(H^+)$ bound per gram of protein at each pH, are shown in Figure 5. In this plot, the values in the ordinate were calculated by formula $H = T - (M + N)$, where $T$ represents the total milliequivalents
Figure 5. Influence of pH on the acid binding power of squash seed globulin.

Ordinate values are milliequivalents of hydrogen ions bound by 1 g. of globulin. 1 g. of globulin dissolved in 100 ml. of 0.02 M HCl, potentiometrically titrated with 0.05 M NaOH.
of hydrochloric acid added to the solution, $M$, the total milliequivalents of base added at each point, and $N$, the milliequivalents of free ($H^+$) present in the medium, as obtained from the pH reading. Although determinations of this nature are usually carried out under a nitrogen atmosphere, this measure was deemed dispensable in this case, since the titration was not carried beyond a pH of approximately five, which is acid enough to prevent the presence of more than minimal amounts of carbon dioxide in the solution. The smooth character of the curve of Figure 5, indicates that no irregular changes in the acid binding power of the protein occur in the range between 3.5 and 4.75, the region of enzymatic activity.

Steinberger and Westheimer (16), had shown that the metal-ion catalysed decarboxylation of dimethyl oxalacetic acid can be accelerated by pyridine, and postulated that for this, the metal-ion coordinated with two molecules of pyridine. Also, it had been shown by Byerrum, Brown and Ball (15), that a metallo-protein was involved in the enzymatic decarboxylation of oxalacetic acid. By analogy and as an inference from these conclusions, it may therefore be postulated that for the enzymatic reaction, a coordinate link between the metal-ion attached to the oxygen atoms of the carbonyl and carboxyl groups of oxalacetic acid, and a basic group on the protein, is formed. Therefore, changes in the charge of these groups with pH, might be expected to influence the rate of the enzymatic reaction. However, in the pH range between 3.5 and 4.75, essentially only the carboxyl groups on the protein are being titrated, and no, or very little influence is exerted on the dissociation of ($-\text{NH}_3^+$) or other
basic groups on the protein, since all of these groups have $pK'$ values above seven (21). Of course, the effect of changes of pH on other groups on the protein cannot be predicted so readily, and their influence on the activity of the enzyme may be more or less significant. However, the even shape of the titration curve, makes it seem reasonable to admit that changes in the acid-base binding power of the protein over the pH range between 3.5 and 4.75, would only affect the enzymatic activity to a very slight extent.

III - The Influence of pH on the Ionic Dissociation of Oxalacetic Acid and the Relation of this Factor to Enzymatic Activity.

In looking further for an explanation for the increase in enzymatic activity between pH 3.5 and 4.5, it was considered of interest to examine whether this behaviour could be due to a specific affinity of the enzyme for one of the three forms in which oxalacetic acid can be present in solution, namely the undissociated acid, $H_2A$, the monovalent anion, $HA^-$, and the divalent anion, $A^{2-}$. Since a search of the literature showed that no titration curves, nor pH values for oxalacetic acid had been made available so far, it was decided, for this work, to determine these values.

The electrometric titration of oxalacetic acid was carried out in the following way: 50 milligrams of twice recrystallized oxalacetic acid was dissolved in 50 milliliters of distilled water and the solution placed in a water-bath maintained at 20°C. The titration was carried out by adding measured amounts of 0.038 normal sodium hydroxide to the solution and taking the pH of the mixture after each addition. A laboratory type Beckmann pH meter was used for all measurements. Mixing of the
Figure 6. Titration curve of Oxalacetic Acid

Abscissa values are ml. of base added at each pH. 50 ml. of 0.0073 M oxalacetic acid titrated with 0.038 M NaOH. Temperature 20.0° (±0.1°) C.
Figure 7. Titration curve of Oxalacetic Acid.

Abscissa values are ml. of base added at each pH. 50 ml. of 0.0071 M oxalacetic acid titrated with 0.038 M NaOH. Temperature 30.0° (± 0.1°)C.
solutions was provided by passing a continuous stream of nitrogen through the mixture. The whole process was carried out as rapidly as possible, in order to prevent a significant decrease in the concentration of the oxalacetic acid by spontaneous decarboxylation. The results, as well as those of a second series for titrations carried out at 30°C (21), using otherwise identical conditions, are plotted in Figures 6 and 7, respectively. It can be seen that in both cases the inflection points occurred between pH 7.5 and 8.0; this point was therefore considered the equivalence point and the exact molarity of the oxalacetic acid was calculated from the milliequivalents of sodium hydroxide added up to this point. Since the smooth character of the titration curves made the graphical evaluation of the dissociation constants impossible, an algebraic method was employed (22). Combining the expressions of the law of mass action with experimental data obtained from the titration curves, the values for $K_1$ and $K_2$ could be evaluated. An example of such a calculation is presented in the appendix of this work. The values for $K_1$ and $K_2$ obtained are summarized in Table I:

### Table I

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<th>(20^\circ C) (±1)</th>
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<td>Dissociation Constant</td>
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(15)
Figure 7a. Influence of pH on the dissociation of oxalacetic acid.

Ordinate values are concentration of the monovalent \([HA^-]\) and divalent \([A^2-]\) anions of oxalacetic acid in moles per liter. Total concentration of oxalacetic acid 2.52 M/l; temperature 30.0°(± 0.1°)C. ○ concentrations of \([A^2-]\); ○ concentrations of \([HA^-]\).
It should be noted that these values do not represent true "activity" dissociation constants, but should rather be regarded as "concentration" constants, since no corrections for activities of the ions involved in the calculations, were made. The errors in the determinations thus produced are however, appreciably lessened by the fact that very dilute solutions of oxalacetic acid were used.

It may be observed that the pH value of oxalacetic acid is 3.96, which is approximately in the middle of the pH range (3.5 - 4.3) over which the rate of the enzymatic decarboxylation of this substrate was increasing, as shown in Figure 1. Figure 1 shows the plot of pH against the concentration of the mono and divalent anions of oxalacetic acid calculated from the experimental pH values. An examination of these data suggested that the divalent anion of oxalacetic acid is the species which undergoes enzymatic decarboxylation, and that the rate of this reaction depends on the availability of this ion.

To provide experimental evidence for this postulate, it was considered of interest to determine the variation in the rate of the enzymatic reaction in experiments at different pH, in which the initial concentration of the divalent anion ([A²⁻]), was maintained the same at each pH. The amounts of undissociated acid (H₂A), which had to be added in each case, to obtain a mixture, 1.20 x 10⁻³ molar in (A⁻), at the various pH, were calculated by the equation:

\[
[H₂A] = [A⁻] \left( \frac{[H^+]^2}{K_1 K_2} + \frac{[H^+]}{K_2} + 1 \right)
\]

(16)

This formula was obtained by solving equations A) and B) below, for [H₂A].
Figure 8. Activity of oxalacetic acid carboxylase in solutions of different pH, containing equal amounts of the divalent anion (A²) of oxalacetic acid.

1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, 3/4 neutralized with sodium hydroxide; acetate buffer ionic strength 0.02; final volume in flask 3 ml.; final concentration of dion (A²) 1.20 x 10⁻³ M/1. ◆ pH 3.72; ○ pH 3.89; ♦ pH 4.16; ● pH 4.43; ▲ heated enzyme.
Figure 9. Activity of oxalacetic acid carboxylase in solutions of different pH, to which equal initial amounts of undissociated oxalacetic acid (H$_2$A) were added.

1 ml. of enzyme (3.12 mg.); 0.1 ml. (1.3 mg., 9.85 μM) oxalacetic acid, 3/4 neutralized with sodium hydroxide; acetate buffer ionic strength 0.02; final volume in flask 3 ml.; Θ pH 3.72; ● pH 4.16; ○ pH 4.43; ▲ heated enzyme.
The rates of the reactions were determined using the Warburg procedure already described (p. 7). Acetate buffers of ionic strength 0.02 were used. This ionic strength value represents a compromise between a buffer which would be dilute enough not to inhibit enzymatic activity, but at the same time able to prevent significant changes in the pH of the reaction, as a result of the change of oxalacetic into pyruvic acid. The results of these determinations are shown in Figure 8, in which reaction rates are expressed as $\mu$ liters of carbon dioxide evolved after given times of reaction. It was considered admissible to use this form of expressing the initial velocity of the reactions, since Figure 8 had shown that approximately a straight line was obtained when $\mu$ liters of carbon dioxide were plotted against time for less than three minutes of reaction.

The results of a series of determinations, run under conditions identical with those in the last experiments, but in which the concentration of the undissociated acid added was kept constant over the pH range, are plotted in Figure 9. It should be noted that if the same amount of undissociated acid is added to buffer mixtures of different pH, different amounts of the divalent anion ($A^-$) will be present in each case, according to expression (C).

The values for decarboxylation in the presence of heat inactivated enzyme are also shown in Figure 9. The amounts of carbon dioxide evolved in these experiments, which were essentially produced by the spontaneous
decarboxylation of the substrate, were always very small, since the rate of the reactions was followed only for short times. This induced a rather large percentage discrepancy between duplicate determinations at the same pH. These differences, at a given pH, were often larger than the differences between the averaged results of two series of determinations made at different pH's. Since the evolutions observed in these determinations were always small, when compared with those obtained, using the non-boiled, active enzyme, it was considered admissible to average all values obtained after given times of reaction and plot them on the corresponding curve of Figure 9. It will be noted that although blank determinations, employing boiled enzyme solutions, were made for each of the experiments outlined in this work, the values thus obtained were merely listed, but never subtracted from values corresponding to the evolutions of carbon dioxide obtained when non-boiled, active enzyme was used. Since Steinberger and Westheimer had indicated that, in the absence of a catalyst, the decarboxylation of dimethyl oxalacetic acid proceeds chiefly through the monovalent anion (H$_2$CO$_2^-$), it did not seem valid to assume that a similar process occurred during the enzymatic reaction. Although experimental evidence is definitely lacking at this point, it is believed that during the enzymatic process no reaction comparable to the uncatalyzed decomposition of oxalacetic acid occurs. Only on the basis of these assumptions did the neglect of the values for the carbon dioxide evolution, using boiled enzyme solution, seem justified.

For the purposes of comparison the results of Figures 8 and 9, are combined in Figure 10. In this graph, values on the ordinate represent
Figure 10. Comparison of the influence of pH on the activity of oxalacetic acid carboxylase acting on solutions containing respectively varying and constant concentrations of the divalent anion ($A^2$) of oxalacetic acid.

Ordinate is activity expressed as μl of CO$_2$ evolved in the initial 150 sec. of the reaction (Q CO$_2$). 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, $3/4$ neutralized with sodium hydroxide; acetate buffer ionic strength 0.02; final volume in flask 3 ml.

- Final concentration of diion $1.20 \times 10^{-3}$ M/l.
- 9.85 M of oxalacetic acid added.
...liters of carbon dioxide evolved after 150 seconds of reaction. The pH of the corresponding reactions is shown on the abscissa. These results show that the availability of the divalent anion of oxalacetic acid, is not the only factor responsible for the increase in the rate of the enzymatic reaction between pH 3.7 and 4.3. It can be seen that when the concentration of this ion is maintained constant, changes in pH still result in variations in the rate of the corresponding reactions.

However, if the results of Figures 8 and 9 are compared as in Figure 10, it is seen that the rates of reactions, in which the concentration of the divalent anion was the same in every case, changed more slowly with pH, indicating that although the availability of the \( A^2 \) ion influenced the rate of the enzymatic reaction, this factor could not be the only one responsible for this effect. In consequence, it seemed necessary to look for other variables of the system, which were also affected by changes in pH within the range of enzymatic activity, and as a result could be expected to influence the overall variation in the rate of enzymatic reaction. Since the change of the charges on the enzyme with pH, had appeared not to affect the rate of the reaction to an appreciable extent, it was postulated that the effect shown in Figure 1, could be produced by an inhibition of the reaction by the monovalent anion of oxalacetic acid. Supporting this hypothesis, an examination of Figure 7 showed that the concentration of the monovalent ion present in solutions of different pH, decreased most rapidly between pH 3.5 and 4.5. Therefore, if an inhibitory influence could be ascribed to this ion, this effect would be more pronounced at regions of lower pH, and would decrease with a decrease in...
acidity. Assuming then, that the availability of the two forms of oxalacetic acid, \((HA^-)\) and \((A^-)\) existing in a mixture at a given pH, are the factors determining the rate of the enzymatic reaction between pH 3.7 and 4.3, an equation of the form \(\frac{dCO_2}{dt} = k \left( \frac{[A^-]^m}{[HA^-]^n} \right)\) should express the rate of the carbon dioxide evolution.

The possible inhibition of the reaction by \((HA^-)\), could be evaluated from the data given in Figure 8, in which the concentration of this ion is varied, but \((A^-)\) remains constant over the pH range. To accomplish this, Figure 11 was plotted, in which the log of \([HA^-]\) present in a mixture at a given pH, is plotted against the log of the rate of decarboxylation. Again, the \([HA^-]\) present in each case was obtained from formulas A) and B) (p. 17). The slope of the line thus obtained was evaluated by the method of least squares, and found to be -0.23. Therefore:

\[
\log \text{rate} = -0.23 \log [HA^-] + \log k_2, \quad \text{and} \quad \text{under conditions of constant } [A^-], \quad \text{Rate} = k_2 [HA^-]^{-0.23}, \quad \text{where rate is expressed as } \mu \text{ liters of carbon dioxide evolved per 150 seconds of reaction, and under these conditions } k_2 = 0.38, \quad \text{is the intercept of the line of Figure 11, on the vertical axis. Thus, the exponent of the denominator of the combined rate expression above, would be 0.23. It will be noted that in this and in subsequent experiments the rate of the reaction is expressed as } \mu \text{ liters of carbon dioxide evolved after 150 seconds of reaction. As explained on (p. 17), this value was considered to adequately express the rate of the reactions, when obtained during the first three minutes of the reactions.}

To evaluate the proportionality between the rate of the reaction and the concentration of the divalent anion of oxalacetic acid present in
Figure 11. Influence of the concentration of the monovalent anion of oxalacetic acid on the rate of the enzymatic reaction.

Ordinate is log rate of the reaction expressed as $\mu$l of $CO_2$ evolved in the initial 150 sec. of the reaction ($log Q CO_2$). Abscissa is log concentration of the monovalent anion of oxalacetic acid ($log [HA^-]$) 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid $3/4$ neutralized with sodium hydroxide; acetate buffer ionic strength 0.02; final volume in flask 3 ml.; final concentration of diion ($A^2$) $1.2 \times 10^{-3}$ M/l.
the mixture, a series of experiments were made at different pH, in which the amounts of oxalacetic acid initially added in each case, had been calculated to give equal concentrations of the monovalent ion (HA\(^{-}\)) in the final mixtures. For this calculation, the formula,

\[
[H_2A] = [HA^-] \left( \frac{[H^+]^2}{K_1} + \frac{K_2}{[H^+] + 1} \right)
\]

which had been obtained from formulas A) and B), in a similar way as formula C), was used. Rates of the reactions were measured in the Warburg apparatus in the manner already described. Acetate buffers of ionic strength 0.02, were used. Since the ionic strength contribution of the (A\(^{2-}\)) ions, present in different amounts at each pH, would be expected to be significantly large in this case, a correction measure was adopted. For this, the final ionic strength in each flask, was brought up to 0.02bb, by adding calculated amounts of a 0.02 molar solution of potassium nitrate to each mixture. As shown by Byerrum, Brown and Ball, potassium nitrate, being the salt of a monovalent cation and a monovalent, non-basic anion, would only influence the system insofar as its contribution to the total ionic strength was concerned. In Figure 12, where the results of this determination are shown, reaction rates are again expressed as \(\nu\) liters of carbon dioxide, evolved after given time intervals. The relationship between rate and the concentration of the divalent anion of oxalacetic acid available in the mixture, is shown in Figure 13. The slope of the line obtained, when the log of carbon dioxide evolved per 150 seconds of reaction is plotted against log of \([A^{2-}]\), was evaluated by the method of least squares, and found to be 1.31. Therefore, the expression of the rate of the enzymatic reaction of mixtures containing equal concentrations of the monovalent
Figure 12. Activity of oxalacetic acid carboxylase in solutions of different pH, containing equal amounts of the monovalent anion (HA\(^-\)) of oxalacetic acid.

1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, 3/4 neutralized with sodium hydroxide; acetate buffer of ionic strength 0.02; 0.02 M KNO\(_3\) solution added; final volume in flask 3 ml.; final ionic strength 0.026. Final concentration of monovalent anion of oxalacetic acid 7.0 \(\times\) 10\(^{-4}\) M. ○ pH 3.70; ○ pH 3.90; ● pH 4.06; ● pH 4.28.
anion of oxalacetic acid would be: Rate = k₂[A²⁻]. k₂, the value of the intercept on Figure 13 was found to be 2.76, under these conditions.

By combining the data obtained from Figures 11 and 13, expression E) was obtained thus: \( \frac{dCO₂}{dt} = k \left( \frac{[A]^{1.31}}{[HA^-]^{0.23}} \right) \). In this formula, k represents \((k_1 \times k_2)\), and \( \frac{dCO₂}{dt} \) is the \( \mu \) liters of carbon dioxide evolved per initial 150 seconds of the reaction. If it may be assumed that changes in the charge of the enzyme between pH 3.7 and 4.3, do not influence the rate of the catalytic reaction to any appreciable extent, the rate of the enzymatic decarboxylation could be considered to be a function of the concentrations of the mono- and divalent anions of oxalacetic acid present in each case, and expressed by E). It may be noted however, that formula E) is only valid as an expression for rates observed during the first three minutes of the reaction. By proper integration, its validity could be extended, but even in this case, its applicability would be limited to the time interval in which, as shown in Figure 3, the evolution of carbon dioxide follows first order kinetics.

It was considered of interest to use formula E) to attempt to explain the variation in the rates of the enzymatic reactions shown on Figure 1, between pH 3.7 and the optimum points of activity. Therefore, in Figure 14, the \( \mu \) liters of carbon dioxide used to evaluate the rate constants shown in Figure 1, are plotted against the ratio \( \left[ A^{2-} \right]^{1.31}/\left[ HA^- \right]^{0.23} \). \( [A^{2-}] \) and \( [HA^-] \) are the concentrations of the two anions of oxalacetic acid present in the mixtures at each pH. The straight lines shown in Figure 14, illustrating these rate relationships, for reactions in buffers of ionic strength 0.0096 and 0.012, would indicate that expression E) can also be
Figure 13. Influence of the concentration of the divalent anion of oxalacetic acid on the rate of the enzymatic reaction.

Ordinate is log rate of the reaction expressed as \( \nu \) of CO\(_2\) evolved in the initial 150 sec. of the reaction (log \( Q_{\text{CO}_2} \)). Abscissa is log concentration of the divalent anion of oxalacetic acid (log \( \Lambda^2 \)). 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, 3/4 neutralized with sodium hydroxide; acetate buffer ionic strength 0.02; 0.02 M KNO\(_3\) solution added; final volume in flask 3 ml.; final ionic strength 0.0266. Final concentration of the monovalent anion of oxalacetic acid 7.0 x 10\(^{-4}\) M.
Figure 14. Activity of oxalacetic acid carboxylase, in solutions of different pH, containing varying concentrations of the mono and divalent anions of oxalacetic acid.

$[\text{HA}^-]^{1.31}/[\text{HA}^+]^{2.3} \times 10^5$

$Q_{\text{CO}_2}$

$2.4$ $6.8$ $10$

$2$ $4$ $6$ $8$

$[\text{HA}^-]$ is concentration of the monovalent anion, $[\text{HA}^+]$ is concentration of the divalent anion of oxalacetic acid. Ordinate is activity expressed as μl of CO$_2$ evolved in the initial 150 sec. of the reaction ($Q_{\text{CO}_2}$). 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, 3/4 neutralized with sodium hydroxide; final volume in flask 3 ml. ○ Final ionic strength of acetate buffer 0.0096; ● Final ionic strength of buffer 0.012.
Figure 15. Activity of oxalacetic acid carboxylase, in solutions of different pH, containing varying concentrations of the mono and divalent anions of oxalacetic acid.

\[ \frac{[A^-]}{[HA^-]} \times 10^5 \]

\( Q_{CO_2} \) is concentration of the monovalent anion, \([A^-]\) is concentration of the divalent anion of oxalacetic acid. Ordinate is activity, expressed as \( \mu \) l of CO\(_2\) evolved in the initial 150 sec. of the reaction \( (Q_{CO_2}) \). 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid, \( 7/4 \) neutralized with sodium hydroxide; final volume in flask 3 ml. Final ionic strength of acetate buffer 0.02.
used to express the rates of the reactions in these cases.

Examining this question further, the slopes of the lines on Figure 14 were evaluated, and found to be 3.6 for the determinations made in a buffer of ionic strength 0.012, and 4.6 for the determinations conducted in buffers of ionic strength 0.0096. These values are appreciably different from the k value of 1.44 calculated in formula E) on page 22. It will be noted however, that while this value had been calculated from data obtained from experiments run at a total ionic strength close to 0.025, the values reported on Figures 1 and 14 had been obtained from reactions run in media of total ionic strength 0.0155 and 0.0135. These differences, which had at first appeared to be insignificant, do, in all probability play an important role in influencing the rate of the enzymatic reactions and further experiments to clarify this question, although not attempted in this work, are clearly indicated. A few more considerations on this question can however be presented here. In Figure 15, the rates of a series of enzymatic reactions at different pH, conducted in buffers of ionic strength 0.02, in which the total ionic strength values ranged from 0.0247 at pH 3.70 to 0.0259 at pH 4.10, are plotted against the ratio \([A^-]^{1.31}/[HA^-]^{2.33}\). \([A^-]\) and \([HA^-]\) express the concentrations of the mono- and divalent anions present in the mixtures at the corresponding pH. A straight line was obtained, (Figure 15), of slope 1.30. This value is sufficiently close to the calculated value of \(k = 1.44\) to fall within the experimental error. Since the experiments which had led to the calculation of this value, (Figures 11 and 13), had been conducted in mixtures of total ionic strength close to 0.025, and this value is
Figure 16. Activity of oxalacetic acid carboxylase, in solutions of identical pH, containing varying concentrations of the mono and divalent anions of oxalacetic acid.

\([\cdot HA^-]\) is concentration of the monovalent anion, \([\cdot A^2^-]\) is concentration of the divalent anion of oxalacetic acid. Ordinate is activity expressed as \(\mu l\) of CO\(_2\) evolved in the initial 150 sec. of the reaction (Q\(_{CO_2}\)). 1 ml. of enzyme (3.12 mg.); 0.1 ml. of oxalacetic acid 3/4 neutralized with sodium hydroxide; 0.02 M KNO\(_3\) solution added; acetate buffer of ionic strength 0.01; final volume in flask 3 ml.; pH 4.0; final ionic strength 0.02.
rather close to the values listed above, it seems valid to assume that
the difference in the slopes of the lines of Figure 14 is chiefly due
to differences in the ionic strength of the reactions. Since it had been
shown by Byerrum, Brown and Ball (15), that enzymatic activity is in-
versely proportional to the cube of ionic strength, this fact could be
used as a further indication for the validity of the foregoing assumption.

It could be shown that when the concentrations of the \((A^2)\) and \((HA^-)\)
anions of oxalacetic acid were made to vary, not by changes in \(pH\), but
by the addition of varying amounts of undissociated substrate \((H_2A)\), to
identical solutions of the enzyme in a buffer of \(pH\) four, expression \(E\)
could also be applied. The results of such a determination are shown in
Figure 16. Rates, expressed as \(\mu\) liters of carbon dioxide evolved in the
initial 150 seconds of the reactions are plotted against the ratio
\([A^-]^{1.31}/[HA^-]^{0.23}\), where \([HA^-]\) and \([A^-]\) are the concentrations of
the mono- and divalent anions present in each case. All reactions were
run in acetate buffers of ionic strength 0.01, and in every case the
total ionic strength of the mixtures was corrected to a final value of
0.02, by the addition of appropriate amounts of a 0.02 molar solution
of potassium nitrate. The slope of the straight line obtained was cal-
culated and found to be 1.80. Therefore it seems apparent that expression
\(E\) could also be used to explain the variation in the rates of reactions
run at constant \(pH\) and constant enzyme concentration, when the concen-
tration of the substrate was varied between \(4 \times 10^{-4}\) molar and \(4 \times 10^{-3}\) molar.
DISCUSSION

From the foregoing results, it appears that the shape of the pH vs. activity curve of oxalacetic acid can be explained as the result of pH induced, progressive changes in the properties of three components of the enzymatic system: the monovalent anion of oxalacetic acid (HA	extsuperscript{-}), the diion (A	extsuperscript{2-}), and the enzyme itself. Thus, the increase in enzymatic activity between pH 3.5 and 4.3 is mainly a result of the increasing availability of the divalent anion, which, as the enzyme-specific form of the substrate, undergoes the enzymatic decarboxylation proper. It is postulated that this effect is decreased by the monovalent ion (HA	extsuperscript{-}), which functions as an enzyme inhibitor, and significantly reduces the rate of the reaction.

If the effects of these two ions are evaluated separately, and the results combined, the initial rate of the enzymatic reaction can be expressed by formula \( \frac{dCO_2}{dt} = k \left( \frac{[A^2]}{[HA^-]} \right)^{1.31} \), in which (A	extsuperscript{2-}) and (HA	extsuperscript{-}), for a given amount of substrate, are a function of the pH of the reaction. When an enzyme concentration of 1.7 milligrams per milliliter is used, this equation is shown to be valid for reactions in which substrate concentrations, ranging from \( 4 \times 10^{-3} \) molar to \( 4 \times 10^{-4} \) molar are employed. The value of the proportionality constant k, is markedly influenced by the ionic strength of the buffer system used, and it appears that, the greater the ionic strength of the medium, the smaller the value of k.

The decrease in enzymatic activity between pH 4.3 and 5.0, appears
to be principally a result of the progressive insolubility of the enzyme in acetate buffers of this pH range. It is probable therefore, that the combination of the three factors above, will explain the formation of the optimum pH for enzymatic activity shown in Figure 1.

It will be noted that, since oxalacetic acid (H₂A) is almost completely dissociated at pH above 3.5 to give either a monovalent or divalent anion, its participation as a substrate for the enzymatic decarboxylation is not likely to take place.

The inhibitory effect of the \((HA^-)\) ion, is believed to take place by one of the following mechanisms: i) the enzyme may form an \((E-S)\) complex with this ion, which however does not decarboxylate, and only functions as a means of removing part of the enzyme from the medium, or, ii) the \((E-HA^-)\) complex, in view of its similarity to the admittedly "active" complex \((E-A)\), undergoes enzymatic decarboxylation, but at a slower rate than this form. In either case, the amount of inhibition produced at each pH would decrease with increasing basicity, over the range of enzymatic activity.

The inhibitory effect of the \((HA^-)\) ion was further examined in the light of the results presented by Steinberger and Westheimer, already referred to (1b). According to these authors, the metal-ion catalysed decarboxylation of dimethyl oxalacetic acid, takes place by the following reactions:

\[
\begin{align*}
\text{I} & \quad \text{II} & \quad \text{III} \\
\text{O} & \text{C} & \text{O} & \text{C(CH₃)₂CO₂⁻} & \text{O} & \text{C} & \text{O} & \text{C(CH₃)₂CO₂⁻} & \text{O} & \text{C} & \text{O} & \text{C(CH₃)₂} \\
\text{H₂O} & \text{OH₂} & \rightarrow & \text{H₂O} & \text{OH₂} & \text{H}^+ & \rightarrow & \text{2H₂O} & + & \text{Cu(OH₂)}^{₄+} \\
\end{align*}
\]

Byerrum, Brown and Ball (15) had shown that the protein fraction of
squash seeds which possess oxalacetic acid decarboxylating activity, is a metallo-protein, containing chiefly manganese. Westheimer and Steinberger had also observed that when pyridine was added to a solution of dimethyl oxalacetic acid containing Cu^{++}, the rate of decarboxylation of this acid was increased over and above the amount expected for the catalysis by the Me^{++} alone. In consequence, the authors had postulated that this activation was due to the formation of a complex between the metal-ion attached in a chelate type of ring to the acid, and two moles of pyridine. A similar structure is proposed here for the (E-S) complex of the diion of oxalacetic acid, which, in analogy with compound I, might be:

\[
\begin{align*}
\text{Me} & \quad \text{IV} \\
\text{(Protein)} & \quad \text{IV}
\end{align*}
\]

In this formulation, it is believed that basic groups on the protein coordinate with the Me^{++} ion, in a manner similar with that shown for pyridine, by Steinberger and Westheimer.

Although the enzyme-substrate complex IV, is the intermediate form through which the enzymatic decarboxylation is believed to take place, it seems plausible to admit that a similar complex can be formed between the metallo-protein and the monovalent anion of oxalacetic acid: \((\text{OOC-CO-CH}_2\text{-COOH})\). However, the complex thus formed, by not having a free negative charge on its carboxyl group, might be expected either to be unable to, or, to decarboxylate more slowly than the corresponding complex formed by the divalent anion. For this hypothesis it must be admitted of course, that the enzymatic decarboxylation of oxalacetic acid follows the same scheme of reactions, outlined by Steinberger and Westheimer.
for the Me-ion catalysed decarboxylation of dimethyl oxalacetic acid.

It is ventured here to give an explanation of the role of the other monovalent anion produced as a result of the dissociation of oxalacetic acid, namely: \((\text{HOOC-CO}-\text{CH}_2-\text{COO})^-\). According to Steinberger and Westheimer, the decarboxylation of the mono-ester of dimethyl oxalacetic acid (\(\text{ROOC-CO-CH}_2-\text{COO}^-\)) cannot be accelerated by Me\(^{++}\) ions, at any pH. Therefore, if an analogy to oxalacetic acid can be drawn, it could be assumed that the monovalent anion of this acid, which has its negative charge on the carboxyl group \(\delta\) to the carbonyl group, behaves essentially like the monoanion of the ester. If this is true, its influence as an inhibitor of the enzymatic reaction would be ruled out, and this effect would be solely due to the effect of the \((\text{COO}-\text{CO-CH}_2-\text{COOH})\) ion.
The pH vs. activity curve of the carboxylase of squash seed globulin, acting on a substrate of oxalacetic acid, was examined, and the possible factors, whose variation with pH, might be responsible for the shape of this curve between pH 3.7 and 5.0 have been discussed.

1. It is shown that the shape of the curve remains essentially unchanged, regardless of whether the reactions were conducted within a certain range of buffers of constant ionic strength, or of constant molarity. However, ionic strength has a definite effect on the overall rate of the enzymatic reactions.

2. The decrease in enzymatic activity between the pH of optimum activity and pH five, was explained by the increasing insolubility of the enzyme in the pH range between 4.5 and 5.0. The parallelism shown by the curves expressing enzyme activity and enzyme solubility, in this range, brought experimental evidence for this assumption.

3. The influence of changes in pH, on the acid-base binding power of the protein, was examined, and the bearing of this factor on the enzymatic activity was discussed. It was shown experimentally, that no irregular changes in the titration curve of the protein, occur within the range of enzymatic activity.

4. The relationship between the availability of the mono- and divalent anions of oxalacetic acid, and the rate of the enzymatic reaction at given pH's between 3.7 and 4.3, was examined. From the results obtained, it was postulated that the decarboxylating species is chiefly the divalent

(29)
anion \( (A^-) \), and that the reaction is inhibited, to some extent, by the monovalent anion \( (HA^-) \). The influence of each of these ions was evaluated in separate experiments and the combined rate equation

\[
\frac{dCO_2}{dt} = k \frac{[A^-]^{1.31}}{[HA^-]^{0.23}}
\]

was set up.

6. This expression was employed in attempts to explain the variation in the rate of three series of enzymatic reactions, conducted at different ionic strength, over a pH range from 3.5 to 4.3.

The validity of the expression was also tested, by attempts to use it to explain the variation of the rates of a series of enzymatic reactions, conducted at constant pH and enzyme concentration, but in mixtures containing varying concentrations of the substrate.
Preliminary Investigations on the Activity of Oxalacetic Acid Carboxylase from Squash Seeds in Phosphate Buffers of pH Ranging from 6.0 to 7.8.

In 1949, Vennesland (12) had noticed that the decarboxylating activity of the crystalline globulin fraction obtained from extracts of cucurbit seeds, might not be of physiological significance, and that this fraction represented merely a storage protein. This assumption was based on the fact that the globulin was non-Mg-ion activable, and inactive in acetate buffers of pH above 5.5, which was considered to be too far from seven to be of physiological importance. It will be noted however, that enzyme preparations obtained by Ochoa from pigeon liver (10), and by Veiga, Salles and Ochoa (8), from Micrococcus luteus, show pH optima at 4.5 and 5.2 respectively. It seems admissible, therefore, to believe that a relatively low pH of optimum does not rule out the biological significance of the enzyme from squash seeds.

As a preliminary experiment in this work, it was considered of interest to examine whether the globulin preparation from squash seeds, prepared according to the method of Vickery et al. (19), would retain its decarboxylating activity in a phosphate buffer of pH seven. Activity determinations, made by the method of Warburg, already explained, showed that no significant increase in the rate of decarboxylation of oxalacetic acid could be observed, when experiments using, heat-inactivated and unheated enzyme were compared. This fact was attributed to the extreme insolubility of the globulin in phosphate buffers of pH between 6.0 and 7.8.
Speck (13), had shown that the activity of oxalacetic acid carboxylase from parsley roots, could be significantly enhanced by divalent cobalt ions. It was therefore considered of interest to investigate whether such an effect could be shown for the enzyme from squash seeds, acting on the substrate, in a phosphate buffer of pH seven. For this, a suspension of cobalt phosphate, prepared by adding disodium hydrogen phosphate to a solution of cobalt sulfate in redistilled water, was added to the reaction mixture. Cobalt ions were added as cobalt phosphate, since the addition of a solution of cobalt sulfate to the reaction mixture directly, would have caused an appreciable decrease in the concentration of the buffer, in consequence of the removal of phosphate ions to form insoluble cobalt phosphate. Although by this measure, the values for the evolution of carbon dioxide obtained, were larger than those shown for the previous experiments, this result could not be ascribed to an activating effect of the metal on the enzyme, since control runs, containing cobalt phosphate alone, showed a comparable increase in the rates of the reaction. Obviously, the cobalt salt, had had no other effect on the reaction, than that of enhancing its rate by a strictly metal-ion catalysis as described by Speck (13).

Attempts to add a solution of cobalt sulfate to a solution of the enzyme in 0.002 normal nitric acid, in order to provide for a "saturation" of the protein with cobalt ions, proved unsuccessful. When an aliquot of this solution was added to the phosphate buffer in the reaction vessel, and allowed to act on the substrate, no significant increase in the rate of the enzymatic decarboxylation could be achieved.
The experiments were therefore abandoned, since the insolubility of both the enzyme and the metal ion in phosphate buffers, seemed to rule out any possibility for enzymatic activity under these conditions.
APPENDIX

Calculation of the Dissociation Constants of Oxalacetic Acid from its Titration Curve.

Let \( C \) = total concentration of acid, \( a \) = total concentration of added alkali and \( h \) = hydrogen-ion concentration (calculated from \( pH \) value).

Oxalacetic acid as a dibasic acid (\( H_2A \)), will dissociate thus:

\[
\begin{align*}
(1) \quad & H_2A & \rightleftharpoons H + HA^- , \\
(1i) \quad & HA^- & \rightleftharpoons H + A^- , \text{ therefore:} \\
K_1 = & \frac{h [HA^-]}{[H_2A]} \quad \text{and} \quad K_2 = \frac{h [A^-]}{[HA^-]} \\
\end{align*}
\]

Also: \( [H_2A] + [HA^-] + [A^-] = C \), and \( [HA^-] + 2[A^-] = a + h \).

By eliminating the three unknown concentrations, \([H_2A] \), \([HA^-] \), and \([A^-] \) from these four equations, and solving for \( K_1 \) and \( K_2 \) we obtain:

\[
\begin{align*}
K_1 &= h^2(a + h) / K_2(2C - a - h), \quad \text{and} \\
K_2 &= h^2(a + h) + K_1(ah + h^2 - hC) / K_1(2C - a - h).
\end{align*}
\]

Putting:

\[
\begin{align*}
& a_n h_n + h_n^2 - h_n C_n = A_n, \quad 2C_n - a_n - h_n = B_n \quad \text{and} \quad h_n^2 (a_n + h_n) = D_n, \\
\end{align*}
\]

we can write:

\[
\begin{align*}
K_1 &= D_n / (K_2B_n - A_n) \quad \text{and} \quad K_2 = (D_n + K_1A_n) / K_1B_n.
\end{align*}
\]

Hence by taking any two points on the titration curve whose parameters are respectively \( A_1, B_1, D_1, \) and \( A_2, B_2, D_2 \) the values of \( K_1 \) and \( K_2 \) can be found thus:

\[
\begin{align*}
K_1 &= \frac{(B_1D_2 - B_2D_1)}{(A_1B_2 - A_2B_1)} \quad \text{and} \quad K_2 = \frac{(A_1D_2 - A_2D_1)}{(B_1D_2 - B_2D_1)}.
\end{align*}
\]
Fitting actual data to these formulas, a sample calculation of the values of $K_1$ and $K_2$ is shown below:

**Sample Calculation:**

50 ml. of 0.0073 M solution of oxalacetic acid titrated with 0.038 M sodium hydroxide at 20° C (1.1).

<table>
<thead>
<tr>
<th>No.</th>
<th>ml. NaOH Volume added of Solution (ml)</th>
<th>Total Volume of NaOH (M)</th>
<th>Final Concentration of Oxalacetic Acid (C) (M)</th>
<th>pH</th>
<th>[H+] (h)</th>
<th>Final Concentration of NaOH (M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>6</td>
<td>56</td>
<td>4.08 x 10^{-3}</td>
<td>2.81</td>
<td>1.55 x 10^{-3}</td>
<td>6.55 x 10^{-3}</td>
<td>2.51</td>
</tr>
<tr>
<td>(2)</td>
<td>12</td>
<td>62</td>
<td>7.33 x 10^{-3}</td>
<td>3.70</td>
<td>1.99 x 10^{-4}</td>
<td>5.91 x 10^{-3}</td>
<td></td>
</tr>
</tbody>
</table>

Applying $\Box$ we get:

$A_1 = 3.60 x 10^{-6}$

$B_1 = 8.24 x 10^{-3}$

$D_1 = 3.58 x 10^{-2}$

$A_2 = 3.03 x 10^{-4}$

$B_2 = 5.43 x 10^{-3}$

$D_2 = 1.29 x 10^{-9}$

and by $\Box$

$K_1 = \frac{5.49 x 10^{-11}}{8.50 x 10^{-9}} = 6.46 x 10^{-3}$

$K_2 = \frac{4.95 x 10^{-15}}{5.86 x 10^{-11}} = 8.44 x 10^{-5}$
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