THE KINETICS OF THE ENZYME-SUBSTRATE COMPOUND OF PEROXIDASE

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Studies on the over-all kinetics of enzyme action revealed in the majority of cases and over certain concentration ranges that the enzymatic activity was related linearly to the enzyme concentration and hyperbolically to the substrate concentration. On the basis of such evidence Michaelis and Menten (13) showed that such relationships were explained on the assumption that an intermediate compound of enzyme and substrate was formed: $E + S \rightarrow ES \rightarrow E + P$. As the rate of formation of such a compound was assumed to be quite rapid, the rate of breakdown was the rate-determining step. This theory was extended by Briggs and Haldane (2) who pointed out that the rate of formation of the intermediate compound could in certain cases be limited by the number of collisions of enzyme and substrate, and modified the Michaelis theory accordingly. The resulting theory has been extremely useful as a first approximation in the explanation of enzyme action and has given a basis for the comparison of different enzymes in terms of their affinity and activity.

The reaction velocity constants are, however, lumped into one term, the Michaelis constant, and are not separately determined. It is the purpose of this research to determine these constants separately, and to show whether the Michaelis theory is an adequate explanation of enzyme mechanism. Moreover, studies on the over-all enzyme activity do not permit a determination of whether the enzyme-substrate compound exists in fact and, if it exists, whether such a compound is responsible for the enzyme activity.

Several attempts have been made to identify enzyme-substrate compounds. Stern (16) made direct spectroscopic measurements of the compound of catalase and ethyl hydroperoxide and found that this compound was unstable and decomposed after several minutes in the presence of 1 M ethyl hydroperoxide. This was interpreted to indicate that the intermediate compound was responsible for the decomposition of all the ethyl hydroperoxide in this period. Although independent tests showed that ethyl hydroperoxide was decomposed by catalase, no data were given on the amount or rate of decomposition of ethyl hydroperoxide in the spectroscopic experiment (Green (8)).

Keilin and Mann (11) studied the compound of peroxidase and hydrogen peroxide by visual spectroscopy. Their observations include the fact that a spectroscopically defined compound of peroxidase and hydrogen peroxide is formed and that this compound rapidly decomposes in the presence of an oxygen acceptor. While these experiments indicate the existence of an unstable intermediate compound, no direct relation between this intermediate compound and the enzymatic activity is given. A conclusive proof of the Michaelis theory rests on such evidence.

This paper describes a detailed study of the compound of horseradish peroxidase and hydrogen peroxide, an enzyme-substrate compound. The enzyme activity in the presence of leucomalachite green, an acceptor, and hydrogen peroxide, a substrate, has been studied in the usual manner and the Michaelis constant determined. A new apparatus and a new method of studying the kinetics of rapid reactions have been developed and used to measure directly the reaction velocity constants which compose the Michaelis constant. These are the rates of formation and breakdown of the enzyme-substrate compound. The equilibrium of enzyme and substrate in the absence of an acceptor has also been studied. These new data have then been compared with the Michaelis constant which has been determined in the classical manner. A point by point comparison between experiment and theory has been made possible by solutions of the differential equations representing the Briggs and Haldane modifications of the Michaelis theory. In this way, the validity of the Michaelis theory has been clearly demonstrated, and the important relationship between the enzyme-substrate compound and its activity has been clearly shown. A preliminary report of this work was given earlier (Chance (4)).

Preparation and Standardization—The method of Elliott and Keilin (7) was used for the preparation of peroxidase. The first alcohol precipitate was usually discarded and in a particular case 1 gm. of enzyme, $PZ^1 = 256$, was obtained from 7 kilos of horseradish. The enzyme was kept in a volume of 75 cc. and was tested periodically for hematin iron and *PZ*. As neither the apparatus nor the information was available at the time, the peroxidase was not purified further in the manner recently indicated by Theorell (18).

A typical preparation contained 5×10^{-5} M hematin iron. The light absorption was measured at 640 and 400 mμ with a grating photoelectric spectrophotometer and it was found that $\epsilon_{640} = 12 \pm 2$ and $\epsilon_{410} = 125 \pm 12$

[1] *PZ* or purpurogallin number indicates peroxidase activity in terms of mg. of purpurogallin formed from pyrogallol in 5 minutes at 20° per mg. of dry weight of enzyme preparation. 12.5 mg. of H_2O_2 and 1.25 gm. of pyrogallol in 500 cc. of water are used.

(c = 1 mM, d = 1 cm.) at pH 6.2 in 0.01 M phosphate buffer on the basis of total hematin iron.[2] The extinction coefficients given do not represent those of a pure peroxidase.

Perhydrol, diluted to 1 M and kept at 0°, was tested periodically by permanganate titration. Further dilutions were freshly made up before each experiment.

A slightly oxidized saturated solution of leucomalachite green in 0.05 M acetic acid was standardized by oxidation in the presence of peroxidase and hydrogen peroxide. The light absorption at 610 mμ was measured and the concentration determined in terms of a standard solution of malachite green ($\epsilon_{614} \doteq 50$). The pH was maintained by 0.05 M acetate buffer at 4.1.

Method

This is set forth elsewhere (Chance (3, 5, 6)). The Hartridge-Roughton (10) flow method has been modified to give fluid economy and photoelectric resolution greatly exceeding the designs of Roughton and Millikan (15) and adequate for the direct measurement of the kinetics of the hematin compounds in a 1 mm. bore observation tube at concentrations of 1×10^{-6} mole of hematin Fe per liter. The apparatus is shown in Fig. 1, and details of the various parts may be obtained in the references above.

Controls—Detailed controls on the efficient mixing by this apparatus have been described in a previous paper (Chance (3)), indicating that the mixing was essentially complete in 2×10^{-4} second for the highest values of flow velocity. In these experiments the times were long compared to the minimum time range of the apparatus.

Controls on the linearity of the photoelectric system were carried out by plotting deflection of the recorder against concentration of the reactant and a linear relationship was obtained, as the light absorption was very small.

Under certain conditions, the production of malachite green may interfere with the measurement of the kinetics of the intermediate compound. The absorption of the dye is rather high at 420 mμ, as shown in Fig. 2, and would add to the absorption of the enzyme. A 4×10^{-6} M malachite green solution would cause a 3 per cent error in the measurement of 1×10^{-6} M hematin Fe peroxidase solution. This sets a limit to the amount of malachite green formed in the presence of a given amount of enzyme.

A compensation for the effect of malachite green absorption was effected by varying the relative amounts of light incident on the 370 and 430 mμ filter combinations so that the absorption of malachite green affected each photocell equally.

[2] ϵ (extinction coefficient) $= \dfrac{\log_{10} I_0/I}{d \text{ (cm.)} \times c \text{ (mM per liter)}}$.

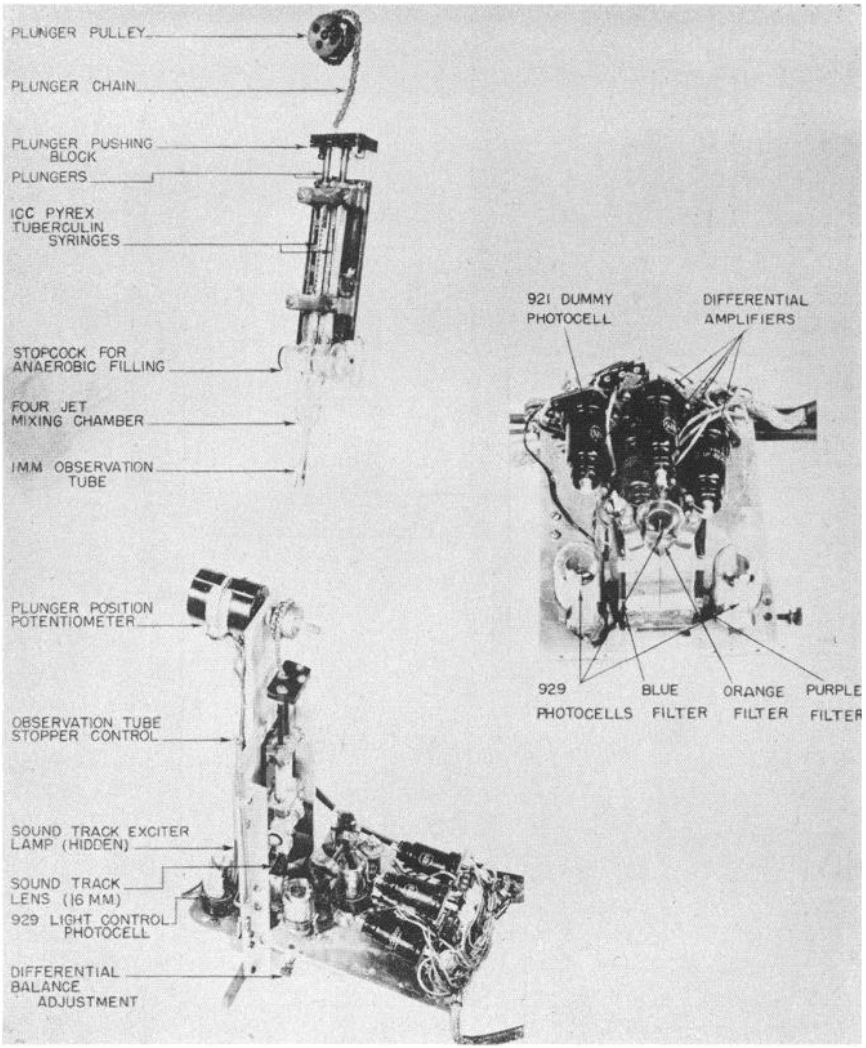

FIG. 1. Syringe unit, photocell unit, and assembled apparatus. Light and electrostatic shields are removed.

Procedure

In order to explain the experimental method more clearly the procedure used to obtain the data of Fig. 3 will be outlined. The enzyme solution was centrifuged before experiment in order to remove denatured protein and give a clear brown solution. Shortly before an experiment, the enzyme

was diluted to 2×10^{-6} M hematin Fe. Hydrogen peroxide was diluted to 16×10^{-6} M just previous to an experiment. A saturated solution of leucomalachite green in 0.05 M acetic acid was diluted to 60×10^{-6} M in acetate buffer to make the final pH 4.0.

The syringes shown in Fig. 1 were thoroughly rinsed with cleaning solution and carefully flushed out with water in order that there might be no trace

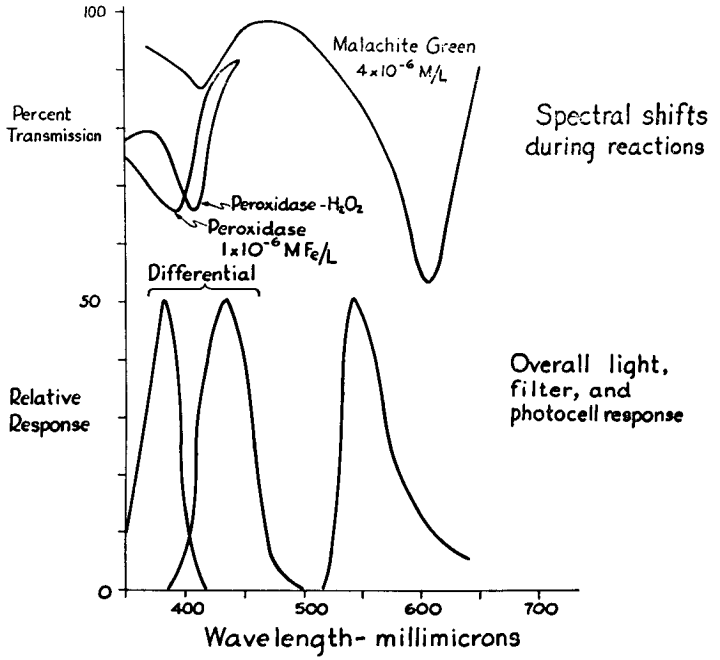

FIG. 2. The upper curves give the light transmission of enzyme, enzyme-substrate compound, and oxidized acceptor under the conditions of Fig. 3. The lower curves give the filter combinations used to measure the kinetics of the reactions. The trough depth was 16 times that of the 1 mm. observation tube of the rapid reaction apparatus. The spectral interval was approximately 8 mμ. The wave-length markers read 15 mμ low.

of the enzyme in the tube which was to be filled with substrate and acceptor. The right-hand syringe was then filled with a mixture of 8×10^{-6} M hydrogen peroxide and 30×10^{-6} M leucomalachite green in 0.05 M acetate buffer. These reactants were squirted into the top of the syringe while the outlet was held closed with a small rubber pad mounted on a lever shown in Fig. 1. The syringe plunger was then entered in the barrel and held in place at the top of the syringe by means of a plunger driving block. The left-hand syringe was flushed out with water and filled with 2×10^{-6} M

enzyme solution while the outlet tube was again held closed by means of the stopper. The plunger for the left syringe was then entered and fitted into the driving block. Both plungers were carefully pushed a few mm. down their respective barrels to make sure that they were running smoothly and were accurately aligned. The zero point of the recording mirror oscillograph was checked and a trial run was made by sharply pushing the driving block approximately 1 cm. This caused the reactants to be mixed and to flow down the observation tube very rapidly and, at the end of the

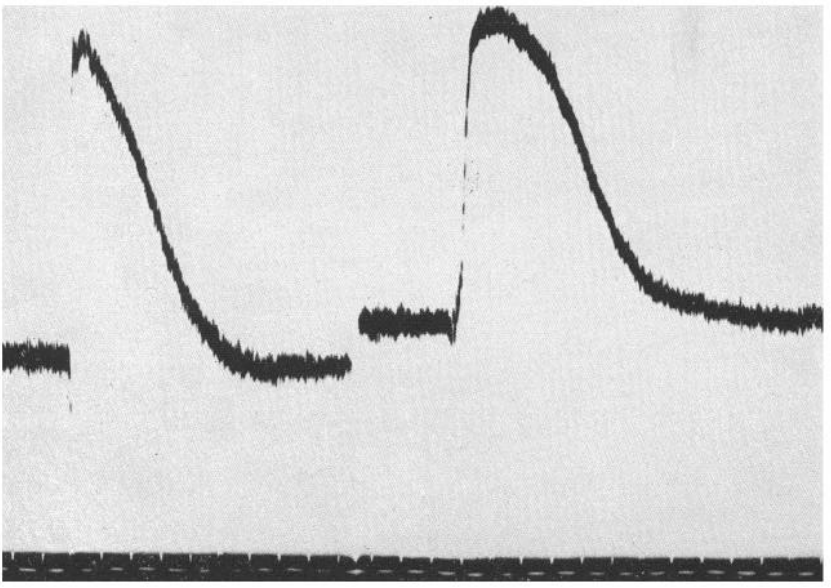

FIG. 3. Mirror oscillograph recording of the production of malachite green (left) and the corresponding kinetics of the enzyme-substrate compound (right). Time markers, 0.2 second. Peroxidase = 1×10^{-6} mole of hematin Fe per liter, H_2O_2 = 4×10^{-6} mole per liter, leucomalachite green = 15×10^{-6} mole per liter, pH = 4.0.

discharge, to stop before the photocell and light beam. The progress of the reaction that ensued in the portion of liquid stopped in the path of the light beam was measured directly by the photoelectric amplifiers. Either Amplifier 1 or 2 could be used, as shown in Chance (5). If the deflection was too large, the amplifier gain was readjusted so that the picture was approximately three-quarters of full linear scale. If it was then considered that the experiment was suitable for recording, the camera attached to the mirror oscillograph was set in operation, and the syringe plungers were given a second sharp push which caused the kinetic curves to repeat themselves. In this way the kinetics of the intermediate compound and the over-all

reaction were recorded. This process was repeated until the syringes were completely discharged, and in most cases it was found that three to six curves could be obtained from one filling of the syringes.

A second experiment was carried out immediately to calibrate the maximum concentration of the enzyme-substrate compound. This was done in the same manner as the first experiment except that the leucomalachite green was omitted. Hence the substrate concentration would be sufficient to saturate the enzyme completely, as was indicated by independent experiment. This reaction was also recorded photographically. The deflection corresponded to 1×10^{-6} M hematin Fe enzyme-substrate compound and is marked on Fig. 10.

A third experiment was necessary to calibrate the amount of malachite green formed. Malachite green, formed by peroxidase action, was diluted to 4×10^{-6} mole per liter and used to calibrate the photoelectric amplifier of the system measuring the rapid reaction. The right-hand syringe was filled with the malachite green solution, and the left-hand syringe was filled with water. These two solutions were pushed down, not simultaneously, but alternately, so that the observation tube was filled first with malachite green and then water. The resulting deflection was recorded photographically and gave the deflection corresponding to 4×10^{-6} M malachite green. In this way, the amount of malachite green which had been formed in the experiment was accurately determined. This calibration point appears in Fig. 10. These calibrations were made so that it was unnecessary to rely upon any long time stability of the photoelectric amplifier or recording system.

Results

Equilibrium of Enzyme and Substrate

$$\text{Peroxidase} + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{peroxidase}\cdot H_2O_2 \qquad (1)$$

This reaction was studied by direct photoelectric measurements of the equilibrium concentration of enzyme-substrate compound as a function of substrate concentration. If hydrogen peroxide is mixed with peroxidase, the spectrum changes as in Fig. 2 and the compound denoted peroxidase-H_2O_2, Complex I (Keilin and Mann (11)), is formed, as the substrate is not in great excess.

In order to measure this equilibrium it is essential that k_3, the first order velocity constant for the enzymatic breakdown of the intermediate compound, be negligible compared to k_1, the second order constant for the combination of enzyme and substrate, and k_2, the first order constant for the reversible breakdown of the enzyme-substrate compound. As Keilin has

pointed out, the small amount of acceptor present in the enzyme preparation may be oxidized by the addition of hydrogen peroxide and under these conditions the enzymatic breakdown of the enzyme-substrate compound is small. Under these conditions the intermediate compound appeared moderately stable at pH 6.2, although its concentration remained constant for only 5 to 10 seconds at pH 4.2. However, complete stability was not essential for measurements in the rapid reaction apparatus, and it was desired to carry out these reactions at the same pH as the other studies (4.0).

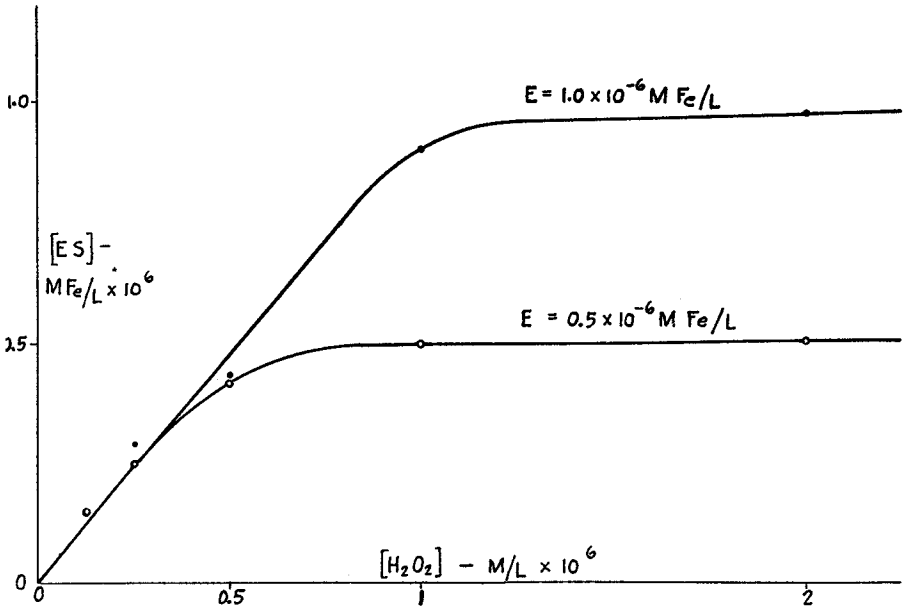

FIG. 4. Equilibrium of enzyme and substrate in absence of acceptor. Ordinate, intermediate compound as total hematin iron; abscissa, initial H_2O_2. pH = 4.2.

The experiments were carried out in this manner. The left-hand syringe was filled with varying concentrations of substrate, while the right-hand syringe was filled with a known concentration of enzyme. Both syringe plungers were then pushed downward in short, sharp pushes so that the observation tube was filled with mixed but unchanged enzyme and substrate, and, after the flow had stopped, the photoelectric system measured and recorded the rate of formation of the intermediate compound and the equilibrium concentration of enzyme-substrate compound. This experiment was repeated for different initial substrate concentrations, and the equilibrium value of the enzyme-substrate compound is plotted in Fig. 4 against initial substrate concentration. It is assumed that the maximum ordinate

corresponds to complete conversion of enzyme into enzyme-substrate compound of concentration equal to the independently determined molar hematin iron.

The data of Fig. 4 indicate very small dissociation of the intermediate compound, and the equilibrium constant estimated from two points on Fig. 4 giving finite values is 2×10^{-8}. As the enzymatic breakdown of the enzyme-substrate compound was not zero, this figure should be regarded as a minimum value. Evidently the enzyme was nearly completely converted into its enzyme-substrate compound by an equimolal concentration of substrate. This indicates that all this hematin iron existed as compounds capable of reacting similarly with hydrogen peroxide, *i.e.* forming a spectroscopically defined intermediate compound.

Rate of Formation of Enzyme-Substrate Compound

$$\text{Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{peroxidase}\cdot H_2O_2 \qquad (2)$$

The rate of this reaction has been determined in the manner described before; namely, the right-hand syringe is filled with a 2×10^{-6} M hydrogen peroxide solution, while the left-hand syringe is filled with a 2×10^{-6} M hematin iron enzyme solution. The syringe plungers are again pushed down rapidly, and the reaction was measured after the flow had stopped in the observation tube. The half time of this reaction was 0.1 second. The experiment was then repeated with substrate concentrations from 0.5 to 8×10^{-6} M. The half time and curve shapes of these data were measured, and it was found that a bimolecular equation approximately satisfied the variation of rate with substrate concentration. Higher substrate concentrations have not been used to a great extent, as there is some question whether or not a compound of different spectral absorption denoted peroxidase-H_2O_2, Complex II (Keilin and Mann (11)), might be formed. There is also slight evidence to lead one to believe that the reaction might not follow a bimolecular course at substrate concentrations greater than 10×10^{-6} mole per liter. Experiments in which concentrations of substrate lower than 0.5×10^{-6} mole per liter are employed involved larger experimental errors, owing to the small changes in light transmission.

The data fit a second order kinetic equation, as Fig. 5 shows. Over a range of enzyme concentrations from 1 to 2×10^{-6} mole of hematin Fe per liter and a range of substrate concentrations from 0.5 to 4×10^{-6} mole per liter the mean value of the second order velocity constant was 1.2×10^7 liter mole^{-1} sec.$^{-1}$. The mean error is 0.4×10^7. The previous section gave the ratio of k_2 to k_1 as 2×10^{-8}, or larger; hence k_2 is 0.2 sec.$^{-1}$ or less.

It is now apparent that the enzyme and substrate unite with extreme

rapidity to form a relatively tight complex, and it is interesting to note that the ratio of k_2/k_1 is considerably smaller than the Michaelis constant determined by measurement of the over-all enzyme action (5×10^{-6}, Mann (12)). k_3 is possibly far greater than k_2 in the case of peroxidase, and this will be shown to be true in the next section.

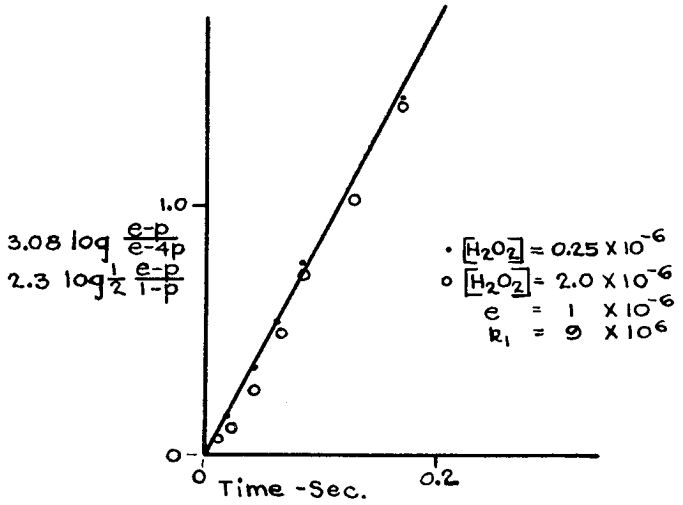

FIG. 5. Kinetics of formation of intermediate compound plotted for two values of substrate concentration according to the second order equation. $k_1 = 9 \times 10^6$ liter mole^{-1} sec.$^{-1}$, pH = 4.0.

Rate of Breakdown of Enzyme-Substrate Compound

$$A + \text{peroxidase}\cdot H_2O_2 \xrightarrow{k_3} \text{peroxidase} + H_2O + AO \qquad (3)$$

The decomposition of the intermediate compound in the presence of an oxygen acceptor is shown schematically by Equation 3. We will choose an oxygen acceptor in the presence of which peroxidase has a high activity. The oxidation products must not interfere with the measurement of the enzyme-substrate compound. This restriction eliminates acceptors like pyrogallol, hydroquinone, and guiacol, while leucomalachite green and ascorbic acid were found to be most satisfactory. In order to demonstrate the effect of such oxygen acceptors on the enzyme-substrate compound, the enzyme is mixed with substrate and acceptor, and the kinetics of the intermediate compound are observed. In Fig. 6 the concentration of the intermediate compound is recorded as a function of time for various concentrations of ascorbic acid. (In contrast to the results of Tauber (17) a polyphenol was not essential in this process.) The right-hand syringe is

filled with a mixture containing 8×10^{-6} M H_2O_2, 0.05 M acetate buffer, pH 4.2, and varying concentrations of ascorbic acid. The left-hand syringe is filled with 2×10^{-6} M enzyme solution. The curves show that in the presence of 2.9×10^{-6} mole of ascorbic acid, the intermediate compound is stable for a long period of time. The stability of the compound is indicated, of course, by the length of time required for its concentration to fall to zero, for this is taken to mean that all the substrate has been con-

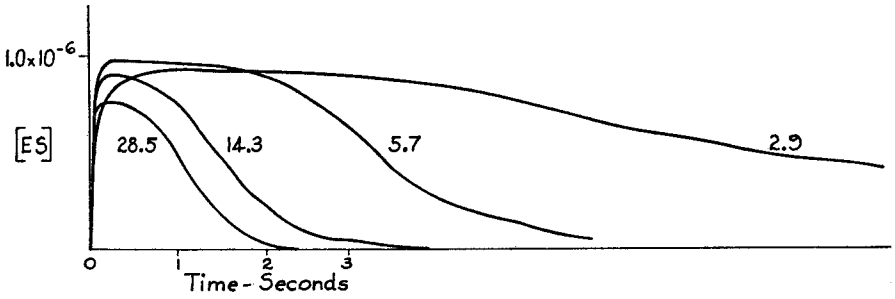

FIG. 6. The effect of an acceptor on the kinetics of the enzyme-substrate compound. $E = 1 \times 10^{-6}$ mole of hematin Fe per liter, $H_2O_2 = 4 \times 10^{-6}$ mole per liter, ascorbic acid as indicated in micromoles per liter, pH = 4.2.

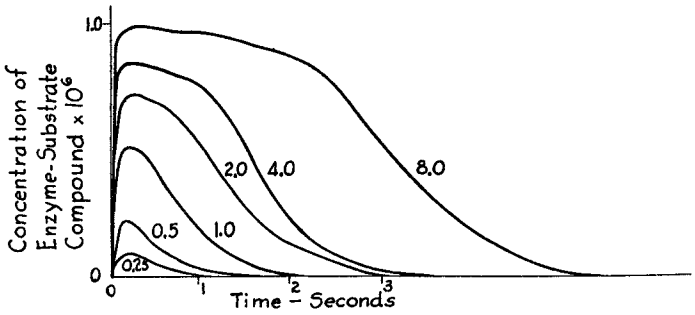

FIG. 7. The effect of substrate on the kinetics of the enzyme-substrate compound. $E = 1 \times 10^{-6}$ mole of hematin Fe per liter, ascorbic acid approximately 14×10^{-6} mole per liter, initial H_2O_2 as indicated in micromoles per liter, pH = 4.2.

sumed. The curves of Fig. 6 for higher concentrations of ascorbic acid clearly show a marked decrease in this interval. The curves also indicate a decrease in the maximum concentration of the enzyme-substrate compound, $p_{max.}$, with increasing ascorbic acid concentration. This decrease in $p_{max.}$ is due to the higher rate of breakdown of the intermediate compound. The low value of $p_{max.}$ in the 2.9×10^{-6} M ascorbic acid curve is believed due to experimental error.

The effect of the substrate concentration is shown in Fig. 7, when the acceptor concentration has been maintained in excess of the substrate con-

centration. The first interesting feature of this family of curves is the variation of height of the curves with substrate concentration, giving a method of directly studying enzyme-substrate affinity from measurements of the enzyme-substrate compound rather than from the over-all enzyme action. It is seen, for the particular value of ascorbic acid concentration, that the enzyme is one-half saturated by 1×10^{-6} M *initial* substrate concentration. It should also be noted that the area under each curve increases regularly with the initial substrate concentration. One would expect this, as k_3, the rate of breakdown of the enzyme-substrate compound, should be constant as the acceptor concentration is maintained constant and it is found that the area under the curve is proportional to the total amount of hydrogen peroxide consumed.

While k_3 can be determined from the kinetics shown above, we have yet to devise an experiment in which the rate of breakdown of the intermediate compound is determined from both enzyme-substrate kinetics and the rate of production of oxidized acceptor. This experiment is of great importance in determining the relation between the over-all reaction and the kinetics of the enzyme-substrate compound. The rate of disappearance of ascorbic acid could not be measured with this apparatus, as it was not adaptable for wave-lengths below 350 mμ. Leucomalachite green was used as an oxygen acceptor for the following reasons. (1) The mechanism of its oxidation appears simple compared to that of pyrogallol. (2) The absorption is quite strong and does not seriously interfere with the measurement of the enzyme absorption. (3) The linearity between enzyme concentration and rate of formation of malachite green is quite good.

One experimental difficulty in the use of leucomalachite green is a variation in the amount of the dye formed. Only when the leuco base is partially oxidized is the full amount realized and not even then at higher enzyme concentrations. This phenomenon is not completely understood.

On the right-hand side of Fig. 3 are shown the kinetics of the intermediate compound recorded by a photokymograph. The time is read from left to right with markers every 0.2 second. The break in the base-line corresponds to the moment when the syringe plungers were pushed downwards and, after 0.1 second, the flow stops and the reaction of enzyme, substrate, and acceptor proceeds. The formation of the intermediate compound occurs quite rapidly, as is indicated by the abrupt upward deflection of the tracing. Within 0.1 second the enzyme-substrate compound has reached its maximum concentration ($p_{\text{max.}}$), and it maintains a steady state for 0.2 second. After this time the substrate concentration has fallen to such a value that the rate of formation of the intermediate compound no longer balances its rate of breakdown. Hence its concentration decreases rapidly and in 1 second has fallen to zero, and the enzyme is all liberated.

The calibrations above indicated that $p_{max.} = 0.85 \times 10^{-6}$ mole of hematin Fe per liter in this experiment.

On the left side of Fig. 3 is shown the rate of production of malachite green by the enzyme system under identical conditions. Here again the break in the base-line indicates a push of the syringe plungers. However, the very rapid upward deflection in this case simply represents clearing out malachite green from the previous run. After 0.1 second the flow stops and the production of malachite green begins just as soon as the intermediate compound has formed. The reaction continues at nearly constant velocity as long as the concentration of the intermediate compound is constant. (The slight variation in slope is due to experimental error.) As this falls, so falls the rate of the over-all reaction, and both reach zero at approximately the same time. Calibrations given above indicated that 4×10^{-6} mole of malachite green was formed in this experiment.

This very simple experiment gives qualitative indication that the relationship between the kinetics of the enzyme-substrate compound and the over-all enzyme activity is that predicted by the Briggs and Haldane modifications of the Michaelis theory.

These experiments have been carried out for substrate concentrations ranging from 5×10^{-7} to 8×10^{-6} mole per liter. At the lower concentrations the error in recording was somewhat large, and at those higher than 6×10^{-6} mole per liter the transmission change due to the formation of the quantity of malachite green interfered with measurements of the enzyme kinetics (see "Controls" above). Enzyme concentrations ranged from 2.5×10^{-7} to 2×10^{-6} M hematin Fe. Lack of an adequate supply of enzyme limited the highest concentrations to 2×10^{-6} M hematin Fe.

Interpretation

Calculation of k_3—The "Appendix" gives methods for determining k_3 from the over-all reaction (Equations 9 and 12) and from the enzyme-substrate kinetics (Equations 11, 13, and 16).

The rate of the *over-all reaction* is 4.3×10^{-6} mole of malachite green per second and $p_{max.} = 0.85 \times 10^{-6}$ mole per liter. From Equations 9 and 12, $k_3 = 5.1$ sec.$^{-1}$.

From the *enzyme-substrate kinetics* there are available the following data for Equation 13.

$k_1 = 1 \times 10^7$ liter mole^{-1} sec.$^{-1}$; $x_0 = 4 \times 10^{-6}$ mole per liter.

$p_{max.} = 0.85 \times 10^{-6}$ mole per liter and $k_2 = 0.2$ sec.$^{-1}$.

$\int_0^t p\,dt$ is evaluated graphically at $t = 0.24$ second when $p = p_{max.}$ and found to be 0.17×10^{-6} mole second; hence $k_3 = 4.3$ sec.$^{-1}$ for $k_2 = 0$ and 4.2 sec.$^{-1}$ for $k_2 = 0.2$ sec.$^{-1}$.

$\int_0^t pdt$ also may be evaluated graphically at $t = \infty$ when $p = 0$ and $x = 0$. The integral is found to be 0.84×10^{-6} mole second and on substitution in Equation 11, $k_3 = 4.8$ sec.$^{-1}$.

According to Equation 16, the value of k_3 is given by $x_0/(p_{max.} \cdot t_{\frac{1}{2}})$. As $t_{\frac{1}{2}} = 0.9$ second, k_3 is calculated to be 5.2 sec.$^{-1}$.

The rate of breakdown of the enzyme-substrate compound in the presence of ascorbic acid is determined from the data of Fig. 6. Using convenient Equation 16, we find in Fig. 8 that the variation of k_3 with ascorbic acid is of such a nature that k_3 divided by the ascorbic acid concentration gives a constant indicative of a second order combination of acceptor and enzyme-substrate compound. The same relationship held for leucomalachite green, and the corresponding quotient is 3×10^5 liter mole^{-1} sec.$^{-1}$.

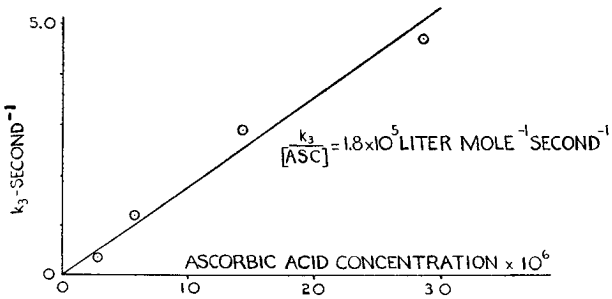

FIG. 8. Variation of k_3 with acceptor concentration. k_3 was obtained by Equation 16 from data of Fig. 6.

The constancy of k_3 for a given acceptor concentration is given in Fig. 9 for the data of Fig. 6 on the basis of Equation 16. The experimental check of the equation is satisfactory although the acceptor concentration was somewhat depleted in two reactions with higher substrate concentrations.

A particular curve for $x_0 = 1.0 \times 10^{-6}$ mole per liter has been examined and k_3 at 14×10^{-6} M ascorbic acid is found to be 2.2, 2.0, and 2.5 sec.$^{-1}$ from Equations 16, 11, and 13 respectively.

There is then substantial agreement between values of k_3 calculated from three different points of the enzyme-substrate kinetics corresponding to the times $p = p_{max.}$, $p = p_{max.}/2$, and $p = 0$ ($t = \infty$) and between values of k_3 determined from the over-all reaction.

Calculation of Michaelis Constant—There are three ways by which we can determine the Michaelis constant and thereby check the validity of the theory.

The first method is to calculate this constant from k_2, k_3, and k_1 which have all been experimentally determined. k_3, calculated solely *from the kinetics of the enzyme-substrate compound* above, is found to be 4.2 sec.$^{-1}$. k_1 is found to be 1×10^7 liter mole^{-1} sec.$^{-1}$ and k_2 a minimum value of 0.2 sec.$^{-1}$. The Michaelis constant is then calculated to be 0.44×10^{-6} from Equation 8.

This value may also be calculated according to Equation 8 from concentrations which obtain during the steady state. The saturation of the enzyme, $p_{max.}$, is known from the experiment and the corresponding value of x may be readily determined. It is important to note that the value of x is not the initial concentration of substrate as is usually

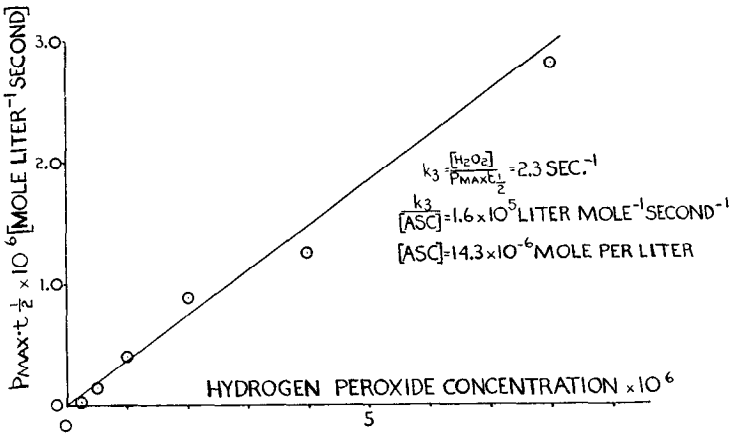

FIG. 9. Experimental test of Equation 16 indicating constancy of k_3 for varying substrate concentration and fixed acceptor concentration. From the data of Fig. 7

the case when this initial concentration is so large compared with the enzyme concentration that the amount of substrate combined with enzyme is relatively small. The value of x when p reaches $p_{max.}$ is calculated from the experimental data in three ways which follow: (*a*) x is readily calculated from Equation 11, as we have already determined the necessary quantities. $p_{max.}$ is equal to 0.85×10^{-6} mole per liter, $\int_0^{0.24} pdt$ is 0.17×10^{-6} mole second, and k_3 is 4.2 sec.$^{-1}$. x is 2.4×10^{-6} mole per liter and Km, calculated from Equation 8, is 0.43×10^{-6} mole per liter. This method is, of course, not independent of the calculation of k_3 shown previously; nevertheless, *all* the data used to determine Km in this manner are obtained from concentration measurements of the enzyme-substrate compound during the steady state. (*b*) The amount of sub-

strate which has been consumed by the time p reaches its maximum value can be determined from Fig. 3 (right) by a second graphical method. The area under the whole curve for the kinetics of the enzyme-substrate compound represents the disappearance of 4×10^{-6} mole of substrate. In fact, this is true for $\int_0^\infty pdt = 0.84 \times 10^{-6}$ mole second and the mean value of $k_3 = 4.9$ sec.$^{-1}$, whence $k_3 \int_0^\infty pdt = 4.0 \times 10^{-6}$ mole, the initial substrate concentration. The area under the curve from time zero until p reaches its maximum value is representative of the amount of substrate which has been decomposed during that time and this is 0.9×10^{-6} mole of decomposed substrate. To this we must add the amount of substrate which is combined with the enzyme, $p_{max.}$. From this, x is readily calculated and the Michaelis constant is found to be 0.40×10^{-6}. This method is completely independent of a determination of k_3, as this quantity appears in both numerator and denominator. (*c*) If we assume that for each molecule of malachite green formed 1 molecule of substrate has been decomposed, we have directly the amount of substrate that disappeared enzymatically. At 0.24 second this is 0.9×10^{-6} mole. When $p_{max.}$ is added to this, the Michaelis constant is calculated to be 0.40×10^{-6}, which agrees very closely with the other values determined independently.

The classical determination of the Michaelis constant by Mann (12) gives 5×10^{-6} mole per liter at pH 4.0 and an acceptor concentration of 0.007 per cent. This constant varied linearly with acceptor concentration over this range. These data also indicated a linear relationship. Hence Mann's value of *Km* was reduced to our acceptor concentration by dividing by the concentration differential, 10. This gives 0.5×10^{-6}, which agrees fairly well with the above independently determined values in view of the widely different enzyme and substrate concentrations.

Correlation with Complete Solutions of Michaelis Theory—While previous data suggest the validity of the Michaelis theory, a much more convincing proof is furnished by the data on the superposition of the differential analyzer (see "Appendix") and direct experimental curves.

The solid curves in Fig. 10 show the kinetics of the enzyme-substrate compound (right) and the over-all reaction (left) for the following values of reaction velocity constants and concentrations: $e = 1 \times 10^{-6}$ mole per liter, $x_0 = 4 \times 10^{-6}$ mole per liter, $k_1 = 0.9 \times 10^7$ liter mole^{-1} sec.$^{-1}$, $k_2 = 0$ sec.$^{-1}$, $k_3 = 4.5$ sec.$^{-1}$. The experimental curves of Fig. 3 ($e = 1 \times 10^{-6}$ mole of hematin Fe per liter, $x_0 = 4 \times 10^{-6}$ mole per liter, leucomalachite green $= 15 \times 10^{-6}$ mole per liter, pH = 4.0) are plotted as circles to the proper scale in Fig. 10. The independently determined

values of reaction velocity constants are $k_1 = 1.2 \times 10^7$ liter mole^{-1} sec.$^{-1}$, $k_2 \leqq 0.2$ sec.$^{-1}$, $k_3 = 4.9$ sec.$^{-1}$ (mean). Remarkably good agreement is obtained in view of the possible error in all experimental quantities required to determine the mathematical solution.

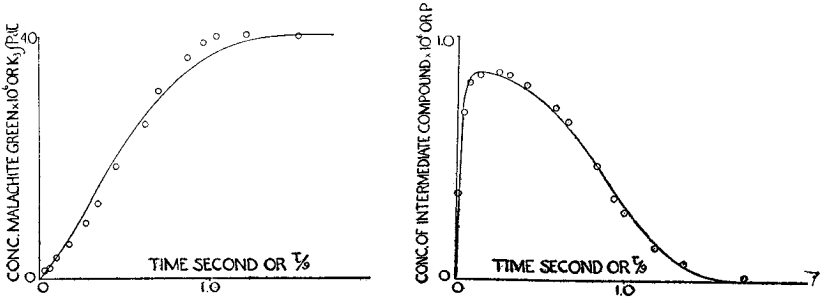

FIG. 10. A comparison of experimental enzyme-substrate and "over-all" kinetics (circles) with a mathematical solution of the Michaelis theory for experimentally determined reaction velocity constants and concentrations (solid lines).

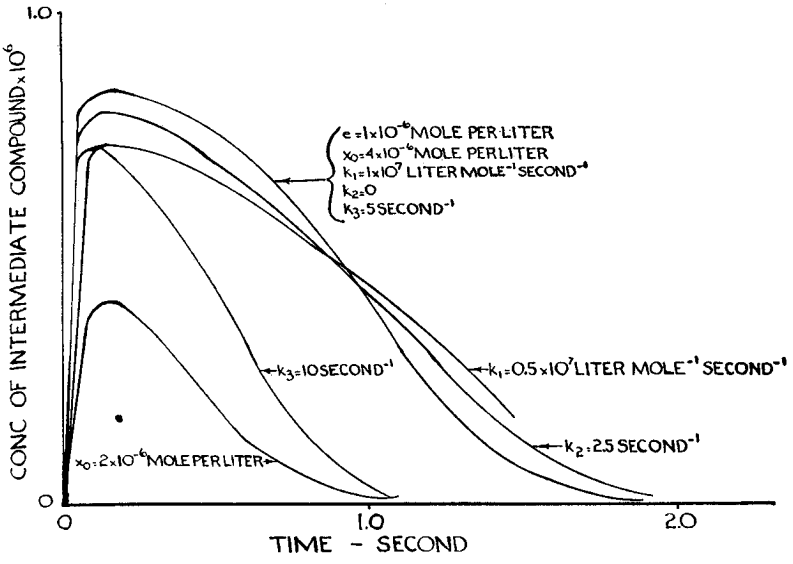

FIG. 11. Effect of variation of reaction velocity constants and concentrations on the shape of the mathematical solutions of the Michaelis theory.

The overshoot in the experimental points (Fig. 10, right) is possibly due to inadequate speed of response in the photocell amplifier. The scatter of points (Fig. 10, left) is thought to represent an instrumental rather than intrinsic irregularity.

Calculations show that the mathematical solutions are quite sensitive to changes in the experimental values. x_0 and k_3 cause large changes in $t_{\frac{1}{2}}$, while k_1 and k_2 affect $p_{max.}$ and the shape of the curve as shown in Fig. 11. The effect of enzyme and leucomalachite green concentration is not given by the mathematical solutions but would be large.

DISCUSSION

The extreme rapidity of the union of enzyme and substrate is indicated by the second order rate constant of 1×10^7 liter mole^{-1} sec.$^{-1}$. It is very interesting to note the similarity of this rate to the measured value for oxygen and muscle hemoglobin, 1.9×10^7 liter mole^{-1} sec.$^{-1}$ (Millikan (14)). Haldane's (9) calculated minimum rate for the union of catalase and hydrogen peroxide of 0.76×10^7 liter mole^{-1} sec.$^{-1}$ is quite similar also. If it is assumed that the reversible breakdown of the enzyme-substrate compound is also slow in the case of catalase and hydrogen peroxide, the similarity between the catalase and peroxidase values is more striking. Such concordance in the rates of union of small molecules and proteins would lead one to question whether or not these reaction velocities were limited by the number of collisions. While experiments made at 0° indicate but little change in the rate of formation of the enzyme-substrate compound, these results are preliminary and should not be used to substantiate the conclusion that the joining of enzyme and substrate is a collision-limited process.

The equilibrium of enzyme and substrate was directly studied as the irreversible breakdown of the intermediate compound (k_3) was quite small in the absence of acceptor. The equilibrium constant was found to have a minimum value of 2×10^{-8}. This indicates an extremely tight complex of enzyme and substrate, and this value is of the same order as that for CO hemoglobin, although the individual rates differ considerably. Cytochrome *c* peroxidase-hydrogen peroxide evidently dissociates more readily (1).

The studies on the enzymatic function of the enzyme-substrate compound were also carried out with ascorbic acid and leucomalachite green as acceptor, the latter over a rather narrow range, as the absorption of dye interfered with the measurement of the enzyme-substrate compound. The Michaelis theory has been checked by various determinations of the Michaelis constant. The first method is from *kinetic* data; namely, the rate of formation of the enzyme-substrate compound, the rate of reversible breakdown, and the rate of its irreversible breakdown into free enzyme and altered substrate. The Michaelis constant was determined from the sum of the last two divided by the first. This was also determined from *concentration measurements* at the steady state, when the concen-

tration of the intermediate compound passed through its maximum value. From this maximum value and the corresponding substrate concentration we can again directly calculate the Michaelis constant. The third method is the classical one wherein the rate of the over-all reaction is measured, and the concentration of substrate giving one-half maximal activity is determined. To these three methods a fourth one has been added to take advantage of the fact that the data are complete kinetic curves of the enzyme-substrate compound, and therefore, with complete solutions of the Michaelis equation we may compare, point by point, theory and experiment.

In all cases in the range of experimental concentrations the kinetics of the intermediate compound were related to the kinetics of the over-all reaction in a manner explained by the Michaelis theory, substantiating the conclusion that the mechanism of a second order combination of enzyme and substrate followed by a first order decomposition is essentially correct for peroxidase action at this particular acceptor concentration.

The rate of breakdown of the intermediate compound of peroxidase and hydrogen peroxide is very small compared to that of catalase $k_3 = 3 \times 10^5$ sec.$^{-1}$. The value for catalase assumes that the Michaelis theory holds and that a chain mechanism is not responsible for the enzyme action. The peroxidase kinetics indicate that a chain mechanism plays no prominent part, if any, as the induction period in the production of dye is no longer than is required by the formation of the enzyme-substrate compound and there is also no further production of dye after the enzyme-substrate compound has disappeared. It is possible that the difference between these two enzymes lies mainly in the slower breakdown of the peroxidase intermediate compound.

It is of considerable interest to know whether there is a bimolecular combination of the enzyme-substrate compound and the acceptor. No spectroscopic evidence of such compound formation from 360 to 600 mμ was found. However, kinetic evidence for such a combination is given by the variation of the enzyme activity with acceptor concentration. The rate of production of malachite green and the effect of ascorbic acid on the kinetics of the enzyme-substrate compound strongly suggest a bimolecular combination with acceptor in accordance with Mann (12).

The mechanism by which the acceptor is oxidized is still obscure. As this may take place through single electron changes involving the formation of a free radical of the triphenylmethyl type in the case of malachite green, studies were made[3] to find spectroscopic evidence for such intermediates. While no data were obtained in the visible spectrum, the question is still open.

[3] Dr. Fred Karush collaborated in this study.

SUMMARY

Under the narrow range of experimental conditions, and at a temperature of approximately 25°, the following data were obtained.

1. The equilibrium constant of peroxidase and hydrogen peroxide has a minimum value of 2×10^{-8}.

2. The velocity constant for the formation of peroxidase-H_2O_2 Complex I is 1.2×10^7 liter mole^{-1} sec.$^{-1}$, $\pm 0.4 \times 10^7$.

3. The velocity constant for the reversible breakdown of peroxidase-H_2O_2 Complex I is a negligible factor in the enzyme-substrate kinetics and is calculated to be less than 0.2 sec.$^{-1}$.

4. The velocity constant, k_3, for the enzymatic breakdown of peroxidase-H_2O_2 Complex I varies from nearly zero to higher than 5 sec.$^{-1}$, depending upon the acceptor and its concentration. The quotient of k_3 and the leucomalachite green concentration is 3.0×10^5 liter mole^{-1} sec.$^{-1}$. For ascorbic acid this has a value of 1.8×10^5 liter mole^{-1} sec.$^{-1}$.

5. For a particular acceptor concentration, k_3 is determined solely from the enzyme-substrate kinetics and is found to be 4.2 sec.$^{-1}$.

6. For the same conditions, k_3 is determined from a simple relationship derived from mathematical solutions of the Michaelis theory and is found to be 5.2 sec.$^{-1}$.

7. For the same conditions, k_3 is determined from the over-all enzyme action and is found to be 5.1 sec.$^{-1}$.

8. The Michaelis constant determined from kinetic data alone is found to be 0.44×10^{-6}.

9. The Michaelis constant determined from steady state measurements is found to be 0.41×10^{-6}.

10. The Michaelis constant determined from measurement of the over-all enzyme reaction is found to be 0.50×10^{-6}.

11. The kinetics of the enzyme-substrate compound closely agree with mathematical solutions of an extension of the Michaelis theory obtained for experimental values of concentrations and reaction velocity constants.

12. The adequacy of the criteria by which experiment and theory were correlated has been examined critically and the mathematical solutions have been found to be sensitive to variations in the experimental conditions.

13. The critical features of the enzyme-substrate kinetics are $p_{max.}$ and curve shape, rather than $t_{\frac{1}{2}}$. $t_{\frac{1}{2}}$ serves as a simple measure of dx/dt.

14. A second order combination of enzyme and substrate to form the enzyme-substrate compound, followed by a first order breakdown of the compound, describes the activity of peroxidase for a particular acceptor concentration.

15. The kinetic data indicate a bimolecular combination of acceptor and enzyme-substrate compound.

It is a very great pleasure to acknowledge the aid of Dr. F. J. W. Roughton, Dr. F. A. Cajori, Dr. G. A. Millikan, and Dr. J. G. Brainerd, and the keen interest of Dr. D. W. Bronk in this research. The aid of the American Philosophical Society is gratefully acknowledged. It is also a source of regret that the problem could not be concluded where it was initiated.

Appendix

Extension of Michaelis Theory

These reactions are represented by Briggs and Haldane as the bimolecular combination of the enzyme, E, and substrate, S, to form an intermediate compound, ES, followed by a monomolecular decomposition into free enzyme and activated or altered substrate, Q, representative of the products of the "over-all" enzyme action.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + Q \tag{4}$$

If e is the total molar enzyme concentration, x the molar substrate concentration, p the molar concentration of ES, k_1 the second order rate constant, and k_2 and k_3 the first order rate constants, then

$$\frac{dp}{dt} = k_1 x(e - p) - (k_2 + k_3)p \tag{5}$$

$$\frac{dx}{dt} = -k_1 x(e - p) + k_2 p \tag{6}$$

These two equations represent the rate of formation of the intermediate compound and the rate of disappearance of the substrate.

The solution of these equations has been already obtained by Briggs and Haldane for the special conditions of the steady state, when

$$p = p_{\max.}, \qquad \frac{dp}{dt} = 0, \qquad \text{and} \qquad \frac{p_{\max.}}{e} = \frac{x}{x - Km} \tag{7}$$

where

$$Km = \frac{k_2 + k_3}{k_1} = x\,\frac{(e - p_{\max.})}{p_{\max.}} \tag{8}$$

A further solution valid during the steady state is obtained by adding Equations 5 and 6,

$$\frac{dx}{dt} = -k_3 p_{\max.} \tag{9}$$

where dx/dt is the rate of disappearance of substrate. This equation is useful for determining k_3.

In addition to these solutions for the steady state the general solution of these differential equations can be indicated thus:

$$\frac{d(p + x)}{dt} = -k_3 p \tag{10}$$

and is obtained by adding Equations 5 and 6. As $p = 0$ and $x = x_0$ when $t = 0$, we have, on integrating,

$$x = x_0 - p - k_3 \int_0^t p dt \tag{11}$$

When $dp/dt = 0$,

$$\frac{dx}{dt} = -k_3 \frac{d \int_0^t p dt}{dt} \tag{12}$$

The right-hand member may represent the rate of appearance of oxidized substrate such as malachite green. We may then use Equation 9 to calculate k_3.

Also when $dp/dt = 0$, $p = p_{max.}$ and at this time Equation 7 is valid. On solving Equation 7 for x and substituting in Equation 12, we have, after simplification,

$$k_3 = \frac{k_1(x_0 - p_{max.})(e - p_{max.})}{p_{max.} + k_1(e - p_{max.}) \int_0^t p dt} - \frac{k_2 p_{max.}}{p_{max.} + k_1(e - p_{max.}) \int_0^t p dt} \tag{13}$$

This equation is useful to calculate k_3 when the curve of p against t is known as in the case of Fig. 3.

As a check on the mathematics, let us substitute in Equation 13 the condition that $p_{max.}$ is nearly equal to e, t is small, and x is nearly equal to x_0. It will be seen that Equation 7 is obtained as would be expected.

Complete Solutions for Michaelis Theory[4]—Under the experimental conditions it was found that the steady state existed only for a fraction of a second. In order to determine whether the transient portions of the curves satisfied the Michaelis theory, solutions of differential Equations 5 and 6 were required. For satisfactory solutions from the differential analyzer, the following substitutions were necessary. Let

$$K_2 = 10^6 \frac{k_2}{k_1}, \qquad X = 10^6 x, \qquad \tau = k_1 et$$

$$K_3 = 10^6 \frac{k_3}{k_1}, \qquad P = \frac{p}{e}, \qquad \Theta = k_1 t 10^{-6}$$

[4] These solutions were obtained with the aid of Dr. J. G. Brainerd, Moore School, University of Pennsylvania.

$$\therefore \frac{dP}{d\Theta} = (1 - P)X - (K_2 + K_3)P \tag{14}$$

$$\frac{dX}{d\tau} = -(1 - P)X + K_2 P \tag{15}$$

When e is equal to 1×10^{-6} mole per liter, the experimental value, then τ is equal to Θ and a series of solutions of these equations may be obtained for this particular value of the enzyme concentration. The solutions have been carried out in this manner, and it should be noted that they are valid only for this enzyme concentration.

The mathematical solutions are given in Fig. 12, and solutions have been obtained for $K_2 = 0$; $K_3 = 0$ to 2; and $X_0 = 1$ to 8. Solutions for $K_2 = 0$ to 8 and $K_3 = 0$ to 8 were also made.

The upper portions of Fig. 12 show the disappearance of substrate, X, and production of "over-all" products, Q. The substrate concentration starts at its initial value, $X = X_0$, and falls to zero. The "over-all" production, represented by $K_3 \int_0^\tau P d\tau$, begins at zero and continues until the substrate is exhausted.

The lower left portion of Fig. 12 gives the ordinary solutions of the bimolecular reaction of enzyme and substrate, when K_2 and K_3 are zero, for four values of the initial substrate concentration, X_0. 1 unit of the ordinate corresponds to $p = e$; *i.e.*, complete conversion of the enzyme into the intermediate compound. The abscissae, plotted in units of τ, are converted into time units by the appropriate values of k_1. Directly above this is the disappearance of substrate due to the bimolecular reaction.

In the remaining lower portions of Fig. 12, K_2, the velocity constant of the reversible reaction is zero and K_3 has a finite value. The concentration of the intermediate compound increases, passes through as a maximum, and then falls to zero. The maximum concentration and area under the curve increase with the substrate concentration. Directly above are the corresponding curves for the over-all reaction. The initial rush in the kinetics of the disappearance of substrate and the induction period in the formation of "over-all" products are significant features.

The abscissae in all cases are represented in units of τ and therefore a wide range of values of k_1 can be used. The range of values of K_2, K_3, and X_0 is that corresponding to the values for which solutions have been obtained. However, to determine k_2 or k_3, K_2 or K_3 is multiplied by k_1.

For larger values of k_1 and X_0, and smaller values of K_3, dp/dt will become quite small for a considerable time and solutions corresponding to $dp/dt = 0$ can easily be obtained, as shown by Briggs and Haldane (2).

The families of curves obtained from the differential analyzer are obviously applicable to any reversible bimolecular combination and a con-

secutive monomolecular breakdown of the intermediate compound. It should be noted that these mathematical solutions do not completely describe peroxidase action, as they do not include the acceptor process. Hence they are valid only for fixed acceptor concentrations giving constant k_3.

A useful feature of these mathematical solutions is that they reveal arbitrary relationships between members of the families of curves. As an example it is found from measurements of the mathematical solutions

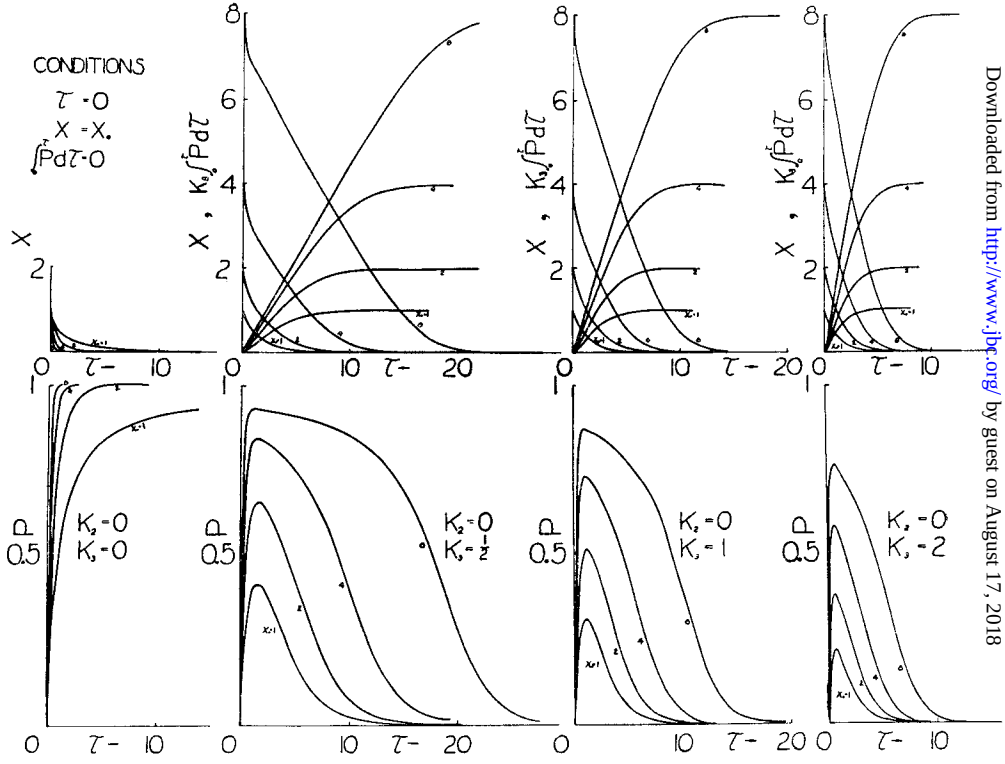

FIG. 12. Differential analyzer solutions of the Michaelis theory for the over-all reaction (upper group) and the kinetics of the enzyme-substrate compound (lower group).

for the kinetics of the enzyme-substrate compound that k_3 can be determined directly by this formula if the acceptor concentration is constant.

$$k_3 = \frac{x_0}{p_{\max.}\, t_{\frac{1}{2}}} \tag{16}$$

$p_{\max.}$ is the maximum value of p. $t_{\frac{1}{2}}$ is the time required for p to fall from $p_{\max.}$ to $p_{\max.}/2$. This relationship has been tested for experimental curves and found very satisfactory, as shown in Fig. 9.

If Equation 16 is combined with Equation 9, we find

$$t_{\frac{1}{2}} = -\frac{x_0}{dx/dt} \tag{17}$$

This indicates that there are a variety of curves of the same half width that would satisfy our experimental data. The shape and $p_{\max.}$ would be quite different.

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