ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

F. F. Nord, Founding Editor

MECHANISM OF ENZYME ACTION, PART A
Volume 73

Edited by DANIEL L. PURICH
University of Florida College of Medicine
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CONTENTS

PREFACE

The systematic investigation of enzyme reaction mechanisms remains a topic of vital interest throughout the molecular life sciences. The nature and origin of the enormous catalytic rate enhancements achieved by enzymes endures as a central focus in both chemistry and biology. It is also clear that metabolic inhibitors and activators are best comprehended in terms of their ability to interfere with or to augment the catalytic capacities of the individual enzymes that mediate the reactions within metabolic pathways. Likewise, one cannot dispute the fact that the study of enzyme mechanism still affords the best conceptual window for glimpsing the chemical features of what have proven to be some of the most potent and selective pharmacological agents. Chapters on enzyme mechanism have always graced the pages of *Advances in Enzymology*, but until now there has never been a single volume entirely devoted to mechanistic inquiries of enzyme catalysis.

Mechanism of Enzyme Action, Part A (Volume 73) inaugurates what should become a most valuable sub-series centering on enzyme catalysis. Each chapter has been written in an integrated manner, as would be true for any well conceived and documented review article. However, in keeping with a longstanding tradition, only about one-third of each Advances chapter is expected to consider the fuller context of the research literature on the topic. Authors were specifically requested to concentrate largely on work achieved in their laboratory, thereby allowing sufficient space and opportunity to divulge the hypotheses and scaffolding of logic that led them to new insights regarding the action of a particular enzyme. The goal is to be sufficiently clear and instructive, such that both experienced and aspiring enzymologists, as well as other non-experts, can follow the logic and trajectory of the experiments and mechanistic inferences.

The chapters organized in this volume should earn broad appeal in the molecular life sciences, while remaining of particular interest to practicing enzymologists, pharmacologists, and medicinal chemists in both academic and industrial settings. The outstanding scientists who prepared these chapters are internationally acknowledged leaders in mechanistic enzymology, and their substantial contributions impart significant momentum to an effort

that already includes the preparation of two additional monographs also dealing with other contemporary topics in enzymic catalysis.

Daniel L. Purich

Gainesville, Florida
March 1999

ABSTRACTS

Rethinking Fundamentals of Enzyme Action
Dexter B. Northrop

Despite certain limitations, investigators continue to gainfully employ concepts rooted in steady-state kinetics in efforts to draw mechanistically relevant inferences about enzyme catalysis. By reconsidering steady-state enzyme kinetic behavior, this review develops ideas that allow one to arrive at the following new definitions: (a) V/K, the ratio of the maximal initial velocity divided by the Michaelis-Menten constant, is the apparent rate constant for the capture of substrate into enzyme complexes that are destined to yield product(s) at some later point in time; (b) the maximal velocity V is the apparent rate constant for the release of substrate from captured complexes in the form of free product(s); and (c) the Michealis-Menten constant K is the ratio of the apparent rate constants for release and capture. The physiologic significance of V/K is also explored to illuminate aspects of antibiotic resistance, the concept of "perfection" in enzyme catalysis, and catalytic proficiency. The conceptual basis of congruent thermodynamic cycles is also considered in an attempt to achieve an unambiguous way for comparing an enzyme-catalyzed reaction with its uncatalyzed reference reaction. Such efforts promise a deeper understanding of the origins of catalytic power, as it relates to stabilization of the reactant ground state, stabilization of the transition state, and reciprocal stabilizations of ground and transition states.

Adenylosuccinate Synthetase: Recent Developments
Richard B. Honzatko, Mark M. Stayton, and Herbert J. Fromm

By exerting strategic control on purine nucleotide biosynthesis, and by engaging GTP-dependent transphosphorylation of IMP to activate loss of an oxygen atom during catalysis, adenylosuccinate synthetase remains an enzyme that justifiably fascinates students of enzyme catalysis. This review describes how the balanced application of X-ray crystallography and enzyme kinetics has advanced the comprehension of the catalytic and regulatory properties of adenylosuccinate synthetase. Detailed analysis has

demonstrated the formation of 6-phosphoryl-IMP, an intermediate originally postulated over 40 years ago on the basis of oxygen-18 exchange experiments showing that position-6 oxygen of IMP becomes incorporated into phosphate. Inferences about the participation of amino acid side-chains that stabilize 6-P-IMP during catalysis have also been confirmed by site-directed mutagenesis and examination of such mutations on various kinetic parameters. Moreover, the action of certain regulatory ligands have also been viewed at atomic level resolution. For example, magnesium ion and GDP can induce conformational changes linked to the stabilization of one of two known conformations of the so-called 40s loop. Another significant finding is that two magnesium ions play fundamental roles: one binding with high affinity to the substrate GTP, and a second binding with lower affinity to the co-substrate aspartate. These structural and kinetic studies have also formed the basis for clarifying the action of various inhibitors and potentially important pharmacologic agents with this key regulatory enzyme. Finally, this review explores the current status of investigations on gene structure and gene expression in a number of organisms.

Nucleoside Monophosphate Kinases: Structure, Mechanism, and Substrate Specificity

Honggao Yan and Ming-Daw Tsai

The catalytic mechanisms of adenylate kinase, guanylate kinase, uridylate kinase, and cytidylate kinase are reviewed in terms of kinetic and structural information that has been obtained in recent years. All four kinases share a highly related tertiary structure, characterized by a central five-stranded parallel beta-sheet with helices on both sides, as well as the three regions designated as the CORE, NMPbind, and LID domains. The catalytic mechanism continues to be refined to higher levels of resolution by iterative structure-function studies, and the strengths and limitations of site-directed mutagenesis are well illustrated in the case of adenylate kinase. The identity and roles of active site residues now appear to be resolved, and this review describes how specific site substitutions with unnatural amino acid side-chains have proven to be a major advance. Likewise, there is mounting evidence that phosphoryl transfer occurs by an associative transition state, based on (a) the stereochemical course of phosphoryl transfer, (b) geometric considerations, (c) examination of likely electronic distributions, (d) the orientation of the phosphoryl acceptor relative to the phosphoryl being transferred, (e) the most likely role of magnesium ion, (f) the lack of re-

stricted access of solvent water, and (g) the results of oxygen-18 kinetic isotope effect experiments.

Enzymology of NAD^+ Synthesis

Giulio Magni, Adolfo Amici, Monica Emanuelli, Nadia Raffaelli, and Silverio Ruggieri

Beyond its role as an essential coenzyme in numerous oxidoreductase reactions as well as respiration, there is growing recognition that NAD^+ fulfills many other vital regulatory functions both as a substrate and as an allosteric effector. This review describes the enzymes involved in pyridine nucleotide metabolism, starting with a detailed consideration of the anaerobic and aerobic pathways leading to quinolinate, a key precursor of NAD^+. Conversion of quinolinate and 5'-phosphoribosyl-1'-pyrophosphate to NAD^+ and diphosphate by phosphoribosyltransferase is then explored before proceeding to a discussion of the molecular and kinetic properties of NMN adenylyltransferase. The salient features of NAD^+ synthetase as well as NAD^+ kinase are likewise presented. The remainder of the review encompasses the metabolic steps devoted to (a) the salvaging of various niacin derivatives, including the roles played by NAD^+ and NADH pyrophosphatases, nicotinamide deamidase, and NMN deamidase, and (b) utilization of niacins by nicotinate phosphoribosyltransferase and nicotinamide phosphoribosyltransferase.

Solution Structure and Mechanism of the MutT Pyrophosphohydrolase

A. S. Mildvan, D. J. Weber, and C. Abeygunawardana

The MutT enzyme plays a central role in preventing mutations resulting from oxidative damage to DNA, and the enzyme exhibits the capacity to remove mutations from DNA, thereby effecting its repair. The reaction is unusual because a nucleoside-5'-triphosphate is hydrolyzed to form a nucleoside-5'-monophosphate plus diphosphate. This review considers the chemical and catalytic mechanism of the reaction, beginning with (a) oxygen-18 experiments to identify the site of bond cleavage, (b) comments on the catalytic power of pyrophosphoryl group transferring enzymes, and (c) the likely roles of multiple divalent metal ions in catalysis. Emphasis is given to the deduced solution structures of the free MutT enzyme and the quaternary complex-containing enzyme, manganese ion, 5'-p(CH_2)ppA, and a second divalent cation. The active site of the MutT enzyme is explored in detail, especially as regards the triphosphate site, and

mechanism of catalysis that is consistent with the previously mentioned solution structures is also presented. The rate acceleration of a billion achieved by the MutT enzyme is ascribed to: catalysis by approximation and polarization of the attacking water molecule by an enzyme-bound metal ion (resulting in an enhanced reactivity of 10^5); activation of the NMP leaving group by Lys-39 (contributing a further factor of ten); charge neutralization of the nucleotide-bound metal ion (adding another factor of 10); as well as orientation and/or deprotonation of the attacking water molecule by Glu-53 (a step that also yields better than a factor of ten).

The Enzymes of Glutathione Synthesis: γ-Glutamylcysteine Synthetase
Owen W. Griffith and R. Timothy Mulcahy

The metabolite glutathione fulfills many important and chemically complex roles in protecting cellular components from the deleterious effects of toxic species. GSH combines with hydroxyl radical, peroxynitrite, and hydroperoxides, as well as reactive electrophiles, including activated phosphoramide mustard. This thiol-containing reductant also maintains so-called thiol-enzymes in their catalytically active form, and maintains vitamins C and E in their biologically active forms. The key step in glutathione synthesis, namely the ATP-dependent synthesis of γ-glutamylcysteine, is the topic of this review. Details are presented on (a) the enzyme's purification and protein chemistry, (b) the successful cDNA cloning, sequencing, and expression of this enzyme, and (c) the cloning and characterization of the genes responsible for the biosynthesis of this enzyme. After considering aspects of the role of overexpression of this synthetase in terms of cancer chemotherapy, attention is focused on post-translational regulation. The remainder of the review deals with the catalytic mechanism (including substrate specificity, reactions catalyzed, steady-state kinetics, and chemical mechanism) as well as the inhibition of the enzyme (via feedback inhibition, reaction with S-alkyl homocysteine sulfoximine inhibitors, the clinical use of buthionine sulfoximine with cancer patients, and inactivation by cystamine, chloroketones, and various nitric oxide donors).

CLASSICS IN ENZYMOLOGY

Reprint of Chance's Germinal Paper Demonstrating That Rapid Reaction Kinetic Experiments Provide New Insights About the Nature of Enzymic Catalysis from Journal of Biological Chemistry 151, 553–573 (1943) (reproduced here by permission of The American Society for Biochemistry and Molecular Biology, Inc.):

THE KINETICS OF THE ENZYME-SUBSTRATE COMPOUND OF PEROXIDASE

By BRITTON CHANCE, *Johnson Research Foundation, University of Pennsylvania, Philadelphia, and the Physiological Laboratory, University of Cambridge, Cambridge, England*

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THE KINETICS OF THE ENZYME-SUBSTRATE COMPOUND OF PEROXIDASE

By BRITTON CHANCE

(From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, and the Physiological Laboratory, University of Cambridge, Cambridge, England)

(Received for publication, May 26, 1943)

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) $= \dfrac{\log_{10} I_0/I}{d \text{ (cm.)} \times c \text{ (mM per liter)}}$.

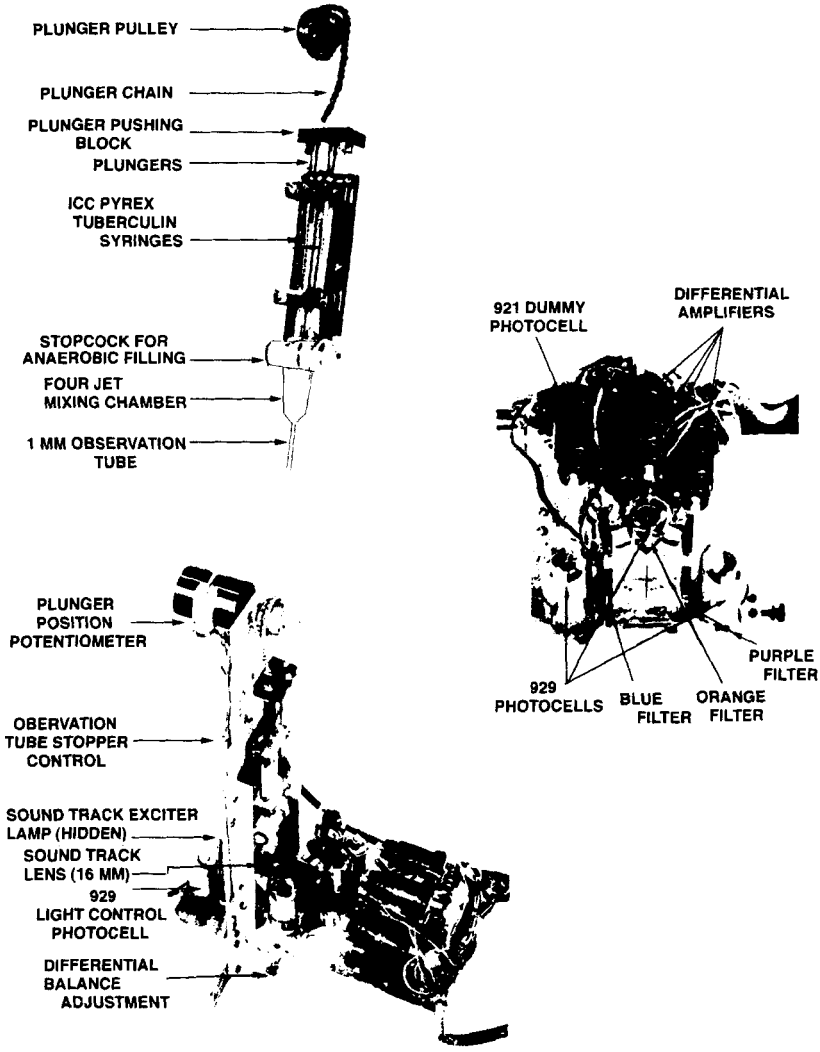

FIG. 1. Syringe unit, photocell unit, and assembled apparatus. Light and electrostatic shields are removed.

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

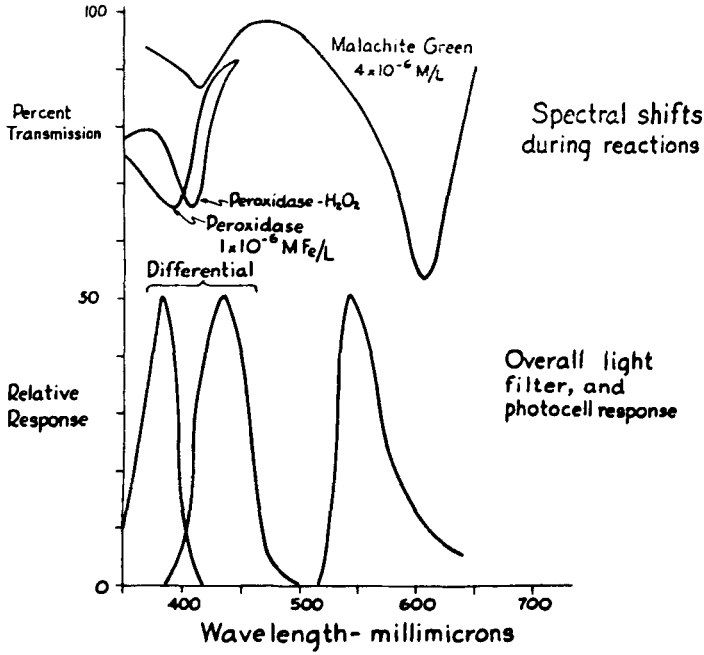

FIG. 2. The upper curves give the light transmission of enzyme, enzyme-substrate compound, and oxidized acceptor under the conditions of Fig. 3. The lower curves give the filter combinations used to measure the kinetics of the reactions. The trough depth was 16 times that of the 1 mm. observation tube 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).

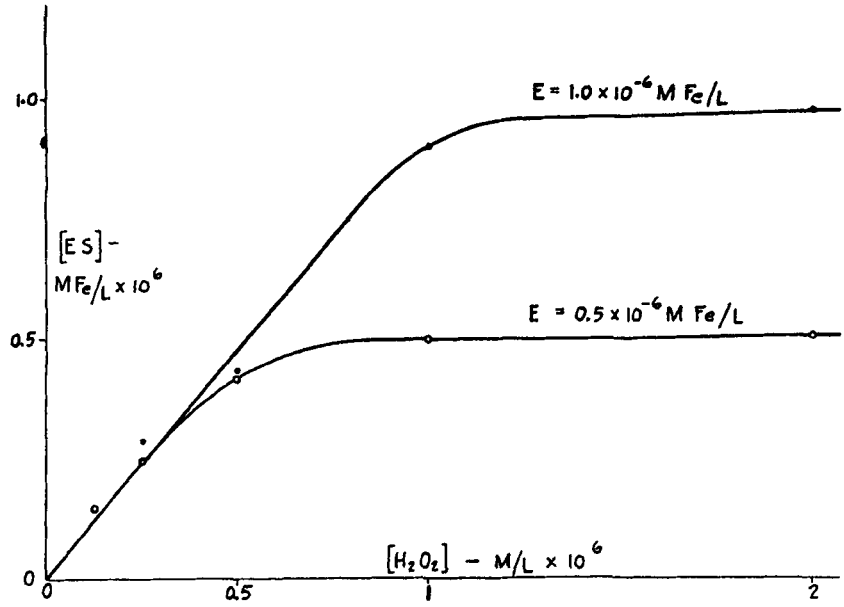

FIG. 4. Equilibrium of enzyme and substrate in absence of acceptor. Ordinate, intermediate compound as total hematin iron; abscissa, initial H_2O_2. pH = 4.2.

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.

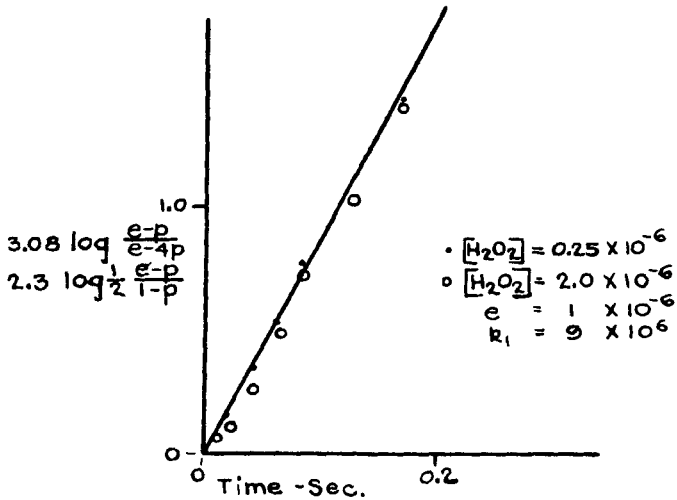

FIG. 5. Kinetics of formation of intermediate compound plotted for two values of substrate concentration according to the second order equation. $k_1 = 9 \times 10^6$ liter mole^{-1} sec.$^{-1}$, pH = 4.0.

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-

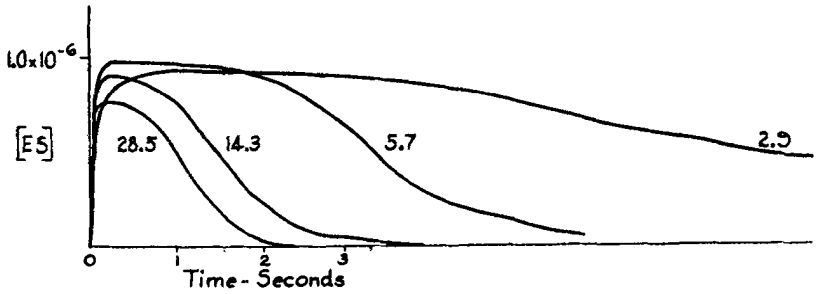

FIG. 6. The effect of an acceptor on the kinetics of the enzyme-substrate compound. $E = 1 \times 10^{-6}$ mole of hematin Fe per liter, $H_2O_2 = 4 \times 10^{-6}$ mole per liter, ascorbic acid as indicated in micromoles per liter, pH = 4.2.

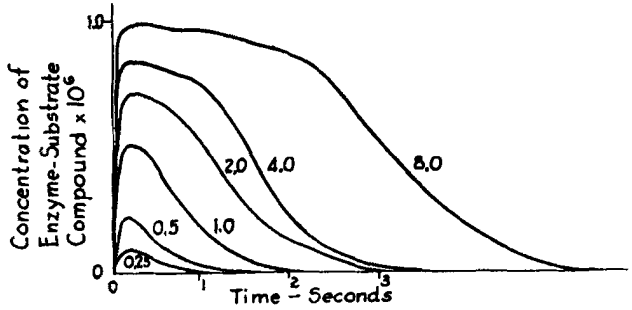

FIG. 7. The effect of substrate on the kinetics of the enzyme-substrate compound. $E = 1 \times 10^{-6}$ mole of hematin Fe per liter, ascorbic acid approximately 14×10^{-6} mole per liter, initial H_2O_2 as indicated in micromoles per liter, pH = 4.2.

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 ($p_{\max.}$), 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.$^{-1}$.

$\int_0^t pdt$ 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.$^{-1}$.

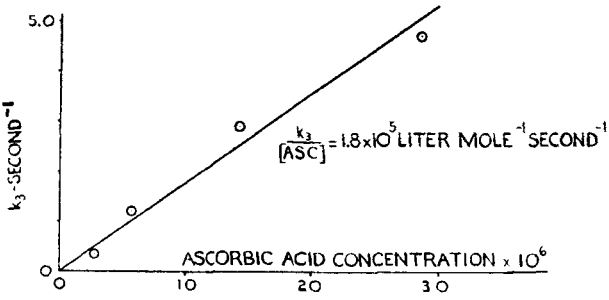

FIG. 8. Variation of k_3 with acceptor concentration. k_3 was obtained by Equation 16 from data of Fig. 6.

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.