KINETIC STUDIES OF THE DECOMPOSITION OF HYDROGEN PEROXIDE BY WHEAT CATALASE

by

Walter Bushuk

Being a thesis submitted to the Faculty of Graduate Studies and Research, in partial fulfillment of the requirements for the degree of Master of Science

University of Manitoba

April, 1953

ACKNOWLEDGEMENTS

The author is indebted to Dr. J. A. Anderson, Chief Chemist of the Grain Research Laboratory, who made available the facilities of the laboratory for this research project. Grateful acknowledgement is made to Dr. G. N. Irvine, Chemist at the Grain Research Laboratory, for his helpful suggestions in the course of the investigation and for his many constructive criticisms in the writing of the thesis.

To Dr. A. D. Robinson, Professor of Agricultural Biochemistry, for his keen interest in the project and for his helpful suggestions in the writing of the thesis the author offers his thanks.

Financial assistance was provided by the Associate Committee on Grain Research in the form of summer grants for 1951 and 1952 and by the National Research Council in the form of a bursary for 1952-53.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Cont.)

LIST OF FIGURES

LIST OF FIGURES (Cont.)

INTRODUCTION

Chemical kinetics is the branch of physical chemistry which is concerned with the measurement of velocities of chemical reactions. This is always the first step towards the elucidation of the mechanism of any chemical process. Kinetic studies of biological reactions have somewhat lagged behind the rapid advances made in other aspects of chemistry. The reason for this is that in studying biological processes one is often faced with consecutive or concurrent reactions which are further complicated by the presence of a multitude of other substances, the effects of which are seldom known. Although a considerable amount of research has been done on the mechanisms of biological processes only a few of them are understood clearly. The importance of kinetic studies in such investigations cannot be over emphasized.

The food of plants and animals which consists mainly of carbohydrates, proteins and fats is of no value until it is changed into a condition suitable for assimilation by the living cell. Outside of the living cell these changes could be readily effected by various chemical reagents or by high temperatures. The effect of such factors on the living cell are obvious. How then does the assimilation of the various biological substances proceed in the living cell?

Berzelius was the first to observe what is now termed the process of catalysis. He found that some chemical reactions are readily accelerated by certain foreign substances which themselves do not undergo any changes. These foreign substances have been called catalysts. For instance, it is by virtue of the countless biological catalysts or enzymes that it is possible for the living animal body to oxidize sugars to carbon dioxide and water. The same end products are readily obtainable by burning sugar in an open flame. There is not a single biological process which can occur without enzymes.

A group of enzymes which are involved in the germination and respiration processes of plants are the oxidizing enzymes. These can oxidize their substrates by the addition of oxygen or by the removal of hydrogen. The substance on which the enzyme acts is known as its substrate. Catalase is one of the oxidizing enzymes. The reaction which it effects is the decomposition of

hydrogen peroxide into water and molecular oxygen. Hydrogen peroxide then is the substrate of the enzyme, catalase. The apparent role of catalase in the biological system is to protect the living cell from the harmful effects of hydrogen peroxide which is produced by the action of certain oxidizing enzymes.

This investigation was undertaken as a part of a general study of the oxidizing systems of wheat with two principal objectives. First of all, it was hoped that such a study would be helpful in elucidating the mechanism of catalase action under biological conditions. The active enzyme extract used throughout this study contained representative concentrations of other water-soluble materials in wheat; the effects of these, if any, are incorporated in the experimental results. In this respect the system studied is similar to the process occurring under "in vivo" conditions. A mechanism based on the kinetic study by which the decomposition of hydrogen peroxide occurs under the conditions studied is postulated and its applicability to the biological process is discussed. Secondly, it was hoped that a kinetic study might indicate where the reaction effected by catalase fits into the numerous reactions which occur during the development of the wheat kernel or during processing of the wheat products. This is of prime importance because Canadian cereal chemists are continuously striving to find out why a certain variety of wheat is considered a good variety and another may be a poor one. Hence a comprehensive study of any chemical reaction which may occur during the development of the wheat plant would be helpful in solving this major problem. The action of the enzyme catalase definitely falls into this group.

To complete the study a number of experiments using crystalline catalase were carried out. These results are compared to those obtained with wheat catalase extracts. Catalase activities of a number of Durum wheat varieties, Canadian and American bread wheats and a number of grade samples were determined and tabulated.

SURVEY OF LITERATURE ON CATALASE

A vast amount of literature on catalase has accumulated during the past fifty years. Only a relatively brief review is within the scope of this thesis. It seemed that the most useful presentation would be of the chronological type. This is subdivided into the discovery of the enzyme, its distribution throughout the living tissues, the isolation of crystalline enzyme from natural sources and the chemical nature of the enzyme. The methods used for estimating its activity and the proposed mechanisms of its action are discussed briefly.

In 1811 Thenard noticed that plant and animal tissues as well as certain finely divided metals were able to decompose hydrogen peroxide with the evolution of oxygen. Schönbein, in 1863 (37) working with crude extracts of various tissues, concluded that all enzymes showed this property. This idea was proven to be wrong in 1892 by Jacobson (23) and again in 1901 by Low (32) who showed that the destruction of hydrogen peroxide was brought about by a single enzyme. Low called the enzyme catalase.

Catalase has been found in nearly all living tissues. Within a single species the concentrations are strikingly different in the various organs. Certain microorganisms which normally do not contain any catalase may produce it after coming into contact with hydrogen peroxide (27). Most of the available literature deals with catalases from animal sources. This is mainly because the catalase content of animal organs such as the liver is much higher than of most plant tissues. Hennichs (19), Stern (41), Chance (9), Heppel and Porterfield (20), Sumner and Dounce (46) and Beers and Sizer (4) all obtained the catalase for their studies from beef liver. Laskowski and Sumner (29) isolated catalase from beef erythrocytes whereas Morgulis et al (35) prepared their catalase from beef kidney. Marks and Fox (33) investigated the inactivation of mussel catalase by oxygen.

The amount of literature on plant catalases is not so extensive. The catalase activity of wheat and wheat products has been studied by a number of investigators. Among these are Bailey (3) and Blish and Bode (5). Bailey determined

the catalase activities of a series of American wheat flours using a crude manometric method. For his enzyme preparation, he used a mixture of flour and water. He made an extensive study of catalase activity as related to the ash content of the flour. Although he found a strikingly close parallelism he could not generalize the relationship for all the samples. The work of Blish and Bode showed that catalase activity was highest in the bran fraction and lowest in the endosperm. Of 36 samples used, they found that in general the activity of Canadian wheats was higher than the activity of American wheats and that spring varieties produced higher values than winter wheats grown under similar climatic conditions. Bailey (3) suggested that catalase activity be used as an index of the grade of flours since he observed that the activity increased as grade decreased. Blish and Bode (5) declared that the knowledge of the locality where the wheat originated would be essential if the activity is to be used as a measure of the grade.

Further experiments of Blish and Bode (5) have shown that catalase activity is decreased by fine grinding and also by the addition of commercial bleaching agents such as nitrogen trichloride or benzoyl peroxide. Prolonged storing at room temperature had a similar effect. Bach et al (2) observed that during germination of wheat the catalase activity at first increased and then decreased. Other investigators obtained similar results. Takasugi and Yamazaki (48) investigated the fluctuation of enzymes and other important substances during wheat germination and have postulated a mechanism for the appearance and disappearance of catalase activity. They found that the catalase activity of wheat grains reached a maximum on the fifth day of germination and then decreased. This effect was not observed when the germination took place in an atmosphere of hydrogen. They showed also that the activity is concentrated in the scutelum and aleurone layer of the wheat kernel. The investigators speculate that these parts play an important role in the formation of catalase. A number of investigators have suggested that the reason for the variation of catalase activity in the plant during the growing period is due to the development within the plant of activating or inhibiting substances.

Appleman (1) studied the relation of chlorophyll and catalase in barley seedlings. He found that when a sudden chlorophyll synthesis occurs catalase activity decreases. If the synthesis of the pigment is blocked the catalase activity increases at a high rate if it is at a low level and remains approximately constant if already at a high level. In 1943 Yamafuzi et al (56) reported that sugar cane leaves which have been infected with plant virus have a lower catalase activity than normal leaves.

Catalase is one of the few enzymes which have been isolated in crystalline form. In 1911 Wolff and de Stoechlin (55) prepared a catalase solution from erythrocytes which was completely free from hemoglobin and, three years later, Waentig and Gierisch (53) were able to concentrate their catalase preparation by repeated alcoholic precipitations, dialysis and adsorption. However it was not until 1937 that crystalline catalase was isolated. In that year Sumner and Dounce (46) succeeded in obtaining pure beef liver catalase in the form of needle or platelet crystals. Their method of preparation consisted of extraction and fractional precipitation with diluted dioxane, followed by crystallization of the precipitate from chilled ammonium sulphate solution. In 1942 Dounce (15) used alcohol and acetone as solvents with equal success. His enzyme was identical with the sample prepared by the dioxane method.

Since the development of the above method crystalline catalase has been isolated from a variety of sources. In 1941, Laskowski and Sumner (29) used beef erythrocytes as their starting materials and in 1942 Dounce (15) used lamb liver as the source of his enzyme. Brown (7) in 1952, using the original method of Sumner and Dounce, prepared crystalline catalase from ox liver. He claims to have isolated two catalases characterized by different activities. Some investigators believe that this may have been caused by some form of enzyme denaturation during the separation. Herbert and Puisent (21) obtained the crystalline enzyme from certain bacteria and human erythrocytes. These had properties identical to those of other preparations. No one has succeeded in isolating crystalline catalase from plant sources. The relative concentration of the enzyme in plant tissues is exceedingly low, consequently a large amount of

plant material would have to be processed to obtain a reasonable amount of the crystalline enzyme. This would be very impractical and in some cases would be quite expensive.

The literature on the chemical nature of catalase is quite extensive. Only some of the more pertinent papers will be reviewed here. Long before the isolation of the crystalline enzyme, Zeile and Hellstrom (58), using a spectrophotometric method, established that the active group of catalase is a ferroporphyrinic combination. These observations were supported by further studies by Zeile (57). In 1935 Stern (41) showed that the hematin group of catalase was derived from aetioporphyrin III and possesses a porphyrin group with the same arrangement of the side chains as found in the natural blood pigment. He suggested that the high activity of catalase was due to a special type of combination of the hematin with a particular protein. Analysis of the crystalline enzyme prepared by Sumner and Dounce in 1937 gave the following results:

isoelectric point	pH 5.7
iron content	0.1%
ash	0.5%
absorption bands	627, 536 mμ.

In 1938 Stern and Wyckoff (44) studied crystalline catalase in the ultra centrifuge and obtained the value 250,000 - 300,000 for its molecular weight. Later in the same year, Sumner and Gralen (47) published the value 248,000. They suggested also that the enzyme contains four hematin groups. Recently Sumner (45) published an excellent and rather complete review on the chemical nature of catalase.

The reaction affected by catalase is the decomposition of hydrogen peroxide into water and molecular oxygen according to the following equation,

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Accordingly, catalase activity may be estimated in two ways, firstly, by determining the amount of hydrogen peroxide decomposed after a definite reaction period or, secondly, by measuring directly the amount of oxygen liberated.

There are various methods by which the decrease in the hydrogen peroxide concentration may be determined. The procedure used by von Euler and Josephson (52) was to stop the enzymic reaction by the addition of sulfuric acid and titrate the residual peroxide with standard permanganate solution. Golblith and Proctor (17) added an excess of permanganate which was determined colorimetrically. These methods are reasonably satisfactory except for reaction periods of less than one minute. Lemberg and Foulkes (30) developed a micro-method by which they succeeded in obtaining data every ten seconds. This technique has limited application because considerable error is involved in taking samples at such short time intervals. Direct measurements of the disappearance of hydrogen peroxide by a polarographic method were carried out by Bonnischen et al (6). This method gave good results for the first minute of reaction and first order kinetics were observed. It was pointed out by the same authors that the polarographic method invariably gave lower results than the titration method. They could not explain the cause of this discrepancy. Chance (9) and Chance and Herbert (13) followed the breakdown of hydrogen peroxide by observing the decrease in light absorption of peroxide solutions in the ultra-violet region. Beers and Sizer (4) investigated the potentitalities of this method and developed a quantitative, spectrophotometric tehhnique for following the decomposition of hydrogen peroxide which could be used for routine studies.

Manometric methods are satisfactory for following the destruction of hydrogen peroxide if the reaction proceeds at a moderate rate. If the decomposition occurs at a rapid rate then the diffusion of oxygen from the solution may become a limiting factor and the measurement of the rate of evolution of oxygen is then no longer a measure of the rate of the decomposition of hydrogen peroxide. The rate of agitation is also an important factor in the manometric technique.

Manometric methods were gradually improved with the development of various types of manometers. Dixon (14) gives a detailed discussion of the three main types of manometers. These are the constant-pressure, the constant-volume and the differential types. In the first type the gas in the flask is kept at constant pressure by adjusting the liquid in a graduated tube connected to the

flask. The change in volume is read from the tube. The second type is commonly known as the Warburg manometer although it was originally developed by Barcroft and Haldane. Here the reaction flask is attached to one end of a U-shaped calibrated manometer tube, while the other remains open to the atmosphere. The liquid in the tube is adjusted to keep the gas in the flask at constant volume and the change in pressure is read on the open end. The amount of gas absorbed or evolved may be readily calculated from the pressure change. In the differential type both the volume and the pressure change simultaneously. This is generally known as the Barcroft manometer as it was developed mainly by him.

Bailey (3) used the first type of manometer to determine the catalase activities of American wheat flours. The method was quite satisfactory since he was only interested in the total volume of oxygen evolved after a definite reaction period. Sizer (39) in his studies of temperature effects on crystalline catalase used the Warburg type of manometer. His results were in good harmony with the Arrhenius equation. Later he confirmed some of these findings using the spectrophotometric method. The Warburg type of manometer has been used throughout in the study on which this thesis is based.

A considerable amount of research has been done on the kinetics of reactions catalyzed by enzymes. Very often the experimental conditions have been so complicated that little information could be obtained concerning the mechanism involved. The kinetics of the decomposition of hydrogen peroxide by catalase have received considerable attention. In order to interpret the experimental data kinetically, various mechanisms have been proposed and it seems appropriate to review some of these at this point. Haber and Willstatter (18) suggested that the decomposition of hydrogen peroxide by catalase occurs by a chain mechanism. During the reaction the catalase iron is reduced from Fe^{+++} to Fe^{++}. The over-all process involves the following reactions:

$$H_2O_2 + Fe^{+++} = \nearrow O.OH + Fe^{++} + H^+$$

$$\nearrow O.OH + H_2O_2 = O_2 + \nearrow OH + H_2O$$

$$\nearrow OH + H_2O_2 = H_2O + \nearrow O.OH$$

According to the first of the above equations, hydrogen peroxide is a reducing agent. Other investigators have shown that hydrogen peroxide cannot reduce ferric iron to the ferrous state and hence the above mechanism is not probable.

The question whether the valence of the iron in catalase changes during the destruction of hydrogen peroxide has received considerable attention. Most investigators believed that the valence did not change and in 1932 Stern (42) proposed the following modification of the above mechanism in which the iron remains trivalent. He suggested that in the first reaction hydrogen peroxide was degraded into two hydroxyl radicals which initiated the decomposition of the second molecule of hydrogen peroxide. The mechanism may be illustrated by the following equations:

$$H_2O_2 + Fe^{+++} = Fe^{+++} + 2\nearrow OH$$

$$\nearrow OH + H_2O_2 = H_2O + \nearrow O.OH$$

$$\nearrow O.OH + H_2O_2 = O_2 + \nearrow OH + H_2O$$

Using a manometric method, Keilin and Hartree (25) observed that catalase was completely inactive in the total absence of oxygen. They immediately put forth the theory that the oxygen was necessary to activate the enzyme by oxidizing the ferrous iron to the ferric state which in turn oxidizes the hydrogen peroxide by the following mechanism:

$$4Fe^{+++} + 2H_2O_2 = 4Fe^{++} + 4H^+ + 2O_2$$

$$4Fe^{++} + 4H + O_2 = 4Fe^{++} + 2H_2O$$

Johnson and van Schouwenbourg (24) made an attempt to repeat the experiments of Keilin and Hartree but were unable to do so. In 1939, Weiss and Weil-Malherbe (54) carried out manometric studies still trying to confirm the findings of Keilin and Hartree. They passed nitrogen through their apparatus until no oxygen could be detected by the ferrous pyrophosphate method. They found no decrease in activity. The authors suggested that the reaction,

$$4Fe^{++} + 4H^{+} + O_2 = 4Fe^{+++} + 2H_2O$$

is very unlikely since the second product would be hydrogen peroxide and not water. In 1943, Keilin and Hartree (26) withdrew their claims that catalase action does not proceed under anaerobic conditions. About the same time it was shown by paramagnetic measurements that the iron in catalase remains in the ferric state throughout the reaction. Hence, the mechanism proposed by Keilin and Hartree could no longer be considered valid.

It will be opportune at this point to review briefly the classical treatment of enzyme kinetics published in 1913 by Michaelis and Menten (34). This treatment applies to enzymes systems in general. They suggested that the first reaction is the combination of the enzyme and the substrate to form an activated complex which then decomposes into the active enzyme and the products. The over-all scheme may be represented by the following equation,

$$E + S \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} E + P$$

in which the E is the enzyme, S is the substrate, ES is the activated enzyme-substrate complex and P are the products of reaction whatever they may be. The above investigators derived a numerical expression, Km, for the extent of the complex formation. Assuming that at high substrate concentration all of the enzyme will be in the form of the complex and the reaction will proceed at maximum velocity Vm, Michaelis and Menten obtained the following relationship,

$$Km = S\left(\frac{Vm}{v} - 1\right) \qquad (1)$$

in which v is the velocity at substrate concentration S. Km is commonly known as the Michaelis constant for the enzyme reaction. We can readily see that Km is numerically equal to the concentration of substrate which gives one-half the maximum initial velocity. Van Slyke and Cullen (51) produced essentially the same equation considering the initial velocities of the reactions involved.

Since the formulation of the Michaelis-Menten theory a large portion of research work on enzyme kinetics was directed towards the identification of

the activated enzyme-substrate complex. In this respect the investigators have been more successful with the catalase system than with any other enzyme. In 1936 Stern (42) demonstrated the formation of an intermediate compound between catalase and monoethylhydrogen peroxide. A solution of his purified enzyme showed absorption maxima at 650, 646-620 and at 610 mμ. When the substrate was added the solution showed a change in color and a shift in absorption maxima to 576-564 and 540-528 mμ. After the reaction was complete, the solution of the enzyme showed its original properties.

Britton Chance (10) has published a series of papers in which he discusses the experimental evidence for the existence of an intermediate complex between catalase and its substrate. He used a microspectrophotometric adaptation of the apparatus designed for kinetic studies by Hartridge and Roughton. He found that approximately 1.2 ± 0.1 hematin groups of the enzyme are occupied by hydrogen peroxide in forming the enzyme-substrate complex. He also showed that hydrogen peroxide and the cyanide radical combine competitively with the iron atom in catalase. The non-competitive inhibition by cyanide, he states, demonstrates that catalase hematins not involved in the catalase-hydrogen peroxide complex are required for the destruction of hydrogen peroxide.

In 1950 Britton Chance (11) investigated the conversion of the active complex of catalase and hydrogen peroxide into an inactive form similar to the secondary complex formed by catalase with hydrogen peroxide in the presence of alkyl hydrogen peroxide. The same author (10) cited experimental evidence for a ternary complex of catalase, hydrogen peroxide and certain alcohols. This reaction tends to occur when catalase is in the presence of considerable concentration of the alcohol and only a trace of hydrogen peroxide. By a very ingenious method Chance (10) was able to titrate solutions of catalase with hydrogen peroxide solutions and from these data he calculated the dissociation constants for the enzyme-substrate complex of catalases from various sources. A number of the very low values could not be calculated directly from the titration data and these had to be determined from the kinetic studies assuming a second-order reaction. The values quoted are in the range 10^{-9} to 10^{-8}.

In 1949 Chance (12) postulated the following mechanism for the decomposition of hydrogen peroxide by solutions of crystalline catalase;

$FeOH + H_2O_2$ (first molecule = hydrogen acceptor) = $FeOOH + H_2O$

$FeOOH + H_2O_2$ (second molecule = hydrogen donor) = $FeOH + H_2O$ & O_2

or in general,

$$E + S \rightleftharpoons ES \text{ (active or inactive)}$$

$$ES + S \longrightarrow E + P$$

in which E is the enzyme and S is the substrate or more specifically, catalase and hydrogen peroxide. The complex ES may be enzymically active or inactive. He points out that the above mechanism is supported by the demonstration of the presence of the intermediate enzyme-substrate complex, the lack of the Michaelis constant for the system, and the peroxidation reactions of the catalase-hydrogen peroxide complex. This mechanism is also supported by the fact that the rate of dissociation and of formation of the enzyme-substrate complex is independent of the hydrogen ion concentration.

To explain their particular experimental results some investigators suggested that catalase may exist in two forms, one being slightly more active than the other. Morgulis et al (35) seem to favour the idea that catalase occurs in nature in an inactive form but quickly changes to the active form when brought into contact with the substrate. The experimental evidence for this, they state, is the irregular variation of activity with pH and temperature. In 1947, George (16) observed that the reaction between catalase and hydrogen peroxide consists of two phases; a rapid initial reaction decreasing exponentially with time and practically non-existent after two minutes and a superimposed, slower reaction. This, he states, is due to the existence of catalase in two forms. However, it is now accepted that there is only one active form of crystalline catalase, although it may have suffered some transformation or change of activity during the course of its isolation from biological materials.

The above review of literature indicates that the catalase system has been fairly well defined. However complete kinetic studies of plant catalases are lacking.

It is known that catalase activity is different at various stages of plant development. Little is known of the physiological reasons for this. Most of the studies have been carried out using pure or almost pure crystalline enzyme so that very little information has been obtained as to what effect other biological substances would have on its activity. The author is of the opinion that further kinetic studies of the decomposition of hydrogen peroxide by water extracts of plant materials would be helpful in elucidiating the mode of action of plant catalase under physiological conditions.

EXPERIMENTAL

KINETIC STUDIES OF THE CATALASE SYSTEM OF DURUM WHEAT

Methods

Extraction of the Enzyme. Crude active extracts of durum wheat endosperm may be obtained by the following method. Ten grams of semolina are triturated with five grams of sand and 20 ml. of distilled water for five minutes in a mechanical mortar grinder. The mass is transferred to a 50 ml. round-bottom centrifuge tube and spun for 10 minutes at top speed in a clinical centrifuge. The supernatant liquid is the active extract. This will be known as the regular extract. Most of the work reported in this thesis was done with semolina milled from two pure varieties, Pelissier and Mindum. In experiments with bread wheats or whole wheats 10 gm. of flour or ground whole wheat replaces the semolina in the above procedure.

Extracts prepared by this method are highly reproducible so that it is not necessary to store them from day to day. If desired these extracts may be kept in a refrigerator for several days with no noticeable decrease in activity. No attempt was made to purify or concentrate the extracts in any way, so that the exact concentration of the enzyme was never known. In a number of cases, especially with the whole wheats, the regular extract was too active and so had to be diluted accordingly. The terms wheat or enzyme extract, wheat catalase and regular extract are used synonymously throughout this thesis.

Preparation of the Substrate. The substrate used throughout this study was hydrogen peroxide solution. It was prepared by diluting the available Merck "superoxol", assayed at 14.8% hydrogen peroxide, with distilled water. The exact concentration of the prepared substrate was checked by titrating with standard permanganate solution. For routine work 1.0 ml. of "superoxol" was diluted with 100 ml. of distilled water. The concentration of this preparation is approximately 4.64×10^{-2}M. Complete decomposition of 1.0 ml. of this substrate preparation results in the evolution of about 523 µl. of oxygen. If the dilute hydrogen peroxide solution is stored in a refrigerator its concentration remains essentially the same for as long as two weeks. Oxygen evolution owing to the spontaneous

decomposition of hydrogen peroxide is negligible when 1.0 ml. of the above preparation is used in a total volume of 7.0 ml. at the normal experimental temperature of 25°C. At higher substrate levels the spontaneous decomposition cannot be neglected and must be corrected for by proper controls. The buffer solution used in this study was prepared by mixing proper amounts of 0.067M NaH_2PO_4 and 0.067M K_2HPO_4 solutions which were prepared using pure anhydrous solids.

Manometric Measurement of the Evolution of Oxygen. Throughout this study, the reaction was followed by measuring the amount of oxygen liberated during the reaction with a Warburg constant volume respirometer. The Warburg apparatus is a 20 manometer, rotary type manufactured by Precision Scientific Co. The temperature range of the instrument is up to 60°C, readily varied by a Philadelphia micro-set thermoregulator. This thermostat controls the temperature of the water bath to within $\pm$0.02°F which is adequate enough for the purposes of this investigation. Fifty milliliter Warburg reaction vessels with a single side-arm and no centre-well were used throughout the study. The exact volumes of the vessels were determined by the method of Scholander et al (36). These values were then used to calculate the flask constants using the technique of Umbreit et al (49). The flask constant is required to convert the pressure change within the reaction vessel, which is obtained in terms millimeters of manometer fluid, to microliters of oxygen evolved. All the vessels, manometers and side-arm stoppers were numbered before calibration to eliminate any possible error due to slight differences in the particular parts.

Unless stated otherwise, the experimental temperature was 25.0°C. As will be seen later, this is slightly above the optimum temperature for the reaction but was found most practical since it did not require the use of the refrigeration unit. In determining the temperature effects it was necessary to work at temperatures considerably below that of the room. This was done with the aid of a special, portable refrigeration unit, designed and built at the Grain Research Laboratory. The shaking rate throughout the investigation was 130 oscillations per minute through an amplitude of 4.0 cm.

The procedure employed in setting up the system for the measurement of oxygen evolution is similar to the one outlined by Umbreit et al (49) for the measurement of cell respiration. The stepwise procedure is as follows:

1. Set up the clean, dry, Warburg vessels in order in which they will be filled.
2. In the main compartment place 5.0 ml. of the phosphate buffer solution of proper pH and add 1.0 ml. of substrate of desired concentration.
3. Add 1.0 ml. of enzyme extract to the side-arm.
4. Grease and insert the side-arm stoppers.
5. Grease the attachment joint on the manometer and attach the filled reaction vessel.
6. Place in the constant temperature bath.
7. Repeat the above procedure until all the reaction vessels are in position in the bath. Leave the three-way stopcock open to atmosphere.
8. Equilibrate by shaking for 10 minutes.
9. Adjust the manometer fluid to 5.0 cm. in the closed side of the manometer.
10. Read the open side of the manometer.
11. Stop the shaking long enough to close the stopcocks.
12. Initiate the reaction by tipping the enzyme extract into the buffered peroxide solution.
13. Take readings at appropriate time intervals, from the time the reactants are mixed (every 5 minutes or less depending on the rate of the reaction).

A control vessel is used which contains the same components in the main compartment but the enzyme extract in the side-arm is replaced by an equal volume of distilled water. The purpose of this flask is to correct for any gaseous exchange within the reaction vessel which is not due to the enzyme. For instance, there may be spontaneous decomposition of hydrogen peroxide. Another manometer is set up containing only a small amount of water

in the main compartment of the vessel. This is known as the thermobarometer and it enables one to make accurate corrections for any external changes in temperature and pressure. This is necessary because the flask constants were calculated on the assumption that the temperature and atmospheric pressure remain constant throughout the experiment.

In determining the effect of an added substance on the enzymic activity a slight modification in the procedure outlined is necessary. In this case the 1.0 ml. of the regular extract together with the added substance are placed in the main compartment of the reaction vessel. The 1.0 ml. of substrate of desired concentration is pipetted into the side-arm. The remainder of the procedure is identical to that given above.

The pressure change within the reaction flask is read off the manometer in terms of millimeters of manometer fluid. Then it is converted to microliters of oxygen by multiplying by the appropriate flask constant. After a number of preliminary experiments it was found that changes occuring within the control vessel were negligible. That is, the control could be completely left out and we need only consider the external changes which are corrected for by the thermobarometer.

The volume of oxygen evolved as determined in microliters may be converted to moles of hydrogen peroxide decomposed. This is possible since 1 mole of hydrogen peroxide gives rise to 0.5 moles of oxygen. The molar volume at the particular experimental conditions may be determined knowing that 1 mole of oxygen occupies 22.4 l. at S.T.P. In this study it was found unnecessary to carry out this final conversion and consequently all calculations were done in terms of microliters of oxygen.

Results and Discussion

Preliminary Considerations. To carry out an extensive kinetic study it is of initial importance to know the order of the reaction which is to be investigated. Preliminary examination of the reaction curves suggested that the decomposition of the hydrogen peroxide by wheat extracts proceeded according to first or second order kinetics. There is no experimental evidence that the

reaction may be of zero order even at relatively high substrate concentrations. Accordingly, the experimental data were tested using the equations developed theoretically for the two probable orders. When the logarithm of the residual substrate concentration is plotted against the reaction time, a linear curve results. This immediately suggested the following relationship;

$$\ln (a-x) = k t + k^1 \qquad (2)$$

which differentiates to

$$- \frac{d (a-x)}{(a-x)} = k dt \qquad (3)$$

Rearranging equation (3) we get

$$- \frac{d (a-x)}{dt} = k (a-x) \qquad (4)$$

In equation (4), (a-x) is the concentration of the reacting substance at time t and k is a constant which for this particular case is known as the first order specific rate constant expressed in reciprocal seconds.

An equivalent treatment may be formulated when the rate is expressed in terms of the product of the reaction, that is in this case we have

$$\frac{dx}{dt} = k (a-x) \qquad (5)$$

in which a is the initial concentration of the reactant and x is the amount reacted after time t.

Equation (5) may be rearranged to

$$\frac{dx}{a-x} = k dt$$

which integrates to

$$-\ln (a-x) = kt + k^1 \qquad (6)$$

The constant of integration k^1, is evaluated by setting x = o at t = o; hence $k^1 = -\ln a$. Substituting for k^1 and rearranging (6) we have

$$\ln \left(\frac{a}{a-x}\right) = kt$$

or

$$k = \frac{1}{t} \ln \left(\frac{a}{a-x}\right) = \frac{2.3026}{t} \log_{10} \left(\frac{a}{a-x}\right) \qquad (7)$$

So that for a first order reaction, the value of k as calculated with the aid of equation (7) should remain a constant as long as the reaction remains first order.

Six values of the specific rate constant as calculated from actual experimental results are shown in Table I.

Table I. First Order Specific Rate Constant as a Function of Reaction Time

t, sec	a-x, µl of O_2	k x 10^3
0	466	- -
300	343	1.02
600	244	1.08
900	180	1.07
1200	130	1.06
1500	96	1.05
1800	71	1.05

In the above experiment 1.0 ml. of the regular extract from the variety Pelissier was used in conjunction with 1.0 ml. of 0.046 M hydrogen peroxide solution and 5.0 ml. of buffer solution of pH7.3. In Table I a-x is expressed in terms of microliters of oxygen. This was found advantageous because it did not require a conversion of the volume of oxygen liberated to moles per liter of hydrogen peroxide and hence eliminated this step as a source of error. The value of a, or the substrate concentration when t = o, is obtained by allowing the reaction to proceed until all the hydrogen peroxide is decomposed. It is equal to the total volume of oxygen liberated. The amount of substrate remaining after time t is then simply equal to a-x, where x is the volume of oxygen liberated in t seconds. It is not necessary to express a and x in terms of moles per liter to be able to use equation (7). Since we are only interested in the ratio $\frac{a}{a-x}$, a and x could be expressed in any units as long as they are the same in both cases.

Referring to Table I again, we see that k is essentially constant until at least 85% of the substrate is used up. This result is as good as has been observed with any other chemical reaction which is of first order. For reasons which will

become apparent when more of the experimental results are considered it is advantageous to use k, the specific rate constant, as an index of enzymic activity. Accordingly, in all further work the enzymic activity is expressed in terms of k. Generally it is calculated with the aid of equation (7). A more accurate method is to plot ln (a-x) against t, a straight line should result, the slope of which is numerically equal to the specific rate constant.

Effect of Hydrogen Ion Concentration. The influence of hydrogen ion concentration on the activity of an enzyme is of prime importance in kinetic studies of enzyme reactions. Every enzyme system shows its highest activity at some characteristic pH. This is known as the optimum pH for the particular enzyme. Some investigators believe that an enzyme acts best at that hydrogen ion concentration at which its protein portion is at its isoelectric point. If the activity is plotted against the pH of the reaction medium the result is a curve showing a maximum. A number of other factors may influence the optimum pH. These are the nature of substrate, purity of the enzyme and the length of time over which it acts. Even the type of buffer employed or its ionic concentration may have considerable effect.

In 1909 Sörensen (40) found that crude beef liver catalase shows its maximum activity at pH 6.8. Similar results were obtained later by Sumner and Dounce (46) with crystalline catalase. We have found a slightly higher optimum pH with crude wheat extracts.

In the optimum pH experiment 1.0 ml. of regular extract was used with 1.0 ml. of 0.046 M hydrogen peroxide and 5.0 ml. of 0.067M phosphate buffer of various hydrogen ion concentrations. The results are tabulated in Table II and are represented graphically in Figure 1. The enzymatic activity is expressed in terms of the specific rate constant, k. The optimum activity occurs at pH 7.33. Accordingly, all further work was done using a buffer solution of this pH.

Table II. Enzymic Activity as a Function of pH.

pH	Activity, k x 10^3
5.80	0.74
5.98	0.89
6.35	1.01
6.83	1.07
7.33	1.11
8.13	1.02
9.15	0.66

Effect of Temperature. The experimental temperature has a marked effect on the rate of enzymic reaction. In general, if the activity is plotted against the temperature an optimum type of curve is obtained. Figure 2 shows that wheat catalase activity at first increased due to a rise in temperature but above 23°C its activity is diminished by a further rise in temperature. Hence the best temperature for the particular enzyme and substrate levels is about 23°C.

Table III. Enzymic Activity at Various Experimental Temperatures.

Temperature	Activity, k x 10^4
10	4.40
15	4.92
20	5.10
25	5.00
30	4.00

The results in Table III were obtained using 1.0 ml. of regular extract and 1.0 ml. of 0.046M hydrogen peroxide solution.

The rapid decrease in activity above 23°C may be explained on the basis of two factors. Catalase must be very thermolabile and hence when a certain temperature is reached the enzyme begins to suffer thermal inactivation. In this case this effect becomes predominant at a relatively low temperature. It is also known that catalase is destroyed by hydrogen peroxide. If the activation energy of this secondary reaction is high, then a small increase in temperature would produce a considerable increase in the rate of this secondary reaction.

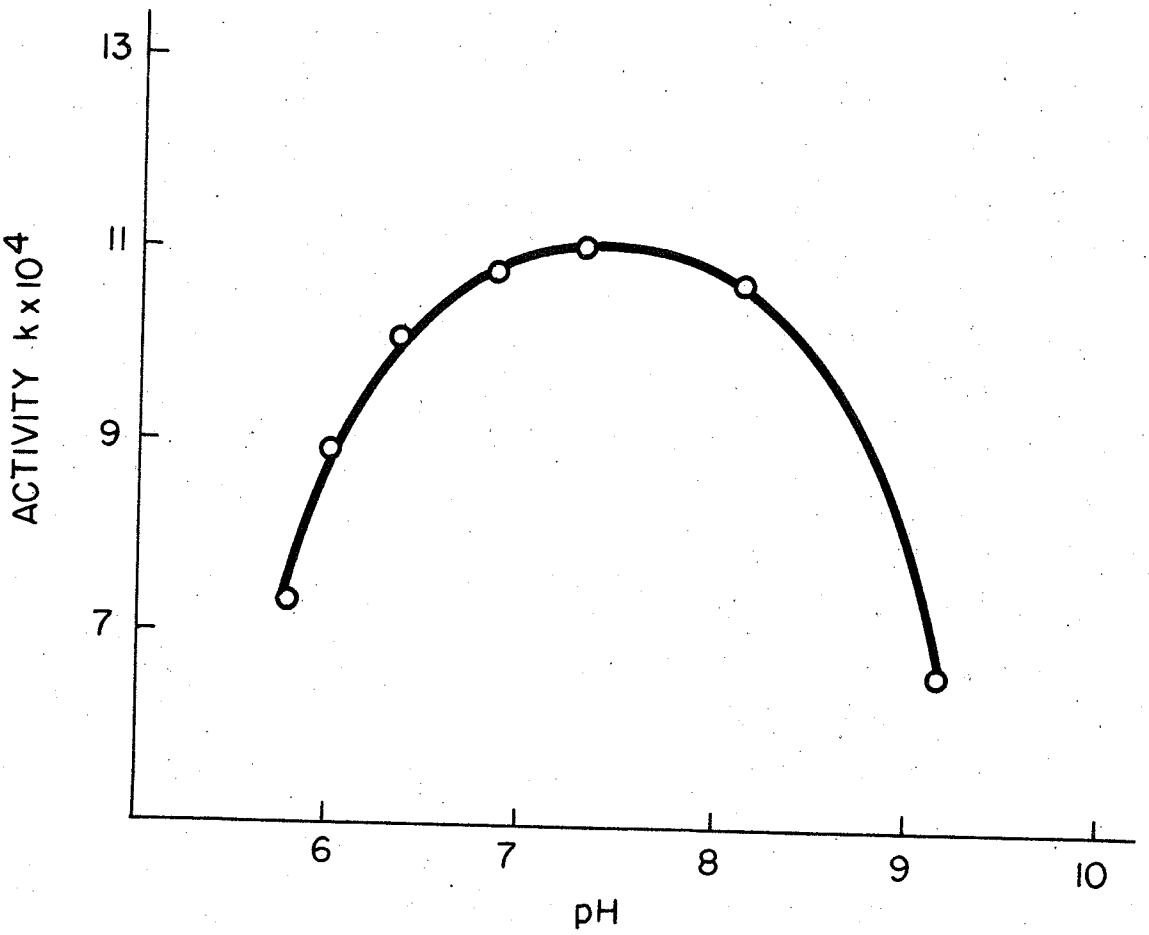

Figure 1. The influence of hydrogen ion concentration on the activity of crude wheat catalase.

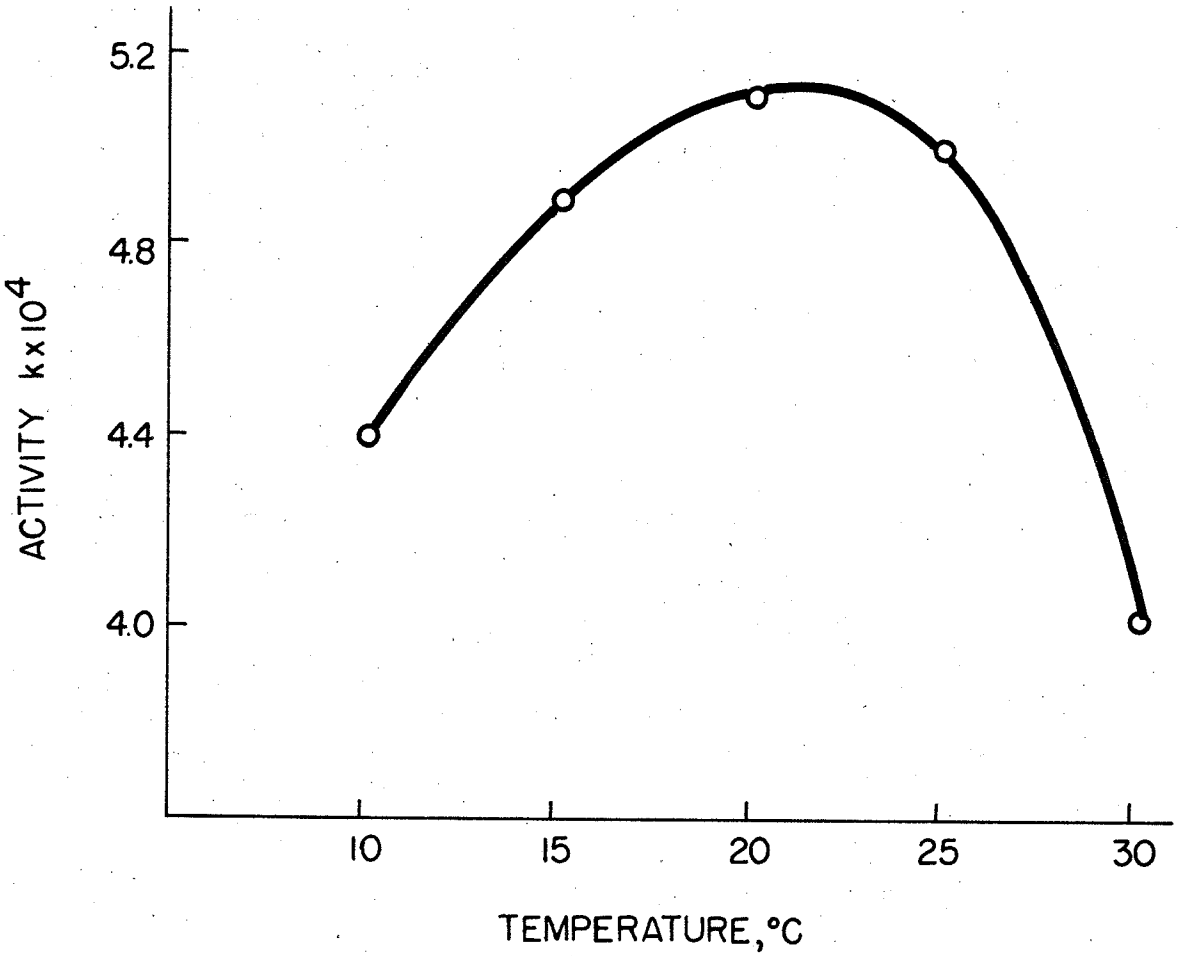

Figure 2. The effect of temperature on the activity of crude wheat catalase.

All the experimental work on which this thesis is based was done at 25.0°C. This may be about 2° above the best temperature but was the most practical temperature to work at since it did not require the use of the refrigeration unit. The refrigeration unit must be used to obtain temperatures below room temperature.

If the enzyme extract is incubated at 40°C we note a rapid decrease in activity due to thermal inactivation of the enzyme. The results obtained are shown in Table IV.

Table IV. Thermal Inactivation of Wheat Catalase at 40°C.

Incubation Period, Min.	Activity, k x 10^4
0	5.00
20	4.38
40	3.78
60	3.30

Another way to demonstrate the effect of temperature on the enzymic activity is to note the decrease in the specific rate constant during the reaction period at a particular temperature. As will be seen later, the specific rate constant is directly proportional to the active enzyme concentration so that any change in the constant will reflect a parallel change in the active enzyme concentration. The results of one such experiment at 40°C are given in Table V.

Table V. Variation of the Specific Rate Constant with Reaction Time at 40°C.

Reaction Time, Min.	k x 10^4
5	3.35
10	3.64
15	3.43
20	3.26
25	3.04
30	2.86

In the above experiment the reaction flask was equilibrated for 5 minutes by shaking in the bath at 40°C before the reaction was initiated. The

discrepancy between the 5 and 10 minute readings may be explained as follows: The temperature of the reaction flask drops considerably when it is removed from the bath to mix the enzyme and the substrate. This is mainly due to the rapid evaporation of water from the surface of the flask. After the flask is replaced into the water bath, it seems that 5 minutes is not sufficient time for the flask to reach equilibrium conditions and hence the first reading includes an error due to this effect. The magnitude of this discrepancy depends on the difference between the temperature of water bath and room temperature. At 25°C it is almost negligible. From Table V again we see that the amount of inactivation is almost directly proportional to the reaction time if we neglect the value at 5 minutes.

Effect of Varying the Enzyme Concentration. The concentration effects on the rate of a chemical process may be determined by several methods. One procedure is to study the effects on the initial rate, the other is to consider the overall reaction. The results by both methods should be the same if the kinetic order of the reaction remains constant throughout the period over which the investigations are made.

In this study the range of enzyme concentrations is limited by the size of the reaction flasks. Considerable error is introduced if the reaction is too rapid as is the case at very high enzyme concentrations or if the reaction is too slow as is the case with low enzyme concentrations. However, between the two limits, the variation is wide enough to justify the conclusions made concerning the effect of varying the enzyme concentration.

In this phase of the study, experiments were carried out using 1.0 ml. of 0.046M hydrogen peroxide solution with varying amounts of active enzyme extracts. The enzyme concentration is expressed in terms of milliliters of regular extract. Typical reaction curves for an eight-fold variation in the enzyme concentration are shown in Figure 3, the volume of oxygen evolved being plotted against reaction time, t. If the logarithm of the residual substrate concentration is plotted against time for the six enzyme levels, six straight lines of various slopes are obtained. These are shown in Figure 4. The specific rate constant for each enzyme concentration was determined from the slope of the particular

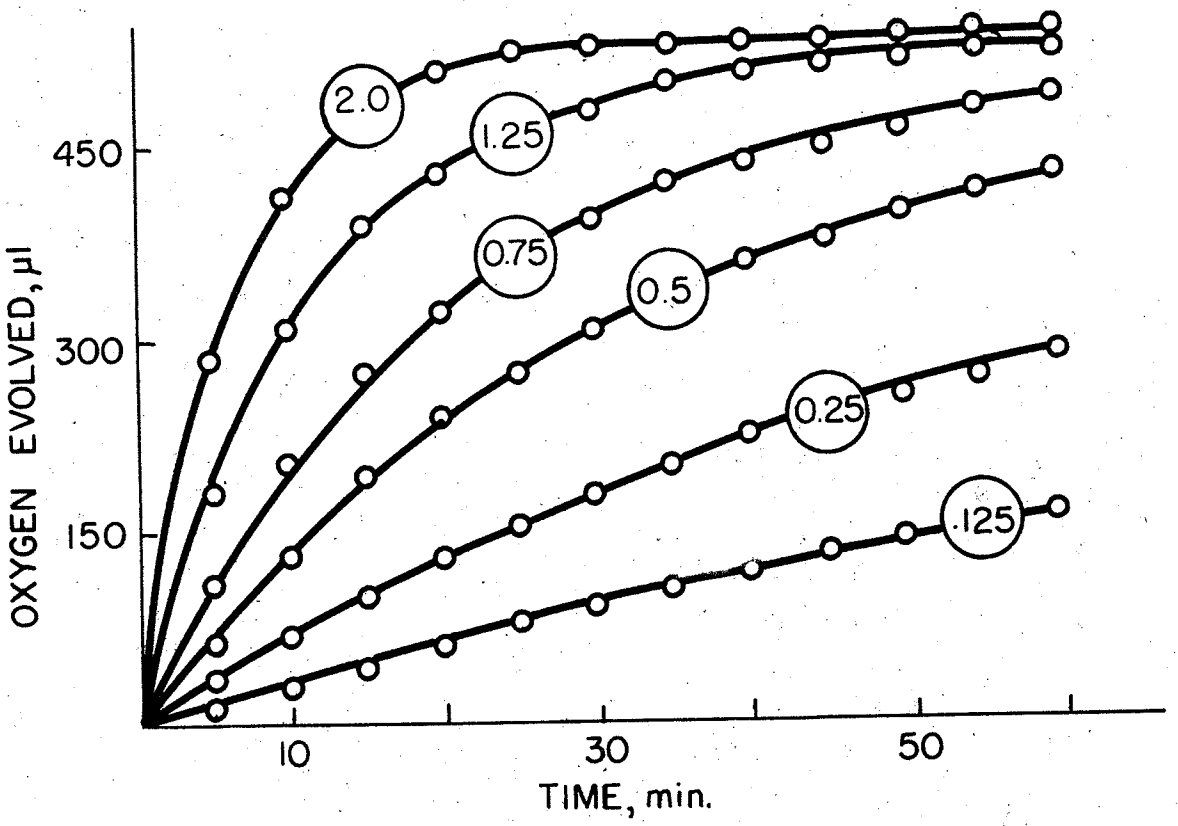

Figure 3. The evolution of oxygen at six different enzyme concentrations.

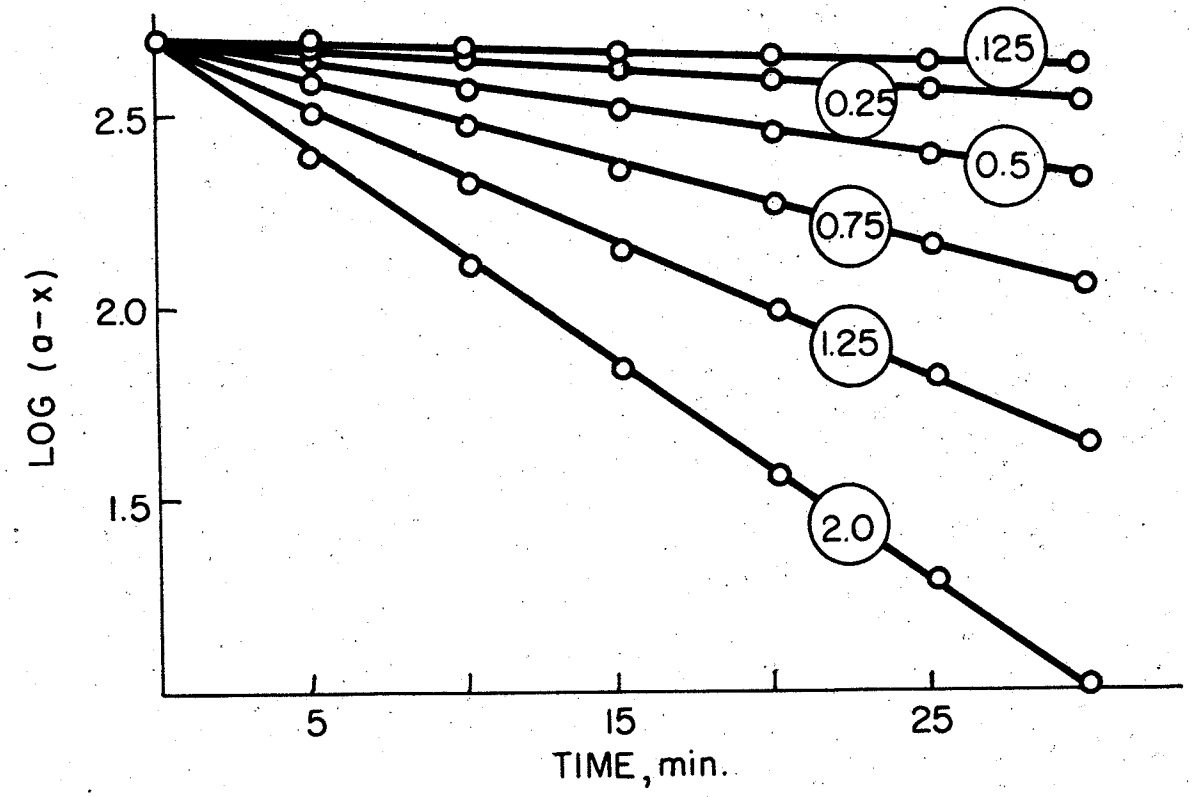

Figure 4. The relation between the logarithm of the residual substrate concentration and the reaction time for six different enzyme concentrations.

straight line in Figure 4. The results are tabulated in Table VI and shown graphically in Figure 6. Figure 5 shows the relationship between the initial rate and the enzyme concentration.

Table VI. Initial Velocities and Specific Rate Constants at Various Enzyme Concentrations.

Enzyme (ml.)	$k \times 10^3$	Initial Velocity $\times 10^8$ (moles / sec.)
.125	0.101	0.47
.25	0.229	1.06
.50	0.50	2.28
.75	0.80	3.69
1.25	1.43	6.64
2.00	2.37	11.51

The results obtained in this phase of the study may be summarized as follows:

1. The decomposition of hydrogen peroxide proceeds according to first order kinetics over the range of enzyme concentrations investigated. (Fig. 4)
2. The initial velocity is directly proportional to the active enzyme concentration. (Fig. 5)
3. The specific rate constant k, is also directly proportional to the active enzyme concentration. This is the reason for expressing enzymic activity in terms of the specific rate constant. (Fig. 6)

Effect of Varying the Substrate Concentration. In order to carry out a complete kinetic study of any chemical reaction, it is necessary to know what effect the variation of each of the reacting substances has on the reaction rate. The effect of hydrogen peroxide on the rate of its decomposition has been studied in some detail. Typical reaction curves for five substrate concentration levels are shown in Figure 7. The concentration of hydrogen peroxide was varied from 0.58×10^{-3}M to 4.64×10^{-3}M, an eight-fold variation, while the amount of enzyme remained constant at 1.0 ml. of the regular extract. Figure 8 shows the type of curves which are invariably obtained when the logarithm of the residual substrate concentration is plotted against time for a single enzyme

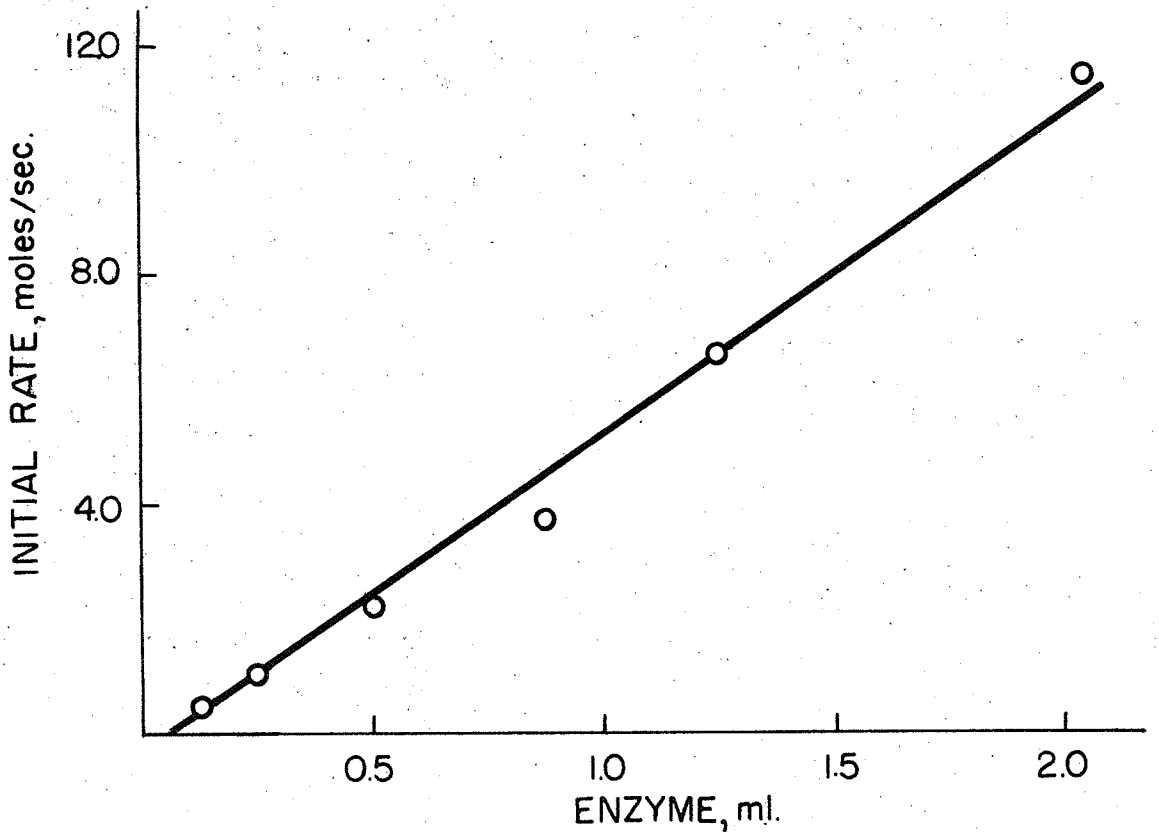

Figure 5. Initial rate as a function of the enzyme concentration.

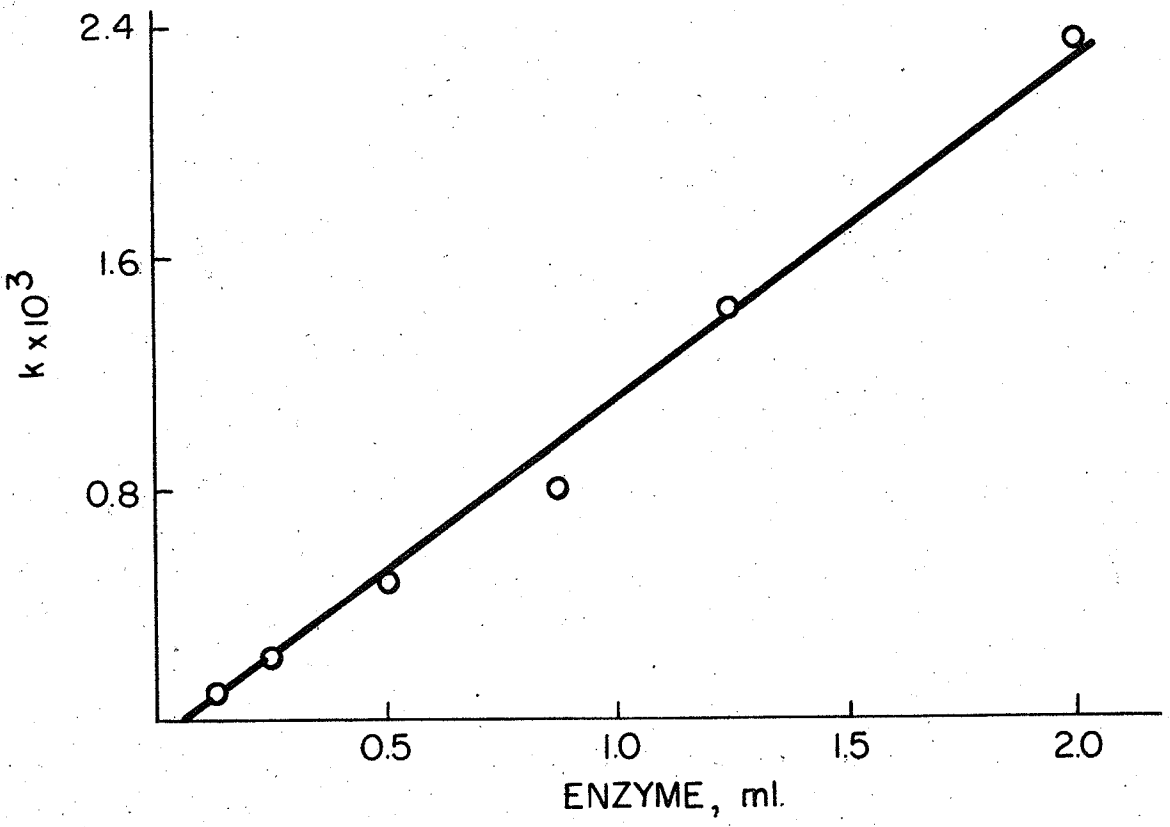

Figure 6. First order specific rate constant as a function of the enzyme concentration.

concentration. In this case, five essentially parallel, straight lines are obtained. The significance of these curves is two-fold. First of all, the decomposition of hydrogen peroxide proceeds according to first order kinetics over the range of concentrations investigated and, secondly, the specific rate constant, which is directly proportional to the slopes of these lines, is independent of the initial substrate concentration. The values of the specific rate constant for the various substrate concentrations are given in Table VII.

Table VII. Specific Rate Constant at Various Initial Substrate Concentrations.

Substrate x 10^3 (M)	k x 10^4
4.64	7.53
2.78	7.51
1.86	7.43
1.16	6.99
0.58	7.71

Figure 9 shows the relationship between the initial rate and the substrate concentration. The rate is directly proportional to the amount of substrate. This relationship holds only for low concentration of substrate. The results are given in Table VIII.

Table VIII. Initial Rate at Various Initial Substrate Concentrations at Low Substrate Levels.

Substrate x 10^3 (M)	Initial Rate x 10^9 (Moles / Sec.)
4.64	3.49
2.78	2.09
1.86	1.38
1.16	0.81
0.58	0.45

At much higher substrate concentrations the relationship is no longer linear. This is shown in Figure 10. The results are tabulated in Table IX.

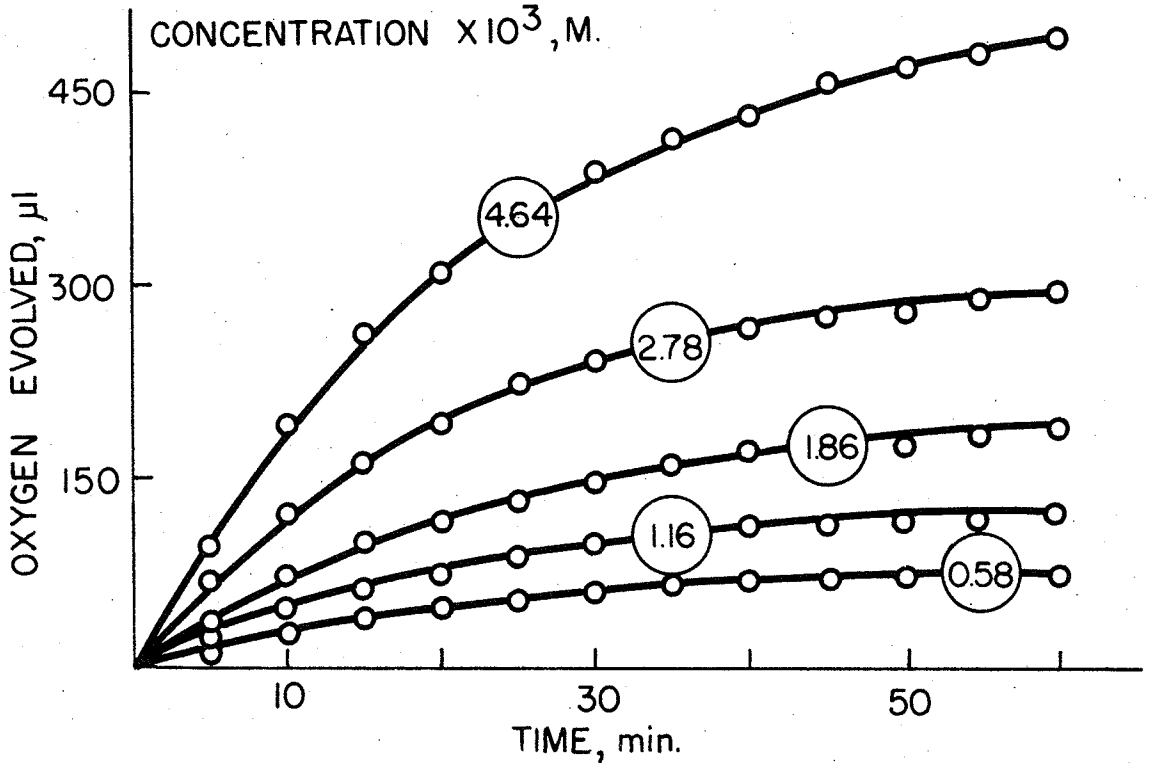

Figure 7. The evolution of oxygen at five different initial substrate concentrations.

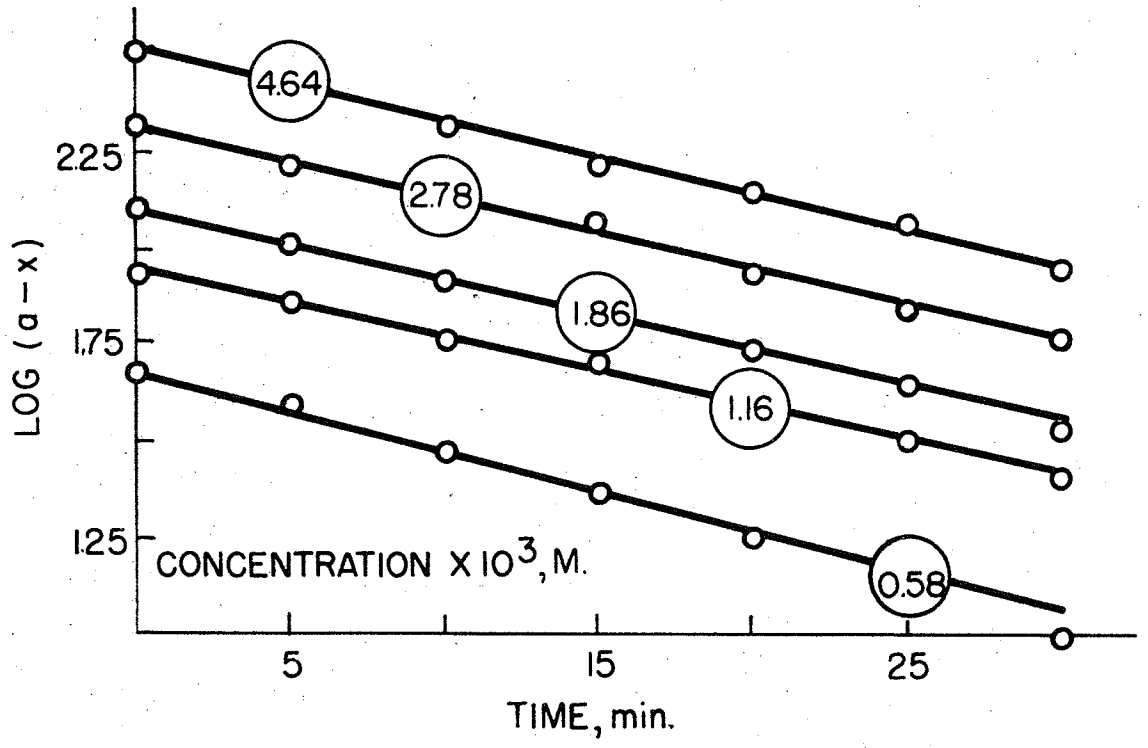

Figure 8. The relation between the logarithm of the residual substrate concentration and the reaction time for five different initial substrate levels.

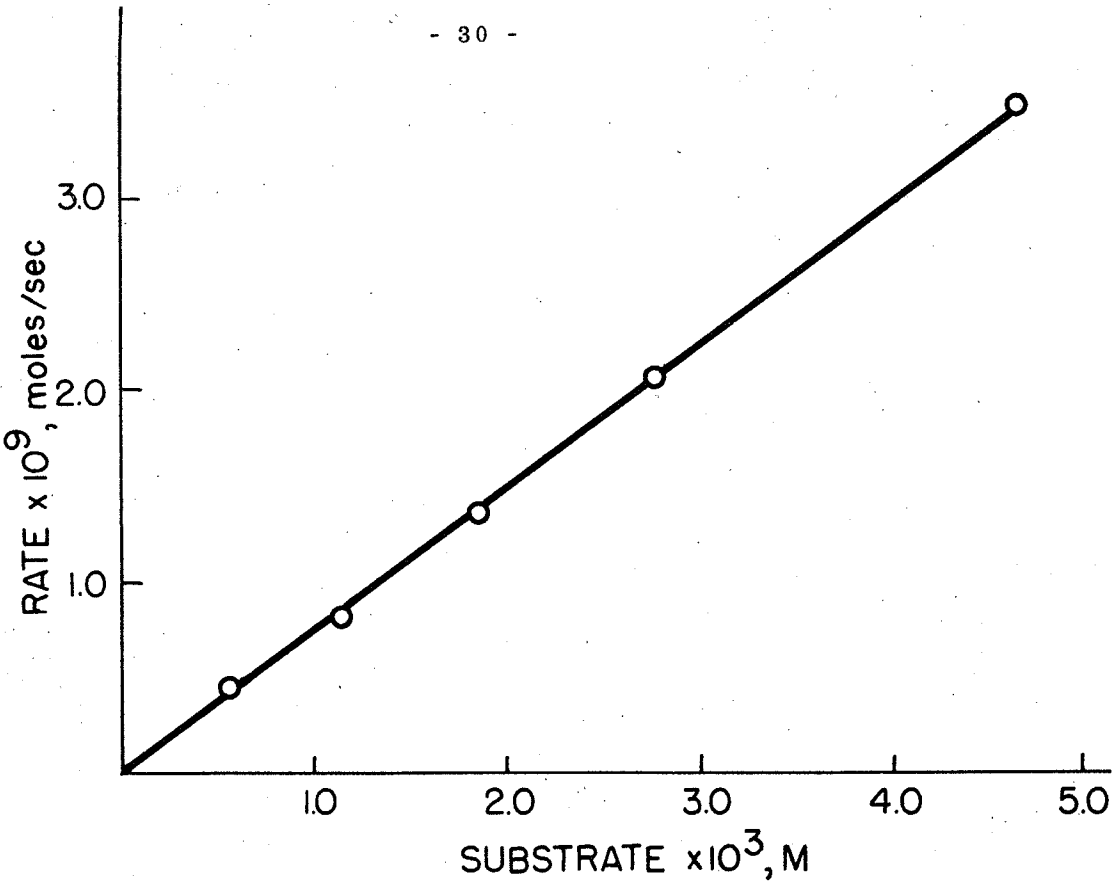

Figure 9. Rate of decomposition of hydrogen peroxide by wheat catalase as a function of substrate concentration at low substrate level.

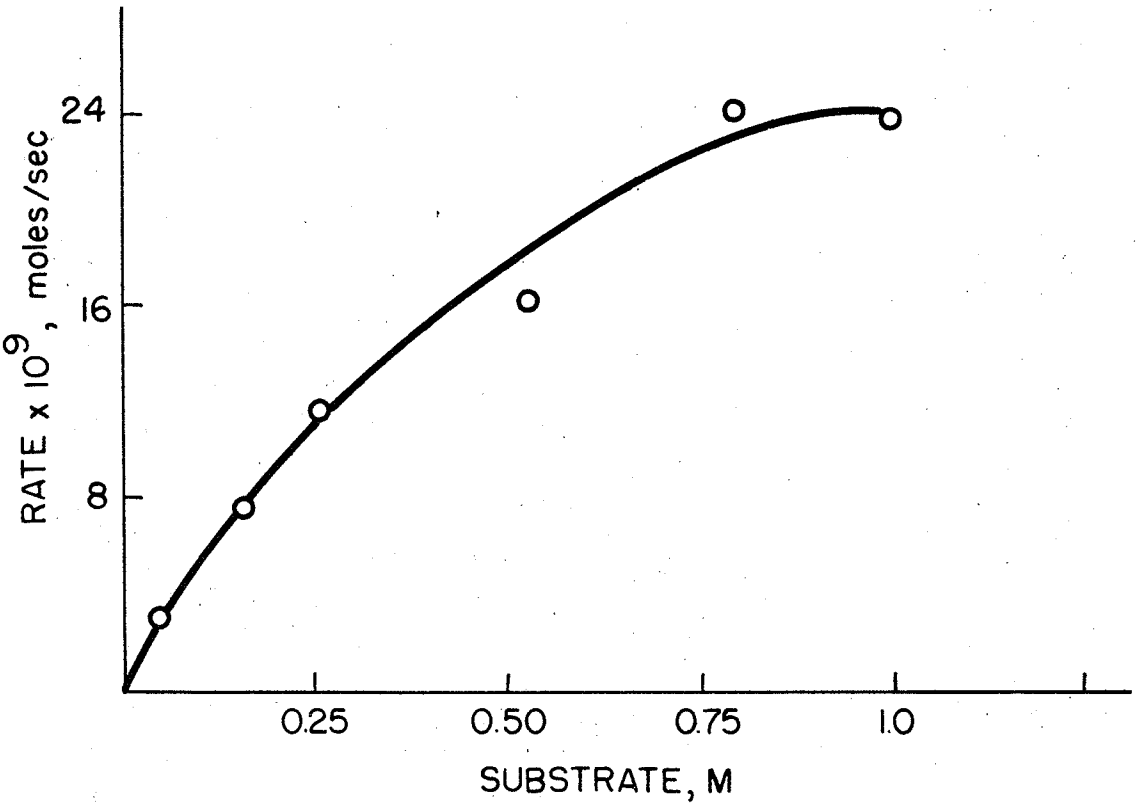

Figure 10. Rate of decomposition of hydrogen peroxide by wheat catalase as a function of substrate concentration at high substrate level.

Table IX. Initial Rate at Various Initial Substrate Concentrations at High Substrate Levels

Substrate (M)	Initial Rate x 10^9 (Moles / Sec.)
0.053	3.35
0.159	7.82
0.265	11.65
0.530	16.75
0.795	24.20
1.060	23.80

In most enzyme systems the rate levels off at high substrate levels owing to saturation of the enzyme by the substrate, that is, when the enzyme becomes completely saturated by its substrate, then a further increase in the substrate concentration will have no effect on the rate. The rate versus substrate curve will exhibit a horizontal portion when saturation is reached. The situation with catalase is somewhat different. This enzyme has a high turnover number and hence saturation can only be realized to a certain extent at very low temperatures and high substrate levels. The peculiar results which have been obtained in this study can be explained by the fact that catalase is destroyed by hydrogen peroxide. The extent of this destruction increases with increasing substrate concentration. Accordingly, the rate-substrate curve shows a maximum beyond which enzyme inactivation by the substrate becomes predominant. When 1.0 ml. of 0.046 M hydrogen peroxide solution is used as the substrate the amount of enzyme destroyed is negligible. This has been demonstrated by the constancy of k, the specific rate constant as shown in Table I.

The problem now is to examine kinetically the results obtained when the initial substrate concentration was varied. To determine the order of the reaction with respect to substrate concentration it is necessary to consider first of all a general case. It may be shown that for any chemical reaction we have,

$$\log \left[- \frac{d(a-x)}{dt} \right] = n \log (a-x) + K \qquad (8)$$

in which $- \frac{d(a-x)}{dt}$ is the rate of the reaction when the residual concentration

of the reactant is $(a-x)$, n is the order of the reaction and K is a constant. Provided that the order remains the same, a plot of log $-\frac{d(a-x)}{dt}$ against log $(a-z)$ should give a straight line of slope equal to n. This then becomes a practical method for determining the order of a reaction with respect to concentration.

Figure 11 shows the relationship between the logarithm of the rate and the logarithm of the residual substrate concentration for the decomposition of hydrogen peroxide by wheat catalase. The enzyme concentration was 1.0 ml. of the regular extract and the substrate concentration was 1.0 ml. of 0.046 M hydrogen peroxide solution. The data on which Figure 11 is based are shown in Table X.

Table X. Data for the Determination of the Order of Reaction with Respect to Substrate Concentration.

$a-x$				
µl. O_2	moles x 10^{-5}	Log $(a-x)$	$-\frac{d(a-x)}{dt}$ x 10^8 (moles / sec.)	log $\left[-\frac{d(a-x)}{dt}\right]$
520	4.64	-4.33	4.85	-7.31
312	2.78	-4.56	2.71	-7.57
208	1.86	-4.73	2.15	-7.67
130	1.16	-4.93	1.07	-7.97
65	0.58	-5.24	.62	-8.20

In the above table $(a-x)$ represents the residual hydrogen peroxide and $-\frac{d(a-x)}{dt}$ is the rate at that substrate concentration.

The slope of the curve in Figure 11 is equal to one. Therefore the decomposition of hydrogen peroxide by wheat catalase proceeds according to first order kinetics with respect to peroxide concentration. Figures 4 and 8 infer that the reaction is of first order with respect to time. That is, the order with respect to substrate concentration is the same as the order with respect to time. A mathematical interpretation of these results will be considered later.

Determination of the Activation Energy for the Decomposition of Hydrogen Peroxide by Wheat Catalase. Activation energy studies are of prime importance in determining the kinetics of relatively simple reactions. However,

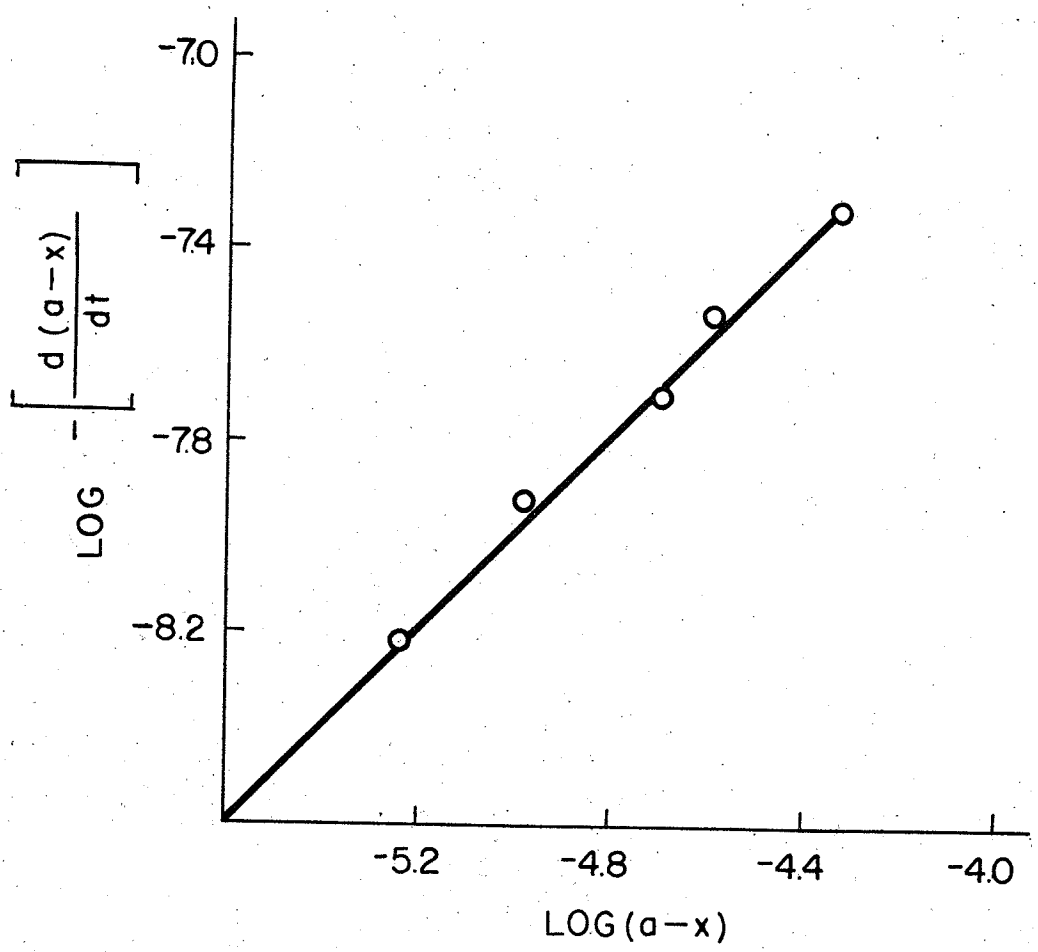

Figure 11. The logarithm of the rate as a function of the logarithm of the residual substrate concentration. The slope equal to unity, is the order of reaction.

activation energy values lose their significance when we deal with complex or oonsecutive reactions. For instance, suppose we are dealing with a system which can be arbitrarily represented as:

$$A + B \rightleftharpoons AB \qquad (9)$$

$$AB \longrightarrow C + D \qquad (10)$$

By measuring the rate of disappearance of A or B or the rate of appearance of C or D at various experimental temperatures we can determine the activation energy for the system. This is equal to the sum of the activation energies of the two consecutive reactions involved. However we seldom know what fraction of the activation energy is for reaction (9) or for reaction (10). So that for such cases activation energy values have limited application in determining the mechanisms of the various processes.

Enzyme reactions generally fall into this group of complex or consecutive reactions. Activation energies have been determined for most of the known enzyme systems, but very little significance is attached to them. Generally they are only used to compare the results for a single enzyme system which have been obtained by two different investigators.

The standard technique for determining the activation energy of a reaction is to obtain its velocity at a number of different temperatures keeping all other variables constant. The activation energy is then evaluated with the aid of the Arrhenius equation. A form of the Arrhenius equation may be written as

$$\log k = \frac{-E}{2.303R} \cdot \frac{1}{T} + C \qquad (11)$$

in which k is the specific rate constant, E is the activation energy, R is the gas constant, T is the temperature in absolute degrees and C is a constant. If we plot log k against $\frac{1}{T}$ we should get a straight line, the slope of which will be equal to $\frac{-E}{2.303R}$. Knowing the value of R, we can calculate E. A more direct, but a less accurate method is to calculate E from the following modification of the Arrhenius equation.

$$E = \log \frac{k_2}{k_1} \times 2.303 \times 1.987 \times \frac{T_2 T_1}{T_2 - T_1} \qquad (12)$$

in which k_1 and k_2 are the specific rate constants at temperatures T_1 and T_2.

The specific rate constants for the decomposition of 1.0 ml. of 0.046 M hydrogen peroxide by 1.0 ml. of regular extract were determined at four different temperatures - 15°, 20°, 25° and 30°C. The data are shown in Table XI.

Table XI. Data for Determining the Activation Energy of the Wheat Catalase System.

T°K	$\frac{1}{T} \times 10^3$	k x 10^3	log k	4 + log k
288	3.48	1.88	-2.726	1.274
293	3.42	2.05	-2.688	1.312
298	3.36	2.21	-2.655	1.344
303	3.30	2.37	-2.625	1.374

Figure 12 shows the relation between the logarithm of the specific rate constant and the reciprocal of the absolute temperature. The value of the activation energy obtained from the slope of the average line through the points is 2,800 cal/mole. Values of activation energy calculated from equation (12) are given in Table XII.

Table XII. Activation Energies Over Various Temperature Ranges.

Temperature Interval (°C)	Temperature (°C)	E (cal/mol)
15-20	17.5	2890
15-25	20.0	2765
15-30	22.5	2681
20-25	22.5	2605
20-30	25.0	2556
25-30	27.5	2494

It will be noted from Table XII that the activation energy decreases as the temperature increases. This effect has been observed in a number of

enzymic reactions and has been attributed to the decrease in the amount of active enzyme owing to its thermal inactivation. This effect would be noticeable in Figure 12 if the curve was drawn directly through the points.

The literature contains no reference as to the magnitude of the activation energy for the decomposition of hydrogen peroxide by wheat extracts. A large number of values are quoted for the decomposition of the peroxide by liver catalase. These vary from 600 cal/mole as given by Beers and Sizer (4) to as high as 5,500 cal/mole quoted by Laidler (28).

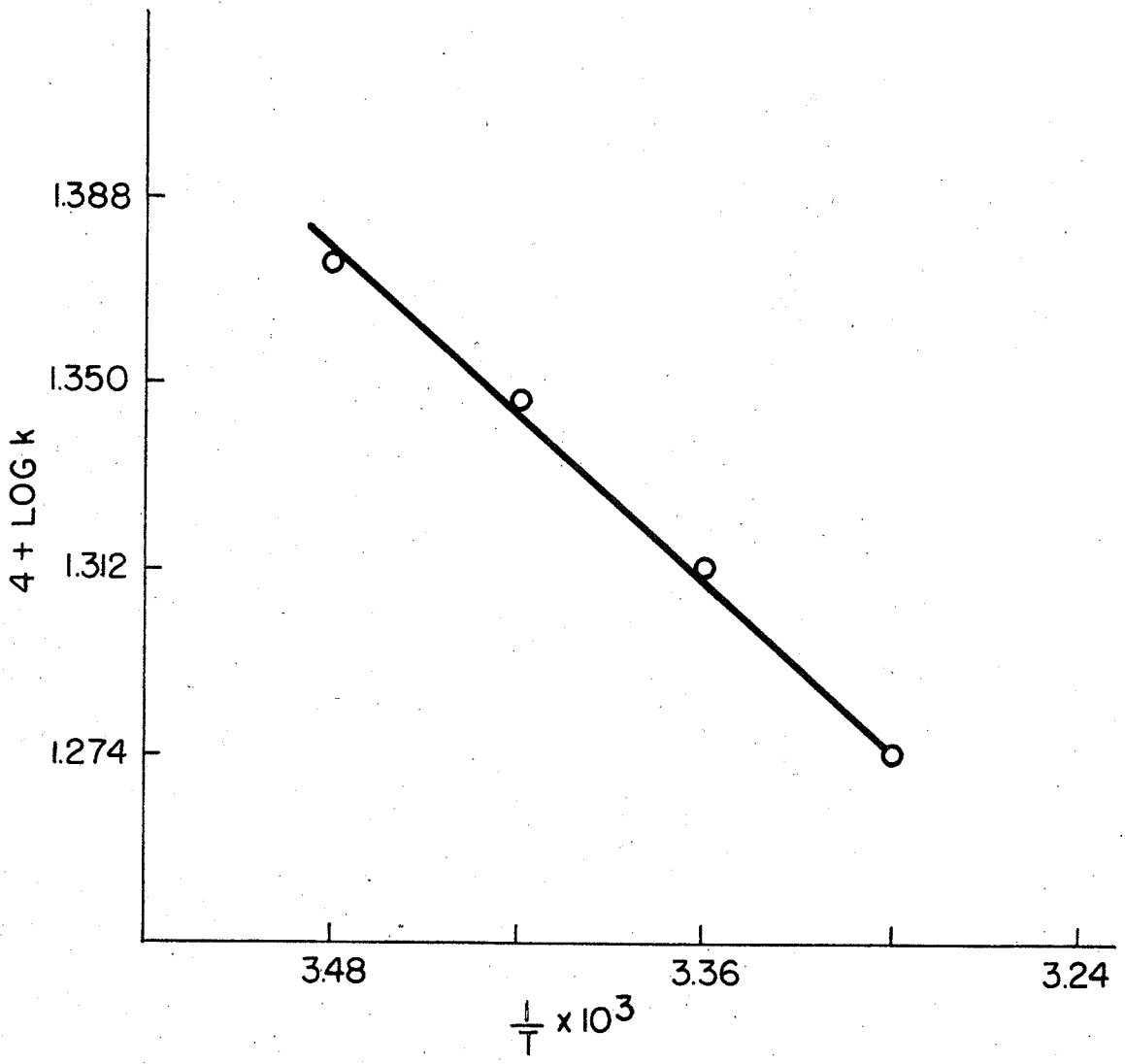

Figure 12. A plot of $4 + \log_{10} k$ against $1/T$ for the decomposition of hydrogen peroxide by wheat catalase. The slope is equal to $\frac{E}{2,303\,R}$, whence $E = 2,800$ cal.

THE DECOMPOSITION OF HYDROGEN PEROXIDE BY CRYSTALLINE CATALASE.

A sample of crystalline catalase of unknown source was obtained from General Biochemicals Incorporated and a preliminary study of its mode of action was carried out. The solution which was used as the active enzyme contained 1.0×10^{-3} gm. of crystalline catalase per liter. Its activity was studied manometrically by the method described for wheat catalase. The reaction affected by the crystalline enzyme follows a slightly different mechanism from the reaction catalysed by wheat catalase. There are two general differences. First of all, the effect of hydrogen ion concentration on crystalline catalase is not as marked as on the activity of wheat extracts. Crystalline enzyme activity remained essentially the same over a pH range from 5.8 to 7.0. Secondly, the reaction cannot be described on the basis of first order kinetics.

The experimental data for crystalline catalase are not too extensive, but it is possible to distinguish between the two enzymes with the aid of the available information. Curve 1 in Figure 13 is a typical reaction curve for the decomposition of hydrogen peroxide by the pure enzyme. Analysis of this curve suggests that the enzyme must be inactivated in some way. It is definite that the substrate is not the limiting factor since a complete decomposition of the hydrogen peroxide used would produce approximately 450 µl. of oxygen. Preliminary experiments showed that molecular oxygen or the phosphate buffer have no inhibitive effect on the enzyme so that the immediate conclusion is that the enzyme must be destroyed by hydrogen peroxide. This is in accord with the observations of other investigators.

Curve 1 in Figure 14 gives the relationship between the logarithm of the residual substrate concentration and the reaction time. We see that in this case we do not have a first order reaction. This would be the result if the concentration of the enzyme was continually decreasing. An attempt was made to analyse the results according to second order kinetics but the available data does not lend itself to such an analysis very easily. If the logarithm of the rate is plotted against the logarithm of the residual substrate concentration an approximately straight line of slope equal to 1.86 is obtained. The significance of such a kinetic order of a chemical reaction cannot be readily explained.

If we add a small amount of filtrate from a sample of boiled wheat extract to the crystalline catalase solution, the destruction of the enzyme by hydrogen peroxide no longer occurs. The filtrate alone has no effect on hydrogen peroxide. Curve 2 in Figure 13 is a typical reaction curve for such an experiment. In this case the substrate is the final limiting factor. If the logarithm of the residual substrate concentration is plotted against reaction time a straight line is the result. This is shown by Curve 2 in Figure 14. That is, we now have a first order reaction as was invariably obtained in wheat catalase studies. The apparent conclusion from this phase of the investigation is that wheat extracts contain some thermostable material which behaves like an enzyme protector. These results are in accord with the findings of Blish and Bode (5) who noted that the destruction of crystalline catalase by hydrogen peroxide may be prevented by the addition of dextrose or liver extracts. This is another reason why it is important to use crude extracts rather than the pure enzyme if one desires a fundamental knowledge about the enzymic reaction under natural conditions.

The turnover number of crystalline catalase may be calculated from the data obtained in this study. A calculation was made using the value 2.48×10^5 for the molecular weight of catalase and assuming that the available sample is of 100% purity. The value obtained is approximately 2.5×10^6 moles of hydrogen peroxide decomposed by a mole of catalase in 1 minute. This is a relatively high figure in comparison to literature values for other enzyme systems but is in the range of values quoted for catalase. The above figure was evaluated from the volume of oxygen evolved during the first two minutes of reaction when the filtrate from the boiled wheat extract was used in conjunction with crystalline catalase. As will be seen later, a high turnover number is essential to the development of rate expressions for the decomposition of hydrogen peroxide by wheat catalase.

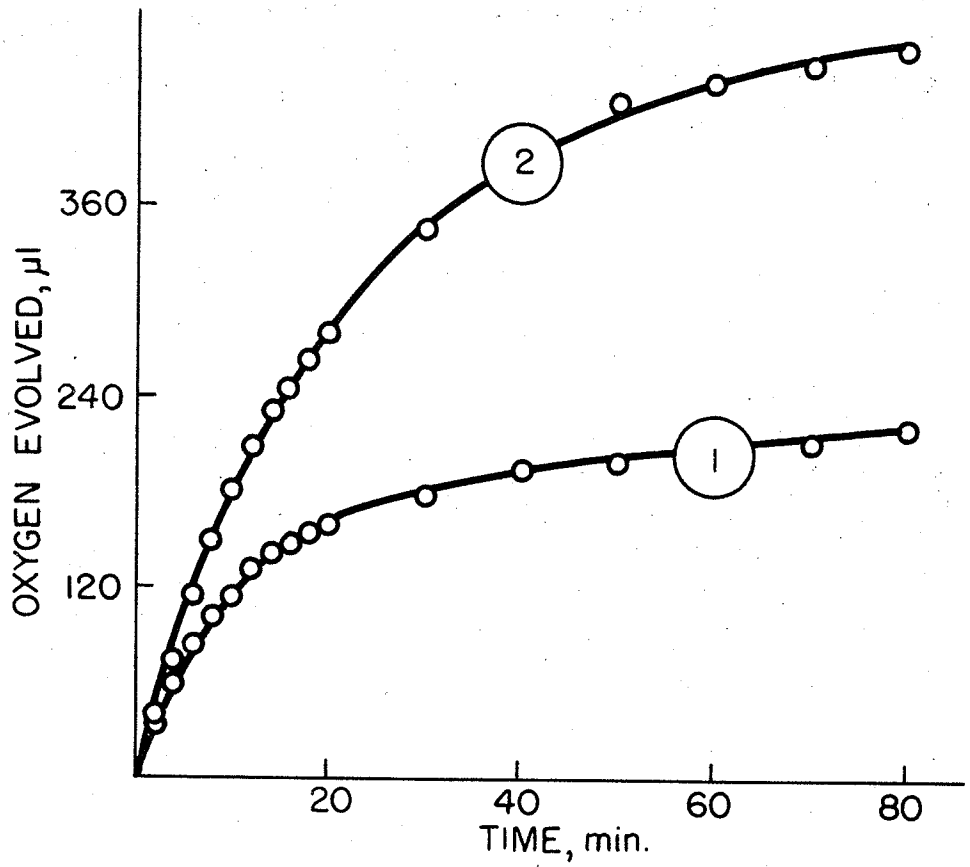

Figure 13. The evolution of oxygen by crystalline catalase; 1. Typical curve for crystalline catalase; 2. Typical curve for crystalline catalase plus a small amount of filtrate from boiled wheat extracts.

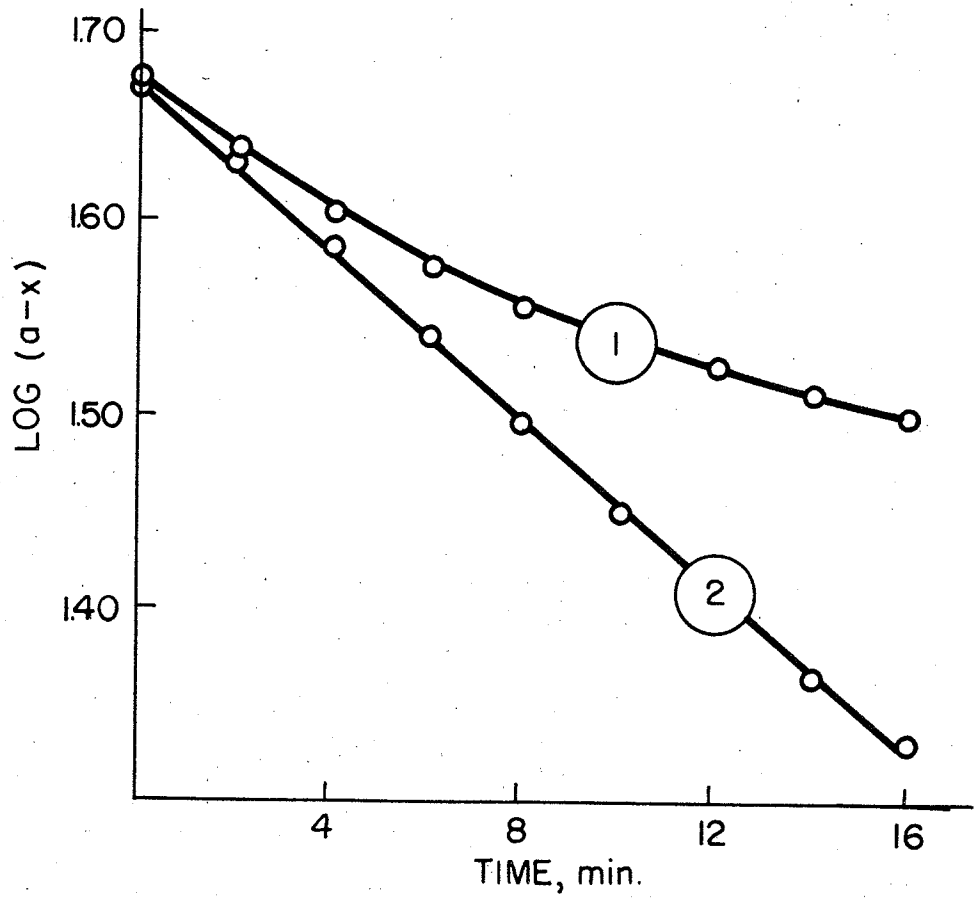

Figure 14. The relation between the logarithm of residual substrate concentration and reaction time for the reactions shown in Figure 13.

DESTRUCTION OF WHEAT CATALASE BY HYDROGEN PEROXIDE.

A number of investigators (8, 35, 43) have observed that crystalline catalase is readily destroyed by hydrogen peroxide. It is not known what the mechanism of this destruction is. Chance (9) stated that he obtained experimental evidence of the formation of an inactive complex between catalase and hydrogen peroxide. Others have suggested that the inactivation may occur by virtue of the oxygen which is liberated when the hydrogen peroxide is decomposed. In 1942, Blish and Bode (5) discovered that the destruction of catalase by hydrogen peroxide could be prevented by the addition of dextrose or liver extracts.

In this study the following technique was employed to determine whether wheat catalase is destroyed by hydrogen peroxide. In the main compartment of flask No. 1 were placed 4.0 ml. buffer solution, 1.0 ml. of 0.046 M hydrogen peroxide solution, and 1.0 ml. of regular enzyme extract in that order. An additional 1.0 ml. of 0.046 M hydrogen peroxide was added to the side-arm. In the control vessel or flask No. 2 the peroxide solution in the main compartment was replaced by 1.0 ml. of distilled water. Flasks No. 3 and 4 were set up similarly but these contained 0.5 ml. instead of 1.0 ml. of 0.046 M hydrogen peroxide solution. The vessels were attached to their respective manometers and placed in the bath at 25°C. After approximately 3 hours of shaking, the liquids in the manometers were adjusted to the proper levels and the stopcocks were closed after making sure that all of the hydrogen peroxide in flasks Nos. 1 and 3 were used up. The side-arm contents were tipped into the main compartment and rate of oxygen evolution was recorded. The results obtained on the basis of a 60-minute reaction period are shown in Table XIII.

Table XIII. Destruction of Wheat Catalase by Hydrogen Peroxide.

Flask No.	Activity, k x 10^4
1	7.86
2	9.40
3	8.07
4	9.44

The enzymes in flasks Nos. 1 and 3 had previously catalyzed the decomposition of 1.0 and 0.5 ml. of 0.046 M hydrogen peroxide, respectively. We see from the results in Table XIII that although the decrease in activity is not too large, it cannot be neglected. However from results quoted previously, for example, Table I, and Figures 4 and 8, it is apparent that the destruction of wheat catalase by hydrogen peroxide of the concentration used is negligible during the first 30 minutes. The possibility that wheat extracts contain some protecting material has been considered in the last section.

EVALUATION OF THE MICHAELIS CONSTANT.

Since the formulation of the classical Michaelis-Menten treatment of enzyme kinetics the Michaelis constant has become the most important single factor in describing enzyme systems. Each system has a characteristic Michaelis constant. Originally the Michaelis constant was the equilibrium constant for the dissociation of the enzyme-substrate complex. If we look at the proposed Michaelis-Menten mechanism, which is,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (13)$$

$$ES \xrightarrow{k_3} E + P \qquad (14)$$

the Michaelis constant Km is given by $\frac{k_2}{k_1}$. However it is not the true dissociation constant of the complex which is given by $\frac{k_2 + k_3}{k_1}$. This becomes equal to the original Michaelis constant if $k_3 \ll k_2$. Any further reference to this constant in this thesis will be to the one which is equivalent to $\frac{k_2 + k_3}{k_1}$.

It may be shown that for enzyme reactions on the basis of the Michaelis-Menten mechanism the following relationship holds;

$$v = \frac{Vm \cdot S}{S + Km} \qquad (15)$$

in which v is the velocity when the substrate concentration is S, Vm is the maximum velocity which is reached when the enzyme is completely saturated with substrate and Km is the Michaelis constant. In order to evaluate Km, Lineweaver and Burk (31) proposed the following modifications of the above equation

$$\frac{1}{v} = \frac{1}{Vm} + \frac{Km}{Vm} \cdot \frac{1}{S} \qquad (16)$$

and

$$\frac{s}{v} = \frac{1}{Vm} S + \frac{Km}{Vm} \qquad (17)$$

We see from equation (16) that if we plot $\frac{1}{v}$ against $\frac{1}{S}$, a straight line should result. The slope and the intercept are given by $\frac{Km}{Vm}$ and $\frac{1}{Vm}$ respectively, from which the Michaelis constant could be evaluated. Using equation (17) we can plot $\frac{s}{v}$ against S and again obtain a straight line, the slope of which will be equal to $\frac{1}{Vm}$ and $\frac{Km}{Vm}$ will be the intercept. In 1952 Hoptee (22) pointed out the disadvantages of equations (16) and (17) and proposed a third modification of equation (15). His equation is

$$v = Vm - \frac{v}{s} Km \qquad (18)$$

If we plot v against $\frac{v}{s}$, the ordinate intercept will be given by Vm and the abscissa intercept will be equal to $\frac{Vm}{Km}$.

In order to obtain experimentally the data required for the evaluation of the Michaelis constant it is necessary to determine reaction velocities over a wide range of substrate concentration. This was rather difficult with the hydrogen peroxide system. Because of the very high turnover number of catalase it was necessary to work in the region of very high peroxide concentration to reach the point where the velocity was no linger linear with substrate concentration. In this region two factors become important. First of all, the destruction of the enzyme by the substrate is no longer negligible and, secondly, the oxygen evolution owing to the spontaneous decomposition of hydrogen peroxide becomes quite high. Even with the appropriate controls it is impossible to make accurate corrections for the above effects. It was hoped that the error might be decreased by using very short reaction times.

An experiment was carried out using 1.0 ml. of the regular extract, 1.0 ml. of substrate of various concentrations and 5.0 ml. of buffer solution. The results obtained and the data necessary for evaluating the Mihhaelis Constant by the three graphical methods are given in Table XIV.

Table XIV. Data Used to Evaluate the Michaelis Constant.

Substrate x 10^3 (M)	Velocity x 10^8 (moles / sec)	$\frac{1}{v} \times 10^{-7}$	$\frac{s}{v} \times 10^{-4}$	$\frac{1}{s} \times 10^{-2}$	$\frac{v}{s} \times 10^{6}$
.77	1.48	6.76	5.20	12.99	19.20
1.54	1.63	6.14	9.45	6.49	10.58
3.08	2.53	3.95	12.17	3.25	8.21
6.16	2.96	3.38	20.81	1.62	4.80
9.24	3.05	3.28	30.30	1.08	3.30
12.32	2.83	3.53	43.53	.81	2.30

Figure 15 shows the relationship between the initial velocity and the initial substrate concentration. Figures 16, 17, and 18 are the plots of equations 18, 16, and 17 respectively. The values of the Michaelis Constant evaluated using equations 18, 16, and 17 with the aid of the Figures 16, 17, and 18 are listed in Table XV.

Table XV. Values of the Michaelis Constant Obtained by Various Graphical Methods.

Equation No.	Figure No.	Vm x 10^8 (moles / sec)	Km x 10^3 M
18	16	3.40	1.02
16	17	3.16	0.87
17	18	3.40	1.01

There is only one reference in the literature which gives a Michaelis Constant for the hydrogen peroxide-catalase system. The value determined by Shirakawa (38) is 0.35 M. This value was obtained using the equations of Lineweaver and Burk. Most of the other investigators are of the opinion that this system has no Michaelis Constant. The question which arises at this point is; what is the significance of the values of the Michaelis Constant obtained in this study?

In most enzyme studies the relation of the initial velocity to substrate concentration becomes non-linear at high substrate concentrations owing to the saturation of the enzyme by the substrate. However, for the

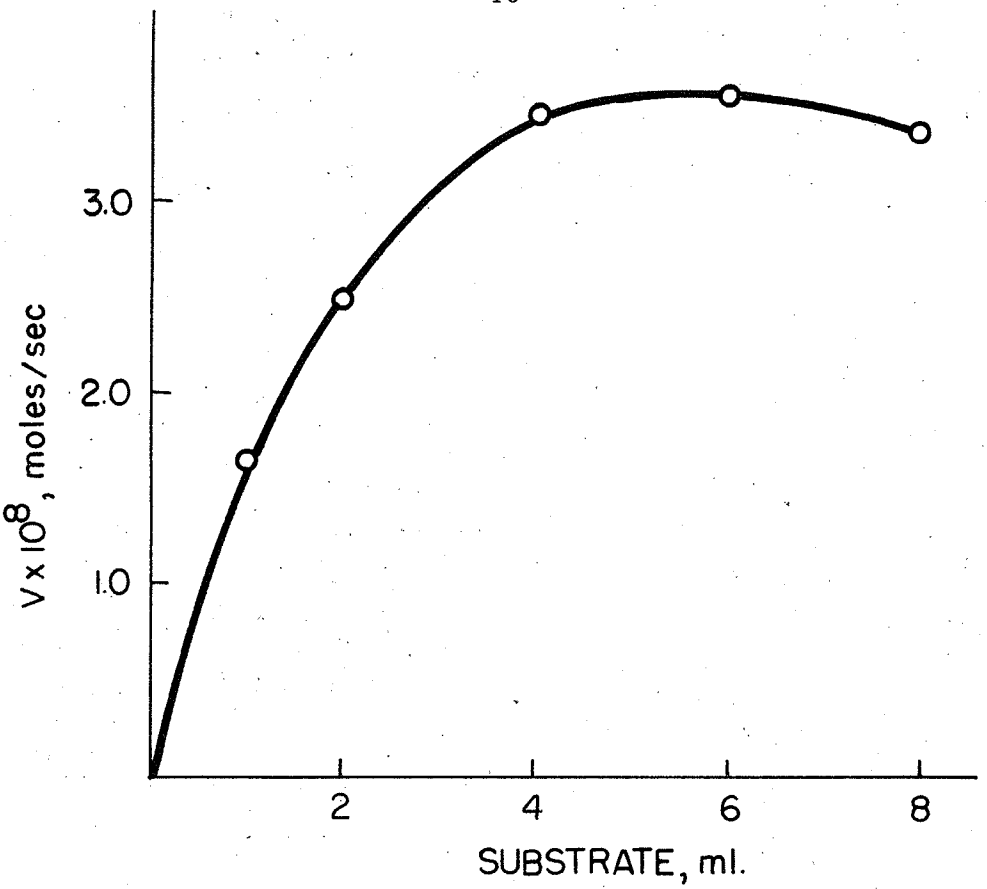

Figure 15. Reaction velocity as a function of initial substrate concentration from data used to determine the Michaelis constant.

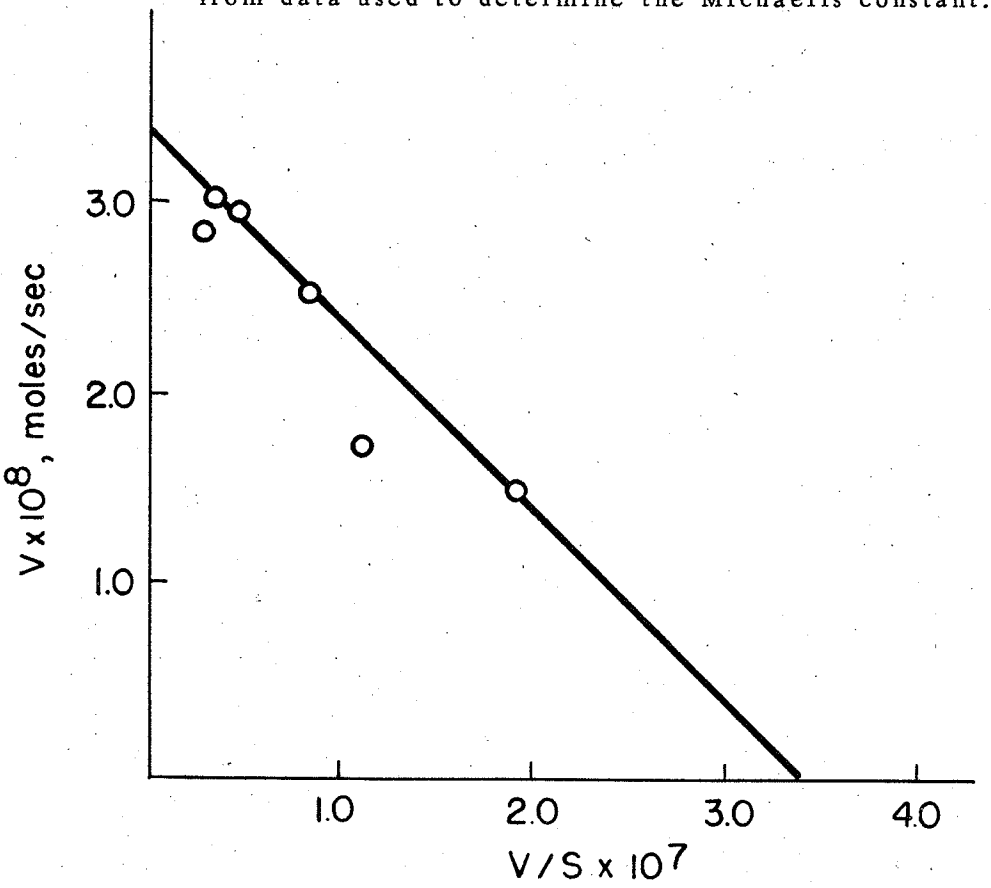

Figure 16. A plot of equation (18).

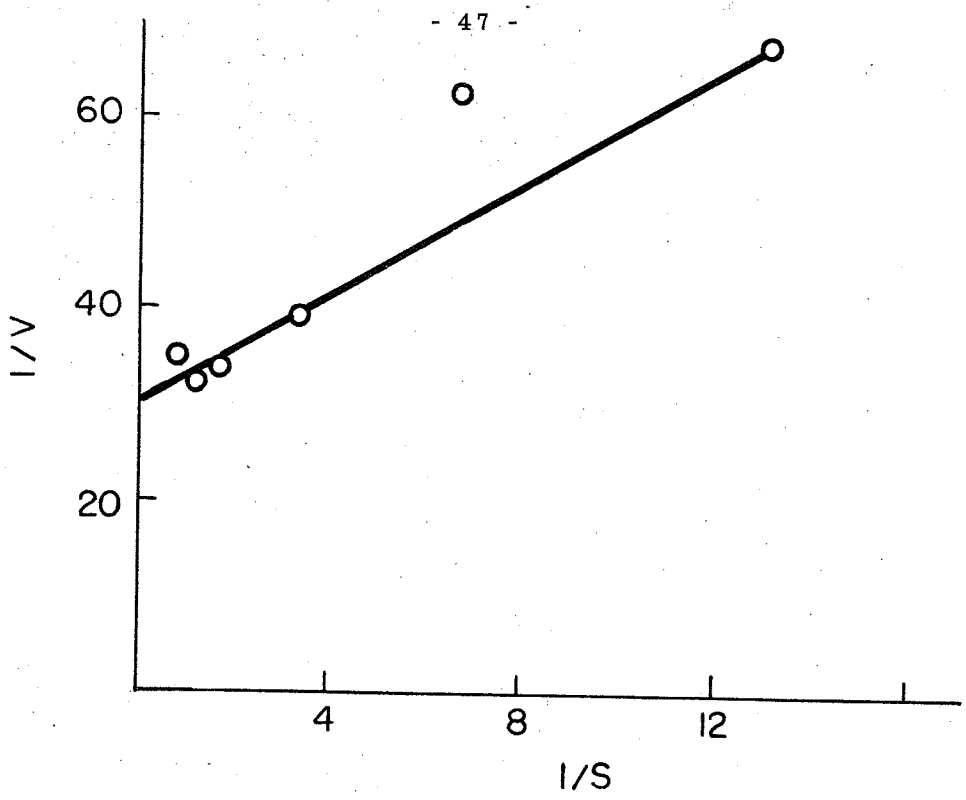

Figure 17. A plot of equation (16).

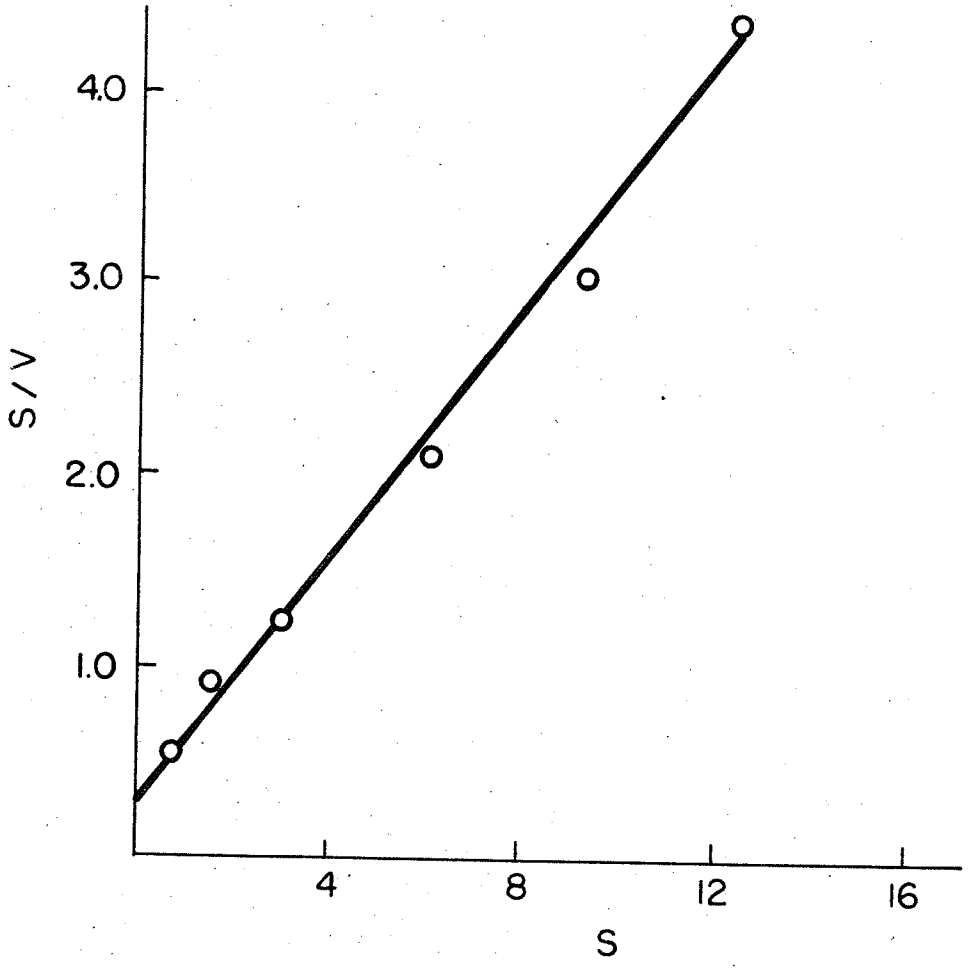

Figure 18. A plot of equation (17).

decomposition of hydrogen peroxide by wheat catalase, the velocity versus substrate plot shows a maximum as in Figure 15. This is typical of enzyme systems in which the enzyme is destroyed by the substrate. Up to the point of the maximum the above effect is similar to the saturation effect generally observed. Hence the Michaelis Constant could be evaluated, but in this case it has a different significance. If the investigation is made under conditions where there is no destruction of the enzyme by the substrate, it will be impossible to obtain a value for the Michaelis Constant. The reason for this is that the velocity will be directly proportional to the substrate concentration and hence a plot of equation (16) will produce a straight line through the origin. Since the ordinate intercept is given by $\frac{1}{Vm}$, Vm must be approaching infinity, that is, it is impossible to saturate the enzyme with substrate. Although a value of the slope may be readily obtained, Km cannot be evaluated since Vm is not known.

INHIBITION EXPERIMENTS

Introduction

Chemical substances which depress the action of enzymes are called inhibitors. This action of inhibitors is sometimes called the poisoning of enzymes. These inhibiting substances are of various kinds and their modes of action may be different. However, their over-all effect is that they reduce the enzymic activity. In this study the effects of two distinct types of inhibitors were investigated. The two inhibitors are potassium cyanide and ethyl alcohol.

It is perhaps pertinent at this point to review briefly the various types of inhibited enzymic reactions. In general, uninhibited enzyme reactions may be represented by equation (16). Inhibited reactions are usually classified into three general cases.

Case I - Competitive Inhibition

Competitive inhibition occurs when the inhibitor competes with the substrate for the active centre on the enzyme. This can be represented by the equation

$$\frac{1}{v} = \frac{1}{Vm} + Km\left(1 + \frac{i}{Ki}\right)\frac{1}{s} \qquad (19)$$

in which Ki is the dissociation constant of the enzyme-inhibitor complex and i is the concentration of the inhibitor. The other terms have their previous meanings.

Case II - Non-competitive Inhibition

In non-competitive inhibition the inhibitor combines with the enzyme at a different active centre than the substrate. The reaction velocity can be represented by the following relation:

$$\frac{1}{v} = \frac{1}{Vm}\left(1 + \frac{i}{Ki}\right) + \frac{Km}{Vm}\left(1 + \frac{i}{Ki}\right)\frac{1}{s} \qquad (20)$$

Case III - Uncompetitive Inhibition

In uncompetitive inhibition the inhibitor combines with the enzyme-substrate complex. This case may be represented by the following equation:

$$\frac{1}{v} = \frac{1}{Vm}\left(1 + \frac{i}{Kesi}\right) + \frac{Km}{Vm}\frac{1}{s} \qquad (21)$$

In the above equation Kesi is the dissociation constant for the enzyme-substrate-inhibitor complex.

If the reciprocal of the velocity, $\frac{1}{v}$, is plotted against the reciprocal of the substrate concentration, $\frac{1}{s}$, for each of the above cases, straight lines should result having the characteristics shown in Table XVI. These curves are used to determine what type of inhibition occurs with various inhibitors.

Table XVI. Summary of the Characteristics of Plots of 1/v against 1/s for Uninhibited and Inhibited Enzymic Reactions.

Type of Inhibition	Intercept	Slope
Uninhibited	$\frac{1}{Vm}$	$\frac{Km}{Vm}$
Competitive	$\frac{1}{Vm}$	$\frac{Km}{Vm}\left(1 + \frac{i}{Ki}\right)$
Noncompetitive	$\frac{1}{Vm}\left(1 + \frac{i}{Ki}\right)$	$\frac{Km}{Vm}\left(1 + \frac{i}{Ki}\right)$
Uncompetitive	$\frac{1}{Vm}\left(1 + \frac{i}{Kesi}\right)$	$\frac{Km}{Vm}$

The Cyanide Effect

Addition of low concentrations of potassium cyanide has a marked effect on the activity of wheat catalase. The results of an experiment in which the concentration of cyanide was varied are shown in Table XVII. The table shows that the amount of inhibition is not directly proportional to the cyanide concentration.

Table XVII. Wheat Catalase Activity as a Function of Cyanide Concentration.

Cyanide Concentration x 10^4 M	Activity, k x 10^4
0	11.00
1.43	6.46
2.86	4.52
8.58	2.00
11.44	1.64
16.02	1.10
18.30	0.89

The following quantitative, theoretical treatment of the inhibitive effect is readily applicable to the cyanide inhibition. Consider the over-all process as comprising the following reactions:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\,S \qquad (22)$$

$$ES + S \xrightarrow{k_3} E + \text{Products} \qquad (23)$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E\,I \qquad (24)$$

in which I is the concentration of the inhibitor. If the concentration of the substrate is much greater than the enzyme, then the velocity of substrate breakdown will be

$$v = k_3\,(ES) \qquad (25)$$

and the maximum velocity will be

$$V_{max} = k_3\,E \qquad (26)$$

which occurs when approximately all of the enzyme is in the form of the complex.

If an inhibitor is present it will form an inactive complex EI according to equation (24) and the amount of the enzyme left to combine with the substrate will be E-EI. The velocity will then be

$$v = k_3 (E - EI) \qquad (27)$$

Dividing (25) by (26) we get

$$\frac{v}{V\,max} = \frac{E - EI}{E} = 1 - \frac{EI}{E} \qquad (28)$$

and rearranging we have that

$$EI = E \left(1 - \frac{v}{V\,max}\right) \qquad (29)$$

For the inhibitor-enzyme reaction we have

$$K = \frac{(EI)}{(E-EI)\ (I-EI)} \qquad (30)$$

in which K is the equilibrium constant.

Substituting for EI and rearranging we have:

$$K = \frac{1 - \frac{v}{V\,max}}{I - E\left(1 - \frac{v}{V\,max}\right)\left(\frac{v}{V\,max}\right)} \qquad (31)$$

If we have a value of V max and values of v at two levels of cyanide concentration for a single concentration of enzyme, we can calculate the equilibrium constant. Then, the enzyme concentration may be determined indirectly. Using the values of I = CN = 1.43×10^{-4} M and 2.86×10^{-4} M the equilibrium constant, K, which is obtained is 5.18×10^{3} and the enzyme concentration is 2.11×10^{-5} M. These values are within the region of possible values.

An attempt was made to determine the type of inhibition which occurs with cyanide. This aspect of the study presented considerable difficulty. In enzyme systems in which it is possible to saturate the enzyme with substrate with considerable ease, a plot of equation (16) gives a straight line with a positive ordinate intercept. The situation with wheat catalase is totally different. As

stated previously because of the high turnover number of the enzymes it is not readily possible to reach the saturation point. That is, the initial velocity is directly proportional to the hydrogen peroxide concentration. For this case a plot of equation (16) will give a straight line through the origin. If an inhibitor is added, and whether it acts according to the competitive or noncompetitive mechanism, the only significant change will be in the slope if the plots of equations (19) and (20) are compared to the plot of equation (16) since all the curves will go through the origin.

Table XVI shows that the plots of equations (16), (19) and (20) for the normal, competitively inhibited, and noncompetitively inhibited, respectively, are characterized by slopes equal to $\frac{Km}{Vm}$, $\frac{Km}{Vm}\left(1 + \frac{i}{Ki}\right)$ and $\frac{Km}{Vm}\left(1 + \frac{i}{Ki}\right)$, so that for inhibition of catalase activity it is impossible to differentiate between competitive and noncompetitive inhibition by the suggested graphical method. Knowing the concentration of the inhibitor (i), we can evaluate the dissociation constant, Ki, of the enzyme-inhibitor complex from the ratio of the slopes of the plots of equations (16), (19) and (20). Theoretically the dissociation constant is equal to the reciprocal of the equilibrium constant for the formation of the complex.

In order to plot equations (19) or (20) it is necessary to determine the effect of a constant amount of inhibitor at various concentrations of hydrogen peroxide. The results of such an experiment and other pertinent data for the cyanide effect are given in Table XVIII. One ml. of regular extract was used with 1.0 ml. of hydrogen peroxide of desired concentration in a total volume of 7.0 ml. The cyanide level was 2.86×10^{-4} M.

Table XVIII. Cyanide Inhibition at Various Substrate Concentrations.

Substrate x 10^3 (M)	$\frac{1}{S} \times 10^{-2}$	Velocity x 10^5 moles/l/sec.		$\frac{1}{V} \times 10^{-3}$	
		Uninhibited Reaction	Inhibited Reaction	Uninhibited Reaction	Inhibited Reaction
.66	15.20	3.36	1.48	29.76	67.56
1.33	7.52	6.68	3.06	14.97	32.67
3.32	3.01	16.77	7.60	5.96	13.16
4.97	2.01	26.10	10.63	3.83	9.41
6.63	1.51	32.29	13.99	3.10	7.15

Plots of the reciprocal of velocity against the reciprocal of substrate concentration for the uninhibited and the inhibited reactions are shown in Figure 19. The results are two straight lines through the origin. From the ratio of the slopes of the two lines we have

$$\left(1 + \frac{i}{Ki}\right) = \frac{44}{20} = 2.2$$

and setting $i = 2.86 \times 10^{-4}$

$$Ki = 2.38 \times 10^{-4}$$

The value of K, the equilibrium constant for the formation of the complex between enzyme and cyanide, was found to be 5.18×10^{3}. Theoretically we have that $Ki = \frac{1}{K}$ so that from the above value of K,

$$Ki = \frac{1}{5.18 \times 10^{3}} = \underline{\underline{1.93 \times 10^{-4}}}$$

The agreement is surprisingly good since the values of Ki and K have been determined from two totally different sets of data. Another value of Ki obtained by the graphical method from another set of results using a lower cyanide concentration is also 2.38×10^{-4}. The value 1.93×10^{-4} for Ki is undoubtedly more accurate because in determining K the amount of cyanide which went into the formation of the complex was considered in the calculation. In evaluating Ki by the graphical method we assumed that the decrease in cyanide concentration owing to the formation of the complex is negligible. We may assume that the enzyme concentration is in the range of 2×10^{-5} M as obtained in evaluating K. Then if all the enzyme went into the formation of enzyme-cyanide complex, that is for a case of total inhibition, the cyanide concentration would decrease from 2.86×10^{-4} M to 2.66×10^{-4} M. So we see that the error, if any, is not very large.

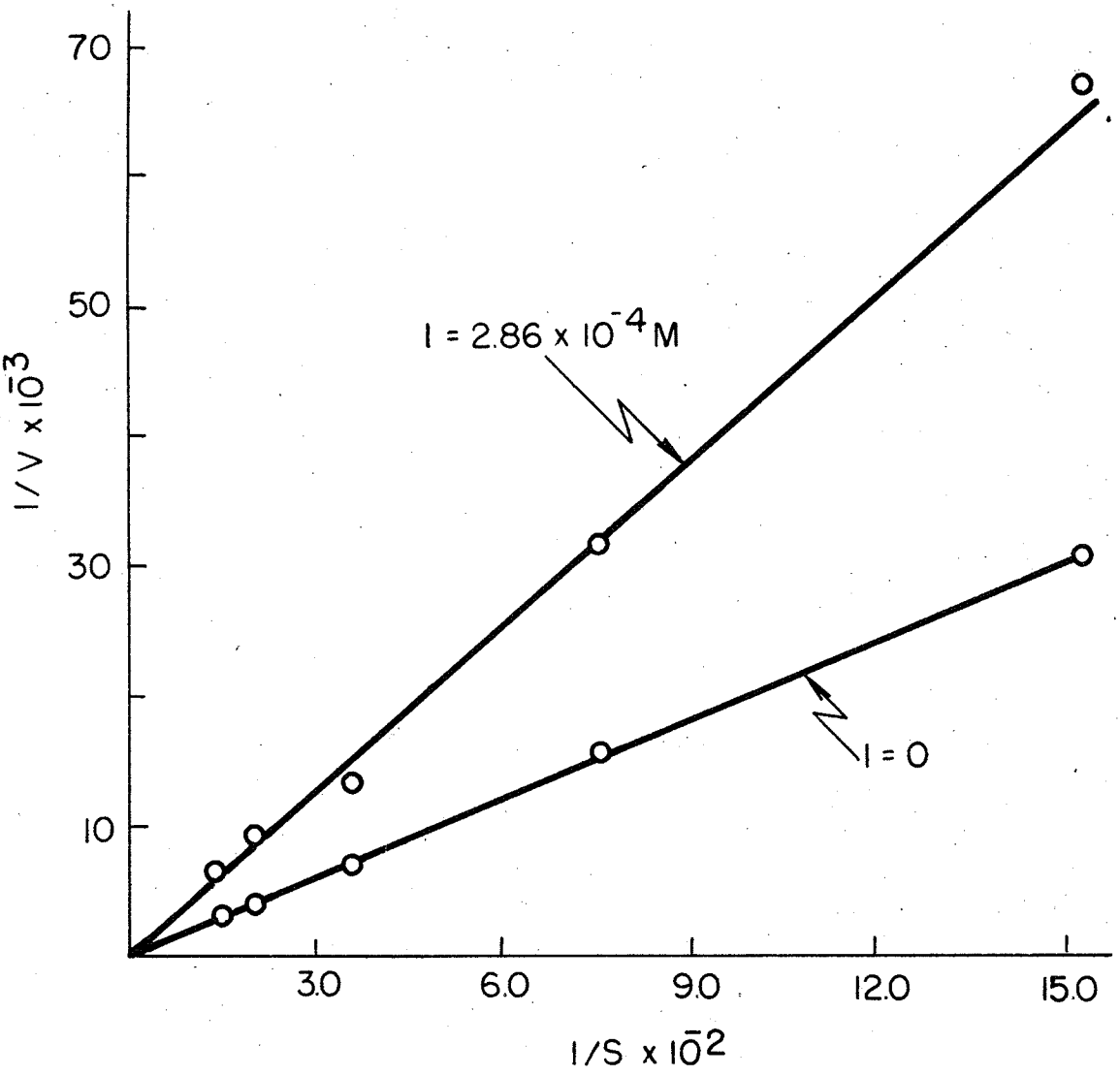

Figure 19. A plot of 1/v against 1/s for the normal and cyanide inhibited decomposition of hydrogen peroxide.

The Alcohol Effect

Ethyl alcohol has an inhibitive effect on almost all enzyme systems. Little is known about the mechanism of this inhibition. In general, alcohol in relatively high concentrations will denature proteins and it is believed that the effect on enzymes is similar to the protein denaturing effect.

The effect of ethyl alcohol on wheat catalase has been investigated. In these experiments it was found that as soon as the contents of the side-arm, regardless of its nature, are tipped into the alcoholic solution in the main compartment of the reacting vessel, there occurs a sudden decrease in pressure. This decrease is equivalent to approximately 27 ul. of oxygen when the main compartment of the vessel contains 0.4 ml. of absolute alcohol in a total volume of 6.0 ml. There is no obvious explanation for this but the effect could be readily corrected with appropriate controls. The data given in Tables XIX and XX include this correction.

Table XIX gives the results of an experiment in which the amount of alcohol was varied. The enzyme level was 1.0 ml. of regular exact and the amount of substrate was 1.0 ml. of 0.046 M hydrogen peroxide. Figure 20 shows that the amount of inhibition is not directly proportioned to alcohol concentration. This suggests that an equilibrium is set up between the enzyme and alcohol.

Table XIX. Effect of Ethyl Alcohol on Wheat Catalase Activity.

Alcohol Concentration, M	Activity, k x 10^4
0	10.60
.476	8.32
.952	5.63
1.428	3.92
1.904	3.37
2.380	1.81
2.856	1.36
3.332	0.94

In another experiment, the hydrogen peroxide concentration was varied while the amounts of alcohol and enzyme were kept constant. The

alcohol concentration was 0.759 M and the enzyme concentration was 1.0 ml. of the regular extract. The results and other pertinent data are given in Table XX.

Table XX. Alcohol Inhibition at Various Substrate Concentrations

Substrate x 10^4 (M)	$\frac{1}{S} \times 10^{-3}$	Velocity x 10^7, moles/l/sec.		$\frac{1}{v} \times 10^{-5}$	
		Uninhibited Reaction	Inhibited Reaction	Uninhibited Reaction	Inhibited Reaction
0.29	34.50	2.06	1.10	49.54	90.91
0.58	17.20	3.48	2.20	28.74	45.46
1.16	8.61	6.61	4.52	15.13	22.10
2.32	4.30	12.29	8.12	8.14	12.31
9.28	1.08	36.19	26.91	2.76	3.69

Figure 21 gives relationship between the reciprocal of the velocity and the reciprocal of the substrate concentration for the inhibited and the uninhibited reactions. Comparing Figure 19 and 21 we see that the alcohol effect is essentially the same as the cyanide effect. It is suspected that alcohol would inhibit catalase activity by a noncompetitive mechanism, whereas cyanide inhibition would be of the competitive type. For the reasons cited previously it is impossible to distinguish between these using the graphical method. The value of the dissociation constant of the enzyme-alcohol complex (if one exists) obtained from the slopes of the curves in Figure 21 is 1.33. However, if the alcohol acts as a denaturing agent then there is no complex formation.

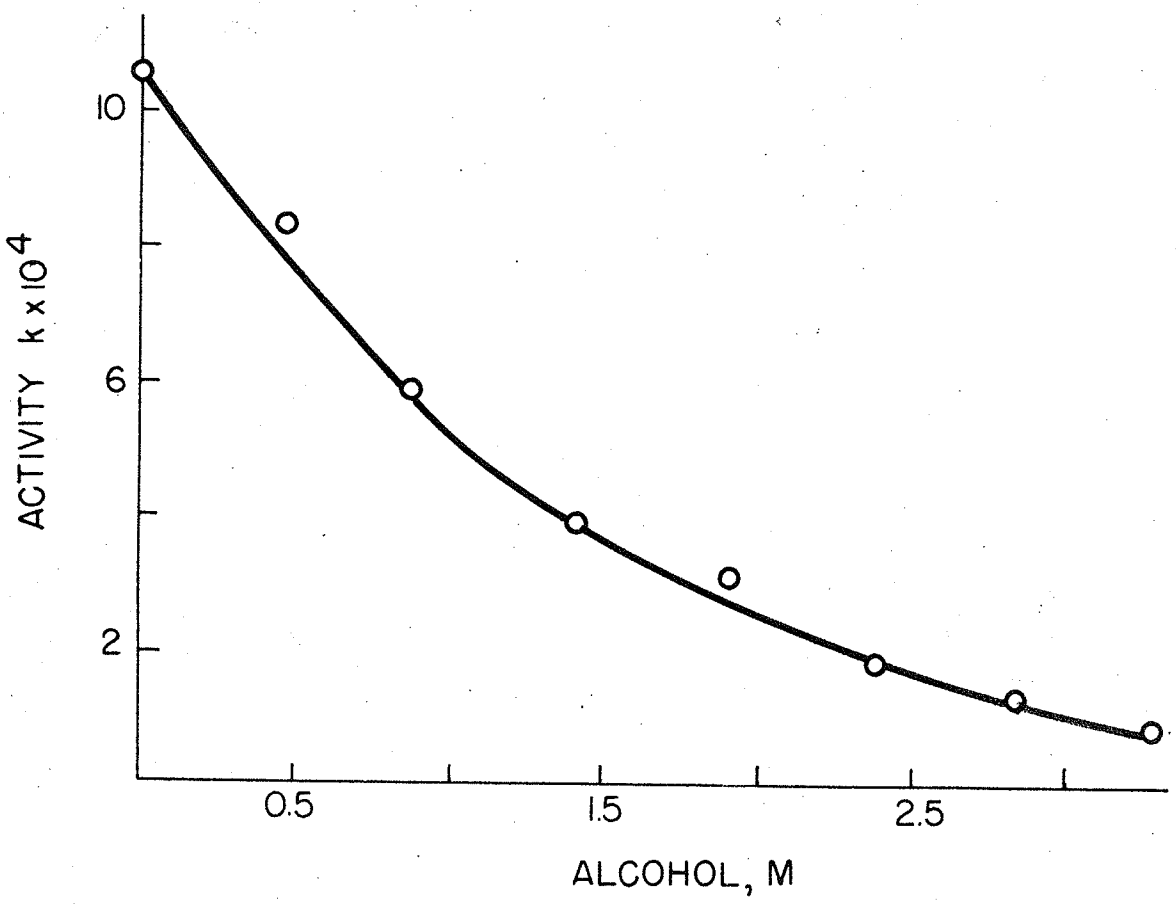

Figure 20. The influence of ethyl alcohol on wheat catalase activity.

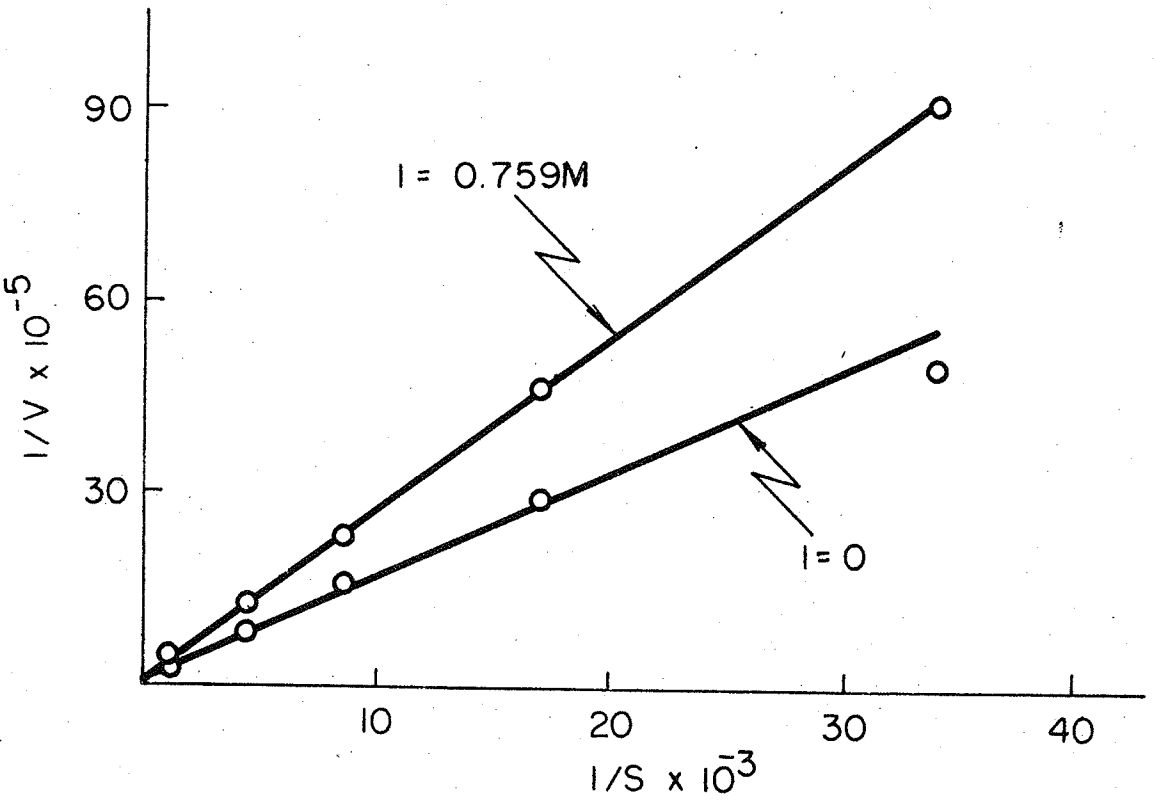

Figure 21. A plot of 1/v against 1/s for the normal and alcohol inhibited decomposition of hydrogen peroxide.

PROPOSED MECHANISM FOR THE DECOMPOSITION OF HYDROGEN PEROXIDE BY CRUDE WHEAT CATALASE

A great amount of research has been done on the mechanism of catalase action. Some of the more important treatments have been reviewed briefly under Survey of Literature on Catalase. Most of the investigators have worked with the crystalline enzyme so that it is doubtful if the postulated mechanisms would apply to a crude enzyme extract such as has been used in the study on which this thesis is based. The consecutive reaction theory proposed by Chance (10) seems to fit our system most satisfactorily. However the reaction studied by Chance was one of second order.

Let us review briefly some of the characteristics of the hydrogen peroxide-wheat catalase system. There is sufficient experimental evidence to establish that the decomposition of hydrogen peroxide proceeds according to first order kinetics at least over the range of concentrations investigated. The specific rate constant is directly proportional to the active enzyme concentration but does not depend on the amount of substrate. The initial velocity or rate is directly proportional to both the enzyme and the substrate concentrations, except in the region of very high substrate concentrations where the destruction of the enzyme by hydrogen peroxide becomes evident. Throughout the investigation the concentration of hydrogen peroxide was at least 10^3 times the enzyme concentration. The amount of enzyme could not be determined directly but an indirect calculation suggests that it is in the range of 2×10^{-5}. Preliminary experiments with crystalline catalase showed that the ratio of substrate to enzyme is probably higher than the value given above. This factor is important in the development of the rate expressions for the mechanism postulated.

The mechanism given here assumes one factor for which no experimental evidence has been obtained in this study. That is the formation of the active wheat catalase — hydrogen peroxide complex. This assumption is justified to the extent that most enzyme systems obey the Michaelis-Menten treatment and, furthermore, an active complex between hydrogen peroxide and crystalline catalase has been identified experimentally by a number of investigators (10, 20). The overall process may be represented by two equations, as follows:

$$\text{Catalase} + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Catalase} - H_2O_2 \text{ (active complex)}$$

$$\text{Complex} + H_2O_2 \xrightarrow{k_3} \text{Catalase} + H_2O + O_2$$

In general the above mechanism may be written as

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\,S \text{ (active complex)} \qquad (32)$$

$$E\,S + S \xrightarrow{k_3} E + P \qquad (33)$$

in which E is the enzyme or wheat catalase, S is the substrate or hydrogen peroxide, E S is the active enzyme substrate complex and P are the products, being water and oxygen in this particular case. The rate expressions will be derived in terms of the general mechanism.

The decomposition of hydrogen peroxide by crystalline catalase can be explained on the basis of the above mechanism with an additional reaction. This is the combination of hydrogen peroxide with crystalline catalase, say at active centre No. 2, to form an enzymically inactive complex. If this active centre is blocked, either through a chemical or physical bond, by such a substance which itself does not render the enzyme inactive, then the inactive complex with the substrate cannot form. There is sufficient experimental evidence to lead one to believe that this is the case with wheat catalase. The substance which occupies this active centre No. 2 has been termed protector. At very high substrate concentrations the protector may be displaced to a certain extent and inactivation by the substrate will take place. This has been observed experimentally with wheat catalase. By adding a small amount of the protector in form of filtrate from boiled wheat extracts the destruction of crystalline catalase by hydrogen peroxide may be prevented. It seems likely that under natural conditions catalase co-exists with another thermostable substance which has this protecting property. The protector is probably removed or destroyed during isolation of crystalline catalase. This is another reason for believing that the bond between catalase and the protecting substance may be of the physical type.

There are two standard procedures for developing theoretically the rate expressions for catalytic processes. Since enzymic reactions are considered to be catalytic processes these procedures should definitely apply to the wheat catalase system. The two procedures are commonly known as the steady-state and the equilibrium treatments. The application of each to the wheat catalase system will be considered in some detail. The treatments will apply only to the case in which the initial concentration of the substrate is much greater than the enzyme. As stated previously, this is the region which has been investigated experimentally.

The steady-state treatment assumes that the concentration of the enzyme-substrate complex is constant during the greater part of the reaction. That is, for the mechanism given above the necessary relationship would be

$$\frac{d\,(ES)}{dt} = k_1\,(E)\,(S) - k_2\,(E\,S) - k_3\,(E\,S)\,(S) = o \qquad (34)$$

The terms k_1, k_2 and k_3 are the velocity constants indicated in the mechanism.

Substitution of $(E_o) - (ES)$ for (E) and $(S_o) - (ES)$ for (S)

in equation (34) gives

$$k_1\left[(E_o) - (ES)\right]\left[(S_o) - (ES)\right] - k_2\,(ES) - k_3\,(ES)\,(S) = o \qquad (35)$$

where (E_o) and (S_o) are the initial concentrations of enzyme and substrate. Since (ES) is small, the term $(ES)^2$ may be neglected; with this approximation the above equation gives rise to

$$(ES) = \frac{k_1\,(E_o)\,(S_o)}{k_1\,(E_o) + k_1\,(S_o) + k_2 + k_3\,(S)} \qquad (36)$$

The rate of reaction from equation (33) is

$$\frac{-d\,(S)}{dt} = k_3\,(ES)\,(S) \qquad (37)$$

which on substituting for (ES) becomes

$$\frac{-d\,(S)}{dt} = \frac{k_1\ k_3\ (E_o)\ (S_o)\ (S)}{k_1\left[(E_o) + (S_o)\right] + k_2 + K_3\ (S)} \qquad (38)$$

Equation (38) indicates that at low concentrations of enzyme and of substrate, the rate varies linearly with the concentration of each of these, but at higher concentrations of either the rate will become independent of that concentration.

The situation with wheat catalase is a bit more complex. Rate expressions given above may be made to fit the experimental results with a few small approximations. We have shown that the turnover number of catalase is very high. On this basis we are justified in assuming that k_1 is very much greater than either k_2 or k_3. Furthermore, the substrate concentration was invariably much greater then the enzyme. On these assumptions equation (38) approximates to

$$\frac{-d\,(S)}{dt} = \frac{k_1\ k_3\ (E_o)\ (S_o)\ (S)}{k_1\ (S_o)}$$

or

$$\frac{-d\,(S)}{dt} = k_3\ (E_o)\ (S) \qquad (39)$$

If the enzyme concentration remains constant throughout the reaction, that is, if it is not inactivated by products or the substrate

$$k_3\ (E_o) = k \qquad (40)$$

where k is a characteristic constant for the particular enzyme concentration. Substituting into equation (39) we have

$$\frac{-d\,(S)}{dt} = k\ (S) \qquad (41)$$

The above equation is a rate expression for a reaction which is kinetically of first order, with k as the specific rate constant. The above treatment shows that

the initial velocity or rate depends on the concentrations of both enzyme and substrate (equation (39)) and that the specific rate constant, k, is directly proportional to the active enzyme concentration (equation (40)). This is in agreement with experimental results.

The equilibrium treatment considers equilibrium concentrations of all the substances involved. Final rate expressions given by this treatment are the same as obtained by the steady-state treatment. Consider the first reaction of the over-all mechanism. We have that

$$K = \frac{k_1}{k_2} = \frac{(E\ S)}{(E)\ (S)} \qquad (42)$$

where K is the equilibrium constant for the particular reaction. In order for this reaction to be in equilibrium the turnover number of the enzyme must be high, that is k_1 must be greater than k_2 or k_3.
Substituting $(E) = (E_o) - (E\ S)$ and $(S) = (S_o) - (E\ S)$ equation (42) becomes

$$K = \frac{(E\ S)}{\left[(E_o) - (E\ S)\right]\left[(S_o) - (E\ S)\right]} \qquad (43)$$

For our case the initial concentration of substrate, (S_o), is much greater than the initial concentration of enzyme, (E_o). We can then say, without introducing too much error, that

$$(S_o) - (E\ S) = (S_o) \qquad (44)$$

Also, (E S) cannot be greater than (E_o) but its magnitude approaches that of (E_o) when the substrate is in excess. That is $(E_o) - (E\ S)$ approaches zero. Accordingly, equation (43) approximates to,

$$K = \frac{(E\ S)}{\left[(E_o) - (E\ S)\right]\ (S_o)} \qquad (45)$$

or

$$(E\ S) = \frac{K\ (E_o)\ (S_o)}{1 + K\ (S_o)} \qquad (46)$$

Let us examine equation (45) qualitatively. We see that the value of K will be very large numerically and will approach infinity as (E_o) - $(E\ S)$ approaches zero when $(S_o) \ll (E_o)$. We need not concern ourselves with the limit values. However, it is pertinent to the development of this treatment to see that the value of K will be very large.

Now, let us consider the second equation of the proposed mechanism. From this we have

$$\frac{-d\ (S)}{dt} = k_3\ (S)\ (E\ S) \qquad (47)$$

Substituting the value of a $(E\ S)$ given by equation (46) the above equation becomes

$$\frac{-d\ (S)}{dt} = \frac{k_3\ K\ (E_o)\ (S_o)\ (S)}{1 + K\ (S_o)} \qquad (48)$$

Now, we can assume that $1 + K\ (S_o) = K\ (S_o)$ since K is very large, then

$$\frac{-d\ (S)}{dt} = k_3\ (E_o)\ (S) \qquad (49)$$

Equation (49) is the same as equation (39) obtained by the steady-state treatment. From this point on the two treatments are identical.

A rather more direct equilibrium treatment may be given as follows. If k_1 is greater than either k_2 or k_3 then the second reaction of the mechanism will be the slower of the two. Therefore the over-all kinetics of the process will be controlled by the second reaction. The rate of substrate decomposition will be

$$\frac{-d\ (S)}{dt} = k_3\ (ES)\ (S) \qquad (50)$$

At equilibrium (E S) will be constant and for the case where the initial substrate concentration is much greater than that of the enzyme it will be approximately equal to (E_o). Equation (50) then becomes

$$\frac{-d\,(S)}{dt} = k_3\,(E_o)\,(S) \qquad (51)$$

which is identical to the one obtained by the other, much longer treatments.

As pointed out previously, the above mechanism holds only for the case in which the initial substrate concentration is much greater than the enzyme. It is difficult to say for how long a single reaction proceeds according to the mechanism outlined. The substrate concentration is continually decreasing while the enzyme level remains constant so that a point will be reached where the assumptions made are no longer justified. At this point the proposed mechanism would no longer apply. However the treatments are valid if we consider only the initial portion of the reaction.

How well the outlined mechanism fits the decomposition of hydrogen peroxide under physiological conditions is open to question. As in most other kinetic studies of enzyme reactions it is difficult to predict the effects of other biological substances which are present under natural conditions. In this study a crude water extract was employed as the active enzyme extract. The extracts were prepared by a limited number of operations so that it is probable that the fraction of the enzyme that is extracted is not altered in any manner. The extracts also include representative concentrations of other water soluble materials contained in wheat endosperm. Their effects, if any, were included in the experimental results. There is no doubt in the mind of the author that if crystalline catalase was isolated from wheat it would show identical properties to crystalline catalase from other sources. There definitely is a difference between water extracts of wheat endosperm and water solutions of the crystalline enzyme. Some of these differences have been pointed out in another section of this thesis. This contrast may be due to one or more water-soluble substances. In this respect the enzyme system studied compares to the "in vivo" system. On this basis we can say that the mechanism given in this thesis is more applicable to the biological decomposition of hydrogen peroxide than any of the mechanisms based on studies using crystalline catalase.

OTHER RELATED STUDIES

Catalase Activity of Various Wheat Varieties

Preliminary tests with a number of amber durum varieties revealed marked differences in their catalase activities. An over-all study was therefore carried out to compare the activities of representative varieties of several classes of wheat. The wheats used for this study were obtained from experimental stations for the purpose of co-operative testing and other studies being carried out at the Grain Research Laboratory. The over-all study includes four sets of experimental results. These are the activities of fifteen amber durum varieties, twelve bread wheats of various classes, nine grade average samples and seven pure amber durum varieties grown at seven stations. The last group includes the activities of the ground whole wheats as well as the activities of the semolina.

The catalase activity was determined manometrically by the procedure described previously. All activities are expressed in terms of the specific rate constant, which in each case represents the mean of six values calculated by using the first six 5-minute readings of the volume of oxygen evolved. Regular extracts were obtained using 10 gm. of semolina for the amber durum wheats, 10 gm. of flour for the bread wheats or 10 gm. of ground whole wheat for the whole wheats. The substrate concentration was 1.0 ml. of 0.046 M hydrogen peroxide solution throughout the study.

Table XXI shows the activities of the fifteen amber durum varieties. These are pure varieties grown at a single station.

Table XXI. Catalase Activities of Fifteen Amber Durum Wheat Varieties

R.L. No.	Variety	Activity, k x 10^3
1073	Heiti	0.85
-74	P.I. 94701	1.80
-75	Chapinge	4.40
-76	Carleton	1.47
-77	Golden Ball x 1317	1.28
-78	Iumillo	2.21
-79	Golden Ball x 1317	1.42
-80	Gaza	2.02
-81	Chapinge x 1317	1.60
-82	Obispado de Medina	3.74
-83	Pelissier	2.41
-84	Beloturka	3.89
-85	Tremes Preto	3.00
-86	Mindum x Beloturka	1.04
-87	C.I. 3255	1.62

The catalase activities of thirteen bread wheat flours were determined for the purpose of comparison. These included nine American and four Canadian varieties of various classes. The activities were determined by the regular method and are expressed in terms of the specific rate constant. Table XXII shows the results obtained. The wheats are listed in order of increasing activity.

Table XXII. Catalase Activities of Flours of Twelve Bread Wheats of Various Classes.

R.L. No.	Class	Variety	Activity, k x 10^3
1028	Hard Red Winter	Pawnee	0.320
1029	Hard Red Winter	Commanche	0.416
1026	Soft Red Winter	Vigo	0.420
1027	Soft Red Winter	Thorne	0.741
1033	Soft White Winter	Elmar	0.851
1031	Soft White Spring	Idaed	0.881
1030	Hard White Spring	Baart	1.33
545	Hard Red Spring	Redman	1.91
1032	Soft White Winter	Golden	2.30
546	Hard Red Spring	Thatcher	2.56
544	Hard Red Spring	Marquis	2.92
547	Hard Red Spring	Lee	3.09

Table XXII shows that the trend in general is for the Canadian wheats to have a higher catalase activity than American varieties. The four Canadian wheats are Redman, Thatcher, Marquis and Lee. The second obvious trend is that spring varieties are higher in activities than winter varieties. These results are in accord with observations of other investigators (5) but so far no one has offered an adequate explanation why this is so. The author speculates that the moisture content during the early stages of plant development might be one of the factors effecting the catalase level in the seed. To follow up this speculation would require a project extending over several growing seasons.

Table XXIII gives the catalase activities of nine grade samples including four grades. Each grade sample is comprised of an unknown number of varieties grown in different parts of Western Canada.

Table XXIII. Catalase Activities of Nine Grade Samples Covering Four Grades.

R.L. No.	Grade	Activity, k x 10^4
2658	2 A.D.	4.12
590	2 A.D.	5.25
2689	2 A.D.	5.96
591	3 A.D.	7.15
2659	3 A.D.	8.12
2690	3 A.D.	8.31
2610	4 A.D.	7.31
592	4 A.D.	7.15
2661	5 A.D.	6.49

Going from top to bottom of Table XXIII we see that there is no overall trend. Closer examination of the data reveals a tendency which is of considerable significance. We see that at first the activity increases as the grade decreases and then decreases as the grade decreases beyond 3 A.D. It shows that all the samples of the same grade are in one range of activity. The possibility of using the catalase activity as an index of the grade has been considered by a number of investigators. (3, 5) but so far no one has developed an accepted method of correlation. It is not known if the differences in activities shown in Table XXIII are due to grade differences or due to varietal differences. As will be noted later, the origin also has a marked effect on catalase activity. A more comprehensive study would be to determine the activities of different grades of a single pure variety. It was impossible to obtain such samples and hence such a study could not be carried out.

The fourth set of data includes the catalase activities of both semolina and ground whole wheat of seven pure varieties grown at seven stations. Varieties which were chosen include Mindum, Golden Ball, Carleton, Nugget, D.T. 122, D.T. 125 and D.T. 208. These were grown during the 1952 growing season at the following experimental stations: Brandon, Morden and Melita in Manitoba, Indian Head, Saskatoon and Swift Current in Saskatchewan, and Lethbridge in Alberta. The activities were determined by the regular technique and are expressed on the basis of activity of 1.0 ml. of regular extract used in conjunction with 1.0 ml. of 0.046 M hydrogen peroxide solution. The results obtained are shown in Table XXIV.

The large differences in the activity of a single variety grown at different stations is very striking and may contain the key to the problem of the effects of environment on the development of catalase. It seems likely that the moisture content is responsible, to a limited extent, for this effect. As an example, we can compare the means of wheat and semolina activities for Lethbridge, Swift Current and Saskatoon to those for the three Manitoba stations. The agreement between the means and composites for each variety is very good and is quoted in support of the method of assay. The ratio of the semolina activity to the whole wheat activity is essentially constant, with the exception of Mindum, and is approximately equal to 6. The reason for knowing the origin as well as variety of the wheat if the catalase activity is to be used as an index of the grade, is readily apparent from the results shown in Table XXIV.

Table XXIV. Catalase Activities of Semolinas and Ground Whole Wheats of Seven Durum Varieties Grown at Seven Stations.

(Wheat: Activity, k x 10^3; Semolina: Activity, k x 10^4)

Variety	Brandon	Indian Head	Sask-atoon	Leth-bridge	Morden	Swift Current	Melita	Mean	Com-posites
Mindum									
Wheat	2.60	2.12	5.26	2.94	3.64	5.16	3.20	3.56	3.64
Semolina	3.21	2.36	5.29	5.35	4.50	6.91	4.35	4.57	4.37
Golden Ball									
Wheat	4.64	4.43	8.06	5.42	7.60	4.30	8.76	6.60	6.70
Semolina	10.40	6.82	12.71	9.56	12.60	10.20	12.00	10.61	10.39
Carleton									
Wheat	4.28	2.87	5.24	5.18	4.50	5.16	3.82	4.43	4.22
Semolina	5.60	3.99	7.76	8.12	4.73	11.60	5.49	6.75	6.65
Nugget									
Wheat	4.14	2.58	5.50	4.78	4.14	3.56	5.40	4.30	3.68
Semolina	6.92	4.18	9.34	9.29	5.65	10.80	5.12	7.31	7.18
D.T. 122									
Wheat	4.68	2.51	6.50	7.52	4.81	3.44	3.60	4.72	5.02
Semolina	5.64	3.74	8.88	11.60	6.00	12.80	7.32	8.00	7.57
D.T. 125									
Wheat	4.60	2.46	7.02	5.56	6.64	4.74	4.18	5.03	5.10
Semolina	6.56	3.96	8.16	11.40	7.29	15.70	7.22	8.68	7.82
D.T. 208									
Wheat	3.51	1.71	4.70	6.20	2.90	3.72	2.18	3.56	3.88
Semolina	5.07	2.48	6.90	7.87	3.78	9.92	3.98	5.71	5.82
Mean									
Wheat	4.06	2.67	6.04	5.37	4.89	4.29	4.45	-	-
Semolina	6.20	3.93	8.43	9.03	6.36	11.13	6.57	-	-

Effect of Certain Flour Improvers on Wheat Catalase Activity

In 1932 Van der Lee (50) found that chlorates, perchlorates, bromates, iodates, periodates and persulfates inhibited catalase activity of flour. Soluble compounds of the above radicals when added to bread flours are said to have an "improver" effect. That is, they tend to improve the bread making qualities of the particular flour. So far, no one has offered a satisfactory explanation of the mechanism of this improver action.

The effect of three common improvers on the activity of wheat catalase was studied manometrically. In this case the improver and 1.0 ml. of regular enzyme extract were placed with the buffer into the main compartment of the reaction vessel. One ml. of 0.046 M hydrogen peroxide solution was pipetted into the side-arm. In each experiment control vessels were set up to detect any change in pressure due to the effect of the improver on either the enzyme or the substrate alone. All activities are expressed in terms of the specific rate constant. The results obtained are given in Table XXV.

Table XXV. Effect of Improvers on Wheat Catalase Activity/

Improver and Amount	Activity, $k \times 10^3$
None	6.60
0.5 ml. of 0.1 M $KBrO_3$	4.20
0.2 ml. of 0.1 M KIO_3	4.14
0.2 ml. of 2 gm./1. $NaClO_2$	2.42

Table XXV. shows that the particular improvers have a definite inhibitive effect. However, the amounts used are approximately ten times the amounts normally used to improve the baking qualities of flour. Preliminary experiments with approximately the same levels of the improvers as are added to bread flours showed no significant inhibitive effects. Accordingly, it seems doubtful that inhibition of catalase activity plays any direct part in the mechanism of flour improvement.

Effect of Ball Milling and Particle Size on Wheat Catalase

Two samples of durum semolina were ball milled for 24 hours in a laboratory ball mill. Extracts were prepared by the regular procedure using 10 gm. of the flour. The activities of these extracts are compared to the activities of regular extracts of normal semolina. Table XXVI shows the results obtained. The decrease in activity as a result of ball milling is probably due to physical destruction of the enzyme.

Table XXVI. Effect of Ball Milling on Wheat Catalase Activity.

Sample No.	Semolina Activity ($k \times 10^4$)	Activity of ball milled sample ($k \times 10^4$)
3101	4.37	1.57
3105	4.00	1.34

Catalase activities were determined for a series of semolina particle-size fractions obtained by fractionating Pelissier semolina on a Ro-Tap sifter. The series consisted of five particle size fractions. These were extracted by the regular method and the activities of 1.0 ml. of the extracts were determined. The results are given in Table XXVII.

Table XXVII. Catalase Activity of Various Semolina Particle Size Fractions.

Fraction	Activity, $k \times 10^3$
Held on 60	0.71
Thru 60 held on 80	0.80
Thru 80 held on 100	1.03
Thru 100 held on 120	1.32
Thru 120 (flour)	2.73

Table XXVII shows that enzymic activity tends to increase with decreasing particle size. The low activities of the largest particles seems to suggest that these particles originated from the central endosperm core. On the other hand, the increase in enzymic activity with decreasing particle size may be due to increase in surface area. This would result in a more efficient extraction of the enzyme.

SUMMARY AND CONCLUSIONS

The kinetics of the decomposition of hydrogen peroxide by water extracts of durum wheat semolina have been studied. As far as is known this is the first such study using durum wheat as the source of the enzyme catalase. The results obtained may be summarized as follows:

(1) The reaction proceeds according to first order kinetics over the range of enzyme and substrate concentrations investigated.

(2) The first order specific rate constant is directly proportional to the amount of active enzyme; it does not vary with the substrate concentration.

(3) The initial velocity is directly proportional to both enzyme and substrate concentrations. The linearity breaks down at very high substrate concentration owing to the destruction of the enzyme by the substrate.

(4) Hydrogen ion concentration has a marked effect on wheat catalase activity. Optimum activity for the particular experimental conditions occurs at pH 7.3.

(5) Above 23°C. wheat catalase readily suffers inactivation. From 10-25° the experimental results are in harmony with the Arrhenius equation and the activation energy is 2,800 calories.

(6) The apparent Michaelis Constant for the wheat catalase-hydrogen peroxide system is approximately 1×10^{-3} M.

(7) Cyanide and ethyl alcohol were found to inhibit wheat catalase activity. The inhibition mechanisms could not be explained by the standard methods.

(8) Crystalline catalase was found significantly different from wheat catalase. It is readily destroyed by hydrogen peroxide.

(9) Catalase activity of wheat varies markedly with variety, origin and grade.

(10) Fine grinding in a ball mill tends to decrease the catalase activity of wheat.

(11) Catalase activity of semolina tends to increase as the particle size decreases.

(12) Potassium bromate, potassium iodate and sodium perchlorate inhibited the action of the catalase of semolina.

(13) A mechanism which is in harmony with the experimental results has been postulated. A modification was suggested to cover the case of crystalline catalase.

BIBLIOGRAPHY

1. Appleman, D. Plant Physiol. 27, 613 (1952); Chem. Abstracts 46, 10316 (1952).

2. Bach, A., Oparin, O., and Vener, R. A. Trans. Karpov. Chem. Inst. 5, 62 (1926); Chem. Abstracts 22, 2186 (1926).

3. Bailey, C.H. J. Biol. Chem. 32, 539 (1917).

4. Beers, R. F., and Sizer, I. W. J. Biol. Chem. (1952) 133 (1952).

5. Blish, H. J., and Bode, C. E. Cereal Chem. 12, 133 (1935).

6. Bonnischen, R. K., Chance, B., and Theorell, H. Acta. Chem. Scand. 179, 1299 (1949); J. Biol. Chem. 195, 133 (1952).

7. Brown, G. L. Biochem. J. 51, 569 (1952).

8. Burk, O. J. Phys. Chem. 34, 1207 (1930).

9. Chance, B. J. Biol. Chem. 179, 1311 (1949).

10. Chance, B. Advances in Enzymology, Vol. 12, Interscience Publishers, Inc. (1951), New York.

11. Chance, B. Biochem. J. 46, 387 (1950).

12. Chance, B. J. Biol. Chem. 180, 947 (1949).

13. Chance, B. and Herbert, D. Biochem. J. 46, 402 (1950).

14. Dixon, M. Manometric Methods, Cambridge (1934), (New York: MacMillan Co.)

15. Dounce, A. L. J. Biol. Chem. 143, 499 (1942).

16. George, P. Nature 160, 41 (1947).

17. Goldblith, S. A. and Proctor, B. E. J. Biol. Chem. 187, 705 (1950).

18. Haber, F. and Willstatter, R. Ber. 64, 2844 (1931); Advances in Enzymology, Vol. I, Interscience Publishers, Inc. (1941), New York.

19. Hennichs, S. Biochem. Z. 171, 314 (1926); Advances in Enzymology, Vol. I, Interscience Publishers, Inc. (1941), New York.

20. Heppel, L. A. and Porterfield, Virginnia T. J. Biol. Chem. 121, 417 (1931).

21. Herbert, D. and Puisent, J. Biochem. J. 43, 193 (1948).

22. Hoptee, B. H. J. Science 116, 329 (1952).

23. Jacobson, W. Z. Physiol. Chem. 16, 340 (1892); Advances in Enzymology, Vol. I, Interscience Publishers, Inc. (1941), New York.

24. Johnson, F. H. and Van Schouwenburg, K. L. Nature 144, 634 (1939).

25. Keilin, D. and Hartree, E. F. Proc. Roy. Soc. (London), (B) 124, 397 (1938).

26. Keilin, D. and Hartree, E. F. Nature 152, 626 (1943).

27. Kitavin, G. S. Biokhimiya 17, 336 (1952); Chem. Abstracts 46, 10238 (1952).

28. Laidler, K. J. Chemical Kinetics, McGraw-Hill Book Co. (1950), New York.

29. Laskowski, M. and Sumner, J. B. Science 94, 615 (1941).

30. Lemberg, R. and Foulkes, E. C. Nature, 161, 131 (1948).

31. Lineweaver, H. and Burk, D. J.A.C.S. 56, 661 (1934).

32. Löw, O. U.S. Dept. Agr. Report No. 68 (1901); Advances in Enzymology, Vol. I., Interscience Publishers, Inc. (1941), New York.

33. Marks, G. W. and Fox, D. L. J. Biol. Chem. 103, 269 (1933).

34. Michaelis, L. and Menten, M. L. Biochem. Z. 49, 333 (1913); Haldane, J. B. S. Enzymes. Longmans, Green and Co., (1930), Toronto.

35. Morgulis, S., Beber, M., and Rabkin, I. J. Biol. Chem. 68, 521 (1926).

36. Scholander, P. F., Niemeyer, H., and Lloyd Claff C. Science 112, 437 (1950).

37. Schonbein, C. F. J. Prakt, Chem. 89, 334 (1863); Advances in Enzymology, Vol. I., Interscience Publishers, Inc. (1941), New York.

38. Shirakawa, M. J. Agr. Chem. Soc. Japan. 25, 166 (1951); Chem. Abstracts, 46, 11296 (1951).

39. Sizer, I. W. J. Biol. Chem. 154, 461 (1944).

40. Sörensen, S. P. L. Biochem. Z. 21, 131 (1909); Haldane, J. B. S. Enzymes. Longmans, Green and Co., (1930), Toronto.

41. Stern, K. G. Nature 136, 302 (1935).

42. Stern, K. G. J. Biol. Chem. 114, 473 (1936).

43. Stern, K. G. Z. Physiol. Chem. 209, 176 (1932); J.A.C.S. 56, 661 (1934).

44. Stern, K. G. and Wychoff, R. W. G. J. Biol. Chem. 124, 573 (1938).

45. Sumner, J. B. Advances in Enzymology, Vol. I, Interscience Publishers, Inc. (1941), New York.

46. Sumner, J. B. and Dounce, A. L. J. Biol. Chem. 121, 417 (1937).

47. Sumner, J. B. and Gralen, N. J. Biol. Chem. 125, 33 (1938).

48. Takasugi, N. and Yamazaki, I. J. Agr. Chem. Soc. Japan, 25, 113 (1951); Chem. Abstracts 46, 10305 (1952).

49. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Tissue Metabolism, Burgess Publishing Co. (1949), Minneapolis, Minn.

50. Van Der Lee, G. U.S. Pat. 1,866,412, July 5, 1932; Anderson, J. A. Enzymes and Their Role in Wheat Technology, Interscience Publishers, Inc. (1946), New York.

51. Van Slyke, D. D. and Cullen, G. E. J. Biol. Chem. 19, 141 (1942).

52. Von Euler, H. and Josephson, K. Ann. Chem. 452, 158 (1927); J. Biol. Chem. 195, 133 (1952).

53. Waentig, P. and Gierisch, W. Fermentforschung. 1, 165 (1914-16); Advances in Enzymology, Vol. I. Interscience Publishers, Inc. (1941), New York.

54. Weiss, J. and Weil-Malherbe, H. Nature 144, 866 (1939).

55. Wolff, J. and de Stoechlin, E. Compt. rend. 152, 729 (1911); Advances in Enzymology, Vol. I. Interscience Publishers, Inc. (1941), New York.

56. Yamafuzi, K., So, K., and Nagano, K. Biochem. Z. 315, 405 (1911) Ann. Rev. of Biochem. Vol. XIV. Annual Reviews, Inc. (1945), Stanford University P.O., Calif.

57. Zeile, K. Z. Physiol. Chem. 195, 139 (1930-31); Ann. Rev. of Biochem. Vol. I. Stanford University Press (1932), Stanford University, California.

58. Zeile, K. and Hellstrom, H. Z. Z. Physiol. Chem. 192, 171 (1930); Advances in Enzymology, Vol. I. Interscience Publishers, Inc. (1941), New York.