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I hereby recommend that the thesis prepared under my supervision by William Ragland Brown

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Approved by:

Albert P. Mathews

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THE HYDROLYSIS OF STARCH BY HYDROGEN PEROXIDE AND FERROUS SALTS.

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William Ragland Brown
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INTRODUCTION

The phenomena of enzyme action are of primary importance in the economy of the living cell, and a vast amount of work has been done in attempts to elucidate their nature and mode of action. In spite of this, there are still many points concerning them which cause one to pause and wonder. An enzyme can, at low temperatures, in very feebly acid or alkaline solutions, and in very low concentrations, effect a transformation which, in order to be duplicated by purely chemical means, requires quite drastic measures such as high temperatures, strong acids or alkalis and relatively high concentrations. This gives rise to the old, still unanswered question, "How may these things be?"

The explanation offered by Michaelis (43) on the basis of specific electric charges, and those of Bayliss (5) and Fodor (18) on the basis of adsorption on colloids with consequent increase of active surface, have not been supported by later research. Especially has the latter fallen into disrepute since it has been shown that some, possibly all, enzymes are relatively simple substances, only bound to colloidal "carriers", and not dependent upon these colloids for their activity (40) (67).

That there is a combination between the enzyme and substrate seems to be accepted at the present time. Upon this basis, Euler and Josephson (16) have advanced the idea that a specific group, possibly of the nature of the aldehyde group, attaches the enzyme to the substrate. Euler's theory maintains that the substrate is split at some point other than the one to which the enzyme is attached. Thus there must be some secondary linkage between the enzyme and substrate in order that the hydrolysis take place.

Hugounenq and Loiseleur (25) (26) showed, by experiments with pepsin and trypsin, that each enzyme showed a specificity for definite groups. By methods which blocked amino and carboxyl groups of proteins, they showed that pepsin combines with the amino group, while trypsin combines at the carboxyl. Balls and Kohler (3) have confirmed this work in their researches upon the polypeptidases, showing that these enzymes may be differentiated by their point of attachment to the polypeptide, whether to the amino or the carboxyl group, the secondary linkage being the imino-group. The great bulk of investigation has been directed toward the chemical nature of the active principles of the various enzymes, or the mechanism of their specificity. The former consideration is of primary importance, for until the chemical nature of the active principle is definitely known, chemical concepts of their specificity and mode of action must necessarily be built upon speculation and circumstantial evidence.

An interesting and important phase of the study of enzymes is the fact that practically all enzymes require the presence of substances, or types of substances, which act as specific activators. These substances vary in nature with the various enzymes, ranging from salts on the one hand to very complicated organic compounds. One of the most interesting substances of this group is glutathione, a tripeptide composed of glycine, glutamic acid, and cysteine. This substance is widely distributed in living matter, and has the ability to activate a number of different enzymes. The mechanism of the activation is obscure, but the evidence points to the fact that the enzyme and activator form a system which gives greater facility to the transfer of energy to the substrate.

Dr. A.P. Mathews (41)(42) has advanced the view that substances exist in two forms, according to the energy content of the molecule. The katakinetic, or energy-poor, form is stable, and the addition of energy is necessary to raise it to the anakinetic, or reactive form. He suggests that enzymes act to transfer energy from some external source (oxygen, radiant energy, or the kinetic energy of surrounding molecules) to the stable molecule, causing it to be raised to the anakinetic or unstable state, in which it undergoes spontaneous hydrolysis. The enzyme molecule having transferred its energy becomes inactive, dissociates, is reactivated, and combines with another molecule of substrate and repeats the transfer of energy. Many instances of this reversibility between active and inactive forms are known.

If no substrate, to which the enzyme may carry the energy received, be present, the absorbed energy may destroy the enzyme itself; while in the presence of its substrate the life of the enzyme is longer. The sensitivity of enzymic action to temperature up to the point of inactivation adds evidence for the theory of energy transfer, since by increasing the kinetic energy of the solution, the chances for the reactivation of the enzyme molecule are greatly enhanced.

The work done in this laboratory by Dr. M.J. Boyd (7) showed that the energy of light and oxygen, in the presence of hematoporphyrin, could be utilized for the splitting of fibrinogen and serum albumin. Dr. E.S. Hill (24) succeeded in causing the hydrolysis of serum albumin by means of dialuric acid and oxygen. Mr. A. Sigal showed that hydrogen peroxide, in the presence of ferrous sulfate, gave an appreciable hydrolysis of serum albumin. These researches have added evidence to the idea that enzymes act in the transfer of energy to the substrate. In all these instances the transfer of energy took the form of a reversible oxidation.

The present study was undertaken at the suggestion of Dr. A.P. Mathews, to ascertain if by the action of the peroxide-iron system upon starch the latter could be hydrolysed in the hope that if this were so it might throw some additional light upon the manner in which amylase accomplishes a similar hydrolysis.

It will be recalled that the formation of starch is just the reverse of the process here imagined as occurring in its hydrolysis and decomposition.

In the process of photosynthesis, formaldehyde is formed from CO_2 and H_2O by the absorption of the energy of light. This formaldehyde, possibly through a process of hydration followed by a subsequent dehydration between adjacent molecules condenses into glucose. Six molecules of formaldehyde have been made, under laboratory conditions, to condense into a hexose isomeric with glucose. Since starch is a polymer of glucose, it is postulated that similar condensation of glucose residues produces starch. Accompanying the condensation by dehydration, there is a successive loss of energy in each product. There is less free energy in each polymer, as evidenced by the fact that the heat of combustion of each successive product is less than the heats of combustion of the molecules from which it was composed. To rehydrolize, and thus split the starch back into sugar or formaldehyde, this energy must be supplied whatever the agent of the transformation may be. The chemical process of hydrolysis consists in interposing a molecule of water between the structural units of the starch molecule. It is still somewhat uncertain whether the energized molecule decomposes first and the fragments combine with water; or the hydration occurs first and the lysis secondarily. The structure of the starch molecule is still a matter of conjecture, and in just how many steps the hydrolysis proceeds is unknown. But, to repeat, it is evident , from a consideration of the foregoing, that energy must be supplied to the starch molecule from some external source in order to accomplish the splitting, and since this splitting is easily accomplished by the various amylolytic enzymes, they must in some way make available to the starch molecule sources of energy not otherwise open to it. The question naturally arises then, "Where does the enzyme obtain the necessary energy, and how does it effect the transfer?" Since the commonest source of energy in living things is oxygen, the possibility exists that amylase is part of an oxidation-reduction system; or is activated itself by such a system. It was desirable to show that starch might be hydrolysed by an artificial oxidation-reduction chain, and hydrogen peroxide and iron were accordingly chosen.

Hydrogen peroxide, as is well known, is an unstable compound of hydrogen and oxygen, breaking down very easily with the formation of water and oxygen. This decomposition of the peroxide is catalyzed by the presence of inorganic compounds or enzymes called catalase reaction following one of two distinct paths, depending upon the nature of the catalyst present. In the presence of catalase or manganese dioxide, the decomposition is known to result in the formation of water and molecular oxygen; while in the presence of peroxidase, or iron or copper salts, it might be represented as resulting in the formation of water and atomic oxygen. The breakdown of the peroxide is accompanied by the setting free of energy, energy which might be absorbed by the starch. The presence of the iron increases the rate of decomposition of the peroxide, hence the rate of production of energy, and possibly the iron acts in the system to transport this energy to a substrate, making possible the utilization of the free energy to accomplish the work of splitting the substrate. With these ideas in view, the study of the action of hydrogen peroxide and ferrous sulfate was undertaken.

HISTORICAL DISCUSSION

Starch hydrolysis came into prominence in chemistry as a result of the researches of Kirchhoff (32), who reported to the Imperial Academy of Sciences at St. Petersburg in 1811 that sugars could be prepared from starch by the action of dilute acids. Three years later the same investigator (32) wrote the first clearly characterized description of enzyme action in reporting that the glutinous extract of wheat meal caused the saccharification of a solution of starch. These classical papers were the beginning of a vast amount of literature on the acid, enzymic, and numerous other methods of starch hydrolysis.

Although the fact that glucose appears in the fermentation of starch had already been noted by Guyton (21) in 1798, and Kirchhoff (32) had already written of the enzymic saccharification of starch, it was not until 1833 that Payen and Persoz (53) actually succeeded in isolating impure diastase and showing the principal products, dextrin and starch sugar, of its reaction with starch. In 1845, Dubrunfaut (14) described a sugar from the action of diastase upon starch which we now recognise as maltose, but for a number of years was considered to be a mixture of dextrin and glucose.

The first theory as to the action of diastase upon starch was proposed by Musculus (45) in 1860. He postulated that the digestion of starch by diastase was a process of hydration and subsequent decomposition into dextrins of lower molecular weight, glucose being split off in the process. In 1876, O'Sullivan (51) showed that the sugar called maltose by Dubrunfaut was the characteristic sugar produced in the enzymic hydrolysis, and an intermediate in the acid hydrolysis, of starch. In 1878, Musculus and Gruber (46) revised the earlier theory of Musculus to conform with the results of O'Sullivan. They succeeded in isolating from the reaction the following products: soluble starch, erythrodextrin, alpha- and beta-achroodextrins, maltose and glucose. The work of Horace T. Brown (1879-1899) and his associates (10) in general confirmed this work, though they were able to obtain other dextrinous products by varying the conditions of the experiments.

Bourquelot (8) in 1887, suggested that the transformation of starch took place in two steps: the starch being first hydrated by water, and the hydrated starch then saccharified by the amylase. He found that low concentrations of acids, alkalis, salts, and saliva ash had no effect upon the reaction.

Lintner and Dull (1892)(38) isolated isomaltose from the reaction of diastase upon starch, and postulated that it was a normal intermediate in the process of saccharification. They also isolated it from the products of the acid hydrolysis of starch. They maintained that in the enzymic saccharification there were but five products: maltodextrin, erythrodextrin, achroodextrin, isomaltose, and maltose, and that the saccharification followed these steps. They showed also that the acid hydrolysis produced several erythrodextrins and achroodextrins, isomaltose and glucose, but no maltose.

Senff (62)(1892) concluded that the action of diastase upon starch produced erythrodextrin, dextrin, and isomaltose. Further action of the enzyme converted the erythrodextrin to maltose, the dextrin and isomaltose remaining unchanged.

Jalowetz (28)(1895), Ulrich (65)(1895) and Ost (50)(1904) were unable to find isomaltose among the products of the reaction. Prior (58)(1896) obtained "Achroodextrin III", and maintained that isomaltose was a mixture of this substance with maltose.

The work of Ling and Baker (35)(1895 et seq.) showed that the limited action of amylase upon starch results in the formation of maltose, maltodextrin-a, maltodextrin-B, and a simple dextrin isomeric with maltose. Ling (36) first explained isomaltose as a reversion product of glucose, since he found glucose as a product of amylase upon dextrins at 70°. The work of Ling and Nanji (37), published in 1925, shows that alphahexaamylose is converted quantitatively into maltose by the action of malt diastase, while the alpha-beta-hexaamylose from amylopectin is converted by the malt diastase into maltose and isomaltose.

In 1922, Perger (54) studied the acid hydrolysis of starch, followed by precipitation of the products with magnesium sulfate. He concluded that the hydrolysis proceeds by the asymmetric cleavage of compounds of varying molecular size, the rate of hydrolysis increasing as the molecular weight of the products becomes less.

Pringsheim and his associates (57)(1912 et seq.) have shown the course of starch degradation by the enzymes of bacteria, particularly those of Bacillus Macerans. Among the products they were able to isolate and identify a diamylose, a triamylose, and a tetraamylose in addition to the dextrinous substances formed.

So far as hydrolysis by hydrogen peroxide is concerned, a search of the literature resulted in the discovery that several authors had recorded the formation of such products of the reaction as dextrin and glucose.

Then in 1889, Wurster (70) noted that starch is not attacked by hydrogen peroxide at ordinary temperatures, but in boiling acid or alkaline solution starch is converted by peroxide to glucose and dextrin. Three years later, von Asboth (2)(1892) reported that treatment of starch with hydrogen peroxide, made slightly alkaline with ammonia, causes liquefaction of the solution, and carbon dioxide and oxygen are evolved.

Fernbach and Wolff (17) in 1909 noted that hydrogen peroxide caused the liquefaction of starch, and observed that formic acid is produced in the reaction. The first attempt to hydrolyse starch by hydrogen peroxide, instead of oxidizing it, was made by Gatin-Gruzewska (19) in 1910. By regulating the conditions, she was able to separate erythrodextrin, achroodextrins, and maltose, but not glucose. The reaction required several days to reach the achromic point, and further action gave the oxidation products of the sugars. She concluded that the action of hydrogen peroxide upon starch is analogous to the action of diastatic enzymes.

Neuberg and Miura (48) in 1911 noted that starch was among the substances acted upon by hydrogen peroxide. Gerber (20) in 1912, confirmed the work of Gatin-Gruzewska and showed that dilute solutions of hydrogen peroxide hydrolyzed starch to dextrins and maltose, and that excess peroxide further oxidized the maltose formed. He concluded also that the process is quite similar to the action of the diastatic enzymes.

Durieux (15)(1913) reported that while starch is very slowly acted upon by hydrogen peroxide or ferric chloride alone, a mixture of the two caused more rapid hydrolysis, as measured by the fall in optical rotation and the production of reducing substances. He observed also that hydrogen peroxide will inhibit the action of diastase, but that the latter undergoes no decomposition in the reaction. In hydrolysing by a mixture of hydrogen peroxide and ferric chloride, the amount of reducing substances formed was found to depend upon the quantity of peroxide decomposed in the reaction.

Biedermann and Jenerkoff (6)(1924) carried the matter further and concluded that the action of amylase itself is due to a peroxidase component, and that the oxidation of the glucose and the hydrolysis of the starch are the result of the action of the same enzyme, a peroxidase. Omori (49) however, in 1931, while showing that the hydrolysis of starch by hydrogen peroxide is catalyzed by the addition of heavy metal salts: ferrous, ferric, copper, vanadium, nickel, cobalt, and manganese, in the descending order of their reactivity; and also that the reaction is hastened by the presence of oxyphenols does not confirm the conclusion of Biedermann as to the presence of a peroxidase in amylase; and concludes that the starch hydrolysis by hydrogen peroxide-heavy metal combination is not analogous to diastatic hydrolysis since diastase does not depend in its action on oxygen. He says, "From my investigations I conclude that starch may indeed be split by a heavy metal-H_2O_2 system, or still more by an addition of an oxyphenol to this system; but the mechanism of the action is quite a different one from that of diastase." He later (1932) shows that the addition of tyrosine or cystine to H_2O_2-Fe system greatly hastens the approach of the achromic points. He did not, however, isolate the decomposition-products.

The effects of other oxidizing agents upon starch have been tried by various workers. Liebig (34) in 1829 found that starch is slightly affected by chlorine or hypochlorous acid. Pelouze (71) in 1838 showed that nitric acid produced xyloidin, a deliquescent acid, and oxalic acid from starch. DeChalmot (13) in 1895 noted that the action of bromine and sodium hydroxide gave products which formed a hydrazone and reduced Fehling's solution. Neither bromine nor sodium hydroxide alone gave this effect. Palit and Dhar (52) in 1925 found that carbohydrates could be oxidized to carbon dioxide and water by a stream of air in the presence of freshly precipitated cerous or ferrous hydroxide.

Starch was among the substances most easily oxidized by this method.

In 1916, Woker (69) reported that she had been able to effect the hydrolysis of starch with formaldehyde, basing her conclusions upon the disappearance of the iodine reaction, and the appearance of reducing power. She was supported by Maggi (39), who wrote a rather interesting discussion on the theoretical function of an aldehyde group in diastase, catalase, and peroxidase. Their results were vigorously attacked by a number of workers (31)(1918), (59)(1919), (60)(1920), (29), (68), These workers showed that the reducing power was due to the presence of the formaldehyde, and the disappearance of the iodine reaction was due to the formation of an addition compound of formaldehyde with the starch. Complete removal of the formaldehyde restores the iodine color, and causes the disappearance of any reducing properties of the solution.

Haehn (22), and Haehn and Berentzen (23) reported the hydrolysis of starch by the system: neutral salt-amino acid-peptone. Ito (27)(1932) reported the hydrolysis of starch by a mixture of sugar, salts, amino acids, and peptone, provided the mixture had been "harmonized" by the action of a weak electric currect for several hours prior to the experiment.

There have been numerous attempts to utilize various sources of energy for the hydrolysis of starch. Notable among these is the work of Semmens (61) and Baly and Semmens (4), who showed that the hydrolytic cleavage of the starch grain by amylase is considerably increased by exposing the solution to plane polarized light. This effect is not so apparent in the acid hydrolysis, but long exposure to the polarized light alone will effect a partial hydrolysis of starch grains. Jones (30), and Bunker and Anderson (12) failed to confirm these results. Abelous and Aloy (1) reported in 1920 that they obtained partial hydrolysis of a solution of starch by mechanical agitation. Szalay (64) in 1933 reported that ultrasonic waves, of a frequency of 722,000 cycles, caused a degradation of starch.

The experimental study was undertaken to discover just how closely the hydrolysis of starch by energy secured from hydrogen peroxide and iron parallels the hydrolysis by amylase. This required the isolation and identification of the hydrolytic products.

EXPERIMENTAL

In the dissertation presented for the degree of Master of Science in 1932 (11), the following facts concerning the action of hydrogen peroxide and ferrous salts upon starch were reported:

1. A solution of starch, containing hydrogen peroxide and small amounts of ferrous salts, loses its opalescence, becomes less viscous, and undergoes a series of changes in the color produced with iodine, from blue through violet and red to colorless. There are also produced, in the course of the reaction, substances which reduce alkaline copper salts. These changes are those produced also by amylase.

2. From the reaction at the achromic point there may be separated a clear, gummy material, which is very soluble in water, giving a clear solution. This substance will give a faint red coloration with iodine, has some reducing power, and upon boiling with hydrochloric acid, gives a strong reduction of Benedict's solution. It seemed to be erythrodextrin. The residual solution after the separation of this substance possesses reducing properties, and upon heating to dryness turns black and gives the odor of burnt sugar. No osazone could be isolated, however, from the reaction mixture.

3. The reaction proceeds in the presence of the peroxide alone, but is much more rapid in the presence of the added ferrous salt. The ferrous salt alone showed no action in 72 hours, while in the presence of the peroxide and ferrous salt, the re-

action reached the achromic point in less than thirty minutes. Other salts also will catalyze the splitting, ferric being almost as active as ferrous. Cupric salt showed very little catalysis of the reaction. The complex salts, ferro- and ferricyanides, are also very weak in their action.

4. The rate of splitting increases with increase in acidity up to a certain point, and with elevation of the temperature. There is also a direct proportion between the rate of splitting and the concentrations of the reagents. The reaction was buffered by acetate buffers at pH 3.6 -3.8, and the experiments were carried out in the neighborhood of 40°C. The reaction proceeds at this pH and temperature fairly rapidly, and neither the acidity nor the temperature is sufficiently high to cause by itself any splitting within the time at which the achromic point is reached in the peroxide-iron system. The concentrations chosen were: one percent starch solution and one percent hydrogen peroxide, usually in the presence of about 10 milligrams of ferrous sulfate.

In the present experiments the following solutions and methods were used:

(a) One percent starch solution, made by suspending 5 grams soluble starch (Kahlbaum) in a little cold water, and pouring this into 500 cc. boiling water. Boiled 15 to 20 minutes, cooled, made up to 500 cc., and covered with toluene. A slightly opalescent solution was obtained which gave a clear blue color with iodine, and a reduction so slight that it could not be measured by the Bertrand sugar method.

(b) Ferrous Sulfate. Made by dissolving 0.695 gr. C.P. ferrous sulfate (Baker) in exactly 250 cc. distilled water to make M/100 solution. Added a drop of concentrated sulfuric acid to prevent precipitation as ferric oxide.

(c) Hydrogen peroxide. Commercial 30% solution (Coleman and Bell), diluted to 1%, and strength checked by permanganate titration.

(d) Buffers. Walpole acetate series (0.185 mol. acetic acid plus 0.015 mol. sodium acetate to give pH 3.6). These were checked against quinhydrone electrode.

Other solutions were made as needed.

The iodine reaction was carried out as follows: Tenth normal iodine in potassium iodide (12.685 gr. I plus 18 gr. KI made to 1 liter.) was diluted one hundred times with water, and a drop of this placed in one of the depressions of a spot plate. A drop of the reaction mixture was obtained on the end of a stirring rod or a clean pipette, and mixed with the iodine solution on the plate. A second drop of iodine on the plate served as a basis of comparison.

The optical rotation of the solutions was measured by means of a Hilger triple-field polariscope, with a vernier marked to 0.01°, using a tube nine inches (23 cm.) in length, and the mercury arc lamp as the source of light (wave length 5461 Angstrom units).

The reducing power of the solutions was determined by the Bertrand modification of the Munson and Walker cuprous oxide method, as described in Mathews' Physiological Chemistry, 5th edition.

The attempts at osazone formation were according to the usual methods and also by the method of Quagliariello and Caponetto (55), using the free phenylhydrazine base, which was shown by Rossi (56) to be far more sensitive than the hydrochloride. Rossi showed that this method could be used to detect glucose in concentrations of 1:20,000.

As a safeguard against bacterial infection, the starch solutions were preserved with toluol, and controls, containing starch and buffer, and starch with iron and buffer added, were run with each experiment to show that the solution did not become contaminated during the course of the reaction.

PART I. THE NATURE OF THE REACTION

1. The Degradation of Starch by Hydrogen Peroxide and Ferrous Salts.

The addition of dilute hydrogen peroxide and small amounts of ferrous salts to a solution of starch causes the starch solution to rapidly lose its opalescence and viscosity just as does the addition of saliva or other amylase. Coincident with this change small samples removed for testing show a series of changes in the color produced by adding iodine to them. The color at the start is blue, and changes to red and finally to colorless as the reaction proceeds. There is also a decrease in the angle of rotation of plane polarized light as the peroxide-iron system acts upon the starch. The reducing powers of the solution increase. The addition of a small amount of the reacting mixture to a solution of alkaline copper sulfate results in the production of cuprous oxide, showing that reducing substances have been formed by the action of the system upon starch. In all these respects the transformation by hydrogen peroxide-iron parallels the hydrolytic transformation by amylase. There are, however, additional changes in this system, changes due to oxidation of the sugar, not found in the amylolytic system. The results of these experiments are shown in Tables I, II, and III.

Insert Tables I, II and III.

The loss of opalescence and viscosity occur at the beginning of the reaction, the solution being clear and watery in a

TABLE I

Time	Begin	15	40	60	120
Starch plus H_2O_2 plus Fe 3%	106.8 seconds	103.2 seconds	100.3 seconds	98.5 seconds	93.8 seconds
Starch alone	106.8 seconds				106.8 seconds
Water alone	89.8 seconds				89.8 seconds

Viscosity measured by capillary viscosimeter at 25.5° C.

TABLE II

Experiment	cc M/100 - $FeSO_4$ added	Vol. cc.	Iodine Reaction	Angle of Rotation	Angle of Rotation	Angle of Rotation	Reduction End of reaction	Fuchsin Test End of reaction
1 (No H_2O_2)	2cc.	30	20 hr. blue	Beginning 1.50°	3 hr 1.50°	20 hr 1.49°	-	-
2 (No H_2O_2)	2cc.	25	24 hr blue	Begin 1.79°	4 hr 1.80°	24 hr 1.80°	-	-
3	0	25	24 hr deep violet	Begin 1.80°	3 hr 1.81°	1.81°	-	-
4	0	30	28 hr red	Begin 1.45°	20 hr 1.40°	28 hr 1.27°	+	-
5	2cc.	30	30 min no color	Begin 1.50°	3 hr 1.27°	20 hr 0.30°	+	+
6	2cc.	25	30 min no color	Begin 1.80°	4 hr 0.90°	24 hr 0.35°	+	+
7	4cc.	30	15 min pink	Begin 1.45°	15 min 1.14°	2.5 hr 0.24°	+	+
8	6cc.	30	15 min pink	Begin 1.45°	15 min 0.99°	2.5 hr 0.23°	+	+
9	8cc.	30	20 min no color	Begin 1.45°	15 min 0.91°	2 hr 0.26°	+	+
10	10cc	32	20 min no color	Begin 1.42°	25 min 0.41°	1.75 hr 0.26°	+	+

Reaction Mixture - 10cc. 1% Starch, 2cc. Acetate Buffer (pH 3.8). 10cc. 1% Hydrogen Peroxide, M/100 $FeSO_4$ added as indicated.

TABLE III

Expt.	Begin	15 min.	45 min.	80 min.	125 min.	155 min.	235 min.	24 hr.
1	0.70°	0.68°	0.64°	0.53°	0.37°	0.26°	0.20°	0.14°
2	0.70°	0.67°	0.63°	0.55°	0.38°	0.30°	0.14°	0.14°
3	0.70°	0.68°	0.64°	0.53°	0.37°	0.28°	0.18°	0.13°
4	0.70°	0.67°	0.63°	0.52°	0.37°	0.28°	0.16°	0.15°
Av.	0.70°	0.675°	0.635°	0.532°	0.372°	0.28°	0.17°	0.14°

Reaction - 10cc. Starch (0.95%), 10cc. H_2O_2 (1%), 2cc Buffer (3.8), 5cc. M/100 $FeSO_4$. Total volume - 27cc.

Temperature - 24.5°C. ± .25°

very short time after the addition of the peroxide, showing that the first action of the peroxide-iron system is the liquefaction of the starch solution. The change of the iodine reaction, and the decrease in optical rotation show that the starch is split into substances of lower optical rotation, presumably into simpler substances, producing in the course of the reaction the erythrodextrin and achroodextrins. Table II further shows that the rate of hydrolysis is dependent upon the concentration of ferrous sulfate added, and the reaction is only a little more than half completed, meacured by the fall in rotation, when the ability of the solution to produce a color with iodine is lost. (Table III shows rate of fall of rotation against time.).

2. The Production of Acid and Reducing Substances.

During the course of the reaction there is a steady increase in the acidity of the unbuffered solution, shown in Table IV. According to Nef (47) there are a number of acids produced by the action of hydrogen peroxide upon glucose and other sugars, and the acidity of the reaction mixture is probably due to the action of the peroxide upon the sugars formed from the starch. No effort was made to isolate and identify the acids in the reaction mixture, except a volatile acid found in the distillate in the concentration of the solution in vacuo. One hundred cubic centimeters of 2% starch, subjected to the action of hydrogen peroxide and ferrous sulfate, produced in twenty-four hours an acidity equivalent to approximately 350 cc. N/100 NaOH. This solution was concentrated under reduced pressure at 60°C., without the use of a capillary tube. The distillate was collected, and was found to have an acidity equivalent to 1.3 cc. N/100 NaOH per cc. This distillate gave a strong reduction of ammoniacal silver nitrate, did not reduce alkaline copper sulfate, did not restore the color to the fuchsin-SO_2 reagent, and gave no precipitate with sodium bisulfite. These results indicate the presence of formic acid, which agrees with the observations of Fernbach and Wolff (17).

The reaction mixture when past the achromic point will reduce an alkaline solution of copper sulfate in the cold in about one minute. This indicates the presence of a much stronger reducing substance than glucose. The mixture will also restore the color to decolorized fuchsin, producing the bluish-red color characteristic of aldehydes, and will give a precipitate when added to a solution of sodium bisulfite. These tests present evidence for the presence of free aldehydes in the reaction mixture, and show that the sugar is not glucose only, if at all. The data of these experiments are shown in Table V.

Insert Tables IV and V.

The presence of formic acid and free aldehyde groups may account for the abnormal reactivity of the reaction mixture to alkaline cupric salts. These products also present evidence that glucose is an intermediate in the degradation of the starch. Two possible paths by which they might be formed from glucose are shown in the following scheme:

$$\text{glucose} \xrightarrow{+O_2} \text{glucosone} \xrightarrow{+H_2O} \text{aldehyde} + \text{formic acid.}$$

$$\text{glucose} \xrightarrow{+O_2} \text{a-hydroxy acid} \xrightarrow{+H_2O} \text{aldehyde} + \text{formic acid.}$$

TABLE IV

CC. N/100 NaOH Required to Neutralize 10cc. of the Reaction Mixture.

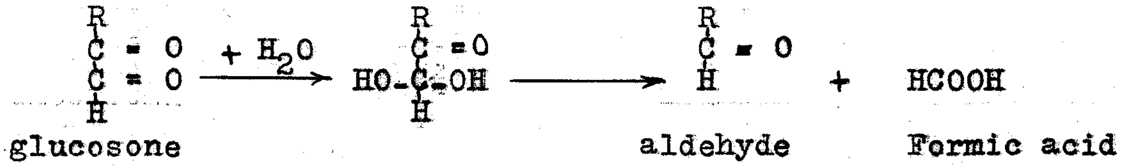
Time	5 min	15 min	30 min	45 min	60 min	75 min	90 min	105 min	120 min	150 min	180 min	240 min	24 hrs
Expt I	3.7	4.1	4.7	5.3	5.9	6.4	7.1	x	x	x	x	9.3	32.2
Expt II	2.8	3.2	4.2	4.9	5.9	x	x	8.0	8.9	10.0	11.6	13.3	32.6
Expt III	3.2	4.1	4.9	x	x	7.4	8.0	8.8	10.1	10.8	x	x	34.1
Expt IV	3.3	4.0	4.9	x	5.8	7.7	x	9.6	x	x	x	x	33.9
Expt V	3.4	4.0	4.7	5.4	5.9	x	7.4	8.4	9.6	x	x	12.9	34.0

Reaction Mixture - 25cc 2% Starch, 1cc. M/100 $FeSO_4$, 1cc. 30% H_2O_2.

TABLE V

Reaction	Time	cc N/20 $KMnO_4$	Mgm Cu	Iodine Reaction
Starch alone	24 hr.	0.60	1.89	Blue
Starch plus H_2O_2	24 hr	6.65	20.94	Deep Violet
Starch plus H_2O_2 plus 5cc. $FeSO_4$ M/100	24 hr	54.3	171.05	Colorless
Starch plus H_2O_2 plus 5cc.M/100 $FeSO_4$	20 hr	51.0	160.7	Colorless
Starch plus H_2O_2 plus 5cc. M/100 $FeSO_4$	18 hr	52.9	166.6	Colorless
Starch plus H_2O_2 plus 5cc. $FeSO_4$	4 hr	48.0	151.2	Colorless

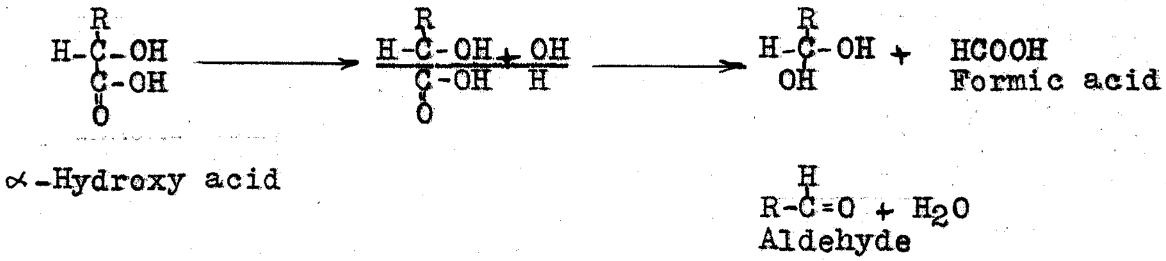
That hydrogen peroxide and ferrous salts attack glucose to form glucosone was shown by Morrel and Crofts (44). Hydrolysis of the glucosone would proceed according to the equation -



$$\begin{array}{c}\mathrm{R}\\ \mathrm{C=O}\\ \mathrm{C=O}\\ \mathrm{H}\end{array} \xrightarrow{+\,H_2O} \begin{array}{c}\mathrm{R}\\ \mathrm{C=O}\\ \mathrm{HO{-}C{-}OH}\\ \mathrm{H}\end{array} \longrightarrow \begin{array}{c}\mathrm{R}\\ \mathrm{C=O}\\ \mathrm{H}\end{array} + \mathrm{HCOOH}$$

glucosone aldehyde Formic acid

Oxidation of the aldehyde group of glucose gives one of the several a-hydroxyacids which may be formed from glucose, which upon hydrolysis produces formic acid and a straight chain aldehyde, according to the scheme,

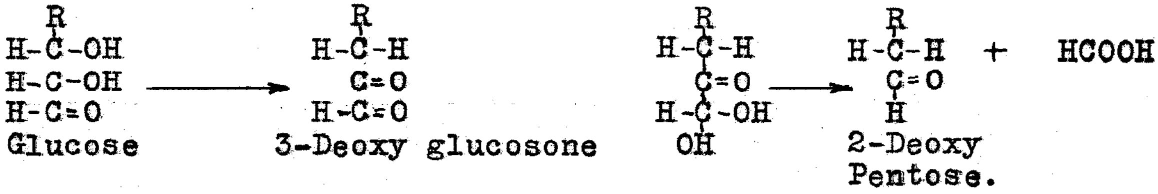


$$\begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}OH}\\ \mathrm{C{-}OH}\\ \mathrm{O}\end{array} \longrightarrow \begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}OH + OH}\\ \mathrm{C{-}OH\ \ H}\\ \mathrm{O}\end{array} \longrightarrow \begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}OH}\\ \mathrm{OH}\end{array} + \mathrm{HCOOH}$$

$$\begin{array}{c}\mathrm{H}\\ \mathrm{R{-}C{=}O}\end{array} + H_2O$$

α-Hydroxy acid Formic acid Aldehyde

An interesting point in this connection is the possibility suggested by Professor Mathews of the formation of a deoxy pentose from glucose by this mechanism. Simultaneous reduction of the third carbon atom with oxidation of the first and second carbons would give a 3-deoxy glucosone, which upon hydrolysis according to the scheme above would give 2-deoxypentose and formic acid. This may be seen from the equations:



$$\begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}OH}\\ \mathrm{H{-}C{-}OH}\\ \mathrm{H{-}C{=}O}\end{array} \longrightarrow \begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}H}\\ \mathrm{C{=}O}\\ \mathrm{H{-}C{=}O}\end{array} \qquad \begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}H}\\ \mathrm{C{=}O}\\ \mathrm{H{-}C{-}OH}\\ \mathrm{OH}\end{array} \longrightarrow \begin{array}{c}\mathrm{R}\\ \mathrm{H{-}C{-}H}\\ \mathrm{C{=}O}\\ \mathrm{H}\end{array} + \mathrm{HCOOH}$$

Glucose 3-Deoxy glucosone 2-Deoxy Pentose.

This 2-deoxy ribose has been isolated from thymo-nucleic acid by Levene and his co-workers (33). It would be extremely interesting if it were produced by this oxidation of starch. Attempts to isolate it from the mixture were not successful, but the difficulties of the separation are such that we do not feel certain that it was not present.

3. The Action of Phenylhydrazine with the Reaction Mixture.

In order to ascertain if glucose, as such, were present in the reaction mixture, an attempt was made to prepare the phenylosazone. The method using the phenylhydrazine-HCl and sodium acetate mixture was substituted by the method of Guagliariello and Caponetto (55), using the free phenylhydrazine base, which was shown by Rossi (56) to be sensitive to 1 part of glucose in 20,000.

In every case in which the reaction mixture was used, there was formed by the addition of the phenylhydrazine a bright yellow to orange precipitate which upon standing changed to a dark reddish brown residue which settled to the bottom of the tube. In no case, however, did there appear the characteristic crystals of phenylglucosazone or phenylmaltosazone. Microscopic observation of the precipitate showed no crystalline form whatever, but the orange colored material appeared as small globules varying in color from yellow to brown, depending upon the time of standing before observation. This was separated, redissolved and attempts were made to obtain it crystalline, but always in vain.

The characteristic phenylglucosazone was easily obtained by this technique from solutions of pure glucose, though the addition of large amounts of iron salts inhibited the formation of the characteristic crystals. That there was no substance present in the reaction mixture which would inhibit the formation of the crystals, were glucosazone present, was shown by the fact that small amounts of glucose (10 mg.), added to the reaction mixture just prior to the osazone test, could easily be identified by the formation of the osazone.

The fact that the crystalline osazone could not be isolated from the reaction mixture confirms the observation of Durieux (15) who obtained a reddish precipitate, by the reaction of phenylhydrazine with a solution of starch which had been acted upon by hydrogen peroxide and ferric chloride, which could not be made to crystallize.

The failure of glucosazone or maltosazone to appear indicates that glucose or maltose, if produced in the degradation of starch, are immediately attacked by the peroxide-iron system, and their concentration is kept below the limits of the sensitivity of the osazone reaction. Two-deoxyribose, if present, should give a hydrazone but not osazone.

That there is a reaction between the phenylhydrazine and some substance, or substances, in the reaction mixture, cannot be doubted. There is the change in color of the mixture from a pale yellow to a deep orange which takes place even in the cold. The production of a colored precipitate indicates the formation of an osazone, one which will not crystallize. The reaction requires an alcohol group adjacent to a carbonyl group, or two adjacent carbonyl groups, and there are several possibilities for the formation of substances of this nature from glucose.

The characteristic crystals of phenylglucosazone were obtained when a solution of dextrin isolated from the reaction was hydrolyzed by HCl, or when the dialysate of the dextrin solution was treated with HCl. Only under these conditions was there a crystalline osazone obtained from any product of the reaction. The data concerning the formation of the osazones is shown in Table VI.

Insert Table VI.

4. The Formation of Dextrins and Reducing Sugars.

The addition, at any stage in the reaction, of four volumes of 95% ethyl alcohol and a small quantity of sodium chloride, or half saturation with ammonium sulfate, causes the precipitation of substances which show the characteristics of dextrins. They are soluble in water, giving a clear solution, are optically active, and give a reaction with iodine which varies from bluish red to colorless, depending upon the time the reaction was allowed to proceed before the addition of the precipitant. The solution obtained by dissolving this gummy precipitate in water gives a positive Molisch test, is dextrorotatory, and shows considerable cupric reducing power. Dialysis through cellophane does not change the Molisch and iodine reactions, but causes a decrease both in optical rotation and reducing power.

Four volumes of 95% ethyl alcohol and five cubic centimeters of saturated NaCl were added to 400 cc. starch solution containing 20 grams of starch, which had reacted with 15 cc. of 30%

TABLE VI

Expt.	Description	Precipitate with PhNHNH$_2$	Osazone Crystals
1	Glucose alone - 1 : 4000	+	+
2	Starch + H_2O_2+$FeSO_4$ 24 hours	+	-
3	Starch + H_2O_2+$FeSO_4$ (24 hrs) + 25mg Glucose	+	+
4	Residual sol'n after p'ption of Dextrins from R.M.	+	-
5	Ether Extract of Residual Solution	+	-
6	Dialysate of Dextrin Solution	+	-
7	Dextrin Solution p'pt'd from R.M.	+	-
8	Dextrin Hydrolysed with HCl	+	+
9	Dialysate hydrolysed with HCl	+	+

hydrogen peroxide and 10 cc M/100 ferrous sulfate until the color produced with iodine was deep red. A white precipitate appeared, and settled out on standing, forming a clear gummy residue in the bottom of the flask. The alcohol solution was poured off, the residue washed with a small amount of cold water, and dissolved in warm water. The precipitation was repeated. This precipitate, dissolved in water, gave a strong Molisch, a deep red color with iodine, and considerable reduction of Benedict's solution. The solution was divided, both portions placed in cellophane, and one dialysed against running tap water, the other against distilled water. The dialysate of the latter was concentrated and preserved. In both cases there was a diminution in both optical rotation and reducing power.

The same procedure was repeated using a solution which had been allowed to react until there was no color produced with iodine. The procedure is outlined schematically in Table VII.

Insert Table VII.

Dialysis of the red dextrin for ten days against running water caused a complete loss of the reducing power, though there was still a red color produced with iodine, and rotation of polarized light was still evident. Evaporation of a portion of the dialysed red dextrin solution showed that there was 1.15 grams organic solids per 100 cc. solution remaining in it. This solution gave an observed angle of rotation of +2.23° (using a 1 dcm. tube and light of wave length 5461 Angstrom units). From the formula $(\alpha)_{5461} = \frac{100\alpha}{lc}$, the specific rotation of the dextrin was

TABLE VII

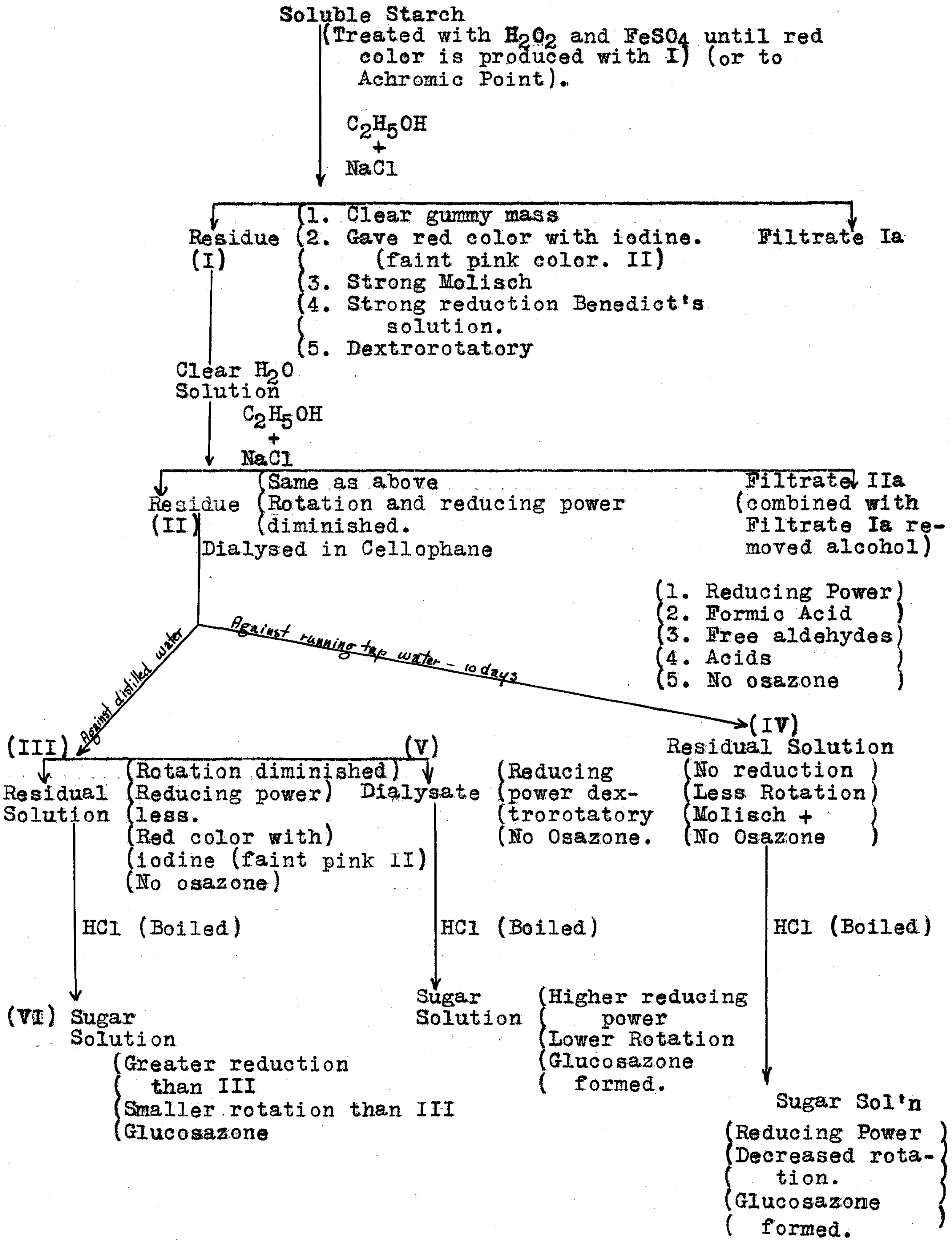


DIAGRAM OF PRECIPITATION AND DIALYSIS OF DEXTRINS PRECIPITATED FROM REACTION.

calculated, $(\alpha)^{24}_{5461} = +193.8^{\circ}$ (calculating total solids as dextrin). The specific rotation of the soluble starch originally used was found to be + 203°. The rotation of this substance corresponds to the amylodextrin- isolated by Brown and Morris (9).

The dextrin isolated after the achromic point had been past, could not be entirely freed of a substance which gave a red color with iodine. This solution, before precipitation, gave no color with iodine, but when the precipitated dextrins were dissolved in a small amount of water there was a faint pink color produced, showing that a little of the erythrodextrin remained.

The dialysis of the solution of colorless dextrin against running water resulted in the complete loss of reducing power. A portion was evaporated to dryness, and 0.25 gram of organic solid per 100 cc. was found. This amount of solids gave an observed angle of rotation of 0.45° (1 dcm. tube), and no reduction by the Bertrand method. The specific rotation of this solution was calculated to be +180°. This is a little high for the substance variously termed amylodextrin-B (Ling and Baker), achroodextrin II (Hertzfeld), and achroodextrin B (Musculus and Gruber).

Hydrolysis of the dextrins, both red and colorless by acid, resulted in the production of glucose. The cupric reducing power is increased and the optical rotation decreased when the solution is boiled with a few cubic centimeters of hydrochloric acid. The original solution in the impure state gave the orange precipitate with phenylhydrazine, but did not show a definite crystalline structure when examined microscopically. The dialyzed dextrin solutions gave little or no precipitate with phenylhydrazine. After treatment with acid, the dialyzed dextrin solutions produced the characteristic crystals of phenylglucosazone. That the hydrolysis was carried completely to glucose is shown by the fact that the rotation and reducing power of the solution after hydrolysis are in close agreement with these figures for a solution of glucose alone. The results of these calculations is shown in Table VIII.

Insert Table VIII.

The concentrated diffusate of the dextrin solutions were found to possess rather high optical rotation and some reducing power. That they do not contain glucose or maltose or a mixture of the two is shown by the fact that no osazone could be isolated from the dialysates. The high optical rotation and low reducing power indicate that they contain higher polysaccharides which are diffusible through cellophane. That this was true was shown by the action of acids upon the dialysates. In both cases there was a fall in the angle of rotation and an increase in the cupric reducing power, until these factors corresponded to the rotation and reducing power of a solution of glucose. At this point the characteristic phenylglucosazone was obtained.

The dialysate from the red dextrin was evaporated very carefully to dryness, and the solid residue weighed. One hundred cubic centimeters of the dialysate contained 0.794 grams of solid

TABLE VIII

	Mg. Cu. Reduction	Angle of Rotation	Reduction Calculated as Glucose	Rotation Calculated as Glucose
1. Red Dextrin (Impure)	289.0	4.15°	150 mg.	2910.0 mg.
2. Red Dextrin plus HCl	1986.0	1.45°	1035 mg.	1017.0 mg.
3. Colorless Dextrin	356.0	4.80°	185 mg.	3366.0 mg.
4. Colorless Dextrin plus HCl	1669.5	1.30°	875 mg.	911.0 mg.

organic material, giving a solution of approximately 0.8%. This solution gave a rotation of 1.10° (1 dcm., 5461 A.u.) and a reduction of 214.2 mgm. Cu.

Specific Rotation $^{25^\circ}_{5461} = 138.5^\circ \left\{ (a)_D = 122.4 \right\}$

Reduction = 23.8 (Maltose = 100)

The constants for this solution are in fair agreement with those of amylotriose obtained by Pringsheim (57), which are:

Specific Rotation $^{20^\circ}_{D} = 124.5$

Reduction = 22.5 (Maltose = 100)

The dialysate from the colorless dextrins was found to contain 1.58 grams solid organic material per 100 cc. This gave a rotation of 0.81° (1 dcm.) and a reduction of 72.45 mgm. Cu. for the total sample.

Specific rotation $^{25^\circ}_{5461} = 52.53^\circ \left\{ (a)_D = 46.6^\circ \right\}$

Reduction 4.1 = (Maltose = 100)

This solution is undoubtedly a mixture of the oxidation products of glucose, possibly containing a small amount of reducing substance of fairly high rotation.

Insert Table IX.

The solution, from which the dextrins and concomitant materials were precipitated, was evaporated under reduced pressure to remove the alcohol and excess water. This concentrated solution was found to be rather strongly acid, and gave a very strong coloration to a solution of decolorized fuchsin. There was some dextrinous material left in this solution, for the precipitation

TABLE IX

		Cu Reducing Power (mg. cu)	Angle of Rotation	Reduction Calc. as Glucose (mg.)	Reduction Calc. as Maltose (mg)	(α) Calc. as Glucose	(α) Calc. as Maltose	Osazone
Dialysate from Red Dextrins	(A) Before Hydrolysis	236.3	2.35°	120.5	208.7	1648.0	666.6	-
	(B) After Hydrolysis	740.0	0.56°	394.8	655.8	392.7	160.9	+
Dialysate from Colorless Dextrins	(A) Before Hydrolysis	168.5	2.45°	84.3	148.6	1711.0	691.7	+
	(B) After Hydrolysis	573.4	0.38°	297.4	507.4	266.4	107.3	+

Took 100 cc. each Dialysate (conc.) and divided into two equal parts. To Part (B) added 10cc. Conc. HCl and boiled 10 minutes. Neutralized and diluted both to the mark. Calculated as undiluted.

of dextrins by alcohol is not quantitative. The solution gave strong reduction of copper, and a very heavy precipitate with phenylhydrazine in the cold. This precipitate was a bright orange color, but did not show the osazone of glucose or maltose. After distillation in vacuo at 60° there was a strong reduction of silver nitrate by the distillate, but no restoration by it of the color to decolorized fuchsin. With the phloroglucinol reagent of Tollens, there was produced a red coloration which, upon spectroscopic examination, failed to show any absorption bands, indicating that this color was not due to glycuronic acid or pentose. There was also a red coloration produced with Seliwanoff's resorcinol reagent, though the color was not produced as rapidly as with levulose.

The tests given by this concentrated filtrate were identical with those given by the reaction mixture. The substance which caused the restoration of color to decolorized fuchsin was not identified, and attention is called to the fact that this test is given by the 2-deoxy pentoses. The possibility of the formation of the deoxy sugars in the reaction has been discussed.

5. Effect of Added Substances upon the Reaction.

The addition of glucose in small amounts does not affect the reaction, but larger amounts cause a definite retardation in the rate of fall of optical rotation, and lengthen the time to the achromic point. There is an increase in the amount of color restored to decolorized fuchsin which seems to be proportional to the concentration of the added glucose. There is also an increase in the amount of silver nitrate reduced by the reaction mixture. There is a slight increase in the acidity produced, though this is less than would be expected. The results of these experiments are shown in Table X.

Insert Table X.

The explanation of these results appears to be that glucose is more reactive toward the peroxide-iron system than is starch, thus slowing the rate at which the starch is split. Since the specific rotation of glucose is so much less than that of starch, the same amount of energy acting upon glucose would cause a much smaller decrease in the optical rotation than if the starch were split. Diverting the energy from the starch to the glucose would naturally increase the time to the achromic point. The peroxide is used up by the glucose and less remains for the starch. Since there is an increase in the amount of formic acid and free aldehyde produced with an increase in the concentration of glucose added, it is evident that the glucose is attacked and lends support to the hypothesis that the aldehyde and formic acid formed in the reaction of starch alone come from glucose; and that glucose is formed in the breakdown of the starch molecule but at once oxidized. A copious orange precipitate was obtained upon the addition of phenylhydrazine to the reaction mixture to which glucose had been added at the beginning of the reaction,

TABLE X

Expt.	Mg. Glucose added	Iodine Reaction	Begin Rotation	End Rotation (3hr)	Drop in Rotation	% Drop in Rotation	Fuchsin Test	Acidity (24 hr) cc.N/100 NaOH
Starch alone	0	27 hr Blue	1.21°	1.21°	0	0	-	---
RM	0	30 min Colorless	1.21°	0.39°	0.82°	67.8%	Faint	21.0
RM	180	30 min Pink	1.84°	0.96°	0.88°	47.8%	+	22.4
RM	360	30 min Red	2.56°	1.79°	0.77°	30.0%	+ +	24.1
RM	540	30 min Red	3.16°	2.41°	0.73°	23.2%	+ + +	26.3

RM = 10 cc. 1% Starch plus 10 cc. 1% H_2O_2 plus 5 cc. M/100 $FeSO_4$, Sugar added and made up to 50 cc. with H_2O. Temperature 37°C. No buffer added. Tube = 2.3 dcm. (9 in.). Wave length = 5461 Au.

but phenylglucosazone could not be identified. The precipitate appeared as spherical yellow particles of varying sizes. That the precipitate was not formed from glucose is evidenced by the fact that the precipitate formed in a few minutes in the cold, indicating a much more reactive substance than glucose which needs to be heated with phenylhydrazein and for some minutes. The precipitate had the same microscopical appearance whether formed in the cold or heated.

Urea, in quite high concentrations, has no effect upon the rate of fall of optical rotation nor upon the time required to reach the achromic point. Solid urea, dissolved in concentrated hydrogen peroxide, forms an addition compound with the peroxide which may be obtained as a powder. This powder is quite stable while dry, but in water solution it decomposes with the liberation of urea and hydrogen peroxide. A small quantity of this powder, added to a solution of starch containing ferrous sulfate, will cause the hydrolysis of the starch as when the liquid peroxide is added.

6. The Effect of other Salts upon the Hydrolysis.

Besides ferrous sulfate, which has been shown to be very active in the catalysis of the action of hydrogen peroxide upon starch, there are other salts which effect the same catalysis. With the exception of the ferric salts, these compounds fall far below the ferrous salts in effectiveness. Cupric sulfate and manganese chloride have greatly inferior action compared with iron. Potassium ferrocyanide and lead nitrate have a very weak effect, while manganese dioxide has no effect whatever. Table XI shows the effect of the various salts upon the hydrolysis.

Insert Table XI.

From these data one is led to conclude that, although the iron does not have an absolute specificity in this particular reaction, that there is certainly a relative specificity shown by the ferrous or ferric salts. The presence of iron seems to be necessary for efficient splitting of the starch by hydrogen peroxide. This result has been obtained by others. Omori (49) showed that copper is far less efficient than iron.

TABLE XI

Expt.	Salt	Iodine Reaction	Rotation	Rotation	Rotation
1.	$FeSO_4$	18 min. Colorless	1 hr 0.83°	7 hr 0.60°	27 hr 0.40°
2.	$CuSO_4$	27 hr Faint pink	1 hr 1.90°	7 hr 1.85°	27 hr 1.18°
3.	$FeCl_3$	20 min Colorless	1 hr 0.85°	6 hr 0.68°	26 hr 0.61°
4.	$K_4Fe(CN)_6$	26 hr Red		6 hr 1.89°	26 hr 1.48°
5.	$Pb(NO_3)_2$	26 hr Red		6 hr 1.84°	26 hr 1.49°
6.	$MnCl_2$	27 hr Pink	1 hr 1.92°	7 hr 1.83°	27 hr 1.15°
7.	MnO_2	27 hr Blue			27 hr 1.92°
8.	$Fe_2(SO_4)_3$	27 hr Colorless	1 hr 1.90°	7 hr 1.70°	27 hr 0.41°
9.	No salt	27 hr Blue		7 hr 1.94°	27 hr 1.90°
10.	No H_2O_2	27 hr Blue		7 hr 1.94°	27 hr 1.94°

Reaction: 10 cc. Starch (1%) plus 2 cc. Acetate Buffer plus 10 cc. H_2O_2 (1%) plus 5 cc. M/100 Salt. In case of MnO_2, o.5 gr. was used.

Beginning Rotation = 1.94° Temperature = 37°C. pH - 3.8

Rotation Tube = 2.3 dcm. (9 in)

SUMMARY OF PART I.

From the experiments just described, the following facts may be briefly summarized:

1. When a solution of starch is treated with hydrogen peroxide and iron salts, there is a loss of opalescence and a decrease in the viscosity of the solution, just as when starch is treated with one of the many amylolytic enzymes.

2. There is a change in the color produced by the solution with iodine, varying from blue through red to colorless, as in the acid and enzymic hydrolyses of starch. There is also a decrease in the angle of rotation of plane polarized light, showing that the starch is indeed undergoing a change into substances of lower optical rotatory power, presumably simpler substances.

3. By the use of ethyl alcohol, there may be precipitated from the reaction substances which have the properties of the dextrins obtained in the hydrolysis by acids or enzymes. By precipitating these substances while the solution gave a red color with iodine, there was obtained a gummy residue which had a specific rotatory power of $+193^{\circ}$ (5461 A.u.). This substance in water solution gave a deep red color with iodine, could be dialysed free from reducing power, and on acid hydrolysis gave a quantitative yield of glucose. This substance is quite likely a mixture of dextrins, containing a considerable proportion of the erythrodextrin.

Precipitation at the achromic point gave a residue quite similar to the one just described, except that only a faint color was produced by the concentrated solution with iodine. This substance had a special rotation of +180° (5461 A.u.), could be dialysed free from cupric reducing substances, and upon hydrolysis with acid produced a quantitative yield of glucose. This substance was chiefly achroodextrin, containing only a small amount of the dextrin giving the red color with iodine.

Dialysis of the red dextrin gave a substance which had a specific rotation of + 138.5° (5461 A.U.), and a reducing power equivalent to 23.8 percent of that of maltose. This substance agrees quite closely in optical rotation and reducing power to the amylotriose identified by Pringsheim from the action of bacterial enzymes on starch.

Dialysis of the colorless dextrin gave a solution showing a specific rotation of +52.53° (5461 A.u.), and a reducing power 4.1 percent of that of maltose. This solution is obviously a mixture of the degradation products of glucose.

4. In the reaction mixture, and in the concentrated filtrate from the precipitation of the dextrins, are found substances which will restore the color to decolorized fuchsin, and give a precipitate with sodium bisulfite. Hence free aldehyde groups are present. The reaction mixture becomes distinctly acid, and formic acid may be distilled from it. These reactions are increased by the addition of glucose or maltose to the reaction mix-

ture, and are given by a solution of glucose or maltose alone, treated with hydrogen peroxide and ferrous salts. From these facts it is concluded that glucose, and possibly maltose, are intermediate products in the reaction.

From these facts the conclusion is reached that the reaction of hydrogen peroxide and ferrous salts upon starch is a typical hydrolysis, analogous in every respect to that produced by enzymes, as malt amylase or ptyalin, differing only in the fact that the reaction proceeds further, producing hydrolysis and oxidation of the monosaccharides formed in the hydrolysis.

PART II. THE MECHANISM OF THE REACTION.

The experiments of the foregoing section have established the fact that the action of hydrogen peroxide and ferrous salts upon starch is a typical hydrolysis, analogous to that effected by the various amylolytic enzymes. The question as to the mechanism of the reaction naturally arises. How does the peroxide-iron mixture cause the splitting of the starch molecule?

The question of the mechanism of the reaction resolves itself, in the final analysis, to the transfer of energy from the peroxide to the starch. Starch is stable because the energy level of the molecule is low. Energy must be supplied in order to make the molecule unstable and reactive (Cf. Mathews (41). When the energy level has been raised to that of the reactive molecule, the starch splits and combines with water to give the hydrolytic products. The real mechanism of the starch hydrolysis, then, is the transfer of energy, and the question to be dealt with here is "How is the energy carried to the starch molecule?"

There are two main possibilities for the transfer of energy, which will be considered in some detail: (a) radiation, and (b) conduction.

(a) Radiation.

Modern research has shown that many substances, particularly certain dyes, may absorb radiant energy and become activated.

The fundamental principle of photosynthesis is the absorption of solar energy by chlorophyll to form sugars from carbon dioxide and water. The mechanism of these reactions consists in the absorption of energy of a definite wave length, with the consequent activation of the molecule. In many cases there is required the presence of some intermediate compound which is capable of absorbing the radiant energy and giving it over to the molecule in question. Thus chlorophyll absorbs energy but passes it to the carbon dioxide. Theoretically every molecule is capable of absorbing energy of a definite wave length, the amount of energy absorbed being proportional to the wave length used, according to the quantum equation, $E = hv$.

To explain the mechanism of starch hydrolysis on the basis of absorption of radiated energy, there are several possible ways in which the reaction may proceed. First there is the possibility that the starch is able to absorb the energy from the peroxide breakdown, and that the iron acts to increase the amout of energy produced in a given time. Or, the iron might act to absorb the energy from the peroxide, changing it to a wave length capable of being absorbed by the starch. In either case, one would expect, were this the case, that the reaction would proceed when the starch is separated from the peroxide-iron mixture by a glass or quartz partition, for whether the energy system were separated or in physical contact with the molecule would make no difference, except possibly in the intensity of the reaction.

In order to test this point, the following experiment was carried out: Ten cubic centimeters of 1% starch were placed in each of two quartz tubes, and a like amount in a glass tube. To one of the quartz tubes was added 1 cc. M/100 $FeCl_3$. All three tubes were immersed in a beaker containing 200 cc. 1% H_2O_2 to which had been added 10 cc. M/100 $FeCl_3$. Evolution of oxygen from the peroxide was quite evident. A control tube, containing starch solution, to which the peroxide and iron had been added, was also run. The results are shown in Table XII.

Insert Table XII.

The results of this table show that there was no change in the iodine coloration, nor in the angle of rotation of the solutions in the beaker, though all the peroxide had decomposed (24 hours). The control tube reached the achromic point in 30 minutes, and at the end of 24 hours showed considerable reduction of rotatory power.

This experiment indicates that the hydrolysis of the starch cannot be explained by direct radiation of the energy from the peroxide to the starch molecule, but that the presence of the peroxide and iron in the solution of starch is necessary for the splitting.

TABLE XII

	Vol. cc.	I_2KI Color	(begin)	20 hr.	Reduction
Control 10cc. Starch + 10cc. 2% H_2O_2 + Buffer + 5 cc. $FeSO_4$	27 cc.	30 min. Colorless	0.76°	0.30°	+
10cc. Starch in Quartz vessel. Immersed in H_2O_2 containing $FeCl_3$	10 cc.	20 hr. Deep Blue	2.05°	2.06°	-
10cc. Starch + 1cc. $FeCl_3$ in Quartz tube, immersed as above.	11 cc.	20 hr. Deep Blue	1.85°	1.75°	-
10cc. Starch in Glass tube. Immersed as above	10 cc.	20 hr. Deep Blue	2.05°	1.95°	-
10cc. Starch Untreated	10 cc.	20 hr. Deep Blue	2.05°	2.05°	-

(b) Conduction.

The second possibility for the transfer of energy, and the one which is the most logical explanation of the starch hydrolysis, is the conduction of the energy by means of intermediate chemical combinations. These combinations are probably through the sharing of electrons, in which the resistance to the passage of energy is diminished. According to this mechanism, there is a peroxide-iron-starch linkage, which permits of the flow of energy from the peroxide into the starch.

That the effect of the iron is not primarily due to the catalysis of the liberation of energy, but that the iron is a vital part in the chain for the transfer of energy, is adduced from the following evidence:

1. The presence of iron or similar metal seems to be necessary for the hydrolysis. Though iron-free starch was not used in the experiments, the content of iron in the reaction mixture, in the absence of added iron, was approximately 0.01 mgm.. The rate of hydrolysis by H_2O_2 with this amount of iron was very slow, requiring several days to reach the achromic point. The dependence of the speed of hydrolysis upon the concentration of iron as shown in Table I indicates that the complete removal of iron would cause the reaction to be immeasurably slow.

2. The speed and amount of hydrolysis produced by various salts is not in the order of their ability to catalyze the decomposition of hydrogen peroxide. The effect of various salts upon

the reaction was shown in Table IX. These salts were tested by the benzidine reaction for their ability to cause the decomposition of peroxide. This reaction was carried out as follows: One cubic centimeter of alcoholic benzidine (1% containing 1.36 gr. Na Acetate) was diluted to ten cubic centimeters with distilled water, and to this was added 1 cc. of 1% hydrogen peroxide. A solution of the salt was added until a detectable blue color was produced. The results are shown in Table XIII.

Insert Table XIII.

This test shows that copper produces a blue color in the highest dilutions, followed by ferrous and ferric salts. There is no action of the other salts upon the benzidine.

A glance at Table IX shows that copper, which gives the benzidine reaction in the highest dilutions, is far inferior to iron in the hydrolysis of starch, being about equal to manganese chloride, which gives no color to benzidine in quite high concentrations.

3. The catalysis of the decomposition of hydrogen peroxide is inversely proportional to the concentration of hydrogen ions (72), while the hydrolysis of starch by hydrogen peroxide and iron increases with an increase in acidity up to about a pH of 3.3 (Omori).

The conclusion reached from this evidence is that the action of iron in the catalysis of the decomposition of the peroxide is of no importance in the consideration of the effect of the iron upon the rate of starch hydrolysis. The evidence points to

TABLE XIII

Salt	Color	Amount
$FeSO_4$	Deep Blue	0.5 cc. M/100
$Cu\ SO_4$	Deep Blue	0.1 cc. M/100
$FeCl_3$	Blue	0.5 cc. M/100
$K_4Fe(CN)_6$	Pale Green	2 cc. M/100
$Pb(NO_3)_2$	No Color	- - -
$MnCl_2$	No Color	- - -
MnO_2	No Color	- - -
$K_2Cr_2O_7$ (no H_2O_2)	Deep Blue	0.1 cc. N/10
Potato ext. (peroxidase)	Deep Blue	0.1 cc. Aq. ext.

Reaction: Benzidine (in Alc. NaAc) plus Salt plus H_2O_2.

the conclusion that there is not a direct combination between the peroxide and the starch, but that the combination is between the starch and the iron, the iron being the link which serves to deliver the energy directly to the starch molecule. The peroxide probably combines with the iron, passing its energy through the iron to the starch, though it may activate the iron by radiation, the activated iron conducting its energy to the starch by virtue of its combination with the starch. Which of these methods of the activation of iron is the true one is uncertain.

The combination between the starch and iron seems to be through the residual valences of the oxygen of the glucoside linkage, for it is at this point at which the starch molecule is split. That the union is with the alcoholic hydroxyl groups is not likely, since these compounds are formed only in alkaline solution, and the hydrolysis of the starch was conducted in acid medium. The union of the iron through the residual valence of the oxygen of the glucoside linkage forms an unstable compound, allowing for the passage of energy into the starch molecule.

The following explanation of the mechanism of the starch hydrolysis is put forth: The iron atom forms an unstable combination through the residual valences of the oxygen of the glucoside linkage in the starch molecule. The iron passes its energy into the starch molecule, raising its energy level and making it more reactive. The iron, even in its highest energy level, does not contain enough energy to split the starch, except at a very slow rate. The decomposition of the peroxide gives large quantities of energy, which is taken up to form activated iron and its energy is passed into the starch molecule. Enough energy is put into the molecule to cause a quite rapid splitting of starch. The iron, upon giving the energy to the starch molecule, reverts to a lower energy level, is again activated by the peroxide, and in turn, passes this energy to another molecule of starch. The reaction appears to be a true catalysis, since the iron is left available for many transfers of energy.

This is in line with Warburg's (73) suggestion that Fe^{IV} is the energy-rich form, which hydrolyses the starch.

$$\begin{tikzcd} Fe^{II} \arrow[r] & Fe^{III} \arrow[r] & Fe^{IV} \arrow[ll, bend left] \end{tikzcd}$$

$$Fe^{IV} + \text{Starch} + H_2O \longrightarrow Fe^{II} + \left\{\begin{array}{l}\text{dextrins}\\ \text{glucose and}\\ \text{oxidation}\\ \quad\text{products.}\end{array}\right.$$

That the iron does not remain in a stable condition during the reaction is indicated by the effect of cyanide upon the reaction. The addition of cyanide at the beginning causes the formation of the deep brown coloration due to ferric ferricyanide, which, as the reaction proceeds, assumes a greenish tint due to the formation of either ferrous ferricyanide or ferric ferrocyanide. Addition of the cyanide after the reaction has proceeded for some time results in the more rapid production of the blue color. The formation of the blue color indicates probably that both ferrous and ferric ions are present in the reaction mixture. This fact adds evidence for the supposition that the iron alternates between the energy-rich and energy-poor forms, that is, from a higher to a lower state of oxidation.

SUMMARY

The mechanism of the hydrolysis of starch by hydrogen peroxide and ferrous salts resolves itself to the conduction of energy from the peroxide to the starch, causing the starch molecule to become reactive and to split into simpler substances. Some of these substances are further oxidized.

The following theory is offered to explain the mechanism of the reaction: The break-down of the peroxide liberates energy, which is absorbed by the iron atom, with a consequent activation of the iron atom, possibly forming a tetravalent atom. The activated, or tetravalent, iron unites with the starch, possibly thru the residual valences of the oxygen of the glucoside linkage, and passes its energy into the starch molecule. The starch molecule is raised to a reactive form, and splits spontaneously, adding water in the process to undergo hydrolysis.

The iron, having given up its energy, reverts to the stable form and dissociates from the linkage. It may be reactivated by the peroxide and may repeat the energy transfer.

DISCUSSION.

The peroxide-iron-starch system appears to be identical with the hydrolytic enzymes, except the fact that the supply of available energy is limited to the amount of peroxide present, and the chain may be called an artificial enzyme system. The analogy of its action to that of enzymes is so striking that the application of the mechanism of the artificial enzyme to the action of the natural enzymes seems logical.

That the enzyme enters into chemical union with the substrate seems established (5)(43). By application of the mechanism of the artificial system, the enzyme acts as does the iron, absorbing energy from an external source, and conducting it to the molecule of the substrate, causing activation and splitting of the substrate. The source of energy of the enzyme is uncertain, being in some instances from light, oxygen, or the kinetic energy of surrounding molecules. The enzyme molecule varies between the anakinetic and katakinetic states just as does the iron. When the energy level of the enzyme is high, the energy flows to the lower level of the substrate. The substrate activated, splits; the inactive enzyme dissociates, picks up energy, combines with another molecule of substrate, and the transfer of energy is repeated.

The discovery of activators and coenzymes points to the conclusion that enzyme activity is not ascribable to a single substance, but to a system. According to the energy transfer view any substance which favors the formation of the enzyme-substrate combination, or by its presence facilitates the transfer of energy to the enzyme, may be classed in the category of activator or coenzyme. Thus the wide diversity of substances, ranging from ordinary salts to complex organic compounds, which act to increase enzymic activity, is clarified.

CONCLUSIONS.

On the basis of the foregoing, the following conclusions seem warranted:

1. Hydrogen peroxide and ferrous salts produce liquefaction and splitting of a solution of starch. The splitting proceeds through the formation of erythrodextrin, achroodextrin, diffusible polysaccharides, and glucose. Possibly maltose is also an intermediate in the process.

2. The reaction is an hydrolysis, which, in the fall in rotation, change in iodine reaction, and in the production of the dextrins and sugars, is analogous to the hydrolysis produced by amylolytic enzymes, as malt amylase or ptyalin. It differs from these, however, in the fact that it proceeds further and produces hydrolysis and oxidation of the monosaccharides formed.

3. The reaction probably proceeds by the conduction of energy from the peroxide, to the iron and from it, by means of an unstable chemical combination of the iron with the starch, into the starch molecule. The activated starch molecule becomes unstable and splits at the glucoside linkage, adding water to give the hydrolysis.

4. The mechanism of the reaction may be applied to the action of enzymes, giving support to the theory of Professor Mathews that enzymes act by the transfer of energy from some external source to the molecule of the substrate.

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